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ARTICLE

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

Cite this: DOI: 10.1039/xoxxoooooox

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Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

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.

Introduction

L-Glutamic acid (L-Glu, Figure 1) is the main excitatory neurotransmitter in the central nervous system (CNS), where it is involved in the modulation of many physiological processes such as learning, memory, and synaptic plasticity.[1] Once released from the presynaptic neurons into the glutamatergic synaptic cleft, L-Glu activates two main classes of receptors: G-protein-coupled metabotropic Glu receptors (mGluRs) and ligand-gated ionotropic Glu receptors (iGluRs). On the basis of the agonist selectivity, iGluRs have been named *N*-methyl-D-aspartic acid (NMDA) receptors, (*RS*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) receptors, and kainic acid (KA) receptors.[1-5] Each of these receptor classes is in turn composed of several receptor subtypes, depending on subunit composition.

The availability of highly selective agonists and antagonists for the different receptor subtypes represents a primary target to understand their physiological role and their pharmacological relevance. Also from a therapeutic point of view, the more interesting compounds are undoubtedly those characterized by high selectivity for a specific receptor subtype, since this feature allows to minimize the possible side effects encountered with unselective compounds.

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 distal carboxylate (*e.g.*, phosphonic acid, tetrazol, 3-hydroxy-isoxazole/isoxazoline).[6]

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.[7] 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

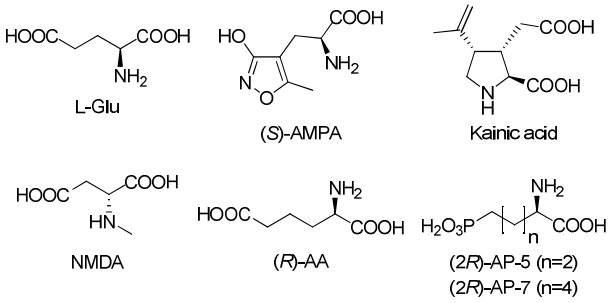

Figure 1. Structure of representative iGluR ligands.

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*-amino 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 and

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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]

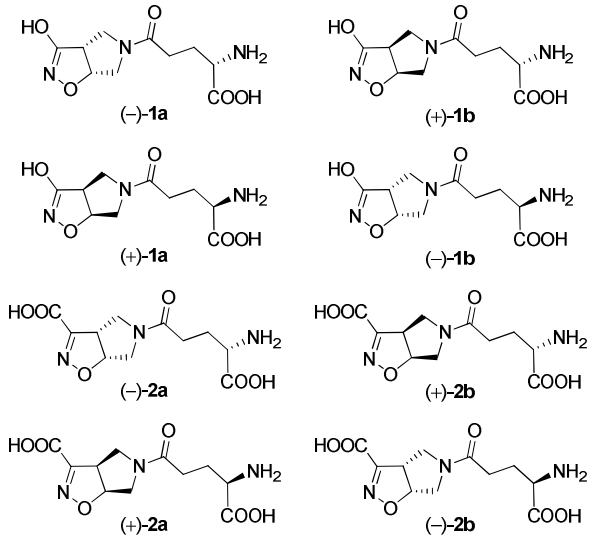
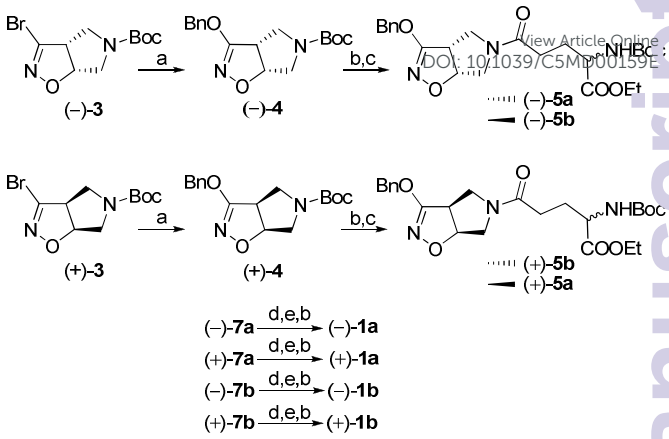


Figure 2. Structure of the target compounds.

Results and discussion

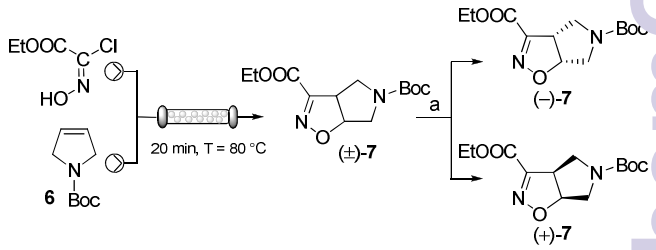
Dipeptides (–)-**1a**, (+)-**1a**, (–)-**1b** and (+)-**1b** were synthetized from the enantiomerically pure compounds (–)-**3** and (+)-**3**, which were obtained as recently reported by us.[10] Intermediates (–)-**3** and (+)-**3** were submitted to a nucleophilic substitution at the C-3, in the presence of benzyl alcohol and sodium hydride, to obtain the desired 3-benzyloxy-substituted intermediates (–)-**4** and (+)-**4**, respectively.[9b,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).



Scheme 1. Reagents and conditions: a) BnOH, NaH 60% in mineral oil, dry THF; b) 30% TFA, CH_2Cl_2 c) Boc-L-Glu-OEt or Boc-D-Glu-OEt, HOBt, HBTU, DIPEA; CH_2Cl_2; d) 1N NaOH, EtOH e) H_2, 5% Pd/C, MeOH.

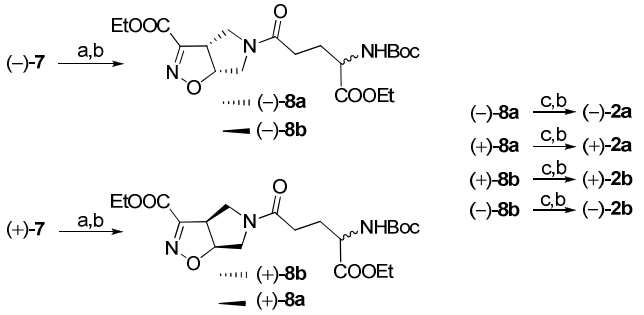
The final dipeptides (–)-**1a**, (+)-**1a**, (–)-**1b** and (+)-**1b** were obtained after deprotection of intermediates (–)-**5a**, (+)-**5a**, (–)-**5b** and (+)-**5b**. In particular, after the hydrolysis of the amino acidic ester with 1N NaOH at room temperature, the *O*-benzyl group was removed by catalytic hydrogenation with 5% Pd/C. Finally, treatment with a 30% trifluoroacetic acid solution in CH_2Cl_2 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 (treating with 1N NaOH at 60 °C) led to degradation of the dipeptidic structure.

The synthesis of compounds (–)-**2a**, (+)-**2a**, (–)-**2b** and (+)-**2b** was accomplished starting from cycloadducts (–)-**7** or (+)-**7**, which were obtained through resolution of the corresponding racemic mixture (±)-**7**, by chiral semi-preparative HPLC. Compound (±)-**7** was synthetized 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 (e.e. >99%) of racemic (±)-**7** was achieved using a *tris*-(2-methyl-5-chloro-phenyl)carbamoyl amylose chiral stationary phase.



Scheme 2. a) Semi-preparative HPLC separation; chiral stationary phase: *tris*-(2-methyl-5-chloro-phenyl)carbamoyl amylose; eluent: 7:3 *n*-hexane/*i*PrOH; 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**.



Scheme 3. Reagents and conditions. a) 30% TFA, CH_2Cl_2 b) Boc-L-Glu-OEt or Boc-D-Glu-OEt, HOBt, HBTU, DIPEA; CH_2Cl_2; 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 (2*S*,7*S*,8*R*) to the enantiomer (+)-**2b** (Figure 3). Consequently, the absolute configuration to derivatives (–)-**2b,** (+)-**2a** and (–)-**2a** was assigned**.**

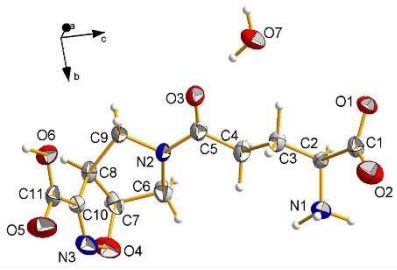


Figure 3. Asymmetric unit of (+)-**2b**, with the atom numbering scheme. A co-crystallized, ordered water molecule is also present. Thermal ellipsoids at RT were drawn at the 50 % probability level.

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 [^{3}H]CGP39653, 5 nM [^{3}H]AMPA, and 5 nM [^{3}H]KA (Table 1).[14]

Table 1. Receptor binding affinities at native rat iGluRs.[a]

Cmpd	[^{3}H]AMPA IC_{50} (μM)	[^{3}H]KAIN IC_{50} (μM)	[^{3}H]CGP39653 K_i (μM)
(–)-**1a**	43 [4.37±0.01]	37 [4.43±0.05]	25 [4.63±0.08]
(+)-**1a**	> 100	> 100	> 100
(–)-**1b**	> 100	> 100	> 100
(+)-**1b**	48 [4.36±0.12]	59 [4.23±0.01]	41 [4.41±0.08]
(–)-**2a**	46 [4.34±0.02]	66 [4.20±0,11]	24 [4.64±0.10]
(+)-**2a**	> 100	> 100	58[4.24±0.04]
(–)-**2b**	34 [4.48±0.06]	40 [4.39±0.01]	21 [4.68±0.03]
(+)-**2b**	67 [4.18±0.03]	56 [4.25±0.03]	28 [4.56±0.07]

[a] Data are given as mean [pIC_{50} mean ± SEM or pK_i mean ± SEM] of three independent experiments each conducted in triplicate.

Unfortunately, pharmacologically investigation at native iGluRs did not highlight any ligand endowed with a worthy 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 (–)-**2** compounds having an *R* configuration at the α-amino acidic 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 synthesis 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 iGluR. 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. ^{1}H NMR and ^{13}C 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 rotati

determinations were carried out using a Jasco P-1010 spectropolarimeter, coupled with a Haake N3-B thermostat. TLC analyses were performed on commercial silica gel 60 F_{254} aluminium sheets; spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution or ninhydrin. Chiral HPLC analyses were performed with 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 × 150 mm, 5 μm) at a flow rate of 1 mL/min using *n*-hexane/*i*PrOH 8:2 as eluent. Preparative HPLC was performed with a 1525 Extended Flow Binary HPLC Pump, equipped with a Waters 2489 UV-vis detector and a Phenomenex Lux Amylose-2 column (21.2 × 250 mm) at a flow rate of 15 mL/min using *n*-hexane/*i*PrOH 7:3 as eluent. MS analyses were performed on a Varian 320-MS triple quadrupole mass spectrometer with ESI source. Microanalyses (C, H, N) of new compounds were within ±0.4% of theoretical values. The continuous-flow cycloaddition reaction was performed using a R2+/R4 flow reactor, commercially available from Vapourtec equipped with Omnifit 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 Et_2O (3 × 10 mL). The organic phase was dried over Na_2SO_4 and concentrated under vacuum. The residue was then purified by flash chromatography (cyclohexane/EtOAc 9:1).

(3a*R*,6a*R*)-*tert*-Butyl 3-(benzyloxy)-6,6a-dihydro-3a*H*-pyrrolo[3,4-*d*]isoxazole-5(4*H*)-carboxylate (−)-4.

Yield: 94%; R_f = 0.28 (cyclohexane/EtOAc 8:2); crystallized from *n*-hexane/EtOAc as colourless prisms; m.p.: 152-153 °C; $[\alpha]^{20}_D$= – 90.1 (*c* = 0.85 in $CHCl_3$); ^{1}H-NMR (300 MHz, $CDCl_3$): 1.45 (s, 9H); 3.41 (dd, *J* = 8.3, 12.8, 1H); 3.48−3.62 (m, 1H); 3.75 (ddd, *J* = 2.2, 8.3, 8.3, 1H); 3.83 (dd, *J* = 1.9, 12.8, 1H); 3.88−4.02 (m, 1H); 5.14 (s, 2H); 5.21 (ddd *J* = 1.9, 6.1, 8.3, 1H); 7.35−7.42 (m, 5H); ^{13}C-NMR (75 MHz, $CDCl_3$): 28.60; 48.24; 49.29; 53.87; 72.69; 80.40; 84,81; 128.59; 128.86; 128.90; 135.50; 154.23; 166.93; MS: 319.2 $[M+H]^+$; Anal. calcd for $C_{17}H_{22}N_2O_4$: C, 64.13; H, 6.97; N, 8.80; found: C, 63.84; H, 7.03; N, 8.70

(3a*S*,6a*S*)-*tert*-Butyl 3-(benzyloxy)-6,6a-dihydro-3a*H*-pyrrolo[3,4-d]isoxazole-5(4*H*)-carboxylate (+)-4.

$[\alpha]^{20}_D$= + 90.4 (*c* = 0.90 in $CHCl_3$); Anal. calcd for $C_{17}H_{22}N_2O_4$: C, 64.13; H, 6.97; N, 8.80; found: C, 63.90; H, 7.00; N, 8.68.

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 CH_2Cl_2 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 CH_2Cl_2 (3 × 20 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, evaporated to dryness and the residue was purified by flash chromatography (CH_2Cl_2/MeOH 9:1).

b) Boc-L-Glu-OEt or Boc-D-Glu-OEt (1.0 mmol) was dissolved in CH_2Cl_2 (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 CH_2Cl_2 (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 H_2O (5 mL), dried over Na_2SO_4 and concentrated under vacuum. The crude material was purified by flash chromatography (cyclohexane/EtOAc 3:7).

(*S*)-Ethyl 5-((3a*R*,6a*R*)-3-(benzyloxy)-3a*H*-pyrrolo[3,4-d]isoxazol-5(4*H*,6*H*,6a*H*)-yl)-2-(*tert*-butoxycarbonylamino)-5-oxopentanoate (−)-5a.

Overall yield: 60%; R_f = 0.30 (cyclohexane/EtOAc 9:1); crystallized from *n*-hexane/EtOAc as colourless prisms; m.p.: 138-140 °C $[\alpha]^{20}_D$= – 61.7 (*c* = 0.5 in $CHCl_3$); ^{1}H-NMR (300 MHz, $CDCl_3$): 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); ^{13}C-NMR (75 MHz, $CDCl_3$): 14.39; 27.71; 28.54; 31.01; 47.92; 48.54; 50.10; 53.87; 61.68; 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. calcd for $C_{24}H_{33}N_3O_7$: C, 60.62; H, 6.99; N, 8.84; found: C, 60.84; H, 7.03; N, 8.80.

(*R*)-Ethyl 5-((3a*S*,6a*S*)-3-(benzyloxy)-3a*H*-pyrrolo[3,4-*d*]isoxazol-5(4*H*,6*H*,6a*H*)-yl)-2-(*tert*-butoxycarbonylamino)-5-oxopentanoate (+)-5a.

$[\alpha]^{20}_D$= + 62.1 (*c* = 0.5 in $CHCl_3$); Anal. calcd for $C_{24}H_{33}N_3O_7$: C, 60.62; H, 6.99; N, 8.84; found: C, 60.80; H, 7.03; N, 8.78.

(*S*)-Ethyl 5-((3a*S*,6a*S*)-3-(benzyloxy)-3a*H*-pyrrolo[3,4-*d*]isoxazol-5(4*H*,6*H*,6a*H*)-yl)-2-(*tert*-butoxycarbonylamino)-5-oxopentanoate (+)-5b.

Overall yield: 58%; R_f = 0.30 (cyclohexane/EtOAc 9:1); crystallized from *n*-hexane/EtOAc as colourless prisms; m.p.: 45-47 °C $[\alpha]^{20}_D$= + 83.7 (*c* = 0.55 in $CHCl_3$); ^{1}H-NMR (300 MHz, $CDCl_3$): 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); 3.70–4.10 (m, 3H); 4.18–4.35 (m, 3H); 5.09–5.20 (m, 2H), 5.20–5.34 (m, 2H); 7.32–7.42 (m, 5H); ^{13}C-NMR (75 MHz, $CDCl_3$): 14.41; 27.77; 28.54; 30.78; 47.93; 48.57; 50.10; 53.79; 61.70; 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. calcd for $C_{24}H_{33}N_3O_7$: C, 60.62; H, 6.99; N, 8.84; found: C, 60.88; H, 7.06; N, 8.74.

(*R*)-Ethyl 5-((3a*R*,6a*R*)-3-(benzyloxy)-3a*H*-pyrrolo[3,4-*d*]isoxazol-5(4*H*,6*H*,6a*H*)-yl)-2-(*tert*-butoxycarbonylamino)-5-oxopentanoate (−)-5b.

$[\alpha]^{20}_D$= – 84.0 (*c* = 0.50 in $CHCl_3$); Anal. calcd for $C_{24}H_{33}N_3O_7$: C, 60.62; H, 6.99; N, 8.84; found: C, 60.80; H, 7.04; N, 8.72.

General deprotection procedure 1.

a) Protected intermediate **5a** or **5b** (0.4 mmol) was dissolved in EtOH (1.2 mL) and treated with 1N aqueous NaOH (0.6 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 Et_2O (3 mL), made acidic (pH = 2) with 2N aqueous HCl and extracted with EtOAc (3 × 10 mL). The organic phase was dried over Na_2SO_4 and concentrated under vacuum.

b) The crude acidic product obtained in the previous step was dissolved in MeOH (3 mL) and 10% w/w of 5% Pd/C was added. The mixture was stirred at rt for 30 min under H_2 atmosphere and the reaction was followed by TLC (CH_2Cl_2/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.

c) The obtained intermediate was treated with a 30% trifluoroacetic acid (10 eq.) solution in CH_2Cl_2 at 0 °C. The solution was stirred at rt for 3 h and the reaction was followed by TLC (CH_2Cl_2/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-((3a*R*,6a*R*)-3-hydroxy-3a*H*-pyrrolo[3,4-d]isoxazol-5(4*H*,6*H*,6a*H*)-yl)-5-oxopentanoic acid (−)-1a.

Overall yield: 45%; R_f = 0.11 (*n*-butanol/H_2O/AcOH 4:2:1); white solid; m.p.: T > 60 °C dec.; $[\alpha]^{20}_D$= – 14.0 (*c* = 0.12 in H_2O); ^{1}H-NMR (300 MHz, D_2O): 2.02–2.14 (m, 2H); 2.38–2.62 (m, 2H) 3.44–3.94 (m, 5H); 5.26–5.38 (m, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): 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. calcd for $C_{10}H_{15}N_3O_5$: C, 46.69; H, 5.88; N, 16.33; found: C, 46.55; H, 5.98; N, 16.08.

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

$[\alpha]^{20}_D$= + 14.1 (*c* = 0.15 in H_2O); Anal. calcd for $C_{10}H_{15}N_3O_5$: C, 46.69; H, 5.88; N, 16.33; found: C, 46.59; H, 5.96; N, 16.12.

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

Yield: 48%; white solid; R_f = 0.11 (*n*-butanol/H_2O/AcOH 4:2:1); m.p.: T > 60 °C dec.; $[\alpha]^{20}_D$ = +51.9 (*c* = 0.14 in H_2O); ^{1}H NMR (300 MHz, D_2O): 2.00–2.15 (m, 2H); 2.40–2.62 (m, 2H); 3.45–3.98 (m, 5H); 5.25–5.40 (m, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): 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. calcd for $C_{10}H_{15}N_3O_5$: C, 46.69; H, 5.88; N, 16.33; found: C, 46.45; H, 6.08; N, 16.04.

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

$[\alpha]^{20}_D$ = – 51.5 (*c* = 0.15 in H_2O); Anal. calcd for $C_{10}H_{15}N_3O_5$: C, 46.69; H, 5.88; N, 16.33; found: C, 46.50; H, 6.05; N, 16.10.

View Article Online
DOI: 10.1039/C5MD00159E

Synthesis of (3a*S*,6a*R*)-5-*tert*-butyl 3-ethyl 6,6a-dihydro-3a*H*-pyrrolo[3,4-*d*]isoxazole-3,5(4*H*)-dicarboxylate (–)-7 and (3a*R*,6a*S*)-5-*tert*-butyl 3-ethyl 6,6a-dihydro-3a*H*-pyrrolo[3,4-*d*]isoxazole-3,5(4*H*)-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 (1.5 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.× 100 mm length) filled with K_2CO_3 and heated at 80 °C at a total flow rate of 0.16 mL min^{-1}, equating 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–EtOAc 8:2) to yield racemic (±)-**7** in 62% yield. Yellow oil; R_f = 0.[illegible] (cyclohexane/EtOAc 8:2): 0.17; ^{1}H-NMR (300 MHz, CDCl3): 1.35 (t, *J* = 7.2 Hz, 3H), 1.42 (s, 9H), 3.41–3.54 (m, 2H), 3.7[illegible] 4.08 (m, 1H), 3.80–4.10 (m, 2H), 4.35 (q, *J* = 7.2 Hz, 2H), 5.32 (dd, *J* = 5.4, 9.6 Hz, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): 14.26; 28.48; 49.49; 50.75; 53.44; 62.45; 80.59; 87,74; 152.52, 154.28; 160.36; MS: 285.0 $[M+H]^+$.

Enantiomerically pure (–)-**7** and (+)-**7** were obtained from (±)-**7** by preparative chiral HPLC. Column: Lux 2-amycoat (21.2 × 250 mm, 5 μm); λ = 220 nm; eluent: *n*-hexane/*i*PrOH 7:3; flow rate: 15 mL/min; t_r (–)-**9**: 9.58 min; t_r (+)-**9**: 13.40 min.

(–)-**7**: $[\alpha]^{20}_D$ = – 172.4 (c = 0.76 in $CHCl_3$).

(+)-**7**: $[\alpha]^{20}_D$ =+173.0 (c = 0.74 in $CHCl_3$).

(3a*S*,6a*R*)-Ethyl 5-((*S*)-4-(*tert*-butoxycarbonylamino)-5-ethoxy-5-oxopentanoyl)-4,5,6,6a-tetrahydro-3a*H*-pyrrolo[3,4-*d*]isoxazole-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; R_f = 0.30 (cyclohexane/EtOAc 3:7); $[\alpha]^{20}_D$= – 131.9 (*c* = 0.10 in $CHCl_3$); ^{1}H-NMR (300 MHz CD_3OD): 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); ^{13}C-NMR (75 MHz, CD_3OD): 13.22; 13.3[illegible]; 26.51; 27.57; 49.69; 49.82; 51.31; 52.56; 53.53; 61.14; 62.0[illegible]; 79.46; 87.48; 152.80; 156.89; 160.31; 171.93; 172.79; MS: 442.4 $[M+H]^+$; Anal. calcd for $C_{20}H_{31}N_3O_8$: C, 54.41; H, 7.08, N, 9.52; found: C, 54.50; H, 7.05; N, 9.25.

(3a*R*,6a*S*)-Ethyl 5-((*R*)-4-(*tert*-butoxycarbonylamino)-5-ethoxy-5-oxopentanoyl)-4,5,6,6a-tetrahydro-3a*H*-pyrrolo[3,4-*d*]isoxazole-3-carboxylate (+)-8a.

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

$[\alpha]^{20}_D$= + 132.5 (*c* = 0.10 in $CHCl_3$); Anal. calcd for $C_{20}H_{31}N_3O_8$: C, 54.41; H, 7.08; N, 9.52; found: C, 54.20; H, 7.00; N, 9.30.

(3a*R*,6a*S*)-Ethyl 5-((*S*)-4-(tert-butoxycarbonylamino)-5-ethoxy-5-oxopentanoyl)-4,5,6,6a-tetrahydro-3a*H*-pyrrolo[3,4-d]isoxazole-3-carboxylate (+)-8b.

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

Overall yield: 48%; yellow oil; R_f = 0.30 (cyclohexane/EtOAc 3:7); $[\alpha]^{20}_D$= + 120.5 (*c* = 0.10 in $CHCl_3$); ^{1}H-NMR (300 MHz, CD_3OD): 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); 3.42–3.62 (m, 1H); 3.68–3.80 (m, 1H); 3.84–4.26 (m, 6H); 4.26–4.40 (m, 2H) 5.38–3.50 (m, 1H); 6.92–7.02 (m, 1H); ^{13}C-NMR (75 MHz, CD_3OD): 13.19; 13.34; 26.53; 27.53; 49.71; 49.83; 51.28; 52.51; 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 for $C_{20}H_{31}N_3O_8$: C, 54.41; H, 7.08; N, 9.52; found: C, 54.35; H, 6.98; N, 9.38.

(3a*S*,6a*R*)-Ethyl 5-((*R*)-4-(tert-butoxycarbonylamino)-5-ethoxy-5-oxopentanoyl)-4,5,6,6a-tetrahydro-3a*H*-pyrrolo[3,4-*d*]isoxazole-3-carboxylate (−)-8b.

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

$[\alpha]^{20}_D$= – 122.0 (*c* = 0.15 in $CHCl_3$); Anal. calcd for $C_{20}H_{31}N_3O_8$: C, 54.41; H, 7.08; N, 9.52; found: C, 54.25; H, 6.94; N, 9.40.

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 Et_2O (3 mL), made acidic (pH = 2) with 2N aqueous HCl and extracted with EtOAc (3 × 10 mL). The organic phase was dried over Na_2SO_4 and concentrated under vacuum.

b) The diacidic product obtained in the previous step was treated with a 30% trifluoroacetic acid (10 eq.) solution in CH_2Cl_2 at 0 °C. The solution was stirred at rt for 3 h and the reaction was followed by TLC (CH_2Cl_2/MeOH 9:1 + 1% AcOH). The volatiles were removed under reduced pressure and the solid residue was taken up with MeOH and filtered.

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

Overall yield: 55%; white solid; R_f = 0.11 (*n*-butanol/H_2O/AcOH 4:2:1); m.p.: T > 80 °C dec.; $[\alpha]^{20}_D$= – 100.8 (*c* = 0.10 in H_2O); ^{1}H-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); ^{13}C-NMR (75 MHz, D_2O): 25.33; 30.09; 48.87; 50.15; 51.72; 52.99; 53.89; 86.95; 156.47; 164.37; 172.61; 172.69; MS: 286.0 $[M+H]^+$; Anal. calcd for $C_{11}H_{15}N_3O_6$: C, 46.32; H, 5.30; N, 14.73; found: C, 46.52; H, 5.50; N, 14.53.

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

$[\alpha]^{20}_D$= + 101.2 (*c* = 0.10 in H_2O); Anal. calcd for $C_{11}H_{15}N_3O_6$: C, 46.32; H, 5.30; N, 14.73; found: C, 46.49; H, 5.48; N, 14.58.

(3a*R*,6a*S*)-5-((*S*)-4-Amino-4-carboxybutanoyl)-4,5,6,6a-tetrahydro-3a*H*-pyrrolo[3,4-*d*]isoxazole-3-carboxylic acid (+)-2b

Overall yield: 62%; white solid; R_f = 0.1 (*n*-butanol/H_2O/AcOH 4:2:1); m.p.: T > 80 °C dec.; $[\alpha]^{20}_D$= + 125.7 (*c* = 0.10 in H_2O); ^{1}H-NMR (300 MHz, D_2O): 1.94–2.12 (m, 2H); 2.38–2.62 (m, 2H); 3.37–3.52 (m, 1H); 3.62–3.74 (m, 1H); 3.78–4.00 (m, 3H); 4.04–4.20 (m, 1H); 5.28–5.44 (m, 1H); ^{13}C-NMR (75 MHz, D_2O): 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 for $C_{11}H_{15}N_3O_6$: C, 46.32; H, 5.30; N, 14.73; found: C, 46.45; H, 5.53; N, 14.48.

(3a*S*,6a*R*)-5-((*R*)-4-Amino-4-carboxybutanoyl)-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-3-carboxylic acid (-)-2b

$[\alpha]^{20}_D$= – 124.6 (*c* = 0.10 in H_2O); Anal. calcd for $C_{11}H_{15}N_3O_6$: C, 46.32; H, 5.30; N, 14.73; found: C, 46.48; H, 5.50; N, 14.52.

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 H_2O:CH_3CN. 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 ω-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.[15,16] The structure was solved by direct methods through SHELXS–2013[16] and refined by the full-matrix least-squares procedure implemented in SHELXL–2014/4.[17,18] The agreement factors for the final least-squares model were *R1(F)* = 0.0496 for 1573 $F_o > 4\sigma(F_o)$ and *wR*(F^2)= 0.099 for all the measured data, whereas the maximum and minimum Fourier residuals in the unit cell were as low as +0.20/–0.19 e Å^{-3}. Crystal data for compound (+)-**2b** at rt: $C_{11}H_{14}N_3O_6$, M = 284.429 amu, orthorhombic, space group $P2_12_12_1$, nº 19, acentric, *a* = 5.4380(7) Å, *b* = 10.423(2) Å, *c* = 22.933(3) Å, *V* = 1299.9(4) Å3, *Z* = 4, *Z'* = 1; ρ_{calcd} = 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.

Acknowledgements

The financial support to the present research by the University of Milan (Finanziamenti di Ateneo per la ricerca – Piano sviluppo Unimi – Linea B) is acknowledged.

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