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Chemoenzymatic Synthesis of New 2,4-*syn*-Functionalized (*S*)-Glutamate Analogues and Structure−Activity Relationship Studies at Ionotropic Glutamate Receptors and Excitatory Amino Acid Transporters

Zeinab Assaf,[‡,§] Anja P. Larsen,[†] Raminta Venskutonytė,[†] Liwei Han,[†] Bjarke Abrahamsen,[†] Birgitte Nielsen,[†] Michael Gajhede,[†] Jette S. Kastrup,[†] Anders A. Jensen,[†] Darryl S. Pickering,[†] Karla Frydenvang,*[,†] Thierry Gefflaut,*[,‡,§] and Lennart Bunch*[,†]

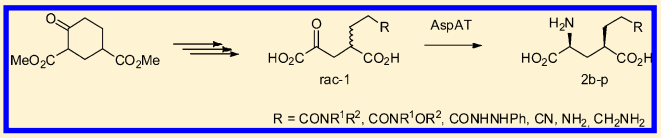
[†]Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, 2100 Copenhagen Ø, Denmark

[‡]Clermont Université, Université Blaise Pascal, Institut de Chimie de Clermont-Ferrand, BP 10448, F-63000 Clermont-Ferrand, France

[§]CNRS, UMR 6296, ICCF, 63177 Aubière, France

Supporting Information

ABSTRACT: In the mammalian central nervous system, (*S*)-glutamate (Glu) is released from the presynaptic neuron where it activates a plethora of pre- and postsynaptic Glu receptors. The fast acting ionotropic Glu receptors (iGluRs) are ligand gated ion channels and are believed to be involved in a vast number of neurological functions such as memory and learning, synaptic plasticity, and motor function. The synthesis of 14 enantiopure 2,4-*syn*-Glu analogues **2b−p** is accessed by a short and efficient chemoenzymatic approach starting from readily available cyclohexanone **3**. Pharmacological characterization at the iGluRs and EAAT1−3 subtypes revealed analogue **2i** as a selective GluK1 ligand with low nanomolar affinity. Two X-ray crystal structures of the key analogue **2i** in the ligand-binding domain (LBD) of GluA2 and GluK3 were determined. Partial domain closure was seen in the GluA2-LBD complex with **2i** comparable to that induced by kainate. In contrast, full domain closure was observed in the GluK3-LBD complex with **2i**, similar to that of GluK3-LBD with glutamate bound.



INTRODUCTION

In the mammalian central nervous system (CNS), (*S*)-glutamate (Glu) functions as the major excitatory neurotransmitter. Once released from the presynaptic neuron into the synapse, Glu activates a number of pre- and postsynaptic Glu receptors. On the basis of pharmacological function and signal transduction pathway, the Glu receptors have been grouped into two main classes: the fast acting ionotropic Glu receptors (iGluRs), which are ion channels,[1] and the G-protein-coupled metabotropic Glu receptors (mGluRs), which produce a much slower signal transduction through second messenger systems.[2] Uptake of Glu from the synaptic cleft is mediated by the excitatory amino acid transporters (EAATs), also termed the Glu transporters.[3−5] On the basis of phylogeny and ligand affinity studies, the iGluRs are divided into three groups named the 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoic acid (AMPA) receptors (subunits GluA1−4), kainic acid or kainate (KA) receptors (subunits GluK1−5), and the *N*-methyl-D-aspartate (NMDA) receptors (subunits GluN1, GluN2A−D, and GluN3A,B). Functional iGluRs are homo- or heterotetrameric in subunit composition, which has been shown to influence greatly the function of the receptor. Discovery of subtype selective ligands for the iGluRs is a highly challenging task but essential, as such ligands are exceedingly valuable compounds to be used in the study of the function of a specific iGluR in normal and diseased states.

In this paper the chemoenzymatic synthesis of a new series of 2,4-*syn* substituted Glu analogues is presented together with their pharmacological characterization at the iGluRs and the EAAT1−3 subtypes. Furthermore, crystal structures of the GluA2 ligand-binding domain (GluA2-LBD) construct and the GluK3 ligand-binding domain (GluK3-LBD) construct both in complex with **2i** were obtained and new insight into the structure−activity relationship of the AMPA and KA receptors is disclosed.

RESULTS AND DISCUSSION

Chemistry. Aminotransferases (ATs) are ubiquitous enzymes involved in proteinogenic α-amino acid metabolism by catalyzing the reversible conversion of 2-oxocarboxylic acids into L-α-amino acids. Most naturally occurring ATs accept Glu and its ketoacid homologue α-ketoglutarate (KG) as preferred substrates but are generally characterized by a relatively broad

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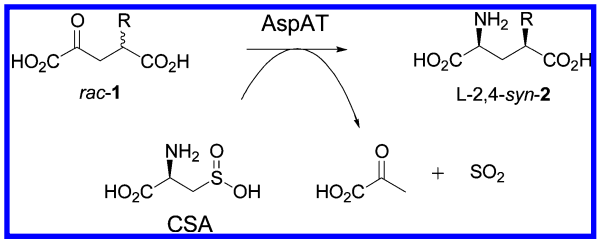
substrate spectrum.[6] Therefore, several ATs have proved to be useful for the stereoselective synthesis of Glu analogues. Notably, aspartate aminotransferase (AspAT) isolated from *Escherichia coli* has been shown to be highly effective as a catalyst for the stereospecific synthesis of a wide range of L-2,4-*syn*-substituted Glu analogues from racemic KG **1** (Scheme 1)

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

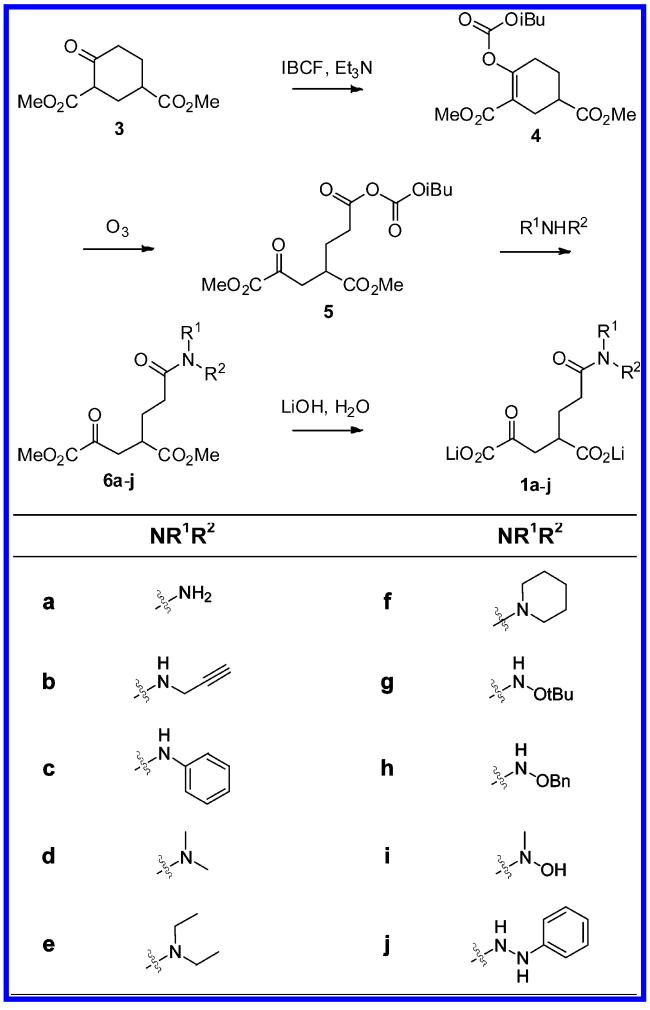


by kinetic resolution.[7−11] Moreover, AspAT accepts cysteine sulfinic acid (CSA) as amino donor substrate. This close analogue of aspartate, readily prepared from L-cystine,[12] is converted into pyruvylsulfinic acid, which is highly unstable and thus decomposes into pyruvic acid and sulfur dioxide, forcing the needed shift in equilibrium of the transamination reaction.[13] All herein reported new 2,4-*syn*-Glu analogues **2b−o** were prepared by enzymatic transamination of the corresponding 4-substituted-2-oxoglutarates **1a−j** (Scheme 1).

Previously, several pathways have been explored for the synthesis of the various substituted KG analogues needed as substrates of the transamination reaction. While a Claisen−Johnson rearrangement proved efficient to create the carbon skeleton of 4-alkyl analogues,[11,14] alternative methodologies were needed to prepare derivatives comprising functionalized substituents at the 4-position.[9,15] The new KG analogues **1a−j** described in the present study were prepared following an original and highly efficient synthetic route giving access to a variety of new functionalized derivatives.

Synthesis of 2-Oxoglutaric Acids. As shown in Scheme 2, the new KG analogues **1a−j** were prepared in four steps from cyclic *β*-keto ester **3**, itself readily prepared following a described procedure from methyl malonate and methyl acrylate.[16] Compound **3** was reacted with isobutyl chloroformate (IBCF) and Et_3N to give the corresponding enol carbonate **4** in quantitative yield. Oxidative cleavage of **4** with ozone afforded *α*-ketoester **5**, also comprising a mixed carbonic anhydride functionality. NMR analysis of the crude product indicated that **5** was formed in high yield (>90%), and this reactive intermediate was generally used directly in the following step without isolation. However, a CH_2Cl_2 solution of **5** could be stored at −20 °C for several weeks without noticeable degradation. Anhydride **5** appears as a key intermediate, as it can be reacted with numerous nucleophiles offering an easy access to chemical diversity: The use of various amines, hydroxylamines, and one hydrazine afforded the corresponding amides **6a−f**, hydroxamates **6g−i**, and hydrazide **6j** isolated after chromatography in 52−94% overall yield from **3**. Finally, the lithium salts **1a−j** were obtained in quantitative yield by selective ester hydrolysis using a stoichiometric amount of LiOH. Previously, compound **1a** was prepared from direct ozonolysis of the enamine formed by reaction of **3** with ammonia.[9] However, this approach suffered from the low yield of the enamine ozonolysis and **1a** was obtained in only 55%

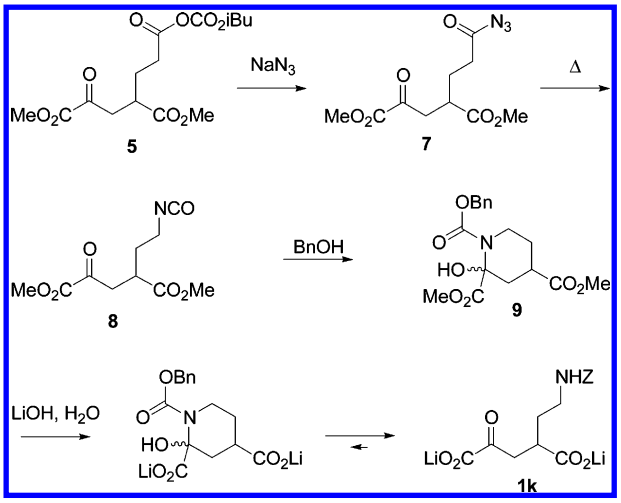
Scheme 2. Synthesis of 4-Substituted KG Analogues 1a−j



yield from **3**. Our new approach involving anhydride **5** gave **1a** with an overall yield of 84%. This result clearly demonstrates the efficiency of this new chemical route.

As shown in Scheme 3, anhydride **5** was also reacted with sodium azide to give the acyl azide intermediate **7**, which upon heating was converted into the isocyanate **8** via a Curtius rearrangement. As judged by ^1H NMR analysis of the crude

Scheme 3. Synthesis of the Carbamate 1k



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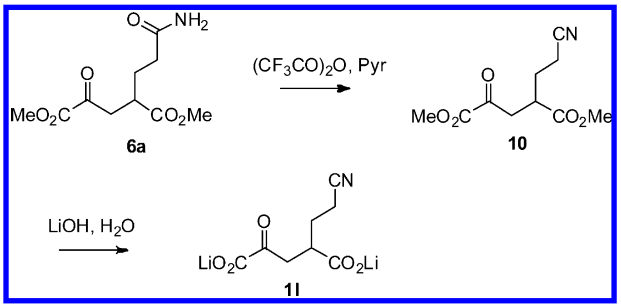
products, **7** and **8** were formed in nearly quantitative yield. Isocyanate **8** was used in the next reaction without purification, and reaction with benzyl alcohol afforded the KG **9** bearing a benzyloxycarbonyl (Cbz) substituted amino group, in an overall yield of 53% from **3** (five steps). It was then converted to the dilithium salt **1k** in quantitative yield. ^{1}H NMR analyses showed that **9** and **1k** exist in solution as cyclic forms resulting from the addition of the carbamate nitrogen to the ketone. For cyclic dimethyl KG **9**, one diastereoisomer was the major form (>85%) in $CDCl_3$ whereas KG **1k** was mainly present in D_2O in its acyclic form.

Finally, KG **10** was obtained by dehydration of the amide functionality of **6a** in the presence of trifluoroacetic anhydride and pyridine in 87% yield and was converted quantitatively to the dilithium salt **1l** without alteration of the nitrile functionality (Scheme 4).

Scheme 4. Synthesis of the Nitrile 1l

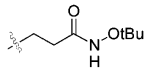
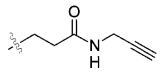
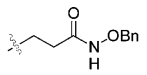
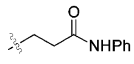
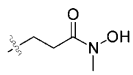
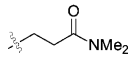
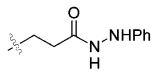
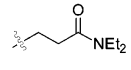
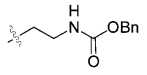
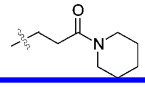
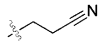


Transamination Reactions. The substituted KGs **1b–l** were evaluated as substrate of *E. coli* AspAT on the basis of the Michaelis–Menten model. Rate measurement of the AAT catalyzed transaminations between aspartate (40 mM) and KG substrates **1b–l** in variable concentrations allowed for the estimation of the kinetic parameters K_m and k_{cat} (Table 1).

These results are fully consistent with the previously observed substrate specificity of AspAT. All the newly prepared KG analogues proved to be substrates with K_m values in the millimolar range and k_{cat} of 2–112% relative to the natural substrate KG. We have observed by molecular modeling of the enzyme active site that substituents at position 4 fit in the hydrophobic channel connecting the active site pocket to the solvent (data not shown). This explains how bulky groups and long chains can be accepted by the enzyme, with a preference for hydrophobic moieties. KG analogues **1b–j** bearing an amide, hydroxamate, or hydrazide functionality are thus relatively poor substrates compared with the 4-alkyl derivatives described in earlier studies.[11] However, the measured activities are still valuable for synthetic purposes. The K_m and k_{cat} values measured for **1k** and **1l** are noteworthy, as they fall into the same range as the natural substrate KG. The kinetic parameters measured for nitrile **1l** were not surprising, with values comparable to those of the 4-propyl KG analogue (K_m = 0.4 mM, k_{cat} = 53%). However, the high k_{cat} value measured for **1k** (112%) was quite unexpected when compared to that of the structurally close analogue **1h** (4%).

Preparative scale transaminations were easily carried out for **1b–l** following the procedure previously described.[11] Cysteine sulfinic acid (CSA) was used as an irreversible amino donor, and the reaction was monitored by enzymatic titration of pyruvate formed from CSA. The reaction was stopped at approximately 40% conversion in order to perform the kinetic resolution of the racemic substrates. Glu analogues **2b–f**, **2k**, and **2l** were isolated after selective adsorption on sulfonic

Table 1. Kinetic Parameters of AspAT Catalyzed Transaminations with KG Analogues 1b–l[a]

compd		K_m (mM)	k_{cat} rel. (%)	compd		K_m (mM)	k_{cat} rel. (%)
KG		0.2 ± 0.1	100[b] ± 3.0	**1g**		2.1 ± 0.1	1.9 ± 0.2
1b		2.7 ± 0.2	34.8 ± 2.8	**1h**		4.3 ± 1.0	4.0 ± 0.9
1c		2.7 ± 0.2	3.9 ± 1.5	**1i**		4.7 ± 1.0	5.4 ± 1.2
1d		4.2 ± 1.0	13.9 ± 3.2	**1j**		3.2 ± 0.3	5.4 ± 0.5
1e		0.7 ± 0.1	21.2 ± 1.4	**1k**		0.9 ± 0.3	112 ± 13
1f		0.8 ± 0.1	7.6 ± 1.0	**1l**		0.7 ± 0.1	69.6 ± 2.0

[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 with KG was 39.6 ± 1.2 s^{-1}.

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Dowex 50 resin (H^+ form) and elution with aqueous ammonia. They were further purified by ion exchange chromatography on cationic Dowex 1 resin (AcO^- form) by elution with an AcOH gradient. Slightly different purification protocols were used for **2g–j**. Indeed, these derivatives proved to be unstable in basic and/or acidic solutions; **2g–j** were partially hydrolyzed to the carboxylic acid derivative in aqueous ammonia, whereas **2i** also proved unstable in aqueous AcOH solution. The first chromatographic step on Dowex 50 was thus omitted for **2g**, **2h**, and **2j**, which were instead purified by a single chromatographic step using Dowex 1. In the case of **2i** both chromatographic steps proved to be necessary, and elution with neutral aqueous ammonium bicarbonate solutions from Dowex 50 (H^+ form) and Dowex 1 (HCO_3^- form) was performed. Glu analogues **2b–l** were isolated in 35–45% yield from the corresponding racemic KG analogues **1b–l**. In all cases, only one stereoisomer was isolated with a diastereomeric excess over 98% as judged by NMR. AspAT was previously shown to display a very high stereoselectivity in favor of the L-2,4-*syn* configuration. This stereoselectivity was unambiguously demonstrated for the previously prepared 2-alkyl analogues[11] as well as for functionalized analogues including the amide or ester functionalities.[9,10] This stereoselectivity was again confirmed for four representative compounds of this series (**1c**, **1i**, **1h**, and **1l**), which gave after acidic hydrolysis the already described L-2,4-*syn*-4-carboxyethyl glutamate analogue (data not shown). This last compound was also obtained by hydrolysis of a previously prepared amide for which the L-2,4-*syn* configuration was demonstrated by NMR.[9] As shown in Scheme 5, free hydroxamate **2m** and amino derivatives **2n** and **2o** were prepared from **2h**, **2k**, and **2l**, respectively.

First attempts of acid catalyzed removal of the *tert*-butyl group of **2g** were unsuccessful resulting in hydrolysis of the hydroxamate functionality to the carboxylic acid and formation of the corresponding pyroglutamate analogue. Conversely, Pd/C catalyzed hydrogenation of **2h** proved to be more efficient. Debenzylation leading to **2m** was the main observed reaction. Although a small quantity (<5%) of primary amide was formed as a result of N–O cleavage, both compounds could easily be separated by ion exchange chromatography on Dowex 1 and **2m** was isolated in 85% yield.

Hydrogenation of **2k** in the presence of Pd/C also gave **2n** in quantitative yield. Attempts to prepare the amino homologue **2o** by direct hydrogenation of the nitrile **2l** gave mixtures of **2o** and the azepane **2p**, which was isolated in yield up to 56%. Moreover, the reaction required several days for completion and partial epimerization was observed at position 2 (up to 10%). To circumvent these problems, the amino group of **2l** was protected with a BOC group to give **11** in 95% yield. Nitrile reduction was then performed in the presence of Pd hydroxide and was complete within 90 min. Cleavage of the BOC group of **12** with 1 M HCl gave **2o** which was finally isolated after ion exchange chromatography with an overall yield of 80% from **2l**. No epimerization at the C2 position was observed under these conditions.

Pharmacology. The newly synthesized L-2,4-*syn*-4-substituted Glu analogues **2b–p** were evaluated as ligands for the iGluRs. In binding studies at native AMPA, KA, and NMDA receptors (Table 2) the Glu analogues showed mid- to high-range micromolar affinities (IC_{50} of 6.5 to >100 μM) and low receptor group selectivity except for cyano analogue **2l**, which displayed mid-nanomolar affinity (IC_{50} = 73 nM) at native KA receptors corresponding to a >90-fold selectivity over native AMPA and NMDA receptors.

Subsequently, binding affinities of **2b–p** were determined at recombinant homomeric rat KA subtypes GluK1–3 and AMPA subtype GluA2 expressed in *Sf9* cell membranes (Table 2). The affinity profile of all tested analogues **2b–p** showed the same trend across the three subtypes GluK1–3, displaying a relatively highest affinity for GluK1, lowest for GluK2, and intermediate affinity for GluK3 (Figure 1). This trend is often observed for orthosteric GluK1–3 agonists exemplified by a large number of L-2,4-*syn* Glu analogues[10,11,17] as well as conformationally restricted[18–20] Glu analogues.[21] Noteworthy, of the new series reported here, *N*-methyl hydroxamate **2i** displays low nanomolar affinity for GluK1 (K_i = 3.4 nM) with a 200- and 35-fold selectivity over GluK2 and GluK3, respectively (Table 2).

Subsequently, representative compounds of the Glu analogues **2b–p** were characterized pharmacologically at the human EAAT1,2,3 subtypes stably expressed in HEK293 cells in a [^{3}H]-D-aspartate uptake assay (Table 3).[24] While the majority of the analogues were without inhibitory activity at any of the three EAAT1–3 subtypes, analogue **2l** displayed activity at EAAT2, exhibiting an IC_{50} of 24 μM at this subtype and 9- and >40-fold lower inhibitory potencies at EAAT1 and EAAT3, respectively (Table 3). A comparison with previously reported EAAT inhibitory profiles of a large number of L-2,4-*syn*-Glu analogues suggests that the observed preference for EAAT2 is a trend for this class of compounds, although the structural basis for this remains unclear.[9,11] Since analogue **2l** shows nanomolar affinity for the KA receptors and micromolar affinity for the AMPA receptors (Table 2), it is not suitable as a pharmacological tool for the investigation of EAAT2 function.[5] However, in the same series we have previously shown that extending the side chain results in a selective EAAT2 inhibitor with low affinity for all of the iGluRs.[9]

Scheme 5. Synthesis of Hydroxamate 2m and Amino Derivatives 2n and 2o

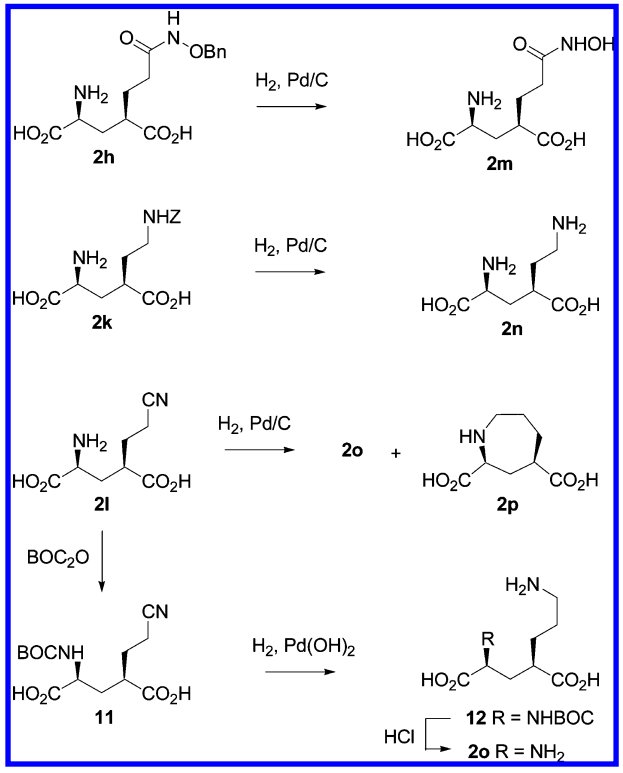

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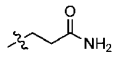
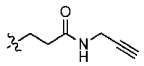
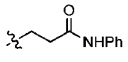
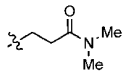
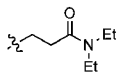
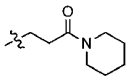
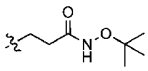
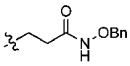
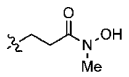
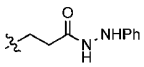
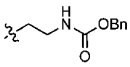
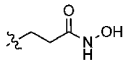
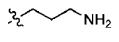
Table 2. Pharmacological Evaluation of 2b−p at Native and Recombinant Homomeric Rat iGluR

		AMPA[a] IC_{50} [μM]	KA[a] IC_{50} [μM]	NMDA[a] K_i [μM]	GluA2[b] K_i [μM]	GluK1[b] K_i [μM]	GluK2[b] K_i [μM]	GluK3[b] K_i [μM]
Glu	H	0.34[c]	0.38[c]	0.20[d]	0.282 ± 0.090	0.140 ± 0.003	0.331 ± 0.012	0.494 ± 0.031
2a[e]		25	16	>100	10.5	0.0075	9.7	2.8
2b		20 [4.71±0.07]	35 [4.46±0.03]	41 [4.39±0.04]	8.05 ± 1.11	0.096 ± 0.014	14.2 ± 3.4	0.185 ± 0.005
2c		59 [4.25±0.09]	78 [4.11±0.04]	> 100	56.2 ± 2.4	0.144 ± 0.023	34.6 ± 4.8	4.66 ± 0.09
2d		nt	nt	nt	49.2 ± 6.0	0.037 ± 0.003	>100	5.39 ± 0.29
2e		67 [4.18±0.05]	29 [4.54±0.02]	> 100	>100	0.413 ± 0.047	8.65 ± 0.10	3.29 ± 0.27
2f		60 [4.26±0.09]	50 [4.30±0.04]	> 100	71.1 ± 1.6	0.583 ± 0.147	30.9 ± 4.1	5.78 ± 0.60
2g		> 100	> 100	> 100	>100	3.18 ± 1.30	>100	44.5 ± 2.0
2h		> 100	> 100	> 100	>100	0.82 ± 0.25	28.4 ± 6.3	2.81 ± 0.21
2i		74 [4.14±0.05]	12 [4.94±0.04]	> 100	40.8 ± 3.9	0.0034 ± 0.0009	0.700 ± 0.062	0.123 ± 0.033
2j		> 100	> 100	> 100	53.3 ± 4.6	0.382 ± 0.136	>100	5.66 ± 0.29
2k		> 100	26 [4.60±0.06]	82 [4.09±0.03]	nt	0.340 ± 0.058	3.04 ± 0.32	0.217 ± 0.047
2l		6.5 [5.19±0.01]	0.073 [7.14±0.03]	> 100	nt	0.0172 ± 0.0032	0.560 ± 0.083	0.0345 ± 0.0024
2m		> 100	> 100	> 100	56.5 ± 3.5	0.0131 ± 0.0016	3.27 ± 0.38	0.239 ± 0.043
2n		> 100	19 [4.73±0.03]	> 100	nt	1.46 ± 0.08	10.9 ± 1.0	0.340 ± 0.016
2o		> 100	89 [4.05±0.01]	> 100	nt	5.53 ± 0.89	83.1 ± 6.1	10.2 ± 0.5
2p	Azepane	>100	25 [4.61±0.02]	67 [4.17±0.02]	nt	0.906 ± 0.146	21.8 ± 0.8	2.50 ± 0.29

[a]Data are mean values of three to four individual experiments performed in triplicate. For AMPA and KA: pIC_{50} values with SEM in brackets. For NMDA: pK_i values with SEM in brackets. [b]Mean ± SEM from at least three separate experiments, conducted in triplicate at 12−16 ligand concentrations. [c]Data taken from ref 22. [d]Data taken from ref 23. [e]Data taken from ref 10.

X-ray Crystal Structure Study. To explore in detail the structural basis for the high affinity binding of **2i** to GluK1 and the subtype selectivity determinants, an X-ray crystallographic study was undertaken. However, despite several attempts to crystallize **2i** in GluK1-LBD, crystals of suitable quality were not obtained. In contrast, crystals of **2i** in the GluK3-LBD and GluA2-LBD were achieved and their structures solved.

Structure of GluA2-LBD with 2i. The structure of GluA2-LBD in complex with **2i** was solved at 1.24 Å resolution (space group $P2_12_12$) with one molecule in the asymmetric unit of the crystal (Figure 2A). Generally, the electron density is very well-defined (for further information on data collection and refinement statistics, see Supporting Information). In the binding site, electron density outside the Glu parental skeleton was clearly identified and thus **2i** could be modeled herein (Figure 2B). The ligand **2i** induces only partial domain closure (13.8°) of the GluA2-LBD (Figure 2A), which has also been observed for the X-ray structure of the partial agonist KA in

dx.doi.org/10.1021/jm301433m | J. Med. Chem. 2013, 56, 1614−1628

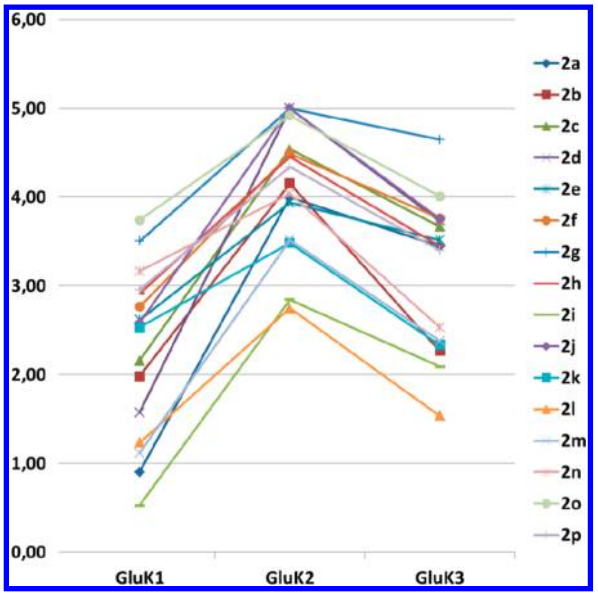


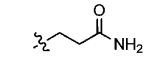
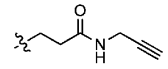
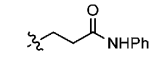
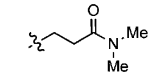
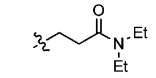
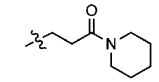
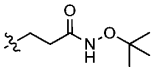
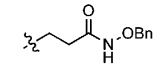
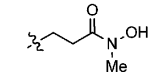
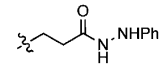
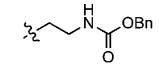
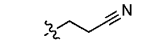
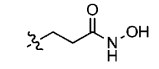
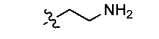
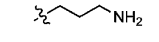
Figure 1. The log(K_i) (in nM) of Glu analogues **2a**–**p** at GluK1–3 subtypes.

GluA2-LBD (12.2°, PDB code 1FW0).[25] In this respect the binding affinity of **2i** at the GluA2-LBD construct was determined to be in the micromolar range (K_i = 126 ± 17 μM, n_H = 0.91 ± 0.10 (n = 3)), as also observed for KA at GluA2-LBD (K_i = 6.3 ± 1.5 μM, n_H = 1.14 ± 0.06 (n = 3)) The side chain comprising the hydroxamic acid functionality was built to point toward residues Tyr471 and Pro499. However, alternative conformations of **2i** with the hydroxamic acid moiety pointing toward residues Glu423 and Ser673 might also be present, but because of weak electron density, alternative conformations could not be modeled satisfactorily (see Supporting Information).

A careful examination of the crystal structure reveals that the amino acid part of **2i** forms almost identical contacts with surrounding residues (Pro499, Thr501, Arg506, Ser675, Thr676, and Glu726; full-length numbering including signal peptide) and water molecules (Figure 2B), as are observed in the structure of GluA2-LBD in complex with Glu (PDB code 1FTJ).[25] However, one contact within 3.5 Å is not seen for **2i**; the γ-carboxylate group of **2i** forms hydrogen bonds to two water molecules, whereas Glu establishes contact with three water molecules. This difference can be explained by the partial domain closure induced by **2i**, which allows the water molecule to move further away from the Glu-like part of **2i** in order to maintain contacts to protein backbone atoms. The distal carboxylate group forms strong hydrogen bonding interaction with the NH and the side chain OH of Thr676 and two water molecules of which one is bridged to the hydroxamic acid carbonyl group. The hydroxamic acid moiety of **2i** is accommodated in the binding site near residues Glu423, Tyr426, Tyr471, Pro499, Thr707, Thr728, Met729, and Tyr753. The carbonyl oxygen accepts two hydrogen bonds from nearby water molecules, whereas the N-hydroxyl group is in close contact with the hydroxyl group of Tyr471.

An analysis of the side chain conformation in residue Met729 in GluA2-LBD disclosed it to adapt distinct conformations depending on the bound agonist.[26] This is also observed in the GluA2-LBD:**2i** complex, where the hydroxamic acid moiety of **2i** causes Met729 to adopt a different conformation in the GluA2-LBD compared to structures with Glu and KA (Figure 2C).[25]

Table 3. Pharmacological Evaluation of 2b–c, 2e, 2f, 2h, 2i, and 2k–p at EAAT1–3

		EAAT1 IC_{50} [μM]	EAAT2 IC_{50} [μM]	EAAT3 IC_{50} [μM]
2a[a]		~3000	~1000	>3000
2b		>300	>300	>300
2c		>300	>300	>300
2d		nt	nt	nt
2e		>300	>300	>300
2f		>300	>300	>300
2g		nt	nt	nt
2h		>1000	~300	>1000
2i		>3000	~1000	>3000
2j		nt	nt	nt
2k		~100	~300	~100
2l		~200	24 [5.63 ± 0.06]	>1000
2m		>1000	~700	>1000
2n		>1000	>1000	>1000
2o		>1000	>1000	>1000
2p	Azepane	>300	>300	>300

[a]Data taken from ref 10.

*Structure of GluK3-LBD with **2i**.* The structure of GluK3-LBD in complex with **2i** was solved at 2.65 Å resolution (Figure 2A). The complex crystallized in space group $P4_122$ and contains one molecule in the asymmetric unit of the crystal (for further information on data collection and refinement statistics, see Supporting Information). The binding affinity of **2i** at the GluK3-LBD construct was determined to be in the high nanomolar range (K_i = 900 ± 230 nM, n_H = 0.93 ± 0.08 (n =

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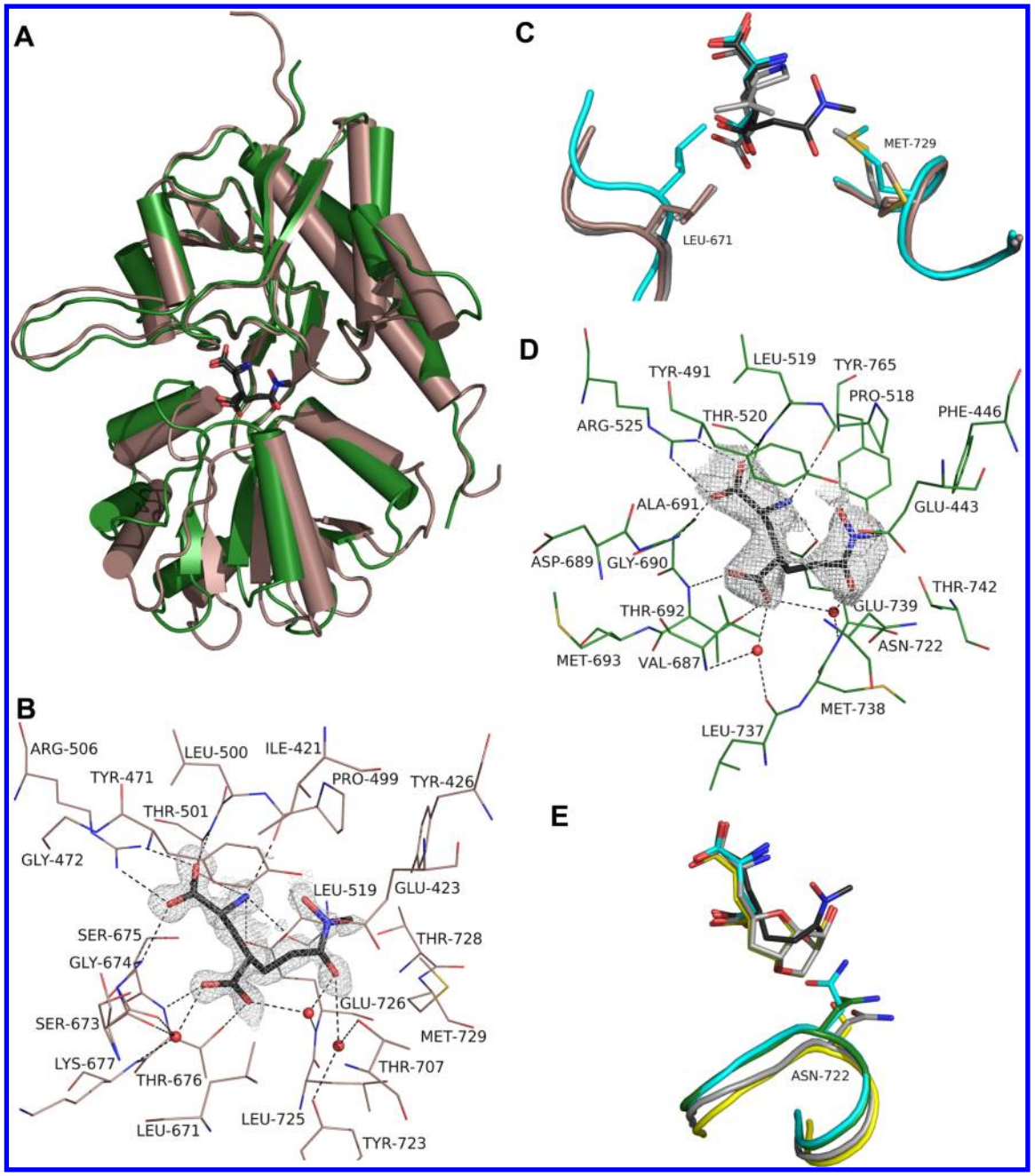

Figure 2. Structures of GluA2-LBD and GluK3-LBD, both in complex with **2i**. (A) The structures of GluA2-LBD and GluK3-LBD are shown in cartoon representation (superimposed on lobe D1 residues) to illustrate the different degree of domain closure induced by **2i** in GluA2-LBD and GluK3-LBD. GluA2-LBD is shown in dark pink and GluK3-LBD in dark green. **2i** in both structures is dark gray. (B) Zoom on the ligand-binding site of GluA2-LBD with **2i** and $2F_o - F_c$ omit map at 1σ carved around the ligand at 1.4 Å radius. Potential hydrogen bonds within 3.2 Å to water molecules (red spheres) and surrounding residues are shown as black, dashed lines. (C) Overlay of GluA2-LBD in complex with **2i**, glutamate (cyan, PDB code 1FTJ),[24] and KA (light gray, PDB code 1FW0).[24] The differing amino acids Leu671 and Met729 of GluA2 are shown as sticks. (D) The ligand-binding site of GluK3-LBD with **2i** and $2F_o - F_c$ omit map at 1σ carved around the ligand at 1.6 Å radius. Potential hydrogen bonds within 3.2 Å to water molecules and surrounding residues are shown. (E) Structures of GluK3-LBD in complex with **2i** and glutamate (in cyan, PDB code 3S9E),[26] with focus on Asn722 shown in stick representation. Structures of GluK1-LBD and GluK2-LBD both in complex with neodysiherbaine are shown in yellow and light gray, respectively (PDB codes 2ZNU and 3QXM).[27] The residues corresponding to those of Asn722 are shown in sticks.

4)), which is approximately 10-fold lower than the binding affinity of **2i** at full-length GluK3 (123 nM). This trend has previously been observed for the endogenous agonist Glu (7.4 μM versus 0.50 μM)[27] and the conformationally restricted Glu analogue (2*S*,1′*R*,2′*S*)-2-(2′-carboxycyclobutyl)glycine (CBG-IV) (4.2 μM versus 0.33 μM).[28] Similarly, a comparison of binding affinities of agonists and antagonists at GluA2-LBD and full-length GluA2 tabulated in Pøhlsgaard et al.[25] shows that the GluA2-LBD/full-length ratio varies between 0.3 and 5.2 for agonists and 0.8−28.3 for antagonists. The origin for these differences is yet to be fully understood.

Similar to Glu, the ligand **2i** induces full domain closure of the GluK3-LBD (25.2° and 24.3° for Glu and **2i**, respectively) (Figure 2A). In comparison, **2i** and KA induce partial domain

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closure when crystallized in the GluA2-LBD. This difference may be due to an otherwise buildup of a steric clash between the terminal side chain carbon of Leu671 and the 3′-carbon atom of **2i** or the isopropenyl group of KA in GluA2-LBD (Figure 2C). The corresponding residue in GluK3 is the less bulky Val687, which strengthens this hypothesis, as full domain closure of the GluK3-LBD with **2i** or KA bound is indeed observed.[29] In further support of this hypothesis, it has previously been shown that mutation of Leu671 to the less bulky amino acid Thr in GluA2[30] and Val in GluA4[31] leads to receptors with improved potency and efficacy of KA.

Excess electron density outside the Glu scaffold could clearly be identified in the binding site of GluK3, corresponding to **2i** (Figure 2D). Because of the resolution (2.65 Å) of the GluK3-LBD:**2i** structure, only two water molecules could be located in the binding site. Similar interactions are observed between the amino acid part of **2i** and the nearby residues (Pro518, Thr520, Arg525, Ala691, Thr692, and Glu739; full-length numbering including signal peptide), as was observed in the GluK3-LBD:Glu structure (PDB code 3S9E).[27] The hydroxamic acid moiety is accommodated in the binding pocket by displacement of two water molecules (W1 and W2 in GluK3-LBD with Glu). Similar to the structure of the GluA2-LBD:**2i** complex, the hydroxyl group is in the vicinity of Tyr491 (2.6 Å). The carbonyl oxygen only establishes contact with one visible water molecule within 3.5 Å (Figure 2D).

Compared to the structure of GluK3-LBD:Glu and GluK3-LBD:CBG-IV[28] complexes, the side chain of Asn722 adopts a different conformation, pointing away from the hydroxamic acid moiety of **2i** in the GluK3-LBD:**2i** complex. A similar alteration in the conformation of Asn722 is observed in the structure of GluK2-LBD (Asn721 in GluK2) in complex with neodysiherbaine (Figure 2E).[32] The high binding affinity of **2i** at GluK1 (K_i = 3.4 nM) and the selectivity versus the GluK2 and GluK3 subtypes (~200-fold and ~35-fold, respectively) are remarkable. On the basis of the GluK3-LBD structure with **2i** and the GluK2 structure with neodysiherbaine, it can be rationalized that a key factor in the origin of this selectivity may be due to the less bulky Ser residue in GluK1 compared to Asn in GluK2 and GluK3 (Figure 2E).

CONCLUSION

We have presented the chemoenzymatic enantioselective synthesis of 14 new 2,4-*syn* substituted Glu analogues **2b−o**. A short and efficient synthetic approach was developed based on readily available cyclic *β*-keto ester **3** to access racemic 4-substituted ketoglutarate analogues. In a highly stereoselective way, the obtained keto acids were converted to the corresponding Glu analogues having the L-2,4-*syn* configuration. Pharmacological characterization at the iGluRs and EAAT1−3 disclosed new insight into the SAR of these receptors and transporters, and notably analogue **2i** was identified as a high affinity GluK1 ligand displaying high subtype and iGluR class selectivity. From the two X-ray structures obtained, **2i** was found to induce partial domain closure in GluA2-LBD, whereas at GluK3-LBD full domain closure was seen. In all, the data presented herein add important information to the SAR of Glu ligands and will aid future rational design of subtype selective Glu receptor and transporter ligands.

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 F_{254} plates were used for column and TLC chromatography. HPLC analyses were performed using a C18 column (Uptisphere ODB, 5 μm, 250 mm × 4.6 mm) in the following conditions: flow rate 0.5 (**2o**, **2e**) or 0.8 (**2i**) mL·min^{-1}; UV detection (210−214 nm); 30 °C; eluent consisting of H_2O/CH_3CN 95:5 (**2i**, **2o**) or 20:80 (**2e**). All solvents were purified by distillation following usual procedures. Cysteine sulfinic acid was prepared from cystine following a described procedure.[12] Bovine heart malic dehydrogenase and rabbit muscle lactic dehydrogenase were purchased from Sigma. *E. coli* AspAT was produced and purified following described procedures from overexpressing *E. coli* strains JM103 transformed with pUC119-*aspC* (AspAT).[33,34] 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), keto acid substrate (0.1−10 mM), AspAT (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}. All compounds undergoing pharmacological characterization were of ≥95% purity determined by elemental analyses or HPLC.

Dimethyl 4-(Isobutoxycarbonyloxy)cyclohex-3-ene-1,3-dicarboxylate 4. To a solution of **3**[16] (3.8 g, 17.7 mmol) in anhydrous toluene (60 mL) were added Et_3N (3.7 mL, 26.6 mmol) and isobutyl chloroformate (2.55 mL, 19.6 mmol). The mixture was stirred at room temperature for 3 h. After filtration and concentration under reduced pressure, **4** was isolated as a slightly yellow oil in quantitative yield (5.6 g). IR (neat film) 1731, 1667 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.96 (2H, d, J = 6.7 Hz), 3.69 (3H, s), 3.68 (3H, s), 2.74 (1H, m), 2.58 (2H, m), 2.38 (2H, m), 2.08 (1H, m), 1.98 (1H, m), 1.83 (1H, m), 0.95 (6H, d, J = 6.7 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.5, 165.3, 154.9, 152.3, 116.3, 74.9, 52.0, 51.8, 38.3, 28.1, 27.8, 27.4, 24.4, 18.9; HRMS (ES+) m/z 337.1263 ([M + Na]$^+$ $C_{15}H_{22}NaO_7$ requires 337.1257).

Isobutylcarbonic-7-methoxy-4-methoxycarbonyl-6,7-dioxoheptanoic Anhydride 5. A solution of enol carbonate **4** (1.5 g, 4.8 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 10 min, the excess ozone was eliminated by oxygen bubbling. Dimethyl sulfide (0.35 mL, 4.7 mmol) was added, and the reaction mixture was allowed to warm to room temperature. The solution of anhydride **5** was used directly in the next reactions. An aliquot was concentrated under reduced pressure for analyses. IR (neat film) 1820, 1732 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.03 (2H, d, J = 6.6 Hz), 3.89 (3H, s), 3.68 (3H, s), 3.35 (1H, m), 2.97 (2H, m), 2.55 (2H, m), 2.07−1.89 (3H, m), 0.95 (6H, d, J = 6.7 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.6, 173.9, 167.2, 160.7, 149.0, 75.7, 53.2, 52.3, 40.9, 38.9, 31.3, 27.7, 25.9, 18.8; HRMS (ES+) m/z 369.1162 ([M + Na]$^+$ $C_{15}H_{22}NaO_9$ requires 369.1148).

General Procedure for the Synthesis of 6a−j. To the solution of anhydride **5** in CH_2Cl_2 was added the nucleophile HNR_1R_2 (1.1 mol equiv). NH_3 and $HNMe_2$ were added as 0.5−2 M solutions in THF. MeNHOH was added as a mixture of the corresponding hydrochloride, DIEA (1.1 mol equiv), and DMSO (3 mL). H_2NOBn and H_2NOtBu were both obtained as solutions in CH_2Cl_2 (50 mL) from an aqueous solution of the corresponding hydrochlorides after adjusting the pH to 10 with 1 M NaOH and extraction with CH_2Cl_2 (2 × 25 mL). The reaction mixture was stirred at room temperature for 10 min to 2 h before concentration under reduced pressure. **6a−j** were purified by flash chromatography (eluent, cyclohexane−EtOAc, 5:5 to 2:8, v/v for **6a−c,e−g,j**; CH_2Cl_2−MeOH, 98:2 to 97:3 for **6d**

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and **6h**; EtOAc to EtOAc−MeOH, 9:1 for **6i**) and isolated as colorless oils (**6a,b,d−j**) or as a white solid (**6c**).

Dimethyl 2-(3-Amino-3-oxopropyl)-4-oxoglutarate 6a. **6a** was isolated as a slightly yellow oil from **4** (3.30 g, 10.5 mmol): yield 2.72 g, 84% from **3**; IR (neat film) 3453, 3363, 3206, 1730, 1667 cm^{-1}; ^1H 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-Oxo-4-(3-oxo-3-(prop-2-ynylamino)propyl)-glutarate 6b. **6b** was isolated as a colorless oil from **4** (728 mg, 2.32 mmol): yield 616 mg, 94% from **3**; IR (solution in CCl_4) 3461, 2268, 1736 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.78 (1H, s, broad), 4.04 (2H, dd, J = 2.5 and 5.3 Hz), 3.87 (3H, s), 3.68 (3H, s), 3.32 (1H, dd, J = 10.0 and 19.7 Hz), 2.96 (2H, m), 2.24 (1H, m), 2.23 (1H, t, J = 2.5 Hz), 1.98 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.0, 174.4, 171.4, 160.9, 79.5, 71.8, 53.3, 52.3, 41.1, 39.3, 33.5, 29.4, 27.2; HRMS (ES+) m/z 306.0940 ($[M + Na]^+$, $C_{13}H_{17}NaNO_6$ requires 306.0954). Anal. ($C_{13}H_{17}NO_6$) C, H, N.

Dimethyl 2-Oxo-4-(3-oxo-3-phenylaminopropyl)glutarate 6c. **6c** was isolated as a white solid from **4** (1.00 g, 3.18 mmol): yield 920 mg, 90% from **3**; IR (neat film) 3374, 1724, 1686, 1598 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.09 (1H, s, broad), 7.47 (2H, d, J = 8.0 Hz), 7.24 (2H, t, J = 7.9 Hz), 7.03 (1H, t, J = 7.4 Hz), 3.81 (3H, s), 3.61 (3H, s), 3.28 (1H, dd, J = 10.2 and 19.9 Hz), 2.92 (2H, m), 2.38 (2H, m), 1.97 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.0, 174.5, 170.4, 160.7, 137.9, 128.9, 124.3, 120.0, 53.1, 52.2, 40.9, 39.2, 34.5, 27.1; HRMS (ES+) m/z 322.1281 ($[M + H]^+$, $C_{16}H_{20}NO_6$ requires 322.1291). Anal. ($C_{16}H_{19}NO_6$) C, H, N.

Dimethyl 2-(3-Dimethylamino-3-oxopropyl)-4-oxoglutarate 6d. **6d** was isolated as a slightly yellow oil from **4** (1.50 g, 4.77 mmol): yield 1.15 g, 88% from **3**; IR (neat film) 1731, 1644 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.84 (3H, s), 3.64 (3H, s), 3.29 (1H, dd, J = 10.0 and 19.6 Hz), 2.99−2.90 (2H, m), 2.96 (3H, s), 2.90 (3H, s), 2.34 (2H, t, J = 7.2 Hz), 1.85−1.99 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.9, 174.6, 171.7, 160.9, 53.1, 52.0, 41.0, 39.3, 37.1, 35.5, 30.4, 26.8; HRMS (ES+) m/z 274.1280 ($[M + H]^+$, $C_{12}H_{20}NO_6$ requires 274.1291). Anal. ($C_{12}H_{19}NO_6 \cdot 0.25H_2O$) C, H, N.

Dimethyl 2-(3-Diethylamino-3-oxopropyl)-4-oxoglutarate 6e. **6e** was isolated as a colorless oil from **4** (1.00 g, 3.18 mmol): yield 526 mg, 54% from **3**; IR (solution in CCl_4) 1761, 1741, 1667 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.84 (3H, s), 3.64 (3H, s), 3.28 (5H, m), 2.95 (2H, m), 2.33 (2H, t, J = 7.2 Hz), 1.95 (2H, m), 1.14 (3H, t, J = 7.1 Hz), 1.07 (3H, t, J = 7.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.0, 174.7, 170.7, 160.9, 53.1, 52.0, 41.9, 41.0, 40.3, 39.4, 30.2, 27.0, 14.3, 13.1; HRMS (ES+) m/z 302.1589 ($[M + H]^+$, $C_{14}H_{24}NO_6$ requires 302.1604). Anal. ($C_{14}H_{23}NO_6 \cdot 0.25H_2O$) C, H, N.

Dimethyl 2-Oxo-4-(3-oxo-3-(piperidin-1-yl)propyl)glutarate 6f. **6f** was isolated as a colorless oil from **4** (1.50 mg, 4.77 mmol): yield 1.25 g, 83% from **3**; IR (neat film) 1729, 1646 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.86 (3H, s), 3.66 (3H, s), 3.6−3.3 (4H, m), 3.31 (1H, dd, J = 10.1 and 19.7 Hz), 2.97 (2H, m), 2.36 (2H, m), 2.04−1.86 (2H, m), 1.63 (2H, m), 1.53 (4H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.0, 174.6, 169.8, 160.9, 53.1, 52.0, 40.9, 39.4, 30.4, 27.0, 24.5; HRMS (ES+) m/z 314.1595 ($[M + H]^+$, $C_{15}H_{24}NO_6$ requires 314.1604). Anal. ($C_{15}H_{23}NO_6$) C, H, N.

Dimethyl 2-Oxo-4-(3-oxo-3-*tert*-butoxyaminopropyl)-glutarate 6g. **6g** was isolated as a colorless oil from **4** (840 mg, 2.67 mmol): yield 0.50 g, 59% from **3**. Two rotamers were identified on NMR spectra. IR (neat film) 3200, 1732, 1664 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.04 and 7.88 (1H, 2 × s broad), 3.86 (3H, s), 3.67 (3H, s), 3.31 (1H, dd, J = 10.2 and 19.6 Hz), 2.95 (2H, m), 2.47 and 2.17 (2H, 2 × m), 1.95 (2H, m), 1.25 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.9, 174.4, 170.9, 160.8, 82.0, 53.1, 52.2, 41.0, 39.3, 30.7, 29.0, 27.3, 26.3; HRMS (ES+) m/z 318.1553 ($[M + H]^+$, $C_{14}H_{24}NO_7$ requires 318.1553). Anal. ($C_{14}H_{23}NO_7 \cdot 0.25H_2O$) C, H, N.

Dimethyl 2-(3-Benzyloxyamino-3-oxopropyl)-4-oxoglutarate 6h. **6h** was isolated as a colorless oil from **4** (1.20 g, 3.82 mmol): yield 792 mg, 59% from **3**. Two rotamers were identified on NMR spectra. IR (neat film) 3221, 1732, 1666 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.39 and 8.02 (1H, 2 × s broad), 7.37 (5H, m), 4.89 (2H, m), 3.86 (3H, s), 3.64 (3H, s), 3.28 (1H, dd, J = 10.0 and 19.2 Hz), 2.91 (2H, m), 2.39 and 2.11 (2H, 2 × m), 1.94 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.9, 174.4, 169.7, 160.8, 135.4, 129.3, 128.7, 78.2, 53.2, 52.2, 40.9, 39.2, 30.4, 27.0; HRMS (ES+) m/z 352.1388 ($[M + H]^+$, $C_{17}H_{22}NO_7$ requires 352.1396). Anal. ($C_{17}H_{21}NO_7 \cdot 0.5H_2O$) C, H, N.

Dimethyl 2-(3-Hydroxy(methyl)amino-3-oxopropyl)-4-oxo-glutarate 6i. **6i** was isolated as a colorless oil from **4** (1.50 g, 4.77 mmol): yield 672 mg, 52% from **3**. Two rotamers were identified on NMR spectra. IR (neat film) 3266, 1732, 1625 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.42 (1H, s broad), 3.85 (3H, s), 3.65 (3H, s), 3.32 and 3.20 (3H, 2 × s), 3.28 (1H, dd, J = 9.2 and 19.6 Hz), 2.95 (2H, m), 2.51 and 2.38 (2H, 2 × m), 1.93 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.07, 191.9, 174.8, 174.4, 173.1, 160.8, 53.2, 52.2, 40.9, 39.5, 39.2, 36.2, 29.4, 26.5; HRMS (ES+) m/z 276.1069 ($[M + H]^+$, $C_{11}H_{18}NO_7$ requires 276.1083). Anal. ($C_{11}H_{17}NO_7 \cdot 0.25H_2O$) C, H, N.

Dimethyl 2-Oxo-4-(3-oxo-3-(2-phenylhydrazinyl)propyl)-glutarate 6j. **6j** was isolated as a colorless oil from **4** (1.50 g, 4.77 mmol): yield 932 mg, 58% from **3**. Two rotamers were identified on NMR spectra. IR (solution in CCl_4) 3434, 3351, 1732, 1699, 1604 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.12 and 7.37 (1H, 2 × s), 7.20 and 7.17 (2H, 2 × t, J = 8.0 Hz), 6.87 and 6.85 (1H, 2 × t, J = 7.6 Hz), 6.76 and 6.68 (2H, 2d, J = 8.4 Hz), 5.97 and 4.98 (1H, 2 × s, broad), 3.82 and 3.81 (3H, 2 × s), 3.64 and 3.56 (3H, 2 × s), 3.26 (1H, m), 2.89 (2H, m), 2.45 and 2.25 (2H, 2 × m), 2.00−1.80 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.0, 191.9, 178.0, 174.6, 174.5, 172.3, 160.8, 148.0, 147.2, 129.5, 129.2, 121.2, 121.1, 113.5, 112.5, 53.2, 52.2, 52.1, 40.9, 40.8, 39.3, 39.2, 31.4, 28.4, 27.0, 26.3; HRMS (ES+) m/z 359.1201 ($[M + Na]^+$, $C_{16}H_{20}N_2NaO_6$ requires 359.1219). Anal. ($C_{16}H_{20}N_2O_6$) C, H, N.

Dimethyl 2-(3-Azido-3-oxopropyl)-4-oxoglutarate 7. To a solution of anhydride **5** (prepared from 1 g of **4**, 3.18 mmol) in THF (15 mL) at −10 °C was added a solution of NaN_3 (0.191 g, 3.18 mmol) in H_2O (1 mL). The mixture was stirred at −10 °C for 15 min and then at room temperature for 1 h. The reaction mixture was diluted with EtOAc (40 mL), dried over $MgSO_4$, and concentrated under reduced pressure to give quantitatively a 80:20 mixture of acyl azide **7** and isocyanate **8** isolated as a colorless oil. IR (neat film) 2273, 2141, 1732 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.87 (3H, s), 3.68 (3H, s), 3.31 (1H, m), 3.05−2.90 (2H, m), 2.43 (2H, m), 2.05−1.85 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.6, 179.5, 174.0, 160.7, 53.1, 52.2, 40.7, 38.9, 34.1, 26.2.

Dimethyl 2-(2-Isocyanatoethyl)-4-oxoglutarate 8. A solution of acyl azide **7** (prepared from 1 g of **4**, 3.18 mmol) in CH_2Cl_2 (10 mL) was refluxed for 24 h. The solvent was removed under reduced pressure to give **8** isolated quantitatively as a yellow oil. IR (neat film) 2273, 1732 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.87 (3H, s), 3.69 (3H, s), 3.42 (2H, t, J = 6.7 Hz), 3.31 (1H, dd, J = 7.9 and 18.0 Hz), 3.05 (1H, m), 2.97 (1H, dd, J = 4.9 and 18.3 Hz), 1.98 (1H, m), 1.82 (1H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.6, 174.1, 160.9, 122.4, 53.3, 52.4, 40.8, 40.6, 37.3, 32.8.

1-Benzyl-2,4-dimethyl 2-Hydroxypiperidine-1,2,4-tricarboxylate 9. To a solution of isocyanate **7** (0.73 g, 3 mmol) in toluene (50 mL) was added benzyl alcohol (0.31 mL, 3 mmol). The mixture was stirred at 80 °C for 72 h before concentration under reduced pressure. Flash chromatography (eluent, cyclohexane−EtOAc, 7:3) afforded carbamate **9** isolated as a pale yellow oil (474 mg, 53% from **3**). NMR analyses showed **9** to be mainly present as one cyclic isomer (>85%). IR (neat film) 3450, 1731, 1634 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.33 (5H, m), 5.17−5.05 (2H, m), 4.32 (1H, s, broad), 3.92 (1H, m), 3.73 (3H, s), 3.68 (3H, s), 3.30 (1H, m), 2.86 (1H, m), 2.1−1.99 (3H, m), 1.76 (1H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.7, 173.1, 156.4, 135.8, 128.7, 128.4, 128.3, 82.4, 68.0, 53.5, 52.1, 41.3, 37.3, 35.1,

26.7; HRMS (ES+) m/z 374.1210 ([M + Na]$^+$, $C_{17}H_{21}NNaO_7$ requires 374.1216).

Dimethyl 2-Cyanoethyl-4-oxoglutarate 10. To a solution of **6a** (2.3 g, 9.38 mmol) in anhydrous THF (25 mL) at 0 °C were added pyridine (1.52 mL, 18.8 mmol) and trifluoroacetic anhydride (1.44 mL, 10.2 mmol). The mixture was stirred at 0 °C for 20 min and then at room temperature for 2 h. After addition of brine (15 mL), the reaction mixture was extracted with EtOAc (2 × 25 mL). The combined organic phases were dried over $MgSO_4$ and concentrated under reduced pressure. Flash chromatography (eluent, cyclohexane−EtOAc, 1:1) afforded nitrile **10** (1.84 g, 87%) isolated as a colorless oil. IR (neat film) 2220, 1731 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.88 (3H, s), 3.71 (3H, s), 3.33 (1H, m), 3.03 (2H, m), 2.47 (2H, t, J = 8.0 Hz), 2.05 (1H, m), 1.92 (1H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.3, 173.3, 160.7, 118.8, 53.4, 52.6, 40.5, 38.8, 27.2, 15.4; HRMS (ES+) m/z 250.0698 ([M + Na]$^+$, $C_{10}H_{13}NNaO_5$ requires 250.0691). Anal. ($C_{10}H_{13}NO_5$) C, H, N.

General Procedure for the Synthesis of Lithium Oxoglutarates 1b−l. To a solution of **6b−j**, **9**, or **10** (1 mmol) in MeOH (5 mL) was added dropwise a freshly prepared 0.4 M solution of LiOH (5.25 mL, 2.1 mmol). The mixture was stirred at room temperature for 16 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−l** were isolated in quantitative yields as white or yellow solids.

Dilithium 2-Oxo-4-(3-oxo-3-(prop-2-ynylamino)propyl)-glutarate 1b. 1b was isolated as a yellow solid from **6b** (518 mg, 2.29 mmol): yield 489 mg, 99%; ^{1}H NMR (400 MHz, D_2O) δ 3.95 (2H, s), 3.06 (1H, dd, J = 8.4 and 18.4 Hz), 2.87 (1H, dd, J = 5.8 and 18.4 Hz), 2.63 (1H, m), 2.28 (3H, m), 1.78 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 202.3, 180.5, 173.9, 167.8, 77.3, 69.7, 40.5, 39.9, 31.5, 26.6, 26.1; HRMS (ES+) m/z 262.0919 ([M − Li + 2H]$^+$, $C_{11}H_{13}LiNO_6$ requires 262.0903).

Dilithium 2-Oxo-4-(3-oxo-3-phenylaminopropyl)glutarate 1c. 1c was isolated as a white solid from **6c** (473 mg, 1.47 mmol): yield 448 mg, 99%; ^{1}H NMR (400 MHz, D_2O) δ 7.43 (4H, m), 7.27 (1H, m), 3.11 (1H, dd, J = 8.5 and 18.5 Hz), 2.92 (1H, dd, J = 5.6 and 18.5 Hz), 2.72 (1H, m), 2.44 (2H, m), 1.89 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 204.4, 182.7, 174.9, 169.9, 136.8, 129.2, 125.6, 122.2, 42.7, 42.1, 34.5, 28.3; HRMS (ES+) m/z 300.1065 ([M − Li + 2H]$^+$, $C_{14}H_{15}LiNO_6$ requires 300.1059).

Dilithium 2-(3-Dimethylamino-3-oxopropyl)-4-oxoglutarate 1d. 1d was isolated as a yellow solid from **6d** (824 mg, 3.02 mmol): yield 760 mg, 98%; ^{1}H NMR (400 MHz, D_2O) δ 3.05 (3H, s), 3.04 (1H, dd, J = 8.4 and 18.4 Hz), 2.90 (3H, s), 2.86 (1H, dd, J = 5.6 and 18.4 Hz), 2.65 (1H, m), 2.40 (2H, m), 1.74 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 204.4, 182.6, 175.5, 169.9, 42.6, 41.7, 37.6, 35.5, 30.9, 27.8; HRMS (ES+) m/z 252.1059 ([M − Li + 2H]$^+$, $C_{10}H_{15}LiNO_6$ requires 252.1059).

Dilithium 2-(3-Diethylamino-3-oxopropyl)-4-oxoglutarate 1e. 1e was isolated as a white solid from **6e** (341 mg, 1.13 mmol): yield 307 mg, 95%; ^{1}H NMR (400 MHz, D_2O) δ 3.35 (2H, q, J = 7.2 Hz), 3.30 (2H, q, J = 7.2 Hz), 3.03 (1H, dd, J = 8.4 and 18.4 Hz), 2.84 (1H, dd, J = 5.6 and 18.4 Hz), 2.63 (1H, m), 2.37 (2H, m), 1.73 (2H, m), 1.15 (3H, t, J = 7.2 Hz), 1.05 (3H, t, J = 7.2 Hz); ^{13}C NMR (100 MHz, D_2O) δ 204.3, 182.6, 174.7, 169.8, 43.0, 42.7, 42.1, 40.7, 30.7, 28.3, 13.2, 12.0; HRMS (ES+) m/z 274.1303 ([M − 2Li + 3H]$^+$, $C_{12}H_{20}NO_6$ requires 274.1291).

Dilithium 2-Oxo-4-(3-oxo-3-(piperidin-1-yl)propyl)glutarate 1f. 1f was isolated as a white solid from **6f** (1.16 g, 3.70 mmol): yield 1.09 g, 99%; ^{1}H NMR (400 MHz, D_2O) δ 3.49 (4H, m), 3.07 (1H, dd, J = 8.4 and 18.4 Hz), 2.88 (1H, dd, J = 5.6 and 18.4 Hz), 2.67 (1H, m), 2.43 (2H, m), 1.75 (2H, m), 1.70−1.50 (6H, m); ^{13}C NMR (100 MHz, D_2O) δ 204.4, 182.6, 173.5, 169.9, 47.5, 43.4, 42.8, 42.1, 31.1, 28.3, 26.1, 25.3, 23.7; HRMS (ES+) m/z 292.1388 ([M − Li + 2H]$^+$, $C_{13}H_{19}LiNO_6$ requires 292.1372).

Dilithium 2-Oxo-4-(3-oxo-3-*tert*-butoxyaminopropyl)-glutarate 1g. 1g was isolated as a white solid from **6g** (400 mg, 1.26 mmol): yield 367 mg, 97%; ^{1}H NMR (400 MHz, D_2O) δ 3.05 (1H, dd, J = 8.4 and 18.4 Hz), 2.87 (1H, dd, J = 5.6 and 18.4 Hz), 2.65 (1H, m), 2.22 (2H, m), 1.80 (2H, m), 1.25 (9H, s); ^{13}C NMR (100 MHz, D_2O) δ 204.3, 182.4, 173.4, 169.8, 83.9, 42.5, 41.8, 30.6, 28.2, 25.4; HRMS (ES+) m/z 290.1244 ([M − 2Li + 3H]$^+$, $C_{12}H_{20}NO_7$ requires 290.1240).

Dilithium 2-(3-Benzyloxyamino-3-oxopropyl)-4-oxoglutarate 1h. 1h was isolated as a yellow solid from **6h** (634 mg, 1.80 mmol): yield 594 mg, 98%; ^{1}H NMR (400 MHz, D_2O) δ 7.44 (5H, m), 4.88 (2H, s), 2.98 (1H, dd, J = 8.4 and 18.4 Hz), 2.78 (1H, dd, J = 5.6 and 18.4 Hz), 2.56 (1H, m), 2.10 (2H, t, J = 8.0 Hz), 1.67 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 204.6, 182.3, 172.1, 169.8, 134.6, 129.8, 129.2, 128.7, 78.1, 42.3, 41.7, 30.4, 28.0; HRMS (ES+) m/z 290.1244 ([M − 2Li + 3H]$^+$, $C_{12}H_{20}NO_7$ requires 290.1240).

Dilithium 2-(3-Hydroxy(methyl)amino-3-oxopropyl)-4-oxoglutarate 1i. 1i was isolated as a yellow solid from **6i** (430 mg, 1.56 mmol): yield 395 mg, 98%; ^{1}H NMR (400 MHz, D_2O) δ 3.36 and 3.22 (3H, 2 × s), 3.04 (1H, dd, J = 9.0 and 18.4 Hz), 2.87 (1H, dd, J = 5.6 and 18.4 Hz), 2.66 (1H, m), 2.52 and 2.40 (2H, 2 × m), 1.85−1.60 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 204.4, 182.8, 175.1, 169.7, 42.6, 42.0, 35.9, 29.6, 27.4; HRMS (ES+) m/z 254.0863 ([M − Li + 2H]$^+$, $C_9H_{13}LiNO_7$ requires 254.0852).

Dilithium 2-Oxo-4-(3-oxo-3-(2-phenylhydrazinyl)propyl)-glutarate 1j. 1j was isolated as a yellow solid from **6j** (537 mg, 1.59 mmol): yield 491 mg, 96%; ^{1}H NMR (400 MHz, D_2O) δ 7.33 (2H, t, J = 7.6 Hz), 6.99 (1H, t, J = 7.6 Hz), 6.91 (2H, d, J = 7.6 Hz), 3.11 (1H, dd, J = 8.4 and 18.4 Hz), 2.91 (1H, dd, J = 5.6 and 18.4 Hz), 2.71 (1H, m), 2.38 (2H, m), 1.86 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 204.3, 182.5, 176.1, 169.8, 147.3, 129.5, 121.2, 113.4, 42.6, 42.0, 31.8, 28.2; HRMS (ES+) m/z 315.1170 ([M − Li + 2H]$^+$, $C_{14}H_{16}LiN_2O_6$ requires 315.1168).

Dilithium 2-(2-Benzyloxycarbonylaminoethyl)-4-oxoglutarate 1k. 1k was isolated as a yellow solid from **9** (472 mg, 1.34 mmol): yield 440 mg, 98%. NMR analyses showed **1k** to be mainly present in D_2O as a 7:3 mixture of the linear form and one cyclic form. Linear form: ^{1}H NMR (400 MHz, D_2O) δ 7.39 (5H, m), 5.06 (2H, s), 3.10 (2H, m), 3.02 (1H, dd, J = 8.8 and 18.8 Hz), 2.83 (1H, dd, J = 5.6 and 18.8 Hz), 2.63 (1H, m), 1.75−1.55 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 204.3, 182.9, 169.7, 158.2, 136.5, 128.7, 128.3, 127.6, 66.8, 42.0, 40.7, 38.7, 31.9. Cyclic form: ^{1}H NMR (400 MHz, D_2O) δ 7.39 (5H, m), 5.10 (2H, s), 3.86 (1H, m), 3.20 (1H, m), 2.51 (1H, m), 1.95 (3H, m), 1.67 (1H, m); ^{13}C NMR (100 MHz, D_2O) δ 183.8, 178.2, 157.4, 136.1, 128.7, 128.3, 127.7, 84.2, 67.6, 41.8, 38.6, 37.9, 27.1; HRMS (ES−) m/z 322.0927 ([M − 2Li + H]$^-$, $C_{15}H_{15}LiNO_7$ requires 322.0927).

Dilithium 2-Cyanoethyl-4-oxoglutarate 1l. 1l was isolated as a yellow solid from **10** (1.20 g, 5.28 mmol): yield 1.10 g, 99%; ^{1}H NMR (400 MHz, D_2O) δ 2.95 (1H, dd, J = 8.4 and 18.8 Hz), 2.77 (1H, dd, J = 5.6 and 18.8 Hz), 2.59 (1H, m), 2.37 (2H, m), 1.73 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 203.8, 181.8, 169.5, 121.2, 42.2, 41.7, 27.5, 14.8; HRMS (ES−) m/z 198.0392 ([M − 2Li + H]$^+$, $C_8H_8NO_5$ requires 198.0402).

General Procedure for the Synthesis of L-2,4-*syn*-Glu Analogues 2b−l. To a solution of racemic **1b−l** (0.5−5 mmol) in water (25−250 mL) was added cysteine sulfinic acid (0.5−5 mmol). The pH of the solution was adjusted to 7.6 with 1 M NaOH before the addition of *E. coli* AspAT (10−200 units; the quantity of enzyme was adjusted to attain 40% conversion in 2−4 h). The mixture was stirred slowly at room temperature, and the reaction was monitored by titration of pyruvate. The 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 containing **2b−f,k,l** 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) before adsorption on a column of Dowex 1X8 resin (200−400 Mesh, AcO$^-$

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form, 1.5 × 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 reaction mixtures containing **2g,h,j** were purified directly on Dowex 1X8 resin as described above. In the case of **2i**, elution from Dowex 50WX8 resin was done with 1 M ammonium bicarbonate and elution from the Dowex 1X8 resin (HCO_3^- form) was done with an ammonium bicarbonate gradient (0.1−0.5 M). All the residues obtained after chromatography were dissolved in water (5 mL), and the solutions were lyophilized to afford **2b−l** as white solids.

(2*S*,4*R*)-4-(3-Oxo-3-(prop-2-ynylamino)propyl)glutamic Acid 2b. **2b** was isolated from **1b** (254 mg, 0.95 mmol): yield 95 mg, 39%; mp 110 °C; $[\alpha]^{25}_D$ +1.0 (*c* 1.5, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 3.95 (2H, d, *J* = 2.5 Hz), 3.79 (1H, dd, *J* = 5.4 and 8.3 Hz), 2.62 (1H, m), 2.61 (1H, t, *J* = 2.4 Hz), 2.40−2.25 (3H, m), 2.00−1.86 (3H, m); ^{13}C NMR (100 MHz, D_2O) δ 178.6, 175.3, 173.6, 79.6, 71.7, 53.0, 41.5, 32.8, 32.3, 28.7, 27.8; HRMS (ES+) *m*/*z* 257.1149 ([M + H]$^+$, $C_{11}H_{17}N_2O_5$ requires 257.1137). Anal. ($C_{11}H_{16}N_2O_5\cdot0.5H_2O$) C, H, N.

(2*S*,4*R*)-4-(3-Oxo-3phenylaminopropyl)glutamic Acid 2c. **2c** was isolated from **1c** (215 mg, 0.70 mmol): yield 85 mg, 41%; mp 172 °C; $[\alpha]^{25}_D$ −44.6 (*c* 0.8, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 7.38 (4H, m), 7.20 (1H, m), 3.70 (1H, dd, *J* = 3.4 and 9.0 Hz), 2.45−2.3 (3H, m), 2.18 (1H, m), 1.95−1.75 (3H, m); ^{13}C NMR (100 MHz, D_2O) δ 182.3, 174.7, 174.4, 136.7, 129.1, 125.6, 122.1, 53.3, 44.6, 34.3, 33.1, 28.5; HRMS (ES+) *m*/*z* 295.1286 ([M + H]$^+$, $C_{14}H_{19}N_2O_5$ requires 295.1294). Anal. ($C_{14}H_{18}N_2O_5$) C, H, N.

(2*S*,4*R*)-4-(3-Dimethylamino-3-oxopropyl)glutamic Acid 2d. **2d** was isolated from **1d** (211 mg, 0.82 mmol): yield 85 mg, 42%; mp 145 °C; $[\alpha]^{25}_D$ −7.0 (*c* 0.7, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 3.81 (1H, dd, *J* = 5.2 and 8.0 Hz), 3.07 (3H, s), 2.93 (3H, s), 2.65 (1H, m), 2.49 (2H, t, *J* = 7.6 Hz), 2.29 (1H, ddd, *J* = 5.6, 9.6, and 14.8 Hz), 2.00−1.85 (3H, m); ^{13}C NMR (100 MHz, D_2O) δ 178.7, 174.9, 173.6, 53.0, 41.6, 37.5, 35.5, 32.4, 30.2, 27.5; HRMS (ES−) *m*/*z* 245.1143 ([M − H]$^-$, $C_{10}H_{17}N_2O_5$ requires 245.1137). Anal. ($C_{10}H_{18}N_2O_5\cdot H_2O$) C, H, N.

(2*S*,4*R*)-4-(3-Diethylamino-3-oxopropyl)glutamic Acid 2e. **2e** was isolated from **1e** (131 mg, 0.46 mmol): yield 44 mg, 35%; mp 145 °C; $[\alpha]^{25}_D$ +42.1 (*c* 1, 6N HCl); ^{1}H NMR (400 MHz, D_2O) δ 3.79 (1H, dd, *J* = 5.5 and 8.3 Hz), 3.39 (2H, q, *J* = 7.2 Hz), 3.34 (2H, q, *J* = 7.2 Hz), 2.66 (1H, m), 2.47 (2H, m), 2.30 (1H, ddd, *J* = 5.5, 9.6, and 14.9 Hz), 2.00−1.85 (3H, m), 1.17 (3H, t, *J* = 7.2 Hz), 1.09 (3H, t, *J* = 7.2 Hz); ^{13}C NMR (100 MHz, D_2O) δ 178.7, 173.9, 173.5, 52.9, 42.9, 41.6, 40.8, 32.4, 29.9, 27.8, 13.1, 12.0; HRMS (ES+) *m*/*z* 297.1418 ([M + Na]$^+$, $C_{12}H_{22}N_2NaO_5$ requires 297.1426). Anal. Calcd for $C_{12}H_{22}N_2O_5\cdot0.5H_2O$: C, 50.87, H, 8.18, N, 9.88. Found: C, 50.98, H, 7.75, N, 9.92. 95.4% purity by HPLC analysis.

(2*S*,4*R*)-4-(3-Oxo-3-(piperidin-1-yl)propyl)glutamic Acid 2f. **2f** was isolated from **1f** (255 mg, 0.86 mmol): yield 86 mg, 35%; mp 170 °C; $[\alpha]^{25}_D$ +41.2 (*c* 0.8, 6 N HCl); ^{1}H NMR (400 MHz, D_2O) δ 3.79 (1H, dd, *J* = 5.4 and 8.3 Hz), 3.49 (4H, m), 2.64 (1H, m), 2.49 (2H, t, *J* = 7.8 Hz), 2.30 (1H, ddd, *J* = 5.4, 9.6, and 14.9 Hz), 2.00−1.85 (3H, m), 1.70−1.50 (6H, m); ^{13}C NMR (100 MHz, D_2O) δ 178.6, 173.5, 172.8, 52.9, 47.3, 43.4, 41.6, 32.4, 30.2, 25.9, 25.2, 23.6; HRMS (ES+) *m*/*z* 309.1430 ([M + Na]$^+$, $C_{13}H_{22}N_2NaO_5$ requires 309.1426). Anal. ($C_{13}H_{22}N_2O_5$) C, H, N.

(2*S*,4*R*)-4-(3-Oxo-3-*tert*-butoxyaminopropyl)glutamic Acid 2g. **2g** was isolated from **1b** (300 mg, 1.00 mmol): yield 116 mg, 40%; mp 137 °C; $[\alpha]^{25}_D$ +7.6 (*c* 0.7, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 3.79 (1H, dd, *J* = 5.5 and 8.2 Hz), 2.61 (1H, m), 2.28 (3H, m), 1.92 (3H, m), 1.24 (9H, s); ^{13}C NMR (100 MHz, D_2O) δ 178.3, 173.6, 172.9, 84.3, 52.9, 41.5, 32.7, 30.3, 28.2, 25.3; HRMS (ES+) *m*/*z* 291.1546 ([M + H]$^+$, $C_{12}H_{23}N_2O_6$ requires 291.1556). Anal. ($C_{12}H_{22}N_2O_6\cdot H_2O$) C, H, N.

(2*S*,4*R*)-4-(3-Benzyloxyamino-3-oxopropyl)glutamic Acid 2h. **2h** was isolated from **1h** (246 mg, 0.73 mmol): yield 100 mg, 42%; mp 137 °C; $[\alpha]^{25}_D$ −5.1 (*c* 0.8, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 7.48 (5H, m), 4.91 (2H, s), 3.67 (1H, dd, *J* = 3.8 and 9.3 Hz), 2.30 (1H, m), 2.18−2.05 (3H, m), 1.86 (1H, ddd, *J* = 4.0, 9.2, and 13.6 Hz), 1.82−1.65 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 182.0, 174.4, 172.1, 134.6, 129.9, 129.2, 128.7, 78.2, 53.3, 44.4, 32.9, 30.4, 28.3; HRMS (ES-) *m*/*z* 323.1235 ([M − H]$^-$, $C_{15}H_{19}N_2O_6$ requires 323.1243). Anal. ($C_{15}H_{20}N_2O_6\cdot0.25H_2O$) C, H, N.

(2*S*,4*R*)-4-(3-Hydroxy(methyl)amino-3-oxopropyl)glutamic Acid 2i. **2i** was isolated from **1i** (211 mg, 0.82 mmol): yield 78 mg, 39%; mp 96 °C; $[\alpha]^{25}_D$ +8.0 (*c* 0.5, H_2O). NMR analyses showed **2i** to exist as a 80:20 mixture of rotamers in D_2O. ^{1}H NMR (400 MHz, D_2O) δ 3.71 (1H, dd, *J* = 3.9 and 9.3 Hz), 3.37 and 3.23 (3H, 2 × s), 2.52 (2H, t, *J* = 8 Hz), 2.39 (1H, m), 2.18 (1H, ddd, *J* = 3.9, 9.8, and 14.2 Hz), 1.91 (1H, ddd, *J* = 4.4, 9.3, and 14.3 Hz), 1.88−1.66 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 182.0, 175.1, 174.3, 53.3, 44.3, 38.7, 35.9, 33.1, 33.0, 30.2, 29.5, 27.8, 27.6; HRMS (ES−) *m*/*z* 247.0934 ([M − H]$^-$, $C_9H_{15}N_2O_6$ requires 247.0930). Anal. Calcd for $C_9H_{16}N_2O_6\cdot0.75NH_3\cdot H_2O$: C, 38.74, H, 7.31 N, 13.80. Found: C, 38.81, H, 6.87, N, 14.17. 99.1% purity by HPLC analysis.

(2*S*,4*R*)-4-(3-Oxo-3-(2-phenylhydrazinyl)propyl)glutamic Acid 2j. **2j** was isolated from **1j** (141 mg, 0.44 mmol): yield 53 mg, 39%; mp 136 °C; $[\alpha]^{25}_D$ +8.0 (*c* 0.6, MeOH). NMR analyses showed **2j** to exist as 93:7 mixture of rotamers in D_2O. ^{1}H NMR (400 MHz, D_2O) δ 7.31 (2H, m), 6.97 (1H, m), 6.89 (2H, m), 3.86 and 3.77 (1H, 2 × dd, *J* = 6.0 and 8.0 Hz), 2.68 (1H, m), 2.53 and 2.42 (2H, 2 × m), 2.33 and 2.25 (1H, 2 × ddd, *J* = 5.8, 9.6, and 14.9 Hz), 2.03−1.83 (3H, m); ^{13}C NMR (100 MHz, D_2O) δ 178.1, 175.4, 173.0, 147.2, 129.6, 129.5, 121.2, 120.9, 113.4, 112.6, 52.6, 41.2, 32.2, 30.9, 27.7; HRMS (ES+) *m*/*z* 310.1408 ([M + H]$^+$, $C_{14}H_{20}N_3O_5$ requires 310.1403). Anal. ($C_{14}H_{19}N_3O_5\cdot1.5H_2O$) C, H, N.

(2*S*,4*R*)-4-(2-Benzyloxycarbonylaminoethyl)glutamic Acid 2k. **2k** was isolated from **1k** (410 mg, 1.23 mmol): yield 159 mg, 40%; mp 97 °C; $[\alpha]^{25}_D$ +8.1 (*c* 0.5, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 7.43 (5H, m), 5.10 (2H, s), 3.74 (1H, dd, *J* = 5.6 and 7.7 Hz), 3.19 (2H, m), 2.62 (1H, m), 2.25 (1H, ddd, *J* = 5.2, 9.6, and 14.8 Hz), 1.99−1.71 (3H, m); ^{13}C NMR (100 MHz, D_2O) δ 179.0, 173.6, 158.3, 136.5, 128.8, 128.3, 127.6, 66.9, 53.0, 39.7, 38.1, 32.3, 31.7; HRMS (ES−) *m*/*z* 323.1259 ([M − H]$^-$, $C_{15}H_{19}N_2O_6$ requires 323.1243). Anal. ($C_{15}H_{20}N_2O_6\cdot0.25H_2O$) C, H, N.

(2*S*,4*R*)-4-(2-Cyanoethyl)glutamic Acid 2l. **2l** was isolated from **1l** (960 mg, 4.55 mmol): yield 364 mg, 40%; mp 110 °C; $[\alpha]^{25}_D$ +15.8 (*c* 0.7, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 3.79 (1H, dd, *J* = 5.6 and 7.6 Hz), 2.68 (1H, m), 2.56 (2H, m), 2.28 (1H, ddd, *J* = 5.6, 9.6, and 15.0 Hz), 2.06−1.88 (3H, m); ^{13}C NMR (100 MHz, D_2O) δ 177.8, 173.4, 120.8, 52.8, 41.2, 32.1, 27.3, 14.4; HRMS (ES−) *m*/*z* 199.0726 ([M − H]$^-$, $C_8H_{11}N_2O_4$ requires 199.0719). Anal. ($C_8H_{12}N_2O_4$) C, H, N.

(2*S*,4*R*)-4-(3-Hydroxyamino-3-oxopropyl)glutamic Acid 2m. To a solution of **2h** (60 mg, 0.185 mmol) in H_2O (10 mL) was added 10% Pd/C (40 mg). The mixture was stirred for 2 h under hydrogen atmosphere, using a balloon. Pd/C was removed by filtration through a membrane (0.2 μm), and the filtrate was concentrated under reduced pressure. Ion exchange chromatography on Dowex 1X8, performed as described for **2h**, afforded **2m** (37 mg, 85%) as a pale yellow solid. Mp 87 °C; $[\alpha]^{25}_D$ +5.4 (*c* 0.5, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 3.79 (1H, dd, *J* = 5.4 and 8.2 Hz), 2.61 (1H, m), 2.34−2.17 (3H, m), 2.00−1.85 (3H, m); ^{13}C NMR (100 MHz, D_2O) δ 178.6, 173.6, 172.0, 53.0, 41.5, 32.3, 29.8, 27.7; HRMS (ES−) *m*/*z* 233.0773 ([M − H]$^-$, $C_8H_{13}N_2O_6$ requires 233.0774). Anal. ($C_8H_{14}N_2O_6\cdot0.5H_2O$) C, H, N.

(2*S*,4*R*)-4-(2-Aminoethyl)glutamic Acid 2n. To a solution of **2k** (87 mg, 0.27 mmol) in H_2O (5 mL) was added 10% Pd/C (30 mg). The mixture was stirred for 3 h at room temperature under hydrogen atmosphere, using a balloon. Pd/C was removed by filtration through a membrane (0.2 μm), and the filtrate was concentrated under reduced pressure. The residue was dissolved in H_2O (2 mL) and the solution poured on a column of Dowex 1X8 (200−400 mesh, AcO^- form, 1.5 cm × 10 cm). The column was eluted with H_2O, and the ninhydrin positive fractions were combined and concentrated under reduced pressure. The residue was dissolved in H_2O and the solution was lyophilized to afford **2n** as a slightly yellow solid (50 mg, 97%). Mp 98 °C; $[\alpha]^{25}_D$ −37.5 (*c* 0.6, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 3.74 (1H, dd, *J* = 4.3 and 8.0 Hz), 3.00 (2H, t, *J* = 7.9 Hz), 2.41 (1H, m), 2.16 (1H, ddd, *J* = 4.3, 9.2, and 14.7 Hz), 2.00−1.77 (3H, m); ^{13}C NMR (100 MHz, D_2O) δ 181.5, 174.3, 53.2, 42.4, 37.8, 32.9, 29.9;

HRMS (ES−) m/z 189.0883 ([M − H]$^-$, $C_7H_{13}N_2O_4$ requires 189.0875). Anal. ($C_7H_{14}N_2O_4 \cdot 0.75H_2O$) C, H, N.

(2*S*,4*R*)-4-(2-Cyanoethyl)-*N*-*tert*-butoxycarbonylglutamic Acid 11. To a solution of **2l** (200 mg, 1 mmol) in H_2O (6 mL) was added $NaHCO_3$ (504 mg, 6.0 mmol). The solution was cooled to 0 °C, and a solution of BOC_2O (0.55 g, 2.5 mmol) in 1,4-dioxane (2.5 mL) was added. The mixture was stirred at room temperature for 72 h and then poured on a column of Dowex 1X8 (200−400 mesh, HCO_3^- form, 1.5 cm × 5 cm). The column was washed with water (25 mL) and then eluted with an ammonium bicarbonate gradient (0.1−1 M). The ninhydrin positive fractions were combined and concentrated under reduced pressure to afford **11** (270 mg, 90%) as a white solid. NMR analyses showed **11** to exist as a 1:3 mixture of rotamers in D_2O. Mp 87 °C; $[\alpha]^{25}_D$ +1.0 (*c* 2, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 3.76 (1H, m), 2.53−2.32 (3H, m), 1.98 (1H, m), 1.80 (2H, m), 1.61 (1H, m), 1.40 (9H, s); ^{13}C NMR (100 MHz, D_2O) δ 183.3, 180.0, 157.8, 157.6, 121.3, 56.1, 55.0, 45.2, 34.6, 34.3, 28.2, 27.7, 27.5, 14.9; HRMS (ES−) m/z 299.1253 ([M − H]$^-$, $C_{13}H_{19}N_2O_6$ requires 299.1243).

(2*S*,4*R*)-4-(3-Aminopropyl)-*N*-*tert*-butoxycarbonylglutamic Acid 12. To a solution of **11** (270 mg, 0.9 mmol) in MeOH (10 mL) and AcOH (10 mL) was added 20% $Pd(OH)_2/C$ (50 mg). The mixture was stirred for 4 h at room temperature under hydrogen atmosphere, using a balloon. $Pd(OH)_2/C$ was removed by filtration through a membrane (0.2 μm), and the filtrate was concentrated under reduced pressure. The residue was diluted in water (5 mL) before adsorption on a column of Dowex 1X8 resin (200−400 mesh, AcO^- form, 1.5 cm × 6 cm). The column was washed with water (25 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 H_2O (5 mL) and the solution was lyophilized to afford **12** as a white solid (256 mg, 93%). Mp 127 °C; $[\alpha]^{25}_D$ +0.8 (*c* 1, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 3.95 (1H, dd, J = 4.2 and 9.4 Hz), 3.00 (2H, t, J = 6.2 Hz), 2.48 (1H, m), 2.14 (1H, m), 1.77 (1H, m), 1.72−1.56 (4H, m), 1.43 (9H, s); ^{13}C NMR (100 MHz, D_2O) δ 180.5, 178.2, 157.4, 157.6, 81.1, 53.8, 42.8, 39.2, 33.9, 28.9, 27.7, 24.7; HRMS (ES−) m/z 303.1547 ([M − H]$^-$, $C_{13}H_{23}N_2O_6$ requires 303.1556).

(2*S*,4*R*)-4-(3-Aminopropyl)glutamic Acid 2o. A solution of 12 (125 mg, 0.41 mmol) in 1 M HCl (5 mL) was stirred at room temperature for 3 h before concentration under reduced pressure. The residue was dissolved in H_2O (10 mL) and the pH adjusted to 12 with 0.2 M NaOH. The solution was poured on a column of Dowex 50WX8 resin (H^+ form, 1.5 cm × 10 cm). The column was washed with H_2O (50 mL) and then eluted with 1 M NH_3. The ninhydrin-positive fractions were combined and concentrated under reduced pressure. The residue was dissolved in H_2O (2 mL) and the solution poured on a column of Dowex 1X8 (200−400 mesh, AcO^- form, 1.5 cm × 10 cm). The column was eluted with H_2O, and the ninhydrin-positive fractions were combined and concentrated under reduced pressure. The residue was dissolved in H_2O (4 mL) and the solution was lyophilized to afford **2o** (73 mg, 80%) as a white solid. Mp 140 °C; $[\alpha]^{25}_D$ −9.0 (*c* 0.6, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 3.72 (1H, dd, J = 4.3 and 8.4 Hz), 3.00 (2H, t, J = 7.1 Hz), 2.38 (1H, m), 2.14 (1H, ddd, J = 4.3, 9.0, and 14.1 Hz), 1.92 (1H, ddd, J = 4.9, 8.4, and 14.0 Hz), 1.72−1.50 (4H, m); ^{13}C NMR (100 MHz, D_2O) δ 182.7, 174.4, 53.3, 44.4, 39.2, 33.0, 29.0, 24.8; HRMS (ES−) m/z 203.1045 ([M − H]$^-$, $C_8H_{15}N_2O_4$ requires 203.1032). Anal. Calcd for $C_8H_{16}N_2O_4 \cdot 1.5H_2O$: C, 41.55, H, 8.28, N, 12.11. Found: C, 41.62, H, 7.65, N, 12.03. 96.9% purity by HPLC analysis.

(2*S*,4*R*)-Azepane-2,4-dicarboxylic Acid 2p. To a solution of **2l** (160 mg, 0.8 mmol) in H_2O (20 mL) was added 10% Pd/C (100 mg). The mixture was stirred for 72 h at room temperature under hydrogen atmosphere, using a balloon. Pd/C was removed by filtration through a membrane (0.2 μm), and the filtrate was concentrated under reduced pressure. The residue was dissolved in H_2O (2 mL) and the solution poured on a column of Dowex 1X8 (200−400 mesh, AcO^- form, 1.5 cm × 15 cm). The column was first eluted with H_2O to afford **2o** (65 mg, 39%) as a 85:15 mixture of diastereomers. Further elution with an AcOH gradient afforded **2p** (89 mg, 59%) as a 94:6 mixture of diastereomers. A second chromatography on Dowex 1X8 allowed the isolation of a fraction (60 mg, 40%) with a diastereomeric excess over 98% as judged by ^{1}H NMR. **2p** was isolated as a white solid. Mp 70 °C; $[\alpha]^{25}_D$ −29.2 (*c* 0.5, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 3.82 (1H, d, J = 11.2 Hz), 3.30 (2H, m), 2.76 (1H, m), 2.62 (1H, m), 2.16 (1H, m), 2.01 (2H, m), 1.93−1.70 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ 179.3, 173.8, 59.5, 44.8, 43.4, 31.2, 29.2, 22.6; HRMS (ES−) m/z 186.0763 ([M − H]$^-$, $C_8H_{12}NO_4$ requires 186.0766). Anal. ($C_8H_{13}NO_4 \cdot 0.5H_2O$) C, H, N.

Binding Pharmacology. *Native iGluRs.* All native receptor binding experiments were performed using rat brain synaptic membranes of cortex and the central hemispheres from male SPRD rats with tissue preparation as described in the literature.[35] Characterization at AMPA, KA, and NMDA receptors was determined using 5 nM [^{3}H]AMPA,[36] 5 nM [^{3}H]KA,[37] and 2 nM [^{3}H]CGP 39653[38] with some modifications: On the day of the assay, frozen membranes were quickly thawed and homogenized in 50 volumes of ice-cold 30 mM Tris-HCl buffer, pH 7.4, containing 2.5 mM $CaCl_2$ (for [^{3}H]AMPA binding), 50 mM Tris-HCl buffer, pH 7.4 (for [^{3}H]KA binding), or 50 mM Tris-HCl buffer, pH 7.4, containing 2.5 mM $CaCl_2$ (for [^{3}H]CGP 39653 binding), before centrifugation (48000*g* for 10 min). This washing step was repeated four times. In the [^{3}H]AMPA binding experiment, 100 mM KSCN was added to the buffer during the final wash and during incubation. The final pellet was resuspended in ice-cold buffer corresponding to approximately 0.4−0.5 mg of protein/mL. The binding was carried out in 96-wells plates by incubation of 200 μL of membrane suspension, 25 μL of [^{3}H]ligand, and 25 μL of test substance in various concentrations at 0 °C for 30 min ([^{3}H]AMPA binding) or for 60 min ([^{3}H]KA and [^{3}H]CGP binding). The reaction was terminated by rapid filtration through GF/C filters (Perkin-Elmer Life Sciences), using a 96-well Packard FilterMate cell harvester, followed by washing with 3 × 250 μL of ice-cold binding buffer. To the dried filters was added Microscint scintillation fluid (Perkin-Elmer Life Sciences), and the amount of filter bound radioactivity was quantified in a Packard TopCount microplate scintillator counter. The experiments were performed in triplicate at least three times for each compound. Nonspecific binding was determined using 1.0 mM Glu. The binding data were analyzed by a nonlinear regression curve-fitting procedure using GraphPad Prism, version 5.04 (GrapPad Software, CA, U.S.).

Recombinant iGluRs. Radioligand binding assays were performed as previously described at full length recombinant rat iGluRs: GluA2(R)$_o$, GluK1(Q)$_{1b}$, GluK2(VCR)$_a$, GluK3$_a$, expressed in *Sf*9 cell membranes.[39] (*RS*)-[^{3}H]AMPA (45.8 Ci/mmol, PerkinElmer, Waltham, MA) was used as the radioligand for GluA2(R)$_o$ (K_d = 16.8 nM). [^{3}H]-(2*S*,4*R*)-4-Methylglutamic acid (40 Ci/mmol, ARC, St. Louis, MO) was used as the radiolabel for GluK1−3: GluK1(Q)$_{1b}$, K_d = 0.66 nM; GluK2(VCR)$_a$, K_d = 17 nM; GluK3$_a$, K_d = 5.7 nM.

Soluble iGluR LBDs. Binding of **2i** was carried out at the LBD constructs as previously described.[40,41] GluK3 LBD protein (50 ng) was incubated with 5−10 nM [^{3}H]-(2*S*,4*R*)-4-methylglutamic acid for 1 h at 4 °C in assay buffer (50 mM Tris-HCl, 10% (v/v) glycerol, pH 7.1 at 4 °C); GluK3 LBD K_d = 63 nM.[26] Nonspecific binding was determined in the presence of 1 mM (*S*)-glutamate. Competition experiments were carried out in the presence of 16 concentrations of **2i** (10 nM to 0.25 mM) in triplicate. Samples were filtered onto Whatman (Dassel, Germany) 0.2 μm ME 24 mixed cellulose ester filters and were washed twice with 1.5 mL of ice-cold assay buffer. Filters were dried at 70 °C for 1 h, and 1.5 mL of Filter-Count (PerkinElmer) scintillation fluid added. After dissolution of the filters radioactivity was determined by liquid scintillation counting (TriCarb 2900, PerkinElmer).

GluA2 LBD protein (50 ng) binding was similarly measured in assay buffer (50 mM Tris-HCl, 100 mM KSCN, 2.5 mM $CaCl_2$, 10% (v/v) glycerol, pH 7.2 at 4 °C) using 2−5 nM (*RS*)-[5-methyl-^{3}H]-AMPA at 11 concentrations of **2i** (1 μM to 1 mM) in triplicate. The radioligand K_d value (12.8 nM) at GluA2 LBD was previously determined.[42]

Competition data were analyzed using Grafit, version 3.00 (Erithacus Software Ltd., Horley, U.K.), and fit as previously described[43] to determine **2i** K_i.

EAAT Pharmacology. *Cell Culture.* The construction and maintenance of stable HEK293 cell lines expressing the human EAAT subtypes EAAT1, EAAT2, and EAAT3 have been described previously.[24] The cell lines were grown in culture medium [Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 5% dialyzed fetal bovine serum] supplemented with 1 mg/mL G-418 at 37 °C in a humidified 5% CO_2 incubator.

[^{3}H]-D-Asp Uptake Assay. The characterization of the compounds was performed in a [^{3}H]-D-Asp uptake assay essentially as previously described.[24] Briefly, cells were split into white 96-well plates (PerkinElmer, Boston, MA). At 16−24 h later the culture medium was aspirated, and cells were washed twice with 75 μL of assay buffer (Dulbecco's phosphate buffered solution supplemented with 20 mM HEPES, 2 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4). Then 50 μL of assay buffer supplemented with 30 nM [^{3}H]-D-Asp and the various compounds were added to the various wells, and the plate was incubated at 37 °C for 6 min. The assay mixtures were quickly removed from the wells, which were then washed with 3 × 75 μL of ice-cold assay buffer, and 150 μL of Microscint scintillation fluid (PerkinElmer, Boston, MA) was added to each well. The plate was shaken for at least 1 h and counted in a TopCounter (PerkinElmer, Boston, MA). The experiments were performed in duplicate 3−4 times for each compound. Concentration−inhibition curves were generated by nonweighted least-squares fits using the program GraphPad Prism (GraphPad Software, CA, U.S.).

X-ray Structure Determinations. *Crystallization of GluA2-LBD in Complex with* ***2i****.* The rat GluA2-LBD (GluR2-S1S2J)[25] has been expressed and purified essentially as previously described[44] except that trypsin digestion was used to remove the His-tag (buffer: 20 mM NaOAc, 1 mM Glu, 10 mM EDTA, 5 mM (*RS*)-methionine, 10 mM NaCl, pH 5.5) followed by ion-exchange chromatography (HiTrap SP).[28] GluA2-LBD in complex with **2i** was crystallized using the hanging drop vapor diffusion method at 6 °C. The drop contained 1 μL of complex solution (4.9 mg/mL GluA2-LBD and 8.4 mM **2i** in 10 mM HEPES, pH 7.0, 20 mM NaCl, and 1 mM EDTA) and 1 μL of reservoir solution of 15.2% PEG4000, 0.1 M Li_2SO_4, 0.1 M phosphate−citrate, pH 4.5. Reservoir volume was 0.5 mL. The crystals appeared within 1 week.

Crystallization of GluK3-LBD in Complex with ***2i****.* The rat GluK3-LBD (GluK3-S1S2) was prepared and purified as previously reported.[27] Crystallization of GluK3-LBD with Glu was carried out by the hanging drop vapor diffusion method at 6 °C. The protein−Glu solution contained 6.2 mg/mL GluK3-LBD in 10 mM HEPES, 20 mM NaCl, and 1 mM Glu, pH 7.0. Crystals were grown in drops of 1 μL of protein solution and 1 μL of reservoir solution of 1.8 M sodium/potassium phosphate, pH 8.2. The reservoir volume was 0.5 mL. After growth, the drops containing crystals were added 1 μL of 20 mM **2i** in 10 mM HEPES, 20 mM NaCl, and 1 mM EDTA, pH 7.0, and were allowed to equilibrate for 3 weeks at 6 °C.

Data Collection and Processing. Crystals of both complexes were flash cooled in liquid nitrogen after soaking them into cryo buffer consisting of reservoir solution plus 20% glycerol. X-ray data were collected at the I911-3 beamline (MAX-Lab, Lund, Sweden) at a wavelength of 1.0000 Å. Full data sets were collected to 1.24 Å resolution for the GluA2 complex and to 2.65 Å resolution for the GluK3 complex. Data processing was performed using iMosflm[45] and the CCP4 suite of programs.[46] Structure determination was carried out using the program PHASER[47] implemented in CCP4. The GluA2-LBD with (*S*)-ACPA (PDB entry 1M5E, molA)[42] was used as a search model for the GluA2 complex, and the structure of GluK3-LBD in complex with Glu (PDB entry 3S9E, molA)[27] was used as search model for the GluK3 complex, including protein atoms only. Further model building was performed by the program ArpWarp[48] in CCP4 (GluA2 complex) and Autobuild in PHENIX[49] (GluK3 complex). A few residues that could not be built in automatically were added manually using the program COOT.[50] The ligand coordinates were created in Maestro [*Maestro*, version 9.2; Schrödinger, LLC: New York, NY, 2011] and fitted into the electron density. Topology and parameter files for **2i** were obtained using eLBOW[51] after geometry optimization (MMFF94s [*MacroModel*, version 9.9; Schrödinger, LLC: New York, NY, 2011]). The two structures were refined using PHENIX, and between refinements the model was inspected and corrected in COOT. The GluA2-LBD complex was refined using anisotropic *B*-factors and riding hydrogen atoms, whereas the GluK3-LBD structure was refined using TLS and grouped *B*-factors. **2i**, water, and glycerol molecules, as well as chloride, lithium, and potassium ions, were manually modeled into the electron densities. For statistics on data collection and refinements, see Table S1 in Supporting Information.

Structure Analysis. Domain closure of GluA2-LBD and GluK3-LBD in complex with **2i**, both compared to the apo structure of GluA2 (PDB entry 1FTO, molA),[25] was calculated using the DynDom server.[52] Ligand−protein interactions were identified and distances measured in COOT. Figures were prepared using PyMOL [*The PyMOL Molecular Graphics System*; Schrödinger, LLC: New York, NY, 2006].

■ ASSOCIATED CONTENT

Supporting Information

Combustion analysis data for **2b−p**, **6a−j**, and **10**; ^{1}H and ^{13}C NMR spectra of compounds **2b−p**; HPLC analysis results of **2e**, **2i**, and **2o**; statistics on X-ray data collection and refinements as well as Figure S1 with zoom on the ligand-binding site of GluA2-LBD with **2i** illustrating the alternative conformation. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The coordinates and structure factors have been deposited in the Protein Data Bank: GluA2-LBD with **2i** (PDB entry 4IGT) and GluK3-LBD with **2i** (PDB entry 4IGR).

■ AUTHOR INFORMATION

Corresponding Author

*For K.F. (structural biology work): phone, +45 35336207; e-mail, karla.frydenvang@sund.ku.dk. For T.G. (synthetic work): phone, +33 473407866; fax, +33 473407717; e-mail, thierry.gefflaut@univ-bpclermont.fr. For L.B.: phone, +45 35336244; e-mail, lebu@sund.ku.dk.

Notes

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■ ABBREVIATIONS

KG, α-ketoglutarate; AMPA, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid; AspAT, aspartate aminotransferase; CSA, cysteine sulfinic acid; EAAT, excitatory amino acid transporter; iGluR, ionotropic Glu receptor; KA, kainic acid; LBD, ligand binding domain; mGluR, metabotropic Glu receptor; NMDA, *N*-methyl-D-aspartate

■ REFERENCES

(1) Traynelis, S. F.; Wollmuth, L. P.; McBain, C. J.; Menniti, F. S.; Vance, K. M.; Ogden, K. K.; Hansen, K. B.; Yuan, H.; Myers, S. J.;

Dingledine, R. Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol. Rev.* **2010**, *62*, 405−496.
(2) Ferraguti, F.; Shigemoto, R. Metabotropic glutamate receptors. *Cell Tissue Res.* **2006**, *326*, 483−504.
(3) Campiani, G.; Fattorusso, C.; De Angelis, M.; Catalanotti, B.; Butini, S.; Fattorusso, R.; Fiorini, I.; Nacci, V.; Novellino, E. Neuronal high-affinity sodium-dependent glutamate transporters (EAATs): targets for the development of novel therapeutics against neurodegenerative diseases. *Curr. Pharm. Des.* **2003**, *9*, 599−625.
(4) Seal, R. P.; Amara, S. G. Excitatory amino acid transporters: a family in flux. *Annu. Rev. Pharmacool. Toxicol.* **1999**, *39*, 431−456.
(5) Bunch, L.; Erichsen, M. N.; Jensen, A. A. Excitatory amino acid transporters as potential drug targets. *Expert Opin. Ther. Targets* **2009**, *13*, 719−31.
(6) Hwang, B.-Y.; Cho, B.-K.; Yun, H.; Koteshwar, K.; Kim, B.-G. Revisit of aminotransferase in the genomic era and its application to biocatalysis. *J. Mol. Catal. B: Enzym.* **2005**, *37*, 47−55.
(7) Helaine, V.; Bolte, J. Chemoenzymatic synthesis of (4*S*)- and (4*R*)-4-methyl-2-oxoglutaric acids, precursors of glutamic acid analogues. *Eur. J. Org. Chem.* **1999**, 3403−3406.
(8) Helaine, V.; Rossi, J.; Gefflaut, T.; Alaux, S.; Bolte, J. Synthesis of 4,4-disubstituted L-glutamic acids by enzymatic transamination. *Adv. Synth. Catal.* **2001**, *343*, 692−697.
(9) 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.
(10) Sagot, E.; Pickering, D. S.; Pu, X.; Umberti, M.; Stensbøl, T. B.; Nielsen, B.; Chapelet, M.; Bolte, J.; Gefflaut, T.; Bunch, L. Chemoenzymatic 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. *J. Med. Chem.* **2008**, *51*, 4093−4103.
(11) 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.
(12) Emiliozzi, R.; Pichat, L. A simple method for the preparation of cysteinesulfinic acid. *Bull. Soc. Chim. Fr.* **1959**, 1887−1888.
(13) Jenkins, W. T.; D'Ari, L. Preparation of the phosphopyridoxamine form of the glutamic-aspartic transaminase. *Biochem. Biophys. Res. Commun.* **1966**, *22*, 376−82.
(14) Helaine, V.; Rossi, J.; Bolte, J. A new access to alkyl-alpha-ketoglutaric acids, precursors of glutamic acid analogues by enzymatic transamination. Application to the synthesis of (2*S*,4*R*)-4-propyl-glutamic acid. *Tetrahedron Lett.* **1999**, *40*, 6577−6580.
(15) 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.
(16) Chakrabortty, P. N.; Dasgupta, R.; Dasgupta, S. K.; Ghosh, S. R.; Ghatak, U. R. Synthetic studies towards complex diterpenoids—VI: New synthetic routes to tetracyclic bridged-bicyclo[3.2.1]octane intermediates by intramolecular alkylation reactions through α-diazomethyl ketones of hydroaromatic γ,δ-unsaturated acids. *Tetrahedron* **1972**, *28*, 4653−4665.
(17) Bunch, L.; Gefflaut, T.; Alaux, S.; Sagot, E.; Nielsen, B.; Pickering, D. S. Pharmacological characterization of (4*R*)-alkyl glutamate analogues at the ionotropic glutamate receptors. Focus on subtypes iGlu(5-7). *Eur. J. Pharmacol.* **2009**, *609*, 1−4.
(18) Bunch, L.; Nielsen, B.; Jensen, A. A.; Brauner-Osborne, H. Rational design and enantioselective synthesis of (1*R*,4*S*,5*R*,6*S*)-3-azabicyclo[3.3.0]octane-4,6-dicarboxylic acid—a novel inhibitor at human glutamate transporter subtypes 1, 2, and 3. *J. Med. Chem.* **2006**, *49*, 172−178.
(19) Faure, S.; Jensen, A. A.; Maurat, V.; Gu, X.; Sagot, E.; Aitken, D. J.; Bolte, J.; Gefflaut, T.; Bunch, L. Stereoselective chemoenzymatic synthesis of the four stereoisomers of L-2-(2-carboxycyclobutyl)glycine and pharmacological characterization at human excitatory amino acid transporter subtypes 1, 2, and 3. *J. Med. Chem.* **2006**, *49*, 6532−6538.
(20) Conti, P.; De Amici, M.; De Sarro, G.; Rizzo, M.; Stensbol, T. B.; Brauner-Osborne, H.; Madsen, U.; Toma, L.; De Micheli, C. Synthesis and enantiopharmacology of new AMPA-kainate receptor agonists. *J. Med. Chem.* **1999**, *42*, 4099−4107.
(21) Bunch, L.; Krogsgaard-Larsen, P. Subtype selective kainic acid receptor agonists: discovery and approaches to rational design. *Med. Res. Rev.* **2009**, *29*, 3−28.
(22) Johansen, T. N.; Janin, Y. L.; Nielsen, B.; Frydenvang, K.; Brauner-Osborne, H.; Stensbol, T. B.; Vogensen, S. B.; Madsen, U.; Krogsgaard-Larsen, P. 2-Amino-3-(3-hydroxy-1,2,5-thiadiazol-4-yl)-propionic acid: resolution, absolute stereochemistry and enantiopharmacology at glutamate receptors. *Bioorg. Med. Chem.* **2002**, *10*, 2259−2266.
(23) Clausen, R. P.; Hansen, K. B.; Cali, P.; Nielsen, B.; Greenwood, J. R.; Begtrup, M.; Egebjerg, J.; Brauner-Osborne, H. The respective *N*-hydroxypyrazole analogues of the classical glutamate receptor ligands ibotenic acid and (*RS*)-2-amino-2-(3-hydroxy-5-methyl-4-isoxazolyl)-acetic acid. *Eur. J. Pharmacol.* **2004**, *499*, 35−44.
(24) Jensen, A. A.; Bräuner-Osborne, H. Pharmacological characterization of human excitatory amino acid transporters EAAT1, EAAT2 and EAAT3 in a fluorescence-based membrane potential assay. *Biochem. Pharmacol.* **2004**, *67*, 2115−2127.
(25) Armstrong, N.; Gouaux, E. Mechanisms for activation and antagonism of an AMPA-sensitive glutamate receptor: crystal structures of the GluR2 ligand binding core. *Neuron* **2000**, *28*, 165−181.
(26) Pøhlsgaard, J.; Frydenvang, K.; Madsen, U.; Kastrup, J. S. Lessons from more than 80 structures of the GluA2 ligand-binding domain in complex with agonists, antagonists and allosteric modulators. *Neuropharmacology* **2011**, *60*, 135−150.
(27) Venskutonyte, R.; Frydenvang, K.; Gajhede, M.; Bunch, L.; Pickering, D. S.; Kastrup, J. S. Binding site and interlobe interactions of the ionotropic glutamate receptor GluK3 ligand binding domain revealed by high resolution crystal structure in complex with (*S*)-glutamate. *J. Struct. Biol.* **2011**, *176*, 307−314.
(28) Juknaite, L.; Venskutonyte, R.; Assaf, Z.; Faure, S.; Gefflaut, T.; Aitken, D. J.; Nielsen, B.; Gajhede, M.; Kastrup, J. S.; Bunch, L.; Frydenvang, K.; Pickering, D. S. Pharmacological and structural characterization of conformationally restricted (*S*)-glutamate analogues at ionotropic glutamate receptors. *J. Struct. Biol.* **2012**, *180*, 39−46.
(29) Venskutonyte, R.; Frydenvang, K.; Hald, H.; Rabassa, A. C.; Gajhede, M.; Ahring, P. K.; Kastrup, J. S. Kainate induces various domain closures in AMPA and kainate receptors. *Neurochem. Int.* **2012**, *61*, 536−545.
(30) Armstrong, N.; Mayer, M.; Gouaux, E. Tuning activation of the AMPA-sensitive GluR2 ion channel by genetic adjustment of agonist-induced conformational changes. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 5736−5741.
(31) Madden, D. R.; Cheng, Q.; Thiran, S.; Rajan, S.; Rigo, F.; Keinanen, K.; Reinelt, S.; Zimmermann, H.; Jayaraman, V. Stereochemistry of glutamate receptor agonist efficacy: engineering a dual-specificity AMPA/kainate receptor. *Biochemistry* **2004**, *43*, 15838−15844;Erratum: *Biochemistry* **2006**, *45*, 3472.
(32) Unno, M.; Shinohara, M.; Takayama, K.; Tanaka, H.; Teruya, K.; Doh-ura, K.; Sakai, R.; Sasaki, M.; Ikeda-Saito, M. Binding and selectivity of the marine toxin neodysiherbaine A and its synthetic analogues to GluK1 and GluK2 kainate receptors. *J. Mol. Biol.* **2011**, *413*, 667−683.
(33) 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.

(34) Gefflaut, T.; Assaf, Z.; Sancelme, M. Preparation of Glutamate Analogues by Enzymatic Transamination. In *Unnatural Amino Acids*; Pollegioni, L., Servi, S., Eds.; Humana Press: New York, 2012; Vol. *794*, pp 55−72.
(35) Ransom, R. W.; Stec, N. L. Cooperative modulation of [H-3] Mk-801 binding to the *N*-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine, and polyamines. *J. Neurochem.* **1988**, *51*, 830−836.
(36) Honore, T.; Nielsen, M. Complex structure of quisqualate-sensitive glutamate receptors in rat cortex. *Neurosci. Lett.* **1985**, *54*, 27−32.
(37) Braitman, D. J.; Coyle, J. T. Inhibition of [H-3] kainic acid receptor-binding by divalent-cations correlates with ion affinity for the calcium-channel. *Neuropharmacology* **1987**, *26*, 1247−1251.
(38) Sills, M. A.; Fagg, G.; Pozza, M.; Angst, C.; Brundish, D. E.; Hurt, S. D.; Wilusz, E. J.; Williams, M. [H-3] Cgp-39653—a new *N*-methyl-D-aspartate antagonist radioligand with low nanomolar affinity in rat-brain. *Eur. J. Pharmacol.* **1991**, *192*, 19−24.
(39) Clausen, R. P.; Naur, P.; Kristensen, A. S.; Greenwood, J. R.; Strange, M.; Brauner-Osborne, H.; Jensen, A. A.; Nielsen, A. S.; Geneser, U.; Ringgaard, L. M.; Nielsen, B.; Pickering, D. S.; Brehm, L.; Gajhede, M.; Krogsgaard-Larsen, P.; Kastrup, J. S. The glutamate receptor GluR5 agonist (*S*)-2-amino-3-(3-hydroxy-7,8-dihydro-6*H*-cyclohepta[*d*]isoxazol-4-yl)propionic acid and the 8-methyl analogue: synthesis, molecular pharmacology, and biostructural characterization. *J. Med. Chem.* **2009**, *52*, 4911−4922.
(40) Nielsen, B. B.; Pickering, D. S.; Greenwood, J. R.; Brehm, L.; Gajhede, M.; Schousboe, A.; Kastrup, J. S. Exploring the GluR2 ligand-binding core in complex with the bicyclical AMPA analogue (*S*)-4-AHCP. *FEBS J.* **2005**, *272*, 1639−1648.
(41) Frydenvang, K.; Lash, L. L.; Naur, P.; Postila, P. A.; Pickering, D. S.; Smith, C. M.; Gajhede, M.; Sasaki, M.; Sakai, R.; Pentikainen, O. T.; Swanson, G. T.; Kastrup, J. S. Full domain closure of the ligand-binding core of the ionotropic glutamate receptor iGluR5 induced by the high affinity agonist dysiherbaine and the functional antagonist 8,9-dideoxyneodysiherbaine. *J. Biol. Chem.* **2009**, *284*, 14219−14229.
(42) Hogner, A.; Kastrup, J. S.; Jin, R.; Liljefors, T.; Mayer, M. L.; Egebjerg, J.; Larsen, I. K.; Gouaux, E. Structural basis for AMPA receptor activation and ligand selectivity: crystal structures of five agonist complexes with the GluR2 ligand-binding core. *J. Mol. Biol.* **2002**, *322*, 93−109.
(43) 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.
(44) Ramanoudjame, G.; Du, M.; Mankiewicz, K. A.; Jayaraman, V. Allosteric mechanism in AMPA receptors: a FRET-based investigation of conformational changes. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 10473−10478.
(45) Leslie, A. G. W.; Powell, H. R. Processing Diffraction Data with Mosflm. *Evol. Methods Macromol. Crystallogr.* **2007**, *245*, 41−51.
(46) Winn, M. D.; Ballard, C. C.; Cowtan, K. D.; Dodson, E. J.; Emsley, P.; Evans, P. R.; Keegan, R. M.; Krissinel, E. B.; Leslie, A. G. W.; McCoy, A.; McNicholas, S. J.; Murshudov, G. N.; Pannu, N. S.; Potterton, E. A.; Powell, H. R.; Read, R. J.; Vagin, A.; Wilson, K. S. Overview of the CCP4 suite and current developments. *Acta Crystallogr., Sect D: Biol. Crystallogr.* **2011**, *67*, 235−242.
(47) McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J. Phaser crystallographic software. *J. Appl. Crystallogr.* **2007**, *40*, 658−674.
(48) Cohen, S. X.; Ben Jelloul, M.; Long, F.; Vagin, A.; Knipscheer, P.; Lebbink, J.; Sixma, T. K.; Lamzin, V. S.; Murshudov, G. N.; Perrakis, A. ARP/wARP and molecular replacement: the next generation. *Acta Crystallogr., Sect D: Biol. Crystallogr.* **2008**, *64*, 49−60.
(49) Adams, P. D.; Afonine, P. V.; Bunkoczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L. W.; Kapral, G. J.; Grosse-Kunstleve, R. W.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R.; Read, R. J.; Richardson, D. C.; Richardson, J. S.; Terwilliger, T. C.; Zwart, P. H. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr., Sect D: Biol. Crystallogr.* **2010**, *66*, 213−221.
(50) Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K. Features and development of Coot. *Acta Crystallogr., Sect D: Biol. Crystallogr.* **2010**, *66*, 486−501.
(51) Moriarty, N. W.; Grosse-Kunstleve, R. W.; Adams, P. D. Electronic ligand builder and optimization workbench (eLBOW): a tool for ligand coordinate and restraint generation. *Acta Crystallogr., Sect D: Biol. Crystallogr.* **2009**, *65*, 1074−1080.
(52) Hayward, S.; Lee, R. A. Improvements in the analysis of domain motions in proteins from conformational change: DynDom version 1.50. *J. Mol. Graphics Modell.* **2002**, *21*, 181−183.