Chemo-Enzymatic Synthesis of a Series of 2,4-*Syn*-Functionalized (*S*)-Glutamate Analogues: New Insight into the Structure−Activity Relation of Ionotropic Glutamate Receptor Subtypes 5, 6, and 7

Emanuelle Sagot,[‡] Darryl S. Pickering,[§] Xiaosui Pu,[||] Michelle Umberti,[||] Tine B. Stensbøl,[⊥] Birgitte Nielsen,[†] Marion Chapelet,[‡] Jean Bolte,[‡] Thierry Gefflaut,*[,‡] and Lennart Bunch*[,†]

Department of Medicinal Chemistry, The Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark, Département de Chimie, Université Blaise Pascal, 63177 Aubière Cedex, France, Department of Pharmacology and Pharmacotherapy, The Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark, Biological Research, H. Lundbeck A/S, 215 College Road, Paramus, New Jersey 07652, Department of Neurobiology, H. Lundbeck A/S, Ottiliavej 9, DK-2500 Valby, Denmark

Received January 30, 2008

(*S*)-Glutamic acid (Glu) is the major excitatory neurotransmitter in the central nervous system (CNS) activating the plethora of ionotropic Glu receptors (iGluRs) and metabotropic Glu receptors (mGluRs). In this paper, we present a chemo-enzymatic strategy for the enantioselective synthesis of five new Glu analogues **2a**−**f** (**2d** is exempt) holding a functionalized substituent in the 4-position. Nine Glu analogues **2a**−**j** are characterized pharmacologically at native 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA), kainic acid (KA), and *N*-methyl-D-aspartic acid (NMDA) receptors in rat synaptosomes as well as in binding assays at cloned rat iGluR5−7 subtypes. A detailed in silico study address as to why **2h** is a high-affinity ligand at iGluR5−7 (K_i = 3.81, 123, 57.3 nM, respectively), while **2e** is only a high affinity ligand at iGluR5 (K_i = 42.8 nM). Furthermore, a small series of commercially available iGluR ligands are characterized in iGluR5−7 binding.

Introduction

(*S*)-Glutamic acid (Glu) is the major excitatory neurotransmitter in the central nervous system (CNS) activating the plethora of ionotropic Glu receptors (iGluRs)[a] and metabotropic Glu receptors (mGluRs).[1,2] While the iGluRs are ion channels and thus mediate a fast excitatory response (Na^+, K^+, Ca^{2+} flux), the mGluRs are classified as G-protein coupled receptors and produce a slower signal transduction through second messenger systems. Termination of the excitatory signal is controlled by uptake of Glu from the synaptic cleft by the excitatory amino acid transporters (EAATs).[3] On the basis of pharmacological studies, the iGluRs are further divided into: 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) receptors (homo- or heteromeric receptors comprising the subunits iGluR1−4), kainic acid (KA) receptors (homo- or heteromeric receptors comprising the subunits iGluR5−7 and KA1,2),[4] and the *N*-methyl-D-aspartic acid (NMDA) receptors (heteromeric receptors comprising the subunits NR1, 2A-D, 3A-C). The mGluRs are divided into eight homodimeric subtypes, mGluR1−8, which are grouped with respect to the second messenger system involved with receptor signaling, pharmacology, and molecular biology (group I: mGluR1,5; group II: mGluR2,3; group III: mGluR4,6−8).[5]

To study a specific iGluR subtype, when functioning in its intrinsic biological environment, the employment of iGluR subtype selective ligands (agonists, partial agonists, and antagonists) is a frequently applied strategy. In this paper, we present the synthetic strategy which allowed the preparation of Glu analogues **2a**−**f** (**2d** is exempt), and the pharmacological evaluation of the nine 2,4-*syn*-4-substituted Glu analogues **2a**−**j** at iGluRs. New insight into the structure−activity relation for KA receptor subtypes iGluR5−7 is presented.

Chemistry

Glu analogues **2a**−**j** were all prepared following a chemoenzymatic route involving a stereoselective transamination reaction as the key step (Scheme 1). Aspartate aminotransferase (AAT) from *Escherichia coli* was shown to be a very effective catalyst for the conversion of a variety of substituted α-ketoglutarates (KG) into L-Glu analogues. A very high stereoselectivity was observed in favor of the L-2,4-*syn* Glu analogues obtained through the kinetic resolution of racemic KGs.[6]

Synthesis of 2-Oxoglutaric Acids. Two synthetic approaches were developed for the preparation of the oxoglutarates **1a**−**j**. Substrates **1g**−**j** bearing the amide functionality were prepared via a short three-step procedure from the readily available dimethyl 4-oxocyclohexane-1,3-dicarboxylate.[7] However, this method was not feasible for introduction of the functionalities present in **1a**−**f**.

As shown in Scheme 2, the syntheses of **1a**−**c** were performed by following a recently described procedure:[8] the glutarate carbon skeleton was obtained by a Michael reaction between methyl acetoacetate and an α-substituted acrylate,

* Author to whom correspondence should be addressed. For L.B.: phone, +45 35336244; fax, +45 35336040; E-mail, lebu@farma.ku.dk. For T.G. (for correspondence regarding the synthetic work): phone, +33 473407866; fax, +33 473407717; E-mail, thierry.gefflaut@univ-bpclermont.fr.

‡ Département de Chimie, Université Blaise Pascal.

§ Department of Pharmacology and Pharmacotherapy, The Faculty of Pharmaceutical Sciences, University of Copenhagen.

|| Biological Research, H. Lundbeck A/S.

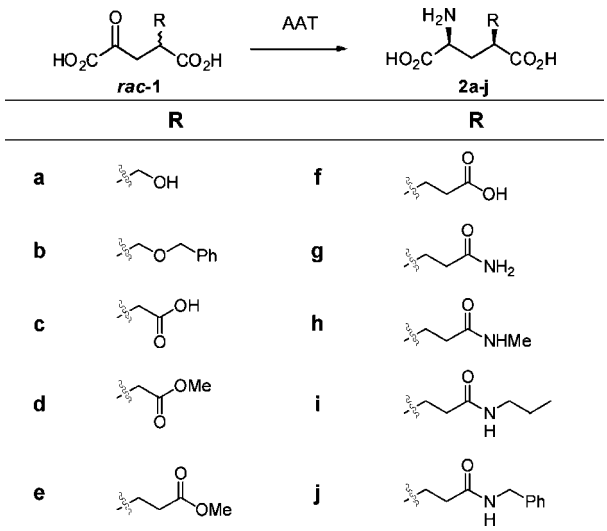
⊥ Department of Neurobiology, H. Lundbeck A/S.

† Department of Medicinal Chemistry, The Faculty of Pharmaceutical Sciences, University of Copenhagen.

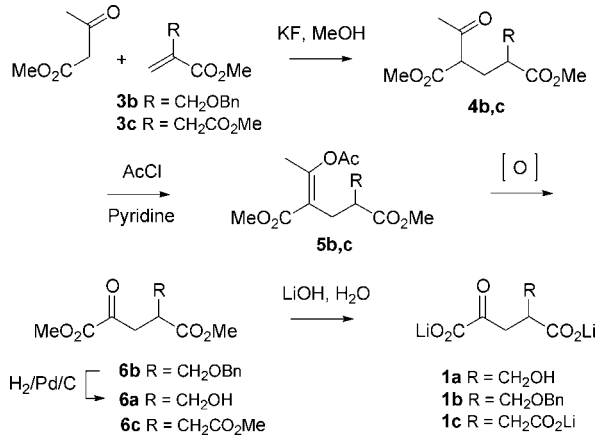
[a] Abbreviations: iGluR, ionotropic Glu receptors; mGluR, metabotropic Glu receptors; EAAT, excitatory amino acid transporter; KG, α-ketoglutarates; AAT, aspartate aminotransferase; DHK, dihydrokainic acid; TBOA, *threo*-benzyloxyaspartate; AMPA, 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic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

10.1021/jm800092x CCC: $40.75
Published on Web 06/25/2008

Scheme 1. AAT Catalyzed Synthesis of 4-Substituted Glu Analogues



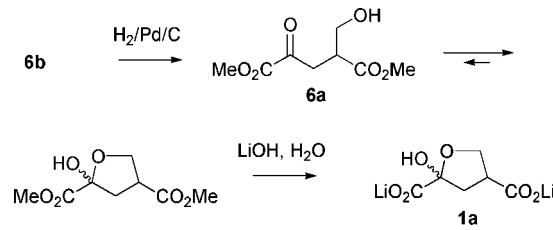
Scheme 2. Synthesis of 4-Substituted KG **1a**−**c**



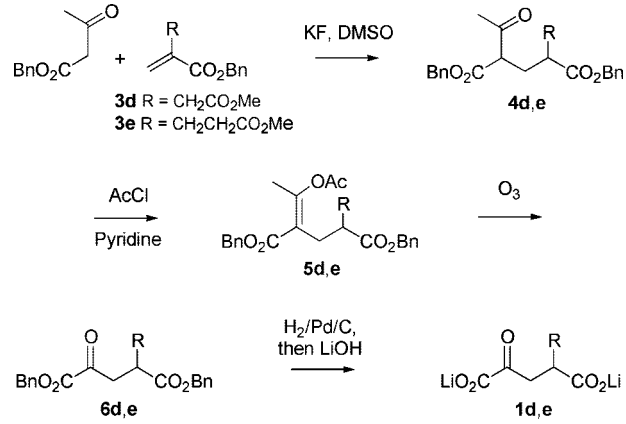
whereas ozonolysis of an enol acetate intermediate afforded the α-keto ester moiety. This strategy, already used for the synthesis of 3-alkyl KGs,[8] appeared very general and compatible with the functionalities of **1a**−**c**.

2-(Benzyloxymethyl)acrylate (**3b**) was prepared from methyl (bromomethyl)acrylate[9] by reaction with benzyl alcohol in the presence of base. Among the several bases tested, 1,4-diazabicyclo[2.2.2]octane (DABCO) gave the best results for the preparation of **3b**, which was isolated in 80% yield. The Michael condensations of **3b**,**c** with methyl acetoacetate were done in mild conditions using KF as the base in MeOH. The Michael products **4b** and **4c** were both isolated as 1:1 mixtures of diastereomers and in 95% and 78% yields, respectively. These β-keto esters were then treated with acetyl chloride and pyridine to form the enol acetates **5b** and **5c** isolated after flash chromatography in 64% and 86% yields, respectively. Finally, these intermediates were oxidized by ozone to form the oxoglutarates **6b** and **6c**. A yield of 72% was obtained for **6c**. Unfortunately, using the same ozonolysis procedure, the oxoglutarate **6b** was isolated with a low yield of 17%. This result was mainly due to an unexpected oxidation of the benzyl ether group to a benzoate isolated as the major byproduct in 45% yield. The oxidative cleavage of **5b** was tried using $NaIO_4$ and catalytic RuO_2 in biphasic conditions using a $CHCl_3/CH_3CN/H_2O$ mixture. However, hydrolysis of the enol acetate **5b** was observed in these conditions and the yield of **6b** could only be

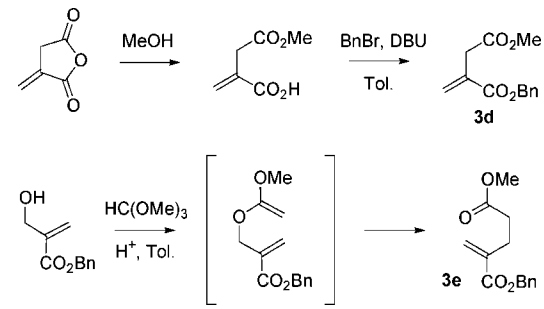
Scheme 3. Synthesis of 4-Hydroxymethyl KG **1a**



Scheme 4. Synthesis of **1d** and **1e**



Scheme 5. Synthesis of Acrylates **3d** and **3e**

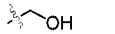
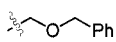
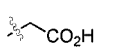
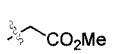


increased to 43%. The benzyl group of **6b** was cleaved by hydrogenolysis to furnish **6a** in quantitative yield. Finally, the lithium salts **1a**−**c** were prepared quantitatively from **6a**−**c** by basic hydrolysis using a stoichiometric amount of LiOH. Interestingly, NMR analyses indicated that both compounds **6a** and **1a** exist in solution as a 1:1 mixture of hemiketal isomers, coming from the addition of the hydroxyl group on the carbonyle at position 2 (Scheme 3).

The synthesis of ketoglutarates **1d** and **1e** bearing an ester functionality was performed following a slightly different procedure (Scheme 4): the Michael condensation of **3d**,**e** with benzylacetoacetate was done in DMSO in order to avoid the transesterification reaction occurring when MeOH was used as the solvent. The ketoesters **6d** and **6e** were thus obtained from **3d** and **3e** in 40% overall yield. Finally, the methyl ester functionality of **1d** and **1e** could be preserved through the selective hydrogenolysis of the benzyl esters of **6c** and **6d** in the presence of Pd/C.

As shown in Scheme 5, the unsymmetrical diester **3d** was obtained from itaconic anhydride: regioselective addition of MeOH on the nonconjugated carbonyl of the anhydride allowed the formation of itaconic acid monoester at position 1. Consecutive esterification with benzyl bromide and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) afforded 1-methyl-4-benzyl itaconate **3d** isolated in 95% overall yield. The methyl ester formation was highly

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

Substrate	R	K_m (mM)	k_{cat} rel.(%)
KG	-	0.23 ± 0.05	100 ± 3[b]
1a		4.7 ± 0.8	1.6 ± 0.3
1b		0.09 ± 0.04	11.0 ± 0.8
1c		30.1 ± 106	1.1 ± 0.06
1d		1.8 ± 0.4	41.0 ± 2.0
1e		0.61 ± 0.03	33.6 ± 0.6

[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}.

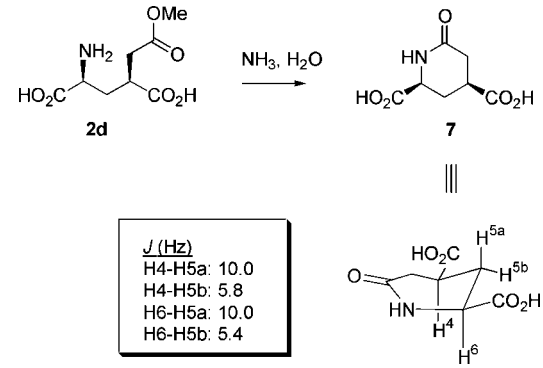
regioselective, giving a 97:3 mixture of **3d** and 1-benzyl-4-methyl itaconate. Acrylate **3e** was prepared in 73% yield from benzyl α-(hydroxymethyl)acrylate[10] via the Claisen−Johnson rearrangement of the ketene ketal formed in the presence of trimethyl orthoacetate.[11]

Transamination Reactions. The substituted KGs **1a**−**e** were evaluated as substrate for *E. coli* AAT on the basis of the Michaelis−Menten model: rate measurement of the AAT catalyzed transaminations between aspartate (40 mM) and **1a**−**e** in variable concentrations allowed estimation of the kinetic parameters reported in Table 1.

These results offer a new highlight on the substrate specificity of AAT: the parameters measured for **1a** and, even more, for **1c** clearly indicate that the introduction of a polar group at position 4 of KG results in a dramatic drop in affinity as well as in k_{cat} value. The low activity of AAT toward **1a** could be the result of the cyclization mentioned above, hiding the carbonyl group of this substrate. The low affinity of AAT toward substrate, which contain polar functional groups, was also noticed with the amide derivatives precursors of **2 g**−**j**.[7] However, when the alcohol and carboxylic acid functionalities are masked as benzyl ether or methyl ester in compounds **1b**, **1d**, and **1e**, the activity is restored. These results are consistent with the previous finding that the AAT active site accommodates various hydrophobic groups substituting the pro-*R* hydrogen atom at position 4 of KG.[6]

Preparative scale transaminations were easily carried out in the case of the 3 protected derivatives **1b**, **1d**, and **1e** following the procedure described previously:[6,8] cysteine sulfinic acid (CSA) was used as an irreversible amino donor, and the reaction was stopped near 40% conversion. Therefore, the kinetic resolution of the racemic substrates **1b**,**d**,**e** was performed, resulting in the stereoselective formation of L-2,4-*syn* Glu analogues **2b**,**d**,**e**. The Glu analogue **2b** was isolated after selective adsorption on sulfonic dowex 50 resin (H^+ form) and elution with aqueous ammonia. It was further purified by ion exchange chromatography on cationic dowex 1 resin (AcO^- form): elution with an AcOH gradient afforded **2b** isolated in 43% yield and with a diastereomeric excess over 98%. Hydrogenation of **2b** in the presence of Pd/C gave **2a** in quantitative yield. In this way, the low activity of AAT toward **1a** could be bypassed through "substrate tailoring" by the design

Scheme 6. Instant Cyclization of **2d** into Lactam **7**



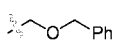
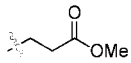
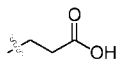
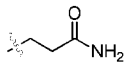
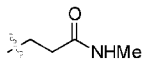
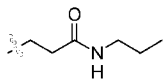
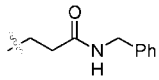
and synthesis of **1b** behaving as a very good substrate of AAT. The same strategy was applied for the tricarboxylic acid **2c**: after the transamination reaction involving the ester **1d**, the crude product was refluxed with 6N HCl to hydrolyze the ester group of **2d**. The Glu analogue **2c** was then purified by ion exchange chromatography as described for **2b** and isolated in 41% yield from *rac*-**1d**. All attempts to isolate **2d** were unsuccessful due to its rapid base catalyzed cyclization into lactame **7**. NMR analysis of the cyclic derivative **7** allowed the confirmation of the pseudoaxial position of H^4 and H^6, i.e., a 2,4-*syn* configuration in **2c** and **2d**, which is in agreement with the previously observed stereoselectivity of AAT toward 4-substituted Glu analogues (Scheme 6).

The tricarboxylic Glu analogue **2f** was prepared from **2e** following the procedure described above for **2c**: HCl catalyzed hydrolysis of **2e** before purification by ion exchange chromatography afforded **2f** in 40% yield. Contrary to **2d**, the Glu analogue **2e** bearing the methyl ester functionality could be isolated because the cyclization giving a seven-membered ring lactame was not favored in this case. **2e** was isolated in 39% yield after purification on the cationic dowex 1 resin. In this case, the first chromatographic step on dowex 50 was omitted in order to avoid the ester hydrolysis during elution with aqueous ammonia.

Pharmacology. The pharmacological properties of the nine 2,4-*syn*-4-substituted Glu analogues **2a**−**j** were first evaluated for binding affinities at native AMPA, KA, and NMDA receptor,[12] and results are summarized in Table 2. In [^{3}H]AMPA binding, all nine Glu analogues showed mid-to-high range micromolar affinities (IC_{50} = 25 to >100 μM). The same trend was observed in [^{3}H]KA binding experiments, except for **2a**, which was found to exhibit high nanomolar affinity (IC_{50} = 0.24 μM). Binding affinities for NMDA receptors were determined by means of the radioactive ligand [^{3}H]CGP39653. Again we observed mid-to-high micromolar affinities (K_i = 15 to >100 μM) for the nine compounds.

We subsequently investigated the binding affinities of **2a**−**j** at recombinant homomeric rat iGluR5, iGluR6, and iGluR7 expressed in *Sf*9 cell membranes (Table 2). Compounds **2b**,**c**,**f** were found to be low affinity ligands (low−high micromolar range) at all three subtypes, iGluR5−7. However, methylester analogue **2e** and amide analogue **2g** displayed high binding affinity for the iGluR5 subtype (42.8 and 7.45 nM, respectively), whereas they exhibited low affinity for the two other subtypes, iGluR6,7 (1−10 μM range). Unexpectedly, this subtype affinity profile was discontinued for the methylamide analogue, **2h**, which showed high affinity for all three subtypes, iGluR5−7 (3.81, 123, 57.3 nM, respectively). Furthermore, upon extending the *N*-alkyl substituent of **2h** to an *N*-*n*-propyl group, **2i**, or a

Table 2. Binding Affinities at Native iGluRs (Rat Synaptosomes) and at Cloned Rat iGluR5-7 Subtypes[a]

Compound (R-group)		[^{3}H]AMPA IC_{50} (μM)[a]	[^{3}H]KA IC_{50} (μM)[a]	NMDA[d] K_i (μM)[a]	Rat iGluR5 K_i (nM)[a,b]	Rat iGluR6 K_i (nM)[a,b]	Rat iGluR7 K_i (nM)[a,b]
2a		44 [4.35 ± 0.01]	0.24 [6.62 ± 0.04]	15 [4.82 ± 0.01]	n.t.	n.t.	n.t.
2b		>100	12 [4.93 ± 0.01]	26 [4.59 ± 0.05]	c)	c)	c)
2c		>100	39 [4.41 ± 0.01]	> 100	c)	c)	c)
2e		31 [4.51 ± 0.02]	7.9 [5.10 ± 0.01]	>100	42.8 [7.38 ± 0.06]	6,670 [5.21 ± 0.10]	1,010 [6.02 ± 0.11]
2f		>100	>100	52 [4.28 ± 0.05]	c)	c)	c)
2g		25 [4.60 ± 0.05]	16 [4.80 ± 0.05]	>100	7.45 [8.18 ± 0.12]	9,660 [5.02 ± 0.02]	2,820 [5.59 ± 0.15]
2h		32 [4.51 ± 0.07]	29 [4.54 ± 0.03]	>100	3.81 [8.42 ± 0.01]	123 [6.92 ± 0.04]	57.3 [7.30 ± 0.13]
2i		74 [4.13 ± 0.04]	41 [4.39 ± 0.04]	>100	641 [6.20± 0.04]	10,600 [4.99 ± 0.06]	3,460 [5.49 ± 0.10]
2j		>100	>100	72 [4.14 ± 0.04]	138 [6.86 ± 0.03]	>100,000	21,400 [4.67 ± 0.02]

[a] Data are given as mean [mean pIC_{50} or mean pK_i(M) ± S.E.M.] of at least three independent experiments. [b] Hill-values are close to unity for all compounds. [c] The compound displayed weak binding affinity to this iGluR subtype (low-high micro-molar range). [d] Radio ligand: [^{3}H]CGP 39653. n.t. = not tested.

benzyl group, **2j**, the preference for subtype iGluR5 over iGluR6,7 was observed again. However, with lower affinities for iGluR5 (641 and 138 nM, respectively), as compared with **2e**,**g**,**h**.

The four Glu analogues **2e**,**g**,**h**,**i** (Figure 1) were further characterized in a functional assay at subtypes mGluR1,5 (group I), mGluR2 (group II), and mGluR4 (group III). None of the analogues displayed agonist, antagonist, nor modulatory activities at these mGluR subtypes (EC_{50} >100 μM). Moreover, these four compounds exhibited low affinity (K_i >1000 μM) for subtypes mGluR2,3 (group II) in binding assays.

Discussion

The orthosteric binding pocket of the KA receptor subtypes KA1,2 and iGluR5−7 are highly confined compartments. The key amino acid residues involved in the molecular recognition of Glu are highly conserved and can be identified from receptor protein crystallographic studies[13,14] or receptor subtype homology models built from such data (Table 3). In detail (numbering for iGluR5 used, Figure 2), the α-carboxylate group of Glu forms hydrogen bonds to the guanidinium side chain of Arg538 and backbone amide-NH of Thr533 and Ser704. The α-ammonium group of Glu hydrogen bonds to the γ-carboxylate group of Glu738 and amide-carbonyl of backbone residue Pro531, whereas the γ-carboxylate group of Glu hydrogen bonds to the β-OH and amide-NH of Thr705. Residue Tyr488 does not form specific interactions with Glu but is significant in the sense that it participates strongly in the occluding of the binding pocket (Table 3).

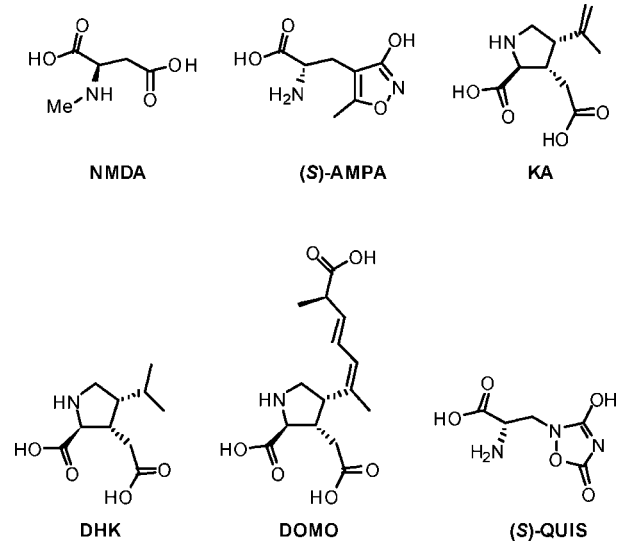


Figure 1. Commercially available standard GluR ligands: NMDA, (*S*)-AMPA, KA, DHK, DOMO, and (*S*)-QUIS.

In contrast to the above-described structural similarities of the KA receptor binding pockets, the most prominent residues of differentiation are: Thr706(KA1), Thr705(KA2), Ser736(iGluR5), Asn721(iGluR6), and Asn723(iGluR7). The residues are found

Table 3. Distinct Amino Acid Residues in the Binding Pocket of KA[a] Preferring iGluRs (Subtypes KA1,2 and iGluR5-7)

	conserved ligand−amino acid residue interactions (CI)[c]					
	α-COO^-	α-NH_3^+	γ-COO^-	conserved residues(CR)		residues of differentiation (RD)
KA1[b]	Arg507 (γ-GH^+) Thr502 (A-NH) Ser674 (A-NH)	Glu723 (γ-COO^-) Gly500 (A-CO)	Ser675 (β-OH/A-NH)	Tyr473	Glu425	Thr706
KA2[b]	Arg506 (γ-GH^+) Thr501 (A-NH) Ser673 (A-NH)	Glu722 (γ-COO^-) Ala500 (A-CO)	Thr674 (β-OH/A-NH)	Tyr472	Glu424	Thr705
iGluR5	Arg538 (γ-GH^+) Thr533 (A-NH)Ser704 (A-NH)	Glu753 (γ-COO^-) Pro531 (A-CO)	Thr705 (β-OH/A-NH)	Tyr504	Glu456	Ser736
iGluR6[b]	Arg523 (γ-GH^+) Ala518 (A-NH) Ala689 (A-NH)	Glu738 (γ-COO^-) Pro516 (A-CO)	Thr690 (β-OH/A-NH)	Tyr488	Glu440	Asn721
iGluR7[b]	Arg525 (γ-GH^+) Thr520 (A-NH) Ala691 (A-NH)	Glu740 (γ-COO^-) Pro518 (A-CO)	Thr692 (β-OH/A-NH)	Tyr491	Glu443	Asn723

[a] The amino acid residues within a distance of 4.5 Å from the Glu ligand occludes the binding pocket. [b] On the basis of homology modeling starting from iGluR5-S1S2 construct crystallized with Glu (PDB code: 1txf). [c] (GH^+) = guanidinium; A-CO = amide-CO; A-NH = amide-NH.

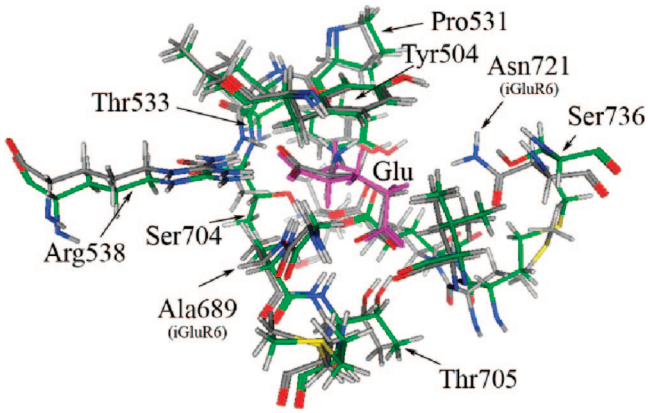


Figure 2. Superimposition of the binding pockets of rat iGluR5 (amino acid residues in green; Glu ligand in pink; PDB code: 1txf) and rat iGluR6 (amino acid residues in type code; Glu ligand in type code; PDB code: 1s50). See Experimental Section for details. Amino acid residue numbering according to iGluR5 unless otherwise noted.

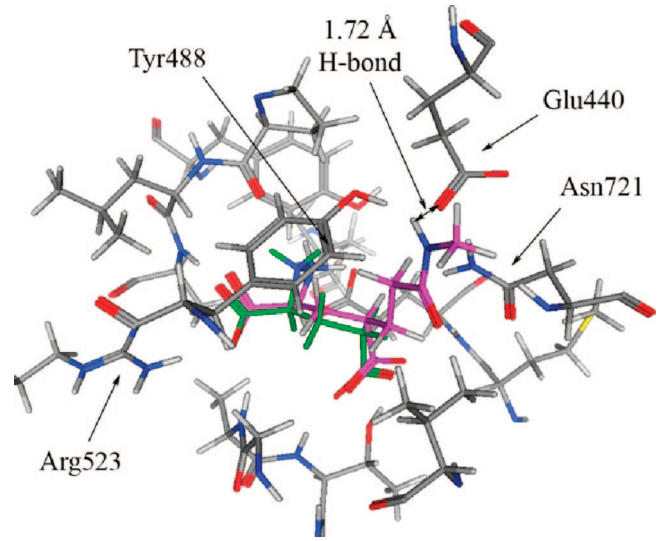


Figure 3. Docking of methylamide **2h** (purple) into full agonist state of the iGluR6 receptor (PDB code:1s50, cocrystallized Glu ligand, in green). The favorable hydrogen bond interaction (1.72 Å) between the amide-NH of **2h** and the γ-carboxylate group of residue Glu440, is indicated by the double-headed black arrow.

in the same area of space (see Figure 2 for illustrative example with iGluR5,6) and form a hydrogen bond with the γ-carboxylate group of a Glu amino acid residue (iGluR5:Glu456; iGluR6: Glu440, for clarity, *not* shown in Figure 2). Intrinsically, residue Ser736(iGluR5) is less voluminous as compared with residues Thr706(KA1), Thr705(KA2), Asn721(iGluR6), and Asn723(iGluR7). Thus, subtype iGluR5 may accommodate larger substituents in the (2*S*)-2,4-*syn* position of Glu, compared to KA1, KA2, iGluR6, and iGluR7.

To address as to what extent residues Ser736(iGluR5), Asn721(iGluR6), and Asn723(iGluR7) play a role in the observed altering of the iGluR5/6/7 subtype selectivity profile of structurally very close Glu analogues **2e**,**h**, we conducted an in silico study using the built-in docking procedure in the comprehensive modeling software package MOE (see Experimental Section for details). We first performed an automated docking (see Experimental Section for details) of methylester **2e**, amide **2g**, methylamide **2h**, and *n*-propylamide **2i** to the full-agonist state of iGluR5 (PDB code: 1txf). The Glu analogues: methylester **2e**, amide **2g**, and methylamide **2h** are all well accommodated by the iGluR5 subtype, whereas *n*-propylamide analogue **2i** induces severe steric clashes with the iGluR5 protein. These findings correlate well with our experimentally determined binding affinities (Table 2).

To investigate the structural origin underlying the unexpected high binding affinity of methylamide **2h** to iGluR6 (and iGluR7), we performed an automated docking of Glu analogues **2e**,**h**,**i** into the full-agonist state of the iGluR6 subtype. We were not able to identify successful Glu binding poses for either methylester **2e** or *n*-propylamide **2i**, a result that is in agreement with the experimental data (Table 2). In contrast, docking of methylamide **2h** proved to be successful. This is because of the increased steric hindrance (residue Asn721) in iGluR6, forcing the *N*-methylamide side chain of **2h** upward, which then can participate in a favorable hydrogen bond to the γ-carboxylate group of residue Glu440 (1.72 Å, Figure 3). For methylester **2e**, such a ligand pose would induce disfavored electron−electron repulsion between the ester-oxygen: γ-carboxylate of Glu440. On the other hand, a disadvantage to this binding mode of **2h** is its amide-NH close encounter with Tyr488 (approximately 1.36 Å). However, we are not concerned by this relatively short distance as in silico docking experiments are static representations of dynamic processes and the mobility of residue Tyr488 is well-documented (see Figure 4). In summary, we thus uphold this amide-NH−γ-carboxylate hydrogen bond interaction to play a crucial role as to why methylamide **2h** shows unexpected nanomolar binding affinity to iGluR6 (and iGluR7, as amino acid residues are identical with iGluR6).

While the amide-NH_2 of analogue **2g** is indeed also capable of hydrogen bonding to the γ-carboxylate group of Glu440, it is nevertheless experimentally shown to be a low affinity ligand at iGluR6,7 (Table 2). The structural difference of **2g**, as compared with **2h**, is solely the *N*-methyl group. We have investigated this issue in silico by looking at specific favored and disfavored interactions as well as the influence of this group on the low-energy conformation of the Glu analogues **2g**,**h**. Regrettably, we are not able to put forward conclusive remarks at this point and we believe X-ray crystallographic studies are the method of choice to verify our hypothesis for the high affinity binding of **2h** at iGluR6,7 and eventually guide us to understand why amide **2g** is yet a low affinity ligand at iGluR6,7. This work is ongoing in our laboratories.

We decided to also characterize a small series of standard commercially available iGluR ligands at iGluR7 and for direct

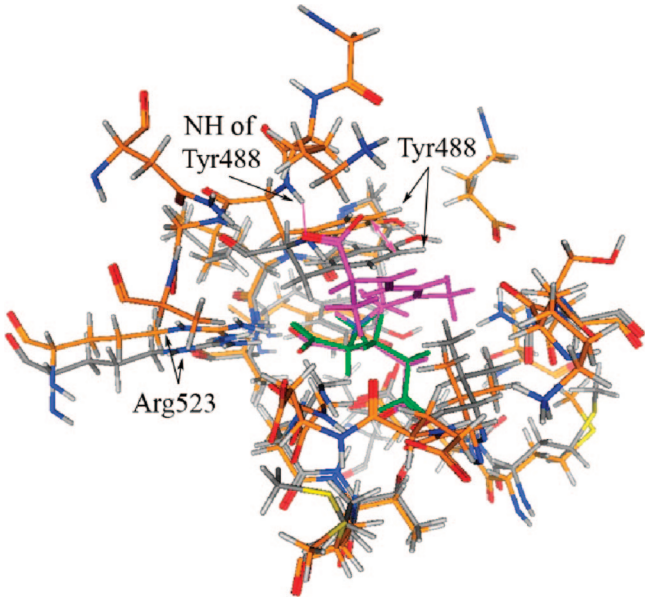


Figure 4. Superimposition of the binding pockets of iGluR6 crystallized with Glu (amino acid residues in type code; Glu ligand in green; PDB code: 1s50) and crystallized with DOMO (amino acid residues in orange; DOMO ligand in pink; PDB code: 1yae).[15] The carboxylate group of the side chain of DOMO hydrogen bond (pink line) to the backbone NH of residue Tyr488, thus stabilizing a more voluminous state of the iGluR6 binding pocket (pink arrow: Tyr488 is moved 1.85 Å up-and-backward).

comparison also at iGluR5,6 (Table 4 and Figure 1). The binding affinity of KA at iGluR7 (K_i = 32.8 nM) is comparable with the affinities for iGluR5,6 and KA1,2. In contrast, DHK shows high nanomolar affinity for iGluR7 (K_i = 376 nM). This is a 20−30 fold increase in affinity as compared with iGluR5,6 (K_i = 8−12 μM), which indicates that the iGluR7 binding pocket is more voluminous. The sterically more bulky KA like ligand, domoric acid (DOMO), exhibits nanomolar binding affinity for iGluR7 (K_i = 3.84 nM) comparable with values found for iGluR5,6 (K_i = 1−6 nM). Quisqualic acid (QUIS) is, however, 10-fold less prone to bind to iGluR7 as compared with iGluR5,6. Finally, the binding affinity for the endogenous ligand of the iGluR7 subtype, Glu, was determined (K_i = 494 nM), a value comparable to that of iGluR5,6 (K_i = 140−331 nM). In general, the profile of our pharmacological data for this small series of commercially available iGluR ligands is in agreement with data reported earlier using [^{3}H]KA as the radioligand at cloned iGluR5−7 expressed in HEK293 cells.[16]

Conclusion

In conclusion, we have presented the chemo-enzymatic enantioselective synthesis of five (2*S*)-2,4-*syn*-4-substituted Glu analogues **2a**−**c**,**e**,**f** and the subsequent pharmacological evaluation of nine Glu analogues **2a**−**j** as potential AMPA, KA, and NMDA ligands in rat synaptosomal binding assays. Furthermore, **2b**−**j** were characterized in binding assays at cloned rat KA receptor subtypes iGluR5−7. Methylester **2e** was shown to have high binding affinity (low nanomolar) at iGluR5 with 120- and 25-fold selectivity over iGluR6,7, respectively. What was quite unexpected was that fact that methylamide **2h** proved to be a high affinity ligand at all three subtypes iGluR5−7. We performed an in silico study to address this finding and have put forward a hypothesis as to why this is. In addition, we have presented iGluR7 binding data for a selected series of commercially available iGluR ligands and shown that Glu itself is a ligand at iGluR7 with affinity comparable with values found for iGluR5,6.

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. ^{1}H 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 $F2_{54}$ plates were used for column and TLC chromatographies. 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. *E. 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 ϵ_{NADH} = 6220 cm^{-1}·M^{-1}.

The synthesis of **2 g**−**j** was described previously by us.[7]

Methyl 2-(benzyloxymethyl)acrylate 3b. To a solution of methyl 2-(bromomethyl)acrylate[9] (5 g, 27.9 mmol) and benzyl alcohol (5.4 mL, 51.8 mmol) in THF (5 mL) cooled at 0 °C was added dropwise a solution of DABCO (4.37 g, 39 mmol) in THF (20 mL). The suspension of white solid was heated at 70 °C for 24 h. After filtration, the solution was washed with an aqueous 1% HCl solution (2 × 20 mL), an aqueous saturated $NaHCO_3$ solution (20 mL), with brine (20 mL), dried over $MgSO_4$ and concentrated under reduced pressure. Flash-chromatography (eluent, CH_2Cl_2) afforded **3b** as a colorless liquid (4.6 g, 80%). IR (neat film) 1720, 1637 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 (5H, m), 6.26 (1H, d, J = 0.8 Hz), 5.87 (1H, d, J = 0.8 Hz), 4.52 (2H,s), 4.17 (3H, s), 3.69 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.2, 138.2, 137.6, 128.3, 127.9, 127.5, 125.6, 72.8, 68.5, 51.5.

1-Benzyl-4-methyl Itaconate 3d. A solution of itaconic anhydride (6 g, 53.5 mmol) in MeOH (27 mL) was refluxed for 30 h. MeOH was evaporated, and the white residual solid was dissolved in toluene (40 mL). To this solution was added 1,8 diazabicyclo[5.4.0]undec-7-ene (DBU, 9.0 mL, 53.5 mmol) and then, dropwise, benzylbromide (6.35 mL, 53.5 mmol). The reaction mixture was stirred at room temperature for 70 min. The solution was then washed with an aqueous saturated $NaHCO_3$ solution (20 mL), with brine (20 mL), dried over $MgSO_4$, and concentrated under reduced pressure to give **3d** isolated as a colorless liquid (11.8 g, 95%). IR (neat film) 1732, 1641 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (5H, m), 6.39 (1H, d, J = 0.8 Hz),5.73 (1H, td, J = 0.8 and 1,2 Hz), 5.21 (2H,s), 3.64 (3H, s), 3.36 (2H, d, J = 1.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.1, 165.9, 135.8, 133.7, 128.9, 128.5, 128.2, 128.1, 66.8, 52.0, 37.6. HRMS (ES+) m/z 257.0784 ([M + Na]$^+$, $C_{13}H_{14}NaO_4$ requires 257.0790). Anal. ($C_{13}H_{14}O_4$): C, H, N.

1-Benzyl-5-methyl 2-methylideneglutarate 3e. To a solution of benzyl 2-(hydroxymethyl)acrylate[10] (5 g, 26.0 mmol) in toluene (60 mL) were added trimethyl orthoacetate (6.6 mL, 52.0 mmol) and propionic acid (0.2 mL, 2.6 mmol). The reaction mixture was heated at 100 °C for 1.5 h before methanol was slowly removed by azeotropic distillation. The solution was concentrated under reduced pressure. Flash-chromatography (eluent, cyclohexane−AcOEt, 8:2, v/v) afforded **3e** isolated as colorless liquid. (4.71 g, 73%). IR (neat film) 1738, 1720, 1632 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 (5H, m), 6.27 (1H, s), 5.65 (1H, s), 5.23 (2H, s), 3.69 (3H, s), 2.69 (2H, t, J = 7.5 Hz), 2.54 (2H, t, J = 7.5 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.1, 166.4, 138.8, 135.9, 128.6, 128.2, 128.1, 126.3, 66.5, 51.6, 32.9, 27.3. HRMS (ES+) m/z

Table 4. Binding Affinities of Commercially Available Standard iGluR Ligands: KA, DHK, DOMO, and QUIS at Cloned Homomeric Rat KA1,2 and Homomeric Rat iGluR5-7 Subtypes[a]

compound	rat KA1 K_i (nM)	rat KA2 K_i (nM)	rat iGluR5 K_i (nM)	rat iGluR6 K_i (nM)	rat iGluR7 K_i (nM)
KA	4.7[b]	15[c]	75.9 [7.15 ± 0.08]	12.7 [7.91 ± 0.06]	32.8 [8.42 ± 0.04]
DHK	nd	nd	8180 [5.09 ± 0.02]	12300 [4.92 ± 0.05]	376 [6.44 ± 0.07]
DOMO	40[b]	nd	1.11 [8.97 ± 0.09]	6.04 [8.24 ± 0.08]	3.84 [8.42 ± 0.04]
QUIS	nd	nd	171 [6.77 ± 0.01]	134 [6.88 ± 0.05]	1670 [5.86 ± 0.13]
Glu	nd	nd	140 [6.86 ± 0.01]	331 [6.48 ± 0.02]	494 [6.31 ± 0.03]
(2*S*,4*R*)-Me-Glu	nd	nd	0.663 [9.18 ± 0.02]	17.0 [7.83 ± 0.09]	5.69 [8.33 ± 0.10]

[a] Mean value from at least three experiments conducted in triplicate is shown [pK_i (M) ± S.E.M.]. nd = no data available. [b] Value taken from ref 17. [c] Value taken from reference 18.

271.0959 ([M + Na]$^+$, $C_{14}H_{16}NaO_4$ requires 271.0946). Anal. ($C_{14}H_{16}O_4$): C, H, N.

General Procedure for the Synthesis of 4b−e. To a solution of acrylate **3b−e** (20 mmol) in MeOH (**3b** and **3c**) or DMSO (**3d** and **3e**) (40 mL) were added KF (3.5 g, 60 mmol) and methyl or benzyl acetoacetate (30 mmol). The reaction mixture was heated at 65 °C for 1−16 h until the disappearance of acrylate **3**. The reaction mixture was then diluted with brine (40 mL) and extracted with AcOEt (4 × 50 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. Flash chromatography (eluent, cyclohexane−AcOEt, 8:2 or CH_2Cl_2−MeOH, 99:1 for **4b**, v/v) afforded **4b−e** as colorless liquids and as 1:1 mixtures of diastereomers.

Dimethyl 2-Acetyl-4-(benzyloxymethyl)glutarate 4b. Yield 95%. IR (neat film) 1738 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (2 × 5H, m), 4.49 (2 × 2H, m), 3.73 (3H, s), 3.70 (3H, s), 3.69 (2 × 3H, s), 3.76−3.57 (2 × 3H, m), 2.69 (2 × 1H, m), 2.24 (3H, s), 2.22 (3H, s), 2.30−2.05 (2 × 2H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.5, 173.7, 169.5, 137.8, 128.4, 127.7, 127.6, 73.1, 70.7, 70.3, 57.2, 56.8, 52.5, 51.9, 43.6, 43.5, 29.5, 29.0, 26.9, 26.8. HRMS (ES+) *m*/*z* 345.1306 ([M + Na]$^+$, $C_{17}H_{22}NaO_6$ requires 345.1314).

Trimethyl 5-Oxohexane-1,2,4-tricarboxylate 4c. Yield 78%. IR (neat film) 1737 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.72 (3H, s), 3.69 (3H, s), 3.66 (3H, s), 3.63 (2 × 3H, s), 3.63−3.57 (2 × 1H, m), 2.75 (2 × 1H, m), 2.75−2.35 (2 × 2H, m), 2.24 (3H, s), 2.21 (3H, s), 2.15−2.00 (2 × 2H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.0, 201.8, 174.2, 171.7, 169.4, 169.3, 57.3, 56.6, 52.6, 52.0, 51.8, 51.7, 39.2, 39.0, 36.3, 36.0, 29.8, 29.7, 29.5, 29.0. HRMS (ES+) *m*/*z* 297.0935 ([M + Na]$^+$, $C_{12}H_{18}NaO_7$ requires 297.0950).

2,4-Dibenzyl-1-methyl 5-oxohexane-1,2,4-tricarboxylate 4d. Yield 66%. IR (neat film) 1745, 1712 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (4 × 5H, m), 5.12 (4 × 2H, m), 3.62 (2 × 1H, m), 3.60 (2 × 3H, s), 2.84 (2 × 1H, m), 2.72 (2 × 1H, m), 2.48 (2 × 1H, m), 2.13 (3H, s), 2.10 (3H, s), 2.20−2.00 (2 × 2H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 201.9, 201.7, 173.6, 171.6, 168.8, 168.6, 135.6, 135.1, 128.7, 128.6, 128.4, 128.2, 128.1, 128.0, 67.4, 66.8, 57.4, 56.6, 51.8, 39.3, 39.1, 36.5, 36.1, 29.7, 29.6, 29.5, 29.0. HRMS (ES+) *m*/*z* 449.1566 ([M + Na]$^+$, $C_{24}H_{26}NaO_7$ requires 449.1576).

3,5-Dibenzyl-1-methyl 6-oxoheptane-1,3,5-tricarboxylate 4e. Yield 75%. IR (neat film) 1737 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (4 × 5H, m), 5.05 (4 × 2H, m), 3.56 (2 × 3H, s), 3.43 (2 × 1H, m), 2.34 (2 × 1H, m), 2.02 (2 × 3H, s), 2.25−1.70 (2 × 6H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.0, 201.8, 174.3, 173.0, 168.9, 168.7, 135.7, 135.2, 135.1, 128.6, 128.5, 128.4, 67.31, 67.28, 66.6, 57.4, 56.6, 51.6, 42.5, 42.3, 31.4, 31.3, 29.8, 29.0, 27.5, 27.1. HRMS (ES+) *m*/*z* 463.1724 ([M + Na]$^+$, $C_{25}H_{28}NaO_7$ requires 463.1733).

General Procedure for the Synthesis of 5b−e. To a solution of **4b−e** (10 mmol) in anhydrous pyridine (20 mL) was added acetyl chloride (1.1 mL, 15 mmol). The mixture was stirred at room temperature for 3−48 h until the complete disappearance of **4b−e**. The solution was then diluted with Et_2O (50 mL), washed with an aqueous saturated solution of $CuSO_4$ (2 × 50 mL), with water (50 mL), dried over $MgSO_4$ and concentrated under reduced pressure. Flash chromatography (eluent, cyclohexane−AcOEt, 8:2, v/v) afforded **5b−e** isolated as colorless liquids and as a single stereomer.

Dimethyl 2-(1-Acetoxyethylidene)-4-(benzyloxymethyl) Glutarate 5b. Yield 64%. IR (neat film) 1762, 1723, 1653 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 (5H, m), 4.47 (2H, m), 3.73 (3H, s), 3.66 (3H, s), 3.60 (1H,dd, J = 7.2 and 9.0 Hz), 3.52 (1H, dd, J = 5.50 and 9.2 Hz), 2.81 (1H, m), 2.57 (2H, m), 2.28 (3H, s), 2.10 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.1, 168.1, 167.5, 158.7, 138.1, 128.3, 127.5, 119.2, 73.0, 70.1, 51.8, 45.0, 26.4, 20.8, 19.6. HRMS (ES+) *m*/*z* 387.1427 ([M + Na]$^+$, $C_{19}H_{24}NaO_7$ requires 387.1420).

Trimethyl 5-Acetoxyhex-4-ene-1,2,4-tricarboxylate 5c. Yield 86%. IR (neat film) 1760, 1732, 1651 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.73 (3H, s), 3.64 (3H, s), 3.62 (3H, s), 2.92 (1H, m), 2.62 (2H, m), 2.43 (2H, m), 2.26 (3H, s), 2.16 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.6, 172.1, 167.9, 167.2, 159.2, 118.6, 51.9, 51.8, 51.7, 40.2, 35.0, 29.1, 20.8, 19.6. HRMS (ES+) *m*/*z* 339.1057 ([M + Na]$^+$, $C_{14}H_{20}NaO_8$ requires 339.1056).

2,4-Dibenzyl-1-methyl 5-acetoxyhex-4-ene-1,2,4-tricarboxylate 5d. Yield 86%. IR (neat film) 1760, 1732, 1650 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (10H, m), 5.18 (2H, s), 5.08 (2H, s), 3.58 (3H, s), 3.01 (1H, m), 2.68 (2H, m), 2.46 (2H, m), 2.26 (3H, s), 2.14 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.5, 173.9, 168.0, 166.6, 159.5, 135.9, 135.6, 128.6, 128.5, 128.3, 128.1, 118.8, 66.8, 66.6, 51.7, 40.4, 35.1, 29.3, 20.8, 19.7. HRMS (ES+) *m*/*z* 491.1692 ([M + Na]$^+$, $C_{26}H_{28}NaO_8$ requires 491.1682).

3,5-Dibenzyl-1-methyl 6-acetoxyhept-5-ene-1,3,5-tricarboxylate 5e. Yield 91%. IR (neat film) 1758, 1736, 1651 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (10H, m), 5.17 (2H, m), 5.05 (2H, s), 3.62 (3H, s), 2.68 (1H, dd, J = 8.2 and 13.1 Hz), 2.59 (1H, m), 2.43 (1H, dd, J = 6.1 and 13.1 Hz), 2.26 (3H, s), 2.22 (2H, m), 2.15 (3H, s), 1.95−1.75 (2H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.6, 173.1, 168.0, 166.7, 159.0, 135.9, 135.7, 128.6, 128.5, 128.3, 128.2, 119.2, 66.7, 66.4, 51.6, 43.9, 31.6, 30.0, 27.0, 20.8, 19.7. HRMS (ES+) *m*/*z* 505.1847 ([M + Na]$^+$, $C_{27}H_{30}NaO_8$ requires 505.1838).

General Ozonolysis Procedure for the Synthesis of 6c−e. A solution of enol acetate **5c−e** (5 mmol) in anhydrous CH_2Cl_2 (30 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. Dimethyl sulfide (0.55 mL, 7.5 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, 8:3, v/v or AcOEt for **6c**) afforded **6c−e** as colorless liquids.

Trimethyl 4-Oxobutane-1,2,4-tricarboxylate 6c. Yield 72%; IR (neat film) 1732 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.84 (3H, s), 3.65 (2 × 3H, s), 3.35 (2H, m) 3.02 (1H, m), 2.73 (1H,

dd, J = 5.8 and 16.8 Hz), 2.60 (1H, dd, J = 6.8 and 16.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.2, 173.2, 171.6, 160.7, 53.1, 52.4, 51.9, 39.9, 36.2, 34.8. HRMS (ES+) m/z 269.0634 ($[M + Na]^+$, $C_{10}H_{14}NaO_7$ requires 269.0637). Anal. ($C_{10}H_{14}O_7$): C, H, N.

2,4-Dibenzyl-1-methyl 4-oxobutane-1,2,4-tricarboxylate 6d. Yield 73%; IR (neat film) 1736 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 (10H, m), 5.27 (2H, s), 5.12 (2H, m), 3.63 (3H, s), 3.42 (2H, m) 3.09 (1H, m), 2.78 (1H, dd, J = 5.6 and 16.8 Hz), 2.64 (1H, dd, J = 6.4 and 16.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.5, 172.7, 171.6, 160.1, 135.4, 134.4, 128.8, 128.7, 128.6, 128.3, 128.2, 68.1, 67.1, 51.9, 40.0, 36.4, 34.9. HRMS (ES+) m/z 421.1270 ($[M + Na]^+$, $C_{22}H_{22}NaO_7$ requires 421.1263). Anal. ($C_{22}H_{22}O_7$): C, H, N.

1,3-Dibenzyl-5-methyl 1-oxopentane-1,3,5-tricarboxylate 6e. Yield 59%; IR (neat film) 1736 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 (10H, m), 5.24 (2H, s), 5.08 (2H, s), 3.62 (3H, s), 3.30 (2H, dd, J = 8.4 and 18.1 Hz), 2.98 (1H, m), 2.93 (1H, dd, J = 4.7 and 18.1 Hz), 2.33 (2H, m), 2.05−1.80 (2H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.7, 173.6, 172.9, 160.1, 135.5, 134.3, 128.8, 128.7, 128.6, 128.3, 128.2, 68.1, 66.9, 51.7, 40.8, 39.2, 31.3, 26.6. HRMS (ES+) m/z 435.1423 ($[M + Na]^+$, $C_{23}H_{24}NaO_7$ requires 435.1420). Anal. ($C_{23}H_{24}O_7$): C, H, N.

Dimethyl 2-(Benzyloxymethyl)-4-oxoglutarate 6b. To a solution of **5b** (0.83 g, 2.3 mmol) in $CHCl_3$ (8 mL) were added CH_3CN (8 mL), H_2O (15 mL), $NaIO_4$ (1.95 g, 9.1 mmol), and RuO_2 (60 mg, 0.45 mmol). The mixture was stirred vigorously at room temperature for 4 h. After filtration through celite, the organic layer was isolated and the aqueous layer extracted with $CHCl_3$ (10 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. Flash chromatography (eluent, cyclohexane−AcOEt, 7:3, v/v) afforded **6b** as a colorless liquid (0.29 g, 43%). IR (neat film) 1736 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 (5H, m), 4.50 (2H, s), 3.86 (3H, s), 3.72 (3H, s), 3.71 (2H, m), 3.44 (1H, dd, J = 8.1 and 18.1 Hz), 3.31 (1H, m), 3.10 (2H, dd, J = 5.3 and 18.1 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.9, 172.5, 160.9, 137.6, 128.4, 127.8, 127.6, 73.1, 69.4, 53.0, 52.2, 41.2, 38.1. HRMS (ES+) m/z 317.1008 ($[M + Na]^+$, $C_{15}H_{18}NaO_6$ requires 317.1001). Anal. ($C_{15}H_{18}O_6$): C, H, N.

Dimethyl 2-(Hydroxymethyl)-4-oxoglutarate 6a. To a solution of **5b** (0.14 g, 0.47 mmol) in MeOH (8 mL) was added 10% Pd/C (20 mg). The solution was degassed under reduced pressure and then flushed with hydrogen using a balloon. The suspension was stirred at room temperature for 1 h before filtration on a 0.2 μm membrane and concentration under reduced pressure. Flash chromatography (eluent, cyclohexane−AcOEt, 5:5, v/v) afforded **6a** as a colorless liquid (93 mg, 97%). As shown by NMR, **6a** exists in solution as a 1:1 mixture of hemiacetals. IR (neat film) 3458, 1737 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.44 (1H, s), 4.30 (1H, t, J = 8.4 Hz), 4.29 (1H, dd, J = 6.3 and 8.7 Hz), 4.17 (1H, t, J = 8.6 Hz), 4.15 (1H, t, J = 8.5 Hz), 4.12 (1H, s), 3.81 (2 × 3H, s), 3.73 (3H, s), 3.71 (3H, s), 3.43 (1H, m), 3.29 (1H, m), 2.71 (1H, dd, J = 9.4 and 13.8 Hz), 2.68 (1H, dd, J = 9.2 and 13.9 Hz), 2.42 (1H, dd, J = 5.7 and 13.7 Hz), 2.32 (1H, dd, J = 8.4 and 13.1 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.4, 172.7, 170.5, 170.2, 102.6, 102.2, 70.4, 53.3, 53.2, 52.5, 52.3, 43.6, 42.7, 38.3. HRMS (ES+) m/z 227.0536 ($[M + Na]^+$, $C_8H_{12}NaO_6$ requires 227.0532). Anal. ($C_8H_{12}O_6$): C, H, N.

General Procedure for the Synthesis of Lithium Oxoglutarates 1a, 1b, and 1c. To a solution of **6a**, **6b**, or **6c** (1 mmol) in MeOH (5 or 7.5 mL for **6c**) was added dropwise a 0.4 M solution of LiOH (5.25 mL, 2.1 mmol or 7.75 mL, 3.1 mmol for **6c**). The mixture was stirred at room temperature for 24 h. 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 of the water under reduced pressure. **1a**, **1b**, and **1c** were isolated in quantitative yields as white solids.

Dilithium 2-(Hydroxymethyl)-4-oxoglutarate 1a. ^{1}H NMR (400 MHz, D_2O) δ 4.40 (1H, t, J = 8.6 Hz), 4.34 (1H, t, J = 8.3 Hz), 4.24 (1H, dd, J = 6.5 and 8.4 Hz), 4.13 (1H, t, J = 8.7 Hz), 3.43 (1H, m), 3.30 (1H, m), 2.55 (1H, dd, J = 9.3 and 13.5 Hz), 2.39 (1H+2 × 1H, m). ^{13}C NMR (100 MHz, D_2O) δ 182.1, 181.5, 176.9, 176.7, 104.5, 104.1, 71.3, 71.2, 46.3, 45.3, 40.5, 40.1. HRMS (ES−) m/z 175.0236 ($[M - 2Li + H]^-$, $C_6H_7O_6$ requires 175.0243).

Dilithium 2-(Benzyloxymethyl)-4-oxoglutarate 1b. ^{1}H NMR (400 MHz, D_2O) δ 7.38 (5H, m), 4.52 (2H, s), 3.68 (1H, dd, J = 6.0 and 9.7 Hz), 3.59 (1H, dd, J = 6.7 and 9.7 Hz), 3.05 (1H, dd, J = 7.2 and 17.1 Hz), 2.96 (1H, m), 2.84 (1H, dd, J = 4.8 and 17.2 Hz). ^{13}C NMR (100 MHz, D_2O) δ 204.1, 180.6, 169.4, 137.4, 128.7, 128.4, 128.1, 72.6, 71.1, 43.6, 39.3. HRMS (ES−) m/z 271.0795 ($[M - Li]^-$, $C_{13}H_{12}LiO_6$ requires 271.0794).

Trilithium 4-Oxobutane-1,2,4-tricarboxylate 1c. ^{1}H NMR (400 MHz, D_2O) δ 3.00 (1H, dd, J = 5.9 and 16.4 Hz), 2.94 (1H, m), 2.84 (1H, dd, J = 5.0 and 16.4 Hz), 2.56 (1H, dd, J = 5.5 and 15.3 Hz), 2.28 (1H, dd, J = 8.6 and 15.3 Hz). ^{13}C NMR (100 MHz, D_2O) δ 202.6, 181.6, 179.6, 168.4, 41.1, 40.6, 40.1. HRMS (ES−) m/z 203.0196 ($[M - 2Li + H]^-$, $C_7H_7O_7$ requires 203.0192).

General Procedure for the Synthesis of Lithium Oxoglutarates 1d and 1e. To a solution of **6d** or **6e** (2 mmol) in MeOH (20 mL) was added 10% Pd/C (50 mg). The solution was degassed under reduced pressure and then flushed with hydrogen using a balloon. The suspension was stirred at room temperature for 30 min before filtration on a 0.2 μm membrane and concentration under reduced pressure. The residue was dissolved in H_2O (10 mL) and the solution brought to pH 7.6 by addition of an aqueous 0.4 M solution of LiOH. Water was evaporated under reduced pressure to give **1d** or **1e** isolated in quantitative yields as white solids.

Dilithium 2-(2-Methoxy-2-oxoethyl)-4-oxoglutarate 1d. ^{1}H NMR (400 MHz, D_2O) δ 3.66 (3H, s), 3.10 (1H, dd, J = 7.1 and 18.0 Hz), 3.05 (1H, m), 2.92 (1H, dd, J = 7.2 and 17.6 Hz), 2.61 (1H, dd, J = 7.8 and 16.4 Hz), 2.52 (1H, dd, J = 6.2 and 16.3 Hz). ^{13}C NMR (100 MHz, D_2O) δ 203.8, 181.4, 175.4, 169.4, 52.2, 41.4, 39.3, 36.3. HRMS (ES−) m/z 217.0351 ($[M - 2Li + H]^-$, $C_8H_9O_7$ requires 217.0348).

Dilithium 2-(3-Methoxy-3-oxopropyl)-4-oxoglutarate 1e. ^{1}H NMR (400 MHz, D_2O) δ 3.69 (3H, s), 3.04 (1H, dd, J = 7.8 and 17.5 Hz), 2.86 (1H, dd, J = 6.1 and 17.6 Hz), 2.65 (1H, m), 2.42 (2H, t, J = 7.8 Hz), 1.79 (2H, m). ^{13}C NMR (100 MHz, D_2O) δ 204.8, 182.1, 177.1, 170.2, 52.8, 42.4, 42.1, 32.1, 27.0. HRMS (ES−) m/z 231.0514 ($[M - 2Li + H]^-$, $C_9H_{11}O_7$ requires 231.0505).

(2*S*,4*S*)-4-(Benzyloxymethyl)glutamic Acid 2b. To a solution of racemic **1b** (154 mg, 0.55 mmol) in water (25 mL) was added cysteine sulfinic acid (84 mg, 0.55 mmol). The pH of the solution was adjusted to 7.6 with 1 M NaOH before the addition of *E. coli* AAT (0.4 mg, 20 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}·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 (0.1−1 M). The ninhydrin positive fractions were combined and concentrated under reduced pressure. The residue was dissolved in water (4 mL) and the solution was lyophilized to afford **2b** (64 mg, 43%) as a white solid; mp 144 °C; $[\alpha]^{25}_D$ = +31.0 (c 1.0, 6 N HCl). ^{1}H NMR (400 MHz, D_2O) δ 7.41 (5H, m), 4.58 (2H, s), 3.98 (1H, t, J = 6.8 Hz), 3.81 (1H, dd, J = 5.1 and 10.0 Hz), 3.76 (1H, dd, J = 5.8 and 10.0 Hz), 2.97 (1H, m), 2.38 (1H, m), 2.04 (1H, m). ^{13}C NMR (100 MHz, D_2O) δ 180.1, 174.2, 137.4, 128.7, 128.4, 128.2, 72.8, 71.4, 53.2, 44.8, 30.0. HRMS (ES−) m/z 266.1033 ($[M - H]^-$, $C_{13}H_{16}NO_5$ requires 266.1028). Anal. ($C_{13}H_{17}NO_5$·0.1H_2O) C, H, N.

(2*S*,4*R*)-4-(3-Methoxy-3-oxopropyl)glutamic Acid 2e. It was prepared following the procedure already described for **2b**, except for the first chromatography step on dowex 50WX8 that was omitted. **2e** was isolated as a white solid (45 mg, 39%); mp 135 °C; $[\alpha]^{25}_D = +29.5$ (*c* 1.0, 6 N HCl). ^{1}H NMR (400 MHz, D_2O) δ 3.75 (1H, dd, $J = 5.4$ and 8.6 Hz), 3.70 (3H, s), 2.58 (1H, m), 2.47 (2H, m), 2.25 (1H, ddd, $J = 5.2$, 9.6 and 14.8 Hz), 1.92 (3H, m). ^{13}C NMR (100 MHz, D_2O) δ 179.2, 176.2, 173.8, 53.1, 52.2, 41.8, 32.5, 31.2, 27.0. HRMS (ES+) *m*/*z* 234.0982 ($[M + H]^+$, $C_9H_{16}NO_6$ requires 234.0978). Anal. ($C_9H_{15}NO_6 \cdot 0.66H_2O$) C, H, N.

(2*S*,4*S*)-4-(Hydroxymethyl)glutamic Acid 2a. To a solution of **2b** (48 mg, 0.18 mmol) in H_2O (5 mL) was added 10% Pd/C (10 mg). The solution was degassed under reduced pressure and then flushed with hydrogen using a balloon. The suspension was stirred at room temperature for 4 h before filtration on a 0.2 μm membrane and concentration under reduced pressure. **2a** was purified by chromatography on dowex 1 × 8 as described for **2b** and isolated as a white solid (31 mg, 97%); mp 145 °C; $[\alpha]^{25}_D = +26.0$ (*c* 1.1, 6 N HCl). ^{1}H NMR (400 MHz, D_2O) δ 3.81 (3H, m), 2.76 (1H, m), 2.30 (1H, ddd, $J = 5.4$, 9.4 and 14.8 Hz), 1.99 (1H, ddd, $J = 4.6$, 8.0 and 14.8 Hz). ^{13}C NMR (100 MHz, D_2O) δ 180.6, 174.4, 63.3, 53.3, 47.0, 29.8. HRMS (ES+) *m*/*z* 178.0719 ($[M + H]^+$, $C_6H_{12}NO_5$ requires 178.0715). Anal. ($C_6H_{11}NO_5 \cdot 0.33H_2O$) C, H, N.

(2*S*,4*S*)-4-(2-Hydroxy-2-oxoethyl)glutamic Acid 2c. The ketoacid **1c** (170 mg, 0.74 mmol) was used in a transamination reaction as described for **2b**. When a conversion rate close to 40% was reached, an aqueous 6N HCl solution (25 mL) was added to the reaction mixture, which was then heated at 100 °C for 4 h before concentration under reduced pressure. The residue was dissolved in water (10 mL) and the pH adjusted to 7 before the two-step purification done as described for **2b**. **2c** was thus isolated as a white solid (63 mg, 41%); mp 145 °C; $[\alpha]^{25}_D = +8.0$ (*c* 1.0, 6 N HCl). ^{1}H NMR (400 MHz, D_2O) δ 3.88 (1H, dd, $J = 5.4$ and 8.1 Hz), 2.68 (1H, m), 2.98 (1H, m), 2.74 (2H, m), 2.34 (1H, ddd, $J = 5.7$, 10.0, 15.0 Hz). ^{13}C NMR (100 MHz, D_2O) δ 177.1, 175.4, 171.3, 51.2, 37.5, 35.6, 31.1. HRMS (ES+) *m*/*z* 206.0672 ($[M + H]^+$, $C_7H_{12}NO_6$ requires 206.0665). Anal. ($C_7H_{11}NO_6 \cdot 1.5H_2O$) C, H, N.

(2*S*,4*R*)-4-(3-Hydroxy-3-oxopropyl)glutamic Acid 2f. It was prepared from **1e** (250 mg, 1.0 mmol) following the procedure described for **2c**. **2f** was isolated as a white solid (90 mg, 41%); mp 95 °C; $[\alpha]^{25}_D = +6.4$ (*c* 1.1, 6 N HCl). ^{1}H NMR (400 MHz, D_2O) δ 3.77 (1H, dd, $J = 5.6$ and 8.2 Hz), 2.61 (1H, m), 2.41 (2H, m), 2.25 (1H, ddd, $J = 5.6$, 9.7 and 15.0 Hz), 1.89 (3H, m). ^{13}C NMR (100 MHz, D_2O) δ 178.5, 177.6, 173.4, 52.8, 41.2, 32.2, 31.2, 26.9. HRMS (ES+) *m*/*z* 220.0815 ($[M + H]^+$, $C_8H_{15}NO_6$ requires 220.0821). Anal. ($C_8H_{14}NO_6 \cdot 1.2H_2O$) C, H, N.

6-Oxopiperidine-2,4-dicarboxylic Acid 7. The ketoacid **1d** (250 mg, 1.08 mmol) was used in a transamination reaction as described for **2b**. After elution of the dowex 50WX8 resin with aqueous 1 M ammonia, the fractions containing **7** were concentrated under reduced pressure. The lactame **7** was isolated as a white solid (70 mg, 35%). mp 225 °C; $[\alpha]^{25}_D = -25.4$ (*c* 0.8, H_2O). ^{1}H NMR (400 MHz, D_2O) δ 4.20 (1H, dd, $J = 5.4$ and 10.2 Hz), 3.03 (1H, tdd, $J = 3.6$, 6.0, 9.6 Hz), 2.67−2.50 (3H, m), 2.74 (2H, m), 1.92 (1H, td, $J = 10.0$ and 13.6 Hz). ^{13}C NMR (100 MHz, D_2O) δ 177.2, 175.8, 173.5, 54.4, 36.9, 32.0, 27.3. HRMS (ES+) *m*/*z* 253.0674 ($[M + Na]^+$, $C_{10}H_{14}NaO_6$ requires 253.0688).

AMPA, KA, and NMDA Binding Assays. Glu analogues **2a**−**j** were evaluated for AMPA, KA, NMDA (CGP 39653) binding affinity in native rat synaptosomes, in accordance with previously described experimental procedures.[12]

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 assay buffer (50 mM Tris-HCl, pH 7.1 at 4 °C) at a protein concentration from 10−50 μg/mL, incubating for 1−2 h at 4 °C. Bound and free radiolabels were separated by cold filtration through GF/B glass fiber filters (UniFilter-96, Perkin-Elmer, Waltham MA) on a Perkin-Elmer FilterMate manifold using two washes with cold assay buffer. Nonspecific binding was determined in the presence of 1 mM *L*-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 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.[21]

mGluR Assays. Mammalian Cell Tissue Culture and Transfection. CHO cells were grown in T150 flasks in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) with supplements (10% bovine calf serum, 4 mM glutamine, 100 units/mL penicillin 100 μg/mL streptomycin) at 37 °C, 5% CO_2. Stock plates of CHO cells were trypsinized and split 1:6 every 3−4 days. COS-7 cells were grown in 225 cm2 flasks in Dulbecco's modified Eagle medium (DMEM) with supplements (10% bovine calf serum, 4 mM glutamine, 100 units/mL penicillin/100 μg/mL streptomycin) at 37 °C, 5% CO_2. Stock plates of COS-7 cells were trypsinized and split 1:6 every 3−4 days.

The rat metabotropic glutamate receptors 2 and 3 were cloned as follows; the coding regions of mGluR2 and mGluR3 were amplified by PCR from rat brain cDNA in 3 fragments that were ligated using internal restriction sites. A Kozak consensus sequence was included in the first PCR primer. The full-length receptor cDNAs were subcloned into mammalian expression vectors suitable for stable and transient expression. The chimera, C elegans G_α Qi3, cDNA was cloned by a PCR approach and sequenced by Lundbeck Research, USA, as previously described.[22,23] Both cDNAs were transiently transfected into CHO cells in T150 flasks by the Lipofectamine Plus (Invitrogen Carlsbad, California) method using a total of 10 μg of DNA/ ~ 7 × 10^6 cells. The standard cDNA transfection ratio was 8:2 (8 μg GPCR cDNA and 2 μg chimera Gα cDNA). The cDNAs for rat metabotropic glutamate receptors 1 and 5 were generous gifts from Prof. S. Nakanishi (Kyoto University, Kyoto, Japan). These cDNAs were transiently transfected into HEK293 cells using the same method.

Membrane Preparation and [^{3}2H]LY43915 Binding. CHO cells were transiently transfected as described above. They were harvested 48 h after transfection and washed 3 times with cold PBS. The cells were centrifuged at 1200 RPM, 4 °C, for 10 min, and the pellet was resuspended in wash buffer A (20 mM HEPES, 10 mM EDTA, pH 7.4). The pellet was sonicated on the Virsonic 300 model, 4 times in 30 s intervals on ice, centrifuged for 10 min at 1200 RPM, 4 °C. The supernatant was collected and centrifuged for 20 min at 17000 RPM. The pellet was resuspended in wash buffer B (20 mM HEPES, 0.1 mM EDTA, pH 7.4) and homogenized for 10 s. Protein concentration was determined by Bradford method.[24] The membrane was stored at −80 °C prior to use.

Binding assays were performed as described with slight modifications.[25] Briefly, after thawing, the membrane homogenates resuspended in 50 mM Tris-HCl, 2 mM $MgCl_2$, and 2 mM $CaKCl_2$ binding buffer at pH 7.0 to a final assay concentration of 10 μg protein/well for [^{3}H] LY341495 scintillation proximity assay (SPA) binding. Incubations included 2 nM [^{3}H] LY341495, 1.0 mg of WGA-SPA beads (GE Health, Buckinghamshire,UK).

Each well contained either buffer or varying concentrations of compound. Nonspecific binding was defined with 10 μM LY341495. After 45 min incubation on ice, the 96-well SPA microplate was further incubated at 4 °C overnight and counted with Wallac MicroBeta TriLux. At the K_d value, the nonspecific binding for [^{3}H]LY341495 was approximately 5%. IC_{50} values were derived from the inhibition curve and K_i values were calculated according to Cheng and Prusoff equation $K_i = IC_{50}/(1+ [L]/K_d)$ (Cheng and Prusoff 1973), where [L] is the concentration of radioligand and K_d is its dissociation constant at the receptor, derived from the saturation isotherm.

Calcium Mobilization Assay. Twenty-four hours after transient transfection, cells were seeded into 384-well black wall microtiter plates coated with poly-D-lysine for assay the following day. Just prior to assay, media was aspirated and cells were dye-loaded (25 μL/well) with 3 μM Fluo-4/0.01% pluronic acid in assay buffer (HBSS = Hank's buffered saline-check: 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, plus 20 mM HEPES, pH 7.4, 0.1% BSA and 2.5 mM probenicid) for 1 h in 5% CO_2 at 37 °C. After excess dye was discarded, cells were washed in assay buffer and layered with a final volume equal to 30 μL/well. Basal fluorescence was monitored in a fluorometric imaging plate reader (FLIPR, Molecular Devices) with an excitation wavelength of 488 nm and an emission range of 500 to 560 nm. Laser excitation energy was adjusted so that basal fluorescence readings were approximately 10000 relative fluorescent units. Cells were stimulated with an EC_{80} or EC_{10} concentration of glutamate in the presence of a test compound, both diluted in assay buffer, and relative fluorescent units were measured at defined intervals (exposure = 0.6 s) over a 3 min period at room temperature. Basal readings derived from negative controls were subtracted from all samples. Maximum change in fluorescence was calculated for each well. Concentration−response curves derived from the maximum change in fluorescence were analyzed by nonlinear regression (Hill equation).

In Silico Studies. The modeling software MOE (version 2006.08, Chemical Computing Group) was used with the built-in mmff94x force field and GB/SA solvation model. iGluR X-ray crystal structures (Brookhaven Protein Data Bank (PDB) data files) were submitted to the following preparative procedure: addition of hydrogen atoms, fixing heavy atoms, energy minimization, unfixing heavy atoms. Docking studies were carried out with parameter setup: explicit water molecules were excluded, ligand conformational database (LCD); placement methodology: *alpha triangle* with PH4-pharmacophore filtering (α-ammonium group, 1.6 vol) and pocket atom as restrains; London-dG scoring-function (readout: free energy of binding in kcal/mol). LCDs were generated by introducing two dihedral angle restrains to the Glu analogue to impose a *folded* conformation Glu scaffold, then subsequently performing a stochastic conformational search with standard parameter setup. (Dihedrals: $^+$NH3CH-CH2CHR: $-79.2 \pm 5°$, weight 100.000; CHCH2-CHRCOO$^-$: $-66.6 \pm 5°$, weight 100.000. Dihedral angle values adapted from Glu when crystallized with iGluR6, PDB code 1s50). Homology models: receptor protein sequences KA1(Q01812; 956aa), KA2(NP_113696; 979aa), iGluR5(P22756; 949aa), iGluR6-(P42260; 908aa), iGluR7(P42264; 919aa) were obtained from the Brookhaven Protein Data Bank and homology models built onto iGluR5 (PDB code: 1txf) using the built-in alignment algorithm (standard parameter setup).

Acknowledgment. We thank The Carlsberg Foundation, the Lundbeck Foundation, The Danish Medical Research Council, the French National Center for Scientific Research (CNRS), and the Novo Nordisk Foundation for funding. We are grateful to Pr. Kagamiyama's group (Osaka Medical College) for providing us with the AAT overexpressing *E. coli* strain. Dr. Susan Amara is thanked for providing us with the EAAT cDNAs.

Supporting Information Available: Combustion analysis data for **2a**−**f** and **6a**−**e**, ^{1}H and ^{13}C NMR spectra of compounds **2a**−**f**. Figures 2−4 as PDB and MOE file formats. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Meldrum, B. S. Glutamate as a neurotransmitter in the brain: Review of physiology and pathology. *J. Nutr.* **2000**, *130*, 1007S–1015S.
(2) Bräuner-Osborne, H.; Egebjerg, J.; Nielsen, E. O.; Madsen, U.; Krogsgaard-Larsen, P. Ligands for glutamate receptors: Design and therapeutic prospects. *J. Med. Chem.* **2000**, *43*, 2609–2645.
(3) Beart, P. M.; O'Shea, R. D. Transporters for L-glutamate: An update on their molecular pharmacology and pathological involvement. *Br. J. Pharmacol.* **2007**, *150*, 5–17.
(4) Wisden, W.; Seeburg, P. H. A Complex Mosaic of High-Affinity Kainate Receptors in Rat Brain. *J. Neurosci.* **1993**, *13*, 3582–3598.
(5) Ferraguti, F.; Shigemoto, R. Metabotropic glutamate receptors. *Cell Tissue Res.* **2006**, *326*, 483–504.
(6) Alaux, S.; Kusk, M.; Sagot, E.; Bolte, J.; Jensen, A. A.; Brauner-Osborne, H.; Gefflaut, T.; Bunch, L. Chemoenzymatic synthesis of a series of 4-substituted glutamate analogues and pharmacological characterization at human glutamate transporters subtypes 1−3. *J. Med. Chem.* **2005**, *48*, 7980–7992.
(7) Sagot, E.; Jensen, A. A.; Pickering, D. S.; Pu, X.; Umberti, M.; Stensbøl, T. B.; Nielsen, B.; Assaf, Z.; Aboab, B.; Bolte, J.; Gefflaut, T.; Bunch, L. 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. *J. Med. Chem.* **2008**, *51*, 4085–4092.
(8) Xian, M.; Alaux, S.; Sagot, E.; Gefflaut, T. Chemoenzymatic Synthesis of Glutamic Acid Analogues: Substrate Specificity and Synthetic Applications of Branched Chain Aminotransferase from *Escherichia coli*. *J. Org. Chem.* **2007**, *72*, 7560–7566.
(9) Borrell, J. I.; Teixido, J.; Martinez-Teipel, B.; Matallana, J. L.; Copete, M. T.; Llimargas, A.; Garcia, E. Synthesis and Biological Activity of 4-Amino-7-oxo-Substituted Analogues of 5-Deaza-5,6,7,8-tetrahydrofolic Acid and 5,10-Dideaza-5,6,7,8-tetrahydrofolic Acid. *J. Med. Chem.* **1998**, *41*, 3539–3545.
(10) O'Leary, B. M.; Szabo, T.; Svenstrup, N.; Schalley, C. A.; Lutzen, A.; Schafer, M.; Rebek, J. "Flexiball" Toolkit: A Modular Approach to Self-Assembling Capsules. *J. Am. Chem. Soc.* **2001**, *123*, 11519–11533.
(11) Johnson, W. S.; Werthema, L.; Bartlett, W. R.; Brocksom, T. J.; Li, T. T.; Faulkner, D. J.; Petersen, M. R. A Simple Stereoselective Version Of Claisen Rearrangement Leading To *trans*-Trisubstituted Olefinic Bonds. Synthesis Of Squalene. *J. Am. Chem. Soc.* **1970**, *92*, 741–743.
(12) Hermit, M. B.; Nielsen, B.; Greenwood, J. R.; Bunch, L.; Jorgensen, C. G.; Vestergaard, H. T.; Stensbol, T. B.; Sanchez, C.; Krogsgaard-Larsen, P.; Madsen, U.; Bräuner-Osborne, H. Ibotenic acid and thioibotenic acid: a remarkable difference in activity at group III metabotropic glutamate receptors. *Eur. J. Pharmacol.* **2004**, *486*, 241–250.
(13) Naur, P.; Vestergaard, B.; Skov, L. K.; Egebjerg, J.; Gajhede, M.; Kastrup, J. S. Crystal structure of the kainate receptor GluR5 ligand-binding core in complex with (*S*)-glutamate. *FEBS Lett.* **2005**, *579*, 1154–1160.
(14) Mayer, M. L. Crystal structures of the GluR5 and GluR6 ligand binding cores: molecular mechanisms underlying kainate receptor selectivity. *Neuron* **2005**, *45*, 539–552.
(15) Nanao, M. H.; Green, T.; Stern-Bach, Y.; Heinemann, S. F.; Choe, S. Structure of the kainate receptor subunit GluR6 agonist-binding domain complexed with domoic acid. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 1708–1713.
(16) Bettler, B.; Egebjerg, J.; Sharma, G.; Pecht, G.; Hermansborgmeyer, I.; Moll, C.; Stevens, C. F.; Heinemann, S. Cloning of a putative glutamate receptor: a low affinity kainate-binding subunit. *Neuron* **1992**, *8*, 257–265.
(17) Werner, P.; Voigt, M.; Keinanen, K.; Wisden, W.; Seeburg, P. H. Cloning of A Putative High-Affinity Kainate Receptor Expressed Predominantly in Hippocampal CA3 Cells. *Nature* **1991**, *351*, 742–744.
(18) Herb, A.; Burnashev, N.; Werner, P.; Sakmann, B.; Wisden, W.; Seeburg, P. H. The KA-2 subunit of excitatory amino acid receptors shows widespread expression in brain and forms ion channels with distantly related subunits. *Neuron* **1992**, *8*, 775–785.
(19) Emiliozzi, R.; Pichat, L. A simple method for the preparation of cysteinesulfinic acid. *Bull. Soc. Chim. Fr.* **1959**, 1887–1888.
(20) Kamitori, S.; Hirotsu, K.; Higuchi, T.; Kondo, K.; Inoue, K.; Kuramitsu, S.; Kagamiyama, H.; Higuchi, Y.; Yasuoka, N.; Kusunoki, M.; Matsuura, Y. Overproduction And Preliminary-X-Ray Characterization Of Aspartate Aminotransferase From *Escherichia Coli*. *J. Biochem.* **1987**, *101*, 813–816.

(21) Nielsen, B. S.; Banke, T. G.; Schousboe, A.; Pickering, D. S. Pharmacological properties of homomeric and heteromeric GluR1(o) and GluR3(o) receptors. *Eur. J. Pharmacol.* **1998**, *360*, 227–238.

(22) Walker, M. W.; Jones, K. A.; Tamm, J.; Zhong, H. L.; Smith, K. E.; Gerald, C.; Vaysse, P.; Branchek, T. A. Use of *Caenorhabditis elegans* G alpha(q) chimeras to detect G-protein-coupled receptor signals. *J. Biomol. Screening* **2005**, *10*, 127–136.

(23) Coward, P.; Chan, S. D. H.; Wada, H. G.; Humphries, G. M.; Conklin, B. R. Chimeric G proteins allow a high-throughput signaling assay of G(i)-coupled receptors. *Anal. Biochem.* **1999**, *270*, 242–248.

(24) Bradford, M. M. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(25) Johnson, M. P.; Barda, D.; Britton, T. C.; Emkey, R.; Hornback, W. J.; Jagdmann, G. E.; McKinzie, D. L.; Nisenbaum, E. S.; Tizzano, J. P.; Schoepp, D. D. Metabotropic glutamate 2 receptor potentiators: receptor modulation, frequency-dependent synaptic activity, and efficacy in preclinical anxiety and psychosis model(s). *Psychopharmacology* **2005**, *179*, 271–283.

JM800092X