Synthesis and Enantiopharmacology of New AMPA-Kainate Receptor Agonists

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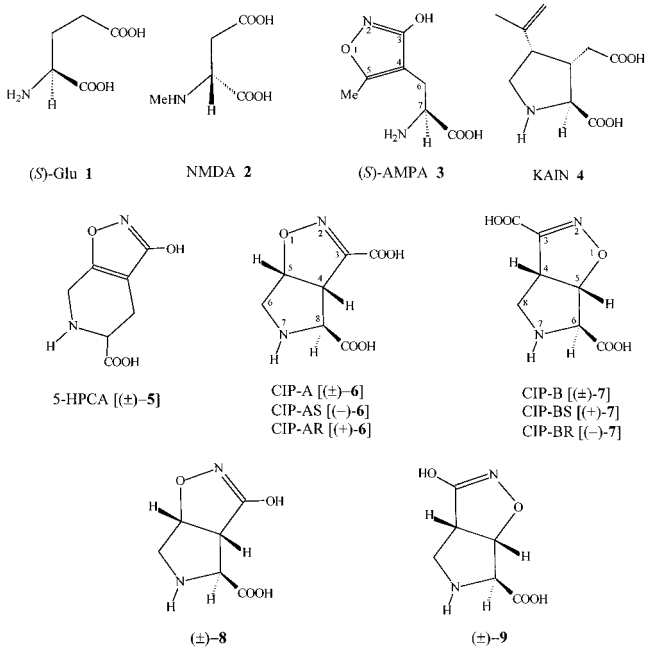
Regioisomeric 3-carboxyisoxazolinyl prolines [CIP-A (±)-**6** and CIP-B (±)-**7**] and 3-hydroxyisoxazolinyl prolines [(±)-**8** and (±)-**9**] were synthesized and assayed for glutamate receptor activity. The tests were carried out in vitro by means of receptor binding techniques, second messenger assays, and the rat cortical wedge preparation. CIP-A showed a good affinity for both 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) and kainic acid (KAIN) receptors. These results were confirmed in the cortical slice model where CIP-A displayed an EC_{50} value very close to that of AMPA. The convulsant properties of all the compounds were evaluated in vivo on DBA/2 mice after icv injection. CIP-A showed a convulsant activity, measured as tonus and clonus seizures, 18−65 times higher than that produced by AMPA. It was also quite active after ip administration, since it induced seizures in mice at doses as low as 3.2 nmol/mouse. On the basis of the above-reported results we prepared and tested the enantiomers of CIP-A and CIP-B, obtained by reacting (*S*)-3,4-didehydroproline and (*R*)-3,4-didehydroproline, respectively, with ethoxycarbonylformonitrile oxide. In all the tests the *S*-form, CIP-AS [(−)-**6**], emerged as the eutomer evidencing common stereochemical requirements with the reference compounds AMPA and KAIN. Through modeling studies, carried out on CIP-A, AMPA, and KAIN, active conformations for CIP-AS and AMPA at AMPA receptors as well as for CIP-AS and KAIN at KAIN receptors are suggested.

Introduction

Excitatory neuronal transmission within the central nervous system (CNS) is mediated predominantly by the amino acid (*S*)-glutamate (Glu, **1**) which plays a role of utmost importance in many physiological processes such as neural plasticity, memory, and learning.[1−3] An imbalance of excitatory pathways seems to be implicated in the pathogenesis of a number of acute and chronic neurological and psychiatric disorders, e.g. epilepsy, cerebral ischemia, stroke, hypoxia, and schizophrenia, as well as chronic neurodegenerative pathologies, e.g. neuropathic pain, amyotrophic lateral sclerosis, and Huntington's, Parkinson's, and Alzheimer's diseases.[4]

Glu activates two families of receptors: the ionotropic (iGlu)[5,6] and metabotropic (mGlu)[7,8] receptors. The iGluRs are multimeric Glu-gated channels which control the flux of cations (Na^+, K^+, and Ca^{2+}) across the postsynaptic membrane. They are responsible for the fast depolarization of postsynaptic cells. On the basis of the pharmacological and functional properties of selective agonists, they have been classified into *N*-methyl-D-aspartic acid (NMDA, **2**), (*S*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA, **3**), and kainic acid (KAIN, **4**) receptors (Chart 1). On the other hand, the mGluRs belong to the superfamily of G protein-coupled receptors and modulate the activity of phospholipase C (PLC) or adenylyl cyclase (AC). To date, eight distinct metabotropic glutamate ($mGlu_{1-8}$) receptors have been cloned and classified based on their amino acid sequence homology, signal transduction mechanism, and pharmacology.[9−11] The eight mGlu receptors have been grouped into three subsets termed group I ($mGlu_{1,5}$), linked to PLC stimulation, and group

Chart 1



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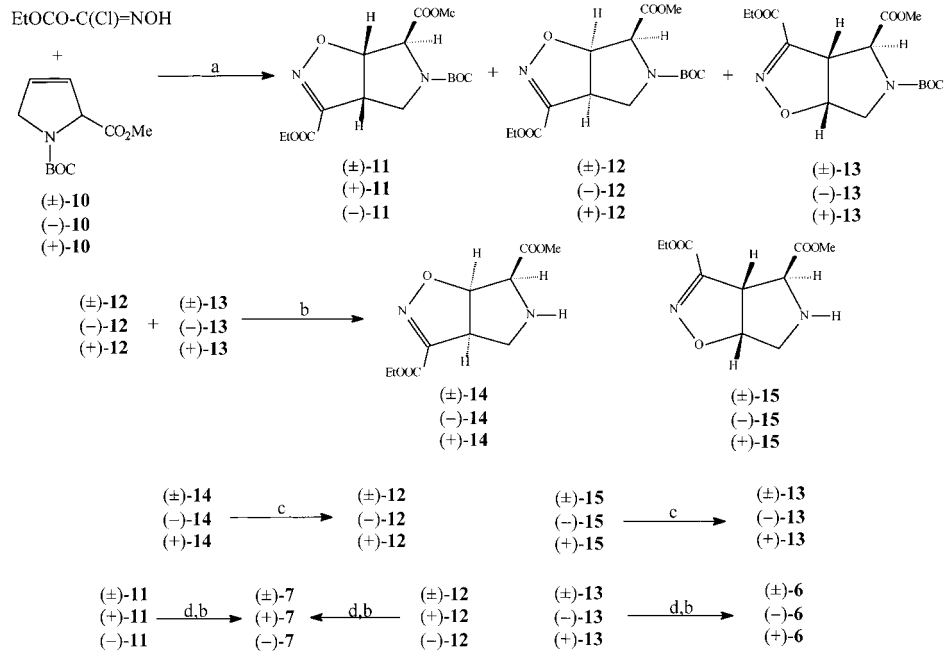
Scheme 1[a]



[a] Reagents and Conditions: (a) $NaHCO_3$/EtOAc; (b) CF_3COOH/CH_2Cl_2; (c) BOC_2O-NEt_3/CH_2Cl_2; (d) $NaOH/H_2O$-MeOH.

II ($mGlu_{2,3}$) and group III ($mGlu_{4,6,7,8}$), both negatively coupled to AC.

A prerequisite for the determination of the physiological role and pharmacological relevance of the subgroups of iGlu receptors as well as the subtypes of mGlu receptors is the availability of highly selective agonists and antagonists.[12] In previous studies a number of agonists and antagonists were developed, which allowed the pharmacological characterization and structure−activity studies of iGlu receptor subtypes, notably the AMPA receptors.[13−15] In this context conformationally restricted AMPA agonists have previously been synthesized, notably the bicyclic (±)-3-hydroxy-4,5,6,7-tetrahydro-isoxazolo[5,4-c]pyridine-5-carboxylic acid (5-HPCA, **5**).[16] This paper deals with the synthesis of the bicyclic Glu analogues CIP-A [3a,5,6,6a-tetrahydro-4*H*-pyrrolo[3,4-*d*]isoxazole-3,4-dicarboxylic acid, (±)-**6**] and CIP-B [3a,5,6,6a-tetrahydro-4*H*-pyrrolo[3,4-*d*]isoxazole-3,6-dicarboxylic acid, (±)-**7**] as well as their enantiomers CIP-AS/CIP-AR[17] [(−)-**6**/(+)-**6**] and CIP-BS/CIP-BR[17] [(+)-**7**/(−)-**7**]. The above-reported compounds and related hydroxy derivatives (±)-**8** and (±)-**9**[18] have been tested for activity at Glu receptors both in in vitro receptor binding techniques, second messenger assays, and the rat cortical wedge preparation and in in vivo tests on DBA/2 mice.

Chemistry

The key step in the synthesis of target compounds (±)-**6** and (±)-**7** is represented by the 1,3-dipolar cycloaddition of ethoxycarbonylformonitrile oxide, generated in situ by treatment of ethyl 2-chloro-2-(hydroxyimino)acetate with a base, to the suitably protected racemic 3,4-didehydroproline [(±)-**10**]. As shown in Scheme 1, the pericyclic reaction allows the characterization of three out of the four possible stereoisomers. Column chromatography of the reaction mixture yielded two fractions containing pure (±)-**11** and an inseparable mixture of (±)-**12** and (±)-**13**. The treatment of the mixture of (±)-**12** and (±)-**13** with excess trifluoroacetic acid afforded the corresponding secondary amines (±)-**14** and (±)-**15** which were separated by column chromatography and reconverted, under standard conditions, into the single *N*-*tert*-butyl carbamates (±)-**12** and (±)-**13**, respectively. Final compounds (±)-**6** and (±)-**7** were obtained through the alkaline hydrolysis of the two ester groups of cycloadducts (±)-**11**, (±)-**12**, and (±)-**13** followed by treatment of the diacidic intermediates with a 30% dichloromethane solution of trifluoroacetic acid. Noteworthy, the alkaline hydrolysis of cycloadducts (±)-**11** and (±)-**12** produced the same final derivative (±)-**7** since the hydrolysis of derivative (±)-**12** with sodium hydroxide is accompanied by an inversion of the chiral center at C-6 (see Chart 1 for numbering). This result can be rationalized on the basis of the higher thermodynamic stability of the 5,6-trans isomer [(±)-**11**] versus the 5,6-cis one [(±)-**12**]. The assignment of the structure to all the synthesized compounds is based on the ^1H NMR spectra. The ^1H NMR resonances of cycloadducts (±)-**11**, (±)-**12**, and (±)-**13** were assigned by standard methods that rely on correlation through chemical bonds (COSY). The coupling constants of H-5 proved highly diagnostic for structural attributions. As a matter of fact, such a proton appears as a doublet in cycloadduct (±)-**11**, as a double doublet in (±)-**12**, and as an eight-line signal (double doublet of doublets) in (±)-**13**.

The same reaction sequence was also carried out on (*S*)-3,4-didehydroproline [(−)-**10**] and (*R*)-3,4-didehydroproline [(+)-**10**] to yield the two couples of diastereoisomers CIP-AS/CIP-BS [(−)-**6**/(+)-**7**] and CIP-AR/CIP-BR [(+)-**6**/(−)-**7**], respectively. Also in this case the hydrolysis of the cis derivatives (−)-**12** and (+)-**12** is accompanied by the inversion of the chiral center at C-6, namely the stereogenic center of the proline moiety. Therefore CIP-BR [(−)-**7**] can be obtained either from (*R*)-3,4-didehydroproline through trans intermediate (−)-**11** or via a base-catalyzed isomerization of cis derivative (−)-**12** which, in turn, can be prepared by a

Table 1. Receptor Binding and Electropharmacological Data (Values ± SEM, $n = 3-4$)

compd	receptor binding, IC_{50} (μM)			electro-pharmacology EC_{50} (μM)
	[^{3}H]AMPA	[^{3}H]KAIN	[^{3}H]CPP	
CIP-A, (±)-**6**	1.3 ± 0.5	0.48 ± 0.11	>100	5.4 ± 0.6[a]
CIP-AS, (−)-**6**	0.54 ± 0.12	0.23 ± 0.03	>100	3.5 ± 0.2[a]
CIP-AR, (+)-**6**	>100	>100	>100	>1000
CIP-B, (±)-**7**	24.7 ± 6.2	48.0 ± 4.4	>100	284 ± 30[b]
CIP-BS, (+)-**7**	11.2 ± 1.9	67.0 ± 9.0	>100	251 ± 26[b]
CIP-BR, (−)-**7**	>100	>100	>100	>1000
(±)-**8**	42.6 ± 11.2	7.9 ± 1.8	>100	∼1000[c]
(±)-**9**	34.9 ± 18.3	45.3 ± 4.3	>100	∼1000[c]
AMPA, **3**	0.040 ± 0.014	>100	>100	3.5 ± 0.2[b]
KAIN, **4**	4.0 ± 1.2	0.007 ± 0.002	>100	25 ± 3[d]
NMDA, **2**				11 ± 3[c]

[a] Partially antagonized by 5 μM NBQX and fully antagonized by 20 μM NBQX. [b] Fully antagonized by 5 μM NBQX. [c] Antagonized by 10 μM CPP. [d] KAIN (10 μM) is not antagonized by 5 μM NBQX (<20% reduction) but fully antagonized by 20 μM NBQX (>80% reduction).

1,3-dipolar cycloaddition of ethoxycarbonylformonitrile oxide to (*S*)-3,4-didehydroproline. In analogy, CIP-BS [(+)-**7**] can be obtained either via trans cycloadduct (+)-**11**, derived from (*S*)-3,4-didehydroproline, or through cis cycloadduct (+)-**12**. The couples of enantiomers CIP-AS/CIP-AR [(−)-**6**/(+)-**6**] and CIP-BS/CIP-BR [(+)-**7**/(−)-**7**] were baseline separated by chiral HPLC using a column which contained the macrocyclic glycopeptide Teicoplanin as the chiral selector; CIP-AS and CIP-BR were the less retained enantiomers. In all the HPLC analyses we detected the presence of a single enantiomer; consequently, to the enantiomers was assigned a value of enantiomeric excess >99.9%. The synthesis of 3-hydroxy derivatives (±)-**8** and (±)-**9** was accomplished following the reaction sequence previously reported.[18]

Pharmacological Results

The two regioisomeric 3-carboxyisoxazolinyl prolines [CIP-A, (±)-**6**, and CIP-B, (±)-**7**] as well as their chiral forms [CIP-AS/CIP-AR and CIP-BS/CIP-BR] and 3-hydroxyisoxazolinyl prolines [(±)-**8** and (±)-**9**] were assayed in vitro by means of receptor binding techniques, second messenger assays, and the rat cortical wedge preparation. The convulsant activity of amino acids **6**, **7**, **8**, and **9** was also evaluated in vivo on DBA/2 mice, a suitable animal model to study the tonic−clonic seizures induced by agonists acting at AMPA and KAIN receptors.[19,20]

The receptor affinity of **6**, **7**, **8**, and **9** for NMDA, AMPA, and KAIN receptors was determined by using the radioligands [^{3}H]CPP, [^{3}H]AMPA, and [^{3}H]KAIN, respectively.[21−23] The activity of the same compounds at metabotropic Glu receptors was evaluated at $mGlu_{1\alpha}$, $mGlu_2$, and $mGlu_{4a}$, expressed in CHO cells, as representatives for group I, II, and III metabotropic receptors, respectively.[24] None of the compounds, tested up to a 1 mM concentration, showed significant activity at the above-mentioned metabotropic receptors, neither as an agonist nor as an antagonist.

As shown in Table 1, compounds **6** and **7** display affinity for both AMPA and KAIN receptors whereas none of them bind to the NMDA receptor complex. The affinity of CIP-A [(±)-**6**] was shown to reside exclusively in its (*S*)-form [CIP-AS]. These results are confirmed in the cortical slice model[25] where CIP-A and CIP-AS display EC_{50} values very close to that of AMPA (Table 1). The depolarization evoked by CIP-A and its eutomer CIP-AS was only partially antagonized by 6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline-2,3-dione (NBQX, 5 μM) whereas virtually full antagonism was observed with 20 μM NBQX, further evidencing the involvement of KAIN receptors. On the other hand, (±)-**8** and (±)-**9** display low affinity for AMPA and KAIN receptors and no affinity for NMDA receptors. Their weak electrophysiological responses (at 1000 μM) were partially antagonized (>50%) by 3-[(±)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid (CPP, 10 μM) but could not be fully blocked by a further addition of 20 μM NBQX. It is well-known that weak NMDA agonists do not show any affinity at the CPP binding site. Experiments with the cortical slice model[24] allowed the assignment to (±)-**8** and (±)-**9** an approximate EC_{50} value of 1000 μM, though a full dose−response curve could not be obtained due to their weak activity. On the other hand, at concentrations of 100 μM, (±)-**8** and (±)-**9** did not behave as antagonists of neither AMPA (5 μM) nor KAIN (10 μM).

Even more striking are the data collected on in vivo assays (Table 2) where an icv injection of CIP-A [(±)-**6**] showed convulsant properties, measured as tonus and clonus seizures, 18−65 times higher than that produced by AMPA and only 2−5 times lower than those induced by KAIN. CIP-AS [(−)-**6**] turned out to be the eutomer with a convulsant potency 90−117 times higher than that of AMPA and similar to that displayed by KAIN (Table 2). The convulsant properties displayed by CIP-B and CIP-BS are 2 orders of magnitude lower than those shown by their stereoisomers CIP-A and CIP-AS. Derivatives (+)-**6**, (−)-**7**, (±)-**8**, and (±)-**9** are almost inactive, consequently they were not tested in vivo in the presence of AMPA or NMDA antagonists. CIP-A [(±)-**6**] is also quite active by ip administration since it is able to induce seizures in mice at doses as low as 3.2 nmol/mouse.

Discussion

The present results suggest the following considerations. CIP-A and CIP-AS are provided with a remarkable convulsant activity evaluated in vivo on DBA/2 mice, at variance with the results collected in in vitro tests where they showed a moderate binding affinity at both AMPA and KAIN receptors. Such a discrepancy may be rationalized by taking into account a synergistic activation of AMPA and KAIN receptors, as evident from the binding data showing CIP-A and CIP-AS to have an affinity profile somewhat similar to that of KAIN. Alternatively, CIP-A and its eutomer may interfere with the transporter mechanism for endogenous neurotransmitters.

Since AMPA and its bicyclic analogues are characterized by the presence of a 3-hydroxyisoxazole nucleus, we can deduce that such a structural feature can lead to compounds characterized by a selective affinity for the AMPA receptor complex. The replacement of the 3-hydroxyisoxazole nucleus of AMPA-selective ligands with the 3-carboxyisoxazolinyl moiety gives compounds, e.g. CIP-A and CIP-AS, in which the spatial arrangement of the pharmacophoric groups is suitable for an additional interaction with the KAIN receptor subsites. As shown in Table 1, bicyclic isoxazolinyl derivatives

Table 2. CD_{50} Values of (±)-**6**, (−)-**6**, (+)-**6**, (±)-**7**, (+)-**7**, (−)-**7**, (±)-**8**, (±)-**9**, KAIN, and AMPA in the Absence and in the Presence of CPP, GYKI 52466, and NBQX

	CD_{50} values[a] (±95% confidence limits)		
treatment	clonus	tonus	potency ratio
CIP-A, (±)-**6**	0.027 (0.016−0.044)	0.16 (0.087−0.296)	
CIP-A + CPP	0.039 (0.013−0.120)	0.19 (0.130−0.296)	1.4−1.2
CIP-A + GYKI 52466	0.50 (0.16−2.27)	1.26 (0.61−2.62)	18.5−7.9
CIP-A + NBQX	0.43 (0.19 + 1.00)	0.99 (0.43−1.53)	15.9−6.2
CIP-AS, (−)-**6**	0.021 (0.010−0.044)	0.048 (0.037−0.062)	
CIP-AS + GYKI 52466	0.131 (0.069−0.247)	0.232 (0.173−0.287)	6.2−4.8
CIP-AS + NBQX	0.071 (0.038−0.133)	0.094 (0.066−0.135)	3.4−2.0
CIP-AR, (+)-**6**	116.2 (75.2−179.5)	235.5 (143.5−386.4)	
CIP-B, (±)-**7**	8.5 (4.9−18.8)	39.9 (18.6−85.5)	
CIP-B + CPP	24.8 (18.5−33.3)	134.1 (88.8−202.6)	2.9−3.4
CIP-B + GYKI 52466	17.6 (12.1−25.6)	54.3 (33.3−88.5)	2.1−1.4
CIP-B + NBQX	9.3 (4.2−20.4)	53.9 (35.4−81.1)	1.1−1.3
CIP-BS, (+)-**7**	3.3 (1.4−7.6)	23.3 (15.4−35.3)	
CIP-BS + CPP	7.8 (3.0−20.6)	47.3 (31.8−70.3)	2.4−2.0
CIP-BS + GYKI 52466	14.5 (9.7−21.7)	53.2 (37.4−75.6)	4.4−1.2
CIP-BS + NBQX	11.9 (7.8−18.2)	44.8 (28.3−70.9)	3.6−1.9
CIP-BR, (−)-**7**	160.4 (117.4−219.1)	337.2 (242.1−469.5)	
(±)-**8**	217.2 (150.1−314.3)	>300	
(±)-**9**	128.7 (89.3−185.6)	205.4 (148.1−284.8)	
KAIN, **4**	0.015 (0.010−0.024)	0.032 (0.017−0.060)	
KAIN + CPP	0.024 (0.016−0.036)	0.071 (0.038−0.133)	1.6−2.2
KAIN + GYKI 52466	0.071 (0.053−0.095)	0.232 (0.184−0.292)	4.7−7.2
KAIN + NBQX	0.094 (0.066−0.134)	0.468 (0.382−0.573)	6.3−14.6
AMPA, **3**	1.76 (1.06−3.07)	2.90 (1.83−4.58)	
AMPA + CPP	2.69 (1.45−5.0)	3.18 (2.48−4.06)	1.5−1.1
AMPA + GYKI 52466	7.61 (4.75−12.2)	16.1 (8.9−29.3)	4.3−5.5
AMPA + NBQX	6.70 (4.0−11.1)	15.7 (11.4−21.8)	3.8−5.4

[a] All the data are expressed as nmol/mouse. The CD_{50} values are related to icv administration of the convulsant. The potency ratio is the ratio between the CD_{50} value of the drug in the presence of an antagonist versus its CD_{50} value.

CIP-A and CIP-B bind, in fact, to both AMPA and KAIN receptors. The affinity of CIP-A and its eutomer CIP-AS for the KAIN receptor complex is noteworthy. Moreover, the spatial arrangement of the groups around the chiral centers of CIP-AS is identical to that of the eutomer of AMPA [(*S*)-AMPA] and of natural KAIN. As a consequence, the stereochemical features of CIP-AS are suitable for a productive interaction with both AMPA and KAIN receptors. Therefore, further investigations by means of molecular mechanics calculations were carried out and the results are discussed later on. On the contrary, the replacement the 3-hydroxyisoxazole nucleus of AMPA with the 3-hydroxy-Δ^2-isoxazolinyl moiety, e.g. (±)-**8** and (±)-**9**, almost abolishes the affinity for both AMPA and KAIN receptors even in the case of (±)-**9** whose through-bond connection of the pharmacophoric groups matches that of Glu. Two conceivable explanations can be proposed to account for the lack of activity of (±)-**9**. The first one is based on a different spatial arrangement of the pharmacophoric groups between (±)-**9** and the reference compounds AMPA and KAIN. A comparative conformational profile of (±)-**9** with that of model compounds was undertaken, and the results will be subsequently discussed. The second hypothesis takes into account the acidity of the ω-hydroxyl group which could be significantly different in a 3-hydroxyisoxazole derivative, e.g. AMPA, and in a 3-hydroxy-Δ^2-isoxazoline derivative, e.g. (±)-**9**. To test the assumption that a 3-hydroxy-Δ^2-isoxazoline is a good bioisoster of the 3-hydroxyisoxazole nucleus and both are bioisosters of the carboxylate moiety, we measured the pK_a values of (±)-**9**, CIP-A, CIP-B, and AMPA, and their values were compared to those reported in the literature for AMPA,[26] KAIN,[27] and Glu[28] (Table 3). We carried out the measurement on regioisomer (±)-**9** since it contains the same through-bond connection as Glu.

Table 3. pK_a Values of (±)-**6**, (±)-**7**, (±)-**9**, AMPA, KAIN, and Glu Measured at 20 °C in Water

compd	pK_{a1} (α-CO_2H)	pK_{a2} (ω-CO_2H or OH)	pK_{a3} (NH_3^+)
(±)-**6**	<2.0	2.15	7.4
(±)-**7**	<2.0	2.6	7.3
(±)-**9**	2.0	5.1	8.3
AMPA	2.3 (2.5)[25]	5.3 (4.8)[25]	9.7 (10.0)[25]
KAIN	2.1[26]	4.3[26]	10.1[26]
Glu	2.39[27]	4.21[27]	9.54[27]

The data of Table 3 put in evidence that whereas the value of pK_{a1} is almost the same for all four compounds, significant differences were detected in the values of pK_{a2} and pK_{a3}. In particular, the ω-carboxylic group of KAIN and Glu is roughly 10 times more acidic than the hydroxyl group of AMPA and (±)-**9** and about 100 times less acidic than the corresponding carboxylic group of CIP-A and CIP-B. Nevertheless, AMPA is a very potent Glu receptor agonist and is able to discriminate among different iGlu receptor subtypes. In this context, the close value of pK_{a2} of (±)-**9** and AMPA indicate that the 3-hydroxy-Δ^2-isoxazoline moiety can surrogate the acidity of the 3-hydroxyisoxazole nucleus in ligands behaving as analogues of Glu. As a consequence, the acidity of the hydroxyl group of (±)-**9** cannot be taken as the cause of its inactivity at AMPA and KAIN receptors.

To gain further insight into the structural requirements for ligand potency and selectivity among iGluR subsets, AMPA (**3**), KAIN (**4**), CIP-A (**6**), CIP-B (**7**), (±)-**8**, and (±)-**9** were submitted to a modeling study with the aid of theoretical calculations. The zwitterionic form of all the compounds with the ω-carboxylate group (or the 3-hydroxyl group of isoxazole or Δ^2-isoxazoline) in

Table 4. Relative Energies (kcal/mol) and Selected Geometrical Features for the Conformers of Compounds **3**, **4**, **6**, **7**, and **8** in a Range of 2 kcal/mol above the Global Minima

conformer	E_{rel}	d_1[a]	d_2[b]	χ_1[c]	χ_2[d]	pyrr conf[e]	isoprop conf[f]
6A	0.00	4.18	4.40	148	−73	E_7	
6B	0.15	4.54	3.84	108	−66	^7E	
7A	0.00	4.04	5.79			E_7	
7B	0.30	4.55	6.05			^7E	
8A	0.00	3.12	3.82			E_7	
8B	0.21	3.56	3.51			^7E	
9A	0.00	3.07	4.56	−90	−121	E_7	
9B	0.39	3.56	4.74	−145	−121	^7E	
3A	0.00	4.80	3.57	63	−104		
3B	0.04	3.34	4.87	178	102		
3C	0.07	4.63	4.01	63	81		
3D	0.13	3.20	4.08	−60	−89		
3E	0.14	4.00	3.35	−59	89		
3F	0.21	3.88	4.73	178	−82		
3G	0.72	4.54	3.39	63	18		
3H	0.77	3.23	4.60	180	−20		
4A	0.00	4.41	3.75	119	−62	E_5	108
4B	0.54	4.83	4.63	74	180	E_2	60
4C	0.56	4.86	4.72	107	−165	E_5	92
4D	0.77	4.86	4.74	99	−176	^5E	−107
4E	1.15	4.87	4.69	99	−169	E_5	−85
4F	1.21	4.84	4.78	101	−179	^5E	48
4G	1.22	4.92	4.61	81	−172	E_4	81
4H	1.23	4.05	4.46	101	71	E_5	107
4I	1.24	4.83	4.61	74	−177	E_2	−105
4J	1.34	4.46	3.56	109	−64	E_5	−77
4K	1.53	4.54	3.53	103	−65	^5E	−106
4L	1.72	4.57	3.27	76	−73	E_2	66
4M	1.98	4.56	3.55	103	−66	^5E	42

[a] α-NH_2^+/γ-COOH distance (Å) for **4**−**6** and α-NH_3^+/C3 distance for **3**. [b] α-COO^-/γ-COOH distance (Å) for **4**−**6** and α-COO^-/C3 distance for **3**. [c] Torsional angle (deg) C8-C7-C6-C4 for **3**, C6-C2-C3-C7 for **4**, C10-C6-C4-C3 for **5**. [d] Torsional angle (deg) C7-C6-C4-C3 for **3**, C2-C3-C7-C8 for **4**, C6-C4-C3-C9 for **5**. [e] Conformational type of the pyrrolidine ring for **4**, **5**, and **6**. [f] Orientation of the isopropenyl substituent in **4**: torsional angle (deg) C3-C4-C9-C10.

the protonated form was considered. The preferred conformations of all the compounds were determined by using a full geometry optimization carried out with the MM^+ force field implemented in the HyperChem program.[29] All the degrees of conformational freedom were examined, with particular attention to the rotation around the acyclic single bonds and the pseudorotation of the pyrrolidine ring. Table 4 reports the relative energies of the low energy conformers together with selected geometrical parameters.

Compound **6** is a quite rigid molecule; its isoxazoline ring is planar and induces the fused pyrrolidine ring to assume only the two envelope conformations E_7 and ^7E instead of the 10 envelope and 10 twisted conformations usually present in the pseudorotational path of a five-membered ring. Conformers **6A** and **6B** (Chart 2) are almost isoenergetic, and consequently, they are equally populated. In both cases, rotation of the two carboxylate groups around the bonds linking them to the bicyclic system is quite easy, so that **6A** and **6B** represent families of conformers. In Table 4 are reported the distances d_1 (α-NH_2^+/ω-COOH) and d_2 (α-COO^-/ω-COOH) between the three pharmacophoric groups as well as the two torsional angles χ_1 and χ_2 of the sequence of bonds along the Glu skeleton for conformers **6A** and **6B**.

The conformational profiles of CIP-B [(±)-**7**)], (±)-**8**, and (±)-**9** are very similar to that of CIP-A [(±)-**6**]. In all cases two conformers were evidenced. A comparison of the conformational parameters of **6** and **7** (Table 4) shows that the value of d_1 is about the same for both compounds whereas the value of d_2 is higher for derivative **7**. The same analysis carried out on derivatives **8** and **9** shows that while **8** is characterized by short distances (d_1 and d_2) among the pharmacophoric groups, compound **9** displays values of the torsional angles χ_1 and χ_2 significantly different from those observed in derivative **6**. The same torsional angles cannot be determined for derivatives **7** and **8** since the through-bond distance between the two carboxylate groups is one bond longer and one bond shorter, respectively, than in Glu.

AMPA (**3**) has a higher conformational mobility due to an easy rotation around the single bonds C4−C6 and C6−C7 (see Chart 1 for numbering). In a range of 1 kcal/mol above the global minimum, eight conformers were detected. By comparing the values of d_1, d_2, χ_1, and χ_2 of the eight conformers of **3** with **6A** and **6B** we can deduce that **3F** can be assimilated to **6A** whereas it is rather difficult to find a conformer of **3** which corresponds to **6B**. To evaluate more precisely the similarity between the conformers of **3** with **6A** and **6B**, the RMS deviations among the five carbon atoms and the nitrogen atom of the Glu skeleton of **6A** and **6B** with respect to the eight conformers of **3** have been determined. Table 5 shows, as the best fits, **6A** vs **3F** and **6B** vs **3G**. Chart 2 reports the three-dimensional superimpositions of the Glu skeleton of **6A** with **3F**.

KAIN (**4**) is another quite flexible molecule as its degrees of conformational freedom rely on the pseudorotation of the pyrrolidine ring and on the rotation around the single bonds of its substituents. A detailed modeling study of **4** through theoretical calculations and NMR studies had already been performed.[30] In solution the pyrrolidine ring assumes conformations in the range $^4E-E_5$ whereas a wider range of conformations is reachable during molecular dynamics simulations indicating a large flexibility of the molecule.[30] The present data confirm such a flexibility, as indicated by the large number of conformers located in a range of 2 kcal/mol above the global minimum (Table 4). The RMS deviations of the five carbon atoms and the nitrogen atom of the Glu skeleton of **4** with respect to **5** (Table 5) show for the global minimum of KAIN (**4A**) a very good superimposition with conformer **6B** of compound **6** (see also Chart 2). Also conformers **4J**, **4K**, and **4M**, (Table 4) which differ from **4A** in the orientation of the isopropenyl group and/or the pyrrolidine conformation, show a similar good fit with **6B**. This result, coupled with the noteworthy activity of **6** at KAIN receptors, suggests that the active conformation of **6** at KAIN receptors should be **6B** while **4A** (or one of the three other conformers **4J**, **4K**, and **4M**) is the active conformation of **4.** On the basis of the above-reported results, the two conformers **6A** and **6B** of CIP-A appear as good models for a selective interaction with AMPA and KAIN receptors, respectively.

In conclusion, the results of the present study indicate that the lack of activity of stereoisomers (±)-**8** and (±)-**9** at iGlu receptors cannot be attributed to the inability of their 3-hydroxy-Δ^2-isoxazoline moiety to behave as an efficient bioisoster of the ω-carboxylate group of Glu

Chart 2

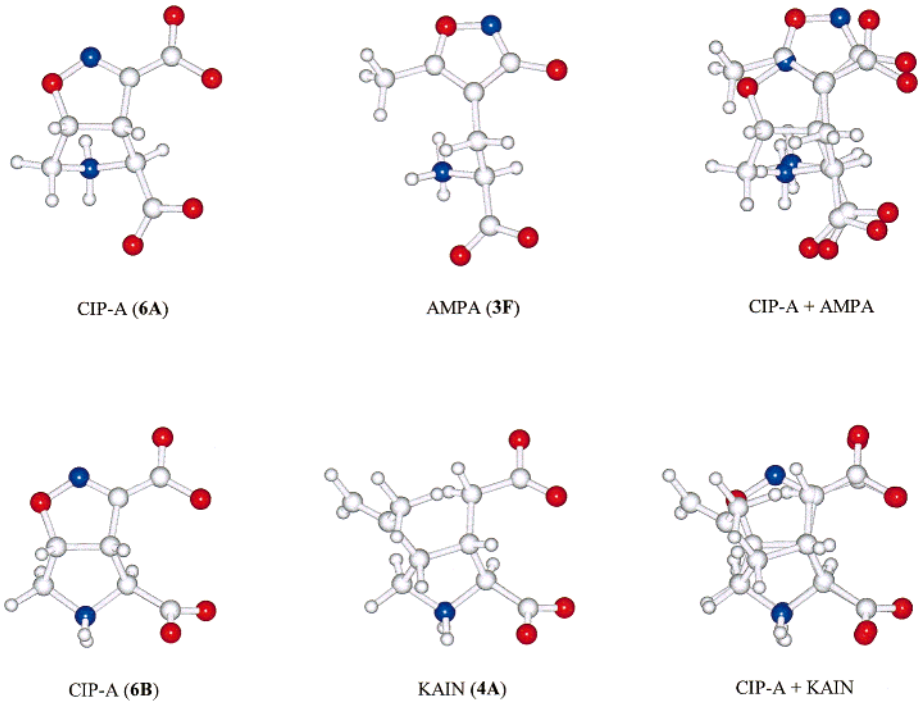


Table 5. RMS Deviations (Å) between the Five Carbon Atoms Plus the α-Nitrogen Atom of the Glutamate Skeleton of the Conformers of **6** and **9** with Respect to the Conformers of **3** and **4**

	6A	6B	9A	9B		6A	6B	9A	9B
3A	0.751	0.433	1.174	1.076	**4A**	0.333	0.127	1.091	0.769
3B	0.786	1.036	0.584	0.590	**4B**	0.685	0.606	0.922	0.882
3C	0.504	0.403	1.026	0.869	**4C**	0.559	0.510	0.979	0.782
3D	1.081	1.124	0.472	0.836	**4D**	0.613	0.567	0.954	0.816
3E	0.989	0.813	0.883	0.991	**4E**	0.594	0.529	0.977	0.816
3F	0.265	0.620	0.621	0.277	**4F**	0.610	0.580	0.941	0.803
3G	0.573	0.310	1.185	0.980	**4G**	0.666	0.566	0.974	0.887
3H	0.502	0.827	0.566	0.424	**4H**	0.440	0.599	0.874	0.700
					4I	0.691	0.602	0.966	0.889
					4J	0.426	0.122	1.157	0.848
					4K	0.469	0.133	1.181	0.884
					4L	0.706	0.362	1.294	1.063
					4M	0.473	0.132	1.180	0.805

but, more likely, to an inappropriate spatial arrangement of their pharmacophoric groups. On the other hand, the capability of CIP-A and, especially, of CIP-AS to activate both the AMPA and KAIN receptors has to be ascribed to the presence in its structure of two conformations which are equally populated and mimic an active conformation of AMPA and KAIN, respectively.

Experimental Section

Materials and Methods. Ethyl 2-chloro-2-(hydroxyimino)-acetate[31] and racemic 3,4-didehydroproline[32] were prepared according to literature procedures. The synthesis of the ester moiety and the protection of the secondary amine of racemic 3,4-didehydroproline were accomplished along standard methodologies. Amino acids (±)-**8** and (±)-**9** were synthesized according to a published procedure.[18] ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker AC-E 300 (300 MHz) spectrometer in toluene-d_8 at 80 °C or in $CDCl_3$ (or CF_3COOD) solution at 20 °C; chemical shifts (δ) are expressed in ppm and coupling constants (J) in hertz. Chiral HPLC analyses were conducted on a chromatograph equipped with a column of spherical silica Si 100, 5 μm (4.0 × 250 mm), containing Teicoplanin as the chiral selector. In the case of enantiomers (−)-**6** and (+)-**6**, the column was eluted at 25 °C with 1.00 mL/min of 20 mM NH_4OAc/acetonitrile−water 4:1 (v/v) at a pressure of 10^3 psi. Enantiomers (−)-**7** and (+)-**7** were separated under the following conditions: eluant, 50 mM NH_4OAc in [EtOH−MeOH 3:2]/water 4:1 (v/v) with 0.1% acetic acid; flow rate, 1.00 mL/min; pressure, 10^3 psi; temperature, 20 °C. In both cases the chromatograph was equipped with the evaporative light scattering detector PL-EMD 960 operating at 80 °C with an air flow of 6.0 L/min. Potentiometric titrations were performed with the GLpK_a apparatus (Sirius Analytical Instruments Ltd, Forrest Row, East Sussex, U.K.) equipped with a pH electrode, a temperature probe, an overhead stirrer, and precision dispensers for automated distribution of the diluent (0.15 M KCl in water) and titrants (0.5 M HCl, 0.5 M KOH). Four separate 15 mL aqueous solutions were added to weighted samples (1−10 mg) and acidified to pH 1.8 with HCl. The solutions were then titrated with standardized KOH to pH 8, under nitrogen at 25.0 ± 0.1 °C. The initial estimates of the pK_a values were obtained by difference plots (Bjerrum plots).[33] These values were then refined by a weighted nonlinear least-squares procedure. TLC was performed on commercial silica gel 60 F_{254} aluminum sheets; spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution. Melting points were determined on a Büchi apparatus and are uncorrected. Microanalyses of new compounds agreed with theoretical value ±0.4%.

Synthesis of Methyl *N*-BOC-3,4-Didehydro-(*S*)-prolinate and Methyl *N*-BOC-3,4-Didehydro-(*R*)-prolinate (−)-10 and (+)-10. The synthesis of (−)-**10** and (+)-**10** was accomplished by using commercially available *trans*-4-hydroxy-L-proline and *cis*-4-hydroxy-D-proline as starting materials following the procedure reported by Rüeger et al.[34]

Methyl *N*-BOC-3,4-Didehydro-(*S*)-prolinate (−)-10: $[\alpha]^{20}_D$ −262.0 (*c* 0.992, $CHCl_3$) [lit.[35] $[\alpha]^{20}_D$ −232 (*c* 0.99, CH_3OH)].

Methyl *N*-BOC-3,4-Didehydro-(*R*)-prolinate (+)-10: $[\alpha]^{20}_D$ +259.1 (*c* 1.002, $CHCl_3$).

1,3-Dipolar Cycloaddition of Ethoxycarbonylformonitrile Oxide to (±)-10, (−)-10, and (+)-10. To a solution of methyl (±)-*N*-BOC-3,4-dehydroprolinate (±)-**10** (3.1 g, 13.7 mmol) in ethyl acetate (50 mL) were added ethyl chlorooximinoacetate (6.2 g, 41.1 mmol) and $NaHCO_3$ (15 g). The mixture was vigorously stirred for 3 days, then another 3 equiv (6.2 g, 41.1 mmol) of ethyl chlorooximinoacetate was added, and the

mixture was stirred for an additional 3 days. The progress of the reaction was monitored by TLC (petroleum ether/ethyl acetate 7:3). Water was added to the reaction mixture, and the organic layer was separated and dried over anhydrous sodium sulfate. The crude material, obtained after evaporation of the solvent, was chromatographed on silica gel (eluant: petroleum ether/ethyl acetate 7:3) to give 1.30 g of unreacted olefin, 0.90 g of (±)-**11** as a yellowish solid, and 1.71 g of a mixture of cycloadducts (±)-**12** and (±)-**13**. Overall yield: 56%.

The same procedure was applied to the cycloaddition of ethoxycarbonylformonitrile oxide to (−)-**10** and (+)-**10** to yield the mixture of cycloadducts (+)-**11**/(−)-**12**/(−)-**13** and (−)-**11**/(+)-**12**/(+)-**13**, respectively, which were separated into two fractions by column chromatography as described above.

Compound (±)-**11** crystallized from diisopropyl ether as colorless prisms: mp 78−80 °C; R_f (petroleum ether/ethyl acetate 7:3) 0.30; ^{1}H NMR (C_7D_8) 1.00 (t, 3, CH_2CH_3; J = 7.1); 1.35 (s, 9, tBu); 3.37 (s, 3, OCH_3); 3.47 (dddd, 1, H-4; J = 0.8, 2.1, 7.6 and 9.5); 3.63 (dd, 1, H-8a; J = 8.0 and 11.6); 3.98 (m, 2, CH_2CH_3); 4.09 (bd, 1, H-8b; J = 11.6); 4.80 (bs, 1, H-6); 4.88 (bd, 1, H-5; J = 9.5). Anal. ($C_{15}H_{22}N_2O_7$) C, H, N.

Mixture (±)-**12** and (±)-**13**: R_f (petroleum ether/ethyl acetate 7:3) 0.23.

Compound (+)-**11**: colorless prisms from diisopropyl ether, mp 77.5−79.5 °C; $[\alpha]^{20}_D$ +77.6 (*c* 1.005, $CHCl_3$).

Compound (−)-**11**: $[\alpha]^{20}_D$ −78.4 (*c* 1.012, $CHCl_3$). Anal. ($C_{15}H_{22}N_2O_7$) C, H, N.

Synthesis of (±)-3-Ethoxycarbonyl-6-methoxycarbonyl-3a,5,6,6a-tetrahydro-4*H*-pyrrolo [3,4-*d*]isoxazole and (±)-3-Ethoxycarbonyl-4-methoxycarbonyl-3a,5,6,6a-tetrahydro-4*H*-pyrrolo[3,4-*d*]isoxazole [(±)-14 and (±)-15]. The mixture of (±)-**12** and (±)-**13** (1.71 g, 5.0 mmol) was treated with a 30% dichloromethane solution of trifluoroacetic acid (12.7 mL) at 0 °C. The reaction mixture was stirred at room temperature until TLC showed the disappearance of the starting material (2 h). The volatiles were removed under vacuum, and the residue was treated with a 10% potassium carbonate solution (30 mL) and extracted with ethyl acetate (3 × 10 mL). The pooled organic extracts were dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was purified by silica gel column chromatography to give 0.33 g of (±)-**14** and 0.66 g of (±)-**15** as yellowish oils in 82% overall yield.

The same reaction was also carried out on the mixtures of (−)-**12**/(−)-**13** and (+)-**12**/(+)-**13** to give, in comparable yield, the mixtures of (−)-**14**/(−)-**15** and (+)-**14**/(+)-**15**, respectively, which were separated into the single enantiomers by column chromatography as described above.

Compound (±)-**14**: R_f (petroleum ether/ethyl acetate 1:4) 0.15; ^{1}H NMR ($CDCl_3$) 1.36 (t, 3, CH_2CH_3; J = 6.9); 2.10 (bs, 1, NH); 3.08 (dd, 1, H-8a; J = 7.3, and 12.9); 3.49 (bd, 1, H-8b; J = 12.9); 3.81 (s, 3, OCH_3); 3.87 (d, 1, H-6; J = 4.7); 3.97 (m, 2, CH_2CH_3); 4.07 (dd, 1, H-4; J = 7.3 and 7.5); 5.48 (dd, 1, H-5; J = 4.7 and 7.5).

Compound (±)-**15**: R_f (petroleum ether/ethyl acetate 1:4) 0.33; ^{1}H NMR ($CDCl_3$) 1.21 (t, 3, CH_2CH_3, J = 6.9); 2.35 (bs, 1, NH); 3.01 (dd, 1; H-6a; J = 4.1, and 13.1); 3.26 (bd, 1, H-6b; J = 13.1); 3.60 (s, 3, OCH_3); 3.98 (bs, 1, H-8); 4.16 (m, 3, CH_2-CH_3 and H-4); 5.21 (bdd, 1, H-5; J = 4.1 and 8.9).

Compound (−)-**14**: colorless needles from 2-propanol, mp 81−82 °C; $[\alpha]^{20}_D$ −375.2 (*c* 1.018, $CHCl_3$).

Compound (−)-**15**: colorless prisms from 2-propanol, mp 71−72 °C; $[\alpha]^{20}_D$ −141.4 (*c* 1.023, $CHCl_3$).

Compound (+)-**14**: $[\alpha]^{20}_D$ +376.1 (*c* 1.012, $CHCl_3$).

Compound (+)-**15**: $[\alpha]^{20}_D$ +140.2 (*c* 1.018, $CHCl_3$).

Synthesis of (±)-3-Ethoxycarbonyl-4-methoxycarbonyl-5-*tert*-butoxycarbonyl-3a,5,6,6a-tetrahydro-4*H*-pyrrolo[3,4-*d*]isoxazole [(±)-13]. To a solution of (±)-**15** (0.66 g, 2.73 mmol) in dichloromethane (6.5 mL) was added triethylamine (0.57 mL, 4.1 mmol) at 0 °C followed by a solution of BOC_2O (0.895 g, 4.1 mmol) in dichloromethane (6.5 mL). The reaction mixture was stirred at room temperature until disappearance of the starting material, then treated with 3 N HCl (5 mL), and washed with water. The organic layer was separated, dried over anhydrous sodium sulfate, and concentrated under vacuum. The residue was purified by silica gel column chromatography (eluant: petroleum ether/ethyl acetate 7:3) to give 0.90 g of (±)-**13** as a colorless viscous oil in 96% yield.

The same treatment carried out on amines (−)-**15** and (+)-**15** gave pure cycloadducts (−)-**13** and (+)-**13**, respectively, in comparable yield.

Compound (±)-**13**: ^{1}H NMR (C_7D_8) 1.02 (t, 3, CH_2CH_3; J = 6.9); 1.35 (s, 9, tBu); 3.39 (s, 3, OCH_3); 3.56 (dd, 1; H-6a; J = 6.0, and 12.6); 3.68 (bd, 1, H-4; J = 10.0); 3.84 (dd, 1, H-6b; J = 0.7 and 12.6); 3.97 (m, 2, CH_2CH_3); 4.69 (ddd, 1, H-5; J = 0.7, 6.0 and 10.0); 4.90 (bs, 1, H-8). Anal. ($C_{15}H_{22}N_2O_7$) C, H, N.

Compound (−)-**13**: $[\alpha]^{20}_D$ −151.06 (*c* 1.042, $CHCl_3$). Anal. ($C_{15}H_{22}N_2O_7$) C, H, N.

Compound (+)-**13**: $[\alpha]^{20}_D$ +147.7 (*c* 1.023, $CHCl_3$). Anal. ($C_{15}H_{22}N_2O_7$) C, H, N.

Synthesis of (±)-3-Ethoxycarbonyl-6-methoxycarbonyl-5-*tert*-butoxycarbonyl-3a,5,6,6a-tetrahydro-4*H*-pyrrolo[3,4-*d*]isoxazole [(±)-12]. The above-described procedure, carried out on amine (±)-**14**, gave pure cycloadduct (±)-**12** in 95% yield.

Compound (±)-**12**: ^{1}H NMR (C_7D_8) 0.98 (t, 3, CH_2CH_3; J = 6.9); 1.37 (s, 9, tBu); 3.46 (s, 3, OCH_3); 3.48 (ddd, 1; H-4; J = 5.2, 9.0 and 10.5); 3.70 (dd, 1, H-8a; J = 9.0 and 11.5); 3.82 (dd, 1, H-8b; J = 5.2 and 11.5); 3.95 (m, 2, CH_2CH_3); 4.48 (d, 1, H-6; J = 8.0); 4.80 (dd, 1, H-5; J = 8.0 and 10.5). Anal. ($C_{15}H_{22}N_2O_7$) C, H, N.

The same treatment carried out on amines (−)-**14** and (+)-**14** gave pure cycloadducts (−)-**12** and (+)-**12**, respectively, in comparable yield.

Compound (−)-**12**: $[\alpha]^{20}_D$ −154.83 (*c* 1.025, $CHCl_3$). Anal. ($C_{15}H_{22}N_2O_7$) C, H, N.

Compound (+)-**12**: $[\alpha]^{20}_D$ +155.15 (*c* 1.126, $CHCl_3$). Anal. ($C_{15}H_{22}N_2O_7$) C, H, N.

Synthesis of 3a,5,6,6a-Tetrahydro-4*H*-pyrrolo[3,4-*d*]-isoxazole-3,4-dicarboxylic Acid [(±)-6]. To a solution of (±)-**13** (0.90 g, 2.63 mmol) in methanol (7.9 mL) was added a 1 N NaOH solution (7.9 mL), and the mixture was stirred at room temperature for 12 h. Methanol was evaporated under vacuum, and the aqueous layer was extracted with ethyl acetate (2 × 5 mL), acidified with 3 N HCl, and extracted with ethyl acetate (3 × 5 mL). The pooled organic extracts were dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was taken up with a 30% dichloromethane solution of trifluoroacetic acid (5.6 mL) at 0 °C. The reaction mixture was stirred at room temperature until disappearance of the starting material (2 h). The volatiles were removed under vacuum, and the residue was washed with methanol and filtered under vacuum to give 0.330 g (40% overall yield) of (±)-**6** as colorless prisms.

The above-reported treatment carried out both on cycloadducts (−)-**13** and (+)-**13** gave final derivative (−)-**6** and (+)-**6** in 43% yield.

Compound (±)-**6**: R_f (butanol/H_2O/acetic acid 60:25:15) 0.11; mp 190−222 °C dec; ^{1}H NMR (CF_3COOD) 4.03 (dd, 1; H-6a; J = 4.3, and 13.7); 4.20 (bd, 1, H-6b; J = 13.7); 4.88 (bd, 1, H-4; J = 9.7); 5.26 (bs, 1, H-8); 5.74 (bdd, 1, H-5; J = 4.3 and 9.7); ^{13}C NMR (CF_3COOD) 56.3 (C-4); 56.7 (C-6); 66.6 (C-8); 89.5 (C-5); 152.6 (C-3); 171.9 (*C*OOH). Anal. ($C_7H_8N_2O_5$) C, H, N.

Compound (−)-**6**: $[\alpha]^{20}_D$ −53.7 (*c* 0.108, H_2O); chiral HPLC $k_1' = 3.40$, $\alpha = 1.73$; ee >99.9%. Anal. ($C_7H_8N_2O_5$) C, H, N.

Compound (+)-**6**: $[\alpha]^{20}_D$ +49.0 (*c* 0.104, H_2O); ee >99.9%. Anal. ($C_7H_8N_2O_5$) C, H, N.

Synthesis of 3a,5,6,6a-Tetrahydro-4*H*-pyrrolo[3,4-*d*]-isoxazole-3,6-dicarboxylic Acid [(±)-7]. The above-reported treatment carried out both on cycloadducts (±)-**11** and (±)-**12** gave final derivative (±)-**7** in 47% yield.

The same treatment carried out on cycloadducts (+)-**11** and (−)-**11** gave final derivative (+)-**7** and (−)-**7**, respectively, in similar yield. On the other hand, the same procedure applied to (−)-**12** gave (−)-**7** and that applied to (+)-**12** yielded (+)-**7**.

Compound (±)-**7**: R_f (butanol/H_2O/acetic acid 60:25:15) 0.11; mp 155−160 °C dec; ^{1}H NMR (CF_3COOD) 4.36 (dd, 1; H-8a; J = 7.8 and 12.5); 4.62 (bd, 1, H-8b; J = 12.5); 4.93 (bdd, 1, H-4; J = 7.8 and 9.7); 5.40 (bs, 1, H-6); 6.29 (bd, 1, H-5; J = 9.7); ^{13}C NMR (CF_3COOD) 52.9 (C-4); 53.2 (C-8); 70.8 (C-6); 91.6 (C-5); 153.7 (C-3); 170.6 (*C*OOH). Anal. ($C_7H_8N_2O_5$) C, H, N.

Compound (+)-**7**: $[\alpha]^{20}_D$ +145.8 (*c* 0.114, H_2O); chiral HPLC $k_1' = 0.97$, $\alpha = 1.40$; ee >99.9%. Anal. ($C_7H_8N_2O_5$) C, H, N.

Compound (−)-**7**: $[\alpha]^{20}_D$ −144.0 (*c* 0.103, H_2O); ee >99.9%. Anal. ($C_7H_8N_2O_5$) C, H, N.

Biological Testing. Receptor Binding. Affinity for NMDA, AMPA, and kainic acid receptors were determined using the ligands [^{3}H]CPP,[21] [^{3}H]AMPA,[22] and [^{3}H]KAIN,[23] respectively. The membrane preparations used in all the receptor binding experiments were prepared according to the method of Ransom and Stec.[36]

In Vitro Electrophysiology. A rat cortical slice preparation for determination of EAA-evoked depolarizations described by Harrison and Simmonds[25] 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^{2+} free (and Ca^{2+} 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 was recorded on a chart recorder. Applications of agonists were made for 90 s at each concentration tested, typically at 15 min intervals. The sensitivity of agonist effects to CPP (10 μM) or NBQX (5 or 20 μM) was tested at agonist concentrations producing at least 50% of maximal responses. In experiments designed to detect antagonist effects, the potential antagonist was applied alone for 90 s followed by co-application of agonists (NMDA, AMPA, or kainic acid) and the potential antagonist for another 90 s.

Metabotropic Testing. Three metabotropic subtypes $mGluR_{1\alpha}$, $mGluR_2$, or $mGluR_{4a}$ were expressed in chinese hamster ovary (CHO) cell lines and used as representatives for group I, II, and III metabotropic receptors.[24]

In Vivo Pharmacology. Male DBA/2 mice (12−22 g; 4−6 weeks old) were used. The animals were housed in groups of 10 in PVC cages (260 mm × 440 mm long x 120 mm high) with a temperature of 21−23 °C and a relative humidity of 57 ± 2%; a 12 h light/dark cycle was applied (light on in the interval 07:00 a.m. to 07:00 p.m.). Food and water were available ad libitum.

Apparatus. A 50 μL Hamilton microsyringe was adapted for constant depth icv injections using a Butterfly-25 short winged needle infusion set (Abbott, Rome, Italy). A needle of 0.5 mm external diameter was inserted into a polyethylene cannula leaving 3 mm of the needle exposed. A new infusion set was employed for each compound and for the different dosages studied.

Procedure. For icv injection, groups of at least 10 mice were anesthetized with diethyl ether and the drug was injected as a 67 mM phosphate buffer solution. The following amounts were used: AMPA 0.25−15.0 nmol, KAIN 0.003−0.1 nmol, CIP-A 0.01−10.0 nmol, CIP-AS 0.003−3.3 nmol, CIP-AR 10−400 nmol, CIP-B 5−200 nmol, CIP-BS 1−100 nmol, CIP-BR 50−500 nmol, (±)-**8** 10−300 nmol, (±)-**9** 10−300 nmol. The injection site was 1 mm anterior to bregma, 1 mm lateral to the midline, and 3 mm below the surface of the cranium. The animals were then observed for 60 min and the induced seizures detected and characterized.

The anticonvulsant effects of CPP (5 mg/kg), ip administered 60 min before the icv injection of AMPA, KAIN, CIP-A, or CIP-B, were evaluated. The antiseizure activity of GYKI 52466 (30 mg/kg ip, 15 min in advance) and NBQX (30 mg/kg ip, 30 min in advance) was also evaluated. The incidence of a clonic and tonic seizure response for 50% of mice (CD_{50} values) with 95% confidence limits was estimated by using the method of Litchfield and Wilcoxon.[37] The relative potency ratios are the ratio between the CD_{50} value of the drug in the presence of an antagonist, e.g. CPP, GYKI 52466, or NBQX, versus its CD_{50} value.

Statistical Analysis. The data of the convulsant tests were statistically analyzed according to the method of Litchfield and Wilcoxon.[37] In Table 2, the 95% confidence limits of the CD_{50} values are shown.

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