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4-Phenylbutanoyl-2(*S*)-acylpyrrolidines and 4-Phenylbutanoyl-L-prolyl-2(*S*)-acylpyrrolidines as Prolyl Oligopeptidase Inhibitors

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Abstract—New 4-phenylbutanoyl-2(*S*)-acylpyrrolidines and 4-phenylbutanoyl-L-prolyl-2(*S*)-acylpyrrolidines were synthesized. Their inhibitory activity against prolyl oligopeptidase from pig brain was tested in vitro. In the series of 4-phenylbutanoyl-2(*S*)-acylpyrrolidines, the cyclopentanecarbonyl and benzoyl derivatives were the best inhibitors having IC_{50} values of 30 and 23 nM, respectively. This series of compounds shows that the P1 pyrrolidine ring, which is common in most POP inhibitors, can be replaced by either a cyclopentyl ring or a phenyl ring, causing only a slight decrease in the inhibitory activity. In the series of 4-phenylbutanoyl-L-prolyl-2(*S*)-acylpyrrolidines the cyclopentanecarbonyl and benzoyl derivatives were not as active as in the series of 4-phenylbutanoyl-2(*S*)-acylpyrrolidines. The hydroxyacetyl derivative did however show high inhibitory activity. This compound is structurally similar to JTP-4819, which is one of the most potent prolyl oligopeptidase inhibitors. The acyl group in the two series of new compounds seems to bind to different sites of the enzyme, since the second series of new compounds did not show the same cyclopentanecarbonyl or benzoyl specificity as the first series.

Introduction

The serine protease prolyl oligopeptidase (POP, previously called prolyl endopeptidase or post-proline cleaving enzyme, *EC 3.4.21.26*) is a large intracellular enzyme (80 kDa) that preferentially hydrolyses proline-containing oligopeptides at the carboxyl side of a prolyl residue. It is presumably involved in the maturation and degradation of peptide hormones and neuropeptides. In the central nervous system POP degrades proline-containing neuropeptides involved in the processes of learning and memory.[1]

Low level of substance P is characteristic in the brains of Alzheimer patients. Supportingly, administration of β-amyloids decrease substance P levels in the brain. Vice versa, administration of substance P is able to alleviate amyloid peptide induced toxicity.[2] There is no firm evidence of increased POP activitiy in Alzheimer patients. In contrast, rather low enzyme activities are correlated with the severity of Alzheimer's disease.[3] To add to the confusion, in aged rats POP gene levels are many-fold increased in several brain areas.[4] Correspondingly, in mice subjected to an enriched environment, the POP gene levels are decreased.[5] As a whole, it seems that enhanced levels of neuroactive peptides like substance P, angiotensins, vasopressin, oxytocin and thyrotropine releasing hormone, that are all substrates of POP, may be beneficial in old age and in patients with cognitive disturbances. Indeed, POP inhibitors have been able to reverse scopolamine-induced amnesia in rats.[6–8] Centrally acting POP inhibitors, that increase neuropeptide concentrations,[9] may therefore be worthy of testing in Alzheimer's disease.

Several low molecular weight POP inhibitors have been reported earlier. One well studied POP inhibitor

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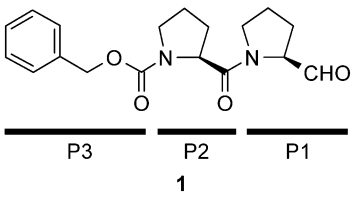

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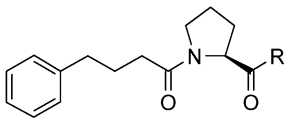

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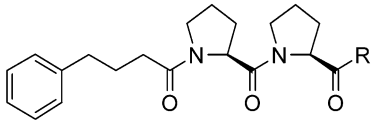
Figure 1. *Z*-L-Pro-L-prolinal **1** and its P1, P2 and P3 binding sites.

is *Z*-L-Pro-L-prolinal **1** (Fig. 1). The crystal structure of the enzyme with this inhibitor has been published.[10] In **1**, the L-prolinal group is the P1 site, the L-prolyl residue the P2 site and the benzyloxycarbonyl group the P3 site. The P1 site binds to the enzyme S1 binding site, and the aldehyde group of the inhibitor forms a hemiacetal adduct with the active serine residue (Ser554) of the enzyme. The P2 and P3 sites binds to the S2 and S3 sites of the enzyme, respectively.

Most of the reported POP inhibitors have in common an acyl-L-prolyl-pyrrolidine structure, wherein the acyl moiety is preferably an aralkanoyl, a benzyloxycarbonyl or a benzylcarbamoyl group. The main objective for this study was to modify the common acyl-L-prolyl-pyrrolidine structure and to study the effects of the modifications on the inhibitory activity.

Our first series of compounds are 4-phenylbutanoyl-2(*S*)-acylpyrrolidines **2**. The strategy was to reduce the number of amide bonds in the acyl-L-prolyl-pyrrolidine structure by focusing on the P1–P2 amide bond. The amide bond nitrogen atom was replaced by a carbon atom, and the amide group was thereby changed to a ketone group. Modification of the amide group by replacing the carbonyl group for a methylene group has been reported to completely reduce the POP inhibitory activity.[11]

Our second series of compounds are 4-phenylbutanoyl-L-prolyl-2(*S*)-acylpyrrolidines **3**. The only difference between two compounds of series **2** and **3** with the same R group are that the end groups are one prolyl unit further apart in series **3**. This means that the two series of compounds can bind to the enzyme either in a way where the R groups or the 4-phenylbutanoyl ends will be superimposed.



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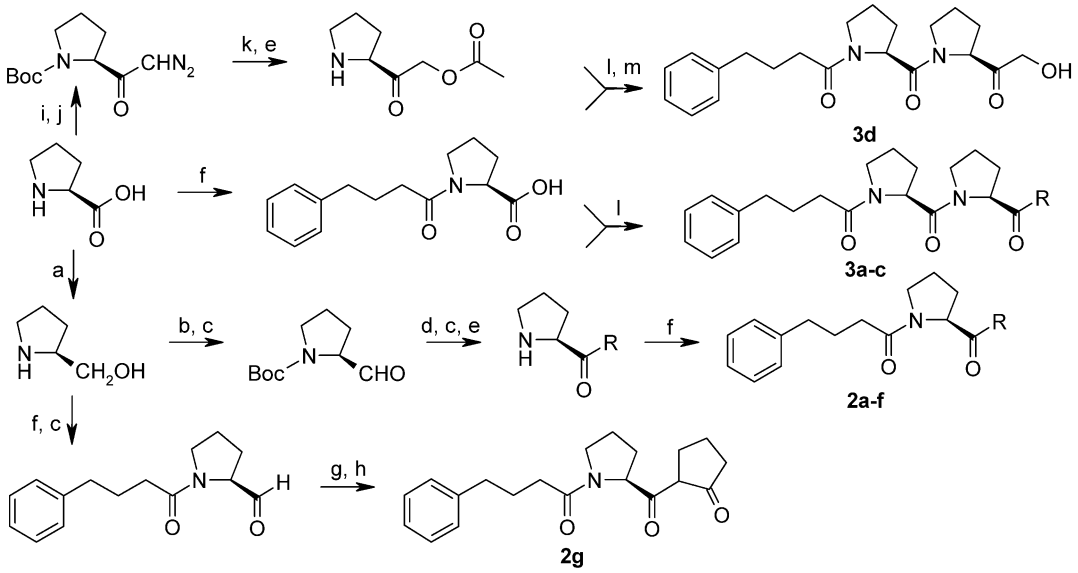
Synthesis

The synthesis routes for the new compounds are presented in Scheme 1. The synthesis of the compounds with unsubstituted hydrocarbon acyl groups **2a**–**f** and **3a**–**c** were performed by a reaction of *N*-BOC-L-prolinal with the appropriate Grignard reagent yielding a secondary alcohol, which was then oxidized to the corresponding ketone.[12,13] The BOC protecting group was removed and the resulting amine was coupled with the appropriate carboxylic acid.[14]



Scheme 1. (a) $LiAlH_4$/THF; (b) Boc_2O, Et_3N/DCM; (c) SO_3–pyridine, Et_3N, DMSO; (d) RMgCl/THF; (e) TFA/DCM; (f) (1) Na_2CO_3/H_2O; (2) 4-phenylbutanoyl chloride/Et_2O; (g) (cyclopent-1-enyloxy)trimethylsilane, $TiCl_4$/DCM; (h) trifluoroacetic anhydride, Et_3N, DMSO/DCM; (i) (1) NaOH/H_2O; (2) Boc_2O/Et_2O; (j) (1) ethyl chloroformate, Et_3N/THF; (2) diazomethane/Et_2O; (k) AcOH; (l) (1) 'the carboxylic acid', Et_3N, pivaloyl chloride/DCM; (2) 'the amine', Et_3N/DCM; (m) K_2CO_3/MeOH, H_2O.

The 2-oxocyclopentanecarbonyl derivative **2g** was synthesized starting from the silylenolether of cyclopentanone and 4-phenylbutanoyl-L-prolinal.[15,16] These two compounds yielded a secondary alcohol, which was then oxidized to the product.[17]

The hydroxyacetyl derivative **3d** was synthesized via BOC-2(*S*)-(diazoacetyl)pyrrolidine, which was reacted with acetic acid to yield BOC-2(*S*)-(acetoxyacetyl)pyrrolidine.[12] The BOC protecting group was removed and the resulting amine was coupled with 4-phenylbutanoyl-L-proline. The final product was obtained by removal of the acetyl group.

The three reference compounds **1** (*Z*-L-Pro-L-prolinal),[18] **4** (SUAM-1221),[14,19] and **5** (JTP-4819)[12] were synthesized using slightly modified reported methods.

In vitro assay for POP activity

The inhibitory effect of the novel compounds on POP activity of pig brain was determined by a method based on that of Toide et al.[20] The substrate Suc-Gly-Pro-7-amido-4-methylcoumarin was used and the formation of 7-amido-4-methylcoumarin was determined fluorometrically.

Results and Discussion

The new compounds and their inhibitory activities against POP are presented in Tables 1 and 2. In the first series of compounds **2** the cyclopentanecarbonyl derivative **2a** shows a high inhibitory activity. Enlarging the ring by making the cyclohexanecarbonyl derivative **2b** or opening the ring by making the isobutanoyl derivative **2c**, clearly results in reduced activity.

The inhibitory activity of the cyclopentanecarbonyl derivative **2a** is only slightly lower than from the reference compound **4** (Table 3). These two compounds can be compared because the two compounds only differ by one atom, the pyrrolidine ring nitrogen atom in **4** is replaced by a carbon atom in **2a**.

Compounds **2a** and **4** show that the pyrrolidine nitrogen atom at the P1 site is not essential for the inhibitory activity. The five membered ring structure is important for binding to the S1 site of the POP enzyme. Adding an oxo group to the cyclopentyl ring reduces the activity strongly, as is shown for compound **2g**. These results are in agreement with an earlier study of the P1 pyrrolidine moiety. In this study enlarging the pyrrolidine ring or adding an oxo substituent to the 2-position of the ring also decreased the inhibitory activity.[14,21]

The benzoyl derivative **2d** has a very high activity, the inhibitory activity being even slightly better than for the cyclopentanecarbonyl derivative **2a**. Tethering the phenyl group with a methylene group reduces the activity strongly, which is shown by the phenylacetyl derivative **2e**. Prolonging the tether to an ethylene group, as in the 3-phenylpropionyl derivative **2f,** removes the activity completely.

The fact that the cyclopentanecarbonyl and benzoyl derivatives are very active, indicates that the indole ring of the tryptophan residue of the enzyme (Trp595), which is crucial for recognition of the P1 L-prolyl residue in the substrate,[10] is able to interact favorably with both the cyclopentyl ring and the phenyl ring.

In the second series of compounds **3**, neither the cyclopentanecarbonyl derivative **3a** nor the benzoyl derivative **3b** show very high inhibitory activities. Interestingly, the acetyl derivative **3c** is the most active inhibitor in the series **3a**–**c**, although its activity is also relatively low. The hydroxyacetyl derivative **3d** has an extremely high inhibitory activity, which was also expected, since it is structurally related to reference compound **5** (Table 3). Compound **3d** differs only by having a 4-phenylbutanoyl group instead of a benzylcarbamoyl group. In the series of compounds **3,** the hydroxyacetyl group is clearly required for high inhibitory activity.

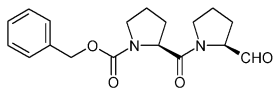
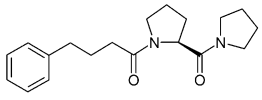
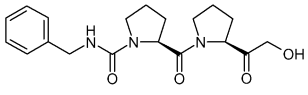
Table 1. Compounds of structure **2**, and their inhibitory activity against POP from pig brain

Compound	Acyl group (COR)	IC_{50} (nM)
2a	Cyclopentanecarbonyl	30
2b	Cyclohexanecarbonyl	1100
2c	Isobutanoyl	620
2d	Benzoyl	23
2e	Phenylacetyl	3600
2f	3-Phenylpropionyl	24,000
2g	2-Oxocyclopentanecarbonyl	260

Table 2. Compounds of structure **3**, and their inhibitory activity against POP from pig brain

Compound	Acyl group (COR)	IC_{50} (nM)
3a	Cyclopentanecarbonyl	1010
3b	Benzoyl	210
3c	Acetyl	170
3d	Hydroxyacetyl	0.2

Table 3. Inhibitory activity of three reference compounds against POP from pig brain

Compound	Structure	IC_{50} (nM)
1 (*Z*-L-Pro-L-prolinal)		0.4
4 (SUAM-1221)		2.0
5 (JTP-4819)		0.2

Comparison of the two new series of compounds shows that in series **2** the R group is most likely bound to the S1 site of the enzyme, thereby being the P1 site in the inhibitor structure. The five-membered ring preference and the relative position of the R group (comparing the structure with the general acyl-L-prolyl-pyrrolidine structure for the known inhibitors) clearly indicates this.

On the other hand, the series of compounds **3** shows none of these features, and most probably the position for the R group is in the S1′ binding site of the enzyme. The S1′ binding site of the enzyme binds the amino acid residue after the cleavage site in the substrate. The hydroxyacetyl group in **3d** and reference compound **5** most probably interacts with the active serine residue (Ser554), and increases the inhibitory activity in the same way as the formyl group in reference compound **1**.

Conclusions

A series of 4-phenylbutanoyl-2(*S*)-acylpyrrolidines **2** have been made, of which the cyclopentanecarbonyl and benzoyl derivatives **2a** and **2d** are very potent POP inhibitors. Compared to the general acyl-L-prolyl-pyrrolidine structure for POP inhibitors, the pyrrolidine moiety is replaced by either a cyclopentyl or a phenyl group in these two compounds.

The series of 4-phenylbutanoyl-L-prolyl-2(*S*)-acylpyrrolidines **3** did not show the same preference for the cyclopentanecarbonyl and benzoyl groups. Furthermore, these compounds were less active inhibitors compared to compounds **2a** and **2d**. Only the hydroxyacetyl derivative **3d**, structurally related to reference compound **5** (JTP-4819), is a highly potent POP inhibitor.

Based on our results, we conclude that the acyl groups of the two series of new compounds bind to different binding sites of the enzyme. In compounds from series **2**, the acyl group is bound to the S1 site of the enzyme. In compounds from series **3** the acyl group is bound to a different location, most probably the S1′ site of the enzyme.

Experimental

Analytical

NMR spectra were recorded on a Bruker Avance 500 spectrometer (500.1 MHz for ^{1}H and 125.8 MHz for ^{13}C), $CDCl_3$ was used as solvent and chemical shifts are expressed in ppm relative to tetramethylsilane as internal standard. Positive ion mass spectra were acquired with ESI-MS, using a Finnegan MAT LCQ quadropole ion trap mass spectrometer equipped with an ESI source. Combustion analysis for CHN were measured on an EA1110 ThermoQuest CE Instruments elemental analysator.

Synthesis

All chemicals and solvents were of commercial quality and were purified if necessary following standard procedures. Some intermediate products and all end products were purified by flash chromatography using 30–60 μm silica gel (J.T. Baker) with a suitable eluent.

Procedure A: Grignard reaction. 1.8–3.0 mmol Grignard reagent is added to a solution of 1.0 mmol *N*-BOC-L-prolinal in anhydrous tetrahydrofuran at −80 °C. The reaction proceeds 30–120 min at −80 to −60 °C, after which the reaction is stopped by adding 0.9–1.5 mL saturated NH_4Cl. The reaction mixture is warmed to rt. Water is added and the product is extracted with ethyl acetate. The organic phase is washed with 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The organic phase is dried with anhydrous Na_2SO_4 and evaporated. The product is purified (mainly from unreacted *N*-BOC-L-prolinal) by flash chromatography.

Procedure B: Parikh–Doering oxidation of primary and secondary alcohols to aldehydes and ketones, respectively. A solution of 3.0 mmol sulfur trioxide pyridine complex in 3 mL anhydrous dimethyl sulfoxide is slowly added to a solution of 1.0 mmol alcohol and 3.0 mmol triethyl amine in 3 mL anhydrous dimethyl sulfoxide at rt. The reaction is stirred 1–2 h at rt, and subsequently poured into 30 mL ice water. The product is extracted with dichloromethane or chloroform. The organic phase is washed with 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The organic phase is dried with anhydrous Na_2SO_4 and evaporated. The product is purified by flash chromatography.

Procedure C: Removal of the BOC amino protecting group. 1.0 mmol BOC protected amine is dissolved in 5–10 mL dichloromethane and 2.0–4.0 mL trifluoroacetic acid is added at 0 °C. The reaction is stirred at 0 °C for 2.0–2.5 h. The solvent and the excess of trifluoroacetic acid are evaporated.

Procedure D: Coupling of a carboxylic acid chloride and an amine trifluoroacetic acid salt under basic aqueous conditions. 1.0 mmol Amine trifluoroacetic acid salt is dissolved in 1 M Na_2CO_3 (or 1 M NaOH). 1.0–2.0 mmol Carboxylic acid chloride is dissolved in diethyl ether and added at 0 °C to the reaction mixture while stirring vigorously, the temperature is raised to rt and the reaction is further stirred vigorously for 1–2 h. The phases are separated and the diethylether phase is washed with 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The diethylether phase is dried with anhydrous Na_2SO_4 and evaporated. The product is purified by flash chromatography.

Procedure E: Coupling 4-phenylbutanoyl-L-proline and an amine trifluoroacetic acid salt with pivaloyl chloride. A solution of 1.0 mmol pivaloyl chloride in dichloromethane is added to a solution of 1.0 mmol 4-phenylbutanoyl-L-proline and 1.1 mmol triethyl amine in dichloromethane at 0 °C. The reaction mixture is stirred 1 h at 0 °C. 2.2–3.3 mmol Triethyl amine and 1.0 mmol

amine trifluoroacetic acid in dichloromethane are added at 0 °C. The reaction mixture is stirred 2 h or overnight at rt. The dichloromethane solution is washed with 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The dichloromethane phase is dried with anhydrous Na_2SO_4 and evaporated. The product is purified by flash chromatography.

L-Prolinol. 17.3 g (150 mmol) L-Proline was added to a suspension of 8.5 g (225 mmol) $LiAlH_4$ in 250 mL anhydrous tetrahydrofuran at 0 °C. The reaction mixture was refluxed 2 h. After cooling to rt, the excess of $LiAlH_4$ was destroyed with 18–20 mL 20% KOH. The reaction mixture was filtered and the residue was refluxed with a new portion of tetrahydrofuran for 30 min. The reaction mixture was filtered. The combined tetrahydrofuran layers were dried with anhydrous Na_2SO_4 and evaporated. Yield 14.3 g (141 mmol, 94%).

***N*-BOC-L-prolinal.** 30.8 g (141 mmol) di-*tert*-Butyl dicarbonate was added to a solution of 14.3 g (141 mmol) L-prolinol and 21.7 mL (156 mmol) triethyl amine in dichloromethane at 0 °C. The reaction was left overnight at rt. The dichloromethane phase was washed with 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The organic phase was dried with anhydrous Na_2SO_4 and evaporated. Yield 23.9 g (119 mmol, 84%) *N*-BOC-L-prolinol. The product was oxidized according to procedure B. Yield 17.7 g (89.0 mmol, 75%).

***N* - BOC - 2(*S*) - (cyclopentanecarbonyl)pyrrolidine.** The synthesis was performed according to procedures A and B starting from 5.16 g (25.9 mmol) *N*-BOC-L-prolinal and 26 mL 2 M (52 mmol) cyclopentylmagnesium chloride. Yield 2.12 g (7.9 mmol, 31%).

***N* - BOC - 2(*S*) - (cyclohexanecarbonyl)pyrrolidine.** The synthesis was performed according to procedures A and B starting from 1.99 g (10.0 mmol) *N*-BOC-L-prolinal and 15 mL 2 M (30 mmol) cyclohexylmagnesium chloride. Yield 760 mg (2.7 mmol, 27%).

***N*-BOC-2(*S*)-isobutanoylpyrrolidine.** The synthesis was performed according to procedures A and B starting from 4.0 g (20.1 mmol) *N*-BOC-L-prolinal and 30.1 mL 2 M (60.2 mmol) isopropylmagnesium chloride. Yield 1.15 g (4.8 mmol, 24%).

***N*-BOC-2(*S*)-benzoylpyrrolidine.** The synthesis was performed according to procedures A and B starting from 1.99 g (10.0 mmol) *N*-BOC-L-prolinal and 10 mL 2 M (20 mmol) phenylmagnesium chloride. Yield 1.65 g (6.0 mmol, 60%).

***N*-BOC-2(*S*)-(phenylacetyl)pyrrolidine.** The synthesis was performed according to procedures A and B starting from 3.46 g (17.4 mmol) *N*-BOC-L-prolinal and 29 mL 2 M (58 mmol) benzylmagnesium chloride. Yield 1.03 g (3.5 mmol, 20%).

***N*-BOC-2(*S*)-(3-phenylpropionyl)pyrrolidine.** The synthesis was performed according to procedures A and B starting from 1.79 g (9.0 mmol) *N*-BOC-L-prolinal and 18 mL 1 M (18 mmol) phenylethylmagnesium chloride. Yield 550 mg (1.8 mmol, 20%).

***N*-BOC-2(*S*)-acetylpyrrolidine.** The synthesis was performed according to procedures A and B starting from 8.25 (41.4 mmol) *N*-BOC-L-prolinal and 25 mL 3 M (75 mmol) methylmagnesium chloride. Yield 5.61 g (26.3 mmol, 64%).

4-Phenylbutanoyl chloride. A flask containing 3.28 g 4-phenylbutanoic acid was put in a 70 °C oil bath. After the starting material had melted 1.61 mL thionyl chloride was added. The reaction mixture was stirred at 70 °C for 1 h and then 10 min at 90 °C. The excess of thionyl chloride was removed under vacuum.

4-Phenylbutanoyl-2(*S*)-(cyclopentanecarbonyl)pyrrolidine (2a). The synthesis was performed according to procedures C and E starting from 1.3 g (5.0 mmol) *N*-BOC-2(*S*)-(cyclopentanecarbonyl)pyrrolidine. Yield 840 mg (2.7 mmol, 54%). ^{1}H NMR: δ 1.4–2.4 (m, 16H), 2.67 (t, 2H, $J=7.5$ Hz), 2.87 (qui, 0.1H, $J=8.0$ Hz), 3.05 (qui, 0.9H, $J=8.0$ Hz), 3.3–3.7 (m, 2H), 4.37 (dd, 0.1H, $J=9.1$ Hz, $J=3.4$ Hz), 4.68 (dd, 0.9H, $J=8.7$ Hz, $J=4.3$ Hz), 7.1–7.3 (m, 5H). ^{13}C NMR: δ 24.66, 25.97, 26.01, 26.16, 28.24, 28.48, 29.53, 33.41, 35.11, 47.24, 49.11, 63.76, 125.82, 128.29, 128.55, 141.76, 171.18, 211.39. ESI-MS: $m/z=314$ $(M+H)^+$. Anal. ($C_{20}H_{27}NO_2 \cdot 0.1H_2O$) Calcd C: 76.20, H: 8.70, N: 4.44; found C: 75.97, H: 8.74, N: 4.40.

4-Phenylbutanoyl-2(*S*)-(cyclohexanecarbonyl)pyrrolidine (2b). The synthesis was performed according to procedures C and D starting from 760 mg (2.7 mmol) *N*-BOC-2(*S*)-(cyclohexanecarbonyl)pyrrolidine. Yield 730 mg (2.2 mmol, 83%). ^{1}H NMR: δ 1.1–2.5 (m, 18H), 2.5–2.62 (m, 1H), 2.66 (t, 2H, $J=7.5$ Hz), 3.3–3.7 (m, 2H), 4.43 (dd, 0.1H, $J=9.2$ Hz, $J=3.2$ Hz), 4.69 (dd, 0.9H, $J=8.6$ Hz, $J=4.6$ Hz), 7.1–7.4 (m, 5H). ^{13}C NMR: δ 24.75, 25.72, 25.73, 25.88, 26.06, 28.34, 28.40, 28.71, 33.45, 35.12, 47.40, 49.02, 63.01, 125.92, 128.38, 128.58, 141.73, 171.65, 212.42. ESI-MS: $m/z=328$ $(M+H)^+$. Anal. ($C_{21}H_{29}NO_2 \cdot 0.1H_2O$) Calcd C: 76.60, H: 8.94, N: 4.25; found C: 76.44, H: 8.85, N: 4.08.

4-Phenylbutanoyl-2(*S*)-isobutanoylpyrrolidine (2c). The synthesis was performed according to procedures C and D starting from 340 mg (1.4 mmol) *N*-BOC-2(*S*)-isobutanoylpyrrolidine. Yield 270 mg (0.94 mmol, 68%). ^{1}H NMR: δ 1.01 (d, 0.3H, $J=7.0$ Hz), 1.07 (d, 0.3H, $J=6.8$ Hz), 1.12 (d, 2.7H, $J=7.0$ Hz), 1.19 (d, 2.7H, $J=6.8$ Hz), 1.7–2.4 (m, 8H), 2.67 (t, 2H, $J=7.6$ Hz), 2.84 (qui, 1H, $J=7.0$ Hz), 3.3–3.7 (m, 2H), 4.44 (dd, 0.1H, $J=9.1$ Hz, $J=3.2$ Hz), 4.71 (dd, 0.9H, $J=8.5$ Hz, $J=4.6$ Hz), 7.1–7.3 (m, 5H). ^{13}C NMR: δ 18.05, 18.48, 24.80, 25.99, 28.44, 33.40, 35.12, 38.95, 47.23, 62.76, 125.83, 128.31, 128.55, 141.76, 171.16, 212.76. ESI-MS: $m/z=288$ $(M+H)^+$. Anal. ($C_{21}H_{29}NO_2 \cdot 0.3H_2O$) Calcd C: 73.84, H: 8.81, N: 4.78; found C: 73.61, H: 8.78, N: 4.54.

4-Phenylbutanoyl-2(*S*)-benzoylpyrrolidine (2d). The synthesis was performed according to procedures C and D

starting from 990 mg (3.6 mmol) *N*-BOC-2(*S*)-benzoylpyrrolidine. Yield 620 mg (1.9 mmol, 53%). ^{1}H NMR: δ 1.8–2.5 (m, 8H), 2.69 (t, 2H, $J=7.5$ Hz), 3.4–3.8 (m, 2H), 5.17 (dd, 0.1H, $J=9.3$ Hz, $J=3.0$ Hz), 5.52 (dd, 0.9H, $J=9.1$ Hz, $J=3.8$ Hz), 7.0–8.1 (m, 10H). ^{13}C NMR: δ 24.70, 26.04, 29.13, 33.40, 35.14, 47.20, 60.83, 125.81, 128.31, 128.54, 128.59, 128.62, 133.20, 135.30, 141.87, 171.23, 198.10. ESI-MS: $m/z=322$ $(M+H)^+$. Anal. ($C_{21}H_{23}NO_2 \cdot 0.2H_2O$) Calcd C: 77.60, H: 7.26, N: 4.31; found C: 77.68, H: 7.29, N: 4.33.

4-Phenylbutanoyl-2(*S*)-(phenylacetyl)pyrrolidine (2e). The synthesis was performed according to procedures C and D starting from 1.03 g (3.5 mmol) *N*-BOC-2(*S*)-(phenylacetyl)pyrrolidine. Yield 880 mg (2.6 mmol, 72%). ^{1}H NMR: δ 1.7–2.5 (m, 8H), 2.46 (s, 2H), 2.69 (t, 2H, $J=7.5$ Hz), 3.4–3.8 (m, 2H), 5.04 (dd, 0.1H, $J=9.4$ Hz, $J=2.6$ Hz), 5.30 (dd, 0.9H, $J=8.6$ Hz, $J=4.0$ Hz), 7.1–7.9 (m, 10H). ^{13}C NMR: δ 20.68, 24.64, 26.08, 28.58, 33.42, 35.14, 47.32, 63.29, 125.61, 125.82, 128.30, 128.58, 128.64, 131.23, 131.59, 136.86, 138.21, 141.84, 171.30, 202.50. ESI-MS: $m/z=336$ $(M+H)^+$. Anal. ($C_{22}H_{25}NO_2 \cdot 0.1H_2O$) Calcd C: 78.35, H: 7.53, N: 4.15; found C: 78.27, H: 7.69, N: 3.96.

4-Phenylbutanoyl-2(*S*)-(3-phenylpropionyl)pyrrolidine (2f). The synthesis was performed according to procedures C and D starting from 550 mg (1.8 mmol) *N*-BOC-2(*S*)-(3-phenylpropionyl)pyrrolidine. Yield 320 mg (0.92 mmol, 51%). ^{1}H NMR: δ 1.6–2.4 (m, 8H), 2.68 (t, 2H, $J=7.5$ Hz), 2.7–3.0 (m, 4H), 3.3–3.6 (m, 2H), 4.20 (dd, 0.1H, $J=9.3$ Hz, $J=3.3$ Hz), 4.55 (dd, 0.9H, $J=8.6$ Hz, $J=4.6$ Hz), 7.1–7.3 (m, 10H). ^{13}C NMR: δ 24.73, 26.02, 27.80, 29.43, 33.37, 35.13, 41.79, 47.20, 64.36, 125.87, 126.05, 128.34, 128.37, 128.44, 128.54, 141.26, 141.71, 171.51, 208.08. ESI-MS: $m/z=350$ $(M+H)^+$. Anal. ($C_{23}H_{27}NO_2 \cdot 0.1H_2O$) Calcd C: 78.64, H: 7.80, N: 3.99; found C: 78.60, H: 7.89, N: 3.88.

4-Phenylbutanoyl-L-prolinal. 1.04 g (10.3 mmol) L-Prolinol was dissolved in 20 mL 10% Na_2CO_3. A solution of 4-phenylbutanoyl chloride, prepared from 1.64 g (10.0 mmol) 4-phenylbutanoic acid, in diethyl ether was added at 0 °C. The reaction was stirred for 2 h at rt. Diethyl ether was added and the phases were separated. The diethyl ether phase was washed with 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The organic phase was dried with anhydrous Na_2SO_4 and evaporated. Yield 1.58 g (6.4 mmol, 64%). 1.53 g (6.2 mmol) 4-Phenylbutanoyl-L-prolinol was oxidized according to synthesis procedure B. Yield 830 mg (3.6 mmol, 55%).

(Cyclopent-1-enyloxy)trimethylsilane. A solution of 4.66 g (31 mmol) NaI in 31 mL acetonitrile was added to a mixture of 2.2 mL (25 mmol) cyclopentanone, 4.3 mL (31 mmol) triethylamine and 3.9 mL (31 mmol) chlorotrimethylsilane. The reaction was allowed to proceed 2 h at rt. 25 mL Cold hexane and 25 mL cold water was added, and the aqueous phase was extracted with hexane. The organic phases were combined and washed with 10% NH_4Cl until the organic phase was neutral. The organic phase was dried with anhydrous Na_2SO_4 and evaporated. The product was vacuum distilled at 45 °C/10 mbar. Yield 2.34 g (15.0 mmol, 60%).

4 - Phenylbutanoyl - 2(*S*) - (2 - oxocyclopentanecarbonyl) - pyrrolidine (2g). A solution of 0.53 mL (2.96 mmol) (cyclopent-1-enyloxy)trimethylsilane in 13 mL dichloromethane was added to a solution of 800 mg (3.26 mmol) 4-phenylbutanoyl-L-prolinal and 0.36 mL (3.26 mmol) titanium tetrachloride. The reaction was allowed to proceed 1 h at −80 °C. 6 mL Water was added at −80 °C and the reaction was stirred 10 min at −80 °C before it was warmed to rt. The phases were separated and the aqueous phase was extracted with dichloromethane. The organic phases were combined and washed with water. The organic phase was dried with anhydrous Na_2SO_4 and evaporated. The intermediate product was purified by flash chromatography. Yield 550 mg (1.67 mmol, 56%). 0.21 mL (1.46 mmol) Trifluoroacetic anhydride was added to a solution of 0.14 mL dimethylsulfoxide and 1 mL dichloromethane at −65 °C. After 10 min, 320 mg intermediate product dissolved in 1 mL dichloromethane was added. After 30 min, 0.4 mL triethyl amine was added and the reaction mixture was stirred at rt for 40 min. The reaction mixture was washed with water and the organic phase was dried with anhydrous Na_2SO_4. The product was purified by flash chromatography. Yield 150 mg (0.46 mmol, 47%). ^{1}H NMR: δ 1.8–2.9 (m, 16H), 3.3–3.7 (m, 2H), 3.89 (t, ∼0.3H, $J=8.4$ Hz), 4.2–5.0 (m, 1H), 7.1–7.3 (m, 5H), 13.7 (s very broad ∼0.7H). ESI-MS: $m/z=328$ $(M+H)^+$. Anal. ($C_{20}H_{25}NO_3 \cdot 0.2H_2O$) Calcd C: 72.57, H: 7.73, N: 4.23; found C: 72.39, H: 7.70, N: 4.23.

4-Phenylbutanoyl-L-proline. 2.30 g (20.0 mmol) L-Proline was dissolved in 40 mL 1 M Na_2CO_3 at 0 °C. 4-Phenylbutanoyl chloride, prepared from 3.28 g (20.0 mmol) 4-phenylbutanoic acid, was dissolved in 5 mL diethylether and the solution was added at 0 °C. The reaction mixture was then stirred vigorously 2 h at rt. The phases were separated and the aqueous phase was washed once with dietylether. The aqueous phase was then made acidic with 2 M HCl. The product was extracted with diethyl ether. The diethyl ether phase (after the acidification) was dried with anhydrous Na_2SO_4 and evaporated. Yield 3.97 g (15.2 mmol, 76%).

4-Phenylbutanoyl-L-prolyl-2(*S*)-(cyclopentanecarbonyl)-pyrrolidine (3a). The synthesis was performed according to procedures C and E starting from 600 mg (2.3 mmol) *N*-BOC-2(*S*)-(cyclopentanecarbonyl)pyrrolidine. Yield 780 mg (1.9 mmol, 83%). ^{1}H NMR: δ 1.5–2.4 (m, 20H), 2.66 (dt, 2H, $J=7.5$ Hz, $J=2.1$ Hz), 3.01 (qui, 1H, $J=8.1$ Hz), 3.2–3.9 (m, 4H), 4.28 (dd, 0.1H, $J=8.7$ Hz, $J=3.2$ Hz), 4.65 (dd, 1H, $J=8.0$ Hz, $J=3.9$ Hz), 4.77 (dd, 0.9H, $J=8.8$ Hz, $J=4.5$ Hz), 7.1–7.3 (m, 5H). ^{13}C NMR: δ 24.75, 24.86, 25.90, 26.00, 26.12, 27.90, 28.30, 28.57, 29.61, 33.57, 35.26, 47.01, 47.28, 49.09, 57.60, 63.86, 125.80, 128.29, 128.53, 141.86, 170.45, 171.43, 211.18. ESI-MS: $m/z=411$ $(M+H)^+$. Anal. ($C_{25}H_{34}N_2O_3 \cdot 0.3H_2O$) Calcd C: 72.19, H: 8.38, N: 6.73; found C: 72.14, H: 8.37, N: 6.67.

4-Phenylbutanoyl-L-prolyl-2(*S*)-benzoylpyrrolidine (3b). The synthesis was performed according to procedures C and E starting from 600 mg (2.3 mmol) *N*-BOC-2(*S*)-(cyclopentanecarbonyl)pyrrolidine. Yield 780 mg (1.9 mmol, 83%). ^{1}H NMR: δ 1.8–2.4 (m, 12H), 2.67 (t, 2H, $J=7.5$ Hz), 3.3–4.0 (m, 4H), 4.3–4.7 (m, 0.1H), 4.74 (dd, 0.9H, $J=7.7$ Hz, $J=3.6$ Hz), 5.47 (dd, 0.1H, $J=9.2$ Hz, $J=3.7$ Hz), 5.60 (dd, 0.9H, $J=9.1$ Hz, $J=4.1$ Hz), 7.1–8.0 (m, 10H). ^{13}C NMR: δ 24.67, 24.95, 25.94, 28.49, 28.77, 33.56, 35.23, 46.93, 47.27, 57.61, 60.99, 125.79, 128.28, 128.53, 128.63, 133.27, 135.17, 141.84, 170.46, 171.54, 198.23. ESI-MS: $m/z=419$ $(M+H)^+$. Anal. ($C_{26}H_{30}N_2O_3$·0.2H_2O) Calcd C: 73.98, H: 7.26, N: 6.64; found C: 73.91, H: 7.29, N: 6.64.

4-Phenylbutanoyl-L-prolyl-2(*S*)-acetylpyrrolidine (3c). The synthesis was performed according to procedures C and E starting from 900 mg (4.2 mmol) *N*-BOC-2(*S*)-acetylpyrrolidine. Yield 970 mg (2.7 mmol, 65%). ^{1}H NMR: δ 1.7–2.4 (m, 12H), 2.19 (s, 3H), 2.67 (t, 2H, $J=7.5$ Hz), 3.42 (m, 1H), 3.5–3.7 (m, 2H), 3.89 (m, 1H), 4.6–4.7 (m, 2H), 7.1–7.3 (m, 5H). ^{13}C NMR: δ 24.70, 24.97, 25.92, 27.50, 27.61, 28.52, 33.53, 35.20, 46.92, 47.22, 57.44, 64.70, 125.80, 128.28, 128.52, 141.80, 170.79, 171.47, 206.75. ESI-MS: $m/z=357$ $(M+H)^+$. Anal. ($C_{21}H_{28}N_2O_3$·0.4H_2O) Calcd C: 69.36, H: 7.98, N: 7.70; found C: 69.59, H: 7.77, N: 7.59.

***N*-BOC-2(*S*)-(acetoxyacetyl)pyrrolidine.** The solutions of 0.8 g (14 mmol) KOH in 20 mL anhydrous ethanol and 4.28 g (20 mmol) *N*-methyl-*N*-nitroso-4-toluenesulfonamide in 60 mL diethyl ether were mixed in the reactor tube of the diazomethane distillation apparatus at 0 °C. The reactor tube was kept 5 min at 0 °C, and then it was placed into a 60 °C oil bath. The distillation was stopped when about 40 mL of diethyl ether containing the diazomethane was distilled over. 2.19 mL (22 mmol) Ethyl chloroformate was added to a solution of 4.3 g (20 mmol) BOC-L-proline and 3.1 mL (22 mmol) triethyl amine in 70 mL tetrahydrofuran at −20 °C. The reaction mixture was stirred at −20 °C for 30 min. Then the diazomethane solution was added to the reaction mixture at −20 °C. The reaction mixture was stirred for 1 h at −20 °C, and the flask was kept without stirring at −20 °C overnight. The solvents were evaporated and the residue was dissolved in dichloromethane. The dichloromethane solution was washed with saturated $NaHCO_3$ and water. The organic phase was dried with anhydrous Na_2SO_4 and evaporated yielding 4.9 g BOC-2(*S*)-(diazoacetyl)pyrrolidine. The BOC-2(*S*)-(diazoacetyl)pyrrolidine and 20 mL acetic acid were stirred at 100 °C for 10 min. The reaction mixture was evaporated. The residue was dissolved in ethyl acetate and the solution was washed with saturated $NaHCO_3$ and water. The ethyl acetate phase was dried with anhydrous Na_2SO_4 and evaporated. The product was purified by flash chromatography. Yield 580 mg (2.1 mmol, 11%).

4-Phenylbutanoyl-L-prolyl-2(*S*)-(acetoxyacetyl)pyrrolidine. The synthesis was performed according to procedures C and E starting from 580 mg (2.1 mmol) *N*-BOC-2(*S*)-(acetoxyacetyl)pyrrolidine. Yield 295 mg (0.71 mmol, 33%).

4-Phenylbutanoyl-L-prolyl-2(*S*)-(hydroxyacetyl)pyrrolidine (3d). 295 mg (0.71 mmol) 4-Phenylbutanoyl-L-prolyl-2(*S*)-(acetoxyacetyl)-pyrrolidine was dissolved in 3 mL 50% methanol in water and 113 mg anhydrous potassium carbonate was added at 0 °C. The mixture was stirred at rt for 1 h. The product was extracted with dichloromethane from the aqueous phase. The organic phase was washed with 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The organic phase was dried with anhydrous Na_2SO_4 and evaporated. The product was purified by flash chromatography. Yield 134 mg (0.36 mmol, 51%). ^{1}H NMR: δ 1.8–2.4 (m, 12H), 2.66 (t, 2H, $J=7.5$ Hz), 3.17 (s, 1H), 3.42 (m, 1H), 3.5–3.7 (m, 2H), 3.93 (m, 1H), 4.31 (d, 1H, $J=18.9$ Hz), 4.42 (d, 1H, $J=18.9$ Hz), 4.63 (dd, 1H, $J=7.8$ Hz, $J=3.8$ Hz), 4.69 (dd, 1H, $J=8.4$ Hz, $J=5.2$ Hz), 7.1–7.3 (m, 5H). ^{13}C NMR: δ 24.79, 25.34, 25.94, 28.20, 28.56, 33.53, 35.19, 47.09, 47.24, 57.43, 61.06, 67.10, 125.85, 128.32, 128.52, 141.74, 171.27, 171.63, 209.09. ESI-MS: $m/z=373$ $(M+H)^+$. Anal. ($C_{21}H_{28}N_2O_4$·0.2H_2O) Calcd C: 67.07, H: 7.61, N: 7.45; found C: 67.13, H: 7.74, N: 7.38.

In vitro assay for POP activity

The whole pig brains, excluding cerebellum and most of the brain stem, of three pigs were placed in liquid nitrogen within 30 min from killing and stored at −80 °C until homogenized. The brains were homogenized in 3 volumes (w/v) of ice-cold 0.1 M sodium–potassium phosphate buffer (pH 7.0) and the homogenates were centrifuged for 20 min at 4 °C at 10,000*g*. The supernatants were collected, pooled and stored in small aliquots at −80 °C until used. The supernatant was thawed in ice just before usage in the activity assay and diluted in a ratio 1:2 with homogenization buffer.

In the microplate assay procedure, 10 μL of the enzyme preparation was preincubated with 460 μL of 0.1 M sodium–potassium phosphate buffer (pH 7.0) and 5 μL of a solution of the compound dissolved in DMSO and diluted with 0.1 M sodium–potassium phosphate buffer at 30 °C for 30 min. The controls contained 10 μL enzyme preparation and 465 μL of 0.1 M sodium–potassium phosphate buffer (pH 7.0). The reaction was initiated by adding 25 μL of 4 mM Suc-Gly-Pro-7-amido-4-methylcoumarin dissolved in 0.1 M sodium–potassium phosphate buffer (pH 7.0), and the mixture was incubated at 30 °C for 60 min. The reaction was terminated by adding 500 μL of 1 M sodium acetate buffer (pH 4.2).

Formation of 7-amido-4-methylcoumarin was determined fluorometrically with microplate fluorescence reader (excitation at 360 nm and emission at 460 nm). The final concentration of the compounds in the assay mixture varied from 10^{-12} to 10^{-4} M.

The inhibitory activities (percent of control) were plotted against the log concentration of the compound, and the IC_{50} value was determined by non-linear regression utilizing GraphPad Prism 3.0 software.

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