CHAPTER 2

PHOSPHORUS-CONTAINING INHIBITORS OF PROTEOLYTIC ENZYMES

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1. INTRODUCTION

The dipeptidyl peptidase IV (DP IV, EC 3.4.14.5) is a proline specific serine protease, which cleaves Xaa-Pro dipeptides from the N-terminus of oligo- and polypeptides (for review Lambeir *et al* 2003).

During the last years our attention was directed to the development of DP IV inhibitors and their specificity. It is well-known, that Xaa-Pro dipeptides which are products of substrate hydrolysis are competitive inhibitors of DP IV (Rahfeld 1989). Furthermore, the product analogous amino acid pyrrolidides (Pyrr), e.g. **1** (figure 1), and thiazolidides are also known as potent competitive inhibitors (Born *et al* 1994). Previously, we synthesized thioxo amino acid amides as amide bond isosteres of amino acid pyrrolidides and thiazolidides (Stöckel-Maschek *et al* 2000a). Moreover, analogous phosphonamidates **2** were synthesized as amide bond isosteres (figure 1).

Peptides containing a 3-amino-2-hydroxy acid like bestatin and amastain were described as inhibitors of different aminopeptidases (Umezawa *et al* 1976, Nishizawa *et al* 1977, Rich *et al* 1984). Peptides and amides containing a N-terminal 3-amino-2-hydroxy acid and a penultimate proline or proline-analogous structure were described as the first potent inhibitors of aminopeptidase P (APP) (Prechel *et al* 1995; Stöckel *et al* 1997). Recently, we showed, that these 3-amino-2-hydroxy acid containing APP inhibitors (**3**, figure 1) are also able to inhibit other aminopeptidases and dipeptidyl peptidase IV (Stöckel-Maschek *et al* in preparation). In a further study, the carbonyl group of the 3-amino-2-hydroxy acid was replaced by a tetrahedral phosphorus moiety. The concept of transition-state analogues has been very useful in designing potent inhibitors of proteolytic enzymes. Tetrahedral phosphorus derivatives are stable mimics of a tetrahedral intermediate that lies along the proteolytic reaction coordinate.

Thus, we have synthesized 2-amino-1-hydroxy phosphonic acid derived amides and peptides (compounds **4**, figure 1). The title compounds were tested as inhibitors of the aminopeptidases APP, aminopeptidase M (APM), leucine aminopeptidase (LAP) as well as DP IV.

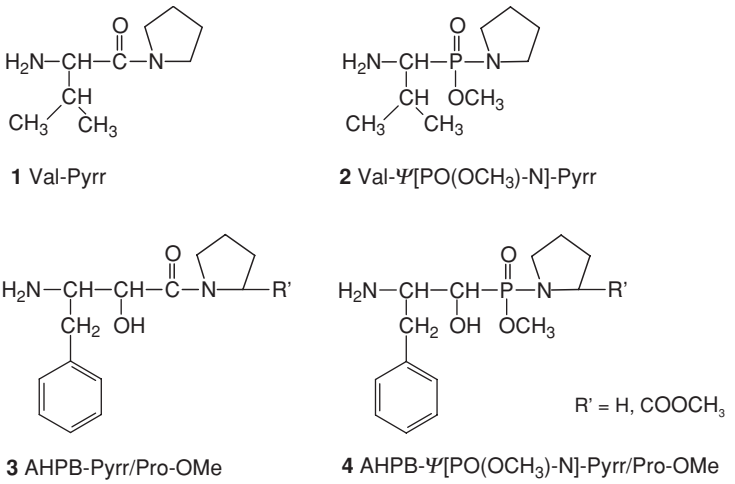


Figure 1. Structure of amino acid pyrrolidides and dipeptides as well as isosteric phosphonamidates - (AHPB, 3-amino-2-hydroxy-4-phenylbutanoic acid)

2. MATERIALS AND METHODS

The phosphonamidate **2** (figure 1) was synthesized starting from Z-protected phosphonovaline dimethylester, which was obtained by reaction of benzyl carbamate, isobutyraldehyde and dimethyl phosphite using the Oleksyszyn reaction (Oleksyszyn *et al* 1979). Partial dealkylation yielded the Z-protected monomethylester. Subsequently, methylester chloride was formed using $(COCl)_2$/DMF. The methylester chloride was converted into the desired phosphonamidate. It was obtained in a diastereomeric mixture and the diastereomers were separated by HPLC. Hydrogenolytic deprotection of the N-terminus provided the expected final compound Val-ψ[$PO(OCH_3)$-N]-Pyrr.

β-amino-α-hydroxy phosphonic acid derived amides and peptides **4** (figure 1) were synthesized starting from an appropriate Z-protected (*R*)- or (*S*)-α-amino alcohol. Oxidation followed by addition of dimethyl phosphite resulted in two diastereomers of Z-protected β-amino-α-hydroxy phosphonic acid dimethylesters (Patel *et al* 1990). After protection of the α-hydroxy group the dimethyl esters were converted into the corresponding amides/peptides *via* monomethylester and methylester chloride, as described above. The β-amino-α-hydroxy phosphonic acid derived amides and peptides were obtained in form of 4 diastereomers, because the compounds contain three asymmetric atoms. The configuration of the C2-atom is defined from the starting material. The diastereomers were separated by HPLC. The α-hydroxy group and the N-terminus were deprotected by well established methods.

DP IV from pig kidney was isolated according to Wolf *et al* (1978). LAP as well as APM both from pig kidney were obtained from Sigma-Aldrich. Recombinant APP from *E.coli* was a gift from Prof. T. Yoshimoto (Japan).

Except APP, the enzymatic hydrolysis of the substrates was monitored at a wavelength of 390 nm ($\varepsilon = 11\ 500\ M^{-1}cm^{-1}$) on a Beckman DU-650 UV/VIS spectrophotometer at 30 °C. Generally, the reaction was initiated by adding the enzyme and was followed over a time interval in which less than 10 % cleavage of substrate occurred.

The mechanism of inhibition of DP IV and the K_i values were determined from the enzyme-catalyzed hydrolysis of Gly-Pro-4NA (4NA: 4-nitroanilide) in the absence and the presence of inhibitor. The incubation mixture (1 ml) contained 40 mM Tris buffer pH 7.6, 0.125 mM NaCl, various concentrations of Gly-Pro-4NA and various concentrations of inhibitor around the expected inhibition constant. The final concentration of DP IV was 1.4 nM.

The activity of recombinant APP from *E.coli* was determined using the substrate Lys(Abz)-Pro-Pro-4NA (Abz, 2-aminobenzoyl, $K_m = 40.7\mu M$). The fluorescence of released Lys(Abz) was monitored at an excitation wavelength of 310 nm and an emission wavelength of 410 nm at 30 °C on a Perkin-Elmer LS-50B luminescence spectrometer (Stöckel-Maschek *et al* 2003). Lyophilized APP (0.1 mg) was dissolved in Tricine-citrate buffer (3 ml, 40 mM Tricine, 0.6 mM citrate, pH 7.4) containing $MnCl_2$ (3 mM). At first, APP was preincubated for 1 h at 30°C. A typical reaction mixture (1 ml) consisted of Tris buffer (40 mM, pH 7.4), $MnCl_2$ (0.75 mM), Lys(Abz)-Pro-Pro-4NA in various concentrations (4-15 μM) and different concentrations of inhibitor. The final enzyme concentration was 24 nM [MW: 49 650 Da (Yoshimoto *et al* 1989)].

The activity of LAP was determined from the enzyme-catalyzed hydrolysis of the substrate Leu-4NA ($K_m = 1.05$ mM) in the absence and presence of inhibitor. At first, LAP (100 μl) was incubated for 2 h at 40 °C in Tricin buffer (900 μl, pH 8.0) and $MnCl_2$ (100 μl, 15 mM). The reaction mixture (1 ml) contained Tris/HCl buffer (40 mM, pH 8.0), various concentrations of Leu-4NA (0.5–2 mM) as well as different concentrations of inhibitor around the expected inhibition constant. The final enzyme concentration was 24 μg/ml.

The substrate Ala-4NA ($K_m = 0.235$ mM) was employed for the determination of APM activity. The reaction mixture contained Tris/HCl buffer (40 mM, pH 7.2), various concentrations of Ala-4NA (0.12-0.5 mM) and different concentrations of inhibitor around the expected inhibition constant. The final enzyme concentration was 1.5 μg/ml.

The steady state kinetics were analyzed using the following equation for competitive and also linear mixed-type inhibition.

$$\frac{1}{v} = \frac{\left(1 + \dfrac{\alpha K_m}{[S]}\right)}{\alpha K_i V_{\max}}[I] + \frac{1}{V_{\max}}\left(1 + \frac{K_m}{[S]}\right) \tag{1}$$

K_i is the competitive inhibition constant and factor α multiplied with K_i represents the uncompetitive inhibition constant. For the distinction between competitive and linear mixed-type inhibition slopes and intercepts were replotted versus 1/[S] to complete the inhibition mechanism and the constants K_i as well as α (equations 2 and 3) (Segel 1993).

$$slope = \frac{K_m}{K_i V_{\max}}\frac{1}{[S]} + \frac{1}{\alpha K_i V_{\max}} \tag{2}$$

$$intercept = \frac{K_m}{V_{\max}}\frac{1}{[S]} + \frac{1}{V_{\max}} \tag{3}$$

In case of hyperbolic mixed-type inhibition the following equation was used to plot the data (Segel 1993).

$$\frac{1}{v} = \frac{\alpha K_m}{V_{\max}}\left(\frac{[I] + K_i}{\beta[I] + \alpha K_i}\right)\frac{1}{[S]} + \frac{1}{V_{\max}}\left(\frac{[I] + \alpha K_i}{\beta[I] + \alpha K_i}\right) \tag{4}$$

For the calculation of the inhibition constants K_i, α and β, the slopes and intercepts were replotted versus $[I]$ (equations 5 and 6) (Segel 1993).

$$slope = \frac{\alpha K_m}{V_{\max}} \left(\frac{[I] + K_i}{\beta[I] + \alpha K_i} \right) \tag{5}$$

$$intercept = \frac{1}{V_{\max}} \left(\frac{[I] + \alpha K_i}{\beta[I] + \alpha K_i} \right) \tag{6}$$

The progress curves of a slow-binding inhibition are described by equation (7)

$$P = v_s \cdot t + (v_i - v_s) \cdot (1 - e^{-k_{obs} \cdot t})/k_{obs} + d \tag{7}$$

Equation (7) allows the determination of the initial velocity v_i, the steady-state velocity v_s and the first-order rate constant k_{obs} for the approach of the steady-state velocity v_s of a single progress curve (Morrison & Walsh 1988, Szedlacsek & Duggleby 1995). A secondary plot of v_i and v_s versus [I] gives the dissociation constant K_i for the initial complex and the overall inhibition constant K_i*, respectively. The detailed type of the slow-binding inhibition as well as k_{off} and k_{on} were determined with equation (8) and (9), respectively.

$$k_{obs} = k_{off} + k_{on} \frac{[I]/K_i}{1 + [S]/K_m + [I]/K_i^*} \tag{8}$$

$$\frac{k_{on}}{k_{off}} = \left(\frac{K_i}{K_i^*} \right) - 1 \tag{9}$$

The kinetic data were analyzed with GraFit 3.0 (Erithacus Software Ltd., England) and SigmaPlot 5.0 (SPSS Inc., USA).

3. RESULTS AND DISCUSSION

Amino acid pyrrolidides (**1**) and thiazolidides are known as potent product analogous inhibitors of DP IV. Generally, these compounds inhibit DP IV competitively (Born *et al* 1994; Stöckel-Maschek *et al* 2000a). The two diastereomers of the isosteric Val-ψ[PO(OCH_3)-N]-Pyrr **2** are able to inhibit DP IV (figure 1, table 1). Interestingly, both diastereomers show different inhibition mechanisms for the inhibition of DP IV. Diastereomer 1 is a poor linear mixed-type inhibitor of DP IV with a K_i-value of 1800 μM, whereas diastereomer 2 is a more potent, competitive inhibitor with a K_i-value of 14.7 μM. Nevertheless, diastereomer 2 of Val-ψ[PO(OCH_3)-N]-Pyrr is an around 100 times less potent inhibitor of DP IV than the corresponding amino acid amide Val-Pyrr ($K_i = 0.255$ μM, Stöckel-Maschek *et al* 2000a). It seems, that the DP IV can not completely tolerate the chemical and stereochemical differences between amide and phosphonamide bond.

DP IV and APP have the same substrate specificity recognizing N-terminal Xaa-Pro dipeptides. APP is also inhibited by the both diastereomers of Val-ψ[PO(OCH_3)-N]-Pyrr **2** with inhibition constants in the micromolar range and the inhibition mechanisms are identical for both enzymes (table 1). In contrast to DP IV, APP is inhibited better by the phosphonamidates **2** than by the amino acid amides Ile-Thia and Phe-Thia or the isosteric thioxo amide Val-ψ[CS-N]-Thia, which have inhibition constants only in the millimolar range (Stöckel-Maschek *et al* 2000a).

Table 1. Inhibition by phosphonamidates Val-ψ[PO(OCH_3)-N]-Pyrr.

Enzyme	Diastereomer 1 K_i (μM)		Mechanism	Diastereomer 2 K_i (μM)		Mechanism
DP IV[1]	1800	$\alpha = 10$	linear mixed-type	14.7		competitive
APP	195	$\alpha = 0.5$	linear mixed-type	294		competitive
LAP	0.218	$\alpha = 5$ $\beta = 0.7$	hyperbolic mixed-type	7.8	$\alpha = 3$ $\beta = 0.3$	hyperbolic mixed-type
APM	341		competitive	$K_i = 546$ μM, $K_i^* = 291$ μM $k_{on} = 0.00015\ s^{-1}$, $k_{on}/K_i^* = 0.5\ s^{-1}M^{-1}$		slow-binding

[1] Stöckel-Maschek *et al* 2000b

Although, the investigated compounds do not correspond to the substrate specificity of LAP and APM, they were investigated as inhibitors of both enzymes, because it is known, that compounds containing a penultimate proline residue are able to inhibit both aminopeptidases (Cushmann *et al* 1977; Xu *et al* 1995; Prechel *et al* 1995). Contrary to our expectations, LAP was inhibited most efficiently from all investigated enzymes by Val-ψ[PO(OCH_3)-N]-Pyrr (**2**). The enzyme was inhibited by the hyperbolic mixed-type inhibition mechanism. This is a partial inhibition mechanism meaning, that the EIS-complex is catalytically active. The inhibition constants are in the low micromolar range (table 1). Otherwise, the well-known DP IV inhibitors Phe-Pyrr (1 mM) and Phe-Thia (1 mM) are not able to inhibit LAP.

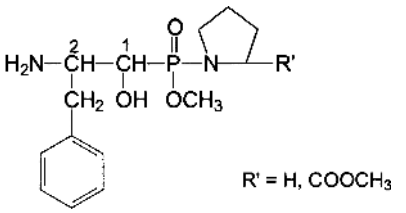
APM is inhibited according to the classical competitive inhibition mechanism by diastereomer 1 of Val-ψ[PO(OCH_3)-N]-Pyrr. On the other hand, the enzyme is inhibited in a time-dependent manner by diastereomer 2. The inhibition constants are in the high micromolar range. APM does not prefer one of the isomers. The amino acid pyrrolidides Ala-Pyrr (1 mM) and Ser-Pyrr (1 mM) are not able to inhibit the enzyme.

Generally, it seems, that inhibition of the metal-dependent aminopeptidases APP, LAP and APM by the phosphonamidate **2** is caused by chelation.

Recent studies showed, that 3-amino-2-hydroxy acid pyrrolidides **3** (figure 1) and thiazolidides as well as 3-amino-2-hydroxy acylproline derivatives are potent inhibitors of the aminopeptidases APP, APM, LAP and DP IV with inhibition constants in the micromolar up to the nanomolar range (Stöckel *et al* 1997; Stöckel-Maschek *et al* in preparation). These investigations were continued by the synthesis of analogous 2-amino-1-hydroxy-phosphonamidates **4** and the enzymatic characterization of the compounds as inhibitors of DP IV, APP, APM and LAP.

DP IV is inhibited by all diastereomers of AHPB-ψ[PO(OCH_3)-N]-Pyrr with inhibition constants in the micromolar range (table 2) according to the competitive inhibition mechanism. The inhibition constants differ around 20 times between the diastereomers. The inhibition constants of AHPB-ψ[PO(OCH_3)-N]-Pyrr are better or in the same range like the constant of (2*S*,3*R*)-AHPB-Pyrr ($K_i = 220$ μM, Stöckel-Maschek *et al* in preparation). The best inhibitors are the diastereomers 4 of (2*R*)- and (2*S*)-AHPB-ψ[PO(OCH_3)-N]-Pyrr with K_i-values of 8.8 μM and 13.7 μM, respectively. The enzyme does not show a preference for the (2*R*)- or (2*S*)-configuration, whereas in case of classical α-amino acids DP IV prefers the (*S*)-isomer. Kim *et al* (2005) have shown by X-ray crystal structure determination, that the β-amino acid-based DP IV inhibitor (2*R*)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-α]pyrazin-7,8*H*)-yl]-1-(2,4,5-trifluorophenyl) butan-2-amine (MK-0431) is bound exhibiting the amide

Table 2. Inhibition of DPIV and APP by 2-amino-1-hydroxy-phosphonamidates of the general structure AHPB–ψ[PO(OCH_3)-N]-R.



R	Configuration of C2-atom	Diastereomer	DP IV K_i (μM) competitive	APP K_i (μM) linear mixed-type	α
Pyrr	(S)	1	336	850	2.7
		2	23.9	1180	2.
		3	62.5	2240	3.2
		4	13.7	61.2	3.1
Pyrr	(R)	1	147	174	0.71
		2	76.7	1860	1.1
		3	22.5	181	0.97
		4	8.8	117	0.58
Pro-OCH_3	(R)	1	52.6	n.d.	
		2	11.5	n.d.	
		3	23.4	n.d.	
		4	57 %, ([I] = 1 mM, [S] = 0.1 mM)	n.d.	

moiety in the opposite orientation in comparison with substrates and inhibitors containing an α-amino acid. In case of the β-amino acid derived inhibitor MK-0431, the (*S*)-isomer is less potent.

The phosphonamidates **4** are 10 to 100 times less potent inhibitors of APP than 3-amino-2-hydroxy acid amides and dipeptides **3** (table 2). For example, (2*S*,3*R*)-AHPB-Pyrr inhibits APP with an inhibition constant of 19.8 μM and is a 3 times better inhibitor of APP than the best phosphonamidate diastereomer 4 of (2*S*)-AHPB-ψ[PO(OCH_3)-N]-Pyrr with an inhibition constant of 61.2 μM. Phosphonamidates **4** as well as 3-amino-2-hydroxy acid amides and dipeptides **3** are linear mixed-type inhibitors of APP.

APM and LAP are inhibited only in the millimolar range by single diastereomers (table 3). Most compounds are not able to inhibit the aminopeptidases efficiently. In contrast, (2*S*,3*R*)-AHPB-Pyrr inhibit LAP according to a competitive slow-binding mechanism with an overall inhibition constant K_i^* of 2.46 μM. APM was only slightly inhibited by (2*S*,3*R*)-AHPB-Pyrr.

4. CONCLUSIONS

The investigated enzymes DP IV, APP, LAP and APM are inhibited by both diastereomers of Val-ψ[PO(OCH_3)-N]-Pyrr. Unexpectedly, LAP is inhibited most efficiently with inhibition constants in the nanomolar and micromolar range. The other enzymes are inhibited with inhibition constants in the micromolar range.

Table 3. Inhibition of APM and LAP by 1-hydroxy-2-amino-phosphonamidates of the general structure AHPB-ψ[PO(OCH_3)-N]-R.

R	Configuration of C2-atom	Diastereomere	APM K_i (μM)	LAP K_i (μM)
Pyrr	(*S*)	1	n.I.	743 (competitive)
		2	560 (competitive)	n.I.
		3	n.d.	n.d.
		4	13% inhibition ([I] = 1 mM, [S] = 0.2 mM)	54% inhibition ([I] = 1mM, [S] = 1 mM)
Pyrr	(*R*)	1	n.I.	514 a = 4.5 (linear mixed-type)
		2	n.I.	1650 (competitive)
		3	4% inhibition ([I] = 1 mM, [S] = 0.2 mM)	30% inhibition ([I] = 1 mM, [S] = 1 mM)
		4	n.I.	32% inhibition ([I] = 1 mM, [S] = 1 mM)
Pro-OCH_3	(*R*)	1	54% inhibition ([I] = 1 mM, [S] = 0.12 mM)	18% inhibition ([I] = 1 mM, [S] = 1 mM)
		2	54% inhibition ([I] = 1 mM, [S] = 0.12 mM)	17% inhibition ([I] = 1 mM, [S] = 1 mM)
		3	54% inhibition ([I] = 1 mM, [S] = 0.12 mM)	26% inhibition ([I] = 1 mM, [S] = 1 mM)
		4	n.I.	51% inhibition ([I] = 1 mM, [S] = 1 mM)

DP IV is inhibited by most of the 2-amino-1-hydroxy-phosphonamidates with inhibition constants in the micromolar range. In contrast, these compounds are only very poor inhibitors of the aminopeptidases LAP and APM or they are not able to inhibit both enzymes. Originally, these compounds were designed as potential inhibitors of APP. Nevertheless, these compounds are less potent than the corresponding 3-amino-2-hydroxy acyl-proline derivatives and 3-amino-2-hydroxy acid amides. These results show, that it was not possible to get effective inhibitors of the metal-dependent aminopeptidases APP, LAP and APM using 2-amino-1-hydroxy-phosphonamidates as amide bond isosteres.

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