Accounts in Drug Discovery
Case Studies in Medicinal Chemistry

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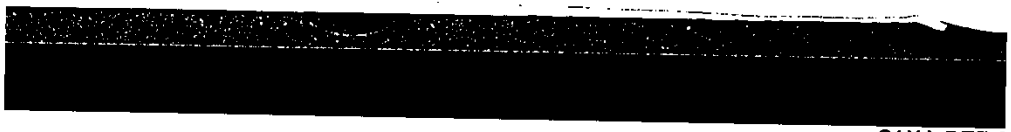

CHAPTER 1

The Discovery of the Dipeptidyl Peptidase-4 (DPP4) Inhibitor Onglyza™: From Concept to Market

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1.1 Introduction

The prevalence of diabetes in developed and now emerging countries represents a significant health burden to a large portion of the world's population. Type-2 diabetic patients, characterized in part by elevated fasting plasma glucose of $> 125\,\mathrm{mg\,dL^{-1}}$ ($7.0\,\mathrm{mmol\,L^{-1}}$) and glycosylated hemoglobin (HbA1c) $\geq 6\%$, are at increased risk for the development of both microvascular (retinopathy, neuropathy, nephropathy) as well as macrovascular complications (myocardial infarction, stroke). As such, diabetes is the leading cause of blindness, kidney failure, and limb amputation worldwide.[1] Diabetes is a progressive disease, with morbidity and mortality risk increasing with both duration and severity of hyperglycemia. Additionally, diabetes is also now impacting different population sectors (adolescents, developing countries) not typically associated with the disease 30 years ago. Consequently, the continually increasing diabetes prevalence is placing greater strain on both health care systems and economies on a global scale. In 2007 alone, studies have shown that diabetes cost the US

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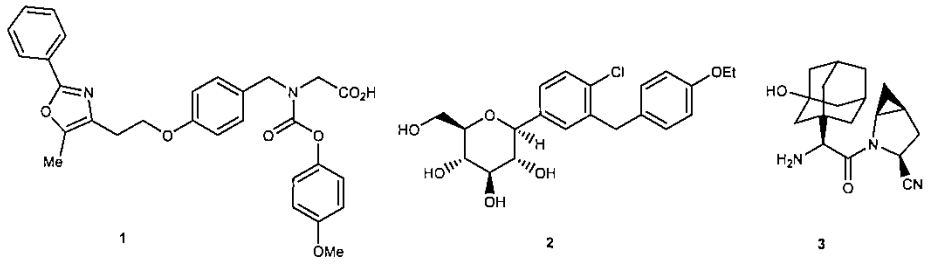

1 2 3

Figure 1.1 Late-stage development candidates from the Bristol-Myers Squibb diabetes research portfolio.

economy $174 billion in medical expenses and lost productivity.[2] While death rates related to heart disease, stroke, and cancer have all decreased since 1987, the death rate due to diabetes has increased by 45% during this same period.[3] Thus, the discovery and development of new therapies for treating and preventing diabetes continue to be a major emphasis of health care companies.

In response to this landscape, the Discovery organization at Bristol-Myers Squibb (BMS) made the strategic decision to refocus efforts in the late 1990's towards identifying and progressing novel targets for the treatment of diabetes. This was in part aimed at building upon BMS's already established presence in the anti-diabetes market through the Glucophage™ franchise and in recognition of the significant unmet medical need for novel, more efficacious, and well tolerated treatments for the disease. It was from these efforts that advanced clinical candidates such as muraglitazar (**1**, Pargluva™, dual PPAR agonist),[4] dapagliflozin (**2**, SGLT2 inhibitor),[5] and saxagliptin (**3**, Onglyza™, DPP4 inhibitor)[6] were discovered within the BMS Discovery organization (Figure 1.1).

1.2 Modulation of GLP-1 in the Treatment of Diabetes

At the start of this effort, several oral anti-diabetic agents (OADs) were available to patients suffering from type-2 diabetes. These included hepatic glucose suppressors (*e.g.* metformin), insulin secretagogues (*e.g.* sulfonylureas), glucose absorption inhibitors (*e.g.* acarbose), and insulin sensitizers (*e.g.* thiazolidinediones or TZDs such as rosiglitazone and pioglitazone). While all have shown utility in lowering HbA1c levels in diabetic patients, current OADs come with a variety of safety and/or tolerability issues. The biguanindes such as metformin, currently the most widely prescribed therapy for diabetes, have issues related to gastrointestinal (GI) tolerability and lactic acidosis.[7] Sulfonylurea treatment is often accompanied by higher incidences of hypoglycemia and weight gain,[8] while glucose absorption inhibitors exhibit modest efficacy and GI disturbance.[9] Finally, TZDs have been associated with edema, worsening of congestive heart failure, negative effects on bone fracture rate, and, in recent studies, mixed results regarding cardiovascular (CV) safety profiles.[10]

With this background in mind, we sought to identify new targets which would not only provide an efficacious alternative mechanism for lowering blood glucose and HbA1c levels, but would also present an opportunity for achieving a superior safety and tolerability profile when compared to current standards of care. Ideally, such a drug would be suitable for combination with existing agents, as poly-pharmacology with multiple OADs is emerging as the standard treatment paradigm for type-2 diabetes therapy.

Glucagon like peptide-1 (GLP-1) is a 30-amino acid peptide incretin hormone derived from processing of pro-glucagon and is secreted by the L-cells of the intestinal mucosa in response to glucose stimulation. Since the early 1990's, GLP-1 had been known to be a potent insulin secretagogue and glucagon suppressor, with robust anti-diabetic and pro-satiety effects in diabetic humans,[11,12] but efforts to advance GLP-1 itself as a pharmaceutical agent were hampered by its extremely short pharmacokinetic half-life *in vivo* (plasma $t_{1/2} \approx 2$ min). As a result, considerable effort in the drug discovery community was expended toward the identification of small-molecule GLP-1 receptor agonists that would capture the beneficial effects of GLP-1 while exhibiting oral bioavailability and a superior pharmacokinetic duration of action. Unfortunately, efforts to identify such small-molecule agonists have to date been unsuccessful, due in part to a dearth of viable *bona fide* screening hits.[13] In light of this shortcoming, a number of pharmaceutical and biotech companies have advanced subcutaneously administered, peptide GLP-1 receptor agonists with superior duration of action *in vivo*. Among the most advanced agents are exenatide (Byetta™)[14] and liraglutide (Victoza™),[15] both of which have been approved by regulatory agencies for the treatment of type-2 diabetes. While these drugs are effective in lowering HbA1c and demonstrate a net beneficial effect on weight gain and other CV risk factors, they require parenteral administration (once or twice daily dosing), and patient uptake of these agents has been limited despite their robust efficacy and promising safety profile.

1.3 Dipeptidyl Peptidase-4 as a Target for Diabetes Treatment

While the advancement of orally active, small-molecule GLP-1 receptor agonists remains elusive, another opportunity to modulate GLP-1 receptor activity *in vivo* focused on preventing the degradation of endogenous GLP-1 with small-molecule inhibitors of the primary peptidase responsible for the *in vivo* degradation of GLP-1, dipeptidyl peptidase-4 (DPP4), a non-classical serine protease.[16] Our initial interest in DPP4 inhibitors was piqued by a report from Holst and Deacon, wherein the authors outlined a compelling argument for the utility of DPP4 inhibition in the treatment of diabetes, primarily *via* the potentiation of endogenous GLP-1.[17] DPP4 belongs to a family of aminodipeptidases and is both a cell surface and circulating enzyme. Historically, it had also been identified as the lymphocyte cell surface marker CD26, and as such DPP4/CD26 exhibits pharmacology related to cell membrane-associated

activation of intracellular signal transduction pathways and cell–cell interaction in addition to its peptidase enzymatic activity.[18] It is widely believed that the signaling function of DPP4/CD26 is distinct from its enzymatic function.

The concept of generating targeted protease/peptidase inhibitors as therapeutic agents is well documented in the literature.[19] In the majority of cases, selective enzyme inhibitors have been used to prevent the conversion of an endogenous "non-functional" peptide/protein precursor (*e.g.* angiotensin I) to a physiologically active peptide/protein (*e.g.* angiotensin II), thereby attenuating formation of the protagonist bio-active enzyme product to effect amelioration of the disease state. Use of such approaches has led to marketed drugs for numerous indications, including ACE and renin inhibitors for hypertension,[20] HIV protease inhibitors for AIDS,[21] and thrombin inhibitors for DVT.[22] Less prevalent are approaches targeting proteases/peptidases that degrade endogenous substrates which are known to exert a beneficial effect. For example, neutral endopeptidase (NEP) proteolytically degrades the endogenous vasodilator atrial natriuretic peptide (ANP) to inactive fragments. By retarding this degradation, NEP inhibitors have found use in the treatment of hypertension.[23] In common with NEP, where inhibition of protease mediated protein degradation was the pharmacological objective, BMS and several other research groups engaged in the search for DPP4 inhibitors to maximize the beneficial effects of endogenously released GLP-1.

1.4 Early Inhibitors of DPP4

To jump-start the BMS DPP4 chemistry program, the group was able to capitalize on groundwork laid in the mid-late 1990's when several potent inhibitors of DPP4/CD26 were reported that could be classified as either "irreversible" or "reversible", depending on the mechanism of inhibition (Figures 1.2 and 1.3). Hydroxamates such as **4** were proposed to be both substrates and inhibitors of DPP4, presumably *via* direct covalent modification of the enzyme through the active-site serine residue (Ser630).[24] Phosphate-based inhibitors such as **5** were also reported to undergo covalent addition to DPP4 but exhibited weak potency.[25] The interesting boronate-based inhibitors (*e.g.* **6**), originally advanced by the Tufts University and Boehringer Ingelheim groups, exhibited exceptionally high inhibitory activity *in vitro*, presenting

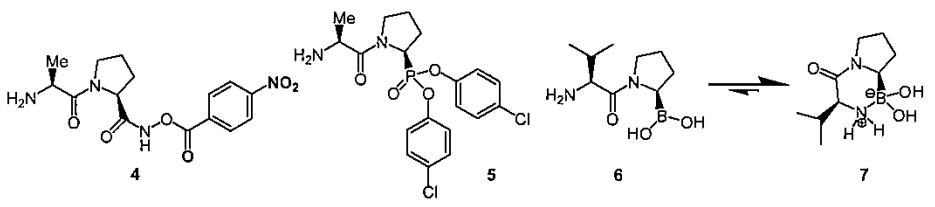

Figure 1.2 Early examples of "irreversible" DPP4 inhibitors.

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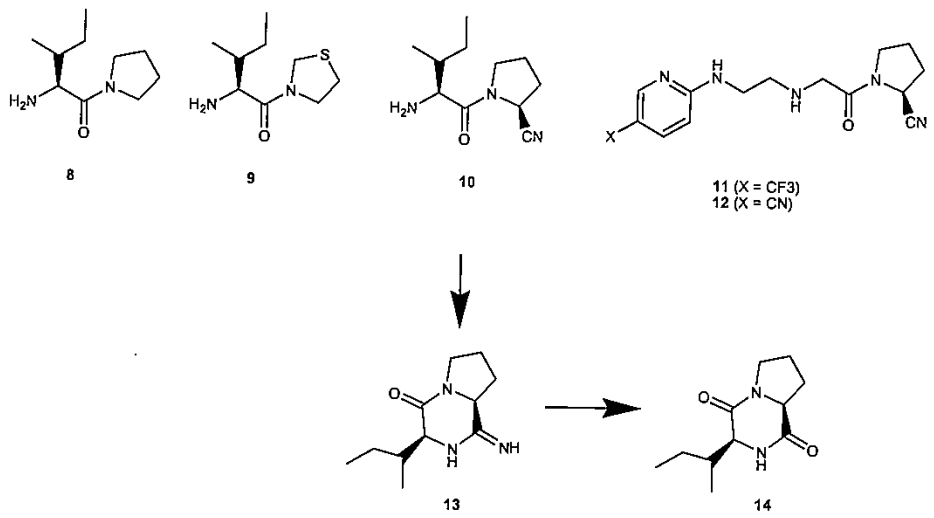

Figure 1.3 Early examples of "reversible" DPP4 inhibitors

themselves as "transition-state" inhibitors which presumably form tetrahedral boronate esters involving the Ser630 hydroxyl group.[26–28] As such, many of these compounds exhibited slow tight-binding kinetics with K_{off} rates of several days (*versus* seconds/minutes with non-covalently bound inhibitors). However, these compounds also suffered from poor solution stability arising from intramolecular cyclization of the terminal primary amine with the boronate, affording an inactive product (*e.g.* **7**). The propensity of compounds of this chemotype to undergo internal cyclization limited their viability as drug candidates.

Due to the uncertain risks associated with advancing irreversible inhibitors as drug candidates, the team viewed the reversible inhibitors exploited by the Mount Sinai, Probiodrug, Ferring Research, and Novartis scientific teams as more attractive starting points for a lead finding effort (Figure 1.3). Probiodrug had described simple Ile-pyrrolidides (**8**) and Ile-thiazolidides (**9**, later advanced to the clinic by Merck and Probiodrug as P32/98) which exhibited *in vitro* potency in the nanomolar range, were chemically stable, and, in the case of **9**, demonstrated glucose area under the curve (AUC) reductions in Zucker$^{fa/fa}$ rats in an oral glucose tolerance test (oGTT).[29–31] Reports from Li *et al.* at Mount Sinai highlighted early examples of nitrilo-pyrrolidines specifically designed as inhibitors of DPP4.[32] Equally intriguing was the work described by Ferring in which nitrilo-pyrrolidines such as **10** were identified as exceptionally potent inhibitors of the enzyme.[33,34] Initially targeted as agents for immunomodulation (via CD26 inhibition), these compounds represented "drug-like" scaffolds and exhibited exceptional inhibitory potency. Additionally, a contemporaneous patent application from Novartis[35] described the structure of compound **11** (a related analogue **12** would later be disclosed as Novartis' first clinical compound, DPP-728)[36] and its ability to increase plasma insulin in

fasted, high-fat fed rats in an oGTT. The inhibitors represented by compounds **8–12** provided useful insights for the design of DPP4 inhibitors at BMS.

At the initiation of the program, there were still many unknown factors related to the pharmacology and safety of DPP4 inhibition. It was clear from earlier work that Fischer 344 rats possessing a naturally occurring loss-of-function mutation of the DPP4 gene were healthy, viable, and free of serious immunological complications.[37] It was later shown that these rats also exhibited a favorable metabolic phenotype on a high-fat diet and demonstrated improved glucose tolerance and GLP-1 secretion.[38] Thus, complete ablation of DPP4 did not appear to represent a serious safety concern in rats, but rather the Fischer rat provided support for the concept that inhibition of DPP4 could be both safe and efficacious. Others questions still remained. Was high selectivity for DPP4 *versus* other related peptidases (*e.g.* DPP8, DPP9, FAP, *etc.*) an absolute requirement for this target? Would inhibition of DPP4 potentiate other endogenous peptides, leading to unintended deleterious (or beneficial) consequences? Would potentiating endogenous GLP-1 (*versus* exogenous administration) be sufficient to affect a robust anti-diabetic response in humans? Finally, what potential mechanism-based toxicological effects, if any, would be seen upon chronic administration of a DPP4 inhibitor?

In light of limited literature in the field, and with no reports of a compound having advanced to clinical trials, these questions would ultimately need to be addressed during the execution of our discovery and subsequent clinical development programs. Despite the unknowns, the positive aspects of this target were numerous. Potent small-molecule inhibitors with systemic exposure upon oral dosing were known. Although limited, DPP4 inhibitors had demonstrated pharmacodynamic efficacy in genetic animal models of type 2 diabetes in preliminary pharmacological studies. Preclinical proof-of-concept for the anti-diabetic actions of GLP-1 was already established and suggested a low potential for hypoglycemia. *In vitro* assays and several *in vivo* models were already described in the literature, enabling rapid program initiation. It was against this backdrop that significant medicinal chemistry and biology resources at BMS were deployed on this newly emerging target in the early months of 1999.

1.5 Design of BMS's DPP4 Medicinal Chemistry Program

Given the attractiveness of DPP4 as a therapeutic target, it was anticipated that this field would soon become highly competitive, and we therefore sought to accelerate the program. High-throughput screening (HTS), routinely a key component of drug discovery programs, was deemed as too time consuming to rapidly afford a chemical starting point. Thus, we decided to initially adopt a design optimization approach, improving upon the leads reported by the Probiodrug, Ferring, and Novartis research groups; HTS would later be used to provide leads for a second-generation effort. From a potency perspective, the

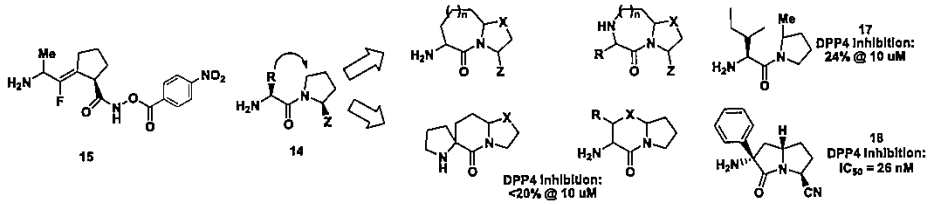

Figure 1.4 Rationale for generation of conformationally restricted DPP4 inhibitors.

nitrilo-pyrrolidines were deemed to be highly attractive, but were reported to exhibit modest pharmacokinetic duration of action and suffered from chemical instability.[33,34] In solution, the proximal amino group attacks the nitrile functionality (see Figure 1.3), eventually leading to the intermediate cyclic imidate **13** and ultimately the diketopiperzine **14**, both of which are inactive *versus* DPP4. Addressing this issue was viewed as a critical component of the medicinal chemistry effort due to considerations regarding both the half-life of the compound *in vivo*, and for high purity processing of the active pharmaceutical ingredient (API) on large scale in a drug manufacturing setting.

From earlier work by Lin *et al.*,[39] it was demonstrated that replacement of the prolyl amide bond with a fluoroalkene isostere resulted in the generation of potent DPP4 inhibitor **15** (Figure 1.4). This finding was significant in that it suggested that the critical prolyl and amino pharmacophores in **16** may be conformationally locked in an extended arrangement which is favorable for enzyme inhibition. In addition, because incorporation of the alkene prohibits intramolecular attack of the amine onto the acyl hydroxamate, the finding suggested novel paths for inhibitor design to retard intramolecular cyclization. Taking a cue from earlier work performed at BMS in the design of dual ACE/NEP inhibitors,[40] we applied the concept of conformationally restricted dipetide mimetics in our search for novel inhibitor chemotypes. Many of these cores seemed to possess the critical elements required for DPP4 inhibition, including a prolyl amide group and a charged amino functionality at the P2 position. It was hoped that locking the inhibitor conformation by this approach would not only enhance binding affinity, but also prevent inactivating cyclization in compounds possessing an electrophilic pharmacophore (*e.g.* nitrile, phosphate, *etc.*) on the proline ring. Unguided by the availability of a DPP4 X-ray crystal structure at that time, our design efforts led to a variety of different bi- and monocyclic dipetide mimetics, generically represented in Figure 1.4. Unfortunately, all of the compounds generated in this series were inactive against DPP4, which we attributed to either incorrect conformational geometry required for inhibition, or to steric intolerance for substitution on the proline ring. The latter hypothesis was supported by the poor activity exhibited by the simple methyl-substituted prolyl derivative **17** as compared to its unsubstituted counterpart **8**. Interestingly, a recent report from Phenomix disclosing 5,5-fused bicyclic lactams such as **18** as potent DPP4 inhibitors provides validation for this initial approach.[41]

1.6 Design of Cyclopropyl-fused Nitrilo-pyrrolidines

In concert with the effort described above, the discovery team examined alternative approaches to the generation of novel inhibitors that might minimize or obviate the undesired cyclization reaction. Cognizant of the impact of pyrrolidine substitution on DPP4 activity (*e.g.* **17**), we proposed that cyclopropyl fusion to the prolyl ring, represented by **19**,[42] might represent a new and viable approach (Figure 1.5). Incorporation of a fused cyclopropane ring constituted a minimal steric burden at P1, would impact the planarity of the prolyl ring, and importantly have the potential to conformationally retard intramolecular attack of the amine to the nitrile. Hence, a series of cyclopropane-fused nitrilo-pyrrolidines **20–23** were synthesized and assessed for both DPP4 inhibition and solution stability (pH 7.2 phosphate buffer, 39.5 °C).[43] As compared to the unsubstituted prototype **10**, the *trans*-4,5- and *trans*-2,3-isomers **22** and **23**, respectively, were not well tolerated by the enzyme, negating further evaluation of these as viable program leads. In contrast, both the *cis*-4,5- (**20**) and the *cis*-3,4- (**21**) analogues exhibited acceptable, though slightly diminished, activity as compared to **10**. More interestingly, **20** exhibited a significant enhancement in solution stability as compared to either **10** or **21**, confirming that *cis*-4,5-cyclopropyl fusion did indeed retard the intramolecular cyclization process. Expanding upon this finding, compounds which incorporated a corresponding *tert*-leucine at P2 as in **24–26** minimized potency differences between the respective cores, though solution stability was still

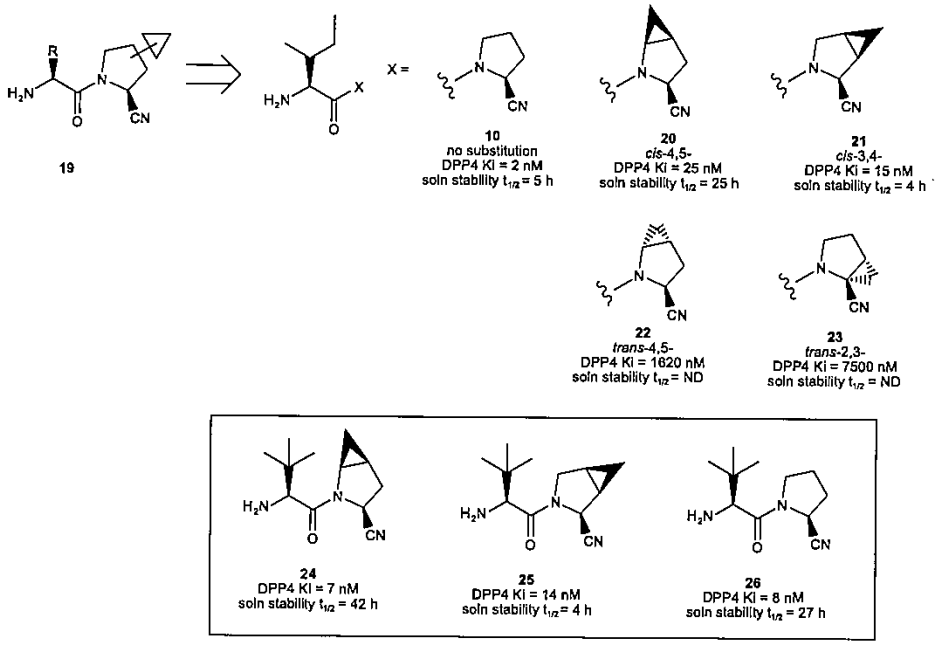

Figure 1.5 Genesis of cyclopropyl-fused nitrilo-pyrrolidine chemotype.

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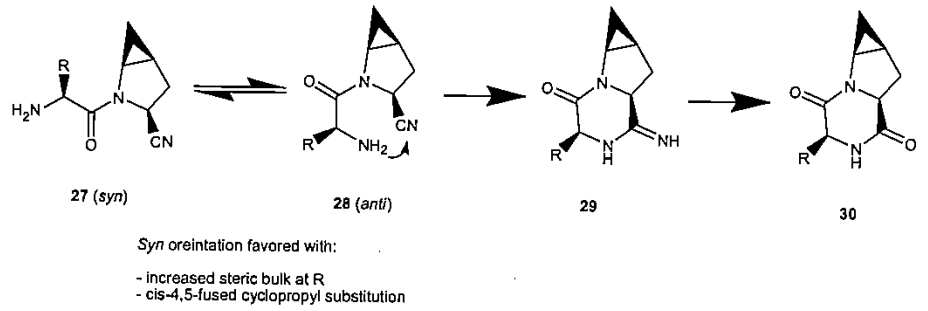

Figure 1.6 Factors determining *syn versus anti* orientation of the methano-nitrilo-pyrrolidine chemotype.

favorably preserved in the *cis*-4,5-methano core (*e.g.* **24**). These and other data suggested that increased steric and lipophilic bulk at the P2 position of the inhibitor (*e.g.* ethyl *vs.* isobutyl *vs. tert*-butyl, *etc.*) enhanced both *in vitro* potency as well as solution stability. A similar trend was also observed by the Ferring Research group in the simple nitrilo-pyrrolidine series.[34]

Clearly both the steric bulk of the P2 side-chain and the cyclopropyl fusion on the pyrrolidine ring cooperatively enhanced comformational stability. Computational analyses later demonstrated that, primarily because of van der Waals interactions, increasing the size of the side-chain R (Figure 1.6) disfavored the *anti* orientation **28** required for irreversible intramolecular cyclization to the inactive products **29** and **30**. For example, where R is Me *versus tert*-butyl, the $\Delta\Delta H$ between the *anti* (**28**) and *syn* (**27**) orientations were calculated to be 0.8 kcal mol^{-1}. Furthermore, addition of the *cis*-4,5-cyclopropyl moiety also disfavors adoption of the *anti* orientation by an additional 0.6 kcal mol^{-1} as compared to the unsubstituted nitrilo-pyrrolidine ring. Thus, by combining these conformational elements, we were able to identify novel inhibitors of DPP4 with high *in vitro* potency and enhanced solution stability.

Compound **24** represented a major breakthrough in the chemistry program. Profiling of this lead demonstrated a low potential for off-target liabilities [hERG inhibition, cytochrome P450 (CYP450) inhibition, broad receptor screening, *etc.*], as well as favorable pharmacokinetic properties in the rat ($F = 77\%$, $t_{1/2} = 2.8$ h). Additionally, compound **24** was efficacious in Zucker$^{fa/fa}$ rats, reducing glucose AUC in an acute oGTT ($ED_{50} = 3.3$ mg kg^{-1}, glucose challenge 30 min post-dose) when compared to vehicle control. While the duration of action of **24** was not particularly long ($ED_{50} = 92$ mg kg^{-1}, glucose challenge 5 h post-dose), the *in vivo* potency was still significantly greater than that of the literature thiazolidine lead **9** ($ED_{50} = 38$ mg kg^{-1}, glucose challenge 30 min post-dose). On the basis of these findings, additional staff were assigned to the program to permit a detailed and robust exploration of the structure–activity relationship (SAR), primarily focusing on further modifications at P2.

1.7 SAR Optimization Leading to the Discovery of Saxagliptin

Building upon the *cis*-4,5-methano-2-nitrilopyrrolidine core, a wide variety of substituents, represented generically in **31**, were explored (Figure 1.7). As highlighted earlier, greater potency and stability were realized via introduction of highly β-branched P2 side-chains. Due to the very limited commercial availability of β-quaternary α-amino acids, the team devised an efficient strategy to generate novel P2 units by several complementary paths, each deriving from cyclic and/or symmetrical ketones to avoid unnecessary introduction of additional stereocenters.[6,43] In one arm of this approach, ketones underwent Knoevenagel condensation with a malonate diester, and the resulting Michael acceptor was subjected to conjugate addition to introduce the alkyl-substituted quaternary center. Mono-hydrolysis and subsequent Curtius rearrangement yielded the desired β-branched amino acids. A parallel approach began with Horner–Emmons condensation of the ketone to give an α,β-unsaturated ester. Reduction of the ester to the primary allylic alcohol and esterification with Boc-Gly set up a zinc-mediated ester enolate Claisen rearrangement, which provided the desired amino acids with a vinyl functional handle at the β-position. Alternatively, a standard Strecker synthesis could be used on available aldehydes to ultimately afford the desired amino acid building blocks. In all cases, the racemic amino acids were then coupled to the

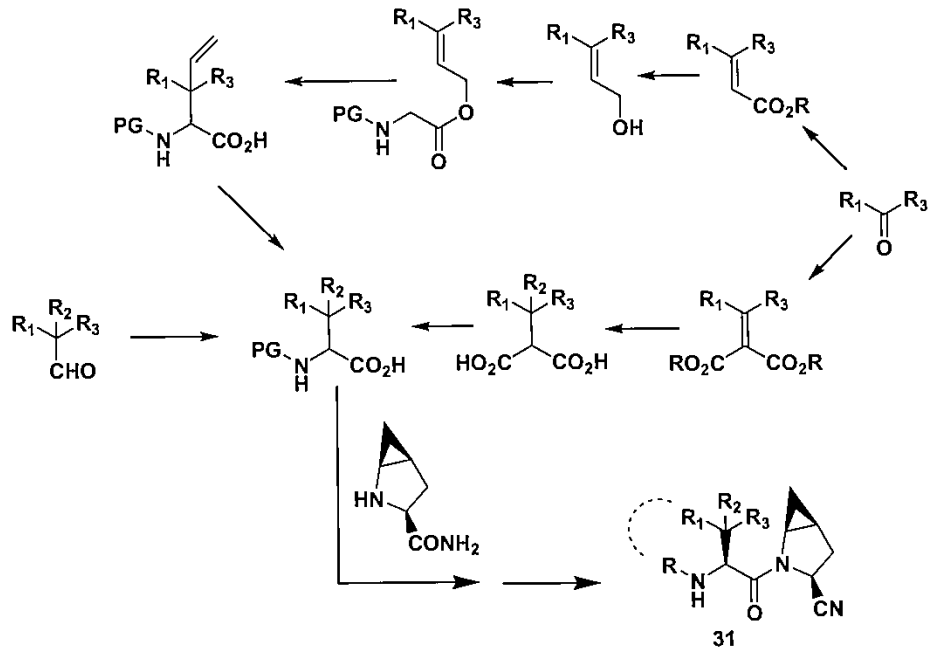

Figure 1.7 Various synthetic routes for the generation of highly β-branched amino acids.

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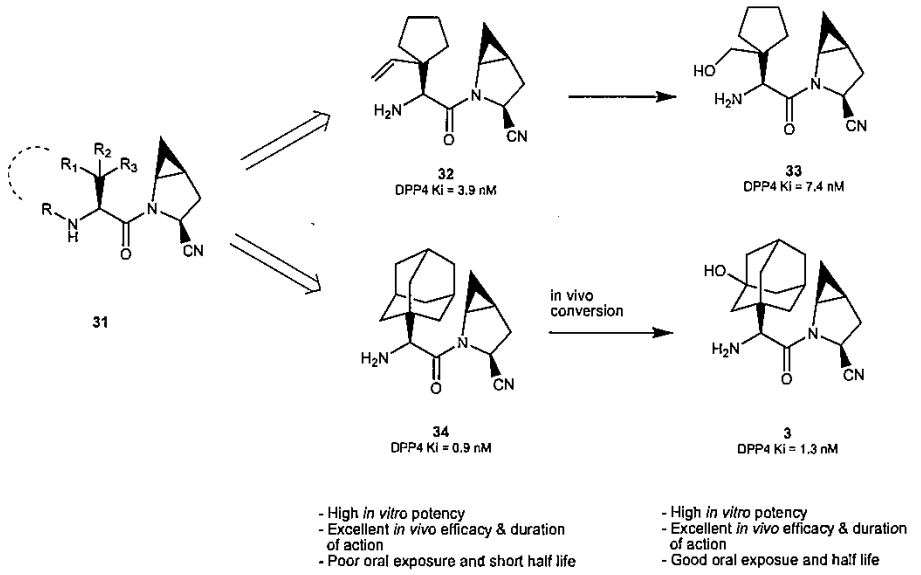


Figure 1.8 Classical PK/PD disconnect in rats leads to hypothesis of active metabolite generation *in vivo*, resulting in the discovery of saxagliptin.

enantiomerically pure P1 nitrilo-methanopyrrolidine fragment and the resulting diastereomers were chromatographically resolved.

From this effort emerged two compounds of particular interest, inhibitors **32** and **34** (Figure 1.8). In the case of **32**, this compound demonstrated superior *in vivo* efficacy and a sustained duration of response for inhibition of plasma DPP4 in normal fasted SD rats at a dose of 4 μmol kg^{-1} (71% @ 30 min post-dose and 64% @ 4 h post-dose). Duration of the pharmacodynamic (PD) response was particularly long when compared to compounds generated earlier in the program. Intriguingly, this extended PD response was most often observed in analogues containing a vinyl substituent (*e.g.* **32**) at P2. Despite its robust *in vivo* activity, compound **32** demonstrated low oral bioavailability (5%) and a short half-life ($t_{1/2} = 1.2$ h) in rats. In contrast, the related saturated analogue of **32** (vinyl replaced with ethyl) exhibited significantly higher bioavailability (31%) and greater *in vitro* stability in rat and human liver microsomes, but a weaker response in the rat plasma DPP4 inhibition assay (despite equivalent *in vitro* potency). The disconnect between the pharmacokinetic and pharmacodynamic profile of **32** immediately suggested *in vivo* conversion to an active metabolite possessing superior target potency and/or reduced clearance profile. Based on this hypothesis, a variety of putative or surrogate hydroxylated metabolites related to **32** were prepared. While unequivocal characterization of **33** as an active metabolite of **32** was never established, the activity of **33** mirrored that of **32** in the aforementioned PD model. While the rat half-life of **33** ($t_{1/2} = 1.3$ h) was not significantly longer when compared to that of **32**, the absolute bioavailability increased 10-fold to 53%. A more

striking finding was observed with compound **34**. In concordance with established SAR, incorporation of the bulkier adamantyl hydrophobic group in the P2 side-chain enhanced *in vitro* potency ($K_i = 0.9$ nM) as compared to compounds with smaller fragments in this position. In the normal rat, this compound (4 μmol kg^{-1}, po) afforded robust plasma DPP4 inhibition (84% @ 0.5 h post-dose and 83% @ 4 h post-dose) despite exceptionally low bioavilability (2%) and modest half-life ($t_{1/2} = 1.4$ h). Capitalizing on the learnings from compound **33**, and considering the propensity of adamantyl groups to undergo CYP-mediated hydroxylation, the corresponding metabolite **3** (saxaglipitin) was synthesized. The compound proved to be highly potent *in vitro*, exhibited good pharmacokinetic properties in the rat ($F = 75\%$, $t_{1/2} = 2.1$ h) and effected near maximal (~87%) plasma DPP4 inhibition in the rat at both 0.5 and 4 h post-dose when administered at 4 μmol kg^{-1}.

In addition to saxagliptin, a number of other inhibitors incorporating P2 side-chains with various substituted adamantanes were prepared and evaluated, including other positional isomers of hydroxyadamantane, dihydroxyadamantane, and fluoroadamantane. While these other adamantane-derived compounds also exhibited potent DPP4 inhibition *in vitro*, saxagliptin provided a comparatively superior PK and PD profile, and was ultimately chosen for development.

1.8 Binding of Saxagliptin to Human DPP4: A Slow Tight-binding Inhibitor

Through more extensive characterization of the binding kinetics of saxagliptin (and select analogs) to DPP4, it was noted that several compounds showed evidence of slow tight-binding. Further analysis of SAR patterns relating to this slow on/off-rate feature revealed correlations with both steric bulk in the P2 side-chain (specifically β-quaternary substitution) and the presence of a nitrile functionality. Prior to our undertaking of detailed kinetic studies, it had been speculated by several groups that a transient covalent bond was formed between the hydroxyl group of the active-site Ser630 of the DPP4 catalytic triad and the nitrile carbon of nitrile-based inhibitors, yet early X-ray co-crystal structures with such compounds lacked adequate resolution to confirm appropriate electron density where such a bond would exist.[44] Our findings were consistent with a hypothesis whereby the bulky P2 side-chain's displacement of water in the S2 pocket drove entropic aspects governing on-rate (for **3**, $K_{on} = 4.6 \times 10^5$ M^{-1} s^{-1}), and, once anchoring the inhibitor in the active site, covalent interaction with the nitrile drove enthalpic aspects governing off-rate (for **3**, $K_{off} = 23 \times 10^{-5}$ s^{-1}), resulting in overall K_i enhancement for certain inhibitors possessing these features.[45] As a result, the $t_{1/2}$ for dissociation of saxagliptin from DPP4 was determined to be ~50 min at 37 °C (~250 min at room temperature). In comparison to the rapid off rates of non-nitrilo-pyrrolidines such as sitagliptin, the slow off-rates exhibited by saxagliptin and, to a lesser extent, other nitrilo-pyrrolidines such as **12**,[45b,46] proved to be a unique

attribute of saxagliptin among the clinically advanced inhibitors. The slow off-rate kinetics exhibited by saxagliptin likely serves to enhance saxagliptin's pharmacodynamic potency and duration *in vivo*, and may also contribute to its enzymatic selectivity.[47,48]

Further understanding of this finding was obtained through: (1) a high-resolution X-ray co-crystal structure of saxagliptin bound to DPP4, definitively revealing the presence of a covalent O–C bond; (2) a series of biophysical studies performed using wild-type and mutant DPP4 proteins S630A and H740Q characterizing interactions with both saxagliptin and its de-nitrilo analogue; and (3) comparative ^{1}H NMR studies of DPP4 in apo form and in complex with saxagliptin,[45,49] revealing the characteristic downfield shift indicative of a short, strong H-bond upon complex formation.[50,51] The X-ray co-crystal structure of saxagliptin bound to DPP4 (Figure 1.9) reveals several key features: (1) the ionic interaction of the primary amine with the Glu205 and Glu206 residues; (2) hydrogen bonding of the adamantyl hydroxyl with Tyr547; (3) efficient hydrophobic space-filling by the adamantyl and methano-pyrrolidine groups; and (4) the aforementioned covalent bonding of the catalytic Ser630 hydroxyl with the pendant nitrilo functional group. Despite the clear covalent nature of the bond, this interaction was shown to be fully reversible (as demonstrated by complete recovery of enzyme activity upon dialysis) and enzymatically unproductive (since no saxagliptin hydrolysis products were

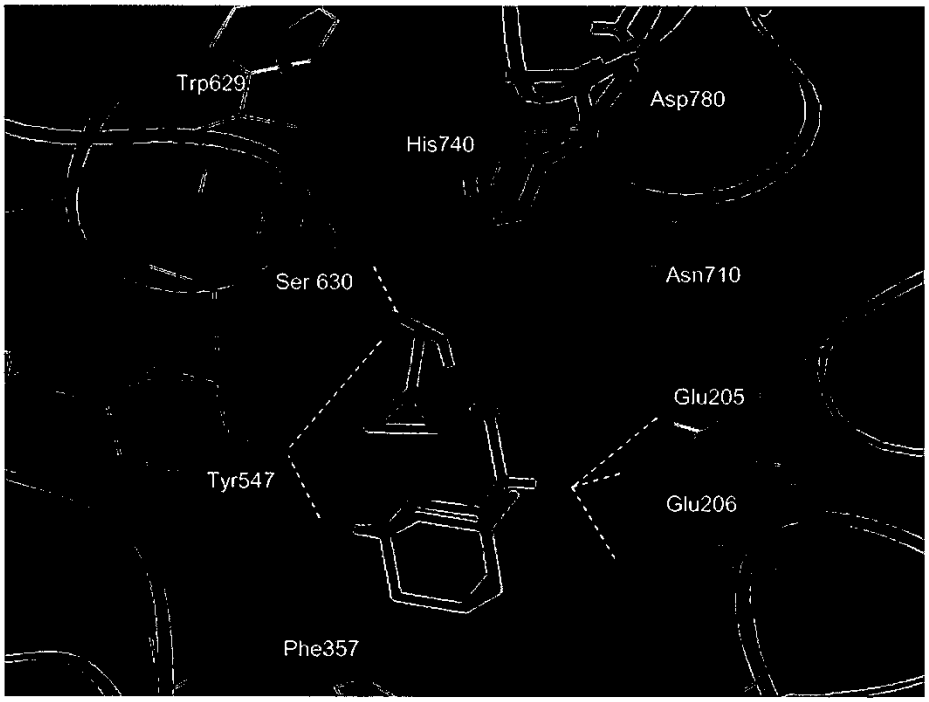


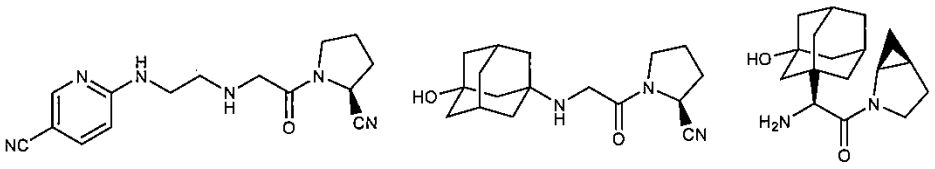
Figure 1.9 X-Ray co-crystal structure of saxagliptin bound to human DPP4.

SAXA-DEF-00338

formed). It is speculated that the methano ring of saxagliptin displaces any active-site water molecules required for hydrolysis, preventing the inhibitor from being a substrate for the enzyme. A unique constellation of features exhibited by both saxagliptin and its biologically active, circulating metabolite (5-hydroxy saxagliptin), including: (1) slow tight-binding kinetics; (2) low nM *in vitro* potency; (3) good pharmacokinetics; and (4) high tissue distribution at the primary site of action (GI tract),[52] all contribute to saxagliptin's low clinically efficacious human dose.

1.9 Chemical Stability of Saxagliptin and Analogs

As highlighted earlier, a property shared by many of the nitrilo-pyrrolidine based inhibitors bearing an amine at the P2 N-terminus is the propensity for intramolecular cyclization at or above neutral pH (see Figures 1.3 & 1.6). Such instability could complicate manufacturing and formulation, and potentially shorten pharmacokinetic half-life. As a result, the stability of the BMS nitrilo-pyrrolidine analogs were routinely assessed in pH 7.4 buffer. While introduction of bulky alkyl substitution on the terminal amine of Novartis' DPP-728 (**12**)[36] and LAF-237 (**35**, vildagliptin)[53] greatly retarded intramolecular cyclization, incorporating a secondary amine structural element into the BMS methano-pyrrolidine series routinely attenuated potency (Figure 1.10). While the solution stability of saxagliptin ($t_{1/2} \approx 4$ days @ pH 7.4/37 °C) bode well for manufacturing and drug pharmacokinetics, lingering formulation concerns spurred later discovery efforts to obviate any unforeseen chemical stability issues associated with the nitrile functionality. Interestingly, wholesale removal of the nitrile moiety from saxagliptin afforded BMS-538305 (**36**), a highly potent ($K_i = 10$ nM) inhibitor of the enzyme with a favorable *in vitro* and *in vivo* development profile.[54] SAR in this non-nitrile series was narrow, with a precipitous drop-off of activity accompanying even modest structural changes. Because BMS-538305 was completely stable in solution, it was nominated as a back-up development candidate and went on to exhibit robust pharmacodynamic efficacy in humans, although it was later suspended from further development, in part due to a clear lack of superiority *versus* saxagliptin.



12 35 36

Figure 1.10 Structure of nitrilo-pyrrolidine-based DPP4 inhibitors with enhanced solution stability.

SAXA-DEF-00339

The potential for formulation-related challenges with saxagliptin also spurred the application of a unique formulation strategy which may ultimately translate into a significant advantage for the development of future single-tablet saxagliptin drug combinations. It was discovered that in the process of compressing the saxagliptin API salt together with excipients to develop a standard tablet formulation, unacceptably high levels (2%) of the undesired cyclization products were produced. A pharmaceutics solution to the problem, made possible by the low dose (2.5–10 mg) of saxagliptin required to effect maximal HbA1c lowering in humans, was realized by spray coating the drug substance (sandwiched between two polymeric vapor barrier layers) onto an inert tablet core. This formulation is currently used in the marketed brand of saxagliptin, Onglyza™. It is anticipated that the spray coating technique, a procedurally simple technology, will greatly facilitate the development of future fixed-dose combinations of saxagliptin with other OADs.[55] As proof, saxagliptin has been readily formulated with extended-release metformin, leading to the most clinically advanced once-daily DPP4/metformin single-tablet combination. This innovative combination was submitted for FDA regulatory review in December 2009.

1.10 *In vivo* Efficacy of Saxagliptin

As SAR within the series developed, a simple *ex vivo* plasma DPP4 inhibition assay was used to efficiently differentiate among the growing number of potent compounds. To further understand particularly promising compounds, a clinically relevant pharmacodynamic assay (an oGTT in Zucker$^{fa/fa}$ rats or ob/ob mice) was also utilized. These models served as an efficient means to phenotypically stratify compounds without the need for prior, time-consuming PK assessments, thereby reducing cycle times. In these models, saxagliptin afforded quite robust effects on glucose excursion and insulin release at doses as low as 0.3–1 $\mu mol\,kg^{-1}$.[6] However, preclinical demonstration of reduced fasting plasma glucose and/or HbA1c in subacute or chronic disease models proved to be elusive. In-house experience gained in earlier discovery programs targeting PPARα agonists led the team to evaluate the chronic dosing of saxagliptin in a variety of genetic models of type-2 diabetes, such as in ZDF and Zucker$^{fa/fa}$ rats. Despite numerous attempts to optimize these models, the group was unable to demonstrate statistically significant, dose-dependent efficacy (fasting plasma glucose lowering), irrespective of the model or dosing duration. Despite this lack of validation in pre-clinical disease models, timely disclosures of clinical data by Novartis helped to bolster internal confidence in the mechanism. Novartis scientists had recently reported promising reductions in HbA1c for DPP-728 (**12**) in a 12-week Phase IIa trial in diabetic subjects.[56] Based on our belief that saxagliptin exhibited superior potency and pharmacokinetic profiles compared to DPP-728, the decision was made to forego further efforts to develop chronic *in vivo* efficacy models and to move forward into clinical development. Despite more than 10 years of pre-clinical testing of DPP4

inhibitors *in vivo*, there are scant reports in the literature regarding the efficacy of chronic DPP4 inhibition in rodent models. These observations highlight the limitations of these pre-clinical disease-pertinent models in fully or accurately mirroring the pathophysiology of human disease.

1.11 Peptidase Selectivity of Saxagliptin

At program initiation, the precise requirements for DPP4 selectivity *versus* inhibition of related proteases were not entirely clear. SAR progression and compound optimization outpaced the full implementation of our protease selectivity panel, and it was not until identification of saxagliptin as a development candidate that all elements of the selectivity panel were in place. Saxagliptin (similar to other analogs within the series) was found to be highly selective (>4000-fold) for inhibition of DPP4 as compared to a variety of other proteases, including FAP, PEP, and DPP2. Against the most closely related enzymes DPP8 and DPP9, selectivity was attenuated but still high (400- and 75-fold, respectively).[45,57] Contributing to saxagliptin's enhanced selectivity is the observation that, unlike its slow tight-binding kinetics for the inhibition of DPP4, saxagliptin did not display slow tight-binding when inhibiting DPP8 or DPP9. Additionally, since DPP8 and DPP9 are intracellular enzymes, the poor cellular permeability of saxagliptin attenuates access to these off-target proteases. As a result, saxagliptin behaves as a highly selective inhibitor of DPP4 with little or no propensity for off-target protease inhibition.

In 2005, scientists at Merck published results of two-week rat (10, 30, and 100 mg kg^{-1}) and single-dose dog (10 mg kg^{-1}) toxicity studies with a cohort of compounds which included a selective DPP4 inhibitor closely related to sitagliptin, a selective inhibitor of DPP8 and DPP9 (L-*allo*-isoleucyl isoindolide), and a somewhat weak and non-selective inhibitor of all three enzymes (L-*allo*-isoleucyl thiazolidide), as well as several other controls.[58] Both the DPP8/9 selective compound and the weak non-selective compound (which share a common structural element in the L-*allo*-isoleucine moiety) caused alopecia, thrombocytopenia, anemia, reticulocytopenia, splenomegaly, and mortality in rats, and bloody diarrhea in dogs. None of these effects were observed in animals dosed with the DPP4 selective compound. Based on these empirical findings, the Merck team concluded that the observed toxicities were a direct result of inhibition of DPP8 and/or DPP9. In formal toxicology studies, neither saxagliptin nor vildagliptin produced the spectrum of pathology changes ascribed by Merck to DPP8/9 inhibition, even at doses predicted to give plasma levels much higher than the K_i's required for inhibition of DPP8/9. Thus, the findings suggested both saxagliptin and vildagliptin are highly selective DPP4 inhibitors *in vivo*, and/or the theory regarding the consequences of DPP8/9 inhibition proposed by the Merck group may be incorrect. An alternate hypothesis, that the observed toxicology profiles of the Merck tool compounds were structure (compound) related, rather than based on their DPP8/9 inhibition profiles, was not initially embraced as a possible cause of the observed

toxicities. The Merck findings undoubtedly influenced the inhibitor design efforts of nearly every DPP4 discovery program in the industry, resulting in a proliferation of varied chemotypes with "atypical" protease inhibitor structural features as compared to the more dipeptidic motifs shared by saxagliptin and vildagliptin.

Several groups attempting to better understand the consequences of DPP8/9 inhibition in animals have subsequently published their findings using more structurally diverse inhibitors of DPP8/9 as tool molecules. Rosenblum *et al.* recently showed that treatment of dogs with high doses of a selective, cell-permeable DPP4/8/9 inhibitor (producing extensive inhibition of DPP4/8/9 in GI tissues) did not afford toxicological responses similar to those observed by Merck with their tool compounds.[59] In a separate study, Chen *et al.* demonstrated that repeated exposure (iv, QD for 14 days) of IG244, a potent and cell-permeable DPP8/9 inhibitor, did not result in severe toxicity in rats, despite complete DPP8/9 inhibition achieved with this inhibitor.[60] Additionally, Burkey *et al.* demonstrated that chronic dosing of vildagliptin (achieving plasma levels well above its K_i for DPP8 and DPP9 inhibition) failed to recapitulate the toxicity profile observed with the Merck DPP8/9 inhibitor.[61] Collectively, these empirical studies offer little to mechanistically connect the inhibition of DPP8 or DPP9 to a phenotype, and as such, the functional consequences of DPP8 and DPP9 inhibition in animals and humans remains unknown.[62] Thus, while DPP4 selectivity has become cemented as an intrinsic component of most DPP4 inhibitor programs industry-wide, the need for exquisitely high selectivity against the DPP8 and DPP9 proteases remains an unproven concept in DPP4 inhibitor design.

1.12 Synthesis of Saxagliptin

The structural complexity of saxagliptin posed some synthesis development challenges, in that the molecule possesses four asymmetric centers, including a neopentyl carbon center, where only one of the stereochemical centers was available through a commercially available chiral building block. However, the team's willingness to employ challenging synthetic chemistry to solve medicinal chemistry problems was key to the discovery of highly potent, more stable, and patentable lead compounds. The discovery synthesis of saxagliptin, used to prepare ~100–200 g of material for pre-development work, was in need of substantial optimization for large-scale production of this rather complex molecule. In particular, the need to perform a zinc-mediated cyclopropanation at low temperature with high diastereoselectivity, and the development of a robust large-scale synthesis of the chiral adamantyl fragment, proved to be a daunting task. The process chemistry group at BMS tackled each portion of this molecule with creative solutions to provide an efficient and reproducible manufacturing route. Key synthetic transformations are depicted in Figure 1.11, and include: (1) a Reformatsky reaction of bromoadamantane with a silyl-protected dichloroketene acetal, followed by oxidation and hydrolysis to give keto

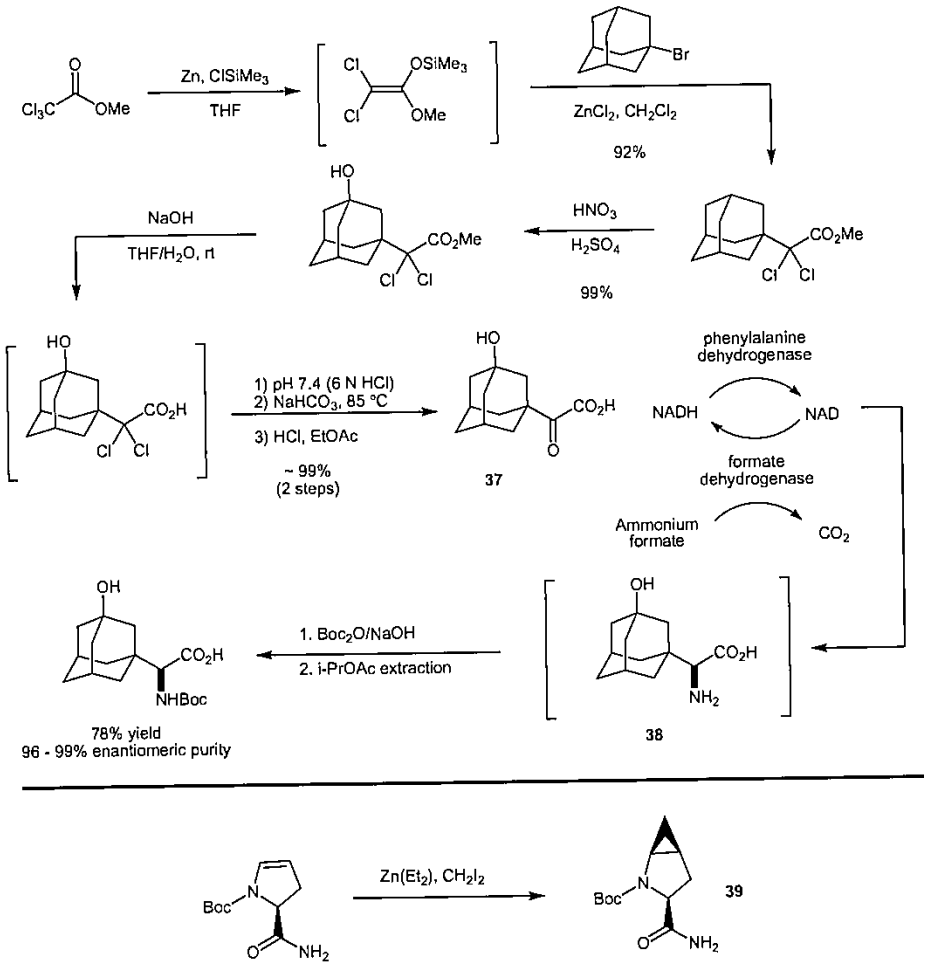


Figure 1.11 Development synthesis of the hydroxyadamantylglycine and methanoproline fragments of saxagliptin.

acid **37**;[63] (2) an enzymatic asymmetric reductive amination with ammonia to give the requisite unnatural amino acid **38**;[64] and (3) a continuous flow process for large-scale diethylzinc-mediated diastereoselective cyclopropanation of a protected dehydroprolinamide **39**.[65]

1.13 Saxagliptin Development

Saxagliptin formally entered development at BMS in April of 2001, and the first human clinical dose was administered to normal healthy volunteers in December of that same year. As highlighted earlier, genetic rodent models of diabetes proved unsuitable for accurately assessing the chronic efficacy of DPP4 inhibitors, and so the translational leap from acute plasma enzyme inhibition and

post-prandial antihyperglycemic effects to a registrational Phase IIb clinical trial would require more detailed dose-ranging efforts early on. Because the majority of the preclinical efficacy data generated for saxagliptin was in an acute setting, accurately projecting an efficacious human clinical dose was somewhat challenging. Saxagliptin was very well tolerated in Phase I/II single and multiple ascending dose studies ranging up to 100 mg and exhibited 24 h maximal inhibition of plasma DPP4 activity at steady state, beginning with the 5 mg dose. Additional higher dose panels (up to 600 mg) were added at a later date to examine the possibility of achieving greater efficacy. As the top marketed dose of saxagliptin is only 5 mg once-daily, the high doses tested in the Phase I clinical program provided the development team with a deeper understanding of and confidence in the compound's safety margin. Dose ranging proof-of-concept studies with saxagliptin followed a standard randomized, double-blind, placebo-controlled Phase IIb protocol in drug naïve type-2 diabetic patients measuring HbA1c reductions.[66] Five doses of saxagliptin were studied (2.5, 5, 10, 20, and 40 mg, q.d.) and compared with placebo in an active 12-week treatment period. All doses of saxagliptin significantly lowered HbA1c from baseline (mean baseline HbA1c 7.9%) compared with placebo, and placebo-subtracted adjusted mean changes from baseline ranged from -0.45 to -0.63%, with no apparent statistically significant dose-relationship in this study. These results supported saxagliptin's advancement to Phase III, where additional efficacy and safety data were generated in >3000 drug-treated patients to support the registrational package.[67] In January of 2007, BMS entered into a co-development and co-marketing agreement with Astra-Zeneca encompassing saxagliptin and the SGLT2 inhibitor dapagliflozin, as well as the back-ups for each. This partnership was established in order to share in the late-stage development costs/risks and to broaden the sales and marketing capacity required to effectively reach the target clinical population, generally served by primary care physicians. Ultimately, the BMS/Astra-Zeneca collaboration was successful in obtaining regulatory approval from both the FDA (July 2009) and the EMEA (October 2009) for the use of saxagliptin, either as monotherapy or in combination with metformin,[68,69] sulfonylureas,[70] or TZDs, in the treatment of type-2 diabetes. The retrospective CV safety package generated to support the filing indicated an acceptable CV safety risk in the patient population studied, though further prospective post-marketing studies will be conducted in other patient populations to more comprehensively assess the impact of chronic saxagliptin treatment on CV outcomes. Interestingly, trends towards reduced events in this analysis have prompted BMS and Astra-Zeneca to initiate larger, more extensive CV safety trials, with the hope of demonstrating a statistically significant and meaningful reduction in CV morbidity and mortality in diabetic patients.

1.14 Summary

In the late 1960's, the pioneering team of Miguel Ondetti and David Cushman gave birth to the concept of rational drug design with the invention of

captopril, a small-molecule, peptidomemtic inhibitor of angiotensin-converting enzyme (ACE). Using a novel approach based on knowledge of a target's relevance to a disease state, a mechanistic understanding of biological function of the target, and an ability to design small molecules to interact with critical elements of the target's active site (transition-state pharmacophores, hydrophobic, hydrogen binding, and ionic interactions), they demonstrated that rational drug design (*versus* an empirical approach) can lead to new medicines. As a result, modern drug discovery was born, and continues to evolve, from this approach. Nearly 40 years after the discovery of captopril, these same principles are still being applied in medicinal chemistry, as exemplified in part with the discovery and development of saxagliptin.

Our interest in DPP4 as a target coincided with the completion of several other metabolic disease discovery programs at BMS, thereby permitting a near immediate influx of staffing for the nascent program. Saxagliptin was discovered and advanced for development by utilizing an efficient and timely expenditure of resources (approximately 320 analogs, 26 months, 6–7 full-time chemists, and 20 total full-time scientist-years across chemistry, biology, ADME and pharmaceutics). Prior to submission of saxagliptin for regulatory approval, the development team also contended with several hurdles, including synthetic and formulation challenges and a continually evolving competitive and regulatory landscape. Now that compounds such as saxagliptin, sitagliptin (Januvia™, Merck),[71] and vildagliptin (Galvus™, Novartis, marketed ex-US) have entered the market, with others likely to follow, DPP4 inhibitors have robustly entered the pharmacopeia of diabetes treatments. The efficacy and safety profiles to date for agents acting by this mechanism appear promising, and future studies may elucidate differentiation of the clinical profiles of these agents, as well as establish their potential for long-term benefit in the treatment of diabetes.

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A computer graphics representation of the active site of a carbon–carbon bond breaking enzyme (human aldolase) highlighting the residues important for catalysis and the substrate fructose-1,6-bisphosphate in space filling mode. Courtesy of J.A. Littlechild and H.C. Watson (1991) *J. Mol. Biol.* 219, 573–576.

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–9–

Angiotensin-Converting Enzyme (ACE) Inhibitors and the Design of Cilazapril

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I. Introduction

Despite an enormous research effort, the fundamental cause of hypertension is still not known. What is known is that high blood pressure, whatever the underlying cause, leads to increased morbidity and mortality as a consequence of arterial disease. If untreated over long periods of time, hypertension increases the risk of myocardial infarction, cerebral haemorrhage and renal failure. Gradually the heart and vascular muscle becomes hypertrophic, or thickened, and atherosclerotic changes occur in the blood vessels. Reduction of blood pressure to normal levels has been shown to lessen the incidence of coronary heart disease, stroke and kidney failure, thereby providing justification for therapy, even if the hypertension itself is asymptomatic.

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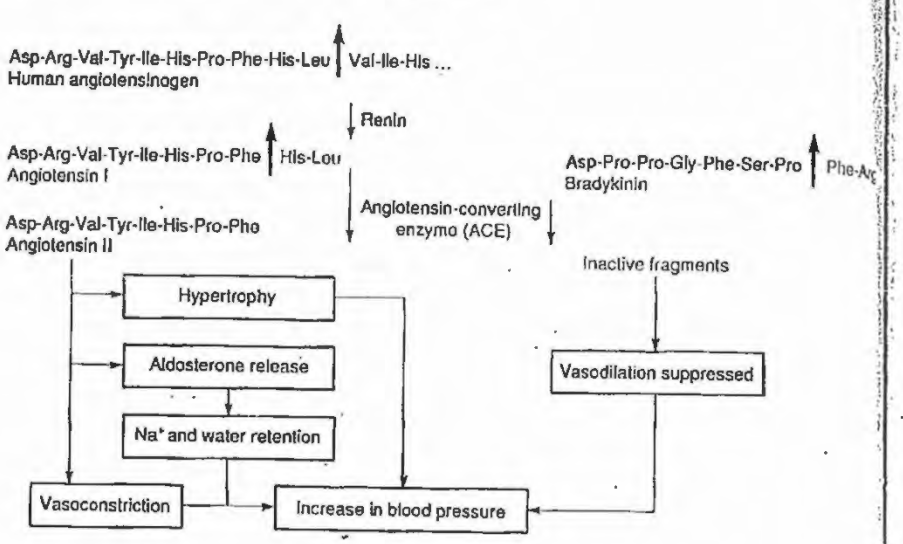


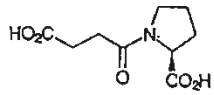
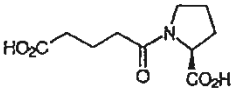
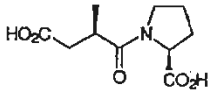
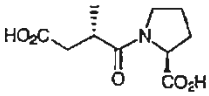
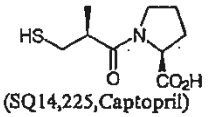
Fig. 9.1 The renin–angiotensin system.

II. The Renin–Angiotensin System

In 1827, Bright at Guy's Hospital in London was amongst the first to recognize that renal disease was often accompanied by high blood pressure. Over a century of investigation subsequently unravelled the details of the renin–angiotensin system so that by the early 1960s the biochemical pathway was well understood. A diagrammatic representation of the renin–angiotensin system is shown in Fig. 9.1. The Leu-Val bond of a circulating globular protein known as angiotensinogen, or renin substrate, is hydrolysed specifically by the aspartic acid proteinase, renin, which is produced by the kidney. This releases the *N*-terminal decapeptide, angiotensin I, which has no known biological activity. Angiotensin-converting enzyme (ACE) then cleaves a two-amino acid fragment from the free *C*-terminus of this decapeptide to give the octapeptide, angiotensin II, which is responsible for the full pressor effect of the renin–angiotensin system. Besides acting directly via receptors on vascular smooth muscle to constrict the arteries and arterioles, angiotensin II also stimulates the adrenal cortex to release aldosterone which induces sodium and water retention, resulting in a further hypertensive effect through increased plasma volume. ACE has also been identified as kininase II (Yang and Erdos, 1967; Nakajima *et al.*, 1973), the enzyme that degrades the vasodepressor peptide, bradykinin, to produce inactive fragments.

Although the biochemical details of the renin–angiotensin system had been worked out by the early 1960s, its relevance to the control of blood pressure under either normal or pathological conditions was not well understood. Indeed, many researchers were of the opinion that the role of the renin–angiotensin system in blood pressure regulation was likely to be a very minor one under any circumstances, and it was only the

TABLE 9.1
In vitro Activities of Inhibitors of Angiotensin-Converting Enzyme Illustrating the Effects of Chain Length and Substitution on Inhibitory Potency

Compound	Structure	IC_{50} (μM)
1	< Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (SQ20,881,Teprotide)	0.56
2		630
3		70
4		52
5		1470
6	 (SQ14,225,Captopril)	0.023

development of specific means of blocking the system that allowed its full importance to be appreciated.

Angiotensin II antagonists such as saralasin ([1-*sar*cosine, 8-*ala*nine]angioten*sin*) were the first agents to be used to probe the renin–angiotensin system. Early results with saralasin (Brunner *et al.*, 1973) clearly showed the therapeutic potential of blocking the system, and a sustained effort has been directed towards identifying potent orally bioavailable antagonists of angiotensin II. Much attention has also been focused on the design of inhibitors of the first enzyme of the cascade, renin. Many pharmaceutical companies have identified potent inhibitors of this highly specific enzyme, but the clinical development of these has been impeded so far by disappointing oral activity. In the late 1960s, the first peptidic inhibitors of the converting enzyme became available. These inhibitors were initially isolated from the venom of the Brazilian viper, *Bothrops jararaca* (Cushman and Ondetti, 1979) and one of them, teprotide [SQ20,811, compound (1), Table 9.1], was shown to have exciting potential as an antihypertensive agent (Engel *et al.*, 1972; Gavras *et al.*, 1974). The lack of oral bioavailability of these early peptidic ACE inhibitors again precluded their use as long-term therapy and prompted Cushman and Ondetti at the Squibb Institute to begin the search for an orally active compound. This search was to herald the dawn of a new era in medicinal chemistry: a largely empirical quest for new drugs was to become a true scientific discipline based on mechanistic theories and design.

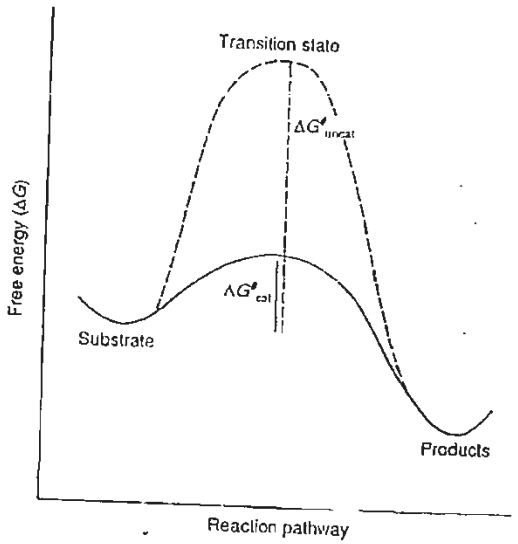


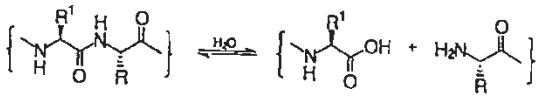
Fig. 9.2 Enzymes catalyse reactions by reducing ΔG. ——, Catalysed reaction; – – – –, uncatalysed reaction.

III. Proteolytic Enzymes

Chemical reactions in biological systems rarely occur in the absence of a catalyst. This essential role is performed by proteins known as enzymes, a term coined in 1878 by Friedrich Wilhelm Kühne from the Greek *en*, in, and *zyme*, leaven. Enzymes have truly awesome catalytic abilities; reactions are accelerated by factors of at least a million-fold. Since enzymes are catalysts, they do not alter the equilibrium of a chemical reaction; the attainment of equilibrium is accelerated, but the position of that equilibrium is not shifted. A chemical reaction, A → B, goes through a transition state which has a higher energy than that of A or B. The binding of a substrate by an enzyme is not equivalent to chemical activation, and in fact, results in stabilization of the substrate. The cardinal property of an enzyme is not, then, its ability to bind the substrate, but rather its ability to discriminate between the substrate and the transition state, binding the latter more tightly, and reducing the difference in energy that limits the rate of the reaction. Enzymes accelerate reactions, then, by decreasing ΔG, the activation barrier. The combination of enzyme and substrate creates a new reaction pathway, the transition state of which is of lower energy than that for the reaction taking place in the absence of enzyme (Fig. 9.2).

The making and breaking of chemical bonds by the enzyme is preceded by the formation of an enzyme–substrate (ES) complex. The substrate is bound to a specific region of the enzyme which is termed the active site. This region of the enzyme encompasses those residues that directly participate in the making and breaking of bonds, and which are known as the catalytic groups. The specificity of binding to the enzyme depends on the precisely defined arrangement of the residues forming the active site.

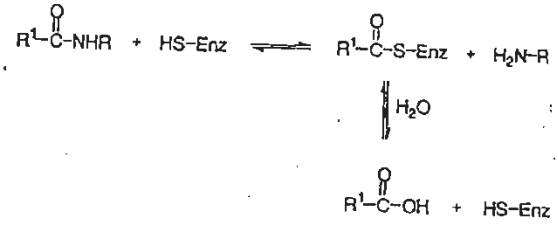
The chemical reaction carried out by proteolytic enzymes is the hydrolysis of the amide bonds linking amino acids in peptides and proteins:



Substrate Products

Proteinases differ enormously in how fastidious they are in their choice of reagent or substrate. At one end of the spectrum, the subtilisins, which are present in certain bacteria, are so undiscriminating that they can be used as ingredients in the so-called 'biological detergents' to digest all manner of protein residues, while at the other extreme, thrombin, one of the enzymes involved in blood clotting, will hydrolyse only peptide bonds between an arginine residue and a glycine residue in its target protein.

A number of devices have evolved to enable hydrolysis of the substrate to take place and proteolytic enzymes are classified into four categories depending upon their catalytic mechanism. The serine and cysteine proteinases are named after the nucleophilic residues in the active sites which form an initial acyl-enzyme intermediate. This intermediate is then hydrolysed by an activated water molecule to release the enzyme and products. The pathway for a cysteine proteinase is illustrated below:



Examples of cysteine proteinases include papain and cathepsin H. Important members of the serine proteinase family are the mammalian enzymes trypsin, chymotrypsin and elastase, as well as the bacterial enzyme, subtilisin.

It is generally held that the enzymes comprising the other two classes, the aspartic proteinases and the metalloproteinases, do not form an initial acyl-enzyme intermediate as described above. The preferred mechanism is for specific residues in the active sites of these enzymes to act as general bases and in this way to 'pep up' the nucleophilicity of a bound water molecule which then attacks the scissile amide bond directly. Important aspartic proteinases include renin and also the proteinase encoded by the human immunodeficiency virus (HIV) which is essential for viral replication (Kohl *et al.*, 1988; Peng *et al.*, 1989). A commercially important aspartic proteinase is chymosin which is used extensively in the cheese-making industry. Metalloproteinases include ACE and collagenase as well as some of the β-lactamases, which, although not strictly speaking proteinases, are important in the development of resistance to antibiotics by bacteria.

IV. ACE and the Zinc Metalloproteinases

ACE (EC 3.4.15.1) is a glycoprotein which is widely distributed in mammals. It is primarily a membrane-bound enzyme of vascular endothelial cells, the pulmonary endothelial cells being a particularly important source.

The difficulties inherent in working with a large (1.3–1.6 kDa) membrane-bound and heavily glycosylated protein have made structure elucidation a Herculean task; the amino acid sequence was not determined until 1988 (Soubrier *et al.*), long after the first inhibitors had been marketed! Even today, the structural information available about this protein is not useful for inhibitor design and the most relevant factors remain a knowledge of the enzyme's substrate specificity together with an understanding of the catalytic mechanism.

The presence of an essential zinc ion in ACE encourages comparison with other zinc metalloproteinases. Carboxypeptidase A (Quiocho and Lipscomb, 1971), carboxypeptidase B (Schmid and Herriot, 1976) and thermolysin (Matthews *et al.*, 1974; Kester and Matthews, 1977) are zinc-dependent enzymes, the structures of which have been determined using X-ray crystallography. The binding of substrates and inhibitors to these enzymes has also been studied crystallographically, and this structural information, together with extensive mechanistic studies (Hartsuck and Lipscomb, 1971), has allowed an accurate picture of the active sites of these enzymes to be developed. A schematic representation of the catalytic site of carboxypeptidase A is shown in Fig. 9.3.

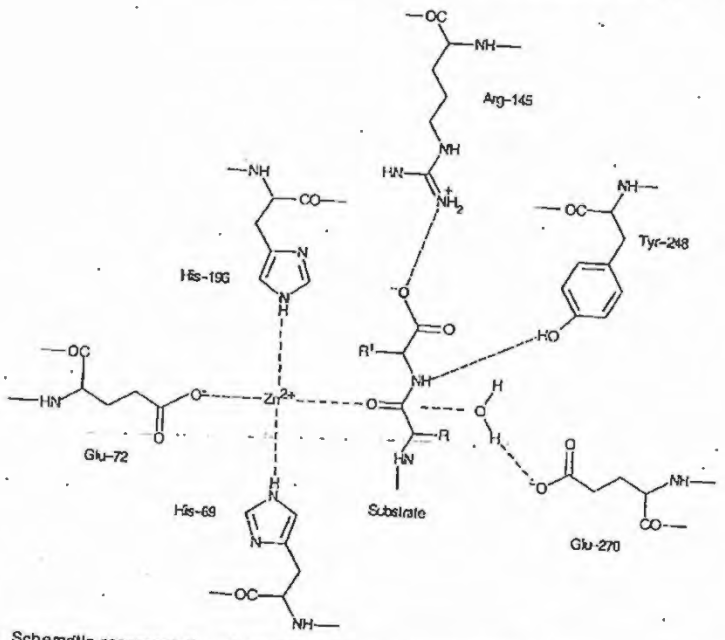


Fig. 9.3 Schematic representation of the active site of carboxypeptidase A showing a bound substrate.

Crystallographic studies on enzyme–substrate complexes show an electrostatic interaction between the positively charged guanidino function of Arg-145 and the negatively charged free carboxy group of a bound substrate.

An adjacent hydrophobic pocket (not shown) is responsible for the substrate specificity of carboxypeptidase A. The side chains of Glu-72, His-69 and His-196 form ligands to the zinc ion, and the scissile amide becomes polarized by forming a fourth ligand to the zinc, displacing a water molecule from the native enzyme as it does so. The mechanism of hydrolysis of the amide bond has been the subject of some controversy: direct attack of Glu-270 on the amide to form an anhydride-like intermediate, action of Glu-270 as a general base to activate a water molecule, and activation of a water molecule by the zinc ion have all been proposed. Studies using $H_2{}^{18}O$ (Breslow and Wernick, 1976) support the general base mechanism: Tyr-248 donates a proton to the released amino function, the resulting phenolate anion is positioned to accept a proton from the same water molecule which attacks the amide bond.

V. Assays for ACE

A reliable, and preferably high-throughput, assay is crucial to any enzyme inhibitor programme. Although a whole cell system or animal model can be used if the target enzyme is unavailable, these methods are less than ideal since they introduce complicating factors such as bioavailability, metabolism and cell penetration. These additional factors can have pronounced effects on activity, and as they are not easily taken into account, can generate very misleading structure–activity relationships (SARs).

Purified preparations of ACE are available and various assay systems have been devised to measure the potency of inhibitors. The most widely used of these involves spectrophotometric determination of the hippuric acid released from the *N*-terminal-protected tripeptide substrate hippuryl-L-histidyl-L-leucine [Fig. 9.4(a)], either alone (Cushman and Cheung, 1971), or as the complex formed with cyanuric chloride (Hayakari *et al.*, 1978).

A schematic representation of the assay procedure using the cyanuric chloride complex method is shown in Fig. 9.4(b). The experiments are repeated at a number of different inhibitor concentrations and a graph is then plotted of the enzyme activity (determined by the amount of hippurylhistidine produced in the reaction) against the concentration of inhibitor present. This produces a sigmoidal curve as shown in Fig. 9.4(c).

The activity of an inhibitor is then usually quoted as an IC_{50} value which can be determined directly from the graph. The IC_{50} value thus represents the concentration of a given inhibitor that will reduce the activity of the enzyme by 50%.

VI. The Design of ACE Inhibitors

A. Using a Two-dimensional Model of the Enzyme Active Site

1. Captopril

In 1972, Byers and Wolfenden discovered a potent inhibitor of carboxypeptidase A. The activity of this compound, L-benzylsuccinic acid, was rationalized on the basis of its

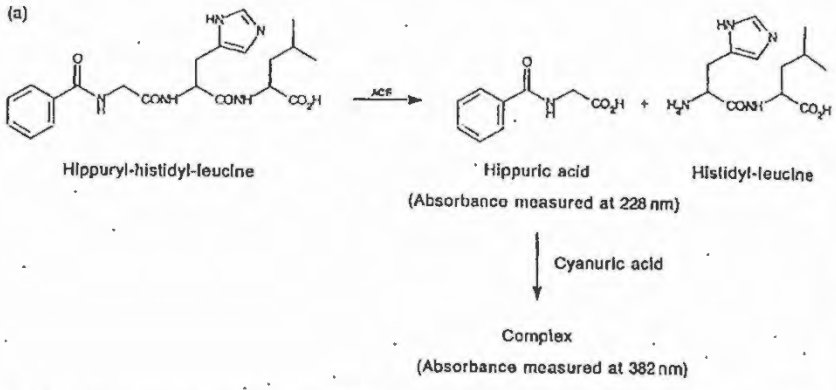


Fig. 9.4 (a) Biochemistry of the ACE assay; (b) the ACE assay; (c) Plot of enzyme activity against inhibitor concentration. (b) and (c) on facing page.

resemblance to the substrates for the reverse enzymic reaction, i.e. to the products of the peptide hydrolysis. The benzyl group was considered to occupy the S_1' subsite, with the adjacent carboxylate anion forming an electrostatic interaction with the enzymic Arg-145 residue. The second carboxylate group could then act as a ligand to the active-site zinc ion (Fig. 9.5).

The first potent inhibitors of ACE were identified as a result of studies on the bradykinin-potentiating properties of peptides isolated from the venom of *Bothrops jararaca*. Knowledge gained from these studies about the chemical and enzymic properties of ACE, together with insights drawn from Wolfenden's recently published work on the biproduct inhibitors of carboxypeptidase A, allowed Cushman and Ondetti at the Squibb Institute to develop a two-dimensional model of the active site of ACE (Ondetti *et al.*, 1977).

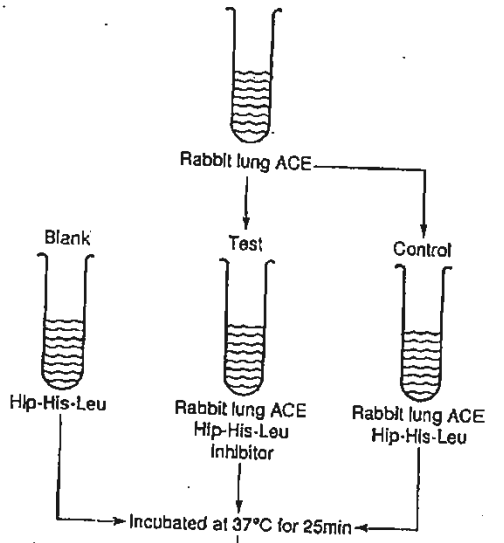
Since ACE is a dipeptidylcarboxypeptidase, Ondetti and his colleagues reasoned that the distance between the cationic binding site and the zinc ion in ACE must be greater than in carboxypeptidase A, by approximately the length of one amino acid residue. It then followed that a succinyl derivative of an amino acid, rather than a simple succinic acid, should form the prototype for inhibitors of ACE (Fig. 9.6).

To test this hypothesis, they initially prepared succinyl-*S*-proline (2) choosing proline as the *C*-terminal amino acid since this residue occurs at the free *C*-terminus of all the naturally occurring peptidic inhibitors.

Although not exceptionally potent (IC_{50} 630 μM), this compound provided support for the original hypothesis, as it was a specific inhibitor of the enzyme. After exploring the effect of different structural modifications, including length and substitution of the acyl group, 2-*R*-methylsuccinyl-*S*-proline (4) was identified as a significantly more active inhibitor (IC_{50} 52 μM). The diastereoisomer (5) with the *S*-configuration at the methyl group was found to be much less active.

The Squibb group then reasoned that if the interaction of the carboxy group of such compounds with the zinc ion in the enzyme active site was crucial for inhibitory potency, then replacement of the carboxylate by another group capable of acting as a ligand to

(b)



Reactions stopped by addition of ice-cold 200mM-phosphate buffer (pH 8.3). Rabbit lung ACE added to blank assay. Then 3% solution of cyanuric chloride in dioxan added and reactions incubated at 20°C for 15 min. Tubes centrifuged.

Absorbance measured at 382nm.

(c)

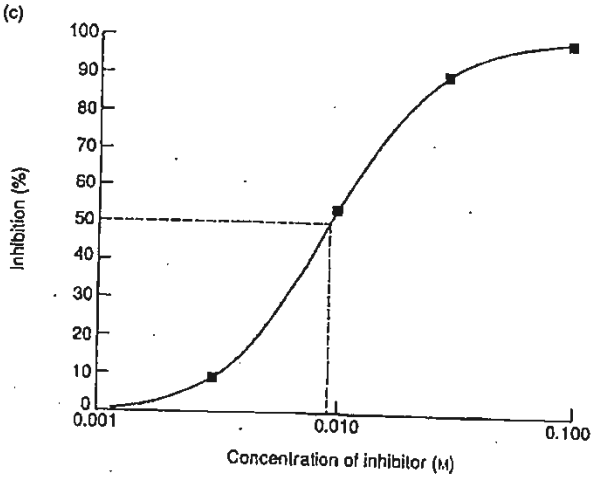


Fig. 9.4 Continued.

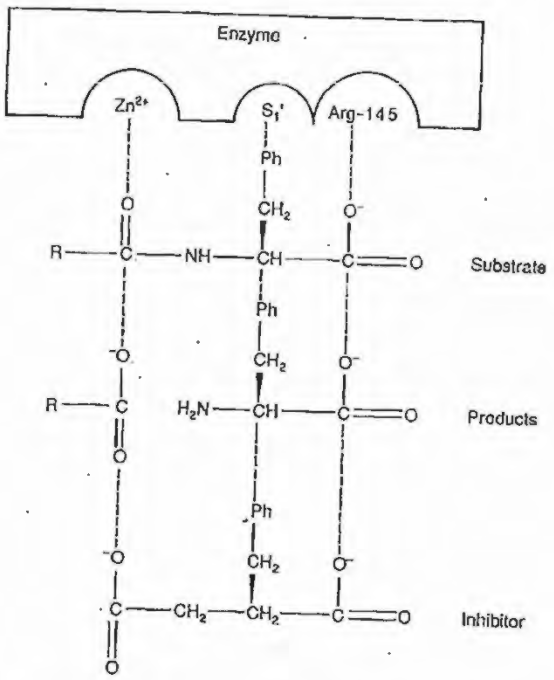


Fig. 9.5 Schematic representation of the binding of substrate, product and inhibitor molecules to the active site of carboxypeptidase A.

the zinc ion should result in a compound of equal or greater potency. Replacement of the carboxy group by nitrogen-containing functionalities (amines, amides or guanidines) gave no improvement in potency, but replacement by a thiol group led to a dramatic enhancement of activity. The most potent inhibitor of this series (6, SQ14,225) with an IC_{50} value of 23 nM was, on a molar basis, more potent than the previously described nonapeptide, teprotide (1). More importantly, this small non-peptidic inhibitor showed good oral bioavailability, and as *captopril* was destined to become the first commercially available ACE inhibitor.

2. *Enalapril*

The design of enalapril also grew out of Wolfenden's biproduct inhibitor hypothesis, but Patchett and colleagues (1980) at Merck used a different approach to improve the potency of glutarylproline (3), choosing to elaborate the molecule in such a way as to provide additional interactions with putative sites on the enzyme. In order to visualize some of the possibilities, they considered 2-methylglutarylproline as a biproduct inhibitor of the hydrolysis of *N*-acyl-Phe-Ala-Pro (Fig. 9.7).

It was hypothesized that incorporation of an NH group, a hydrophobic side chain or an RCONH group into the prototype inhibitor, 2-methylglutarylproline, should increase

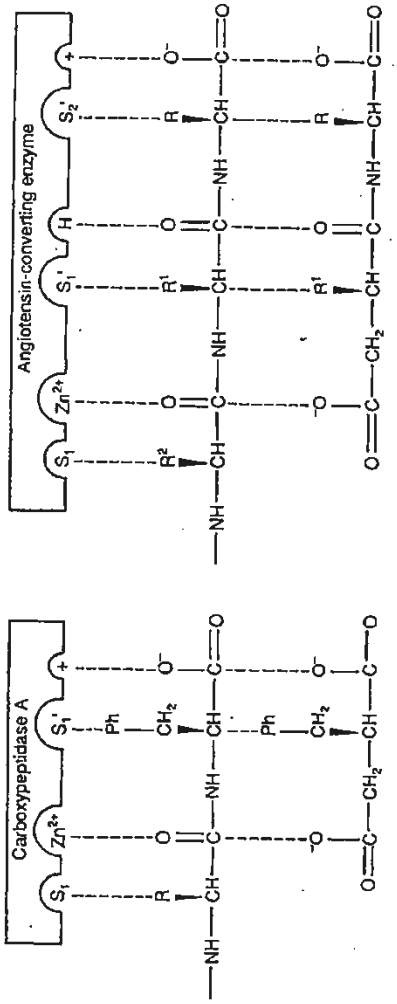


Fig. 9.6 Schematic representation of the binding of substrates and inhibitors to the active site of carboxypeptidase A, and to the hypothetical active site of angiotensin-converting enzyme.

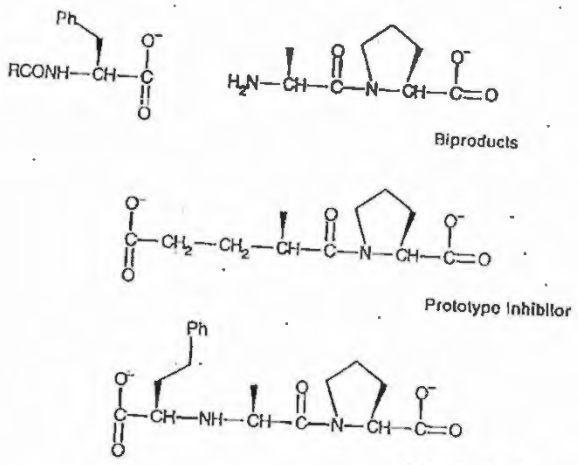
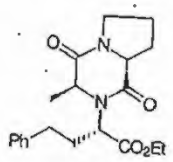


Fig. 9.7 Schematic representation of 2-methylglutarylproline as a biproduct inhibitor of the enzymic cleavage of *N*-acyl-Phe-Ala-Pro.

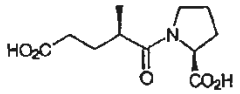
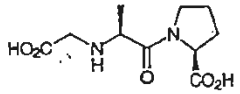
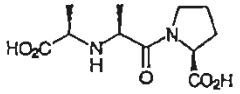
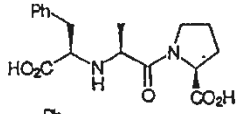
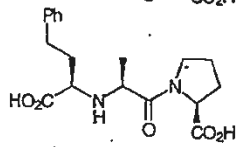
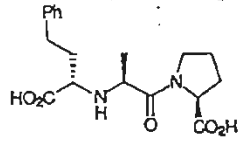
its resemblance to the products of the enzymic hydrolysis, and thus enhance inhibitory potency. Introduction of an NH group did not significantly improve activity (compound 8 compared with compound 7), but incorporation of a hydrophobic substituent next to the *N*-terminal carboxy group (a manoeuvre suggested by the known preference of ACE for substrates with a hydrophobic amino acid in the antipenultimate position) led to a dramatic increase in potency (compounds 9–11) (Table 9.2). The most potent compound (11), designated MK422, was that in which a phenethyl group was used as the hydrophobic side chain. The diastereisomer (12) with the *R*-configuration at the centre bearing the carboxylate group is a much less potent inhibitor.

MK422 is not well absorbed orally in laboratory animals or in man, but esterification of the *N*-carboxyalkyl group provided a simple solution to this problem. The ethyl ester, MK421, *enalapril*, is, as expected, a much less potent inhibitor of ACE, but *in vivo* the action of esterases rapidly converts the prodrug into the active diacid. It is fortunate that satisfactory oral absorption can be achieved with the monoester since esterification of the proline carboxy group strongly promotes the irreversible formation of the diketopiperazine (13).



(13)

TABLE 9.2
In vitro Activities of Inhibitors of Angiotensin-Converting Enzyme Illustrating the Effects of Additional Binding Groups on Inhibitory Potency

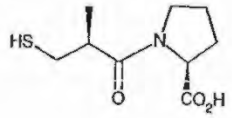
Compound	Structure	IC_{50} (μM)
7		4.9
8		2.4
9		0.09
10		0.04
11		0.001
12		0.8

B. Using a Three-dimensional Model of the Enzyme Active Site

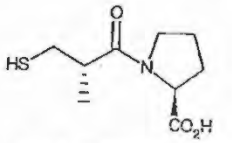
1. Thiol-containing Inhibitors

Design approaches using a two-dimensional schematic representation of the enzyme active site have proved to be very effective, and, as in the case of captopril and enalapril, have led to potent enzyme inhibitors. Several groups, including our own at Roche, believed that it should be possible to achieve further improvements in potency if the bioactive conformation of these inhibitors could be used in a more rigid molecule. The importance of the three key binding groups, i.e. the carboxy group, the amide group and the zinc ligand, was well recognized, but information about their relative spatial orientation in the bound state was not accessible directly, since no X-ray crystallographic data were available, for either the native enzyme or enzyme–inhibitor complexes.

We addressed this problem by attaching the three important binding groups to a rigid template which could be varied systematically in order to change the relative spatial orientation of these groups (Hassall *et al.*, 1984). We began by imposing the constraint that the amide bond of captopril should remain in the *trans* conformation in the



trans 0.8 mol fraction



cis 0.2 mol fraction

Fig. 9.8 *Trans* and *cis* conformers of captopril.

enzyme–inhibitor complex. This seemed to be a reasonable supposition since the *trans* form is clearly favoured both in solution and in the crystal (Fig. 9.8).

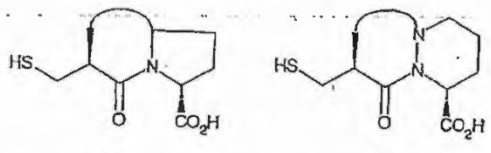
As our template, we chose a bicyclic system which is notionally formed by bridging the proline ring δ-carbon on to the alanylmethyl group [Fig. 9.9(a)]. In practice, we preferred to replace proline by hexahydropyridazine-3-carboxylic acid [Fig. 9.9(b)], since this avoids the inconvenience of an additional chiral centre and also provides the basis of a relatively straightforward route to a range of bicyclic compounds (Table 9.3).

We initially chose compounds which would place the thiol group in very different positions to allow us to map a large volume of space quickly.

In order to learn more about the bioactive conformation of captopril, we then needed to compare the positions of the three key binding groups (the thiol group, the amide group and the carboxy group) in each of our conformationally restricted inhibitors with the full range of spatial orientations possible for captopril. Keeping the amide bond of captopril in what we believed was the bioactive *trans* conformation, it was possible, using molecular graphics, to effect rotations about the CH_2-CH(Me) and CH(Me)-C(O) bonds (Fig. 9.10).

This generated the locus for the captopril S-atom depicted in Fig. 9.11(a). Using conventional molecular mechanics calculations, it was then possible to exclude high-energy (> 50 kcal/mol) conformations, leaving the mesh plot shown in Fig. 9.11(b) to represent the most likely positions of the S-atom relative to the amide and carboxy groups.

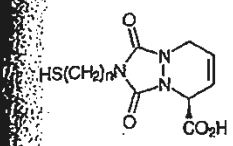
By comparing the restricted S-atom locus of each of our conformationally restricted inhibitors with the captopril S-atom locus, we were able to associate biological activities



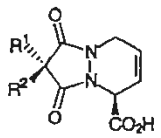
(a) (b)

Fig. 9.9 (a) *Trans* conformer of captopril showing link required to form bicyclic derivative; and (b) preferred bicyclic derivative.

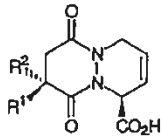
TABLE 9.3
In vitro Activities of Conformationally Restricted Thiol-containing Inhibitors of Angiotensin-Converting enzyme



Compound	n	IC_{50} (μM)
14	1	0.7
15	2	0.5
16	3	6.0



Compound	R^1	R^2	IC_{50} (μM)
17	$HSCH_2CH_2$	H_3C	0.1
18	H_3C	$HSCH_2CH_2$	43
19	$HSCH_2$	H_3C	1.0



Compound	R^1	R^2	IC_{50} (μM)
20	$HSCH_2$	H	0.1
21	H	$HSCH_2$	0.04

with the different regions of this locus, and hence identify the probable bioactive conformations of captopril.

Figure 9.12(a) represents the superposition of the mesh plot for compound (21) on that for captopril. The carboxy and amide groups of the two molecules have been aligned, and conformationally feasible positions for the thiol groups then traced. Figure 9.12(b) shows a scale representation of the portion of the mesh trace common to compound 21 and captopril. This process can be repeated for the individual conformationally restricted inhibitors (compounds 14–21), producing in each case a restricted locus which corresponds to only part of the whole potential locus for the sulphur atom of captopril. If it is assumed that the enzyme inhibitory activity of the conformationally restricted inhibitors is directly dependent upon their ability to achieve the best fit in the active site, the mesh plot for the sulphur atom can be related to the biological activity (I) of each compound (Fig. 9.13).

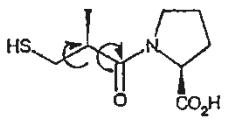


Fig. 9.10 Rotation about the CH_2-CH(Me) and CH(Me)-CO bonds of captopril allows generation of the mesh plot depicted in Figure 9.11(a).

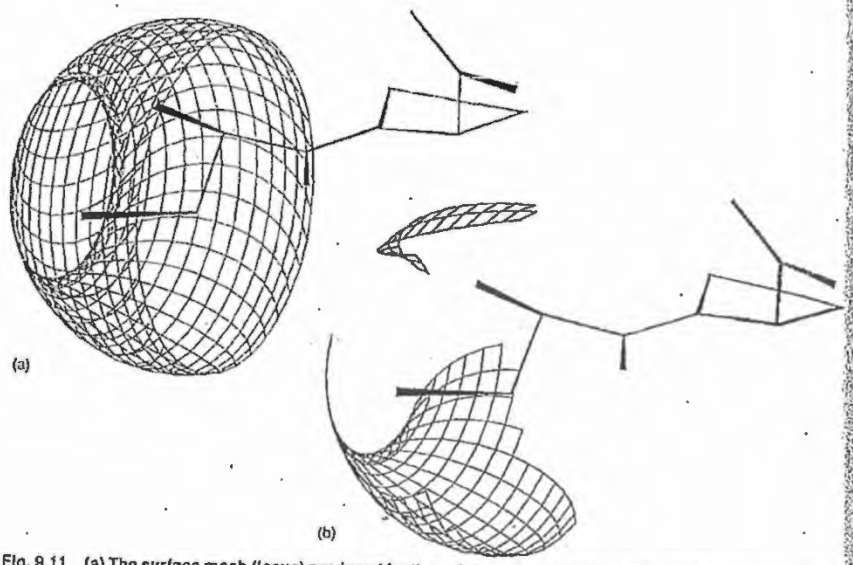


Fig. 9.11 (a) The surface mesh (locus) produced for the sulphur atom of captopril by allowing free rotation about the CH_2-CH(Me) and CH(Me)-CO bonds; and (b) the reduced locus after rejecting conformations > 50 kcal/mol in total energy.

A consideration of our initial thiol-containing compounds ascribed highest potency to two regions of the locus, Γ_4.

Although compounds 20 and 21 are reasonably potent enzyme inhibitors (IC_{50} 100 and 40 nM), they are less potent than captopril (IC_{50} 23 nM), indicating that the three key binding groups in these rigid molecules are not fixed in precisely the right orientation for optimal binding to ACE. This is probably because the torsion angle ψ (Fig. 9.14) is not optimal in these compounds which thus mimic relatively high-energy conformations of alanylproline. In these conformers, the methyl group of the alanine residue almost eclipses the δ-carbon atom of the proline ring. The energy profile for alanylproline does in fact show two rather broad energy minima, both of which correspond to ψ values outside the range calculated for compounds 20 and 21.

2. *Cilazapril*

Having used our conformationally restricted thiol-containing inhibitors to map likely positions of the active site zinc ion relative to the hydrogen bond donor and cationic binding sites, we then further refined and extended our model to include other zinc ligands (Attwood *et al.*, 1986). We prepared a series of *N*-carboxyalkyl-bicyclic lactams in which the torsion angle, ψ, was varied systematically (Table 9.4).

The most potent ACE inhibitors (24 and 28) were those incorporating a seven-membered lactam with a torsion angle, ψ, of 164–166°. Interestingly, this value of approximately 165°, which appears to be optimal for binding to ACE, is different from that adopted by *N*-[1-carboxy-3-phenylpropyl]-L-leucyl-L-tryptophan (ψ = 316°) as

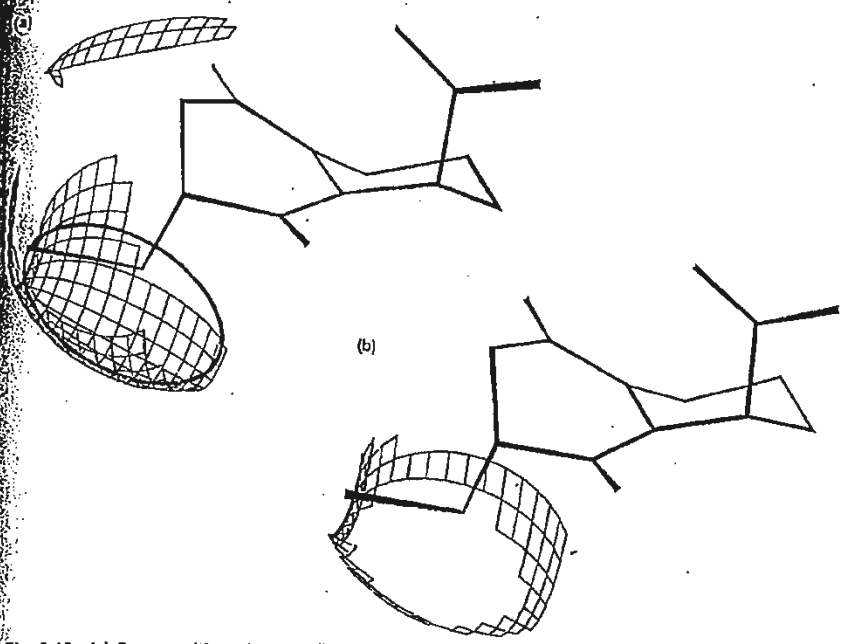


Fig. 9.12 (a) Superposition of captopril and bicyclic compound (21); and (b) the common locus of the sulphur atoms in compound (21) and captopril.

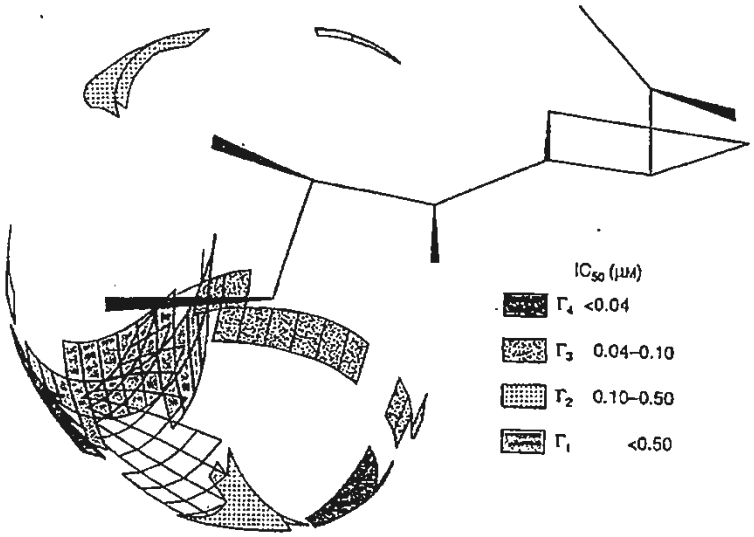


Fig. 9.13 Biological activities assigned to different regions of the captopril sulphur atom locus.

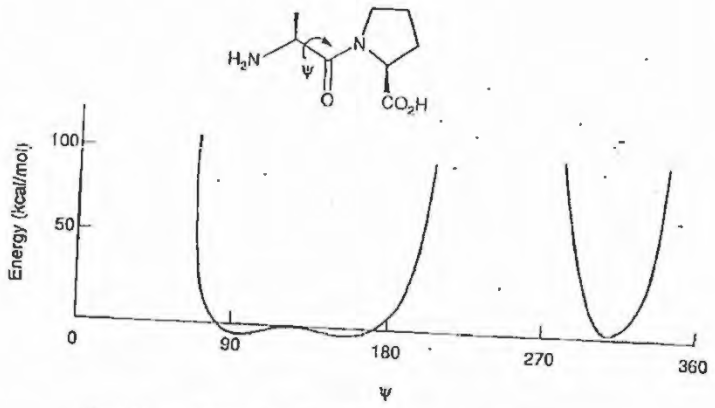


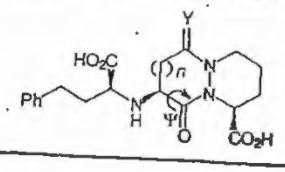
Fig. 9.14 The ECEPP energy profile of the H-Ala-Pro-OH fragment.

determined by X-ray crystallography in the complex with thermolysin (Monzingo and Matthews, 1984). This suggests that ACE binds inhibitors (and presumably substrates) in one low-energy conformation whilst the related metalloproteinase, thermolysin, binds its substrates in an alternative low-energy conformation. The proposed enzyme-bound conformation of compound **24** (cilazaprilat) is depicted in Fig. 9.15.

In common with other non-thiol-containing ACE inhibitors, the parent diacid, cilazaprilat is poorly absorbed orally. Conversion to the monoethyl ester affords the prodrug, cilazapril (**30**). Cilazapril shows excellent oral bioavailability and, once absorbed, the

TABLE 9.4

In vitro Activities of Conformationally Restricted N-carboxyalkyl Inhibitors of Angiotensin-Converting Enzyme Showing Variation with Torsion Angle, Ψ



Y = H_2				Y = O			
Compound	*n*	Ψ	IC_{50} (nM)	Compound	*n*	Ψ	IC_{50} (nM)
22	0	254.9°	8000	**26**	0	237.6°	
23	1	245.6°	28	**27**	1	196.6°	20
24	2	163.9°	1.6	**28**	2	165.3°	4
25	3	142.1°*	4.5	**29**	3	146.4°*	15

Ψ values obtained from crystal structures of model bicyclic compounds.
*Values calculated by Modified Neglect of Diatomic Overlays (MNDO) (Dewar and Thiel, 1977).
The Ψ value ~ 165° seems optimal for ACE. The value 316° was found for the thermolysin/*N*-(1-carboxy-3-phenylpropyl)-L-leucyl-L-tryptophan complex. These values are within the ranges of the two energy minima for alanylproline.

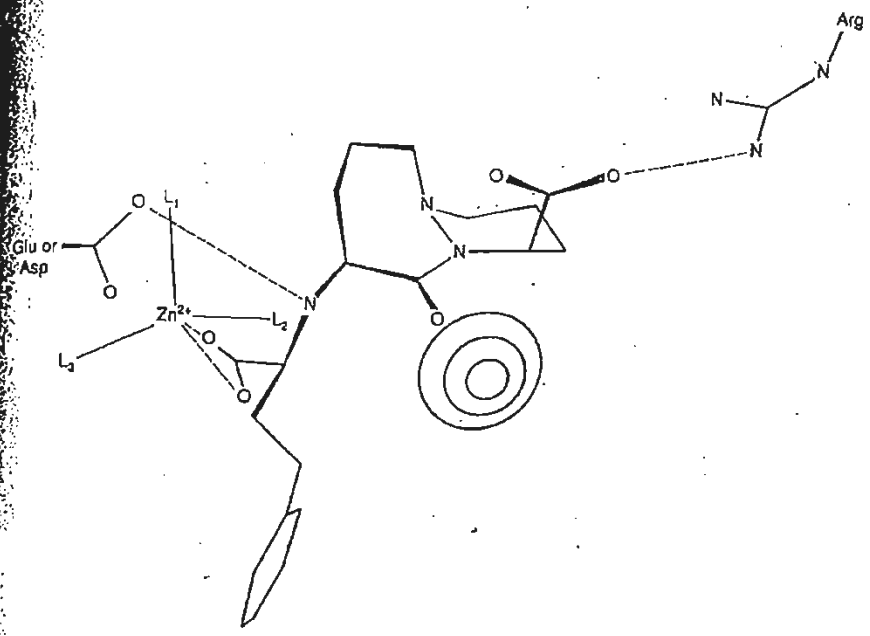
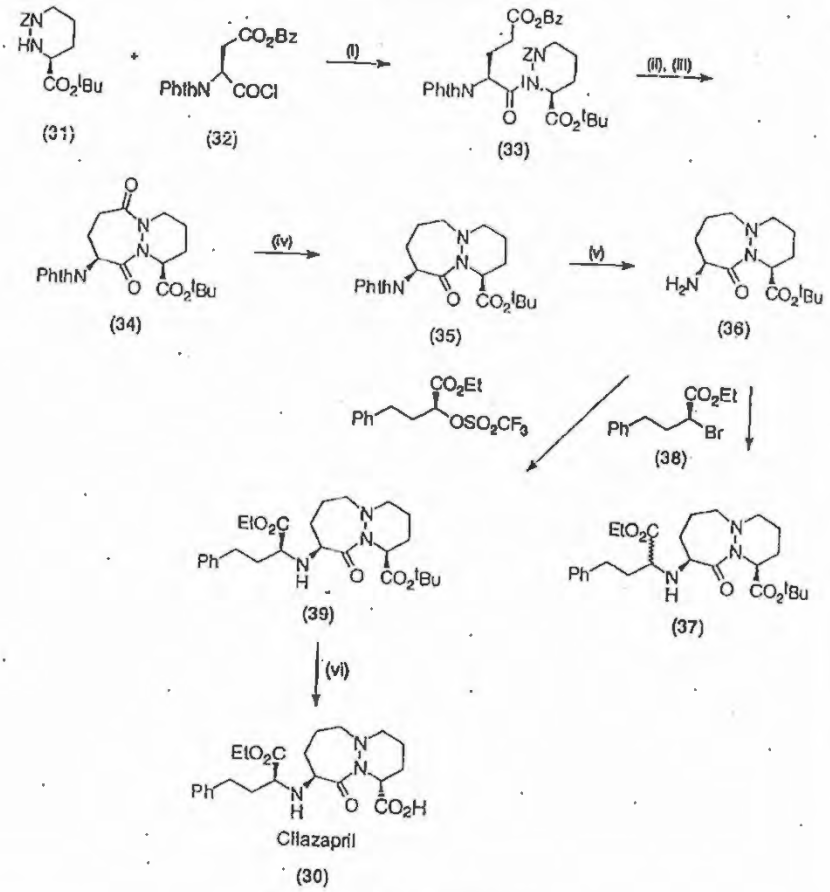


Fig. 9.15 The proposed orientation of cilazaprilat for binding to the catalytic site of ACE.

ethyl ester is rapidly hydrolysed by plasma esterases to liberate the parent diacid, cilazaprilat.

3. *Synthesis of Cilazapril*

The synthesis of cilazapril is set out in Scheme 9.1. The *t*-butyl ester of (N-1) protected hexahydropyridazine-3(*S*)-carboxylic acid (31) was acylated at N-2 using the protected glutamic acid derivative (32) to give compound 33. The protecting groups were removed simultaneously by hydrogenolysis and the resulting acid was cyclized via the acid chloride to give the protected dipeptide mimetic 34. We were gratified to find that reduction of compound 34 with borane/tetrahydrofuran (H_3B/THF) was highly regioselective, possibly because the second amide is in a rather crowded environment. Deprotection of intermediate 35 by hydrazinolysis then afforded the key dipeptide mimetic (36). Two methods of attaching the carboxyalkyl side chain were investigated. In our hands, reductive alkylation using keto acids or esters gave poor yields with little evidence of asymmetric induction at the newly formed chiral centre. Initial studies using racemic ethyl 2-bromo-4-phenylbutanoate afforded, in good yield, the required product (37) as a 1 : 1 mixture of epimers. Much to our chagrin, the same 1 : 1 mixture of epimers was obtained following alkylation with the hard-earned chiral bromo ester (38). The racemization was presumed to stem from nucleophilic exchange of bromide ion, and was avoided by the use of trifluoromethanesulphonate (triflate) as the leaving group. Our



Scheme 9.1 Synthesis of cilazapril.

(i) Toluene, aqueous $NaHCO_3$; (ii) H_2, Pd/C, EtOH; (iii) $SoCl_2$, CH_2Cl_2; (iv) H_3B/THF; (v) N_2H_4. H_2O, EtOH; (vi) HCl/CH_2Cl_2.

experience was subsequently confirmed by the findings of Effenberger *et al.* (1983) who used triflate esters to prepare a range of chiral *N*-substituted α-amino carboxylic esters. Deprotection of the butyl ester (39) using anhydrous hydrogen chloride in dichloromethane then gave cilazapril (30).

VII. Biological Properties of ACE Inhibitors

A. Pharmacokinetics

In contrast with the position with enzyme inhibition, it is still barely possible to apply design principles to overcome pharmacokinetic problems, and serendipity remains the major operator. Fortune has favoured the medicinal chemist in the field of ACE inhibitors since captopril is well absorbed orally, and those inhibitors that use a carboxy group as the zinc ligand can be rendered orally bioavailable by the simple expedient of monoesterification. When cilazapril is administered orally to rats, approximately 98% of the dose is absorbed (Attwood *et al.*, 1984). The action of esterases then releases the parent diacid, cilazaprilat.

B. Efficacy In Animal Models of Hypertension

Cilazapril, and other ACE inhibitors, have long been established to lower blood pressure in a range of hypertensive animal models. Cilazapril lowers established high blood pressure in spontaneously hypertensive rats (SHRs) (Natoff *et al.*, 1985), and prevents the development of hypertension in young SHRs (Hefti *et al.*, 1986). In this animal model, cilazapril lowers blood pressure largely by a decrease in total peripheral resistance with little or no effect on heart rate or cardiac output.

C. Clinical Results

1. Hypertension

Numerous clinical trials have clearly demonstrated the efficacy of ACE inhibitors as monotherapy in mild, moderate and even severe hypertension. Although early theories suggested that angiotensin II might be important only in relatively rare forms of hypertension, e.g. renal hypertension, it is now known that ACE inhibitors, in conjunction with diuretics if necessary, will lower blood pressure in over 80% of patients.

2. Cardiac and Vascular Hypertrophy

High blood pressure damages the heart by causing thickening (hypertrophy) of the heart muscle (myocardium) and also of the walls of the blood vessels supplying the heart. These changes reduce the ability of the heart to increase its capacity beyond its normal work load (coronary reserve), and can also lead to an inadequate blood supply to the heart muscle itself (myocardial ischaemia). These pathological changes predispose the patient to heart failure, so the aim of any long-term therapy should be to reverse ventricular hypertrophy and improve coronary vascular reserve (Motz and Strauer, 1990). Cilazapril has been shown to prevent the development of cardiac and vascular hypertrophy and can also improve coronary reserve once hypertension is established (Clozel and Hefti, 1988).

3. Reperfusion Arrhythmias

Contrary to apparent logic, restoration of blood flow to the ischaemic heart is more likely to cause fibrillation and death than the period of ischaemia itself. Reperfusion can occur spontaneously or as a result of medical intervention and frequently leads to

life-threatening arrhythmias. Studies have shown that ACE inhibitors protect against reperfusion arrhythmias (Van Gilst *et al.*, 1984; Lad and Manning, 1987), whereas angiotensin I and angiotensin II potentiate these arrhythmias (Van Gilst *et al.*, 1984).

4. *Congestive Heart Failure*

Congestive heart failure occurs more frequently with increasing age and is often linked to disease of the heart muscle caused by inadequate blood supply (ischaemic cardiomyopathy) or to hypertension. In order to maintain blood flow to vital organs, a number of compensatory mechanisms are triggered. Initially the loss of contractility is countered by cardiac dilation, ventricular hypertrophy and increased catecholamine production. As the condition progresses, decreased cardiac output and increased filling pressures result in activation of the sympathetic nervous and renin–angiotensin systems which in turn causes vasoconstriction and increased blood volume. This then sets up a vicious circle in which the failing heart has to work against increasing blood pressure. Recently interest has focused on the use of ACE inhibitors in congestive heart failure since a fall in systemic vascular resistance should reduce the excessive pre- and after-loads seen in this disease. ACE inhibition has been shown to be beneficial in congestive heart failure and it is becoming evident that early treatment may even prevent the development of overt heart failure.

5. *Inhibition of Neo-intimal Thickening*

In atherosclerosis, the blood vessels are narrowed by deposits of lipid and collagen which later become calcified. Atherosclerotic vascular stenosis, vascular surgery and percutaneous transluminal coronary angioplasty (PTCA) (a technique which attempts to clear extensively blocked arteries) are all accompanied by proliferation of the underlying smooth muscle cells (Ross, 1986) which further narrows the blood vessel. The walls of the large arteries and veins contain local angiotensin systems which seem to be involved in the thickening of the blood vessel walls seen in hypertension since ACE inhibitors such as cilazapril have been shown (Clozel *et al.*, 1989) to reverse this thickening.

In animal experiments, Powell and colleagues (1989) have shown that cilazapril similarly prevents this smooth muscle cell proliferation after vascular injury. The outcome of clinical studies in PTCA patients will determine whether these impressive results are reproduced in man.

VIII. Conclusions

Although early ideas about hypertension suggested that ACE inhibitors would be useful only in certain rather rare circumstances, this has proved to be an oversimplification, and ACE inhibitors have become an important class of drugs for controlling the commonly encountered forms of hypertension. Moreover, ACE inhibitors will also probably prove beneficial to patients suffering from myocardial infarction, congestive heart failure and possibly atherosclerosis.

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United States Patent [19]

Villhauer

[11] Patent Number: 6,166,063

[45] Date of Patent: Dec. 26, 2000

[54] N-(SUBSTITUTED GLYCYL)-2-CYANOPYRROLIDINES, PHARMACEUTICAL COMPOSITIONS CONTAINING THEM AND THEIR USE IN INHIBITING DIPEPTIDYL PEPTIDASE-IV

[75] Inventor: Edwin Bernard Villhauer, Morristown, N.J.

[73] Assignee: Novartis AG, Basel, Switzerland

[21] Appl. No.: 09/458,224

[22] Filed: Dec. 9, 1999

Related U.S. Application Data

[63] Continuation of application No. 09/209,068, Dec. 10, 1998, abandoned.

[51] Int. Cl.[7] A61K 31/401; A61P 5/48; C07D 207/12; C07D 207/14

[52] U.S. Cl. 514/423; 514/428; 548/528; 548/530

[58] Field of Search 514/423, 428; 548/530, 528

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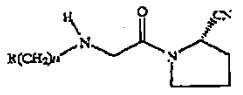
Attorney, Agent, or Firm—Joseph J. Borovian

[57] ABSTRACT

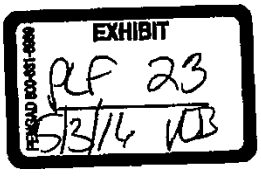
The present invention relates to a compound of formula (I)

I



wherein R is substituted adamantyl; and n is 0 to 3; in free form or in acid addition salt form. Compounds of formula I inhibit DPP-IV (dipeptidyl-peptidase-IV) activity. They are therefore indicated for use as pharmaceuticals in inhibiting DPP-IV and in the treatment of conditions mediated by DPP-IV, such as non-insulin-dependent diabetes mellitus, arthritis, obesity, osteoporosis and further conditions of impaired glucose tolerance.

9 Claims, No Drawings



AZ-SAXA-8024001

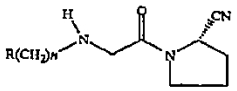
N-(SUBSTITUTED GLYCYL)-2-CYANOPYRROLIDINES, PHARMACEUTICAL COMPOSITIONS CONTAINING THEM AND THEIR USE IN INHIBITING DIPEPTIDYL PEPTIDASE-IV

This application claims the benefit of U.S. application Ser. No. 09/209,068, filed Dec. 10, 1998, now abandoned, and which is incorporated herein by reference.

The present invention provides new dipeptidyl peptidase-IV (DPP-IV) inhibitors which are effective in treating conditions mediated by DPP-IV. More recently, it was discovered that DPP-IV is responsible for inactivating glucagon-like peptide-1 (GLP-1). Since GLP-1 is a major stimulator of pancreatic insulin secretion and has direct beneficial effects on glucose disposal, DPP-IV inhibition appears to represent an attractive approach for treating conditions such as non-insulin-dependent diabetes mellitus (NIDDM).

The instant invention relates to novel N-(substituted glycyl)-2-cyanopyrrolidines of formula I:

I



wherein

R is substituted adamantyl; and

n is 0 to 3; in free form or in acid addition salt form.

The compounds of formula I can exist in free form or in acid addition salt form. Pharmaceutically acceptable (i.e., non-toxic, physiologically acceptable) salts are preferred, although other salts are also useful, e.g., in isolating or purifying the compounds of this invention. Although the preferred acid addition salts are the hydrochlorides, salts of methanesulfonic, sulfuric, phosphoric, citric, lactic and acetic acid may also be utilized.

The compounds of the invention may exist in the form of optically active isomers or diastereoisomers and can be separated and recovered by conventional techniques, such as chromatography.

Listed below are definitions of various terms used to describe this invention. These definitions apply to the terms as they are used throughout this specification, unless otherwise limited in specific instances, either individually or as part of a larger group.

The term "alkyl" refers to straight or branched chain hydrocarbon groups having 1 to 10 carbon atoms, preferably 1 to 7 carbon atoms, most preferably 1 to 5 carbon atoms. Exemplary alkyl groups include methyl, ethyl, propyl, isopropyl, n-butyl, t-butyl, isobutyl, pentyl, hexyl and the like.

The term "alkanoyl" refers to alkyl-C(O)—.

The term "substituted adamantyl" refers to adamantyl, i.e., 1- or 2-adamantyl, substituted by one or more, for example two, substituents selected from alkyl, —OR_1 or —NR_2R_3; where R_1, R_2 and R_3 are independently hydrogen, alkyl, (C_2–C_8-alkanoyl), carbamyl, or —CO—NR_4R_5; where R_4 and R_5 are independently alkyl, unsubstituted or substituted aryl and where one of R_4 and R_5 additionally is hydrogen or R_4 and R_5 together represent C_2–C_7alkylene.

The term "aryl" preferably represents phenyl. Substituted phenyl preferably is phenyl substituted by one or more, e.g., two, substituents selected from, e.g., alkyl, alkoxy, halogen and trifluoromethyl.

The term "alkoxy" refers to alkyl-O—.

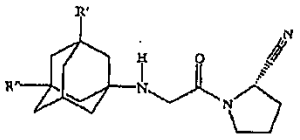
The term "halogen" or "halo" refers to fluorine, chlorine, bromine and iodine.

The term "alkylene" refers to a straight chain bridge of 2 to 7 carbon atoms, preferably of 3 to 6 carbon atoms, most preferably 5 carbon atoms.

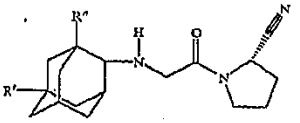
A preferred group of compounds of the invention is the compounds of formula I wherein the substituent on the adamantyl is bonded on a bridgehead or a methylene adjacent to a bridgehead. Compounds of formula I wherein the the glycyl-2-cyanopyrrolidine moiety is bonded to a bridgehead, the R' substituent on the adamantyl is preferably 3-hydroxy. Compounds of formula I wherein the the glycyl-2-cyanopyrrolidine moiety is bonded at a methylene adjacent to a bridgehead, the R' substituent on the adamantyl is preferably 5-hydroxy.

The present invention especially relates to a compound of formulae (I A) or (I B)

(IA)



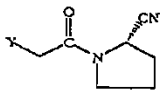
(IB)



wherein R' represents hydroxy, C_1–C_7alkoxy, C_1–C_8-alkanoyloxy, or R_3R_4N—CO—O—, where R_4 and R_5 independently are C_3–C_7alkyl or phenyl which is unsubstituted or substituted by a substituent selected from C_1–C_7alkyl, C_1–C_7alkoxy, halogen and trifluoromethyl and where R_4 additionally is hydrogen; or R_4 and R_5 together represent C_3–C_6alkylene; and R" represents hydrogen; or R' and R" independently represent C_1–C_7alkyl; in free form or in form of a pharmaceutically acceptable acid addition salt.

The compounds of the invention may be prepared e.g. by a process which comprises coupling a reactive (2-cyanopyrrolidino)carbonylmethylene compound with an appropriate substituted amine; more particularly, for the preparation of the compounds of formula I, it comprises reacting a compound of formula II.

II



wherein Y is a reactive group (preferably a halogen such as bromine, chlorine or iodine) with a compound of formula III

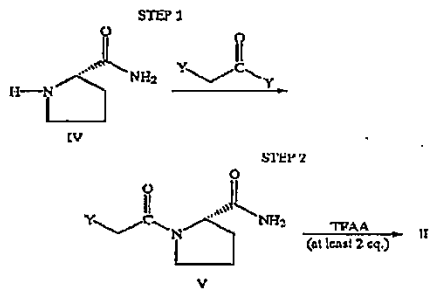
$$NH_2(CH_2)_n—R \qquad III$$

wherein R is as defined above, and recovering the resultant compound of formula I in free form or in acid addition salt form.

The process of the invention may be effected in conventional manner. For example, the compound of formula II is reacted with 1 to 3 equivalents, preferably 3 equivalents, of a primary amine of formula III. The reaction is conveniently conducted in the presence of an inert, organic solvent, such as methylene chloride or a cyclic ether such as tetrahydrofuran. The temperature preferably is of from about 0° to about 35° C., preferably between about 0° and about 25° C.

The compounds of the invention may be isolated from the reaction mixture and purified in conventional manner, e.g. by chromatography.

The starting materials may also be prepared in conventional manner. The compounds of formula II may be prepared by the following two-step reaction scheme:



Step 1 involves the reaction of the pyrrolidine of formula IV with a slight molar excess of a haloacetylhalide such as bromoacetylbromide or chloroacetylchloride and a base such as potassium carbonate or triethylamine. The reaction conveniently is conducted in the presence of an inert, organic solvent, such as tetrahydrofuran or a chlorinated, aliphatic hydrocarbon such as methylene chloride, at a temperature of from about 0° to about 25° C., preferably at a temperature between about 0° and about 15° C.

Step 2 concerns the dehydration of the compound of formula V, prepared in Step 1, with 1 to 2 equivalents of trifluoroacetic anhydride (TFAA). The dehydration preferably is conducted in the presence of an inert, organic solvent such as tetrahydrofuran or a chlorinated, aliphatic hydrocarbon such as methylene chloride, at a temperature of from about 0° to about 25° C., preferably at a temperature between about 0° and about 15° C.

Insofar as its preparation is not particularly described herein, a compound used as starting material is known or may be prepared from known compounds in known manner or analogously to known methods or analogously to methods described in the Examples.

For example, the primary amine compounds of formula III are known and may be prepared by procedures documented in the literature, for example, Khim. -Farm. Zh. (1986), 20(7), 810–15.

Finally, compounds of the invention are either obtained in the free form, or as a salt thereof if salt forming groups are present.

Compounds of the invention having basic groups can be converted into acid addition salts, especially pharmaceutically acceptable acid addition salts. These are formed, for example, with inorganic acids, such as mineral acids, for example sulfuric acid, a phosphoric or hydrohalic acid, or with organic carboxylic acids. Preferred are salts formed with hydrochloric acid.

In view of the close relationship between the free compounds and the compounds in the form of their salts, whenever a compound is referred to in this context, a corresponding salt is also intended, provided such is possible or appropriate under the circumstances.

The compounds, including their salts, can also be obtained in the form of their hydrates, or include other solvents used for their crystallization.

The instant invention also includes pharmaceutical compositions, for example, useful in inhibiting DPP-IV, comprising a pharmaceutically acceptable carrier or diluent and a therapeutically effective amount of a compound of formula I, or a pharmaceutically acceptable acid addition salt thereof.

In still another embodiment, the instant invention provides a method of inhibiting DPP-IV comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound of formula I, or a pharmaceutically acceptable acid addition salt thereof.

In a further embodiment, the instant invention provides a method of treating conditions mediated by DPP-IV inhibition comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound of formula I above, or a pharmaceutically acceptable acid addition salt thereof.

The present invention also relates to the use of a compound according to the instant invention or a pharmaceutically acceptable salt thereof, e.g., for the manufacture of a medicament for the prevention or treatment of diseases or conditions associated with elevated levels of DPP-IV.

As indicated above, all of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, are useful in inhibiting DPP-IV. The ability of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, to inhibit DPP-IV may be demonstrated employing the Caco-2 DPP-IV Assay which measures the ability of test compounds to inhibit DPP-IV activity from human colonic carcinoma cell extracts. The human colonic carcinoma cell line Caco-2 was obtained from the American Type Culture Collection (ATCC HTB 37). Differentiation of the cells to induce DPP-IV expression was accomplished as described by Reisher, et al. in an article entitled "Increased expression of intestinal cell line Caco-2" in Proc. Natl. Acad. Sci., Vol. 90, pgs. 5757–5761 (1993). Cell extract is prepared from cells solubilized in 10 mM Tris HCl, 0.15 M NaCl, 0.04 t.i.u. aprotinin, 0.5% nonidet-P40, pH 8.0, which is centrifuged at 35,000 g for 30 min. at 4° C. to remove cell debris. The assay is conducted by adding 20 μg solubilized Caco-2 protein, diluted to a final volume of 125 μl in assay buffer (25 mM Tris HCl pH 7.4, 140 mM NaCl, 10 mM KCl, 1% bovine serum albumin) to microtiter plate wells. After a 60 min. incubation at room temperature, the reaction is initiated by adding 25 μl of 1 mM substrate (H-Alanine-Proline-pNA; pNA is p-nitroaniline). The reaction is carried out at room temperature for 10 minutes after which time a 19 μl volume of 25% glacial acetic acid is added to stop the reaction. Test compounds are typically added as 30 μl additions and the assay buffer volume is reduced to 95 μl. A standard curve of free p-nitroaniline is generated using 0–500 μM solutions of free pNA in assay buffer. The curve generated is linear and is used for interpolation of substrate consumption (catalytic activity in nmoles substrate cleaved/min). The endpoint is determined by measuring absorbance at 405 nm in a Molecular Devices UV Max microtiter plate reader.

The potency of the test compounds as DPP-IV inhibitors, expressed as IC_{50}, is calculated from 8-point, dose-response curves using a 4-parameter logistic function.

The following IC_{50} was obtained:

Compound	Caco-2 DPP-IV (nM)
Ex. 1	3.5 ± 1.5
Ex. 4	8

The ability of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, to inhibit DPP-IV may also be demonstrated by measuring the effects of test compounds on DPP-IV activity in human and rat plasma employing a modified version of the assay described by Kubota, et al. in an article entitled "Involvement of dipeptidylpeptidase IV in an in vivo immune response" in Clin. Exp. Immunol., Vol. 89, pgs. 192–197 (1992). Briefly, 5 μl of plasma are added to 96-well flat-bottom microtiter plates (Falcon), followed by the addition of 5 μl of 80 mM $MgCl_2$ in incubation buffer (25 mMHEPES, 140 mM NaCl, 1% RIA-grade BSA, pH 7.8). After a 60 min. incubation at room temperature, the reaction is initiated by the addition of 10 μl of incubation buffer containing 0.1 mM substrate (H-Glycine-Proline-AMC;AMC is 7-amino-4-methylcoumarin). The plates are covered with aluminum foil (or kept in the dark) and incubated at room temperature for 20 min. After the 20 min. reaction, fluorescence is measured using a CytoFluor 2350 fluorimeter (Excitation 380 nm Emission 460 nm; sensitivity setting 4). Test compounds are typically added as 2 μl additions and the assay buffer volume is reduced to 13 μl. A fluorescence-concentration curve of free AMC is generated using 0–50 μM solutions of AMC in assay buffer. The curve generated is linear and is used for interpolation of substrate consumption (catalytic activity in omoles substrate cleaved/min). As with the previous assay, the potency of the test compounds as DPP-IV inhibitors, expressed as IC_{50}, is calculated from 8-point, dose-response curves using a 4 parameter logistic function.

The following IC_{50} was obtained:

Compound	human plasma DPP-IV (nM)	rat plasma DPP-IV (nM)
Ex. 1	2.7 ± 0.1	2.3 ± 0.1
Ex. 8	6	12

In view of their ability to inhibit DPP-IV, the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, are useful in treating conditions mediated by DPP-IV inhibition. Based on the above and findings in the literature, it is expected that the compounds disclosed herein are useful in the treatment of conditions such as non-insulin-dependent diabetes mellitus, arthritis, obesity, allograft transplantation and calcitonin-osteoporosis. In addition, based on the roles of glucagon-like peptides (such as GLP-1 and GLP-2) and their association with DPP-IV inhibition, it is expected that the compounds disclosed herein are useful for example, to produce a sedative or anxiolytic effect, or to attenuate post-surgical catabolic changes and hormonal responses to stress, or to reduce mortality and morbidity after myocardial infarction,or in the treatment of conditions related to the above effects which may be mediated by GLP-1 and/or GLP-2 levels.

More specifically, for example, the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, improve early insulin response to an oral glucose challenge and, therefore, are useful in treating non-insulin-dependent diabetes mellitus. The ability of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, to improve early insulin response to an oral glucose challenge may be measured in insulin resistant rats according to the following method:

Male Sprague-Dawley rats that had been fed a high fat diet (saturated fat=57% calories) for 2–3 weeks were fasted for approximately 2 hours on the day of testing, divided into groups of 8–10, and dosed orally with 10 μmol/kg of the test compounds in CMC. An oral glucose bolus of 1 g/kg was administered 30 minutes after the test compound directly into the stomach of the test animals. Blood samples, obtained at various timepoints from chronic jugular vein catheters, were analyzed for plasma glucose and immunoreactive insulin (IRI) concentrations, and plasma DPP-IV activity. Plasma insulin levels were assayed by a double antibody radioimmunoassay (RIA) method using a specific anti-rat insulin antibody from Linco Research (St. Louis, Mo.). The RIA has a lower limit of detection of 0.5 μU/mL with intra- and inter-assay variations of less than 5%. Data are expressed as % increase of the mean of the control animals. Upon oral administration, each of the compounds tested amplified the early insulin response which led to an improvement in glucose tolerance in the insulin resistant test animals. The following results were obtained:

Compound	Increase of Insulin Response at 10 μmol/kg
Ex. 1	64%

The precise dosage of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, to be employed for treating conditions mediated by DPP-IV inhibition depends upon several factors, including the host, the nature and the severity of the condition being treated, the mode of administration and the particular compound employed. However, in general, conditions mediated by DPP-IV inhibition are effectively treated when a compound of formula I, or a corresponding pharmaceutically acceptable acid addition salt, is administered enterally, e.g., orally, or parenterally, e.g., intravenously, preferably orally, at a daily dosage of 0.002–5, preferably 0.02–2.5 mg/kg body weight or, for most larger primates, a daily dosage of 0.1–250, preferably 1–100 mg. A typical oral dosage unit is 0.01–0.75 mg/kg, one to three times a day. Usually, a small dose is administered initially and the dosage is gradually increased until the optimal dosage for the host under treatment is determined. The upper limit of dosage is that imposed by side effects and can be determined by trial for the host being treated.

The compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, may be combined with one or more pharmaceutically acceptable carriers and, optionally, one or more other conventional pharmaceutical adjuvants and administered enterally, e.g., orally, in the form of tablets, capsules, caplets, etc. or parenterally, e.g., intravenously, in the form of sterile injectable solutions or suspensions. The enteral and parenteral compositions may be prepared by conventional means.

The compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, may be formulated into enteral and parenteral pharmaceutical compositions containing an amount of the active substance that is effective for treating conditions mediated by DPP-IV

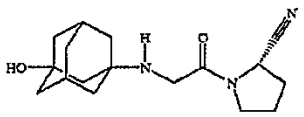
inhibition, such compositions in unit dosage form and such compositions comprising a pharmaceutically acceptable carrier.

The compounds of formula I (including those of each of the subscopes thereof and each of the examples) may be administered in enantiomerically pure form (e.g., ee >98%, preferably >99%) or together with the R enantiomer, e.g., in racemic form. The above dosage ranges are based on the compounds of formula I (excluding the amount of the R enantiomer).

The following examples show representative compounds encompassed by this invention and their synthesis. However, it should be clearly understood that they are for purposes of illustration only.

EXAMPLE 1

Pyrrolidine, 1-[(3-hydroxy-1-adamantyl)amino]acetyl-2-cyano-, (S)



A. 1-Aminoadamantane-3-ol

Slight modifications to the synthesis found in Khim.-Farm. Zh. (1986), 20(7), 810–15, may be used.

To a rapidly stirred, clear and colorless, ice-water chilled mixture of concentrated sulfuric acid 96% (210 mL; 3,943 mmol) and 65% nitric acid (21.0 mL; 217.0 mmol) is added 21.0 g (112.0 mmol) of 1-adamantylamine HCl (99%), in small portions over 30 minutes. Upon adamantylamine hydrochloride addition, slight bubbling occurs and the reaction is slightly exothermic. This bubbling, yellow solution is stirred at ice-water temperature for about 2 hours and then at room temperature for 30 hours. This clear, light yellow reaction is then poured into about 100 g of ice and the resulting solution is clear green-blue.

The solution is placed in an ice-water bath and allowed to stir for 30 minutes. Approximately 550 g of 89% pure KOH (8.74 mol) is then added in small portions over 45 minutes. During this addition, the reaction is exothermic; reaching 80° C. and producing copious amounts of brown NO_2 gas. By the end of the addition, the reaction is thick with white solids (both product and salts). The resulting white paste is then poured onto a buchner funnel/celite pad and washed with 1.2 L of CH_2Cl_2. The CH_2Cl_2 layer is then extracted from the water layer and dried over Na_2SO_4. The solution is then filtered and concentrated (rotovap/pump) to provide 1-aminoadamantane-3-ol as a white solid.

Alternatively, the reaction may be carried out using n-butanol as solvent instead of methylene chloride.

Alternatively, 1-aminoadamantane-3-ol can be prepared e.g. as follows: A 2-L, 4-necked, round-bottomed flask is thoroughly flushed with nitrogen. The flask is charged under nitrogen with 420 mL of conc. sulfuric acid (98%). The contents are cooled to 8° C., then slowly (slightly exothermic & HCl gas evolution) 100.8 g of 1-aminoadamantane hydrochloride are added into the mixture in 8 portions at 9–10° C. over 20 min (minutes), then the hazy contents are stirred at 9–10° C. for 20 min to obtain a homogenous mixture. 72 mL of conc. (concentrated) nitric acid (70%) are added (very exothermic) dropwise into the mixture maintaining inner temperature at 14–15° C. with efficient cooling (at this scale 20 min. needed for this addition). The mixture is stirred at 14–15° C. for 20 min, the temperature is allowed to raise to 25° C. over 1 h (hour) (15–20° C. for 30 min, and 20–25° C. for 30 min), then the contents a stirred at 24–25.5° C. for 5 h (external cooling is needed). 1.7 L of water are charged into a 5-L, 4-necked flask, the water is cooled to 10° C., then the reaction mixture is slowly poured (very exothermic, some NO_2 gas evolution) over 25 min. maintaining the internal temperature below 35° C. to give a blue-green homogenous solution. The original 2-L flask (slightly exothermic) is rinsed once with 0.3 L of water and the water wash is poured into the 5-L flask. Slowly 900 mL of 50% sodium hydroxide aqueous solution are added (very exothermic, some NO_2 gas evolution) into the 5-L flask over 30 min. at 65–70° C. to bring the pH of the mixture to 13. 800 mL of 1-butanol and 200 mL of toluene are added (not exothermic) under vigorous stirring and allow the mixture to reach 30° C. The bottom aqueous layer is separated for proper disposal. The organic layer is once washed with 100 mL of saturated sodium chloride solution. The saturated sodium chloride wash is saved for disposal. The organic layer is concentrated at 60–85° C. (20–200 mbar) to give a pale yellow viscous oil. 600 mL of heptane and 50 mL of methanol are added into and the mixture is maintained at 40–50° C. for 15 min to give a thick suspension. The slurry is cooled to 12° C. and maintained at 12–14° C. for 15 min. The solids are filtered off through a polypropylene pad and Buchner funnel, then the flask and filter cake are washed once with 80 mL of heptane. The methanol/heptane filtrate is saved. The filter cake is dried at 55–60° C. (30 mbar) for 16 h to afford 1-aminoadamantane-3-ol as an off-white solid.

B. 1-Chloroacetyl-2-cyanopyrrolidine

To a mechanically stirred solution of 20.0 g (180.0 mmol) of chloroacetylchloride and 97 g (0.70 mmol) of potassium carbonate in 150 mL of tetrahydrofuran is added a solution of L-prolinamide 20.0 g (180.0 mmol) in 500 mL of tetrahydrofuran in a dropwise fashion over 45 minutes. This reaction is then mechanically stirred for an additional two hours at room temperature. The reaction is then filtered to remove potassium salts and the filtrate is dried over Na_2SO_4. The Na_2SO_4 is then removed via filtration and to this colorless filtrate is added trifluoroacetic anhydride (25.0 mL, 0.180 mmol) in one portion. The reaction is then magnetically stirred for 1 hour at room temperature and the resulting clear yellow/orange solution is concentrated via rotovap. The excess trifluoroacetic anhydride is removed by adding ethyl acetate to the concentrated oil and reconcentrating via rotovap. This removing operation is performed three times.

The resulting oil is partitioned between ethyl acetate and water. The product is then extracted into the ethyl acetate and the aqueous layer is then washed twice with ethyl acetate. The combined organic layers are then washed successively with water and brine dried over magnesium sulfate, filtered and concentrated to obtain 1-chloroacetyl-2-cyanopyrrolidine as a yellow solid.

Alternatively, the reaction may be carried out by using, as base, a mixture, e.g. 2-ethyl-hexanoic acid/sodium hydride.

C. Pyrrolidine, 1-[(3-hydroxy-1-adamantyl)amino]acetyl-2-cyano-, (S)

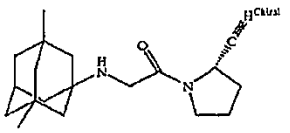
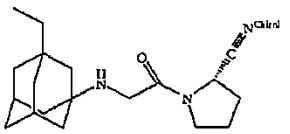
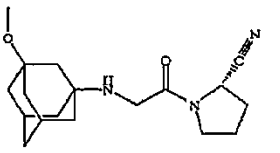
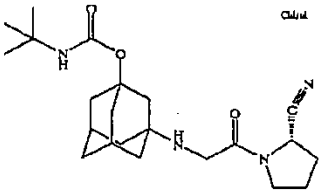
To a heterogeneous solution of the title A compound (1-aminoadamantane-3-ol (5.80 g, 34.7 mmol) in CH_2Cl_2 (68.0 mL) is added 9.6 g (69 mmol) of K_2CO_3. This heterogeneous mixture is then cooled in an ice-water bath and a solution of 3.0 g (17 mmol) of the title B compound (1-chloroacetyl-2-cyanopyrrolidine) dissolved in 25.0 mL of CH_2Cl_2 is added dropwise over a period of 30 minutes. The resulting mixture is stirred for 2 hours at 0° C. and at room temperature for 6 days. The reaction is then concentrated to

obtain a yellow pasty material which is purified on silica gel employing a SIMS/Biotage Flash chromatography system and a 7% solution of methanol in methylene chloride as the eluent to yield the title compound in free base form as a white crystalline solid (melting point 138° C.–140° C., ^{13}CNMR (ppm)=119.59).

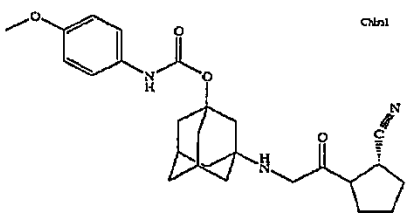
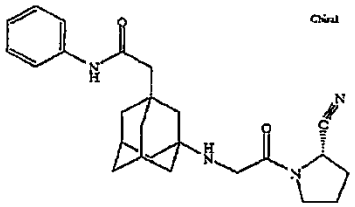
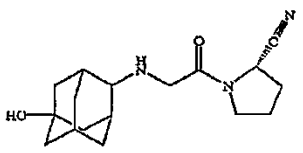
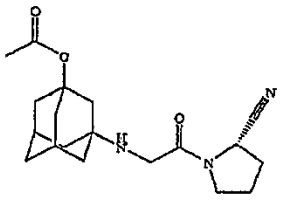
Alternatively, the reaction may be carried out using tetrahydrofuran as solvent instead of methylene chloride; furthermore, the chromatography step may be eliminated.

EXAMPLES 2 TO 12

The following compounds are prepared analogous to the method of Example 1 (especially Step C):

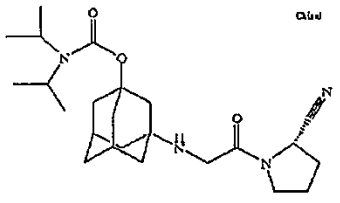
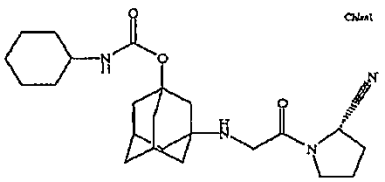
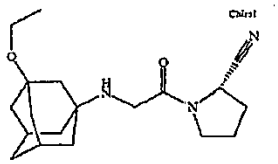
Example	Structure	M.P. [° C.]
2		103–105 (HCl)
	Pyrrolidine, 1-[[(3,5-dimethyl-1-adamantyl)amino]-acetyl]-2-cyano-, (S)-	
3		212–214 (HCl)
	Pyrrolidine, 1-[[(3-ethyl-1-adamantyl)amino]acetyl]-2-cyano-, (S)-	
4		92–94 (HCl)
	Pyrrolidine, 1-[[(3-methoxy-1-adamantyl)amino]-acetyl]-2-cyano-, (S)-	
5		210–212 (HCl)
	Pyrrolidine, 1-[[[3-[[(t-butylamino)carbonyl]oxy]-1-adamantyl]amino]acetyl]-2-cyano-, (S)-	

-continued

Example	Structure	M.P. [° C.]
6	 Chiral	212–214 (HCl)
	Pyrrolidine, 1-[[[3-[[[(4-methoxyphenyl)amino]-carbonyl]oxy]-1-adamantyl]amino]acetyl]-2-cyano-, (S)-	
7	 Chiral	205–207 (HCl)
	Pyrrolidine, 1-[[[(3-[[(phenylamino)carbonyl]oxy]-1-adamantyl]amino]acetyl]-2-cyano-, (S)-	
8		[^{13}C NMR (CN group): 121.56 (ppm)] (HCl)
	Pyrrolidine, 1-[[(5-hydroxy-2-adamantyl)amino]-acetyl]-2-cyano-, (S)-	
9		[^{13}C NMR (CN group): 118.54 (ppm)]
	Pyrrolidine, 1-[[(3-acetyloxy-1-adamantyl)amino]-acetyl]-2-cyano-, (S)-	

AZ-SAXA-8024007

-continued

Example	Structure	M.P. [° C.]
10		143–150 (HCl)
	Pyrrolidine, 1-[[[3-[[[(diisopropyl)amino]carbonyl]-oxy]-1-adamantyl]amino]acetyl]-2-cyano-, (S)-	
11		155–157 (HCl)
	Pyrrolidine, 1-[[[3-[[[(cyclohexyl)amino]carbonyl]-oxy]-1-adamantyl]amino]acetyl]-2-cyano-, (S)-	
12		[^{13}C NMR (CN group): 119.31 (ppm)] (HCl)
	Pyrrolidine, 1-[[(3-ethoxy-1-adamantyl)amino]-acetyl]-2-cyano-, (S)-	

(HCl) = as hydrochloride

All HCl salts of final products are prepared by passing HCl gas through a 0.1 Molar solution of the free base in tetrahydrofuran until solution is clearly acidic followed by removal of the solvent (rotovap/pump).

The amino-adamantane starting materials are known in the literature or can be prepared as follows:

The manufacture of 3,5-dimethyl-1-adamantylamine is described in J. Med. Chem, 25; 1; 1982; 51–56.

The manufacture of 3-ethyl-1-adamantylamine is described in J. Med. Chem, 25; 1; 1982; 51–56.

3-Methoxy-1-adamantylamine can be prepared as follows

To a stirred, ice-water chilled suspension of potassium hydride (0.680 gm; 5.95 mmol) in 15.0 ml of tetrahydofuran is added a mixture of 1-aminoadamantane-3-ol (1.00 g; 5.95 mmol) and 15.0 ml of tetrahydrofuran dropwise over 30 minutes. The resulting mixture is then stirred for an addition 30 minutes and iodomethane (0.370 ml; 5.95 mmol) is then added dropwise over one minute. The resulting opaque white reaction is then stirred at room temperature for 18 hours. The mixture is then diluted with 50 ml of methylene chloride and filtered to remove the inorganic impurities. The filtrate is then concentrated and purified on silica gel employing a SIMS/Biotage apparatus and 19% methanol and 19% ammonium hydroxide in methylene chloride as eluent to yield 3-methoxy-1-adamantylamine as an opaque oil.

Synthesis of 3-[[(tertbutylamino)carbonyl]oxy]-1-aminoadamantane

To a mixture of 1-aminoadamantane-3-ol (5.00 g; 30.0 mmol) and potassium carbonate (6.20 g; 45 mmol) in 150 ml of tetrahydrofuran is added benzylchloroformate (4.70 g, 33.0 mmol) in dropwise fashion over a 10 minute period. The mixture is then stirred at room temperature for 2 h and then partitioned between ethyl acetate and water. The product is then extracted into the ethyl acetate and the aqueous layer is washed twice with ethyl acetate (100 ml). The combined organic layers are then washed successively with 100 ml of aqueous 2 N sodium hydroxide, water and brine, dried over sodium sulfate, filtered and concentrated (rotovap/pump) to provide 1-benzylcarbamoyladamantane-3-ol as a white solid in 85% yield.

To a clear solution of 1-benzylcarbamoyladamantane-3-ol (1.00 g; 3.32 mmol) and tert-butylisocyanate (380 μl, 3.32 mmol) in 30 ml of methylene chloride is syringe-added

trimethylsilyl chloride (20.0 µl, 0.17 mmol). This reaction is then stirred at room temperature for 18 hours, concentrated (rotovap) and purified on silica gel employing a SIMS/Biotage apparatus and 20% ethyl acetate in hexane as eluent to yield 3-[[(tertbutylamino)carbonyl]oxy]-1-benzylcarbamoyladamantane as a white solid in quantitative yield.

To a mixture of 3-[[(tertbutylamino)carbonyl]oxy]-1-benzylcarbamoyladamantane (1.50 g, 3.75 mmol) and 10% palladium on carbon (400 mg) in ethanol (150 ml) in a 1-liter parr hydrogenation flask is added hydrogen (50 psi). This opaque black mixture is then shaken for 24 h. The reaction is then filtered through celite to remove the palladium catalyst and concentrated (rotovap/pump) to provide 3-[[(tertbutylamino)carbonyl]oxy]-1-aminoadamantane as a clear oil in 99% yield.

The procedure for the synthesis of 4-[[[(methoxyphenyl) amino]carbonyl]oxy]-1-aminoadamantane is essentially the procedure of 3-[[(tertbutylamino)carbonyl]oxy]-1-aminoadamantane except in the second step where an equivalent of 4-methoxyphenyl isocyanate replaces tert-butylisocyanate, 1,2-dichloroethane is used as solvent instead of methylene chloride and the reaction is stirred at 50° C. for 18 hours. The final amine intermediate is provided as an oil.

The procedure for the synthesis of 3-[[(phenylamino) carbonyl]oxy]-1-aminoadamantane is essentially the procedure of 3-[[(tertbutylamino)carbonyl]oxy]-1-aminoadamantane except in the second step where an equivalent of phenyl isocyanate replaces the tert-butylisocyanate, 1,2-dichloroethane is used as solvent instead of methylene chloride and the reaction is stirred at 50° C. for 18 hours. The final amine intermediate is provided as a clear oil.

The procedure to make 2-aminoadamantane-5-ol is the same as in Example 1 except that the starting material is 2-aminoadamantane instead of 1-aminoadamantane.

The procedure for the synthesis of the nucleophile 3-acetoxy-1-aminoadamantane is essentially the procedure of 3-[[(tertbutylamino)carbonyl]oxy]-1-aminoadamantane except for a standard acylation of 1-benzylcarbamoyladamantane-3-ol using 1.2 eq of acetyl chloride, 3.0 eq. of pyridine, 0.1 eq of 4-dimethylaminopyridine and 1,2 dichloroethane which are all stirred at room temperature for 24 hours. The final amine is provided as a thick oil.

The procedure for the synthesis of 3-[[[(diisopropyl) amino]carbonyl]oxy]-1-amino-adamantane is essentially the procedure of 3-[[(tertbutylamino)carbonyl]oxy]-1-aminoadamantane except in the second step where an equivalent of diisopropylcarbamoyl chloride replaces the tert-butylisocyanate, 1,2-dichloroethane is used as solvent instead of methylene chloride and the reaction is stirred at 85° C. for 18 hours. The final amine intermediate is provided as a gray solid.

The procedure for the synthesis of 3-[[[(cyclohexyl) amino]carbonyl]oxy]-1-aminoadamantane is essentially the procedure of 3-[[(tertbutylamino)carbonyl]oxy]-1-aminoadamantane except in the second step where an equivalent of cyclohexylisocyanate replaces the tert-butylisocyanate, 1,2-dichloroethane is used as solvent instead of methylene chloride and the reaction is stirred at 50° C. for 18 hours. The final amine intermediate is provided as a thick clear oil.

The procedure to make 3-ethoxy-1-adamantylamine (a clear oil) is the same as for 3-methoxy-1-adamantylamine except that iodoethane (1.3 equivalent) is used instead of iodomethane.

Formulation Example

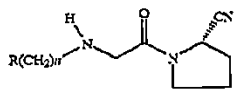
Tablets, each containing 50 mg of active ingredient, for example, (S) 1[(3-hydroxy-1-adamantyl)amino]acetyl-2-cyano-pyrrolidine, can be prepared as follows:

Composition (for 10,000 tablets)	
Active ingredient	500.0 g
Lactose	500.0 g
Potato starch	352.0 g
Gelatin	8.0 g
Talc	60.0 g
Magnesium stearate	10.0 g
Silica (highly disperse)	20.0 g
Ethanol	q.s.

The active ingredient is mixed with the lactose and 292 g of potato starch, and the mixture is moistened using an alcoholic solution of the gelatin and granulated by means of a sieve. After drying, the remainder of the potato starch, the talc, the magnesium stearate and the highly disperse silica are admixed and the mixture is compressed to give tablets of weight 145.0 mg each and active ingredient content 50.0 mg which, if desired, can be provided with breaking notches for finer adjustment of the dose.

What is claimed is:

1. A compound of formula I:



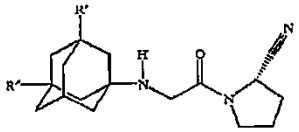
(I)

wherein

R is substituted adamantyl; and

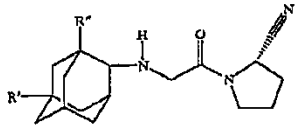
n is 0 to 3; in free form or in acid addition salt form.

2. A compound according to claim 1 of formula (I A) or (I B)



(IA)

or



(IB)

wherein R' represents hydroxy, C_1–C_7alkoxy, C_1–C_8-alkanoyloxy, or R_4R_5N—CO—O——, where R_4 and R_5 independently are C_1–C_7alkyl or phenyl which is unsubstituted or substituted by a substituent selected from C_1–C_7alkyl, C_1–C_7alkoxy, halogen and trifluoromethyl and where R_4 additionally is hydrogen; or R_4 and R_5 together represent C_3–C_6alkylene; and R" represents hydrogen; or R'

and R^a independently represent C_1–C_2alkyl; in free form or in form of a pharmaceutically acceptable acid addition salt.

3. A compound according to claim 1 selected from the group consisting of:

pyrrolidine, 1-[[(3,5-dimethyl-1-adamantyl)amino]-acetyl]-2-cyano-, (S)-;

pyrrolidine, 1-[[(3-ethyl-1-adamantyl)amino]acetyl]-2-cyano-, (S)-;

pyrrolidine, 1-[[(3-methoxy-1-adamantyl)amino]-acetyl]-2-cyano-, (S)-;

pyrrolidine, 1-[[[3-[[(t-butylamino)carbonyl]oxy]-1-adamantyl]amino]acetyl]-2-cyano-, (S)-;

pyrrolidine, 1-[[[3-[[[(4-methoxyphenyl)amino]-carbonyl]oxy]-1-adamantyl]amino]acetyl]-2-cyano-, (S)-;

pyrrolidine, 1-[[[(3-[[(phenylamino)carbonyl]oxy]-1-adamantyl]amino]acetyl]-2-cyano-, (S)-;

pyrrolidine, 1-[[(5-hydroxy-2-adamantyl)amino]-acetyl]-2-cyano-, (S)-;

pyrrolidine, 1-[[(3-acetyloxy-1-adamantyl)amino]acetyl]-2-cyano-, (S)-;

pyrrolidine, 1-[[[3-[[[(diisopropyl)amino]carbonyl]oxy]-1-adamantyl]amino]acetyl]-2-cyano-, (S)-

pyrrolidine, 1-[[[3-[[[(cyclohexyl)amine]carbonyl]oxy]-1-adamantyl]amino]acetyl]-2-cyano-, (S)-; and

pyrrolidine, 1-[[(3-ethoxy-1-adamantyl)amino]acetyl]-2-cyano-, (S)-;

or, in each case, a pharmaceutically acceptable acid addition salt thereof.

4. A compound according to claim 1 which is pyrrolidine, 1-[(3-hydroxy-1-adamantyl)amino]acetyl-2cyano-, (S), or a pharmaceutically acceptable acid addition salt thereof.

5. A pharmaceutical composition comprising a compound according to claim 1 in free form or in pharmaceutically acceptable acid addition salt form, together with at least one pharmaceutically acceptable carrier or diluent.

6. A method of inhibiting dipeptidyl peptidase-IV comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound according to claim 1, or a pharmaceutically acceptable acid addition salt thereof.

7. A method of treating conditions mediated by dipeptidyl peptidase-IV inhibition comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound according to claim 1, or a pharmaceutically acceptable acid addition salt thereof.

8. The method according to claim 7 wherein the condition treated is non-insulin-dependent diabetes mellitus.

9. The method according to claim 7 wherein the condition treated is obesity.

* * * * *

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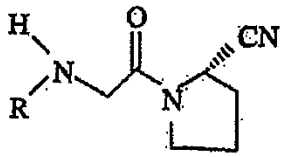
(54) Title: N-SUBSTITUTED 2-CYANOPYRROLIDINES

(57) Abstract

N-(N'-substituted glycyl)-2-cyanopyrrolidines, e.g. the compounds of formula (I) wherein R has various significances, are novel. They inhibit DPP-IV (dipeptidyl-peptidase-IV) activity. They are therefore indicated for use as pharmaceuticals in inhibiting DPP-IV and in the treatment of conditions mediated by DPP-IV, such as non-insulin-dependent diabetes mellitus, arthritis, obesity, osteoporosis and further conditions of impaired glucose tolerance.



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N-SUBSTITUTED 2-CYANOPYRROLIDINES

Field

The present invention relates to N-substituted 2-cyanopyrrolidines. More particularly, it provides novel N-glycyl-2-cyanopyrrolidine derivatives.

Background

Dipeptidyl peptidase-IV (**DPP-IV**) is a serine protease which cleaves N-terminal dipeptides from a peptide chain containing, preferably, a proline residue in the penultimate position. Although the biological role of DPP-IV in mammalian systems has not been completely established, it is believed to play an important role in neuropeptide metabolism, T-cell activation, attachment of cancer cells to the endothelium and the entry of HIV into lymphoid cells. DPP-IV is responsible for inactivating glucagon-like peptide-1 (**GLP-1**). More particularly, DPP-IV cleaves the amino-terminal His-Ala dipeptide of GLP-1, generating a GLP-1 receptor antagonist, and thereby shortens the physiological response to GLP-1. Since the half-life for DPP-IV cleavage is much shorter than the half-life for removal of GLP-1 from circulation, a significant increase in GLP-1 bioactivity (5- to 10-fold) is anticipated from DPP-IV inhibition. Since GLP-1 is a major stimulator of pancreatic insulin secretion and has direct beneficial effects on glucose disposal, DPP-IV inhibition appears to represent an attractive approach for treating non-insulin-dependent diabetes mellitus (**NIDDM**).

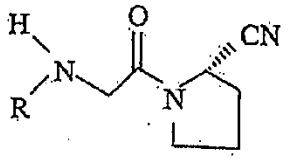
Although a number of DPP-IV inhibitors have been described, all have limitations relating to potency, stability or toxicity. Accordingly, a great need exists for novel DPP-IV inhibitors which are useful in treating conditions mediated by DPP-IV inhibition and which do not suffer from the above-mentioned limitations.

Summary of the invention

The invention provides novel N-(N'-substituted glycyl)-2-cyanopyrrolidines which are effective as DPP-IV inhibitors in treating conditions mediated by DPP-IV. It also concerns corresponding pharmaceutical compositions, a process for their preparation, a method of inhibiting DPP-IV comprising administering to a patient in need of such treatment a therapeutically effective amount thereof, the compounds for use as a pharmaceutical, and their use in a process for the preparation of a medicament for treating a condition mediated by DPP-IV.

Detailed description

The invention concerns N-(N'-substituted glycyl)-2-cyanopyrrolidines, hereinafter briefly named "**the compounds of the invention**"; more particularly, it concerns compounds of formula I:



I

wherein **R** is:

a) $R_1R_{1a}N(CH_2)_m$- wherein

R_1 is a pyridinyl or pyrimidinyl moiety optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy, halogen, trifluoromethyl, cyano or nitro; or phenyl optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen;

R_{1a} is hydrogen or (C_{1-8})alkyl; and

m is 2 or 3;

b) (C_{3-12})cycloalkyl optionally monosubstituted in the 1-position with (C_{1-3})hydroxyalkyl;

c) $R_2(CH_2)_n$- wherein either

R_2 is phenyl optionally mono- or independently di- or independently trisubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy, halogen or phenylthio optionally monosubstituted in the phenyl ring with hydroxymethyl; or is (C_{1-8})alkyl; a [3.1.1]bicyclic carbocyclic moiety optionally mono- or plurisubstituted with (C_{1-8})alkyl;

a pyridinyl or naphthyl moiety optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen; cyclohexene; or adamantyl; and

n is 1 to 3; or

R_2 is phenoxy optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen; and

n is 2 or 3;

d) $(R_3)_2CH(CH_2)_2$- wherein each R_3 independently is phenyl optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen;

e) $R_4(CH_2)_p$- wherein R_4 is 2-oxopyrrolidinyl or (C_{2-4})alkoxy and

p is 2 to 4;

f) isopropyl optionally monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;

g) R_5 wherein R_5 is: indanyl; a pyrrolidinyl or piperidinyl moiety optionally substituted with benzyl; a [2.2.1]- or [3.1.1]bicyclic carbocyclic moiety optionally mono- or plurisubstituted with (C_{1-8})alkyl; adamantyl; or (C_{1-8})alkyl optionally mono- or independently plurisubstituted with hydroxy, hydroxymethyl or phenyl optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen;

in free form or in acid addition salt form.

The compounds of formula I can exist in free form or in acid addition salt form. Salt forms may be recovered from the free form in known manner and vice-versa. Acid addition salts may e.g. be those of pharmaceutically acceptable organic or inorganic acids. Although the preferred acid addition salts are the hydrochlorides, salts of methanesulfonic, sulfuric, phosphoric, citric, lactic and acetic acid may also be utilized.

The compounds of the invention may exist in the form of optically active isomers or diastereoisomers and can be separated and recovered by conventional techniques, such as chromatography.

"Alkyl" and "alkoxy" are either straight or branched chain, of which examples of the latter are isopropyl and tert-butyl.

R preferably is a), b) or e) as defined above. R_1 preferably is a pyridinyl or pyrimidinyl moiety optionally substituted as defined above. R_{1a} preferably is hydrogen. R_2 preferably is phenyl optionally substituted as defined above. R_3 preferably is unsubstituted phenyl.

R_4 preferably is alkoxy as defined above. R_5 preferably is optionally substituted alkyl as defined above. m preferably is 2. n preferably is 1 or 2, especially 2. p preferably is 2 or 3, especially 3.

Pyridinyl preferably is pyridin-2-yl; it preferably is unsubstituted or monosubstituted, preferably in 5-position. Pyrimidinyl preferably is pyrimidin-2-yl. It preferably is unsubstituted or monosubstituted, preferably in 4-position. Preferred as substitutents for pyridinyl and pyrimidinyl are halogen, cyano and nitro, especially chlorine.

When it is substituted, phenyl preferably is monosubstituted; it preferably is substituted with halogen, preferably chlorine, or methoxy. It preferably is substituted in 2-, 4- and/or 5-position, especially in 4-position.

(C_{3-12})cycloalkyl preferably is cyclopentyl or cyclohexyl. When it is substituted, it preferably is substituted with hydroxymethyl. (C_{1-4})alkoxy preferably is of 1 or 2 carbon atoms, it especially is methoxy. (C_{2-4})alkoxy preferably is of 3 carbon atoms, it especially is isopropoxy. Halogen is fluorine, chlorine, bromine or iodine, preferably fluorine, chlorine or bromine, especially chlorine. (C_{1-8})alkyl preferably is of 1 to 6, preferably 1 to 4 or 3 to 5, especially of 2 or 3 carbon atoms, or methyl. (C_{1-4}) alkyl preferably is methyl or ethyl, especially methyl. (C_{1-3})hydroxyalkyl preferably is hydroxymethyl.

A [3.1.1]bicyclic carbocyclic moiety optionally substituted as defined above preferably is bicyclo[3.1.1]hept-2-yl optionally disubstituted in 6-position with methyl, or bicyclo[3.1.1]hept-3-yl optionally trisubstituted with one methyl in 2-position and two methyl groups in 6-position. A [2.2.1]bicyclic carbocyclic moiety optionally substituted as defined above preferably is bicyclo[2.2.1]hept-2-yl.

Naphthyl preferably is 1-naphthyl. Cyclohexene preferably is cyclohex-1-en-1-yl. Adamantyl preferably is 1- or 2-adamantyl.

A pyrrolidinyl or piperidinyl moiety optionally substituted as defined above preferably is pyrrolidin-3-yl or piperidin-4-yl. When it is substituted it preferably is N-substituted.

A preferred group of compounds of the invention is the compounds of formula I wherein R is R' (**compounds Ia**), whereby R' is:

- R_1'$NH(CH_2)_2$- wherein R_1' is pyridinyl optionally mono- or independently disubstituted with halogen, trifluoromethyl, cyano or nitro; or unsubstituted pyrimidinyl;
- (C_{3-7})cycloalkyl optionally monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;
- R_4'$(CH_2)_3$- wherein R_4' is (C_{2-4})alkoxy; or
- R_5, wherein R_5 is as defined above;

in free form or in acid addition salt form.

More preferred compounds of the invention are those compounds of formula I wherein R is R'' (**compounds Ib**), whereby R'' is:

- R_1''$NH(CH_2)_2$- wherein R_1'' is pyridinyl mono- or independently disubstituted with halogen, trifluoromethyl, cyano or nitro;
- (C_{4-6})cycloalkyl monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;
- R_4'$(CH_2)_3$- wherein R_4' is as defined above; or
- R_5' wherein R_5' is a [2.2.1]- or [3.1.1]bicyclic carbocyclic moiety optionally mono- or plurisubstituted with (C_{1-8})alkyl; or adamantyl;

in free form or in acid addition salt form.

Even more preferred compounds of the invention are the compounds of formula I wherein R is R''' (**compounds Ic**), whereby R''' is:

- R_1''$NH(CH_2)_2$- wherein R_1'' is as defined above;
- (C_{4-6})cycloalkyl monosubstituted in 1-position with hydroxymethyl;
- R_4'$(CH_2)_3$- wherein R_4' is as defined above; or
- R_5'' wherein R_5'' is adamantyl;

in free form or in acid addition salt form.

A further group of compounds of the invention is **compounds Ip,** wherein R is R^P, which is:

a) $R_1^P NH(CH_2)_2$- wherein R_1^P is a pyridinyl or pyrimidinyl moiety optionally mono- or independently disubstituted with halogen, trifluoromethyl, cyano or nitro;

b) (C_{3-7})cycloalkyl optionally monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;

c) $R_2^P(CH_2)_2$- wherein R_2^P is phenyl optionally mono- or independently di- or independently trisubstituted with halogen or (C_{1-3})alkoxy;

d) $(R_3^P)_2CH(CH_2)_2$- wherein each R_3^P independently is phenyl optionally monosubstituted with halogen or (C_{1-3})alkoxy;

e) $R_4(CH_2)_3$- wherein R_4 is as defined above; or

f) isopropyl optionally monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;

in free form or in pharmaceutically acceptable acid addition salt form.

A further group of compounds of the invention is **compounds Is,** wherein R is R^S, which is:

a) $R_1^S R_{1a}^S (CH_2)_{ms}$- wherein R_1^S is pyridinyl optionally mono- or independently disubstituted with chlorine, trifluoromethyl, cyano or nitro; pyrimidinyl optionally monosubstituted with chlorine or trifluoromethyl; or phenyl;

R_{1a}^S is hydrogen or methyl; and

ms is 2 or 3;

b) (C_{3-12})cycloalkyl optionally monosubstituted in 1-position with hydroxymethyl;

c) $R_2^S(CH_2)_{ns}$- wherein either

R_2^S is phenyl optionally mono- or independently di- or independently trisubstituted with halogen, alkoxy of 1 or 2 carbon atoms or phenylthio monosubstituted in the phenyl ring with hydroxymethyl; (C_{1-6})alkyl; 6,6-dimethylbicyclo[3.1.1]hept-2-yl; pyridinyl; naphthyl; cyclohexene; or adamantyl; and ns is 1 to 3; or

R_2^S is phenoxy; and ns is 2;

d) (3,3-diphenyl)propyl;

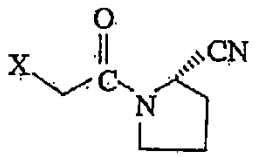
e) $R_4^s(CH_2)_{ps}$ wherein R_4^s is 2-oxopyrrolidin-1-yl or isopropoxy and
ps is 2 or 3;

f) isopropyl optionally monosubstituted in 1-position with hydroxymethyl;

g) R_5^s wherein R_5^s is: indanyl; a pyrrolidinyl or piperidinyl moiety optionally N-substituted with benzyl; bicyclo[2.2.1]hept-2-yl; 2,6,6-trimethylbicyclo-[3.1.1]hept-3-yl; adamantyl; or (C_{1-8})alkyl optionally mono- or independently disubstituted with hydroxy, hydroxymethyl or phenyl;

in free form or in acid addition salt form.

The compounds of the invention may be prepared by a process which comprises coupling a reactive (2-cyanopyrrolidino)carbonylmethylene compound with an appropriate substituted amine; more particularly, for the preparation of the compounds of formula I it comprises reacting a compound of formula II



II

wherein X is a reactive group;

with a compound of formula III

NH_2R III

wherein R is as defined above,

and recovering the resultant compound of formula I in free form or in acid addition salt form.

X preferably is a halogen such as bromine, chlorine or iodine.

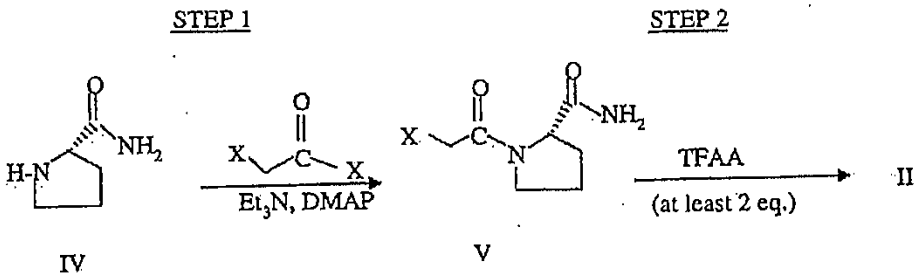
The process of the invention may be effected in conventional manner.

The compound of formula II is preferably reacted with at least 3 equivalents of a primary amine of formula III. The reaction is conveniently conducted in the presence of an inert, organic solvent, preferably a cyclic ether such as tetrahydrofuran. The temperature preferably is of from about 0° to about 35°C, preferably between about 0° and about 25°C.

The compounds of the invention may be isolated from the reaction mixture and purified in conventional manner, e.g. by chromatography.

The starting materials may also be prepared in conventional manner.

The compounds of formula II may e.g. be prepared by the following two-step reaction scheme:



Step 1 involves the reaction of the pyrrolidine of formula IV with a slight molar excess of a haloacetylhalide such as bromoacetylbromide or chloroacetylchloride and triethylamine and a catalytic amount of dimethylaminopyridine (DMAP). The reaction conveniently is conducted in the presence of an inert, organic solvent, preferably a chlorinated, aliphatic hydrocarbon such as methylene chloride, at a temperature of from about 0° to about 25°C, preferably at a temperature between about 0° and about 15°C.

Step 2 concerns the dehydration of the compound of formula V, prepared in Step 1, with at least 2 equivalents of trifluoroacetic anhydride (TFAA). The dehydration preferably is conducted in the presence of an inert, organic solvent such as tetrahydrofuran or a chlorinated, aliphatic hydrocarbon such as methylene chloride, at a temperature of from about 0° to about 25°C, preferably at a temperature between about 0° and about 15°C.

Insofar as its preparation is not particularly described herein, a compound used as starting material is known or may be prepared from known compounds in known manner or analogously to known methods or analogously to methods described in the Examples.

The following Examples illustrate the invention. All temperatures are in degrees Celsius.

Example 1: 1-[2-[(5-Chloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine

To a 500 ml flask is added 16.6 g of **2-[(5-chloropyridin-2-yl)amino]ethylamine** and 100 ml of tetrahydrofuran and the mixture is cooled in an ice bath. To the cooled mixture is added 7.0 g of **(2-cyanopyrrolidino)carbonylmethylene-(S)-bromide** dissolved in 30 ml of tetrahydrofuran. The resultant mixture is stirred for 2 hours at 0°, the solvent is removed by rotovaping and the mixture is partitioned between ethyl acetate and water. The product is then extracted into the ethyl acetate layer and the aqueous layer is then washed twice with ethyl acetate. The combined organic layers are washed successively with water and brine, dried over sodium sulfate and concentrated to obtain the desired free base compound in crude form. The crude form is then purified on silica gel employing a mixture of 5% methanol in methylene chloride as the eluent to yield the **title compound in free base form** as a light brown oil.

After dissolving the **free base** in 30 ml of dry tetrahydrofuran, **hydrogen chloride** gas is bubbled into the solution for five seconds. The off-white precipitate that forms is filtered, washed with dry tetrahydrofuran and the solvent is removed by high vacuum pumping to obtain the **title compound in dihydrochloride acid addition salt form** (off-white solid; m.p. 265°-267°; NMR: see * at bottom of Table hereunder).

The starting material is obtained as follows:

a) 22.37 g of **(S)-2-carbamoylpyrrolidine**, 30.1 ml of triethylamine and 30.0 mg of dimethylaminopyridine **(DMAP)** are dissolved in 200 ml of methylene chloride and the solution is then added, dropwise, to an ice-cold solution of 18.8 ml of **bromoacetylbromide** in 192 ml of methylene chloride, over a period of 60 minutes under a calcium sulfate drying tube. The resultant solution is stirred for 2 hours at ice-water temperature under a calcium sulfate drying tube, then poured into 3.5 liters of ethyl acetate. The resultant precipitate is filtered, washed with ethyl acetate, and the filtrate is concentrated to obtain **(2-carbamoylpyrrolidino)-carbonylmethylene-(S)-bromide** (hard yellow taffy).

b) 50.0 g of the **bromide compound** prepared in a) above is dissolved in 300 ml of methylene chloride and the solution is cooled in an ice water bath under a calcium sulfate drying tube. The cooled solution is then poured into 60.2 ml of **trifluoroacetic anhydride** over a 2 minute period, the resultant solution is stirred at ice-water temperature under a calcium sulfate drying tube for 4 hours, and partitioned between methylene chloride and saturated aqueous sodium bicarbonate. The product is extracted into the methylene chloride layer and the aqueous layer is washed twice with methylene chloride. The combined organic layers are washed successively with water and brine and then dried over sodium sulfate. The solution is filtered and the solvent is removed by rotovaping and high vacuum pumping to obtain **(2-cyanopyrrolidino)carbonylmethylene-(S)-bromide** (dark yellow solid).

The following compounds of the invention, of formula I, are obtained in analogous manner by reacting a corresponding compound of formula II with a corresponding compound of formula III (in the following Table, where only an acid addition salt form of a compound of the invention is mentioned, the compound is recovered from the free base without isolation thereof):

Example No.	R	Form	Analogously to Ex. No.	Characterization data
2	2-[(5-CF_3-pyridin-2-yl)amino]ethyl	b	1[1)]	golden oil; NMR*
3	2-[(5-cyanopyridin-2-yl)amino]ethyl	b	1	golden oil
		dch	1	off-white precipitate, m.p. 155-157°; NMR*; $[\alpha]_D^{20}$= - 77.2° (c=0.012, MeOH)
4	2-[(pyrimidin-2-yl)amino]ethyl	b	2[1a)]	golden oil; NMR*
5	(1-hydroxymethyl)cyclopent-1-yl	b	1[2)]	yellow solid; m.p. 65-67°; NMR*
6	2-[(pyridin-2-yl)amino]ethyl	b	2[3)]	golden oil; NMR*
7A	2-[(4-chloropyrimidin-2-yl)amino]ethyl	b	2	tan solid; NMR*
7B	2-[(3-chloropyridin-2-yl)amino]ethyl	b	2	golden oil; NMR*
7C	2-[(4-CF_3-pyrimidin-2-yl)amino]ethyl	b	2	golden oil; NMR*
7D	2-(2-chlorophenyl)ethyl	b	2	NMR*
7E	(3,3-diphenyl)propyl	b	2	NMR*
8	2-[(5-nitropyridin-2-yl)amino]ethyl	b	5[3a)]	bright yellow thick oil; NMR*
9A	2-[(3-chloro-5-CF_3-pyridin-2-yl)amino]ethyl	b	2[3b)]	golden oil; NMR*
9B	2-[(3-CF_3-pyridin-2-yl)amino]ethyl	b	2[3b)]	golden oil; NMR*
9C	2-[(3,5-dichloropyridin-2-yl)amino]ethyl	b	2[3b)]	golden oil; NMR*
10	cyclopent-1-yl	b	2	tan solid
		ch	1	white solid; NMR*
11	2-(2-bromo-4,5-dimethoxyphenyl)ethyl	b	5[3a)]	clear light yellow, thick oil; NMR*
12	3-(isopropoxy)propyl	b	1	brown oil
		ch	1	white solid, m.p. 174-176°; NMR*

Example No.	R	Form	Analogously to Ex. No.	Characterization data
13	2-hydroxy-1,1-dimethylethyl	b	2[4]	golden oil
		ch	1	brown solid; NMR*
14	3-(2-oxopyrrolidin-1-yl)propyl	b	2[5]	golden oil
		ch	1	tan solid; NMR*
15	1-hydroxymethylcyclohexyl	b	1[6]	yellow waxy solid; m.p. 93°; ^{13}C-NMR: 118.1 (ppm)
16	2-(4-ethoxyphenyl)ethyl	ch	1	white solid; m.p. 182-184°; ^{13}C-NMR: 121.4 (ppm)
17	1-phenylmethyl-3-(R)-pyrrolidinyl	dch	1[7]	off-white solid; m.p. 175-177°; ^{13}C-NMR: 121.5 (ppm)
18	2-(4-methoxyphenyl)ethyl	ch	1	white solid; m.p. 185-187°; ^{13}C-NMR: 121.4 (ppm)
19	2-(3-methoxyphenyl)ethyl	ch	1	light yellow solid; m.p. 172-174°; ^{13}C-NMR: 119.25 (ppm)
20	(1-naphthalenyl)methyl	ch	1	light yellow solid; m.p. 130-135°; ^{13}C-NMR: 119.29 (ppm)
21	3-phenylpropyl	ch	1	off-white fluffy solid; ^{13}C-NMR: 119.26 (ppm)
22	3-[(phenyl)(methyl)amino]propyl	dch	1	white solid; m.p. 96-98° (foams); ^{13}C-NMR: 121.6 (ppm)
23	2-(3,4-dimethoxyphenyl)ethyl	ch	1	white solid; m.p. 170-172°; ^{13}C-NMR: 121.5 (ppm)
24	cycloheptyl	ch	1	white solid; m.p. 68-70°; ^{13}C-NMR: 121.4 (ppm)
25	[1S[1α,2α(S*),5α]]-(6,6-dimethylbicyclo[3.1.1]-hept-2-yl)methyl	ch	1[8]	white solid; m.p. 275-279° (dec.); ^{13}C-NMR: 119.17 (ppm)
26	2-(2,5-dimethoxyphenyl)ethyl	ch	1	white fluffy solid; m.p. 65-67°; ^{13}C-NMR: 119.25 (ppm)
27	2-(1-cyclohexen-1-yl)ethyl	ch	1	off-white fluffy solid; m.p.162-164°; ^{13}C-NMR: 119.27 (ppm)
28	cyclohexyl	ch	1	white fluffy solid;m.p.182-184°; ^{13}C-NMR: 119.28 (ppm)
29	[1S[1α, 2α(S*),5α]]-bicyclo[2.2.1]hept-2-yl	ch	1[9]	white solid; m.p. 98-100°; ^{13}C-NMR: 118.36 (ppm)
30	2-(2-pyridinyl)ethyl	dch	1	white solid; m.p. 95-97°; ^{13}C-NMR: 121.5 (ppm)
31	(2-phenylamino)ethyl	dch	1	white solid; m.p. 124-126°; ^{13}C-NMR: 121.4 (ppm)
32	3,3-dimethylbutyl	ch	1	white solid; m.p. 164-166°; ^{13}C-NMR: 121.5 (ppm)
33	[1S[1α,2β,3α(S*),5α]]-2,6,6-trimethylbicyclo[3.1.1]-hept-3-yl	ch	1[10]	white solid; m.p. 82-84°; ^{13}C-NMR: 121.5 (ppm)

Example No.	R	Form	Analogously to Ex. No.	Characterization data
34	**[S,S]-(1-hydroxymethyl)propyl**	ch	1[11]	off-white solid; m.p. 80-82°; ^{13}C-NMR: 118.2 (ppm)
35	**[2-[(2-hydroxymethyl)phenyl]thio]phenylmethyl**	ch	1[12]	yellow solid; m.p. 65-67°; ^{13}C-NMR: 121.4 (ppm)
36	**2-(2-methoxyphenyl)ethyl**	ch	1	off-white solid; m.p. 174-176°; ^{13}C-NMR: 121.7 (ppm)
37	**5-hydroxypentyl**	ch	1	sticky light-green solid; ^{13}C-NMR: 121.67 (ppm)
38	**cyclobutyl**	ch	1	off-white solid; m.p. 274-278° (dec.); ^{13}C-NMR: 121.64 (ppm)
39	**2-(2,4-dichlorophenyl)ethyl**	ch	1	white fluffy solid;m.p.154-156°; ^{13}C-NMR: 121.48 (ppm)
40	**1-(S)-(+)-hydroxymethyl-3-methylbutyl**	ch	1[13]	light yellow solid; m.p. 65-66°; ^{13}C-NMR: 117.99 (ppm)
41	**[1R*,2S*]-2-hydroxy-2-phenylethyl**	ch	1[14]	light yellow solid; m.p. 82-83°; ^{13}C-NMR: 118.35 (ppm)
42	**2-(2-fluorophenyl)ethyl**	ch	1	white fluffy solid;m.p.160-162°; ^{13}C-NMR: 121.70 (ppm)
43	**cyclopropyl**	ch	1	off-white solid; m.p. 170-172°; ^{13}C-NMR: 121.62 (ppm)
44	**[1S[1S,2S,3S,5R]]-2,6,6-trimethylbicyclo-[3.1.1]hept-3-yl**	ch	1[15]	white solid; m.p. 84-86°; ^{13}C-NMR: 121.8 (ppm)
45	**(2-phenoxy)ethyl**	ch	1	sticky golden solid; ^{13}C-NMR: 121.7 (ppm)
46	**2-(3,5-dimethoxyphenyl)ethyl**	ch	1	white fluffy solid; m.p. 74-76°; ^{13}C-NMR: 121.66 (ppm)
47	**1-adamantyl**	ch	1	white solid; m.p. 240-242°; ^{13}C-NMR: 121.80 (ppm)
48	**1,1,3,3-tetramethylbutyl**	ch	1	white fluffy solid; m.p. 68-70°; ^{13}C-NMR: 121.55 (ppm)
49	**2-adamantyl**	ch	1	off-white solid; m.p. 122-124°; ^{13}C-NMR: 121.69 (ppm)
50	**1,1-dimethylpropyl**	ch	1	white fluffy solid; m.p. 62-64°; ^{13}C-NMR: 121.53 (ppm)
51	**benzyl**	ch	1	white solid; m.p. 58-60°; ^{13}C-NMR: 121.38 (ppm)
52	**1,1-dimethylethyl**	ch	1	white solid; m.p. 226-228°; ^{13}C-NMR: 121.56 (ppm)
53	**(2-adamantyl)methyl**	ch	1	white solid; m.p. 158-160°; ^{13}C-NMR: 121.53 (ppm)
54	**2-phenylethyl**	ch	1	white solid; m.p. 275-280° (dec.); ^{13}C-NMR: 121.52 (ppm)
55	**pentyl**	ch	1	white solid; m.p. 176-178°; ^{13}C-NMR: 121.67 (ppm)
56	**butyl**	ch	1	white solid; m.p. 180-182°; ^{13}C-NMR: 121.53 (ppm)

Example No.	R	Form	Analogously to Ex. No.	Characterization data
57	**cyclododecyl**	ch	1	white fluffy solid; ^{13}C-NMR: 121.52 (ppm)
58	**cyclooctyl**	ch	1	white fluffy solid; ^{13}C-NMR: 121.64 (ppm)
59	**propyl**	ch	1	white solid; m.p. 193-194°; ^{13}C-NMR: 121.57 (ppm)
60	**ethyl**	ch	1	off-white sticky solid; ^{13}C-NMR: 121.67 (ppm)
61	**heptyl**	ch	1	white solid; m.p. 170-172°; ^{13}C-NMR: 121.7 (ppm)
62	**hexyl**	ch	1	white solid; m.p. 174-176°; ^{13}C-NMR: 121.75 (ppm)
63	**3-[(5-cyano-2-pyridinyl)amino]propyl**	dch	1[16)]	white sticky solid; m.p. 210-212°; ^{13}C-NMR: 119.33 (ppm)
64	**1-ethylpropyl**	ch	1	white fluffy sticky solid; ^{13}C-NMR: 119.35 (ppm)
65	**2,3-dihydro-1H-inden-2-yl**	ch	1	white solid; m.p. 182-184°; ^{13}C-NMR: 121.38 (ppm)
66	**1-benzylpiperidin-4-yl**	ch	1	white solid; m.p. 280-283° (dec.); ^{13}C-NMR: 121.39 (ppm)

b = in free base form ch= in monohydrochloride form; dch = in dihydrochloride form
dec.= decomposes; m.p.= melting point

[1)] starting from **2-[(5-trifluoromethylpyridin-2-yl)amino]ethylamine** in tetrahydrofuran, using 5 % methanol in methylene chloride as eluent
[1a)]using 10 % methanol in methylene chloride as eluent
[2)] reacting **(1-hydroxymethyl)cyclopentylamine** in anhydrous tetrahydrofuran for 18 h at room temperature under a calcium sulfate drying tube
[3)] using methylene chloride, methanol and ammonium hydroxide 90:10:0.5 as eluent
[3a)]using flash chromatography, employing a mixture of 5 % methanol in methylene chloride as eluent
[3b)]using 3 % methanol in methylene chloride as eluent
[4)] using methylene chloride, methanol and ammonium hydroxide 80:20:1 as eluent
[5)] using methylene chloride, methanol and ammonium hydroxide 90:10:1 as eluent
[6)] reacting **(1-aminocyclohexane)methanol** in tetrahydrofuran with **1-chloroacetyl-2-(S)-cyanopyrrolidine** (prepared from chloroacetylchloride and L-prolinamide and reaction of the resultant product with trifluoroacetic anhydride) at ice-temperature under a calcium sulfate drying tube, and purifying the reaction mixture on silicagel employing 5 % methanol in methylene chloride as eluent

[7] using (3R)-(-)-1-benzyl-3-aminopyrrolidine as starting material
[8] using (-)-cis-myrtanylamine as starting material
[9] using (+/-)-exo-2-aminonorbornane as starting material
[10] using (1R,2R,3R,5S)-(-)-isopinocampheylamine as starting material
[11] using (S)-(+)-2-amino-1-butanol as starting material
[12] using 2-(2-aminomethyl)phenylthio)benzyl alcohol as starting material
[13] using (S)-(+)-leucinol as starting material
[14] using (1R,2S)-(-)-norephedrine as starting material
[15] using (1S,2S,3S,5R)-(+)-isopinocampheylamine as starting material
[16] using 2-(3-aminopropylamino)-5-cyanopyridine as starting material

* NMR:

Compound #	^{13}C-NMR (MHz, solvent) d ppm (CN)	
Ex. 5	^{13}C NMR (75 MHz, CD_3OD)	d 119.64 ppm (CN)
Ex. 12	^{13}C NMR (75 MHz, D_2O)	d 121.63 ppm (CN)
Ex. 1	^{13}C NMR (75 MHz, D_2O)	d 121.60 ppm (CN)
Ex. 3	^{13}C NMR (75 MHz, D_2O)	d 120.42 ppm (CN)
Ex. 8	^{13}C NMR (75 MHz, DMSO)	d 119.13 ppm (CN)
Ex. 7B	^{13}C NMR (75 MHz, $CDCl_3$)	d 118.23 ppm (CN)
Ex. 9A	^{13}C NMR (75 MHz, CD_3OD)	d 119.68 ppm (CN)
Ex. 9B	^{13}C NMR (75 MHz, CD_3OD)	d 119.66 ppm (CN)

Compound #	^{13}C NMR (MHz, solvent) d ppm (CN)
Ex. 9C	^{13}C NMR (75 MHz, CD_3OD) d 119.68 ppm (CN)
Ex. 6	^{13}C NMR (75 MHz, CD_3OD) d 119.84 ppm (CN)
Ex. 7C	^{13}C NMR (75 MHz, $CDCl_3$) d 118.23 ppm (CN)
Ex. 2	^{13}C NMR (75 MHz, CD_3OD) d 119.68 ppm (CN)
Ex. 7A	^{13}C NMR (75 MHz, CD_3OD) d 119.66 ppm (CN)
Ex. 4	^{13}C NMR (75 MHz, CD_3OD) d 119.66 ppm (CN)
Ex. 10	^{13}C NMR (75 MHz, D_2O) d 121.69 ppm (CN)
Ex. 11	^{13}C NMR (75 MHz, $CDCl_3$) d 118.31 ppm (CN)
Ex. 7D	^{13}C NMR (75 MHz, CD_3OD) d 119.63 ppm (CN)
Ex. 7E	^{13}C NMR (75 MHz, CD_3OD) d 119.64 ppm (CN)
Ex. 13	^{13}C NMR (75 MHz, D_2O) d 121.52 ppm (CN)
Ex. 14	^{13}C NMR (75 MHz, D_2O) d 121.52 ppm (CN)

The compounds of the invention in free form or in pharmaceutically acceptable acid addition salt form, hereinafter briefly named "**the agents of the invention**", in particular, the compounds of formula I in free form or in pharmaceutically acceptable acid addition salt form, possess pharmacological activity. They are therefore indicated for use as pharmaceuticals.

In particular, they inhibit DPP-IV. This activity may be demonstrated employing the **Caco-2 DPP-IV assay,** which measures the ability of test compounds to inhibit DPP-IV activity from human colonic carcinoma cell extracts. The human colonic carcinoma cell line Caco-2 can be obtained from the American Type Culture Collection (ATCC HTB 37). Differentiation of the cells to induce DPP-IV expression is accomplished as described by Reisher et al. in Proc.Natl.Acad.Sci.USA 90 (1993) 5757-5761. Cell extract is prepared from cells solubilized in 10 mM Tris-HCl, 0.15 M NaCl, 0.04 t.i.u. (trypsin inhibitor unit) aprotinin, 0.5% non-ionic detergent P40, pH 8.0, which is centrifuged at 35 000 g for 30 min at 4°C to remove cell debris. The assay is conducted by adding 20 mg solubilized Caco-2 protein, diluted to a final volume of 125 ml in assay buffer (25 mM Tris-HCl pH 7.4, 140 mM NaCl, 10 mM KCl, 1% bovine serum albumin) to microtiter plate wells. The reaction is initiated by adding 25ml of 1 mM substrate (H-Alanine-Proline-pNA; pNA is *p*-nitroaniline). The reaction is run at room temperature for 10 minutes after which time a 19 ml volume of 25% glacial acetic acid is added to stop the reaction. Test compounds are typically added as 30 ml additions and the assay buffer volume is reduced to 95 ml. A standard curve of free *p*-nitroaniline is generated using 0-500 mM solutions of free pNA in assay buffer. The curve generated is linear and is used for interpolation of substrate consumption (catalytic activity in nmoles substrate cleaved /min). The endpoint is determined by measuring absorbance at 405 nm in a Molecular Devices UV Max microtiter plate reader. The potency of the test compounds as DPP-IV inhibitors, expressed as IC_{50}, is calculated from 8-point, dose-response curves using a 4-parameter logistic function.

In the above test, IC_{50} values of from about 10 nM to about 900 nM are obtained with the agents of the invention, e.g. 22 nM for the agent of Example 3.

The DPP-IV inhibition may also be demonstrated by measuring the effects of test compounds on DPP-IV activity in human and rat plasma employing a modified version of the assay described by Kubota et al. in Clin.Exp.Immunol. 89 (1992) 192-197. Briefly, five ml of plasma are added to 96-well flat-bottom microtiter plates (Falcon), followed by the addition

of 5 ml of 80 mM $MgCl_2$ in incubation buffer (25 mM HEPES, 140 mM NaCl, 1 % RIA-grade BSA, pH 7.8). After a 5 min incubation at room temperature, the reaction is initiated by the addition of 10 ml of incubation buffer containing 0.1 mM substrate (H-Glycine-Proline-AMC; AMC is 7-amino-4-methylcoumarin). The plates are covered with aluminum foil (or kept in the dark) and incubated at room temperature for 20 min. After the 20 min reaction, fluorescence is measured using a CytoFluor 2350 fluorimeter (Excitation 380 nm Emission 460 nm; sensitivity setting 4). Test compounds are typically added as 2 ml additions and the assay buffer volume is reduced to 13 ml. A fluorescence-concentration curve of free AMC is generated using 0-50 mM solutions of AMC in assay buffer. The curve generated is linear and is used for interpolation of substrate consumption (catalytic activity in nmoles substrate cleaved/min). As with the previous assay, the potency of the test compounds as DPP-IV inhibitors, expressed as IC_{50}, is calculated from 8-point, dose-response curves using a 4 parameter logistic function.

In the above assay, IC_{50} values of from about 7 nM to about 2000 nM are obtained in human plasma, and of from about 3 nM to about 400 nM in rat plasma, e.g., for the agent of Example 3, of 7 nM in human and 6 nM in rat plasma, respectively.

In view of their ability to **inhibit DPP-IV**, the agents of the invention are indicated for use in **treating conditions mediated by DPP-IV.** It is expected that the compounds disclosed herein are useful in the treatment of **non-insulin-dependent diabetes mellitus, arthritis, obesity, and osteoporosis such as calcitonin-osteoporosis.** The agents of the invention improve early insulin response to an oral glucose challenge and, therefore, are particularly indicated for use in treating **non-insulin-dependent diabetes mellitus and further conditions of impaired glucose tolerance (IGT).**

The ability of the agents of the invention to improve early insulin response to an oral glucose challenge may e.g. be measured in insulin resistant rats according to the following method:

Male Sprague-Dawley rats that have been fed a high fat diet (saturated fat = 57 % calories) for 2-3 weeks are fasted for approximately 2 hours on the day of testing, divided into groups of 8-10, and dosed orally with 10 mmol/kg of the test compounds in carboxymethylcellulose (CMC). An oral glucose bolus of 1 g/kg is administered 30 min after

the test compound directly into the stomach of the test animals. Blood samples, obtained at various timepoints from chronic jugular vein catheters are analyzed for plasma glucose and immunoreactive insulin (IRI) concentrations, and plasma DPP-IV activity. Plasma insulin levels are assayed by a double antibody radioimmunoassay (RIA) method using a specific anti-rat insulin antibody from Linco Research (St. Louis, MO, USA). The RIA has a lower limit of detection of 0.5 mU/ml with intra- and inter-assay variations of less than 5%. Data are expressed as % increase of the mean of the control animals.

It was found that upon oral administration, each of the compounds tested amplified the early insulin response which led to an improvement in glucose tolerance in the insulin resistant test animals. The following results were obtained:

Compound	Increase of insulin response at 10 mmol/kg
Ex. 1	61 %
Ex. 3	66 %
Ex. 5	108 %
Ex. 8	144 %
Ex. 12	59 %

The precise dosage of the agents of the invention to be employed for treating conditions mediated by DPP-IV inhibition depends upon several factors, including the host, the nature and the severity of the condition being treated, the mode of administration and the particular compound employed. However, in general, conditions mediated by DPP-IV inhibition are effectively treated when an agent of the invention is administered enterally, e.g. orally, or parenterally, e.g. intravenously, preferably orally, at a daily dosage of from about 0.002 mg/kg to about 5 mg/kg, preferably of from about 0.02 mg/kg to about 2.5 mg/kg body weight or, for most larger primates, a daily dosage of from about 0.1 mg to about 250 mg, preferably from about 1 mg to about 100 mg. A typical oral dosage unit is from about 0.01 mg/kg to about 0.75 mg/kg, one to three times a day. Usually, a small dose is administered initially and the dosage is gradually increased until the optimal dosage for the host

under treatment is determined. The upper limit of dosage is that imposed by side effects and can be determined by trial for the host being treated.

The agents of the invention may be combined with one or more pharmaceutically acceptable carriers and, optionally, one or more other conventional pharmaceutical adjuvants and administered enterally, e.g. orally, in the form of tablets, capsules, caplets, etc., or parenterally, e.g. intravenously, in the form of sterile injectable solutions or suspensions. The enteral and parenteral compositions may be prepared by conventional means.

The agents of the invention may be formulated into enteral and parenteral pharmaceutical compositions containing an amount of the active substance that is effective for treating conditions mediated by DPP-IV, such compositions in unit dosage form and such compositions comprising a pharmaceutically acceptable carrier.

Those agents of the invention which are e.g. of formula I may be administered in enantiomerically pure (S) form (e.g. ≥ 98 %, preferably ≥ 99 % pure) or together with the other enantiomer, e.g. in racemic form. The above dosage ranges are based on the compounds of formula I (excluding the amount of R enantiomer).

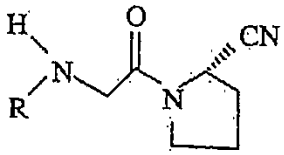
The invention thus also comprises **an agent of the invention**, in particular, a compound of formula I as defined above, in free form or in pharmaceutically acceptable acid addition salt form, **for use as a pharmaceutical**. It further includes a **pharmaceutical composition** comprising an agent of the invention, in particular a compound of formula I as defined above, in free form or in pharmaceutically acceptable acid addition salt form, together with at least one pharmaceutically acceptable carrier or diluent. It further comprises the use of an agent of the invention, in particular, a compound of formula I as defined above, in free form or in pharmaceutically acceptable acid addition salt form **in the preparation of a medicament** for inhibiting DPP-IV or treating conditions mediated by DPP-IV, by a process comprising mixing an agent of the invention with a pharmaceutically acceptable carrier or diluent. It further provides a **method of inhibiting DPP-IV, or of treating conditions mediated by DPP-IV**, which comprises administering to a patient in need of such treatment a therapeutically effective amount of a compound of the invention, in particular, of formula I in free form or in pharmaceutically acceptable acid addition salt form.

The agents of Examples 1, 3, 5, 8 and 12 are the preferred agents of the invention, particularly those of Examples 1, 3, 5 and 12, preferably in hydrochloride acid addition salt form, especially the agent of Example 3, namely 1-[2-[(5-cyanopyridin-2-yl)amino]-ethylamino]acetyl-2-cyano-(S)-pyrrolidine, preferably in dihydrochloride acid addition salt form. It has been determined that in hydrochloride form they have an IC_{50} value in the Caco-2 DPP-IV assay of, respectively, 36, 22, 26, 8 and 279 nM, and in the modified Kubota assay above, an IC_{50} value for, respectively, human and rat plasma DPP-IV, of 27 and 22 nM (Example 1); 7 and 6 nM (Example 3); 37 and 18 nM (Example 5); 12 and 11 nM (Example 8); and 95 and 38 nM (Example 12). It is, therefore, indicated that for the above uses the compounds of Examples 1, 3, 5, 8 and 12 may be administered to larger mammals, for example humans, by similar modes of administration at similar dosages than conventionally employed with metformin.

Claims:

1. An N-(N'-substituted glycyl)-2-cyanopyrrolidine.

2. A compound of formula I:



I

wherein R is:

a) $R_1R_{1a}N(CH_2)_m$- wherein

R_1 is a pyridinyl or pyrimidinyl moiety optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy, halogen, trifluoromethyl, cyano or nitro; or phenyl optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen;

R_{1a} is hydrogen or (C_{1-8})alkyl; and

m is 2 or 3;

b) (C_{3-12})cycloalkyl optionally monosubstituted in the 1-position with (C_{1-3})hydroxyalkyl;

c) $R_2(CH_2)_n$- wherein either

R_2 is phenyl optionally mono- or independently di- or independently trisubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy, halogen or phenylthio optionally monosubstituted in the phenyl ring with hydroxymethyl; or is (C_{1-8})alkyl; a [3.1.1]bicyclic carbocyclic moiety optionally mono- or plurisubstituted with (C_{1-8})alkyl; a pyridinyl or naphthyl moiety optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen; cyclohexene; or adamantyl; and

n is 1 to 3; or

R_2 is phenoxy optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen; and

n is 2 or 3;

d) $(R_3)_2CH(CH_2)_2$- wherein each R_3 independently is phenyl optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen;

e) $R_4(CH_2)_p$- wherein R_4 is 2-oxopyrrolidinyl or (C_{2-4})alkoxy and
p is 2 to 4;

f) isopropyl optionally monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;

g) R_5 wherein R_5 is: indanyl; a pyrrolidinyl or piperidinyl moiety optionally substituted with benzyl; a [2.2.1]- or [3.1.1]bicyclic carbocyclic moiety optionally mono- or plurisubstituted with (C_{1-8})alkyl; adamantyl; or (C_{1-8})alkyl optionally mono- or independently plurisubstituted with hydroxy, hydroxymethyl or phenyl optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen;

in free form or in acid addition salt form,

3. A compound according to claim 2 (a **compound Ip**) wherein R is $\mathbf{R^P}$, which is:

a) $R_1^PNH(CH_2)_2$- wherein R_1^P is a pyridinyl or pyrimidinyl moiety optionally mono- or independently disubstituted with halogen, trifluoromethyl, cyano or nitro;

b) (C_{3-7})cycloalkyl optionally monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;

c) $R_2^P(CH_2)_2$- wherein R_2^P is phenyl optionally mono- or independently di- or independently trisubstituted with halogen or (C_{1-3})alkoxy;

d) $(R_3^P)_2CH(CH_2)_2$- wherein each R_3^P independently is phenyl optionally monosubstituted with halogen or (C_{1-3})alkoxy;

e) $R_4(CH_2)_3$- wherein R_4 is as defined above; or

f) isopropyl optionally monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;

in free form or in acid addition salt form.

4. A compound according to claim 2 (a **compound Is**), wherein R is $\mathbf{R^s}$, which is:

a) $R_1^sR_{1a}^s(CH_2)_{ms}$- wherein R_1^s is pyridinyl optionally mono- or independently disubstituted with chlorine, trifluoromethyl, cyano or nitro; pyrimidinyl optionally monosubstituted with chlorine or trifluoromethyl; or phenyl;

R_{1a}^s is hydrogen or methyl; and

ms is 2 or 3;

b) (C_{3-12})cycloalkyl optionally monosubstituted in 1-position with hydroxymethyl;

c) $R_2^5(CH_2)_{ns}$- wherein either

R_2^5 is phenyl optionally mono- or independently di- or independently trisubstituted with halogen, alkoxy of 1 or 2 carbon atoms or phenylthio monosubstituted in the phenyl ring with hydroxymethyl; (C_{1-6})alkyl; 6,6-dimethylbicyclo[3.1.1]hept-2-yl; pyridinyl; naphthyl; cyclohexene; or adamantyl; and ns is 1 to 3; or

R_2^5 is phenoxy; and ns is 2;

d) (3,3-diphenyl)propyl;

e) $R_4^5(CH_2)_{ps}$ wherein R_4^5 is 2-oxopyrrolidin-1-yl or isopropoxy and ps is 2 or 3;

f) isopropyl optionally monosubstituted in 1-position with hydroxymethyl;

g) R_5^5 wherein R_5^5 is: indanyl; a pyrrolidinyl or piperidinyl moiety optionally N-substituted with benzyl; bicyclo[2.2.1]hept-2-yl; 2,6,6-trimethylbicyclo-[3.1.1]hept-3-yl; adamantyl; or (C_{1-8})alkyl optionally mono- or independently disubstituted with hydroxy, hydroxymethyl or phenyl;

in free form or in acid addition salt form.

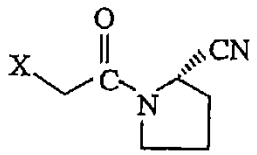
5. The compound according to claim 2 wherein R is 2-[(5-cyanopyridin-2-yl)amino]ethyl, i.e. 1-[2-[(5-cyanopyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine, in free form or in acid addition salt form, especially in dihydrochloride acid addition salt form, or a compound according to claim 2 which is of formula I wherein R is either

- 2-[(5-chloropyridin-2-yl)amino]ethyl, or
- (1-hydroxymethyl)cyclopent-1-yl, or
- 2-[(5-nitropyridin-2-yl)amino]ethyl, or
- 3-(isopropoxy)propyl,

in free form or in acid addition salt form.

6. A process for the preparation of a compound according to claim 1 which comprises coupling a reactive (2-cyanopyrrolidino)carbonylmethylene compound with an appropriate substituted amine or, for the preparation of a compound according to claim 2, which comprises

reacting a compound of formula II



II

wherein X is a reactive group,

with a compound of formula III

NH_2R III

wherein R is as defined in claim 2,

and recovering the resultant compound in free form or in acid addition salt form.

7. A pharmaceutical composition comprising a compound according to claim 1 in free form or in pharmaceutically acceptable acid addition salt form, together with at least one pharmaceutically acceptable carrier or diluent.

8. A compound according to claim 1 in free form or in pharmaceutically acceptable acid addition salt form, for use as a pharmaceutical.

9. Use of a compound according to claim 1 in free form or in pharmaceutically acceptable acid addition salt form in the preparation of a medicament for inhibiting DPP-IV or treating conditions mediated by DPP-IV.

10. A method of inhibiting DPP-IV, or of treating a condition mediated by DPP-IV, which comprises administering a therapeutically effective amount of a compound according to claim 1 in free form or in pharmaceutically acceptable acid addition salt form to a patient in need of such treatment.

US006011155A

United States Patent [19]

Villhauer

[11] Patent Number: 6,011,155

[45] Date of Patent: Jan. 4, 2000

[54] N-(SUBSTITUTED GLYCYL)-2-CYANOPYRROLIDINES, PHARMACEUTICAL COMPOSITIONS CONTAINING THEM AND THEIR USE IN INHIBITING DIPEPTIDYL PEPTIDASE-IV

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[21] Appl. No.: 08/962,168

[22] Filed: Oct. 31, 1997

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[51] Int. Cl.[7] C07D 207/34; C07D 207/42; C07D 401/06; C07D 405/10; C07D 409/06

[52] U.S. Cl. 544/333; 544/326; 544/330; 546/208; 546/276.4; 546/279.1; 548/530; 548/540

[58] Field of Search 548/530, 540, 548/517; 546/276.4, 279.1, 208; 544/516, 330, 328, 333

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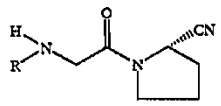
[57] ABSTRACT

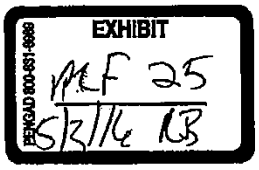
N-(N'-substituted glycyl)-2-cyanopyrrolidines of formula I

I



Compounds of formula I inhibit DPP-IV (dipeptidyl-peptidase-IV) activity. They are therefore indicated for use as pharmaceuticals in inhibiting DPP-IV and in the treatment of conditions mediated by DPP-IV, such as non-insulin-dependent diabetes mellitus, arthritis, obesity, osteoporosis and further conditions of impaired glucose tolerance.

11 Claims, No Drawings



N-(SUBSTITUTED GLYCYL)-2-CYANOPYRROLIDINES, PHARMACEUTICAL COMPOSITIONS CONTAINING THEM AND THEIR USE IN INHIBITING DIPEPTIDYL PEPTIDASE-IV

This application claims the benefit of Provisional Application number 60/030,570 filed on Nov. 7, 1996.

FIELD OF THE INVENTION

The present invention relates to the area of dipeptidyl peptidase-TV (DPP-IV) inhibition. DPP-IV is a serine protease which cleaves N-terminal dipeptides from a peptide chain containing, preferably, a proline residue in the penultimate position. Although the biological role of DPP-IV in mammalian systems has not been completely established, it is believed to play an important role in neuropeptide metabolism, T-cell activation, attachment of cancer cells to the endothelium and the entry of HIV into lymphoid cells.

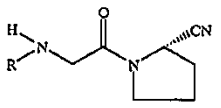
More recently, it was discovered that DPP-IV is responsible for inactivating glucagon-like peptide-1 (GLP-1). More particularly, DPP-IV cleaves the amino-terminal His-Ala dipeptide of GLP-1, generating a GLP-1 receptor antagonist, and thereby shortens the physiological response to GLP-1. Since the half-life for DPP-IV cleavage is much shorter than the half-life for removal of GLP-1 from circulation, a significant increase in GLP-1 bioactivity (5- to 10-fold) is anticipated from DPP-IV inhibition. Since GLP-1 is a major stimulator of pancreatic insulin secretion and has direct beneficial effects on glucose disposal, DPP-IV inhibition appears to represent an attractive approach for treating non-insulin-dependent diabetes mellitus (NIDDM).

SUMMARY OF THE INVENTION

The present invention provides new DPP-IV inhibitors which are effective in treating conditions mediated by DPP-IV. More particularly, the present invention relates to certain N-(substituted glycyl)-2-cyanopyrrolidines which inhibit DPP-IV. In addition, the present invention provides pharmaceutical compositions useful in inhibiting DPP-IV comprising a therapeutically effective amount of a N-(substituted glycyl)-2-cyanopyrrolidine disclosed herein. Moreover, the present invention provides a method of inhibiting DPP-IV comprising administering to a mammal in need of such treatment a therapeutically effective amount of a N-(substituted glycyl)-2-cyanopyrrolidine.

DETAILED DESCRIPTION OF THE INVENTION

The instant invention relates to novel N-(substituted glycyl)-2-cyanopyrrolidines of formula I:



I

wherein R is:

a) $R_1R_{1a}N(CH_2)_m$- wherein

R_1 is a pyridinyl or pyrimidinyl moiety optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy, halogen, trifluoromethyl, cyano or nitro; or phenyl optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen;

R_{1a} is hydrogen or (C_{1-8})alkyl; and

m is 2 or 3;

b) (C_{3-12})cycloalkyl optionally monosubstituted in the 1-position with (C_{1-3})hydroxyalkyl;

c) $R_2(CH_2)_n$- wherein either

R_2 is phenyl optionally mono- or independently di- or independently trisubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy, halogen or phenylthio optionally monosubstituted in the phenyl ring with hydroxymethyl; or is (C_{1-8})alkyl; a [3.1.1]bicyclic carbocyclic moiety optionally mono- or plurisubstituted with (C_{1-8})alkyl; a pyridinyl or naphthyl moiety optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen; cyclohexene; or adamantyl; and

n is 1 to 3; or

R_2 is phenoxy optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen; and

n is 2 or 3;

d) $(R_3)_2CH(CH_2)_2$- wherein each R_3 independently is phenyl optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen;

e) $R_4(CH_2)_p$- wherein R_4 is 2-oxopyrrolidinyl or (C_{2-4})alkoxy and p is2to4;

f) isopropyl optionally monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;

g) R_5 wherein R_5 is: indanyl; a pyrrolidinyl or piperidinyl moiety optionally substituted with benzyl; a [2.2.1]- or [3.1.1]bicyclic carbocyclic moiety optionally mono- or plurisubstituted with (C_{1-8})alkyl; adamantyl; or (C_{1-8})alkyl optionally mono- or independently plurisubstituted with hydroxy, hydroxymethyl or phenyl optionally mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen;

in free form or in acid addition salt form.

The compounds of formula I can exist in free form or in acid addition salt form. Salt forms may be recovered from the free form in known manner and vice-versa. Acid addition salts may e.g. be those of pharmaceutically acceptable organic or inorganic acids. Although the preferred acid addition salts are the hydrochlorides, salts of methanesulfonic, sulfuric, phospboric, citric, lactic and acetic acid may also be utilized.

The compounds of the invention may exist in the form of optically active isomers or diastereoisomers and can be separated and recovered by conventional techniques, such as chromatography.

"Alkyl" and "alkoxy" are either straigbt or branched chain, of which examples of the latter are isopropyl and tert-butyl.

R preferably is a), b) or e) as defined above. R_1 preferably is a pyridinyl or pyrimidinyl moiety optionally substituted as defined above. R_{1a} preferably is hydrogen. R_2 preferably is phenyl optionally substituted as defined above. R_3 preferably is unsubstituted phenyl. R_4 preferably is alkoxy as defined above. R_5 preferably is optionally substituted alkyl as defined above. m preferably is 2. n preferably is 1 or 2, especially 2. p preferably is 2 or 3, especially 3.

Pyridinyl preferably is pyridin-2-yl; it preferably is unsubstituted or monosubstituted, preferably in 5-position. Pyrimidinyl preferably is pyrimidin-2-yl. It preferably is unsubstituted or monosubstituted, preferably in 4-position. Preferred as substitutents for pyridinyl and pyrimidinyl are halogen, cyano and nitro, especially chlorine.

When it is substituted, phenyl preferably is monosubstituted; it preferably is substituted with halogen, preferably

chlorine, or methoxy. It preferably is substituted in 2-, 4- and/or 5-position, especially in 4-position.

(C_{3-12})cycloalkyl preferably is cyclopentyl or cyclohexyl. When it is substituted, it preferably is substituted with hydroxymethyl. (C_{1-4})alkoxy preferably is of I or 2 carbon atoms, it especially is methoxy. (C_{2-4})alkoxy preferably is of 3 carbon atoms, it especially is isopropoxy. Halogen is fluorine, chlorine, bromine or iodine, preferably fluorine, chlorine or bromine, especially chlorine. (C_{1-8})alkyl preferably is of 1 to 6, preferably I to 4 or 3 to 5, especially of 2 or 3 carbon atoms, or methyl. (C_{1-4}) alkyl preferably is methyl or ethyl, especially methyl. (C_{1-3})hydroxyalkyl preferably is hydroxymethyl.

A [3.1.1]bicyclic carbocyclic moiety optionally substituted as defined above preferably is bicyclo[3.1.1]hept-2-yl optionally disubstituted in 6-position with methyl, or bicyclo [3.1.1]hept-3-yl optionally trisubstituted with one methyl in 2-position and two methyl groups in 6-position. A [2.2.1] bicyclic carbocyclic moiety optionally substituted as defined above preferably is bicyclo[2.2.1]hept-2-yl.

Naphthyl preferably is 1-naphthyl. Cyclohexene preferably is cyclohex-1-en-1-yl. Adamantyl preferably is 1- or 2-adamantyl.

A pyrrolidinyl or piperidinyl moiety optionally substituted as defined above preferably is pyrrolidin-3-yl or piperidin-4yl. When it is substituted it preferably is N-substituted.

A preferred group of compounds of the invention is the compounds of formula I wherein R is R' (compounds Ia), whereby R' is:

- R_1'$NH(CH_2)_2$- wherein R_1' is pyridinyl optionally mono- or independently disubstituted with halogen, trifluoromethyl, cyano or nitro; or unsubstituted pyrimidinyl;
- (C_{3-7})cycloalkyl optionally monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;
- R_4'$(CH_2)_3$- wherein R_4' is (C_{2-4})alkoxy; or
- R_5, wherein R_5 is as defined above;

in free form or in acid addition salt form.

More preferred compounds of the invention are those compounds of formula I wherein R is R" (compounds Ib), whereby R" is:

- R_1"$NH(CH_2)_2$- wherein R_1" is pyridinyl mono- or independently disubstituted with halogen, trifluoromethyl, cyano or nitro;
- (C_{4-6})cycloalkyl monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;
- R_4'$(CH_2)_3$- wherein R_4' is as defined above; or
- R_5' wherein R_5' is a [2.2.1]- or [3.1.1]bicyclic carbocyclic moiety optionally mono- or plurisubstituted with (C_{1-8})alkyl; or adamantyl;

in free form or in acid addition salt form.

Even more preferred compounds of the invention are the compounds of formula I wherein R is R'" (compounds Ic), whereby R'" is:

- R_1"$NH(CH_2)_2$- wherein R_1" is as defined above;
- (C_{4-6})cycloalkyl monosubstituted in 1-position with hydroxymethyl;
- R_4'$(CH_2)_3$- wherein R_4' is as defined above; or
- R_5" wherein R_5" is adamantyl;

in free form or in acid addition salt form.

A further group of compounds of the invention is compounds Ip, wherein R is R^p, which is:

a) $R_1{}^p NH(CH_2)_2$- wherein $R_1{}^p$ is a pyridinyl or pyrimidinyl moiety optionally mono- or independently disubstituted with halogen, trifluoromethyl, cyano or nitro;

b) (C_{3-7})cycloalkyl optionally monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;

c) $R_2{}^p(CH_2)_2$- wherein $R_2{}^p$ is phenyl optionally mono- or independently di- or independently trisubstituted with halogen or (C_{1-3})alkoxy;

d) $(R_3{}^p)_2CH(CH_2)_2$- wherein each $R_3{}^p$ independently is phenyl optionally monosubstituted with halogen or (C_{1-3})alkoxy;

e) $R_4(CH_2)_3$- wherein R_4 is as defined above; or

f) isopropyl optionally monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;

in free form or in pharmaceutically acceptable acid addition salt form.

A further group of compounds of the invention is compounds Is, wherein R is R^s, which is:

a) $R_1{}^s R_{1a}{}^s(CH_2)_{ms}$- wherein $R_1{}^s$ is pyridinyl optionally mono- or independently disubstituted with chlorine, trifluoromethyl, cyano or nitro; pyrimidinyl optionally monosubstituted with chlorine or trifluoromethyl; or phenyl; $R_{1a}{}^s$ is hydrogen or methyl; and ms is 2or3;

b) (C_{3-12})cycloalkyl optionally monosubstituted in 1-position with hydroxymethyl;

c) $R_2{}^s(CH_2)_{ns}$- wherein either
   $R_2{}^s$ is phenyl optionally mono- or independently di- or independently trisubstituted with halogen, alkoxy of 1 or 2 carbon atoms or phenylthio monosubstituted in the phenyl ring with hydroxymethyl; (C_{1-6})alkyl; 6,6-dimethylbicyclo[3.1.1]hept-2-yl; pyridinyl; naphthyl; cyclohexene; or adamantyl; and
   ns is 1 to 3; or
   $R_2{}^s$ is phenoxy; and ns is 2;

d) (3,3-diphenyl)propyl;

e) $R_4{}^s(CH_2)_{ps}$wherein $R_4{}^s$ is 2-oxopyrrolidin-1 -yl or isopropoxy and ps is2or3;

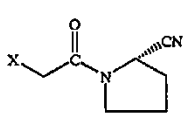
f) isopropyl optionally monosubstituted in 1-position with hydroxymetbyl;

g) $R_5{}^s$ wherein $R_5{}^s$ is: indanyl; a pyrrolidinyl or piperidinyl moiety optionally N-substituted with benzyl; bicyclo[2.2.1]hept-2-yl; 2,6,6trimethylbicyclo-[3.1.1] hept-3-yl; adamantyl; or (C_{1-8})alkyl1 optionally mono- or independently disubstituted with hydroxy, hydroxymethyl or phenyl;

in free form or in acid addition salt form.

The compounds of the invention may be prepared by a process which comprises coupling a reactive (2-cyanopyrrolidino)carbonylmethylene compound with an appropriate substituted amine; more particularly, for the preparation of the compounds of formula I it comprises reacting a compound of formula II

II



wherein X is a reactive group,
with a compound of formula III

NH_2R III

wherein R is as defined above,
and recovering the resultant compound of formula I in free form or in acid addition salt form.

X preferably is a halogen such as bromine, chlorine or iodine.

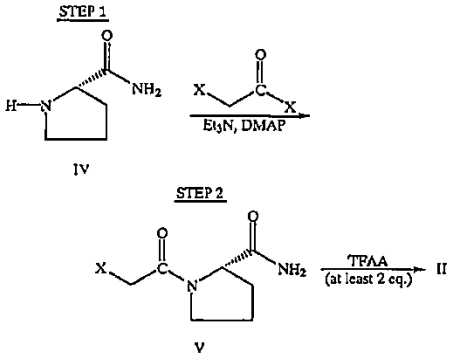
The process of the invention may be effected in conventional manner.

The compound of formula II is preferably reacted with at least 3 equivalents of a primary amine of formula III. The reaction is conveniently conducted in the presence of an inert, organic solvent, preferably a cyclic ether such as tetrahydrofuran. The temperature preferably is of from about 0° to about 35° C., preferably between about 0° and about 25° C.

The compounds of the invention may be isolated from the reaction mixture and purified in conventional manner, e.g. by chromatography.

The starting materials may also be prepared in conventional manner.

The compounds of formula II may e.g. be prepared by the following two-step reaction scheme:

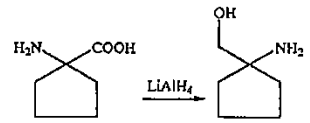


Step 1 involves the reaction of the pyrrolidine of formula IV with a slight molar excess of a haloacetylhalide such as bromoacetylbromide or chloroacetylchloride and triethylamine and a catalytic amount of dimethylaminopyridine (DMAP). The reaction conveniently is conducted in the presence of an inert, organic solvent, preferably a chlorinated, aliphatic hydrocarbon such as methylene chloride, at a temperature of from about 0° to about 25° C., preferably at a temperature between about 0° and about 15° C.

Step 2 concerns the dehydration of the compound of formula V, prepared in Step 1, with at least 2 equivalents of trifluoroacetic anhydride (TFAA). The dehydration preferably is conducted in the presence of an inert, organic solvent such as tetrahydrofuran or a chlorinated, aliphatic hydrocarbon such as methylene chloride, at a temperature of from about 0° to about 25° C., preferably at a temperature between about 0° and about 15° C.

Insofar as its preparation is not particularly described herein, a compound used as starting material is known or may be prepared from known compounds in known manner or analogously to known methods or analogously to methods described in the Examples.

For example, the primary amine compounds of formula III are known and may be prepared by procedures documented in the literature. More particularly,: a) 1-hydroxymethylcyclopentylamine can be prepared by the reduction of 1-amino-1-cyclopentane carboxylic acid with lithium aluminum hydride as set forth below:



The reduction is conducted in the presence of an inert, organic solvent, preferably a cyclic ether such as tetrahydrofuran, at the reflux temperature of the solvent for a period of between 14 and 24 hours. (b) 2-[(5-chloropyridin-2-yl)amino]ethylamine can be prepared by refluxing a mixture of 2,5-dichloropyridine with ethylenediamine in an oil bath for a period of between 6 and 12 hours. (c) Similarly, 2-[(5-trifluoromethylpyridin-2-yl) amino]ethylamine can be prepared by refluxing a mixture of 2-chloro-5-trifluoromethyl pyridine with ethylenediamine in an oil bath for a period of between 6 and 12 hours. (d) 2-[(5-cyanopyridin-2-yl)amino]-ethylamine can be prepared by stirring a mixture of 2-chloropyridine-5-carbonitrile and ethylenediamine at a temperature between 20° and 30° C., for a period of between 4 and 6 hours. (e) 2-[(pyrimidin-2-yl)amino]ethylamine can be prepared by adding ethylenediamine to ice-bath cooled 2-chloropyrimidine and allowing the mixture to react at a temperature between 20° and 30° C., for a period of between 12 and 20 hours. (f) 1-amino-1-cyclohexanemethanol can be prepared by the reduction of 1-amino-1-cyclohexane carboxylic acid with lithium aluminum hydride. The reduction is conducted in the presence of an inert, organic solvent, preferably a cyclic ether such as tetrahydrofuran, at the reflux temperature of the solvent for a period of between 14 and 24 hours. (g) 2(3-aminopropylamino)-5-cyanopyridine can be prepared by refluxing a mixture of 2,5-dichloropyridine with 1,3 propyl diamine in an oil bath for a period of between 6 and 12 hours. Alternatively, the above examples (a) through (g) may be carried out at room temperature.

The instant invention also includes pharmaceutical compositions useful in inhibiting DPP-IV comprising a pharmaceutically acceptable carrier or diluent and a therapeutically effective amount of a compound of formula I, or a pharmaceutically acceptable acid addition salt thereof.

In still another embodiment, the instant invention provides a method of inhibiting DPP-IV comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound of formula I, or a pharmaceutically acceptable acid addition salt thereof.

In a further embodiment, the instant invention provides a method of treating conditions mediated by DPP-IV inhibition comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound of formula I above, or a pharmaceutically acceptable acid addition salt thereof.

As indicated above, all of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, are useful in inhibiting DPP-IV. The ability of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, to inhibit DPP-IV may be demonstrated employing the Caco-2 DPP-IV Assay which measures the ability of test compounds to inhibit DPP-IV activity from human colonic carcinoma cell extracts. The human colonic carcinoma cell line Caco-2 was obtained from the American Type Culture Collection (ATCC HTB 37). Differentiation of the cells to induce DPP-IV expression was accomplished as described by Reisher, et al. in an article entitled "Increased expression of . . . intestinal

cell line Caco-2" in Proc. Natl. Acad. Sci., Vol. 90, pgs. 5757–5761 (1993). Cell extract is prepared from cells solubilized in 10 mM Tris-HCl, 0.15 M NaCl, 0.04 t.i.u. aprotinin, 0.5% nonidet-P40, pH 8.0, which is centrifuged at 35,000 g for 30 min. at 4° C. to remove cell debris. The assay is conducted by adding 20 μg solubilized Caco-2 protein, diluted to a final volume of 125 pi in assay buffer (25 mM Tris-HCl pH 7.4, 140 mM NaCl, 10 mM KCl, 1% bovine serum albumin) to microtiter plate wells. The reaction is initiated by adding 25 μl of 1 mM substrate (H-Alanine-Proline-pNA; pNA is p-nitroaniline). The reaction is run at room temperature for 10 minutes after which time a 19 μl volume of 25% glacial acetic acid is added to stop the reaction. Test compounds are typically added as 30 μl additions and the assay buffer volume is reduced to 95 μl. A standard curve of free p-nitroaniline is generated using 0–500 μM solutions of free pNA in assay buffer. The curve generated is linear and is used for interpolation of substrate consumption (catalytic activity in nmoles substrate cleaved/min). The endpoint is determined by measuring absorbance at 405 nm in a Molecular Devices UV Max microfiter plate reader. The potency of the test compounds as DPP-IV inhibitors, expressed as IC_{50}, is calculated from 8-point, dose-response curves using a 4-parameter logistic function.

The following IC_{50}s were obtained:

Compound	Caco-2 DPP-IV (nM)
Ex. 1	36
Ex. 2	176
Ex. 3	22
Ex. 4	140
Ex. 5	26
Ex. 6	50
Ex. 7A	165
Ex. 8	8
Ex. 7B	175
Ex. 9A	990
Ex. 7C	290
Ex. 9C	295
Ex. 10	54
Ex. 11	215
Ex. 7D	382
Ex. 7E	388
Ex. 12	279
Ex. 13	227
Ex. 14	110
Ex. 15	150
Ex. 16	130
Ex. 17	60
Ex. 18	100
Ex. 19	120
Ex. 20	90
Ex. 21	390
Ex. 22	150
Ex. 23	50
Ex. 24	70
Ex. 25	140
Ex. 26	170
Ex. 27	310
Ex. 28	90
Ex. 29	130
Ex. 30	650
Ex. 31	500
Ex. 32	150
Ex. 33	10
Ex. 34	37
Ex. 35	130
Ex. 36	160
Ex. 37	220
Ex. 38	50
Ex. 39	380
Ex. 40	240
Ex. 41	140
Ex. 42	240
Ex. 43	850
Ex. 44	5
Ex. 45	700
Ex. 46	150
Ex. 47	10
Ex. 48	35
Ex. 49	12
Ex. 50	23
Ex. 51	250
Ex. 52	20
Ex. 53	860
Ex. 54	240
Ex. 55	270
Ex. 56	350
Ex. 57	470
Ex. 58	50
Ex. 59	390
Ex. 60	600
Ex. 61	310
Ex. 62	270
Ex. 63	46
Ex. 64	220
Ex. 65	80
Ex. 66	60

The ability of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, to inhibit DPP-IV may also be demonstrated by measuring the effects of test compounds on DPP-IV activity in human and rat plasma employing a modified version of the assay described by Kubota, et al. in an article entitled "Involvement of dipeptidylpeptidase IV in an in vivo immune response" in Clin. Exp. Immunol., Vol. 89, pgs. 192–197 (1992). Briefly, five μl of plasma are added to 96-well flat-bottom microtiter plates (Falcon), followed by the addition of 5 μl of 80 mM $MgCl_2$ in incubation buffer (25 mM HEPES, 140 mM NaCl, 1% RIA-grade BSA, pH 7.8). After a 5 min. incubation at room temperature, the reaction is initiated by the addition of 10 μl of incubation buffer containing 0.1 mM substrate (H-Glycine-Proline-AMC; AMC is 7-amino4-methylcoumarin). The plates are covered with aluminum foil (or kept in the dark) and incubated at room temperature for 20 min. After the 20 min. reaction, fluorescence is measured using a CytoFluor 2350 fluorimeter (Excitation 380 nm Emission 460 nm; sensitivity setting 4). Test compounds are typically added as 2 μl additions and the assay buffer volume is reduced to 13 μl. A fluorescence-concentration curve of free AMC is generated using 0–50 μM solutions of AMC in assay buffer. The curve generated is linear and is used for interpolation of substrate consumption (catalytic activity in nmoles substrate cleaved/min). As with the previous assay, the potency of the test compounds as DPP-IV inhibitors, expressed as IC_{50}, is calculated from 8-point, dose-response curves using a 4 parameter logistic function.

The following IC_{50}s were obtained:

Compound	human plasma DPP-IV (nM)	rat plasma DPP-IV (nM)
Ex. 1	27	22
Ex. 3	7	6
Ex. 4	40	23
Ex. 5	37	18
Ex. 6	22	32
Ex. 8	12	11
Ex. 10	51	19
Ex. 12	95	38

-continued

Compound	human plasma DPP-IV (nM)	rat plasma DPP-IV (nM)
Ex. 14	95	24
Ex. 15	70	40
Ex. 16	170	60
Ex. 17	250	120
Ex. 18	160	70
Ex. 19	180	50
Ex. 20	180	150
Ex. 21	210	110
Ex. 22	170	60
Ex. 23	40	40
Ex. 24	32	19
Ex. 25	110	140
Ex. 26	240	70
Ex. 27	150	160
Ex. 28	180	60
Ex. 29	28	9
Ex. 30	80	90
Ex. 31	80	100
Ex. 32	160	130
Ex. 33	20	10
Ex. 34	277	161
Ex. 35	1090	340
Ex. 36	170	80
Ex. 37	100	150
Ex. 38	65	23
Ex. 39	220	200
Ex. 40	340	370
Ex. 41	100	50
Ex. 42	140	180
Ex. 43	240	120
Ex. 44	10	10
Ex. 45	2130	390
Ex. 46	280	60
Ex. 47	11	5
Ex. 48	60	30
Ex. 49	8	3
Ex. 50	60	40
Ex. 51	180	150
Ex. 52	20	10
Ex. 53	490	400
Ex. 54	90	60
Ex. 55	140	90
Ex. 56	140	100
Ex. 57	420	150
Ex. 58	20	100
Ex. 59	280	130
Ex. 60	250	110
Ex. 61	260	80
Ex. 62	190	100
Ex. 63	60	30
Ex. 64	150	60
Ex. 65	90	40
Ex. 66	130	40

In view of their ability to inhibit DPP-IV, the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, are useful in treating conditions mediated by DPP-IV inhibition. Based on the above and findings in the literature, it is expected that the compounds disclosed herein are useful in the treatment of conditions such as non-insulin-dependent diabetes mellitus, arthritis, obesity, allograft transplantation, and calcitonin-osteoporosis. More specifically, for example, the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, improve early insulin response to an oral glucose challenge and, therefore, are useful in treating non-insulin-dependent diabetes mellitus. The ability of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, to improve early insulin response to an oral glucose challenge may be measured in insulin resistant rats according to the following method:

Male Sprague-Dawley rats that had been fed a high fat diet (saturated fat=57% calories) for 2–3 weeks were fasted for approximately 2 hours on the day of testing, divided into groups of 8–10, and dosed orally with 10 μmol/kg of the test compounds in CMC. An oral glucose bolus of 1g/kg was administered 30 minutes after the test compound directly into the stomach of the test animals. Blood samples, obtained at various timepoints from chronic jugular vein catheters were analyzed for plasma glucose and immunoreactive insulin (IRI) concentrations, and plasma DPP-IV activity. Plasma insulin levels were assayed by a double antibody radioimmunoassay (RIA) method using a specific anti-rat insulin antibody from Linco Research (St. Louis, Mo.). The RIA has a lower limit of detection of 0.5 μU/ml with intra- and inter-assay variations of less than 5%. Data are expressed as % increase of the mean of the control animals. Upon oral administration, each of the compounds tested amplified the early insulin response which led to an improvement in glucose tolerance in the insulin resistant test animals. The following results were obtained:

Compound	Increase of Insulin Response at 10 μmol/kg
Ex. 1	61%
Ex. 3	66%
Ex. 5	108%
Ex. 8	144%
Ex. 12	59%

The precise dosage of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, to be employed for treating conditions mediated by DPP-IV inhibition depends upon several factors, including the host, the nature and the severity of the condition being treated, the mode of administration and the particular compound employed. However, in general, conditions mediated by DPP-IV inhibition are effectively treated when a compound of formula I, or a corresponding pharmaceutically acceptable acid addition salt, is administered enterally, e.g., orally, or parenterally, e.g., intravenously, preferably orally, at a daily dosage of 0.002–5, preferably 0.02–2.5 mg/kg body weight or, for most larger primates, a daily dosage of 0.1–250, preferably 1–100 mg. A typical oral dosage unit is 0.01–0.75 mg/kg, one to three times a day.

Usually, a small dose is administered initially and the dosage is gradually increased until the optimal dosage for the host under treatment is determined. The upper limit of dosage is that imposed by side effects and can be determined by trial for the host being treated.

The compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, may be combined with one or more pharmaceutically acceptable carriers and, optionally, one or more other conventional pharmaceutical adjuvants and administered enterally, e.g., orally, in the form of tablets, capsules, caplets, etc. or parenterally, e.g., intravenously, in the form of sterile injectable solutions or suspensions. The enteral and parenteral compositions may be prepared by conventional means.

The compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, may be formulated into enteral and parenteral pharmaceutical compositions containing an amount of the active substance that is effective for treating conditions mediated by DPP-IV inhibition, such compositions in unit dosage form and such compositions comprising a pharmaceutically acceptable carrier.

The compounds of formula I (including those of each of the subscopes thereof and each of the examples) may be administered in enantiomerically pure form (e.g., ee≧98%,

preferably ≧99%) or together with the R enantiomer, e.g., in racemic form. The above dosage ranges are based on the compounds of formula I (excluding the amount of the R enantiomer).

Prior US Provisional Application number 60/030,570, filed on Nov. 7, 1996 is incorporated by reference herein, in it's entirety.

The following examples show representative compounds encompassed by this invention and their synthesis. However, it should be clearly understood that they are for purposes of illustration only.

EXAMPLE 1

1-[2-[(5-chloropyridin-2-yl)amino]ethylamino] acetyl-2-cyano-(S)-pyrrolidine dihydrochloride

A. Preparation of 2-carbamoylpyrrolidine-carbonylmethylene-(S)-bromide

22.37 g (196 mmol) of (S)-2-carbamoylpyrrolidine, 30.1 ml (216 mmol) of triethylamine and 30.0 mg of dimethylaminopyridine (DMAP) are dissolved in 200 ml of methylene chloride and the solution is then added, dropwise, to an ice-cold solution of 18.8 ml (216 mmol) of bromoacetylbromide in 192 ml of methylene chloride, over a period of 60 minutes under a calcium sulfate drying tube. The resultant solution is then stirred for 2 hours at ice-water temperature under a calcium sulfate dying tube, after which time it is poured into 3.5 liters of ethyl acetate. The resultant precipitate is filtered, washed with ethyl acetate, and the filtrate is concentrated to obtain the desired compound as a bard yellow taffy.

B. Preparation of 2-Cyanopyrrolidino-Carbonylmethylene-(S)Bromide

50.0 g (213 mmol) of the bromide compound prepared in a) above is dissolved in 300 ml of methylene chloride and the solution is cooled in an ice water bath under a calcium sulfate drying tube. The cooled solution is then poured into 60.2 ml (426 mmol) of trifluoroacetic anhydride over a 2 minute period and the resultant solution is then stirred at ice-water temperature under a calcium sulfate drying tube for 4 hours and partitioned between methylene chloride and saturated aqueous sodium bicarbonate. The product is then extracted into the methylene chloride layer and tbe aqueous layer is then washed twice with methylene chloride. The combined organic layers are then washed successively with water and brine and then dried over sodium sulfate. The solution is then filtered and tbe solvent is removed by rotovaping and high vacuum pumping to obtain the desired compound as a dark yellow solid.

C. Preparation of the Title Compound in Free Base Form

To a 500 ml flask is added 16.6g (97.2 mmol) of 2-[(5-chloropyridin-2-yl)amino]ethylamine and 100 ml of tetrahydrofuran and the mixture is cooled in an ice bath. To the cooled mixture is added 7.0 g (32.4 mmol) of the bromide compound prepared in b) above dissolved in 30 ml of tetrahydmfuran. The resultant mixture is stirred for 2 hours at 0° C., the solvent is removed by rotovaping and the mixture is partitioned between ethyl acetate and water. The product is then extracted into the ethyl acetate layer and the aqueous layer is then washed twice with ethyl acetate. The combined organic layers are then washed successively with water and brine, dried over sodium sulfate and concentrated to obtain the desired compound in crude form. The crude form is then purified on silica gel employing a mixture of 5% methanol in methylene chloride as the eluent to yield the desired compound as a light brown oil.

D. Preparation of the Title Compound

After dissolving the free base compound prepared in c) above in 30 ml of dry tetrahydrofuiran, hydrogen chloride gas is bubbled into the solution for five seconds. The off-white precipitate that forms is tben filtered, washed with dry tetrahydrofuran and the solvent is removed by high vacuum pumping to obtain the title compound as an off-white solid, m.p. 265°–267° C.

EXAMPLE 2

1-[2-[(5-trifluoromethylpryrdin-2-yl)amino] ethylamino]acetyl-2-cyano-(S)-pyrrolidine

To a 25 ml. flask is added 1.15 g (5.61 mmol) of 2-[(5-trifluoromethylpyrdin-2-yl)-amino]ethylamine and 10 ml of tetrahydrofuran and the mixture is cooled in an ice bath. To the cooled mixture is added 0.404 g (1.87 mmol) of the bromide compound of Example 1b) dissolved in 5 ml of tetrahydrofuran. The resultant mixture is stirred for 2 hours at 0° C., the solvent is removed by rotovaping and the mixture is partitioned between ethyl acetate and water. The product is then extracted into the ethyl acetate layer and the aqueous layer is then washed twice with etbyl acetate. The combined organic layers are then washed successively with water and brine, dried over sodium sulfate and concentrated to obtain the desired compound in crude form. The crude form is then purified on silica gel employing a mixture of 5% methanol io methylene chloride as the eluent to yield the title compound as a golden oil.

EXAMPLE 3

1-[2-[(5-cyanopyridin-2-yl)amino]ethylamino] acetyl-2-cyano-S)-pyrrolidine dihydrochloride

A. Preparation of the Title Compound in Free Base Form

Following essentially the procedure of Example 1c), and using in place of the amine therein, an equivalent amount of 2-[(5-cyanopyridin-2-yl)amino]-ethylamine, the desired compound is obtained as a golden oil.

B. Preparation of the Title Compound

Following essentially the procedure of Example 1d), and using in place of the free base compound prepared in Example 1c), an equivalent amount of the free base compound prepared in a) above, the title compound is obtained as an off-white precipitate, m.p. 155°–157° C.

EXAMPLE 4

1[-2-[(pyrimidin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine

Following essentially the procedure of Example 2, and using in place of the amine therein, an equivalent amount of 2-[(pyrimidin-2-yl)amino]ethylamine, and using in place of the eluent therein, a mixture of 10% methanol in methylene chloride, the title compound is obtained as a golden oil.

EXAMPLE 5

1-[(1 -hydroxymethylcyclopent-1-yl)amino]acetyl-2-cyano-(S)-pyrrolidine

To 1.5 g of (1-hydroxymethyl)cyclopentylamine in 40 ml of anhydrous tetrahydrofuran is added, dropwise via an addition funnel over 40 minutes, 0.93 g (4.35 mmol) of the bromide compound of Example 1b) under a calcium sulfate drying tube. The resultant mixture is then stirred at room temperature for 18 hours under a calcium sulfate drying tube, after whicb time hydrogen chloride gas is bubbled in for ~5 seconds. The resultant gum is then separated from the solution by decanting and washed with 25 ml of tetrahy-

drofuran. The solution is then decanted and the gum is partitioned between methylene chloride and saturated aqueous sodium bicarbonate. The product is then extracted into the methylene chloride layer and the aqueous layer is then washed twice with methylene chloride. The combined organic layers are then washed successively with water and brine and then dried over sodium sulfate. The solution is then filtered and the solvent is removed by rotovaping and high vacuum pumping to obtain the title compound as a clear yellow oil which solidifies to a yellow solid, m.p. 65°–67° C.

EXAMPLE 6

1-[2-[(pyridin-2-yl)amino]ethylamino]acetyl-2-cyano(S)-pyrrolidine

Following essentially the procedure of Example 2, and using in place of the amine therein, an equivalent amount of 2-[(pyridin-2-yl)amino]ethylamine, and using in place of the eluent therein, a 90:10:0.5 mixture of methylene chloride, methanol and ammonium hydroxide, the title compound is obtained as a golden oil.

EXAMPLE 7

Following essentially the procedure of Example 2, and using in place of the amine therein, an equivalent amount of:

a) 2-[(4-chloropyrimidin-2-yl)amino]ethylamine;

b) 2-[(3-chloropyridin-2-yl)amino]ethylamine;

c) 2-[(4-trifluoromethylpyrimidin-2-yl)amino]ethylamine;

d) (2-chlorophenyl)ethylamine; and

e) (3,3-biphenyl)propylamine;

there is obtained:

A) 1-[2-[(4-chloropyrimidin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine as a tan solid;

B) 1-[2-[(3-chloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine as a golden oil;

C) 1-[2-[4-trifluoromethylpyrimidin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine as a golden oil;

D) 1-[(2-chlorophenyl)ethylamino]acetyl-2-cyano-(S)-pyrrolidine; and

E) 1-[(3,3-diphenyl)propylamino]acetyl-2-cyano-(S)-pyrrolidine, respectively.

EXAMPLE 8

1-[2-[(5-nitropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)pyrrolidine

To 83.6 ml of anhydrous tetrahydrofuran is added 4.54 g (24.9 mmol) of 2-[(5-nitropyridin-2-yl)amino]ethylamine, and the resultant mixture is heated slightly then stirred at room temperature under a calcium sulfate drying tube. 1.80 g (8.3 mmol) of the bromide compound of Example 1b) in 20 ml of anhydrous tetrahydrofuran is then added, over a period of 30 minutes, under a calcium sulfate drying tube. The resultant mixture is then stirred at room temperature for 2 hours under a calcium sulfate drying tube and concentrated via rotovaping. The resultant paste is then partitioned between methylene chloride and saturated aqueous sodium bicarbonate. The product is then extracted into the methylene chloride layer and the aqueous layer is then washed twice with methylene chloride. The combined organic layers are then washed successively with water and brine and then dried over sodium sulfate. The solution is then filtered and the solvent is removed by rotovaping and high vacuum pumping to obtain the crude form of the title compound as a dark yellow-orange clear thick oil. The crude form is then flash chromatographed employing a mixture of 5% methanol in methylene chloride as the eluent to obtain the title compound as a bright yellow thick oil.

EXAMPLE 9

Following essentially the procedure of Example 2, and using in place of the amine therein, an equivalent amount of:

a) 2-[(3-chloro-5-trifluoromethylpyridin-2-yl)amino]ethylamine;

b) 2-[(3-trifluoromethylpyridin-2-yl)amino]ethylamine; and

c) 2-[(3,5-dichloropyridin-2-yl)amino]ethylamine;

and using in place of the eluent therein, a mixture of 3% methanol in methylene chloride, there is obtained:

A) 1-[2-[(3-chloro-5-trifluoromethylpyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine as a golden oil;

B) 1-[2-[(3-trifluoromethylpyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)pyrrolidine as a golden oil; and

C) 1-[2-[(3,5-dichloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine as a golden oil.

EXAMPLE 10

1-[(cyclopent-1-yl)amino-acetyl-2-cyano-(S)-pyrrolidine monohydrochloride

A. Preparation of the Title Compound in Free Base Form

Following essentially the procedure of Example 2, and using in place of the amine therein, an equivalent amount of (cyclopent-1-yl)amine, the desired compound is obtained as a tan solid.

B. Preparation of the Title Compound

Following essentially the procedure of Example 1d), and using in place of the free base compound therein, an equivalent amount of the compound prepared in a) above, the title compound is obtained as a white solid.

EXAMPLE 11

1-[2-(2-bromo4,5-dimethoxyphenyl)ethylamino]acetyl-2-cyano-(S)-pyrrolidine

To 15 ml of anhydrous tetrahydrofuran is added 1.44 g (5.52 mmol) of 2-(2-bromo4,5-dimethoxy)ethylamine, and the resultant mixture is heated slightly under a calcium sulfate drying tube. 0.4 g (1.84 mmol) of the bromide compound of Example 1b) is then added, dropwise, over a period of 10 minutes. The resultant mixture is then stirred at room temperature for 18 hours under a calcium sulfate drying tube, concentrated via rotovaping and partitioned between methylene chloride and saturated aqueous sodium bicarbonate. The product is then extracted into the methylene chloride layer and the aqueous layer is then washed twice with methylene chloride. The combined organic layers are then washed successively with water and brine and then dried over sodium sulfate. The solution is then filtered and the solvent is removed by rotovaping and high vacuum pumping to obtain the crude form of the title compound as a clear yellow oil. The crude form is then flash chromatographed employing a mixture of 5% methanol in methylene chloride as the eluent to obtain the title compound as a clear, light yellow, thick oil.

EXAMPLE 12

1-[3-(isopropoxy)propylamino]acetyl-2-cyano-(S)-pyrrolidine monohydrochloride

A. Preparation of the Title Compound in Free Base Form

Following essentially the procedure of Example 1c), and using in place of the amine therein, an equivalent amount of 3-(isopropoxy)propylamine, the desired compound is obtained as a brown oil.

B. Preparation of the Title Compound

Following essentially the procedure of Example 1d), and using in place of the free base compound therein, an equivalent amount of the compound prepared in a) above, the title compound is obtained as a white solid, m.p. 174°–176° C.

EXAMPLE 13

1-1(2-hydroxy-1,1-dimethylethylamino)]acetyl-2-cyano-(S)-pyrrolidine monohydrochloride

A. Preparation of the Title Compound in Free Base Form

Following essentially the procedure of Example 2, and using in place of the amine therein, an equivalent amount of 2-hydroxy-1,1 -dimethylethylamine, and using in place of the eluent therein, an 80:20:1 mixture of methylene chloride, methanol and ammonium hydroxide, the title compound is obtained as a golden oil.

B. Preparation of the Title Compound

Following essentially the procedure of Example 1d), and using in place of the free base compound therein, an equivalent amount of the compound prepared in a) above, the title compound is obtained as a brown solid.

EXAMPLE 14

1-[3-(2-oxo-pyrrolidin-1-yl)propylamino]acetyl-2-cyano-(S)-pyrrolidine monohydrochloride

A. Preparation of the Title Compound in Free Base Form

Following essentially the procedure of Example 2, and using in place of the amine therein, an equivalent amount of 3-(2-oxo-pyrrolidin-1-yl) propylamine, and using in place of the eluent therein, a 90:10:1 mixture of methylene chloride, methanol and ammonium hydroxide, the desired compound is obtained as a golden oil.

B. Preparation of the Title Compound

Following essentially the procedure of Example 1d), and using in place of the free base compound therein, an equivalent amount of the compound prepared in a) above, the title compound is obtained as a tan solid.

Below are the ^{13}C NMR signals for tie nitrile functionalities of the specific synthesized compounds described above:

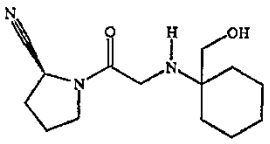
Compound #	^{13}C NMR (MHz, solvent) δ ppm (CN)
Ex. 5	^{13}C NMR (75 MHz, CD_3OD) δ 119.64 ppm (CN)
Ex. 12	^{13}C NMR (75 MHz, D_2O) δ 121.63 ppm (CN)
Ex. 1	^{13}C NMR (75 MHz, D_2O) δ 121.60 ppm (CN)
Ex. 3	^{13}C NMR (75 MHz, D_2O) δ 120.42 ppm (CN)
Ex. 8	^{13}C NMR (75 MHz, DMSO) δ 119.13 ppm (CN)
Ex. 7B	^{13}C NMR (75 MHz, $CDCl_3$) δ 118.23 ppm (CN)
Ex. 9A	^{13}C NMR (75 MHz, CD_3OD) δ 119.68 ppm (CN)
Ex. 9B	^{13}C NMR (75 MHz, CD_3OD) δ 119.66 ppm (CN)
Ex. 9C	^{13}C NMR (75 MHz, CD_3OD) δ 119.68 ppm (CN)
Ex. 6	^{13}C NMR (75 MHz, CD_3OD) δ 119.84 ppm (CN)
Ex. 7C	^{13}C NMR (75 MHz, $CDCl_3$) δ 118.23 ppm (CN)
Ex. 2	^{13}C NMR (75 MHz, CD_3OD) δ 119.68 ppm (CN)
Ex. 7A	^{13}C NMR (75 MHz, CD_3OD) δ 119.66 ppm (CN)

-continued

Compound #	^{13}C NMR (MHz, solvent) δ ppm (CN)
Ex. 4	^{13}C NMR (75 MHz, CD_3OD) δ 119.66 ppm (CN)
Ex. 10	^{13}C NMR (75 MHz, D_2O) δ 121.69 ppm (CN)
Ex. 11	^{13}C NMR (75 MHz, $CDCl_3$) δ 118.31 ppm (CN)
Ex. 7D	^{13}C NMR (75 MHz, CD_3OD) δ 119.63 ppm (CN)
Ex. 7E	^{13}C NMR (75 MHz, CD_3OD) δ 119.64 ppm (CN)
Ex. 13	^{13}C NMR (75 MHz, D_2O) δ 121.52 ppm (CN)
Ex. 14	^{13}C NMR (75 MHz, D_2O) δ 121.52 ppm (CN)

EXAMPLE 15

1-[(1-hydroxymethylcylohexyl)amino]acetyl-2-cyano-(S)-pyrrolidine



A. Preparation of 1-chloroacetyl-2-cyanopyrrolidine

To a mechanically stirred solution of 20.0 g (180.0 mmol) of chloroacetylchloride and 97 g (0.70 mmol) of potassium carbonate in 150 ml of tetrahydrofuran was added a solution of L-prolinamide 20.0 g (180.0 mmol) in 500 ml of tetrahydrofuran in a dropwise fashion over 45 minutes: This reaction was then mechanically stirred for an additional two hours. The reaction was then filtered to remove potassium salts and the filtrate was dried over Na_2SO_4. The Na_2SO_4 was then removed via filtration and to this colorless filtrate was added trifluoroacetic anbydride (25.0 ml, 0.180 mmol) in one portion. The reaction is then magnetically stirred for 1 hour and the resulting clear yellow/orange solution is concentrated via rotovap. The excess trifluoroacetic anhydride is chased by adding ethyl acetate to the concentrated oil and reconcentrating via rotovap. This operation is performed three times.

The resulting oil is partitioned between ethyl acetate and water. The product is then extracted into the ethyl acetate and the aqueous layer is then washed twice with ethyl acetate. The combined organic layers are then washed successively with water and brine dried over magnesium sulfate, filtered and concentrated to obtain 17.0 g (98.6 mmol) of 1-chloroacetyl-2-cyanopyrrolidine as a yellow solid.

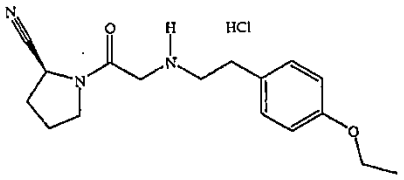
B. Preparation of the Title Compound

To a 100 ml flask is dissolved 1.2 g (8.70 mmol) of 1-amino-1-cyclohexanemethanol (amine nucleophile; preparation described above) into 20 ml of tetydrofuran. Potassium carbonate (1.60 g, 11.6 mmol) is then added and the solution is cooled in an ice-water bath. To this cooled mixture is added a solution of 0.50 g (2.89 mmol) of 1 -chloroacetyl-2-cyanopyrrolidine in 10 ml of tetrahydrofuran over 20 minutes. The reaction is then stirred at ice-water temperature for two hours under a calcium sulfate drying tube and then allowed to stir at room temperature for 18 hours. The reaction is then filtered with THF washing to remove the potassium salts and concentrated via rotovap to provide an opaque, light-yellow oil. The crude form is then purified on silica gel employing a mixture of 5% methanol in methylene chloride as the eluent to yield the free base of the title compound as a yellow waxy solid. Melting point= softens at 93° C. ^{13}C NMR(ppm)=118.1.

Using the procedures described in the above examples, and/or with minor modifications thereto as noted below, the following additional compounds were prepared:

EXAMPLE 16

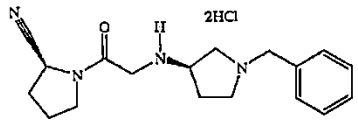
Pyrrolidine, 1-[[2-(4-ethoxyphenyl)ethyl]amino]acetyl-2-cyano-,(S)-,monohydrochloride



4-Ethoxyphenethylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=182°–184° C. ^{13}C NMR (ppm)=121.4.

EXAMPLE 17

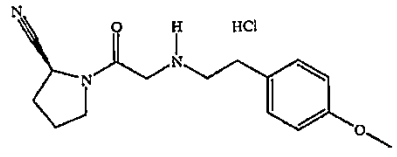
Pyrrolidine, 1-[(1-phenylmethyl-3-pyrrolidinyl) amino]acetyl-2-cyano-,(S)-(R)-,dihydrochloride



(3R)-(−)-1-Benzyl-3-aminopyrrolidine (commercially available) was used as the amine nucleophile. The title compound was an off white solid. Melting point=175° C.–177° C. ^{13}C NMR (ppm)=121.5.

EXAMPLE 18

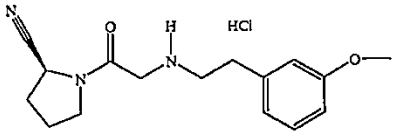
Pyrrolidine, 1-[[2-(4-methoxyphenyl)ethyl]amino] acetyl-2-cyano-,(S)-,monohydrochloride



4-Methoxyphenethylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=185° C.–187° C. ^{13}C NMR (ppm)=121.4.

EXAMPLE 19

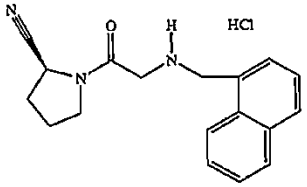
Pyrrolidine, 1-[[2-(3-methoxyphenyl)ethyl]amino]acetyl-2-cyano-,(S)-,monohydrochloride



3-Methoxyphenethylamine (commercially available) was used as the amine nucleophile. The title compound was a light yellow solid. Melting point=172° C.–174° C. ^{13}C NMR (ppm)=119.25.

EXAMPLE 20

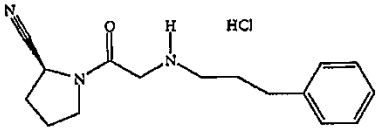
Pyrrolidine, 1-[[(1-naphthalenyl)methyl]amino] acetyl-2-cyano-,(S)-,monohydrochloride



1-Naphthalenemethylamine (commercially available) was used as the amine nucleophile. The title compound was a light yellow solid. Melting point=130° C.–135° C. ^{13}C NMR (ppm)=119.29.

EXAMPLE 21

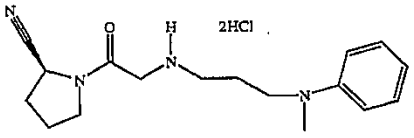
Pyrrolidine, 1-[(3-phenylpropyl)amino]acetyl-2-cyano-,(S)-,monohydrochloride



3-Phenyl-1-propylamine (commercially available) was used as the amine nucleophile. The title compound was an off-white fluffy solid. ^{13}C NMR (ppm)=119.26.

EXAMPLE 22

Pyrrolidine, 1-[[3-[(phenyl)(methyl)amino]propyl] amino]acetyl-2-cyano-, (S)-,dihydrochloride

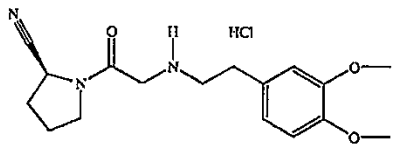


N-(3-Aminopropyl)-N-methylaniline (commercially available) was used as the amine nucleophile. The title

compound was a white solid. Melting point=96° C.–98° C. (foams). ^{13}C NMR (ppm)–121.6.

EXAMPLE 23

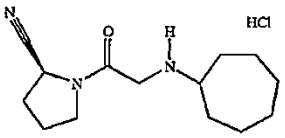
Pyrrolidine, 1-[2-[(3,4-dimethoxyphenyl)ethyl] amino]acetyl-2-cyano-,(S)-,monohydrochloride



3,4-Dimethoxyphenethylamine (commercially available) was used as the amine nucleophile. The Title compound was a white solid. Melting point=170° C.–172° C. ^{13}C NMR (ppm)=121.5.

EXAMPLE 24

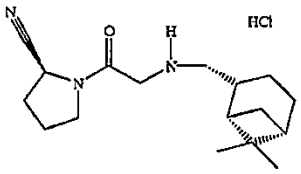
Pyrrolidine, 1-(acycloheptylamino)acetyl-2-cyano-, (S)-,monohydrochloride



Cycloheptylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=68° C.–70° C. ^{13}C NMR (ppm)=121.4.

EXAMPLE 25

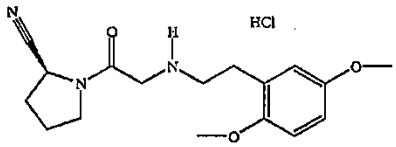
Pyrrolidine, 1-[[(6,6-dimethylbicyclo[3.1.1]hept-2-yl)methyl]amino]acetyl-2-cyano-[1S[1α,2α(S*), 5α]]-(S)-,monohydrochloride



(–)-Cis-myrtanylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=275° C.–279° C., decomposed. ^{13}C NMR (ppm)=119.17.

EXAMPLE 26

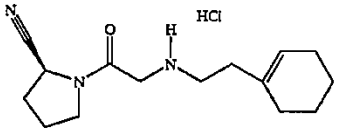
Pyrrolidine, 1-[[2-(2,5-dmethoxyphenyl)ethyl] amino]acetyl-2-cyano-,(S)-,monohydrochloride



2,5-Dimethoxyphenethylamine (commercially available) was used as the amine nucleophile. The title compound was a white fluffy solid. Melting point=65° C.–67° C. ^{13}C NMR(ppm)=119.25.

EXAMPLE 27

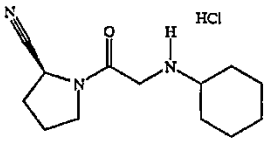
Pyrrolidine, 1-[[2-(1-cyclohexen-1-yl)ethyl]amino] acetyl-2-cyano-,(S)-,monohydrochloride



2-(1-Cyclohexenyl) ethylamine (commercially available) was used as the amine nucleophile. The title compound was an off-white fluffy solid. Melting point=162° C.–164° C. ^{13}C NMR (ppm)=119.27.

EXAMPLE 28

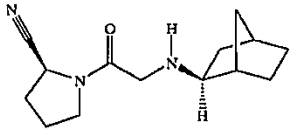
Pyrrolidine, 1-(cyclohexylamino)acetyl-2-cyano-, (S)-,monohydrochloride



Cyclohexylamine (commercially available) was used as the amine nucleophile. The title compound was a white fluffy solid. Melting point=182° C.–184° C. ^{13}C NMR (ppm)=119.28.

EXAMPLE 29

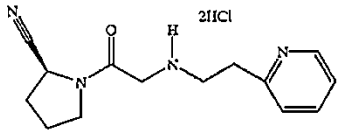
Pyrrolidine, 1-[(bicyclo[2.2.1]hept-2-yl)amino]acetyl-2-cyano-[1S[1α,2α(S*),5α]]-(S)-,monohydrochloride



(±)-Exo-2-aminonorbornane (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=98° C.–100° C. ^{13}C NMR (ppm)=118.36.

EXAMPLE 30

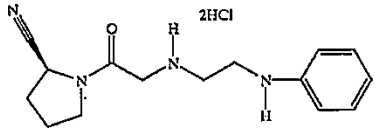
Pyrrolidine, 1-[[2-(2-pyridinyl)ethyl]amino]acetyl-2-cyano-, (S)-,dihydrochloride



2-(2-Aminoethyl) pyridine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=95° C.–97° C. ^{13}C NMR (ppm)=121.5.

EXAMPLE 31

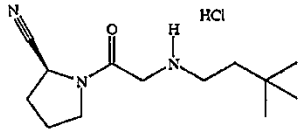
Pyrrolidine, 1-[[(2-phenylamino)ethyl]amino]acetyl-2-cyano-,(S)-,dihydrochloride



N-phenylethylenediamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=124° C.–126° C. ^{13}C NMR (ppm)=121.4.

EXAMPLE 32

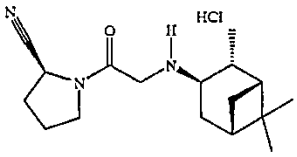
Pyrrolidine, 1-[(3,3-dimethylbutyl)amino]acetyl-2-cyano-,(S)-,monohydrochloride



3,3-Dimethylbutylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=164° C.–166° C. ^{13}C NMR (ppm)=121.5.

EXAMPLE 33

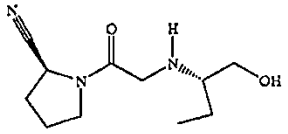
Pyrrolidine,1-[(2,6,6-trimethylbicyclo[3.1.1]hept-3-yl) amino]acetyl-2-cyano-, (S)[1S [1α,2β,3α(S*),5α]]-monohydrochloride



(1R,2R,3R,5S)-(−)-Isopinocampheylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=82° C.—84° C. ^{13}C NMR (ppm)=121.5.

EXAMPLE 34

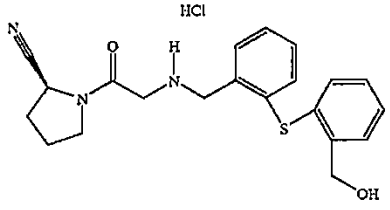
Pyrrolidine, 1-[[(1-hydroxymethyl)propyl]amino] acetyl-2-cyano-[S,S)]-



(S)-(+)-2-Amino-1-butanol (commercially available) was used as the amine nucleophile. The title compound was used as an off-white solid. Melting point=80° C.–82° C. ^{13}C NMR (ppm)=118.2.

EXAMPLE 35

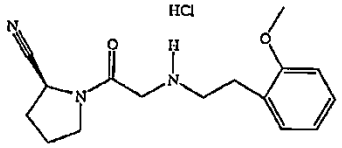
Pyrrolidine, 1-[[[2-[(2-hydroxymethyl)phenyl]thio] phenylmethyl]amino]acetyl-2-cyano-,(S)-, monohydrochloride



2-(2-(Aminomethyl) phenylthio) benzyl alcohol (commercially available) was used as the amine nucleophile. The title compound was a yellow solid. Melting point=65° C.–67° C. ^{13}C NMR (ppm)=121.4.

EXAMPLE 36

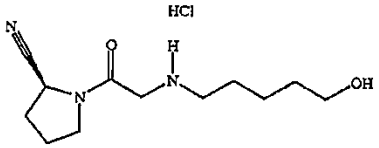
Pyrrolidine, 1-[[2-(2-methoxyphenyl)ethyl]amino] acetyl-2-cyano-,(S)-,monohydrochloride



2-Methoxyphenethylamine (commercially available) was used as the amine nucleophile. The title compound was an off white solid. Melting point=174° C. –176° C. ^{13}C NMR (ppm)=121.7.

EXAMPLE 37

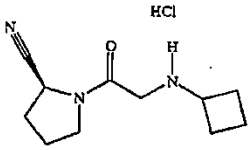
Pyrrolidine, 1-[(5-hydroxypentyl)amino]acetyl-2-cyano-,(S)-,monohydrochloride



5-Amino-1-pentanol (commercially available) was used as the amine nucleophile. The title compound was a sticky light-green solid. ^{13}C NME (ppm)=121.67.

EXAMPLE 38

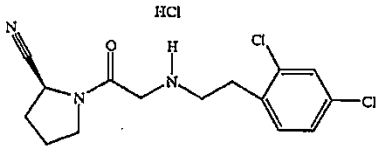
Pyrrolidine, 1-(cyclobutylamino)acetyl-2-cyano-, (S)-monohydrochloride



Cyclobutylamine (commercially available) was used as the amine nucleophile. The title compound was an off-white solid. Melting point=274° C.–278° C. decomposed. ^{13}C NMR (ppm)=121.64.

EXAMPLE 39

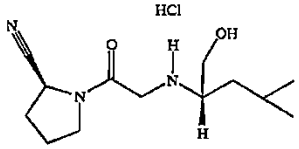
Pyrrolidine, 1-[[2-(2,4-dichlorophenyl)ethyl]amino] acetyl-2-cyano-,(S),monohydrochloride



2,4-Dichlorophenethylamine (commercially available) was used as the amine nucleophile. The title compound was a white fluffy solid. Melting point=154° C.–156° C. ^{13}C NMR (ppm)=121.48.

EXAMPLE 40

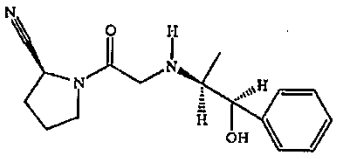
Pyrrolidine, 1-[(1-hydroxymethyl]-3-methylbutyl) amino]acetyl-2-cyano-,O-,(S)-,



(S)-(+)-Leucinol (commercially available) was used as the amine nucleophile. The title compound was a light yellow solid. Melting point=65° C.–66° C. ^{13}C NMR (ppm)=117.99.

EXAMPLE 41

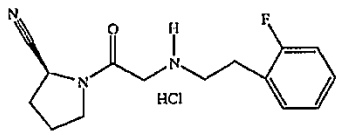
Pyrrolidine, 1-[(2-hydroxy-2-phenylethyl)amino] acetyl-2-cyano-[2S-[1R*,2S*]-monohydrochloride



(1R,2S)-(–)-Norephedrine (commercially available) was used as the amine nucleophile. The title compound was a light yellow solid. Melting point=82° C.–83° C. ^{13}C NMR (ppm)=118.35.

EXAMPLE 42

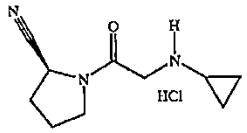
Pyrrolidine, 1-[[2-(2-fluorophenyl)ethyl]amino] acetyl-2-cyano-,(S)-,monohydrochloride



2-Fluorophenethylamine (commercially available) was used as the amine nucleophile. The title compound was a white fluffy solid. Melting point=160° C.–162° C. ^{13}C NMR (ppm)=121.70.

EXAMPLE 43

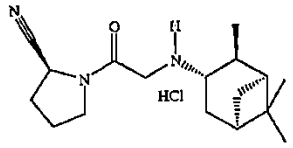
Pyrrolidine, 1-(cyclopropylamino)acetyl-2-cyano-,(S)-, monohydrochloride



Cyclopropylamine (commercially available) was used as the amine nucleophile. The title compound was an off-white solid. Melting point=170° C.–172° C. ^{13}C NMR (ppm)=121.62.

EXAMPLE 44

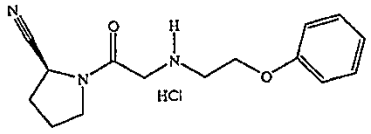
Pyrrolidine, 1-[(2,6,6-trimethylbicyclo[3.1.1]hept-3-yl)amino]acetyl-2-cyano-,[1S[1α,2α,3β(S*),5α]]-monohydrochloride



(1S,2S,3S,5R)-(+)-Isopinocampheylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=84° C.–86° C. ^{13}C NMR (ppm)=121.8.

EXAMPLE 45

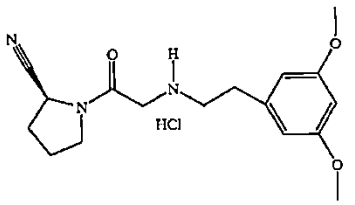
Pyrrolidine, 1-[[(2-phenoxy)ethyl]amino]acetyl-2-cyano-,(S)-,monohydrochloride



2-Phenoxyethylamine (commercially available) was used as the amine nucleophile. The title compound was a sticky golden solid. ^{13}C NMR (ppm)=121.7.

EXAMPLE 46

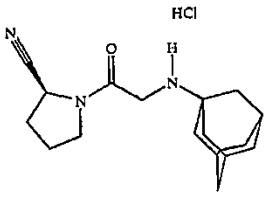
Pyrrolidine, 1-[2-[(3,5-dimethoxyphenyl)ethyl] amino]acetyl-2-cyano-,(S)-,monohydrochloride



3,5-Dimethoxyphenethylamine (commercially available) was used as the amine nucleophile. The title compound was a white fluffy solid. Melting point=74° C.–76° C. ^{13}C NMR (ppm)=121.66.

EXAMPLE 47

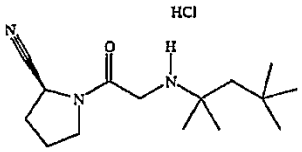
Pyrrolidine, 1-[(1-adamantyl)amino]acetyl-2-cyano-, (S)-,monohydrochloride



1-Adamantanamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=240° C.–242° C. ^{13}C NMR (ppm)=121.80.

EXAMPLE 48

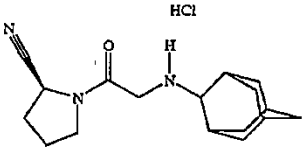
Pyrrolidine, 1-[(1,1,3,3-tetramethylbutyl)amino] acetyl-2-cyano-,(S)-,monohydrochloride



1,1,3,3-Tetramethylbutylamine (commercially available) was used as the amine nucleophile. The title compound was a white fluffy solid. Melting point=68° C.–70° C. ^{13}C NMR (ppm)=121.55.

EXAMPLE 49

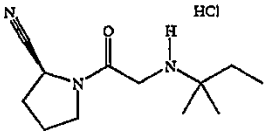
Pyrrolidine, 1-[(2-adamantyl)amino]acetyl-2-cyano-, (S)-,monohydrochloride



2-Adamantanamine (commercially available) was used as the amine nucleophile. The title compound was an off-white fluffy solid. Melting point=122° C.–124° C. ^{13}C NMR= (ppm)=121.69.

EXAMPLE 50

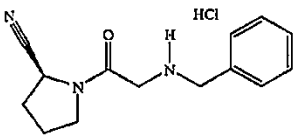
Pyrrolidine, 1-[(1,1-dimethylpropyl)amino]acetyl-2-cyano-,(S)-,monohydrochloride



1,1-Dimethylpropylamine (commercially available) was used as the amine nucleophile. The title compound was a white fluffy solid. Melting point=62° C.–64° C. ^{13}C NMR (ppm)=121.53.

EXAMPLE 51

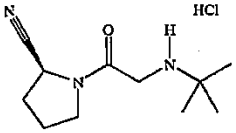
Pyrrolidine, 1-[(phenylmethyl)amino]acetyl-2-cyano-,(S)-,monohydrochloride



Benzylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=58° C.–60° C. ^{13}C NMR (ppm)=121.38.

EXAMPLE 52

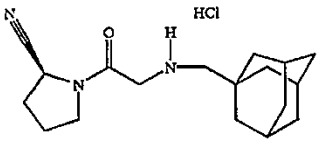
Pyrrolidine, 1-[(1,1-dimethylethyl)amino]acetyl-2-cyano-,(S),monohydrochloride



Tert-butylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=226° C.–228° C. ^{13}C NMR ppm)=121.56.

EXAMPLE 53

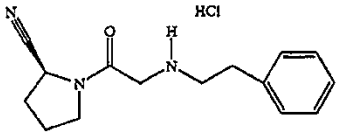
Pyrrolidine, 1-[[(2-adamantyl)methyl]amino]acetyl-2-cyano-,(S)-,monohydrochloride



1-Adamnantanemethylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=158° C.–160° C. ^{13}C NMR= 121.56.

EXAMPLE 54

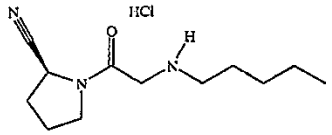
Pyrrolidine, 1-[(2-phenylethyl)amino]acetyl-2-cyano-,(S)-,monohydrocbloride



Phenethylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=275° C.–280° C. decomposed. ^{13}C NMR (ppm)=121.52.

EXAMPLE 55

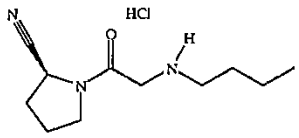
Pyrrolidine, 1-(pentylamino)acetyl-2-cyano-,(S)-, monohydrochloride



Pentylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point 176° C.–178° C. ^{13}C NMR (ppm)=121.67.

EXAMPLE 56

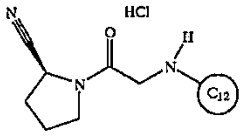
Pyrrolidine, 1-(butylamino)acetyl-2-cyano-,(S)-, monohydrochloride



Butylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=180° C.–182° C. ^{13}C NMR (ppm)=121.56.

EXAMPLE 57

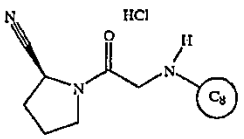
Pyrrolidine, 1-(cyclododecylamino)acetyl-2-cyano-, (S)-,monohydrochloride



Cyclododecylamine (commercially available) was used as the amine nucleophile. The title compound was a white fluffy solid. ^{13}C NMR (ppm)=121.52.

EXAMPLE 58

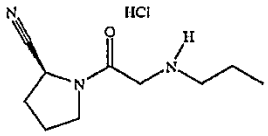
Pyrrolidine, 1-(cyclooctylamino)acetyl-2-cyano-, (S)-,monohydrochloride



Cyclooctylamine (commercially available) was used as the amine nucleophile. The title compound was a white fluffy solid. ^{13}C NMR (ppm)=121.64.

EXAMPLE 59

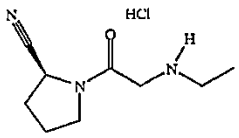
Pyrrolidine, 1-(propylamino)acetyl-2-cyano-,(S)-, monohydrochloride



Propylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=193° C.–194° C. ^{13}C NMR (ppm)=121.57.

EXAMPLE 60

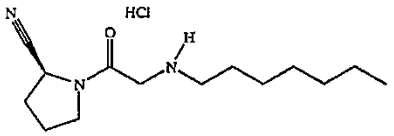
Pyrrolidine, 1-(ethylamino)acetyl-2-cyano-,(S)-, monohydrochloride



Ethylamine (commercially available) was used as the amine nucleophile. The title compound was an off-white stick solid. ^{13}C NMR (ppm)=121.67.

EXAMPLE 61

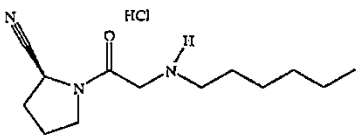
Pyrrolidine, 1-(heptylamino)acetyl-2-cyano-,(S)-, monohydrochloride



Heptylamine (commercially available) was used as the amine nucleopbile. The title compound was a white solid. Melting point=170° C.–172° C. ^{13}C NMR (ppm)=121.7.

EXAMPLE 62

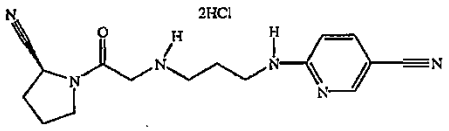
Pyrrolidine, 1-(hexylamino)acetyl-2-cyano-,(S)-, monohydrochloride



Hexylamine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=174° C.–176° C. ^{13}C NMR (ppm)=121.75.

EXAMPLE 63

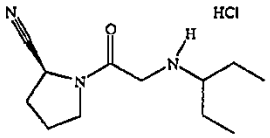
Pyrrolidine, 1-[[3-[(5-cyano-2-pyridinyl)amino] propyl]amino]acetyl-2-cyano-,(S)-, dihydrochloride



2(3-Aminopropylamino)-5-cyanopyridine (preparation described above) was used as the amine nucleophile. The title compound was a white sticky solid. Melting point=210° C.–212° C. ^{1}C NMR(pm)=119.33.

EXAMPLE 64

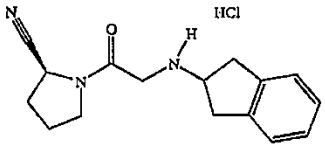
Pyrrolidine, 1-[(1-etbylpropyl)amino]acetyl-2-cyano-,(S)-,monohydrochloride



3-Aminopentane (commercially available) was used as the amine nucleophile. The title compound was a white fluffy sticky solid. ^{13}C NMR ppm)=119.35.

EXAMPLE 65

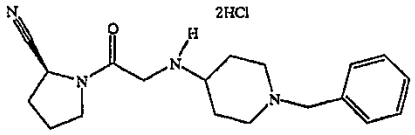
Pyrrolidine, 1-[(2,3-dihydro-1H-inden-2-yl)amino] acetyl-2-cyano-,(S)-,monohydrochloride



2-Aminoindan (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=182° C.–184° C. ^{13}C NMR (ppm)=121.38.

EXAMPLE 66

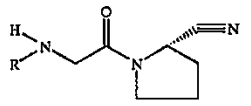
Pyrrolidine, 1-[(1-phenylmethyl-4-piperidinyl) amino]acetyl-2-cyano-,(S)-,-monohydrochloride



4-Amino-1-benzylpiperidine (commercially available) was used as the amine nucleophile. The title compound was a white solid. Melting point=280° C.–283° C. decomposed. ^{13}C NMR (ppm)=121.39.

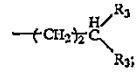
What is claimed is:

1. A compound of formula I:



wherein

R is a group $-(CH_2)_m-N-R_1$; an unsubstituted (C_{3-12})-cycloaklyl ring; a (C_{3-12})cycloalkyl ring substituted in the 1-position by a hydroxy(C_{1-3})alkyl group; a group $-(CH_2)_n-R_2$; a group



a group $-(CH_2)_p-R_4$; an isopropyl group; an isopropyl group substituted in the i-position by a hydroxy(C_{1-3})alkyl group; or R_5;

R_1 is an unsubstituted pyridine ring; a pyridine ring mono- or di-substituted by halo, trifluorometbyl, cyano or nitro; an unsubstituted pyrimidine ring; a pyrimidine ring monosubstituted by halo, trifluoromethyl, cyano or nitro; or an unsubstituted phenyl ring;

R_2 is an unsubstituted phenyl ring; a phenyl ring mono-, di- or tri-substituted by halo, (C_{1-3})alkoxy or a phenyl sulfide (optionally substituted with a (C_{1-8})alkoxy); phenoxy; (C_{1-8})alkyl; 3,1,1 bicyclic ring system (optionally substituted with 1 or more (C_{1-8}) alkyl groups); unsubstituted pyridine ring; naphthyl, cyclohexene; or adamantyl;

each R_3, independently, is an unsubstituted phenyl ring; or a phenyl ring mono-substituted by halo or (C_{1-3}) alkoxy;

R_4 is a 2-oxopyrrolidine group or a (C_{2-4})alkoxy group;

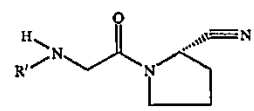
R_5 is indan; pyrrolidine (unsubstituted or substituted with —CH_2-phenyl); piperidine (unsubstituted or substituted with —CH_2-phenyl); a 2.2,1 bicyclic ring system (unsubstituted or substituted with 1 or more (C_{1-6}) alkyl groups); adamantyl; a straight or branched chain (C_{1-8}) alkyl (unsubstituted or substituted by one or more substituents selected from hydroxy, —CH_2OH and phenyl); or a 3,1,1 bicyclic ring system (unsubstituted or substituted with 1 or more (C_{1-8})alkyl groups); and

m and n independently are integers of 1 to 3;

p is an integer of 2 to 4;

or a pharmaceutically acceptable acid addition salt thereof.

2. A compound according to claim 1 of formula Ia:



where

R' is a group $-(CH_2)-N-R_1'$; an unsubstituted (C_{3-7}) cycloalkyl ring; a (C_{3-7}) cycloalkyl ring substituted in

the 1-position by a hydroxy(C_{1-3})alkyl group; a group $-(CH_2-)_3R_4'$; or a group R_5 as defined in claim 1;

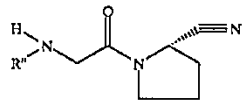
R_1' is an unsubstituted pyridine ring; a pyridine ring mono- or di-substituted by halo, trifluoromethyl, cyano or nitro; or an unsubstituted pyrimidine ring; and

R_4' is a (C_{2-4})alkoxy group;

or a pharmaceutically acceptable acid addition salt thereof.

3. A compound according to claim 2 of formula Ib:

Ib



where

R" is a group $-(CH_2-)_2N-R_1"$; a (C_{4-6})cycloalkyl ring substituted in the 1-position by a hydroxy(C_{1-3})alkyl group; a group $CH_2-)_3R_4'$; or a group R_5';

$R_1"$ is a pyridine ring mono- or di-substituted by halo, trifluoromethyl, cyano or nitro;

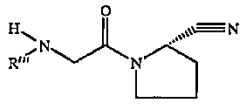
R_4' is as defined in claim 2; and

R_5' is a 3,1,1 bicyclic ring system (unsubstituted or substituted with 1 or more (C_{1-8}) alkyl groups; a 2,2,1 bicyclic ring system (optionally substituted with 1 or more (C_{1-8}) alkyl groups; or adamantyl;

or a pharmaceutically acceptable acid addition salt thereof.

4. A compound according to claim 3 of formula Ic:

Ic



where

R'" is a group $-(CH_2-)_2N-R_1"$; a (C_{4-6})cyclonlkly ring substituted in the 1-position by a hydroxymethyl group; a group $-(CH_2-)_3R_4"$; or a group $R_5"$;

$R_1'"$ is a pyridine ring monosubstituted by halo, trifluoromethyl, cyano or nitro;

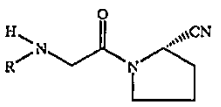
R_4' is as defined in claim 3; and

R_5&41 is adamantyl;

or a pbarmaceutically acceptable acid addition salt thereof.

5. A compound of formula I:

I



wherein R is:

a) $R_1R_{1a}N(CH_2)_m$- wherein

R_1 is a pyridinyl or pyrimidinyl moiety unsubstituted or mono- or independently disubstituted with (C_{1-4}) alkyl, (C_{1-4})alkoxy, halogen, trifluoromethyl, cyano or nitro; or phenyl unsubstituted or- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen;

R_{2a} is hydrogen or (C_{1-8}alkyl; and

m is 2 or3;

b) (C_{3-12})cycloalkyl unsubstituted or monosubstituted in the 1-position with (C_{1-3})hydroxyalkyl;

c) $R_2(CH_2)_n$- wherein either

R_2 is phenyl unsubstituted or mono- or independently di- or independently trisubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy, halogen or phenylthio unsubstituted or monosubstituted in the phenyl ring with hydroxymethyl; or is (C_{1-8})alkyl; a [3.1.1]bicyclic carbocyclic moiety unsubstituted or mono- or plurisubstituted with (C_{1-8})alkyl; a pyridinyl or naphthyl moiety unsubstituted or mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen; cyclohexene; or adamantyl; and

n is 1 to 3; or

R_2 is phenoxy unsubstituted or mono- or independently disubstituted with (C_{1-4}), (C_{1-4})alkoxy or halogen; and

n is 2or3;

d) $(R_3)_2CH(CH_2)_p$- wherein each R_3 independently is phenyl unsubstituted or mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4})alkoxy or halogen;

e) $R_4(CH_2)_p$- wherein R_4 is 2-oxopyrrolidinyl or (C_{2-4}) alkoxy and p is2to4;

f) isopropyl unsubstituted or monosubstituted in the 1 -position with (C_{1-3})hydroxyalkyl;

g) R_5 wherein R_5 is: indanyl; a pyrrolidinyl or piperidinyl moiety unsubstituted or substituted with benzyl; a [2.2.1]- or [3.1.1]bicyclic carbocyclic moiety unsubstituted or mono- or plurisubstituted with (C_{1-8})alkyl; adamantyl; or (C_{1-8})alkyl unsubstituted or mono- or independently plurisubstituted with hydroxy, hydroxymethyl or phenyl unsubstituted or mono- or independently disubstituted with (C_{1-4})alkyl, (C_{1-4}) alkoxy or halogen;

in free form or in acid addition salt form.

6. A compound according to claim 5 (a compound Ip) wherein R is R^p, which is:

a) $R_1^pNH(CH_2)_2$- wherein R_1^p is a pyridinyl or pyrimidinyl moiety unsubstituted or mono- or independently disubstituted with halogen, trifluoromethyl, cyano or nitro;

b) (C_{3-7})cycloalkyl unsubstituted or the monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;

c) $R_2^p(CH_2)_2$- wherein R_2^p is phenyl unsubstituted or mono- or independently di- or independently trisubstituted with halogen or (C_{1-3})alkoxy;

d) $(R_3^p)_2CH(CH_2)_2$- wherein each R_3^p independently is phenyl substituted or monosubstituted with halogen or (C_{1-3})alkoxy;

e) $R_4(CH_2)_3$- wherein R_4 is as defined in claim 5; or

f) isopropyl unsubstituted or monosubstituted in 1-position with (C_{1-3})hydroxyalkyl;

in free form or in acid addition salt form.

7. A compound according to claim 5 (a compound Is), wherein R is R^s, which is:

a) $R_1^sR_{1a}^s(CH_2)_{ms}$- wherein R_1^s is pyridinyl unsubstituted or mono- or independently disubstituted with chlorine, trifluoromethyl, cyano or nitro; pyrimidinyl unsubstituted or monosubstituted with chlorine or trifluoromethyl; or phenyl; R_{1a}^s is hydrogen or methyl; and ms is2or3;

b) (C_{3-12})cycloalkyl unsubstituted or monosubstituted in the 1-position with hydroxymethyl;

c) $R_2^s(CH_2)_{ns}$- wherein either R_2^s is phenyl unsubstituted or mono- or independently di- or independently trisub-

stituted with halogen, alkoxy of 1 or 2 carbon atoms or phenylthio monosubstituted in the phenyl ring with hydroxymethyl; (C_{1-6})alkyl; 6,6-dimethylbicyclo [3.1.1]hept-2-yl; pyridinyl; naphthyl; cyclohexene; or adamantyl; and ns is 1 to 3; or R_2^s is phenoxy; and ns is 2;

d) (3,3-diphenyl)propyl;

e) $R_4^s(CH_2)_{ps}$ wherein R_4^s is 2-oxopyrrolidin-1-yl or isopropoxy and ps is2or3;

f) isopropyl unsubstituted or monosubstituted in 1-position with hydroxymethyl;

g) R_5^s wherein R_5^s is: indanyl; a pyrrolidinyl or piperidinyl moiety unsubstituted or N-substituted with benzyl; bicyclo[2.2.1]hept-2-yl; 2,6,6-trimethylbicyclo-[3.1.1] hept-3-yl; adamantyl; or (C_{1-8})alkyl optionally mono- or independently disubstituted with hydroxy, hydroxymethyl or phenyl;

in free form or in acid addition salt form.

8. The compound according to claim 5 wherein R is 2-[(5-cyanopyridin-2-yl)amino]ethyl, i.e. 1-[2-[(5-cyanopyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine, in free form or in acid addition salt form, or a compound according to claim 5 which is of formula I wherein R is either

2-[(5-chloropyridin-2-yl)amino]ethyl, or
(1-hydroxymethyl)cyclopent-1-yl, or
2-[(5-nitropyridin-2-yl)amino]ethyl, or
3-(isopropoxy)propyl,

in free form or in acid addition salt form.

9. A compound according to claim 5 which is:

1-[2-[(5-chloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine dihydrochloride;

1-[2-[(5-trifluoromethylpryridin-2-yl)amino]ethylamino] acetyl-2-cyano-(S)-pyrrolidine;

1-[2-[(5-cyanopyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine dihydrochloride;

1-[2-[(pyrimidin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine;

1-[(1-hydroxymethylcyclopent-1-yl)amino]acetyl-2-cyano-(S)-pyrrolidine;

1-[2-[(pyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine;

1-[2-[(4-chloropyrimidin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine;

1-[2-[(3-chloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine;

1-[2-[4-trifluoromethylpyrimidin-2-yl)amino]ethylamino] acetyl-2-cyano-(S)-pyrrolidine;

1-[(2-chlorophenyl)ethylamino]acetyl-2-cyano-(S)-pyrrolidine;

1-[(3,3-diphenyl)propylamino]acetyl-2-cyano-(S)-pyrrolidine;

1-[2-[(5-nitropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)pyrrolidine;

1-[2-[(3-chloro-5-trifluoromethylpyridin-2-yl)amino] ethylamino]acetyl-2-cyano-(S)-pyrrolidine;

1-[2-[(3-trifluoromethylpyridin-2-yl)amino]ethylamino] acetyl-2-cyano-(S)pyrrolidine;

1-[2-[(3,5-dichloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine;

1-[(cyclopent-1-yl)amino-acetyl-2-cyano-(S)-pyrrolidine monohydrochloride;

1-[2-(2-bromo-4,5-dimethoxyphenyl)ethylamino]acetyl-2-cyano-(S)-pyrrolidine;

1-[3-(isopropoxy)propylamino]acetyl-2-cyano-(S)-pyrrolidine monohydrochloride;

1-[(2-hydroxy-1,1-dimethylethylamino)]acetyl-2-cyano-(S)-pyrrolidine monohydrochloride;

1-[3-(2-oxo-pyrrolidin-1-yl)propylamino]acetyl-2-cyano-(S)-pyrrolidine monohydrochloride;

1-[(1-hydroxymethylcylohexyl)amino]acetyl-2-cyano-(S)-pyrrolidine;

Pyrrolidine, 1-[[2-(4-ethoxyphenyl)ethyl]amino]acetyl-2-cyano-,(S)-,monohydrochloride;

Pyrrolidine, 1-[(1-phenylmethyl-3-pyrrolidinyl)amino] acetyl-2-cyano-,(S)-(R)-,dihydrochloride;

Pyrrolidine, 1-[[2-(4-methoxyphenyl)ethyl]amino]acetyl-2-cyano-,(S)-,monohydrochloride;

Pyrrolidine, 3-[[2-(3-methoxyphenyl)ethyl]amino]acetyl-2-cyano-,(S)-,monohydrochloride;

Pyrrolidine, 1-[[(1-naphthalenyl)methyl]amino]acetyl-2-cyano-,(S)-,monohydrochloride;

Pyrrolidine, 1-[(3-phenylpropyl)amino]acetyl-2-cyano-,(S)-,monohydrochloride;

Pyrrolidine, 1-[[3-[(phenyl)(methyl)amino]propyl]amino] acetyl-2-cyano-,(S)-,dihydrochloride;

Pyrrolidine, 1-[2-[(3,4-dimethoxyphenyl)ethyl]amino] acetyl-2-cyano-,(S)-,monohydrochloride;

Pyrrolidine, 1-(acycloheptylamino)acetyl-2-cyano-,(S)-, monohydrochloride;

Pyrrolidine, 1-[[(6,6-dimethylbicyclo[3.1.1]hept-2-yl) methyl]amino]acetyl-2-cyano-[1S[1α,2α(S*),5α]]-(S)-, monohydrochloride;

Pyrrolidine, 1-[[2-(2,5-dmethoxyphenyl)ethyl]amino] acetyl-2-cyano-,(S)-,monohydrochloride;

Pyrrolidine, 1-[[2-(1-cyclohexen-1-yl)ethyl]amino]acetyl-2-cyano-,(S)-;

Pyrrolidine, 1-(cyclohexylamino)acetyl-2-cyano-,(S)-, monohydrochloride;

Pyrrolidine, 1-[(bicyclo[2.2.1]hept-2-yl)amino]acetyl-2-cyano-[1S[1α,2α(S*),5α]]-(S)-,monohydrochloride;

Pyrrolidine, 1-[[2-(2-pyridinyl)ethyl]amino]acetyl-2-cyano-,(S)-,dihydrochloride;

Pyrrolidine, 1-[[(2-phenylamino)ethyl]amino]acetyl-2-cyano-,(S)-,dihydrochloride;

Pyrrolidine, 1-[(3,3-dimethylbutyl)amino]acetyl-2-cyano-, (S)-,monohydrochloride;

Pyrrolidine, 1-[(2,6,6-trimethylbicyclo[3.1.1]hept-3-yl) amino]acetyl-2-cyano-,(S)[1S[1α,2β,3α(S*),5α]]-monohydrochloride;

Pyrrolidine, 1-[[(1 -hydroxymethyl)propyl]amino]acetyl-2-cyano-[S,S)]-;

Pyrrolidine, 1-[[[2-[(2-hydroxymethyl)phenyl]thio] phenylmetbyl]amino]acetyl-2-cyano-,(S)-, monohydrochloride;

Pyrrolidine, 1-[[2-(2-methoxyphenyl)ethyl]amino]acetyl-2-cyano-,(S)-,monohydrochloride;

Pyrrolidine, 1-[(5-hydroxypentyl)amino]acetyl-2-cyano-, (S)-,monohydrochloride;

Pyrrolidine, 1-(cyclobutylamino)acetyl-2-cyano-,(S)-monohydrochloride;

Pyrrolidine, 1-[[2-(2,4dichlorophenyl)ethyl]amino]acetyl-2-cyano-,(S),monohydrochloride;

Pyrrolidine, 1-[(1-hydroxymethyl]-3-methylbutyl)amino] acetyl-2-cyano-,O-,(S)-;

Pyrrolidine, 1-[(2-hydroxy-2-phenylethyl)amino]acetyl-2-cyano-[2S-[1R*,2S*]-monohydrochloride;

Pyrrolidine, 1-[[2-(2-fluorophenyl)ethyl]amino]acetyl-2-cyano-,(S)-,monohydrochloride;

Pyrrolidine, 1-(cyclopropylamino)acetyl-2-cyano-,(S)-, monohydrochloride;

Pyrrolidine, 1-[(2,6,6-trimethylbicyclo[3.1.1]hept-3-yl) amino]acetyl-2-cyano-[1S[1α,2α,3β(S*),5α]]-monohydrochloride;

Pyrrolidine, 1-[[(2-phenoxy)ethyl]amino]acetyl-2-cyano-, (S)-,monohydrochloride;

Pyrrolidine, 1-[2-[(3,5-dimethoxyphenyl)ethyl]amino] acetyl-2-cyano-,(S)-,monohydrochloride;

Pyrrolidine, 1-[(1 -adamantyl)amino]acetyl-2-cyano-,(S)-, mooohydrochloride;

Pyrrolidine, 1-[(1,1,3,3-tetramethylbutyl)amino]acetyl-2-cyano-,(S)-,monohydrochloride;

Pyrrolidine, 1-[(2-adamantyl)amino]acetyl-2-cyano-,(S)-, monohydrochloride;

Pyrrolidine, 1-[(1,1-dimethylpropyl)amino]acetyl-2-cyano-, (S)-,monohydrochloride;

Pyrrolidine, 1-[(phenylmethyl)amino]acetyl-2-cyano-,(S)-, monohydrochloride;

Pyrrolidine, 1-[(1,1 -dimethylethyl)amino]acetyl-2-cyano-, (S),monohydrochloride;

Pyrrolidine, 1-[[(2-adamantyl)methyl]amino]acetyl-2-cyano-,(S)-,monohydrochloride;

Pyrrolidine, 1-[(2-phenylethyl)amino]acetyl-2-cyano-, (S)-, monohydrochloride;

Pyrrolidine, 1-(pentylamino)acetyl-2-cyano-,(S)-, monohydrochloride;

Pyrrolidine, 1-(butylamino)acetyl-2-cyano-,(S)-, monohydrochloride;

Pyrrolidine, 1-(cyclododecylamino)acetyl-2-cyano-,(S)-, monohydrochloride;

Pyrrolidine, 1-(cyclooctylamino)acetyl-2-cyano-,(S)-, monohydrochloride;

Pyrrolidine, 1-(propylamino)acetyl-2-cyano-,(S)-, monohydrochloride;

Pyrrolidine, 1-(ethylamino)acetyl-2-cyano-,(S)-, monohydrochloride;

Pyrrolidine, 1-(heptylamino)acetyl-2-cyano-,(S)-, monohydrochloride;

Pyrrolidine, 1-(hexylamino)acetyl-2-cyano-,(S)-, monohydrochloride;

Pyrrolidine, 1-[[3-[(5-cyano-2-pyridinyl)amino]propyl] amino]acetyl-2-cyano-,(S)-,dihydrochloride;

Pyrrolidine, 1-[(1-ethylpropyl)amino]acetyl-2-cyano-,(S)-, monohydrochloride,

Pyrrolidine, 1-1(2,3-dihydro-1H-inden-2-yl)amino]acetyl-2-cyano-,(S)-,monohydrochloride;

Pyrrolidine, 1-[(1-phenylmethyl-4-piperidinyl)amino] acetyl-2-cyano-,(S)-,

monohydrochloride; or a pharmaceutically acceptable salt of any of the above compounds which are in free form.

10. A pharmaceutical composition comprising a compound according to claim 5 in free form or in pharmaceutically acceptable acid addition salt form, together with at least one pharmaceutically acceptable carrier or diluent.

11. A compound according to claim 8 which is 1-[2-[(5cyanopyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine dihydrochloride.

* * * * *

PCT/EP97/06125

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APPLICATION NUMBER: *08/746,295*
FILING DATE: *November 7, 1996*

PRIORITY DOCUMENT

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

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Certifying Officer

EXHIBIT
PLF 26
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N-(SUBSTITUTED GLYCYL)-2-CYANOPYRROLIDINES, PHARMACEUTICAL COMPOSITIONS CONTAINING THEM AND THEIR USE IN INHIBITING DIPEPTIDYL PEPTIDASE-IV

FIELD OF THE INVENTION

The present invention relates to the area of dipeptidyl peptidase-IV inhibition and, more particularly, relates to certain N-(substituted glycyl)-2-cyanopyrrolidines, pharmaceutical compositions containing said compounds, and the use of said compounds in inhibiting dipeptidyl peptidase-IV.

BACKGROUND OF THE INVENTION

Dipeptidyl peptidase-IV (DPP-IV) is a serine protease which cleaves N-terminal dipeptides from a peptide chain containing, preferably, a proline residue in the penultimate position. Although the biological role of DPP-IV in mammalian systems has not been completely established, it is believed to play an important role in neuropeptide metabolism, T-cell activation, attachment of cancer cells to the endothelium and the entry of HIV into lymphoid cells.

More recently, it was discovered that DPP-IV is responsible for inactivating glucagon-like peptide-1 (GLP-1). More particularly, DPP-IV cleaves the amino-terminal His-Ala dipeptide of GLP-1, generating a GLP-1 receptor antagonist, and thereby shortens the physiological response to GLP-1. Since the half-life for DPP-IV cleavage is much shorter than the half-life for removal of GLP-1 from circulation, a significant increase in GLP-1 bioactivity (5- to 10-fold) is anticipated from DPP-IV inhibition. Since GLP-1 is a major stimulator of pancreatic insulin secretion and has direct beneficial effects on glucose disposal, DPP-IV inhibition appears to represent an attractive approach for treating non-insulin-dependent diabetes mellitus (NIDDM).

Case 600-7247/R

Although a number of DPP-IV inhibitors have been described in the literature, all have limitations relating to potency, stability or toxicity. Accordingly, it is clear that a great need exists for novel DPP-IV inhibitors which are useful in treating conditions mediated by DPP-IV inhibition and which do not suffer from the above-mentioned limitations of known DPP-IV inhibitors.

DESCRIPTION OF THE PRIOR ART

WO 95/15309 discloses certain peptide derivatives which are inhibitors of DPP-IV and, therefore, are useful in treating a number of DPP-IV mediated processes.

WO 95/13069 discloses certain cyclic amine compounds which are useful in stimulating the release of natural or endogenous growth hormone.

European Patent 555,824 discloses certain benzimidazolyl compounds which prolong thrombin time and inhibit thrombin and serine-related proteases.

Archives of Biochemistry and Biophysics, Vol. 323, No. 1, pgs. 148-154 (1995) discloses certain aminoacylpyrrolidine-2-nitriles which are useful as DPP-IV inhibitors.

Journal of Neurochemistry, Vol. 66, pgs. 2105-2112 (1996) discloses certain Fmoc-aminoacylpyrrolidine-2-nitriles which are useful in inhibiting prolyl oligopeptidase.

Bulletin of the Chemical Society of Japan, Vol. 50, No. 7, pgs. 1827-1830 (1977) discloses the synthesis of an aminohexapeptide, viz., Z-Val-Val-ImPro-Gly-Phe-Phe-OMe, and its related aminopeptides. In addition, the antimicrobial properties of said compounds were examined.

Bulletin of the Chemical Society of Japan, Vol. 51, No. 3, pgs. 878-883 (1978) discloses the synthesis of two known peptide antibiotics, viz., Bottromycins B_1 and B_2 according to the structures proposed by Nakamura, et al. However, since the resultant compounds were devoid of antimicrobial properties, it was concluded that the structures proposed by Nakamura, et al. were erroneous.

WO 90/12005 discloses certain amino acid compounds which inhibit prolylendopeptidase activity and, therefore, are useful in treating dementia or amnesia.

Chemical Abstracts 95: 302548 discloses certain N-(aryl(alkyl)carbonyl) substituted heterocyclic compounds which are cholinesterase activators with enhanced peripheral selectivity useful in treating conditions due to the lowering of cholinesterase activity.

Chemical Abstracts 84: 177689 discloses certain 1-acyl-pyrrolidine-2-carbonitrile compounds which are useful as intermediates for proline compounds exhibiting angiotensin converting enzyme (ACE) inhibiting activity.

Chemical Abstracts 96: 116353 discloses certain 3-amino-2-mercapto-propyl-proline compounds which are Ras farnesyl-transferase inhibitors useful in treating various carcinomas or myeloid leukemias.

WO 95/34538 discloses certain pyrrolidides, phosphonates, azetidines, peptides and azaprolines which inhibit DPP-IV and, therefore, are useful in treating conditions mediated by DPP-IV inhibition.

WO 95/29190 discloses certain compounds characterized by a plurality of KPR-type repeat patterns carried by a peptide matrix enabling their multiple presentation to, and having an affinity for, the enzyme DPP-IV, which compounds exhibit the ability to inhibit the entry of HIV into cells.

WO 91/16339 discloses certain tetrapeptide boronic acids which are DPP-IV inhibitors useful in treating autoimmune diseases and conditions mediated by IL-2 suppression.

WO 93/08259 discloses certain polypeptide boronic acids which are DPP-IV inhibitors useful in treating autoimmune diseases and conditions mediated by IL-2 suppression.

WO 95/11689 discloses certain tetrapeptide boronic acids which are DPP-IV inhibitors useful in blocking the entry of HIV into cells.

German Patent 158109 discloses certain N-protected peptidyl-hydroxamic acids and nitrobenzoyloxamides which are useful as, inter alia, DPP-IV inhibitors.

WO 95/29691 discloses, inter alia, certain dipeptide proline phosphonates which are DPP-IV inhibitors useful in the treatment of immune system disorders.

German Patent 296075 discloses certain amino acid amides which inhibit DPP-IV.

Biochimica et Biophysica Acta, Vol. 1293, pgs. 147-153 discloses the preparation of certain di- and tri-peptide p-nitroanilides to study the influence of side chain modifications on their DPP-IV and PEP-catalyzed hydrolysis.

Bioorganic and Medicinal Chemistry Letters, Vol. 6, No. 10, pgs. 1163-1166 (1996) discloses certain 2-cyanopyrrolidines which are inhibitors of DPP-IV.

J. Med. Chem., Vol. 39, pgs. 2087-2094 (1996) discloses certain prolineboronic acid-containing dipeptides which are inhibitors of DPP-IV.

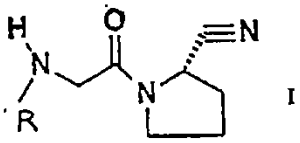
Diabetes, Vol. 44, pgs. 1126-1131 (Sept.'96) is directed to a study which demonstrates that GLP-I amide is rapidly degraded when administered by subcutaneous or intravenous routes to diabetic and non-diabetic subjects.

SUMMARY OF THE INVENTION

The present invention provides new DPP-IV inhibitors which are effective in treating conditions mediated by DPP-IV inhibition. More particularly, the present invention relates to certain N-(substituted glycyl)-2-cyanopyrrolidines which inhibit DPP-IV. In addition, the present invention provides pharmaceutical compositions useful in inhibiting DPP-IV comprising a therapeutically effective amount of a certain N-(substituted glycyl)-2-cyano-pyrrolidine. Moreover, the present invention provides a method of inhibiting DPP-IV comprising administering to a mammal in need of such treatment a therapeutically effective amount of a certain N-(substituted glycyl)-2-cyano-pyrrolidine.

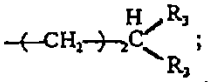
DETAILED DESCRIPTION OF THE INVENTION

The essence of the instant invention is the discovery that certain N-(substituted glycyl)-2-cyanopyrrolidines are useful in inhibiting DPP-IV. In one embodiment, the present invention provides compounds of formula I:



I

wherein R is a group $-(CH_2)_2\overset{H}{N}-R_1$; an unsubstituted (C_{3-7})-cycloalkyl ring; a (C_{3-7})cycloalkyl ring substituted in the 1-position by a hydroxy(C_{1-3})alkyl group; a group $-(CH_2)_2R_2$; a group



; a group $-(CH_2)_3R_4$; an isopropyl group; or an isopropyl

group substituted in the 1-position by a hydroxy(C_{1-3})alkyl group;

R_1 is an unsubstituted pyridine ring; a pyridine ring mono- or di-substituted by halo, trifluoromethyl, cyano or nitro; an unsubstituted pyrimidine ring; or a pyrimidine ring monosubstituted by halo, trifluoromethyl, cyano or nitro;

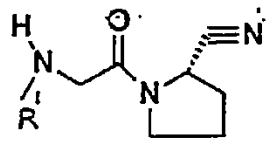
R_2 is an unsubstituted phenyl ring; or a phenyl ring mono-, di- or tri-substituted by halo or (C_{1-3})alkoxy;

each R_3, independently, is an unsubstituted phenyl ring; or a phenyl ring mono-substituted by halo or (C_{1-3})alkoxy; and

R_4 is a 2-oxopyrrolidine group or a (C_{2-4})alkoxy group;

or a pharmaceutically acceptable acid addition salt thereof.

Preferred compounds are those of formula Ia:



Ia

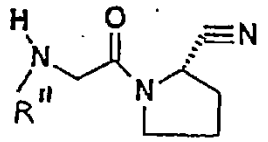
where R' is a group $-(CH_2)_2\overset{H}{N}-R_1'$; an unsubstituted ($C_{3-7}$)cycloalkyl ring; a ($C_{3-7}$) cycloalkyl ring substituted in the 1-position by a hydroxy(C_{1-3})alkyl group; or a group $-(CH_2)_3R_4'$;

R_1' is an unsubstituted pyridine ring; or a pyridine ring mono- or di-substituted by halo, trifluoromethyl, cyano or nitro; and

R_4' is a (C_{2-4})alkoxy group;

or a pharmaceutically acceptable acid addition salt thereof.

More preferred compounds are those of formula Ib:



Ib

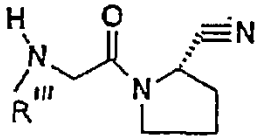
where R" is a group $-(CH_2)_2\overset{H}{N}-R_1^{II}$; a ($C_{4-6}$)cycloalkyl ring substituted in the 1-position by a hydroxy(C_{1-3})alkyl group; or a group $-(CH_2)_3R_4^I$;

R_1^{II} is a pyridine ring mono- or di-substituted by halo, trifluoromethyl, cyano or nitro; and

R_4^I is as defined above;

or a pharmaceutically acceptable acid addition salt thereof.

Even more preferred compounds are those of formula Ic:



Ic

where R'" is a group $-(CH_2)_2\overset{H}{N}-R_1^{III}$; a ($C_{4-6}$)cycloalkyl ring substituted in the 1-position by a hydroxymethyl group; or a group $-(CH_2)_3R_4^I$;

R_1^{III} is a pyridine ring monosubstitued by halo, trifluoromethyl, cyano or nitro; and

R_4^I is as defined above;

or a pharmaceutically acceptable acid addition salt thereof.

In another embodiment, the instant invention provides pharmaceutical compositions useful in inhibiting DPP-IV comprising a pharmaceutically acceptable carrier or diluent and a therapeutically effective amount of a compound of formula I above, or a pharmaceutically acceptable acid addition salt thereof, preferably a compound of formula Ia above, or a pharmaceutically acceptable acid addition salt thereof, more preferably a compound of formula Ib above, or a pharmaceutically acceptable acid addition salt thereof, and even more preferably a compound of formula Ic above, or a pharmaceutically acceptable acid addition salt thereof.

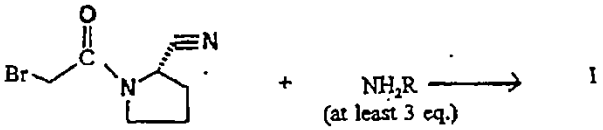
In still another embodiment, the instant invention provides a method of inhibiting DPP-IV comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound of formula I above, or a pharmaceutically acceptable acid addition salt thereof, preferably a compound of formula Ia above, or a pharmaceutically acceptable acid addition salt thereof, more preferably a compound of formula Ib above, or a pharmaceutically acceptable acid addition salt thereof, and even more preferably a compound of formula Ic above, or a pharmaceutically acceptable acid addition salt thereof.

In a further embodiment, the instant invention provides a method of treating conditions mediated by DPP-IV inhibition comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound of formula I above, or a pharmaceutically acceptable acid addition salt thereof, preferably a compound of formula Ia above, or a pharmaceutically acceptable acid addition salt thereof, more preferably a compound of formula Ib above, or a pharmaceutically acceptable acid addition salt thereof, and even more preferably a compound of formula Ic above, or a pharmaceutically acceptable acid addition salt thereof.

In the above definitions, it should be noted that the "alkoxy" significance is either straight or branched chain, of which examples of the latter are isopropyl and t-butyl.

The acid addition salts of the compounds of formula I may be those of pharmaceutically acceptable organic or inorganic acids. Although the preferred acid addition salts are the hydrochlorides, salts of methanesulfonic, sulfuric, phosphoric, citric, lactic and acetic acid may also be utilized.

The compounds of formula I may be prepared as depicted below:

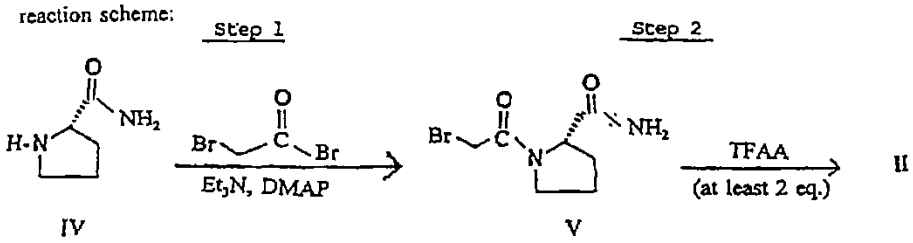


II III

where R is as defined above.

In the above reaction, the bromide compound of formula II is reacted with at least 3 equivalents of a primary amine compound of formula III to obtain an N-(substituted glycyl)-2-cyanopyrrolidine compound of formula I. The reaction is conducted in the presence of an inert, organic solvent, preferably a cyclic ether such as tetrahydrofuran, at a temperature of from 0° to 35°C., preferably at a temperature between 0° and 25°C., for a period of between 1 and 20 hours.

The bromide compound of formula II may be prepared by the following two-step reaction scheme:

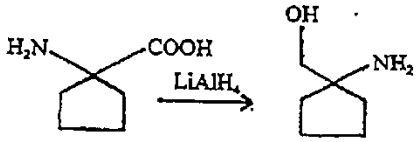


IV V

As to the individual steps, Step 1 involves the reaction of the pyrrolidine compound of formula IV with a slight molar excess of bromoacetylbromide and triethylamine and a catalytic amount of dimethylaminopyridine (DMAP) to obtain the bromide compound of formula V. The reaction is conducted in the presence of an inert, organic solvent, preferably a chlorinated, aliphatic hydrocarbon such as methylene chloride, at a temperature of from 0° to 25°C, preferably at a temperature between 0° and 15°C, for a period of between 2 and 6 hours, preferably between 2 and 4 hours.

Step 2 concerns the dehydration of the bromide compound prepared in Step 1, i.e., the compound of formula V, with at least 2 equivalents of trifluoroacetic anhydride to obtain the bromide compound of formula II. The dehydration is conducted in the presence of an inert, organic solvent, preferably a chlorinated, aliphatic hydrocarbon such as methylene chloride, at a temperature of from 0° to 25°C, preferably at a temperature between 0° and 15°C, for a period of between 3 and 8 hours, preferably between 3 and 6 hours.

The primary amine compounds of formula III are known and may be prepared by procedures well documented in the literature. For example: a) 1-hydroxymethylcyclopentylamine can be prepared by the reduction of 1-amino-1-cyclopentane carboxylic acid with lithium aluminum hydride as set forth below:



The reduction is conducted in the presence of an inert, organic solvent, preferably a cyclic ether such as tetrahydrofuran, at the reflux temperature of the solvent for a period of between 14 and 24 hours. (b) 2-[(5-chloropyridin-2-yl)amino]ethylamine can be prepared by refluxing a mixture of 2,5-dichloropyridine with ethylenediamine in an oil bath for a period of between 6 and 12 hours. (c) Similarly, 2-[(5-trifluoromethylpyridin-2-yl)amino]ethylamine can be

prepared by refluxing a mixture of 2-chloro-5-trifluoromethyl pyridine with ethylenediamine in an oil bath for a period of between 6 and 12 hours. (d) 2-[(5-cyanopyridin-2-yl)amino]-ethylamine can be prepared by stirring a mixture of 2-chloropyridine-5-carbonitrile and ethylenediamine at a temperature between 20° and 30°C, for a period of between 4 and 6 hours. (e) 2-[(pyrimidin-2-yl)amino]ethylamine can be prepared by adding ethylenediamine to ice-bath cooled 2-chloropyrimidine and allowing the mixture to react at a temperature between 20° and 30°C, for a period of between 12 and 20 hours.

As indicated above, the compounds of formula I form pharmaceutically acceptable acid addition salts. For example, the free base of a compound of formula I can be reacted with hydrochloric acid in gaseous form to form the corresponding mono- and di-hydrochloride salt forms, whereas reacting the free base with methanesulfonic acid forms the corresponding mesylate salt form. All pharmaceutically acceptable acid addition salt forms of the compounds of formula I are intended to be embraced by the scope of this invention.

As indicated above, all of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, are useful in inhibiting DPP-IV. The ability of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, to inhibit DPP-IV may be demonstrated employing the Caco-2 DPP-IV Assay which measures the ability of test compounds to inhibit DPP-IV activity from human colonic carcinoma cell extracts. The human colonic carcinoma cell line Caco-2 was obtained from the American Type Culture Collection (ATCC HTB 37). Differentiation of the cells to induce DPP-IV expression was accomplished as described by Reisher, et al. in an article entitled "Increased expression of ... intestinal cell line Caco-2" in Proc. Natl. Acad. Sci., Vol. 90, pgs. 5757-5761 (1993). Cell extract is prepared from cells solubilized in 10mM Tris-HCl, 0.15 M NaCl, 0.04 t.i.u. aprotinin, 0.5% nonidet-P40, pH 8.0, which is centrifuged at 35,000 g for 30 min. at 4°C. to remove cell debris. The assay is conducted by adding 20 µg solubilized Caco-2 protein, diluted to a final volume of 125 µl in assay buffer (25 mM Tris-HCl pH 7.4, 140 mM NaCl, 10 mM KCl, 1% bovine serum albumin) to microtiter plate wells. The reaction is initiated by adding 25µl of 1 mM substrate (H-Alanine-Proline-pNA; pNA is

p-nitroaniline). The reaction is run at room temperature for 10 minutes after which time a 19 µl volume of 25% glacial acetic acid is added to stop the reaction. Test compounds are typically added as 30 µl additions and the assay buffer volume is reduced to 95 µl. A standard curve of free *p*-nitroaniline is generated using 0-500 µM solutions of free pNA in assay buffer. The curve generated is linear and is used for interpolation of substrate consumption (catalytic activity in nmoles substrate cleaved /min). The endpoint is determined by measuring absorbance at 405 nm in a Molecular Devices UV Max microtiter plate reader. The potency of the test compounds as DPP-IV inhibitors, expressed as IC_{50}, is calculated from 8-point, dose-response curves using a 4-parameter logistic function.

The following IC_{50}s were obtained:

Compound	Caco-2 DPP-IV (nM)
Ex. 1	36
Ex. 2	176
Ex. 3	22
Ex. 4	140
Ex. 5	26
Ex. 6	50
Ex. 7A	165
Ex. 8	8
Ex. 7B	175
Ex. 9A	990
Ex. 7C	290
Ex. 9C	295
Ex. 10	54
Ex. 11	215
Ex. 7D	382
Ex. 7E	388
Ex. 12	279
Ex. 13	227
Ex. 14	110

The ability of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, to inhibit DPP-IV may also be demonstrated by measuring the effects of test compounds on DPP-IV activity in human and rat plasma employing a modified version of the assay described by Kubota, et al. in an article entitled "Involvement of dipeptidylpeptidase IV in an in vivo immune response" in Clin. Exp. Immunol., Vol. 89, pgs. 192-197 (1992). Briefly, five µl of plasma are added to 96-well flat-bottom microtiter plates (Falcon), followed by the addition of 5 µl of 80 mM $MgCl_2$ in incubation buffer (25 mM HEPES, 140 mM NaCl, 1% RIA-grade BSA, pH 7.8). After a 5 min. incubation at room temperature, the reaction is initiated by the addition of 10 µl of incubation buffer containing 0.1 mM substrate (H-Glycine-Proline-AMC; AMC is 7-amino-4-methylcoumarin). The plates are covered with aluminum foil (or kept in the dark) and incubated at room temperature for 20 min. After the 20 min. reaction, fluorescence is measured using a CytoFluor 2350 fluorimeter (Excitation 380 nm Emission 460 nm; sensitivity setting 4). Test compounds are typically added as 2 µl additions and the assay buffer volume is reduced to 13 µl. A fluorescence-concentration curve of free AMC is generated using 0-50 µM solutions of AMC in assay buffer. The curve generated is linear and is used for interpolation of substrate consumption (catalytic activity in nmoles substrate cleaved/min). As with the previous assay, the potency of the test compounds as DPP-IV inhibitors, expressed as IC_{50}, is calculated from 8-point, dose-response curves using a 4 parameter logistic function.

The following IC_{50}s were obtained:

Compound	human plasma DPP-IV (nM)	rat plasma DPP-IV (nM)
Ex. 1	27	22
Ex. 3	7	6
Ex. 4	40	23
Ex. 5	37	18
Ex. 6	22	32
Ex. 8	12	11
Ex. 10	51	19
Ex. 12	95	38
Ex. 14	95	24

In view of their ability to inhibit DPP-IV, the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, are useful in treating conditions mediated by DPP-IV inhibition. For example, the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, improve early insulin response to an oral glucose challenge and, therefore, are useful in treating non-insulin-dependent diabetes mellitus. The ability of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, to improve early insulin response to an oral glucose challenge may be measured in insulin resistant rats according to the following method:

Male Sprague-Dawley rats that had been fed a high fat diet (saturated fat = 57% calories) for 2-3 weeks were fasted for approximately 2 hours on the day of testing, divided into groups of 8-10, and dosed orally with 10μmol/kg of the test compounds in CMC. An oral glucose bolus of lg/kg was administered 30 minutes after the test compound directly into the stomach of the test animals. Blood samples, obtained at various timepoints from chronic jugular vein catheters were analyzed for plasma glucose and immunoreactive insulin (IRI) concentrations, and plasma DPP-IV activity. Plasma insulin levels were assayed by a double antibody radioimmunoassay (RIA) method using a specific anti-rat insulin antibody from Linco Research (St. Louis, MO). The RIA has a lower limit of detection of 0.5 μU/ml with intra- and inter-assay variations of less than 5%. Data are expressed as % increase of the mean of the control animals. Upon oral administration, each of the compounds tested amplified the early insulin response which led to an improvement in glucose tolerance in the insulin resistant test animals. The following results were obtained:

Compound	Increase of Insulin Response at 10μmol/kg
Ex. 1	61%
Ex. 3	66%
Ex. 5	108%
Ex. 8	144%
Ex. 12	59%

The precise dosage of the compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, to be employed for treating conditions mediated by DPP-IV inhibition depends upon several factors, including the host, the nature and the severity of the condition being treated, the mode of administration and the particular compound employed. However, in general, conditions mediated by DPP-IV inhibition are effectively treated when a compound of formula I, or a corresponding pharmaceutically acceptable acid addition salt, is administered enterally, e.g., orally, or parenterally, e.g., intravenously, preferably orally, at a daily dosage of 0.002-5, preferably 0.02-2.5 mg/kg body weight or, for most larger primates, a daily dosage of 0.1-250, preferably 1-100 mg. A typical oral dosage unit is 0.01-0.75 mg/kg, one to three times a day.

Usually, a small dose is administered initially and the dosage is gradually increased until the optimal dosage for the host under treatment is determined. The upper limit of dosage is that imposed by side effects and can be determined by trial for the host being treated.

The compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, may be combined with one or more pharmaceutically acceptable carriers and, optionally, one or more other conventional pharmaceutical adjuvants and administered enterally, e.g., orally, in the form of tablets, capsules, caplets, etc. or parenterally, e.g., intravenously, in the form of sterile injectable solutions or suspensions. The enteral and parenteral compositions may be prepared by conventional means.

The compounds of formula I, and their corresponding pharmaceutically acceptable acid addition salts, may be formulated into enteral and parenteral pharmaceutical compositions containing an amount of the active substance that is effective for treating conditions mediated by DPP-IV inhibition, such compositions in unit dosage form and such compositions comprising a pharmaceutically acceptable carrier.

The compounds of formula I (including those of each of the subscopes thereof and each of the examples) may be administered in enantiomerically pure form (e.g., ee≥ 98%, preferably ≥ 99%) or together with the R enantiomer, e.g., in racemic form. The above dosage ranges are based on the compounds of formula I (excluding the amount of the R enantiomer).

The following examples show representative compounds encompassed by this invention and their synthesis. However, it should be clearly understood that they are for purposes of illustration only.

EXAMPLE 1

1-[2-[(5-chloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine dihydrochloride.

a) Preparation of 2-carbamoylpyrrolidine-carbonylmethylene-(S)-bromide.

22.37g (196 mmol) of 2-carbamoylpyrrolidine, 30.1 ml (216 mmol) of triethylamine and 30.0 mg of dimethylaminopyridine (DMAP) are dissolved in 200 ml of methylene chloride and the solution is then added, dropwise, to an ice-cold solution of 18.8 ml (216 mmol) of bromoacetylbromide in 192 ml of methylene chloride, over a period of 60 minutes under a calcium sulfate drying tube. The resultant solution is then stirred for 2 hours at ice-water temperature under a calcium sulfate drying tube, after which time it is poured into 3.5 liters of ethyl acetate. The resultant precipitate is filtered, washed with ethyl acetate, and the filtrate is concentrated to obtain the desired compound as a hard yellow taffy.

b) Preparation of 2-cyanopyrrolidino-carbonylmethylene-(S)bromide.

50.0 g (213 mmol) of the bromide compound prepared in a) above is dissolved in 300 ml of methylene chloride and the solution is cooled in an ice water bath under a calcium sulfate drying tube. The cooled solution is then poured into 60.2 ml (426 mmol) of trifluoroacetic anhydride over a 2 minute period and the resultant solution is then stirred at ice-water temperature under a calcium sulfate drying tube for 4 hours and partitioned between methylene chloride and saturated aqueous sodium bicarbonate. The product is then extracted into the methylene chloride layer and the aqueous layer is then washed twice with methylene chloride. The combined organic layers are then washed successively with water and brine and then dried over sodium sulfate. The solution is then filtered and the solvent is removed by rotovaping and high vacuum pumping to obtain the desired compound as a dark yellow solid.

c) Preparation of the title compound in free base form.

To a 500 ml flask is added 16.6g (97.2 mmol) of 2-[(5-chloropyridin-2-yl)amino]ethylamine and 100 ml of tetrahydrofuran and the mixture is cooled in an ice bath. To the cooled mixture is added 7.0 g (32.4 mmol) of the bromide compound prepared in b) above dissolved in 30 ml of tetrahydrofuran. The resultant mixture is stirred for 2 hours at 0°C., the solvent is removed by rotovaping and the mixture is partitioned between ethyl acetate and water. The product is then extracted into the ethyl acetate layer and the aqueous layer is then washed twice with ethyl acetate. The combined organic layers are then washed successively with water and brine, dried over sodium sulfate and concentrated to obtain the desired compound in crude form. The crude form is then purified on silica gel employing a mixture of 5% methanol in methylene chloride as the eluent to yield the desired compound as a light brown oil.

d) Preparation of the title compound.

After dissolving the free base compound prepared in c) above in 30 ml of dry tetrahydrofuran, hydrogen chloride gas is bubbled into the solution for five seconds. The off-white precipitate that forms is then filtered, washed with dry tetrahydrofuran and the solvent is removed by high vacuum pumping to obtain the title compound as an off-white solid, m.p. 265°-267°C.

EXAMPLE 2

1-[2-[(5-trifluoromethylpryridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine.

To a 25 ml. flask is added 1.15g (5.61 mmol) of 2-[(5-trifluoromethylpyridin-2-yl)-amino]ethylamine and 10 ml of tetrahydrofuran and the mixture is cooled in an ice bath. To the cooled mixture is added 0.404g (1.87 mmol) of the bromide compound of Example 1b) dissolved in 5 ml of tetrahydrofuran. The resultant mixture is stirred for 2 hours at 0°C., the solvent is removed by rotovaping and the mixture is partitioned between ethyl acetate and water. The product is then extracted into the ethyl acetate layer and the aqueous layer is then washed twice with ethyl acetate. The combined organic layers are then washed successively with water and brine, dried over sodium sulfate and concentrated to obtain the desired compound in crude form. The crude form is then purified on silica gel employing a mixture of 5% methanol in methylene chloride as the eluent to yield the title compound as a golden oil.

EXAMPLE 3

1-[2-[(5-cyanopyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine dihydrochloride.

a) Preparation of the title compound in free base form.

Following essentially the procedure of Example 1c), and using in place of the amine therein, an equivalent amount of 2-[(5-cyanopyridin-2-yl)amino]-ethylamine, the desired compound is obtained as a golden oil.

b) Preparation of the title compound.

Following essentially the procedure of Example 1d), and using in place of the free base compound prepared in Example 1c), an equivalent amount of the free base compound prepared in a) above, the title compound is obtained as an off-white precipitate, m.p. 155°-157°C.

EXAMPLE 4

1-[2-[(pyrimidin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine.

Following essentially the procedure of Example 2, and using in place of the amine therein, an equivalent amount of 2-[(pyrimidin-2-yl)amino]ethylamine, and using in place of the eluent therein, a mixture of 10% methanol in methylene chloride, the title compound is obtained as a golden oil.

EXAMPLE 5

1-[(1-hydroxymethylcyclopent-1-yl)amino]acetyl-2-cyano-(S)-pyrrolidine.

To 1.5g of (1-hydroxymethyl)cyclopentylamine in 40 ml of anhydrous tetrahydrofuran is added, dropwise via an addition funnel over 40 minutes, 0.943g (4.35 mmol) of the bromide compound of Example 1b) under a calcium sulfate drying tube. The resultant mixture is then stirred at room temperature for 18 hours under a calcium sulfate drying tube, after which time hydrogen chloride gas is bubbled in for ~ 5 seconds. The resultant gum is

then separated from the solution by decanting and washed with 25 ml of tetrahydrofuran. The solution is then decanted and the gum is partitioned between methylene chloride and saturated aqueous sodium bicarbonate. The product is then extracted into the methylene chloride layer and the aqueous layer is then washed twice with methylene chloride. The combined organic layers are then washed successively with water and brine and then dried over sodium sulfate. The solution is then filtered and the solvent is removed by rotovaping and high vacuum pumping to obtain the title compound as a clear yellow oil which solidifies to a yellow solid, m.p. 65°-67°C.

EXAMPLE 6

1-[2-[(pyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine.

Following essentially the procedure of Example 2, and using in place of the amine therein, an equivalent amount of 2-[(pyridin-2-yl)amino]ethylamine, and using in place of the eluent therein, a 90:10:0.5 mixture of methylene chloride, methanol and ammonium hydroxide, the title compound is obtained as a golden oil.

EXAMPLE 7

Following essentially the procedure of Example 2, and using in place of the amine therein, an equivalent amount of:

a) 2-[(4-chloropyrimidin-2-yl)amino]ethylamine;
b) 2-[(3-chloropyridin-2-yl)amino]ethylamine;
c) 2-[(4-trifluoromethylpyrimidin-2-yl)amino]ethylamine;
d) (2-chlorophenyl)ethylamine; and
e) (3,3-biphenyl)propylamine;

there is obtained:

A) 1-[2-[(4-chloropyrimidin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine as a tan solid;

B) 1-[2-[(3-chloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine as a golden oil;

C) 1-[2-[4-trifluoromethylpyrimidin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine as a golden. oil;

D) 1-[(2-chlorophenyl)ethylamino]acetyl-2-cyano-(S)-pyrrolidine; and

E) 1-[(3,3-diphenyl)propylamino]acetyl-2-cyano-(S)-pyrrolidine, respectively.

EXAMPLE 8

1-[2-[(5-nitropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)pyrrolidine.

To 83.6 ml of anhydrous tetrahydrofuran is added 4.54g (24.9 mmol) of 2-[(5-nitropyridin-2-yl)amino]ethylamine, and the resultant mixture is heated slightly then stirred at room temperature under a calcium sulfate drying tube. 1.80g (8.3 mmol) of the bromide compound of Example 1b) in 20 ml of anhydrous tetrahydrofuran is then added, over a period of 30 minutes, under a calcium sulfate drying tube. The resultant mixture is then stirred at room temperature for 2 hours under a calcium sulfate drying tube and concentrated via rotovaping. The resultant paste is then partitioned between methylene chloride and saturated aqueous sodium bicarbonate. The product is then extracted into the methylene chloride layer and the aqueous layer is then washed twice with methylene chloride. The combined organic layers are then washed successively with water and brine and then dried over sodium sulfate. The solution is then filtered and the solvent is removed by rotovaping and high vacuum pumping to obtain the crude form of the title compound as a dark yellow-orange clear thick oil. The crude form is then flash chromatographed employing a mixture of 5% methanol in methylene chloride as the eluent to obtain the title compound as a bright yellow thick oil.

EXAMPLE 9

Following essentially the procedure of Example 2, and using in place of the amine therein, an equivalent amount of:

a) 2-[(3-chloro-5-trifluoromethylpyridin-2-yl)amino]ethylamine;

b) 2-[(3-trifluoromethylpyridin-2-yl)amino]ethylamine; and

c) 2-[(3,5-dichloropyridin-2-yl)amino]ethylamine;

and using in place of the eluent therein, a mixture of 3% methanol in methylene chloride, there is obtained:

A) 1-[2-[(3-chloro-5-trifluoromethylpyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine as a golden oil;

B) 1-[2-[(3-trifluoromethylpyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)pyrrolidine as a golden oil; and

C) 1-[2-[(3,5-dichloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine as a golden oil.

EXAMPLE 10

1-[(cyclopent-1-yl)amino-acetyl-2-cyano-(S)-pyrrolidine monohydrochloride.

a) Preparation of the title compound in free base form.

Following essentially the procedure of Example 2, and using in place of the amine therein, an equivalent amount of (cyclopent-1-yl)amine, the desired compound is obtained as a tan solid.

b) Preparation of the title compound.

Following essentially the procedure of Example 1d), and using in place of the free base compound therein, an equivalent amount of the compound prepared in a) above, the title compound is obtained as a white solid.

EXAMPLE 11

1-[2-(2-bromo-4,5-dimethoxyphenyl)ethylamino]acetyl-2-cyano-(S)-pyrrolidine.

To 15 ml of anhydrous tetrahydrofuran is added 1.44g (5.52 mmol) of 2-(2-bromo-4,5-dimethoxy)ethylamine, and the resultant mixture is heated slightly under a calcium sulfate drying tube. 0.4g (1.84 mmol) of the bromide compound of Example 1b) is then added, dropwise, over a period of 10 minutes. The resultant mixture is then stirred at room temperature for 18 hours under a calcium sulfate drying tube, concentrated via rotovaping and partitioned between methylene chloride and saturated aqueous sodium bicarbonate. The product is then extracted into the methylene chloride layer and the aqueous layer is then washed twice with methylene chloride. The combined organic layers are then washed successively with water and brine and then dried over sodium sulfate. The solution is then filtered and the solvent is removed by rotovaping and high vacuum pumping to obtain the crude form of the title compound as a clear yellow oil. The crude form is then flash chromatographed employing a mixture of 5% methanol in methylene chloride as the eluent to obtain the title compound as a clear, light yellow, thick oil.

EXAMPLE 12

1-[3-(isopropoxy)propylamino]acetyl-2-cyano-(S)-pyrrolidine monohydrochloride.

a) Preparation of the title comound in free base form.

Following essentially the procedure of Example 1c), and using in place of the amine therein, an equivalent amount of 3-(isopropoxy)propylamine, the desired compound is obtained as a brown oil.

b) Preparation of the title compound.

Following essentially the procedure of Example 1d), and using in place of the free base compound therein, an equivalent amount of the compound prepared in a) above, the title compound is obtained as a white solid, m.p. 174°-176°C.

EXAMPLE 13

1-[(2-hydroxy-1,1-dimethylethylamino)]acetyl-2-cyano-(S)-pyrrolidine monohydrochloride.

a) Preparation of the title compound in free base form.

Following essentially the procedure of Example 2, and using in place of the amine therein, an equivalent amount of 2-hydroxy-1,1-dimethylethylamine, and using in place of the eluent therein, an 80:20:1 mixture of methylene chloride, methanol and ammonium hydroxide, the title compound is obtained as a golden oil.

b) Preparation of the title compound.

Following essentially the procedure of Example 1d), and using in place of the free base compound therein, an equivalent amount of the compound prepared in a) above, the title compound is obtained as a brown solid.

EXAMPLE 14

1-[3-(2-oxo-pyrrolidin-1-yl)propylamino]acetyl-2-cyano-(S)-pyrrolidine monohydrochloride.

a) Preparation of the title compound in free base form.

Following essentially the procedure of Example 2, and using in place of the amine therein, an equivalent amount of 3-(2-oxo-pyrrolidin-1-yl) propylamine, and using in place of the eluent therein, a 90:10:1 mixture of methylene chloride, methanol and ammonium hydroxide, the desired compound is obtained as a golden oil.

b) Preparation of the title compound.

Following essentially the procedure of Example 1d), and using in place of the free base compound therein, an equivalent amount of the compound prepared in a) above, the title compound is obtained as a tan solid.

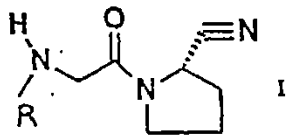
Below are the ^{13}C NMR signals for the nitrile functionalities of the specific synthesized compounds described above:

Compound #	^{13}C NMR (MHz, solvent) δ ppm (CN)	
Ex. 5	^{13}C NMR (75 MHz, CD_3OD)	δ 119.64 ppm (CN)
Ex. 12	^{13}C NMR (75 MHz, D_2O)	δ 121.63 ppm (CN)
Ex. 1	^{13}C NMR (75 MHz, D_2O)	δ 121.60 ppm (CN)
Ex. 3	^{13}C NMR (75 MHz, D_2O)	δ 120.42 ppm (CN)
Ex. 8	^{13}C NMR (75 MHz, DMSO)	δ 119.13 ppm (CN)
Ex. 7B	^{13}C NMR (75 MHz, $CDCl_3$)	δ 118.23 ppm (CN)
Ex. 9A	^{13}C NMR (75 MHz, CD_3OD)	δ 119.68 ppm (CN)
Ex. 9B	^{13}C NMR (75 MHz, CD_3OD)	δ 119.66 ppm (CN)
Ex. 9C	^{13}C NMR (75 MHz, CD_3OD)	δ 119.68 ppm (CN)
Ex. 6	^{13}C NMR (75 MHz, CD_3OD)	δ 119.84 ppm (CN)

Compound #	^{13}C NMR (MHz, solvent) δ ppm (CN)	
Ex. 7C	^{13}C NMR (75 MHz, $CDCl_3$)	δ 118.23 ppm (CN)
Ex. 2	^{13}C NMR (75 MHz, CD_3OD)	δ 119.68 ppm (CN)
Ex. 7A	^{13}C NMR (75 MHz, CD_3OD)	δ 119.66 ppm (CN)
Ex. 4	^{13}C NMR (75 MHz, CD_3OD)	δ 119.66 ppm (CN)
Ex. 10	^{13}C NMR (75 MHz, D_2O)	δ 121.69 ppm (CN)
Ex. 11	^{13}C NMR (75 MHz, $CDCl_3$)	δ 118.31 ppm (CN)
Ex. 7D	^{13}C NMR (75 MHz, CD_3OD)	δ 119.63 ppm (CN)
Ex. 7E	^{13}C NMR (75 MHz, CD_3OD)	δ 119.64 ppm (CN)
Ex. 13	^{13}C NMR (75 MHz, D_2O)	δ 121.52 ppm (CN)
Ex. 14	^{13}C NMR (75 MHz, D_2O)	δ 121.52 ppm (CN)

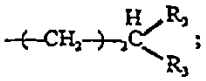
WHAT IS CLAIMED IS:

1. A compound of formula I:



I

wherein R is a group $-(CH_2)_2-\overset{H}{N}-R_1$; an unsubstituted ($C_{3-7}$)cycloalkyl ring; a ($C_{3-7}$)cycloalkyl ring substituted in the 1-position by a hydroxy(C_{1-3})alkyl group; a group $-(CH_2)_2-R_2$; a group



a group $-(CH_2)_3-R_4$; an isopropyl group; or an isopropyl group substituted in the 1-position by a hydroxy-(C_{1-3})alkyl group;

R_1 is an unsubstituted pyridine ring; a pyridine ring mono- or di-substituted by halo, trifluoromethyl, cyano or nitro; an unsubstituted pyrimidine ring; or a pyrimidine ring monosubstituted by halo, trifluoromethyl, cyano or nitro;

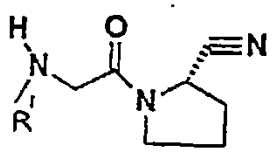
R_2 is an unsubstituted phenyl ring; or a phenyl ring mono-, di- or tri-substituted by halo or (C_{1-3})alkoxy;

each R_3, independently, is an unsubstituted phenyl ring; or a phenyl ring mono-substituted by halo or (C_{1-3})alkoxy; and

R_4 is a 2-oxopyrrolidine group or a (C_{2-4})alkoxy group;

or a pharmaceutically acceptable acid addition salt thereof.

2. A compound according to Claim 1 of formula Ia:



Ia

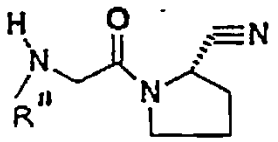
where R^I is a group $-(CH_2)_2N(H)-R_1$; an unsubstituted (C_{3-7})cycloalkyl ring; a (C_{3-7}) cycloalkyl ring substituted in the 1-position by a hydroxy(C_{1-3})alkyl group; or a group $-(CH_2)_3R_4$;

R_1^I is an unsubstituted pyridine ring; or a pyridine ring mono- or di-substituted by halo, trifluoromethyl, cyano or nitro; and

R_4^I is a (C_{2-4})alkoxy group;

or a pharmaceutically acceptable acid addition salt thereof.

3. A compound according to Claim 2 of formula Ib:



Ib

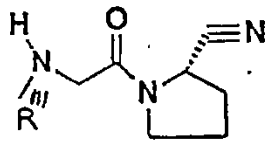
where R" is a group $-(CH_2)_2N(H)-R_1^{II}$; a (C_{4-6})cycloalkyl ring substituted in the 1-position by a hydroxy(C_{1-3})alkyl group; or a group $-(CH_2)_3R_4^I$;

R_1^{II} is a pyridine ring mono- or di-substituted by halo, trifluoromethyl, cyano or nitro; and

R_4^I is as defined in Claim 2;

or a pharmaceutically acceptable acid addition salt thereof.

4. A compound according to Claim 3 of formula Ic:



Ic

where R"' is a group $-(CH_2)_2-\overset{H}{N}-R_1^{III}$; a ($C_{4-6}$)cycloalkyl ring substituted in the 1-position by a hydroxymethyl group; or a group $-(CH_2)_3R_4^1$;

R_1^{III} is a pyridine ring monosubstituted by halo, trifluoromethyl, cyano or nitro; and

R_4^1 is as defined in Claim 3;

or a pharmaceutically acceptable acid addition salt thereof.

5. A compound according to Claim 4 which is 1-[2-[(5-chloropyridin-2-yl)amino]-ethylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

6. The compound according to Claim 5 which is 1-[2-[(5-chloropyridin-2-yl)amino]-ethylamino]acetyl-2-cyano-(S)-pyrrolidine dihydrochloride.

7. A compound according to Claim 4 which is 1-[2-[(5-cyanopyridin-2-yl)amino]-ethylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

8. The compound according to Claim 7 which is 1-[2-[(5-cyanopyridin-2-yl)amino]-ethylamino]acetyl-2-cyano-(S)-pyrrolidine dihydrochloride.

9. A compound according to Claim 4 which is 1-[(1-hydroxymethylcyclopent-1-yl)amino]-acetyl-2-cyano-(*S*)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

10. The compound according to Claim 9 which is 1-[(1-hydroxymethylcyclopent-1-yl)amino]acetyl-2-cyano-(S)-pyrrolidine.

11. A compound according to Claim 4 which is 1-[3-(isopropoxy)propylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

12. The compound according to Claim 11 which is 1-[3-(isopropoxy)propylamino]acetyl-2-cyano-(S)-pyrrolidine monohydrochloride.

13. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and a therapeutically effective amount of a compound according to Claim 1, or a pharmaceutically acceptable acid addition salt thereof.

14. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and a therapeutically effective amount of a compound according to Claim 2, or a pharmaceutically acceptable acid addition salt thereof.

15. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and a therapeutically effective amount of a compound according to Claim 3, or a pharmaceutically acceptable acid addition salt thereof.

16. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and a therapeutically effective amount of a compound according to Claim 4, or a pharmaceutically acceptable acid addition salt thereof.

17. A pharmaceutical composition according to Claim 16 comprising a therapeutically effective amount of 1-[2-[(5-chloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

18. A pharmaceutical composition according to Claim 17 comprising a therapeutically effective amount of 1-[2-[(5-chloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine dihydrochloride.

19. A pharmaceutical composition according to Claim 16 comprising a therapeutically effective amount of 1-[2-[(5-cyanopyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

20. A pharmaceutical composition according to Claim 19 comprising a therapeutically effective amount of 1-[2-[(5-cyanopyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine dihydrochloride.

21. A pharmaceutical composition according to Claim 16 comprising a therapeutically effective amount of 1-[(1-hydroxymethylcyclopent-1-yl)amino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

22. A pharmaceutical composition according to Claim 21 comprising a therapeutically effective amount of 1-[(1-hydroxymethylcyclopent-1-yl)amino]acetyl-2-cyano-(S)-pyrrolidine.

23. A pharmaceutical composition according to Claim 16 comprising a therapeutically effective amount of 1-[3-(isopropoxy)propylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

24. A pharmaceutical composition according to Claim 23 comprising a therapeutically effective amount of 1-[3-isopropoxy)propylamino]acetyl-2-cyano-(S)-pyrrolidine monohydrochloride.

25. A method of inhibiting dipeptidyl peptidase-IV comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound according to Claim 1, or a pharmaceutically acceptable acid addition salt thereof.

26. A method of inhibiting dipeptidyl peptidase-IV comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound according to Claim 2, or a pharmaceutically acceptable acid addition salt thereof.

27. A method of inhibiting dipeptidyl peptidase-IV comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound according to Claim 3, or a pharmaceutically acceptable acid addition salt thereof.

28. A method of inhibiting dipeptidyl peptidase-IV comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound according to Claim 4, or a pharmaceutically acceptable acid addition salt thereof

29. A method according to Claim 28 comprising administering a therapeutically effective amount of 1-[2-[(5-chloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

30. A method according to Claim 29 comprising administering a therapeutically effective amount of 1-[2-[(5-chloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine dihydrochloride.

31. A method according to Claim 28 comprising administering a therapeutically effective amount of 1-[2-[(5-cyanopyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

32. A method according to Claim 31 comprising administering a therapeutically effective amount of 1-[2-[(5-cyanopyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine dihydrochloride.

33. A method according to Claim 28 comprising administering a therapeutically effective amount of 1-[(1-hydroxymethylcyclopent-1-yl)amino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

34. A method according to Claim 33 comprising administering a therapeutically effective amount of 1-[(1-hydroxymethylcyclopent-1-yl)amino]acetyl-2-cyano-(S)-pyrrolidine.

35. A method according to Claim 28 comprising administering a therapeutically effective amount of 1-[3-(isopropoxy)propylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

36. A method according to Claim 35 comprising administering a therapeutically effective amount of 1-[3-(isopropoxy)propylamino]acetyl-2-cyano-(S)-pyrrolidine monohydrochloride.

37. A method of treating conditions mediated by dipeptidyl peptidase-IV inhibition comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound according to Claim 1, or a pharmaceutically acceptable acid addition salt thereof.

38. A method of treating conditions mediated by dipeptidyl peptidase-IV inhibition comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound according to Claim 2, or a pharmaceutically acceptable acid addition salt thereof.

39. A method of treating conditions mediated by dipeptidyl peptidase-IV inhibition comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound according to Claim 3, or a pharmaceutically acceptable acid addition salt thereof.

40. A method of treating conditions mediated by dipeptidyl peptidase-IV inhibition comprising administering to a mammal in need of such treatment a therapeutically effective amount of a compound according to Claim 4, or a pharmaceutically acceptable acid addition salt thereof.

41. A method according to Claim 40 comprising administering a therapeutically effective amount of 1-[2-[(5-chloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

42. A method according to Claim 41 comprising administering a therapeutically effective amount of 1-[2-[(5-chloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

43. A method according to Claim 40 comprising administering a therapeutically effective amount of 1-[2-[(5-cyanopyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

44. A method according to Claim 43 comprising administering a therapeutically effective amount of 1-[2-[(5-cyanopyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine dihydrochloride.

45. A method according to Claim 40 comprising administering a therapeutically effective amount of 1-[(1-hydroxymethylcyclopent-1-yl)amino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

46. A method according to Claim 45 comprising administering a therapeutically effective amount of 1-[(1-hydroxymethylcyclopent-1-yl)amino]acetyl-2-cyano-(S)-pyrrolidine.

47. A method according to Claim 40 comprising administering a therapeutically effective amount of 1-[3-(isopropoxy)propylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

48. A method according to Claim 47 comprising administering a therapeutically effective amount of 1-[3-(isopropoxy)propylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

49. A method according to Claim 37 wherein the condition treated is non-insulin-dependent diabetes mellitus.

50. A method according to Claim 38 wherein the condition treated is non-insulin-dependent diabetes mellitus.

51. A method according to Claim 39 wherein the condition treated is non-insulin-dependent diabetes mellitus.

52. A method according to Claim 40 wherein the condition treated is non-insulin-dependent diabetes mellitus.

53. A method according to Claim 52 comprising administering a therapeutically effective amount of 1-[2-[(5-chloropyridin-2-yl)amino]ethylamino]-acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

54. A method according to Claim 53 comprising administering a therapeutically effective amount of 1-[2-[(5-chloropyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine dihydrochloride.

55. A method according to Claim 52 comprising administering a therapeutically effective amount of 1-[2-[(5-cyanopyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

56. A method according to Claim 55 comprising administering a therapeutically effective amount of 1-[2-[(5-cyanopyridin-2-yl)amino]ethylamino]acetyl-2-cyano-(S)-pyrrolidine dihydrochloride.

57. A method according to Claim 52 comprising administering a therapeutically effective amount of 1-[(1-hydroxymethylcyclopent-1-yl)amino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

58. A method according to Claim 57 comprising administering a therapeutically effective amount of 1-[(1-hydroxymethylcyclopent-1-yl)amino]acetyl-2-cyano-(S)-pyrrolidine.

59. A method according to Claim 52 comprising administering a therapeutically effective amount of 1-[3-(isopropoxy)propylamino]acetyl-2-cyano-(S)-pyrrolidine, or a pharmaceutically acceptable acid addition salt thereof.

60. A method according to Claim 59 comprising administering a therapeutically effective amount of 1-[3-(isopropoxy)propylamino]acetyl-2-cyano-(S)-pyrrolidine monohydrochloride.

Abstract of the Disclosure:

The invention discloses certain N-(substituted glycyl)-2-cyanopyrrolidines, pharmaceutical compositions containing said compounds as an active ingredient thereof, and the use of said compounds in inhibiting dipeptidyl peptidase-IV.

Case No. 600-7247/R

DECLARATION AND POWER OF ATTORNEY FOR
UNITED STATES PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, and

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if more than one name is listed below) of the subject matter which is claimed and for which a United States patent is sought on the invention entitled

N-(SUBSTITUTED GLYCYL)-2-CYANOPYRROLIDINES, PHARMACEUTICAL COMPOSITIONS CONTAINING THEM AND THEIR USE IN INHIBITING DIPEPTIDYL PEPTIDASE-IV

the specification of which
[X] is attached hereto.

[ ] was filed on , 19 as application Serial No. / , , and, if these brackets contain an X [ ], was amended on , 19 .

[ ] was filed as Patent Cooperation Treaty international application No. on , 19 , if these brackets contain an X [ ], was amended under Patent Cooperation Treaty Article 19 on , 19 , if these brackets contain an X [ ], entered the national stage in the United States on , 19 and was accorded Serial No. / , and, if these brackets contain an X [ ], was amended on , 19 .

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge my duty to disclose all information which is known by me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim the benefit under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate indicated below and of any Patent Cooperation Treaty international application(s) designating at least one country other than the United States indicated below and have also identified below any foreign application(s) for patent or inventor's certificate and Patent Cooperation Treaty international application(s) designating at least one country other than the United States for the same subject matter and having a filing date before that of the application for said subject matter the priority of which is claimed:

Case No. 600-7247/R

Country	Number	Filing Date	Priority Claimed
			[ ] Yes [ ] No
			[ ] Yes [ ] No
			[ ] Yes [ ] No
			[ ] Yes [ ] No
			[ ] Yes [ ] No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and of any Patent Cooperation Treaty international application(s) designating the United States listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in said prior application(s) in the manner required by the first paragraph of Title 35, United States Code, §112, I acknowledge my duty to disclose all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date(s) of the prior application(s) and the national or Patent Cooperation Treaty international filing date of this application:

Application Serial No.	Filed	Status (Pending, Abandoned or Patented)

I hereby appoint the following:

ROBERT S. HONOR	Reg. No. 22,801
THOMAS O. MCGOVERN	Reg. No. 25,741
MELVYN M. KASSENOFF	Reg. No. 26,389
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DIANE E. FURMAN	Reg. No. 31,104
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JOHN L. CHIATALAS	Reg. No. 31,818
CAROL A. LOESCHORN	Reg. No. 35,590
MICHAEL P. MORRIS	Reg. No. 34,513
THOMAS C. DOYLE	Reg. No. 22,340

respectively and individually, as my attorneys and/or agents, with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. Please address all communications to ROBERT S. HONOR, SANDOZ CORPORATION, 59 Route 10, East Hanover, New Jersey 07936-1080, whose telephone number is 201-503-8485.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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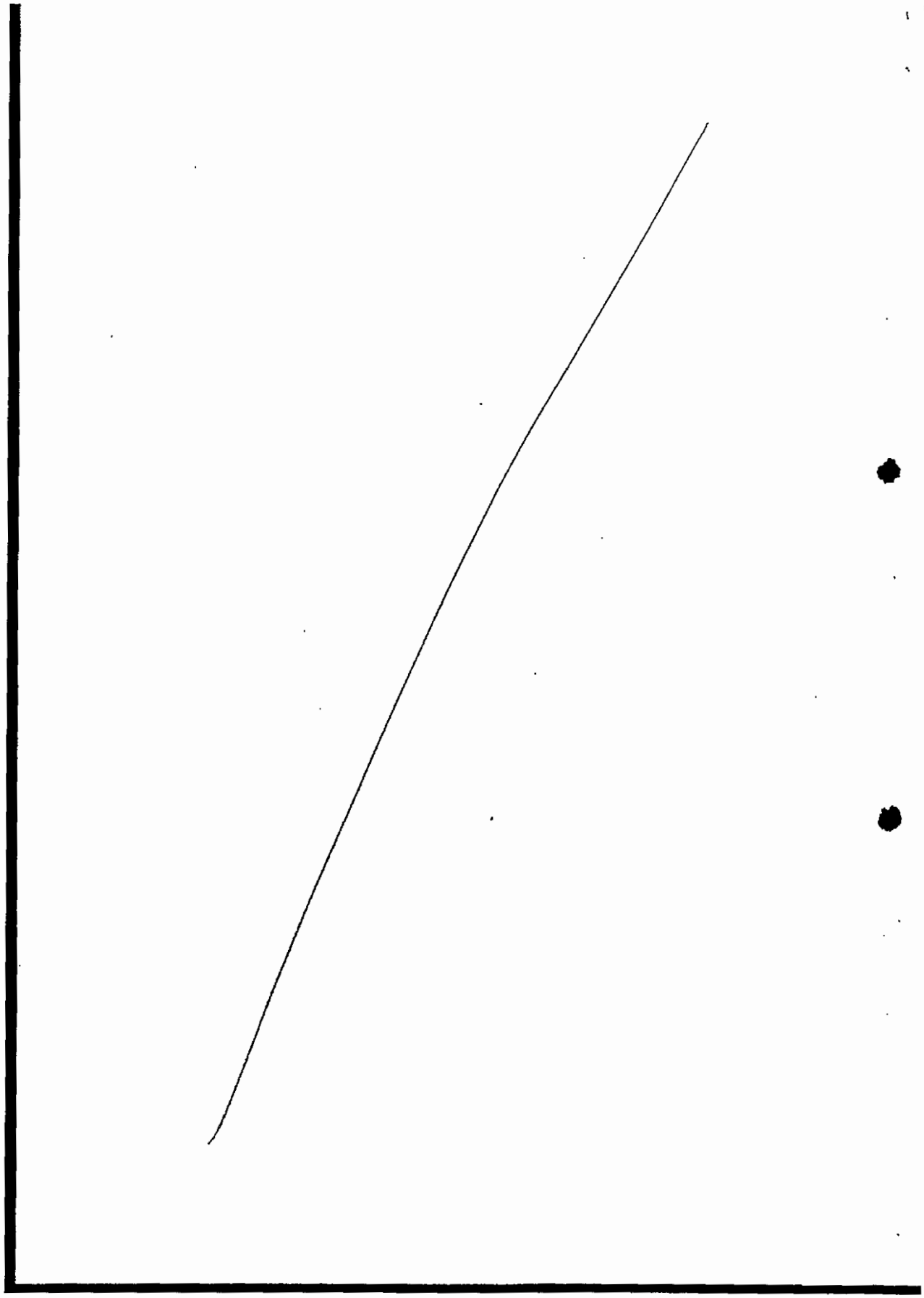
Second joint inventor, if any:

Full name	:	
Signature	:	_____
Date	:	
Citizenship	:	
Residence	:	
P.O. Address	:	

IMPORTANT: *Before this declaration is signed, the patent application (the specification, the claims and this declaration) must be read and understood by each person signing it, and no changes may be made in the application after this declaration has been signed.*



Articles

NVP-DPP728 (1-[[[2-[(5-Cyanopyridin-2-yl)amino]ethyl]amino]acetyl]-2-cyano-(*S*)-pyrrolidine), a Slow-Binding Inhibitor of Dipeptidyl Peptidase IV

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ABSTRACT: Inhibition of dipeptidyl peptidase IV (DPP-IV) has been proposed recently as a therapeutic approach to the treatment of type 2 diabetes. *N*-Substituted-glycyl-2-cyanopyrrolidide compounds, typified by NVP-DPP728 (1-[[[2-[(5-cyanopyridin-2-yl)amino]ethyl]amino]acetyl]-2-cyano-(*S*)-pyrrolidine), inhibit degradation of glucagon-like peptide-1 (GLP-1) and thereby potentiate insulin release in response to glucose-containing meals. In the present study NVP-DPP728 was found to inhibit human DPP-IV amidolytic activity with a K_i of 11 nM, a k_{on} value of 1.3×10^5 M^{-1} s^{-1}, and a k_{off} of 1.3×10^{-3} s^{-1}. Purified bovine kidney DPP-IV bound 1 mol/mol [^{14}C]-NVP-DPP728 with high affinity (12 nM K_d). The dissociation constant, k_{off}, was 1.0×10^{-3} and 1.6×10^{-3} s^{-1} in the presence of 0 and 200 μM H-Gly-Pro-AMC, respectively (dissociation $t_{1/2}$ ~10 min). Through kinetic evaluation of DPP-IV inhibition by the D-antipode, des-cyano, and amide analogues of NVP-DPP728, it was determined that the nitrile functionality at the 2-pyrrolidine position is required, in the L-configuration, for maximal activity (K_i of 11 nM vs K_i values of 5.6 to >300 μM for the other analogues tested). Surprisingly, it was found that the D-antipode, despite being ~500-fold less potent than NVP-DPP728, displayed identical dissociation kinetics (k_{off} of 1.5×10^{-3} s^{-1}). NVP-DPP728 inhibited DPP-IV in a manner consistent with a two-step inhibition mechanism. Taken together, these data suggest that NVP-DPP728 inhibits DPP-IV through formation of a novel, reversible, nitrile-dependent complex with transition state characteristics.

Dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5)[1] is a post-proline cleaving serine protease with significant sequence and structural similarity to other α-β-hydrolases (e.g., prolyl oligopeptidase, acetylcholinesterase). DPP-IV is found throughout the body, both circulating in plasma and as a type II membrane protein produced by a variety of tissues, including kidney, liver, and intestine. DPP-IV may play a role in cleavage and inactivation of biologically active peptides with accessible amino-terminal Xaa-Pro- or Xaa-Ala- sequences (*1*, *2*). Indeed, DPP-IV degrades and regulates the activity of several regulatory peptides in man (including the gut peptide "incretin" hormone glucagon-like peptide-1 (GLP-1), growth hormone-releasing hormone, and gastric inhibitory polypeptide). Due to the impressive antidiabetic actions of GLP-1, DPP-IV inhibition has been proposed as an intriguing new approach to the therapy of type 2 diabetes mellitus (*3*).

Several classes of DPP-IV inhibitors bearing transition state mimics have been identified, and their kinetic properties have been extensively investigated. Peptidyl (α-aminoalkyl)-phosphate diphenyl ester inhibitors of DPP-IV bind with low affinity (10^{-4} M IC_{50} values) but rapidly form highly stable covalent complexes with the active site serine residue of DPP-IV (*4*). Pro-boroPro and related analogues bind with high affinity (K_i ~10^{-11} M) in a reversible manner (half-life of enzyme−inhibitor complex ~ 150 min; *5*). The boronic acid analogues, however, are unstable in solution due to reversible intramolecular cyclization, and also inhibit dipeptidyl peptidase II, a related serine protease. Irreversible "suicide substrate" methylsulfonio cyclopeptide inhibitors have been described (*6*) as mechanistic tools but may not be suitable for therapeutic use. Although a series of (*N*-hydroxyacyl amide) aminodicarboxylic acid pyrrolidides have been described, only relatively unselective inhibitors with micromolar potency have been prepared (*7*). Conformationally constrained fluoroolefin-containing peptidyl-hydroxylamine inhibitors also have been described (*8*), but isolation of enantiomerically pure compounds requires tedious separation of diastereomers. For these reasons, new chemical classes of selective and potent DPP-IV inhibitors are of interest and needed in order to evaluate the feasibility and efficacy of DPP-IV inhibition as a therapeutic approach.

Recently, DPP-IV inhibitors with 2-cyanopyrrolidide P1 substituents have been reported (*9*, *10*). These compounds

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[1] Abbreviations: NVP DPP728, 1 [[[2 [(5 cyanopyridin 2 yl)amino] ethyl]amino]acetyl]-2-cyano-(*S*)-pyrrolidine; DPP-IV, dipeptidyl peptidase IV; pNA, *p*-nitroaniline.

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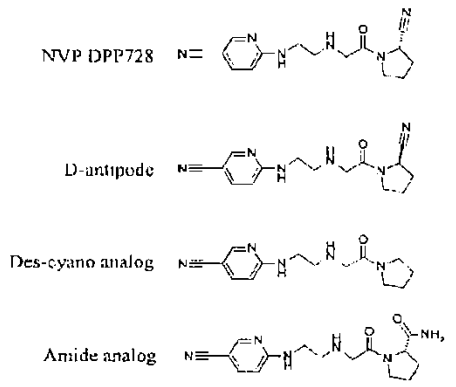
AZ-SAXA-8023676

Chart 1. Structures of NVP-DPP728 and Analogs



bind to DPP-IV several orders of magnitude more tightly than the corresponding pyrrolidide analogues (e.g., K_i values for isoleucine-2-cyanopyrrolidide and isoleucine pyrrolidide are 2 and 400 nM, respectively; *10*, *11*). Similar potency is observed with a new class of cyanopyrrolidide inhibitors, termed *N*-substituted-glycyl-2-cyanopyrrolidide compounds (*12*). Recently, NVP-DPP728 (see Chart 1), a novel derivative of this class, has been identified as a potent and selective DPP-IV inhibitor for use in the treatment of diabetes mellitus (example no. 5 in ref *12*, described also in ref *13*). NVP-DPP728 inhibits human and rat plasma DPP-IV with IC_{50} values in the range of 5−10 nM with >15 000-fold selectivity relative to DPP−II and a range of proline-cleaving proteases (*13*). This compound shows promise as an antidiabetic agent due to its ability to preserve the integrity of GLP-1 (*13*) and improve glucose tolerance (*14*).

While dipeptide-like pyrrolidide compounds (e.g., valine pyrrolidide) inhibit DPP-IV through simple reversible competitive binding (*11*), the kinetic properties of nitrile-containing inhibitors have not been rigorously evaluated. In a brief communication, Li and colleagues (*9*) reported that dipeptide cyanopyrrolidide compounds generate competitive or mixed inhibition profiles and postulated that the mechanism of inhibition involves formation of an imidate intermediate, (comparable to the thioimidate intermediate state known for nitrile−cysteine protease complexes; *15*, *16*).

We hypothesized that imidate formation for nitrile DPP-IV inhibitors, if analogous to cysteine protease inhibition, should display slow-binding inhibition kinetics. A series of studies, focused on NVP-DPP728, have been undertaken to define the kinetic and molecular mechanisms for responsible for the potent inhibition of DPP-IV by cyanopyrrolidine compounds. Here we report that NVP-DPP728 inhibits DPP-IV by a slow-binding mechanism and that the rate of inactivation is dependent upon L-chirality of the pyrrolidine nitrile functionality. The binding affinity and rate of dissociation of bound inhibitor determined by kinetic experiments were further confirmed by direct binding measurements using radiolabeled NVP-DPP728 and DPP-IV highly purified from bovine kidney cortex.

EXPERIMENTAL PROCEDURES

Materials. Bovine serum albumin, bromelain, calf intestinal adenosine deaminase, CNBr-activated Sepharose 4B, and *p*-nitroaniline were from Sigma (St. Louis, MO). H-Ala-Pro-pNA was from Bachem (King of Prussia, PA). Bovine kidney cortices were obtained from Pell Freeze Biological (Rogers, AR). The human colonic carcinoma cell line Caco-2 was obtained from the American Type Culture Collection (ATCC HTB 37).

Inhibitors. NVP-DPP728 was prepared as described (compound no. 5 in ref *12*). The D-antipode, des-cyano, and amide analogues of NVP-DPP728 were prepared as described (*13*). Dr. Tapan Ray (Novartis Radiosynthesis Laboratory), incorporating the label at the carbonyl carbon, kindly provided [^{14}C]-NVP-DPP728 (specific activity 49 mCi/mmol).

Preparation of Human and Bovine DPP-IV. Where indicated, human DPP-IV preparations consisted of extracts of Caco-2 cells (*17*), cultured as previously described to induce differentiation (*18*). Cell extract containing human DPP-IV was prepared from cells solubilized in 10 mM Tris-HCl, 0.15 M NaCl, 0.04 tiu aprotinin, 0.5% nonidet-P40, pH 8.0, by centrifugation at 35 000*g* for 30 min at 4 °C to remove cell debris. The preparations contained approximately 30 mU DPP-IV/mg (~0.6 μg/mg of protein; 1 unit cleaves 1 μmol of H-Ala-Pro-pNA/min; enzyme content derived from V_{max} determined using Gly-Pro-4-nitroaniline, using a theoretical maximal activity of 55 U/mg as described (*19*)). Bovine DPP-IV was purified from kidney cortex using adenosine deaminase (ADA) affinity chromatography as previously described (*20*). Following digestion of a microsomal membrane fraction with bromelain, the resulting soluble protein was resolved by sequential Q-Sepharose, ADA-Sepharose 4B, and Mono-Q chromatography to yield a >90% pure DPP-IV enzyme preparation with a molecular weight by SDS−PAGE of 105 kDa (specific activity was 20 units/mg of protein).

Kinetics of Inhibition of DPP-IV. The progress of DPP-IV inhibition by the indicated compounds was measured under pseudo-first-order inhibition conditions, i.e., $[I_0] \geq 10\text{-}[E_0]$, by reacting DPP-IV with a mixture of inhibitor and substrate and recording the liberation of free pNA at 405 nm. Unless otherwise indicated, all reactions were conducted using 20 μg of extract protein in 25 mM Tris-HCl, 140 mM NaCl, 10 mM KCl, 1% bovine serum albumin, pH 7.4, at 25 °C (referred to as "assay buffer"). Under these conditions, K_m for H-Ala-Pro-pNA was 73 μM. Reaction progress was monitored using a Molecular Devices SpectraMax Plus microplate spectrophotometer (Sunnyvale CA). Reactions were 0.15 mL of final volume, initiated by the addition of a 5 μL aliquot of enzyme stock and mixed using the automated mixing feature of the SpectraMax reader. Total elapsed time between enzyme addition and the initiation of data collection was less than 30 s. Readings were taken every 10 s for a total of 1000 s, and initial (blank) absorbance values were subtracted from the data prior to subsequent calculations. Data were exported to Microsoft Excel and subsequently into the data analysis package Origin (Microcal Software Inc., Northampton, MA) where curve fitting was performed. Data were fitted to the integrated rate equation for slow binding inhibition (eq 1) according to the method described by

$$A = v_s t + (v_0 - v_s)(1 - e^{-k't})/k' + A_0$$

Williams and Morrison (1979), by nonlinear regression analysis. Values for v_0 (initial rate), v_s (final steady-state rate),

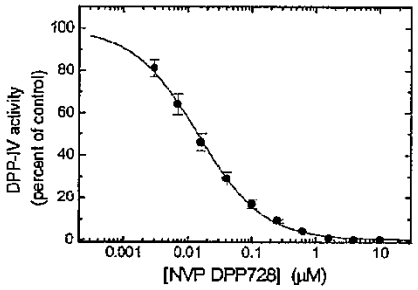


FIGURE 1: Dose−response curve of DPP-IV inhibition by NVP-DPP728. Following a 10-min preincubation of human DPP-IV with the indicated concentrations of inhibitor, the reaction was initiated by the addition of H-Ala-Pro-pNA (166 μM final concentration). Values are means ± SEM of three independent experiments. The line represents the logistic function with upper and lower asymptotes and slope fixed at 100, 0, and 1, respectively. The IC_{50} value derived from these data is 14 nM.

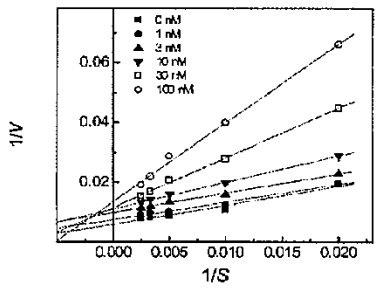


FIGURE 2: Lineweaver−Burk plot of DPP-IV activity measured in the presence of varied concentrations of NVP-DPP728 and substrate. Inhibitor effects were assessed as described in the legend to Figure 1, except both inhibitor and substrate concentrations were varied. Symbols correspond to different inhibitor concentrations as indicated in the legend. Values are means of triplicate determinations in which the standard deviations were less than 5% of the mean values. Lines shown are the least-squares linear regression lines.

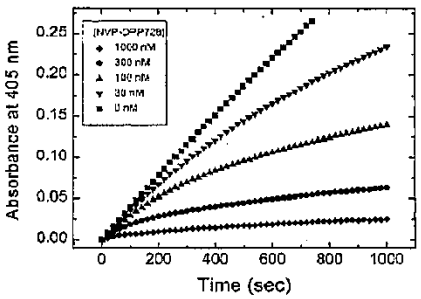


FIGURE 3: Slow-binding kinetics for the inhibition of DPP-IV by NVP-DPP728. Progress curves for pNA generation were recorded over 1000 s (16.7 min) at 405 nm. Measurement was done in 25 mM Tris-HCl pH 7.4, 140 mM NaCl, 10 mM KCl, and 1 wt %/vol bovine serum albumin in the presence of 166 μM H-Ala-Pro-pNA. Values are shown corrected for background absorbance (approximately 0.03 AU). Symbols correspond to different inhibitor concentrations as indicated in the legend. Values are from one of three replicate studies.

K' (apparent rate constant for the transition from v_0 to v_s), and A_0 (the initial absorbance at 405 nm) were obtained for each progress curve. These values were subsequently used to generate k_{on} (association rate constant), k_{off} (dissociation rate constant), and K_i values as described in the Results.

Radiolabeled Inhibitor Binding. Binding and dissociation of [^{14}C]-NVP-DPP728 were studied by incubating 2.5 μg (23 pmol) of purified bovine kidney DPP-IV with inhibitor in a volume of 4 mL of 50 mM Tris-HCl, pH 8.0, for 5 min at 25 °C, followed by capture on DEAE cellulose membrane disks (25 mm diameter, Schleicher & Schuell). Bound enzyme was rapidly washed with 1 mL of the same buffer at 4 °C, and both bound and eluted ^{14}C were quantified by liquid scintillation counting in a Beckman (Columbia, MD) LS6000IC scintillation counter with quench correction (counting time was 20 min or 2% of σ). Nonspecific binding, less than 10% of the total bound activity, was determined in the presence of a 1000-fold excess of nonradioactive NVP-DPP728. For determination of dissociation rates of the enzyme−inhibitor complex, bovine kidney DPP-IV was incubated (2.5 μg/time point) as above with 1000 nM [^{14}C]-NVP-DPP728 for 10 min, followed by capture with 100 μL of a 5:1 (gel:buffer) slurry of ADA-Sepharose 4B. The samples were then incubated with mixing for 20 min, and enzyme-bound inhibitor was collected on a 0.45 μm nylon-66 membrane (Rainin, Woburn MA). The resin (with immobilized labeled inhibitor) was resuspended in 10 mL of buffer ([EI] after dilution was 2.3 nM). At the indicated time points, samples were removed and quickly filtered through Whatman type 1 filter paper disks (2.5 cm). The trapped resin was rapidly washed with 1 mL of ice-cold assay buffer, and both trapped (enzyme bound) and eluted (free) inhibitor were quantified by scintillation counting. Blanks containing radiolabeled inhibitor, but no enzyme, were subtracted from both the bound and free counts and were less than 10% of the total radioactivity. Dissociation curves were plotted as the log of the fraction of initial bound enzyme versus time following dilution. Off-rates were calculated as the slope of these plots.

Inhibitor Stability. Under the conditions employed, NVP-DPP728 undergoes intramolecular cyclization, yielding a cyclic imidate product, with a half-life of approximately 72 h. Accordingly, less than 1% of the compound is expected to cyclize during the time frame of the current investigations.

RESULTS

NVP-DPP728 fully inhibited H-Ala-Pro-pNA cleavage by DPP-IV derived from human colonic adenocarcinoma cells with an IC_{50} value of 14 nM (Figure 1). NVP-DPP728 displayed complex inhibition kinetics when assessed graphically by Lineweaver−Burk analysis (shown for illustrative purposes in Figure 2), consistent with results reported (*9*) for Xaa-cyanopyrrolidide compounds. Assessment of reaction progress curves in the presence of varied inhibitor concentrations revealed a clear time-dependent approach to steady state, characteristic of slow binding inhibition kinetics (Figure 3). These progress curves were fitted to eq 1 to determine values for k_{on}', the association rate constant for inhibitor binding. Values for k' were plotted against the inhibitor concentration, $[I_0]$ (Figure 4). A linear dependency between $[I_0]$ and k' was observed and fitted (eq 2) to obtain estimates

Table 1: Kinetic Constants for DPP-IV Inhibition by Pyrrolidine Compounds[a]

compd	P1′ substituent[b] S	R	K_i (μM)	k_{on} (10^3 M^{-1} s^{-1})	k_{off} (s^{-1})	EI half-life (h)
NVP-DPP728	CN	H	0.011 ± 0.004	127 ± 27	(1.3 ± 0.2) × 10^{-3}	0.14
D-antipode	H	CN	5.6 ± 1.4	0.27 ± 0.03	(1.5 ± 0.2) × 10^{-3}	0.13
des-cyano	H	H	15.6 ± 3.6	rapid	rapid	<0.01
amide	$CONH_2$	H	320 ± 118	ND	ND	ND
Pro-boroPro[c]	$B(OH)_2$	H	0.000016	5000	0.078 × 10^{-3}	2.5
Pro-Pro(OPh)$_2$[d]	$Pro(OPh)_2$	H	70	0.02	irreversible	>672

[a] Reactions were performed at 25 °C in 25 mM Tris-HCl, pH 7.4, containing 140 mM NaCl, 10 mM KCl, 1% bovine serum albumin, and 166 μM H-Ala-Pro-pNA. Values are means ± standard deviations for three experiments. [b] Functional group present at the pyrrolidine-2-position. [c,d] Values are from Gutheil and Bachovchin (*5*) and from Lambeir et al. (*23*), respectively. ND: not determined. EI half-life values were calculated as the ratio of 0.693/k_{off}.

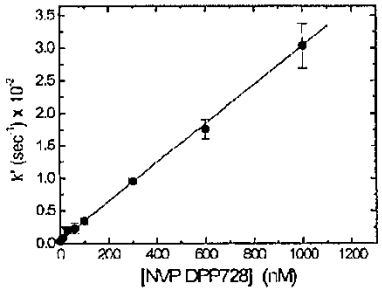


FIGURE 4: Determination of the association rate constant k_{on}' from a plot of k' vs $[I_0]$. The line represents a least squares linear fit of the indicated k' and I (NVP-DPP728 concentration) values. k' values were calculated according to eq 2. The line predicts a slope (k_{on}') of 0.47 μM^{-1} s^{-1}. Values are means ± SEM of three separate experiments.

$$k' = k_{off} + k_{on}'[I_0] \quad (2)$$

of k_{on}' and k_{off}. The rate constant k_{on}' was subsequently corrected for the competition of the substrate using eq 3,

$$k_{on} = k_{on}'(1 + [S_0]/K_m) \quad (3)$$

where $[S_0]$ is the concentration of the chromogenic substrate and K_m is the separately determined Michaelis–Menton constant. K_i' values were determined using a direct, nonlinearizing plot of v_s vs I, fitted to eq 4.

$$v_s = v_0/(([I_0]/K_i') + 1) \quad (4)$$

K_i was subsequently calculated from K_i' according to eq 5.

$$K_i = K_i'/(1 + [S_0]/K_m) \quad (5)$$

The inhibition constants for NVP-DPP728, its D-antipode, and its des-cyano and amide analogues, determined in three separate experiments, are shown in Table 1. Potency of NVP-DPP728 was strongly dependent upon the presence and chirality of the P1 nitrile functionality. By alteration of the orientation (L- to D-) of the nitrile–pyrrolidine bond, approximately 500-fold loss of potency was observed. By removal of the nitrile substituent altogether (hydrogen replacement), a 1000-fold loss of potency resulted.

Similarly, placement of a more bulky amide substituent with substantially less dipole character in place of the nitrile resulted in a 30 000-fold loss of potency. These results

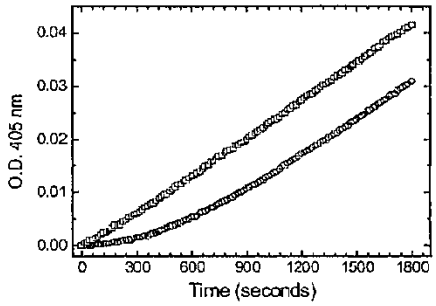


FIGURE 5: Dissociation of the NVP-DPP728–DPP-IV complex following dilution into substrate. An aliquot of DPP-IV enzyme previously incubated in the presence of 0 (squares) or 300 (circles) nM L-NVP-DPP728 was diluted 100-fold into 1 mM H-Ala-Pro-pNA, in assay buffer. Dissociation was monitored by substrate hydrolysis (absorbance at 405 nm). Absorbance readings were taken every 15 s for 30 min.

indicated that the nitrile functionality, in the L- (or S-) configuration, imparts approximately 3.9 kcal/mol of binding energy compared to the des-cyano (–H) analogue.

Inhibitor dissociation was studied by diluting the preformed NVP-DPP728/DPP-IV complex into a concentrated substrate solution such that the complex concentration was approximately 150-fold less than K_i' and the S/K_m ratio was >10. Figure 5 shows that the DPP-IV enzymatic activity was slowly recovered from the inhibitory complex, indicated by the nonlinear increase in rate relative to the control curve.

A value for k' was determined from the upwardly concave curve by fitting the data to eq 1, in which k' represents the rate for reestablishment of the steady-state equilibrium between DPP-IV and NVP-DPP728/DPP-IV complexes following dilution. A value for k_{-2} (Table 2, 1.4 ± 0.5 × 10^{-3} s^{-1}) was then derived by linear regression from a plot of k' against I (not shown), where the y-intercept is taken as the rate constant for decay of the NVP-DPP728/DPP-IV complex, as described (*22*). Comparison of disassociation rates calculated for the des-cyano analogue of NVP-DPP728 and the amide analogue (Table 1) indicate that the presence of the nitrile functionality of NVP-DPP728 imparts potency by promoting formation of a relatively long-lived complex.

Equilibrium binding experiments were carried out using [^{14}C]-labeled NVP-DPP728 in order to confirm the results obtained by kinetic methods and to assess the potential for effects of substrate on enzyme–inhibitor dissociation. The compound was bound to bovine kidney DPP-IV, and EI

Table 2: NVP-DPP728 Affinity and Dissociation Constants Determined by Kinetic and Binding Methods

method	[S$_0$] (μM)	K_i (nM)	k_{off} (s^{-1})
Kinetic Methods			
initiated by enzyme addition[a]	166	11 ± 4	(1.3 ± 0.2) × 10^{-3}
	1000	15 ± 1	(1.5 ± 0.3) × 10^{-3}
initiated by dilution of EI[b]	166	16 ± 4	(1.5 ± 0.5) × 10^{-3}
Binding[c] Methods			
without substrate	0	12 ± 2	(1.0 ± 0.1) × 10^{-3}
with substrate	200	ND	(1.6 ± 0.1) × 10^{-3} [c]

[a] Reactions were initiated by the addition of enzyme and were performed at 25 °C in 25 mM Tris-HCl, pH 7.4, containing 140 mM NaCl, 10 mM KCl, 1% bovine serum albumin, and H-Ala-Pro-pNA as indicated. [b] Reactions were initiated by 100-fold dilution of enzyme preincubated for 60 min with NVP-DPP728 into buffer containing substrate. Inhibition constants were determined as described in the Results. [c] Binding and dissociation of [^{14}C]-NVP-DPP728 to purified bovine kidney DPP-IV was measured at 25 °C in 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl. Following washing of DPP-IV saturated with [^{14}C]-NVP-DPP728 and dilution into buffer containing 0 or 200 μM Gly-Pro-AMC, bound and free inhibitor were collected by filtration and quantitated by scintillation counting. [c] Significantly different from 0 substrate value ($p < 0.0001$ by Student's t test). ND: not determined.

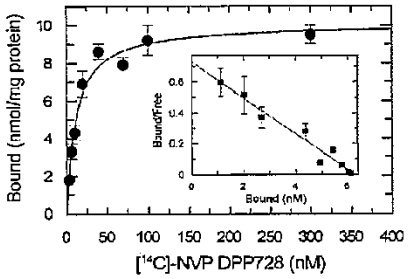


FIGURE 6: Equilibrium binding of [^{14}C]-NVP-DPP728 to bovine kidney DPP-IV. Aliquots (2.5 μg) of bovine DPP-IV were diluted into 50 mM Tris-HCl, pH 8.0, containing 0−300 nM [^{14}C]-NVP-DPP728. Following a 10-min incubation, enzyme-bound inhibitor was separated from free inhibitor and quantified by scintillation counting. The data represent the mean (SEM) of three independent experiments.

complexes were adsorbed onto DEAE cellulose disks. After subtraction of nonspecific binding, the data were fit (Figure 6) according to eq 6, where [EI] is the concentration of

$$[EI] = [E_{total}][I]/(K_d + [I]) \quad (6)$$

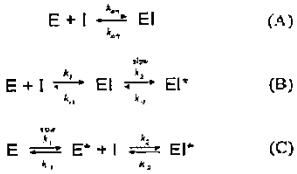
enzyme−inhibitor complex, [I] is the free inhibitor concentration, [E_{total}] is the enzyme concentration, and K_d is the dissociation constant (equivalent to K_i).

The calculated K_d and [E_{total}] derived from these data were 11.5 ± 1.8 nM and 10.0 ± 0.4 nmol/mg of protein, respectively. On the basis of a unit molecular weight of 110 000 Da, approximately 1 mol of binding was observed per mol of enzyme. The data also were plotted according to the method of Scatchard (*24*) (see eq 7, inset to Figure 6),

$$[EI]/[I] = ([EI]_{max} - [EI])/K_d \quad (7)$$

where the slope of the fit line is equal to $1/K_d$ and the x-intercept is equal to $[EI]_{max}$. The K_d value obtained by this method was 8.6 nM, in agreement with the value obtained by both the saturation binding and kinetic methods. The

Scheme 1



x-intercept (6.3 nM), equal to the concentration of binding sites, agreed well with the enzyme concentration of 5.7 nM. Thus, using equilibrium binding measurements with radiolabeled compound, it was possible to confirm the affinity measurements obtained using kinetic methods and to substantiate a model for single-site, competitive binding.

Since the both the D-antipode and the des-cyano analogue of NVP-DPP728 were found to have similar, low potency (K_i values of 5.6 and 15.6 μM, respectively), it appeared that L-chirality was required for high-affinity binding of the nitrile functionality. Surprisingly, evaluation of the inhibition kinetics for the D-antipode revealed essentially identical dissociation rates (k_{off} values of 1.5 and 1.3 × 10^{-3} s^{-1} for the D- and L-isomers, respectively). Because the pair of inhibitors bind with markedly different association rates (127 × 10^3 vs 0.27 × 10^3 M^{-1} s^{-1}), but dissociate with identical kinetics, a series of experiments were performed to dissect the mechanism of slow binding.

As described above, reaction progress curves obtained in the presence of a range of inhibitor concentrations indicated that NVP-DPP728 obeyed slow-binding inhibition kinetics. This behavior was indicated by the observation that NVP-DPP728-mediated inhibition of H-Ala-Pro-pNA cleavage approached steady-state equilibrium on a time scale of minutes under the conditions employed and the data could be fitted robustly to the slow-binding equation (*22*). Three mechanisms have been proposed that describe slow-binding behavior (Scheme 1, after Cha (*25*)).

In mechanism A, enzyme (E) binds to the inhibitor (I) in a slow step to form a tight EI complex. In mechanism B, a loose EI complex forms rapidly and is followed by a (relatively) slow isomerization to a tight EI* complex. Mechanism C describes a slow isomerization of free enzyme (E) to form E* which can rapidly and tightly bind I, forming a tight EI* complex. To discriminate between the binding mechanisms, the relationships observed between I and k', and between I and v_0, were assessed. The initial velocity (v_0) was found to be significantly inhibited in proportion to the inhibitor concentration (Figure 7) for NVP-DPP728, a finding inconsistent with mechanism A in which v_0 is predicted to be unaffected by the concentration of inhibitor (*22*).

The observation that the first-order rate constant k' increased with increasing inhibitor concentration (illustrated in Figure 4) was consistent with mechanisms A and B but not mechanism C, in which k' should decline with increasing inhibitor concentration. For this reason, mechanism B appears to best explain inhibition of DPP-IV by NVP-DPP728.

Dissociation studies employing radiolabeled NVP-DPP728 were conducted with purified bovine DPP-IV to confirm the binding constants determined by kinetic means using DPP-IV contained in cell extracts. For these studies, purified

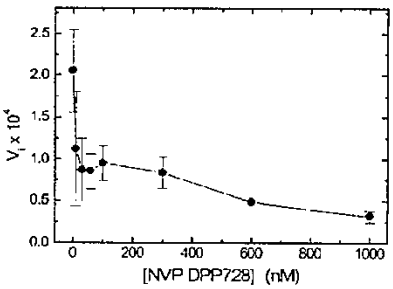


FIGURE 7: Dependence of initial velocities (v_0) on NVP-DPP728 concentration. Data represent initial velocity (v_i) values derived using eq 1 from progress curves measured as described in Figure 3. Measurement was done in 25 mM Tris-HCl pH 7.4, 140 mM NaCl, 10 mM KCl, and 1 wt %/vol bovine serum albumin in the presence of 1 mM H-Ala-Pro-pNA. Values shown are means (SEM) of three experiments.

bovine kidney DPP-IV was saturated with [^{14}C]-labeled NVP-DPP728, trapped with ADA-Sepharose, washed, and resuspended in buffer (with or without 0.2 mM H-Gly-Pro-AMC) such that the concentrations of enzyme and inhibitor were $\leq 0.2\ K_i$. The concentrations of free and bound inhibitor were determined at 0, 1, 2, 3, 4, 6, 8, and 10 min after resuspension in buffer. The data (percent bound vs time) were fitted to a single-exponential decay curve (eq 8), where [EI],

$$[EI] = [EI]_0 e^{-kt} \qquad (8)$$

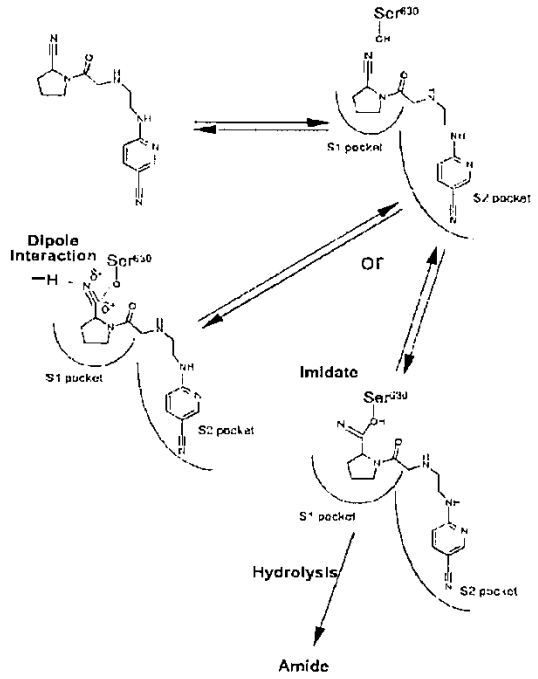
$[EI]_0$, and k are the concentration of enzyme−inhibitor complex at time t, the concentration of complex at time 0, and the rate constant, respectively. The half-life for inhibitor dissociation was taken as the natural log of 2 (0.693) divided by the rate constant and was determined to be 11.9 and 7.2 min in the absence and presence of substrate, respectively ($p < 0.0001$ by Student's t test, Table 2). These results indicated that, although the effects were relatively minor and although the dissociation of the inhibitor from the enzyme−inhibitor complex occurred more rapidly in the presence of the dipeptide substrate than in its absence, the enzymatically determined kinetic results represented a reasonable prediction of dissociation kinetics.

DISCUSSION

We have identified a new class of potent cyanopyrrolidine inhibitors in which a glycyl Xaa amine moiety is substituted with aliphatic and aromatic substituents (*12*, *13*). These inhibitors are remarkably specific for inhibition of DPP-IV relative to other post-proline and -alanine cleaving enzymes (e.g., prolyl oligopeptidase, aminopeptidase P, and DPP-II). We have assessed kinetic behavior of this series in detail, focusing in this report on NVP-DPP728.

Through kinetic evaluation of DPP-IV inhibition by NVP-DPP728, as well as by direct measurement of radiolabeled inhibitor binding in the presence and absence of substrate, we have established that NVP-DPP728 derives its potency through a slow-binding inhibition mechanism. Formation of the high-affinity complex is dependent upon the nitrile functionality within this series. Substitution with a variety of other substituents (e.g., amide, hydrogen) is associated with a significant loss of inhibitory potency as well as a clear

Scheme 2. Proposed Model for Inhibition of DPP-IV by NVP-DPP728



loss of time-dependent function. Interestingly, while moving the nitrile from the L- to the D-configuration substantially reduces the overall potency of the compound, this loss of potency is due to a ~500-fold slower binding rate (k_{on}, Table 1). Indeed, the dissociation kinetics for the L- and D-enantiomers are identical, indicating that once formed, reversal of the high-affinity complex is independent of the nitrile orientation.

While it is not presently possible to precisely determine the mechanism of binding of NVP-DPP728 to DPP-IV, structure−activity relationships support the involvement of several key interactions. First, the pyrrolidide ring interacts with the S1 pocket, through van der Waals or hydrophobic interactions. Second, hydrogen-bonding and ionic interactions stabilize the peptide bond carbonyl and the P2 site basic nitrogen functionality, respectively. Third, hydrophobic interactions stabilize P2 site side-chain binding in the S2 pocket. These interactions may occur equally with nitrile and non-nitrile inhibitors. The negative charge derived from the acid−base−nucleophile (Asp-His-Ser) charge relay in the vicinity of the nitrile carbon drives a dipole−hydrogen bond interaction (interactions with both a hydrogen bond donor and the negatively charged active site serine) or transient imidate intermediate. The free energy change associated with the nitrile functionality, approximately 3.9 kcal/mol, may be adequately explained by either approach. These alternative high affinity state models are depicted in Scheme 2.

Although additional and novel inhibition mechanisms can potentially be forwarded, several consequences of the model shown in Scheme 2 can be feasibly approached and will be

addressed in subsequent communications. First, formation of an imidate intermediate should frequently proceed via hydration to yield a transformed amide byproduct (as observed for nitrile cysteine protease inhibitors; *26*). A hydrogen-bond-stabilized dipole interaction would, in contrast, be readily reversible, and inhibitor dissociation should generate unchanged parent compound only. Second, it should be possible to identify, through site-directed mutagenesis or through X-ray crystallography, the involvement of residues acting as hydrogen bond donors, capable of stabilizing nitrile inhibitor interactions. Recently, a high-resolution X-ray crystallographic structure of prolyl oligopeptidase has been reported (*27*) in which a tyrosine hydroxyl residue has been demonstrated to participate in stabilization of the oxyanion intermediate formed during binding of Z-pro-prolinaldehyde, a highly potent slow-binding inhibitor. Prolyl oligopeptidase is a member of the α,β-hydrolase family, closely related to DPP-IV. The observation that hydrogen-bonding interactions may contribute to the stabilization of catalytic intermediates could potentially extrapolate to DPP-IV. Indeed, the finding that DPP-IV and prolyl oligopeptidase (*28*), both serine proteases, are strongly inhibited by nitrile-based inhibitors indicates that significant mechanistic differences may emerge which distinguish DPP-IV and other α,β-hydrolase enzymes from the major classes of serine proteases.

As an agent under consideration for therapeutic utility in the management of a chronic disease (type 2 diabetes mellitus), NVP-DPP728 offers high potency, competitive behavior, and rapid reversibility. Since the long-term safety and side-effect profile of DPP-IV inhibitors has not been established in the human population, particularly with regard to inhibitor effects on peptide substrates other than GLP-1, compounds with rapid reversibility may have a safety advantage due to the ability of DPP-IV activity to recover on a daily basis. This may be of particular importance with regard to inhibitor effects on natural peptide substrates other than GLP-1 including growth hormone-releasing hormone (*2*) glucagon-like peptide-2 (29) and neuropeptides such as Substance P (*30*). Use of NVP-DPP728 as an antidiabetic agent is predicated on the therapeutic effects of glucagon-like peptide-1 (GLP-1), a small peptide hormone produced by the intestine during the absorption of nutrients. GLP-1, with a His-Ala- amino terminus, is rapidly cleaved and inactivated by DPP-IV (*2*, *31*). By preserving GLP-1 activity in circulation, DPP-IV inhibitors are expected to improve insulin release following ingestion of meals, to suppress glucagon, a stimulus for hepatic glucose production, and to augment satiety (*3*). Thus rapidly reversible inhibitors that inhibit GLP-1 degradation, which are present during meal absorption and which are readily cleared from circulation, will exert a therapeutic effect while allowing other DPP-IV-mediated functions to be minimally affected. As an inhibitor with high potency and rapidly reversible inhibition kinetics, NVP-DPP728 will allow for the safety and tolerability of DPP-IV inhibition to be evaluated under conditions allowing intermittent recovery of enzyme function.

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DIAEAZ 49 Supplement (1) 1–A524 (2000)

PDX-29

decreases of ≥ -0.7% for the 5mg or 10mg doses. The overall tolerability profile of GI262570 was similar to that observed with other PPARγ agonists, with dose-related weight gain, hemoglobin decreases, and peripheral edema. These data suggest that GI262570 will have clinical utility in the treatment of T2DM.

Parameter	P	1mg	2mg	5mg	10mg
Number of subjects (Intent-to-Treat)	67	59	61	58	67
Baseline (B) FSG (mg/dL)	201	205	208	204	206
FSG Δ from B at 4 weeks	+21	-10[†]	-20[†]	-34[*]	-54[†]
FSG Δ from B at 12 weeks	+22	-8[†]	-28[†]	-48[*]	-66[†]
% of subjects with ≥ -30 mg/dL FSG Δ from B	11%	33%[*]	48%[†]	74%[†]	85%[†]
Baseline HbA1c (%)	8.1	7.8	8.0	8.1	8.1
HbA1c Δ vs. P at 12 weeks	—	-0.4[ψ]	-0.8[†]	-1.4[†]	-1.9[†]
HbA1c Δ from B at 12 weeks	+1.1	+0.7	+0.3	-0.3	-0.7
% of subjects with ≥ -0.7% HbA1c Δ from B	3%	9%	19%[*]	41%[†]	54%[†]

Significance levels vs. P: [ψ] = < 0.05 [*] = < 0.005 [†] = < 0.001

158-OR

Monotherapy with GI262570, a Tyrosine-Based Non-Thiazolidinedione PPARγAgonist, Significantly Reduces Triglyceride and Increases HDL-C Concentrations in Patients with Type 2 Diabetes Mellitus

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GI262570 is a novel, non-thiazolidinedione, L-tyrosine-based peroxisome proliferator-activated receptor gamma (PPARγ) agonist that is ~1000-fold more selective for human PPARγ compared to the human PPARα isoform. A total of 376 Type 2 diabetes mellitus (T2DM) patients were randomized to receive either placebo (P) or one of 5 daily doses of GI262570 (0.25, 1, 2, 5, or 10mg) in a 12-week double-blind placebo-controlled study. Mean baseline fasting triglyceride (TG) and high-density lipoprotein cholesterol (HDL-C) concentrations are listed in the table. Approximately 60% of T2DM patients had mild fasting hypertriglyceridemia (HT) defined as TG > 150 mg/dL. Twelve weeks of treatment with GI262570 resulted in significant metabolic improvement in fasting TG and HDL-C (see table) along with changes in glycemic parameters that are described in a separate abstract. The maximum reduction in TG was achieved within 4 weeks and the effect was maintained over 12 weeks. While metabolic improvement in TG and HDL-C levels was observed across the range of T2DM patients regardless of baseline TG status, the greatest reductions were observed in subjects with mild/moderate HT. Apolipoprotein B levels fell by 8-14% for the 5mg and 10mg doses and there was also a non-significant decrease in LDL-C at these two GI262570 doses. These findings suggest that GI262570 will enhance overall metabolic control for T2DM patients by improving the high TG/low HDL-C dyslipidemia associated with T2DM as well as by improving hyperglycemia.

PARAMETER	P	1mg	2mg	5mg	10mg
Number of subjects (Intent-to-Treat)	67	59	61	58	67
Baseline (B) TG (mg/dL)	170	200	185	199	179
% TG Δ from B at 12 weeks	+3%	-6%	-13%[ψ]	-30%[†]	-43%[†]
Baseline TG (mg/dL) HT group	269	302	261	322	268
% TG Δ from B at 12 wks HT group	-7%	-18%	-19%	-44%	-53%
Baseline HDL-C (mg/dL)	42	42	42	40	42
% HDL-C Δ from B at 12 weeks	0%	+4%	+13%[†]	+12%[*]	+15%[†]

Significance levels vs. P: [ψ] = p < 0.05 [*] = p < 0.005 [†] = p < 0.001

159-OR

Rosiglitazone Liver Safety Update

HAROLD E. LEBOVITZ,[1,2] ALAN SALZMAN,[1,2] *Brooklyn, NY; Collegeville, PA*

Rosiglitazone is a potent thiazolidinedione for the treatment of type 2 diabetes. The thiazolidinedione troglitazone has been associated with hepatotoxicity, including liver failure and hepatic-related deaths. To date, no signal of hepatotoxicity has been seen with rosiglitazone, which has been extensively evaluated.

The incidence in rosiglitazone-treated patients of ALT elevations greater than 3x the upper limit of normal (ULN) was low and similar to placebo/comparators at the time of FDA filing in November 1998, at which time total exposure to rosiglitazone was 3600 patient years. Subsequently, exposure to rosiglitazone in clinical trials has substantially increased and as of November 1999 comprised over 5000 patient years including more than 1000 patients treated for ≥ 2 years. For all rosiglitazone-treated patients (including monotherapy and combination with SU or metformin), the rate of ALT levels > 3x the ULN is 0.30 cases per 100 patient years (see Table below). This compares to 0.59 cases per 100 patient years for placebo-treated patients and 0.73 cases per 100 patient years for SU- or metformin-treated patients. These are similar to the rates seen 1 year prior.

Rates of ALT Levels > 3x ULN in the Rosiglitazone Trial Program (expressed as cases per 100 patient years)

	Rosiglitazone*	Placebo	SU or Metformin
November 1998	0.35	0.59	0.78
November 1999	0.30	0.59	0.73

*Includes monotherapy and combination with SU or metformin

In addition, rosiglitazone has been prescribed to over 250,000 patients, and thus far the clinical trial experience has been predictive of the rosiglitazone safety experience in the marketplace.

In conclusion, the current clinical trial and postmarketing experience with rosiglitazone indicate no evidence of troglitozone-like hepatotoxicity.

160-OR

Treatment with a DPP-IV Inhibitor, NVP-DPP728, Increases Prandial Intact GLP-1 Levels and Reduces Glucose Exposure in Humans

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NVP-DPP728 is a highly selective, orally active inhibitor of dipeptidyl peptidase-IV (DPP-IV) designed to augment the glucose-lowering activity of endogenously secreted GLP-1. Recent studies have demonstrated that NVP-DPP728 prevents N-terminal degradative inactivation of GLP-1 and improves glucose tolerance in insulin-resistant rats. The present crossover trial evaluated the single dose pharmacodynamics of NVP-DPP728 administered to 12 healthy normoglycemic volunteers. After an overnight fast, subjects were administered 100 mg NVP-DPP728 or placebo, followed 30 minutes later by a 1000 kcal solid meal (23g protein, 42g fat, 36g carbohydrate, standard FDA breakfast). Blood samples were obtained predose and for up to 24 hours after each dose for analysis of plasma glucose and insulin levels and also for active, undegraded GLP-1 levels by direct ELISA (Linco Research). NVP-DPP728 increased peak plasma levels of active GLP-1 (15±2 vs. 9±2 pmol/l, p = 0.018) and also increased active GLP-1 prandial exposures, $AUC_{GLP-1(0-4h)}$ (28±4 vs. 14±4 pmol.l^{-1}.h^{-1}, p < 0.0001). Prandial glucose excursions above baseline were reduced by NVP-DPP728 relative to placebo (12±3 vs. 20±4 mg.dl^{-1}.h^{-1}, respectively, p = 0.04), while glucose excursions below baseline were unchanged (-22±4 vs. -16±4, p=0.3). Insulin excursions were not affected. No clinically significant adverse events were observed. In addition, administration of NVP-DPP728 to fasting individuals was unaccompanied by clinically significant changes in plasma glucose levels.

Thus NVP-DPP728 increased prandial active GLP-1 levels with concomitant reduction in prandial glucose exposure in normal subjects without causing hypoglycemia. These results provide the first direct clinical demonstration that DPP-IV inhibition is a viable new pharmacological approach for potentiating endogenous GLP-1 activity, and support the investigation of the glucose-lowering potential of NVP-DPP728 for the treatment of type 2 diabetics.

A numeral beside an author's name indicates a duality of interest. See page 93.

MAY 2000 VOLUME 49 SUPPLEMENT 1

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413-P

Single Dose Treatment of Diabetic Patients by the DP IV Inhibitor P32/98

HANS-U. DEMUTH, TORSTEN HOFFMANN, KONRAD GLUND, CHRISTOPHER H. S. MCINTOSH, RAYMOND A. PEDERSON, KATJA FUECKER, SABINE FISCHER, MARKOLF HANEFELD; *Halle (Saale), Germany; Vancouver, Canada; Dresden, Germany*

The DP IV inhibitor Di-[3N-((2S,3S)-2-amino-3-methyl-pentanoyl) 1,3-thiazolidine] fumarate (P32/98) improves glucose tolerance (GI) by an incretin-mediated enhanced insulin response in normal and diabetic rodents, as well as in human volunteers. Within the clinical program, a pilot study in diabetic patients on different therapies was designed. Goal of the open investigation was the evaluation of patients response to a single dose of 60 mg P32/98 15 min prior to an OGTT (75 g) after over-night fasting and 12 hour post-medication (diet, acarbose, metformin, glibenclamide or insulin). Patients (n=20, men) were allocated according to there current medication to 5 groups, each receiving placebo and OGTT at the beginning of the experiment. Seven days later, again after over-night fasting and 12 hours post-medication, 15 min prior OGTT one tablet containing 60 mg P32/98 was administered. Glucose response was recorded every 15 min in an interval of -15 to 300 min. Blood samples were taken to all that time points for determination of P32/98, glucose, insulin, proinsulin, C-peptide, GLP-1, glucagon, FFA and leptin. As expected, a profound GI improvement caused by P32/98 was observed in patients being treated with acarbose or glibenclamide. In these cases the glucose tolerance improvement was 20.6% and 31.3%, respectively. These values parallel the elevated insulin responses observed after P32/98 treatment in these patients. In contrast, in diabetics on insulin therapy, the acute GI improvement after a single dose of P32/98 was 8.8% only (assessed by area under the GI curve). Whether insulin resistance can be reduced or islet responsiveness will improve, mediated by DP IV inhibition, remains to be proven by longer term application of P32/98 in such patients.

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413-P

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The DP IV inhibitor DI-[3N-((2S,3S)-2-amino-3-methyl-pentanoyl) 1,3-thiazolidine] fumarate (P32/98) improves glucose tolerance (Gt) by an incretin-mediated enhanced insulin response in normal and diabetic rodents, as well as in human volunteers. Within the clinical program, a pilot study in diabetic patients on different therapies was designed. Goal of the open investigation was the evaluation of patients response to a single dose of 60 mg P32/98 15 min prior to an OGTT (75 g) after over-night fasting and 12 hour post-medication (diet, acarbose, metformin, glibenclamide or insulin). Patients (n=20, men) were allocated according to there current medication to 5 groups, each receiving placebo and OGTT at the beginning of the experiment. Seven days later, again after over-night fasting and 12 hours post-medication, 15 min prior OGTT one tablet containing 60 mg P32/98 was administered. Glucose response was recorded every 15 min in an interval of -15 to 300 min. Blood samples were taken to all that time points for determination of P32/98, glucose, insulin, proinsulin, C-peptide, GLP-1, glucagon, FFA and leptin. As expected, a profound Gt improvement caused by P32/98 was observed in patients being treated with acarbose or glibenclamide. In these cases the glucose tolerance improvement was 20.6% and 31.3%, respectively. These values parallel the elevated insulin responses observed after P32/98 treatment in these patients. In contrast, in diabetics on insulin therapy, the acute Gt improvement after a single dose of P32/98 was 8.8% only (assessed by area under the Gt curve). Whether insulin resistance can be reduced or islet responsiveness will improve, mediated by DP IV inhibition, remains to be proven by longer term application of P32/98 in such patients.

otides in length. In vitro "evolution" was done on this region at 30% mutagenesis, and four more rounds of in vitro selection followed before this second population was cloned. From these sequences, a consensus region was discovered. Certainly though this work is a pioneering achievement in the field, it is an example of how the conventional protocol is significantly more involved than that presented here.

In summary, a novel in vitro selection protocol has been designed to take advantage of a combinatorial library of small size that has multiple copies of every distinct sequence. The method condensed the many days of a typical screening strategy to less than two days. This was a proof-of-concept experiment that showed that the new method succeeds by creating a large number of copies of individual sequences in the initial random pool, consistently reducing the level of nonspecific binding sequences per selection round, and effectively amplifying the few surviving sequences.

Since only the original synthesized sequences were used for all he screenings, the technique should allow for the iterative n vitro selection of modified oligonucleotides that previously ould not undergo this powerful process.[10] Hence, this method hould significantly increase the power of the in vitro selection nethod and is the direction that we are currently investigating.

Experimental Section

The DNA library[4] (31 mg) was labeled at the 5'-end with [γ-^{32}P]ATP, purified by el chromatography, and suspended in 300 mL of folding buffer (300 mM KCl, 5 mM $MgCl_2$, 20 mM Tris, pH 7.5). After cooling down to room temperature following lenaturation at 75 °C, the ^{32}P-labeled DNA was loaded onto an acetate-agarose recolumn (300 μL), which was attached directly to a 2.5 mM ATP-agarose column 800 μL, Sigma). The precolumn was washed with 600 μL of buffer, and the eluted DNA was allowed to equilibrate on the ATP-agarose column for 10 min. The precolumn was discarded after a single use as were all subsequent columns. After equilibration, the ATP-agarose column was washed with 4 mL of folding buffer to elute unbound or weakly bound oligonucleotides. The retained DNA was eluted with 3 mL of the ATP elution buffer (5 mM ATP in folding buffer) and collected in 300 μL fractions.

In order to perform another round of selection, the ATP had to be removed. Hence, the eluted fractions were collected directly into Microcon-3 microcentrifuge devices (3000 D cutoff, Amicon). After membrane diafiltration, about 98% of the total ATP was removed. The filtered fractions were then pooled, and folding buffer was added until a final volume of 10 mL was attained. The concentration of contaminating ATP concentration was 30 μM for the DNA sample, which was over 80 times more dilute than that of the 2.5 mM ATP-agarose column. Each cycle of selection started with a new set of stacked affinity columns, i.e. a precolumn attached to a ligand column. The screening cycles for the ATP aptamers are summarized in Table 1.

The rare-DNA PCR was performed as follows: On the last cycle the DNA was eluted from the ATP-agarose column with 3 mL of 10 mM ATP in 20 mM Tris, pH 7.5. This last fraction was precipitated twice from ethanol, and the PCR reagents (50 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 20 mM Tris, pH 8.8 at 25 °C, 200 μM dNTPs, 0.1% Triton X-100, 20 units of Deep Vent (exo-) DNA polymerase 0.5 μg 5'-primer, 0.5 μg 3'-primer) were added. Thermal cycling (94 °C for 45 s; 42 °C for 90 s; 60 °C for 45 s; 45 cycles) was done in a microcentrifuge tube that had first been irradiated with UV light. A positive control containing a dilute solution (~20000 molecules) of a 52-mer, and a negative control containing no DNA also underwent the same amplification protocol. Gel electrophoresis after amplification showed DNA in all lanes except the negative control.

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Keywords: aptamers · combinatorial chemistry · in vitro selection · nucleotides · polymerase chain reaction

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The Synthesis of Enantiopure ω-Methanoprolines and ω-Methanopipecolic Acids by a Novel Cyclopropanation Reaction: The "Flattening" of Proline**

Stephen Hanessian,* Ulrich Reinhold, and Gabriella Gentile

Proline occupies a prominent position in the hierarchy of natural amino acid constituents of mammalian proteins.[1] As part of a peptidic motif, its unique structure results in secondary amide bonds, leading to important conformational and functional consequences.[2] For example, the well-known *cis–trans* isomerism in prolylamides is associated with vitally important biological phenomena and functions, such as protein folding,[3] hormone regulation,[4] recognition,[5] and transmembrane signaling[6] to mention a few. The importance of *cis–trans* conformational changes is manifested by the role that peptidyl prolyl isomerases such as the immunophilins play in immunoregulation.[7] Proline has also figured prominently as a component of therapeutic agents,[8] in drug design,[9] and in probing enzyme activity.[10]

Conformationally constrained analogues of proline have been used extensively in connection with peptidomimetic research.[11] Although 2,3- and 3,4-methanoprolines have been

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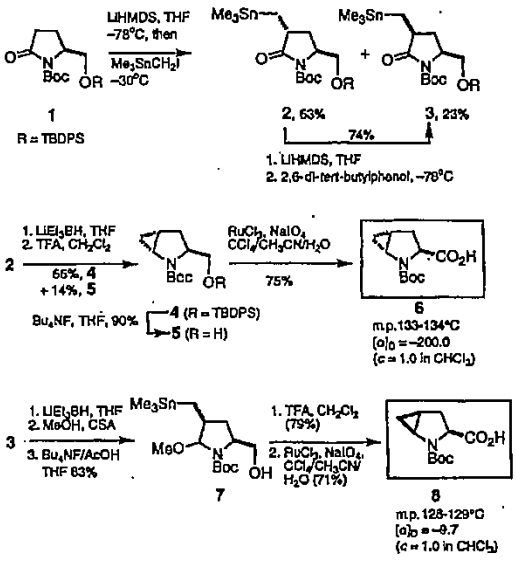
described,[12,13] the 4,5-methanoprolines are relatively unexplored.[14] Furthermore, structural investigations that study the consequences of introducing strain and its effects on the configuration and stability of amide linkages are not available to compare such systems to their proline counterparts.

We describe herein highly stereocontrolled syntheses of the diastereomeric 4,5-methano-L-prolines and 5,6-methano-L-pipecolic acids by a novel intramolecular cyclopropanation reaction of iminium ions and the extension of the methodology to other congeners.[15,16]

Treatment of the readily available lactam 1[17a] with lithium hexamethyldisilazide (LiHMDS) and Me_3SnCH_2I gave the α-alkylated products 2 ($[\alpha]_D = -15.3$, $c = 0.43$ in $CHCl_3$) and 3 ($[\alpha]_D = -16.0$, $c = 1.23$ in $CHCl_3$) in 63% and 23% yields, respectively (Scheme 1). The *syn*-isomer 3 could be easily obtained

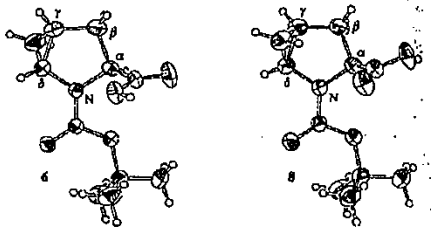


Scheme 1. TBDPS = *t*-BuPh$_2$Si, TFA = trifluoroacetic acid, Boc = *tert*-butoxycarbonyl, CSA = camphor-10-sulfonic acid.

by treatment of the enolate from 2 with the proton source 2,6-di-*tert*-butylphenol.[18,19] Generation of the hemiaminal from 2 and treatment with TFA led to the (4*R*,5*R*)-methanopyrrolidine derivative 4 ($[\alpha]_D = -69.3$, $c = 1.41$ in $CHCl_3$), which was smoothly deprotected to 5, and the latter oxidized to give the crystalline (4*R*,5*R*)-methano-*N*-Boc-L-proline in excellent overall yield.

Similar treatment of the *syn*-isomer 3 gave the diastereomeric crystalline acid 8 via its methylaminal derivative 7. The structures and conformations of 6 and 8 in the solid state were unambiguously confirmed by single-crystal X-ray analysis. Table 1 lists selected torsion angles for compounds 6 and 8, where considerable "flattening" of the pyrrolidine ring is observed relative to *N*-Boc-L-proline,[20] particularly in the case of 6. The flattening of the pyrrolidine ring in 6 is also manifested in the root-mean-square value of 0.003 Å for the C_β and N atoms from the plane defined by C_β,C_γ,C_δ, and N (0.013 Å in 8). The lowest deviation of 0.018 Å in the case of *N*-Boc proline was found for C_α and C_γ atoms in the plane C_α,N,C_δ,C_γ; in this case C_β was distinctly above the plane (0.521 Å). This differs substantially

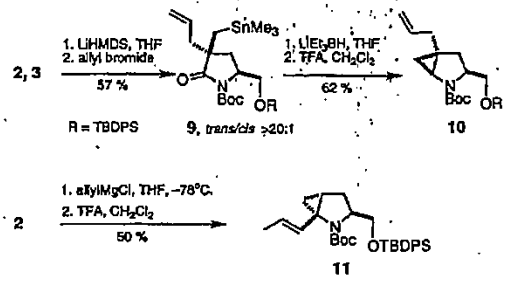
Table 1. Selected torsion angles and root-mean-square deviations from fitted atoms in a given plane of X-ray crystal structures, and ^{13}C NMR chemical shifts ($CDCl_3$).



	N-Boc-L-proline [20]	6	8
τ(NC$_\alpha$)	−17	−5.6	−14.4
τ(C$_\alpha$C$_\beta$)	+31	+4.8	+15.3
τ(C$_\beta$C$_\gamma$)	−35	−2.6	−11.4
τ(C$_\gamma$C$_\delta$)	+24	−0.7	+2.9
τ(C$_\delta$N)	−4	+4.1	+7.6
τ(BocNC$_\alpha$CO$_2$H)	−72	−64.0	−67.1
rms deviation of fitted atoms	0.018 C$_\alpha$, N, C$_\delta$, C$_\gamma$	0.003 C$_\beta$, C$_\gamma$, C$_\delta$, N	0.013 C$_\beta$, C$_\gamma$, C$_\delta$, N
	δ(*cis*/*trans*)	δ(*cis*/*trans*)	δ(*cis*/*trans*)
COOH	178.35/176.60	177.7/175.5	179.1/176.1
NC=O	153.95/155.39	157.1/154	155.7/154.1
C$_\alpha$	58.8	60.8/60.1	59.5/59.1
C$_\beta$	30.75/29.53	32.0	31.5/29.1

from 6 and 8, in which the out-of-plane carbon atom was the one bearing the carboxyl group (0.082 Å and 0.235 Å respectively).

Intermediates 2 and 3 could also be subjected to further stereocontrolled branching leading to the α-C-allyl derivative 9, which upon controlled reduction and acid-catalyzed destannylation led to the branched 4,5-methano-L-proline precursor 10 ($[\alpha]_D = +4.3°$, $c = 0.72$ in $CHCl_3$; Scheme 2). Treatment of 2



Scheme 2.

with allylmagnesium chloride, followed by trifluoroacetic acid (TFA), led to the (*S*)-5-(2-propenyl)-4,5-methano-L-proline derivative 11 ($[\alpha]_D = -27.0$, $c = 0.57$ in $CHCl_3$) on migration of the double bond. Compounds 10 and 11 represent uniquely functionalized precursors to constrained ω-methanoprolines.

The versatility and generality of the intramolecular carbocyclization reaction with appended trimethylstannylalkyl groups via incipient iminium ions can be demonstrated in the synthesis of bicyclic proline congeners (Scheme 3). These compounds are related to the antihypertensive agent ramipril.[21] A highly stereoselective allylation of the enolate from 1 gave 12 ($[\alpha]_D = -45.0$, $c = 1.0$ in $CHCl_3$), which was subjected to a photoinduced trimethylstannylation[22] to give 13 ($[\alpha]_D = -23.6$,

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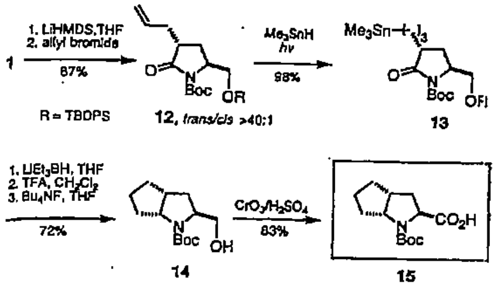
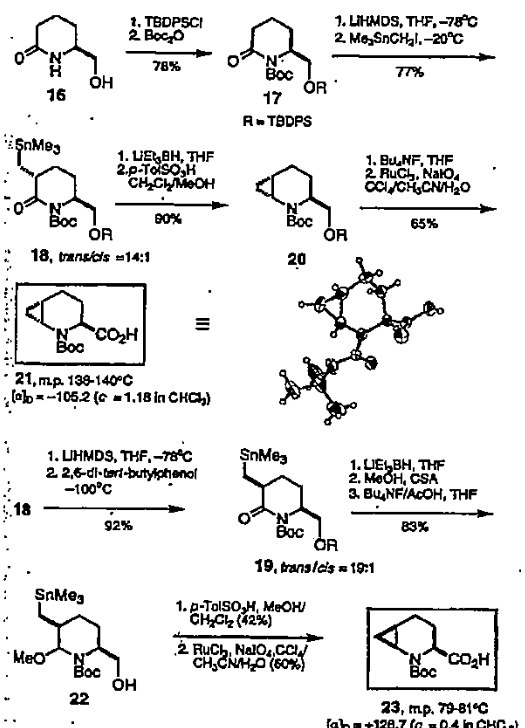


Scheme 3.

$c = 0.92$ in $CHCl_3$). Formation of the hemiaminal, followed by acid-catalyzed cyclization and deprotection, led to the bicyclic prolinol derivative 14 ($[\alpha]_D = -97.3$, $c = 1.38$ in $CHCl_3$). Finally, oxidation under Jones conditions gave the immediate precursor to the *N*-Boc-(4*R*,5R)-ramipril diastereomer 15 (m.p. 61–63 °C; $[\alpha]_D = -126.7$, $c = 0.46$ in $CHCl_3$).

It is also of interest to view compounds 6, 8, 10, and 11 as precursors to constrained analogues or precursors to L-pipecolic acid. The extension of the cyclopropanation reaction to the pipecolic acid series is shown in Scheme 4. Trimethylstannylmethylation of the lithium enolate derived from the readily available 17[23] gave the *anti*-isomer 18. Reduction to the hemiaminal and acid-catalyzed cyclization led to 20 ($[\alpha]_D = -56.0$, $c = 1.02$ in $CHCl_3$), which was deprotected and oxidized to the crystalline (5*R*,6*S*)-methano-*N*-Boc-L-pipecolic acid 21 (m.p. 138–140 °C; $[\alpha]_D = -105.2$, $c = 1.17$ in $CHCl_3$). Epimerization of 18 to the *syn*-isomer 19 by diastereoselective protonation, followed by functional group manipulation as described above, led to the crystalline diastereomeric (5*S*,6*S*)-methano-*N*-Boc-pipecolic acid 23 (m.p. 79–81 °C; $[\alpha]_D = -126.7°$, $c = 0.40$ in $CHCl_3$). The structure of crystalline 21 was unambiguously established by X-ray analysis. It is of interest to note that while the proline derivatives 6 and 8 adopt a *cis*-*N*-Boc proline orientation in the solid state (Table 1), the corresponding 4,5-methanopipecolic acid analogue 21 exhibits a *trans* orientation (Scheme 4). The presence of *cis* and *trans* isomers of 6 and 8 in $CDCl_3$ was evidenced by the corresponding ^{13}C NMR shifts, as in the case of *N*-Boc-L-proline (Table 1).



Scheme 4.

Pipecolic acid is an important constituent of the immunosuppressive agents FK-506[24] and rapamycin,[25] in which its α-ketoamide portion is intimately involved in an "active complex" with the target enzyme.[26] It is also involved in the metabolism of L-lysine, an essential amino acid for mammalian growth and development.[27] Functionalized pipecolic acids are also considered strained analogues of lysine with applications in drug design and peptidomimetic research,[28] as well as in the inhibition of L-pipecolate oxidase.[27]

It is our belief that the replacement of L-proline and L-pipecolic acid by conformationally altered ring variants represented by the methano congeners described in this work could have important consequences in biological recognition, in *cis–trans* conformational changes, in the susceptibility of the secondary amide bonds to enzymatic cleavage, and in related processes or phenomena. Studies that address these issues will be reported in due course.

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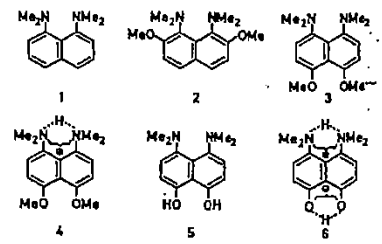
1,8-Bis(dimethylamino)-4,5-dihydroxynaphthalene, a Neutral, Intramolecularly Protonated "Proton Sponge" with Zwitterionic Structure**

Heinz A. Staab,* Claus Krieger, Gisela Hieber, and Klaus Oberdorf

The interaction of basic groups in close proximity to each other may lead, as in the case of 1,8-bis(dimethylamino)-naphthalene (**1**), to unusually high basicities ("proton sponges").[1] The influences of gradually changed distances and orientations of the basic centers as well as of inductive, mesomeric, and steric effects on the basicity of such compounds have been thoroughly studied.[2] In comparison to the basicity of **1** [$pK_a \approx 12.1$ (H_2O); 7.5 (DMSO)],[3] that of 2,7-dimethoxy-1,8-bis(dimethylamino)naphthalene (**2**) is found to be increased by four powers of ten [$pK_a \approx 16.1$ (H_2O); 11.5 (DMSO)].[3] To separate the mesomeric effect of the two methoxy groups from their steric effect on the dimethylamino groups, we were interested in **3**, an isomer of **2** in which the two methoxy groups are not in the 2,7-positions but in the opposite *peri*-positions. In fact, 1,8-bis(dimethylamino)-4,5-dimethoxynaphthalene (**3**) is considerably less basic [$pK_a \approx 13.9$ (H_2O); 9.3 (DMSO)] than the isomer **2**, indicating that the main reason for the high basicity of **2** is the steric effect of the methoxy groups in *ortho*-positions to the dimethylamino groups. Irrespective of this primarily intended basicity-comparison of **2** and **3**, the synthesis of **3** should allow the easy preparation of the corresponding 4,5-dihydroxy compound **5**, which by intramolecular proton displacement may lead to a new type of neutral, yet zwitterionic "proton sponge" (formula **6**).



For the synthesis of **3**, 1,8-dihydroxynaphthalene[4] was methylated to give 1,8-dimethoxynaphthalene, which was nitrated (conc. nitric acid, glacial acetic acid/dichloromethane, 9:5) to yield 1,8-dimethoxy-4,5-dinitronaphthalene (39%; m.p. 278 °C); the isomeric 2,5-dinitro product (m.p. 151–153 °C) was separated by chromatography on silica gel with dichloromethane as eluent. Catalytic hydrogenation (10% Pd/C, tetrahydrofuran (THF), 20 °C) resulted in the formation of 1,8-diamino-4,5-dimethoxynaphthalene (97%; m.p. 83–95 °C, decomp), which was N-methylated according to the method of Quast et al.[5] to give **3** (71%; m.p. 75 °C, from *n*-hexane/ethyl

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2-CYANOPYRROLIDIDES AS POTENT, STABLE INHIBITORS OF DIPEPTIDYL PEPTIDASE IV

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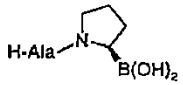
Abstract: A novel series of stable, potent inhibitors of dipeptidyl peptidase IV has been developed. A number of dipeptide analogues, incorporating a 2-cyanopyrrolidide, were found to have K_i values of less than 5 nM versus human DP-IV and half-lives of >48h in aqueous solution (pH 7.4). Copyright © 1996 Elsevier Science Ltd

Dipeptidyl peptidase IV (DP-IV, EC 3.4.14.5) is a serine protease which catalyses the cleavage of dipeptides from the N-terminus of proteins with the sequence H-X-Pro-Y- or H-X-Ala-Y- (where X, Y= any amino acid, Y≠ Pro).[1] DP-IV is widely distributed in mammalian tissues and is found in great abundance in the kidney, liver, intestinal epithelium and placenta.[2] In the human immune system, DP-IV is identical to the T cell activation marker, CD26. Recent evidence has also shown CD26 to be an activation marker of natural killer cells[3] and of a main population of B cells.[4]

Our interest in DP-IV was stimulated by the publication of data which showed that either simple inhibitors or antibodies of the enzyme were effective as inhibitors of T cell proliferation and were thus potential immunomodulators.[5-7]

Substrates and inhibitors of DP-IV require a free N-terminus, which means that potential dipeptide serine protease inhibitors (e.g. C-terminal aldehydes, boronic acids, α-ketoacids, trifluoromethylketones, or chloromethylketones) are inherently unstable at neutral pH due to intramolecular cyclisation.[8]



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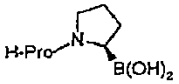

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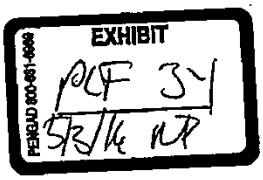
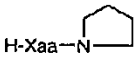
The most potent DP-IV inhibitors reported to date are the boroproline analogues 1, (K_i=2nM) and 2, (K_i=3nM). However, these boronic acids are unstable at neutral pH ($t_{½}$=30min and 90min for 1 and 2



respectively).[7] Other, more stable classes of DP-IV inhibitors have been reported. These include tripeptides;[8] aminoacyl pyrrolidides and thiazolidides;[6] dipeptide phosphonates;[9,10] azaprolines;[11] and the irreversible *N*-peptidyl-*O*-aroylhydroxylamines.[12] Although specific for DP-IV, these compounds exhibit, at best, only modest levels of inhibition.

We felt that it was necessary to develop more potent, stable inhibitors of DP-IV. These would help elucidate the physiological role of the enzyme and may have therapeutic potential in a number of disease states such as inflammation, graft versus host disease (GVHD), cancer, or AIDS.[5]

Table I. *Inhibition of human DP-IV by aminoacyl pyrrolidides.*[13]



Compound N°	Xaa	K_i (μM)[13]
5	Cyclohexylglycine [Chg]	0.064 ± 0.01
6	(R,S)-Cyclopentylglycine [Cpg]	0.21 ± 0.04
7	Ile	0.41 ± 0.01
8	*allo*-Ile	0.44 ± 0.04
9	Val	0.47 ± 0.02
10	Lys(Cbz)	0.52 ± 0.07
11	tert-Butylglycine [Tbg]	0.88 ± 0.20
12	Thr(Me)	0.90 ± 0.15
13	Orn(Cbz)	0.91 ± 0.20
14	2-Aminohexanoic acid [Aha]	1.20 ± 0.20
15	Glu	2.00 ± 0.40
16	Pro	2.10 ± 0.20
17	Cyclohexylalanine [Cha]	2.15 ± 0.50
18	Glu(OBn)	2.70 ± 0.30
19	Thr	4.90 ± 0.90
20	Phenylglycine [Phg]	5.30 ± 0.10
21	Ser(Bn)	6.00 ± 1.50
22	Ala	7.00 ± 1.00
23	Asp	14.50 ± 1.90

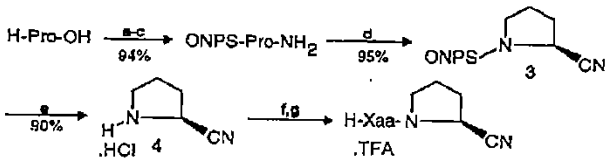
Our attention was drawn by a patent claiming 2-cyanopyrrolidides as inhibitors of prolyl endopeptidase[14] (PEP, EC 3.4.21.26), an enzyme belonging to the same subfamily of serine proteases as DP-IV. PEP differs from DP-IV by being an endopeptidase but the two enzymes share the common specificity for cleaving peptides at the carboxyl side of proline peptidyl bonds.

We now wish to report on the synthesis and biological activity of a series of dipeptide nitriles[15] with potencies versus human DP-IV comparable to the boroprolines 1 and 2 but with superior stability in aqueous solution. One other group has recently described similar compounds as inhibitors of DP-IV[16] but whereas they confirm that such derivatives possess good stability, their series exhibit only modest potency (four compounds with K_i values versus rat DP-IV of 0.19-1.2 μM).

To establish an optimal *N*-terminal residue, we prepared a series of amino acid pyrrolidides.[6] These compounds were prepared by reaction of the *O*-succinimide, (ONSu), ester of the required Boc protected amino acid with a slight excess of pyrrolidine in dichloromethane. Subsequent acid catalysed deprotection (4N HCl/dioxane) afforded the inhibitor as its hydrochloride salt. As expected, from the substrate specificity of DP-IV, only (S)-amino acid derivatives showed any activity and, as can be seen in **Table I**, lipophilic amino acids gave more potent compounds. In particular, β-branched α-amino acid derivatives were the most potent compounds with the non-proteinogenic amino acid, (S)-cyclohexylglycine providing the most active pyrrolidide (compound **5** possessing a K_i value of 64 nM).

We then applied these findings to a series of 2-cyanopyrrolidides. The preparation of these compounds required a large scale synthesis of 2-cyanopyrrolidine **4** (Scheme I). *N*-Boc-2-cyanopyrrolidine was readily prepared from Boc-Pro-NH_2 using a dehydrating mixture of phosphorous oxychloride, pyridine and imidazole but the usual acidic conditions required to remove the Boc protecting group led to decomposition of the 2-cyanopyrrolidide. Employment of the *o*-nitrophenylsulfenyl (ONPS) protecting group[17] however, enabled a very mild deprotection to be used in the final step. Adding three equivalents of 4N HCl/dioxane to **3** in a large volume of diethyl ether afforded the hydrochloride salt **4** as an off-white precipitate in excellent yield.

Scheme I. *Preparation of dipeptide nitriles.*

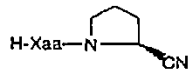


Reagents: a.ONPS-Cl, 2N NaOH. b. HONSu, Water soluble carbodiimide. c conc. NH_4OH, dioxane.
d. imidazole (2 equiv.), $POCl_3$ (4 equiv.), pyridine. e. 4N HCl/dioxane (3 equiv.), diethyl ether.
f. Boc-Xaa-OH, pyBop, NEt_3, CH_2Cl_2. g. Trifluoroacetic acid.

The series of dipeptide nitriles described in **Table II** were prepared via a pyBop[18] mediated coupling of **4** with the required Boc protected amino acid, followed by deprotection with TFA (**Scheme I**).

We were gratified to find that these compounds were potent inhibitors of DP-IV. The S.A.R. for the N-terminal residue developed in the pyrrolidide series correlated well for the dipeptide nitrile series and the most potent compounds **24**, **25**, **26** and **27** possessed activity comparable to the boroprolines, **1** and **2**. Stability studies[19] revealed excellent half-lives ($t_{1/2}$) in aqueous solution (pH 7.4) at room temperature (**Table II**) with several examples having $t_{1/2}$ greater than 48h. Further work on optimisation of the pyrrolidine ring will be reported shortly.

Table II. *Dipeptide nitriles: Potency versus human DP-IV and stability in aqueous solution (pH 7.4).*



Compound N°	Xaa	K_i(nM)[15]	$t_{½}$(h)[19]
24	Cpg	1.1 ± 0.2	48
25	Chg	1.4 ± 0.5	>48
26	Ile	2.2 ± 0.5	48
27	Tbg	3.8 ± 0.8	>48
28	Lys(Z)	5.2 ± 1.0	24
29	Pro	22.0 ± 4.0	7.5

These compounds were found to be non-toxic in T cell assays up to 72h and inhibitor **26** had no acute toxicity when injected into mice (up to 10mg/Kg). We are currently exploring the effects of these compounds on lymphocytes (*e.g.* proliferation and cytokine release) and further details will be reported in due course.

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13) All compounds were tested in vitro against pure human DP-IV (purchased from M&E, Copenhagen, Denmark). Inhibition was determined using the fluorogenic substrate, H-Ala-Pro-AFC at three concentrations per inhibitor. A typical assay (total volume 0.4 mL) comprised sodium HEPES 83.3 mM, EDTA 1.67 mM, BSA 1.5 mg mL^{-1}, pH 7.8, DP-IV 25 μU mL^{-1}, inhibitor (in 10 mM acetate pH 4.0). The reaction was started by the addition of substrate and readings taken every 30 sec for 7.5 min, excitation at 395 nm, emission 450nm. K_i values were determined using Dixon plots.
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19) The stability of the inhibitors in buffered, aqueous solution (100 mM Tris, pH 7.4) was monitored by reverse-phase HPLC.

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4-CYANOTHIAZOLIDIDES AS VERY POTENT, STABLE INHIBITORS OF DIPEPTIDYL PEPTIDASE IV

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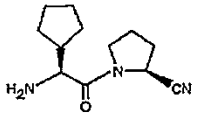
Abstract: A series of stable, very potent inhibitors of dipeptidyl peptidase IV has been developed. A number of dipeptide analogues, incorporating a 4-cyanothiazolidide, were found to have K_i values of less than 1 nM versus human DP-IV and half-lives of between 5 and 27h in aqueous solution (pH 7.4).

The serine protease dipeptidyl peptidase IV (DP-IV, EC 3.4.14.5)[1,2] which is identical to the T cell activation marker CD26 has been the subject of intense scrutiny because it was recently shown that inhibitors or antibodies of this enzyme can inhibit T cell proliferation.[3,4] However, the physiological role of DP-IV in the immune system and the molecular events mediated by this enzyme are only partly established[5] and we felt that it was necessary to develop potent, stable inhibitors of DP-IV to help elucidate the biological role of the enzyme and to investigate their therapeutic use in a number of disease states such as inflammation, graft versus host disease (GVHD), cancer or AIDS.



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We recently reported a series of aminoacyl-2-cyanopyrrolidides[6,7] which possess K_i values of less than 5 nM versus human DP-IV[8] and half-lives ($t_{1/2}$) of greater than 48h in aqueous solution (pH 7.4).[9] This series of inhibitors is exemplified by 1 which has a K_i value of 1.1 nM versus human DP-IV and a half-life of 48h in aqueous buffer (pH 7.4).

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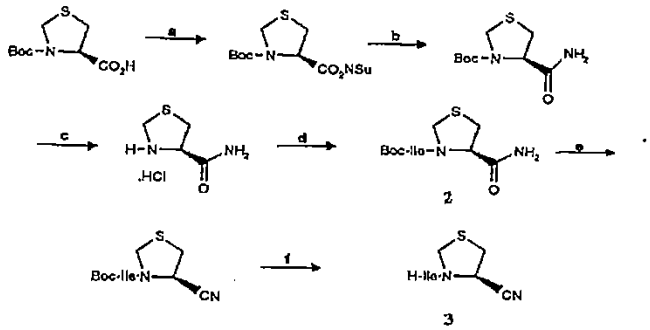
In a quest to improve the potency of this class of inhibitors, we investigated replacing the pyrrolidide ring with other nitrogen heterocycles. We chose isoleucine (Ile) as a standard *N*-terminal residue as it was the most potent natural amino acid in the 2-cyanopyrrolidide series.[7] The preparation of 3 (Scheme I) illustrates the general route to the series of cyano compounds described in **Table I.**

Scheme I. *Preparation of 3-isoleucyl-4-cyanothiazolidide.*

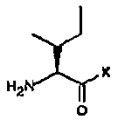


Reagents and Yields: a. *N*-hydroxysuccinimide (HONSu), water soluble carbodiimide, CH_2Cl_2. 99%. b. conc. NH_4OH, dioxane. 96%. c. 4N HCl/dioxane. 99%. d. Boc-Ile-OH, PyBop, CH_2Cl_2, NEt_3. 38%. e. $POCl_3$, imidazole, pyridine. 53%. f. Trifluoroacetic acid. 75%.

A pyBop[10] mediated coupling of 4-amidothiazolidide with Boc protected isoleucine afforded the dipeptide mimic 2 in modest yield. Dehydration of the primary amide function to a nitrile and subsequent acid catalysed deprotection yielded the trifluoroacetate salt of 3.[11] From a range of compounds with various heteroatoms in 5- or 6-membered rings, we were pleased to find that the 4-cyanothiazolidide analogue 3 was approximately 5-fold more active than the 2-cyanopyrrolidide inhibitor 5[7] (Table I). However, this increase in activity was accompanied by a slight decrease in stability.

Having established 4-cyanothiazolidide as an optimum *C*-terminal residue, we prepared further analogues with the best *N*-terminal α-amino acids from the pyrrolidide series.[7] These compounds were prepared as described in Scheme I but Boc-Ile-OH, in step d, was replaced with the required Boc-Xaa-OH. A number of analogues were prepared with sub-nanomolar activity against DP-IV and good stability in aqueous buffer (pH 7.4). (Table II)

Table I. *Isoleucyl heterocyclic nitriles: Potency versus human DP-IV and stability in aqueous solution.*



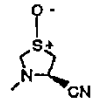
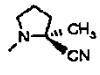
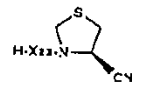
Compound No.	X	K_i (nM)	$t_{1/2}$ (h)
3		0.41 ± 0.15	27
4[12]		1.70 ± 0.50	3
5		2.2 ± 0.50	48
6		21.0 ± 5.0	4
7[13]		34.0 ± 7.0	1.25
8		260 ± 50	>48
9[13]		440 ± 200	1.5
10		450 ± 100	>48
11		4,200 ± 900	48
12		6,000 ± 1,500	>48

Table II. *3-Aminoacyl-4-cyanothiazolidides: Potency versus human DP-IV and stability in aqueous solution (pH 7.4).*



Compound No.	Xaa	K_i (nM)[a]	$t_{1/2}$ (h)[b]
3	Ile	0.41 ± 0.15	27
13	Cyclopentylglycine	0.50 ± 0.10	5
14	Cyclohexylglycine	0.80 ± 0.20	16
15	Lys(Cbz)	5.00 ± 1.00	>48

These new, stable, low molecular weight inhibitors should offer the opportunity to study the physiological role of DP-IV and possibly have therapeutic benefits. We are currently exploring the effects of these compounds on lymphocytes (e.g. proliferation and cytokine release) and further details will be reported in due course.

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9) The stability of the inhibitors in buffered, aqueous solution (100 mM Tris, pH 7.4) was monitored by reverse-phase HPLC. The inhibitors with half-lives of less than 48h decompose to multiple products which have not been characterised.
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11) All compounds were >95% pure by HPLC and were characterised by a combination of ^1H NMR, ^{13}C NMR and FAB mass spectrometry.
12) Compound 4 is the active diastereomer of a pair separated by preparative HPLC and was assigned the (S,S) stereochemistry. The (S,R) diastereomer was inactive versus human DP-IV.
13) Compounds 7 and 9 are separated diastereomers (preparative HPLC) whose absolute stereochemistry was not determined.

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The Unique Properties of Dipeptidyl-peptidase IV (DPP IV / CD26) and the Therapeutic Potential of DPP IV Inhibitors

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Abstract: This review deals with the properties and functions of dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5). This membrane anchored ecto-protease has been identified as the leukocyte antigen CD26. The following aspects of DPP IV/CD26 will be discussed : the structure of DPP IV and the new family of serine proteases to which it belongs, the substrate specificity, the distribution in the human body, specific DPP IV inhibitors and the role of CD26 in the intestinal and renal handling of proline containing peptides, in cell adhesion, in peptide metabolism, in the immune system and in HIV infection. Especially the latest developments in the search for new inhibitors will be reported as well as the discovery of new natural substrates for DPP IV such as the glucagon-like peptides and the chemokines. Finally the therapeutical perspectives for DPP IV inhibitors will be discussed.

Introduction

Dipeptidyl peptidase IV (DPP IV/CD26) attracts a lot of interest in current research. The biochemical properties (ref. 1 and 113) and the functions of the molecule in the immunological system and in metabolism (ref. 111, 112 and 113) are extensively reviewed. Here we deal with the reported DPP IV inhibitors and their properties, the recent discoveries of new substrates for DPP IV, such as the glucagon-like peptides and the chemokines, and the latest developments concerning the role of CD26 in immunology and HIV infection.

Proline and Proline-specific Peptidases

Due to the unique structure of proline among the amino acids, the peptide bond before or after a proline residue is relatively resistant to breakdown by common proteases. Therefore, it is not surprising that specific enzymes participate in the cleavage of such bonds. These proline-specific proteases play an important role in the regulation of the life-time of biologically active peptides [1]. Remarkably, most of these proteases are exopeptidases. Besides the dipeptidases prolidase (EC 3.4.13.9) and prolinase (EC 3.4.13.8) only a few proline-specific enzymes have been demonstrated. These include the *N*-terminal exopeptidases proline iminopeptidase (EC 3.4.11.5), aminopeptidase P (EC 3.4.11.9), dipeptidyl peptidase II (EC 3.4.14.2, DPP II) and IV (EC 3.4.14.5, DPP IV), and the *C*-terminal exopeptidases carboxypeptidase P (EC 3.4.17.16) and prolyl carboxypeptidase (EC 3.4.16.2). The only known endopeptidase capable of hydrolysing a Pro-X bond is prolyl oligopeptidase (EC 3.4.21.26, PO) [2]. The specific cleavage points of these proteases are illustrated schematically in Fig. (**1**).

Structure and Classification of DPP IV

Dipeptidyl-peptidase IV (DPP IV) is a highly specific aminopeptidase cleaving off dipeptides from the amino terminus of peptides with preferentially proline at the penultimate position. It is a type II transmembrane protein having a cytoplasmic tail of only 6 highly conserved amino acids [3]. The hydrophobic transmembrane region is located at the N-terminus (7-29). A flexible stalk links the membrane anchor with a large glycosylated region (48-324), a cysteine region (325-552) and the C-terminal domain containing the catalytic triad (553-766) [4]. The purified enzyme is found to be dimeric, comprising two identical subunits of 110-130 kDa, each.

A truncated form of DPP IV/CD26 (cleaved in the flexible stalk region) exists in serum and other biological fluids. The presence in human serum of yet another form of DPP IV has been reported. This enzyme is not a

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PDX-37

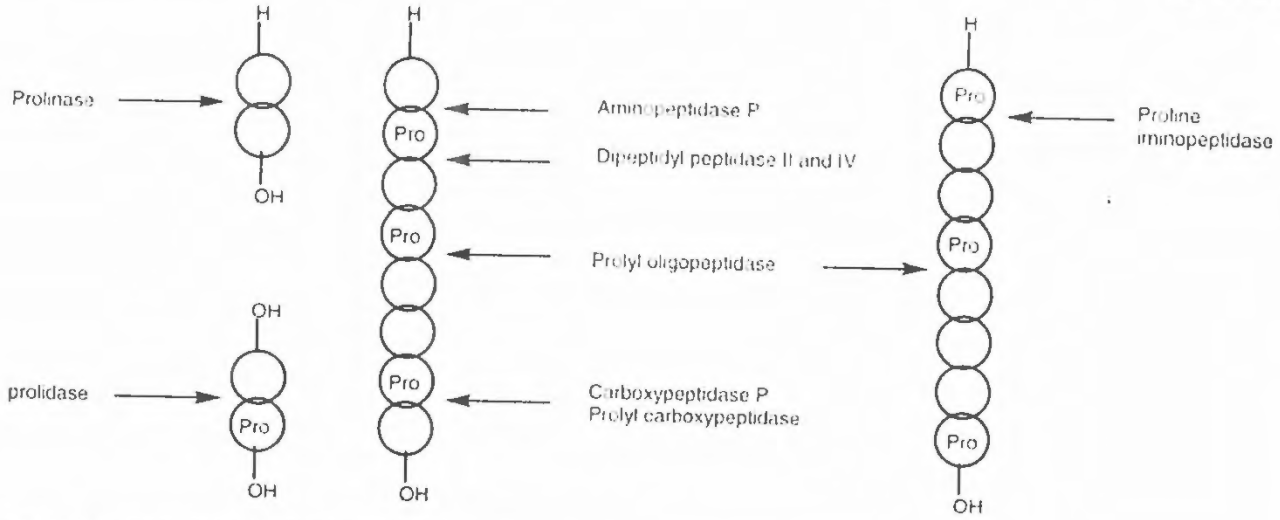


Fig. (1). Proline-specific peptidases and their cleavage points.

breakdown product of bound DPP IV, but shows a significant structural similarity [5]. Another novel cell-surface-expressed protein with DPP IV activity is also described, and is referred to as DPP IV-β [6]. Recently, a membrane-bound enzyme with gelatinase activity, seprase, was found with high homology to DPP IV but with differences in substrate specificity [7]. The expression of this protease correlates with the invasive phenotype of human melanoma and carcinoma cells. Another recently discovered homologous protein is the fibroblast-activation protein (FAP), selectively expressed by reactive stromal fibroblasts on epithelial cancers and healing wounds [8].

Identification of the active site residues in rat DPP IV by affinity labelling and site-directed mutagenesis allowed to conclude that the active site serine is found in the sequence Gly-Trp-Ser-Tyr-Gly, which corresponds to the active site motif Gly-X-Ser-X-Gly proposed for serine proteases [9]. However, DPP IV does not exhibit sequence similarity with any of the members of the classical serine protease families, chymotrypsin and subtilisin. There is circumstantial evidence that DPP IV is a member of a new family of serine proteases, the prolyl oligopeptidase family [10]. Also prolyl oligopeptidase (PO) and acylaminoacyl-peptidase belong to this family, sharing a conserved C-terminal stretch of approximately 200 amino acids containing the catalytic triad. The order of the catalytic triad residues is different in the three families : His-Asp-Ser in chymotrypsin, Asp-His-Ser in subtilisin and Ser-Asp-His in mouse DPP IV [11]. A similar catalytic triad was found in the α/β hydrolases which regroup very diverse enzymes such as lipases, cholinesterases, dienelactone hydrolases, dehalogenases and serine carboxypeptidases [10,12]. Moreover, the predicted secondary structural organisation of DPP IV and the X-ray structure of porcine muscle PO [13], exhibiting a C-terminal enzymatic domain made up of alternative α-helix and β-sheet segments, is strikingly similar to that provided by the three-dimensional structural analysis of α/β hydrolases [14-17]. Likewise, the three-dimensional structure of proline iminopeptidase shows two domains, with the larger domain containing the catalytic triad and showing the general topology of the α/β hydrolase fold [17].

Substrate Specificity

The catalytic mechanism of a serine protease is depicted in Fig. (2). The three amino acids involved in the catalytic reaction are serine, histidine and aspartic acid. They form the catalytic triad in which the nucleophilic properties of the serine alcohol increase drastically. Substrate binding induces a conformational change in the enzyme that leads to a proton shift of serine to histidine. The formed histidinium ion is stabilised by the aspartate moiety. The activated serine attacks the carbonyl group of the scissile amide bond of the substrate to give a first tetrahedral intermediate. The negatively charged tetrahedral adduct is stabilised by a hydrogen bonding system in the oxyanion hole. Decomposition of this intermediate gives an acylated enzyme, which is subsequently hydrolysed by water via a second tetrahedral intermediate to the carboxylic acid product and the active enzyme.

The substrate specificity of DPP IV has been well characterised. Besides proline at the penultimate position (P_1 position) it can accommodate, although less efficiently, alanine, dehydroproline and hydroxyproline [18]. In an extended investigation of the substrate specificity using Ala-X-4-nitroanilides, it was concluded that the enzyme can hydrolyse some substrates in which the ring structure of proline in

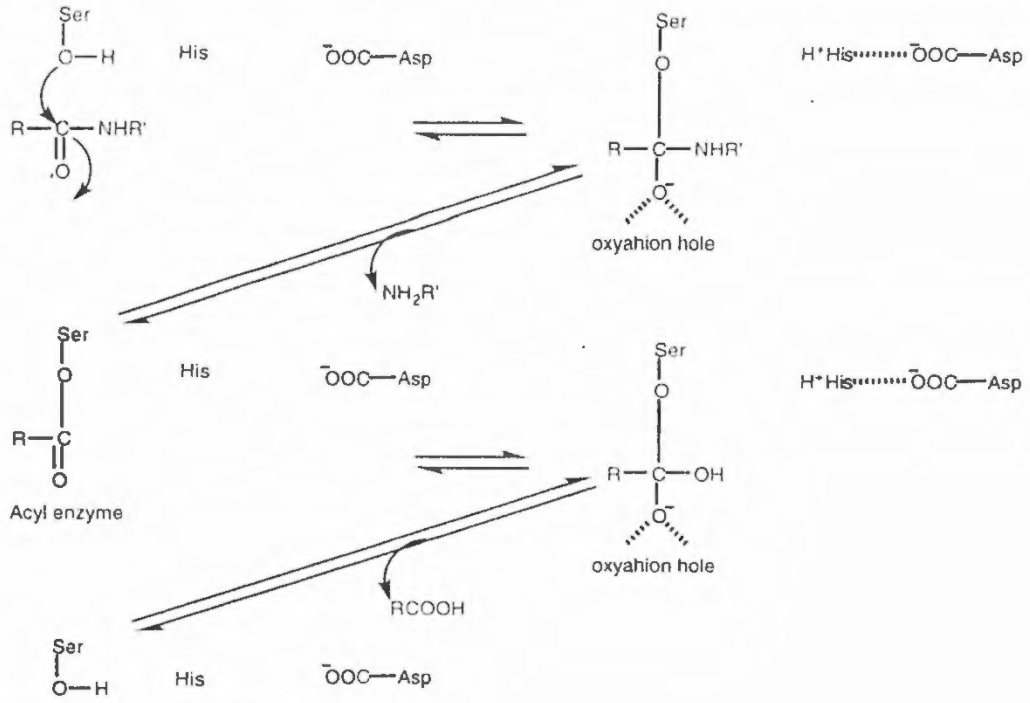


Fig. (2). Catalytic mechanism of serine proteases.

position P_1 has been modified into a cyclic imino acid as azetidine-2-carboxylic acid and piperidine-2-carboxylic acid or an oxa- or thia analogue of proline. The replacement of the proline residue by (*S*)-azetidine-2-carboxylic acid gives the best substrate according to its k_{cat}/K_m value. This substrate has a low affinity that is counteracted by a high catalytic turnover. On the contrary, the substrate with piperidine-2-carboxylic acid has higher affinity, but the lowest k_{cat} value, resulting in poor substrate properties. Substituting the methylene group by sulphur or oxygen at position 4 of the pyrrolidine ring slightly affects the k_{cat}/K_m value. The affinity, however, is slightly decreased for the oxa analogue and increased for the thia analogue. [19]. The rate-limiting step for the hydrolysis of substrates with Pro in P_1 is the deacylation, and for Ala in P_1 is the acylation reaction [18].

Studies with short synthetic peptides show that the P'_1 position accepts all amino acids, except secondary amines such as *N*-methylated amino acids, proline and hydroxyproline. Any L-amino acid can be placed at the P_2 position, provided that the *N*-terminal amino function is free and protonated. In general, the nature of the side chain of the P_2 amino acid has only limited influence on binding. With proline as the P_1 amino acid, the P_2 amino acid needs an L-configuration, with alanine at P_1 it also accepts D-amino acids at the *N*-terminal position [18]. The conformation around the peptide bond between P_2 and P_1 has to be trans for catalytic activity [20]. DPP IV can utilise substrates with a thioamide peptide bond between P_2 and P_1, but with a 100-1000 fold reduced k_{cat}/K_m [21].

Based on this information the probable conformation of substrates when bound in the active site of DPP IV has been predicted [22] and a model of the active site was proposed using comparative molecular field analysis and molecular modelling [23]. A new mechanism in serine protease catalysis exhibited by DPP IV was also presented, involving the stabilisation of the tetrahedral intermediate by the formation of an oxazolidine ring with the P_2-P_1 amide bond and compensation of the negative charge by a proton transfer from the positively charged aminoterminus [24].

Distribution

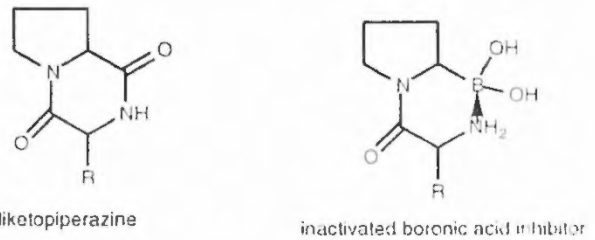
DPP IV is expressed ubiquitously in mammalian tissues and organs. It is found in the kidney cortex, kidney microvilli, liver, microvilli of the small intestine, pancreas, submaxillary gland and placenta. It is expressed in high density in membrane regions organised as microvilli such as the brush-border regions of the small intestine and kidney. Comparison of DPP IV activity in human intestine revealed that the activity was highest in ileum and jejunum, low in duodenum but not detectable in colon [25]. The organ with the highest specific activity is the kidney, where it is located primarily in the cortex and is abundant in brush-border membranes [26].

The enzyme has been reported in body fluids such as human serum, saliva, urine and synovial fluid. Compared to other human body fluids, only low DPP IV activity has been found in cerebrospinal fluid. Prostasomes, which are prostate-derived multi-layered membrane vesicles occurring freely in seminal plasma, contain a very high DPP IV specific activity [26].

In the hematopoietic system, DPP IV was identified as the leukocyte antigen CD26, where its expression depends strongly on the differentiation and activation status. First described as a T-cell activation antigen, CD26 was found on subsets of both activated CD4+ and CD8+ T cells and CD26 expression was associated with the capacity of the T cell to produce interleukin-2 [27]. Detailed analysis of subsets of CD4+ lymphocytes indicates that CD26 appears to be more restricted than most accessory molecules since it is expressed only on the CD4 memory/helper (CD45RO+CD29+) population. This unique population of human CD4+ cells is the only one that can respond to recall antigens, induce B-cell immunoglobulin (IgG) synthesis and activate MHC-restricted cytotoxic T cells [28,29]. More recently, low-density levels of CD26 have been detected on other hematopoietic cells, especially natural killer cells [30], B lymphocytes [31] and myeloid cells [32].

DPP IV Inhibitors

DPP IV is inhibited by the typical serine protease marker diisopropyl fluorophosphate [9]. A wide variety of inhibitors for serine proteases have been reported [33], and most of them are peptide analogues that have an electrophilic group at the P_1 amino acid that replaces the normally cleaved amide bond. In the case of DPP IV, a cyclization reaction can occur between the free amino group of the P_2 amino acid and the electrophile attached to the proline mimic in P_1, causing serious stability problems (Fig. (3)). This is not surprising since it is well known that peptides with proline at the penultimate position form relatively easy diketopiperazines, arising from the nucleophilic attack of the *N*-terminal nitrogen on the amide carbonyl



diketopiperazine inactivated boronic acid inhibitor

Fig. (3). Instability of some DPP IV inhibitors due to intramolecular attack of the N-terminal nitrogen.

between the second and the third amino acid [34]. Therefore only a few classes of the classical serine protease inhibitors are effective for DPP IV inhibition. Typical examples of DPP IV inhibitors are shown in Fig. (4).

Competitive Reversible Inhibitors

These are usually substrate or product analogues. **Dipeptides** (1) resulting from the hydrolytic actions of the enzyme are the only known endogenous DPP IV inhibitors. Inhibition constants of these competitive product inhibitors are in the range of K_i = 10-2000 μM [1,23]. **Oligopeptides** with N-terminal X-Pro sequences are also sometimes inhibitors. Diprotin A (Ile-Pro-Ile, 2) is a competitive inhibitor with a K_i = 2.2 μM [35]. This result is rather surprising since the compound has the overall substrate-like structure needed for DPP IV hydrolysis. A more recent report showed that those tripeptides are indeed substrates with a low turnover rate (k_{cat}) [36].

Product-like compounds lacking the carbonyl function of the proline residue, such as **Aminoacyl pyrrolidines and thiazolidines** were reported as potent and stable DPP IV inhibitors [23,37,38]. The most potent compound of this series was *N*-isoleucylthiazolidine (3) with an IC_{50} value of 2.8 μM. Also *N*-isoleucylpyrrolidine (4) and the analogous *N*-(ε-(4-nitrobenzyloxycarbonyl)lysyl) compounds 5 and 6 are of similar potency. In a recent structure-activity relationship investigation of these compounds it was shown that changing the 5-membered ring of pyrrolidine or thiazolidine to their 4-, 6-, or 7-membered analogues or to their unsaturated analogues decreases potency [39]. The same is true for the acyclic analogues. Introduction of a substituent at the 3-position of the pyrrolidine ring generally decreased the inhibitory activity. Only a small substituent such as fluorine is allowed (7). To establish an optimal *N*-terminal residue, a series of aminoacyl pyrrolidines were prepared, showing that lipophilic amino acids gave more potent compounds [40]. In particular, β-branched α-amino acid derivatives were the most potent compounds with the non-proteinogenic amino acid, cyclohexylglycine providing the most active pyrrolidide (8).

These findings were applied to a series **pyrrolidine-2-nitriles** [40,41]. The structure-activity relationship for the *N*-terminal residue developed in the pyrrolidide series correlated well for the dipeptide nitriles, with the most potent compound being the cyclopentylglycine derivative **9**. This compound has a K_i = 1.1 nM and an excellent stability at pH = 7.4 ($t_{1/2}$ = 48 h). In analogy with the pyrrolidines and thiazolidines, replacement of the pyrrolidine-2-nitrile to the

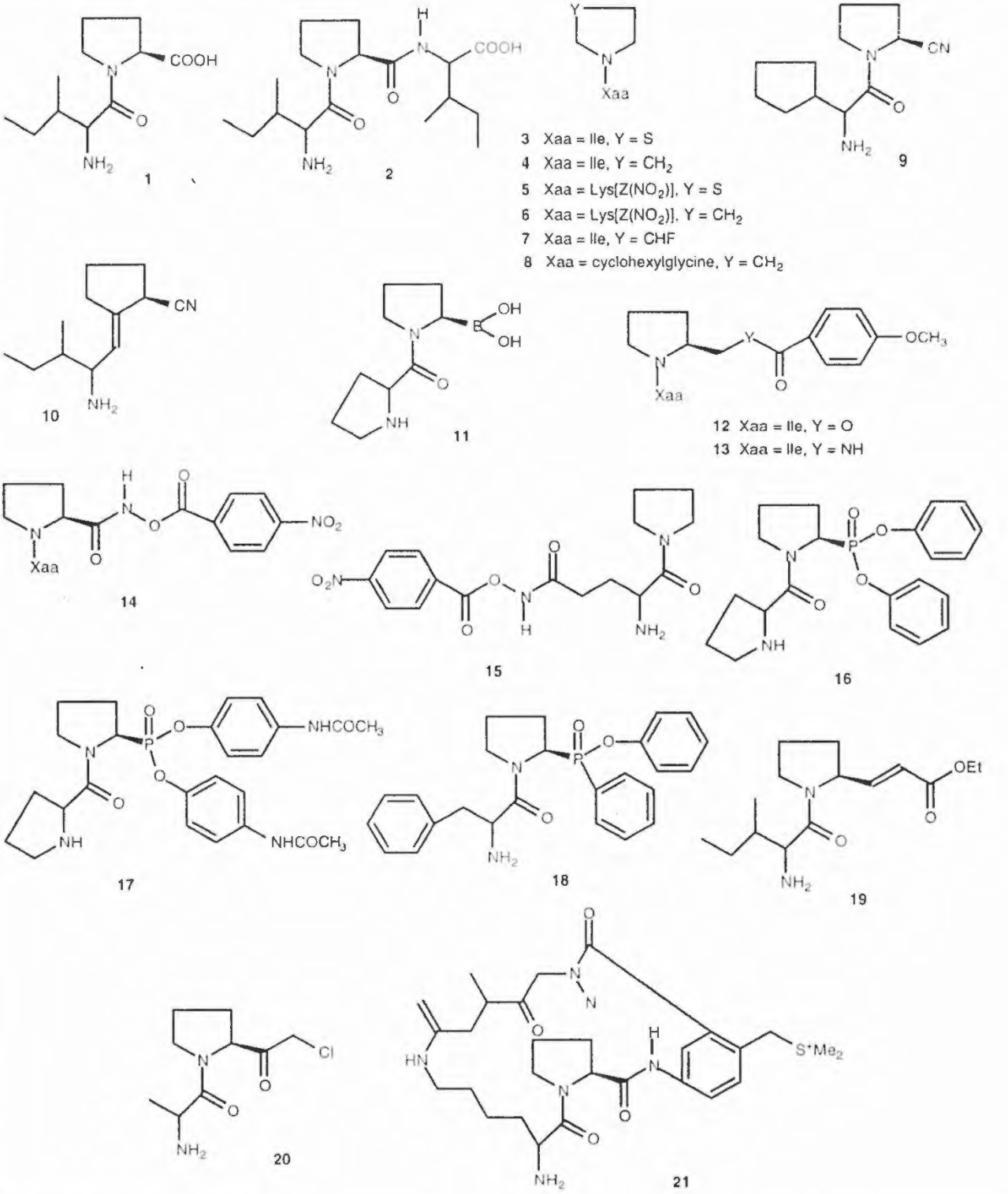


Fig. (4). Selected DPP IV inhibitors.

thiazolidine-4-nitrile enhanced potency [42]. Replacement of the amide bond with an isosteric alkene in the isoleucyl analogue (**10**) decreased potency a 1000 fold (K_i = 1.7 μM) [43].

The **HIV-1 Tat (1-86)** protein has been reported as a DPP IV inhibitor, suggesting that the immunosuppressive effects of Tat on non-HIV-1-infected T cells could be mediated by DPP IV [44,45]. It

was shown that the ***N*-terminal Xaa-Xaa-Pro sequence** of this protein was important and that other proteins with this sequence and at least 6 amino acids in length are competitive inhibitors [46,47].

Transition-state Analogues

Xaa-boroPro dipeptides (11) were reported among the most potent inhibitors with K_i values in the nanomolar range. These compounds are reversible transition state analogues with slow, tight-binding kinetics [48]. The empty P-orbital at boron is thought to interact with the catalytic serine to form a stable "ate" complex which mimics the transition state of amide hydrolysis. Separation of L-Pro-DL-boroPro into its diastereoisomers showed that the L-L isomer has a K_i value of 16 pM [49]. Unfortunately, they have a short half-life at neutral pH, caused by cyclisation of the terminal aminofunction with the boronic acid, forming a cyclic, inactive species containing a B-N bond (Fig. (**3**)) [50-52]. In a structure-activity relationship of boronic acid inhibitors it was shown that a wide variety of L-amino acids are accepted at the P_2 position, but D-amino acids, α,α-disubstituted amino acids and glycine are not tolerated [53]. The specificity of these compounds for DPP IV inhibition was investigated, and it was shown that most compounds that were active on DPP IV were also potent inhibitors of DPP II. On the contrary the IC_{50} values for PO inhibition were 30 to 1000 fold higher.

Acyl Enzyme Inhibitors

Acyl enzyme inhibitors cause the formation of an acyl enzyme which deacylates more slowly than the parent enzyme-product complexes. **Azapeptides** are peptides in which an α-carbon is substituted for a N-atom. 4-Nitrophenylesters of such peptides are inhibitors of several serine proteases due to acylation of the active site serine forming a stable acylated enzyme [54]. Glycyl- and alanyl-azaprolylphenylesters were found to acylate DPP IV, but the deacylation rates were high and these compounds could not be considered good inhibitors [55]. Some members of a series of semicarbazide, carbazate, acylhydrazine and sulphonylhydrazine derivatives of Xaa-azaPro were also poor inhibitors, showing inhibitory activity in the millimolar range [56].

Synthetic peptides containing a *C*-terminal β-amino alcohol linked to *p*-methoxybenzoic acid via an ester linkage have been found to be potent irreversible inhibitors of chymotrypsin and elastase. These inactivated proteases regain activity slowly, presumably due to slow hydrolysis of a long-lived *p*-methoxybenzoyl enzyme intermediate [57]. However, compounds designed following this principle are not very active as DPP IV inhibitors (**12** : IC_{50} = 2.1 mM, **13**: IC_{50} = 1.7 mM, unpublished results).

Irreversible Inhibitors

***N*-peptidyl - *O* - (4-nitrobenzoyl) hydroxylamines** are enzyme-activated inhibitors for serine proteases [58]. After the attack of the active site serine, the inhibitor forms a latent chemically reactive intermediate, which forms a stable covalent bond with a functional group at or near the active site, thus leading to an irreversibly modified enzyme. Xaa-Pro-NHO-(4-NO_2)-benzoyl (**14**) is such an irreversible inhibitor of DPP IV [59,60]. Similar compounds with this reactive functionality attached to the carboxylic acid of the side chain of the P_2 amino acid of aminoacyl pyrrolidines (**15**) are not irreversible, but competitive reversible inhibitors with K_i values in the micromolar range [61].

Dipeptide-derived diphenyl phosphonate esters are potent, irreversible inhibitors of DPP IV [62], probably because they lead to a phosporylated serine at the active site (Fig. (**5**)). With these inhibitors only a fraction (10 %) of the enzyme activity was regained after 4 weeks *in vitro*, indicating the strong irreversible inhibition [63]. In a study on the role of the P_2 amino acid in dipeptide diphenyl phosphonates [63,64], it was shown that proline in this position gives

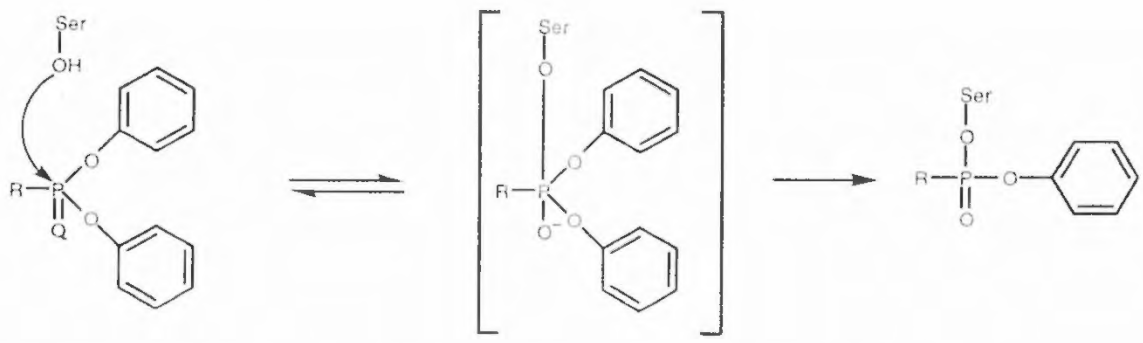


Fig. (5) Proposed mechanism for the irreversible inhibition of DPP IV with diphenyl phosphonates.

one of the most potent inhibitors (**16**). A major advantage of **16** was its improved stability in human citrated plasma ($t_{1/2}$ = 5 h) compared to the other dipeptide derivatives. The stability of **16** in plasma equals its stability in buffer, whereas the other compounds display reduced stability in plasma compared to buffer. This reflects the relative stability of a Pro-Pro amino acid sequence to proteolytic breakdown, and indicates that the decrease in activity of **16** is mainly caused by hydrolysis of the phosphonate ester. Intravenous injection of a single dose of **16** (1, 5 or 10 mg) in rabbits reduces the plasma DPP IV activity with more than 80 %, and it takes more than 20 days for complete recovery. Not only plasma DPP IV was inhibited, but also DPP IV in circulating lymphocytes and peripheral tissues [65]. The role of the P_1 amino acid was also investigated showing that a 6-membered analogue (homoproline) increased activity [62], whereas alanine decreased potency [63]. With **16** as lead compound, we recently synthesised a series of diaryl 1-(*S*)-prolylpyrrolidine-2-(*R,S*)-phosphonates with different substituents on the aryl rings (hydroxyl, methoxy, acylamino, sulfonylamino, ureyl, methoxycarbonyl and alkylaminocarbonyl) (unpublished results). A good correlation was found between the electronic properties of the substituent and the inhibitory activity and stability. This means that electron-withdrawing substituents increase potency, but also decrease stability. The most striking divergence of this correlation was the high potency combined with a high stability of the 4-acetylamino substituted derivative (**17**, IC_{50} = 0.4 μM, $t_{1/2}$ = 320 min). This compound is specific for DPP IV, shows low cytotoxicity in human peripheral blood mononuclear cell and has favourable properties *in vivo*. Therefore, we consider di(4-acetamidophenyl) 1-(*S*)-prolylpyrrolidine-2-(*R,S*)-phosphonate (**17**) as a major improvement and a highly valuable DPP IV inhibitor for further studies on the biological function of the enzyme and the therapeutic value of its inhibition. **Phosphinates** similar to these phosphonates were also prepared, but were poor inhibitors of DPP IV (**18** : IC_{50} = 2.4 mM, unpublished results).

Another approach to inactivate the enzyme is the use of **Michael addition substrates**. These compounds could potentially react with the active site serine via a Michael addition reaction. On the other hand, the double bond can serve as an amide bond mimetic. A series was prepared, but most compounds behaved as competitive inhibitors in the millimolar range. The most potent compound was the isoleucyl derivative (**19**, IC_{50} = 90 μM), that showed a time-dependent inhibition and that was only partially competitive. Although a rapid equilibrium is established between enzyme and inhibitor, some slow steps could be observed that were probably occuring during the dissociation step (unpublished results).

Chloromethyl ketones are known as affinity labelling inhibitors of serine proteases. They are substrate analogues containing a chemically reactive substance, which diffuses into the active site. There, it reacts with one of the essential catalytic groups of the enzyme and irreversibly blocks enzyme activity. **Dipeptide chloromethyl ketones** (**20**) for inhibition of DPP IV are rapidly inactivated in solution by an intramolecular cyclisation, with half-times of around 10 minutes [66]. Likewise, **dipeptidyl ammonium and pyridinium methyl ketones** are inhibitors in the micromolar range with slow-binding mechanism and half-times around 10 minutes [67,68].

Recently, a new type of irreversible **cyclopeptide inhibitors** was reported, containing a latent quinoniminium methide electrophile (**21**) responsible for inactivation [69]. This compound has a K_i = 85 nM, and is specific for DPP IV, giving a rapid irreversible inhibition that lasts for several hours. Remarkably, DPP IV-β is much less affected, confirming that it is a different protein. **16** is an enzyme-activated inhibitor, since attack of serine at the carbonyl of the cyclopeptide would unmask a P'_1 aniline having a good benzylic leaving group. Following a fast elimination of dimethylsulfide this should give a quinoniminium methide ion, tethered in the active site during the lifetime of the acyl-enzyme by means of the peptide chain. A Michael-type addition of a second nucleophile in the vicinity of the active site on this demasked electrophile would establish a covalent enzyme-inhibitor bond. The linear analogues do not inhibit the enzyme, probably because a fast diffusion of the electrophile out of the active center may occur. In addition, the cyclic structure prevents decomposition by cyclisation due to attack of the free amine on the carbonyl.

Intestinal and Renal Handling of Proline Containing Peptides

The absorption or recycling of proline containing peptides is a vital process. DPP IV's contribution to this process is twofold due to its brush border localisation in the small intestine and the kidney (*vide supra*). It plays an obligatory role in the breakdown of peptides in the intestinal and renal tubular lumen, generating the substrates for the peptide specific transport systems [70]. Using rats that were genetically deficient in DPP IV, the functional role of this enzyme in the hydrolysis and transport of radiolabeled β-casomorphin (1-5) (Tyr-Pro-[^{3}H]Phe-Pro-Gly) in renal brush-border membrane vesicles was studied. The membrane vesicles from DPP IV-positive rats were able to hydrolyse the pentapeptide to di- and tripeptides with the subsequent active transport of these products via the H^+ gradient-dependent transporter, that accepts di-

and tripeptides, but not tetrapeptides or longer. On the contrary, DPP IV-negative rats failed to hydrolyse the pentapeptide and hence lacked the ability to transport the radiolabeled peptide [71]. Furthermore, urine analysis revealed an increased excretion of proline and hydroxyproline in DPP IV-deficient rats. Also, following intravenous administration of Tyr-Pro-Phe-Pro-NH_2, a peptide that is exclusively hydrolysed by DPP IV, urinary excretion of the intact peptide was many-fold greater in these DPP IV-negative rats.

DPP IV in the small intestine has a similar function. *In vivo* jejunal perfusion of a model tetrapeptide, Leu-Pro-Gly-Gly, showed that Leu-Pro and Gly-Gly were the major hydrolytic products found in the lumen [72]. Experiments using the DPP IV-negative rats also confirmed the role of DPP IV in the hydrolysis of prolyl peptides and the assimilation of proline-rich proteins. There was no difference in the growth rate between DPP IV-negative and control rats fed on a reference diet not rich in proline. When the protein source was changed to gliadin, a proline-rich protein, the control rats maintained their body weight, whereas the DPP IV-deficient rats experienced a significant weight loss [73].

Role in Cell Adhesion

The ability of DPP IV to interact with proteins of the extracellular matrix as a cell adhesion molecule has been emphasised by the basic observation that the DPP IV substrate Gly-Pro-Ala interferes *in vitro* with the initial spreading of rat hepatocytes on a matrix consisting of fibronectin and collagen [74]. Moreover, an antibody against DPP IV delayed fibronectin-mediated adhesion of rat DPP IV^+-hepatocytes on denatured collagen [75]. It was reported that *in vitro* fibronectin binding of DPP IV was independent of its ectopeptidase activity [76]. Likewise, DPP IV on murine fibroblasts acted as a collagen receptor [77]. On human T cells, DPP IV has been demonstrated to be a functional collagen receptor leading to cell activation [78]. These results show conclusively that DPP IV is able to bind collagen, and it has been reported more recently that the binding site is found in the cysteine-rich region of DPP IV [79].

Due to the presence of Gly-Pro sequences in collagen and the higher secretion of proline- and hydroxyproline-containing peptides in the urine of DPP IV-negative rats, it has been suggested that DPP IV plays a role in collagen metabolism. This gelatinase activity would be possible if the enzyme has endopeptidase activity, next to the well established exopeptidase activity. Indeed, it has been reported very recently that rat DPP IV exhibits weak endopeptidase activity, able to cleave denatured fibrillar collagens, but not native collagens [80]. It has been suggested, that the endo- and exopeptidase activity reside in a common active site, and that endopeptidase activity should be seen in context with other gelatinases and collagenases, or alternatively, that this activity might have auxiliary functions in cell-matrix adhesion processes by unmasking binding sites for the integrin receptors. The detection of this gelatinolytic activity, makes it acceptable to propose that DPP IV and the homologous proteins, seprase and FAPα, represent a new subfamily of gelatinolytic integral membrane serine proteases [7,80].

Role in Peptide Metabolism

A lot of cytokines, growth factors and some neuropeptides share an Xaa-Pro motif at their amino terminus [2]. This sequence may not only contribute to the biological activity, but can also serve as a structural protection against non-specific proteolytic degradation. The striking degree of conservation seems to reflect an evolutionary pressure toward this Xaa-Pro motif. Only two proline-specific aminopeptidases, respectively aminopeptidase P and dipeptidyl peptidase IV have a substrate specificity towards this *N*-terminal Xaa-Pro motif (Fig. (**1**)), and may therefore be important in the modulation of the biological activity of some of the cytokines, growth factors and neuropeptides. Another interesting observation was that phosphorylation of serine or threonine in a Ser-Pro or Thr-Pro substrate decreased the k_{cat}/K_m value with more than two orders of magnitude [81]. This could indicate that posttranslational phosphorylation of peptide sequences may modulate their proteolytic stability. We will discuss hereafter the biologically active peptides which are proven to be hydrolysed by DPP IV (Table (**1**)).

Cytokines

It was demonstrated that oligopeptides with sequences analogous to the *N*-terminal part of human interleukin-1β (IL-1β), IL-2, tumor necrosis factor-β (TNF-β) and murine IL-6 were hydrolysed by purified DPP IV [82]. In contrast to these results no degradation was found for the intact recombinant cytokines. Since three-dimensional structures show that the N-terminal part is at the surface of these proteins and hence should be accessible to exopeptidases, additional steric factors must influence their susceptibility to DPP IV.

Neuropeptides

It was shown that purified DPP IV hydrolyses the neuropeptide **substance** P [83]. The undecapeptide

Table 1. Biologically Active Peptides with Proven DPP IV Susceptibility

Name and N-Terminal Sequence	Length	References
Substance P Arg-Pro*Lys-Pro*Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	11	[83-5]
β-Casomorphin (1-5) Tyr-Pro*Phe-Pro*Gly	5	[71,73,86-88]
Morphiceptin Tyr-Pro*Phe-Pro-NH_2	4	[71,73]
Human Neuropetide Y Tyr-Pro*Ser-Lys-Pro-Asp-Asn-Pro-Gly-	36	[89-92]
Human Peptide YY Tyr-Pro*Ile-Lys-Pro-Glu-Ala-Pro-Gly-	36	[89-2]
Enterostatin Val-Pro*Asp-Pro*Arg	5	[93]
Human growth hormone-releasing factor Tyr-Ala*Asp-Ala-Ile-Phe-Thr-Asn-Ser-	44	[94-97]
Human glucose-dependent insulinotropic polypeptide Tyr-Ala*Glu-Gly-Thr-Phe-Ile-Ser-Asp-	42	[100-102]
Human glucagon-like peptide-1 (7-36) His-Ala*Glu-Gly-Thr-Phe-Thr-Ser-Asp-	30	[100-102]
Human glucagon-like peptide-2 His-Ala*Asp-Gly-Ser-Phe-Ser-Asp-Glu-	33	[106]
Human peptide histidine methionine His-Ala*Asp-Gly-Val-Phe-Thr-Ser-Asp-	27	[100]
Human RANTES Ser-Pro*Tyr-Ser-Ser-Asp-Thr-Thr-Pro-	68	[107,108]
Human stromal cell-derived factor-1α Lys-Pro*Val-Ser-Leu-Ser-Tyr-Arg-Cys-	68	[109,110]

The susceptible bonds are indicated with an asterisk.

was converted to the fragment (3-11) and (5-11) by the sequential removal of Arg-Pro and Lys-Pro. In addition to plasma metabolism of substance P by angiotensin converting enzyme (ACE), the majority of hydrolysis was due to DPP IV. In turn, the substance P (5-11) was rapidly hydrolysed by aminopeptidase M to substance P (6-11) [84]. Using DPP IV-negative rats, it was shown that also *in vivo*, DPP IV was responsible for substance P hydrolysis [85]. A combination of an ACE-inhibitor (captopril) and a DPP IV inhibitor (diprotin A, **2**) prevented to a large extent substance P degradation in plasma [84,85].

β-Casomorphin (1-5), a peptide with potent morphine-like biological activity derived from milk proteins, is rapidly hydrolysed by DPP IV to Tyr-Pro, Phe-Pro and Gly. This cleavage completely abolished the activity [86]. Changing Pro to D-Pro improved the stability both *in vitro* and *in vivo* [86,87]. DPP IV is also involved in the hydrolysis and renal uptake of this opioid peptide [71,88]. Together with ACE and carboxypeptidase P, DPP IV is responsible for the intestinal degradation of β-casomorphin (1-5) [73].

The same studies report the importance of renal DPP IV hydrolysis for **morphiceptin** (Tyr-Pro-Phe-Pro-NH_2). Also, in situ perfusion experiments in intact animals, revealed that the ability of the opiate agonist morphiceptin, when administered into the intestinal lumen, to block the cholera toxin-induced water secretion, was significantly greater in DPP IV-negative rats [73].

Members of the **pancreatic polypeptide family** are also substrates for DPP IV. Purified DPP IV liberated Tyr-Pro from both, **neuropeptide Y** and **peptide YY** to their (3-36) fragments, but almost no Ala-Pro from pancreatic polypeptide [89]. This was

confirmed in serum, and the hydrolysis was blocked by specific DPP IV inhibitors. Removal of this *N*-terminal dipeptide inactivates these peptides for binding to one receptor subtype, and is therefore important for the biological activity. Beside DPP IV, aminopeptidase P and endopeptidase-24.11 are also involved in processing of these peptides [90-92]. Peptide YY (3-36), which has an *N*-terminal Xaa-Xaa-Pro sequence has been reported as a competitive inhibitor of DPP IV [46], giving some kind of feedback regulation.

The degradation of **enterostatin**, a potent inhibitor of food intake, by intestinal brush-border membranes and brain membranes involves both DPP IV and carboxypeptidase P. In serum, degradation was mainly due to DPP IV [93].

The Glucagon/Secretin/Vasoactive-intestinal Peptide Family

Peptides of this family either start with Tyr-Ala, His-Ala or His-Ser and are sometimes physiologically more important substrates than what could be expected from the substrate specificity of DPP IV.

The plasma protease responsible for primary proteolytic cleavage of **growth-hormone-releasing factor** (GRF) was shown to be DPP IV, liberating Tyr-Ala to give GRF (3-44)-NH_2. This degradation is responsible for the inactivation of GRF both *in vitro* and *in vivo* [94,95], and could be overcome by inhibition with diprotin A, or by substitution of the first or second amino acid with a D-amino acid. A detailed kinetic analysis of DPP IV proteolysis of GRF and analogues showed some interesting results concerning the substrate specificity at the P_1 position. Values of k_{cat}/K_m for different P_1-substituted analogues varied as follows : Abu > Pro > Ala >> Ser > Gly = Val >> Leu. This means that k_{cat}/K_m is at a maximum for a hydrophobic P_1 side-chain of about 0.25 nm in length, i.e., the ethyl side-chain of α-aminobutyric acid (Abu) is very close to optimal [96]. A similar study with Ser, Thr or Val at the penultimate position showed some DPP IV cleavage [97]. The detailed investigation of GRF degradation led to the development of GRF analogues with enhanced metabolic stability [98,99].

Other members of this family, i.e., **glucose-dependent insulinotropic polypeptide** (GIP), **glucagon-like peptide-1 (7-36)** (GLP-1 (7-36)) and **peptide histidine methionine** (PHM) are hydrolysed by purified DPP IV, and also in serum [100]. Degradation in serum could be prevented by the addition of Lys-pyrrolidide or diprotin A as inhibitors. Since an intact *N*-terminus is obligatory for the biological activity of the members of this family, DPP IV action inactivates these peptide hormones. *In vivo*, using DPP IV-negative rats, it was shown that GIP and GLP-1 (7-36) are degraded by DPP IV [101]. Mass spectrometry indicated that incubation of these peptides in serum resulted mainly in cleavage by DPP IV, with only minor secondary degradation due to other serum protease activities [102]. This knowledge led to the development of GLP-1 analogues with extended metabolic stability and improved biological activity [103]. Furthermore, oral administration of the DPP IV inhibitor Ile-thiazolidine (**3**) to Zucker rats increased the stability of GIP and GLP-1 [104]. A similar effect was seen with an inhibitor in anaesthetised pigs [105]. **Glucagon-like peptide-2** (GLP-2) is degraded and inactivated by DPP IV *in vivo*, which led to the development of a more potent and stable analogue of GLP-2 [106].

Chemokines

N-terminal truncation of the C-C chemokine **RANTES (1-68)** (regulated on activation normal T cells expressed and secreted) to RANTES (3-68) by DPP IV converted a potent chemoattractant of monocytes (the former) to a chemotaxis inhibitor (the latter) [107,108]. Also, Rantes (3-68) inhibited infection of mononuclear cells by an M-tropic HIV-1 strain 5-fold more efficiently than intact RANTES. Thus proteolytic processing of RANTES may constitute an important regulatory mechanism during anti-inflammatory and antiviral responses.

On the contrary, the truncation of the C-X-C chemokine **granulocyte chemotactic protein-2** (GCP-2) by DPP IV did not result in a loss of chemotactic properties [108]. Other C-X-C chemokines, i.e., **stromal cell-derived factor** 1α (SDF-1α) and 1β (SDF-1β) are also substrates for DPP IV. Their hydrolysis results in complete abrogation of the chemotactic properties. In contrast to RANTES, truncation of SDF-1 leads to a loss of anti-HIV-1 properties [109,110].

Role in Immunological Processes

The CD3/T-cell receptor (TcR) complex plays a central role in T-cell activation and function. However, T cells require a second co-stimulatory signal which can be provided by a number of accessory molecules, including CD26. The importance of CD26 in the immune response has recently been reviewed extensively [111-113].

A number of observations have linked CD26 to the functions of the immune system and, in particular, to the functions of T cells. First, CD26 appears to be more restricted than other accessory molecules since it is

expressed only on the CD4 memory/helper (CD45RO+CD29+) population. These cells are the only one that can respond to recall antigens, induce B-cell immunoglobulin (IgG) synthesis and activate MHC-restricted cytotoxic T cells (*vide supra*). Second, certain antibodies against CD26 can activate T cells. Third, other antibodies and specific inhibitors of DPP IV enzymatic activity have been shown to inhibit mitogen- and antigen-induced T-cell proliferation *in vitro and in vivo*. In 1985 it was shown that a diacylhydroxylamine inhibitor (**14**) was able to suppress the proliferation of human lymphocytes stimulated with pokeweed mitogen or allogeneic cells [114]. These inhibitors and also pyrrolidides and thiazolidides (**3-6**) inhibit DNA synthesis and cytokine production (such as IL-2, IL-10 and IL-12) of stimulated lymphocytes [37,115-117]. Pro-boroPro (**11**) inhibits antigen-induced proliferation and interleukin 2 production in T cells, but does not inhibit the response of these T cells to mitogens [48,118]. This is in contrast with the previous studies where also mitogen-induced proliferation was inhibited. Boronic acid dipeptides also inhibit the mixed lymphocyte reaction, and this inhibition was correlated with the amount of DPP IV inhibition [53].

In vivo studies with inhibitors confirm the role of CD26 in immunological processes. Pro-boroPro (**11**), administered subcutaneously, was found to inhibit DPP IV in serum and spleen cell suspensions, and suppress antibody production in mice immunised with bovine serum albumin [119]. Alkyldiamine-induced arthritis in rats, a model that shares several pathological features associated with rheumatoid arthritis, was suppressed by several DPP IV inhibitors in a dose dependent manner, i.e. Ala-boroPro, a thiazolidide (**5**) and a diacylhydroxylamine (**14**) [120]. Cardiac transplantation in rats resulted in an early increase in cellular CD26 expression, followed by a rise in DPP IV serum activity, which peaked at day 6, i.e., before the time of actual graft loss. The use of a dipeptide diphenyl phosponate ester (**16**) abrogated acute rejection and prolonged cardiac allograft survival from 7 to 14 days [121]. It prevented the early peak of cellular CD26 expression and thoroughly suppressed systemic DPP IV activity. The inhibition of DPP IV was associated with severely impaired host cytotoxic T lymphocyte responses *in vitro*. These results demonstrate the role of CD26 in allo-antigen mediated immune regulation *in vivo* and provide the first direct evidence that CD26 plays an important role in the mechanism of allograft rejection.

A fourth evidence for the role of CD26 in the immune system is the fact that a recombinant soluble CD26, that comprises only the extracellular domain, enhances the proliferative response of human T cells to tetanus toxoid antigen [122]. Fifth, CD 26 is associated on the cell surface with the adenosine deaminase (ADA) protein [123-125] and with CD45 [126. It is well known that the deficiency of ADA results in severe combined immunodeficiency in humans, and that CD45 is a tyrosine phosphatase involved in T-cell activation, indicating that CD26 might have a modulating effect on T-cell signalling.

Thus, the above results demonstrate that CD26 plays an important role in T-cell co-stimulation, but the exact mechanism remains unknown. CD26 has a cytoplasmic region of only 6 amino acids, which may be too short to explain its signal-transducing activity. This suggests that CD26 might be associated with other molecules that are capable of transmitting signals from the outside of the cell to the inside. One of the possibilities is CD45, that has a cytoplasmic domain with protein tyrosine phosphatase activity [112]. The hypothesis is that CD26-induced modulation of the enzymatic activity of CD45 would account for the increased activity of the tyrosine kinase $p56^{lck}$, which itself might be involved in the CD3 ζ chain phosphorylation. This process could amplify the immune response, since it is known that the CD3 ζ chain of the TcR complex is required for signalling via CD26 [127]. In this respect, it is worthwhile mentioning that DPP IV inhibitors (e.g. **5**) strongly suppress the PMA induced hyperphosphorylation of $p56^{lck}$ in a dose dependent manner [128].

Another candidate for the signal transduction molecule is the ADA protein. It binds to the extracellular domain of CD26 [129] and the exact amino acids of CD26 involved in ADA binding are determined [130]. ADA catalyses the conversion of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. High concentrations of adenosine inhibit the proliferation of T cells, and this inhibitory effect of adenosine is markedly potentiated by inhibitors of ADA. Studies with mutated CD26 tranfectants unable to bind ADA show that these transfectants were much more sensitive to the inhibitory effect of adenosine on IL-2 production. However the same mutated transfectants produced the same amount of IL-2 than the wild type transfectants following stimulation with anti-CD3 and PMA. This means that ADA is not directly involved in T cell activation after such stimulation. However, ADA expressed on the T-cell surface can aid the cell in resisting an inhibitory effect of adenosine by deaminating adenosine to inactive inosine, thereby reducing the effective extracellular concentration of adenosine [130].

Another possibility is that CD26 participates in the control of T-cell function, not via signal transduction molecules, but through DPP IV enzyme processing of biologically active molecules. This is still controversial and a matter of much debate [131,132], but lately a lot of evidence is produced that enzymatic activity is

important. In favour of this theory is the fact that DPP IV inhibitors are able to suppress T-cell proliferation *in vitro* and *in vivo*. Hyperphosphorylation can be modulated with DPP IV specific inhibition (*vide supra*). DPP IV-negative soluble CD26 could not enhance the proliferation of peripheral blood lymphocytes induced by the recall antigen tetanus toxoid, implying a requirement for enzymatic activity [122]. Definitive proof came from an experiment with mutated CD 26 transfectants, where human CD26 cDNA was mutated so that the active-site serine was replaced by alanine, abolishing enzymatic activity [133]. After stimulation, wild-type transfectants (CD26+, DPP IV+) produced substantially more IL-2 than did mutant (CD26+, DPP IV-) or control (CD26-) transfectants. Nevertheless, the mutant transfectants (CD26+, DPP IV-) still produced significantly more IL-2 than did the control transfectants. These results suggest that DPP IV enzyme activity plays an important but not absolute role in the co-stimulatory activity of CD26 in this system.

Role in HIV Infection

It is well known that CD4 lymphocytes in patients with AIDS have an intrinsic defect in their ability to recognise and respond to recall antigens before the reduction in the total number of CD4 cells [134]. This immune dysfunction in infected individuals, even in the presence of normal CD4 numbers is called the "AIDS paradox". Since the response to recall antigens is clearly a property of CD4+CD26+ cells, it is not surprising that a selective decrease in CD26+ T cells prior to a general reduction in CD4+ T cells is reported in HIV-1 infected individuals [135]. Furthermore, the HIV-1 Tat protein, which is a regulatory protein and is essential for transactivation of viral genes as well as for viral replication, has been shown to have a potent immunosuppressive effect on lymphocyte proliferation induced by soluble antigen [136]. Linking these observations together, it was investigated if Tat could mediate its immunosuppressive effect through CD26/DPP IV. Indeed, it has been reported that Tat is a DPP IV inhibitor, suggesting that the immunosuppressive effects of Tat on non-HIV-1-infected T cells could be mediated by DPP IV [44,45]. It was shown that the *N*-terminal Xaa-Xaa-Pro sequence of this protein was important for DPP IV inhibition and for suppression of CD26-dependent T cell growth [46,47].

It has been proposed that CD26 serves as an essential co-factor for HIV-1 entry [137,138], but this proposal has been seriously challenged [139-142]. Studies with the same transfectants as mentioned above, showed that (CD26-) and (CD26+, DPP IV-) transfectants were more readily infected than the (CD26+, DPP IV+) transfectants, showing that CD26/DPP IV is not a necessary co-factor for HIV infection [143]. On the other hand, experiments indicate that CD26 expression correlates with entry, replication and cytopathicity of monocytotropic (M-tropic), but not T-cell line-tropic (T-tropic), HIV-1 strains in a T-cell line [144]. So, the role of CD26 in HIV entry is still controversial.

Another important feature of CD26 is its possible involvement in HIV infection and related apoptosis. A selective loss of CD26+ T cells prior to a general reduction of CD4+ T cells in HIV-1 infected individuals was noticed (*vide supra*). More importantly, the same authors demonstrated that HIV-1 preferentially infected CD4+CD26- T cells. It was concluded that CD26- T cells were a principal reservoir of HIV-1 *in vivo* [135]. This also points to the fact that CD26 could not be an essential co-factor for entry. Moreover, *in vitro* coculture experiments of HIV-1 infected and uninfected cells have indicated that while uninfected cells die by apoptosis, HIV-infected cells are resistant to HIV-induced cell death [145]. This phenomenon is obviously favourable for the survival of the virus-producing cells, and could explain the selective loss of CD26+ uninfected cells. But how can it be explained that CD26+ cells die by apoptosis and that the infected CD26- cells are resistant to cell death? It is suggested that the HIV Tat protein is not only responsible for immunosuppression, but also contributes to the induction of apoptosis [143]. In the regulation of this apoptosis Tat plays a dual role : 1) Extracellular Tat induces apoptosis of HIV-uninfected cells, and 2) Tat protects cell death of HIV-1-infected cells. This was confirmed by the protection from apoptosis for transfectants that constitutively expressed Tat [146]. This modulation of apoptosis might be due to the interaction of Tat with CD26/DPP IV.

Another proof for the connection of CD26 and HIV is that ADA binding to CD26 is inhibited by HIV-1 envelope glycoprotein gp120 [147]. Also, two types of DPP IV inhibitors are inhibitors of HIV infection, i.e., pyrrolidine-2-nitriles (**9**) [148] and the irreversible cyclopeptide inhibitor **21** [69], showing the implication of CD26 in HIV infection.

The link between CD26/DPP IV, chemokines and HIV is also remarkable in view of a possible role for CD26 in HIV infection. HIV infection is initiated by interaction of the virion envelope glycoproteins (gp120/41) with at least two cellular receptors : the CD4 molecule and a seven-transmembrane domain G-protein coupled chemokine receptor. Macrophage-tropic (M-tropic) strains of HIV-1 replicate in macrophages and CD4+ T cells and use the CC chemokine receptor CCR5. T-tropic isolates of HIV-1 replicate primarily in CD4+ T cells, as well as in macrophages. These viruses use the CXC chemokine

receptor CXCR4. Although CCR5 and CXCR4 are believed to be the primary receptor for HIV-1 entry, additional chemokine receptors have also been shown to serve as co-receptors. [149,150]. The chemokines that are the natural ligands of these chemokine receptors are able to compete with HIV-1 envelope glycoprotein for binding to the receptor, and hence can inhibit HIV-1 entry. The CC chemokines MIP-1α, MIP-1β and RANTES inhibit the replication of M-tropic HIV-1 strains that use the CCR5 receptor, and the CXC chemokine SDF-1 blocks the viral entry of T-tropic HIV-1 strains that use the CXCR4 receptor. It has been shown that some of these chemokines are substrates for DPP IV (*vide supra*). Amino-terminal truncation of RANTES by DPP IV converses RANTES (1-68) into RANTES (3-68), an even more potent inhibitor of HIV-1 infection [107,108]. In contrast to RANTES (3-68), SDF-1α (3-68) has diminished potency to inhibit HIV-1 infection [109,110]. Thus, CD26 plays a dual role in HIV-infection and AIDS.

All the above elements suggest that CD26/DPP IV plays a role in HIV infection, but the exact mechanism remains to be elucidated.

Therapeutic Perspectives

The exact biological functions of CD26/DPP IV are still under investigation, but considerable evidence exists for the therapeutic potential of DPP IV inhibitors. As mentioned earlier DPP IV is involved in the metabolic processing of several biologically active peptides, and DPP IV inhibitors could be used to modulate their metabolism. For instance, co-administration of a DPP IV inhibitor and the opiate peptide morphiceptin could be used in case of diarrhoea, as the experiment with DPP IV-negative rats showed [73]. Since GRF is also degraded by DPP IV, the use of a DPP IV inhibitor together with GRF could be useful to treat children with growth hormone deficiency.

More importantly, DPP IV is also involved in the degradation of GIP and GLP-1 *in vitro* and *in vivo*. GIP and GLP-1 are considered to be the most important insulin-releasing hormones (incretins) comprising the enteroinsular axis. The term enteroinsular axis refers to the signalling pathways between the gut and pancreatic islets that amplify the insulin response to absorbed nutrients. It has been shown that *N*-terminal truncation by DPP IV abolishes insulinotropic activity and numerous studies show that this hydrolysis is the primary mechanism of their inactivation *in vivo* (vide supra). Inhibition of circulating DPP IV with orally administered Ile-thiazolidine (**3**) enhanced insulin secretion and improved glucose tolerance in response to an oral glucose challenge in lean and obese Zucker rats. The enhanced incretin response was greater in obese than in lean animals, with a more profound improvement in glucose tolerance [104]. This was attributed to disruption of DPP IV inactivation of GIP and GLP-1, resulting in amplification of the enteroinsular axis. The Zucker obese rat exhibits abnormalities in glucose metabolism that characterises non-insulin dependent diabetes mellitus (NIDDM), i.e., insulin secretory defects as well as insulin resistance, leading to glucose intolerance. Therefore, it is believed that inhibitors of DPP IV, alone or in combination with GLP-1, could be used for lowering glucose levels in NIDDM and other disorders involving glucose intolerance [151].

Likewise, DPP IV hydrolysis of GLP-2 is also responsible for its inactivation. GLP-2 has recently been shown to display intestinal growth factor activity in rodents, raising the possibility that GLP-2 may be therapeutically useful for enhancement of mucosal regeneration in patients with intestinal disease [151]. The use of [Gly^2]GLP-2, resistant to DPP IV hydrolysis, increases small bowel weight in mice, predominantly due to a significant increase in villous height [106,152,153]. Therefore, DPP IV inhibitors might be useful to increase the intestinotrophic properties of GLP-2.

It has been shown that CD26 plays an important role in the immune system by a number of possible mechanism. The exact mechanism remains to be elucidated, but a few examples are reported where DPP IV inhibitors are useful immunosuppressants *in vivo*. A dipeptide diphenyl phosphonate ester (**16**) was able to abrogate acute rejection and prolong allograft cardiac survival [121]. Alkyldiamine-induced arthritis in rats, a model that shares several pathological features associated with rheumatoid arthritis, was suppressed by several DPP IV inhibitors in a dose dependent manner, i.e. Ala-boroPro, a thiazolidide (**5**) and a diacylhydroxylamine (**14**) [120]. It was reported that T cells with high levels of CD26 may preferentially migrate into the rheumatoid synovium to induce inflammation and tissue destruction [154]. This proves the potential of DPP IV inhibitors as immunosuppressants for the treatment of autoimmune diseases and for the prevention of transplant rejection.

The role of CD26 in HIV infection is also not completely clear yet, but seems nevertheless important. Some DPP IV inhibitors are reported to inhibit HIV infection such as pyrrolidine-2-nitriles (**9**) [148] and an irreversible cyclopeptide inhibitor (**21**) [69].

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Abbreviations

DPP II	=	Dipeptidyl peptidase II
DPP IV	=	Dipeptidyl peptidase IV
FAP	=	Fibroblast-activation protein
GCP-2	=	Granulocyte chemotactic protein-2
GIP	=	Glucose-dependent insulinotropic polypeptide
GLP-1	=	Glucagon-like peptide-1
GLP-2	=	Glucagon-like peptide-2
IL	=	Interleukin
NIDDM	=	Non-insulin dependent diabetes mellitus
PHM	=	Peptide histidine methionine
PO	=	Prolyl oligopeptidase
RANTES	=	Regulated on activation normal T cells expressed and secreted
SDF	=	Stromal cell-derived factor

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The Synthesis of 4,5-Methano Congeners of α-Kainic and *α-allo*-Kainic Acids as Probes for Glutamate Receptors

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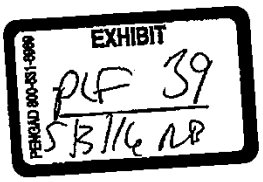


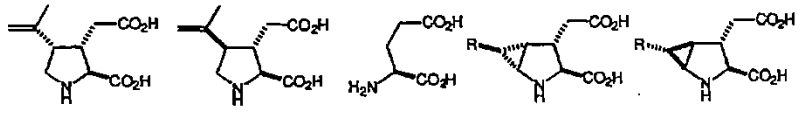
Abstract: The synthesis of diastereomeric 4,5-methano-L-proline 3-acetic acids is described starting from D-serine. The key reactions include a free-radical carbocyclization and an acid-catalyzed desilanylative cyclopropanation of an iminium ion intermediate.

The synthesis of biologically relevant α-amino acids in which the carbon skeleton has been rigidified has been an area of active research for some years.[1] 2,3-Methano amino acids, also known as "methanologs" have attracted considerable attention in this regard.[2] Many of these compounds were prepared with the intention of probing spatial, conformational and functional features of the natural substrates at their biological receptor sites. Methano[2,3] and related[4] analogs of L-glutamic acid have been of particular interest because of the importance of this amino acid in the CNS. Indeed, the glutamate receptor is considered to be an important target in the quest for therapeutically effective drugs in the cardiovascular and related areas[5] Cyclopropane α-amino acids are also natural products[2] or components of more elaborate structures.[6]

Receptors to excitatory amino acids include among others, those that have an affinity to α-kainic acid **1**,[7] which, viewed in a different perspective, can be considered as a constrained L-glutamic acid **3**. Indeed, in addition to a plethora of total[8] and partial syntheses[8] of α-kainic acid **1** and *allo*-kainic acid **2**, there are several reports of kainoid analogs[8,9] aimed at finding new bioactive compounds in this series.

We report in this Letter, the design and synthesis of structurally novel 4,5-methanoproline 3-acetic acid analogs **4** and **5** in enantiomerically pure form (Figure 1). Examples of methanoprolines are rare,[10,11] and to the best of our knowledge compounds like **4** and **5** which are structurally and stereochemically related to α-kainic and *allo*-kainic acids, are unprecedented.

Figure 1



1, α-kainic acid 2, *allo*-kainic acid 3, *L*-Glutamic acid **4**, R=H **4a**, R=vinyl **5**, R=H **5a**, R=vinyl

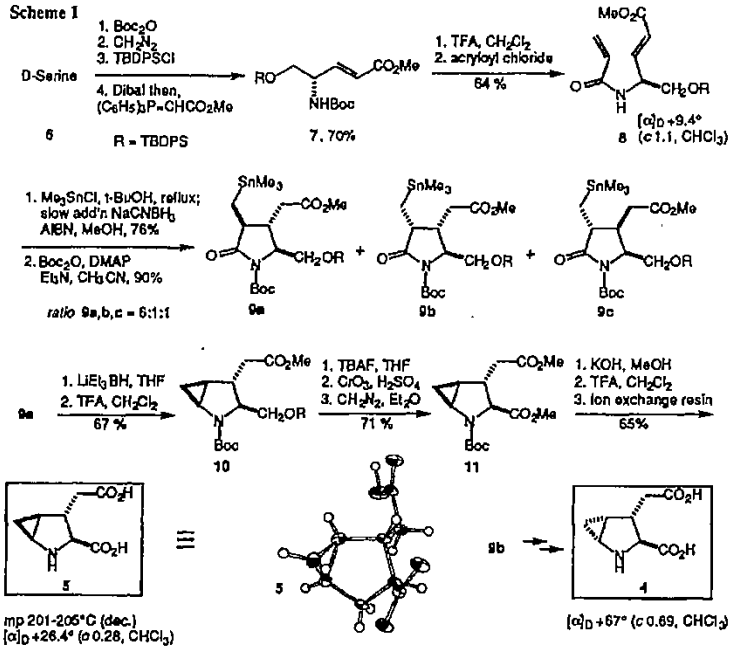
In order to have access to both isomers **4** and **5**, we chose a method of synthesis that produced stereoisomeric intermediates from a common precursor (Scheme 1). D-Serine **6** was elaborated upon by a series of standard manipulations to give the diene **8**. Treatment with trimethyltin hydride[12,13] led to a mixture of pyrrolidinones, which could be separated into the three isomers **9a**, **9b**, and **9c** after conversion to

the N-boc derivatives and column chromatography.[14] The stereochemical outcome favoring the major *trans*-isomer **10a** has been rationalized based on the prevalence of a late transition state.[11,15]

In a key transformation, the lactam was sequentially reduced to the hemiaminal, then treated with acid to give the 4,5-methano derivative **10** *via* intermolecular alkylation of the corresponding N-Boc iminium ion.[11] Subsequent steps relied on functional group manipulations to afford the diester **11** which was in turn hydrolyzed to the crystalline 4*S*,5*S*-methano-3*S*-carboxylmethyl-L-proline, **5**. An X-ray crystal analysis provided conclusive proof for its structure and stereochemistry.

The isomeric 4*R*,5*R*-analog **4** was prepared in a similar manner, to give an amorphous product, whose stereochemistry was rigorously assigned by detailed n.O.e. studies.

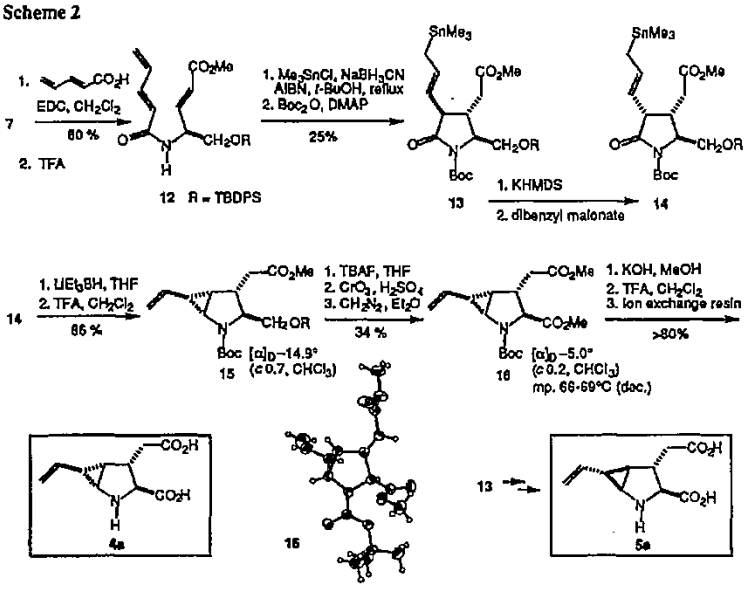
Scheme 1



The radical carbocyclization reaction can also be done on an extended dienic system[12a] which can eventually lead to the functionalized 4,5-methano analogs **4a** and **5a** (Figure 1).

The readily available diene **12** was subjected to the free radical carbocyclization reaction to give mainly the all-*trans*-pyrrolidinone which was isolated as the N-Boc derivative **13** (Scheme 2). Quenching the potassium dienolate of the all-*trans*-isomer **13** with dibenzylmalonate[16] as a proton source led to the isomeric product **14** as the major product. Formation of the N-Boc iminium ion by the method described above led to the vinyl cyclopropane **15** which was in turn converted into the α-kainic acid congener **4a**, isolated as an amorphous solid. Application of the same methodology to the isomeric **13** gave **5a**, also isolated as an amorphous solid. The structures in this series were unambiguously established by detailed N.M.R. studies and by an X-ray crystal analysis of **16**.

Scheme 2



Compounds 4, 4a, 5 and related amino acids from another series[11] were tested for their binding as antagonists and agonists in five receptor assays.[5a] Unfortunately, no significant binding affinity was found at 1 μM in the DCKA (^{3}H-5,7-dichlorokynurenic acid) assay for the glycine recognition site of the NMDA receptor. When tested in the AMPA, kainate, and other receptor binding assays at concentrations of 1 μM and 10 μM, again, activity was surprisingly low compared to standards.[17]

Clearly, the structural requirements for effective binding to these receptors have not been satisfied by our methano analogs in spite of their novel structures. The lack of activity in the kainate receptor and the glutamate recognition site of N-methyl-D-aspartate receptor (CGP 39653) are reflective of the lack of our understanding for specific spatial requirements and hydrophobic interactions of the appended cyclopropane in analogs 4, 4a, 5 vis-a-vis the 2-propenyl group in α-kainic acid itself.

We are presently developing alternative, highly stereocontrolled methods for the synthesis of conformationally constrained analogs of L-proline and L-pipecolic acid. These should find specific applications in the design of peptidomimetics aimed at probing enzymatic reactions that involve *cis/trans* amide-bond isomerization[18] such as in the immunophilins,[19] as well as in the study of secondary and tertiary local structures of certain peptides.[20]

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Chronic Inhibition of Circulating Dipeptidyl Peptidase IV by FE 999011 Delays the Occurrence of Diabetes in Male Zucker Diabetic Fatty Rats

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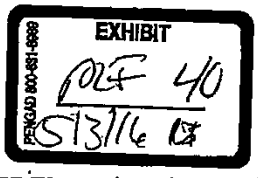

Acute suppression of dipeptidyl peptidase IV (DPP-IV) activity improves glucose tolerance in the Zucker fatty rat, a rodent model of impaired glucose tolerance, through stabilization of glucagon-like peptide (GLP)-1. This study describes the effects of a new and potent DPP-IV inhibitor, FE 999011, which is able to suppress plasma DPP-IV activity for 12 h after a single oral administration. In the Zucker fatty rat, FE 999011 dose-dependently attenuated glucose excursion during an oral glucose tolerance test and increased GLP-1(7-36) release in response to intraduodenal glucose. Chronic treatment with FE 999011 (10 mg/kg, twice a day for 7 days) improved glucose tolerance, as suggested by a decrease in the insulin-to-glucose ratio. In the Zucker diabetic fatty (ZDF) rat, a rodent model of type 2 diabetes, chronic treatment with FE 999011 (10 mg/kg per os, once or twice a day) postponed the development of diabetes, with the twice-a-day treatment delaying the onset of hyperglycemia by 21 days. In addition, treatment with FE 999011 stabilized food and water intake to prediabetic levels and reduced hypertriglyceridemia while preventing the rise in circulating free fatty acids. At the end of treatment, basal plasma GLP-1 levels were increased, and pancreatic gene expression for GLP-1 receptor was significantly upregulated. This study demonstrates that DPP-IV inhibitors such as FE 999011 could be of clinical value to delay the progression from impaired glucose tolerance to type 2 diabetes. *Diabetes* 51:1461–1469, 2002

Dipeptidyl peptidase IV (DPP-IV) (CD26, EC.3.4.14.5) is a membrane-bound and circulating serine protease with a restricted substrate specificity, hydrolyzing peptides after a penultimate NH_2-terminal proline or alanine residue (1). Mice or rats bearing null mutation of the DPP-IV gene have increased glucose tolerance (2,3). Acute inhibition of DPP-IV activity by specific inhibitors such as isoleucine-thiazolidide, NVP-DPP728, or valine-pyrrolidide has been recently shown to significantly reduce glucose excursion in Zucker fatty (*fa/fa*) rats and high fat–fed glucose-intolerant mice during oral or intraduodenal glucose tolerance tests (4–6). Whatever the animal model used, insulin secretion following glucose administration was enhanced by DPP-IV inhibition, and this has been attributed to the role played by DPP-IV in the early inactivation of the two incretins glucagon-like peptide (GLP)-1 and gastric inhibitory peptide/glucose-dependent insulinotropic polypeptide (GIP) (7). These gastrointestinal peptides are released postprandially from intestinal L-cells and duodenal K-cells, respectively (8), and potentiate glucose-induced insulin secretion by sensitizing pancreatic β-cells to stimulation by glucose (9–11). Inactivation of these peptides can be successfully prevented by DPP-IV inhibitors, leading to potentiation of their biological activity (12).

The Zucker fatty rat is obese and insulin resistant, with normal or slightly elevated glucose concentrations, reflecting only mild glucose intolerance. Obesity in these animals is due to a mutation in the leptin receptor gene (13). Like the Zucker fatty rat, the Zucker diabetic fatty (ZDF) rat displays glucose intolerance, marked insulin resistance, and hyperlipidemia, but only the ZDF rat becomes overtly diabetic after 8 weeks of age if fed a diet containing 6.5% fat (14). In the prediabetic state, the male ZDF rat experiences a steady increase in basal insulinemia and plasma free fatty acid (FFA) levels. Hyperglycemia develops between 8 and 10 weeks of age, leading to overt diabetes and collapsing insulin secretion (15). This is similar to the progressive loss of glucose-stimulated insulin secretion in human type 2 diabetes, and thus ZDF rats represent a good animal model for this form of human diabetes (16). Interestingly, GLP-1 retains its potency of enhancing glucose-stimulated insulin release in prediabetic and diabetic ZDF rats (17,18), and chronic administration of exendin-4, a peptide showing agonistic activity at the GLP-1 receptor, demonstrated antidiabetic efficacy in these animals, improving glycemic control and insulin sensitivity (19). Similarly, in type 2 diabetic patients, GLP-1 has been successfully used to normalize fasting and prandial glycemia (20–23). Recently, it has been shown that GLP-1 and analogs also stimulate growth and differentiation of β-cell progenitor cells in the pancreas and may therefore restore

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AFC, 7-amino-4-trifluoromethylcoumarin; DPP-IV, dipeptidyl peptidase IV; ELISA, enzyme-linked immunosorbent assay; FFA, free fatty acid; GIP, gastric inhibitory peptide; GLP, glucagon-like peptide; GLP-1R, GLP-1 receptor; IC_{50}, half-maximal inhibitory concentration; IDGTT, intraduodenal glucose tolerance test; OGTT, oral glucose tolerance test; RT, reverse transcription; TG, triglyceride.

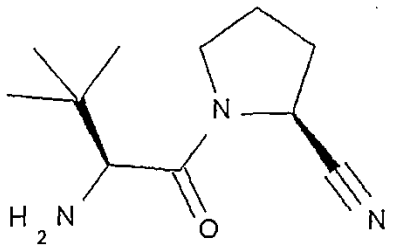

FIG. 1. Chemical structure of FE 999011.

functional β-cell mass when administered to individuals with diabetes (24,25). Taken together, these observations suggest that inappropriate incretin action at the level of the islet cells could be one important factor participating in the development of diabetes. We thus postulated that by chronically increasing endogenous GLP-1 bioactivity in the ZDF rat through constant inhibition of DPP-IV, a similar improvement in glycemic control could be obtained.

In the present study, we have investigated the effects of chronic administration of the new DPP-IV inhibitor FE 999011 [(2S)-1-([2′S]-2′-amino-3′,3′dimethyl-butanoyl)-pyrrolidine-2-carbonitrile] (Fig. 1) in obese Zucker fatty and ZDF rats. FE 999011 is a reversible DPP-IV inhibitor, inhibiting human and rat DPP-IV with half-maximal inhibitory concentration (IC_{50}) values of 7 and 3 nmol/l, respectively (26).

RESEARCH DESIGN AND METHODS

Animals. Male Zucker fatty rats were purchased from Iffa-Credo (L'Arbresle, France) at 8–10 weeks of age; housed in a temperature-, humidity-, and light-controlled room (21–23°C, 12–12 h light-dark cycle); and given free access to food and water. Obese male ZDF rats (n = 24) and lean controls (n = 8), received at 6 weeks of age from Genetic Models (Indianapolis, IN), were individually housed. They were fed ad libitum with Purina 5008 (6.5% fat).

Experimental protocols concerning the use of laboratory animals were reviewed by the University of Geneva School of Medicine Ethics Committee for Animal Experimentation and approved by the State of Geneva Veterinary Office.

Effects of FE 999011 and NVP-DPP728 on plasma DPP-IV activity in the Zucker fatty rat. Three groups of fed male Zucker fatty rats, 11 weeks of age, received an oral administration of FE 999011 or NVP-DPP728 at 10 mg/kg or vehicle (sterile distilled water). Blood samples (200–250 μl) were collected before dosing and then 10, 30, and 60 min and 3, 6, 10, 12, and 24 h postdosing. Tail blood was collected in heparinized tubes (30 units/ml), and plasma was extracted after centrifugation at 3,000 rpm for 10 min and stored at −20°C until determination of DPP-IV activity.

Effects of FE 999011 on glucose excursion during an oral glucose tolerance test orally in the Zucker fatty rat. Four groups of overnight-fasted male Zucker fatty rats, 19–20 weeks of age, were injected with FE 999011 (1, 3, or 10 mg/kg) or vehicle (sterile distilled water). Eight hours later, all animals were administered oral glucose (1 g/kg) as a 40% solution (wt/vol). Blood samples (200–250 μl) were collected before dosing and 10, 20, 30, 60, and 120 min after dosing.

Effects of FE 999011 on the glucose excursion and plasma levels in GLP-1 and insulin following intraduodenal glucose tolerance test in the Zucker fatty rat. Two groups of overnight-fasted obese male Zucker rats and one group of lean rats, 19–20 weeks of age, were anesthetized with pentobarbital (Narcoren, 5 mg/100 g body wt i.p.). A cannula was quickly inserted into the right jugular vein, and the abdomen was exposed by laparotomy. In one obese group of rats, FE 999011 prepared in distilled water was injected intravenously (3 mg/kg), whereas the other rats received a vehicle injection. Fifteen minutes later, a 40% glucose solution (2.5 ml/kg) was injected into the first loop of the duodenum. Blood samples (300 μl) were collected before the glucose load and 4, 7, 10, 13, 16, 20, 30, 60, and 90 min thereafter. For plasma determination of glucose and insulin, 100 μl was taken in heparinized tubes; for plasma determination of GLP-1[(7-36), (7-37)], 200 μl was taken in Eppendorf tubes containing EDTA and diprotin A (Sigma, St. Louis, MO) to achieve a final concentration of 2 mg EDTA and 50 μmol diprotin A per milliliter of blood.

Chronic effects of FE 999011 on glucose and insulin excursion during an oral glucose tolerance test in the Zucker fatty rat. Two groups of male Zucker fatty rats, 15 weeks of age, received FE 999011 (10 mg/kg per os, twice a day) or vehicle (sterile distilled water) during 7 days. Seventeen hours after the final administration of FE 999011, an oral glucose tolerance test (OGTT) was performed as described above, with or without coadministration of FE 999011.

Chronic effects of FE 999011 in the ZDF rat. At 6 weeks of age, obese (*fa/fa*) rats were weighed and randomized into three groups of 8. Two groups were treated with FE 999011 orally once a day (10 mg/kg, 6:00 P.M.) or twice a day (10 mg/kg, 8:00 A.M and 6:00 P.M.), and one group was injected orally with sterile distilled water. A group of eight lean (*fa/+* or +/+) rats was included as controls for metabolic and molecular parameters. Tail blood was collected from rats in the fed state into heparinized tubes three times a week before the first daily treatment with FE 999011, for plasma determinations of glucose, insulin, triglycerides (TGs), and FFAs. Body weight and food and water consumption were recorded throughout the experiment. The day before the rats were killed, blood samples were taken to determine plasma GLP-1 levels in the fed state. At the end of the study, the rats were killed, and trunk blood was collected in heparinized tubes for plasma determination of DPP-IV activity. Inguinal and retroperitoneal adipose tissues were dissected out and weighed. Pancreata were dissected out and frozen for GLP-1 receptor mRNA measurements.

Plasma analyses. Glycemia was assayed by using a standard glucose-oxidase technique (Boehringer Mannheim, Mannheim, Germany). Plasma insulin concentration was determined by radioimmunoassay (Linco Research, St. Charles, MO). Plasma GLP-1 concentration was determined by an enzyme-linked immunosorbent assay (ELISA) technique that measured the biologically active GLP-1 forms GLP-1(7-36) and GLP-1(7-37) amide in plasma (Linco Research). Plasma TG and FFA concentrations were measured using an enzymatic colorimetric test (Triglyceride L-Type and NEFA C; Wako Chemicals, Richmond, VA).

Plasma DPP-IV activity was measured using a fluorometric assay. The plasma samples were centrifuged at 4°C, 10,000*g*, for 1 min; 0.5 μl supernatant was added to 49 μl assay buffer (HEPES 83.3 mmol/l, EDTA 2 mmol/l, pH 7.8, with 1.5% BSA); and the reaction was started by addition of 0.5 μl substrate Ala-Pro-AFC (7-amino-4-trifluoromethylcoumarin, 20 μmol/l final concentration; Enzyme Systems Products, Livermore, CA) in assay buffer. The initial rate of AFC release was measured at 20°C over 3 min using a Labsystems Fluoroskan Ascent FL instrument at 410 nm excitation/510 nm emission.

Gene expression assays using competitive quantitative RT-PCR. Expression of the gene encoding GLP-1 receptor (GLP-1R) was evaluated by competitive RT-PCR. Tissue was quickly dissected, frozen in liquid nitrogen, then stored at −80°C until processing using a rotor-stator homogenizer. RNA was extracted by using the Qiagen RNeasy Mini Kit method, and then checked for purity and quantified using ultraviolet spectroscopy.

RNase-resistant competitor RNA was synthesized for this gene using the RT-PCR Competitor Construction Kit (Ambion, Austin, TX) and supplied protocol. Using PCR with P3 and P4 primers specific for the gene, a cDNA template was created that was identical to the mRNA but contained the T7 RNA polymerase promoter sequence on the 5′ end, ~50 bp of additional sequence on the 3′ end for efficient reverse transcription (RT) priming, and an internal deletion of 10% to allow for agarose gel size separation of the competitor- and mRNA-derived PCR products. A trace amount of [^{32}P]GTP was added to the synthesis reaction to allow for accurate quantification of the acrylamide gel–purified, full-length competitor.

RT of RNA was done under standard conditions using Moloney murine leukemia virus RT (Life Technologies, Rockville, MD) and random decamer priming. Sample RNA was reverse-transcribed together with known amounts of synthetic RNA competitor. Both the gene-specific and competitor-derived cDNAs were then coamplified using PCR with a specific primer pair for each gene of interest, P1 and P2. PCR was done under standard conditions using *Taq* polymerase (Sigma), prebound with TaqStart antibody (Clontech, Palo Alto, CA) to increase specificity of the reaction. A PE9700 thermocycler (PE Biosystems, Foster City, CA) was used for 30 cycles. A touchdown procedure for primer annealing was used. Cycles consisted of denaturation at 94°C for 10 s; primer annealing at 65 to >60°C for 15 s; and extension at 72°C for 30 s. Primer pairs were designed to span at least one intron so that any PCR products derived from contaminating genomic DNA were easily distinguished from RNA-derived products. Also, PCR extension times were minimized to further reduce the chance of amplification of longer, genomic-DNA derived products.

Two PCR products were obtained for each reaction, and mRNA content was quantified by comparing the amount of DNA in each product band.

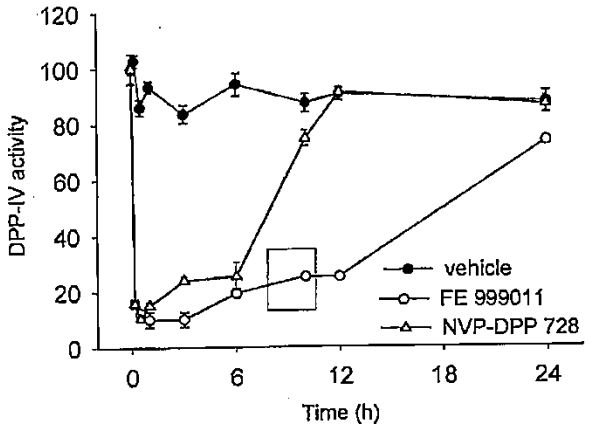

FIG. 2. Effect of FE 999011 and NVP-DPP728 on plasma DPP-IV activity in the Zucker fatty rat. Plasma DPP-IV activity was determined after oral administration of 10 mg/kg FE 999011 (○), 10 mg/kg NVP-DPP728 (△), or vehicle (●). The rectangle indicates when the OGTT was performed as illustrated in Fig. 3. Data are the means ± SE, $n = 6$ rats per group.

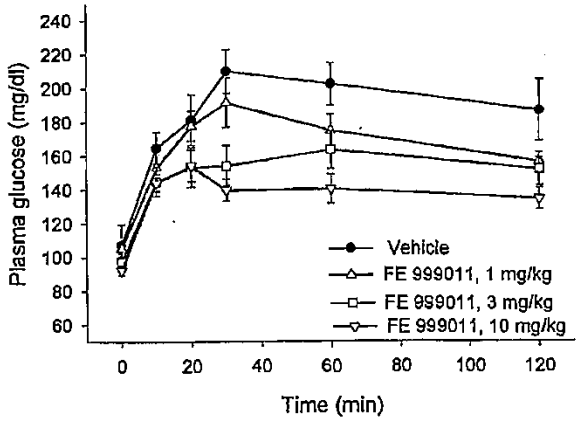

FIG. 3. Effect of previous oral administration of the DPP-IV inhibitor FE 999011 on glucose tolerance in the Zucker fatty rat. Plasma glucose excursion was assessed in response to oral glucose loading (1 g/kg) in the absence (●) or presence of three dosages (1 [△], 3 [□], and 10 [▽] mg/kg) of FE 999011 administered 8 h before. Data are the means ± SE, $n = 6$ rats per group.

Competitor- and mRNA-derived product sizes were 202 and 225 bp, respectively. Coamplification and comparison using another standard (e.g., 18S RNA) is not required for quantification using this technique. Amplified DNA samples were prestained by adding SYBR green dye (Molecular Probes, Eugene, OR) to the loading buffer and electrophoresed on a 3% agarose gel. Gels were analyzed using blue light epi-illumination with a Fuji LAS-1000 digital camera system and quantified using Aida 1D software (Raytest, Wilmington, NC).

The sequences of the primers used are as follows:

GLP-1R P1: 5′-AAT CGG GGT CAA CTT CCT TGT CTT CAT-3′;

GLP-1R P2: 5′-GTG AAG GAG AGC TCT GTG AAC AGC TTG-3′;

GLP-1R P3: 5′-GCG TAA TAC GAC TCA CTA TAG GGA GAG GAG AAT CGG GGT CAA CTT CCT TGT CTT CAT GCC AAG CTG AAG GCT AAT CTC ATG TGT-3′;

GLP-1R P4: 5′-GAC AAA GCA GTA CAA GAC AGC CAC CAT-3′.

Genotyping of rats for leptin receptor mutation. Genomic DNA was extracted from 50–200 μl whole blood using the Qiagen QIAamp DNA Blood Mini Kit and amplified by PCR using primers flanking the nucleotide substitution in the leptin receptor coding sequence that creates the *fa* mutation (5′ primer, 5′-CGT ATG GAA GTC ACA GAT GAT GGT AAT-3′; 3′ primer, 5′-CCT CTC TTA CGA TTG TAG AAT TCT CT-3′). The resulting 118-bp product was digested with *MspI* and electrophoresed on a 3% agarose gel. Amplicons from the normal allele are not digested and migrate as a single 118-bp band, whereas the mutant allele is cleaved, resulting in 39- and 79-bp fragments, thus allowing for unambiguous genotyping.

Statistics. All data are presented as means ± SE. They were subjected to ANOVA followed by Student-Newman-Keuls test. $P < 0.05$ was considered significant.

RESULTS

Studies in Zucker fatty rats

Plasma DPP-IV activity following FE 999011 and NVP-DPP728 administration. Oral administration of FE 999011 and NVP-DPP728 to Zucker fatty rats produced an immediate suppression of plasma DPP-IV activity (Fig. 2). Maximal inhibition was obtained 30 min postinjection for NVP-DPP728 and 1 h postinjection for FE 999011. FE 999011 and NVP-DPP728 significantly reduced DPP-IV activity for at least 12 h and 6 h, respectively ($F_{26,45} = 58.763$, $P < 0.001$). Twenty-four hours postinjection, plasma DPP-IV activity in rats treated with FE 999011 had returned to control values.

Effect of FE 999011 on glucose excursion during an OGTT. Pretreatment 8 h before the glucose load with FE 999011 at doses ranging from 1 to 10 mg/kg produced a dose-dependent reduction in plasma glucose excursion during the OGTT (Fig. 3). At the time when the glucose load was given, circulating DPP-IV activity was still suppressed (rectangle in Fig. 2). The integrated glucose responses were 10,046 ± 410 mg · dl^{-1} · 120 min^{-1} for the vehicle group and 7,643 ± 1,032, 6,821 ± 747, and 5,522 ± 644 mg · dl^{-1} · 120 min^{-1} for the groups treated with 1, 3, and 10 mg/kg, respectively. The observed decrease in integrated glucose response by graded doses of FE 999011 was highly significant ($F_{3,20} = 6.57$, $P < 0.01$).

Effect of FE 999011 on glucose excursion, plasma GLP-1, and insulin responses during an intraduodenal glucose tolerance test. A bolus intraduodenal glucose load (1 g/kg) was given to anesthetized lean or obese Zucker rats. One group of obese rats was pretreated with FE 9999011 (3 mg/kg intravenously) 15 min before the glucose load. A steady increase in glycemia was seen in all three groups, with a peak at 20 min for the lean group followed by a slow decrease. The glucose excursion in untreated obese rats was much larger, with a peak at 30 min (area under the curve 3,520.9 ± 382.6 vs. 2,684.0 ± 531.3 mg · dl^{-1} · 60 min^{-1} in lean controls). Glucose excursion in obese Zucker rats pretreated with FE 999011 was indistinguishable from that of lean controls (Fig. 4). The insulin rise in lean controls peaked at 2.1 ± 0.5 ng/ml at 30 min. In untreated obese Zucker rats, insulinemia rose within 20 min from 4.7 ± 2.1 to 13 ± 2.5 ng/ml. Upon treatment with FE 999011, this insulin rise was enhanced, with a peak value at 10 min and a plateau at ~25 ng/ml, persisting until 30 min. Basal values for plasma GLP-1 were indistinguishable between lean and obese untreated rats (2 pmol/l), with no visible increase during the intraduodenal glucose tolerance test (IDGTT). Conversely, pretreatment with FE 999011 produced a brisk rise in plasma GLP-1, with a peak at 12 min (Fig. 4).

Effects of chronic treatment with FE 999011 on glucose excursion and insulin response during an OGTT. The glucose excursion and insulin response of the

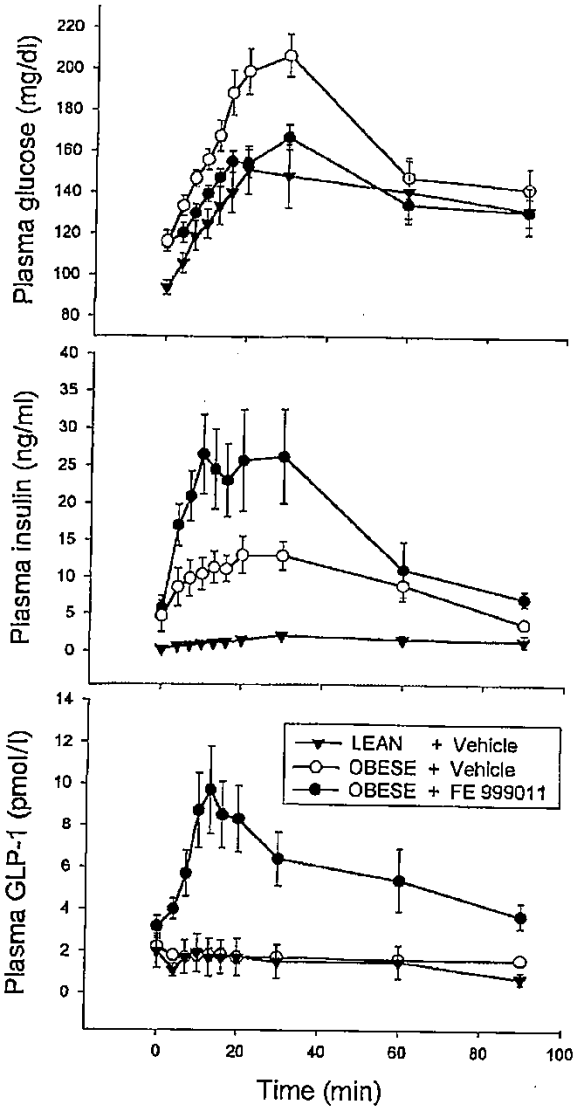

FIG. 4. Effect of previous intravenous administration of the DPP-IV inhibitor FE 999011 on glucose excursion, and plasma insulin and GLP-1 responses during an IDGTT in the Zucker fatty rat. Plasma glucose, insulin, and GLP-1 excursions were assessed in response to an intraduodenal glucose load (1 g/kg) in the absence or presence of 3 mg/kg FE 999011 administered intravenously 15 min before. The ELISA technique used for the measurement of bioactive GLP-1 equally detects GLP-1(7-36) and GLP-1(7-37) amide moieties but none of the inactive forms of GLP-1. Data are the means ± SE, n = 8 rats per group.

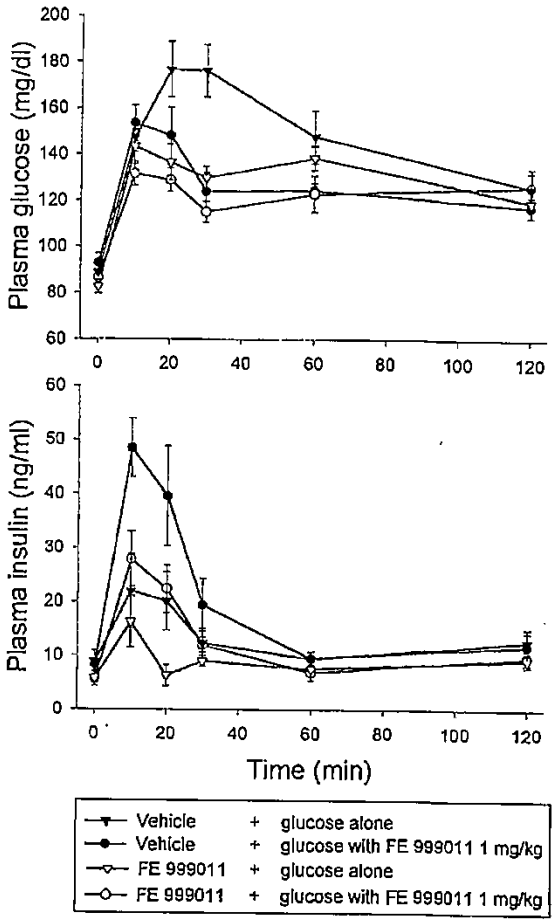

FIG. 5. Effect in the Zucker fatty rat of repeated oral administration for 7 days of FE 999011, 10 mg/kg twice a day (▽, ○), or vehicle (▼, ●) on glucose excursion and insulin response during an OGTT (1 g/kg) performed on the eighth day. At the time of oral glucose administration, rats received either FE999011 (1 mg/kg) with glucose (○, ●) or glucose alone (▽, ▼). Data are the means ± SE, n = 8 rats per group.

control rats that were pretreated with vehicle are shown in Fig. 5 (▼); they exhibit the typical pattern of glucose intolerance known for naive Zucker fatty rats. The addition of FE 999011 (1 mg/kg) to the glucose load in rats that were pretreated with vehicle improved glucose tolerance and enhanced insulin secretion (Fig. 5 [●]). Pretreatment with FE 999011 significantly improved glucose tolerance even when no inhibitor was added to the glucose load (Fig. 5 [▽]). Such improvement in glucose tolerance compared with vehicle-treated rats (▼) was possible with a lower insulin response, suggesting improved insulin sensitivity after 7 days of treatment. Basal insulinemia was decreased in FE 999011 (5.8 ± 0.8 ng/ml) compared with vehicle-treated rats (8.6 ± 1.1 ng/ml, $P < 0.05$). Combination of chronic treatment with FE 999011 for 7 days and addition of FE 999011 to the glucose load produced the lowest glucose excursion (Fig. 5 [○]). In comparison to the rats challenged with glucose alone (▽, ▼), glucose excursion was reduced in rats previously chronically treated with FE 999011 ($F_{11,60} = 14.4$, $P < 0.001$), glycemia at 20 and 30 min postglucose being significantly decreased in the FE 999011–treated group (▽ vs. ▼). The insulin responses were also reduced in rats repeatedly treated with FE 999011 ($F_{11,60} = 2.607$, $P < 0.01$). Insulin-to-glucose ratios were 0.12 ± 0.04 and 0.04 ± 0.01 for rats previously treated with vehicle and FE 999011, respectively. Chronic treatment with FE 999011 had no effect on food intake, water intake, or body weight gain.

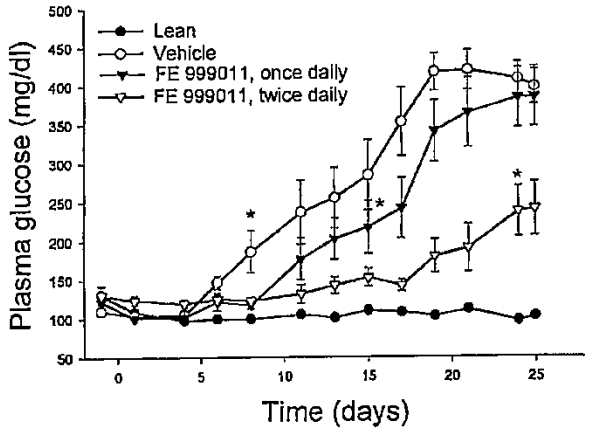

FIG. 6. Pattern of changes in glycemia in lean rats (●), and obese ZDF rats receiving vehicle (○) or treated with 10 mg/kg FE 999011 once a day in the evening (▼) or twice a day, morning and evening (▽). In this model, untreated ZDF rats become diabetic between 6 and 10 weeks of age. At time 0, rats were 6.5 weeks old. Data are the means ± SE, n = 8 rats per group.

Studies in male ZDF rats. The leptin receptor mutation was confirmed by genotyping all obese ZDF rats. Lean rats carried either the heterozygote *fa*/+ or the homozygote +/+ genotype.

Pattern of changes in glycemia. At the beginning of the study, glycemia in lean and obese rats was not different (Fig. 6). Eight days later, the obese control group developed hyperglycemia, with plasma levels for glucose of 186.4 ± 27.1 mg/dl in obese rats and 99.4 ± 0.9 mg/dl in lean rats ($P < 0.05$). At day 19, plasma glucose in the control obese group reached a plateau at 417.9 ± 24.1 mg/dl. Treatment with FE 999011 once or twice a day significantly delayed this rise in glycemia in the obese ZDF rats. Plasma glucose levels became significantly increased over lean rat values at day 15 for the once-a-day treated group (217.8 ± 34.0 vs. 110.1 ± 1.9 mg/dl, $P < 0.05$), but only at day 24 for the twice-a-day treated group (238.3 ± 32.4 vs. 97.3 ± 1.6 mg/dl, $P < 0.05$). Overall changes in plasma glucose levels over the 26 days of treatment with FE 999011 (Fig. 6) were highly significant ($F_{51,364} = 21.967$, $P < 0.001$). At the end of the study, glycemia remained highly significantly decreased by the twice-a-day treatment (241.7 ± 34.7 mg/dl) compared with the untreated obese group (399.2 ± 22.4 mg/dl, $P < 0.001$).

Pattern of changes in body weight gain and food and water intake. At the end of the experiment, the cumulative body weight gain was similar for the three obese groups (Fig. 7), but throughout the course of the experiment, body weight gain was differentially affected by treatment ($F_{63,448} = 302.371$, $P < 0.001$). The lean control group gained less weight than the obese groups. From day 11 of treatment, FE 999011 twice a day significantly stabilized weight gain. There was no significant difference between the control obese group and the obese group treated with FE 999011 once a day at any time point. Retroperitoneal and inguinal fat masses were increased in obese compared with lean rats. Treatment with FE 999011 had no effect on fat pad weight. Food consumption in the four groups clearly differed throughout the time course of

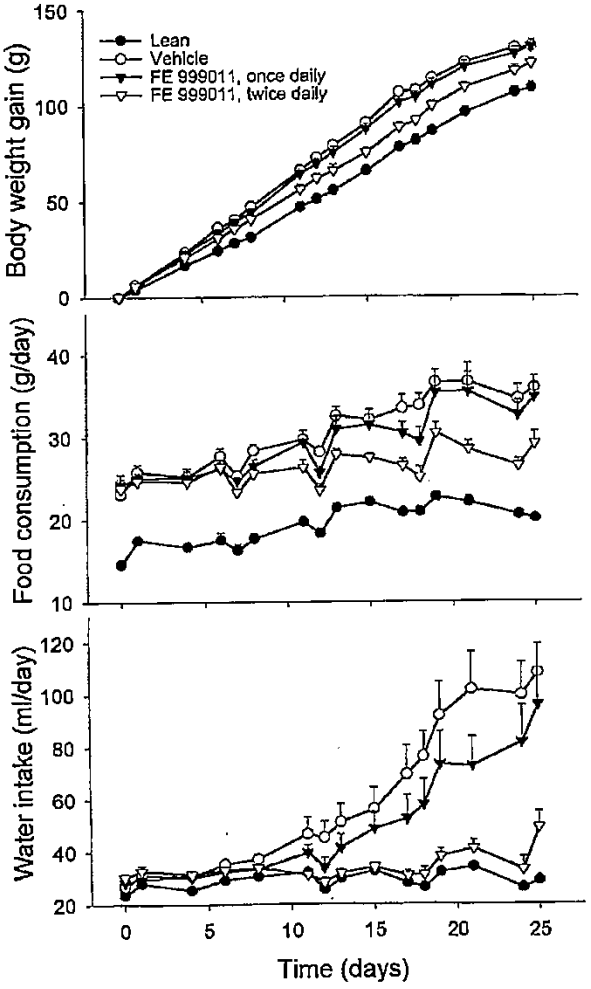

FIG. 7. Pattern of changes in body weight gain, food intake, and water intake of lean rats (●) and obese ZDF rats receiving vehicle (○) or treated with 10 mg/kg FE 999011 once a day (▼) or twice a day (▽). Data are the means ± SE, n = 8 rats per group.

the experiment ($F_{63,447} = 22.8$, $P < 0.001$). The obese groups ate significantly more than the lean control group. Food consumption increased with time in the control obese group and the group treated once a day with FE 999011. From day 17 to the end of the treatment period, FE 999011 twice a day stabilized food intake to pretreatment level. Water intake in the four groups strikingly differed throughout the time course of the experiment ($F_{63,444} = 14.9$, $P < 0.001$). Ten days after the start of the experiment, water intake steadily increased in the control obese group and the group treated with FE 999011 once a day. FE 999011 twice a day fully prevented this large increase in water consumption.

Pattern of changes in insulinemia. The three obese groups had increased basal plasma insulin levels at the beginning of the experiment in comparison to lean controls (Fig. 8). Insulinemia in the control obese group gradually increased to a peak value of 23.3 ± 3.6 ng/ml 8

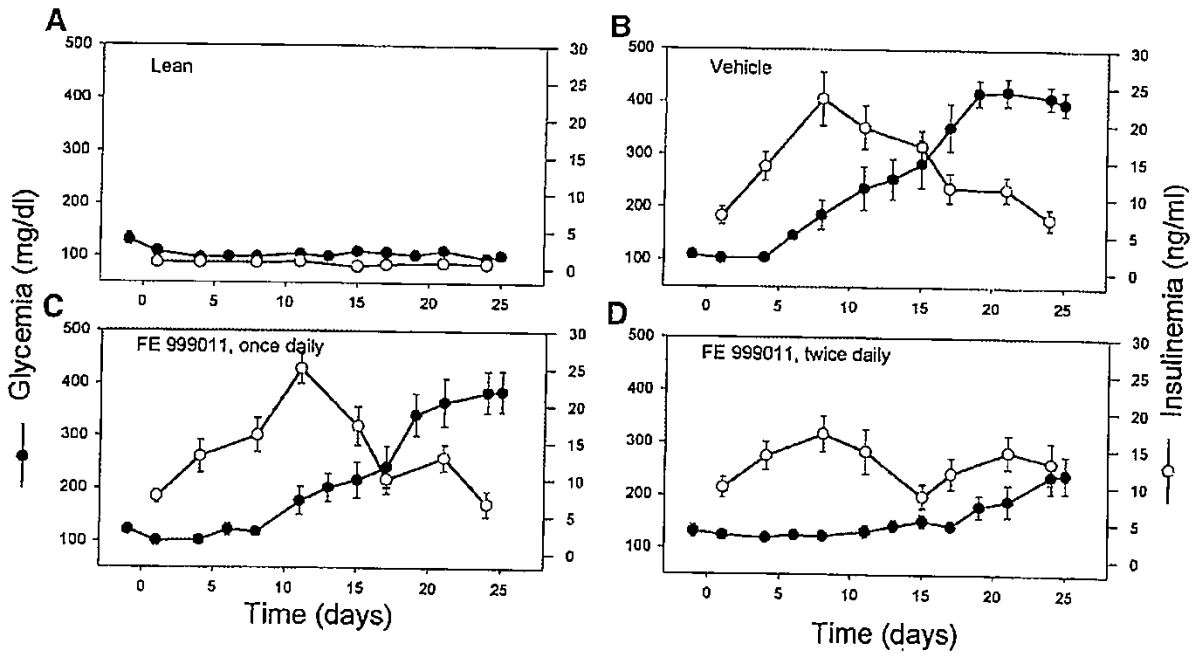

FIG. 8. Comparison of the pattern of changes in glycemia (●) and insulinemia (○) measured in lean control rats (*A*) and obese male ZDF rats receiving vehicle (*B*) or treated with 10 mg/kg FE 999011 once a day (*C*) or twice a day (*D*). Data are the means ± SE, n = 8 rats per group.

days after the initiation of treatment. Then, plasma insulin steadily decreased to a value of 7.2 ± 1.4 ng/ml at day 24 of the study. Obese rats treated with FE 999011 once a day showed a similar pattern of insulin secretion during this treatment period, with a peak of 24.9 ± 2 ng/ml at day 11 and a low value of 6.7 ± 1.7 ng/ml at day 24. The fall in insulinemia observed in the vehicle and once-a-day treated rats clearly coincided with the appearance of overt diabetes characterized by escalating plasma glucose levels. In obese rats treated with FE 999011 twice a day, basal insulin levels were generally lower and exhibited a biphasic pattern that differed from the two other obese groups. A first peak of insulinemia was seen at day 8 (17 ± 2.4 ng/ml), a trough after 15 days, and a second peak (14.6 ± 2.3 ng/ml) 21 days after initiation of treatment.

Plasma GLP-1 levels. Basal plasma levels for GLP-1 were measured in the fed state 25 days after initiation of treatment. Mean plasma level was lowest in untreated, obese diabetic rats (4.0 ± 0.1 pmol/l). Chronic treatment with FE 999011 increased basal GLP-1 plasma concentration to 5.8 ± 0.5 pmol/l in once-a-day treated rats and 6.2 ± 0.5 pmol/l in twice-a-day treated rats, with both increases being significant versus vehicle-treated obese rats ($F_{3,27}$ = 3.649, $P < 0.025$). The value for lean rats was 5.2 ± 0.7 pmol/l.

Plasma DPP-IV activity. At the end of the study (day 26), plasma DPP-IV activity was 0.79 ± 0.03 in lean and 1.02 ± 0.05 rate/min in untreated obese rats, whereas a value of 0.23 ± 0.02 rate/min was measured in obese rats injected with FE 999011 twice a day ($F_{2,21}$ = 105.8, $P <$ 0.001).

Plasma FFA and TG levels. Plasma FFA and TG levels in the three obese groups were significantly higher than in lean rats (Fig. 9 and Table 1). Throughout the study, in vehicle-treated rats, there was a large increase in plasma levels of both FFAs and TG, whereas in the lean group, no change in plasma FFAs and a small increase in TGs were observed ($F_{15,112}$ = 18.5, $P < 0.001$; $F_{10,119}$ = 42.4, $P <$ 0.001 for FFAs and TGs, respectively). Interestingly, mean plasma FFA levels did not increase significantly with the twice-a-day treatment (1.52 ± 0.16 to 1.84 ± 0.18 mEq/l), thus remaining in the concentration range seen in the prediabetic state (1.81 ± 0.17 mEq/l). Treatment with FE

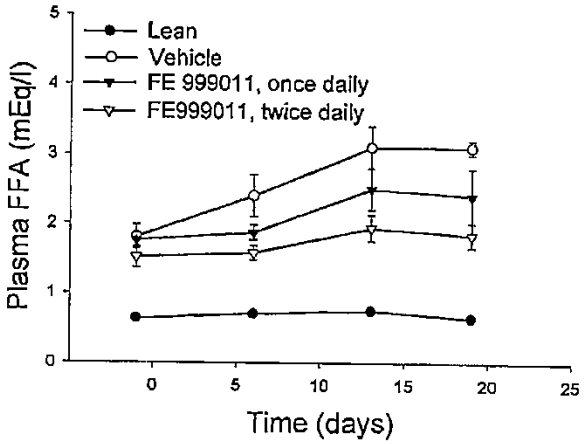

FIG. 9. Pattern of changes in plasma FFAs in lean rats (●) and obese ZDF rats receiving vehicle (○) or treated with 10 mg/kg FE 999011 once a day (▼) or twice a day (▽). Data are the means ± SE, n = 8 rats per group.

TABLE 1
Plasma FFA and TG levels in ZDF rats

	Plasma FFA (mEq/l)		Plasma TG (ng/ml)	
	Day −1	Day 19	Day −1	Day 19
Lean FA/?				
No treatment	0.64 ± 0.04	0.65 ± 0.04	61.6 ± 4.1	94.5 ± 4.6
Obese *fa/fa*				
Vehicle	1.81 ± 0.17*	3.10 ± 0.1*	348.6 ± 16.7*	1254.5 ± 45*
FE 999011 once a day	1.76 ± 0.1*	2.40 ± 0.4*†	342.2 ± 13.6*	993.6 ± 130.9*†
FE 999011 twice a day	1.52 ± 0.16*	1.84 ± 0.18*†	327.3 ± 39.7*	672.4 ± 69*†‡

*$P < 0.05$ vs. lean; †$P < 0.05$ vs. obese vehicle; ‡$P < 0.05$ vs. obese FE 999011 once daily.

999011 significantly and dose-dependently attenuated the increase in TG values in the obese rats.

Gene expression for GLP-1 receptor in the pancreas. Gene expression for GLP-1R (as characterized by the number of mRNA copies per microgram of extracted total RNA from the whole pancreas) was increased in obese compared with lean control rats (Fig. 10). Treatment of obese rats with FE 999011 increased gene expression from $4.9 \pm 1.0 \times 10^6$ to $7.8 \pm 1.2 \times 10^6$ mRNA copies/μg total RNA in the once-a-day treated obese rat group (NS) and to 14.4 ± 3.2 in the twice-a-day treated group, a significant increase over the untreated group ($F_{3,26} = 49.0, P < 0.001$).

DISCUSSION

The new DPP-IV inhibitor FE 999011 described in this article was able to inhibit circulating DPP-IV activity and therefore improve GLP-1 secretion after a glucose load; it then effectively reduced the glucose excursion following OGTT in Zucker fatty rats to values in lean controls.

One major advantage of FE 999011 resides in its long duration of action, which extends ~12 h, so that twice-a-day oral administration continuously inhibits DPP-IV activity. Such chronic administration of FE 999011 to Zucker fatty rats led to a robust improvement in glucose tolerance: after 7 days of twice-a-day oral administration, the glucose excursion following OGTT was normalized even in absence of inhibitor given at the time of the glucose load. The fact that such improved glucose tolerance occurred with a reduced insulin surge suggests a significant improvement in insulin sensitivity. In the male ZDF rat, chronic treatment with FE 999011 significantly delayed the rise in glycemia. Our data clearly indicate that prevention of the diabetic situation was possible only with the twice-a-day administration of FE 999011, suggesting that continuous inhibition of circulating DPP-IV activity is required for optimal efficacy. With the once-a-day treatment, only a short-lived delay in onset of hyperglycemia was observed, and the pattern of changes in insulin secretion, food consumption, water intake, and body weight gain were not modified compared with untreated ZDF rats. With the twice-a-day treatment, euglycemia was maintained for at least 21 days with low basal insulin secretion, suggesting improved insulin sensitivity. Normal control of water intake, a hallmark of adequate control of glucose metabolism in diabetic rats (27), was another sign of treatment efficacy. During the last 10 days of treatment, both basal insulin and glucose levels started to rise moderately.

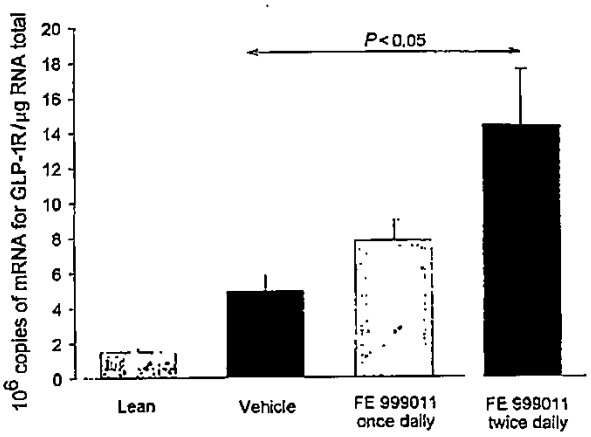

FIG. 10. Pattern of changes in pancreatic gene expression for GLP-1 receptor (GLP-1R), as quantified by RT-PCR. Data are the means ± SE, $n = 8$ rats per group.

One key outcome in this study was the improvement in glucose tolerance when circulating DPP-IV activity was chronically inhibited. This was clearly observed in both Zucker fatty and ZDF rats. It can be suggested that improvements of both glucose-induced insulin secretion and insulin sensitivity were determining factors for the prevention of diabetes in male ZDF rats. Stabilization of circulating FFAs to pretreatment levels obtained with the twice-a-day treatment was probably another determining factor for the maintenance of islet cell function in this model. The improvement of insulin secretion and efficacy observed in this study could have been mediated by enhanced GLP-1 secretion. Enhanced GLP-1 action at the level of rat pancreatic islets using GLP-1 analog infusion can increase insulin release (28). Plasma GLP-1 response upon an intraduodenal glucose load was greatly improved in FE999011-treated Zucker rats, and basal plasma GLP-1 levels were significantly increased by FE 999011, 25 days after initiation of treatment of ZDF rats.

GLP-1R is expressed in pancreatic islets and hypothalamus (29,30). In our study, gene expression for GLP-1R in the whole pancreas was found to be significantly increased in untreated obese compared with lean animals and was further upregulated by the twice-a-day FE 999011 treatment. The latter observation could suggest an increased sensitivity to the action of GLP-1, consistent with the improvement in insulin secretion and action. Because gene expression analysis was performed in total RNA extracted from the whole pancreas, GLP-1R mRNAs present in all types of islet cells (31,32) were detected.

Therefore, such an increase could have happened on each islet cell type, apparently improving each GLP-1 action at the pancreatic level. Another word of caution relates to whether increased gene expression automatically means increased cell sensitivity to the ligand. Regulation of GLP-1 action also depends on postreceptor signaling pathways. In the case of GLP-1, it has been shown in vitro that overexpression of GLP-1R can lead to desensitization (33). In view of the marked improvement in control of glucose homeostasis in this rat model following stabilization of GLP-1 action, one can hypothesize that an increased pancreatic GLP-1R population can be considered a positive factor. However, the mechanism and meaning of such change clearly require further evaluation.

GLP-1 is also known to reduce feeding through a brain-originating peptide acting on hypothalamic GLP-1 receptors (34,35). In this study, a hypothalamic anorectic effect of GLP-1 is very unlikely as a primary cause for the prevention of diabetes by FE 999011, as no anorectic effect was observed in Zucker rats after 7 days of treatment or at any time in ZDF rats chronically treated with FE 999011 twice a day. The role of circulating FFAs and TGs in the occurrence of the diabetic syndrome in leptin-resistant ZDF rats has been well discussed in the literature (36,37). In particular, the unusual accumulation of TGs in islet cells due to the absence of leptin action has been postulated to represent a major cause of failure of β-cells to produce and release insulin (38). Nonadipose tissues of normal rodents have a low TG content, even when fed a high-fat diet. In contrast, in rodents with a loss-of-function mutation in the leptin receptor, the TG content of certain nonadipose tissues is markedly increased, even on a normal fat intake (39). In ZDF rats, a 10- to 50-fold increase in TG content of the pancreatic islets has been reported (39). This is associated with impairment of β-cell function and ultimate loss of β-cells through lipoapoptosis, which results in diabetes (39,40). Thus lack of leptin action at the level of the β-cells in the ZDF rat and the resulting increase in pancreatic TG content could be the primary cause for loss of glucose-induced insulin secretion. During the course of our study, plasma levels for FFAs and TGs in the obese ZDF rat increased by two- and fourfold, respectively. The increase in plasma FFAs was prevented by the twice-a-day treatment with FE 999011 and maintained below the 2 mEq/l level that is toxic for β-cells (39,41). With the once-a-day treatment, prevention of this increase in plasma FFAs was less efficient. With the prevention of overt diabetes thanks to adequate insulin secretion and improvement of insulin sensitivity, insulin action on lipid metabolism is also enhanced and leads to a maintenance of moderate circulating FFA and TG levels. A combination of decrease in FFA and TG levels and enhanced GLP-1 action, both leading to preservation of β-cell function, represents the likely mechanism for the successful prevention of diabetes by FE 999011.

This study demonstrated that chronic inhibition of DPP-IV by FE 999011 can delay the occurrence of type 2 diabetes in ZDF rats. Permanent inhibition of circulating DPP-IV activity most likely leads to a sustained action of GLP-1 and possibly GIP at the pancreatic level. GLP-1 also stimulates increases in β-cell mass and insulin synthesis and thus favors adequate insulin stores in pancreatic islets. Furthermore, GLP-1 can enhance insulin action by inhibiting glucagon secretion (42). It is therefore speculated that in the clinic, DPP-IV inhibitors such as FE 999011 could delay or even prevent the progression from impaired glucose tolerance to type 2 diabetes by improving glucose tolerance and preserving β-cell function. Of importance for a DPP-IV–based treatment is assessment of its effect on the immune response (43,44) and also determination of whether other endocrine axes are modified, since inhibition of DPP-IV activity may enhance the action of other polypeptide hormones that are susceptible to cleavage and inactivation, such as neuropeptide Y (45) or growth hormone–releasing hormone (46).

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RESEARCH ARTICLE Open Access

Potency, selectivity and prolonged binding of saxagliptin to DPP4: maintenance of DPP4 inhibition by saxagliptin in vitro and ex vivo when compared to a rapidly-dissociating DPP4 inhibitor

Aiying Wang[1], Charles Dorso[1], Lisa Kopcho[2], Gregory Locke[2], Robert Langish[3], Eric Harstad[4], Petia Shipkova[3], Jovita Marcinkeviciene[2], Lawrence Hamann[5] and Mark S Kirby[1*]

Abstract

Background: Dipeptidylpeptidase 4 (DPP4) inhibitors have clinical benefit in patients with type 2 diabetes mellitus by increasing levels of glucose-lowering incretin hormones, such as glucagon-like peptide -1 (GLP-1), a peptide with a short half life that is secreted for approximately 1 hour following a meal. Since drugs with prolonged binding to their target have been shown to maximize pharmacodynamic effects while minimizing drug levels, we developed a time-dependent inhibitor that has a half-life for dissociation from DPP4 close to the duration of the first phase of GLP-1 release.

Results: Saxagliptin and its active metabolite (5-hydroxysaxagliptin) are potent inhibitors of human DPP4 with prolonged dissociation from its active site (Ki = 1.3 nM and 2.6 nM, $t_{1/2}$ = 50 and 23 minutes respectively at 37°C). In comparison, both vildagliptin (3.5 minutes) and sitagliptin (< 2 minutes) rapidly dissociated from DPP4 at 37°C. Saxagliptin and 5-hydroxysaxagliptin are selective for inhibition of DPP4 versus other DPP family members and a large panel of other proteases, and have similar potency and efficacy across multiple species.
Inhibition of plasma DPP activity is used as a biomarker in animal models and clinical trials. However, most DPP4 inhibitors are competitive with substrate and rapidly dissociate from DPP4; therefore, the type of substrate, volume of addition and final concentration of substrate in these assays can change measured inhibition. We show that unlike a rapidly dissociating DPP4 inhibitor, inhibition of plasma DPP activity by saxagliptin and 5-hydroxysaxagliptin in an ex vivo assay was not dependent on substrate concentration when substrate was added rapidly because saxagliptin and 5-hydroxysaxagliptin dissociate slowly from DPP4, once bound. We also show that substrate concentration was important for rapidly dissociating DPP4 inhibitors.

Conclusions: Saxagliptin and its active metabolite are potent, selective inhibitors of DPP4, with prolonged dissociation from its active site. They also demonstrate prolonged inhibition of plasma DPP4 ex vivo in animal models, which implies that saxagliptin and 5-hydroxysaxagliptin would continue to inhibit DPP4 during rapid increases in substrates in vivo.

Background

Diabetes is a worldwide epidemic, with the World Health organization estimating that more than 220 million people have diabetes worldwide http://www.who.int/mediacentre/factsheets/fs312/en/index.html, with greater than 90% of those having type 2 diabetes mellitus (T2DM). T2DM is thought to develop as a combination of insulin resistance and pancreatic β-cell failure [1]. Therefore, identification of novel treatments that would increase pancreatic insulin secretion while protecting pancreatic β-cells are of great interest.

Incretin hormones, such as glucagon-like peptide-1 (GLP-1), are secreted from cells in the gastrointestinal (GI) tract into the circulation in response to nutrient

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absorption. They are a major component of the mechanism regulating post-prandial insulin secretion when it is needed following meals [2]. Incretins account for up to 60% of the post-prandial insulin secretion in healthy individuals, but the incretin response is impaired in T2DM [3]. Incretin effects do not lead to insulin release per se, but potentiate the physiological release of insulin from the pancreas in response to increases in plasma glucose. Since GLP-1 has been shown to have the major incretin effect on glucose homeostasis in patients with type 2 diabetes [4], much work has been done to understand the effects of this incretin hormone on normal and pathophysiological glucose homeostasis.

Following its secretion, dipeptidylpeptidase-4 (DPP4) rapidly metabolizes the intact form of GLP-1 (GLP-1_{7-36}) to inactive GLP-1_{9-36} with a half-life of 1 to 2 minutes in vivo [5]. Therefore, two approaches have been taken to increase activity of the incretin axis, parenteral administration of DPP4-resistant GLP-1 analogues or oral administration of DPP4 inhibitors. DPP4 inhibitors have minimal risk of hypoglycemia because they enhance glucose-dependent insulin secretion and glucagon reduction. They are also weight neutral; i.e., they do not promote weight gain that is typically seen with many other antidiabetic agents. DPP4 inhibitors are also effective in combination with several other diabetes drug classes [6-8]. Finally, data from animal models indicate that GLP-1 is a trophic factor for β-cells, and potentiating endogenous incretins with DPP4 inhibitors does increase β-cell function and number, thereby contributing to improvement of β-cell function over the long-term [9].

There are many examples of enzyme inhibitors displaying time-dependence (e.g. [10,11]), with several becoming marketed drugs, including members of the DPP4 inhibitor class [12-14]. In many cases, prolonged pharmacodynamic effects on the target enzyme (when compared to the pharmacokinetics of the drug) confers an advantage over rapidly dissociating compounds, because time-dependent drugs typically require lower plasma levels and reduced drug peak-to-trough ratios, reducing the risk of off-target toxicity [11,15]. In humans, peak GLP-1 secretion occurs during the first phase of secretion, which occurs rapidly following a meal, giving a 2- to 3-fold increase that lasts 30 to 60 minutes [3]. This can be followed by a prolonged phase that gives a small increase in GLP-1 levels above fasting levels for up to 2 hours [reviewed in [16]. Therefore, we hypothesized that if a time-dependent inhibitor has a half-life for dissociation close to the duration of the first phase of GLP-1 secretion, the majority of the enzyme-inhibitor complex would not dissociate during the release of GLP-1 and this would maximize the compound's beneficial effects while minimizing plasma drug levels. DPP4 also has many other substrates in vitro, although only a few have been shown to be cleaved by DPP4 in vivo (reviewed in [17]). Therefore, it would be ideal if binding did not extend past the duration of first phase GLP-1 secretion, such that the inhibitor activity would follow its pharmacokinetics for inhibition of cleavage of other substrates of DPP4, should any such substrates have more prolonged in vivo half-lives relative to GLP-1.

Here we describe the inhibitory properties of saxagliptin and its 5-hydroxy metabolite, which are both slow binding DPP4 inhibitors with extended off-rates from DPP4 at 37°C, similar to the duration of the first phase of release of GLP-1 in vivo. We also use the ex vivo plasma DPP assays that are used in preclinical animal models and the clinic as a biomarker for efficacy, to demonstrate how slow binding compounds such as saxagliptin differ from rapidly dissociating DPP4 inhibitors. We show that saxagliptin does not have a dilution artifact or a large dependence on the pseudo-substrate used in the assay, unlike rapidly dissociating DPP4 inhibitors, and we discuss the significance of these findings.

Results

Saxagliptin is a potent inhibitor of human DPP4 in vitro irrespective of substrate

We measured the IC_{50} for inhibition of substrates across a range of substrate concentrations (10 μM to 1000 μM, dependent on substrate) that straddled the Km for the pseudo-substrate gly-pro-pNA (Km 180 ± 8 μM, kcat 40 ± 9 s^{-1}, room temp, n = 3), and GLP-1 (Km 24 ± 16 μM, kcat 2.9 ± 0.9 s^{-1}; room temp, n = 5), then calculated the Ki for inhibition of cleavage by each substrate (Table 1).

Each of the DPP4 inhibitors tested were equipotent inhibitors of GLP-1 and gly-pro-pNA, as expected for inhibitors that are competitive with substrate and bind in the active site of DPP4. Therefore, we used gly-pro-pNA as a substrate in subsequent experiments. Saxagliptin was approximately 10-fold more potent than vildagliptin or sitagliptin under these conditions at room temperature.

Potency and selectivity of saxagliptin and 5-hydroxysaxagliptin for human enzymes in vitro at 37°C

Routine screening was performed at room temperature. However, as DPP4 inhibition in vivo occurs at 37°C, we measured the potency and selectivity of DPP4 inhibitors in vitro at that temperature (Table 2). The Km and turnover rate of gly-pro-pNA pseudo-substrate for DPP4 increased (Km = 209 ± 18 μM; kcat = 67 ± 4 s^{-1}, n = 3), as did the Ki values for inhibition of DPP4 by DPP4 inhibitors (Table 2). Saxagliptin was 10-fold more potent than either vildagliptin or sitagliptin at 37°C. Saxagliptin generates an active metabolite in vivo [18], 5-hydroxysaxagliptin; it was 2-fold less potent than saxagliptin.

Table 1 Inhibition of isolated cloned human DPP4 at room temperature

compound	GLP-1 K_i (nM)	gly-pro-pNA K_i (nM)
Saxagliptin	0.41 ± 0.1 (7)	0.45 ± 0.1 (5)
Sitagliptin	2.5 ± 0.7 (4) ***	8 ± 1 (5)***
Vildagliptin	1.5 ± 0.5 (4) ***	7 ± 2 (5)***

mean ± standard deviation (number of independent experiments). ***P < 0.001 versus saxagliptin

The gly-pro-pNA pseudo-substrate is not specific to DPP4 and is also cleaved by other enzymes, including DPP8 (Km = 792 ± 60 μM; kcat = 5.1 ± 0.4 s^{-1}, n = 3) and DPP9 (Km = 221 ± 27 μM; kcat = 3.7 ± 0.7 s^{-1}, n = 3), although the cleavage rate of both enzymes is 20-fold lower than DPP4. Therefore, we also used this substrate to investigate inhibition of DPP8 and DPP9 by DPP4 inhibitors. Saxagliptin is approximately 400-fold selective and 75-fold selective for DPP4 versus DPP8 and DPP9, respectively, with the 5-hydroxymetabolite having approximately twice the selectivity (DPP8 approximately 950-fold and DPP9 approximately 160-fold). In comparison, vildagliptin had 400-fold selectivity for DPP8 and 20-fold selectivity for DPP9, while sitagliptin had 1900-fold selectivity for DPP8 and 3000-fold selectivity for DPP9.

Saxagliptin and 5-hydroxysaxagliptin were tested against multiple other enzymes (at room temperature). Both compounds had > 1000-fold selectivity against FAP, and > 6000-fold selectivity against DPP2 and all other proteases tested: these included neutral endopeptidase, angiotensin converting enzyme, aminopeptidase P, prolidase, prolyl carboxypeptidase, activated protein C, chymotrypsin, factor IXa, Factor VIIa, Factor Xa, Factor XIa, factor XIIa, plasma kallikrein, plasmin, thrombin, tissue kallikrein, tissue plasminogen activator, trypsin and urokinase (data not shown). They also had > 10,000-fold selectivity against a panel of 39 unrelated proteins that included 15 G-protein coupled receptors, 4 nuclear hormone receptors, 6 ion channels, 4 other enzymes and 10 transporters (data not shown).

Potency and selectivity of saxagliptin and 5-hydroxysaxagliptin for inhibition of cynomolgus monkey DPP enzymes in vitro at 37°C

The potency and selectivity for all 4 compounds for inhibition of cynomolgus monkey (rhesus monkey has the same DPP4 DNA sequence) DPP4, DPP8 and DPP9 is shown in Table 3 and is very similar to that found versus the human enzymes.

Similar data were also obtained for mouse and rat enzymes (data not shown). Therefore, we confirmed that the potency and specificity of saxagliptin and its 5-hydroxymetabolite were similar across species in vitro. We did not investigate the effects of DPP4 inhibitors on other peptidases from other species because no effect of saxagliptin and 5-hydroxysaxagliptin were seen on the human proteins tested.

Saxagliptin and 5-hydroxysaxagliptin are long-acting DPP4 inhibitors in vitro

During the course of initial experiments, we noticed that there was time dependence to inhibition of DPP4 by some DPP4 inhibitors. In order to determine time-dependence, we preincubated DPP4 inhibitors with DPP4 and measured the rate of dissociation of DPP4 inhibitors from DPP4 using an 'infinite dilution' method.

The data in table 4 show that saxagliptin and 5-hydroxysaxagliptin have slow binding when tested at 37°C, with $t_{1/2}$ for dissociation of 50 minutes and 23 minutes, respectively. While vildagliptin shows some evidence of slow binding ($t_{1/2}$ = 3.5 minutes), this was much less pronounced. Sitagliptin showed no time dependence (within the limitations of the experimental protocol at < 2 minutes). The time dependence was only found for inhibition of DPP4 and was not seen during experiments investigating the inhibition of DPP8 and DPP9; therefore, these prolonged effects would only relate to inhibition of cleavage of DPP4 substrates by DPP4.

Saxagliptin does not have a dilution artifact in plasma DPP assays in vitro

The ex vivo assay measuring inhibition of plasma DPP4 activity has been used as a key biomarker assay for DPP4 inhibitor assessment by multiple groups in both animal models and in the clinic. Given that the duration of the ex vivo assay is typically between 10 and 20 minutes, there would be no dilution artifact predicted for ex vivo determination of inhibition from saxagliptin dosed animals, because negligible dissociation of saxagliptin from DPP4 would occur over the time frame of the experiment (Table 4 and the rate of dissociation is

Table 2 Inhibition of isolated, cloned human DPP4, DPP8 and DPP9 at 37°C

	DPP4 K_i (nM)	DPP8 K_i (nM)	DPP9 K_i (nM)
Saxagliptin	1.3 ± 0.3 (12)	508 ± 174 (13)	98 ± 44 (11)
5-hydroxysaxagliptin	2.6 ± 1.0 (12)***	2495 ± 727 (14)*	423 ± 64 (12)
Vildagliptin	13 ± 2.8 (12)***	5218 ± 2319 (14)***	258 ± 93 (12)
Sitagliptin	18 ± 1.6 (12)***	33780 ± 5532 (12)***	55142 ± 19414 (11)***

mean ± standard deviation (number of independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001 versus saxagliptin

Table 3 Inhibition of isolated, cloned cynomolgus monkey DPP4, DPP8 and DPP9 at 37°C

	DPP4 K_i (nM)	DPP8 K_i (nM)	DPP9 K_i (nM)
Saxagliptin	1.1 ± 0.2 (14)	390 ± 82 (6)	61 ± 5 (6)
5-hydroxysaxagliptin	2.9 ± 1.1 (13)***	2061 ± 658 (6)***	323 ± 60 (6)*
Vildagliptin	6.8 ± 2.0 (14)***	3692 ± 917 (7)***	125 ± 39 (7)***
Sitagliptin	15.6 ± 3.6 (14)***	21949 ± 17461 (6)***	65757 ± 7966 (6)***

mean ± standard deviation (number of independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001 versus saxagliptin

decreased with lower temperature). To confirm this for saxagliptin, we took naive human plasma and compared samples with just the addition of compound to plasma only, to samples where compound was added to both plasma and buffer (such that total concentration of drug was kept constant during the 'dilution step').

Saxagliptin is unaffected by the 3-fold dilution in the human plasma assay (Figure 1a) using ala-pro-AFC as substrate. However, sitagliptin clearly has a dilution artifact (Figure 1b). When sitagliptin was added only to the plasma, the inhibition curve shifted 3-fold to the right compared to when compound was added to both the plasma and the dilution buffer (IC_{50} = 152 ± 41 nM versus 414 ± 116 nM: mean ± s.d., n = 3), except where there is virtually no inhibition or full inhibition, consistent with the 3-fold dilution during substrate addition. This is presumably due to a new equilibrium being rapidly established following dilution, such that the potency of sitagliptin will be underestimated when compound is only present in the plasma. Similar data were also obtained using both cynomolgus and rhesus monkey plasma (data not shown).

Maximum inhibition of plasma DPP4 activity in plasma samples by DPP4 inhibitors differs between species

Untreated human plasma samples gave a plasma DPP enzyme activity rate of 5.0 ± 0.6 nmoles/min per ml plasma (mean ± s.d., n = 3 independent experiments) when ala-pro-AFC was used as substrate. Untreated cynomolgus and rhesus monkey plasma DPP4 rates were similar to those seen in human, with rates of 5.2 ± 0.3 and 7.3 ± 0.2 nmoles/min per ml plasma, respectively. However, the ability of DPP4 inhibitors to inhibit cleavage of peudo-substrates differs among species. Figure 2 shows that the maximum inhibition of plasma DPP activity seen with saxagliptin was approximately 85% in rhesus and 80% in cynomolgus monkeys, but > 95% in humans (Figure 2). Like human, rodent (mouse and rat) and dog plasma DPP is inhibited > 95% by saxagliptin (data not shown).

These effects were shown to be independent of DPP4 inhibitor and similar data were obtained with gly-pro-pNA as the pseudo-substrate (Table 5; Figure 3). Since the pseudo-substrates are not specific for DPP4, presumably these findings reflect a species difference in the relative activity of all the other plasma peptidases that cleave these pseudo-substrates.

The in vitro IC_{50} for inhibition of human, rhesus monkey and cynomolgus monkey plasma DPP activity by saxagliptin, vildagliptin and sitagliptin was similar across species (Table 5). Therefore, although there were different amounts of maximal inhibition among species, DPP4 inhibitors have similar potency in rhesus monkey, cynomolgus monkey and human plasma for inhibition of plasma DPP activity.

Table 4 On and off rates of DPP4 inhibitors at 37°C

Compound	kon, 10^5 M^{-1} s^{-1}	koff, 10^{-5} s^{-1}	$t_{1/2}$ (min.)
	37°C		
Saxagliptin	4.6 ± 0.6	23 ± 1	50
5-hydroxysaxagliptin	0.7 ± 0.1	50 ± 2	23
Vildagliptin	1.2 ± 0.2	330 ± 30	3.5
Sitagliptin	> 100	> 580	< 2

mean ± standard error. Standard errors for kon were calculated from equations (2), (3) and (4), and for koff are from the fits to equation (5)

Choice of assay affects the IC_{50} measured for inhibition of plasma DPP activity by DPP4 inhibitors at steady-state in vitro

The measured IC_{50} varies with the ratio of substrate concentration to substrate Km for competitive inhibitors (see Methods). Using the assays described here, Km values were calculated as 57 ± 13 μM (n = 3 independent experiments) for the ala-pro-AFC assay, and 180 ± 10 μM (n = 3) for gly-pro-pNA assay in human plasma. Km values were similar in cynomolgus monkey plasma, at 35 ± 6 μM (n = 3) for ala-pro-AFC assay and 134 ± 5 μM (n = 3) for gly-pro-pNA assay. Since the majority of DPP4 inhibitors are competitive with substrate, a difference in substrate concentration will affect the measured IC_{50} of these inhibitors. In the two pseudo-substrate assays we used to measure inhibition of plasma DPP activity, the ratio of Km to substrate concentration is approximately 7-fold in the ala-pro-AFC assay (370 μM substrate concentration), but is only 2-fold in the gly-pro-pNA assay (400 μM substrate concentration). Therefore, using gly-pro-pNA as substrate would give an apparent increase in potency of DPP4 inhibitors compared to ala-pro-AFC. Further, the difference in temperature (30°C versus room temperature) and pH (7.4

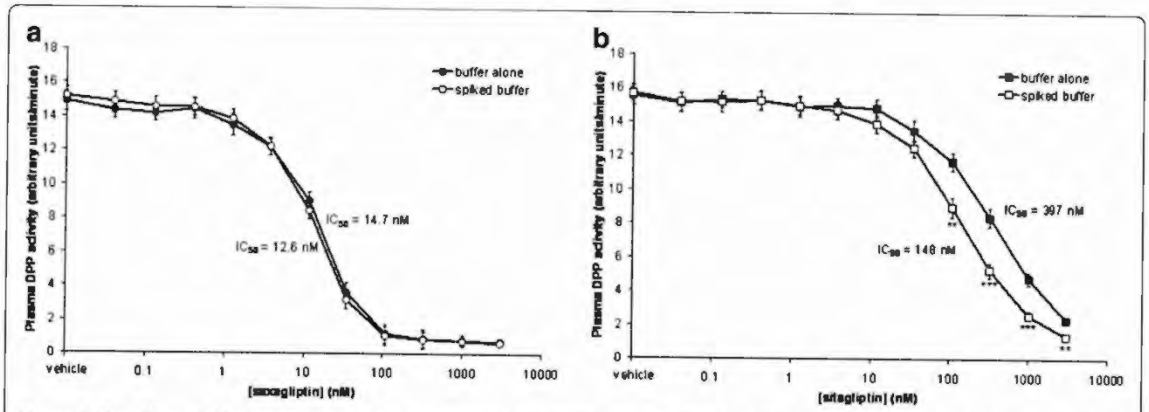

Figure 1 The effect of dilution on the IC_{50} for inhibition of human plasma DPP activity by saxagliptin (Panel A) and sitagliptin (Panel B) using ala-pro-AFC as substrate (activity is measured as arbitrary fluorescence units. Mean ± s.e.m., n = 3 independent experiments, 3 replicates per experiment).

versus 7.9) of the two assays would also affect the measured IC_{50}. Figure 3 shows the inhibition of cynomolgus monkey plasma DPP activity in vitro for saxagliptin and sitagliptin under pseudo-steady-state conditions. There was a small change in IC_{50} for saxagliptin (2.5 ± 0.2 nM to 9.8 ± 0.3 nM, $P < 0.0001$), with a narrow concentration range over which a difference would be seen between the two assays (1 to 10 nM. Figure 3A). This presumably reflects differences in temperature and pH between the two assays. However, at concentrations of sitagliptin between 5 and > 3000 nM, much more inhibition of DPP activity is seen with the gly-pro-pNA assay than with the ala-pro-AFC assay (Figure 3B). Further, the IC_{50} for inhibition in the ala-pro-AFC assay was significantly increased 26-fold compared to the gly-pro-pNA assay, from 17 ± 2 nM to 440 ± 163 nM ($P < 0.0001$). Similar data were obtained using human plasma (data not shown).

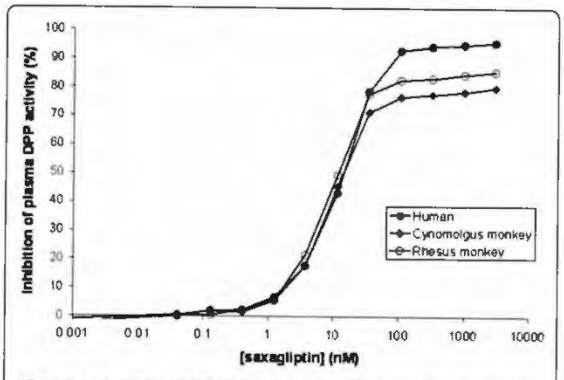

Figure 2 Percent inhibition of plasma DPP activity in human, rhesus monkey and cynomolgus monkey plasma by saxagliptin using ala-pro-AFC as substrate.

Measurement of plasma DPP activity in ex vivo assays

The differences in dissociation rate from DPP4 and the substrate used have substantial implications for measurement of activity following dosing in animals and humans. Figure 4 shows data from an in vivo study where various doses of saxagliptin and sitagliptin were given to cynomolgus monkeys and plasma DPP inhibition was measured after 24 hours, at trough.

When the ala-pro-AFC assay was used to measure plasma DPP inhibition ex vivo, saxagliptin treatment resulted in close to maximal inhibition of the inhibitable plasma DPP activity at its highest doses, with the 1, 3 and 10 mg/kg doses being statistically different from vehicle. However, sitagliptin treatment had no effect on plasma DPP activity at any of the doses. When the gly-pro-pNA assay was run on exactly the same samples, similar results were obtained for saxagliptin and there was no statistical difference between the data obtained with either assay at any dose. In contrast to the ala-pro-AFC assay, sitagliptin treatment gave statistically significant inhibition of plasma DPP activity at 3, 10 and 40 mg/kg doses when compared to vehicle in the gly-pro-pNA assay. Further, plasma DPP inhibition in the gly-pro-pNA assay at the 10 and 40 mg/kg doses were statistically significantly different from those obtained using the ala-pro-AFC assay ($P = 0.07$ for the 3 mg/kg dose). However, the highest dose tested still did not give maximal inhibition of plasma DPP activity. Therefore, choice of assay had significant relevance for the interpretation of inhibition by sitagliptin in this study.

Discussion

Saxagliptin (BMS-477118) is a potent inhibitor of DPP4 that is approximately 10-fold more potent than vildagliptin or sitagliptin. Saxagliptin also has an active

Table 5 Maximum levels of inhibition of human and monkey plasma DPP activity using ala-pro-AFC as substrate

	Rhesus		Cyno		Human	
	IC_{50} (nM)	Max % inhib	IC_{50} (nM)	Max % inhib	IC_{50} (nM)	Max % inhib
saxagliptin	29 ± 03	86 ± 1†††	24 ± 02	81 ± 6††	40 ± 08	100 ± 0
vildagliptin	17 ± 5**	85 ± 1†††	13 ± 4**	79 ± 6††	20 ± 7*	98 ± 1
sitagliptin	17 ± 2***	87 ± 1†††	17 ± 2***	80 ± 7††	22 ± 3***	98 ± 0

N = 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus saxagliptin. $^{††}P < 0.01$, $^{†††}P < 0.001$ versus human

metabolite, 5-hydroxysaxagliptin, which is present in human plasma at levels between 2- and 7-fold higher than saxagliptin (Fura 2009). 5-hydroxysaxagliptin is 2-fold less potent than saxagliptin, but approximately 5-fold more potent than sitagliptin and vildagliptin.

The data here shows that temperature and the choice of substrate can have significant effects on IC_{50} values, accounting for some of the differences in values typically reported (reviewed in [17]). However, Thomas et al. [14] reported very high IC_{50} values for saxagliptin and vildagliptin in particular. In the case of saxagliptin, the value was 50-fold higher than the Ki value reported here and 50-fold higher than the value reported for their DPP4 inhibitor, linagliptin. Saxagliptin (Onglyza$^{®}$) is available commercially in many world markets at doses of 2.5 mg and 5 mg [6] and the minimal efficacious dose of linagliptin is 5 mg [19]. It is difficult to conceive how saxagliptin could be 50-fold less potent than linagliptin in vitro, but at least equipotent in the clinic; therefore, it seems likely that the data reported here are more accurate determinations of the activity of saxagliptin and vildagliptin. A known concentration of pure DPP4 is typically used in the majority of these in vitro studies (we used 100 pM of isolated cloned human DPP4) because a low concentration of DPP4 is important for avoiding obtaining artifactually high inhibition values. Part of the reason for the discrepancy may be that Thomas et al. [14] are the only group to use "DPP4 extracted from Caco cell membranes", with DPP4 purity and concentration undisclosed.

The increased potency and prolonged binding of saxagliptin compared to sitagliptin and vildagliptin may be a reflection of its strong interactions with both Ser^{630} and Glu^{205}/Glu^{206}, whereas sitagliptin interacts primarily with Glu^{205}/Glu^{206}, and vildagliptin with Ser^{630} [17]. Further, we showed that saxagliptin demonstrated slow-onset inhibition at room temperature that was partially due to a covalent, but reversible enzyme-adduct formation at Ser^{630}, but also partially due to another conformational change induced by saxagliptin [20]. Both saxagliptin and 5-hydroxysaxagliptin also have slow binding kinetics at 37°C and would be expected to have prolonged pharmacodynamic effects in vivo.

The data in Figure 4 shows that saxagliptin bound to DPP4 prior to substrate addition remained bound during the time course of the ex vivo assay. The half life for dissociation of saxagliptin from DPP4 ($t_{1/2}$ of 50 minutes at 37°C) is similar to the duration of first phase of elevated GLP-1 in the plasma following a meal in humans [4]. This may be relevant to the cleavage of endogenous substrate(s) because saxagliptin (and 5-hydroxysaxagliptin) given before a meal will already be bound to DPP4 at its site(s) of action. When incretins are released in response to a meal, incretins will increase and compete with inhibitors with rapid off-rates. However, this would be unlikely to

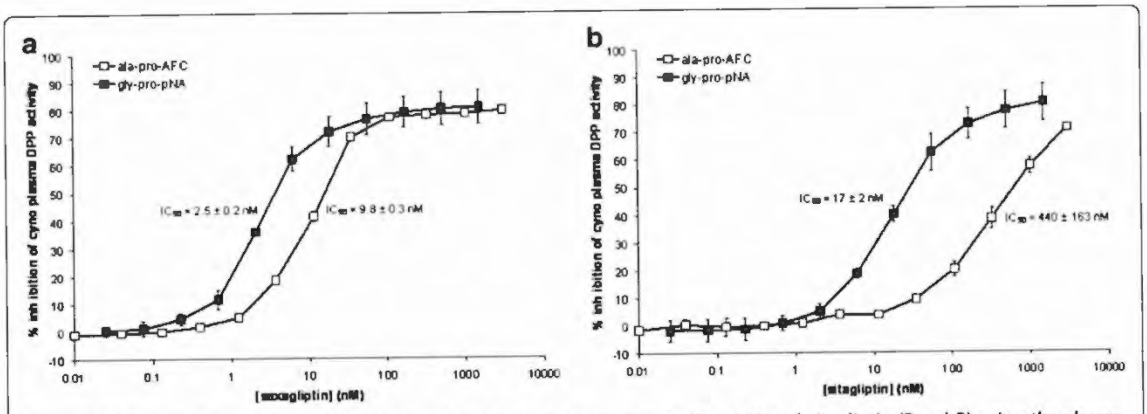

Figure 3 Inhibition of cynomolgus monkey plasma DPP activity by saxagliptin (Panel A) and sitagliptin (Panel B) using the ala-pro-AFC and gly-pro-pNA assays (mean of 3 independent experiments).

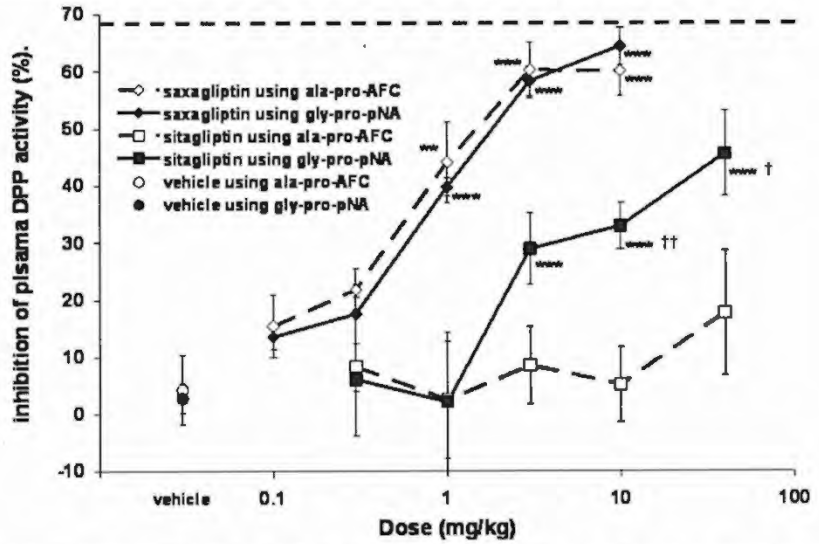

Figure 4 The effect of pseudo-substrate choice on the measurement of plasma DPP activity ex vivo in cynomolgus monkeys 24 hours post dose. **P < 0.01, ***P < 0.001 versus vehicle. †P < 0.05, ††P < 0.01, versus ala-pro-AFC at the same dose. The dashed line represents the average maximum inhibition seen (68.9 ± 3.9%, 1 hour post dose at the highest doses tested).

occur for saxagliptin, unless substrate concentrations were raised for significantly longer than the duration of the first phase of GLP-1 secretion, when saxagliptin would equilibrate with substrate as with the other DPP4 inhibitors. In clinical practice, DPP4 inhibitors currently have a placebo-like side-effect profile in trials up to 2 years in length. Therefore, rapidly dissociating DPP4 inhibitors can be given at high enough doses that any theoretical advantages from extended binding are currently not seen in the clinic. However, the chronic adverse event profiles of current DPP4 inhibitors are not yet fully defined and differences between DPP4 inhibitors may yet be seen.

Saxagliptin and 5-hydroxysaxagliptin are selective for DPP4 versus DPP8 and DPP9 at 37°C. Neither saxagliptin nor 5-hydroxysaxagliptin exhibited slow binding kinetics to DPP8 or DPP9, so any DPP8/9 related pharmacodynamics would closely match their pharmacokinetics. The potential for inhibition of DPP8 and DPP9 in vivo is difficult to assess in the absence of a known physiologically relevant substrate and knowledge of the specific tissues and cells where either may play a physiological role. Further, as DPP8 and DPP9 are cytosolic enzymes [21], the cytosolic concentration of saxagliptin and 5-hydroxysaxagliptin in those cells would also be required to accurately predict the potential for inhibition. Since this information is unknown (and to test for other off-target issues), extensive toxicity studies are typically undertaken in several species and adverse events are scrutinized in clinical studies. As discussed previously (reviewed in [17]), the preponderance of evidence for saxagliptin would show that there is no toxicity attributable to inhibition of DPP8 or DPP9 at clinically relevant doses across species.

There was no significant species difference for potency of inhibition of human and cynomolgus monkey DPP4, DPP8, DPP9 or plasma DPP activity for three DPP4 inhibitors (saxagliptin, vildagliptin or sitagliptin). However, there were significant differences in the maximum amount of inhibition of plasma DPP activity seen between rhesus and cynomolgus monkey plasma (80 to 85%) and the other species tested (95%). This presumably shows a difference between the levels of dipeptidylpeptidases found in the plasma of the two species. The identities of the peptidases that underlie this difference are unknown and were not a focus of these experiments. However, as there was no differentiation between saxagliptin, vildagliptin and sitagliptin, it is unlikely to be attributable to species differences in DPP8 or DPP9 expression (also consistent with current understanding that DPP8 and DPP9 are not secreted or thought to be present in plasma [17]).

Based on the data presented here, interpretation of data from ex vivo assays for DPP4 inhibitors should be done with caution because the choice of substrate, as well as the nature of the ex vivo assay (such as dilution artifact), can all affect measured efficacy. The difference in plasma DPP inhibition for sitagliptin between these two assays (Figure 4) can be explained by sitagliptin's

rapid dissociation and reassociation with the active site of DPP4. Due to the difference in the ratio of Km to substrate concentration between the two assays, one substrate is less effective at competing out the inhibitor and the inhibitor appears more potent in that assay. Consistent with this conclusion was the in vitro data that showed that this issue would most likely manifest itself across the range of plasma concentrations typically found at trough (24-hour post dose) for sitagliptin in cynomolgus monkeys. In contrast, saxagliptin would not dissociate appreciably from the active site during the time course of the assay and there would be no inhibitor-substrate competition.

Krishna et al. [22] recently illustrated the benefits of accelerating drug development using biomarkers with the example of DPP4 inhibitors and the plasma DPP4 assay. While this approach can be fundamentally useful, some important caveats to this approach have been demonstrated here. The pseudo-substrate gly-pro-pNA was used in both clinical assays to determine plasma DPP inhibition for saxagliptin and sitagliptin. However, the conditions are very different: i.e. the substrate concentration is 2000 μM (10-fold the Km), with an 11-fold dilution step and 120 minute assay duration in the saxagliptin assay [23]; the substrate concentration is 400 μM (2-fold Km), with a 2.5-fold dilution step and a 14 minute assay duration in the sitagliptin assay [24]. The data presented here show that, given the comparably longer duration of the assay, the larger dilution factor and the higher substrate concentration in the saxagliptin clinical assay, it is not possible to perform a meaningful direct comparison between clinical data for the two drugs using these different assays. This may explain why Krishna et al. [22] reported that they need 80% inhibition of plasma DPP4 activity at trough (24-hours post dose) to obtain maximal glucose lowering for sitagliptin. However, only 50% to 60% inhibition of plasma DPP activity was seen 24-hours post dose for vildagliptin at the 100 mg dose [25] and for saxagliptin at the 5 mg dose [23]. Despite these differences, all the DPP4 inhibitors have similar glucose-lowering efficacy in the clinic at those doses, leading Neumiller et al. [6] to conclude that "there does not appear to be a compelling advantage of one DPP-4 inhibitor over another".

Further, saxagliptin is localized to the GI tract when given intra-arterially in animal models [18]. Holst and Deacon [26] proposed that GLP-1 cleavage in the GI tract is important: therefore, saxagliptin, by virtue of its location in the GI tract, may give rise to total inhibition of GLP-1 cleavage, while cleavage of another substrate in a different tissue could be unaffected, and the ex vivo plasma DPP assay shows only partial inhibition of DPP4. Further, the tissue distribution of all DPP4 inhibitors is unlikely to be the same and different DPP4 inhibitors may have different effects if there is tissue-specific localization of inhibitor or substrate. Thus, the action of a DPP4 inhibitor in the relevant tissue can only be approximated by the evaluation of ex vivo plasma DPP activity.

Conclusions

Saxagliptin and its active metabolite are potent, selective inhibitors of DPP4, with prolonged dissociation from its active site. They also demonstrate prolonged inhibition of plasma DPP4 ex vivo in animal models, which implies that saxagliptin and 5-hydroxysaxagliptin would continue to inhibit DPP4 during rapid increases in endogenous substrates (such as GLP-1 during its physiologic release). However, this remains to be demonstrated for a physiologic substrate in vivo.

Interpretation of data from ex vivo assays for DPP4 inhibitors should be done with caution because the choice of substrate, as well as the nature of the ex vivo assay (such as dilution artifact), can all affect measured efficacy. Therefore, while ex vivo plasma DPP assays may be useful as a biomarker for a compound, for the reasons discussed in this paper, clinically relevant efficacy, efficacy for endogenous substrates, or relative efficacy across DPP4 inhibitors cannot be predicted solely using a plasma DPP activity assay.

Methods

Reagents

Saxagliptin (Onglyza®, BMS-477118, or (1 *S*,3 *S*,5 *S*)-2-[(2 *S*)-2-Amino-2-(3-hydroxytricyclo[3.3.1.1^{3,7}]dec-1-yl) acetyl]-2-azabicyclo[3.1.0]hexane-3-carbonitrile, monohydrate [13], vildagliptin [27], sitagliptin [28], active and DPP4 cleaved glucagon-like-peptide-1 (GLP-1$_{7-36}$ and GLP-1$_{9-36}$, respectively) were synthesized at Bristol-Myers Squibb. All other chemicals were reagent grade, obtained from Sigma (St Louis, MO). For the in vitro assays, plasma from rhesus and cynomolgus monkeys was obtained frozen from Bioreclamation Inc. (Hicksville, NY). Plasma from human blood was obtained from healthy volunteers within BMS (after obtaining written consent, following the BMS standard operating procedure RD-DIR-003, in accordance with the Helsinki Declaration) by collecting blood into EDTA tubes. These tubes were kept on ice and were centrifuged at 3000 rpm, 4°C for 15 minutes (within 15 minutes of collection). Plasma was aliquoted into polypropylene tubes, quick-frozen in powdered dry ice, and stored at -80°C until the assay was performed.

Steady-state inhibition of isolated cloned human and cynomolgus monkey DPP4, 8 and 9 in vitro at 37°C

All solutions were preheated to 37°C. Inhibitors were preincubated with enzyme for 40 minutes prior to assay initiation. The assay was initiated by substrate addition and contained: 100 mM Aces, 52 mM Tris, 52 mM

ethanolamine, pH 7.4, enzyme, substrate (gly-pro-pNA at either 120 μM or 1000 μM), inhibitor (various concentrations) and 1% DMSO (final volume of 100 μl). Production of p-nitroaniline was measured at 405 nm at 30 second intervals over 40 minutes using a Spectramax 340 plate reader (Molecular Probes, Carlsbad, CA). Softmax Pro software from Molecular Devices (Sunnyvale, CA) was used to obtain the initial slope of the reaction in each well, and the fits were inspected to insure that the reactions were linear to a correlation coefficient of 0.99. An Excel template with XLFit (IDBS, Guildford, U. K.) was used for determination of the IC_{50} for inhibition at each substrate concentration. Three models of enzyme inhibition (competitive, uncompetitive, and non-competitive) were used, but all of the inhibitors in our DPP4 program yielded competitive inhibition against gly-pro-pNA. Therefore, IC_{50} was then converted to K_i assuming competitive inhibition according to:

$$Ki = \frac{IC50}{\left(1 + \frac{S}{Km}\right)} \quad (1)$$

The calculated K_i values for competitive inhibition at each substrate concentration were averaged and reported. DPP8 and DPP9 were tested under identical conditions. The in vitro assays were also used to determine substrate Km, by varying substrate concentration across an appropriate range. Km was calculated using GraFit software (Erithacus, Surrey U.K.)

Slow binding kinetics at 37°C for human DPP4

All solutions were preheated to 37°C and the assay was run at 37°C. When slow onset inhibition occurs, final Ki* is a function of the koff and kon (Ki* = koff/kon). Progress of DPP4 inhibition was measured under steady-state conditions (E < < I/(1 + S/Km)), initiating the reaction by adding the enzyme to the substrate and inhibitor mixture. Progress curve analysis was performed as described previously [11] to derive k_{obs} values:

$$P = v_s t + \frac{(v_i - v_s)}{k_{obs}} \left(1 - \exp(-k_{obs} t)\right) \quad (2)$$

where P is reaction product, v_i and v_s are initial and steady-state velocities, respectively, and k_{obs} is the pseudo-first order rate constant for the approach to steady-state. These values were subsequently used to generate k_{on}, k_{off} and final equilibrium Ki^*. In the cases where k_{obs} was linearly dependent upon I/(1 + S/Km), k_{on} and k_{off} were calculated from the slope and intercept of the line:

$$k_{obs} = k_{on} I / \left(1 + S/Km\right) + k_{off} \quad (3)$$

When k_{obs} dependence on effective inhibitor concentration was hyperbolic, k_{on} and k_{off}

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E * I$$

were calculated using following equation according to Scheme 1:

$$k_{obs} = \frac{k_3 I}{Ki\left(1 + \frac{S}{Km}\right) + I} + k_4 \quad (4)$$

where Ki is k_2/k_1, $k_{on} = k_3/Ki$, k_4 is k_{off} and final $Ki^* = k_{off}/k_{on}$.

We measured the inhibitor dissociation constant independently using dilution experiments because calculating the inhibitor dissociation constant from the final complex (k_4) is subject to large errors,. Briefly, enzyme (20 nM) and inhibitor (500 nM) were preincubated for 30 minutes at 37°C to allow complete equilibration, then put through a spin column P-30 (BioRad, Hercules, CA) to eliminate excess free inhibitor. The resulting solution was immediately diluted (~ 10-fold) into an activity assay containing an excess of substrate (250 μM, Km = 60 μM). k_{off} was then calculated by fitting the subsequent time course with the equation:

$$P = v_s t + \frac{(v_i - v_s)}{k_{off}} \left(1 - \exp(-k_{off} t)\right) \quad (5)$$

Inhibition of DPP4 cleavage of active GLP-1 by human DPP4

An LC/MS/MS assay was used to measure GLP-1 cleavage in vitro at room temperature. To limit losses of cleaved GLP-1 ($GLP\text{-}1_{9\text{-}36}$) due to aggregation and/or adsorption to the walls of the vial, samples were diluted to 40% acetonitrile composition. To determine Km, various concentrations of active GLP-1 ($GLP\text{-}1_{7\text{-}36}$; concentration range 10 to 240 μM) were incubated with DPP4 (2 nM) in 20 mM phosphate buffer, pH 7.4. Total reaction volume was 125 μl. Every 5 minutes, 28 μl samples were withdrawn and quenched with 28 μl stop buffer (80% acetonitrile, 18% water, 2% formic acid). To determine Ki, two concentrations of active GLP-1 (10 and 50 μM) were incubated with DPP4 (0.45 nM) in 20 mM phosphate buffer, pH 7.4. Total reaction volume was 40 μl. After 90 min, 40 μl stop buffer was added to quench the reaction.

A Thermo Fisher LTQ Orbitrap mass spectrometer in positive electrospray mode was used to detect $GLP\text{-}1_{9\text{-}36}$. The chromatography system was a Waters Acquity UPLC with a Waters UPLC BEH C18, 1.7 μm, 2.1 × 50 mm column. A short gradient was employed (0% B to 100% B in 4 minutes) using 98/2 water/acetonitrile (10 mM ammonium acetate) as solvent A and 2/98 water/acetonitrile (10 mM ammonium acetate) as solvent B. A flow rate of 0.8 ml/min was used and 5 μl were injected onto the

column. Under these conditions, GLP-1_{9-36} was chromatographically separated from GLP-1_{7-36}. GLP-1_{9-36} quantitation was performed by integrating the sum of the 2+, 3+ and 4+ charged ions. Concentrations of GLP-1_{9-36} were calculated from a $1/x^2$ quadratic fit calibration curve (10 nM - 2,000 nM).

Cynomolgus monkey ex vivo plasma DPP inhibition assay

Male and female cynomolgus monkeys (Charles River BRF, Houston, TX), were housed in an AAALAC-accredited facility (on a 12-hour light/dark cycle) in stainless-steel cages. They had access to purified tap water ad libitum and were fed Harlan Diet 2050 C (Certified 20% protein primate diet). Six monkeys per dose group (3 to 4 males and 2 to 3 females per group) were administered single oral gavage doses of saxagliptin (0.1, 0.3, 1, 3, or 10 mg/kg), sitagliptin (0.3, 1, 3, 10, or 40 mg/kg), or vehicle alone (water, final pH 5.0) at a dose volume of 2 mL/kg. Doses were administered once weekly using a crossover design such that no monkeys received the same dose twice. For the ex vivo cynomolgus monkey assays, blood samples (approximately 0.5 ml) were collected prior to dosing and 1, 2, 4, 6, 8, and 24 hours post dosing in tubes containing K_2EDTA. Tubes were inverted several times to ensure mixing, and placed on ice. Samples were centrifuged at 5000 rpm for 10 minutes at 4°C for plasma isolation. Plasma was stored at -20°C until analyzed for DPP activity. No difference in DPP inhibition was noted between males and females and so data were combined for analyses.

Ala-pro-AFC plasma DPP inhibition assay

For the ex vivo assays, plasma from cynomolgus monkeys was diluted with buffer alone. For the in vitro assays, plasma 'spiked' with compound to be tested (10 μl) was added to buffer alone or buffer with drug 'spiked' at concentrations that would yield the same concentration as the 'spiked' plasma in the well (10 μl), followed by the substrate (10 μl, final concentrations were ala-pro-AFC 370 μM and DMSO 3%). This procedure produced a 3-fold dilution of plasma. The buffer contained (final concentration) 33 mM HEPES and 140 mM NaCl (no BSA) at pH 7.9, and the assay was run at room temperature (22°C). A 384-well microplate format was employed to measure plasma dipeptidyl peptidase activity. Activity, the rate of release of fluorescence from the pseudo-substrate, was assayed using a Fluoroskan Ascent microplate reader with rate-determining software. Readings were taken after a 10 second shaking step, at 405 nm excitation and 510 nm emission, with subsequent reads every 1 minute for 18 minutes. Rates were determined from the slope of the regression line fitted to the linear portion of the data (a minimum of 8 minutes and a maximum of 12 minutes from the start of the experiment) showing the increase in fluorescence due to cleavage of substrate over time. Results were expressed as arbitrary units because standard AFC curves were not run for all experiments. The extent of DPP inhibition, derived from the reduction in the initial rate observed at each drug concentration with respect to untreated plasma, were determined and IC_{50} values were calculated using XLfit.

Gly-pro-pNA Plasma DPP inhibition assay

This assay was reported by Merck as used in their clinical trials [24], using gly-pro-pNA as substrate. The buffer contained 100 mM HEPES with 0.1 mg/ml BSA and the assay was run at 30°C in 96-well microplate format (Costar 9017, Corning. Corning, NY). For the ex vivo assays, plasma from cynomolgus monkeys was diluted with buffer alone. For the in vitro assays, 'spiked' plasma (40 μl) was added to buffer alone or buffer with drug 'spiked' at concentrations that would yield the same concentration as the 'spiked' plasma in the well (40 μl), followed by the substrate (20 μl, final concentration of gly-pro-pNA is 400 μM). Activity, the rate of release of free pNA from the pseudo-substrate, was assayed at 30°C, using a Spectramax 340 plate reader (Molecular Probes) with rate determining software. Absorbance at 390 nm was measured. Results were expressed as arbitrary units because standard pNA curves were not run for all experiments. The extent of DPP inhibition and IC_{50} values were calculated as described above.

Statistical analysis

The SAS JMP statistical package was used for all statistical analyses. A Dunnett's test was used for comparison to vehicle and all pairs were compared with a Tukey HSD.

Abbreviations

DPP: Dipeptidylpeptidase; (1S,3S,5S)-2-[(2S)-2-Amino-2-(3-hydroxytricyclo [3.3.1.1^{3,7}]dec-1-yl)acetyl]-2-azabicyclo[3.1.0]hexane-3-carbonitrile, monohydrate; Saxagliptin, Onglyza™ or BMS-477118; GLP-1: Glucagon-like peptide-1.

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Authors' contributions

AW carried out some of the in vitro enzyme measurements and the ex vivo assays and contributed to writing the manuscript. CD carried out some of the in vitro enzyme measurements and the ex vivo assays and contributed to writing the manuscript. LK carried out some of the in vitro time-dependence assays. GL carried out some of the in vitro time-dependence assays. RL measured GLP-1 concentrations and contributed to writing the manuscript. EH conceived and ran the cynomolgus monkey studies and contributed to writing the manuscript. PS conceived the GLP-1 assay. JM conceived and designed the in vitro time-dependence assays and contributed to writing the manuscript. LH conceived and designed the compounds, some of the in vitro assays and contributed to writing the manuscript. MK conceived and designed these studies, coordinated the work and was the primary author of the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

All the authors were employees of Bristol-Myers Squibb at the time that this work was performed.

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Characterization of the In Vitro and In Vivo Metabolism and Disposition and Cytochrome P450 Inhibition/Induction Profile of Saxagliptin in Human[S]

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ABSTRACT:

Saxagliptin is a potent dipeptidyl peptidase-4 inhibitor approved for the treatment of type 2 diabetes mellitus. The pharmacokinetics and disposition of [^{14}C]saxagliptin were investigated in healthy male subjects after a single 50-mg (91.5 μCi) oral dose. Saxagliptin was rapidly absorbed (T_{max}, 0.5 h). Unchanged saxagliptin and 5-hydroxy saxagliptin (M2), a major, active metabolite, were the prominent drug-related components in the plasma, together accounting for most of the circulating radioactivity. Approximately 97% of the administered radioactivity was recovered in the excreta within 7 days postdose, of which 74.9% was eliminated in the urine and 22.1% was excreted in the feces. The parent compound and M2 represented 24.0 and 44.1%, respectively, of the radioactivity recovered in the urine and feces combined. Taken together, the excretion data suggest that saxagliptin was well absorbed and was subsequently cleared by both urinary excretion and metabolism; the formation of M2 was the major metabolic pathway. Additional minor metabolic pathways included hydroxylation at other positions and glucuronide or sulfate conjugation. Cytochrome P450 (P450) enzymes CYP3A4 and CYP3A5 metabolized saxagliptin and formed M2. Kinetic experiments indicated that the catalytic efficiency (V_{max}/K_m) for CYP3A4 was approximately 4-fold higher than that for CYP3A5. Therefore, it is unlikely that variability in expression levels of CYP3A5 due to genetic polymorphism will impact clearance of saxagliptin. Saxagliptin and M2 each showed little potential to inhibit or induce important P450 enzymes, suggesting that saxagliptin is unlikely to affect the metabolic clearance of coadministered drugs that are substrates for these enzymes.

Introduction

The dipeptidyl peptidase-4 (DPP4) inhibitors are promising recent additions to the arsenal of therapies available for the treatment of type 2 diabetes mellitus (Scheen, 2012). The DPP4 enzyme is responsible for degrading and inactivating glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide, incretins that regulate blood glucose levels. GLP-1 is released postprandially and stimulates meal-induced insulin secretion and contributes to glucose homeostasis (Kieffer and Habener, 1999; Gorrell, 2005). By inhibiting the DPP4 enzyme, GLP-1 is sustained, thereby leading to increased activity and improved glycemic control in patients with type 2 diabetes (McIntosh et al., 2005). Because this mechanism results in a glucose-dependent release of insulin, DPP4 inhibitors are expected to offer important advantages over traditional diabetes treatments including low risk for hypoglycemia and weight gain (Gallwitz, 2008).

Saxagliptin (Onglyza; Bristol-Myers Squibb, Princeton NJ and AstraZeneca, Wilmington, DE) (Fig. 1) is an orally administered, small molecule, reversible DPP4 inhibitor approved for the treatment of type 2 diabetes mellitus. It was specifically designed for enhanced potency and selectivity and to provide extended inhibition of the DPP4 enzyme (Augeri et al., 2005). The ability of saxagliptin to affect reductions in glycosylated hemoglobin (HbA_{1c}) and fasting plasma glucose in type 2 diabetes patients has been demonstrated in multiple

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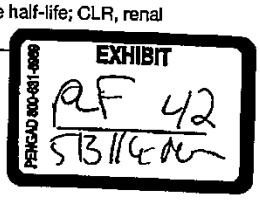
ABBREVIATIONS: DPP4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; P450, cytochrome P450; HPLC, high-performance liquid chromatography; HLM, human liver microsomes; LSC, liquid scintillation counting; LC-MS/MS, liquid chromatography with tandem mass spectrometry; MRM, multiple reaction monitoring; AUC, area under plasma concentration-time curve; T-HALF, terminal phase half-life; CLR, renal clearance; DMSO, dimethyl sulfoxide; 3-MC, 3-methylcholanthrene; PB, phenobarbital; RIF, rifampicin; C_T, threshold cycle.



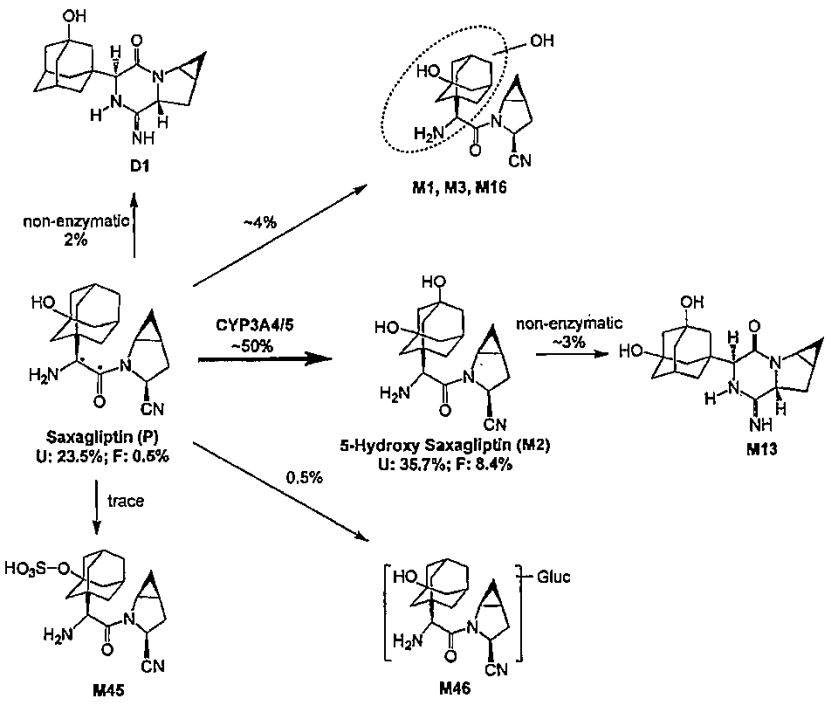


FIG. 1. Primary pathways for biotransformation of [^{14}C]saxagliptin in humans. The C-14 label was evenly distributed between the carbonyl carbon and the adjacent carbon, as indicated by asterisk (*) on the saxagliptin structure. The percentage of administered radioactivity recovered in urine and feces as saxagliptin and M2 is indicated underneath the structures. The estimated flux through each pathway (also reported as percentage of administered radioactivity) is indicated next to the corresponding arrow. D1, a degradant known to form in solution, was found in small quantities in all samples.

Phase III clinical trials, both as a single agent and in combination regimens with metformin, a sulfonylurea or a thiazolidinedione (Kania et al., 2011). The most commonly used clinical dose of saxagliptin in adults is 5 mg, once daily (United States prescribing information for Onglyza, http://www.packageinserts.bms.com/pi/pi_onglyza.pdf).

In nonclinical pharmacokinetic studies, saxagliptin was rapidly absorbed and showed good oral bioavailability in rats (75%), dogs (76%), and monkeys (51%). A significant portion (33–60%) of the administered dose was excreted as unchanged drug in the urine in these species. Formation of 5-hydroxy saxagliptin (M2) was a major metabolic pathway, and this metabolite was a major circulating metabolite in all species (Fura et al., 2009). Metabolite M2 was pharmacologically active, with an in vitro DPP4 inhibitory activity approximately half that of saxagliptin (Augeri et al., 2005; Fura et al., 2009).

The purpose of the current study was to investigate the in vivo disposition of saxagliptin and to determine its major metabolic pathways in healthy male subjects after administration of a single 50-mg (91.5 μCi) p.o. dose of [^{14}C]saxagliptin. In addition, a series of in vitro studies were conducted to gain insight regarding possible cytochrome P450 (P450)-based drug-drug interactions between saxagliptin and potential comedications. These included the identification of enzymes involved in the metabolism of saxagliptin and formation of M2 and the determination of the potential of saxagliptin and M2 to inhibit or induce P450 enzymes.

Materials and Methods

Chemicals. [^{14}C]Saxagliptin (radiochemical purity 99.86%, specific activity 1.83 μCi/mg) with the C-14 label distributed between the carbonyl carbon and the adjacent carbon (Fig. 1) and stable-labeled $^{13}C_4$,^{15}N-saxagliptin, and $^{13}C_4$,^{15}N-5-hydroxy saxagliptin [internal standards for high-performance liquid chromatography (HPLC) analysis] were synthesized by the Radiochemistry Group of the Department of Chemical Synthesis, Bristol-Myers Squibb Research (Princeton, NJ) (Cao et al., 2007). Unlabeled saxagliptin (P, (1*S*,3*S*,5*S*)-2-((*S*)-2-amino-2-(-3-hydroxyadamantan-1-yl)acetyl)-2-azabicyclo[3.1.0]hexane-3-carbonitrile); and reference standards for 5-hydroxy saxagliptin, (M2, (1*S*,3*S*,5*S*)-2-((*S*)-2-amino-2-((1r,3*R*,5*S*,7*S*)-3,5-dihydroxyadamantan-1-yl)acetyl)-2-azabicyclo[3.1.0]hexane-3-carbonitrile); degradant (D1, (1a*S*,4*S*,6a*R*,7a*S*)-4-(-3-hydroxyadamantan-1-yl)-6-iminohexahydro-1*H*-cyclopropa[4,5]pyrrolo[1,2-a]pyrazin-3(1a*H*)-one); the *S,R,S,S* and *S,S,S,R* diastereomers of saxagliptin (Supplemental Fig. S1) were supplied by the Departments of Chemical Development or Chemical Synthesis (Bristol-Myers Squibb).

Selective chemical inhibitors of P450 enzymes for reaction phenotyping experiments were obtained from Sigma-Aldrich (St. Louis, MO), with the exception of montelukast, which was purchased from Sequoia Research Products (Pangbourne, UK), and benzylnirvanol (BD Biosciences, Woburn, MA). Chemical inducers, inhibitors, substrates, and metabolites of P450 enzymes and internal standards used in experiments to evaluate whether saxagliptin and M2 were inhibitors or inducers of P450 enzymes were procured by CellzDirect (Pittsboro, NC). All chemicals were of the highest purity available.

Human liver microsomes (HLM; 19 donors male/female) and individual human cDNA-expressed cytochrome P450 enzymes were purchased from BD Biosciences. Individual lots (n = 16) of HLM, for which the vendor had determined the activities of various P450 enzymes, were purchased as a Reaction Phenotyping kit (version 7) from XenoTech, LLC (Lenexa, KS). Monoclonal antibodies with inhibitory activity for specific P450 enzymes were obtained from Kristopher W. Krausz at the Laboratory of Metabolism, National Institutes of Health (Bethesda, MD).

Ecolite scintillation cocktail was obtained from MP Biomedicals (Irvine, CA), and Emulsifier-Safe and Permofluor E+ scintillation fluid were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Deionized water was prepared using a MilliQ ultrapure water system (Millipore Corporation, Billerica, MA). All organic solvents were HPLC grade, and other regents were reagent grade or better.

Clinical Study Design, Dosing, and Sample Collection. The clinical phase of the study was conducted at the Bristol-Myers Squibb Clinical Research Center (Hamilton, NJ). This was an open-label, nonrandomized single dose study. Six healthy males participated in the study. The mean age was 29 years

(range, 25 to 37 years), and the mean body mass index was 23.6 kg/m^2 (range, 19.7 to 28.1 kg/m^2). The study was conducted in accordance with the Declaration of Helsinki and guidelines on Good Clinical Practice. Before study initiation, the study protocol and informed consent documents were approved by the Institutional Review Board of the New England Institutional Review Board (Wellesley, MA). All study participants provided written informed consent before the initiation of study-specific procedures.

After a 10-h fast, each subject received a single 50-mg p.o. solution dose of [^{14}C]saxagliptin containing 91.5 μCi of radioactivity, immediately followed by 240 ml of water on day 1. Blood samples were collected at selected time points via an indwelling catheter or direct venipuncture into Vacutainers (BD Biosciences Medical Supplies, Franklin Lakes, NJ) containing K_3EDTA and were centrifuged to obtain plasma for pharmacokinetic and biotransformation analysis. The total urine and fecal output was collected for the duration of the study (0–168 h). On the morning of day 6, a single 30-ml oral dose of Milk of Magnesia was administered to each subject to facilitate defecation before release from the clinical facility. All subjects were released on the morning of day 8.

Blood samples (6 ml total per time point) for the plasma pharmacokinetic analysis of saxagliptin, M2, and radioactivity were collected predose and at 0.25, 0.5, 0.75, 1, 1.5, 2.0, 2.5, 3, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120, 144, and 168 h postdose. Additional aliquots (10 ml per time point) for metabolite profiling were collected in conjunction with the pharmacokinetic samples at predose and at 1, 2, 4, 8, 12, 24, 48, 96, 144, and 168 h postdose.

Cumulative urine was collected predose and over 0 to 12 h, 12 to 24 h, and thereafter in 24-h intervals through 168 h for determining saxagliptin, M2, and radioactivity concentrations and for metabolite profiling. Feces were collected predose and over 24-h intervals postdose for the measurement of radioactivity concentrations and for metabolite profiling. All samples were stored at −20°C or below until analysis.

Radioactivity Analysis. Radioactivity in plasma, urine, and feces was measured by liquid scintillation counting (LSC) on a Model LS 6500 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Plasma and urine were mixed with Emulsifier-Safe scintillation fluid (PerkinElmer Life and Analytical Sciences) and counted directly. Water was added to each fecal sample to form an approximately 20% (w/w) feces mixture, which was homogenized using a probe-type homogenizer (Kinematica Polytron model no. PT 45-80; Brinkman Instruments, Westbury, NY). Aliquots of fecal homogenate were then combusted using a sample oxidizer before counting by LSC, as described previously (Christopher et al., 2008).

Quantification of Saxagliptin and M2 in Plasma and Urine Samples. The concentrations of saxagliptin and M2 in individual plasma and urine were determined with validated liquid chromatography/tandem mass spectrometry (LC-MS/MS) methods. In brief, after the addition of stable-labeled internal standards ($^{13}C_4$,^{15}N-saxagliptin and $^{13}C_4$,^{15}N-5-hydroxy saxagliptin) to each plasma or urine sample, the analytes were isolated by solid-phase extraction (Waters Oasis HLB, 10 mg; Waters, Milford, MA). The eluates were evaporated to dryness, reconstituted in mobile phase, and then applied to an Atlantis dC18, 2.1 × 50 mm, 5-μm HPLC column (Waters). The LC-MS system used for plasma samples consisted of LC10AD delivery pumps (Shimadzu Corporation, Columbia, MD) and a Series 200 Autosampler (PerkinElmer Life and Analytical Sciences). The HPLC system was interfaced to either a Quattro Premier mass spectrometer (Waters Corporation, Manchester, UK) for the plasma method or an API3000 mass spectrometer (AB Sciex, Foster City, CA) for the urine method. The mass spectrometers were operated in positive ion electrospray mode, and analytes were monitored by multiple reaction monitoring (MRM) with transitions that were characteristic for each analyte. For saxagliptin and M2, the standard curve ranges were 5 to 1000 and 10 to 2000 ng/ml, respectively, for the plasma method and 25 to 5000 and 50 to 10,000 ng/ml, respectively, for the urine method.

Pharmacokinetic Analysis of Saxagliptin, M2, and Total Radioactivity. The noncompartmental pharmacokinetic parameters of saxagliptin, M2, and total radioactivity were determined from plasma concentration versus time profiles and urine concentrations with cumulative urinary excretion volumes using noncompartmental methods with Kinetica 4.2 in eToolbox (Thermo Fisher Scientific, Waltham, MA). The single-dose pharmacokinetic parameters determined included the following: maximum observed concentration (C_{max}); time of maximum concentration (T_{max}); area under the plasma concentration-time curve (AUC) between time 0 and the last quantifiable concentration [AUC(0-T)]; and AUC between time 0 to infinity AUC(INF), terminal phase half-life (T-HALF), renal clearance (CLR), and percentage of urinary excretion. The percentage of fecal excretion was determined for total radioactivity only, and the calculation was based on cumulative fecal weights and fecal total radioactivity concentrations. For the percentage of dose excreted in urine and feces, the actual dose of saxagliptin administered to each subject was determined by subtracting the weight of the dosing syringe (in grams) after dosing from the weight of the dosing syringe (in grams) before dosing and multiplying by the density of the dosiag solution (1.0 g/ml) and the concentration of the dosing solution (5 mg/ml).

Preparation of Samples for Biotransformation Profiling and Identification of Metabolites. Representative pools of plasma, urine, and feces were prepared for metabolite profiling and identification experiments. Plasma samples were segregated by collection time (i.e., 1, 2, 4, and 8 h), and equal volumes from all subjects were combined. Plasma samples collected after 8 h were not analyzed because the radioactivity in these samples was too low to produce meaningful profiles. Urine and fecal homogenate pools (0–168 h) were prepared across all subjects by combining a percentage of the volume (urine) or weight (fecal homogenate) proportional to the total amount excreted over each interval.

Pooled plasma and fecal homogenate samples were each extracted with three volumes of methanol/acetonitrile 50:50 (v/v). After centrifugation at 2500*g* for 40 min, the pellets were extracted an additional two times with methanol/acetonitrile/water (25:25:50, v/v/v). The supernatants from each extraction step were combined and evaporated to dryness under nitrogen. The dried residues were reconstituted in methanol/acetonitrile/water (~10:20:70, v/v/v), and the resulting supernatants were analyzed by HPLC with offline radioactivity detection or LC-MS/MS. The recovery of radioactivity from extracted plasma and fecal samples was approximately 100%. Pooled urine samples were centrifuged at 11,000*g* to remove any particulates and analyzed without additional processing.

LC-Radiochromatographic and LC-MS/MS Methods for Metabolite Profiling of In Vivo Samples and Identification of Metabolites. Samples for metabolite profiling were analyzed on a Shimadzu LC-10AD HPLC system (Shimadzu Corporation), equipped with two 10AD VP pumps, a SIL-10AD autoinjector, a model SCL-10A system controller, and an SPD-M10A photodiode array detector. A Zorbax 4.6 × 250 mm, 5-μm, RX-C8 column (Agilent Technologies, Santa Clara, CA) maintained at 30°C was used to separate drug-related components. The mobile phase consisted of two solvents: 1) mobile phase (A) 0.1% formic acid and 1% acetonitrile in water and 2) mobile phase (B) 0.1% formic acid in acetonitrile. The mobile phase flow rate was 0.5 ml/min. The gradient program used for sample elution was as follows: hold isocratic at 0% B (0–5 min); linear gradient from 0 to 20% B (5–35 min); hold isocratic at 20% B (35–42 min); linear gradient from 20 to 30% B (42–45 min); hold isocratic at 30% B (45–50 min); linear gradient from 30 to 40% B (50–52 min); linear gradient from 40 to 80% B (52–55 min); hold isocratic at 80% B (55–60 min); return to 0% B (60–62 min); re-equilibrate at 0% B for 10 min before the next injection.

For quantification of metabolites by radioactivity, the HPLC eluate was collected in 0.25-min intervals on Wallac ScintiPlate-96-well plates with a Gilson Model FC 204 fraction collector (Gilson, Middleton, WI). The plates were evaporated to dryness on a Savant Speed-Vac (Savant Instruments Inc., Holbrook, NY) and counted for 10 min/well with a PerkinElmer 1450 MicroBeta Wallac TRILUX Liquid Scintillation and Luminescence Counter (PerkinElmer Life Sciences, Turku, Finland) to quantify radioactivity. Radioprofiles were prepared by plotting the net counts per minute values obtained from the MicroBeta versus time after injection using Microsoft Excel (Microsoft Corporation, Redmond, WA). The metabolites were quantified based on the percentage of total radioactivity in each peak relative to the entire radiochromatogram.

Mass spectral analysis was performed on a Finnigan LCQ Deca XP ion trap mass spectrometer equipped with an electrospray ionization probe (Thermo Fisher Scientific). Analyses were performed in the positive ion mode. Samples were introduced into the mass-spectrometer after chromatographic separation, using the same HPLC method used for radioprofiling. High purity nitrogen was used as the sheath and the auxiliary gas with levels at 60 and 10 (relative flow rate), respectively. The capillary temperature was 350°C. The nitrogen gas

flow rate, spray current, and voltages were adjusted as required to achieve maximum sensitivity or optimal fragmentation of drug-related components. Reference standards, available for saxagliptin, M2, D1, M13, and the *S,R,S,S* and *S,S,S,R* epimers of saxagliptin were used to confirm the retention time and mass-spectral fragmentation patterns of these analytes.

Identification of Enzymes Involved in the Metabolism of Saxagliptin and in the Formation of M2. [^{14}C]Saxagliptin (10 μM) was incubated at 37°C with pooled HLM (1 mg/ml protein) and individually expressed human P450 enzymes (500 pmol/ml each, including CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5) in 100 mM phosphate buffer (pH 7.4), fortified with 1 mM NADPH to evaluate which enzymes were capable of metabolizing the compound and forming the major metabolite, M2. After the incubation period (30 min for expressed enzymes; 60 min for HLM), the reactions were terminated by adding 1 to 2 volumes of ice-cold acetonitrile. The samples were then centrifuged, and the resulting supernatants were analyzed by LC-MS/MS and off-line radioanalysis. The analytical methodology was similar to that described for biotransformation profiling and metabolite identification of in vivo samples, with the exception that drug-related components were separated on a YMC ODS-AQ *S*-3 120A column, maintained at 30°C, and the mobile phase gradient was modified, with a shorter run time (48 min).

A correlation analysis between the formation of M2 and P450 activity was conducted by incubating saxagliptin, at concentrations of 1 and 10 μM, in singlicate with individual lots of HLM from 16 different donors. For each lot, the activities of CYP1A2, -2A6, -2B6, -2C8, -2C9, -2C19, -2D6, -2E1, -3A4/5, and -4A11 had been determined by the vendor using marker substrates specific for each enzyme (Technical Information for Reaction Phenotyping Kit version 7, ref. 0510189; XenoTech, LLC). The reaction mixtures contained 0.25 mg/ml of HLM, 1 or 10 μM saxagliptin, 1 mM NADPH, and 0.1 M phosphate buffer with 2 mM $MgCl_2$ (pH 7.4). Incubations (final volume, 0.5 ml) were conducted for 30 min at 37°C in a shaking water bath. Reactions were stopped with the addition of ice-cold acetonitrile. The concentration of M2 in each sample was then determined by analyzing an aliquot of the resulting supernatant by LC-MS/MS with multiple reaction monitoring as described below. Plots of M2 versus marker substrate activity were prepared, and r values were calculated with Microsoft Excel (Office 2003; Microsoft Corporation).

The metabolism of saxagliptin to M2 by specific P450 enzymes was also investigated with HLM in the presence of specific chemical or monoclonal antibody inhibitors of P450 enzymes. Chemical inhibitors included direct inhibitors, tranylcypromine (2 μM, CYP2A6), montelukast (3 μM, CYP2C8), sulfaphenazole (10 μM, CYP2C9), benzylnirvanol (1 μM, CYP2C19), quinidine (1 μM, CYP2D6), ketoconazole (1 μM, CYP3A4/5) and time-dependent inhibitors, furafylline (10 μM, CYP1A2), orphenadrinie (50 μM, CYP2B6), diethyldithiocarbamate (50 μM, CYP2E1), troleandomycin (20 μM, CYP3A4/5), and 1-aminobenzotriazole (1000 μM, all P450s). Anti-P450 monoclonal antibodies included anti-CYP1A2, anti-CYP2B6, anti-CYP2C8, anti-CYP2C19, anti-CYP2D6, and anti-CYP3A4/5. A final concentration of 5 to 7 μl of antibody mixture was used per incubation. The antibody solutions were used as received; the concentration of each of the antibodies was not provided.

Saxagliptin, at concentrations of 1 and 10 μM, was incubated in triplicate with pooled HLM (0.25 mg/ml), 1 to 2 mM NADPH, and 2 mM $MgCl_2$ in 0.1 M phosphate buffer (pH 7.4), in the presence or absence of chemical or anti-P450 monoclonal antibody inhibitors. The final volume of the reaction mixtures was 1 ml for the chemical inhibitor experiments and 0.25 ml for the anti-P450 antibody experiments. For incubations with direct chemical inhibitors, all ingredients except NADPH were added to the incubation tubes, and the samples were equilibrated at 37°C for 2 to 3 min before incubation. Then, NADPH was added to initiate the reactions. For incubations with time-dependent chemical inhibitors, all ingredients except saxagliptin were added to the incubation tubes. The samples were equilibrated at 37°C for 2 to 3 min, and then 1 mM NADPH was added to initiate a 15-min preincubation with the inhibitors. After the preincubation period, saxagliptin and an additional 1 mM NADPH were added to initiate the reactions. For incubations with anti-P450 antibodies, the antibodies were preincubated with HLM in phosphate buffer on ice for 20 min, and then warmed at 37°C for 10 min. Saxagliptin and NADPH were added to the incubation mixtures to initiate the reactions. To establish the initial rate of metabolism of saxagliptin to M2 in HLM, incubations without chemical inhibitors or with antibodies against egg lysozyme (Hy-Hei-9) rather than anti-P450 antibodies were conducted. The rate of M2 formation in other incubations was normalized to the appropriate control incubation. Negative control incubations were carried out in the same manner, but they either lacked NADPH or contained heat-inactivated microsomes (boiled for 5 min).

After the appropriate equilibrations and preincubation periods, HLM incubations were carried out for 30 min at 37°C. An equivalent volume of ice-cold acetonitrile was added to stop each reaction. Samples were vortex mixed and centrifuged to precipitate proteins. The concentration of M2 in each sample was then determined by analyzing an aliquot of the resulting supernatant by LC-MS/MS.

Concentration-Dependent Metabolism of Saxagliptin to M2. The kinetics for the formation of M2 were determined in pooled HLM and expressed CYP3A4 and CYP3A5. Incubations (0.25 ml total volume, in triplicate) contained 1 mM NADPH, 2 mM $MgCl_2$, 0.1 mM phosphate buffer (pH 7.4), saxagliptin, and HLM (0.25 mg protein/ml), or expressed CYP3A4 or CYP3A5 (10 pmol P450 enzyme/ml). Twelve concentrations of saxagliptin from 1 to 800 μM were evaluated. The HLM incubations were conducted at 37°C. After the designated incubation period (30 min for HLM, 10 min for CYP3A4 and CYP3A5), reactions were quenched by adding an equal volume of ice-cold acetonitrile (0.25 ml). The quenched reaction mixtures were vortexed to mix and centrifuged. The concentration of M2 in each sample was then determined by analyzing an aliquot of the resulting supernatant by LC-MS/MS.

LC-MS/MS Method for Quantification of M2 in In Vitro Samples. Internal standard, $^{13}C_4$,^{15}N-5-hydroxy saxagliptin, was added to the quenched reaction mixtures from the in vitro incubations before LC-MS/MS analysis. The LC/MS system used for quantitation of M2 in in vitro samples consisted of two 10AD-VP pumps, a model SCL system controller and a degasser (Shimadzu Corporation), a LEAP HTC PAL autosampler equipped with a cooling stack maintained at 10°C (CTC Analytics, Carrboro, NC), and a Micromass Quattro Ultima triple quadrupole mass spectrometer (Waters). MassLynx software (version 4.0 or 4.1; Waters) was used to control the instrumentation and acquire data. Chromatographic separation of M2 from other mono-hydroxylated metabolites was achieved on an Agilent Zorbax SB-C8 column (4.6 × 75 mm, 3.5-μm particle size) (Agilent, Wilmington, DE) maintained at ambient temperature. The mobile phase consisted of two solvents: mobile phase (A), 0.1% formic acid in water; and mobile phase (B), 0.1% formic acid in acetonitrile. The mobile phase flow rate was 0.3 ml/min. The linear gradient program used for elution of the sample components was as follows: hold isocratic at 15% B (0–0.1 min); from 15 to 30% B (0.1–3 min); from 30 to 38% B (3–4 min); from 38 to 40% B (4–5 min); from 40 to 80% B (5–5.5 min); hold isocratic at 80% B (5.5–6.5 min); return to 15% B (6.5–7 min); re-equilibrate at 0% B for 5 min before the next injection.

The Micromass Quattro Ultima mass spectrometer was operated in positive electrospray ionization mode. Ultrahigh purity nitrogen was used for the nebulizing and desolvation gases at flow rates of approximately 85 and 1000 l/h, respectively. The capillary voltage was 3.5 kV, the cone voltage was 36V, and the collision energy was 45 eV. The desolvation temperature was 300°C, and the source temperature was 150°C. Detection of 5-hydroxysaxagliptin and its internal standard were achieved through MRM. The individual selected reaction monitoring transitions were 332 → 196 for M2 and 335 → 196 for the internal standard.

Assessment of Potential of Saxagliptin and M2 to Inhibit P450 Enzymes. The potential for saxagliptin and M2 to inhibit P450 enzymes in a direct or time-dependent manner was assessed with HLM (n = 15 donors, mixed gender pool; CellzDirect, Durham, NC). IC_{50} values for nine enzymes were determined using probe substrates specific for each of the enzymes evaluated. The metabolic reactions monitored and probe substrate concentrations used were phenacetin O-deethylation (50 μM, CYP1A2), coumarin 7-hydroxylation (1 μM, CYP2A6), bupropion hydroxylation (20 μM, CYP2B6), paclitaxel 6-hydroxylation (5 μM, CYP2C8), tolbutamide hydroxylation (140 μM, CYP2C9), *S*-mephenytoin 4′-hydroxylation (50 μM, CYP2C19), bufuralol 1′-hydroxylation (40 μM, CYP2D6), chlorzoxazone 6-hydroxylation (50 μM, CYP2E1), midazolam 1′-hydroxylation and testosterone 6β-hydroxylation (5 and 50 μM, respectively, CYP3A). The final concentration of each probe substrate was near the experimentally determined K_m value for the indicated enzyme.

To evaluate whether saxagliptin or M2 were competitive inhibitors of P450 enzymes, saxagliptin (at concentrations of 0, 0.1, 1, 5, 20, and 50 μM), M2 (at concentrations of 0, 0.1, 1, 10, 50, and 200 μM), or prototypical P450 inhibitors (positive controls) were mixed with HLM, and the probe substrates in 100 mM phosphate buffer (pH 7.4) in a total volume of approximately 0.5 ml. After a 3-min equilibration at 37°C, 1 mM NADPH was added to initiate the reactions. The reactions were carried out using previously established conditions to ensure linearity with respect to protein concentration and incubation time. Incubations were stopped with addition of organic solvents. To assess the time-dependent inhibition, saxagliptin, M2, or positive control time-dependent inhibitors were preincubated for 15 min at 37°C with pooled human liver microsomes in the presence and absence of 1 mM NADPH. After the preincubation, P450-specific probe substrates were added to the incubation mixtures at the same concentrations used above. Metabolite formation in incubations with test compounds and control inhibitors was assessed with validated LC-MS/MS methods for each of the reaction products as described in Supplemental Table S1. Then, the percentage remaining activity was determined by comparison of probe substrate metabolism in incubations containing NADPH but without test compounds or control inhibitors. If inhibition reached significant levels (i.e., the percentage remaining activity was <50%), IC_{50} values were reported.

Assessment of Potential of Saxagliptin and M2 to Induce P450 Enzymes. The potential of saxagliptin and M2 to induce the expression of mRNA levels and/or P450 enzyme activity of CYP1A2, -2B6, and -3A4 was investigated in primary cultures of freshly isolated human hepatocytes, as described previously (Hong et al., 2011). Human hepatocytes isolated from three individual donors (lots Hu 211, Hu 223, and Hu 224; CellzDirect) were used. Donor information is provided in Supplemental Table S3. The cultured human hepatocytes were treated once daily for three consecutive days with either saxagliptin (0.2, 1, 5, and 25 μM), M2 (0.2, 1, 10, and 100 μM), solvent control [0.1% dimethyl sulfoxide (DMSO)] or known prototypical inducers, 3-methylcholanthrene [(3-MC) 2 μM, a prototypical CYP1A2 inducer], phenobarbital [(PB) 1000 μM, a prototypical CYP2B6 inducer], and rifampicin [(RIF) 10 μM, a prototypical CYP3A4 inducer].

At the end of the treatment period, microsomes were isolated from a subset of the various hepatocyte incubations. Enzyme activity was determined by incubating microsomal samples with probe substrates specific for each P450 enzyme and then measuring the formation of marker metabolites by LC-MS/MS, as described in Supplemental Table S1. The probe substrate concentration and quantity of microsomal protein in each assay were as follows: 100 μM phenacetin and 0.02 mg/ml protein for CYP1A2; 250 μM bupropion and 0.02 mg/ml protein for CYP2B6; and 200 μM testosterone and 0.01 mg/ml protein for CYP3A4. The relative fold induction in enzymatic activity was calculated by comparing the rate of metabolite formation for treatment groups to that of the negative control group (0.1% DMSO).

Another subset of the various hepatocyte incubations were used for mRNA determination. After cell lysis, total mRNA was isolated using the RNeasy Mini or Midi Kit (QIAGEN, Valencia, CA) cDNA, according to the manufacturer's instructions. Total RNA was quantified using the RiboGreen RNA Quantitation reagent and kit (Invitrogen, Carlsbad, CA). Samples were subsequently diluted to the desired RNA concentration (10 ng/μl). TaqMan real-time quantitative polymerase chain reaction was then used to determine the levels of CYP1A2, CYP2B6, and CYP3A4 present in each sample using primers and probes specific for each enzyme (Applied Biosystems, Foster City, CA). The reactions were characterized during the point in the polymerase chain reaction cycle at which the amplification product was first detected [threshold cycle (C_T)]. C_T values, baseline, and threshold levels were automatically calculated by the ABI 7500 Sequencer software (Applied Biosystems). A difference in one C_T was considered equivalent to a 2-fold difference in gene expression (i.e., an exponential relationship, $2^{\Delta CT}$). Relative-fold mRNA content was determined for each treatment group relative to the endogenous control gene expression and 0.1% DMSO vehicle control for each sample.

Results

Excretion of the Radioactive Dose. After administration of a single 50-mg, 91.5 μCi oral dose of [^{14}C]saxagliptin to healthy male subjects, the mean cumulative recovery of radioactivity over the study duration (168 h) was ~97% (Table 1). The majority of the radioactivity (mean value, ~75%) was excreted in the urine. Approximately 22% was recovered in the feces.

For one subject (subject 6), the total radioactivity recovered (~55%) was substantially lower than the recovery for the other subjects, which ranged from 84 to 106%. Although the amount of radioactivity recovered in the feces for this subject (21.5%) was similar to the other subjects (~9–33%), the urinary recovery of radioactivity was only ~34%, compared with ~70 to 81% for the other subjects. This difference was evident from the first, 0- to 12-h urine collection, where the recovery for subject 6 was only ~28%, compared with ~65 to 72% for the other subjects. Pharmacokinetic analysis of plasma exposures of saxagliptin and M2 indicated that this subject had plasma exposures that were similar to the other five subjects (data not shown). These data are consistent with a possible sampling error for an early urine collection for subject 6. The data from subject 6 were therefore excluded from the calculation of the mean and S.D. values for urinary excretion reported in Table 1. However, the data from this subject were included in the calculations of fecal recovery and pharmacokinetic parameters (Table 2).

Pharmacokinetic Parameters. The mean plasma concentration versus time profiles for saxagliptin, M2, a summation of saxagliptin and M2, and total radioactivity, after administration of [^{14}C]saxagliptin, are shown in Fig. 2. The mean pharmacokinetic parameters are presented in Table 2. Saxagliptin was rapidly absorbed with a T_{max} of ~0.5 h postdose. T_{max} values for M2 and total radioactivity occurred

TABLE 1

Excretion of radioactivity in human urine and feces after administration of a single oral dose of [^{14}C]saxagliptin

Values in italics represent total excretion values over the entire study period.

Matrix	Time (h)	% Recovered Per Collection Interval						
		Subject Number						Mean ± S.D.[a]
		1	2	3	4	5	6	
Urine	0–12	65.88	66.70	64.89	71.64	71.63	27.65	68.15 ± 3.25
	12–24	0.63	4.27	1.65	5.26	4.47	1.02	3.26 ± 2.00
	24–48	2.29	0.75	1.84	2.53	2.07	4.02	1.90 ± 0.69
	48–168	2.13	0.99	1.83	1.60	1.43	1.26	1.60 ± 0.43
	0–168	*70.93*	*72.71*	*70.21*	*81.03*	*79.60*	*33.95*	*74.89 ± 5.05*
Feces	0–24	<LOQ	30.64	0.04	0.07	11.14	1.40	7.22 ± 12.27
	24–48	NS	2.06	16.26	9.02	8.51	14.88	8.46 ± 6.56
	48–72	<LOQ	0.25	9.95	0.24	1.79	4.90	2.86 ± 3.94
	72–96	5.61	0.06	NS	NS	1.11	NS	1.13 ± 2.24
	96–144	7.96	<LOQ	5.60	<LOQ	0.46	0.33	2.39 ± 3.48
	0–144	*13.57*	*33.01*	*31.85*	*9.33*	*23.01*	*21.51*	*22.05 ± 9.50*
Total	0–168	*84.50*	*105.72*	*102.06*	*90.36*	*102.61*	*55.46*	*97.05 ± 9.13*

NS, no sample; <LOQ, below the limit of quantification.

[a] $n = 5$; the values for subject 6 were not included in the calculation of mean urinary excretion or total excretion; mean fecal excretion values include data from all six subjects.

TABLE 2

Mean pharmacokinetic parameters for saxagliptin, M2, and radioactivity after administration of a single oral dose of [^{14}C]saxagliptin

PK Parameter	Saxagliptin	M2[a]	Radioactivity
C_{max}, ng/ml	279	606	779
Mean, %CV	(13)	(11)	(10)
AUC(INF), ng · h/ml	845	2943	3874
Mean, %CV	(17)	(7)	(9)
T_{max}, h	0.50	1.50	1.50
Median (min, max)	(0.25, 0.50)	(1.00, 2.00)	(1.00, 1.50)
T-HALF, h	2.45	2.69	2.77
Mean (S.D.)	(0.31)	(0.31)	(0.22)
CLR, ml/min	234	102	165
Mean (S.D.)[b]	(23)	(10)	(14)
%Urinary excretion	23.5	35.7	74.9
Mean (S.D.)[b]	(3.1)	(3.0)	(5.1)
%Fecal excretion	0.5	8.4	22.1
Mean (S.D.)	(N.A.)[c]	(N.A.)[c]	(9.5)

%CV, percentage coefficient of variation; N.A., not applicable.

[a] Values reported for M2 may include small amounts of other hydroxylated metabolite isomers.

[b] n = 5; CLR and urinary excretion values do not include data for subject 6.

[c] Determined via biotransformation profiling of pooled 0- to 168-h urine or fecal samples; therefore, S.D. was not determined.

at approximately 1.5 h postdose. Thereafter, the concentrations of saxagliptin, M2, and total radioactivity all declined rapidly with elimination half-life values of <3 h. The AUC(INF) values indicated saxagliptin and M2 together comprised almost all of the circulating radioactivity; based on their molar ratios, they represented approximately 22 and 72%, respectively, of the AUC of total radioactivity. On the basis of measurements with the validated LC-MS/MS assay, approximately 23.4 and 35.7% of the administered radioactivity was excreted in the urine as unchanged saxagliptin and M2, respectively.

Subsequent to the analysis for this study, it was determined that the validated LC-MS/MS method used for quantitation was not completely specific for the measurement of M2. Other mono-hydroxylated metabolites with the same MRM transition (i.e., M1, M3, and M13) eluted in close proximity to M2 under the chromatographic conditions used and may have been included in the M2 plasma measurements. Therefore, the reported values for M2 may be overestimated by up to 9% (based on radioprofiling data from plasma and urine samples) due to interference from other hydroxylated metabolites.

Identification of Metabolites. The structures of the metabolites of saxagliptin were characterized by LC-MS/MS analysis on a LCQ Deca XP ion trap mass spectrometer. Similarities in HPLC retention time and mass spectrometric fragmentation patterns with authentic standards facilitated the identification of saxagliptin, M2, and the cyclic amidine degradant (D1) in the human samples. The structures of other metabolites were proposed based on their mass spectrometric fragmentation patterns relative to saxagliptin or the other available reference standards. The mass spectrometric data supporting the structural elucidation of the metabolites are summarized in Table 3.

Saxagliptin had a protonated molecule $[M+H]^+$ of *m/z* 316 and one major MS^2 fragment at *m/z* 180 resulting from cleavage of the carbon-carbon bond between the carbonyl carbon and the carbon of the methanamine group. The metabolites generally underwent similar fragmentation, enabling the localization of structural modifications on either the amino-methyl-adamantyl group or the formyl-methanoprolinenitrile moiety.

Metabolite M2 had a protonated molecule $[M+H]^+$ of *m/z* 332 (16 Da higher than saxagliptin). The product ion mass spectrum showed a major MS^2 fragment at *m/z* 196, consistent with hydroxylation on the amino-methyl-adamantyl group. Like M2, metabolites M1, M3, and M16 each had a protonated molecular ion $[M+H]^+$ of *m/z* 332 (16 Da higher than saxagliptin) and a major MS^2 fragment at *m/z* 196. These MS data suggested that these metabolites were positional isomers of mono-hydroxylated saxagliptin with the site of the hydroxylation for all three metabolites on the amino-methyl-adamantyl group. Metabolite M16 had an additional MS^2 major fragment at *m/z* 314 (loss of −18 Da from the protonated molecular ion), indicative of a loss of water. This fragment was not observed as a significant fragment in the MS^2 spectra for either M1, M2, or M3.

Degradant D1, an isomer of saxagliptin with a keto-iminopiperazine ring structure, is a known impurity that forms as a degradation product in solution (Jones et al., 2011). D1 had a protonated molecule $[M+H]^+$ of *m/z* 316, identical to the mass of saxagliptin. A normalized collision energy of 23%, which produced a large MS^2 fragment at *m/z* 180 for saxagliptin, resulted in little or no fragmentation of D1 (data not shown). However, at a higher normalized collision energy of 30%, two major MS^2 fragment ions of D1 were observed at *m/z* 288 and 180. The fragment ion at *m/z* 288 (28 Da less than the molecular ion) corresponded to loss of the carbonyl group, whereas the ion at *m/z* 180 most likely resulted from an additional cleavage to form a fragment similar to that observed for saxagliptin.

M13 had a protonated molecule $[M+H]^+$ of *m/z* 332 (16 Da higher than saxagliptin), suggesting that it was a hydroxylated metabolite. Like D1, M13 did not show fragmentation when subjected to a normalized collision energy of 23% in the mass spectrometer, but at 30%, two major MS^2 fragment ions were observed at *m/z* 196 (M + 16) and 304 (loss of carbonyl group). Based on the MS data, and comparison of LC-MS/MS data to a subsequently synthesized reference standard, M13 is proposed to be a cyclized product of M2, having a keto-iminopiperazine ring structure.

M45 had a protonated molecule $[M+H]^+$ of *m/z* 396, 80 Da higher than saxagliptin, which is characteristic of a sulfate conjugate. The MS^2 spectra for M45 showed major fragment ions at *m/z* 298 and 260. The fragment at *m/z* 298 (neutral loss of −98 Da from the protonated molecular ion) was indicative of loss of H_2SO_4, whereas the fragment at *m/z* 260 resulted from cleavage of the carbon-carbon bond between the carbonyl carbon and the carbon of the methanamine group. Based on the MS data, M45 was identified as a sulfate conjugate of saxagliptin with the site of sulfation most likely on the hydroxyl group of the adamantyl ring.

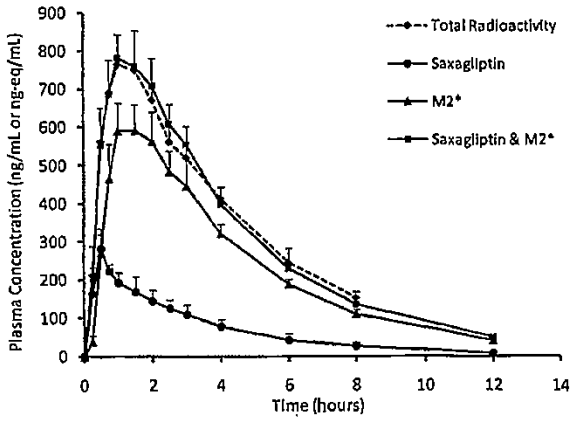
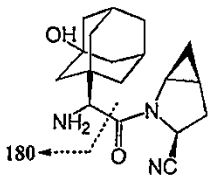


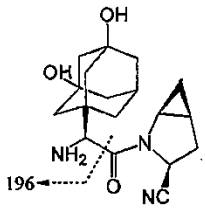
FIG. 2. Mean concentration versus time profiles of total radioactivity, saxagliptin, M2, and the summed contribution of saxagliptin and M2, in plasma from healthy male subjects after oral administration of 50 mg (91.5 μCi) of [^{14}C]saxagliptin. *, the concentration values reported for M2 may contain small amounts of other hydroxylated metabolites; based on radioprofiling results of plasma samples, the contribution of these metabolites is expected to be <9% of the reported M2 concentration at each time point.

TABLE 3

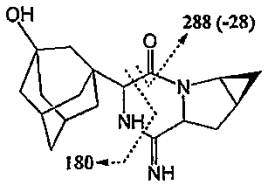
Mass-spectral characterization of primary metabolites of saxagliptin identified in human plasma samples after administration of a single oral dose of [^{14}C]saxagliptin to healthy volunteers



Saxagliptin (P)



5-Hydroxysaxagliptin (M2)



D1

Metabolite	[M + H]$^+$	Major Fragment Ions	Metabolic Pathway(s)
Saxagliptin	316	180	
M1	332	196	Hydroxylation
5-Hydroxy saxagliptin (M2)	332	196	Hydroxylation
M3	332	196	Hydroxylation
M13	332	304, 196	Hydroxylation, cyclization
M16	332	314, 196	Hydroxylation
M45	396	298, 260	Sulfation
M46	492	474, 316, 298	Glucuronidation
D1	316	288, 180	Cyclization

Metabolites M46 had a protonated molecule $[M+H]^+$ of *m/z* 492, 176 Da higher than saxagliptin. The MS^2 spectra showed a major fragment ion at *m/z* 316, corresponding to a loss of glucuronic acid. These data suggested that M46 is a direct glucuronide conjugate of the parent compound; however, the exact site of glucuronidation could not be localized based on the MS fragmentation data.

Metabolite Profiles in Plasma. Metabolite profiles from pooled plasma collected at 4 h postdose are shown in Fig. 3A. Similar profiles were obtained at 1, 2, and 8 h postdose. Consistent with the pharmacokinetic data, parent drug and the M2 metabolite were the major circulating components, representing ~20 to 30% and ~60 to 70% of the plasma radioactivity at the 1- to 8-h time points, respectively. Additional minor circulating metabolites that each represented <4% of the plasma radioactivity at each time point included the following: hydroxylated metabolites (M1, M3, M13), a sulfate conjugate (M45), and a glucuronide conjugate (M46). A small amount of degradant D1 (<2%) was also detected in the plasma at each time point.

Metabolite Profiles in Urine and Feces. The metabolite profile of 0- to 168-h pooled urine (Fig. 3B) was similar to the plasma profiles, with saxagliptin and M2 accounting for the majority of urinary radioactivity and the remainder comprised of multiple minor metabolites, each representing <4% of the administered radioactive dose. In 0- to 168-h feces (Fig. 3C), M2 was the most abundant drug-related component, representing 8.4% of the administered radioactivity. Metabolites M1 and M13 coeluted and together represented 2.5% of the administered radioactivity, whereas the parent compound accounted for only 0.5%. Multiple additional fecal metabolites, each representing <2% of the administered dose, could not be identified due to high levels of mass-spectral background interference.

Potential for Chiral Interconversion of Saxagliptin. To determine whether any of the chiral centers of saxagliptin were susceptible to chiral inversion, the metabolite profiles of in vivo samples were examined for the presence of diastereomers of saxagliptin. Other than saxagliptin and D1, no additional peaks corresponding to the protonated molecule $[M+H]^+$ of *m/z* 316 were observed. In addition, there were no peaks eluting at the retention time of authentic reference standards for the *S,R,S,S* and *S,S,S,R* epimers of saxagliptin. These two epimers were chromatographically resolved from saxagliptin on the chromatographic method used for metabolite profiling (data not shown). Of all the saxagliptin diastereomers (Supplemental Fig. S1), formation of these two would be the most probable, because they are the product of inversion at only one of each of the two independent stereogenic sites.

Identification of Cytochrome P450 Enzymes Involved in the Metabolism of Saxagliptin. The biotransformation of [^{14}C]saxagliptin was investigated in HLM and a panel of recombinant P450 enzymes (Fig. 4, A–C). At a substrate concentration of 10 μM, the turnover of saxagliptin in HLM was ~32% after a 1-h incubation. The M2 metabolite was the primary metabolite formed, along with several additional minor mono-hydroxylated metabolites. The saxagliptin metabolite profiles with CYP3A4 and CYP3A5 after a 30-min incubation were similar to that observed with the HLM, indicating that these enzymes were capable of catalyzing the formation of M2 and other minor metabolites. There was no significant turnover of saxagliptin with any of the other P450 enzymes evaluated. Consistent with these observations, the rate of formation of M2 in a panel of 16 lots of HLM from individual donors showed good correlation with the vendor-reported CYP3A activity, which was based on rates of testosterone

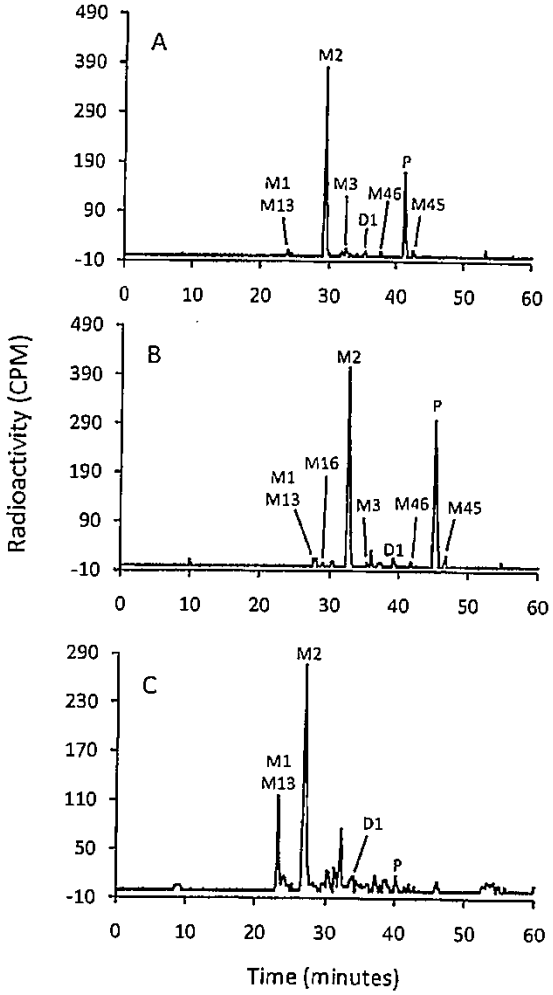


FIG. 3. Representative biotransformation profiles of saxagliptin in pooled plasma collected at 4 h postdose (A), pooled urine collected at 0- to 168-h postdose (B), and pooled fecal homogenate collected at 0- to 168-h postdose (C) after oral administration of a single dose of [^{14}C]saxagliptin to human subjects.

6β-hydroxylation (r = 0.985; Fig. 4D) or midazolam 1′-hydroxylation (r = 0.949; Fig. 4E).

In HLM incubations conducted in the presence of the chemical inhibitor 1-aminobenzotriazole (a general P450 inhibitor), or in the absence of NADPH, M2 formation was diminished by >99%, indicating that the metabolism of saxagliptin to M2 was a P450-mediated process. The CYP3A inhibitors ketoconazole and troleandomycin both inhibited the formation of M2 from saxagliptin (1 μM) in human liver microsomes by ≥97%, and an anti-CYP3A4/5 antibody inhibited M2 formation by >89% (Supplemental Fig. S2).

Kinetic experiments indicated that the formation of M2 from saxagliptin in HLM and expressed CYP3A4 and CYP3A5 followed Michaelis-Menten kinetics (Fig. 5, A–C). The K_m values for the formation of M2 were 94.8 μM in HLM and 81.7 and 252 μM in CYP3A4 and CYP3A5 incubations, respectively (Fig. 5D). The V_{max} values were 496 pmol M2/mg protein/min in HLM, 31.7 pmol M2/pmol P450/min in CYP3A4, and 24.0 pmol M2/pmol P450/min in CYP3A5. The catalytic efficiency (V_{max}/K_m) for CYP3A4 (0.388 μl/pmol P450/min) was approximately 4-fold higher than for CYP3A5 (0.095 μl/pmol P450/min), suggesting that CYP3A4 was the major P450 enzyme involved in the formation of M2.

Assessment of the Potential of Saxagliptin and M2 to Inhibit P450 Enzymes. Saxagliptin and M2 were incubated with HLMs in the presence of NADPH to assess their potential to inhibit P450 enzymes. For all P450 enzymes investigated, the IC_{50} values for direct inhibition (no preincubation) were greater than the highest concentrations tested, >50 μM for saxagliptin and >200 μM for its M2 metabolite. When saxagliptin and M2 were preincubated with HLM in the presence of NADPH for 15 min, before the reporting phase of the incubation, the IC_{50} values were also greater than the highest concentrations tested, >50 and >200 μM, respectively (Supplemental Table S2). These data suggest that at concentrations exceeding 100-fold of their clinically relevant concentrations, saxagliptin and its M2 metabolite were not direct or time-dependent inhibitors of P450 enzymes.

Assessment of the Potential of Saxagliptin and M2 to Induce P450 Enzymes. The enzyme activities and mRNA levels of CYP1A2, CYP2B6, and CYP3A4 were determined in primary cultured human hepatocytes obtained from three individual donors after treatment with either saxagliptin (0.2, 1.0, 5.0, and 25 μM), M2 (0.2, 1.0, 10, and 100 μM), or prototypical inducers 3-MC (positive control inducer for CYP1A2), PB (positive control inducer for CYP2B6), and RIF (positive control inducer for CYP3A4). The mean enzyme activity and mRNA data from three donors are summarized in Fig. 6, and the individual data for each donor are provided in Supplemental Tables S4 and S5. At the concentrations tested, saxagliptin and M2 were not cytotoxic as indicated by morphological integrity and lactate dehydrogenase activity assays (data not shown). Treatment with saxagliptin and M2 at concentrations up to 25 or 100 μM, respectively, did not result in meaningful increases of CYP1A2, CYP2B6, or CYP3A4 enzyme activity or mRNA content, suggesting that these compounds are not inducers of these P450 enzymes at clinically relevant concentrations.

Discussion

After administration of a single, oral 50-mg dose of [^{14}C]saxagliptin (91.5 μCi) to healthy human subjects, saxagliptin was rapidly absorbed into the systemic circulation. The T_{max} value was 0.5 h for the parent compound and 1.5 h for both the M2 metabolite and total radioactivity. After reaching T_{max}, the concentrations for all three analytes declined rapidly, each with elimination half-life values of <3 h (Table 2). Consistent with the short half-life, nearly 80% of the administered radioactivity was recovered within the first 24 h after dosing. By the end of the 7-day study, a total of 97% of the administered dose was recovered, of which 74.9% was eliminated in the urine and 22.1% was excreted in the feces (Table 1). In urine and feces, respectively, approximately 23.5 and 0.5% of the drug-related radioactivity was recovered as saxagliptin and 35.7 and 8.4% was recovered as M2 (Table 2; Fig. 1). These data indicate that saxagliptin was well absorbed (≥74.9%) and was subsequently cleared by both renal excretion and metabolism, with the formation of M2 representing the major metabolic pathway. Minor metabolic pathways included hydroxylation at other positions and glucuronide or sulfate conjugation. In addition, small amounts of both saxagliptin and M2 underwent intramolecular cyclization to form compounds with a keto-iminopiperazine ring structure, D1 and M13, respectively (~2–3% of the dose each; Fig. 1). These cyclic amidine products are known degradation products of this chemotype, and their formation is favored under aqueous, alkaline conditions (Jones et al., 2011). In vitro, the formation of

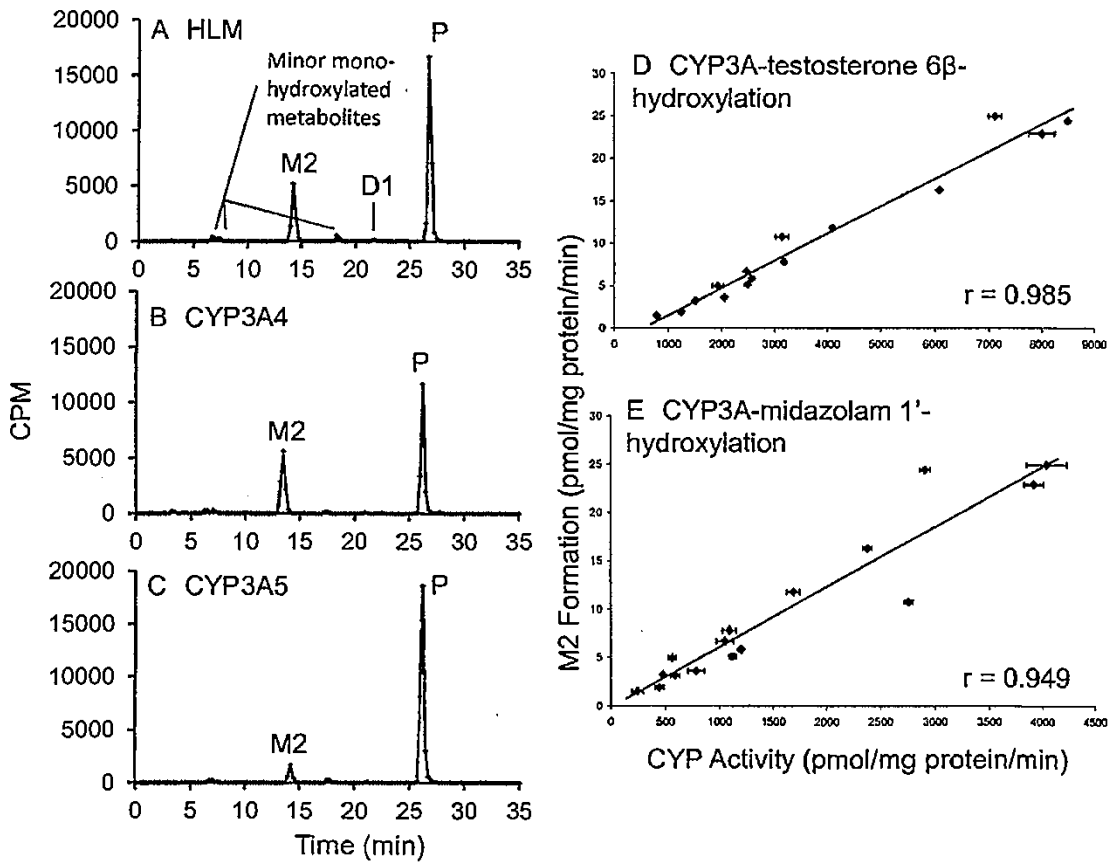
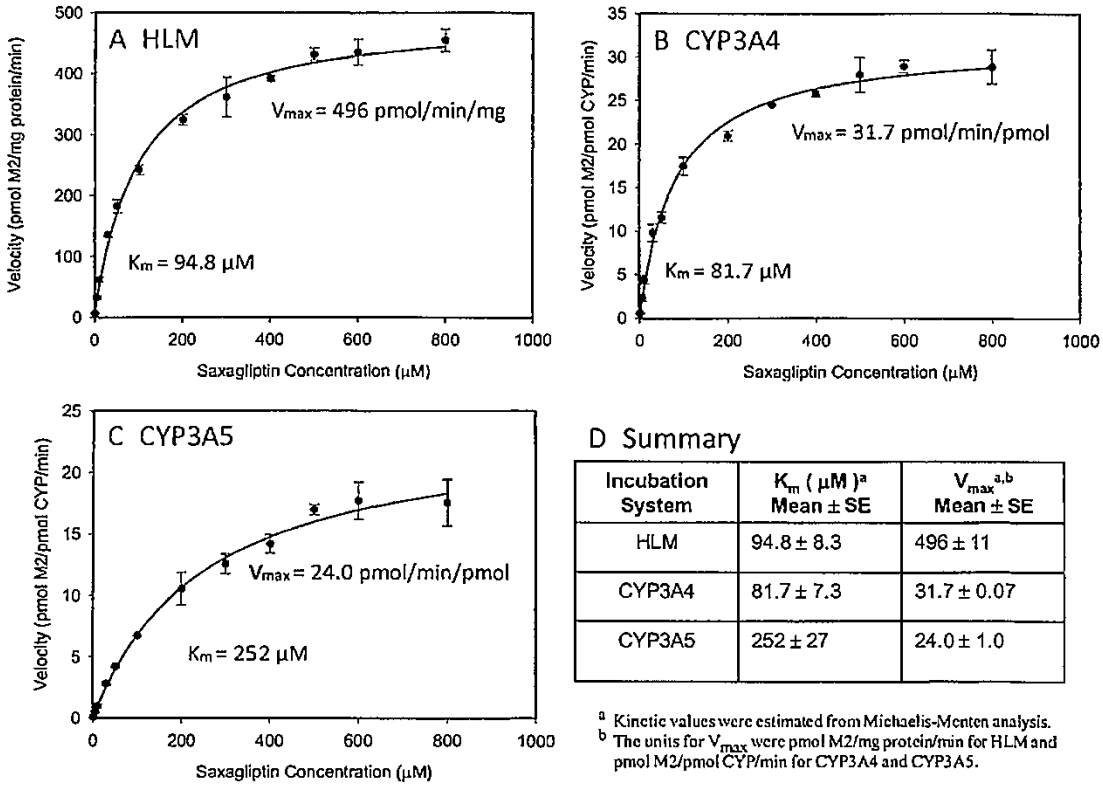


FIG. 4. Metabolite profiles after incubation of [^{14}C]saxagliptin (30 μM) with human liver microsomes (A), CYP3A4 (B), and CYP3A5 (C). In these incubations, saxagliptin (P) was mainly metabolized to M2; other minor monohydroxylated metabolites were also formed. No metabolism of saxagliptin was observed in incubations with expressed CYP1A2, -2A6, -2B6, -2C8, -2C9, -2C18, -2C19, -2D6, or -2E1 enzymes (data not shown). Plots show the correlation between M2 formation from saxagliptin (1 μM) and reported activities of CYP3A4/5 in a panel of HLM from 16 individual donors. D, correlation based on reported testosterone 6β-hydroxylation activity; E, correlation based on midazolam 1'-hydroxylation activity.

D1 or M13 in HLM incubations with saxagliptin or M2, respectively, was low (approximately <1%) and was similar to negative controls containing only compound and buffer (data not shown). Therefore, we concluded that these cyclization reactions are nonenzymatic processes. Based on the structures proposed from LC-MS/MS analyses (Table 3), all of the metabolites and degradants retained both of the C-14 labels, thus confirming the suitability of the labeling scheme for [^{14}C]saxagliptin used in this study.

Both pharmacokinetic data and biotransformation profiling results (Figs. 2 and 3A) indicated that unchanged saxagliptin and M2 were the most prominent drug-related components in the plasma; together, the two compounds accounted for almost all of the circulating radioactivity; based on their molar ratios, they represented approximately 22 and 72%, respectively, of the AUC of total radioactivity. Minor drug-related species in plasma included other hydroxylated metabolites, glucuronide- and sulfate-conjugated metabolites, and rearrangement products, D1 and M13. The M2 is pharmacologically active, with an in vitro potency for DDP4 inhibition that was approximately half that of saxagliptin (Fura et al., 2009). The serum protein binding of saxaglitpin and its M2 metabolite are both negligible (Fura et al., 2009); therefore, their relative in vivo potencies are expected to be similar to the values determined in vitro. Because of its potency and high plasma concentrations, M2 is expected to significantly contribute to the observed pharmacological effect of saxagliptin. Other metabolites and degradants, due to their low abundance in plasma and/or significant structural modifications, are not expected to contribute to the pharmacological activity of saxagliptin. All human circulating metabolites were represented in one or more of the preclinical species (rat, dog, and monkey), with no disproportionate human metabolites observed (H. Su, personal communication). Metabolite M2 was the predominant metabolite found in all species (Fura et al., 2009), and each of the toxicology species provided >2-fold exposure multiples for this metabolite, relative to a 5-mg human dose, which was sufficient for toxicological evaluation.

Saxagliptin has four chiral centers. Although chiral inversion of saxagliptin was not expected to occur through traditional metabolic mechanisms, i.e., oxidation of a secondary alcohol or conjugation of a carboxylic acid with acetyl CoA (Wsol et al., 2004), chiral inversion might occur via a chemical mechanism, either in vivo or ex vivo, during sample storage or processing. Therefore, we examined the in vivo samples for the presence of diastereomers of saxagliptin. No peaks with the same protonated molecule as saxagliptin and D1 were observed in the LC-MS/MS chromatograms of the plasma, urine, or feces samples. Furthermore, there were no



D Summary

Incubation System	K_m (μM)[a] Mean ± SE	V_{max}[a,b] Mean ± SE
HLM	94.8 ± 8.3	496 ± 11
CYP3A4	81.7 ± 7.3	31.7 ± 0.07
CYP3A5	252 ± 27	24.0 ± 1.0

[a] Kinetic values were estimated from Michaelis-Menten analysis.
[b] The units for V_{max} were pmol M2/mg protein/min for HLM and pmol M2/pmol CYP/min for CYP3A4 and CYP3A5.

FIG. 5. Concentration-dependent formation of M2 from saxagliptin in incubations with HLM (A), CYP3A4 (B), and CYP3A5 (C); and summary of kinetic parameters (D). Data were fitted to a Michaelis-Menten substrate binding curve: $V = (V_{max} \times S)/(K_m + S)$. The values of K_m and V_{max} are shown.

peaks corresponding to the retention time of authentic reference standards for the *S,R,S,S* and *S,S,S,R* epimers in the pooled urine, feces, or plasma samples. Of all the saxagliptin diastereomers, the formation of these two were considered most likely, because they were each the product of inversion at only one of the two independent stereogenic sites; the other two chiral centers were conformationally locked within the cyclopropyl ring system. These data suggest that saxagliptin does not undergo measurable chiral inversion either in vivo, after administration to humans, or during subsequent sample processing.

Although the dose used in the clinical portion of the current study was higher than the one ultimately selected as the prescription dose (5 mg), saxagliptin showed predictable, dose-proportional, multiple-dose pharmacokinetics over the range of 5 to 400 mg with minimal accumulation when dosed once daily (Boulton and Geraldes, 2007). It is therefore expected that the pharmacokinetic, metabolism, and excretion results from the current study will be applicable to lower doses within this range.

Consistent with the in vivo data, M2 was the predominant metabolite of saxagliptin formed in human liver microsomes (Fig. 4A). P450 enzymes CYP3A4 and CYP3A5 were both capable of metabolizing saxagliptin to M2 (Fig. 4, B and C); little or no turnover was observed with other P450 enzymes. Experiments with human liver microsomes and chemical and monoclonal antibody inhibitors for specific P450 enzymes supported the role of CYP3A4/5 in the formation of M2. In addition, the formation of M2 correlated highly ($r \geq 0.949$) with the reported testosterone 6β-hydroxylation and midazolam 1′-hydroxylation activities in a panel of human liver microsomes from 16 individual donors (Fig. 4, D and E). Kinetic experiments indicated that catalytic efficiency (V_{max}/K_m) for M2 formation by CYP3A4 was approximately 4-fold higher than for CYP3A5 (Fig. 5), suggesting that the formation of M2 will be predominantly mediated by CYP3A4, even in subjects with high expression levels of CYP3A5. The *CYP3A5*3* variant results in functionally defective CYP3A5 enzyme; whites are known to have a higher frequency (~90%) of the *CYP3A5*3* allele, compared with blacks (~32%), Hispanics (~63%), and Asians (~73%) (Xie et al., 2004). Population analysis of data from Phase II/III clinical trials suggested that race (whites, blacks, Hispanic, or Asian) had no impact on exposures of either saxagliptin or M2 (D. Boulton, personal communication), confirming the minimal contribution of CYP3A5 in the metabolism of saxagliptin.

When saxagliptin (100 mg) was coadministered with ketoconazole (200 mg, once every 12 h) in a clinical drug-drug interaction study, the AUC of saxagliptin increased by 145% and the AUC of M2 decreased by 88% (Patel et al., 2011). In addition, when saxagliptin (5 mg) was coadministered with rifampin at steady state (600 mg, once daily for 6 days), the AUC of saxagliptin decreased by 76% (Upreti et al., 2011). Thus, the role of CYP3A enzymes in the metabolism of saxagliptin was confirmed in these two clinical studies.

Saxagliptin and M2 each demonstrated little potential to inhibit or induce P450 enzymes in primary cultures of human hepatocytes, suggesting that saxagliptin is unlikely to affect the metabolic clearance of coadministered drugs that are substrates for P450 enzymes. The lack of an inhibitory effect of saxagliptin on CYP3A was con-

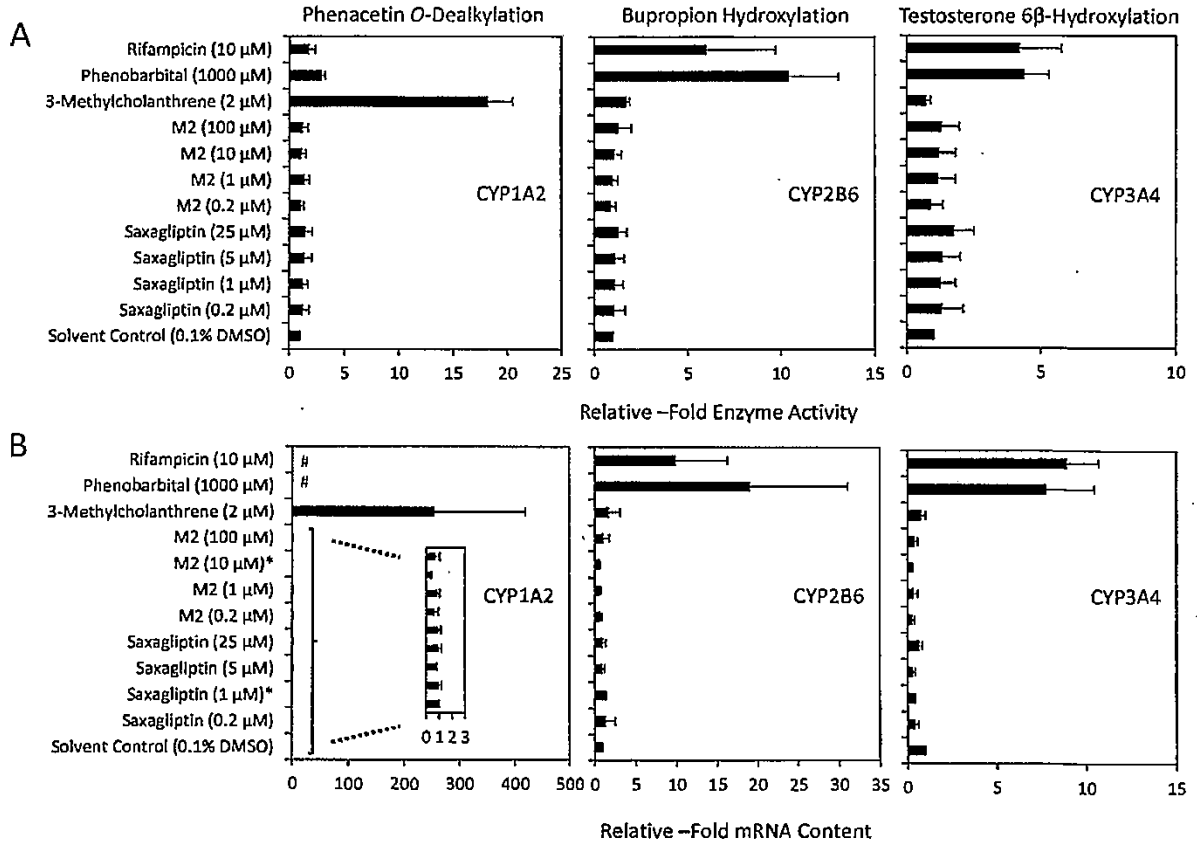


FIG. 6. Evaluation of the potential induction effects of saxagliptin and M2 on P450 enzymes in primary cultures of human hepatocytes. A, fold increase in CYP1A2, CYP2B6, and CYP3A4 enzyme activity relative to solvent control. B, fold increase in CYP1A2, CYP2B6, and CYP3A4 mRNA content relative to solvent control. Positive control inducers were 3-MC, PB, and RIF for CYP1A2, CYP2B6, and CYP3A4, respectively. The results presented represent the average ± S.D. from three individual preparations of human hepatocyte donors. *, where indicated, mRNA could not be obtained from one of the donor hepatocytes; therefore, these values represent the average of the remaining two donors. #, the CYP1A2 mRNA levels were not determined in hepatocyte preparations treated with either PB or RIF.

firmed in a clinical drug-drug interaction study between saxagliptin and simvastatin, where little or no increase in the AUC values of simvastatin and simvastatin acid (4 and 16%, respectively) was observed when 40 mg of simvastatin was administered together with a 10-mg dose of saxagliptin (Patel et al., 2011).

In a separate clinical study, the absolute bioavailability of saxagliptin in healthy subjects was 50% after administration of a single 5-mg dose (Xu et al., 2011). The current in vivo and in vitro experimental data support this result. Although at least 74.9% of the saxagliptin dose was absorbed after oral administration, metabolite profiling results suggested that a substantial portion of the administered dose was metabolized to M2.

In summary, after a 50-mg (91.5 μCi) p.o. dose to healthy male volunteers, [^{14}C]saxagliptin was rapidly absorbed, and recovery of the administered radioactivity was good (97%). At least 74.9% of the dose was absorbed based on the amount of radioactivity recovered in urine. Saxagliptin and its active M2 metabolite were the predominant circulating components, together comprising almost all of the plasma radioactivity. Elimination of saxagliptin occurred via both renal excretion and metabolism, with the formation of M2 by CYP3A4/5 representing the major metabolic pathway. Neither saxagliptin nor M2 inhibited or induced P450 enzymes in vitro, suggesting that saxagliptin is unlikely to alter the clearance of comedications that are metabolized by these enzymes.

Acknowledgments

We acknowledge Dr. Tong Li and colleagues at the Bristol-Myers Squibb Clinical Pharmacology Unit (Hamilton, NJ) for overseeing the clinical portion of the study and Christopher Black and the staff at CellzDirect for conducting the in vitro studies on the inhibition and induction potential of saxaglitpin.

Authorship Contributions

Participated in research design: Su, Boulton, Iyer, Humphreys, and Christopher.
Conducted experiments: Su, Barros Jr., Wang, and Christopher.
Contributed new reagents or analytic tools: Cao and Bonacorsi Jr.
Performed data analysis: Su, Boulton, Iyer, and Christopher.
Wrote or contributed to the writing of the manuscript: Su, Boulton, Iyer, Humphreys, and Christopher.

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Absorption, Metabolism, and Excretion of [^{14}C]Vildagliptin, a Novel Dipeptidyl Peptidase 4 Inhibitor, in Humans

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ABSTRACT:

The absorption, metabolism, and excretion of (1-[[3-hydroxy-1-adamantyl) amino] acetyl]-2-cyano-(S)-pyrrolidine (vildagliptin), an orally active and highly selective dipeptidyl peptidase 4 inhibitor developed for the treatment of type 2 diabetes, were evaluated in four healthy male subjects after a single p.o. 100-mg dose of [^{14}C]vildagliptin. Serial blood and complete urine and feces were collected for 168 h postdose. Vildagliptin was rapidly absorbed, and peak plasma concentrations were attained at 1.1 h postdose. The fraction of drug absorbed was calculated to be at least 85.4%. Unchanged drug and a carboxylic acid metabolite (M20.7) were the major circulating components in plasma, accounting for 25.7% (vildagliptin) and 55% (M20.7) of total plasma radioactivity area under the curve. The terminal half-life of vildagliptin was 2.8 h. Complete recovery of the dose was achieved within 7 days, with 85.4% recovered in urine (22.6% unchanged drug) and the remainder in feces (4.54% unchanged drug). Vildagliptin was extensively metabolized via at least four pathways before excretion, with the major metabolite M20.7 resulting from cyano group hydrolysis, which is not mediated by cytochrome P450 (P450) enzymes. Minor metabolites resulted from amide bond hydrolysis (M15.3), glucuronidation (M20.2), or oxidation on the pyrrolidine moiety of vildagliptin (M20.9 and M21.6). The diverse metabolic pathways combined with a lack of significant P450 metabolism (1.6% of the dose) make vildagliptin less susceptible to potential pharmacokinetic interactions with comedications of P450 inhibitors/inducers. Furthermore, as vildagliptin is not a P450 inhibitor, it is unlikely that vildagliptin would affect the metabolic clearance of comedications metabolized by P450 enzymes.

Dipeptidyl peptidase 4 (DPP-4, DPP-IV) is a highly specialized aminopeptidase that is present in plasma, the kidney, and the intestinal brush-border membranes, as well as on the surface of capillary endothelial cells, hepatocytes, and a subset of T lymphocytes (Deacon et al., 1995; Mentlein, 1999). DPP-4 is responsible for the rapid inactivation of the incretin glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide. GLP-1, which is released postprandially, stimulates meal-induced insulin secretion and contributes to glucose homeostasis (Gutniak et al., 1997; Kieffer and Habener, 1999). Circulating GLP-1 is rapidly degraded and inactivated by DPP-4 (Deacon et al., 1995; Mentlein, 1999). With the inhibition of the DPP-4 enzyme activity, GLP-1 activity increases markedly, improving glycemic control in experimental and human studies (Balkan et al., 1999; Ahrén et al., 2002, 2004; Reimer et al., 2002). Therefore, administration of a DPP-4 inhibitor to diabetic patients augments endogenous GLP-1 activity, which in turn produces a clinically significant lowering of diabetic glycemia comparable with that observed when GLP-1 is administered by direct infusion (Gutniak et al., 1992; Drucker, 2003; Mest and Mentlein, 2005).

Vildagliptin (Galvus, Novartis, East Hanover, NJ; (1-[[3-hydroxy-1-adamantyl) amino] acetyl]-2-cyano-(*S*)-pyrrolidine) is a potent, orally active, highly selective inhibitor of DPP-4 (Villhauer et al., 2003) and is marketed as an antidiabetic drug in this novel class of action mechanisms (He et al., 2007b). Based on an in vitro recombinant DPP-4 assay, the IC_{50} for vildagliptin is 2 nM. In humans, the efficacy of vildagliptin against the DPP-4 enzyme also shows a low in vivo inhibitory constant (IC_{50} 4.5 nM), a value that suggests higher potency than that reported for another DPP-4 inhibitor, sitagliptin (IC_{50} 26 nM) (Herman et al., 2005; He et al., 2007b). Vildagliptin has shown the ability to inhibit DPP-4, increase plasma concentrations of intact GLP-1 and glucose-dependent insulinotropic peptide, decrease fasting and postprandial glucose, and suppress plasma glucagons in clinical trial in patients with type 2 diabetes. The pharmacokinetics and pharmacodynamics of vildagliptin after various dosing regimens in healthy volunteers and patients with type 2 diabetes have been previously reported (He et al., 2007a,b, 2008; Sunkara et al., 2007).

The purpose of this study was to investigate the disposition and

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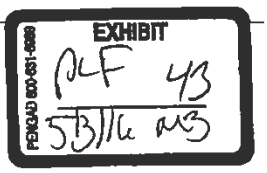
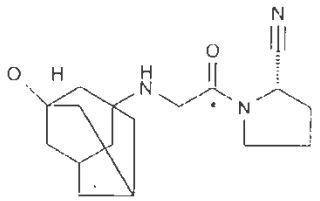
ABBREVIATIONS: DPP-4, dipeptidyl peptidase 4; GLP-1, glucagon-like peptide 1; vildagliptin, (1-[[3-hydroxy-1-adamantyl) amino] acetyl]-2-cyano-(S)-pyrrolidine; [^{14}C]vildagliptin, (1-[3-hydroxy-adamant-1-yl-amino)-acetyl]-pyrrolidine-2(S)-carbonitrile; LSC, liquid scintillation counting; LC/MS/MS, liquid chromatography/tandem mass spectrometry; IS, internal standard; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; DMSO, dimethyl sulfoxide; CID, collision-induced dissociation; P450, cytochrome P450; UGT, UDP glucuronosyltransferase; AUC, area under the curve; amu, atomic mass unit.





* indicates position of C-14 label

FIG. 1. Chemical structure of [^{14}C]vildagliptin.

biotransformation of vildagliptin in healthy male volunteers after a single 100-mg (47 μCi) p.o. dose of [^{14}C]vildagliptin [(1-[3-hydroxy-adamant-1-yl-amino)-acetyl]-pyrrolidine-2(*S*) carbonitrile]. A daily dose of 100 mg is the recommended human efficacious dosing regimen for vildagliptin, and no pharmacokinetic gender difference has been observed (He et al., 2007b, 2008). [^{14}C]Vildagliptin has been shown to be highly absorbed in both rats and dogs (He et al., 2009). Vildagliptin was mainly metabolized before excretion in rats and dogs. One major metabolite in excreta involved hydrolysis at the cyano moiety to yield a carboxylic acid metabolite (M20.7) in rats and dogs. Another predominant metabolic pathway included the hydrolysis of the amide bond (M15.3) in the dog.

Materials and Methods

Study Drug. [^{14}C]Vildagliptin (specific activity 0.47 μCi/mg, radiochemical purity >99%) was synthesized by the Isotope Laboratory of Novartis Pharmaceuticals Corporation (East Hanover, NJ). The chemical structure of vildagliptin and the position of the radiolabel are shown in Fig. 1.

Metabolites. Synthetic standards of metabolites M20.2, M20.7, and M15.3 were also obtained from Novartis Pharmaceuticals Corporation.

Human Studies. The study protocol and the informed consent document were approved by an independent institutional review board. The written informed consent was obtained from all the subjects before enrollment.

Four healthy, nonsmoking, male white subjects, age 18 to 45 years, with weights ranging from 77 to 93 kg, participated in the study. Subjects were confined to the study center for at least 20 h before administration of the study drug until 168 h (7 days) postdose. After an overnight fast, the subjects were given a single p.o. 100-mg dose of [^{14}C]vildagliptin as a 250-ml drinking solution. The radioactive dose given per subject was 47 μCi (1.85 MBq). After administration, the subjects continued to abstain from food for an additional 4 h.

Blood was collected at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 36, 48, 72, 96, 120, 144, and 168 h postdose by either direct venipuncture or an indwelling cannula inserted in a forearm vein. Eighteen milliliters of venous blood was collected at each time point in heparinized tubes. Plasma was separated from whole blood by centrifugation, transferred to a screw-top polypropylene tube, and immediately frozen.

Urine samples were collected at predose and at 0 to 4, 4 to 8, 8 to 12, 12 to 16, 16 to 24, 24 to 36, 36 to 48, 48 to 72, 72 to 96, 96 to 120, 120 to 144, and 144 to 168 h postdose. Feces were collected as passed from time of dosing until at least 168 h postdose. All of the samples were stored at −20°C or less until analysis.

Radioactivity Analysis of Blood, Plasma, Urine, and Feces Samples. Radioactivity was measured in plasma and blood by liquid scintillation counting (LSC) on a liquid scintillation analyzer (Tri-CARB 2500; Canberra Industries, Meriden, CT). Plasma was mixed with scintillant and counted directly; whole blood samples were digested with tissue solubilizer (Soluene 350; PerkinElmer Life and Analytical Sciences, Waltham, MA), decolorized with hydrogen peroxide, stored in the dark to reduce luminescence, and then counted. Radioactivity in urine and feces was also assessed by LSC. Urine was mixed with liquid scintillant and counted directly. Feces was homogenized in water (approximately 1 + 2, w/v). Aliquots of feces homogenates were then combusted with a biological oxidizer (Packard Oxidizer 306, PerkinElmer Life and Analytical Sciences) before LSC.

The total radioactivity given with the dose was set to 100%. The radioactivity at each sampling time for urine and feces was defined as the percentage of dose excreted in the respective matrices. The radioactivity measured in plasma was converted to nanogram-equivalents of vildagliptin based on the specific activity of the dose.

Analysis of Unchanged Vildagliptin. Amounts of unchanged vildagliptin in plasma and urine were measured quantitatively using a validated liquid chromatography/tandem mass spectrometry (LC/MS/MS) assay. Aliquots of plasma (200 μl) or human urine (100 μl diluted with 100 μl of water) and 200 μl of internal standard (IS) solution ($^{13}C_5{}^{15}N$-vildagliptin) were transferred to individual wells in a 1-ml, 96-well polypropylene plate. Extraction of the samples was performed using a Quadra-96 model 320 workstation (TomTec, Hamden, CT). Before extraction of samples, a 10-mg Oasis HLB 96-well solid-phase extraction plate (Waters, Milford, MA) was conditioned with 300 μl of methanol, followed by 300 μl of water. The samples were applied to the preconditioned extraction plate. The plate was washed with 300 μl of 5% methanol (containing 2% ammonium hydroxide), 300 μl of 20% methanol (containing 2% ammonium hydroxide), and 300 μl of water. After vacuum-drying each well, the samples were eluted with 2 × 75 μl of 80% methanol (containing 0.1% trifluoroacetic acid) and evaporated under nitrogen (35°C) to a volume of ~50 μl using an Evaporex solvent evaporator (Apricot Designs, Monrovia, CA). The samples were diluted with 50 μl of 15% methanol (containing 0.5% ammonium hydroxide) and mixed before injection.

Samples were analyzed on a Micromass Quattro LC (Waters) operated in multiple reaction monitoring mode with electrospray ionization (ESI$^+$) as an interface. Vildagliptin and IS were separated on a Polaris 5-μm C18-A 50 × 2.0-mm column (45°C) (Metachem Technologies, Torrance, CA) with isocratic elution. The mobile phase of A/B (1:3, v/v) was used, where A was methanol/10 mM ammonium acetate, pH 8.0 (5:95, v/v), and B was acetonitrile/methanol (10:90, v/v). The flow rate was maintained at 0.2 ml/min with an injection volume of 10 μl. Multiple reaction monitoring transitions for the drug and IS were m/z 304.2 → m/z 154.1 and m/z 310.3 → m/z 160.0, respectively. The dynamic range of the assay was from 1.93 to 2020 ng/ml for plasma and 5.13 to 5010 ng/ml for urine.

Sample Preparation of Plasma, Urine, and Feces for Metabolite Investigation. Semiquantitative determination of main and trace metabolites was obtained for plasma, urine, and feces (based on peak areas) using high-performance liquid chromatography (HPLC)-radiodetection with off-line microplate solid scintillation counting and structural characterization by liquid chromatography/mass spectrometry (LC/MS). Plasma samples (3.5–4.5 ml) from each subject at 0.5, 1, 2, 3, 6, 12, 16, and 24 h postdose were protein-precipitated with acetonitrile/ethanol (90:10 v/v) containing 0.1% acetic acid and removed by centrifugation. Recoveries of radioactivity after plasma sample preparation averaged 95%. The supernatant was evaporated to near dryness under a stream of nitrogen using the Zymark Turbo-Vap LV (Zymark Corp., Hopkinton, MA), and the residues were reconstituted in acetonitrile/5 mM ammonium acetate containing 0.1% trifluoroacetic acid (10:90 v/v). Aliquots (80–85 μl) of concentrated plasma extracts were injected onto the HPLC column. For urine analysis, a pool of equal percent volume from the 0- to 48-h fractions (10% of urine volume from each time point, e.g., 0–24 and 24–48 h) was prepared for each subject. An aliquot was centrifuged, and 100 μl was injected onto the HPLC column without further purification. Recoveries of radioactivity after centrifugation of urine samples were 100%. Feces homogenates were pooled from 0 to 96 h at equal percent weight for each subject (10% of feces homogenates from each time point, e.g., 0–24, 24–48, and 48–72 h) and extracted twice with methanol by vortexing and centrifugation. The average recovery of sample radioactivity in the methanolic extracts was 87%. Aliquots of combined supernatant (5 ml) were evaporated to dryness under a stream of nitrogen using the Zymark Turbo-Vap LV, and the residues were reconstituted in 0.2 ml of acetonitrile/5 mM ammonium acetate containing 0.1% trifluoroacetic acid (10:90 v/v). Aliquots (60–80 μl) of concentrated fecal extracts were injected onto the HPLC column.

HPLC Instrumentation for Metabolite Pattern Analysis. Vildagliptin and its metabolites in urine, plasma, and feces were analyzed by HPLC with off-line radioactivity detection using a Waters Alliance 2690 HPLC system equipped with a Phenomenex (Torrance, CA) Synergy Hydro-RP column

(4.6 × 150 mm, 4 μm, maintained at 30°C) and a guard column of the same type. The mobile phase consisted of 5 mM ammonium acetate containing 0.1% trifluoroacetic acid, pH 2.3 (solvent A), and acetonitrile (solvent B), and a gradient method was used. The mobile phase was initially composed of solvent A (100%) and held for 4 min. The mobile phase composition was then linearly programmed to solvent A/solvent B (87:13) over 26 min and held for 2 min and to solvent A/solvent B (40:60) in 0.5 min and held for 4 min. A short gradient was programmed to solvent A/solvent B (5:95) over 0.5 min, and these conditions were held for 4 min. The mobile phase condition was returned to the starting solvent mixture over 0.5 min. The system was allowed to equilibrate for 10 min before the next injection. A flow rate of 1.0 ml/min was used for all the analyses. The HPLC effluent was fractionated into a 96-deep-well Lumaplate (PerkinElmer Life and Analytical Sciences) using a fraction collector (FC 204; Gilson Inc., Middleton, WI) with a collection time of 8.4 s/well. Samples were dried under a stream of nitrogen, sealed, and counted for 1 to 15 min/well on a TopCount microplate scintillation counter (PerkinElmer Life and Analytical Sciences).

The amounts of metabolites of parent drug in plasma or excreta were derived from the radiochromatograms (metabolite patterns) by dividing the radioactivity in original sample in proportion to the relative peak areas. Parent drug or metabolites were expressed as concentrations (in nanogram-equivalent per milliliter) in plasma or as percentage of dose in excreta. These values are to be considered as semiquantitative only, unlike those determined by the validated quantitative LC/MS/MS assay.

In Vitro Human Blood Distribution and Protein Binding. A single 10-μl aliquot of the stock solution in ethanol containing [^{14}C]vildagliptin (35×10^6 dpm) and known amounts of unlabeled vildagliptin was spiked to 1 ml of human fresh blood or plasma ($n = 3$) to achieve final concentrations of 10 to 10,000 ng/ml.

For the blood distribution study, triplicate determinations of the hematocrit in blood were made, and single blood samples (1 ml) were prepared from each of the three male human volunteers. After gentle mixing, a single 200-μl aliquot of blood containing [^{14}C]vildagliptin was pipetted for radioactivity analysis. Then the blood samples were incubated at 37°C for 30 min and centrifuged at ~3000*g* for ~15 min at 37°C. The resultant plasma was analyzed for radioactivity using a single 200-μl aliquot.

For the protein binding study, single plasma samples (1 ml) were prepared from each of the three male human volunteers. The pH was adjusted to 7.4 by adding 10 μl of 0.1 N HCl/ml plasma and gently vortexing the sample. After a single aliquot of plasma containing [^{14}C]vildagliptin (200 μl) was pipetted for radioactivity analysis, each sample (~0.8-ml aliquot) was transferred to the sample reservoir of individual micropartition centrifuge tube (Centrifree Micropartition Centrifuge Tube; Millipore Corporation, Billerica, MA). The membrane had a molecular mass cutoff of 30,000 Da. Samples were centrifuged for 20 min at ~1000*g* at 37°C. The ultrafiltrate contained the free fraction, and 200-μl aliquots were analyzed for radioactivity. Nonspecific binding studies were conducted in 0.2 M sodium phosphate buffer, pH 7.4, under the same conditions described above.

For radioactivity analysis of blood or plasma samples in the blood distribution study, 200 μl of blood was pipetted onto individual Combusto-Pads (PerkinElmer Life and Analytical Sciences), air-dried, and combusted in a Packard 308 oxidizer before counting in a liquid scintillant (PerkinElmer Life and Analytical Sciences). For radioactivity analysis in the protein binding study, aliquots (200 μl) of plasma samples and filtrates were mixed with 2 ml of a liquid scintillant (NEN Formula 989; PerkinElmer Life and Analytical Sciences) in a vial for direct counting. The radioactivity in all the samples was determined by LSC in a Packard spectrometer (Hewlett Packard, Palo Alto, CA). M20.7 human protein binding was also determined over the concentration range from 10 to 1000 ng/ml using the same ultrafiltration method as described above.

In Vitro Metabolism in Human Liver Slices. [^{14}C]Vildagliptin was incubated with liver slice preparations from one human subject. The human tissue was obtained through the Association of Human Tissue Users (Tucson, AZ). Each of the human organs had been perfused with Belzer's University of Wisconsin solution but was rejected for transplantation. The incubations were carried out at 5 and 20 μM substrate concentrations for 1, 2, 4, 10, 18, and 24 h. The incubates were analyzed by HPLC with online radioactivity detection. Metabolites formed from the incubations were characterized by LC/MS. Human liver slices with a diameter of approximately 200 ± 25 μm were prepared from 8-mm diameter tissue cores using a Vitron (Tucson, AZ) sterile tissue slicer. The individual organs were cored transversely and sliced using a Vitron sterile tissue slicer in ice-cold oxygenated (95% O_2/5% CO_2) V7 preservation media. The viability of the human liver slices was assessed by determining the intracellular K^+ content and measurement of ATP content in 0.1% dimethyl sulfoxide (DMSO) and vildagliptin-exposed slice incubates. The slices were placed onto roller culture inserts and maintained at 37°C in Dulbecco's modified Eagle's/F-12 media without phenol red (Invitrogen, Carlsbad, CA) and supplemented with 10 ml/l Antibiotic AntiMycotic solution (Invitrogen), 10% Nu Serum, and Mito/Serum Extender, 1 ml/l (BD Biosciences, Franklin Lakes, NJ). After a preincubation period of 90 min, fresh media containing [^{14}C]vildagliptin in 0.1% DMSO were added. At the various time points, the slice and media were transferred to separate vials, and the roller culture vial and insert were bathed in methanol (3.0 ml), which was then collected. Before HPLC analysis, the human liver slices were disrupted by homogenization with MeOH/H_2O (50:50) followed by brief sonication. The incubation media were extracted with methanol, and the methanol wash was evaporated to dryness. All the fractions were pooled, and the protein was pelleted at approximately 40,000*g* for 10 min at 20°C. The pellet was re-extracted with methanol, and the resultant supernatant was evaporated to dryness and combined with the pooled sample.

Structural Characterization of Metabolites by LC/MS/MS. Metabolite characterization was conducted with a Finnigan LCQ ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with an ESI source. The effluent from the HPLC column was split, and approximately 500 μl/min was introduced into the atmospheric ionization source after diverting to waste during the first 4 min of each run to protect the mass spectrometer from nonvolatile salts. The electrospray interface was operated at 5000 V, and the mass spectrometer was operated in the positive ion mode. Collision-induced dissociation (CID) studies were performed using helium gas at the collision energy of 35% (arbitrary unit).

Metabolism of Vildagliptin in Human Liver Microsomes and by Recombinant Cytochromes P450. The metabolism of [^{14}C]vildagliptin (specific activity of 154.5 μCi/mg) was examined in pooled human liver microsomes ($n = 46$ donors, mixed gender) and in microsomal preparations from baculovirus-infected insect cells expressing recombinant human cytochrome P450 (P450) enzymes (BD Gentest, Woburn, MA). The recombinant P450 enzymes examined in this study were CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, and CYP4A11. Human liver microsomes (1 mg of microsomal protein/ml) or recombinant P450 enzymes (100 pmol of P450/ml) were preincubated with [^{14}C]vildagliptin (47 μM, 0.5% final DMSO concentration, v/v) in 100 mM potassium phosphate buffer, pH 7.4, and 5 mM $MgCl_2$, final concentrations, at 37°C for 3 min. The reactions were initiated by the addition of NADPH (1 mM, final concentration) and incubated for 30 min; reactions were then quenched by the addition of half the reaction volume of cold acetonitrile. The precipitated protein was removed by centrifugation, and an aliquot of each sample was analyzed by HPLC with in-line radioactivity detection as described above.

P450 Inhibition Assessment by Vildagliptin and M20.7. The ability of vildagliptin and its metabolite M20.7 to inhibit P450 enzyme activity was assessed using pooled human liver microsomes ($n = 50$ donors, mixed gender; XenoTech, LLC, Lenexa, KS). To determine individual P450 activities, several probe substrate reactions were used that are known to be P450 enzyme-selective. The reactions used and corresponding probe substrate concentrations included phenacetin *O*-deethylation (5 μM, CYP1A2), bupropion hydroxylation (25 μM, CYP2B6), paclitaxel 6α-hydroxylation (10 μM, CYP2C8), diclofenac 4′-hydroxylation (5 μM, CYP2C9), *S*-mephenytoin 4′-hydroxylation (15 μM, CYP2C19), bufuralol 1′-hydroxylation (5 μM, CYP2D6), chlorzoxazone 6-hydroxylation (10 μM, CYP2E1), and midazolam 1′-hydroxylation (5 μM, CYP3A4/5). The probe concentrations used were less than or approximately equal to their reported K_m values. The conditions for each probe reaction were previously established to ensure linearity with time and protein concentration and to limit probe substrate turnover to ~<10% (results not shown). Increasing concentrations of the vildagliptin or authentic synthetic M20.7 (up to 100 μM) were incubated at 37°C individually with human liver microsomes (0.2 or 0.5 mg/ml) and one probe substrate in (final concentration)

100 mM potassium phosphate buffer, pH 7.4, 1 mM NADPH, 5 mM $MgCl_2$, 1 mM EDTA, and 0.2% DMSO. After incubation, the reactions were quenched by addition of an equal volume of cold acetonitrile. Probe substrate turnover was determined by LC/MS/MS analysis (PE Sciex API300 mass spectrometer; Applied Biosystems, Foster City, CA; Shimadzu LC, Shimadzu, Kyoto, Japan) of metabolite formation. Reference standards for probe metabolites were obtained from commercial sources as follows: acetaminophen (Sigma-Aldrich, St. Louis, MO); 1′-hydroxybufuralol, hydroxybupropion, 6-hydroxychlorzoxazone, 4′-hydroxy-*S*-mephenytoin, and 1′-hydroxymidazolam (Ultrafine Chemicals, Manchester, UK); and 6-hydroxypaclitaxel and 4′-hydroxydiclofenac (BD Biosciences, San Jose, CA). Chromatographic separation was achieved on a Supelco (Bellefonte, PA) Discovery DP-Amide C16 column (50 × 2.1 mm, 4 μm, 0.3 ml/min flow rate, 25°C). The chromatographic solvents were as follows: A = 0.1% formic acid in 10 mM ammonium acetate, pH ~4.7, B = acetonitrile; the gradient elution program (%B) was 0→1 min (5%), 1→4 min (from 5% to 95%), 4→6 min (95%), 6→6.5 min (from 95% to 5%). Probe metabolites derived from phenacetin, bupropion, midazolam, bufuralol, and paclitaxel were analyzed using ESI in positive ion mode, whereas the metabolites of the remaining probes (diclofenac, chlorzoxazone, *S*-mephenytoin) were analyzed in negative ion mode.

UDP Glucuronosyltransferase Enzyme Involvement in the Glucuronidation of Vildagliptin. The enzymes involved in the glucuronidation of vildagliptin to form M20.2 were determined using a panel of recombinant human UDP glucuronosyltransferase (UGT) enzymes, including UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17 (BD Gentest). In the initial assessment, incubations (100 μl, 37°C) consisted of (final concentrations): vildagliptin (20 μM), UDP-glucuronic acid (5 mM), alamethicin (0.25 mg/mg protein), enzyme protein (1 mg/ml), $MgCl_2$ Tris-HCl buffer (pH 7.6, 50 mM), and acetonitrile (<0.2%). The enzyme protein had been preincubated with the alamethicin for 15 min on ice immediately before the experiments. The reactions were initiated by the addition of UDP-glucuronic acid after a 3-min preincubation and terminated after 60 min by the addition of acetonitrile (200 μl). The incubation samples were evaporated, reconstituted in 100 μl of 10:90 (v/v) acetonitrile/water, and centrifuged at ~4000*g* (10 min). A 25-μl aliquot was analyzed by LC/MS (Finnigan hybrid LTQ ion trap, ESI, positive mode). Chromatographic separation was achieved using an Ace3 C18 (50 × 3 mm, 3.5 μm, 0.25 ml/min flow rate, 35°C). The chromatographic solvents were as follows: A = 0.1% formic acid in 10 mM ammonium acetate, pH ~4.7, B = acetonitrile; the gradient elution program (%B) was 0→1 min (10%), 1→5 min (from 10 to 30%), 5→6 min (from 30 to 98%), 6→7 min (98%), and 7→8 min (from 98 to 10%).

DPP-4-Catalyzed Formation of M20.7. Vildagliptin (0.1 μM) was incubated with human recombinant DPP-IV (expressed in Sf9 cells; Sigma-Aldrich) in Tris-HCl buffer (50 mM, pH 8.0) at 37°C. After 60 min, the reaction was terminated by the addition of acetonitrile (200 μl). The incubation samples were evaporated, reconstituted in 100 μl of 10:90 (v/v) acetonitrile/water, and centrifuged at ~4000*g* (10 min). A 25-μl aliquot was analyzed by LC/MS. The LC/MS method was as described above (see UGT involvement).

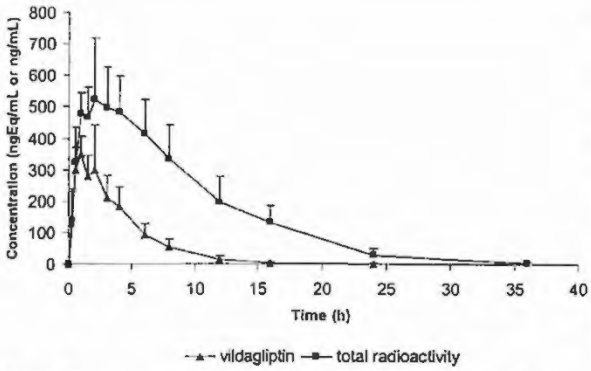


FIG. 2. Plasma concentrations of radioactivity (squares) and vildagliptin parent drug (triangles) after a single 100-mg p.o. dose of [^{14}C]vildagliptin to humans.

TABLE 1

Pharmacokinetic parameters (mean ± S.D.) after a single 100-mg p.o. dose of [^{14}C]vildagliptin

Pharmacokinetic Parameters[a]	Plasma Vildagliptin[b]	Plasma Radioactivity[c]	Blood Radioactivity[c]
C_{max} (ng/ml) or (ngEq/ml)	397 ± 92	594 ± 153	470 ± 87
t_{max} (h)	1.1 ± 0.6	2.1 ± 1.3	2.0 ± 1.4
AUC_{0-t} (ng · h/ml) or (ngEq · h/ml)[d]	1620 ± 460		
$AUC_{0-\infty}$ (ng · h/ml) or (ngEq · h/ml)	1610 ± 460	6000 ± 1610	3850 ± 1580
$t_{1/2}$ (h)	2.8 ± 1.0	4.6 ± 0.3	5.1 ± 2.5
CL/F (l/h)	65.2 ± 15.5		
Vz/F (l)	269 ± 125		

[a] The abbreviation definitions for pharmacokinetic parameters, e.g., C_{max}, t_{max}, AUC, $t_{1/2}$, CL/F, and Vz/F are denoted in the *Pharmacokinetic Analysis* under *Materials and Methods*.
[b] Vildagliptin was determined by validated LC/MS/MS.
[c] Total radioactivity was determined by LSC.
[d] t was 24 or 48 h.

Pharmacokinetic Analysis. The following pharmacokinetic variables were determined by fitting the concentration-time profiles to a noncompartmental model with an iterative nonlinear regression program (WinNonlin software version 4.0; Pharsight, Mountain View, CA): area under the blood or plasma drug concentration-time curve between time 0 and time t (AUC_{0-t}); AUC until time infinity ($AUC_{0-\infty}$); highest observed blood or plasma drug concentration (C_{max}); time to highest observed drug concentration (t_{max}); apparent terminal half-life ($t_{1/2}$); apparent volume of distribution of parent drug (V_z/F) calculated as dose/(AUC · λ_z), where F is bioavailability and λ_z is the terminal rate constant; and apparent clearance (CL/F), calculated as dose/$AUC_{0-\infty}$.

Results

In Vitro Human Blood Distribution and Protein Binding. The mean human blood/plasma ratio (C_b/C_p) and fraction of [^{14}C]vildagliptin hound to red blood cells (f_{BC}) were 1.0 and 0.44, respectively, indicating approximately equal distribution between plasma and blood cells. The blood distribution was independent of concentration between 10 and 10,000 ng/ml.

The mean plasma protein binding of vildagliptin in humans was low (9.3%) and also independent of concentration. The nonspecific binding of the compound to centrifuge tubes and/or membranes was low (<12%), suggesting that ultrafiltration is a suitable method. In addition, M20.7 showed no plasma protein binding in humans over the concentration range of 10 to 1000 ng/ml and was independent of concentration.

Absorption. The absorption of vildagliptin was rapid after oral administration, with tbe peak plasma concentration of vildagliptin observed at an average of 1.1 h (range, 0.5–2 h). The percentage of drug absorbed was estimated to be at least 85.4%, because this amount of the radioactivity was recovered in urine.

Pharmacokinetics of Vildagliptin and Total Radioactivity. The mean plasma concentration-time profiles and pharmacokinetic parameters of total radioactivity and unchanged vildagliptin in healthy male volunteers after a single oral dose of [^{14}C]vildagliptin are shown in Fig. 2 and Table 1, respectively. The highest concentrations in plasma (C_{max}) were achieved at 2.1 h postdose with the mean value 594 ng-Eq/ml (total radioactivity) and at 1 h postdose with the mean value of 397 ng/ml (vildagliptin) in all four subjects. Radioactivity and parent levels at 48 h were below the limit of quantification. The terminal elimination half-life ($t_{1/2}$) of radioactivity and vildagliptin averaged 4.6 and 2.8 h, respectively. Based on $AUC_{0-\infty}$ values, approximately 25.7 and 55% of the circulating radioactivity were attributable to unchanged vildagliptin and its major metabolite M20.7,

TABLE 2

Cumulative excretion of ^{14}C radioactivity in urine and feces after a single p.o. 100-mg dose of [^{14}C]vildagliptin to humans, mean ± S.D.

Time Period (h)	Urine	Feces	Total
h	*% dose*	*% dose*	*% dose*
0–24	72.7 ± 4.8	1.37 ± 2.0	
0–48	81.6 ± 4.2	9.93 ± 8.0	
0–72	83.8 ± 4.4	13.2 ± 4.8	
0–96	84.7 ± 4.4	14.3 ± 3.7	
0–168	85.4 ± 4.4	14.8 ± 3.5	100 ± 1

respectively. In addition, mean blood-to-plasma ratios of radioactivity calculated at specific time points (between 0.25 and 1 h) averaged near 1.1, indicating that vildagliptin distributed almost equally between blood cells and plasma as the main circulating component was the parent drug at the early time points, consistent with the in vitro finding (C_b/C_p ratio of vildagliptin ~1). However, the total radioactivity AUC_b/AUC_p ratio was 0.64, suggesting that metabolite(s) should be distributed more to plasma than blood cells. The CL/F and V_z/F values of vildagliptin were 65.2 l/h and 269 liters, respectively. With a human bioavailability of 84%, the estimated CL was 55 l/h, which is slightly higher than that after an intravenous infusion dose (41 l/h) (He et al., 2007a). A measured V_z value has not been reported in the literature, but V_z can be estimated to be 229 liters based on data from this study.

Excretion and Mass Balance in Urine and Feces. After a single p.o. 100-mg dose of [^{14}C]vildagliptin, radioactivity was excreted predominantly in the urine (Table 2). At 168 h after dosing, the excretion in urine and feces averaged 85.4 ± 4.4 and 14.8 ± 3.5% of the administered dosed, respectively. In total, the cumulative excretion of radioactivity was complete in all four subjects, averaging 100 ± 1.10%. Elimination of radioactivity was rapid. More than 90% of the entire radioactivity recovered in urine and feces was excreted in the first 48 h after dose administration.

Metabolite Profiling. Tables 3 and 4 summarize the pharmacokinetics of vildagliptin and its metabolites in plasma and percentage of vildagliptin and its metabolites in excreta, respectively, after a single p.o. 100-mg dose of [^{14}C]vildagliptin to humans. A representative HPLC radiochromatogram of circulating metabolites is shown in Fig. 3A. In plasma, major circulating components were unchanged drug and M20.7 (a carboxylic metabolite), accounting for 25.7% (vildagliptin) and 55% (M20.7) of the total plasma exposure. At early time points (up to 1 h) postdose, the predominant radiolabeled plasma component was vildagliptin, accounting for 70.2 to 88.3% of the total radioactivity. At later time points (12–24 h), the predominant component was M20.7, accounting for 78.4 to 89.9% of the total radioactivity. Other metabolites detected in plasma included M15.3 (carboxylic metabolite formed from hydrolysis of the amide bond) and M20.2 (glucuronic acid conjugate of vildagliptin), accounting for 8.1 and 9.3%, respectively, of the total plasma exposure.

Representative HPLC radiochromatograms for metabolites in urine and feces are shown in Fig. 3, B and C, respectively. Approximately 27.1% of the administered dose was excreted as unchanged drug in urine and feces. In addition to the unchanged drug, five metabolites were radiochemically quantifiable in the urine or feces. In urine, M20.7 represented the major metabolite (49.6% of the administered dose), and all the other metabolites were minor (each <4.5% of the dose). Unchanged vildagliptin in urine averaged at 22.6% of the dose. In the feces, M20.7 was also the major metabolite, accounting for 6.89% of the dose; unchanged vildagliptin accounted for 4.54% of the dose. In summary, the urinary and fecal metabolites totally accounted for >90% of the total radioactivity recovered.

In Vitro Metabolism in Human Liver Slices. Incubation of [^{14}C]vildagliptin with human liver slices formed three metabolites: M15.3, M20.7, and M20.2 (formed at late time points in the incubations at 20 μM substrate concentration).

Metabolite Characterization by Mass Spectrometry. Metabolite structures were characterized by LC/MS/MS using a combination of full and product ion scanning techniques. The structure of major metabolites, where possible, was supported by comparisons of their retention times on HPLC and mass spectra with those of synthetic standards (vildagliptin, M20.2, M20.7, and M15.3).

Vildagliptin displayed a protonated molecular ion (MH^+) at *m/z* 304. The product ion spectrum of *m/z* 304 showed major fragment ions at *m/z* 97 and 154 (Fig. 4). The ion at *m/z* 154 corresponded to the amino acetyl pyrrolidine carbonitrile moiety, and *m/z* 97 corresponded to the pyrrolidine carbonitrile moiety.

Metabolite M15.3. M15.3 was found in urine, feces, and plasma. Mass spectral analysis showed a protonated molecular ion at *m/z* 226 [78 atomic mass units (amu) lower than the parent drug]. The product ion mass spectrum of *m/z* 226 showed fragment ions at *m/z* 76 and 151. The ion at *m/z* 151 corresponded to the hydroxy adamantyl moiety, suggesting modification had occurred at the other half of vildagliptin. Based on comparison of the HPLC retention time and CID spectrum with the synthetic standard, M15.3 was identified as the carboxylic acid metabolite resulting from hydrolysis at the amide bond.

Metabolite M20.2. M20.2 was found in urine and plasma. Its full-scan mass spectrum displayed a protonated molecular ion at *m/z* 480, 176 amu higher than that of the parent drug, suggesting that it was a glucuronide conjugate. Based on comparison of the HPLC retention time and CID spectrum with the synthetic standard, M20.2 was identified as the *O*-glucuronide of vildagliptin.

Metabolite M20.7. M20.7 was found in urine, feces, and plasma. Its full-scan MS displayed a protonated molecular ion at *m/z* 323, 19 amu higher than that of the parent drug. The product ion mass spectrum of *m/z* 323 showed fragment ions at *m/z* 305, 173, and 116. The ion at *m/z* 305 occurred from the loss of water in the molecule. The ions at *m/z* 173 and 116 were 19 amu higher than those observed in the parent drug (*m/z* 154 and 97, respectively), suggesting modification had occurred at the pyrrolidine carbonitrile moiety. Based on comparison of the HPLC retention time and CID spectrum with the synthetic

TABLE 3

Pharmacokinetic parameters (mean ± S.D.) of vildagliptin and its metabolites in plasma after a single p.o. 100-mg dose of [^{14}C] vildagliptin

Pharmacokinetic Parameters[a]	M15.3[b]	M20.2[b]	M20.7[b]	Vildagliptin[b]
C_{max} (ngEq/ml)	54.9 ± 20.4	90.9 ± 23.1	230 ± 50	399 ± 100
t_{max} (h)	3.8 ± 1.5	1.8 ± 0.5	6.0 ± 0.0	1.3 ± 0.5
$AUC_{0-168\ h}$ (ngEq · h/ml)	489 ± 166	559 ± 120	3310 ± 1190	1550 ± 520
$AUC_{0-\infty}$ (ngEq · h/ml)	490 ± 164	572 ± 124	3350 ± 970	1550 ± 510
AUC (%)	8.1	9.3	55	25.7

[a] The abbreviation definitions for pharmacokinetic parameters, e.g., C_{max}, t_{max}, and AUC are denoted in the *Pharmacokinetic Analysis* under *Materials and Methods*.
[b] Vildagliptin and its metabolites were determined by HPLC with radio detection.

TABLE 4

Amount of vildagliptin and metabolites (expressed as percentage of dose) in urine (0–48 h) and feces (0–96 h) after a single p.o. 100-mg dose of [^{14}C]vildagliptin, mean ± S.D.

Matrices	M15.3	M20.2	M20.7	M20.9	M21.6	Vildagliptin
Urine	3.40 ± 1.1	4.40 ± 0.47	49.6 ± 3.6	0.95 ± 0.18	0.64 ± 0.50	22.6 ± 5.3
Feces	0.26 ± 0.07	—	6.89 ± 2.0	—	—	4.54 ± 2.9
Urine + feces	3.7 ± 1.1	4.4 ± 0.5	56.5 ± 3.5	1.0 ± 0.2	0.6 ± 0.5	27.1 ± 2.5

—, not detected.

standard, M20.7 was identified as the carboxylic acid metabolite resulting from hydrolysis of the cyano moiety.

Metabolite M20.9. M20.9 was found in urine and plasma. Its full-scan MS displayed a protonated molecular ion at *m/z* 336, 32 amu higher than that of the parent drug. The product ion mass spectrum of *m/z* 336 showed fragment ions at *m/z* 318, 186, and 129. The ion at *m/z* 318 occurred from the loss of water in the molecule. The ions at *m/z* 186 and 129 were 32 amu higher than those observed in the parent drug (*m/z* 154 and 97, respectively), suggesting modification (dioxygenation) had occurred at the pyrrolidine carbonitrile moiety. Based on these data, M20.9 was tentatively identified as dihydroxy vildagliptin.

Metabolite M21.6. M21.6 was found in urine and plasma. Its full-scan MS displayed a protonated molecular ion at *m/z* 311, 7 amu

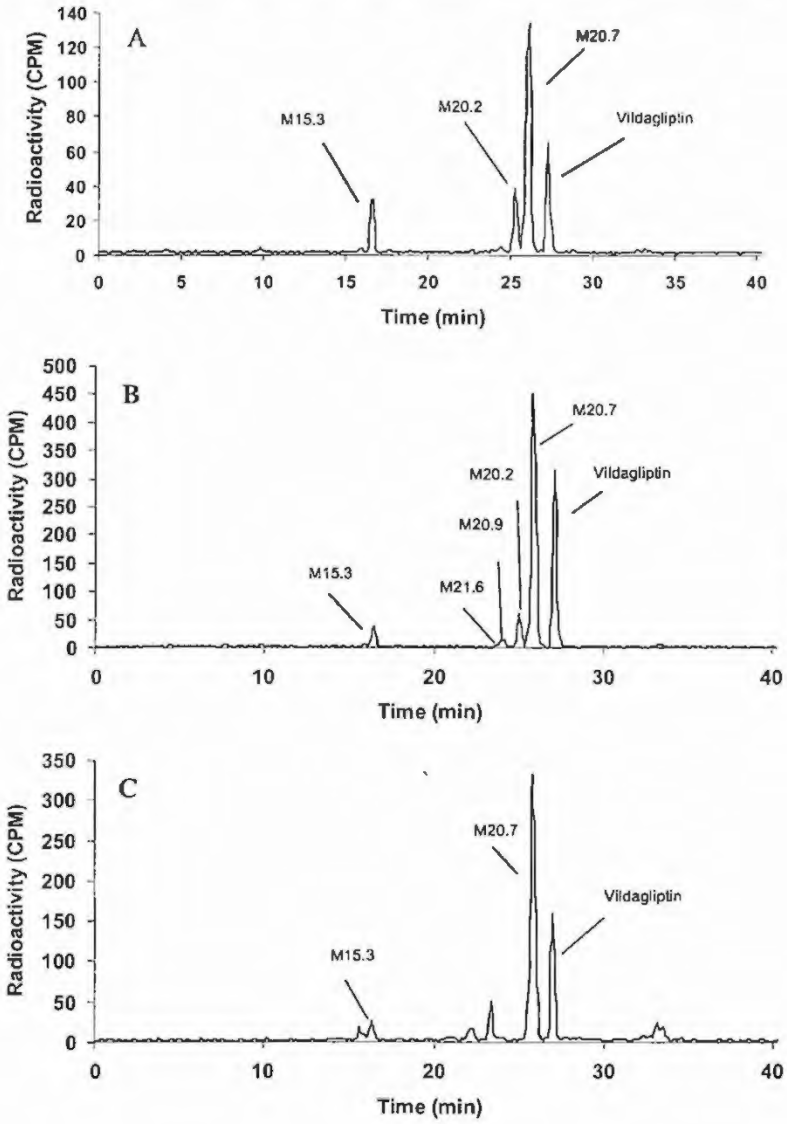


FIG. 3. Representative radiochromatograms of vildagliptin in plasma 6 h postdose (A), urine 0 to 48 h (B), and feces 0 to 96 h (C) after a single oral dose of [^{14}C]vildagliptin to humans. Note that M15.3, M20.2, and M20.7 were identified by retention times and CID product ion spectra that were similar to those of their synthetic standards, whereas the other metabolite structures were tentatively assigned as described under *Results*.

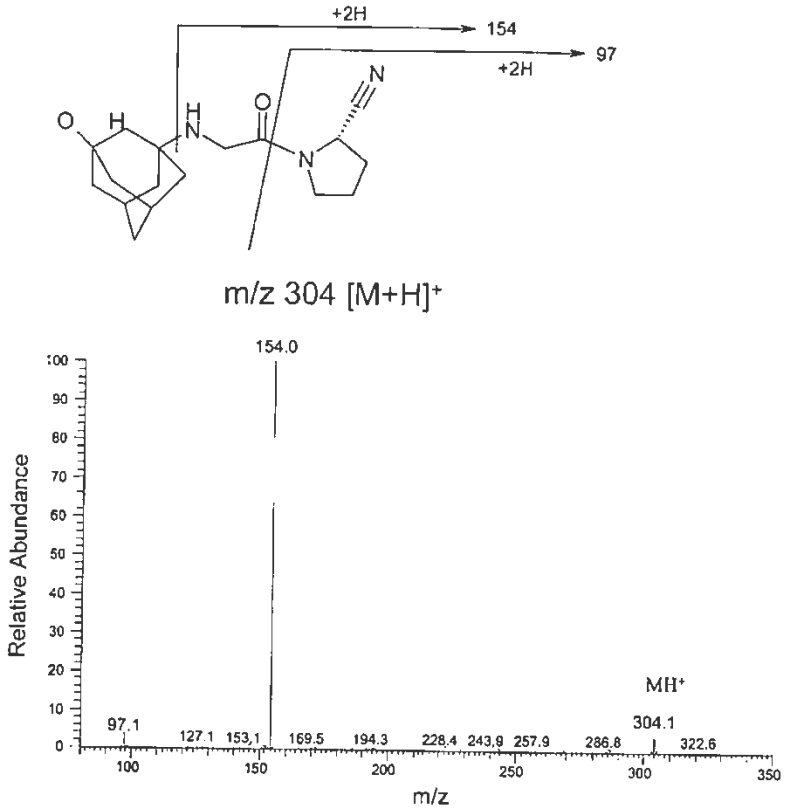


FIG. 4. CID mass spectrum of vildagliptin.

higher than that of the parent drug. Because its protonated molecular ion is an odd number, the molecule must contain an even number of nitrogen. The product ion mass spectrum of *m/z* 311 showed fragment ions at *m/z* 293, 161, and 143. The ion at *m/z* 161 was 7 amu higher than that observed in the parent drug (*m/z* 154), suggesting modification had occurred at the pyrrolidine carbonitrile moiety. Based on these data, M21.6 was identified as the carboxylic acid metabolite resulting from opening of the pyrrolidine moiety.

Identification of P450 Enzymes Involved in the Metabolism of Vildagliptin. The oxidative metabolism of [^{14}C]vildagliptin at 47 μM was evaluated in human liver microsomes and by individual P450 enzymes. The results indicated that [^{14}C]vildagliptin was not metabolized in human liver microsomes nor by any P450 enzymes examined to any quantifiable extent in the presence of NADPH.

DPP-4-Catalyzed Conversion of Vildagliptin to Form M20.7. Incubation of vildagliptin (0.1 μM) with recombinant human DDP-4 (0.1 mg/ml) for 60 min resulted in approximately 1.5% turnover to the carboxylic acid metabolite, M20.7, supporting the contribution of this enzyme to this metabolic pathway.

Identification of the UGT Enzymes Involved in Formation of M20.2. Incubation of vildagliptin (20 μM) with a panel of recombinant UGT enzymes indicated that vildagliptin *O*-glucuronide (M20.2) seems to be primarily catalyzed by UGT2B7. Minor contributions of UGT enzymes UGT2B17 and UGT2B4 were also observed (Fig. 6).

Inhibition of P450 by Vildagliptin and M20.7. Vildagliptin and M20.7 showed little or no inhibition of P450 enzyme activities (IC_{50} values > 100 μM), including CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4/5, when tested at concentrations of up to 100 μM.

Discussion

In the present study, the absorption and disposition of vildagliptin were investigated in four male healthy volunteers after a single p.o. 100-mg dose of [^{14}C]vildagliptin (47 μCi), which was well tolerated in all the subjects. Vildagliptin was rapidly absorbed with a short mean t_{max} of 1.1 h. Based on the amount of dose recovered in urine (85.4%) and with the finding of only a small amount of unchanged drug in feces (4.54% of the dose), vildagliptin absorption in humans was estimated to be at least 85.4%. This result is in agreement with the high absolute oral bioavailability of vildagliptin determined in healthy volunteers (85%) based on the single i.v. 25-mg dose (He et al., 2007a), suggesting a low first-pass metabolism and high intestinal absorption.

At t_{max} for the radioactivity, vildagliptin accounted for approximately 67% of the radioactivity in plasma. Thereafter, the parent drug concentrations decreased more rapidly than the total radioactivity, reflecting the increasing presence of metabolites in the circulation, predominantly metabolite M20.7. The terminal half-lives of both parent drug (average $t_{1/2}$ 2.8 h) and radioactivity (average $t_{1/2}$ 4.6 h) in plasma were short, consistent with previous findings in healthy volunteers and patients (He et al., 2007b). However, this short half-life does not affect the DPP-4 inhibition as vildagliptin inhibited DPP-4 up to 24 h (90%) (He et al., 2007b).

Four metabolic pathways are involved in the in vivo biotransformation of vildagliptin (Fig. 5): 1) cyano group hydrolysis, leading to

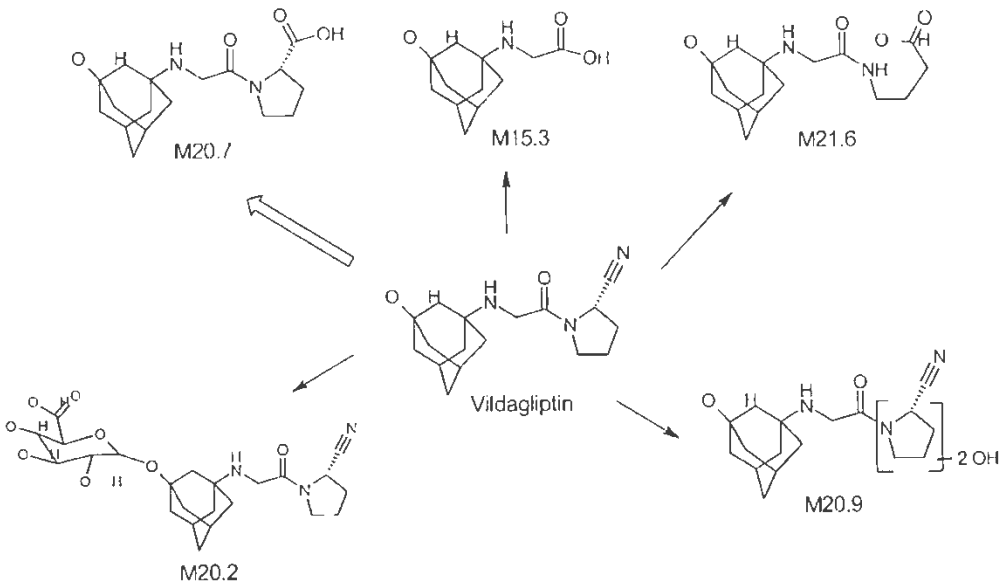


FIG. 5. Metabolism of vildagliptin in humans. The major route is indicated by a large arrow.

formation of M20.7; 2) amide bond hydrolysis, leading to formation of M15.3; 3) glucuronidation, leading to formation of M20.2; and 4) oxidation on the pyrrolidine, leading to the formation of M20.9 and M21.6. The latter is thought to be derived from the decarboxylation of M20.7 and/or the loss of cyano group of the parent drug.

The primary route of elimination of radioactivity was via urinary excretion (85.4%), and vildagliptin was mainly eliminated in humans through hydrolysis reactions and excretion of unchanged drug. Hydrolysis of the cyano group, resulting in the M20.7, accounted for elimination of 56.5% of the dose (49.6% in urine and 6.89% in feces). M20.7 was not detected in the liver microsome incubation in the presence of NADPH, suggesting P450 is not involved in the hydrolysis of the cyano group. However, M20.7 was observed in the human liver slice, confirming the hydrolysis occurs in liver, possibly in the cytosolic fraction. Furthermore, based on findings from the in vivo metabolism data in DPP-4-deficient rats (He et al., 2009), approximately 20% of the cyano group hydrolysis reaction (formation of main metabolite M20.7) may be attributable to the DPP-4 enzyme. Indeed, the formation of M20.7 was found to be catalyzed by human recombinant DDP-IV in vitro, supporting the contribution of this enzyme to this metaholic transfomation.

Approximately 27.1% of vildagliptin was excreted as unchanged (22.6% in urine and 4.54% in feces). Given negligihle protein binding (9.3% for vildagliptin and no binding for M20.7), the renal clearance was estimated to be 14.6 and 15.0 l/h for vildagliptin and M20.7, respectively, using the ratio of the total amount in urine (22.6 mg for vildagliptin and 49.6 mg for M20.7) relative to total AUC in plasma (1550 ng · h/ml for vildagliptin and 3350 ng · h/ml for M20.7). Similar renal clearances of vildagliptin (13.4 l/h) have also heen reported after an intravenous dose (He et al., 2007a). With the protein binding correction (fu = 90.7% for vildagliptin and 100% for M20.7), the unbound renal clearance of vildagliptin and M20.7 was estimated to be 16 and 15 l/h, respectively. Thus, the unbound renal clearance of vildagliptin and M20.7 was 167 and 150% higher than the glomerular filtration rate (6.0 l/h) in healthy humans, respectively (Rule et al., 2004), suggesting possible involvement of renal transporters in the elimination process.

Vildagliptin was also metabolized to form M20.2 (vildagliptin *O*-glucuronide), only accounting for 4.4% of the dose. The direct glucuronidation of vildagliptin was found to be primarily catalyzed by UGT2B7, with minor contributions by UGT2B17 and UGT2B4 (Fig. 6). The formation of minor metabolites, M20.9 and M21.6, is probably mediated by P450s. However, these pathways accounted for only approximately 1.6% of the dose, indicating a lack of significant P450 involvement. The oxidative metabolism of vildagliptin was confirmed in human liver microsomes and by individual human recombinant P450 enzymes. Consistent with the in vivo findings, results from the in vitro studies indicated that vildagliptin was not metabolized by P450 enzymes to any quantifiable extent. Therefore, it is unlikely that inhibitors or inducers of P450 enzymes would have a significant drug-drug interaction effect on the metabolic clearance of vildagliptin in humans. Moreover, as both vildagliptin and M20.7 showed no inhibition on P450 isoenzyme activities (IC_{50} values $\geq$100 μM) and given that the plasma C_{max} values of vildagliptin and M20.7 in humans after a single oral dose of 100 mg (the clinical efficacious dose) is much lower (2–10 μM) than the IC_{50} values, it is unlikely that vildagliptin and M20.7 would inhibit the metabolic clearance of potential comedications metabolized by P450 enzymes.

The elimination of vildagliptin in humans was comparable with that in rats and dogs, being primarily metabolized to M20.7 and excreted as unchanged drug (He et al., 2009). The other major pathway in dogs was the hydrolysis at the amide bond (M15.3). Overall, all the metabolites observed in human plasma and excreta were also found in rat and dog.

In summary, vildagliptin is rapidly absorbed in fasting humans after a single oral dose of 100 mg; the absorption was high (>85.4%), and dose recovery was complete. The major circulating components in the plasma were unchanged drug and metabolite M20.7. Elimination of vildagliptin in humans mainly involved renal excretion of unchanged parent drug and cyano group hydrolysis with little P450 involvement, suggesting a low potential for drug-drug interaction when coadmin-

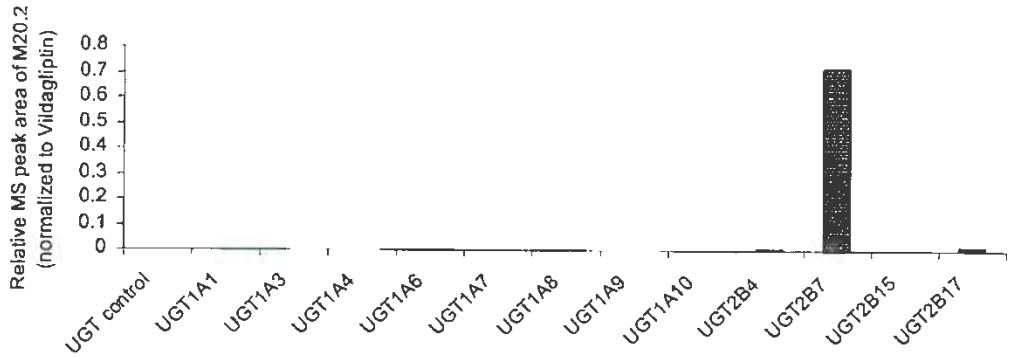


FIG. 6. Glucuronidation of vildagliptin by human recombinant UGT enzymes. Vildagliptin was incubated with recombinant human UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17 at 37°C for 60 min. A substrate concentration of 20 μM and a protein concentration of 1 mg/ml were used.

istered with P450 inhibitors/inducers. Moreover, vildagliptin is unlikely to inhibit the metabolic clearance of comedications metabolized by P450 enzymes.

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Pharmacokinetics of the Dipeptidyl Peptidase 4 Inhibitor Saxagliptin in Rats, Dogs, and Monkeys and Clinical Projections

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Pharmaceutical Candidate Optimization (A.F., A.K., B.K., S.-Y.C., C.C., L.J.C., W.G.H.), Preclinical and Pharmaceutical Development Informatics (V.V.), Discovery Medicine and Clinical Pharmacology (D.W.B.), Drug Safety Evaluation (K.D.C.), Discovery Chemistry (L.G.H.), and Metabolic Diseases (M.K.), Bristol-Myers Squibb, Research and Development, Princeton, New Jersey

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ABSTRACT:

Saxagliptin is a potent, selective, reversible dipeptidyl peptidase 4 (DPP4) inhibitor specifically designed for extended inhibition of the DPP4 enzyme and is currently under development for the treatment of type-2 diabetes. The pharmacokinetics of saxagliptin were evaluated in rats, dogs, and monkeys and used to predict its human pharmacokinetics. Saxagliptin was rapidly absorbed and had good bioavailability (50–75%) in the species tested. The plasma clearance of saxagliptin was higher in rats (115 ml/min/kg) than in dogs (9.3 ml/min/kg) and monkeys (14.5 ml/min/kg) and was predicted to be low to moderate in humans. The plasma elimination half-life was between 2.1 and 4.4 h in rats, dogs, and monkeys, and both metabolism and renal excretion contributed to the overall elimination. The primary metabolic clearance pathway involved the formation of a significant circulating, pharmacologically active hydroxylated metabolite, M2. The volume of distribution values observed in rats, dogs, and monkeys (1.3–5.2 l/kg) and predicted for humans (2.7 l/kg) were greater than those for total body water, indicating extravascular distribution. The in vitro serum protein binding was low (≤30%) in rats, dogs, monkeys, and humans. After intra-arterial administration of saxagliptin to Sprague-Dawley and Zucker diabetic fatty rats, higher levels of saxagliptin and M2 were observed in the intestine (a proposed major site of drug action) relative to that in plasma. Saxagliptin has prolonged pharmacodynamic properties relative to its plasma pharmacokinetic profile, presumably due to additional contributions from M2, distribution of saxagliptin and M2 to the intestinal tissue, and prolonged dissociation of both saxagliptin and M2 from DPP4.

The inhibition of dipeptidyl peptidase 4 (DPP4) is a novel and promising treatment for type 2 diabetes. DPP4 is a proline-specific serine protease enzyme that is known to rapidly degrade the incretin hormones gastric inhibitory polypeptide and glucagon-like peptide-1. Incretins are essential for regulating both fasting and postprandial plasma glucose by stimulating insulin secretion, supporting β-cell mass, and inhibiting glucagon production by the α-cells to reduce glucose production by the liver (Barnett, 2006; Deacon et al., 2008). DPP4 inhibitors augment glucose homeostasis by preventing the degradation of these incretins (Deacon et al., 2008). Clinical trials of DPP4 inhibitors have demonstrated significant glycemic efficacy with a low risk of hypoglycemia, a neutral effect on body weight, and the potential, based on animal and in vitro studies, for preservation or enhancement of β-cell function (Barnett, 2006; Amori et al., 2007; Drucker, 2007; Raz et al., 2008).

Saxagliptin, also known as BMS-477118 (Fig. 1), is a potent and selective inhibitor of DPP4 with an inhibition constant (K_i) value of 1.3 nM and is currently under development for the treatment of type 2 diabetes (Augeri et al., 2005; Metzler et al., 2008; Rosenstock et al., 2008). Saxagliptin was designed to provide high potency and selectivity and extended inhibition of DPP4. It has been shown to significantly reduce DPP4 activity in healthy human subjects and lower plasma glucose in patients with type 2 diabetes mellitus (Rosenstock et al., 2008). The pharmacokinetics of saxagliptin were evaluated in rats, dogs, and monkeys to support compound selection and pharmacodynamic and preclinical toxicology studies. Tissue distribution of saxagliptin and its major, pharmacologically active metabolite, M2 (BMS-510849), that is also a potent, selective, reversible DPP4 inhibitor (K_i of 2.6 nM against DPP4) (Fig. 1) in Sprague-Dawley (SD) rats and Zucker diabetic fatty (ZDF) rats after intra-arterial administration was also evaluated to begin to assess how tissue concentrations

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ABBREVIATIONS: DPP4, dipeptidyl peptidase 4; BMS-477118, (*S*)-3-hydroxyadamantylglycine-L-*cis*-4,5-methanoprolinenitrile, saxagliptin; BMS-510849, (*S*)-3,5-dihydroxyadamantylglycine-L-*cis*-4,5-methanoprolinenitrile, metabolite M2; SD, Sprague-Dawley; ZDF, Zucker diabetic fatty; LC-MS/MS, liquid chromatography-tandem mass spectrometry; BDC, bile duct-cannulated; SAD, single ascending dose; SIT, species invariant time; ROE, rule of exponent.

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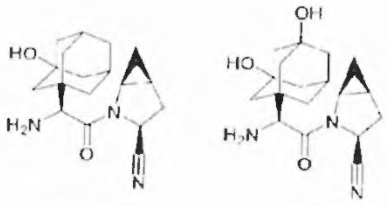

FIG. 1. Molecular structures of saxagliptin and M2.

are related to efficacy endpoints including plasma DPP4 inhibition. Important human pharmacokinetic parameters were also predicted based on the preclinical in vivo pharmacokinetic data using in vivo scaling methodologies and compared with actual clinical data.

Materials and Methods

Chemicals and Reagents. Saxagliptin [(*1S*)-3-hydroxyadamantylglycine-(*cis*-4,5-methanoprolinenitrile] and M2 [(*1S*)-3,5-dihydroxyadamantylglycine-(*cis*-4,5-methanoprolinenitrile] were synthesized by the Discovery and Process Chemistry Departments of Bristol-Myers Squibb (Princeton, NJ). All the other reagents and chemicals were purchased and were reagent grade.

Serum Protein Binding. The extent of serum protein binding of saxagliptin and M2 was determined by equilibrium dialysis using a Dianorm dialysis system (Harvard Bioscience, Holliston, MA) and Diachema dialysis membranes (mol. wt. cutoff, 12,000) at relevant toxicological and clinical concentrations. The pooled serum was purchased from Bioreclamation, Inc. (Hicksville, NY). Before the experiments, the dialysis membranes were preconditioned with deionized water and subsequently with 0.133 M sodium phosphate buffer (pH 7.4). Serum from different species containing 100 ng/ml (human and monkey), 5000 ng/ml (dog and rat), and 25,000 ng/ml (mouse) of saxagliptin or M2 was added to one side of the cell, and an equal volume of buffer (0.133 M sodium phosphate buffer, pH 7.4) was added to the opposite side of the cell. Equilibrium was achieved by rotating the cells at 3 to 5 rpm at 37°C for 4 h. After incubation, samples from the serum and the buffer sides were collected separately and mixed with an equal volume of the opposite matrix. Human, monkey, dog, and rat samples were further diluted 10-fold and mouse samples 100-fold with mixed matrix (50% buffer-50% mixed serum from all five species at equal volume). The concentrations of saxagliptin and M2 in the samples were determined using a liquid chromatography/tandem mass spectrometry (LC-MS/MS) method.

Animal Studies. All procedures involving animals used in this study were consistent with the guidelines set by the National Institute of Health (National Institutes of Health Publication 85-23, revised in 1985) and approved by Bristol-Myers Squibb Institutional Animal Care and Use Committee. In all animal studies described below, saxagliptin was administered in solution in 100% water unless otherwise noted. The anticoagulant used for the preparation of plasma was either heparin or EDTA. The blood samples were centrifuged to obtain plasma, and the plasma samples were frozen at −20°C until analysis. Plasma, urine, bile, and tissue samples collected in these studies were analyzed for the concentrations of saxagliptin and M2 by LC-MS/MS as described below.

Pharmacokinetic studies. Male rats (SD, 0.26–0.30 kg, $n = 2$ per route) received a single intravenous bolus or oral dose of saxagliptin, after an overnight fast (approximately 10 h) in a parallel study design. The intravenous and oral doses were 10.0 and 8.0 mg/kg, respectively, and the animals were fed 4 h after dosing. Serial blood samples were collected at 0.03 (i.v. only), 0.17, 0.33, 0.67, 1, 1.5, 2, 4, 6, 8, and 10 h postdose. Urine samples were also collected over 10 h postdose.

To further evaluate exposure-dose relationship over a wide dose range, the compound was administered orally as a suspension in 1.25% aqueous Avicel to three groups of 10 male SD rats at doses of 2, 20, and 200 mg/kg. Blood samples were obtained from the tail at 0.5 and 4 h for the first group ($n = 3$), 1 and 8 h for the second group ($n = 3$), and 2 and 24 h for the third group ($n = 4$) of animals at each dose level; plasma was harvested as described above and analyzed for saxagliptin.

Male beagles (13.5–15.5 kg, $n = 2$ per route) received a single i.v. (10-min infusion) or oral dose of saxagliptin after an overnight fast (approximately 10 h) in a crossover study design, with a 1-week washout period between dosing. The intravenous and oral doses were 5.9 and 5.2 mg/kg, respectively, and the animals were fed 4 h after dosing. After intravenous and oral dosing, serial blood samples were collected via the jugular vein at predose and at 0.17, 0.33, 0.67, 1, 2, 4, 6, 8, and 24 h. Urine samples were collected over 24 h postdose.

Systemic exposures of saxagliptin and M2 after oral administration of saxagliptin to male dogs were also determined as part of the toxicokinetic studies. In these studies, gelatin capsules were filled with saxagliptin in 1.25% Avicel and administered orally to groups of dogs ($n = 3$ per dose) at 1, 5, and 25 mg/kg, and the animals were fed 8 h after dosing. Blood samples were obtained from the external jugular vein at 0.5, 1, 2, 4, 8, and 24 h postdose, and plasma was prepared as described above.

Male cynomolgus monkeys (5.9–8.1 kg, $n = 1$, i.v. dose and $n = 2$, oral dose) received a single i.v. (10-min infusion) or oral dose of saxagliptin after an overnight fast (approximately 10 h) in a parallel study design. The intravenous and oral doses were 3.4 mg/kg, and the animals were fed 4 h after dosing. Serial blood samples were collected at predose and at 0.17 (i.v. only), 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 10, and 24 h. Blood samples were collected from the femoral artery. Urine samples were collected over 24h postdose.

Systemic exposures to saxagliptin and M2 after oral administration of saxagliptin to male monkeys were also determined as part of the toxicokinetic studies. In these studies, suspensions of saxagliptin in 1.25% Avicel were administered by oral gavage to monkeys ($n = 3$ per dose) at 5 and 25 mg/kg, and the animals were fed 8 h after dosing. Blood samples were obtained from the femoral vein at 1, 2, 4, 8, and 24 h postdose, and plasma was prepared as described above.

Bile duct-cannulated rat study. The in vivo disposition of saxagliptin was determined using bile duct-cannulated (BDC) rats. Male SD rats received a single i.v. or oral dose ($n = 1$ per route, 10 mg/kg) of saxagliptin after an overnight fast. After dosing, bile was collected over 0 to 5 and 5 to 10 h intervals, and urine was collected over the 0 to 10 h interval postdose. All samples were frozen at −20°C until analysis.

Tissue distribution studies in rats. Male SD and ZDF rats were fasted overnight before dosing and remained fasted during the study. Saxagliptin was administered as a single intra-arterial bolus dose of either 0.1 or 2.5 mg/kg to three rats per dose level per strain. The rats were euthanized 1 h postdose. Blood samples were collected just before euthanasia and processed for plasma. Duodenum, small intestine (minus duodenum), large intestine, kidney, spleen, heart, pancreas, brain, and skeletal muscles were harvested after euthanasia. Each tissue was blotted dry and weighed. Tissues were homogenized after the addition of 3 ml of water per g of tissue. The weight of individual tissue homogenate was recorded, and all homogenates were stored at −20°C until analysis. Samples were analyzed for the concentrations of saxagliptin and M2 by LC-MS/MS, using separate standard calibration curves for each of the tissues prepared in their respective matrices. The total amount of each compound in each tissue was calculated by multiplying the concentration in each tissue homogenate with the volume of the tissue homogenate. The level of the compounds in a specific tissue was then calculated by dividing the total amount of compound in the tissue with the weight of the tissue. However, because absolute extraction efficiency for each tissue was not determined, saxagliptin and M2 concentrations in each tissue could be underestimated.

Human Pharmacokinetic Study. The clinical pharmacokinetic data for saxagliptin were obtained from the initial single ascending dose (SAD) study in healthy subjects ($n = 6$ per dose). The compound was administered orally over a wide dose range after a ≥10 h fast. Saxagliptin was administered orally as an aqueous solution for doses less than 5 mg but in dry blend capsule formulations for the 5-mg and higher doses. Blood and urine samples were collected over 48 h after saxagliptin administration. The concentrations of saxagliptin and M2 in plasma and urine samples were determined using validated LC-MS/MS methods.

Analytical Methods. Samples from the pharmacokinetic, BDC rat, tissue distribution, and plasma protein binding studies were analyzed for saxagliptin and M2 using high-performance liquid chromatography and LC-MS/MS meth-

TABLE 1

Key pharmacokinetic parameters of saxagliptin in rats, dogs, and monkeys

Data are mean values

Species	Route	Dose	C_{max}	t_{max}	$AUC_{0-\infty}$	$t_{1/2}$	CL	V_{ss}	F
		mg/kg	ng/ml	h	μg · h/ml	h	ml/min/kg	l/kg	%
Rat (n = 2, i.v. and p.o.)	i.v.	10.0	5.2		1.6	2.1	115	5.2	
	p.o.	8.0	0.5	0.7	0.9				75
Dog (n = 2, i.v. and p.o.)	i.v.	5.9	10.1		10.7	3.0	9.3	1.3	
	p.o.	5.2	2.7	1.2	7.3				76
Monkey (n = 1, i.v.; n = 2 p.o.)	i.v.	3.4	5.4		3.0	4.4	14.5	1.8	
	p.o.	3.4	1.0	1.0	2.0				51

ods. In all cases, a high-performance liquid chromatography system equipped with a reverse-phase column was interfaced to a triple quadrupole tandem mass spectrometer with an electrospray interface. Data were acquired via selected reaction monitoring. Ions representing the $(M + H)^+$ species for both the analytes and internal standards were selected in MS1 and collisionally dissociated with argon to form specific product ions, which were subsequently monitored by MS2 using reaction monitoring of a transition unique to each of the three compounds. The selected reaction monitoring transitions used for saxagliptin and M2 were 316.2 → 180.1 and 332.2 → 196.1, respectively. Standards were analyzed in duplicate or triplicate to prepare calibration curves for each analyte. The standard calibration curves were fitted with quadratic regressions weighted by reciprocal concentration ($1/x$). Quality control samples at three concentrations within the range of the calibration curve were also analyzed in duplicate or triplicate with each analytical set. The analytical assay performance was determined based on the predicted quality control concentrations to be within 20% of the nominal concentration, for acceptable assay performance.

Pharmacokinetic Data Analysis. Plasma concentrations versus time data of saxagliptin and M2 were analyzed by noncompartmental methods using the KINETICA software program (version 4.2; InnaPhase Corporation, Philadelphia, PA). The maximum concentration (C_{max}) and time of C_{max} (t_{max}) were recorded directly from experimental observations. The area under the curve from time 0 to the last measurable concentration (AUC_{0-t}) and the area under the curve from time 0 to infinity ($AUC_{0-\infty}$) were calculated using a combination of linear and log trapezoidal summations. The total body clearance (CL) and the volume of distribution at steady-state (V_{ss}) were calculated after intravenous administration. The first-order elimination rate constant (k_{el}) was estimated from the terminal log-linear phase of the concentration-time curve and was identified by best linear fit of at least three data points. The half-life of the terminal log-linear phase was calculated as $\ln(2)/k_{el}$, where k_{el} is the absolute value of the slope of the terminal log-linear phase. The fractions (f_e) of saxagliptin and M2 recovered in bile and urine were calculated as the cumulative amount of unchanged saxagliptin and M2 recovered in each biological fluid divided by the dose of saxagliptin administered. Saxagliptin renal clearance (CLr) values were determined from the total plasma CL and the fraction excreted in urine. The oral bioavailability (expressed as a percentage) was estimated by taking the ratio of dose-normalized AUC values after oral doses of saxagliptin to those after intravenous doses of saxagliptin.

Prediction of Human Pharmacokinetics. In vivo based scaling methods were used to predict various human pharmacokinetics.

Prediction of human clearance and volume of distribution. Simple allometric scaling (Mahmood and Balian, 1996) and species invariant time (SIT) (Dedrick et al., 1970; Bonenbaum and Ronfeld, 1983; Hutchaleelaha et al., 1997; Mahmood and Yuan, 1999) methods were used to predict plasma clearance and volume of distribution values in humans. In the simple allometric scaling method, body weights of the animals were used to allometrically scale total plasma clearance (or renal clearance) values from rats, dogs, and monkeys to humans by using eq. 1, where W is species body weight and a and b are the allometric coefficient and exponent, respectively:

$$CL = aW^b \quad (1)$$

Similarly, allometric scaling was used to estimate human V_{ss} by using body weight of the animal (rat, dog, and monkey) and the corresponding animal steady-state volume of distribution (eq. 2) as follows:

$$V_{ss} = aW^b \quad (2)$$

where W is species body weight and a and b are the allometric coefficient and exponent, respectively. The human plasma clearance and volume of distribution were also estimated from the human intravenous pharmacokinetics of saxagliptin predicted with the SIT method. In this approach, the intravenous plasma concentrations (C) obtained from various species (rat, dog, and monkey) were first normalized with respect to dose (milligrams per kilogram). The dose-normalized plasma concentrations (y-axis) and time (t) (x-axis) were then transformed by dividing by the body weight (W) to power functions (c and d) (eqs. 3 and 4) determined by simultaneous fitting of the three species profiles to a two-compartment model using KINETICA software (eq. 5).

$$y\text{-axis} = C/(\text{Dose}/W^c) \quad (3)$$

$$x\text{-axis} = t/W^d \quad (4)$$

$$C/(\text{Dose}/W^c) = A \times \exp(-\alpha t/W^d) + B \times \exp(-\beta t/W^d) \quad (5)$$

where A and B are the intercepts of the y-axis and α and β are the initial and terminal phase elimination rate constants.

The human intravenous plasma concentration-time profile was then simulated by applying the best fit of body weight exponents. The intravenous data were then analyzed using a noncompartmental analysis to estimate the human intravenous pharmacokinetic parameters such as clearance and volume of distribution.

Prediction of human oral clearance and bioavailability. The oral bioavailability (F) of saxagliptin in human was estimated from the mean values observed in preclinical species. This human oral bioavailability estimate, in combination with the predicted CL values obtained from the simple allometric and the SIT methods discussed above, were used to predict the human apparent oral clearance (CL/F). The first-order oral absorption rate constant (k_a) in humans was estimated from the mean value of the absorption rate constants obtained from the deconvolution analysis of rat, dog, and monkey pharmacokinetics using the standard KINETICA software. The k_a values were calculated from fitting the deconvolution absorption-time profile to a monoexponential function.

Prediction of human oral plasma concentration-time profile. The human intravenous pharmacokinetic profile predicted by the SIT method (vide supra), in combination with the estimates of the extent (human bioavailability value) and rate of absorption (mean absorption rate constant), was used to simulate the oral concentration-time profile in humans using a multicompartment model. Key oral pharmacokinetic parameters (C_{max}, C_{max}, AUC, and $t_{1/2}$) in humans were then determined with noncompartmental analysis and compared with the actual clinical pharmacokinetic data obtained from the SAD studies.

Results

Pharmacokinetics of Saxagliptin in Rats, Dogs, and Monkeys. The pharmacokinetic parameters of saxagliptin calculated after single intravenous and oral doses in rats, dogs, and monkeys are summarized in Table 1. The total plasma clearance of saxagliptin was higher in rats (115 ml/min/kg) than in dogs (9.3 ml/min/kg) and monkeys (14.5 ml/min/kg). The terminal plasma elimination half-lives ranged from 2.1 to 4.4 h in the three species. The steady-state volume of distribu-

TABLE 2

Plasma exposure of saxagliptin and M2 in pre-clinical species after oral administration

Species	Dose	$AUC_{0-\infty}$[a]	
		Saxagliptin	M2
	mg/kg	ng · h/ml	
Rat[b]	2	0.19	0.06
	20	1.36	0.4
	200	18.01	4.46
Dog (n = 3/dose)	1	1.41 ± 0.36	1.18 ± 0.57
	5	8.69 ± 1.28	12.07 ± 1.03
	25	46.50 ± 26.08	17.47 ± 0.37
Monkey (n = 3/dose)	5	2.33 ± 0.91	15.14 ± 2.45
	25	13.77 ± 2.13	88.38 ± 10.37

[a] Calculated from time 0 h to the time of last measurable concentration; range is between 8 and 24 h.

[b] AUC is the result of the composite data of total of 10 rats; hence SD is not given (see *Materials and Methods* for the details of the study design).

tion values in dogs, monkeys, and rats were in the range of 1.3 to 5.2 l/kg, higher than those for total body water (0.7 l/kg). The compound was rapidly (t_{max} = 0.7–1.2 h) and well absorbed in rats, dogs, and monkeys with absolute oral bioavailability values of 75, 76, and 51%, respectively. As summarized in Table 2, the increase in plasma exposure (AUC) to saxagliptin was approximately dose proportional in the dose ranges of 1 to 200 mg/kg (rats), 1 to 25 (dogs), and 5 to 25 mg/kg (monkeys), indicating linear pharmacokinetics.

Human Pharmacokinetic Data. Important human pharmacokinetic parameters of saxagliptin and its major metabolite, M2, obtained over a dose range of 2.5 to 50 mg of saxagliptin during the initial SAD study in healthy subjects are summarized in Table 3. The compound was rapidly absorbed (t_{max} ~0.6–1 h) with the apparent oral plasma elimination half-life of ~2 to 2.8 h over this dose range. In addition, approximately dose proportional exposure was obtained over a wide dose range, similar to that obtained in preclinical species.

In Vivo Metabolism and Excretion. After intravenous administration of saxagliptin to intact rats, dogs, and monkeys, respectively, 33, 40, and 60% of the dose was excreted unchanged in urine. Thus, saxagliptin renal clearance values in rats, dogs, and monkeys were 38, 3.7, and 8.7 ml/min/kg, respectively. After a 10 mg/kg i.v. or oral administration of saxagliptin to BDC rats, ~2% of the dose was excreted unchanged in bile. M2 (a hydroxy metabolite) was the major metabolite observed, although other minor oxidative metabolites (data not shown) were also observed in the urine and bile samples in rats. No conjugated metabolites were detected in the BDC rat samples (bile, urine, and plasma). M2 was also observed as a circulating metabolite in rats, dogs, and monkeys. After 10 (i.v.) and 8 (oral) mg/kg doses of saxagliptin to rats, the ratio of plasma exposure of M2 to saxagliptin was approximately 20 to 40%. The plasma molar exposure to M2 after oral administration of saxagliptin to dogs (1, 5, and 25 mg/kg) and monkeys (5 and 25 mg/kg), respectively, was ~0.4- to 1.3- and ~5- to 6-fold of the exposure to saxagliptin (Table 2). M2 was also the major metabolite observed in human (Table 3). The plasma molar exposure to M2 was ~3- to 7-fold higher than that of saxagliptin in human, and the apparent oral plasma elimination half-life of M2 was approximately 3 to 4 h (Table 3). In addition, 7 to 21% and 16 to 51% of the dose was eliminated over 48 h as saxagliptin and M2 (Table 3), respectively, in the urine of human subjects after oral administration of saxagliptin (2.5–50 mg). Therefore, the major excretion and metabolic pathways observed for saxagliptin in preclinical species are consistent with those observed in human.

Tissue Distribution of Saxagliptin and M2 in SD Rats. The plasma concentration and tissue levels of saxagliptin in SD rats after 0.1 and 2.5 mg/kg i.a. doses of saxagliptin were determined 1 h postdose and are summarized in Table 4. Saxagliptin distributed into various tissues after 0.1 or 2.5 mg/kg doses with the tissue concentrations being lowest in brain and highest in the kidney and intestine. For example, the exposure to the intestinal tissues is 10- to 46-fold higher than that in the plasma for saxagliptin.

The distribution of M2 into various tissues of SD rats after a 0.1 and 2.5 mg/kg dose of saxagliptin administration is also summarized in Table 4. The concentrations of M2 in SD rats after saxagliptin doses were low in the brain and higher in the intestinal tissues relative to the concentrations in plasma.

Tissue Distribution of Saxagliptin and M2 in ZDF Rats. The plasma and tissue concentrations of saxagliptin in ZDF rats after 0.1 or 2.5 mg/kg i.a. doses were determined at 1 h post dose and are summarized in Table 5. At these doses, concentrations of saxagliptin were lower in brain and higher in kidney, pancreas, or intestinal tissues relative to that observed in plasma. For example, the intestinal tissue/plasma ratio of saxagliptin was as high as 17 in the ZDF rats.

Plasma concentration and tissue distribution data for M2 in ZDF rats after 0.1 and 2.5 mg/kg i.a. doses of saxagliptin are also summarized in Table 5. After 0.1 or 2.5 mg/kg doses of saxagliptin, the concentrations of M2 were lower in brain and higher in intestinal tissues compared with the concentrations observed in plasma.

Serum Protein Binding. The in vitro protein binding of saxagliptin and its major metabolite, M2, in mouse, rat, dog, monkey, and human sera was determined by equilibrium dialysis. The serum protein binding of saxagliptin was very low in all species tested, with a free fraction of 100% in dog and human and 73, 82, and 80% in mouse, rat, and monkey sera, respectively. The serum protein binding of M2 was also very low in all species tested, with a free fraction of 100% in mouse, rat, dog, and human and 89% in monkey.

Prediction of Human Pharmacokinetic Parameters. Human CL and V_{ss} values predicted for saxagliptin by allometric scaling and SIT methods are summarized in Table 6. The human plasma CL and V_{ss} values predicted for saxagliptin with simple allometry were 3.4 ml/min/kg and 0.8 l/kg. The coefficient (a) and exponent (b) of the simple allometric equation used for the estimation of CL were 44.88 and 0.35, respectively, with a correlation coefficient (r^2) of 1.00. The exponent (b) of the allometric equation used for the estimation of V_{ss} was 0.66 with a correlation coefficient (r^2) of 1.00. To determine human plasma clearance and volume of distribution with the SIT method, prediction of the intravenous plasma concentration-time profile from preclinical data was undertaken. To predict this, the dose (milligrams per kilogram) normalized plasma concentration-time profiles obtained from the preclinical species (rat, dog, and monkey) were first transformed by dividing both dose-normalized concentration and time by the body weights to power functions (c and d) (Fig. 2) that were obtained from the best fit of the profiles of the three species simultaneously to a two-compartment model. For saxagliptin, the weight exponent of the time axis converged to a value of 0.32, whereas the body weight exponent for the y-axis converged to a value of 5×10^{-9}, which for all practical purposes is zero ($W^0 = 1$), therefore resulting in only the need to normalize the concentration by dose (milligrams per kilogram). The human intravenous plasma concentration-time profile was simulated after conversion of the x-axis back to time by multiplying it by 3.89 (= $70^{0.32}$), assuming a 70-kg human. Basic pharmacokinetic parameters such as clearance and volume of distribution were then calculated using a noncompartmental analysis.

TABLE 3

Pharmacokinetic parameters of saxagliptin and M2 in human after oral administration of saxagliptin during a single ascending dose study

Dose	Compound	Pharmacokinetic Parameters				
		C_{max}	AUC_{0-t}	t_{max}	$t_{1/2}$	f_e
mg		ng/ml	ng · h/ml	h		%
2.5	Saxagliptin	0.007 ± 0.001	0.009 ± 0.002[a]	0.56 ± 0.13	N.A.	6.7 ± 2.2
	M2	0.024 ± 0.007	0.063 ± 0.05[a]	1.46 ± 0.87	N.A.	15.6 ± 3.3
5	Saxagliptin	0.021 ± 0.003	0.06 ± 0.02	0.88 ± 0.38	2.0 ± 0.3	11.4 ± 4.2
	M2	0.092 ± 0.022	0.43 ± 0.11	1.67 ± 0.61	2.8 ± 0.6	34.9 ± 11.5
10	Saxagliptin	0.045 ± 0.009	0.16 ± 0.03	0.75 ± 0.16	2.8 ± 0.7	17.8 ± 3.4
	M2	0.184 ± 0.067	0.93 ± 0.28	1.42 ± 0.38	3.0 ± 0.3	42.6 ± 7.0
20	Saxagliptin	0.082 ± 0.028	0.28 ± 0.09	0.79 ± 0.19	2.3 ± 0.2	15.4 ± 3.7
	M2	0.369 ± 0.090	1.94 ± 0.59	1.75 ± 0.27	2.9 ± 0.2	44.0 ± 10.8
30	Saxagliptin	0.178 ± 0.062	0.51 ± 0.2	0.67 ± 0.41	2.5 ± 0.4	16.2 ± 3.3
	M2	0.503 ± 0.095	2.86 ± 0.75	1.38 ± 0.44	3.6 ± 1.1	39.8 ± 9.0
50	Saxagliptin	0.234 ± 0.067	0.83 ± 0.09	1.04 ± 0.60	2.5 ± 0.3	21.3 ± 3.4
	M2	0.717 ± 0.251	4.56 ± 1.78	2.08 ± 0.74	4.1 ± 1.1	51.2 ± 13.4

f_e, the fraction of saxagliptin or M2 in urine calculated as the cumulative amount recovered in urine divided by the dose of saxagliptin administered; N.A., not available because of insufficient data at the terminal phase (< below lower limit of quantification).
[a] AUC_{0-t} : the area under the curve from time zero to the last measurable concentration.

TABLE 4

Mean concentrations of saxagliptin and M2 in tissues at 1 h after 0.1 or 2.5 mg/kg i.v. doses of saxagliptin to SD rats (n = 3)

S.D. is not given when at least one of the rats was found to have a concentration BLLQ in any given tissue. Lower limit of quantification: large intestine, 8.6 ng/g; heart, 10.3 ng/g; brain, 2 ng/g; and muscle, 1.8 ng/g.

Tissue	Dose	Saxagliptin		M2	
		Average Conc	Tissue/Plasma Ratio	Average Conc	Tissue/Plasma Ratio
	mg/kg	ng/g		ng/g	
Plasma	0.1	4 ± 2	1	1.3 ± 0.4	1
	2.5	97 ± 23	1	48 ± 18	1
Small intestine	0.1	159 ± 15	46 ± 18	28 ± 5	24 ± 10
	2.5	942 ± 193	10 ± 1	2396 ± 348	55 ± 21
Large intestine	0.1	71 ± 13	20 ± 5	BLLQ	
	2.5	2205 ± 411	24 ± 8	113 ± 20	3 ± 1
Duodenum	0.1	50 ± 22	15 ± 9	15 ± 1	14
	2.5	134 ± 10	1 ± 0.3	329 ± 166	7 ± 1
Kidney	0.1	1243 ± 76	349 ± 102	68 ± 9	53 ± 9
	2.5	1373 ± 282	14 ± 1	417 ± 110	9 ± 2
Spleen	0.1	80 ± 23	21 ± 3	2	1
	2.5	523 ± 50	4 ± 1	13 ± 9	0.3 ± 0.1
Heart	0.1	39 ± 7	11 ± 3	BLLQ	
	2.5	224 ± 34	2 ± 0.3	27 ± 8	0.6 ± 0.1
Pancreas	0.1	26 ± 5	7 ± 2	12	10
	2.5	186 ± 20	2 ± 0.3	32 ± 5	0.7 ± 0.2
Brain	0.1	1	0.1	BLLQ	
	2.5	8 ± 1	0.08 ± 0.01	BLLQ	
Muscle	0.1	11 ± 0.5	3 ± 1	BLLQ	
	2.5	291 ± 21	3 ± 1	7 ± 4	0.1 ± 0.04

BLLQ, below lower limit of quantification.

CL/*F* was also predicted for saxagliptin using the CL values obtained from the two scaling methodologies discussed above and *F*. The human oral bioavailability value was estimated to be 67% from the average of the absolute bioavailability values observed across preclinical species. The predicted human apparent oral clearance values are summarized in Table 6 and compared with the value calculated from the pharmacokinetic data obtained from the single ascending dose clinical studies. As shown in Table 6, the predicted human oral clearance value estimated using the systemic CL value obtained from the SIT method was very close to that observed in human. On the other hand, the apparent oral clearance estimated using a CL value from simple allometry was significantly lower than the observed value.

Because the observed urinary elimination of the intact saxagliptin across preclinical species was significant, the renal components of the systemic clearance observed in animal species were allometrically scaled to human to assess the human renal clearance of the compound. This resulted in predicted human renal clearance and apparent oral renal clearance (CL$_r$/*F*) values of 1.9 and 2.8 ml/min/kg, respectively. The predicted oral renal clearance was very close to the actual human oral renal clearance (CL$_r$/*F* = 2.5 ml/min/kg) calculated from the total oral CL (discussed above) and the mean urinary recovery of intact saxagliptin (14.8% of total dose) over a dose range of 2.5 to 50 mg (Table 3).

Relatively similar oral k_a values for saxagliptin in rats (0.92 h^{-1}), monkeys (1.25 h^{-1}), and dogs (1.14 h^{-1}) were obtained from deconvolution analyses. Therefore, the human oral absorption kinetics of saxagliptin was simulated using the average k_a value (1.11 h^{-1}) observed across these preclinical species.

Prediction of the Human Oral Concentration-Time Profile. The oral pharmacokinetic profile of saxagliptin in human was simulated using a combination of predicted human intravenous pharmacokinetic

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TABLE 5

Concentrations of saxagliptin and M2 in tissues at 1 h after 0.1 or 2.5 mg/kg p.o. dose of saxagliptin to ZDF rats (n = 4)

SD is not given when at least one of the rats was found to have a concentration less than BLLQ in any given tissue. Lower limit of quantification: spleen, 1.8 ng/g; heart, 7.5 ng/g; pancreas, 22.3 ng/g; muscle, 0.8 ng/g; and brain, 0.77 ng/g.

Tissue	Dose	Saxagliptin		M2	
		Average Conc	Tissue/Plasma Ratio	Average Conc	Tissue/Plasma Ratio
	mg/kg	ng/g		ng/g	
Plasma	0.1	14 ± 11		0.7 ± 0.03	1
	2.5	148 ± 70		34 ± 7	1
Small intestine	0.1	164 ± 21	7 ± 9	29 ± 10	39 ± 15
	2.5	759 ± 392	6 ± 4	943 ± 475	28 ± 15
Large intestine	0.1	81 ± 43	10 ± 8	18 ± 8	24 ± 11
	2.5	1547 ± 524	13 ± 8	140 ± 65	1 ± 3
Duodenum	0.1	60 ± 13	6 ± 4	19 ± 8	21 ± 9
	2.5	179 ± 74	1 ± 0.2	254 ± 119	7 ± 2
Kidney	0.1	923 ± 316	87 ± 34	20 ± 0.2	27 ± 1
	2.5	2662 ± 340	21 ± 11	271 ± 41	8 ± 2
Spleen	0.1	139 ± 43	16 ± 12	BLLQ	
	2.5	1300 ± 656	9 ± 1	22 ± 9	0.7 ± 0.3
Heart	0.1	25 ± 9	8 ± 5	BLLQ	
	2.5	236 ± 108	3 ± 1	17 ± 5	0.5 ± 0.1
Pancreas	0.1	115 ± 70	36 ± 30	BLLQ	
	2.5	910 ± 671	10 ± 8	BLLQ	
Brain	0.1	BLLQ		9	
	2.5	10 ± 1	0.07 ± 0.02	0.8	0.02
Muscle	0.1	27 ± 2	2 ± 1	BLLQ	
	2.5	500 ± 170	4 ± 0.5	56 ± 2	0.2 ± 0.04

BLLQ, below lower limit of quantification.

TABLE 6

Saxagliptin predicted human CL and CL_r, oral CL/F and CL_r/F, and V_{ss} values obtained from scaling of in vivo pre-linical data

The actual observed mean plasma and renal oral clearance values are also shown.

Method	Pharmacokinetic Parameters				
	CL	CL_r	CL/F	CL_r/F	V_{ss}
	ml/min/kg				l/kg
SA	3.4	1.9	5.1	2.8	0.8
SIT	11.8		15.0		2.7
Observed			17.1	2.5	

SA, simple allometry; SIT, species invariant time method.

data, oral bioavailability and the mean absorption rate constant. The resulting human projected oral pharmacokinetic profile of saxagliptin for a 1 mg/kg dose is summarized in Fig. 3 and Table 7 and compared with the actual clinical data normalized to 1 mg/kg. As discussed above, the clinical data were obtained from the first-in-human SAD studies, in which saxagliptin showed approximately linear oral pharmacokinetics over a wide dose range (2.5–50 mg). To compare the clinical data to the predicted values at the same dose, the observed pharmacokinetic data at every dose were normalized to a 1 mg/kg dose (for a 70-kg human) and then averaged over the dose range studied.

With this approach, the predicted oral plasma AUC, C_{max}, concentration at 12 h postdose ($C_{12\,h}$), and oral $t_{1/2}$ values directly calculated from the projected oral plasma concentration-time profile were about factors of 1.2-, 1.7-, 1.1-, and 1.0-fold of the actual human observed values, indicating that the prediction accuracy of the important human pharmacokinetic parameters was very good. At 1 mg/kg, the predicted concentration of saxagliptin at 24 h postdose was 2.1 ng/ml. The concentration at 24 h postdose in the actual clinical samples was below the limit of quantification (10 ng/ml) of the LC-MS/MS method used for the analysis of the human plasma samples.

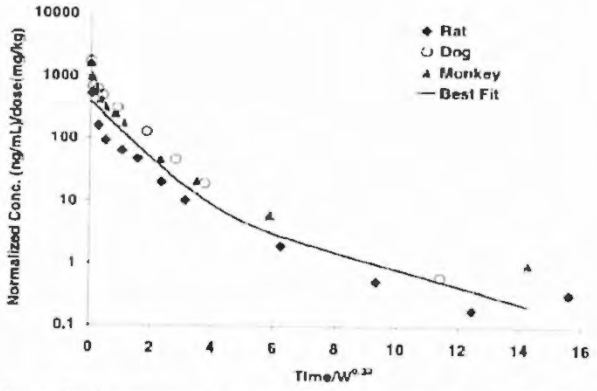

FIG. 2. The dose-normalized plasma intravenous concentration-time profiles of rat, dog, and monkey obtained by dividing by the body weights to power functions determined from the best fit of the profiles of the three species simultaneously to a two-compartment model.

Discussion

Saxagliptin had good oral bioavailability and demonstrated dose-dependent increases in exposure over a wide dose range in rats, dogs, and monkeys. In all three species, the compound was well distributed extravascularly as indicated by high volumes of distribution. After intra-arterial administration of saxagliptin to SD and ZDF rats, saxagliptin and M2 concentrations were lowest in the brain and highest in the intestine and kidney. In these studies, the small intestine/plasma ratio for saxagliptin was ≥10-fold in SD rats and ≥6-fold in ZDF rats. For M2, the small intestine/plasma ratio ranged from ≥24-fold in SD rats to ≥28-fold in ZDF rats. This increased intestinal exposure is due to extravascular distribution and not contamination from gut contents. Saxagliptin was cleared rapidly in rats and at a low to moderate rates in dogs and monkeys. Biliary excretion in rats was a minor route of

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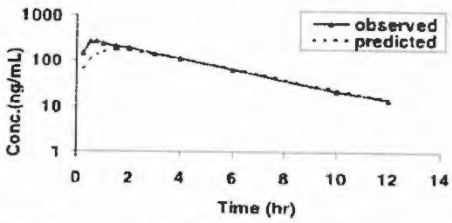

FIG. 3. Predicted versus observed oral plasma concentration-time profiles of saxagliptin in human at a 1 mg/kg oral dose. ▲——▲, observed human clinical data; - - -, predicted human concentration-time profile.

TABLE 7

Predicted and observed human pharmacokinetic parameters of saxagliptin at 1 mg/kg oral dose

Prediction fold errors are also included.

Pharmacokinetic Parameter	Observed	Predicted	Prediction Fold Error
$AUC_{0-\infty}$ (ng/ml × h)	1110	988	1.2
C_{max} (ng/ml)	270	163	1.7
$C_{12\,h}$ (ng/ml)*	14.4	15.1	1.1
$t_{1/2}$ (h)	2.7	2.7	1.0

* $C_{12\,h}$ values reflect the concentration at 12 h postdose (the last measurable concentration at this dose).

elimination. Between 33 and 60% of the dose was excreted unchanged in rat, dog, and monkey urine after intravenous administration, indicating significant renal elimination in all species examined. The hydroxylation of saxagliptin to form M2 was the major metabolic pathway in all of the species tested. This metabolite was pharmacologically active and was observed to circulate in plasma at significant levels in all species. Taken together, the preclinical pharmacokinetic parameters as well as safety and efficacy results led to the selection of saxagliptin for clinical development.

As a result of the dual elimination pathways (metabolic and renal) of saxagliptin, scaling methods based on preclinical in vivo data (instead of in vitro metabolic clearance) were used to predict human plasma clearance. Simple allometric scaling is more successful in the prediction of human clearance for compounds that show similar interspecies hepatic clearance, for which the allometric exponent, *b*, is in the range of 0.55 to 0.71, but tends to under- or overpredict if the *b* value is less than or greater than this range [rule of exponents (ROE)] (Mahmood and Balian, 1996). For saxagliptin, the exponent, *b*, was 0.35, and hence based on the ROE, the clearance value (3.4 ml/min/kg) predicted using simple allometry is expected to underpredict the human clearance value for the compound. Correction with empirical correction factors such as maximum life span or brain weight would not have been useful according to the ROE with such a value of *b* (Mahmood and Balian, 1996).

On the other hand, in rat, dog, and monkey, renal clearance is approximately 33 to 60% of the total clearance of saxagliptin. Allometric scaling of these renal clearance values resulted in human renal clearance of ~1.9 ml/min/kg. This value is expected to reflect the actual human renal clearance value, as renal clearance is considered to be fairly scalable across species. In fact, consistent with this assertion, the predicted oral renal clearance (~2.8 ml/min/kg) was found to be similar to the actual oral renal clearance (~2.5 ml/min/kg) observed in humans. However, hepatic clearance is also considered to contribute significantly (~40–67%) to the total clearance of saxagliptin (assuming that liver and kidney are the major organs of elimination) in the three species tested. As described above, simple allometry does not work well for compounds such as saxagliptin that show substantial interspecies difference in hepatic clearance.

Volume of distribution values are usually fairly scalable across animal species based on body weight, with the *b* value of the allometric exponent approaching unity. However, the exponent *b* (0.66) for saxagliptin is on the low side; hence, the allometry method may have underestimated the actual V_{ss} value.

Consequently, saxagliptin human CL and V_{ss} values were further assessed using the human intravenous concentration-time profile predicted by the SIT method. The CL (11.5 ml/min/kg) and V_{ss} (~2.7 l/kg) values obtained by this method were significantly higher than those obtained from simple allometry. The SIT method was based on the concept of physiological time and on the assumption that the human concentration-time profile can be generated from the transformed chronological time units with the power functions obtained from the simultaneous fitting of animal concentration-time profiles. The SIT method is considered to take into account the variations in biological structure and metabolic rates across various species and thus is assumed to show improvement in the prediction of human pharmacokinetics (Boxenbaum and Ronfeld, 1983; Mohammed and Yuan, 1999).

The human CL and V_{ss} values predicted by the SIT method suggest moderate plasma clearance and significant extravascular distribution in humans. The apparent human oral clearance projected for saxagliptin using the predicted bioavailability (67%) and human CL values obtained by the SIT method was very close to the actual value. The human CL/*F* predicted using the CL value from the simple allometry was significantly lower. Consistent with these data, the predicted human oral concentration-time profile (AUC, C_{max}, C_{min}, and $t_{1/2}$) using the SIT method was similar to that observed in human as shown in Fig. 3 and Table 7.

In humans, consistent with the observations in preclinical species, both renal excretion and metabolism were the major elimination pathways for saxagliptin. The compound had an apparent oral plasma elimination half-life of ~2 to 2.8 h (Table 3) in humans, similar to that predicted by the SIT method (~2.7 h). The formation of M2 was also the major metabolic pathway in humans. This metabolite circulates in human plasma at significant levels similar to those observed in preclinical species.

Saxagliptin has a plasma pharmacokinetic half-life of 2 to 5 h in multiple species including humans. However, saxagliptin has demonstrated robust efficacy in clinical trials as well as in preclinical animal testing at relatively low doses after once-daily dosing. There are a number of factors that could contribute to the sustained duration of pharmacological action of saxagliptin. First, there is likely to be a contribution from an active metabolite, M2, to the overall efficacy of the compound. This metabolite, which is also a potent and specific inhibitor of DPP4, circulates in significant concentrations in human plasma (Table 3). Second, both saxagliptin and M2 display prolonged binding to the catalytic site of DPP4 (Kirby et al., 2008; Metzler et al., 2008). The extended rate of dissociation of saxagliptin and M2 from the DPP4 active site would be expected to give hysteresis between plasma concentration and DPP4 inhibition, which has been observed in animal models and humans, providing a longer pharmacodynamic half-life than pharmacokinetic half-life for the compound. For example, the trough concentration ($C_{24\,h}$) (~0.5 nM, projected) for saxagliptin at a 5-mg once daily oral dose was less than its K_i (1.3 nM) for DPP4 inhibition (the $C_{24\,h}$ in the actual clinical samples was below the limit of quantification). Conversely, the trough concentration of sitagliptin (another DPP4 inhibitor currently in clinical use), which does not show extended binding to DPP4 (Kirby et al., 2008) in humans is 6-fold above its DPP4 K_i value at the dose (100 mg) that gives

equivalent efficacy to saxagliptin at 5 mg (Herman et al., 2005; Rosenstock et al., 2008).

Finally, the extravascular distribution of saxagliptin and its active metabolite to the intestinal tissues (proposed major site of pharmacological action) as demonstrated in this study could also play a significant role in its extended therapeutic effect. One of the hypotheses for the mechanism of action of incretins, in addition to the effect they elicit after reaching the general circulation, is that after release from L cells, incretins activate afferent sensory nerve fibers arising from the nodose ganglion in the intestinal interstitium (Holst and Deacon, 2005). In this hypothesis, DPP4 within the interstitium would be important for regulating the neuron loop-mediated release of insulin from the pancreas. This suggests that at least part of the high glycemic efficacy and potent modulation of key hormones by saxagliptin in humans may be due to its extravascular uptake and localization in the intestinal tissues. This assertion would thus predict the dissociation between inhibition of plasma DPP4 activity and glucose lowering for compounds that had extravascular uptake into the intestinal tissues, when comparing plasma DPP4 inhibition at trough across similar clinical trials with equivalent maximal glucose-lowering efficacy.

In conclusion, the objective of the preclinical animal studies was to use an adequate group size to be able to make a reasonable early estimate of saxagliptin human pharmacokinetics and was not meant to capture interindividual variability characteristics. As such, the mean data were presented here and were used fairly well for the prediction of the pharmacokinetics of saxagliptin in humans. The data from the preclinical studies showed that saxagliptin has a desirable pharmacokinetic profile with good oral bioavailability and low plasma protein binding and thus was an attractive candidate for further development. The compound is currently in late-stage clinical development and has displayed robust antidiabetic activity (Rosenstock et al., 2008). As demonstrated in this study, the compound has very good distribution into tissues and was found at high concentrations in intestinal tissues relative to plasma. This finding also suggests that local concentration of drug in the intestine as well as inhibition of DPP4 in the plasma may be important in driving overall efficacy.

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A comparative study of the binding modes of recently launched dipeptidyl peptidase IV inhibitors in the active site

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ABSTRACT

In recent years, various dipeptidyl peptidase IV (DPP-4) inhibitors have been released as therapeutic drugs for type 2 diabetes in many countries. In spite of their diverse chemical structures, no comparative studies of their binding modes in the active site of DPP-4 have been disclosed. We determined the co-crystal structure of vildagliptin with DPP-4 by X-ray crystallography and compared the binding modes of six launched inhibitors in DPP-4. The inhibitors were categorized into three classes on the basis of their binding subsites: (i) vildagliptin and saxagliptin (Class 1) form interactions with the core S_1 and S_2 subsites and a covalent bond with Ser630 in the catalytic triad; (ii) alogliptin and linagliptin (Class 2) form interactions with the S'_1 and/or S'_2 subsites in addition to the S_1 and S_2 subsites; and (iii) sitagliptin and teneligliptin (Class 3) form interactions with the S_1, S_2 and S_2 extensive subsites. The present study revealed that the additional interactions with the S'_1, S'_2 or S_2 extensive subsite may increase DPP-4 inhibition beyond the level afforded by the fundamental interactions with the S_1 and S_2 subsites and are more effective than forming a covalent bond with Ser630.

1. Introduction

Dipeptidyl peptidase IV (DPP-4, EC 3.4.14.5) inhibitors are a new class of oral anti-hyperglycemic agents for the treatment of type 2 diabetes. The glucose lowering effect of DPP-4 inhibitors is mediated by suppressing the degradation of the incretin hormone glucagon-like peptide-1 and stimulating insulin secretion in response to increased blood glucose levels [1]. Prescriptions for recently launched DPP-4 inhibitors for type 2 diabetes have been expanding because of their high effectiveness and safety.

Among the recently marketed DPP-4 inhibitors (Table 1), vildagliptin [2], saxagliptin [3] and teneligliptin [4] are peptide mimetic compounds, which have been discovered by replacing segments of peptide-based substrates [5]. In contrast, sitagliptin [6], alogliptin [7] and linagliptin [8] are non-peptide mimetic compounds, which have been discovered by optimization of the initial lead compounds identified by random screening [5]. Therefore, their chemical structures are diverse, suggesting that each of their binding modes in DPP-4 would be unique.

DPP-4 is a highly specific serine protease that recognizes an amino acid sequence having proline or alanine at the N-terminal penultimate (P_1) position and inactivates or generates biologically active peptides [9]. The amino acid sequence and three-dimensional structure of DPP-4 are well known [10,11]. The structure comprises a β-propeller domain and a catalytic domain, which together embrace an internal cavity housing the active center. This cavity is connected to the bulk solvent by a "propeller opening" and a "side opening" [12]. The conventional hypothesis suggests that substrates and inhibitors enter or leave the active site via the side opening [12,13].

While some comparative studies on the pharmacological effects of DPP-4 inhibitors have been reported [14], there have been no reports comparing their binding modes in DPP-4. X-ray co-crystal structures of five inhibitors, sitagliptin [6], saxagliptin [15], alogliptin [16], linagliptin [8] and teneligliptin [4], with DPP-4 were determined by each originator except vildagliptin. Because these inhibitors have diverse chemical structures, a comparative study of their binding modes in DPP-4 is of considerable interest. Although it is well known that all DPP-4 inhibitors bind to the S_1 and S_2 subsites in common, it has not been systematically understood whether other subsites exist and whether each inhibitor binds to these in a distinct manner. In this study, we determined the co-crystal structure of vildagliptin with DPP-4, analyzed those

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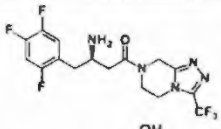
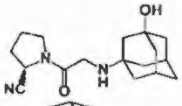
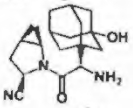
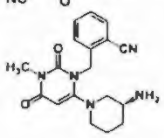
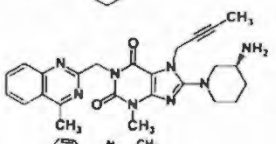
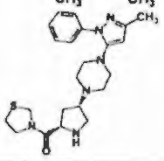
Table 1
Recently launched DPP-4 inhibitors.

Compound name	Chemical structure	Release year	Originator
Sitagliptin		2006	Merck & co.
Vildagliptin		2007	Novartis
Saxagliptin		2009	Astrazeneca and bristol-myers squibb
Alogliptin		2010	Takeda
Linagliptin		2011	Boehringer ingelheim
Teneligliptin		2012	Mitsubishi tanabe pharma

Data were collected in Thomson Reuters Integrity on February 4, 2013.

of the six inhibitors in parallel and studied the relationships between their binding interactions with DPP-4 and their inhibitory activity.

2. Materials and methods

2.1. Synthesis of vildagliptin

Vildagliptin was prepared according to the method described by Villhauer et al. [2].

2.2. X-ray crystallographic studies

The protein of human DPP-4 (33-766) secreted from insect cells was purified and crystallized according to the method reported by Hiramatsu et al. [17] The protein–inhibitor complex was obtained by soaking a preformed DPP-4 crystal in the presence of vildagliptin and preserving it in liquid nitrogen for data collection at 100 K. X-ray diffraction data were collected at the High Energy Accelerator Research Organization (KEK) beam line BL5 and processed using the program HKL2000 [18]. The structure of the DPP-4–inhibitor complex was solved by molecular replacement with the program PHASER [19], utilizing the previously determined coordinates of DPP-4 with the Protein Data Bank (PDB) accession code 3VJK. Data collection and model refinement statistics are summarized in Table 2.

2.3. Comparison of X-ray complex structures

The co-crystal structures of five inhibitors with human DPP-4 have been reported [PDB: 1X70 (sitagliptin), 3BJM (saxagliptin), 3G0B (alogliptin), 2RGU (linagliptin), and 3VJK (teneligliptin)]. They were superimposed on the co-crystal structure of a substrate peptide, diprotin A with DPP-4 (PDB: 1NU8) to analyze the binding subsites. The molecular modeling software Molecular Operating Environment version 2011.10 (Chemical Computing Group, Inc., Montreal, Canada) was used for analysis and graphical visualization of the X-ray co-crystal structures.

The contact area between the inhibitor and DPP-4 was calculated using the molecular modeling software Discovery Studio version 3.5 (Accelrys, Inc., San Diego, USA). For each co-crystal structure, the molecular surface area of the inhibitor, and its solvent-exposed surface area in DPP-4 were calculated. The difference between these areas was defined as the contact area.

3. Results and discussion

3.1. X-ray co-crystal structures of six inhibitors with DPP-4

3.1.1. Definition of subsites in the active site of DPP-4

In the active site of a protease, subsites are generally defined by the binding site of the substrate peptide [20]. The amino acids in the substrate peptide are numbered from the point of cleavage

Table 2
Data collection and refinement statistics.

	Vildagliptin
PDB entry code	3W2T
Crystal	
Space group	$P2_12_12_1$
Unit cell parameters a (Å)	118.22
b (Å)	126.24
c (Å)	138.09
Data	
Resolution (Å)	50.00–2.36 (2.44–2.36)
Unique reflections	82418 (7691)
Redundancy	5.0 (4.6)
Completeness (%)	97.1 (91.7)
R_{merge}[a]	0.080 (0.234)
I/σ (I)	16.3 (6.96)
Refinement	
Resolution (Å)	30.00–2.36 (2.42–2.36)
Unique reflections	78,227 (5407)
Completeness (%)	97.3 (92.1)
Data in the test set	4103 (265)
R-work	0.180 (0.206)
R-free	0.231 (0.287)
R.m.s.d. bond lengths (Å)	0.011
R.m.s.d. bond angles (°)	1.319
Ramachandran plot	
Favored regions (%)	96.1
Allowed regions (%)	3.9
No. of non-H atoms/average B (Å²)	
Protein	12,228/32.7
Ligand	44/23.7
Water	1027/34.7

Values in parentheses are for the highest-resolution shell.
[a] $R_{merge} = \sum|I - \langle I \rangle| / \sum(I)$, where I is the observed intensity.

(P_2, P_1, P'_1, P'_2), and the protein subsites occupied by the respective amino acids are also numbered in the same fashion (S_2, S_1, S'_1, S'_2). In the case of DPP-4, the N-terminus of the substrate peptide is recognized by Glu205 and Glu206, and Ser630 cleaves at the N-terminus penultimate position (P_1). Although, in principle, no subsites are defined after S_2 in DPP-4, our recent study has shown that not the substrates but the inhibitors can bind well beyond the S_2 subsite to increase their inhibitory activity [4,21]. We therefore defined the site beyond S_2 as the S_2 extensive subsite, which is composed of Val207, Ser209, Phe357 and Arg358.

3.1.2. Binding mode of vildagliptin

The co-crystal structure of vildagliptin with DPP-4 is shown in Fig. 1(A). The cyanopyrrolidine binds to the S_1 subsite, with the nitrile forming a covalent imidate adduct with the hydroxyl of Ser630 in the catalytic triad. The imidate nitrogen forms a hydrogen bond with the side-chain hydroxyl of Tyr547. The remaining part including the adamantane binds to the S_2 subsite, where the carbonyl group forms a hydrogen bond with Asn710 and the amino group forms salt bridges with Glu205 and Glu206. The hydroxyl group on the adamantyl moiety forms hydrogen bonds with His126 and Ser209 via the water molecules.

3.1.3. Categorization of the six inhibitors on the basis of their binding subsites

The co-crystal structures of the six inhibitors with DPP-4 superimposed on that of the substrate peptide (diprotin A, Ile-Pro-Ile) are shown in Fig. 1(B)–(H). We categorized the six inhibitors into three classes on the basis of their binding subsites. (i) Vildagliptin and saxagliptin have the most basic binding modes, binding to only the S_1 and S_2 subsites (Class 1). (ii) Alogliptin and linagliptin bind to the S_1, S_2 and S'_1 subsites. Moreover, only linagliptin additionally binds to the S'_2 subsite (Class 2). (iii) Sitagliptin and teneligliptin bind to the S_1, S_2 and S_2 extensive subsites (Class 3). Fig. 2 shows the concept of this categorization.

3.2. Relationship between the inhibitory activity and the binding mode of each class

We focus on the characteristic binding interactions with DPP-4 because other details have been described in previous studies [4,6,8,15,16]. It is well known that all the DPP-4 inhibitors form salt bridges with Glu205 and Glu206 in the S_2 subsite, which have vital roles in the inhibitory activity. The potency of the six DPP-4 inhibitors is shown in Table 3 [22].

3.2.1. Class 1: vildagliptin and saxagliptin

Because vildagliptin and saxagliptin were designed as peptide mimetics, they overlap with the P_1 and P_2 residues of the substrate peptide. As described above, their cyanopyrrolidine moieties bind to the S_1 subsite, forming a covalent bond between the nitrile group and Ser630, and their hydroxy adamantyl groups bind to the S_2 subsite. While they bind in almost the same mode, one of the reasons why saxagliptin has 5-fold higher activity than vildagliptin is attributed to the cyclopropanated cyanopyrrolidine of saxagliptin. Although it was originally intended to enhance the chemical stability of the cyanopyrrolidine [3], introduction of the cyclopropane moiety afforded an additional hydrophobic interaction with the side chain of Tyr666 in the S_1 subsite. Moreover, the direct hydrogen bond between the hydroxyl group of saxagliptin and the side chain of Tyr547 may also contribute to its higher potency.

3.2.2. Class 2: alogliptin and linagliptin

The chemical structures of alogliptin and linagliptin are far different from those of the substrate peptides. The cyanobenzyl group of alogliptin and the butynyl group of linagliptin bind to the S_1 subsite. Their uracil rings form π–π interactions with Tyr547, which undergoes a conformational change in the S'_1 subsite. One of the reasons why linagliptin has 8-fold higher activity than alogliptin may be because only linagliptin binds to the S'_2 subsite. The phenyl component of the quinazoline substituent forms a π–π interaction with Trp629 located in the S'_2 subsite [23]. Eckhardt et al. reported that the introduction of the quinazoline moiety improved its potency 88-fold [8].

3.2.3. Class 3: sitagliptin and teneligliptin

The trifluorophenyl moiety of sitagliptin and the thiazoline moiety of teneligliptin bind to the S_1 subsite. The triazolopyrazine moiety and trifluoromethyl substituent of sitagliptin and the (1-phenylpyrazol-5-yl) piperazine moiety, referred to here as the "anchor lock domain," of teneligliptin bind to the S_2 extensive subsite. Although both inhibitors appear to bind to the subsites in the same manner, teneligliptin has 5-fold higher activity. We suggest three potential reasons for the difference. The first reason may be related to their chemical structures. Because teneligliptin consists of a considerably rigid "J-shaped" structure formed by five rings, four of which are directly connected, the loss in entropy is small upon binding to DPP-4. The second reason may be related to the binding interactions with the S_2 subsite. The carbonyl group of teneligliptin, derived from the peptide mimetics, forms a hydrogen bond with the side chain of Asn710. The third reason may be related to the binding to the S_2 extensive subsite. As shown in Fig. 3, for teneligliptin, introduction of the "anchor lock domain", which binds to the S_2 extensive subsite, increased the activity by 1500-fold over the corresponding fragment that binds to S_1 and S_2 only

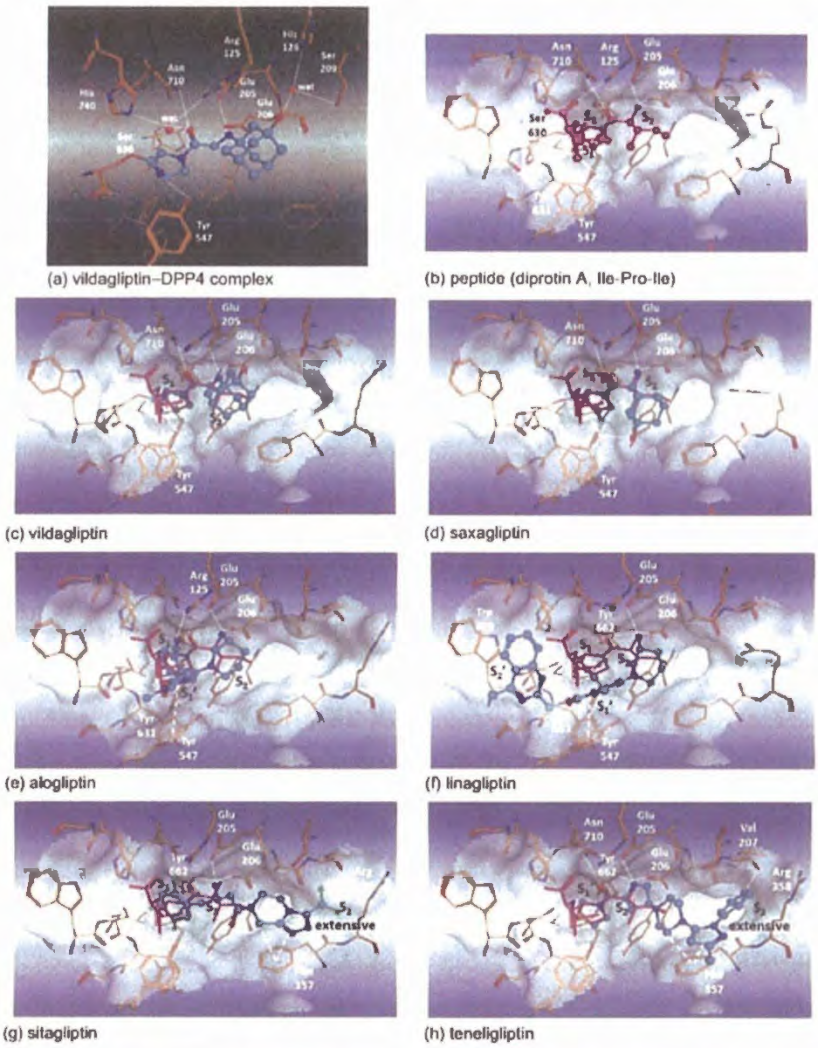


Fig. 1. Binding modes of each inhibitor in the active site of DPP-4. (A) Co-crystal structure of vildagliptin (cyan) bound to DPP-4 (orange) (PDB: 3W2T). (B) Co-crystal structure of the substrate peptide, diprotin A (magenta) bound to DPP-4 (orange). (C)–(H) Co-crystal structures of each inhibitor (cyan) bound to DPP-4 (orange) superimposed on the substrate peptide (magenta). The active site of DPP-4 is shown as a gray-colored surface. Blue, red, yellow and green colors indicate nitrogen, oxygen, sulfur and fluorine atoms respectively, and others indicate carbon atoms. Interactions between inhibitors and water molecules are not shown in (B)–(H). PDB codes are noted in Section 2.

[4,24]. On the other hand, for sitagliptin, previous studies revealed that the introduction of the substituent binding to the S_2 extensive subsite increased the activity by 7-fold [6,25]. To investigate the reason for the difference in increased activity, we applied the estimation method (see Section 2) to the calculation of contact areas in the S_2 extensive subsite. The results showed that teneligliptin has a contact area of 0.92 nm^2 (total contact area, 2.08 nm^2), while sitagliptin has a contact area of 0.71 nm^2 (total contact area, 1.90 nm^2). This result indicates that teneligliptin may bind more tightly to the S_2 extensive subsite as a result of stronger hydrophobic interactions mediated by the "anchor lock domain". Binding of the anchor lock domain may relate to the residence time of the inhibitor in DPP-4 and the long in vivo duration of action.

3.3. *Particularity of the S_2 extensive subsite*

As mentioned above, the S_2 extensive subsite, which is not involved in substrate binding, contributes to increase the inhibitory activity for some DPP-4 inhibitors, but the particularity of the S_2 extensive subsite has not been well known. In other related prolyl peptidases, including DPP-8, DPP-9 and fibroblast activation

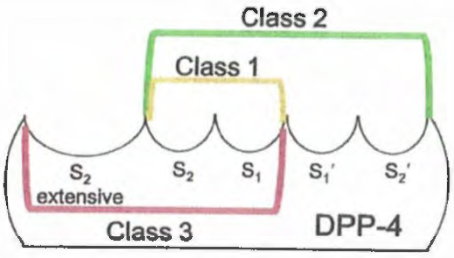


Fig. 2. The concept of three classes on the basis of the inhibitor's binding subsites.

Table 3
The DPP-4 inhibitory activity [22].

Compound	DPP-4 inhibition, IC_{50} (nmol/L)
Vildagliptin	29.2
Saxagliptin	6.3
Alogliptin	4.9
Linagliptin	0.6
Sitagliptin	10.3
Teneligliptin	1.9

protein (FAP, PDB: 1Z68), the S_2 extensive subsite cannot be clearly defined. As a result of our comparison of the corresponding amino acid sequences and three-dimensional structures [26,27] of these proteins, it was found that inhibitors cannot have sufficient hydrophobic interactions with the region beyond S_2 in DPP-8 and DPP-9, and FAP does not have the subsite where the inhibitors can bind beyond S_2. Binding to the S_2 extensive subsite, DPP-4 inhibitors can increase not only their inhibitory activity but also their selectivity against other related prolyl peptidases.

In conclusion, we comparatively present X-ray co-crystal structures of six inhibitors with DPP-4 and categorized them into three classes on the basis of their binding subsites. As a result of the comparative study of the three classes, it is suggested that DPP-4 inhibition tended to increase with an increase in the number of binding subsites. Furthermore, the additional interactions with the S_1', S_2' or S_2 extensive subsite may increase DPP-4 inhibition beyond the level afforded by the fundamental interactions with the S_1 and S_2 subsites and are more effective than forming a covalent bond with Ser630.

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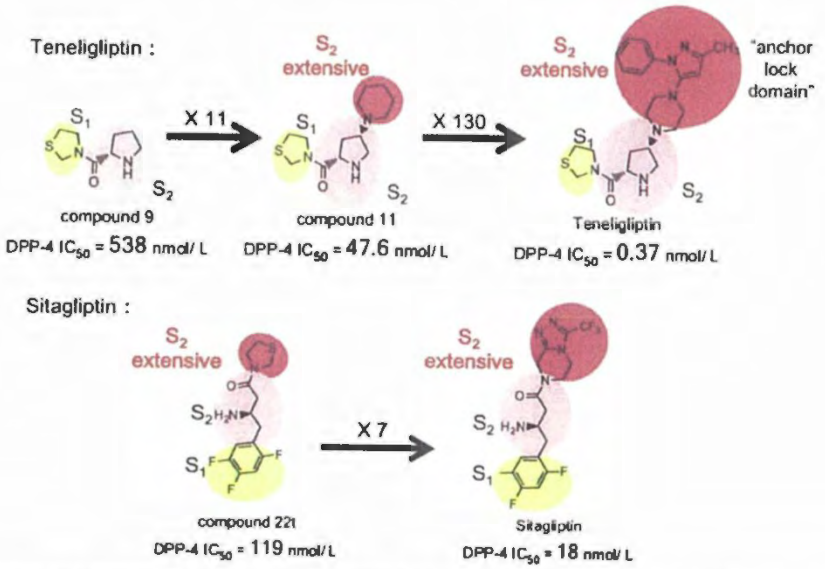

Fig. 3. The effect of binding to the S_2 extensive subsite. The activity of compounds 9 and 11 is obtained from [24] and that of teneligliptin is obtained from [4]. They were assayed in the same system. The activity of compound 22t and sitagliptin is obtained from [25] and [6], respectively.

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Inhibition of dipeptidyl peptidase IV by fluoroolefin-containing *N*-peptidyl-*O*-hydroxylamine peptidomimetics

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ABSTRACT **Dipeptidyl peptidase IV (EC 3.4.14.5; DPP IV), also known as the leukocyte differentiation antigen CD26 when found as an extracellular membrane-bound proline specific serine protease, cleaves a dipeptide from the N terminus of a polypeptide chain containing a proline residue in the penultimate position. Here we report that known (*Z*)-Ala-ψ[CF=C]-Pro dipeptide isosteres 1 and 2, which contain *O*-acylhydroxylamines, were isolated as diastereomeric pairs *u*-1, *l*-1, and *l*-2. The effect of each diastereomeric pair as an inhibitor of human placental dipeptidyl peptidase DPP IV has been examined. The inhibition of DPP IV by these compounds is rapid and efficient. The diastereomeric pair *u*-1 exhibits very potent inhibitory activity with a K_i of 188 nM. Fluoroolefin containing *N*-peptidyl-*O*-hydroxylamine peptidomimetics, by virtue of their inhibitory potency and stability, are superior to *N*-peptidyl-*O*-hydroxylamine inhibitors derived from an Ala-Pro dipeptide.**

Dipeptidyl peptidase IV (EC 3.4.14.5; DPP IV; CD26), discovered in 1966 (1), is a transmembrane serine peptidase found in a variety of human tissues and organs (2–4). In particular, DPP IV, when expressed on the surface of $CD4^+$ T cells, is identical with the leukocyte differentiation antigen CD26 and is considered to be a lymphocyte activation marker (5, 6). Although the involvement of DPP IV in the immune response and regulation of lymphocyte activation has been implicated, the mechanism of the involvement is not clear (7, 8). Of the many functions that have been postulated (9–14), the most intriguing is the role of DPP IV in T-cell activation and in the regulation of T-cell proliferation (13, 15–18). Recognized as a cell surface activation marker of lymphocytes (19), the failure to observe CD26 implies a reduced immune response (20). The presence of DPP IV is associated with the capacity of cells to produce interleukin 2 and to proliferate strongly in response to mitogen stimulation (20, 21). Importantly, binding of mAbs to CD26 suppresses interleukin 2 production (21). CD26 modulation also can lead to enhanced cell proliferation preceded by an increase in Ca^{2+} mobilization (22). CD26 is associated physically with CD45, which regulates T-cell activation pathways through protein tyrosine phosphatase action. CD26 apparently modulates the activity of CD45 by affecting the accessibility of critical substrates, with the result that the CD2/CD3 path amplifies the immune response (23). DPP IV, known to be localized on the surface of T cells with adenosine deaminase, seems to form a complex with adenosine deaminase that is involved in an important immunoregulatory mechanism involving T-cell proliferation (24, 25). DPP IV appears to be not only up-regulated among proliferating thymocytes but also by those undergoing programmed cell death (26). The involvement of CD26 in HIV infection has been the subject of investigation for some time, with the initial report (27) that CD26 was a cofactor facilitating HIV entry in $CD4^+$ cells refuted (28–30). Reports that DPP IV enzymatic activity may decrease the efficiency of HIV infection (31) may be related to the binding of HIV glycoproteins gp120 and gp41, which have been shown to be responsible for cell killing by apoptosis in $CD4^+$ cells (32). The Tat protein of HIV-1, known to be capable of suppressing CD3 activation of T cells, also has been shown to bind to DPP IV with effects on cytokine production and DNA synthesis, implying that the DPP IV plays a role in Tat immunosuppression (33, 34).

DPP IV will cleave the dipeptides Xaa-Pro from the N terminus of a polypeptide while recognizing several key structural features in substrate proteins or peptides. It has been postulated (35) that DPP IV substrates require the presence of a proline at the P_1 position as well as a protonated free N terminus (36, 37). It also has been proposed that DPP IV possesses a high conformational specificity for a trans amide bond between the P_1 and N-terminal P_2 residues (38). There is the additional requirement for the L configuration of the amino acid residue, both in the penultimate and the N-terminal position (39, 40).

Obviously, inhibition of CD26 may critically affect T-cell activation and function and may potentially have therapeutic utility in the modulation of the immune response. Relatively few of the compounds reported thus far are effective inhibitors of DPP IV, with most inhibitors suffering from either instability or low reactivity. *N*-Peptidyl-*O*-acylhydroxylamines irreversibly inhibit DPP IV, but most of the inhibitor is enzyme-hydrolyzed during the inactivation process (41, 42). The boronic acids Ala-*boro*Pro, Pro-*boro*Pro, and Val-*boro*Pro are potent and specific reversible inhibitors of DPP IV with K_i values in the nanomolar range. However, these compounds lose their inhibitory activity in aqueous solution at neutral pH because of the formation of cyclic species in which the N-terminal amine nitrogen coordinates to the boron atom (37, 43–45). Peptidyl (α-aminoalkyl) phosphonate esters (46) and diphenyl phosphonate esters (47) are moderate and specific DPP IV inhibitors. These compounds are quite stable because phosphonate esters are relatively unreactive with nitrogen nucleophiles or N-terminal amines. Aminoacylpyrrolidine-2-nitriles (48) and 4-cyanothiazolidides (49, 50) recently were reported as very potent and rather stable inhibitors of DPP IV. They were found to have K_i values in the nanomolar to low submicromolar range and half-lives between 27 and 72 h.

Many of the problems associated with inefficient inactivation of DPP IV are a consequence of the importance of the trans conformation of the P_1-P_2 amide bond and the requirement for a protonated free N terminus. The cyclization

Abbreviation: DPP IV, dipeptidyl peptidase IV.

Data deposition: The atomic coordinates and structure factors have been deposited in the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, United Kingdom (reference 103375).

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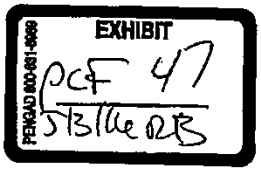

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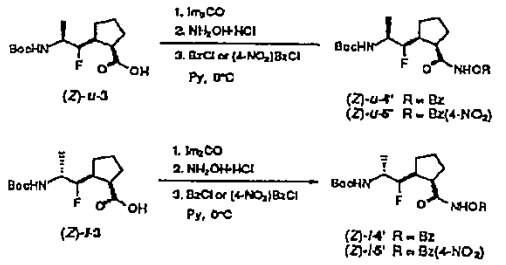
reaction of the free N-terminal amino group with the reactive site of the inhibitor does, however, require the molecule to assume the cis conformation, the conformation previously proposed to be unreactive with DPP IV (38). To obviate this mode of inactivation and to rigorously examine the cis–trans selectivity of DPP IV, we have prepared a series of conformationally constrained fluoroolefin dipeptide isosteres. The fluoroolefin dipeptide isostere was proposed as early as 1984 (51) as a superior isoelectronic and isosteric replacement for the amide bond. Theoretical studies strongly have supported the original hypothesis behind introduction of the fluoroolefin amide surrogate (52–54). The syntheses of fluoroolefin dipeptide surrogates, Ala-ψ[CF=C]-Pro containing *N,O*-diacylhydroxamic acid type protease inhibitors **1** and **2**, were reported recently by our laboratory (55).



SCHEME 1.

MATERIALS AND METHODS

Materials. Human placenta dipeptidyl peptidase IV (EC 3.4.14.5) was purchased from Calbiochem–Novabiochem (La Jolla, CA). The specific activity is 8,333 milliunits per milligram of protein. One milliunit, specified by Calbiochem–Novabiochem, is defined as the amount of enzyme that will hydrolyze 1.0 μM of Ala-Pro-7-amino-4-trifluoromethyl coumarin per minute at 30°C, pH 7.8. The DPP IV substrate Gly-Pro-*p*-nitroanilide was obtained from Sigma. The phosphate buffer (KH_2PO_4-$NaHPO_4$, 90 mM, pH 7.6) and Tris·HCl buffer (20 mM, pH 7.8) were prepared in our laboratory.

^1H, ^{13}C, ^{19}NMR spectra were recorded on a Gemini-300 NMR spectrometer (Varian) with CD_3OD as solvent and residual methanol or $CFCl_3$ as the internal standard. Thin layer chromatography was performed with F_{254} (Merck) silica gel as the adsorbent on 0.2-mm thick, plastic-backed plates. The chromatograms were visualized under UV (254 nm) and by spraying with a 95:5 mixture of 0.2% ninhydrin in *n*-butanol and 10% aqueous acetic acid followed by heating. The UV-visible spectra were determined by using a Shimadzu UV-visible recording spectrophotometer (UV-160).

Syntheses of Inhibitors 1–2. The general procedure for amine deprotection is shown in Scheme 3. Compound *l*-4 (14.7 mg, 0.025 mmol), prepared as described (55), was treated with 1 M HCl in acetic acid (1 ml), stirring at room temperature for 1–2 h. The solvent was removed under high vacuum. Diethyl ether (2 ml) was added to the residue. This mixture was stored at −4°C overnight. After the supernatant was decanted, the resultant white solid was washed with ether, and dried, to yield the sufficiently pure hydrochloride salts *(Z)-(R, R)*, *(Z)-(S, S)*-1-[(1′-fluoro-2′-amino)propylidene]-2-cyclopentane-*O*-benzoyl hydroxamate hydrochloride (*l*-1) (10.7 mg, 86%). Data for *l*-1: ^{19}NMR (CD_3OD) δ -124.37 (d, J = 27.1 Hz); ^1H NMR (CD_3OD) δ 8.07 (d, 2H, J = 7.3 Hz), 7.69 (t, 1H, J = 7.4 Hz), 7.36 (t, 2H, J = 7.8 Hz), 4.29 (dq, 1H, J = 27.2, 6.9 Hz), 3.66–3.58 (m, 1H), 2.55–2.45 (m, 2H), 2.39–1.96 (m, ^3H), 1.84–1.72 (m, 1H), 1.47 (d, ^3H, J = 6.8 Hz); ^{13}C NMR (CH_3OD) δ 171.81, 164.08, 148.52 (d, J = 249.7 Hz), 133.67, 129.14, 128.29, 126.74, 123.34 (d, J = 13.6 Hz), 44.71, 43.23 (d, J = 27.6 Hz), 31.76, 28.03 (d, J = 1.8 Hz), 25.26, 14.46.

(Z)-(R, S), *(Z)-(S, R)*-1-[(1′-fluoro-2′-amino)propylidene]-2-cyclopentane-*O*-benzoyl hydroxamate hydrochloride (*u*-1) was prepared in the same manner from *u*-4 in 51% yield. Data for *u*-1: ^{19}NMR (CD_3OD) δ -124.42 (d, J = 24.4 Hz); ^1H NMR (CD_3OD) δ 8.07 (d, 2H, J = 8.5 Hz), 7.69 (t, 1H, J = 7.5 Hz), 7.53 (t, 2H, J = 7.3 Hz), 4.29 (dq, 1H, J = 26.8, 6.9 Hz), 3.68–3.59 (m, 1H), 2.59–2.39 (m, 2H), 2.26–1.93 (m, 4H), 1.84–1.68 (m, 1H), 1.49 (d, ^3H, J = 6.9 Hz); ^{13}C NMR (CD_3OD) δ 172.84, 165.57, 149.98 (d, J = 249.7 Hz),135.13, 130.67, 129.78, 128.30, 124.69 (d, J = 13.7 Hz), 47.35 (d, J = 27.5 Hz), 45.30, 33.20, 29.67 (d, J = 3.2 Hz), 26.99, 16.32.

(Z)-(R, R), *(Z)-(S, S)*-1-[(1′-fluoro-2′-amino)propylidene]-2-cyclopentane-(4-nitro)-*O*-benzoyl hydroxamate hydrochloride (*l*-2) was prepared in the same manner from *l*-5 in 63% yield. Data for *l*-2: ^{19}NMR (CD_3OD) δ -124.33 (d, J = 27.1 Hz); ^1H NMR (CD_3OD) δ 8.39 (d, 2H, J = 9.1 Hz), 8.29 (d, 2H, J = 9.0 Hz), 4.29 (dq, 1H, J = 27.1, 6.9 Hz), 3.67–3.59 (m, 1H), 2.62–2.40 (m, 2H), 2.26–2.11 (m, 1H), 2.10–1.93 (m, 2H), 1.87–1.70 (m, 1H), 1.48 (d, ^3H, J = 6.9 Hz); ^{13}C NMR (CD_3OD) δ 173.22, 164.03, 152.51, 150.00 (d, J = 249.7 Hz), 133.66, 132.03, 124.84 (d, J = 13.7 Hz), 124.83, 47.47 (d, J = 27.1 Hz), 45.07, 33.43, 29.70 (d, J = 2.8 Hz), 26.93, 16.16.

Crystal Structure Determination of *l*-3. Diastereomeric pair *l*-3 prepared as described (55) was recrystallized from a mixture of hexanes and ethyl acetate (1:1). Crystal data: $C_{14}H_{21}FNO_3$, M = 287.3, monoclinic, space group $P2_1/n$, a = 9.607 (4) Å, b = 9.300 (3) Å, c = 17.204 (6) Å, β = 95.66 (3) °, V = 1529.7 (9) Å^3, Z = 4, D_c = 1.248 g cm^{-1}, μ = 0.98 cm^{-1}, λ(MoKα) = 0.71073 Å, F(000) = 616, T = 298 K. Nicolet R3 m/V diffractometer was used to collect 2,038 reflections ($3° < 2\theta < 45°$) on a colorless crystal 0.15 × 0.15 × 0.40 mm^3. Of these, 1,961 were unique and 1,193 were observed ($F_o > 6\sigma F_o$). Lorentz and polarization corrections were applied to the data. The non-hydrogen atoms were located by direct methods. R = 0.070, R_w = 0.068, GOF = 2.14.

Inactivation Assays. Method A (inactivation in the absence of substrate): The inhibitory activity of the compounds, *u*-1, *l*-1, and *l*-2, was estimated from the residual activity of DPP IV in a solution of the substrate Gly-Pro-*p*-nitroanilide. An aliquot of inhibitor (20 μl, from 50 μM stock solution in water) was added to 80 μl of a buffered enzyme solution [0.2 milliunit in Tris·HCl buffer (pH 7.6)] to initiate the inactivation reaction. The concentration of inhibitor in the incubation mixture (total volume 100 μl) was 10 μM. After the enzyme and inhibitor were incubated for either 2 or 30 min at 30°C, the incubation mixture was added to a 1-ml cuvette containing 900 μl of substrate Gly-Pro-*p*-nitroanilide (0.1 mM) in 45 mM phosphate buffer (pH 7.6, μ = 0.123). The measuring cell had been equilibrated thermally in the spectrophotometer for 2 min before enzyme-inhibitor preincubation solution was added. The rate of change in UV absorbance at 385 nm, with respect to a cuvette containing only 0.1 mM substrate in 45 mM buffer, gave a straight line with the slope proportional to the enzyme activity. The residual enzyme activity is expressed relative to a DPP IV control, which was prepared by adding only enzyme to the substrate solution. The percentage inhibition (% *I*) was calculated as $\% I = [(1 - v_i/v_o)] \times 100\%$, where v_i and v_o are the rate of change in absorbance at 385 nm, with and without inhibitor, respectively. The percentage inhibition (% *I*) at other inhibition concentrations was measured by the same method.

Method B (inactivation in the presence of substrate): To a cuvette containing 5 to 20 μl of appropriate concentration of inhibitor, 20 μl of 5 mM substrate Gly-Pro-*p*-nitroanilide, 500 μl of 90 mM phosphate buffer (pH 7.6), and enough water to bring the final volume to 1 ml was added 20 μl of enzyme

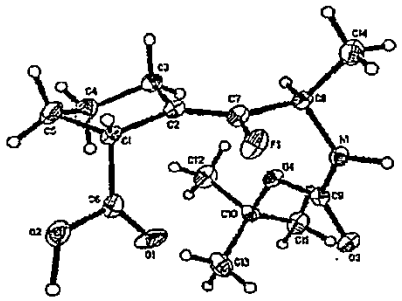


FIG. 1. ORTEP drawing of the x-ray structure of (*Z*)-*l*-Boc-Ala-ᵠ[CF=C]-Pro (*l*-3).

solution (0.2 milliunit) in pH 7.6 Tris buffer. The rate of change in the absorbance at 385 nm, with respect to a cuvette containing the same amount of inhibitor and substrate in buffer, gave the inactivation progress curves. All inhibition experiments were monitored by using a Shimadzu UV-160 at 385 nm and 30 ± 0.1°C.

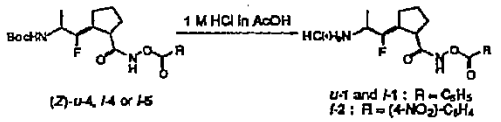
Determination of K_i Values. For inhibitors *u*-1, the data for two Dixon plots (1/V vs. [I]) were obtained by repeating method B at two concentrations of substrate (0.2 mM and 0.4 mM) and six to seven different inhibitor concentrations (0, 0.25, 0.5, 0.75, 1.0, 5.0, and 10.0 μM). The correlation coefficients of both lines were >0.994. The K_i value of *u*-1 was calculated according to the method of Dixon (56). The K_i value for compound *l*-1 was determined by the method described above.

Determination of the Half-Life of Inhibitor *u*-1. An aliquot (40 μl) of inhibitor *u*-1 from 5 mM stock solution in H_2O was added to a 1-ml cuvette containing 500 μl of 90 mM phosphate buffer solution (pH 7.6) and 460 μl of water at 30°C, such that the final inhibitor concentration was 0.2 mM. Spontaneous decomposition was monitored by following the decrease in absorbance at 229 nm at various time intervals. The absorbance data points at 229 nm were recorded and plotted as function of time, which gave a spontaneous degradation curve. The half-life was obtained from first-order plot of $\ln(A/A_0)$ vs. time, where A is the absorbance of the mixture at time t, and A_0 is the absorbance at initial time ($t = 0$).

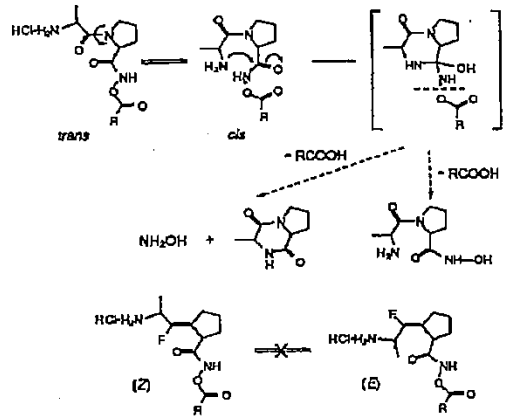
RESULTS AND DISCUSSION

Chemistry. (*Z*)-*N*-*tert*-Butyloxycarbonyl-1-[(1′-fluoro-2′-amino)propylidene]-2-cyclopentane carboxylic acid 3 was synthesized and isolated as two diastereomeric pairs 3′ and 3″, as described in a previous report (55). The relative stereochemistry of these diastereomeric pairs was determined by single crystal x-ray diffraction studies. The structure of compound 3′, crystallized from a mixture of hexanes and ethyl acetate (1:1), is shown in Fig. 1. The absolute configurations at C1 and C8 of 3′ were confirmed as *R* and *R* or *S* and *S*, respectively. Therefore, 3′ can be designated as the "like" diastereomeric pair (*l*-3). Obviously, compound 3″, as "unlike" diastereomeric pair (*u*-3), contains the *S*, *R* isomer corresponding to the natural amino acid L-(*S*)-Ala-L-(*S*)-Pro and would be predicted to have higher biological activity.

The two diastereomeric pairs were converted independently to *l*-4 and *u*-4 after three step transformation (55). The analog *l*-5 was prepared in the same manner by using 4-nitrobenzoyl chloride instead of benzoyl chloride, as shown in Scheme 2. Removal of the Boc-groups was accomplished by using 1 M HCl in AcOH to give compounds 1 and 2 (Scheme 3).



SCHEME 2. 1. Im_2CO; 2. NH_2OH·HCl; 3. BzCl or (4-NO_2)BzCl, Py, 0°C.



SCHEME 3.

Inhibition of DPP IV. The results of initial inhibition studies of DPP IV by diastereomeric pairs *u*-1, *l*-1, and *l*-2 are shown in Table 1. At the same inhibitor concentration with 2-min incubation time or 30-min incubation time, the percentages of inhibition of DPP IV by *u*-1, *l*-1, and *l*-2 unexpectedly remained the same or changed only slightly with an increase in incubation time.

The results presented in Table 1 revealed the following phenomena: (*i*) Inactivation of DPP IV by *u*-1 did not follow pseudo-first order reaction kinetics. The inactivation process was principally dependent on inhibitor concentration and independent of incubation time. At a concentration of 0.01 mM, inhibitor *u*-1 showed nearly the same percentages of inhibition, 42% and 39% inhibition, at both 2 min and 30 min incubation time, respectively. At 0.25 mM, after both 2 min and 30 min incubation time, 100% inhibition of the activity of DPP IV was observed. For inhibitors *l*-1 and *l*-2, the percentages of inhibition (% *I*) increased or remained the same within experimental error with increasing incubation time at the same inhibitor concentrations (shown in Table 1). (*ii*) Inhibitory potency of *u*-1 was much greater than that of the other diastereomeric pair *l*-1. At a concentration of 0.01 mM and a 2-min incubation time, compound *u*-1 inhibited 42% of the enzymatic activity of DPP IV; however, the isomer *l*-1 was nearly ineffective (4% inhibition) under the same conditions. As mentioned above, DPP IV has an absolute requirement for the L configuration of the amino acid residue, both in penultimate and N-terminal positions. Because the pair *u*-1 contains the compounds with the desired configuration (L, L), it was more reactive with DPP IV. (*iii*) Replacement of the benzoyl group (*l*-1) by a *para*-nitrobenzoyl group (*l*-2) enhanced the inhibitory activity slightly. This may be because the electron withdrawing group (4-NO_2) facilitates the rate-determining *N*-*O* fission. (*iv*) Inhibitors *u*-1, *l*-1, and *l*-2 all exhibited

Table 1. Inhibition of DPP IV by fluoroolefin-containing *N*-peptidyl-*O*-hydroxylamines

Inhibitors	[I], mM	% Inhibition* 2 min	% Inhibition* 30 min
l-1 (*Z*)-Ala-ψ[CF=C]-Pro-NHO-Bz	0.01	4	1
	0.50	17	25
l-2 (*Z*)-Ala-ψ[CF=C]-Pro-NHO-Bz(4-NO_2)	0.50	22	34
u-1 (*Z*)-Ala-ψ[CF=C]-Pro-NHO-Bz	0.01	42	39
	0.25	100	100
Ala-Pro-NHO-Bz(4-NO_2)	1.10	29†	60

*Percentage inhibition was measured after 2- or 30-min incubation in 45 mM phosphate buffer, pH 7.6, at 30°C. Gly-Pro-*p*-nitroanilide was used as substrate.
†Incubation time was 10 min (41).

inhibitory activity superior to the previously prepared Ala-Pro-NHO-Bz(4-NO_2) compounds (41, 42) It has been proposed that the trans P_2-Pro peptide bonds of the substrates are essential to the reactivity of enzyme DPP IV. The enhancement in inhibitory potency of our fluoroolefin containing dipeptide isosteres can be attributed to the efficient mimicking of the trans P_1-P_2 amide bonds of the original dipeptides by the (*Z*) fluoroolefin double bond conformation.

The effect of inhibitor *u*-1 on the hydrolysis of substrate (S) by DPP IV (E) was demonstrated in two different ways: enzyme-initiated assay, (S + I) + E, and substrate initiated assay, (E + I) + S (Fig. 2). The results were unexpected. In both cases (curves B and C), the rates of hydrolysis of substrate by DPP IV increased linearly over 50 min and were nearly identical (curves B and C overlapped). The initial rate (u) of hydrolysis of substrate in an enzyme initiated assay is often larger than that found in a substrate initiated assay (57). The curves B and C shown in Fig. 1 indicate that inhibitor *u*-1 very rapidly inactivates DPP IV in a process much faster than the rate of hydrolysis of the substrate by DPP IV; thus, the presence of competing substrate did not slow down the inactivation process.

To determine the values of the inhibition constant K_i for both compound *u*-1 and *l*-1, the rates of DPP IV-catalyzed hydrolysis of Gly-Pro-*p*-nitroanilide substrate were estimated at six to seven different concentrations for each inhibitor (0.25 to 10 μM) in a competitive hydrolysis fashion. The K_i values reported in Table 3 for compounds *u*-1 and *l*-1 were obtained

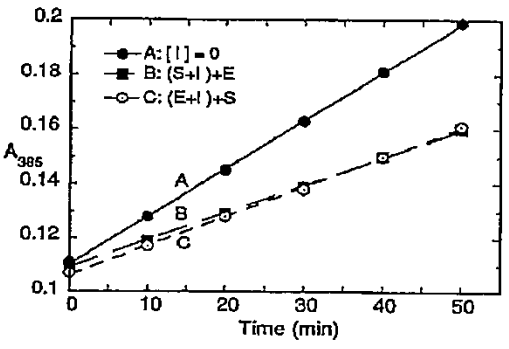


FIG. 2. Hydrolysis of substrate Gly-Pro-4-nitroanilide was monitored by the change in absorbance at 385 nm with time. In all cases, the final concentrations S, E, and I were 0.1 mM, 0.2 milliunit, and 1 μM, respectively.

Table 2. Inhibition constants of inhibitors of DPP IV, *l*-1, and *u*-1

Inhibitors	K_i, nM
l-1 (*Z*)-Ala-ψ[CF=C]-Pro-NHO-Bz	14,400
u-1 (*Z*)-Ala-ψ[CF=C]-Pro-NHO-Bz	188
Ala-Pro-NHO-Bz(4-NO_2)	30,000 (58)

from the plots 1/v versus [I] according to the method of Dixon (56). The Dixon plot revealed that compounds *u*-1 and *l*-1 act as competitive inhibitors.

As shown in Table 2, the diastereomeric pair *u*-1 (containing the L, L isomer) exhibited very potent inhibitory activity with a small K_i value in the nanomolar range (0.19 μM). The affinity of this isomer for DPP IV is two orders of magnitude greater than the other diastereomeric pair *l*-1 (K_i = 14.4 μM). The Demuth's inhibitor, Ala-Pro-NHO-Bz(4-NO_2), also has a larger K_i value (30 μM) (58) and is a poorer inhibitor.

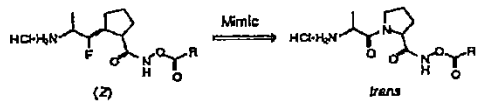
Stability of Inhibitors. As shown in Table 3, inhibitor *u*-1 was very stable in buffer (pH 7.6) with a decomposition rate constant k_d of 1.1×10^{-4} min^{-1} and a half-life of 103 h. In contrast, the stability of inhibitor Ala-Pro-NHO-Bz(4-NO_2) under assay conditions was limited because of a 10-fold higher decomposition rate constant k_d (1.3×10^{-3} min^{-1}), resulting in a shorter half-life of only 8.8 h (42).

We believe that intramolecular cyclization is probably responsible for facile, spontaneous degradation of natural peptide-based hydroxamic acid inhibitors. In a manner similar to enzyme-induced *N-O* fission, the free amino group at the N terminus nucleophilically attacks the amide carbonyl carbon, thus forming a tetrahedral intermediate and thereby promoting *N-O* scission and subsequent generation of the reactive acylnitrene intermediate. The six-membered cyclic intermediate is hydrolyzed further either to hydroxamic acid or diketopiperazine products (Scheme 4). Clearly, the findings of our study support this postulate. Considerable improvement in stability of our compounds in buffer at neutral pH can be ascribed to the constrained double bond conformation of (*Z*)-fluoroolefin, excluding the possibility of intramolecular cyclization caused by the amide bond rotation in the dipeptides.

(*Z*)-Fluoroolefin-containing dipeptides *u*-1, *l*-1, and *l*-2, designed as the mimics of *N*-peptidyl-*O*-acylhydroxylamines, have been synthesized and tested as inhibitors of dipeptidyl peptidase DPP IV. One diastereomeric pair *u*-1 exhibits very potent inhibitory activity with a K_i of 188 nM. The inhibitory potency of this isomer is ≈70-fold higher than the other diastereomer *l*-1 (K_i = 14, 400 nM). In comparison with the Ala-Pro-NHO-Bz(4-NO_2) analog, the dipeptide isosteres 1 and 2 are better inhibitors of DPP IV by virtue of their superior inhibitory potency and stability; presumably, the (*Z*) double bond conformation of the fluoroolefin dipeptide isosteres efficiently mimics the trans P_2-Pro amide bonds of the original dipeptides. In addition, the rates of inactivation of DPP IV by compounds 1 and 2 appeared to be very fast. More detailed biological studies, kinetic analysis for inactivation rate constant k_{inact}, and investigations of inhibition mechanism are in progress at present. The results of this study reveal that a series of known inhibitors of DPP IV such as dipeptide boronic acids (43–45), dipeptide phosphonates (46, 47), peptidyl nitriles (49–51), and others can be modified by replacing the amide bonds by fluoroolefin moieties. Because of the anticipated high affinity and stability, the fluoroolefin containing dipeptide peptidomimetics should prove to be very promising inhibitors of

Table 3. Spontaneous degradation rate constants k_d and half-life $t_{1/2}$

Inhibitors	$k_d \cdot 10^4$, min^{-1}	$t_{1/2}$, h
u-1 (*Z*)-Ala-ψ[CF=C]-Pro-NHO-Bz	1.1	103 ± 3
Ala-Pro-NHO-Bz(4-NO_2)	13.0	8.8



SCHEME 4.

DPP IV and therefore helpful in elucidating the biological functions of DPP IV in T-cell activation. These agents may also be potential therapeutic agents useful in modifying and controlling the immune response.

Dedicated to Professor Dieter Seebach on the occasion of his 60th birthday. Financial support of this work by the National Science Foundation Grant CHE 9413004 and National Institutes of Health Grant AI33690 is gratefully acknowledged.

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IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

ASTRAZENECA AB, Plaintiff, v. AUROBINDO PHARMA LTD. and AUROBINDO PHARMA U.S.A., INS., Defendants.	C.A. No. 14-664-GMS (consolidated)
ASTRAZENECA AB, Plaintiff, v. MYLAN PHARMACEUTICALS INC., Defendants.	C.A. No. 14-696-GMS

EXPERT REPORT OF DAVID P. ROTELLA, PH.D.
ON THE INVALIDITY OF U.S. PATENT NO. RE 44,186

DX-57

I. INTRODUCTION

1. I, David P. Rotella, have been retained by counsel for Mylan Pharmaceuticals Inc. in connection with the above-captioned litigation matter to provide expert testimony concerning U.S. Patent No. RE 44,186 ("the '186 patent") by Robl *et al.*, and entitled "Cyclopropyl-fused pyrrolidine-based inhibition of dipeptidyl peptidase IV and method." This report presents my opinions regarding the invalidity of claims 8, 9, 25, and 26 (collectively, "the asserted claims") of the '186 patent. For the reasons detailed below, it is my opinion that the asserted claims of the '186 patent would have been obvious in view of the prior art to a person of ordinary skill in the art at the time of the claimed invention.

2. This expert report, submitted pursuant to Fed. R. Civ. P. 26(a)(2)(B), sets forth the opinions as to which, if asked, I will testify at trial with respect to the '186 patent. In addition, if asked, I may respond to the opinions and testimony of Plaintiff's witnesses regarding issues within my area of expertise. I reserve the right to adjust, modify, or supplement my opinions in light of any response, critique, or comments on my report or different opinions made by or on behalf of other parties to this litigation, including, but not limited to, any deposition testimony or rebuttal reports that other parties submit.

A. Qualifications and Experience

3. I received my B.S. Pharm. from the University of Pittsburgh in 1981 and Ph.D. in Medicinal Chemistry from The Ohio State University in 1985. I was a Postdoctoral Scholar in the Department of Chemistry at The Pennsylvania State University from 1985 to 1987.

4. I am currently the Margaret and Herman Sokol Professor of Chemistry in the Department of Chemistry and Biochemistry and in the Sokol Institute of Pharmaceutical Life

Sciences at Montclair State University. I have been a member of the faculty of this university since 2011.

5. I am also currently an adjunct professor in the Department of Pharmaceutical Sciences at the University of Pittsburgh, in the Center for Drug Discovery at Northeastern University, and in the Department of Medicinal Chemistry at the University of Mississippi. I have been a member of the faculty of these departments since 2010, 2010, and 2009, respectively.

6. I am currently a registered pharmacist in the Commonwealth of Pennsylvania.

7. I was formerly a research scientist at multiple pharmaceutical companies during the years 1991-2010, including at Bristol-Myers Squibb PRI, Lexicon Pharmaceuticals, and Wyeth Research/Pfizer. My industry experience focused on drug discovery and development.

8. My current research focuses on protein kinase inhibitors for anti-infective and anti-inflammatory applications. Specifically, I work on the discovery of new agents useful for the potential treatment of parasitic and neurodegenerative diseases, including the synthesizing of new analogs of a lead structure as potential protein kinase inhibitors and investigation of structure-activity relationships in a product that has HSP90 inhibitor activity.

9. I have authored or co-authored more than 20 abstracts for presentation at professional meetings, 40 peer-reviewed journal articles, and seven book chapters. I have also edited or co-edited five books in the field of Medicinal Chemistry. I have received numerous honors, fellowships and awards, and am an inventor or co-inventor on seven granted patents.

10. A summary of my education, experience, publications, awards and honors, patents, publications, and presentations is provided in my CV, a copy of which is provided as Exhibit A, attached hereto.

B. Compensation

11. I am being compensated at the rate of $500/hour for my work in connection with this litigation. My compensation is not dependent on the outcome of this lawsuit. I do not have any financial interest in the outcome of this matter.

C. Prior Testimony

12. I have not appeared as an expert, either at trial or by deposition, within the last 5 years.

D. Materials Considered

13. In forming my opinions and preparing this report, I reviewed and considered the materials cited in this report and those materials listed in Exhibit B to this report. I have further relied on my knowledge, education, and training as reflected in my qualifications and credentials set forth above and in my *curriculum* vitae.

14. Additionally, I may use the materials cited or listed to assist me in preparing demonstratives such as graphics and animations for my testimony or in the event that I am asked to provide testimony or a technology tutorial.

II. APPLICABLE LEGAL STANDARDS

15. I have been advised that the following legal standards are applicable to this report.

16. I understand that patents are presumed valid and the party challenging validity has the burden of proving invalidity by clear and convincing evidence. I further understand that an invalidity analysis involves two steps: first, ascertaining the proper meaning and scope of the patent claims; and second, determining whether the limitations of the claims, as properly interpreted, are disclosed in the prior art.

17. I have been advised that, in the present case, the '186 patent claims are to be given their broadest reasonable interpretation in view of the specification. I also understand that, absent some reason to the contrary, claim terms are typically given their ordinary and accustomed meaning as would have been understood by one of ordinary skill in the art. I have followed these principles in my analysis described throughout this declaration. The '186 patent provides definitions for certain claim terms. In my opinion, those definitions are conventional.

A. Priority Date

18. I have been advised that the '186 patent claims priority to U.S. provisional application number 60/188,555 ("the '555 application)," which was filed on March 10, 2000. I further understand, however, that the '186 patent may not be entitled to that 2000 priority date. Each of the opinions expressed in this declaration apply regardless of whether the priority date is March 10, 2000 (the filing date of the '555 application) or February 16, 2001 (the filing date of the '173 application).

B. Level of Ordinary Skill in the Art

19. I am informed by counsel that a patent is to be interpreted from the perspective of a person of ordinary skill in the art ("POSA") to which the patent pertains, as assessed at the time of the claimed invention. I am further informed that a determination of the level of ordinary skill is based on, among other things, the educational level of the inventors, the types of problems encountered in the art, prior art solutions to those problems, the rapidity with which innovations are made, the sophistication of the technology, and the educational level of active workers in the field. I further understand that a person of ordinary skill is also a person of ordinary creativity.

20. Counsel has informed me that a POSA is a hypothetical person that may have the combined understanding of those of ordinary skill in various fields pertinent to the subject matter

of the patent. In this case I have been asked to presume that the POSA is aware of all prior art available as of March 10, 2000. Therefore, I have been asked to opine on the qualifications of a POSA as of March 10, 2000. My opinion regarding the level of skill of a POSA, detailed *infra* in Section V., thus regards a person of ordinary skill in the relevant field as of March 10, 2000.

C. Obviousness

21. I have been informed that a claimed invention is not patentable under 35 U.S.C. § 103 for obviousness, if the differences between the invention and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the field to which the subject matter pertains.

22. I have been informed that a determination of obviousness involves four factual inquiries: (1) the scope and content of the prior art; (2) the differences between the claimed subject matter and the prior art; (3) the level of ordinary skill in the art at the time of the alleged invention; and (4) secondary considerations of non-obviousness.

23. I understand from counsel that a claim can be found to have been obvious if all the claimed elements were known in the prior art and one skilled in the field could have combined the elements as claimed by known methods with no change in their respective functions, and the combination would have yielded nothing more than predictable and expected results to one of ordinary skill in the art. I also understand that improper hindsight must not be used when comparing the prior art to the invention for obviousness. Thus, a conclusion of obviousness must be firmly based on knowledge and skill of a person of ordinary skill in the field at the time the invention was made without the use of post-filing knowledge.

24. I understand from counsel that in order for a claimed invention to be considered obvious, there must be some supporting rationale for combining cited references as proposed. I

have been informed that obviousness may be established by showing that it would have been obvious to combine the teachings of more than one item of prior art. In determining whether a piece of prior art could have been combined with other prior art or with other information within the knowledge of one of ordinary skill in the art, the following are examples of approaches and rationales that may be considered: (i) combination of prior art elements according to known methods to yield predictable results; (ii) simple substitution of one known element for another to obtain predictable results; (iii) use of a known technique to improve similar methods or products in the same way; (vi) application of a known technique to a known method or product ready for improvement to yield predictable results; (vii) application of a technique or approach that would have been "obvious to try" (choosing from a finite number of identified, predictable solutions, with a reasonable expectation of success); (viii) known work in one field of endeavor may prompt variations of it for use in either the same field or a different one based on design incentives or other market forces if the variations would have been predictable to one of ordinary skill in the art; or (ix) some teaching, suggestion, or motivation in the prior art that would have led one of ordinary skill to modify the prior art reference or to combine prior art reference teachings to arrive at the claimed invention.

25. I understand from counsel that multiple factors may guide the analysis of whether it would be obvious to a person of ordinary skill to select a particular prior art compound as a lead compound for further modification. I understand that these factors may include positive attributes such as activity and potency, negative attributes such as toxicity, or any other relevant characteristics that would have been known to a person of ordinary skill from the prior art.

26. I understand from counsel that an analysis of whether there are any relevant differences between the prior art and the claimed subject matter is performed from the view of a

person having ordinary skill in the art at the time of the alleged invention. I understand that, in analyzing any differences between the claimed subject matter and the prior art, inferences and creative steps a person of ordinary skill in the art would have employed in reviewing the prior art at the time of the alleged invention may be taken into account.

27. I understand from counsel that evidence of "secondary considerations" may be weighed against evidence of the scope and content of, and the level of skill in, the art to rebut a conclusion of *obviousness* where appropriate. I understand that such secondary considerations when in evidence may include: (i) commercial success of a product due to the merits of the claimed invention; (ii) a long-felt, but unsatisfied need for the invention; (iii) failure of others to find the solution provided by the claimed invention; (iv) deliberate copying of the invention by others; (v) unexpected results achieved by the invention; (vi) praise of the invention by others skilled in the art; (vii) lack of independent simultaneous invention within a comparatively short space of time; and (viii) teaching away from the invention in the prior art. Secondary considerations are relevant where there is a nexus, or relationship, between the objective evidence and the claimed invention.

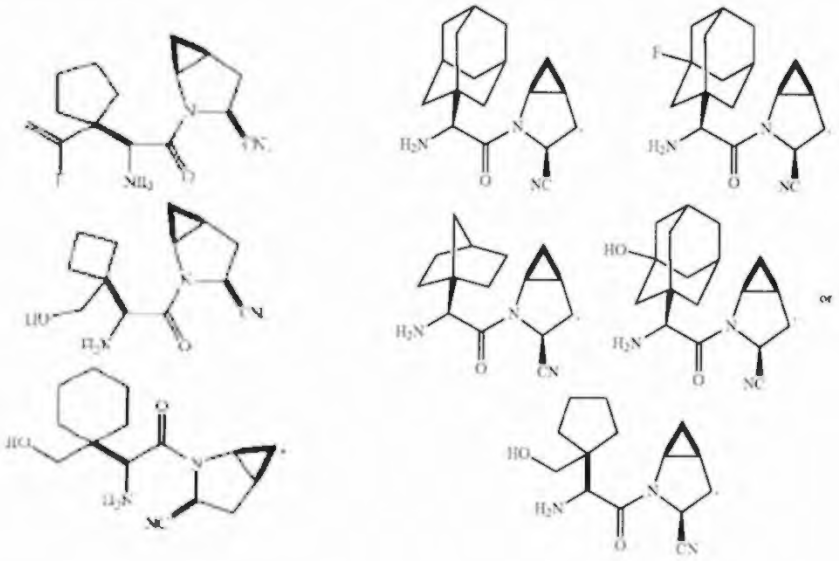
III. OVERVIEW OF THE '186 PATENT

28. The '186 patent is entitled "Cyclopropyl-Fused Pyrrolidine-Based Inhibitors of Dipeptidyl Peptidase IV and Method" and was issued on April 30, 2013. I have been advised that the '186 patent issued from U.S. Application No. 13/308,658, which was filed on December 1, 2011, as a reissued application of U.S. Application No. 09/788,173, which was filed on February 16, 2001 and issued as U.S. Patent No. 6,395,767 on May 28, 2002. I have also been advised that U.S. Application No. 09/788,173 claims priority to U.S. Provisional Application No. 60/188,555, which was filed on March 10, 2000.

29. The '186 patent is generally directed to cyclopropyl-fused pyrrolidine-based compounds with a variety of optional substituents, as well as pharmaceutical combinations and methods for treating diabetes and additional diseases. According to the '186 patent, the "cyclopropyl-fused pyrrolidine-based compounds [of the '186 patent are] inhibitors of dipeptidyl peptidase IV [(DP-IV)] . . . for treating diabetes, especially Type II diabetes." '186 patent at col. 1, ll. 19-21. The '186 patent describes the mechanism by which DP-IV inhibition treats type 2 diabetes as follows: "[DP- IV] has been shown to be the primary degrading enzyme of GLP-1(7-36) in vivo . . . [t]hus, inhibition of [DP-IV] in vivo should potentiate endogenous levels of GLP-1(7-36) and . . . thus serve to ameliorate the diabetic condition." *Id.* at 1:59-67.

30. Independent claim 1 discloses a genus of chemical compounds comprising a cyclopropyl-fused pyrrolidine-based core with a variety of optional substituents. Dependent claims 2-7 and independent claims 8 and 10 further limit the substituent groups of the compound of claim 1. For example, independent claim 8 recites the following:

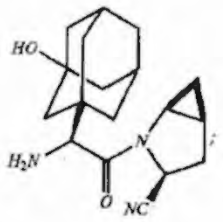
A compound having the structure:



or a pharmaceutically acceptable salt thereof.

Dependent claim 9 is directed to the hydrochloride or trifluoroacetic acid salts of the compounds of claim 8. Dependent claim 11 is directed to a pharmaceutical composition comprising a compound within the scope of claim 1. Claims 12-21 recite pharmaceutical combinations comprising a compound within the scope of claim 1 and an antidiabetic agent for treating diabetes and/or additional agents for treating related diseases. Claim 22 recites pharmaceutical combinations comprising a compound within the scope of claim 1 and an agent for treating obesity or other related diseases. Claims 23 and 24 were canceled upon reissue.

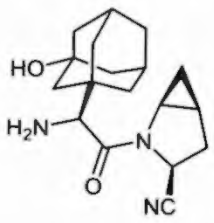
31. Independent claim 25 recites a single compound as follows:



or a pharmaceutically acceptable salt thereof.

The compound of claim 25 also falls within the scope of composition claims 1, 2, 4, 6, 7, 8, and 10. Dependent claim 26 recites the hydrochloride salt of the compound of claim 25. Dependent claims 27 and 28 further recite pharmaceutical compositions of the compound of claim 25. Dependent claims 29-31 recite a combination of the compound of claim 25 and an antidiabetic agent other than a DP-IV inhibitor. Claims 32-43 recite various methods of treatment using the compound of claim 25, alone or in combination with an antidiabetic agent other than a DP-IV inhibitor.

32. The compound of claim 25 is also known as (1S,3S,5S)-2-[(2S)-2-amino-2-(3-hydroxy-1-adamantyl) acetyl]-2-azabicyclo[3.1.0]hexane-3-carbonitrile. For convenience, this compound will be referred to as "saxagliptin" as shown below:



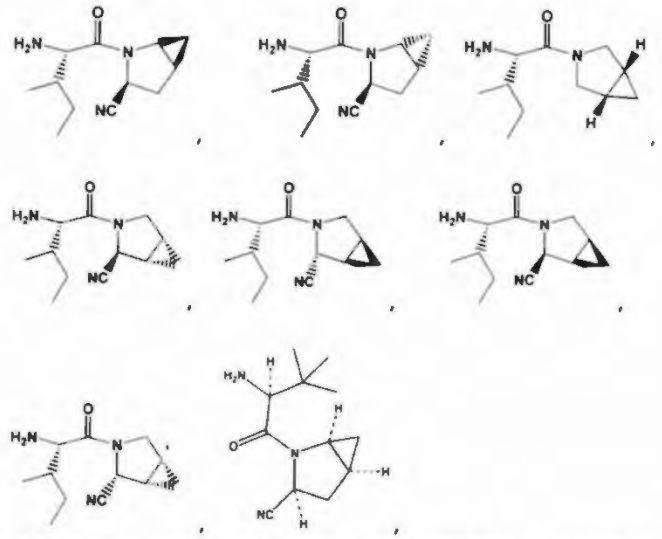
33. In my opinion, and as explained in detail below, the claims of the '186 patent would have been obvious to individuals of ordinary skill in the field prior to and at the time of the earliest possible priority filing date of the '186 patent, i.e., prior to March 10, 2000.

34. Several claim elements of the '186 patent are not described in the'555 application, and instead appear for the first time in the later-filed '173 application. For example, there is no description in the '555 application of: (i) the genus of compounds recited in claim 6; (ii) the specific compounds recited in claim 8 or (iii) the specific compound of claim 25.

35. There is no disclosure in the'555 application of the specific genus of claim 6, in which claim 1 is further limited to:

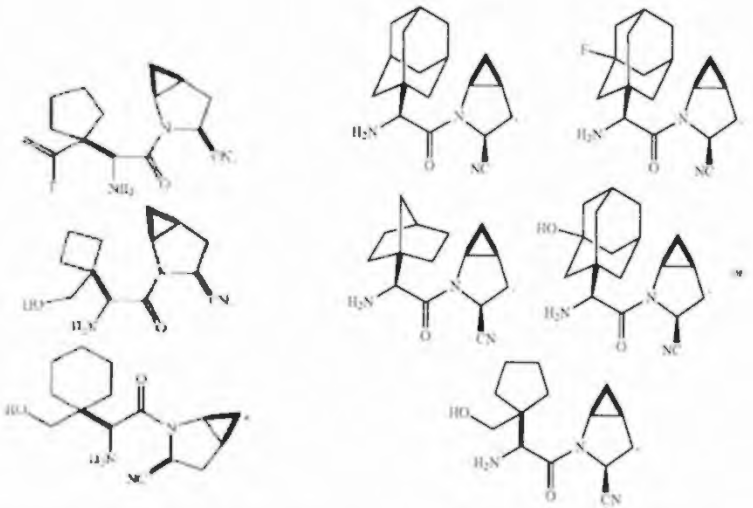
> R^3 is H, R^1 is H, alkyl, cycloalkyl, bicycloalkyl, tricycloalkyl, alkylcycloalkyl, hydroxyalkyl, hydroxyalkylcycloalkyl, hydroxycycloalkyl, hydroxybicycloalkyl, or hydroxytricycloalkyl,
>
> R^2 is H or alkyl, n is 0,
>
> X is CN.

See, e.g., the '555 Application, at p. 6, ll. 10-18; '186 patent, claim 6. Further, the '555 application exemplifies only short, straight and/or branched alkyl substituents at the 2-position as shown below:



See, e.g., *id.* at p. 54, claim 13. Accordingly, one of ordinary skill in the art would have understood that the inventors were not in possession of the genus recited in claim 6 at the time of the '555 application's filing.

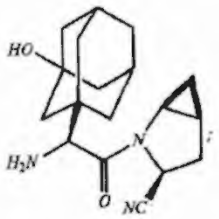
36. Claim 8 of the '186 patent recites a compound as defined in claim 1 having the structure:



or a pharmaceutically acceptable salt thereof. '186 patent at 88:43-89:29.

The only specific compounds described in the '555 application are directed to short, straight and/or branched alkyl substituents at the 2-position. *See*, e.g., the '555 Application at p. 54, claim 13. The compounds of claim 8 were first disclosed in the '173 application. *See*, e.g., U.S. Patent No. 6,395,767 File History, Application filed 2/16/01 at p. 8.

37. Similarly, the compound of claim 25 is also not specifically disclosed in the specification of the '555 application. Independent claim 25 recites the following:



or, a pharmaceutically acceptable salt thereof.

As discussed above, the only specific compounds described in the '555 application are directed to short, straight and/or branched alkyl substituents at the 2-position as depicted in the preceding paragraph. *See*, e.g., the '555 Application at p. 54, claim 13. The compound of claim 25 was first disclosed in the '173 application. *See*, e.g., '767 FH, Application filed 2/16/01 at p. 8. Accordingly, one of ordinary skill in the art could surmise that the inventors were not in possession of the compounds of claims 8 or 25 at the time of the '555 application's filing.

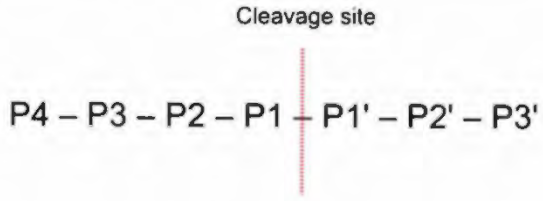
38. As discussed in detail above, several claim elements of the '186 patent are not described in the earliest-filed '555 application, and instead appear for the first time in the later-filed '173 application. Accordingly, one of ordinary skill in the art would have understood that the inventors were not in possession of the claimed subject matter at the time of the '555 application's filing.

IV. RELEVANT TECHNICAL BACKGROUND

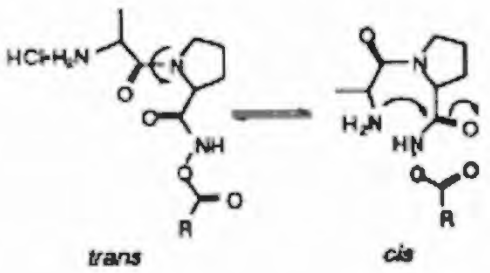
39. Below I describe the details of what was generally known in the art as of March 2000, including: (i) the structure and activity of known DP-IV inhibitors; (ii) principles for improving a compound's drug-likeness and suitability as an orally bioavailable drug and (iii) standard strategies for assessing and modulating compound potency.

40. As set forth above, the Background section of the '186 patent discloses that "inhibitors of dipeptidyl peptidase IV [(DP-IV) are known to] . . . treat[ ] diabetes, especially Type II diabetes." '186 patent at 1:19-21. The Background of the '186 patent also describes what was well known about the mechanism by which DP-IV inhibitors treat type 2 diabetes: "[DP-IV] has been shown to be the primary degrading enzyme of GLP-1(7-36) in vivo . . . [t]hus, inhibition of [DP-IV] in vivo should potentiate endogenous levels of GLP-1(7-36) and . . . thus serve to ameliorate the diabetic condition." *Id.* at 1:59-67.

41. Lin describes what was well known in the art in March 2000 regarding the substrate structural elements required by DP-IV. Lin, et al., "Inhibition of dipeptidyl peptidase IV by fluoroolefin-containing N-peptidyl-O-hydroxylamine peptidomimetics," *Proc. Natl. Acad. Sci. USA*, 95:14020-14024 (1998) at 14020 ("Lin"). For example, Lin reports that "[DP-IV] substrates require the presence of a proline at the P_1 position as well as a protonated free N terminus." *Id.* The P_1, etc. positions are depicted schematically below:



42. Lin also describes what was generally known in the art in March 2000 regarding DP-IV's preferred substrate and inhibitor conformations. For example, Lin reports that "[DP-IV] possesses a high conformational specificity for a trans amide bond between the P1 and N-terminal P2 residues." *Id.* at 14020. Where the P1 residue is the C-terminal residue and the P2 residue is the N-terminal residue (see schematic above for P1 and P2 position designations). The trans and cis conformations of the Lin prolyl dipeptides are depicted below:



Id. at 14022. Lin addresses the importance of the trans conformation for compound stability and its effect on DP-IV inhibition as follows:

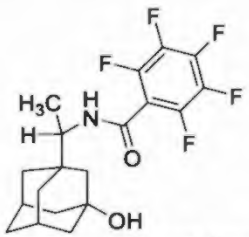
> Many of the problems associated with inefficient inactivation of [DP-IV] are a consequence of the **importance of the trans conformation** of the P_1-P_2 amide bond and the requirement for a protonated free N terminus. **The cyclization reaction of the free N-terminal amino group with the reactive inhibitor . . . require[s] the molecule assume the cis conformation**.

Id. at 14020-14021. Lin reports K_i values of 14,000 nM for a fluoro-olefin dipeptide with the cis conformation and 188 nM for a fluoro-olefin dipeptide isomer with the trans conformation. Comparison of these K_i values for DP-IV inhibition reveals the significantly greater potency of, and preference for, inhibitors in the trans conformation. *See*, e.g., *id.* at 14023 and Table 2.

43. Lin also describes what was well known in the art in March 2000 regarding the effect of conformation on dipeptide stability. Specifically, Lin describes "the cyclization

reaction of the free N-terminal amino acid group with the reactive site of the inhibitor" for related dipeptide compounds (*id.* at 14020-14021), which negatively impacts chemical stability. Lin also observed that intramolecular cyclization was minimized by selecting the trans instead of cis conformation. *Id.* A person of ordinary skill in the art would have also known that having a sterically large substituent at the C2 position would favor a trans conformation and disfavor a cis configuration. *See*, e.g., Pal, D. & P. Chakrabarti, "Cis Peptide Bonds in Proteins: Residues Involved, their Conformations, Interactions and Location," *J. Mol. Biol.*, 294:271-288 (1999), at 274 ("Pal"). Thus from these teachings, a person of ordinary skill in the art would have understood that intramolecular cyclization could be reduced by both selecting against a conformation that favors intramolecular cyclization (i.e., the cis conformation) and through the addition of a large, steric group to the compound, which would also limit the interaction between the free N-terminal amine and the reactive inhibitor.

44. Hoffman, et al., "Pharmacokinetics and Metabolism of Rimantadine Hydrochloride in Mice and Dogs," *Antimicrobial Agents & Chemotherapy*, 32(11):1699-1704 (1988) ("Hoffman"), describes the use of a large, steric adamantyl group in the antiviral drug, rimantadine. As described by Hoffman, the adamantyl moiety was known to be metabolized to a hydroxylated derivative at the 3-position as shown below:



3-hydroxy rimantidine

Hoffman thus serves as further basis that adamantane was generally known in the art to undergo metabolism to yield a 3-hydroxylated adamantyl group.

45. Lipinski, Hansch, Cates, and other references available to one of ordinary skill in the art describe well-known guidelines and strategies for enhancing the drug-like properties of a compound, such as by reducing a compound's partition coefficient, and thus potentially increasing its solubility in aqueous solution. The partition coefficient (P) is a ratio of concentrations of un-ionized compound between two immiscible liquid phases (1-octanol and water), and Log P is a measure of a compound's lipophilicity. For example, addition of a hydroxyl group at the 3-position of an adamantyl moiety would improve the Lipinski parameters, such as reducing Log P and improving water solubility as discussed below.

46. According to Lipinski, et al., "Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings," *Adv. Drug Deliv. Rev.*, 23:3-25 (1996) ("Lipinski"), the use of hydroxyl groups on drugs can increase their water solubility. *See*, e.g., *id.* at 17. Also according to Lipinski, adding a hydroxyl group to a molecule should also reduce the compound's Log P and thus improve its solubility. *See*, e.g., *id.* at 8, 15. These positions are consistent with the knowledge generally available in the art, as evidenced by at least by the Lipinski, Hansch or Cates disclosures.

47. Hansch, et al., "Cluster Analysis and the Design of Congener Sets," *Substituent Constants for Correlation Analysis in Chemistry & Biology*, CH. 6 (John Wiley & Sons 1979) ("Hansch"), describes a process of drug design, in particular molecular modification. *Id.* at 48-49. Hansch describes well-characterized Log P fragment values (Fr) for aliphatic substituents (e.g., hydroxyl = -1.64). *Id.* at 52. Thus according to Hansch, hydroxyl groups have the effect of lowering Log P when added as a compound substituent.

48. Cates, L.A., "Calculation of Drug Solubilities by Pharmacy Students," *Am. J. Pharma. Ed.*, 45:11-13 (1981) ("Cates"), describes a process of approximating whether or not a

drug is soluble in water and how modifications to such a drug will affect the solubility of the drug. *See*, e.g., *id.* at 11-12. Cates discloses, "[t]his procedure involves correlation of partition coefficient values using the octanol/water system and aqueous solubility." *Id.* at Abstract. Referring to partition coefficients based on the octanol/water system expressed as log P, Cates discloses, "[a]lthough this is a measure of the solubility characteristics of the whole molecule, one normally uses the sum of the fragments of the molecule which have been assigned relative hydrophilic-lipophilic values, (π), to calculate log P. Using this procedure, a positive value for π means the substituent, relative to H, favors the octanol phase (i.e., lipophilic). And negative π value indicates its greater affinity for water (*i.e.*, hydrophilic)." *Id.* at 11. Cates also discloses a number of π values that can be used for calculations involving whole molecules, and thus to calculate an approximate log P, which will be indicative of relative solubility. Thus, Cates discloses a method of approximating solubilities, and in particular teaches that addition of hydrophilic substituents increases the solubility of a drug in water. Furthermore, in March 2000, it was also known that an understanding of candidate drug metabolites "can guide structural modifications, thereby improving the activity and/or bioavailability." *See* Korfmacher, et al., "HPLC-API/MS/MS: a powerful tool for integrating drug metabolism into the drug discovery process," *Drug Disc. Today*, 2(12):532-537 (1997) ("Korfmacher"). 3-Hydroxy adamantane is a known metabolite of adamantane. Further, Korfmacher teaches the following advantages of metabolite identification:

> Metabolite identification in drug discovery provides early information that can lead to structural changes in the current lead compound, improving such pharmacokinetic parameters as oral bioavailability, half-life ($t_{1/2}$), or C_{max}. Often these parameters can be changed by improving the metabolic stability of the compound. In order to improve metabolic stability, it is very important to know how a compound is metabolized. The goal of drug discovery is to progress a lead compound into a final candidate drug that can be placed in the development stage. . . **Early metabolite identification can provide information on how to improve**

> **the metabolic stability of the lead structure. In this way, future lead compounds might be a metabolite identified from the previous lead drug or an analog of the previous drug designed to block the major route of metabolism.**

Id. at 534 (emphasis added). Thus according to Korfmacher, one of ordinary skill in the art would have been motivated to make and test a known or potential metabolite when optimizing a lead.

49. As discussed above, the significance of dipeptide conformation on the effective interaction between DP-IV and its substrates and inhibitors was well understood in the art. For example, Lin reported that "[DP-IV] possesses a high conformational specificity for a trans amide bond" and noted "the importance of the trans conformation." Lin at 14020. As would have been understood by one of ordinary skill in the art, the orientation of certain functional groups in the DP-IV inhibitor would be distinct for the cis and trans conformations. *Id.* at 14020-14021.

50. A standard strategy in March 2000 for modulating the orientation of a ring-bound substituent by one of ordinary skill in the art would have been through fusion of the substituent-bearing ring with another ring. *See*, e.g., Chiou, et al., "The Cholinergic Effects and Rates of Hydrolysis of Conformationally Rigid Analogs of Acetylcholine," *J. Pharm. Exp. Ther.*, 166(2):243-248 (1969), at 243 ("Chiou"). Fusion between two rings can result in significant changes in ring flexibility, including ring flattening. *Id.* These changes in turn would have been expected by one of ordinary skill in the art to affect the orientation of ring-bound substituents. *Id.* The smallest ring that can be used for fusion is a cyclopropyl ring. *Id.*

51. Cyclopropyl is one of the most commonly used ring fusion agents because: (i) addition of a cyclopropyl ring has a negligible effect on compound molecular weight, which is an important contributor to better drug-like qualities (*see*, e.g., *id.* at 243); (ii) cyclopropyl has an

exceedingly small footprint, meaning it adds a minimal steric effect on the the compound to which it is attached (*see*, e.g., *id.*) and (iii) cyclopropyl provides greater conformational restriction than larger ring fusions, resulting in fewer conformations in the bicyclic structure (*see*, e.g., *id.*).

52. Chiou explored the fusion of a cyclopropyl ring onto acetylcholine (ACh) in order to produce a compound that was "1) structurally as close to ACh as possible and 2) conformationally as rigid as possible." According to Chiou, cyclopropyl rings "are considered the smallest chemical structure . . . capable of conferring conformational rigidity." *Id.* at 243.

53. As would have been well appreciated by one of ordinary skill in the art in March 2000, an enzyme and its respective substrates and inhibitors typically fit together in a manner analogous to a hand in a glove. *See*, e.g., Koshland, D.E., "The Key-Lock Theory and the Induced Fit Theory, *Angew. Chem. Int. Ed. Engl.*, 33:2375-2378 (1994) at 2377. Closer degrees of matching often result in greater affinity (with respect to a substrate) or greater inhibition (with respect to an inhibitor). *See*, e.g. *id.* at 2376. Thus inhibitor conformation and functional group orientation are important to effective interactions between enzymes and their inhibitors. *See*, e.g., *id.* Like other enzymes, DP-IV inhibition would have been expected to be improved when the active site and inhibitor fit more closely.

V. LEVEL OF ORDINARY SKILL IN THE ART

54. As discussed above, a POSA is a hypothetical person who is presumed to have typical knowledge and experience in the relevant field or fields at the time of the invention. I have been asked to use the time prior to March 10, 2000,[1] as the relevant timeframe for

[1] References herein to "March 2000," may be understood as referring specifically to the time period on or before March 10, 2000.

assessing validity of the '186 patent, and thus to opine on the qualifications of a POSA as of March 10, 2000. I have also been asked to presume that the POSA is aware of all prior art available as of March 10, 2000. To help me ascertain the qualifications of a POSA, I reviewed the '186 patent.

55. By virtue of my education, experience, and training, I am familiar with the level of skill in the field of the '186 patent on or about March 10, 2000.

56. One of ordinary skill in the field would likely have some combination of the following skills and experience: (i) designing target compounds towards drug discovery; (ii) designing and preparing formulations of drugs that exhibit inhibitory activity; (iii) understanding the biological aspects of drug development, including the drug's effect on the whole animal; and (iv) understanding work presented or published by others in the field, including the patents and printed publications discussed in this declaration.

57. It is my opinion that, as of March 10, 2000, a POSA would be a person with an advanced degree (e.g., a Ph.D.) in pharmaceutics, pharmaceutical chemistry, medicinal chemistry or a related field and at least 2-3 years of practical experience in the design of drugs. Alternatively, a POSA could have had less education but considerable professional experience.

58. My understanding of the level of ordinary skill in the art is corroborated by the specification of the '186 patent, which in many instances provides general rather than specific guidance regarding how the invention would be practiced. For example, the '186 patent lacks specific guidance of the various pharmaceutical combinations that it claims. There are no validated or tested dosages for those combinations, nor any examples describing any actual combinations produced by the inventors. Rather than providing specific guidance regarding dosages for the claimed combinations, the '186 patent invites those of ordinary skill in the art to

turn to the knowledge and resources readily available to them when selecting and formulating appropriate combinations of known drugs. For example, rather than providing specific guidance for the combination dosages, the '186 patent provides very broad dosage ranges (*see*, e.g., '186 patent at 4:48-53), which provide essentially no guidance for selecting actual dosages or treatment regimens. The lack of specific guidance provided in the specification reflects the high level of skill in the art.

59. WO Patent App. Pub. No. 98/19998 (published May 14,1998) ("Villhauer") similarly indicates a high level of skill in the art by relying on that skill to select from the many options disclosed in the specification or known to those in the art. *See*, e.g., *id.* at 2-3 (disclosing large and diverse R groups), *id.* at 3, ll. 20-27 (disclosing pharmaceutically acceptable salts and isomers), *id.* at 7, ll. 22 (teaching that "[t]he process of the invention may be effected in conventional manner."), *id.* at 8, ll. 1-10 (disclosing starting materials known or prepared in known or conventional manner) and *id.* at 20 (disclosing pharmaceutically acceptable carriers, adjuvants and modes of administration, and conventional preparation of same). Villhauer thus reflects the conventional approach in the art to prepare promising variants of lead compounds and compare the results.

60. Claims 13-22 of the '186 patent recite various combinations of DP-IV inhibitors and additional therapeutic agents (e.g., other antidiabetic agents, anti-obesity agents, lipid-modulating agents, etc.). At multiple instances, the '186 patent invites those of ordinary skill in the art to select additional agents or mechanisms beyond those disclosed in the specification for use in combination with the claimed DP-IV inhibitors, for example:

> The term "lipid-modulating" agent as employed herein refers to agents which lower LDL and/or raise HDL and/or lower triglycerides and/or lower total cholesterol **and/or other known mechanisms** for therapeutically treating lipid disorders.

'186 patent at 4:43-47 (emphasis added).

> The other antidiabetic agent may also preferably be a sulfonyl urea . . ., **other known sulfonylureas or other antihyperglycemic agents which act on the ATP-dependent channel of the β-cells** . . .

Id. at 15:5-11 (emphasis added).

> The squalene synthetase inhibitors suitable for use herein include, but are not limited to, a-phosphono-sulfonates . . . **as well as other known squalene synthetase inhibitors** . . .

Id. at 17:47-52 (emphasis added).

> Other hypolipidemic agents suitable for use herein include, but are not limited to, fibric acid derivatives . . . **and other known serum cholesterol lowering agents**.

Id. at 18:1-20 (emphasis added).

> The beta 3 adrenergic agonist which may be optionally employed in combination with a compound of formula I may be AJ9677 (Takeda/Dainippon), L750355 (Merck), or CP331648 (Pfizer) **or other known beta 3 agonists** . . .

Id. at 20:12-18 (emphasis added). The '186 patent's repeated reliance on knowledge available outside the specification itself reflects the high level of skill in the art.

61. Furthermore, the '186 patent defers in several instances to resources readily available to those of ordinary skill in the art for determining dosages and other treatment parameters for the claimed combinations, including 15 citations to the Physician's Desk Reference (PDR). *See*, e.g., *id.* at 15:60-61; 16:4-5; 19:3, 35; 20:43, 50, 57, and 67; 21:9, 15, 24, 41, 47, and 54. For example, the '186 patent states that "[t]he amounts and dosages employed will be as indicated in the Physician's Desk Reference . . ." *id.* at 19:2-4. I agree that the PDR is a resource often used in the art for established dosing and treatment regimens for FDA approved drugs.

62. The '186 patent provides a reasonable expectation of success for practicing the claimed invention to the extent it is enabled by the specification. The background section of the

'186 patent defines the mechanism by which DP-IV inhibitors treat type 2 diabetes, for example, by providing that "[DP-IV] has been shown to be the primary degrading enzyme of GLP-1(7-36) in vivo . . . [t]hus, inhibition of [DP-IV] in vivo should potentiate endogenous levels of GLP-1(7-36) and . . . thus serve to ameliorate the diabetic condition." *Id.* at 1:59-67. Villhauer provides evidence for what was generally known in the art by teaching that because "GLP-1 is a major stimulator of pancreatic insulin secretion and has direct beneficial effects on glucose disposal, [DP-IV] inhibition . . . represent[s] an attractive approach for treating non-insulin-dependent diabetes mellitus (NIDDM)." Villhauer at 1. In addition, the '186 patent teaches that the claimed DP-IV inhibitors can be used "for treating diabetes, especially Type II diabetes." '186 patent at 3:53-4:1. Given the high level of skill in the art and the advanced knowledge in the art regarding the activity of DP-IV inhibitors, one of ordinary skill in the art would have had a reasonable expectation for treating diabetes to the same extent the claims of the '186 are enabled by the specification.

VI. OVERVIEW OF PRIOR ART REFERENCES

63. I have reviewed several references that I believe teach or suggest the compounds, pharmaceutical compositions, pharmaceutical combinations, and methods recited in the asserted claims of the '186 patent. By the virtue of their publication dates, I understand that these references, described in more detail below, are considered prior art to the '186 patent.

A. Ashworth, et al., "2-cyanopyrrolidides as potent, stable inhibitors of dipeptidyl peptidase IV," *Bioorg. & Med. Chem. Ltrs.*, 6(10):1163-166 (1996) ("Ashworth")

64. Ashworth shows a publication date of May 21, 1996. I understand that Ashworth can be applied in assessing the validity of the '186 patent claims.

65. Ashworth discloses a series of stable, potent inhibitors of dipeptidyl peptidase IV (DP-IV), which incorporate a 2-cyanopyrrolidide moiety into the structure of the inhibitor. Ashworth describes DP-IV inhibitors as having the following activity:

> [DP-IV] is a serine protease which catalyses the cleavage of dipeptides from the N-terminus of proteins with the sequence H-X-Pro-Y or H-X-Ala-Y (where X, Y= any amino acid, Y ≠ Pro).

Id. at 1163.

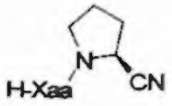
66. DP-IV inhibitors were known to be useful for the treatment of conditions such as type 2 diabetes. *See*, e.g., Villhauer at Abstract.

67. DP-IV inhibitors are typically evaluated and optimized for a variety of properties including their stability and potency. *See*, e.g., Ashworth at Table II. Stability is a sum of chemical and metabolic stability, both of which ultimately impact the quantity of active inhibitor available for interaction with an enzyme target. Chemical stability of DPP IV inhibitors is assessed in solution using a well known set of conditions (see e.g. Ashworth I) and includes solid state stability as required by FDA in accordance with ICH guidelines. *See*, e.g., Ariëns, E.J. & A. M. Simonis, "Optimalization of Pharmacokinetics – An Essential Aspect of Drug Development – by 'Metabolic Stabilization'," *Strategy in Drug Research*, pp. 165-178 (Elsevier Sci. Pub. Co. 1982), at 173-178 ("Ariens"); Dept. of Health and Human Services, "Int'l Conf. on Harmonisation; Stability Testing of New Drug Substances and Products; Guideline; Availability," *Federal Register*, 59:48754-48759 (Sept. 22, 1994) ("FDA Stability Guidelines"). Inhibitor metabolic stability is generally measured in terms of an inhibitor's half-life ($t_{1/2}$), with longer half-lives representing greater stability. *See*, e.g., Obach, R.S., "Prediction of Human Clearance of Twenty-nine Drugs from Hepatic Microsomal Intrinsic Clearance Data: An Examination of In Vitro Half-life Approach and Nonspecific Binding to Microsomes," *Drug*

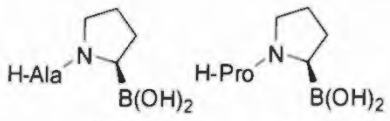
Metab. Dispos., 27(11):1350-1359 (1999), at 1354-1355 ("Obach"). Potency reflects the degree to which an inhibitor acts on its target. Inhibitor potency is generally measured in terms of its dissociation constant (K_i) or using an IC_{50} concentration (i.e., a concentration of a potential inhibitor at which enzyme activity is reduced by 50% from control), which represents the propensity of dissociation between an inhibitor and its target, with smaller K_i or IC_{50} values representing greater potency. Cheng, Y. & W.H. Prusoff, "Relationship Between the Inhibition Constant (K_I) and the Concentration of Inhibitor which Causes 50 Per Cent Inhibition (I_{50}) of an Enzymatic Reaction," *Biochem. Pharmacol.*, 22:3099-3108 (1973), at 3099 ("Cheng").

68. Some of the compounds described in Ashworth have K_i values of less than 5 nM versus human DP-IV and chemical stability half-lives of greater than 48 hours in aqueous solution at pH 7.4. Ashworth at 1163. Thus some of the compounds of Ashworth have both high potency and good chemical stability in solution, making them desirable drug leads.

69. Ashworth discloses 29 compounds, six of which contain the 2-cyanopyrrolidide moiety, as follows:



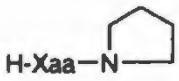
where Xaa represents various amino acid substituents as specified in Table II. *Id.* at 1166. Ashworth discloses that the most potent DP-IV inhibitors previously known were the boroproline analogues **1**, and **2**,



1 **2**

which had K_i values of 2 nM and 3 nM respectively. *Id.* at 1163. Ashworth concludes that the 2-cyanopyrrolidide analogues "possessed activity comparable to the boroprolines, 1 and 2." *Id.* at 1165. In addition, Ashworth teaches that the 2-cyanopyrrolidide analogues exhibited "superior stability in aqueous solution" relative to boroprolines **1** and **2**. *Id.* at 1164.

70. Ashworth discloses the DP-IV inhibition activity of 19 aminoacyl pyrrolidides (lacking the 2-cyano moiety) in Table I:

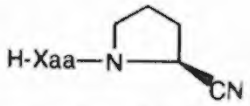


Compound N°	Xaa	K_i (μM)[13]
5	Cyclohexylglycine [Chg]	0.064 ± 0.01
6	(R,S)-Cyclopentylglycine [Cpg]	0.21 ± 0.04
7	Ile	0.41 ± 0.01
8	*allo*-Ile	0.44 ± 0.04
9	Val	0.47 ± 0.02
10	Lys(Cbz)	0.52 ± 0.07
11	tert-Butylglycine [Tbg]	0.88 ± 0.20
12	Thr(Me)	0.90 ± 0.15
13	Orn(Cbz)	0.91 ± 0.20
14	2-Aminohexanoic acid [Aha]	1.20 ± 0.20
15	Glu	2.00 ± 0.40
16	Pro	2.10 ± 0.20
17	Cyclohexylalanine [Cha]	2.15 ± 0.50
18	Glu(OBn)	2.70 ± 0.30
19	Thr	4.90 ± 0.90
20	Phenylglycine [Phg]	5.30 ± 0.10
21	Ser(Bn)	6.00 ± 1.50
22	Ala	7.00 ± 1.00
23	Asp	14.50 ± 1.90

Id. at 1164. The compounds of Table I exhibit inhibition activity in the 0.064 μM to 14.50 μM range. Ashworth notes that the most active of these compounds was compound **5**, "[i]n particular, β-branched α-amino acid derivatives were the most potent compounds with the non-

proteinogenic amino acid, **(S)-cyclohexylglycine providing the most active pyrrolidide** (compound **5** possessing a K_i value of 64 nM)."[2] *Id.* at 1165 (emphasis added).

71. By comparison, Table II of Ashworth discloses the DP-IV inhibition activity of six analogues having the 2-cyano moiety:



Compound N°	Xaa	K_i (nM)[13]	$t_{1/2}$ (h)[19]
24	Cpg	1.1 ± 0.2	48
25	Chg	1.4 ± 0.5	>48
26	Ile	2.2 ± 0.5	48
27	Tbg	3.8 ± 0.8	>48
28	Lys(Z)	5.2 ± 1.0	24
29	Pro	22.0 ± 4.0	7.5

Id. at 1166. The compounds of Table II (having a 2-cyanopyrrolidide moiety) exhibit inhibition activity in the 1.1 nM to 22 nM range, which in some cases equates to a 50-fold increase in inhibition activity relative to compound **5**, which lacks the 2-cyano moiety. This clearly shows that the cyano moiety is engaged in an interaction with DP-IV that improves affinity of a potential inhibitor with the enzyme.

72. Ashworth teaches that, "[s]ubstrates and inhibitors of DP-IV require a free N-terminus, which means that potential dipeptide serine protease inhibitors (e.g., C-terminal aldehydes, boronic acids, a-ketoacids, trifluoromethylketones, or chloromethylketones) are inherently unstable at neutral pH due to intramolecular cyclisation" likely via a cis conformation of the dipeptide. *Id.* at 1163.

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[2] The value of 0.064 μM, as given for Ashworth compound **5** in Table I, can alternatively be written as 64 nM; both these notations refer to the same amount, and should be understood to be interchangeable in both Ashworth and my report.

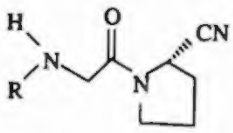
73. Table II of Ashworth shows the stability data of DP-IV inhibitors in aqueous solution (pH of 7.4), as measured in half-life ($t_{1/2}$). *Id.* at 1166. When changing the substituent at the 2-position of the acetyl-pyrrolidine-2-carbonitrile from a straight chain alkyl moiety, for example compound **28** (lysine moiety), to a moiety having at least one branch at the α-carbon, such as the cyclohexyl moiety in Ashworth compound **25**, the stability increases from 24 hours to greater than 48 hours. *Id.* at 1166. Ashworth discloses that substitution at this position improves the potency of an aminoacyl pyrrolidide compound as shown in Table I of Ashworth. *Id.* at 1163 and 1166 (compare Tables I and II).

74. Specifically, compound **5** (having a cyclohexyl group) of Table I shows at least a 100-fold increase in potency compared to compound **7** (having a 1-methyl propyl group) or compound **9** (having an isopropyl group). Ashworth confirms this observation by noting, "[a]s expected, from the substrate specificity of DP-IV, only (S)-amino acid derivatives showed any activity and, as can be seen in Table I, lipophilic amino acids gave more potent compounds. In particular, β-branched α-amino acid derivatives were the most potent compounds with the non-proteinogenic amino acid, (S)-cyclohexylglycine providing the most active pyrrolidide (compound **5** possessing a K_i value of 64 nM)." *Id.* at 1165. Thus the combined properties of potency and solution stability make compound **25** an attractive lead that can be further improved.

B. WO Patent App. Pub. No. 98/19998 ("Villhauer")

75. Villhauer was published May 14, 1998. Thus, I understand that Villhauer can be applied in assessing the validity of the '186 patent claims.

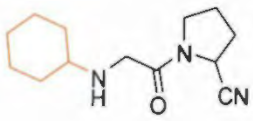
76. Villhauer discloses numerous DP-IV serine protease inhibitor compounds. The dipeptide compounds of Villhauer are N-(N'-substituted glycyl)-2-cyanopyrrolidines having the following structure of formula (I):



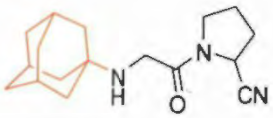
(I)

Id. at Abstract.

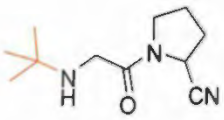
77. Villhauer discloses various substituents at position "R" of formula (I) above. For example, Villhauer discloses compounds having cycloalkyl groups attached to the amino moiety of 2-cyano pyrrolidides, including a cyclohexyl group:



(*id.* at 12, Example No. 28); adamantyl groups attached to the amino moiety of 2-cyano pyrrolidines:



(*id.* at 13, Example No. 47); and alkyl groups attached to the amino moiety of 2-cyano pyrrolidides:



(*id.* at 13, Example No. 52). Villhauer also teaches how the compounds shown directly above were prepared. *Id.* at 8-10. Each of the variations cited above are considered bulky lipophilic groups.

78. Villhauer discloses the use of DP-IV inhibitors, such as N-(N'-substituted glycyl)-2-cyanopyrrolidines, for "the treatment of conditions mediated by DPP-IV, such as non-insulin-

dependent diabetes melitus." *Id.* at Abstract. As disclosed in Villhauer, "DPP-IV is responsible for inactivating glucagon-like peptide-1 (GLP-1) . . . [and] [s]ince GLP-1 is a major stimulator of pancreatic insulin secretion and has direct beneficial effects on glucose disposal, DPP-IV inhibition [is] an attractive approach for treating non-insulin-dependent diabetes mellitus (NIDDM)." *Id.* at 1, ll. 6-13.

79. Villhauer discloses that the DP-IV inhibitor compound may be in free form or in acid addition salt form, where the salt could be from any pharmaceutically acceptable acid, with hydrochloride as a preferred option. *Id.* at 3. Similarly, Villhauer discloses that any pharmaceutically acceptable carriers, adjuvants and enteral or parenteral administration forms (prepared by conventional means) could be used with any of the disclosed agents of the invention. *Id.* at 20.

80. Villhauer further discloses the use of DP-IV inhibitors, such as N-(N'-substituted glycyl)-2-cyanopyrrolidines, for "use in **treating conditions mediated by DPP-IV** [such as] **non-insulin-dependent diabetes mellitus** . . . [and] **obesity** . . ." *Id.* at 18, ll. 18-21 (emphasis in original).

81. In an effort to modify Ashworth compound **25**, a POSA would be motivated to investigate the observations of Villhauer to explore additional lipophilic substituents at C2 of compound **25**, including an adamantyl moiety and other β-branched amino acids. This optimization would include exploration of solid state stability as required by FDA regulations.

C. Raag, R. & T.L. Poulos, "Crystal Structure of Cytochrome P-450$_{CAM}$ Complexed with Camphane, Thiocamphor, and Adamantane: Factors Controlling P-450 Substrate Hydroxylation," *Biochem.*, 30:2674-2684 (1991) ("Raag")

82. Raag shows a publication date of March 1, 1991. Thus, I understand that Raag can be applied in assessing the validity of the '186 patent claims.

83. Raag describes a study of the metabolism of adamantane by cytochrome P-450$_{cAm}$ and corresponding X-ray crystal structures for complexes of cytochrome P450$_{cAm}$ with adamantane as the substrate. Raag describes the metabolic hydroxylation of adamantane, as well as other substrates. Table III from Raag, presented below, shows the various molecules that underwent hydroxylation, where the adamantane group is shown in the third column:

Table III. Various Substrate-Dependent Parameters[a]

	camphor	adamantanone	adamantane	thiocamphor	camphor Y96F	norcamphor	camphane
molec vol	315 Å^3	300 Å^3	293 Å^3	322 Å^3	315 Å^3	236 Å^3	309 Å^3
hydrogen bond to Y96	yes[a]	yes[b]	no	no	no	yes[a]	no
no. of iron ligands	5[a]	5[b]	6	6		6[d]	6
redox pot. Fe^{3+}/Fe^{2+}	−170 mV[c]	−175 mV[c]				−206 mV[c]	
high-spin %	94–97%[c–e]	96–98%[c,d]	99%[d]	65%[e]	59%[e]	46%[e]	46%[e]
regiospecif of substr hydroxylatn	5-exo (100%)[d–f]	5 (100%)[d]	1 (100%)[d]	5-exo (64%)[e] 6-exo (34%) 3-exo (2%)	5-exo (92%)[e,f] 4 (1%) 6-exo (2–4%) 3-exo (0–4%) 9 (<1%)	5-exo (45%)[f] 6-exo (47%) 3-exo (8%)	5-exo (90%)[e] 6-exo (10%)
substr temp factor (Fe^{3+})	16.2 Å^2[a]	16.5 Å^2[b]	24.7 Å^2	23.5 Å^2		33.5 Å^2[b]	30.1 Å^2
substr hydrophilic groups	yes	yes	no	yes	yes	yes	no
hydroxylatn "efficiency"	100%[c,f]			98%[e]	100%[e]	12%[f]	8%[e]
L6–substr dist	NA	NA	2.63 Å	2.35 Å (70%) 3.35 Å (30%)		3.0 Å[b]	2.88 Å
L6–iron dist	NA	NA	1.95 Å	1.35 Å		1.73 Å[b]	1.67 Å
L6 occupancy	NA	NA	1.00	0.90		0.97[b]	1.00
L6 temp factor	NA	NA	14.3 Å^2	19.6 Å^2		3.8 Å^2[b]	7.7 Å^2
cation occupancy	1.00[a]	1.00[b]	0.89	0.91		1.00[b]	0.72
cation temp factor	12.1 Å^2[a]	10.0 Å^2[b]	15.5 Å^2	14.2 Å^2		7.9 Å^2[b]	21.7 Å^2

[a] Poulos et al. (1985, 1987). [b] Raag and Poulos (1989a). [c] Fisher and Sligar (1985). [d] White et al. (1984). [e] Atkins and Sligar (1988b). [f] Atkins and Sligar (1989). [g] Carbon numbering for each substrate begins with C-1 at the top of the six-membered ring which is in the plane of the table. Numbering proceeds counterclockwise such that the carbonyl carbon is C-2 and C-5 is in the lower right-hand portion of the ring. Note that C-5 is a secondary carbon in some substances and a tertiary carbon in others.

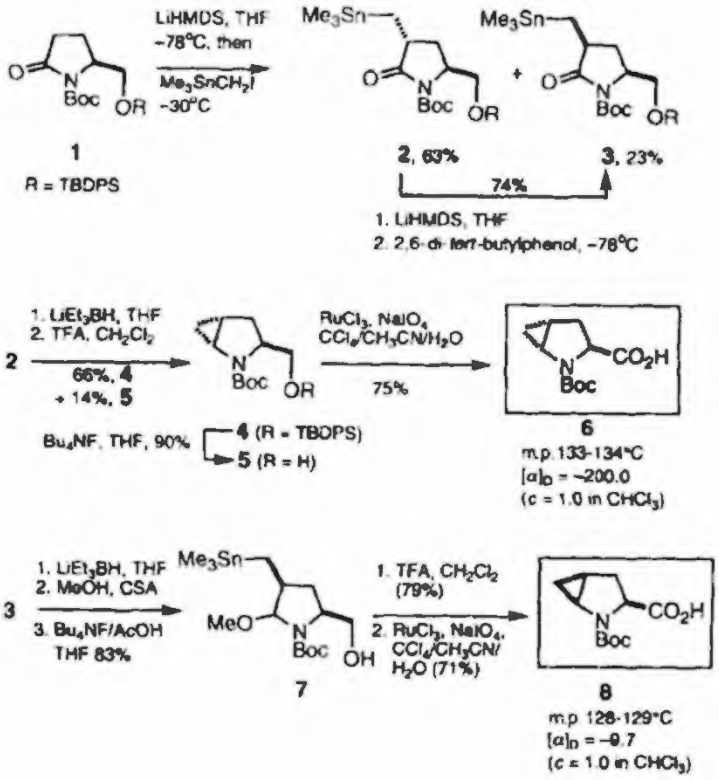
84. Raag, Table III, shows that substrates that undergo regiospecific hydroxylation of adamantane by cytochrome P450 (a xenobiotic metabolizing enzyme). Hydroxylation occurs at a specific position, i.e., at a tertiary carbon site (and not a secondary carbon site). Raag also teaches that "[a]damantane is . . . metabolized to a single product despite having a relatively high active site mobility. The single product can be attributed to the existence of only two types of unique carbon atoms in adamantane, together with the greater reactivity of tertiary versus secondary carbons." *Id.* at 2678.

85. With this knowledge, a POSA would be motivated to investigate an hydroxyl-adamantyl derivative of Ashworth compound **25** to improve water solubility and metabolic stability of a lead molecule. *See* Lipinski at 8, 15; Ariens.

D. Hanessian, et al., "The Synthesis of Enantiopure ω-Methanoprolines and ω-Methanopipecolic Acids by a Novel Cyclopropanation Reaction: The 'Flattening' of Proline," *Angew. Chem. Int. Ed. Engl.*, 36(17):1881-1884 (1997) ("Hanessian")

86. Hanessian shows a publication date of September 17, 1997. Thus, I understand that Hanessian can be applied in assessing the validity of the '186 patent claims.

87. Hanessian describes the synthesis of enantiopure ω-methanoproline acids by a cyclopropanation reaction. Hanessian teaches that the use of conformationally constrained analogues of proline were well known in peptidomimetic research at the time the application which led to the '186 patent was filed. Hanessian describes the "highly stereocontrolled syntheses of the diastereomeric 4,5-methano-L-prolines and 5,6-methano-L-pipecolic acids by a novel intramolecular cyclopropanation reaction of iminium ions . . . ." *Id.* at 1882. One such example shown by Hanessian is compound **8** which was synthesized according to Scheme 1 below:



Id.

88. The structure and conformation of compound 8 in the solid state were unambiguously confirmed by single crystal X-ray analysis. Further, Table 1 of Hanessian, as reproduced below, shows selected torsion angles for compound 8 where "considerable 'flattening' of the pyrrolidine ring is observed relative to N-Boc-L-proline." *Id.* Thus, Hanessian provides evidence for adjusting the orientation of substituents in the pyrrolidine ring resulting from cyclopropane fusion. Further, a POSA would recognize that cyclopropane fusion to a pyrrolidine ring would result in a ring system with limited conformational flexibility compared to a simple pyrrolidine ring.

Table 1. Selected torsion angles and root-mean-square deviations from fitted atoms in a given plane of X-ray crystal structures, and ^{13}C NMR chemical shifts ($CDCl_3$).

6

8

	N-Boc-L-proline [20]	6	8
τ(NC$_\alpha$)	−17	−5.6	−14.4
τ(C$_\alpha$C$_\beta$)	+31	+4.8	+15.3
τ(C$_\beta$C$_\gamma$)	−35	−2.6	−11.4
τ(C$_\gamma$C$_\delta$)	+24	−0.7	+2.9
τ(C$_\delta$N)	−4	+4.1	+7.6
τ(BocNC$_\alpha$CO$_2$H)	−72	−64.0	−67.1
rms deviation of fitted atoms	0.018 C$_\alpha$, N, C$_\delta$, C$_\gamma$	0.003 C$_\beta$, C$_\gamma$, C$_\delta$, N	0.013 C$_\beta$, C$_\gamma$, C$_\delta$, N
	δ(cis/trans)	δ(cis/trans)	δ(cis/trans)
COOH	178.35/176.60	177.7/175.5	179.1/176.1
NC=O	153.95/155.39	157.1/154	155.7/154.1
C$_\alpha$	58.8	60.8/60.1	59.5/59.1
C$_\beta$	30.75/29.53	32.0	31.5/29.1

Id.

89. Hanessian confirms the "flattening" of the pyrrolidine ring by determining the root-mean-square values for the C_β and N atoms from the plane defined by C_β, C_γ, C_δ and N (*see* Table 1 above). *Id.* Hanessian determined that for compound **8**, the out-of-plane carbon was the carbon bearing the carboxyl group (C_α) when compared to *N*-Boc proline. *Id.* Hanessian thus observed that by flattening the pyrrolidine ring, the orientation of the out-of-plane carbon was modified with respect to the ring.

90. A POSA would recognize that a substituent bonded at this C_α carbon would be expected to have its orientation in space modified compared to proline and because of the limited conformational flexibility of this cyclopropyl-fused pyrrolidine ring.

E. WO Patent App. Pub. No. 99/38501 ("Bachovchin")

91. Bachovchin was published August 5, 1999. Thus, I understand that Bachovchin can be applied in assessing the validity of the '186 patent claims.

92. Bachovchin discloses improved methods for reducing in animal subjects (including humans) at least one type of insulin resistance, hyperinsulinemia, and Type II diabetes. In particular, Bachovchin describes the use of DP-IV inhibitors in an amount effective to treat, among other indications, Type II diabetes. Claim 4 of Bachovchin recites, "[a] method for treating Type II diabetes, comprising administering to an animal a composition including one or more inhibitors of dipeptidylpeptidase IV [(DP-IV)]." *Id.* at claim 4. Moreover, various examples of DP-IV inhibitors are listed in Bachovchin. For example, a class of DP-IV inhibitors shown in Bachovchin are compounds based on the dipeptide (D)-Ala-(L)-Ala. Bachovchin also discloses the use of pharmaceutical compositions of DP-IV inhibitors, "another aspect of the present invention relates to pharmaceutical compositions of dipeptidylpeptidase inhibitors, particularly [DP-IV] inhibitors." *Id.* at 7, ll. 29-30. Thus, the use of DP-IV inhibitors for treating type 2 diabetes was well known at least one year before the application which led to the '186 patent was filed.

93. Bachovchin also discloses that "[i]n particular, it is an object of the invention to provide methods for producing long lasting beneficial changes . . . to provide effective treatments for diabetes [and] obesity . . ." *Id.* at 4, ll. 7-11. Bachovchin also discloses "administration of a [DP-IV] inhibitor . . . in an amount effective to improve one or more aberrant indices associated with glucose metabolism disorders . . . [such as] Type II diabetes . . . [and] obesity." *Id.* at 6, ll. 16-22.

94. Furthermore, the use of DP-IV inhibitors in combination therapy with various antidiabetic agents other than a DP-IV inhibitor for treating type 2 diabetes is also described extensively in Bachovchin:

> The [DP-IV] inhibitors used according to the invention can also be used conjointly with agents acting on the ATP-dependent potassium channel of the β-cells, such as glibenclamide, **glipizide**, **gliclazide** and AG-EE 623 ZW. The DPIV inhibitors may also advantageously be applied in combination with other oral agents such as **metformin** and related compounds or glucosidase inhibitors as, for example, **acarbose**.

Id. at 46 (emphasis added.)

F. XENICAL Label (available by FOIA Aug. 9, 1999) ("the Xenical Label")

95. The Xenical Label was published August 9, 1999 following FDA approval of Xenical. Thus, I understand that the Xenical Label can be applied in assessing the validity of the '186 patent claims.

96. The Xenical Label discloses the FDA-approved uses of the anti-obesity agent, Xenical (i.e., orlistat), and supporting studies. In one such study, patients with type 2 diabetes were treated with Xenical. As indicated in the Xenical Label, "XENICAL is indicated for obesity management . . . in the presence of other risk factors (e.g., . . . diabetes)." *Id.* at Label, p. 8. According to the Xenical Label, "epidemiological studies have established a relationship between obesity and visceral fat and the risks for cardiovascular disease [and] type 2 diabetes." *Id.* at p. 3. The Xenical Label also provides dosages for Xenical as follows: "[t]he recommended dose of XENICAL is one 120 mg capsule three times a day." *Id.* at p. 14.

G. MEVACOR Label (available by FOIA Sept. 15, 1994) ("the Mevacor Label")

97. The Mevacor Label was published September 13, 1994 following FDA approval of Mevacor. Thus, I understand that the Mevacor Label can be applied in assessing the validity of the '186 patent claims.

98. The Mevacor Label discloses the FDA-approved uses of the lipid-modulating agent, Mevacor (i.e., lovastatin), and supporting studies. As indicated in the Mevacor label, "poorly controlled diabetes mellitus" was considered a "secondary cause[ ] for hypercholesterolemia . . ." *Id.* at Package Insert, p. 4. The Mevacor Label also provides dosages for Mevacor as follows: "[t]he recommended dosing range is 20-80 mg/day in a single or two divided doses; the maximum recommended dose is 80 mg/day." *Id.* at p. 8.

H. GLUCOPHAGE Label (available by FOIA Jan. 8, 1998) ("the Glucophage Label")

99. The Glucophage Label was published January 8, 1998 following FDA approval of Glucophage. Thus, I understand that the Glucophage Label can be applied in assessing the validity of the '186 patent claims.

100. The Glucophage Label discloses the FDA-approved uses of the antihyperglycemic agent, Glucophage (i.e., metformin), and supporting studies. The Glucophage label describes a "29-week double-blind, placebo-controlled study of GLUCOPHAGE and glyburide, alone and in combination . . . in obese patients with type 2 diabetes," where glyburide is also an antihyperglycemic agent for use in treating patients with type 2 diabetes. *Id.* at Package Insert, p. 4. The Glucophage Label also provides dosages for Glucophage as follows: "[i]n general, elinically significant responses are not seen at doses below 1500 mg per day . . . [and] GLUCOPHAGE may be given to a maximum daily dose of 2550 mg per day." *Id.* at p. 35.

VII. THE ASSERTED CLAIMS OF THE '186 PATENT ARE OBVIOUS OVER THE PRIOR ART

101. As explained in detail below, it is my opinion that each element of claims 8, 9, 25, and 26 is taught in the combination of Ashworth, Villhauer, Raag, and Hanessian, and that one of

ordinary skill in the art would have been motivated to combine these teachings by March 10, 2000.

102. Each of claims 8, 9, 25, and 26 is presented below in bold text followed by my analysis of the claims. The analysis below identifies exemplary disclosure of the cited references with respect to the corresponding claim elements, and is not meant to be exclusive or exhaustive.

103. Claims 8 and 25 of the '186 patent each disclose a specific chemical compound or a pharmaceutically acceptable salt of that compound, while claims 9 and 26 depend from these respective claims, further specifying one or more particular pharmaceutically acceptable salts. Therefore, claims 8 and 25 will be addressed first, followed by claims 9 and 26.

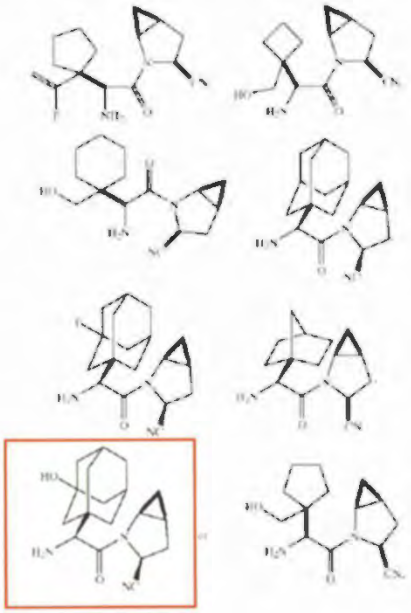
104. As discussed above, Ashworth, Villhauer, Raag, and Hanessian can be considered prior art to the '186 patent.

105. Where reference is made to additional publications below, those publications are intended to provide additional bases regarding what was generally known in the art in March 2000 and provide factual basis for my statements. Those references are not intended to serve as additional grounds for invalidity.

A. Each Feature of Claims 8 and 25 is Disclosed by Ashworth, Villhauer, Raag, and Hanessian

Claim 8:

"A compound having the structure:

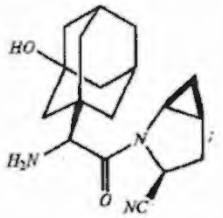


or a pharmaceutically acceptable salt thereof."

The red box outline is provided for illustration purposes and does not appear in the original claim, but the structure shown therein is equivalent to the compound of claim 25.

Claim 25:

"A compound that is

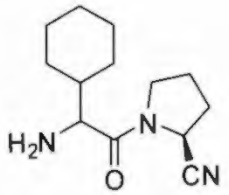


or a pharmaceutically acceptable salt thereof."

106. As will be discussed in greater detail below, the prior art provides specific motivation to select Ashworth compound 25 as a lead compound for further optimization. One of ordinary skill in the art would understand that certain core features of Ashworth compound 25 should be retained in the ultimate compound, including the glycyl-proline core, free amine, and cyano components of Ashworth compound 25. As explained below, one of ordinary skill also would have been motivated to favor a structure in which the trans orientation of the dipeptide in order to enhance its activity and chemical stability. *See* Lin; FDA Stability Guidelines. There was also motivation to substitute the cyclohexyl group of Ashworth compound **25** with a hydroxylated adamantyl group in order to improve the compound's chemical and metabolic stability. *See* Ashworth; Raag; Villhauer. One would have further been motivated to fuse a cyclopropyl ring onto the pyrrolidine ring of Ashworth compound 25 as a means to improve the interaction between the active 2-cyano moiety with the DP-IV enzyme. *See* Hanessian and Ashworth. Thus, as evidenced by prior art references (including Ashworth, Villhauer, Raag and Hanessian), claims 8 and 25 of the '186 patent would be considered obvious to one of ordinary skill in the art in view of the knowledge and motivation provided by the references to the skilled artisan.

1. A POSA Would Have Been Motivated to Select Ashworth's Compound 25 as a Lead Compound

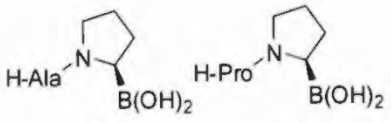
107. The prior art provided ample motivation for one of skill in the art to select the Ashworth's compound **25** as a lead compound to develop a composition as an inhibitor of DP-IV enzyme, as claimed in claims 8 and 25 of the '186 patent.



Ashworth Compound **25**

108. Ashworth discloses potent dipeptide analogues incorporating a 2-cyanopyrrolidide moiety useful as inhibitors of dipeptidyl peptidase IV. Ashworth at 1163 (disclosing, "[a] novel series of stable, potent inhibitors of [DP-IV] has been developed. A number of dipeptide analogues, incorporating a 2-cyanopyrrolidide, were found to have K_i values of less than 5 nM versus human DP-IV and half-lives of >48h in aqueous solution . . . ."). Because Ashworth explicitly discloses the use of the 2-cyanopyrrolidide compounds as inhibitors of DP-IV, a person of skill in the art had express motivation to select the disclosed compounds with a reasonable expectation of success for developing improved compositions useful as inhibitors of DP-IV.

109. Further, Ashworth provides motivation to select the 2-cyanopyrrolidine analogues. For example, Ashworth teaches that the 2-cyanopyrrolidide analogues "possessed activity comparable to the boroprolines, 1 and 2" which were previously known as the most potent DP-IV inhibitors as shown below:



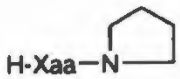
1 **2**

Id. at 1165. The boroprolines **1** and **2** had K_i values of 2 nM and 3 nM respectively. A POSA would recognize a molecule such as Ashworth compound **25**, with molecular weight less than 400 daltons and a K_i value less than 3 nM as a potential lead compound. *Id.* at 1163. Because

the Ashworth reference teaches that 2-cyanopyrrolidide analogues have activity comparable to the boroproline analogues, one of ordinary skill in the art had express motivation to select the 2-cyanopyrrolidide analogues as lead compounds to arrive at the compound in claims 8 and 25 of the '186 patent with a reasonable expectation of success. Furthermore, one of ordinary skill in the art would select the 2-cyanopyrrolidide analogues as lead compounds because they were more synthetically accessible than the boroprolines and metabolic considerations (boronic acid vs. cyano group) would make them more desirable. *Id.*

110. Further, Ashworth provided ample motivation to specifically select compound **25** as a lead from the limited compounds described therein (*see* below, *id.* at 1166, Table II) because (i) compound **25** is one of only six 2-cyanopyrrolidide analogues (i.e., dipeptide nitriles) described by Ashworth (*see id.*, Table II, listing only six compounds); and (ii) compound **25**, which contains a 2-cyano moiety, exhibits high potency compared to aminoacyl pyrrolidides lacking the 2-cyano moiety (*id.* at 1164 and 1166, compare Tables I and II) and good solution stability compared to other analogs lacking a β-branched moiety at C2. Structures of this type with sterically large groups at C2 prefer to exist in a trans conformation. *See*, e.g., Pal at 274.

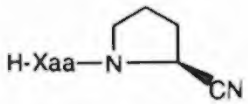
111. For example, Ashworth discloses the DP-IV inhibition activity of 19 aminoacyl pyrrolidides (lacking the 2-cyano moiety) in Table I:



Compound N°	Xaa	K_i (μM)[13]
5	Cyclohexylglycine [Chg]	0.064 ± 0.01
6	(R,S)-Cyclopentylglycine [Cpg]	0.21 ± 0.04
7	Ile	0.41 ± 0.01
8	*allo*-Ile	0.44 ± 0.04
9	Val	0.47 ± 0.02
10	Lys(Cbz)	0.52 ± 0.07
11	tert-Butylglycine [Tbg]	0.88 ± 0.20
12	Thr(Me)	0.90 ± 0.15
13	Orn(Cbz)	0.91 ± 0.20
14	2-Aminohexanoic acid [Aha]	1.20 ± 0.20
15	Glu	2.00 ± 0.40
16	Pro	2.10 ± 0.20
17	Cyclohexylalanine [Cha]	2.15 ± 0.50
18	Glu(OBn)	2.70 ± 0.30
19	Thr	4.90 ± 0.90
20	Phenylglycine [Phg]	5.30 ± 0.10
21	Ser(Bn)	6.00 ± 1.50
22	Ala	7.00 ± 1.00
23	Asp	14.50 ± 1.90

Id. at 1164. The compounds of Table I (i.e., dipeptides lacking the 2-cyano moiety) have inhibition activity in the 0.064 μM to 14.50 μM range. Ashworth notes that the most active of these compounds was compound **5**, "[i]n particular, β-branched α-amino acid derivatives were the most potent compounds with the non-proteinogenic amino acid, **(S)-cyclohexylglycine providing the most active pyrrolidide** (compound **5** possessing a K_i value of 64 nM)." *Id.* at 1165 (emphasis added).

112. In comparison, Table II of Ashworth discloses the DP-IV inhibition activity of six analogues having the 2-cyano moiety:



Compound N°	Xaa	K_i (nM)[13]	$t_{1/2}$ (h)[19]
24	Cpg	1.1 ± 0.2	48
25	Chg	1.4 ± 0.5	>48
26	Ile	2.2 ± 0.5	48
27	Tbg	3.8 ± 0.8	>48
28	Lys(Z)	5.2 ± 1.0	24
29	Pro	22.0 ± 4.0	7.5

Id. at 1166. The six compounds of Table II (all having a 2-cyanopyrrolidide moiety) have inhibition activity in the 1.1 nM to 22 nM range, which in some cases equates to a 50-fold increase in inhibition activity over the same compounds lacking the 2-cyano moiety. *Id.* at 1164, 1166. For example, compound **5** has a K_i value of 64 nM, which decreases to 1.4 nM in compound **25** solely through the addition of a 2-cyano moiety. This decrease in inhibition activity represents significantly greater DP-IV inhibition potency for compound **25** relative to compound **5**. This illustrates the beneficial effect on potency of the cyano moiety in DP-IV inhibitors. This further suggests to a POSA that the cyano moiety interacts with DP-IV to form an attractive interaction and provides motivation to optimize this to further improve potency.

113. Taken as a whole, the Ashworth reference contains ample and explicit motivation for one of skill in the art to select compound **25** as a lead for designing DP-IV inhibitors such as the compounds recited in claims 8 and 25 of the '186 patent. As evidenced by Ashworth, the inventors of the '186 patent were not the first to recognize the use of this core structure in designing DP-IV inhibitor compounds.

2. A POSA Would Have Been Motivated Modify Ashworth Compound 25 While Retaining Certain Structural Elements

114. One of ordinary skill in the art would have been motivated to retain certain structural elements of Ashworth compound **25** when optimizing it toward the ultimate

compound. For example, by March 2000, it was well known in the art that proline dipeptides and mimetics were therapeutically useful for a variety of purposes, including DP-IV inhibition. *See*, e.g., Ashworth. Early work with pyrrolidinyl dipeptides revealed high inhibitor potency when coupling a pyrrolidine-containing amino acid with cyclohexylglycine. Villhauer at 1165. Villhauer also identified dipeptide glycyl-cyanopyrrolidines as inhibitors of DP-IV. *See, id.* at Abstract. Therefore, one of ordinary skill in the art would have been motivated to retain the dipeptide structure of Ashworth compound **25**.

115. One of ordinary skill in the art would have been motivated to retain the P_1 proline, free amine, and 2-cyano moieties of the Ashworth compound **25** based on the knowledge generally available in the art. For example, Ashworth states that "[s]ubstrates and inhibitors of DP-IV require a free N-terminus" (at 1163) and describes superior potency with the 2-cyano moiety versus without it (at 1164 and 1166, compare Tables I and II). Thus one of ordinary skill in the art would have been motivated to retain the free amine and 2-cyano moieties in the lead compound and would have had a reasonable expectation of success that the ultimate compound having these features would be an active DP-IV inhibitor.

116. One of ordinary skill in the art would have been motivated to select structures in which the trans conformation predominates when optimizing Ashworth compound **25**. It was well known in the art in March 2000 that DP-IV has selectivity for substrates and inhibitors in the trans conformation relative to the cis conformation. Lin represents what was generally known in the art in March 2000 regarding DP-IV's preferred substrate and inhibitor conformations. Lin reports that "[DP-IV] possesses a high conformational specificity for a trans amide bond between the P_1 and N-terminal P_2 residues." Lin at 14020. Lin reports K_i values of 14,000 nM for fluoro olefin dipeptides in the cis conformation and 188 nM for isomeric fluoro

olefin dipeptides in the trans conformation. Comparison of these K_i values for DP-IV inhibition reveals the significantly greater potency of, and preference for, inhibitors in the trans conformation. *See*, e.g., *id.* at 14023 and Table 2.

a. A POSA Would Have Been Motivated To Increase Chemical Stability By Limiting Intramolecular Cyclization

117. One of ordinary skill in the art would have been motivated to limit intramolecular cyclization when optimizing Ashworth compound **25**. It was generally understood in the art that intramolecular cyclization was a source of dipeptide instability in DP-IV inhibitors. For example, Ashworth teaches that, "[s]ubstrates and inhibitors of DP-IV require a free N-terminus, which means that potential dipeptide serine protease inhibitors (e.g., C-terminal aldehydes, boronic acids, α-ketoacids, trifluoromethylketones, or chloromethylketones) are inherently unstable at neutral pH due to intramolecular cyclisation." Ashworth at 1163. As further basis for what was known in the art, Lin describes "the cyclization reaction of the free N-terminal amino acid group with the reactive site of the inhibitor" for related dipeptide compounds (Lin at 14020-14021), which negatively impacts compound stability. Lin further teaches that "[t]he cyclization reaction of the free N-terminal amino group with the reactive inhibitor . . . require[s] the molecule assume the cis conformation. *Id.*

118. One of ordinary skill would have understood that intramolecular cyclization could be reduced by selecting against a conformation that favors intramolecular cyclization (i.e., selecting against the cis conformation), which can be achieved by the addition of a large, steric group to the compound at C2. *See*, e.g., Pal at 274. The trans conformation advantageously places the reactive cyano and amine groups farther from each other, thereby limiting intramolecular cyclization. Adding a large group such as adamantane to the compound would be expected to restrict its range of motion, thereby limiting intramolecular cyclization. *Id.*

119. One of ordinary skill in the art would have understood that a large group could be added to Ashworth compound **25** in order to increase its chemical stability. Further, one of ordinary skill in the art would have been motivated to substitute the cyclohexyl group at the 2-position of the acetyl-pyrrolidine-2-carbonitrile compound **25** with a large tricyclo[3.3.1.1]dec-1-yl group (also referred to as the "adamantyl group") in order to optimize the compound, e.g., to increase the stability of the compound and to bias the compound toward the trans conformation. Ashworth provides the motivation to do so because it shows increased stability for compounds having larger substituents at the 2-position. Furthermore it is reasonable to expect that an increase in size of a group at the 2-position on a cyanopyrrolidinyl glycine derivative would further increase chemical stability. *See*, e.g., Ashworth at 1166, Table II (compare compounds **24** and **25**).

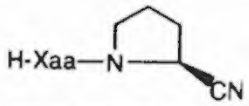
120. One of ordinary skill in the art would also recognize that there are regulatory guidelines for chemical stability that must be met. *See* FDA Stability Guidelines. These guidelines indicate that the stability of a drug candidate under "the same storage conditions as applied to the drug product" should be studied for a time period "sufficient to cover storage, shipment, and subsequent use" of the drug substance in question. *Id.* at 4. The FDA Stability Guidelines also specify that, unless a drug is intended to be stored in a freezer or refrigerator, stability of the drug substance should be tested under the following criteria:

	Conditions	**Minimum time period at submission**
Long-term testing	25°C ±2°C/60% RH ±5%	12 Months
Accelerated testing	40°C ±2°C/75% RH ±5%	6 Months

Id. In order to submit and initial Registration Application for a new drug, the applicant will need to have "a minimum of 6 months of data from a 12-month study." *Id* at 5. Additionally, a POSA

would recognize the relationship between chemical stability and impurities that can arise during storage as degradation products. *Id.* at 3; Dept. of Health & Human Services, "Int'l Conf. on Harmonisation; Guideline on Impurities in New Drug Substances; Availability," *Federal Register*, 61(3):372-376 (Jan. 4, 1996) ("FDA Impurities Guidelines"), at 373. The FDA may require reporting, identification, and qualification of impurities that arise from degradation during storage even if they are present in very small amounts. FDA Impurities Guidelines at 373-374. For example, for a drug substance that is dosed at less than 2 grams per day, the FDA may require qualification of any impurity present at 0.1% or greater. *Id.* at 374. A more stable compound would be expected to give rise to fewer degradation products over time and/or lower quantities of degradation products over time, and thus would be expected to increase the purity of drug substance and decrease the analytical burden associated with producing each batch of the drug substance. As such, a POSA would be motivated to develop compounds with good chemical stability, hoping to produce drug substances that would continue to adhere to their specifications after many months of storage under the required conditions.

121. Ashworth provides motivation to optimize the 2-position of the acetyl-pyrrolidine-2-carbonitrile compound **25** with a suitable substituent to enhance stability. Table II of Ashworth shows the chemical stability data of DP-IV inhibitors in aqueous solution (pH of 7.4), as measured in half-life ($t_{1/2}$).



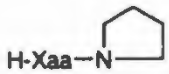
Compound N°	Xaa	K_i (nM)[13]	$t_{1/2}$ (h)[19]
24	Cpg	1.1 ± 0.2	48
25	Chg	1.4 ± 0.5	>48
26	Ile	2.2 ± 0.5	48
27	Tbg	3.8 ± 0.8	>48
28	Lys(Z)	5.2 ± 1.0	24
29	Pro	22.0 ± 4.0	7.5

Id. at 1166.

122. When changing the substituent at the 2-position of the acetyl-pyrrolidine-2-carbonitrile from a straight chain alkyl moiety, for example compound **28** (a lysine moiety), to a more bulky cycloalkyl moiety such as a cyclohexyl moiety, for example compound **25**, the stability dramatically increases from 24 hours to greater than 48 hours. Given this teaching, one of ordinary skill in the art would have been motivated to try even larger, bulkier alkyl groups, such as substituting the cyclohexyl group at the 2-position of the acetyl-pyrrolidine-2-carbonitrile compound **25** with an adamantyl group, in order to further minimize cyclization and increase the stability of the compound under similar conditions. Adamantyl was well known and studied in the art. For example, as discussed in more detail below, Raag, published in 1991, taught hydroxylated adamantane. Raag at 2678.

b. A POSA Would Have Been Motivated To Increase Potency

123. One of ordinary skill in the art also would have been motivated to substitute the cyclohexyl group at the 2-position of the acetyl-pyrrolidine-2-carbonitrile compound **25** with an adamantyl group because, e.g., Ashworth discloses that substitution at this position improves the potency of an aminoacyl pyrrolidide compound as shown in Table 1 of Ashworth:



Compound N°	Xaa	K_i (μM)[13]
5	Cyclohexylglycine [Chg]	0.064 ± 0.01
6	(R,S)-Cyclopentylglycine [Cpg]	0.21 ± 0.04
7	Ile	0.41 ± 0.01
8	*allo*-Ile	0.44 ± 0.04
9	Val	0.47 ± 0.02
10	Lys(Cbz)	0.52 ± 0.07
11	tert-Butylglycine [Tbg]	0.88 ± 0.20
12	Thr(Me)	0.90 ± 0.15
13	Orn(Cbz)	0.91 ± 0.20
14	2-Aminohexanoic acid [Aha]	1.20 ± 0.20
15	Glu	2.00 ± 0.40
16	Pro	2.10 ± 0.20
17	Cyclohexylalanine [Cha]	2.15 ± 0.50
18	Glu(OBn)	2.70 ± 0.30
19	Thr	4.90 ± 0.90
20	Phenylglycine [Phg]	5.30 ± 0.10
21	Ser(Bn)	6.00 ± 1.50
22	Ala	7.00 ± 1.00
23	Asp	14.50 ± 1.90

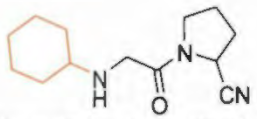
Id. at 1164. Specifically, compound **5** (having a cyclohexyl group) of Table I shows at least a 100-fold increase in potency compared to compound **7** (having a 1-methyl propyl group) or compound **9** (having an isopropyl group). Ashworth confirms this observation by noting, "[a]s expected, from the substrate specificity of DP-IV, only (S)-amino acid derivatives showed any activity and, as can be seen in Table I, lipophilic amino acids gave more potent compounds. In particular, β-branched α-amino acid derivatives were the most potent compounds with the non-proteinogenic amino acid, **(S)-cyclohexylglycine providing the most active pyrrolidide** (compound **5** possessing a K_i value of 64 nM)." *Id.* at 1165.

124. Therefore, Ashworth describes and teaches: (i) the advantage of replacing a smaller **straight chain alkyl moiety** (i.e., a 4-amino-butyl group) at the 2-position of an aminoacyl pyrrolidine with **a larger cycloalkyl moiety** (e.g., a cyclohexyl group) to produce an aminoacyl pyrrolidine-carbonitrile with **increased stability** under physiological pH conditions;

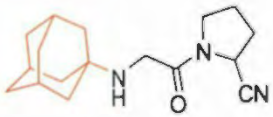
and (ii) the advantage of replacing a **smaller branched alkyl moiety** (i.e.,1-methyl propyl group or isopropyl group) at the 2-position of an aminoacyl pyrrolidine by a **larger cycloalkyl moiety** (cyclohexyl group) to produce an aminoacyl pyrrolidine with **superior activity** in inhibiting DP-IV.

3. A POSA Would Have Been Motivated To Replace The Cyclohexyl Group On Compound 25 With An Adamantyl Group

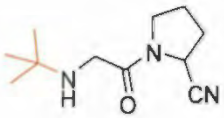
125. Additionally, one of ordinary skill in the art would have been motivated to substitute the cyclohexyl group at the 2-position of compound **25** with an adamantyl group because the art taught adamantyl as an obvious alternative to cyclohexyl. For example, Villhauer discloses compounds having cycloalkyl (e.g., adamantyl groups) or alkyl groups attached to the amino moiety of 2-cyano pyrrolidides for use as DP-IV inhibitors. Specifically, Villhauer discloses a cyclohexyl group attached to the amino moiety of 2-cyano pyrrolidides:



(Villhauer at 12, Example No. 28); adamantyl groups attached to the amino moiety of 2-cyano pyrrolidides:



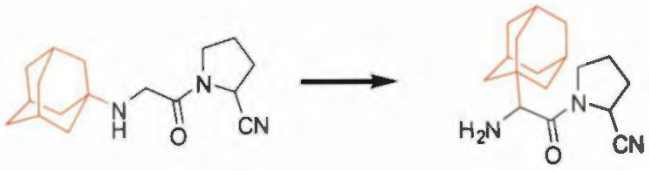
(*id.* at 13, Example No. 47) and alkyl groups attached to the amino moiety of 2-cyano pyrrolidides:



(*id.* at 13, Example No. 52). Villhauer performed a pairwise comparative analysis between cyclohexyl and adamantyl groups, along with a small number of other substituents. Villhauer thus provides motivation for selecting adamantyl as an alternative to cycloalkyl in a DP-IV inhibitor.

126. Given the disclosures of Ashworth and Villhauer, one of ordinary skill in the art would have been motivated in March 2000 to make the adamantyl substituted 2-cyano pyrrolidide (i.e., adamantyl substituted Ashworth compound **25**) because Villhauer discloses the adamantyl group attached to the amino moiety 2-cyano pyrrolidide compound.

127. Ashworth provides motivation to position the adamantyl group of Villhauer at the 2-position of the aminoacyl pyrrolidide of Ashworth compound **25** given that the cyclohexyl moiety was also positioned at the 2-position. Thus one of ordinary skill in the art would easily surmise that the adamantyl-substituted Ashworth compound **25** (shown on the right-hand side below) is merely a logical extension of what was previously known at the time of filing (the compound on the left hand side below):

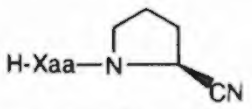


Accordingly, well before March 2000, it would have been obvious to one of ordinary skill in the art to substitute the cyclohexyl group at the 2-position of the Ashworth lead compound **25** with an adamantyl group. One of ordinary skill in the art would have expected the modification to improve the characteristics of the compound, and particularly, to increase the potency and stability of the compound.

128. In the context of the '186 patent, one of ordinary skill in the art would only need to verify the readily predicted results of adding an adamantyl group and removing the cyclohexyl group. Such a modification requires less experimentation than is invited by the specification of the '186 patent.

4. A POSA Would Have Been Motivated To Add A Hydroxyl At The 3-Position Of The Adamantyl Group

129. One of ordinary skill in the art would have been motivated to add a hydroxyl group at the 3-position of the adamantyl substituent of the modified Ashworth lead compound **25** in order to improve the compound's characteristics. For example, addition of a hydroxyl moiety would have been expected to reduce the compound's partition coefficient (thereby enhancing its solubility and permeability) and potentially increase its metabolic stability. Specifically, one of ordinary skill in the art would have expected the hydroxyl group to potentially increase compound solubility and possibly increase absorption of the compound without diminishing its activity.



Compound N°	Xaa	K_i (nM)[13]	$t_{1/2}$ (h)[19]
24	Cpg	1.1 ± 0.2	48
25	Chg	1.4 ± 0.5	>48
26	Ile	2.2 ± 0.5	48
27	Tbg	3.8 ± 0.8	>48
28	Lys(Z)	5.2 ± 1.0	24
29	Pro	22.0 ± 4.0	7.5

Ashworth at 1066.

130. Also, it would have been within the general knowledge of one of ordinary skill in the art—as evidenced by Lipinski, Hansch, Cates, and/or other references available to one of

ordinary skill in the art—to incorporate a hydroxyl group in the adamantyl-modified Ashworth lead compound, e.g., to reduce the partition coefficient of the compound, and thus potentially increase the solubility in aqueous solution. Thus, the teachings of Ashworth in combination with such knowledge would have motivated one of ordinary skill in the art to make such a modification.

131. Raag provides additional motivation for incorporation of a hydroxyl group at a specific position on the adamantyl ring. Raag discloses a study of the metabolism of adamantane by cytochrome P-450$_{CAM}$. Raag at Abstract. Table III of Raag, shows the substrates that are subject to hydroxylation, including adamantane and other substrates:

Table III. Various Substrate-Dependent Parameters[a]

	camphor	adamantanone	adamantane	thiocamphor	camphor Y96F	norcamphor	camphane
molec vol	315 Å^3	300 Å^3	293 Å^3	322 Å^3	315 Å^3	236 Å^3	309 Å^3
hydrogen bond to Y96	yes[a]	yes[b]	no	no	no	yes[b]	no
no. of iron ligands	5[a]	5[b]	6	6		6[b]	6
redox pot. Fe^{3+}/Fe^{2+}	-170 mV[c]	-175 mV[c]				-206 mV[c]	
high-spin %	94–97%[c,e]	96–98%[c,e]	99%[e]	65%[e]	59%[e]	46%[e]	46%[e]
regiospecif of substr hydroxylatn	5-exo (100%)[e,f]	5 (100%)[e]	1 (100%)[d]	5-exo (64%)[e] 6-exo (34%) 3-exo (2%)	5-exo (92%)[e,f] 4 (1%) 6-exo (2–4%) 3-exo (0–4%) 9 (<1%)	5-exo (45%)[f] 6-exo (47%) 3-exo (8%)	5-exo (90%)[e] 6-exo (10%)
substr temp factor (Fe^{2+})	16.2 Å^2[a]	16.5 Å^2[b]	24.7 Å^2	23.5 Å^2		33.5 Å^2[b]	30.1 Å^2
substr hydrophilic groups	yes	yes	no	yes	yes	yes	no
hydroxylatn "efficiency"	100%[e,f]			98%[e]	100%[e]	12%[f]	8%[e]
L6–substr dist	NA	NA	2.63 Å	2.35 Å (70%) 3.35 Å (30%)		3.0 Å[b]	2.88 Å
L6–iron dist	NA	NA	1.95 Å	1.35 Å		1.73 Å[b]	1.67 Å
L6 occupancy	NA	NA	1.00	0.90		0.97[b]	1.00
L6 temp factor	NA	NA	14.3 Å^2	19.6 Å^2		3.8 Å^2[b]	7.7 Å^2
cation occupancy	1.00[a]	1.00[b]	0.89	0.91		1.00[b]	0.72
cation temp factor	12.1 Å^2[a]	10.0 Å^2[b]	15.5 Å^2	14.2 Å^2		7.9 Å^2[b]	21.7 Å^2

[a] Poulos et al. (1985, 1987). [b] Raag and Poulos (1989a). [c] Fisher and Sligar (1985). [d] White et al. (1984). [e] Atkins and Sligar (1988b). [f] Atkins and Sligar (1989). [g] Carbon numbering for each substrate begins with C-1 at the top of the six-membered ring which is in the plane of the table. Numbering proceeds counterclockwise such that the carbonyl carbon is C-2 and C-5 is in the lower right-hand portion of the ring. Note that C-5 is a secondary carbon in some substances and a tertiary carbon in others.

Id. at 2678. Column 7 of Table III describes the regiospecific hydroxylation of adamantane at a specific 1-position, i.e., at a tertiary carbon site (and not a secondary carbon site). *Id.* This position corresponds directly to the hydroxylation site (i.e., the 3-position on the adamantyl ring)

in saxagliptin and the location of the hydroxyl group of the adamantyl-modified Ashworth compound **25**.

132. Raag also teaches that "[a]damantane is . . . metabolized to a single product despite having a relatively high active site mobility. The single product can be attributed to the existence of only two types of unique carbon atoms in adamantane, together with the greater reactivity of tertiary versus secondary carbons." *Id.* As additional evidence for the teachings of Raag, Hoffman describes the antiviral drug rimantadine, which contains an adamantane moiety that was known to be metabolized to a hydroxylated derivative. Hoffman serves as further basis of what was generally known in the art regarding the tendency for adamantane to preferentially undergo hydroxylation at the 3-position.

133. Therefore, it was known long before March 2000 that 3-hydroxylated adamantyl groups were used in drugs and that adamantyl groups were subject to hydroxylation at the same site as a result of normal metabolism. Further, one of ordinary skill in the art, given the teaching of Raag (and as evidenced by Hoffman), would have been motivated to block metabolism of a substituted adamantyl ring at the 3-position by placing a group such as a hydroxyl group at that position. Blocking metabolism at the 3-position would result in greater metabolic stability.

134. Furthermore, at the time the application of the '186 patent was filed, it was also known that information on metabolites of candidate drugs "can guide structural modifications, thereby improving the activity and/or bioavailability." *See* Korfmacher at 532. Korfmacher teaches the following advantages of metabolite identification:

> Metabolite identification in drug discovery provides early information that can lead to structural changes in the current lead compound, improving such pharmacokinetic parameters as oral bioavailability, half-life ($t_{1/2}$), or C_{max}. Often these parameters can be changed by improving the metabolic stability of the compound. In order to improve metabolic stability, it is very important to know how a compound is metabolized. The goal of drug discovery is to progress a lead

> compound into a final candidate drug that can be placed in the development stage. . . **Early metabolite identification can provide information on how to improve the metabolic stability of the lead structure. In this way, future lead compounds might be a metabolite identified from the previous lead drug or an analog of the previous drug designed to block the major route of metabolism.**

Id. at 534 (emphasis added).

135. Therefore, it was well known that metabolites (such as 3-hydroxy substituted adamantyl compounds) could provide improved metabolic stability to a compound.

136. Additionally, having a hydroxyl group at the 3-position would also improve the Lipinski parameters, such as reducing Log P and improving permeability as discussed below. The partition coefficient (P) is a ratio of concentrations of un-ionized compound between two liquid phases, and Log P is as a measure of compound lipophilicity. One of ordinary skill in the art would have been motivated to add a hydroxyl group at the 3-position of the adamantyl-substituted moiety of the modified Ashworth lead compound **25** because the use of hydroxyl groups on drugs increases the solubility of the drugs in water. This position is supported by the knowledge of one of ordinary skill in the art, as evidenced by at least by the Lipinski, Hansch and/or Cates disclosures as discussed in details in ¶¶ 50-51 above.

137. One of ordinary skill in the art would have been motivated to improve characteristics or properties of the lead compound, e.g., the solubility thereof, particularly wherein the prior art indicates a potential problem with such a characteristic or property, e.g., very low solubility, as predicted by one of ordinary skill in the art, for example using information and teachings available, such as evidenced by Lipinski, Hansch and Cates.

138. Accordingly, before 2000, in view of the knowledge of one of ordinary skill in the art, as seen in the teachings of Lipinski, Hansch and Cates, it would have been obvious to one of ordinary skill in the art to add a hydroxy group at the 3-position of the adamantyl modified

compound **25**. A person of ordinary skill in the art would have had reason to try such an addition with an expectation of success in preparing a DP-IV inhibitor with improved water solubility, as well as increased stability (as taught in Korfmacher), permeability and bioavailability.

5. A POSA Would Have Been Motivated To Modify Ashworth's Compound 25 By Cyclopropanation Of The Pyrrolidine Ring

139. As discussed above, one of ordinary skill in the art would have understood the significance of dipeptide conformation on the effective interaction between DP-IV and its substrates and inhibitors. As would have been appreciated by one of ordinary skill in the art, the orientation of certain functional groups in the ultimate compound (e.g., the free amine and 2-cyano moieties) would be controlled, at least in part, by biasing the molecule to prefer a trans versus cis conformation of the dipeptide core structure. As discussed above, it was generally known in the art that the trans conformation would be favored over the cis conformation for a DP-IV inhibitor. *Id.* at 14020. This can be accomplished by substitution of a large group, such as hydroxyadamantyl at the C2 position of the core structure. *See* Pal at 274.

140. One of ordinary skill in the art also would have been motivated to optimize the interaction between the cyano functional group and the DP-IV enzyme. Specifically, a person of ordinary skill would have been motivated to optimize the interaction between DP-IV and the cyano group by exploring variations in the point of attachment on the pyrrolidine ring as well as the orientation of the cyano group on the pyrrolidine ring. One of ordinary skill in the art would have selected as a starting point the 2-cyano position found in both Ashworth compound **25** and Villhauer. The cyano moiety in saxagliptin shares the same pyrrolidine ring position described in these prior art references.

141. A person of ordinary skill would have also sought to modulate the orientation of the 2-cyano moiety on the pyrrolidine ring in order to optimize interaction with and inhibition of

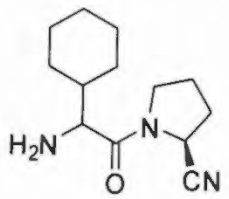
the DP-IV enzyme. A standard strategy in March 2000 for modulating the orientation of a ring-bound substituent would have been through fusion of the pyrrolidine ring with another ring. *See*, e.g., Chiou at 243. Fusion between two rings results in significant changes in ring flexibility, including as discussed above, ring flattening in a cyclopropyl fused derivative. These changes in turn would be expected by one of ordinary skill in the art to affect the orientation of ring-bound substituents (e.g., the 2-cyano moiety of Ashworth, compound **25**). The smallest ring that can be used for fusion is a cyclopropyl ring. *Id.*

142. Cyclopropyl rings are one of the most commonly used ring fusion agents because: (i) addition of a cyclopropyl ring has a negligible effect on compound molecular weight, which can translate to better drug-like qualities; (ii) cyclopropane has an exceedingly small footprint, meaning it exerts a minimal steric effect on the rest of the compound to which it is attached and (iii) cyclopropane provides greater conformational restriction than larger ring fusions, resulting in fewer possible conformations. *See*, e.g., *id.*

143. One of ordinary skill in the art would have been motivated to select cyclopropyl fusion as a means for modulating the interaction between a DP-IV inhibitor and DP-IV. Chiou represents what was generally known in the art in March 2000 regarding the effects of cyclopropyl ring fusion. Specifically, Chiou explored the fusion of a cyclopropyl ring onto acetylcholine (ACh) in order to produce a compound that was "1) structurally as close to ACh as possible and 2) conformationally as rigid as possible." According to Chiou, cyclopropyl rings "are considered the smallest chemical structure . . . capable of conferring conformational rigidity." *Id.* at 243. Like Chiou, one of ordinary skill in the art would have been motivated to start with a compound having known activity (e.g., Ashworth compound **25**) and to optimize it by making small, incremental changes to its size and rigidity near the active 2-cyano group.

144. For the above reasons, cyclopropyl fusion to the pyrrolidine ring of Ashworth compound **25** would have been one of the first modifications a person of ordinary skill would have chosen for its optimization in March 2000. Moreover, the pool of compounds that would have been tried by a person of ordinary skill would have been quite small given the likelihood of selecting the cyclopropyl fusion modification.

145. Selection of the point of attachment for the cyclopropyl ring would have been straightforward for one of ordinary skill in the art in March 2000. As can be seen in the pyrrolidine ring of Ashworth compound **25** below, there are only three possible cyclopropyl fusion sites at the pyrrolidine ring:



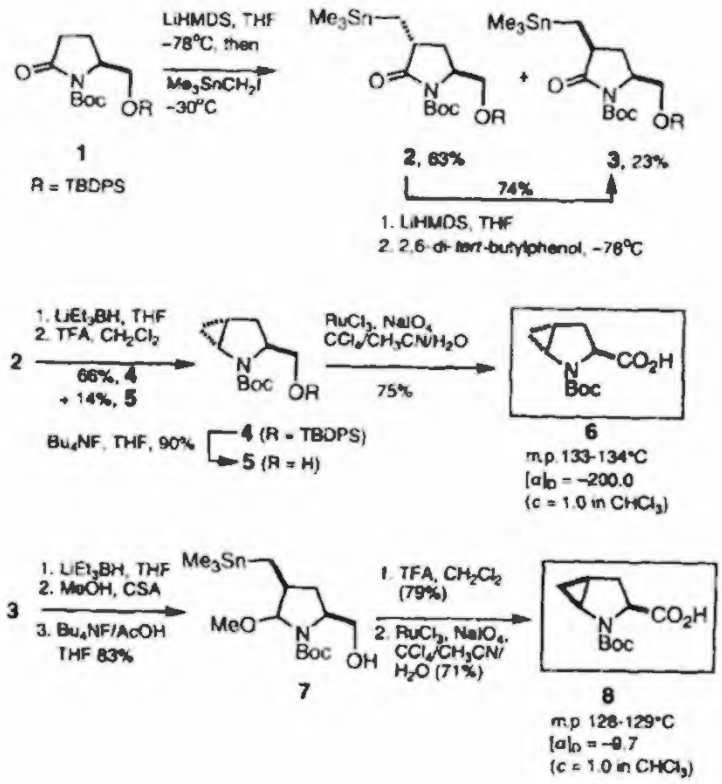
Ashworth Compound **25**

The resulting fusion would thus be one of: 1,2-methanopyrrolidine, 3,4-methanopyrrolidine or 4,5-methanopyrrolidine. One of ordinary skill in the art would have understood in March 2000 that two possible orientations of the fused cyclopropane ring could exist for each of the above three positions ring. *See*, e.g., Hanessian at 1882, compounds **6** and **8**.

146. One of ordinary skill in the art would have prioritized fusion of the cyclopropane ring at the two positions farthest from the carbon bearing the cyano moiety. Thus the skilled artisan would have conservatively started with the 4,5-methanopyrrolidine and 3,4-methanopyrrolidine fusions because the prior art provided expeditious synthetic methods for their preparation. *See* Hanessian; Tverezovsky, et al., "Synthesis of (2S, 3R, 4S)-3,4-

Methanoproline and Analogues by Cyclopropylidene Insertion," *Tetrahedron*, 53(43):14773-14792 (1997). Therefore, the 4,5-methanopyrrolidine, as present in saxagliptin, would have been one of only a small set of compounds that would have been investigated by one of ordinary skill in the art.

147. Hanessian provides motivation for producing a fused pyrrolidine-cyclopropyl ring system as found in saxagliptin. Specifically, Hanessian teaches that the use of conformationally constrained analogues of proline were well known in the peptidomimetic research area well before March 2000. Hanessian describes the "highly stereocontrolled syntheses of the diastereomeric 4,5-methano-L-prolines and 5,6-methano-L-pipecolic acids by a novel intramolecular cyclopropanation reaction of iminium ions . . ." *Id.* at 1882. One such example shown by Hanessian is compound **8**, which was synthesized according to Scheme 1 below:



Id.

148. The structure and conformation of compound 8 in the solid state were unambiguously confirmed by single X-ray analysis. *Id.* Further, Table 1 of Hanessian shows selected torsion angles for compound 8 where "considerable 'flattening' of the pyrrolidine ring is observed relative to *N*-Boc-L-proline." *Id.*

Table 1. Selected torsion angles and root-mean-square deviations from fitted atoms in a given plane of X-ray crystal structures, and ^{13}C NMR chemical shifts ($CDCl_3$).

6

8

	N-Boc-L-proline [20]	6	8
$\tau(NC_\alpha)$	−17	−5.6	−14.4
$\tau(C_\alpha C_\beta)$	+31	+4.8	+15.3
$\tau(C_\beta C_\gamma)$	−35	−2.6	−11.4
$\tau(C_\gamma C_\delta)$	+24	−0.7	+2.9
$\tau(C_\delta N)$	−4	+4.1	+7.6
$\tau(BocNC_\alpha CO_2H)$	−72	−64.0	−67.1
rms deviation	0.018	0.003	0.013
of fitted atoms	C_α, N, C_δ, C_γ	C_β, C_γ, C_δ, N	C_β, C_γ, C_δ, N
	δ(cis/trans)	*δ(cis/trans)*	*δ(cis/trans)*
COOH	178.35/176.60	177.7/175.5	179.1/176.1
NC=O	153.95/155.39	157.1/154	155.7/154.1
C_α	58.8	60.8/60.1	59.5/59.1
C_β	30.75/29.53	32.0	31.5/29.1

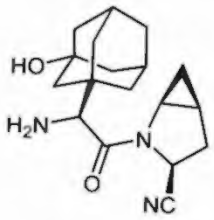
Id. Hanessian confirms the "flattening" of the pyrrolidine ring by determining the root-mean-square values for the C_β and N atoms from the plane defined by C_β, C_γ, C_δ and N (*see* Table 1 above). It was determined that for compound **8**, the out-of-plane carbon was the carbon bearing the carboxyl group when compared to *N*-Boc proline. *Id.* Therefore, Hanessian taught that modification of a substituted proline ring (such as a 2-carboxyl substituted proline) to a 4,5-methanoproline ring system "flattens" the ring. Ring flattening would also be expected to result in a modified orientation of the 2-cyano group. Modifying the orientation of the 2-cyano group in Ashworth compound **25** would likely result in a modified interaction with DP-IV that may improve potency and possibly cause it to resist intramolecular reaction with the free amine group found in DP-IV inhibitors (i.e., increased stability).

149. One of ordinary skill in the art would have had reason to modify the 2-cyano substituted proline portion of the Ashworth lead compound **25** to a 4,5-methanoproline ring system in order to enhance compound stability. For example, Ashworth teaches that, "[s]ubstrates and inhibitors of DP-IV require a free N-terminus, which means that potential dipeptide serine protease inhibitors (e.g., C-terminal aldehydes, boronic acids, α-ketoacids, trifluoromethylketones, or chloromethylketones) are inherently unstable at neutral pH due to intramolecular cyclisation." Ashworth at 1163. One of ordinary skill in the art would have had reason to try modifying the 2-cyano substituted proline portion of the Ashworth compound **25**, to produce a 4,5-methanoproline ring system in order to "flatten" the proline ring as taught in Hanessian, thereby adjusting the orientation of the cyano substituent to the proline ring and minimizing or preventing intramolecular cyclization, as taught by Ashworth. Furthermore one of ordinary skill in the art may expect that a cyclopropyl fused pyrrolidine may also have improved chemical stability in FDA-mandated stability testing. *See* FDA Stability Guidelines; FDA Impurities Guidelines.

150. Hanessian describes and teaches: (i) modification of a substituted proline ring (such as a 2-carboxyl substituted proline) (ii) to produce a 4,5-methano-modified substituted proline ring with the 2-substituent orientation modified with respect to the proline ring.

151. Accordingly, before 2000, it would have been obvious to one of ordinary skill in the art to modify the substituted proline ring of the Ashworth compound to a 4,5-methano-modified compound. Such chemist would have had reason to make such a modification with a reasonable expectation of success for improving the characteristics of the compound. For example, according to the teachings of Ashworth, one of ordinary skill in the art would have expected success in preparing the DP-IV inhibitor with improved stability properties.

152. By making these commonly known modifications at the positions indicated above, routine experimentation would have led to the following molecule:



153. This molecule is identical to the compounds recited in claims 8 and 25 of the '186 patent and encompassed by claims 1, 2, 4, 6, 7, 9-11, 26-28, 32-35, and 39-40 of the '186 patent.

154. Taken together, the prior art provided reason: 1) to select the core structure of Ashworth compound **25** to form a DP-IV inhibitor; 2) to substitute the cyclohexyl group at the 2-position of the acetyl-pyrrolidine-2-carbonitrile of the lead compound with tricyclo[3.3.1.1]dec-1-yl group (adamantyl group); 3) to add a hydroxyl group at the 3-position of the adamantyl group and 4) to form a 4,5-methanoproline moiety of the lead compound. Thus, claims 8 and 25 of the '186 patent are unpatentably obvious over Ashworth in view of the knowledge and motivation of one of ordinary skill in the art, as evidenced by prior art references (including Villhauer, Raag, and Hanessian).

B. Each Additional Limitation Of Dependent Claims 9 And 26 Is Also Disclosed By The Prior Art

Claim 9:

"The compound as defined in claim 8 wherein the pharmaceutically acceptable salt is the hydrochloride salt or the trifluoroacetic acid salt."

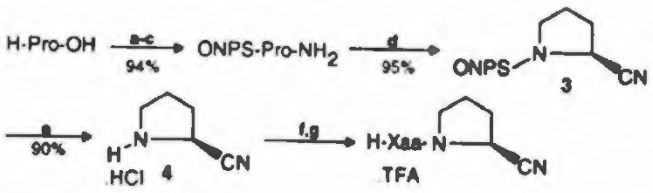
Claim 26:

"The compound as defined in claim 25, wherein the pharmaceutically acceptable salt is the hydrochloride salt."

155. Claim 9 of the '186 patent recites the compound as defined in claim 8 and further claims the hydrochloride salt or the trifluoroacetic acid salt as the pharmaceutically acceptable salt. Claim 26 of the '186 patent recites the compound as defined in claim 25 and further claims the hydrochloride salt as the pharmaceutically acceptable salt. It would have been obvious to one of ordinary skill in the art to make and use the hydrochloride salt or the trifluoroacetic acid salt because it was previously disclosed in Ashworth. Further, making the salt would have been an ordinary exercise or task one of ordinary skill in the art would have performed at the time the application which led to the '186 patent was filed.

156. Ashworth teaches the formation of DP-IV pyrrolidides as both the hydrochloride salt and trifluoroacetic acid salt. For example, Ashworth describes the "subsequent acid catalyzed deprotection (4N HCl/dioxane) afforded the inhibitor as its **hydrochloride salt**." Ashworth at 1165 (emphasis added). Ashworth also describes the preparation of dipeptide nitriles as shown in Scheme I, below.

Scheme I. *Preparation of dipeptide nitriles.*



Reagents: a. ONPS-Cl, 2N NaOH. b. HONSu, Water soluble carbodiimide. c conc. NH_4OH, dioxane. d. imidazole (2 equiv.), $POCl_3$ (4 equiv.), pyridine. e. 4N HCl/dioxane (3 equiv.), diethyl ether. f. Boc-Xaa-OH, pyBop, NEt_3, CH_2Cl_2. g. Trifluoroacetic acid.

Id.

157. Here, step g of Scheme I shows the formation of the trifluoroacetic acid salt. *Id.* Because it would have been obvious to one of skill in the art to modify the lead compound **25** of Ashworth, as described above, the hydrochloride salt or trifluoroacetic acid salt of claim 9 or the

hydrochloride salt of claim 26 of the '186 patent would be obvious for at least the reasons set forth above with respect to claims 8 and 25, given the disclosure that hydrochloride salts or trifluoroacetic acid salts were synthesized in Ashworth. Further, it would have been within the ordinary routine of one of skill in the art to make pharmaceutically acceptable salts, such as hydrochloride salts or trifluoroacetic acid salts once the base compound was synthesized. Villhauer also teaches the use of pharmaceutically acceptable salt, including the hydrochloride salt.

VIII. CONCLUSION

158. For the foregoing reasons it is my opinion that claims 8, 9, 25, and 26 of the '186 patent would have been obvious to a person of ordinary skill in the art at the time of the claimed invention.

Executed on this 21 day of January, 2016.

David P. Rotella, Ph.D.

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

ASTRAZENECA AB, Plaintiff, v. AUROBINDO PHARMA LTD. and AUROBINDO PHARMA U.S.A., INS., Defendants.	C.A. No. 14-664-GMS (consolidated)
ASTRAZENECA AB, Plaintiff, v. MYLAN PHARMACEUTICALS INC., Defendants.	C.A. No. 14-696-GMS

EXPERT REPORT OF DAVID P. ROTELLA, PH.D.
ON THE INVALIDITY OF U.S. PATENT NO. RE 44,186

EXHIBIT 57-A
WIT: _____
DATE: _____
TOM FRASIK, CSR 6961

I. INTRODUCTION

1. I, David P. Rotella, have been retained by counsel for Mylan Pharmaceuticals Inc. in connection with the above-captioned litigation matter to provide expert testimony concerning U.S. Patent No. RE 44,186 ("the '186 patent") by Robl *et al.*, and entitled "Cyclopropyl-fused pyrrolidine-based inhibition of dipeptidyl peptidase IV and method." This report presents my opinions regarding the invalidity of claims 8, 9, 25, and 26 (collectively, "the asserted claims") of the '186 patent. For the reasons detailed below, it is my opinion that the asserted claims of the '186 patent would have been obvious in view of the prior art to a person of ordinary skill in the art at the time of the claimed invention.

2. This expert report, submitted pursuant to Fed. R. Civ. P. 26(a)(2)(B), sets forth the opinions as to which, if asked, I will testify at trial with respect to the '186 patent. In addition, if asked, I may respond to the opinions and testimony of Plaintiff's witnesses regarding issues within my area of expertise. I reserve the right to adjust, modify, or supplement my opinions in light of any response, critique, or comments on my report or different opinions made by or on behalf of other parties to this litigation, including, but not limited to, any deposition testimony or rebuttal reports that other parties submit.

A. Qualifications and Experience

3. I received my B.S. Pharm. from the University of Pittsburgh in 1981 and Ph.D. in Medicinal Chemistry from The Ohio State University in 1985. I was a Postdoctoral Scholar in the Department of Chemistry at The Pennsylvania State University from 1985 to 1987.

4. I am currently the Margaret and Herman Sokol Professor of Chemistry in the Department of Chemistry and Biochemistry and in the Sokol Institute of Pharmaceutical Life

Sciences at Montclair State University. I have been a member of the faculty of this university since 2011.

5. I am also currently an adjunct professor in the Department of Pharmaceutical Sciences at the University of Pittsburgh, in the Center for Drug Discovery at Northeastern University, and in the Department of Medicinal Chemistry at the University of Mississippi. I have been a member of the faculty of these departments since 2010, 2010, and 2009, respectively.

6. I am currently a registered pharmacist in the Commonwealth of Pennsylvania.

7. I was formerly a research scientist at multiple pharmaceutical companies during the years 1991-2010, including at Bristol-Myers Squibb PRI, Lexicon Pharmaceuticals, and Wyeth Research/Pfizer. My industry experience focused on drug discovery and development.

8. My current research focuses on protein kinase inhibitors for anti-infective and anti-inflammatory applications. Specifically, I work on the discovery of new agents useful for the potential treatment of parasitic and neurodegenerative diseases, including the synthesizing of new analogs of a lead structure as potential protein kinase inhibitors and investigation of structure-activity relationships in a product that has HSP90 inhibitor activity.

9. I have authored or co-authored more than 20 abstracts for presentation at professional meetings, 40 peer-reviewed journal articles, and seven book chapters. I have also edited or co-edited five books in the field of Medicinal Chemistry. I have received numerous honors, fellowships and awards, and am an inventor or co-inventor on seven granted patents.

10. A summary of my education, experience, publications, awards and honors, patents, publications, and presentations is provided in my CV, a copy of which is provided as Exhibit A, attached hereto.

B. Compensation

11. I am being compensated at the rate of $500/hour for my work in connection with this litigation. My compensation is not dependent on the outcome of this lawsuit. I do not have any financial interest in the outcome of this matter.

C. Prior Testimony

12. I have not appeared as an expert, either at trial or by deposition, within the last 5 years.

D. Materials Considered

13. In forming my opinions and preparing this report, I reviewed and considered the materials cited in this report and those materials listed in Exhibit B to this report. I have further relied on my knowledge, education, and training as reflected in my qualifications and credentials set forth above and in my *curriculum* vitae.

14. Additionally, I may use the materials cited or listed to assist me in preparing demonstratives such as graphics and animations for my testimony or in the event that I am asked to provide testimony or a technology tutorial.

II. APPLICABLE LEGAL STANDARDS

15. I have been advised that the following legal standards are applicable to this report.

16. I understand that patents are presumed valid and the party challenging validity has the burden of proving invalidity by clear and convincing evidence. I further understand that an invalidity analysis involves two steps: first, ascertaining the proper meaning and scope of the patent claims; and second, determining whether the limitations of the claims, as properly interpreted, are disclosed in the prior art.

17. I have been advised that, in the present case, the '186 patent claims are to be given their broadest reasonable interpretation in view of the specification. I also understand that, absent some reason to the contrary, claim terms are typically given their ordinary and accustomed meaning as would have been understood by one of ordinary skill in the art. I have followed these principles in my analysis described throughout this declaration. The '186 patent provides definitions for certain claim terms. In my opinion, those definitions are conventional.

A. Priority Date

18. I have been advised that the '186 patent claims priority to U.S. provisional application number 60/188,555 ("the '555 application)," which was filed on March 10, 2000. I further understand, however, that the '186 patent may not be entitled to that 2000 priority date. Each of the opinions expressed in this declaration apply regardless of whether the priority date is March 10, 2000 (the filing date of the '555 application) or February 16, 2001 (the filing date of the '173 application).

B. Level of Ordinary Skill in the Art

19. I am informed by counsel that a patent is to be interpreted from the perspective of a person of ordinary skill in the art ("POSA") to which the patent pertains, as assessed at the time of the claimed invention. I am further informed that a determination of the level of ordinary skill is based on, among other things, the educational level of the inventors, the types of problems encountered in the art, prior art solutions to those problems, the rapidity with which innovations are made, the sophistication of the technology, and the educational level of active workers in the field. I further understand that a person of ordinary skill is also a person of ordinary creativity.

20. Counsel has informed me that a POSA is a hypothetical person that may have the combined understanding of those of ordinary skill in various fields pertinent to the subject matter

of the patent. In this case I have been asked to presume that the POSA is aware of all prior art available as of March 10, 2000. Therefore, I have been asked to opine on the qualifications of a POSA as of March 10, 2000. My opinion regarding the level of skill of a POSA, detailed *infra* in Section V., thus regards a person of ordinary skill in the relevant field as of March 10, 2000.

C. Obviousness

21. I have been informed that a claimed invention is not patentable under 35 U.S.C. § 103 for obviousness, if the differences between the invention and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the field to which the subject matter pertains.

22. I have been informed that a determination of obviousness involves four factual inquiries: (1) the scope and content of the prior art; (2) the differences between the claimed subject matter and the prior art; (3) the level of ordinary skill in the art at the time of the alleged invention; and (4) secondary considerations of non-obviousness.

23. I understand from counsel that a claim can be found to have been obvious if all the claimed elements were known in the prior art and one skilled in the field could have combined the elements as claimed by known methods with no change in their respective functions, and the combination would have yielded nothing more than predictable and expected results to one of ordinary skill in the art. I also understand that improper hindsight must not be used when comparing the prior art to the invention for obviousness. Thus, a conclusion of obviousness must be firmly based on knowledge and skill of a person of ordinary skill in the field at the time the invention was made without the use of post-filing knowledge.

24. I understand from counsel that in order for a claimed invention to be considered obvious, there must be some supporting rationale for combining cited references as proposed. I

have been informed that obviousness may be established by showing that it would have been obvious to combine the teachings of more than one item of prior art. In determining whether a piece of prior art could have been combined with other prior art or with other information within the knowledge of one of ordinary skill in the art, the following are examples of approaches and rationales that may be considered: (i) combination of prior art elements according to known methods to yield predictable results; (ii) simple substitution of one known element for another to obtain predictable results; (iii) use of a known technique to improve similar methods or products in the same way; (vi) application of a known technique to a known method or product ready for improvement to yield predictable results; (vii) application of a technique or approach that would have been "obvious to try" (choosing from a finite number of identified, predictable solutions, with a reasonable expectation of success); (viii) known work in one field of endeavor may prompt variations of it for use in either the same field or a different one based on design incentives or other market forces if the variations would have been predictable to one of ordinary skill in the art; or (ix) some teaching, suggestion, or motivation in the prior art that would have led one of ordinary skill to modify the prior art reference or to combine prior art reference teachings to arrive at the claimed invention.

25. I understand from counsel that multiple factors may guide the analysis of whether it would be obvious to a person of ordinary skill to select a particular prior art compound as a lead compound for further modification. I understand that these factors may include positive attributes such as activity and potency, negative attributes such as toxicity, or any other relevant characteristics that would have been known to a person of ordinary skill from the prior art.

26. I understand from counsel that an analysis of whether there are any relevant differences between the prior art and the claimed subject matter is performed from the view of a

person having ordinary skill in the art at the time of the alleged invention. I understand that, in analyzing any differences between the claimed subject matter and the prior art, inferences and creative steps a person of ordinary skill in the art would have employed in reviewing the prior art at the time of the alleged invention may be taken into account.

27. I understand from counsel that evidence of "secondary considerations" may be weighed against evidence of the scope and content of, and the level of skill in, the art to rebut a conclusion of *obviousness* where appropriate. I understand that such secondary considerations when in evidence may include: (i) commercial success of a product due to the merits of the claimed invention; (ii) a long-felt, but unsatisfied need for the invention; (iii) failure of others to find the solution provided by the claimed invention; (iv) deliberate copying of the invention by others; (v) unexpected results achieved by the invention; (vi) praise of the invention by others skilled in the art; (vii) lack of independent simultaneous invention within a comparatively short space of time; and (viii) teaching away from the invention in the prior art. Secondary considerations are relevant where there is a nexus, or relationship, between the objective evidence and the claimed invention.

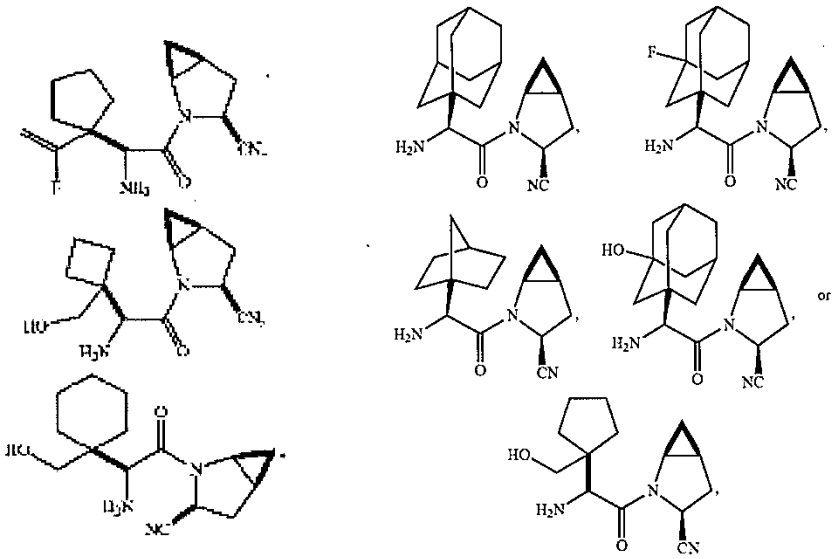
III. OVERVIEW OF THE '186 PATENT

28. The '186 patent is entitled "Cyclopropyl-Fused Pyrrolidine-Based Inhibitors of Dipeptidyl Peptidase IV and Method" and was issued on April 30, 2013. I have been advised that the '186 patent issued from U.S. Application No. 13/308,658, which was filed on December 1, 2011, as a reissued application of U.S. Application No. 09/788,173, which was filed on February 16, 2001 and issued as U.S. Patent No. 6,395,767 on May 28, 2002. I have also been advised that U.S. Application No. 09/788,173 claims priority to U.S. Provisional Application No. 60/188,555, which was filed on March 10, 2000.

29. The '186 patent is generally directed to cyclopropyl-fused pyrrolidine-based compounds with a variety of optional substituents, as well as pharmaceutical combinations and methods for treating diabetes and additional diseases. According to the '186 patent, the "cyclopropyl-fused pyrrolidine-based compounds [of the '186 patent are] inhibitors of dipeptidyl peptidase IV [(DP-IV)] . . . for treating diabetes, especially Type II diabetes." '186 patent at col. 1, ll. 19-21. The '186 patent describes the mechanism by which DP-IV inhibition treats type 2 diabetes as follows: "[DP- IV] has been shown to be the primary degrading enzyme of GLP-1(7-36) in vivo . . . [t]hus, inhibition of [DP-IV] in vivo should potentiate endogenous levels of GLP-1(7-36) and . . . thus serve to ameliorate the diabetic condition." *Id.* at 1:59-67.

30. Independent claim 1 discloses a genus of chemical compounds comprising a cyclopropyl-fused pyrrolidine-based core with a variety of optional substituents. Dependent claims 2-7 and independent claims 8 and 10 further limit the substituent groups of the compound of claim 1. For example, independent claim 8 recites the following:

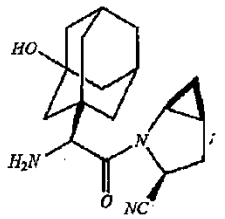
A compound having the structure:



or a pharmaceutically acceptable salt thereof.

Dependent claim 9 is directed to the hydrochloride or trifluoroacetic acid salts of the compounds of claim 8. Dependent claim 11 is directed to a pharmaceutical composition comprising a compound within the scope of claim 1. Claims 12-21 recite pharmaceutical combinations comprising a compound within the scope of claim 1 and an antidiabetic agent for treating diabetes and/or additional agents for treating related diseases. Claim 22 recites pharmaceutical combinations comprising a compound within the scope of claim 1 and an agent for treating obesity or other related diseases. Claims 23 and 24 were canceled upon reissue.

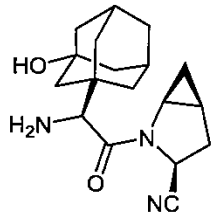
31. Independent claim 25 recites a single compound as follows:



or a pharmaceutically acceptable salt thereof.

The compound of claim 25 also falls within the scope of composition claims 1, 2, 4, 6, 7, 8, and 10. Dependent claim 26 recites the hydrochloride salt of the compound of claim 25. Dependent claims 27 and 28 further recite pharmaceutical compositions of the compound of claim 25. Dependent claims 29-31 recite a combination of the compound of claim 25 and an antidiabetic agent other than a DP-IV inhibitor. Claims 32-43 recite various methods of treatment using the compound of claim 25, alone or in combination with an antidiabetic agent other than a DP-IV inhibitor.

32. The compound of claim 25 is also known as (1S,3S,5S)-2-[(2S)-2-amino-2-(3-hydroxy-1-adamantyl) acetyl]-2-azabicyclo[3.1.0]hexane-3-carbonitrile. For convenience, this compound will be referred to as "saxagliptin" as shown below:



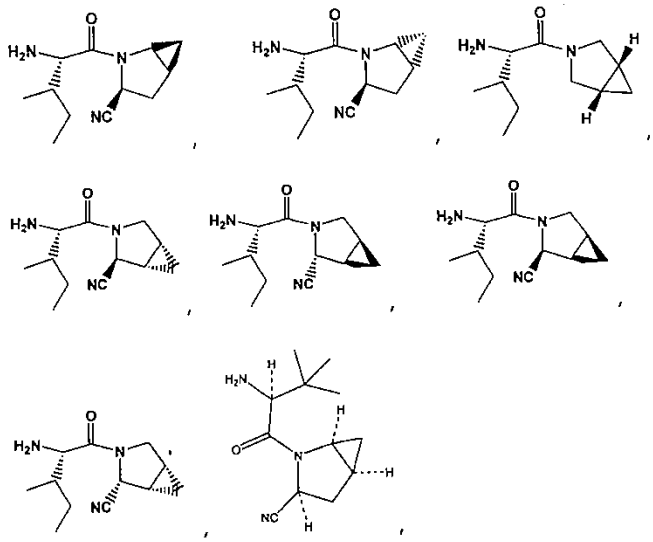
33. In my opinion, and as explained in detail below, the claims of the '186 patent would have been obvious to individuals of ordinary skill in the field prior to and at the time of the earliest possible priority filing date of the '186 patent, i.e., prior to March 10, 2000.

34. Several claim elements of the '186 patent are not described in the'555 application, and instead appear for the first time in the later-filed '173 application. For example, there is no description in the '555 application of: (i) the genus of compounds recited in claim 6; (ii) the specific compounds recited in claim 8 or (iii) the specific compound of claim 25.

35. There is no disclosure in the'555 application of the specific genus of claim 6, in which claim 1 is further limited to:

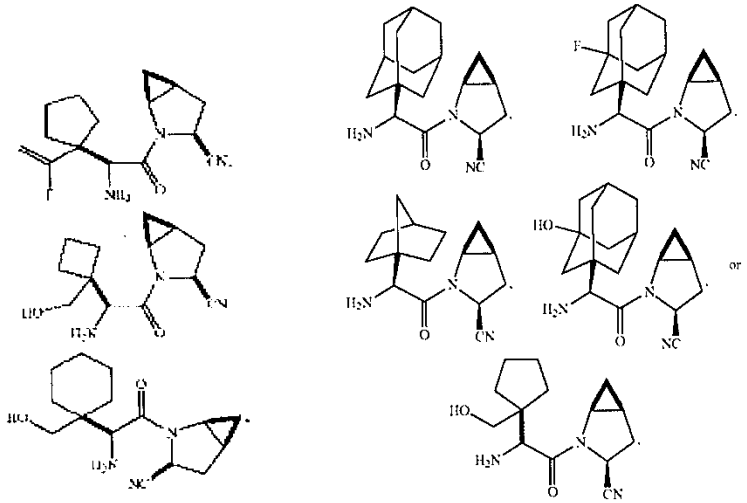
> R^3 is H, R^1 is H, alkyl, cycloalkyl, bicycloalkyl, tricycloalkyl, alkylcycloalkyl, hydroxyalkyl, hydroxyalkylcycloalkyl, hydroxycycloalkyl, hydroxybicycloalkyl, or hydroxytricycloalkyl,
>
> R^2 is H or alkyl, n is 0,
>
> X is CN.

See, e.g., the '555 Application, at p. 6, ll. 10-18; '186 patent, claim 6. Further, the '555 application exemplifies only short, straight and/or branched alkyl substituents at the 2-position as shown below:



See, e.g., *id.* at p. 54, claim 13. Accordingly, one of ordinary skill in the art would have understood that the inventors were not in possession of the genus recited in claim 6 at the time of the '555 application's filing.

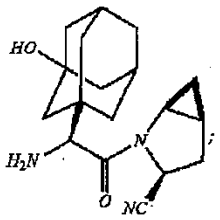
36. Claim 8 of the '186 patent recites a compound as defined in claim 1 having the structure:



or a pharmaceutically acceptable salt thereof. '186 patent at 88:43-89:29.

The only specific compounds described in the '555 application are directed to short, straight and/or branched alkyl substituents at the 2-position. *See*, e.g., the '555 Application at p. 54, claim 13. The compounds of claim 8 were first disclosed in the '173 application. *See*, e.g., U.S. Patent No. 6,395,767 File History, Application filed 2/16/01 at p. 8.

37. Similarly, the compound of claim 25 is also not specifically disclosed in the specification of the '555 application. Independent claim 25 recites the following:



or, a pharmaceutically acceptable salt thereof.

As discussed above, the only specific compounds described in the '555 application are directed to short, straight and/or branched alkyl substituents at the 2-position as depicted in the preceding paragraph. *See*, e.g., the '555 Application at p. 54, claim 13. The compound of claim 25 was first disclosed in the '173 application. *See*, e.g., '767 FH, Application filed 2/16/01 at p. 8. Accordingly, one of ordinary skill in the art could surmise that the inventors were not in possession of the compounds of claims 8 or 25 at the time of the '555 application's filing.

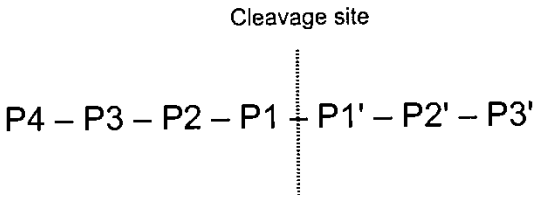
38. As discussed in detail above, several claim elements of the '186 patent are not described in the earliest-filed '555 application, and instead appear for the first time in the later-filed '173 application. Accordingly, one of ordinary skill in the art would have understood that the inventors were not in possession of the claimed subject matter at the time of the '555 application's filing.

IV. RELEVANT TECHNICAL BACKGROUND

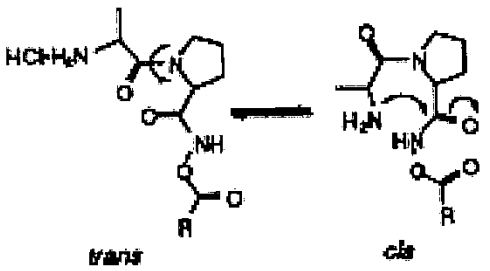
39. Below I describe the details of what was generally known in the art as of March 2000, including: (i) the structure and activity of known DP-IV inhibitors; (ii) principles for improving a compound's drug-likeness and suitability as an orally bioavailable drug and (iii) standard strategies for assessing and modulating compound potency.

40. As set forth above, the Background section of the '186 patent discloses that "inhibitors of dipeptidyl peptidase IV [(DP-IV) are known to] . . . treat[ ] diabetes, especially Type II diabetes." '186 patent at 1:19-21. The Background of the '186 patent also describes what was well known about the mechanism by which DP-IV inhibitors treat type 2 diabetes: "[DP-IV] has been shown to be the primary degrading enzyme of GLP-1(7-36) in vivo . . . [t]hus, inhibition of [DP-IV] in vivo should potentiate endogenous levels of GLP-1(7-36) and . . . thus serve to ameliorate the diabetic condition." *Id.* at 1:59-67.

41. Lin describes what was well known in the art in March 2000 regarding the substrate structural elements required by DP-IV. Lin, et al., "Inhibition of dipeptidyl peptidase IV by fluoroolefin-containing N-peptidyl-O-hydroxylamine peptidomimetics," *Proc. Natl. Acad. Sci. USA*, 95:14020-14024 (1998) at 14020 ("Lin"). For example, Lin reports that "[DP-IV] substrates require the presence of a proline at the P_1 position as well as a protonated free N terminus." *Id.* The P_1, etc. positions are depicted schematically below:



42. Lin also describes what was generally known in the art in March 2000 regarding DP-IV's preferred substrate and inhibitor conformations. For example, Lin reports that "[DP-IV] possesses a high conformational specificity for a trans amide bond between the P1 and N-terminal P2 residues." *Id.* at 14020. Where the P1 residue is the C-terminal residue and the P2 residue is the N-terminal residue (see schematic above for P1 and P2 position designations). The trans and cis conformations of the Lin prolyl dipeptides are depicted below:



Id. at 14022. Lin addresses the importance of the trans conformation for compound stability and its effect on DP-IV inhibition as follows:

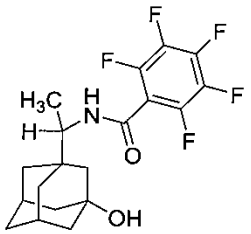
> Many of the problems associated with inefficient inactivation of [DP-IV] are a consequence of the **importance of the trans conformation** of the P_1-P_2 amide bond and the requirement for a protonated free N terminus. **The cyclization reaction of the free N-terminal amino group with the reactive inhibitor . . . require[s] the molecule assume the cis conformation.**

Id. at 14020-14021. Lin reports K_i values of 14,000 nM for a fluoro-olefin dipeptide with the cis conformation and 188 nM for a fluoro-olefin dipeptide isomer with the trans conformation. Comparison of these K_i values for DP-IV inhibition reveals the significantly greater potency of, and preference for, inhibitors in the trans conformation. *See*, e.g., *id.* at 14023 and Table 2.

43. Lin also describes what was well known in the art in March 2000 regarding the effect of conformation on dipeptide stability. Specifically, Lin describes "the cyclization

reaction of the free N-terminal amino acid group with the reactive site of the inhibitor" for related dipeptide compounds (*id.* at 14020-14021), which negatively impacts chemical stability. Lin also observed that intramolecular cyclization was minimized by selecting the trans instead of cis conformation. *Id.* A person of ordinary skill in the art would have also known that having a sterically large substituent at the C2 position would favor a trans conformation and disfavor a cis configuration. *See*, e.g., Pal, D. & P. Chakrabarti, "Cis Peptide Bonds in Proteins: Residues Involved, their Conformations, Interactions and Location," *J. Mol. Biol.*, 294:271-288 (1999), at 274 ("Pal"). Thus from these teachings, a person of ordinary skill in the art would have understood that intramolecular cyclization could be reduced by both selecting against a conformation that favors intramolecular cyclization (i.e., the cis conformation) and through the addition of a large, steric group to the compound, which would also limit the interaction between the free N-terminal amine and the reactive inhibitor.

44. Hoffman, et al., "Pharmacokinetics and Metabolism of Rimantadine Hydrochloride in Mice and Dogs," *Antimicrobial Agents & Chemotherapy*, 32(11):1699-1704 (1988) ("Hoffman"), describes the use of a large, steric adamantyl group in the antiviral drug, rimantadine. As described by Hoffman, the adamantyl moiety was known to be metabolized to a hydroxylated derivative at the 3-position as shown below:



3-hydroxy rimantidine

Hoffman thus serves as further basis that adamantane was generally known in the art to undergo metabolism to yield a 3-hydroxylated adamantyl group.

45. Lipinski, Hansch, Cates, and other references available to one of ordinary skill in the art describe well-known guidelines and strategies for enhancing the drug-like properties of a compound, such as by reducing a compound's partition coefficient, and thus potentially increasing its solubility in aqueous solution. The partition coefficient (P) is a ratio of concentrations of un-ionized compound between two immiscible liquid phases (1-octanol and water), and Log P is a measure of a compound's lipophilicity. For example, addition of a hydroxyl group at the 3-position of an adamantyl moiety would improve the Lipinski parameters, such as reducing Log P and improving water solubility as discussed below.

46. According to Lipinski, et al., "Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings," *Adv. Drug Deliv. Rev.*, 23:3-25 (1996) ("Lipinski"), the use of hydroxyl groups on drugs can increase their water solubility. *See*, e.g., *id.* at 17. Also according to Lipinski, adding a hydroxyl group to a molecule should also reduce the compound's Log P and thus improve its solubility. *See*, e.g., *id.* at 8, 15. These positions are consistent with the knowledge generally available in the art, as evidenced by at least by the Lipinski, Hansch or Cates disclosures.

47. Hansch, et al., "Cluster Analysis and the Design of Congener Sets," *Substituent Constants for Correlation Analysis in Chemistry & Biology*, CH. 6 (John Wiley & Sons 1979) ("Hansch"), describes a process of drug design, in particular molecular modification. *Id.* at 48-49. Hansch describes well-characterized Log P fragment values (Fr) for aliphatic substituents (e.g., hydroxyl = -1.64). *Id.* at 52. Thus according to Hansch, hydroxyl groups have the effect of lowering Log P when added as a compound substituent.

48. Cates, L.A., "Calculation of Drug Solubilities by Pharmacy Students," *Am. J. Pharma. Ed.*, 45:11-13 (1981) ("Cates"), describes a process of approximating whether or not a

drug is soluble in water and how modifications to such a drug will affect the solubility of the drug. *See*, e.g., *id.* at 11-12. Cates discloses, "[t]his procedure involves correlation of partition coefficient values using the octanol/water system and aqueous solubility." *Id.* at Abstract. Referring to partition coefficients based on the octanol/water system expressed as log P, Cates discloses, "[a]lthough this is a measure of the solubility characteristics of the whole molecule, one normally uses the sum of the fragments of the molecule which have been assigned relative hydrophilic-lipophilic values, (π), to calculate log P. Using this procedure, a positive value for π means the substituent, relative to H, favors the octanol phase (i.e., lipophilic). And negative π value indicates its greater affinity for water (*i.e.*, hydrophilic)." *Id.* at 11. Cates also discloses a number of π values that can be used for calculations involving whole molecules, and thus to calculate an approximate log P, which will be indicative of relative solubility. Thus, Cates discloses a method of approximating solubilities, and in particular teaches that addition of hydrophilic substituents increases the solubility of a drug in water. Furthermore, in March 2000, it was also known that an understanding of candidate drug metabolites "can guide structural modifications, thereby improving the activity and/or bioavailability." *See* Korfmacher, et al., "HPLC-API/MS/MS: a powerful tool for integrating drug metabolism into the drug discovery process," *Drug Disc. Today*, 2(12):532-537 (1997) ("Korfmacher"). 3-Hydroxy adamantane is a known metabolite of adamantane. Further, Korfmacher teaches the following advantages of metabolite identification:

> Metabolite identification in drug discovery provides early information that can lead to structural changes in the current lead compound, improving such pharmacokinetic parameters as oral bioavailability, half-life ($t_{1/2}$), or C_{max}. Often these parameters can be changed by improving the metabolic stability of the compound. In order to improve metabolic stability, it is very important to know how a compound is metabolized. The goal of drug discovery is to progress a lead compound into a final candidate drug that can be placed in the development stage. . . **Early metabolite identification can provide information on how to improve**

> **the metabolic stability of the lead structure. In this way, future lead compounds might be a metabolite identified from the previous lead drug or an analog of the previous drug designed to block the major route of metabolism.**

Id. at 534 (emphasis added). Thus according to Korfmacher, one of ordinary skill in the art would have been motivated to make and test a known or potential metabolite when optimizing a lead.

49. As discussed above, the significance of dipeptide conformation on the effective interaction between DP-IV and its substrates and inhibitors was well understood in the art. For example, Lin reported that "[DP-IV] possesses a high conformational specificity for a trans amide bond" and noted "the importance of the trans conformation." Lin at 14020. As would have been understood by one of ordinary skill in the art, the orientation of certain functional groups in the DP-IV inhibitor would be distinct for the cis and trans conformations. *Id.* at 14020-14021.

50. A standard strategy in March 2000 for modulating the orientation of a ring-bound substituent by one of ordinary skill in the art would have been through fusion of the substituent-bearing ring with another ring. *See*, e.g., Chiou, et al., "The Cholinergic Effects and Rates of Hydrolysis of Conformationally Rigid Analogs of Acetylcholine," *J. Pharm. Exp. Ther.*, 166(2):243-248 (1969), at 243 ("Chiou"). Fusion between two rings can result in significant changes in ring flexibility, including ring flattening. *Id.* These changes in turn would have been expected by one of ordinary skill in the art to affect the orientation of ring-bound substituents. *Id.* The smallest ring that can be used for fusion is a cyclopropyl ring. *Id.*

51. Cyclopropyl is one of the most commonly used ring fusion agents because: (i) addition of a cyclopropyl ring has a negligible effect on compound molecular weight, which is an important contributor to better drug-like qualities (*see*, e.g., *id.* at 243); (ii) cyclopropyl has an

exceedingly small footprint, meaning it adds a minimal steric effect on the the compound to which it is attached (*see*, e.g., *id.*) and (iii) cyclopropyl provides greater conformational restriction than larger ring fusions, resulting in fewer conformations in the bicyclic structure (*see*, e.g., *id.*).

52. Chiou explored the fusion of a cyclopropyl ring onto acetylcholine (ACh) in order to produce a compound that was "1) structurally as close to ACh as possible and 2) conformationally as rigid as possible." According to Chiou, cyclopropyl rings "are considered the smallest chemical structure . . . capable of conferring conformational rigidity." *Id.* at 243.

53. As would have been well appreciated by one of ordinary skill in the art in March 2000, an enzyme and its respective substrates and inhibitors typically fit together in a manner analogous to a hand in a glove. *See*, e.g., Koshland, D.E., "The Key-Lock Theory and the Induced Fit Theory, *Angew. Chem. Inl. Ed. Engl.*, 33:2375-2378 (1994) at 2377. Closer degrees of matching often result in greater affinity (with respect to a substrate) or greater inhibition (with respect to an inhibitor). *See*, e.g. *id.* at 2376. Thus inhibitor conformation and functional group orientation are important to effective interactions between enzymes and their inhibitors. *See,* e.g., *id.* Like other enzymes, DP-IV inhibition would have been expected to be improved when the active site and inhibitor fit more closely.

V. LEVEL OF ORDINARY SKILL IN THE ART

54. As discussed above, a POSA is a hypothetical person who is presumed to have typical knowledge and experience in the relevant field or fields at the time of the invention. I have been asked to use the time prior to March 10, 2000,[1] as the relevant timeframe for

[1] References herein to "March 2000," may be understood as referring specifically to the time period on or before March 10, 2000.

assessing validity of the '186 patent, and thus to opine on the qualifications of a POSA as of March 10, 2000. I have also been asked to presume that the POSA is aware of all prior art available as of March 10, 2000. To help me ascertain the qualifications of a POSA, I reviewed the '186 patent.

55. By virtue of my education, experience, and training, I am familiar with the level of skill in the field of the '186 patent on or about March 10, 2000.

56. One of ordinary skill in the field would likely have some combination of the following skills and experience: (i) designing target compounds towards drug discovery; (ii) designing and preparing formulations of drugs that exhibit inhibitory activity; (iii) understanding the biological aspects of drug development, including the drug's effect on the whole animal; and (iv) understanding work presented or published by others in the field, including the patents and printed publications discussed in this declaration.

57. It is my opinion that, as of March 10, 2000, a POSA would be a person with an advanced degree (e.g., a Ph.D.) in pharmaceutics, pharmaceutical chemistry, medicinal chemistry or a related field and at least 2-3 years of practical experience in the design of drugs. Alternatively, a POSA could have had less education but considerable professional experience.

58. My understanding of the level of ordinary skill in the art is corroborated by the specification of the '186 patent, which in many instances provides general rather than specific guidance regarding how the invention would be practiced. For example, the '186 patent lacks specific guidance of the various pharmaceutical combinations that it claims. There are no validated or tested dosages for those combinations, nor any examples describing any actual combinations produced by the inventors. Rather than providing specific guidance regarding dosages for the claimed combinations, the '186 patent invites those of ordinary skill in the art to

turn to the knowledge and resources readily available to them when selecting and formulating appropriate combinations of known drugs. For example, rather than providing specific guidance for the combination dosages, the '186 patent provides very broad dosage ranges (*see*, e.g., '186 patent at 4:48-53), which provide essentially no guidance for selecting actual dosages or treatment regimens. The lack of specific guidance provided in the specification reflects the high level of skill in the art.

59. WO Patent App. Pub. No. 98/19998 (published May 14,1998) ("Villhauer") similarly indicates a high level of skill in the art by relying on that skill to select from the many options disclosed in the specification or known to those in the art. *See*, e.g., *id.* at 2-3 (disclosing large and diverse R groups), *id.* at 3, ll. 20-27 (disclosing pharmaceutically acceptable salts and isomers), *id.* at 7, ll. 22 (teaching that "[t]he process of the invention may be effected in conventional manner."), *id.* at 8, ll. 1-10 (disclosing starting materials known or prepared in known or conventional manner) and *id.* at 20 (disclosing pharmaceutically acceptable carriers, adjuvants and modes of administration, and conventional preparation of same). Villhauer thus reflects the conventional approach in the art to prepare promising variants of lead compounds and compare the results.

60. Claims 13-22 of the '186 patent recite various combinations of DP-IV inhibitors and additional therapeutic agents (e.g., other antidiabetic agents, anti-obesity agents, lipid-modulating agents, etc.). At multiple instances, the '186 patent invites those of ordinary skill in the art to select additional agents or mechanisms beyond those disclosed in the specification for use in combination with the claimed DP-IV inhibitors, for example:

> The term "lipid-modulating" agent as employed herein refers to agents which lower LDL and/or raise HDL and/or lower triglycerides and/or lower total cholesterol **and/or other known mechanisms** for therapeutically treating lipid disorders.

'186 patent at 4:43-47 (emphasis added).

> The other antidiabetic agent may also preferably be a sulfonyl urea . . ., **other known sulfonylureas or other antihyperglycemic agents which act on the ATP-dependent channel of the β-cells** . . .

Id. at 15:5-11 (emphasis added).

> The squalene synthetase inhibitors suitable for use herein include, but are not limited to, a-phosphono-sulfonates . . . **as well as other known squalene synthetase inhibitors** . . .

Id. at 17:47-52 (emphasis added).

> Other hypolipidemic agents suitable for use herein include, but are not limited to, fibric acid derivatives . . . **and other known serum cholesterol lowering agents.**

Id. at 18:1-20 (emphasis added).

> The beta 3 adrenergic agonist which may be optionally employed in combination with a compound of formula I may be AJ9677 (Takeda/Dainippon), L750355 (Merck), or CP331648 (Pfizer) **or other known beta 3 agonists** . . .

Id. at 20:12-18 (emphasis added). The '186 patent's repeated reliance on knowledge available outside the specification itself reflects the high level of skill in the art.

61. Furthermore, the '186 patent defers in several instances to resources readily available to those of ordinary skill in the art for determining dosages and other treatment parameters for the claimed combinations, including 15 citations to the Physician's Desk Reference (PDR). *See*, e.g., *id.* at 15:60-61; 16:4-5; 19:3, 35; 20:43, 50, 57, and 67; 21:9, 15, 24, 41, 47, and 54. For example, the '186 patent states that "[t]he amounts and dosages employed will be as indicated in the Physician's Desk Reference . . ." *id.* at 19:2-4. I agree that the PDR is a resource often used in the art for established dosing and treatment regimens for FDA approved drugs.

62. The '186 patent provides a reasonable expectation of success for practicing the claimed invention to the extent it is enabled by the specification. The background section of the

'186 patent defines the mechanism by which DP-IV inhibitors treat type 2 diabetes, for example, by providing that "[DP-IV] has been shown to be the primary degrading enzyme of GLP-1(7-36) in vivo . . . [t]hus, inhibition of [DP-IV] in vivo should potentiate endogenous levels of GLP-1(7-36) and . . . thus serve to ameliorate the diabetic condition." *Id.* at 1:59-67. Villhauer provides evidence for what was generally known in the art by teaching that because "GLP-1 is a major stimulator of pancreatic insulin secretion and has direct beneficial effects on glucose disposal, [DP-IV] inhibition . . . represent[s] an attractive approach for treating non-insulin-dependent diabetes mellitus (NIDDM)." Villhauer at 1. In addition, the '186 patent teaches that the claimed DP-IV inhibitors can be used "for treating diabetes, especially Type II diabetes." '186 patent at 3:53-4:1. Given the high level of skill in the art and the advanced knowledge in the art regarding the activity of DP-IV inhibitors, one of ordinary skill in the art would have had a reasonable expectation for treating diabetes to the same extent the claims of the '186 are enabled by the specification.

VI. OVERVIEW OF PRIOR ART REFERENCES

63. I have reviewed several references that I believe teach or suggest the compounds, pharmaceutical compositions, pharmaceutical combinations, and methods recited in the asserted claims of the '186 patent. By the virtue of their publication dates, I understand that these references, described in more detail below, are considered prior art to the '186 patent.

A. Ashworth, et al., "2-cyanopyrrolidides as potent, stable inhibitors of dipeptidyl peptidase IV," *Bioorg. & Med. Chem. Ltrs.*, 6(10):1163-166 (1996) ("Ashworth")

64. Ashworth shows a publication date of May 21, 1996. I understand that Ashworth can be applied in assessing the validity of the '186 patent claims.

65. Ashworth discloses a series of stable, potent inhibitors of dipeptidyl peptidase IV (DP-IV), which incorporate a 2-cyanopyrrolidide moiety into the structure of the inhibitor. Ashworth describes DP-IV inhibitors as having the following activity:

> [DP-IV] is a serine protease which catalyses the cleavage of dipeptides from the N-terminus of proteins with the sequence H-X-Pro-Y or H-X-Ala-Y (where X, Y= any amino acid, Y ≠ Pro).

Id. at 1163.

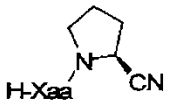
66. DP-IV inhibitors were known to be useful for the treatment of conditions such as type 2 diabetes. *See*, e.g., Villhauer at Abstract.

67. DP-IV inhibitors are typically evaluated and optimized for a variety of properties including their stability and potency. *See*, e.g., Ashworth at Table II. Stability is a sum of chemical and metabolic stability, both of which ultimately impact the quantity of active inhibitor available for interaction with an enzyme target. Chemical stability of DPP IV inhibitors is assessed in solution using a well known set of conditions (see e.g. Ashworth I) and includes solid state stability as required by FDA in accordance with ICH guidelines. *See*, e.g., Ariëns, E.J. & A. M. Simonis, "Optimalization of Pharmacokinetics – An Essential Aspect of Drug Development – by 'Metabolic Stabilization'," *Strategy in Drug Research*, pp. 165-178 (Elsevier Sci. Pub. Co. 1982), at 173-178 ("Ariens"); Dept. of Health and Human Services, "Int'l Conf. on Harmonisation; Stability Testing of New Drug Substances and Products; Guideline; Availability," *Federal Register*, 59:48754-48759 (Sept. 22, 1994) ("FDA Stability Guidelines"). Inhibitor metabolic stability is generally measured in terms of an inhibitor's half-life ($t_{1/2}$), with longer half-lives representing greater stability. *See*, e.g., Obach, R.S., "Prediction of Human Clearance of Twenty-nine Drugs from Hepatic Microsomal Intrinsic Clearance Data: An Examination of In Vitro Half-life Approach and Nonspecific Binding to Microsomes," *Drug*

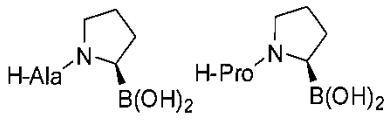
Metab. Dispos., 27(11):1350-1359 (1999), at 1354-1355 ("Obach"). Potency reflects the degree to which an inhibitor acts on its target. Inhibitor potency is generally measured in terms of its dissociation constant (K_i) or using an IC_{50} concentration (i.e., a concentration of a potential inhibitor at which enzyme activity is reduced by 50% from control), which represents the propensity of dissociation between an inhibitor and its target, with smaller K_i or IC_{50} values representing greater potency. Cheng, Y. & W.H. Prusoff, "Relationship Between the Inhibition Constant (K_I) and the Concentration of Inhibitor which Causes 50 Per Cent Inhibition (I_{50}) of an Enzymatic Reaction," *Biochem. Pharmacol.*, 22:3099-3108 (1973), at 3099 ("Cheng").

68. Some of the compounds described in Ashworth have K_i values of less than 5 nM versus human DP-IV and chemical stability half-lives of greater than 48 hours in aqueous solution at pH 7.4. Ashworth at 1163. Thus some of the compounds of Ashworth have both high potency and good chemical stability in solution, making them desirable drug leads.

69. Ashworth discloses 29 compounds, six of which contain the 2-cyanopyrrolidide moiety, as follows:



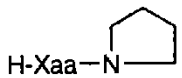
where Xaa represents various amino acid substituents as specified in Table II. *Id.* at 1166. Ashworth discloses that the most potent DP-IV inhibitors previously known were the boroproline analogues **1**, and **2**,



1 **2**

which had K_i values of 2 nM and 3 nM respectively. *Id.* at 1163. Ashworth concludes that the 2-cyanopyrrolidide analogues "possessed activity comparable to the boroprolines, 1 and 2." *Id.* at 1165. In addition, Ashworth teaches that the 2-cyanopyrrolidide analogues exhibited "superior stability in aqueous solution" relative to boroprolines **1** and **2**. *Id.* at 1164.

70. Ashworth discloses the DP-IV inhibition activity of 19 aminoacyl pyrrolidides (lacking the 2-cyano moiety) in Table I:

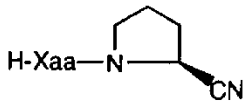


Compound N°	Xaa	K_i (μM)[13]
5	Cyclohexylglycine [Chg]	0.064 ± 0.01
6	(R,S)-Cyclopentylglycine [Cpg]	0.21 ± 0.04
7	Ile	0.41 ± 0.01
8	*allo*-Ile	0.44 ± 0.04
9	Val	0.47 ± 0.02
10	Lys(Cbz)	0.52 ± 0.07
11	tert-Butylglycine [Tbg]	0.88 ± 0.20
12	Thr(Me)	0.90 ± 0.15
13	Orn(Cbz)	0.91 ± 0.20
14	2-Aminohexanoic acid [Aha]	1.20 ± 0.20
15	Glu	2.00 ± 0.40
16	Pro	2.10 ± 0.20
17	Cyclohexylalanine [Cha]	2.15 ± 0.50
18	Glu(OBn)	2.70 ± 0.30
19	Thr	4.90 ± 0.90
20	Phenylglycine [Phg]	5.30 ± 0.10
21	Ser(Bn)	6.00 ± 1.50
22	Ala	7.00 ± 1.00
23	Asp	14.50 ± 1.90

Id. at 1164. The compounds of Table I exhibit inhibition activity in the 0.064 μM to 14.50 μM range. Ashworth notes that the most active of these compounds was compound **5**, "[i]n particular, β-branched α-amino acid derivatives were the most potent compounds with the non-

proteinogenic amino acid, **(S)-cyclohexylglycine providing the most active pyrrolidide** (compound **5** possessing a K_i value of 64 nM)."[2] *Id.* at 1165 (emphasis added).

71. By comparison, Table II of Ashworth discloses the DP-IV inhibition activity of six analogues having the 2-cyano moiety:



Compound N°	Xaa	K_i (nM)[13]	$t_{1/2}$ (h)[19]
24	Cpg	1.1 ± 0.2	48
25	Chg	1.4 ± 0.5	>48
26	Ile	2.2 ± 0.5	48
27	Tbg	3.8 ± 0.8	>48
28	Lys(Z)	5.2 ± 1.0	24
29	Pro	22.0 ± 4.0	7.5

Id. at 1166. The compounds of Table II (having a 2-cyanopyrrolidide moiety) exhibit inhibition activity in the 1.1 nM to 22 nM range, which in some cases equates to a 50-fold increase in inhibition activity relative to compound **5**, which lacks the 2-cyano moiety. This clearly shows that the cyano moiety is engaged in an interaction with DP-IV that improves affinity of a potential inhibitor with the enzyme.

72. Ashworth teaches that, "[s]ubstrates and inhibitors of DP-IV require a free N-terminus, which means that potential dipeptide serine protease inhibitors (e.g., C-terminal aldehydes, boronic acids, a-ketoacids, trifluoromethylketones, or chloromethylketones) are inherently unstable at neutral pH due to intramolecular cyclisation" likely via a cis conformation of the dipeptide. *Id.* at 1163.

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[2] The value of 0.064 μM, as given for Ashworth compound **5** in Table 1, can alternatively be written as 64 nM; both these notations refer to the same amount, and should be understood to be interchangeable in both Ashworth and my report.

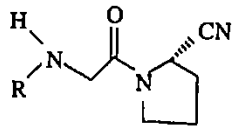
73. Table II of Ashworth shows the stability data of DP-IV inhibitors in aqueous solution (pH of 7.4), as measured in half-life ($t_{1/2}$). *Id.* at 1166. When changing the substituent at the 2-position of the acetyl-pyrrolidine-2-carbonitrile from a straight chain alkyl moiety, for example compound **28** (lysine moiety), to a moiety having at least one branch at the α-carbon, such as the cyclohexyl moiety in Ashworth compound **25**, the stability increases from 24 hours to greater than 48 hours. *Id.* at 1166. Ashworth discloses that substitution at this position improves the potency of an aminoacyl pyrrolidide compound as shown in Table I of Ashworth. *Id.* at 1163 and 1166 (compare Tables I and II).

74. Specifically, compound **5** (having a cyclohexyl group) of Table I shows at least a 100-fold increase in potency compared to compound **7** (having a 1-methyl propyl group) or compound **9** (having an isopropyl group). Ashworth confirms this observation by noting, "[a]s expected, from the substrate specificity of DP-IV, only (S)-amino acid derivatives showed any activity and, as can be seen in Table I, lipophilic amino acids gave more potent compounds. In particular, β-branched α-amino acid derivatives were the most potent compounds with the non-proteinogenic amino acid, (S)-cyclohexylglycine providing the most active pyrrolidide (compound **5** possessing a K_i value of 64 nM)." *Id.* at 1165. Thus the combined properties of potency and solution stability make compound **25** an attractive lead that can be further improved.

B. WO Patent App. Pub. No. 98/19998 ("Villhauer")

75. Villhauer was published May 14, 1998. Thus, I understand that Villhauer can be applied in assessing the validity of the '186 patent claims.

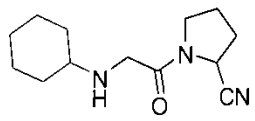
76. Villhauer discloses numerous DP-IV serine protease inhibitor compounds. The dipeptide compounds of Villhauer are N-(N'-substituted glycyl)-2-cyanopyrrolidines having the following structure of formula (I):



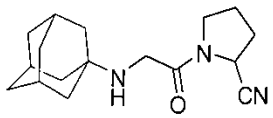
(I)

Id. at Abstract.

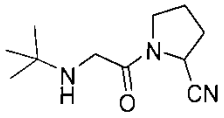
77. Villhauer discloses various substituents at position "R" of formula (I) above. For example, Villhauer discloses compounds having cycloalkyl groups attached to the amino moiety of 2-cyano pyrrolidides, including a cyclohexyl group:



(*id.* at 12, Example No. 28); adamantyl groups attached to the amino moiety of 2-cyano pyrrolidines:



(*id.* at 13, Example No. 47); and alkyl groups attached to the amino moiety of 2-cyano pyrrolidides:



(*id.* at 13, Example No. 52). Villhauer also teaches how the compounds shown directly above were prepared. *Id.* at 8-10. Each of the variations cited above are considered bulky lipophilic groups.

78. Villhauer discloses the use of DP-IV inhibitors, such as N-(N'-substituted glycyl)-2-cyanopyrrolidines, for "the treatment of conditions mediated by DPP-IV, such as non-insulin-

dependent diabetes melitus." *Id.* at Abstract. As disclosed in Villhauer, "DPP-IV is responsible for inactivating glucagon-like peptide-1 (GLP-1) . . . [and] [s]ince GLP-1 is a major stimulator of pancreatic insulin secretion and has direct beneficial effects on glucose disposal, DPP-IV inhibition [is] an attractive approach for treating non-insulin-dependent diabetes mellitus (NIDDM)." *Id.* at 1, ll. 6-13.

79. Villhauer discloses that the DP-IV inhibitor compound may be in free form or in acid addition salt form, where the salt could be from any pharmaceutically acceptable acid, with hydrochloride as a preferred option. *Id.* at 3. Similarly, Villhauer discloses that any pharmaceutically acceptable carriers, adjuvants and enteral or parenteral administration forms (prepared by conventional means) could be used with any of the disclosed agents of the invention. *Id.* at 20.

80. Villhauer further discloses the use of DP-IV inhibitors, such as N-(N'-substituted glycyl)-2-cyanopyrrolidines, for "use in **treating conditions mediated by DPP-IV** [such as] **non-insulin-dependent diabetes mellitus** . . . [and] **obesity** . . ." *Id.* at 18, ll. 18-21 (emphasis in original).

81. In an effort to modify Ashworth compound **25**, a POSA would be motivated to investigate the observations of Villhauer to explore additional lipophilic substituents at C2 of compound **25**, including an adamantyl moiety and other β-branched amino acids. This optimization would include exploration of solid state stability as required by FDA regulations.

C. Raag, R. & T.L. Poulos, "Crystal Structure of Cytochrome P-450$_{CAM}$ Complexed with Camphane, Thiocamphor, and Adamantane: Factors Controlling P-450 Substrate Hydroxylation," *Biochem.*, 30:2674-2684 (1991) ("Raag")

82. Raag shows a publication date of March 1, 1991. Thus, I understand that Raag can be applied in assessing the validity of the '186 patent claims.

83. Raag describes a study of the metabolism of adamantane by cytochrome P-450_{cAm} and corresponding X-ray crystal structures for complexes of cytochrome $P450_{cAm}$ with adamantane as the substrate. Raag describes the metabolic hydroxylation of adamantane, as well as other substrates. Table III from Raag, presented below, shows the various molecules that underwent hydroxylation, where the adamantane group is shown in the third column:

Table III: Various Substrate-Dependent Parameters[a]

	 camphor	 adamantanone	 adamantane	 thiocamphor	 camphor/Y96F	 norcamphor	 camphane
molec vol	315 Å^3	300 Å^3	293 Å^3	322 Å^3	315 Å^3	236 Å^3	309 Å^3
hydrogen bond to Y96	yes[a]	yes[b]	no	no	no	yes[b]	no
no. of iron ligands	5[a]	5[b]	6	6		6[b]	6
redox pot. Fe^{3+}/Fe^{2+}	−170 mV[c]	−175 mV[c]				−206 mV[c]	
high-spin %	94–97%[c–e]	96–98%[c,d]	99%[d]	65%[e]	59%[e]	46%[c]	46%[e]
regiospecif of substr hydroxylatn	5-exo (100%)[d–f]	5 (100%)[d]	1 (100%)[d]	5-exo (64%)[e] 6-exo (34%) 3-exo (2%)	5-exo (92%)[e,f] 4 (1%) 6-exo (2–4%) 3-exo (0–4%) 9 (<1%)	5-exo (45%)[f] 6-exo (47%) 3-exo (8%)	5-exo (90%)[e] 6-exo (10%)
substr temp factor (Fe^{3+})	16.2 Å^2[a]	16.5 Å^2[b]	24.7 Å^2	23.5 Å^2		33.5 Å^2[b]	30.1 Å^2
substr hydrophilic groups	yes	yes	no	yes	yes	yes	no
hydroxylatn "efficiency"	100%[e,f]			98%[e]	100%[e]	12%[f]	8%[e]
L6–substr dist	NA	NA	2.63 Å	2.35 Å (70%) 3.35 Å (30%)		3.0 Å[b]	2.88 Å
L6–iron dist	NA	NA	1.95 Å	1.35 Å		1.73 Å[b]	1.67 Å
L6 occupancy	NA	NA	1.00	0.90		0.97[b]	1.00
L6 temp factor	NA	NA	14.3 Å^2	19.6 Å^2		3.8 Å^2[b]	7.7 Å^2
cation occupancy	1.00[a]	1.00[b]	0.89	0.91		1.00[b]	0.72
cation temp factor	12.1 Å^2[a]	10.0 Å^2[b]	15.5 Å^2	14.2 Å^2		7.9 Å^2[b]	21.7 Å^2

[a] Poulos et al. (1985, 1987). [b] Raag and Poulos (1989a). [c] Fisher and Sligar (1985). [d] White et al. (1984). [e] Atkins and Sligar (1988b). [f] Atkins and Sligar (1989). [g] Carbon numbering for each substrate begins with C-1 at the top of the six-membered ring which is in the plane of the table. Numbering proceeds counterclockwise such that the carbonyl carbon is C-2 and C-5 is in the lower right-hand portion of the ring. Note that C-5 is a secondary carbon in some substances and a tertiary carbon in others.

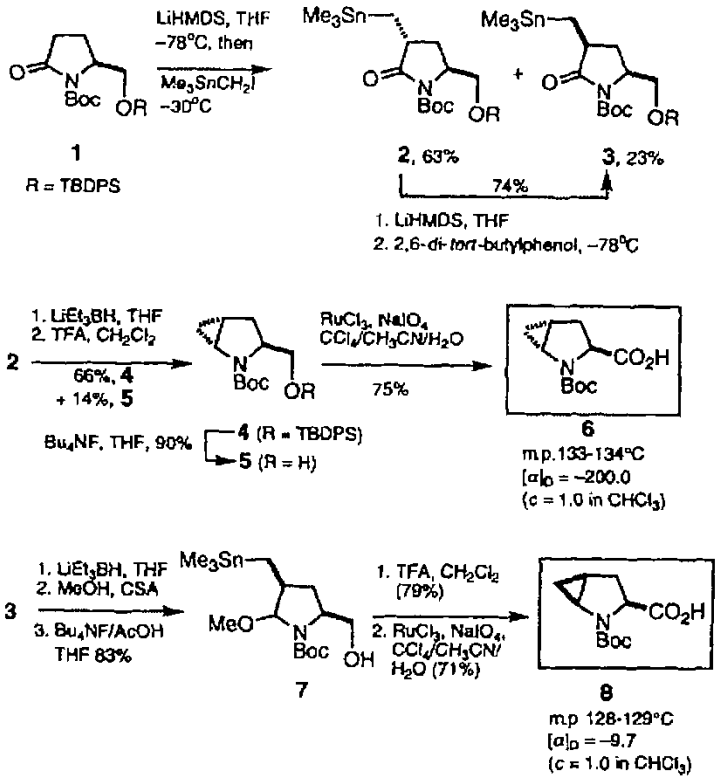
84. Raag, Table III, shows that substrates that undergo regiospecific hydroxylation of adamantane by cytochrome P450 (a xenobiotic metabolizing enzyme). Hydroxylation occurs at a specific position, i.e., at a tertiary carbon site (and not a secondary carbon site). Raag also teaches that "[a]damantane is . . . metabolized to a single product despite having a relatively high active site mobility. The single product can be attributed to the existence of only two types of unique carbon atoms in adamantane, together with the greater reactivity of tertiary versus secondary carbons." *Id.* at 2678.

85. With this knowledge, a POSA would be motivated to investigate an hydroxyl-adamantyl derivative of Ashworth compound **25** to improve water solubility and metabolic stability of a lead molecule. *See* Lipinski at 8, 15; Ariens.

D. Hanessian, et al., "The Synthesis of Enantiopure ω-Methanoprolines and ω-Methanopipecolic Acids by a Novel Cyclopropanation Reaction: The 'Flattening' of Proline," *Angew. Chem. Int. Ed. Engl.*, 36(17):1881-1884 (1997) ("Hanessian")

86. Hanessian shows a publication date of September 17, 1997. Thus, I understand that Hanessian can be applied in assessing the validity of the '186 patent claims.

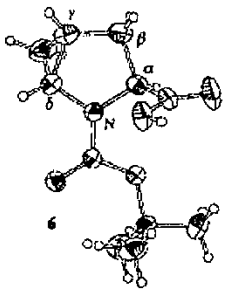
87. Hanessian describes the synthesis of enantiopure ω-methanoproline acids by a cyclopropanation reaction. Hanessian teaches that the use of conformationally constrained analogues of proline were well known in peptidomimetic research at the time the application which led to the '186 patent was filed. Hanessian describes the "highly stereocontrolled syntheses of the diastereomeric 4,5-methano-L-prolines and 5,6-methano-L-pipecolic acids by a novel intramolecular cyclopropanation reaction of iminium ions . . . ." *Id.* at 1882. One such example shown by Hanessian is compound **8** which was synthesized according to Scheme 1 below:



Id.

88. The structure and conformation of compound **8** in the solid state were unambiguously confirmed by single crystal X-ray analysis. Further, Table 1 of Hanessian, as reproduced below, shows selected torsion angles for compound **8** where "considerable 'flattening' of the pyrrolidine ring is observed relative to N-Boc-L-proline." *Id.* Thus, Hanessian provides evidence for adjusting the orientation of substituents in the pyrrolidine ring resulting from cyclopropane fusion. Further, a POSA would recognize that cyclopropane fusion to a pyrrolidine ring would result in a ring system with limited conformational flexibility compared to a simple pyrrolidine ring.

Table 1. Selected torsion angles and root-mean-square deviations from fitted atoms in a given plane of X-ray crystal structures, and ^{13}C NMR chemical shifts ($CDCl_3$).



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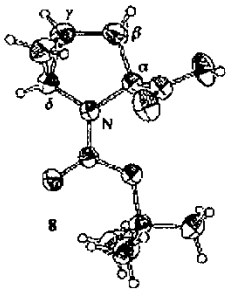


8

	N-Boc-L-proline [20]	6	8
τ(NC$_\alpha$)	−17	−5.6	−14.4
τ(C$_\alpha$C$_\beta$)	+31	+4.8	+15.3
τ(C$_\beta$C$_\gamma$)	−35	−2.6	−11.4
τ(C$_\gamma$C$_\delta$)	+24	−0.7	+2.9
τ(C$_\delta$N)	−4	+4.1	+7.6
τ(BocNC$_\alpha$CO$_2$H)	−72	−64.0	−67.1
rms deviation of fitted atoms	0.018 C$_\alpha$, N, C$_\delta$, C$_\gamma$	0.003 C$_\beta$, C$_\gamma$, C$_\delta$, N	0.013 C$_\beta$, C$_\gamma$, C$_\delta$, N
	δ(cis/trans)	*δ(cis/trans)*	*δ(cis/trans)*
COOH	178.35/176.60	177.7/175.5	179.1/176.1
NC=O	153.95/155.39	157.1/154	155.7/154.1
C$_\alpha$	58.8	60.8/60.1	59.5/59.1
C$_\beta$	30.75/29.53	32.0	31.5/29.1

Id.

89. Hanessian confirms the "flattening" of the pyrrolidine ring by determining the root-mean-square values for the C_β and N atoms from the plane defined by C_β, C_γ, C_δ and N (*see* Table 1 above). *Id.* Hanessian determined that for compound **8**, the out-of-plane carbon was the carbon bearing the carboxyl group (C_α) when compared to *N*-Boc proline. *Id.* Hanessian thus observed that by flattening the pyrrolidine ring, the orientation of the out-of-plane carbon was modified with respect to the ring.

90. A POSA would recognize that a substituent bonded at this C_α carbon would be expected to have its orientation in space modified compared to proline and because of the limited conformational flexibility of this cyclopropyl-fused pyrrolidine ring.

E. WO Patent App. Pub. No. 99/38501 ("Bachovchin")

91. Bachovchin was published August 5, 1999. Thus, I understand that Bachovchin can be applied in assessing the validity of the '186 patent claims.

92. Bachovchin discloses improved methods for reducing in animal subjects (including humans) at least one type of insulin resistance, hyperinsulinemia, and Type II diabetes. In particular, Bachovchin describes the use of DP-IV inhibitors in an amount effective to treat, among other indications, Type II diabetes. Claim 4 of Bachovchin recites, "[a] method for treating Type II diabetes, comprising administering to an animal a composition including one or more inhibitors of dipeptidylpeptidase IV [(DP-IV)]." *Id.* at claim 4. Moreover, various examples of DP-IV inhibitors are listed in Bachovchin. For example, a class of DP-IV inhibitors shown in Bachovchin are compounds based on the dipeptide (D)-Ala-(L)-Ala. Bachovchin also discloses the use of pharmaceutical compositions of DP-IV inhibitors, "another aspect of the present invention relates to pharmaceutical compositions of dipeptidylpeptidase inhibitors, particularly [DP-IV] inhibitors." *Id.* at 7, ll. 29-30. Thus, the use of DP-IV inhibitors for treating type 2 diabetes was well known at least one year before the application which led to the '186 patent was filed.

93. Bachovchin also discloses that "[i]n particular, it is an object of the invention to provide methods for producing long lasting beneficial changes . . . to provide effective treatments for diabetes [and] obesity . . ." *Id.* at 4, ll. 7-11. Bachovchin also discloses "administration of a [DP-IV] inhibitor . . . in an amount effective to improve one or more aberrant indices associated with glucose metabolism disorders . . . [such as] Type II diabetes . . . [and] obesity." *Id.* at 6, ll. 16-22.

94. Furthermore, the use of DP-IV inhibitors in combination therapy with various antidiabetic agents other than a DP-IV inhibitor for treating type 2 diabetes is also described extensively in Bachovchin:

> The [DP-IV] inhibitors used according to the invention can also be used conjointly with agents acting on the ATP-dependent potassium channel of the β-cells, such as glibenclamide, **glipizide**, **gliclazide** and AG-EE 623 ZW. The DPIV inhibitors may also advantageously be applied in combination with other oral agents such as **metformin** and related compounds or glucosidase inhibitors as, for example, **acarbose**.

Id. at 46 (emphasis added.)

F. XENICAL Label (available by FOIA Aug. 9, 1999) ("the Xenical Label")

95. The Xenical Label was published August 9, 1999 following FDA approval of Xenical. Thus, I understand that the Xenical Label can be applied in assessing the validity of the '186 patent claims.

96. The Xenical Label discloses the FDA-approved uses of the anti-obesity agent, Xenical (i.e., orlistat), and supporting studies. In one such study, patients with type 2 diabetes were treated with Xenical. As indicated in the Xenical Label, "XENICAL is indicated for obesity management . . . in the presence of other risk factors (e.g., . . . diabetes)." *Id.* at Label, p. 8. According to the Xenical Label, "epidemiological studies have established a relationship between obesity and visceral fat and the risks for cardiovascular disease [and] type 2 diabetes." *Id.* at p. 3. The Xenical Label also provides dosages for Xenical as follows: "[t]he recommended dose of XENICAL is one 120 mg capsule three times a day." *Id.* at p. 14.

G. MEVACOR Label (available by FOIA Sept. 15, 1994) ("the Mevacor Label")

97. The Mevacor Label was published September 13, 1994 following FDA approval of Mevacor. Thus, I understand that the Mevacor Label can be applied in assessing the validity of the '186 patent claims.

98. The Mevacor Label discloses the FDA-approved uses of the lipid-modulating agent, Mevacor (i.e., lovastatin), and supporting studies. As indicated in the Mevacor label, "poorly controlled diabetes mellitus" was considered a "secondary cause[ ] for hypercholesterolemia . . ." *Id.* at Package Insert, p. 4. The Mevacor Label also provides dosages for Mevacor as follows: "[t]he recommended dosing range is 20-80 mg/day in a single or two divided doses; the maximum recommended dose is 80 mg/day." *Id.* at p. 8.

H. GLUCOPHAGE Label (available by FOIA Jan. 8, 1998) ("the Glucophage Label")

99. The Glucophage Label was published January 8, 1998 following FDA approval of Glucophage. Thus, I understand that the Glucophage Label can be applied in assessing the validity of the '186 patent claims.

100. The Glucophage Label discloses the FDA-approved uses of the antihyperglycemic agent, Glucophage (i.e., metformin), and supporting studies. The Glucophage label describes a "29-week double-blind, placebo-controlled study of GLUCOPHAGE and glyburide, alone and in combination . . . in obese patients with type 2 diabetes," where glyburide is also an antihyperglycemic agent for use in treating patients with type 2 diabetes. *Id.* at Package Insert, p. 4. The Glucophage Label also provides dosages for Glucophage as follows: "[i]n general, clinically significant responses are not seen at doses below 1500 mg per day . . . [and] GLUCOPHAGE may be given to a maximum daily dose of 2550 mg per day." *Id.* at p. 35.

VII. THE ASSERTED CLAIMS OF THE '186 PATENT ARE OBVIOUS OVER THE PRIOR ART

101. As explained in detail below, it is my opinion that each element of claims 8, 9, 25, and 26 is taught in the combination of Ashworth, Villhauer, Raag, and Hanessian, and that one of

ordinary skill in the art would have been motivated to combine these teachings by March 10, 2000.

102. Each of claims 8, 9, 25, and 26 is presented below in bold text followed by my analysis of the claims. The analysis below identifies exemplary disclosure of the cited references with respect to the corresponding claim elements, and is not meant to be exclusive or exhaustive.

103. Claims 8 and 25 of the '186 patent each disclose a specific chemical compound or a pharmaceutically acceptable salt of that compound, while claims 9 and 26 depend from these respective claims, further specifying one or more particular pharmaceutically acceptable salts. Therefore, claims 8 and 25 will be addressed first, followed by claims 9 and 26.

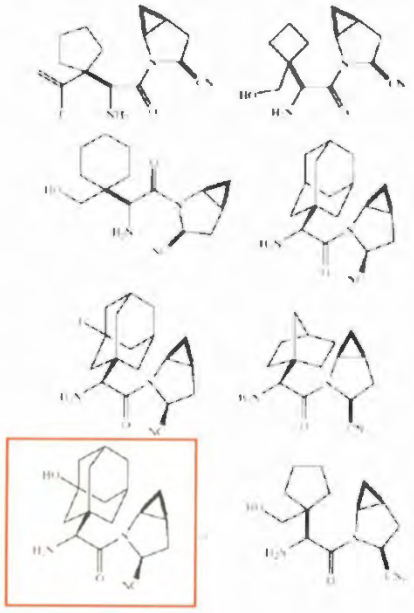
104. As discussed above, Ashworth, Villhauer, Raag, and Hanessian can be considered prior art to the '186 patent.

105. Where reference is made to additional publications below, those publications are intended to provide additional bases regarding what was generally known in the art in March 2000 and provide factual basis for my statements. Those references are not intended to serve as additional grounds for invalidity.

A. Each Feature of Claims 8 and 25 is Disclosed by Ashworth, Villhauer, Raag, and Hanessian

Claim 8:

"A compound having the structure:

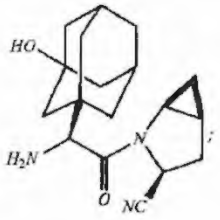


or a pharmaceutically acceptable salt thereof."

The red box outline is provided for illustration purposes and does not appear in the original claim, but the structure shown therein is equivalent to the compound of claim 25.

Claim 25:

"A compound that is

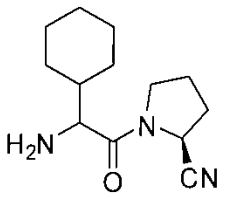


or a pharmaceutically acceptable salt thereof."

106. As will be discussed in greater detail below, the prior art provides specific motivation to select Ashworth compound 25 as a lead compound for further optimization. One of ordinary skill in the art would understand that certain core features of Ashworth compound 25 should be retained in the ultimate compound, including the glycyl-proline core, free amine, and cyano components of Ashworth compound 25. As explained below, one of ordinary skill also would have been motivated to favor a structure in which the trans orientation of the dipeptide in order to enhance its activity and chemical stability. *See* Lin; FDA Stability Guidelines. There was also motivation to substitute the cyclohexyl group of Ashworth compound **25** with a hydroxylated adamantyl group in order to improve the compound's chemical and metabolic stability. *See* Ashworth; Raag; Villhauer. One would have further been motivated to fuse a cyclopropyl ring onto the pyrrolidine ring of Ashworth compound 25 as a means to improve the interaction between the active 2-cyano moiety with the DP-IV enzyme. *See* Hanessian and Ashworth. Thus, as evidenced by prior art references (including Ashworth, Villhauer, Raag and Hanessian), claims 8 and 25 of the '186 patent would be considered obvious to one of ordinary skill in the art in view of the knowledge and motivation provided by the references to the skilled artisan.

1. A POSA Would Have Been Motivated to Select Ashworth's Compound 25 as a Lead Compound

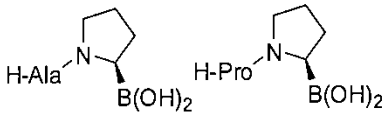
107. The prior art provided ample motivation for one of skill in the art to select the Ashworth's compound **25** as a lead compound to develop a composition as an inhibitor of DP-IV enzyme, as claimed in claims 8 and 25 of the '186 patent.



Ashworth Compound **25**

108. Ashworth discloses potent dipeptide analogues incorporating a 2-cyanopyrrolidide moiety useful as inhibitors of dipeptidyl peptidase IV. Ashworth at 1163 (disclosing, "[a] novel series of stable, potent inhibitors of [DP-IV] has been developed. A number of dipeptide analogues, incorporating a 2-cyanopyrrolidide, were found to have K_i values of less than 5 nM versus human DP-IV and half-lives of >48h in aqueous solution . . . ."). Because Ashworth explicitly discloses the use of the 2-cyanopyrrolidide compounds as inhibitors of DP-IV, a person of skill in the art had express motivation to select the disclosed compounds with a reasonable expectation of success for developing improved compositions useful as inhibitors of DP-IV.

109. Further, Ashworth provides motivation to select the 2-cyanopyrrolidine analogues. For example, Ashworth teaches that the 2-cyanopyrrolidide analogues "possessed activity comparable to the boroprolines, 1 and 2" which were previously known as the most potent DP-IV inhibitors as shown below:



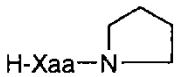
1 **2**

Id. at 1165. The boroprolines **1** and **2** had K_i values of 2 nM and 3 nM respectively. A POSA would recognize a molecule such as Ashworth compound **25,** with molecular weight less than 400 daltons and a K_i value less than 3 nM as a potential lead compound. *Id.* at 1163. Because

the Ashworth reference teaches that 2-cyanopyrrolidide analogues have activity comparable to the boroproline analogues, one of ordinary skill in the art had express motivation to select the 2-cyanopyrrolidide analogues as lead compounds to arrive at the compound in claims 8 and 25 of the '186 patent with a reasonable expectation of success. Furthermore, one of ordinary skill in the art would select the 2-cyanopyrrolidide analogues as lead compounds because they were more synthetically accessible than the boroprolines and metabolic considerations (boronic acid vs. cyano group) would make them more desirable. *Id.*

110. Further, Ashworth provided ample motivation to specifically select compound **25** as a lead from the limited compounds described therein (*see* below, *id.* at 1166, Table II) because (i) compound **25** is one of only six 2-cyanopyrrolidide analogues (i.e., dipeptide nitriles) described by Ashworth (*see id.*, Table II, listing only six compounds); and (ii) compound **25**, which contains a 2-cyano moiety, exhibits high potency compared to aminoacyl pyrrolidides lacking the 2-cyano moiety (*id.* at 1164 and 1166, compare Tables I and II) and good solution stability compared to other analogs lacking a β-branched moiety at C2. Structures of this type with sterically large groups at C2 prefer to exist in a trans conformation. *See*, e.g., Pal at 274.

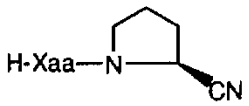
111. For example, Ashworth discloses the DP-IV inhibition activity of 19 aminoacyl pyrrolidides (lacking the 2-cyano moiety) in Table I:



Compound N°	Xaa	K_i (μM)[13]
5	Cyclohexylglycine [Chg]	0.064 ± 0.01
6	(R,S)-Cyclopentylglycine [Cpg]	0.21 ± 0.04
7	Ile	0.41 ± 0.01
8	*allo*-Ile	0.44 ± 0.04
9	Val	0.47 ± 0.02
10	Lys(Cbz)	0.52 ± 0.07
11	tert-Butylglycine [Tbg]	0.88 ± 0.20
12	Thr(Me)	0.90 ± 0.15
13	Orn(Cbz)	0.91 ± 0.20
14	2-Aminohexanoic acid [Aha]	1.20 ± 0.20
15	Glu	2.00 ± 0.40
16	Pro	2.10 ± 0.20
17	Cyclohexylalanine [Cha]	2.15 ± 0.50
18	Glu(OBn)	2.70 ± 0.30
19	Thr	4.90 ± 0.90
20	Phenylglycine [Phg]	5.30 ± 0.10
21	Ser(Bn)	6.00 ± 1.50
22	Ala	7.00 ± 1.00
23	Asp	14.50 ± 1.90

Id. at 1164. The compounds of Table I (i.e., dipeptides lacking the 2-cyano moiety) have inhibition activity in the 0.064 μM to 14.50 μM range. Ashworth notes that the most active of these compounds was compound **5**, "[i]n particular, β-branched α-amino acid derivatives were the most potent compounds with the non-proteinogenic amino acid, **(S)-cyclohexylglycine providing the most active pyrrolidide** (compound **5** possessing a K_i value of 64 nM)." *Id.* at 1165 (emphasis added).

112. In comparison, Table II of Ashworth discloses the DP-IV inhibition activity of six analogues having the 2-cyano moiety:



Compound N°	Xaa	K_i (nM)[13]	$t_{1/2}$ (h)[19]
24	Cpg	1.1 ± 0.2	48
25	Chg	1.4 ± 0.5	>48
26	Ile	2.2 ± 0.5	48
27	Tbg	3.8 ± 0.8	>48
28	Lys(Z)	5.2 ± 1.0	24
29	Pro	22.0 ± 4.0	7.5

Id. at 1166. The six compounds of Table II (all having a 2-cyanopyrrolidide moiety) have inhibition activity in the 1.1 nM to 22 nM range, which in some cases equates to a 50-fold increase in inhibition activity over the same compounds lacking the 2-cyano moiety. *Id.* at 1164, 1166. For example, compound **5** has a K_i value of 64 nM, which decreases to 1.4 nM in compound **25** solely through the addition of a 2-cyano moiety. This decrease in inhibition activity represents significantly greater DP-IV inhibition potency for compound **25** relative to compound **5**. This illustrates the beneficial effect on potency of the cyano moiety in DP-IV inhibitors. This further suggests to a POSA that the cyano moiety interacts with DP-IV to form an attractive interaction and provides motivation to optimize this to further improve potency.

113. Taken as a whole, the Ashworth reference contains ample and explicit motivation for one of skill in the art to select compound **25** as a lead for designing DP-IV inhibitors such as the compounds recited in claims 8 and 25 of the '186 patent. As evidenced by Ashworth, the inventors of the '186 patent were not the first to recognize the use of this core structure in designing DP-IV inhibitor compounds.

2. A POSA Would Have Been Motivated Modify Ashworth Compound 25 While Retaining Certain Structural Elements

114. One of ordinary skill in the art would have been motivated to retain certain structural elements of Ashworth compound **25** when optimizing it toward the ultimate

compound. For example, by March 2000, it was well known in the art that proline dipeptides and mimetics were therapeutically useful for a variety of purposes, including DP-IV inhibition. *See*, e.g., Ashworth. Early work with pyrrolidinyl dipeptides revealed high inhibitor potency when coupling a pyrrolidine-containing amino acid with cyclohexylglycine. Villhauer at 1165. Villhauer also identified dipeptide glycyl-cyanopyrrolidines as inhibitors of DP-IV. *See, id.* at Abstract. Therefore, one of ordinary skill in the art would have been motivated to retain the dipeptide structure of Ashworth compound **25**.

115. One of ordinary skill in the art would have been motivated to retain the P_1 proline, free amine, and 2-cyano moieties of the Ashworth compound **25** based on the knowledge generally available in the art. For example, Ashworth states that "[s]ubstrates and inhibitors of DP-IV require a free N-terminus" (at 1163) and describes superior potency with the 2-cyano moiety versus without it (at 1164 and 1166, compare Tables I and II). Thus one of ordinary skill in the art would have been motivated to retain the free amine and 2-cyano moieties in the lead compound and would have had a reasonable expectation of success that the ultimate compound having these features would be an active DP-IV inhibitor.

116. One of ordinary skill in the art would have been motivated to select structures in which the trans conformation predominates when optimizing Ashworth compound **25**. It was well known in the art in March 2000 that DP-IV has selectivity for substrates and inhibitors in the trans conformation relative to the cis conformation. Lin represents what was generally known in the art in March 2000 regarding DP-IV's preferred substrate and inhibitor conformations. Lin reports that "[DP-IV] possesses a high conformational specificity for a trans amide bond between the P_1 and N-terminal P_2 residues." Lin at 14020. Lin reports K_i values of 14,000 nM for fluoro olefin dipeptides in the cis conformation and 188 nM for isomeric fluoro

olefin dipeptides in the trans conformation. Comparison of these K_i values for DP-IV inhibition reveals the significantly greater potency of, and preference for, inhibitors in the trans conformation. *See*, e.g., *id.* at 14023 and Table 2.

a. A POSA Would Have Been Motivated To Increase Chemical Stability By Limiting Intramolecular Cyclization

117. One of ordinary skill in the art would have been motivated to limit intramolecular cyclization when optimizing Ashworth compound **25**. It was generally understood in the art that intramolecular cyclization was a source of dipeptide instability in DP-IV inhibitors. For example, Ashworth teaches that, "[s]ubstrates and inhibitors of DP-IV require a free N-terminus, which means that potential dipeptide serine protease inhibitors (e.g., C-terminal aldehydes, boronic acids, α-ketoacids, trifluoromethylketones, or chloromethylketones) are inherently unstable at neutral pH due to intramolecular cyclisation." Ashworth at 1163. As further basis for what was known in the art, Lin describes "the cyclization reaction of the free N-terminal amino acid group with the reactive site of the inhibitor" for related dipeptide compounds (Lin at 14020-14021), which negatively impacts compound stability. Lin further teaches that "[t]he cyclization reaction of the free N-terminal amino group with the reactive inhibitor . . . require[s] the molecule assume the cis conformation. *Id.*

118. One of ordinary skill would have understood that intramolecular cyclization could be reduced by selecting against a conformation that favors intramolecular cyclization (i.e., selecting against the cis conformation), which can be achieved by the addition of a large, steric group to the compound at C2. *See*, e.g., Pal at 274. The trans conformation advantageously places the reactive cyano and amine groups farther from each other, thereby limiting intramolecular cyclization. Adding a large group such as adamantane to the compound would be expected to restrict its range of motion, thereby limiting intramolecular cyclization. *Id.*

119. One of ordinary skill in the art would have understood that a large group could be added to Ashworth compound **25** in order to increase its chemical stability. Further, one of ordinary skill in the art would have been motivated to substitute the cyclohexyl group at the 2-position of the acetyl-pyrrolidine-2-carbonitrile compound **25** with a large tricyclo[3.3.1.1]dec-1-yl group (also referred to as the "adamantyl group") in order to optimize the compound, e.g., to increase the stability of the compound and to bias the compound toward the trans conformation. Ashworth provides the motivation to do so because it shows increased stability for compounds having larger substituents at the 2-position. Furthermore it is reasonable to expect that an increase in size of a group at the 2-position on a cyanopyrrolidinyl glycine derivative would further increase chemical stability. *See*, e.g., Ashworth at 1166, Table II (compare compounds **24** and **25**).

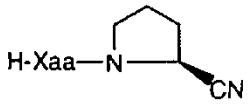
120. One of ordinary skill in the art would also recognize that there are regulatory guidelines for chemical stability that must be met. *See* FDA Stability Guidelines. These guidelines indicate that the stability of a drug candidate under "the same storage conditions as applied to the drug product" should be studied for a time period "sufficient to cover storage, shipment, and subsequent use" of the drug substance in question. *Id.* at 4. The FDA Stability Guidelines also specify that, unless a drug is intended to be stored in a freezer or refrigerator, stability of the drug substance should be tested under the following criteria:

	Conditions	**Minimum time period at submission**
Long-term testing	25°C ±2°C/60% RH ±5%	12 Months
Accelerated testing	40°C ±2°C/75% RH ±5%	6 Months

Id. In order to submit and initial Registration Application for a new drug, the applicant will need to have "a minimum of 6 months of data from a 12-month study." *Id* at 5. Additionally, a POSA

would recognize the relationship between chemical stability and impurities that can arise during storage as degradation products. *Id.* at 3; Dept. of Health & Human Services, "Int'l Conf. on Harmonisation; Guideline on Impurities in New Drug Substances; Availability," *Federal Register*, 61(3):372-376 (Jan. 4, 1996) ("FDA Impurities Guidelines"), at 373. The FDA may require reporting, identification, and qualification of impurities that arise from degradation during storage even if they are present in very small amounts. FDA Impurities Guidelines at 373-374. For example, for a drug substance that is dosed at less than 2 grams per day, the FDA may require qualification of any impurity present at 0.1% or greater. *Id.* at 374. A more stable compound would be expected to give rise to fewer degradation products over time and/or lower quantities of degradation products over time, and thus would be expected to increase the purity of drug substance and decrease the analytical burden associated with producing each batch of the drug substance. As such, a POSA would be motivated to develop compounds with good chemical stability, hoping to produce drug substances that would continue to adhere to their specifications after many months of storage under the required conditions.

121. Ashworth provides motivation to optimize the 2-position of the acetyl-pyrrolidine-2-carbonitrile compound **25** with a suitable substituent to enhance stability. Table II of Ashworth shows the chemical stability data of DP-IV inhibitors in aqueous solution (pH of 7.4), as measured in half-life ($t_{1/2}$).



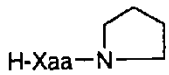
Compound N°	Xaa	K_i (nM)[13]	$t_{1/2}$ (h)[19]
24	Cpg	1.1 ± 0.2	48
25	Chg	1.4 ± 0.5	>48
26	Ile	2.2 ± 0.5	48
27	Tbg	3.8 ± 0.8	>48
28	Lys(Z)	5.2 ± 1.0	24
29	Pro	22.0 ± 4.0	7.5

Id. at 1166.

122. When changing the substituent at the 2-position of the acetyl-pyrrolidine-2-carbonitrile from a straight chain alkyl moiety, for example compound **28** (a lysine moiety), to a more bulky cycloalkyl moiety such as a cyclohexyl moiety, for example compound **25**, the stability dramatically increases from 24 hours to greater than 48 hours. Given this teaching, one of ordinary skill in the art would have been motivated to try even larger, bulkier alkyl groups, such as substituting the cyclohexyl group at the 2-position of the acetyl-pyrrolidine-2-carbonitrile compound **25** with an adamantyl group, in order to further minimize cyclization and increase the stability of the compound under similar conditions. Adamantyl was well known and studied in the art. For example, as discussed in more detail below, Raag, published in 1991, taught hydroxylated adamantane. Raag at 2678.

b. A POSA Would Have Been Motivated To Increase Potency

123. One of ordinary skill in the art also would have been motivated to substitute the cyclohexyl group at the 2-position of the acetyl-pyrrolidine-2-carbonitrile compound **25** with an adamantyl group because, e.g., Ashworth discloses that substitution at this position improves the potency of an aminoacyl pyrrolidide compound as shown in Table 1 of Ashworth:



Compound N°	Xaa	K_i (μM)[13]
5	Cyclohexylglycine [Chg]	0.064 ± 0.01
6	(R,S)-Cyclopentylglycine [Cpg]	0.21 ± 0.04
7	Ile	0.41 ± 0.01
8	*allo*-Ile	0.44 ± 0.04
9	Val	0.47 ± 0.02
10	Lys(Cbz)	0.52 ± 0.07
11	tert-Butylglycine [Tbg]	0.88 ± 0.20
12	Thr(Me)	0.90 ± 0.15
13	Orn(Cbz)	0.91 ± 0.20
14	2-Aminohexanoic acid [Aha]	1.20 ± 0.20
15	Glu	2.00 ± 0.40
16	Pro	2.10 ± 0.20
17	Cyclohexylalanine [Cha]	2.15 ± 0.50
18	Glu(OBn)	2.70 ± 0.30
19	Thr	4.90 ± 0.90
20	Phenylglycine [Phg]	5.30 ± 0.10
21	Ser(Bn)	6.00 ± 1.50
22	Ala	7.00 ± 1.00
23	Asp	14.50 ± 1.90

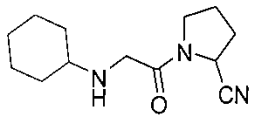
Id. at 1164. Specifically, compound **5** (having a cyclohexyl group) of Table I shows at least a 100-fold increase in potency compared to compound **7** (having a 1-methyl propyl group) or compound **9** (having an isopropyl group). Ashworth confirms this observation by noting, "[a]s expected, from the substrate specificity of DP-IV, only (S)-amino acid derivatives showed any activity and, as can be seen in Table I, lipophilic amino acids gave more potent compounds. In particular, β-branched α-amino acid derivatives were the most potent compounds with the non-proteinogenic amino acid, **(S)-cyclohexylglycine providing the most active pyrrolidide** (compound **5** possessing a K_i value of 64 nM)." *Id.* at 1165.

124. Therefore, Ashworth describes and teaches: (i) the advantage of replacing a smaller **straight chain alkyl moiety** (i.e., a 4-amino-butyl group) at the 2-position of an aminoacyl pyrrolidine with **a larger cycloalkyl moiety** (e.g., a cyclohexyl group) to produce an aminoacyl pyrrolidine-carbonitrile with **increased stability** under physiological pH conditions;

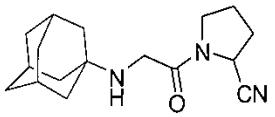
and (ii) the advantage of replacing a **smaller branched alkyl moiety** (i.e.,1-methyl propyl group or isopropyl group) at the 2-position of an aminoacyl pyrrolidine by a **larger cycloalkyl moiety** (cyclohexyl group) to produce an aminoacyl pyrrolidine with **superior activity** in inhibiting DP-IV.

3. A POSA Would Have Been Motivated To Replace The Cyclohexyl Group On Compound 25 With An Adamantyl Group

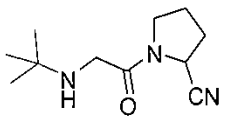
125. Additionally, one of ordinary skill in the art would have been motivated to substitute the cyclohexyl group at the 2-position of compound **25** with an adamantyl group because the art taught adamantyl as an obvious alternative to cyclohexyl. For example, Villhauer discloses compounds having cycloalkyl (e.g., adamantyl groups) or alkyl groups attached to the amino moiety of 2-cyano pyrrolidides for use as DP-IV inhibitors. Specifically, Villhauer discloses a cyclohexyl group attached to the amino moiety of 2-cyano pyrrolidides:



(Villhauer at 12, Example No. 28); adamantyl groups attached to the amino moiety of 2-cyano pyrrolidides:



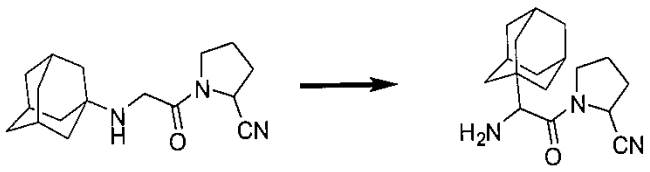
(*id.* at 13, Example No. 47) and alkyl groups attached to the amino moiety of 2-cyano pyrrolidides:



(*id.* at 13, Example No. 52). Villhauer performed a pairwise comparative analysis between cyclohexyl and adamantyl groups, along with a small number of other substituents. Villhauer thus provides motivation for selecting adamantyl as an alternative to cycloalkyl in a DP-IV inhibitor.

126. Given the disclosures of Ashworth and Villhauer, one of ordinary skill in the art would have been motivated in March 2000 to make the adamantyl substituted 2-cyano pyrrolidide (i.e., adamantyl substituted Ashworth compound **25**) because Villhauer discloses the adamantyl group attached to the amino moiety 2-cyano pyrrolidide compound.

127. Ashworth provides motivation to position the adamantyl group of Villhauer at the 2-position of the aminoacyl pyrrolidide of Ashworth compound **25** given that the cyclohexyl moiety was also positioned at the 2-position. Thus one of ordinary skill in the art would easily surmise that the adamantyl-substituted Ashworth compound **25** (shown on the right-hand side below) is merely a logical extension of what was previously known at the time of filing (the compound on the left hand side below):

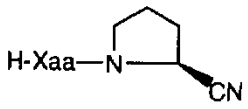


Accordingly, well before March 2000, it would have been obvious to one of ordinary skill in the art to substitute the cyclohexyl group at the 2-position of the Ashworth lead compound **25** with an adamantyl group. One of ordinary skill in the art would have expected the modification to improve the characteristics of the compound, and particularly, to increase the potency and stability of the compound.

128. In the context of the '186 patent, one of ordinary skill in the art would only need to verify the readily predicted results of adding an adamantyl group and removing the cyclohexyl group. Such a modification requires less experimentation than is invited by the specification of the '186 patent.

4. A POSA Would Have Been Motivated To Add A Hydroxyl At The 3-Position Of The Adamantyl Group

129. One of ordinary skill in the art would have been motivated to add a hydroxyl group at the 3-position of the adamantyl substituent of the modified Ashworth lead compound **25** in order to improve the compound's characteristics. For example, addition of a hydroxyl moiety would have been expected to reduce the compound's partition coefficient (thereby enhancing its solubility and permeability) and potentially increase its metabolic stability. Specifically, one of ordinary skill in the art would have expected the hydroxyl group to potentially increase compound solubility and possibly increase absorption of the compound without diminishing its activity.



Compound N°	Xaa	K_i (nM)[13]	$t_{1/2}$ (h)[19]
24	Cpg	1.1 ± 0.2	48
25	Chg	1.4 ± 0.5	>48
26	Ile	2.2 ± 0.5	48
27	Tbg	3.8 ± 0.8	>48
28	Lys(Z)	5.2 ± 1.0	24
29	Pro	22.0 ± 4.0	7.5

Ashworth at 1066.

130. Also, it would have been within the general knowledge of one of ordinary skill in the art—as evidenced by Lipinski, Hansch, Cates, and/or other references available to one of

ordinary skill in the art—to incorporate a hydroxyl group in the adamantyl-modified Ashworth lead compound, e.g., to reduce the partition coefficient of the compound, and thus potentially increase the solubility in aqueous solution. Thus, the teachings of Ashworth in combination with such knowledge would have motivated one of ordinary skill in the art to make such a modification.

131. Raag provides additional motivation for incorporation of a hydroxyl group at a specific position on the adamantyl ring. Raag discloses a study of the metabolism of adamantane by cytochrome P-450$_{CAM}$. Raag at Abstract. Table III of Raag, shows the substrates that are subject to hydroxylation, including adamantane and other substrates:

Table III: Various Substrate-Dependent Parameters[a]

	 camphor	 adamantanone	 adamantane	 thiocamphor	 camphor/Y96F	 norcamphor	 camphane
molec vol	315 Å^3	300 Å^3	293 Å^3	322 Å^3	315 Å^3	236 Å^3	309 Å^3
hydrogen bond to Y96	yes[a]	yes[b]	no	no	no	yes[b]	no
no. of iron ligands	5[a]	5[b]	6	6		6[b]	6
redox pot. Fe^{3+}/Fe^{2+}	−170 mV[c]	−175 mV[c]				−206 mV[c]	
high-spin %	94–97%[c–e]	96–98%[c,d]	99%[d]	65%[e]	59%[e]	46%[c]	46%[e]
regiospecif of substr hydroxylatn	5-exo (100%)[d–f]	5 (100%)[d]	1 (100%)[d]	5-exo (64%)[e] 6-exo (34%) 3-exo (2%)	5-exo (92%)[e,f] 4 (1%) 6-exo (2–4%) 3-exo (0–4%) 9 (<1%)	5-exo (45%)[f] 6-exo (47%) 3-exo (8%)	5-exo (90%)[e] 6-exo (10%)
substr temp factor (Fe^{3+})	16.2 Å^2[a]	16.5 Å^2[b]	24.7 Å^2	23.5 Å^2		33.5 Å^2[b]	30.1 Å^2
substr hydrophilic groups	yes	yes	no	yes	yes	yes	no
hydroxylatn "efficiency"	100%[e,f]			98%[e]	100%[e]	12%[f]	8%[e]
L6–substr dist	NA	NA	2.63 Å	2.35 Å (70%) 3.35 Å (30%)		3.0 Å[b]	2.88 Å
L6–iron dist	NA	NA	1.95 Å	1.35 Å		1.73 Å[b]	1.67 Å
L6 occupancy	NA	NA	1.00	0.90		0.97[b]	1.00
L6 temp factor	NA	NA	14.3 Å^2	19.6 Å^2		3.8 Å^2[b]	7.7 Å^2
cation occupancy	1.00[a]	1.00[b]	0.89	0.91		1.00[b]	0.72
cation temp factor	12.1 Å^2[a]	10.0 Å^2[b]	15.5 Å^2	14.2 Å^2		7.9 Å^2[b]	21.7 Å^2

[a] Poulos et al. (1985, 1987). [b] Raag and Poulos (1989a). [c] Fisher and Sligar (1985). [d] White et al. (1984). [e] Atkins and Sligar (1988b). [f] Atkins and Sligar (1989). [g] Carbon numbering for each substrate begins with C-1 at the top of the six-membered ring which is in the plane of the table. Numbering proceeds counterclockwise such that the carbonyl carbon is C-2 and C-5 is in the lower right-hand portion of the ring. Note that C-5 is a secondary carbon in some substances and a tertiary carbon in others.

Id. at 2678. Column 7 of Table III describes the regiospecific hydroxylation of adamantane at a specific 1-position, i.e., at a tertiary carbon site (and not a secondary carbon site). *Id.* This position corresponds directly to the hydroxylation site (i.e., the 3-position on the adamantyl ring)

in saxagliptin and the location of the hydroxyl group of the adamantyl-modified Ashworth compound **25**.

132. Raag also teaches that "[a]damantane is . . . metabolized to a single product despite having a relatively high active site mobility. The single product can be attributed to the existence of only two types of unique carbon atoms in adamantane, together with the greater reactivity of tertiary versus secondary carbons." *Id.* As additional evidence for the teachings of Raag, Hoffman describes the antiviral drug rimantadine, which contains an adamantane moiety that was known to be metabolized to a hydroxylated derivative. Hoffman serves as further basis of what was generally known in the art regarding the tendency for adamantane to preferentially undergo hydroxylation at the 3-position.

133. Therefore, it was known long before March 2000 that 3-hydroxylated adamantyl groups were used in drugs and that adamantyl groups were subject to hydroxylation at the same site as a result of normal metabolism. Further, one of ordinary skill in the art, given the teaching of Raag (and as evidenced by Hoffman), would have been motivated to block metabolism of a substituted adamantyl ring at the 3-position by placing a group such as a hydroxyl group at that position. Blocking metabolism at the 3-position would result in greater metabolic stability.

134. Furthermore, at the time the application of the '186 patent was filed, it was also known that information on metabolites of candidate drugs "can guide structural modifications, thereby improving the activity and/or bioavailability." *See* Korfmacher at 532. Korfmacher teaches the following advantages of metabolite identification:

> Metabolite identification in drug discovery provides early information that can lead to structural changes in the current lead compound, improving such pharmacokinetic parameters as oral bioavailability, half-life ($t_{1/2}$), or C_{max}. Often these parameters can be changed by improving the metabolic stability of the compound. In order to improve metabolic stability, it is very important to know how a compound is metabolized. The goal of drug discovery is to progress a lead

> compound into a final candidate drug that can be placed in the development stage. . . **Early metabolite identification can provide information on how to improve the metabolic stability of the lead structure. In this way, future lead compounds might be a metabolite identified from the previous lead drug or an analog of the previous drug designed to block the major route of metabolism.**

Id. at 534 (emphasis added).

135. Therefore, it was well known that metabolites (such as 3-hydroxy substituted adamantyl compounds) could provide improved metabolic stability to a compound.

136. Additionally, having a hydroxyl group at the 3-position would also improve the Lipinski parameters, such as reducing Log P and improving permeability as discussed below. The partition coefficient (P) is a ratio of concentrations of un-ionized compound between two liquid phases, and Log P is as a measure of compound lipophilicity. One of ordinary skill in the art would have been motivated to add a hydroxyl group at the 3-position of the adamantyl-substituted moiety of the modified Ashworth lead compound **25** because the use of hydroxyl groups on drugs increases the solubility of the drugs in water. This position is supported by the knowledge of one of ordinary skill in the art, as evidenced by at least by the Lipinski, Hansch and/or Cates disclosures as discussed in details in ¶¶ 50-51 above.

137. One of ordinary skill in the art would have been motivated to improve characteristics or properties of the lead compound, e.g., the solubility thereof, particularly wherein the prior art indicates a potential problem with such a characteristic or property, e.g., very low solubility, as predicted by one of ordinary skill in the art, for example using information and teachings available, such as evidenced by Lipinski, Hansch and Cates.

138. Accordingly, before 2000, in view of the knowledge of one of ordinary skill in the art, as seen in the teachings of Lipinski, Hansch and Cates, it would have been obvious to one of ordinary skill in the art to add a hydroxy group at the 3-position of the adamantyl modified

compound **25**. A person of ordinary skill in the art would have had reason to try such an addition with an expectation of success in preparing a DP-IV inhibitor with improved water solubility, as well as increased stability (as taught in Korfmacher), permeability and bioavailability.

5. A POSA Would Have Been Motivated To Modify Ashworth's Compound 25 By Cyclopropanation Of The Pyrrolidine Ring

139. As discussed above, one of ordinary skill in the art would have understood the significance of dipeptide conformation on the effective interaction between DP-IV and its substrates and inhibitors. As would have been appreciated by one of ordinary skill in the art, the orientation of certain functional groups in the ultimate compound (e.g., the free amine and 2-cyano moieties) would be controlled, at least in part, by biasing the molecule to prefer a trans versus cis conformation of the dipeptide core structure. As discussed above, it was generally known in the art that the trans conformation would be favored over the cis conformation for a DP-IV inhibitor. *Id.* at 14020. This can be accomplished by substitution of a large group, such as hydroxyadamantyl at the C2 position of the core structure. *See* Pal at 274.

140. One of ordinary skill in the art also would have been motivated to optimize the interaction between the cyano functional group and the DP-IV enzyme. Specifically, a person of ordinary skill would have been motivated to optimize the interaction between DP-IV and the cyano group by exploring variations in the point of attachment on the pyrrolidine ring as well as the orientation of the cyano group on the pyrrolidine ring. One of ordinary skill in the art would have selected as a starting point the 2-cyano position found in both Ashworth compound **25** and Villhauer. The cyano moiety in saxagliptin shares the same pyrrolidine ring position described in these prior art references.

141. A person of ordinary skill would have also sought to modulate the orientation of the 2-cyano moiety on the pyrrolidine ring in order to optimize interaction with and inhibition of

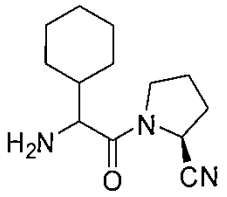
the DP-IV enzyme. A standard strategy in March 2000 for modulating the orientation of a ring-bound substituent would have been through fusion of the pyrrolidine ring with another ring. *See*, e.g., Chiou at 243. Fusion between two rings results in significant changes in ring flexibility, including as discussed above, ring flattening in a cyclopropyl fused derivative. These changes in turn would be expected by one of ordinary skill in the art to affect the orientation of ring-bound substituents (e.g., the 2-cyano moiety of Ashworth, compound **25**). The smallest ring that can be used for fusion is a cyclopropyl ring. *Id.*

142. Cyclopropyl rings are one of the most commonly used ring fusion agents because: (i) addition of a cyclopropyl ring has a negligible effect on compound molecular weight, which can translate to better drug-like qualities; (ii) cyclopropane has an exceedingly small footprint, meaning it exerts a minimal steric effect on the rest of the compound to which it is attached and (iii) cyclopropane provides greater conformational restriction than larger ring fusions, resulting in fewer possible conformations. *See*, e.g., *id.*

143. One of ordinary skill in the art would have been motivated to select cyclopropyl fusion as a means for modulating the interaction between a DP-IV inhibitor and DP-IV. Chiou represents what was generally known in the art in March 2000 regarding the effects of cyclopropyl ring fusion. Specifically, Chiou explored the fusion of a cyclopropyl ring onto acetylcholine (ACh) in order to produce a compound that was "1) structurally as close to ACh as possible and 2) conformationally as rigid as possible." According to Chiou, cyclopropyl rings "are considered the smallest chemical structure . . . capable of conferring conformational rigidity." *Id.* at 243. Like Chiou, one of ordinary skill in the art would have been motivated to start with a compound having known activity (e.g., Ashworth compound **25**) and to optimize it by making small, incremental changes to its size and rigidity near the active 2-cyano group.

144. For the above reasons, cyclopropyl fusion to the pyrrolidine ring of Ashworth compound **25** would have been one of the first modifications a person of ordinary skill would have chosen for its optimization in March 2000. Moreover, the pool of compounds that would have been tried by a person of ordinary skill would have been quite small given the likelihood of selecting the cyclopropyl fusion modification.

145. Selection of the point of attachment for the cyclopropyl ring would have been straightforward for one of ordinary skill in the art in March 2000. As can be seen in the pyrrolidine ring of Ashworth compound **25** below, there are only three possible cyclopropyl fusion sites at the pyrrolidine ring:



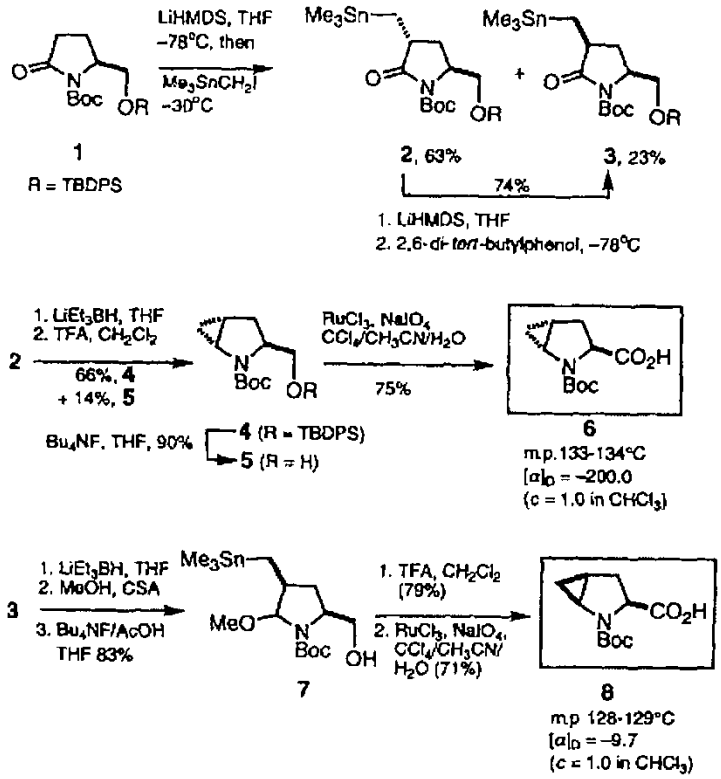
Ashworth Compound **25**

The resulting fusion would thus be one of: 1,2-methanopyrrolidine, 3,4-methanopyrrolidine or 4,5-methanopyrrolidine. One of ordinary skill in the art would have understood in March 2000 that two possible orientations of the fused cyclopropane ring could exist for each of the above three positions ring. *See*, e.g., Hanessian at 1882, compounds **6** and **8**.

146. One of ordinary skill in the art would have prioritized fusion of the cyclopropane ring at the two positions farthest from the carbon bearing the cyano moiety. Thus the skilled artisan would have conservatively started with the 4,5-methanopyrrolidine and 3,4-methanopyrrolidine fusions because the prior art provided expeditious synthetic methods for their preparation. *See* Hanessian; Tverezovsky, et al., "Synthesis of (2S, 3R, 4S)-3,4-

Methanoproline and Analogues by Cyclopropylidene Insertion," *Tetrahedron*, 53(43):14773-14792 (1997). Therefore, the 4,5-methanopyrrolidine, as present in saxagliptin, would have been one of only a small set of compounds that would have been investigated by one of ordinary skill in the art.

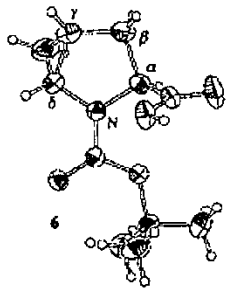
147. Hanessian provides motivation for producing a fused pyrrolidine-cyclopropyl ring system as found in saxagliptin. Specifically, Hanessian teaches that the use of conformationally constrained analogues of proline were well known in the peptidomimetic research area well before March 2000. Hanessian describes the "highly stereocontrolled syntheses of the diastereomeric 4,5-methano-L-prolines and 5,6-methano-L-pipecolic acids by a novel intramolecular cyclopropanation reaction of iminium ions . . ." *Id.* at 1882. One such example shown by Hanessian is compound **8**, which was synthesized according to Scheme 1 below:



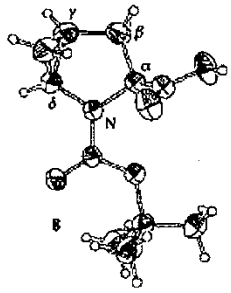
Id.

148. The structure and conformation of compound **8** in the solid state were unambiguously confirmed by single X-ray analysis. *Id.* Further, Table 1 of Hanessian shows selected torsion angles for compound **8** where "considerable 'flattening' of the pyrrolidine ring is observed relative to *N*-Boc-L-proline." *Id.*

Table 1. Selected torsion angles and root-mean-square deviations from fitted atoms in a given plane of X-ray crystal structures, and ^{13}C NMR chemical shifts ($CDCl_3$).



6



8

	N-Boc-L-proline [20]	6	8
τ(NC_α)	−17	−5.6	−14.4
τ($C_\alpha C_\beta$)	+31	+4.8	+15.3
τ($C_\beta C_\gamma$)	−35	−2.6	−11.4
τ($C_\gamma C_\delta$)	+24	−0.7	+2.9
τ($C_\delta N$)	−4	+4.1	+7.6
τ($BocNC_\alpha CO_2H$)	−72	−64.0	−67.1
rms deviation of fitted atoms	0.018 C_α, N, C_δ, C_γ	0.003 C_β, C_γ, C_δ, N	0.013 C_β, C_γ, C_δ, N
	δ(cis/trans)	*δ(cis/trans)*	*δ(cis/trans)*
COOH	178.35/176.60	177.7/175.5	179.1/176.1
NC=O	153.95/155.39	157.1/154	155.7/154.1
C_α	58.8	60.8/60.1	59.5/59.1
C_β	30.75/29.53	32.0	31.5/29.1

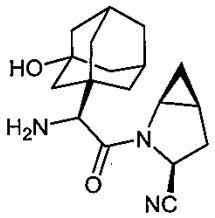
Id. Hanessian confirms the "flattening" of the pyrrolidine ring by determining the root-mean-square values for the C_β and N atoms from the plane defined by C_β, C_γ, C_δ and N (*see* Table 1 above). It was determined that for compound **8**, the out-of-plane carbon was the carbon bearing the carboxyl group when compared to *N*-Boc proline. *Id.* Therefore, Hanessian taught that modification of a substituted proline ring (such as a 2-carboxyl substituted proline) to a 4,5-methanoproline ring system "flattens" the ring. Ring flattening would also be expected to result in a modified orientation of the 2-cyano group. Modifying the orientation of the 2-cyano group in Ashworth compound **25** would likely result in a modified interaction with DP-IV that may improve potency and possibly cause it to resist intramolecular reaction with the free amine group found in DP-IV inhibitors (i.e., increased stability).

149. One of ordinary skill in the art would have had reason to modify the 2-cyano substituted proline portion of the Ashworth lead compound **25** to a 4,5-methanoproline ring system in order to enhance compound stability. For example, Ashworth teaches that, "[s]ubstrates and inhibitors of DP-IV require a free N-terminus, which means that potential dipeptide serine protease inhibitors (e.g., C-terminal aldehydes, boronic acids, α-ketoacids, trifluoromethylketones, or chloromethylketones) are inherently unstable at neutral pH due to intramolecular cyclisation." Ashworth at 1163. One of ordinary skill in the art would have had reason to try modifying the 2-cyano substituted proline portion of the Ashworth compound **25**, to produce a 4,5-methanoproline ring system in order to "flatten" the proline ring as taught in Hanessian, thereby adjusting the orientation of the cyano substituent to the proline ring and minimizing or preventing intramolecular cyclization, as taught by Ashworth. Furthermore one of ordinary skill in the art may expect that a cyclopropyl fused pyrrolidine may also have improved chemical stability in FDA-mandated stability testing. *See* FDA Stability Guidelines; FDA Impurities Guidelines.

150. Hanessian describes and teaches: (i) modification of a substituted proline ring (such as a 2-carboxyl substituted proline) (ii) to produce a 4,5-methano-modified substituted proline ring with the 2-substituent orientation modified with respect to the proline ring.

151. Accordingly, before 2000, it would have been obvious to one of ordinary skill in the art to modify the substituted proline ring of the Ashworth compound to a 4,5-methano-modified compound. Such chemist would have had reason to make such a modification with a reasonable expectation of success for improving the characteristics of the compound. For example, according to the teachings of Ashworth, one of ordinary skill in the art would have expected success in preparing the DP-IV inhibitor with improved stability properties.

152. By making these commonly known modifications at the positions indicated above, routine experimentation would have led to the following molecule:



153. This molecule is identical to the compounds recited in claims 8 and 25 of the '186 patent and encompassed by claims 1, 2, 4, 6, 7, 9-11, 26-28, 32-35, and 39-40 of the '186 patent.

154. Taken together, the prior art provided reason: 1) to select the core structure of Ashworth compound **25** to form a DP-IV inhibitor; 2) to substitute the cyclohexyl group at the 2-position of the acetyl-pyrrolidine-2-carbonitrile of the lead compound with tricyclo[3.3.1.1]dec-1-yl group (adamantyl group); 3) to add a hydroxyl group at the 3-position of the adamantyl group and 4) to form a 4,5-methanoproline moiety of the lead compound. Thus, claims 8 and 25 of the '186 patent are unpatentably obvious over Ashworth in view of the knowledge and motivation of one of ordinary skill in the art, as evidenced by prior art references (including Villhauer, Raag, and Hanessian).

B. Each Additional Limitation Of Dependent Claims 9 And 26 Is Also Disclosed By The Prior Art

Claim 9:

"The compound as defined in claim 8 wherein the pharmaceutically acceptable salt is the hydrochloride salt or the trifluoroacetic acid salt."

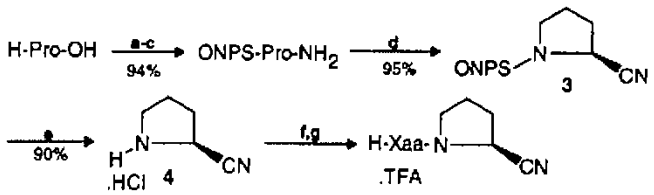
Claim 26:

"The compound as defined in claim 25, wherein the pharmaceutically acceptable salt is the hydrochloride salt."

155. Claim 9 of the '186 patent recites the compound as defined in claim 8 and further claims the hydrochloride salt or the trifluoroacetic acid salt as the pharmaceutically acceptable salt. Claim 26 of the '186 patent recites the compound as defined in claim 25 and further claims the hydrochloride salt as the pharmaceutically acceptable salt. It would have been obvious to one of ordinary skill in the art to make and use the hydrochloride salt or the trifluoroacetic acid salt because it was previously disclosed in Ashworth. Further, making the salt would have been an ordinary exercise or task one of ordinary skill in the art would have performed at the time the application which led to the '186 patent was filed.

156. Ashworth teaches the formation of DP-IV pyrrolidides as both the hydrochloride salt and trifluoroacetic acid salt. For example, Ashworth describes the "subsequent acid catalyzed deprotection (4N HCl/dioxane) afforded the inhibitor as its **hydrochloride salt**." Ashworth at 1165 (emphasis added). Ashworth also describes the preparation of dipeptide nitriles as shown in Scheme I, below.

Scheme I. *Preparation of dipeptide nitriles.*



Reagents: a. ONPS-Cl, 2N NaOH. b. HONSu, Water soluble carbodiimide. c conc. NH_4OH, dioxane. d. imidazole (2 equiv.), $POCl_3$ (4 equiv.), pyridine. e. 4N HCl/dioxane (3 equiv.), diethyl ether. f. Boc-Xaa-OH, pyBop, NEt_3, CH_2Cl_2. g. Trifluoroacetic acid.

Id.

157. Here, step g of Scheme I shows the formation of the trifluoroacetic acid salt. *Id.* Because it would have been obvious to one of skill in the art to modify the lead compound **25** of Ashworth, as described above, the hydrochloride salt or trifluoroacetic acid salt of claim 9 or the

hydrochloride salt of claim 26 of the '186 patent would be obvious for at least the reasons set forth above with respect to claims 8 and 25, given the disclosure that hydrochloride salts or trifluoroacetic acid salts were synthesized in Ashworth. Further, it would have been within the ordinary routine of one of skill in the art to make pharmaceutically acceptable salts, such as hydrochloride salts or trifluoroacetic acid salts once the base compound was synthesized. Villhauer also teaches the use of pharmaceutically acceptable salt, including the hydrochloride salt.

VIII. CONCLUSION

158. For the foregoing reasons it is my opinion that claims 8, 9, 25, and 26 of the '186 patent would have been obvious to a person of ordinary skill in the art at the time of the claimed invention.

Executed on this 29 day of January, 2016.

David P. Rotella, Ph.D.

EXHIBIT A

DAVID P. ROTELLA, Ph.D.
Margaret & Herman Sokol Professor of Medicinal Chemistry
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Summary of Accomplishments:

- **Montclair State University**-Obtained $2.5MM, 5 year drug discovery research grant from Defense Threat Reduction Agency and an additional $300K in pharmaceutical industry research funding in three years.
- **Wyeth Research**-led chemistry teams in CNS drug discovery projects and key leader for collaboration with Solvay Pharmaceuticals. Delivered a clinical candidate, managed chemists in group that delivered another.
- **Lexicon Pharmaceuticals**- Beginning from a screening hit, in less than one year, led project team that identified potent, selective, orally bioavailable inhibitors of PDE7A.
- **Bristol-Myers Squibb**-First to publish the discovery of novel phosphodiesterase type 5 inhibitors with better *in vitro* potency and selectivity compared to sildenafil. Contributed to discovery of 2 clinical candidates (PDE5 inhibitor, DPP4 inhibitor).
- **Cephalon**-Responsible for initial conception and development of several programs. Key leader in collaborations with Kyowa Hakko and Schering Plough. Discovered CEP 1347, which advanced to phase III trials for Parkinson's Disease.

Experience:

- **Montclair State University July 2011-present**
  Margaret and Herman Sokol Professor of Chemistry, Department of Chemistry and Biochemistry; joint appointment in Sokol Institute of Pharmaceutical Life Sciences
- **Independent Consultant, February 2010-present**
  Established consulting agreements with pharmaceutical companies and law firms to advance drug discovery programs and provide expert information on selected topics in drug development
- **Wyeth Research/Pfizer, 2005-February 2010**
  Principal Research Scientist III, chemistry team leader. Directed up to 20 chemists. Member of Princeton Chemical Science leadership team.
- **Lexicon Pharmaceuticals, 2003-2005**
  Senior Group Leader, responsible for multiple drug discovery programs. Directed up to 18 FTEs with 4 direct reports. Member of department leadership team.
- **Bristol-Myers Squibb PRI, 1997-2003**
  Principal Scientist, cardiovascular and metabolic disease drug discovery
- **Cephalon, Incorporated, 1991-1997**
  Group Leader, CNS and cancer drug discovery.
- **School of Pharmacy, University of Mississippi**

Assistant Professor, Department of Pharmacognosy 1987-1991
Adjunct Professor, Department of Medicinal Chemistry, 2009-present

- **School of Pharmacy, University of Pittsburgh**, 2010-present
  Adjunct Professor, Department of Pharmaceutical Sciences
- **Center for Drug Discovery, Northeastern University**, 2010-present
  Adjunct Professor
- Registered pharmacist, Pennsylvania, 1981-1991, 2010-present

Education:

- Postdoctoral Scholar, Department of Chemistry, The Pennsylvania State University, 1985-1987, under the direction of Prof. K. S. Feldman.
- Ph.D. Medicinal Chemistry, The Ohio State University, 1985, under the direction of Prof. D. T. Witiak.
- B.S. Pharm., Magna cum laude, School of Pharmacy, University of Pittsburgh, April 1981.

Professional Service:

American Chemical Society, Organic and Medicinal Chemistry Divisions
Fellow, Royal Society of Chemistry

Division of Medicinal Chemistry, American Chemical Society:

- Five year term as Vice Chair/Long Range Planning Committee chair, Program Chair, Chair and past Chair (2004-2008). These roles required leadership and collaborative interactions nationally and internationally.
- Three year term as academic councilor (2012-2014)
- Treasurer, 2015-2017

Gordon Research Conference on Medicinal Chemistry

- 2012 vice chair elect
- 2013 vice chair
- 2014 chair

Co-editor, 3rd edition, Comprehensive Medicinal Chemistry 2014-present

Co-editor, 7th edition, Burger's Medicinal Chemistry 2007-present

Senior Editor, Royal Society of Chemistry series on Drug Discovery, 2008-present

Co-editor, "Successful Drug Discovery", (2014), Wiley VCH

Co-editor, "Analogue-Based Drug Discovery", volume 3, (2012), Wiley VCH

Program co-chair, National Medicinal Chemistry Symposium (2010)

Scientific Advisory Board National Medicinal Chemistry Symposium (2014)

Scientific Advisory Board Frontiers in Medicinal Chemistry 2014-2015

Organizer and conference co-chair for "Frontiers in CNS and Oncology Medicinal Chemistry", Siena, Italy, October 7-9, 2007, jointly organized with European Federation for Medicinal Chemistry.

Current Research Funding:

- Discovery of Novel Botulinum Toxin Protease A Inhibitors, 9/29/14-9/28/19, $2.5MM, Defense Threat Reduction Agency
- Protein Kinase Inhibitors for Parasitic Diseases, 3/1/14-2/28/15, $90,000, Celgene Corporation
- Research Support, 9/1/11-8/31/16, $50,000 annually, Margaret and Herman Sokol Endowment

Past Research Funding:

- Protein Kinase Inhibitors for Parasitic Diseases, 3/1/13-2/28/14, $115,000, Celgene Corporation
- Protein Kinase Inhibitors for Parasitic Diseases, 3/1/12-2/28/13, $90,000, Celgene Corporation
- Purchase of LCMS, 10/1/13, $70,000, Shimadzu Corporation
- Lactam Inhibitors of Phospholipase A2, 7/1/88-6/30/90, direct costs $25,000, Mississippi Affiliate, American Heart Association
- Novel Calmodulin Inhibitors, 7/1/89-6/30/91, direct costs $35,000, Elsa U Pardee Foundation
- Phospholipase A2 Inhibitors as Novel Anti-inflammatory Agents, 7/1/89-6/30/91, direct costs $200,000, American Lung Association

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2. "Synthesis and P-388 Antitumor Properties of the Four Diastereomeric Dichloro 1-Hydroxy-3,4-diaminocyclohexane Pt(II) Complexes" , Donald T. Witiak, David P. Rotella, Yong Wei, Joyce A. Filppi and Judith C. Gallucci *J. Med. Chem.* **32,** 214 (1989).

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4. "Homoallylically Controlled Epoxidation of Δ^4-*cis*-1,2-Disubstituted Cyclohexenes", David P. Rotella, *Tetrahedron Letters*, 1913 (1989).

5. "Application of an Intramolecular Tropone-Alkene Photocyclization to the Total Synthesis of (±) Dactylol", Ken S. Feldman, Ming-Jung Wu and David P. Rotella, *J. Am. Chem. Soc.* **111**, 6457 (1989).

6. "Chloroperoxidase Mediated Halogenation of Phenols", Cheryl F. Wannstedt, David P. Rotella and Jerome F. Siuda, *Bull. Contamin. Environ. Toxicol.* **44**, 282 (1990).

7. "Stereocontrolled Iodolactonization of *Erythro* and *Threo* Tertiary Amides", David P. Rotella and Xun Li, *Heterocycles* **31**, 1205 (1990).

8. "The Total Synthesis of (±) Dactylol and Related Studies", Ken S. Feldman, Ming-Jung Wu and David P. Rotella, *J. Am. Chem. Soc.* **112**, 8490 (1990).

9. "Synthesis and Structural Analysis of Stereospecific 3,4,5-Trisubstituted γ-Butyrolactone Phospholipids", Xun Li and David P. Rotella, *Lipids* **29**, 211-224 (1994).

10. "The Effect of Pyrrolo[3,4-c]Carbazole Derivatives on Spinal Cord ChAT Activity" David P. Rotella, Marcie A. Glicksman, J. Eric Prantner, Nicola Neff and Robert L Hudkins, *Bioorganic and Medicinal Chemistry Letters.* **5**,1167-1170 (1995).

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15. "Solid Phase Synthesis of Olefin and Hydroxyethylene Peptidomimetics", David P. Rotella, *J. Am. Chem. Soc.* **118**, 12246-12247 (1996).

16. "Neurotrophic 3,9-Bis[(alkylthio)methyl]- and -Bis(alkoxymethyl)-K-252a Derivatives", Masami Kaneko, Yutaka Saito, Hiromitsu Saito, Tadashi Matsumoto, Yuzuru Matsuda, Jeffry L. Vaught, Craig A. Dionne, Thelma S. Angeles, Marcie A. Glicksman, Nicola T. Neff, David P. Rotella, James C. Kauer, John P. Mallamo, Robert L. Hudkins, Chikara Murakata, *J. Med. Chem.* **40**, 1863-1869 (1997).

17. "An Update on COX-2 and Farnesyltransferase Inhibitor Development", David P. Rotella, *Curr. Opin. Drug Discovery and Development*, **1**, 165-174 (1998).

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39. "Structure-activity Studies of (-)-Epigallocatechin Gallate Derivatives as HCV Entry Inhibitors" Rohit Bhat, Amna Adam, Jungeun Jasmine Lee, Gaspard Deloison, Yves Rouillé, Karin Séron, David P. Rotella, *Bioorg. Med. Chem. Lett.* **2014**, 24, 4162-4165.

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2. "K252a Functional Derivatives Potentiate Neurotrophin-3 Activity for the Treatment of Neurological Disorders" Marcie A. Glicksman, Robert L. Hudkins, David P. Rotella, Nicola Neff and Chikara Murakata, US 5,468,872.

3. "K252 Derivatives Which Enhance Neurotrophin-Induced Activity" Marcie A. Glicksman, Robert L. Hudkins, David P. Rotella, Nicola Neff and Chikara Murakata, US 5,516,772.

4. "Quinazolinone Inhibitors of cGMP Phosphodiesterase", David P. Rotella, John E. Macor, David Cushman, Joseph Yevich, US 6,087,368

5. "Quinoline Inhibitors of cGMP Phosphodiesterase", Yingzhi Bi, David P. Rotella, Guixue Yu, John E. Macor, US 7,378,430 .

6. "2-Substituted cyclic amines as calcium sensing receptor modulators", Ashvinikumar Gavai, Roy J. Vaz, John K. Dickson, Jacques Y. Roberge, Wu Wang, Timur Gungor, James R. Corte, David P. Rotella, Yufeng Wang, Wu Yang, US 7,105,537.

7. "Preparation of substituted piperidines and pyrrolidines as calcium sensing receptor modulators", John K. Dickson, Michael R. Lawrence, Jacques Y. Roberge, David P. Rotella, Wu Yang, US 7,265,145.

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"Recent Results in Phosphodiesterase Inhibitor Development and CNS Applications","Cyclic-Nucleotide Phosphodiesterases in the Central Nervous System: From Biology to Drug Discovery", John Wiley and Sons, **2014** 115-144, Anthony West, Nicholas J. Brandon, editors.

"Monoaminergic Targets for Treatment of Schizophrenia", "New Approaches to Psychiatric Drug Discovery", Royal Society of Chemistry Press, **2012**, 33-47, Zoran Rankovic, editor.

"Pioneer and Analogue Drugs", "Analogue-based Drug Discovery", volume 3, Wiley VCH, **2012**, 3-18, edited by Janos Fischer, C. Robin Ganellin, David P. Rotella

"SSRIs" David P. Rotella and Wayne E. Childers, "Analogue-Based Drug Discovery, volume 2, Wiley-VCH, **2010**, 269-296, Janos Fischer and Robin Ganellin, editors.

"Medicinal Chemistry Challenges in the Discovery of Novel Antidepressants", "Next Generation Antidepressants", **2010,** 102-118, Cambridge University Press, Chad E. Beyer, Stephen M. Stahl, editors.

"Drug Discovery for Non-Life Threatening Disorders", "Burger's Medicinal Chemistry", volume 5, 7th edition, John Wiley and Sons, **2010**, 711-728, Donald J. Abraham and David P. Rotella, co-editors.

"Phosphodiesterase Enzymes, Inhibitors and Therapeutic Applications" "Comprehensive Medicinal Chemistry II" Elsevier Science, **2006**, 919-957, Walter H. Moos editor.

Abstracts and Presentations:

"A Natural Product Template for Medicinal Chemistry" Merck Research Laboratories, Kenilworth NJ, March 2014.

"Progress Toward the Identification of Drug-Like Hsp90 Inhibitors based on (-)-Epigallocatechin Gallate", Department of Medicinal Chemistry, University of Illinois at Chicago, October 2013.

"Design, Synthesis and Use of Novel Diamine Templates in Medicinal Chemistry" Eli Lilly Research Laboratories, May 2013.

"Structure-activity Relationships of Novel Inhibitors of the *Brugia malayi* Stress-activated Protein Kinase, Bm-MPK1" David P. Rotella, Sreedhar R. Tummalapalli, Agnieska Chojnowski, Tamara W. Kreiss, Deborah S. Mortensen, Veronique Plantevin, Stacie Canan, Vikram Khetani, Jerome B. Zeldis, Ronald Goldberg, John Siekierka, poster presentation American Society of Tropical Medicine and Health national meeting, Washington DC November 5, 2013.

"Novel inhibitors of the *Brugia malayi* stress-activated protein kinase, Bm-MPK1", Mortensen DS, Khetani V, Satoh Y, Cathers B, Canan S, Zeldis J, **Chojnowski AN**, Patel A, Goldberg R, Rotella D, Siekierka J. poster presentation American Society of Tropical Medicine and Hygiene national meeting, Atlanta, GA, November 11-15, 2012.

"Antipsychotic-like profile of WS-50030, a combined partial D_2 receptor agonist and selective serotonin reuptake inhibitor", Julie Brennan, Karen Marquis, Claudine Pulichiccio, Mark Pausch, Steven Grauer, David P. Rotella, Rolf Feenstra, Andrew McCreary, poster presentation, Society for Neuroscience meeting 2008, Washington DC, November 2008.

"PDE5 Inhibitors: Comparison of Current Agents and Future Prospects", David P. Rotella, 2nd Annual Phosphodiesterases in Drug Discovery and Development, November 8-9, 2004.

"Phosphodiesterase Type 5 Inhibitors: Is There Room for More?" David P. Rotella, American Society of Andrology 29th National Meeting, April 18, 2004.

"Effects of the M3 antagonist, darafenicin, on food intake and body weight in two models of obesity." M.J. Cullen, M.A. Pelleymounter, D. Rotella, K Carlson and K. Behnia, Keystone Symposium: Obesity: New Insights into Pathogenesis and Treatment, Keystone, CO, January 21-26, 2003.

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"The Effect of Hydroxyl Protection on Stereochemistry and Stereocontrol During Amide Iodolactonization" David P. Rotella, 202nd National Meeting of the American Chemical Society, August 25-30, 1991, New York, NY, ORGN 258.

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"Synthesis and Preliminary ^{31}P NMR Evaluation of Lactones as Substrates for Phospholipase A_2" David P. Rotella, Xun Li and John K. Baker, invited poster presentation to the National Meeting of the American Association of Colleges of Pharmacy, Salt Lake City, UT, July 8-11, 1990.

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"Homoallylically Controlled Epoxidation of Δ^4-*cis*-1,2-Disubstituted Cyclohexenes", David P. Rotella, 197th National Meeting of the American Chemical Society, Dallas, TX , April 9-14, 1989, ORGN 34.

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"Synthesis and Antimetastatic Properties of Stereoisomeric Bisdioxopiperazines", D.T. Witiak, B.S. Zwilling, B.K. Trivedi, R.V. Nair, and D.P. Rotella, 183rd National Meeting of the American Chemical Society, Kansas City, MO, August 25-30, 1982.

EXHIBIT B

EXHIBIT B

Materials Considered by David P. Rotella, Ph.D.

DESCRIPTION	BATES
U.S. Patent No. 6,395,767	MYL_SAX0045556 – MYL_SAX0045607
U.S. Patent No. RE 44,186	MYL_SAX0081678 – MYL_SAX0081725
U.S. Provisional Application No. 60/188,555	MYL_SAX0298655 – MYL_SAX0298716
U.S. Patent No. 6,395,767 File History	MYL_SAX0297174 – MYL_SAX0297744
U.S. Patent No. RE 44,186 File History	MYL_SAX0297745 – MYL_SAX0298376
WO Patent App. Pub. No. 98/19998	SAXA-DEF-00103 – SAXA-DEF-00129
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