Chemo-Enzymatic Synthesis of (2*S*,4*R*)-2-Amino-4-(3-(2,2-diphenylethylamino)-3-oxopropyl)pentanedioic Acid: A Novel Selective Inhibitor of Human Excitatory Amino Acid Transporter Subtype 2

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In the mammalian central nervous system (CNS), the action of sodium dependent excitatory amino acid transporters (EAATs) is responsible for termination of glutamatergic neurotransmission by reuptake of (*S*)-glutamate (Glu) from the synaptic cleft. Five EAAT subtypes have been identified, of which EAAT1−4 are present in the CNS, while EAAT5 is localized exclusively in the retina. In this study, we have used an enantioselective chemo-enzymatic strategy to synthesize 10 new Glu analogues **2a**−**k** (**2d** is exempt) with different functionalities in the 4*R*-position and characterized their pharmacological properties at the human EAAT1−3. In particular, one compound, **2k**, displayed a significant preference as inhibitor of the EAAT2 subtype over EAAT1,3. The compound also displayed very low affinities toward ionotropic and metabotropic Glu receptors, making it the most selective EAAT2 inhibitor described so far.

Introduction

In the mammalian central nervous system (CNS) glutamatergic neurotransmission is terminated by reuptake of (*S*)-glutamate (Glu) from the synaptic cleft by the action of a family of sodium dependent excitatory amino acid transporters (EAATs).[1,2] To date, five transporter subtypes have been identified, of which four, EAAT1−4, are present in the mammalian CNS while EAAT5 is localized exclusively in the retina.[a] EAAT1−3 are localized on neurons and/or in astroglial cells and exhibit high capacity for transporting Glu across the membrane, whereas EAAT4 and EAAT5 predominately function as Glu-gated chloride channels.[1] In the healthy CNS, activation of Glu receptors is involved in important neurophysiological processes underlying memory and learning, motor functions, and neural plasticity and development.[3] However, under conditions of metabolic stress and oxygen deprivation, Glu is a neurotoxic agent. Thus, it is believed that neurodegenerative diseases such as Alzheimer's disease, dementia, Huntington's disease, amyotrophic lateral sclerosis, epilepsy, and cerebral stroke may be related to disordered glutamatergic neurotransmission originating from dysfunction of either the ionotropic Glu receptors (iGluR), the metabotropic Glu receptors (mGluR), or the Glu reuptake system (EAATs).[3,4]

In several recent studies, we have explored the structure−activity relationship (SAR) of ligands targeting human EAATs, with the ultimate goal of identifying new EAAT subtype selective inhibitors and substrates.[5−8] In this paper, we present the pharmacological evaluation of Glu analogues **2a**−**k** (**2b** is exempt) at human EAAT1−3 and the chemo-enzymatic synthetic strategy developed for the preparation of amide analogues **2 g**−**k**. Furthermore, a modeling study addressing the observed structure−activity relations is presented.

Chemistry

The enzyme aspartate aminotransferase (AAT), isolated from *E. coli*, may catalyze the stereoselective conversion of substituted α-ketoglutarates (KG) into their corresponding L-Glu analogues. Using this approach, we have previously prepared a series of L-2,4-*syn*-4-alkyl Glu analogues starting from their corresponding racemic KGs,[6] thus demonstrating the KG substrate tolerance and that AAT is highly enantioselective. In this paper, we show that this methodology can be extended to also cover a range of functionalized side chains in the 4-position of KG (Scheme 1).

Whereas the enantioselective preparation of Glu analogues **2a**−**f**, including their corresponding KG precursors, is reported elsewhere by us,[9] the synthesis of 4-amide functionalized KGs **1 g**−**k** is based on an original approach starting from the cyclohexanone **3** (Scheme 2). The well-known disubstituted cyclohexanone **3** was readily prepared from methyl malonate and methyl acrylate following a described four-step procedure involving a double Michael condensation and a Dieckmann reaction.[10] This cyclic β-keto ester was then converted in high yield to the enamines **4g**−**k** by treatment with ammonia or several primary amines. Oxidative cleavage by ozone of the various enamines **4g**−**k** afforded simultaneously the α-keto ester

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[a] Abbreviations: iGluR, ionotropic Glu receptors; mGluR, metabotropic Glu receptors; EAAT, excitatory amino acid transporter; KG, α-ketoglutarates; AAT, aspartate aminotransferase; DHK, dihydrokainic acid; TBOA, *threo*-benzyloxyaspartate; AMPA, 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid; KA, kainic acid; NMDA, *N*-methyl-D-aspartic acid; FMP, FLIPR membrane potential.

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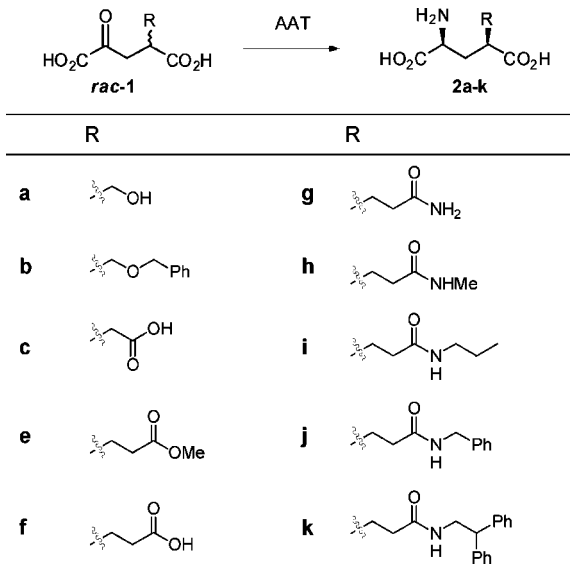
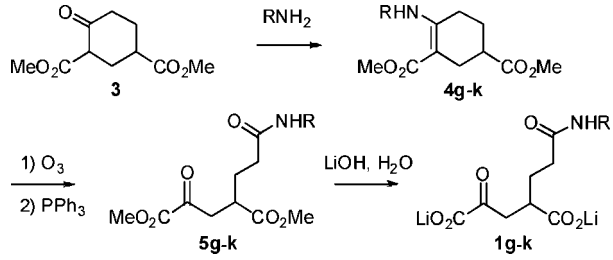
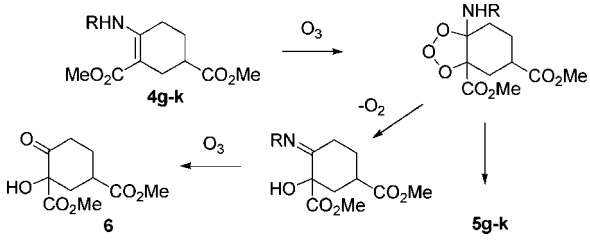
Scheme 1. AAT Catalyzed Synthesis of 4-Substituted Glu Analogues **2a**−**k**



Scheme 2. Synthesis of 4-Amide Functionalized KGs **1g**−**k**



Scheme 3. Oxidation of Enamines **4g**−**k**

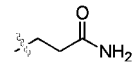
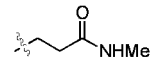
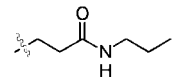
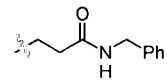
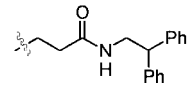


moiety and the amide functionality. The ketoglutarates **5g**−**k** were thus isolated with overall yields of 25−55% from **3**. This modest yield resulted from a competitive oxidation reaction, giving, in every case, the 2-hydroxy cyclohexanone **6**, presumably via an elimination of O_2 from the primary ozonides formed from **4g**−**k** (Scheme 3). Despite this side reaction, the procedure described in Scheme 2 offers an easy and short route to a variety of amide-substituted KGs. Ester hydrolysis was finally done with a stoichiometric amount of LiOH and gave the desired KGs **1g**−**k** in quantitative yields.

The substituted KGs were evaluated as substrate for *E. coli* AAT on the basis of the Michaelis−Menten model. Table 1 summarizes the kinetic constants measured for the transamination reaction of **1g**−**k** in the presence of aspartic acid used as the amino donor in quasisaturating concentration (40 mM).

The K_m values measured with **1g**−**k** are in the mmol range, 1 to 2 orders of magnitude higher than that of the natural substrate (KG). This result contrasts with the observations made previously with the 4-alkyl derivatives, which showed very good affinities for the enzyme active site.[6] More interestingly,

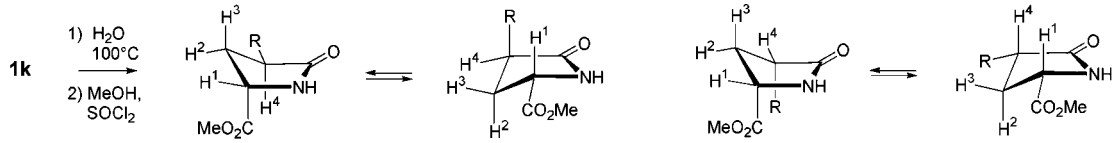
Table 1. Kinetic Parameters of AAT Catalyzed Transaminations of KG Analogues **1g**−**k**[a]

Substrate	R	K_m (mM)	k_{cat} rel.(%)
KG	--	0.23 ± 0.05	100[b] ± 3
1g		4.2 ± 0.3	24.6 ± 3.2
1h		4.6 ± 1.2	10.3 ± 1.7
1i		14.5 ± 0.8	7.6 ± 0.6
1j		2.9 ± 0.5	15.0 ± 2.2
1k		2.3 ± 0.1	10.7 ± 1.2

[a] Values and standard errors were calculated from the Hanes−Woolf plot according to the least-squares method and Gauss's error propagation law. [b] The absolute k_{cat} value measured in our experimental conditions was 39.6 ± 1.2 s^{-1}.

however, the k_{cat} measured with **1g**−**k** are all close to 10% of that of KG thus offering the opportunity of synthetic applications. Preparative scale transaminations were carried out as previously described, by an equilibrium shifted reaction using cysteine sulfinic acid (CSA) as the amino donor substrate: during the reaction, CSA is converted into the very unstable pyruvyl sulfinic acid that decomposes into sulfur dioxide and pyruvic acid, which is not a substrate for AAT. The transamination reaction was monitored by enzymatic titration of pyruvic acid using lactate dehydrogenase and NADH. When a conversion rate around 40% was reached, the reaction was stopped to achieve the kinetic resolution of the keto acid substrates. Glu analogues were selectively adsorbed on a short column of sulfonic resin (H^+ form) and then eluted with aqueous ammonia. Finally, ion exchange chromatography on cationic dowex 1 resin (AcO^- form) afforded **2g**−**k**, each compound being isolated as a single stereomer in 35−46% yield. AAT was previously shown to display a very high stereoselectivity in favor of the L-2,4-*syn*-4-alkyl Glu derivatives.[6,11] The L-2,4-*syn* configuration was thus expected for the new Glu analogues **2g**−**k**. The configuration was demonstrated, as an example, in the case of the amide **2k**: an aqueous solution of this compound was heated to form a lactame which was then converted into the ester **7** in the presence of methanol and thionyl chloride. NMR analysis of this pyro-Glu derivative confirmed the expected *trans* configuration (Scheme 4): the measured coupling constants are in agreement with the expected preferred conformation of (3*S*,5*S*)-**7**. On the contrary, these data are inconsistent with the (3*R*,5*S*)-*cis* configuration for which H^2 (and H^3) would present close values of coupling constants with H^1 and H^4. These experiments confirmed previous findings with the 4-alkyl-Glu derivatives and were considered to be demonstrative of the general

Scheme 4. Synthesis and NMR Analysis of Lactam **7**



Protons interaction	*Model Coupling constant*[a] (Hz): (3*S*,5*S*)-7 **I**	(3*S*,5*S*)-7 **II**	(3*R*,5*S*)-7 **III**	(3*R*,5*S*)-7 **IV**	*Experimental Coupling constant*
H^1-H^2	2.1	9.2	1.6	9.9	3.5
H^1-H^3	9.2	7.7	8.7	7.2	9.0
H^2-H^4	8.8	9.2	1.3	10.1	8.8
H^3-H^4	8.6	1.6	8.9	7.8	8.6

[a] Coupling constants were estimated from the preferred calculated conformations **I**−**IV**.

stereoselectivity of AAT in favor of the L-2,4-*syn* configuration of Glu analogues substituted at position 4.

Pharmacology

The Glu analogues **2a**−**k** (**2d** is exempt) were characterized pharmacologically at human EAAT1, EAAT2, and EAAT3 stably expressed in HEK293 cells using the FLIPR membrane potential (FMP) assay (see Experimental Section for details). The assay was performed essentially as described previously,[12] and results are summarized in Table 2. All analogues were either inactive or displayed very weak activities as nonsubstrate inhibitors at EAAT1 and EAAT3. At EAAT2, compounds **2a**,**b**,**e**,**g**,**h**,**j**,**k** displayed weak-to-moderate inhibitory activities, whereas **2k**, which holds a bulky substituent on the amide nitrogen (*N*-2,2-diphenylethyl), displayed a significant preference for inhibition of EAAT2 over EAAT1 and EAAT3, with a midrange micromolar potency ($IC_{50} = 75\ \mu M$).

The Glu analogues **2a**−**k** were also characterized at the EAAT-HEK293 cell lines in a conventional [^{3}H]-D-Asp assay (see Experimental Section for details). The inhibition profiles exhibited by the compounds in this assay correlated well with the results from the FMP assay (Table 3). For example, **2k** displayed a 10−30 fold higher inhibitory potency at EAAT2 compared with EAAT1 and EAAT3.

In other studies, dihydrokainic acid (DHK, Table 2) has been shown to be a selective inhibitor of the EAAT2 transporter subtype in medium-range micromolar potency.[12,13] However, DHK also displays moderate affinity ($IC_{50} = 6\ \mu M$) for native kainic acid receptors as well as the cloned homomeric subtypes iGluR5−7 (Table 4).[13] On the other hand, the well-described EAAT inhibitor, L-*threo*-benzyloxyaspartate[13] (TBOA) displays very low affinity for the iGluRs (Table 4) but also no selectivity among the EAAT subtypes (Table 2).

These facts prompted us to investigate **2k** at native 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) receptors, kainic acid (KA, Table 2) receptors, and the *N*-methyl-D-aspartic acid (NMDA) receptors in a binding assay using rat brain homogenate. As summarized in Table 4, **2k** displayed low affinities ($K_i > 100\ \mu M$) for all three groups of native iGluRs. Given the fact that affinities for KA receptor subunits iGluR5−7 are poorly estimated in the [^{3}H]KA binding assay, we further investigated **2k**, and DHK, in binding assays at cloned rat iGluR5−7 subtypes. Whereas DHK displayed low micromolar affinity for iGluR5−7, compound **2k** showed only midrange micromolar affinity for iGluR5 and no affinity for receptor subtypes iGluR6 and iGluR7. We also characterized **2k** in a functional assay at the metabotropic Glu receptors (mGluRs) subtypes mGluR1 and mGluR5 (group I), mGluR2 (group II), and mGluR4 (group III).[9] In these assays, **2k** proved unable to agonize, antagonize, or modulate any of aforementioned mGluR subtypes. Furthermore, in binding assays at cloned human mGluR2 and mGluR3 subtypes, **2k** proved without affinity (both $IC_{50} > 1000\ \mu M$).

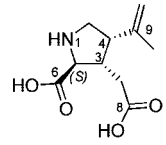
The EAAT2-Inhibitor Pharmacophore. On the basis of several SAR studies, we[5−8] and others[14−16] have proposed that EAAT substrates and inhibitors, which contain a Glu skeleton, bind in two distinct ways: while substrates bind to the transporter in an *extended* Glu conformation, the binding conformation of inhibitors is assumed to be equivalent to the *folded* Glu conformation previously identified as the agonist binding conformation at the iGluRs by X-ray crystallographic studies[17] and rational ligand design studies.[18]

To address the origin of the inhibitory preference for EAAT2 by **2k**, we submitted the compounds **2j**, **2k**, and DHK to a stochastic conformational search (see Experimental Section for details). The observed low-energy *folded* Glu conformations (up to +1 kcal/mol) of **2j**, **2k**, and DHK were superimposed by fitting the ammonium and the two carboxylate groups (Figure 1). Interestingly, the isopropyl group of DHK, which is believed to be the molecular feature determining the EAAT2 subtype selectivity of the compound,[5] does not occupy the same area in space as the bulky substituent on the amide nitrogen (*N*-2,2-diphenylethyl) of **2k**. This suggests that the EAAT2 preference displayed by **2k** arises from a different molecular mechanism than that of DHK. The lack of exact three-dimensional structural information of EAAT2 makes it impossible for us to point out the specific structural features of neither the EAAT2 protein nor the **2k** ligand, which underlie the EAAT2 selectivity profile of this novel compound.

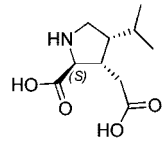
Conclusion

In conclusion, we have described a chemo-enzymatic enantioselective synthetic route toward compounds **2g**−**k** and presented the pharmacological characteristics of Glu analogues **2a**−**k** at human EAAT1−3. Notably, **2k** was found to be a novel inhibitor with significant preference for EAAT2 over EAAT1 and EAAT3. In comparison with the often used EAAT2-selective inhibitor, DHK, compound **2k** displays a significantly higher degree of selectivity toward EAAT2 over iGluRs and mGluRs. Thus **2k** may be a more useful pharmacological tool

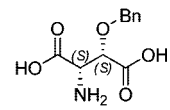
Table 2. Inhibition of Glu-Induced Depolarization in EAAT1-, EAAT2-, and EAAT3-HEK293 Cells in the FMP Assay[a]



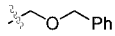
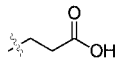
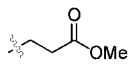
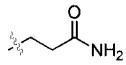
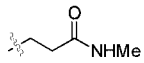
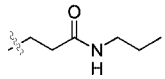
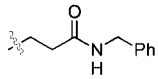
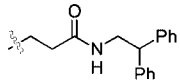
KA



DHK



L-TBOA

	R	EAAT1	EAAT2	EAAT3
Glu	H	**7.9 [5.1 ± 0.03]**	**21 [4.7 ± 0.02]**	**9.9 [5.0 ± 0.02]**
2a		~3000 [~ 2.5]	~300 [~ 3.5]	~3000 [~ 2.5]
2b		~1000 [~ 3]	~300 [~ 3.5]	~3000 [~ 2.5]
2c		>3000 [< 2.5]	>3000 [< 2.5]	>3000 [< 2.5]
2f		~3000 [~ 2.5]	~3000 [~ 2.5]	~3000 [~ 2.5]
2e		~1000 [~ 3]	~300 [~ 3.5]	>1000 [< 3]
2g		~3000 [~ 2.5]	~1000 [~ 3]	>3000 [~ 2.5]
2h		>1000 [< 3]	~1000 [~ 3]	>1000 [< 3]
2i		>3000 [< 2.5]	~3000 [~ 2.5]	~3000 [~ 2.5]
2j		~1000 [~ 3]	~300 [~ 3.5]	~1000 [~ 3]
2k		~1000 [~ 3]	75 [4.1 ± 0.05]	~1000 [~ 3]
DHK[b]	--	>3000 [< 2.5]	89 [4.1 ± 0.05]	>3000 [< 2.5]
DL-TBOA[b]	--	2.9 [5.5 ± 0.09]	2.2 [5.7 ± 0.07]	9.3 [5.0 ± 0.06]

[a] The IC_{50} values of the nonsubstrate inhibitors were obtained using $EC_{80}-EC_{90}$ concentrations of Glu at the three EAAT subtypes. The K_m values for substrates (bold) and IC_{50} values for inhibitors, are given in μM (with pK_m ± SEM and pIC_{50} ± SEM values in brackets, respectively). [b] Values taken from ref 12.

for the investigation of the physiological roles governed by the EAAT2. The design and synthesis of analogues of **2k**, which may improve potency, is currently in progress in our laboratories.

Experimental Section

Chemistry. Melting points were determined on a Reichert hot-stage apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 801 spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer. Chemical shifts are reported in ppm (δ) relative to TMS as internal standard. HRMS were recorded on a q-tof Micromass spectrometer. Optical rotations were determined with a JASCO DIP 370 polarimeter and are reported at the sodium D line (589 nm). Elemental analyses were performed at the Service Central d'Analyze du CNRS, Solaize, France. Silicagel 60 (Merck, 40−63 μm) and precoated F_{254} plates were used for column and TLC chromatography. All solvents were purified by distillation following usual procedures. Cysteine sulfinic acid was prepared from cystine following a described procedure.[19] Bovine heart malic dehydrogenase and rabbit muscle lactic dehydrogenase were purchased from Sigma. *Escherichia. coli* AAT was produced and purified following described procedures from overexpressing *E. coli* strains JM103 transformed with pUC119-*aspC* (AAT).[20] Enzyme kinetic measurements were performed at 25 °C in 0.1 M potassium phosphate buffer, pH 7.6, Asp (40 mM), NADH (0.2 mM), ketoacid substrate (0.1−10 mM), AAT (0.05 UI), and malic dehydrogenase (2 UI) in a total volume of 1 mL. Rates were calculated from the OD linear decay at 340 nm using $\epsilon_{NADH} = 6220\ cm^{-1}\cdot M^{-1}$.

Dimethyl 4-Aminocyclohex-3-ene-1-3-dicarboxylate 4g. To a solution of **3**[10] (1 g, 4.6 mmol) in MeOH (5 mL), was added a 20% aqueous solution of NH_3 (1.3 mL, 13.8 mmol). The reaction mixture was stirred at room temperature for 48 h. After addition of brine (10 mL), the aqueous solution was extracted with AcOEt (3 × 10 mL). The combined organic layers were dried over $MgSO_4$

Table 3. Inhibition of [^{3}H]-D-Asp Uptake in EAAT1-, EAAT2-, and EAAT3-HEK293 Cells[a]

	EAAT1	EAAT2	EAAT3
Glu	35 [4.5 ± 0.04]	62 [4.2 ± 0.03]	51 [4.3 ± 0.02]
2a	~3000 [~2.5]	140 [3.9 ± 0.05]	~3000 [~2.5]
2b	450 [3.3 ± 0.05][b]	290 [3.5 ± 0.03][b]	1000−3000 [2.5−3]
2c	>3000 [<2.5]	>3000 [<2.5]	>3000 [<2.5]
2f	>3000 [<2.5]	>3000 [<2.5]	>3000 [<2.5]
2e	1000−3000 [2.5−3]	180 [3.8 ± 0.04]	~3000 [~2.5]
2g	>3000 [<2.5]	290 [3.5 ± 0.04][b]	>3000 [<2.5]
2h	>3000 [<2.5]	680 [3.2 ± 0.05][b]	>3000 [<2.5]
2i	>3000 [<2.5]	1000−3000 [2.5−3]	>3000 [<2.5]
2j	~1000 [~3]	410 [3.4 ± 0.05][b]	~3000 [~2.5]
2k	1000−3000 [2.5−3]	95 [4.0 ± 0.04]	~3000 [~2.5]

[a] The K_i values are given in μM with pK_i ± SEM values in brackets. [b] The specific [^{3}H]-D-Asp uptake at the EAAT was not completely inhibited by the maximal concentration of the compound (3 mM), and thus the K_i value is determined from the fit of the concentration−inhibition curve for the compound.

and concentrated under reduced pressure. **4g** was isolated as a white solid (0.98 g) and used in the next reaction without further purification; mp 56 °C (lit.[21] mp 58−60.5 °C). ^{1}H NMR (400 MHz, D_2O) δ 7.5−4.5 (2H, s, broad), 3.69 (3H, s), 3.68 (3H, s), 2.66 (1H, dd, J = 5.1 and 15.6 Hz), 2.52 (1H, tdd, J = 2.9, 5.3 and 10.3 Hz) 2.40−2.30 (2H, m), 2.25 (1H, ddd, J = 3.6, 5.7 and 16.8 Hz), 2.00 (1H, m), 1.74 (1H, tdd, J = 5.8, 10.9 and 13.0 Hz). ^{13}C NMR (100 MHz, D_2O) δ 175.8, 170.2, 155.9, 89.9, 51.7, 50.4, 39.4, 29.4, 25.9, 24.2.

General Procedure for the Synthesis of 4h−k. To a solution of **3** (1 g, 4.6 mmol) in anhydrous toluene (4 mL) was added 4 Å molecular sieves (1 g) and the amine (6.9 mmol). For **4h** synthesis, a 2 M solution of $MeNH_2$ in THF was used. In the case of **4k** synthesis, 4.6 mmol of $Ph_2CHCH_2NH_2$ and 40 mL of toluene were used. The reaction mixture was stirred at room temperature for 48 h. After filtration, the solution was concentrated under reduced pressure. Compounds **4h−k** were isolated as white solids (**4h** and **4k**) or as colorless liquids (**4i** and **4j**) and were used in the next reaction without further purification. In every case, ^{1}H NMR analysis indicated a yield over 90%.

Dimethyl 4-Methylaminocyclohex-3-ene-1-3-dicarboxylate 4h. Melting point 68−70 °C. ^{1}H NMR (400 MHz, D_2O) δ 8.76 (1H, s, broad), 3.61 (3H, s), 3.57 (3H, s), 2.79 (3H, d, J = 5.2 Hz), 2.66 (1H, dd, J = 4.5 and 15.0 Hz), 2.50−2.15 (4H, m), 1.98 (1H, m), 1.65 (1H, m). ^{13}C NMR (100 MHz, D_2O) δ 175.6, 170.4, 159.1, 87.4, 51.6, 50.1, 39.2, 29.0, 29.4, 26.4, 25.2, 24.2.

Dimethyl 4-Propylaminocyclohex-3-ene-1-3-dicarboxylate 4i. ^{1}H NMR (400 MHz, D_2O) δ 8.93 (1H, s, broad), 3.69 (3H, s), 3.66 (3H, s), 3.11 (2H, m), 2.68 (1H, dd, J = 4.8 and 15.0 Hz), 2.56−2.24 (4H, m), 2.05 (1H, m), 1.70 (1H, tdd, J = 5.8, 11.0 and 13.1 Hz), 1.58 (2H, hex, J = 7.2 Hz), 0.97 (3H, t, J = 7.3 Hz). ^{13}C NMR (100 MHz, D_2O) δ 175.7, 170.8, 158.7, 87.1, 51.7, 50.3, 44.1, 39.3, 26.4, 25.6, 24.3, 23.5, 11.5.

Dimethyl 4-Benzylaminocyclohex-3-ene-1-3-dicarboxylate 4j. ^{1}H NMR (400 MHz, D_2O) δ 9.31 (1H, s, broad), 7.35−7.10 (5H, m), 4.37 (2H, m), 3.66 (6H, s), 2.68 (1H, dd, J = 4.4 and 14.8 Hz), 2.55−2.35 (3H, m), 2.24 (1H, ddd, J = 6.3, 10.8 and 17.5 Hz), 1.98 (1H, m), 1.66 (1H, tdd, J = 5.8, 10.9 and 13.1 Hz). ^{13}C NMR (100 MHz, D_2O) δ 175.6, 170.7, 158.4, 139.1, 128.8, 127.2, 126.7, 88.7, 51.7, 50.5, 46.2, 39.3, 26.4, 25.4, 24.3.

Dimethyl 4-(2,2-Diphenylethylamino)cyclohex-3-ene-1-3-dicarboxylate 4k. Melting point 145 °C. ^{1}H NMR (400 MHz, D_2O) δ 8.83 (1H, t, J = 5.0 Hz), 7.25−7.10 (10H, m), 4.06 (1H, t, J = 7.2 Hz), 3.71 (2H, m), 3.60 (3H, s), 3.51 (3H, s), 2.56 (1H, dd, J = 4.2 and 14.7 Hz), 2.40−2.15 (3H, m), 2.08 (1H, m), 1.90 (1H, m), 1.55 (1H, m). ^{13}C NMR (100 MHz, D_2O) δ 175.7, 170.4, 157.7, 141.9, 128.7, 128.1, 126.8, 88.2, 52.1, 51.7, 50.4, 47.4, 39.2, 26.5, 25.7, 24.3.

General ozonolysis procedure for the synthesis of 5g−k. A solution of enamine **4g−k** (approximately 4 mmol) in anhydrous CH_2Cl_2 (40 mL) was treated at −70 °C with a mixture of O_2 and O_3 at a rate of 10 L/h until saturation (blue coloration of the solution). After 30 min, the excess ozone was eliminated by oxygen bubbling. Triphenylphosphine (1.2 g, 4.5 mmol) was added, and the reaction mixture was allowed to warm to room temperature. The solution was washed with water (20 mL), brine (20 mL), dried over $MgSO_4$, and concentrated under reduced pressure. Flash chromatography (eluent, cyclohexane−AcOEt, 7:3 to AcOEt−MeOH, 9:1, v/v) afforded **5g−k** as colorless liquids. **6** was also isolated as a 1:2 mixture of isomers.

Dimethyl 2-(3-Amino-3-oxopropyl)-4-oxoglutarate 5g. Yield 55% from **3**. IR (neat film) 3453, 3363, 3206, 1730, 1667 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.73 (2H, s, broad), 3.86 (3H, s), 3.67 (3H,s), 3.31 (1H, dd, J = 10.1 and 20.0 Hz) 2.96 (1H, dd, J = 4.9 and 20.0 Hz), 2.97 (1H, m), 2.29 (2H, m), 1.95 (2H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.9, 174.3, 174.2, 160.8, 53.1, 52.2, 40.9, 39.2, 32.8, 26.9. HRMS (ES+) m/z 268.0798 ([M + Na]$^+$, $C_{10}H_{15}NaNO_6$ requires 268.0797). Anal. ($C_{10}H_{15}NO_6$): C, H, N.

Dimethyl 2-(3-Methylamino-3-oxopropyl)-4-oxoglutarate 5h. Yield 35% from **3**. IR (neat film) 3393, 3313, 3103, 1735, 1652 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.03 (1H, s, broad), 3.88 (3H, s), 3.67 (3H, s), 3.32 (1H, dd, J = 10.2 and 19.8 Hz) 2.96 (1H, dd, J = 4.6 and 19.8 Hz), 2.96 (1H, m), 2.81 (3H, d, J = 4.8 Hz), 2.23 (2H, m), 1.97 (2H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.9, 174.4, 172.4, 160.8, 53.1, 52.1, 40.9, 39.2, 33.6, 27.3, 26.3. HRMS (ES+) m/z 282.0968 ([M + Na]$^+$, $C_{11}H_{17}NaNO_6$ requires 282.0954). Anal. ($C_{11}H_{17}NO_6$): C, H, N.

Dimethyl 2-(3-Propylamino-3-oxopropyl)-4-oxoglutarate 5i. Yield 35% from **3**. IR (neat film) 3389, 3306, 3100, 1732, 1648 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.64 (1H, s, broad), 3.85 (3H, s), 3.66 (3H, s), 3.30 (1H, dd, J = 10.1 and 19.7 Hz), 3.18 (2H, q, J = 6.8 Hz), 2.94 (1H, dd, J = 4.6 and 19.8 Hz), 2.92 (1H, m), 2.20 (2H, m), 1.93 (2H, m), 1.49 (2H, hex, J = 7.2 Hz), 0.89 (3H, t, J = 7.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.9, 174.4, 171.5, 160.8, 53.1, 52.1, 41.3, 40.9, 39.2, 33.4, 27.4, 22.8, 11.3. HRMS (ES+) m/z 310.1274 ([M + Na]$^+$, $C_{13}H_{21}NaNO_6$ requires 310.1267). Anal. ($C_{11}H_{17}NO_6$): C, H, N.

Dimethyl 2-(3-Benzylamino-3-oxopropyl)-4-oxoglutarate 5j. Yield 43% from **3**. IR (neat film) 3384, 3303, 1734, 1654 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28 (5H, m), 6.19 (1H, s, broad), 4.39 (2H, d, J = 5.7 Hz), 3.85 (3H, s), 3.64 (3H, s), 3.29 (1H, dd, J = 10.2 and 19.8 Hz), 2.94 (1H, m), 2.93 (1H, dd, J = 4.4 and 19.8 Hz), 2.25 (2H, m), 1.96 (2H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.9, 174.4, 171.5, 160.8, 138.2, 128.7, 127.8, 127.5, 53.1, 52.1, 43.6, 40.9, 39.2, 33.6, 27.3. HRMS (ES+) m/z 358.1267 ([M + Na]$^+$, $C_{17}H_{21}NaNO_6$ requires 358.1267). Anal. ($C_{11}H_{17}NO_6$): C, H, N.

Dimethyl 2-(2,2-Diphenylethylamino-3-oxopropyl)-4-oxoglutarate 5k. Yield 25% from **3**. IR (CCl_4) 3450, 1735, 1668 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25 (10H, m), 5.50 (1H, s, broad), 4.18 (1H, t, J = 7.8 Hz), 3.90 (2H, m), 3.87 (3H, s), 3.62 (3H, s), 3.26 (1H, dd, J = 8.8 and 18.6 Hz), 2.89 (1H, dd, J = 4.3 and 18.5 Hz), 2.83 (1H, m), 2.11 (2H, m), 1.85 (2H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.8, 174.3, 171.6, 160.8, 141.8, 128.7, 128.0, 126.8, 53.1, 52.0, 50.6, 43.8, 40.9, 39.1, 33.6, 27.2. HRMS (ES+) m/z 426.1933 ([M + H]$^+$, $C_{24}H_{28}NO_6$ requires 426.1917). Anal. ($C_{11}H_{17}NO_6$, 0.25H_2O): C, H, N.

Dimethyl 3-Hydroxy-4-oxocyclohexanedicarboxylate 6. Yield 20−50%. IR (neat) 3456, 1728 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.39 (1H, s), 4.00 (1H, s), 3.83 (3H, s), 3.78 (3H, s), 3.70 (2 × 3H, s), 3.08 (2 × 1H, m), 2.89 (2 × 1H, m), 2.67 (1H, m), 2.50 (3 × 1H, m), 2.38−2.23 (3 × 1H, m), 2.03−1.81 (3 × 1H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 205.7, 205.2, 174.2, 173.4, 171.9, 169.8, 79.7, 78.9, 53.3, 53.2, 52.2, 52.0, 39.5, 39.1, 37.7, 37.1, 37.0, 36.2, 29.1, 28.4. HRMS (ES+) m/z 253.0674 ([M + Na]$^+$, $C_{10}H_{14}NaO_6$ requires 253.0688).

General Procedure for the Synthesis of Dilithium Oxoglutarate 1g−k. To a solution of **5g−k** (2 mmol) in MeOH (10 mL) was added dropwise a 0.4 M solution of LiOH (10.5 mL, 4.2 mmol). The mixture was stirred at room temperature for 5 to 15 h, until completion. After evaporation of MeOH, the pH of the aqueous solution was adjusted to 7.6 by addition of dowex 50WX8 resin (H^+ form). The resin was removed by filtration before evaporation

Table 4. Binding Affinities of 2k, DHK, and TBOA at Native AMPA-, KA-, NMDA Receptors (Rat Synaptosomes) as well as at Rat Homomeric iGluR5-7

compound	AMPA IC_{50} (μM)	KA IC_{50} (μM)	NMDA IC_{50} (μM)	iGluR5 K_i (μM)	iGluR6 K_i (μM)	iGluR7 K_i(μM)
2k	>100	>100	>100	37.2 [4.44 ± 0.05]	>100	>100
DHK	1100[a]	6[a]	350[a]	8.18 [5.09 ± 0.02]	12.3 [4.92 ± 0.05]	0.376 [6.44 ± 0.07]
TBOA	>1000[a]	550[a]	470[a]	nd	nd	nd

[a] Data are taken from ref 13. Mean pK_i (M) ± SEM is given in square brackets (3−5 experiments conducted in triplicate). Hill values were unity for DHK and **2k** at iGluR5−7. nd: no data available.

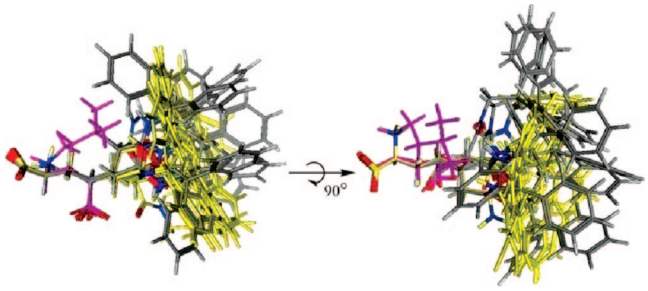


Figure 1. Superimposition of calculated low-energy conformations (up to +1 kcal/mol) of **2k** (type code), **2j** (yellow), and DHK (purple), by fitting ammonium groups and two carboxylate groups.

of the water under reduced pressure. **2g−k** were isolated in quantitative yield as white solids.

Dilithium 2-(3-Amino-3-oxopropyl)-4-oxoglutarate 1g. ^{1}H NMR (400 MHz, D_2O) δ 3.02 (1H, dd, J = 9.0 and 19.5 Hz), 2.84 (1H, dd, J = 6.7 and 19.2 Hz), 2.61 (1H, m), 2.24 (2H, m), 1.74 (2H,m). ^{13}C NMR (100 MHz, D_2O) δ 204.3, 182.6, 179.2, 167.9, 42.6, 42.0, 33.0, 28.2. HRMS (ES−) m/z 216.0509 ([M − 2Li + H]$^-$, $C_8H_{10}NO_6$ requires 216.0508).

Dilithium 2-(3-Methylamino-3-oxopropyl)-4-oxoglutarate 1h. ^{1}H NMR (400 MHz, D_2O) δ 3.02 (1H, dd, J = 8.8 and 18.4 Hz), 2.83 (1H, dd, J = 5.4 and 18.6 Hz), 2.68 (3H, s), 2.59 (1H, m), 2.21 (2H, m), 1.73 (2H,m). ^{13}C NMR (100 MHz, D_2O) δ 204.4, 182.9, 176.5, 169.7, 42.6, 42.0, 33.7, 28.4, 25.8. HRMS (ES−) m/z 236.0761 ([M − 2Li + H]$^-$, $C_9H_{12}NO_6$ requires 236.0746).

Dilithium 2-(3-Propylamino-3-oxopropyl)-4-oxoglutarate 1i. ^{1}H NMR (400 MHz, D_2O) δ 3.12 (3H, t, J = 6.8 Hz), 3.05 (1H, dd, J = 8.4 and 18.4 Hz), 2.86 (1H, dd, J = 5.2 and 18.4 Hz), 2.62 (1H, m), 2.24 (2H, m), 1.75 (2H,m), 1.50 (2H, hex, J = 7.4 Hz), 0.87 (3H, t, J = 7.4 Hz). ^{13}C NMR (100 MHz, D_2O) δ 204.4, 181.6, 175.7, 170.8, 41.5, 41.2, 33.7, 27.9, 21.7, 10.6. HRMS (ES−) m/z 258.0995 ([M − 2Li + H]$^-$, $C_{11}H_{16}NO_6$ requires 258.0978).

Dilithium 2-(3-Benzylamino-3-oxopropyl)-4-oxoglutarate 1j. ^{1}H NMR (400 MHz, D_2O) δ 7.35 (5H, m), 4.36 (2H, s), 3.03 (1H, dd, J = 9.1 and 17.6 Hz), 2.84 (1H, dd, J = 5.5 and 17.8 Hz), 2.63 (1H, m), 2.29 (2H, m), 1.78 (2H,m). ^{13}C NMR (100 MHz, D_2O) δ 204.3, 182.3, 175.8, 170.0, 137.9, 128.7, 127.4, 127.2, 43.0, 42.3, 41.7, 33.8, 28.2. HRMS (ES−) m/z 306.0991 ([M − 2Li + H]$^-$, $C_{15}H_{16}NO_6$ requires 306.0978).

Dilithium 2-(2,2-Diphenylethylamino-3-oxopropyl)-4-oxoglutarate 1k. ^{1}H NMR (400 MHz, D_2O) δ 7.30 (10H, m), 4.28 (1H, t, J = 8.2 Hz), 3.86 (2H, m), 2.93 (1H, dd, J = 8.6 and 18.7 Hz), 2.71 (1H, dd, J = 5.5 and 18.7 Hz), 2.44 (1H, m), 2.09 (2H, t, J = 8.0 Hz), 1.54 (1H,m), 1.50 (1H, m). ^{13}C NMR (100 MHz, D_2O) δ 205.6, 184.3, 177.2, 171.2, 143.6, 130.3, 129.6, 128.4, 51.6, 45.2, 44.4, 43.6, 35.5, 30.4. HRMS (ES−) m/z 396.1451 ([M − 2Li + H]$^-$, $C_{22}H_{22}NO_6$ requires 396.1447).

General Procedure for the Synthesis of Substituted Glutamic Acids 2g−k. To a solution of racemic **1g−k** (0.5 mmol) in water (25 mL) was added cysteine sulfinic acid (0.5 mmol). The pH of the solution was adjusted to 7.6 with 1 M NaOH before the addition of *E. coli* AAT (20−100 Units). The reaction was stirred slowly at room temperature and monitored by titration of pyruvate: 5 μL aliquots of the reaction mixture were added to 995 μL of 0.1 M potassium phosphate buffer, pH 7.6 containing NADH (0.2 mM), and lactate dehydrogenase (1 unit). Pyruvate concentration was calculated from the ΔOD measured at 340 nm using ϵ_{NADH} = 6220 $M^{-1}\cdot cm^{-1}$. When a conversion rate of 40% was reached, the reaction mixture was rapidly passed through a column of dowex 50WX8 resin (H^+ form, 25 mL). The column was then washed with water (100 mL) until complete elution of CSA and then eluted with 1 M NH_4OH. The ninhydrin positive fractions were combined and concentrated to dryness under reduced pressure. The residue was diluted in water (5 mL) and, if necessary, the pH adjusted to 7.0 with 1 M NaOH before adsorption of the product on a column of dowex 1 × 8 resin (200−400 mesh, AcO^- form, 1.5 cm × 12 cm). The column was washed with water (50 mL) and then eluted with an AcOH gradient (0.1−1M) and, in the case of **2k**, with 1 M AcOH in H_2O−MeOH (1:1, v/v). The ninhydrin positive fractions were combined and concentrated under reduced pressure. The oily residue was dissolved in water (4 mL), and the solution was lyophilized to afford **2g−k** as hygroscopic white solids.

(2*S*,4*R*)-4-(3-Amino-3-oxopropyl)glutamic Acid 2g. Yield 35 mg, 35%; mp 120 °C; $[\alpha]^{25}_D$ = +34.2 (c 1.0, 6 N HCl). ^{1}H NMR (400 MHz, D_2O) δ 3.78 (1H, dd, J = 5.4 and 8.3 Hz), 2.62 (1H, m), 2.34 (2H, m), 2.29 (1H, ddd, J = 5.5, 10.0 and 15.0 Hz), 1.92 (3H, m). ^{13}C NMR (100 MHz, D_2O) δ 178.7, 178.5, 173.6, 53.0, 41.6, 32.3, 27.8. HRMS (ES−) m/z 217.0838 ([M − H]$^-$, $C_8H_{13}N_2O_5$ requires 217.0824). Anal. ($C_8H_{14}N_2O_5\cdot 0.75H_2O$) C, H, N.

(2*S*,4*R*)-4-(3-Methylamino-3-oxopropyl)glutamic Acid 2h. Yield 74 mg, 46%; mp 124 °C; $[\alpha]^{25}_D$ = +30.8 (c 1.0, 6 N HCl). ^{1}H NMR (400 MHz, D_2O) δ 3.75 (1H, dd, J = 5.0 and 8.5 Hz), 2.71 (3H, s), 2.54 (1H, m), 2.29 (2H, m), 2.25 (1H, ddd, J = 5.2, 9.7 and 14.8 Hz), 1.89 (3H, m). ^{13}C NMR (100 MHz, D_2O) δ 179.4, 176.0, 173.9, 53.1, 42.2, 33.1, 32.5, 28.2, 25.8. HRMS (ES+) m/z 255.0961 ([M + Na]$^+$, $C_9H_{16}NaN_2O_5$ requires 255.0957). Anal. ($C_9H_{16}N_2O_5$) C, H, N.

(2*S*,4*R*)-4-(3-Propylamino-3-oxopropyl)glutamic acid 2i. Yield 94 mg, 38%; mp 145 °C; $[\alpha]^{25}_D$ = +27.6 (c 1.0, 6 N HCl). ^{1}H NMR (400 MHz, D_2O) δ 3.76 (1H, dd, J = 5.3 and 8.4 Hz), 3.11 (2H, t, J = 6.9 Hz), 2.57 (1H, m), 2.28 (2H, m), 2.27 (1H, ddd, J = 5.2, 9.6 and 15.0 Hz), 1.90 (3H, m), 1.48 (hex, J = 7.3 Hz), 0.87 (3H, t, J = 7.4 Hz). ^{13}C NMR (100 MHz, D_2O) δ 178.9, 175.3, 173.7, 53.1, 41.8, 41.2, 33.2, 32.4, 28.2, 21.7, 10.6. HRMS (ES+) m/z 283.1277 ([M + Na]$^+$, $C_{11}H_{20}NaN_2O_5$ requires 283.1270). Anal. ($C_{11}H_{20}N_2O_5$) C, H, N.

(2*S*,4*R*)-4-(3-Benzylamino-3-oxopropyl)glutamic acid 2j. Yield 70 mg, 39%; mp 110 °C; $[\alpha]^{25}_D$ = +22.0 (c 1.1, 6 N HCl). ^{1}H NMR (400 MHz, D_2O) δ 7.33 (5H, m), 4.33 (2H, s), 4.03 (1H, t, J = 7.2 Hz), 2.68 (1H, m), 2.34 (3H, m), 1.96 (1H, ddd, J = 4.4, 7.2 and 14.4 Hz), 1.92 (2H, m). ^{13}C NMR (100 MHz, D_2O) δ 177.8, 175.1, 171.4, 137.9, 128.8, 127.5, 127.3, 51.4, 43.0, 40.9, 32.8, 31.6, 27.9. HRMS (ES+) m/z 331.1268 ([M + Na]$^+$, $C_{15}H_{20}NaN_2O_5$ requires 331.1270); Anal. ($C_{15}H_{20}N_2O_5\cdot 0.75H_2O$) C, H, N.

(2*S*,4*R*)-4-(2,2-Diphenylethylamino-3-oxopropyl)glutamic acid 2k. Yield 108 mg, 37%; mp 135 °C; $[\alpha]^{25}_D$ = +16.9 (c 0.9, 6 N HCl). ^{1}H NMR (400 MHz, D_2O) δ 7.33 (8H, m), 7.25 (2H, m), 4.24 (1H, t, J = 8.3 Hz), 3.94 (1H, t, J = 7.1 Hz), 3.82 (2H, d, J = 8.3 Hz), 2.50 (1H, m), 2.25 (1H, ddd, J = 7.0, 9.3 and 14.8 Hz), 2.12 (2H, m), 1.87 (1H, ddd, J = 4.4, 7.3 and 14.7 Hz), 1.68 (2H, m). ^{13}C NMR (100 MHz, D_2O) δ 177.8, 175.0, 171.4, 142.1, 128.9, 127.9, 127.0, 51.3, 49.9, 43.3, 40.7, 32.8, 31.3, 27.9. HRMS (ES+) m/z 421.1742 ([M + Na]$^+$, $C_{22}H_{26}NaN_2O_5$ requires 421.1739). Anal. ($C_{22}H_{26}N_2O_5\cdot 2.75\ H_2O$) C, H, N.

Methyl (2*S*,4*R*)-4-(2,2-Diphenylethylamino-3-oxopropyl)pyroglutamate 7. A solution of **2k** (20 mg, 0.05 mmol) in water (5 mL) was heated under reflux for 48 h until complete disappearance of **2k**. After evaporation of water under reduced pressure, the solid residue was dried by addition and evaporation of MeOH (2 × 5 mL). It was then dissolved in MeOH (2 mL) and $SOCl_2$ (25 μL, 0.35 mmol) was added. The mixture was stirred at room temperature for 18 h before concentration under reduced pressure. Flash chromatography (eluent, AcOEt/acetone, 8:2, v/v) afforded **7** (11 mg, 56%) isolated as a colorless viscous liquid: $[\alpha]^{25}_D = +20.8$ (*c* 0.6, $CHCl_3$). ^{1}H NMR (400 MHz, C_6D_6) δ 7.14 (8H, m), 7.02 (2H, m), 5.98 (1H, s, broad), 5.65 (1H, t, $J = 5.5$ Hz); 4.18 (1H, t, $J = 8.0$ Hz), 3.93 (1H, ddd, $J = 6.3$, 7.9 and 13.8 Hz), 3.77 (1H, ddd, $J = 5.6$, 7.8 and 13.5 Hz), 3.39 (1H, dd, $J = 3.5$ and 9.0 Hz), 3.22 (3H, s), 2.09−1.93 (3H, m); 1.85 (1H, ddd, $J = 3.5$, 8.8 and 12.8 Hz), 1.69 (2H, m), 1.43 (1H, td, $J = 8.7$ and 13.0 Hz). ^{13}C NMR (100 MHz, D_2O) δ 178.9, 172.5, 171.7, 142.8, 128.9, 128.6, 126.8, 53.4, 51.7, 51.2, 44.2, 38.1, 33.8, 31.7, 27.7. HRMS (ES+) *m*/*z* 395.1976 ($[M + H]^+$, $C_{23}H_{27}N_2O_4$ requires 395.1971).

Modeling

In Silico Study of 4-Substituted Glu Analogues and Selected Ligands. The modeling study was performed using the software package MOE (Molecular Operating Environment, v2006.08, Chemical Computing Group, 2006) using the built-in mmff94x forcefield and the GB/SA continuum solvent model. *General procedure for compounds* **2g−k**: The γ-carboxylate group was protonated and the compound submitted to a stochastic conformational search (standard setup). Conformations which hold intramolecular hydrogen bond(s) or π-cation interactions were discarded. Superimpositions of selected conformations were carried out using the built-in function in MOE by fitting the ammonium group and the two carboxylate groups.

Pharmacology

FLIPR Membrane Potential (FMP) Assay. The construction of Human Embryonic Kidney 293 (HEK293) cell lines stably expressing human EAAT1, EAAT2, and EAAT3 have been reported previously, and the pharmacological characterization of **2a−k** (**2d** is exempt) at the cell lines in the FMP assay was performed essentially as described.[12] Briefly, cells were split into poly-D-lysine (PDL)-coated black 96-well plates with a clear bottom (BD Biosciences, Palo Alto, CA) in Dulbecco's modified Eagle medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), 10% dialyzed fetal bovine serum, and 1 mg/mL G-418 (culture medium). At 16−24 h later, the medium was aspirated and the cells were washed with 100 μL Krebs buffer [140 mM NaCl/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/11 mM HEPES/10 mM D-glucose, pH 7.4]. Then 50 μL of Krebs buffer was added to each well (in the characterization of nonsubstrate inhibitors, the inhibitors were added to this buffer). Then 50 μL of Krebs buffer supplemented with FMP assay dye was then added to each well (in the experiments with the nonsubstrate inhibitors, the compounds were added to this solution), and the plate was incubated at 37 °C for 30 min. The plate was assayed at 30 °C in a NOVOstar plate reader measuring emission at 560 nm caused by excitation at 530 nm before and up to 1 min after addition of 33 μL of substrate solution. The experiments were performed in duplicate at least three times for each compound. For the characterization of nonsubstrate inhibitors, 30 μM Glu was used as substrate concentration at the EAAT1- and EAAT3-cell lines, whereas 50 μM Glu was used as substrate concentration at the EAAT2-cell line.

[^{3}H]-D-Asp Uptake Assay. The EAAT1-, EAAT2-, and EAAT3-HEK293 cell lines were characterized pharmacologically in a [^{3}H]-D-Asp assay essentially as previously described.[12] Briefly, cells were split into PDL-coated white 96-well plates (Perkin-Elmer, Boston, MA) in culture medium. At 16−24 h later, the medium was aspirated, and cells were washed 3 times with 100 μL of assay buffer (Hanks Buffered Saline Solution supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$). Then 50 μL of assay buffer supplemented with 30 nM [^{3}H]-D-Asp and various concentrations of different ligands was added to each well, and the plate was incubated at 37 °C for 7 min. The wells were then washed with 3 × 100 μL of ice-cold assay buffer, and 150 μL of Microscint20 scintillation fluid (Perkin-Elmer) was added to each well. The plate was shaken for 1 h and counted in a TopCounter (Perkin-Elmer). Nonspecific [^{3}H]-D-Asp transport in the cell lines was determined using 3 mM Glu.

AMPA, KA and NMDA Binding Assays. Glu analogues were evaluated for AMPA, KA, NMDA (CGP 39653) binding affinity in native rat synaptosomes, in accordance with previously described experimental procedure.[22]

iGluR5−7 Binding Assay. Rat iGluR5(Q)$_{1b}$, iGluR6(*V*,*C*,*R*)a, and iGluR7a were inserted into recombinant baculoviruses, receptors expressed by infection of *Sf*9 insect cells and infected *Sf*9 cell membranes utilized for radioligand binding assays. Cells were maintained in BaculoGold Max-XP serum-free medium (BD Biosciences-Pharmingen, San Diego, CA) according to standard protocols in "Guide to Baculovirus Expression Vector Systems and Insect Cell Culture Techniques" (Life Technologies, Paisley, UK) and "Baculovirus Expression Vector System: Procedures and Methods Manual", 2nd ed., (Pharmingen). [^{3}H]-SYM2081 ([^{3}H]-(2*S*,4*R*)-4-methylglutamic acid) (47.9 Ci/mmol; ARC Inc., St. Louis, MO) microtiter plate binding assays were performed in 250 μL of assay buffer (50 mM Tris-HCl, pH 7.1 at 4 °C) at a protein concentration from 10 to 50 μg/mL, incubating for 1−2 h at 4 °C. Bound and free radiolabel were separated by cold filtration through GF/B glass fiber filters (UniFilter-96, Perkin-Elmer) on a Perkin-Elmer FilterMate manifold using two washes with cold assay buffer. Nonspecific binding was determined in the presence of 1 mM Glu. Competition studies were performed using 1−5 nM radiolabel in the presence of 10 nM to 1 mM cold ligand. Filters were dried 1 h at 50 °C and then 50 μL/well of Microscint 20 was added. Radioactivity was detected as DPM using a TopCounter. The affinity of the radiolabel for the kainate receptors was determined from saturation binding experiments, K_d (mean ± SEM): iGluR5(Q)$_{1b}$, 0.663 ± 0.035 nM; iGluR6(*V*,*C*,*R*)a, 17.0 ± 3.0 nM; iGluR7a, 5.69 ± 1.12 nM. Competition data were analyzed using Grafit v3.00 (Erithacus Software Ltd., Horley, UK) and fit to equations as previously described for the determination of K_i.[23]

mGluR Assays. Binding and functional assays were carried out as described in ref 9.

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Supporting Information Available: Combustion analysis data of compounds **2g−k** and **5g−k**, and ^{1}H and ^{13}C NMR spectra of compounds **2 g−k**. HPLC analyses of compound **2k**. This material is available free of charge via the Internet at http://pubs.acs.org.

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