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ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 7581–7589
Bioorganic & Medicinal Chemistry

1-Aminocyclopentane-1,2,4-tricarboxylic acids screening on glutamatergic and serotonergic systems

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Received 1 February 2007; revised 1 August 2007; accepted 7 September 2007
Available online 12 September 2007

Abstract—Enantiopure constrained 1-aminocyclopentane-1,2,4-tricarboxylic acids containing the glutamic acid skeleton were prepared as two diastereomers characterized by having the carboxylic groups in position two and four *cis*-oriented to each other and *trans* with respect to 1-carboxylic group and all *cis*-oriented carboxylic groups, respectively. A biochemical screening of activity of the above amino acids was investigated on glutamate and 5-HT receptors to find a possible metabotropic agonist, acting on the serotoninergic system.

1. Introduction

Glutamate, a major excitatory neurotransmitter in the brain, participates in the production of several physiological and pathological conditions. Altered glutamatergic neurotransmission has been suggested to play a central role in several neurological and psychiatric disorder, including neurodegenerative diseases, epilepsy, schizophrenia, and anxiety.[1] Glutamate receptors are currently classified into two major types: ionotropic glutamate receptors and metabotropic glutamate receptors. Metabotropic glutamate receptors (mGluRs) are guanine nucleotide-binding protein (G-protein)-coupled receptors that primarily serve to modulate excitatory neurotransmission in the brain.[2] Eight mGluR subtypes have been identified and they are classified into three groups based on sequence homology, pharmacology and signal transduction mechanism. Group I consists of mGluR1 and mGluR5, which are coupled to the $G\alpha_q$ protein that activates phospholipase C, with subsequent formation of the secondary messengers inositol triphosphate and diacylglycerol. Group II comprises the subtypes 2 and 3, and the Group III the subtypes 4, 6, 7, and 8. Both Groups II and III are coupled to the $G\alpha_i$ protein and inhibit adenylyl cyclase thereby reducing intracellular cAMP formation. Group I mGluRs are located mostly postsynaptically, whereas group II and group III ones occur chiefly presynaptically.[3] Taking account of the physiological functions and distribution of mGluR subtypes in glutamatergic synapses in the central nervous system (CNS), a therapeutic application of group I mGluR antagonists and groups II and III mGluR agonists for a variety of brain diseases is proposed.

The use of amino acids as drugs acting selectively on different neuronal receptors is well documented. Recently, synthesis of 2-substituted derivatives of (*S*)-4-carboxyphenylglycine (4CPG) revealed (*R*,*S*)-2-amino-2-(4-carboxyphenyl)-3-(thioxanth-9-yl)-propionic acid (LY367366) as a potent antagonist at both mGlu1a and mGlu5a receptors, with IC50 values of 6.6 and 5.6 μM, respectively.[4] The binomium activity-stereochemistry of amino acids is very important[5] for the selective interaction with the neuronal receptors as documented above and for different constrained carbocyclic amino acids.[6] This is the case of the stereoisomeric 1-amino-cyclopentane-1,3-dicarboxylic acids[7] (ACPD) and 1-amino-cyclopentane-1,3,4-tricarboxylic acids[8] (ACPT) which are characterized by a selective activity

Keywords: Aminocyclopentane-1,2,4-tricarboxylic acids; Chemical resolution; Serotoninergic receptors; Glutamatergic receptors.
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doi:10.1016/j.bmc.2007.09.004

on metabotropic mGluRs subtypes depending on their stereochemistry. ACPTs are analogues of ACPDs, the first described selective agonists for mGluRs, in which an additional carboxylic group has been introduced at position 4 in cyclopentane ring. (1*R*,3*R*,4*S*)-ACPT-II isomer was found to be a general competitive antagonist for metabotropic receptors and exhibited a similar affinity for mGluR1a, mGluR2, and mGluR4a, the representative members of group I, II and III mGluRs, respectively. Two other isomers, (1*S*,3*R*,4*S*)-ACPT-I and (+)-(3*S*,4*S*)-ACPT-III, were potent agonists at the group III receptor mGluR4a and competitive antagonists with low affinity for mGluR1a and mGluR2. (−)-(3*R*,4*R*)-ACPT-III was a competitive antagonist with poor but significant affinity for mGluR4a. These results demonstrated that either the addition of a group or the stereoisomeric isoforms can modify the activity of ACPT from agonist to antagonist, increasing or decreasing its selectivity and/or affinity for the various mGluR subtypes.[8]

Kawashima et al.[9] demonstrated interaction between group II mGluR and the serotonergic neural system. The mechanism by which group II mGluR antagonists activate the serotonergic nervous system is unclear. There are multiple interactions between glutamatergic and serotonergic nervous systems in the prefrontal cortex.[10] It has been reported that local injection of 5-HT2a/2c agonists into medial prefrontal cortex (mPFC) activates dorsal raphe nucleus (DRN) serotonergic neural activity and increases 5-HT release in mPFC indirectly, via stimulation of glutamate release and subsequent activation of AMPA/kainate receptors on the pyramidal neurons projecting to the DRN.[11] Moreover, high concentrations of the 5-HT1_A receptor agonist, 8-hydroxy-2-(di-*n*-propylamino)tetraline (8-OH-DPAT), completely abolished the rise of extracellular glutamate induced by *N*-methyl-D-aspartate (NMDA) receptor antagonists, as the 3-[(*R*)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP). Literature data have provided evidence that antagonist of group I and agonist of group II/III metabotropic glutamate receptors show anxiolytic-like properties in preclinical study.[12] Moreover, anatomical, behavioral, and electrophysiological evidence jointly supports the role of central serotonin in the modulation of anxiety.

Stachowicz et al.[13] showed that a (*R*,*S*)-α-cyclopropyl-4-phosphophenylglycine injection exhibited a potent anxiolytic-like action, which was blocked by a concomitant administration of ACPT-I. Moreover, the benzodiazepine receptor antagonist flumazenil did not change the anxiolytic-like effect of CPPG, but this effect was abolished by the non-selective antagonist of 5-HT receptor metergoline and the antagonist of 5-HT$_{2A}$ receptor ritanserin.

These findings suggest that the blockade of group III mGluR in the amygdala is responsible for anxiolysis and that serotonergic system, but not the benzodiazepine recognition site of the GABAergic system, is involved in the anxiolytic-like response induced by group III mGluR antagonist.

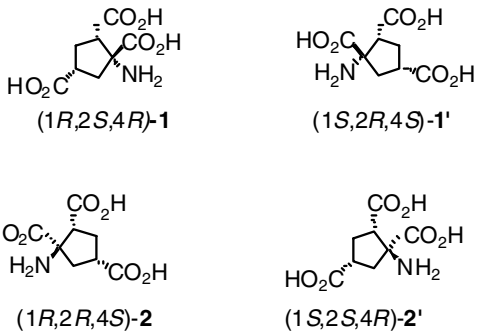


Figure 1. 1-Aminocyclopentane-1,2,4-tricarboxylic acids.

Recently,[14] we designed and synthesized a couple of diastereomeric 1-aminocyclopentane-1,2,4-tricarboxylic acids (compounds **1′** and **2′**, Fig. 1). Diastereomer **1′** possesses the carboxyl groups in position two and four *cis*-oriented to each other and *trans* with respect to 1-carboxylic group. Instead, all *cis*–oriented carboxylic groups characterize isomer **2′** (Fig. 1).

Since, as pointed out before for 1-amino-cyclopentane-1,3,4-tricarboxylic acids, the stereochemistry plays an important role toward the biological activity and selectivity on mGluRs, here we report on a general synthetic procedure to obtain each enantiomer.

The activity of amino acids **1′** and **1**, as well as of **2′** and **2** (Fig. 1), was investigated on glutamate and 5-HT systems to find a possible metabotropic agonist, with activity on serotonergic system.

2. Chemistry

Amino acids **1′** and **2′** were prepared from *exo*- and *endo*-2-acetylamino-bicyclo[2.2.1]hept-5-ene-2-carboxylates obtained in enantiopure form using, as chiral auxiliary, the (−)-8-phenylmenthol and, as key reaction, a Diels–Alder cycloaddition.

In view of biological tests, the availability of each diastereomer is very important in the form of both pure enantiomers. Even if the above cycloaddition reaction worked efficiently, some limits are met in this synthetic approach such as the need to repeat twice the whole synthetic protocol to obtain both enantiomers, the unfavorable ratio in the cycloaddition process of the endo norbornene adduct (exo/endo, 4.9:1). Furthermore, 8-phenyl-menthol is a very expensive chiral auxiliary and this is detrimental for large-scale synthesis. For this reason, it was planned to use racemic methyl 2-acetylamino-bicyclo[2.2.1]hept-5-ene-2-carboxylates (*exo*-**3**) and (*endo*-**4**) as the starting materials, obtained in a most favorable endo ratio, and the chemical resolution technique was chosen to prepare each enantiopure compound.

Cycloadducts (±)-*exo*-**3** and (±)-*endo*-**4** (2.3:1 ratio, 97%) were prepared under mild conditions according to a known synthetic protocol followed for the cycloaddition of β-heterosubstituted-aminoacrylates,[15] starting

from the commercially available methyl 2-acetylaminoacrylate and cyclopentadiene using dichloromethane as solvent, $Mg(ClO_4)_2$ (0.3 eq) as catalyst, and operating in the presence of ultrasound (14 h). The chromatographic separation on silica gel afforded pure cycloadducts *exo*-**3** (68%) and *endo*-**4** (29%).

Compound (±)-*exo*-**3** was then treated with an aqueous solution of potassium permanganate (3.3 eq) at 0 °C in acetone affording tricarboxylic acid derivative (±)-(1*R**,2*S**,4*R**)-**5** which was isolated, after chromatography on a Dowex resin, in good yield (92%) (Scheme 1).

In a first experiment we tested the chemical resolution of racemic compound (±)-**5** using different commercially available enantiopure amines such as brucine, (+)-1-naphtylethylamine, and (+)-1-phenylethylamine. In all cases attempts to isolate the enantiopure corresponding salt failed because of the low yield of precipitated salt. The possibility to perform an enzymatic resolution by hydrolyzing the ester function was also considered starting from **5** and by using both acylase from *Aspergillus melleus* and acylase I from *Porcine kidney*. In both cases the reaction was unsuccessful, probably because of the α,α-disubstitution of the amino acid function, and the starting material was recovered in quantitative yield.

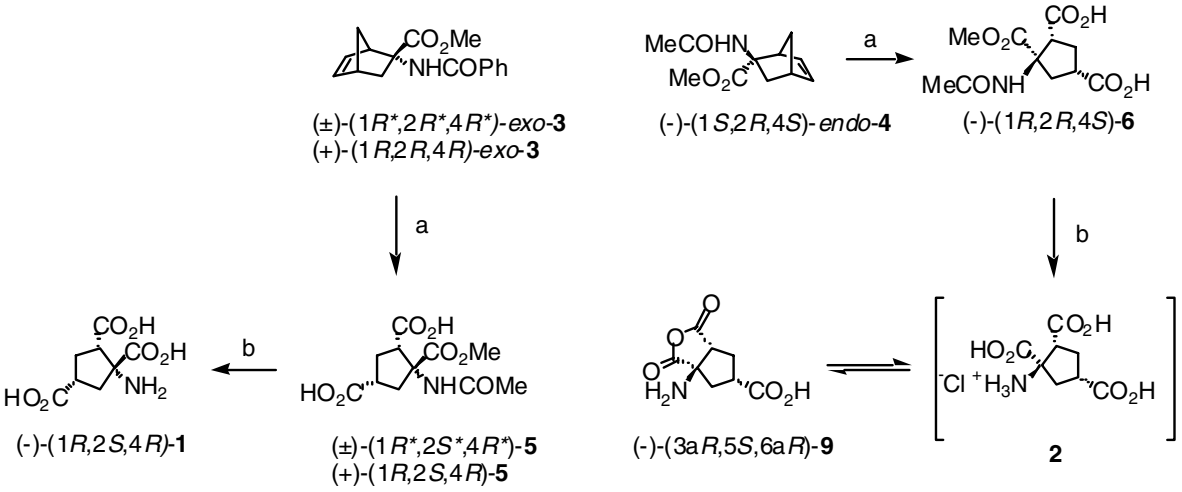
Finally, we focused our attention on the resolution of the cycloadducts **3** and **4** with previous hydrolysis of methyl ester function. Even if this class of amino acids has been known since the 1980s, only the chemical resolution of the corresponding 2-amino-bicyclo[2.2.1]heptane-2-carboxylic acid is reported in the literature.[16]

Racemic esters (±)-*exo*-**3** and (±)-*endo*-**4** were hydrolyzed into the corresponding acids (±)-*exo*-**7** (92%) and (±)-*endo*-**8** (86%) using KOH in EtOH. The (+)-(*R*)-1-phenylethylamine was selected to resolve the racemic mixture of each diastereomer because it is a commercially available cheap reagent. The reaction of (±)-*exo*-**7** with the amine in MeOH led to the formation of the corresponding diastereomeric salts which were separated by crystallization. The precipitated salt was collected and transformed, after acidification, into acid (−)-(1*S*,2*S*,4*S*)-*exo*-**7′** (yield: 37%). From the mother liquor, after acidification, acid (+)-(1*R*,2*R*,4*R*)-*exo*-**7** (yield: 31%) was isolated. Starting from (±)-*endo*-**8** and operating as described before, acid (−)-(1*S*,2*R*,4*S*)-*endo*-**8** (yield: 40%) was obtained as pure salt. Enantiomer (−)-(1*R*,2*S*,4*R*)-*endo*-**8′** (yield: 42%) was isolated from the mother liquor. Both acids (+)-(1*R*,2*R*,4*R*)-*exo*-**7** and (−)-(1*S*,2*R*,4*S*)-*endo*-**8** were transformed into the corresponding methyl esters (+)-(1*R*,2*R*,4*R*)-*exo*-**3** (98%) and (−)-(1*S*,2*R*,4*S*)-*endo*-**4** (85%), respectively, by reaction with thionyl chloride in methanol at reflux (Scheme 2).

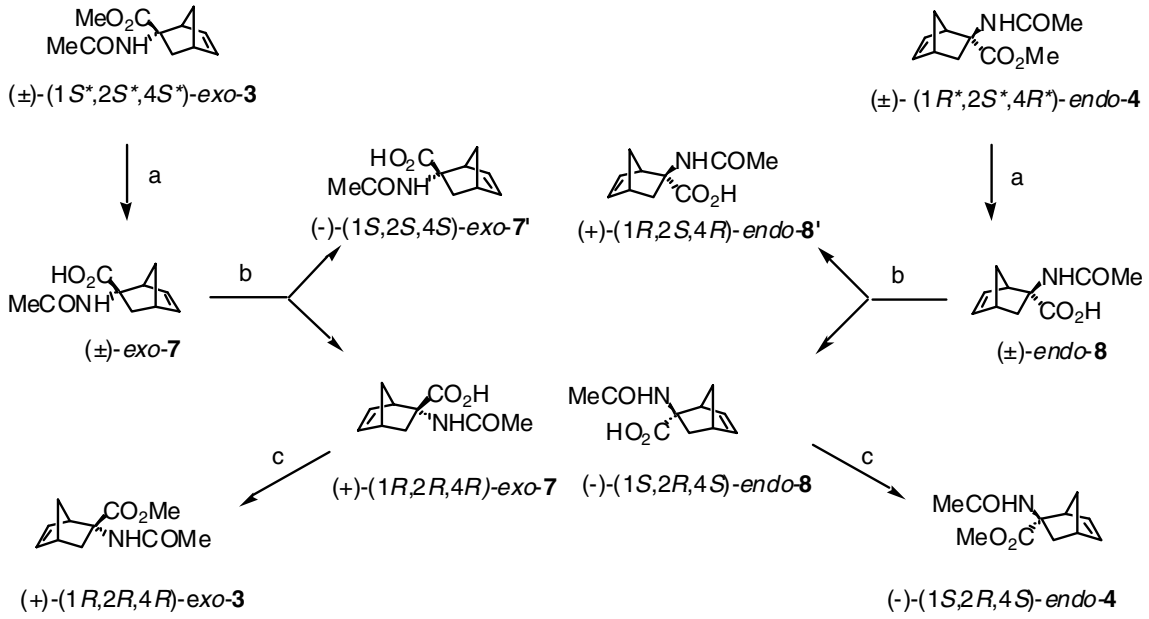
Following the above synthetic protocol, norbornene derivatives (+)-*exo*-**3** and (−)-*endo*-**4** were oxidized and cyclopentyltricarboxylic acid derivatives (+)-(1*R*,2*S*,4*R*)-**5** and (−)-(1*R*,2*R*,4*S*)-**6** were isolated in good yield (92% and 87%, respectively) (Scheme 1).

The hydrolysis of the protecting groups was achieved in acid conditions allowing the deprotection of both methyl ester and amide function (Scheme 1). Starting from (+)-**5** the deprotection was done with 6 N HCl at reflux for 14 h. Amino acid (−)-(1*R*,2*S*,4*R*)-**1** was isolated in 77% yield after purification on ionic exchange Dowex resin. From diastereomer (−)-**6**, amino acid **2** was first formed which was directly transformed into the anhydride (−)-(3a*R*,5*S*,6a*R*)-**9** (88%) when warming during the work-up.[14]

The formation of the anhydride was confirmed by IR spectroscopy of compounds **9** in which an absorption at 1713 cm^{-1} was present, corresponding to the carbonyl of an anhydride function. As expected, compound **9** was very hygroscopic. When a sample of **9** was not stored in a dry atmosphere and the IR spectrum then recorded, a new absorption at 1594 cm^{-1} was present instead of that at 1713 cm^{-1}, the same absorbtion observed in the IR spectrum of diastereomer **1**. We can conclude that the formation of anhydride **9** or of tricarboxylic acid **2** is strictly dependent on the presence or absence of moisture).



Scheme 1. Reagent and conditions: (a) $KMnO_4$, acetone, 0 °C; (b) 6 N HCl, reflux.



Scheme 2. Reagent and condition: (a) KOH, EtOH, reflux, then H_3O^+; (b) (+)-(*R*)-1-Phenylethylamine, MeOH, then H_3O^+; (c) $SOCl_2$, MeOH, reflux.

It is pointed out that during the hydrolysis process, epimerization was not observed as shown in ^{1}H NMR spectra (see Supporting Informations).

The whole synthetic procedure could be used to prepare isomers **1′** and **2′**.

The absolute configuration of each stereocenter of final amino acids was unequivocally determined using the $[\alpha]_D$ correlation between **1′**[14] and **1**, as well as between **2′**[14] and **2**.

3. Biological results

Each diastereomer, in the form of both pure enantiomers (−)-(1*R*,2*S*,4*R*)-**1**/(+)-(1*S*,2*R*,4*S*)-**1′** and (1*R*, 2*R*, 4*S*)-**2**/(1*S*, 2*S*, 4*R*)-**2′** (derived from anhydrides **9** and **9′** by hydrolysis in water solution), was tested on mGluR1 by mean PI hydrolysis assay, and on mGluR2 and mGluR6 by mean CAMP assay. Moreover the enantiomers were tested on 5-HT_{1A} and 5-HT_{2A} receptors and on serotonin transporter (SERT) using binding assays.

1-Aminocyclopentane-1,2,4-tricarboxylic acids were tested for agonist and antagonist activity on CHO cells expressing mGlu1 receptor in a PI hydrolysis assay. When tested in absence of agonist, the **1,1′** and **2,2′** stereoisomers did not change basal PI hydrolysis at concentration of 10 μM (Fig. 2a). Stimulation of PI hydrolysis by glutamate 30 μM was not inhibited by all enantiomers at a concentration of 10 μM (Fig. 2b).

When 1-aminocyclopentane-1,2,4-tricarboxylic acids were tested for agonist and antagonist activity on CHO expressing mGlu2 and mGlu6 receptors in a cAMP assay, it was found that **1**, and **2**, **2′** stereoisomers

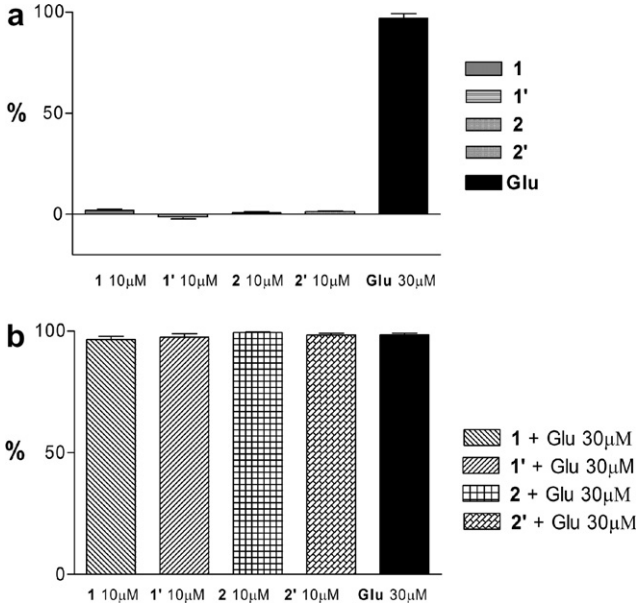


Figure 2. Effects of 10 μM stereoisomers as agonists on basal PI hydrolysis in CHO mGluR1 receptor. Data are expressed as percentage (mean ± S.E.M. (n = 6)) of activation on PI hydrolysis. Glutamate 30 μM represented the agonist standard (a). Effects of 10 μM stereoisomers as antagonists (b) on PI hydrolysis in CHO mGluR1 receptor. Data are expressed as percentage (mean ± S.E.M. (n = 6)) of inhibition on PI hydrolysis induced by 30 μM glutamate (b).

at 10 μM did not affect the forskolin-stimulated cAMP accumulation respect on standard, on both receptors (Figs. 3 and 4). Moreover, when applied alone, **1′** enantiomer showed an inhibitory effect (30% of cAMP decrease at 10 μM) at the human mGlu2 (Fig. 3) and mGlu6 receptors (Fig. 4). When tested as antagonists **1**, **1′**, and **2**, **2′** stereoisomers did not show a capacity to reverse agonists response (glutamate and AP4, respectively) (Figs. 5 and 6).

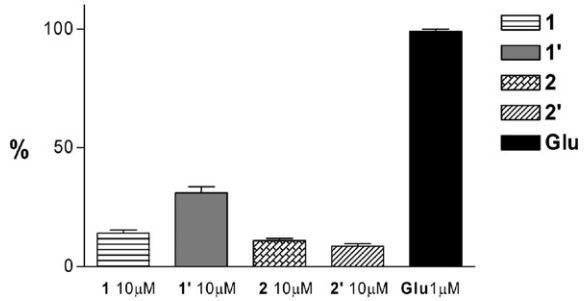


Figure 3. Effects of 10 μM stereoisomers as agonists on cAMP in CHO mGluR2 receptor. Data are expressed as percentage of cAMP decrease (mean ± S.E.M. (n = 6)) respect on standard.

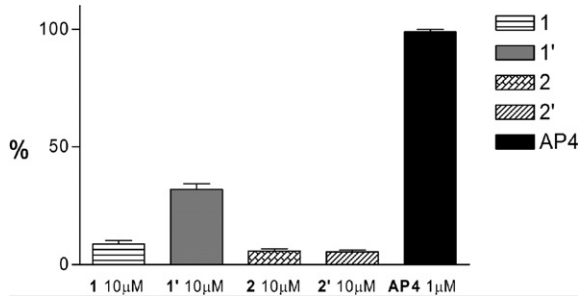


Figure 4. Effects of 10 μM stereoisomers as agonists on cAMP in CHO mGluR6 receptor. Data are expressed as percentage cAMP decrease (mean ± S.E.M. (n = 6)) respect on standard.

All isomers were tested on 5-HT_{1A} receptor, using [^{3}H]8-OH-DPAT binding on rat cortex membrane and on 5-HT_{2A} receptor, using [^{3}H]Ketanserin binding on rat cortex membrane. K_d and B_{max} values of 5-HT_{1A} receptors obtained on rat cortex membranes were 1.8 ± 0.21 nM and 140 ± 8.5 fmol/mg, respectively, and of 5-HT_{2A} receptors obtained on rat cortex membranes were 0.49 ± 0.09 nM and 490 ± 85 fmol/mg respectively. At 50 μM concentration all the compounds did not show a significant affinity for both receptors, in comparison to standard (Table 1).

Finally, the above compounds were tested on serotonin transporter (SERT), using [^{3}H]Paroxetine binding on platelets membrane. K_d and B_{max} values of 5-HT_{2A} receptors obtained on human platelet membranes were 0.08 ± 0.01 nM and 1260 ± 53.2 fmol/mg, respectively. Up to 50 μM concentration **1**, **1′** and **2** compounds did not showed a significant affinity for the transporter, in comparison to standard. (Table 1) The (1*R*,2*R*,4*S*)-**2** isomer evidenced a K_i of 10 μM (Table 1).

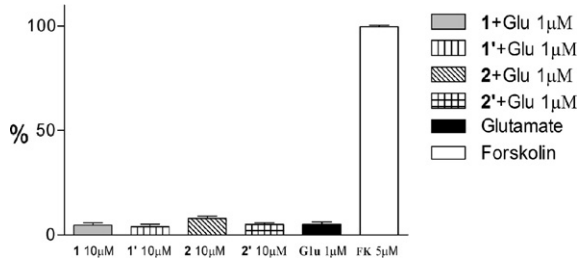


Figure 5. Effects of 10 μM stereoisomers as antagonists, in the presence of Glutamate 1 μM, on cAMP accumulation in CHO mGluR2 receptor (mean ± S.E.M. (n = 6)).

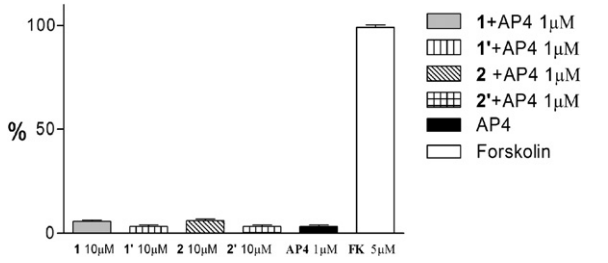


Figure 6. Effects of 10 μM stereoisomers, in the presence of AP4 1 μM, as antagonists on cAMP accumulation in CHO mGluR6 receptor (mean ± S.E.M. (n = 6)).

Table 1. K_i (nM) values of binding assay on 5-HT_{1A}, 5-HT_{2A} receptors and serotonin transporter (SERT) (n = 3)

Compound	5-HT_{1A} K_i (nM)	5-HT_{2A} K_i (nM)	SERT K_i (nM)
1	>50000	>50000	>50000
1′	>50000	>50000	>50000
2	>50000	>50000	10000
2′	>50000	>50000	>50000
8-OH-DPAT	0.64 ± 0.1	—	—
Ketanserin	—	0.49 ± 0.05	—
Paroxetine	—	—	0.09 ± 0.01

Based on the above data, we can conclude that agonist activities of **1, 1′** and **2, 2′** enantiomers observed at concentration of 10 μM were not significant at the human mGlu1R. Stimulation of PI hydrolysis by glutamate 30 μM was non inhibited by each enantiomer at a concentration of 10 μM. When applied alone at 10 μM, (+)-(1*S*,2*R*,4*S*)-**1′** enantiomer showed an agonist action (about 30% of maximal cAMP decrease) at the human mGlu2 and mGlu6 receptors. No significant antagonist effect was evidenced on both glutamate receptor subtypes. We therefore confirmed the impact of stereochemistry either on activity or selectivity of a compound toward metabotropic receptors.

Binding assays of the **1**,**1′** and **2**,**2′** isomers on 5-HT_{1A} receptors did not evidence activity at 50 μM. These screening results suggested that K_i of these compounds was over 50 μM. Moreover, all stereoisomers were tested on 5-HT_{2A} receptor, using [^{3}H]Ketanserin binding on rat cortex membranes and at 50 μM concentration all the compounds did not show a significant affinity for the receptor. At this concentration, **1**, **1′** and **2′** compounds did not show a significant affinity for the serotonin transporter too, except the (1*R*,2*R*,4*S*)-**2** isomer that showed a K_i of 10 μM.

4. Experimental

4.1. General methods

Mps (melting points) were determined using a Büchi 510 (capillary) apparatus. ^{1}H NMR spectra were recorded

with an AVANCE 500 Bruker at 500 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. Chemical shifts, relative to TMS as internal standard, are given in δ values. *J* are given in Hz. TLC: ready-to-use silica gel plates. Column chromatography: silica gel [Kieselgel 60-70 230 ASTM (Merck)] with the eluant indicated. IR spectra were taken with a Perkin-Elmer 1725X FT-IR spectrophotometer. $[\alpha]_D$ were measured with a Perkin-Elmer MODEL343 Plus Polarimeter. Dowex 50WX 4-50 and Dowex 1x4-400 column ion-exchange resins were used.

4.2. Methyl 2-acetylamino-bicyclo[2.2.1]hept-5-ene-2-carboxylates

Operating under nitrogen atmosphere, to a stirred solution of methyl 2-acetylaminoacrylate (5 g, 35 mmol) in anhydrous CH_2Cl_2 (200 mL), freshly distilled cyclopentadiene (12 mL, 140 mmol) and $MgClO_4$ (2.33 g, 10 mmol) were added. The reaction was sonicated for 14 h. After solvent evaporation, the reaction mixture was flash chromatographed on silica gel (CH_2Cl_2/AcOEt, 2:1). Cycloadducts *exo*-**3** (5 g, 68%) and *endo*-**4** (2.1 g, 29%) were obtained.

4.2.1. Methyl 2-acetylamino-bicyclo[2.2.1]hept-5-ene-2-carboxylate *exo*-3. Mp: 148 °C (AcOEt); Lit. 148–150 °C.[17]

4.2.2. Methyl 2-acetylamino-bicyclo[2.2.1]hept-5-ene-2-carboxylate *endo*-4. Mp: 131 °C (AcOEt); Lit. 131–133 °C).[17]

4.3. 2-Acetylamino-bicyclo[2.2.1]hept-5-ene-2-carboxylic acids

Operating in a screw-cap tube, to a stirred solution of (±)-*exo*-**3** (544 mg, 2.6 mmol) or (±)-*endo*-**4** (544 mg, 2.6 mmol) in EtOH (5 mL, 95%), KOH (292 mg, 5.2 mmol) was added. The reaction mixture was stirred and heated at 110 °C for 2 h. After solvent evaporation, the crude material was dissolved in water and was acidified to pH 5 with 2 N HCl. A solid was separated, filtered, washed with cold water, and dried. Acids (±)-*exo*-**7** (468 mg, 92%) or (±)-*endo*-**8** (436 mg, 86%) were isolated, respectively, as colorless crystals. Spectroscopic data are in agreement with the reported data.[19]

(±)-*exo*-**7**: mp 227 °C (EtOH). Lit: mp 224–226 °C.[18]

(±)-*endo*-**8**: mp 225 °C (EtOH). Lit: mp 221 °C.[18]

4.4. General procedure for acid separation

Acid (±)-*exo*-**7** (300 mg, 1.54 mmol) and (*R*)-α-methylbenzylamine (187 mg, 1.54 mmol) were stirred in methanol (20 mL) at room temperature. A white precipitate was separated immediately. The mixture was heated at reflux for 10 min and then cooled to 25 °C. The insoluble salt was filtered out and recrystallized twice in MeOH. The resulting solid was suspended in MeOH (10 mL) and treated with 2 N HCl (0.7 mL). After 30 min under stirring, the solution was evaporated under reduced pressure. The solid was suspended in water and the pH was adjusted to 5 with 1 N NaOH. A solid was separated, filtered, washed with cold water, and dried. A further recrystallization from absolute EtOH gave pure (−)-*exo*-**7′** (111 mg, 37%) as a white solid. The mother liquor derived from the separation of (−)-enantiomer was further cooled at 20 °C for 2 h, filtered, and evaporated. The resulting solid was treated as reported for (−)-*exo*-**7′** and acid (+)-*exo*-**7** (93 mg, 31%) was isolated as a white solid. Acid (±)-*endo*-**8** (300 mg, 1.54 mmol) were separated as reported for (±)-*exo*-**7**. Compound (−)-*endo*-**8** (120 mg, 40%) was isolated as a white solid. From the mother liquor, enantiomer (+)-*endo*-**8′** (129 mg, 42%) was isolated. Spectroscopic data are in agreement with the reported data.[19]

4.4.1. (1*S*,2*S*,4*S*)-2-Acetylamino-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid exo-7′. Mp: 224 °C (EtOH); $[\alpha]_D^{25}$ −102.3 (c 0.5, MeOH). Lit: $[\alpha]_D^{25}$ −106 (c 1.2, MeOH).[18]

4.4.2. (1*R*,2*R*,4*R*)-2-Acetylamino-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid *exo*-7. Mp: 220 °C (EtOH); $[\alpha]_D^{25}$ +101 (c 0.5, MeOH).

4.4.3. (1*S*,2*R*,4*S*)-2-acetylamino-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid *endo*-8. Mp: 215 °C (EtOH); $[\alpha]_D^{25}$ −148 (c 0.5, MeOH).

4.4.4. (1*R*,2*S*,4*R*)-2-acetylamino-bicyclo[2.2.1]hept-5-ene-2-carboxylic acid *endo*-8′. Mp: 206 °C (EtOH); $[\alpha]_D^{25}$ + 130 (c 0.5, MeOH); Lit.: $[\alpha]_D^{25}$ + 156 (c 1.14, MeOH).[18]

4.5. General procedure for the esterification reaction

A stirred solution of (+)-*exo*-**7** (161 mg, 0.8 mmol) or (−)-*endo*-**8** (161 mg, 0.8 mmol) in MeOH (5 mL) was cooled at 0 °C and thionyl chloride (0.072 mL, 1 mmol) in MeOH (2 mL) was added dropwise. The reaction mixture was left under stirring at room temperature for 20 h after which the solvent was evaporated and the crude material was dissolved in CH_2Cl_2 (5 mL) and washed with $NaHCO_3$. The organic layer was dried over Na_2SO_4 and evaporated under vacuum. After crystallization compounds (+)-*exo*-**3** (149 mg, 89%) or (−)-*endo*-**4** (142 mg, 85%) were isolated.

(+)-(1*R*,2*R*,4*R*)-*exo*-**3**: mp 140 °C (AcOEt); $[\alpha]_D^{25}$ + 95.0 (c 0.3, MeOH). Lit. (−) enantiomer: mp 132 °C; $[\alpha]_D^{25}$ −97.3 (c 1.79, MeOH).[18]

(−)-(1*S*,2*R*,4*S*)-*endo*-**4**: mp 125 °C (AcOEt); $[\alpha]_D^{25}$ −71.8 (c 0.5, MeOH). Lit. (+) enantiomer: mp 123 °C; $[\alpha]_D^{25}$ + 72.5 (c 1.275, MeOH).[18]

4.6. General procedure for the oxidation reaction

A solution of racemic (±)-*exo*-**3** or pure cycloadduct (+)-*exo*-**3** or (−)-*endo*-**4** (148 mg, 0.71 mmol) in acetone (10 mL) was added dropwise at 0 °C under stirring to a mixture of potassium permanganate (370 mg, 2.34 mmol) in water (2 mL). The temperature was maintained below 5 °C during the addition and then was allowed to rise at 25 °C. After 3 h, $Na_2S_2O_5$ (444 mg,

2.34 mmol) was added and the mixture was stirred for additional 20 min. The solution was carefully acidified to pH 2 with HCl (37%). After solvent evaporation, the crude reaction mixture was dissolved in degased water (5 mL) and the pH was brought to 9 with NaOH (1M). The resulting precipitate was removed by filtration and the clear solution was loaded on a Dowex 1x4-400 column (AcO^-, 200–400 mesh, 1×10 cm). The resin was rinsed with degased water (100 mL) and the compound was eluted with AcOH (2M). Evaporation of the AcOH solution gave the desired compound. Racemic tricarboxylic derivative (±)-*exo*-**5** (180 mg, 93%) was isolated from (±)-*exo*-**3**. Compound (+)-**5** (178 mg, 92%) or (−)-**6** (170 mg, 87%) was obtained from (+)-*exo*-**3** and (−)-*endo*-**4**, respectively.

4.6.1. (1*R*,2*S*,4*R*) Methyl 1-acetylaminocyclopentane-1,2,4-tricarboxylate (+)-5. Mp: 145 °C (THF). $[\alpha]_D^{25}$ + 29.5 (c 0.34, MeOH); IR (Nujol) 3500–3000, 1727, 1630 cm^{-1}; ^1H NMR (CD_3OD) δ 3.70 (s, 3H), 3.05-2.85 (m, 2H), 2.71–2.60 (m, 1H), 2.60–2.50 (m, 1H), 2.40–2.31 (m, 2H), 1.95 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 177.5, 174.7, 172.4, 171.6, 66.9, 54.1, 52.0, 42.5, 39.2, 33.5, 21.9. Anal. Calcd for $C_{11}H_{15}NO_7$: C, 48.35; H, 5.53; N, 5.13. Found: C, 48.28; H, 5.70; N, 5.06.

4.6.2. (1*R*,2*R*,4*S*) Methyl 1-acetylaminocyclopentane-1,2,4-tricarboxylate (−)-6. Mp: 197 °C (THF). $[\alpha]_D^{25}$ −17 (c 0.26, MeOH); IR (Nujol) 3500–3000, 1738, 1620 cm^{-1}; ^1H NMR (D_2O) δ 3.67 (s, 3H), 3.36–3.27 (m, 1H), 3.20–3.00 (m, 1H), 2.63 (dd, *J* 14.0, 9.9, 1H), 2.43-2.29 (m, 3H), 1.98 (s, 3H); ^{13}C NMR (D_2O) δ 176.3, 173.6, 172.3, 172.2, 67.5, 52.9, 51.8, 41.5, 39.4, 31.7, 21.5. Anal. Calcd for $C_{11}H_{15}NO_7$: C, 48.35; H, 5.53; N, 5.13. Found: C, 48.40; H, 5.41; N, 5.20.

4.7. 1-Aminocyclopentane-1,2,4-tricarboxylic acids

Operating in a screw-cap tube, a solution of (+)-**5** (160 mg, 0.58 mmol) or (−)-**6** (140 mg, 0.51 mmol) in 6 N HCl (1 mL) was stirred and heated at 120 °C for 14 h. HCl was removed by evaporation and the crude material was dissolved in water (10 mL). The resulting solution was adjusted to pH 4 with 1 N NaOH and deposited on a Dowex 50×4 column (H^+, 20–50 mesh, 1×10 cm). The resin was rinsed with water and the amino acid was eluted with aqueous NH_4OH (0.5 M). Ninhydrin positive fractions (TLC CH_2Cl_2/MeOH/NH_4OH(15%) = 5:3:0.9) were pooled and evaporated. Amino acid (−)-**1** (97 mg, 77%) and (−)-**9** (90 mg, 88%) were obtained from (+)-**5** and (−)-**6** as a solid, respectively. Spectroscopic data are in agreement with those reported in the literature.[14]

4.7.1. (1*R*,2*S*,4*R*)-1-Aminocyclopentane-1,2,4-tricarboxylic acids (−)-1. $[\alpha]_D^{25}$ −3.3 (c 0.27, H_2O); (+)-**1′**: $[\alpha]_D^{25}$ + 3.1 (c 0.26, H_2O).[14]

4.7.2. (1*R*,2*R*,4*S*)-1-Aminocyclopentane-1,2,4-tricarboxylic acid (−)-2. $[\alpha]_D^{25}$ −6.1 (c 0.29, H_2O). Lit.: (+)-**2:** $[\alpha]_D^{25}$ +6.7 (c 0.25, H_2O).[14] The [α] value was taken starting from **9** which in water solution was transformed into **2**.

5. Biological assay

5.1. PI hydrolysis (phospholipase C-coupled mGlu receptors)

Cells, cultured in 96-well plates to confluency (3–4 days), were incubated overnight in glutamine-free culture medium supplemented with 0.5 μCi *myo*-[^{3}H]inositol (NEN) to label the cell membrane phosphoinositides. After washing the cultures, incubations with new compounds (10 μM) were carried out for 45 min at 37 °C in Locke's buffer (156 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.6 mM Glucose and 20 mM Hepes, pH 7.4) containing 20 mM LiCl to block the degradation of inositol phosphates (IPs). The reaction was terminated by aspiration, and IPs were extracted with 0.1 M HCl. Antagonist action of new compounds was measured as percentage of inhibition on PI stimulation induced by 30 μM glutamate. Agonist action was measured as percentage of activation on basal PI hydrolysis. The separation of [^{3}H] IPs was performed by anion exchange chromatography. This assay was used with CHO cells expressing mGluR1 (with 30 μM glutamate) receptors. This cell line was created by co-transfection of a chimeric G-protein (Gqi9) which allowed the coupling of these receptors to phospholipase C and the measuring of PI hydrolysis.

5.2. Measurements of cAMP formation (mGlu receptor negatively coupled with adenylyl-cyclase)

These assays involved mGluR2 and mGluR6 receptors expressed in CHO cells. The activity of agonists on these receptors was determined by measurements of their ability to decrease the forskolin-induced elevation of cyclic AMP formation. Cells cultured in 96-well culture plates were preincubated for 10 min at 37 °C in Locke's medium containing 300 μM *iso*-butylmethylxanthine to inhibit the activity of phosphodiesterases which degrade cAMP. About 5 μM forskolin was added, with or without mGluR new ligands (10 μM), and the incubation was performed for 10 min. After the incubation, the medium was rapidly aspirated and cAMP was extracted with 0.1 M HCl. cAMP was measured by radioimmunoassay, using a magnetic Amerlex RIA kit (Amersham). These studies are performed in the presence and in absence of standard agonists 1 μM glutamate for mGluR2 and 0.5 μM-1 μM of 4-aminophosphonbutyrate (AP4) for mGluR6.

5.3. Rat cortex membrane preparation

Cerebral cortex was dissected from rat brain, and the tissues were homogenized in 10 volumes of ice-cold 50 mM Tris–HCl buffer at pH 7.4. The homogenate was centrifuged at 48,000*g* for 15 min at 4 °C. The resulting pellet was suspended in 35 volumes of 50 mM Tris–HCl buffer (T1), incubated at 37 °C for 10 min to remove endogenous 5-HT, and successively centrifuged at 48,000*g* for 15 min at 4 °C. The obtained pellet was frozen at −80 °C. Protein concentration was determined according to the method of Lowry et al.[20] using BSA as standard.

5.4. 5-HT$_{1A}$ receptor binding

K_d and B_{max} of [^{3}H]-8-OH-DPAT (Perkin-Elmer Life Science, a.s. 106 Ci/mmol) were determinated using saturation curve with crescent concentrations (0.1–20 nM) of radioligand. Membranes prepared with methods previously described were suspended in T1 buffer and homogenized with ultraturrax. Suspension (0.3–0.4 mg of proteins) was incubated with [^{3}H]-8-OH-DPAT at 37 °C for 30 min in a final volume of 1 mL. Aspecific bound was determinated in the presence of 8-OH-DPAT 10 μM. The binding reaction was stopped by filtration through Whatman GF/C glass fiber filters, under reduced pressure. Kinetic parameters at equilibrium were obtained using Scatchard analysis.

5.5. 5-HT$_{1A}$ receptor binding in the presence of new compounds

Binding assay was performed according to the method of Dalpiaz et al.[21] incubating aliquots of membrane fractions (0.2–0.3 mg of proteins/tube) in T1 buffer, with 0.5 nM [3H]-8-OH-DPAT in a final volume of 1 mL. Non-specific bind was determined in the presence of 10 μM 8-OH-DPAT non-labeled. New compounds were dissolved in buffer and added to assay mixture. The inhibition of specific binding was determinated in the presence of a single concentration (50 μM) of each enantiomer. Incubation was carried out at 37 °C for 30 min. The binding reaction was stopped by filtration through Whatman GF/C glass fiber filters, under reduced pressure. Filtrates were washed four times with 5-ml aliquots of ice-cold buffer and placed in pony vials with 4 mL of scintillation liquid. Specific binding was obtained by subtracting non-specific binding from total binding.

5.6. 5-HT$_{2A}$ receptor binding

K_d and B_{max} of [^{3}H]-Ketanserin (Perkin-Elmer Life Science, a.s. 77.2 Ci/mmol) were determinated using saturation curve with crescent concentrations (0.01–1.2 nM) of radioligand. Membranes prepared with methodic previously described, were suspended in T1 buffer and homogenized with ultraturrax. Suspension (0.3–0.4 mg of proteins) was incubated with [^{3}H]-Ketanserin at 37 °C for 15 min in a final volume of 1ml. Aspecific bound was determinated in the presence of Spiperon 10 μM. The binding reaction was stopped by filtration through Whatman GF/C glass fiber filters, under reduced pressure. Kinetic parameters at equilibrium were obtained using Scatchard analysis.

5.7. 5-HT$_{2A}$ receptor binding in the presence of new compounds

Binding assay was performed according to the method of Leysen et al.,[22] incubating aliquots of membrane fractions (0.2–0.3 mg of proteins) in Tris–HCl buffer 50 mM pH 7.4, with 0.4 nM [^{3}H]Ketanserin in a final volume of 1 ml. Non-specific bind was determined in the presence of 10 μM Spiperon non-labeled. New compounds were dissolved in buffer and added to assay mixture. The inhibition of specific binding was determined in the presence of a single concentration (50 μM) of each enantiomer. Incubation was carried out at 37 °C for 15 min. The binding reaction was stopped by filtration through Whatman GF/C glass fiber filters, under reduced pressure. Filtrates were washed four times with 5 mL aliquots of ice-cold buffer and placed in pony vials with 4 mL of scintillation liquid. Specific bound was measured as described above.

5.8. Platelets separation

Platelet-rich plasma (PRP) was obtained by centrifugation at 200*g* for 10 min at 4 °C of blood recruited from fasting healthy volunteers, between 8 and 9 a.m., in order to avoid the possible interference of circadian rhythms. Platelets were precipitated by centrifugation of PRP at 1000*g* for 15 min at 4 °C and the pellets were then stored at −80 °C until the assay, which was performed within a week. Protein concentration was determined according to the method of Lowry et al.[20] using BSA as standard.

5.9. Platelet membranes preparation

Pellets were lysed and homogenized in 10 mL buffer (5 mM Tris–HCl, pH 7.4, containing protease inhibitors: 200 μg/mL Bacitracine, 160 μg/mL Benzamidine, 20 μg/ml Trypsin inhibitor) with an Ultraturrax homogenizer for few minutes at low speed and centrifuged twice at 48,000*g* for 15 min at 4 °C. The resulting pellets were resuspended in ice-cold 50 mM Tris–HCl buffer, pH 7.4, and centrifuged at 48,000*g* for 15 min at 4 °C.

5.10. SERT binding assay to platelet membranes.

K_d and B_{max} of [^{3}H]Paroxetine (Perkin-Elmer Life Science, a.s. 24.4 Ci/mmol) were determinated using saturation curve with crescent concentrations (0.01–1.5 nM) of radioligand. Membranes prepared with methodic previously described, were suspended in assay buffer (50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, pH 7.4) and homogenized with ultraturrax. Suspension (0.05–0.1 mg proteins/tubes) was incubated with [^{3}H]Paroxetine at 22 °C for 60 min in a final volume of 2 mL. Aspecific bound was determinated in the presence of Fluoxetine 10 μM. The binding reaction was stopped by filtration through Whatman GF/C glass fiber filters, under reduced pressure. Kinetic parameters at equilibrium were obtained using Scatchard analysis.

5.11. SERT binding assay to platelet membranes in the presence of new compounds

The incubation mixture consisted of 100 μL of platelet membranes (0.05–0.1 mg proteins/tube), [^{3}H]Paroxetine 0.1 nM, in 1 mL of final volume. Specific binding was obtained as the binding remaining in the presence of 10 μM Fluoxetine as a displacer. New compounds were dissolved in buffer and added to assay mixture. The inhibition of specific binding was determined in the presence of a single concentration (50 μM) of each enantiomers. All samples were assayed in duplicate and incubated at

22 °C for 1 h. The incubation was halted while adding 5 mL of cold assay buffer. The content of the tubes was immediately filtered under vacuum through glass fibre filters GF/C and washed three times with 5 mL of assay buffer using Brandel cell harvester. Filters were then placed in vials with 4 mL of scintillation cocktail, and radioactivity was measured by means of a beta-counter (Packard LS 1600).

Acknowledgments

We thank Cariplo Foundation for financial support and Psychoactive Drug Screening Program (Case Western Reserve University, Cleveland, USA) for performing screening on mGlu receptors.

Supplementary data

Spectroscopic and analytical data for compounds *exo*-**3**, *endo*-**4**, *exo*-**7**/**7′**, *endo***8**/**8′**, (−)-**1** and (−)-**9**. ^{1}H NMR spectra of **1** and of mixture **2**,**9**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.09.004.

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