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SYNTHESIS OF PEPTIDYL ACETALS AS INHIBITORS OF PROLYL ENDOPEPTIDASE

Koen Augustyns[1], Marianne Borloo[1], Alexander Belyaev[1], Padinchare Rajan[1], Filip Goossens[2], Dirk Hendriks[2], Simon Scharpé[2], Achiel Haemers[1,*]

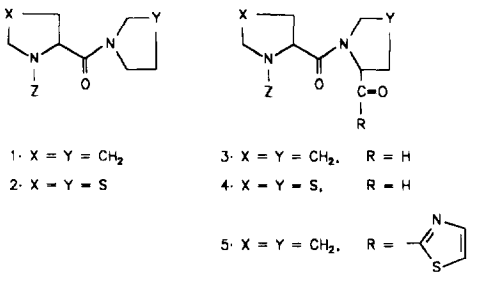
Department of Pharmaceutical Chemistry[1] and Department of Medical Biochemistry[2], University of Antwerp (U.I.A.), Universiteitsplein 1, B-2610 Wilrijk, Belgium

Abstract: Several acyclic and cyclic dipeptidyl acetals were synthesized. Among these, *N*-[*N*-benzyloxycarbonyl-(*S*)-prolyl]-(*S*)-prolinal dimethyl acetal **8** was shown to be a potent inhibitor of prolyl endopeptidase.

Prolyl endopeptidase (PEP, EC 3.4.21.26) was identified as a cytoplasmic serine endoprotease using active site specific inhibitors[1] and using sequence analysis[2] . PEP is fairly ubiquitously distributed throughout the body and its activity is relatively high within the brain[3]. It cleaves amide bonds at the carboxyl side of prolyl residues within peptides that are up to approximately 30 amino acids long, but does not cleave larger proteins[4] .

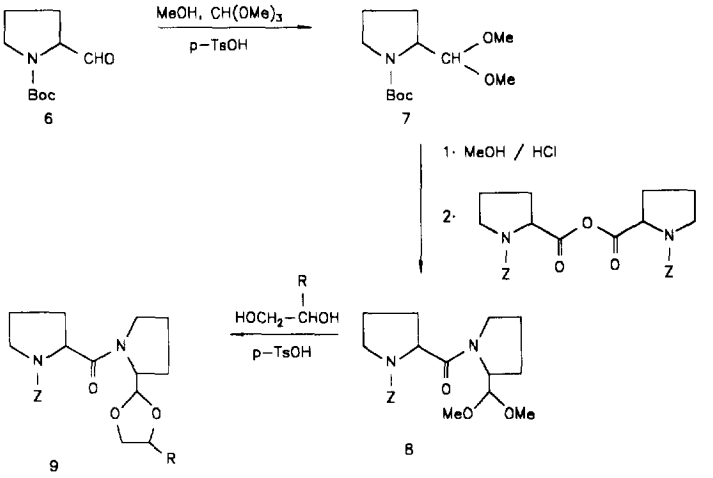
Although the exact physiological function is not known, PEP is thought to be involved in the degradation and metabolism of neuropeptides, such as thyrotropin releasing hormone (TRH), vasopressin and substance P[5,6]. TRH stimulates the release of acetylcholine and a non-peptide PEP inhibitor potentiated this action *in vivo* due to the prevention of TRH inactivation[7]. This may have therapeutical applications since a deficit in central cholinergic function contributes to the cognitive dysfunction in both aging and Alzheimer's disease[8]. Furthermore the central action of vasopressin facilitates learning and memory[9]. Substance P has a protective activity to the neurodegenerative effect of β-amyloid in an *in vivo* model[10]. PEP can also contribute directly to Alzheimer's disease as the amyloid generating enzyme, although the activity of PEP is very low as the cleaving enzyme of amyloid precursor protein[11]. Considering all this, it is believed that PEP inhibitors are able to improve memory and learning. Indeed, some inhibitors are reported to prevent scopolamine induced amnesia in a passive avoidance test using rats, and this effect paralleled their *in vitro* PEP inhibitory activity[12]. Some nootropics such as pramiracetam and aniracetam also inhibit PEP[13]. It is therefore hypothesized that the anti-amnesic effect is related to the inhibitory effect on PEP. These studies suggest that PEP inhibitors could be useful for the treatment of cognitive dysfunction in aging and neurodegenerative diseases including Alzheimer's disease.

SCHEME 1



There are several inhibitors described containing pyrrolidine or thiazolidine at the P1 position (e.g. **1** and **2**, scheme 1). Introduction of sulfur in the pyrrolidine ring occupying P1 and P2 sites increased the inhibitory activity[14]. The peptidyl aldehydes such as **3** are very potent transition state inhibitors forming a tetrahedral intermediate with the serine of the active site[15]. Likewise, introduction of sulfur (**4**) increased potency[14]. Dipeptides containing other electrophilic groups are also potent PEP inhibitors, e.g. chloromethyl ketones (Z-Gly-ProCH$_2$Cl)[1], diazomethylketones (Z-Gly-ProCHN$_2$)[16] and α-keto heterocycles (**5**)[17]. However, these reactive electrophiles possibly limit their potential in biological systems. Peptidyl aldehydes, e. g. , have a very short half-life *in vivo* due to rapid racemization and oxidation to the acid[18]. To enhance the metabolic stability we converted the aldehydes to acetals[19], what resulted in a new class of potent PEP inhibitors.

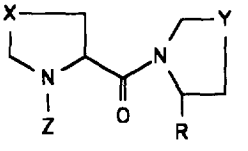
SCHEME 2

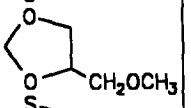


The aldehyde **6** was converted to the dimethyl acetal **7**, followed by cleavage of the N-protecting group and coupling with a symmetrical anhydride to afford a dipeptidyl dimethyl acetal **8** (scheme 2). This acetal was converted to cyclic acetals **9** by acid catalyzed transacetalization. Sulfur containing analogs of **8** were synthesized analogously from thioproline derivatives. The purity of **8** was carefully checked by HPLC in order to exclude the presence of aldehyde **3**, which might be a degradation product of **8**[20] .

Enzyme inhibition assays were carried out with PEP purified from human peripheral blood mononuclear cells[21]. The results indicate that the acyclic dimethyl acetal **8** is more active than the cyclic acetals (table 1). In contrast with previously reported results[14], introduction of sulfur in the pyrrolidine rings (**13** and **14**) afforded less active compounds.

TABLE 1



	X	Y	R	IC_{50} (nM)
8	CH_2	CH_2	$CH(OMe)_2$	120
10	CH_2	CH_2		700
11	CH_2	CH_2		6500
12	CH_2	CH_2		420
13	CH_2	S	$CH(OMe)_2$	350
14	S	S	$CH(OMe)_2$	2600
1	CH_2	CH_2	H	600
4	S	S	CHO	0,5
15	CH_2	CH_2	CH_2OH	14000

The stability of the acetal **8** in the buffer used in the enzyme inhibition assays (potassium phosphate 100 mM pH = 7.5, EDTA 1 mM, NaN_3 1 mM) was controlled with HPLC[20]. The acetal seemed completely stable up to 24 h at room temperature. Also in the enzyme assay, the inhibitory properties of **8** remained the same after incubation for 24 h at 25 °C, indicating no breakdown of the acetals. This is in agreement with the results obtained by HPLC and proves that the acetal **8** acts as a PEP inhibitor and not as a prodrug for the aldehyde **3**.

It can be concluded that the acetals are not as active as the aldehydes, but they are probably more stable. Still *N*-[*N*-benzyloxycarbonyl-(*S*)-prolyl]-(*S*)-prolinal dimethyl acetal **8** is slighly more active than the reference pyrrolidine derivative **1**, and far more active than the alcohol **15**. So acetals represent a group of new potent PEP inhibitors. The mode of inhibition and further stability studies will be reported *in extenso* elsewhere[22].

Typical procedure:

***N*-(*tert*- Butyloxycarbonyl)-(*S*)-prolinal dimethyl acetal (7)**

A mixture of *N*-(*tert*-butyloxycarbonyl)-(*S*)-prolinal[23] **6** (4.38 g, 22 mmol), methanol dry (8.9 mL, 220 mmol), trimethyl orthoformate (12.2 mL, 110 mmol) and p-toluenesulfonic acid monohydrate (80 mg, 0.4 mmol) in toluene (100 mL) was stirred for 18 h at room temperature. After concentration under reduced pressure the residue was dissolved in dichloromethane (200 mL) and washed with sodium bicarbonate (5%, 2 x 50 mL) and brine (50 mL). The organic layer was dried and evaporated yielding 5.25 g (21.4 mmol, 97%) of the title compound as an oil.

^{1}H NMR ($CDCl_3$, 60MHz) δ 1.50 (s, 9 H, $(CH_3)_3$), 1.70-2.25 (m, 4 H, β-H, γ-H), 3.15-3.60 (m, 2 H, δ-H), 3.43 (s, 6 H, OCH_3), 3.65-4.00 (m, 1 H, α-H), 4.45-4.70 (m, 1 H , CHO_2).

***N*-[*N*-Benzyloxycarbonyl-(*S*)-prolyl]-(*S*)-prolinal dimethyl acetal (8)**

N-(*tert*-Butyloxycarbonyl)-(*S*)-prolinal dimethyl acetal **7** (3.19 g, 13 mmol) was dissolved in HCl-MeOH (1.8 N, 100 mL) and stirred for 9 h at room temperature. The mixture was evaporated, and coevaporated with MeOH (3 x) and toluene yielding 2.36 g (13 mmol, 100%) of (*S*)-prolinal dimethyl acetal hydrochloride as a solid . This solid was used as such in a coupling reaction with the symmetrical anhydride of *N*-benzyloxycarbonyl-(*S*)-proline.

N-Benzyloxycarbonyl-(*S*)-proline (7.84 g , 31.5 mmol) was dissolved in tetrahydrofuran dry (30 mL) and N,N'-dicyclohexylcarbodiimide (3.08 g , 14.9 mmol) was added. After 15 min stirring at room temperature, the dicyclohexylurea was filtered off and washed with tetrahydrofuran dry (15 mL). To this solution the above prepared acetal [dissolved in dimethylformamide (45 mL) and neutralized with triethylamine (1.8 mL, 13 mmol)] was added and stirred for 24 h at room temperature. Water (250 mL) was added and the mixture was extracted with EtOAc (3 x 100 mL). The combined organic layer was washed with sodium bicarbonate (5%, 2 x 50 mL) , brine (50 mL), dried, evaporated and purified by column chromatography [silicagel H,

CH_2Cl_2; CH_2Cl_2- MeOH (99-1); CH_2Cl_2-MeOH (98-2)]yielding 3.46 g (9.2 mmol, 71%) of the title compound as a solid. An analytical sample was obtained by crystallization from EtOAc/n-hexane.

^{1}H NMR ($CDCl_3$, 300 MHz, mixture of cis and trans conformational isomers): δ 1.69 and 1.80-2.30 (m, 8 H, β-H, γ-H) , 3.30-3.85 (m, 4 H, δ-H), 3.38, 3.41, 3.45 and 3.46 (4 x s, 6 H, OCH_3), 4.10 and 4.31 (2 x m, 1 H, α-H of P1), 4.45 and 4.56 (2 x m, 1 H, α-H of P2), 4.68 (d, J = 2.6 Hz) and 4.74 (d, J = 2.7 Hz) (1 H, CHO_2), 4.95-5.25 (m , 2 H, $CH_2C_6H_5$), 7.25-7.35 (m, 5 H, C_6H_5).

2-[*N*-(*N*-Benzyloxycarbonyl-(*S*)-prolyl)-2(*S*)-pyrrolidinyl]-1.3-dioxolane (10)

A mixture of *N*-[*N*-benzyloxycarbonyl-(*S*)-prolyl]-(*S*)-prolinal dimethyl acetal **8** (0.94 g, 2.5 mmol), ethylene glycol (0.78 g, 12.5 mmol) and p-toluenesulfonic acid monohydrate (30 mg, 0.16 mmol) in toluene (15 mL) was stirred overnight at room temperature, followed by reflux for 3 h. After concentration under reduced pressure the residue was dissolved in dichloromethane and washed with sodium bicarbonate (5%, twice) and water. The organic layer was dried, evaporated and purified by column chromatography [silicagel H, CH_2Cl_2; CH_2Cl_2 - MeOH (99-1); CH_2Cl_2 - MeOH (98-2); CH_2Cl_2 - MeOH (97-3)] yielding 0.78 g (2.09 mmol, 83%) of the title compound as a solid.

^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.77 and 1.80-2.30 (m, 8 H, β-H, γ-H) , 3.30-3.75 (m, 4 H, δ-H), 3.75-4.10 (m, 4 H, OCH_2CH_2O), 4.15-4.70 (m, 2 H, α-H), 4.95-5.20 (m, 3 H, CHO_2, $CH_2C_6H_5$), 7.25-7.40(m, 5 H, C_6H_5).

Acknowledgement

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20. **HPLC procedure** : HPLC separations were performed with a Merck-Hitachi 655A-11 liquid chromatograph and a L-4000 UV detector. Experiments were performed isocratically using CH_3CN-H_2O (3-7) on a RP-18 column with flow-rate = 2 mL/min and were monitored at 220 nm (retention time **8** = 7.9 min, retention time **3** = 2.7 min).
21. **Enzyme inhibition** : A mixture of 100 µL buffer (potassium phosphate 100 mM pH = 7.5, EDTA 1 mM, NaN_3 1mM), 10 µL PEP enzyme (6 mU) and 3 µL inhibitor (solutions in MeOH) was incubated for 20 min at 37°C. Substrate stock solution was then added (5 µL of a 5.7 mM solution of Z-Gly-Pro-MCA in DMSO) and incubation at 37°C was continued for 20 min. The reaction was stopped by the addition of 500 µL acetic acid (1.5 M) and fluorescence was measured at λ_{ex} 370 nm and λ_{em} 440 nm. Coefficient of variation of the enzyme assay was 5.6 % within run and 11.1 % between runs. Inhibition assays were performed three times.
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