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Synthesis of new β- and γ-benzyloxy-*S*-glutamic acid derivatives and evaluation of their activity as inhibitors of excitatory amino acid transporters

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ABSTRACT

An efficient stereoselective preparation of the two enantiomerically pure diasteroisomers of γ-benzyloxy-*S*-glutamic acid was performed using *trans*-4-hydroxy-L-proline as a source of chirality, while the *erythro* and *threo* isomers of β-benzyloxy-*S*-glutamic acid were prepared starting from (*R*)-Garner's aldehyde. All new derivatives were tested for their inhibitory activity against excitatory amino acid transporters in a rat synaptosomal preparation and their IC_{50} values were compared to that of **TBOA**, a one carbon lower homologue commonly used as the reference blocker of glutamate transporters.

1. Introduction

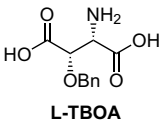
L-Glutamate (Glu) is an important nutrient involved in several biochemical pathways such as gluconeogenesis and ammonia detoxification.[1] In the mammalian central nervous system (CNS) Glu is the most abundant excitatory neurotransmitter; it is involved in physiological functions of utmost importance such as neuronal development and synaptic plasticity, learning and memory, cognition, and pain perception (nociception).[2] An abnormal increase in the extracellular concentration of Glu induces toxicity (excitotoxicity) leading to neuronal cell death.

Under physiological conditions, the concentration of Glu in the synaptic cleft is kept at low level by a number of plasma membrane proteins acting as excitatory amino acid transporters (EAATs).[3] So far, five different human EAATs (EAAT1–5) have been cloned. EAATs are mainly localised at the glial level; once taken up in the glial cells, the neurotransmitter is then recycled through intracellular metabolism and returned to the nerve endings in the form of glutamine (Gln), its precursor.[4] In contrast, the fraction of Glu removed by the EAATs located in the presynaptic nerve endings is directly stored in presynaptic vescicles, ready to be released in the extracellular space.

The rapid removal of glutamate from the synaptic cleft by high-affinity uptake is thought to influence the kinetics of Glu receptor activation, and to contribute to (i) the termination of the excitatory signal, (ii) the maintenance of extracellular glutamate levels below excitotoxic concentrations, though maintaining synaptic communication, and (iii) the recycling of the transmitter via the glutamine cycle.

In pathological conditions in which energy levels fall and the transmembrane gradient of Na^+ collapses (e.g., ischaemia, neurotrauma), EAATs release additional Glu through the reversed mode of operation, thus contributing to neuronal cell death.[5,6] This scenario supports the idea that the blockade of EAATs by non-transportable inhibitors (blockers) could be a useful therapeutic approach to prevent glutamate release and neuronal death after cerebral ischaemia.[7–9] Unfortunately, EAAT blockers are likely to be neurotoxic under physiological conditions.[10,11]

Selective and potent inhibitors are needed to identify the physiological roles of transporters in the regulation of synaptic transmission or in the pathogenesis of neurological diseases. An interesting compound is L-**TBOA**, a non-selective but potent EAAT blocker, which contains the aspartate backbone functionalised with a benzyloxy moiety at the β position (Fig. 1). A survey of the literature shows that the same type of functionalisation applied to glutamic acid has never been investigated, even though it is known that the insertion of a substituent such a methyl in the β position of Glu gives rise to a selective EAAT2 blocker.[12] Substitution at the γ position of Glu was recently reported:[13] a series of 4-alkyl-substituted glutamic acid derivatives proved to act as EAAT inhibitors, in particular when a bulky substituent was introduced.



L-TBOA

Figure 1. Model compound.

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Again, the impact of the benzyloxy group on the biological activity was never investigated. Therefore, it appeared highly relevant to synthesise both β and γ benzyloxy glutamic acid derivatives and to test their activity as EAAT inhibitors.

Since a common feature of EAAT inhibitors is the *S* configuration at the α-amino acidic centre, we planned the synthesis of the α*S* diastereomers, and we varied the sole configuration at the 3- or 4-position, thus obtaining the four possible isomers (2*S*,4*R*)-**1**, (2*S*,4*S*)-**2**, (2*S*,3*R*)-**3** and (2*S*,3*S*)-**4** (Fig. 2).

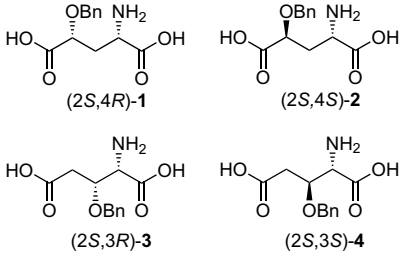


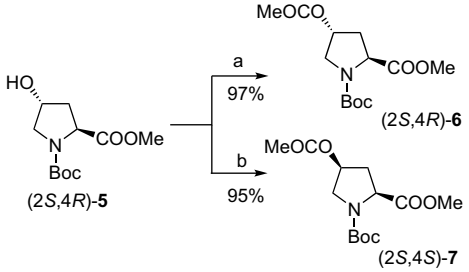
Figure 2. Target compounds.

2. Results

The stereoselective synthesis of enantiomerically pure amino acids (2*S*,4*R*)-**1** and (2*S*,4*S*)-**2** was achieved using as a source of chirality *trans*-4-hydroxy-L-proline, which is commercially available.

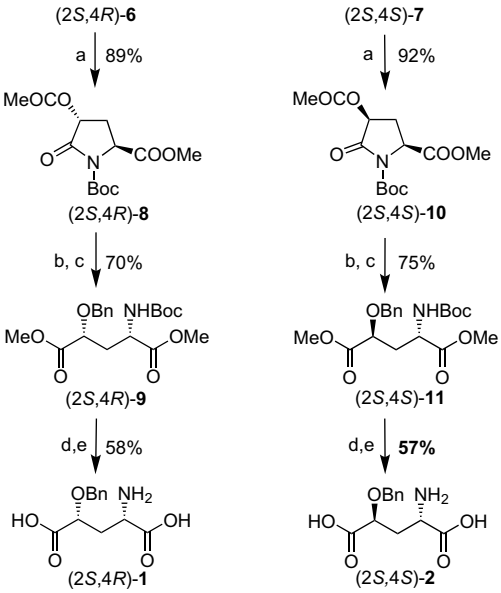
trans-4-Hydroxy-L-proline was protected at the carboxylic acid and amino functions, following the procedure reported in the literature[14] to afford compound (2*S*,4*R*)-**5** (Scheme 1). This intermediate was in part acetylated using acetic anhydride and dimethylamino pyridine (DMAP) to give compound **6**, having a 2*S*,4*R* configuration at the stereogenic centres, thus being a suitable precursor of the final amino acid (2*S*,4*R*)-**1**.

Alternatively, intermediate (2*S*,4*R*)-**5** was submitted to a Mitsunobu reaction with acetic acid, triphenylphosphine, and diethylazodicarboxylate (DEAD), to induce a complete inversion of configuration at the stereogenic centre in position 4, leading to compound (2*S*,4*S*)-**7**, the suitable precursor of amino acid (2*S*,4*S*)-**2** (Scheme 1).



Scheme 1. Reagents and conditions: (a) Ac_2O, DMAP, MeCN; (b) HAc, Ph_3P, DEAD, THF.

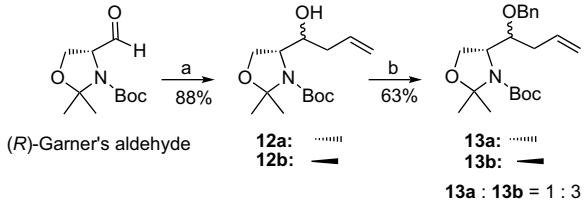
Intermediates (2*S*,4*R*)-**6** and (2*S*,4*S*)-**7** were oxidised with a catalytic amount of hydrated ruthenium(IV) oxide and a 10% aqueous solution of sodium periodate in a biphasic system water/ethyl acetate.[15] Treatment of (2*S*,4*R*)-**8** and (2*S*,4*S*)-**10** with polymer supported sodium carbonate (PS-CO_3) in methanol allowed the direct deprotection of the hydroxy group in position 4 and the opening of the lactam ring, to give the corresponding methyl esters, which were directly benzylated using freshly prepared silver oxide and benzyl bromide in diethyl ether[16] to yield intermediates (2*S*,4*R*)-**9** and (2*S*,4*S*)-**11**. The latters were then hydrolysed and deprotected at the amine function to produce the desired enantiopure amino acids (2*S*,4*R*)-**1** and (2*S*,4*S*)-**2** (Scheme 2).



Scheme 2. (a) RuO_2xH_2O, $NaIO_4$, H_2O, AcOEt; (b) PS-CO_3, MeOH; (c) Ag_2O, $PhCH_2Br$, Et_2O; (d) NaOH, MeOH; (e) 30% CF_3COOH/CH_2Cl_2.

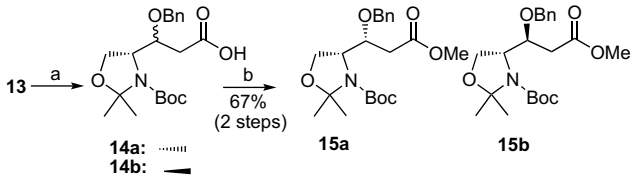
In a first attempt, the preparation of amino acids (2*S*,3*R*)-**3** and (2*S*,3*S*)-**4** was tackled by using *trans*-3-hydroxy-L-proline as starting material following the synthetic strategy described above. Unfortunately, we have been unable to prepare the β-benzyloxy-glutamate derivative since the substrate proved to be unreactive when treated with the mixture of benzyl bromide and silver oxide, whereas, under conventional reaction conditions, i.e., benzyl bromide/sodium hydride, it gave the corresponding unsaturated derivative.

Consequently, we moved on to another route starting from (*R*)-Garner's aldehyde, obtained following a literature procedure.[17] The aldehyde was submitted to a Grignard's reaction, using allyl magnesium chloride in anhydrous tetrahydrofuran, to obtain the mixture of the two diasteroisomers **12a**,**b**. At this stage the two isomers could not be separated and were used as such in the next step. The mixture of **12a**,**b** was alkylated by using benzyl bromide/sodium hydride in anhydrous tetrahydrofuran to yield the mixture of the corresponding benzyl derivatives **13a**,**b** (Scheme 3). Also at this stage the two isomers could not be separated by column chromatography; nevertheless we could determine their 1:3 relative ratio by HRGC analysis.



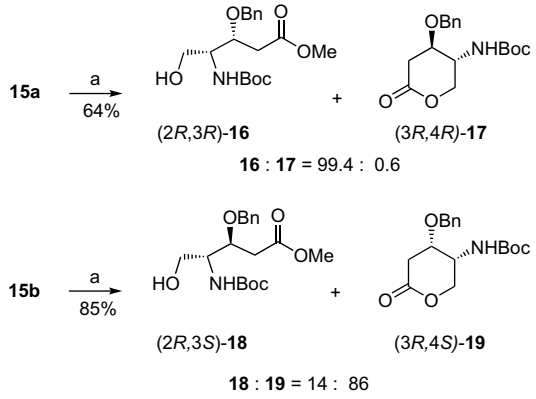
Scheme 3. (a) $CH_2{=}CHCH_2MgCl$, $ZnCl_2$, THF; (b) BnBr, NaH 60%, THF.

The mixture of intermediates **13a**,**b** was then oxidised to the corresponding diols by using 4-methylmorpholine-*N*-oxide and a 4% aqueous solution of OsO_4. The crude reaction mixture was treated with sodium periodate, to yield the aldehyde, followed by treatment with sodium chlorite and sulfamic acid to produce the mixture of carboxylic acids **14a**,**b**. After the usual reaction work-up, the mixture of **14a**,**b** was converted into the corresponding methyl esters **15a**,**b** by treating with iodomethane and potassium carbonate. At this stage, the two diasteroisomers **15a** and **15b** were separated by flash chromatography (Scheme 4).



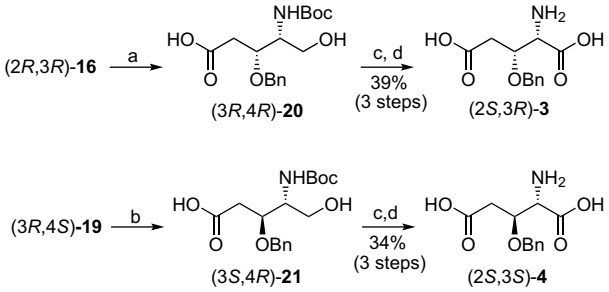
Scheme 4. (a) (i) 4-Methylmorpholine-*N*-oxide, 4% OsO_4 in H_2O, 1,4-dioxane, H_2O; (ii) $NaIO_4$; (iii) $NaOCl_2$, NH_2SO_3H; (b) CH_3I, K_2CO_3, acetone.

Compounds **15a** and **15b** were treated with a weakly acidic solution of acetic acid and water (5:1),[18] in order to remove the acetonide protective group. Compound **15a** gave almost exclusively the expected intermediate **16** with trace amounts of lactone **17**, derived from the intramolecular cyclisation of the alcohol. On the contrary, the same transformation carried out on derivative **15b** yielded lactone **19** as the major product and alcohol **18** as the byproduct (ratio **19**/**18**=86:14) (Scheme 5). This different outcome could be due to the different spatial orientation of the benzyloxy group.



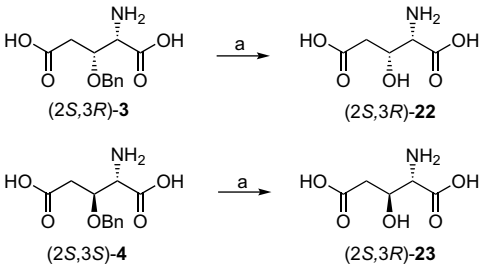
Scheme 5. (a) H_2O/AcOH (1:5).

The two major fractions, i.e., (2*R*,3*R*)-**16** and (3*R*,4*S*)-**19**, were then hydrolysed to carboxylic acids (3*R*,4*R*)-**20** and (3*S*,4*R*)-**21**, respectively. The ω-alcohol function of **20** and **21** was oxidised to the corresponding carboxylate group by using pyridinium dichromate in *N*,*N*-dimethylformamide and, subsequently, the amine was deprotected by treatment with a 30% solution of trifluoroacetic acid in dichloromethane, to give final amino acids (2*S*,3*R*)-**3** and (2*S*,3*S*)-**4** (Scheme 6).



Scheme 6. (a) NaOH 1 N, MeOH; (b) NaOH 1 N, 1,4-dioxane; (c) PDC, DMF; (d) 30% CF_3COOH/CH_2Cl_2.

In order to assign the relative stereochemistry to stereoisomeric amino acids (2*S*,3*R*)-**3** and (2*S*,3*S*)-**4** as well as to their precursors, they were submitted to hydrogenation with a catalytic amount of 5% palladium on charcoal to produce the corresponding β-hydroxy amino acids (2*S*,3*R*)-**22** and (2*S*,3*S*)-**23** (Scheme 7), whose structure was previously assigned, and their ^1H NMR spectra were compared to those reported in the literature.[19]



Scheme 7. (a) H_2, Pd/C 5%, MeOH/H_2O.

Amino acids **1**–**4** were tested as inhibitors of [^{3}H]L-glutamate uptake in a crude synaptosomal fraction (P2) of the rat brain cortex and their IC_{50} values are reported in Table 1, compared to the activity of the reference compound (±)-**TBOA**.

Table 1
Inhibition of the EAATs-mediated uptake

Compounds	[^{3}H]L-Glutamate uptake in synaptosomes IC_{50} (μM)
(2*S*,4*R*)-**1**	>1000
(2*S*,4*S*)-**2**	213.5 (103–442)[a]
(2*S*,3*R*)-**3**	187.7 (163–216)[a]
(2*S*,3*S*)-**4**	>300
(±)-**TBOA**	5.4 (4.3–6.7)[a]

[a] 95% Confidence intervals (C.I.).

As shown in Table 1, all new derivatives are significantly less active than **TBOA** as inhibitors of the Glu reuptake, demonstrating that homologation of the amino acidic chain worsens the interaction with the EAA transporter proteins. Among the four tested compounds, the most active stereoisomers (lowest IC_{50} values) are (2*S*,4*S*)-**2** and (2*S*,3*R*)-**3.** Noteworthy, compound (2*S*,3*R*)-**3** shares with **TBOA** the *threo* arrangement of the amino and the benzyloxy groups while the *erythro* counterpart (2*S*,3*S*)-**4** is basically inactive. This outcome confirms the high stereoselectivity of the interaction between the transporters and their inhibitors.

3. Conclusion

The present work describes a very efficient stereoselective preparation of the two enantiomerically pure diastereoisomers of γ-benzyloxy glutamic acid, using as a source of chirality *trans*-4-hydroxy-L-proline. Moreover, starting from (*R*)-Garner's aldehyde, the *erythro* and *threo* isomers of β-benzyloxy glutamic acid, in an enantiomeric pure form, were prepared. The four amino acids are one carbon higher homologues of **TBOA**, which is widely considered the reference compound in the field of glutamate transporter inhibitors due to its high potency as an EAAT blocker, even if it is unable to discriminate the different EAAT subtypes. Unfortunately, our new derivatives showed a markedly decreased activity as inhibitors of glutamate reuptake. Nevertheless, the four stereoisomers confirm the importance of the stereochemistry in the interaction of the inhibitors with the active site of the excitatory amino acid transporters.

4. Experimental

4.1. Material and methods

L-Glutamic acid was obtained from Sigma (St Louis, MO). [^{3}H]L-Glutamate was purchased from GE Healthcare (Chalfont St. Giles, UK). TBOA was obtained from Tocris (Ellisville, MO).

IR spectra were registered with a Perkin–Elmer FT-IR spectrometer.

^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts (δ) are expressed in parts per million and coupling constants (J) in hertz.

Rotary power determinations were carried out with a Jasco J-810 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 with ninhydrin. Melting points were determined on a model B 540 Büchi apparatus and are uncorrected. Microanalyses (C, H, N) of new compounds agreed with the theoretical value within ±0.4%.

The HRGC-FID analysis was performed using a Trace GC (Thermo Electron) equipped with a split–splitless injector and flame ionisation detector (FID). The column used was a Varian VF-5ms (5% phenyl 95% dimethylpolysiloxane) fused-silica capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness). He was used as the carrier gas at a flow rate of 1.0 mL/min. The temperature was programmed as follows: from 70 °C to 180 °C at 40 °C/min, from 180 °C to 300 °C at 10 °C/min, then at 300 °C for 7.25 min. The injector and detector temperatures were 250 and 280 °C, respectively. The injector was operated in split mode with a split rate of 30:1.

4.2. Synthesis of (2*S*,4*R*)-1-*tert*-butyl 2-methyl 4-acetoxypyrrolidine-1,2-dicarboxylate (2*S*,4*R*)-6

To a solution of compound (2*S*,4*R*)-**5** (245 mg, 1.00 mmol) in MeCN (2.5 mL) were added DMAP (61 mg, 0.50 mmol) and Ac_2O (0.38 mL, 4.00 mmol). The reaction mixture was stirred for 2 h at room temperature. The solvent was evaporated and the crude material was purified by column chromatography (eluent: petroleum ether/AcOEt 8:2), obtaining 278 mg of compound (2*S*,4*R*)-**6** (0.97 mmol, 97% yield).

4.2.1. Compound (2*S*,4*R*)-*6*

Pale yellow oil; R_f 0.37 (petroleum ether/AcOEt 8:2); $[\alpha]_D^{20}$ −42.3 (*c* 1.00, $CHCl_3$); IR (neat, cm^{-1}) ν_{max}: 3339, 2853, 1734, 1654, 1426, 1232, 1161; ^{1}H NMR (DMSO-d_6, T=100 °C): 1.38 (s, 9H), 2.00 (s, 3H), 2.18 (m, 1H), 2.31 (m, 1H), 3.44 (d, J=12.0, 1H), 3.60 (dd, J=4.9, 12.0, 1H), 3.68 (s, 3H), 4.28 (t, J=7.6, 1H), 5.20 (ddd, J=2.9, 4.9, 7.9, 1H); ^{13}C NMR (DMSO-d_6, T=100 °C): 21.01, 28.8, 39.2, 52.3, 55.1, 58.4, 68.9, 79.4, 154.3, 172.0, 174.5. Anal. Calcd for $C_{13}H_{21}NO_6$ (287.21): C, 54.35; H, 7.37; N, 4.88. Found: C, 54.49; H, 7.52; N, 4.75.

4.3. Synthesis of (2*S*,4*R*)-1-*tert*-butyl 2-methyl 4-acetoxy-5-oxopyrrolidine-1,2-dicarboxylate (2*S*,4*R*)-8

To a magnetically stirred 10% aqueous solution of $NaIO_4$ (10 mL), hydrated RuO_2 (13 mg, 0.10 mmol) was added. Such a mixture was immediately poured into a well stirred solution of (2*S*,4*R*)-6 (278 mg, 0.97 mmol) in AcOEt (2.5 mL) in an Erlenmeyer flask. The resulting biphasic mixture was stirred for 48 h at room temperature. Isopropanol was added until the solution became black. The mixture was filtered through a Celite pad and washed with AcOEt. The aqueous layer was extracted with AcOEt (3×15 mL). The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated under vacuum. The crude material was purified by flash chromatography (eluent: petroleum ether/AcOEt 7:3) to give 260 mg of compound (2*S*,4*R*)-**8** (89% yield).

4.3.1. Compound (2*S*,4*R*)-*8*

Colourless oil; R_f: 0.31 (petroleum ether/AcOEt 6:4); $[\alpha]_D^{20}$ +20.8 (*c* 1.00, $CHCl_3$); IR (neat, cm^{-1}) ν_{max}: 2980, 1800, 1750, 1371, 1288, 1228, 1151; ^{1}H NMR (DMSO-d_6, T=100 °C): 1.40 (s, 9H), 2.05 (s, 3H), 2.36 (ddd, J=9.9, 9.9, 13.4, 1H), 2.49 (ddd, J=1.9, 8.8, 13.4, 1H), 3.75 (s, 3H), 4.70 (dd, J=1.9, 9.9, 1H), 5.35 (dd, J=8.8, 9.9, 1H); ^{13}C NMR (DMSO-d_6, T=100 °C): 20.7, 28.2, 53.0, 55.8, 56.0, 69.8, 83.9, 149.1, 168.7, 169.7, 171.7. Anal. Calcd for $C_{13}H_{19}NO_7$ (301.29): C, 51.82; H, 6.36; N, 4.65. Found: C, 52.02; H, 6.53; N, 4.54.

4.4. Synthesis of *tert*-butyl (1*S*,3*R*)-1,3-di(methoxycarbonyl)-3-(benzyloxy)propylcarbamate (2*S*,4*R*)-9

(a) To a solution of compound (2*S*,4*R*)-**8** (260 mg, 0.86 mmol) in 1.5 mL of methanol was added PS-CO_3 (245 mg, 0.86 mmol, 3.5 mmol/g). The reaction mixture was stirred for 1 h at room temperature. PS-CO_3 was filtered off and washed with MeOH. The solvent was evaporated to give a pale yellow oil (227 mg, 0.78 mmol), which was directly submitted to the next step.

(b) The crude derivative obtained from the previous step (227 mg, 0.78 mmol) was dissolved in 3 mL of anhydrous Et_2O and benzyl bromide (0.093 mL, 0.78 mmol) followed by freshly prepared Ag_2O (180 mg, 0.78 mmol) were added. The reaction mixture was stirred for 48 h at room temperature. After disappearance of the starting material, Ag_2O was filtered off and washed with Et_2O. The solvent was evaporated and the crude material was purified by column chromatography (eluent: petroleum ether/AcOEt 9:1) to give 230 mg of compound (2*S*,4*R*)-**9** (70% overall yield).

4.4.1. Compound (2*S*,4*R*)-*9*

Crystallised from diisopropyl ether as white prisms; mp>234 °C dec; R_f: 0.42 (petroleum ether/AcOEt 8:2); $[\alpha]_D^{20}$ +82.3 (*c* 1.00, $CHCl_3$); IR (neat, cm^{-1}) ν_{max}: 3368, 2977, 1750, 1718, 1522, 1368, 1224, 1162; ^{1}H NMR (DMSO-d_6, T=100 °C): 1.38 (s, 9H), 2.05 (ddd, J=7.0, 7.6, 14.3, 1H), 2.18 (ddd, J=5.2, 6.4, 14.3, 1H), 3.56 (s, 3H), 3.67 (s, 3H), 4.14 (dd, J=5.2, 7.0, 1H), 4.18 (dd, J=6.4, 7.6, 1H), 4.44 (d, J=11.1, 1H), 4.58 (d, J=11.1, 1H), 6.72 (br s, 1H), 7.25–7.35 (m, 5H); ^{13}C NMR (DMSO-d_6, T=100 °C): 28.8, 34.9, 50.5, 52.4, 52.5, 72.2, 75.2, 79.0, 128.3, 128.5, 128.8, 138.2, 155.8, 172.5, 172.9. Anal. Calcd for $C_{19}H_{27}NO_7$ (381.42): C, 59.83; H, 7.14; N, 3.67. Found: C, 59.83; H, 7.20; N, 3.64.

4.5. Synthesis of (2*S*,4*R*)-2-amino-4-(benzyloxy)pentanedioic acid (2*S*,4*R*)-1

(a) Compound (2*S*,4*R*)-**9** (230 mg, 0.60 mmol) was dissolved in MeOH (2 mL) and 1 N NaOH (0.60 mL) was added. The mixture was stirred overnight at room temperature. The solvent was evaporated, water (6 mL) was added and the aqueous phase was extracted with Et_2O (1×4 mL). The aqueous layer was made acidic with 2 N HCl and newly extracted with AcOEt (4×3 mL). The organic extracts were pooled and dried over anhydrous sodium sulfate. The solvent was removed under vacuum to give 212 mg (100% yield) of the carboxylic acid intermediate, which was directly submitted to next step.

(b) The crude derivative obtained from the previous step (212 mg, 0.60 mmol) was treated with a 30% dichloromethane solution of trifluoroacetic acid (1.60 mL) at 0 °C. The reaction mixture was stirred at room temperature until disappearance of the starting material (3 h). The volatiles were removed under vacuum and the residue was taken up with acetone and filtered under vacuum to give 89 mg (58% yield) of (2*S*,4*R*)-**1** as white foam.

4.5.1. Compound (2*S*,4*R*)-*1*

White foam (hygroscopic); R_f: 0.46 (BuOH/H_2O/AcOH 4:2:1); $[\alpha]_D^{20}$ +53.5 (*c* 0.55, H_2O); IR (neat, cm^{-1}) ν_{max}: 3246, 2361, 2343, 1718, 1429, 1267, 1214, 1118; ^{1}H NMR (DMSO-d_6): 2.02 (ddd, J=4.6, 10.2, 14.6, 1H), 2.16 (ddd, J=3.2, 8.5, 14.6, 1H), 3.82 (dd, J=4.6, 8.5, 1H), 4.23 (dd, J=3.2, 10.2, 1H), 4.42 (d, J=11.1, 1H), 4.58 (d, J=11.1, 1H), 7.22–7.40 (m, 5H); ^{13}C NMR (DMSO-d_6): 34.2, 50.5, 72.2, 75.5,

128.1, 128.3, 128.8, 138.5, 171.2, 173.6. Anal. Calcd for $C_{12}H_{15}NO_5$ (253.25): C, 56.91; H, 5.97; N, 5.53. Found: C, 56.72; H, 6.03; N, 5.48.

4.6. Synthesis of (2*S*,4*S*)-1-*tert*-butyl 2-methyl 4-acetoxypyrrolidine-1,2-dicarboxylate (2*S*,4*S*)-7

To a solution of compound (2*S*,4*R*)-**5** (245 mg, 1.00 mmol) in anhydrous THF (15 mL) was added triphenylphosphine (1.0 g, 4.00 mmol) followed by glacial acetic acid (0.114 mL, 2.00 mmol). The mixture was cooled to 0 °C and a solution of diethylazadicarboxylate (solution 40% in toluene, 1.7 mL, 4.00 mmol) in 4 mL of anhydrous THF was added dropwise over 10 min. The reaction mixture was allowed to warm at room temperature and stirred for 6 h. The solvent was evaporated and the crude material was purified by flash chromatography (eluent: petroleum ether/AcOEt 8:2) to give 273 mg of compound (2*S*,4*S*)-**7** (95% yield).

4.6.1. Compound (2*S*,4*S*)-*7*

Pale yellow oil; R_f: 0.20 (petroleum ether/AcOEt 8:2); $[\alpha]_D^{20}$ −34.0 (*c* 1.00, $CHCl_3$); IR (neat, cm^{-1}) ν_{max}: 3341, 2852, 1736, 1651, 1429, 1230, 1158; ^1H NMR (DMSO-d_6, *T*=100 °C): 1.38 (s, 9H), 1.85 (s, 3H), 2.05 (m, 1H), 2.54 (ddd, *J*=5.5, 9.3, 14.0, 1H), 3.32 (dd, *J*=2.5, 12.0, 1H), 3.66 (s, 3H), 3.68 (dd, *J*=5.8, 12.0, 1H), 4.34 (dd, *J*=2.9, 9.3, 1H), 5.15 (ddd, *J*=2.5, 5.8, 8.2, 1H); ^{13}C NMR (DMSO-d_6, *T*=100 °C): 21.1, 28.4, 35.9, 52.1, 52.3, 57.9, 72.6, 79.9, 153.7, 170.2, 172.0. Anal. Calcd for $C_{13}H_{21}NO_6$ (287.21): C, 54.35; H, 7.37; N, 4.88. Found: C, 54.47; H, 7.50; N, 4.76.

4.7. Synthesis of (2*S*,4*S*)-1-*tert*-butyl 2-methyl 4-acetoxy-5-oxopyrrolidine-1,2-dicarboxylate (2*S*,4*S*)-10

The same procedure used for the synthesis of (2*S*,4*R*)-1-*tert*-butyl 2-methyl 4-acetoxy-5-oxopyrrolidine-1,2-dicarboxylate (2*S*,4*R*)-**8** was applied to compound (2*S*,4*S*)-**7** (273 mg, 0.95 mmol) to give compound (2*S*,4*S*)-**10** (263 mg, 92% yield).

4.7.1. Compound (2*S*,4*S*)-*10*

Crystallised from diisopropyl ether as white prisms, mp 68–71 °C; R_f: 0.26 (petroleum ether/AcOEt 8:2); $[\alpha]_D^{20}$ −33.8 (*c* 1.07, $CHCl_3$); IR (neat, cm^{-1}) ν_{max}: 2981, 1797, 1750, 1370, 1291, 1220, 1153; ^1H NMR ($CDCl_3$): 1.50 (s, 9H), 1.96 (ddd, *J*=8.4, 8.4, 13.5, 1H), 2.14 (s, 3H), 2.82 (ddd, *J*=8.4, 8.4, 13.5, 1H), 3.80 (s, 3H), 4.50 (dd, *J*=8.4, 8.4, 1H), 5.38 (dd, *J*=8.4, 8.4, 1H); ^{13}C NMR ($CDCl_3$): 20.7, 28.5, 52.8, 55.6, 55.7, 69.7, 84.6, 149.0, 168.4, 169.9, 170.9. Anal. Calcd for $C_{13}H_{19}NO_7$ (301.29): C, 51.82; H, 6.36; N, 4.65. Found: C, 51.98; H, 6.49; N, 4.56.

4.8. Synthesis of *tert*-butyl (1*S*,3*S*)-1,3-di(methoxycarbonyl)-3-(benzyloxy)propylcarbamate (2*S*,4*S*)-11

The same procedure used for the synthesis of *tert*-butyl (1*S*,3*R*)-1,3-di(methoxycarbonyl)-3-(benzyloxy)propylcarbamate (2*S*,4*R*)-**9** was applied to compound (2*S*,4*S*)-**10** (263 mg, 0.87 mmol) to give compound (2*S*,4*S*)-**11** (249 mg, 75% yield).

4.8.1. Compound (2*S*,4*S*)-*11*

Colourless oil; R_f: 0.31 (petroleum ether/AcOEt 8:2); $[\alpha]_D^{20}$ −35.5 (*c* 1.00, $CHCl_3$); IR (neat, cm^{-1}) ν_{max}: 3369, 2976, 1744, 1718, 1498, 1366, 1210, 1163; ^1H NMR (DMSO-d_6, *T*=100 °C): 1.40 (s, 9H), 2.08 (m, 2H), 3.60 (s, 3H), 3.70 (s, 3H), 4.06 (dd, *J*=6.0, 8.2, 1H), 4.30 (m, 1H), 4.42 (d, *J*=11.2, 1H), 4.62 (d, *J*=11.2, 1H), 6.80 (br s, 1H), 7.20–7.35 (m, 5H); ^{13}C NMR (DMSO-d_6, *T*=100 °C): 28.7, 34.8, 51.1, 52.1, 52.3, 72.8, 75.6, 79.1, 128.3, 128.8, 129.2, 138.3, 155.9, 172.5, 173.0. Anal. Calcd for $C_{19}H_{27}NO_7$ (381.42): C, 59.83; H, 7.14; N, 3.67. Found: C, 59.83; H, 7.23; N, 3.62.

4.9. Synthesis of (2*S*,4*S*)-2-amino-4-(benzyloxy)pentanedioic acid (2*S*,4*S*)-2

The same procedure used for the synthesis of (2*S*,4*R*)-2-amino-4-(benzyloxy)pentanedioic acid (2*S*,4*R*)-**1** was applied to compound (2*S*,4*S*)-**11** (249 mg, 0.65 mmol) to give compound (2*S*,4*S*)-**2** (93 mg, 57% yield).

4.9.1. Compound (2*S*,4*S*)-*2*

White foam (hygroscopic); R_f: 0.50 ($BuOH/H_2O/AcOH$ 4:2:1); $[\alpha]_D^{20}$ −40.3 (*c* 0.255, H_2O); IR (neat, cm^{-1}) ν_{max}: 3247, 2359, 2340, 1718, 1425, 1259, 1216, 1128; ^1H NMR (DMSO-d_6): 2.33 (m, 2H), 3.92 (dd, *J*=6.9, 6.9, 1H), 4.26 (dd, *J*=5.5, 7.9, 1H), 4.51 (d, *J*=11.2, 1H), 4.60 (d, *J*=11.2, 1H), 7.22–7.42 (m, 5H); ^{13}C NMR (DMSO-d_6): 34.0, 49.9, 71.8, 74.4, 128.2, 128.4, 128.8, 138.5, 171.1, 173.3. Anal. Calcd for $C_{12}H_{15}NO_5$ (253.25): C, 56.91; H, 5.97; N, 5.53. Found: C, 56.74; H, 6.03; N, 5.48.

4.10. Synthesis of (*R*)-*tert*-butyl 4-((*R*)-1-hydroxybut-3-enyl)-2,2-dimethyloxazolidine-3-carboxylate 12a and (*R*)-*tert*-butyl 4-((*S*)-1-hydroxybut-3-enyl)-2,2-dimethyloxazolidine-3-carboxylate 12b

To a stirred mixture of (*R*)-Garner aldehyde (3.5 g, 15.3 mmol) and $ZnCl_2$ (3.2 g, 22.9 mmol) in anhydrous THF (32 mL) at −78 °C, was added dropwise a solution of vinyl magnesium bromide 2 M in THF (11.4 mL, 22.9 mmol). The suspension was allowed to warm at room temperature and then stirred for additional 2 h. The reaction was quenched with a saturated solution of NH_4Cl (20 mL) and the aqueous layer was extracted with Et_2O (3×30 mL). The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated under vacuum. The crude material was purified by flash chromatography (eluent: petroleum ether/AcOEt 85:15) to give compounds **12a** and **12b** as an unsplittable mixture (3.6 g, 88% yield).

Compounds **12a**,**b:** pale yellow oil; R_f: 0.20 (petroleum ether/AcOEt 85:15).

4.11. Synthesis of (*R*)-*tert*-butyl 4-((*R*)-1-(benzyloxy)but-3-enyl)-2,2-dimethyloxazolidine-3-carboxylate 13a and (*R*)-*tert*-butyl 4-((*S*)-1-(benzyloxy)but-3-enyl)-2,2-dimethyloxazolidine-3-carboxylate 13b

To a solution of **12a**,**b** (3.6 g, 13.4 mmol) in anhydrous THF (26 mL) was added NaH (60% in mineral oil, 804 mg, 20.1 mmol) at 0 °C and the mixture was stirred for 10 min under nitrogen atmosphere. Then, benzyl bromide (2.4 mL, 20.1 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 12 h and then was quenched with water (20 mL). The aqueous layer was extracted with Et_2O (3×20 mL). The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated under vacuum. The crude material was purified by flash chromatography (eluent: petroleum ether/AcOEt 98:2) to give an unsplittable mixture of compounds **13a** and **13b** (3.0 g, 63% yield).

Compounds **13a**,**b:** yellow oil; R_f: 0.19 (petroleum ether/AcOEt 99:1).

4.12. Synthesis of (*R*)-*tert*-butyl 4-((*R*)-2-(methoxycarbonyl)-1-(benzyloxy)ethyl)-2,2-dimethyloxazolidine-3-carboxylate 15a and (*R*)-*tert*-butyl 4-((*S*)-2-(methoxycarbonyl)-1-(benzyloxy)-ethyl)-2,2-dimethyloxazolidine-3-carboxylate 15b

(a) To a stirred solution of **13a**,**b** (3.0 g, 8.4 mmol) in dioxane (12 mL) and H_2O (12 mL) were added 4-methyl-morpholine *N*-oxide (1.5 g, 12.6 mmol) and osmium tetraoxide (4% in H_2O, 1.1 mL). After stirring at room temperature for 1 h, $NaIO_4$ (2.7 g, 12.6 mmol) was added, and the suspension was stirred at room temperature for additional 1.5 h. The reaction mixture was cooled to 0 °C and sodium chlorite (3.0 g, 33.6 mmol) and sulfamic acid (3.3 g,

33.6 mmol) were added, and the resulting mixture was removed from the cold bath and stirred for 2 h. To the mixture was added 5% aqueous HCl (10 mL), and the resulting solution was extracted with CH_2Cl_2 (3×20 mL). The combined organic extracts were washed with 5% aqueous HCl, dried over Na_2SO_4 and the solvent was evaporated under vacuum to give the mixture of the acids as a yellow oil (2.7 g, 7.1 mmol).

(b) The crude material was treated with iodomethane (0.88 mL, 14.2 mmol) and K_2CO_3 (1.9 g, 14.2 mmol) in refluxing acetone (20 mL) for 1 h. The solvent was evaporated under reduced pressure and the residue was dissolved in H_2O (20 mL); the aqueous layer was extracted with Et_2O (3×15 mL) and the organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated under vacuum. The crude material was purified by flash chromatography (eluent: petroleum ether/AcOEt 95:5) to give compounds **15a** (0.55 g, 1.40 mmol) and **15b** (1.65 g, 4.20 mmol). Overall yield: 67%.

*4.12.1. Compound **15a***

Crystallised from hexane as white prisms; mp 57–58 °C; R_f: 0.33 (petroleum ether/AcOEt 9:1); $[\alpha]_D^{20}$ +7.3 (*c* 0.98, $CHCl_3$); IR (neat, cm^{-1}) ν_{max}: 2978, 1739, 1697, 1454, 1366, 1254, 1172, 1096, 1072; ^1H NMR (DMSO-d_6, 100 °C): 1.40 (s, 3H), 1.42 (s, 9H), 1.50 (s, 3H), 2.44 (m, 1H), 2.57 (dd, *J*=3.8, 15.9, 1H), 3.58 (s, 3H), 3.88–4.02 (m, 2H), 4.14 (m, 1H), 4.30 (m, 1H), 4.50 (d, *J*=11.8, 1H), 4.58 (d, *J*=11.8, 1H), 7.20–7.38 (m, 5H); ^{13}C NMR (DMSO-d_6): 22.8, 26.5, 28.6, 35.7, 52.0, 55.5, 57.1, 63.5, 72.1, 75.8, 80.1, 94.3, 128.0, 128.1, 128.8, 138.9, 152.1, 172.4. Anal. Calcd for $C_{21}H_{31}NO_6$ (393.47): C, 64.10; H, 7.94; N, 3.56. Found: C, 64.49; H, 8.14; N, 3.49.

*4.12.2. Compound **15b***

Colourless oil; R_f: 0.25 (petroleum ether/AcOEt 9:1); $[\alpha]_D^{20}$ +30.1 (*c* 1.02, $CHCl_3$); IR (neat, cm^{-1}) ν_{max}: 2980, 1738, 1697, 1450, 1368, 1251, 1166, 1091, 1067; ^1H NMR (DMSO-d_6, 100 °C): 1.42 (s, 15H), 2.54 (m, 2H), 3.59 (s, 3H), 3.88–3.98 (m, 3H), 4.26 (m, 1H), 4.54 (s, 2H), 7.20–7.35 (m, 5H); ^{13}C NMR (DMSO-d_6): 23.7, 26.9, 28.7, 37.7, 52.1, 60.3, 64.0, 73.0, 75.6, 80.1, 94.2, 128.2, 128.5, 128.8, 138.9, 152.0, 172.0. Anal. Calcd for $C_{21}H_{31}NO_6$ (393.47): C, 64.10; H, 7.94; N, 3.56. Found: C, 64.44; H, 8.11; N, 3.50.

4.13. Synthesis of *tert*-butyl (2*R*,3*R*)-4-(methoxycarbonyl)-3-(benzyloxy)-1-hydroxybutan-2-yl carbamate (2*R*,3*R*)-16

Compound **15a** (550 mg, 1.40 mmol) was dissolved in a 1:5 mixture of H_2O and acetic acid (14 mL). The solution was stirred at room temperature for 24 h. After disappearance of the starting material, the solvent was evaporated under reduced pressure and the crude material was purified by flash chromatography (eluent: petroleum ether/AcOEt 7:3), obtaining the amino alcohol (2*R*,3*R*)-**16**, as the major product (317 mg, 64% yield) and lactone (3*R*,4*R*)-**17** as the minor product (0.4% yield).

*4.13.1. Compound (2R,3R)-**16***

Colourless oil; R_f: 0.21 (petroleum ether/AcOEt 7:3); $[\alpha]_D^{20}$ +4.60 (*c* 0.99, $CHCl_3$); IR (neat, cm^{-1}) ν_{max}: 3338, 2923, 2852, 1718, 1499, 1458, 1366, 1248, 1165, 1061; ^1H NMR ($CDCl_3$): 1.42 (s, 9H), 2.59 (dd, *J*=6.0, 16.2, 1H), 2.69 (dd, *J*=6.8, 16.2, 1H), 3.60 (m, 1H), 3.65 (s, 3H), 3.70–3.83 (m, 2H), 4.23 (m, 1H), 4.51 (d, *J*=11.0, 1H), 4.63 (d, *J*=11.0, 1H), 5.04 (br d, *J*=8.8, 1H), 7.25–7.40 (m, 5H); ^{13}C NMR ($CDCl_3$): 28.5, 37.0, 52.0, 55.1, 63.3, 73.3, 75.3, 80.0, 128.1, 128.2, 128.7, 137.9, 156.6, 172.1. Anal. Calcd for $C_{18}H_{27}NO_6$ (353.41): C, 61.17; H, 7.70; N, 3.96. Found: C, 61.14; H, 7.86; N, 3.85.

4.14. Synthesis of (2*S*,3*R*)-2-amino-3-(benzyloxy)pentanedioic acid (2*S*,3*R*)-3

(a) Compound (2*S*,3*R*)-**16** (317 mg, 0.90 mmol) was dissolved in MeOH (2 mL) and 1 N NaOH (0.9 mL) was added. The mixture was stirred at room temperature for 3 h. The solvent was evaporated, water (6 mL) was added and the aqueous phase was extracted with Et_2O (1×4 mL). The aqueous layer was made acidic with 2 N HCl and newly extracted with AcOEt (4×3 mL). The organic extracts were pooled and dried over anhydrous sodium sulfate. The solvent was removed under vacuum to give 298 mg (98% yield) of the carboxylic acid intermediate, which was directly submitted to next step.

(b) To a solution of the crude derivative obtained from the previous step (298 mg, 0.88 mmol) in DMF (4.4 mL) pyridinium dichromate (PDC) (4.4 g, 13.2 mmol) was added and the mixture was stirred at room temperature for 4 h. The progress of the reaction was monitored by TLC ($CHCl_3$/MeOH 9:1+1% acetic acid). Water was added (6 mL) and the mixture was extracted with AcOEt (3×5 mL). The organic layer was extracted with $NaHCO_3$ (3×5 mL) and the aqueous phase was made acidic with 2 N HCl and extracted with AcOEt (3×5 mL). The organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and the solvent evaporated to give the carboxylic acid (282 mg, 90% yield).

(c) The intermediate from the previous step (282 mg, 0.81 mmol) was treated with a 30% CH_2Cl_2 solution (0.15 mL) of trifluoroacetic acid (0.048 mL, 7.8 mmol) at 0 °C. The solution was stirred at room temperature for 4 h until disappearance of the starting material. The volatiles were removed under vacuum and the residue was taken up with MeOH, filtered, washed with MeOH and Et_2O, and dried under vacuum to give amino acid (2*S*,3*R*)-**3** (79 mg, 39% yield).

*4.14.1. Compound (2S,3R)-**3***

White powder; mp>155 °C dec; R_f: 0.35 (BuOH/H_2O/AcOH 3:2:1); $[\alpha]_D^{20}$ +3.3 (*c* 0.28, H_2O); IR (neat, cm^{-1}) ν_{max}: 2945, 1699, 1547, 1420, 1215, 1181, 1063; ^1H NMR (D_2O): 2.68 (d, *J*=6.4, 2H), 3.83 (d, *J*=3.2, 1H), 4.44 (ddd, *J*=3.2, 6.4, 6.4, 1H), 4.52 (s, 2H), 7.23–7.38 (m, 5H); ^{13}C NMR (D_2O): 37.5, 57.1, 73.1, 73.8, 128.7, 128.8, 128.9, 137.0, 171.7, 174.5. Anal. Calcd for $C_{12}H_{15}NO_5$ (253.25): C, 56.91; H, 5.97; N, 5.53. Found: C, 56.61; H, 6.07; N, 5.44.

4.15. Synthesis of *tert*-butyl (3*R*,4*S*)-4-(benzyloxy)-tetrahydro-6-oxo-2H-pyran-3-ylcarbamate (3*R*,4*S*)-19

The same procedure used for the synthesis of (2*R*,3*R*)-**16** was applied to compound **15b** (1.6 g, 4.2 mmol) to give compound (3*R*,4*S*)-**19** (984 mg, 73% yield) and compound (2*R*,3*S*)-**18** (12% yield) as the minor product.

*4.15.1. Compound (3R,4S)-**19***

Crystallised from diisopropyl ether as white prisms, mp 85–86 °C; R_f: 0.16 (petroleum ether/AcOEt 7:3); $[\alpha]_D^{20}$ +49.4 (*c* 1.00, $CHCl_3$); IR (neat, cm^{-1}) ν_{max}: 3361, 2360, 2325, 1756, 1682, 1527, 1364, 1222, 1177, 1072; ^1H NMR ($CDCl_3$): 1.42 (s, 9H), 2.70 (dd, *J*=4.1, 18.1, 1H), 2.92 (dd, *J*=3.3, 18.1, 1H), 3.92 (m, 1H), 4.14 (m, 1H), 4.22–4.42 (m, 2H), 4.50 (d, *J*=11.6, 1H), 4.70 (d, *J*=11.6, 1H), 4.84 (br s, 1H), 7.23–7.42 (m, 5H); ^{13}C NMR ($CDCl_3$): 28.5, 34.3, 46.6, 67.1, 71.3, 71.9, 80.6, 128.0, 128.5, 128.9, 137.1, 155.3, 168.3. Anal. Calcd for $C_{17}H_{23}NO_5$ (321.27): C, 63.54; H, 7.21; N, 4.36. Found: C, 63.44; H, 7.38; N, 4.25.

4.16. Synthesis of (2*S*,3*S*)-2-amino-3-(benzyloxy)pentanedioic acid (2*S*,3*S*)-4

The same procedure used for the synthesis of amino acid (2*S*,3*R*)-**3** was applied to compound (3*R*,4*S*)-**19** (984 mg, 3.0 mmol) to give compound (2*S*,3*S*)-**4** (258 mg, 34% yield).

*4.16.1. Compound (2S,3S)-**4***

White powder; mp>140 °C dec; R_f: 0.37 (BuOH/H_2O/AcOH 3:2:1); $[\alpha]_D^{20}$ −15.0 (*c* 0.25, H_2O); IR (neat, cm^{-1}) ν_{max}: 2943, 1700, 1551, 1419, 1227, 1186, 1069; ^1H NMR (D_2O): 2.50 (m, 1H), 2.53 (m, 1H), 4.08 (d, *J*=3.8, 1H), 4.34 (ddd, *J*=3.8, 5.5, 7.6, 1H), 4.52 (d, *J*=11.7, 1H), 4.62 (d, *J*=11.7, 1H), 7.25–7.38 (m, 5H); ^{13}C NMR (D_2O): 38.3, 58.4, 74.4, 74.8, 74.5, 131.3, 131.3, 131.5, 139.5, 173.5, 177.2. Anal. Calcd for $C_{12}H_{15}NO_5$ (253.25): C, 56.91; H, 5.97; N, 5.53. Found: C, 56.64; H, 6.06; N, 5.45.

4.17. Synthesis of (2*S*,3*R*)-2-amino-3-hydroxypentanedioic acid (2*S*,3*R*)-22

Amino acid (2*S*,3*R*)-**3** (30 mg, 0.12 mmol) was dissolved in methanol (1.0 mL) and a catalytic amount of 5% Pd/C was added. The mixture was hydrogenated for 10 h. After disappearance of the starting material, the catalyst was filtered off, and the solvent was evaporated under vacuum to give 19.4 mg of the corresponding hydroxy amino acid (0.12 mmol, 100% yield) as a colourless foam.

*4.17.1. Compound (2S,3R)-**22***

Mp 180–182 °C; $[\alpha]_D^{20}$ +21.0 (*c* 0.25, H_2O); ^1H NMR (D_2O): 2.55 (dd, *J*=8.80, 16.22, 1H), 2.72 (dd, *J*=4.13, 16.22, 1H), 3.66 (d, *J*=5.22, 1H), 4.39 (ddd, *J*=4.13, 5.22, 8.80, 1H); ^{13}C NMR (D_2O): 39.3, 58.8, 66.4, 172.1, 175.0. Anal. Calcd for $C_5H_9NO_5$ (163.13): C, 36.81; H, 5.56; N, 8.59. Found: C, 36.64; H, 5.59; N, 8.55.

4.18. Synthesis of (2*S*,3*S*)-2-amino-3-hydroxypentanedioic acid (2*S*,3*S*)-23

The same procedure used for the synthesis of (2*S*,3*R*)-**22** was applied to amino acid (2*S*,3*S*)-4 (30 mg, 0.12 mmol) to give compound (2*S*,3*S*)-**23** (19.4 mg, 100% yield).

*4.18.1. Compound (2S,3S)-**23***

Mp 172–174 °C dec; $[\alpha]_D^{20}$ +19.8 (*c* 0.25, H_2O); ^1H NMR (D_2O): 2.58 (d, *J*=6.74, 2H); 3.81 (d, *J*=3.52, 1H); 4.41 (ddd, *J*=3.52, 6.74, 6.74, 1H); ^{13}C NMR (D_2O): 37.7, 58.4, 66.3, 171.0, 175.2. Anal. Calcd for $C_5H_9NO_5$ (163.13): C, 36.81; H, 5.56; N, 8.59. Found: C, 36.59; H, 5.60; N, 8.53.

4.19. Biological assays

[^{3}H]Glutamate uptake was measured as previously described[20] in a crude synaptosomal fraction (P2) obtained from rat brain cortex. Adult male CRL:CD(SD)BR rats (Charles River, Calco, Italy) were used. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n.116G.U., suppl. 40, 1992 Feb 18) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, 1987 Dec 12; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996). Rats were killed by decapitation and their brain cortices homogenised in 40 volumes of ice-chilled 0.32 M sucrose, pH 7.4, in a glass homogeniser with a Teflon pestle. The homogenates were centrifuged at 1000×*g* for 5 min and the supernatants centrifuged again at 12,000×*g* for 20 min at 4 °C. The pellets (P2) were diluted to a concentration of about 3 mg of tissue/mL, wet weight, in 10 mM Tris-acetate, pH 7.4, containing 128 mM NaCl, 10 mM D-glucose, 5 mM KCl, 1.5 mM NaH_2PO_4, 1 mM $MgSO_4$ and 1 mM $CaCl_2$. Samples of 0.5 mL were preincubated for 7 min at 35 °C in a water bath with or without the compounds to be tested. Nonspecific uptake was determined in the presence of 300 μM L-glutamate. Uptake was started by adding 10 μM [^{3}H]L-glutamate (initial specific activity, 49 Ci/mmol; specific activity after isotopic dilution with unlabelled Glu, 0.049 Ci/mmol) and was stopped 4 min later by adding 2 ml of ice-chilled assay buffer. Samples were immediately filtered through 0.65 μm cellulose mixed ester filters (*Millipore Corporation, Cork, Ireland*), which were washed with 2 mL of assay buffer and counted for radioactivity in 4 mL of Ultima Gold MV (*PerkinElmer Life and Analytical Sciences, Waltham, MA*), in a Tri-Carb 2800TR rack-beta liquid scintillation counter (*PerkinElmer Life and Analytical Sciences*) with a counting efficiency of about 60%.

For each drug, the percentages inhibition of specific uptake were determined for three to six concentrations in three different experiments and the inhibition curves were fitted using the 'one-site competition' equation built into Prism version 5.0 for Windows (GraphPad Software, San Diego, CA). This analysis gives the IC_{50} (i.e., the drug concentration inhibiting specific binding by 50%), with its 95% confidence intervals (C.I.).

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