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Aminobicyclo[2.2.1.]heptane dicarboxylic acids (ABHD), rigid analogs of ACPD and glutamic acid: synthesis and pharmacological activity on metabotropic receptors mGluR1 and mGluR2

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Abstract

Isomeric norbornane-derived rigid analogs mimicking different potential conformations of ACPD (1-aminocyclopentane-1,3-dicarboxylic acid) and glutamic acid have been synthesized, via the hydantoin route, to be used as conformational probes for bioactive conformations at the glutamatergic receptors of the central nervous system. Activities on metabotropic receptors mGluR1 and mGluR2 are reported and discussed.

Keywords: Norbornane analog, aminocyclopentane-1,3-dicarboxylic acid (ACPD), glutamic acid, conformation, metabotropic receptors.

1. Introduction

L-Glutamate, the neurotransmitter of the vast majority of fast excitatory synapses in the central nervous system plays an important role in synaptic plasticity [1], neurotoxicity and probably in some neurodegenerative disorders [2]. Glutamate binds to its receptor protein via its three ionisable groups, namely α-amino, α- and γ-carboxylates, through hydrogen bondings [3,4] or electrostatic interactions. Glutamic acid is a flexible molecule and various conformations corresponding to different spatial disposition of these three groups may define different bioactive conformations that activate each single type of glutamate receptors [5].

Five receptor classes [6] have been identified and named according to their selective agonists which all display three ionisable functions: NMDA (N-methyl-D-aspartic acid), AMPA (L-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid), KA (kainic acid), *trans*-ACPD (1*S*,3*R*-1-aminocyclopentane-1,3-dicarboxylic acid), and L-AP4 (L-2-amino-4-phosphonobutyric acid). Whereas the NMDA, AMPA, and kainate receptors are ligand-gated channels, the *trans*-ACPD and L-AP4 receptors are coupled to second messenger pathways via the activation of G-proteins and also called metabotropic receptors or mGluRs [7,8].

The *trans*-ACPD class is constituted of the 4 mGluRs activated by *trans*-ACPD but not L-AP4 and that have been further subdivided in two groups according to their transduction mechanism and pharmacological profile [7,8]. Members of the Group-I are mGluR1 and

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L-*trans*-ACPD (1*S*,3*R*) L-*cis*-ACPD (1*S*,3*S*)

Fig. 1. Structures of 1-aminocyclopentane 1,3-dicarboxylic acids.

mGluR5 that activate PLC, whereas members of Group-II are linked to adenyl-cyclase inhibition, and include mGluR2 and mGluR3. (1*S*,3*R*)-ACPD or L-*trans*-isomer (Fig. 1) is a selective agonist of group-I and -II with EC_{50} of 9 μM for mGluR1 and 20 μM for mGluR2, whereas (1*S*,3*S*)-ACPD or L-*cis*-isomer (Fig. 1) is a weak agonist of mGluR1 ($EC_{50} > 300\,\mu M$) and a good agonist of mGluR2 ($EC_{50} = 13\,\mu M$) (Table 1) [9,10]. The L-AP4 class corresponds to the group-III mGluRs, and is constituted of 4 receptors (mGluR4, mGluR6-8) selectively activated by L-AP4, and not or poorly by *trans*-ACPD [8].

In a recent study by NMR and molecular modeling [11], these two isomers of ACPD have been shown to be rather flexible. They can adopt several envelop conformations which mimic four glutamic acid conformations named A to D, which are characterized by two distances d_1 (α-amino-γ-carboxyl) and d_2 (α-carboxyl-γ-carboxyl) (Figs 2 and 3). *trans*-ACPD can adopt conformations of A, B, and C families, whereas *cis*-ACPD can only adopt conformations of B and D families [11,12].

It can be speculated that ACPD isomers bind to the receptors in a prior conformation and that a different geometry is then induced, due to interactions with specific residues of the receptor protein. In order to gain more insight into the bound conformation(s), we have locked the four possible conformation families of ACPD in completely rigid analogs, namely amino bicyclo[2.2.1]heptane dicarboxylic acids (ABHDs) [13]. Each conformational family is represented by a pair of isomers: ABHD-IV and -VIII for family A (characterized by minimal d_1), ABHD-II and -VI for family B (characterized by maximal d_2), ABHD-I and -V for family C (characterized by maximal d_1), ABHD-III and -VII for family D (characterized by minimal d_2) (Figs 2 and 3). Racemic mixtures of each ABHD isomer display the same distances d_1 and d_2 with the three ionisable groups in the same relative spatial disposition but with a different steric environment and different dihedral angles χ_1, χ_2. Thus each conformational family is composed of four ABHD stereomers. This is illustrated in Fig. 4 with conformational family C [14].

As mentioned above the ABHD isomers can be considered as combined conformational and topological probes. Several other amino acids have been previously constrained in a norbornyl structure for the study of enzyme mechanisms in which they are involved [15–27]. For example, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid, which mimics leucine, isoleucine or valine, has allowed a discrimination among the transport systems of neutral amino acids [19,28–31].

In this paper we present the synthesis and pharmacological activities of racemic mixtures of seven of the ABHD isomers on mGluR1 and mGluR2 as representatives of pharmacological groups-I and -II

Table 1
Activities of ACPD and (±) ABHD isomers on mGluR1 and mGluR2

Effector[a]	Conformational family[b]	Activity on mGluR1a	Activity on mGluR2
(1*S*,3*R*)-ACPD	A,B,C	9[c]	20[c]
(1*S*,3*S*)-ACPD	B,D	> 300[c]	13[c]
(1*R*,3*S*)-ACPD	A,B,C	128[c]	100[c]
(1*R*,3*R*)-ACPD	B,D	inactive	⩾1000
ABHD-I[d]	C	antagonist (Kb 300 μM)	agonist
ABHD-V	C	agonist	antagonist
ABHD-II[d]	B	partial agonist	agonist
ABHD-VI[d]	B	antagonist	agonist
ABHD-III	D	—	—
ABHD-VII	D	antagonist	inactive
ABHD-IV	A	antagonist	noncompetitve antagonist
ABHD-VIII	A	antagonist	inactive

[a](±) ABHD tested at 3 mM concentration.
[b]Conformational families defined according to d_1 and d_2 distances in glutamic acid [11].
[c]Agonist EC_{50} (μM) [9,10].
[d]No agonist activity was detected with mGluR4 representing pharmacological group III.

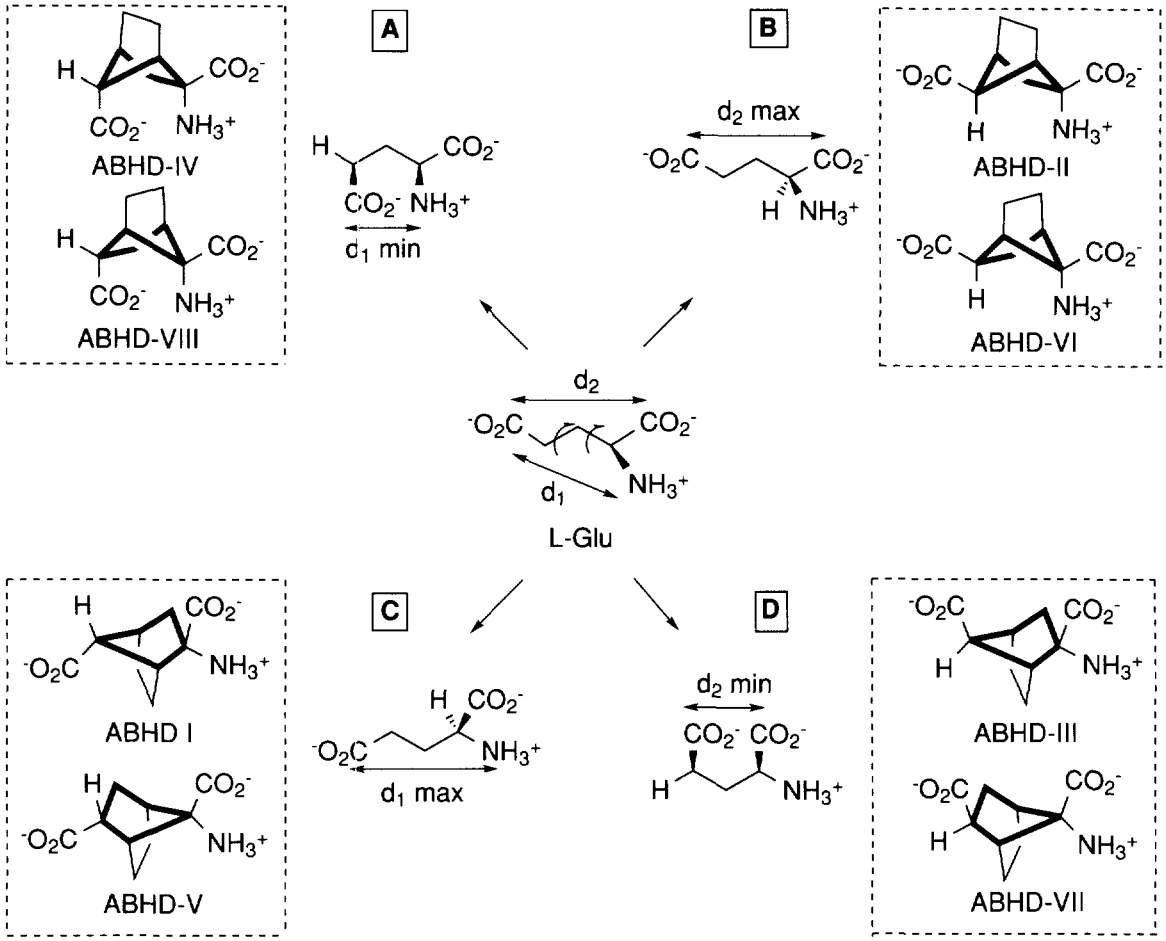


Fig. 2. Structures of ABHD I-VIII displaying the four conformational families A–D related to glutamic acid and ACPD conformations. ACPDs included in the ABHD structures are drawn in bold lines. Only L-isomers are represented.

trans-ACPD receptors. These receptors where chosen because of the complete characterization of their pharmacology [8], allowing a better comparison of the properties of the ABHD molecules with those of well characterized drugs. Conformational family(ies) of the receptor bound glutamate will be tentatively deduced therefrom.

2. Results

2.1 Chemistry

The methodology used to obtain these amino acids analogs was based on a classical hydantoin formation according to Bucherer-Bergs' method [19,32], from adequate oxocarboxylic acids. The amino acids were then obtained by acidic or alkaline hydrolysis of the spirohydantoins and separated by ion exchange

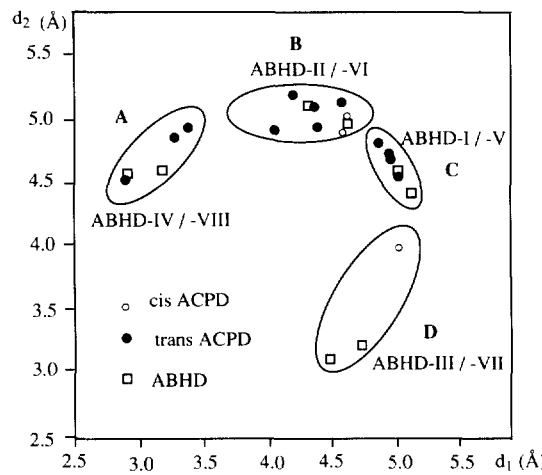


Fig. 3. Conformational families A–D (defined by distances d_1, d_2) including *cis* and *trans* ACPD and ABHD I-VIII.

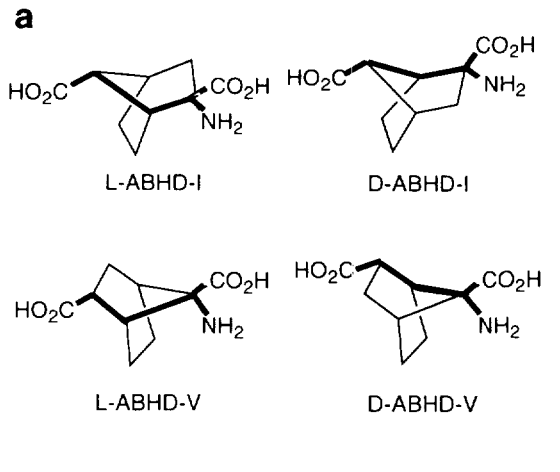


Fig. 4. (a) The four ABHD stereomers belonging to conformational family C and (b) the superposition of their three functional groups. Glutamic acid included in the ABHD structure is drawn in bold lines, hydrogens have been removed for clarity and atoms that have been superposed are starred [14].

chromatography. The relative stereochemistry of amino and carboxyl groups was deduced from lactam formation or from NMR nOe experiments, and from reciprocal interconversions, making use of the characteristic acid-catalyzed epimerization of the distal carboxyl-bearing carbon atom, previously described in γ-alkyl glutamic acids [33–36]. A prerequisite for such syntheses was a convenient preparation of the corresponding carboxy-substituted norbornanones **1–4** (Fig. 5).

Oxoacids **1** and **2** were prepared according to the literature [37–39]. Oxoacid **3** was obtained (see experimental procedure) starting from the Diels-Alder reaction of 2,3,4,5-tetrachloro-1,1-dimethoxycyclopentadiene with acrylic acid [40,41]. This procedure was stereospecific, as previously shown [40], and thus did not allow to obtain the diastereomeric oxoacid **4**, precursor of ABHD-VII and -VIII. Nevertheless, an epimerization of the distal carboxyl-bearing position, at the subsequent amino acid level, made their preparation possible (see below). The Bucherer-Bergs' reaction has been previously used for the synthesis of norbornyl derived amino acids [19–21,25,26]. In all cases, the amino acid resulting from an exo attack of cyanide on the (substituted) norbornanone was obtained as a major [19,21,26] or unique [20,25] compound. However, if steric hindrance and conformational features of the starting ketone have been put forward to predict the product stereochemistry, there are no existing data about the possible role of charged substituent groups on the ketone. Thus the assignement of the relative configuration of the newly introduced amino and carboxy groups, with regards to the preexisting carboxylic group of the ketone, will be a major concern, including the possible epimerization reaction during hydantoins hydrolysis to amino acids.

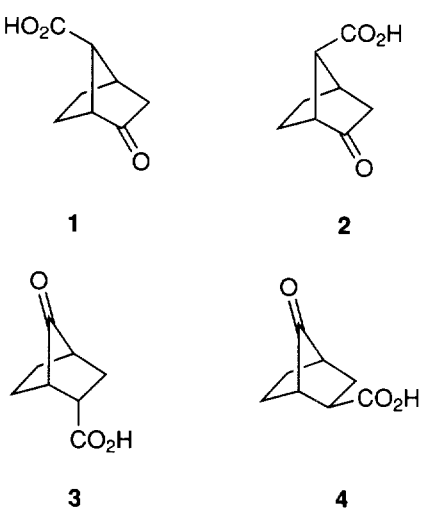


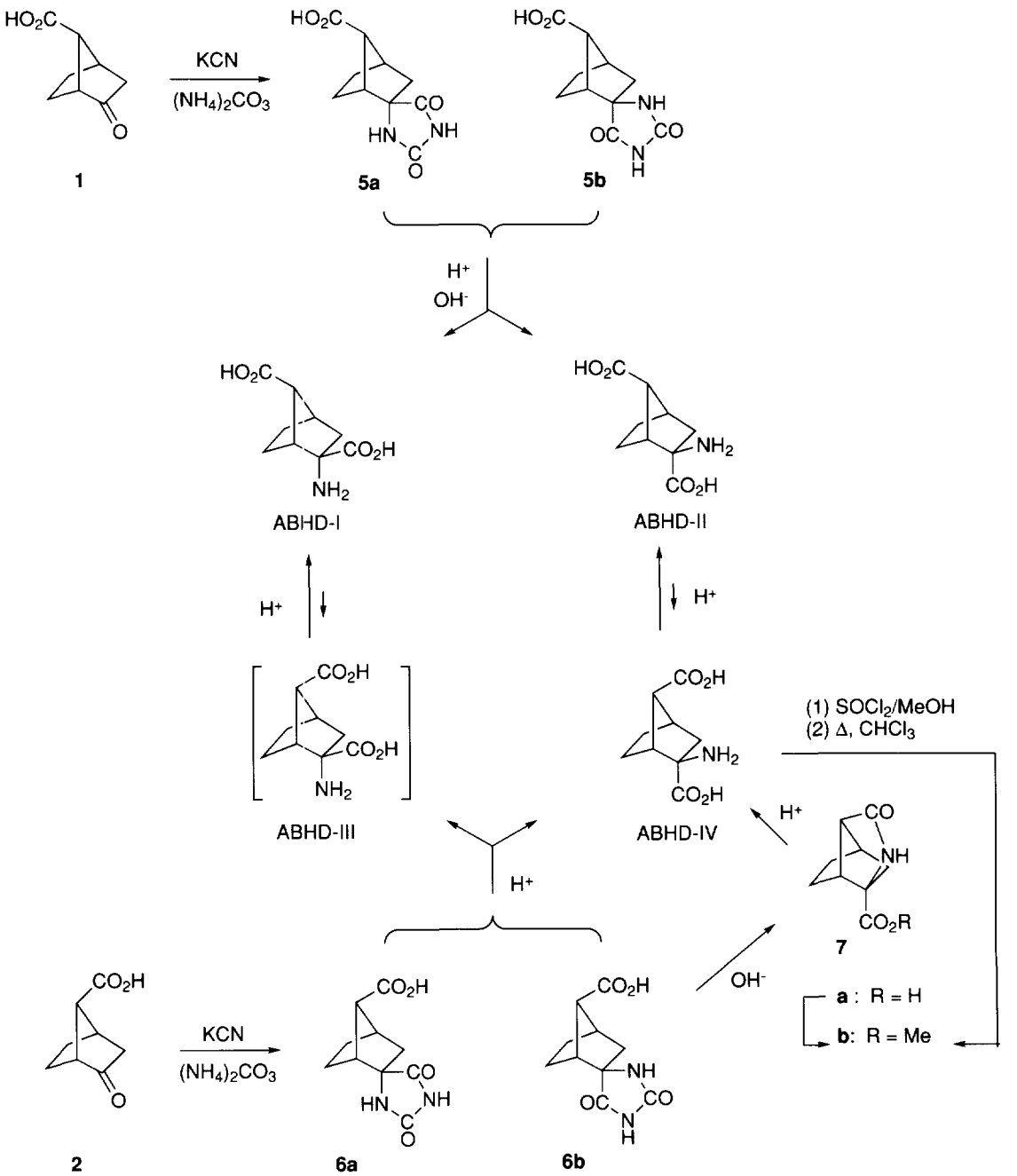
Fig. 5. Carboxy-substituted norbornanones **1–4** required for hydantoin synthesis.

Oxoacid **1** led to isomeric hydantoins **5a** and **5b** (Scheme 1) in a 9:1 ratio deduced from ^{1}H NMR spectrum. This ratio resulted from the major attack of cyanide on the exo side of the norbornane ring, as described above. Acidic or alkaline hydrolysis of the mixture afforded diastereomeric ABHD-I and ABHD-II in the same 9:1 ratio.

From oxoacid **2** was obtained a mixture of hydantoins **6a** and **6b** (Scheme 1) in a 5:95 ratio. In this case, the *exo* approach of cyanide was prevented by steric hindrance and electrostatic repulsion of the negatively charged carboxylate substituent of **2**. Thus reaction occurred mostly from the *endo* side at a slower rate (48 h compared to 5 h for **1**). Acidic hydrolysis afforded mainly ABHD-IV and small amounts of ABHD-I and ABHD-II (90:5:5) (Scheme 1). The latter amino acids resulted from an acidic epimerization of ABHD-III and -IV, respectively. Alkaline hydrolysis (which is not prone to epimerization) of the (**6a** + **6b**) mixture afforded mainly the lactam **7a** and a small amount of ABHD-III (95:5), which could not be isolated and rapidly epimerized upon workup to ABHD-I. The lactam was characterized as its methyl ester **7b** by ^{1}H and ^{13}C NMR, MS and

IR spectra (see Experimental). The crude alkaline hydrolysis mixture could also be converted to ABHD-IV by mild acidic hydrolysis (HCl, 2 N, 8 h, 100°C), together with small amounts of ABHD-I and -II, in the same 90:5:5 ratio that was obtained by direct acidic hydrolysis of hydantoins **6**. The relative stereochemistry of ABHD-I to -IV was established from the structure of lactam **7** which could be hydrolyzed to ABHD-IV, which in turn could be epimerized to ABHD-II, while ABHD-I, the major isomer of the Bucherer-Bergs' reaction on **1**, could be epimerized to ABHD-III (see below).

Oxoacid **3** gave a 1:1 mixture of hydantoins **8a** and **8b** which were completely destroyed by HCl hydrolysis. Alkaline hydrolysis afforded ABHD-V and -VI (Scheme 2). Their structure assignment was obtained from NOE

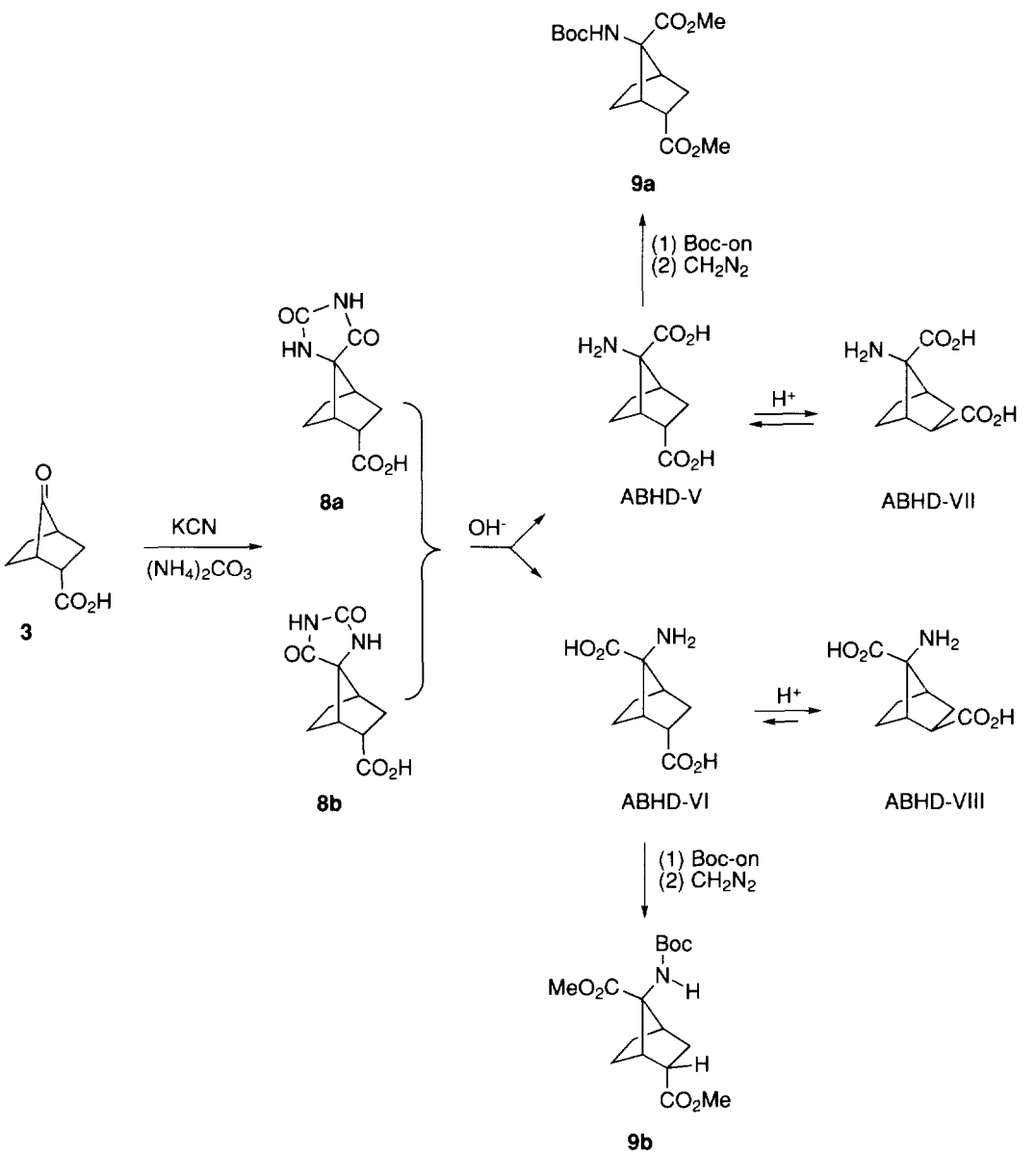


Scheme 1. Synthesis of ABHD-I to -IV.

measurements, after derivatization to the corresponding N-Boc-dimethyl esters **9a** and **9b**: a significant NOE effect between the C-2 and N-bound protons could be observed with the ABHD-VI derivative; no such effect was observed with the ABHD-V derivative (Scheme 2). Slow acidic epimerization (see below) of ABHD-V and -VI in 25% H_2SO_4 at 120°C afforded ABHD-VII and -VIII, respectively.

All obtained amino acids were purified and completely separated by ion exchange chromatography. They were then characterized by ^1H and ^{13}C NMR, GC-MS, and HPLC after derivatization [42,43]. An analytical resolution of four of the eight ABHD racemates (ABHD-II, -III, -V, and -VII) was obtained by HPLC using the chiral OPA-NAcCysteine adduct previously described [43].

The Bucherer-Bergs' synthesis from oxoacids **1–3** afforded different amounts of ABHD isomers. Nevertheless, we anticipated that yields of the minor ones should be increased by acidic epimerization of the major ones. Therefore, epimerization of ABHD-I, -IV, -V, and -VI was monitored by ^1H NMR. Samples of ABHDs (5 mg) were dissolved in a concentrated H_2SO_4:D_2O (1:3) solution and warmed at 120°C in order to follow



Scheme 2. Synthesis of ABHD-V to -VIII.

both the rate of the exchange of the hydrogen atom adjacent to the carboxylic group, and the rate of the epimerization reaction. In the case of ABHD-V and ABHD-VI, the deuterium exchange reaction was fast and completed in 24 h, while the epimerization equilibrium was only reached in 8 days with the following ratios: ABHD-V/ABHD-VII = 50/50 and ABHD-VI/ABHD-VIII = 25/75. In the case of ABHD-I and -IV, two months were necessary to reach full exchange. Epimerization of ABHD-I into ABHD-III was characterized by the appearance of a broad singlet at 3.12 ppm in the ^{1}H NMR spectrum of the mixture. Small amounts of ABHD-III were rapidly (3 days) detected but after 3 months the ABHD-I/ABHD-III ratio remains only 9/1. In the case of ABHD-IV, the ABHD-IV/ABHD-II ratio was 65:35 after 3 months but epimerization equilibrium was not reached. These data justified the use of epimerization reactions for the preparation of the ABHD-V to -VIII series, but not in the ABHD-I to -IV series.

2.2 *Biological results*

The effects of ABHD-I, -II and -IV to -VIII were examined on mGluR1 and mGluR2, the representatives of group-I and group-II mGluRs, respectively. These receptors were transiently expressed in HEK-293 cells as previously described [9] and the total inositol phosphate production resulting from the receptor activation were determined. Since mGluR2 is not normally coupled to PLC but rather inhibit adenylyl cyclase, this coupling was made possible by co-expressing this receptor with the chimeric G-protein alpha subunit Gαqi [9,44]. We previously reported that this assay gave more accurate results than the classical measurement of the inhibition of the forskolin-activated adenylyl cyclase activity, and that the pharmacology of mGluR2 determined using this assay was identical to that reported by others [9].

2.2.1 *mGluR1*

None of the ABHD molecules tested had potent agonist activity at this mGluR subtype (Fig. 6(a)). When tested at 3 mM, only ABHD-II and ABHD-V significantly increased IP production in mGluR1 expressing cells. However, this effect was much smaller than the maximal effect obtained with Glu. Although small, the stimulatory activities of ABHD-II and -V likely resulted from a direct activation of mGluR1, and not from an increase in the extracellular Glu concentration due to an inhibition of Glu uptake or to an heteroexchange with intracellular Glu. Indeed the small stimulatory effects of ABHD-II and V were not inhibited by the Glu degrading enzyme Glutamate-pyruvate transaminase in the presence of 2 mM sodium pyruvate (data not shown).

The different ABHD molecules were then tested for their antagonist activity at mGluR1. A concentration of Glu close to its EC_{50} value (3 μM) was chosen to activate the receptor. As shown on Fig. 6(b), ABHD-I was the most potent derivative able to inhibit the Glu-induced IP formation in cells expressing mGluR1. Using a different cell line to express mGluR1, we previously reported that ABHD-I was a competitive antagonist with a Kb value of 300 μM [13]. ABHD-V was without effect, but ABHD-II, IV, VI, VII, and VIII significantly inhibited the Glu effect at 3 mM (Fig. 6(b)). Since ABHD-II was also able to slightly stimulate IP formation in mGluR1-expressing cells, it is possible that ABHD-II is a partial agonist at this receptor subtype.

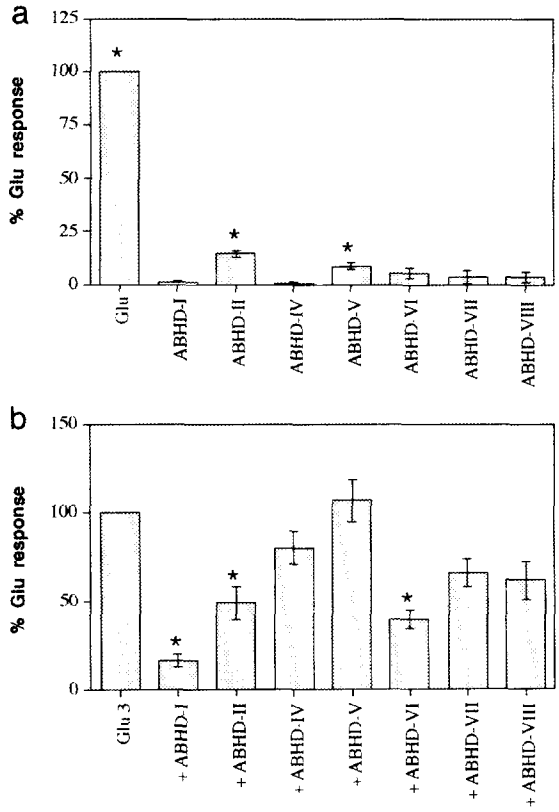


Fig. 6. Activity of ABHD derivatives on mGluR1. In (a) mGluR1-expressing cells were stimulated for 30 min with glutamate (1 mM) or each ABHD compound (3 mM), and the total IP formation was determined. In (b) mGluR1-expressing cells were stimulated for 30 min with glutamate (3 μM) in the absence or in the presence of the indicated ABHD compound (3 mM). Values are the IP formation induced by the agonists over basal value, and are expressed as percentage of that obtained with glutamate alone. Values are means ± SEM of 3 to 5 independent experiments performed in triplicates. $^*P < 0.01$ compared with untreated cells (a) or with glutamate-stimulated cells (b).

2.2.2 mGluR2

Among the different ABHD molecules, only ABHD-I, -II, and -VI activated mGluR2 when tested at 3 mM (Fig. 7(a)). No such effect was observed in control cells not expressing mGluR2 (data not shown). The effects of ABHD-I, II and VI were dose-dependent (data not shown). However, the EC_{50} values could not be determined because it was not possible to determine the maximal effect of these drugs. The activity of ABHD-I, -II, and -VI was not inhibited by the Glu degrading enzyme indicating that it resulted from a direct activation of mGluR2 (data not shown).

Among the different ABHD molecules, ABHD-IV and -V were able to antagonise the effect of 30 μM Glu on mGluR2-expressing cells (Fig. 7(b)). Full dose-response curves of Glu were then performed in the presence of ABHD-IV or ABHD-V (3 mM). A clear shift to the right of the Glu-dose response curve, without a change in the maximal response was observed with ABHD-V, indicating that it is a competitive antagonist (data not shown). However, ABHD-IV was found to decrease the maximal effect of glutamate without modifying its EC_{50} value, consistent with ABHD-IV being a non competitive antagonist (data not shown).

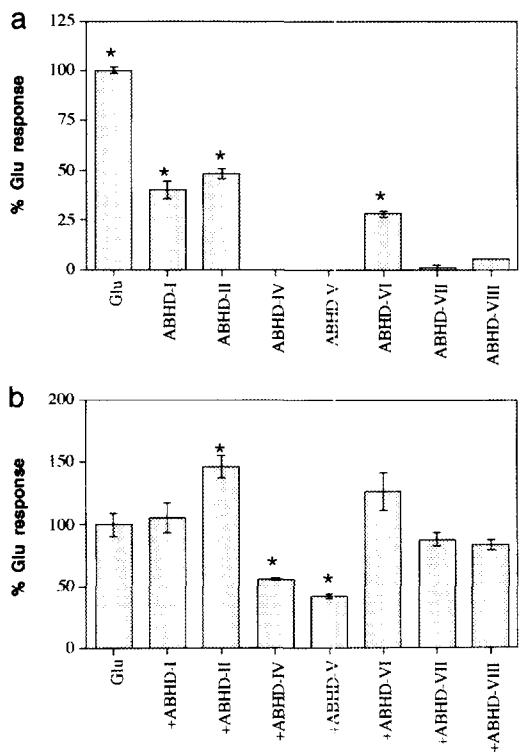


Fig. 7. Activity of ABHD derivatives on mGluR2. In (a) cells expressing mGluR2 and Gαqi were stimulated for 30 min with glutamate (1 mM) or each ABHD compound (3 mM), and the total IP formation was determined. In (b) cells were stimulated for 30 min with glutamate (30 μM) in the absence or in the presence of the indicated ABHD compound (3 mM). Values are the IP formation induced by the agonists over basal values and are expressed as percentage of that obtained with glutamate alone. Values are means ± sem of triplicate determinations from a typical experiment. $^*P < 0.01$ compared with untreated cells (a) or with glutamate-stimulated cells (b).

3. Discussion

All ABHDs are less active effectors of mGluR1 and mGluR2 than expected, probably because of steric hindrance. As an analogous binding site has been assumed for the bacterial leucine-binding protein and the amino terminal domain of mGluR1 [3], it is relevant to note that the leucine-binding protein which binds neutral β-branched amino acids with affinity in the μM range [45], exhibits a 100-fold decrease in affinity with norbornyl analogs [28]. Nevertheless valuable conclusions regarding conformational family(ies) of bound glutamate can be drawn.

3.1 mGluR1

Fig. 6 and Table 1 show that ABHD-I is the best effector on mGluR1 among the ABHDs tested. Thus we can deduce that the extended conformation (family C) of *trans*-ACPD and glutamic acid is probably recognized by the protein. The L isomer is supposed to be the most active enantiomer because L-*trans*-ACPD is ten times more potent than its D isomer (Table 1) and the *trans*-ACPD structure is part of ABHD-I (Fig. 2). Ionisable groups of ABHD-V can be perfectly superposed to the same groups of ABHD I (Fig. 4). The difference originates from the added bridge on ACPD which is linked to carbons 2 and 4 in ABHD-I and carbons 2 and 5 in ABHD-V. The resulting steric bulk would be responsible for the lower affinity of ABHD-V and unexpectedly allows the switch from antagonist to agonist. Table 1 shows that ABHD-II is a weak partial agonist of mGluR1. ABHD-II is a rigid analog of *cis*-ACPD which is also a weak agonist of mGluR1 compared to *trans*-ACPD (L isomers) (Table 1). Thus ABHD-II could be considered as the locked bound conformation of *cis*-ACPD. Moreover ABHD-VI of the same conformational family is a weak antagonist (Table 1) which would mean that different steric bulk turned the partial agonist ABHD-II into an antagonist, as described above for the ABHD-V/ABHD-I couple.

When comparing the conformational families of ABHD-I, -V (C), and of ABHD-II, -VI (B) it appears that they are quite close (Figs 4 and 8) and it can be assumed that effectors of family C can adapt to family B requirements and vice versa.

Finally, it can be noted that ABHD-IV, -VII, and -VIII slightly antagonize the effect of glutamate at high concentration. ABHD-III was not tested because it was

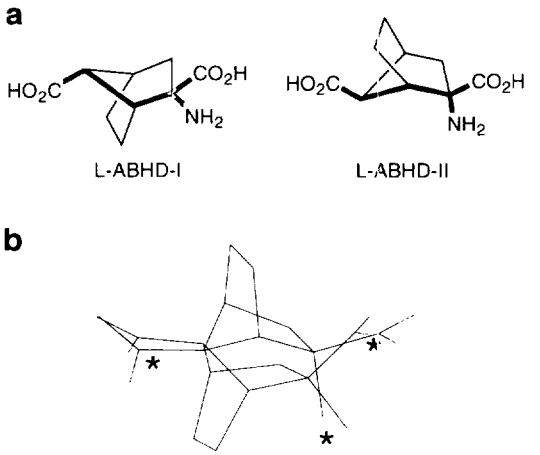


Fig. 8. (a) L-ABHD-I and L-ABHD-II and (b) the superposition of their three functional groups. Glutamic acid included in the ABHD structure is drawn in bold lines, hydrogens have been removed for clarity and atoms that have been superposed are starred [14].

not obtained in sufficient amount to be isolated. Interestingly PCCG-6 (Fig. 9), an mGluR1a antagonist recently described by Pellicciari and coworkers [46], displays the same tridimensional disposition of the functional groups as in ABHD-IV and -VIII (folded conformational family A, data not shown [14]). Moreover, these authors stated on the basis of an homology modeling study, that agonists such as glutamic acid, quisqualic acid and (1*S*,3*R*)-ACPD could bind to the receptor in a folded conformation and that antagonists of the phenylglycine family which mimic an extended glutamate conformation, would bind differently to the protein [47]. Thus we can speculate that different conformations of *trans*-ACPD and glutamic acid would be recognized by the protein [12]. However, because of too low affinity of ABHD-IV, -VII, and -VIII, we did not determine whether they are competitive or non competitive antagonists, which was not either performed for PCCG-6. We are currently investigating this question of a possible folded bound conformation, with less hindered probes.

3.2 mGluR2

ABHD-I, -II, and -VI are modest agonists of mGluR2 and ABHD-V a modest competitive antagonist. ABHD-II and -VI belong to conformational family B and are rigid analogs of *cis*-ACPD, whereas ABHD-I and -V belong to conformational family C and are rigid analogs of *trans*-ACPD. As mentioned earlier conformational families B and C are close (Figs 4 and 8). It can be speculated that the bound conformation of *cis*-ACPD and *trans*-ACPD is of conformational family B, as it is the only common family to both isomers [11] (Table 1).

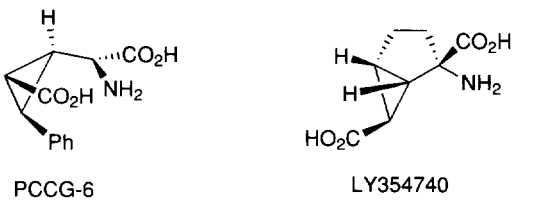


Fig. 9. Structure of (2*R*,1′*S*,2′*R*,3′*R*)-2-(2′-carboxy-3′-phenylcyclopropyl)glycine (PCCG-6) [46] and of (1*S*, 2*S*, 5*R*, 6*S*)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate (LY354740) [50].

This result would be in agreement with the bioactive conformation described by Pellicciari [48], Ohfune [49], and Monn [50]. Indeed, the three functional groups of LY354740 (Fig. 9), a new conformationally constrained and highly potent mGluR2 agonist [50] can be perfectly superposed on those of ABHD-II and -VI [14]. Interestingly, the two ABHD members of the same conformational family (B or C) can be both agonists (ABHD-II/ABHD-VI) or one agonist (ABHD-I) and the other antagonist (ABHD-V) as noted above for mGluR1. The comparison of these two activities (agonist and antagonist) shows that flexibility would not be required for agonist activity and that, in this case, antagonist activity would be the result of adequate positioning of the functional groups at the binding stage, together with steric hindrance preventing subsequent conformational changes of the receptor.

The other ABHD isomers are either noncompetitive antagonists or inactive, showing that conformational family B and C would be the only ones that have some affinity for the receptor.

The general decrease of ABHDs' affinities compared to ACPDs', can be explained by negative steric interactions in particular on the β position as it has been shown that β-methyl glutamate (2*S*,3*S*) and (2*S*,3*R*) isomers are poor agonists of mGluR1 and mGluR2 (EC_{50} > 150 μM) (J.-P. Pin, unpublished results).

We have thus demonstrated that glutamic acid would bind to mGluR1 and mGluR2 in an extended conformation that could be of family C for the former and family B for the latter, although modest activities for ABHDs are described. Further confirmation of the bioactive conformations and steric requirements of mGluRs are under study using other less hindered probes.

4. Experimental

4.1 Chemistry

Melting points were determined with a Büchi capillary tube melting point apparatus. Elemental analyses were carried out by the Service Central de Microanalyse du CNRS (Gif-s-Yvette, France). ^{1}H NMR

(250.13 MHz) and ^{13}C NMR (62.9 MHz) spectra were recorded on a ARX 250 Bruker Spectrometer; Chemical shifts [δ (ppm)] were given with reference to residual ^{1}H or ^{13}C of deuterated solvents ($CDCl_3$, 7.24, 77.00; DMSO-d_6, 2.49, 39.7) or to 3-(trimethylsilyl)[2,2,3,3-$^{2}H_4$] propionic acid sodium salt in a sealed capillary for D_2O solutions. Multiplicities [J (Hz)] are reported as br (broad), s (singlet), d (doublet), t (triplet), q (quadruplet), and m (multiplet). NOE experiments were performed on an ARX 500 Bruker spectrometer. TLC was performed on MercK $60F_{254}$ precoated silica–gel plates (0.2 mm thick, 8 cm migration) with the indicated solvent systems. Products were visualized by UV light (254 nm), 2% (w/v) ninhydrin in ethanol and TDM reagent [51]. HPLC of hydantoins was performed with an Altex chromatem 380 pump equipped with a 20 μl loop, a Pye-Unicam LC-UV detector set at 210 nm, and a Shimadzu CR-3A integrator, using a Nucleosil 5 C_{18} (25 cm×4 mm I.D.) column equilibrated with 0.1 M NaH_2PO_4 buffer pH 3-MeOH (9:1) at a 0.6 ml. min^{-1} flow rate. The purity of amino acids was checked by HPLC after derivatization to *o*-phthalaldehyde/N-Ac-Cys adducts [43] with the same apparatus but with a Shimadzu RF-530 flow spectrofluorometer detector (excitation 345 nm; emission 445 nm) and using a Nucleosil 5 C_{18} (25 cm×4 mm I.D.) column equilibrated with 0.1 M Na,KH_2PO_4 buffer pH 7.5:MeOH (75:25) at a 0.8 ml min^{-1} flow rate. Gas chromatography (OV-1701) of amino acids was performed after derivatization to *N*-trifluoroacetyl-*O*-isopropyl esters [42]: in most cases only >98% pure fractions were considered for pooling. Mass spectra were recorded on a Hewlett-Packard 5890-II/5972 fitted with a GC-mass coupling (30 m capillary HP1 column): inj. temp. 250°C; det. temp. 280°C; method A: column temp. 90°C (2 min), 90°C to 250°C (8°C/min), 250°C (5 min); method B: column temperature 120°C (2 min), 120°C to 300°C (10°C/min), 300°C (5 min). High-resolution mass spectrometry was carried out by the Spectrometry Laboratory, Université Pierre et Marie Curie (Paris). Infrared spectra were measured on a Perkin-Elmer 783 spectrometer and reported in cm^{-1}.

4.1.1 cis- *and* trans-*Bicyclo[2.2.1]heptan-2-one-7-carboxylic acid (**1-2**)*

Oxoacids **1** and **2** were obtained starting from norbornadiene. According to published procedure [37], 5-oxotricyclo[2.2.2.1]heptane-3-carboxylic acid, a common intermediate for both oxoacids, was first prepared. Fom this tricyclic product either oxoacid **1** [38] or oxoacid **2** [39] were obtained by described methods.

4.1.2 endo-*Bicyclo[2.2.1]heptan-7-one-2-carboxylic acid (**3**)*

Oxoacid **3** was prepared starting from Diels-Alder reaction of 2,3,4,5-tetrachloro-1,1-dimethoxycyclopentadiene with acrylic acid [40,41]. The tetrachlorinated product led after dechlorination [41,52] to 7,7-dimethoxybicyclo[2.2.1]hept-5-ene-2-carboxylic acid. The latter was then successively hydrogenated and deprotected according to a common procedure previously used for the synthesis of bicyclo[2.2.1]hept-2-ene-7-one [53,54]. In a hydrogenation flask, 0.2 g of 10% palladium on carbon was added to 7,7-dimethoxybicyclo[2.2.1]hept-5-ene-2-carboxylic acid (1 g, 5 mmol) in ethanol (40 ml). This mixture was stirred under hydrogen at room temperature. After 24 h, the catalyst was removed by filtration and the solvent evaporated to give 1 g of crystalline 7,7-dimethoxybicyclo[2.2.1]heptane-2-carboxylic acid. Melting point 82°C, after recrystallization in Et_2O. GC-MS (method B): t_R 8.2 min; m/z (%) 53, 55, 77, 79, 101, 127, 145, 155 (100), 169, 200 $[M]^+$ (1). ^{1}H NMR ($CDCl_3$) δ 1.25–1.45 (m, 2H), 1.65–2 (m, 4H), 2.1 (m, 1H), 2.4 (m, 1H), 3.1 (m, 1H), 3.26 (s, 3H), 3.27 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 22.55 (CH_2), 27.00 (CH_2), 29.43 (CH_2), 37.87 (CH), 41.26 (CH), 43.22 (CH), 50.44 (CH_3), 50.60 (CH_3), 114.43 (quat. C), 180.88 (CO_2H).

The crude ketal (1 g, 5 mmol) was added to 25 ml of 5% H_2SO_4 solution. Stirring was maintained for 48 h at room temperature. The mixture was poured into ice and extracted with AcOEt. The organic phase was dried over $MgSO_4$. After evaporation of the solvent, 0.8 g (quantitative crude yield) of oxoacid **3** was obtained. This product was found relatively unstable and was immediately used without purification in the next step. TLC: CH_2Cl_2:AcOEt:AcOH (60:40:1), R_f 0.40. ^{1}H NMR ($CDCl_3$) δ 1.55–2.35 (m, 6H), 3.05–3.25 (m, 2H), 4.10 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 18.99 (CH_2), 23.52 (CH_2), 26.73 (CH_2), 38.58 (CH), 38.70 (CH), 41.20 (CH), 178.39 (CO_2H), 213.76 (CO).

4.1.3 trans- *and* cis-*Bicyclo[2.2.1]heptan-7-carboxylic acid-2-spiro-5′-hydantoins (**5a** + **5b**) and (**6a** + **6b**); endo-bicyclo[2.2.1]heptan-2-carboxylic acid-7-spiro-5′-hydantoins (**8a** + **8b**)*

To a solution of oxoacid **1**, **2**, or **3** (0.15 g, 1 mmol) in 4 ml of EtOH:water (1:1) were sucessively added $(NH_4)_2CO_3$ (0.48 g, 5 mmol) and KCN (0.08 g, 1.2 mmol). The mixture was heated with magnetic stirring at 55–60°C (5 h for **1** and **3**, 48 h for **2**). The temperature was then raised to 90°C for 1 h to eliminate excess ammonium carbonate. After evaporation of EtOH, the reaction mixture was acidified with HCl (for **1** and **2**) or H_2SO_4 (for **3**) and evaporated to dryness. The residue was extracted with EtOH and the solution filtered to eliminate salts. After evaporation of the solvent, crude crystalline hydantoins were obtained and used without purification.

Spirohydantoins (**5a** + **5b**): quantitative crude yield. TLC: CH_2Cl_2:MeOH:33%aqNH_3 (65:35:10), R_f 0.50; HPLC: t_R 9.0 and 11.5 min. Spirohydantoin **5a**:

^{1}H NMR (DMSO-d_6) δ 1.25 (d, 1H), 1.3–1.4 (m, 1H), 1.5–1.8 (m, 3H), 2.03 (dm, 1H), 2.07 (s, 1H), 2.36 (br s, 1H), 3.32 (s, 1H), 8.25 (s, 1H), 10.56 (br s, 1H), 12.03 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 21.82 (CH_2), 26.37 (CH_2), 37.98 (CH), 41.50 (CH_2), 47.86 (CH), 52.27 (CH), 66.05 (quat. C), 156.70 (CONH), 172.92 (CO), 179.43 (CO). Spirohydantoin **5b**: ^{1}H NMR (DMSO-d_6) δ 8.32 (s, 1H), 10.56 (br s, 1H).

Spirohydantoins (**6a** + **6b**): quantitative crude yield. TLC: CH_2Cl_2:MeOH:33%aqNH_3 (65:35:10), R_f 0.49; HPLC: t_R 21.5 min. Spirohydantoin **6a**: ^{1}H NMR (DMSO-d_6) δ 1.2–2.2 (m, 6H), 2.5–2.8 (m, 3H), 6.89 (s, 1H), 10.84 (s, 1H), 12.77 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 22.07 (CH_2), 26.97 (CH_2), 40.69 (CH), 42.53 (CH_2), 49.09 (CH), 56.76 (CH), 66.78 (quat. C), 155.97 (CONH), 175.84 (CO), 177.44 (CO). Spirohydantoin **6b**: ^{1}H NMR (DMSO-d_6) δ 8.21 (*s*, 1H), 10.48 (*s*, 1H).

Spirohydantoins (**8a** + **8b**): TLC: CH_2Cl_2:MeOH: 33%aqNH_3 (65:35:10), R_f 0.49; HPLC: t_R 12.8 and 14.0 min. Characterization of these products by NMR could not be performed.

4.1.4 ABHD-I, ABHD-II, ABHD-IV, ABHD-V, and ABHD-VI.

The spirohydantoins (**5a** + **5b**) and (**6a** + **6b**) (obtained from 1 mmol of oxoacid **1** or **2**) were heated in HCl solution (6 N, 20 ml) at 120°C in a tightly closed pyrex bottle for 5 days. The solution was then evaporated to dryness to afford the crude mixture of amino acids. The hydrolysis of (**5a** + **5b**) and (**6a** + **6b**) led respectively to a mixture of (ABDH-I + ABHD-II) and (ABDH-I + ABHD-II + ABHD-IV).

The spirohydantoins (**8a** + **8b**) (obtained from 1 mmol of oxoacid **3**) were suspended in H_2O (20 ml) with BaO (3.1 g, 20 mmol) (1 N solution) in a tightly closed bottle. The temperature was raised to 120°C with magnetic stirring for 48 h. After cooling, the pH of the suspension was ajusted to 4 with H_2SO_4 solution and the barium sulfate precipitate eliminated by filtration and washed with water. The filtrate was then evaporated to dryness to afford a crude mixture of (ABHD-V + ABHD-VI).

In all cases, the resulting amino acid mixtures dissolved in H_2O (200 ml) were first brought to pH 4 and deposited on a Bio-Rad AG 50W-X4 (H^+ form, 20–50 mesh, 25×2 cm) column. After washing with water (200 ml) amino acids were eluted with 0.5 N NH_4OH (200 ml). The ninhydrin positive fractions were evaporated to dryness under vacuum. These amino acids mixtures were then separated by anion exchange chromatography, on a Bio-Rad AG 1-X4 (AcO^- form, 200–400 mesh, 17×2 cm) column. The mixture was dissolved in boiled water (200 ml), the solution brought to pH 9 with 1 N NaOH and deposited on the column. After washing with boiled water, the various amino acids were eluted with increasing concentrations of AcOH. The ninhydrin positive fractions were evaporated to dryness under vacuum. Yields are based on the starting oxoacid. In the case of the mixture obtained from hydantoins (**5a** + **5b**) **ABHD-I** (130 mg, 65% yield) was eluted with 0.2 N AcOH (300 ml) and **ABHD-II** (14 mg, 7% yield) with 0.3 N AcOH (100 ml). In the case of the mixture obtained from hydantoins (**6a** + **6b**), **ABHD-I** (about 5 mg) was eluted with 0.2 N AcOH (300 ml), **ABHD-II** (about 5 mg) with 0.3 N AcOH (200 ml) and **ABHD-IV** (140 mg, 70% yield) with 0.7 N AcOH (300 ml). In the case of the mixture obtained from hydantoins (**8a** + **8b**), elution was performed with 0.1 N AcOH (200 ml), 0.2 N AcOH (200 ml) affording **ABHD-V** (65 mg, 32% yield) and 0.3 N AcOH (200 ml) affording **ABHD-VI** (65 mg, 32% yield).

4.1.5 ABHD-VII and ABHD-VIII

ABHD-V and ABHD-VI (40 mg, 0.2 mmol) were respectively heated with a 25% H_2SO_4 solution (4 ml) in a tightly closed bottle at 120°C for 6 days. When epimerization (checked by ^{1}H NMR) was completed, the solution was first ajusted to pH 1 by dilution with water (400 ml) and percolated through a Dowex 1X8 (AcO^- form, 50–100 mesh, 13×3 cm) column in order to remove H_2SO_4. The resin was rinsed with 0.5 N AcOH. The mixtures of amino acids (ABHD-V and ABHD-VII or ABHD-VI and ABHD-VIII) were recovered in the eluent (ninhydrin detection) and then further separated by anion exchange chromatography as previously described for ABHD-I to -VI. In the case of epimerization of ABHD-V, successive elutions with 0.1 N AcOH (200 ml) afforded **ABHD-VII** (20 mg, 50% yield) followed by 0.2 N AcOH (200 ml) affording **ABHD-V** (20 mg, 50% yield). Similarly, in the case of epimerization of ABHD-VI, successive elutions with 0.3 N AcOH (200 ml) affording **ABHD-VI** (10 mg, 25% yield), 0.4 N AcOH (200 ml) and 0.7 N AcOH (200 ml) affording **ABHD-VIII** (30 mg, 75% yield) were performed.

4.1.6 ABHD-I

HPLC: t_R (s) 9.8 min; GC-MS (method A): t_R 17.44 min; *m/z* (%) 67 (22), 91 (28), 177 (28), 204 (53), 232 (40), 250 (66), 278 (2), 292 (100), 320 [M-OiPr]$^+$ (13), 379 [M]$^+$ (1); ^{1}H NMR (D_2O, HCl-salt) δ 1.42 (d, 1H, H_{3b}), 1.35–1.50 (m, 1H, H_{5b}), 1.50–1.67 (m, 1H, H_{6b}), 1.87 (m, 1H, H_{5a}), 1.89 (m, 1H, H_{6a}), 2.43 (dm, 1H, H_{3a}), 2.55 (m, 1H, H_4), 2.72 (m, 1H, H_1), 3.41 (br s, 1H, H_7), H_{3a}/H_{3b} = 13.7 Hz, H_{3a}/H_4 = 4.0 Hz; ^{13}C NMR (D_2O, HCl-salt) δ 23.53 (CH_2), 28.12 (CH_2), 40.77 (CH), 41.87 (CH_2), 49.57 (CH), 56.06 (CH), 60.04 (quat. C), 176.52 and 178.31 (2×CO_2H); Anal. calcd for $C_9H_{13}NO_4$: C, 54.26; H, 6.58; N, 7.03. Found: C, 47.55; H, 6.43; N, 5.37.

4.1.7 ABHD-II

HPLC: t_R (d) 6.8 and 7.7 min; GC-MS (method A): t_R 17.0 min; *m/z* (%) 67 (9), 91 (16.5), 177 (19), 204 (12),

232 (100), 250 (76), 278 (9), 292 (15), 320 [M-OiPr]$^+$ (1); ^{1}H NMR (D_2O, HCl-salt) δ 1.38–1.55 (m, 2H, H_{5b} and H_{6b}), 1.72-1.83 (m, 2H, H_{5a} and H_{6a}), 1.76 (dd, 1H, H_{3a}), 2.30 (br d, 1H, H_{3b}), 2.64–2.67 (2m, 2H, H_1 and H_4), 3.00 (br s, 1H, H_7), $H_{3a}/H_{3b} = 13.7$ Hz, $H_{3a}/H_4 = 4.2$ Hz; ^{13}C NMR (D_2O, HCl-salt) δ 25.03 (CH_2), 27.44 (CH_2), 41.72 (CH), 41.81 (CH_2), 50.05 (CH), 55.67 (CH), 68.54 (quat. C), 174.81 and 177.94 (2×CO_2H); Anal calcd for $C_9H_{13}NO_4$: C, 54.26; H, 6.58; N, 7.03. Found: C, 50.31; H, 6.18; N, 6.48.

4.1.8 ABHD-III

HPLC: t_R (d) 6.4 and 7.6 min. GC-MS (method A): t_R 17.37 min; *m/z* (%) 66 (36), 91 (35), 177 (33), 204 (66), 232 (47), 250 (74), 277 (11), 292 (100), 320 [M-OiPr]$^+$ (16.5).

4.1.9 ABHD-IV

HPLC: t_R (s) 6.1 min; GC-MS (method A): t_R 17.81 min; *m/z* (%) 67 (13), 91 (16.5), 177 (18), 204 (14), 232 (100), 250 (74), 278 (12), 292 (26), 320 [M-OiPr]$^+$ (5), 379 [M]$^+$ (1); ^{1}H NMR (D_2O, HCl-salt) δ 1.30–1.50 (m, 2H, H_{5b} and H_{6b}), 1.58 (m, 1H, H_{6a}), 1.78 (m, 1H, H_{5a}), 1.80 (dm, 1H, H_{3a}), 2.41 (dd, 1H, H_{3b}), 2.66–2.67 (2m, 2H, H_1 and H_4), 2.78 (br s, 1H, H_7); $H_{3a}/H_{3b} = 13.7$ Hz, $H_{3a}/H_4 = 3.5$ Hz; ^{13}C NMR (D_2O, HCl-salt) δ 27.75 (CH_2), 28.95 (CH_2), 40.54 (CH_2), 44.93 (CH), 49.45 (CH), 58.62 (CH), 68.09 (quat. C), 175.16 and 182.10 (2×CO_2H); Anal. calcd for $C_9H_{13}NO_4$: C, 54.26; H, 6.58; N, 7.03. Found: C, 47.79; H, 5.92; N, 6.04.

4.1.10 ABHD-V

HPLC: t_R (d) 8.3 and 9.6 min; ^{1}H NMR (D_2O, NH_4^+ salt) δ 1.55–1.70 (m, 2H, H_{5b} and H_{6b}), 1.74 (dd, 1H, H_{3b}), 1.75–1.88 (m, 2H, H_{5a} and H_{6a}), 2.16 (dddd, 1H, H_{3a}), 2.43 (dd, 1H, H_4), 2.65 (dd, 1H, H_1), 3.13 (dddd, 1H, H_{2a}); $H_{3a}/H_{3b} = 12.9$ Hz, $H_{3a}/H_{2a} = 11.4$ Hz, $H_{2a}/H_{3b} = 5.5$ Hz, $H_{3a}/H_4 = 4.5$ Hz, $H_{2a}/H_1 = 4.2$ Hz, $H_1/H_{6a} = 4.0$ Hz, $H_4/H_{5a} = 4.0$ Hz, $H_{3a}/H_{5a} = 2.0$ Hz, $H_{2a}/H_{6a} = 1.9$ Hz; ^{13}C NMR (D_2O, NH_4^+ salt) δ 24.73 (CH_2), 29.15 (CH_2), 34.01 (CH_2), 43.93 (CH), 47.37 (CH), 48.73 (CH), 76.96 (quat. C), 177.08 and 184.49 (2×CO_2H); Anal. calcd for $C_9H_{13}NO_4$: C, 54.26; H, 6.58; N, 7.03. Found: C, 51.76; H, 6.26; N, 6.99.

4.1.11 ABHD-VI

HPLC: t_R (s) 6.3 min; ^{1}H NMR (D_2O, NH_4^+ salt) δ 1.45–1.55 (m, 2H, H_{5b} and H_{6b}), 1.88–2.00 (m, 2H, H_{5a} and H_{6a}), 1.91 (dd, 1H, H_{3b}), 2.03 (dddd, 1H, H_{3a}), 2.42 (dd, 1H, H_4), 2.64 (dd, 1H, H_1), 2.99 (dddd, 1H, H_{2a}); $H_{3a}/H_{3b} = 13.6$ Hz, $H_{3a}/H_{2a} = 11.2$ Hz, $H_{2a}/H_{3b} = 5.8$ Hz, $H_{3a}/H_4 = 4.2$ Hz, $H_1/H_{6a} = 4.0$ Hz, $H_4/H_{5a} = 4.0$ Hz, $H_{2a}/H_1 = 4.0$ Hz, $H_{3a}/H_{5a} = 2$ Hz, $H_{2a}/H_{6a} = 2$ Hz; ^{13}C NMR (D_2O, NH_4^+ salt) δ 25.96 (CH_2), 30.11 (CH_2), 32.56 (CH_2), 44.15 (CH), 47.27 (CH), 48.37 (CH), 77.78 (quat. C), 177.05 and 184.01 (2×CO_2H); Anal. calcd for $C_9H_{13}NO_4$: C, 54.26; H, 6.58; N, 7.03. Found: C, 52.05; H, 6.34; N, 6.82.

4.1.12 ABHD-VII

HPLC: t_R (d) 19.4 and 21.9 min; ^{1}H NMR (D_2O, NH_4^+ salt) δ 1.55–1.70 (m, 2H, H_{5b} and H_{6b}), 1.77 (dd, 1H, H_{3b}), 1.82–1.95 (m, 2H, H_{5a} and H_{6a}), 2.40 (dm, 1H, H_{3a}), 2.45 (m, 1H, H_4), 2.56 (dd, 1H, H_{2b}), 2.84 (m, 1H, H_1); $H_{3a}/H_{3b} = 12$ Hz, $H_{2b}/H_{3b} = 10$ Hz, $H_{2b}/H_{3a} = 6.1$ Hz; ^{13}C NMR (D_2O, NH_4^+ salt) δ 28.48 (CH_2) , 30.07 (CH_2), 35.25 (CH_2), 44.25 (CH), 46.77 (CH), 50.86 (CH), 75.58 (quat. C), 176.70 and 183.44 (2×CO_2H); Anal. calcd for $C_9H_{13}NO_4$: C, 54.26; H, 6.58; N, 7.03. Found: C, 50.77; H, 6.35; N, 6.48.

4.1.13 ABHD-VIII

HPLC: t_R (s) 5.8 min; ^{1}H NMR (D_2O, NH_4^+ salt) δ 1.47–1.63 (m, 2H, H_{5b} and H_{6b}), 1.86–1.98 (m, 1H, H_{6a}), 1.98–2.10 (m, 1H, H_{5a}), 2.10–2.15 (m, 2H, H_{3a} and H_{3b}), 2.68 (m, 1H, H_4), 2.84 (d, 1H, H_1), 2.89 (dd, 1H, H_{2b}); $H_{2b}/H_{3b} = 8$ Hz, $H_{2b}/H_{3a} = 8$ Hz, $H_1/H_{6a} = 3$ Hz; ^{13}C NMR (D_2O, NH_4^+ salt) δ 28.63 (CH_2), 30.57 (CH_2), 35.12 (CH_2), 43.74 (CH), 48.10 (CH), 48.86 (CH), 74.54 (quat. C), 173.96 and 183.89 (2×CO_2H); Anal. calcd for $C_9H_{13}NO_4$: C, 54.26; H, 6.58; N, 7.03. Found: C, 50.81; H, 6.18; N, 6.52.

*4.1.14 Tricyclo[2.2.1.2 [2,5]]nonane-2'-oxo-3'-aza-5-carboxylic acid methyl ester (**7b**)*

To a cooled solution of ABHD-IV (14 mg, 0.07 mmol) in MeOH (4 ml) was added thionyl chloride (0.2 ml) and the stirred mixture was then allowed to warm up to room temperature. After 12 h, the solvents were removed and the amine hydrochloride dissolved in AcOEt (3 ml) and saturated aqueous Na_2CO_3 solution. This mixture was extracted with AcOEt. The organic phase was dried over Na_2SO_4 and concentrated in vacuo to give the free amine ester as a white solid (14.7 mg, 95% yield); Melting point 102–104°C.

The amine ester (14.7 mg, 0.065 mmol) was heated in $CHCl_3$ (5 ml) at 60°C. Every day, the solvents were evaporated in order to remove MeOH, and $CHCl_3$ (5 ml) added again. The progress of cyclization was followed by TLC [CH_2Cl_2:AcOEt (8:2), R_f free amine 0.46; R_f lactam 0.23]. After 5 days, the reaction was completed, the solvents were evaporated. The crude residue was dissolved in AcOEt and washed with saturated aqueous NaCl solution. The organic phase was dried over Na_2SO_4 and concentrated in vacuo to give white cristals of lactam **7b** (8.7 mg, 69% yield). Melting point 128°C; IR ($CHCl_3$) ν: 3420 (NH), 1745 (shoulder CO_2Me) and 1715 (CONH); ^{1}H NMR ($CDCl_3$) δ 1.75–2 (m, 6H), 2.45 (m, 1H), 2.60 (s, 1H), 2.80 (m, 1H), 3.78 (s, 3H), 5.67 (s, 1H); ^{13}C NMR δ 19.71 (CH_2), 33.17

(CH_2), 36.85 (CH), 41.29 (CH_2), 52.43 (CH_3), 56.60 (CH), 57.16 (CH), 68.88 (quat. C), 170.41 (CO), 176.85 (CO_2H); HRMS (EI): calcd for $C_{10}H_{13}O_3N$ $[M]^+$ m/z 195.089541; found m/z 195.089503; GC-MS (method A): t_R 15.8 min; m/z (%) 67 (100), 136 (81), 154 (60), 167 $[M-CO]^+$ (43), 195 $[M]^+$ (79).

4.1.15 N-t-*Boc-dimethyl esters of ABHD-V (**9a**) and ABHD-VI (**9b**)*

To a sample of ABHD-V or -VI (8 mg) dissolved in a mixture of water (300 μl), triethylamine (30 μl), and dioxane (300 μl), Boc-on [2-(*tert*-butoxy-carbonyloxyimino)-2-phenyl acetonitrile], (45 mg×2, second addition after 2 days) was added and the resulting suspension heated at 30°C. The reaction was followed by TLC [CH_2Cl_2:MeOH:33%aqNH_3 (60:40:15), R_f AA 0.12, R_f Boc-AA 0.42]. After 4 days, saturated aqueous $NaHCO_3$ solution (200 μl) was added to the cooled solution, which was extracted with AcOEt (3×6 ml), acidified to pH 3 with solid $KHSO_4$ and extracted with AcOEt (3×8 ml). The acidic organic phase was dried on Na_2SO_4 and evaporated to yield a clear oil (4.7 mg) which was dissolved in CH_2Cl_2 and esterified with diazomethane. Purification by chromatography on a Sep-Pack column (Silica Plus, Waters) eluting with CH_2Cl_2 and CH_2Cl_2:AcOEt (9:1) gave clean Boc diesters (3.7 mg).

9a: ^{1}H NMR ($CDCl_3$) δ 1.2–1.5 (m, 2H), 1.41 (s, 9H, CH_3C), 1.65–2.0 (m, 4H), 2.46 (br s, 1H, H_4), 2.90 (br s, 1H, H_1), 3.24 (m, 1H, H_{2a}), 3.67 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 4.90 (br s, 1H, NH).

9b: ^{1}H NMR ($CDCl_3$) δ 1.2–1.5 (m, 2H), 1.42 (s, 9H, CH_3C), 1.6–2.05 (m, 4H), 2.46 (br s, 1H, H_4), 2.93 (br s, 1H, H_1), 3.08 (m, 1H, H_{2a}), 3.68 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 4.93 (br s, 1H, NH).

4.2 Biological assays

4.2.1 Culture and trans*fection of HEK 293 cells*

HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal calf serum and transfected by electroporation as previously described [9]. Electroporation was carried out in a total volume of 300 μL with 10 μg carrier DNA, plasmid DNA containing mGluR1 (0.3 μg) or mGluR2 (2 μg) and 10 million cells. To allow mGluR2 to activate PLC, an effect easier to measure than the inhibition of cAMP production, this receptor was co-expressed with the chimeric G-protein Gqo5 as previously described [9].

4.2.2 Determination of inositol phosphates (IP) accumulation

Determination of IP accumulation in transfected cells was performed as previously described after labelling the cells overnight with [^{3}H]-myo-inositol (23.4 Ci/mol, NEN, France) [9]. The stimulation was conducted for 30 min in a medium containing 10 mM LiCl and 1 mM Glu. The basal IP formation was determined after 30 min incubation in the presence of 10 mM LiCl and the Glu degrading enzyme glutamate pyruvate transaminase (1 U/ml) and 2 mM pyruvate to avoid the possible action of Glu released from the cells. Results are expressed as the amount of IP produced over the radioactivity present in the membranes.

4.3 Statistical analysis

Statistical differences were checked by analysis of variance with Fisher's PLSD test using the StatView Student program (Abacus Concepts, Inc., Berkeley, CA 1991).

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