Notes

(*S*)-Homo-AMPA, a Specific Agonist at the mGlu$_6$ Subtype of Metabotropic Glutamic Acid Receptors

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Received May 29, 1997⊗

Our previous publication (*J. Med. Chem.* **1996**, *39*, 3188−3194) described (*RS*)-2-amino-4-(3-hydroxy-5-methylisoxazol-4-yl)butyric acid (Homo-AMPA) as a highly selective agonist at the mGlu$_6$ subtype of metabotropic excitatory amino acid (EAA) receptors. Homo-AMPA has already become a standard agonist for the pharmacological characterization of mGlu$_6$ (*Trends Pharmacol. Sci. Suppl.* **1997**, 37−39), and we here report the resolution, configurational assignment, and pharmacology of (*S*)- (**6**) and (*R*)- (**7**) Homo-AMPA. Using the "Ugi four-component condensation", 3-(3-ethoxy-5-methylisoxazol-4-yl)propanal (**10**) was converted into the separable diastereomeric derivatives of **6** and **7**, compounds **12** and **11**, respectively. Deprotection of **12**, in one or two steps, gave extensively racemized **6**, which was converted in low yield into **6** (99.0% ee) through several crystallizations. **6** (99.7% ee) and **7** (99.9% ee) were finally obtained by preparative chiral HPLC. The configurational assignments of **6** and **7** were based on ^{1}H NMR spectroscopic studies on **12** and **11**, respectively, and circular dichroism studies on **6** and **7**. Values of optical rotations using different solvents and the chiral HPLC elution order of **6** and **7** supported the results of the spectroscopic configurational assignments. The activities of **6** and **7** at ionotropic EAA (iGlu) receptors and at mGlu$_{1-7}$ were studied. (*S*)-Homo-AMPA (**6**) was shown to be a specific agonist at mGlu$_6$ (EC$_{50}$ = 58 ± 11 μM) comparable in potency with the endogenous mGlu agonist (*S*)-glutamic acid (EC$_{50}$ = 20 ± 3 μM). Although Homo-AMPA did not show significant effects at iGlu receptors, (*R*)-Homo-AMPA (**7**), which was inactive at mGlu$_{1-7}$, turned out to be a weak *N*-methyl-D-aspartic acid (NMDA) receptor antagonist (IC$_{50}$ = 131 ± 18 μM).

Introduction

Based on pharmacological, electrophysiological, and molecular cloning studies, the excitatory amino acid (EAA) receptors, operated by (*S*)-glutamic acid [(*S*)-Glu, **1**], have been classified into two major classes: the ionotropic EAA (iGlu) receptors and the metabotropic EAA (mGlu) receptors coupled to G-proteins. The former class of EAA receptors comprises the *N*-methyl-D-aspartic acid (NMDA), (*RS*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA), and kainic acid subgroups of receptors, all of which are heterogeneous.[1−4] So far, eight different subtypes of mGlu receptors (mGlu$_{1-8}$) have been cloned.[5−8]

A prerequisite for the determination of the physiological role and pharmacological importance of the subgroups of iGlu receptors and the subtypes of mGlu receptors is the availability of highly selective agonists and antagonists.[6,9] We have previously shown that (*R*)-2-amino-2-(3-hydroxy-5-methylisoxazol-4-yl)acetic acid [(*R*)-AMAA, **2**] is a selective NMDA agonist,[10] whereas the *S*-form of the homologous 3-isoxazolol amino acid, (*S*)-AMPA (**3**), is a specific AMPA receptor agonist[11] (Chart 1). The higher homologue of AMPA, (*RS*)-2-amino-4-(3-hydroxy-5-methylisoxazol-4-yl)butyric acid (Homo-AMPA), on the other hand, does not interact significantly with iGlu receptors,[12] but Homo-AMPA was shown to be a highly selective mGlu$_6$ receptor agonist.[13] Homo-AMPA has already become a standard ligand for the pharmacological characterization of mGlu$_6$ receptors,[14] and we here report the preparation, configurational assignment, and pharmacology of (*S*)-Homo-AMPA (**6**) and (*R*)-Homo-AMPA (**7**). The pharmacological effects of **6** and **7** are compared with those of the *S*- and *R*-forms of the glutamic acid homologue 2-aminoadipic acid, compounds **4** and **5**, respectively (Chart 1).

Results

Chemistry and Stereochemistry. Under "Ugi four-component condensation" reaction conditions,[15] using (*S*)-1-phenylethylamine [(*S*)-PEA] as a chiral auxiliary, 3-(3-ethoxy-5-methylisoxazol-4-yl)propanal (**10**) was converted into (2*R*)-*N*-*tert*-butyl-4-(3-ethoxy-5-methylisoxazol-4-yl)-2-[*N*-[(*S*)-1-phenylethyl]benzamido]butyramide (**11**) and the 2*S*-diastereomeric compound **12** (Scheme 1). Compounds **11** and **12** were readily sepa-

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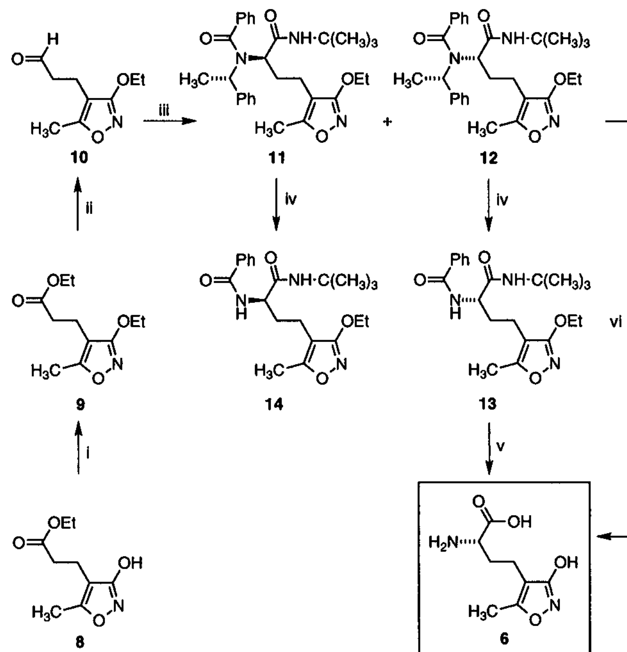
⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

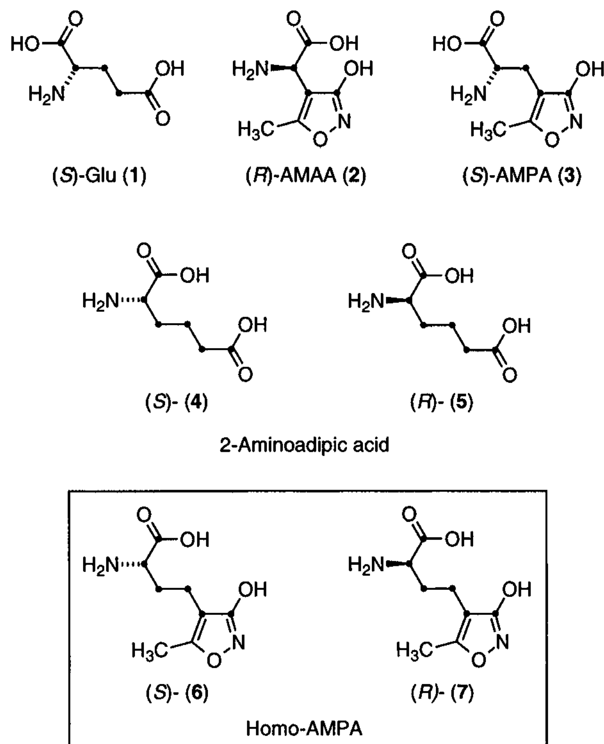
S0022-2623(97)00359-2 CCC: $14.00 © 1997 American Chemical Society

Scheme 1[a]



[a] Reagents: (i) EtBr, K_2CO_3; (ii) diisobutylaluminum hydride; (iii) (*S*)-PEA, benzoic acid, *tert*-butylisonitrile; (iv) formic acid; (v) 6 M HCl; (vi) 48% aqueous HBr.

Chart 1. Structures of (*S*)-Glutamic Acid [(*S*)-Glu, **1**] and a Number of Analogues and Homologues



rable by silica gel column chromatography (CC). The ^{1}H NMR chemical shifts of the *tert*-butyl groups of this type of diastereomeric products, containing a (*S*)-PEA unit, have empirically been used to assign their relative, and consequently their absolute, stereochemistry.[15−17] Thus, a resonance signal for this group in the region of 1.3−1.4 ppm is consistent with *R*,*S*-configuration, whereas a signal near 1.1 ppm indicates *S*,*S*-configuration. On the basis of an ^{1}H NMR spectroscopic analysis, compound **11** (1.37 ppm) and compound **12** (1.13 ppm) were assigned the 2*R*- and 2*S*-configurations, respectively.

Compound **12** was subjected to one- and two-step deprotection reactions to give (*S*)-Homo-AMPA (**6**) under previously reported conditions.[15,17] In the two-step procedure, **12** was debenzylated using formic acid to give **13**, which was subsequently converted into **6**·HCl by treatment with 6 M hydrochloric acid at 105−110 °C for 16 h. Chiral HPLC analysis of the fully deprotected product before recrystallization disclosed pronounced racemization, the crude **6**·HCl showing an enantiomeric excess (ee) of 62%. ^{1}H NMR analyses of the starting material **12**, to which different amounts of **11** were added, indicated a diastereomeric excess (de) of **12** higher than 90%. The stereochemical purity of **13** was analyzed by chiral HPLC. To be able to identify the enantiomeric impurity in the HPLC chromatogram of compound **13**, the enantiomer **14** was synthesized as a reference compound. Compound **14** was synthesized in analogy with **13** by treatment of compound **11** with formic acid (Scheme 1). The HPLC analysis showed both compounds **13** and **14** to have ee ≥ 96%, indicating that no significant racemization takes place during the formic acid treatment.

In the one-step deprotection reaction, a solution of **12** in 48% hydrobromic acid was heated at 100 °C for 1 h providing extensively racemized crude **6**·HBr (45% ee). Additional heating of this solution at 100 °C for more than 3 h did not result in further racemization, indicating stereochemical stability of **6** under strongly acidic conditions. After four crystallizations, crude **6**·HCl (62% ee) was converted into **6**·HCl of relatively high optical purity (99% ee) but at a low yield (20%). The results prompted us to develop a chromatographic procedure for the preparation of **6** and **7**.

The preparative resolution of Homo-AMPA was carried out on a preparative Crownpak CR(+) column (150 × 10 mm) containing 18-crown-6-type crown ether as the chiral selector. The column was eluted at 0−1 °C with aqueous acetic acid. Under these conditions, 10 mg samples of racemic compound dissolved in the mobile phase could be separated with baseline resolution in 30 min. Collection and evaporation of appropriate fractions gave after recrystallization **6** (99.7% ee) and **7** (99.9% ee) in 73% and 80% yields, respectively (Figure 1).

It has been shown that *R*-forms of α-amino acids elute faster than the corresponding *S*-forms on Crownpak CR(+) columns,[18] and accordingly, compound **6**, which was assigned the *S*-configuration on the basis of ^{1}H NMR studies, was eluted more slowly than **7**, assigned the *R*-configuration, using the Crownpak CR(+) column. The elution sequence of **6** and **7** was reversed, when the enantiomeric Crownpak CR(−) column was used (Figure 1). Furthermore, the elution order of **6** and **7** was studied using a chiral ligand exchange HPLC column containing (*S*)-pipecolic acid bound to silica gel and chelated with Cu^{2+}. In agreement with the general observations that (*R*)-α-amino acids elute before the corresponding *S*-forms using a similar (*S*)-proline-based column,[19,20] **7** eluted before **6** on the (*S*)-pipecolic acid-based column.

CD spectra of **6** and **7**, recorded in 0.1 M hydrochloric acid, clearly are mirror images (Figure 2), demonstrating the enantiomeric relationship. The positive Cotton

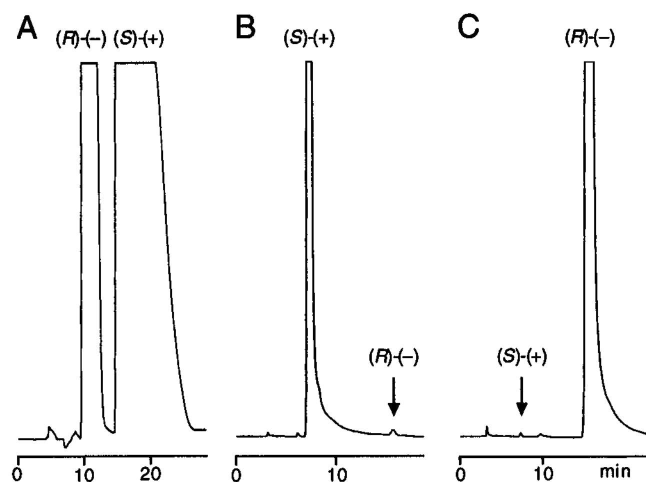


Figure 1. Chromatographic separation of 10 mg of Homo-AMPA on a 150 × 10 mm Crownpak CR(+) column eluted at 1.5 mL/min with 15 mM aqueous AcOH at 0−1 °C (A). Chiral HPLC analyses of the chromatographically resolved (*S*)-(+)-Homo-AMPA (**6**) (B) and (*R*)-(−)-Homo-AMPA (**7**) (C) on a 150 × 4 mm Crownpak CR(−) column eluted at 0.4 mL/min with aqueous $HClO_4$, pH 2, at room temperature. Spike experiments using Homo-AMPA confirmed the identity of the enantiomeric impurities indicated in panels B and C.

effect of **6** at 210 nm ($\Delta\epsilon = +0.14$ m^2/mol) is in agreement with published data describing positive Cotton effects of (*S*)-α-amino acids in acidic solutions.[21,22] Under similar conditions, (*S*)-AMPA (**3**) also showed a positive Cotton effect ($\Delta\epsilon = +0.19$ m^2/mol) (Figure 2), supporting the configurational assignment of **6**.

The chiral chromatographic behavior, IR spectrum, optical rotations in hydrochloric acid, and CD spectrum of **6**·HCl, prepared from **6** obtained by preparative chiral HPLC, were identical with those of **6**·HCl obtained by deprotection of compound **12** (Scheme 1) and crystallized to an ee of 99%.

***In Vitro* Pharmacology.** So far, eight different mGlu receptors ($mGlu_{1-8}$) have been cloned.[5−8] In this study we tested the effects of (*S*)-Homo-AMPA (**6**) and (*R*)-Homo-AMPA (**7**) at $mGlu_{1-7}$ essentially following published procedures,[23−25] whereas $mGlu_8$ was not available to us. Initially, we tested **6** and **7** at 1 mM concentrations (10 mM in the case of $mGlu_7$) alone and in the presence of 30 μM (*S*)-Glu (**1**) [in the case of $mGlu_7$, 1 mM of (*S*)-2-amino-4-phosphonobutyric acid [(*S*)-AP4] was used due to the low potency of (*S*)-Glu (**1**)]. Compound **6** showed agonist potency at $mGlu_6$ but did not show significant activities at the remaining mGlu receptors, neither as an agonist nor as an antagonist (Table 1). As shown in Figure 3 and Table 1, **6** had an EC_{50} value of 58 ± 11 μM at $mGlu_6$, being almost equipotent with (*S*)-Glu (**1**) at this subtype of mGlu receptors. Whereas (*S*)-2-aminoadipic acid (**4**) previously has been shown to activate $mGlu_2$ as well as $mGlu_6$, (*R*)-2-aminoadipic acid (**5**) is inactive at these receptors,[13] and in the present studies, compound **7** was inactive at all seven mGlu receptors, when tested for both agonist and antagonist effects (Table 1 and Figure 3).

Homo-AMPA has previously been shown to be inactive when tested as an agonist (at 1000 μM) and as an antagonist (at 100 μM) at iGlu receptors.[12] Nevertheless, (*S*)- (**6**) and (*R*)- (**7**) Homo-AMPA were tested for agonist or antagonist effects at these receptors using the rat cortical wedge preparation.[26] Compound **6** was inactive when tested as an agonist ($EC_{50} > 1000$ μM) or an antagonist ($IC_{50} > 1000$ μM) at iGlu receptors. Compound **7** marginally ($IC_{50} > 500$ μM) reduced depolarizations induced by AMPA (5 μM) or kainic acid (10 μM) and turned out to be a weak antagonist at NMDA receptors ($IC_{50} = 131 \pm 18$ μM).

Discussion

The preparation, configurational assignment, and *in vitro* pharmacology of (*S*)-Homo-AMPA (**6**) and (*R*)-Homo-AMPA (**7**) are described. The "Ugi four-component condensation"[15] reaction was used to synthesize the diastereomeric condensation products **11** and **12**, which were separated chromatographically. Deprotection of **12** to give **6** using one- or two-step procedures, both involving hydrolysis under strongly acidic conditions (Scheme 1), provided extensively racemized **6**. This amino acid was shown to be stereochemically stable under these reaction conditions indicating that an as yet unidentified intermediate in the transformation of **12** into **6** must be susceptible to acid-catalyzed racemization. In any case, the "Ugi four-component condensation" reaction does not seem to be a versatile procedure for the synthesis of optically pure α-amino acids. Compounds **6** and **7** of high enantiomeric purities and in good yields were finally obtained by preparative chiral HPLC separation of Homo-AMPA[12] (Figure 1).

The configurational assignments of **6** and **7** were based on ^{1}H NMR chemical shift values for the *tert*-butyl groups of **12** and **11**. These values have been shown to be predictive of the absolute configuration of the α-amino acid units of such condensation products.[15−17] The results of this empirical determination of the absolute stereochemistry of **6** and **7** were corroborated by the results of comparative CD spectral analyses of **6**, **7**, and (*S*)-AMPA (**3**) (Figure 2) and by elution orders of **6** and **7** under different chiral HPLC conditions (Figure 1). Finally, the signs of optical rotations of **6** and **7**, recorded in water and hydrochloric acid, were in accordance with the empirical Clough−Lutz−Jirgenson's rule.[27] These results together are consistent with (+)-Homo-AMPA (**6**) having *S*-configuration and (−)-Homo-AMPA (**7**) *R*-configuration.

(*S*)-Glu (**1**) is capable of activating all iGlu and mGlu receptors, and (*S*)-Glu (**1**) probably is the endogenous ligand for all of these receptors.[1−7] In light of this total lack of selectivity of (*S*)-Glu (**1**), it is remarkable that (*S*)-AMPA (**3**), which is the 3-isoxazolol bioisostere of (*S*)-Glu (**1**) (Chart 1), is a specific agonist at AMPA receptors.[4] The higher homologue of (*S*)-Glu (**1**), (*S*)-2-aminoadipic acid (**4**), interacts with a number of glutamatergic synaptic mechanisms,[13] including activation of $mGlu_2$ and $mGlu_6$.[13] We have now shown that the 3-isoxazolol bioisostere of **4**, (*S*)-Homo-AMPA (**6**) (Chart 1), is a specific agonist at $mGlu_6$, approximately equipotent with (*S*)-Glu (**1**) (Table 1).

(*R*)-Homo-AMPA (**7**) turned out to be a weak NMDA antagonist reflecting the structural relationship of **7** with (*R*)-2-aminoadipic acid (**5**) (Chart 1), which has previously been shown to be an NMDA antagonist.[2]

Experimental Section

General Methods. Melting points were determined in capillary tubes and are uncorrected. CC was performed on Merck silica gel 60 (0.063−0.200 mm). Compounds were

Table 1. Pharmacological Profiles of (*S*)- (**6**) and (*R*)- (**7**) Homo-AMPA and Some Structurally Related Amino Acids at the Cloned mGlu Receptors

compound	EC$_{50}$ (μM)[a]						
	mGlu$_{1\alpha}$[b]	mGlu$_2$[c]	mGlu$_3$[c]	mGlu$_{4a}$[d]	mGlu$_5$[b]	mGlu$_6$[d]	mGlu$_7$[d]
(*S*)-Glu (**1**)	10 ± 2	4.0 ± 0.5	9.0 ± 4.4	12 ± 2	4.4 ± 0.8	20 ± 3	5400 ± 2800
(*S*)-2-aminoadipic acid (**4**)[e]	>1000	35 ± 1	nd	>3000	nd	140 ± 35	nd
(*R*)-2-aminoadipic acid (**5**)[e]	>1000	>1000	nd	nd	nd	>1000	nd
(*S*)-AP4	>1000	>1000	nd	0.91 ± 0.50	>1000	0.57 ± 0.23	252 ± 23
Homo-AMPA[e]	>1000	>1000	nd	>3000	nd	82 ± 15	nd
(*S*)-Homo-AMPA (**6**)	>1000	>1000	>1000	>1000	>1000	58 ± 11	>5000
(*R*)-Homo-AMPA (**7**)	>1000	>1000	>1000	>1000	>1000	>1000	>5000

[a] EC$_{50}$ values ± SEM of at least two independent experiments. [b] mGlu$_{1\alpha}$ and mGlu$_5$ (group I) are positively coupled to the hydrolysis of phosphatidylinositol (PI), and agonism at these receptors was tested as the fold increase in PI level as compared to control. [c] mGlu$_2$ and mGlu$_3$ (group II) are negatively coupled to the formation of cyclic AMP, and agonism at these receptors was tested as percent decrease of cyclic AMP level, in the presence of forskolin, as compared to control. [d] mGlu$_{4a}$, mGlu$_6$, and mGlu$_7$ (group III) are also negatively coupled to the formation of cyclic AMP, and agonism at these receptors was tested as indicated above for group II mGlu receptors (for details, see ref 13). [e] Data from ref 13.

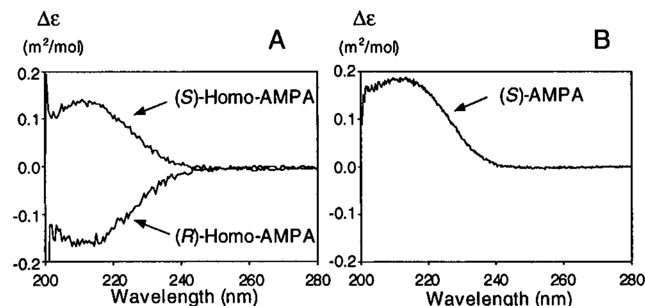


Figure 2. CD spectra of the chromatographically resolved (*S*)-Homo-AMPA (**6**) and (*R*)-Homo-AMPA (**7**) (A) and (*S*)-AMPA (**3**) (B) in 0.1 M HCl.

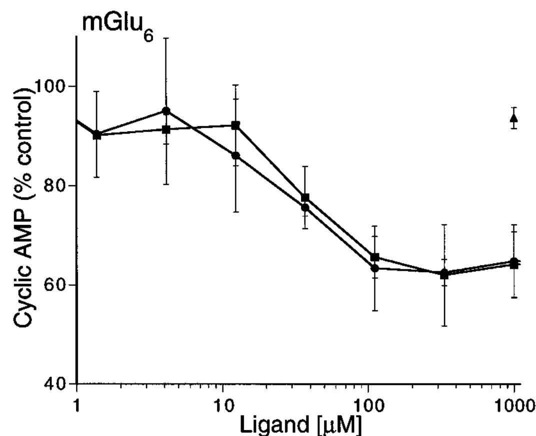


Figure 3. Dose–response curves of (*S*)-Glu (**1**) (●), (*S*)-Homo-AMPA (**6**) (■), and (*R*)-Homo-AMPA (**7**) (▲) at CHO cells expressing mGlu$_6$ receptors. Cells were incubated with the agonists in the presence of 10 μM forskolin for 10 min, and cyclic AMP levels were determined by a RIA assay (Amersham). Data are the mean (±SD) of a representative experiment performed in triplicate.

visualized on TLC plates (Merck silica gel 60 F$_{254}$) using UV light or a KMnO$_4$ spraying reagent. Compounds containing an amino group or 3-isoxazolol moiety were visualized using a ninhydrin or FeCl$_3$ spraying reagent, respectively; 4 Å molecular sieves were used to dry solvents at least 2 days prior to use. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 200F (200 MHz) spectrometer using TMS or 1,4-dioxane as internal standards for spectra recorded in CDCl$_3$ or D$_2$O, respectively. IR spectra were recorded from KBr disks on a Perkin-Elmer 781 grating infrared spectrophotometer. Optical rotations were determined on a Perkin-Elmer 241 polarimeter. CD spectra were recorded at room temperature in 1.0 cm cuvettes on a Jasco J-720 spectropolarimeter. Elemental analyses were performed at Analytical Research Department, H. Lundbeck A/S, Denmark, or by Mrs. K. Linthoe, Department of Chemistry, University of Copenhagen, Denmark, and are within ±0.4% of the calculated values unless otherwise stated. The HPLC system for the preparative resolution and the analytical determinations of the enantiomeric purities of **6** and **7** consisted of a Jasco 880 pump, a Rheodyne 7125 injector, and a Waters 480 UV detector (220 nm), connected to a Hitachi D-2000 Chromato-Integrator. A Waters HPLC system consisting of a M510 pump, a U6K injector, and a M991 photodiode array detector was used for the analytical separation of **13** and **14**. The ee values were calculated from peak areas. The preparative chromatographic resolution of **6** and **7** was performed on a 150 × 10 mm Crownpak CR(+) column equipped with a 10 × 4.0 mm Crownpak CR(+) guard column, both obtained from Daicel and eluted with 1.5 mL/min aqueous AcOH (15 mM) at 0–1 °C. Chiral HPLC analyses of **13** and **14** were performed on a 100 × 4.0 mm CHIRAL-AGP column (ChromTech, Sweden) eluted at ambient temperature with 0.5 mL/min of 10% 2-PrOH in aqueous potassium phosphate (50 mM), pH 7.0, and the HPLC analyses of **6** and **7** were performed on a 150 × 4.0 mm Crownpak CR(−) column (Daicel) eluted with 0.4 mL/min aqueous HClO$_4$, pH 2, at ambient temperature.

Ethyl 3-(3-Ethoxy-5-methylisoxazol-4-yl)propionate (9). A mixture of ethyl 3-(3-hydroxy-5-methylisoxazol-4-yl)propionate (**8**)[28] (15.0 g, 75.4 mmol) and K$_2$CO$_3$ (20.8 g, 150.8 mmol) in acetone (600 mL) was heated at 60 °C for 30 min. After the mixture cooled to 21 °C, ethyl bromide (12.3 g, 113 mmol) was added dropwise, and the resulting suspension was heated at 60 °C for 24 h. The reaction mixture was cooled to 21 °C, filtered, and concentrated *in vacuo*. CC [toluene (tol)–EtOAc (20:1)] of the residue gave **9** (8.0 g, 35.2 mmol) as a slightly yellow oil in 48% yield. A small sample was distilled in a Kugelrohr apparatus (200 °C, 0.1 mmHg): ^{1}H NMR (CDCl$_3$) δ 1.23 (3H, t, J = 7.2 Hz), 1.40 (3H, t, J = 7.1 Hz), 2.26 (3H, s), 2.51–2.56 (4H, m), 4.10 (2H, q, J = 7.2 Hz), 4.28 (2H, q, J = 7.1 Hz); ^{13}C NMR (CDCl$_3$) δ 11.2, 13.4, 14.5, 16.5, 32.8, 60.2, 65.2, 103.0, 165.8, 170.5, 172.5. Anal. (C$_{11}$H$_{17}$NO$_4$) C, H, N.

3-(3-Ethoxy-5-methylisoxazol-4-yl)propanal (10). Compound **9** (5.5 g, 24.0 mmol) was dissolved in dry tol (120 mL) under a N$_2$ atmosphere, and the solution was cooled to −78 °C. Diisobutylaluminum hydride (60 mL of a 1 M solution in hexane, 60.0 mmol) was added, and after 6 min the reaction was quenched with CH$_3$OH (6 mL) followed by addition of a Rochelle salt solution (45 mL of a saturated aqueous solution of sodium potassium tartrate). The mixture was allowed to warm to 25 °C, and the product was extracted with 3 × 150 mL of Et$_2$O. The combined organic phases were dried (MgSO$_4$), filtered, and concentrated *in vacuo* to give **10** (3.0 g, 68%) as a yellow oil: ^{1}H NMR (CDCl$_3$) δ 1.40 (3H, t, J = 7.1 Hz), 2.27 (3H, s), 2.56 (2H, m), 2.68 (2H, m), 4.28 (2H, q, J = 7.1 Hz), 9.77 (1H, t, J = 1.0 Hz). Crude **10** was, without further purification, converted into a mixture of **11** and **12**.

(2*R*)- and (2*S*)-*N*-*tert*-Butyl-4-(3-ethoxy-5-methylisoxazol-4-yl)-2-[*N*-[(*S*)-1-phenylethyl]benzamido]butyramide (11 and 12). A mixture of **10** (3.0 g, 16.4 mmol) and (*S*)-PEA (1.98 mL, 16.4 mmol) in CH$_3$OH (28 mL) was refluxed at 100 °C for 30 min. After the mixture cooled to 25 °C, benzoic acid (2.0 g, 16.4 mmol) and *tert*-butylisonitrile (1.36 g, 16.4

mmol) were added, and the resulting solution was stirred at 25 °C for 24 h. Evaporation of the solvent followed by CC [tol−EtOAc (9:1)] of the residue produced **11** (3.0 g, 38%) as a yellow oil: $[\alpha]^{23}_{D} = -120°$, $[\alpha]^{23}_{365} = -490°$ ($c = 0.52$, EtOH); ^{1}H NMR ($CDCl_3$) δ 1.37 (12H, s and t, $J = 7.1$ Hz), 1.62 (3H, d, $J = 7.0$ Hz), 1.70 (1H, m), 1.90 (2H, m), 2.08 (3H, s), 2.65 (1H, m), 3.35 (1H, m), 4.19 (2H, q, $J = 7.1$ Hz), 5.05 (1H, q, $J = 7.0$ Hz), 7.29 (5H, m), 7.46 (5H, bs), 8.0 (1H, bs); ^{13}C NMR ($CDCl_3$) δ 11.1, 14.5, 17.3, 17.9, 28.4, 30.3, 50.6, 58.6, 60.2, 64.9, 103.3, 125.4, 127.0, 128.0, 128.3, 128.8, 129.5, 137.0, 138.5, 165.4, 170.3, 171.3, 173.0. Anal. ($C_{29}H_{37}N_3O_4$) H, N; C: calcd, 70.85; found, 69.44.

Further elution gave after recrystallization (Et_2O−light petroleum ether) **12** (2.0 g, 25%): mp 108−110 °C; $[\alpha]^{23}_{D} = -34.8°$, $[\alpha]^{23}_{365} = -142°$ ($c = 0.51$, EtOH); ^{1}H NMR ($CDCl_3$) δ 1.13 (9H, s), 1.40 (3H, t, $J = 7.1$ Hz), 1.46 (3H, d, $J = 7.0$ Hz), 2.0 (1H, m), 2.20 (3H, s), 2.45 (2H, t, $J = 8.7$ Hz), 2.72 (1H, m), 3.45 (1H, m), 4.30 (2H, q, $J = 7.1$ Hz), 5.10 (1H, q, $J = 7.0$ Hz), 7.14−7.40 (5H, m), 7.47 (5H, bs); ^{13}C NMR ($CDCl_3$) δ 11.3, 14.7, 16.9, 18.8, 28.4, 29.9, 50.3, 57.7, 59.3, 65.2, 103.8, 125.7, 127.3, 128.3, 128.8, 128.9, 129.6, 137.0, 166.3, 169.5, 171.0, 172.0. Anal. ($C_{29}H_{37}N_3O_4$) C, H, N.

(*S*)-*N*-*tert*-Butyl-2-benzamido-4-(3-ethoxy-5-methylisoxazol-4-yl)butyramide (13). A solution of **12** (0.40 g, 0.81 mmol) in 98% formic acid (20 mL) was stirred at 21 °C for 30 min and then at 60 °C for 2.5 h. After the mixture cooled to 21 °C and was concentrated *in vacuo*, the residue was purified by CC [tol−EtOAc (3:1)] to give **13** (0.20 g, 64%): mp 142−143 °C; $[\alpha]^{23}_{D} = -3.9°$, $[\alpha]^{23}_{365} = -17.9°$ ($c = 0.38$, EtOH); 97% ee; ^{1}H NMR ($CDCl_3$) δ 1.35 (12H, s and t, $J = 7.1$ Hz), 2.05 (2H, m), 2.20 (3H, s), 2.36 (2H, m), 4.24 (2H, q, $J = 7.1$ Hz), 4.60 (1H, dd, $J = 12.7, 6.0$ Hz), 6.40 (1H, s), 7.45 (5H, m); ^{13}C NMR ($CDCl_3$) δ 11.4, 14.6, 17.1, 28.6, 31.6, 51.6, 53.2, 65.4, 103.4, 127.0, 128.3, 128.5, 130.0, 131.8, 133.7, 165.8, 167.2, 170.4, 170.6. Anal. ($C_{21}H_{29}N_3O_4 \cdot 0.25H_2O$) C, H, N.

(*R*)-*N*-*tert*-Butyl-2-benzamido-4-(3-ethoxy-5-methylisoxazol-4-yl)butyramide (14). Compound **14** (0.11 g, 63%) with ee = 96% was synthesized from **11** (0.23 g, 0.45 mmol) by a method similar to that used for compound **13**. Compound **14**: mp 141−143 °C; $[\alpha]^{23}_{D} = +3.3°$, $[\alpha]^{23}_{365} = +16.0°$ ($c = 0.36$, EtOH); 96% ee; ^{1}H and ^{13}C NMR spectra of **14** were identical to those obtained for compound **13**.

(*S*)-2-Amino-4-(3-hydroxy-5-methylisoxazol-4-yl)butyric Acid [(*S*)-Homo-AMPA, 6], Hydrochloride. A solution of **13** (0.20 g, 0.52 mmol) in 6 M HCl (15 mL) was heated at 105−110 °C for 16 h, then cooled to 21 °C, washed with 3 × 15 mL of CH_2Cl_2, and concentrated *in vacuo* to give 100 mg of crude product with an ee of 62%. Recrystallization from AcOH gave 50 mg of colorless crystals with an ee of 88%. This procedure was repeated three times to afford 20 mg of **6**·HCl in 20% yield with an ee of 99%: mp 164−165 °C dec; $[\alpha]^{23}_{D} = +27.9°$, $[\alpha]^{23}_{365} = +103°$ [$c = 0.408$ (concentration of zwitterion), 0.1 M HCl]; ^{1}H NMR (D_2O) δ 2.12 (2H, m), 2.22 (3H, s), 2.43 (2H, t, $J = 7.4$ Hz), 4.00 (1H, t, $J = 6.4$ Hz); ^{13}C NMR (D_2O) δ 11.7, 17.0, 29.7, 53.4, 105.4, 169.9, 171.3, 173.2; IR 3450 (m), 3200−2900 (s), 1720 (m), 1620 (s), 1520 (s) cm^{-1}.

Crude (*S*)-2-Amino-4-(3-hydroxy-5-methylisoxazol-4-yl)butyric Acid [(*S*)-Homo-AMPA, 6], Hydrobromide. A solution of **12** (5 mg, 0.40 mmol) in 48% aqueous HBr (0.5 mL) was heated at 115 °C for 1.5 h, then cooled to 20 °C, and concentrated *in vacuo* to give 4 mg of crude **6**·HBr with an ee of 45%. This crude product was redissolved in 48% aqueous HBr, and after heating at 115 °C for 3 h, this solution was evaporated *in vacuo* to give crude **6**·HBr with an ee of 45%. This sample of crude **6**·HBr, which cochromatographed [Crownpak CR(−)] with **6**·HCl prepared from compound **13**, was not worked up.

(*R*)-2-Amino-4-(3-hydroxy-5-methyl-4-isoxazolyl)butyric Acid [(*R*)-(−)-Homo-AMPA, 7]. Homo-AMPA[12] (173 mg) was dissolved in 15 mM aqueous AcOH (34.5 mL) by ultrasonication and heating and filtered through a 0.45 μm membrane filter (Millex-HV, Millipore). This amount of Homo-AMPA was resolved in 20 injections, each of 1.0−2.0 mL of solution. The collected fractions from each of the two peaks were pooled and evaporated. These two residues were re-evaporated twice from water and dried *in vacuo*. The pooled and dried chiral HPLC fractions of the first peak ($t_R = 10$ min, 81.6 mg, corresponding to 94% yield) with an enantiomeric purity of 99.5% were dissolved in H_2O, filtered, evaporated, and recrystallized in H_2O/EtOH to give 69.3 mg of **7** (80%) with an ee of 99.9%: mp 211−212 °C dec; $[\alpha]^{25}_{D} = -17.4°$, $[\alpha]^{25}_{365} = -61.1°$ ($c = 0.44$, H_2O); IR 3280 (m), 3100−2800 (multiple, m), 1585 (s), 1510 (s), 1460 (s), 1400 (s), 1290 (s) cm^{-1}. Anal. ($C_8H_{12}N_2O_4$) H, N; C: calcd, 48.00; found, 48.61.

Recrystallization (H_2O/EtOH) of the mother liquor furnished an additional 7.8 mg of **7** (total yield: 89%) with an ee of 99.4%: mp 210−211 °C dec; IR spectrum identical with that of the first crop of crystals.

(*S*)-2-Amino-4-(3-hydroxy-5-methyl-4-isoxazolyl)butyric Acid [(*S*)-(+)-Homo-AMPA, 6]. The pooled and dried chiral HPLC fractions of the second peak from the separation procedure described above for **7** ($t_R = 16$ min, 81.5 mg, corresponding to 94% yield) with an enantiomeric purity of 99.5% were dissolved in H_2O, filtered, and evaporated. Recrystallization (H_2O/EtOH) gave 63.1 mg of **6** (73%) with an ee of 99.7%: mp 211−212 °C dec; $[\alpha]^{25}_{D} = +17.4°$, $[\alpha]^{25}_{365} = +61.0°$ ($c = 0.41$, H_2O), $[\alpha]^{23}_{D} = +29.1°$, $[\alpha]^{23}_{365} = +103°$ ($c = 0.37$, 0.1 M HCl); ^{1}H NMR (D_2O) δ 2.0 (2H, m), 2.22 (3H, s), 2.36 (2H, m), 3.67 (1H, t, $J = 6.4$ Hz); IR spectrum identical with that of **7**.

The mother liquor furnished after recrystallization (H_2O/EtOH) an additional 9.2 mg of **6** (total yield: 84%) with an ee of 99.0%: mp 208−210 °C dec; IR spectrum identical with that of the first crop of crystals of **6** and with that of **7**. The solution used for optical rotation in 0.1 M HCl was evaporated once and recrystallized from AcOH to give 2.9 mg of **6**·HCl. The IR spectrum was identical with that of **6**·HCl obtained after deprotection of compound **13**.

Cell Culture. The Chinese hamster ovary (CHO) cell lines expressing $mGlu_{1\alpha}$, $mGlu_2$, $mGlu_3$, $mGlu_{4a}$, $mGlu_5$, $mGlu_6$, and $mGlu_7$ were maintained as described previously.[29−34] The cell lines were grown in a humidified 5% CO_2/95% air atmosphere at 37 °C in DMEM containing a reduced concentration of (*S*)-glutamine (2 mM), 1% proline, penicillin (100 U/mL), streptomycin (100 mg/mL), and 10% dialyzed fetal calf serum (all GIBCO, Paisley, Scotland). Two days before assay 1.8×10^6 cells were divided into the wells of 24-well plates.

Measurement of PI Hydrolysis and Cyclic AMP Formation. PI hydrolysis was essentially measured as described previously.[23−25] Briefly, the cells were labeled with [^{3}H]inositol (2 μCi/mL) 24 h prior to the assay. For agonist assay, the cells were incubated with ligand dissolved in PBS−LiCl for 20 min, and agonist activity was determined by measurement of ^{3}H-labeled mono-, bis-, and tris-inositol phosphates by ion exchange chromatography. For antagonist assay, the cells were preincubated with the ligand dissolved in PBS−LiCl for 20 min prior to incubation with ligand and 30 μM (*S*)-Glu (**1**). The antagonist activity was then determined as the inhibitory effect of the (*S*)-Glu (**1**)-mediated response. The assay of cyclic AMP formation was performed as described previously.[23−25] Briefly, the cells were incubated for 10 min in PBS containing the ligand, 10 μM forskolin, and 1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma Chemicals, St. Louis, MO). The agonist activity was then determined as the inhibitory effect of the forskolin-induced cyclic AMP formation. For antagonist assay, the cells were preincubated with ligand dissolved in PBS containing 1 mM IBMX for 20 min prior to a 10 min incubation in PBS containing the ligand, 30 μM (*S*)-Glu (**1**) [in the case of $mGlu_7$, 1 mM (*S*)-AP4 was used], 10 μM forskolin, and 1 mM IBMX. Cyclic AMP levels were determined by use of a RIA assay (Amersham). All experiments were performed at least twice in triplicate.

Electrophysiology *in Vitro*. A rat cortical wedge preparation[26] was used for the determination of the depolarizing effects of the EAA amino acid analogues under study, as described previously.[12]

Acknowledgment. This work was supported by a postdoctoral grant to H.B.-O. from H. Lundbeck A/S, by the Lundbeck Foundation, and by the Danish State Biotechnology Programme (1991−1995). The collabora-

tion with Dr. S. E. Harnung and Mrs. K. Jørgensen, Department of Chemistry, University of Copenhagen, on the recording and interpretation of the CD spectra, the secretarial assistance of Mrs. Anne Nordly, and the technical assistance of Heidi Petersen are gratefully acknowledged.

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JM9703597