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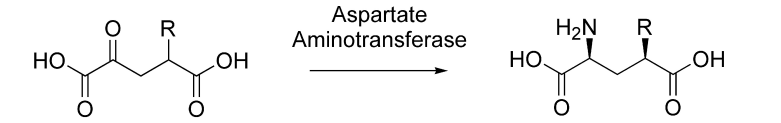

rac-4-alkyl-2-oxoglutaric acids

L-2,4-*syn*-4-alkylglutamic acids

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Chemoenzymatic Synthesis of a Series of 4-Substituted Glutamate Analogues and Pharmacological Characterization at Human Glutamate Transporters Subtypes 1−3

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A series of nine L-2,4-*syn*-4-alkylglutamic acid analogues (**1a**−**i**) were synthesized in high yield and high enantiomeric excess (>99% ee) from their corresponding 4-substituted ketoglutaric acids (**2a**−**i**), using the enzyme aspartate aminotransferase (AAT) from pig heart or *E. coli*. The synthesized compounds were evaluated as potential ligands for the glutamate transporters EAAT1, EAAT2, and EAAT3 (excitatory amino acid transporter, subtypes 1−3) in the FLIPR membrane potential (FMP) assay. We found a distinct change in the pharmacological profile when the 4-methyl group (compound **1a**, an EAAT1 substrate and EAAT2,3 inhibitor) was extended to a 4-ethyl group, compound **1b**, as this analogue is an inhibitor at all three subtypes, EAAT1−3. Furthermore, we conclude that both large and bulky hydrophobic substituents in the 4-position of L-2,4-*syn* Glu are allowed by all three glutamate transporter subtypes EAAT1−3 while maintaining inhibitory activity.

Introduction

In the mammalian central nervous system (CNS), L-glutamate (Glu) functions as the major excitatory neurotransmitter. Once released from the presynaptic neuron into the glutamatergic synapse, Glu activates a number of pre- and postsynaptic glutamate receptors. On the basis of pharmacological profile and ligand selectivity, these receptors have been grouped in two classes: the fast acting *ionotropic receptors* (the iGluRs, which are further divided into *N*-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, and kainate receptors) and the *metabotropic receptors* (mGluR1−8), which are G-protein-coupled and thus produce a much slower signal transduction through second messenger systems.[1,2] The glutamatergic neurotransmission is terminated by reuptake of Glu from the synaptic cleft by the action of sodium-dependent excitatory amino acid transporters (EAATs).[3,4] To this date, five subtypes have been identified of which four, EAAT1−4, are present in the mammalian CNS, while EAAT5 is located exclusively in the retina. EAAT1−3 are localized on neurons and/or astroglial cells and exhibit high capacity for transporting Glu across the membrane, whereas EAAT4 and EAAT5 function predominately as chloride channels. In the healthy CNS, activation of Glu receptors is involved in important neurophysiological processes such as memory and learning, motor functions, and neural plasticity, and development.[1] However, under conditions of metabolic stress and oxygen deprivation, Glu is a neurotoxic agent. Thus, it is believed that neurodegenerative diseases such as Alzheimer's disease, dementia, Huntington's disease, amyotrophic lateral sclerosis (ALS), epilepsy, and cerebral stroke may be directly related to disordered glutamatergic neurotransmission originating from dysfunction of either the Glu receptors (iGluR or mGluR) or the Glu reuptake system (EAATs).[1]

Considering the essential role played by the EAATs in the maintenance of synaptic Glu concentrations below neurotoxic levels, surprisingly little attention has been paid to the transporters as potential drug targets in the treatment of the plethora of neurotoxic and neurodegenerative disorders mentioned above.[5,6] Recently, it has been suggested that the EAATs may exhibit reverse transport of Glu under ischemic conditions and that selective inhibitors may prevent this.[7] At present, only a limited amount of EAAT inhibitors and substrates have been described, all which, except for dihydrokainic acid (DHK), do not show high EAAT subtype selectivity. In a previous study, Vandenberg et al.[8] characterized the pharmacological profiles of L-2,4-*syn*/*anti*-4-methyl and L-2,4-*syn*/*anti*-4-hydroxy Glu analogues at EAAT1 and EAAT2 (Figure 1, Table 1). In the present study, we have explored the SAR of EAAT1−3 through the design and synthesis of a series of L-2,4-*syn*-4-alkyl Glu analogues **1b**−**i** (Figure 1).

Chemistry

The stereoselective synthesis of 4-alkylated Glu analogues **1a**−**i** was considered using a transamination-based chemoenzymatic approach (Scheme 1). Aminotransferases catalyze the reversible transfer of the amino group from a donor amino acid to an acceptor α-keto acid with an equilibrium constant near unity. These broad substrate spectrum enzymes have proven useful for the synthesis of unnatural amino acids from the corresponding α-keto acids, provided that an equi-

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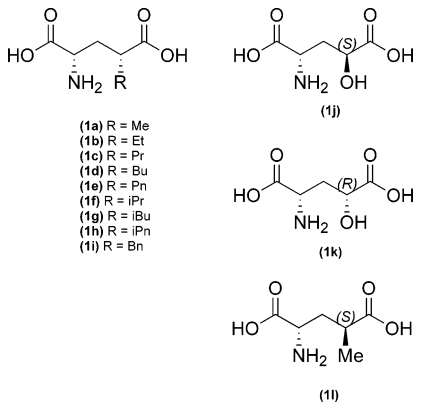


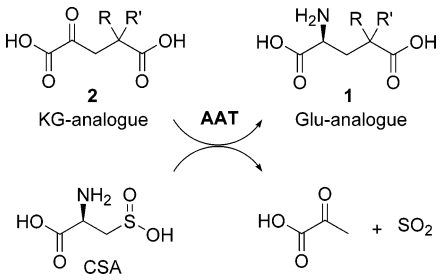
Figure 1. Chemical structures of novel and previously reported Glu analogues **1a**−**l**.

Table 1. Reported Pharmacology of Glu Analogues **1a**, **1j**, **1k**, and **1l** at EAAT1,2[a]

	4-substitution	EAAT1	EAAT2
Glu	H	*20 ± 3*	*18 ± 3*
1a	2,4-*syn*-Me	*54 ± 17*[b]	3.4 ± 0.2
1j	2,4-*anti*-OH	*61 ± 14*[c]	*48 ± 5*[c]
1k	2,4-*syn*-OH	*>1000*	*>1000*
1l	2,4-*anti*-Me	no activity	no activity

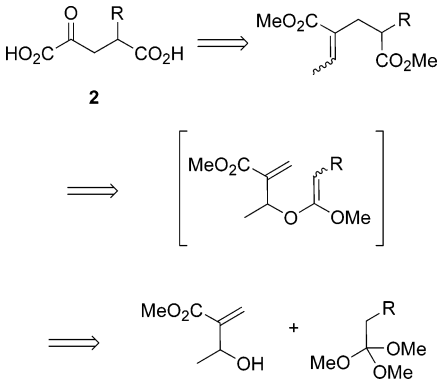
[a] K_m values for substrates (in italics) and K_i values for nonsubstrate inhibitors are given in μM. Data are taken from ref 8. [b] The I_{max} value of **1a** at EAAT1 was 80 ± 5% of the I_{max} for (*S*)-Glu. [c] The I_{max} values of **1j** at EAAT1 and EAAT2 were 78 ± 6% and 90 ± 4%, respectively, of the I_{max} values for (*S*)-Glu.

Scheme 1. AAT-Catalyzed Synthesis of 4-Substituted L-Glu from the Corresponding KG Analogues



librium shift is performed.[9−18] Because glutamic acid is a preferred substrate for most naturally occurring aminotransferases, these enzyme are of particular interest for the synthesis of L-Glu analogues from the corresponding substituted ketoglutaric acids (KG). Aspartate aminotransferase (AAT, also called glutamic oxaloacetic transaminase) from pig heart was shown to be active toward 4-substituted ketoglutaric acids **2** and was used to prepare both isomers of 4-hydroxy-L-Glu[19] **1j** and **1k**, 4-methyl, 4-ethyl,[20] and 4-propyl derivatives[21] **1a**, **1b**, **1c** and **1l**, as well as the disubstituted 4-OH,4-Me and 4,4-diMe analogues.[22] In the case of the monosubstituted 4-alkyl derivatives, AAT showed a marked enantioselectivity in favor of the 4*R* isomer, allowing the kinetic resolution of the racemic keto acid precursors and providing a stereoselective route to the L-2,4-*syn* stereomers. These syntheses were carried out according to Scheme 1. Cysteine sulfinic acid (CSA), a close analogue of aspartic acid, was used as the amino donor. The ketoacid produced from CSA is the very unstable pyruvylsulfinic acid, which undergoes a rapid decomposition into pyruvate and sulfurous anhydride. An equilibrium shift is thus obtained because pyruvate is not a substrate for AAT.

Scheme 2. Retrosynthesis of KG Analogues **2** through Claisen−Johnson Rearrangement



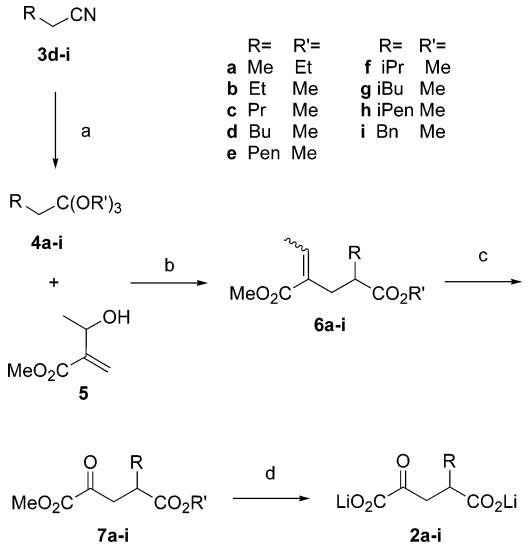
A few synthetic approaches to alkyl-substituted KGs **2** have been described so far. The Claisen condensation of the alkyl succinic ester onto diethyl oxalate gives a mixture of 3- and 4-alkyl-KGs and requires the previous synthesis of the corresponding 2-alkyl succinic ester. The direct alkylation at position 4 of protected α-ketoglutaric acid was also tried;[23] however, this method is efficient only for monomethylation. Substituted KGs **2** have also been obtained by a new route in only a few steps and in good yields through a Claisen−Johnson rearrangement, as depicted in the retro synthetic Scheme 2.[21]

In the present study, we extended this methodology to the synthesis of nine substituted α-ketoglutaric acids **2a**−**i** and their stereoselective transamination into the corresponding L-2,4-*syn*-4-alkylglutamic acids **1a**−**i**, catalyzed by isolated pig heart or *E. coli* AAT.

Results and Discussions

Synthesis of 4-Alkyl-2-oxoglutaric Acids 2a−i. The syntheses of 4-alkyl-KG analogues **2a**−**i** were carried out according to Scheme 3. The key step is the acid-catalyzed reaction of the allylic alcohol **5** and ortho esters **4a**−**i** in refluxing toluene. After transesterification and elimination of methanol, a ketene ketal is formed that irreversibly undergoes a Claisen−Johnson rearrangement,[24] leading to dimethyl 2-ethylidene-4-alkylglutarate **6a**−**i** in 70−95% yields. In every case, a mixture of *Z* and *E* isomers was obtained with a ratio close to 4/1 except **6i** (3/2) as determined by ^{1}H NMR. As previously shown, ozonolysis of the C−C double bond can provide the 2-ketoglutarates **7a**−**c**.[21] Alternatively, the oxidative cleavage was accomplished with sodium periodate in the presence of a catalytic amount of ruthenium tetroxide.[25,26] The α-keto esters **7a**−**i** were thus isolated in 70−90% yields. Ester hydrolysis was performed with a stoechiometric amount of LiOH and afforded the desired substituted lithium 2-ketoglutarates **2a**−**i** in quantitative yields. Methyl 2-(1-hydroxyethyl)acrylate **5** was easily prepared by the Baylis−Hillman reaction between methyl acrylate and acetaldehyde in the presence of DABCO.[27,28] Propanoic, butanoic, and pentanoic ortho esters were commercially available. Ortho esters **4d**−**i** were prepared from the corresponding nitriles using the Pinner method.[29] Nitriles **3d**−**i** were synthesized by standard, described

Scheme 3. Synthesis of 4-Alkyl-2-oxoglutaric Acids **2a**−**i**[a]



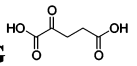
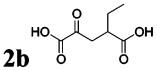
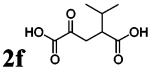
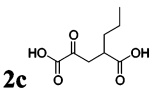
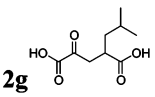
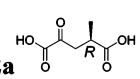
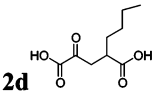
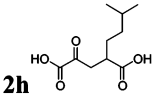
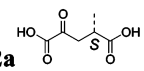
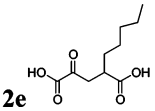
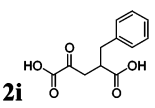
[a] Reagents and conditions: (a) (i) MeOH, HCl, 0 °C; (ii) MeOH, cyclohexane, reflux; (b) $EtCO_2H$, $PhCH_3$, reflux; (c) $NaIO_4$, RuO_4, H_2O, CCl_4, CH_3CN, room temp; (d) LiOH, H_2O, THF, room temp.

methods. This synthesis offers a very general access to the 4-alkyl-2-ketoglutaric acids. It should be noted that it is applicable to 4,4-disubstituted derivatives as well as 3-substituted analogues provided that the corresponding ortho ester or the 3-substituted acrylate is available. Moreover, the concerted nature of the Claisen rearrangement could allow the development of a stereoselective version using enantiomerically pure allylic alcohol **5**.

Aspartate Aminotransferase Activity toward 2-Ketoglutaric Acid Analogues. Kinetic constants for the transamination reaction of the KG analogues **2a**−**i** were estimated using aspartic acid as the donor amino acid in quasi-saturating concentration (40 mM). The oxalacetate produced from Asp by transamination was reduced by NADH in the presence of malic dehydrogenase. The optical density decrease consecutive to NADH oxidation was measured at 340 nm and allowed to monitor the transamination reaction. Rate measurements for various substrate concentrations allowed us to estimate the apparent Michaelis constants (K_m) and maximum velocity ($V_m = k_{cat}(E)$) in the defined experimental conditions on the basis of the Michaelis−Menten model for enzyme-catalyzed reactions. Results are reported in Table 2.

The activity of pig heart AAT for both enantiomers of **2a** was already described.[23] The *R* isomer is a very good substrate, with K_m and k_{cat} values in the same range as those measured for the natural substrate. The *S* isomer was characterized by higher K_m and lower k_{cat}, resulting in a lower activity, which is still valuable for synthetic purposes. This AAT enantioselectivity (E > 600) allowed the kinetic resolution of rac-**2a**, **2b**,[20] and **2c**.[21] We have now observed that when larger substituents are introduced in position 4 of KG, the K_m values suffer no great changes. Except for the branched substituted **2f** showing lower affinity and, to a lesser extent, **2g**, 4-alkyl analogues are bound equally well as KG itself, even the bulky pentyl, isopentyl, or benzyl derivatives. Furthermore, rate measurements were performed with the racemic derivatives and we can assume that the determined K_m values correspond to the best recognized 4*R* isomer (4*S* for **2f**), as was observed for the 4-methyl analogue **2a**. This hypothesis was confirmed by the structure of the transamination products (vide infra). The K_m values determined from the racemic concentration should thus be divided by a factor of 2 because only one enantiomer is a good substrate. Comparison with the natural substrate then indicates that hydrophobic substituents at position 4 can actually increase the enzyme−substrate affinity. The k_{cat} values also remain close to that of KG, so the k_{cat}/K_m value, indicating the overall enzyme efficiency for the tested reaction, is in the same order of magnitude as for the natural substrate with the exception of **2f**. Transamination of KG analogues **2a**−**i** catalyzed by *E. coli* AAT

Table 2. Kinetic Parameters for Pig Heart AAT Transamination of **2a**−**i**[a]

compd[b]	K_m (mM)	k_{cat}[c] (%)	k_{cat}/K_m[c] (%)	compd	K_m (mM)	k_{cat}[c] (%)	k_{cat}/K_m[c] (%)	compd	K_m (mM)	k_{cat}[c] (%)	k_{cat}/K_m[c] (%)
KG 	0.23 ± 0.04	100 ± 3	100 ± 18	**2b** 	0.50 ± 0.06	130 ± 5	47 ± 10	**2f** 	4.76 ± 0.27	11 ± 0.6	0.5 ± 0.1
				2c 	0.50 ± 0.02	82 ± 1	37 ± 7	**2g** 	1.48 ± 0.09	62 ± 2	9.6 ± 1.8
***R*-2a** 	0.19 ± 0.02	120 ± 4	149 ± 30	**2d** 	0.28 ± 0.05	58 ± 2	48 ± 12	**2h** 	0.47 ± 0.08	84 ± 4	41 ± 10
***S*-2a** 	9 ± 0.8	9 ± 0.4	0.2 ± 0.05	**2e** 	0.25 ± 0.05	89 ± 7	83 ± 22	**2i** 	0.28 ± 0.03	82 ± 6	68 ± 15

[a] Values and standard errors were calculated from the Hanes−Woolf plot according to the least-squares method and Gauss' error propagation law. [b] Parameters for already described enantiomers of **2a**[23] are included for comparison. [c] k_{cat} and k_{cat}/K_m are expressed as percent relative to the natural unsubstituted KG substrate.

was also studied. The K_m and relative k_{cat} values measured with the bacterial enzyme are very similar to those reported in Table 1 for the pig heart enzyme. This high activity of AAT toward the 4-substituted analogues is somewhat surprising; however, Kirsch and co-workers already pointed out that the presence of a long aliphatic chain on substrates does not decrease the *E. coli* enzyme−ligand complex stabilization.[30] Numerous X-ray structures of AAT complexed with substrates or inhibitors have been published.[31−34] A careful inspection of a chicken mitochondrial enzyme−substrate structure[35] (PDB code 1MAQ) allowed us to observe that the 4-pro*R* hydrogen is pointed to the outside of the active site and is surrounded by hydrophobic side chains creating a favorable environment for alkyl group stabilization in that position.

Synthesis of Glutamic Acid Analogues. The enzymatic transaminations were performed according to Scheme 1 as described above. In early experiments, we observed a loss of enzyme activity as the reaction proceeded. This was not considered before as a major obstacle for small-scale syntheses; however, we tried in this study to minimize enzyme inhibition. It was attributed to the use of CSA as the amino donor substrate. Indeed, sulfur dioxide produced from CSA in the reaction medium is hydrated into sulfites and the strongly nucleophilic HSO_3^- anions interact with the pyridoxal coenzyme bound at the active site as several imine intermediates. *E. coli* enzyme proved to be less sensitive to inhibition than pig heart AAT. Moreover, we have found that this inactivation can be limited by the addition of acetaldehyde to the reaction mixture; the aldehyde acts as a hydrogenosulfite scavenger through bisulfitic adduct formation and allows faster reactions and yield improvements. The 4-alkyl-KG derivatives **2a**−**i** were submitted to the action of pig heart or *E. coli* AAT in the presence of CSA and acetaldehyde. The initial concentration was 20 mM in both substrates and 40 mM in acetaldehyde. The reaction was monitored by enzymatic assay of pyruvic acid produced from CSA, using lactic acid dehydrogenase and NADH. The reaction was stopped when a conversion rate between 40% and 50% was reached, to achieve a good kinetic resolution of the racemic substrates. The reaction mixture was then poured onto a short column of a sulfonic resin (H^+ form). The column was washed with water to elute the unreacted KG analogue and then CSA, which was only weakly adsorbed because of the strong acidity of the sulfinic moiety. Glu analogue **1** was then eluted with diluted ammonia. The amino acid containing fractions were concentrated, and the desired Glu analogues were thus isolated in 32−42% yields from racemic KGs and with purities over 90%. The purity was further increased to more than 98% by a second chromatography on anion-exchange resin (AcO^- form) using an acetic acid gradient for elution. For each synthesis one stereomer only was obtained with diastereomeric excess over 98% as determined by ^1H NMR and HPLC analyses. Since AAT is known to be highly stereoselective toward amino acids of the L series, an *S* configuration was assigned to carbon 2. Carbon 4 configuration was expected to be *R* in any case except for **1f** by analogy with the well described 4-methyl and 4-ethyl analogues already obtained by AAT transamination.[20] However, we have been looking for additional evidence of the stereochemical assignments. **2a**−**e** and **2i** were submitted to reductive amination by reaction with ammonia and reduction of the imine with $NaBH_4$. The four-stereomer mixture thus obtained for each compound was analyzed by chiral HPLC on a Crownpack column. Under the used experimental conditions and for all the studied amino acids, D-isomers were always eluted first. The chromatogram obtained with the 4-methylglutamic acids **1a** showed four signals with 8, 9, 18, and 28 min retention times. Peaks observed at 18 and 28 min were attributed to the L-2,4-*syn* and L-2,4-*anti* isomers, respectively, by comparison with authentic samples previously prepared with AAT. For each mixture studied, conditions were found to resolve the four stereomers and similar chromatographic profiles were observed. Analyses of the samples obtained by enzymatic transamination showed only one signal always corresponding to the third eluted isomer of the corresponding mixture obtained by chemical amination. Since the order of elution cannot be considered as absolute evidence for the absolute configuration, a complementary experiment was performed in the case of the bulky 4-pentyl-L-glutamic acid **1e**. This compound was converted into 4-pentyl-L-pyroglutamic acid ethyl ester **8** by reaction with thionyl chloride and EtOH. This constrained derivative was then studied by NMR spectroscopy. Comparison of the observed coupling constants with the theoretical values estimated by molecular modeling for the expected (3*R,*5*S*)-**8**, as well as experimental NOE, is reported in Figure 2.

The measured coupling constants are in good agreement with the calculated values for the expected preferred conformation **II** of (3*R,*5*S*)-**8**. Analysis of the NOE is also consistent with the proposed structure. In contrast, the experimental data cannot account for the proposed structure of the (3*S,*5*S*)-stereomer and especially for the expected antiperiplanar position of H^3 toward H^1 and H^4 in structure **III**. These experiments confirmed previous findings and were considered to be demonstrative of the general stereoselectivity of the AAT catalyzed transamination providing L-2,4-*syn*-4-alkylglutamic acids.

Pharmacology and Modeling. All synthesized 4-substituted Glu analogues **1a**−**k** were characterized at the glutamate transporter subtypes EAAT1, EAAT2, and EAAT3, using a FLIPR membrane potential (FMP) assay (see Experimental Section for details). The assay was performed essentially as described previously,[36] and the results are summarized in Table 3. Compound **1a** was found to be a substrate at EAAT1, whereas it was an inhibitor at the two other EAAT subtypes. The 4-hydroxy Glu analogues **1j** and **1k** were substrates at all three EAAT subtypes (EAAT1−3), the L-2,4-*anti* diastereomer **1j** being considerably more potent than the L-2,4-*syn* diastereomer **1k** (Table 3). The pharmacological properties of **1a**, **1j**, and **1k** in the FMP assay were in good agreement with data previously obtained for these Glu analogues in electrophysiological recordings on EAAT1 and EAAT2 expressed in *Xenopus* oocytes (Table 1).[8] The eight novel 4-substituted Glu analogues presented in this study (compounds **1b**−**h**) were all inhibitors at all three EAAT subtypes, where they displayed K_i values in the mid-to-high micromolor

Protons interaction	Model dihedral angle for (*3R,5S*)-**8** (°)	Model coupling constant for (*3R,5S*)-**8** (Hz)	Measured coupling constant (Hz)	NOE (%)
H_1-H_2	32	7.5	9.0	10
H_1-H_3	88	1.2	2.5	1,2
H_2-H_3	-	-	13.0	24
H_2-H_4	160	11.1	9.0	0
H_3-H_4	40	6.5	9.0	6

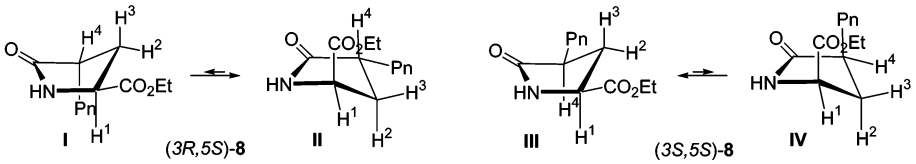


Figure 2. Conformational and NMR analyses of compound **8**.

Table 3. Pharmacological Characteristics of (*S*)-Glu and Its 4-Substituted Analogues **1a**−**k** at Human EAAT1, EAAT2, and EAAT3 in the FMP Assay[a]

	4-substitution	EAAT1	EAAT2	EAAT3
(*S*)-Glu	H	*9.2 [5.04 ± 0.05]*	*21 [4.69 ± 0.05]*	*25 [4.61 ± 0.06]*
1a	L-2,4-*syn*-Me	*13 [4.92 ± 0.11]*[b]	13 [4.93 ± 0.16]	6.6 [5.27 ± 0.20]
1b	L-2,4-*syn*-Et	23 [4.64 ± 0.01]	14 [4.97 ± 0.23]	38 [4.43 ± 0.07]
1c	L-2,4-*syn*-Pr	13 [4.99 ± 0.20]	22 [4.68 ± 0.12]	32 [4.50 ± 0.07]
1d	L-2,4-*syn*-Bu	50 [4.32 ± 0.09]	42 [4.42 ± 0.12]	80 [4.15 ± 0.14]
1e	L-2,4-*syn*-Pn	120 [3.93 ± 0.04]	77 [4.17 ± 0.16]	98 [4.06 ± 0.14]
1f	L-2,4-*syn*-^{i}Pr	35 [4.54 ± 0.15]	19 [4.79 ± 0.17]	51 [4.30 ± 0.06]
1g	L-2,4-*syn*-^{i}Bu	55 [4.39 ± 0.20]	22 [4.66 ± 0.07]	300 [3.53 ± 0.06]
1h	L-2,4-*syn*-^{i}Pn	29 [4.54 ± 0.03]	26 [4.66 ± 0.19]	81 [4.18 ± 0.20]
1i	L-2,4-*syn*-Bn	13 [4.94 ± 0.12]	36 [4.49 ± 0.12]	66 [4.21 ± 0.11]
1j	L-2,4-*anti*-OH	*140 [4.06 ± 0.28]*[c]	*67 [4.21 ± 0.13]*[c]	*81 [4.16 ± 0.13]*[c]
1k	L-2,4-*syn*-OH	*~1000 [~3.0]*	*~1,000 [~3.0]*	*~1000 [~3.0]*

[a] K_m values for substrates (in italics) and K_i values for nonsubstrate inhibitors are given in μM with pK_m ± SEM and pK_i ± SEM values in brackets. [b] The R_{max} value of **1a** at EAAT1 was 48 ± 8% of the R_{max} for (*S*)-Glu. [c] The R_{max} values of **1j** at EAAT1, EAAT2, and EAAT3 were 82 ± 3%, 68 ± 9%, and 87 ± 2%, respectively, of the R_{max} values for (*S*)-Glu.

ranges (Table 3). Compound **1g** displayed a slightly higher K_i value at EAAT3 than at the two other transporters, but overall the eight ligands were nonselective among the three EAATs.

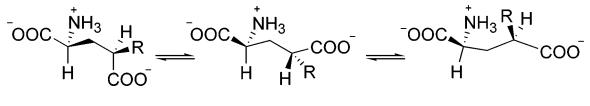
Two major observations can be extracted from the characterization of the 11 4-substituted Glu analogues **1a**−**k** at EAAT1−3. First, extension of the L-2,4-*syn*-4-alkyl substituent from a methyl group, compound **1a**, to an ethyl group, compound **1b**, marks a clear change in the pharmacological profile from substrate to inhibitor at the EAAT1 subtype. This, with the inhibitory activity at EAAT2 and EAAT3, is maintained. Second, this study shows that both large and bulky substituents of a hydrophobic nature in the 4-position, compounds **1b**−**h**, are readily accommodated by all three EAAT subtypes investigated here (EAAT1−3). Thus, we predict that EAAT subtype selectivity is likely not obtained by introduction of another alkyl substituent in the L-2,4-*syn*-4-position of Glu.

Structure−Activity-Relation Study. It has been proposed by other research groups that substrates and inhibitors bind in two distinct ways at the EAATs.[5,37,38] While substrates are believed to bind in an extended Glu conformation, the binding conformation of inhibitors is assumed to be equivalent to the folded Glu conformation previously identified as the agonist binding conformation at the iGluRs by X-ray crystallographic studies[39] and rational ligand design studies.[40]

To identify biologically relevant conformational minima, we submitted 4-alkyl Glu analogues **1a**−**h** to a stochastic conformational search in the modeling software MOE (see Experimental Section for details). For each compound except **1f** and **1i**, we located three important low-energy conformations: one folded and two extended conformations (Table 4). On the other hand, the stochastic conformational search of compounds **1f** and **1i** returned only two low-energy conformation: one folded and one extended (denoted A). For all nine Glu analogues, the folded conformation is favored by an average of 2.1 kcal/mol over the extended A conformation and by an average 3.4 kcal/mol (compounds **1i** and **1f** not included) over the extended B conformation (Table 4). It is noteworthy that the extended Glu conformation, denoted A, is similar to the conformation of Glu found when crystallized together with the extracellular domain of the metabotropic Glu receptor mGluR1.[41]

EAAT Inhibitor Pharmacophore. To clarify the size and bulkiness of the 4-alkyl substituent allowed while maintaining inhibitory activity at EAAT1−3, the low-energy folded conformation of compounds **1a**−**i**, were superimposed by fitting the ammonium and the

Table 4. Biologically Important Low-Energy Conformations of 4-Alkyl Glu Analogues **1a**−**i**



	total free conformational energy (kcal/mol)[a]				
	Δ*G*-(folded)	Δ*G*-(extended A)	Δ*G*-(extended B)	$\Delta\Delta G_A$[b]	$\Delta\Delta G_B$[c]
1a	−90.20	−87.50	−86.53	−2.70	−3.67
1b	−89.34	−86.95	−85.73	−2.39	−3.61
1c	−88.77	−86.76	−85.74	−2.01	−3.03
1d	−88.68	−86.81	−85.37	−1.87	−3.04
1e	−88.41	−86.72	−85.30	−1.69	−3.11
1f	−83.11	−80.65	cnf[d]	−2.46	<−7.0
1g	−83.04	−80.96	−79.56	−2.08	−3.48
1h	−83.46	−81.16	−79.50	−2.30	−3.96
1i	−75.03	−73.20	cnf[d]	−1.83	<−7.0

[a] For details, see Experimental Section. [b] $\Delta\Delta G_A$ calculated from Δ*G*(folded) − Δ*G*(extended A). [c] $\Delta\Delta G_B$ calculated from Δ*G*(folded) − Δ*G*(extended B). [d] cnf: conformation not found.

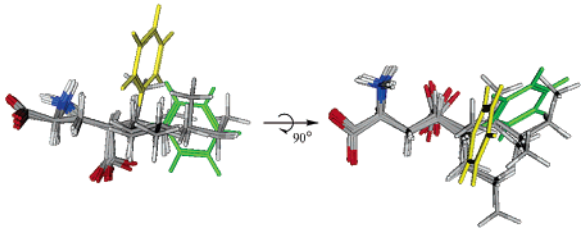


Figure 3. Superimposition by fitting the ammonium group and the two carboxylate groups of calculated low-energy folded conformation of **1a**−**i**. Two conformations of the phenyl ring in compound **1i** are highlighted: lowest energy conformation (yellow); +0.66 kcal/mol (green).

two carboxylate groups (Figure 3). In eight of the nine compounds, the 4-alkyl substituents arrange themselves in a flat planar fashion away from the Glu backbone, except for the 4-benzyl Glu analogue **1i** (benzyl group in yellow, Figure 3), of which the 4-benzyl group is oriented perpendicular. To fit the orientation of the 4-benzyl group to the other 4-alkyl substituents, a slightly higher energy conformation (+0.66 kcal/mol) of **1i** is used (benzyl group in green, Figure 3). On the other hand, if a +1 kcal/mol margin of error is allowed, the number of conformations found for the 4-alkyl substituents is highly increased. Superimposition of all of these conformations suggests a 4-alkyl region, which is now best characterized as spherical (Figure 4). On the basis of these modeling considerations and the fact that we do not see significant EAAT subtype selectivity, we put forward that these 4-alkyl substituents are likely oriented out in space rather than into a pocket of the EAAT protein. Thus, we suggest that the observed decrease in potency as the alkyl group is grown longer (compounds **1a**−**e**) is primarily due to loss of entropic energy.

Recently, some aryl analogues of L-*threo*-*β*-benzyloxyaspartate (TBOA) were published as being highly potent, yet not subtype selective, EAAT inhibitors.[42] We therefore decided to probe if the large arylic substituents of these novel TBOA analogues were likely to occupy the same area in space as our 4-alkyl substituents. Superimposition of low-energy conformations of TBOA with low-energy conformations of **1a**−**i** (up to +1 kcal/mol) suggests that the phenyl ring in TBOA is oriented

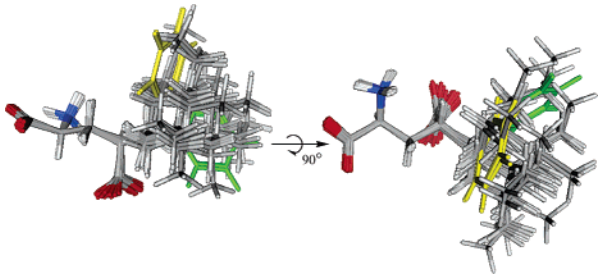


Figure 4. Superimposition by fitting the ammonium group and the two carboxylate groups of calculated low-energy folded conformations (up to +1 kcal/mol) of compounds **1a**−**i**. The two conformations of the phenyl ring in compound **1i** are highlighted: lowest energy conformation (yellow); +0.66 kcal/mol (green).

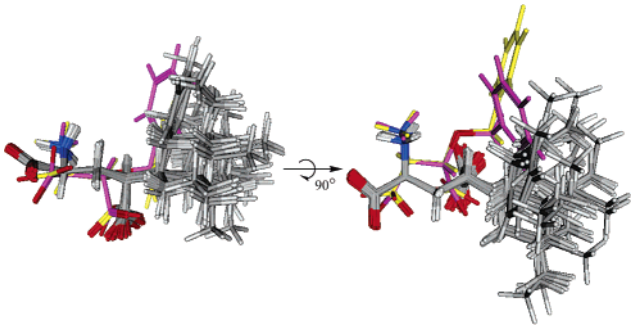


Figure 5. Superimposition by fitting the ammonium group and the two carboxylate groups of calculated low-energy folded conformations (up to +1 kcal/mol) of compounds **1a**−**i** and ;-*threo*-*β*-benzyloxyaspartate (TBOA) (low-energy conformation, yellow; +0.93 kcal/mol, purple).

on the brink of our 4-alkyl substituents (Figure 5). This supports our hypothesis that the 4-alkyl substituents (compounds **1a**−**i**) are most likely directed into a larger area in space rather than into a pocket of the EAAT protein.

EAAT Substrate Pharmacophore. It is important to stress that any compound that can be accommodated by the EAAT substrate pocket is to be characterized as a substrate, even though its folded conformation (inhibitory conformer) is energetically favored. An example is the L-2,4-*syn*-4-methyl analogue **1a**, which is a substrate at EAAT1, while it is an inhibitor at EAAT2 and EAAT3 (Tables 3 and 4). For an improved understanding of the pharmacophore of the EAAT substrate binding pocket, it is essential to determine which extended conformer, extended A or extended-B, is the biologically active one. On the basis of total free energy calculations, this question cannot be answered because the difference amounts to only <1 kcal/mol (Table 4). We therefore turned to investigate the low-energy conformations of L-2,4-*anti*-4-methyl Glu (**1l**), which is completely inactive at all EAAT subtypes (Table 1). When this compound is submitted to a stochastic conformational search, only one low-energy extended conformation is found (denoted extended AA). Superimposition of this conformer with the extended A conformer of **1a** and the two well-described rigid EAAT substrates *t*-PDC and L-2,4-MPDC shows that the two diastereomeric 4-methyl substituents occupy the same area in space (Figure 6). Since **1l** is inactive at EAAT1−3, the extended-A/extended-AA Glu conformations are likely not the bioactive substrate conformers, and we suggest that the

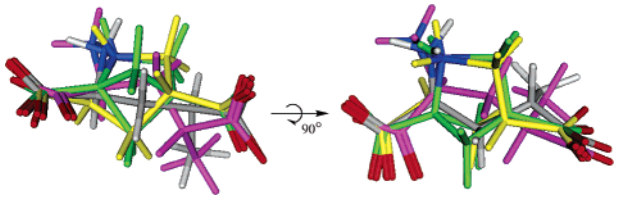


Figure 6. Pharmacophore model A of substrate binding conformation. Superimposition was done by fitting the ammonium group and the two carboxylate groups of calculated low-energy conformations of L-2,4-MPDC (green), *t*-PDC (yellow), extended A **1a** (purple), extended AA **1l** (type code).

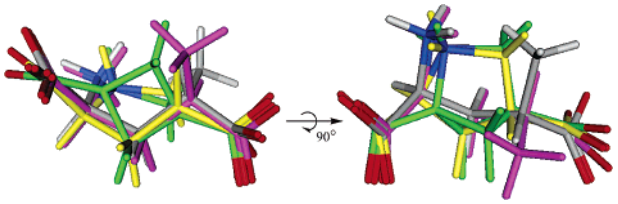


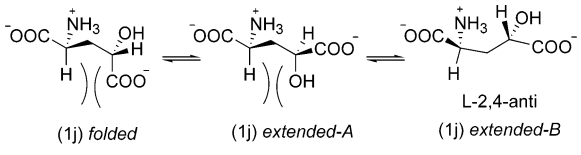
Figure 7. Pharmacophore model B of substrate binding conformation. Superimposition was done by fitting the ammonium group and the two carboxylate groups of calculated low-energy conformations of L-2,4-MPDC (green), *t*-PDC (yellow), extended B **1a** (purple), extended B **1l** (type code).

area in space occupied by these two methyl groups is most likely forbidden in the EAAT substrate pharmacophore.

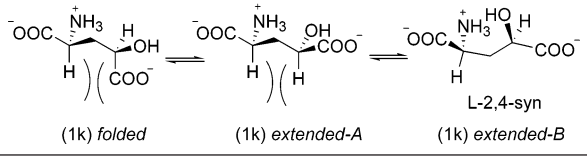
Also, the conformer of **1l**, which resembles the extended B conformer of **1a**, can only be considered at the theoretical level because of the imposed highly disfavored interaction between the L-2,4-*anti*-4-methyl group and the ammonium group (Figure 7). Thus, we suggest that the extended B conformer of **1a** (Figure 7) best describes the substrate binding conformation at the EAATs (pharmacophore model B) and that the origin of the observed difference in pharmacology of compounds **1a** and **1b** is due to differentiated structural features of the substrate binding pocket in each EAAT subtype. It remains to be identified which exact amino acids in the EAAT proteins are involved.

We then turned to investigate the origin of difference in pharmacology of the two diastereomeric 4-OH Glu analogues **1j** and **1k**. First, we wanted to address why **1j** is a substrate despite the fact that its 4-methyl replacement analogue **1l** is completely inactive. Second, we wanted to investigate why **1k** is a low potent substrate even though its 4-methyl replacement analogue **1a** is indeed a potent substrate at EAAT1. Thus, amino acid alcohols **1j** and **1k** were submitted to a stochastic conformational search in MOE, which revealed three low-energy conformations for each compound (Table 5). The conformational energies calculated for **1j** suggest the folded and extended B conformations to exist in about 1:1 equilibrium, while the extended A conformation is unfavored by +6.52 kcal/mol (relative to the extended B conformation). Because the extended B conformation is believed to be the active substrate binding conformation, these free energy calculations are thus in line with the observed substrate character of **1j** at EAAT1−3. The conformational energies calculated for **1k** show a clear preference for the extended A conformation by −4.26 kcal/mol (relative to the folded conformation) and as much as −12.44 kcal/mol over the extended B conformation. The highly favorable hydrogen bond interaction between the ammonium group and the alcohol in the extended A conformation is largely accountable for this observation. However, **1k** is a low potent substrate at EAAT1−3, which may be explained from a superimposition study of the known rigid substrates L-2,4-MPDC and *t*-PDC with extended B **1j** and extended A **1k** (Figure 8).

Table 5. Biological Relevant Low-Energy Conformations of **1j** and **1k**



(1j) *folded* (1j) *extended-A* (1j) *extended-B*



(1k) *folded* (1k) *extended-A* (1k) *extended-B*

	total free conformational energy (kcal/mol)[a]				
	Δ*G*- (folded)	Δ*G*- (extended A)	Δ*G*- (extended B)	$\Delta\Delta G_A$[b]	$\Delta\Delta G_B$[c]
1j	−70.33	−64.23	−70.75	−6.10	+0.42
1k	−64.74	−69.00	−56.56	+4.26	−8.18

[a] For details, see Experimental Section. [b] $\Delta\Delta G_A$ calculated from Δ*G*(folded) − Δ*G*(extended A). [c] $\Delta\Delta G_B$ calculated from Δ*G*(folded) − Δ*G*(extended B).

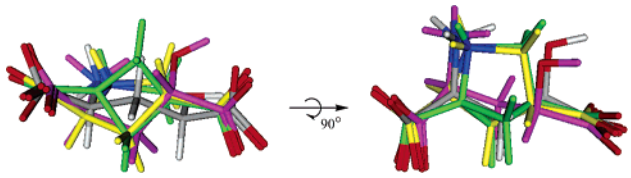


Figure 8. Pharmacophore model of substrate binding conformation, showing three-point superimposition of calculated low-energy conformations of L-2,4-MPDC (green), *t*-PDC (yellow), extended B **1j** (purple), extended A **1k** (type code).

It is evident that the 4-hydrogen in the extended A conformer of **1k** is found in the area previously identified as forbidden (see above and Figure 6). We thus suggest that this observation and the fact that the extended B conformer of **1k** is not energetically favored are the two major reasons for the observed low substrate potency of **1k** at EAAT1−3.

Conclusion

In conclusion, nine Glu analogues were synthesized in a stereoselective fashion taking advantage of a chemoenzymatic strategy. Aspartate aminotransferase proved to be useful for the preparation of L-2,4-*syn*-4-alkylglutamic acid analogues from the corresponding racemic ketoglutarates. These latter compounds were prepared by a simple and general procedure. The 11 compounds were then characterized at human EAAT1, EAAT2, and EAAT3 in the FMP assay. A distinct change in the pharmacological profile was observed when the 4-methyl group (compound **1a**, an EAAT1 substrate and EAAT2,3 inhibitor) was extended to a 4-ethyl group, compound **1b**, as this analogue is an inhibitor at all three subtypes EAAT1−3. We have shown that quite large and bulky hydrophobic substituents in the 4-position of Glu are accommodated by

EAAT1−3, maintaining the inhibitory activity. Our modeling study presents new insight on the origin of the diverse pharmacological profiles of structurally close Glu analogues **1a**, **1j**, **1k**, and **1l**. Furthermore, our two suggested binding conformations, one for substrates and one for inhibitors, clarify the EAAT1−3 structure−activity relations and are in agreement with results from earlier reports.

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 experiments were performed at the Centre Regional de Mesures Physiques de l'Université de Rennes, France, using an MS/MS ZABspec 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'Analyse du CNRS, Solaize, France. Silica gel 60 (Merck, 40−63 μm) and precoated F_{254} plates were used for column and TLC chromatographies. All solvents were purified by distillation following the usual procedures. HPLC analyses of **1a**−**e** and **1i** were performed at 220 nm using a Crownpack column (Daicel, 4.0 mm × 150 mm) with the following conditions: **1a**, $HClO_4$, pH 1, 0.3 mL min^{-1}, 25 °C; **1b**, $HClO_4$, pH 1, 0.5 mL min^{-1}, 25 °C; **1c**−**d**, $HClO_4$, pH 1, 15% MeOH (v/v), 1 mL min^{-1}, 25 °C; **1e**, $HClO_4$, pH 1, 15% MeOH (v/v), 1.6 mL min^{-1}, 43 °C; **1i**, $HClO_4$, pH 2, 15% MeOH (v/v), 1.3 mL min^{-1}, 25 °C. Racemic reference samples were prepared from **2a**−**i** using a described procedure for reductive amination.[43]

Nitrile **3d** and ortho esters **4a**−**c** were purchased from Aldrich. 3-Phenylpropanenitrile **3i** was prepared from cinnamonitrile following a described procedure.[44] Cysteine sulfinic acid was prepared from cystine following a described procedure.[45] Pig heart aspartate aminotransferase, bovine heart malic dehydrogenase, and rabbit muscle lactic dehydrogenase were purchased from Sigma and Roche. *E. coli* aspartate aminotransferase was produced and purified following a described procedure from an overexpressing *E. coli* strain TY103 transformed with pUC19-aspC.[46,47] Enzyme kinetic measurements were performed at 25 °C in 0.1 M potassium phosphate buffer, pH 7.6, Asp (40 mM), NADH (0.2 mM), ketoacid substrate (0.1−10 mM), AAT (0.05 IU), and malic dehydrogenase (2 IU) 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}.

General Procedure for the Syntheses of Nitriles 3e−g. A mixture of finely powdered KCN (48.8 g, 0.375 mol), water (13.4 mL, 0.375 mol), bromoalcane (0.5 mol), and Aliquat 336 (4.6 mL, 10 mmol) was heated at 85 °C. The reaction was monitored by TLC for 4−48 h. After addition of Et_2O (100 mL), the mixture was filtered on Florisil, and the filtrate was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was distilled under reduced pressure to give the desired nitrile isolated as a colorless liquid.

Heptanenitrile 3e. Yield 50.5 g, 91%; bp 82−85 °C (30 mmHg); IR (neat film) 2247 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.34 (2H, t, J = 7.0 Hz), 1.66 (2H, quint, J = 7.0 Hz), 1.45 (2H, m), 1.32 (4H, m), 0.90 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 119.6, 30.7, 28.1, 25.1, 22.1, 16.8, 13.7.

3-Methylbutanenitrile 3f. Yield 30.8 g, 74%; bp 56−58 °C (16 mmHg); IR (neat film) 2247 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.20 (2H, d, J = 7.0 Hz), 1.95 (1H, non., J = 7.0 Hz), 1.00 (6H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 118.7, 25.8, 25.7, 21.5.

4-Methylpentanenitrile 3g. Yield 37.8 g, 78%; bp 70−75 °C (16 mmHg); IR (neat film) 2251 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.30 (2H, t, J = 7.5 Hz), 1.68 (1H, non., J = 7.0 Hz), 1.50 (2H, q, J = 7.5 Hz), 0.89 (6H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 119.8, 33.6, 27.1, 21.6, 14.9.

5-Methylhexanenitrile 3h. Sodium (23 g, 1 mol) was added to absolute EtOH (500 mL). After complete reaction, ethyl cyanoacetate (200 g, 1.77 mol) was added slowly before 1-bromo-3-methylbutane (151 g, 1 mol). The mixture was stirred under reflux for 10 h until neutralization of the reaction mixture was indicated. After cooling, the mixture was filtered on Celite and ethanol was evaporated under reduced pressure. The residue was diluted with water (500 mL) and extracted with Et_2O (3 × 100 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was distilled under reduced pressure to give 115 g of ethyl 2-cyano-5-methylhexanoate (88−91 °C, 10 mmHg). To a solution of this product (50 g, 0.27 mol) in DMSO (250 mL) were added water (10.7 mL, 0.6 mol) and NaCl (5.26 g, 0.09 mol). The mixture was heated under reflux for 2 h, then diluted with water (100 mL) and extracted with Et_2O (3 × 100 mL). The organic layer was washed with brine (2 × 50 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was distilled under reduced pressure (75−80 °C, 30 mmHg) to give **3h** (24.7 g, 52% overall yield) as a colorless liquid: IR (neat film) 2244 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.29 (2H, t, J = 7.0 Hz), 1.65−1.48 (3H, m), 1.28 (2H, m), 0.86 (6H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 119.7, 37.7, 27.3, 23.2, 22.2, 17.2.

General Procedure for the Synthesis of Trimethyl-ortho Esters 4d−i. To a solution of nitrile (0.2 mol) and MeOH (9.7 mL, 0.24 mol) in anhydrous Et_2O (200 mL) was added gaseous HCl (0.6 mol) dried by passing through concentrated H_2SO_4 and generated in a separated vessel by dropwise addition of concentrated HCl (50 mL) into concentrated H_2SO_4 (75 mL). The solution obtained was kept at 4 °C for 2−5 days to complete the imidate hydrochloride crystallization. The solid was isolated by filtration under N_2, washed with anhydrous Et_2O, and dried under reduced pressure in the presence of KOH pellets. MeOH (41.1 mL, 0.6 mol) was added to a suspension of the imidate hydrochloride in anhydrous hexane (400 mL). The mixture was stirred at room temperature for 48 h. The precipitated ammonium chloride was discarded by filtration, and K_2CO_3 (50 mg, 0.36 mmol) was added to the filtrate before concentration under reduced pressure. The residue was distilled under reduced pressure to give the ortho ester isolated as a colorless liquid.

Trimethyl Orthohexanoate 4d. Yield 24.8 g, 70%; bp 64−68 °C (16 mmHg); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.20 (9H, s), 1.68 (2H, m), 1.28 (6H, m), 0.88 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 115.7, 49.1, 31.8, 30.2, 22.5, 22.4, 13.8.

Trimethyl Orthoheptanoate 4e. Yield 16.4 g, 43%; bp 75−80 °C (18 mmHg); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.18 (9H, s), 1.67 (2H, m), 1.25 (8H, m), 0.85 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 115.8, 49.2, 31.7, 30.3, 29.1, 22.7, 22.5, 14.0.

Trimethyl 3-Methylorthobutanoate 4f. Yield 17.5 g, 54%; bp 46−48 °C (12 mmHg); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.20 (9H, s), 1.77 (1H, non., J = 7.0 Hz), 1.60 (2H, d, J = 7.0 Hz), 0.95 (6H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 115.7, 49.1, 38.1, 23.4, 23.2.

Trimethyl 4-Methylorthopentanoate 4g. Yield 12.7 g, 38%; bp 65−67 °C (15 mmHg); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.20 (9H, s), 1.69 (2H, m), 1.50 (1H, non., J = 7.0 Hz), 1.18 (2H, m), 0.88 (6H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 116.0, 49.3, 31.6, 28.1, 27.9, 22.5.

Trimethyl 5-Methylorthohexanoate 4h. Yield 19.0 g, 50%; bp 80−84 °C (12 mmHg); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.22 (9H, s), 1.69 (2H, m), 1.53 (1H, non., J = 6.5 Hz), 1.32 (2H, m), 1.18 (2H, m), 0.87 (6H, d, J = 6.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 115.9, 49.4, 38.9, 30.7, 28.0, 22.6, 20.6.

Trimethyl 3-Phenylorthopropanoate 4i. Yield 19.7 g, 47%; bp 114−120 °C (10 mmHg); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34−7.20 (5H, m), 3.31 (9H, s), 2.71 (2H, m), 2.09 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 141.6, 128.1, 128.0, 125.8, 115.3, 50.4, 32.1, 29.2.

General Procedure for the Synthesis of Dialkyl 2-Ethylidene-4-alkylglutarate 6a−i. To a solution in anhydrous toluene (25 mL) of methyl 2-(1-hydroxyethyl)acrylate **5** (3.25 g, 25 mmol) prepared following a described procedure[48] were added the ortho ester (50 mmol) and propionic acid (0.18 mL, 2.5 mmol). The mixture was heated under reflux for 1 h before methanol was slowly removed by azeotropic distillation. The solution was concentrated under reduced pressure. Flash chromatography (eluent, cyclohexane−AcOEt, 9:1, v/v) afforded **6a−i** isolated as colorless liquids and consisting of a mixture of *Z* and *E* isomers in an approximately 4/1 ratio except for **6i** (0.64/0.36).

Methyl, Ethyl 2-Ethylidene-4-methylglutarate 6a. Yield 4.18 g, 78%; IR (neat film) 1731, 1714, 1647 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.95 and 6.07 (1H, q, $J = 7.0$ Hz, *E* and *Z* isomers), 4.09 (2H, q, $J = 7.0$ Hz), 3.74 and 3.73 (3H, s, *Z* and *E* isomers), 2.71−2.30 (3H, m), 1.97 and 1.82 (3H, d, $J = 7.0$ Hz, *Z* and *E* isomers), 1.20 (3H, t, $J = 7.0$ Hz), 1.12 and 1.10 (3H, d, $J = 7.0$ Hz, *E* and *Z* isomers); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.0, 167.8, 139.7, 139.5, 130.4, 129.8, 60.1, 51.6, 51.1, 38.9, 38.7, 30.2, 16.8, 15.7, 14.5, 14.2.

Dimethyl 2-Ethylidene-4-ethylglutarate 6b. Yield 3.92 g, 73%; IR (neat film) 1736, 1719, 1647 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.87 and 6.02 (1H, q, $J = 7.0$ Hz, *E* and *Z* isomers), 3.69 and 3.68 (3H, s, *Z* and *E* isomers), 3.58 and 3.57 (3H, s, *Z* and *E* isomers), 2.60−2.40 (3H, m), 1.92 and 1.75 (3H, d, $J = 7.0$ Hz, *Z* and *E* isomers), 1.60−1.40 (2H, m), 0.85 and 0.83 (3H, t, $J = 7.0$ Hz, *E* and *Z* isomers); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.0, 175.9, 167.8, 139.6, 139.4, 130.5, 129.9, 51.6, 51.3, 51.2, 51.1, 46.7, 46.5, 37.1, 29.0, 25.3, 25.2 15.8, 14.4, 11.7, 11.6.

Dimethyl 2-Ethylidene-4-propylglutarate 6c. Yield 4.10 g, 72%; IR (neat film) 1735, 1718, 1647 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.90 and 6.04 (1H, q, $J = 7.0$ Hz, *E* and *Z* isomers), 3.73 and 3.71 (3H, s, *Z* and *E* isomers), 3.60 and 3.59 (3H, s, *Z* and *E* isomer), 2.60−2.40 (3H, m), 1.94 and 1.77 (3H, d, $J = 7.0$ Hz, *Z* and *E* isomers), 1.60−1.20 (4H, m), 0.86 and 0.85 (3H, t, $J = 7.0$ Hz, *E* and *Z* isomers); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.3, 176.2, 167.9, 139.7, 139.5, 130.5, 129.9, 51.7, 51.33, 51.26, 51.2, 45.0, 44.7, 37.5, 34.5, 34.4, 29.3, 20.6, 20.5, 15.9, 14.4, 13.9.

Dimethyl 2-Ethylidene-4-butylglutarate 6d. Yield 4.10 g, 72%; IR (neat film) 1737, 1725, 1647 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.92 and 6.05 (1H, q, $J = 7.0$ Hz, *E* and *Z* isomers), 3.75 and 3.72 (3H, s, *Z* and *E* isomers), 3.62 and 3.61 (3H, s, *Z* and *E* isomer), 2.65−2.40 (3H, m), 1.96 and 1.80 (3H, d, $J = 7.0$ Hz, *Z* and *E* isomers), 1.65−1.15 (6H, m), 0.86 (3H, t, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.0, 175.9, 167.6, 139.7, 139.2, 130.4, 129.8, 51.7, 51.4, 51.1, 51.0, 45.0, 44.7, 37.4, 34.2, 31.8, 31.2, 29.4, 29.3, 22.4, 22.2, 15.6, 14.2, 13.7.

Dimethyl 2-Ethylidene-4-pentylglutarate 6e. Yield 5.89 g, 92%; IR (neat film) 1737, 1722, 1647 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.91 and 6.05 (1H, q, $J = 7.0$ Hz, *E* and *Z* isomers), 3.72 and 3.70 (3H, s, *Z* and *E* isomers), 3.60 and 3.59 (3H, s, *Z* and *E* isomer), 2.65−2.30 (3H, m), 1.95 and 1.78 (3H, d, $J = 7.0$ Hz, *Z* and *E* isomers), 1.65−1.15 (8H, m), 0.85 (3H, t, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.0, 175.8, 167.5, 139.6, 139.2, 130.4, 129.7, 51.4, 51.04, 50.96, 50.9, 45.0, 44.7, 37.4, 32.2, 32.0, 31.5, 29.2, 26.8, 26.7, 22.3, 15.6, 14.2, 13.7.

Dimethyl 2-Ethylidene-4-isopropylglutarate 6f. Yield 4.28 g, 75%; IR (neat film) 1737, 1724, 1647 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.90 and 6.05 (1H, q, $J = 7.0$ Hz, *E* and *Z* isomers), 3.74 and 3.71 (3H, s, *Z* and *E* isomers), 3.60 and 3.58 (3H, s, *Z* and *E* isomers), 2.65−2.30 (3H, m), 1.95 and 1.80 (3H, d, $J = 7.0$ Hz, *Z* and *E* isomers), 1.85 (1H, m), 1.00 and 0.95 (3H, d, $J = 7.0$ Hz, *E* and *Z* isomers), 0.94 and 0.90 (3H, d, $J = 7.0$ Hz, *E* and *Z* isomers); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.6, 175.4, 167.8, 139.7, 139.4, 130.7, 130.1, 52.2, 51.7, 51.6, 51.2, 51.1, 34.9, 30.8, 30.6, 26.9, 20.4, 20.3, 20.1, 15.9, 14.3.

Dimethyl 2-Ethylidene-4-isobutylglutarate 6g. Yield 4.98 g, 82%; IR (neat film) 1737, 1723, 1647 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.92 and 6.05 (1H, q, $J = 7.0$ Hz, *E* and *Z* isomers), 3.74 and 3.73 (3H, s, *Z* and *E* isomers), 3.64 and 3.60 (3H, s, *E* and *Z* isomers), 2.75−2.25 (3H, m), 1.96 and 1.80 (3H, d, $J = 7.0$ Hz, *Z* and *E* isomers), 1.54 (2H, m), 1.25 (1H, m), 0.88 and 0.86 (6H, d, $J = 7.0$ Hz, *E* and *Z* isomers); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.4, 176.3, 167.8, 139.8, 139.5, 130.4, 129.8, 51.6, 51.4, 51.3, 51.2, 43.3, 42.9, 41.6, 41.4, 37.8, 29.7, 26.2, 26.1, 22.2, 22.1, 15.9, 14.4.

Dimethyl 2-Ethylidene-4-isopentylglutarate 6h. Yield 5.0 g, 78%; IR (neat film) 1737, 1723, 1647 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.94 and 6.08 (1H, q, $J = 7.0$ Hz, *E* and *Z* isomers), 3.76 and 3.74 (3H, s, *Z* and *E* isomers), 3.64 and 3.63 (3H, s, *Z* and *E* isomers), 2.70−2.25 (3H, m), 1.98 and 1.82 (3H, d, $J = 7.0$ Hz, *Z* and *E* isomers), 1.70−1.10 (5H, m), 0.88 and 0.87 (6H, d, $J = 7.0$ Hz, *E* and *Z* isomers); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.1, 176.0, 167.8, 139.6, 139.3, 130.4, 129.8, 51.5, 51.24, 51.18, 51.1, 45.3, 45.0, 38.3, 36.3, 36.2, 30.2, 30.0, 29.3, 27.9, 22.5, 22.3, 15.7, 14.3.

Dimethyl 2-Ethylidene-4-benzylglutarate 6i. Yield 6.53 g, 95%; IR (neat film) 1735, 1720, 1648, 1604 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.35−7.10 (5H, m), 6.95 and 6.07 (1H, q, $J = 7.0$ Hz, *E* and *Z* isomers), 3.73 and 3.72 (3H, s, *Z* and *E* isomers), 3.54 and 3.53 (3H, s, *Z* and *E* isomers), 3.00−2.45 (5H, m), 1.98 and 1.76 (3H, d, $J = 7.0$ Hz, *Z* and *E* isomers); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.3, 175.2, 167.5, 140.2, 139.8, 139.0, 130.7, 129.5, 128.8, 128.3, 126.3, 51.6, 51.33, 51.25, 51.2, 47.1, 46.8, 38.4, 38.2, 37.3, 29.2, 15.9, 14.3.

General Procedure for the Synthesis of Dimethyl 4-Alkyl-2-oxoglutarates 7a−i. To a solution of **6a−i** (20 mmol) in a mixture of CCl_4 (45 mL) and CH_3CN (45 mL) were added a solution of $NaIO_4$ (17.1 g, 80 mmol) in water (60 mL) and RuO_2 (270 mg, 2 mmol). The mixture was stirred at room temperature for 24 h and then filtered on Celite. The organic layer was isolated, and the aqueous phase was extracted with CH_2Cl_2 (2 × 25 mL). The combined organic layers were washed with brine (50 mL), dried over $MgSO_4$, and concentrated under reduced pressure. Flash chromatography (eluent, cyclohexane−AcOEt, 8:2, v/v) afforded **7a−i** isolated as colorless liquids.

Methyl, Ethyl 4-Methyl-2-oxoglutarate 7a. Yield 3.24 g, 80%; IR (neat film) 1730 cm^{-1} (broad); ^1H NMR (400 MHz, $CDCl_3$) δ 4.12 (2H, q, $J = 7.0$ Hz), 3.86 (3H, s), 3.31 (1H, dd, $J = 8.0$ and 18.0 Hz), 2.99 (1H, m), 2.86 (1H, dd, $J = 5.0$ and 18.0 Hz), 1.23 (3H, d, $J = 8.0$ Hz), 1.23 (3H, t, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.1, 174.8, 161.0, 60.8, 52.9, 42.3, 34.8, 16.9, 14.0.

Dimethyl 4-Ethyl-2-oxoglutarate 7b. Yield 2.90 g, 71%; IR (neat film) 1732 cm^{-1} (broad); ^1H NMR (400 MHz, $CDCl_3$) δ 3.83 (3H, s), 3.64 (3H, s), 3.26 (1H, dd, $J = 10.0$ and 19.5 Hz), 2.86 (1H, dd, $J = 4.5$ and 19.5 Hz), 2.85 (1H, m), 1.70−1.50 (2H, m), 0.89 (3H, t, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.3, 174.9, 161.0, 53.0, 51.8, 41.2, 40.2, 24.8, 11.2.

Dimethyl 4-Propyl-2-oxoglutarate 7c. Yield 2.90 g, 70%; IR (neat film) 1733 cm^{-1} (broad); ^1H NMR (400 MHz, $CDCl_3$) δ 3.85 (3H, s), 3.66 (3H, s), 3.23 (1H, dd, $J = 8.0$ and 17.5 Hz), 2.93 (1H, m), 2.88 (1H, dd, $J = 5.0$ and 17.5 Hz), 1.65 (1H, m), 1.50 (1H, m), 1.34 (2H, hex., $J = 7.0$ Hz), 0.91 (3H, t, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.2, 175.0, 160.9, 52.9, 51.8, 40.6, 39.5, 33.8, 20.0, 13.7.

Dimethyl 4-Butyl-2-oxoglutarate 7d. Yield 3.65 g, 79%; IR (neat film) 1732 cm^{-1} (broad); ^1H NMR (400 MHz, $CDCl_3$) δ 3.87 (3H, s), 3.67 (3H, s), 3.30 (1H, dd, $J = 10.5$ and 19.5 Hz), 2.92 (1H, m), 2.90 (1H, dd, $J = 4.5$ and 19.5 Hz), 1.67 (1H, m), 1.55 (1H, m), 1.30 (4H, m), 0.89 (3H, t, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.3, 175.1, 161.0, 53.1, 52.0, 40.7, 39.8, 31.4, 29.1, 22.5, 13.9.

Dimethyl 4-Pentyl-2-oxoglutarate 7e. Yield 3.02 g, 62%; IR (neat film) 1731 cm^{-1} (broad); ^1H NMR (400 MHz, $CDCl_3$) δ 3.86 (3H, s), 3.68 (3H, s), 3.30 (1H, dd, $J = 10.5$ and 20.0 Hz), 2.93 (1H, m), 2.90 (1H, dd, $J = 4.5$ and 20.0 Hz), 1.65 (1H, m), 1.55 (1H, m), 1.28 (6H, m), 0.87 (3H, t, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.4, 175.2, 161.1, 53.1, 52.0, 40.8, 39.9, 31.7, 31.6, 26.6, 22.5, 14.0.

Dimethyl 4-Isopropyl-2-oxoglutarate 7f. Yield 3.55 g, 82%; IR (neat film) 1732 cm^{-1} (broad); ^1H NMR (400 MHz, $CDCl_3$) δ 3.88 (3H, s), 3.68 (3H, s), 3.34 (1H, dd, J = 11.5 and 19.5 Hz), 2.85 (1H, dd, J = 3.5 and 19.5 Hz), 2.84 (1H, m), 2.07 (1H, m), 0.95 (3H, d, J = 7.0 Hz), 0.92 (3H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.7, 174.4, 161.0, 53.0, 51.8, 46.0, 37.6, 29.8, 20.1, 19.3.

Dimethyl 4-Isobutyl-2-oxoglutarate 7g. Yield 3.36 g, 73%; IR (neat film) 1732 cm^{-1} (broad); ^1H NMR (400 MHz, $CDCl_3$) δ 3.88 (3H, s), 3.68 (3H, s), 3.26 (1H, dd, J = 9.0 and 18.0 Hz), 2.99 (1H, m), 2.92 (1H, dd, J = 4.5 and 18.0 Hz), 1.60 (2H, m), 1.32 (1H, m), 0.94 (3H, d, J = 6.5 Hz), 0.89 (3H, d, J = 6.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.3, 175.5, 161.0, 53.1, 52.0, 41.2, 41.0, 38.1, 25.8, 22.4, 22.3.

Dimethyl 4-Isopentyl-2-oxoglutarate 7h. Yield 3.32 g, 68%; IR (neat film) 1732 cm^{-1} (broad); ^1H NMR (400 MHz, $CDCl_3$) δ 3.87 (3H, s), 3.68 (3H, s), 3.30 (1H, dd, J = 10.5 and 19.5 Hz), 2.90 (2H, m), 1.75−1.50 (3H, m), 1.20 (2H, m), 0.87 (3H, d, J = 6.5 Hz), 0.86 (3H, d, J = 6.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.4, 175.1, 161.0, 53.0, 51.9, 40.7, 40.1, 35.9, 29.6, 27.9, 22.44, 22.40.

Dimethyl 4-Benzyl-2-oxoglutarate 7i. Yield 3.17 g, 60%; IR (neat film) 1732 cm^{-1} (broad); ^1H NMR (400 MHz, $CDCl_3$) δ 7.35−7.10 (5H, m), 3.84 (3H, s), 3.67 (3H, s), 3.35−3.25 (2H, m), 3.10 (1H, dd, J = 6.0 and 13.5 Hz), 2.90−275 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.2, 174.3, 160.9, 137.9, 129.0, 128.7, 126.9, 53.1, 52.1, 42.0, 39.8, 37.5.

General Procedure for the Synthesis of Dilithium 4-Alkyl-2-oxoglutarate 2a−i. To a solution of **7a−i** (10 mmol) in THF or MeOH (50 mL) was added dropwise a 0.4 M solution of LiOH (52.5 mL, 21 mmol). The mixture was stirred at room temperature for 3−4 h until completion. After evaporation of the organic solvent, the pH of the aqueous solution was adjusted to 7.6 by addition of Dowex 50X8 resin (H^+ form). The resin was removed by filtration before evaporation of the water. **2a−i** were isolated in quantitative yields as white solids.

Dilithium 4-Methyl-2-oxoglutarate 2a. ^1H NMR (400 MHz, D_2O) δ 2.99 (1H, dd, J = 8.0 and 17.5 Hz), 2.70 (1H, dd, J = 6.0 and 17.5 Hz), 2.62 (1H, m), 1.05 (3H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 205.1, 184.8, 170.2, 43.8, 37.5, 17.4.

Dilithium 4-Ethyl-2-oxoglutarate 2b. ^1H NMR (400 MHz, D_2O) δ 3.07 (1H, dd, J = 8.5 and 18.0 Hz), 2.88 (1H, dd, J = 5.5 and 18.5 Hz), 2.63 (1H, m), 1.56 (2H, quint, J = 7.0 Hz), 0.93 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 205.3, 183.8, 170.2, 44.6, 42.0, 25.3, 11.1.

Dilithium 4-Propyl-2-oxoglutarate 2c. ^1H NMR (400 MHz, D_2O) δ 3.06 (1H, dd, J = 8.5 and 18.5 Hz), 2.90 (1H, dd, J = 5.5 and 18.0 Hz), 2.70 (1H, m), 1.50 (2H, m), 1.35 (2H, m), 0.94 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 208.2, 186.8, 172.9, 45.8, 45.1, 37.3, 22.8, 16.1.

Dilithium 4-Butyl-2-oxoglutarate 2d. ^1H NMR (400 MHz, D_2O) δ 3.05 (1H, dd, J = 8.5 and 18.5 Hz), 2.89 (1H, dd, J = 5.5 and 18.5 Hz), 2.67 (1H, m), 1.52 (2H, m), 1.32 (4H, m), 0.90 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 207.9, 186.8, 172.8, 45.9, 45.0, 34.5, 31.7, 24.7, 15.9.

Dilithium 4-Pentyl-2-oxoglutarate 2e. ^1H NMR (400 MHz, D_2O) δ 3.09 (1H, dd, J = 8.5 and 18.0 Hz), 2.93 (1H, dd, J = 6.0 and 18.0 Hz), 2.72 (1H, m), 1.56 (2H, m), 1.36 (6H, m), 0.94 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 208.1, 186.8, 173.0, 46.0, 45.1, 34.9, 33.8, 29.0, 24.5, 16.1.

Dilithium 4-Isopropyl-2-oxoglutarate 2f. ^1H NMR (400 MHz, D_2O) δ 3.07 (1H, dd, J = 9.5 and 18.5 Hz), 2.97 (1H, dd, J = 4.5 and 18.5 Hz), 2.52 (1H, m), 1.91 (1H, oct, J = 7.0 Hz), 0.99 (3H, d, J = 7.0 Hz), 0.95 (3H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 208.3, 186.1, 172.8, 52.5, 42.2, 32.8, 22.9, 21.8.

Dilithium 4-Isobutyl-2-oxoglutarate 2g. ^1H NMR (400 MHz, D_2O) δ 3.04 (1H, dd, J = 8.5 and 18.0 Hz), 2.90 (1H, dd, J = 5.5 and 18.0 Hz), 2.79 (1H, m), 1.65−1.45 (2H, m), 1.31 (1H, m), 0.96 (3H, d, J = 7.0 Hz), 0.92 (3H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 207.8, 187.1, 172.8, 45.4, 44.4, 44.3, 28.3, 24.8, 24.4.

Dilithium 4-Isopentyl-2-oxoglutarate 2h. ^1H NMR (400 MHz, D_2O) δ 3.09 (1H, dd, J = 8.5 and 18.0 Hz), 2.93 (1H, dd, J = 6.0 and 18.0 Hz), 2.69 (1H, m), 1.57 (3H, m), 1.25 (2H, m), 0.93 (6H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 208.1, 186.8, 172.9, 46.1, 45.1, 38.6, 32.8, 30.0, 24.7, 24.5.

Dilithium 4-Benzyl-2-oxoglutarate 2i. ^1H NMR (400 MHz, D_2O) δ 7.50−7.30 (5H, m), 3.15−2.75 (5H, m); ^{13}C NMR (100 MHz, D_2O) δ 207.5, 185.5, 172.5, 142.6, 131.9, 131.3, 129.2, 48.0, 44.4, 40.9.

General Procedure for the Synthesis of (2*S*,4*R*)-4-Alkylglutamic Acids 1a−i. To a solution of racemic **2a−i** (2.5 mmol) in water (125 mL) were added cysteine sulfinic acid (2.5 mmol) and acetaldehyde (0.3 mL, 5 mmol). The pH of the solution was adjusted to 7.6 with 1 M NaOH before the addition of pig heart or *E. coli* AAT (20−100 units). The mixture was stirred slowly at room temperature and monitored by titration of pyruvate in the following way: 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 rabbit muscle lactate dehydrogenase (1 unit). Pyruvate concentration was calculated from the ΔOD measured at 340 nm using ϵ_{NADH} = 6220 M^{-1} cm^{-1}. When a conversion rate of 40% was reached, the reaction mixture was rapidly passed through a column of Dowex 50X8 resin (H^+ form, 25 mL). The column was then washed with water (100 mL) until complete elution of CSA and then eluted with 1 M NH_4OH. The ninhydrin positive fractions were combined and concentrated to dryness under reduced pressure. The residue was diluted in water (5 mL), and if necessary, the pH was adjusted to 7.0 with 1 M NaOH before adsorption of the product on a column of Dowex 2X8 resin (200−400 mesh, AcO^- form, 1.5 cm × 12 cm). The column was washed with water (50 mL) and then eluted with an AcOH gradient (0.1−0.5 M). The ninhydrin positive fractions were combined and dried under reduced pressure to afford **1a−i** as white solids.

(2*S*,4*R*)-4-Methylglutamic Acid 1a. Yield 165 mg, 41%; mp 178 °C (lit.[49] mp 177 °C); $[\alpha]^{25}_D$ +24.0° (*c* 1.3, 6 N HCl) (lit.[50] $[\alpha]^{25}_D$ +23.2° (*c* 0.7, 6 N HCl)); ^1H NMR (400 MHz, D_2O) δ 3.83 (1H, dd, J = 4.5 and 8.5 Hz), 2.58 (1H, m, J = 5.0, 7.0, 8.5), 2.23 (1H, ddd, J = 5.0, 8.5, and 14.0 Hz), 1.96 (1H, ddd, J = 5.0, 8.5, and 13.5 Hz), 1.25 (3H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 185.3, 178.5, 53.9, 39.5, 36.9, 17.8. Anal. ($C_6H_{11}NO_4$) C, H, N.

(2*S*,4*R*)-4-Ethylglutamic Acid 1b. Yield 174 mg, 40%; mp 175 °C (lit.[20] mp 179 °C); $[\alpha]^{25}_D$ +31.0° (*c* 1.3, 6 N HCl); ^1H NMR (400 MHz, D_2O) δ 3.80 (1H, dd, J = 4.0 and 9.0 Hz), 2.55 (1H, m, J = 4.0, 7.0, and 10.0 Hz), 2.25 (1H, ddd, J = 4.0, 10.0, and 14.0 Hz), 1.95 (1H, ddd, J = 4.0, 9.0, and 14.0 Hz), 1.65 (2H, quint, J = 7.0 Hz), 0.90 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 186.7, 177.8, 56.3, 46.5, 35.0, 28.4, 13.6. Anal. ($C_7H_{13}NO_4$) C, H, N.

(2*S*,4*R*)-4-Propylglutamic Acid 1c. Yield 198 mg, 42%; mp 164 °C; $[\alpha]^{25}_D$ +34.5° (*c* 1.0, 6 N HCl); ^1H NMR (400 MHz, D_2O) δ 3.79 (1H, dd, J = 4.0 and 9.0 Hz), 2.52 (1H, m), 2.24 (1H, ddd, J = 4.0, 10.0, and 14.5 Hz), 1.98 (1H, ddd, J = 4.5, 9.0, and 14.0 Hz), 1.70−1.50 (2H, m), 1.39 (2H, hex., J = 7.0 Hz), 0.98 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 186.3, 177.1, 55.8, 47.3, 36.9, 35.6, 22.5, 15.7. Anal. ($C_8H_{15}NO_4$) C, H, N.

(2*S*,4*R*)-4-Butylglutamic Acid 1d. Yield 163 mg, 32%; mp 162 °C; $[\alpha]^{25}_D$ +29.7° (*c* 1.2, 6 N HCl); ^1H NMR (400 MHz, D_2O) δ 3.79 (1H, dd, J = 4.5 and 9.0 Hz), 2.55 (1H, m), 2.26 (1H, ddd, J = 4.5, 9.5, and 14.5 Hz), 1.99 (1H, ddd, J = 4.5, 9.0, and 14.0 Hz), 1.75−1.55 (2H, m), 1.45−1.30 (4H, m), 0.96 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 185.0, 177.2, 56.1, 46.6, 35.6, 34.7, 31.5, 24.7, 15.9; HRMS (EI, positive ion) *m/z* 204.1235 ($[MH]^+$, $C_9H_{18}NO_4$ requires 204.1236). Anal. ($C_9H_{17}NO_4 \cdot 0.33H_2O$) C, H, N.

(2*S*,4*R*)-4-Pentylglutamic Acid 1e. Yield 195 mg, 36%; mp 157 °C (lit.[51] mp 160−1 °C); $[\alpha]^{25}_D$ +18.6° (*c* 1.1, 6 N HCl); ^1H NMR (400 MHz, D_2O) δ 3.78 (1H, dd, J = 4.0 and 9.0 Hz), 2.51 (1H, m), 2.25 (1H, ddd, J = 4.5, 9.5, and 14.5 Hz), 1.98 (1H, ddd, J = 4.5, 9.0, and 14.0 Hz), 1.70−1.50 (2H, m), 1.45−1.30 (6H, m), 0.94 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz,

D_2O) δ 183.3, 176.8, 56.0, 45.3, 35.3, 34.7, 33.6, 28.6, 24.5, 16.0. HRMS (FAB, positive ion) *m/z* 262.1034 ($[M - H + 2Na]^+$, $C_{10}H_{18}NO_4Na_2$ requires 262.1031). Anal. ($C_{10}H_{19}NO_4 \cdot 0.33H_2O$) C, H, N.

(2*S*,4*S*)-4-Isopropylglutamic Acid 1f. Yield 195 mg, 41%; mp 178 °C (lit.[52] mp 175−177 °C); $[\alpha]^{25}_D$ +31.0° (*c* 1.0, 6 N HCl); ^{1}H NMR (400 MHz, D_2O) δ 3.81 (1H, dd, J = 4.5 and 9.0 Hz), 2.48 (1H, ddd, J = 3.0, 6.0, and 9.0 Hz), 2.35 (1H, ddd, J = 5.0, 11.0, and 15.0 Hz), 2.14−2.02 (2H, m), 1.06 (3H, d, J = 7.0 Hz), 1.04 (3H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 185.5, 177.4, 56.4, 54.6, 32.9, 32.8, 23.1, 21.7. HRMS (FAB, positive ion) *m/z* 190.1080 ($[M + H]^+$, $C_8H_{16}NO_4$ requires 190.1079). Anal. ($C_8H_{15}NO_4$) C, H, N.

(2*S*,4*R*)-4-Isobutylglutamic Acid 1g. Yield 213 mg, 42%; mp 165 °C; $[\alpha]^{25}_D$ +26.0° (*c* 1.1, 6 N HCl); ^{1}H NMR (400 MHz, D_2O) δ 3.75 (1H, dd, J = 5.5 and 8.5 Hz), 2.66 (1H, m), 2.22 (1H, ddd, J = 5.0, 9.5, and 15.0 Hz), 1.92 (1H, ddd, J = 4.5, 8.5, and 15.0 Hz), 1.58 (2H, m), 1.38 (1H, m), 0.90 (3H, d, J = 7.0 Hz), 0.88 (3H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 186.4, 177.3, 56.2, 45.8, 44.4, 36.3, 29.4, 25.0, 24.3. HRMS (FAB, positive ion) *m/z* 204.1233 ($[M + H]^+$, $C_9H_{18}NO_4$ requires 204.1236). Anal. ($C_9H_{17}NO_4 \cdot 0.5H_2O$) C, H, N.

(2*S*,4*R*)-4-Isopentylglutamic Acid 1h. Yield 185 mg, 34%; mp 150 °C; $[\alpha]^{25}_D$ +26.4° (*c* 1.1, 6 N HCl); ^{1}H NMR (400 MHz, D_2O) δ 3.80 (1H, dd, J = 4.0 and 9.0 Hz), 2.58 (1H, m), 2.30 (1H, ddd, J = 5.5, 9.0, and 15.0 Hz), 1.92 (1H, ddd, J = 4.0, 8.0, and 15.0 Hz), 1.66 (3H, m), 1.29 (2H, m), 0.98 (6H, d, J = 7.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 184.1, 177.0, 56.1, 46.2, 38.3, 35.5, 32.8, 30.0, 24.6, 24.4. HRMS (FAB, positive ion) *m/z* 218.1399 ($[M + H]^+$, $C_{10}H_{20}NO_4$ requires 218.1392). Anal. ($C_{10}H_{19}NO_4 \cdot 0.5H_2O$) C, H, N.

(2*S*,4*R*)-4-Benzylglutamic Acid 1i. Yield 250 mg, 42%; mp 161 °C; $[\alpha]^{25}_D$ +36.7° (*c* 1.0, 6 N HCl); ^{1}H NMR (400 MHz, D_2O) δ 7.45 (5H, m), 3.82 (1H, dd, J = 4.5 and 9.0 Hz), 3.03 (1H, dd, J = 8.5 and 13.5 Hz), 2.94 (1H, dd, J = 6.5 and 13.5 Hz), 2.82 (1H, m), 2.29 (1H, ddd, J = 4.5, 10.0, and 14.5 Hz), 2.01 (1H, ddd, J = 4.5, 9.0, and 15.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 184.3, 177.1, 142.2, 131.8, 131.3, 129.3, 56.2, 49.2, 41.2, 35.4; HRMS (EI, positive ion) *m/z* 238.1083 ($[M + H]^+$, $C_{12}H_{16}NO_4$ requires 238.1079). Anal. ($C_{12}H_{15}NO_4 \cdot 0.33H_2O$) C, H, N.

Ethyl (2*S*,4*R*)-4-Pentylpyroglutamate 8. A solution of **1e** (45 mg, 0.2 mmol) in water (15 mL) was heated under reflux for 48 h until complete disappearance of **1e**. After evaporation of water under reduced pressure, the solid residue was diluted with absolute EtOH (10 mL). $SOCl_2$ (100 μL, 1.4 mmol) was added. The mixture was stirred at room temperature for 20 h before concentration under reduced pressure. The residue was chromatographed on silica gel to give **8** (29 mg, 64%) isolated as a colorless oil. $[\alpha]^{25}_D$ + 9.3° (*c* 1.5, MeOH); IR (neat film) 3235, 1742, 1708 cm^{-1}; ^{1}H NMR (400 MHz, C_6D_6) δ 7.85 (1H, s), 3.88 (2H, q, J = 7.0 Hz), 3.70 (1H, dd, J = 2.5 and 9.0 Hz), 2.30 (1H, m), 2.05 (1H, ddd, J = 2.5, 9.0, and 13.0 Hz), 1.82 (1H, m), 1.55 (1H, td, J = 9.0 and 13.0 Hz), 1.12 (7H, m), 0.92 (3H, t, J = 7.0 Hz), 0.81 (3H, t, J = 7.0 Hz); ^{13}C NMR (100 MHz, C_6D_6) δ 180.2, 172.5, 61.1, 54.1, 39.9, 32.0, 31.6, 31.2, 27.1, 22.8, 14.2, 14.0; HRMS (EI, positive ion) *m/z* 228.1611 ($[M + H]^+$, $C_{12}H_{22}NO_3$ requires 228.1600).

Pharmacological Characterization of 4-Substituted Glu Analogues 1a−k at Human EAATs. The pharmacological properties of 4-substituted Glu analogues at human EAAT1, EAAT2, and EAAT3 were determined in the FLIPR membrane potential (FMP) assay. The construction of human embryonic kidney 293 (HEK293) cell lines stably expressing human EAAT1, EAAT2, and EAAT3 has been reported previously, and the pharmacological characterization of the 4-substituted Glu analogues was performed essentially as described here.[36] Briefly, cells were split into poly-D-lysine-coated black walled clear-bottom 96-well plates in Dulbecco's modified Eagle medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), 10% dialyzed fetal bovine serum, and 1 mg/mL G-418. After 16−24 h, the medium was aspirated, and the cells were washed with 100 μL of Krebs buffer [140 mM NaCl/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/11 mM HEPES/10 mM D-glucose, pH 7.4]. Then 50 μL of Krebs buffer was added to each well (in the characterization of nonsubstrate inhibitors, the inhibitors were added to this buffer). An amount of 50 μL of Krebs buffer supplemented with FMP assay dye was then added to each well, and the plate was incubated at 37 °C for 30 min. The plate was assayed at room temperature in a NOVOstar plate reader measuring emission at 560 nm caused by excitation at 530 nm before and up to 1 min after addition of 25 μL of substrate solution. The experiments were performed in duplicate at least three times for each compound. For the characterization of nonsubstrate inhibitors 30 μM Glu was used as substrate concentration. Concentration−response curves were constructed on the basis of the maximal responses obtained for seven different concentrations of each compound. IC_{50} values were converted to K_i values by the use of the Cheng−Prusoff equation.[53]

Molecular Modeling Study of 4-Substituted Glu Analogues 1a−1l and Other Selected Ligands. The modeling study was performed using the software package MOE (Molecular Operating Environment, version 2004.03, Chemical Computing Group, 2004) using the built-in mmff94x force field and the GB/SA continuum solvent model. Each compound was submitted to a stochastic conformational search and with respect to its global minimum returned (ΔG in kcal/mol); conformations above +7 kcal/mol were discarded. For Glu analogues **1a−i,l**, the γ-carboxylate group was protonated prior to execution of the conformational search because this gave a larger and thus more reliable number of output conformations. Superimpositions of ligands were carried out using the built-in function in MOE, by fitting the ammonium group and the two carboxylate groups.

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

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