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Original article

# Synthesis of new isoxazoline-based acidic amino acids and investigation of their affinity and selectivity profile at ionotropic glutamate receptors

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ABSTRACT

The synthesis of four new isoxazoline-based amino acids being analogues of previously described glutamate receptor ligands is reported and their affinity for ionotropic glutamate receptors is analyzed in comparison with that of selected model compounds. Molecular modelling investigations have been carried out to rationalize the interaction with the NMDA receptors.



## 1. Introduction

(*S*)-Glutamic acid (Glu, **1**, Fig. 1) exerts its functions as the major central excitatory neurotransmitter by interaction with two classes of receptors: the ionotropic Glu receptors (iGluRs) and the metabotropic Glu receptors (mGluRs). The iGluRs are multimeric proteins which mediate fast excitatory neuronal transmission *via* ligand-operated cation channels within the central nervous system. Based primarily on studies using selective agonists and antagonists, iGluRs have been classified into *N*-methyl-D-aspartic acid (NMDA), 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) and kainic acid (KA) receptors [1,2]. Eight mGlu receptor subtypes have been cloned and subdivided into group I (mGlu1,5), group II (mGlu2,3) and group III (mGlu4,6,7,8) based on pharmacology, signal transduction pathway, and sequence homology [3,4]. Excitatory imbalance of these receptors is involved in the development of a range of neurological disorders including epilepsy [5], cerebral ischemia [6], schizophrenia [7], as well as neurodegenerative pathologies such as Parkinson's [8] and Alzheimer's [9] diseases. Therefore, both ionotropic and metabotropic receptors are potential therapeutic targets for the treatment of these CNS disorders, making design and synthesis of selective ligands for Glu receptors highly interesting [10].

We previously reported the synthesis and pharmacological characterization of the quite potent NMDA receptor antagonist (3a*S**,5*R**,6a*S**)-5-amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*] isoxazole-3,5-dicarboxylic acid (±)-**2** [11] (Fig. 1), which is a higher homologue of glutamic acid, in which the amino acid skeleton is embedded into the bicyclic structure. Based on this compound we decided to synthesize and test the two ring opened derivatives (±)-**5** and (±)-**6**. Compound (±)-**5** is also structurally related to (±)-**3** (ACPA) [12], which is a potent AMPA receptor agonist. On the other hand, derivative (±)-**6** is structurally related to compound (±)-**4**, which is a selective NMDA antagonist [13].

With the aim to get a better understanding of the relationship between both the conformational freedom of the amino acid side chain and the relative orientation of the three pharmacophoric groups (i.e. α-$NH_2$, α-COOH and γ-COOH) versus the activity/selectivity at iGluRs, we planned the synthesis of all four isomers (±)-**5a**, (±)-**5b**, (±)-**6a**, and (±)-**6b**. The derivatives were submitted to biological evaluation at iGluRs and the results compared to those previously obtained for model compounds. Finally the results were further substantiated by means of molecular modelling investigations.

## 2. Results and discussion

The usual synthetic strategy to produce $\Delta^2$-isoxazolines is the 1,3-dipolar cycloaddition between an appropriately substituted

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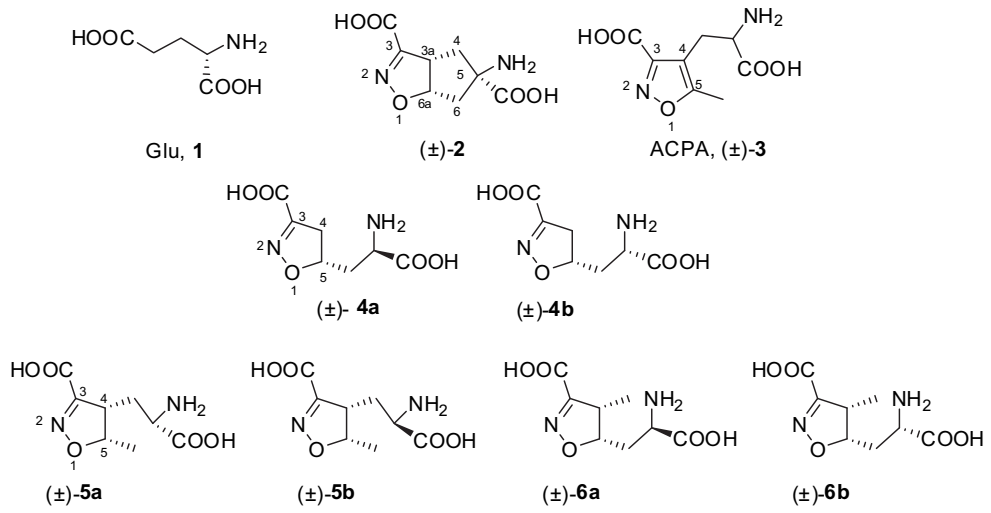
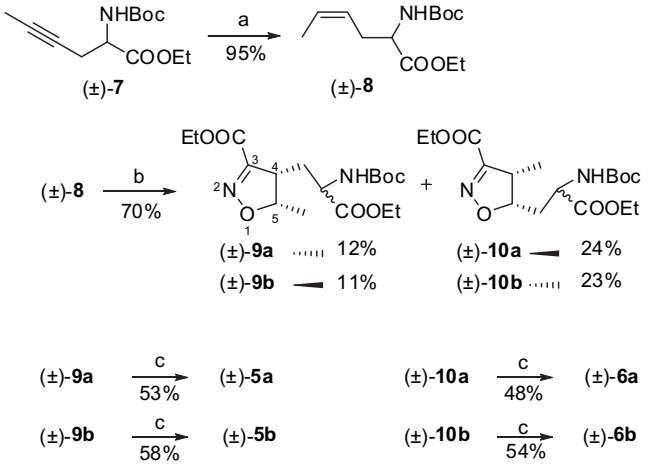



**Fig. 1.** Structure of model and target compounds.

alkene and a suitable nitrile oxide [14]. The advantage of this methodology is the complete translocation of the structural features of the reagents into the final products. As a matter of fact, by reacting a nitrile oxide with a *cis*-1,2-disubstituted alkene, it is possible to obtain solely the *cis*-4,5-disubstituted-$\Delta^2$-isoxazoline [14]. We therefore decided to perform the cycloaddition reaction between (*Z*)-ethyl 2-(*tert*-butoxycarbonylamino)hex-4-enoate (±)-**8** and ethoxycarbonylformonitrile oxide, generated *in situ* from a stable precursor. The desired *cis*-alkene (±)-**8** was prepared by converting commercially available 1-bromo-2-butyne into ethyl 2-(*tert*-butoxycarbonylamino)hex-4-ynoate (±)-**7** [15], which was then hydrogenated in the presence of Lindlar's catalyst [16] and quinoline. The obtained *cis*-alkene (±)-**8** was reacted with ethoxycarbonylformonitrile oxide, generated *in situ* from ethyl 2-chloro-2-(hydroxyimino)acetate and solid $NaHCO_3$. The mixture was heated for 2 h under microwave irradiation (Scheme 1). It has previously been documented that a pericyclic reaction carried out on a 1,2-disubstituted alkene produces a mixture of the two possible *cis*-regioisomers in a variable ratio [14]. In our case, due to the presence of an additional stereocenter (i.e. the α-amino acid carbon), the outcome of the reaction was a mixture of the four

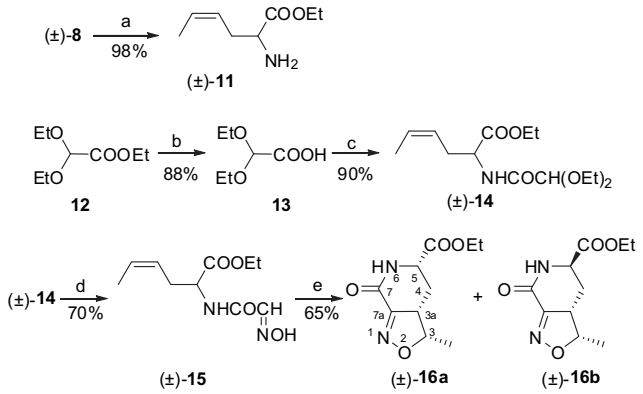



**Scheme 1.** (a) $H_2$, Lindlar's catalyst, quinoline, EtOAc/MeOH (1:1); (b) EtOOCC(Cl)=NOH, $NaHCO_3$, EtOAc, 80 °C, μW; (c) (i) NaOH 1 N, EtOH; (ii) 30% $CF_3COOH/CH_2Cl_2$.

stereoisomers (±)-**9a**, (±)-**10a**, (±)-**9b**, (±)-**10b** in a 1:2:1:2 relative ratio, as determined by HPLC analysis carried out on the crude reaction mixture. The four compounds were then separated by flash chromatography.

The regiochemistry of the new cycloadducts was easily established by $^1$H NMR spectroscopy. A more challenging task was the assignment of the relative stereochemistry to the two pairs of diastereoisomers (±)-**9a** *versus* (±)-**9b** and (±)-**10a** *versus* (±)-**10b**.

The relative configuration (4*S**,5*S**,α*R**) was assigned to the three stereogenic centers of (±)-**10a** by comparing its $^1$H NMR spectrum with that of the structurally related precursor of **4a** [13]; consequently, configuration (4*S**,5*S**,α*S**) was assigned to its diastereoisomer (±)-**10b**. The structure of reference compound **4a** was previously unambiguously assigned by an X-ray analysis [13]. For the two diastereoisomers (±)-**9a** and (±)-**9b**, we did not have a reference compound, and the assignment was based on comparison between experimental and theoretical values of the coupling constants of selected diagnostic protons. This approach can be successfully applied to highly rigidified compounds, in order to reduce the number of conformations to be taken into account. Therefore, we decided to prepare the bicyclic derivatives (±)-**17a** and (±)-**17b**, which could be used for the stereochemical assignment purpose and then easily re-converted into derivatives (±)-**9a** and (±)-**9b**. Despite numerous attempts using different solvents (i.e. AcOEt, toluene, DMSO) and temperatures (rt − 100 °C), the direct lactamization of the free amines, obtained after Boc-deprotection of (±)-**9a** and (±)-**9b**, never occurred. Consequently, we devised a new synthetic route to prepare derivatives (±)-**16a**–**b**, by exploiting the intramolecular nitrile oxide cycloaddition (INOC) methodology. Such a methodology is a powerful tool to control the regiochemistry [17]. We identified the unsaturated oxime (±)-**15** as the key INOC intermediate. The synthesis of (±)-**15** was achieved starting from (±)-**8** following the reaction sequence reported in Scheme 2. The amino group of intermediate (±)-**8** was deprotected by treatment with a 30% solution of trifluoroacetic acid in dichloromethane and the free amine (±)-**11** was then condensed with 2,2-diethoxyacetic acid **13**, in turn obtained by alkaline hydrolysis of the commercially available ethyl ester **12**. The diethylacetal moiety of (±)-**14** was efficiently transformed into the corresponding aldehyde by treatment with a catalytic amount of $I_2$ in refluxing acetone, using a slightly modified version of the published procedure [18]. Noteworthy, the double bond was not affected under these conditions [18]. After evaporation of the



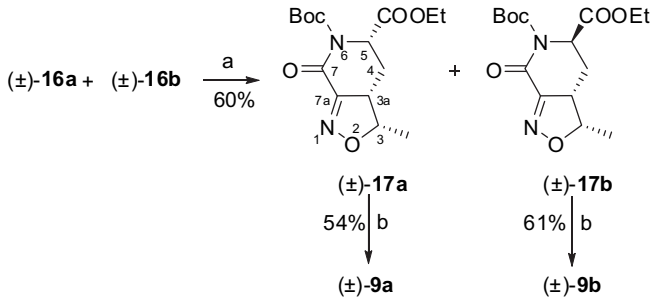

**Scheme 2.** (a) 30% TFA, $CH_2Cl_2$; (b) NaOH, EtOH; (c) (i) TEA, ClCOOEt, THF, 0 °C, (ii) (±)-**11**; (d) (i) $I_2$, acetone, Δ; (ii) $NH_2OH^*HCl$, pyridine, MeOH; (e) 13% NaOCl, $CH_2Cl_2$.

solvent, the crude aldehyde was used directly and converted into the corresponding oxime (±)-**15** by treatment with a methanolic solution of hydroxylamine (Scheme 2). The final intramolecular cycloaddition reaction took place in good yield by treatment of the oxime (±)-**15** with a 13% NaOCl solution to give (±)-**16a** and (±)-**16b** (Scheme 2) [19].

Protection of the lactam nitrogen as a *tert*-butyl carbamate allowed the separation of the two desired diastereoisomers (±)-**17a** and (±)-**17b** by flash chromatography (Scheme 3).

At this level, it was possible to assign the relative stereochemistry by comparing the experimental $^1$H NMR coupling constants of vicinal protons H5–H4 and H5–H4′ with the corresponding calculated values. In fact, as shown in Table 1, the two diastereoisomers showed quite different coupling constant patterns. Conformational analysis carried out at the B3LYP/6-31g* level confirmed the existence of four geometries for each diastereoisomer due to the conformational freedom of the ethoxycarbonyl and the *tert*-butylcarbamoyl moieties. The weighted mean values of the coupling constants, calculated for the conformers by the Haasnot et al. equation [20], are reported in Table 1. The close agreement between experimental and calculated data allowed us to assign the (3*S**,3a*S**,5*S**) configuration to diastereoisomer (±)-**17a** and, consequently, the configuration (3*S**,3a*S**,5*R**) to diastereoisomer (±)-**17b**. Separately, the two stereoisomers (±)-**17a** and (±)-**17b** were treated with $K_2CO_3$ in EtOH to give key intermediates (±)-**9a** and (±)-**9b**, respectively (Scheme 3).

The final amino acids (±)-**5a**, (±)-**5b**, (±)-**6a**, and (±)-**6b** were prepared from (±)-**9a**, (±)-**9b**, (±)-**10a**, (±)-**10b** by alkaline hydrolysis of the ester functions followed by treatment with a 30% solution of trifluoroacetic acid in dichloromethane to remove the *N*-Boc protective group (Scheme 1).




**Scheme 3.** (a) $Boc_2O$, DMAP, THF, Δ; (b) $K_2CO_3$, EtOH.

**Table 1**
Diagnostic experimental $^1$H NMR coupling constants for compounds (±)-**17a** and (±)-**17b** in comparison with the calculated values.

| | $J_{5,4}$ | $J_{5,4'}$ |
|---|---|---|
| **17a** (exp) | 11.0 | 5.5 |
| **17a** (calc) | 11.2 | 4.8 |
| **17b** (exp) | 5.2 | 2.5 |
| **17b** (calc) | 4.3 | 2.3 |

The four new compounds were submitted to receptor binding assays at iGluRs. The binding affinities for NMDA, AMPA and KA receptors were measured on rat cortical membranes using the radioligands, [$^3$H]CGP39653, [$^3$H]AMPA and [$^3$H]kainic acid, respectively [21–23].

The data reported in Table 2 show that compounds (±)-**5a**, (±)-**5b** have no affinity ($IC_{50} > 100$ μM) for AMPA and KA receptors and possess low affinity for NMDA receptors. Thus, the replacement of the isoxazole ring of ACPA with the isoxazoline moiety completely abolishes the activity at AMPA and KA receptors. Moreover, if we compare the biological profile of (±)-**5a** and (±)-**5b** with that of bicyclic analogue (±)-**2**, we observe that ring opening leads to a 50–150 times drop in the NMDA receptor affinity (Table 2). On the other hand, a comparison of the binding affinity profile of regioisomers (±)-**6a** and (±)-**6b** with that of their 4-demethyl-analogues (±)-**4a** and (±)-**4b** shows a 5–15-fold reduction in the NMDA receptor affinity. Notably, it can also be observed that (±)-**6a** and (±)-**6b** gained some affinity for AMPA (and KA) receptors.

In order to account for the observed decrease in the NMDA receptor affinity of (±)-**6a** and (±)-**6b**, in comparison to their 4-demethyl-analogues, we performed a molecular modelling investigation. We previously reported that, as with most of the NMDA antagonists, the biological activity of (±)-**4a** and (±)-**4b** resides exclusively on the enantiomer having an R configuration at the α-amino acid carbon, i.e. (5*S*,α*R*)-**4a** and (5*R*,α*R*)-**4b** [24]. Thus, in analogy, we selected for the docking experiments the enantiomers (4*S*,5*S*,α*R*)-**6a** and (4*R*,5*R*,α*R*)-**6b**, having the absolute configuration at C-5 and C-α identical to that of the eutomers of **4a** and **4b**. For this study we used the homology model of the NR2A subunit previously reported by us [25].

In the best docking pose found for (4*S*,5*S*,α*R*)-**6a** the three ionized pharmacophoric groups (α-COOH, α-$NH_2$, γ-COOH) interact with the binding pocket in a way similar to that reported for (5*S*,α*R*)-**4a** [24]: the α-COOH creates a salt bridge with the guanidine group of the Arg518 and a hydrogen bond with the Thr513 side chain (Fig. 2A). In addition, the amino group generates hydrogen

**Table 2**
Receptor binding affinities at native rat iGluRs.

| Compounds | [$^3$H]CGP 39653 $K_i$ (μM)[a] | [$^3$H]AMPA $IC_{50}$ (μM)[a] | [$^3$H]KAIN $IC_{50}$ (μM)[a] |
|---|---|---|---|
| (±)-**2**[b] | 0.37 | >100 | >100 |
| (±)-**3** (ACPA)[c] | >100[d] | 0.020 | 6.3 |
| (±)-**4a**[b] | 0.21 | >100 | >100 |
| (±)-**4b**[b] | 0.96 | >100 | >100 |
| (±)-**5a** | 18 [17;19] | >100 | >100 |
| (±)-**5b** | 60 [53;68] | >100 | >100 |
| (±)-**6a** | 3.0 [2.6;3.4] | 4.1 [4.0;4.2] | >100 |
| (±)-**6b** | 5.1 [4.8;5.4] | 31 [28;33] | 56 [53;58] |

[a] Values are expressed as the antilog to the log mean of at least three individual experiments. The numbers in brackets [min;max] indicate ± SEM according to a logarithmic distribution.
[b] Data from ref. [13].
[c] Data from ref. [12].
[d] [$^3$H]CPP as radioligand.

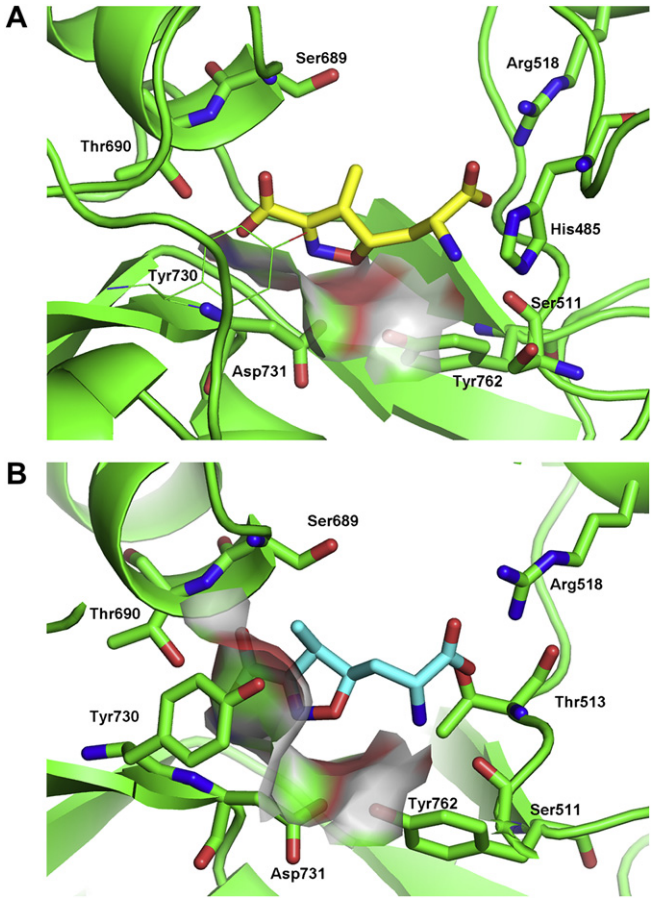



**Fig. 2.** Binding of compounds (4*S*,5*S*,α*R*)-**6a** (A) and (4*R*,5*R*,α*R*)-**6b** (B) in the active site of the NMDA/NR2A receptor. The receptor model residues are depicted as stick model and carbon atoms are colored in green. The ligands' carbon atoms are colored in yellow and cyan for (4*S*,5*S*,α*R*)-**6a** and (4*R*,5*R*, α*R*)-**6b**, respectively. For the sake of clarity, some residues have been omitted and Tyr730 is shown as lines (A).

bond with the backbone of Ser511 and a cation–π interaction with the side chain of His485. The distal COOH group makes electrostatic interactions with the macro dipole located at the amino terminal region of the α-helix including the Ser689 and Thr690 residues. At variance with (5*S*,α*R*)-**4a**, a steric hindrance between the 4-methyl group and the side chain of Ser689 forces the isoxazoline ring of (4*S*,5*S*,α*R*)-**6a** to rotate producing two effects: (i) disruption of a hydrogen bond between the distal acidic group and the side chain of Ser689 previously observed for (5*S*,α*R*)-**4a** and, (ii) a repulsive interaction between the negatively charged side chain of Asp731 and the lone pairs of the oxygen belonging to the isoxazoline ring. These effects may be responsible for the 15-fold reduction of the NMDA receptor affinity of (±)-**6a** compared to (±)-**4a** ($K_i$ 3.0 μM *vs.* 0.21 μM).

In the best scored pose for (4*R*,5*R*,α*R*)-**6b** the amino acid moiety is anchored to residues Arg518, Thr513 and Ser511 but the distal carboxylic group does not interact with Ser689 and Thr690 residues, being oriented towards the interface between the S1 and S2 soluble portions. Therefore, we have examined a second hypothetical binding mode, more similar to that reported for (4*S*,5*S*,α*R*)-**6a**: in this case, (4*R*,5*R*,α*R*)-**6b** (Fig. 2B) adopts an eclipsed conformation (torsion angle between the α-COOH and the C-5) in which the 4-methyl group creates a steric hindrance with the side chain of Tyr730. It can be concluded that the reduced NMDA affinity of (±)-**6b**, compared to its 4-demethyl analogue (±)-**4b** ($K_i$ 5.1 μM *vs.* 0.96 μM), is due to the unfavourable geometry necessary for the ligand to adopt in order to allow the three pharmacophoric groups to interact with the complementary binding sites.

## 3. Conclusion

We have synthesized and tested the receptor binding affinities of four new isoxazoline-based amino acids mimicking known glutamate receptor ligands acting at AMPA or at NMDA receptors. Based on the finding that replacement of the aromatic isoxazole ring of ACPA with the quite similar $\Delta^2$-isoxazoline ring completely abolishes the affinity for AMPA receptors, we have demonstrated that this is an essential structural feature for activation of AMPA receptors.

On the other hand, even though the isoxazoline ring is well tolerated by NMDA receptors, as shown by model compounds **4a** and **4b**, a small modification, such as the insertion of a methyl group in the 4-position of the heterocycle, is enough to produce a marked decrease of the NMDA receptor affinity, due to a different orientation of the pharmacophoric groups and to steric hindrance.

## 4. Experimental section

### 4.1. Materials and methods

All reagents were purchased from Sigma. Ethyl 2-(*tert*-butoxycarbonylamino)hex-4-ynoate (±)-7 [15] and ethyl 2-chloro-2-(hydroxyimino)acetate [26] were prepared according to literature procedures. Microwave-assisted cycloaddition reaction was carried out on a CEM Discovery microwave synthesizer. 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 ($\delta$) are expressed in ppm and coupling constants ($J$) in hertz. 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. HPLC analyses were performed with a Jasco PU-980 pump equipped with a UV–vis detector Jasco UV-975 (wavelength: 220 nm) using the following column: Hypersil APS-2 (250 × 4.6 mm 5 μm); eluent, hexane/isopropanol 98:2 (v/v); flow rate, 1.00 mL/min.

#### 4.1.1. (Z)-ethyl 2-(tert-butoxycarbonylamino)hex-4-enoate [(±)-8]

To a solution of compound (±)-**7** (2.60 g, 10.2 mmol) in a 1:1 EtOAc/MeOH mixture (50 mL) quinoline (1.20 mL, 10.2 mmol) and Lindlar's catalyst (1.56 g; ~5% Pd) were added. The resulting mixture was vigorously stirred overnight under $H_2$ at rt and atmospheric pressure. The catalyst was filtered off and washed with EtOAc. The solvent was evaporated and the crude material was purified by column chromatography (cyclohexane/EtOAc 9:1), to give 2.50 g of compound (±)-**8** (9.7 mmol, 95% yield) as a colorless oil. $R_f$ = 0.30 (cyclohexane/EtOAc 8:2); IR (neat, cm$^{-1}$) $\nu_{max}$: 3416, 2978, 2931, 1740, 1693, 1518, 1210, 1120, 1060; $^1$H NMR (300 MHz, $CDCl_3$) $\delta$: 1.22 (t, $J$ = 7.2 Hz, 3H); 1.38 (s, 9H); 1.58 (d, $J$ = 7.5 Hz, 3H); 2.35–2.50 (m, 1H); 2.52–2.62 (m, 1H); 4.15 (q, $J$ = 7.2 Hz, 2H); 4.25–4.35 (m, 1H); 5.05 (bd, $J$ = 7.3 Hz, 1H); 5.20–5.32 (m, 1H,); 5.52–5.67 (m, 1H); $^{13}$C NMR (75.5 MHz, $CDCl_3$) $\delta$: 13.0, 14.3, 28.4, 29.9, 53.3, 61.4, 79.8, 123.8, 128.3, 155.3, 172.3. Anal. calcd for $C_{13}H_{23}NO_4$ (257.33): C, 60.68; H, 9.01; N, 5.44; found: C, 60.85; H, 9.21; N, 5.26.

#### 4.1.2. Cycloaddition of ethyl 2-chloro-2-(hydroxyimino)acetate to alkene (±)-8

To a solution of alkene (±)-**8** (2.0 g, 7.8 mmol) in EtOAc (40 mL), were added $NaHCO_3$ (3.3 g, 39.0 mmol) and ethyl 2-chloro-2-

(hydroxyimino)acetate (2.4 g, 15.6 mmol) and the mixture was heated in a sealed vessel under microwave irradiation (80 °C, 1 h). A second portion of ethyl 2-chloro-2-(hydroxyimino)acetate (2.4 g, 15.6 mmol) was added and the mixture was irradiated for one additional hour. After cooling, solid $NaHCO_3$ was filtered off, the solvent was evaporated under vacuum and the crude material was purified by flash chromatography (cyclohexane/EtOAc 8:2) to give (±)-**9a** (348 mg, 0.93 mmol, 12% yield), (±)-**10a** (697 mg, 1.87 mmol, 24% yield), (±)-**9b** (335 mg, 0.90 mmol, 11% yield), and (±)-**10b** (668 mg, 1.79 mmol, 23% yield). Overall yield: 70%.

#### 4.1.3. (4S*,5S*)-Ethyl 4-[(2S*)-2-(tert-butoxycarbonylamino)-2-ethoxycarbonylethyl]-5-methyl-4,5-dihydroisoxazole-3-carboxylate [(±)-**9a**]

Yellow oil; $R_f = 0.35$ (Cyclohexane/EtOAc 7:3); IR (neat, $cm^{-1}$) $\nu_{max}$: 3370, 2968, 2920, 1721, 1527, 1365, 1170, 1034; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$: 1.26 (t, $J = 7.2$ Hz, 3H); 1.36 (t, $J = 7.2$ Hz, 3H); 1.44 (s, 9H); 1.53 (d, $J = 6.6$ Hz, 3H); 1.85 (ddd, $J = 2.5$, 12.1, 14.0 Hz, 1H); 2.16 (ddd, $J = 2.5$, 10.1, 14.0 Hz, 1H); 3.48 (ddd, $J = 2.5$, 10.1, 12.1 Hz, 1H); 4.20 (q, $J = 7.2$ Hz, 2H); 4.26–4.34 (m, 1H); 4.33 (q, $J = 7.2$ Hz, 2H); 4.90 (dq, $J = 6.6$, 10.1 Hz, 1H); 5.17 (bd, $J = 12.1$ Hz, 1H); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) $\delta$: 14.1, 14.1, 28.9, 29.7, 44.2, 51.9, 61.8, 62.1, 77.2, 80.3, 83.0, 154.6, 155.1, 160.8, 171.8. $t_R$: 5.858 min. Anal. calcd for $C_{17}H_{28}N_2O_7$ (372.41): C, 54.83; H, 7.58; N, 7.52; found: C, 54.93; H, 7.61; N, 7.35.

#### 4.1.4. (4S*,5S*)-Ethyl 4-[(2R*)-2-(tert-butoxycarbonylamino)-2-ethoxycarbonylethyl]-5-methyl-4,5-dihydroisoxazole-3-carboxylate [(±)-**9b**]

Yellow oil; $R_f = 0.27$ (cyclohexane/EtOAc 7:3); IR (neat, $cm^{-1}$) $\nu_{max}$: 3379, 2979, 2919, 1718, 1507, 1370, 1163, 1023; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$: 1.28 (t, $J = 7.0$ Hz, 3H); 1.34 (t, $J = 7.2$ Hz, 3H); 1.42 (s, 9H); 1.45 (d, $J = 6.6$ Hz, 3H); 2.00–2.09 (m, 2H); 3.43–3.54 (m, 1H); 4.20 (q, $J = 7.0$ Hz, 2H); 4.24–4.30 (m, 1H); 4.33 (q, $J = 7.2$ Hz, 2H); 4.83 (dq, $J = 6.6$, 10.0 Hz, 1H); 5.20 (bd, $J = 7.5$ Hz, 1H); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) $\delta$: 14.1, 14.2, 28.3, 29.0, 44.8, 52.3, 61.7, 62.1, 77.3, 80.2, 83.1, 154.9, 155.1, 160.1, 171.7. $t_R$: 9.283 min. Anal. calcd for $C_{17}H_{28}N_2O_7$ (372.41): C, 54.83; H, 7.58; N, 7.52; found: C, 54.79; H, 7.49; N, 7.53.

#### 4.1.5. (4S*,5S*)-Ethyl 5-[(R*)-2-(tert-butoxycarbonylamino)-2-ethoxycarbonylethyl]-4-methyl-4,5-dihydroisoxazole-3-carboxylate [(±)-**10a**]

Yellow oil; $R_f = 0.32$ (cyclohexane/EtOAc 7:3); IR (neat, $cm^{-1}$) $\nu_{max}$: 3375, 2976, 2924, 1716, 1518, 1360, 1178, 1040; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$: 1.18 (d, $J = 7.2$ Hz, 3H); 1.30 (t, $J = 7.1$ Hz, 3H); 1.35 (t, $J = 7.1$ Hz, 3H); 1.43 (s, 9H); 2.10–2.24 (m, 1H); 2.26–2.37 (m, 1H); 3.50 (dq, $J = 7.2$, 9.0 Hz, 1H); 4.24 (q, $J = 7.1$ Hz, 2H); 4.34 (q, $J = 7.1$ Hz, 2H); 4.35–4.45 (m, 1H); 4.70 (ddd, $J = 5.2$, 9.0, 9.0 Hz, 1H); 5.34 (bd, $J = 4.7$ Hz, 1H); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) $\delta$: 14.3, 25.4, 28.5, 29.9, 43.3, 51.6, 62.1, 62.2, 68.5, 80.4, 83.3, 152.7, 156.7, 160.7, 171.8. $t_R$: 6.900 min. Anal. calcd for $C_{17}H_{28}N_2O_7$ (372.41): C, 54.83; H, 7.58; N, 7.52; found: C, 54.99; H, 7.65; N, 7.87.

#### 4.1.6. (4S*,5S*)-Ethyl 5-[(S*)-2-(tert-butoxycarbonylamino)-2-ethoxycarbonylethyl]-4-methyl-4,5-dihydroisoxazole-3-carboxylate [(±)-**10b**]

Yellow oil; $R_f = 0.24$ (cyclohexane/EtOAc 7:3); IR (neat, $cm^{-1}$) $\nu_{max}$: 3385, 2983, 2920, 1721, 1523, 13680, 1168, 1034; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$: 1.17 (d, $J = 7.1$ Hz, 3H), 1.29 (t, $J = 7.1$ Hz, 3H); 1.36 (t, $J = 7.1$ Hz, 3H); 1.42 (s, 9H); 2.05–2.30 (m, 2H); 3.46 (dq, $J = 7.1$, 9.2 Hz, 1H); 4.22 (q, $J = 7.1$ Hz, 2H); 4.34 (q, $J = 7.1$ Hz, 2H); 4.40–4.55 (m, 1H); 4.68 (ddd, $J = 4.1$, 9.2, 9.2 Hz, 1H), 5.30 (bs, 1H); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) $\delta$: 14.3, 14.6, 28.2, 29.9, 43.4, 51.9, 61.9, 62.2, 78.6, 80.6, 83.7, 153.2, 156.8, 160.7, 172.0. $t_R$: 10.517 min. Anal. calcd for $C_{17}H_{28}N_2O_7$ (372.41): C, 54.83; H, 7.58; N, 7.52; found: C, 54.56; H, 7.32; N, 7.34.

#### 4.1.7. (Z)-Ethyl 2-aminohex-4-enoate [(±)-**11**]

Compound (±)-**8** (2.5 g, 9.7 mmol) was treated with a 30% dichloromethane solution of trifluoroacetic acid (25.7 mL) at 0 °C. The reaction mixture was stirred at rt until disappearance of the starting material (2 h). The volatiles were removed under vacuum. Water was added (10 mL), made basic with $NaHCO_3$ and extracted with EtOAc (3 × 10 mL). The organic extracts were dried over anhydrous $Na_2SO_4$. The solvent was removed under vacuum to give 1.56 g (98% yield) of the primary amine (±)-**11** as a pale yellow oil. $R_f = 0.26$ (EtOAc); IR (neat, $cm^{-1}$) $\nu_{max}$: 3298, 2918, 2846, 1737, 1657, 1543, 1375, 1201, 1137; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$: 1.22 (t, $J = 7.0$ Hz, 3H); 1.58 (d, $J = 6.6$ Hz, 3H); 2.35–2.58 (m, 2H); 3.15–3.40 (bs, 2H); 3.50–3.65 (m, 1H); 4.15 (q, $J = 7.0$ Hz, 2H); 5.25–5.38 (m, 1H); 5.55–5.70 (m, 1H); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) $\delta$: 13.0, 14.3, 31.6, 54.1, 61.4, 124.3, 128.4, 174.6. Anal. calcd for $C_8H_{15}NO_2$ (157.21): C, 61.12; H, 9.62; N, 8.91; found: C, 60.83; H, 9.60; N, 8.61.

#### 4.1.8. 2,2-Diethoxyacetic acid (**13**)

Ethyl 2,2-diethoxyacetate **12** (3.8 g, 21.6 mmol) was dissolved in EtOH (10 mL) and 1 N NaOH (21.6 mL) was added. The mixture was stirred for 1 h at rt. The organic solvent was evaporated and the aqueous phase was extracted with $Et_2O$ (1 × 20 mL). The aqueous layer was made acidic with 2 N HCl and extracted with EtOAc (4 × 20 mL). The organic extracts were dried over anhydrous $Na_2SO_4$. The solvent was removed under vacuum to give 2.8 g (88% yield) of compound **13** as a colorless oil. $R_f = 0.63$ ($CH_2Cl_2$/MeOH 9:1 + 1% AcOH); IR (neat, $cm^{-1}$) $\nu_{max}$: 3472, 2980, 2935, 2899, 1747, 1223, 1118, 1064; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$: 1.18–1.30 (m, 6H); 3.60–3.80 (m, 4H); 4.98 (s, 1H); 10.15 (bs, 1H); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) $\delta$: 15.1, 63.0, 97.2, 171.5. Anal. calcd for $C_6H_{12}O_4$ (148.16): C, 48.64; H, 8.16; found: C, 48.70; H, 8.14.

#### 4.1.9. Ethyl (4Z)-2-[(diethoxyacetyl)amino]hex-4-enoate [(±)-**14**]

Compound **13** (2.8 g, 19.0 mmol) was dissolved in 50 mL of anhydrous THF and TEA (5.3 mL, 38.0 mmol) followed by ethyl chloroformate (1.8 mL, 19.0 mmol) were added dropwise at 0 °C. After 30 min, a solution of amine (±)-**11** (1.56 g, 9.5 mmol) in anhydrous THF (20 mL) was added dropwise. The reaction mixture was allowed to warm at rt and stirred for 3 h. After disappearance of the starting material, the solvent was evaporated and the crude material was purified by column chromatography (eluent:cyclohexane/EtOAc 8:2) to give 2.46 g of compound (±)-**14** (90% yield) as a pale yellow oil. $R_f = 0.48$ (cyclohexane/EtOAc 7:3); IR (neat, $cm^{-1}$) $\nu_{max}$: 3416, 2978, 2932, 1740, 1693, 1518, 1210, 1120, 1059; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$: 1.18–1.30 (m, 9H); 1.58 (d, $J = 6.7$ Hz, 3H); 2.45–2.70 (m, 2H); 3.50–3.76 (m, 4H); 4.18 (q, $J = 7.3$ Hz, 2H); 4.60 (ddd, $J = 5.6$, 5.6, 8.1 Hz, 1H); 4.80 (s, 1H); 5.20–5.35 (m, 1H); 5.62 (dq, $J = 6.7$, 11.0 Hz, 1H); 7.10 (bd, $J = 8.1$ Hz, 1H); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) $\delta$: 13.0, 14.3, 15.2, 29.6, 51.7, 61.7, 62.5, 98.3, 123.6, 128.6, 167.8, 171.6. Anal. calcd for $C_{14}H_{25}NO_5$ (287.35): C, 58.52; H, 8.77; N, 4.87; found: C, 58.19; H, 8.69; N, 4.86.

#### 4.1.10. Ethyl (4Z)-2-{[2-(hydroxyimino)acetyl]amino}hex-4-enoate [(±)-**15**]

(a) Compound (±)-**14** (2.46 g, 8.6 mmol) was dissolved in acetone (30 mL) and a catalytic amount of $I_2$ was added. The mixture was refluxed for 4 h. The solvent was removed under vacuum to give the crude aldehyde, which was directly submitted to next step. (b) The crude derivative obtained from the previous step was dissolved in MeOH (30 mL) and a solution of $NH_2OH^*HCl$ (0.59 g, 8.6 mmol) and pyridine (0.69 mL, 8.6 mmol) in MeOH

(30 mL) were added. The reaction mixture was stirred at rt for 1 h. The solvent was removed under vacuum and the residue was purified by column chromatography to give 1.36 g (70% overall yield) of (±)-**15** as a yellow oil. $R_f = 0.48$ (cyclohexane/EtOAc 7:3); IR (neat, $cm^{-1}$) $\nu_{max}$: 3283, 2981, 2930, 1738, 1664, 1531, 1446, 1212, 1136, 1023; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$: 1.28 (t, $J = 7.2$ Hz, 3H); 1.59 (d, $J = 6.8$ Hz, 3H); 2.59 (ddd, $J = 5.6$, 6.5, 14.0 Hz, 1H); 2.67 (ddd, $J = 5.6$, 6.5, 14.0 Hz, 1H); 4.20 (q, $J = 7.2$ Hz, 2H); 4.70 (ddd, $J = 5.6$, 5.6, 7.9 Hz, 1H); 5.30 (ddd, $J = 6.5$, 6.5, 11.0 Hz, 1H); 5.65 (dq, $J = 6.8$, 11.0 Hz, 1H); 7.16 (bd, $J = 7.9$ Hz, 1H); 7.50 (s, 1H); 9.34 (bs, 1H); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) $\delta$: 13.1, 14.3, 29.6, 52.0, 62.0, 123.3, 128.9, 144.0, 162.3, 172.2. Anal. calcd for $C_{10}H_{16}N_2O_4$ (228.25): C, 52.62; H, 7.07; N, 12.27; found: C, 52.40; H, 7.17; N, 12.15.

#### *4.1.11. (3S*,3aS*,5S*)-Ethyl 3-methyl-7-oxo-3,3a,4,5,6,7-hexahydroisoxazolo[3,4-c]pyridine-5-carboxylate [(±)-**16a**] and (3S*,3aS*,5R*)-ethyl 3-methyl-7-oxo-3,3a,4,5,6,7-hexahydroisoxazolo[3,4-c]pyridine-5-carboxylate [(±)-**16b**]*

To a solution of compound (±)-**15** (1.36 g, 6.0 mmol) in $CH_2Cl_2$ (50 mL) was added a 13% solution of NaOCl (15.6 mL). The reaction mixture was stirred for 3 h at rt. The organic phase was washed with $H_2O$ (3 × 20 mL), dried over anhydrous $Na_2SO_4$ and the solvent was evaporated under vacuum. The crude material was purified by flash chromatography (petroleum ether/EtOAc 8:2) to give compounds (±)-**16a** and (±)-**16b** as an inseparable mixture (0.88 g, 65% yield).

#### *4.1.12. (3S*,3aS*,5S*)-5-Ethyl N-tert-butoxycarbonyl-3-methyl-7-oxo-3,3a,4,5,6,7-hexahydroisoxazolo[3,4-c]pyridine-5-carboxylate [(±)-**17a**] and (3S*,3aS*,5R*)-5-ethyl N-tert-butoxycarbonyl-3-methyl-7-oxo-3,3a,4,5,6,7-hexahydroisoxazolo[3,4-c]pyridine-5-carboxylate [(±)-**17b**]*

To a stirred solution of the mixture of derivatives (±)-**16a,b** (0.67 g, 3.0 mmol) in THF (20 mL), a catalytic amount of DMAP and $Boc_2O$ (0.98 g, 4.5 mmol) were added. The reaction mixture was refluxed for 3 h. The solvent was evaporated under reduced pressure and the crude residue was purified by column chromatography (petroleum ether/EtOAc 8:2) to give compound (±)-**17a** (0.28 g, 0.85 mmol) and (±)-**17b** (0.31 g, 0.95 mmol) (60% overall yield).

*4.1.12.1. (±)-**17a**.* Crystallized from diisopropyl ether as colorless prisms; Mp. 119–122 °C; $R_f = 0.33$ (petroleum ether/EtOAc 7:3); IR (neat, $cm^{-1}$) $\nu_{max}$: 3374, 2912, 2845, 1785, 1720, 1472, 1370, 1285, 1191, 1140; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$: 1.25 (d, $J = 6.9$ Hz, 3H); 1.28 (t, $J = 6.9$ Hz, 3H); 1.51 (s, 9H); 1.90 (ddd, $J = 11.0$, 12.3, 12.9 Hz, 1H); 2.45 (ddd, $J = 5.5$, 5.5, 12.9 Hz, 1H); 3.60 (ddd, $J = 5.5$, 12.3, 12.5 Hz, 1H); 4.20 (q, $J = 6.9$ Hz, 2H); 4.60 (dd, $J = 5.5$, 11.0 Hz, 1H); 5.10 (dq, $J = 6.9$, 12.5 Hz, 1H); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) $\delta$: 14.0, 15.6, 25.6, 27.8, 44.5, 59.1, 62.1, 82.3, 85.0, 151.5, 151.7, 156.8, 170.3. Anal. calcd for $C_{15}H_{22}N_2O_6$ (326.34): C, 55.21; H, 6.79; N, 8.58; found: C, 55.01; H, 6.63; N, 8.41.

*4.1.12.2. (±)-**17b**.* Crystallized from diisopropyl ether as colorless prisms; Mp. 119–122 °C; $R_f = 0.43$ (petroleum ether/EtOAc 7:3); IR (neat, $cm^{-1}$) $\nu_{max}$: 3379, 2924, 2852, 1776, 1731, 1457, 1370, 1287, 1200, 1149, 890; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$: 1.18 (d, $J = 6.6$ Hz, 3H); 1.27 (t, $J = 7.2$ Hz, 3H); 1.51 (s, 9H); 2.14 (ddd, $J = 5.2$, 13.7, 13.7 Hz, 1H); 2.40 (ddd, $J = 2.5$, 4.7, 13.7 Hz, 1H); 3.47 (ddd, $J = 4.7$, 10.7, 13.7 Hz, 1H); 4.23 (q, $J = 7.2$ Hz, 2H); 4.95 (dd, $J = 2.5$, 5.2 Hz, 1H); 5.08 (dq, $J = 6.6$, 10.7 Hz, 1H); $^{13}C$ NMR (75.5 MHz, $CDCl_3$) $\delta$: 14.2, 15.7, 24.5, 27.9, 43.7, 58.3, 62.4, 82.1, 84.8, 151.4, 151.7, 156.6, 170.3. Anal. calcd for $C_{15}H_{22}N_2O_6$ (326.34): C, 55.21; H, 6.79; N, 8.58; found: C, 54.95; H, 6.61; N, 8.40.

#### *4.1.13. (4S*,5S*)-Ethyl 4-[(2S*)-2-(tert-butoxycarbonylamino)-2-ethoxycarbonylethyl]-5-methyl-4,5-dihydroisoxazole-3-carboxylate [(±)-**9a**] from (±)-**17a***

Compound (±)-**17a** (0.23 g, 0.7 mmol) was dissolved in EtOH (3 mL) and $K_2CO_3$ (0.10 g, 0.7 mmol) was added. The mixture was stirred at rt for 1 h. The solvent was evaporated, $Et_2O$ was added and the solid was filtered off. The solvent was removed under vacuum and the crude material was purified by column chromatography (eluent:petroleum ether/EtOAc 8:2) to give 0.14 g of compound (±)-**9a** (54% yield).

#### *4.1.14. (4S*,5S*)-Ethyl 4-[(2R*)-2-(tert-butoxycarbonylamino)-2-ethoxycarbonylethyl]-5-methyl-4,5-dihydroisoxazole-3-carboxylate [(±)-**9b**] from (±)-**17b***

Compound (±)-**17b** (0.23 g, 0.7 mmol) was treated as described above for (±)-**17b** to give compound (±)-**9b** (0.16 g, 61% yield).

#### *4.1.15. (4S*,5S*)-4-[(2S*)-2-Amino-2-carboxyethyl]-5-methyl-4,5-dihydroisoxazole-3-carboxylic acid [(±)-**5a**]*

(a) Compound (±)-**9a** (140 mg, 0.38 mmol) was dissolved in EtOH (1 mL) and 1 N NaOH (0.76 mL) was added. The mixture was stirred at rt for 3 h. The solvent was evaporated, water (3 mL) was added and the aqueous phase was extracted with $Et_2O$ (1 × 3 mL). The aqueous layer was made acidic with 2 N HCl and extracted with EtOAc (4 × 3 mL). The organic extracts were pooled and dried over anhydrous $Na_2SO_4$. The solvent was removed under vacuum to give 0.11 g of the carboxylic acid intermediate, which was directly submitted to next step. (b) The intermediate from the previous step (110 mg, 0.35 mmol) was treated with a 30% $CH_2Cl_2$ solution of trifluoroacetic acid (0.89 mL) at 0 °C. The solution was stirred at rt 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 (±)-**5a** (44 mg, 53% overall yield) as colorless prisms. Crystallized from MeOH. Mp: (dec) > 180 °C; $R_f = 0.20$ (BuOH/$H_2O$/AcOH 4:2:1); IR (neat, $cm^{-1}$) $\nu_{max}$: 3400, 1630, 1415, 1120; $^1H$ NMR (300 MHz, $D_2O$) $\delta$: 1.31 (d, $J = 6.2$ Hz, 3H); 1.92–2.03 (m, 1H); 2.21–2.33 (m, 1H); 3.49–3.60 (m, 1H); 3.95 (dd, $J = 6.6$, 6.6 Hz, 1H); 4.79–4.92 (m, 1H); $^{13}C$ NMR (75.5 MHz, $D_2O$) $\delta$: 13.3, 27.2, 45.4, 52.7, 83.1, 159.1, 165.1, 172.5. Anal. calcd for $C_8H_{12}N_2O_5$ (216.19): C, 44.44; H, 5.59; N, 12.96; found: C, 44.23; H, 5.61; N, 12.80.

#### *4.1.16. (4S*,5S*)-4-[(2R*)-2-Amino-2-carboxyethyl]-5-methyl-4,5-dihydroisoxazole-3-carboxylic acid [(±)-**5b**]*

The same procedure used for the synthesis of (±)-**5a** was applied to compound (±)-**9b** (160 mg, 0.43 mmol) to give compound (±)-**5b** (54 mg, 58% overall yield) as colorless prisms. Crystallized from MeOH. Mp (dec) >145 °C; $R_f = 0.23$ (BuOH/$H_2O$/AcOH 4:2:1); IR (neat, $cm^{-1}$) $\nu_{max}$: 3410, 1626, 1407, 1204, 1129; $^1H$ NMR (300 MHz, $D_2O$) $\delta$: 1.27 (d, $J = 6.6$ Hz, 3H); 1.98 (ddd, $J = 6.3$, 8.8, 14.8 Hz, 1H); 2.08 (ddd, $J = 6.1$, 8.8, 14.8 Hz, 1H); 3.36 (ddd, $J = 6.3$, 8.5, 8.8 Hz, 1H); 3.82 (dd, $J = 6.1$, 8.8 Hz, 1H); 4.77 (dq, $J = 6.6$, 8.5 Hz, 1H); $^{13}C$ NMR (75.5 MHz, $D_2O$) $\delta$: 12.5, 27.0, 44.2, 52.0, 83.4, 159.9, 165.7, 172.9. Anal. calcd for $C_8H_{12}N_2O_5$ (216.19): C, 44.44; H, 5.59; N, 12.96; found: C, 44.11; H, 5.65; N, 12.82.

#### *4.1.17. (4S*,5S*)-5-[(2R*)-2-Amino-2-carboxyethyl]-4,5-dihydro-4-methylisoxazole-3-carboxylic acid [(±)-**6a**]*

The same procedure used for the synthesis of (±)-**5a** was applied to compound (±)-**10a** (200 mg, 0.54 mmol,) to give compound (±)-**6a** (56 mg, 48% overall yield) as colorless prisms. Crystallized from MeOH. Mp: (dec) >140 °C; $R_f = 0.19$ (BuOH/$H_2O$/AcOH 4:2:1); IR (neat, $cm^{-1}$) $\nu_{max}$: 3406, 1632, 1412, 1210, 1125; $^1H$ NMR (300 MHz, $D_2O$) $\delta$: 1.06 (d, $J = 7.3$ Hz, 3H); 2.08 (ddd, $J = 8.4$,

10.4, 15.2 Hz, 1H); 2.32 (ddd, $J = 2.6$, 5.5, 15.2 Hz, 1H); 3.48–3.61 (m, 1H); 3.97 (dd, $J = 5.5$, 8.4 Hz, 1H); 4.85 (ddd, $J = 2.6$, 10.4, 10.4 Hz, 1H); $^{13}$C NMR (75.5 MHz, $D_2O$) $\delta$: 10.4, 30.0, 44.5, 52.9, 83.6, 160.9, 165.0, 172.7. Anal. calcd for $C_8H_{12}N_2O_5$ (216.19): C, 44.44; H, 5.59; N, 12.96; found: C, 44.67; H, 5.69; N, 13.09.

#### 4.1.18. (4S*,5S*)-5-[(2S*)-2-Amino-2-carboxyethyl]-4,5-dihydro-4-methylisoxazole-3-carboxylic acid [(±)-6b]

The same procedure used for the synthesis of (±)-**5a** was applied to compound (±)-**10b** (200 mg, 0.54 mmol) to give compound (±)-**6b** (63 mg, 54% overall yield) as colorless prisms. Crystallized from MeOH. Mp: (dec) >135 °C; $R_f = 0.28$ (BuOH/$H_2O$/AcOH 4:2:1); IR (neat, cm$^{-1}$) $\nu_{max}$: 3412, 1629, 1415, 1215, 1118; $^1$H NMR (300 MHz, $D_2O$) $\delta$: 1.02 (d, $J = 8.1$ Hz, 3H); 2.14–2.22 (m, 2H); 3.42–3.53 (m, 1H); 4.01 (dd, $J = 5.1$, 5.1 Hz, 1H), 4.61 (dd, $J = 1.5$, 8.1 Hz, 1H); $^{13}$C NMR (75.5 MHz, $D_2O$) $\delta$: 10.4, 29.2, 44.4, 52.2, 82.2, 160.1, 164.8, 172.5. Anal. calcd for $C_8H_{12}N_2O_5$ (216.19): C, 44.44; H, 5.59; N, 12.96; found: C, 44.56; H, 5.70; N, 12.84.

### 4.2. Molecular modelling

The DFT/B3lyp/6-31g* calculations were performed with GAUSSIAN03 package [27]. The structures of (±)-**17a** and (±)-**17b** were built with GAUSSVIEW3.0 and, after a systematic conformational analysis, the $^1$H NMR vicinal coupling constants were calculated by the Haasnoot et al. equation [20] for each conformer. The obtained $^1$H NMR vicinal coupling constants were weight averaged on the basis of the population percentages.

The ligands (4*S*,5*S*,α*R*)-**6a** and (4*R*,5*R*,α*R*)-**6b**, docked into the NMDA/NR2A binding cleft, were built by GAUSSVIEW3.0 and preliminarily minimized at the DFT/b3lyp/6-31g* level as implemented in GAUSSIAN03 [27]. The carboxylic and the amine groups were considered in the ionized form to better simulate the physiological conditions. Docking experiments of the tested ligands were performed by means of the program GOLD 4.0 [28]; the goldscore fitness function and the distribution of torsion angles were chosen as indicators of the quality of the docking results. Van der Waals and hydrogen bonding radii were set at 4.0 and 3.0 Å, respectively while genetic algorithm parameters were kept at the default value. Fig. 2 was acquired by the PyMOL software [29].

### 4.3. iGlu receptor binding assays

All binding assays were performed using rat brain synaptic membranes from male Sprague Dawley rats, and tissue preparations were prepared as earlier described [30]. Affinities for NMDA [21], AMPA [22], and KA [23] receptors were determined using 2 nM [$^3$H]CGP 39653 (50 Ci/mmol, $K_d = 6$ nM), 5 nM [$^3$H]AMPA (45.5 Ci/mmol), and 5 nM [$^3$H]KA (58 Ci/mmol), respectively, with some modifications. In brief, on the day of the assay, frozen membranes were quickly thawed and homogenised in 40 volumes of ice-cold buffer, pH 7.4 (50 mM Tris–HCl + 2.5 mM $CaCl_2$, 30 mM Tris–HCl + 2.5 mM $CaCl_2$, or 50 mM Tris–HCl 50, for [$^3$H]CGP 39653, [$^3$H]AMPA, or [$^3$H]KA, binding, respectively), and centrifuged (48,000×*g* for 10 min). This step was repeated four times. In [$^3$H]AMPA binding experiments, 100 mM KSCN was added to the buffer during the final wash and during incubation. The final pellet was re-suspended in ice-cold buffer, corresponding to approx. 0.4–0.5 mg protein/mL. [$^3$H]CGP 39653, [$^3$H]AMPA, and [$^3$H]KA binding were carried out in aliquots consisting of 25 μL [$^3$H]ligand, 25 μL test substance in varying concentrations, and 200 μL membrane suspension and incubated for 60 min, 30 min, and 60 min, respectively. Non-specific binding was determined using 1 mM Glu. Binding was terminated by rapid filtration through Whatman GF/B filters, using a 96-well Packard Filter-Mate Cell Harvester, followed by washing with 3 × 250 μL of ice-cold binding buffer. After drying, 25 μL Microscint 0 (Perkin–Elmer) per well was added and the amount of filterbound radioactivity was quantified in a Packard TopCount microplate scintillation counter.

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