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Design of (ω-*N*-(*O*-acyl)hydroxy amid) aminodicarboxylic acid pyrrolidides as potent inhibitors of proline-specific peptidases

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A novel class of competitive, acylating inhibitors for the proline-specific peptidases: dipeptidyl peptidase IV, dipeptidyl peptidase II and prolyl endopeptidase, has been developed. The inhibitor molecules combine the efficacy of aminoacyl pyrrolidides and the potential transacylating capability of diacyl hydroxyl amines. The N-terminal deblocked inhibitors are potent reversible inhibitors of porcine kidney dipeptidyl peptidase IV, human placenta dipeptidyl peptidase II exhibiting K_i values in the μM range. Boc-protected (ω-*N*-hydroxy acyl amid) aminodiacarboxylic acid pyrrolidides inhibit substrate hydrolysis by prolyl endopeptidases from different sources competitively reaching K_i values of 30 nM to 60 μM. Additionally, α-*N*-BOC-(ω-*N*-hydroxy acetyl) glutaminyl pyrrolidide modifies human placenta prolyl endopeptidase in a time-dependent reaction.

Prolyl endopeptidase; Dipeptidyl peptidase IV; Inhibition; Pyrrolidide

1. INTRODUCTION

Peptide bonds involving proline residues are fairly resistant to the action of endo- and exopeptidases with broader substrate specificity. These proline bonds are exposed cleavage points for proline-specific peptidases (Scheme 1). Thus, these enzymes seem to play an important role in maturation and degradation of peptide hormones and neuropeptides [1,2], and on the other hand they serve as invasion factors for numerous pathogenic microorganisms [3,4]. Physiological processes as protein folding and signal transmission as well as undesirable processes like HIV maturation, caused by -X-Pro-specific aspartate protease, are related to the physical properties of proline. Work dealing with mechanistic features of the proline-specific enzymes including inhibitor design is therefore of general interest [5,6]. Among these enzymes, prolyl endopeptidase (PEP) and dipeptidyl peptidase IV (DP IV) exhibit certain similarities in their catalytic behavior, i.e. similar rate constants for substrate hydrolysis, preference of proline over alanine in the P_1 position of similar substrates and drastic discrimination of other amino acids at the cleavage site [7]. Recent studies suggest that PEP is an enzyme involved in learning and memory process [8]. Possibly, it could be involved in the generation of the β-amyloid A4 peptide in Alzheimer's disease [9,10]. DP IV seems to be involved in signal transmission during the immune response [11–13].

To perform detailed investigations of the physiological role of regulatory peptides, stable, specific and selective inhibitors are useful tools. Our studies on mechanism-based protease inhibitors show that, in contrast to other serine and cysteine proteases which are inactivated efficiently by *N*-peptidyl-*O*-acyl hydroxylamines, PEP and DP IV mainly hydrolyze these compounds as substrates [14–16].

Proline-containing peptide aldehydes [17] and boronic acids [3] have been tested as inhibitors of proline-specific peptidases, but their instability due to the reactive electrophiles within their structures should limit their potential to be used in biological systems. Both enzyme types are inhibited by diisopropylfluorophosphate (DFP) and DEP and have been characterized to be serine proteases [2,7,18]. In contrast to the bacterial enzymes, vertebrate PEPs have sensitive thiol residues in or near their active site [19].

Hauzer and co-workers [19] showed that the natural substrates of PEP, the intramolecular disulfide-bridged peptide hormones oxytocin (OT: Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2) and vasopressin (VP: Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg- Gly-NH_2) are already inhibitors of the enzyme. The mechanism of the inactivation suggested is a thiol–disulfide exchange between the substrate and a thiol group in/near the active site of vertebrate PEP.

Since *N*-peptidyl, *O*-acyl hydroxylamines are known

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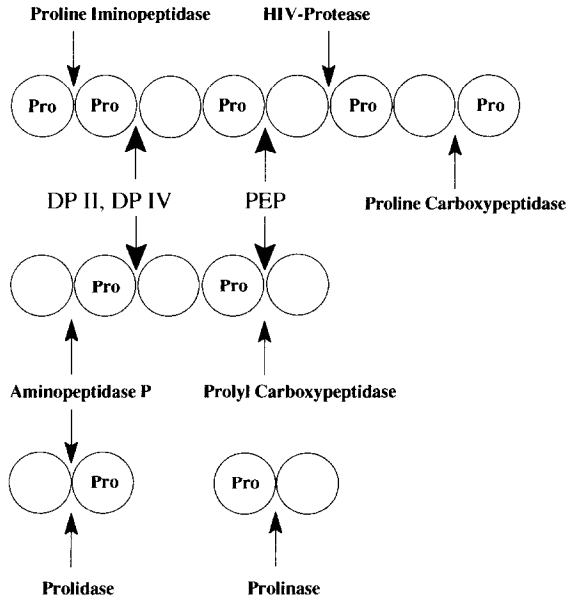
Abbreviations: BOC, tert. butoxycarbonyl; PEP, prolyl endopeptidase; DP IV, dipeptidyl peptidase IV; DP II, dipeptidylpeptidase II; PepX, X-prolyl dipeptidyl aminopeptidase from *Lactococcus lactis*; Ac, acetyl; Bz, benzoyl; DFP, diisopropylfluorophosphate; DEP, diethyl pyrocarbonate; OT, oxytocin; VP, vasopressin; Pyr, pyrrolidide.



Scheme 1. Substrate specificity of proline-specific peptidases.

to be effective and selective inhibitors of thiol-dependent enzymes [15], we anticipated a potential modification of the active site thiol group of vertebrate PEP incorporating the inhibiting -NH-*O*-acyl-moiety into a substrate analog structure.

2. MATERIALS AND METHODS

2.1. *General, synthesis and product analysis*

All reagents for synthesis were purchased from commercial sources and, in the case of solvents, dried using common procedures. α-*N*-BOC-, ω-*O*Me aminodicarboxylic acid pyrrolidides were obtained according to known standard procedures as described in [14,20]. The products were transformed into the appropriate hydroxamic acids by treatment of the corresponding methyl esters with 3.5 M hydroxylamine in a methanol solution followed by acetylation or benzoylation according to [14]. After purification and characterization of the inhibitors for the PEPs (compounds 1–4, Table I), the N-terminal deprotected inhibitors of the dipeptidyl peptidases (compounds 5–8, Table I) were obtained by removing the BOC groups in 1.1 N HCl/glacial acetic acid for 30 min and washing of the products using diethyl ether. After drying and recrystallization the purity of the products was checked by HPLC. The structures and molecular weights were confirmed by ^{13}C NMR, mass spectroscopy and elemental analysis. HPLC analysis of all compounds was performed using a Merck-Hitachi system equipped with a photodiode array detector. ^{13}C NMR spectra were recorded on a Bruker WP-200 spectrometer. Mass spectra were taken in positive mode on a FISONS VG-BIO-Q triple quadrupole tandem mass spectrometer equipped with an electrospray atmospheric pressure LC interface (Manchester, UK). Data were acquired and processed on a Intel-486 personal computer system.

2.2. *Enzymes and substrates*

Preparation of substrates has been described previously [7,11,18]. DP IV from pig kidney and PepX from *Lactococcus lactis* were prepared according to earlier work [14,21]. The specific activity using H-Gly-Pro-4-NA as substrate was 33.1 U/mg and 12.7 U/mg, respectively. Dipeptidyl peptidase II (DP II) from human placenta was a gift of Dr. J. Rahfeld, Max-Planck-Research Group, Halle (Saale) and exhibited a specific activity of 8.2 U/mg using H-Ala-Pro-4-NA as substrate.

PEP from *Flavobacterium meningosepticum* (PEP bact.) was from Miles, USA. PEP from pig brain (PEP porc.) was purified according to [25]. PEP from human placenta (PEP hum.) was purified to homogeneity using a slightly modified preparation protocol [25]. The specific activities using Suc-Ala-Pro-4-NA as a substrate [25] were 14.5, 24.7 and 3.1 U/mg, respectively.

2.3. *Kinetic measurements*

The activity of the PEPs and the dipeptidyl peptidases DP IV and PepX was analyzed at 30°C, in 0.04 M sodium phosphate buffer, pH 7.6, ionic strength of 0.125. DP II, according to its acid activity optimum, was investigated in 0.04 M sodium acetate buffer, pH 5.5. Enzyme-catalyzed hydrolysis of the substrates succinyl-alanyl-prolyl-4-nitroanilide and H-glycyl-prolyl-4-nitroanilide by the PEPs and the dipeptidyl peptidases, respectively, was analyzed on a Kontron Uvicon 630 UV-vis spectrophotometer. Estimation of the inhibition constants K_i was performed according to Dixon, essentially as described in [7,25]. The progress curves for the inhibition of PEPs in the presence of substrate were monitored using succinyl-glycyl-prolyl-4-methyl-7-coumarylamide (Sigma) as a substrate at 30°C, in 0.04 M tricine buffer, pH 7.6, ionic strength of 0.125, on a Kontron SFM 25 fluorimeter using 383 nm as excitation and 450 nm as emission wavelength. The pseudo-first order rate constants of the time-dependent decrease in the enzyme activity were calculated by applying non-linear regression programs to the fluorescence–time values as described in [14,15].

2.4. *Stability of the inhibitors*

Incubation of the compounds in test buffer solution at 30°C over a time period of up to 24 h and proving the integrity of the products using UV-spectroscopy and HPLC indicated no significant decomposition of the inhibitors within the first 4 h of incubation time. Addition-

Table I

Analytical data of (ω-*N*(*O*-acyl) hydroxyamid) aminodicarboxylic acid pyrrolidides

No.	Inhibitor	Mol. wt.	MS (M+H)**	Fp. (°C)*	HPLC-R_f (min)**
1	BOC-Glu(NHO-Bz) Pyr	419.20	420.96	158	19.62
2	BOC-Asp(NHO-Bz) Pyr	405.19	406.02	138	19.01
3	BOC-Glu(NHO-Ac) Pyr	357.19	357.99	124	14.48
4	BOC-Asp(NHO-Ac) Pyr	343.17	344.09	116	14.59
5	H-Glu(NHO-Bz) Pyr/HCl	354.81	319.41	140	17.84
6	H-Asp(NHO-Bz) Pyr/HCl	340.78	305.09	136	17.29
7	H-Glu(NHO-Ac) Pyr/HCl	292.74	257.18	137	9.11
8	H-Asp(NHO-Ac) Pyr/HCl	278.72	244.53	oil	8.76

*Uncorrected values.

**Retention time (R_f) on a SERVA HPLC-column; Si 100; 5 × 200 mm; 10 μm particle size; solvent system, acetonitrile/water containing 0.04% trifluoroacetic acid gradient, 20–80% acetonitrile in 20 min; flow rate, 0.5 ml/min.

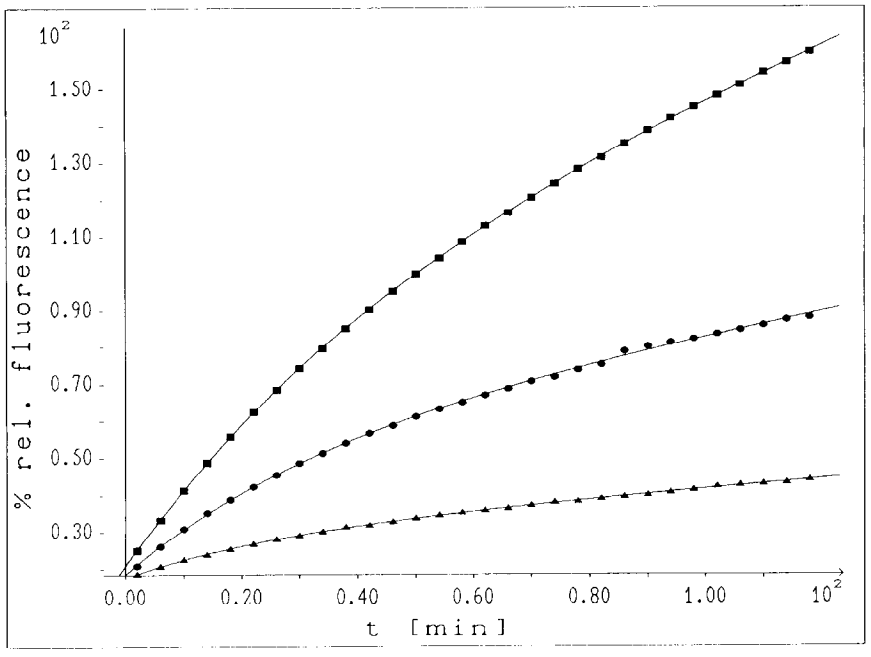


Fig. 1. Progress curves of the PEP-catalyzed hydrolysis of succinyl-glycyl-prolyl-4-methyl-7-coumarylamide in the presence of different concentrations of the inhibitor BOC-Glu(NH-O-Ac) pyrrolidide. ▼, 4.68 μM; ●, 1.56 M; ■, 0.52 M. Suc-Gly-Pro-MCA was 35 μM (see section 2).

ally, no significant loss of the inhibitory potency of solutions of H-Glu(NHO-Bz) pyrrolidide and BOC-Glu(NHO-Bz) pyrrolidide against DP IV and PEP porc. during the first 4 h of incubation was observed, confirming the above result. The release of the benzoic acid residue from H-Glu(NHO-Bz) pyrrolidide and H-Asp(NHO-Ac) pyrrolidide was followed as described in [14] and resulted in pseudo-first order rate constants of the decomposition of 0.028 h^{-1} and 0.044 h^{-1}, respectively.

2.5. *Reversibility of the modification*

To demonstrate chemical modification of PEP from human placenta by BOC-Glu(NHO-Ac) pyrrolidide the enzyme was incubated for 3 h with 2.6–69 μM inhibitor resulting in a residual activity of the enzyme of 3.5%. The solution was given to Centricell molecular filter tubes (Polyscience) of an exclusion molecular weight of 10,000 Da. Protein and inhibitor were separated by repeated centrifugation for 1 h at 5,000 rpm and washing with buffer solution. The activity of the protein solution and of controls was estimated as described above.

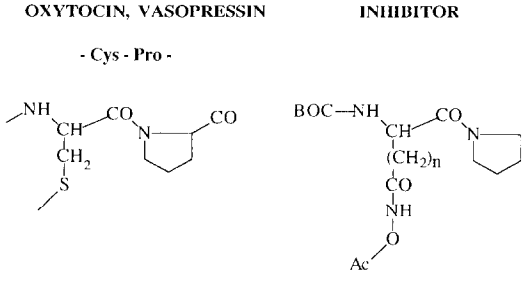
3. RESULTS AND DISCUSSION

Mimicking the parts of the molecules of oxytocin (OT) and vasopressin (VP) responsible for substrate recognition and enzyme inhibition we analyzed several α-*N*-BOC-(ω-*N*-(*O*-acyl) hydroxyamid) aminodiacarboxylic acid pyrrolidides of the following structure as potential inhibitors of proline-specific enzymes (Scheme 2).

Since aminoacyl pyrrolidides and thiazolidides are known to be potent reversible inhibitors of PEP and DP IV [22–24], we used aspartyl pyrrolidide and glutamyl pyrrolidide as basis compounds for further inhibitor design. We attached an *O*-acyl hydroxylamine residue to the carboxyl side chain functionality of the amino acid in position P_2, hoping that interaction of the hydroxylamine linkage would lead to a chemical reaction [22] if positioned near the active site thiol group during non-covalent binding of the inhibitor to vertebrate PEP.

Since no crystal structure of any of the proline-specific peptidases is available on which to perform some initial theoretical design, we varied the length and size of the side chain by using glutamic or aspartic acid in position P_2 and acetyl (Ac) and benzoyl (Bz) groups as *O*-acyl residues (Table I). The synthesized compounds were tested as competitive inhibitors of three PEPs and three dipeptidyl peptidases from different sources. Table II lists the inhibition constants of the non-covalent E · I complexes formed. The inhibition is, in all but one case, competitive and reversible. The inhibitors are among the most potent and stable reversible inhibitors of their target enzymes.

While the inhibitory activity of the 4 inhibitors tested is almost identical for the dipeptidyl peptidases, the PEPs from various organisms interact with their effec-



Scheme 2. Substrate and inhibitor structures.

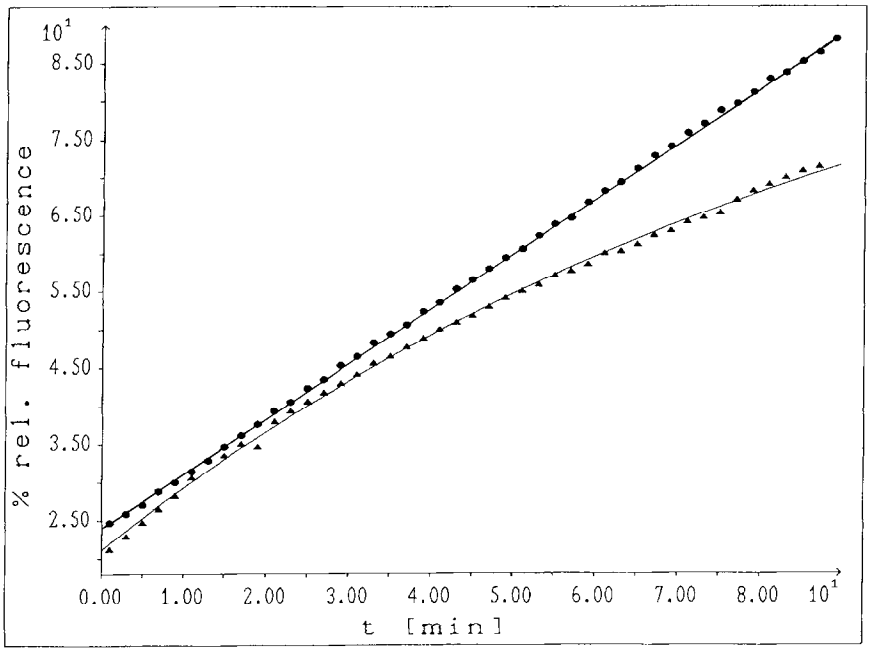


Fig. 2. Progress curve of the PEP-catalyzed hydrolysis of succinyl-glycyl-prolyl-4-methyl-7-coumarylamide (35 μM) in the presence of 2.58 μM BOC-Glu(NH-*O*-Ac) pyrrolidide (▼) and progress curve (●) of the PEP-catalyzed hydrolysis of succinyl-glycyl-prolyl-4-methyl-7-coumarylamide (35 μM) after separation of the inhibited enzyme from excess inhibitor by ultrafiltration and repetitive incubation by 2.58 μM BOC-Glu-(NHO-O-Ac) pyrrolidide (see section 2).

tors remarkably differently. PEP bact. (*Flavobacterium meningosepticum*) and PEP hum. (human placenta) are discriminated for by more than 3 orders of magnitude by BOC-Glu(NHO-Bz) pyrrolidide. This result might reflect greater differences in the spatial structure around the active site of the enzymes. Generally, hydrophobic residues attached to the side chain of the amino acid in position P_2 increase the affinity between enzyme and inhibitor in both groups of peptidases. In contrast to the dipeptidyl peptidases and the bacterial PEP the vertebral PEPs are known to be sensitive towards thiol modifying reagents [7,19,25]. This reactive thiol group, thought to be located near the active site, might serve as target in inhibitor design.

For one of the two vertebrate enzymes tested, we found the anticipated time-dependent loss of activity of the target enzyme applying our potential modifying reagents. Incubation of PEP hum. with BOC-Glu(NH-O-Ac) pyrrolidide in the presence of substrate led also to an inhibitor concentration-dependent decrease of the enzyme activity (Fig. 1). Analyzing this time-dependent reaction (see [14,15]) using an inhibitor concentration range of 20 nM–70 μM we found a maximum rate constant of $k = 4.0 \cdot 10^{-4}$ s^{-1} and an apparent binding constant of $K_i = 28$ nM.

Three possible mechanisms could explain the effect: (i) slow binding inhibition, (ii) chemical modification of the inhibitor (after binding to the target protein) resulting in higher affinity of the modified inhibitor to the enzyme, or (iii) chemical modification of the enzyme by the inhibitor resulting a new enzyme species with modified kinetic properties.

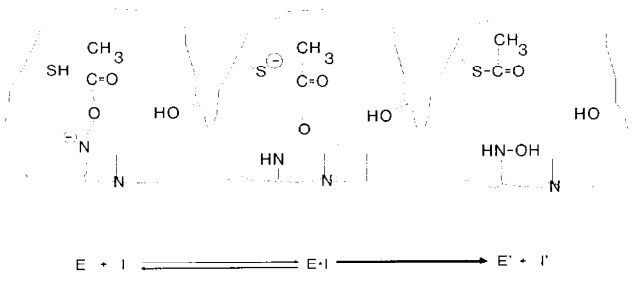
Separation of the inhibited enzyme from excess inhibitor by ultrafiltration did not restore the full activity of the inhibitor-treated enzyme. Incubating PEP hum. with BOC-Glu(NH-O-Ac) pyrrolidide and extensive filtration and washing yielded only partially (up to 62%) reactivated enzyme. Incubation of this enzyme with fresh inhibitor solution did not result in a reproducible time-dependent loss of activity, as shown in Fig. 2.

Table II

Inhibition constants (K_i in μM) of proline-specific peptidases by (ω-*N*(*O*-acyl) hydroxyamid) aminodicarboxylic acid pyrrolidides

Inhibitor	Enzyme		
	PEP bact.	PEP porc.	PEP hum.
1	11.7	0.1	0.03
2	21.5	5.4	0.5
3	49.5	13.8	1.5
4	59.2	25.6	2.8
	DP IV	PepX	DP II
5	1.0	2.2	3.8
6	4.2	1.9	4.1
7	13.0	22.8	29.9
8	24.0		40.9

Proposed inactivation mechanism of PEP by Boc-Glu(NHO-Ac)-Pyrrolidide



Scheme 3. Proposed inactivation mechanism of PEP by BOC-Glu(NH-O-Ac) pyrrolidide.

From this result we propose that, during interaction of PEP hum. with BOC-Glu(NH-O-Ac) pyrrolidide, modification of the target protein occurs. The following sequence of events might explain the observed effect (Scheme 3): (i) substrate–analog binding of the inhibitor into the active site of PEP hum.; (ii) deprotonation and activation of the thiol group by the negatively charged hydroxyamide group; (iii) nucleophilic attack of sulfur on the carbonylcarbon of the *O*-acetyl residue; (iv) transacylation of the acetyl residue to the enzyme forming a thiolester derivative.

Currently the structure of the enzyme–inhibitor complex formed and other modifying attachments to the competitive inhibitors are under investigation.

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