DOI: 10.1002/cmdc.200700118

Synthesis of Conformationally Constrained Glutamic Acid Homologues and Investigation of Their Pharmacological Profiles

Paola Conti,*[a] Andrea Pinto,[a] Lucia Tamborini,[a] Giovanni Grazioso,[a] Giovambattista De Sarro,[b] Hans Bräuner-Osborne,[c] Geza Szabo,[d] László Gábor Hársing,[d] and Carlo De Micheli[a]

*Homologation of the glutamic acid chain together with conformational constraint is a commonly used strategy to achieve selectivity towards different types of glutamate receptors. We investigated the effects of a further increase in the distance between the amino acid moiety and the distal carboxylate group of model compounds (±)-**1** and (±)-**2** on their activity/selectivity profiles. We therefore synthesized new derivatives (±)-**3**–(±)-**6**, which are homologues of glutamic acid containing three additional carbon units. Moreover, because the potency of NMDA antagonists can be markedly increased by replacing the distal carboxylate with the bioisosteric phosphonate group, we also prepared the corresponding phosphonate derivatives (±)-**7**–(±)-**10**. All new compounds were submitted to binding assays with iGluRs, and derivatives (±)-**3**–(±)-**6** were also tested in second messenger assays at representative mGluR subtypes. All the applied structural modifications were detrimental to the interaction with NMDA receptors. Conversely, structural variation of the nonselective mGluR ligand (±)-**2** led to derivative (±)-**5**, which behaved as a selective group I metabotropic receptor antagonist. Notably, upon i.c.v. administration in DBA/2 mice, amino acid (±)-**5** produced a significant protection against audiogenic seizures, whereas it was inactive after i.p. administration.*

Introduction

L-Glutamic acid (*S*-Glu) is the main excitatory neurotransmitter in the central nervous system (CNS), where it is involved in the modulation of many physiological processes such as learning, memory, and synaptic plasticity.[1] However, a massive influx of Glu into the synapses can lead to acute and chronic neurodegenerative diseases (for example, cerebral ischemia, traumatic brain injury, spinal injury, epilepsy, and Parkinson's, Alzheimer's and Huntington's diseases).[2] Glu operates through four different receptor classes: three heterogeneous classes of ionotropic glutamate receptors (iGluRs), named after the selective agonists *N*-methyl-D-aspartic acid (NMDA), (*R*,*S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA), and kainic acid (KA),[1,3] and a heterogeneous class of G-protein-coupled glutamate receptors (mGluRs).[4,5] The iGluRs are heterotetramers: to date, seven NMDA receptor subunits (NR1, NR2A–2D, and NR3A and 3B), four AMPA receptor subunits (iGluR1–4), and five subunit building blocks for KA-preferring receptors (iGluR5–7, KA1, and KA2) have been cloned and characterized, but the number of functional NMDA, AMPA, and KA receptors present in the CNS has not been established. In addition, eight subtypes of the seven transmembrane mGluRs have been cloned and categorized in three groups according to their sequence homology, second-messenger coupling, and pharmacology, that is, group I (mGluRs 1 and 5), group II (mGluRs 2 and 3), and group III (mGluRs 4, 6, 7, and 8).[4,5]

The availability of highly selective agonists and antagonists for the different receptor subtypes represents a primary target to understand their physiological role and their pharmacological relevance. From a therapeutic point of view, the most promising compounds are undoubtedly those characterized by high selectivity for a specific receptor subtype, as the side effects usually associated with unselective ligands could be minimized. As an example, the NMDA antagonist CP-101,606, which is highly selective for the NR2B subtype, showed direct antiparkinsonian actions in both rodents and monkeys, and was devoid of any drug-related side effects.[6] Focusing our attention on competitive GluR agonists and antagonists, two strategies were set forth to achieve receptor selectivity: 1) con-

[a] *Prof. P. Conti, Dr. A. Pinto, Dr. L. Tamborini, Dr. G. Grazioso, Prof. C. De Micheli*
Istituto di Chimica Farmaceutica e Tossicologica "Pietro Pratesi"
Università degli Studi di Milano
Via Mangiagalli 25, 20133 Milano (Italy)
Fax: (+39) 02-50319326
E-mail: paola.conti@unimi.it

[b] *Prof. G. De Sarro*
Dipartimento di Medicina Sperimentale e Clinica
Università di Catanzaro
Via T. Campanella 115, 88100 Catanzaro (Italy)

[c] *Prof. H. Bräuner-Osborne*
Department of Medicinal Chemistry
Faculty of Pharmaceutical Sciences, University of Copenhagen
Universitetsparken 2, 2100 Copenhagen (Denmark)

[d] *Dr. G. Szabo, Dr. L. Gábor Hársing*
Division of Preclinical Research, EGIS Pharmaceuticals PLC
P.O. Box 100, 1475 Budapest (Hungary)

formational constraint and 2) homologation of the glutamate skeleton. Through the combined application of these approaches, a large number of potent and selective competitive NMDA antagonists were developed. Most of them are acidic amino acids bearing a phosphonate group as a bioisostere of the distal carboxylate. The proximal and the distal acidic groups are typically connected by a spacer of four or six carbon atom units[7,8] which, in some cases, is incorporated into a ring to decrease conformational freedom. Figure 1 shows some of the most striking NMDA antagonists, such as (*R*)-AP5, (*R*)-AP7, (*R*)-CPP,[9] (*R*)-CPPene,[10] and LY235959.[11] In other cases, homologation of the amino acid backbone produced selective mGluR ligands such as (*S*)-homo-AMPA,[12] which behaves as a highly selective agonist at the mGluR6 subtype.

In the recent past, we used such a strategy to design the new amino acids (±)-**1** and (±)-**2** (Figure 1),[13] which are homologues of glutamic acid containing one additional carbon unit and an amino acid skeleton locked in a bicyclic structure. These novel amino acids show a very interesting pharmacological profile. In particular, (±)-**1** is characterized by a remarkable affinity for the NMDA receptor ($K_i = 0.37$ μM, [^{3}H]CGP 39653), weak antagonism at mGluR1 ($K_i = 94$ μM) and mGluR5 ($K_i = 640$ μM), and partial agonism at mGluR2 (1 mM (±)-**1** produced 36±7% activation). We subsequently found that one enantiomer, (+)-**1**, is a quite potent and selective NMDA competitive antagonist, whereas the other, (−)-**1**, is responsible for the activity at metabotropic receptors.[14] Conversely, amino acid (±)-**2** is devoid of any activity at iGluRs, whereas it displays antagonistic activity at both mGluR1 ($K_i = 27$ μM) and mGluR5 ($K_i = 440$ μM), coupled to mGluR2 agonist activity ($EC_{50} = 16$ μM);[13] these biological activities reside in the dextrorotatory enantiomer.[14] As reported, the potency of NMDA antagonists of the "AP5 type" can be substantially increased through further homologation to give "AP7 type" ligands, for example, on passing from 4-phosphonomethylpiperazine-2-carboxylic acid to 4-phosphonopropylpiperazine-2-carboxylic acid (CPP).[7] Moreover, the introduction of an unsaturation in the chain can yield a further increase in potency, for example, on passing from CPP to CPPene.[7] Because derivatives (±)-**1** and (±)-**2** are characterized by the presence of a four-carbon-atom linker between the proximal and distal acidic groups, we designed the amino acids (±)-**3**–(±)-**6** reported herein (Figure 1), which are characterized by a six-carbon-atom spacer. In addition, as the experimental evidence suggests that the potency of NMDA antagonists can be markedly increased by replacing the distal carboxylic acid with the bioisosteric phosphonic acid group, we prepared and tested the phosphonate analogues (±)-**7**–(±)-**10** against NMDA receptors (Figure 1).

Results and Discussion

The synthesis of amino acids (±)-**3**–(±)-**10** was carried out by using bicyclic isoxazoline derivatives (±)-**11a** and (±)-**11b**[13] as starting materials, which were separately submitted to a selective reduction of the 3-ethoxycarbonyl group, performed with $NaBH_4$, to give primary alcohols (±)-**12a** and (±)-**12b** (Scheme 1). These intermediates were then oxidized with pyridinium chlorochromate (PCC) to yield the corresponding aldehydes (±)-**13a** and (±)-**13b**, which were directly submitted to a Wittig olefination with methyl(triphenylphosphoranylidene)-acetate to produce unsaturated derivatives (±)-**14** and (±)-**16**, respectively. In both cases we isolated only one stereoisomer to which we assigned the *E* configuration by taking into account the high value of the ^{1}H NMR coupling constant ($J = 16.5$ Hz) between the two olefinic protons. Stereoisomers (±)-**14** and (±)-**16** were then submitted to two different reaction sequences. In one case, they were converted into final amino acids (±)-**3** and (±)-**5** by alkaline hydrolysis with a mixture of 1 N aqueous NaOH and EtOH, followed by *N*-Boc deprotection with a solution of 30% trifluoroacetic acid in dichloromethane. In the other case, (±)-**14** and (±)-**16** were catalytically hydrogenated to yield the saturated intermediates (±)-**18** and (±)-**19**, which were then transformed into the final derivatives (±)-**4** and (±)-**6** following the procedure described for the unsaturated analogues.

The synthesis of the phosphonate derivatives (±)-**7**–(±)-**10** took advantage of the same key

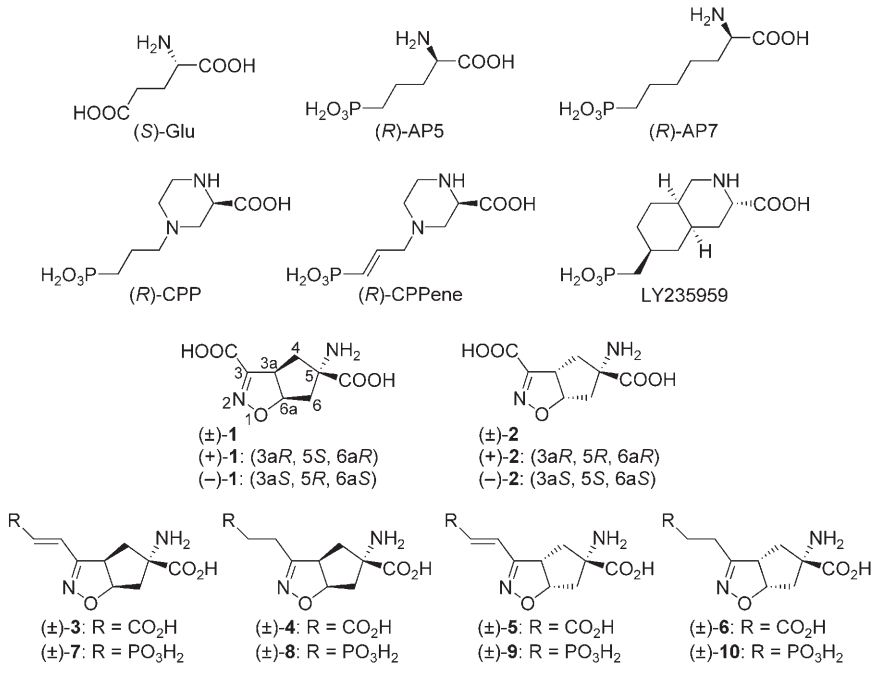
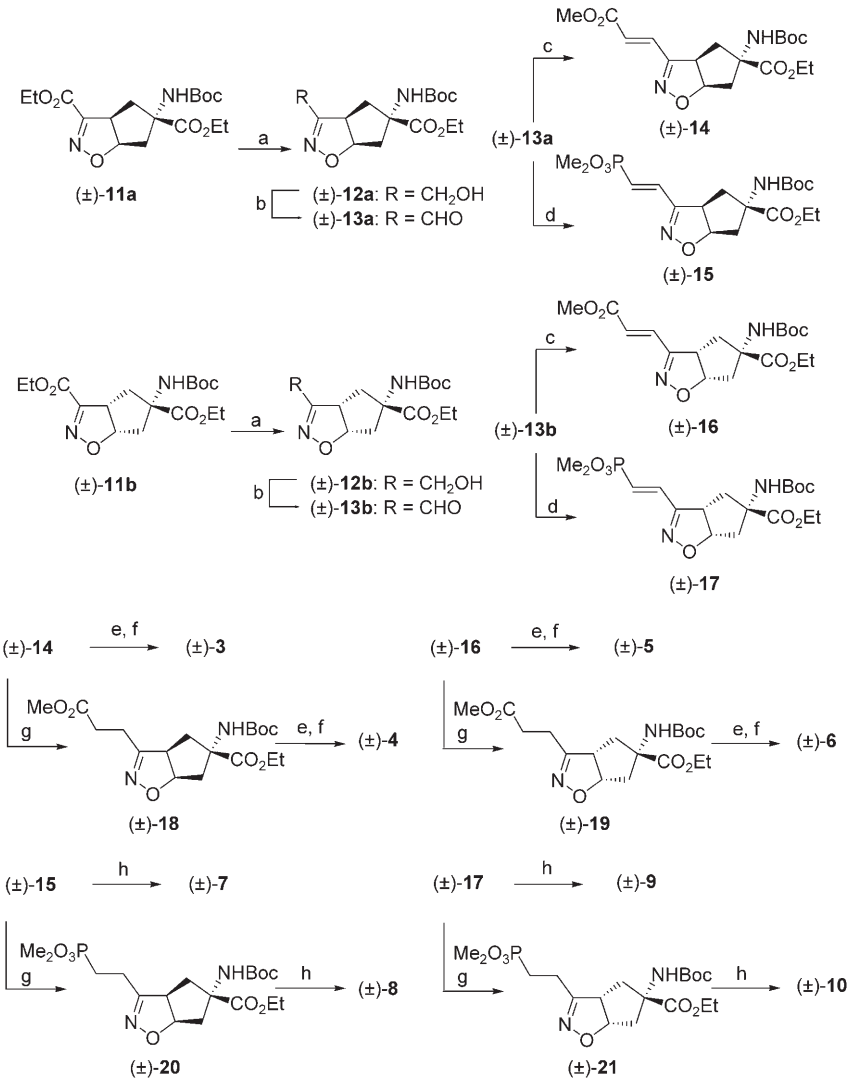


Figure 1. Structure of model and target compounds.



Scheme 1. Reagents and conditions: a) $NaBH_4$, EtOH; b) PCC, NaOAc, CH_2Cl_2; c) $Ph_3P{=}CHCO_2Me$, THF; d) $CH_2[PO(OEt)_2]_2$, NaH, THF; e) 1 N NaOH, EtOH; f) 30% TFA, CH_2Cl_2; g) 5% Pd/C, H_2, EtOAc or THF; h) 4 N HCl, EtOH, reflux.

intermediates, namely the aldehydes (±)-**13a** and (±)-**13b**, which were treated this time with tetraethyl diphosphomalonate in the presence of sodium hydride, to give compounds (±)-**15** and (±)-**17**, respectively. Similarly to the olefination reported above, we did not observe the formation of the *Z* stereoisomers. As described above, intermediates (±)-**15** and (±)-**17** were in part converted into their saturated analogues (±)-**20** and (±)-**21** by catalytic hydrogenation. Finally, phosphonate intermediates (±)-**15**, (±)-**17**, (±)-**20**, and (±)-**21** were transformed into the target amino acids (±)-**7**–(±)-**10** by treatment with 4 N HCl at reflux. This effected, in one step, cleavage of the *N*-Boc group and hydrolysis of both the carboxylic and phosphonic acid ester functions.

All new compounds were submitted to binding assays with iGluRs and second-messenger assays with representative mGluRs subtypes. The binding affinities for NMDA, AMPA, and KA receptors were measured on rat forebrain homogenates using [^{3}H]CGP 39653, [^{3}H]AMPA, and [^{3}H]kainic acid as respective radioligands.[15–17] Moreover, the affinities for the Gly binding site and for the noncompetitive Glu binding site were measured with [^{3}H]Gly and [^{3}H]MK-801, respectively.[18,19] Analogously to the model compound (±)-**1**, homologues (±)-**3** and (±)-**4** interact with the NMDA receptor, albeit with a significantly (40–65-fold) lower affinity (Table 1). They behave as competitive ligands, as they bind exclusively to the Glu binding site. In fact, at 10 μM, they interact neither with the Gly binding site nor with the MK-801 site, located inside the channel. Like (±)-**1**, they do not reveal any affinity for AMPA or KA receptors when tested at 10 μM, and interestingly, in contrast to their model compound, they are also inactive as agonists or antagonists toward representative mGluR subtypes at a concentration up to 1000 μM. Thus, (±)-**3** and (±)-**4** can be classified as selective but low-affinity ligands for the NMDA receptor. Unfortunately, our attempt to improve their NMDA affinity by replacing the distal carboxylate moiety with the bioisosteric phosphonate group was unsuccessful. In fact, the pharmacological profile of (±)-**7** and (±)-**8** is very similar to that of the carboxylate analogues (±)-**3** and (±)-**4**; their affinity for the NMDA receptor is even slightly lower (Table 1).

Taking into account the biological activity of amino acids (±)-**5** and (±)-**6**, the higher homologues of (±)-**2**, it is interesting to observe that the unsaturated derivative (±)-**5** shows a pharmacological profile strictly related to that of the parent compound, while the saturated analogue (±)-**6** is completely inactive at both iGlu and mGlu receptors. Interestingly, derivative (±)-**5** behaves as a selective mGluR ligand devoid of any affinity for iGluRs. In this case, the homologation left the potency substantially unaffected and gave rise to a selectivity for group I over group II mGluRs. In fact, (±)-**5** is a selective antagonist for group I mGluRs: $K_i = 36$ μM toward mGluR1 and $K_i = 160$ μM toward mGluR5.

Because group I mGlu receptor antagonists are known to possess anticonvulsant activity,[20] we tested (±)-**5** in vivo on DBA/2 mice to evaluate its ability to protect the animals against audiogenic seizures. Amino acid (±)-**5**, microinjected focally into the cerebral ventricles, was able to decrease the severity of seizures in a dose-dependent fashion. In fact, microin-

Table 1. Rat forebrain NMDA receptor binding, activity at cloned rat mGlu receptors expressed in CHO cells, and anticonvulsant activity in DBA/2 mice after i.c.v. administration.

Compd	[^{3}H]CGP 39653	mGluR1		mGluR2		mGluR4		mGluR5		ED_{50} [μmol mouse^{-1}][e]	
	K_i [μM]	K_i [μM]	EC_{50} [μM]	K_i [μM]	EC_{50} [μM]	K_i [μM]	EC_{50} [μM]	K_i [μM]	EC_{50} [μM]	clonus	tonus
(±)-**1**[a]	0.37 ± 0.03	94 ± 16	–	–	36 ± 7%[b]	> 1000	> 1000	640 ± 140	–	NT	NT
(±)-**2**[a]	> 100	27 ± 7	–	–	16 ± 3[c]	> 1000	> 1000	440 ± 70	–	0.56 (0.34–0.94)	0.20 (0.13–0.31)
(±)-**3**	26 (21–39)[d]	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	NT	NT
(±)-**4**	16 (13–19)[d]	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	NT	NT
(±)-**5**	> 100	36 ± 17	–	> 1000	> 1000	> 1000	> 1000	160 ± 62	–	0.69 (0.40–1.19)	0.20 (0.12–0.33)
(±)-**6**	> 100	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	NT	NT
(±)-**7**	37 (30–47)[d]	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
(±)-**8**	31 (25–38)[d]	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
(±)-**9**	> 100	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
(±)-**10**	> 100	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT

[a] Data from reference [12], affinity for NMDA receptors was measured on rat cortical membranes; [b] Percent activation relative to full L-Glu response; [c] Max = 59 ± 7%; [d] Data are the mean of three individual experiments, values in parentheses indicate the 95% confidence interval; [e] Anticonvulsant activity: ED_{50} values (i.c.v. administration) against audiogenic seizures induced in DBA/2 mice. NT: Not tested.

jection of (±)-**5** at doses of 1, 3, 7, and 10 μmol mouse^{-1} effected a marked protection against tonic and clonic seizures elicited by audiogenic stimuli. A lower dose (0.3 μmol mouse^{-1}) was only able to significantly protect the animals against the tonic phase of the audiogenic seizure. The ED_{50} values for clonus and tonus were 0.69 (0.40–1.19) and 0.20 (0.12–0.33) μmol mouse^{-1}, respectively. The observed anticonvulsant activity is similar to that of (±)-**2**, which gave ED_{50} values for clonus and tonus of 0.56 (0.34–0.94) and 0.20 (0.13–0.31) μmol mouse^{-1}, respectively. The toxic effects of the compounds are minimal, as no gross behavioral changes were observed after intracerebroventricular (i.c.v.) administration of either (±)-**2** or (±)-**5**. Despite the parallel behavior of (±)-**2** and (±)-**5** after i.c.v. administration, it is worth pointing out that whereas the former possesses significant anticonvulsant activity after i.p. administration, as previously reported,[13] amino acid (±)-**5** is unable to protect DBA/2 mice against the clonic phase of audiogenic seizures when administered i.p. at doses of 50, 75, 100, and 200 μmol kg^{-1}. A substantial protection against the clonic phase was observed, after i.p. administration, only at 300 μmol kg^{-1}.

Based on these results, it is evident that the increase in the length of the linker, while leaving the potency at mGluRs unaffected, drastically decreases the ability of the molecule to reach the CNS, presumably owing to a loss of affinity for the proteins involved in the transport of amino acids across the blood–brain barrier (BBB).

To explain the difference in biological behavior between (±)-**5** and (±)-**6**, we performed computational studies on the eutomer of model compound (3a*R*,5*R*,6a*R*)-(+)-**2** and on its related higher homologues (3a*R*,5*R*,6a*R*)-**5** and (3a*R*,5*R*,6a*R*)-**6.** Docking experiments were carried out with GOLD 3.1[21] using both the open (functionally inactive) and closed (active) conformations of the mGluR1 receptor, the coordinates of which were retrieved from the crystal structure (PDB code: 1ewk). As shown in Figure 2A, the amino acid moiety of (+)-**2** docked into the open form binds to residues T188 and S165 present in lobe I of mGluR1; an adjunctive hydrogen bond involving the distal carboxylic group could be created by rotating the side chain of S186, a residue conserved in both mGluR1 and mGluR5 subtypes. The distal carboxylate moiety is in close proximity to the side chain of the highly conserved K409, thus suggesting a hydrogen bond between these two groups. Moreover, a water molecule seems essential to mediate a hydrogen bond in the distal carboxylate group's interaction with the highly conserved R78 residue. On the other hand, to activate the mGlu1 receptor, the preferred interactions of (+)-**2** with lobe II should involve residues D318 and Y236. However, when we tried to dock the compound into the closed form, a steric clash between the cyclopentane ring of the ligand and the aromatic ring of tyrosine was observed, which prevents the closure of the binding cleft. Furthermore, an additional steric hindrance could arise from the protein loop formed by G319.

Based on the binding mode of (+)-**2**, the structurally related amino acid (3a*R*,5*R*,6a*R*)-**5** could bind to mGluR1 by adopting a similar network of bonds (Figure 2B); in this case, in contrast to (+)-**2**, the distal carboxylic group binds to R78 without the intermediacy of a water molecule. This selective contact is likely mediated by Y74, which is a distinctive feature of the mGluR1 and mGluR5 subtypes. Finally, to explain the inactivity of compound (3a*R*,5*R*,6a*R*)-**6**, we carried out a conformational analysis at the DFT/B3LYP/6-31g(d) level. The high flexibility of the side chain appended at position 3 of the isoxazoline ring allows a strong intramolecular ionic interaction between the α-amino and the distal carboxylic acid groups. The preferred torsion angle of the side chain results in a (−)-*gauche* conformation (Figure 2C), which is not suitable to establish the productive interactions needed to fit the binding sites of the receptor, thus making (±)-**6** biologically inactive.

Conclusions

Starting from model compounds (±)-**1** and (±)-**2**, which are conformationally constrained glutamic acid homologues, we designed a set of new amino acids characterized by a further increase in the distance between the two pharmacophoric entities, that is, the amino acid and the distal carboxylate moiety.

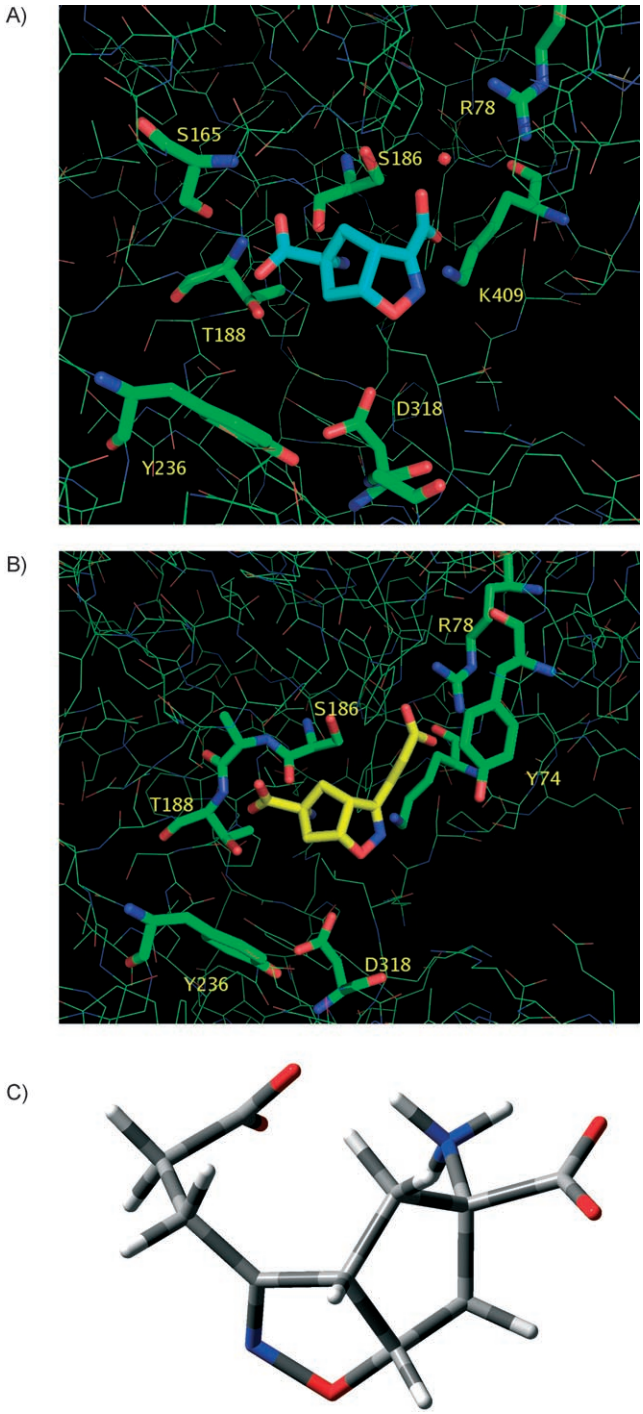


Figure 2. Schematic representations of A) (3a*R*,5*R*,6a*R*)-(+)-**2**, carbon atoms colored in blue and B) (3a*R*,5*R*,6a*R*)-**5**, carbon atoms colored in yellow, docked into the open form of mGluR1. C) Graphic representation of the preferred conformer of (3a*R*,5*R*,6a*R*)-**6**.

Additional modifications involved the insertion of a double bond in the side chain and the bioisosteric replacement of the distal carboxylic acid with a phosphonic acid group.

As far as the affinity for NMDA receptors is concerned, none of the modifications mentioned above turned out to be productive; all derivatives structurally related to the NMDA antagonist (±)-**1**, that is, (±)-**3**, (±)-**4**, (±)-**7**, and (±)-**8**, possess a reduced affinity for the same receptor complex. Therefore, we can conclude that, in this set of derivatives, the amino acid moiety and the distal acidic group are too far away for an optimal interaction with the binding sites of the NMDA receptor. Conversely, the same structural modifications applied to the nonselective mGluR ligand (±)-**2** produced divergent results. The saturated derivative (±)-**6** was completely inactive at all iGlu and mGlu receptors; this result has been rationalized with the presence of a folded conformation that is inadequate to fit the mGluR and iGluR binding pockets. By contrast, the unsaturated derivative (±)-**5** behaves as a selective group I mGlu receptor antagonist and, interestingly, it shows anticonvulsant activity after i.c.v. administration to DBA/2 mice. The lack of activity after i.p. administration may be the consequence of decreased affinity for the amino acid transporters, which mediate passage through the BBB. Because group I mGluRs are also expressed at the peripheral terminals of sensory neurons,[22] the latter result suggests the possible use of (±)-**5** to prevent and treat inflammatory pain.

Experimental Section

Materials and Methods

All reagents were purchased from Sigma. [^{3}H]CGP 39653 was purchased from New England Nuclear (NEN). (±)-(3a*S**,5*R**,6a*S**)-5-*tert*-Butoxycarbonylamino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,5-dicarboxylic acid diethyl ester (±)-**11a** and (±)-(3a*S**,5*S**,6a*S**)-5-*tert*-butoxycarbonylamino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,5-dicarboxylic acid diethyl ester (±)-**11b** were prepared as previously described.[13] ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mercury 300 spectrometer in $CDCl_3$ or D_2O at 20 °C. Chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hz. TLC analyses were performed on commercial silica gel 60 F_{254} aluminum sheets; spots were visualized by spraying with a dilute alkaline solution of $KMnO_4$ or with ninhydrin. Melting points were determined on a model B 540 Büchi apparatus and are uncorrected. Microanalyses (C, H, N) of new compounds agreed with the theoretical values within ±0.4%.

Synthesis of (±)-(3a*S,5*R**,6a*S**)-5-*tert*-butoxycarbonylamino-3-hydroxymethyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-5-carboxylic acid ethyl ester (±)-12a**: $NaBH_4$ (0.25 g, 6.6 mmol) was added to a solution of (±)-**11a**[13] (2.22 g, 6.0 mmol) in EtOH (80 mL). The reaction was vigorously stirred for 10 h, and the progress of the reaction was followed by TLC (petroleum ether/EtOAc 4:1). After that time the mixture was adjusted to pH 7 with 2 N aqueous HCl, and the solvent was evaporated. The aqueous layer was extracted with CH_2Cl_2, and the organic layer was dried over anhydrous Na_2SO_4. The crude material was purified by column chromatography on silica gel (petroleum ether/EtOAc 7:3) to give (±)-**12a** (1.02 g, 3.1 mmol, 52%) as a colorless oil; R_f = 0.57 (CH_2Cl_2/MeOH 95:5); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.25 (t, 3H, J = 7.0), 1.42 (s, 9H), 1.62 (br s, 1H), 2.28–2.40 (m, 1H), 2.43 (d, 2H, J = 5.8), 2.72 (dd, 1H, J = 5.8, 14.0), 3.91 (ddd, 1H, J = 5.8, 10.0, 10.0), 4.18 (q, 2H, J = 7.0), 4.45 (s, 2H), 5.01 (br s, 1H), 5.22 ppm (ddd, 1H, J = 5.8, 5.8, 10.0).

Synthesis of (±)-(3a*S,5*R**,6a*S**)-5-*tert*-butoxycarbonylamino-3-(2-methoxycarbonylvinyl)-4,5,6,6a-tetrahydro-3a*H*-cyclopenta-[*d*]isoxazole-5-carboxylic acid ethyl ester (±)-14**: 1) NaOAc (1.02 g, 12.4 mmol) and PCC (3.34 g, 15.5 mmol) were added to a

stirred solution of (±)-**12a** (1.02 g, 3.1 mmol) in CH_2Cl_2 (30 mL). The reaction was stirred at room temperature for 2 h until completion. Et_2O was added, and the reaction mixture was filtered under vacuum through a FLORISIL column. The column was then washed with Et_2O (3×100 mL). The organic solvent was evaporated under reduced pressure to give the intermediate aldehyde (±)-**13a** as a yellow oil, which was used as a crude material in the next step.

2) Methyl(triphenylphosphoranylidene)acetate (1.04 g, 3.1 mmol) was added to a stirred solution of (±)-**13a** in anhydrous THF (30 mL), and the reaction was stirred at room temperature for 2 h until completion. The crude material, obtained after evaporation of the solvent, was purified by column chromatography on silica gel (petroleum ether/EtOAc 7:3) to give (±)-**14** (770 mg, 2.01 mmol) as a white solid. Overall yield: 65%, crystallized from *i*PrOH; mp: (dec) >200 °C; R_f=0.28 (petroleum ether/EtOAc 7:3); ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.22 (t, 3H, J=7.0), 1.42 (s, 9H), 2.41 (dd, 1H, J=5.5, 14.0), 2.48 (dd, 1H, J=7.0, 14.0), 2.60 (m, 2H), 3.80 (s, 3H), 3.95 (ddd, 1H, J=7.5, 9.0, 9.5), 4.18 (q, 2H, J=7.0), 4.98 (br s, 1H), 5.35 (ddd, 1H, J=5.5, 7.0, 9.5), 6.08 (d, 1H, J=16.5), 7.55 ppm (d, 1H, J=16.5); anal. calcd for $C_{18}H_{26}N_2O_7$: C 56.53%, H 6.85%, N 7.33%, found: C 56.61%, H 7.01%, N 7.12%.

Synthesis of (±)-(3a*S,5*R**,6a*S**)-5-amino-3-(2-carboxyvinyl)-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-5-carboxylic acid (±)-3**: 1) Derivative (±)-**14** (370 mg, 0.97 mmol) was dissolved in EtOH (2.5 mL) and treated with 1 N aqueous NaOH (2.5 mL). The reaction was stirred at room temperature for 5 h until completion. The disappearance of the starting material was monitored by TLC (petroleum ether/EtOAc 7:3). After evaporation of EtOH the aqueous layer was washed with Et_2O, made acidic with 2 N aqueous HCl and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and, after evaporation of the solvent, the diacid intermediate (330 mg, 0.88 mmol) was obtained as a white solid, which was directly submitted to the next step.

2) The diacid intermediate (330 mg, 0.88 mmol) was treated with trifluoroacetic acid (TFA, 30%, 8.8 mmol) in CH_2Cl_2 at 0 °C. The solution was stirred at room temperature for 4 h, and the reaction was followed by TLC ($CHCl_3$/CH_3OH 9:1 + 1% HOAc). The volatiles were removed under reduced pressure, and the residue was crystallized from EtOH and Et_2O to give (±)-**3** (127 mg, 55% overall yield) as white prisms; mp: (dec) >190 °C; R_f=0.40 (BuOH/H_2O/HOAc 4:2:1); ^{1}H NMR (300 MHz, D_2O): δ=2.42–2.50 (m, 2H), 2.52–2.58 (m, 2H), 4.18 (ddd, 1H, J=9.5, 9.5, 9.5), 5.36 (ddd, 1H, J=5.8, 5.8, 9.5), 6.19 (d, 1H, J=16.5), 7.30 ppm (d, 1H, J=16.5); ^{13}C NMR (75.5 MHz, D_2O): δ=39.7, 43.8, 50.6, 67.4, 88.3, 127.6, 131.6, 160.0, 169.9, 174.2 ppm; anal. calcd for $C_{10}H_{12}N_2O_5$: C 50.00%, H 5.04%, N 11.66%, found: C 49.88%, H 5.12%, N 11.53%.

Synthesis of (±)-(3a*S,5*R**,6a*S**)-5-*tert*-butoxycarbonylamino-3-(2-methoxycarbonylethyl)-4,5,6,6a-tetrahydro-3a*H*-cyclopenta-[*d*]isoxazole-5-carboxylic acid ethyl ester (±)-18**: 5% Pd/C (50 mg) was added to a solution of (±)-**14** (400 mg, 1.05 mmol) in THF (30 mL). The mixture was hydrogenated for 6 h until completion. The catalyst was then filtered off, and the solvent was evaporated under reduced pressure. The crude material was purified by column chromatography on silica gel (petroleum ether/EtOAc 4:1) to give the corresponding saturated derivative (±)-**18** (280 mg, 70% yield) as a colorless oil; R_f=0.33 (petroleum ether/EtOAc 4:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.24 (t, 3H, J=7.3), 1.42 (s, 9H), 2.30 (dd, 1H, J=5.5, 14.2), 2.44 (dd, 1H, J=6.5, 14.2), 2.45–2.76 (m, 6H), 3.68 (s, 3H), 3.73 (ddd, 1H, J=8.8, 8.8, 8.8), 4.16 (q, 2H, J=7.3), 5.01 (br s, 1H), 5.14 ppm (ddd, 1H, J=5.5, 6.5, 8.8); anal. calcd for $C_{18}H_{28}N_2O_7$: C 56.24%, H 7.34%, N 7.29%, found: C 56.45%, H 7.01%, N 7.47%.

Synthesis of (±)-(3a*S,5*R**,6a*S**)-5-amino-3-(2-carboxyethyl)-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-5-carboxylic acid (±)-4**: 1) Derivative (±)-**18** (280 mg, 0.73 mmol) was dissolved in EtOH (2.5 mL) and treated with 1 N aqueous NaOH (2.2 mL). The reaction was stirred at room temperature for 4 h until completion. The disappearance of the starting material was monitored by TLC (petroleum ether/EtOAc 7:3). After evaporation of EtOH the aqueous layer was washed with Et_2O, made acidic with 2 N aqueous HCl, and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and, after evaporation of the solvent, the diacid intermediate (220 mg, 0.64 mmol) was obtained as a white solid.

2) The diacid (220 mg, 0.64 mmol) was treated with TFA (30%, 6.4 mmol) in CH_2Cl_2 at 0 °C. The solution was stirred at room temperature for ~3 h, and the reaction was followed by TLC ($CHCl_3$/CH_3OH 9:1 + 1% HOAc). The volatiles were removed under reduced pressure, and the residue was crystallized from EtOH and Et_2O to give (±)-**4** (108 mg, 62% overall yield) as white prisms; mp: (dec) >175 °C; R_f=0.31 (BuOH/H_2O/HOAc 4:2:1); ^{1}H NMR (300 MHz, D_2O): δ=2.30 (dd, 1H, J=9.5, 14.6), 2.38–2.70 (m, 7H), 3.93 (ddd, 1H, J=5.8, 9.5, 9.5), 5.17 ppm (ddd, 1H, J=5.8, 5.8, 9.5); ^{13}C NMR (75.5 MHz, D_2O): δ=21.78, 30.43, 38.49, 43.76, 54.91, 67.11, 85.06, 162.30, 174.60, 177.06 ppm; anal. calcd for $C_{10}H_{14}N_2O_5$: C 49.58%, H 5.83%, N 11.56%, found: C 49.43%, H 6.01%, N 11.50%.

Synthesis of (±)-(3a*S,5*S**,6a*S**)-5-*tert*-butoxycarbonylamino-3-hydroxymethyl-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-5-carboxylic acid ethyl ester (±)-12b**: (±)-**12b** was obtained from (±)-**11b**,[13] following the procedure described for the synthesis of (±)-**12a**, in comparable yield: R_f=0.36 (CH_2Cl_2/MeOH 95:5); ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.24 (t, 3H, J=7.0), 1.38 (s, 9H), 1.60 (br s, 1H), 2.24–2.42 (m, 2H), 2.53 (dd, 1H, J=9.3, 14.3), 2.81 (m, 1H), 3.91 (ddd, 1H, J=1.0, 9.3, 9.3), 4.18 (m, 2H), 4.40 (d, 1H, J=12.1), 4.41 (d, 1H, J=12.1), 5.14 (br s, 1H), 5.2 ppm (ddd, 1H, J=2.6, 5.1, 9.3).

Synthesis of (±)-(3a*S,5*S**,6a*S**)-5-*tert*-butoxycarbonylamino-3-(2-methoxycarbonylvinyl)-4,5,6,6a-tetrahydro-3a*H*-cyclopenta-[*d*]isoxazole-5-carboxylic acid ethyl ester (±)-16**: (±)-**16** was obtained from (±)-**12b** in two steps, following the procedure described for the synthesis of (±)-**14**, in comparable yield: crystallized from *i*PrOH; mp: 172–174 °C; R_f=0.28 (petroleum ether/EtOAc 7:3); ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.22 (t, 3H, J=7.2), 1.38 (s, 9H), 2.38 (m, 2H), 2.70 (m, 2H), 3.78 (s, 3H), 3.92 (ddd, 1H, J=6.5, 9.5, 9.5), 4.18 (m, 2H), 5.00 (br s, 1H), 5.30 (ddd, 1H, J=4.5, 4.5, 9.5), 6.10 (d, 1H, J=16.5), 7.50 ppm (d, 1H, J=16.5); anal. calcd for $C_{18}H_{26}N_2O_7$: C 56.53%, H 6.85%, N 7.33%, found: C 56.66%, H 7.12%, N 7.13%.

Synthesis of (±)-(3a*S,5*S**,6a*S**)-5-amino-3-(2-carboxyvinyl)-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-5-carboxylic acid (±)-5**: Amino acid (±)-**5** was obtained in two steps, starting from (±)-**16**, following the procedure described for the synthesis of (±)-**3**, in comparable yield: white prisms; mp: (dec) >200 °C; R_f=0.45 (BuOH/H_2O/HOAc 4:2:1); ^{1}H NMR (300 MHz, D_2O): δ=2.02 (dd, 1H, J=5.5, 14.5), 2.24 (dd, 1H, J=3.2, 15.0), 2.69 (m, 2H), 4.13 (ddd, 1H, J=5.5, 9.5, 9.5), 5.35 (ddd, 1H, J=3.2, 5.6, 9.5), 6.22 (d, 1H, J=16.5), 7.27 ppm (d, 1H, J=16.5); ^{13}C NMR (75.5 MHz, D_2O): δ=39.7, 43.8, 50.6, 67.4, 88.3, 127.6, 131.6, 160.0, 169.9, 174.2 ppm; anal. calcd for $C_{10}H_{12}N_2O_5$: C 50.00%, H 5.04%, N 11.66%, found: C 49.88%, H 5.27%, N 11.51%.

Synthesis of (±)-(3a*S*,5S*,6aS)-5-*tert*-butoxycarbonylamino-3-(2-methoxycarbonylethyl)-4,5,6,6a-tetrahydro-3a*H*-cyclopenta-[*d*]isoxazole-5-carboxylic acid ethyl ester (±)-19**: (±)-**19** was obtained from (±)-**16**, following the procedure described for the synthesis of (±)-**18**, in comparable yield: colorless oil; R_f = 0.24 (petroleum ether/EtOAc 4:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.22 (t, 3H, J = 7.0), 1.39 (s, 9H), 2.30 (d, 2H, J = 3.6), 2.45–2.56 (m, 6H), 3.67 (s, 3H), 3.72 (dd, 1H, J = 9.5, 9.5), 4.16 (m, 2H), 5.12 (ddd, 1H, J = 3.6, 3.6, 9.5), 5.19 ppm (br s, 1H); anal. calcd for $C_{18}H_{28}N_2O_7$: C 56.24%, H 7.34%, N 7.29%, found: C 56.54%, H 7.01%, N 7.10%.

Synthesis of (±)-(3a*S*,5S*,6aS)-5-amino-3-(2-carboxyethyl)-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-5-carboxylic acid (±)-6**: Amino acid (±)-**6** was obtained in two steps, starting from (±)-**19**, following the procedure described for the synthesis of (±)-**4**, in comparable yield: white prisms; mp: (dec) > 185 °C; R_f = 0.37 (BuOH/H_2O/HOAc 4:2:1); ^{1}H NMR (300 MHz, D_2O): δ = 2.03 (dd, 1H, J = 4.5, 15.0), 2.13 (dd, 1H, J = 3.0, 15.4), 2.62 (m, 6H), 3.92 (ddd, 1H, J = 4.5, 9.2, 9.2), 5.19 ppm (ddd, 1H, J = 3.0, 6.2, 9.2); ^{13}C NMR (75.5 MHz, D_2O): δ = 22.0, 30.2, 38.9, 42.8, 54.7, 67.1, 87.0, 163.8, 175.2, 177.0 ppm; anal. calcd for $C_{10}H_{14}N_2O_5$: C 49.58%, H 5.83%, N 11.56%, found: C 49.37%, H 5.91%, N 11.50%.

Synthesis of (±)-(3a*S*,5R*,6aS)-5-*tert*-butoxycarbonylamino-3-[(*E*)-2-(dimethoxyphosphoryl)vinyl]-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-5-carboxylic acid ethyl ester (±)-15**: Tetraethyl methylenediphosphonate (1.5 mL, 6.2 mmol) was added to a suspension of NaH (75 mg, 3.1 mmol) in benzene (25 mL). The mixture was stirred for 30 min at room temperature. After that time a solution of aldehyde **13a** (3.1 mmol) in benzene (25 mL) was added dropwise, and the reaction mixture was stirred for 1 h until completion. The solvent was then evaporated under reduced pressure, and the crude material was purified by column chromatography on silica gel (petroleum ether/EtOAc 3:7) to give (±)-**15** (1.1 g, 2.5 mmol, 81% yield) as a colorless oil; R_f = 0.26 (petroleum ether/EtOAc 3:7); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.22 (t, 3H, J = 6.9), 1.32 (t, 6H, J = 6.9), 1.42 (s, 9H), 2.35 (m, 2H), 2.60 (m, 2H), 3.93 (m, 1H), 4.10 (m, 6H), 5.30 (m, 1H), 5.94 (dd, 1H, J = 17.5, 17.8), 7.25 ppm (dd, 1H, J = 17.5, 21.6); anal. calcd for $C_{18}H_{29}N_2O_8P$: C 50.00%, H 6.76%, N 6.48%, found: C 49.88%, H 7.11%, N 6.37%.

Synthesis of (±)-(3a*S*,5R*,6aS)-5-amino-3-[(*E*)-2-(phosphonovinyl)]-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-5-carboxylic acid (±)-7**: 4 N aqueous HCl (20 mL) was added to a solution of (±)-**15** (500 mg, 1.1 mmol) in EtOH (10 mL). The reaction mixture was held at reflux for 5 days. The solvent was then removed under reduced pressure, and the solid residue was taken up with MeOH, filtered, washed with MeOH and Et_2O, and dried to give (±)-**7** (106 mg, 35% yield) as a white solid; mp: (dec) > 184 °C; R_f = 0.46 (BuOH/H_2O/HOAc 3:2:2); ^{1}H NMR (300 MHz, D_2O): δ = 2.28–2.60 (m, 4H), 4.10 (m, 1H), 5.22 (m, 1H), 6.14 (dd, 1H, J = 14.5, 17.5), 6.76 ppm (dd, 1H, J = 17.5, 17.5); ^{13}C NMR (75.5 MHz, D_2O): δ = 39.2, 43.6, 50.8, 66.0, 87.4, 129.0, 130.7, 161.1, 172.9 ppm; anal. calcd for $C_9H_{13}N_2O_6P$: C 39.14%, H 4.74%, N 10.14%, found: C 38.90%, H 4.79%, N 10.08%.

Synthesis of (±)-(3a*S*,5R*,6aS)-5-*tert*-butoxycarbonylamino-3-[(*E*)-2-(dimethoxyphosphoryl)ethyl]-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-5-carboxylic acid ethyl ester (±)-20**: 5% Pd/C (60 mg) was added to a solution of (±)-**15** (600 mg, 1.3 mmol) in EtOAc (30 mL). The reaction mixture was hydrogenated overnight. The catalyst was then filtered off, and the solvent evaporated under reduced pressure. The crude material was purified by column chromatography on silica gel (petroleum ether/EtOAc 3:7) to give (±)-**20** (460 mg, 77% yield): colorless oil; R_f = 0.14 (petroleum ether/EtOAc 3:7); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.25 (t, 3H, J = 5.0), 1.33 (t, 6H, J = 5.0), 1.43 (s, 9H), 2.10 (m, 2H), 2.30–2.80 (m, 6H), 3.76 (ddd, 1H, J = 7.7, 8.0, 10), 4.12 (m, 6H), 4.94 (s, 1H), 5.20 ppm (ddd, 1H, J = 6.0, 6.2, 10); anal. calcd for $C_{18}H_{31}N_2O_8P$: C 49.77%, H 7.19%, N 6.45%, found: C 49.98%, H 7.02%, N 6.40%.

Synthesis of (±)-(3a*S*,5R*,6aS)-5-amino-3-[(*E*)-2-(phosphonoethyl)]-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-5-carboxylic acid (±)-8**: 4 N aqueous HCl (20 mL) was added to a solution of (±)-**20** (460 mg, 0.1 mmol) in EtOH (8 mL). The reaction mixture was held at reflux for 5 days. The solvent was then removed under reduced pressure, and the solid residue was taken up with MeOH, filtered under vacuum, washed with MeOH and Et_2O, and dried to give (±)-**8** (97 mg, 35% yield) as white prisms; mp: (dec) > 190 °C; R_f = 0.44 (BuOH/H_2O/HOAc 3:2:2); ^{1}H NMR (300 MHz, D_2O): δ = 1.78 (m, 2H), 2.18 (dd, 1H, J = 10.2, 14.6), 2.30–2.58 (m, 5H), 3.90 (m, 1H), 5.08 ppm (m, 1H); ^{13}C NMR (75.5 MHz, D_2O): δ = 21.0, 25.5, 38.3, 43.8, 54.5, 66.7, 85.1, 164.1, 174.9 ppm; anal. calcd for $C_9H_{15}N_2O_6P$: C 38.86%, H 5.43%, N 10.07%, found: C 38.58%, H 5.49%, N 9.99%.

Synthesis of (±)-(3a*S*,5S*,6aS)-5-*tert*-butoxycarbonylamino-3-[(*E*)-2-(dimethoxyphosphoryl)vinyl]-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-5-carboxylic acid ethyl ester (±)-17**: (±)-**17** was obtained from (±)-**13b**, following the procedure described for the synthesis of (±)-**15**, in comparable yield: colorless oil; R_f = 0.28 (petroleum ether/EtOAc 3:7); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.22 (t, 3H, J = 6.9), 1.30 (t, 6H, J = 6.9), 1.38 (s, 9H), 2.35 (m, 2H), 2.64 (m, 2H), 3.90 (ddd, 1H, J = 4.8, 9.4, 9.6), 4.00–4.12 (m, 6H), 5.02 (s, 1H), 5.24 (ddd, 1H, J = 4.5, 4.7, 9.6), 5.98 (dd, 1H, J = 17.6, 17.6), 7.22 ppm (dd, 1H, J = 17.6, 17.6); anal. calcd for $C_{18}H_{29}N_2O_8P$: C 50.00%, H 6.76%, N 6.48%, found: C 49.87%, H 7.14%, N 6.31%.

Synthesis of (±)-(3a*S*,5S*,6aS)-5-amino-3-[(*E*)-2-(phosphonovinyl)]-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-5-carboxylic acid (±)-9**: Amino acid (±)-**9** was obtained from (±)-**17**, following the procedure described for the synthesis of (±)-**7**, in comparable yield: white prisms; mp: (dec) > 185 °C; R_f = 0.46 (BuOH/H_2O/HOAc 4:2:1); ^{1}H NMR (300 MHz, D_2O): δ = 2.02 (dd, 1H, J = 5.9, 14.6), 2.20 (dd, 1H, J = 3.2, 14.9), 2.74 (m, 2H), 4.12 (ddd, 1H, J = 5.9, 9.4, 10.0), 5.27 (ddd, 1H, J = 3.2, 5.9, 9.4), 6.16 (dd, 1H, J = 14.6, 17.9), 6.78 ppm (dd, 1H, J = 17.9, 19.9); ^{13}C NMR (75.5 MHz, D_2O): δ = 40.0, 42.7, 50.6, 66.5, 88.8, 128.6, 131.6, 162.0, 174.1 ppm; anal. calcd for $C_9H_{13}N_2O_6P$: C 39.14%, H 4.74%, N 10.14%, found: C 38.82%, H 4.82%, N 10.05%.

Synthesis of (±)-(3a*S*,5S*,6aS)-5-*tert*-butoxycarbonylamino-3-[(*E*)-2-(dimethoxyphosphoryl)ethyl]-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-5-carboxylic acid ethyl ester (±)-21**: (±)-**21** was obtained from (±)-**17**, following the procedure described for the synthesis of (±)-**20**, in comparable yield: colorless oil; R_f = 0.14 (petroleum ether/EtOAc 3:7); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.18 (t, 3H, J = 7.0), 1.28 (t, 6H, J = 7.0), 1.36 (s, 9H), 2.00–2.60 (m, 8H), 3.70 (dd, 1H, J = 9.1, 9.5), 4.04 (m, 6H), 5.10 (m, 1H), 5.17 ppm (s, 1H); $C_{18}H_{31}N_2O_8P$: C 49.77%, H 7.19%, N 6.45%, found: C 49.94%, H 7.27%, N 6.30%.

Synthesis of (±)-(3a*S*,5S*,6aS)-5-amino-3-[(*E*)-2-(phosphonoethyl)]-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-5-carboxylic acid (±)-10**: Amino acid (±)-**10** was obtained starting from (±)-**21**, following the procedure described for the synthesis of (±)-**8**, in comparable yield: white prisms; mp: (dec) > 195 °C; R_f = 0.44 (BuOH/H_2O/HOAc 3:2:2); ^{1}H NMR (300 MHz, D_2O): δ = 1.73 (m, 2H), 2.05 (dd, 1H, J = 5.0, 14.6), 2.15 (dd, 1H, J = 3.2, 15.8), 2.50 (m, 4H), 3.86 (m, 1H), 5.13 ppm (m, 1H); ^{13}C NMR (75.5 MHz, D_2O):

δ = 21.0, 25.2, 38.7, 42.5, 54.1, 66.3, 86.8, 164.4, 174.2 ppm; anal. calcd for $C_9H_{15}N_2O_6P$: C 38.86%, H 5.43%, N 10.07%, found: C 38.65%, H 5.50%, N 10.01%.

Pharmacology

Receptor binding at iGluRs: The membrane preparations used in glutamate receptor binding experiments were prepared according to Murphy et al.[23] Affinity for [^{3}H]CGP 39653 was determined in Tris buffer (50 mM, pH 7.4, on ice) containing 4 nM [^{3}H]CGP 39653 ligand for 60 min.[15] Assays were carried out in triplicate; L-Glu (0.1 mM) was used to define nonspecific binding. Bound radioactivity was separated from free radioactivity by vacuum filtration through Whatman GF/B glass fiber filters, and the radioactivity was determined using a Packard TriCarb Scintillation Analyzer 2900TR. IC_{50} determinations were performed with seven different concentrations over the range of 1–50 μM. Binding data were analyzed by nonlinear regression using GraphPad Prism 4.03 (GraphPad Software, San Diego, CA, USA). Data were fitted to sigmoidal dose–response curves. For K_i determinations, the following equation was used: $K_i = IC_{50} \times K_d/(K_d+L)$, for which K_d = 6 nM[15] and L = 4 nM. Affinities for AMPA,[16] KA,[17] glycine,[18] and noncompetitive NMDA[19] receptor sites were determined using 2 nM [^{3}H]AMPA, 1 nM [^{3}H]KA, 10 nM [^{3}H]glycine, and 2 nM [^{3}H]MK-801, respectively.

mGluR Activity: Chinese hamster ovary (CHO) cell lines stably expressing rat mGluR1a, mGluR2, mGluR4a, and mGluR5a were prepared as previously described.[24] Measurement of intracellular Ca^{2+} levels and cyclic AMP formation: pharmacological activity at mGluR1a was assessed by measurement of intracellular Ca^{2+} levels as previously described.[24] Pharmacological activity at mGluR2 and mGluR4a was assessed by measuring intracellular cAMP levels as previously described.[25]

In vivo pharmacology: DBA/2 mice (8–12 g, 22–25 days old) were purchased from Charles River (Calco, Como, Italy). Procedures involving animals and their care were conducted in conformity with international and national law and policies (European Communities Council Directive of 24th November 1986, 86/609EEC). Groups of 10 mice of either sex were exposed to auditory stimulation 30 min following the administration of vehicle or each dose of the test drug. The compounds were given i.p. (0.1 mL per 10 g of body weight of the mouse) as a freshly prepared solution in 50:50 DMSO/NaCl (sterile 0.9% solution) or i.c.v. (10 μL mouse^{-1}). Individual mice were placed under an hemispheric perspex dome (∅ = 58 cm), and 60 s were allowed to accustom and to assess locomotor activity. Auditory stimulation (12–16 kHz, 109 dB) was applied for 60 s or until tonic extension occurred and induced a sequential seizure response in control DBA/2 mice, consisting of an early wild running phase, followed by generalized myoclonus and tonic flexion and extension, sometimes followed by respiratory arrest.[26] Behavioral changes were observed and recorded during the period between drug administration and auditory testing. The ED_{50} values of each phase of the audiogenic seizure were determined for each dose of administered compound; dose–response curves were fitted using a computer program by the method of Litchfield and Wilcoxon.[27]

Molecular Modeling

Ligand geometry and conformation: The conformational space of (3a*R*,5*R*,6a*R*)-**6** was explored with the DFT/B3LYP approach at the 6-31g(d) level as implemented in Gaussian 03.[28] The amino and the two carboxylic acid groups were considered in the ionized states to better simulate physiological conditions. All starting geometries deriving from the pseudorotational path of the five-membered carbocyclic ring and from rotation around the exocyclic single bonds were fully optimized. The energy of the conformations were recalculated in a polarizable conductor-like solvation model (C-PCM)[29] to obtain values compatible with a water solution. The preferred conformation of the side chain is (−)-*gauche*, the energy of which is 1.31 kcal mol^{-1} lower than that of the (+)-*gauche* conformation and 13.51 kcal mol^{-1} lower than the *anti* one. Although these energy differences might be overestimated, they give a clear idea of the ionic interaction between the amino and the distal carboxylate groups. The ligands docked into the receptors were built by Sybyl 7.3 (Tripos Inc., 1699 South Hanley Rd., St. Louis, MO 63144, USA) and preliminarily minimized by Gaussian 03[28] at the DFT/B3LYP/6-31g(d) level.

Docking calculations at mGluR1: The two conformational states of the mGluR1 were retrieved from the Protein Data Bank (code: 1ewk). Docking calculations were performed with the GOLD 3.1 program[21] to optimize the Tyr, Thr, and Ser hydroxy hydrogen atom located in the binding pocket. Lone pairs were automatically added with the default geometry. The GoldScore fitness function and the distribution of torsion angles were chosen to evaluate the quality of the docking results. Van der Waals and hydrogen bonding radii were set at 4.0 and 3.0 Å, respectively; genetic algorithm parameters were kept at the default values.

To evaluate the quality of the 100 poses obtained by GOLD 3.1, the Autonomous Hierarchical Agglomerative Cluster Analysis (AClAP 1.0)[30] was applied. The recently developed AClAP 1.0 applies the average linkage role with the KGS penalty function, as was shown by Bottegoni et al.[31] to be the best combination for a robust clustering protocol. The poses shown in Figure 2 A and B are representative of the most populated clusters, which were also the lowest-energy conformers according to Gold Scoring Functions. Finally, the results were visually inspected by Sybyl 7.3 to evaluate both the quality and accordance of the resulting binding mode with the experimental biological data.

Acknowledgments

This work was financially supported by MIUR (PRIN 2005, Rome), Università degli Studi di Milano (FIRST) and the Danish Medical Research Council (to H.B.O.). G.G. thanks Professors Maurizio Recanatini and Andrea Cavalli (Dipartimento di Scienze Farmaceutiche, Università degli Studi di Bologna, Italy) for their kind support in the use of AClAP and MatLab softwares.

Keywords: amino acids · anticonvulsants · glutamic acid · mGluR antagonists · NMDA receptors

[1] *Excitatory Amino Acids and Synaptic Transmission* (Eds.: H. V. Wheal, A. M. Thomson), Academic Press, London, **1995**.
[2] H. Bräuner-Osborne, J. Egebjerg, E. Ø. Nielsen, U. Madsen, P. Krogsgaard-Larsen, *J. Med. Chem.* **2000**, *43*, 2609 – 2645.
[3] *The Ionotropic Glutamate Receptors* (Eds.: D. T. Monaghan, R. J. Wenthold), Humana, Totowa, **1997**.
[4] *The Metabotropic Glutamate Receptors* (Eds.: P. J. Conn, J. Patel), Humana, Totowa, **1994**.
[5] *Metabotropic Glutamate Receptors and Brain Function* (Eds.: F. Moroni, F. Nicoletti, D. E. Pellegrini-Giampietro), Portland, London, **1998**.
[6] K. Steece-Collier, L. K. Chambers, S. S. Jaw-Tsai, F. S. Menniti, J. T. Greenamyre, *Exp. Neurol.* **2000**, *163*, 239 – 243.

[7] G. Johnson, P. L. Ornstein, *Curr. Pharm. Des.* **1996**, *2*, 331 – 356.
[8] P. L. Ornstein, V. J. Klimkowski in *Excitatory Amino Acid Receptors: Design of Agonists and Antagonists* (Eds.: P. Krogsgaard-Larsen, J. J. Hansen), Ellis Horwood, Chichester, **1992**, pp. 183 – 200.
[9] J. Lehmann, J. Schneider, S. E. McPherson, D. E. Murphy, P. Bernard, C. Tsai, D. A. Bennet, G. Pastor, M. Schmutz, C. M. Sinton, D. J. Steele, C. Bohem, D. L. Cheney, J. M. Lieberman, M. Williams, P. L. Wood, *J. Pharmacol. Exp. Ther.* **1987**, *240*, 737 – 746.
[10] B. Aebischer, P. Frey, H. P. Haerter, P. L. Herrling, W. Mueller, H. J. Olverman, J. C. Watkins, *Helv. Chim. Acta* **1989**, *72*, 1043 – 1051.
[11] P. L. Ornstein, D. D. Schoepp, M. B. Arnold, N. K. Augenstein, D. Lodge, J. D. Millar, J. Chambers, J. Campbell, J. W. Paschal, D. L. Zimmerman, J. D. Leander, *J. Med. Chem.* **1992**, *35*, 3547 – 3560.
[12] H. Ahmadian, B. Nielsen, H. Bräuner-Osborne, T. N. Johansen, T. B. Stensbøl, F. A. Sløk, N. Sekiyama, S. Nakanishi, P. Krogsgaard-Larsen, U. Madsen, *J. Med. Chem.* **1997**, *40*, 3700 – 3705.
[13] P. Conti, M. De Amici, S. Joppolo di Ventimiglia, T. B. Stensbøl, U. Madsen, H. Bräuner-Osborne, E. Russo, G. De Sarro, G. Bruno, C. De Micheli, *J. Med. Chem.* **2003**, *46*, 3102 – 3108.
[14] P. Conti, M. De Amici, G. Grazioso, G. Roda, A. Pinto, K. B. Hansen, B. Nielsen, U. Madsen, H. Bräuner-Osborne, J. Egebjerg, V. Vestri, D. E. Pellegrini-Giampietro, P. Sibille, F. C. Acher, C. De Micheli, *J. Med. Chem.* **2005**, *48*, 6315 – 6325.
[15] M. A. Sills, G. Fagg, M. Pozza, C. Angst, D. E. Brundish, S. D. Hurt, E. J. Wilisz, M. Williams, *Eur. J. Pharmacol.* **1991**, *192*, 19 – 24.
[16] D. E. Murphy, E. W. Snowhill, M. Williams, *Neurochem. Res.* **1987**, *12*, 775 – 782.
[17] D. R. Hampson, D. Huie, R. J. Wenthold, *J. Neurochem.* **1987**, *49*, 1209 – 1215.
[18] K. Ogita, T. Suzuki, Y. Yoneda, *Neuropharmacology* **1989**, *28*, 1263 – 1270.
[19] A. C. Foster, E. H. Wong, *Br. J. Pharmacol.* **1987**, *91*, 403 – 409.
[20] R. X. Moldrich, A. G. Chapman, G. De Sarro, B. S. Meldrum, *Eur. J. Pharmacol.* **2003**, *476*, 3 – 16.
[21] GOLD 3.1, Cambridge Crystallographic Data Centre: Cambridge (UK).
[22] K. S. Lee, J. Kim, Y. W. Yoon, M.-G. Lee, S. K. Hong, H. C. Han, *Neurosci. Lett.* **2007**, *416*, 123 – 127.
[23] D. E. Murphy, A. J. Hutchison, S. D. Hurt, M. Williams, M. A. Sills, *Br. J. Pharmacol.* **1988**, *95*, 932 – 938.
[24] E. J. Bjerrum, A. S. Kristensen, D. S. Pickering, J. R. Greenwood, B. Nielsen, T. Liljefors, A. Schousboe, H. Bräuner-Osborne, U. Madsen, *J. Med. Chem.* **2003**, *46*, 2246 – 2249.
[25] H. Bräuner-Osborne, P. Krogsgaard-Larsen, *Br. J. Pharmacol.* **1998**, *123*, 269 – 274.
[26] G. B. De Sarro, M. J. Croucher, B. S. Meldrum, *Neuropharmacology* **1984**, *23*, 525 – 530.
[27] J. T. Litchfield, Jr., F. Wilcoxon, *J. Pharmacol. Exp. Ther.* **1949**, *96*, 99 – 103.
[28] Gaussian 03, Revision B.04: M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, J. A. Montgomery, Jr., T. Vreven, K. N. Kudin, J. C. Burant, J. M. Millam, S. S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G. A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J. E. Knox, H. P. Hratchian, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, P. Y. Ayala, K. Morokuma, G. A. Voth, P. Salvador, J. J. Dannenberg, V. G. Zakrzewski, S. Dapprich, A. D. Daniels, M. C. Strain, O. Farkas, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. V. Ortiz, Q. Cui, A. G. Baboul, S. Clifford, J. Cioslowski, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, M. Challacombe, P. M. W. Gill, B. Johnson, W. Chen, M. W. Wong, C. Gonzalez, J. A. Pople, Gaussian, Inc., Wallingford CT (USA), **2004**.
[29] V. Barone, M. Cossi, *J. Phys. Chem. A* **1998**, *102*, 1995 – 2001.
[30] G. Bottegoni, W. Rocchia, M. Recanatini, A. Cavalli, *Bioinformatics* **2006**, *22*, e58 – e65.
[31] G. Bottegoni, A. Cavalli, M. Recanatini, *J. Chem. Inf. Model.* **2006**, *46*, 852 – 862.

Received: May 18, 2007
Revised: June 27, 2007
Published online on September 11, 2007

