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

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The four stereoisomers of L-2-(2-carboxycyclobutyl)glycine, L-CBG-I, L-CBG-II, L-CBG-III, and L-CBG-IV, were synthesized in good yield and high enantiomeric excess, from the corresponding *cis* and *trans*-2-oxalylcyclobutanecarboxylic acids **5** and **6** using the enzymes aspartate aminotransferase (AAT) and branched chain aminotransferase (BCAT) from *Escherichia coli*. The four stereoisomeric compounds were evaluated as potential ligands for the human excitatory amino acid transporters, subtypes 1, 2, and 3 (EAAT1, EAAT2, and EAAT3) in the FLIPR membrane potential assay. While the one trans-stereoisomer, L-CBG-I, displayed weak substrate activity at all three transporters, EAAT1−3, we found a particular pharmacological profile for the other trans-stereoisomer, L-CBG-II, which displayed EAAT1 substrate activity and inhibitory activity at EAAT2 and EAAT3. Whereas L-CBG-III was found to be a weak inhibitor at all three EAAT subtypes, the other cis-stereoisomer L-CBG-IV was a moderately potent inhibitor with 20−30-fold preference for EAAT2/3 over EAAT1.

Introduction

In the mammalian central nervous system (CNS) glutamatergic neurotransmission is terminated by reuptake of L-glutamate (Glu) from the synaptic cleft, by the action of sodium dependent excitatory amino acid transporters (EAATs).[1,2] To date, five transporter subtypes have been identified of which four, EAAT1−4, are present in the mammalian CNS, while EAAT5 is localized exclusively in the retina. EAAT1−3 are localized on neurons and/or astroglial cells and exhibit high capacity for transporting Glu across the membrane, whereas EAAT4 and EAAT5 predominately function as chloride channels.[1] 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.[3] However, under conditions of metabolic stress and oxygen depravation, 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).[3,4] We have recently reported a numbers of studies in which we have explored the structure−activity relationship (SAR) of ligands at the human EAAT1−3, with the ultimate goal of identifying new EAAT subtype selective inhibitors and substrates.[5−7] For this purpose, constrained cyclic Glu analogues are of particular interest and many different compounds have been prepared and evaluated for their activity at the glutamatergic neurotransmitter system. Although L-2-(2-carboxycyclopropyl)glycine (L-CCG) isomers have been the subject of several studies,[8−10] very limited work has been done with the cyclobutane analogues L-CBG. Only three isomers have been prepared to date: L-CBG-I, L-CBG-III,[11] and, recently, L-CBG-II.[12] We present here the stereoselective synthesis of all four stereoisomers of L-2-(2-carboxycyclobutyl)-glycine (L-CBG-I−IV) (Figure 1) and the pharmacological characterization of these four compounds at human EAAT1−3.

Chemistry

Enzymatic transamination is a valuable approach for the stereoselective preparation of amino acids. Aminotransferases are very common enzymes with broad substrate spectra, and a variety of biologically active compounds have been prepared, including unnatural L- and D-α-amino acids[13−20] as well as β-amino acids or simple amines.[21−24] Most naturally occurring L-α-aminotransferases accept L-Glu as a preferred substrate. Among them, aspartate aminotransferase (AAT) has proven useful for the stereoselective preparation of Glu analogues from the corresponding 2-oxoglutaric acid (KG) derivatives.[5,25−28] AAT offers the opportunity to shift the transamination equilibrium through the use of aspartic acid or cysteine sulfinic acid[29] (CSA) as the amino donor substrate: an unstable keto acid is produced in both cases, which is decomposed into pyruvate, which is not a substrate for the enzyme. AAT is active toward many 4-substituted KG analogues, and its enantiopreference allows the stereoselective syntheses of a variety of Glu analogues and especially L-2,4-*syn*-4-alkylglutamic acids (Scheme 1).[5]

Recently, we have shown in a preliminary communication that the scope of AAT transamination can be extended to constrained Glu analogues with the synthesis of L-CBG-II from (±)-*trans*-2-oxalylcyclobutanecarboxylic acid **5**. Branched chain aminotransferase (BCAT) from *Escherichia coli* allowed the preparation of the L-CBG-I stereomer from the same keto acid.[12] In the present study, we present the synthesis of the *cis*- and *trans*-cyclobutane keto acids **5** and **6** and their stereoselective conversion into the four stereomers L-CBG-I to IV using *E. coli* AAT and BCAT (Scheme 2).

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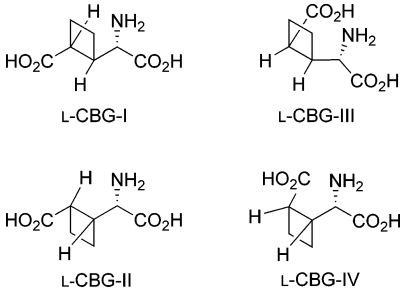
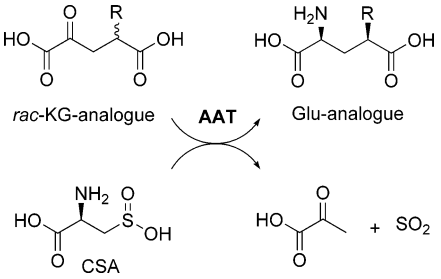
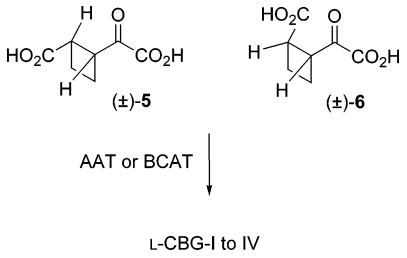


Figure 1. L-CBG isomers.

Scheme 1. AAT-Catalyzed Synthesis of L-2,4-*syn*-4-Alkyl-Glu from the Corresponding KG Analogues



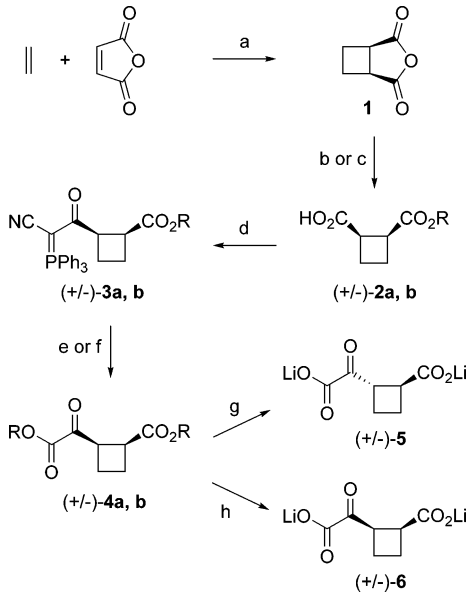
Scheme 2. Synthesis of L-CBG Isomers by Enzymatic Transamination



Results and Discussion

Synthesis of 2-Oxalylcyclobutanecarboxylic Acids 5 and 6. Both cis- and trans-isomers of cyclobutane α-ketoglutaric acids **5** and **6** were synthesized from *cis*-1,2-cyclobutane dicarboxylic anhydride **1** as described in Scheme 3. A modification of the [2 + 2] photocycloaddition reaction described by Bloomfield and Owsley[30] afforded *cis*-1,2-cyclobutanedicarboxylic anhydride **1** in 75% yield. Methanolysis of the anhydride moiety gave (±)-*cis*-2-methoxycarbonylcyclobutane carboxylic acid **2a** in good yield. Homologation of acid **2a** into α-keto ester was performed using cyanoketophosphorane methodology developed by Wasserman's group:[31] First, the carboxylic acid **2a** was coupled with (cyanomethylene)triphenylphosphorane to give the α-keto cyanophosphorane **3a** in 91% yield. Ozonolysis of compound **3a** then generated a diketo nitrile, which was trapped in situ by methanol to provide (±)-*cis*-2-oxalylcyclobutane carboxylic dimethyl ester **4a** in 81% yield. Final ester hydrolysis was carried out in the presence of a stoichiometric amount of lithium hydroxide in MeOH. Under these reaction conditions, regiospecific epimerization α to the ketone functionality was observed to furnish thermodynamically more stable *trans*-cyclobutane. The dilithium salt of **5** was isolated as a 90:10 trans/cis mixture determined by ^{1}H NMR. To obtain the cis isomer of cyclobutane α-ketoglutaric acid (±)-**6**, we decided to modify the above sequence to accommodate a benzyl ester moiety that should be cleaved under neutral conditions, thus avoiding epimerization. Opening of the anhydride moiety of **1** with benzyl alcohol catalyzed by DMAP gave (±)-*cis*-2-benzyloxycarbonylcyclobutane carboxylic acid **2b** in 63% yield.[32] The α-keto cyanophosphorane **3b**, obtained by coupling, was submitted to ozonolysis followed by addition of benzyl alcohol to provide (±)-*cis*-2-oxalylcyclobutanecarboxylic dibenzyl ester **4b** in 62% yield. Quantitative hydrogenolysis of the benzyl group in the presence of Pd/C followed by generation of the dilithium salt by careful addition of lithium hydroxide gave an aqueous solution of the cis isomer of cyclobutane α-ketoglutaric acid **6**. To minimize the isomerization process this solution was used directly in the transamination reaction. ^{1}H NMR analysis after concentration first revealed a 80:20 cis/trans mixture rapidly evolving in a few hours to the major trans isomer. The transamination substrates **5** and **6** were thus obtained in five steps with respective overall yields of 42% and 27% from maleic anhydride.

Scheme 3. Synthesis of *cis*- and *trans*-Cyclobutane α-Ketoglutaric Acids **5** and **6**[a]

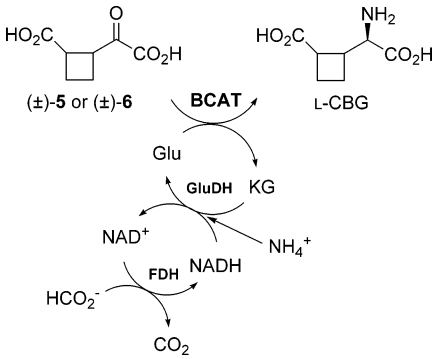


[a] Reagents and conditions: (a) acetophenone, CH_3CN, rt, hν, Pyrex, 5h, 75%; (b) MeOH, reflux, 16 h, 77%; (c) BnOH, cat. DMAP, rt, 20 h, 63%; (d) (triphenylphosphoranylidene)acetonitrile, EDCI, DMAP, CH_2Cl_2, rt, 4 h, 91%; (e) O_3, CH_2Cl_2/MeOH 3:1, −78 °C, 1 h, 81%; (f) O_3, CH_2Cl_2, −78 °C, 1 h then BnOH, 2 h, 62%; (g) LiOH, MeOH, rt, 2 h then Dowex resin H^+, 100%; (h) H_2, Pd/C, AcOEt, rt, 3 h then LiOH, H_2O, 100%.

Synthesis of Glutamic Acid Analogues. Since the cyclobutane KG analogues **5** and **6** were obtained as an enriched mixture and are prone to isomerization under neutral conditions, we could not determine reliable kinetic parameters for the aminotransferase-catalyzed reactions. However, analytical measurements indicated that both racemates are substrates for *E. coli* AAT and BCAT with k_{cat} of around 1% of that of the natural substrate KG. Because of the high specific activity of these aminotransferases, this modest activity proved to be sufficient for synthetic purposes.

AAT-catalyzed reactions were performed according to the method depicted in Scheme 1 and previously described:[5,12] an aqueous solution of (±)-**5** or (±)-**6** (approximately 2 mmol) was reacted with a stoechiometric amount of CSA in the presence of AAT at pH 7.6. The reaction was monitored by enzymatic titration of pyruvic acid formed from CSA and was stopped when a conversion rate of approximately 40% was reached, to observe a possible kinetic resolution. Glu analogues were selectively adsorbed on a short column of sulfonic resin (H^+ form) and recovered by elution with 1 M aqueous ammonium hydroxide. They were finally purified by adsorption on a strongly basic Dowex 2 resin (AcO^- form) followed by

Scheme 4. BCAT-Catalyzed Synthesis of L-CBG



elution with an AcOH gradient. Three L-CBG isomers were thus isolated with a very good purity. L-CBG-II was isolated in 32% yield from (±)-**5**. This result indicates that AAT is enantioselective toward **5**, allowing the kinetic resolution of this racemic substrate. Reaction of (±)-**6** produced L-CBG-III and -IV in 19% and 17% respective yields. The two cis isomers were consecutively eluted from the basic resin and were thus easily separated. A small amount of L-CBG-II (10% yield) was also isolated in that reaction and easily separated from the cis isomers. L-CBG-II was likely formed from the isomerized *trans*-keto acid present in the reaction mixture. In the case of the cis derivative **6**, the enzyme enantioselectivity is very low, affording access to both stereomers L-CBG-III and IV. These products should be isolated in better yields after a complete conversion of (±)-**6** substrate.

The transaminations with BCAT were performed on a smaller scale (0.3 mmol) according to Scheme 4 as previously described.[12]

As CSA is not a substrate for BCAT, Glu was used as catalytic amino donor and was regenerated by reductive amination of KG using glutamic dehydrogenase (GluDH), NADH, and NH_4^+ ions. NADH itself was used catalytically and regenerated using the equilibrium-shifted oxidation of formic acid catalyzed by formate dehydrogenase (FDH). The reaction was followed by TLC and stopped after 2−5 days. Glu analogues were isolated as previously described for AAT-catalyzed reactions. Starting from **5** or **6**, the same L-CBG-I isomer was isolated in 26% and 16% respective yields. This result indicates that BCAT exhibits an enantioselectivity toward **5** that is complementary to that of AAT. Unfortunately, in the conditions used, isomerization of **6** into **5** was faster than its transamination and did not allow an access to *cis*-L-CBG derivatives.

Configurations were attributed by comparison to the physicochemical properties and spectroscopic data of the already described L-CBG-I and L-CBG-III isomers.[11] As these known derivatives are respectively a trans and a cis isomer, it was easy to attribute configurations to our two new analogues, corresponding to *trans*-L-CBG-II and *cis*-L-CBG-IV. The L-CBG-II stereochemistry was further confirmed, as previously described,[12] by the synthesis of (1*R*,2*R*)-**5** and confirmation of the AAT enantiopreference for this enantiomer. Because *E. coli* aminotransferases are highly specific for L-amino acids, all CBG analogues prepared in the present study were isolated with very high enantiopurity. Table 1 summarizes the results obtained for AAT- and BCAT-catalyzed transamination of **5** and **6**. Our chemoenzymatic approach appeared shorter than the previous described syntheses[11] of L-CBG-I and -III isomers. Moreover, it allowed the preparation of the two new stereomers L-CBG-II and -IV.

Table 1. L-CBG Isomers Prepared by Transamination

substrate	product with AAT	product with BCAT
(±)-**5**	L-CBG-II	L-CBG-I
(±)-**6**	L-CBG-III	−
	L-CBG-IV	

Table 2. Pharmacological Characterization of L-CBG-I, L-CBG-II, L-CBG-III, and L-CBG-IV at Human Excitatory Amino Acid Transporters EAAT1, EAAT2, and EAAT3 in the FMP Assay[a]

	EAAT1	EAAT2	EAAT3
Glu	**7.9 (5.1 ± 0.03)**	**21 (4.7 ± 0.02)**	**9.9 (5.0 ± 0.02)**
L-CBG-I	**300−1000**[b]	**300−1000**[b]	**300−1000**[b]
L-CBG-II	**96 (4.0 ± 0.03)**[c]	22 (4.7 ± 0.01)	49 (4.3 ± 0.03)
L-CBG-III	∼200	110 (3.9 ± 0.02)	52 (4.3 ± 0.02)
L-CBG-IV	∼200	6.6 (5.2 ± 0.04)	10 (5.0 ± 0.03)

[a] The K_m values for substrate (in bold) and the K_i values for inhibitors are given in μM (with pK_m ± SEM and pK_i ± SEM values in parentheses, respectively). [b] The concentration−response curves for the substrate L-CBG-I at the three transporters did not reach saturation in the concentration ranges used. The highest concentration of L-CBG-I used (1000 μM) elicited substrate responses of 72 ± 6%, 21 ± 3% and 48 ± 4% of the R_{max} values of Glu at EAAT1, EAAT2 and EAAT3, respectively. [c] L-CBG-II was a "full substrate" at EAAT1, eliciting a maximal response of 94 ± 7% of that of Glu.

Pharmacology and Modeling. The four stereoisomeric Glu analogues L-CBG-I−IV were characterized pharmacologically at EAAT1−3, using a FLIPR membrane potential (FMP) assay (see Experimental Section for details). The assay was performed essentially as described previously,[33] and results are summarized in Table 2. The trans-stereoisomer L-CBG-I was found to be a weak substrate at all three EAAT subtypes. In contrast, the stereoisomeric trans substituted analogue, L-CBG-II, was found to be a substrate at EAAT1 (K_m = 96 μM) and an inhibitor at subtypes EAAT2 and EAAT3 (K_i = 22 and 49 μM, respectively). A similar pharmacological profile (EAAT1 substrate vs EAAT2 and EAAT3 inhibitor) has previously been observed by us and others for (*R*)-4-methyl Glu.[5,34] The cis substituted analogue L-CBG-III was a weak inhibitor at all three subtypes EAAT1, -2, and -3 (K_i = ∼200, 110, and 52 μM, respectively), whereas the other cis stereoisomer, L-CBG-IV, was found to a weak inhibitor at EAAT1 (K_i ∼ 200) but a moderately potent inhibitor at EAAT2 and EAAT3 (K_i = 6.6 and 10 μM, respectively).

To explain and eventually expand the SAR for substrates and inhibitors at EAAT1−3, we carried out a modeling study of L-CBG-I−IV. We have previously argued for a differentiation of substrate and inhibitor activity at EAAT1−3 on the basis of conformations of a set of diverse ligands. An extended Glu conformation appears to be essential to allow substrate transport, whereas inhibitors bind to the transporters in a folded Glu conformation.[5] Thus, we submitted the four stereoisomeric analogues, L-CBG-I−IV, to a stochastic conformational search to identify relevant low-energy folded and extended Glu conformations, and results are summarized in Table 3. The two trans stereoisomers, L-CBG-I and L-CBG-II, can both attain low-energy extended and folded Glu conformations. Furthermore, L-CBG-II is found in a −3 kcal/mol lower energy pseudoextended Glu conformation compared with the extended one. The two cis stereoisomers, L-CBG-III and L-CBG-IV, are both found to attain a folded Glu conformation, exclusively.

By superimposition of the ammonium group and the two carboxylate groups in the two trans stereoisomers, L-CBG-I and L-CBG-II, it becomes evident that the two cyclobutane rings occupy very different areas in space (Figure 2). We propose

Table 3. Calculated Low-Energy Conformations[a]

	folded	extended
L-CBG-I	−117.255	−115.902
L-CBG-II	−118.028	−114.908 (−117.957[b])
L-CBG-III	−112.491	cnf[c]
L-CBG-IV	−115.599	cnf

[a] All calculated ΔG values in kcal/mol, using the mmff94x force field and gb/sa solvation model. For further details, see the Experimental Section. [b] Energy of a pseudoextended conformation that fits the substrate EAAT pharmacophore poorly. [c] Conformation not found.

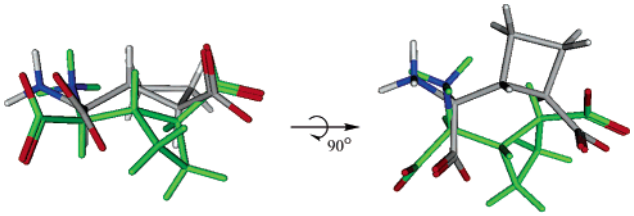


Figure 2. Superimposition by fitting the ammonium group and the two carboxylate groups of calculated low-energy extended Glu conformations of L-CBG-I (green) and L-CBG-II (type code).

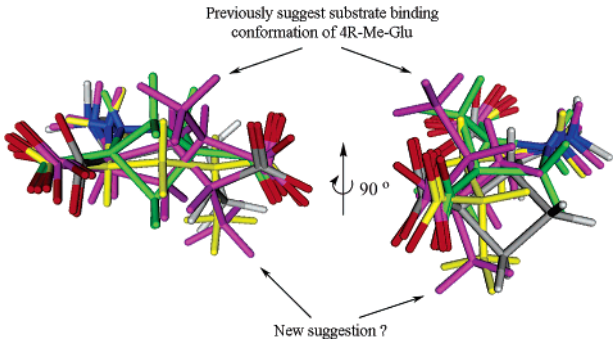


Figure 3. EAAT1 substrate pharmacophore model. Superimposition by fitting the ammonium group and the two carboxylate groups of calculated low-energy extended Glu conformations of L-CBG-II (type code) and L-2,4-methanopyrrolidine-2,4-dicarboxylate (L-2,4-MPDC) (green), (4*R*)-4-Me-Glu (two conformations) (purple), and (4*S*)-4-Me-Glu (yellow).

this to be the origin of the different potencies observed for these two substrates at EAAT1 (Table 2).

The distinct pharmacological profile of L-CBG-II, being an EAAT1 substrate and an EAAT2/3 inhibitor, has also been observed for (4*R*)-4-methyl-Glu.[34] In contrast, (4*S*)-4-methyl-Glu is neither a substrate nor an inhibitor at EAAT1−3.[34] In our previous study, we concluded that when (4*S*)-4-methyl Glu adapts its only extended Glu conformation found, there is inadequate space in the EAAT1−3 proteins, because the 4-methyl group is pointing down. As a consequence of this we put forward the hypothesis that (4*R*)-4-methyl-Glu binds in its extended Glu conformation with the 4-methyl group pointing up, and not down, and thus becomes a substrate at EAAT1.[5]

On superimposition and comparison of the extended Glu conformation of L-CBG-II with the inactive ligand (4*S*)-4-methyl-Glu, it becomes evident that part of the cyclobutane ring in L-CBG-II occupies approximately the same region in space as the 4-methyl substituent of (4*S*)-4-methyl-Glu (Figure 3). Furthermore, L-CBG-II does not reach into the region in space defined by the 4-methyl group of (4*R*)-4-methyl-Glu. Consequently, we have to revisit the premises of our previous hypothesis to address the following: What is the molecular basis for (4*S*)-4-methyl-Glu being inactive at EAAT1[34] while L-CBG-II is a substrate? And which of the two previously proposed

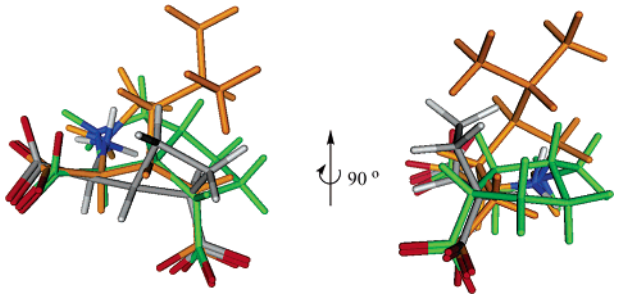


Figure 4. EAAT1−3 inhibitory pharmacophore model. Superimposition by fitting the ammonium group and the two carboxylate groups of calculated low-energy folded Glu conformations of L-CBG-II (type code), dihydrokainate (DHK) (orange), and (1*R*,4*S*,5*R*,6*S*)-3-azabicyclo-[3.3.0]octane-4,6-dicarboxylic acid (ABOD) (green).

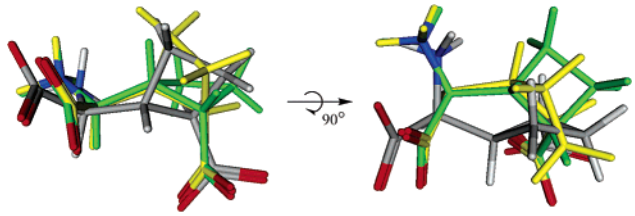


Figure 5. EAAT1−3 inhibitory pharmacophore model. Superimposition by fitting the ammonium group and the two carboxylate groups of calculated low-energy folded Glu conformations of L-CBG-II (type code), L-CBG-III (yellow), and L-CBG-IV (green).

extended Glu conformations does (4*R*)-4-methyl-Glu adapt,[5] when transported by EAAT1? First, a molecular modeling study like the one presented here provides static representations of the dynamic transport process of a ligand across a membrane. Second, ligand−protein interactions are acutely sensitive to disfavored van der Waals forces. Hence, the minor differences in spatial orientation that we predict between the cyclobutane ring of L-CBG-II and the methyl group of (4*S*)-4-methyl-Glu may be of detrimental nature to the transport of the ligand (4*S*)-4-methyl-Glu.

Since L-CBG-II is an inhibitor at EAAT2/3, the two subtypes are clearly not capable of facilitating transport, in contrast to the substrate properties observed for this ligand at EAAT1. To address this observation, we bring to the readers attention that L-CBG-II, in addition to an extended Glu conformation, can also adapt a folded Glu conformation (Table 3). Comparing the folded L-CBG-II conformation with our previously published inhibitory pharmacophore models of EAAT2 and EAAT3, a clear resemblance between folded L-CBG-II and conformationally restricted EAAT inhibitors (1*R*,4*S*,5*R*,6*S*)-3-azabicyclo-[3.3.0]octane-4,6-dicarboxylic acid (ABOD)[7] and dihydrokainate (DHK) can be observed (Figure 4).

Superimposition of the two cis-stereoisomers, L-CBG-III and L-CBG-IV, in their folded Glu conformation (Figure 5), together with folded L-CBG-II, provides a clear three-point overlap and unambiguously explains the pharmacological profile of L-CBG-III and L-CBG-IV as EAAT1−3 inhibitors. Although DHK has been reported to be selective EAAT2 inhibitors, most published EAAT inhibitors to date show no or little subtype selectivity.[33] In this context, the pharmacological profile of L-CBG-IV, with its 20−30-fold preference for EAAT2/3 over EAAT1, is remarkable.

Conclusion

Our chemoenzymatic approach allowed a convenient access to the four stereomers of L-CBG, including the two new derivatives L-CBG-II and IV. These Glu analogues were isolated

in good yields and very high purity. The four compounds were characterized at human EAAT1, EAAT2, and EAAT3, in the FMP assay. Notably, a distinct pharmacological profile was observed for the one trans-stereoisomer, L-CBG-II, which displayed EAAT1 substrate activity and EAAT2/3 inhibitory activity. The SAR study of this ligand adds new insight to the EAAT1 substrate pharmacophore. The cis-stereoisomer L-CBG-IV was found to be a moderately potent inhibitor with 20−30-fold preference for EAAT2/3 over EAAT1.

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 for 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 flash column and TLC chromatographies. All solvents were purified by distillation following usual procedures. Cysteine sulfinic acid was prepared from cystine following a described procedure.[35] Rabbit muscle lactic dehydrogenase, glutamic dehydrogenase from bovine liver, and formate dehydrogenase from *Candida boidinii* were purchased from Sigma. *E. coli* AAT and BCAT were produced and purified following described procedures from overexpressing *E. coli* strains TY103 transformed with pUC19-*aspC* (AAT) and pUC19-*ilvE* (BCAT).[36−38] Activities expressed as IU refers to V_{max} measured with natural substrates (Asp and KG for AAT, Glu and 4-methyl-2-oxopentanoic acid for BCAT) in 0.1 M phosphate buffer, pH 7.6, as previously described.[5] Both *E. coli* aminotransferases are commercially available from Biocatalytics.

***cis*-Cyclobutanecarboxylic Anhydride 1.** Maleic anhydride (0.981 g, 10 mmol) was introduced into a cylindrical reactor containing acetonitrile (500 mL) with acetophenone (117 μL, 1 mmol). The mixture was stirred at room temperature, degassed with argon for 30 min, and then saturated with ethylene for 30 min. While ethylene bubbling continued, the mixture was irradiated with a 400 W medium-pressure mercury lamp fitted with a Pyrex filter for 5 h. The solvent was evaporated and the solid residue was recrystallized from a cyclohexane/ether solution. The cyclobutane adduct **1** was obtained as a white solid (0.763 g, 75%): mp 74−75 °C; IR (KBr pellet) 1854, 1782 cm^{-1}; ^{1}H NMR (400 MHz, acetone-d_6) δ 2.25−2.45 (m, 2H), 2.70−2.85 (m, 2H), 3.65−3.72 (m, 2H); ^{13}C NMR (100 MHz, acetone-d_6) δ 23.2, 40.0, 206.3.

(±)-*cis*-2-Methoxycarbonylcyclobutanecarboxylic Acid 2a. A solution of *cis*-cyclobutane carboxylic anhydride **1** (0.240 g, 2.35 mmol) in methanol (5 mL) was stirred under reflux for 18 h. The solvent was evaporated under reduce pressure. Flash chromatography on silica gel (eluent cyclohexanes−ethyl acetate 1:1) afforded **2a** as a colorless liquid (0.287 g, 77%): IR (neat film) 3200−2860, 1735, 1705 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.18−2.30 (m, 2H), 2.36−2.46 (m, 2H), 3.41−3.48 (m, 2H), 3.69 (s, 3H), 10.9 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.9, 22.1, 40.4, 40.5, 51.8, 173.7, 179.3.

(±)-*cis*-2-Benzyloxycarbonylcyclobutanecarboxylic Acid 2b. A mixture of anhydride **1** (0.600 g, 5.88 mmol), benzyl alcohol (1.2 mL, 11.75 mmol), and 4-(dimethylamino)pyridine (0.043 g, 0.35 mmol) was stirred under argon at room temperature during 20 h. A saturated aqueous solution of $NaHCO_3$ (10 mL) was added. The aqueous layer was washed three times with ether and then acidified to pH 3 with a 1 M HCl solution. After extraction with EtOAc, organic layers were washed with brine, dried over $MgSO_4$, and concentrated under reduce pressure to afford **2b** as a white solid (0.872 g, 63%): mp 67−69 °C; IR (KBr pellet) 3500−2850, 1743, 1698 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.17−2.32 (m, 2H), 2.38−2.50 (m, 2H), 3.43−3.49 (m, 2H), 5.06 (d, J = 12,2 Hz, 1H), 5.15 (d, J = 12.2 Hz, 1H), 7.26−7.36 (m, 5H), 10.10 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.1, 22.2, 40.4, 40.5, 66.6, 128.2, 128.3, 128.5, 135.7, 173.0, 179.4.

General Procedure for Cyanophosphorane Formation. To a solution of acid **2** (5 mmol) in CH_2Cl_2 (40 mL) under argon at 0 °C were added DMAP (0.5 mmol) and EDCI (5.5 mmol) followed by (triphenylphosphoranylidene)acetonitrile (7.5 mmol). The reaction mixture was stirred for 20 min at 0 °C and then 5 h at room temperature. It was added to CH_2Cl_2/H_2O (1:1; 30 mL) and the aqueous phase was extracted again with CH_2Cl_2 (20 mL). Combined organic extracts were washed with brine, dried over $MgSO_4$, and concentrated under reduce pressure to afford **3**, which was purified by flash chromatography on silica gel (eluent cyclohexanes−ethyl acetate 1:1).

(±)-*cis*-2-(Cyanotriphenylphosphorane)ketomethylcyclobutanecarboxylic Acid Methyl Ester 3a. Compound **3a** was isolated as a white foam from (±)-*cis*-cyclobutane 1,2-dicarboxylic acid monomethyl ester **2a** (0.780 g, 4.93 mmol): yield 1.97 g, 91%; IR (KBr pellet) 2180, 1725, 1580 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.05−2.20 (m, 1H), 2.24−2.43 (m, 3H), 3.35−3.45 (m, 1H), 3.48 (s, 3H), 4.10−4.17 (m, 1H), 7.48−7.64 (m, 15H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.8, 23.3, 40.4, 45.0, 47.5 (d, J = 125 Hz), 51.3, 122.2 (d, J = 17 Hz), 123.3 (d, J = 93 Hz), 129.1, 129.2, 133.0, 133.5, 133.6, 174.6, 195.9 (d, J = 3 Hz).

(±)-*cis*-2-(Cyanotriphenylphosphorane)ketomethylcyclobutanecarboxylic Acid Benzyl Ester 3b. Compound **3b** was isolated as a white foam from (±)-*cis*-cyclobutane 1,2-dicarboxylic acid monobenzyl ester **2b** (0.896 g, 3.82 mmol): yield 1.802 g, 91%; mp 131−133 °C; IR (KBr pellet) 2175, 1739, 1581 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.14−2.44 (m, 4H), 3.45−3.50 (m, 1H), 4.11−4.18 (m, 1H), 5.85 (d, J = 13 Hz, 1H), 5.10 (d, J = 13 Hz, 1H), 7.24−7.32 (m, 5H), 7.44−7.65 (m, 15H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.8, 23.3, 40.8, 45.4, 47.8 (d, J = 125 Hz), 65.6, 122.2 (d, J = 16 Hz), 123.3 (d, J = 92 Hz), 127.6, 128.3, 129.0, 129.1, 133.0, 133.6, 133.7, 174.1, 195.9.

(±)-*cis*-2-Oxalylcyclobutanecarboxylic Acid Dimethyl Ester 4a. The α-keto cyanophosphorane **3a** (1.932 g, 4.38 mmol) in a 7:3 mixture of CH_2Cl_2/MeOH (50 mL) at −78 °C, was treated with ozone at a rate of 10L/h during 25 min. The excess of ozone was eliminated by oxygen bubbling and then dimethylsulfur was added and the reaction mixture was allowed to warm to room temperature during 2 h. After evaporation of the solvent, the residue was dissolved in CH_2Cl_2 (20 mL) and was washed three times with water. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. A short flash chromatography (eluent, cyclohexanes−EtOAc acetate 7:3) gave **4a** isolated as a colorless oil (0.707 g, 81%): IR (neat film) 1733, 1277, 1207 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.00−2.15 (m, 2H), 2.22−2.35 (m, 1H), 2.41−2.51 (m, 1H), 3.58 (s, 3H), 3.61−3.70 (m, 1H), 3.80 (s, 3H), 3.85−3.92 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.4, 22.7, 43.2, 43.3, 52.1, 52.8, 160.7, 173.9, 192.7.

(±)-*cis*-2-Oxalylcyclobutanecarboxylic Acid Dibenzyl Ester 4b. A solution of **3b** (1.801 g, 3.48 mmol) in CH_2Cl_2 (40 mL) at −78 °C was treated with ozone at a rate of 10L/h during 1 h. The excess of ozone was eliminated by argon bubbling and benzyl alcohol (720 μL, 6.96 mmol) was added under argon. The reaction mixture was stirred at −78 °C for 2 h and then triphenylphosphine (1.826 g, 6.96 mmol) was added and the solution was allowed to warm to room temperature over a period of 2 h. After evaporation of the solvent, the residue was dissolved in CH_2Cl_2 (20 mL). The solution was washed three times with water (3 × 20 mL), dried over $MgSO_4$, and concentrated under reduced pressure. Purification by flash chromatography on silica gel (eluent, cyclohexanes−EtOAC 8:2) afforded **4a** as a yellow semisolid (0.761 g, 62%): IR (film) 1732, 1270 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.14−2.24 (m, 2H), 2.32−2.37 (m, 1H), 2.53−2.58 (m, 1H), 3.69−3.71 (m, 1H), 3.94−3.96 (m, 1H), 4.98 (d, J = 12.4 Hz, 1H), 5.06 (d, J = 12,4 Hz, 1H), 5.18 (s, 2H), 7.28−7.38 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.6, 22.7, 43.5, 67.2, 67.7, 128.5, 128.2, 128.3, 128.4, 128.6, 128.7, 134.8, 135.5, 159.8, 173.4, 192.6.

(±)-*trans*-2-Oxalylcyclobutanecarboxylic Acid Dilithium Salt 5. To a solution of α-keto ester **4a** (0.204 g, 1.02 mmol) in methanol (5 mL) was added dropwise an aqueous solution of LiOH (0.4 M, 5.4 mL). The reaction mixture was stirred for 2 h and then MeOH was evaporated under reduced pressure. The pH was adjusted to 7.6 by addition of H^+ resin (Dowex 50Wx8−100). Filtration and evaporation of water under reduced pressure furnished quantitatively compound **5** (187 mg) as a white solid and as a 10:90 cis/trans mixture: ^{1}H NMR (400 MHz, D_2O) δ 1.91−2.31 (m, 4H), 3.24 (q, J = 8.8 Hz, 1H), 3.80 (q, J = 8.8 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) δ 21.4, 22.3, 41.3, 45.3, 170.4, 182.8, 206.1.

(±)-*cis*-2-Oxalylcyclobutanecarboxylic Acid Dilithium Salt 6. A mixture of α-keto ester **4b** (0.530 g, 1.5 mmol) with palladium on activated carbon (10% Pd, 50 mg) in EtOAc (50 mL) was stirred under pressure of hydrogen (40 psi) during 3 h. After filtration and evaporation of the solvent, the residue was dissolved in water and the pH adjusted to 7.6 by careful addition of lithium hydroxide (0.4 M). This solution was directly used for the transamination reaction. A sample was concentrated under reduced pressure and rapidly analyzed. A 80:20 cis/trans mixture was thus characterized by NMR analysis: ^{1}H NMR (400 MHz, D_2O) δ 1.92−2.37 (m, 4H), 3.43 (td, J = 8.8 and 6.4 Hz, 1H), 3.74−3.89 (m, 1H); ^{13}C NMR (100 MHz, D_2O) δ 20.9, 22.8, 44.8, 45.2, 168.2, 182.6, 205.6.

AAT-Catalyzed Transaminations. To an aqueous solution of racemic **5** or **6** (1.9−2.8 mmol) were added stoechiometric amounts of cysteine sulfinic acid and acetaldehyde. The pH of the solution was adjusted to 7.6 with 1 M NaOH and the volume adjusted to 100 mL with water before addition of AAT (250 IU, approximately 5 mg). The reaction was stirred slowly at room temperature and monitored by titration of pyruvate: 5-μL aliquots of the reaction mixture were added to 995 μL of 0.1 M potassium phosphate buffer, pH 7.6, containing NADH (0.2 mM) and rabbit muscle lactate dehydrogenase (1 unit). Pyruvate concentration was calculated from the ΔDO measured at 340 nm using ϵ_{NADH} = 6220 M^{-1} cm^{-1}. When a conversion rate of 40−45% was reached within approximately 4 h, the reaction mixture was rapidly passed through a column of Dowex 50 × 8 resin (H^+ form, 25 mL). The column was then washed with water (100 mL) until complete elution of CSA and then eluted with 1 M NH_4OH. The ninhydrin-positive fractions were combined and concentrated to dryness under reduced pressure. The residue was diluted in water (5 mL) and, if necessary, the pH adjusted to 7.0 with 1 M NaOH before adsorption of the product on a column of Dowex 2 × 8 resin (200−400 mesh, AcO^- form, 1.5 × 12 cm). The column was washed with water (50 mL) and then eluted with an AcOH gradient (0.05−1 M). The ninhydrin-positive fractions were combined and dried under reduced pressure to afford L-CBG derivatives: L-CBG-II was isolated from (±)-**5** while L-CBG-IV, L-CBG-II, and L-CBG-III were consecutively eluted from the basic resin in the case of (±)-**6** reaction.

(2*S*,1′*R*,2′*R*)-2-(2′-Carboxycyclobutyl)glycine L-CBG-II: yield 157 mg, 32%; mp 164−6 °C; $[\alpha]^{20}_D$ −53.0° (*c* 0.7, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 1.79−2.13 (4H, m), 2.86 (1H, dq, J = 8.0, 9.2 Hz), 3.07 (1H, q, J = 9.2 Hz), 3.68 (1H, d, J = 7.6 Hz); ^{13}C NMR (100 MHz, D_2O) δ 178.5, 172.8, 57.4, 41.3, 38.0, 21.6, 21.0; HRMS (ES, negative ion) *m*/*z* 172.0600 ([M − H]$^-$, $C_7H_{10}NO_4$ requires 172.0610). Anal. ($C_7H_{11}NO_4$) C, H, N.

(2*S*,1′*S*,2′*R*)-2-(2′-Carboxycyclobutyl)glycine L-CBG-III: yield 63 mg, 19%; mp 175−6 °C (lit.[11] mp 165−6 °C); $[\alpha]^{20}_D$ +3.7° (*c* 1.0, H_2O) (lit.[11] $[\alpha]^{20}_D$ +3.7° (c 0.5, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 2.05−2.32 (4H, m), 2.95−3.05 (1H, m), 3.37−3.43 (1H, m), 3.90 (1H, d, J = 11.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 178.7, 172.8, 55.8, 41.0, 37.4, 23.1, 21.4; HRMS (ES, negative ion) *m*/*z* 172.0614 ([M − H]$^-$, $C_7H_{10}NO_4$ requires 172.0610). Anal. ($C_7H_{11}NO_4$·0.2H_2O) C, H, N.

(2*S*,1′*R*,2′*S*)-2-(2′-Carboxycyclobutyl)glycine L-CBG-IV: yield 57 mg, 17%; mp 165−6 °C; $[\alpha]^{20}_D$ +42.9° (*c* 1.1, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 2.02−2.30 (4H, m), 2.95−3.09 (1H, m), 3.30−3.40 (1H, m), 4.06 (1H, d, J = 11.0 Hz); ^{13}C NMR (100 MHz, D_2O) δ 178.7, 173.0, 54.9, 42.0, 37.1, 23.6, 21.3; HRMS (ES, negative ion) *m*/*z* 172.0613 ([M − H]$^-$, $C_7H_{10}NO_4$ requires 172.0610). Anal. ($C_7H_{11}NO_4$·0.5H_2O) C, H, N.

BCAT-Catalyzed Transaminations. To an aqueous solution of racemic **5** or **6** (0.3 mmol) were added glutamic acid (9 mg, 0.06 mmol), NADH (10 mg, 0.014 mmol), and HCO_2NH_4 (38 mg, 0.6 mmol). The pH of the solution was adjusted to 7.6 with 1 M NaOH and the volume adjusted to 15 mL with water before addition of glutamate dehydrogenase (1 mg, 8 IU), formate dehydrogenase (15 mg, 15 IU), and BCAT (2 mg, 4 IU). The reaction was stirred slowly at room temperature for 2−7 d and the reaction mixture was then passed through a column of Dowex 50 × 8 resin (H^+ form, 25 mL). L-CBG-I was isolated in both cases as previously described for AAT transaminations.

(2S,1′*R*,2′*R*)-2-(2′-Carboxycyclobutyl)glycine L-CBG-I: yield 14 mg, 26% from **5**; mp >245 °C (dec) (lit.[11] mp 258 °C (dec)); $[\alpha]^{20}_D$ +96.4° (*c* 0.7, H_2O) (lit.[11] $[\alpha]^{20}_D$ +97.0° (*c* 0.5, H_2O)); ^{1}H NMR (400 MHz, D_2O) δ 1.79−1.95 (2H, m), 1.96−2.11 (2H, m), 2.78 (1H, dq, J = 8.8, 9.2 Hz), 3.15 (1H, q, J = 9.2 Hz), 3.67 (1H, d, J = 8.8 Hz); ^{13}C NMR (100 MHz, D_2O) δ 178.5, 172.6, 57.8, 41.7, 38.9, 21.4, 20.9; HRMS (ES, negative ion) *m*/*z* 172.0617 ([M − H]$^-$, $C_7H_{10}NO_4$ requires 172.0610). Anal. ($C_7H_{11}NO_4$· H_2O) C, H, N: calcd, 43.98, 6.85, 7.33; found, 43.66, 6.10, 7.41.

Molecular Modeling. The modeling study was performed using the software package MOE (Molecular Operating Environment, v2005.06, Chemical Computing Group) using the build-in mmff94x force field and the GB/SA continuum solvation model. Relevant low-energy conformations were sought by first methylating the γ-carboxylate group to avoid the return of a large number of collapsed conformations (holding intramolecular hydrogen bonds). Each compound was then submitted to a stochastic conformational search, and conformations above +7 kcal/mol were discarded. The γ-carboxylate group was demasked and the structure energy-minimized and used in the following superimposition studies. The superimposition of ligands was carried out using the built-in function in MOE, by fitting the ammonium group and the two carboxylate groups.

Pharmacological Characterization at Human EAAT1−3. The pharmacological properties of l-CBG-I−IV 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 have been reported previously, and the pharmacological characterization of L-CBG-I−IV was performed essentially as described here.[33] 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. Then 16−24 h later the medium was aspirated, and the cells were washed with 100 μL Krebs buffer [140 mM NaCl/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/11 mM HEPES/10 mM D-glucose, pH 7.4]. Next 50 μL of Krebs buffer was added to each well (in the characterization of nonsubstrate inhibitors, the inhibitors were added to this buffer), 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 30 °C in a NOVOstar plate reader measuring emission at 560 nm caused by excitation at 530 nm before and up to 1 min after addition of 33 μL 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. IC_{50} values were converted to K_i values by the use of the Cheng−Prusoff equation.[39]

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Supporting Information Available: A zipped file containing Figures 2−5 in manipulatable 3D coordinates (file formats MOE

and PDB), a pdf file containing combustion analysis data of compounds L-CBG-I−IV, and ^{1}H and ^{13}C NMR spectra of L-CBG-I. This material is available free of charge via the Internet at http://pubs.acs.org.

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