Synthesis of Novel Potent Dipeptidyl Peptidase IV Inhibitors with Enhanced Chemical Stability: Interplay between the N-Terminal Amino Acid Alkyl Side Chain and the Cyclopropyl Group of α-Aminoacyl-L-*cis*-4,5-methanoprolinenitrile-Based Inhibitors

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A series of methanoprolinenitrile-containing dipeptide mimetics were synthesized and assayed as inhibitors of the N-terminal sequence-specific serine protease dipeptidyl peptidase IV (DPP-IV). The catalytic action of DPP-IV is the principle means of degradation of glucagon-like peptide-1, a key mediator of glucose-stimulated insulin secretion, and DPP-IV inhibition shows clinical benefit as a novel mechanism for treatment of type 2 diabetes. However, many of the reversible inhibitors to date suffer from chemical instability stemming from an amine to nitrile intramolecular cyclization. Installation of a cyclopropyl moiety at either the 3,4- or 4,5-position of traditional 2-cyanopyrrolidide proline mimetics led to compounds with potent inhibitory activity against the enzyme. Additionally, *cis*-4,5-methanoprolinenitriles with *β*-branching in the N-terminal amino acid provided enhanced chemical stability and high inhibitory potency. This class of inhibitors also exhibited the ability to suppress prandial glucose elevations after an oral glucose challenge in male Zucker rats.

Introduction

With the spread of Western lifestyles, the prevalence of type 2 diabetes in the world's population is rising.[1] Current treatment strategies include reducing insulin resistance using glitazones,[2] supplementing insulin supplies with exogenous insulin,[3] increasing insulin secretion with sulfonylureas,[4] reducing hepatic glucose output with biguanides,[5] and limiting glucose absorption with glucosidase inhibitors.[6] Promising new targets for drug development are also emerging. Of particular interest is the pharmacology surrounding the incretin hormone glucagon-like peptide 1 (GLP-1).[7] GLP-1 is known to function as a mediator of glucose-stimulated insulin secretion, and several clinical studies have shown that administration of the peptide or its analogues results in antidiabetic action in subjects with type 2 diabetes.[8] Although GLP-1 is secreted as GLP-1 (7−36) amide from the small and large intestines in response to dietary signals, it is rapidly truncated to GLP-1 (9−36) by cleavage of the N-terminal dipeptide residues. The truncated metabolite has antagonist activity against the GLP-1 receptor both in vitro and in vivo.[9] The principle enzyme responsible for the cleavage of GLP-1 (7−36) amide to GLP-1 (9−36) amide is dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5), a nonclassical, sequence-specific serine protease that catalyzes 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).[10] Inhibition of DPP-IV has been shown to be effective at sustaining circulating levels of GLP-1 (7−36) and therefore offers a new therapeutic approach for the treatment of type 2 diabetes.[11]

Early reports of DPP-IV inhibitors included proline-based dipeptide mimics bearing boronic acid[12] (**1**) or diphenyl phosphonate substituents (**2**).[13] These compounds were irreversible inhibitors of DPP-IV or were slow to dissociate from the enzyme. Several first-generation dipeptide surrogates have been disclosed as reversible inhibitors of DPP-IV, including both C-substituted (**3**) and N-substituted (**4**) glycinylprolinenitrile dipeptide analogues.[14,15] These compound classes include many potent inhibitors of the enzyme, but all suffer from chemical instability whereby the N-terminal amine intramolecularly cyclizes onto the nitrile, forming inactive cyclic imidates and/or their diketopiperazine hydrolysis products. However, more recent publications have disclosed a series of more hindered *N*-alkylamines (**5**) that have much greater chemical stability.[16] Thia-

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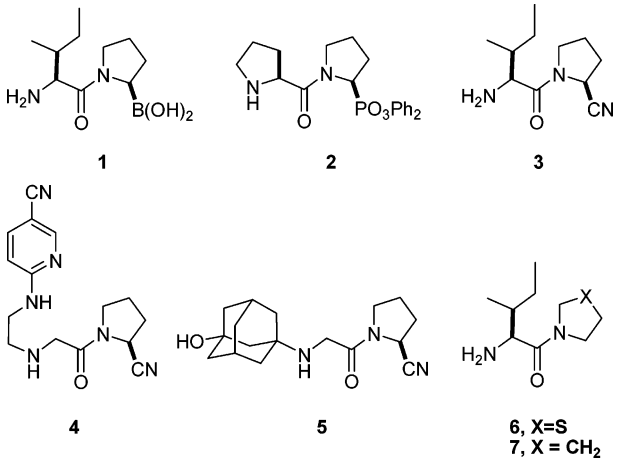


Figure 1. Known proline-derived dipeptidyl peptidase IV inhibitors.

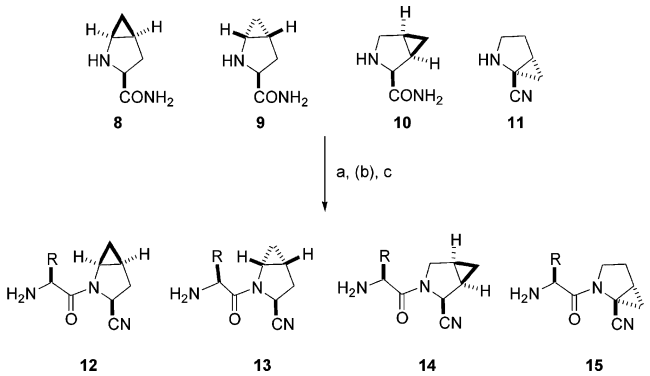
zolidide (**6**) and pyrrolidide (**7**) based inhibitors have also been disclosed that lack the nitrile moiety and thus possess greater stability, though at the cost of reduced potency[17a−c] with a few very recent notable exceptions.[17d−f] These compounds are shown in Figure 1.

We endeavored to synthesize novel dipeptide surrogates containing a proline mimic linked to an N-terminal amino acid that would act as reversible inhibitors of DPP-IV with maximum potency and enhanced solution stability. Hanessian has reported that installation of a 4,5-methano moiety into a proline residue has the effect of flattening the five-membered ring,[18] and one might extend a similar conformational argument to other cyclopropanated prolines as well. By inference, the cyclopropane bridge may occupy the space-filling region that would normally be given to the methylene group in the puckered or "envelope" conformation of a typical five-member ring. With this precedent we sought to establish whether a methanoproline derivative could serve as a viable proline surrogate in a DPP-IV inhibitor. Our strategy was to prepare dipeptide surrogates containing a cyclopropanated prolinenitrile derived from L-proline. The regio- and stereochemical disposition of the cyclopropane bridge was varied in order to identify a compound with maximal stability and potency. Herein, we report the discovery of L-*cis*-4,5-methanoprolinenitrile dipeptides as potent inhibitors of DPP-IV with increased chemical stability and high potency. In addition, we also present data demonstrating that these novel DPP-IV inhibitors effectively lower plasma glucose after a glucose challenge in rodent models.

Chemistry

Dipeptides (**12**−**15**) composed of N-terminal isoleucine appended to cyclopropylprolineamides or nitriles derived from L-proline (**8**−**11**) were targeted to probe the potential for this type of inhibitor scaffold. Isoleucine was selected as the N-terminal residue because it was the most potent natural amino acid reported in the 2-cyanopyrrolidide series.[14a] These inhibitors were expected to provide a dependable inhibitory benchmark for the differing methanoproline structures. The synthesis is outlined in Scheme 1.

Scheme 1[a]



[a] (a) *N*-Boc-amino acid, EDAC, DMAP or PyBop, 50−90%; (b) (for intermediates **8**−**10**) $POCl_3$, pyridine, imidazole, 70−90%; (c) TFA, CH_2Cl_2, TFA or HCl in Et_2O or EtOAc, 70−90%.

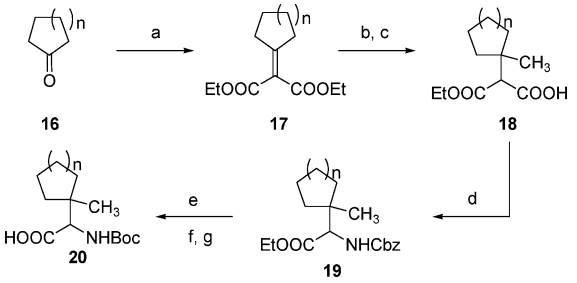
anoproline,[19] and L-2,3-methanoproline[20] are shown in Scheme 1.

Standard conditions were employed to couple the enantiomerically pure L-*cis*-4,5-cyclopropylprolineamide nucleus (**8**) to give the corresponding Boc-protected dipeptides in good to excellent yield.[21] Dehydration[14b,22] ($POCl_3$, pyridine, imidazole) of the amide and removal[23] of the N-terminal Boc protecting group (TFA or HCl) gave the proline dipeptide (**12**) in high yield. Similar protocols were used for the L-*cis*-3,4-methanoproline fragment **10** and the L-4,5-*trans*-methanoproline compound **9**. The known (−)-(2*S*,3*R*)-methanoprolinenitrile (**11**) was prepared according to the method of Hercout.[20a] In this instance, the dehydration reaction was performed prior to introduction of the isoleucine fragment.

Preparation of a library was undertaken to probe structure−activity relationships between the N-terminal amino acid residue and the *cis*-4,5-methanoprolinenitriles. These inhibitors were generated in a three-step sequence in parallel array format in a manner similar to that described in Scheme 1. Initial reaction of the Boc-protected amino acid with methanoprolineamide, EDAC, and DMAP in dichloromethane, and subsequent purification through an SCX ion exchange cartridge, gave good to excellent yields of coupled dipeptides. Dehydration and acid-promoted deprotection (TFA in dichloromethane) yielded the inhibitors as TFA salts. Further purification of the final products was easily accomplished by preparative reverse-phase HPLC or by trituration with Et_2O.

Holmberg has prepared *tert*-alkylmalonic acid derivatives through a $TiCl_4$-mediated Knoevenagel process and subsequent copper-assisted conjugate addition of a Grignard reagent or conjugate reduction. Following this method (Scheme 2), diesters can be converted to the protected amino acids in three steps.[24] This methodology provided an approach to the cycloalkylglycine amino acid derivatives **20**. First, the malonic acid diesters **17** were subjected to conjugate addition of a methyl group or hydride and then hydrolyzed to the corresponding monoacids **18**. Subsequent Curtius rearrangement of **18** by treatment with diphenylphosphoryl azide, followed by trapping of the intermediate isocyanate with benzyl alcohol, provided the Cbz adducts **19**. Finally, the esters were hydrolyzed to give the amino acids **20** in racemic

Scheme 2[a]



[a] (a) $TiCl_4$, THF, CCl_4, diethyl malonate, 0 °C, then pyridine, 0 °C to room temp, 68%; (b) MeMgI, CuCl, Et_2O, 0 °C, 69%; (c) NaOH, THF, EtOH, 78%; (d) (i) diphenylphosphorylazide, NEt_3, PhH, reflux; (ii) BnOH, reflux, 18 h, 100%; (e) H_2, $Pd(OH)_2$, EtOAc, 100%; (f) $(Boc)_2O$, K_2CO_3, THF, H_2O, 92%, two steps; (g) NaOH, MeOH, THF, room temp, then aqueous HCl, 95%.

Processing as previously described (Scheme 1) gave a mixture of diastereomers that were separated at the nitrile stage by silica gel flash chromatography. The later-eluting isomer in each case was consistently identified as the desired L,L-dipeptide.[25]

Results and Discussion

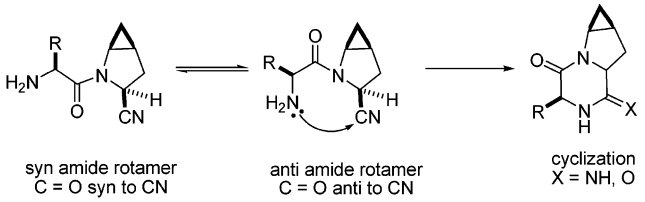
All compounds were tested in vitro against purified porcine DPP-IV. Inhibition was determined against the substrate H-Ala-Pro-*p*NA. Production of *p*-nitroaniline (*p*NA) was measured at 405 nm over 15 min. A comparison of methanoproline-derived isoleucine N-terminal dipeptides shows that the enzyme inhibitory activity is critically dependent on both the position and the orientation of the methano bridge (Table 1). The presence of the methano bridge on the trans side of the prolinenitrile (e.g., **22**, **23**) resulted in a significant loss of activity relative to the unsubstituted prolinenitrile **21**. This result was consistent with early reports where insertion of a methyl group at the 2-postion of 2-cyanopyrrolidides resulted in a 2000-fold decrease in activity.[26] In contrast, when the methano bridge was oriented cis to the nitrile at either the 4,5- or 3,4-position, inhibitory activity was only slightly diminished. This is clearly illustrated with *cis*-4,5-methano inhibitors **24** and **26** and *cis*-3,4-methano inhibitors **25** and **27**, where inhibitory potency resides in the 20 nM range. A more complete exploration of N-terminal amino acids reveals that increasing the degree of β-branching to that of *tert*-Leu (e.g., **29**) further increases potency in the 4,5-methano series to that of the prolinenitrile version (**28**). Replacement of these alkyl substituents with aromatic residues such as Phe (**31**) or Trp (**32**) in the N-terminal position significantly eroded potency. Alkyl substitution on the terminal amine was generally ill-tolerated in this series (e.g., **33**−**35**), though it would appear that the relatively more accessible N-terminus found in proline derivative **34** is capable of restoring a modest degree of inhibitory activity. Replacement of Leu and *tert*-Leu side chains with medium-ring cycloalkyl and methylcycloalkyl (**37**−**42**) generally led to inhibitors with comparable or slightly improved activity, though potency dropped steadily with increasing larger ring size (data not shown). The Cp-Gly and Me-Cp-Gly derived analogues **38** and **41** were among the most potent compound

Table 1. In Vitro Inhibition Constants for Porcine DPP-IV and Solution Stability Half-Lives for Prolinenitrile DPP-4 Inhibitors

compd	N-terminal amino acid[a]	substituted prolinenitrile	K_i (nM)[b]	stability, $t_{1/2}$ (h)[c]
21	Ile	prolinenitrile	2 ± 0.5	5
22	Ile	*trans*-4,5-	1620 ± 80	
23	Ile	*trans*-2,3-	7500 ± 200	
24	Ile	*cis*-4,5-	25 ± 1	22
25	Ile	*cis*-3,4-	15 ± 1	4
26	Val	*cis*-4,5-	29 ± 1	28
27	Val	*cis*-3,4-	12 ± 1	2
28	*tert*-Leu	prolinenitrile	8 ± 0.5	27
29	*tert*-Leu	*cis*-4,5-	7 ± 0.5	42
30	*tert*-Leu	*cis*-3,4-	14 ± 1	4
31	Phe	*cis*-4,5-	65 ± 3	
32	Trp	*cis*-4,5-	230 ± 10	
33	*N*-Me-Val	*cis*-4,5-	1940 ± 80	
34	Pro	*cis*-4,5-	107 ± 5	
35	Pip	*cis*-4,5-	10000	
36	Met	*cis*-4,5-	135 ± 10	
37	Cb-Gly	*cis*-4,5-	12 ± 0.5	
38	Cp-Gly	*cis*-4,5-	4 ± 0.5	
39	Ch-Gly	*cis*-4,5-	15 ± 1	19
40	(1-Me-Cb-1-yl)-Gly	*cis*-4,5-	11 ± 0.5	
41	(1-Me-Cp-1-yl)-Gly	*cis*-4,5-	7 ± 0.5	24
42	(1-Me-Ch-1-yl)-Gly	*cis*-4,5-	8 ± 0.5	

[a] Cb = cyclobutyl; Cp = cyclopentyl; Ch = cyclohexyl. [b] Values represent the mean ± SEM and are at least triplicate determinations. [c] Solution stability data are measured at 39.5 °C and pH 7.2 in phosphate buffer.

Scheme 3



Solution Stability. After the discovery that the L-*cis*-4,5- and L-*cis*-3,4-methanoprolinenitriles were potent inhibitors of DPP-IV in vitro, a comparative study was initiated to investigate aqueous solution stability of these analogues. The N-terminal valine, isoleucine, and *tert*-leucine dipeptide nitriles were selected in order to make direct comparisons between methanoprolinenitrile and unsubstituted prolinenitrile compounds with respect to solution stability. Reaction rates were monitored by following the disappearance of starting material on reverse-phase HPLC at pH 7.2 and 39.5 °C in phosphate buffer. HPLC mass spectral analysis revealed that the two major products that formed during the stability experiments had either an identical mass or an M + 1 mass to the parent starting material. These data are consistent with the formation of intramolecular cyclization products, with the initial cyclic imidate (X = NH) surrendering to the diketopiperazine (X = O) upon hydrolysis (Scheme 3).

Two interesting aspects of the solution half-life data should be noted. The first is that there is increased

cis-3,4-methanoprolinenitrile or the unadorned prolinenitrile compounds. For instance, in the case where the N-terminal amino acid is isoleucine, the isomeric 4,5-methano-substituted compound **24** has a solution half-life of 22 h, which is 5.5-fold longer than the analogous 3,4-methano-substituted compound **25** and approximately 4.5-fold longer than the unadorned prolinenitrile analogue **21**. The second feature is that there is a strong correlation between steric size at the β-position of the N-terminal amino acid and relative solution stability. Increasing the degree of branching at the β-position of the alkylglycine substituent increases solution stability. The most stable compounds within their respective prolinenitrile series have the *tert*-Leu N-terminal amino acid fragment in common. For example, unadorned prolinenitrile **28** ($t_{1/2}$ = 27 h) is more stable than its less branched isomer **21** ($t_{1/2}$ = 5 h). Similarly, *cis*-4,5-methanoprolinenitrile **29** ($t_{1/2}$ = 42 h) is more stable than the less branched dipeptidenitriles **24** and **39** ($t_{1/2}$ = 22 and 19 h, respectively), though the magnitude of the effect of increased β-branching in this series appears to be blunted by the inherent baseline stability imparted by the methano bridge. This observation is in agreement with data reported by Coutes[12a] and Snow[27] where the rates of cyclization for Xaa-boroproline dipeptides were shown to be Gly-BoroPro > Ala-BoroPro > Val-BoroPro.

Computational Analysis. Computational analysis was undertaken to more fully understand the relative stabilities of the methanoprolinenitriles. Ground-state conformations were generated for methanoprolinenitrile and prolinenitrile forms of the N-terminal *tert*-leucine dipeptide compounds. The calculated ground-state structure for the *tert*-leucine dipeptidenitrile is identical to the conformation observed through single-crystal X-ray structural analysis[28] of the TFA salt of **29** (rms = 0.1 Å for calculated and observed heavy atoms; see Figure 2, lower structure). Both have the same syn conformation around the amide bond, characterized by a small C(2)−N−C(8)═O torsional angle (−5° and +2° for the TFA structure). It is of additional interest that a similar conformation has been observed in several recently disclosed cocrystal structures of DPP-IV/inhibitor complexes.[29]

In addition to the syn conformation, there is a calculated local low-energy minimum where the reactive amine and nitrile are close to each other; in this anti conformation (Figure 2, upper structure), the C(2)−N−C(8)═O torsional angle is 180°. Moreover, the angle between the amine N and the C≡N group is 109° ± 1° and the distance between the these reactive partners is 2.95 Å. It is therefore reasonable to assume that the observed intramolecular cyclization is initiated from this conformation. The value of 109° between the amine group and the nitrile is in close agreement with the hypothetical angle of attack of at least 108° reported by Baxter and Connor.[30] It was envisioned that the relative energetic differences between the global minimum and the reactive local minimum would represent a means to evaluate the relative stabilities of compounds in solution.

The calculated conformations and their relative energies due to side chain bulk (specifically β-branching) and the increase in stability upon conversion from prolinenitrile to *cis*-4,5-methanoprolinenitrile. Calculated relative energies between the ground-state conformation and the local low-energy minimum conformation that brings the reactive amine and nitrile in proximity are presented in Table 2 for the methanoprolinenitrile and prolinenitrile forms of N-terminal *tert*-leucine and alanine dipeptidenitriles, as well as for the same prolinenitriles with a simple acetamide cap.

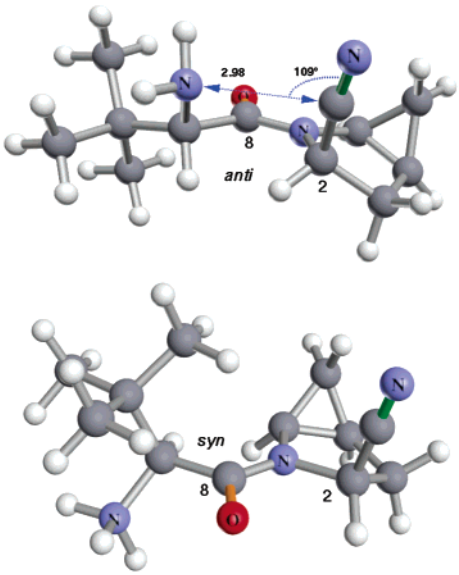
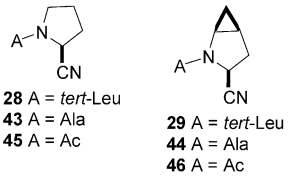


Figure 2. The upper structure depicts the local low-energy minimum for **29** where the reactive amine is close to the nitrile (anti conformation). The lower structure is the solid-state conformation observed in the X-ray crystallographic structure of the TFA salt of compound **29** (syn conformation). Two independent cations were observed in the crystal structure, though because these have identical conformations, only one is shown for purposes of clarity. Carbon atoms C(2) and C(8) are labeled.

Conformational Stability Due to Side Chain Bulk. The values in the first column of Table 2 compare the conformational energy differences between the ground state and the geometry where internal cyclization could occur for the unsubstituted prolinenitrile series. The ab initio (G98) results are expected to be considerably more accurate than the force field values and indicate that the energy required to assume the anti conformation increases with side chain bulk (e.g., 0.3, 1.9, 2.8 kcal/mol for no side chain, the alanine side chain, and the *tert*-leucine side chain, respectively). The force field energy results agree qualitatively with the ab initio values and suggest that the primary contribution is due to van der Waals interactions. Examination of the structures reveals extremely close contacts between two of the side chain methyl groups and the carbonyl oxygen in the anti conformation (approximately 3 Å each) that would increase the energy barrier for internal cyclization and thus lead to greater stability. A similar trend is observed for increasing side chain bulk in the methano-substituted series (column 2), and these values are in good agreement with the solution data where increased β-branching enhances stability.

Conformational Stability Due to Methano Sub-

Table 2. Calculated Energy[a] Differences between the Calculated Ground State and the Local Low-Energy Minimum Required for Cyclization



28 A = *tert*-Leu
43 A = Ala
45 A = Ac

29 A = *tert*-Leu
44 A = Ala
46 A = Ac

28 (Prolinenitrile) and **29** (Methanoprolinenitrile) (A = *tert*-Leu)

method interaction	ΔH(**28**) [b]	ΔH(**29**) [b]	$\Delta\Delta H$(**29** − **28**)
G98 ab initio	2.8	3.4	0.6
Insight CFF force field			
total nonbond	3.5	4.2	0.7
van der Waals	3.3	3.9	0.6
electrostatic	0.2	0.3	0.1

43 (Prolinenitrile) and **44** (Methanoprolinenitrile) (A = Ala)

method interaction	ΔH(**43**) [b]	ΔH(**44**) [b]	$\Delta\Delta H$ (**44** − **43**)
G98 ab initio	1.9	2.6	0.6
Insight CFF force field			
total nonbond	3.3	3.9	0.6
van der Waals	2.9	3.4	0.6
electrostatic	0.4	0.4	0.0

45 (Prolinenitrile) and **46** (Methanoprolinenitrile) (A = Ac)

method interaction	ΔH(**45**) [b]	ΔH(**46**) [b]	$\Delta\Delta H$ (**46** − **45**)
G98 ab initio	0.3	0.9	0.6
Insight CFF force field			
total nonbond	−0.2	0.0	0.2
van der Waals	−0.4	−0.2	0.2
electrostatic	0.2	0.3	0.1

[a] Energies are for the *cis*-4,5-methanoprolinenitrile and prolinenitrile versions of the compounds and are given in kcal/mol. [b] Energies are for the anti conformation (Scheme 3 and Figure 2 upper structure) relative to the global minimum conformation, which corresponds to the syn geometry in Scheme 3 and Figure 2 lower structure.

addition of the methano bridge increases the energy barrier toward adopting the conformation required for cyclization by approximately 0.6 kcal/mol for the N-terminal *tert*-leucine dipeptide compound as well as for the alanine dipeptide and acetamide compounds. The lack of dependence on the nature of the side chain suggests that the observed increase in stability of the *cis*-4,5-methano compounds is due to a local interaction between the methano bridge and the N-terminal amino acid that favors the ground state relative to the anti conformation where cyclization is initiated. The force field energy analysis (Insight CFF) results also agree qualitatively, implying that van der Waals interactions are primarily responsible for the increase in energy. These conclusions are supported by the cyclization rate differences between the isomeric isoleucine derivatives **24** ($t_{1/2}$ = 22 h) and **21** ($t_{1/2}$ = 5 h), where a 4.5-fold increase in stability is observed for the methanoprolinenitrile. This increase in stability is in fair agreement with the calculated value of 0.6 kcal/mol. The calculated $\Delta\Delta H$ and observed solution half-life results support contributions to stability from both β-branching and the presence of the 4,5-methano bridge on the pyrrolidine ring.

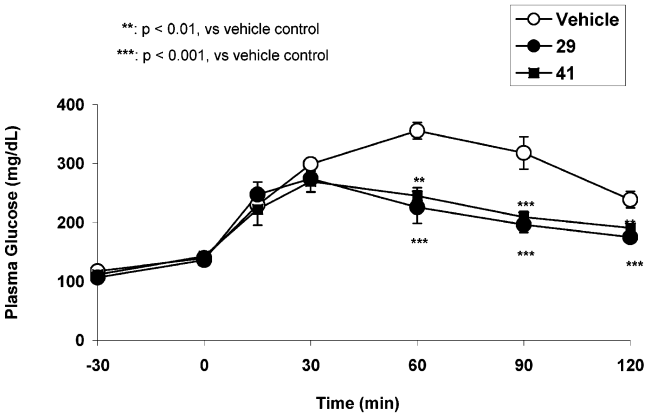


Figure 3. Effects of inhibitors **29** (●) and **41** (■) dosed at 3 μmol/kg po versus vehicle control (○) on plasma DPP-IV activity in Zucker$^{fa/fa}$ rats.

IV inhibition, increases in plasma insulin levels, and an improvement in glucose tolerance.[31] Compounds **29** and **41** were potent inhibitors of DPP-IV in vitro and demonstrated excellent solution stability. As such, these inhibitors were selected to determine the effects of DPP-IV inhibition in vivo on glucose tolerance and insulin secretion in Zucker$^{fa/fa}$ rats. An oral glucose tolerance test (oGTT) in the Zucker$^{fa/fa}$ rat is a frequently used model of hyperglycemia in type 2 diabetes and obesity research. Zucker$^{fa/fa}$ rats are severely hyperphagic, extremely obese, markedly insulin-resistant, and mildly hyperglycemic because of a mutation and loss of function of the leptin receptor gene.[32,33] Fasted male Zucker$^{fa/fa}$ rats were dosed orally with water or with inhibitors **29** and **41** (3 μmol/kg), and an oGTT was conducted 0.5 h after the dosing. Plasma DPP-IV activity, glucose, and insulin levels were then monitored over a 2 h period. Figures 3−5 show the ex vivo plasma DPP-IV activity, insulin response, and glucose excursion curves in response to an oral glucose challenge (2 g/kg). Animals in the control group reached peak plasma glucose levels 60 min after glucose administration, at which point the drug-treated animals exhibited a 30−35% decrease in glucose levels compared to controls (control animals, 356 mg/dL; compound **29** treated animals, 226 mg/dL; compound **41** treated animals, 245 mg/dL). Glucose levels were significantly reduced in the drug-treated animals from 30 min onward, with maximal reductions in glucose observed at 90 min (−34% to −38%). Plasma DPP-IV activity was maximally suppressed (60%) 30 min after dosing (Figure 3), and the effects of these inhibitors were sustained throughout the course of the experiment (60−35%). The insulin response to oral glucose was also enhanced by treatment with DPP-IV inhibitors (Figure 4), demonstrating the link between the glucose-lowering effects and DPP-IV inhibition of these compounds.

Interestingly, a significant decrease in plasma glucose levels occurred when DPP-IV activity in plasma was inhibited only by 35−60%. This finding suggests that it may not be necessary to completely suppress plasma DPP-IV activity in order to achieve antihyperglycemic efficacy in type 2 diabetics. However, differences may exist in the inhibition of the turnover of native sub-

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