Pergamon

SCIENCE DIRECT

Bioorganic & Medicinal Chemistry 11 (2003) 3611–3619

BIOORGANIC & MEDICINAL CHEMISTRY

# Conformationally Rigid *N*-Acyl-5-alkyl-L-prolyl-pyrrolidines as Prolyl Oligopeptidase Inhibitors

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Received 4 April 2003; accepted 28 May 2003

**Abstract**—In the *N*-acyl-L-prolyl-pyrrolidine type of prolyl oligopeptidase inhibitors the L-prolyl group was replaced by different 5-alkyl-L-prolyl groups, resulting in a series of *N*-acyl-5-alkyl-L-prolyl-pyrrolidines. Since *N*-amides of 5-alkyl-L-prolines are conformationally more rigid than those of L-proline, the main objective was to make more rigid prolyl oligopeptidase inhibitors. In the series of compounds where the *N*-acyl group was a Boc group, the 5(*R*)-*tert*-butyl group increased the potency strongly. A similar effect was not observed for the 5(*S*)-*tert*-butyl group. In the series of compounds where the *N*-acyl group was a 4-phenylbutanoyl group, the 5(*R*)-*tert*-butyl, 5(*R*)-methyl and 5(*S*)-methyl groups did not have an effect on the potency [the 5(*S*)-*tert*-butyl group was not tested in this series]. As an additional effect, the 5-*tert*-butyl groups increased the log P of the compounds 1.5 log units, which might be beneficial when targeting the compounds to the brain.


## Introduction

The serine protease prolyl oligopeptidase (POP, previously called prolyl endopeptidase or post-proline cleaving enzyme, EC 3.4.21.26) is a large enzyme of the size of 80 kDa, that preferentially hydrolyses proline-containing oligopeptides at the carboxyl side of a prolyl residue.[1]

Several substrates of POP, such as substance P, vasopressin, neurotensin and thyroliberin, are implicated in learning and memory.[2] Furthermore, low levels of substance P are characteristic in the brains of Alzheimer patients and administration of substance P is able to block β amyloid-induced neurotoxicity.[3] There is no firm evidence of increased POP activity in Alzheimer patients, since rather low POP activities have been correlated with the severity of Alzheimer's disease, which is thought to reflect the degree of neuronal damage.[4] However, it was recently reported that the expression of POP gene was increased many-fold in the hypothalamus and the cortex in aged rats.[5] Furthermore, the expression of POP gene was decreased in rats in an enriched environment that enhances learning and memory.[6] POP inhibitors have been reported to increase the concentrations of substance P, vasopressin and thyroliberin in the brain,[7,8] which is suggested to be beneficial in patients with cognitive disturbances. Indeed, POP inhibitors have been shown to reverse scopolamine-induced amnesia in rats and to improve cognition in MPTP-treated monkeys.[9–11]

In addition to human POP, a parasitic POP has recently also been recognized as a therapeutic target.[12] *Trypanosoma cruzi*, which is the causative agent in Chagas disease, contains a POP which is linked to the invasion of mammalian cells by trypomastigotes.

Many low molecular weight POP inhibitors are based on an *N*-acyl-L-prolyl-pyrrolidine structure.[13] The L-proline moiety at the P2 site has been very challenging to replace. Only a few successful replacements that increased the potency have been reported (Chart 1).

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doi:10.1016/S0968-0896(03)00363-8

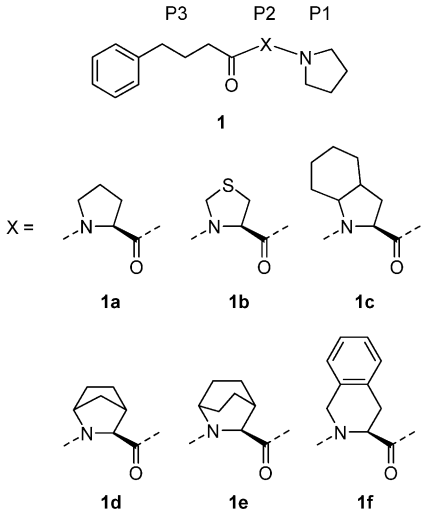
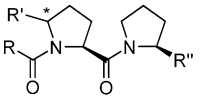



**Chart 1.**

The well known compound SUAM-1221 **1a** has an L-prolyl moiety at the P2 site. Compound **1b** has a L-thioproline moiety at the P2 site, which increased the potency 2–3 times against bovine and canine POP as compared to SUAM-1221 **1a**.[14,15] Compounds **1c–e** have bicyclic L-proline analogues at the P2 site, which increased the potency over 10 times against *Flavobacterium meningoseptum* POP as compared to compound **1b**.[16] Compound **1f** was not so active against human POP, but it had an increased activity against the POP of *T. cruzi*.[12] This compound also demonstrates that a modified L-proline moiety at the P2 site can be used to improve the selectivity between POP of different species.

Due to the cyclic structure of proline, *N*-amide bonds of proline have energetically similar *trans* and *cis* conformations. The ratio can be shifted towards the *cis* conformation by adding a bulky 5-alkyl substituent to the proline ring.[17–21] A bulky 5-alkyl substituent affects the *N*-amide bond of proline by making it conformationally more rigid.

A series of *N*-acyl-5-alkyl-L-prolyl-pyrrolidines **3** were synthesized in order to study the effect of the 5-substituted L-proline moiety at the P2 site of a POP inhibitor. The *tert*-butyl and methyl groups were selected as the 5-substituents, since they are the two opposite extremes in bulkiness of alkyl groups. The 5-substituent introduces a new stereocentre, which has to be controlled in the synthesis. In order to establish the effect of the 5-substituent on the log P value, it was determined for selected compounds.




**3**

RCO = Boc, 4-phenylbutanoyl, $PhCH_2NHCO$, Ac,
3-(2(*S*)-benzoyl-pyrrolidine-1-carbonyl)-benzoyl
R' = *t*-Bu, Me
R'' = H, $COCH_2OH$

## Synthesis

The (5*R*)- and (5*S*)-diastereomers of the *N*-Boc-5-alkyl-L-proline esters **2a–d** were synthesized according to diastereoselective synthetic procedures (Scheme 1). The *N*-Boc-5-alkyl-L-proline esters **2a–d** were used as starting materials for the synthesis of the compounds **3b**, **3c**, **3e–i**, **3k**, **3l** and **3n**.

Compound **2a** was synthesized according to a reported procedure.[18] However, the amino group of the starting material was protected with a trityl group instead of a phenylfluorenyl group according to a general procedure.[22] This modification was made because the trityl group is a less expensive protecting group. Compound **2b** was also synthesized according to a reported procedure.[23] This procedure was modified slightly by performing the last reductive amination step with the methyl ester instead of the carboxylic acid. This modification was made because the methyl esters were assumed to be easier to purify by flash chromatography. Synthesis of compounds **2c** and **2d** followed reported procedures.[24,25]

The *N*-Boc-5-alkyl-L-proline esters **2a–d** were then used as starting materials for the latter part of the synthetic route for the synthesis of compounds **3b**, **3c**, **3e–i**, **3k**, **3l** and **3n**. The ester protecting group was hydrolyzed with LiOH in water-methanol or alternatively with KOH in water–ethanol. The pyrrolidinyl and the 2(*S*)-(acetoxyacetyl)pyrrolidinyl groups were coupled to the carboxylic acid group by activation with pivaloyl chloride in the presence of triethyl amine in dichloromethane [in the case of 2(*S*)-(acetoxyacetyl)pyrrolidinyl the acetyl group was removed subsequently with $K_2CO_3$ in water–methanol in order to obtain compounds **3k** and **3l**]. The Boc protecting group was removed with trifluoroacetic acid in dichloromethane and the resulting amine was reacted with an acid chloride, an anhydride, an isocyanate or alternatively by activating the carboxylic acid with pivaloyl chloride in the presence of triethyl amine. The order of these reactions was not always the same. A general observation from these reactions was that compound **2c** was less reactive than compounds **2a**, **2b** and **2d**.

Since the reactions were not completely diastereoselective, the major diastereomer was purified by flash chromatography at some stage of the synthetic route. The diastereomeric purity was verified by NMR.

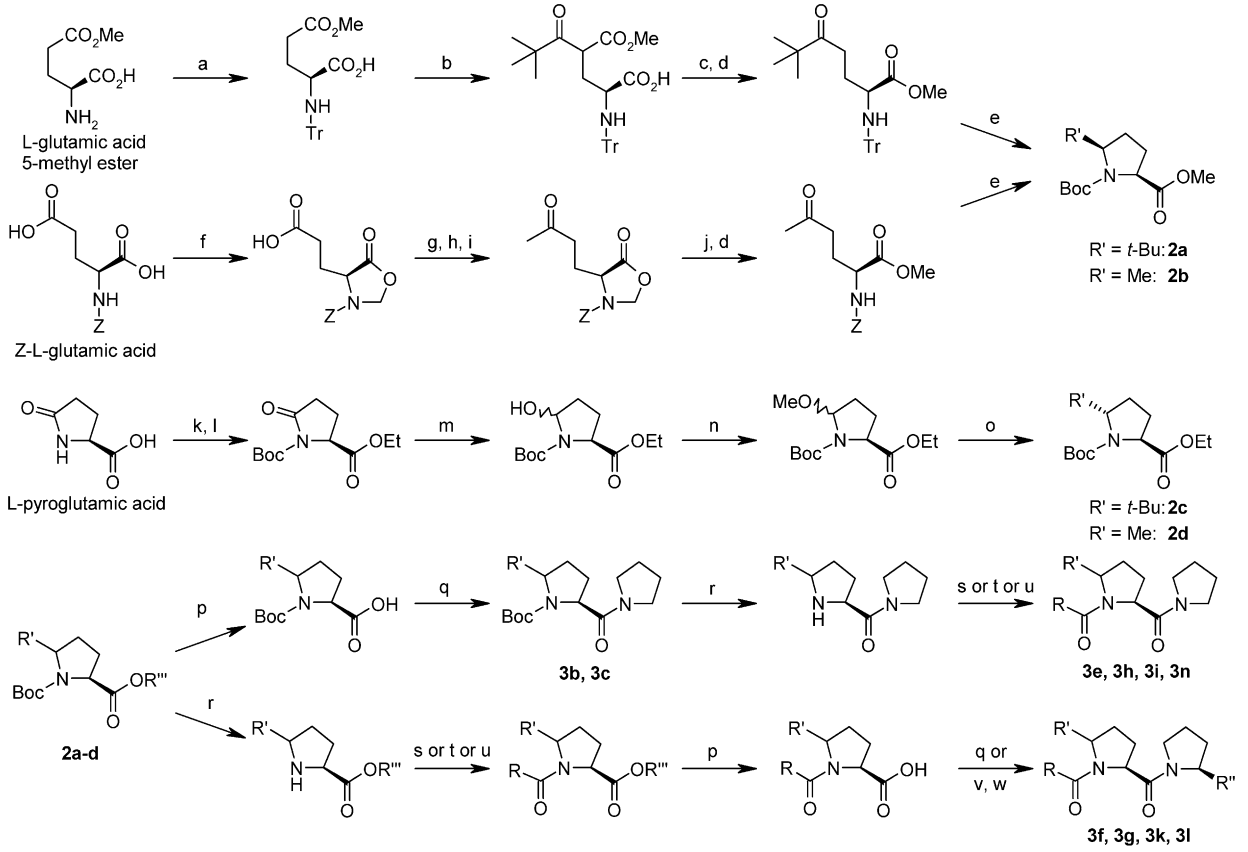
The reference compounds **3a**, **3d**, **3j** and **3m** were synthesized according to reported procedures (not included in Scheme 1).[14,15,26,27]

## In Vitro Assay for POP Activity

The inhibitory effect of the novel compounds on pig brain POP activity was determined according to an earlier described method.[28] Suc-Gly-Pro-7-amino-4-methylcoumarin was used as substrate and the formation of 7-amino-4-methylcoumarin was determined fluorometrically with a microplate fluorescence reader.

## Results and Discussion

The in vitro activities of the compounds of series **3** against pig brain POP were determined (Table 1). The combined effect of a bulky Boc group at the P3 site and a bulky *tert*-butyl substituent at the 5-position of the L-proline group at the P2 site was expected to give an indication which stereoisomer at the 5-position was favoured. The inhibitory activities of compounds Boc-L-prolyl-pyrrolidine **3a**, Boc-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine **3b** and Boc-5(*S*)-*tert*-butyl-L-prolyl-pyrrolidine **3c** were compared. Interestingly, 5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine **3b**, with an $IC_{50}$ value of 2.2 nM, was 13 times more active than Boc-L-prolyl-pyrrolidine **3a**,




**Scheme 1.** (a) (1) $Me_3SiCl$/acetonitrile, DCM; (2) $Et_3N$, TrCl; (3) MeOH; (b) (1) $LiN(SiMe_3)_2$/THF; (2) pivaloyl chloride; (c) NaOH, water/EtOH; (d) $CH_2N_2$/THF; (e) $H_2$, Pd/C, $Boc_2O$/MeOH; (f) paraformaldehyde, *p*-TsOH/benzene; (g) oxalyl chloride, DMF/DCM; (h) $CH_2N_2$/diethylether, benzene; (i) 48% HI; (j) $K_2CO_3$, MeOH, water; (k) EtI, $K_2CO_3$/DMF; (l) $Boc_2O$, DMAP, $Et_3N$/DCM; (m) $LiBEt_3H$/THF; (n) *p*-TsOH, MeOH; (o) R′ = *t*-Bu: (*t*-BuLi, CuBr·$Me_2S$, $BF_3$·$OEt_2$)/THF; R′ = Me: (MeMgBr, CuBr·$Me_2S$, $BF_3$·$OEt_2$)/diethyl ether; (p) LiOH, water/MeOH (or alternatively: KOH, water/EtOH); (q) (1) pivaloyl chloride, $Et_3N$/DCM; (2) pyrrolidine, $Et_3N$; (r) TFA/DCM; (s) $Ph(CH_2)_3COCl$ or $Ac_2O$, $Et_3N$/DCM; (t) $PhCH_2NCO$, $Et_3N$/DMF; (u) (3-(2(*S*)-benzoyl-pyrrolidine-1-carbonyl)-benzoic acid, pivaloyl chloride, $Et_3N$/ DCM), $Et_3N$/ DCM); (v) (1) pivaloyl chloride, $Et_3N$/DCM; (2) 2(*S*)-(acetoxyacetyl)pyrrolidine, $Et_3N$; (w) $K_2CO_3$, water/MeOH.

**Table 1.** The in vitro activities (with 95% confidence intervals given in parentheses) against POP from pig brain and log *P* values for compounds of series **3**

| Compd | RCO | R′ | R″ | $IC_{50}$ (nM) | | $K_i$ (nM) | | Log *P* |
|---|---|---|---|---|---|---|---|---|
| **3a** | Boc | H | H | 29 | (22–38) | | | |
| **3b** | Boc | 5(*R*)-*t*-Bu | H | 2.2 | (1.9–2.5) | | | |
| **3c** | Boc | 5(*S*)-*t*-Bu | H | 9.2 | (7.0–12) | | | |
| **3d** (also **1a**) | 4-Phenylbutanoyl | H | H | 2.2 | (1.9–2.5) | | | 1.8 |
| **3e** | 4-Phenylbutanoyl | 5(*R*)-*t*-Bu | H | 1.2 | (1.0–1.4) | | | 3.3 |
| **3f** | 4-Phenylbutanoyl | 5(*R*)-Me | H | 0.71 | (0.36–1.4) | | | |
| **3g** | 4-Phenylbutanoyl | 5(*S*)-Me | H | 1.4 | (0.94–2.3) | | | |
| **3h** | Benzylcarbamoyl | 5(*R*)-*t*-Bu | H | 2.0 | (0.87–4.4) | | | |
| **3i** | Acetyl | 5(*R*)-*t*-Bu | H | 7100 | (3800–13,000) | | | |
| **3j** | 4-Phenylbutanoyl | H | $COCH_2OH$ | 0.24 | (0.14–0.38) | 0.018 | (0.013–0.023) | |
| **3k** | 4-Phenylbutanoyl | 5(*R*)-*t*-Bu | $COCH_2OH$ | 0.26 | (0.17–0.41) | 0.015 | (0.0006–0.029) | 2.3 |
| **3l** | 4-Phenylbutanoyl | 5(*R*)-Me | $COCH_2OH$ | 0.15 | (0.08–0.29) | 0.026 | (0.015–0.038) | |
| **3m** | 3-(2(*S*)-Benzoyl-pyrrolidine-1-carbonyl)-benzoyl | H | H | 18 | (15–22) | | | |
| **3n** | 3-(2(*S*)-Benzoyl-pyrrolidine-1-carbonyl)-benzoyl | 5(*R*)-*t*-Bu | H | 460 | (370–570) | | | |

with an $IC_{50}$ value of 29 nM, and 3 times more active than Boc-5(*S*)-*tert*-butyl-L-prolyl-pyrrolidine **3c**, with an $IC_{50}$ value of 9.2 nM. This result indicated that the 5(*R*)-*tert*-butyl-L-prolyl group was preferred over 5(*S*)-*tert*-butyl-L-prolyl group and that the 5(*R*)-*tert*-butyl group increased the potency of this type of compounds.

This result is likely to reflect that the conformation of a bulky acyl group at the P3 site, such as the Boc group, is significantly altered by the bulky 5-*tert*-butyl substituent of the L-prolyl group at the P2 site.

The combination of the 4-phenylbutanoyl group at the P3 site and the 5-substituent of the L-prolyl group at the P2 site was studied next. 4-Phenylbutanoyl-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine **3e**, with an $IC_{50}$ value of 1.2 nM, did not show a noticeable increase of the inhibitory activity as compared to SUAM-1221 (4-phenylbutanoyl-L-prolyl-pyrrolidine) **3d**, with an $IC_{50}$ value of 2.2 nM. However, extending this series of compounds to the 5-methyl substituted L-prolyl groups at the P2 site, showed that 4-phenylbutanoyl-5(*R*)-methyl-L-prolyl-pyrrolidine **3f**, with an $IC_{50}$ value of 0.71 nM, was slightly more active, while 4-phenylbutanoyl-5(*S*)-methyl-L-prolyl-pyrrolidine **3g**, with an $IC_{50}$ value of 1.4 nM, was equipotent as compared to SUAM-1221 **3d** and 4-phenylbutanoyl-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine **3e**.

The conformation of a flexible acyl group at the P3 site, such as the 4-phenylbutanoyl group, is hardly affected by the 5-substituent of the L-prolyl group at the P2 site, and it can adopt the optimal conformation for binding to the active site in all cases. The 5(*S*)-*tert*-butyl-L-prolyl moiety was not tested in this group of compounds, since it was expected to be less active based on the results with a Boc group at the P3 site.

The acyl group at the P3 site was further modified. Benzylcarbamoyl-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine **3h**, with an $IC_{50}$ value of 2.0 nM, was equipotent as compared to 4-phenylbutanoyl-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine **3e**. Since the 5(*R*)-*tert*-butyl substituent of the L-prolyl group at the P2 site is a rather large lipophilic group, it was thought to mimic the lipophilic acyl group at the P3 site. However, this was not the case, since acetyl-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine **3i**, with an $IC_{50}$ value of 7100 nM, was almost inactive.

Replacing the pyrrolidinyl group at the P1 site by a 2(*S*)-(hydroxyacetyl)pyrrolidinyl group increased the potency. 4-Phenylbutanoyl-5(*R*)-*tert*-butyl-L-prolyl-2(*S*)-(hydroxyacetyl)pyrrolidine **3k** and 4-phenylbutanoyl-5(*R*)-methyl-L-prolyl-2(*S*)-(hydroxyacetyl)pyrrolidine **3l**, with $IC_{50}$ values of 0.26 and 0.15 nM, respectively, were equipotent as compared to 4-phenylbutanoyl-L-prolyl-2(*S*)-(hydroxyacetyl)pyrrolidine **3j**, with an $IC_{50}$ value of 0.24 nM. However, these values do not give reliable information of the potencies of compounds **3j–l**, since they were found to be tight binding inhibitors of POP (their $IC_{50}$ values are of the same order of magnitude as the enzyme concentration in the reaction mixture). In this case the depletion of the free inhibitor has to be taken into account when measuring the inhibition parameters. When tight binding inhibition was measured utilizing the Morrison equation (see Experimental part), compounds **3j**, **3k** and **3l** were found to be equipotent POP inhibitors with $K_i$ values of 0.018, 0.015 and 0.026 nM, respectively.

Recently, a new type of POP inhibitors based on a isophthalic acid bis(L-prolyl-pyrrolidine) amide structure was presented.[29] These compounds were developed further, resulting in compound **3m** with an $IC_{50}$ value of 18 nM.[27] Incorporating the 5(*R*)-*tert*-butyl-L-proline group into this structure resulted in compound **3n** with an $IC_{50}$ value of 460 nM. It was evident that a bulky 5-substituent of the L-prolyl group was not beneficial in this type of inhibitors.

The log *P* values were determined for selected compounds (Table 1). SUAM-1221 **3d** had a log *P* value of 1.8 and 4-phenylbutanoyl-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine **3e** had a log *P* value of 3.3. The 5-*tert*-butyl group increased the log *P* value by 1.5 log units. The very potent 4-phenylbutanoyl-5(*R*)-*tert*-butyl-L-prolyl-2(*S*)-(hydroxyacetyl)pyrrolidine **3k** had a log *P* value of 2.3, which is very good for a compound that is targeted to the brain.

## Conclusions

Introducing a bulky Boc group at the P3 site showed that the 5(*R*)-*tert*-butyl-L-prolyl group is preferred over the 5(*S*)-*tert*-butyl-L-prolyl group at the P2 site. The inhibitory activity was improved noticeably by a 5(*R*)-*tert*-butyl-L-prolyl group at the P2 site only for compounds with a bulky Boc group at the P3 site. The conformation of a bulky Boc group at the P3 site was most probably significantly altered by the bulky 5-*tert*-butyl substituent of the L-prolyl moiety at the P2 site. A more flexible 4-phenylbutanoyl group at the P3 site does not seem to be affected by a 5(*R*)-*tert*-butyl substituent on the adjacent L-prolyl group. The lipophilic acyl group at the P3 site could not be mimicked by the 5-*tert*-butyl substituent of the L-prolyl moiety at the P2 site. The 5-*tert*-butyl substituent of the L-prolyl group at the P2 site increases the log *P* by 1.5 log units. This is a significant increase in lipophilicity and it may be necessary in order to target the compounds better to the brain. Therefore, these compounds are good candidates for further pharmacological studies.

## Experimental

NMR spectra were recorded on a Bruker Avance 500 spectrometer (500.1 MHz for $^1H$ and 125.8 MHz for $^{13}C$) or a Bruker AM 400 spectrometer (400.1 MHz for $^1H$ and 100.6 MHz for $^{13}C$), $CDCl_3$ was used as solvent if not otherwise noted and chemical shifts are expressed in ppm relative to tetramethylsilane as internal standard. Amides N-terminal to prolyl groups or proline analogue groups have energetically similar *cis* and *trans* isomers (also sometimes referred to as rotamers), which

complicates the NMR spectra. Changing the solvent from $CDCl_3$ to acetone-$d_6$ simplified the NMR spectra for some compounds, for compound **3e** the two isomers had a ratio of 6:4 in $CDCl_3$ and a ratio 8:2 in acetone-$d_6$. When the isomer ratio was 8:2 or larger, the minor isomer could usually not be detected in the $^{13}C$ NMR spectrum.

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 analyses for CHN were measured on an EA1110 ThermoQuest CE Instruments elemental analysator.

All chemicals and solvents were of commercial quality and were purified if necessary following standard procedures.

### Procedure A: General procedure for coupling an amine to a carboxylic acid with pivaloyl chloride

Pivaloyl chloride (1.0 mmol) was added to a solution of the carboxylic acid (1.0 mmol) and triethyl amine (1.1 mmol) in dichloromethane at 0 °C. After 1 h triethyl amine (1.1 mmol, or if the amine is in the form of a HCl or trifluoroacetic acid salt then 3.3 mmol) and the amine (1.0–1.1 mmol) was added, where after the reaction mixture was allowed to react 3–20 h at rt. The dichloromethane solution was washed with 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The dichloromethane phase was dried and evaporated.

### Procedure B: Procedure for hydrolyzing a methyl or ethyl ester group

LiOH (or KOH) (1.5–6.0 mmol) and carboxylic acid ester (1.0 mmol) were dissolved in a small volume of water–MeOH (or water–EtOH in the case of KOH). After the reaction was complete the solvent was evaporated and water was added. The aqueous phase was washed with dichloromethane or diethyl ether. The aqueous phase was then made acidic with hydrochloric acid and the product was extracted with dichloromethane or diethyl ether. The organic phase was dried and evaporated.

### Procedure C: Deprotecting a Boc protected amine

The Boc protected amine (1.0 mmol) was dissolved in dichloromethane (5–10 mL) and trifluoroacetic acid (2–4 mL) was added at 0 °C. The reaction was stirred at 0 °C for 2 h. The solvent was evaporated, yielding the trifluoroacetic acid salt of the amine.

### Procedure D: Hydrolysis of an *O*-acetyl group

$K_2CO_3$ (1.1 mmol) was added to a solution of *O*-acetyl compound (1.0 mmol) in water–methanol (6 mL) at 0 °C. The reaction was stirred 10 min at 0 °C and then 50 min at rt. The solvent methanol was evaporated. Dichloromethane and saturated NaCl were added and the phases were separated. The dichloromethane phase was washed once with saturated NaCl. The dichloromethane phase was dried and evaporated.

***N*-Tr-L-glutamic acid 5-methyl ester.** Prepared according to a reported general procedure.[22]

**Boc-5(*R*)-*tert*-butyl-L-proline methyl ester.** Prepared starting from *N*-Tr-L-glutamic acid 5-methyl ester. according to a reported procedure,[18] with the modification that the Tr group was used instead of the 9-(9-phenylfluorenyl) group to protect the amine. This procedure yields the (5*R*)-diastereomer as the major product, which was isolated by flash chromatography.

**Boc-5(*R*)-*tert*-butyl-L-proline.** The methyl ester group of Boc-5(*R*)-*tert*-butyl-L-proline methyl ester (1.14 g, 4.0 mmol) was hydrolyzed according to procedure B with LiOH in water–MeOH. Yield 0.88 g (3.2 mmol, 80%).

**Boc-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine 3b.** Boc-5(*R*)-*tert*-butyl-L-proline (0.88 g, 3.2 mmol) and pyrrolidine (0.27 mL, 3.2 mmol) were coupled according to procedure A. Purification by flash chromatography, yield 0.87 g (2.7 mmol, 84%). $^1H$ NMR: δ 1.01 (s, 9H), 1.42 (s, 9H), 1.80–2.11 (m, 8H), 3.36–3.70 (m, 4H), 3.79 (d, 1H, $J = 7.9$ Hz), 4.39 (br s, 1H).$^{13}C$ NMR: δ 24.09, 26.35, 27.08, 27.59, 28.38, 28.85, 36.36, 45.96, 45.99, 61.00, 66.69, 79.60, 156.21, 171.15. ESI-MS: *m*/*z* 325 $(M+H)^+$. Anal. ($C_{18}H_{32}N_2O_3$) calcd C: 66.63, H: 9.94, N: 8.63; found C: 66.28, H: 9.95, N: 8.57.

**Boc-5(*S*)-*tert*-butyl-L-proline ethyl ester.** Prepared according to a reported procedure.[25] This procedure yields the (5*S*)-diastereomer as the as the major product. The product was purified without separating the diastereomers by flash chromatography.

**Boc-5(*S*)-*tert*-butyl-L-proline.** The ethyl ester group of Boc-5(*S*)-*tert*-butyl-L-proline ethyl ester (0.51 g, 1.7 mmol) was hydrolyzed according to procedure B with LiOH in water–MeOH. Yield 0.46 g (1.7 mmol, 100%).

**Boc-5(*S*)-*tert*-butyl-L-prolyl-pyrrolidine 3c.** Boc-5(*S*)-*tert*-butyl-L-proline (0.46 g, 1.7 mmol) and pyrrolidine (0.16 mL, 2.1 mmol) were coupled according to procedure A. Purification by flash chromatography, yield 0.050 g (0.15 mmol, 9%). $^1H$ NMR: δ 0.90 (s, 9H), 1.37 (s, 6.3H), 1.46 (s, 2.7H), 1.70–2.23 (m, 8H), 3.35–3.70 (m, 4H), 3.91 (br s, 0.3H), 4.04 (d, 0.7H, $J = 7.8$ Hz), 4.42 (d, 0.7H, $J = 7.8$ Hz), 4.50 (br s, 0.3H). $^{13}C$ NMR: δ 24.19, 25.03, 26.33, 27.52, 28.24, 29.66, 36.89, 45.91, 46.06, 60.18, 66.25, 79.01, 155.79, 172.02. ESI-MS: *m*/*z* 325 $(M+H)^+$. Anal. ($C_{18}H_{32}N_2O_3 \cdot 0.3H_2O$) calcd C: 65.54, H: 9.96, N: 8.49; found C: 65.29, H: 9.87, N: 8.99.

**4-Phenylbutanoyl-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine 3e.** 4-Phenylbutanoylchloride (prepared from 4-phenylbutanoic acid (0.39 g, 2.4 mmol) and thionyl chloride (0.21 mL, 2.9 mmol)) was added to a solution of the 5(*R*)-tert-butyl-L-prolyl-pyrrolidine trifluroacetic acid salt (prepared from Boc-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine (0.63 g, 1.9 mmol) according to procedure C) and triethyl amine (0.89 mL, 6.4 mmol) in dichloromethane at 0 °C. The reaction mixture was stirred at rt for 3 h. The dichloromethane phase was washed with 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The

dichloromethane phase was dried and evaporated. Purification by flash chromatography, yield 0.61 g (1.6 mmol, 84%) $^1$H NMR: δ 1.00 (s, 9H), 1.65–2.47 (m, 12H), 2.57–2.71 (m, 2H), 3.17–3.65 (m, 4H), 3.75–3.81 (m, 0.4H), 4.22 (t, 0.6H, $J=9.0$ Hz, 4.30 (d, 0.6H, $J=8.5$ Hz), 4.59 (t, 0.4H, $J=9.0$ Hz), 7.15–7.28 (m, 5H). $^{13}$C NMR: δ 23.90, 24.09, 25.92, 26.18, 26.34, 26.78, 27.41, 27.68, 27.93, 28.12, 29.60, 29.71, 33.07, 33.88, 35.12, 35.27, 36.44, 36.62, 45.76, 45.97, 46.00, 46.17, 60.82, 60.99, 65.72, 67.04, 125.74, 125.86, 128.25, 128.30, 128.51, 128.62, 141.75, 142.03, 170.34, 170.53, 173.99, 174.26. $^1$H NMR (acetone-$d_6$): δ 0.97 (s, 7.6H), 0.99 (s, 1.4H), 1.73–2.45 (m, 12H), 2.54–2.67 (m, 2H), 3.25–3.50 (m, 4H), 3.73 (br s, 0.2H), 4.23 (d, 0.8H, $J=8.7$ Hz), 4.52 (t, 0.8H, $J=8.8$ Hz), 4.61 (m, 0.2H), 7.15–7.28 (m, 5H). $^{13}$C NMR (acetone-$d_6$): δ 24.49, 26.62, 26.91, 27.23, 28.10, 30.21, 33.64, 35.86, 37.14, 46.30, 46.67, 61.56, 66.09, 126.42, 128.99, 129.27, 143.09, 171.06, 173.94. ESI-MS: $m/z$ 371 $(M+H)^+$. Anal. ($C_{23}H_{34}N_2O_2\cdot0.2H_2O$) calcd C: 73.84, H: 9.27, N: 7.49; found C: 73.91, H: 9.35, N: 7.17.

**(2*S*)-5-Oxo-2-[*N*-(benzyloxycarbonyl)-amino]hexanoic acid.** Prepared according to a reported procedure.[23]

**(2*S*)-5-Oxo-2-[*N*-(benzyloxycarbonyl)-amino]hexanoic acid methyl ester.** (2*S*)-5-Oxo-2-[*N*-(benzyloxycarbonyl)-amino]hexanoic acid (3.45 g, 12.3 mmol) was methylated with an excess of diazomethane in anhydrous tetrahydrofuran at 0 °C. The reaction mixture was left at 4 °C overnight. The solvent was evaporated and the residue was dissolved in diethyl ether. The diethyl ether phase was washed with water and saturated $NaHCO_3$. The diethyl ether phase was dried and evaporated. Purification by flash chromatography, yield 1.5 g (5.1 mmol, 41%).

**Boc-5(*R*)-methyl-L-proline methyl ester.** Prepared by reacting (2*S*)-5-oxo-2-[*N*-(benzyloxycarbonyl)-amino]-hexanoic acid methyl ester 1.5 g (5.1 mmol) and di-*tert*-butyl-dicarbonat (3.1 g, 14.0 mmol) with 10% Pd/C (0.28 g) in methanol under 4 atm pressure of $H_2$ overnight. The solution was filtered through Celite and evaporated. This procedure yields the (5*R*)-diastereomer as the major product, which was isolated by flash chromatography, yield 0.90 g (3.7 mmol, 73%).

**4-Phenylbutanoyl-5(*R*)-methyl-L-proline ethyl ester.** 4-Phenylbutanoylchloride (prepared from 4-phenylbutanoic acid (0.73 g, 4.4 mmol) and thionyl chloride (0.64 mL, 8.9 mmol)) was added to a solution of the 5(*R*)-methyl-L-proline ethyl ester trifluoroacetic acid salt (prepared from Boc-5(*R*)-methyl-L-proline ethyl ester (0.90 g, 3.7 mmol) according to procedure C) and triethyl amine (2.1 mL, 15.0 mmol) in dichloromethane at 0 °C, where after it was stirred at rt for 3 h. The dichloromethane phase was washed with 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The dichloromethane phase was dried and evaporated. Purification by flash chromatography, yield 0.74 g (2.6 mmol, 70%).

**4-Phenylbutanoyl-5(*R*)-methyl-L-proline.** The ethyl ester group of 4-phenylbutanoyl-5(*R*)-methyl-L-proline ethyl ester (0.74 g, 2.6 mmol) was hydrolyzed according to procedure B with KOH in water–EtOH. Yield 0.67 g (2.4 mmol, 92%).

**4-Phenylbutanoyl-5(*R*)-methyl-L-prolyl-pyrrolidine 3f.** 4-Phenylbutanoyl-5(*R*)-methyl-L-proline (0.67 g, 2.4 mmol) and pyrrolidine (0.22 mL, 2.7 mmol) were coupled according to procedure A. Purification by flash chromatography, yield 0.53 g (1.6 mmol, 59%). $^1$H NMR: δ 1.32 (d, 2.6H, $J=6.5$ Hz), 1.37 (d, 0.4H, $J=6.3$ Hz), 1.65–2.20 (m, 10H), 2.30–2.34 (m, 2H), 2.67 (t, 2H, $J=7.6$ Hz), 3.13–3.90 (m, 4H), 3.94–4.00 (m, 0.9H), 4.25–4.33 (m, 0.2H), 4.56 (t, 0.9H, $J=8.1$ Hz), 7.13–7.28 (m, 5H). $^{13}$C NMR: δ 20.51, 24.16, 26.21, 26.22, 26.99, 32.85, 32.89, 35.21, 46.02, 46.35, 54.28, 58.87, 125.80, 128.27, 128.52, 141.75, 170.69, 171.03. ESI-MS: $m/z$ 329 $(M+H)^+$. Anal. ($C_{20}H_{28}N_2O_2\cdot0.3H_2O$) calcd C: 71.95, H: 8.63, N: 8.39; found C: 72.14, H: 8.76, N: 8.34.

**Boc-5(*S*)-methyl-L-proline ethyl ester.** Prepared according to a reported procedure.[24] Purification without separating the diastereomers by flash chromatography. This procedure yielded the (5*S*)-diastereomer as the as the major product.

**4-Phenylbutanoyl-5(*S*)-methyl-L-proline ethyl ester.** 4-Phenylbutanoylchloride (prepared from 4-phenylbutanoic acid (1.42 g, 8.6 mmol) and thionyl chloride (0.93 mL, 13.0 mmol)) was added to a solution of the 5(*S*)-methyl-L-proline ethyl ester trifluroacetic acid salt (prepared from Boc-5(*S*)-methyl-L-proline ethyl ester (1.85 g, 7.2 mmol) according to procedure C) and triethyl amine (4.0 mL, 28.7 mmol) in dichloromethane at 0 °C. The reaction was stirred 3 h in rt. The dichloromethane phase was washed with 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The dichloromethane phase was dried and evaporated. Purification by flash chromatography, yield 1.56 g (5.1 mmol, 71%).

**4-Phenylbutanoyl-5(*S*)-methyl-L-proline.** The ethyl ester group of 4-phenylbutanoyl-5(*S*)-methyl-L-proline ethyl ester (1.54 g, 5.1 mmol) was hydrolyzed according to procedure B with KOH in water–EtOH. Yield 1.36 g (4.9 mmol, 96%).

**4-Phenylbutanoyl-5(*S*)-methyl-L-prolyl-pyrrolidine 3g.** 4-Phenylbutanoyl-5(*S*)-methyl-L-proline (0.67 g, 2.4 mmol) and pyrrolidine (0.20 mL, 2.4 mmol) were coupled according to procedure A. Purification by flash chromatography, yield 0.64 g (2.0 mmol, 83%). $^1$H NMR: δ 1.12 (d, 2.5H, $J=6.4$ Hz), 1.21 (d, 0.5H, $J=6.4$ Hz) 1.47–2.54 (m, 12H), 2.65–2.70 (m, 2H), 3.33–3.43 (m, 2H), 3.54–3.59 (m, 1H), 3.75–3.80 (m, 1H), 4.08–4.15 (m, 1H), 4.26 (d, 0.2H, $J=8.6$ Hz), 4.63 (d, 0.8H, $J=8.9$ Hz), 7.14–7.28 (m, 5H). $^{13}$C NMR: δ 21.72, 24.15, 26.25, 26.51, 26.54, 31.72, 32.99, 35.11, 45.87, 46.22, 53.72, 58.06, 125.76, 128.26, 128.64, 141.95, 170.53, 171.70. ESI-MS: $m/z$ 329 $(M+H)^+$. Anal. ($C_{20}H_{28}N_2O_2\cdot0.2H_2O$) calcd C: 72.34, H: 8.62, N: 8.44; found C: 72.08, H: 8.86, N: 8.55.

**Benzylcarbamoyl-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine 3h.** Benzylisocyanate (0.55 mL, 4.5 mmol) was added to a

solution of the 5(*R*)-*tert*-butyl-L-proline methyl ester trifluroacetic acid salt [prepared from Boc-5(*R*)-*tert*-butyl-L-proline methyl ester (1.46 g, 4.5 mmol) according to procedure C] and triethyl amine (1.9 mL, 13.5 mmol) in dimethylformamide at 0 °C. The reaction was stirred 3 h in rt. The dimethylformamide solution was poured into ice-water and the product was extracted with dichloromethane. The dichloromethane phase was washed with 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The dichloromethane phase was dried and evaporated. Purification by flash chromatography, yield 1.24 g (3.5 mmol, 78%). $^1$H NMR: δ 0.99 (s, 9H), 1.78–2.06 (m, 7H), 2.19–2.26 (m, 1H), 3.35–3.46 (m, 2H), 3.50–3.56 (m, 1H), 3.59–3.65 (m, 1H), 3.91 (d, 2H, $J$ = 8.1 Hz), 4.31 (m, 2H), 4.48–4.53 (m, 1H), 4.98 (br t, 1H, $J$ = 5.3 Hz), 7.21–7.32 (m, 5H). $^{13}$C NMR: δ 23.90, 26.34, 26.84, 27.54, 29.32, 36.46, 44.96, 46.16, 46.33, 62.56, 66.51, 127.07, 127.41, 128.54, 139.56, 160.29, 171.54. ESI-MS: $m/z$ 358 $(M+H)^+$. Anal. ($C_{21}H_{31}N_3O_2$) calcd C: 70.55, H: 8.74, N: 11.75; found C: 70.72, H: 8.85, N: 12.08.

**Acetyl-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine 3i.** Acetic anhydride (0.15 mL, 1.5 mmol) was added to a solution of the 5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine trifluoroacetic acid salt [prepared from Boc-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine (0.25 g, 0.77 mmol) according to procedure C] and triethyl amine (0.40 mL, 3.1 mmol) in dichloromethane at 0 °C. The reaction was stirred at rt for 3 h. The dichloromethane solutution was washed with 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The dichloromethane phase was dried and evaporated. Purification by flash chromatography, yield 0.17 g (0.65 mmol, 84%). $^1$H NMR: δ 1.00 (s, 4.5H), 1.08 (s, 4.5H), 11.76–2.39 (m, 8H), 1.94 (s, 1.5H), 2.16 (s, 1.5H), 3.35–3.81 (m, 4H), 3.72 (d, 0.5H, $J$ = 8.1 Hz), 4.27 (d, 0.5H, $J$ = 8.7 Hz), 4.42 (t, 0.5H, $J$ = 9.2 Hz), 4.62 (t, 0.5H, $J$ = 9.2 Hz). $^{13}$C NMR: δ 22.74, 23.17, 23.94, 24.08, 26.25, 26.29, 26.42, 27.61, 27.95, 28.12, 29.65, 36.62, 36.64, 45.97, 45.98, 46.01, 46.31, 60.78, 61.81, 65.64, 68.18, 170.30, 170.46, 172.00, 172.02 (all except one carbon give double peaks). ESI-MS: $m/z$ 267 $(M+H)^+$. Anal. ($C_{15}H_{26}N_2O_2$) calcd C: 67.63, H: 9.84, N: 10.52; found C: 67.79, H: 10.16, N: 10.68.

**4-Phenylbutanoyl-5(*R*)-*tert*-butyl-L-proline methyl ester.** 4-Phenylbutanoylchloride [prepared from 4-phenylbutanoic acid (0.76 g, 4.6 mmol) and thionyl chloride (0.50 mL, 6.9 mmol)] was added to a solution of the 5(*R*)-*tert*-butyl-L-proline methyl ester trifluroacetic acid salt (prepared from Boc-5(*R*)-*tert*-butyl-L-proline methyl ester (1.1 g, 3.8 mmol) according to procedure C) and triethyl amine (2.1 mL, 15.3 mmol) in dichloromethane at 0 °C. The reaction was stirred 4 h in rt. The dichloromethane solution was washed with 30% citric acid, saturated NaCl and saturated $NaHCO_3$. The dichloromethane phase was dried and evaporated. Purification by flash chromatography, yield 0.73 g (2.2 mmol, 58%).

**4-Phenylbutanoyl-5(*R*)-*tert*-butyl-L-proline.** The methyl ester group of 4-phenylbutanoyl-5(*R*)-*tert*-butyl-L-proline methyl ester (0.68 g, 2.1 mmol) was hydrolyzed according to procedure B with LiOH in water–MeOH. Yield 0.58 g (1.8 mmol, 86%).

**Boc-2(*S*)-(acetoxyacetyl)pyrrolidine.** Prepared according to a reported procedure.[26]

**4-Phenylbutanoyl-5(*R*)-*tert*-butyl-L-prolyl-2(*S*)-(acetoxyacetyl)pyrrolidine.** 4-Phenylbutanoyl-5(*R*)-*tert*-butyl-L-proline (0.58 g, 1.8 mmol) and 2(*S*)-(acetoxyacetyl) pyrrolidine trifluoroacetic acid salt [prepared from Boc-2(*S*)-(acetoxyacetyl)pyrrolidine (0.50 g, 1.8 mmol) according to procedure C] were coupled accoroding to procedure A. Purification by flash chromatography, yield 0.30 g (0.64 mmol, 36%).

**4-Phenylbutanoyl-5(*R*)-*tert*-butyl-L-prolyl-2(*S*)-(hydroxyacetyl)pyrrolidine 3k.** Prepared according to procedure D using 4-phenylbutanoyl-5(*R*)-*tert*-butyl-L-prolyl-2(*S*)-(acetoxyacetyl)pyrrolidine (0.30 g, 0.64 mmol) as starting material. Purification by flash chromatography, yield 0.26 g (0.61 mmol, 95%). $^1$H NMR: δ 0.94 (s, 9H), 1.72 (m, 12H), 2.64 (t, 2H, $J$ = 7.3 Hz), 3.04 (br s, 0.4H), 3.10 (br s, 0.6H), 3.26–4.76 (m, 7H), 7.14–7.28 (m, 5H). $^{13}$C NMR: δ 25.37, 25.42, 25.82, 26.06, 26.76, 27.15, 27.57, 27.82, 28.06, 28.07, 29.15, 29.43, 33.01, 33.79, 34.97, 35.24, 36.43, 36.53, 46.50, 46.79, 60.44, 60.63, 61.24, 61.30, 65.83, 66.90, 66.97, 67.08, 125.77, 125.91, 128.26, 128.33, 128.49, 128.65, 141.64, 141.97, 170.78, 171.01, 173.74, 174.39, 208.42, 209.31. ESI-MS: $m/z$ 429 $(M+H)^+$. Anal. ($C_{25}H_{36}N_2O_4 \cdot 0.1H_2O$) calcd C: 69.77, H: 8.48, N: 6.51; found C: 69.62, H: 8.48, N: 6.73.

**4-Phenylbutanoyl-5(*R*)-methyl-L-prolyl-2(*S*)-(acetoxyacetyl)pyrrolidine.** 4-Phenylbutanoyl-5(*R*)-methyl-L-proline (0.23 g, 0.84 mmol) and 2(*S*)-(acetoxyacetyl)-pyrrolidine trifluoroacetic acid salt (prepared from Boc-2(*S*)-(acetoxyacetyl)pyrrolidine (0.23 g, 0.84 mmol) according to procedure C) were coupled according to procedure A. Purification by flash chromatography, yield 0.23 g (0.54 mmol, 64%).

**4-Phenylbutanoyl-5(*R*)-methyl-L-prolyl-2(*S*)-(hydroxyacetyl)pyrrolidine 3l.** Prepared according to procedure D using 4-phenylbutanoyl-5(*R*)-methyl-L-prolyl-2(*S*)-(acetoxyacetyl)pyrrolidine (0.23 g, 0.54 mmol) as starting material. Purification by flash chromatography, yield 0.11 g (0.29 mmol, 54%). $^1$H NMR: δ 1.28 (d, 3H, $J$ = 6.6 Hz), 1.66–2.21 (m, 10H), 2.29–2.33 (m, 2H), 2.66 (t, 2H, $J$ = 7.4H), 3.05 (t, 1H, $J$ = 5.1 Hz), 3.58–3.63 (m, 1H), 3.93–4.02 (m, 2H), 4.30–4.47 (m, 2H), 4.55 (t, 1H, $J$ = 8.1 Hz), 4.70 (dd, 1H, $J$ = 5.1 Hz, $J$ = 8.4 Hz). $^{13}$C NMR: δ 20.65, 25.34, 26.23, 26.82, 28.25, 32.84, 32.90, 35.23, 47.19, 54.30, 58.56, 61.27, 66.96, 125.88, 128.32, 128.50, 141.66, 171.21, 171.33, 209.05. ESI-MS: $m/z$ 387 $(M+H)^+$. Anal. ($C_{22}H_{30}N_2O_4 \cdot 0.5H_2O$) calcd C: 66.81, H: 7.90, N: 7.08; found C: 66.82, H: 7.83, N: 6.83.

**Boc-2(*S*)-benzoylpyrrolidine.** Prepared according to reported procedure.[26]

**3-(2(*S*)-Benzoylpyrrolidine-1-carbonyl)-benzoic acid methyl ester.** Isophthalic acid mono methyl ester (1.28 g, 7.1 mmol) and 2(*S*)-benzoylpyrrolidine trifluoroacetic acid

salt [prepared from Boc-2(*S*)-benzoyl-pyrrolidine (1.96 g, 7.1 mmol) according to procedure C], were coupled according to procedure A. Purification by flash chromatography, yield 1.1 g (3.3 mmol, 46%).

**3-(2(*S*)-Benzoylpyrrolidine-1-carbonyl)-benzoic acid.** The methyl ester group of 3-(2(*S*)-benzoylpyrrolidine-1-carbonyl)-benzoic acid methyl ester (1.1 g, 3.3 mmol) was hydrolyzed according to procedure B with LiOH in water–MeOH. Yield 0.99 g (3.1 mmol, 94%).

**3-(2(*S*)-Benzoyl-pyrrolidine-1-carbonyl)-benzoyl-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine 3n.** 3-(2(*S*)-Benzoyl-pyrrolidine-1-carbonyl)-benzoic acid (0.35 g, 1.1 mmol) and 5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine trifluoroacetic acid salt (prepared from Boc-5(*R*)-*tert*-butyl-L-prolyl-pyrrolidine (0.36 g, 1.1 mmol) according to procedure C) were coupled according to procedure A. Purification by flash chromatography, yield 0.15 g (0.28 mmol, 25%). $^{1}$H NMR: δ 1.14 (s, 9H), 1.40–2.44 (m, 12H), 2.87–3.91 (m, 6H), 4.19 (br s, 1H), 4.46 (br s, 1H), 5.64–5.69 (m, 1H), 7.14–8.06 (m, 9H). $^{13}$C NMR: δ 197.41, 173.01, 170.81, 168.34, 138.04, 136.48, 135.18, 133.37, 128.71, 128.60, 128.55, 128.29, 128.05, 124.69, 50.19, 46.03, 45.71, 36.48, 29.60, 29.48, 27.66, 26.02, 25.21, 23.88. ESI-MS: *m*/*z* 530 $(M+H)^+$. Anal. ($C_{32}H_{39}N_3O_4 \cdot 1.2H_2O$) calcd C: 69.72, H: 7.57, N: 7.62; found C: 69.90, H: 7.51, N: 7.26.

## In vitro assay for POP activity

The whole pig brains, excluding cerebellum and most of the brain stem, of three pigs were frozen in liquid nitrogen within 30 min from slaughtering 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 pooled and stored in small aliquots at −80 °C until used. The supernatant was thawed in ice and diluted (1:2) with homogenization buffer. In the microplate assay procedure, 10 μL of the enzyme preparation (protein concentration 4.3 mg/mL) 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 (final DMSO concetration was less than 0.1%). 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-amino-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-amino-4-methylcoumarin was determined fluorometrically with microplate fluorescence reader (excitation at 360 nm and emission at 460 nm). 5–7 inhibitor concentrations were used to determine the $IC_{50}$ values and the final concentrations of the compounds in the assay mixture varied from $10^{-12}$ M up to $10^{-5}$ M. 2–4 independent measurements were made for each inhibitor. 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 3.02 software.

The $K_i$ values were determined for the tight binding inhibitors **3j–l** employing the following procedure. 10 μL of enzyme preparation was incubated with 465 μL of 0.1 M sodium–potassium phosphate buffer (pH 7.0) for 2 h in the presence of various concentrations of inhibitors at room temperature. The reaction was started with 25 μL of 4 mM Suc-Gly-Pro-7-amino-4-methylcoumarin and the reaction was monitored every 1 min for 30 min. Over that time scale, the product formations were linear, indicating that the inhibitors did not dissociate markedly from the enzyme. The $K_i$ values were calculated using the Morrison equation that takes the tight binding inhibition into account:[30]

$$\frac{v_i}{v_0} = 1 - \frac{(E + I + K_i) - \sqrt{(E + I + K_i)^2 - 4E \cdot I}}{2E}$$

where $v_0$ and $v_i$ are the reaction velocities in the absence and presence of the inhibitor ($I$), respectively, $K_i$ is the inhibition constant of the inhibitor and $E$ is the active enzyme concentration in the reaction medium. Since the inhibitors did not dissociate from the enzyme during the measurements, competition of binding between substrate and inhibitor did not occur and hence the calculated $K_i$ values are the real dissociation constants of the inhibitors.[31]

## Determination of the log *P* values

A known concentration of an inhibitor in phosphate buffer (saturated with 1-octanol, pH 7.4) was shaken with a suitable volume of 1-octanol for 60 min at room temperature. The phases were separated by centrifugation for 5 min at 2000 rpm and the aqueous phase was analysed. The partition coefficient was calculated in relation to a control that was treated in the same way as the samples but did not contain 1-octanol. Each partition coefficient was determined at least in triplicate. For each HPLC method 20 mM $KH_2PO_4$ of pH 7 was used as the aqueous phase and 90% acetonitrile was used as the organic phase. The HPLC methods were tested for linearity and repeatability. The Merck Hitachi HPLC system consisted of an UV-detector (L-7400), an interface module (D-7000), a pump (L-7100), an autosampler (L-7250) and a Purospher RP-C18e column (125×4 mm, 5 μm).

## Acknowledgements

This research was supported by Finncovery Ltd. and the National Technology Agency in Finland (TEKES). We also thank Ms. Tiina Koivunen, Ms. Helly Rissanen and Ms. Päivi Sutinen for their outstanding technical assistance, Prof. Dr. Jouko Vepsäläinen for his cooperation in the synthesis of the 5(*S*)-*tert*-butyl-L-proline

moiety, Dr. Antti Poso for the discussions regarding the 3D structure of the active site of the enzyme, Dr. Seppo Auriola and Ms. Unni Tengvall, M.Sc., for performing the ESI-MS analysis, and Ms. Piia Palonen, Mr. Pekka Keski-Rahkonen, Mr. Rustam Safin Ms. Anne Riekkinen and Ms. Virpi Turunen for helping us by performing some synthetic steps.

## References and Notes

1. Cunningham, D. F.; O'Connor, B. *Biochim. Biophys. Acta* **1997**, *1343*, 160.
2. Huston, J. P.; Hasenöhrl, R. U. *Behav. Brain. Res.* **1995**, *66*, 117.
3. Kowall, N. W.; Beal, M. F.; Busciglio, J.; Duffy, L. K.; Yankner, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7247.
4. Laitinen, K. S. M.; van Groen, T.; Tanila, H.; Venäläinen, J.; Männistö, P. T.; Alafuzoff, I. *Neuroreport* **2001**, *12*, 3309.
5. Jiang, C. H.; Tsien, J. Z.; Schultz, P. G.; Hu, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *98*, 1930.
6. Rampon, C.; Jiang, C. H.; Dong, H.; Tang, Y. P.; Lockhart, D. J.; Schultz, P. G.; Tsien, J. Z.; Hu, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 12880.
7. Miura, N.; Shibata, S.; Watanabe, S. *Neurosci. Lett.* **1995**, *196*, 128.
8. Toide, K.; Fujiwara, T.; Iwamoto, Y.; Shinoda, M.; Okamiya, K.; Kato, T. *Naunyn-Schmied. Arch. Pharmacol.* **1996**, *353*, 355.
9. Yoshimoto, T.; Kado, K.; Matsubara, F.; Koriyama, N.; Kaneto, H.; Tsuru, D. *J. Pharmacobio-Dyn.* **1987**, *10*, 730.
10. Atack, J. R.; Suman-Chauhan, N.; Dawson, G.; Kulagowski, J. J. *Eur. J. Pharmacol.* **1991**, *205*, 157.
11. Schneider, J. S.; Giardiniere, M.; Morain, P. *Neuropsychopharmacology* **2002**, *26*, 176.
12. Vendeville, S.; Goossens, F.; Debreu-Fontaine, M.-A.; Landry, V.; Davioud-Charvet, E.; Grellier, P.; Scharpé, S.; Sergheraert, C. *Bioorg. Med. Chem.* **2002**, *10*, 1719.
13. De Nanteuil, G.; Portevin, B.; Lepagnol, J. *Drugs Future* **1998**, *23*, 167.
14. Saito, M.; Hashimoto, M.; Kawaguchi, N.; Shibata, H.; Fukami, H.; Tanaka, T.; Higuchi, N. *J. Enzyme Inhibit.* **1991**, *5*, 51.
15. Arai, H.; Nishioka, H.; Niwa, S.; Yamanaka, T.; Tanaka, Y.; Yoshinaga, K.; Kobayashi, N.; Miura, N.; Ikeda, Y. *Chem. Pharm. Bull.* **1993**, *41*, 1583.
16. Portevin, B.; Benoist, A.; Rémond, G.; Hervé, Y.; Vincent, M.; Lepagnol, J.; De Nanteuil, G. *J. Med. Chem.* **1996**, *39*, 2379.
17. Beausoleil, E.; Lubell, W. D. *J. Am. Chem. Soc.* **1996**, *118*, 12902.
18. Beausoleil, E.; L'Archevêque, B.; Bélec, L.; Atfani, M.; Lubell, W. D. *J. Org. Chem.* **1996**, *61*, 9447.
19. Halab, L.; Lubell, W. D. *J. Org. Chem.* **1999**, *64*, 3312.
20. Bélec, L.; Slaninova, J.; Lubell, W. D. *J. Med. Chem.* **2000**, *43*, 1448.
21. Halab, L.; Bélec, L.; Lubell, W. C. *Tetrahedron* **2001**, *57*, 6439.
22. Rapoport, H.; Sim, T. B. *J. Org. Chem.* **1999**, *64*, 2532.
23. Ho, T. L.; Gopalan, B.; Nestor, J. J., Jr. *J. Org. Chem.* **1986**, *51*, 2405.
24. Collado, I.; Ezquerra, J.; Pedregal, C. *J. Org. Chem.* **1995**, *60*, 5011.
25. Wallén, E. A. A.; Christiaans, J. A. M.; Gynther, J.; Vepsäläinen, J. *Tetrahedron Lett.* **2003**, *44*, 2081.
26. Wallén, E. A. A.; Christiaans, J. A. M.; Saario, S. M.; Forsberg, M. M.; Venäläinen, J. I.; Paso, H. M.; Männistö, P. T.; Gynther, J. *Bioorg. Med. Chem.* **2002**, *10*, 2199.
27. Wallén, E. A. A.; Christiaans, J. A. M.; Jarho, E. M.; Forsberg, M. M.; Venäläinen, J. I.; Männistö, P. T.; Gynther, J. *J. Med. Chem.* In press.
28. Venäläinen, J. I.; Juvonen, R. O.; Forsberg, M. M.; Garcia-Horsman, A.; Poso, A.; Wallén, E. A. A.; Gynther, J.; Männistö, P. T. *Biochem. Pharmacol.* **2002**, *64*, 463.
29. Wallén, E. A. A.; Christiaans, J. A. M.; Forsberg, M. M.; Venäläinen, J. I.; Männistö, P. T.; Gynther, J. *J. Med. Chem.* **2002**, *45*, 4581.
30. Copeland, R. A. *Enzymes. A Practical Introduction to Structure, Mechanism and Data Analysis*; Wiley: New York, 2000.
31. Gutheil, W. G.; Bachovchin, W. W. *Biochemistry* **1993**, *32*, 8723.