(*S*)-2-Amino-3-(3-hydroxy-7,8-dihydro-6*H*-cyclohepta[*d*]isoxazol-4-yl)propionic Acid, a Potent and Selective Agonist at the GluR5 Subtype of Ionotropic Glutamate Receptors. Synthesis, Modeling, and Molecular Pharmacology

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We have previously described (*RS*)-2-amino-3-(3-hydroxy-7,8-dihydro-6*H*-cyclohepta[*d*]isoxazol-4-yl)propionic acid (4-AHCP) as a highly effective agonist at non-*N*-methyl-D-aspartate (non-NMDA) glutamate (Glu) receptors in vivo, which is more potent than (*RS*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) but inactive at NMDA receptors. However, 4-AHCP was found to be much weaker than AMPA as an inhibitor of [^{3}H]AMPA binding and to have limited effect in a [^{3}H]kainic acid binding assay using rat cortical membranes. To shed light on the mechanism(s) underlying this quite enigmatic pharmacological profile of 4-AHCP, we have now developed a synthesis of (*S*)-4-AHCP (**6**) and (*R*)-4-AHCP (**7**). At cloned metabotropic Glu receptors mGluR1α (group I), mGluR2 (group II), and mGluR4a (group III), neither **6** nor **7** showed significant agonist or antagonist effects. The stereoisomer **6**, but not **7**, activated cloned AMPA receptor subunits GluR1o, GluR3o, and GluR4o with EC_{50} values in the range 4.5−15 μM and the coexpressed kainate-preferring subunits GluR6 + KA2 (EC_{50} = 6.4 μM). Compound **6**, but not **7**, proved to be a very potent agonist (EC_{50} = 0.13 μM) at the kainate-preferring GluR5 subunit, equipotent with (*S*)-2-amino-3-(5-*tert*-butyl-3-hydroxyisothiazol-4-yl)propionic acid [(*S*)-Thio-ATPA, **4**] and almost 4 times more potent than (*S*)-2-amino-3-(5-*tert*-butyl-3-hydroxyisoxazol-4-yl)propionic acid [(*S*)-ATPA, **3**]. Compound **6** thus represents a new structural class of GluR5 agonists. Molecular modeling and docking to a crystal structure of the extracellular binding domain of the AMPA subunit GluR2 has enabled identification of the probable active conformation and binding mode of **6**. We are able to rationalize the observed selectivities by comparing the docking of **4** and **6** to subtype constructs, i.e., a crystal structure of the extracellular binding domain of GluR2 and a homology model of GluR5.

Introduction

Glutamic acid (Glu) is the major excitatory amino acid neurotransmitter in the central nervous system (CNS) and is implicated in pathological processes leading to a number of neurological and psychiatric diseases.[1−3] The transmitter functions of Glu are mediated by a heterogeneous group of receptors comprising both G-protein-coupled metabotropic receptors (mGluRs) and ionotropic receptors (iGluRs).[4−6] On the basis of structural, functional, and pharmacological characteristics, the eight mGluRs[7−9] are divided into three groups: group I (mGluR1,5), group II (mGluR2,3), and group III (mGluR4,6,7,8).[8−10] The iGluRs are subdivided into three pharmacologically distinct classes of receptors, namely, *N*-methyl-D-aspartate (NMDA), (*RS*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA), and kainate receptors.[11,12] AMPA and kainate receptors were previously termed collectively non-NMDA receptors.[13]

The iGluRs are homo- or heteromeric assemblies of subunits forming ion channels, AMPA receptors being made up of GluR1−4 subunits and kainate receptors of GluR5−7 and KA1,2 subunits.[14,15] Whereas (*S*)-AMPA (**2**, Figure 1) shows low or no affinity for kainate receptors, kainic acid (**1**) exerts a nondesensitizing response at AMPA receptors, resulting in large current flows.[16] The observed effects of **1** at native iGluRs in some cases probably reflect activation of AMPA receptors,[17] and a number of questions regarding the pharmacology of **1** and kainate receptors remain unanswered.

Initial pharmacological studies indicated that the AMPA analogue (*RS*)-2-amino-3-(5-*tert*-butyl-3-hydroxyisoxazol-4-yl)propionic acid (ATPA) was a relatively weak AMPA receptor agonist.[18] However, more recent studies have revealed that ATPA is a selective and highly potent agonist at homomerically expressed GluR5 receptors and that [^{3}H]ATPA binds to cloned human GluR5 with a K_d of 13 nM.[19,20] These effects of ATPA reside with the (*S*)-enantiomer **3**,[21] and (*S*)-2-amino-3-(5-*tert*-butyl-3-hydroxyisothiazol-4-yl)propionic acid [(*S*)-Thio-ATPA, **4**] has recently been characterized as a selective GluR5 agonist of even greater potency than (*S*)-ATPA (**3**).[22] Together with (*S*)-5-iodowillardiine (**5**,[23,24] Figure 1), these compounds form a group of selective and highly potent GluR5 agonist ligands possessing

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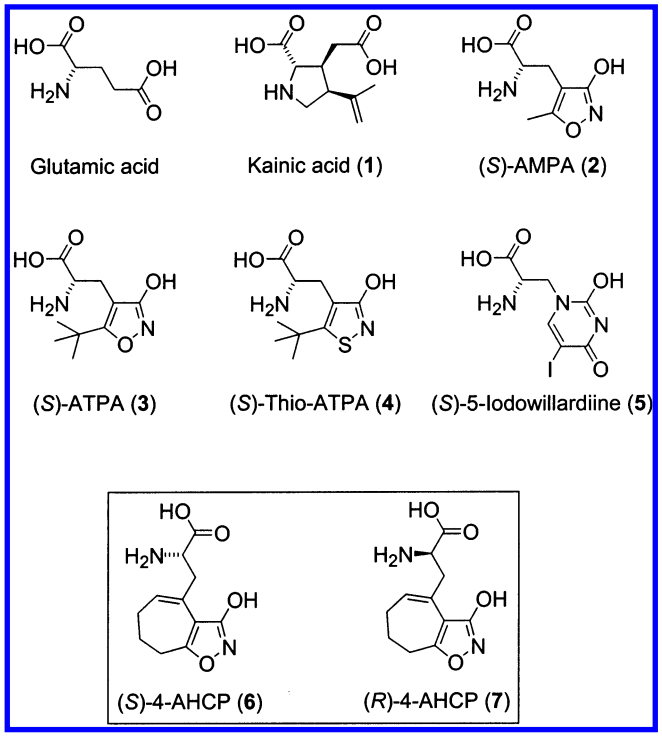


Figure 1. Structures of glutamic acid, a number of agonists at AMPA and kainate receptors, the eutomer (*S*)-4-AHCP (**6**), and the distomer (*R*)-4-AHCP (**7**).

bulbous and relatively bulky substituents in equivalent positions, considered to be an important structural determinant for effective activation of GluR5 receptors. The bicyclic amino acid (*RS*)-2-amino-3-(3-hydroxy-7,8-dihydro-6*H*-cyclohepta[*d*]isoxazol-4-yl)propionic acid (4-AHCP) has previously been described as a highly active non-NMDA receptor agonist in vivo showing limited affinity for [^{3}H]AMPA or [^{3}H]kainic acid binding sites.[25,26] We here describe the synthesis of (*S*)-4-AHCP (**6**) and (*R*)-4-AHCP (**7**) and the pharmacological characterization of **6** as a selective and very potent agonist at cloned GluR5 receptors. The results of molecular modeling and docking experiments have enabled identification of the probable active conformations and binding modes of **4** and **6** at a homology model of the GluR5 recognition site.

Results

Chemistry. The syntheses of (*S*)-4-AHCP (**6**) and (*R*)-4-AHCP (**7**) are outlined in Scheme 1. Compound **8**[27] was protected using isopropyl bromide and potassium carbonate in DMF to give the O-alkylated compound **9** (70−92%), contaminated by a minor amount of the corresponding N-alkylated derivative **10**. Chromic acid oxidation of **9** gave the desired ketone **11** in 47% yield together with **12** (27% yield). By reaction with triethyl phosphonoacetate, ketone **11** was converted into a mixture of isomeric esters **13** (61%) and a 1:1 mixture of **14** and **15** (29%, from ^{1}H NMR). Compound **13** was converted into the isomeric ester **15** by treatment with potassium *tert*-butoxide. Subsequent DIBAL-H reduction of **15** provided aldehyde **17** (72%). Under "Ugi four-component condensation" reaction conditions,[28−30] with (*R*)-1-phenylethylamine [(*R*)-PEA] as a chiral auxiliary, aldehyde **17** was converted into the (2*S*)- and (2*R*)-diastereomeric compounds **18** and **19**, respectively. Compounds **18** (99.9% de) and **19** (99.5% de) were readily separable by column chromatography (CC). The ^{1}H NMR chemical shifts of the *tert*-butyl groups of this type of diastereomeric product containing a PEA moiety have been empirically used to assign the stereochemistry at the α-amino acid centers.[29,31,32] Thus, a resonance signal for the *tert*-butyl groups in the region δ 1.3−1.4 ppm indicates (*S*,*R*)- or (*R*,*S*)-configurations, whereas a signal at about δ 1.1 ppm indicates (*S*,*S*)- or (*R*,*R*)-configurations. On the basis of ^{1}H NMR spectroscopic analyses, compounds **18** (δ 1.39 ppm) and **19** (δ 1.12 ppm) were assigned the (2*S*)- and (2*R*)-configurations, respectively. Compounds **18** and **19** were deprotected in two steps. Formic acid debenzylation provided the respective (*S*)-isomer **20** (99.8% ee) and (*R*)-isomer **21** (99.5% ee) after recrystallization. Subsequently **20** and **21** were treated with a mixture of aqueous HCl and AcOH followed by ion exchange. The fully deprotected crude products of **6** (60% ee) and **7** (40% ee) showed pronounced racemization. Preparative chiral HPLC of these crude products gave pure **6** (99.9% ee) and **7** (98.8% ee). For a large number of α-amino acids, it has been shown that the (*S*)-enantiomer is less retained than the (*R*)-enantiomer on Chirobiotic T[33] and Crownpak CR(−) columns.[34] The elution order of **6** and **7** on these two columns was in accordance with the assigned configurations based on ^{1}H NMR studies. The elution order of **6** and **7** was reversed using a Crownpak CR(+) column. CD spectra of **6** and **7** are mirror images demonstrating the enantiomeric relationship. The respective positive and negative Cotton effects observed for **6** and **7** are in agreement with what has been previously observed for α-amino acids containing an isoxazole moiety, (*S*)- and (*R*)-enantiomers respectively showing positive and negative Cotton effects of around 210−220 nm in acidic solutions.[29,35−37]

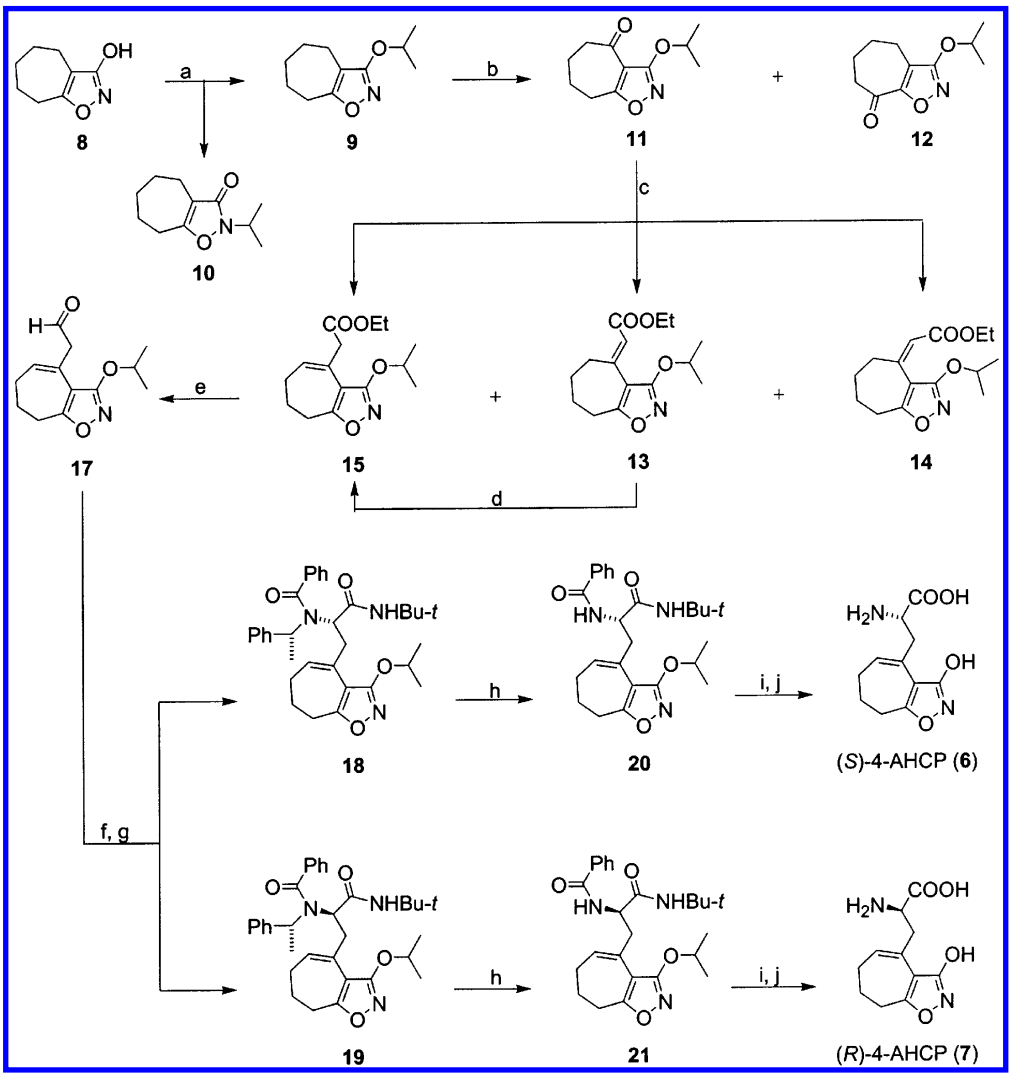
Table 1. Binding Data on Ionotropic Glutamate Receptors in Rat Cortical Membranes

	IC_{50} [a] (μM)		
	[^{3}H]AMPA	[^{3}H]kainic acid	[^{3}H]CGP39653
AMPA[b]	0.039 (0.037, 0.041)	>100	>100[c]
3[b]	1.8 (1.6, 2.0)	23 (22, 24)	>100[c]
4[b]	0.27 (0.22, 0.34)	14 (11, 18)[d]	>100[c]
6	0.28 (0.25, 0.32)	1.6 (1.3, 1.9)[d]	>100
7	33 (29, 38)	>100	28 (25, 32)[e]

[a] Values are expressed as the antilog of the mean of the log of at least three individual experiments. The numbers in parentheses (min, max) indicate ±SEM according to a logarithmic distribution. [b] Data from ref 22. [c] Tested in [^{3}H]CPP binding assay. [d] B_{max1} for **4** and **6** were 53% and 30%, respectively. [e] K_i value. K_d = 6 nM (from ref 55).

In Vitro Pharmacology. The affinities of **6** and **7** for native AMPA, kainate, and NMDA receptors were established using the radioligands [^{3}H]AMPA, [^{3}H]-kainic acid, and [^{3}H]CGP 39653, respectively (Table 1). Compound **6** was found to be the eutomer at AMPA receptors, displacing [^{3}H]AMPA with relatively high affinity (0.28 μM) and displacing [^{3}H]kainic acid with low affinity (Table 1). Compound **6** only displaced 70% of the bound [^{3}H]kainic acid, indicating that the compound is able to discriminate between different [^{3}H]-kainic acid labeled receptor sites, as previously reported for (*S*)-Thio-ATPA (**4**).[22] Compound **7** showed very low displacement of bound [^{3}H]AMPA, [^{3}H]kainic acid, and [^{3}H]CPG 39653.

Scheme 1[a,b]



[a] Reagents and conditions: (a) $(CH_3)_2CHBr$, K_2CO_3, DMF; (b) $Na_2Cr_2O_7$, AcOH, concentrated H_2SO_4; (c) $EtOOCCH_2PO_3(Et)$; (d) *t*-BuOK, DME; (e) DIBAL-H, toluene; (f) (*R*)-PEA, benzoic acid, *tert*-butylisonitrile, MeOH; (g) column chromatography; (h) 98% HCOOH; (i) 23% aqueous HCl, AcOH; (j) chiral HPLC. [b] Compound: **16** is the *tert*-butyl ester analogue of **15**.

Table 2. Electrophysiological Data on Oocytes Expressing Different Homomeric and Heteromeric AMPA and Kainate Receptors[a]

	3[b]			**4**[b]			**6**		
	EC_{50} (μM)	*n*	rel I_{max}	EC_{50} (μM)	*n*	rel I_{max}	EC_{50} (μM)	*n*	rel I_{max}
GluR1o	22 (18, 27)	1.6	0.33 ± 0.11	5.2 (4.8, 5.6)	0.64	0.78 ± 0.026	4.5 (3.9, 5.3)	1.1 (1.0, 1.2)	0.67 ± 0.055
GluR3o	7.9 (6.6, 9.2)	0.84	0.054 ± 0.002	32 (31, 33)	0.90	1.2 ± 0.015	7.2 (6.6, 7.9)	1.4 (1.4, 1.5)	1.04 ± 0.084
GluR4o	7.6 (5.6, 9.6)	0.67	0.34 ± 0.051	20 (15, 27)	0.8	1.7 ± 0.095	15 (13, 17)	1.3 (1.3, 1.3)	1.64 ± 0.22
GluR5	0.48 (0.44, 0.52)	1.0	1.0 ± 0.083	0.10 (0.090, 0.12)	1.5	1.0±0.027	0.13 (0.12, 0.14)	1.0 (1.0, 1.1)	1.61 ± 0.13
GluR6	nr			nr			nr		
GluR6 + KA2	61 (58, 64)	2.0	0.17 ± 0.011	4.9 (4.6, 5.3)	1.9	0.41 ± 0.040	6.4 (5.8, 6.9)	0.9 (0.9, 1.0)	0.53 ± 0.0.064

[a] Values are expressed as the antilog of the mean of the log of at least three individual experiments. The numbers in parantheses (min, max) indicate ±SEM according to a logarithmic distribution. The I_{max} values are relative to maximal steady-state currents evoked by kainic acid for all the receptor combinations except GluR6 + KA2 heteromers, where the amplitudes are relative to the maximal currents evoked by AMPA. (*R*)-4-AHCP (**7**) did not show any agonistic or antagonistic activity that could not be ascribed to contamination with (*S*)-4-AHCP (**6**). nr = no response at 100 μM. [b] Data from ref 22.

Compounds **6** and **7** were tested for pharmacological activity on mGluR1α, mGluR2, and mGluR4a expressing CHO cells, representing groups I, II, and III mGluR receptors, respectively, using conventional second messenger assays. At 1 mM concentrations, no agonist or antagonist (inhibition of 30 μM Glu response) activity was observed.

In Vitro Electrophysiology. Compounds **6** and **7** were applied on two-electrode-voltage-clamped *Xenopus* oocytes, each expressing one of the following: homomeric AMPA receptors composed of GluR1o, GluR3o, or GluR4o; homomeric kainate receptors composed of GluR5 or GluR6; heteromeric GluR6 + KA2 kainate receptors (Table 2). Compound **6** was shown to be an agonist at all of the AMPA receptor subtypes, with EC_{50} values ranging from 4.5 to 15 μM, being most potent at GluR1o. At GluR5 receptors, however, compound **6** was found to be 35−115 times more potent than at AMPA receptors, having an EC_{50} of 0.13 μM and thus being equipotent with **4** at GluR5. The amplitudes of the

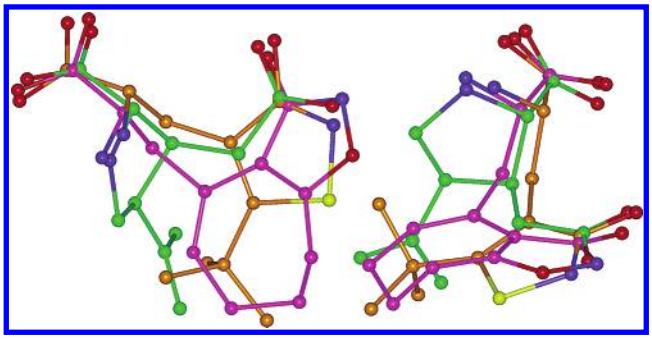


Figure 2. Two views of compounds **1** (green), **4** (orange), and **6** (magenta) superimposed in low-energy conformations on the basis of the five isosteric N and O atoms.

maximal steady-state currents (I_{max}) relative to the maximal responses evoked by kainic acid (**1**) were highest for GluR4o and GluR5 (Table 2). Application of up to 100 μM of **7** resulted in very small responses that could be ascribed to small amounts of **6** present.

Molecular Modeling. Ligand Analysis. Compounds **1**, **4**, and **6**, modeled as tri-ionized species, were subjected to a Monte Carlo conformation search using the MMFFs force field in Macromodel 8.0, including GB/SA treatment of aqueous solvation,[38] and were superimposed by least-squares fitting of the five isosteric oxygen and nitrogen atoms to the known binding mode of **1**, which is also its global energy minimum (Figure 2). The minimum energy conformation of **6** matched **1** closely, apart from a rotation of the α-carboxylate group of **6**; rotating this group to match **1** costs 2.4 kJ/mol. The seven-membered ring prefers to adopt a conformation anti to the α-amino acid side chain. As commonly seen for flexible ionized ligands, intramolecular hydrogen-bonded structures dominate the low-energy conformers of **4** according to the mechanics potential; however, the lowest energy extended conformation matched **1** (at 10.2 kJ/mol above the global minimum for **4**).

Homology Modeling. Sequences were first aligned using BLAST,[39] and an S1S2 sequence construct was built for GluR5 corresponding to GluR2−S1S2J (53% identity; higher within the binding site) (Figure 3). A homology model of GluR5−S1S2J was built on the basis of the **1**:GluR2−S1S2J crystal structure[40] using Swiss-model, first approach mode.[41] Compound **1** was reintroduced to the binding site. Because it was apparent that Swissmodel had modeled the side chain of M733 in a conformation unfavorable for binding, a restrained Monte Carlo search (MMFFs force field[38]) was performed on this residue and the hydrophobic side chains in contact with it. Once a suitable low-energy side chain packing had been found, the entire complex was submitted to a series of restrained minimizations using the impref module in Impact 1.8 (OPLS-AA force field).[38]

Docking. Both complexes (experimental kainic acid:GluR2−S1S2J and the model of kainic acid:GluR5−S1S2J) were prepared according to the method recommended prior to docking using Glide 1.8.[38] Compounds **1**, **4**, and **6** were docked to both models. The results were clear-cut, with all three ligands given highest scores and lowest energies in binding poses corresponding to those observed experimentally for similar ligands.[40,42] As an internal test, the four highest-scoring (Glidescore[38]) poses of **1** docked to GluR2, which also had the four lowest interaction energies (Emodel[38]), had rms deviations from the crystal structure of around 0.5 Å or less. In addition, the docked position of **4** in GluR2 closely resembles the binding mode that has been depicted for the experimental **4**:GluR2−S1S2J complex,[43] giving confidence in Glide's ability to also predict the binding mode of **6**. The results of docking **4** and **6** to GluR2 and GluR5 are depicted in Figures 4 and 5, respectively.

Discussion

The bicyclic Glu homologue (*RS*)-2-amino-3-(3-hydroxy-7,8-dihydro-6*H*-cyclohepta[*d*]isoxazol-4-yl)propionic acid (4-AHCP), derived from the standard AMPA receptor agonist (*S*)-AMPA (**2**) (Figure 1), was originally designed[25] as a tool for indirect mapping of AMPA receptor recognition sites.[44] Using microelectrophoretic techniques, 4-AHCP was shown to be a highly potent agonist at non-NMDA receptors in vivo, markedly more potent than AMPA.[25] However, 4-AHCP displayed only limited affinity for [^{3}H]kainic acid[25] and [^{3}H]AMPA[26] binding sites. For a number of years, attempts to improve the very low-yield synthesis of 4-AHCP[25] failed, and furthermore lack of appropriate pharmacological tools prevented studies on the mechanism(s) underlying this quite enigmatic pharmacological profile of 4-AHCP.

We have previously shown that homologues of Glu and Glu analogues typically show agonist or antagonist effects at subtypes of mGluRs,[29,45−47] encouraging the investigation of 4-AHCP as a potential mGluR ligand. In addition, cloned AMPA receptors (GluR1−4) and kainate receptors (GluR5−7, KA1,2) are now available for pharmacological characterization of Glu receptor ligands. Prompted by these pharmacological advances in the Glu receptor field and by the accumulating evidence of the important role of kainate receptors[17] including GluR5 receptors[48] in CNS disorders such as epilepsy, we decided to synthesize (*S*)-4-AHCP (**6**) and (*R*)-4-AHCP (**7**) with a view to further pharmacological characterization.

GluR2										
402	450	478	485	650	654	686	702	705		723
L **E** S	K **Y** G	A **P** L **T** I	V **R** E	T **L** D S G **S** **T** K		T **T** A	A **Y** L L **E** S T **M** N			G **Y** G
GluR5										
435	483	511	518	679	683	715	731	734		752
L **E** E	K **Y** G	A **P** L **T** I	V **R** E	A **V** R D G **S** **T** M		N **S** D	A **L** L M **E** S T **S** I			G **Y** G
Location										
lock	Pkt	$-NH_3^+$	$-COO^-$	Pkt	distal anion	lock	Pkt	$-NH_3^+$	Pkt	Pkt

Figure 3. Comparison of binding site residues (bold) in GluR2 and GluR5, based on alignment of the S1S2 extracellular ligand binding domains (53% identity). Numbering is derived from ref 40. "lock" = agonist state interdomain hydrogen bond. "Pkt" = binding site pocket. "$-NH_3^+$", "$-COO^-$", and "distal anion" bind the respective charged moieties of the ligands.

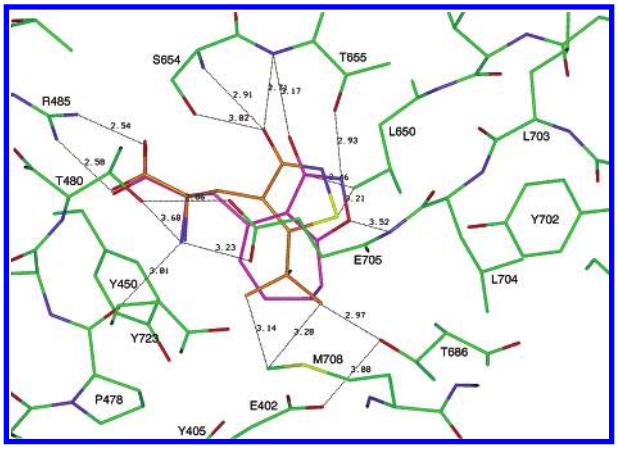


Figure 4. Compounds **4** (orange) and **6** (magenta) docked onto the X-ray crystal structure of the **1**:GluR2−S1S2J complex.

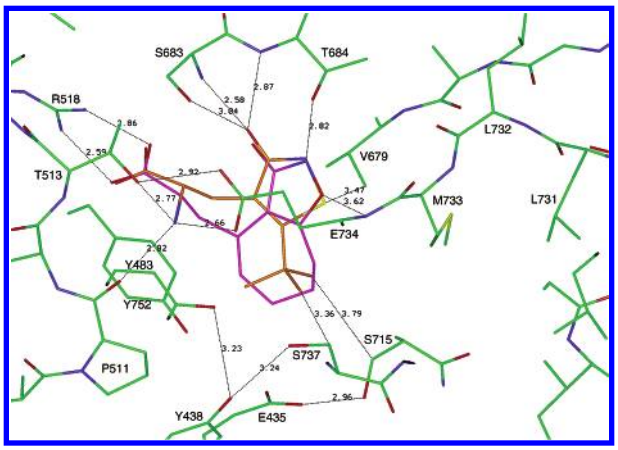


Figure 5. Compounds **4** (orange) and **6** (magenta) docked onto a homology model of GluR5−S1S2J built from the X-ray crystal structure of the **1**:GluR2−S1S2J complex.

Interestingly however, neither **6** nor **7** showed detectable agonist or antagonist effects at mGluR1α, mGluR2, or mGluR4a, representatives of group I, II, and III mGluR, respectively, perhaps because the conformational constraints imposed by the seven-membered ring do not allow 4-AHCP to mimic the extended binding conformation of Glu at mGluRs. Whereas **7** turned out to be essentially inactive at the iGluRs tested (Tables 1 and 2), **6** was shown to be a highly potent agonist at GluR5, equipotent with (*S*)-Thio-ATPA (**4**)[22] and more potent than (*S*)-ATPA (**3**).[21] As observed for **3** and **4** (Table 2), **6** showed much weaker agonist effects at the homomeric AMPA receptors, GluR1o, GluR3o, and GluR4o. The affinity of **6** for kainate receptor sites labeled by [^{3}H]kainic acid (IC_{50} = 1.6 μM) (Table 1) is somewhat higher than that reported previously for (*RS*)-4-AHCP,[25] but still this affinity is surprisingly low compared to the very potent GluR5 agonist potency of **6** (Table 2).

(*S*)-4-AHCP (**6**) represents a new structural class of GluR5 receptor agonists and may be an important lead structure for the design of new GluR5 agonists and antagonists. It is GluR5 antagonists that have attracted particular interest as therapeutic agents.[17,48,49] However, since kainate receptors such as GluR5 desensitize very rapidly and since GluR5 expression is restricted compared with other kainate subunits,[14] the therapeutic prospects are open for developing GluR5 agonists as "functional antagonists".[24] To further the development of this area of Glu receptor pharmacology, we have carried out comparative molecular modeling studies on compounds **1**, **4**, and **6**, mapping out the agonist binding site of GluR5 in detail for the first time.

Analysis of the ligands shows that although the skeleton of **6** has one extra carbon between its α-amino acid and distal anion moieties compared with Glu, **1** and **4**, the side chain is held in an ideal orientation for recognition by iGluRs (but not mGluRs) because of the unsaturated seven-membered ring. In addition, **4** and **6** occupy remarkably similar volumes, in concord with their similar pharmacological profiles. Although **3** also occupies a similar volume, its isoxazole ring oxygen does not protrude as far as the sulfur atom of **4**, nor is it as lipophilic.

With such a high degree of binding-site homology, we can interpret the results of the docking study with confidence. The residues recognizing the α-amino acid and distal anion moieties of agonists at GluR2 and GluR5 are identical. The receptors differ only in the size, shape, and polarity of the cavity beside and "below" the volume occupied by glutamate upon binding (Figures 4 and 5).[40] Compounds **6** and **4** both possess moieties that protrude below the plane of their heterocyclic rings, whereas high-potency AMPA receptor agonists do not.[42] As with **1**, L650 in GluR2 experiences steric repulsion from **6** and **4**, limiting the degree to which the receptor can close tightly around the ligands. Although the relationship between affinity and the macroscopic domain closure measured from the crystal structures is by no means linear,[42] agonists that bind tightly and are strongly desensitizing display tight domain closure.[40,42] In addition to L650, **4** and **6** come close to M708 and T686 in GluR2; the receptor must adapt upon domain closure to accommodate these ligands, with a corresponding drop in potency at GluR2 compared with AMPA and AMPA agonists. Finally, a water molecule observed experimentally in all agonist:GluR2 complexes to date links Y702, T686, and the ligand by a hydrogen bonding network[42,50] (not shown in Figure 4). The hydrophobic volumes of **6** and **4** destabilize this water molecule compared to the effect of potent GluR2 agonists. Ligand selectivity seems to be highly sensitive to the ligand's disposition toward the hydrogen bond network in the direction of Y702,[50] and the reduced selectivity of **3** versus **4** may be linked to the positioning, reduced size, and hydrophobicity of the isoxazole ring oxygen versus sulfur. By contrast with GluR2, the cavity in GluR5 beside and below the binding position of the distal carboxylate of Glu is larger and more hydrophobic overall. The shortening of L650 in GluR2 to V679 in GluR5 allows for better van der Waals contact and tighter domain closure around the bulkier ligands, as does the replacement of T686 by S715. Interestingly, not only does the substitution of M708 (GluR2) for S737 (GluR5) alter the shape of the cavity (more space around the *tert*-butyl group of **4** or the seven-membered ring of **6**) it also enables a new interdomain hydrogen bond network through Y438 (GluR5) upon agonist binding, either directly or through an extra water molecule (not included in the homology model). At the same time the volume between Y450, P478, Y723, and Y405, known to accommodate heteroaromatic substituents in GluR2,[42] seems to be reduced in size and hydrophobicity in

GluR5. Finally, replacement of Y702 by L731 in GluR5 means that instead of a water molecule bound in a fairly rigid hydrogen bond network,[40,42] GluR5 agonists face a more hydrophobic and flexible tunnel in this direction.

In summary, the so-called hydrophobic pocket[44] of agonized GluR2 has changed shape and character in GluR5 as well as shifting position—smaller and less hydrophobic on the left, larger and substantially more hydrophobic below and on the right (Figures 4 and 5). It would also seem that neither **4** nor **6** have exhausted the hydrophobic space available within the binding cavity. The current model may be of assistance in further structure-based design of higher-affinity GluR5 specific agonists. In this context, (*S*)-4-AHCP (**6**) presents a new scaffold for the design of kainate subtype specific ligands.

Experimental Section

Chemistry. General Procedures. Analytical thin-layer chromatography (TLC) was performed on silica gel F_{254} plates (Merck). All compounds were detected using UV light and a $KMnO_4$ spraying reagent. Compounds containing the 3-isoxazolol moiety were also visualized using an $FeCl_3$ spraying reagent. Compounds containing amino groups were also visualized using a ninhydrin spraying reagent. Flash chromatography (FC) was performed on silica gel Matrex LC 60A (0.035−0.070 mm). Column chromatography (CC) was performed on silica gel Matrex LC 60A (0.070−0.200 mm). The light petroleum used had a boiling point of 80−100 °C. Organic phases were dried using $MgSO_4$. Melting points were determined in capillary tubes and are uncorrected. ^{1}H, ^{13}C, and associated proton test (APT) spectra were recorded on a Varian Gemini-2000 BB spectrometer (300 MHz). Unless otherwise stated, $CDCl_3$ was used as a solvent. Chemical shifts are given in ppm (δ) using TMS (^{1}H NMR) or the $CDCl_3$ peak [^{13}C NMR (δ 77.00)] as internal standards. Elemental analyses were performed at Analytical Research Department, H. Lundbeck A/S, Denmark, or by J. Theiner, Microanalytical Laboratory, Department of Physical Chemistry, University of Vienna, Austria, and are within ±0.4% of the theoretical values, unless otherwise stated. Optical rotations were measured in thermostated cuvettes on a Perkin-Elmer 241 polarimeter. Circular dichroism (CD) spectra were recorded in 0.1 M HCl solution in 1.0 cm cuvettes at room temperature on a Jasco J-720 spectropolarimeter.

Preparative chiral HPLC was performed on a Chirobiotic T column (500 mm × 10.0 mm) equipped with a Chirobiotic T guard column (50 mm × 4.6 mm). The column was connected to an HPLC instrumentation consisting of a Jasco 880 pump, a Rheodyne 7125 injector equipped with a 5.0 mL loop, a Shimadzu SPD-6A detector (220 nm), and a Hitachi D-2000 Chromato-Integrator. The column was eluted at 1.5 mL/min with aqueous $AcONH_4$/AcOH (15 mM, pH 4.0) and EtOH (60:40). Preparative reversed-phase HPLC was performed using a Vydac C18 column (250 mm × 22 mm; 10 μm), and the products were eluted with AcOH (15 mM) and MeOH (80:20) at 6.0 mL/min. The column was connected to the same instrumentation as described above except using a TSP detector. Achiral HPLC analyses of **18** and **19** were performed on a Knauer Vertex Sperisorb ODS2 column (120 mm × 4.0 mm; 5 μm) using a Waters M510 pump, a Waters U6K injector, and a Waters 991 photodiode array detector (190 nm). The column was eluted at 1.0 mL/min with H_2O and MeOH (30:70). Chiral HPLC analyses of **20** and **21** were performed using a chiral-AGP column (150 mm × 4.0 mm), and the products were eluted with aqueous KH_2PO_4/K_2HPO_4 (50 mM, pH 7.0) and 2-PrOH (80:20) at 0.5 mL/min. The column was connected to the same instrumentation as described above for the Vydac column, though with a detector wavelength of 200 nm. Analytical chiral HPLC using a Crownpak CR(−) column (150 mm × 4.0 mm) was performed at room temperature with a flow rate of 0.40 mL/min. Analytical HPLC using a Crownpak CR(+) column (150 mm × 10.0 mm) equipped with a guard column (10 mm × 4.0 mm) was performed at 30 °C using an LKB 2155 HPLC column oven, and the products were eluted at 6.0 mL/min. For both columns, products were eluted with aqueous $HClO_4$ (pH 2.0), and the columns were connected to the same instrumentation as described above for the Chirobiotic T column with a detection wavelength of 200 nm. The CR(+) and CR(−) columns were used for the determinations of stereochemical purities of **6** and **7**, respectively. The % ee values were calculated from peak areas.

3-Isopropoxy-5,6,7,8-tetrahydro-4*H*-cyclohepta[*d*]isoxazole (9) and 2-Isopropyl-5,6,7,8-tetrahydro-4*H*-cyclohepta[*d*]isoxazolin-3-one (10). Potassium carbonate (23.1 g, 167 mmol) dried in vacuo was added to a solution of 5,6,7,8-tetrahydro-4*H*-cyclohepta[*d*]isoxazol-3-ol (**8**)[27] (23.3 g, 152 mmol) in dry DMF (200 mL), and the reaction mixture was left to stir for 30 min at 60 °C. Isopropyl bromide (21.3 mL, 227 mmol) was then added, and the resulting reaction mixture was stirred at 60 °C for 3 h (followed by TLC). After the mixture was cooled, water (150 mL) was added and the mixture was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic phases were evaporated, and water (100 mL) was added to the residue. Extraction with Et_2O (3 × 100 mL), drying, evaporation, and FC [light petroleum/AcOEt (9:1)] gave **9** (27 g, 92%) as an oil. A sample was distilled in a Kugelrohr apparatus: bp 185−195 °C/1.3 kPa; ^{1}H NMR δ 1.37 (6H, d, J = 6.2 Hz), 1.62−1.84 (6H, m), 2.31 (2H, m), 2.74 (2H, m), 4.87 (1H, heptet, J = 6.2 Hz); ^{13}C NMR δ 20.6, 21.8, 25.6, 28.0, 28.3, 30.3, 72.5, 106.7, 169.7, 171.0. Anal. ($C_{11}H_{17}NO_2$) C, H, N. Different batches gave yields of **9** in the range 70−92%.

Further elutions gave in different batches yields of the N-alkylated isomer **10** as an oil in the range trace−20%: ^{1}H NMR δ 1.30 (6H, d, J = 6.6 Hz), 1.60−1.84 (6H, m), 2.36 (2H, t, J = 5.7 Hz), 2.61 (2H, t, J = 5.6 Hz), 4.58 (1H, heptet, J = 6.6 Hz); ^{13}C NMR δ 19.7, 21.3, 25.6, 27.7, 28.5, 30.3, 48.6, 110.9, 167.8, 169.3.

3-Isopropoxy-5,6,7,8-tetrahydrocyclohepta[*d*]isoxazol-4-one (11) and 3-Isopropoxy-5,6,7,8-tetrahydrocyclohepta[*d*]isoxazol-8-one (12). To a solution of **9** (7.56 g, 38.7 mmol) in AcOH (99.9%, 10 mL) and concentrated H_2SO_4 (6 mL) kept at 0−10 °C was added, over 20 min, a solution of $Na_2Cr_2O_7 \cdot 2H_2O$ (17.3 g, 58.1 mmol) in AcOH (50 mL). After stirring for 3 h at room temperature, the mixture was neutralized by addition of concentrated NaOH. Extraction with Et_2O (4 × 200 mL), drying, evaporation, and CC [toluene/AcOEt (9:1 → 6:1)] gave crude unreacted **9** (2 g, 26%). Further elution gave **12** (2.2 g, 27%): mp 70.0−71.0 °C [toluene/light petroleum]; ^{1}H NMR δ 1.40 (6H, d, J = 6.1 Hz), 1.96 (4H, m), 2.60 (2H, t, J = 6.1 Hz), 2.77 (2H, m), 4.94 (1H, heptet, J = 6.1 Hz); ^{13}C NMR δ 21.7, 21.9, 22.3, 25.7, 43.2, 73.9, 115.8, 160.3, 169.7, 190.2. Anal. ($C_{11}H_{15}NO_3$) C, H, N.

Later fractions contained **11** (3.8 g, 47%): mp 64.5−66.0 °C [toluene/light petroleum]; ^{1}H NMR δ 1.43 (6H, d, J = 6.1 Hz), 1.88−2.09 (4H, m), 2.72 (2H, m), 3.03 (2H, t, J = 6.2 Hz), 4.94 (1H, heptet, J = 6.1 Hz); ^{13}C NMR δ 21.5, 22.3, 23.7, 28.5, 44.8, 74.0, 109.5, 169.1, 177.8, 194.1. Anal. ($C_{11}H_{15}NO_3$) H, N. C: calcd 63.14; found 62.55.

Ethyl (*E*)- and Ethyl (*Z*)-(3-Isopropoxy-5,6,7,8-tetrahydrocyclohepta[*d*]isoxazol-4-ylidene)acetate (13 and 14) and Ethyl (3-Isopropoxy-7,8-dihydro-6*H*-cyclohepta[*d*]-isoxazol-4-yl)acetate (15). Triethyl phosphonoacetate (5.1 mL, 25.8 mmol) was added dropwise to a stirred solution of potassium *tert*-butoxide (2.4 g, 21.5 mmol) in dry THF (20 mL), and the mixture was stirred at room temperature for 30 min. A solution of **11** (900 mg, 4.30 mmol) in dry THF (5 mL) was then added. After the mixture was stirred for 22 h at 80 °C, a saturated solution of ammonium chloride (10 mL) and H_2O (25 mL) was added. Extraction with AcOEt (3 × 100 mL), drying, and evaporation followed by CC [toluene/AcOEt (9:1)] gave **13** (730 mg, 61%) as an oil: ^{1}H NMR δ 1.31 (3H, t, J = 7.1 Hz), 1.43 (6H, d, J = 6.2 Hz), 1.88 (4H, m), 2.84 (2H, m), 3.12 (2H, m), 4.19 (2H, q, J = 7.1), 4.97 (1H, heptet, J = 6.2 Hz), 6.25 (1H, s); ^{13}C NMR δ 14.1, 21.7, 24.3, 25.8, 26.7, 30.0, 59.6, 73.6, 109.0, 116.3, 147.2, 167.0, 167.8, 173.9. Anal.

($C_{15}H_{21}NO_4$) C, H, N. Further elution gave **14** and **15** as a 1:1 mixture (350 mg, 29%). **14**: ^{1}H NMR δ 1.24 (3H, t, J = 7.2 Hz), 1.34 (6H, d, J = 6.2 Hz), 1.78−1.90 (4H, m), 2.41 (2H, m), 2.83 (2H, m), 4.13 (2H, q, J = 7.2 Hz), 4.86 (1H, heptet, J = 6.2 Hz), 5.83 (1H, s). **15**: ^{1}H and ^{13}C NMR spectra of pure **15** (see synthesis below).

Ethyl and *tert*-Butyl (3-Isopropoxy-7,8-dihydro-6*H*-cyclohepta[*d*]isoxazol-4-yl)acetate (15 and 16). A solution of **13** (2.32 g, 8.31 mmol) in dry DME was added to a stirred solution of potassium *tert*-butoxide (0.93 g, 8.29 mmol) in dry DME (12 mL) at 0 °C and under a N_2 atmosphere. The reaction was left to stir for 1 h (followed by TLC) at room temperature. The reaction mixture was then neutralized by addition of HCl (4 M). Extraction with CH_2Cl_2 (2 × 30 mL), drying, evaporation, and CC [toluene/AcOEt (18:1 → 9:1)] gave crude unreacted **13**, ca. 9%, contaminated with <1% of the analogous *tert*-butyl ester (**16**). Further elution gave **16** (120 mg, 4.7%) as an oil: ^{1}H NMR δ 1.38 (6H, d, J = 6.2 Hz), 1.42 (9H, s), 1.90 (2H, m), 2.32 (2H, m), 2.94 (2H, t, J = 6.5), 3.37 (2H, s), 4.91 (1H, heptet, J = 6.2 Hz), 5.65 (1H, t, J = 6.2 Hz). Later fractions gave **15** (1.00 g, 46%) as an oil: ^{1}H NMR δ 1.25 (3H, t, J = 7.2 Hz), 1.34 (6H, d, J = 6.1 Hz), 1.90 (2H, m), 2.35 (2H, m), 2.95 (2H, t, J = 6.5), 3.40 (2H, s), 4.12 (2H, q, J = 7.2 Hz), 4.91 (1H, heptet, J = 6.1 Hz), 5.65 (1H, t, J = 6.2 Hz); ^{13}C NMR δ 14.0, 21.2, 21.7, 28.5, 29.0, 41.1, 60.3, 73.2, 105.4, 122.4, 131.6, 168.3, 170.9, 172.4. Anal. ($C_{15}H_{21}NO_4$) C, H, N.

(3-Isopropoxy-7,8-dihydro-6*H*-cyclohepta[*d*]isoxazol-4-yl)ethanal (17). Compound **15** (1.85 g, 6.62 mmol) was dissolved in dry toluene (40 mL) under a N_2 atmosphere, and the solution was cooled to −78 °C. DIBAL-H hydride (5.5 mL, 1.2 M in hexane, 6.62 mmol) was added dropwise. After the mixture was stirred for 10 min, the reaction mixture was quenched with EtOH (10 mL) followed by addition of a saturated aqueous solution of ammonium dihydrogen phosphate (20 mL). The reaction mixture was allowed to warm to room temperature, and water (40 mL) was added. Extraction with CH_2Cl_2 (3 × 100 mL), drying, evaporation, and CC [toluene/AcOEt (9:1)] gave **17** (1.12 g, 72%); ^{1}H NMR δ 1.35 (6H, d, J = 6.1 Hz), 1.91 (2H, m), 2.39 (2H, m), 2.97 (2H, t, 6.5 Hz), 3.44 (2H, s br), 4.92 (1H, heptet, J = 6.1 Hz), 5.68 (1H, t, J = 6.0 Hz), 9.71 (1H, t, J = 1.4 Hz); ^{13}C NMR δ 21.2, 21.7, 28.6, 29.0, 49.8, 73.7, 105.0, 121.0, 132.6, 167.5, 171.3, 200.7. Anal. ($C_{13}H_{17}NO_3$) C, H, N.

(2*S*)- and (2*R*)-*N*-*tert*-Butyl-3-(3-isopropoxy-7,8-dihydro-6*H*-cyclohepta[*d*]isoxazol-4-yl)-2-{*N*-[(*R*)-1-phenylethyl]benzamido}propionamide 18 and 19. A solution of **17** (1.12 g, 4.76 mmol) and (*R*)-1-phenylethylamine (606 μL, 4.76 mmol) in dry MeOH (20 mL) was refluxed for ca. 1.5 h. After the mixture was cooled to 10−15 °C, benzoic acid (581 mg, 4.76 mmol) was added, and the reaction mixture was stirred at 10−15 °C for 5 min. *Tert*-butylisonitrile (538 μL, 4.76 mmol) was added to the mixture, and stirring was continued at room temperature overnight. Evaporation followed by CC [toluene/AcOEt (9:1)] gave 374 mg (14.5%) of **18** (99.9% de based on HPLC): ^{1}H NMR δ 1.35 (3H, d br, J = 6.3 Hz), 1.39 (9H, s), 1.47 (3H, d br, J = 6.0 Hz), 1.54 (3H, d br, J = 6.6 Hz), 1.81 (2H, m), 2.24 (2H, m), 2.82 (2H, m), 3.23 (2H, m), 3.84 (1H, m), 4.77 (1H, m), 4.98 (1H, m), 5.59 (1H, m), 7.1−7.2 (5H, m), 7.4−7.5 (5H, m), 8.18 (1H, s br). Further elution gave 399 mg (15.4%) of **19** (99.5% de based on HPLC): ^{1}H NMR δ 1.12 (9H, s), 1.27 (3H, d br, J = 6.0 Hz), 1.42 (3H, d br, J = 6.3 Hz), 1.51 (3H, d br, J = 6.0 Hz), 1.93 (2H, m), 2.35 (2H, m), 2.98 (2H, m), 3.36 (2H, m), 3.91 (1H, m), 5.04 (2 × 1H, m), 5.76 (1H, t, J = 6.0 Hz), 7.0−7.3 (5H, m), 7.4−7.5 (5H, m).

(*S*)-2-Benzamido-*N*-*tert*-butyl-3-(3-isopropoxy-7,8-dihydro-6*H*-cyclohepta[*d*]isoxazol-4-yl)propionamide (20). A solution of **18** (754 mg, 1.39 mmol) in formic acid (98%, 20 mL) was stirred at room temperature for 30 min and then 5.5 h at 60 °C. Evaporation followed by FC [toluene/AcOEt (4:1)] gave 352 mg (58%) of **20** (95% ee based on HPLC). A small sample was recrystallized [CH_2Cl_2/light petroleum]: mp 173−174 °C; 99.8% ee; ^{1}H NMR δ 1.34 (9H, s), 1.40 (3H, d, J = 6 Hz), 1.41 (3H, d, J = 6 Hz), 1.67−1.94 (2H, m), 2.25 (2H, m), 2.71 (1H, dd, J = 9.0 and J = 13.5 Hz), 2.89 (2H, t, J = 6.6 Hz), 3.15 (1H, dd, J = 5.5 and J = 13.5 Hz), 4.53 (1H, m), 4.97 (1H, heptet, J = 6.2 Hz), 5.77 (1H, t, J = 6.2 Hz), 6.02 (1H, s br), 6.65 (1H, d br, J = 7.8 Hz), 7.39−7.55 (3H, m), 7.68−7.74 (2H, m); ^{13}C NMR δ 21.7, 21.9, 22.4, 27.8, 28.49, 28.53, 37.3, 51.2, 53.8, 73.5, 104.9, 125.1, 126.9, 128.4, 131.5, 131.7, 134.0, 167.2, 168.2, 170.7, 171.7. Anal. ($C_{25}H_{33}N_3O_4$) C, H, N.

(*R*)-2-Benzamido-*N*-*tert*-butyl-3-(3-isopropoxy-7,8-dihydro-6*H*-cyclohepta[*d*]isoxazol-4-yl)propionamide (21). Compound **21** was synthesized as described for the synthesis of **20** from **19** (913 mg, 1.68 mmol). Yield: 375 mg (51%) (97% ee based on HPLC). A small sample was recrystallized [CH_2Cl_2/light petroleum]: mp 172−173 °C; 99.5% ee. ^{1}H and ^{13}C NMR spectra were virtually identical with those of the enantiomer compound **20**. Anal. ($C_{25}H_{33}N_3O_4$) C, H, N.

(*S*)- and (*R*)-2-Amino-3-(3-hydroxy-7,8-dihydro-6*H*-cyclohepta[*d*]isoxazol-4-yl)propionic Acid (6 and 7). Compound **20** (325 mg, 0.74 mmol) was dissolved in a mixture of HCl (35%, 20 mL), H_2O (10 mL), and AcOH (99.9%, 10 mL), and the mixture was heated at 130 °C for 1.5 h. After evaporation, the brownish residue was dissolved in H_2O (10 mL) and washed with CH_2Cl_2 (2 × 10 mL), and the pooled CH_2Cl_2 phases were extracted with H_2O (10 mL). The H_2O phases were pooled and evaporated. To remove ca. 1 equiv of $(CH_3)_3CNH_2$·HCl, the residue was subjected to ion exchange [Amberlite IR-400 (OH); eluent AcOH (2 M)]. A solution of crude racemized **6** (135 mg, 66%; 60% ee) in H_2O (15.5 mL) was filtered through a Millex HV filter (0.45 μm, Millipore) and resolved on a Chirobiotic T column (0.6 mL injections). All fractions containing the first eluting enantiomer (**6**) were evaporated and re-evaporated twice from H_2O. To remove $AcONH_4$, the dried oily residue was redissolved in H_2O (5 mL) and subjected to a Vydac (RP-18) column in five injections. Appropriate fractions were pooled, evaporated, re-evaporated from H_2O, and dried in vacuo to give **6** as white crystals (52 mg): >99.7% ee. All fractions containing the second eluting enantiomer were evaporated, re-evaporated twice from H_2O, and dried in vacuo. The semicrystalline residue was dissolved in water (1 mL), and $AcONH_4$ was removed on a Vydac column in three injections. Appropriate fractions were pooled, evaporated, re-evaporated from H_2O, and dried in vacuo to give **7** (11.5 mg): mp 174 °C dec; 97% ee. Compound **21** (350 mg, 0.80 mmol) was treated as **20**. The deprotection led to crude racemized **7** (40% ee), which after resolution gave **6** (26 mg), 99.6% ee, and **7** (57.5 mg), 98.8% ee. **6**: Recrystallization [52 mg, >99.7% ee; 26 mg, 99.6% ee; H_2O (5−10 mL)] gave 57 mg as light-mauve crystals; mp 180 °C dec; 99.9% ee; ^{1}H NMR (D_2O, CH_3CN, δ 2.06) δ 1.83−1.92 (2H, m), 2.27−2.35 (2H, m), 2.84 (1H, dd, J = 13.8 and 8.1 Hz), 2.90 (2H, t, J = 6.5 Hz), 3.15 (1H, dd, J = 6.0 and 13.8 Hz), 3.82 (1H, dd, J = 6.0 and 8.1 Hz), 5.88 (1H, t, J = 6.2 Hz); $[\alpha]^{25}_D$ −0.8° (*c* 0.38, 0.1 M HCl); $\Delta\epsilon$ (210 nm) = +0.2 m^2/mol. Anal. ($C_{11}H_{14}N_2O_4$·H_2O) C, H, N. **7**: White crystals (57.5 mg); mp 174 °C dec; 98.8% ee; $[\alpha]^{25}_D$ +1.0° (*c* 0.40, 0.1 M HCl); $\Delta\epsilon$ (210 nm) = −0.2 m^2/mol. The ^{1}H NMR spectrum was identical with that of the (*S*)-enantiomer **6**. Anal. ($C_{11}H_{14}N_2O_4$·H_2O) H, N. C: calcd 51.56; found 51.09.

Cell Culture and Second Messenger Assays. Chinese hamster ovary (CHO) cell lines expressing mGluR1α, mGluR2, and mGluR4a were maintained as previously described.[9,10,51] Briefly, cells were maintained in a humidified 5% CO_2/95% air atmosphere at 37 °C in DMEM containing a reduced concentration of (*S*)-glutamine (100 mg/mL) and 10% dialyzed fetal calf serum (all GIBCO, Paisley, Scotland). The day before the inositol phosphate assay, two million mGluR1α-expressing cells were divided into the wells of a 96-well plate in inositol-free culture medium containing 4 μCi/mL [^{3}H]inositol. The day before the cyclic AMP-assay, two million mGluR2- or mGluR4a-expressing cells were divided into the wells of a 96-well plate in culture medium. Measurements of inositol phosphate generation and cyclic AMP inhibition were determined by ion-exchange chromotography and scintillation proximity assays as previously described.[47]

Receptor Binding Assays. The membrane preparations used in all receptor binding experiments were prepared according to Ransom and Stec[52] with slight modifications as previously described.[21] Affinities for AMPA, kainic acid, and NMDA receptor sites were determined using 5 nM [^{3}H]-AMPA,[53] 5 nM [^{3}H]kainic acid[54] in the absence of $CaCl_2$, and 2 nM [^{3}H]CGP 39653,[55] respectively. The binding assays were carried out with the modifications previously described.[21] The amount of bound radioactivity was determined using a Packard TOP-COUNT microplate scintillation counter.

Electrophysiology. In vitro cRNA transcription was performed as described previously.[22] *Xenopus laevis* oocytes were isolated and injected as described previously.[22] Currents were recorded 3−14 days after injection using a two-microelectrode clamp (OC-725C oocyte clamp, Warner Instrument). Experiments were performed in low Ca^{2+}-Ringer (10 mM Na-HEPES, pH 7.5, 1165 mM NaCl, 0.1 mM $CaCl_2$, 2.5 mM KCl, 1.8 mM $MgCl_2$) to avoid activation of the endogenous Ca^{2+}-dependent Cl^- channel. The electrodes were filled with 3 M KCl and had a resistance of 0.7−2 MΩ. Oocytes were clamped at −100 to −20 mV, and recordings on homomeric GluR5 and GluR6 were performed after a 10 min treatment with concanavalin A (1 mg/mL, Sigma type IV) to minimize receptor desensitization.

Data Analysis. Binding data were analyzed by the nonlinear curve-fitting program GRAFIT 3.0.[56] Data were fit to the following equation:

$$B = 100 - \frac{100 \times [\text{inhibitor}]^n}{\text{IC}_{50}{}^n + [\text{inhibitor}]^n}$$

where B is the binding as a percentage of total specific binding and n is the Hill coefficient.

Data on the cloned iGluR subtypes were acquired using Clampex 7.0 (Axon Instruments) and subsequently processed with Sigmaplot 3.0 (Jandel Scientific, SPSS). Dose−response data from individual oocytes were fit to the equation

$$I = I_{\max} \frac{[\text{agonist}]^n}{\text{EC}_{50}{}^n + [\text{agonist}]^n}$$

where I is the current, $I_{\max}$ is the maximum current in response to the action of the agonist, and n denotes the Hill coefficient.

Relative maximal currents (relative $I_{\max}$) were calculated as $I_{\max \text{ ligand}}/I_{\max \text{ kainic acid or AMPA}}$ where $I_{\max \text{ ligand}}$ is the fitted $I_{\max}$ according to the equation (see above). $I_{\max \text{ kainic acid or AMPA}}$ is calculated from the current evoked by kainic acid (for GluR1o, GluR3o, GluR4o, and GluR5) or AMPA (for GluR6 + KA2), and the EC_{50} values previously defined in our laboratory for the two agonists. The (kainic acid EC_{50} values)/(n values) for homomeric GluR1o, GluR3o, GluR4o, and GluR5 were determined to be (55.1 μM)/1.28, (27.1 μM)/2.30, (29.8 μM)/1.80, (9.6 μM)/1.38, respectively, and the (AMPA EC_{50} value)/(n value) for heteromeric GluR6 + KA2 was determined to be (110.0 μM)/1.84.

The mean and SEM are calculated assuming a logarithmic distribution of the EC_{50} or IC_{50} values. Here, numbers in parentheses (min, max) indicate ± SEM according to a logarithmic distribution of EC_{50} and IC_{50} values.[57]

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Supporting Information Available: Homology model of GluR5 as Protein Data Bank file. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) *Excitatory Amino Acids and Synaptic Transmission*; Wheal, H. V., Thomson, A. M., Eds.; Academic Press: London, 1995.
(2) *The Ionotropic Glutamate Receptors*; Monaghan, D. T., Wenthold, R. J., Eds.; Humana Press: Totowa, NJ, 1997.
(3) Parsons, C. G.; Danysz, W.; Quack, G. Glutamate in CNS disorders as a target for drug development: an update. *Drug News Perspect.* **1998**, *11*, 523−569.
(4) *The Metabotropic Glutamate Receptors*; Conn, P. J., Patel, J., Eds.; Humana Press: Totowa, NJ, 1994.
(5) Dingledine, R.; Borges, K.; Bowie, D.; Traynelis, S. F. The glutamate receptor ion channels. *Pharmacol. Rev.* **1999**, *51*, 7−61.
(6) Bräuner-Osborne, H.; Egebjerg, J.; Nielsen, E. Ø.; Madsen, U.; Krogsgaard-Larsen, P. Ligands for glutamate receptors: design and therapeutic prospects. *J. Med. Chem.* **2000**, *43*, 2609−2645.
(7) Knöpfel, T.; Kuhn, R.; Allgeier, H. Metabotropic glutamate receptors: novel targets for drug development. *J. Med. Chem.* **1995**, *38*, 1417−1426.
(8) Monn, J. A.; Schoepp, D. D. Metabotropic glutamate receptor modulators: recent advances and therapeutic potential. *Annu. Rep. Med. Chem.* **2000**, *35*, 1−10.
(9) Tanabe, Y.; Masu, M.; Ishii, T.; Shigemoto, R.; Nakanishi, S. A family of metabotropic glutamate receptors. *Neuron* **1992**, *8*, 169−179.
(10) Aramori, I.; Nakanishi, S. Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. *Neuron* **1992**, *8*, 757−765.
(11) *Ionotropic Glutamate Receptors in the CNS*; Jonas, P., Monyer, H., Eds.; Springer: Berlin, 1999.
(12) Egebjerg, J.; Schousboe, A.; Krogsgaard-Larsen, P. *Glutamate and GABA Receptors and Transporters. Structure, Function and Pharmacology*; Taylor and Francis: London, 2002.
(13) Hollmann, M.; Heinemann, S. Cloned glutamate receptors. *Annu. Rev. Neurosci.* **1994**, *17*, 31−108.
(14) Dingledine, R.; Borges, K.; Bowie, D.; Traynelis, S. F. The glutamate receptor ion channels. *Pharmacol. Rev.* **1999**, *51*, 7−61.
(15) Rosenmund, C.; Stern-Bach, Y.; Stevens, C. F. The tetrameric structure of a glutamate receptor channel. *Science* **1998**, *280*, 1596−1599.
(16) Fletcher, E. J.; Lodge, D. New developments in the molecular pharmacology of α-amino-3-hydroxyisoxazole propionate and kainate receptors. *Pharmocol. Ther.* **1996**, *70*, 65−89.
(17) Lees, G. J. Pharmacology of AMPA/kainate receptor ligands and their therapeutic potential in neurological and psychiatric disorders. *Drugs* **2000**, *59*, 33−78.
(18) Lauridsen, J.; Honoré, T.; Krogsgaard-Larsen, P. Ibotenic acid analogues. Synthesis, molecular flexibility, and in vitro activity of agonists and antagonists at central glutamic acid receptors. *J. Med. Chem.* **1985**, *28*, 668−672.
(19) Clarke, V. R. J.; Ballyk, B. A.; Hoo, K. H.; Mandelzys, A.; Pellizzari, A.; Bath, C. P.; Thomas, J.; Sharpe, E. F.; Davies, C. H.; Ornstein, P. L.; Shoepp, D. D.; Kamboj, R. K.; Collingridge, G. L.; Lodge, D.; Bleakman, D. A hippocampal GluR5 kainate receptor regulating inhibitory synaptic transmission. *Nature* **1997**, *389*, 599−603.
(20) Hoo, K.; Legutko, B.; Rizkalla, G.; Deverill, M.; Hawes, C. R.; Ellis, G. J.; Stensbøl, T. B.; Krogsgaard-Larsen, P.; Skolnick, P.; Bleakman, D. [^{3}H]ATPA: a high affinity ligand for GluR5 kainate receptors. *Neuropharmacology* **1999**, *38*, 1811−1817.
(21) Stensbøl, T. B.; Johansen, T. N.; Egebjerg, J.; Madsen, U.; Ebert, B.; Krogsgaard-Larsen, P. Resolution, absolute stereochemistry and molecular pharmacology of the enantiomers of ATPA. *Eur. J. Pharmacol.* **1999**, *380*, 153−162.
(22) Stensbøl, T. B.; Jensen, H. S.; Nielsen, B.; Johansen, T. N.; Egebjerg, J.; Frydenvang, K.; Krogsgaard-Larsen, P. Stereochemistry and molecular pharmacology of (*S*)-thio-ATPA, a new potent and selective GluR5 agonist. *Eur. J. Pharmacol.* **2001**, *411*, 245−253.
(23) Thomas, N. K.; Hawkins, L. M.; Miller, J. C.; Troop, H. M.; Roberts, P. J.; Jane, D. E. Pharmacological differentiation of kainate receptors on neonatal rat spinal motoneurones and dorsal roots. *Neuropharmacology* **1998**, *37*, 1223−1237.
(24) Chittajallu, R.; Braithwaite, S. P.; Clarke, V. R. J.; Henley, J. M. Kainate receptors: subunits, synaptic localization and function. *Trends Pharmacol. Sci.* **1999**, *20*, 26−35.
(25) Krogsgaard-Larsen, P.; Nielsen, E. Ø; Curtis, D. R. Ibotenic acid analogues. Synthesis and biological and in vitro activity of conformationally restricted agonists at central excitatory amino acid receptors. *J. Med. Chem.* **1984**, *27*, 585−591.
(26) Ebert, B.; Madsen, U.; Lund, T. M.; Holm, T.; Krogsgaard-Larsen, P. Molecular pharmacology of cortical and spinal AMPA receptors. *Mol. Neuropharmacol.* **1992**, *2*, 47−49.
(27) Krogsgaard-Larsen, P.; Natova, L.; Christensen, S. B. Muscimol analogues. I. Syntheses of 4- and 8-aminocycloheptено[1,2-*d*]-isoxazol-3-ols and 4-(3-aminopropyl)-5-methyl-3-isoxazolol. *Acta Chem. Scand.* **1977**, *31*, 577−583.

(28) Ugi, I.; Offermann, K.; Herlinger, H.; Marquarding, D. Die Umsetzung von (*S*)-α-Phenyläthylamin und Isobutyraldehyd mit Benzoesäure und *tert*-Butyl-isocyanid als Modellreaktion für stereoselektive Peptid-Synthesen mittels Vierkomponenten-Kondensationen (The reaction of (*S*)-α-phenylethylamine and isobutyraldehyde with benzoic acid and *tert*-butyl isocyanide as a model reaction for stereoselective peptide synthesis through a four-component condensation). *Leibigs Ann. Chem.* **1967**, *709*, 1−10.

(29) Ahmadian, H.; Nielsen, B.; Bräuner-Osborne, H.; Johansen, T. N.; Stensbøl, T. B.; Sløk, F. A.; Sekiyama, N.; Nakanishi, S.; Krogsgaard-Larsen, P.; Madsen, U. (*S*)-Homo-AMPA, a specific agonist at the $mGlu_6$ subtype of metabotropic glutamic acid receptors. *J. Med. Chem.* **1997**, *40*, 3700−3705.

(30) Dömling, A.; Ugi, I. Multicomponent reactions with isocyanides. *Angew. Chem., Int. Ed.* **2000**, *39*, 3168−3210.

(31) Marquarding, D.; Hoffmann, P.; Heitzer, H.; Ugi, I. Stereoselective four-component condensations of α-ferrocenylethylamine and its absolute configuration. *J. Am. Chem. Soc.* **1970**, *92*, 1969−1971.

(32) Semple, J. E.; Wang, P. C.; Lysenko, Z.; Joullié, M. M. Total synthesis of (+)-furanomycin and stereoisomers. *J. Am. Chem. Soc.* **1980**, *102*, 7505−7510.

(33) Berthod, A.; Liu, Y.; Bagwill, C.; Armstrong, D. W. Facile liquid chromatographic enantioresolution of native amino acids and peptides using a teicoplanin chiral stationary phase. *J. Chromatogr. A* **1996**, *731*, 123−137.

(34) Shinbo, T.; Yamaguchi, T.; Nishimura, K.; Sugiura, M. Chromatographic separation of racemic amino acids by use of chiral crown ether-coated reversed-phase packings. *J. Chromatogr.* **1987**, *405*, 145−153.

(35) Johansen, T. N.; Ebert, B.; Falch, E.; Krogsgaard-Larsen, P. AMPA receptor agonists: Resolution, configurational assignment and pharmacology of (+)-(*S*)- and (−)-(*R*)-2-amino-3-[3-hydroxy-5-(2-pyridyl)isoxazol-4-yl]propionic acid (2-Py-AMPA). *Chirality* **1997**, *9*, 274−280.

(36) Falch, E.; Brehm, L.; Mikkelsen, I.; Johansen, T. N.; Skjærbæk, N.; Nielsen, B.; Stensbøl, T. B.; Ebert, B.; Krogsgaard-Larsen, P. Heteroaryl analogues of AMPA. 2. Synthesis, absolute stereochemistry, photochemistry, and structure−activity relationships. *J. Med. Chem.* **1998**, *41*, 2513−2523.

(37) Johansen, T. N.; Ebert, B.; Bräuner-Osborne, H.; Didriksen, M.; Christensen, I. T.; Søby, K. K.; Madsen, U.; Krogsgaard-Larsen, P.; Brehm, L. Excitatory amino acid receptor ligands: resolution, absolute stereochemistry, and enantiopharmacology of 2-amino-3-(4-butyl-3-hydroxyisoxazol-5-yl)propionic acid. *J. Med. Chem.* **1998**, *41*, 930−939.

(38) *Macromodel 7.2−8.0* (2001) and *Glide 1.8* (2002); Schrödinger, Inc., 1500 SW First Avenue, Suite 1180, Portland, OR 97201.

(39) Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. Basic local alignment search tool (BLAST). *J. Mol. Biol.* **1990**, *215*, 403−410.

(40) Armstrong, N.; Gouaux, E. Mechanisms for activation and antagonism of an AMPA-sensitive glutamate receptor: crystal structures of the GluR2 ligand binding core. *Neuron* **2000**, *28*, 165−181.

(41) http://www.expasy.ch/swissmod/SWISS-MODEL.html. Schwede, T.; Diemand, A.; Guex, N.; Peitsch, M. C. Protein structure computing in the genomic era. *Res. Microbiol.* **2000**, *151*, 107−112.

(42) Hogner, A.; Kastrup, J. S.; Jin, R.; Liljefors, T.; Mayer, M. L.; Egebjerg, J.; Larsen, I. K.; Gouaux, E. Structural basis for AMPA receptor activation and ligand selectivity: Crystal structures of five agonist complexes with the GluR2 ligand-binding core. *J. Mol. Biol.* **2002**, *322*, 93−109.

(43) Lunn, M.-L. Ph.D. Thesis, The Royal Danish School of Pharmacy, Copenhagen, Denmark, 2002.

(44) Brehm, L.; Jørgensen, F. S.; Hansen, J. J.; Krogsgaard-Larsen, P. Agonists and antagonists for central glutamic acid receptors. *Drug News Perspect.* **1988**, *1*, 138−144.

(45) Bräuner-Osborne, H.; Sløk, F. A.; Skjærbæk, N.; Ebert, B.; Sekiyama, N.; Nakanishi, S.; Krogsgaard-Larsen, P. A new highly selective metabotropic excitatory amino acid agonist: 2-amino-4-(3-hydroxy-5-methylisoxazol-4-yl)butyric acid. *J. Med. Chem.* **1996**, *39*, 3188−3194.

(46) Bräuner-Osborne, H.; Krogsgaard-Larsen, P. Pharmacology of (*S*)-homoquisqualic acid and (*S*)-2-amino-5-phosphonopentanoic acid [(*S*)-AP5] at cloned metabotropic glutamate receptors. *Br. J. Pharmacol.* **1998**, *123*, 269−274.

(47) Bräuner-Osborne, H.; Nielsen, B.; Krogsgaard-Larsen, P. Molecular pharmacology of homologues of ibotenic acid at cloned metabotropic glutamic acid receptors. *Eur. J. Pharmcol.* **1998**, *350*, 311−316.

(48) Lerma, J.; Paternain, A. V.; Rodríguez-Moreno, A.; López-García, J. C. Molecular physiology of kainate receptors. *Physiol. Rev.* **2001**, *81*, 971−998.

(49) Smolders, I.; Bortolotto, Z. A.; Clarke, V. R. J.; Warre, R.; Khan, G. M.; O'Neill, M. J.; Ornstein, P. L.; Bleakman, D.; Ogden, A.; Weiss, B.; Stables, J. P.; Ho, K. H.; Ebinger, G.; Collingridge, G. L.; Lodge, D.; Michotte, Y. Antagonists of GLU_{K5}-containing kainate receptors prevent pilocarpine-induced limbic seizures. *Nat. Neurosci.* **2002**, *5*, 796−804.

(50) Banke, T. G.; Greenwood, J. R.; Christensen, J. K.; Liljefors, T.; Schousboe, A.; Pickering, D. S. Identification of amino acid residues in GluR1 responsible for ligand binding and desensitisation. *J. Neurosci.* **2001**, *21*, 3052−3062.

(51) Tanabe, Y.; Nomura, A.; Masu, M.; Shigemoto, R.; Mizuno, N.; Nakanishi, S. Signal transduction, pharmacological properties, and expression patterns of two rat metabotropic glutamate receptors, mGluR3 and mGluR4. *J. Neurosci.* **1993**, *13*, 1372−1378.

(52) Ransom, R. W.; Stec, N. L. Cooperative modulation of [^{3}H]MK-801 binding to the *N*-methyl-D-aspartate receptor ion channel complex by L-glutamate, glycine and polyamines. *J. Neurochem.* **1988**, *51*, 830−836.

(53) Honoré, T.; Nielsen, M. Complex structure of quisqualate-sensitive glutamate receptors in rat cortex. *Neurosci. Lett.* **1985**, *54*, 27−32.

(54) Braitman, D. J.; Coyle, J. T. Inhibition of [^{3}H]kainic acid receptor binding by divalent cations correlates with ion affinity for the calcium channel. *Neuropharmacology* **1987**, *26*, 1247−1251.

(55) Sills, M. A.; Fagg, G.; Pozza, M.; Angst, C.; Brundish, D. E.; Hurt, S. D.; Wilusz, E. J.; Williams, M. [^{3}H]CGP 39653: a new *N*-methyl-D-aspartate antagonist radioligand with low nanomolar affinity in rat brain. *Eur. J. Pharmacol.* **1991**, *192*, 19−24.

(56) Leatherbarrow, R. J. *GraFit*, version 3.0; Erithacus Software: Staines, U.K., 1992.

(57) Christopoulus, A. Assessing the distrubution of parameters in models of ligand−receptor interactions: to log or not to log. *Trends Pharmacol. Sci.* **1998**, *19*, 351−357.

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