Design of \((\omega-N-(O\text{-acyl})\text{hydroxy amid})\) aminodicarboxylic acid pyrrolidides as potent inhibitors of proline-specific peptidases

H.-U. Demuth\textsuperscript{a}, D. Schlenzig\textsuperscript{a}, A. Schierhorn\textsuperscript{a}, G. Grosche\textsuperscript{a}, M.-P. Chapot-Chartier\textsuperscript{b} and J.-C. Grippon\textsuperscript{b}

\textsuperscript{a}Department of Biochemistry, Martin Luther-University of Halle, Weinbergweg 16 a, D-O-Halle (Saale), Germany and \textsuperscript{b}Unité d'Enzymologie, Station de Recherches Laitières, INRA, F-78352 Jouy-en Josas Cedex, France

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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 \(\mu M\) range. Boc-protected \((\omega-N\text{-hydroxy acyl amid})\) aminodiacarboxylic acid pyrrolidides inhibit substrate hydrolysis by prolyl endopeptidases from different sources competitively reaching \(K_i\) values of 30 nM to 60 \(\mu M\). Additionally, \(\omega\text{-}N\text{-}BOC\text{-}N\text{-}hydroxy acetyl\) 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 infection, 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 processes [8]. Possibly, it could be involved in the generation of the \(\beta\)-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 \(N\text{-}peptidyl\text{-}O\text{-acetyl}\) 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 \(N\text{-}peptidyl\), \(O\text{-acetyl}\) hydroxylamines are known
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-moietiy 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-, α-OMe aminodicarboxylic acid pyrrolidides were obtained according to [14]. After purification and characterization of the inhibitors within the first 4 h of incubation time. Addition-

After drying and recrystallization the purity of the products was checked by HPLC. The structures and molecular weights were confirmed by 13C NMR, mass spectroscopy and elemental analysis. HPLC analysis of all compounds was performed using a Merck-Hitachi system equipped with a photodiode array detector. 13C 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.

PepX from Flavobacterium meningosepticum (PepX bact.) was from Miles, USA. PepX from pig brain (PepX por.) was purified according to [22]. PepX from human placenta (PepX 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. 13C 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.

2.4. 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

<table>
<thead>
<tr>
<th>No.</th>
<th>Inhibitor</th>
<th>Mol. wt.</th>
<th>MS (M+H)**</th>
<th>Fp. (°C)*</th>
<th>HPLC-R_t (min)**</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>BOC-Glu(NHO-Bz) Pyr</td>
<td>419.20</td>
<td>420.96</td>
<td>158</td>
<td>19.62</td>
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<tr>
<td>2</td>
<td>BOC-Asp(NHO-Bz) Pyr</td>
<td>405.19</td>
<td>406.02</td>
<td>138</td>
<td>19.01</td>
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<tr>
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<td>BOC-Glu(NHO-Ac) Pyr</td>
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<td>357.99</td>
<td>124</td>
<td>14.48</td>
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<tr>
<td>4</td>
<td>BOC-Asp(NHO-Ac) Pyr</td>
<td>343.17</td>
<td>344.09</td>
<td>116</td>
<td>14.59</td>
</tr>
<tr>
<td>5</td>
<td>H-Glu(NHO-Bz) Pyr/HCl</td>
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<td>354.81</td>
<td>140</td>
<td>17.84</td>
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<tr>
<td>6</td>
<td>H-Asp(NHO-Bz) Pyr/HCl</td>
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<td>340.78</td>
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<td>17.29</td>
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<tr>
<td>7</td>
<td>H-Glu(NHO-Ac) Pyr/HCl</td>
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<td>292.74</td>
<td>137</td>
<td>9.11</td>
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<tr>
<td>8</td>
<td>H-Asp(NHO-Ac) Pyr/HCl</td>
<td>278.72</td>
<td>278.72</td>
<td>oil</td>
<td>8.76</td>
</tr>
</tbody>
</table>

*Uncorrected values.
**Retention time (R_t) on a SERVA HPLC-column; Si 100; 5 x 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.
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.

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-(α-N-(O-acyl) hydroxamid) 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 P₂, 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 P₇ 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-
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 $P_1$ 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(NHO-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 and repetitive incubation by 2.58 μM BOC-Glu(NHO-Ac) pyrrolidide (see section 2).

Table I

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Enzyme</th>
<th>(K_i) in μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEP bact</td>
<td>PEP pore.</td>
<td>PEP hum.</td>
</tr>
<tr>
<td>1</td>
<td>11.7</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>21.5</td>
<td>5.4</td>
</tr>
<tr>
<td>3</td>
<td>49.9</td>
<td>15.8</td>
</tr>
<tr>
<td>4</td>
<td>59.2</td>
<td>25.6</td>
</tr>
<tr>
<td>DP IV</td>
<td>PepX</td>
<td>DP II</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>2.2</td>
</tr>
<tr>
<td>6</td>
<td>4.2</td>
<td>1.9</td>
</tr>
<tr>
<td>7</td>
<td>13.0</td>
<td>22.8</td>
</tr>
<tr>
<td>8</td>
<td>24.0</td>
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</tbody>
</table>
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 hydroxamide group; (iii) nucleophilic attack of sulfur on the carbonyl carbon 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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REFERENCES
