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Inhibition of Prolyl Oligopeptidase with a Synthetic Unnatural Dipeptide

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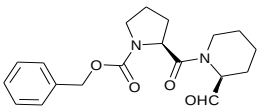
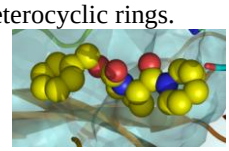
The synthesis of a new inhibitor of prolyl oligopeptidase, containing a piperidine ring, together with an X-ray structure of its complex with the enzyme, is described. This provides evidence that covalent inhibitors of POP do not have to be limited to structures containing 5-membered N-containing heterocyclic rings.

inhibitor:





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BIOORGANIC & MEDICINAL CHEMISTRY

Inhibition of Prolyl Oligopeptidase with a Synthetic Unnatural Dipeptide

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Abstract—A new inhibitor, containing a linked proline-piperidine structure, for the enzyme prolyl oligopeptidase (POP) has been synthesised and demonstrated to bind covalently with the enzyme at the active site. This provides evidence that covalent inhibitors of POP do not have to be limited to structures containing 5-membered N-containing heterocyclic rings.

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Introduction

Prolyl Oligopeptidase[1] (POP; also known as Prolyl Endopeptidase) was first discovered in 1979. The serine type endopeptidase is found in the active form in the cytosol, but does not digest polypeptides containing more than 30 amino acid residues. POP cleaves peptides at the carboxyl group of proline residues, and at a much slower rate at Ala residues[2a] such specificity sharply contrasts with other serine proteases.

The POP crystal structure (Figure 1) was first determined by Fülop et al in 1998.[2a] The enzyme consists of the catalytic and regulatory β-propeller domains. The catalytic domain has an α/β hydrolase fold. Both *N*- and *C*-termini are exposed on the surface of the domain. The *N*-terminus contains two anti-parallel β-sheets and two α-helices that are linked with the non-catalytic domain. The *C*-terminal region of the catalytic domain is substantially larger and contains eight α-helices and an 8-stranded β-sheets fragment. The active site is located at the interface with the central cavity.

The disc-shaped 7-bladed β-propeller is mainly composed of anti-parallel β-strands and loops. Each blade contains 4 twisted anti-parallel β-sheets. Typically, the 'Molecular Velcro' is formed between the *N*- and *C*-termini of the domain, with the first strand located in the centre of the module. However, in POP the "Velcro" is not closed.[2b] At first, it was postulated[2a] that the first and the seventh blades may undergo conformational change to allow the diffusion of the substrate. Alternatively, the loops connecting both domains are situated within a close distance of each other and may act as a hinge.[3] This theory was supported by the crystal structure of the open form of *Sphingomonas capsulata* POP.[4] The two domains are held together in the closed form by salt bridges and other electrostatic interactions. The enzyme specificity for the unstructured oligopeptides containing up to 30 amino acid residues arises from the size of the internal cavity. Peptides possessing ordered secondary structure may not be accommodated at the active site.

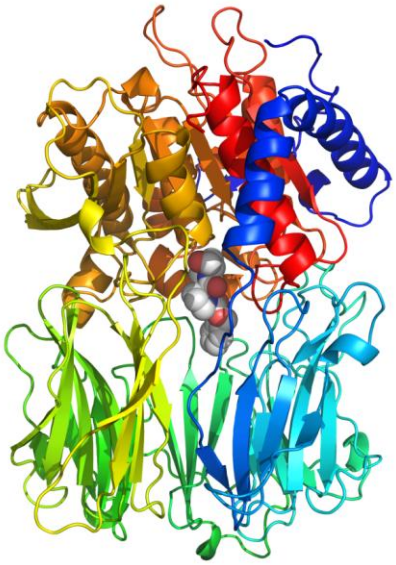


Figure 1: X-ray structure of Prolyl Oligopeptidase (POP).

The protein binds covalently to an inhibitor, Z-Pro-Prolinal **1**. The active site catalytic residues are depicted in Figure 2, which illustrates the binding of Ser554 to **1**. The mechanism of the hemiacetal formation is identical to the first step of the generic peptide hydrolysis mechanism, and the reaction is reversible. However, the tetrahedral complex does not have a good leaving group (except serine) and therefore cannot be recycled. The inhibitor also forms hydrogen bonds with other residues in the oxyanion-binding site and may not freely dissociate.

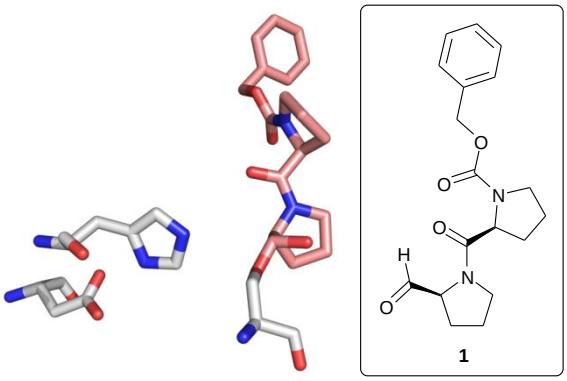
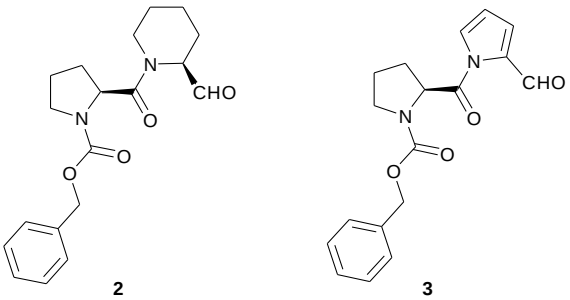


Figure 2; Location of inhibitor **1** relative to the catalytic triad.

Prolyl Oligopeptidase is involved in the maturation and degradation of some neuropeptides and peptide hormones. The enzyme has been linked with the regulation of blood pressure, various central nervous system disorders, including Alzheimer's disease, schizophrenia and depression, and parasitic illnesses, such as Chagas disease (see SI for a summary Table). The development of new potent inhibitors is therefore highly desirable, and a large number of these have been reported, as summarized in a recent review.[1] Known inhibitors which operate by a *covalent mechanism* (i.e. addition of serine OH to a functional group in the inhibitor) contain a five membered, heterocyclic ring, usually a pyrrolidine, structure. These

derivatives are close in structure to the natural substrates, which contain a proline group at the site adjacent to the position of amide cleavage. Furthermore, almost all non-covalently binding inhibitors are also based on five membered heterocyclic ring structures at the position occupied by a proline in the natural substrates.

We wished to establish whether other small molecules, related in structure to Z-Pro-Prolinal **1**, would also act as inhibitors. In particular, we selected compounds **2**, with a piperidine rather than pyrrolidine ring, and **3**, with an aromatic ring bearing the aldehyde (Figure 3). These compounds feature the same conserved Cbz-proline tail but would contain a different aldehyde module, hence offering the opportunity to retain some of the stabilising interactions present in **1**. Inhibition of the enzyme by either **2** or **3** would open a route to several further avenues for inhibitor development. Molecule **3** was of particular interest since, if it acted as an inhibitor, it would demonstrate for the first time that it is not essential to use 5-membered heterocyclic rings, in covalently-bond POP inhibitors.

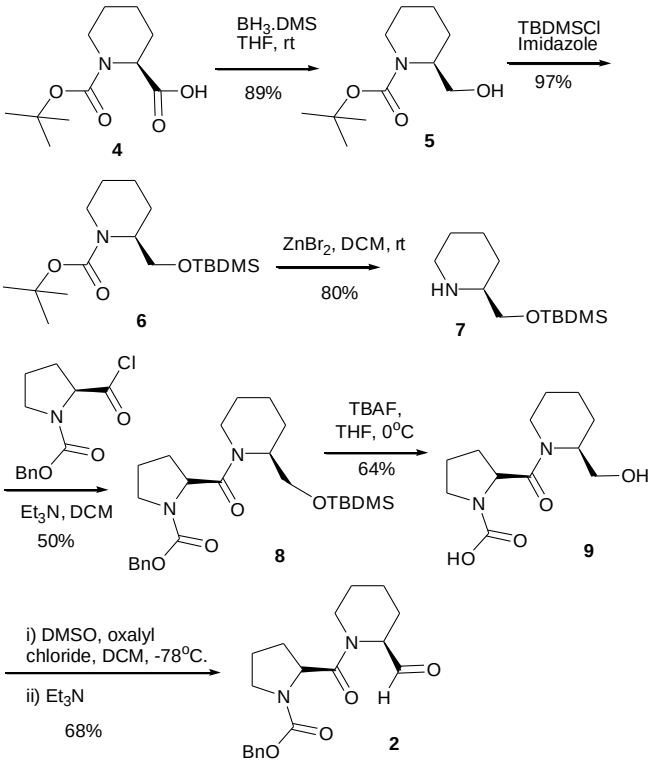


2 3

Figure 3: Potential inhibitors of POP.

Results and Discussion.

Our initial approach to **2** required conversion of carboxylic acid **4** to a protected aldehyde. Reduction of **4** was carried out with $BH_3 \cdot DMS$ complex in THF to give **5** in high yield.[9] Alcohol **5** was oxidised to an aldehyde using Swern conditions in 66% yield. Attempted protection of the aldehyde as an acetal failed to provide any protected product.[10,11] At this point a different route was required. The alcohol of **5** was protected with a TBDMS group to give silyl ether **6**.[12,13] Imidazole was used in the reaction as a catalyst to activate TBDMS-Cl. A small volume of DMF was required to solubilise all the reaction components.



4 5 6 7 8 9 2

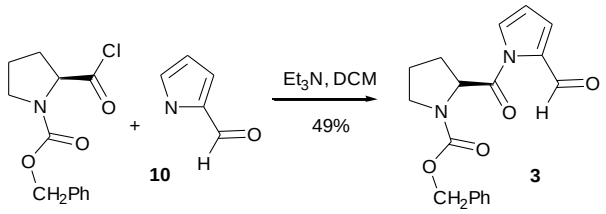
Scheme 1. Synthesis of inhibitor **2**.

Removal of the Boc protecting group was required before the peptide coupling step. Trifluoroacetic acid is commonly used to remove Boc groups.[14] However, upon treatment of **6** with TFA, only a complex mixture of products was obtained. Analytical data suggested that the TBDMS group had been affected during the reaction. Alternative methods for the removal of Boc protecting groups in the presence of acid labile moieties have been described.[15-19] Following some investigations, zinc bromide ($ZnBr_2$) in DCM in the presence of a catalytic amount of H_2O provided amine **7** in 80% yield.

The key step of the synthetic route was the formation of the peptide bond. The reaction was first attempted on amine **7** using equimolar amounts of cbz-proline and slight excess of both DCC and DMAP[20,21] in DCM under an argon atmosphere. This approach was partially successful, producing desired product **8**, albeit in only 15% yield. EDCI.HCl and HOBt[22] were also tried in the coupling reaction, but produced only trace amounts of the desired product. Ultimately, Cbz-proline was treated with thionyl chloride[23] to produce the acid chloride which was then reacted with amine **7** in the presence of triethylamine in DCM to afford amide **8** in 50% yield. Compound **8** was then treated with TBAF in THF remove the TBDMS group,[24] generating alcohol **9** in 64% yield. Swern oxidation of **9** then produced the desired aldehyde product **2** in 68% yield.

The main building blocks of inhibitor **3** were Cbz-proline and pyrrole-2-carboxaldehyde **10**. To prevent any

possible side reactions, an attempt was made to protect the aldehyde group in **10** as an acetal.[10] As before, this failed to provide any of the desired product. There are reports in the literature of pyrrole-2-carboxaldehyde **4** reactions forming porphyrin-related structures, and this is likely to have been the outcome here.[25] An attempt was made to directly couple aldehyde **10** with Cbz-proline,[26] but no product was observed. Cbz-proline was therefore activated with thionyl chloride as before (Scheme 2) and a solution of pyrrole-2-carboxaldehyde **10** and triethylamine in DCM was added.[27]



Scheme 2. Synthesis of (*S*)-benzyl 2-(2-formyl-1H-pyrrole-1-carbonyl)pyrrolidine-1-carboxylate **3**.

The desired product **3** was isolated from this reaction in a moderate yield (49%) after purification on silica, however it proved to be unstable on standing either at rt or -70°C, which hindered accurate binding studies with POP.

Crystallisation studies.

Porcine Prolyl Oligopeptidase (POP) was co-crystallized with inhibitor **2** using the hanging drop-vapour equilibrium method.[28] Full details, and a typical diffraction image, are given in the Supporting Information. All data were indexed, integrated and scaled using the CCP4[29] programs suite. The model was refined against the crystal structure of POP with Z-Pro-prolinal **1** inhibitor (1QFS.pdb) using Refmac.[30] The data collection and processing statistics of a typical crystal are shown in Table 1. The crystal data information has been deposited with the RCSB Protein Data Bank (PDB) and assigned the code **XXXX**.

Table 1. Data collection and processing statistics. Values in parentheses refer to the highest resolution shell (1.42-1.35 Å).

Synchrotron radiation	Diamond
Space group	$P2_12_12_1$
Unit cell parameters	
Molecules per AU	1
Solvent convent by (%)	48
Resolution range (Å)	51.20-1.35
Total observations	796701
Unique reflections	168680
R_{merge} (%)[a]	0.080 (0.648)
Average $I/\sigma(I)$[b]	10.4 (2.1)
Completeness (%)	98.5 (99.5)
R-factor (%)[c]	16.4
R_{free} (%)[d]	18.6

[a] $R_{merge} = \Sigma_i\Sigma_h |I_{h,i} - \langle I_h \rangle| / \Sigma_i\Sigma_h \langle I_h \rangle \times 100$, where $I_{h,i}$ is the j^{th} observation of reflection h, and $\langle I_h \rangle$ is the mean intensity of that reflection.

[b] $I/\sigma(I)$, average of the diffraction intensities, divided by their standard deviations.

[c] *R-factor* $= \Sigma_{hkl} ||F_{obs}| - |F_{calc}|| / \Sigma_{hkl} |F_{obs}|$, where F are structure factors

[d] $R_{free} = \Sigma_{hkl\ \varepsilon T} ||F_{obs}| - |F_{calc}|| / \Sigma_{hkl\ \varepsilon T} |F_{obs}|$, where the test set (4% of the data) is omitted from the refinement in such a way that all structure factors in each of several thin resolution shells were selected

The model of the structure of the POP-**2** complex is shown in Figure 4. β-Propeller and α/β hydrolase catalytic domains are well resolved and in agreement with the previously recorded structures. The β-propeller has 7 blades and the molecular "Velcro" in not closed. The active site of the catalytic domain is buried in the cavity at the interface of the two domains. The inhibitor **2** molecule is in the centre cavity. Thre are no sufficiently large holes in the enzyme that would allow diffusion of the substrate peptides. Therefore Prolyl Oligopeptidase must undergo significant conformational changes to bind the substrate.

The cross-section of the model reveals more features of the active site (Figure 5); the key residues of the catalytic triad are shown as sticks. The Ser554 (cyan) is bound to **2**, while His680 (green) and Asp641 (blue) are in close proximity. The solvent molecules are not displayed. The model (Figure 6) confirmed the structure of **2**. The bond lengths of the hemiacetal group were restrained to their target values (C1-C2 = 1.55Å; C1-O1 = 1.43Å; C1-O-Ser554 = 1.35Å). The hemiacetal group is in the axial position of piperidine ring to accommodate **2** in the active site. The conformation is also enforced by stacking with the aromatic Trp595 indole ring. Tryptophan is important for the specificity of POP for substrates with proline residues. The piperidine ring is similarly sized and fits well into the hydrophobic pocket.

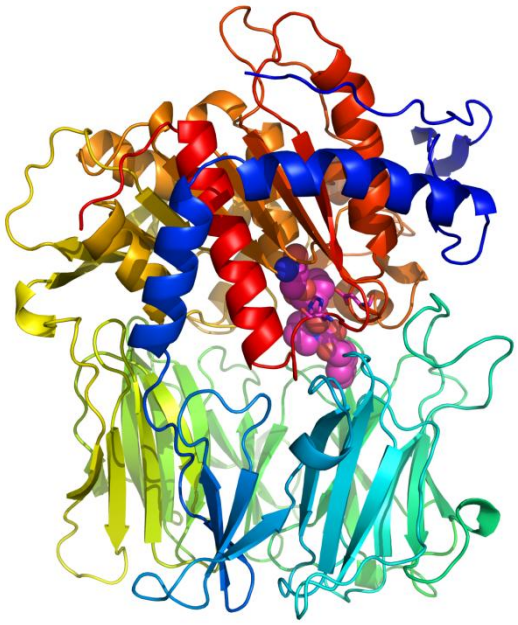


Figure 4. X-Ray crystal structure model of Prolyl Oligopeptidase with bound inhibitor **2** (pink).

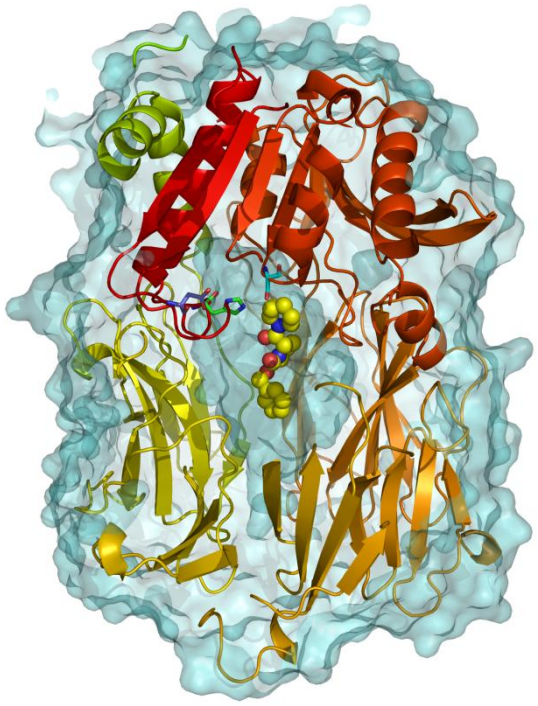


Figure 5. Cross-section of Prolyl Oligopeptidase crystal structure model showing the active site with the bound inhibitor **2** molecule (yellow); the surface of the protein is shown in light blue. Figures drawn with PyMol.[31]

His680 is in close proximity to Ser554 and involved in H-bonding interaction with Asp641. The "oxyanion hole" is formed with Tyr473 residue side chain and the main chain NH group of Asn555. Both groups form hydrogen-bonds with O1 atom of **2**. The binding of the substrate **2** is further stabilised with two hydrogen-bonds between O2 and O3 with side chain NH groups of Arg643 and Trp595, respectively. Although the hemiacetal group is labile, the substrate remains bound in the active and can reform the covalent bond.

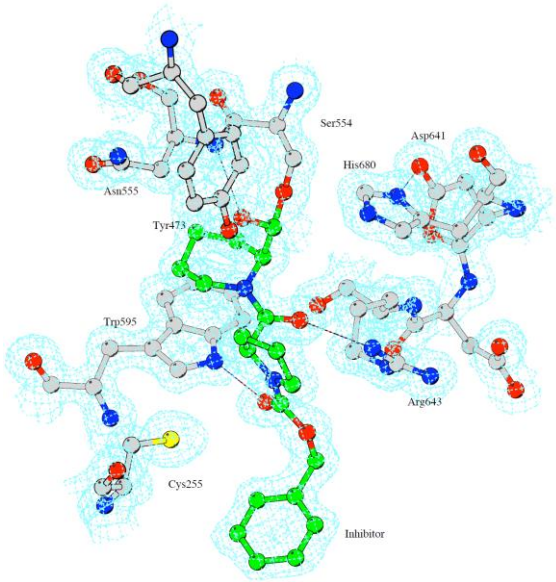
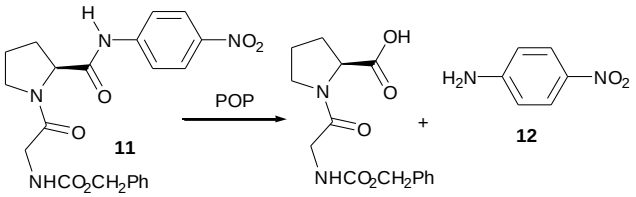


Figure 6. Electron density map and the model of the active site residues of Prolyl Oligopeptidase with covalently bound (S)-benzyl 2-((S)-2-formylpiperidine-1-carbonyl)pyrrolidine-1-carboxylate 2 (green). The SIGMAA weighted 2mFo - ΔFc electron density[32] is contoured at the 1.5σ level, where σ represents the rms electron density for the unit cell. Contours more than 1.4 Å from any of the displayed atoms have been removed for clarity. Figure drawn with MolScript.[33]

Inhibition Assays

Z-Gly-*L*-Pro-4-nitroanilide **11** is a synthetic peptide that can be used for the kinetic activity assays of peptidases (Scheme 3). 4-Nitroaniline **12** is one of the products released from the enzymatic cleavage reaction. Compound **12** strongly absorbs visible light at 410 nm, which allowed collecting the measurements with the UV/VIS spectrophotometer.



Scheme 3. Cbz-Gly-*L*-Pro-4-nitroanilide **11** is cleaved by Prolyl Oligopeptidase. 4-nitroaniline **12** is released in to solution and absorbs strongly at 410 nm (appears yellow).

The optimum concentration of **11** was determined by the kinetic digestion assays (see Supporting Information). The saturation of POP (176 µg/ml) was reached at around 90 µM concentration of the synthetic peptide **12**. K_M = 27 µM was the concentration of the substrate **12** at which the reaction proceeded at the half-maximal velocity. The IC_{50} of **3** could not be precisely determined because it had degraded over 3 weeks (50% purity was determined by ^{1}H NMR). The preliminary measurements indicated the IC_{50} value could be in a range of 10-100 µM. The low activity could be associated with the conjugation of the aldehyde group with the aromatic pyrrole ring. The assays also suggested that the activity of POP was adversely affected by DMSO. However, DMSO was necessary to dissolve organic compounds including **2, 3** and **11**.

Inhibitor **2** was stable and kinetic measurement data could be collected. The Prolyl Oligopeptidase (176 µg/ml) activity was measured with Z-Gly-Pro-4-nitroanilide **11** as the substrate. IC_{50} values were measured by following the residual activity of the enzyme using 100 µM substrate **11** with various concentrations of compound **2** (Figure 7).

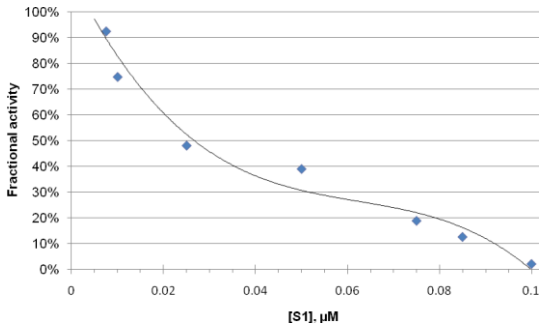


Figure 7. Kinetic measurements of the inhibition of Prolyl Oligopeptidase with **2**.

Compound **2** was a very potent inhibitor ($IC_{50} \approx 26$ nM); however its activity was slightly lower when compared with Z-Pro-prolinal **1**. $K_i = IC_{50}/(1 + [\mathbf{11}]/K_m) = 4.3$ nM .

Conclusions.

In conclusion, two potential POP inhibitors, **2** and **3**, have been synthesised, isolated and characterised. Compound **2** was co-crystallised with Prolyl Oligopeptidase using the hanging drop-vapour equilibrium method. The X-ray crystal structure was successfully determined at 1.35Å maximum resolution. The crystal structure model revealed that inhibitor **2** covalently bound to the Ser554 residue in the active site. This provides direct evidence that POP inhibitor structures need not be limited to five-membered N-containing rings at the position where covalent attachment to an the enzyme takes place. The enzyme inhibition assays revealed that **2** is a potent inhibitor (IC_{50} = 26 nM). This confirms that piperidine ring-containing compounds also act as effective substrate mimics for the POP enzyme, thus opening up new possibilites for the development of new synthetic inhibitors for POP based on alternative ring structures. Compound **3** degraded before it could be co-crystallised with POP. Preliminary kinetics measurements however indicated a low inhibitory potential of **3**.

Experimental

All reactions were carried out under ambient conditions unless otherwise stated in the method. Anhydrous grade solvents were supplied commercially in sealed bottles, while DMSO and triethylamine were distilled. Unless otherwise stated, reactions were monitored by NMR and thin layer chromatography using aluminum-backed silica gel 60 (F_{254}) plates, visualizing with a $KMnO_4$ stain. 60 Å silica gel (Merck) was used for flash column chromatography. NMR spectra were recorded on a Bruker DPX-400 (^{1}H 400 MHz, ^{13}C 100 MHz) and Bruker AVII-700 (^{1}H 700 MHz, ^{13}C 176 MHz) spectrometers unless otherwise specified. Chemical shifts are reported in ppm on the $\boldsymbol{\delta}$ scale standardized against TMS, where $\boldsymbol{\delta}_{\mathbf{TMS}} = 0$ ppm. Coupling constants are measured in Hz. IR spectra were recorded on a Perkin-Elmer Spectrum One FT-IR spectrometer. Low resolution mass spectra were recorded on a Bruker Esquire 2000 mass spectrometer. HR-MS were recorded on Bruker MicroTOF mass spectrometer. Melting points were recorded on a Stuart Scientific SMP 1 instrument and optical rotations on a Perkin-Elmer 241 polarimeter. The kinetics data was recorded with Varian Cary 100 Bio UV/Vis spectrophotometer. The X-ray crystallographic data was acquired on the beamline IO4 at the DIAMOND synchrotron using an ADSC CCD Q315 detector.

(S)-tert-Butyl 2-(hydroxymethyl)piperidine-1-carboxylate ***5***. A solution of (*S*)-(-)-*N*-Boc-carbonyl-2-piperidinine carboxylic acid **4** (2.3 g, 10 mmol, 1.0 equiv) in THF (15 mL) was stirred under a nitrogen atmosphere and cooled to 0 °C, at which point 2M BH_3 DMS in THF (10 mL, 10 mmol, 2.0 equiv) was carefully added over 5 minutes. The reaction mixture was kept at this temperature for additional 5 h, and then left to warm up overnight. The reaction was confirmed to be complete by TLC analysis. Water (40 mL) was slowly added to quench the reaction. Ethyl acetate (220 mL) was added and the mixture was separated. The organic layer was washed with saturated aqueous solutions of NaCl (40 mL), $NaHCO_3$ (40 mL), distilled water (2x 40 mL) and finally brine (40 mL). The organic phase was dried over $MgSO_4$ and was concentrated in vacuo to yield crude alcohol **5** (1.92 g, 8.9 mmol, 89%). Product **5** was used in the next step without purification. White solid (1.917 g, 8.91 mmol, 89%); Mp 80-81 °C; $[\alpha]_D^{23}$ -39.3 (*c* 2.0 in $CHCl_3$) (S), (lit.[34] $[\alpha]_D^{20}$ -31.2 (c 2.00 in $CHCl_3$) (S)); (Found: C, 61.14; H, 9.73; N, 6.51. $C_{11}H_{21}NO_3$ requires C, 61.37; H, 9.83; N, 6.51%); (Found: (ESI+) 238.1410 (M + Na^+). $C_{11}H_{21}NNaO_3$ requires 238.1414); $\boldsymbol{\nu}_{\mathbf{max}}$ 3708 (amide N-H str), 3441 (O-H str), 2940 (C-H str), 2868, 1652 (C=O str), 1414, 1365, 1278, 1048, 871, and 772 cm^{-1}; $\boldsymbol{\delta}_{\mathbf{H}}$ (400 MHz; $CDCl_3$; Me_4Si) 4.30 (1 H, m, N-C***H***), 3.94 (1 H, br d, *J* 12.8, N-HC***H***), 3.82 (1 H, br d, *J* 10.0, –HC***H***-O), 3.61 (1 H, dd, *J* 5.8, 10.9, -HC***H***-O), 2.87 (1 H, br dd, *J* 11.5, N-HC***H***), 2.17 (1 H, br s, -O***H***), 1.72-1.35 (6 H, m, -(C$\boldsymbol{H}_2$)$_3$-) and 1.46 (9 H, s, -C(C$\boldsymbol{H}_3$)$_3$); $\boldsymbol{\delta}_{\mathbf{C}}$ (100 MHz; $CDCl_3$; Me_4Si) 79.86 (O***C***Me_3), 61.81 (-***C***H_2-OH), 52.57 (N-***C***H), 40.06 (N-***C***H_2), 28.47 (3x ***C***H_3), 25.31 (***C***H_2), 25.26 (***C***H_2) and 19.68 (***C***H_2); *m/z* (ESI+) 238 ($M+Na^+$, 100%), 182 (M^+-^{t}Bu + Na^+, 15) and 116 (M^+-BOC, 7). Data is consistent with that reported in the literature[34,35]

(S)-tert-Butyl 2-formylpiperidine-1-carboxylate. To a stirred 2 M solution of oxalyl chloride (10.15 mL, 20.3 mmol, 2.9 equiv) in anhydrous DCM at -78°C was added dropwise a solution of anhydrous DMSO (2.55 mL, 35 mmol, 5.0 equiv) in anhydrous DCM (7.5 mL). After 5 min a solution of (*S*)-*tert*-butyl 2-(hydroxymethyl)piperidine-1-carboxylate **5** (1.5 g, 7 mmol, 1.0 equiv) in anhydrous DCM (7.5 mL) was added dropwise. Triethylamine (8.5 mL, 61 mmol, 8.7 equiv) was added 30 min later. The colourless reaction mixture instantly became yellow. The reaction was allowed to warm up to room temperature. The mixture was then poured into distilled water (40 mL), and the organic layer was separated. The aqueous layer was extracted with dichloromethane (4x 25 mL). The organic fractions were combined and washed with brine (25 mL), saturated $NaHCO_3$ aqueous solution (25 mL), and distilled water (25 mL). The organic layer was dried over magnesium sulphate and concentrated in vacuo to give the

product as a brown oily residue(1.40 g 93% crude). The crude oil was purified on silica using hexane : ethyl acetate (50 : 50) eluent system (R_f = 0.233 with 10 : 90 hexane : ethyl acetate). The compound was isolated as a yellow oil (0.984 mg, 4.61 mmol, 66%, pure). A small amount of starting material **5** was recovered.; $[\alpha]_D^{23}$ -37.7 (*c* 1.48 in $CHCl_3$) (S), (lit.[36] $[\alpha]_D^{20}$ -77.9 (c 1.49 in $CHCl_3$) (S)); (Found: (ESI+) 236.1259 (M + Na^+). $C_{11}H_{19}NNaO_3$ requires 236.1257); $\boldsymbol{\nu}_{max}$ 2975, 2940 (C-H str), 2861, 1733, 1686 (C=O str), 1447, 1392, 1365, 1275, 1249, 1160, 1044, 870, and 770 cm^{-1}; $\boldsymbol{\delta}_H$ (400 MHz; $CDCl_3$; Me_4Si, rotamers) 9.59 (1 H, s, -CO***H***), 4.56 (1 H, m, N-C***H***), 3.95 (1 H, m, N-HC***H***), 2.92 (1 H, m, N-HC***H***), 2.16 (1 H, m, N-CH-HC***H***), 1.50-1.73 (4 H, m, alkyl-***H***), 1.47 (9 H, s, -C(C***H***$_3$)$_3$) and 1.15-1.35 (1 H, m, alkyl-***H***); $\boldsymbol{\delta}_C$ (100 MHz; $CDCl_3$; Me_4Si, rotamers) 201.34 (***C***OH), 80.42 (O***C***Me$_3$), 60.76, 28.26 (3x ***C***H$_3$), 24.73 (***C***H$_2$), 23.64 (***C***H$_2$) and 20.96 (***C***H$_2$); *m*/*z* (ESI+) 236 (M+Na^+, 100%), 180 ([M+Na]$^+$-^{t}Bu, 48) and 114 (M^+-BOC, 69). Data is consistent with that reported in the literature[35,37]

(S)-tert-Butyl-2-((tert-butyldimethylsilyloxy)methyl-)piperidine-1-carboxylate ***6.*** (*S*)-*tert*-Butyl 2-(hydroxymethyl)piperidine-1-carboxylate **5** (1.00 g, 4.64 mmol, 1.0 equiv) was treated with imidazole (0.95 g, 13.9 mmol, 3.0 equiv) and TBDMS-Cl (1.04 g, 6.96 mmol, 1.5 equiv) in a mixture of DCM (60 mL) and dry DMF (17 mL) at room temperature. After 36 hours the reaction was confirmed complete by TLC analysis. Diethyl ether (90 mL) was added, and the precipitate was filtered off. The filtrate was concentrated in vacuo to a crude oily residue **6**. The product was purified on silica using gradient hexane : ethyl acetate eluent system to give **6** (1.48 g, 4.48 mmol, 97%) as a colourless oil; $[\alpha]_D^{23}$ -35.8 (*c* 0.60 in $CHCl_3$) (S), (lit.[38] $[\alpha]_D^{20}$ +34.9 (c 0.59 in $CHCl_3$) (R)); (Found: (ESI+) 352.2276 (M + Na^+). $C_{17}H_{35}NNaO_3Si$ requires 352.2278)); $\boldsymbol{\nu}_{max}$ 2930, 2857, 1692, 1408, 1364, 1251, 1140, 1090, 834, 774 cm^{-1}; $\boldsymbol{\delta}_H$ (400 MHz; $CDCl_3$; Me_4Si) 4.17 (1 H, br, -N-C***H***-), 3.98 (1 H, br d, *J* 11.5, -N-HC***H***-), 3.68 (1 H, dd, *J* 9.2, -HC***H***-O-), 3.58 (1 H, dd, *J* 6.0, 9.7, -HC***H***-O-), 2.73 (1 H, br dd, *J* 12.6, -N-HC***H***-), 1.85 (1 H, br d, *J* 7.5), 1.69-1.25 (5 H, m, alkyl-***H***), 1.45 (9 H, s, BOC -C***H*****$_3$**), 0.88 (9 H, s, TBDMS -(C***H***$_3$)$_3$) and 0.05 (6 H, s, 2x -Si-C***H***$_3$); $\boldsymbol{\delta}_C$ (100 MHz; $CDCl_3$; Me_4Si) 155.23 (-***C***=O), 79.18 (-O-***C***Me$_3$), 60.85 (-***C***H$_2$-O-), 51.84 (-N-***C***H-), 51.73, 40.27 (-N-***C***H$_2$-), 40.01, 28.53 (BOC -***C***H$_3$), 25.94 (TBDMS -(***C***H$_3$)$_3$), 25.36 (***C***H$_2$), 24.49 (***C***H$_2$), 19.16 (***C***H$_2$), 18.28 (-Si-***C***Me$_3$), -5.30 (-Si-***C***H$_3$) and -5.36 (-Si-***C***H$_3$); *m*/*z* (ESI+) 352 (M+Na^+, 100%).

(S)-2-((tert-Butyldimethylsilyloxy)methyl)piperidine ***7.*** To a dry round bottomed flask were charged (*S*)-*tert*-butyl 2-((*tert*-butyldimethylsilyloxy)methyl)piperidine-1-carboxylate **6** (600 mg, 1.82 mmol, 1.0 equiv), zinc bromide (2.06 g, 9.12 mmol, 5.0 equiv) and DCM (13 mL). The reaction was stirred at ambient conditions overnight. The reaction mixture was diluted with diethyl ether (150 mL) and sat. $NaHCO_3$ aq. sol. (150 mL) was added. The organic phase was separated, and the aqueous layer was extracted with further diethyl ether portions (3x 100 mL). The combined organic phases were dried over $MgSO_4$ and concentrated under reduced pressure to give **7** (333 mg, 1.45 mmol, 80%) as a colourless oil. The material **7** was used in the next step without purification. Colourless oil (333 mg, 1.45 mmol, 80%); $[\alpha]_D^{22}$ +3.59 (*c* 1.48 in $CHCl_3$) (*S*); (Found: (ESI+) 230.1940 (M + H^+). $C_{12}H_{28}NOSi$ requires 230.1935)); $\boldsymbol{\nu}_{max}$ 2978, 2857, 1472, 1463, 1446, 1330, 1253, 1087, 1075, 832, 774 cm^{-1}; $\boldsymbol{\delta}_H$ (400 MHz; $CDCl_3$; Me_4Si) 3.52 (1 H, dd, *J* 3.8, 9.6, -O-HC***H***-), 3.38 (1 H, dd, *J* 8.9, 8.9, -O-HC***H***-), 3.06 (1 H, br d, *J* 11.5, -N-HC***H***-), 2.61 (1 H, ddd, *J* 2.6, 11.5, 11.6, -N-HC***H***-), 2.54-2.62 (1 H, m, -N-C***H***-), 2.13 (1 H, br s, N***H***), 1.77 (1 H, br d, *J* 12.5, -N(CH$_2$)$_2$-HC***H***-), 1.58 (1 H, br d, *J* 12.8, -NCH$_2$-HC***H***-), 1.49 (1 H, m, -NCH-HC***H***-), 1.23-1.46 (2 H, m), 1.06 (1 H, dddd, *J* 3.8, 12.3, 12.3, 12.3, -NCH-HC***H***-), 0.88 (9 H, s, TBDMS (C***H***$_3$)$_3$) and 0.03 (6 H, s, 2x -Si-C***H***$_3$); $\boldsymbol{\delta}_C$ (100 MHz; $CDCl_3$; Me_4Si) 67.93 (-***C***H$_2$-O-), 58.32 (-N-***C***H-), 46.67 (-N-***C***H$_2$-), 28.47 (-NCH-***C***H$_2$-), 26.47 (-NCH$_2$-***C***H$_2$-), 25.95 (TBDMS (-***C***H$_3$)$_3$), 24.46 (-N(CH$_2$)$_2$-***C***H$_2$-), 18.32 (-Si-***C***Me$_3$), -5.37 (-Si-***C***H$_3$); *m*/*z* (ESI+) 230 (M+H^+, 100%).

(S)-Benzyl-2-((S)-2-((tert-butyldimethylsilyloxy)methyl)-piperidine-1-carbonyl)pyrrolidine-1-carboxylate **8.** Coupling method A. To a dry, clean round bottomed flask were added cbz-proline (166 mg, 0.668 mmol, 1.0 equiv), HOBt (115 mg, 0.741 mmol, 1.11 equiv), EDCI HCl (157 mg, 0.800 mmol, 1.0 equiv), DMF (0.06 mL) and DCM (1.5 mL). The mixture was degassed, purged with argon, and stirred for 10 min. (S)-2-((tert-butyldimethylsilyloxy)methyl)piperidine **7** was dissolved in DCM (6 mL) and was added to the reaction. The reaction mixture was degassed, purged with argon and stirred at ambient conditions. The progress was monitored by ESI+ MS, and TLC. After 3 days, the desired product **10** was detected in the mixture, however the major component was still the starting amine **7**. Triethylamine (0.07 mL) was added. A day later the reaction composition was unchanged; another 0.5 equiv of HOBt, and EDCI, and 1 mL of triethylamine were charged. A day later NMM (1 mL) was added. After 4 days the reaction did not progress any further. The material was combined with another reaction mixture (NMM was used instead of DMF, no Et_3N, and 1.2 equiv of EDCI), poured into water and extracted with DCM. The organic layer was washed twice with citric acid aq. solution, twice with $NaHCO_3$ aqueous solution, brine, and dried over $MgSO_4$. The organic phase was concentrated in vacuo to yellowish oil (0.247 g), which was purified on silica using gradient hexane : ethyl acetate eluent system to give **8** (0.128 g, 0.277 mmol, 28%) as a clear colourless oil; $[\alpha]_D^{25}$ -30.45 (c 0.56 in $CHCl_3$) (SS); (Found: (ESI+) 483.2636 (M + Na^+). $C_{25}H_{40}N_2NaO_4Si$ requires 483.2650); $\boldsymbol{\nu}_{max}$ 2928, 2855 (C-H str), 1704, 1649 (C=O str), 1498, 1412, 1350, 1248, 1210, 1171, 1103, 1024, 872, 775 and 696 cm^{-1}; $\boldsymbol{\delta}_H$ (700 MHz; $CDCl_3$; Me_4Si, rotamers) 7.25-7.40 (5 H, m, Ph-C**H**), 4.91-5.25 (2 H, m, -C(=O)OC**H$_2$**-), 4.49-4.80 (2 H, m, Pro–N-C**H**-, Pip—N-C**H**- (1 conformer), -N-HC**H**- (1 conformer)), 3.49-3.93 (4 H, m, -C**H$_2$**-OSi- (1½ H), Pip—N-C**H**- (1 conformer), -N-C**H$_2$**- (1½ H), -N-HC**H**- (1 conformer)), 3.04-3.22 (1 H, m, -N-HC**H**- (1 conformer), -C**H$_2$**-OSi- (½ H)), 2.61 (½ H, ddd, J 13.4, 13.4, 2.7, -N-HC**H**- (1 conformer)), 2.43 (½ H,

ddd, J 13.4, 13.4, 2.7, -N-HC**H**- (1 conformer)), 1.12-2.30 (10 H, m, alkyl-**H**), 0.91, 0.89, 0.88 and 0.83 (9 H, 4x s, TBDMS (C**H**$_3$)$_3$) and -0.09-0.10 (6 H, m, 2x -Si-C**H**$_3$); **δ$_C$** (176 MHz; $CDCl_3$; Me_4Si, rotamers) 171.08, 171.06, 170.72 and 170.63 (-N**C**(=O)CH-), 154.88, 154.77, 154.41 and 154.37 (-N**C**(=O)O-), 136.97, 136.81 and 136.73 (Ph-**C1**), 128.46, 128.37, 128.27, 128.25, 128.15, 127.92, 127.87, 127.76 and 127.35 (Ph-**C2,3,4,5,6**), 67.06, 66.95, 66.77 and 66.73 (-C(=O)O**C**H$_2$-), 61.51, 61.35, 60.27 and 59.83 (-SiO-**C**H$_2$-), 57.48, 57.33, 57.13 and 56.86 (-N**C**H-), 53.64, 53.50, 49.64 and 49.60 (-N**C**H-), 47.20, 47.10, 46.57 and 46.56 (-N**C**H$_2$-), 42.16 and 41.78, 38.44 and 38.35 (-N**C**H$_2$-), 30.94, 30.45, 30.07 and 29.49 (-**C**H$_2$-), 25.91, 25.87, 25.84 and 25.80 (TBDMS (-**C**H$_3$)$_3$), 25.58, 25.40, 25.13 and 25.04 (-**C**H$_2$-), 24.67, 24.63, 24.52 and 24.46 (-**C**H$_2$-), 24.11, 24.00, 23.35 and 23.25 (-**C**H$_2$-), 19.49, 19.42, 18.89 and 18.73 (-**C**H$_2$-), 18.23, 18.20 and 18.12 (-Si-**C**Me$_3$), -5.23, -5.42, -5.47 and -5.57 (-Si-**C**H$_3$); m/z (ESI+) 483 (M+Na$^+$, 48%), 461 (M+H$^+$, 100) and 329 (M$^+$-OTBDMS, 6).

(S)-Benzyl-2-((S)-2-((tert-butyldimethylsilyloxy)methyl)-piperidine-1-carbonyl)pyrrolidine-1-carboxylate ***8***. Coupling method B. To a dry, clean round-bottomed flask were added cbz-proline (196 mg, 0.758 mmol, 1.0 equiv), dry toluene (3 mL), and thionyl chloride (0.10 mL, 1.36 mmol, 1.8 equiv). The reaction mixture was degassed and purged with argon two times. The reaction was stirred under reflux conditions under nitrogen atmosphere for 1.5 h. [A colour change was noted shortly after heating began when the mixture became yellow]. The reaction was allowed to cool down to room temperature, and the reaction mixture was concentrated to dark yellow oil in vacuo. In a separate flask a solution of (S)-2-((tert-butyldimethylsilyloxy)-methyl)piperidine **7** (178 mg, 0.78 mmol, 1.0 equiv), and triethylamine (0.33 mL, 2.36 mmol, 3.0 equiv) was prepared in DCM (2 mL). The mixture was carefully added to the acyl chloride. The resulting mixture was degassed and purged with argon. The reaction was stirred for 1 d under argon atmosphere. The mixture was poured into 0.1 M HCl aq. (30 mL). DCM (20 mL) was added and phases were separated. The aqueous layer was extracted with another portion of DCM (20 mL). The organic fractions were combined and washed successively with distilled water (20 mL), brine (25 mL), sat. Na_2CO_3 aq. sol. (2x 30 mL), brine (25 mL), and dried over $MgSO_4$. The organic layer was concentrated in vacuo to crude oil **8** (213 mg). The oil was separated on silica using hexane : ethyl acetate (100 : 0 to 60 : 40) gradient eluent system. The desired product was isolated as colourless oil **8** (175 mg, 0.38 mmol, 50%); $[\alpha]_D^{25}$ -25.38 (c 0.47 in $CHCl_3$) (SS); matching NMR data described in previous section.

(S)-Benzyl-2-((S)-2-(hydroxymethyl)piperidine-1-carbonyl)pyrrolidine-1-carboxylate ***9.*** (*S*)-Benzyl 2-((*S*)-2-((*tert*-butyldimethylsilyloxy)methyl)piperidine-1-carbonyl)-pyrrolidine-1-carboxylate **8** (92 mg, 0.2 mmol, 1.0 equiv) was dissolved in THF (1.2 mL), and cooled in an ice bath. 1 M TBAF solution in THF (0.40 mL, 0.40 mmol, 2.0 equiv) was carefully added, while the reaction was stirred at +4 °C. After 2 h distilled the reaction was complete and water was added to quench the reaction. The suspension was extracted with DCM, and diethyl ether. The organic layer was washed with brine, dried over $MgSO_4$ and was concentrated in vacuo (64 mg). The material **9** was purified on silica using hexane : ethyl acetate eluent system to give the desired product **9** (44 mg, 0.127 mmol, 64%); $[\alpha]_D^{28}$ -34.0 (*c* 0.97 in $CHCl_3$) (*SS*); (Found: (ESI+) 347.1964 (M + Na$^+$) and 369.1795 (M + H$^+$). $C_{19}H_{27}N_2O_4$ requires 347.1965; $C_{19}H_{27}N_2NaO_4$ requires 369.1785); ***ν*$_{max}$** 3413 (O-H str), 2939, 2873 (C-H str), 1697, 1631 (C=O str), 1415, 1352, 1254, 1209, 1167, 1120, 1021, 919, 728 and 697 cm^{-1}; ***δ*$_H$** (700 MHz; $CDCl_3$; Me_4Si, rotamers) 7.19-7.32 (5 H, m, Ph-C***H***), 4.91-5.20 (2 H, m, -C(=O)OC***H*$_2$**-), 4.45-4.91 (2 H, m, -N-C***H***- (½ H), -N-HC***H***- (½ H)), 4.13 (½ H, br s, -N-C***H***- (1 conformer)), 4.06 (½ H, dd, *J* 4.1, 9.9, -HC***H***-OH (1 conformer)), 3.41-3.86 (3½ H, m, -HC***H***-OH (1½ H), -N-HC***H***- (2 H)), 3.24 (¼ H, m, -N-HC***H***- (1 conformer)), 3.07 (¼ H, ddd, *J* 2.5, 12.9, -N-HC***H***- (1 conformer)), 3.00 (¼ H, ddd, *J* 2.5, 12.9, -N-HC***H***- (1 conformer)), 2.69 (½ H, dt, *J* 2.6, 13.4, -N-HC***H***- (1 conformer)), 2.50 (1 H, br s, -O***H***), 2.40 ((¼ H, dt, *J* 2.6, 13.4, -N-HC***H***- (1 conformer)) and 1.10-2.25 (10 H, m, alkyl-***H***); **δ$_C$** (176 MHz; $CDCl_3$; Me_4Si, rotamers) 173.15, 173.04, 172.15 and 171.81 (-N***C***(=O)CH-), 155.41, 154.97, 154.41 and 154.36 (-N***C***(=O)O-), 136.94, 136.80, 136.68 and 136.60 (Ph-***C1***), 128.67, 128.57, 128.50, 128.39, 128.35, 127.99, 127.93, 127.88 and 127.83 (Ph-***C2,3,4,5,6***), 67.34, 67.25, 67.06 and 66.94 (-C(=O)O***C***H$_2$-), 62.33, 62.21, 62.02 and 60.84 (HO-***C***H$_2$-), 57.84, 57.30, 57.20 and 56.79 (-N***C***H-), 55.53, 54.34, 51.18 and 51.08 (-N***C***H-), 47.30, 47.26, 46.99 and 46.75 (-N***C***H$_2$-), 41.56, 41.25 and 37.14 (-N***C***H$_2$-), 30.72, 30.50, 29.98 and 29.50 (-***C***H$_2$-), 27.68, 26.46, 25.45, 25.32, 25.25, 24.95, 24.83, 24.71, 24.12, 23.52, 23.40, 24.11, 24.00, 23.52 and 23.40 (3x -***C***H$_2$-) and 19.94, 19.72, 19.68 and 19.56 (-***C***H$_2$-); *m/z* (ESI+) 385 (M+K$^+$, 3%), 369 (M+Na$^+$, 100), 347 (M+H$^+$, 72) 329 (M$^+$-OH, 21), 303 (M$^+$-43, 48) and 239 (M$^+$-PhCH$_2$O, 3).

(S)-Benzyl-2-((S)-2-formylpiperidine-1-carbonyl)pyrrolidine-1-carboxylate ***2.*** To a stirred 2 M solution of oxalyl chloride (184.5 µl, 0.369 mmol) in anhydrous DCM at -78 °C was added dropwise solution anhydrous DMSO (50 µL, 0.637 mmol) in anhydrous DCM (0.10 mL). After 10 min solution of (*S*)-benzyl 2-((*S*)-2-(hydroxymethyl)piperidine-1-carbonyl)pyrrolidine-1-carboxylate **9** (44 mg, 0.127 mmol) in anhydrous DCM (0.15 mL) was added dropwise. After 3 h a low resolution MS sample was taken; m/z+1=347. Triethylamine (0.154 mL, 1.11 mmol) was added. The colourless reaction mixture instantly became yellow. The reaction was allowed to warm up to room temperature. The mixture was then poured into distilled water (5 mL), and was separated. The aqueous layer was extracted with dichloromethane (3 x 10 mL). The organic fractions were combined and washed with brine, saturated $NaHCO_3$ aqueous solution, distilled water, and brine. The organic layer was dried over magnesium sulphate and concentrated in vacuo to give yellow oily residue **2** (60 mg, crude). Low resolution MS analysis confirmed it as the desired product **2**; m/z+1=301,

345, 367. ^{1}H NMR also confirmed the formation of the product **2**. The crude oil **2** was purified on silica using gradient hexane : ethyl acetate eluent system (R_f = 0.145 with 50 : 50 hexane : ethyl acetate). The compound was isolated as a colourless oil **2** (29.7 mg, 0.086 mmol, 68%); $[\alpha]_D^{23}$ -45.2 (*c* 1.0 in $CHCl_3$) (*SS*), (Found: (ESI+) 367.1630 (M + Na^+). $C_{19}H_{24}N_2NaO_4$ requires 367.1628); $\boldsymbol{\nu}_{max}$ 2943 (C-H str), 2873, 1699, 1648 (C=O str), 1412, 1351, 1237, 1165, 1117, 764, 742, and 697 cm^{-1}; $\boldsymbol{\delta}_H$ (700 MHz; $CDCl_3$; Me_4Si, rotamers) 9.52 and 9.47 (1 H, 2x s, -CO***H***), 7.20-7.45 (5 H, m, Ph-C***H***), 4.91-5.23 (3 H, m, -C(=O)OC***H*****$_2$**, -N-C***H***-), 4.82 (½ H, dd, *J* 2.8, 8.7, -N-C***H***- (1 conformer)), 4.73 (½ H, dd, *J* 2.8, 8.7, -N-C***H***- (1 conformer)), 3.85 (½ H, d, *J* 13.3, -N-HC***H***- (1 conformer)), 3.62-3.73 (1½ H, m, -N-HC***H***- (1½ H)), 3.48-3.62 (1 H, m, -N-HC***H***-), 3.13 (½ H, dd, *J* 2.9, 12.7, -N-HC***H***- (1 conformer)), 3.04 (½ H, dd, *J* 2.9, 12.7, -N-HC***H***- (1 conformer)) and 1.11-2.29 (10 H, m, alkyl-***H***); $\boldsymbol{\delta}_C$ (176 MHz; $CDCl_3$; Me_4Si, rotamers) 202.18, 201.33, 201.08 and 200.14 (-***C***OH), 172.45, 172.35 and 171.94 (-N***C***(=O)CH-), 155.04, 154.99, 154.90 and 154.33 (-N***C***(=O)O-), 136.95 and 136.82 (Ph-***C1***), 128.52, 128.43, 128.06, 128.01, 127.95 and 127.82 (Ph-***C2,3,4,5,6***), 67.12 and 66.97 (-C(=O)O***C***H_2-), 59.43 and 59.20 (-N***C***H-), 57.41 and 56.77 (-N***C***H-), 47.25 and 46.72 (-N***C***H_2-), 44.00 and 43.80 (-N***C***H_2-), 30.59 and 29.50 (-***C***H_2-), 25.08, 25.03, 24.45, 24.11, 23.41 and 23.14 (3x -***C***H_2-) and 21.19 and 21.13 (-***C***H_2-); *m*/*z* (ESI+) 367.1 (M+Na^+, 34%), 345.2 (M+H^+, 100) and 301.2 (M^+-CH_2COH, 16).

(S)-Benzyl 2-(2-formyl-1H-pyrrole-1-carbonyl)pyrrolidine-1-carboxylate ***3.*** To a dry, clean round bottomed flask were added cbz-proline (1.05 g, 4.2 mmol, 1.0 equiv), dry toluene (10 mL), and thionyl chloride (0.55 mL, 7.56 mmol, 1.8 equiv). The reaction mixture was degassed and purged with argon two times. The reaction was stirred under reflux conditions under nitrogen atmosphere for 1.5 h. [A colour change was noted shortly after heating began when the mixture became yellow]. The reaction was allowed to cool down to room temperature, and the reaction mixture was concentrated to a dark yellow oil in vacuo. In a separate flask a solution of pyrrole-2-carboxaldehyde **10** (600 mg, 6.3 mmol, 1.5 equiv), and triethylamine (0.62 mL, 8.4 mmol, 2.0 equiv) were mixed in DCM (10 mL). The mixture was carefully added to the acyl chloride prepared in the previous step. The resulting mixture was degassed and purged with argon. The reaction was stirred for 1 d under nitrogen atmosphere. The mixture was poured into 0.1 M HCl aq. (21 mL). DCM (40 mL) was added and phases were separated. The organic fraction was washed successively with distilled water (20 mL), brine (50 mL), sat. Na_2CO_3 aq. Sol. (150 mL), distilled water (2x 100 mL), brine (100 mL), and dried over $MgSO_4$. The organic layer was concentrated in vacuo to crude dark green oil **3** (1.02 g). The oil was separated on silica using hexane : ethyl acetate (100 : 0 to 50 : 50) gradient eluent system. The desired product **3** (670 mg, 2.05 mmol, 49%) was isolated as colourless oil, which darkened and degraded over time; $[\alpha]_D^{20}$ -101.28 (*c* 1.26 in $CHCl_3$) (S); (Found: (ESI+) 349.1165 (M + Na^+). $C_{18}H_{18}N_2NaO_4$ requires 349.1159); $\boldsymbol{\nu}_{max}$ 3124, 2957, 2885 (C-H str), 1695, 1663 (C=O str), 1547, 1443, 1410, 1353, 1259, 1170, 1110, 1023, 978, 871, 773 and 695 cm^{-1}; $\boldsymbol{\delta}_H$ (400 MHz; $CDCl_3$; Me_4Si, rotamers) 10.30 and 10.11 (1 H, s, -CO***H***), 7.51 and 7.33 (1 H, br d, *J* 1.57, -NC***H***=CH-), 7.10-7.45 (5 H, m, Ph-C***H***), 7.20-7.30 (1 H, m, -NC=***C***H-), 6.42 and 6.34 (1 H, dd, *J* 3.3, NCH=C***H***), 4.98-5.25 (3 H, m, -O***C***H_2- and -NC***H***-CH=O), 3.57-3.85 (2 H, m, -NC***H*****$_2$**-), 2.45 (1 H, m, -NCH***C***H_2-), 1.95-2.23 (3 H, m, -NCH***C***H_2- and -NCH_2***C***H_2-); $\boldsymbol{\delta}_C$ (100 MHz; $CDCl_3$; Me_4Si, rotamers) 182.30 and 181.94 (-***C***OH), 171.81 and 171.57 (-N***C***(=O)CH-), 155.86 and 155.07 (-N***C***(=O)O-), 136.38, 135.93 and 135.72 (***C***-COH and Ph-***C1***), 128.53 and 128.45, 128.13, 127.90 and 127.85 (Ph-***C2,3,4,5,6***), 125.86 and 125.56 (-N***C***H=CH-), 123.28 and 122.98 (-NC=***C***H-), 113.27 and 113.22 (-NCH=***C***H-), 67.41 (-O***C***H_2-), 59.98 and 59.46 (-N***C***H-CH=O), 47.19 and 46.68 (-N***C***H_2-), 31.64 and 30.61 (-NCH***C***H_2-), 24.44 and 23.60 (-NCH_2***C***H_2-); *m*/*z* (ESI+) 349 (M+Na^+, 70%), 327 (M+H^+, 100), 309 (M^+-17, 3), 283 (17) and 255 (10). Compound **3** degraded on standing, and could not be recovered by column chromatography. The sample was stored in a flask filled with argon under -70 °C; however the colourless oil quickly degraded. The compound visually darkened and eventually formed a black gum. The change of the ^{1}H NMR spectrum of **3** after 3 weeks from isolation is shown in the Supporting Information. Compound **3** was unstable which hindered accurate studies of **3** with the POP enzyme.

Crystallisation studies. Crystals were grown using the hanging drop-vapour equilibrium method.[28] The composition of mother liquor in the reservoir was varied around 18-20% methoxy-polyethylene glycol (mPEG) 5K, 20 mM $Ca(OAc)_2$, 0.1 M TRIS pH=8.5 and 15% glycerol. Prolyl Oligopeptidase 8.8 mg/ml solution in 20 mM MES pH=6.5 was mixed with the precipitation buffer in a 2:1 ratio. The 3 µL drops of the mixture were suspended over the wells and left to equilibrate. Small crystals were formed and used as seeds. Prolyl Oligopeptidase 8.8 mg/ml solution and inhibitor **2** 4.4 mM solution in DMSO were mixed in a 10:1 ratio. The enzyme-inhibitor mixture was further diluted with the corresponding buffer solution in a 2:1 ratio. The 3 µL drops were allowed to pre-equilibrate with the reservoir solution and were seeded with the micro crystals of free Prolyl Oligopeptidase. Large crystals of the protein with **2** were formed after the drops were seeded and left to equilibrate with the reservoir solution at +19˚C over a week. Crystals were picked up from the crystallization drop using a nylon loop, transferred directly into liquid nitrogen and stored until needed for data collection. The crystals diffracted well and a data set was collected to a resolution of 1.35Å with an oscillation range of 0.5º per frame on the beamline IO4 at DIAMOND synchrotron, Oxfordshire, using an ADSC CCD detector. The X-ray crystal diffraction pattern of Prolyl Oligopeptidase with bound inhibitor **2** is shown in the Supporting Information.

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Supplementary Material

Electronic Supplementary Information (ESI) available: [Spectra of new compounds, X-ray details and inhibition testing results]. See DOI: 10.1039/b000000x/.