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Synthesis and biological evaluation of (2*S*)-and (2*R*)-2-(3′-phosphonobicyclo[1.1.1]pentyl)glycines as novel group III selective metabotropic glutamate receptor ligands

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Abstract—The synthesis of (2*S*)- and (2*R*)-2-(3′-phosphonobicyclo[1.1.1]pentyl)glycine isomers (**10** and **11**), characterized by the bioisosteric replacement of the distal carboxylic group of 2-(3′-carboxybicyclo[1.1.1]pent-1-yl)glycine by the phosphonate moiety, was accomplished by a stereoselective Ugi condensation. The two isomers were tested for their activity against an array of metabotropic glutamate receptors, and the *S*-isomer (**10**) turned out to be a moderately potent and selective mGluR4 agonist.

1. Introduction

The isosteric replacement of the ω-carboxylate moiety of glutamic acid (L-Glu, **1**, Chart 1) or L-Glu analogs, with a ω-phosphonate group, has represented a productive strategy to obtain classes of acidic aminoacids endowed with specific activities at excitatory aminoacid (EAA) receptors. Thus, extended L-Glu derivatives of the D series, exemplified by D-AP5 (**2**) and D-AP7 (**3**), have been characterized as potent and selective NMDA competitive antagonists,[1] while L-Glu derivatives such as L-AP4 (**4**) and L-SOP (**5**) have been characterized as selective group III metabotropic glutamate receptor agonists.[2] This latter property is particularly interesting since the replacement of the ω-carboxy moiety with a ω-phosphono one is a well-recognized modification which allows for the achievement of group III versus group I/II mGluR selectivity.[3]

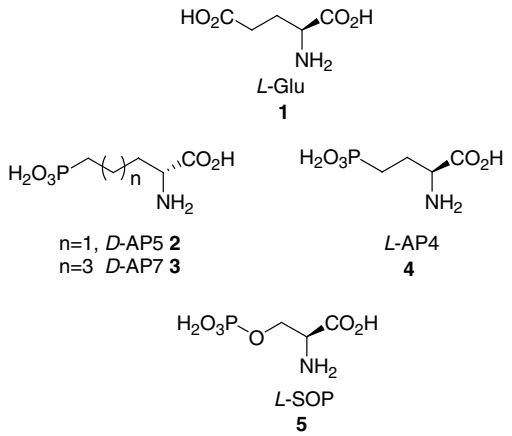

Chart 1.

Several attempts to further exploit this property toward subtype selective ligands have been based on the conformational constraining of ω-phosphonate analogs by embedding the L-AP4 (**4**) skeleton into cyclic, rigid structures.[4]

To date, however, these attempts have substantially failed, as L-AP4 (**4**) is still the most potent group III agonist known, and subtype selectivity has been only seldom obtained. The involvement of group III mGluR

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subtype in a variety of physiological states as well as pathological conditions and diseases, and the need for the further clarification of the specific role of individual subtypes in these physiopathological conditions are at the basis for the continuous quest for subtype selective ligands to be used as pharmacological tools on route toward clinically useful agents.

Conformational constraining is a first-choice strategy to achieve subtype selectivity. Among the strategies pursued for conformationally constrained L-Glu analogs, the incorporation of a phenyl ring between the α-amino acidic and the ω-acidic moieties to give 4-carboxy- or 4-phosphono-phenylglycine (4-CPG, **6** and 4-PPG, **7**, respectively) was recognized early to be a productive one,[5](Chart 2) and CPG derivatives are among the most potent group I antagonists so far described.[6a] Interestingly, the same compounds have no antagonist activity at either group II or group III receptors, while 4-PPG (**7**) turned out to be a potent and group III selective agonist.[6b]

In 1996, we demonstrated that the antagonist profile of 4-CPG (**6**) at mGluR1 could be maintained and even increased if the phenyl ring was substituted by the bicyclo[1.1.1]pentane scaffold to give the corresponding 2-(3′-carboxybicyclo[1.1.1]pentyl)glycine (*S*-CBPG, **8**).[7]

This effect was ascribed to the ability of the bicyclo[1.1.1]pentane scaffold to keep the pharmacophoric groups into a linear, co-planar disposition, similar although shorter to that provided by the phenyl ring. This result, further confirmed by the activity as group I antagonist of *S*-TBPG (**9**), suggested that the phenyl ring of CPGs functions as a spacer between pharmacophoric groups and is not involved in specific, electronic interaction with the receptor.[8]

In order to further prove and to extend this concept, and in search for new group III ligands to be used as pharmacological tools for the characterization of this family of receptors, we report herein the synthesis and the preliminary pharmacological evaluation of the two isomers (2*S*)- and (2*R*)-2-(3′-phosphonobicyclo[1.1.1]pentyl)glycines (*S*-PBPG, **10**) and (*R*-PBPG, **11**), respectively.

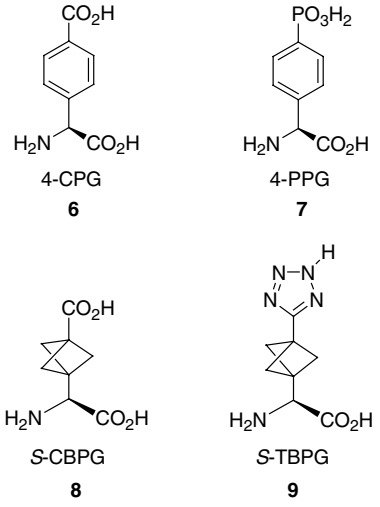
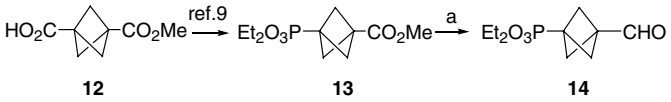


Chart 2.

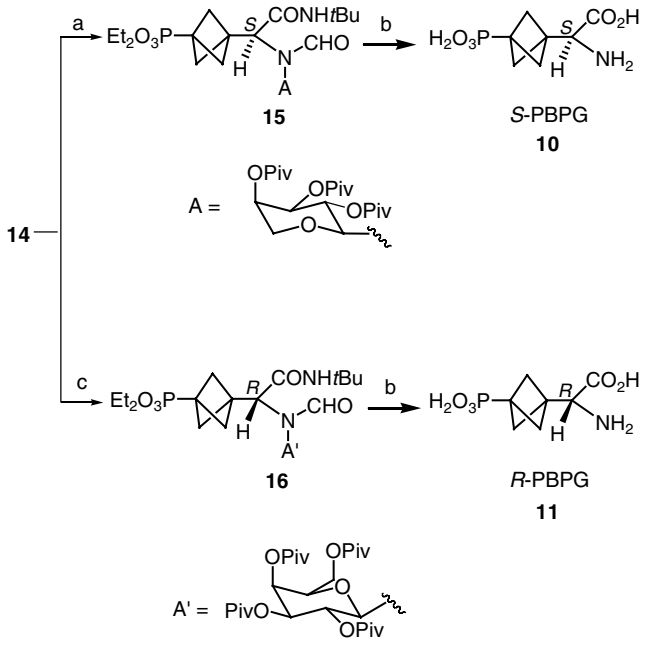
2. Results and discussion

The synthesis of (2S)- and (2*R*)-2-(3′-phosphonobicyclo[1.1.1]pentyl)glycines (**10** and **11**, respectively) involved the methyl 3-(diethoxyphosphoryl) bicyclo[1.1.1]pentane-1-carboxylate (**13**) as a key intermediate.

Briefly, **13** was synthesized (Scheme 1) according to the Michl procedure[9] starting from the monoacid **12**, which was converted, via the acyl chloride, into the corresponding 2-mercaptopyridine *N*-oxide ester (Barton ester). This intermediate was then decomposed photochemically in the presence of triethyl phosphite as a radical trap to give the title compound. DIBAL-H reduction of **13** yielded aldehyde **14** (56% yield), which was then submitted to a diastereoselective Ugi reaction using a carbohydrate-derived amine[10] as a chiral auxiliary (Scheme 2). Thus, the reaction of aldehyde **14** with 2,3,4-tri-*O*-pivaloyl-α-D-arabinopyranosylamine,[10] *tert*-butyl isocyanide, and formic acid in the presence of zinc chloride in THF at −25 °C afforded the mixture of 2*S*- and 2*R*-*N*-formyl-*N*-arabinosyl aminoacid amides in a 85:15 diastereoisomeric ratio (HPLC). Purification by flash chromatography (ethyl acetate–hexane, 80:20) of the crude mixture gave the major diastereoisomer **15** in 28% yield. According to previously reported data,[10] a *S* configuration at the amino acidic center was tentatively assigned to the more abundant amide **15**. The final aminoacid was obtained by a two-step hydrolysis. Treatment of derivative **15** with hydrogen chloride/methanol resulted in the removal of the formyl group. Subsequent addition of water caused the smooth cleavage of the *N*-glycosidic bond. Final hydrolysis of the resulting amide was achieved with 6 N HCl at 80 °C. After purification on Dowex 50WX2-200 with 1 N acetic acid, (2*S*)-2-(3′-phosphonobicyclo[1.1.1]pentyl)glycine (**10**) was obtained in 93% yield. To obtain the corresponding enantiomer (2*R*)-2-(3′-phosphonobicyclo[1.1.1]pentyl) glycine (**11**), an analogous synthetic scheme was followed (Scheme 2) using the commercially available 2,3,4,6-tetra-*O*-pivaloyl-β-D-galactopyranosyl amine as the chiral auxiliary in the diastereoselective Ugi reaction. It has been reported, indeed, that the asymmetric induction can be reversed using the galactose-derived amine in place of the 2,3,4-tri-*O*-pivaloyl-α-D-arabinopyranosylamine. [11] Thus, the reaction of the aldehyde **14** with 2,3,4,6-tetra-*O*-pivaloyl-β-D-galactopyranosylamine, *tert*-butyl isocyanide, and formic acid in the presence of zinc chloride in THF at −25 °C afforded the mixture of 2*R*- and 2*S*-*N*-formyl-*N*-galactopyranosyl aminoacid amides in a 90:10 diastereoisomeric ratio (HPLC). After purification by flash chromatography (ethyl acetate–hexane, 80:20), the major diastereoisomeric 2*R*-*N*-formyl-*N*-galactosyl-aminoacid amide **16** was obtained in 58% yield. The amide **16** was then submitted to the hydrolysis protocol as described above to



Scheme 1. Reagents and condition: (a) DIBAL-H 1.0 M solution in toluene, toluene, −78 °C, 56%.



Scheme 2. Reagents and conditions: (a) i—2,3,4-tri-*O*-pivaloyl-α-D-arabinopyranosylamine, *t*-BuNC, HCO_2H, $ZnCl_2$ 2.2 M in THF, THF, −25 °C to rt; ii—flash chromatography, 28%; (b) i—HClg/MeOH; ii—6 N HCl, 80 °C, 24 h; iii—Dowex 50WX-200, 1 N AcOH; (c) i—2,3,4,6-tetra-*O*-pivaloyl-β-D-galactopyranosylamine, *t*-BuNC, HCO_2H, $ZnCl_2$ 2.2 M in THF, THF, −25 °C to rt; ii—flash chromatography, 58%.

give the final (2*R*)-2-(3′-phosphonobicyclo[1.1.1]pentyl)glycine (**11**) in 26% yield.

The new derivatives **10** and **11** were evaluated as potential mGluR ligands by functional assays (rmGluR1, rmGluR5, hmGluR2, rmGluR4, hmGluR6, and hmGluR7) and binding experiments (rmGluR4 and rmGluR8). As summarized in Tables 1 and 2, the new derivatives showed activity as group III mGluR agonists albeit with significant differences in potency and subtype, whereas both compounds prove to be inactive at group I mGluRs and only very weakly active at mGluR2. The pharmacological profiles of the two enantiomers were examined by evaluating their binding affinity and their functional profile at a broad spectrum of mGlu receptor subtypes.

Table 2. Binding affinity values expressed as K_i (μM), for *S*-PBPG (**10**) and *R*-PBPG (**11**)

Compound	mGluR4	mGluR8
S-PBPG (**10**)	2.4 ± 2.7	4.4 ± 16
R-PBPG (**11**)	13 ± 15	7.6 ± 4
L-Glu (**1**)	3.4 ± 0.5	3.4 ± 1.1
L-AP4 (**4**)	0.16 ± 0.02	1.9 ± 0.3
4-PPG (**7**)	16	3.8

[^{3}H]L-AP4 and [^{3}H]LY341495 binding experiments were performed on rmGluR4 and rmGluR8 receptor-expressing cell membranes (BHK cells) using a SPA assay. Data are means ± SEM of at least three independent experiments performed in triplicate.

Both compounds were able to displace [^{3}H]L-AP4 at mGluR4 and [^{3}H]LY341495 at mGluR8, showing low micromolar affinity (Table 2). Inspection of functional data reported in Table 1 indicates that both compounds were inactive in the intracellular calcium mobilization assay at group I mGluRs, whereas they displayed only very weak activities in the GTPγS assay at the group II mGluR2 subtype.

It can be of some interest the observation that the two enantiomers, *S*-PBPG (**10**) and *R*-PBPG (**11**), have opposite effects at mGluR2, with **10** a weak antagonist and **11** a weak agonist (Fig. 1).

At group III receptors, **10** exhibited agonist activity in the cAMP assay at mGluR4 and mGluR6 with a noticeable 16-fold difference in potencies in favor of mGluR4 (Fig. 2A). **11** showed moderate agonist potency in the cAMP assay at both mGluR4 and 6 (Fig. 2B).

No activity was observed in the cAMP assay at mGluR7 for both enantiomers when tested up to 1000 μM concentration (Fig. 2).

Table 1. EC_{50} (μM) values for *S*-PBPG (**10**) and *R*-PBPG (**11**)

Compound	Group I[a]		Group II[b]		Group III[c]	
	mGluR1	mGluR5	mGluR2	mGluR4	mGluR6	mGluR7
S-PBPG (**10**)	>1000	>1000	310 ± 30	4.2 ± 2.0	66 ± 28	>1000
R-PBPG (**11**)	>1000	>1000	190 ± 42	29 ± 1.3	29 ± 9	>1000
L- Glu (**1**)	7.3 ± 1.0	1.2 ± 0.3	26 ± 2	6.1 ± 1.5	7.6 ± 0.5	>1000
L-AP4 (**4**)	>1000	>1000	>1000	0.51 ± 0.14	0.34 ± 0.07	188 ± 79
4-PPG (**7**)	>1000	>1000	>1000	5.3 ± 0.1	4.7	185

[a] Intracellular Ca^{2+} mobilization assay.
[b] [^{35}S]GTPγS binding assay.
[c] Inhibition of forskolin-stimulated cAMP production.

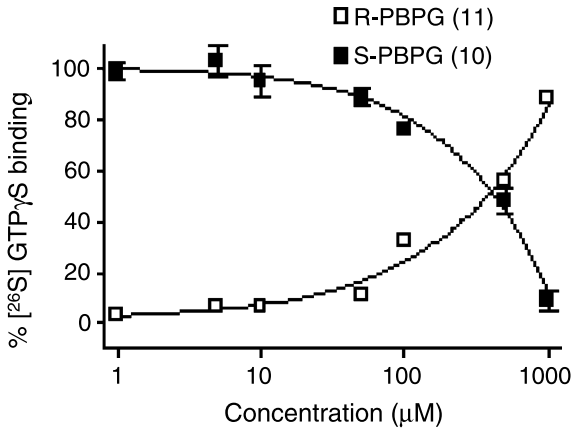


Figure 1. Concentration–response curves from BHK cells expressing hmGluR2 in the GTPγS assay. Data points were normalized to the potentiation obtained by either an EC_{100} value for the agonist *R*-PBPG (**11**) or 100 μM glutamate for the antagonist *S*-PBPG (**10**) and are from representative experiments performed in triplicate.

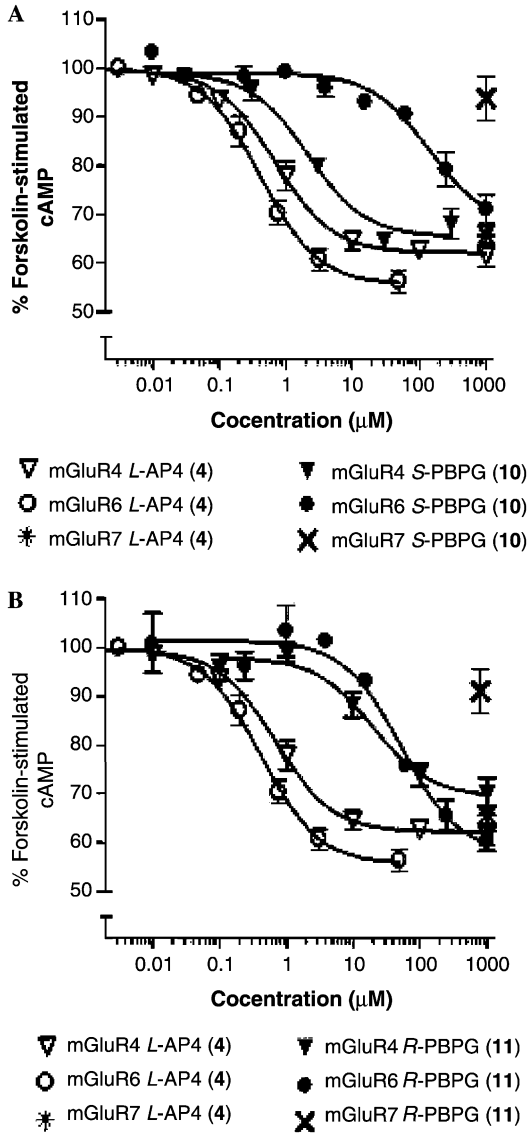


Figure 2. Inhibitory effect of **10** (A) and **11** (B) on cAMP formation given as percent inhibition of 10 μM forskolin-stimulated cAMP production at mGluR4, mGluR6, and mGluR7, respectively.

When compared with reference compounds such as L-AP4 (**4**) or 4-PPG (**7**), the new derivatives **10** and **11** showed an interesting profile. In particular, the *S*-enantiomer **10** is turning out as new potential pharmacological tool for the study of physiopathological role of individual group III mGluR subtypes.

Indeed, although L-AP4 (**4**) is still the most potent agonist at group III mGluRs, our new derivative **10** shows quite a selective profile for mGluR4, being about 16-fold more potent at this receptor subtype than at mGluR6.

In comparison, reference compounds such as 4-PPG (**7**) and L-AP4 (**4**) are equally potent at the two subtypes. Thus, *S*-PBPG (**10**) can be considered as a valuable tool to study the specific role of mGluR4.

From a structural point of view, our present data confirm and bring additional support to the concept of the bioisosterism between the bicyclo[1.1.1]pentane nucleus and the phenyl ring in the case of mGluRs. Indeed, the bicyclo[1.1.1]pentane scaffold is appearing as privileged structure for the orthosteric active site of mGluRs and a series of PBPG derivatives are currently under preparation to explore their potential as potent subtype selective ligands acting at group III mGluRs.

3. Experimental

Flash chromatography was performed on Merck silica gel (0.040–0.063 mm). Melting points were determined in open capillary tubes on a Büchi 535 electrothermal apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were registered on a Bruker AC 200 or Bruker AC 400 using $CDCl_3$ as solvent unless otherwise indicated. Chemical shifts are reported in ppm. The abbreviations used are as follows: s, singlet; d, doublet; m, multiplet. Optical rotations were recorded on a Jasco Dip-360 digital polarimeter. HPLC analyses were carried out on a Shimadzu (Kyoto, Japan) LC-Workstation Class LC-10 equipped with a CBM-10A system controller, two LC-10AD high-pressure binary gradient delivery systems, a SPD-10A variable-wavelength UV–vis detector, and a Rheodyne 7725i injector (Rheodyne, Inc., Cotati, CA, USA) with a 20 μl stainless steel loop. Microanalyses were carried out on a Carlo Erba 1106 elemental analyzer.

3.1. Diethyl 3-formylbicyclo[1.1.1]pentylphosphonate (14)

A −78 °C solution of DIBAL-H (1.0 M in toluene, 2.5 mL) was added dropwise over 30 min to a −78 °C solution of methyl 3-(diethoxyphosphoryl)bicyclo [1.1.1]pentane-1-carboxylate (**13**) (0.650 g, 2.48 mmol) in dry toluene (45 mL). After 30 min, the reaction was quenched with methanol (4 mL) and saturated NH_4Cl solution (20 mL), and then allowed to warm up to room temperature. The organic phase was separated and the aqueous phase was extracted with EtOAc (3× 50 mL). The combined organic phases were washed with water (2× 50 mL), dried (Na_2SO_4), and evaporated. The residue was purified by flash chromatography. Elution with

light petroleum–EtOAc (20:80) gave 0.306 g of the starting material. Following elution with EtOAc–MeOH (80:20) afforded 0.170 g of **14** (56%). ^{1}H NMR (200 MHz) δ 1.17–1.26 (m, 6H, $PO(OCH_2CH_3)_2$), 2,23 (m, 6H, 3× CH_2), 3.97–4.04 (m, 4H, $PO(OCH_2CH_3)_2$), 9.45, (d, J = 2.9 Hz, 1H, CHO); ^{13}C NMR (50 MHz) δ 14.12, 16.49, 29.61, 51.03, 62.05, 195.19; ^{31}P NMR (81 MHz) δ 19.47.

3.2. (2*S*)-*N*-Formyl-*N*-(2,3,4-tri-*O*-pivaloyl-α-D-arabinopyranosyl)-2-[3′-(diethoxyphosphoryl) bicyclo[1.1.1]pentyl)glycine *tert*-butyl amide (15)

Compound **14** (0.184 g, 0.80 mmol) was added to a stirred solution of 2,3,4-tri-*O*-pivaloyl-α-D-arabinopyranosylamine (0.423 g, 0.82 mmol) in THF (6 mL). After 15 min, the reaction mixture was cooled to −25 °C and formic acid (34.7 μl, 0.9 mmol), *t*-BuNC (90.5 μl, 1.46 mmol), and $ZnCl_2$ (2.2 M in THF, 0.37 mL) were added. The resulting mixture was stirred at −25 °C for 3 h. The solvent was evaporated off, the residue dissolved in CH_2Cl_2 and washed with a saturated $NaHCO_3$ solution (10 mL) and brine (10 mL). The organic phase was dried (Na_2SO_4) and concentrated in vacuo to give a residue, which was submitted to flash chromatography. Elution with EtOAc–hexane (80:20) afforded 0.168 g of the amide **15** (28%). ^{1}H NMR (200 MHz) δ (major rotamer) 1.07 (9H, m), 2.0 (6H, m), 3.97 (4H, m), 5.06 (s,1H, 2-CH), 5.85 (d, 1H, J = 7.2 Hz), 8.55 (s, 1H, CHO); ^{31}P NMR δ (81 MHz) 19.76; $[\alpha]_{20}^{D}$ = +44.8 (*c* 1.6, $CHCl_3$).

3.3. (2*S*)-2-(3′-Phosphonobicyclo[1.1.1]pentyl)glycine (10)

A saturated solution of HCl in MeOH (1.2 mL) was added to a solution of the amide **15** (0.168 g, 0.23 mmol) in MeOH (4.0 mL). The reaction mixture was stirred for 1 h at 0 °C and then allowed to warm up to room temperature. H_2O (2.0 mL) was added and the resulting mixture was stirred for 24 h. After evaporation of the solvent, the residue was dissolved in H_2O and extracted with pentane (3× 3 mL). The aqueous solution was evaporated to dryness and the residue was treated with 6 N HCl (10 mL). The resulting solution was stirred at 80 °C for 48 h and then the solvent was evaporated off and the residue was purified by ion-exchange resin chromatography (Dowex 50WX2-200). Elution with 1 N acetic acid afforded 0.023 g of (2*S*)-2-(3′-phosphonobicyclo[1.1.1]pentyl)glycine (**10**) (93%). ^{1}H NMR (400 MHz, D_2O) δ 1.87 (m, 6 H, 3× CH_2), 4.1 (s, 1 H, 2-CH);^{13}C NMR (100 MHz, D_2O) δ 32.92 (d, J = 157 Hz), 39.53 (d, J = 38.1 Hz), 49.43, 53.97 (d, J = 25.9 Hz), 169.93; ^{31}P NMR (81 MHz, D_2O) δ 16.81; $[\alpha]_{20}^{D}$ = +19.6 (*c* 0.15, H_2O); purity 95% [HPLC: Lichospher 100 RP18 (250 × 4.0 mm i.d., 5 μm), MeCN-H_2O 2:8, 0.8 mL/min]; Anal. Calcd for $C_7H_{12}NO_5P$: C, 38.02; H, 5.47; N, 6.33. Found: C, 37.99; H, 5.40; N, 6.30.

3.4. (2*R*)-*N*-Formyl-*N*-(2,3,4,6-tetra-*O*-pivaloyl-β-D-galactopyranosyl)-2-[3′-(diethoxyphosphoryl) bicyclo[1.1.1]pentyl)glycine *tert*-butyl amide (16)

14 (0.250 g, 1.08 mmol) was added to a stirred solution of 2,3,4,6-tetra-*O*-pivaloyl-β-D-galactopyranosylamine (0.537 g, 1.08 mmol) in THF (8 mL). After 15 min, the reaction mixture was cooled to −25 °C and formic acid (43.14 μl, 1.33 mmol), *t*-BuNC (123 μl, 1.08 mmol), and $ZnCl_2$ (2.2 M solution in THF, 0.5 mL) were added. The resulting mixture was stirred at −25 °C for 3 h. The solvent was evaporated off, the residue dissolved in CH_2Cl_2 and washed with a saturated $NaHCO_3$ solution (10 mL) and brine (10 mL). The organic phase was dried (Na_2SO_4) and concentrated in vacuo to give a residue, which was submitted to flash chromatography. Elution with EtOAc–hexane (80:20) afforded 0.530 g of the amide **16** (58%). ^{1}H NMR (200 MHz) δ (major rotamer) 1.07 (12H, m), 1.26 (6H, m), 2.0 (6H, m), 3.97 (4H, m) 5.06 (s,1H, 2-CH), 5,85 (d, 1H, J = 7.2 Hz), 8.36 (s, 1H, CHO); ^{31}P NMR δ (162 MHz) 19.76; $[\alpha]_{20}^{D}$ = −14.2 (*c* 0.1, $CHCl_3$).

3.5. (2*R*)-2-(3′-Phosphonobicyclo[1.1.1]pentyl)glycine (11)

A saturated solution of HCl in MeOH (1.0 mL) was added to a solution of the amide **16** (0.530 g, 0.64 mmol) in MeOH (4 mL). The reaction mixture was stirred for 1 h at 0 °C and then allowed to warm up to room temperature. H_2O (2 mL) was added and the resulting mixture was stirred for 24 h. After evaporation of the solvent, the residue was dissolved in H_2O and extracted with pentane (3× 3 mL). The aqueous solution was evaporated to dryness and the residue was treated with 6 N HCl (10 mL). The resulting solution was stirred at 80 °C for 48 h, then the solvent was evaporated and the residue was purified by ion-exchange resin chromatography (Dowex 50WX2-200). Elution with 1 N acetic acid afforded 0.037 g of (2*R*)-2-(3′-phosphonobicyclo[1.1.1]pentyl)glycine **(11)** (26%). $[\alpha]_{20}^{D}$ = −20.5 (*c* 0.92, H_2O); purity 97% [HPLC: Lichospher 100 RP18 (250 × 4.0 mm i.d., 5 μm), MeCN-H_2O 2:8, 0.8 mL/min]; Anal. Calcd for $C_7H_{12}NO_5P$: C, 38.02; H, 5.47; N, 6.33. Found: C, 37.97; H, 5.41; N, 6.32.

4. Biology

4.1. Materials

(*S*)-2-Amino-4-phosphonobutyric acid (L-AP4), [^{3}H]L-AP4 (45.5 Ci/mmol), L-serine-*O*-phosphate (L-SOP), (2*S*,1′*S*,2′*S*)-2-(9-xanthylmethyl)-2-(2′-carboxy cyclopropyl)glycine (LY341495), and [^{3}H]LY341495 (36.5 Ci/mmol) were purchased from Tocris Cookson (Bristol, UK). [^{35}S]GTPγS (1250 Ci/mmol) was purchased from Amersham Biosciences (Uppsala, Sweden). All other compounds and reagents were purchased from Sigma–Aldrich (Munich, Germany) unless else is mentioned.

4.2. Cell cultures

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing Glutamax-I and supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), and 10% dialyzed fetal calf serum (all Invitrogen, Paisley, UK). In addition, media for rmGlu receptor

subtypes 1a, 5a, and 6 expressing cell lines contained 1% proline, media for hmGluR2, rmGluR6, and hmGluR7 cell lines were supplemented with 0.5 mg/mL G-418 and rmGluR4 media were supplemented with 10 μM methotrexate. Cells were maintained at 37 °C, 95% humidified air, 5% CO_2 in an incubator, and subcultured every 3–4 days.

The Chinese hamster ovary (CHO) cell lines stably expressing the rmGluR1a, rmGluR5a or rmGluR6 receptors have previously been described.[12–14]

The BHK rmGluR4 cell line has also been described,[15] and the BHK hmGluR2 and hmGluR7 expressing cell lines were generated using the same procedure as recently described for hmGluR4.[16] Briefly, the cDNA was transfected into BHK cells using Lipofectamine 2000 according to the manufacturer's instructions (Gibco, UK). Two days later selection media (5 mg/mL G-418) were added and cells were grown for 7 days after which, clones were selected and tested in a cAMP assay.

4.3. Membrane preparation

BHK cells transiently expressing rmGluR8 receptor were used 2 days after transfection using Lipofectamine 2000 as a DNA carrier according to the manufacturer's instructions (Gibco, UK) and a rmGlu8a expression construct.[14] rmGluR2 and rmGluR4 cells were grown to 90% confluence and both rmGluR2/4 and rmGlu8 expressing cells were harvested as previously described.[17] On the day of the assay membranes were thawed, centrifuged, and resuspended in the desired assay binding buffer. Protein concentrations were determined using the Pierce BCA Protein Assay (Pierce Biotechnology, USA) with bovine serum albumin as standard.

4.4. Binding assays

rmGluR4 and rmGluR8 receptor binding experiments were performed using the scintillation proximity assay (SPA) from Amersham, essentially as already described[18] but with some modifications. Briefly, the following were placed in a well of a 96-well white optiplate (Packard, Meriden, CT, USA): membranes (100 μg protein, 50 μl), wheat germ agglutinin SPA beads (1 mg/well, 50 μl), compounds (100 μl), and [^{3}H]L-AP4 (30 nM final concentration, 50 μl)[16] or [^{3}H]LY341495 (10 nM final concentration, 50 μl).[19] The plate was sealed and shaken at room temperature for 1 h before counting. Non-specific binding was measured in the presence of 100 μM L-SOP. The K_D values for L-AP4 at mGluR4 (160 nM) and LY341495 at mGlu8 (20 nM) were determined by homologous displacement curves and in agreement with previous findings.[18–20]

4.5. Intracellular Ca^{2+} mobilization assay

CHO cells expressing rmGluR1 or rmGluR5 were seeded at 12,000 per well in 96-well black/clear-bottomed plates. Two days later the cells were loaded for 1 h at 37 °C with 4 μM Flou-4 acetomethyl ester (Molecular Probes, Leiden, Netherlands) in loading buffer (Hanks' balanced saline solution supplemented with 30 mM HEPES and 2.5 mM Probenecid). The cells were washed three times to remove excess dye and intracellular calcium mobilization was measured using a fluorometric imaging plate reader (FLIPR, molecular probes). First, potential agonist activity of the compounds was measured and then 10 min later potential antagonist potencies were determined by adding 60 μM L-glutamate as agonist.

4.6. Inhibition of forskolin-stimulated cyclic AMP production

BHK cells expressing rmGluR4 or hmGluR7 and CHO cells expressing rmGluR6 were seeded at 8000 and 10,000 per well, respectively, in 96-well plates two days before assaying. On the day of the cyclic AMP assay, cells were preincubated for 10 min in DMEM with 20 mM HEPES and 0.1 mM 3-isobutyl-1-methylxanthine (IBMX). Then the buffer was replaced with the test compound diluted in a similar buffer supplemented with 10 μM forskolin and incubated for 15 min. The reaction was terminated by replacing the media with cold 0.1 M HCl, after which plates were frozen at −80 °C and then thawed again and wells were neutralized with 0.15 M NaOH. Cyclic AMP levels were quantified by use of the Adenylyl Cyclase Flash-Plate® assay as described by the manufacturer (NEN, Belgium).

4.7. [^{35}S]GTPγS binding assay

The assay was essentially performed as previously described[21] but with the following modifications.[16] The assay buffer contained 3 μM GDP and 15 μg membranes per well supplemented with 50 μg/mL saponin, 1 mg/well wheat germ agglutinin-coated SPA beads (RPNQ001, Amersham), and 0.5 nM [^{35}S]GTPγS were used. For antagonist tests compounds were tested in the presence of 100 μM glutamate.

4.8. Data analysis

Pharmacological experiments were performed in duplicate or triplicate in at least three independent experiments. Single concentration tests were performed in triplicate with two independent experiments. Concentration–response and homologous displacement curves were analyzed by non-linear regression using GraphPad Prism (GraphPad Software, San Diego, USA). KD values were estimated using the following equation,[22] where L is the concentration of the radioactive ligand and the IC_{50} was estimated by non-linear regression from homologous displacement curves: $K_D = IC_{50}\text{-}L$. K_i values were calculated from IC_{50} values by use of the Cheng–Prusoff equation.[23] The classic group III selective agonist L-AP4 was tested in all the group assays and results were in agreement with previous findings.[18–20,24,25]

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