Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 3209–3213

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis and Preliminary Evaluation of *trans*-3,4-Conformationally-Restricted Glutamate and Pyroglutamate Analogues as Novel EAAT2 Inhibitors

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Received 13 December 2001; accepted 10 June 2002

Abstract—Select *trans*-4,5-[bi]cyclohexenylglutamic and pyroglutamic acids (3,4-substituted glutamates) were synthesized in three steps and were screened as potential inhibitors of the sodium dependent excitatory amino acid transporters 2 (EAAT2) and 3 (EAAT3), the chloride dependent glial cystine/glutamate exchanger system x_c^-, and the glutamate vesicular transport system (VGLUT). Two glutamate analogues and one pyroglutamate analogue were found to inhibit EAAT2 with activity comparable to dihydrokainate.

The receptors, transporters, and enzymes that bind L-glutamate during the process of excitatory neurotransmission comprise one of the most diverse and broadly functional protein groups in the mammalian central nervous system.[1] The ability to resolve this diversity and delineate these systems dictates the development of selective ligands that are capable of differentiating the binding sites of the various proteins that constitute the excitatory amino acid (EAA) system. L-Glutamic acid (Fig. 1) has a high degree of conformational flexibility and can exist in numerous, low-energy conformations. That L-glutamate can simulate such a large array of structural motifs may explain in part, its ability to interact with such a large number of proteins that can be differentiated pharmacologically.

To address this hypothesis, researchers have attempted to lock the L-glutamate molecule into specific configurations for the purpose of enhancing the selectivity with which the compounds bind to the participating proteins.[2] Conformationally-restricted analogues of L-glutamate have been shown, for example, to pharmacologically differentiate a variety of EAA transporters and receptors.[2–4]

The high selectivity and affinity of these conformationally-restricted glutamate analogues have been particularly helpful in identifying and refining key pharmacophore elements of the substrate binding sites. If one or more of these compounds are found to act at specific transporters, enzymes or receptors, there is, depending upon selectivity, increased potential for experimental and therapeutic use. Several families of compounds have proven valuable in characterizing the high-affinity, sodium dependent excitatory amino acid transporters (EAATs) that mediate the majority of glutamate uptake in the CNS, including: 2,4-pyrrolidinedicarboxylic acids (2,4-PDCs), 2-(carboxycyclopropyl) glycines (L-CCG-I-IV), β-hydroxyaspartate derivatives, methyl-substituted glutamate analogues, and oxazoline-based derivatives.[4–9] In particular, the conformationally

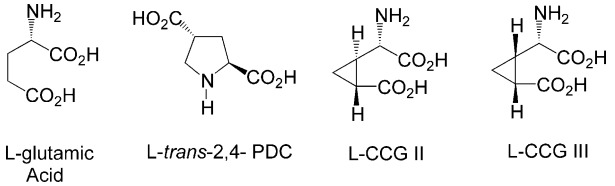

Figure 1. Structures of L-glutamic acid, *trans*-2,4-PDC, L-CCG-II, and L-CCG-III.

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PII: S0960-894X(02)00520-6

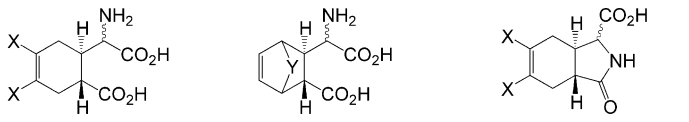


Figure 2. Structures of the target (D/L)-glutamate and pyroglutamate analogues.

restricted PDC- and CCG-based mimics are locked into specific configurations at the subsequent sites C_3 and/or C_4 (Fig. 1).[4–6] Transport assays have demonstrated that L-*trans*-2,4-PDC acts as a substrate of the excitatory amino acid transporter subtypes EAAT1–4 and as a non-substrate inhibitor of the EAAT5 subtype.[10–13] Similarly, the individual stereoisomers of the L-CCGs also exhibit interesting activities at these transporters. For example, L-CCG-II and L-CCG-III (Fig. 1) have both been reported to block the uptake of [^{3}H]-L-glutamate into synaptosomal preparations.[14] More detailed studies using cellular expression systems have demonstrated that L-CCG-III potently inhibits EAAT1, EAAT2, and EAAT3, while L-CCG-IV preferentially inhibits EAAT2.[15,16] Indeed, the L-CCGs represent an excellent template for exploration of glutamate transport inhibitors for a number of reasons: (a) attachment and/or substitution of alkyl groups at the C_3 and C_4 positions do not dramatically alter the chemistry (p*K*a, etc.) of the three glutamate functional groups and (b) only one conformational restriction is imposed, thereby allowing the molecule to appear as a constrained 'set' of conformers. Our approach to further elucidate the stereochemical binding requirements of these transporters is to increase the size of the conformational locking group relative to the L-CCG compounds, namely, the ring fused to the C_3–C_4 bond of glutamate.

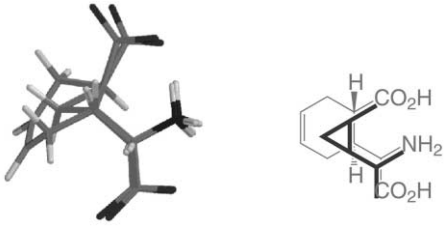


Figure 3. Representative overlays of L-CCG-II and L-*trans*-cyclohexenyl analogue (representative target). Left: models rendered using PC Spartan. Right: CCG carbon backbone overlay.

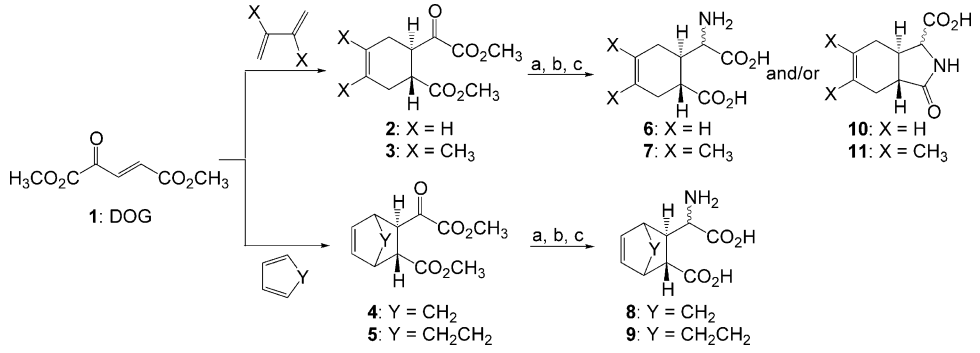
In a recent report, we described the use of dimethyl-4-oxoglutaconate (DOG) in the preparation of vesicular glutamate transport (VGLUT) inhibitors.[3] It occurred to us that DOG was a likely dienophile with the potential for accessing glutamate and pyroglutamate analogues (Fig. 2).

The resultant *trans*-3,4-conformationally-locked glutamate analogues should have excellent structural overlap with L-CCG-II as depicted by the structures of the cyclohexenyl target and L-CCG-II (Fig. 3).

This paper reports: (a) our exploration of DOG as a dienophile, (b) conversion of the Diels–Alder adducts to glutamate and pyroglutamate analogues and (c) the preliminary biological screening of the analogues at four different transport systems that utilize L-glutamate as a substrate (EAAT2, EAAT3, system x_c^-, and VGLUT).

Results and Discussion

The synthesis of the analogues was accessed by a Diels–Alder sequence (Scheme 1) utilizing dimethyl 4-oxoglutaconate (**1**: DOG)[17] as the dienophile. The reaction of select dienes (sulfolene, 2,3-dimethyl-1,3-butadiene, cyclopentadiene, and 1,3-cyclohexadiene) with DOG afforded the cyclohexenyl (**2** and **3**) and bicyclic (**4** and **5**)[18] adducts in 44–99% yield, with DOG showing approximate reactivity to maleic anhydride. Analysis of the ^{1}H NMR coupling constants indicates the expected orientation of the substituents as *trans* ($J \sim 11$ Hz) with respect to attachment to the ring. The ketodiesters (**2**–**5**) were converted to their corresponding dimethyl hydrazones in 55–99% yield. The hydrazones were reduced to the amino diesters (20–80%), lactam esters (30–90%) or lactam acids (1–25%) using sodium hydrosulfite. The amino diesters or lactam esters were subsequently hydrolyzed with NaOH to afford the *trans*-3,4-restricted glutamate (amino acid, **6**–**9**) or pyroglutamate (lactam,



Scheme 1. Synthesis of conformationally-restricted glutamate and pyroglutamic acid analogues via Diels–Alder reactions with DOG [(a) $(CH_3)_2NNH_2$, AcOH (b) $Na_2S_2O_4$ (c) NaOH].

Table 1. Activity of conformationally restricted glutamate (**6–9**) and pyroglutamate (**10** and **11**) analogues as transport inhibitors

Compd (concn)		Synaptosomal (EAAT2)[13] uptake of ^{3}H-D-Asp (5 μM)	C6 cellular (EAAT3)[19] uptake of ^{3}H-D-Asp (25 μM)	SNB-19 cellular (x_C^-)[20,21] uptake of ^{3}H-L-Glu (100 μM)	Synaptic vesicular (VGLUT)[22] uptake of ^{3}H-L-Glu (250 μM)
6	(250 μM)	38±2 (n=3)			85±5 (n=3)
	(500 μM)		95±3 (n=3)	101±7 (n=3)	
7	(250 μM)	71±14 (n=3)			99±5 (n=3)
	(500 μM)		100±1 (n=3)	104±8 (n=3)	
8	(250 μM)	35±5 (n=3)			93±5 (n=3)
	(500 μM)		87±9 (n=3)	88±5 (n=3)	
9	(250 μM)	70±4 (n=3)			104±4 (n=3)
	(500 μM)		97±3 (n=3)	88±5 (n=3)	
10	(250 μM)	53±6 (n=3)			103±2 (n=3)
	(500 μM)		93±2 (n=3)	89±7 (n=3)	
11	(250 μM)	77±7 (n=3)			96±3 (n=3)
	(500 μM)		97±3 (n=3)	103±9 (n=3)	
DHK	(250 μM)	22±3 (n=3)	—	—	—
L-Glu	(25 uM)	28±5 ($n\geq$3)[24]			

Compounds were evaluated at the concentrations indicated as inhibitors of several different glutamate transport systems. Na-dependent uptake of [^{3}H]-D-aspartate into synaptosomes[13] and C6 glioma cells[19] was used to evaluate activity at EAAT2 and EAAT3, respectively. Cl-dependent uptake of [^{3}H]-L-glutamate into SNB19[20,21] was used to determine activity at system x_C^-, while the ATP-dependent uptake of [^{3}H]-L-glutamate by isolated synaptic vesicles[22] was used to quantify activity at the vesicular glutamate transporter. Values shown are reported as mean% of Control±SEM (n ≥3). Dihydrokainate (DHK) and L-glutamate (L-Glu) are included as a reference for inhibition of EAAT2 and EAAT3. Control rates of uptake (pmol/min/mg protein) were as follows: synaptosomes, 1827±184 (n=8); C6 cells, 26±1 (n=8); SNB19 cells, 629±23 (n=7); synaptic vesicles, 3328±373 (n=5). All values have been corrected for non-specific uptake.

10 and **11**) analogues in 80–99% yield. As indicated by Scheme 1, the lactam products (**10** and **11**) formed only from the cyclohexenyl adducts and not from the bicycloheptene or bicyclooctene adducts and was a likely result of the rigidity imparted by the bicyclic system. Surprisingly, a number of procedures failed to convert the ketone diesters to the amine diesters via the C=N linkage including reduction with hydrides, Zn-HOAc, H_2/cat. (also reduced alkene), etc. The final products (**6–11**) were isolated following ion-exchange (amino acids) or normal phase (lactams) chromatography. Six analogues were prepared and their activity at four transporters was determined (Table 1).

The activity of select glutamate (**6–9**) and pyroglutamate (**10** and **11**) analogues was tested as potential inhibitors of EAAT2 by quantifying the ability of the analogues to attenuate the uptake of [^{3}H]-D-aspartate into synaptosomes prepared from rat forebrain. Under the assay conditions employed, the K_m value for the uptake of [^{3}H]-D-aspartate into the synaptosomes was 4.9±1 μM.[13] While EAAT2 is generally considered a glial transporter, previous studies have demonstrated that synaptosomal preparations clearly exhibit a pharmacological profile consistent with EAAT2.[13] As reported in Table 1, compounds **6**, **8**, and **10** markedly reduced uptake below control values. In particular, **6** and **8** proved to be comparable in activity (i.e., 62 and 65% inhibition, respectively) to dihydrokainate (DHK), a well-characterized selective EAAT2 inhibitor. Further, as these analogues were tested as a stereoisomeric mixture, it is quite possible that the observed activity may reside in a single species that would, consequently, possess considerably greater activity. The potential cross reactivity of the analogues was also assessed at: (a) EAAT3, by quantifying the uptake of [^{3}H]-D-aspartate into C6 rat glioma cells[19] (b) the system x_c^- glutamate/cystine exchanger, by quantifying the uptake of [^{3}H]-L-glutamate into SNB-19 human astrocytoma cells[20,21] and (c) the vesicular glutamate transporter, by quantifying the uptake of [^{3}H]-L-glutamate into synaptic vesicles prepared from rat forebrain.[3,22] To provide an indication of activity at these other systems, the K_m values for the respective substrates have been determined to be: 9±2 μM (n=9) for D-aspartate at EAAT3 in C6 cells, 111±10 μM (n=4) for L-glutamate at system x_c^- in SNB-19 cells, and 2.8±0.2 mM for L-glutamate at the vesicular transporter in isolated synaptic vesicles.[23] In each instance (Table 1), the compounds exhibited little or no activity at these alternative transporters.

The activity and selectivity shown by inhibitors **6** and **8** at EAAT2 suggests a well-defined steric relationship. Analogues **7** and **9** differ from analogues **6** and **8** in the incorporation of two methyl groups and insertion of a ring methylene yet **7** and **9** show significantly lower activity than analogues **6** and **8**. Taken together, these results identify a new structural class of compounds that should prove useful in developing novel probes of EAAT2 and help to further delineate a pharmacophore model for the substrate-binding site on the transporter protein. Future studies will focus on isolating and evaluating the activity of the individual isomers as selective inhibitors and substrates of EAAT2.

Acknowledgements

The authors thank Dr. Joseph DeGraw for his suggestions in the synthetic approach. We thank Kimberly Cybulski, Fred Rhoderick, and Brady Warren for carrying out the assays of VGLUT, EAAT3, and system x_c^-, respectively. The authors acknowledge the support of the NIH (NS38248 (C.M.T.), NS30570 (R.J.B.) and NCRR RR15583 (R.J.B.) and NSF MCB9808327 (C.M.T.) and CHE-98076469 (C.M.T.) for financial support.

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18. A representative synthesis is as follows: to a diene (2.0 mmol) in dichloromethane was added dimethyl-4-oxoglutaconate (1.0 mmol). The solution was refluxed (up to 24 h), the solvent and excess diene were removed in vacuo and the residue was chromatographed with EtOAc/hex (1:3) to afford the ketodiester adducts **3** in yields ranging from 55 to 99%. *N*,*N*-Dimethylhydrazine (5.0 mmol) and acetic acid (3.0 mmol) were added to the ketodiesters (1.0 mmol) in dichloromethane. The solution was refluxed (up to 24 h), the solvent, hydrazine and acetic acid were removed in vacuo and the resultant residue was chromatographed with EtOAc/hex (1:3) to afford the hydrazone diesters in yields ranging from 85 to 95%. Sodium hydrosulfite (2.0–3.0 mmol) was added to the hydrazone diester (1.0 mmol) in MeOH/H_2O (1:1). The solution was brought to reflux (4–14 h), the MeOH was removed in vacuo and the insoluble lactam acids collected by vacuum filtration. The remaining aqueous layer was titrated to pH 9 with solid K_2CO_3, extracted with Et_2O, dried with Na_2SO_4 and the solvent was evaporated in vacuo. The resultant residue was chromatographed (MeOH/CH_2Cl_2; 1:19) to afford the amino diesters (in the bicyclic cases, 20–80%) or the lactam esters (in the monocyclic cases, 30–90%). To the amino diesters or lactam esters in MeOH/H_2O (1:3) was added NaOH (1.0 M, excess). The solution was heated to 50 °C and stirred (10 min to overnight), the MeOH was removed in vacuo, water was added and the basic solution was extracted with EtOAc to remove any unreacted ester. The remaining aqueous solution was titrated to ca. pH 7 and added to a sulfonate cation exchange column (Dowex 50WX8-400). The amino acids were eluted with 1.0 N HCl where upon the aqueous was removed using a stream of air at 50 °C to afford the amino acids in 80–99% yield.
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mM $MgCl_2$ and [^{3}H]-L-glutamate (0.25–8 mM). The assays were initiated by the addition of [^{3}H]-L-glutamate ± inhibitors (0.01–5 mM) to the synaptic vesicles (total vol. 100 μL, 0.1 mg protein). Uptake was allowed to proceed at 30 °C for 1.5 min, after which the vesicles were collected by vacuum filtration through Millipore HAWP filters. The filters were rinsed twice, after which the retained radioactivity was quantified by liquid scintillation counting. Nonspecific uptake was determined in the absence of ATP.

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