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Synthesis and pharmacological characterization at glutamate receptors of *erythro*- and *threo*-tricholomic acid and homologues thereof

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Abstract—The *erythro*- and *threo*-amino-(3′-hydroxy-4′,5′-dihydro-isoxazol-5′-yl)-acetic acids, stereoisomers of tricholomic acid, were synthesized along with the corresponding higher homologues *erythro*- and *threo*-amino-(3′-carboxy-4′,5′-dihydro-isoxazol-5′-yl)-acetic acids. The target compounds were prepared via the 1,3-dipolar cycloaddition of a suitable nitrile oxide to (±)-2-*tert*-butoxycarbonyl-amino-3-buten-1-ol. Such a strategy allowed the synthesis of the two stereoisomeric amino acids in comparable amounts. The pharmacological activity of these compounds was investigated at ionotropic and metabotropic glutamate receptors (iGluRs and mGluRs) by means of receptor binding assays to rat cortical membranes, electrophysiological tests and second messenger assays at cloned receptors expressed in CHO cells. Their pharmacological profiles were compared to those of L-glutamate and of the previously described selective NMDA receptor antagonists 5-(2-amino-2-carboxyethyl)-4,5-dihydroisoxazole-3-carboxylic acids in order to highlight the effect of increasing/reducing the distance between the amino acid moiety and the distal acid group, which represent the two pharmacophoric entities.

1. Introduction

L-Glutamate (Glu) is the most widely distributed excitatory neurotransmitter in the mammalian central nervous system (CNS). Once released from the presynaptic neuron into the glutamatergic synapse, Glu activates a number of pre- and post-synaptic Glu receptors.[1,2] On the basis of pharmacological profile and ligand selectivity, these receptors have been grouped in two classes: the fast acting ionotropic receptors (iGluRs), which are further classified into *N*-methyl-D-aspartate (NMDA) receptors, 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionate (AMPA) receptors and kainate (KA) receptors and the metabotropic receptors (mGluR1-8), which are G-protein-coupled and thus produce a much slower signal transduction through second messenger systems.[1,2]

Over stimulation of Glu receptors by endogenous or exogenous substances causes excitotoxicity and gives rise to a number of CNS disorders. It is hoped that novel Glu receptor ligands offer new therapeutic opportunities in disease states such as chronic pain, stroke, epilepsy, drug addiction and dependence, schizophrenia, and Parkinson's disease.[3,4] While an extensive preclinical literature exists showing potential beneficial effects of NMDA-, AMPA-, KA-, and metabotropic receptor ligands, only NMDA receptor antagonists have been clinically investigated to an appreciable degree. Since nonselective NMDA receptor antagonists are characterized by a number of adverse CNS effects, including hallucinations, a centrally mediated increase in blood pressure and, at high doses, catatonia and anesthesia,[5,6] current interest largely centers on the development of potent and NR2B subtype-selective antagonists,[7] e.g., analogues of Ifenprodil. In preclinical tests such compounds appear to lack the side-effects commonly associated with nonselective NMDA receptor antagonists.[8] At present, Memantine is the only drug acting at NMDA receptors as an uncompetitive, low-affinity, open-channel blocker approved by the European Union and FDA for the treatment of Alzheimer's disease.[9]

In a recent paper,[10] we reported the synthesis and the pharmacological characterization of the racemic form of the two acidic monocyclic amino acids, 5-(2-amino-2-carboxyethyl)-4,5-dihydroisoxazole-3-carboxylic acids (±)-**1a** and (±)-**1b** (Fig. 1). Both racemates were endowed with a

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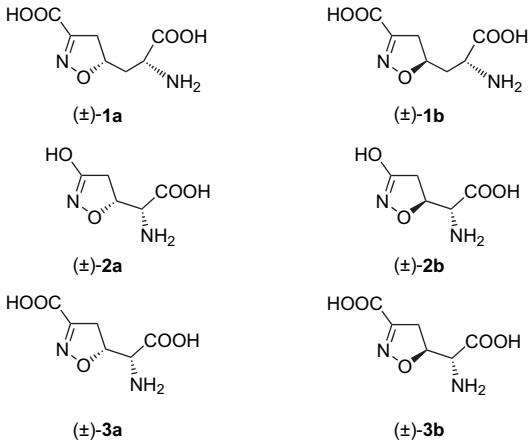


Figure 1. Structures of reference [(±)-**1a** and (±)-**1b**] and target compounds [(±)-**2a**, (±)-**2b**, (±)-**3a**, and (±)-**3b**].

remarkable NMDA antagonist activity and were devoid of any activity at mGluRs.[10] Subsequently, we prepared and tested their single enantiomers and found that the biological activity resided exclusively in the two diastereomers bearing the *R* configuration at the amino acid stereogenic center.[11]

As can be seen from the structures depicted in Figure 1, amino acids (±)-**1a** and (±)-**1b** are the two-carbon homologues of the Glu analogues (±)-**2a** and (±)-**2b**, respectively. It is worth pointing out that the L-enantiomer of (±)-**2a** is a naturally occurring amino acid, termed tricholomic acid. It is a flycidal substance isolated from different species of mushrooms such as *Tricholoma muscarium*,[12] *Amanita strobiliformis*,[13] and *Ustilago maydis*.[14] Its biological activity, evaluated on rat cortical neurones[15] and on giant neurones of an African giant snail (*Achatina fulica*, Férrusac),[16,17] turned out to be similar to that displayed by Glu. Since at that time the classification of the different Glu receptors was unknown, the pharmacological profile of tricholomic acid was not ascertained and it is, at present, undefined. With the aim of investigating in depth its pharmacological profile, we designed the syntheses of the stereoisomers *erythro*-[(±)-**2a**] and *threo*-[(±)-**2b**] tricholomic acids. Furthermore, to compare the structure–activity/selectivity relationships of amino acids (±)-**2a** and (±)-**2b**, with those of the corresponding pair (±)-**1a** and (±)-**1b**, we prepared and tested new derivatives (±)-**3a** and (±)-**3b** (Fig. 1), which represent an intermediate distance among the two pharmacophoric moieties.

This paper reports the syntheses of the two pairs of stereoisomers (±)-**2a**/(±)-**2b**, and (±)-**3a**/(±)-**3b**, and the results of their pharmacological investigation at iGluRs and mGluRs by in vitro binding to rat cortical membranes. Evaluation of functional activity was performed by electrophysiological tests and in second messenger assays at cloned receptors, expressed in Chinese hamster ovary (CHO) cells.

2. Results and discussion

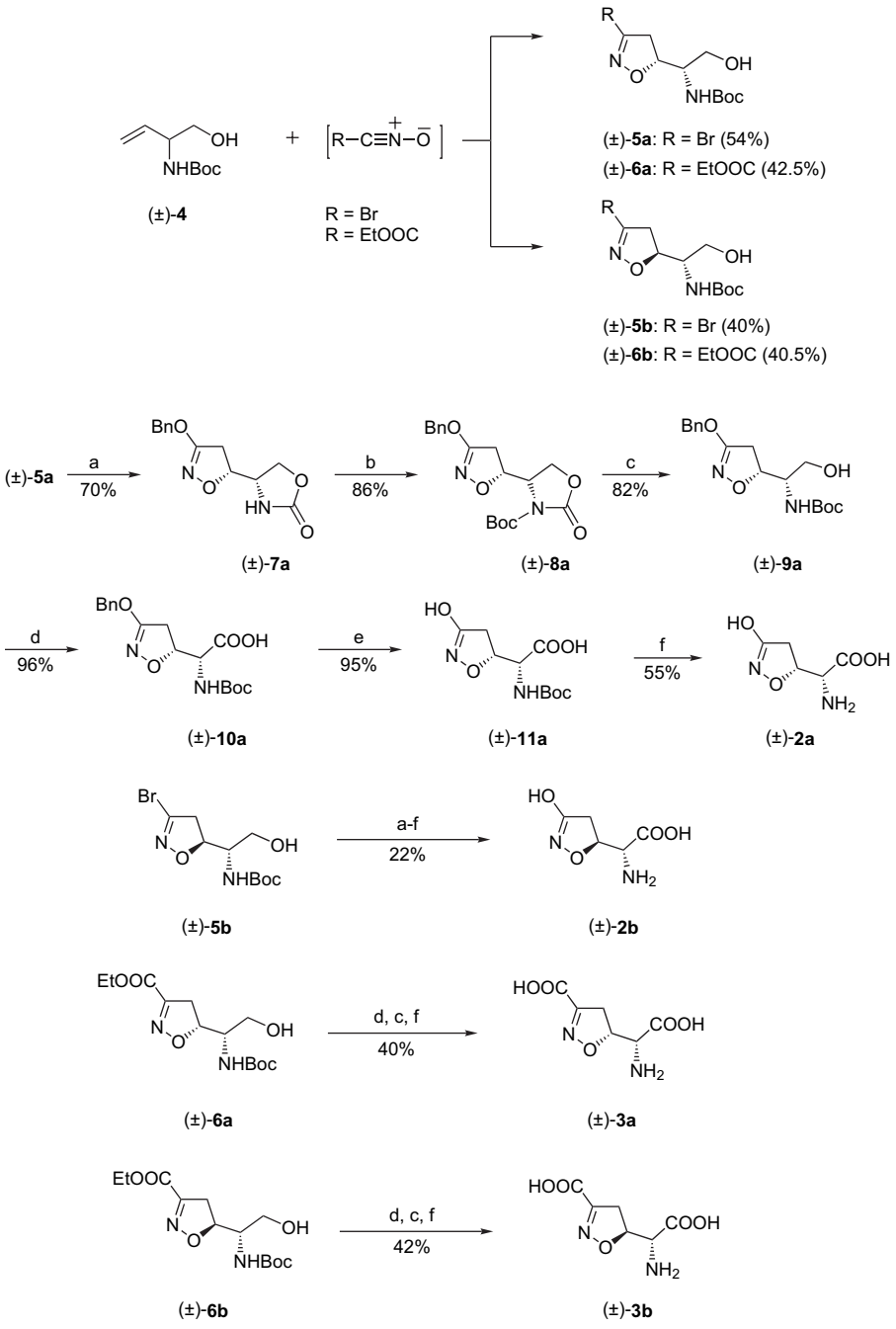
The key step in the syntheses of the target amino acids (±)-**2a**, (±)-**2b**, (±)-**3a**, and (±)-**3b**, is a 1,3-dipolar cycloaddition of bromonitrile oxide or ethoxycarbonylformonitrile oxide to (±)-2-*tert*-butoxycarbonylamino-3-buten-1-ol [(±)-**4**] (Scheme 1). The dipolarophile (±)-**4** was prepared from racemic Garner's aldehyde[18] following the procedure reported for the synthesis of its *S* enantiomer.[19]

The two pairs of diastereomeric cycloadducts *erythro*-(±)-**5a**/*threo*-(±)-**5b** and *erythro*-(±)-**6a**/*threo*-(±)-**6b** were separated by a silica gel column chromatography, and then individually submitted to the reaction sequences depicted in Scheme 1. Surprisingly, the displacement of the 3-bromo moiety of (±)-**5a** (and of (±)-**5b**) by the benzyloxy group gave, besides the expected nucleophilic reaction, formation of the oxazolidin-2-one nucleus, yielding derivative (±)-**7a** (and (±)-**7b**). This intermediate (±)-**7a** (and (±)-**7b**) was re-protected to produce derivative (±)-**8a** (and (±)-**8b**), which was then treated with a 1 M sodium hydroxide solution to give amino alcohol (±)-**9a** (and (±)-**9b**). Intermediates (±)-**9a** and (±)-**9b** were then transformed into final amino acids *erythro*-(±)-**2a** and *threo*-(±)-**2b** via sequential oxidation of the primary alcohols followed by hydrogenolysis of the benzyl group and final treatment with a dichloromethane solution of trifluoroacetic acid.

Since the 1,3-dipolar cycloaddition of bromonitrile oxide to dipolarophile (±)-**4** yields the two diastereomeric cycloadducts *erythro*-(±)-**5a** and *threo*-(±)-**5b** in comparable amounts (ratio 58:42), the synthetic strategy herewith described represents, among the reported methodologies,[20] the most suitable procedure to prepare both the (±)-*erythro*- and the (±)-*threo*-tricholomic acids. The syntheses of amino acids (±)-**3a** and (±)-**3b** were accomplished using amino alcohols (±)-**6a** and (±)-**6b** as key intermediates. These derivatives were oxidized to the corresponding monoacids by treatment with pyridinium dichromate, and then converted into final amino acids (±)-**3a** and (±)-**3b** through hydrolysis of their ester group followed by removal of the *N*-Boc protecting group with a dichloromethane solution of trifluoroacetic acid.

The structure of (±)-**2a** [(±)-*erythro*-tricholomic acid] and (±)-**2b** [(±)-*threo*-tricholomic acid] was assigned by comparing their physical and spectroscopic (^{1}H NMR spectra) data with those previously reported for (±)-**2b**.[21] The coupling constant between protons H-5 and H-α is used for the assignment of *erythro*-/*threo*-isomers, and is 3.3 Hz for the *erythro* isomer and 7.2 Hz for the *threo* isomer. This difference in coupling constants is also shared by amino acids structurally related to (±)-**2a** and (±)-**2b**.[22] The same pattern was observed in the pair of diastereomers (±)-**3a**/(±)-**3b**, and was similarly used for the assignment of the relative configuration of their stereogenic centers.

The two pairs of diastereomeric acidic amino acids (±)-**2a**/(±)-**2b** and (±)-**3a**/(±)-**3b** were assayed in vitro by means of receptor binding techniques, electrophysiological studies and second messenger assays. The receptor affinity for NMDA, AMPA, and KA receptors was determined by use of the radioligands [^{3}H]CGP39653, [^{3}H]AMPA, and [^{3}H]KA, respectively.[23–25] The mGluRs activity of the new compounds was evaluated at rat mGluR1 and mGluR5 (group I), at mGluR2 (representative of group II) and at mGluR4 (representative of group III); all the receptors were expressed in CHO cells.[26]



Scheme 1. (a) $PhCH_2OH$, NaH, THF; (b) $(Boc)_2O$, DMAP, THF; (c) 1 M NaOH, EtOH; (d) PDC, DMF; (e) H_2, Pd/C, MeOH; and (f) TFA 30%, CH_2Cl_2.

As reported in Tables 1 and 2, the two stereoisomers of tricholomic acid (±)-**2a** and (±)-**2b** bind to both iGlu and mGlu receptors. As a consequence, they can be classified as nonselective agonists at the Glu receptors. Furthermore, the *erythro* isomer (±)-**2a** is roughly 10 times more active than its *threo* counterpart (±)-**2b** at iGluRs whereas the opposite holds true for the mGluRs activity (Table 2). Since derivatives (±)-**2a** and (±)-**2b** contain the skeleton of Glu in a conformation partially locked by the 2-isoxazoline ring, it can be noticed that such a rigidification brings about a significant reduction in activity. As a matter of fact, the affinities of the *erythro* isomer (±)-**2a**, the most active diastereomer, are 2–7 times lower than those of Glu[27] at iGluRs. Elongation of the chain connecting the two pharmacophoric moieties, i.e., the amino acid and the distal acidic groups, causes a remarkable increase in selectivity of (±)-**3a** toward AMPA receptors, due to a sharp drop in affinity for KA and NMDA receptors. In particular, if we compare the data of *threo*-tricholomic acid (±)-**2b** with those of the *threo* homologue (±)-**3b**, we notice a three-fold reduction in affinity at AMPA receptors and the disappearance of any affinity at KA and NMDA receptors. On the other hand, by comparing the pair of *erythro* isomers (±)-**2a** and (±)-**3a** we observe that, whereas the affinity at AMPA receptors is unaffected (IC_{50} 1.4 μM), the affinity at the remaining iGluRs is marginal. Electrophysiological experiments, performed on rat cortical slices, showed that (±)-**3a** behaves as an agonist.

Table 1. Rat cortical membrane receptor binding and electrophysiology at iGluRs[a]

Compd	[^{3}H]AMPA IC_{50} (μM)	[^{3}H]KA IC_{50} (μM)	[^{3}H]CGP39653 K_i (μM)	Electrophysiology (rat cortical wedge)
(±)-**1a**[b]	>100	>100	0.21	14.2[c]
(±)-**1b**[b]	>100	>100	0.96	30.5[c]
(±)-**2a**	1.4 [5.86±0.04]	0.76 [6.14±0.08]	1.5 [5.82±0.05]	
(±)-**2b**	19 [4.73±0.04]	6.0 [5.22±0.03]	73 [4.14±0.06]	
(±)-**3a**	1.4 [5.86±0.04]	100	25 [4.61±0.05]	70[d] [4.16±0.05] agonist
(±)-**3b**	64 [4.20±0.04]	>100	>100	
Glu[e]	0.34	0.38	0.20	

[a] Data are given as mean [mean pIC_{50}, mean pK_i or mean pEC_{50}±S.E.M.] of at least three independent experiments.
[b] Data from Ref. 10.
[c] IC_{50} (μM) (NMDA antagonism).
[d] EC_{50} (μM) (mixed AMPA–NMDA agonism).
[e] Data from Ref. 27.

Table 2. Activities at cloned rat mGlu receptors expressed in CHO cells[a]

Compd	mGluR1a EC_{50} (μM)	mGluR2 EC_{50} (μM)	mGluR4 EC_{50} (μM)	mGluR5 EC_{50} (μM)
(±)-**1a**	>1000	>1000	>1000	>1000
(±)-**1b**	>1000	>1000	>1000	>1000
(±)-**2a**	790 [3.1±0.1]	>1000	>1000	300 [3.5±0.01]
(±)-**2b**	110 [4.1±0.3]	100 [4.0±0.1]	>1000	59 [4.2±0.04]
(±)-**3a**	>1000	35±1[b] [4.5±0.01]	>1000	>1000
(±)-**3b**	>1000	>1000	>1000	>1000
Glu	11 [5.0±0.1]	3.8 [5.4±0.1]	17.1 [4.8±0.1]	4.5 [5.4±0.1]

[a] Data are given as mean [mean pEC_{50}±S.E.M.] of three independent experiments.
[b] Maximal response=68±4%. All compounds listed as inactive (>1000) were also inactive when tested as antagonists.

A further increase in the distance between the pharmacophoric groups, i.e., on passing from (±)-**3a**/(±)-**3b** to (±)-**1a**/(±)-**1b**, produces an enhancement in both selectivity and affinity at NMDA receptors (both diastereomers are provided with a submicromolar affinity at NMDA receptors) and, interestingly, a change in the pharmacological profile from agonist to antagonist. This result may be rationalized by the models of the ligand binding domain of some NR2 NMDA receptor subunits.[28–30] It is widely accepted that the ligand binding domain has the form as a hinged clamshell-like structure formed by a region preceding the first membrane spanning domain (termed S1) and a region between the second and third membrane spanning domains (termed S2).[31] The distance between S1 and S2 domains is the critical determinant of the receptor behavior. Glu and Glu-agonists induce the closure of the S1–S2 domain (agonist-bound state) causing the opening of the channel pore and the activation of the receptor. In contrast, ligands significantly longer than Glu keep S1 and S2 domains apart (open form), and behaves as antagonists.[31]

The data reported in Table 2 give an evidence that stereomeric tricholomic acids are provided with a weak activity at mGluRs, definitely lower than that of Glu.[32] The homologation of the Glu backbone abolishes the activity at the mGluRs with the exception of (±)-**3a** at mGluR2.

3. Conclusion

In summary, we herein report the syntheses of two pairs of diastereomeric acidic amino acids and the evaluation of their activity at iGluRs and mGluRs. The two stereoisomers of tricholomic acid (±)-**2a** and (±)-**2b** have been investigated and showed affinities at iGlu as well as mGlu receptors. The pharmacological profile of the two largely resembles that of the endogenous neurotransmitter but with a lower potency. Elongation of the chain connecting the two pharmacophoric groups by a carbon atom, i.e., from (±)-**2a**,**b** to (±)-**3a**,**b**, brings about a noticeable selectivity for AMPA receptors. A further elongation of the chain connecting the two pharmacophoric moieties by one carbon atom, i.e., from (±)-**3a**,**b** to (±)-**1a**,**b**, resulted in a marked increase in both affinity and selectivity for the NMDA receptors accompanied by a switch in the pharmacological behavior, from agonist to antagonist.

4. Experimental

4.1. Material and methods

All reagents were purchased from Sigma. Ethyl 2-chloro-2-(hydroxyimino)acetate[33] and dibromoformaldoxime[34] were prepared according to literature procedures. Dipolarophile (1-hydroxymethyl-allyl)-carbamic acid *tert*-butyl ester (±)-**4** was prepared from racemic Garner's aldehyde[18] following the procedure reported for the synthesis of its *S* enantiomer.[19]

^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer in $CDCl_3$ or D_2O solution at 20 °C. Chemical shifts (δ) are expressed in parts per million and coupling constants (J) in hertz. IR spectra were recorded with a Perkin–Elmer FTIR spectrometer Paragon 1000 PC. TLC analyses were performed on commercial silica gel 60 F_{254} aluminum sheets; spots were visualized 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.

4.1.1. (5′*R,1*S**)[1-(3′-Bromo-4′,5′-dihydro-isoxazol-5′-yl)-2-hydroxy-ethyl]-carbamic acid *tert*-butyl ester and (5′*S**,1*S**)[1-(3′-bromo-4′,5′-dihydro-isoxazol-5′-yl)-2-hydroxy-ethyl]-carbamic acid *tert*-butyl ester [(±)-5a and (±)-5b].** To a solution of (±)-**4** (4.0 g, 21.4 mmol) in AcOEt (80 mL) was added dibromoformaldoxime (5.2 g, 25.7 mmol) and $NaHCO_3$ (5 g). The mixture was vigorously stirred for 5 days at room temperature; the progress of the reaction was monitored by TLC (petroleum ether/AcOEt 7:3). 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 7:3) to give (±)-**5a** (3.6 g, yield 54%) and (±)-**5b** (2.6 g, yield 40%).

(5′*R**,1*S**)[1-(3′-Bromo-4′,5′-dihydro-isoxazol-5′-yl)-2-hydroxy-ethyl]-carbamic acid *tert*-butyl ester (±)-**5a**: crystallized from diisopropyl ether as white prisms; mp 114–117 °C; R_f 0.46 (petroleum ether/AcOEt 1:1); ν_{max} (neat) 3367, 2926, 1690, 1522, 1164; ^{1}H NMR ($CDCl_3$): 1.45 (s, 9H), 1.89 (br s, 1H), 3.25–3.38 (m, 2H), 3.67–3.82 (m, 2H), 3.88–3.99 (m, 1H), 4.77 (ddd, *J*=8.7, 8.7, 8.7, 1H), 5.11 (br s, 1H). Anal. Calcd for $C_{10}H_{17}BrN_2O_4$: C, 38.85; H, 5.54; N, 9.06. Found: C, 38.71; H, 5.64; N, 9.13.

(5′*S**,1*S**)[1-(3′-Bromo-4′,5′-dihydro-isoxazol-5′-yl)-2-hydroxy-ethyl]-carbamic acid *tert*-butyl ester (±)-**5b**: crystallized from diisopropyl ether as white prisms; mp 102–105 °C; R_f 0.39 (petroleum ether/AcOEt 1:1); ν_{max} (neat) 3364, 2980, 1694, 1517, 1165; ^{1}H NMR ($CDCl_3$): 1.45 (s, 9H), 1.83 (br s, 1H), 3.21 (dd, *J*=8.4, 17.5, 1H), 3.32 (dd, *J*=10.5, 17.5, 1H), 3.72 (dd, *J*=6.3, 10.9, 1H), 3.80 (dd, *J*=4.8, 10.9, 1H), 3.80–3.90 (m, 1H), 4.88–5.01 (m, 2H). Anal. Calcd for $C_{10}H_{17}BrN_2O_4$: C, 38.85; H, 5.54; N, 9.06. Found: C, 38.76; H, 5.43; N, 9.02.

4.1.2. (5′*R,4*S**) 4-(3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-oxazolidin-2-one [(±)-7a].** To a suspension of NaH (835 mg, 34.8 mmol) in anhydrous THF (100 mL) in an inert atmosphere, benzyl alcohol (7.5 g, 69.6 mmol) was added dropwise. The mixture was stirred for 20 min at room temperature. A solution of (±)-**5a** (3.6 g, 11.6 mmol) in anhydrous THF was added dropwise and the mixture refluxed overnight. The progress of the reaction was monitored by TLC (petroleum ether/AcOEt 3:2). Water was added and the mixture was made acidic with 2 N HCl. The solvent was evaporated and the aqueous layer extracted with Et_2O. The organic phase was dried over anhydrous Na_2SO_4 and the crude material obtained after evaporation of the solvent, was chromatographed on silica gel (petroleum ether/AcOEt 7:3) to give (±)-**7a** (2.13 g, yield 70%).

(5′*R**,4*S**) 4-(3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-oxazolidin-2-one (±)-**7a**: crystallized from *i*-PrOH as white prisms; mp 172–176 °C dec; R_f 0.25 (petroleum ether/AcOEt 2:3); ν_{max} (neat) 3265, 1759, 1627, 1359, 1236; ^{1}H NMR ($CDCl_3$): 2.91 (dd, *J*=7.8, 16.8, 1H), 3.13 (dd, *J*=10.2, 16.8, 1H), 4.10–4.20 (m, 2H), 4.48–4.56 (m, 1H), 4.65 (ddd, *J*=5.1, 7.8, 10.2, 1H), 5.13 (s, 2H), 5.81 (br s, 1H), 7.35–7.44 (m, 5H). Anal. Calcd for $C_{13}H_{14}N_2O_4$: C, 59.54; H, 5.38; N, 10.68. Found: C, 59.44; H, 5.42; N, 10.60.

4.1.3. (5′*R,4*S**) 4-(3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-2-oxo-oxazolidine-3-carboxylic acid *tert*-butyl ester [(±)-8a].** To a solution of (±)-**7a** (2.13 g, 8.12 mmol) in THF (20 mL) $(Boc_2)O$ (2.66 g, 12.18 mmol) and dimethylaminopyridine (DMAP) (100 mg, 0.82 mmol) were added and the mixture was refluxed overnight. The progress of the reaction was monitored by TLC (petroleum ether/AcOEt 7:3). The solvent was removed under vacuum and the residue chromatographed (petroleum ether/AcOEt 7:3) to give (±)-**8a** (2.53 g, yield 86%).

(5′*R**,4*S**) 4-(3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-2-oxo-oxazolidine-3-carboxylic acid *tert*-butyl ester (±)-**8a**: crystallized from *i*-PrOH as white prisms; mp 152–156 °C; R_f 0.55 (petroleum ether/AcOEt 1:1); ν_{max} (neat) 3423, 2980, 1814, 1720, 1628, 1352; ^{1}H NMR ($CDCl_3$): 1.54 (s, 9H), 3.05 (dd, *J*=8.1, 16.6, 1H), 3.15 (dd, *J*=9.3, 16.6, 1H), 4.28–4.50 (m, 3H), 4.78–4.94 (m, 1H), 5.14 (s, 2H), 7.30–7.42 (m, 5H). Anal. Calcd for $C_{18}H_{22}N_2O_6$: C, 59.66; H, 6.12; N, 7.73. Found: C, 59.50; H, 6.04; N, 7.92.

4.1.4. (5′*R,1*S**)[1-(3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-2-hydroxy-ethyl]-carbamic acid *tert*-butyl ester [(±)-9a].** Derivative (±)-**8a** (2.53 g, 6.98 mmol) was dissolved in EtOH (3.5 mL) and treated with 1 N NaOH (7.0 mL) at room temperature overnight. The disappearance of the starting material was monitored by TLC (petroleum ether/AcOEt 3:2). The aqueous layer was washed with CH_2Cl_2, made acidic with 2 N HCl and extracted with AcOEt. The organic phase was dried over anhydrous Na_2SO_4 and the residue, obtained after evaporation of the solvent, chromatographed (petroleum ether/AcOEt 3:2) to give (±)-**9a** (1.93 g, yield 82%).

(5′*R**,1*S**)[1-(3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-2-hydroxy-ethyl]-carbamic acid *tert*-butyl ester (±)-**9a**: white prisms; R_f 0.44 (petroleum ether/AcOEt 1:1); mp 117–120 °C; ν_{max} (neat) 3368, 2975, 1704, 1626, 1350, 1165; ^{1}H NMR ($CDCl_3$): 1.44 (s, 9H), 2.10 (br s, 1H), 3.05 (dd, *J*=6.6, 16.5, 1H), 3.11 (dd, *J*=9.6, 16.5, 1H), 3.72 (dd, *J*=3.6, 11.4, 1H), 3.73–3.84 (m, 1H), 3.97 (dd, *J*=3.0, 11.4, 1H), 4.66–4.77 (m, 1H), 5.13 (s, 2H), 5.17 (br s, 1H), 7.32–7.44 (m, 5H). Anal. Calcd for $C_{17}H_{24}N_2O_5$: C, 60.70; H, 7.19; N, 8.33. Found: C, 60.77; H, 7.23; N, 8.51.

4.1.5. (5′*R,2*R**) (3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-*tert*-butoxycarbonylamino-acetic acid [(±)-10a].** To a solution of (±)-**9a** (1.93 g, 5.74 mmol) in DMF (40 mL), pyridinium dichromate (PDC) (32.4 g, 86.1 mmol) was added and the mixture was stirred at room temperature for 6 h. The progress of the reaction was monitored by TLC ($CHCl_3$/MeOH 9:1+1% acetic acid). Water was added and the mixture was extracted with AcOEt. The organic layer was washed with $NaHCO_3$ and the aqueous phase was made acidic with 2 N HCl and extracted with AcOEt. The organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and the solvent evaporated to give (±)-**10a** (1.94 g, yield 96%).

(5′*R**,2*R**) (3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-*tert*-butoxycarbonylamino-acetic acid (±)-**10a**: colorless oil; R_f 0.66 ($CHCl_3$/MeOH 9:1+1% acetic acid); ν_{max} (neat) 3325, 2977, 1717, 1628, 1411, 1353, 1163; ^1H NMR ($CDCl_3$): 1.44 (s, 9H), 3.01–3.38 (m, 2H), 4.40–4.55 (m, 1H), 4.82–4.96 (m, 1H), 5.11 (s, 2H), 5.45 (br d, *J*=7.5, 1H), 7.33–7.42 (m, 5H), 7.50 (br s, 1H). Anal. Calcd for $C_{17}H_{22}N_2O_6$: C, 58.28; H, 6.33; N, 8.00. Found: C, 58.43; H, 6.50; N, 7.81.

4.1.6. (5′*R,2*R**) *tert*-Butoxycarbonylamino-(3′-hydroxy-4′,5′-dihydro-isoxazol-5′-yl)-acetic acid [(±)-11a].** To a solution of (±)-**10a** (1.94 g, 5.54 mmol) in MeOH (100 mL), 5% palladium on carbon powder (Engelhard cod.5011, 100 mg) was added and the mixture was stirred in an hydrogen atmosphere at room temperature for 30 min. The progress of the reaction was monitored by TLC ($CHCl_3$/MeOH 9:1+1% CH_3COOH). The mixture was filtered and the solvent evaporated to give (±)-**11a** (1.37 g, yield 95%).

(5′*R**,2*R**) *tert*-Butoxycarbonylamino-(3′-hydroxy-4′,5′-dihydro-isoxazol-5′-yl)-acetic acid (±)-**11a**: colorless oil; R_f 0.38 ($CHCl_3$/MeOH 9:1+1% CH_3COOH); ν_{max} (neat) 3326, 2926, 1701, 1368, 1164; ^1H NMR ($CDCl_3$): 1.44 (s, 9H), 2.10 (br s, 1H), 3.00–3.21 (m, 2H), 4.33 (d, *J*=8.4, 1H), 4.90–5.00 (m, 1H), 5.67 (br d, *J*=6.6, 1H), 7.1 (br s, 1H). Anal. Calcd for $C_{10}H_{16}N_2O_6$: C, 46.15; H, 6.20; N, 10.76. Found: C, 46.27; H, 6.38; N, 10.86.

4.1.7. (5′*R,2*R**) Amino-(3′-hydroxy-4′,5′-dihydro-isoxazol-5′-yl)-acetic acid [(±)]-2a.** Compound (±)-**11a** (1.37 g, 5.26 mmol), was treated with a 30% CH_2Cl_2 solution (13.5 mL) of trifluoroacetic acid (4.0 mL, 52.6 mmol) at 0 °C. The solution was stirred at room temperature for 5 h until disappearance of the starting material (TLC: *n*-BuOH/H_2O/CH_3COOH 4:2:1). 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 (±)-**2a** (464 mg, yield 55%).

(5′*R**,2*R**) Amino-(3′-hydroxy-4′,5′-dihydro-isoxazol-5′-yl)-acetic acid (±)-**2a**: white prisms; mp 180–183 °C dec; R_f 0.32 (*n*-BuOH/H_2O/CH_3COOH 4:2:1); ν_{max} (KBr disc) 3140, 2998, 1696, 1597, 1154, 1034; ^1H NMR (D_2O): 2.82 (dd, *J*=9.0, 16.8, 1H), 2.88 (dd, *J*=9.0, 16.8, 1H), 3.85 (d, *J*=3.3, 1H), 4.96 (ddd, *J*=3.3, 9.0, 9.0, 1H); ^{13}C NMR (D_2O): 33.77, 55.54, 78.33, 169.64, 173.02. Anal. Calcd for $C_5H_8N_2O_4$: C, 37.50; H, 5.04; N, 17.49. Found: C, 37.36; H, 5.08; N, 17.40.

4.1.8. (5′*S,4*S**) 4-(3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-oxazolidin-2-one [(±)]-7b.** Compound (±)-**7b** was prepared starting from (±)-**6b** as described for (±)-**7a**.

(5′*S**,4*S**) 4-(3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-oxazolidin-2-one (±)-**7b**: crystallized from *i*-PrOH as white prisms; mp 114–118 °C; R_f 0.20 (petroleum ether/AcOEt 2:3); ν_{max} (neat) 3264, 1750, 1628, 1413, 1351, 1243; ^1H NMR ($CDCl_3$): 2.62 (dd, *J*=7.8, 16.8, 1H), 3.18 (dd, *J*=10.2, 16.8, 1H), 3.97–4.15 (m, 2H), 4.46–4.54 (m, 1H), 4.59–4.68 (m, 1H), 5.17 (s, 2H), 5.58 (br s, 1H), 7.36–7.42 (m, 5H). Anal. Calcd for $C_{13}H_{14}N_2O_4$: C, 59.54; H, 5.38; N, 10.68. Found: C, 59.71; H, 5.25; N, 10.58.

4.1.9. (5′*S,4*S**) 4-(3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-2-oxo-oxazolidine-3-carboxylic acid *tert*-butyl ester [(±)-8b].** Compound (±)-**8b** was prepared starting from (±)-**7b** as described for (±)-**8a**.

(5′*S**,4*S**) 4-(3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-2-oxo-oxazolidine-3-carboxylic acid *tert*-butyl ester (±)-**8b**: crystallized from *i*-PrOH as white prisms; mp 144–147 °C; R_f 0.55 (petroleum ether/AcOEt 1:1); ν_{max} (neat) 3260, 1815, 1720, 1627, 1351, 1156; ^1H NMR ($CDCl_3$): 1.52 (s, 9H), 2.96 (dd, *J*=8.7, 16.8, 1H), 3.06 (dd, *J*=10.5, 16.8, 1H), 4.29 (dd, *J*=3.3, 9.5, 1H), 4.38 (dd, *J*=9.5, 9.5, 1H), 4.56–4.62 (m, 1H), 5.09–5.18 (m, 1H), 5.14 (s, 2H), 7.34–7.42 (m, 5H). Anal. Calcd for $C_{18}H_{22}N_2O_6$: C, 59.66; H, 6.12; N, 7.73. Found: C, 59.53; H, 6.22; N, 7.87.

4.1.10. (5′*S,1*S**)[1-(3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-2-hydroxy-ethyl]-carbamic acid *tert*-butyl ester [(±)-9b].** Compound (±)-**9b** was prepared starting from (±)-**8b** as described for (±)-**9a**.

(5′*S**,1*S**)[1-(3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-2-hydroxy-ethyl]-carbamic acid *tert*-butyl ester (±)-**9b**: crystallized from *i*-PrOH as white prisms; mp 87–90 °C; R_f 0.35 (petroleum ether/AcOEt 1:1); ν_{max} (neat) 3338, 1702, 1628, 1509, 1350, 1165; ^1H NMR ($CDCl_3$): 1.44 (s, 9H), 2.05 (br s, 1H), 2.99–3.07 (m, 2H), 3.69–3.90 (m, 3H), 4.90 (ddd, *J*=2.4, 9.7, 9.7, 1H), 5.08 (br s, 1H), 5.13 (s, 2H), 7.35–7.43 (m, 5H). Anal. Calcd for $C_{17}H_{24}N_2O_5$: C, 60.70; H, 7.19; N, 8.33. Found: C, 60.85; H, 7.02; N, 8.45.

4.1.11. (5′*S,2*R**) (3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-*tert*-butoxycarbonylamino-acetic acid [(±)-10b].** Compound (±)-**10b** was prepared starting from (±)-**9b** as described for (±)-**10a**.

(5′*S**,2*R**) (3′-Benzyloxy-4′,5′-dihydro-isoxazol-5′-yl)-*tert*-butoxycarbonylamino-acetic acid (±)-**10b**: colorless oil; R_f 0.65 ($CHCl_3$/MeOH 9:1+1% CH_3COOH); ν_{max} (neat) 3327, 1715, 1629, 1509, 1352, 1163; ^1H NMR ($CDCl_3$): 1.44 (s, 9H), 3.04 (dd, *J*=9.0, 16.8, 1H), 3.13 (dd, *J*=9.9, 16.8, 1H), 4.51 (dd, *J*=1.5, 9.0, 1H), 5.12 (s, 2H), 5.21 (ddd, *J*=1.5, 9.0, 9.9, 1H), 5.38 (d, *J*=9.0, 1H), 7.30–7.42 (m, 5H), 10.35 (br s, 1H). Anal. Calcd for $C_{17}H_{22}N_2O_6$: C, 58.28; H, 6.33; N, 8.00. Found: C, 58.04; H, 6.36; N, 7.91.

4.1.12. (5′*S,2*R**) *tert*-Butoxycarbonylamino-(3′-hydroxy-4′,5′-dihydro-isoxazol-5′-yl)-acetic acid [(±)-11b].** Compound (±)-**11b** was prepared starting from (±)-**10b** as described for (±)-**11a**.

(5′*S**,2*R**) *tert*-Butoxycarbonylamino-(3′-hydroxy-4′,5′-dihydro-isoxazol-5′-yl)-acetic acid (±)-**11b**: colorless oil; R_f 0.46 ($CHCl_3$/MeOH 9:1+1% CH_3COOH); ν_{max} (neat) 3283, 1711, 1677, 1529, 1245, 1162; ^1H NMR (CD_3OD): 1.46 (s, 9H), 2.72 (dd, *J*=8.7, 16.5, 1H), 2.87 (dd, *J*=8.7, 16.5, 1H), 4.43 (d, *J*=3.6, 1H), 5.10 (ddd, *J*=3.6, 8.7, 8.7, 1H). Anal. Calcd for $C_{10}H_{16}N_2O_6$: C, 46.15; H, 6.20; N, 10.76. Found: C, 46.00; H, 6.31; N, 10.91.

4.1.13. (5′*S,2*R**) Amino-(3′-hydroxy-4′,5′-dihydro-isoxazol-5′-yl)-acetic acid (±)-2b.** Compound (±)-**2b** was prepared starting from (±)-**11b** as described for (±)-**2a**.

(5′*S**,2*R**) Amino-(3′-hydroxy-4′,5′-dihydro-isoxazol-5′-yl)-acetic acid (±)-**2b**: white prisms; R_f 0.34 (*n*-BuOH/H_2O/CH_3COOH 4:2:1); decomposes in the range 180–186 °C; ν_{max} (KBr disc) 3248, 2995, 1714, 1583, 1157, 1044; ^1H NMR (D_2O): 2.84 (dd, *J*=7.8, 17.1, 1H), 2.93 (dd, *J*=8.7, 17.1, 1H), 3.82 (d, *J*=7.2, 1H), 4.80 (ddd, *J*=7.2, 7.8, 8.7, 1H); ^{13}C NMR (D_2O): 35.53, 55.86, 78.39, 170.23, 173.31. Anal. Calcd for $C_5H_8N_2O_4$: C, 37.50; H, 5.04; N, 17.49. Found: C, 37.33; H, 5.12; N, 17.47.

4.1.14. (5*R,1′*S**) 5-(1′-*tert*-Butoxycarbonylamino-2′-hydroxy-ethyl)-4,5-dihydro-isoxazole-3-carboxylic acid ethyl ester and (5*S**,1′*S**) 5-(1′-*tert*-butoxycarbonylamino-2′-hydroxy-ethyl)-4,5-dihydro-isoxazole-3-carboxylic acid ethyl ester [(±)-6a and (±)-6b].** To a solution of (±)-**4** (1.36 g, 7.26 mmol) in AcOEt (30 mL) was added ethyl 2-chloro-2-(hydroxyimino)-acetate (1.65 g, 10.9 mmol) and $NaHCO_3$ (2.7 g). The mixture was vigorously stirred for 4 days at room temperature; the progress of the reaction was monitored by TLC (petroleum ether/AcOEt 7:3). 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 7:3) to give (±)-**6a** (0.940 g, yield 42.5%) and (±)-**6b** (0.900 g, yield 40.5%).

(5*R**,1′*S**) 5-(1′-*tert*-Butoxycarbonylamino-2′-hydroxy-ethyl)-4,5-dihydro-isoxazole-3-carboxylic acid ethyl ester (±)-**6a**: colorless oil; R_f 0.61 (cyclohexane/AcOEt 2:3); ν_{max} (neat) 3385, 1718, 1522, 1367, 1254, 1166; ^1H NMR ($CDCl_3$): 1.36 (t, *J*=6.9, 3H), 1.44 (s, 9H), 1.70 (br s, 1H), 3.28–3.32 (m, 2H), 3.70–3.80 (m, 2H), 3.90–3.98 (m, 1H), 4.34 (q, *J*=6.9, 2H), 4.90 (ddd, *J*=7.8, 7.8, 7.8, 1H), 5.07 (br d, *J*=4.0, 1H). Anal. Calcd for $C_{13}H_{22}N_2O_6$: C, 51.65; H, 7.33; N, 9.27. Found: C, 51.44; H, 7.22; N, 9.38.

(5*S**,1′*S**) 5-(1′-*tert*-Butoxycarbonylamino-2′-hydroxy-ethyl)-4,5-dihydro-isoxazole-3-carboxylic acid ethyl ester (±)-**6b**: crystallized from diisopropyl ether; white prisms; R_f 0.51 (cyclohexane/AcOEt 2:3); mp 87 °C; ν_{max} (neat) 3343, 1720, 1521, 1367, 1252, 1166; ^1H NMR ($CDCl_3$): 1.38 (t, *J*=6.9, 3H), 1.42 (s, 9H), 2.00 (br s, 1H), 3.20 (dd, *J*=8.7, 18.0, 1H), 3.29 (dd, *J*=11.3, 18.0, 1H), 3.70–3.82 (m, 2H), 3.82–3.95 (m, 1H), 4.35 (q, *J*=6.9, 2H), 4.84 (br d, *J*=4.0, 1H), 5.06 (ddd, *J*=2.9. 8.7, 11.3, 1H). Anal. Calcd for $C_{13}H_{22}N_2O_6$: C, 51.65; H, 7.33; N, 9.27. Found: C, 51.60; H, 7.27; N, 9.20.

4.1.15. (5*R,1′*R**) 5-(Amino-carboxy-methyl)-4,5-dihydro-isoxazole-3-carboxylic acid (±)-3a.** (A) To a solution of (±)-**6a** (940 mg, 3.1 mmol) in DMF (20 mL), PDC (17.5 g, 46.6 mmol) was added and the mixture was stirred at room temperature for 6 h. The progress of the reaction was monitored by TLC ($CHCl_3$/MeOH 7:3+1% acetic acid). Water (10 mL) was added and the mixture was extracted with AcOEt (3×10 mL). The organic layer was extracted with a 5% aqueous solution of $NaHCO_3$ (3×10 mL); the aqueous phase was then made acidic with 2 N HCl and extracted with AcOEt (3×10 mL). The organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and the solvent evaporated to give the crude carboxylic acid as a white powder (930 mg, 95%), which was directly submitted to next step.

(B) The crude carboxylic acid (930 mg, 2.94 mmol) was dissolved in EtOH (15 mL) and treated with 1 N NaOH (3.0 mL) at room temperature overnight. The disappearance of the starting material was monitored by TLC ($CHCl_3$/MeOH 7:3+1% acetic acid). The aqueous layer was made acidic with 2 N HCl and extracted with AcOEt. The organic phase was dried over anhydrous Na_2SO_4 and after evaporation of the solvent the crude dicarboxylic acid was obtained as a white powder (595 mg, 70%).

(C) The crude material, obtained from the previous transformation (595 mg, 2.06 mmol), was treated with a 30% CH_2Cl_2 solution of trifluoroacetic acid (5.3 mL, 20.6 mmol) at 0 °C. The solution was stirred at room temperature for 3 h until disappearance of the starting material (TLC: *n*-butanol/water/acetic acid 4:2:1). 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 (±)-**3a** (232 mg, 60%).

(5*R**,1′*R**) 5-(Amino-carboxy-methyl)-4,5-dihydro-isoxazole-3-carboxylic acid (±)-**3a**: white prisms; R_f 0.33 (*n*-butanol/water/acetic acid 4:2:1); decomposes in the range 160–165 °C; ν_{max} (KBr disc) 3255, 1729, 1647, 1598, 1393, 1300; ^1H NMR (D_2O): 3.24 (dd, *J*=7.9, 18.4, 1H), 3.33 (dd, *J*=11.4, 18.4, 1H), 4.06 (d, *J*=2.9, 1H), 5.14 (ddd, *J*=2.9, 7.9, 11.4, 1H); ^{13}C NMR (D_2O): 37.65, 55.90, 79.95, 157.13, 162.54, 170.75. Anal. Calcd for $C_6H_8N_2O_5$: C, 38.30; H, 4.29; N, 14.89. Found: C, 38.19; H, 4.33; N, 14.82.

4.1.16. (5*S,1′*R**) 5-(Amino-carboxy-methyl)-4,5-dihydro-isoxazole-3-carboxylic acid (±)-3b.** Compound (±)-**3b** was prepared from (±)-**6b** as described for (±)-**3a**.

(5*S**,1′*R**) 5-(Amino-carboxy-methyl)-4,5-dihydro-isoxazole-3-carboxylic acid (±)-**3b**: white prisms; R_f 0.33 (*n*-butanol/water/acetic acid 4:2:1); decomposes in the range 147–152 °C; ν_{max} (KBr disc) 3179, 1710, 1608, 1511, 1377, 1282; ^1H NMR (D_2O): 3.24 (dd, *J*=7.0, 18.6, 1H), 3.36 (dd, *J*=11.0, 18.6, 1H), 3.86 (d, *J*=7.0, 1H), 5.03 (ddd, *J*=7.0, 7.0, 11.0, 1H); ^{13}C NMR (D_2O): 36.35, 55.86, 80.64, 156.05, 163.90, 169.24. Anal. Calcd for $C_6H_8N_2O_5$: C, 38.30; H, 4.29; N, 14.89. Found: C, 38.15; H, 4.39; N, 14.80.

5. Pharmacology

5.1. Receptor binding

Affinity for NMDA, AMPA, and KA receptors was determined using the ligands [^{3}H]CGP39653, [^{3}H]AMPA, and [^{3}H]KA, respectively.[23–25] The membrane preparations used in all the receptor binding experiments were prepared according to the method of Ransom and Stec.[35]

5.2. In vitro electrophysiology

A rat cortical slice preparation for determination of excitatory amino acid-evoked depolarizations described by Harrison and Simmonds[36] was used in a slightly modified version. Wedges (500 μM thick) of rat brain, containing cerebral cortex and corpus callosum, were placed through a grease barrier for electrical isolation with each part in

contact with a DriRef-5SH (World Precision Instruments) electrode. The cortex and corpus callosum parts were constantly superfused with a Mg^{++} free (and Ca^{++} free for the corpus callosum) oxygenated Krebs buffer at room temperature. The test compounds were added to the cortex superfusion medium and the potential difference between the electrodes recorded on a chart recorder. Applications of agonists were made for 90 s at each concentration tested, typically at 15 min intervals. In experiments designed to detect antagonist effects the potential antagonist were applied alone for 90 s followed by co-application of agonists (NMDA, AMPA or KA) and the potential antagonist for another 90 s.

5.3. Metabotropic testing

Four metabotropic subtypes mGluR1a, mGluR2, mGluR4a or mGluR5 were expressed in Chinese hamster ovary cell lines and maintained and assayed as previously described.[26]

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