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2(*S*)-(Cycloalk-1-enecarbonyl)-1-(4-phenyl-butanoyl)pyrrolidines and 2(*S*)-(aroyl)-1-(4-phenylbutanoyl)pyrrolidines as prolyl oligopeptidase inhibitors

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Abstract—In order to replace the P2–P1 amide group, different 1-cycloalkenyls and 2-aryls were studied in the place of the P1 pyrrolidine group of a 4-phenylbutanoyl-L-Pro-pyrrolidine structure, which is a well-known prolyl oligopeptidase inhibitor SUAM-1221. The 1-cyclopentenyl and the 2-thienyl groups gave novel compounds, which were equipotent with the corresponding pyrrolidine-analog SUAM-1221. It was shown that the P2–P1 amide group of POP inhibitors can be replaced by an α,β-unsaturated carbonyl group or the aryl conjugated carbonyl group.

1. Introduction

Human prolyl oligopeptidase (POP, EC 3.4.21.26) is an 80 kDa serine protease that was discovered in the beginning of the 1970s.[1] POP is a proline-specific peptidase that hydrolyzes oligopeptides after prolyl residues and has mainly been described as a cytosolic enzyme. POP has gained pharmaceutical interest because specific POP inhibitors have been shown to ameliorate age-related and induced amnesia in laboratory animals.[2–6] Furthermore, the expression of the POP gene is up-regulated in the hypothalamus[7] and the hippocampus[8] of aged mice. Certain memory-enhancing neuropeptides, like substance P, arginine-vasopressin, and thyroliberin, are in vitro substrates of POP and it has been suggested that POP has an important role in the neuropeptide metabolism.[9] The intracellular functions of POP started to be revealed only in the late 1990s. Inhibition of POP has been shown to elevate the intracellular IP_3 level, which was suggested to explain the memory-enhancing properties of POP inhibitors.[10,11] Recently, a specific POP inhibitor *Z*-Pro-prolinal was shown to prevent the translocation of glyceraldehyde-3-phosphate dehydrogenase from the cytosol to the nucleus and, to prevent the formation of reactive oxygen species in monkey fibroblasts, which had been exposed to 6-hydroxydopamine.[12] Furthermore, POP has been proposed to have a role in protein trafficking and secretion.[13]

POP is a proline-specific peptidase and the S1 binding site of POP has evolved to fit the pyrrolidine ring of an L-prolyl residue.[14] The pyrrolidine group is unquestionably the most utilized structure at the P1 position of POP inhibitors and only few successful replacements of the P1 pyrrolidine ring have been described in the literature.[15,16] An electrophilic substituent, like a 2(*S*)-formyl or a 2(*S*)-cyano group, at the 2-position of the pyrrolidine ring increases significantly the potency.[17,18] However, these groups are chemically reactive, which may limit their usability in drugs.

Keywords: Prolyl oligopeptidase; α,β-Unsaturated carbonyl group; 2-Thiophene; 1-Cyclopentene.
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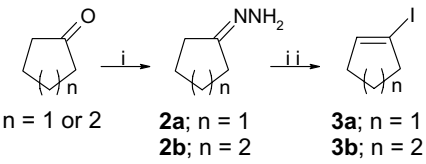
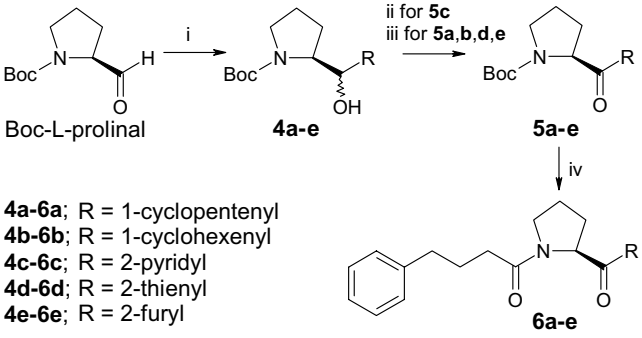
In a peptide chain, a prolyl residue lacks the main chain amide hydrogen. Consequently, the P1 pyrrolidine group of POP inhibitors (or the substrates of POP) is not stabilized by hydrogen bonding.[14,19] We have previously replaced the P1 pyrrolidine group of the well-known reference compound SUAM-1221[20] **1a** by a series of alkyl groups and a phenyl group.[21] To our surprise the cyclopentyl and the phenyl groups gave equipotent compounds **1b** and **1c** (Fig. 1). These compounds were moderately potent but still one order of magnitude less potent than the reference compound **1a**. In the present study, the field between these two replacements was further studied, focusing on mimicking the amide bond geometry.

2. Chemistry

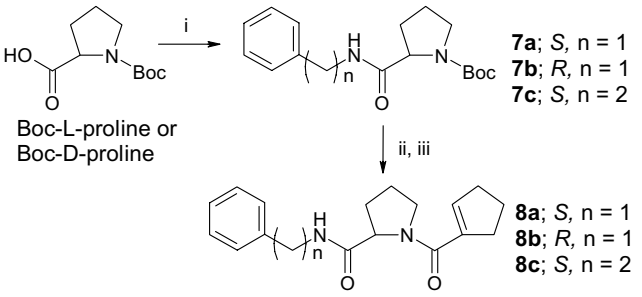
The synthetic routes are presented in Schemes 1–3. Cyclopentylidene-hydrazine (**2a**) and cyclohexylidene-hydrazine (**2b**) were prepared by the reaction of hydrazine monohydrate with cyclopentanone and cyclohexanone, respectively. While the reaction with cyclopentanone gave *N*,*N*′-dicyclopentylidene-hydrazine as a minor side-product (about 5 mol %), the reaction with cyclohexanone produced a significant amount of *N*,*N*′-dicyclohexylidene-hydrazine (about 20 mol %). All attempts to distill **2b** led to the decomposition of the product. The unpurified compounds **2a** and **2b** were reacted with iodine in the presence of tetramethylguanidine to yield 1-iodocyclopentene (**3a**) and 1-iodocyclohexene (**3b**), respectively, which were distilled before further use.[22] Compounds **3a** and **3b** were treated with *tert*-BuLi, and 2-bromopyridine and furan were treated with *n*-BuLi to obtain the corresponding organolithium reagents, which were reacted with Boc-L-prolinal to give compounds **4a-c** and **4e** respectively. Compound **4d** was obtained from the reaction of the commercially available 2-thienyllithium and Boc-L-prolinal. Compounds **4a**–**e** were oxidized with Dess–Martin periodinane or SO_3·pyridine/dimethylsulfoxide and deprotected. The resulting amines were coupled with 4-phenylbutyric acid using EDC and HOBt to yield compounds **6a**–**e**. Boc-L-proline and Boc-D-proline were activated with ethyl chloroformate and reacted with benzylamine or



Scheme 1. Reagents and condition: (i) Hydrazine monohydrate/reflux; (ii) 1—I_2, tetramethylguanidine/Et_2O; 2—compound **2a** or **2b**/Et_2O.



Scheme 2. Reagents and conditions: (i) organolithium reagent (prepared in situ or commercial reagent)/tetrahydrofuran, −80 °C; (ii) Et_3N, SO_3·pyridine/dimethylsulfoxide; (iii) Dess–Martin periodinane/CH_2Cl_2; (iv) 1—trifluoroacetic acid/ CH_2Cl_2; 2—4-phenylbutyric acid, HOBt·H_2O, EDC·HCl, Et_3N/ CH_2Cl_2, 0–20 °C.



Scheme 3. Reagents and conditions: (i) 1—Et_3N, ethyl chloroformate/tetrahydrofuran, −15 °C, 2—benzylamine/tetrahydrofuran, −15 to 20 °C; (ii) 1. trifluoroacetic acid/CH_2Cl_2, 0 °C; (iii) 1. 1-cyclopentene-1-carboxylic acid, Et_3N, trimethylacetyl chloride/CH_2Cl_2, 0 °C; 2—Et_3N, trifluoroacetic acid salt of the deprotected **6** from reaction (ii).

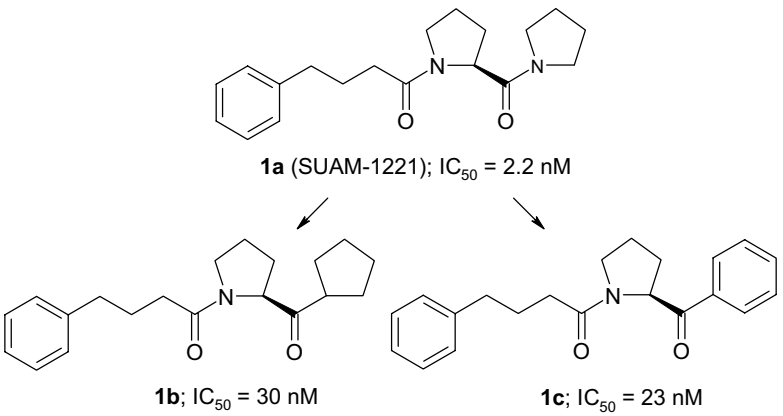


Figure 1. The reference compound **1a**[20] and the previously reported compounds **1b** and **1c**.[21]

phenethylamine to yield compounds **7a**–**c**. 1-Cyclopentene-1-carboxylic acid was activated with trimethylacetyl chloride and reacted with **7a**–**c** after the removal of the Boc-protection group to yield compounds **8a**–**c**.

3. Results and discussion

Compounds **6a**–**e** are presented in Table 1. The P1 pyrrolidine ring of the reference compound **1a** (Fig. 1) has been replaced by a 1-cyclopentene ring in compound **6a**. A similar replacement at the P2 position has previously been shown to give potent POP inhibitors.[23] Indeed, compound **6a** was equipotent with the reference compound **1a**. Compound **6a** was one order of magnitude more potent than the previously reported compound **1b** with the cyclopentyl ring at the P1 site. The difference in their potencies can be explained by differences in geometry and π-stacking. The P1 amide nitrogen is sp^2 hybridized and, although the replacement by an sp^3 carbon was tolerated (IC_{50} of compound **1b** was 30 nM), an sp^2 carbon provides a better mimetic for the amide nitrogen. Furthermore, the double bond may have beneficial π-stacking with the indole ring of Trp595.[14]

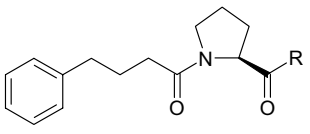
The enlargement of the 1-cyclopentenyl group of compound **6a** resulted in compound **6b** with a 1-cyclohexenyl group at the P1 position. Compound **6b** was 20× less potent than compound **6a**, which reflects the limited space in the S1 pocket.[14] However, we have previously reported that a compound with the same skeleton as **6b** but with a fully saturated cyclohexyl group at the P1 site gives only week POP inhibitory activity (IC_{50} = 1.1 μM). Compound **6b** was 18× more potent than this compound, while it was only 2.6× less potent than the reference compound **1c**, which has the phenyl group at the P1 site. This small decrease in potency in relation to compound **1c** may be explained by the smaller size of the phenyl ring or loss in π-stacking with Trp595.

In order to study different aryls at the P1 position, the 2-pyridyl, 2-thienyl, and 2-furyl groups were selected. The pyridyl group has previously been successfully employed at the P1′ and P3 positions of POP inhibitors.[24,25] As compared to compound **1c** with the phenyl group at the P1 site, compound **6c** with the 2-pyridyl group had strongly decreased inhibitory activity. In contrast, compound **6d**, with the 2-thienyl group at the P1 site, was one order of magnitude more potent than compound **1c** and equipotent with compound **6a**. The difference between compounds **6d** and **1c** can be explained by the smaller size of the 2-thienyl group. The 1-cyclopentenyl and the 2-thienyl groups, instead, are almost equal in size and compound **6d** does not seem to have any beneficial π-stacking over **6a**. The potency of compound **6e** with the 2-furyl group at the P1 site was slightly decreased but of the same order of magnitude as the potency of compound **6d**.

The 2-thienyl group has previously been studied at the P1 position of POP inhibitors but with opposite results. It gave a poor POP inhibitor against *Flavobacterium meningosepticum* POP.[15] On the other hand, an unusual POP inhibitor Y-29794 possesses a 2-thienyl group although it is not known where it binds. Y-29794 was reported to have a K_i value of 0.95 nM against rat POP.[16] The excellent POP inhibitory activity of compound **6d** indicates that the 2-thienyl group of Y-29794 may bind to the S1 pocket of POP. It also seems that the S1 subsites of the mammalian and the bacterial POP have differences.

The use of the 1-cyclopentenyl group at the P1 position allowed further modifications on the backbone. Compounds **8a**–**c** (Table 2) were prepared in order to study whether it is possible to reverse the P2 prolyl residue; the P1–P2 carbonyl group was kept in the original position but the direction of the amide bond was changed. Consequently, compounds **8a–c** possess the α,β-unsaturated amide group instead of the α,β-unsaturated ketone group. α,β-Unsaturated ketones are usually avoided in drug design because of their ability to undergo Michael additions to the double bond. α,β-Unsaturated amides may be less reactive due to the electron-donating effect of the amide nitrogen. Compound **8a** with a reversed L-prolyl residue at the P2 position had an IC_{50} value of 210 nM. The potency was significantly decreased as compared to compound **6a**. A D-prolyl residue was also studied but the resulting compound **8b** was over 200× less active than compound **8a** with the L-prolyl residue. The P3 chain of compound **8a** was elongated with one methylene group resulting in compound **8c**, which had improved inhibitory activity. This reflects the known

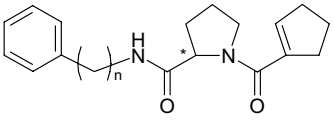
Table 1. Inhibitory activities with 95% confidence intervals of the compounds with different 1-cycloalkenyl and 2-aryl groups at the P1 position



Compound	R	IC_{50}[a] (nM)
6a	1-Cyclopentenyl	3.0 (2.7–3.4)
6b	1-Cyclohexenyl	59 (52–69)
6c	2-Pyridyl	510 (450–570)
6d	2-Thienyl	3.0 (2.7–3.4)
6e	2-Furyl	5.2 (4.3–6.1)

[a] The IC_{50} values were determined against POP from porcine brain.[26]

Table 2. Inhibitory activities with 95% confidence intervals of the compounds with the reversed prolyl residue at the P2 position



Compound	*n*	Stereo center	IC_{50}[a] (nM)
8a	1	*S*	210 (180–240)
8b	1	*R*	44,000 (28,000–67,000)
8c	2	*S*	110 (91–130)

[a] The IC_{50} values were determined against POP from porcine brain.[26]

structure–activity relationships of the P3 site suggesting that the 1-cyclopentenyl group of compounds **8a**–**c** also binds to the S1 site of POP.

4. Conclusions

The 1-cycloalkenyl groups at the P1 position gave better POP inhibitory activities than the corresponding fully saturated cycloalkyl groups. The 1-cyclopentenyl group gave the most potent compound, which was equipotent with the reference compound **1a**. In the series of the aromatic groups, the 2-thienyl group gave the most potent compound. This confirms again that the 5-membered ring is preferred. The reversed P2 L-prolyl residue decreased potency significantly but the effect of the reversed D-prolyl residue was much more pronounced. In conclusion, the P2–P1 amide group of POP inhibitors can be replaced by the α,β-unsaturated carbonyl group or the aryl conjugated carbonyl group.

5. Experimental

5.1. General methods

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 500 spectrometer in $CDCl_3$. Chemical shifts (δ) are given in ppm downfield from the internal standard tetramethylsilane (δ 0.00). The *N*-amide bond of a proline residue has energetically similar *cis* and *trans* isomers (rotamers). These rotamers have slightly different shifts. Minor rotamers that are less than 20% of the major rotamers are not reported for ^{13}C spectra. Electrospray ionization mass spectra (ESI-MS) were obtained on a LCQ ion trap mass spectrometer equipped with an electrospray ionization source (Finnigan MAT, San Jose, CA). Elemental analyses (CHN) were carried out with a Thermo Quest CE Instruments EA 1110 CHNS-O elemental analyzer. Flash chromatography was performed on J. T. Bakers silica gel for chromatography (pore size 60 Å, particle size 50 μm). The silica plate for the chromatotron was made of Merck silica gel 60 PF_{254} containing gypsum (particle size 5–40 μm). Diethyl ether, furan, and tetrahydrofuran (THF) were distilled from sodium and organolithium reactions were performed under argon.

5.2. Method A

5.2.1. 1-Iodo-cyclopentene (3a). *Step 1 (***2a***).* Cyclopentanone (8.8 mL, 100 mmol) was added dropwise to hydrazine monohydrate (30 mL, 620 mmol) under vigorous stirring. The mixture was refluxed for 2 h and cooled down to room temperature (rt). Dichloromethane (DCM) was cautiously added at cold water bath and the phases were separated. The organic phase was washed with saturated (satd) NaCl aq, dried over Na_2SO_4, filtered, and evaporated. The product was used without further purification. Crude product 7.1 g. *Step 2* (**3a**). Tetramethylguanidine (75 mL, 600 mmol) in Et_2O (110 mL) was added dropwise to iodine (28 g, 110 mmol) in Et_2O (160 mL) during 1.5 h and the mixture was stirred for 2.5 h. Compound **2a** (4.9 g, about 50 mmol) in Et_2O (50 mL) was added dropwise during 2 h and the mixture was stirred overnight. The mixture was refluxed for 2 h, cooled down to rt, and filtered. The filtrate was concentrated from Et_2O, heated up to 90–95 °C for 3 h, cooled to rt, and diluted with Et_2O. The organic phase was washed with 1 M HCl aq, 20% $Na_2S_2O_3$, satd $NaHCO_3$ aq, and satd NaCl aq, dried over Na_2SO_4, filtered, and evaporated (with a rotary evaporator at 40 °C and 100 mbar as the lowest pressure point). The product was stored at −18 °C under argon and was distilled before further use at 39 °C and 6 mbar. Yield 5.6 g, 42% for two steps. ^{1}H NMR δ 1.93 (m, 2H), 2.32 (m, 2H), 2.60 (m, 2H), 6.10 (m, 1H).

5.2.2. 1-Iodo-cyclohexene (3b). Prepared according to method A from cyclohexanone (10.4 mL, 100 mmol). *Step 1 (***3a***).* Crude product 10.8 g. *Step 2* (**3b**). Tetramethylguanidine (1.16 mol), I_2 (212 mmol). Compound **3b** was distilled at 50 °C and 1 mbar. Yield 9.9 g, 47% for two steps. ^{1}H NMR δ 1.63–1.72 (m, 4H), 2.09 (m, 2H), 2.50 (m, 2H), 6.34 (m, 1H).

5.3. Method B

5.3.1. ((*S*)-1-Boc-pyrrolidin-2-yl)-cyclopent-1-enyl-methanol (4a). 1.7 M *tert*-BuLi (17 mL, 29 mmol) was added dropwise to **3a** (5.6 g, 29 mmol) in THF (30 mL) and the mixture was stirred for 1 h at −80 °C. Boc-L-prolinal (1.9 g, 9.5 mmol) in THF (10 mL) was added dropwise and the mixture was stirred for 3 h at −80 °C. The reaction was quenched with satd NH_4Cl aq at −80 °C. The mixture was let to warm up to rt, diluted with H_2O, and extracted with DCM. The organic phase was washed with satd NaCl aq, dried over Na_2SO_4, filtered, and evaporated. The product was purified by flash-chromatography (15–50% EtOAc in hexane). Yield 1.9 g, 75%.

5.3.2. ((*S*)-1-Boc-pyrrolidin-2-yl)-cyclohex-1-enyl-methanol (4b). Prepared according to method B from **3b** (3.1 g, 15 mmol) and Boc-L-prolinal (1.0 g, 5 mmol). The product was purified twice by flash-chromatography. One diastereomer was purified with 15% EtOAc in petroleum ether (PE) but the other diastereomer co-eluted with the unreacted Boc-prolinal. The other diastereomer was purified with 5% acetonitrile in DCM. The diastereomers were combined giving the total yield of 0.54 g, 38%.

5.4. ((*S*)-1-Boc-pyrrolidin-2-yl)-pyridin-2-yl-methanol (4c)

To a solution of 2-bromopyridine (3.2 g, 20 mmol) in THF (40 mL) was added dropwise 1.6 M n-BuLi (12.5 mL, 20 mmol) at −70 °C and the mixture was stirred for 1 h. Boc-L-prolinal (3.6 g, 18 mmol) in THF (40 mL) was added and the mixture was stirred for 2 h at −70 °C. The mixture was poured in water and THF was evaporated. The water phase was extracted with DCM and the organic phase was extracted with 0.1 M HCl aq The water phase was made basic with 2 M NaOH aq, extracted with DCM, dried over Na_2SO_4, filtered, and evaporated. Yield 2.8 g, 56%.

5.5. ((*S*)-1-Boc-pyrrolidin-2-yl)-thiophen-2-yl-methanol (4d)

To a solution of Boc-L-prolinal (0.31 g, 1.6 mmol) in THF was added 1 M 2-thienyllithium solution in THF (1.75 mL, 1.75 mmol) at −70 °C. The mixture was stirred for 3 h and quenched with satd NH_4Cl aq at −70 °C. The mixture was let to warm up to rt, diluted with H_2O, and extracted with DCM. The organic phase was washed with satd NaCl aq, dried over Na_2SO_4, filtered, and evaporated. The product was purified by flash-chromatography (1% MeOH in DCM). Yield 0.32 g, 71%.

5.6. Method C

5.6.1. 1-Boc-2(*S*)-(cyclopent-1-enecarbonyl)pyrrolidine (5a). To a solution of Dess–Martin periodinane (DMP) (1.4 g, 3.3 mmol) in DCM (16 mL) was added **4a** (0.80 g, 3.0 mmol) in DCM (10 mL) and the mixture was stirred for 1 h at rt 30% $Na_2S_2O_3$ aq was added and the mixture was stirred for 5 min. The phases were separated and the organic phase was washed with 30% $Na_2S_2O_3$ aq and satd $NaHCO_3$ aq, dried over Na_2SO_4, filtered, and evaporated. The product was purified by flash-chromatography (20% EtOAc in PE). Yield 0.58 g, 73%. ^{1}H NMR δ 1.34 (s, 5H), 1.45 (s, 4H), 1.80–1.96 (m, 5H), 2.13–2.25 (m, 1H), 2.50–2.69 (m, 4H), 3.38–3.61 (m, 2H), 4.78 (dd, J = 4.5, 8.9 Hz, 0.5H), 4.96 (dd, J = 3.5, 9.0 Hz, 0.5H), 6.77 (m, 0.5H), 6.83 (m, 0.5H).

5.6.2. 1-Boc-2(*S*)-(cyclohex-1-enecarbonyl)pyrrolidine (5b). Prepared according to method C from **4b** (0.54 g, 1.9 mmol) using 1.3 equiv of DMP. No chromatographic purification was performed. Yield 0.54 g, 100%. ^{1}H NMR δ 1.34 (s, 4.7H), 1.45 (s, 4.3 H), 1.57–1.69 (m, 4H), 1.75–1.94 (m, 3H), 2.12–2.39 (m, 5H), 3.41 (m, 0.5H), 3.46–3.62 (m, 1.5H), 4.90 (dd, J = 4.1, 8.8 Hz, 0.5H), 5.05 (dd, J = 3.4, 9.1 Hz, 0.5H), 6.90 (m, 0.5H), 6.96 (m, 0.5H).

5.6.3. 1-Boc-2(*S*)-(thiophene-2-carbonyl)pyrrolidine (5d). Prepared according to method C from **4d** (0.32 g, 1.1 mmol) using 1.3 equiv of DMP. The product was purified by flash-chromatography (0.5% MeOH in DCM). Yield 0.27 g, 87%. ^{1}H NMR δ 1.25 (s, 5.4 H), 1.46 (s, 3.6H), 1.87–2.05 (m, 3H), 2.25–2.38 (m, 1H), 3.47 (m, 0.4H), 3.56–3.69 (m, 1.6H), 4.88 (dd, J = 5.0, 8.6 Hz, 0.6H), 5.10 (dd, J = 3.2, 8.9 Hz, 0.4H), 7.13 (m, 0.4H), 7.15 (m, 0.6 H), 7.78 (d, J = 3.8 Hz, 0.6H), 7.81 (d, J = 3.5 Hz, 0.4H).

5.7. 1-Boc-2(*S*)-(pyridine-2-carbonyl)pyrrolidine (5c)

To a solution of **4c** (0.60 g, 2.2 mmol) and Et_3N (0.9 mL, 6.5 mmol) in 2.2 mL of dimethylsulfoxide (DMSO) was added SO_3·pyridine (1.03 g, 6.5 mmol) in DMSO (2.2 mL). The mixture was stirred for 1 h and then poured in ice-cold water (25 mL). The water phase was extracted with $CHCl_3$ and the organic phase was washed with water and satd $NaHCO_3$ aq, dried over Na_2SO_4, filtered, and evaporated. The product was purified with a chromatotron (1% EtOH in $CHCl_3$). Yield 0.37 g, 61%. ^{1}H NMR δ 1.24 (s, 5.4H), 1.46 (s, 3.6H), 1.88–1.98 (m, 3H), 2.38–2.45 (m, 1 H), 3.48–3.70 (m, 2H), 5.69 (dd, 0.6H), 5.78 (dd, 0.4H), 7.44 (ddd, J = 1.0, 4.8, 7.6 Hz, 0.4H), 7.48 (ddd, J = 1.1, 4.8, 7.7 Hz, 0.6H), 7.82 (td, J = 1.6, 7.7 Hz, 0.4H), 7.86 (td, J = 1.7, 7.8 Hz, 0.6H), 8.08 (dt, J = 1.0, 7.8 Hz, 1H), 8.66 (d, 0.4H), 8.68 (d, J = 4.8 Hz, 0.6H).

5.8. 1-Boc-2(*S*)-(furan-2-carbonyl)pyrrolidine (5e)

1.6 M *n*-BuLi (4.7 mL, 7.5 mmol) was added dropwise to furan (10 mL) at 0 °C and the mixture was stirred for 1 h. Boc-L-prolinal (0.30 g, 1.5 mmol) in THF (10 mL) was added dropwise at −80 °C and the mixture was stirred for 3 h. Satd NH_4Cl aq was added and the mixture was let to warm up to rt and diluted with DCM. The organic phase was washed with satd NaCl aq, dried over Na_2SO_4, and evaporated to yield **4e**. Without further purification, the residue was dissolved in DCM, added to a solution of DMP (0.95 g, 2.2 mmol) in DCM (4 mL), and then continued according to method C. The product was purified by flash-chromatography (10% EtOAc in PE). Yield 110 mg, 28% (two steps). ^{1}H NMR δ 1.26 (s, 5.4H), 1.46 (s, 3.6H), 1.87–2.01 (m, 3H), 2.23–2.36 (m, 1H), 3.43–3.67 (m, 2H), 4.90 (dd, J = 4.6, 8.6 Hz, 0.6H), 5.09 (dd, 0.4H), 6.53 (dd, J = 1.5, 3.3 Hz, 0.4H), 6.56 (dd, J = 1.6, 3.5 Hz, 0.6H), 7.23 (d, J = 3.5 Hz, 0.6H), 7.26 (m, 0.4H), 7.58 (d, 0.4H), 7.61 (d, 0.6).

5.9. Method D

5.9.1. 2(*S*)-(Cyclopent-1-enecarbonyl)-1-(4-phenylbutanoyl)pyrrolidine (6a). To a solution of **5a** (110 mg, 0.35 mmol) in DCM (2.5 mL) was added trifluoroacetic acid (TFA) (0.7 mL, 9.4 mmol) at 0 °C. The mixture was stirred for 2.5 h at 0 °C and evaporated. TFA salt of the amine, 4-phenylbutyric acid (58 mg, 0.35 mmol), HOBt·H_2O (81 mg, 0.53 mmol), and EDC·HCl (102 mg, 0.53 mmol) were dissolved/suspended in DCM. Et_3N (240 μL, 1.75 mmol) was added at 0 °C and the mixture was stirred for 0.5 h at 0 °C and for 5 h at rt. The reaction mixture was washed with 30% citric acid, satd NaCl aq, and satd $NaHCO_3$, dried over Na_2SO_4, filtered, and evaporated. The product was purified with a chromatotron (40–50% EtOAc in PE; R_f = 0.12 in 30% EtOAc in PE). Yield 50 mg, 46%. ^{1}H NMR δ 1.81–2.06 (m, 7H), 2.14 (m, 1H), 2.23–2.38 (m, 2H), 2.50–2.69 (m, 6H), 3.43 (m, 0.9H), 3.56-3.62 (m, 1H), 3.67 (m, 0.1H), 4.78 (dd, J = 3.0, 9.2 Hz, 0.1H), 5.16 (dd, J = 3.8, 8.8 Hz, 0.9H), 6.65 (m, 0.1H), 6.87 (m, 0.9H), 7.12–7.29 (m, 5H). ^{13}C NMR δ 22.5, 24.6, 26.0, 29.4, 30.9, 33.3, 34.2, 35.1, 47.2, 61.2, 125.8, 128.3, 128.6, 141.9, 143.4, 144.2, 171.1, 196.4. Anal. Calcd for $C_{20}H_{25}NO_2$·0.1 H_2O: C, 76.69; H, 8.11; N, 4.47. Found C, 76.72; H, 8.19; N, 4.52. ESI-MS *m*/*z* 312.1 $[M+H]^+$.

5.9.2. 2(*S*)-(Cyclohex-1-enecarbonyl)-1-(4-phenylbutanoyl)pyrrolidine (6b). Prepared according to method D from **5b** (325 mg, 1.0 mmol) having a 20-h reaction time. The product was purified by flash chromatography (50% EtOAc in PE; R_f = 0.31). Yield 160 mg, 49%. ^{1}H NMR δ 1.56–1.67 (m, 4H), 1.80 (m, 1H), 1.87–2.05 (m, 4H),

2.09-2.38 (m, 7H), 2.61 (m, 0.3H), 2.68 (t, J = 7.5 Hz, 1.7H), 3.43 (m, 0.9H), 3.56–3.62 (m, 1H), 3.67 (m, 0.1H), 4.88 (dd, J = 2.9, 9.2 Hz, 0.1H), 5.24 (dd, J = 3.7, 8.9 Hz, 0.9H), 6.76 (m, 0.1H), 7.00 (m, 0.9H), 7.13–7.28 (m, 5H). ^{13}C NMR δ 21.5, 21.8, 23.2, 24.6, 26.0, 26.1, 29.5, 33.4, 35.1, 47.1, 59.6, 125.8, 128.3, 128.6, 137.4, 140.5, 141.9, 171.0, 198.6. Anal. Calcd for $C_{21}H_{27}NO_2 \cdot 0.1\ H_2O$: C, 77.08; H, 8.38; N, 4.28. Found C, 76.97; H, 8.50; N, 4.21. ESI-MS m/z 326.1 $[M+H]^+$.

5.9.3. 1-(4-Phenylbutanoyl)-2(*S*)-(pyridine-2-carbonyl)pyrrolidine (6c). Prepared according to method D from **5c** (280 mg, 1.0 mmol) having a 20-h reaction time. The reaction mixture was not washed with 30% citric acid. The product was purified by flash chromatography (50% EtOAc in PE; R_f = 0.14). Yield 130 mg, 40%. ^{1}H NMR δ 1.79–2.17 (m, 5.5H), 2.28–2.49 (m, 2.5H), 2.56 (m, 0.4H) 2.68 (t, J = 7.5 Hz, 1.6H), 3.52 (m, 0.8H), 3.64–3.71 (m, 1H), 3.76 (m, 0.2H), 5.85 (dd, J = 3.0, 9.4 Hz, 0.2H), 5.93 (dd, 0.8H), 7.07 (m, 0.5 H), 7.13–7.21 (m, 2.5H), 7.26–7.29 (m, 2H), 7.46 (ddd, J = 1.2, 4.8, 7.6 Hz, 0.8H), 7.53 (ddd, J = 1.2, 4.7, 7.6 Hz, 0.2 H), 7.83 (td, J = 1.7, 7.7 Hz, 0.8H), 7.89 (td, J = 1.7, 7.7 Hz, 0.2H), 8.04–8.08 (m, 1H), 8.66–8.69 (m, 1H). ^{13}C NMR δ 25.0, 26.1, 29.3, 33.4, 35.1, 47.5, 60.4, 122.7, 125.8, 127.1, 128.3, 128.6, 136.9, 141.9, 148.8, 152.3, 171.0, 198.6. Anal. Calcd for $C_{20}H_{22}N_2O_2$: C, 74.15; H, 6.88; N, 8.69. Found C, 74.36; H, 7.09; N, 8.73. ESI-MS m/z 323.2 $[M+H]^+$.

5.9.4. 1-(4-Phenylbutanoyl)-2(*S*)-(thiophene-2-carbonyl)pyrrolidine (6d). Prepared according to method D from **5d** (270 mg, 1.0 mmol) having a 20-h reaction time and using 2.5 equiv of EDC and HOBt. The product was purified by flash chromatography (15–50% EtOAc in PE; R_f = 0.30 in 50% EtOAc in PE). Yield 155 mg, 47%. ^{1}H NMR δ 1.88–2.15 (m, 5.3H), 2.22–2.44 (m, 2.7H), 2.57 (m, 0.3H), 2.69 (t, J = 7.5 Hz, 1.7H), 3.48 (m, 0.85H), 3.63–3.69 (m, 1H), 3.75 (m, 0.15H), 4.93 (dd, J = 3.2, 9.1 Hz, 0.15H), 5.32 (dd, J = 3.6, 9.0 Hz, 0.85H), 7.07–7.21 (m, 4H), 7.26–7.29 (m, 2H), 7.64 (dd, J = 0.9, 5.0 Hz, 0.85H), 7.66 (dd, 0.15H), 7.72 (dd, J = 0.9, 5.0 Hz, 0.15H), 7.83 (dd, J = 0.9, 3.8 Hz, 0.85H). ^{13}C NMR δ 24.8, 26.0, 29.6, 33.3, 35.1, 47.2, 61.8, 125.8, 128.1, 128.3, 128.6, 132.3, 133.8, 141.7, 141.8, 171.4, 191.1. Anal. Calcd for $C_{19}H_{21}NO_2S \cdot 0.8\ H_2O$: C, 66.75; H, 6.66; N, 4.10. Found C, 66.61; H, 6.34; N, 4.12. ESI-MS m/z 328.1 $[M+H]^+$.

5.9.5. 2(*S*)-(Furan-2-carbonyl)-1-(4-phenylbutanoyl)pyrrolidine (6e). Prepared according to method D from **5e** (100 mg, 0.37 mmol) having a 20-h reaction time. The product was purified by flash chromatography (27% EtOAc in PE; R_f = 0.26 in 50% EtOAc in PE). Yield 42 mg, 36%. ^{1}H NMR δ 1.86–2.12 (m, 5.4H), 2.17–2.40 (m, 2.6 H), 2.58 (m, 0.3H), 2.69 (t, J = 7.5 Hz, 1.7H), 3.47 (m, 0.85H), 3.61–3.67 (m, 1H), 3.73 (m, 0.15H), 4.97 (dd, J = 3.0, 9.2 Hz, 0.15H), 5.30 (dd, J = 3.9, 8.8 Hz, 0.85H), 6.54 (dd, J = 1.6, 3.6 Hz, 0.85H), 6.59 (dd, J = 1.6, 3.5 Hz, 0.15H), 7.09–7.22 (m, 3.7H), 7.26–7.29 (m, 2.3H), 7.59 (d, J = 1.6 Hz, 0.85H), 7.63 (d, J = 1.6 Hz, 0.15H). ^{13}C NMR δ 24.8, 26.0, 29.1, 33.3, 35.1, 47.2, 60.9, 112.3, 117.9, 125.7, 128.2, 128.6, 141.9, 146.5, 151.4, 171.3, 187.1. Anal. Calcd for $C_{19}H_{21}NO_3 \cdot 0.1\ H_2O$: C, 72.87; H, 6.82; N, 4.47. Found C, 72.74; H, 7.10; N, 4.31. ESI-MS m/z 312.1 $[M+H]^+$.

5.10. Method E

5.10.1. 1-Boc-L-proline benzylamide (7a). To a solution of Boc-L-proline (0.43 g, 2.0 mmol) and Et_3N (310 μL, 2.2 mmol) in THF (4 mL) was added ethyl chloroformate (190 μL, 2.0 mmol) in THF (3 mL) dropwise at −15 °C, and the mixture was stirred for 30 min. Benzylamine (440 μL, 4.0 mmol) in THF (1 mL) was added at −15 °C and the mixture was stirred overnight at rt. The mixture was diluted with DCM, washed with 30% citric acid, satd NaCl aq, and satd $NaHCO_3$, dried over Na_2SO_4, filtered, and evaporated. Yield 0.55 g, 90%.

5.10.2. 1-Boc-D-proline benzylamide (7b). Prepared according to method E from Boc-D-proline (0.90 g, 4.2 mmol). At the end of the reaction, the mixture was diluted with EtOAc. Yield 1.08 g, 84%.

5.10.3. 1-Boc-L-proline phenethyl-amide (7c). Prepared according to method E from Boc-L-proline (0.54 g, 2.5 mmol) and phenethylamine (0.63 mL, 5 mmol). At the end of the reaction, the mixture was diluted with EtOAc. Yield 0.80 g, 100%.

5.11. Method F

5.11.1. 1-(Cyclopent-1-enecarbonyl)-L-proline benzylamide (8a). *Step 1.* To a solution of **7a** (0.55 g, 1.8 mmol) in DCM (4 mL) was added TFA (3.6 mL, 48 mmol) in DCM (4 mL) dropwise at 0 °C. The mixture was stirred for 1 h at 0 °C and evaporated. *Step 2.* To a solution of 1-cyclopentene-1-carboxylic acid (0.20 g, 1.8 mmol) and Et_3N (0.28 mL, 2.0 mmol) in DCM (4 mL) was added trimethylacetyl chloride (0.22 mL, 1.8 mmol) in DCM (4 mL) dropwise at 0 °C. The mixture was stirred for 1 h at 0 °C and the ice bath was removed. Et_3N (0.82 mL, 5.9 mmol) and the product of STEP 1 in DCM (4 mL) were added in this order and the mixture was stirred for 2 h. The mixture was washed with 30% citric acid, satd NaCl aq, and satd $NaHCO_3$, dried over Na_2SO_4, filtered, and evaporated. The product was purified by flash-chromatography (40–80% EtOAc in PE; R_f = 0.26 in EtOAc). Yield 380 mg, 71%. Crystallized from EtOAc–hexane, mp 77.1–78.8 °C. ^{1}H NMR δ 1.85–1.98 (m, 4H), 2.10 (m, 1H), 2.45–2.61 (m, 4H), 2.70 (m, 1H), 3.56–3.65 (m, 2H), 4.39 (dd, J = 5.0, 15.1 Hz, 1H), 4.48 (dd, J = 5.7, 15.1 Hz, 1H), 4.72 (m, 1H), 6.16 (m, 1H), 7.24–7.32 (m, 5H), 7.42 (m, 1H). ^{13}C NMR δ 22.7, 25.4, 26.8, 33.7, 33.9, 43.4, 49.2, 59.9, 127.2, 127.4, 128.6, 136.3, 138.5, 139.1, 168.7, 171.2. Anal. Calcd for $C_{18}H_{22}N_2O_2$: C, 72.46; H, 7.43; N, 9.39. Found C, 72.57; H, 7.51; N, 9.46. ESI-MS m/z 299.0 $[M+H]^+$.

5.11.2. 1-(Cyclopent-1-enecarbonyl)-D-proline benzylamide (8b). Prepared according to method F from **7b** (0.30 g, 1.0 mmol). The product was purified by

flash-chromatography (50–60% EtOAc in hexane; $R_f = 0.27$ in EtOAc). Yield 180 mg, 60%. Crystallized from EtOAc–hexane, mp 75.8–78.8 °C. ^{1}H NMR δ 1.85–1.98 (m, 4H), 2.10 (m, 1H), 2.45–2.61 (m, 4H), 2.70 (m, 1H), 3.56–3.65 (m, 2H), 4.39 (dd, $J = 5.2$, 15.1 Hz, 1H), 4.48 (dd, $J = 5.9$, 15.1 Hz, 1H), 4.72 (m, 1H), 6.16 (m, 1H), 7.24–7.32 (m, 5H), 7.42 (m, 1H). ^{13}C NMR δ 22.7, 25.4, 26.8, 33.7, 33.9, 43.4, 49.2, 59.9, 127.2, 127.4, 128.6, 136.3, 138.5, 139.1, 168.7, 171.2. Anal. Calcd for $C_{18}H_{22}N_2O_2$: C, 72.46; H, 7.43; N, 9.39. Found C, 72.30; H, 7.56; N, 9.34. ESI-MS *m/z* 299.0 $[M+H]^+$.

5.11.3. 1-(Cyclopent-1-enecarbonyl)-L-proline phenethylamide (8c). Prepared according to method F from **7c** (0.40 g, 1.25 mmol). The product was purified by flash-chromatography (50% EtOAc in PE; $R_f = 0.11$ in 50% EtOAc in PE). Yield 330 mg, 85%. Crystallized from EtOAc–hexane, mp 95.0–96.2 °C. ^{1}H NMR δ 1.82–2.05 (m, 5H), 2.33–2.73 (m, 5H), 2.75–2.85 (m, 2H), 3.44–3.66 (m, 4H), 4.42 (m, 0.2H), 4.62 (m, 0.8H), 5.91 (m, 0.2H), 6.13 (m, 0.8H), 6.23 (m, 0.2H), 7.04 (m, 0.8H), 7.17–7.21 (m, 3H), 7.25–7.28 (m, 2H). ^{13}C NMR δ 22.7, 25.3, 27.1, 33.7, 33.8, 35.7, 40.6, 49.2, 60.0, 126.3, 128.4, 128.8, 136.4, 139.1, 139.1, 168.3, 171.3. Anal. Calcd for $C_{19}H_{24}N_2O_2$: C, 73.05; H, 7.74; N, 8.97. Found C, 72.88; H, 7.75; N, 8.95. ESI-MS *m/z* 313.0 $[M+H]^+$.

5.12. Determination of the IC_{50} values

The whole brains, excluding cerebellum and most of the brain stem, of three pigs were placed in liquid nitrogen within 30 min after the animals were killed 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 it was used in the activity assay and diluted in a ratio of 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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