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Synthesis of unusual isoxazoline containing β and γ-dipeptides as potential glutamate receptor ligands

Lucia Tamborini,†a Federica Mastronardi,†a Federica Dall’Oglio,a Carlo De Micheli,a Birgitte Nielsen,b Leonardo Lo Presti,c Paola Conti,c and Andrea Pintoa

New unconventional beta and gamma dipeptides, representing conformational constrained higher homologues of glutamic acid, have been prepared and tested as new pharmacological tools to investigate the iGluR binding domain, in the attempt to identify potential selective antagonists.

Starting from the structure of the endogenous ligand L-Glu, the more used molecular manipulation approaches to obtain potent and selective ligands are the conformational rigidification and the bioisosteric substitution, in particular on the diester carboxylate (e.g., phosphonic acid, tetrazol, 3-hydroxyisoxazole/isoxazoline). In addition, homologation of the amino acidic chain is normally the strategy pursued to turn agonists into antagonists, because it prevents the closure of the clamshell like ligand binding domain (LBD), thus leaving the channel pore closed. Finally, it has to be highlighted that, whereas AMPA and KA receptor ligands are usually characterized by an S configuration at the α amino acid stereogenic center, in analogy to the natural ligands, NMDA receptors often exhibit a preference for R-configured amino acids, as in the case of the prototypical agonist NMDA.

Figure 1. Structure of representative iGluR ligands.

A simple chain homologation of glutamic acid leads to an increase of selectivity and to a switch of the pharmacological profile strictly related to the absolute configuration of the amino acidic C-α atom. Homologation of S-Glu leads to S αmino adipic acid, which selectively activates mGluR2 and mGluR6, whereas it has no effect on mGluR1, mGluR4, or mGluR5. On the other hand, while R-Glu is inactive, the R enantiomer of amino adipic acid behaves as a competitive NMDA receptor antagonist, even if with low potency. Very interestingly, bioisosteric substitution of the distal carboxylic group with a phosphonic acid group generates to potent antagonists.
selective competitive antagonists for the NMDA receptor, i.e., (R)-2-amino-5-phosphonopentanoic acid (R-AP-5). Further extension of the backbone chain length gives another potent NMDA antagonist, i.e., (R)-2-amino-5-phosphonoheptanoic acid (R-AP-7) (Figure 1). \(^8\)

On this ground, we planned the synthesis of a series of unusual isoxazoline containing dipeptides as higher homologues of glutamic acid, i.e., compounds 1a, 1b, 2a and 2b (Figure 2), in which the distal carboxylate of glutamic acid was condensed to unconventional isoxazoline-containing beta or gamma amino acids. In this way, we generated partially constrained glutamic acid homologues, of different length, possessing the suitable characteristics to be considered potential selective Glu receptor antagonists (i.e., increased chain length and conformational rigidification). Notably, whereas compounds 2a and 2b have a carboxylate function in the distal position, mimicking that of L-Glu, in compounds 1a and 1b the distal acidic group, which is one of the essential pharmacophoric groups, is represented by the 3-hydroxy-isoxazoline ring, which has already proved to behave as a γ-COOH bioisostere (Figure 2). \(^9\)

**Figure 2. Structure of the target compounds.**

**Results and discussion**

Dipeptides (−)-1a, (+)-1a, (−)-1b and (+)-1b were synthesized from the enantioselectively pure compounds (−)-3 and (+)-3, which were obtained as recently reported by us. \(^10\) Intermediates (−)-3 and (+)-3 were submitted to a nucleophilic substitution at the C-3, in the presence of benzyl alcohol and sodium hydride, to obtain the desired 3-benzoxyl-substituted intermediates (−)-4 and (+)-4, respectively. \(^10,11\) After N-Boc deprotection with a 30% trifluoroacetic acid solution in dichloromethane, the free amines were coupled with the suitable protected Boc-L-Glu-OEt or Boc-D-Glu-OEt, obtained in good yield and high purity following a literature procedure, \(^12\) using HOBt and HBTU as coupling reagents (Scheme 1).

The final dipeptides (−)-1a, (+)-1a, (−)-1b and (+)-1b were obtained after deprotection of intermediates (−)-5a, (+)-5a, (−)-5b and (+)-5b. In particular, after the hydrolysis of the amino acid ester with 1N NaOH at room temperature, the O-benzyl group was removed by catalytic hydrogenation with 5% Pd/C. Finally, treatment with a 30% trifluoroacetic acid solution in CH₂Cl₂ gave the final desired compounds. The substitution of the Br in the C-3 position with the benzyloxy group, a precursor of the desired hydroxyl function, was necessary since the direct substitution with the OH group (treatment with 1N NaOH at 60 °C) led to degradation of the dipeptide structure.

The synthesis of compounds (−)-2a, (+)-2a, (−)-2b and (+)-2b was accomplished starting from cycloducts (−)-7 or (−)-7 which were obtained through resolution of the corresponding racemic mixture (±)-7, by chiral semi-preparative HPLC. Compound (−)-7 was synthesized in a flow chemistry reactor exploiting the 1,3-dipolar cycloaddition reaction of the dipolarophile 6 with ethoxycarbonyl formonitrile oxide generated in situ by treatment of ethyl 2-chloro-2-(hydroxyimino)acetate with solid potassium carbonate, following a procedure recently reported by us (Scheme 2). \(^13\) An excellent enantiomeric separation (ee >99%) of racemic (±)-7 was achieved using a tris(2-methyl-5-chloro-phenyl)carbamoyl amine chiral stationary phase.

**Scheme 1. Reagents and conditions: a) BnOH, NaH 60% in mineral oil, dry THF; b) 30% TFA, CH₂Cl₂; c) Boc-L-Glu-OEt or Boc-D-Glu-OEt, HOBT, HBTU, DIPEA; CH₂Cl₂; d) 1N NaOH, EtOH e) H₂, 5% Pd/C, MeOH.**

**Scheme 2. a) Semi-preparative HPLC separation; chiral stationary phase: tris(2-methyl-5-chloro-phenyl)carbamoyl amine; eluent: 7:3 n-hexane/IPrOH; flow rate: 15 mL/min.**
On both the enantiomers (−)-7 and (+)-7, the N-Boc protecting group was removed under standard conditions to yield the corresponding free amines that were used for the coupling reaction with the protected L-Glu or D-Glu derivative (Scheme 3), as described above. Intermediates were finally deprotected to obtain the desired products (−)-2a, (+)-2a, (−)-2b and (+)-2b.

![Diagram of compounds](image)

**Scheme 3.** Reagents and conditions. a) 30% TFA, CH₂Cl₂; b) Boc-L-Glu-OEt or Boc-D-Glu-OEt, HOBT, HBTU, DIPEA, CH₂Cl₂; c) 1N NaOH, EtOH.

Whereas the absolute configuration of derivatives 1 was determined by the know configurations of the two building blocks (i.e., the amine and the amino acidic portion), the absolute configurations of compounds 2 had to be assigned and it was determined by X-ray analysis of the final compound (+)-2b. Despite the lack of anomalous scatterers in the unit cell, being the absolute configuration at the α amino acidic carbon known to be S, it was sufficient to determine the relative configuration of the three stereogenic centers, to unequivocally assign the absolute configuration (2S,7S,8R) to the enantiomer (+)-2b (Figure 3). Consequently, the absolute configuration to derivatives (−)-2b, (+)-2a and (−)-2a was assigned.

![Asymmetric unit of (+)-2b](image)

**Figure 3.** Asymmetric unit of (+)-2b, with the atom numbering scheme. A co-crystallized, ordered water molecule is also present. Thermal ellipsoids at RT were drawn at the 50% probability level.

All the new compounds were preliminary submitted to binding assays at native iGluRs, using rat brain synaptic membranes from male Sprague–Dawley rats. Affinities for NMDA, AMPA, and KA receptors were determined using 2 nM [³H]CGP39653, 5 nM [³H]AMPA, and 5 nM [³H]KA (Table 1).[^4]

<table>
<thead>
<tr>
<th>Compound</th>
<th>[³H]AMPA IC₅₀ (µM)</th>
<th>[³H]KAIN IC₅₀ (µM)</th>
<th>[³H]CGP39653 IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(−)-1a</td>
<td>43 [4.37]±0.01</td>
<td>37 [4.34]±0.03</td>
<td>49 [4.39]±0.04</td>
</tr>
<tr>
<td>(+)-1a</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>(−)-1b</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>(+)-1b</td>
<td>48 [4.36]±0.12</td>
<td>59 [4.33]±0.01</td>
<td>41 [4.41]±0.08</td>
</tr>
<tr>
<td>(−)-2a</td>
<td>46 [4.34]±0.02</td>
<td>66 [4.20]±0.11</td>
<td>24 [4.64]±0.10</td>
</tr>
<tr>
<td>(+)-2a</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>58 [4.24±0.04]</td>
</tr>
<tr>
<td>(−)-2b</td>
<td>34 [4.48]±0.06</td>
<td>40 [4.39]±0.01</td>
<td>21 [4.68±0.03]</td>
</tr>
<tr>
<td>(+)-2b</td>
<td>67 [4.18±0.03]</td>
<td>56 [4.25±0.03]</td>
<td>28 [4.56±0.07]</td>
</tr>
</tbody>
</table>

[^4]: Data are given as mean [pIC₅₀ mean ± SEM or pKᵢ mean ± SEM] of three independent experiments each conducted in triplicate.

Unfortunately, pharmacologically investigation at native iGluRs did not highlight any ligand endowed with a worth noting affinity or selectivity for a specific iGlu receptor. In fact, most compounds showed a mid-micromolar affinity for all iGlu receptors. As expected, with the only exception of (−)-2a having an R configuration at the α-amino acid center, did not interact with AMPA and KA receptors; (+)-2a weakly bound to NMDA receptors. Conversely, all compounds derived from L-Glu showed a comparable profile, which was not significantly affected by the absolute configuration of the bicyclic scaffold. Functional studies as well as activity at mGluRs remain to be investigated.

**Conclusions**

New unconventional beta and gamma dipeptides, representing conformational constrained higher homologues of glutamic acid, have been prepared and tested as new pharmacological tools to investigate the iGluR binding domain, in the attempt to identify potential selective antagonists. The rationale was based on the use of classical medicinal chemistry strategies, widely applied in the design of glutamatergic ligands. The synthetic entailed the use of a flow-chemistry reactor to perform the 1,3-dipolar cycloaddition to build the rigid isoxazoline bicyclic scaffolds, which were then condensed to the distal carboxylate of L-Glu or D-Glu. All the new derivatives were obtained in enantiomerically pure form and assignment of the absolute configuration relied on X-ray crystal analysis. Based on the available pharmacological data, we can speculate that the conformational constraint imposed by the bicyclic scaffold, which was meant to increase the receptor selectivity, did not favour the correct orientation of the pharmacophoric groups for a fruitful interaction with the D1 and D2 lobes of iGluRs. Alternatively, the distance between the α-amino acidic group and the distal carboxylate may not be optimal. To deepen this particular aspect, shorter derivatives may be designed by substituting L-Asp (or D-Asp) for L-Glu (or D-Glu).

**Experimental**

**Materials and methods**

All reagents were purchased from Sigma. [³H]NMR and CE-NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts (δ) are expressed in ppm and coupling constants (J) are expressed in Hz. Optical rotation...
determinations were carried out using a Jasco P-1010 spectrophotometer, coupled with a Haake N3-B thermostat. TLC analyses were performed on commercial silica gel 60 F254 aluminium sheets; spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution or ninhydrin. Chiral HPLC analyses were performed using a Jasco PU-980 pump equipped with a UV–vis detector Jasco UV-975 (wavelength: 220 nm) and a Phenomenex Lux Amylose-2 column (4.6 x 150 mm, 5 µm) at a flow rate of 1 mL/min using n-hexane/iPrOH 8:2 as eluent. Preparative HPLC was performed with a 1525 Extended Flow Binary HPLC Pump, equipped with a Waters 2489 UV-visual detector and a Phenomenex Lux Amylose-2 column (21.2 x 250 mm) at a flow rate of 15 mL/min using n-hexane/iPrOH 7:3 as eluent. MS analyses were performed on a Varian 320-MS triple quadrupole mass spectrometer with ESI source. Micro analyses (C, H, N) of new compounds were within ±0.4% of the theoretical values. The continuous-flow cycloaddition reaction was performed using a R2+/R4 flow reactor, commercially available from Vapourtec equipped with Omniphi glass column. Cycloadduct (−)-3 and its enantiomer (+)-3 were prepared as previously reported by us. \(^{10}\)

### General procedure for the nucleophilic substitution

To a solution of benzyl alcohol (1.45 mL, 14.0 mmol) in dry THF (50 mL), NaH (60% dispersion in mineral oil; 7.0 mmol) was added in small portions and the mixture was stirred at rt under a nitrogen atmosphere for 30 min. A solution of 3-Br-isoxazoline derivative 3 (2.3 mmol) in dry THF (3.7 mL) was then added and the mixture was refluxed for 3 h. The progress of the reaction was monitored by TLC (cyclohexane/EtOAc 8:2). The reaction was quenched with 2N HCl (5 mL) and, after evaporation of the solvent, the aqueous layer was extracted with EtO (3 x 10 mL). The organic phase was dried over Na2SO4 and concentrated under vacuum. The residue was then purified by flash chromatography (cyclohexane/EtOAc 9:1).

**General procedure for the coupling reaction.**

a) Boc-protected secondary amine 4 (2.0 mmol) was treated with a 30% solution of trifluoroacetic acid (20.0 mmol) in CH2Cl2 at 0 °C and the solution was stirred at rt for 4 h. The volatiles were removed under vacuum, 1N NaOH (5 mL) was added and the aqueous layer was extracted with CH2Cl2 (3 x 20 mL). The organic layer was dried over anhydrous Na2SO4, filtered, evaporated to dryness and the residue was purified by flash chromatography (CH2Cl2/MeOH 9:1).

b) Boc-(3aS,6aS)-Glu-OEt or Boc-(3aS,6aS)-Glu-OEt (1.0 mmol) was dissolved in CH2Cl2 (2.0 mL). HOBT hydrate (2.0 mmol), HBTU (2.0 mmol), DIPEA (2.0 mmol) and a solution of the amine obtained in the previous step (1.0 mmol) in CH2Cl2 (0.5 mL) were added to the solution. Then the reaction was stirred at rt for 24 h. The progress of the reaction was monitored by TLC (cyclohexane/EtOAc 3:7). After removal of the solvent, the residue was diluted with EtOAc (5 mL) and the organic phase was washed with distilled H2O (5 mL), dried over Na2SO4 and concentrated under vacuum. The crude material was purified by flash chromatography (cyclohexane/EtOAc 3:7).

**(S)-Ethyl 5-(3aR,6aR)-3-(benzylxoy)-3aH-pyrrolo[3,4-d]isoxazol-5(4H,6,6aH)-yl)-2-(tert-butoxycarbonylaminoo)-5-oxopentanoate (−)-5a.**

Overall yield: 60%; Rf = 0.30 (cyclohexane/EtOAc 9:1); crystallized from n-hexane/EtOAc as colourless prisms; m.p.: 138-140 °C [α]D20 + 62.1 (c = 0.5 in CHCl3); 3H-NMR (300 MHz, CDCl3): 1.18–1.34 (m, 3H); 1.40–1.52 (m, 9H); 1.84–2.10 (m, 1H); 2.10–2.55 (m, 3H); 3.40–3.73 (m, 2H); 3.74–4.07 (m, 3H); 4.13–4.33 (m, 3H); 5.10–5.14 (m, 2H); 5.22–5.34 (m, 2H); 7.34–7.42 (m, 5H); 13C-NMR (75 MHz, CDCl3): 14.39; 27.71; 28.54; 31.01; 47.92; 48.54; 50.10; 53.87; 61.78; 72.84; 80.10; 84.85; 128.62; 128.90; 129.08; 135.30; 155.80; 166.92; 170.93; 172.55; MS: 476.3 [M+H]+; Anal. calc'd for C24H33N3O7: C, 66.92; H, 6.99; N, 8.84; found: C, 66.80; H, 7.03; N, 8.80.

**((R)-Ethyl 5-(3aS,6aS)-3-(benzylxoy)-3aH-pyrrolo[3,4-d]isoxazol-5(4H,6,6aH)-yl)-2-(tert-butoxycarbonylaminoo)-5-oxopentanoate (+)-5b.**

Overall yield: 58%; Rf = 0.30 (cyclohexane/EtOAc 9:1); crystallized from n-hexane/EtOAc as colourless prisms; m.p.: 45-47 °C [α]D20 + 83.7 (c = 0.5 in CHCl3); 3H-NMR (300 MHz, CDCl3): 1.20–1.32 (m, 3H); 1.41–1.50 (m, 9H); 1.81–2.09 (m, 1H); 2.10–2.42 (m, 3H); 3.40–3.70 (m, 2H); 3.74–4.07 (m, 3H); 4.13–4.35 (m, 3H); 5.10–5.14 (m, 2H); 5.22–5.34 (m, 2H); 7.32–7.42 (m, 5H); 13C-NMR (75 MHz, CDCl3): 14.41; 27.77; 28.54; 30.78; 47.93; 48.57; 50.10; 53.79; 61.78; 72.82; 80.16; 84.89; 128.60; 128.91; 129.09; 135.30; 155.83; 166.97; 170.85; 172.63; MS: 476.3 [M+H]+; Anal. calc'd for C24H33N3O7: C, 66.92; H, 6.99; N, 8.84; found: C, 66.80; H, 7.06; N, 8.74.
dissolved in MeOH (3 mL) and 10% w/w of 5% Pd/C was taken up with MeOH and filtered. The crude acidic product obtained in the previous step was taken up with MeOH and filtered. The solution was stirred at rt for 30 min under H₂ atmosphere and the reaction was followed by TLC (CH₂Cl₂/MeOH 9:1 + 1% AcOH). The mixture was filtered under vacuum on a celite pad to eliminate the catalyst, and the solvent was removed under reduced pressure. The crude intermediate was treated with a 30% trifluoroacetic acid (10 eq.) solution in CH₂Cl₂ at 0 °C. The solution was stirred at rt for 3 h and the reaction was followed by TLC (CH₂Cl₂/MeOH 9:1 + 1% AcOH). The volatiles were removed under reduced pressure and the solid residue was taken up with MeOH and filtered.

(S)-2-Amino-5-((3aR,6aR)-3-hydroxy-3aH-pyrrolo[3,4-d]isoxazol-5(4H,6H,6aH)-yl)-5-oxopentanoic acid (+)-1a.

Overall yield: 45%; R₆ = 0.11 (n-butanol/H₂O/ACO₂H 4:2:1); white solid; m.p.: T > 60 °C dec.; [α]D²₀ = –14.0 (c = 0.12 in H₂O); ¹H-NMR (300 MHz, D₂O): 2.02–2.14 (m, 2H); 2.38–2.62 (m, 2H) 3.44–3.94 (m, 5H); 5.26–5.38 (m, 1H); ¹³C-NMR (75 MHz, CDCl₃): 25.20; 29.95; 46.18; 47.51; 51.88; 52.94; 82.99; 161.80; 172.59; 172.96; MS: 258.1 [M+H]⁺; Anal. calcld for C₁₉H₁₅N₂O₅: C 66.9; H 5.88; N 16.33; found: C 66.59; H 5.96; N 16.12.

(R)-2-Amino-5-((3aS,6aS)-3-hydroxy-3aH-pyrrolo[3,4-d]isoxazol-5(4H,6H,6aH)-yl)-5-oxopentanoic acid (+)-1a.

[α]D²₀ = +14.1 (c = 0.15 in H₂O); Anal. calcld for C₁₉H₁₅N₂O₅: C 66.9; H 5.88; N 16.33; found: C 66.59; H 5.96; N 16.12.

(S)-2-Amino-5-((3aS,6aS)-3-hydroxy-3aH-pyrrolo[3,4-d]isoxazol-5(4H,6H,6aH)-yl)-5-oxopentanoic acid (+)-1b.

Yield: 48%; white solid; R₆ = 0.11 (n-butanol/H₂O/ACO₂H 4:2:1); m.p.: T > 60 °C dec.; [α]D²₀ = +51.9 (c = 0.14 in H₂O); ¹H-NMR (300 MHz, D₂O): 2.00–2.15 (m, 2H); 2.40–2.62 (m, 2H); 3.45–3.98 (m, 5H); 5.25–5.40 (m, 1H); ¹³C-NMR (75 MHz, CDCl₃): 25.20; 29.95; 46.18; 47.37; 51.47; 53.15; 82.35; 163.80; 172.59; 172.96; MS: 258.1 [M+H]⁺; Anal. calcld for C₁₉H₁₅N₂O₅: C 66.9; H 5.88; N 16.33; found: C 66.45; H 6.08; N 16.04.

(R)-2-Amino-5-((3aR,6aR)-3-hydroxy-3aH-pyrrolo[3,4-d]isoxazol-5(4H,6H,6aH)-yl)-5-oxopentanoic acid (-)-1b.

[α]D²₀ = –51.5 (c = 0.15 in H₂O); Anal. calcld for C₁₉H₁₅N₂O₅: C 46.69; H 5.88; N 16.33; found: C 46.50; H 6.05; N 16.10.

Synthesis of (3aS,6aR)-5-tert-butyl 3-ethyl 6,6a-dihydro-3aH-pyrrolo[3,4-d]isoxazol-3,5(4H)-dicarboxylate (-)-7 and (3aR,6aS)-5-tert-butyl 3-ethyl 6,6a-dihydro-3aH-pyrrolo[3,4-d]isoxazol-3,5(4H)-dicarboxylate (+)-7.

A 0.25 M solution of compound 6 (1.0 mmol) in EtOAc (4 mL) and a 0.37 M solution of ethyl chlorooximinoacetate (1.0 mmol) in EtOAc (4 mL) were prepared. The two reactant streams were mixed using a simple T-piece and delivered to a glass column (6.6 mm i.d x 100 mm length) filled with K₃PO₄, and heated at 80 °C at a total flow rate of 0.16 mL min⁻¹, allowing to a residence time of about 20 min. A 100 psi backpressure regulator was applied to the system. The solvent was evaporated, and the crude material was purified by silica gel column chromatography (cyclohexane-ethyl acetate 8:2) to yield racemic (±)-7 in 62% yield. Yellow oil; R₆ = 0.39 (cyclohexane-ethyl acetate 8:2); ¹H-NMR (300 MHz, CDCl₃): 1.35 (t, J = 7.2 Hz, 3H); 1.42 (s, 9H); 3.41–3.54 (m, 2H); 3.7–4.08 (m, 1H); 3.80–4.10 (m, 2H); 4.35 (q, J = 7.2 Hz, 2H); 5.31 (dd, J = 5.4, 9.6 Hz, 1H); ¹³C-NMR (75 MHz, CDCl₃): 14.28; 28.48; 49.49; 50.75; 53.44; 62.45; 80.59; 87.74; 152.52; 154.28; 160.56; MS: 285.0 [M+H]⁺.

Enantiomerically pure (−)-7 and (+)-7 were obtained from (±)-7 by preparative chiral HPLC. Column: Lux 2-amylco (21.2 × 250 mm, 5 μm); λ = 220 nm; eluent: n-hexane/PrOH 7:3; flow rate: 15 mL/min; t (−)-7: 9.58 min; t (+)-7: 13.40 min.

(3aS,6aR)-Ethyl 5-((S)-4-(tert-butoxy carbonyl amino)-5-ethoxy-5-oxopentanoate)-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazol-3-carboxylate (−)-8a.

Compound (−)-8a was obtained following the general procedure for the coupling reaction reported above, coupling (−)-7 with Boc-L-Glu-OEt.

Overall yield: 44%; yellow oil; R₆ = 0.30 (cyclohexane/ethyl acetic acid 3:7); [α]D²₀ = –131.9 (c = 0.10 in CHCl₃); ¹H-NMR (300 MHz, CDCl₃): 1.26 (t, J = 7.15, 3H); 1.30–1.38 (m, 3H); 1.43 (s, 9H); 1.78–1.96 (m, 1H); 2.02–2.16 (m, 1H); 2.30–2.56 (m, 2H); 3.44–3.62 (m, 1H); 3.68–3.80 (m, 1H) 3.84–4.40 (m, 1H); 4.02–4.24 (m, 5H); 4.26–4.38 (m, 2H); 5.37–5.50 (m, 1H); 6.92–7.02 (m, 1H); ¹³C-NMR (75 MHz, CDCl₃): 13.22; 13.72; 26.51; 27.57; 49.69; 49.82; 51.31; 52.56; 53.53; 61.14; 62.43; 79.46; 87.48; 152.80; 156.89; 160.31; 171.93; 172.79; MS: 442.4 [M+H]⁺; Anal. calcld for C₂₉H₂₃N₂O₅: C 54.41; H 7.08; N 9.52; found: C 54.50; H 7.05; N 9.25.

(3aR,6aS)-Ethyl 5-((R)-4-(tert-butoxy carbonyl amino)-5-ethoxy-5-oxopentanoate)-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazol-3-carboxylate (+)-8a.

Compound (+)-8a was obtained following the general procedure for the coupling reaction reported above, coupling (+)-7 with Boc-D-Glu-OEt.
b) The diacidic product obtained in the previous step was treated with 2N aqueous HCl and extracted with EtOAc (3 × 100 mL). The solid residue was taken up with MeOH and filtered. Overall yield: 48%; yellow oil; Rf = 0.30 (cyclohexane/EtOAc 3:7); [α]D = +120.5 (c = 0.10 in CHCl₃); 1H-NMR (300 MHz, CDCl₃): 1.26 (t, J = 7.2, 3H); 1.30–1.38 (m, 3H); 1.42 (s, 9H); 1.80–1.96 (m, 1H); 2.00–2.18 (m, 1H); 2.30–2.54 (m, 2H); 2.42–3.62 (m, 1H); 3.68–3.80 (m, 1H); 3.84–4.26 (m, 6H); 4.26–4.40 (m, 2H). 5.38–5.30 (m, 1H); 6.92–7.02 (m, 1H); 13C-NMR (75 MHz, CDCl₃): 13.19; 13.34; 26.53; 27.53; 49.71; 49.83; 51.25; 53.57; 61.14; 62.03; 79.46; 86.48; 152.81; 156.94; 160.30; 171.97; 172.81; MS: 442.4 [M+H]+. Anal. calcd: C, 45.41; H, 7.08; N, 9.52; found: C, 45.35; H, 6.98; N, 9.38.

**General deprotection procedure 2.**

a) Protected intermediate 8a or 8b (0.4 mmol) was dissolved in EtOH (1.2 mL) and treated with 1N aqueous NaOH (1.2 mL). The mixture was stirred at rt for 1 h and the disappearance of the starting material was monitored by TLC (cyclohexane/EtOAc 3:7). After evaporation of EtOH, the aqueous layer was washed with Et₂O (3 mL), made acidic (pH = 2) with 2N aqueous HCl and extracted with EtOAc (3 × 10 mL). The organic phase was dried over Na₂SO₄ and concentrated under vacuum.

b) The diacidic product obtained in the previous step was treated with a 30% trifluoroacetic acid (10 eq.) solution in CH₂Cl₂ at 0 °C. The solution was stirred at rt for 3 h and the reaction was followed by TLC (CH₂Cl₂/MeOH 9:1 + 1% AcOH). The volatiles were removed under reduced pressure and the solid residue was taken up with MeOH and filtered.

(3aS,6aR)-5-(S)-4-Amino-4-carboxybutanoyl)-4,5,6,6a-tetrahydro-3H-pyrrolo[3,4-d]isoxazole-3-carboxylic acid (4)-2a.

Overall yield: 55%; white solid; Rf = 0.11 (n-butanol/H₂O/ACOH 4:2:1); m.p.: T > 80 °C dec.; [α]D = –100.8 (c = 0.10 in H₂O); 1H-NMR (300 MHz, D₂O): 1.94–2.12 (m, 2H); 2.34–2.60 (m, 2H); 3.36–3.52 (m, 1H); 3.60–3.74 (m, 1H); 3.78–4.00 (m, 3H); 4.04–4.20 (m, 1H); 5.30–5.46 (m, 1H); 5.53 (m, 1H); 3.74 (m, 1H); 3.78–5.20 (m, 3H); 3.78–4.00 (m, 3H); 4.04–4.20 (m, 1H); 5.28–5.44 (m, 1H); 13C-NMR (75 MHz, D₂O): 25.25; 30.03; 49.34; 50.91; 52.81; 53.89; 87.05; 156.20; 164.04; 172.45; 172.73; MS: 286.0 [M+H]+. Anal. calcd: C, 46.32; H, 5.30; N, 14.73; found: C, 46.48; H, 5.50; N, 14.48.

X-ray diffraction analysis of (+)-2b.

Well-formed colorless crystals of the compound (+)-2b were grown by slow evaporation (≈ 7 d) from a 1:1 mixture of H₂O:CH₃CN. A transparent thin plate (0.225 × 0.175 × 0.025 mm) was selected for the analysis and mounted on a glass capillary fiber with perfluorinated oil as glue. X-ray diffraction intensities were collected at room temperature on a three-circle Bruker SMART APEX II diffractometer equipped with a CCD area detector. The data collection consisted of 5 o-scans (0.5 deg/frame, with exposure time ranging from 60 to 90 s/frame) at different φ orientations of the crystal. Graphite-monochromated Mo Kα radiation (λ = 0.71073 Å) was employed throughout. A 100% complete dataset up to a maximum Bragg angle 2θ = 55° was obtained, consisting of 19388 measured reflections (2982 symmetry-independent). Data reduction and correction for beam anisotropy effects were performed by SAINT+ and SADABS, respectively. The structure was solved by direct methods through SHELXS-2013 and refined by the full-matrix least-squares procedure implemented in SHELXL-2014. The agreement factors for the final least-squares model were R(1/F) = 0.0496 for 1573 F₀ > 4σ(F₀) and wR²(F) = 0.099 for all the measured data. The structure unit cell was as low as a = 0.201–0.19 e Å⁻³. Crystal data for compound (+)-2b at rt: C₁₁H₁₃N₂O₄, M = 284.429 amu, orthorhombic, space group P₂₁2₁2₁, n° 19, acenctic, a = 5.4380(7) Å, b = 10.4232(2) Å, c = 22.933(3) Å, V = 1299.9(4) Å³, Z = 4, Z' = 1; μcalc = 1.545 g cm⁻³, μ = 0.13 mm⁻¹. The compound is chiral and crystallizes with an ordered water molecule in the asymmetric unit. CCDC 1060015 contains the supplementary crystallographic data for this paper. These data...
can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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