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Original article

Synthesis and pharmacological evaluation of novel conformationally constrained homologues of glutamic acid

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Abstract

Twelve novel conformationally constrained homologues of glutamic acid have been synthesized and pharmacologically characterized at ionotropic glutamate receptors (iGluRs). Synthesis of the target compounds involved 1,3-dipolar cycloaddition of nitrile oxides to suitable dipolarophiles. The structure to the compounds has been assigned by ^{1}H NMR and, in the case of derivatives (±)-**4a**, (±)-**4b**, (±)-**5a**, and (±)-**5b**, by means of an X-ray crystallographic analysis carried out on intermediate (±)-**12a**. The synthesized amino acids were found to be without affinity (K_i/$IC_{50} > 100$ μM) for iGluRs with the exception of compounds (±)-**4b** and (±)-**5b**, which showed a modest affinity for NMDA receptors ($K_i = 34$ and 13 μM, respectively). The results indicate that the increased conformational constraints introduced by the cyclopropane ring and the spiro-attached proline ring are both detrimental to the pharmacological activity.

Keywords: Glutamic acid homologues; Isoxazoline derivatives; Spirocyclic compounds; Ionotropic glutamate receptors

1. Introduction

Glutamate (Glu, Fig. 1) is the main excitatory neurotransmitter in the mammalian central nervous system (CNS) and mediates essential physiological functions [1–3]. On the other hand, an overactivation of the glutamatergic synapses causes neurotoxicity (excitotoxicity) typically associated with acute and chronic neurodegenerative diseases, e.g. cerebral ischemia, epilepsy, amyotrophic lateral sclerosis, Parkinson's and Alzheimer's diseases [1]. Both physiological and pathophysiological effects of Glu are mediated by either ligand-gated ionotropic receptors (iGluRs) or G-protein coupled receptors (mGluRs) [3]. The two receptor families are heterogeneous in nature since the iGluR family is composed of three members identified according to selective agonists, i.e. *N*-methyl-D-aspartic acid (NMDA), 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA), and kainic acid (KA), whereas the mGluR family contains eight different subtypes grouped into three, i.e. groups I, II, and III, according to protein sequence homology and signal transduction mechanisms.

Modulation of the glutamatergic pathways may represent a relevant therapeutic approach for the treatment of a number of neurodegenerative pathologies, neuropsychiatric diseases, as well as learning and memory impairments [2]. Hence, novel high affinity ligands endowed with family and subtype selectivity are demanded to better characterize the physio-pathological role of a specific iGluR or mGluR and, consequently, to uncover a defined target for pharmacological intervention.

Abbreviations: Glu, glutamate; CNS, central nervous system; iGluRs, ionotropic glutamate receptors; mGluRs, metabotropic glutamate receptors; NMDA, *N*-methyl-D-aspartic acid; AMPA, 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid; KA, kainic acid; (*R*)-AP5, (*R*)-2-amino-5-phosphonopentanoic acid; (*R*)-AP7, (*R*)-2-amino-7-phosphonoeptanoic acid.

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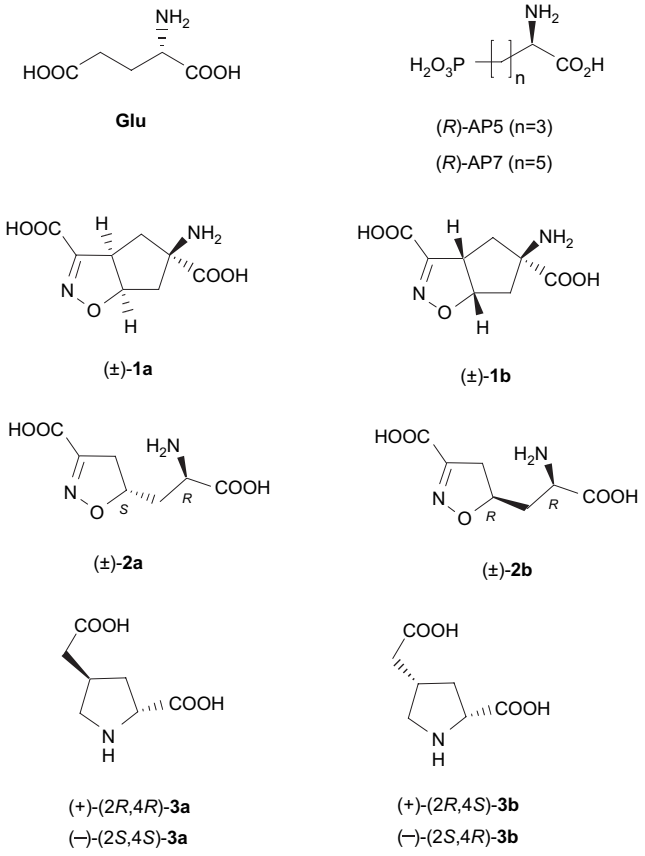


Fig. 1. Structure of Glu and some competitive NMDA receptor antagonists.

Since Glu is a flexible molecule, a number of constrained analogues have been designed, synthesized and tested with the aim to define the stereochemical requirements of the interaction between the ligand and the Glu receptor in question [2]. Such investigations have shown that an extended conformation of Glu is suitable to interact with mGluRs, whereas a folded conformation is better accommodated by the binding sites of iGluRs [4].

Homologation of the Glu chain is another strategy applied to the design of ligands selective for some Glu receptor subtypes. In particular, an increase in the distance between the amino acid moiety and the distal acidic group of Glu has led to selective NMDA antagonists [5]; the most potent NMDA antagonists bear a chain of three or five carbon atoms between the amino acid moiety and the distal acidic group. The distal acidic group can be either a carboxylate or the bioisosteric phosphonate moiety, as in (*R*)-2-amino-5-phosphonopentanoic acid [(*R*)-AP5] and (*R*)-2-amino-7-phosphonoeptanoic acid [(*R*)-AP7] (Fig. 1) [5]. Remarkably, the eutomer of the majority of competitive NMDA receptor antagonists has the *R* configuration at the amino acidic stereogenic center, which is opposite to that of Glu, the endogenous neurotransmitter [6].

On the basis of the above described strategies we previously designed two Glu homologues with a conformation locked by a bicyclic system [(±)-**1a** and (±)-**1b**, Fig. 1] [7]. These two diastereoisomeric amino acids behaved as antagonists at mGluR1,5 and as agonists at mGluR2. Furthermore, whereas (±)-**1a** turned out to be inactive at all ionotropic glutamate receptors, (±)-**1b** displayed a quite potent antagonism at the NMDA receptors [7]. In order to deepen the knowledge of the relationship between conformation and activity, we then prepared and tested derivatives (±)-**2a** and (±)-**2b** which are analogues of (±)-**1a** and (±)-**1b**, characterized by a flexible structure [8]. These two diastereoisomeric amino acids turned out to be inactive at all mGluRs but very potent and selective NMDA antagonists [8].

Since the amino acidic chain appended at the isoxazoline moiety is totally locked in derivatives (±)-**1a** and (±)-**1b** and capable to assume several different conformations in their analogues (±)-**2a** and (±)-**2b**, we planned to generate new compounds characterized by an intermediate flexibility. Furthermore, since it has been reported that three out of the four diastereoisomeric 4-(carboxymethyl)prolines, i.e. (−)-**3a**, (+)-**3b**, and (−)-**3b**, are provided with a moderate affinity for the NMDA receptors [9], we found attractive to project conformationally rigid analogues. Worth noting, the most potent stereoisomer (−)-**3b** is characterized by the *S* configuration at the amino acidic stereogenic center.

To reach the above described goals, we designed amino acids (±)-**4a** and (±)-**4b** (Fig. 2), which are characterized by the presence of a cyclopropane ring, as well as compounds (5*R*,8*S*)-**6a**, (5*S*,8*S*)-**6b**, (5*S*,8*R*)-**6a**, (5*R*,8*R*)-**6b** (Fig. 2), in which the distal carboxylate group is replaced by the spiro 3-hydroxy-2-isoxazoline moiety which, in analogy to literature precedents [10,11], is assumed as a carboxylate bioisoster. Furthermore, to enlarge our knowledge on the relationship between activity at NMDA receptors and distance among the two pharmacophoric entities, we designed the corresponding lower homologues of compounds (±)-**4a**, (±)-**4b**, i.e. derivatives (±)-**5a** and (±)-**5b**, as well as the higher homologues of

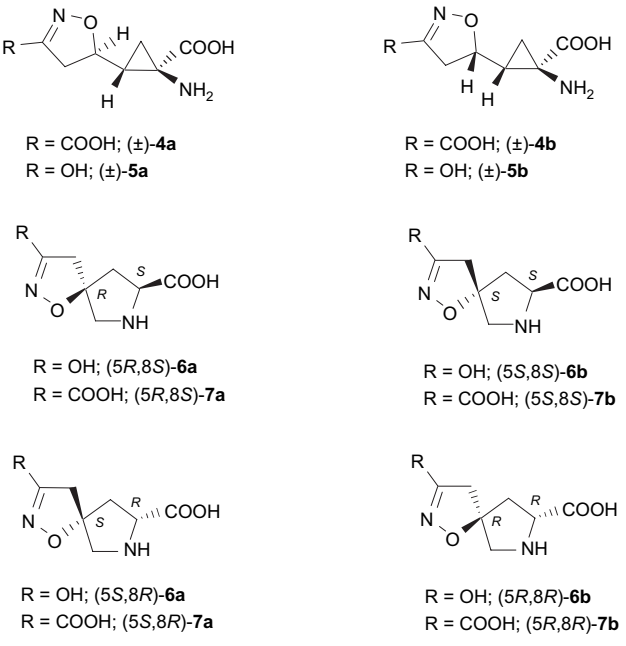


Fig. 2. Target compounds.

compounds **6a** and **6b**, i.e. derivatives (5*R*,8*S*)-**7a**, (5*S*,8*S*)-**7b**, (5*S*,8*R*)-**7a**, and (5*R*,8*R*)-**7b** (Fig. 2).

This paper reports the synthesis and the pharmacological evaluation of stereoisomers of amino acids **4**–**7** (Fig. 2). Affinity of all compounds for iGluRs was investigated by binding assays using [^{3}H]CGP39653, [^{3}H]AMPA and [^{3}H]KA as radioligands for NMDA, AMPA and KA receptors, respectively. Functional activity of compounds with significant affinity was further investigated in a calcium imaging assay.

2. Chemistry

Racemic amino acids (±)-**4a**, (±)-**4b**, (±)-**5a** and (±)-**5b** were synthesized by means of a 1,3-dipolar cycloaddition of dipolarophile (±)-**8**, prepared according to a reported procedure [12], with either ethyl 2-chloro-2-(hydroxyimino)acetate **9** [13] or dibromoformaldoxime **10** [14]. The mixtures of intermediate cycloadducts (±)-**11a**/(±)-**11b** and (±)-**12a**/(±)-**12b** were separated by column chromatography and then transformed into target amino acids through standard reactions (Scheme 1). Since the structure of (±)-**8** is known from literature [12] and its stereochemistry is maintained during the pericyclic reaction, in order to characterize the structure of the pair of cycloadducts (±)-**11a**/(±)-**11b** and (±)-**12a**/(±)-**12b** we needed to assign the relative configuration between C-5 and C-β. Unfortunately, the ^{1}H NMR coupling constant $J_{\beta,5}$ is similar in both stereoisomer pairs (±)-**11a**/(±)-**11b** and (±)-**12a**/(±)-**12b**, i.e. $J_{\beta,5} = 9.5$ Hz for both **11a** and **11b**, $J_{\beta,5} = 8.1$ Hz for **12a** versus $J_{\beta,5} = 9.5$ Hz for **12b**. Consequently, this parameter could not be used in the assignment of the relative stereochemistry. Instead, an X-ray crystallographic analysis allowed the unequivocal assignment of the structure to cycloadduct (±)-**12a** (Fig. 3) [15]. Subsequently, the structure of the pair of stereoisomers (±)-**11a**/(±)-**11b** was assigned by comparing their ^{1}H NMR patterns with those of the related couple (±)-**12a**/(±)-**12b**.

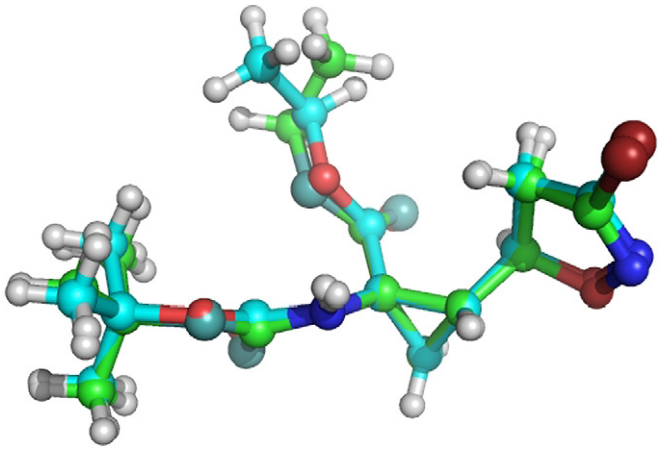
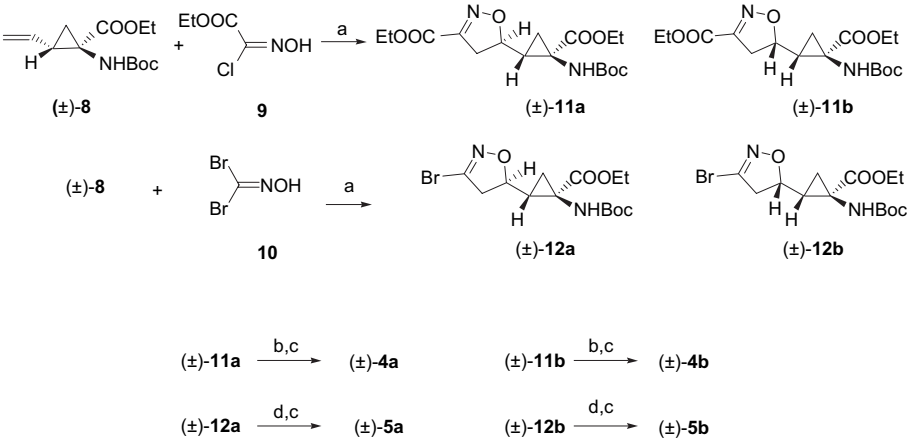


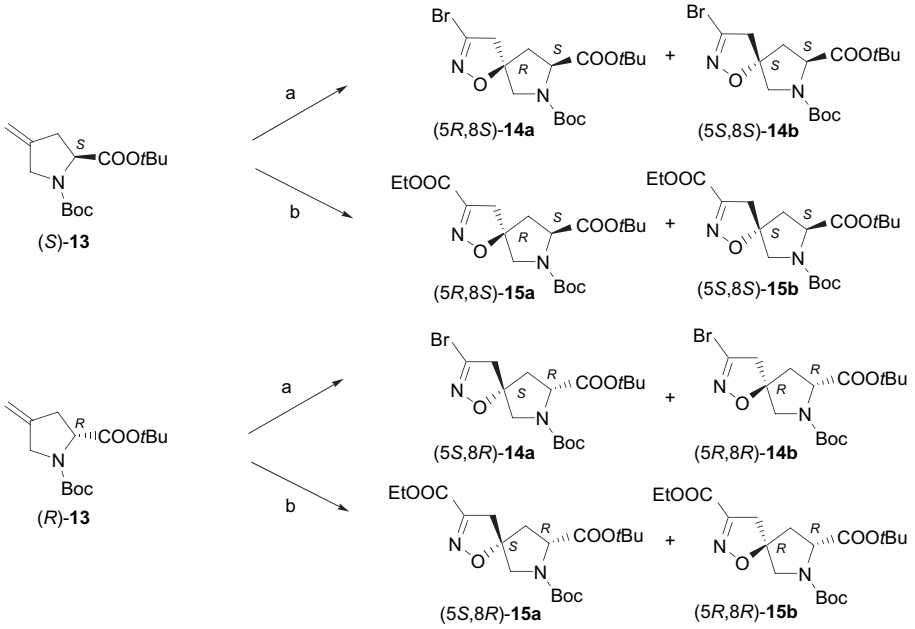
Fig. 3. Perspective view [15] of the molecular structure of compound (±)-**12a**. The two molecules of the asymmetric unit are superimposed. The differences in conformation between the two molecules are observed mainly in the ethyl ester moiety. Green: molecule A, cyan: molecule B. (For interpretation of the references to colour in figure legends, the reader is refered to the web version of this article).

The synthetic strategy described above was also applied to the preparation of amino acids **6a**, **6b**, **7a** and **7b** as single enantiomers. Bromonitrile oxide and ethoxycarbonylformonitrile oxide, respectively, were reacted with either (*S*)-**13** [16] or (*R*)-**13** [16] to yield a 1:3 mixture of stereoisomers (5*R*,8*S*)-**14a**/(5*S*,8*S*)-**14b** [(5*S*,8*R*)-**14a**/(5*R*,8*R*)-**14b**], and a 1:4 mixture of (5*R*,8*S*)-**15a**/(5*S*,8*S*)-**15b** [(5*S*,8*R*)-**15a**/(5*R*,8*R*)-**15b**] as depicted in Scheme 2. It is worth pointing out that the chromatographic separation of the two stereoisomeric cycloadducts was made possible only when the proline moiety carried the *tert*-butyl ester. The intermediate cycloadducts were transformed into final amino acids (5*R*,8*S*)-**6a**, (5*S*,8*S*)-**6b**, (5*S*,8*R*)-**6a**, (5*R*,8*R*)-**6b**, (5*R*,8*S*)-**7a**, (5*S*,8*S*)-**7b**, (5*S*,8*R*)-**7a**, and (5*R*,8*R*)-**7b** following the reaction sequence reported in Scheme 2. The configuration of the new stereogenic center was assigned by comparing the ^{1}H NMR spectra of the two stereoisomeric cycloadducts, i.e. (5*R*,8*S*)-**14a**/(5*S*,8*S*)-**14b**, with those of structural analogues reported in the literature [17].



a: $NaHCO_3$, AcOEt; b: 1M NaOH, EtOH, r.t.; c: 30% CF_3COOH, CH_2Cl_2; d: 1M NaOH, H_2O/dioxane, 60 °C

Scheme 1. Synthesis of (±)-**4a**, (±)-**4b**, (±)-**5a** and (±)-**5b**.



a: **10**, $NaHCO_3$, AcOEt; b: **9**, $NaHCO_3$, AcOEt; c: 1M NaOH, EtOH; d: 30% CF_3COOH, CH_2Cl_2;
e: 1M NaOH, dioxane, 80°C.

Scheme 2. Synthesis of (5*R*,8*S*)-**6a**, (5*S*,8*S*)-**6b**, (5*S*,8*R*)-**6a**, (5*R*,8*R*)-**6b**, (5*R*,8*S*)-**7a**, (5*S*,8*S*)-**7b**, (5*S*,8*R*)-**7a** and (5*R*,8*R*)-**7b**.

3. Pharmacological results and discussion

All the new amino acids prepared were pharmacologically characterized at iGluRs. The receptor affinity for NMDA, AMPA and KA receptors was determined by using the radioligands [^{3}H]CGP39653, [^{3}H]AMPA and [^{3}H]KA, respectively [18–20]. The binding assays showed that none of the novel amino acids interacted with AMPA and KA receptors ($IC_{50} > 100$ μM), a result expected by considering the lack of affinity of the lead compounds for the same receptors. Worth mentioning is the trend noticed in the NMDA binding on passing from the 4-(carboxymethyl)prolines **3a**–**3b** to the corresponding spiro isoxazolinyl-prolines **6a**–**6b**. As evident from the data reported in Table 1, the modest affinity observed in the reference compounds is totally lost in the corresponding rigidified analogues. Furthermore, an increase in the distance between the amino acidic moiety and the distal carboxylate group, i.e. on passing from **6a**–**6b** to **7a**–**7b**, does not change the scenario. Similarly, the remarkable affinity for the NMDA receptors which characterizes amino acids (±)-**2a** and (±)-**2b** is lost or drastically reduced in the corresponding cyclopropyl analogues (±)-**4a** and (±)-**4b**. In this case, a reduction in the distance between the two pharmacophoric entities, i.e. on passing from (±)-**4b** to (±)-**5b**, brings about a three fold increase in affinity (Table 1). As previously reported [10,11], the 3-hydroxy-isoxazoline ring of (±)-**5b** mimics the distal carboxylate group of an acidic amino acid. We also performed functional tests based on the measurement of the increase or reduction of intracellular calcium concentration, an increase indicating NMDA receptor activation. Neither (±)-**4b** nor (±)-**5b** showed any significant activity as agonist or antagonist at 100 μM, which might reflect the modest affinity of the amino acids for the NMDA receptors.

In summary, by using the homologues of Glu **2a**, **2b**, **3a**, and **3b** as reference amino acids, we synthesized novel derivatives with an increased conformational rigidity of the pharmacophoric groups. In the first set of compounds, the amino acidic moiety was attached to a cyclopropane ring, whereas in the second set of derivatives the distal carboxylate group was replaced by a spirocyclic 3-hydroxy-isoxazoline ring. In all the novel compounds we noticed the disappearance or a sharp drop in activity evidencing that the selected

Table 1
Affinity for iGluRs using rat cortical membranes

Compound	[^{3}H]CGP K_i (μM)	[^{3}H]AMPA IC_{50} (μM)	[^{3}H]KA IC_{50} (μM)
(±)-**4a**	>100	>100	>100
(±)-**4b**	34 [31–36][a]	>100	>100
(±)-**5a**	>100	>100	>100
(±)-**5b**	13 [11–16][a]	>100	>100
(5*R*,8*S*)-**6a**	>100	>100	>100
(5*S*,8*S*)-**6b**	>100	>100	>100
(5*S*,8*R*)-**6a**	>100	>100	>100
(5*R*,8*R*)-**6b**	>100	>100	>100
(5*R*,8*S*)-**7a**	>100	>100	>100
(5*S*,8*S*)-**7b**	>100	>100	>100
(5*S*,8*R*)-**7a**	>100	>100	>100
(5*R*,8*R*)-**7b**	>100	>100	>100
(2*R*,4*R*)-**3a**	202 ± 43[b,c]		
(2*R*,4*S*)-**3b**	8.0 ± 2.0[b,c]		
(2*S*,4*S*)-**3a**	8.1 ± 1.4[b,c]		
(2*S*,4*R*)-**3b**	1.7 ± 0.65[b,c]		
(±)-**2a**	0.21 [0.18; 0.23][a,d]	>100	>100
(±)-**2b**	0.96 [0.88; 1.1][a,d]	>100	>100
(*R*)-AP5	0.88 ± 0.45[b,e]	>100	>100
(*S*)-AP5	29 ± 9[b,e]	>100	>100

The numbers in parentheses [min; max] indicate ± SEM according to a logarithmic distribution of K_i.
[a] Values are expressed as the antilog to the log mean of three individual experiments.
[b] [^{3}H]Glu NMDA sensitive binding.
[c] Data from the literature [9].
[d] Data from the literature [8].
[e] Data from the literature [5].

conformations are not suitable for a productive interaction with the binding sites of the NMDA receptors. As an alternative explanation, the extra volume taken up by the cyclopropane or the spirocyclic ring, respectively, is not tolerated by the active binding sites of the NMDA receptors.

4. Experimental protocols

4.1. Chemistry

4.1.1. General

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Mercury 300 (300 MHz) spectrometer at 20 °C. Chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in hertz. TLC analyses were performed on commercial silica gel 60 F_{254} aluminum sheets; spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution or with ninhydrin. Melting points were determined on a B 540 Büchi apparatus and are uncorrected. Microanalyses (C, H, N) of new compounds agreed with the theoretical value within ±0.3%.

Determinations of optical rotations were carried out with a Jasco J-810 spectropolarimeter coupled with a Haake N3-B thermostat. Compounds (5*R*,8*S*)-**14a**, (5*S*,8*S*)-**14b**, (5*S*,8*R*)-**14a**, (5*R*,8*R*)-**14b**, (5*R*,8*S*)-**6a**, (5*S*,8*S*)-**6b**, (5*S*,8*R*)-**6a** and (5*R*,8*R*)-**6b** did not show any significant optical activity at 589 nm, therefore optical rotation was determined at 365 nm.

All reagents were purchased from Aldrich and used without any further purification. Ethyl 2-chloro-2-(hydroxyimino)acetate **9** [13], dibromoformaldoxime **10** [14], 1-*tert*-butoxycarbonylamino-2-vinyl-cyclopropanecarboxylic acid ethyl ester (±)-**8** [12], (*R*)-*N*-Boc-4-methylene-proline *tert*-butyl ester (*R*)-**13** [16] and (*S*)-*N*-Boc-4-methylene-proline *tert*-butyl ester (*S*)-**13** [16] were prepared according to literature procedures.

4.1.2. (1′*R**,2′*R**,5*R**)-5-(2′-tert-Butoxycarbonylamino-2′-ethoxycarbonyl-cyclopropyl)-4,5-dihydro-isoxazole-3-carboxylic acid ethyl ester [(±)-**11a**] and (1′*R**,2′*R**,5*S**)-5-(2′-tert-butoxycarbonylamino-2′-ethoxycarbonyl-cyclopropyl)-4,5-dihydro-isoxazole-3-carboxylic acid ethyl ester [(±)-**11b**]

To a solution of 1-*tert*-butoxycarbonylamino-2-vinyl-cyclopropanecarboxylic acid ethyl ester (±)-**8** (1.0 g, 3.92 mmol) in AcOEt (20 mL) was added ethyl 2-chloro-2-(hydroxyimino)-acetate (**9**) (890 mg, 5.88 mmol) and $NaHCO_3$ (2 g). The mixture was vigorously stirred for 12 h at room temperature; the progress of the reaction was monitored by TLC (petroleum ether/AcOEt 9:1). Further **9** (594 mg, 3.92 mmol) was added and the mixture was stirred for another 24 h. Water was added and the organic layer was separated and dried over anhydrous Na_2SO_4. The crude material, obtained after evaporation of the solvent, was chromatographed on silica gel (petroleum ether/AcOEt 9:1) to give 210 mg of (±)-**11a** and 340 mg of (±)-**11b**. Overall yield 38%.

(±)-**11a**: colourless oil; R_f 0.52 (petroleum ether/AcOEt 7:3). ^{1}H NMR ($CDCl_3$): 1.28 (t, $J = 7.1$, 3H), 1.37 (t, $J = 7.1$, 3H), 1.45 (s, 9H), 1.47 (dd, $J = 5.5$, 9.5, 1H), 1.69 (dd, $J = 5.5$, 8.1, 1H), 1.83 (ddd, $J = 8.1$, 9.5, 9.5, 1H), 3.24 (dd, $J = 10.6$, 16.8, 1H), 3.50 (m, 1H), 4.18 (q, $J = 7.1$, 2H), 4.35 (q, $J = 7.1$, 2H), 5.01 (ddd, $J = 9.5$, 9.5, 10.6, 1H), 5.08 (bs, 1H); Anal. $C_{17}H_{26}N_2O_7$ (C, H, N).

(±)-**11b**: colourless oil; R_f 0.38 (petroleum ether/AcOEt 7:3). ^{1}H NMR ($CDCl_3$): 1.28 (t, $J = 6.9$, 3H), 1.37 (t, $J = 6.9$, 3H), 1.40–1.60 (m, 1H), 1.45 (s, 9H), 1.60–1.69 (m, 2H), 3.05 (dd, $J = 8.1$, 17.9, 1H), 3.37 (dd, $J = 11.0$, 17.9, 1H), 4.15 (q, $J = 6.9$, 2H), 4.35 (q, $J = 6.9$, 2H), 4.88 (ddd, $J = 8.1$, 9.5, 11.0, 1H), 5.22 (bs, 1H); Anal. $C_{17}H_{26}N_2O_7$ (C, H, N).

4.1.3. (1′*R**,2′*R**,5*R**)-5-(2′-Amino-2′-carboxy-cyclopropyl)-4,5-dihydro-isoxazole-3-carboxylic acid [(±)-**4a**]

(A) Derivative (±)-**11a** (210 mg, 0.57 mmol) was dissolved in EtOH (5 mL) and treated with 1 M NaOH (1.7 mL) at room temperature overnight. The disappearance of the starting material was monitored by TLC (petroleum ether/AcOEt 7:3). After evaporation of the solvent, water (5 mL) was added and the aqueous layer was washed with CH_2Cl_2 (1 × 3 mL), made acid with 2 M HCl and extracted with AcOEt (4 × 3 mL). The organic phase was dried over anhydrous Na_2SO_4 and after evaporation of the solvent a white powder was obtained (160 mg, yield 89%).

(B) The crude material, obtained from the previous transformation (160 mg, 0.51 mmol), was treated with a 30%

solution of trifluoroacetic acid in CH_2Cl_2 (1.3 mL) at 0 °C. The reaction mixture was stirred at room temperature until the disappearance of the starting material (2 h). The volatiles were removed under vacuum and the residue was taken up with MeOH, filtered under vacuum and washed with MeOH and diethyl ether to give 70 mg of (±)-**4a**. Yield 55%.

(±)-**4a**: white prisms; R_f 0.45 (*n*-butanol/water/acetic acid 4:2:1); 147–148 °C dec. ^{1}H NMR (D_2O): 1.44 (dd, $J = 7.0$, 9.5, 1H), 1.57 (dd, $J = 7.0$, 7.0, 1H), 1.75 (ddd, $J = 7.0$, 9.5, 9.5, 1H), 2.85 (dd, $J = 7.0$, 17.6, 1H), 3.20 (dd, $J = 10.6$, 17.6, 1H), 4.89 (ddd, $J = 7.0$, 9.5, 10.6, 1H); ^{13}C NMR (D_2O): 19.4, 30.2, 37.3, 40.1, 81.8, 153.0, 161.9, 169.7; Anal. $C_8H_{10}N_2O_5$ (C, H, N).

4.1.4. (1′R,2′R*,5S*)-5-(2′-Amino-2′-carboxy-cyclopropyl)-4,5-dihydro-isoxazole-3-carboxylic acid [(±)-**4b**]*

Amino acid (±)-**4b** was prepared as reported for (±)-**4a**, starting from (±)-**11b**.

(±)-**4b**: white prisms; R_f 0.35 (*n*-butanol/water/acetic acid 4:2:1); 212–213 °C dec. ^{1}H NMR (D_2O): 1.50–1.52 (m, 2H), 1.90 (ddd, $J = 9.5$, 9.5, 9.5, 1H), 3.07 (dd, $J = 7.0$, 18.0, 1H), 3.35 (dd, $J = 11.0$, 18.0, 1H), 4.86 (ddd, $J = 7.0$, 9.5, 11.0, 1H); ^{13}C NMR (D_2O): 16.9, 30.1, 38.5, 39.1, 82.0, 153.3, 162.0, 171.0; Anal. $C_8H_{10}N_2O_5$ (C, H, N).

4.1.5. (1′R,2′R*,5R*)-3-Bromo-5-(2′-tert-butoxycarbonyl-amino-2′-ethoxycarbonyl-cyclopropyl)-4,5-dihydro-isoxazole [(±)-**12a**] and (1′R*,2′R*,5S*)-3-bromo-5-(2′-tert-butoxycarbonylamino-2′-ethoxycarbonyl-cyclopropyl)-4,5-dihydro-isoxazole [(±)-**12b**]*

To a solution of (±)-**8** (1.5 g, 5.87 mmol) in AcOEt (30 mL) was added dibromoformaldoxime (**10**) (1.79 g, 8.81 mmol) and $NaHCO_3$ (3 g). The mixture was vigorously stirred for 12 h at room temperature; the progress of the reaction was monitored by TLC (petroleum ether/AcOEt 9:1). Further **8** (1.19 g, 5.87 mmol) was added and the mixture was stirred for another 24 h. Water was added and the organic layer was separated and dried over anhydrous Na_2SO_4. The crude material, obtained after evaporation of the solvent, was chromatographed on silica gel (petroleum ether/AcOEt 85:15) to give 795 mg of (±)-**12a** and 1.20 g of (±)-**12b**. Overall yield 90%.

(±)-**12a**: colourless prisms from MeOH; mp: 155–156 °C; R_f 0.56 (petroleum ether/AcOEt 7:3). ^{1}H NMR ($CDCl_3$): 1.27 (t, $J = 7.0$, 3H), 1.44 (s, 9H), 1.46 (dd, $J = 5.5$, 9.5, 1H), 1.63 (dd, $J = 5.5$, 8.1, 1H), 1.85 (ddd, $J = 8.1$, 8.1, 9.5, 1H), 3.25 (dd, $J = 10.6$, 17.6, 1H), 3.60 (dd, $J = 8.1$, 17.6, 1H), 4.17 (q, $J = 7.0$, 2H), 4.92 (ddd, $J = 8.1$, 8.1, 10.6, 1H), 5.12 (bs, 1H); Anal. $C_{14}H_{21}BrN_2O_5$ (C, H, N).

(±)-**12b**: yellow oil; R_f 0.40 (petroleum ether/AcOEt 7:3). ^{1}H NMR ($CDCl_3$): 1.28 (t, $J = 7.4$, 3H), 1.45 (s, 9H), 1.40–1.56 (m, 1H), 1.60–1.80 (m, 2H), 3.06 (dd, $J = 7.7$, 17.2, 1H), 3.37 (dd, $J = 10.6$, 17.2, 1H), 4.22 (q, $J = 7.4$, 2H), 4.76 (ddd, $J = 7.7$, 9.5, 10.6, 1H), 5.26 (bs, 1H); Anal. $C_{14}H_{21}BrN_2O_5$ (C, H, N).

4.1.6. (1′R,2′R*,5R*)-5-(2′-Amino-2′-carboxy-cyclopropyl)-3-hydroxy-4,5-dihydro-isoxazole [(±)-**5a**]*

(A) Derivative (±)-**12a** (795 mg, 2.11 mmol) was dissolved in dioxane (5 mL), 1 M NaOH (10.6 mL) was added and the mixture was heated at 80 °C for 5 h. The aqueous layer was washed with diethyl ether (1 × 10 mL), made acid with 2 M HCl and extracted with AcOEt (4 × 10 mL). The organic phase was dried over anhydrous Na_2SO_4 and after evaporation of the solvent a white powder was obtained (477 mg, yield 84%).

(B) The intermediate obtained in the previous step (477 mg, 1.66 mmol) was treated with a 30% solution of trifluoroacetic acid in CH_2Cl_2 (4.2 mL) at 0 °C. The solution was stirred at room temperature for 3 h. The volatiles were removed under vacuum and the residue was taken up in MeOH, filtered under vacuum and washed with MeOH and diethyl ether to give 150 mg of amino acid (±)-**5a**. Yield: 49%.

(±)-**5a**: white prisms; R_f 0.35 (*n*-butanol/water/acetic acid 4:2:1); 200–210 °C dec. ^{1}H NMR (D_2O): 1.42 (dd, $J = 6.6$, 9.9, 1H), 1.52 (dd, $J = 6.6$, 7.3, 1H), 1.86 (ddd, $J = 7.3$, 7.3, 9.9, 1H), 2.59 (dd, $J = 7.3$, 16.8, 1H), 2.85 (dd, $J = 8.4$, 16.8, 1H), 4.72 (ddd, $J = 7.3$, 7.3, 8.4, 1H); ^{13}C NMR (D_2O): 17.0, 26.9, 30.0, 37.7, 80.0, 172.0, 174.1; Anal. $C_7H_{10}N_2O_4$ (C, H, N).

4.1.7. (1′R,2′R*,5S*)-5-(2′-Amino-2′-carboxy-cyclopropyl)-3-hydroxy-4,5-dihydro-isoxazole [(±)-**5b**]*

Amino acid (±)-**5b** was prepared as reported for (±)-**5a**, starting from (±)-**12b**.

(±)-**5b**: white prisms; R_f 0.26 (*n*-butanol/water/acetic acid 4:2:1); 170–180 °C dec. ^{1}H NMR (D_2O): 1.38–1.44 (m, 2H), 1.91 (ddd, $J = 8.1$, 8.1, 8.1, 1H), 2.71 (dd, $J = 7.7$, 16.8, 1H), 2.94 (dd, $J = 8.1$, 16.8, 1H), 4.66 (ddd, $J = 7.7$, 8.1, 8.1, 1H); ^{13}C NMR (D_2O): 16.8, 29.0, 37.3, 38.2, 77.9, 169.6, 173.7; Anal. $C_7H_{10}N_2O_4$ (C, H, N).

*4.1.8. (5R,8S)-3-Bromo-1-oxa-2,7-diaza-spiro[4.4]non-2-ene-7,8-dicarboxylic acid di-tert-butyl ester [(5R,8S)-**14a**] and (5S,8S)-3-bromo-1-oxa-2,7-diaza-spiro[4.4]non-2-ene-7,8-dicarboxylic acid di-tert-butyl ester [(5S,8S)-**14b**]*

To a solution of (*S*)-**13** (2.2 g, 7.76 mmol) in AcOEt (45 mL) was added **10** (2.36 g, 11.64 mmol) and $NaHCO_3$ (5 g). The mixture was vigorously stirred for 12 h at room temperature; the progress of the reaction was monitored by TLC (petroleum ether/AcOEt 9:1). Water was added and the organic layer was separated and dried over anhydrous Na_2SO_4. The crude material, obtained after evaporation of the solvent, was chromatographed on silica gel (petroleum ether/AcOEt 9:1) to give 700 mg of (5*R*,8*S*)-**14a** and 2.2 g of (5*S*,8*S*)-**14b**. Overall yield 92%.

(5*R*,8*S*)-**14a**: colourless oil; R_f 0.25 (petroleum ether/AcOEt 85:15); $[\alpha]^{20}_{365nm} = -25.2$ ($c = 5.0$, $CHCl_3$). ^{1}H NMR (toluene-d_8, 90 °C): 1.43 (s, 9H), 1.48 (s, 9H), 1.53 (dd, $J = 10.0$, 12.2, 1H), 2.13 (dd, $J = 4.0$, 12.2, 1H), 2.17 (d, $J = 18.6$, 1H), 2.33 (d, $J = 18.6$, 1H), 3.27 (d, $J = 10.8$, 1H), 3.65 (d, $J = 10.8$, 1H), 4.11 (dd, $J = 4.0$, 10.0, 1H); Anal. $C_{16}H_{25}BrN_2O_5$ (C, H, N).

(5*S*,8*S*)-**14b**: colourless oil; R_f 0.30 (petroleum ether/AcOEt 85:15); $[\alpha]^{20}_{365nm} = +4.18$ ($c = 5.5$, $CHCl_3$). ^{1}H NMR (toluene-d_8, 90 °C): 1.43 (s, 9H), 1.48 (s, 9H), 1.68 (dd, $J = 5.8$, 13.5, 1H), 2.25 (dd, $J = 8.0$, 13.5, 1H), 2.46 (d, $J = 17.5$, 1H), 2.60 (d, $J = 17.5$, 1H), 3.32 (d, $J = 11.7$, 1H), 3.75 (d, $J = 11.7$, 1H), 4.30 (dd, $J = 5.8$, 8.0, 1H); Anal. $C_{16}H_{25}BrN_2O_5$ (C, H, N).

*4.1.9. (5S,8R)-3-Bromo-1-oxa-2,7-diaza-spiro[4.4]non-2-ene-7,8-dicarboxylic acid di-tert-butyl ester [(5S,8R)-**14a**] and (5R,8R)-3-Bromo-1-oxa-2,7-diaza-spiro[4.4]non-2-ene-7,8-dicarboxylic acid di-tert-butyl ester [(5R,8R)-**14b**]*

Compounds (5*S*,8*R*)-**14a** and (5*R*,8*R*)-**14b** were obtained starting from dipolarophile (*R*)-**13** as described for cycloadducts (5*R*,8*S*)-**14a** and (5*S*,8*S*)-**14b**.

(5*S*,8*R*)-**14a**: colourless oil; $[\alpha]^{20}_{365nm} = +26.55$ ($c = 5.2$, $CHCl_3$).

(5*R*,8*R*)-**14b**: colourless oil; $[\alpha]^{20}_{365nm} = -4.87$ ($c = 5.6$, $CHCl_3$).

*4.1.10. (5R,8S)-3-Hydroxy-1-oxa-2,7-diaza-spiro[4.4]non-2-ene-8-carboxylic acid [(5R,8S)-**6a**]*

(A) Derivative (5*R*,8*S*)-**14a** (500 mg, 1.23 mmol) was dissolved in dioxane (6 mL), 1 M NaOH (12.3 mL) was added and the mixture was heated at 80 °C for 36 h. The aqueous layer was washed with diethyl ether (1 × 10 mL), made acid with 2 M HCl and extracted with AcOEt (4 × 10 mL). The organic phase was dried over anhydrous Na_2SO_4 and after evaporation of the solvent a white powder was obtained (140 mg, yield 40%).

(B) The crude material, obtained from the previous transformation (140 mg, 0.49 mmol), was treated with a 30% solution of trifluoroacetic acid in CH_2Cl_2 (1.25 mL) at 0 °C. The solution was stirred at room temperature for 3 h. The volatiles were removed under vacuum and the residue was taken up with MeOH, filtered under vacuum and washed with MeOH and diethyl ether to give 70 mg of amino acid (5*R*,8*S*)-**5a**. Yield 76%.

(5*R*,8*S*)-**6a**: white prisms; R_f 0.21 (*n*-butanol/water/acetic acid 4:2:1); 225–230 °C dec. $[\alpha]^{20}_{365nm} = -53.0$ ($c = 0.13$, H_2O). ^{1}H NMR (D_2O): 2.25 (dd, $J = 10.3$, 14.6, 1H), 2.78 (ddd, $J = 2.2$, 8.0, 14.6, 1H), 2.90 (d, $J = 17.5$, 1H), 2.92 (d, $J = 17.5$, 1H), 3.45 (d, $J = 13.2$, 1H), 3.80 (dd, $J = 2.2$, 13.2, 1H), 4.37 (dd, $J = 8.0$, 10.3, 1H); ^{13}C NMR (D_2O): 37.2, 37.7, 52.9, 59.3, 90.2, 172.1, 173.3; Anal. $C_7H_{10}N_2O_4$ (C, H, N).

*4.1.11. (5S,8S)-3-Hydroxy-1-oxa-2,7-diaza-spiro[4.4]non-2-ene-8-carboxylic acid [(5S,8S)-**6b**]*

Amino acid (5*S*,8*S*)-**6b** was obtained as described for (5*R*,8*S*)-**6a**, starting from (5*S*,8*S*)-**14b**.

(5*S*,8*S*)-**6b**: white prisms; R_f 0.26 (*n*-butanol/water/acetic acid 4:2:1); 190–200 °C dec. $[\alpha]^{20}_{365nm} = -54.0$ ($c = 0.11$, H_2O). ^{1}H NMR (D_2O): 2.20 (dd, $J = 10.2$, 14.6, 1H), 2.78 (ddd, $J = 2.2$, 8.0, 14.6, 1H), 2.90 (d, $J = 17.5$, 1H), 2.92 (d, $J = 17.5$, 1H), 3.45 (d, $J = 13.2$, 1H), 3.71 (dd, $J = 2.2$, 13.2, 1H), 4.37 (dd, $J = 8.0$, 10.2, 1H); ^{13}C NMR (D_2O): 37.1, 37.7, 52.6, 59.5, 90.8, 171.8, 173.3; Anal. $C_7H_{10}N_2O_4$ (C, H, N).

*4.1.12. (5S,8R)-3-Hydroxy-1-oxa-2,7-diaza-spiro[4.4]non-2-ene-8-carboxylic acid [(5S,8R)-**6a**] and (5R,8R)-3-hydroxy-1-oxa-2,7-diaza-spiro[4.4]non-2-ene-8-carboxylic acid [(5R,8R)-**6b**]*

Amino acids (5*S*,8*R*)-**6a** and (5*R*,8*R*)-**6b** were obtained from (5*S*,8*R*)-**14a** and (5*R*,8*R*)-**14b** as described for (5*R*,8*S*)-**6a**.

(5*S*,8*R*)-**6a**: $[\alpha]^{20}_{365nm} = +52.4$ ($c = 0.125$, H_2O).

(5*R*,8*R*)-**6b**. $[\alpha]^{20}_{365nm} = +55.2$ ($c = 0.12$, H_2O).

*4.1.13. (5R,8S)-1-Oxa-2,7-diaza-spiro[4.4]non-2-ene-3,7,8-tricarboxylic acid 7,8-di-tert-butyl ester 3-ethyl ester [(5R,8S)-**15a**] and (5S,8S)-1-oxa-2,7-diaza-spiro[4.4]non-2-ene-3,7,8-tricarboxylic acid 7,8-di-tert-butyl ester 3-ethyl ester [(5S,8S)-**15b**]*

To a solution of (*S*)-**13** (2.0 g, 7.06 mmol) in AcOEt (40 mL) was added **9** (2.14 g, 14.1 mmol) and $NaHCO_3$ (5 g). The mixture was vigorously stirred for 12 h at room temperature; the progress of the reaction was monitored by TLC (petroleum ether/AcOEt 9:1). Further **9** (2.14 g, 14.1 mmol) was added and the mixture was stirred for another 24 h. Water was added and the organic layer was separated and dried over anhydrous Na_2SO_4. The crude material, obtained after evaporation of the solvent, was chromatographed on silica gel (petroleum ether/AcOEt 85:15) to give 310 mg of (5*R*,8*S*)-**15a** and 1.3 g of (5*S*,8*S*)-**15b**. Overall yield 57%.

(5*R*,8*S*)-**15a**: colourless oil; R_f 0.15 (petroleum ether/AcOEt 85:15); $[\alpha]^{20}_{D} = -9.92$ ($c = 0.987$, $CHCl_3$). ^{1}H NMR (toluene-d_8, 90 °C): 1.36 (t, $J = 7.3$, 3H), 1.43 (s, 9H), 1.45 (s, 9H), 1.55 (dd, $J = 10.2$, 12.2, 1H), 2.13 (dd, $J = 3.8$, 12.2, 1H), 2.17 (d, $J = 18.4$, 1H), 2.33 (d, $J = 18.4$, 1H), 3.27 (d, $J = 11.0$, 1H), 3.65 (d, $J = 11.0$, 1H), 4.11 (dd, $J = 3.8$, 10.2, 1H), 4.34 (q, $J = 7.3$, 2H). Anal. $C_{19}H_{30}N_2O_7$ (C, H, N).

(5*S*,8*S*)-**15b**: colourless oil; R_f 0.20 (petroleum ether/AcOEt 85:15); $[\alpha]^{20}_{D} = -6.82$ ($c = 0.982$, $CHCl_3$). ^{1}H NMR (toluene-d_8, 90 °C): 1.37 (t, $J = 7.8$, 3H), 1.44 (s, 9H), 1.46 (s, 9H), 1.70 (dd, $J = 5.9$, 13.3, 1H), 2.28 (dd, $J = 8.1$, 13.3, 1H), 2.45 (d, $J = 17.6$, 1H), 2.62 (d, $J = 17.6$, 1H), 3.32 (d, $J = 11.5$, 1H), 3.77 (d, $J = 11.5$, 1H), 4.30–4.36 (m, 3H); Anal. $C_{19}H_{30}N_2O_7$ (C, H, N).

*4.1.14. (5S,8R)-1-Oxa-2,7-diaza-spiro[4.4]non-2-ene-3,7,8-tricarboxylic acid 7,8-di-tert-butyl ester 3-ethyl ester [(5S,8R)-**15a**] and (5R,8R)-1-oxa-2,7-diaza-spiro[4.4]non-2-ene-3,7,8-tricarboxylic acid 7,8-di-tert-butyl ester 3-ethyl ester [(5R,8R)-**15b**]*

Compounds (5*S*,8*R*)-**15a** and (5*R*,8*R*)-**15b** were obtained starting from dipolarophile (*R*)-**13** as described for cycloadducts (5*R*,8*S*)-**15a** and (5*S*,8*S*)-**15b**.

(5*S*,8*R*)-**15a**: colourless oil; $[\alpha]^{20}_{D} = +10.55$ ($c = 1.102$, $CHCl_3$).

(5*R*,8*R*)-**15b**: colourless oil; $[\alpha]_D^{20} = +6.22$ ($c = 0.947$, $CHCl_3$).

4.1.15. (5*R*,8*S*)-1-Oxa-2,7-diaza-spiro[4.4]non-2-ene-3,8-dicarboxylic acid [(5*R*,8*S*)-**7a**]

(A) Derivative (5*R*,8*S*)-**15a** (310 mg, 0.78 mmol) was dissolved in EtOH (10 mL) and treated with 1 M NaOH (0.78 mL) at room temperature overnight. The solvent was evaporated under vacuum, water (10 mL) was added and the aqueous layer was washed with CH_2Cl_2 (1 × 4 mL), acidified with 2 M HCl and extracted with AcOEt (3 × 5 mL). The organic phase was dried over anhydrous Na_2SO_4 and after evaporation of the solvent a white powder was obtained (205 mg, yield 71%).

(B) The crude material, obtained from the previous transformation (205 mg, 0.55 mmol), was treated with a 30% solution of trifluoroacetic acid in CH_2Cl_2 (2.8 mL) at 0 °C. The solution was stirred at room temperature for 3 h. The volatiles were removed under vacuum and the residue was taken up with MeOH, filtered under vacuum and washed with MeOH and diethyl ether to give 52 mg of amino acid (5*R*,8*S*)-**7a**. Yield 44%.

(5*R*,8*S*)-**7a:** white prisms; R_f 0.10 (*n*-butanol/water/acetic acid 4:2:1); 270–280 °C dec. $[\alpha]_D^{20} = +10.0$ ($c = 0.10$, DMSO). ^{1}H NMR (DMSO-d_6): 2.50 (dd, $J = 4.2$, 14.2, 1H), 2.59 (dd, $J = 9.6$, 14.2, 1H), 3.34 (d, $J = 17.6$, 1H), 3.38 (d, $J = 17.6$, 1H), 3.44 (d, $J = 12.4$, 1H), 3.58 (d, $J = 12.4$, 1H), 4.60 (dd, $J = 4.2$, 9.6, 1H); ^{13}C NMR (DMSO-d_6): 27.2, 41.3, 55.5, 60.1, 76.4, 118.8, 162.1, 170.4; Anal. $C_8H_{10}N_2O_5$ (C, H, N).

4.1.16. (5*S*,8*S*)-1-Oxa-2,7-diaza-spiro[4.4]non-2-ene-3,8-dicarboxylic acid [(5*S*,8*S*)-**7b**]

Amino acid (5*S*,8*S*)-**7b** was obtained as described for (5*R*,8*S*)-**7a**, starting from (5*S*,8*S*)-**15b**.

(5*S*,8*S*)-**7b** white prisms; R_f 0.13 (*n*-butanol/water/acetic acid 4:2:1); 157–170 °C dec. $[\alpha]_D^{20} = +13.0$ ($c = 0.092$, DMSO). ^{1}H NMR (DMSO-d_6): 2.15 (dd, $J = 10.2$, 13.9, 1H), 2.45 (dd, $J = 8.1$, 13.9, 1H), 3.21 (d, $J = 18.3$, 1H), 3.30 (d, $J = 18.3$, 1H), 3.33 (d, $J = 13.2$, 1H), 3.39 (d, $J = 13.2$, 1H), 3.97 (dd, $J = 8.1$, 10.2, 1H); ^{13}C NMR (DMSO-d_6): 26.9, 41.2, 54.6, 60.0, 79.9, 118.6, 161.8, 170.2; Anal. $C_8H_{10}N_2O_5$ (C, H, N).

4.1.17. (5*S*,8*R*)-1-Oxa-2,7-diaza-spiro[4.4]non-2-ene-3,8-dicarboxylic acid [(5*S*,8*R*)-**7a**] and (5*R*,8*R*)-1-oxa-2,7-diaza-spiro[4.4]non-2-ene-3,8-dicarboxylic acid [(5*R*,8*R*)-**7b**]

Amino acids (5*S*,8*R*)-**7a** and (5*R*,8*R*)-**7b** were obtained from (5*S*,8*R*)-**15a** and (5*R*,8*R*)-**15b** as described for (5*R*,8*S*)-**4a**.

(5*S*,8*R*)-**7a**: $[\alpha]_D^{20} = -9.98$ ($c = 0.105$, DMSO).

(5*R*,8*R*)-**7b**. $[\alpha]_D^{20} = -14.2$ ($c = 0.113$, DMSO).

4.2. Pharmacology

4.2.1. Receptor binding at iGluRs

The membrane preparations used in receptor-binding experiments were prepared according to Ransom and Stec [21] with slight modifications, as previously described [22]. Affinity for NMDA [18], AMPA [19], and KA [20] receptors was determined using 2 nm [^{3}H]CGP39653, 5 nm [^{3}H]AMPA and 5 nM [^{3}H]KA in the absence of $CaCl_2$, respectively, with the modifications described previously [23]. The amount of bound radioactivity was determined using a Packard TOP-COUNT microplate scintillation counter. Binding data were analyzed by nonlinear regression using GraphPad Prism (GraphPad Software, San Diego, USA). Data were fitted to the following equation: $B = B_{max} - (B_{max} \times [\text{inhibition}]^n)/(IC_{50}{}^n + [\text{inhibitor}]^n)$, where B is the binding, expressed as a percentage of total specific binding, and n is the Hill coefficient.

4.2.2. Functional tests

The INS-1E clone from the insulin-secreting cell line INS-1 was cultured in RPMI 1640 medium additioned with 5% fetal calf serum (Euroclone, Milan, Italy), 100 IU/ml penicillin, 10 mg/ml streptomycin, 10 mM HEPES and 1 mM pyruvate at 37 °C and 5% CO_2. Cells were plated on glass cover slips (BDH, Italy) 72 h before single cell calcium imaging recording.

Primary neuronal cultures were prepared from the hippocampi of 18-day-old fetal rats as described [24,25]. Either rat hippocampi were dissociated by treatment with trypsin (0.25% for 15 min at 37 °C), followed by trituration with a fire-polished Pasteur pipette. Dissociated cells were plated on poly-L-lysine-treated (Sigma) glass cover slips in MEM (Invitrogen, Milano, Italy) with 10% horse serum at densities ranging from 10,000 to 20,000 cells/cm^2. After a few hours, cover slips were transferred to dishes containing a monolayer of rat cortical glial cells [26], so that they were suspended over the glial cells, but not in direct contact with them [25]. Cells were maintained in MEM without sera, supplemented with 1% N_2 (Invitrogen), and 1 mg/ml BSA (neuronal medium). Calcium recordings were performed on 8–10-day-old cultures.

Primary hippocampal cultures or INS-1E were loaded for 45 min at 37 °C with 2 μM Fura-2 pentacetoxy methylester in Krebs–Ringer solution buffered with HEPES (KRH) (125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose, and 25 mM HEPES/NaOH, pH 7.4) and transferred to the recording chamber of an inverted microscope (Axiovert 100; Zeiss, New York) equipped with a calcium imaging unit. Polychrome IV (TILL Photonics, Germany) was used as light source. Fura-2 fluorescence images were collected with a PCO Super VGA SensiCam (Axon Instruments, Forest City, CA) and analyzed with Axon Imaging Workbench 2.2 software (Axon Instruments, USA). Images were acquired at 1–2 340/380 ratios/s.

Stimulation with 100 μM NMDA, (±)-**4b** or (±)-**5b** was performed in low Mg^{2+} KRH (0.3 mM) to facilitate NMDA receptor activation, in the presence of 1 μM TTX.

4.3. X-ray analysis of (1′*R**,2′*R**,5*R**)-3-bromo-5-(2′-tert-butoxycarbonylamino-2′-ethoxycarbonyl-cyclopropyl)-4,5-dihydro-isoxazole[(±)-**12a**]

4.3.1. Crystal data

Colourless single crystals were obtained from a solution in MeOH. Crystal data: $C_{14}H_{21}BrN_2O_5$, $M_r = 377.23$, monoclinic,

space group *Cc* (No. 9), $a = 11.8650(10)$ Å, $b = 38.5240(9)$ Å, $c = 9.457(2)$ Å, $\beta = 128.447(10)^\circ$, $V = 3385.4(8)$ Å^3, $Z = 8$, $D_c = 1.480$ mg m^{-3}, $F(000) = 1552$, $\mu(\text{MoK}\alpha) = 2.452$ mm^{-1}, $T = 122.0(5)$ K, crystal dimensions $= 0.46 \times 0.22 \times 0.19$ mm.

4.3.2. Data collection and reduction

Data were collected, using the graphite-monochromated MoKα radiation source ($\lambda = 0.71073$ Å) on a KappaCCD diffractometer. Data collection and cell refinement were performed using COLLECT [27] and DIRAX [28]. Data reduction was performed using EvalCCD [29]. Correction for absorption was performed using NUMABS [30,31].

4.3.3. Structure solution and refinement

Positions of all non-hydrogen atoms were found by direct methods (SIR92 [32] and SHELXS97 [33,34]). Full-matrix least squares refinements (SHELXL97) [35] were performed on F^2, minimizing $\sum w(F_o^2 - kF_c^2)^2$, with anisotropic displacement parameters of the non-hydrogen atoms. The positions of the hydrogen atoms were located in subsequent difference electron density maps. Nearly all hydrogen atoms are included in calculated positions, riding on the parent atoms with fixed isotropic displacement parameters of the parent atom ($U_{iso} = 1.2\,U_{eq}$ for CH_2 and $U_{iso} = 1.5\,U_{eq}$ for CH_3). Only the positions of hydrogen atoms connected to chiral carbons and hydrogen atoms connected to nitrogen atoms are refined with fixed isotropic displacement parameters ($U_{iso} = 1.2\,U_{eq}$ for CH and NH). The refinement (415 parameters, 2 restraints, 7693 reflections) converged at $R_F = 0.0514$, $wRF^2 = 0.1304$ for 7006 reflections with $F_o > 4\sigma(F_o)$; $w = 1/[\sigma^2(F_o^2) + (0.\ 0715P)^2 + 8.0869P]$, where $P = (F_o^2 + 2F_c^2)/3$; $S = 1.143$. In the final difference Fourier map maximum and minimum electron densities were 1.422 and -0.863 e Å^{-3}, respectively. Residual density is observed close to the Bromine atom. High displacement parameters are observed for the *tert*-butyl moieties and for the bromine atom. This could indicate disorder, but it was not possible to split the positions into two alternative solutions. Complex atomic scattering factors for neutral atoms were as incorporated in SHELXL97 [35,36].

Further details of the crystal structure of compound (±)-**12a** have been deposited at the Cambridge Crystallographic Data Center with the following reference number (No. CCDC 616324).

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Appendix

Elemental analyses

Compound	Formula	Molecular weight	Required			Found		
			C	H	N	C	H	N
(±)-**11a**	$C_{17}H_{26}N_2O_7$	370.40	55.13	7.08	7.56	55.38	6.90	7.29
(±)-**11b**	$C_{17}H_{26}N_2O_7$	370.40	55.13	7.08	7.56	55.40	7.02	7.35
(±)-**4a**	$C_8H_{10}N_2O_5$	214.18	44.86	4.71	13.08	44.59	4.76	12.97
(±)-**4b**	$C_8H_{10}N_2O_5$	214.18	44.86	4.71	13.08	44.57	4.85	12.83
(±)-**12a**	$C_{14}H_{21}BrN_2O_5$	377.23	44.57	5.61	7.43	44.62	5.74	7.39
(±)-**12b**	$C_{14}H_{21}BrN_2O_5$	377.23	44.57	5.61	7.43	44.40	5.55	7.39
(±)-**5a**	$C_7H_{10}N_2O_4$	186.17	45.16	5.41	15.05	45.37	5.33	15.16
(±)-**5b**	$C_7H_{10}N_2O_4$	186.17	45.16	5.41	15.05	45.12	5.20	14.98
(5*R*,8*S*)-**15a**	$C_{19}H_{30}N_2O_7$	398.45	57.27	7.59	7.03	57.04	7.66	7.09
(5*S*,8*S*)-**15b**	$C_{19}H_{30}N_2O_7$	398.45	57.27	7.59	7.03	57.22	7.75	7.11
(5*S*,8*R*)-**15a**	$C_{19}H_{30}N_2O_7$	398.45	57.27	7.59	7.03	57.20	7.43	7.15
(5*R*,8*R*)-**15b**	$C_{19}H_{30}N_2O_7$	398.45	57.27	7.59	7.03	57.31	7.46	7.07
(5*R*,8*S*)-**7a**	$C_8H_{10}N_2O_5$	214.18	44.86	4.71	13.08	44.76	4.65	13.16
(5*S*,8*S*)-**7b**	$C_8H_{10}N_2O_5$	214.18	44.86	4.71	13.08	44.99	4.85	13.01
(5*S*,8*R*)-**7a**	$C_8H_{10}N_2O_5$	214.18	44.86	4.71	13.08	44.97	4.69	12.97
(5*R*,8*R*)-**7b**	$C_8H_{10}N_2O_5$	214.18	44.86	4.71	13.08	44.74	4.77	13.19
(5*R*,8*S*)-**14a**	$C_{16}H_{25}BrN_2O_5$	405.28	47.42	6.22	6.91	47.31	6.33	6.78
(5*S*,8*S*)-**14b**	$C_{16}H_{25}BrN_2O_5$	405.28	47.42	6.22	6.91	47.54	6.15	6.81
(5*S*,8*R*)-**14a**	$C_{16}H_{25}BrN_2O_5$	405.28	47.42	6.22	6.91	47.39	6.41	6.98
(5*R*,8*R*)-**14b**	$C_{16}H_{25}BrN_2O_5$	405.28	47.42	6.22	6.91	47.22	6.07	6.95
(5*R*,8*S*)-**6a**	$C_7H_{10}N_2O_4$	186.17	45.16	5.41	15.05	44.98	5.37	15.15
(5*S*,8*S*)-**6b**	$C_7H_{10}N_2O_4$	186.17	45.16	5.41	15.05	45.21	5.44	15.02
(5*S*,8*R*)-**6a**	$C_7H_{10}N_2O_4$	186.17	45.16	5.41	15.05	45.30	5.23	15.14
(5*R*,8*R*)-**6b**	$C_7H_{10}N_2O_4$	186.17	45.16	5.41	15.05	45.27	5.55	14.94

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