Pyrrolidides: synthesis and structure–activity relationship as inhibitors of dipeptidyl peptidase IV

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Summary — Dipeptidyl peptidase IV cleaves specifically the peptide bond at the carboxyl side of a proline at the penultimate N-terminal position of a peptide. It is thought to be important for the regulation of biologically active peptides. Moreover, it has been identified as an activation marker of T-lymphocytes (CD26). Pyrrolidides and thiazolidides are known as reversible inhibitors of DPP IV. Several homologues, unsaturated, open and 3-substituted analogues were synthesized in order to determine the structure–activity relationship of the P 1 site. L-Isoleucine was taken as P-2 amino acid. 1-(L-Isoleucyl)-3(*S*)-fluoropyrrolidine is about as active as the non-fluorinated compound and behaves as a competitive inhibitor. Other changes decrease or abolish the activity.

dipeptidyl peptidase IV / pyrrolidide / serine protease / proline peptidase / protease inhibitor

Introduction

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 are supposed to play an important role in the regulation of the lifetime of biologically active peptides [1].

Dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5) is a serine exopeptidase that cleaves off N-terminal dipeptides specifically at the carboxyl side of proline (X-Pro) [2]. DPP IV is expressed quite ubiquitously in mammalian tissues. On the epithelial cells of intestine and kidney, DPP IV participates in the metabolism and uptake of proline-containing peptides [3]. In human plasma, DPP IV is responsible for the degradation and inactivation of growth hormone-releasing hormone [4]. It may also be involved in the metabolism of several other biologically active peptides [5–7]. On human T-lymphocytes, DPP IV is assigned to the

CD 26 cluster involved in T-cell activation [8]. Furthermore, lymphocytic DPP IV/CD 26 is associated with the adenosine deaminase protein, the deficiency of which is known to result in severe combined immunodeficiency in humans [9]. Development of potent, selective and biocompatible inhibitors of DPP IV contributes to the unravelling of the physiological functions of this intriguing ectopeptidase.

Most of the inhibitors reported for DPP IV are dipeptide analogues. Relevant reference compounds of different classes of DPP IV inhibitors are shown in figure 1. The boronic acid transition state analogues Ala-boroPro, Val-boroPro and Pro-boroPro (**1**) are very potent reversible inhibitors of DPP IV [10]. Unfortunately, they have a very short half-life in aqueous solution at neutral pH due to a cyclization reaction between the free amino group of the P-2 amino acid and the boronic acid [11]. The same instability problems can be expected for other transition-state analogues (such as aldehydes or trifluoromethyl-ketones) that are often used as serine protease inhibitors. Dipeptide diphenylphosphonates (**2**) are irreversible inhibitors leading to a phosphorylated enzyme [12, 13]. They have a half-life of a few hours in plasma. Other irreversible inhibitors are proline derived diacylhydroxylamines [14]. Pyrrolidides
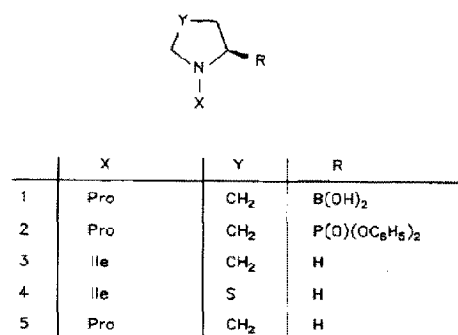
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Fig 1. DPP IV inhibitors.

	X	Y	R
1	Pro	CH_2	$B(OH)_2$
2	Pro	CH_2	$P(O)(OC_6H_5)_2$
3	Ile	CH_2	H
4	Ile	S	H
5	Pro	CH_2	H

(3 and 5) and thiazolidides (4) are reversible, competitive inhibitors of DPP IV [15]. Substitution at position 2 with a nitrile group also affords competitive inhibitors [16].

In order to fully explore the S-1 subsite of the enzyme and because of the chemical and biological instability of reactive electrophiles, we concentrated on potential inhibitors containing substituted pyrrolidines, open pyrrolidines or homologues thereof at the P-1 position (6b–23b) (figs 2 and 3). In this study, L-Ile was used at the P-2 position as this amino acid proved to be an efficient P2-residue. Furthermore, we introduced the constrained L-Leu and L-Ile analogues, L-cyclohexylalanine (24b) and *trans*-3-methyl-L-proline (25c) (fig 4).

Chemistry

Boc-Ile was coupled to an appropriate secondary amine in the presence of benzotriazol-1-yloxy-tris(dimethyl-amino)phosphonium hexafluorophosphate (BOP) to afford compounds 6a–15a. Deprotection with trifluoroacetic acid gave final compounds 6b–15b (fig 2). Coupling of Boc-Ile to 3-hydroxypyrrolidine in the presence of BOP was possible without hydroxyl protection (16a). Various reactions at the hydroxyl function and cleavage of the Boc protecting group resulted in 3-substituted pyrrolidines 16b–23b (fig 3). Chlorine and fluorine were introduced with triphenylphosphine/CCl_4 and diethylaminosulphur trifluoride (DAST), respectively. An azide was obtained from a tosylate intermediate, prepared with tosylsulphonyl chloride and sodium azide. A methoxy group was introduced with methyl iodide. Treatment with benzoyl chloride afforded the benzoate and a periodate-ruthenium oxide oxidation gave rise to the corresponding ketone.
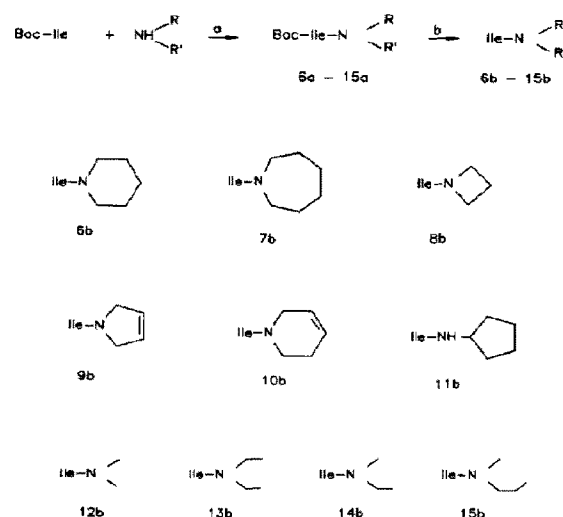

Fig 2. Synthesis and structures of ring-modified pyrrolidides: (a) BOP, piperidine; (b) trifluoroacetic acid.

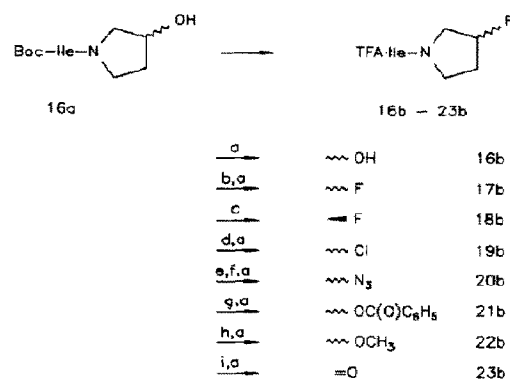|	R	
a	$\sim$ OH	16b
b,a	$\sim$ F	17b
c	$\rightarrow$ F	18b
d,a	$\sim$ Cl	19b
e,f,a	$\sim$ N_3	20b
g,a	$\sim$ $OC(O)C_6H_5$	21b
h,a	$\sim$ OCH_3	22b
i,a	=O	23b

Fig 3. Synthesis and structure of 3-substituted pyrrolidides: (a) trifluoroacetic acid; (b) diethylaminosulfur trifluoride; (c) identical to the synthesis of 17, but starting from the (*R*)-3-hydroxypyrrolidine derivative; (d) $P(C_6H_5)_3$, CCl_4; (e) *p*-toluenesulphonyl chloride, Et_3N; (f) NaN_3; (g) benzoyl chloride, pyridine; (h) 1) NaH; 2) CH_3I; (i) RuO_2, $NaIO_4$.

Examination of the ^{1}H-NMR spectra indicates that several compounds (10b, 14b, 15b, 18b, 23b) exist in solution as a mixture of two rotamers around the amide bond, as is frequently observed for *X*-proline amide bonds.
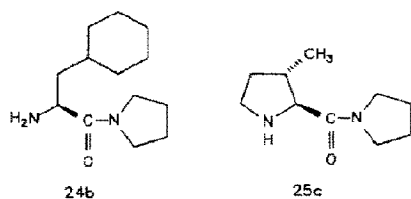
Fig 4. Structures of compounds 24b and 25c.

Compound **24b** was obtained by hydrogenolysis of Z-protected N-L-cyclohexylalanylpyrrolidide **24a**, obtained by BOP-mediated coupling of pyrrolidine and Z-L-cyclohexylalanine. For the synthesis of **25c**, trans-3-L-methylproline [17] was Z-protected (**25a**) and coupled to pyrrolidine with a water soluble carbodiimide. Compound **25c** was obtained by hydrogenolysis.

Biological evaluation

DPP IV was isolated from human seminal plasma as described previously [18]. The specific activity of the preparation was 35 U/mg. One activity unit is defined as the amount of enzyme which catalyzes the conversion of 1 μmol of substrate per minute at 37 °C. The IC_{50} was determined using the fluorogenic substrate Gly-Pro-4-methoxy-2-naphthylamide-HCl. The IC_{50} value was defined as the inhibitor concentration which caused a 50% decrease in the activity. The error on repeated IC_{50} determinations was around 20%.

To determine the type of inhibition and the inhibition constants, the chromogenic substrate Gly-Pro-p-nitroanilide was used. The decrease in initial rate with inhibitor concentration was fitted with equation (1):

$$v_i/v_0 = K_{i,app}/(K_{i,app} + [I]_0) \qquad (1)$$

where v_i and v_0 are the initial rates in the presence and absence of inhibitor, $K_{i,app}$ is the apparent inhibition constant at the substrate concentration used, and $[I]_0$ is the total inhibitor concentration. In case of competitive inhibition, $K_{i,app}$ is defined by equation (2):

$$K_{i,app} = K_i * (1 + [S]_0/K_m) \qquad (2)$$

where K_i is the substrate-independent, 'true' inhibition constant, $[S]_0$ is the initial substrate concentration and K_m is the Michaelis constant of the substrate. Measured in a separate experiment under the same conditions, K_m was 0.34 ± 0.02 mM.

Results and discussion

The IC_{50} values for DPP IV inhibition of the compounds prepared are summarized in table I. Analogues with different ring sizes or an open ring structure are less active than the parent pyrrolidide **3**. The IC_{50} values of compounds **3**, **4** and **6b–9b** show a good correlation with the ring size, indicating an optimal inhibition with pyrrolidine and thiazolidine: the inhibitory capacity increases from azetidine **8b** (270 μM) and pyrroline **9b** (100 μM) to the optimal five-rings pyrrolidine **3** (21 μM) and thiazolidine **4** (18 μM), and decreases when larger rings like tetrahydropyridine **10b** (310 μM), piperidine **6b** (510 μM) or hexamethyleneimine **7b** (2700 μM) are at the P1 position. Replacing the ring with acyclic substituents (**12b–15b**), which could possibly adopt the pyrrolidine conformation, or taking the amino group out of the ring as in **11b** also increase the IC_{50} considerably. Therefore, we believe that the S-1 subsite of DPP IV ideally fits a five-membered saturated ring. This corresponds to the high specificity of the enzyme for proline among the amino acids. In their study on the substrate specificity of DPP IV, Rahfeld et al [19] observed a much higher k_{cat} value for azetidine compared to five-membered rings, but the K_m value, reflecting the affinity of the enzyme for the substrate, was higher. In agreement, we find higher IC_{50} values for the inhibitors with a four-membered ring.

Introduction of a substituent at 3-position of the pyrrolidine ring (fig 3) (**16b–23b**) generally decreased the inhibitory activity (table I). Only a small substituent such as fluorine, isosteric to hydrogen, is allowed. L-Ile-3-fluoropyrrolidide (**17b**) shows about the same activity (27 μM) as its hydrogen (**3**, 21 μM) or thia analogue (**4**, 18 μM). Moreover, the stereochemistry of the fluorine atom was not important (**17b** versus **18b**).

The allowance of a highly electronegative fluorine atom is surprising in view of a recently published model of the active site of DPP IV [20]. By comparative molecular field analysis and molecular modelling, the authors predict that the proline specificity of DPP IV is mainly caused by recognition of proline by the tyrosine side chain near the active serine residue, and that in the vicinity of P1 a positive or an uncharged structure element is favourable. Our data clearly show that the presence of the electronegative fluorine does not interfere with binding, suggesting that the interaction of P1 with the Tyr residue is mostly sterically determined. More bulky substituents as hydroxy, chloro, azido, methoxy, benzoyloxy and oxo are deleterious for the inhibitory activity.

In addition to L-Ile, the prototype P2 amino acid, we synthesised two analogous P2 derivatives: L-cyclohexylalanylpyrrolidide **24b** and trans-3-methyl-L-

Table I. IC_{50} values for DPP IV inhibitors.

| Compound	In 50 mM Tris, pH 8.3			In citrated plasma		
	Range (mM) (n)		IC_{50} (μM)	Range	(n)	IC_{50}
6b	10–0.01	(4)	510 ± 50	10–0.02	(5)	470 ± 180
7b	10–0.01	(4)	2700 ± 600			
8b	10–0.01	(4)	270 ± 40	2–0.005	(5)	460 ± 70
9b	2–0.002	(10)	100 ± 3	1–0.002	(5)	73 ± 7
10b	10–0.01	(10)	310 ± 30	2–0.005	(5)	201 ± 38
11b	10–0.01	(4)	14000 ± 1000			
12b	10–0.5	(5)	3800 ± 900			
13b	10–0.5	(5)	> 10000			
14b	10–0.5	(5)	2200 ± 400			
15b	10–0.5	(5)	3500 ± 600			
16b[a]	10–0.01	(4)	740 ± 80	10–0.02	(5)	1040 ± 70
17b[a]	10–0.01	(4)	27 ± 3	0.2–0.0005	(5)	35 ± 8
18b	1–0.001	(10)	25 ± 2	0.2–0.0005	(5)	22 ± 6
19b[a]	10–0.01	(10)	610 ± 30	10–0.02	(5)	500 ± 100
20b[a]	10–0.01	(10)	1070 ± 80	10–0.02	(5)	490 ± 50
21b[a]	10–0.01	(10)	6200 ± 700			
22b	10–0.001	(5)	> 10000			
23b	10–0.01	(10)	320 ± 30	2–0.005	(5)	380 ± 110
24b	1–0.001	(10)	180 ± 15	2–0.005	(5)	180 ± 40
25c	1–0.001	(10)	300 ± 20	2–0.005	(5)	350 ± 50
3[b]	10–0.01	(4)	21 ± 4	0.2–0.0005	(5)	23 ± 4
4[b]	1–0.01	(7)	18 ± 2	0.2–0.0005	(5)	30 ± 10
5[b]	1–0.001	(10)	126 ± 9	1–0.002	(5)	83 ± 7

The activity versus inhibitor concentration was fitted with the equation of a single saturatable binding site. The errors in the table are the standard errors of the fit. n = number of different concentrations used. [a]Tested as diastereoisomeric mixture; [b]reference compounds (prepared as described) from the literature [15].

prolylpyrrolidide (25c). *trans*-3-Methyl-L-proline, which can be considered as a ring-constrained analogue of L-Ile, gives a ten-fold decreased inhibition. L-Cyclohexylalanine, with its bulky aliphatic side chain, fulfills the proposed requirements for binding to the S2 subsite. However, this could not be experimentally confirmed. Summarizing, the IC_{50} values increase from L-Ile-pyrrolidide (3, 21 μM) via L-Pro-pyrrolidide (5, 126 μM) to L-cyclohexylalanyl pyrrolidide (24b, 180 μM)] and *trans*-3-methyl-L-proline pyrrolidide (25c, 300 μM).

Determination of inhibition constants revealed that the compounds of this study are competitive inhibitors of DPP IV. This is illustrated in figure 5 for the 3-fluoro compound 17b: the apparent inhibition constant increases linearly with the substrate concentration and the presence of the inhibitor causes an increase in K_m but leaves the V_{max} unaffected. The K_i values of a representative set of inhibitors (3, 4 and 17b) were 1.5 ± 0.1, 1.8 ± 0.1 and 2.0 ± 0.1, respectively. The IC_{50} values listed in table I, therefore, are apparent inhibition constants obtained at a $[S]_0 \simeq 10K_m$ (using the fluorogenic substrate). The inhibition constants of

these compounds strictly reflect the affinity of the P1-P2 residues for substrate binding sites. Examining published data, it is clear that a good affinity for the substrate binding site is not always sufficient to make a good substrate or inhibitor. Parameters equally important for a catalytic efficiency are the rate of acylation, the rate of deacylation, or the ability of the substrate to adopt the *trans*-proline conformation. Other types of inhibitors that mimic later steps in the catalytic mechanism in their mode of action may have a different P1–P2 preference than the compounds of this study. A striking example of this is the relative potency of dipeptide-derived diphenyl phosphonate esters, where cyclohexylalanine and proline were better P2 residues than isoleucine [13].

The potency of our compounds to inhibit the intrinsic DPP IV activity present in human plasma is comparable to that obtained in assay buffer using highly purified human CD26 (table I) [18]. Human plasma contains a form of DPP IV which differs from CD26 by the lack of adenosinedeaminase binding and its high subunit molecular weight [21]. The catalytic properties of both forms of DPP IV were very similar
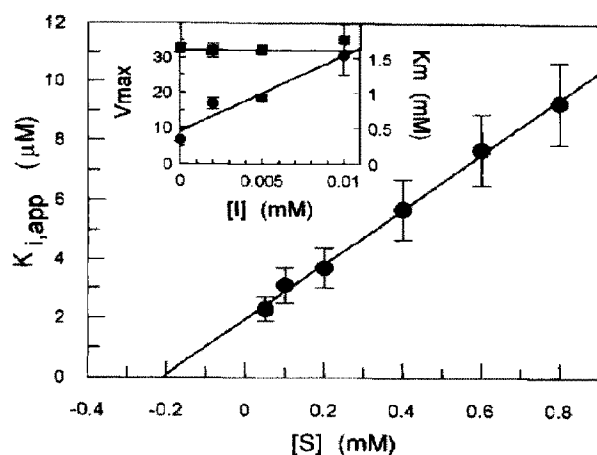
Fig 5. Competitive inhibition of DPP IV activity by compound **17b**. The apparent inhibition constant increases with increasing substrate concentration, as predicted by equation (2). The inset shows the effect of the inhibitor concentration on the K_m (●) and V_{max} (■) of the substrate. V_{max} is expressed in arbitrary units. Conditions are described in the *Experimental protocols*.

using the chromogenic dipeptide substrate Gly-Pro-*p*-nitroanilide. Our results with the inhibitors extend this observation to include the P1 specificity of both enzyme forms.

The stability of the compounds in plasma is an important advantage for their potential use in biological systems.

Experimental protocols

Materials and methods

Amino acids and intermediates were obtained from Bachem Feinchemikalien AG, Switzerland. Reagents and solvents were from ACROS Chimica or Aldrich. Gly-Pro-4-methoxynaphthyl-amide and Gly-Pro-*p*-nitroanilide were from Sigma. Fluorescence was measured in a Model RF-5000 fluorimeter from Shimadzu Corporation (Tokyo, Japan), excitation wavelength was 340 nm, emission wavelength 425 nm.

Melting points were determined on a digital melting point apparatus (Electrothermal) and are uncorrected. The ^{1}H-NMR spectra were determined on a Bruker 300 MHz with tetramethylsilane as internal standard. Column chromatography was performed on Fluka silica gel H (5–40 µm) under vacuum.

Measurement of DPP IV activity

The enzyme was diluted in reaction buffer immediately before the experiment, typically around 5–10 mU/mL. Enzymatic reactions were carried out in 50 mM Tris·HCl buffer, pH 8.3 at 37 °C. Substrate and inhibitors were dissolved in DMSO and diluted in the reaction buffer immediately before the experiment. Substrate stock solutions were stored at -20 °C, inhibitor stock solutions were kept at 4 °C.

Determination of IC_{50} values

To determine the IC_{50}, 5 µL of inhibitor solution (between 100 and 0.01 mM in DMSO) was incubated with 40 µL of DPP IV in reaction buffer (or 40 µL of freshly frozen citrated human plasma) at room temperature for 1 h. The remaining activity was determined by adding 5 µL of 10 mM Gly-Pro-4-methoxy-naphthylamide (25% DMSO), then incubation for 20 min at 37 °C. The reaction was stopped by addition of 0.5 mL of 100 mM citrate pH 4.0 before the fluorescence was measured. Measurements were performed in duplicate and corrected for background fluorescence using a blank with exactly the same composition except that the citrate solution was added before the substrate.

Type of inhibition, determination of inhibition constants

To determine the type of inhibition and the inhibition constants, the chromogenic substrate Gly-Pro-*p*-nitroanilide was used at final concentrations of 0.05, 0.1, 0.2, 0.4, 0.6 and 0.8 mM in 100 mM Tris buffer, pH 8. At every substrate concentration six inhibitor concentrations were used between 0.002 and 0.1 mM (**17b**, **3**, **4**), or between 0.05 and 5 mM (**8b**).

Chemistry

1-[N-(tert-Butyloxycarbonyl)-ʟ-isoleucyl]piperidine 6a

To a mixture of *N*-(*tert*-butyloxycarbonyl)-ʟ-isoleucine (1.16 g, 5 mmol), piperidine (0.43 g, 5 mmol) and BOP (2.43 g, 5.5 mmol) in DMF (20 mL) was added triethylamine (1.5 mL, 11 mmol). After stirring at room temperature overnight, water (100 mL) was added and the mixture was extracted with EtOAc (3 × 50 mL). The combined organic layer was washed with HCl (2 N, 2 × 25 mL), water (25 mL), NaHCO₃ (5%, 2 × 25 mL) and brine (25 mL). The organic layer was dried, evaporated and purified by column chromatography (CH₂Cl₂; CH₂Cl₂/MeOH, 99:1; CH₂Cl₂/MeOH, 98:2) yielding 1.48 g (4.97 mmol, 99%) of the title compound as a solid. ^{1}H-NMR (CDCl₃) δ 0.88 (t, $J = 7.6$ Hz, 3H, δ-CH₃), 0.93 (d, $J = 6.8$ Hz, 3H, γ-CH₃), 1.00–1.20 (m, 1H, γ-CH), 1.43 (s, 9H, C(CH₃)₃), 1.50–1.80 (m, 8H, γ-CH, β-CH, 3-CH₂, 4-CH₂, 5-CH₂), 3.45–3.65 (m, 4H, 2-CH₂, 6-CH₂), 4.49 (dd, $J = 6.3$ and 8.7 Hz, 1 H, α-CH), 5.32 (d, $J = 8.7$ Hz, 1H, NH).

1-(ʟ-Isoleucyl)piperidine trifluoroacetate 6b. General procedure for deprotection with trifluoroacetic acid

The Boc-protected compound **6a** (1.45 g, 4.87 mmol) was treated with trifluoroacetic acid (TFA) (10 g) for 1 h at room temperature. The title compound was obtained in quantitative yield after evaporation, coevaporation with toluene (three times) and MeOH (three times). The compound was crystallized from MeOH/Et₂O. Mp 147–149 °C. ^{1}H-NMR (DMSO-*d₆*) δ 0.85 (t, $J = 7.3$ Hz, 3H, δ-CH₃), 0.94 (d, $J = 6.9$ Hz, 3H, γ-CH₃), 1.00–1.25 (m, 1H, γ-CH), 1.35–1.70 (m, 7H, γ-CH, 3-CH₂, 4-CH₂, 5-CH₂), 1.70–1.85 (m, 1H, β-CH), 3.20–3.65 (m, 4H, 2-CH₂, 6-CH₂), 4.25 (d, $J = 4.9$ Hz, 1H, α-CH), 8.07 (br s, 3H, NH₃⁺). Anal C₁₁H₂₂N₂O·C₂HF₃O₂ (C, H, N).

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