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Design of novel conformationally restricted analogues of glutamic acid

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Abstract—Stereomeric 3-carboxy-Δ^2-isoxazoline–cyclopentane amino acids, which represent restricted conformations of glutamic acid, have been prepared through a strategy based on the 1,3-dipolar cycloaddition of ethoxycarbonylformonitrile oxide to a suitably protected 1-amino-cyclopent-2-enecarboxylic acid. These amino acids, assayed at iGluRs and mGluRs, proved to be inactive. The biological data have been accounted for through the comparison of their conformational profile with that of a 3-carboxy-Δ^2-isoxazolinyl proline (CIP-A) and (±)-1-aminocyclopentane-1,3-dicarboxylic acid.

1. Introduction

L-Glutamic acid (Glu, **1**) (Fig. 1) is widely recognized as the primary excitatory neurotransmitter in the mammalian central nervous system (CNS). Its synaptic actions are mediated by two heterogeneous families of cell membrane-associated receptors: metabotropic Glu receptors (mGluRs) and ionotropic Glu receptors (iGluRs).[1–4] The mGluRs are G-protein-coupled receptors which mainly modulate the fast excitatory effects of Glu,[1] whereas fast excitatory synaptic transmission is due to the ligand-induced opening of transmembrane cation channels.[2–4] Flux of monovalent and divalent cations through the postsynaptic membrane depolarizes the cell and propagates the electrical signal. The iGluRs have been classified according to their sensitivity to the following selective agonists: *N*-methyl-D-aspartate (NMDA, **2**), 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA, **3**), and kainic acid (KA, **4**).[2–4] They are homo- or heteromeric assemblies of four or five subunits, which form an ion channel. A number of iGluR subunits has been cloned and classified in regards to their sequence homology. NMDA-preferring receptors are made up by combinations of seven different subunits (NR1, NR2A-2D, and NR3A-3B).[5] AMPA-preferring receptors are composed of four subunits (iGluR1-4), while five different KA-preferring receptor subunits have been cloned and classified as either high affinity KA1-2 or low affinity iGluR5-7.[6–8] Each subunit contributes to the cation-permeable channel with three transmembrane segments and a membrane-embedded re-entrant loop.[9–10] Such a variety of subunits results in a large structural and functional diversity of the assembled receptors, which is further increased by alternative splicing, e.g. the presence of either a flip or flop sequence,[11] and post-transcriptional RNA editing.[12] At present, the number of functional NMDA, AMPA and KA receptors present in the CNS is unknown.

The activation of both iGluRs and mGluRs gives rise to a variety of physiological functions of utmost importance such as learning, memory and developmental plasticity.[13,14] On the other hand, an overactivation of the same receptors, caused by an excessive release of endogenous Glu, is implicated in the pathogenesis of several acute and chronic diseases.[15]

A prerequisite for the identification of the patho-physiological role played by the subgroups of iGluRs and mGluRs is the availability of highly selective agonists and antagonists. Since Glu is a highly flexible molecule, a number of constrained analogues have been designed and tested with the aim to uncover the conformational requirements needed to activate the different Glu receptor subtypes. It turned out that an extended conformation is required for a fruitful interaction with mGlu receptors, whereas a folded conformation is necessary to fit the binding sites of the iGlu receptors.[16] Nevertheless, not all the extended conformations are capable to bind efficiently to mGlu receptors. The same holds true for the folded conformations in respect to the different iGlu receptors.

Keywords: 1,3-dipolar cycloaddition; bicyclic amino acids; glutamic acid analogues; ionotropic glutamate receptors; metabotropic glutamate receptors.

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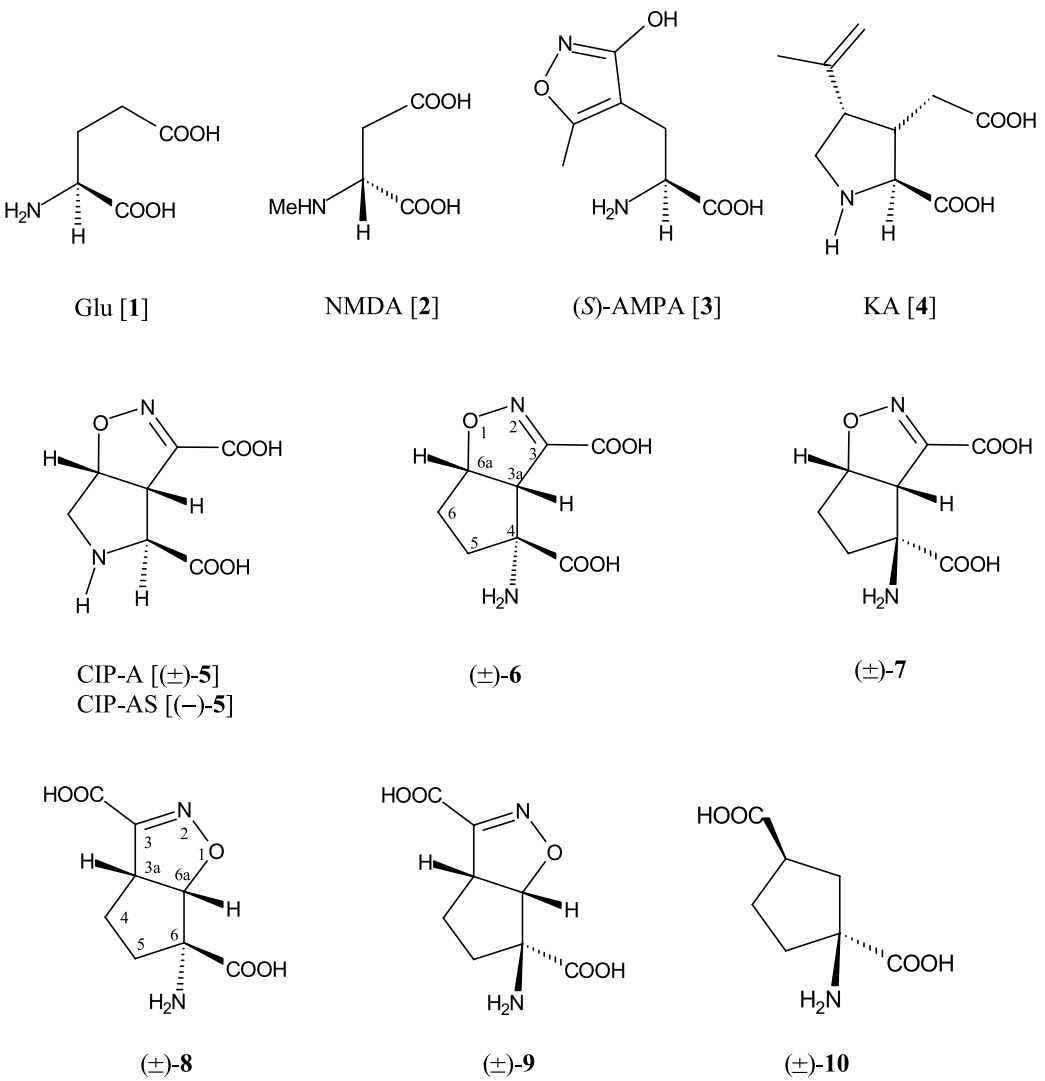


Figure 1. Structure of model and tested compounds.

In our first approach to this field, we designed a 3-carboxyisoxazolinyl proline [CIP-A, (±)-**5**] (Fig. 1), a structural hybrid between AMPA and KA, as a constrained folded conformation of glutamic acid.[17] In the in vitro and in vivo assays, CIP-A displayed a potent agonist activity at both AMPA and KA receptors. Since the eutomer of **5** [CIP-AS; (−)-**5**] has the same stereochemistry at the α-carbon as **3** and **4**,[18] we collected further insights on its conformation through molecular modeling studies. The full geometry optimization of **5**, carried out with the MM^{+} force field implemented in the HyperChem program, showed the presence of two conformations which are almost equally populated and mimic an active conformation of AMPA and KA, respectively.[18] Recently, a further conformational analysis of CIP-AS [(−)-**5**] was performed[19] in aqueous solution by using AM1/SM2 and AM1/SM5.4 and showed, at this level of calculations, the presence of a single stable conformer which corresponds to the global minimum found in the molecular mechanics approach.[18] A superimposition of such a conformation of CIP-AS with the experimentally determined iGluR2-bound conformation of KA evidenced an excellent fit. Since iGluR2 is a subunit of the AMPA receptors, the authors deduced that the selectivity between AMPA and KA receptors is due to differences in the steric requirements of the ligands and/or to additional interactions.[19]

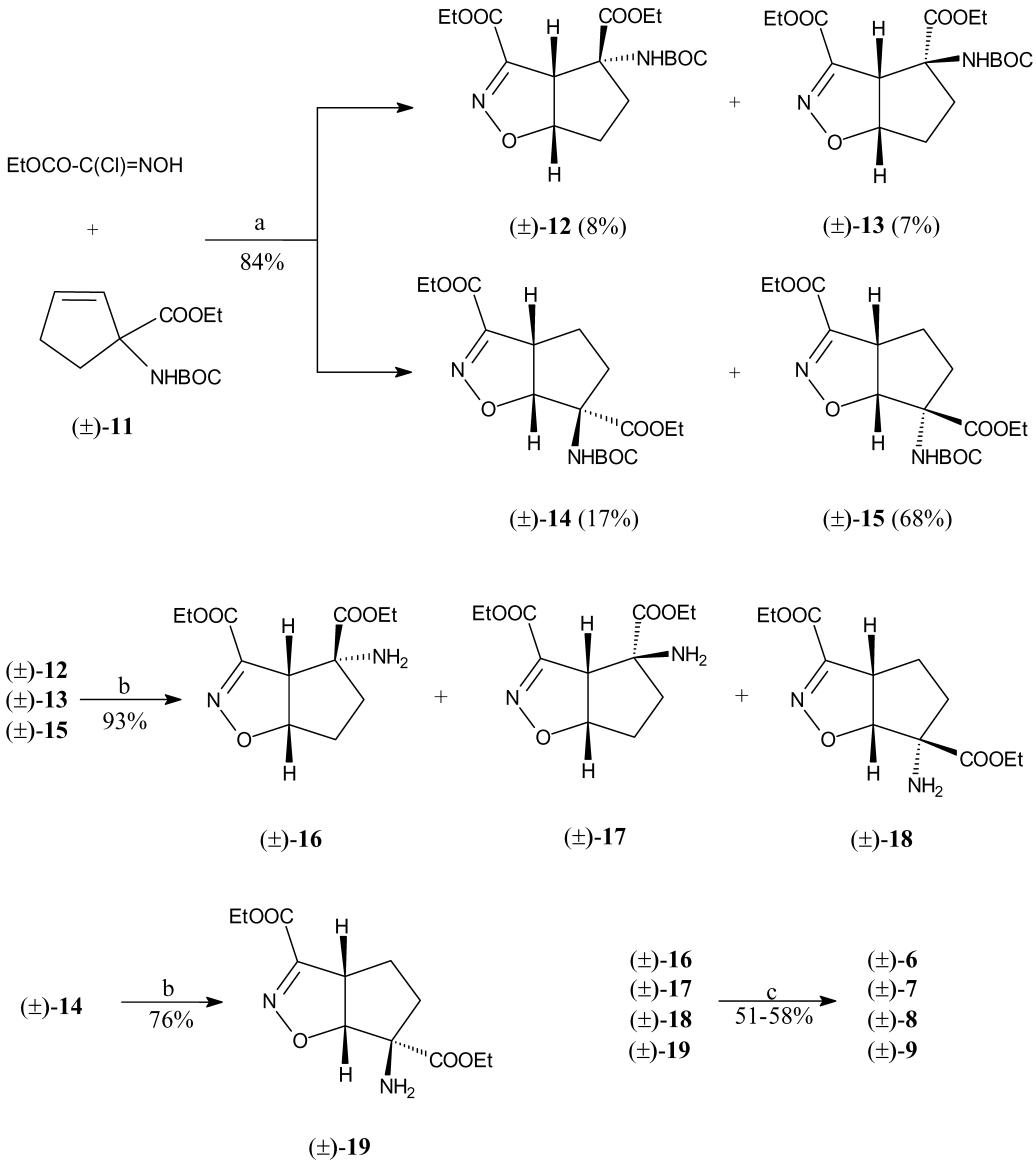
In order to uncover if a folded conformation, different from that found in CIP-A, is able to interact with the iGluRs, we designed the bicyclic amino acids **6** and **7**. On the other hand, the bicyclic derivatives **8** and **9**, representing extended conformations of glutamic acid, may interact with mGlu receptors, in analogy to 1-aminocyclopentane-1,3-dicarboxylic acid [(±)-ACPD, **10**]. This paper deals with the design and the synthesis of the stereoisomers **6** and **7** and of their regioisomers **8** and **9** (Fig. 1). The binding affinity and electrophysiological activity of novel compounds were assayed at NMDA, AMPA, and KA receptors as well at the cloned homomeric iGlu6 receptor. The activity at representative subtypes of mGluRs was also evaluated. A conformational analysis of the new compounds was performed and compared with the most populated conformation of CIP-A and (±)-ACPD.

2. Results and discussion

2.1. Synthesis

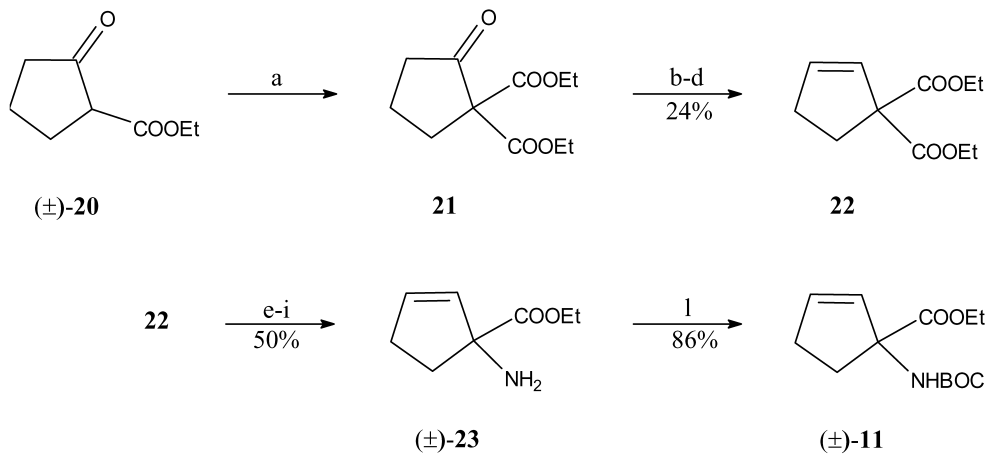
The key step in the synthesis of target compounds (±)-**6**–(±)-**9** is represented by the 1,3-dipolar cycloaddition of ethoxycarbonylformonitrile oxide, generated in situ by treatment of ethyl 2-chloro-2-(hydroxyimino)acetate with a base, to the suitably protected racemic 1-amino-cyclopent-2-enecarboxylic acid [(±)-**11**] (Scheme 1). Dipolarophile (±)-**11** was prepared according to the reaction sequence reported in Scheme 2. Following a procedure reported in the literature,[20] commercially available β-ketoester (±)-**20** was transformed into intermediate **21**. The keto group of **21** was reduced to a secondary alcohol by sodium borohydride, which was transformed into the corresponding methanesulfonate and subsequently removed by a treatment with DBU at reflux in toluene to yield the unsaturated derivative **22**. Intermediate **22** was treated with 1 equiv. of sodium hydroxide to yield the corresponding monoacid, which was then submitted to the Curtius rearrangement to give amino ester (±)-**23**. The primary amino group of (±)-**23** was reacted with di-*tert*-butyl dicarbonate [$(BOC)_2O$] to give the *N*-BOC derivative (±)-**11**.

It is worth pointing out that the pericyclic reaction between (±)-**11** and the in situ generated nitrile oxide (Scheme 1) produces all four possible stereo- and regio-isomers (**12**–**15**). Column chromatography of the reaction mixture yielded two fractions containing pure (±)-**14** and an inseparable mixture of (±)-**12**, (±)-**13**, and (±)-**15**. Treatment of the mixture of (±)-**12**, (±)-**13**, and (±)-**15** with excess trifluoroacetic acid afforded the corresponding primary amines (±)-**16**, (±)-**17**, and (±)-**18**, which were separated by a silica gel column chromatography. The same treatment carried out on cycloadduct (±)-**14** afforded



a: $NaHCO_3$/AcOEt; b: CF_3COOH/CH_2Cl_2; c: NaOH/EtOH-H_2O

Scheme 1.



a: see ref. 20; b: $NaBH_4$/EtOH; c: $MeSO_2Cl/NEt_3$; d: DBU/toluene; e: NaOH/EtOH-H_2O; f: $NEt_3/ClCO_2Et$; g: NaN_3/H_2O; h: heat/benzene; i: HCl/THF-H_2O; l: $(BOC)_2O$-NEt_3/CH_2Cl_2

Scheme 2.

intermediate (±)-**19**. Samples of pure amines (±)-**16**–(±)-**18** were reconverted into the corresponding *N*-BOC derivatives and used as standards in the HPLC analysis of the crude 1,3-dipolar cycloaddition (see Section 4). Final amino acids (±)-**6**, (±)-**7**, (±)-**8**, and (±)-**9** were obtained through the alkaline hydrolysis of the two ester groups of the corresponding amino esters and were purified by cation exchange column chromatography.

The assignment of the structure of all the synthesized compounds is based on their ^{1}H NMR spectra. The ^{1}H NMR resonances of amino acids (±)-**6**, (±)-**7**, (±)-**8**, and (±)-**9** were assigned by standard methods that rely on correlation through chemical bonds (COSY). The outcome of such an analysis was then applied to cycloadducts (±)-**16**, (±)-**17**, (±)-**18**, and (±)-**19**. The multiplicity of H-6a, the most deshielded proton, proved to be highly diagnostic for assignment of the regiochemistry. This proton appears as a doublet in the ^{1}H NMR spectrum of cycloadducts (±)-**18** and (±)-**19**, and as a multiplet in cycloadducts (±)-**16** and (±)-**17**. The structure of the pairs of stereoisomers (±)-**16**/(±)-**17** and (±)-**18**/(±)-**19** was assigned by taking into account the upfield shift of proton H-3a and H-6a, respectively, observed in cycloadducts (±)-**17** and (±)-**19**. Such a shielding effect, reported in the literature as *syn*-upfield rule,[21] is explained by the proximity to the amino group. Thus, the proton H-3a of (±)-**17** resonates at 3.64 ppm versus 4.25 ppm of (±)-**16**; a value similar to that observed in derivatives (±)-**18** and (±)-**19** (4.02 and 4.05 ppm, respectively). The same considerations hold true when the chemical shift of H-6a in cycloadducts (±)-**19** (4.83 ppm) is compared with that detected in derivatives (±)-**18**, (±)-**16**, and (±)-**17** (5.25, 5.33. and 5.40 ppm, respectively). To further support such an assignment, it is worth pointing out that (±)-**18** derives from cycloadduct (±)-**15**, the major stereoisomer of the 1,3-dipolar cycloaddition.

As shown in Figure 2, the hydrogen bond between NH of the carbamate group and the oxygen of the nitrile oxide stabilizes the transition state of the reaction. Such an effect has been observed in 1,3-dipolar cycloadditions of nitrile oxides with alkenes bearing hydroxyl and carbamate groups.[22–25] In a related 1,3-dipolar cycloaddition, we reversed the ratio between stereoisomers, such as **14**/**15**, by replacing the hydrogen of the carbamate moiety with a second BOC group.[26]

2.2. Biological evaluation

The four stereomeric amino acid derivatives **6**–**9** were assayed in vitro by means of receptor binding techniques, the rat cortical wedge preparation, and second messenger tests at both iGlu and mGlu receptors. The receptor affinity of **6**, **7**, **8**, and **9** for NMDA, AMPA, KA, and cloned homomeric iGluR6, a KA receptor subtype, was determined by using the radioligands [^{3}H]CPP, [^{3}H]AMPA, and [^{3}H]KA, respectively.[27–29] As shown in Table 1, none of the compounds, tested up to a 0.1 mM in binding assays and to 1.0 mM in electrophysiological tests, showed significant activity at the above-mentioned iGlu receptors, neither as agonists nor as antagonists.

The same compounds were also assayed at mGluR1a, mGluR2, and mGluR4a, expressed in CHO cells, as representatives for group I, II, and III metabotropic receptors, respectively and turned out to be inactive up to 1.0 mM.[1,4]

In order to rationalize the above-reported data, we carried out a conformational analysis on derivatives **6**–**9** by means of molecular mechanics calculations and the results are reported in Table 2 and compared with the data of the preferred conformations of CIP-A (**5**)[18] and (±)-ACPD

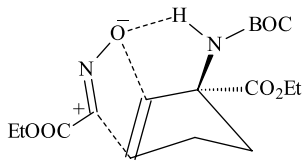


Figure 2. Transition state stabilized by an intermolecular hydrogen bond.

Table 1. Receptor binding and electropharmacological data (mean values±SEM, n=3–4)

Compound	Receptor binding, IC_{50} (μM)				Electropharmacology
	[^{3}H]AMPA	[^{3}H]KA	[^{3}H]KA[a]	[^{3}H]CPP	EC_{50} (μM)
(±)-**6**	>100	>100	>100	>100	>1000
(±)-**7**	>100	>100	>100	>100	>1000
(±)-**8**	>100	>100	>100	>100	>1000
(±)-**9**	>100	>100	>100	>100	>1000
AMPA	0.040±0.014	>100		>100	3.5±0.2
KA	4.0±1.2	0.007±0.002		>100	25±3

[a] The data refer to the binding affinity to homomeric iGluR6.

Table 2. Molecular parameters describing the pharmacophoric distances and the conformational profile of compounds **6**–**9** in comparison with CIP-A and (±)-ACPD

Conformation	$d(N^+-C_\omega)$[a]	$d(C_\alpha-C_\omega)$[a]	ΔE[b]	% Frequency[c]
CIP-A[d]	4.18	4.40	–	–
6A	3.05	4.71	0.00	94.6
6B	3.38	3.88	+1.82	5.4
7A	4.53	3.28	+1.58	8.3
7B	3.82	3.61	0.00	91.7
(±)-ACPD	4.81	4.68	–	–
8A	5.93	5.93	0.00	81.3
8B	4.47	6.10	+1.71	18.7
9A	5.82	6.02	+0.47	32.6
9B	6.04	4.58	0.00	67.4

[a] Distances expressed in Å.
[b] Relative energies (kcal/mol) of the optimized conformers.
[c] Conformational distribution, in percentage, of the two populated conformations in the frames collected during the molecular dynamics simulations at 300 K (see Figure 3).
[d] Values taken from Ref. 18.

(**10**). All compounds **6**–**9** showed two populated conformations, tagged as **A** and **B** and shown in Figure 3, due to the geometry of the cyclopentane ring which can assume either the E_7 or the ^7E conformation. The differences in the conformational profile between derivatives **6**–**9** and the considered templates **5** and **10** can be compared by examining the interatomic distances among the three ionized centers (N^+, C_ω and C_α) which represent the pharmacophoric pattern.[19,30] Such an analysis may explain the inactivity of the new derivatives.

The interatomic distances N^+-C_ω and $C_\alpha-C_\omega$ for the new compounds are reported in Table 2 and compared with those found in the global minimum conformation of the two templates. The distance N^+-C_α has been neglected since it spans a narrow range (2.40–2.50 Å). Table 2 also highlights the conformational profile of derivatives **6**–**9** which is strictly dependent upon the configuration of the aminoacid stereogenic center. If we compare the profiles of diastereomers **6** and **7** we can notice that they are opposite. In fact, if we consider the relative frequency of each conformation (Table 2) we can observe that conformation A is highly preferred (94.6 vs. 5.4%) in derivative **6** while conformation B is dominant in derivative **7** (91.7 vs 8.3). The same holds true for the pair of stereoisomers **8** and **9** where geometry A is dominant in **8** and geometry B in **9**.

The data reported in Table 2 show that the conformation assumed by derivatives **6** and **7** does not match the biologically active one present in CIP-A.[18] In the preferred conformation of compounds **6** and **7** the ammonium and the ω-carboxylate groups are too close to fit the CIP-A model. On the other hand, if we compare the same parameters for compounds **8** and **9** with those of (±)-ACPD we can deduce that their conformation is too extended to mimic the active one.

3. Conclusion

In summary we have shown that the conformational profile of derivatives **6** and **7** is too different from that of CIP-A and may account for their inactivity at iGluRs. Similar arguments hold true for compounds **8** and **9** relative to (±)-ACPD and justify their inability to interact with mGlu receptors.

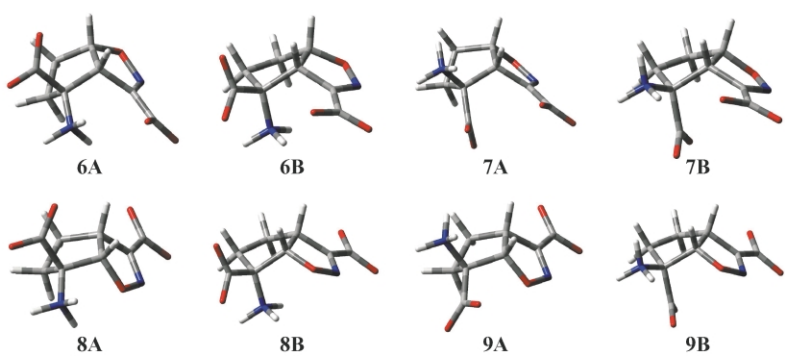


Figure 3. Three-dimensional plots of the populated conformations of compounds **6**–**9**.

4. Experimental

4.1. Materials and methods

Ethyl 2-chloro-2-(hydroxyimino)acetate[31] and 2-oxo cyclopentane-1,1-dicarboxylic acid diethyl ester (**21**)[20] were prepared according to literature procedures. IR spectra were registered with a Perkin–Elmer FT-IR spectrometer; ^{1}H NMR spectra were recorded with a Varian Gemini 200 (200 MHz) spectrometer in $CDCl_3$ (or D_2O) solution at 20°C; signal assignments have been made by a combination of 1D and 2D COSY analyses; chemical shifts (δ) are expressed in ppm and coupling constants (J) in hertz. HPLC analyses were performed using a Jasco PU-980 pump equipped with a detector UV–VIS Jasco UV-975 and a LiChrospher Si 60 column. The components of the reaction mixture were eluted with a mixture of petroleum ether/2-propanol 98:2 at a flow rate of 0.5 mL/min and were detected spectrophotometrically at a wavelength (λ) of 254 nm. TLC analyses were performed on commercial silica gel 60 F_{254} aluminum sheets; spots were further detected by spraying with a dilute alkaline potassium permanganate solution or with ninhydrin. Melting points were determined on a Büchi apparatus and are uncorrected. Elemental analyses (C, H, N) of new compounds were performed by Redox s.n.c. (Milan) and agreed with theoretical value ±0.3%.

4.2. Synthesis of cyclopent-2-ene-1,1-dicarboxylic acid diethyl ester 22

(A) To a stirred solution of **21**[20] (30.7 g, 134.7 mmol) in EtOH (300 mL), cooled at 0°C, sodium borohydride (2.5 g, 67.3 mmol) was added portionwise. After stirring for 30 min at 0°C, the reaction was quenched by a dropwise addition of 1N HCl. After evaporation of the volatiles at reduced pressure, the residue was dissolved in water and extracted with CH_2Cl_2. The organic extracts were dried over anhydrous Na_2SO_4 and concentrated. The crude material was submitted to column chromatography on silica gel (eluant: petroleum ether/ethyl acetate 4:1) to give 14.54 g (yield: 47%) of the corresponding alcohol derivative as yellow oil.

(B) The above prepared alcohol (14.54 g, 63.21 mmol) was reacted with TEA (13.1 mL, 94.8 mmol) and methansulfonylchloride (7.4 mL, 94.8 mmol), in CH_2Cl_2, at 0°C. After stirring for further 30 min at room temperature, 1N HCl (6 mL) was added to the reaction mixture. The organic layer was separated and washed with an aqueous solution of $NaHCO_3$. After drying and evaporation of the organic layer, the crude material was purified by column chromatography on silica gel (eluant: petroleum ether/ethyl acetate 9:1) to give 11.1 g (57% yield) of the corresponding mesylate.

(C) A solution of the mesylate obtained in the previous step (11.1 g, 36.04 mmol) and DBU (27 mL, 180.2 mmol) in toluene (90 mL) was heated at reflux for 48 h. After cooling at room temperature the reaction mixture was washed with 2N HCl (2×30 mL) and with water (30 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude material was purified by column chromatography on silica gel (eluant: petroleum ether/ethyl acetate 95:5) to give 6.88 g (90% yield) of **22** as a colorless oil.

4.2.1. Cyclopent-2-ene-1,1-dicarboxylic acid diethyl ester 22.[32] Bp 70°C/0.35 mbar; R_f (cyclohexane/ethyl acetate 95:5) 0.25; ν_{max} (film) 2923, 2852, 1736, 1617, 1463, 1261, 1066; ^{1}H NMR ($CDCl_3$): 1.24 (t, 6H, J=7.3 Hz, CH_2CH_3); 2.38–2.55 (m, 4H, CH_2CH_2); 4.18 (q, 4H, J=7.3 Hz, OCH_2); 5.78–5.83 (m, 1H, H-3 or H-2); 5.96–6.00 (m, 1H, H-2 or H-3). ^{13}C NMR ($CDCl_3$) 14.47 (CH_3); 31.83 (C-5); 32.33 (C-4); 61.75 (OCH_2); 63.91 (C-1); 129.01 (C-3 or C-2); 135.97 (C-2 or C-3); 171.48 (CO).

4.3. Synthesis of *N*-BOC-cyclopent-2-enecarboxylic acid ethyl ester (±)-11

(A) Alkene **22** (6.88 g, 32.5 mmol) was dissolved in EtOH (30 mL) and treated with 1N NaOH (32.5 mL, 32.5 mmol) at room temperature for 15 h. After evaporation of the ethanol at reduced pressure, the aqueous layer was extracted with CH_2Cl_2 (3×10 mL) to remove unreacted **22**. The aqueous layer was then acidified with 2N HCl and extracted with CH_2Cl_2 (3×15 mL). The organic phase was dried and evaporated to give 4.93 g of the corresponding monoacid of **22**, which were directly used in the next step.

(B) The above-prepared monoacid derivative (4.93, 26.8 mmol) was dissolved in acetone (45 mL) and cooled at 0°C. TEA (4.48 mL, 32.2 mmol) was added at once followed by the dropwise addition of ethyl chloroformate (3.44 mL, 36.1 mmol). The mixture was stirred at room temperature for 30 min. then cooled at 0°C. A solution of sodium azide (2.63 g, 40.4 mmol) in water (5 mL) was slowly added and the mixture was stirred for 1 h at 0°C. The progress of the reaction was monitored by TLC (eluant: petroleum ether/ethyl acetate 4:1). After evaporation of the acetone at reduced pressure, the aqueous phase was extracted with Et_2O (2×10 mL) and the pooled organic extracts were dried and concentrated. The residue, containing the acyl-azide, was dissolved in benzene (100 mL) and refluxed for 3 h to give the corresponding isocyanate. The progress of the reaction was monitored by TLC (eluant: petroleum ether/ethyl acetate 9:1). At the complete conversion of the substrate, the solvent was evaporated under vacuum and the residue was dissolved in THF (100 mL) and reacted with 2N HCl (100 mL) overnight. THF was evaporated at reduced pressure; the aqueous phase was extracted with CH_2Cl_2 (2×30 mL) then made alkaline with solid K_2CO_3. The product was extracted with ethyl acetate (5×30 mL). The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give 2.05 g (50% yield) of amino ester **23** as yellow oil.

(C) To a CH_2Cl_2 solution (30 mL) of **23** (2.05 g, 13.2 mmol) and TEA (2.76 mL, 19.8 mmol), stirred and cooled at 0°C, a solution of $(BOC)_2O$ (4.3 g, 19.8 mmol) in CH_2Cl_2 (10 mL) was added dropwise. The reaction mixture was stirred at room temperature overnight, then acidified with 1N HCl. The organic layer was dried and concentrated under vacuum and the residue was purified by a silica gel column chromatography (eluant: petroleum ether/ethyl acetate 9:1) to give alkene **11** as a yellow oil (2.9 g, 86% yield) which becomes colorless after distillation.

4.3.1. *N*-BOC-cyclopent-2-enecarboxylic acid ethyl ester (±)-11. Bp 170°C/0.27 mbar; R_f (cyclohexane/ethyl acetate 9:1) 0.24; ν_{max} (film) 3368, 2977, 2929, 1736, 1718, 1499, 1366, 1170; ^{1}H NMR ($CDCl_3$) 1.26 (t, 3H, J=7.2 Hz, CH_2CH_3); 1.43 (s, 9H, $C(CH_3)_3$); 1.95–2.10 (m, 1H, H-5); 2.50–2.60 (m, 2H, H-4 and H-4′); 2.63–2.79 (m, 1H, H-5′); 4.19 (q, 2H, J=7.2 Hz, OCH_2); 5.10–5.30 (bs, 1H, NH); 5.62–5.70 (m, 1H, H-3 or H-2); 6.03–6.12 (m, 1H, H-2 or H-3).); ^{13}C NMR ($CDCl_3$) 14.56 (CH_2CH_3); 28.69 (CCH_3 and C-5); 32.39 (C-4); 61.80 (OCH_2); 65.91 (C-1); 69.41 (CCH_3); 130.55 (C-3 or C-2); 137.39 (C-2 or C-3); 154.80 (NCO);173.36 (CO). Anal. calcd for $C_{13}H_{21}NO_4$: C, 61.16; H, 8.29; N, 5.49. Found: C, 60.89; H, 8.41; N, 5.37.

4.4. 1,3-Dipolar cycloaddition of ethoxycarbonylformonitrile oxide to (±)-*N*-BOC-1-amino-cyclopent-2-enecarboxylic acid ethyl ester

To a solution of **11** (1.8 g, 7.06 mmol) in ethyl acetate (40 mL) was added ethyl 2-chloro-2-(hydroxyimino)acetate (1.6 g, 10.59 mmol) and $NaHCO_3$ (5 g). The mixture was vigorously stirred for 5 days, than other 3 equiv. (3.2 g, 21.18 mmol) of ethyl 2-chloro-2-(hydroxyimino)acetate were added and the mixture was stirred for additional 5 days. The progress of the reaction was monitored by TLC (petroleum ether/ethyl acetate 4:1). 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 9:1) to give 1.88 g of an inseparable mixture of **12**, **13** and **15** and 0.306 g of cycloadduct **14**. Overall yield: 84%.

Mixture of **12**, **13**, **15**: R_f (cyclohexane/ethyl acetate 4:1) 0.18.

4.4.1. Diethyl (±)-*N*-BOC-(3a*S*,6*S*,6a*R*)-6-amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,6-dicarboxylate (±)-14. Yellow oil, R_f (cyclohexane/ethyl acetate 4:1) 0.13; ν_{max} (film) 3355, 2971, 1729, 1707, 1581, 1509, 1274, 1241, 1158; ^{1}H NMR ($CDCl_3$) 1.28 (t, 3H, J=7.3 Hz, CH_2CH_3); 1.36 (t, 3H, J=7.1 Hz, CH_2CH_3); 1.43 (s, 9H, $C(CH_3)_3$); 1.53–158 (bs, 2H, H-4 and H-4′); 2.00–2.30 (m, 2H, H-5 and H-5′); 2.42–2.55 (bs, 1H, NH); 3.97–4.10 (bdd, 1H, H-3a, J=9.8, 9.8 Hz); 4.15–4.44 (m, 4H, $2CH_2CH_3$); 5.03 (d, 1H, J=9.8 Hz, H-6a). Anal. calcd for $C_{17}H_{26}N_2O_7$: C, 55.13; H, 7.08; N, 7.56. Found: C, 55.28; H, 6.84; N, 7.39.

4.5. Synthesis of derivatives 16, 17 and 18

The mixture of **12**, **13** and **15** (1.88 g, 5.08 mmol) was treated with a 30% CH_2Cl_2 solution of trifluoroacetic acid (12.9 mL) at 0°C. The reaction mixture was stirred at room temperature until disappearance of the starting material (3 h). The volatiles were removed under vacuum and the residue was treated with a 10% K_2CO_3 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 (eluant: petroleum ether/ethyl acetate 1:4) to give 0.149 g of **16**, 0.990 g of **18**, and 0.136 g of **17** as yellowish oils in 93% overall yield.

4.5.1. Diethyl (±)-(3a*S*,6*S*,6a*R*)-6-amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,6-dicarboxylate (±)-16. R_f (cyclohexane/ethyl acetate 1:4) 0.62; ν_{max} (film) 3392, 2979, 2933, 2872, 1724, 1215, 753; ^{1}H NMR ($CDCl_3$) 1.30 (t, 3H, J=7.1 Hz, CH_2CH_3); 1.36 (t, 3H, J=7.1 Hz, CH_2CH_3); 1.64–1.85 (bs, 4H, H-6, H-6′ and NH_2); 2.07–2.30 (m, 2H, H-5 and H-5′); 4.22 (q, 2H, J=7.1 Hz, OCH_2); 4.25 (d, 1H, J=10.1 Hz, H-3a); 4.33 (q, 2H, J=7.1 Hz, OCH_2); 5.25–5.40 (m, 1H, H-6a). Anal. calcd for $C_{12}H_{18}N_2O_5$: C, 53.33; H, 6.71; N, 10.36. Found: C, 53.51; H, 6.45; N, 10.21.

4.5.2. Diethyl (±)-(3a*S*,6*S*,6a*R*)-6-amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,6-dicarboxylic acid (±)-18. R_f (cyclohexane/ethyl acetate 1:4) 0.32; ν_{max} (film) 3375, 2946, 2931, 2870, 1730, 1250, 853; ^{1}H NMR ($CDCl_3$) 1.31 (t, 3H, J=7.3 Hz, CH_2CH_3); 1.37 (t, 3H, J=7.3 Hz, CH_2CH_3); 1.53–1.77 (bs, 4H, H-4, H-4′ and NH_2); 2.00–2.25 (m, 2H, H-5 and H-5′); 3.94–4.10 (m, 1H, H-3a); 4.22 (q, 2H, J=7.3 Hz, OCH_2); 4.35 (q, 2H, J=7.3 Hz, OCH_2); 5.25 (d, 1H, J=9.8 Hz, H-6a). Anal. calcd for $C_{12}H_{18}N_2O_5$: C, 53.33; H, 6.71; N, 10.36. Found: C, 53.62; H, 6.58; N, 10.30.

4.5.3. Diethyl (±)-*N*-(3a*S*,4*S*,6a*S*)-4-amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,4-dicarboxylate (±)-17. R_f (cyclohexane/ethyl acetate 1:4) 0.14; ν_{max} (film) 3391, 2971, 2924, 2876, 1726, 1213, 758; ^{1}H NMR ($CDCl_3$) 1.26 (t, 3H, J=7.1 Hz, CH_2CH_3); 1.36 (t, 3H, J=7.1 Hz, CH_2CH_3); 1.57–1.77 (bs, 4H, H-6, H-6′ and NH_2); 2.20–2.45 (m, 2H, H-5 and H-5′); 3.64 (d, 1H, J=9.1 Hz, H-3a); 4.07 (q, 2H, J=7.1 Hz, OCH_2); 4.29 (q, 2H, J=7.1 Hz, OCH_2); 5.40 (m, 1H, H-6a). Anal. calcd for $C_{12}H_{18}N_2O_5$: C, 53.33; H, 6.71; N, 10.36. Found: C, 53.54; H, 6.49; N, 10.24.

4.6. Synthesis of derivative 19

Compound **14** (0.306 g, 0.83 mmol) was treated with a 30% CH_2Cl_2 solution of trifluoroacetic acid (2.1 mL) at 0°C. The reaction mixture was stirred at room temperature until disappearance of the starting material (3 h). The volatiles were removed under vacuum and the residue was treated with a 10% potassium carbonate solution (4 mL) and extracted with ethyl acetate (3×2 mL). The pooled organic extracts were dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was purified by silica gel column chromatography (eluant: petroleum ether/ethyl acetate 1:4) to give 0.170 g of **19** (76 % yield).

4.6.1. Diethyl (±)-(3a*S*,6*R*,6a*R*)-6-amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,6-dicarboxylate (±)-19. R_f (cyclohexane/ethyl acetate 1:4) 0.32; ν_{max} (film) 3388, 2969, 2937, 2872, 1723, 1219, 733; ^{1}H NMR ($CDCl_3$) 1.31 (t, 3H, J=7.0 Hz, CH_2CH_3); 1.37 (t, 3H, J=7.0 Hz, CH_2CH_3); 1.53–1.75 (bs, 4H, H-4, H-4′ and NH_2); 2.03–2.25 (m, 2H, H-5 and H-5′); 4.00–4.17 (m, 1H, H-3a); 4.24 (q, 2H, J=7.0 Hz, OCH_2); 4.34 (q, 2H, J=7.0 Hz, OCH_2);

4.83 (d, 1H, J=8.8 Hz, H-6a). Anal. calcd for $C_{12}H_{18}N_2O_5$: C, 53.33; H, 6.71; N, 10.36. Found: C, 53.49; H, 6.43; N, 10.29.

4.7. Conversion of amino esters 16–18 into the corresponding *N*-BOC derivatives 12, 13 and 15

Amino esters **16**–**18** (20 mg) were separately treated with 1.5 equiv. of TEA and 1.5 equiv. of $(BOC)_2O$ in CH_2Cl_2 to give samples of the corresponding *N*-BOC derivatives **12**, **13**, and **15**, which were not isolated but directly used, together with cycloadduct **14**, as standards for HPLC analyses.

Retention times: **12**, 21.73 min; **15**, 24.52 min; **13**, 26.95 min; **14**, 30.57 min.

4.8. Synthesis of (±)-(3a*S*,4*R*,6a*S*)-4-amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,4-dicarboxylic acid (±)-6

A solution of **16** (0.120 g, 0.44 mmol) in methanol (1.3 mL) was treated with 1N NaOH (1.3 mL). The reaction mixture was stirred at room temperature for 24 h and the disappearance of the starting material was monitored by TLC (eluant: cyclohexane/ethyl acetate 1:4). At the disappearance of the substrate, the solution was acidified to pH 2 with 2N HCl, and submitted to a cation exchange chromatography, using Amberlite IR 120 plus. The acidic solution was slowly eluted onto the resin, and then the column was washed with water until the pH was neutral. The compound was then eluted off the resin with 10% aqueous pyridine, and the product-containing fractions (detected with ninhydrin stain on a TLC plate) were combined and concentrated under vacuum. The compound thus obtained was crystallized from water/methanol, filtered, washed sequentially with methanol and ethyl ether and dried in vacuum at 50°C to give 49 mg of **6** as white prisms (yield: 52 %).

4.8.1. (±)-(3a*S*,4*R*,6a*S*)-4-Amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,4-dicarboxylic acid (±)-6.

R_f 0.25 (*n*-butanol/water/acetic acid 50:35:15); mp dec. >180°C; ν_{max} (KBr) 3401, 3233, 2523, 1913, 1677, 1623, 1384; ^{1}H NMR (D_2O): 1.95–2.41 (m, 4H, H-5, H-5′, H-6, and H-6′); 4.31 (d, 1H, J=9.5 Hz, H-3a); 5.37–5.46 (m, 1H, H-6a). Anal. calcd for $C_8H_{10}N_2O_5$: C, 44.86; H, 4.71; N, 13.08. Found: C, 44.58; H, 4.75; N, 12.89.

4.9. Synthesis of (±)-(3a*S*,4*S*,6a*S*)-4-amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,4-dicarboxylic acid (±)-7

Compound **17** (0.110 g, 0.41 mmol), submitted to the procedure described for compound **16**, afforded 45 mg of **7** as white prisms (yield: 51%).

4.9.1. (±)-(3a*S*,4*S*,6a*S*)-4-Amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,4-dicarboxylic acid (±)-7.

R_f 0.22 (*n*-butanol/water/acetic acid 50:35:15); mp dec. >168°C; ν_{max} (KBr) 3429, 3241, 1712, 1626, 1579, 1385; ^{1}H NMR (D_2O) 1.98–2.39 (m, 4H, H-5, H-5′, H-6 and H-6′); 3.96 (d, 1H, J=8.8 Hz, H-3a); 5.29–5.35 (m, 1H, H-6a). Anal. calcd for $C_8H_{10}N_2O_5$: C, 44.86; H, 4.71; N, 13.08. Found: C, 44.56; H, 4.74; N, 12.90.

4.10. Synthesis of (±)-(3a*S*,6*S*,6a*R*)-6-amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,6-dicarboxylic acid (±)-8

Compound **18** (0.960 g, 3.56 mmol), submitted to the procedure described for compound **16**, afforded 442 mg of **8** as white prisms (yield: 58%).

4.10.1. (±)-(3a*S*,6*S*,6a*R*)-6-Amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,6-dicarboxylic acid (±)-8.

R_f 0.11 (*n*-butanol/water/acetic acid 50:35:15); mp dec. >161°C; ν_{max} (KBr) 3869, 3505, 2550, 1890, 1715, 1609; ^{1}H NMR (D_2O) 1.58–1.78 (m, 1H, H-5); 1.90–2.31 (m, 3H, H-4, H-4′ and H-5′); 4.12 (ddd, 1H, J=2.0, 9.2, 9.2 Hz, H-3a); 5.15 (d, 1H, J=9.2 Hz, H-6a). Anal. calcd for $C_8H_{10}N_2O_5$: C, 44.86; H, 4.71; N, 13.08. Found: C, 44.77; H, 4.65; N, 12.92.

4.11. Synthesis of (±)-(3a*S*,6*R*,6a*R*)-6-amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,6-dicarboxylic acid (±)-9

Compound **19** (0.140 g, 0.52 mmol), submitted to the procedure described for compound **16**, afforded 61 mg of **9** as white prisms (yield: 55%).

4.11.1. (±)-(3a*S*,6*R*,6a*R*)-6-Amino-4,5,6,6a-tetrahydro-3a*H*-cyclopenta[*d*]isoxazole-3,6-dicarboxylic acid (±)-9.

R_f 0.17 (*n*-butanol/water/acetic acid 50:35:15); mp dec. >175°C; ν_{max} (KBr) 3865, 3487, 2562, 1890, 1721, 1604; ^{1}H NMR (D_2O) 1.95–2.10 (m, 1H, H-5); 2.15–2.42 (m, 3H, H-4, H-4′ and H-5′); 4.23 (ddd, 1H, J=2.7 Hz, 9.4, 9.4, H-3a); 5.26 (d, 1H, J=9.4 Hz, H-6a). Anal. calcd for $C_8H_{10}N_2O_5$: C, 44.86; H, 4.71; N, 13.08. Found: C, 44.96; H, 4.39; N, 13.18.

4.12. Biological testing

4.12.1. Receptor binding. Affinity for NMDA, AMPA, KA, and iGlu6 receptors was determined using the ligands [^{3}H]CPP,[27] [^{3}H]AMPA[28] and [^{3}H]KA,[29] respectively. The membrane preparations used in all the receptor-binding experiments were prepared according to the method of Ransom and Stec.[33]

4.12.2. In vitro electrophysiology. A rat cortical slice preparation for determination of EAA-evoked depolarizations described by Harrison and Simmonds[34] was used in a slightly modified version. Wedges (500 μM thick) of rat brain, containing cerebral cortex and corpus callosum, were placed through a grease barrier for electrical isolation with each part in contact with a DriRef-5SH (World Precision Instruments) electrode. The cortex and corpus callosum parts were constantly superfused with a Mg^{++} free (and Ca^{++} free for the corpus callosum) oxygenated Krebs buffer at room temperature. The test compounds were added to the cortex superfusion medium and the potential difference between the electrodes recorded on a chart recorder. Applications of agonists were made for 90 s at each concentration tested, typically at 15 min intervals. In experiments designed to detect antagonist effects the

potential antagonist were applied alone for 90 s followed by co-application of agonists (NMDA, AMPA or KA) and the potential antagonist for another 90 s.

4.12.3. Metabotropic testing. Three metabotropic subtypes mGluR1a, mGluR2 or mGluR4a were expressed in Chinese hamster ovary cell lines and used as representatives for group I, II and III metabotropic receptors. Cell lines were maintained and assayed as previously described.[35]

4.13. Computational details

The structure of all the compounds was built with ChemNote module of Quanta and was submitted to a preliminary minimization with MOPAC 6.0[36] (keywords= 'AM1', 'PRECISE', 'GEO-OK') to avoid high energy starting conformations and to set the atomic charges (especially for ionized centers).

The conformational analysis of compounds **6**–**9** was performed with Quanta/CHARMM[37] using molecular dynamics (200 ps) at a high temperature (2000 K), able to span the conformational space of flexible molecules. Simulated annealing gave the best structures, which were minimized with the Newton–Raphson algorithm until RMS reached the value of 0.01. Two distinct minima were located for each compound. In these calculations, the dielectric constant was set to 80 to take into account the presence of the ionized centers.

Molecular dynamics simulations were also performed on compounds **6**–**9** using the following characteristics: constant temperature in the range 300±25 K, integration of Newton's equation every 1 fs according to Verlet's algorithm, calculation of initial atomic velocities according to Boltzmann's equation, frame stored every 1000 iterations (1.0 ps). The simulations were carried out in three phases: initial period of heating from 0 to 300 K over 3000 iterations (3 ps, i.e. 1 K/10 iterations), equilibration period of 250 ps with recalculation of atomic velocities during this period every 0.2 ps, and the monitored phase of simulation of 1 ns. Only the frames memorized during this third phase were considered in the analysis of the conformational behavior. The frames collected during the simulations were analyzed in terms of torsional angles of the cyclopentane ring and allowed to determine the percentage frequency of the **A** and **B** conformations.

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