Selective Antagonists at Group I Metabotropic Glutamate Receptors: Synthesis and Molecular Pharmacology of 4-Aryl-3-isoxazolol Amino Acids

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Homologation of (*S*)-glutamic acid (Glu, **1**) and Glu analogues has previously provided ligands with activity at metabotropic Glu receptors (mGluRs). The homologue of ibotenic acid (**7**), 2-amino-3-(3-hydroxy-5-isoxazolyl)propionic acid (HIBO, **8**), and the 4-phenyl derivative of **8**, compound **9a**, are both antagonists at group I mGluRs. Here we report the synthesis and molecular pharmacology of HIBO analogues **9b**−**h** containing different 4-aryl substituents. All of these compounds possess antagonist activity at group I mGluRs but are inactive at group II and III mGluRs.

Introduction

The central excitatory neurotransmitter (*S*)-glutamic acid (Glu, **1**) (Chart 1) operates through two classes of receptors in the central nervous system (CNS): the ionotropic receptors (iGluRs) and the metabotropic receptors (mGluRs). iGluRs are ligand-gated ion channels and are subdivided into *N*-methyl-D-aspartic acid (NMDA), (*RS*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA), and kainic acid receptors.[1,2] mGluRs belong to the family of G-protein coupled receptors. Currently eight mGluR subtypes are known, which have been classified into three groups on the basis of similarities in pharmacology, sequence homology of the receptor proteins, and second messenger pathways. Group I mGluRs include mGluR1 and mGluR5; group II mGluRs comprise mGluR2 and mGluR3; and mGluR4, mGluR6, mGluR7, and mGluR8 constitute group III.[2,3] It is generally agreed that both iGluRs and mGluRs play important roles in the healthy as well as the diseased CNS and that all subtypes of these receptors are potential therapeutic targets.[4,5] Thus, the design of subtype-selective receptor ligands is of great interest and is the first step in the development of drugs selective for subtypes of iGluRs and mGluRs.

A number of heterocyclic analogues of **1**, notably quisqualic acid (**2**) and (*S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid [(*S*)-AMPA, **3a**], are very potent AMPA receptor agonists, **3a** being highly selective for this type of GluR.[2] A large number of analogues of **3a** have been synthesized and pharmacologically characterized,[2] including (*S*)-2-amino-3-(3-hydroxy-5-phenyl-4-isoxazolyl)propionic acid [(*S*)-APPA, **3b**][6] and (*S*)-2-amino-3-[3-hydroxy-5-(2-pyridyl)-4-isoxazolyl)propionic acid [(*S*)-2-Py-AMPA, **3c**].[7] Whereas **3c** is approximately equipotent with **3a** as an AMPA receptor agonist,[7] the isomeric 3′- and 4′-pyridyl analogues are essentially inactive.[8]

We have previously shown that homologation of **1** as well as of its heterocyclic analogues provided with potent iGluR agonist activity, such as **2** and **3a**, results in compounds interacting predominantly with mGluRs. Thus, (*S*)-aminoadipic acid (**4**) and compound **5**, which is the homologue of the nonselective iGluR agonist **2**, become nonselective mGluR ligands,[9] and (*S*)-homo-AMPA (**6**), derived from the specific AMPA agonist **3a**, turns out to be a specific mGluR6 agonist.[10,11] (*S*)-2-

Chart 1

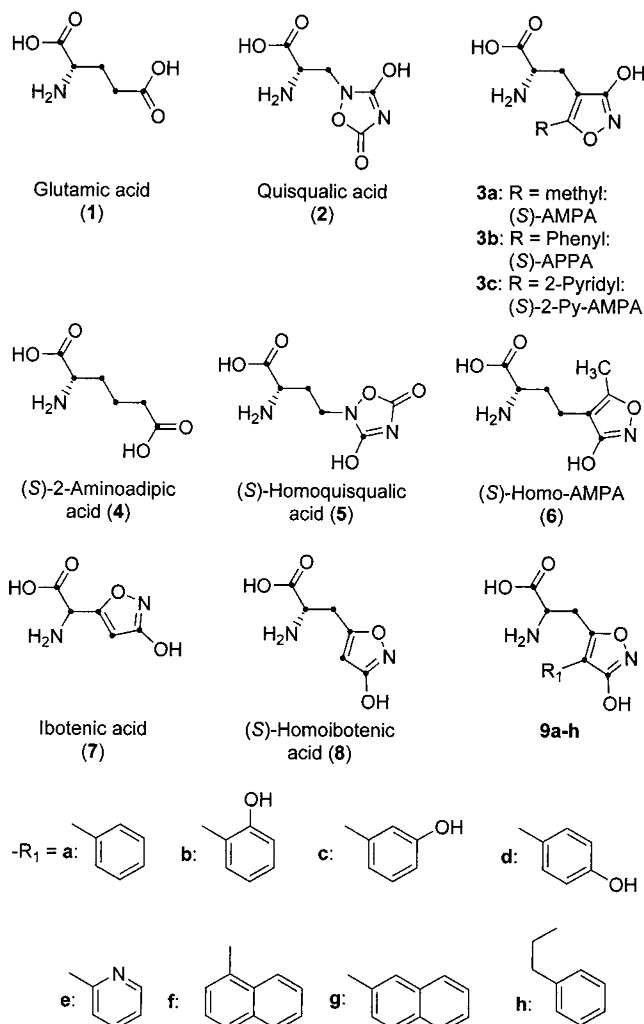

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Table 1. Receptor Binding Affinities, Agonist or Antagonist Effects in the Cortical Wedge Model, and Antagonist Effects at Group I–III mGluR (pIC_{50}, pEC_{50}, and pK_i Values Are ± SEM, $n = 3-4$)

	receptor binding			electropharmacology		cellular pharmacology			
	IC_{50} (μM)/[pIC_{50}]			EC_{50} (μM)/[pEC_{50}]	IC_{50} (μM)/[pIC_{50}]	K_i (μM)/[pK_i] antagonism			
compd	[^{3}H]CPP	[^{3}H]AMPA	[^{3}H]kainic acid	agonism	antagonism	mGluR1α	mGluR5a	mGluR4a	mGluR2
8	>100[a]	0.8[a]	>100[a]	329[a,b]		250[c]	490[c]	>1000[c]	>1000[c]
9a	>100[d]	>100[d]	>100[d]	590[b,d]		160[d]	nd	>1000[d]	>1000[d]
9b	>100	>100	>100	>1000[b]		125 [3.92 ± 0.08]	45 [4.40 ± 0.08]	>1000	>1000
9c	42 [4.41 ± 0.08]	62 [4.22 ± 0.07]	>100	990[b] [3.06 ± 0.10]		226 [3.65 ± 0.06]	nd	>1000	>1000
9d	20 [4.71 ± 0.07]	>100	>100		~1000[e]	71 [4.18 ± 0.12]	65 [4.20 ± 0.02]	>1000	>1000
9e	>100	1.3 [5.84 ± 0.07]	>100	23[b] [4.65 ± 0.06]		396 [3.40 ± 0.04]	224 [3.70 ± 0.08]	>1000	>1000
9f	>100	>100	>100		~1000[f]	119 [3.94 ± 0.08]	62 [4.20 ± 0.04]	>1000	>1000
9g	14 [4.87 ± 0.04]	>100	>100		715[e] [3.15 ± 0.03]	249 [3.60 ± 0.01]	53 [4.30 ± 0.07]	>1000	>1000
9h	>100	>100	>100		>1000	92 [4.10 ± 0.16]	70 [4.20 ± 0.04]	>1000	>1000

[a] Reference 12. [b] AMPA agonist. [c] Reference 15. [d] Reference 13. [e] NMDA antagonist. [f] AMPA and NMDA antagonist.

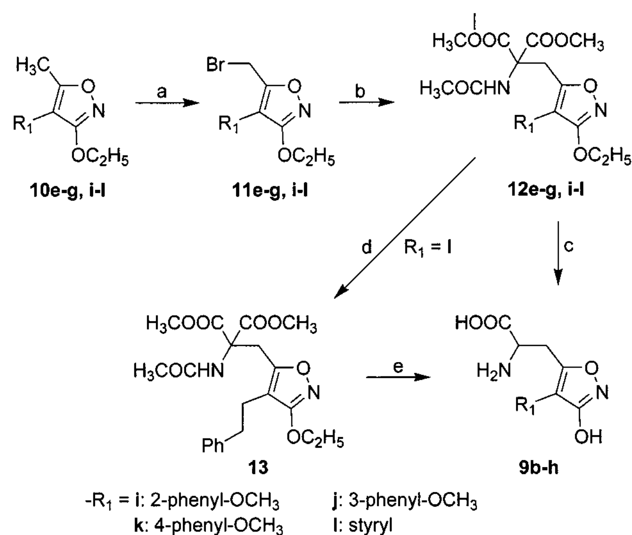
Amino-3-(3-hydroxy-5-isoxazolyl)propionic acid [(*S*)-HIBO, **8**], related to the nonselective iGluR agonist ibotenic acid (**7**), is a weak AMPA agonist and a group I mGluR antagonist.[12,13] In an attempt to eliminate the AMPA agonist effect of **8** and to optimize the mGluR antagonist effects, we have previously reported its 4-phenyl analogue **9a** which is an mGluR antagonist but almost devoid of effect at AMPA receptors (Table 1).[13] This observation prompted us to synthesize and pharmacologically characterize compounds **9b–h** containing aromatic substituents in the 4-position of the 3-isoxazolol ring of **8**.

Results

Chemistry. We have previously reported the palladium-catalyzed reaction of 3-ethoxy-4-iodo-5-methylisoxazole with a number of arylboronic acids or aryltributyltin analogues as a versatile way to synthesize 4-aryl or heteroaryl 3-alkoxy-5-methylisoxazoles.[14] In addition, Heck couplings using 3-ethoxy-4-iodo-5-methylisoxazole have proven useful in synthesizing 4-substituted 3-ethoxy-5-methylisoxazole. The bromination of compounds **10e–g**,**i–l** with *N*-bromosuccinimide (NBS) in the presence of benzoyl peroxide yielded **11e–g**,**i–l**. Compounds **11e–g**,**i–l** were reacted with dimethyl acetamidomalonate in the presence of NaH to give the fully protected compounds **12e–g**,**i–l** (Scheme 1). Reduction of the styryl doublebond of **12l** was performed at 1 atm and room temperature to obtain **13** (Scheme 1). Reflux of **12e–g**,**i–k** with aqueous HBr yielded the target compounds **9b–g** in the zwitterionic form. Compound **13** was refluxed in a 10 to 1 mixture of concentrated HCl and AcOH to give **9h**.

In Vitro Pharmacology. In this study we tested the effects of the synthesized HIBO analogues at mGluR1α, mGluR2, mGluR4a, and mGluR5a (Table 1), using established second messenger assay systems.[10] In agreement with previous studies on HIBO analogues,[13,15,16] compounds **9b–h** were shown to exhibit antagonist effect at the group I receptors, mGluR1α and mGluR5a, while no effect was observed at mGluR2 or mGluR4a (at 1 mM concentrations), representing group II and III receptors, respectively. The most potent compounds at mGluR1α proved to be **9d** and **9h** with K_i values of 71 μM and 92 μM, respectively, being 2–3 times more potent than the (*S*)-form of HIBO (**8**). When tested at mGluR5a, all new compounds but **9e** were shown to be 8–10 times more potent than **8**.

Scheme 1[a]



[a] (a) NBS, $(PhCO)_2O_2$; (b) $AcNHCH(COOCH_3)_2$, NaH; (c) 48% aq HBr; (d) H_2, 5% Pd/C; (e) 37% aq HCl, AcOH.

The synthesized 4-substituted HIBO analogues (**9b–h**) were studied in different receptor binding assays in order to determine their affinity for the iGluRs. The ligands [^{3}H]-(*RS*)-3-(2-carboxy-4-piperazinyl)propyl-1-phosphonic acid ([^{3}H]CPP),[17] [^{3}H]AMPA,[18] and [^{3}H]-kainic acid[19] were used to determine affinity for NMDA, AMPA, and kainic acid receptors, respectively (Table 1). None of the compounds showed detectable affinity toward kainic acid receptors. Compounds **9c**, **9d**, and **9g** possessed comparable although weak affinities in the [^{3}H]CPP binding assay. All other compounds were devoid of affinity toward [^{3}H]CPP labeled binding sites. Compound **9c** showed weak affinity toward AMPA receptors whereas **9e** was found to be a fairly potent inhibitor of [^{3}H]AMPA binding. The receptor binding studies were supported by in vitro electrophysiological experiments, using the rat cortical wedge model.[20,21] In accordance with the binding studies, **9e** proved to be an AMPA receptor agonist ($EC_{50} = 23$ μM). Compounds **9d** and **9g** showed weak NMDA antagonism (IC_{50} values > 1 mM and 715 μM, respectively) while compound **9f** proved to be a very weak NMDA and AMPA antagonist (IC_{50} > 1 mM). Compound **9h** was inactive when tested as an agonist (EC_{50} > 1000 μM) or an antagonist (IC_{50} > 1000 μM) at the iGluRs.

Discussion

All of the phenolyl analogues **9b**−**d** show antagonist effects at group I mGluRs, but whereas **9c**,**d** in addition to the mGluR effect also show effects at iGluRs (Table 1), compound **9b**, containing a 2-phenolyl substituent, does not interact detectably with iGluRs. Thus compound **9b** is a selective group I mGluR antagonist showing marginal preference for mGluR5. The presence of a hydroxy group in the 2′- or 4′-position in compounds **9b** and **9d** seems favorable compared to **9a** and **9c**. This may be due to the ability of the hydroxy group to function as a hydrogen bond acceptor and/or donor. The presence of a potential hydrogen bond acceptor in the same position of the aromatic substituent in **9e** does, on the other hand, not facilitate an antagonist effect at these receptors (Table 1). Compound **9e** is, however, a rather potent AMPA receptor agonist, analogous to the potent AMPA agonist activity observed in (*S*)-2-Py-AMPA (**3c**) containing the same heterocyclic substituent.[7] The activity data of **9b** and **9e** emphasize the different structural requirements necessary to activate AMPA receptors and to block group I mGluRs.

Compounds **9f** and **9g**, containing in the 4-position a 1′-naphthyl and 2′-naphthyl substituent, respectively, show additional weak iGluR antagonist effects (Table 1). The iGluR antagonist effects of **9f** and **9g** were eliminated by replacing the naphthyl moiety with the phenylethyl group to give **9h**.

In conclusion, using the nonselective iGluR and mGluR ligand HIBO (**8**) as a lead, we were able to develop compounds **9b**,**f**,**h** as selective group I mGluR antagonists, showing some (2−5-fold) preference for mGluR5 (Table 1). These compounds illustrate that group I mGluRs can accommodate 3-isoxazolol amino acid ligands containing relatively bulky and lipophilic substituents and may be useful in the attempts to develop therapeutically useful group I mGluR antagonists. These new compounds did not interact detectably with mGluR2 or mGluR4a as representatives for group II and group III mGluRs, respectively.

Experimental Section

Chemistry. 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 amino groups were visualized using a ninhydrin spraying reagent. ^1H, ^{13}C, and associated proton test (APT) spectra were recorded on a 300 MHz Varian Gemini spectrometer or a Bruker AC-200 F spectrometer, using $CDCl_3$ or D_2O/NaOD as the solvent. Chemical shifts are given in ppm (δ) using TMS or dioxane (δ 3.70/67.3) as the internal standard, and coupling constants (J) are given in hertz. Column chromatography (CC) was performed on Merck silica gel 60 (0.063−0.200 mm). Melting points were determined in open capillaries and are uncorrected. All solvents and reagents were obtained from Fluka or Aldrich and used without further purification, except DMF, which was stored over 3 Å molecular sieves. Compounds **10e**−**g**,**i**−**l** were prepared as previously described.[14] Elemental analyses were performed at the Analytical Research Department, H. Lundbeck A/S, Denmark, or by J. Theiner, Microanalytical Laboratory, Institute of Physical Chemistry, University of Vienna, Austria, and are within ±0.4% of the theoretical value unless otherwise stated.

General Procedure for the Bromination of 4-Substituted 3-Ethoxy-5-methylisoxazoles (11e−g,i−l). To a solution of compound **10e**−**g**,**i**−**l** (8.9 mmol) in CCl_4 (40 mL) was added NBS (1.7 g, 9.8 mmol) and dibenzoyl peroxide (50 mg). The reaction was heated at reflux for 6 h, followed by cooling and filtration. Concentration in vacuo gave the crude product that was purified by CC.

5-Bromomethyl-3-ethoxy-4-(2-methoxyphenyl)isoxazole (11i). CC (toluene, 1% AcOH) gave **11i** (63%) as a yellow oil. Recrystallization (diethyl ether/hexane) of a small sample gave **11i** as colorless crystals: mp 48.5−50 °C; ^1H NMR ($CDCl_3$) δ 1.39 (t, 3H, J = 7.0 Hz), 3.85 (s, 3H), 4.35 (s, 2H), 4.36 (dd, 2H, J = 7.0 Hz), 6.99 (d, 1H, J = 8.2 Hz), 7.04 (dt, 1H, J = 7.6 Hz, J = 1.1 Hz), 7.32−7.42 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.46, 19.80, 55.43, 65.91, 105.85, 111.25, 116.29, 120.78, 130.08, 131.26, 156.99, 163.96, 169.78. Anal. ($C_{13}H_{14}NO_3Br$) C, H, Br, N.

General Procedure for the Preparation of Methyl 2-Acetamido-2-(methoxycarbonyl)-3-(4-aryl-3-ethoxy-5-isoxazolyl)propionates (12e−g,i−l). Dimethyl acetamidomalonate (4.2 mmol) was added in small portions to a suspension of NaH (60% suspension in mineral oil) (4.2 mmol) in DMF (10 mL). The mixture was stirred at room temperature for 30 min. A solution of **11e**−**g**,**i**−**l** (4.2 mmol) in DMF (10 mL) was added, and the reaction was stirred overnight at room temperature. The mixture was evaporated, and H_2O was added and extracted with diethyl ether. Drying ($MgSO_4$) of the organic phase, filtration, and concentration in vacuo gave the crude product that was purified by CC.

Methyl 2-Acetamido-2-(methoxycarbonyl)-3-[3-ethoxy-4-(2-methoxyphenyl)-5-isoxazolyl]propionate (12i). CC (toluene/EtOAc 9:1, 1% AcOH) gave **12i** as colorless crystals. Recrystallization (EtOAc/hexane) gave **12i** (83%) as colorless crystals: mp 115−116 °C; ^1H NMR ($CDCl_3$) δ 1.36 (t, 3H, J = 7.1 Hz), 1.60 (s, 3H), 3.66 (s, 6H), 3.83 (s, 3H), 3.86 (s, 2H), 4.30 (dd, 2H, J = 7.1 Hz), 6.55 (bs, 1H), 6.95 (d, 1H, J = 8.3 Hz), 6.99 (t, 1H, J = 7.4 Hz), 7.24 (dd, 1H, J = 7.4 Hz, J = 1.8 Hz), 7.32 (dt, 1H, J = 7.4 Hz, J = 1.8 Hz); ^{13}C NMR ($CDCl_3$) δ 14.42, 22.03, 30.73, 53.49, 55.32, 64.55, 65.61, 106.33, 111.31, 116.64, 120.55, 129.61, 131.44, 150.66, 164.64, 167.44, 169.28, 169.73. Anal. ($C_{20}H_{24}N_2O_8$) C, H, N.

Methyl 2-Acetamido-2-(methoxycarbonyl)-3-(3-ethoxy-4-phenylethyl-5-isoxazolyl)propionate (13). To a solution of compound **12l** (1.93 mmol) in absolute ethanol (20 mL) was added 5% Pd/C (20 mg). The reaction was left under an atmosphere of H_2 for 3 h at room temperature, followed by filtration through a pad of Celite and concentration in vacuo. CC (toluene/EtOAc 9:1) gave **13** (93%) as colorless crystals: mp 101−102 °C; ^1H NMR ($CDCl_3$) δ 1.40 (t, 3H, J = 7.0 Hz), 1.88 (s, 3H), 2.45 (t, 2H, J = 6.9 Hz), 2.72 (t, 2H, J = 6.9 Hz), 3.53 (s, 2H), 3.81 (s, 6H), 4.25 (dd, 2H, J = 7.0 Hz), 5.76 (bs, 1H), 7.09−7.26 (m, 5H). ^{13}C NMR ($CDCl_3$) δ 9.91, 18.72, 25.49, 31.82, 52.03, 63.82, 64.55, 110.06, 129.76, 132.19, 132.21, 145.47, 169.57, 173.99, 176.35, 177.67. Anal. ($C_{21}H_{26}N_2O_7$) C, H, N.

General Procedure for the Preparation of Amino Acid 9b−g. Compound **12e**−**g**,**i**−**k** refluxed for 3 h in aqueous HBr (48%). The reaction mixture was cooled and evaporated. The residue was dissolved in H_2O and evaporated three times.

(*RS*)-2-Amino-3-[3-hydroxy-4-(2-phenolyl)-5-isoxazolyl]-propionic Acid (9b). Compound **12i** (3.2 mmol) was refluxed for 3 h in aqueous HBr (48%, 20 mL). The reaction mixture was cooled and evaporated. The residue was dissolved in H_2O and evaporated three times. The residue was washed several times with hot EtOAc and H_2O giving colorless crystals. Recrystallization (water) gave **9b** (22%) as colorless crystals: mp 233−235 °C dec; ^1H NMR (D_2O/NaOD) δ 2.48 (dd, 1H, J = 15.0 Hz, J = 8.3 Hz), 2.69 (dd, 1H, J = 15.0 Hz, J = 4.9 Hz), 3.24 (dd, 1H, J = 8.3 Hz, J = 4.9 Hz), 6.85−6.93 (m, 2H), 7.05 (dd, 1H, J = 7.5 Hz, J = 1.8 Hz), 7.21 (ddd, 1H, J = 8.3 Hz, J = 7.5 Hz, J = 1.8 Hz). Anal. ($C_{12}H_{12}N_2O$) C, N; H: calcd, 4.58; found, H, 5.01.

(*RS*)-2-Amino-3-(3-hydroxy-4-phenylethyl-5-isoxazolyl)-propionic Acid (9h). Compound **13** (1.9 mmol) was refluxed overnight in HCl (37%, 10 mL) and AcOH (1 mL). Subsequently the mixture was evaporated to dryness, and the residue was washed several times with hot EtOAc and H_2O giving compound **9h** (70%) as colorless crystals. Recrystallization (water) of a small sample gave **9h** as colorless crystals: mp 205−207 °C (dec); ^1H NMR (D_2O/NaOD) δ 2.06 (dd, 1H, J = 15.0 Hz, J = 8.4 Hz), 2.24 (dd, 1H, J = 15.0 Hz, J = 5.1 Hz), 2.33 (dt, 2H, J = 6.6 Hz, J = 2.7 Hz) 2.61 (dt, 2H, J = 6.6 Hz, J = 2.7 Hz), 2.92 (dd, 1H, J = 8.4 Hz, J = 5.1 Hz), 6.99−

7.16 (m, 5H); ^{13}C NMR (D_2O/NaOD) δ 23.89, 31.92, 34.97, 55.02, 108.85, 126.89, 129.31, 128.96, 142.83, 166.56, 178.57, 182.24. Anal. ($C_{14}H_{16}N_2O_4$) C, H, N.

Cell Culture. The Chinese hamster ovary (CHO) cell line expressing mGluR1α, mGluR2, mGluR4, and mGluR5a were maintained as described previously.[22–24] 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 (100 mg/mL) and 10% dialyzed fetal calf serum (all GIBCO, Paisley, Scotland). Two days before the inositol phosphate assay, 1.8×10^6 cells were divided into the wells of 48-well plates; and 2 days before the cyclic AMP-assay, 1.0×10^6 cells were divided into the wells of 96-well plates.

Measurement of PI Hydrolysis and Cyclic AMP Formation. The mGluR subtypes mGluR1α, mGluR2, mGluR4a, and mGluR5a were expressed in CHO cell lines. All compounds were tested for agonist and antagonist activity at 1 mM concentrations unless otherwise stated, by the method previously described.[15] K_i values were calculated from IC_{50} values by use of the Cheng–Prusoff equation.[25]

Receptor Binding Assays. Affinities for NMDA, AMPA, and kainic acid receptors were determined using [^{3}H]CPP,[17] [^{3}H]AMPA,[18] and [^{3}H]kainic acid[19] with the modifications previously described.[6] The membrane preparation used in all the receptor binding experiments were prepared according to the method described by Ransom and Stec.[26] The amount of bound radioactivity was determined using a Packard TOP-COUNT microplate scintillation counter. Data were analyzed using Grafit 3.0 Leatherbarrow software. Data were fitted to the equation, $B = 100 - (100 \times [\text{inhibitor}]^n)/(IC_{50}{}^n + [\text{inhibitor}]^n)$, where B is the binding as a percentage of total specific binding and n the Hill coefficient.

In Vitro Electrophysiology. A rat cortical preparation[20] in a modified version[21] was used for the determination of the depolarizing effects of the excitatory amino acid analogues under study. Agonists were applied for 90 s. Receptor selectivity was determined by antagonizing responses, approximately corresponding to the EC_{50} values of the compounds in question, with 5 μM CPP or 5 μM NBQX for NMDA and AMPA receptors, respectively. Antagonists were applied for 90 s, followed by a coapplication of agonist and antagonist for 90 s. Data were fitted to the equation, % response $= (E_{max} \times [\text{agonist}]^n)/(EC_{50}{}^n + [\text{agonist}]^n)$, where E_{max} is the relative maximal response and n is the Hill coefficient.

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Supporting Information Available: Detailed information on the synthesis and characterization of **9b–g**, **11e–g,j–l**, and **12e–g,j–l**. This material is available free of charge via the Internet at http://pubs.acs.org.

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