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A Virtual Screening Hit Reveals New Possibilities for Developing Group III Metabotropic Glutamate Receptor Agonists

Chelliah Selvam,[†,⊥] Nadia Oueslati,[‡,§] Isabelle A. Lemasson,[†] Isabelle Brabet,[‡,§] Delphine Rigault,[†] Tiphanie Courtiol,[†] Sara Cesarini,[†] Nicolas Triballeau,[†,∥,#] Hugues-Olivier Bertrand,[∥] Cyril Goudet,[‡,§] Jean-Philippe Pin,[‡,§] and Francine C. Acher*[,†]

[†]*Laboratoire de Chimie et de Biochimie Pharmacologiques et Toxicologiques, CNRS UMR8601, Université Paris Descartes, 45 Rue des Saints-Pères, 75270 Paris Cedex 06, France,* [‡]*Institut de Génomique Fonctionnelle, Université de Montpellier, CNRS UMR5203, 34094 Montpellier, France,* [§]*INSERM, U661, 39094 Montpellier, France, and* [∥]*Accelrys SARL, Parc-Club Université, 20 Rue Jean Rostand, 91898 Orsay Cedex, France.* [⊥] *Current address: Department of Chemistry, State University of New York at Binghamton, Binghamton, New York 13902.* [#]*Current address: Galápagos SASU, 102 Avenue Gaston Roussel, 93230 Romainville, France.*

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(*R*)-PCEP (3-amino-3-carboxypropyl-2′-carboxyethyl phosphinic acid, **1**), a new metabotropic glutamate receptor 4 (mGlu4R) agonist, was discovered in a previously reported virtual screening. The (*S*)-enantiomer and a series of derivatives were synthesized and tested on recombinant mGlu4 receptors. A large number of derivatives activated this receptor but was not able to discriminate between mGlu4 and mGlu8 receptors. The most potent ones **6** and **12** displayed an EC_{50} of 1.0 ± 0.2 μM at mGlu4R. Interestingly these agonists with longer alkyl chains revealed a new binding pocket adjacent to the glutamate binding site, which is lined with residues that differ among the mGluR subtypes and that will allow the design of more selective compounds. Additionally **6** was able to activate mGlu7 receptor with an EC_{50} of 43 ± 16 μM and is thus significantly more potent than L-AP4 (EC_{50} of 249 ± 106 μM).

Introduction

Many studies have demonstrated that metabotropic glutamate receptors (mGluR) can serve as new therapeutic targets for a wide panel of brain disorders (ischemia after-effects, convulsions, Parkinson's disease, anxiety, schizophrenia, pain, drug withdraw symptoms, ...). In contrast to glutamate-gated channel receptors (iGluR[a]) that mediate fast synaptic transmission, mGlu receptors fine-tune such transmission by modulating either the release process or the postsynaptic response. These receptors are G-protein-coupled receptors (GPCRs) that belong to class C[1] and are divided into three groups on the basis of sequence similarities, transduction mechanism, and pharmacological profile. Group I receptors (mGlu1 and mGlu5) couple to activation of PLC, while group II (mGlu2, mGlu3) and group III (mGlu4, mGlu6, mGlu7, mGlu8) receptors couple to adenylyl cyclase inhibition. Group I receptors are mostly postsynaptic, while most group II/III receptors are located on the presynaptic terminals of both glutamatergic and nonglutamatergic synapses.[2] Activation of these presynaptic receptors inhibits the release process, as observed with many other Gi/o-coupled presynaptic GPCRs. Such a presynaptic effect may be beneficial in many neurological disorders, stimulating drug discovery programs aimed at identifying new activators or enhancers of group II/III mGlu receptors.

While initially all efforts were devoted to the search for orthosteric agonists by rational design, they shifted to allosteric modulators when these were discovered through high throughput screening campaigns. Indeed allosteric modulators present unquestionable advantages, showing high subtype selectivity among mGluRs and having both druglike structures and the ability to enhance receptor activity only when and where biologically needed.[3,4] However, such drugs may face some drawbacks such as off-target activity, weak solubility, metabolic instability, or poor pharmacokinetic profile. In addition, many of the identified positive allosteric modulators display significant agonist activity, thus limiting the theoretical advantage of the pure allosteric modulation. Conversely, agonists of the amino acid type, which have a highly polar structure, high solubility, and low ability to target other proteins, offer some distinct advantages. Altogether, development of both types of ligands can be appropriate if they fulfill all specifications (selectivity, solubility, brain penetration, metabolic stability).

In the pharmaceutical industry, both orthosteric and allosteric ligands have reached phase II clinical trials with encouraging results in the treatment of Parkinson's disease levodopa-induced dyskinesia (PD-LID) for AFQ056 (Novartis), gastroesophageal reflux disease (GERD)[5] and migraine for ADX10059 (Addex), and schizophrenia for LY404039 (Eli Lilly).[6] AFQ056 and ADX10059 are negative allosteric modulators of mGlu5

*To whom correspondence should be addressed. Phone: +33 (0)1 42 86 33 21. Fax: +33 (0)1 42 86 83 87. E-mail: francine.acher@parisdescartes.fr.

[a]Abbreviations: AIBN, α,α′-azoisobutyronitrile; AMN082, *N*,*N*′-bis(diphenylmethyl)-1,2-ethanediamine dihydrochloride; AP4, 2-amino-4-phosphonobutyric acid; thioAP4, 2-amino-4-thiophosphonobutyric acid; ACPT, 1-aminocyclopentane-1,3,4-tricarboxylic acid; APCPr, 1-amino-2-phosphonomethylcyclopropane carboxylic acid; BSA, *N*,*O*-bis(trimethylsilyl)acetamide; DCG-IV, (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropylglycine; DCPG, 3,4-dicarboxyphenylglycine; FP429, (2*S*,4*S*)-4-amino-1-[(*E*)-3-carboxyacryloyl]pyrrolidine-2,4-dicarboxylic acid; IMP inositol monophosphate; iGluR, ionotropic glutamate receptors; PCEP, 3-amino-3-carboxypropyl-2′-carboxyethylphosphinic acid; PHCCC, *N*-phenyl-7-(hydroxyimino)cyclopropa[*b*]chromen-1a-carboxamide; PPG, 4-phosphonophenylglycine; TMSCl, trimethylsilyl chloride; VU0155041, *cis*-2-(3,5-dichlorophenylcarbamoyl)cyclohexanecarboxylic acid; VU0361737, *N*-(4-chloro-3-methoxyphenyl)picolinamide.

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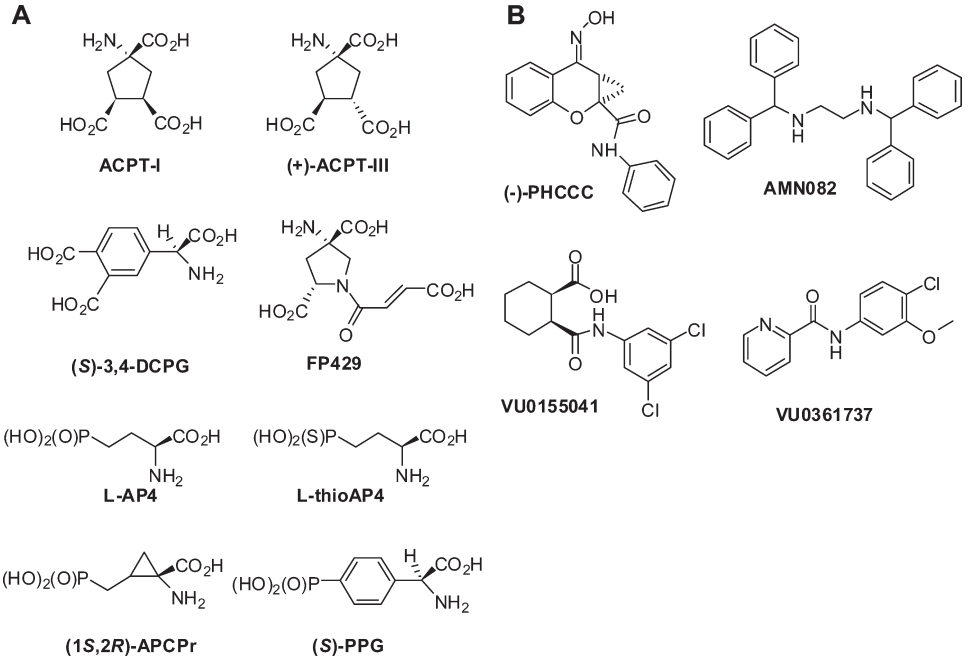
Chart 1. Ligands of Group III mGlu Receptors: (A) Orthosteric Agonists and (B) Positive Allosteric Modulators

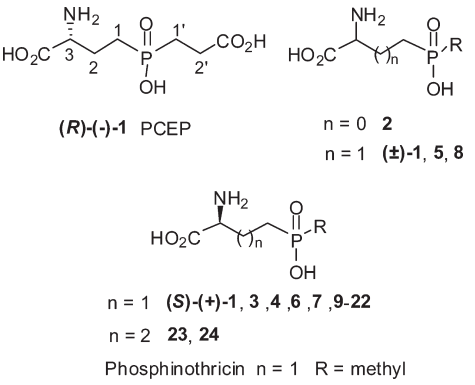


receptor, while LY404039 is an orthosteric agonist of group II receptors. Much less has been discovered regarding the activation or blockade of group III mGluRs.[7] L-AP4 has been known as the most potent group III agonist for many years. Several other agonists of the amino acid type have been described. They hold two acidic functions on their side chain, either two carboxylic groups (ACPT-I, (*S*)-DCPG, FP429, Chart 1A) or a phosphonate (L-Ser-*O*-phosphate, L-thioAP4, (1*S*,2*R*)-APCPr, (*S*)-PPG, Chart 1A).[8] Alternatively, positive allosteric modulators (PAM) have been discovered: PHCCC,[9] VU015504,[10] VU0361737[11] for mGlu4R, and AMN082[12] for mGlu7R (Chart 1B).

One major recognized limitation of orthosteric agonists is their lack of strong subtype selectivity. This likely results from the very high conservation of the glutamate binding site within all mGluRs from a given group. Our aim here was to examine whether longer chain analogues of glutamate could still act as mGlu4R agonists, with a long-term aim of identifying more selective compounds able to reach less conserved regions outside the glutamate binding pocket.

The virtual screening strategy has been used only rarely for discovering mGluR ligands[13] and has not been applied for group III receptors. Consequently, we recently chose this strategy for identifying new possible ligands for activating mGlu4 receptors. We previously described the vHTS workflow that we set up to screen the binding pocket of the mGlu4 receptor.[14] We used our homology models of the binding pocket that had been validated. Out of 720 000 commercially available compounds, we identified five of them that were able to activate the mGlu4 receptor. Yet only one induced full activation when applied at 100 μM.[14] In this article, we disclose its structure and pharmacological properties. In addition, its chemical optimization revealed a noticeable number of new group III mGlu receptor agonists. Considering the limited number of agonists of this type that have been discovered to date (Chart 1), this series (Chart 2) may be considered as a breakthrough in the domain, since it reveals new possibilities for developing original group III mGlu receptor agonists.

Chart 2. Compounds Synthesized and Tested as Group III mGlu Receptor Agonists[a]



[a] For R groups, see Table 1.

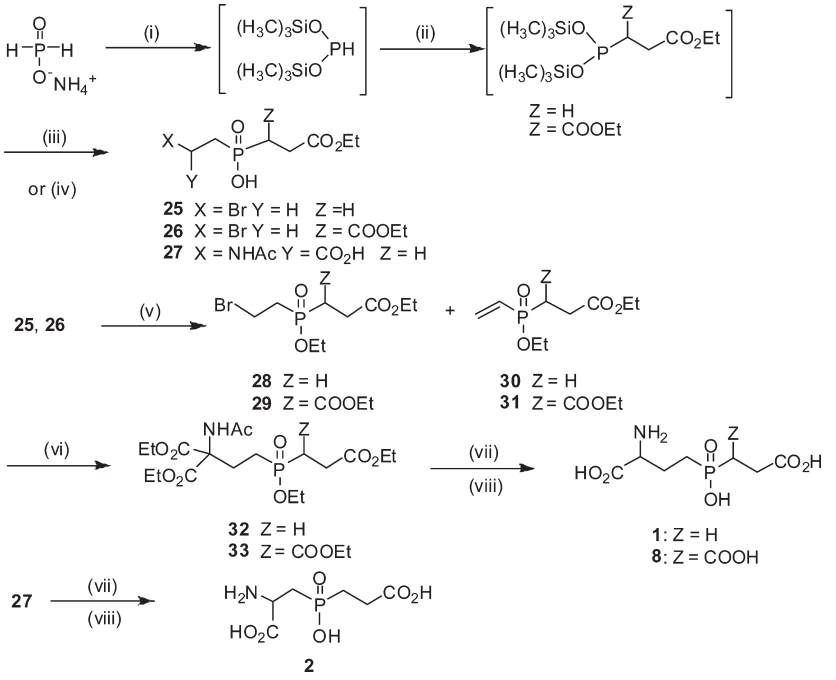
Results

PCEP a New mGlu4 Receptor Agonist. Searching for new mGlu4 receptor agonists by means of a virtual screening approach, we identified (*R*)-PCEP (3-amino-3-carboxyPropyl-2′-CarboxyEthylPhosphinic acid, **1**, Chart 2). This compound was able to fully activate, at 100 μM, the mGlu4 receptor transiently expressed in HEK-293 cells[14] and was selected for chemical optimization. We first synthesized the racemic mixture (*RS*)-PCEP and then the (*S*)-enantiomer that displayed higher potency than the initial hit (see below). Hence, various analogues of (*S*)-PCEP (Chart 2) were designed, prepared, and tested to establish a structure−activity relationship and define the optimal features for mGlu4 receptor activation.

Chemistry. All derivatives of PCEP are phosphinic acids (**1**−**24**, Chart 2), and their syntheses consist of a double alkylation of hypophosphorous acid (H_3PO_2). A retrosynthetic analysis and literature survey suggested various possible approaches among which we chose the synthetic routes depicted in Schemes 1, 2, and 3.

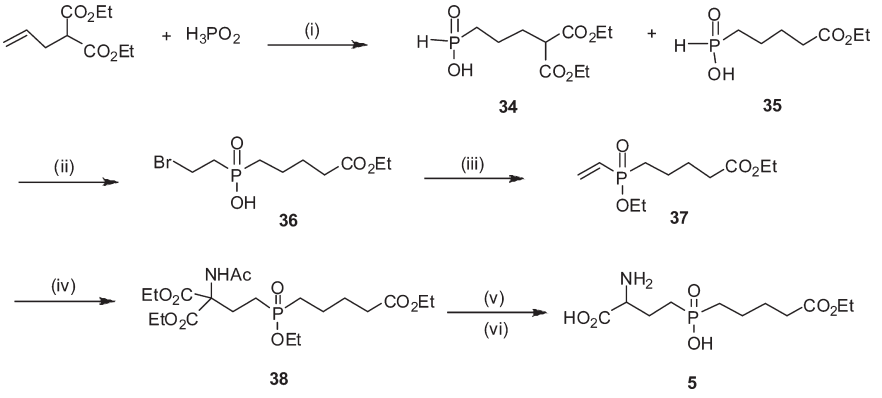
The first strategy (method A, Scheme 1) involves the activation of the phosphorus moiety to trivalent form P^{III} [15,16]

Scheme 1. Method A[a]



[a] Reagents and conditions: (i) HMDS, reflux at 120 °C; (ii) ethyl acrylate or diethyl maleate, 50 °C, 2 h; (iii) dibromoethane, reflux at 120 °C, 5 h, 35−41% yield from hypophosphite; (iv) acetamidoacrylic acid, 60 °C, 4 h, 73% yield from hypophosphite; (v) $CH(OEt)_3$, reflux at 140 °C; (vi) diethyl acetamidomalonate, K_2CO_3, tetrabutylammonium bromide, THF, reflux, 50−77% over two steps; (vii) 8 N HCl, reflux, 15 h; (viii) Dowex AG 50W-X4 (H^+, 50−100 mesh) or AG 1-X4 resin ($HCOO^-$, 200−400 mesh), 11−21% yield.

Scheme 2. Method B[a]



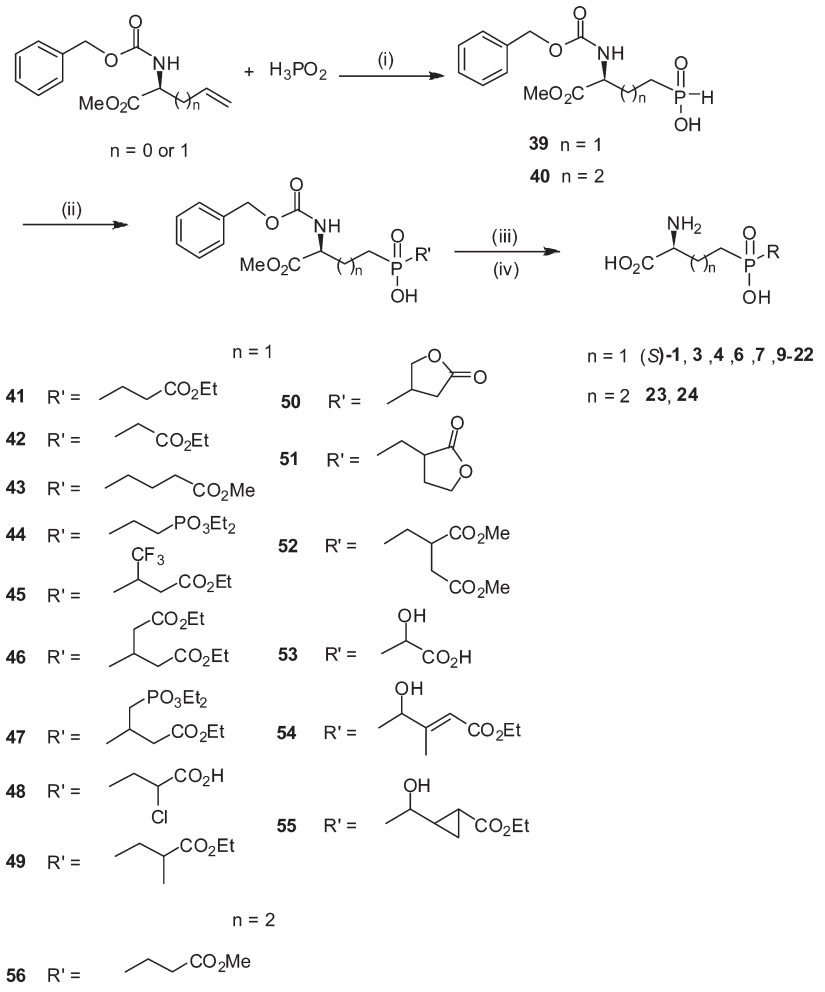
[a] Reagents and conditions: (i) AIBN, CH_3OH, 5 h reflux at 80 °C; (ii) HMDS, dibromoethane, reflux at 120 °C, 9 h; (iii) $CH(OEt)_3$, reflux at 140 °C; (iv) diethyl acetamidomalonate, K_2CO_3, tetrabutylammonium bromide, THF, reflux ; (v) 8 N HCl, reflux, 15 h; (vi) Dowex AG 50W-X4 (H^+, 50−100 mesh) or AG 1-X4 resin ($HCOO^-$, 200−400 mesh), yield < 5%.

and successive reactions with a Michael acceptor and an alkyl halide, followed by incorporation of the amino acid moiety by addition of the anion of diethylacetamidomalonate.[17−19] The silylated P^{III} entity was obtained from heating a mixture of ammonium hypophosphite and hexamethydisilazane (HMDS). Reaction with an acrylate derivative and subsequently dibromoethane were performed without intermediate purification (**25**, **26**). The phosphinic group was then esterified with ethyl orthoformate. At this step a partial elimination of HBr was observed; however, both the bromide (**28**, **29**) and the alkene (**30**, **31**) reacted with diethylacetamidomalonate (**32**, **33**) and yielded the desired amino acids [(±)-**1**, **8**] after acidic deprotection and ion exchange chromatography purification (Scheme 1).[17,20] The second alkylation of the trivalent phosphorus may be performed with acetamidoacrylic acid (**27**), yielding the shorter PCEP derivative **2** after acidic deprotection. This method A is rather straightforward, yet it generates the final amino acids as racemates.

In the second strategy (method B, Scheme 2), the first alkylation of H_3PO_2 is effected by AIBN mediated radical reaction.[21] Such a reaction permits, in contrast to method A, the addition of an unactivated alkene such as diethylallyl malonate. The acidic medium causes the decarboxylation of malonate **34** which leads to **35**. The next steps are identical to method A: reaction with dibromoethane (**36**), esterification (**37**), substitution with acetamidomalonate (**38**), acidic hydrolysis, and purification, affording the longer PCEP derivative **5** (Scheme 2).[20]

In the third strategy (method C, Scheme 3) the first P−C bond formation is similar to method B starting from aqueous

Scheme 3. Method C[a]



[a] Reagents and conditions: (i) AIBN, CH_3OH, 5 h, 52−94% yield; (ii) CH_2Cl_2, TMSCl, Et_3N or *N*,*O*-bis(trimethylsilyl)acetamide (BSA), acrylate or halide or aldehyde, 29−95% yield; (iii) 6 N HCl, reflux; (iv) Dowex AG 50W-X4 (H^+, 50−100 mesh) or AG 1-X4 resin ($HCOO^-$, 200−400 mesh), 3−69% yield.

hypophosphorous acid and protected vinylglycine or allylglycine to give the H-phosphinate derivatives **39** and **40** as previously described.[21] The second P−C bond formation is achieved by in situ generation of P^{III} species by means of TMSCl or BSA[21] followed by a conjugate addition with a Michael acceptor (**41**, **44**−**52**, **56**), a halide (**42**, **43**), or an aldehyde (**53**−**55**). Acidic hydrolysis and ion exchange chromatography purification afforded the desired amino acids [(*S*)-(+)-**1**, **3**, **4**, **6**, **7**, **9**−**24**; Scheme 3]. The alkene derivative of PCEP (**6**) was formed by HCl elimination during the acidic hydrolysis of **48**; **6** and **12** were separated at the final chromatographic purification stage. The hydroxymethyl and hydroxylethyl derivatives of PCEP (**15**, **17**) could be obtained from ring-opening of lactones **14** and **16** under basic conditions. PCEP derivatives **3** and **4** were prepared using alkyl halides in the second step of the synthesis. While ethyl bromoacetate reacted smoothly with **39** to yield **42**, the iodide derivative was required for nonactivated halides (e.g., methyl 4-iodobutyrate for the synthesis of **43**). Phosphinate **55** resulted from the addition of **39** to the aldehyde function of *trans*-ethyl 2-formyl-1-cyclopropanecarboxylate as a mixture of four isomers. After hydrolysis, two sets of diastereoisomers **21** and **22** were separated by ion exchange chromatography. Their absolute configuration was not determined. In method C, the configuration and enantiomeric purity of the final amino acids are the same as for the starting alkene. This is an advantage over the first and second strategies, and most PCEP derivatives were prepared following this method.

Pharmacology. (*R*)-**1** and its derivatives were tested functionally on mGluRs transiently transfected in HEK293 cells, through their coupling to phospholipase C (PLC). Activation of this transduction pathway was determined by two different functional assays, one measuring the production of total radiolabeled inositol phosphates (IP) and the other measuring the intracellular Ca^{2+} release using Fluo-4 as a fluorescent probe. Both types of assays gave comparable results in terms of compound potency (see Experimental Section). For the mGlu receptors that are not naturally coupled to Gq (i.e., mGlu2, -4, -6, -7, and -8 coupled to Gi/o), a chimeric Gq/Gi protein named GqiTOP was co-transfected with the receptor, thereby permitting their efficient activation of PLC. These assays give more accurate results and are easier to handle than determinations of the inhibition of forskolin-induced cAMP production through the natural Gi coupling. Over the recent years, we and others have obtained evidence showing that the pharmacological profiles of these receptors are well conserved in these assays.[22]

Since we were initially searching for mGlu4 receptor agonists, the new compounds were first tested on this receptor. Subsequently we assessed the selectivity of the most potent

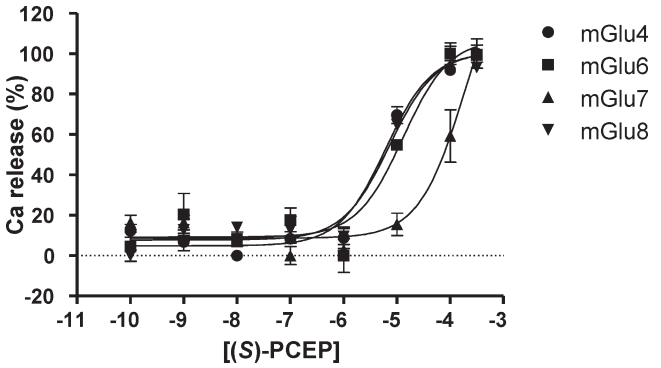


Figure 1. Dose−response curves of (*S*)-**1** on group III mGluRs. Rat clone mGlu4, mGlu6, mGlu7, and mGlu8 receptors were transiently transfected in HEK293 cells together with a chimeric G-protein α subunit and the high affinity glutamate transporter EAAC1. Receptor activities were determined by the release of intracellular calcium resulting from receptor activation by various concentrations of (*S*)-**1**. Each point corresponds to the mean ± SEM of triplicate. Data presented are representative of at least $n = 3$ experiments.

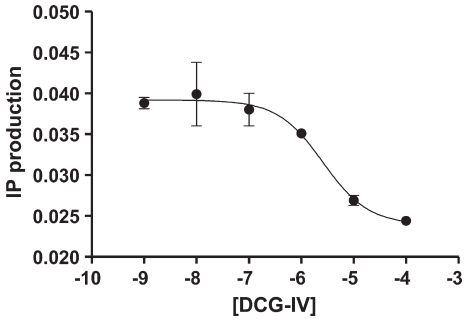


Figure 2. Dose−response curve of the competitive antagonist DCG-IV on mGlu4 receptor activated by 20 μM (±)-**1**. Rat clone mGlu4 receptor was transiently transfected in HEK293 cells together with a chimeric G-protein α subunit and the high affinity glutamate transporter EAAC1. Receptor activity was determined by the accumulation of inositol phosphates (IP) resulting from receptor activation by various concentrations of PCEP. Each point corresponds to the mean ± SEM of triplicate. Data presented are representative of at least $n = 3$ experiments.

ones on the other mGlu subtypes. Pharmacological data are summarized in Table 1.

We first determined the activity of (*R*)-**1**, the initial vHTS hit, on mGlu4. This compound dose-dependently activated mGlu4 with an EC_{50} value of 17 ± 2 μM as determined by IP measurement. We then tested the activity of the PCEP racemate and its (*S*)-enantiomer on mGlu4 and found that both were more potent than (*R*)-**1**. Indeed, (*S*)-**1** was almost 3 times more potent than (*R*)-**1** on this receptor [EC_{50} of 6.4 ± 1.4 μM ($n = 4$) (Figure 1)]. We also verified that the effect of 20 μM of (±)-**1** was dose-dependently inhibited by group III mGluR antagonist DCG-IV, further indicating that (±)-**1** effects are mediated through the activation of mGlu4 (Figure 2).

Compounds **6** (LSP1-3154) and **12** (LSP1-3155) were the most potent derivatives on mGlu4, displaying similar EC_{50} values of 1.0 ± 0.2 μM ($n = 3$) (Table 1). These compounds are in the same rank of potency as ACPT-I (Table 1), a well characterized group III mGluR agonist that is a cyclic derivative of glutamate with an EC_{50} value of 1.74 ± 0.24 μM ($n = 10$) on mGlu4.[23]

Agonist activity of PCEP and its derivatives was tested on the four group III mGluR subtypes. Once it had been tested on mGlu4, the activity of (*S*)-**1** was assayed on mGlu6, mGlu7, and mGlu8 transiently transfected in HEK293 cells (Figure 1, Table 1). This ligand did not discriminate between mGlu4, -6, and -8. Indeed the EC_{50} values determined for mGlu6 and mGlu8 [6.7 ± 3.3 μM ($n = 3$) and 6.6 ± 2.4 μM ($n = 3$), respectively; Table 1] are very similar to those on mGlu4 [6.4 ± 1.4 μM ($n = 4$), Table 1]. Interestingly, the potency of (*S*)-**1** on mGlu7 [89 ± 15 μM ($n = 2$)] is better than that of L-AP4 in the same assay [249 ± 106 μM ($n = 3$), Table 1] making (*S*)-**1** less selective than L-AP4 against mGlu7 receptor. To assess the selectivity of the new compounds on the subtypes of group III receptors, we tested all mGlu4 active compounds on mGlu8, since these two receptors display the highest sequence identity. In this series no selective compounds were found. However, it is interesting to note that, in contrast to compound **1**, compound **12** is 8 times more potent on mGlu4 than on mGlu8 [EC_{50} values of 1.0 ± 0.2 μM ($n = 3$) and 8.3 ± 1.5 μM ($n = 7$), respectively; Table 1] while compound **3** is less potent on mGlu4 than on mGlu8 [EC_{50} values of 7.4 ± 1.8 μM ($n = 8$) and 2.7 ± 0.7 μM ($n = 7$), respectively; Table 1]. This means that it may be possible to design selective compounds deriving from compound **1**.

Potential agonist or antagonist activity of (*S*)-**1** and several derivatives (**3**, **4**, **6**, **12**, and **18**) was then assayed on other mGluR subtypes (mGlu1 and mGlu5 which belong to group I mGluRs, and mGlu2 as a representative of group II mGluRs). None of the tested compounds were active on group I mGluRs. While compounds **4** and **18** were inactive on mGlu2, compounds **1**, **3**, **6**, and **12** presented a low agonist activity on mGlu2 at 100 μM, compared to DCG-IV (a selective group II mGluR agonist) which fully activated the receptor at 100 μM. As determined by intracellular Ca^{2+} measurement experiments, compounds **1**, **3**, **6**, and **12** dose-dependently activated mGlu2 with the following EC_{50} values: compound **1**, 52 ± 21 μM ($n = 3$); compound **3**, 57 ± 30 μM ($n = 3$); compound **6**, 6.7 ± 2.3 μM ($n = 4$); compound **12**, 49 ± 14 μM ($n = 3$) (data not shown). In the same tests, the EC_{50} value determined for DCG-IV was 45 ± 4 nM ($n = 5$) (data not shown).

Structure−Activity Relationship. We first examined the ability of the PCEP series to activate mGlu4 receptor. Interestingly, many of the tested compounds were able to activate the receptor (Table 1). The EC_{50} values of the racemate (±)-**1** and (*S*)-**1** were lower than that of (*R*)-**1**, showing that the (*S*)-enantiomer is the most potent at mGlu4R as expected from reports in the literature.[24] We then studied the optimal chain length of both the amino acid and the acidic substituents of the phosphinic moiety (Chart 2). Shortening the distance separating either the α-carbon (of amino acids) or the distal acidic group from the phosphinate function is deleterious in the first case (**2**, Table 1) but has no significant effect in the second case (**3**). Extending the phosphinic side chains from two methylene groups (**1**) to three (**4** and **24**) is also not tolerated on the amino acid side (**24**) and accommodated on the acidic side (**4**). Thus, we conclude that while the length of the acidic side chain of the phosphinate group may vary from one to three methylenes groups without major effect on the mGlu4 receptor activation, the length of the amino acid side chain must be restricted to two methylenes if activation is to occur. Nevertheless, further elongation of the acidic chain (**5**) is detrimental and replacing the distal carboxylate by a phosphonate (**7**) results in a 23-fold loss of activity. Constraining the carboxyethyl chain in an extended conformation (**6**) affords the most potent agonist of the series, suggesting that PCEP and derivatives may

Table 1. (*S*)-PCEP [(*S*)-**1**] and Derivatives' Agonist Activity at Group III mGlu Receptors and Comparison with L-AP4 and ACPT-I[d]

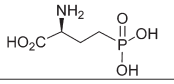
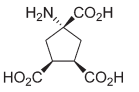
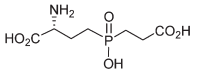
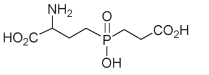
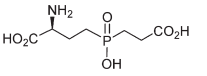
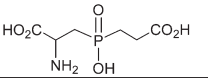
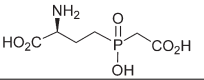
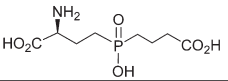
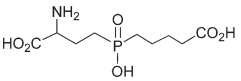
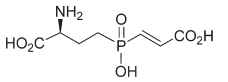
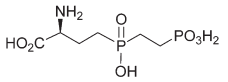
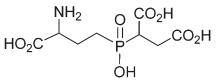
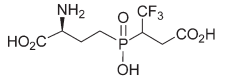
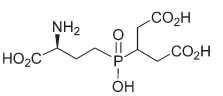
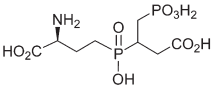
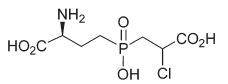
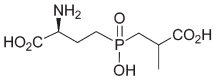
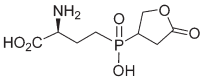
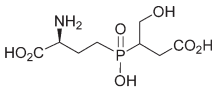
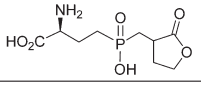
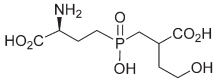
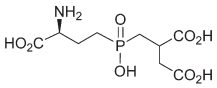
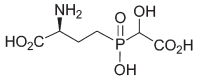
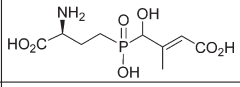
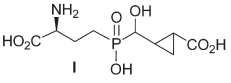
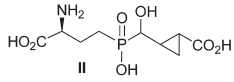
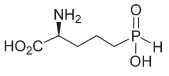
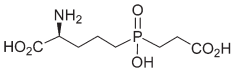
Compd acronyms	compd n°	compound structure	mGlu4 EC_{50} μM (n)	mGlu6 EC_{50} μM (n)	mGlu7 EC_{50} μM (n)	mGlu8 EC_{50} μM (n)
L-AP4			0.096±0.013 (61)[a] 0.13±0.02 (34)[b]	3.3±0.5 (16)[a] 1.0±0.3 (7)[b]	306±70 (10)[a] 249±106 (3)[b]	0.24±0.025 (45)[a] 0.30±0.08 (43)[b]
ACPT-I			1.74±0.24 (10)[a]	10.6±3.3 (6)[a]	280±84 (2)[a]	5.1±0.8 (5)[a]
(*R*)-PCEP	(*R*)-**1**		17±2 (2)[a]	99±9 (3)[a]	-	58±9 (2)[a]
(*RS*)-PCEP	(±)-**1**		7.1±1.5 (5)[a]	32±8 (6)[a]	-	20±5 (5)[a]
(*S*)-PCEP	(*S*)-**1**		6.4±1.4 (4)[a] 6.15±0.01 (3)[b]	31±6 (4)[a] 6.7±3.3 (3)[b]	- 89±15 (2)[b]	7.8±0.5 (4)[a] 6.6±2.4 (3)[b]
LSP1-1102	**2**		inactive at 100 μM[a]	-	-	-
LSP1-1128	**3**		2.4±0.5 (7) 7.4±1.8 (8)[b]	131±28 (2) >100 (3)[b]	inactive inactive	0.91±0.4 (4) 2.7±0.7 (7)[b]
LSP1-2042	**4**		11.4±4.3 (4)[a] 8.5±3.0 (3)[b]	>100[a] 44±14 (3)[b]	- inactive	19±4 (4)[a] 18±5 (3)[b]
LSP1-1117	**5**		inactive at 100 μM[a]	-	-	-
LSP1-3154	**6**		3.0±1.0 (3)[a] 1.0±0.2 (3)[b]	- 1.1±0.2 (3)[b]	43±16 (3)[b]	7.2±1.3 (2)[a] 5.1±0.7 (8)[b]
LSP1-2024	**7**		88±11 (2)[a]	-	-	48±5 (2)[a]
LSP1-1068	**8**		47±13 (4)[a]	113±1 (3)[a]	-	-
LSP1-1191	**9**		26 ±2 (2)[a]	-	-	-
LSP1-1183	**10**		114±10 (2)[a]	-	-	-
LSP1-2029	**11**		33% max at 100 μM (1)[a]	-	-	-
LSP1-3155	**12**		3.8±1.5 (3)[a] 1.0±0.2 (3)[b]	- 8.9±2.5 (3)[b]	- >100 (2)[b]	8.1±1.4 (4)[a] 8.3±1.5 (7)[b]

Table 1. Continued

Compd acronyms	compd n°	compound structure	mGlu4 EC_{50} μM (n)	mGlu6 EC_{50} μM (n)	mGlu7 EC_{50} μM (n)	mGlu8 EC_{50} μM (n)
LSP1-3146	**13**		12.1±2.6 (2)[a]	-	-	53±14 (3)[a]
LSP1-1172	**14**		141±10 (3)[b]	-	-	>100 (3)[b]
LSP3-2174	**15**		not tested			
LSP4-1192	**16**		123±33 (3)[b]	-	-	>100 (3)[b]
LSP4-2037	**17**		20±5 (4)[b]	-	-	76±10 (3)[b]
LSP1-1134	**18**		4.6±0.7 (7)[a] 2.4±0.8 (3)[b]	115±25 (3)[a] 42±12 (3)[b]	- inactive (2)[b]	38±10 (6)[a] 3.5±0.5 (3)[b]
LSP4-2029	**19**		7.2±2.7 (3)[b]	-	-	2.9±0.1 (3)[b]
LSP1-1171	**20**		inactive at 100 μM (1)[a]	-	-	-
LSP1-1158	**21**		5.1±3.7 (2)[a]	-	-	-
LSP1-1159	**22**		18±3 (4)[a,c]	-	-	91±29 (2)[a]
LSP8-1059	**23**		209±116 (3)[b]			73±14 (3)[b]
LSP8-1058	**24**		inactive[b]			inactive[b]

[a] IP production assay. [b] Ca^{2+} release and fluorescence assay. [c] Partial agonist with 71 ± 14% maximal effect. [d] Data are the mean ± SEM of *n* separate experiments.

bind to the receptor in a similarly extended form (see below). We then investigated the effect of various substitutions on that chain (**8**−**13**, **17**−**22**). PCEP derivatives substituted at the 1′ or 2′ position were tested as mixtures of diastereoisomers in a preliminary evaluation. Most of these mixtures did not display significant improved activation of mGlu4 receptor. The 2′-chloro derivative **12** was the most active, but attempts to separate the diastereoisomers were unsuccessful. Nevertheless dockings of both isomers in a 3D model of the binding site (see below and Figure SI-2 of Supporting Information) revealed a similar binding. Thus, no major difference in activity was anticipated for each pure stereoisomer. Lactones **14** and **16** were poor agonists. Among the constrained and/or substituted analogues of **4** (**18**, **20**−**22**), derivative **18** turned out to be 3.5-fold more potent than the parent compound but still weaker than **6** and **12**.

We then evaluated the PCEP series on the other subtypes of group III mGluRs. All compounds displayed similar or slightly weaker potencies at mGlu8R as observed with L-AP4 and ACPT-I (Table 1) with the exception of **3** and **19**, which were somewhat more active. The most active mGlu4R analogues were tested at mGlu6 and mGlu7 receptors. They showed moderate activity on subtype 6 and almost no effect on subtype 7, with the exception of (*S*)-**1** and **6** both of which activate mGlu7 more efficiently than known agonists such as L-AP4, L-thio-AP4,[21] and ACPT-I (Table 1).

Altogether, some variability around the (*S*)-PCEP structure is accommodated by the mGlu4 receptor and the other

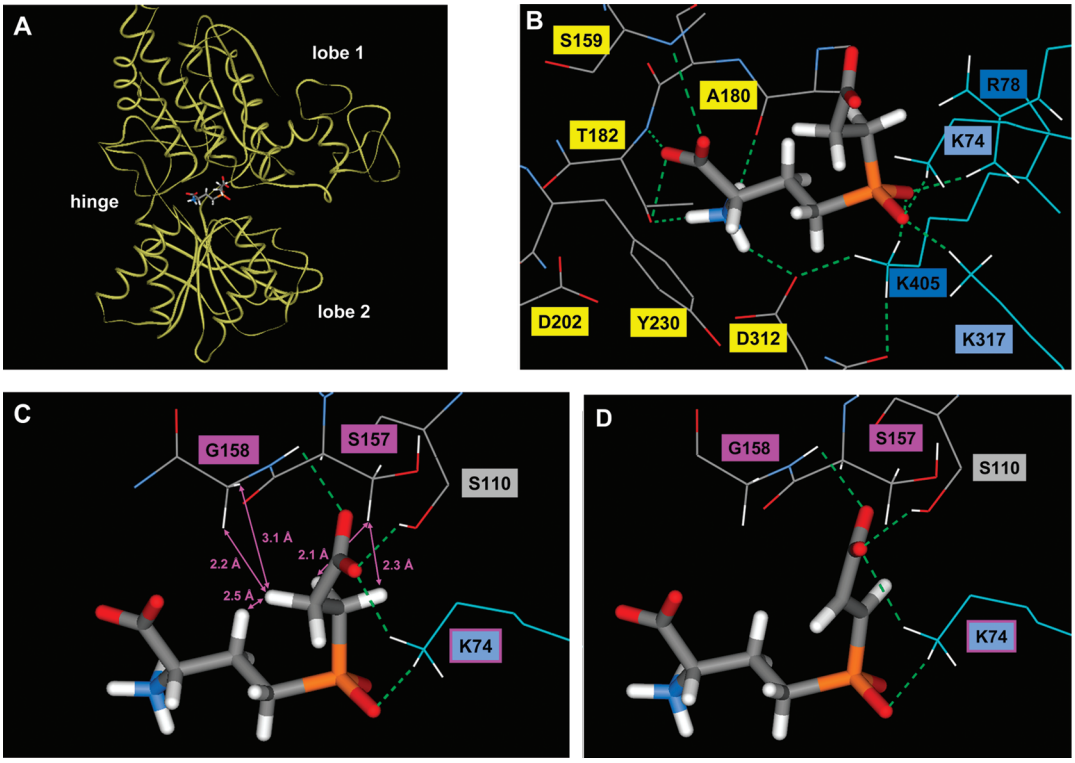


Figure 3. Homology model of mGlu4 receptor ATD docked with (*S*)-**1** and **6**. (A) Backbone of the domain (yellow ribbon) with docked (*S*)-**1** in sticks: carbon, gray; oxygen, red; nitrogen, blue; hydrogen, white; phosphorus. orange. (B) Expanded view of the binding site of (A) where side chains of residues binding the L-AP4-like part of (*S*)-**1** are displayed. Numbers of the signature motif residues binding the α-amino acid moiety are highlighted in yellow, and those of the cluster of basic residues binding the phosphinate moiety are in dark-blue for R78/K405 (residues conserved among mGluRs) and mauve for K74 /K317 (residues found in group III receptors). Atom colors are as in (A) except for the basic cluster where carbons and nitrogens are in cyan. Hydrogens have been removed except for the basic functions of the basic cluster. Hydrogen bonds are shown as green dashed lines. (C) Expanded view of the binding site of (A) where side chains of residues binding the distal carboxylate are displayed. Distances between (*S*)-**1** and S157/G158 protons are indicated. Atom colors are as in (B). Residue numbers are highlighted in magenta for S157 and G158 that differ in mGlu6 and mGlu8, in mauve framed with magenta for K74 that differ in mGlu6 and -7, and in gray for S110 that is conserved in group III mGluRs. (D) Binding site of mGlu4 receptor ATD docked with **6**. Atom colors and residue numbers are similar to those in (C).

group III mGluRs. Actually, (*S*)-**1** is a derivative of phosphinothricin (Chart 2) that is known to weakly activate mGlu4[21,25] and for which the phosphinic methyl group has been replaced by a carboxyethyl chain. The gain of affinity is thus due to the additional acidic function of the various derivatives. However substitutions at the 1′ and 2′ positions of the carboxyethyl chain of (*S*)-**1** are weakly tolerated with the exception of a chlorine atom at 2′ in **12**. The most potent analogue of the series is **6** which has a fixed extended acidic side chain. Interestingly, **24** may be considered as a derivative of (*S*)-AP5[26] or **23** (Table 1), both of which are weak mGlu4R agonists. In this case, the carboxyethyl substituent is unable to restore any activity.

Molecular Modeling. In class C GPCRs, the large amino terminal domain (ATD) folds in two lobes connected by a hinge.[1] The agonist binds to lobe 1 of ATD in an open conformation of this domain and is then trapped in a closed conformation that triggers receptor activation.[27] X-ray structures[28,29] and homology models[30,31] of the two forms of mGluRs'ATD have been disclosed. We used our 3D model of the mGlu4R binding domain in its active closed conformation to set up a virtual screening experiment.[14] (*R*)-PCEP [(−)-**1**] was identified as a virtual hit in our workflow.[14] In the present study we improved our homology model that is now based on mGlu1R[28] and mGlu3R[29] templates and performed docking and molecular dynamics experiments (Figure 3). Combining mGlu1R and mGlu3R in the model generation led to a significant improvement of the backbone and side chain positioning at the binding site. A crystal structure of mGlu7R ATD is available;[29] however, it is in an open form (inactive) and at a crystallographic resolution of 3.00 Å. Hence, it was not considered as a template, although being closer to mGlu4R on the basis of sequence similarity.

In our model, the L-AP4-like part of (*S*)-PCEP binds to the protein in a manner similar to that of L-AP4 (Figure 3B and SI-1): the amino acid entity binds to the signature motif[32] (S159, T180, A182 from lobe 1, D202 from hinge, Y230, D312 from lobe 2) while the two oxygen atoms of the phosphinate bind to the basic residues of the distal pocket (K74, R78, K405 from lobe 1 and K317 from lobe 2).[21] The distal acidic group of (*S*)-PCEP binds to residues belonging to lobe 1 only. This carboxylate makes an ionic interaction with K74 and hydrogen-bonds with the hydroxyl of S110 and the amide NH between S157 and G158 (Figure 3C). The model reveals an extended acidic side chain that is consistent with the activity of the constrained analogue **6** (Figure 3D and Figure SI-3). Interestingly, the four residues found around the distal chain build up a new binding pocket right next to the glutamate binding pocket (Figure 4). Moreover, while S110 is conserved in mGlu receptors of groups II and III, the combination of the three residues aligning with K74, S157, G158 is different in all mGluR subtypes of group III and is not found in group I or II sequences (Figure 5). Yet (*S*)-PCEP and its derivatives only display some selectivity versus mGlu6 and -7 receptors but not

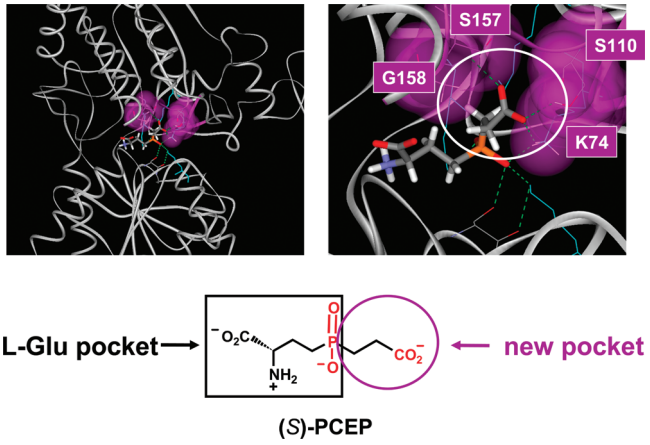


Figure 4. Homology model of mGlu4 receptor ATD docked with (*S*)-**1**. The distal carboxyethyl side chain (circled in magenta in the chemical structure, in white in the model) reveals a new binding pocket adjacent to the glutamate pocket. The shapes (magenta) of the residues lining this pocket are displayed. The protein ribbon is colored in gray, and the atoms and side chains are as in Figure 3.

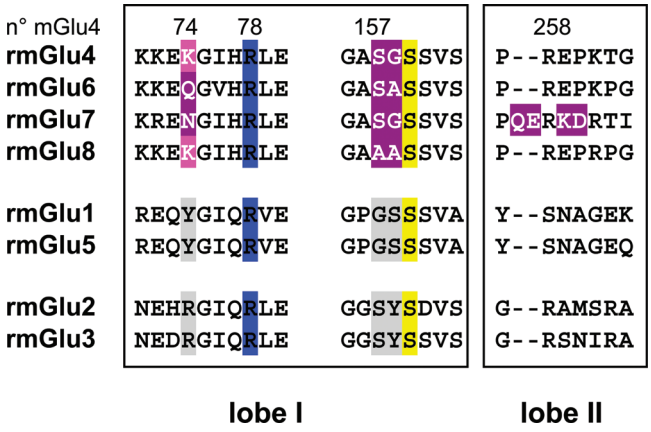


Figure 5. Rat mGlu receptor sequences of three binding loops where subtype selective residues are found. Residue color highlights are the same as in Figure 3B: conserved proximal serine in yellow, conserved distal arginine in blue, group III variable residues in magenta, those that align in other groups in gray.

versus mGlu8 among the four subtypes of group III receptors. This situation may be explained by the lack of ionic interaction with the residues aligning with K74 in mGlu6/7,[33] a longer loop around Q258 in mGlu7R,[33] and orientations of the PCEP substituants that do not allow selective interactions with S157-G158/A154-A155 side chains in mGlu4/8. Indeed distances shown in Figure 3C reveal that substitutions at 1′ or 2′ methylene protons of (*S*)-**1** need to be of small size and will predominantly interact with the pro-R proton of G158 (mGlu4) and A154 (mGlu8) or the same methylene protons of S157 (mGlu4) and A155 (mGlu8). Consequently, even when the resulting (*S*)-PCEP derivatives fit into the new binding pocket, they do not trigger significant selective subtype activation (e.g., **12**). New derivatives of (*S*)-PCEP in which the carboxyethyl chain will be replaced by new moieties will need to be designed in order to take advantage of selective interactions with the three variable residues of the new distal mGluR pocket (Figure 4). Such compounds will be disclosed in due course.

The noticeable EC_{50} value decrease at mGlu7R for (*S*)-**1** and **6** may be due to their longer size that allows additional interaction with the binding site. In order to interpret this result, we generated a homology model of the closed conformation of mGlu7 receptor ATD. We used as templates the crystal structures of mGlu1 and mGlu3 receptors and not that of mGlu7 for the same reasons as for mGlu4R model (see above). We then docked **6**. The L-AP4-like part of **6** binds to the mGlu7 receptor in a manner similar to that described for the binding of (*S*)-PCEP to mGlu4R (see above and Figure SI-4) except for the ionic interaction with N74 found in place of K74. It was previously shown that the lack of this interaction is partly responsible for the weak activation of mGlu7R.[33] However, our model shows that the distal carboxylate of **6** makes new interactions with K71 (lobe 1) and the backbone of N288 and D289 (lobe 2) (Figure 6). Moreover, this carboxylate is part of a network of hydrogen bonds stabilizing the closed conformation of the ATD of the receptor (Figure 6). Q288 and R260 are also found to be part of this network. These residues belong to a small loop between the βG sheet and αVI helix[30] that was suggested to make a protrusion in the cleft between the two lobes preventing complete closure when L-AP4 is bound.[33] This loop is probably quite flexible, since it was not resolved in the

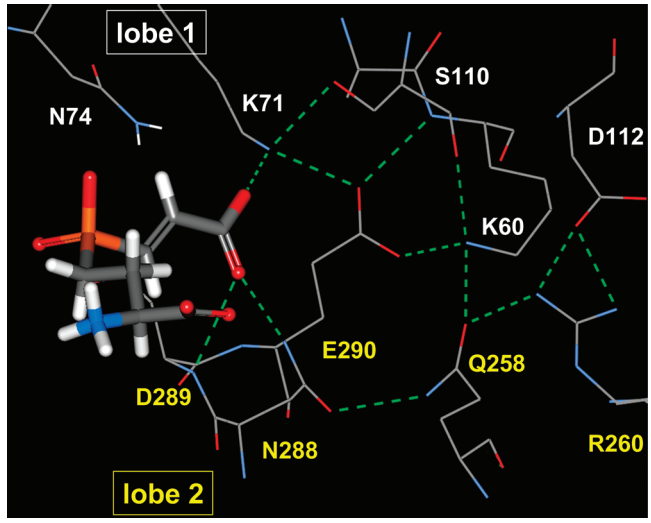


Figure 6. Binding site of the homology model of mGlu7 receptor ATD docked with **6** (sticks and bond order displayed). Atom colors are the same as in Figure 3. Residues numbers are white for lobe 1, yellow for lobe 2. A network of hydrogen bonds between the two lobes stabilizes the closed form of the binding domain. Other interactions are not shown for clarity.

crystal structure of mGlu7R binding domain.[29] The present model of **6** docked at mGlu7R binding site suggests that longer agonists such as **1** or **6** may induce a hydrogen bond network between the two lobes in the remote part of the cleft and cause the Q288 loop to be fixed in a conformation that permits the domain closure. This situation would thus explain the increased apparent affinities of **1** and **6** compared to known agonists at mGlu7R. Data from Table 1 show that only a two-carbon acidic phosphoryl substituent is tolerated, since **3** and **4** are inactive. Substitutions on that chain are also deleterious (Table 1: **12**, **18**).

Discussion

Searching for new mGlu4 receptor agonists, we carried out a virtual screening campaign[14] and identified (*R*)-**1**, named (*R*)-PCEP, as the most potent and optimizable hit. Such pseudopeptides have been widely designed as enzyme

inhibitors, among which are (±)-**1**[17] and (*S*)-**1**,[34,35] both previously synthesized and tested as part of a series of glutamine synthetase inhibitors. Indeed, **1** is a γ-glutamyl analogue that is derived from phosphinothricin (Chart 2), a well-known inhibitor of that enzyme and used as a herbicide.[36] However, phosphinothricin was shown to be a poor agonist of the mGlu4 receptor[21,25] and was not considered as a valuable L-AP4 analogue to be optimized. Other phosphinic acid pseudopeptides were also prepared to inhibit various γ-glutamyl ligases[18,37−39] but were not evaluated at mGlu receptors. It is noted that in early studies, **3** was able to antagonize lateral perforant path-evoked field potentials in a hippocampal slice, similar to L-AP4 but with weaker potency.[40]

Although the initial hit **1** was of the D-configuration, the L-enantiomer proved to be more potent as for all orthosteric mGluR agonists.[24] The docking of (*S*)-**1** (Figure 3) revealed that its distal carboxylic group was able to provide the ionic interaction that was lost with phosphinothricin lacking the second phosphonate acidity of L-AP4. This observation is in agreement with the presence of two acidic functions in group III mGluR agonists (Chart 1A). Hence, we designed a series of phosphinoamino acids holding a distal acidic group (Chart 2). Compounds were obtained through stepwise double alkylation of hypophosphorous acid combining methods obtained from the literature (Schemes 1−3). Variation of the chain length between the amino acid moiety, the phosphinyl and carboxylic groups proved that only two methylenes on the amino acid side of the central phosphorus atom permit mGlu4 receptor activation while one to three methylenes on the acidic side may activate the receptor. Potency of (*S*)-**1** was increased when constraining the carboxyethyl chain in an extended conformation in **6** but not with substitutions in that chain (Table 1). However, introducing a chlorine atom in the 2′ position in **12** induces some differentiation within the group III subtypes responses: **6** and **12** activate mGlu4 and 8 with similar potencies, while **12** displays 9-fold lower potency than **6** on mGlu6, -7, -8R. A fairly larger group such as a methyl in **13** is weakly tolerated, which means that the tuning of (*S*)-**1** derivatives is subtle. On the other hand, **3** and **19**, in which the distal carboxylate is only one atom away from the phosphoryl group, are slightly more active at mGlu8 than at mGlu4, with no effect of the 1′-hydroxyl in **19**. It is noted that as for group III receptors, **6** displays the highest potency at mGlu2R among the tested derivatives. However, substitution as in **12** reduces that effect, making **12** a preferred agonist for mGlu4R compared to **6**.

A major outcome of this study is (1) the demonstration that even after the introduction of a longer side chain into glutamate derivatives, they maintain their agonist activity, allowing the development of larger molecules that are likely to be more specific for mGluRs compared to other glutamate binding proteins and (2) the disclosure of a binding pocket next to the glutamate binding site and surrounded by residues that differ in the various mGlu subtypes (Figures 4 and 5). Compound **1** and its derivatives are longer than glutamate and may thus reach this pocket located in lobe 1 of the ATD. Interestingly, although these compounds are larger than glutamate, they all remain agonists which attests that the full closure of the two lobes occurs after initial binding to lobe 1. In the present series of agonists, we have not yet discovered any one that would clearly take advantage of the variable pocket in order to selectively activate one subtype. Nevertheless, this may be obtained by developing new molecules bearing chemical fragments that would fit this pocket differently.

Another interesting finding is that some of these longer analogues (**1** and **6**) activate the mGlu7 receptor more potently than known agonists. Molecular models reveal that they stabilize the closed conformation of the ATD by making additional interactions with residues further away from the hinge that could not be reached by shorter molecules. Such a stabilization of the closed bilobate domain, the Venus flytrap domain (VFT), has been proposed by Zhang et al. to explain the enhancer effect of IMP for umami taste.[41] This savory taste results from glutamate binding to heteromeric T1R receptors that belong to class C GPCRs. The authors demonstrated that IMP binds to the VFT domain of T1R1 in a site adjacent to glutamate lined with residues from the two lobes. The sequence alignment between mGluRs and T1R1 allows one to define the IMP homologous pocket in mGlu4 and mGlu7 receptors. Comparison with the residues that interact with the distal part of (*S*)-**1** and **6** reveals that both pockets are in proximity (Figure SI-5). Both IMP and the distal chain of PCEP derivatives may play a similar role of enhancing receptor activation by establishing a network of additional interactions that stabilize the active VFT conformation.

In conclusion, we have disclosed a new group III mGlu receptor agonist that was found by virtual high throughput screening. Chemical modulation and testing at cloned receptors proved that some variations in chain length of (*S*)-PCEP [(+)-**1**] are well accommodated and that **6** and **12** are the most potent in the series of agonists. Dockings revealed a new binding pocket adjacent to the glutamate site in the VFT domain. Moving away from the endogenous binding pocket allows to reach residues that are less conserved and may induce some selectivity among mGluR orthosteric agonists. These observations led us to investigate a new series of PCEP derivatives. Longer agonists (e.g., **1** and **6**) are also more potent at mGlu7R, which is of particular interest, since this receptor is difficult to activate.

Experimental Section

Chemistry. General. All chemicals and solvents were purchased from commercial suppliers (Acros, Aldrich) and used as received. *Z*-L-Vinylglycine methyl ester was purchased from Ascent Scientific Ltd. (North Somerset, U.K.) and *Z*-L-allylglycine from Boaopharma Inc. (Woburn, MA). ^{1}H (250.13 or 500.16 MHz), ^{13}C (62.9 or 125.78 MHz), and ^{31}P (101.25 or 202.47 MHz) NMR spectra were recorded on an ARX 250 or an Avance II 500 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 3.31, 49.0; D_2O 4.80) or external reference (H_3PO_4 95%). Product visualization was achieved with 2% (w/v) ninhydrin in ethanol. Optical rotations were measured at the sodium D line (589 nm) or a mercury 546 nm line 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 4.5 kV, injection temperature 240 °C). HPLC analyses were carried out on a Gilson analytical instrument with a 321 pump, column temperature was controlled with an Igloo-CIL Peltier effect thermostat, eluted peaks were detected at 210 and 220 nm by a UV−vis 156 detector, and retention times are reported in minutes. A Daicel Crownpak CR(+) column (150 mm × 4 mm) eluted with pH 1.0 perchloric acid at a 0.4 mL·min^{-1} flow rate and at 4 °C was used. HPLC−MS analyses were performed on a Thermo Finnigan LCQ Advantage Instrument as described above, equipped for HPLC with a nucleosil 100 C18 column (Macherey-Nagel, 250 mm × 4.6 mm, 5 μm). Products were eluted with a

water−acetonitrile mobile phase at 40 °C and a 0.4 mL/min flow rate and detected by mass and UV at 210 nm. The following gradient conditions were used with solvent A (97% H_2O−3% CH_3CN) and solvent B (90% H_2O−10% CH_3CN): 100% A for 7 min, linear increase from 0 to 50% B between 7 and 14.5 min, 50% A−50% B from 14.5 to 20 min. Purity of the tested compounds was established by analytical HPLC−MS and was at least 95%.

Method A. Ethyl 3-[2-Bromoethyl(hydroxy)phosphinyl]propanoate (25). A mixture of ammonium hypophosphite (3.99 g, 48 mmol) and hexamethydisilazane (10.0 mL, 48 mmol) was heated at 120 °C for 1 h under argon. After the mixture was cooled to 0 °C, ethyl acrylate (5.2 mL, 48 mmol) was carefully added dropwise, and the resulting mixture was stirred at 50 °C for 2 h. Then the mixture was cooled to room temperature, dibromoethane (20.0 mL) was added, and the mixture was stirred for 5 h at 120 °C. The formed trimethylbromosilane and excess dibromoethane were removed under vacuum. Then 50 mL of aqueous ethanol (1:1) were added dropwise to the residue and refluxed for 0.5 h. Then the solvent was removed under vacuum and extracted with ethyl acetate. The organic layer was dried over magnesium sulfate and the solvent was removed under vacuum to give **25** (5.42 g, 41% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 1.25 (t, J = 7.1 Hz, 3H), 2.06 (m, 2H), 2.42 (m, 2H), 2.61 (m, 2H), 3.59 (m, 2H), 4.14 (q, J = 7.1 Hz, 2H). ^{31}P NMR (101 MHz, CD_3OD): δ 49.5.

Ethyl 2-[(2-Bromoethyl(hydroxy)phosphinyl)methyl]butan-1,4-dioate (26). The compound was prepared from diethyl maleate by a procedure similar to that for the preparation of compound **25** to afford **26** (oily liquid, 1.21 g, 35% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 1.26 (m, 6H), 2.58 (m, 2H), 2.91 (m, 2H), 3.50 (m, 1H), 3.66 (m, 2H), 4.20 (m, 4H). ^{31}P NMR (101 MHz, CD_3OD): δ 41.9.

Ethyl 3-[(((2-(*N*-Acetyl)amino)-2-carboxy)ethyl)(hydroxy)-phosphinyl]propanoate (27). A mixture of ammonium hypophosphite (498 mg, 6.0 mmol) and hexamethydisilazane (1.3 mL, 6.0 mmol) was heated at 120 °C for 1 h under argon. After the mixture was cooled to 0 °C, ethyl acrylate (0.38 mL, 3.5 mmol) was carefully added dropwise and the resulting mixture was stirred at 50 °C for 2 h. Then the mixture was cooled to room temperature, acetamidoacrylic acid (387 mg, 3.0 mmol) was added, and the mixture was stirred for 5 h at 65 °C. An amount of 10 mL of 2 N HCl was added dropwise to the above residue and extracted with ethylacetate. The aqueous part was evaporated to dryness. Then 50 mL of methanol were added and the solvent was removed at 50 °C under vacuum to afford **27** (645 mg, 73% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 1.28 (t, J = 7.1 Hz, 3H), 2.02 (s, 3H), 2.12 (m, 2H), 2.36 (m, 2H), 2.62 (m, 2H), 4.18 (q, J = 7.1 Hz, 2H), 4.72 (m, 1H). ^{31}P NMR (101 MHz, CD_3OD): δ 48.7.

Ethyl 3-[2-Bromoethyl(ethoxy)phosphinyl]propanoate (28) and Ethyl 3-[Ethoxy(vinyl) phosphinyl]propanoate (30).[17] An amount of 5.42 g of **25** (20 mmol) was treated with 40.0 mL of triethyl orthoformate, and the mixture was refluxed with a Dean−Stark trap to remove ethanol and ethyl formate. Excess triethyl orthoformate was removed under vacuum to give **28** and **30** ([39.5:60.5 ^{31}P NMR ratio], 5.91 g). **30**: ^{1}H NMR (250 MHz, CD_3OD) δ 1.27 (m, 6H), 2.18 (m, 2H), 2.57 (m, 2H), 4.10 (m, 4H), 6.36 (m, 3H). ^{31}P NMR (101 MHz, CD_3OD): δ 44.9. **28**: ^{31}P NMR (101 MHz, CD_3OD) δ 53.3.

Diethyl 2-[Ethoxy(2-bromoethyl)phosphinyl]butan-1,4-dioate (29) and Diethyl 2-[Ethoxy(vinyl)phosphinyl]butan-1,4-dioate (31). 26 was esterified by triethyl orthoformate by a procedure similar to that for the preparation of compounds **28** and **30** to afford **29** and **31** (oily liquid, 1.36 g). ^{31}P NMR (101 MHz, CD_3OD): δ 37.8 (**31**), 48.1 (**29**).

Ethyl 3-[((3-(*N*-Acetyl)amino)-3-(bisethoxycarbonyl)propyl)-(ethoxy)phosphinyl]propanoate (32).[17] Diethylacetamidomalonate (456 mg, 2.1 mmol), potassium carbonate (573 mg, 4.2 mmol), and tetrabutylammonium bromide (32 mg, 0.1 mmol) were mixed with **28** and **30** (500 mg, 1.9 mmol estimated from above ratio) in tetrahydrofuran (2.0 mL). The reaction mixture was refluxed with stirring for 15 h. The residue was extracted with chloroform, washed with water, dried over magnesium sulfate and the solvent was removed under vacuum to give 564 mg of crude product **32**. The residue was purified by column chromatography (silica gel 60 (70−230 mesh), ethyl actetate/methanol, 1:0 to 8:2) to afford **32** (218 mg, 50% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 1.31 (m, 12H), 1.75 (m, 2H), 2.05 (s, 3H), 2.16 (m, 2H), 2.59 (m, 4H), 4.17 (m, 8H). ^{13}C NMR (63 MHz, CD_3OD): δ 13.5, 16.1, 21.6, 22.4 (d, J = 101 Hz), 22.9 (d, J = 93 Hz), 25.8, 26.7, 60.5, 61.1, 62.7, 66.8 (d, J = 17 Hz), 167.6, 171.4, 172.5 (d, J = 14 Hz). ^{31}P NMR (101 MHz, CD_3OD): δ 58.1.

Diethyl 2-[(((3-(*N*-Acetyl)amino)-3-(bisethoxycarbonyl)propyl)(ethoxy)phosphinyl)]butan-1,4-dioate (33). The **29** and **31** mixture (1.0 g) was treated similarly to **28** and **30** without further purification to afford **33** (77% yield over two steps). ^{1}H NMR (250 MHz, CD_3OD): δ 1.33 (m, 15H), 1.88 (m, 2H), 2.07 (s, 3H), 2.57 (m, 2H), 2.92 (m, 2H), 3.56 (m, 1H), 4.22 (m, 10H). ^{31}P NMR (CD_3OD): δ 51.7, 52.2. MS (ESI): m/z 508.1 (M − 1).

3-[((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl]propanoic Acid ((±)-1).[17] Compound **32** (210 mg, 0.5 mmol) was treated with 2.0 mL of 8 N HCl and refluxed for 15 h. The reaction mixture was concentrated under vacuum, and the residue was purified using Dowex AG 50W-X4 cation exchange resin column (H^+, 50−100 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** (24.6 mg, 21% yield). ^{1}H NMR (250 MHz, D_2O): δ 1.66 (m, 2H), 1.85 (m, 2H), 2.06 (m, 2H), 2.51 (m, 2H), 3.96 (t, J = 5.7 Hz, 1H). ^{13}C NMR (63 MHz, D_2O): δ 23.5, 24.3 (d, J = 91 Hz), 25.0 (d, J = 91 Hz), 27.3, 54.1 (d, J = 15 Hz), 172.6, 177.5 (d, J = 15 Hz). ^{31}P NMR (101 MHz, D_2O): δ 57.4. MS (ESI): m/z 238.1 (M − 1). Anal. ($C_7H_{14}NO_6P\cdot 0.25H_2O$) C, H, N.

2-[(((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl)]butan-1,4-dioic Acid (8). The removal of the protecting groups in compound **33** (186 mg, 0.4 mmol) was accomplished using the same procedure as that used for compound (±)-**1** to afford **8**. The residue (83 mg) was purified by anion exchange chromatography. The residue was dissolved in freshly boiled and cooled water (200 mL), the pH was then adjusted to 8−9, and the solution was deposited on a AG 1-X4 resin ($HCOO^-$, 200−400 mesh, 8.5 cm × 1 cm). The resin was washed with freshly boiled and cooled water and was eluted with 0.72−0.73 M HCOOH to afford 11.1 mg of pure **8**. ^{1}H NMR (250 MHz, D_2O): δ 1.78 (m, 2H), 2.14 (m, 2H), 2.81 (m, 2H), 3.18 (m, 1H), 4.05 (t, J = 5.9 Hz, 1H). ^{13}C NMR (63 MHz, D_2O): δ 23.4, 24.7 (d, J = 96 Hz), 30.9, 45.0 (d, J = 77 Hz), 53.7 (d, J = 15 Hz), 172.0, 174.4, 176.2 (d, J = 15 Hz). ^{31}P NMR (101 MHz, D_2O): δ 46.5. HPLC−MS t_R = 4.15 min.

3-[((2-Amino-2-carboxy)ethyl)(hydroxy)phosphinyl]propanoic Acid (2). The removal of the protecting groups in compound **27** (525 mg, 1.8 mmol) was accomplished following the same procedure as that followed for compound (±)-**1**. The compound was purified with a Dowex AG 50W-X4 column as described earlier to afford 56.2 mg of pure **2**. ^{1}H NMR (250 MHz, D_2O): δ 1.93 (m, 2H), 2.06 (m, 1H), 2.32 (m, 1H), 2.56 (m, 2H), 4.19 (m, 1H). ^{13}C NMR (63 MHz, D_2O): δ 25.3 (d, J = 96 Hz), 27.3, 29.4 (d, J = 86 Hz), 49.3, 172.0, 177.3. ^{31}P NMR (101 MHz, D_2O): δ 52.0. MS (ESI): m/z 224.1 (M − 1). HPLC−MS t_R = 3.67 min.

Method B. 5-Ethoxy-4-ethoxycarbonyl-5-oxopentylphosphinic Acid (34) and 5-Ethoxy-5-oxopentylphosphinic Acid (35). A mixture of hypophosphorous acid (3.30 g, 25 mmol, 50% aqueous), diethylallyl malonate (1.00 g, 5.0 mmol), and α,α′-azoisobutyronitrile (AIBN, 41 mg, 0.3 mmol) in methanol (2.0 mL) was refluxed at 80 °C for 5 h. Then methanol was evaporated under vacuum and the residue was extracted with ethyl acetate and dried over magnesium sulfate. The organic layer was evaporated under vacuum to afford **34** and **35** used as such in the next step.

Ethyl 5-[2-Bromoethyl(hydroxy)phosphinyl]pentanoate (36). Crude **34** and **35** (1.34 g) was mixed with dibromoethane (2.4 mL, 28 mmol) and hexamethydisilazane (2.9 mL, 14 mmol) and

heated at 120 °C for 9 h under argon. The formed trimethylbromosilane and excess dibromoethane were removed under vacuum. Then 50 mL of aqueous ethanol (1:1) were added dropwise to the residue and refluxed for 0.5 h. Then the solvent was removed under vacuum and the residue extracted with ethyl acetate. The organic layer was dried over magnesium sulfate and the solvent was removed under vacuum to afford **36** used as such in the next step.

Ethyl 5-[Ethoxy(vinyl)phosphinyl]pentanoate (37). The crude product **36** (270 mg) was treated with 40.0 mL of triethyl orthoformate, and the mixture was refluxed with a Dean−Stark trap to remove ethanol and ethyl formate. Excess triethyl orthoformate was removed under vacuum to afford **37** which was submitted to the next step without purification.

Ethyl 5-[((3-(*N*-Acetyl)amino)-3-(bisethoxycarbonyl)propyl)-(ethoxy)phosphinyl]pentanoate (38). The crude product **37** (200 mg) was mixed with diethyl acetamidomalonate (174 mg, 0.8 mmol), potassium carbonate (221 mg, 1.6 mmol), and tetrabutylammonium bromide (13 mg, 0.04 mmol) in tetrahydrofuran (1.0 mL). The reaction mixture was refluxed with stirring for 15 h. The residue was extracted with chloroform, washed with water, dried over magnesium sulfate and the solvent was removed in vacuum to give **38**. ^{1}H NMR (250 MHz, CD_3OD): δ 1.21 (m, 12H), 2.01 (m, 15H), 4.20 (m, 8H). ^{31}P NMR (101 MHz, D_2O): δ 59.0.

5-[((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl]pentanoic Acid (5). An amount of 190 mg of **38** was treated with 2.0 mL of 8 N HCl and refluxed for 15 h. The reaction mixture was concentrated under vacuum, and the residue was purified using Dowex AG 50W-X4 cation exchange resin column (H^+, 50−100 mesh, 24 cm × 1.7 cm, water elution). The fractions that gave a positive color reaction with ninhydrin were combined and evaporated under vacuum to give 3.2 mg of pure **5**. ^{1}H NMR (250 MHz, D_2O): δ 1.66 (m, 8H), 2.06 (m, 2H), 2.38 (t, J = 7.2 Hz, 2H), 3.94 (t, J = 5.9 Hz, 1H). ^{31}P NMR (101 MHz, D_2O): δ 60.6. HPLC−MS t_R = 5.07 min.

Compound (*S*)-**5** may be prepared following the same procedure as for **4** using methyl 5-iodopentanoate.

Method C. Methyl (2*S*)-2-(*N*-Benzyloxycarbonyl)amino-4-[(hydroxy)phosphinyl]butanoate (39).[21] A mixture of hypophosphorous acid (660 mg, 5.0 mmol, 50% aqueous), *N*-benzyloxycarbonyl-L-α-vinylglycine methyl ester (249 mg, 1.0 mmol), and α,α′-azoisobutyronitrile (AIBN, 8 mg, 0.05 mmol) in methanol (1.0 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 magnesium sulfate, and evaporated under vacuum to afford **39** (296 mg, 94% yield). ^{1}H NMR (250 MHz, 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 (63 MHz, 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 (101 MHz, CD_3OD): δ 35.3. $[\alpha]_D$ −9.7 (*c* 1.17, MeOH).

Methyl (2*S*)-2-(*N*-Benzyloxycarbonyl)amino-5-[(hydroxy)-phosphinyl]pentanoate (40). Methyl (2*S*)-2-(*N*-benzyloxycarbonyl)amino-4-pentenoate (790 mg, 75% yield) was synthesized from and *Z*-L-allylglycine (997 mg, 4 mmol) according to a previously published procedure.[42] Analytical data are in agreement with the data reported in literature.[42−44] ^{1}H NMR (500 MHz, CD_3CO-CD_3): δ 2.45−2.64 (m, 2H), 3.70 (s, 3H), 4.28−4.36 (m, 1H), 5.07−5.17 (m, 2H), 5.09 (s, 2H), 5.78−5.88 (m, 1H), 6.58 (bs, 1H), 7.35−7.42 (m, 5H). ^{13}C NMR (63 MHz, CD_3COCD_3): δ 36.4, 51.8, 54.3, 66.3, 118.0, 128.2, 128.7, 129.0, 133.9, 137.6, 156.4, 172.4. Compound **40** (326 mg, 52% yield) was prepared from methyl (2*S*)-2-(*N*-benzyloxycarbonyl)amino-4-pentenoate (527 mg, 2.0 mmol) following the same procedure employed to prepare **39**. ^{1}H NMR (250 MHz, CD_3COCD_3): δ 1.64−1.94 (m, 6H), 3.70 (s, 3H), 4.20−4.37 (m, 1H), 5.10 (s, 2H), 7.08 (d, J_{HP} = 538 Hz, 1H), 7.21−7.48 (m, 5H). ^{13}C NMR (63 MHz, CD_3COCD_3): δ 17.8, 23.6 (d, J = 133 Hz), 32.5 (d, J = 16 Hz), 51.9, 54.0, 66.3, 128.1, 128.2, 128.7, 137.7, 156.6, 172.9. ^{31}P NMR (101 MHz, CD_3COCD_3): δ 34.3. MS (ESI): m/z 328.9 (M − 1). $[\alpha]_D$ − 10.9 (*c* 1.18, MeOH).

General Procedure A. To a solution of **39** (252 mg, 0.8 mmol) and acrylate or halide or aldehyde (3.0 mmol) in 2.0 mL of methylene chloride at 0 °C under an argon atmosphere was added dropwise *N*,*O*-bis(trimethysilyl)acetamide (BSA) (1.5 mL, 6.0 mmol). The mixture was allowed to warm to room temperature and stirred overnight, then cooled to 0 °C, and 25 mL of 1 N HCl were added, then extracted with ethyl acetate. The organic layer was concentrated in vacuo. This residue was dissolved in 10 mL of water, the pH was adjusted to 7 using saturated sodium hydrogen carbonate solution, then extracted with ethyl acetate (2 × 50 mL). The organic layer was separated, and the aqueous phase was treated with 1 N HCl to adjust the pH to 1. The aqueous phase was extracted with ethyl acetate twice (2 × 50 mL). The combined acidic organic extracts were dried over magnesium sulfate, filtered, and concentrated in vacuo.

Ethyl (3*S*)-3-[((3-(*N*-Benzyloxycarbonyl)amino-3-methoxycarbonyl)propyl)(hydroxy)phosphinyl]propanoate (41). The compound was prepared from **39** (1.2 mmol) and ethyl acrylate (0.53 mL, 4.9 mmol) according to general procedure A. An amount of 280 mg of crude **41** was obtained and purified by anion exchange resin chromatography: The residue was dissolved in 100 mL of freshly boiled and cooled water and 10 mL of methanol (pH 5−6), and the solution deposited on a AG 1-X4 resin ($HCOO^-$, 200−400 mesh, 10 cm × 1.5 cm). The compound was eluted with a 9:1 solution of 3.5 N HCOOH and methanol to afford **41** (150 mg, 29% yield). ^{1}H NMR (500 MHz, CD_3OD): δ 1.23 (t, J = 7.2 Hz, 3H), 1.97 (m, 6H), 2.57 (m, 2H), 3.72 (s, 3H), 4.13 (q, J = 7.2 Hz, 2H), 4.27 (bs, 1H), 5.09 (s, 2H), 7.34 (m, 5H). ^{13}C NMR (126 MHz, CD_3OD): δ 13.0, 23.6 (d, J = 163 Hz), 23.7, 25.6 (d, J = 147 Hz), 25.9, 51.4, 54.2 (d, J = 16 Hz), 60.6, 66.3, 127.3, 127.6, 128.0, 136.6, 157.1, 172.2, 172.8. ^{31}P NMR (101 MHz, CD_3OD): δ 54.0. MS (ESI) m/z 415.9 (M + 1).

Ethyl (3*S*)-2-[((3-(*N*-Benzyloxycarbonyl)amino-3-(methoxycarbonyl)propyl)(hydroxy)phosphinyl]ethanoate (42). The compound was prepared from **39** (0.8 mmol) and ethyl bromoacetate (501 mg, 3.0 mmol) according to general procedure A to afford **42** (60% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 1.28 (t, J = 7.1 Hz, 3H), 2.07 (m, 4H), 3.00 (d, J = 17.3 Hz, 2H), 3.76 (s, 3H), 4.19 (q, J = 7.1 Hz, 2H), 4.31 (m, 1H), 5.13 (s, 2H), 7.34 (m, 5H). ^{13}C NMR (63 MHz, CD_3OD): δ 13.5, 24.2, 25.6 (d, J = 99 Hz), 37.2 (d, J = 82 Hz), 51.9, 54.8, 61.6, 66.8, 127.9, 128.1, 128.5, 137.1, 157.6, 167.1, 172.7. ^{31}P NMR (101 MHz, CD_3OD): δ 45.7. MS (ESI): m/z 400.1 (M − 1).

Methyl (3*S*)-4-[((3-(*N*-Benzyloxycarbonyl)amino-3-methoxycarbonyl)propyl)(hydroxy)phosphinyl]butanoate (43). The compound was prepared from **39** (0.8 mmol) and methyl 4-iodobutyrate (684 mg, 3.0 mmol) according to general procedure A to afford **43** (65% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 2.14 (m, 10H), 3.68 (s, 3H), 3.74 (s, 3H), 4.34 (m, 1H), 5.12 (s, 2H), 7.36 (m, 5H). ^{31}P NMR (101 MHz, CD_3OD): δ 55.4. MS (ESI): m/z 416.1 (M + 1).

Diethyl (3*S*)-2-[((3-(*N*-Benzyloxycarbonyl)amino-3-methoxycarbonyl)propyl)(hydroxy)phosphinyl]ethylphosphonate (44). The compound was prepared from **39** (0.8 mmol) and diethylvinyl phosphonate (492 mg, 3.0 mmol) according to general procedure A to afford **44** (88% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 1.29 (m, 6H), 1.99 (m, 8H), 3.73 (s, 3H), 4.14 (m, 4H), 4.31 (m, 1H), 5.12 (s, 2H), 7.37 (m, 5H). ^{31}P NMR (101 MHz, CD_3OD): δ 32.6 (d, J = 65.9 Hz), 52.3 (d, J = 65.6 Hz). MS (ESI): m/z 480.1 (M + 1).

Ethyl (3*S*)-3-[((3-(*N*-Benzyloxycarbonyl)amino-3-methoxycarbonyl)propyl)(hydroxy)phosphinyl]-3-trifluoromethylpropanoate (45). The compound was prepared from **39** (0.8 mmol) and ethyl 4,4,4-trifluorocrotonate (504 mg, 3.0 mmol) according to general procedure A to afford **45** (64% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 1.24 (m, 3H), 2.02 (m, 4H), 2.87 (m, 3H), 3.74 (s, 3H), 4.17 (q, J = 6.8 Hz, 2H), 4.24 (m, 1H), 5.12 (s, 2H), 7.36 (m, 5H). ^{31}P NMR (101 MHz, CD_3OD): δ 43.2.

Diethyl (3*S*)-3-[((3-(*N*-Benzyloxycarbonyl)amino-3-methoxycarbonyl)propyl)(hydroxy)phosphinyl]pentan-1,5-dioate (46). The compound was prepared from **39** (0.8 mmol) and diethyl glutaconate (558 mg, 3.0 mmol) according to general procedure A to afford **46** (54% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 1.26 (m, 6H), 2.40 (m, 9H), 3.74 (s, 3H), 4.13 (m, 4H), 4.37 (m, 1H), 5.12 (s, 2H), 7.37 (m, 5H). ^{13}C NMR (63 MHz, CD_3OD): δ 13.8, 23.9 (d, J = 91 Hz), 24.1, 31.9 (d, J = 94 Hz), 33.2, 52.1, 54.9, 60.6, 66.8, 128.0, 128.2, 128.7, 137.2, 157.4, 171.8, 172.2, 172.7. ^{31}P NMR (101 MHz, CD_3OD): δ 54.8.

Ethyl (3*S*)-3-[((3-(*N*-Benzyloxycarbonyl)amino-3-methoxycarbonyl)propyl)(hydroxy)phosphinyl]-3-((diethylphosphono)methyl)propanoate (47). The compound was prepared from **39** (0.8 mmol) and triethyl 4-phosphonocrotonate (751 mg, 3.0 mmol) according to general procedure A to afford **47** (84% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 1.28 (m, 9H), 2.19 (m, 6H), 2.81 (m, 3H), 3.73 (s, 3H), 4.12 (m, 6H), 4.32 (m, 1H), 5.12 (s, 2H), 7.37 (m, 5H). ^{31}P NMR (101 MHz, CD_3OD): δ 31.3 (d, J = 57.3 Hz), 54.0 (d, J = 57.3 Hz). MS (ESI): m/z 566.1 (M + 1).

(3*S*)-3-[((3-(*N*-Benzyloxycarbonyl)amino-3-methoxycarbonyl)propyl)(hydroxy)phosphinyl]-2-chloropropanoic Acid (48). The compound was prepared from **39** (0.8 mmol) and 2-chloroacrylic acid (320 mg, 3.0 mmol) according to general procedure A to afford **48** (93% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 2.34 (m, 6H), 3.72 (s, 3H), 4.13 (m, 1H), 4.68 (m, 1H), 5.11 (s, 2H), 7.33 (m, 5H).

Ethyl (3*S*)-3-[((3-(*N*-Benzyloxycarbonyl)amino-3-methoxycarbonyl)propyl)(hydroxy)phosphinyl]-2-methylpropanoate (49). The compound was prepared from **39** (0.8 mmol) and ethyl methacrylate (342 mg, 3.0 mmol) according to general procedure A to afford **49** (95% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 1.27 (m, 6H), 1.99 (m, 6H), 2.86 (m, 1H), 3.74 (s, 3H), 4.15 (q, J = 7.1 Hz, 2H), 4.31 (m, 1H), 5.12 (s, 2H), 7.35 (m, 5H). ^{31}P NMR (101 MHz, CD_3OD): δ 52.8.

(3*S*)-4-[((3-(*N*-Benzyloxycarbonyl)amino-3-methoxycarbonyl)propyl)(hydroxy)phosphinyl]furanone (50). The compound was prepared from **39** (0.8 mmol) and 2-(5*H*)-furanone (252 mg, 3.0 mmol) according to general procedure A to afford **50** (59% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 1.88 (m, 2H), 2.15 (m, 2H), 2.73 (m, 2H), 3.01 (m, 1H), 3.76 (s, 3H), 4.36 (m, 1H), 4.51 (m, 2H), 5.13 (s, 2H), 7.36 (m, 5H). ^{13}C NMR (63 MHz, CD_3OD): δ 24.1, 24.7 (d, J = 81 Hz), 28.6, 34.5 (d, J = 97 Hz), 51.9, 54.9, 66.8, 67.7, 127.9, 128.1, 128.6, 137.1, 157.6, 172.8, 177.3 (d, J = 10 Hz). ^{31}P NMR (101 MHz, CD_3OD): δ 49.2.

(3*S*)-2-[(((3-(*N*-Benzyloxycarbonyl)amino-3-methoxycarbonyl)propyl)(hydroxy)phosphinyl)methyl]oxotetrahydrofuran-3-yl (51). The compound was prepared from **39** (0.8 mmol) and 3-methylenedihydrofuran-2-(3*H*)-one (225 mg, 2.3 mmol) according to general procedure A to afford **51** (66% yield). ^{31}P NMR (101 MHz, CD_3OD): δ 52.0.

Dimethyl (3*S*)-2-[(((3-(*N*-Benzyloxycarbonyl)amino-3-methoxycarbonyl)propyl)(hydroxy)phosphinyl)methyl]butan-1,4-dioate (52). The compound was prepared from **39** (0.8 mmol) and dimethyl itaconate (474 mg, 3.0 mmol) according to general procedure A to afford **52** (61% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 2.10 (m, 6H), 2.83 (m, 2H), 3.20 (m, 1H), 3.75 (s, 3H), 3.72 (s, 3H), 3.71 (s, 3H), 4.30 (m, 1H), 5.13 (s, 2H), 7.38 (m, 5H). ^{13}C NMR (63 MHz, CD_3OD): δ 24.3, 25.9 (d, J = 93 Hz), 29.9 (d, J = 93 Hz), 35.9, 36.3, 51.5, 52.1, 54.1, 54.6, 66.8, 128.0, 128.2, 128.6, 137.2, 157.5, 172.4, 172.7, 174.6 (d, J = 10 Hz). ^{31}P NMR (101 MHz, CD_3OD): δ 52.2.

(3*S*)-2-[((3-(*N*-Benzyloxycarbonyl)amino-3-methoxycarbonyl)propyl)(hydroxy)phosphinyl]-2-hydroxyacetic Acid (53). The compound was prepared from **39** (0.8 mmol) and 2-oxoacetic acid (157 mg, 1.7 mmol) according to general procedure A. The crude product **53** was directly deprotected into **19**.

Ethyl (3*S*)-4-[((3-(*N*-Benzyloxycarbonyl)amino-3-methoxycarbonyl)propyl)(hydroxy)phosphinyl]-4-hydroxy-3-methyl-2-butenoate (54). The compound was prepared from **39** (0.8 mmol) and ethyl 3-methyl-4-oxocrotonate (426 mg, 3.0 mmol) according to general procedure A to afford **54**. ^{1}H NMR (250 MHz, CD_3OD): δ 1.26 (m, 3H), 2.16 (m, 7H), 3.74 (s, 3H), 4.19 (m, 2H), 4.48 (d, J = 13.7 Hz, 1H), 4.60 (m, 1H), 5.12 (s, 2H), 5.86 (m, 1H), 7.36 (m, 5H). ^{13}C NMR (63 MHz, CD_3OD): δ 14.0, 17.7, 22.6 (d, J = 90 Hz), 24.2, 52.2, 54.8, 60.7, 66.9, 74.1 (d, J = 102 Hz), 117.0, 128.0, 128.2, 128.7, 137.1, 155.9, 172.1, 172.8. ^{31}P NMR (101 MHz, CD_3OD): δ 49.0.

Ethyl (3*S*)-2-[(((3-(*N*-Benzyloxycarbonyl)amino-3-methoxycarbonyl)propyl)(hydroxy)phosphinyl)hydroxymethyl]cyclopropan-1-carboxylate (55). The compound was prepared from **39** (0.8 mmol) and *trans*-ethyl 2-formyl-1-cyclopropanecarboxylate (426 mg, 3.0 mmol) according to general procedure A to afford **55** (55% yield). ^{1}H NMR (250 MHz, CD_3OD): δ 1.19 (m, 5H), 1.96 (m, 6H), 3.40 and 3.67 (2m, 1H), 3.73 (s, 3H), 4.12 (m, 2H), 4.29 (m, 1H), 5.11 (s, 2H), 7.37 (m, 5H). ^{31}P NMR (101 MHz, CD_3OD): δ 50.5.

Methyl (4*S*)-3-[((4-(*N*-Benzyloxycarbonyl)amino-4-methoxycarbonyl)butyl)(hydroxy)phosphinyl]propanoate (56). The compound was prepared from **40** (1.0 mmol) and methyl acrylate (0.35 mL, 3.6 mmol) according to general procedure A to afford **56** (67% yield). ^{1}H NMR (250 MHz, CD_3OCD_3): δ 1.59−2.10 (m, 8H), 2.52−2.71 (m, 2H), 3.67 (s, 3H), 3.70 (s, 3H), 4.18−4.36 (m, 1H), 5.10 (s, 2H), 7.24−7.45 (m, 5H). ^{13}C NMR (126 MHz, CD_3OCD_3): δ 19.6, 22.6 (d, J = 97 Hz), 25.5 (d, J = 95 Hz), 27.6, 33.8 (d, J = 17 Hz), 52.6, 55.1, 61.06, 67.3, 129.1, 129.2, 129.7, 138.7, 157.7, 172.5, 174.0. ^{31}P NMR (101 MHz, CD_3COCD_3): δ 54.2. MS (ESI): m/z 414.0 (M − 1).

General Procedure B. A solution of protected compound in 6 N HCl was refluxed overnight and the solution was evaporated to dryness, followed by purification of residue by cation exchange resin chromatography (Dowex AG 50W-X4 column, H^+, 50−100 mesh, 24 cm × 1.7 cm, water elution). Yields were not optimized; only very pure fractions were collected for pharmacological tests.

General Procedure C. A solution of protected compound in 6 N HCl was refluxed overnight and the solution was evaporated to dryness, followed by purification of residue by anion exchange resin chromatography. The residue was dissolved in freshly boiled and cooled water (200 mL), then pH adjusted to 8−9, and the solution was deposited on a AG 1-X4 resin ($HCOO^-$, 200−400 mesh, 8.5 cm × 1 cm). The resin was washed with freshly boiled and cooled water, and the compound was eluted with HCOOH. Yields were not optimized; only very pure fractions were collected for pharmacological tests.

(3*S*)-3-[((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl]propanoic Acid [(*S*)-1].[34,35] The compound was prepared from **41** (130 mg, 0.3 mmol) according to general procedure B to afford 23.8 mg of pure (*S*)-**1**. ^{1}H NMR (500 MHz, D_2O): δ 1.76 (m, 2H), 1.93 (dt, J = 13.5/8.0 Hz, 2H), 2.09 (m, 2H), 2.57 (dt, J = 11.0/8.0 Hz, 2H), 4.03 (t, J = 6.0 Hz, 1H). ^{13}C NMR (126 MHz, D_2O): δ 24.5 (d, J = 2 Hz), 25.4 (d, J = 93 Hz), 26.1 (d, J = 91 Hz), 28.3, 55.1 (d, J = 15 Hz), 173.6, 178.5 (d, J = 15 Hz). ^{31}P NMR (101 MHz, D_2O): δ 57.4. MS (ESI): m/z 238.1 (M − 1). $[\alpha]_D$ +12.8 (*c* 1.0, H_2O), lit.[45] $[\alpha]_D$ +12.5 (*c* 1.2, H_2O), $[\alpha]_{546}$ + 15.0 (*c* 1.0, H_2O), lit.[34] (*R*)-**1** $[\alpha]_{546}$ −13.4 (*c* 1.1, H_2O). HPLC t_R = 2.0 min. HPLC−MS t_R = 4.95 min

(3*S*)-2-[((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl]ethanoic Acid (3).[40,46] The compound was prepared from **42** according to general procedure B to afford 6.6 mg of pure **3**. ^{1}H NMR (250 MHz, D_2O): δ 1.86 (m, 2H), 2.22 (m, 2H), 2.82 (d, J = 16.9 Hz, 2H), 4.08 (t, J = 6.2 Hz, 1H). ^{13}C NMR (63 MHz, D_2O): δ 21.9, 21.2 (d, J = 96 Hz), 36.8 (d, J = 77 Hz), 51.9, 170.3, 170.7. ^{31}P NMR (101 MHz, D_2O): δ 46.6. MS (ESI): m/z 226.1 (M − 1). $[\alpha]_D$ +14.8 (*c* 0.1, H_2O). HPLC−MS t_R = 4.68 min.

(3*S*)-4-[((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl]-butanoic Acid (4). The compound was prepared from **43** according to general procedure B to afford 21.6 mg of pure **4**. ^{1}H NMR (500 MHz, D_2O): δ 1.72 (m, 6H), 2.10 (m, 2H), 2.45 (t, J = 7.0 Hz, 2H), 3.94 (t, J = 6.0 Hz, 1H). ^{13}C NMR (126 MHz, D_2O): δ 18.9 (d, J = 2 Hz), 24.7 (d, J = 2 Hz), 26.0 (d, J = 89 Hz), 29.5

(d, J = 94 Hz), 36.0 (d, J = 15 Hz), 55.6 (d, J = 14 Hz), 174.2, 179.4. ^{31}P NMR (101 MHz, D_2O): δ 58.4. MS (ESI): m/z 252.1 (M − 1). HPLC−MS t_R = 4.68 min

(3*S*)-3-[((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl]propenoic Acid (6). The compound was prepared from **48** according to general procedure B. Partial elimination of HCl occurred during acidic hydrolysis, affording a mixture of **6** and **12** that were eluted together from the cation exchange resin. The mixture was dissolved in water, and the mildly acidic solution was loaded on the anion exchange column. It was eluted with 0.35 M HCOOH to afford 5.4 mg of pure **6**. ^{1}H NMR (500 MHz, D_2O): δ 1.81 (m, 2H), 2.13 (m, 2H), 4.11 (t, J = 6.0 Hz, 1H), 6.48 (t_{app}, J = 17.0 Hz, 1H), 7.03 (dd, J = 17.5/19.0 Hz, 1H). ^{13}C NMR (126 MHz, D_2O): δ 24.7, 27.1 (d, J = 99 Hz), 54.7 (d, J = 15 Hz), 135.1 (d, J = 5 Hz), 141.6 (d, J = 116 Hz), 170.2 (d, J = 22 Hz), 173.0. ^{31}P NMR (101 MHz, D_2O): δ 42.0. MS (ESI): m/z 236.1 (M − 1). HPLC t_R = 12.8 min. HPLC−MS t_R = 5.01 min. $[\alpha]_D$ +3.5 (c 0.4, H_2O).

(3*S*)-2-[((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl]-ethylphosphonate (7).[47] The compound was prepared from **44** according to general procedure C. The anion exchange column was eluted with 0.8−1.0 M HCOOH to afford 90.0 mg of pure **7**. ^{1}H NMR (250 MHz, D_2O): δ 1.75 (m, 6H), 2.00 (m, 2H), 3.97 (t, J = 5.8 Hz, 1H). ^{13}C NMR (63 MHz, D_2O): δ 18.2, 20.8 (d, J = 54 Hz), 22.7, 24.4, 53.1, 171.4. ^{31}P NMR (101 MHz, D_2O): δ 38.2 (d, J = 65.0 Hz), 62.0 (d, J = 65.1 Hz). MS (ESI): m/z 276.1 (M + 1). $[\alpha]_D$ +15.1 (c 0.1, H_2O). HPLC−MS t_R = 4.58 min.

(3*S*)-3-[((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl]-3-trifluoromethypropanoic Acid (9). The compound was prepared from **45** according to general procedure B to afford 8.6 mg of pure **9**. ^{1}H NMR (250 MHz, D_2O): δ 1.81 (m, 2H), 2.17 (m, 2H), 2.79 (m, 2H), 3.15 (m, 1H), 4.07 (m, 1H). ^{13}C NMR (63 MHz, D_2O): 23.3, 25.8 (d, J = 98 Hz), 29.2, 31.0, 41.4 (dq, J = 84/26 Hz), 53.6 (d, J = 15 Hz), 126.5 (q, J = 279 Hz), 172.0, 175.4. ^{31}P NMR (101 MHz, D_2O): δ 43.9. MS (ESI): m/z 306.1 (M − 1). HPLC−MS t_R = 3.90 min.

(3*S*)-3-[(((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl)]-pentan-1,5-dioic Acid (10). The compound was prepared from **46** according to general procedure B. The obtained residue was then purified using an anion exchange column (Dowex AG 1-X4, 200−400 mesh, $HCOO^-$, 0.4 M HCOOH elution) to afford 88.9 mg of pure **10**. ^{1}H NMR (250 MHz, D_2O): δ 1.63 (m, 2H), 2.00 (m, 2H), 2.33 (m, 3H), 2.58 (m, 2H), 3.91 (t, J = 6.1 Hz, 1H). ^{13}C NMR (63 MHz, D_2O): δ 23.2, 23.4 (d, J = 91 Hz), 31.8 (d, J = 93 Hz), 33.6, 53.8 (d, J = 15 Hz), 166.1, 172.2, 176.3 (d, J = 12 Hz). ^{31}P NMR (101 MHz, D_2O): δ 57.1. MS (ESI): m/z 296.1 (M − 1). $[\alpha]_D$ +12.5 (c 1.0, H_2O). HPLC−MS t_R = 3.75 min.

(3*S*)-3-[((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl]-2-(phosphonomethyl)propanoic Acid (11). The compound was prepared from **47** according to general procedure C. The anion exchange column was eluted with 1.0−1.3 M HCOOH to afford 84.0 mg of pure **11**. ^{1}H NMR (250 MHz, D_2O): δ 1.72 (m, 6H), 2.26 (m, 1H), 2.44 (m, 2H), 3.85 (m, 1H). ^{13}C NMR (63 MHz, CD_3OD): δ 22.2 (d, J = 91 Hz), 22.5, 25.1 (d, J = 137 Hz), 29.7 (d, J = 93 Hz), 33.4, 53.0, 171.1, 175.3 (d, J = 8 Hz). ^{31}P NMR (101 MHz, D_2O): δ 37.5 (d, J = 50.7 Hz), 63.7 (d, J = 50.7 Hz). HPLC−MS t_R = 3.51 min.

(3*S*)-3-[((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl]-2-chloropropanoic Acid (12). The compound was prepared from **48** according to general procedure B. During acidic hydrolysis, **12** was partly dehydrohalogenated into **6**; **6** and **12** were easily separated by anion exchange chromatography. The mixture was loaded at pH 5. Elution with 1.0 M HCOOH afforded 7.4 mg of pure **12** after elution of **6**. ^{1}H NMR (250 MHz, D_2O): δ 1.74 (m, 2H), 2.09 (m, 2H), 2.34 (m, 2H), 3.98 (m, 1H), 4.56 (m, 1H). ^{13}C NMR (63 MHz, D_2O): δ 23.5, 26.6 (d, J = 94 Hz), 35.3 (d, J = 89 Hz), 53.2, 53.9 (d, J = 15 Hz), 172.3, 173.8. ^{31}P NMR (101 MHz, D_2O): δ 51.6. MS (ESI): m/z 272.0, 274.0 (M − 1). HPLC t_R = 25.9 and 28.6 min. HPLC−MS t_R = 3.33 min.

(3*S*)-3-[((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl]-2-methylpropanoic Acid (13).[19] The compound was prepared from **49** according to general procedure B to afford 7.2 mg of pure **13**. ^{1}H NMR (250 MHz, D_2O): δ 1.14 (d, J = 7.0 Hz, 3H), 1.82 (m, 6H), 2.68 (m, 1H), 3.88 (t, J = 5.9 Hz, 1H). ^{13}C NMR (63 MHz, D_2O): δ 19.1 (d, J = 11 Hz), 23.7, 25.9 (d, J = 91 Hz), 32.8 (d, J = 93 Hz), 34.6, 54.3 (d, J = 15 Hz), 172.8, 180.9 (d, J = 8 Hz). ^{31}P NMR (101 MHz, D_2O): δ 54.5. MS (ESI): m/z 252.1 (M − 1). HPLC−MS t_R = 2.81 min.

(3*S*)-4-[((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl]-furanone (14). The compound was prepared from **50** according to general procedure B. The obtained residue was then purified using an anion exchange column (Dowex AG 1-X4, 200−400 mesh, acetate form, 0.3 M HCOOH elution) to afford 18.7 mg of pure **14**. ^{1}H NMR (500 MHz, D_2O): δ 1.58 (m, 2H), 2.03 (m, 2H), 2.57 (m, 1H), 2.69 (m, 1H), 2.80 (m, 1H), 3.95 (t, J = 6.0 Hz, 1H), 4.31 (m, 1H), 4.48 (m, 1H). ^{13}C NMR (63 MHz, D_2O): δ 23.2, 24.5 (d, J = 94 Hz), 29.5, 34.9 (d, J = 96 Hz), 54.2, 69.4, 172.0, 180.7 (d, J = 10 Hz). ^{31}P NMR (101 MHz, D_2O): δ 51.0. MS (ESI): m/z 250.1 (M − 1). HPLC−MS t_R = 3.61 min.

(3*S*)-3-[((3-Amino-3-carboxylate)propyl)(hydroxy)phosphinate]-3-hydroxymethylpropanoate Trisodium Salt (15). A sample of **14** (4.0 mg, 0.02 mmol) and sodium hydroxide (2.4 mg, 0.06 mmol) in 0.5 mL of pure water were stirred 36 h at room temperature. Water was removed under vacuum to afford **15**. ^{1}H NMR (250 MHz, D_2O): δ 1.59 (m, 4H), 2.15 (m, 2H), 2.50 (m, 1H), 3.19 (m, 1H), 3.70 (m, 2H). MS (ESI): m/z 268.0 (M − 1).

(3*S*)-2-[(((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl)-methyl]oxotetrahydrofuran-3-yl (16). The compound was prepared from **51** according to general procedure B to afford 59.6 mg of pure **16**. ^{1}H NMR (250 MHz, D_2O): δ 1.88 (m, 3H), 2.14 (m, 4H), 2.53 (m, 1H), 2.95 (m, 1H), 4.09 (m, 1H), 4.27 (m, 1H), 4.42 (m, 1H). ^{13}C NMR (126 MHz, D_2O): δ 24.3, 26.5 (d, J = 92 Hz), 30.5 (d, J = 94 Hz), 30.7, 35.9 (d, J = 2 Hz), 54.7 (d, J = 15 Hz), 69.8, 172.9, 183.9 (d, J = 19 Hz). ^{31}P NMR (101 MHz, D_2O): δ 59.2. MS (ESI): m/z 266.1 (M + 1). HPLC−MS t_R = 3.87 min.

(3*S*)-3-[((3-Amino-3-carboxylate)propyl)(hydroxy)phosphinate]-2-(2-hydroxyethyl)propanoate, Trisodium Salt (17). A sample of **16** (8.0 mg, 0.03 mmol) and sodium hydroxide (4.8 mg, 0.12 mmol) in 1.0 mL of pure water was stirred 24 h at room temperature. Water was removed under vacuum to afford **17**. ^{1}H NMR (250 MHz, D_2O): δ 1.66 (m, 8H), 2.49 (m, 1H), 3.19 (m, 1H), 3.52 (m, 2H). ^{31}P NMR (101 MHz, D_2O): δ 54.7. MS (ESI): m/z 350.1 (M + 1 + 3Na^+). HPLC−MS t_R = 3.82 min.

(3*S*)-2-[(((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl)-methyl]butan-1,4-dioic Acid (18).[19] The compound was prepared from **52** according to general procedure B to afford 72.0 mg of pure **18**. ^{1}H NMR (250 MHz, D_2O): δ 1.82 (m, 3H), 2.17 (m, 3H), 2.78 (m, 2H), 3.08 (m, 1H), 4.04 (t, J = 6.1 Hz, 1H). ^{13}C NMR (63 MHz, D_2O): δ 23.3, 25.5 (d, J = 91 Hz), 30.3 (d, J = 91 Hz), 36.0, 37.0 (d, J = 7 Hz), 53.7 (d, J = 15 Hz), 172.1, 175.9, 178.3 (d, J = 9 Hz). ^{31}P NMR (101 MHz, D_2O): δ 56.4. HPLC−MS t_R = 3.00 min.

(3*S*)-3-[((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl]-2-(hydroxymethyl)ethanoic Acid (19). The compound was prepared from **53** according to general procedure B. The obtained residue was then purified using an anion exchange column (Dowex AG 1-X4, 200−400 mesh, acetate form, 0.5 M HCOOH elution) to afford 40.8 mg of pure **19**. ^{1}H NMR (250 MHz, D_2O): δ 1.89 (m, 2H), 2.14 (m, 2H), 4.10 (bs, 1H), 4.52 (d, J = 13.1 Hz, 1H). ^{13}C NMR (63 MHz, D_2O): δ 24.2, 24.3 (d, J = 90 Hz), 54.5 (d, J = 16 Hz), 72.1 (d, J = 96 Hz), 172.8, 174.7. ^{31}P NMR (101 MHz, D_2O): δ 51.2. MS (ESI): m/z 240.1 (M − 1). HPLC−MS t_R = 4.05 min.

(3*S*)-4-[((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl]-4-hydroxy-3-methyl-2-butenoic Acid (20). Compound **54** (1.0 mmol) was dissolved in 10.0 mL of ethanol and 10.0 mL of water. Lithium hydroxide (144 mg, 6.0 mmol) in 5.0 mL of water was added to the solution, which was stirred at room tempera-

ture for 12 h. Following removal of ethanol under vacuum, the resulting solution pH was adjusted to 1, then extracted with ethyl acetate (2 × 100 mL). The organic layer was washed with brine and dried with anhydrous magnesium sulfate, and the solvent was evaporated under vacuum to afford the benzyloxycarbonyl protected **20**. ^{1}H NMR (250 MHz, CD_3OD): δ 1.88 (m, 7H), 4.21 (m, 1H), 4.39 (d, J = 12.1 Hz, 1H), 5.11 (s, 2H), 5.93 (m, 1H), 7.39 (m, 5H). ^{31}P NMR (101 MHz, CD_3OD): δ 49.4. The removal of the benzyloxycarbonyl group was then accomplished by adding 4 N HCl (5.0 mL). The resulting solution was stirred at 75 °C for 4 h and cooled to room temperature. Volatile organic byproducts and water were removed under vacuum. The compound **20** was purified by anion exchange column (Dowex AG 1-X4, 200−400 mesh, formate form, 0.3 M HCOOH elution) to afford 22.7 mg of pure **20**. ^{1}H NMR (250 MHz, D_2O): δ 1.86 (m, 2H), 2.16 (m, 5H), 4.10 (m, 1H), 4.43 (d, J = 13.3 Hz, 1H), 5.99 (d, J = 3.9 Hz, 1H). ^{13}C NMR (63 MHz, D_2O): δ 17.2, 22.4 (d, J = 100 Hz), 23.2, 53.5, 75.3 (d, J = 107 Hz), 116.4, 157.2, 170.5, 171.8. ^{31}P NMR (101 MHz, D_2O): δ 53.1. HPLC−MS t_R = 5.04 min.

(3*S*)-2-[(((3-Amino-3-carboxy)propyl)(hydroxy)phosphinyl)hydroxymethyl]cyclopropane-1-carboxylic Acid (21 and 22). The compounds were prepared from **55** according to general procedure B. The crude product was a mixture of four diastereoisomers that were separated into two sets **21** and **22** of two diastereoisomers each by cation exchange column (200−400 mesh, 44 cm × 2.2 cm, H^+, water elution). **22** was eluted first, and 3.1 mg of **22** and 4.4 mg of **21** were obtained. **22**: ^{1}H NMR (250 MHz, D_2O) δ 1.12 (m, 1H), 1.25 (m, 1H), 1.80 (m, 4H), 2.13 (m, 2H), 3.40 (m, 1H), 3.96 (m, 1H). ^{31}P NMR (101 MHz, D_2O): δ 51.5. HPLC−MS t_R = 3.71 min. **21**: ^{1}H NMR (250 MHz, D_2O) δ 1.14 (m, 1H), 1.34 (m, 1H), 1.82 (m, 4H), 2.15 (m, 2H), 3.15 (m, 1H), 4.00 (m, 1H). ^{13}C NMR (63 MHz, D_2O): δ 13.3 (d, J = 78 Hz), 18.4 (d, J = 43 Hz), 22.6 (d, J = 79 Hz), 23.2 (d, J = 79 Hz), 23.3, 53.9, 70.6 (d, J = 109 Hz), 72.3 (d, J = 112 Hz), 172.5, 178.5. ^{31}P NMR (101 MHz, D_2O): δ 51.5. HPLC−MS t_R = 4.11 min.

(2*S*)-2-Amino-5-[(hydroxy)phosphinyl]pentanoic Acid (23) and (4*S*)-3-[((4-Amino-4-carboxy)butyl)(hydroxy)phosphinyl]propanoic Acid (24). The compounds were prepared from **56** (166 mg) according to general procedure B. **23** and **24** were separated by cation exchange resin chromatography (Dowex AG 50W-X4 column, H^+, 50−100 mesh, 24 cm × 1.7 cm, water elution). **23** was eluted first, and 34.7 mg of **23** and 15.5 mg of **24** were obtained. **23**: ^{1}H NMR (500 MHz, D_2O): δ 1.52−1.69 (m, 4H), 1.87−1.98 (m, 2H), 3.94−3.97 (m, 1H), 6.93 (d, J_{HP} = 517 Hz, 1H). ^{13}C NMR (126 MHz, D_2O): δ 18.3, 31.4 (d, J = 90 Hz), 32.3 (d, J = 17 Hz), 54.5, 174.0. ^{31}P NMR (101 MHz, D_2O): δ 42.1. MS (ESI): m/z 180.0 (M − 1), 361.0. $[\alpha]_D^{20}$ +3.8 (c 0.5, H_2O). HPLC−MS t_R = 3.03 min. **24**: ^{1}H NMR (250 MHz, D_2O): δ 1.45−1.78 (m, 4H), 1.80−2.12 (m, 4H), 2.41−2.63 (m, 2H), 3.83−4.01 (m, 1H). ^{13}C NMR (63 MHz, D_2O): δ 17.9, 24.2 (d, J = 91 Hz), 27.5, 28.3 (d, J = 92 Hz), 31.4 (d, J = 16 Hz), 53.4, 173.1, 177.5 . ^{31}P NMR (101 MHz, D_2O): δ 61.7. MS (ESI): m/z 252.1 (M − 1). $[\alpha]_D^{20}$ +7.1 (c 0.7, H_2O). HPLC−MS t_R = 3.20 min.

Molecular Modeling. All calculations were performed in Discovery Studio 2.1 (Accelrys Software Inc., San Diego, CA).

Homology Modeling. All mGluR ATD sequences were aligned using Align123. The resulting sequence alignment was further used for comparative modeling. mGlu4R and mGlu7R models were generated using MODELER and based on mGlu1R and mGlu3R (respectively PDB codes 1EWK and 2E4U). For each run, 100 models were generated and subsequently analyzed based on MODELER's probability density function, Profiles_3D scores, and the percentage of amino acids located in the disallowed regions of the Ramachandran plot. Models were selected in order to optimize these parameters.

Docking. For each docking experiment, the ligand was initially positioned in the binding site using CDOCKER. CDOCKER uses a CHARMm-based molecular dynamics scheme to dock ligands into a receptor binding site. Random ligand conformations are generated using high-temperature molecular dynamics. The conformations are then translated into the binding site. Candidate poses are then created using random rigid-body rotations followed by simulated annealing. A final energy minimization is then used to refine the ligand poses. As CDOCKER takes into account only ligand flexibility, protein−ligand interactions were further optimized by 1 ns molecular dynamics using CHARMm. Once the trajectory was equilibrated, snapshots of the trajectory were analyzed in terms of protein−ligand contacts and the selected ones were submitted to energy minimization leading to the figures presented in this article (Figure 3 and 4).

Pharmacology. Cell Culture and Transfection. Pharmacological experiments were performed in HEK293 cells cultured in Dulbecco's modified Eagle's medium (GIBCO-BRL-Life Technologies, Inc., Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS, GIBCO-BRL-Life Technologies, Inc., Cergy Pontoise, France) and antibiotics (penicillin and streptomycin, 100 U/mL final, GIBCO-BRL-Life Technologies, Inc., Cergy Pontoise, France).

Metabotropic glutamate receptors were transiently transfected in HEK293 cells by electroporation as described elsewhere[48] and plated in 96-well microplates. The high affinity glutamate transporter EAAC1 was co-transfected with the receptor in order to avoid any influence of glutamate released by the cells in the assay medium. Group II and group III mGluRs activate naturally Gi/o proteins that modulate the adenylylcyclase pathway. In our experiments, the receptors belonging to these groups were co-transfected with a chimeric G-protein which is recognized by these receptors but couples to the phospholipase C (PLC) pathway. Thus, receptor activation induces production of inositol phosphate (IP) which in turn induces intracellular Ca^{2+} release. Receptor activity was then determined by measurement of the IP production or Ca release as already described.[49]

We previously reported that these assays are more easily handled and gave more accurate results than the classical measurement of the inhibition of the forskolin-activated adenylylcyclase activity and that the pharmacology of these receptors was not altered.[22] For a given compound, the EC_{50} values determined by either IP or Ca^{2+} measurement are comparable. For example, the EC_{50} values of L-AP4 on mGlu4, -6, -7, and -8 obtained by the IP method are 0.096 ± 0.013 μM (n = 61), 3.3 ± 0.5 μM (n = 16), 306 ± 70 μM (n = 10), and 0.24 ± 0.025 μM (n = 45), respectively, while these values are 0.13 ± 0.02 μM (n = 34), 1.0 ± 0.3 (n = 7), 249 ± 106 μM (n = 3), and 0.30 ± 0.08 μM (n = 43) as determined by the Ca^{2+} method.

Inositol Phosphate Determination. Briefly, cells were seeded after transfection in polyornithine-coated 96-well plates and cultured for 24 h in glutamate/glutamine-free medium. Six hours following transfection, cells were incubated overnight with ^{3}H-myoinositol (23.4 Ci/mol, Amersham Saclay, France). The following day, cells were rinsed and ambient glutamate was degraded by incubation in the presence of glutamate−pyruvate transaminase (Roche, Basel, Switzerland). 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, Marnes-La-Coquette, France) and then eluted by a 4 M formate solution and collected in a 96-well sample plate. The radioactivity remaining in the membranes, which is proportional to the quantity of cells in each well, was used to normalize the IP produced. Radioactivity was counted using a Wallac 1450 Microbeta stintillation and luminescence counter (Perkin-Elmer, Courtaboeuf, France). Results are expressed as the ratio between IP and the total radioactivity corresponding to IP plus membrane. All points are realized in triplicate.

Intracellular Calcium Measurements. Cells were seeded after transfection in polyornithine-coated, black-walled, clear-bottom 96-well plates and cultured for 24 h in glutamate/glutamine-free medium. Cells were washed with fresh buffer and loaded with Ca^{2+}-sensitive fluorescent dye Fluo-4 AM (Invitrogen, Cergy Pontoise, France) dissolved in Hanks' balanced salt solution (HBSS, Invitrogen, Cergy Pontoise, France) containing 2.5 mM probenicid (Sigma-Aldrich Chemie, Saint-Quentin Fallavier, France) for 1 h at 37 °C. Cells were washed and incubated with HBSS containing probenecid. A drug plate was prepared with the various concentrations of agonist to be tested, and drug solution was added in each well after 20 s of recording. Fluorescence signals (excitation 485 nm, emission 525 nm) were measured by using the fluorescence microplate reader Flexstation III (Molecular Devices, Saint Grégoire, France) at sampling intervals of 1.5 s for 60 s. 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: Homology models, ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, mass spectra, HPLC chromatograms of **1**, **6**, **12**, **40**. This material is available free of charge via the Internet at http://pubs.acs.org.

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