L-(+)-2-Amino-4-thiophosphonobutyric Acid (L-thioAP4), a New Potent Agonist of Group III Metabotropic Glutamate Receptors: Increased Distal Acidity Affords Enhanced Potency

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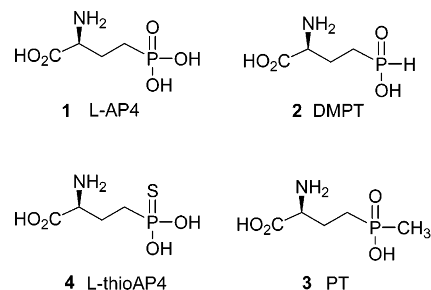
L-2-Amino-4-phosphonobutyric acid (L-AP4), L-2-amino-4-thiophosphonobutyric acid (L-thioAP4), and L-2-amino-4-(hydroxy)phosphinylbutyric acid (desmethylphosphinothricin, DMPT) were synthesized from protected vinylglycine. They were tested as agonists at group III metabotropic glutamate receptors (mGluR) along with phosphinothricin (PT). DMPT and PT display a much lower potency at mGlu4 receptor (EC_{50} = 4.0 and 1100 μM, respectively) in comparison to L-AP4 (EC_{50} = 0.08 μM), whereas L-thioAP4 has a 2-fold higher potency (EC_{50} = 0.039 μM). Similar rank orders of potency were observed at mGlu6,7 and mGlu8 receptors. The higher potency of L-thioAP4 is due to its stronger second acidity compared to L-AP4. These pK_a values of 5.56 and 6.88, respectively, were determined using ^{31}P NMR chemical shift variations. The second distal negative charge of L-AP4/L-thioAP4 probably provides stronger binding to specific basic residues of the binding sites of group III mGluRs, which stabilizes the active conformation of the receptor.

Introduction

Glutamate is the neurotransmitter of most excitatory synapses in the mamalian central nervous system (CNS). It is thus involved in numerous physiological brain functions and CNS pathologies and disorders. Glutamate activates several types of receptors[1] (ionotropic and metabotropic glutamate receptors noted iGluR and mGluR) and is the substrate of transporters and enzymes that all play an important role in synaptic transmission. Consequently, these receptors, transporters, and enzymes are potential therapeutic targets in many pathologies related to glutamatergic transmission.[2] A large number of glutamate analogues have been assayed at these systems in order to identify selective ligands, some of which have proved to be successful[3] and even reached the advanced clinical phase.[2] The mGlu receptors belong to class C among the large G-protein-coupled receptor (GPCR) family[4] and are classified in three groups according to their sequence similarity, transduction mechanism, and pharmacological profile.[5] Group I (mGlu1, mGlu5) receptors activate phospholipase C, whereas group II (mGlu2, mGlu3) and group III (mGlu4, mGlu6−8) receptors inhibit adenylyl cyclase when expressed in heterologous systems. Group III mGlu receptors are mostly presynaptic; their activation reduces glutamate release and postsynaptic activation of iGluRs as a consequence. The therapeutic benefits of agonists result from that modulatory property. It is expected that they will allow a fine-tuning in the CNS that is not possible when targeting iGluRs. Indeed, studies with selective ligands of the mGlu receptors have opened the way to future CNS disorder treatments. With group III receptors, the most promising effects are expected for anxiety,[6] Parkinson's disease,[7] and neuropathic pain relief.[8] It is pointed out that recently these receptors were also shown to control tumor cell growth.[9] The development of new and more potent ligands is still needed for group III receptors. Among other benefits, such compounds may allow binding experiments that are difficult to perform today with available radiolabeled ligands. They may also provide new QSAR data that would lead to the development of improved drugs.

Chart 1[a]



[a] Compounds tested as agonists of mGlu4, 6, 7, 8 Receptors: L-AP4 (2-amino-4-phosphonobutyric acid); L-thioAP4 (2-amino-4-thiophosphonobutyric acid); PT (phosphinothricin, 2-amino-4-(hydroxymethylphosphinyl)butyric acid also named glufosinate); DMPT (desmethylphosphinothricin, 2-amino-4-(hydroxyphosphinyl)butyric acid).

Several glutamate analogues are selective group III receptor agonists. They differ from the other analogues by the presence of an additional distal acidic function that provides both higher affinity and selectivity. That function is either a third carboxylic group as in ACPT-I, (+)-ACPT-III,[10] and (*S*)-3,4-DCPG[11] or the second acidity of a phosphonate group as in L-AP4,[a,12,13] L-SOP,[12,13] (*S*)-PPG,[14,15] and (1*S*,2*R*)-APCPr[12,16] (Chart S1 in Supporting Information). Among these, L-AP4 (**1**) is the most potent group III mGluR agonist with a potency of about 10- to 30-fold that of glutamate.[13] In the present study we have further investigated the chemical features that are responsible for the increased potency of L-AP4. We report on the synthesis of the

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[a] Abbreviations: AP4, 2-amino-4-phosphonobutyric acid; thioAP4, 2-amino-4-thiophosphonobutyric acid; PT, phosphinothricin; DMPT, desmethylphosphinothricin; ACPT, 1-aminocyclopentane-1,3,4-tricarboxylic acid; DCPG, 3,4-dicarboxyphenylglycine; SOP, serine *O*-phosphate; APCPr, 1-amino-2-phosphonomethylcyclopropane carboxylic acid; PPG, 4-phosphonophenylglycine; mGluR, metabotropic glutamate receptor.

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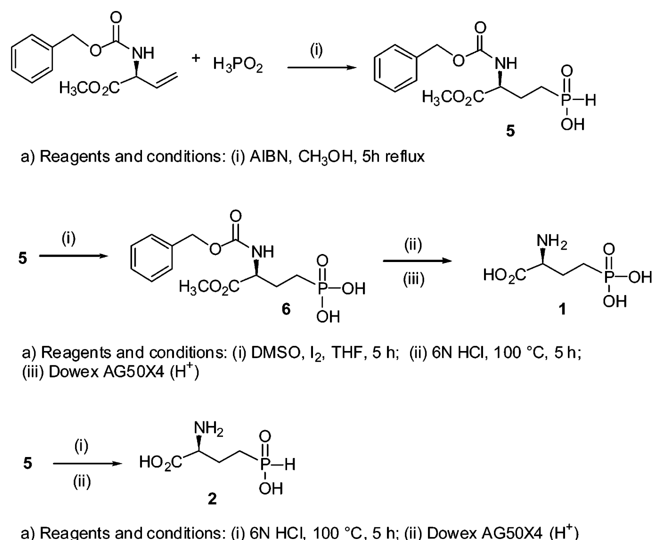
Scheme 1. Synthesis of Target Compounds **1** and **2**[a]



H-phosphinate (**2**), L-AP4 (**1**), and the thiophosphonate analogue of L-AP4 named L-thioAP4 (**4**) (Chart 1). This last compound has been listed in a patent application[17] describing a series of cerebral amyloid angiopathy inhibitors, but no data are available for **4**. The functional activity of **2**, **4**, and phosphinothricine (**3**) at mGlu receptors expressed in HEK cells was compared to that of L-AP4 (**1**) and interpreted on the basis of 3D structures and models.

Results

Chemistry. The γ-phosphinic acid derivative of glutamate **5** is a key intermediate in all the synthetic schemes. It was synthesized from aqueous hypophosphorous acid by a radical addition to the N-Z protected vinylglycine methyl ester[18−21] (Scheme 1). The synthesis of H-phosphinic acid derivatives has been the subject of numerous studies in which the formation of the P−C bond usually results from the addition of a phosphorus(III) moiety to unsaturated systems or activated halides.[22,23] These reactions occur under base- or metal-catalyzed conditions or under radical conditions. When starting from hypophosphorous acid (H_3PO_2), the challenge is to limit the addition to 1 equiv of substituent to obtain monosubstituted phosphinic acid. This problem is faced when bis(trimethylsilyloxy)phosphonite (BTSP)[24,25] is used, where a large excess of BTSP (5 equiv[26]) is required in order to yield only the H-phosphinic derivative. An alternative route has been suggested by Froestl et al.[27] for the synthesis of GABA phosphinic analogues. A temporary protection secured the monoalkylation; however, yields were later reported to be low and not reproducible.[28] The optimal reaction appears to occur under radical conditions.[21,29−32] H-Alkyl phosphonates are cleanly obtained because their radicals may not be formed for the second alkylation step.[23] Thus, we chose to use α,α'-azoisobutyronitrile (AIBN) to initiate the radical condensation of H_3PO_2 to a protected vinylglycine to yield the protected H-phosphinic derivative **5** (Scheme 1).[30] Synthesis of the *R*-enantiomer of compound **5** has been reported by Zeng et al.[33] by the BTSP route, and more recently a synthesis of the *S*-enantiomer in which Et_3B initiated radical addition of ammonium hypophosphite to Z-L-α-vinylGlyOMe has been described.[21]

In view of the high cost of commercially available L-AP4, an alternative efficient synthetic route was developed starting from **5**. Oxidation of the P−H bond of the protected phosphinic acid **5** was achieved by heating compound **5** with 1 equiv of DMSO and a catalytic amount of iodine at 60 °C for 5 h to yield **6**.[34] Deprotection of functional groups was performed by heating **6** in the presence of 6 N HCl for 5 h and gave the desired product **1** in quantitative yield (Scheme 1). We found this method to be advantageous in terms of simplicity and high overall yield of the final product over several other methods.[35,36] Phosphinate **5** was deprotected and purified using the same conditions as **6** to yield **2** (Scheme 1).

The protected H-phosphinic derivative **5** was oxidized to the corresponding thiophosphonate under mild conditions. Compound **5** was silylated to the protected BTSP intermediate with *N*,*O*-bis(trimethylsilyl)acetamide (BSA). In the presence of sulfur powder this intermediate was smoothly oxidized to the protected thiophosphonate **7** (Scheme 2). Deprotection of **7** with 6 N HCl at 90 °C for 3 h resulted in 35% of the desired product **4** and 65% of the hydrolyzed product **1** (ratio measured by ^{31}P NMR). A milder deprotection was thus required. Hydrolysis of the carboxylic methyl ester of **7** was carried out with 3 equiv of LiOH and afforded **8** in good yield (Scheme 2). Hydrogenolysis of **8** in the presence of palladium on charcoal failed to give **4**. Thus, we turned to mild acidic deprotection of the benzyloxycarbonyl group. Treating **8** with 4 N HCl at 75 °C for 3 h afforded the desired product **4** as the major product and **1** in smaller amounts (7:3 ratio mesured by ^{31}P NMR). Compound **4** was purified by water elution on a cation exchange resin column. The purity of **4** was easily checked by ^{31}P NMR because of the large difference in chemical shifts (86.7 ppm for **4** and 35.4 ppm for **1**). This assessment was used to check the stability of **4** after several weeks of storage in aqueous solution at pH 7 at −20 °C.

Because the potency of several group III mGluR agonists (e.g., ACPT-I, DCPG, L-AP4) is related to their additional acidic function, we anticipated that the differences in pharmacological activities of L-AP4 **1** and L-thioAP4 **4** would be due to their different ionization states. In order to compare them, we evaluated the pK_a values of these amino acids. pK_a values may be predicted at the SPARC algorithm Web site[37,38] (Figure 1A). In addition, the ^{31}P NMR chemical shifts of **1** and **4** are sensitive to the ionization state; thus, titration curves may be obtained from their pH dependence[39−41] (Figure 1B). L-AP4 **1** and L-thioAP4 **4** are characterized by four pK_a values corresponding to the acidities of the α-carboxylic, the γ-phosphonate/thiophosphonate (pK_{a1}, pK_{a2}, pK_{a3}), and α-ammonium (pK_{a4}) groups. The pK_{a1} and pK_{a2} values are too close to be determined from the titration curves; however, pK_{a3} and pK_{a4} are easily measured. Values of 6.88 and 9.90 were found for pK_{a3} and pK_{a4} of **1** and 5.56 and 9.70 for **4**. It can be noted that shielding of the phosphorus atom (decrease of the ^{31}P NMR chemical shift) is observed upon ionization of the first three acidic functions while a deshielding effect (increase of the ^{31}P NMR chemical shift) occurs when the amine is deprotonated and its positive charge removed. A similar observation has been reported;[41] the electrostatic effect of the ammonium ion involved in the ion pair with the phosphonate/thiophosphonate increases the electron density around the phosphorus, resulting in shielding. Once the amine is deprotonated, this effect is suppressed and ^{31}P NMR chemical shift increased because the electron density is then located more on the heteroatoms bound to the phosphorus atom. This effect is more pronounced with **1** than with **4** (Figure 1B). Because pK_{a1} and pK_{a2} are well below 7, the corresponding functions (α-carboxylic acid and first acidity of the phosphonate/thiophosphonate group) are totally deprotonated at physiological pH. The second acidity of the phosphonate is only half deprotonated at that pH because the pK_{a3} of **1** is found at 6.88.

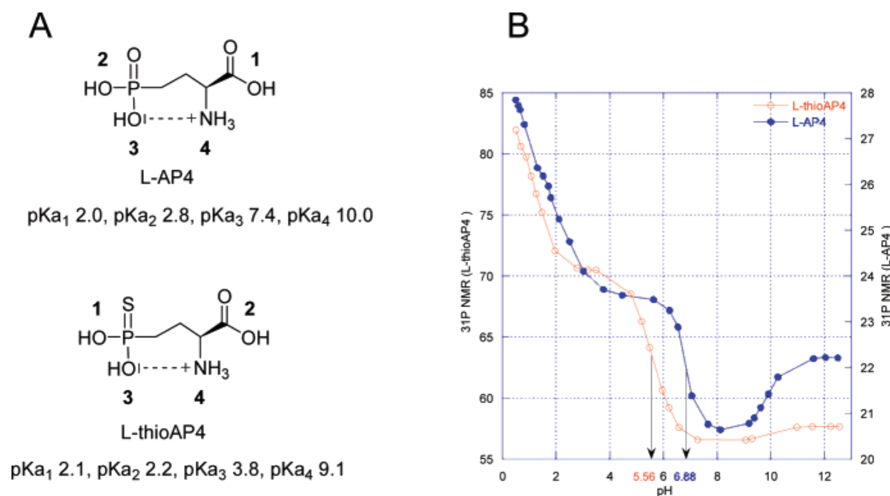
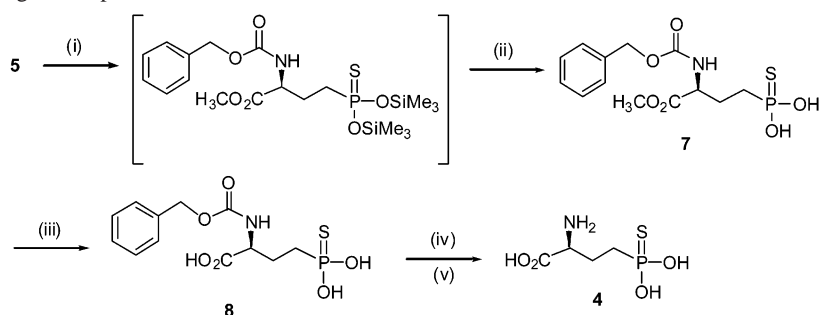


Figure 1. Dissociation constants of L-AP4 (**1**) and L-thioAP4 (**4**). (A) Calculated pK_a values using SPARC on-line calculator.[37] The ionizable functional groups 1−4 correspond to pK_{a1}, pK_{a2}, pK_{a3}, and pK_{a4}. (B) Experimental pK_{a3} values determined by plotting ^{31}P NMR chemical shift versus pH variations for L-AP4 (**1**, blue) and L-thioAP4 (**4**, red). Indicated pK_{a3} values were calculated by nonlinear regression analysis using GraphPad Prism program.

Scheme 2. Synthesis of Target Compound **4**[a]



a) Reagents and conditions: (i) CH_2Cl_2, N,O-bis(trimethylsilyl)acetamide (BSA), S_8, 1 h; (ii) 1N HCl; (iii) LiOH, H_2O, C_2H_5OH, 3 h; (iv) 4N HCl, 75 °C, 3 h; (v) Dowex AG50X4 (H^+)

Table 1. Agonist Activities of L-AP4 Analogues **1**−**4** at Group III mGlu Receptors[a]

agonists	EC$_{50}$ (μM)			
	mGlu4	mGlu6	mGlu7	mGlu8
L-AP4 (**1**)	0.080 ± 0.017 (10)	2.08 ± 0.38 (6)	440 ± 120 (5)	0.128 ± 0.019 (6)
DMPT (**2**)	4.05 ± 0.42 (5)	27.8 ± 2.7 (3)	>500	1.72 ± 0.17 (4)
PT (**3**)	1100 ± 670 (2)[b]	>500	>500	412 ± 72 (2)[b]
L-thioAP4 (**4**)	0.039 ± 0.006 (10)	0.73 ± 0.06 (6)	197 ± 55 (6)	0.054 ± 0.080 (5)

[a] Data are the mean ± SEM of *n* separate experiments. [b] Extrapolated value from partial dose-response curve.

In contrast, the same function is almost completely deprotonated in **4** because of the decrease of the pK_{a3} value to 5.56. As a result, the negative charge that bears the distal group of **4** is significantly increased in comparison to **1** and allows stronger interaction with the basic residues of the binding site.

Pharmacology. The effects of the three L-AP4 (**1**) analogues DMPT (**2**), PT (**3**), and L-thioAP4 (**4**) (Chart 1) were examined on all group III mGlu receptors (mGlu4, mGlu6, mGlu7, and mGlu8) (Table 1). These receptors were transiently expressed in HEK-293 cells as previously described.[42] Since group III mGlu receptors are not naturally coupled to phospholipase C but rather inhibit adenylyl cyclase, receptors were cotransfected with a chimeric G protein α subunit, which is recognized by these receptors but effectively activates the phospholipase C pathway. Thus, the functional assay consisted of measuring the total inositol phosphate production resulting from receptor activation.[42,43] 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 these receptors was not altered.[42]

All L-AP4 analogues exhibited an agonist activity at group III mGlu receptors (Figure 2). Deleting the second acidity of L-AP4 decreased drastically the potency of the resulting agonists **2** and **3**. Yet replacing the OH group by a hydrogen atom as in **2** was better tolerated than by a methyl group as in **3**. The potency of **2** was found in the same range as that of glutamate. In contrast, L-thioAP4 (**4**) turned out to be about 2-fold more active than L-AP4 (**1**) on all subtypes (Student's paired *t* test: $p < 0.05$). None of these analogues displayed any selectivity among group III mGlu receptor subtypes.

Selectivity versus groups I and II mGlu receptors was checked for all L-AP4 analogues. At 100 μM, no agonist or antagonist activities were detected on receptors belonging to these groups, indicating that L-AP4 and its analogues are selective group III mGlu receptor agonists (data not shown).

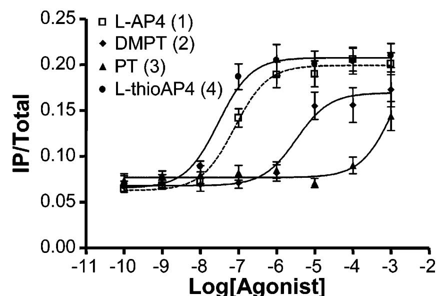


Figure 2. Dose-response curves of L-AP4 (**1**) and its analogues on mGlu4 receptors. Rat clone mGlu4 receptor was transiently transfected in HEK293 cells together with a chimeric G protein α subunit and the high-affinity glutamate transporter EAAC1. Receptor activity was determined by the accumulation of inositol phosphates (IP) resulting from receptor activation by various concentrations of agonists. Each point corresponds to the mean ± SEM of triplicates. Data are representative of at least $n = 3$ experiments.

Molecular Analysis. The molecular basis of the selectivity of **1** and **4** regarding group III mGlu receptors may be explained with the help of crystal structures[44,45] and homology models[46] of the ligand binding domain (LBD). This domain folds in two lobes and adopts open or closed conformations.[44,45] Agonists bind to lobe 1 in the open form of the LBD and are then trapped in the closed form, which was demonstrated to be required for receptor activation.[47] The closed conformation is stabilized by agonist interactions with both lobes. It is assumed that the better that stabilization is, the more potent the agonist is. Herein, we demonstrate that such an interpretation of the rank order of potency applies to the comparison of glutamate, L-AP4 (**1**), and L-thio-AP4 (**4**) bound to the closed form of the LBD of mGlu receptors.

Because of a high receptor similarity among each of the three mGluR groups, we have chosen one subtype of each group (mGlu1, mGlu3, and mGlu4) for our molecular analysis (see sequence alignment in Supporting Information). Thus, we used the X-ray structures of glutamate bound to mGlu1[44] and mGlu3[45] LBD (Protein Data Bank codes 1ewk:A and 2e4u) and a homology model of L-AP4 (**1**) bound to mGlu4 LBD.[46] We found that the crystal structure of mGlu7R bound to 2-(*N*-morpholino)ethanesulfonic acid (MES), a crystallization additive (PDB code 2e4z), was not advantageous to building a new homology model of mGlu4 LBD because it displays an open conformation of the domain and a limited resolution.[45] As a matter of fact, some side chains of residues likely contacting agonists are not solved (e.g., E405, K407, Q258 to R263) and MES does not bind to the signature motif that is found in all mGlu receptors.[48] However, we superimposed the residues (C_α atoms) of lobe 1 in our former 3D model of mGlu4 docked with L-AP4 (**1**) on those of the recent mGlu7R structure. The very good superimposition (rmsd = 1.05 Å) attests to the accuracy of our model (see Figure in Supporting Information). Moreover, side chains of the binding site residues of lobe 1 and hinge are well oriented in our homology model in comparison to those of the X-ray structure (Figure 3).

A close look at the binding residues around L-AP4 (**1**) reveals that five basic residues make ionic interactions with the distal phosphonate group. They are K74, R78, and K405 from lobe 1 and R258 and K317 from lobe 2 (Figure 4). Among them, three of these basic residues (R78, K405, and R258) are simultaneously bound to acidic residues (E403, D312, E287, D288) so that their positive charge is neutralized. The two remaining basic residues (K74 and K317) are neutralized by the negative charges of the phosphonate group. With glutamate holding only one negative distal charge, the electrostatic stabilization is weaker, resulting in a less potent agonist. A similar binding pattern as for L-AP4 (**1**) is found with L-thioAP4 (**4**) bound to mGlu4 receptor. Moreover, since the total charge of the thiophosphonate group is closer to 2 than with the L-AP4 (**1**) phosphonate group (see above), the electrostatic interaction of **4** to both lobes of the LBD is strengthened compared to **1**. The increased stabilization of the closed conformation of the LBD bound to L-thioAP4 (**4**) may explain its higher potency at the mGlu4 receptor binding site. The same interpretation may be suggested for the equally high potency of **4** at mGlu8 receptor because the binding pattern is the same at mGlu4 and mGlu8 binding sites. Interestingly the **1** and **4** EC_{50} increase at mGlu6 and mGlu7 receptors (Table 1) correlates with a nonoptimal stabilization of the LBD in its closed conformation. Indeed, K74

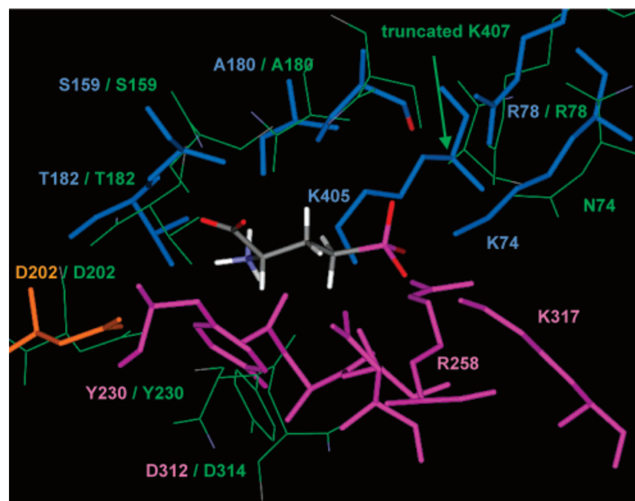


Figure 3. Superimposition of C_α atoms of lobe 1 residues of mGlu7R (X-ray structure PDB code 2e4z)[45] and of mGlu4R docked with L-AP4 (**1**) (homology model).[46] Residues 41–124, 150–202, 339–371 from mGlu4R and residues 41–124, 150–202, 341–373 from mGlu7 were superimposed with an rmsd of 1.05 Å. Only residues of the binding sites are displayed. Those of mGlu7R are colored green, and those of mGlu4R are in blue for lobe 1, in magenta for lobe 2, and in orange for the hinge. The side chain of K407 from mGlu7R is not present in the structure and may not be compared with the homologous residue in mGlu4 receptor. Atom colors for L-AP4 are gray for carbon, red for oxygen, blue for nitrogen, white for hydrogens, magenta for phosphorus. Except for the ligand, hydrogen atoms are not displayed for clarity.

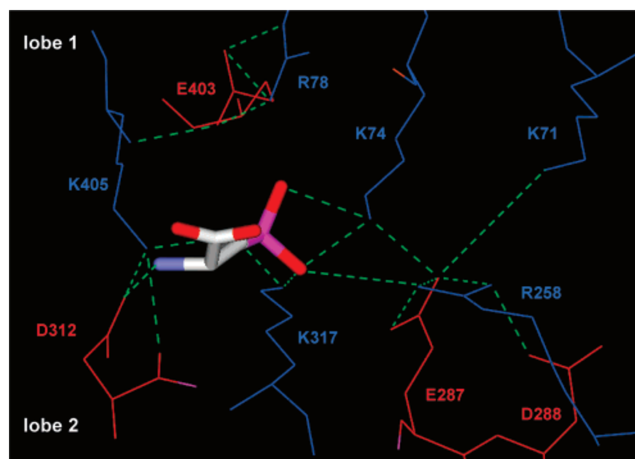


Figure 4. L-AP4 (**1**) docked at mGlu4R LBD. Basic residues are colored blue, acidic ones are in red. For L-AP4, carbon atoms are white, oxygen red, nitrogen blue, phosphorus magenta. Hydrogens have been removed for clarity. Polar interactions (hydrogen bonds and ionic interactions) are shown as green dotted lines. K71 is too far to interact with **1** but participate in the positioning of E287.

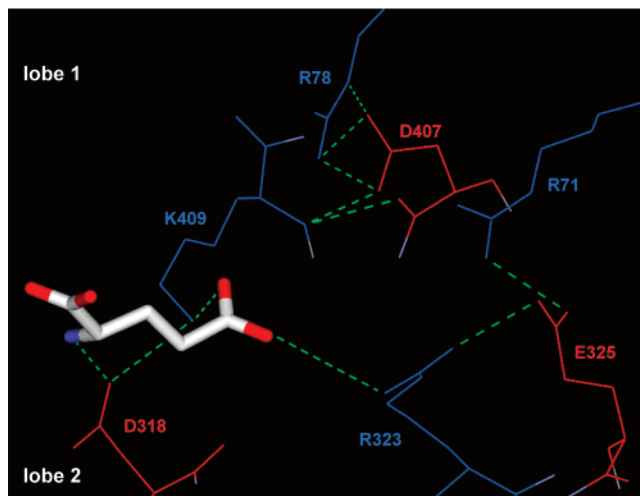


Figure 5. Crystal structure of glutamate bound to the closed form of mGlu1R LBD (PDB code 1ewk:A). Carbon atoms are white, oxygen red, nitrogen blue, phosphorus magenta. Hydrogens have been removed for clarity. Polar interactions (hydrogen bonds and ionic interactions) are shown as green dotted lines. R71 is too far to interact with glutamate but participate in the positioning of E325.

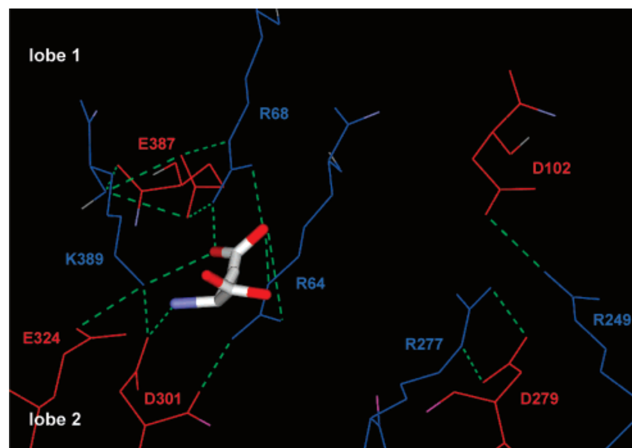


Figure 6. Crystal structure of glutamate bound to the closed form of mGlu3R LBD (PDB code 2e4u). Carbon atoms are white, oxygen red, nitrogen blue, phosphorus magenta. Hydrogens have been removed for clarity. Polar interactions (hydrogen bonds and ionic interactions) are shown as green dotted lines. R249 and R277 are too far to interact with glutamate because of their interactions with D102 and D279.

is replaced by a glutamine (Q58) in mGlu6 and by an aspargine (N74) in mGlu7 (see alignment in Supporting Information) so that one of the ionic interaction is missing in comparison to **1**/**4** bound to mGlu4/8. Rosemond et al. actually showed that mutating those Q/N residues in mGlu6/7 receptors to K increased significantly the affinity of L-AP4 (**1**) in these receptors.[49] In addition, another basic residue analogous to R258 in mGlu4 receptor is missing in mGlu7 receptor (Q258), decreasing even more the stability of the active conformation of the LBD bound to **1** or **4**.[49] Nevertheless, in all group III mGlu receptors, the lysine of lobe 2 (K317 at mGlu4, K306 at mGlu6, K319 at mGlu7, and K314 at mGlu8R) that makes a strong electrostatic interaction with **1** and **4** is conserved. Because this interaction is stronger with **4**, potency of **4** is higher than that of **1** for all group III receptors.

The crystal structure of glutamate bound to mGlu1 receptor in the closed conformation of the LBD[44] shows that glutamate is bound to the conserved R78 and K409 of lobe 1 and to R323 from lobe 2, making a total of three basic residues (Figure 5). Each of these basic residues is also bound to an acidic residue (D407, D318, E325) so that the negative charge of the distal acidic group of glutamate does not seem to be mandatory. Indeed (*S*)-3,5-dihydroxyphenylglycine, a well-known agonist of group I mGlu receptors, does not hold a distal charge. Yet the distal charge of glutamate allows a stronger interaction between K409 and glutamate in the open form of the LBD, in the first step of the activation process. L-AP4 (**1**) and L-thioAP4 (**4**) have no effect at mGlu1/5 receptors. This inactivity may be due to the tetrahedral geometry of the phosphonate (compared to the planar structure of a carboxylate) that would not fit to the hydrogen bond network observed around the distal function of glutamate in the crystal structure.[44] One other possible explanation may be that if the ligand binds to lobe 1 of the LBD, the additional negative charge may then interact with some basic residues of lobe 2 that previously adopted a different conformation (e.g., R323 of mGlu1R). The distal acidic group may then adopt a different position that could also disrupt the hydrogen bond set and destabilize the closed conformation of the LBD.

The crystal structure of glutamate bound to mGlu3 receptor in the closed conformation of the LBD[45] shows that glutamate is bound to R68, K389, and R64 of lobe 1 and to none of the basic residues of lobe 2 (Figure 6). Two of the three basic residues are bound to acidic residues (R68 to E387 and K389 to E324); consequently, only one negative charge is needed on the agonist to afford a neutral system. A similar binding pattern may be expected for mGlu2 receptor according to the sequence alignment (see Supporting Information). Accordingly, the additional negative charges of L-AP4 (**1**) and L-thioAP4 (**4**) in comparison to glutamate do not afford any additional interactions with the protein. On the opposite, once the ligand is bound to the first lobe, the additional negative charge may then interact with some basic residues of lobe 2 that previously adopted a different conformation (e.g., R271 of mGlu2, R277 of mGlu3). The closing of the LBD may be modified preventing the activation of the receptor. As a matter of fact, L-AP4 (**1**) was described as a modest mGlu2 receptor antagonist.[50] It demonstrates that L-AP4 (**1**) is able to bind to lobe 1 but that full closing of the LBD is hampered.

Several mutagenesis studies have been performed to better define the molecular determinants of the mGluR selectivity.[49,51−53] They demonstrate that agonist selectivity is derived from a set of distal residues that is specific to each group of mGlu receptors. The residues discussed in the present study are part of those sets. The geometry and the charge of the phosphonate and thiophosphonate groups of L-AP4 and L-thioAP4 fit best to the group III receptor cluster and not to those of group I/II receptors as explained above. This situation defines the molecular basis of the high potency and selectivity of L-AP4 and L-thioAP4 regarding the activation of group III mGlu receptors.

Discussion

Numerous preparations of L-AP4 have been published over decades; some used natural amino acids as the starting material.[54,55] The synthesis presented herein took advantage of the mild conditions of a radical condensation between vinylglycine and hypophosphorous acid.[21]

Potency and selectivity of group III mGlu receptor agonists are brought about by an additional acidic function that may be a carboxylic acid or the second acidity of a phosphonate group hold by glutamate analogues (Chart S1). Indeed, this acidity seems to be critical because L-homocysteic acid, which is isosteric to L-AP4 (**1**) but with only one distal acidic moiety, shows no enhanced activity compared to glutamate at group III mGlu receptors.[56] The purpose of the present study was to

further demonstrate the requirement for such an additional group for high activity. Accordingly, we were expecting that decreasing the acidity of the L-AP4 phosphonate group would lead to a less potent agonist and that increasing acidity would result in a more potent one.

Replacing the second OH of the phosphonate group of L-AP4 (**1**) by a proton in **2** causes a loss of 12- to 50-fold in activity at group III mGlu receptors, resulting in analogous potency to glutamate or L-homocysteic acid (Table 1). An analogous replacement of the carboxylate function of GABA by a phosphinate resulted in increased activity at the $GABA_B$ receptor and much weaker effect at the $GABA_A$ receptor.[27] Although the geometry of the H-phosphinic group of **2** is tetrahedral and that of γ-carboxylate of glutamate is planar, their global charge (−1) is identical at physiological pH. A similar observation is made with L-homocysteic acid bearing a distal tetrahedral sulfonate group. We conclude that in comparaison with L-AP4, the presence of two negative charges on the distal group of the amino acid ligands is likely to be responsible for the observed increase in potency. We then anticipated that replacing the second OH of the phosphonate group of L-AP4 (**1**) by a hydrophobic group would be deleterious. We chose phosphinothricin (PT, **3**), also named glufosinate[57] a well-known inhibitor of glutamine synthase and as such a widely used herbicide.[58] PT (**3**) is the methyl phosphinate analogue of L-AP4; its distal methyl group completely abolishes activity at group III mGlu receptors (Table 1).[12] This result is consistent with a hydrophilic environment around the phosphonate of L-AP4 at the binding site. In contrast, PT (**3**) is one of the most potent inhibitors of glutamine synthase while L-AP4 is 100-fold weaker.[59] We next synthesized and tested L-thioAP4 (**4**), a sulfur analogue of L-AP4. L-thioAP4 (**4**) was able to activate group III mGlu receptors more efficiently than L-AP4 because of its stronger acidity as detailed below. To date, L-thioAP4 (**4**) is the most potent agonist described for these receptors.

L-thioAP4 (**4**) may exist in two tautomeric thiolo (P−SH) or thiono (P═S) forms in aqueous solution. Studies indicate that the thiono−thiolo equilibrium lies far on the thiono side in phosphonothioic acids[60,61] (structure shown for **4** in Chart 1). Sulfur makes much weaker hydrogen bonds than oxygen; thus, the increased potency of L-thioAP4 may not result from such interactions. The particularity of the sulfur replacing the oxygen atom of the phosphoryl group (P═S replacing P═O) is that it has a major effect on the second acidity of the phosphonate. Calculated pK_a values using the SPARC on-line calculator[37] for the **1** distal group (pK_{a2}, pK_{a3}) are 2.8 and 7.4, while those of **4** (pK_{a1}, pK_{a3}) are 2.1 and 3.8. In contrast, introducing withdrawing substituants in the 4 position of L-AP4 as in 4,4-difluoro substituted L-AP4[36] affects both acidities because predicted pK_a values are 1.15 and 5.80. Dissociation of the second (thio)phosphonate acid group (pK_{a3}) is critical for the charge of this moiety at neutral pH. Experimental values were determined by ^{31}P NMR titration to be 6.88 and 5.56 for **1** and **4**, respectively. Thus, at physiological pH, while L-AP4 (**1**) is only partialy ionized, the thiophosphonate group of L-thioAP4 (**4**) is almost totally deprotonated and allows stronger electrostatic interaction with the five basic residues of the binding site that were identified in the 3D model of L-AP4 docked at the mGlu4 receptor binding site[46] (Figure 4). These interactions stabilize the active conformation of the binding domain, which in turn triggers receptor activation. Most of the basic residues shown in Figure 3 are conserved among the four subtypes of group III mGlu receptors; as a consequence, L-thioAP4 (**4**) is not subtype selective. However, like L-AP4 (**1**), L-thioAP4 (**4**) is a group III selective agonist, displaying no agonist or antagonist activity on other groups of mGlu receptors. The potency and selectivity of L-AP4 (**1**) and L-thioAP4 (**4**) may be explained at the molecular level analyzing X-ray structures[44,45] and homology models.[46] While the negative charges of their distal (thio)phosphonate group allow strong ionic interactions with the highly basic distal pocket of mGlu4/8 receptors, they allow no additional interactions at group I/II receptor binding sites in comparison with bound glutamate. In contrast, for these latter receptors, the extra charge may be deleterious because it may perturb the polar binding network around the ligand and prevent it from reaching the active conformation of the LBD.

L-AP4 does not cross the blood−brain barrier, and it is probable that L-thioAP4 will not either, preventing this compound from becoming a potential medicine. However, because L-thioAP4 is more potent than L-AP4, once radiolabeled, it may become a useful pharmacological tool and allow us to perform binding experiments that were limited up to now. Furthermore, the structure−activity analysis of this compound disclosed new molecular features that will allow the design of more potent group III mGluR agonists, which in turn may be developed as new drugs for psychiatric or neurodegenerative diseases and neuropathic pain relief.

In conclusion, we have demonstrated that replacing the phosphonate by a phosphinic group in L-AP4 (**2** and **3**, Chart 1) resulted in loss of activity. On the other hand, changing the phosphonate to a thiophosphonate (**4**, Chart 1) had the opposite effect. The enhanced potency of **4** is attributed to the increased second acidity of the thiophosphonate group and complete deprotonation of this group at physiological pH. Taken together, these results confirm the critical role of the additional acidic function and its negative charge in glutamate analogues that are group III mGlu receptor agonists and led us to identify L-(+)-2-amino-4-thiophosphonobutyric acid **4** that we named L-thioAP4 as the most potent group III mGlu receptor agonist (EC_{50} = 0.039, 0.73, 197, 0.054 μM at mGlu4, 6, 7, 8 respectively). Structural interpretation of the increased potency of L-thioAP4 disclosed key molecular determinants that will allow the design of future potent agonists.

Experimental Section

Chemistry. All chemicals and solvents were purchased from commercial suppliers (Acros, Aldrich) and used as received. Glufosinate ammonium salt (PT **3**) and Z-L-α-vinylGlyOMe (*N*-benzyloxycarbonyl-α-vinylglycine methyl ester) were purchased from Riedel-de Haën (Sigma-Aldrich) and Ascent Scientific Ltd. (North Somerset, U.K.), respectively. ^{1}H (250.13 MHz), ^{13}C (62.9 MHz), and ^{31}P (101.25 MHz) NMR spectra were recorded on an ARX 250 Bruker spectrometer. Chemical shifts (δ, ppm) are given with reference to residual ^{1}H or ^{13}C of deuterated solvents ($CDCl_3$ 7.24, 77.00; CD_3OD 3.30, 49.0; D_2O 4.80). For ^{31}P NMR chemical shifts, SR values of −16 664.43 Hz in D_2O and −15 643.78 Hz in CD_3OD that were previously determined with an external reference (H_3PO_4 95%) were used for calibration; the external reference was used for the titration experiments. Product visualization was achieved with 2% (w/v) ninhydrin in ethanol. Optical rotations were measured at the sodium D line (589 nm) at room temperature with a Perkin-Elmer 341 polarimeter using a 0.1 or 1 dm path length cell. Mass spectra (MS) were recorded with a LCQ-Advantage (ThermoFinnigan) mass spectrometer with positive (ESI+) or negative (ESI−) electrospray ionization (ionization tension of 4.5 kV, injection temperature of 240 °C). Molecular models and 3D structures were displayed using Discovery Studio 1.6 (Accelrys, San Diego, CA).

Methyl (2*S*)-2-(*N*-Benzyloxycarbonyl)amino-4-[(hydroxy)-phosphinyl]butanoate (5). A mixture of hypophosphorous acid (H_3PO_2, 660 mg, 5 mmol, 50% aqueous), *N*-benzyloxycarbonyl-

L-α-vinylglycine methyl ester (Z-L-α-vinylGlyOMe, 249.3 mg, 1 mmol), and α,α′-azoisobutyronitrile (AIBN, 8.2 mg, 0.05 mmol) in methanol (1 mL) was refluxed at 80 °C for 5 h. Then the methanol was evaporated under vacuum and the residue was treated with 15 mL of water and extracted with ethyl acetate (125 mL). The organic solution was washed with 10 mL of water, dried over anhydrous $MgSO_4$, and evaporated under vacuum to afford **5** (296 mg, 94% yield). ^{1}H NMR (CD_3OD): δ 1.98 (m, 4H), 3.72 (s, 3H), 4.11 (m, 1H), 5.12 (s, 2H), 7.08 (d, J_{PH} = 565 Hz, 1H), 7.34 (m, 5H). ^{13}C NMR (CD_3OD): δ 23.4, 26.1 (d, J = 92 Hz), 52.2, 54.7, 66.9, 128.0, 128.3, 128.7, 137.2, 157.5, 172.7. ^{31}P NMR (CD_3OD): δ 35.3.

(2*S*)-2-(*N*-Benzyloxycarbonyl)amino-4-phosphonobutyric Acid Methyl Ester (6). A mixture of **5** (284 mg, 0.90 mmol), DMSO (70 mg, 0.9 mmol), and iodine (1 mg) in 3 mL of THF was stirred under heating at 60 °C for 5 h. The resulting mixture was evaporated to dryness under vacuum to give **6** (292 mg, 98% yield). ^{1}H NMR (CD_3OD): δ 1.94 (m, 4H), 3.72 (s, 3H), 4.31 (m, 1H), 5.12 (s, 2H), 7.35 (m, 5H). ^{31}P NMR (CD_3OD): δ 29.61. ^{13}C NMR (CD_3OD): δ 23.76 (d, J = 139.79 Hz), 25.50, 52.16, 54.82 (d, J = 18.56 Hz), 66.81, 127.99, 128.25, 128.71, 137.22, 157.53, 172.90.

L-(+)-2-Amino-4-phosphonobutyric Acid (1). Compound **6** was dissolved in 5 mL of 6 N HCl. The mixture was heated at 100 °C for 5 h, and the resulting solution was cooled to room temperature. Volatile organic byproducts and water were removed under vacuum, and the residue was purified using a Dowex AG50X4 cation exchange resin column (H^+, 20−50 mesh, 24 cm × 1.7 cm, water elution). The fractions that gave positive color reaction with ninhydrin were combined and evaporated under vacuum to give **1** (quantitative yield). ^{1}H NMR (D_2O): δ 1.70 (m, 2H), 2.12 (m, 2H), 3.99 (t, J = 6.02 Hz, 1H). ^{31}P NMR (D_2O): δ 35.42. ^{13}C NMR (D_2O): δ 23.61 (d, J = 135.42 Hz), 24.68, 53.91 (d, J = 13.37 Hz), 172.49. MS (ESI−) *m/z* 182.2 (M − 1). $[\alpha]_D^{20}$ +13.2 (*c* 1.0, H_2O), lit.[55] $[\alpha]_D^{20}$ +10.3 (*c* 2.0, H_2O).

L-(+)-2-Amino-4-[(hydroxy)phosphinyl]butyric Acid (2). The removal of the protecting groups in compound **5** (296 mg, 0.94 mmol) was accomplished following the same procedure as that followed for the deprotection of compound **6**, to afford **1**. Compound **2** was purified by water elution on a Dowex AG50X4 column as described for **1** (quantitative yield). ^{1}H NMR (D_2O): δ 1.66 (m, 2H), 2.06 (m, 2H), 4.01 (t, J = 6.07 Hz, 1H), 6.90 (d, J_{PH} = 524.5 Hz, 1H). ^{31}P NMR (D_2O): δ 39.91. ^{13}C NMR (D_2O): δ 22.66, 26.76 (d, J = 88.81 Hz), 53.67 (d, J = 16.16 Hz), 172.20. MS (ESI−) *m/z* 166.3 (M − 1). $[\alpha]_D^{20}$ +7.9 (*c* 0.4, H_2O).

Methyl (2*S*)-2-(*N*-Benzyloxycarbonyl)amino-4-thiophosphonobutanoate (7). To a mixture of **5** (296 mg, 0.94 mmol) and sulfur powder (96 mg, 3 mmol) in 2 mL of methylene chloride at 0 °C under an argon atmosphere was added dropwise *N*,*O*-bis(trimethylsilyl)acetamide (BSA) (814 mg, 4 mmol). The mixture was allowed to warm to room temperature and stirred for 1 h and then cooled to 0 °C, and an amount of 15 mL of 1 N HCl was added, then extracted with ethyl acetate (2 × 100 mL). The combined organic solution was dried over anhydrous $MgSO_4$ and concentrated in vacuo (302 mg, 93%). ^{1}H NMR (CD_3OD): δ 2.10 (m, 4H), 3.78 (s, 3H), 4.32 (m, 1H), 5.11 (s, 2H), 7.33 (m, 5H). ^{31}P NMR (CD_3OD): δ 87.23. ^{13}C NMR (CD_3OD): δ 26.21, 32.46 (d, J = 108.38 Hz), 52.04, 54.51, 66.83, 127.87, 128.13, 128.60, 137.11, 157.58, 173.08.

(2*S*)-2-(*N*-Benzyloxycarbonyl)amino-4-thiophosphonobutyric Acid (8). To a solution of **7** (302 mg, 0.87 mmol) in ethanol (5 mL) at room temperature was added $LiOH \cdot H_2O$ (126 mg, 3 mmol) in ethanol/water (10 + 10 mL). The mixture was stirred at the same temperature for 3 h. Then 15 mL of 1 N HCl was added and extracted with ethyl acetate (2 × 75 mL). The organic extracts were combined, dried over anhydrous $MgSO_4$, and concentrated under vacuum to give **8** (257 mg, 89% yield). ^{1}H NMR (CD_3OD): δ 1.91 (m, 4H), 4.03 (m, 1H), 5.12 (s, 2H), 7.36 (m, 5H). ^{31}P NMR (CD_3OD): δ 87.36. ^{13}C NMR (CD_3OD): δ 26.31, 32.51 (d, J = 108.38 Hz), 54.51 (d, J = 18.12 Hz), 66.80, 127.82, 128.09, 128.56, 137.11, 157.68, 174.30.

L-(+)-2-Amino-4-thiophosphonobutyric Acid (4). The crude compound **8** (147 mg, 0.44 mmol) was treated with 4 mL of 4 N HCl and stirred at 75 °C for 3 h. The reaction mixture was concentrated under vacuum, and the residue was purified using a Dowex AG50X4 cation exchange resin column (H^+, 20−50 mesh, 24 cm × 1.7 cm, water elution, 12 mL fractions, ninhydrin product visualization). Collection of fractions 4−20 gave 53 mg of **4** containing 20% of L-AP4 **1**. This mixture was reloaded on the same column and eluted with water. Fractions 4−9 afforded pure **4** (19 mg, 0.095 mmol, 22% yield) after evaporation. ^{1}H NMR (D_2O): δ 1.97 (m, 2H), 2.24 (m, 2H), 4.16 (t, J = 6.03 Hz, 1H). ^{31}P NMR (D_2O): δ 86.69. ^{13}C NMR (D_2O): δ 25.06, 32.28 (d, J = 103.28 Hz), 53.08, 171.85. MS (ESI+) *m/z* 200.0 (M + 1). $[\alpha]_D^{20}$ +17 (*c* 1.0, H_2O).

pK_a Determination of L-AP4 (1) and L-thioAP4 (4). Each ^{31}P NMR spectrum was acquired at 27 °C with external H_3PO_4 (95%) reference at 0 ppm (sealed capillary). Compound **1** (12 mg) or **4** (6.4 mg) was dissolved in 0.54 mL of H_2O and 0.06 mL of D_2O. The pH of the solution was adjusted with a small volume (1−6 μL) of concentrated HCl or 2 M NaOH solution. A total of 27 spectra (**1**) or 23 spectra (**4**) were recorded over a pH range of 0.50−12.50 (**1**) or 0.51−12.56 (**4**). The ^{31}P NMR chemical shifts of the dianionic and monoanionic forms of the (thio)phosphonate groups were plotted against measured pH. All pK_a values were calculated by nonlinear regression analysis using the GraphPad Prism program (GraphPad Software Inc., San Diego, CA) and the equation $pH = pK_a - \log[(\delta_a - \delta)/(\delta - \delta_b)]$, where δ is the ^{31}P NMR chemical shift at varying pH and δ_a and δ_b are the ^{31}P NMR chemical shifts with titrating group in fully acidic and basic forms, respectively.

Pharmacology. Cell Culture and Transfection. Pharmacological experiments were carried out using HEK293 cells. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin and streptomycin, 100 U/mL final).

Cells were transiently transfected with rat clones of group III mGlu receptors (mGlu4, mGlu6, mGlu7, and mGlu8) by electroporation as described elsewhere[62] and plated in 96-well microplates. The high-affinity glutamate transporter EAAC1 was also cotransfected with the receptor in order to avoid any influence of glutamate released by the cells in the assay medium. Since group III mGluRs activates naturally Gi/o proteins that modulate the adenylyl cyclase pathway, these receptors were cotransfected with a chimeric G protein that is recognized by these receptors but couples to the phospholipase C pathway, thus leading to inositol phosphate (IP) production following receptor activation.[42] Receptor activity was then determined by measurement of the IP production.

Culture medium, FCS, and other products used for cell culture were purchased from GIBCO-BRL-Life Technologies, Inc. (Cergy Pontoise, France). Glutamate pyruvate transaminase (GPT) was purchased from Roche (Basel, Switzerland). ^{3}H-Myoinositol (16 Ci/mmol) was purchased from Amersham (Saclay, France).

Functional Assay. Inositol phosphate determination experiments in 96-well microplates were performed as already described.[63] Briefly, 6 h following transfection, the cell medium was removed and replaced by fresh medium devoid of glutamate, not complemented with FCS, and that contained ^{3}H-myoinositol. Cells were incubated overnight in this medium. The following day, cells were rinsed and ambient glutamate was degraded by incubation in the presence of GPT. Cells were stimulated by agonist for 30 min. Then the medium was removed and cells were incubated for 1 h with cold 0.1 M formic acid, which induced cell lysis. The ^{3}H-IP produced following receptor stimulation was recovered by ion exchange chromatography using a Dowex resin (Biorad). IP kept by the resin was then eluted by a 4 M formate solution (pH 4.4) and collected in a 96-well sample plate. Samples were then mixed with liquid scintillator (Perkin-Elmer). In order to minimize well to well variability due to difference in cell density, the radioactivity remaining in the membranes, which is proportional to the quantity of cells in each well, was used to normalize the IP produced. Membranes were solubilized with a solution of NaOH

(0.1 M) containing 10% of Triton X100 (Sigma). The resulting solution was then collected in a 96-well sample plate and mixed with liquid scintillator. Radioactivity was counted using a Wallac 1450 Microbeta stintillation and luminescence counter (Perkin-Elmer). Results are expressed as the ratio of IP to the total radioactivity corresponding to IP plus membrane (Figure 2). All points are realized in triplicate. The dose-response curves were fitted using the GraphPad Prism program and the following equation: $y = [(y_{max} - y_{min})/(1 + (x/EC_{50})n)] + y_{min}$, where EC_{50} is the concentration of the compound necessary to obtain the half-maximal effect and n is the Hill coefficient.

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Supporting Information Available: Chart S1, structures of group III mGlu receptor agonists (ACPT, DCPG, SOP, APCPr, and PPG); ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, and mass spectra of **1**, **2**, and **4**; sequence alignment of the eight mGlu receptors; superimposition of mGlu7R and mGlu4R amino terminal domain traces. This material is available free of charge via the Internet at http://pubs.acs.org.

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