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# STRUCTURAL DETERMINANTS OF SUBSTRATES AND INHIBITORS: PROBING GLUTAMATE TRANSPORTERS WITH 2,4-METHANOPYRROLIDINE-2,4-DICARBOXYLATE

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**Abstract**: Using an intramolecular [2+2] photocyclization, 2,4-methanopyrrolidine-2,4-dicarboxylate was prepared as a conformationally locked analogue of glutamate. This compound, in combination with two other pyrrolidine dicarboxylates, has been used to define the structural elements that differentiate substrate and non-substrate inhibitors of a high-affinity, sodium-dependent glutamate transporter. 

L-Glutamate is the principle excitatory neurotransmitter in the mammalian CNS.[1] As such, it participates not only in neuronal communication and higher cognitive function, but it is also capable of contributing to CNS pathology. Neuronal damage mediated by L-Glutamate (excitotoxicity) has been linked to both acute and chronic insults to the brain (e.g., stroke, trauma, Huntington's disease, and Alzheimer's disease).[2] Integral membrane transport proteins are credited with regulating extracellular glutamate and protecting neurons from excitotoxicity.[3] In particular, the high-affinity, sodium-dependent excitatory amino acid transporters (EAATs) are believed to play a primary role in regulating CNS glutamate levels.[3,5] Several EAAT subtypes have been identified and cloned (e.g., GLAST, GLT1 and EAAC-1; human counterparts: EAAT1, EAAT2, and EAAT3, respectively).[4,5] Selective inhibitors of these systems will undoubtedly prove useful as probes of transporter pharmacology, mechanisms, and function in glutamate-mediated signaling and pathology.[6,7]

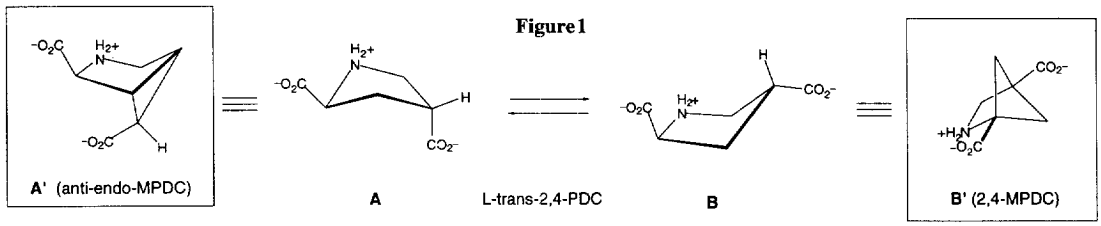


**Figure1**

Previously, we identified L-*trans*-2,4-PDC as a potent competitive inhibitor of the sodium-dependent, high-affinity EAATs present on rat forebrain synaptosomes.[8] It was postulated that the bioactive conformation of L-*trans*-2,4-PDC resembles a "folded" form **A** (Figure 1),[6] as opposed to an "extended" form **B**. This model was based on conformational comparisons of L-*trans*-2,4-PDC with glutamate (g+g+ conformational

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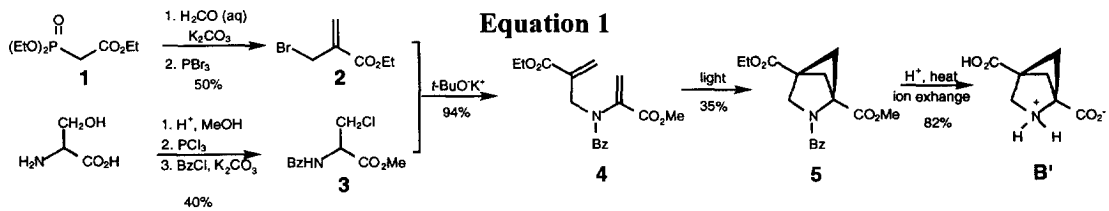
designation[9]) and known inhibitors (e.g., dihydrokainate, DHK).[6] To test this hypothesis, we prepared L-*anti-endo*-(3,4-methanopyrrolidine) dicarboxylate (L-*anti-endo*-3,4-MPDC, **A'**) as a conformationally locked mimic of the folded conformer and demonstrated that it too, was a potent competitive inhibitor.[10] Presently, we have prepared a conformationlly locked mimic of the "extended" conformer, 2,4-methanopyrrolidine-2,4-dicarboxylate (2,4-MPDC, **B'**), and characterized its activity as an EAAT inhibitor and substrate.

The synthesis (Eq 1) of 2,4-MPDC included an intramolecular [2+2] photocyclization, as was used in the preparation of the parent methanoproline derivative.[11] The precursor diene **4** was formed by coupling ethyl 2-(bromomethyl)acrylate[12] **2** and N-benzoyl chloroalanine methyl ester[13] **3** (starting from commercially available triethyl phosphonoacetate **1** and D,L-serine) that, following dehydrohalogenation, afforded **4** in 94% yield. Photolysis gave the desired [2+2] cycloadduct **5** in modest yield. Acidic deprotection of **5**, followed by ion exchange chromatography (Bio-Rad AG-1-X2 acetate) afforded the final product **B'**.

**Equation 1**




Based on the original hypothesis and the activity of L-*anti-endo*-3,4-MPDC (**A'**, Figure 1), it was anticipated that 2,4-MPDC (**B'**) would be less effective as an EAAT inhibitor. We were surprised, however, to find that both **B'** and **A'** attenuated $^3$H-D-aspartate uptake in synaptosomes as effectively as the endogenous substrate, L-glutamate (Table 1). At 250 μM, 2,4-MPDC produced an almost complete inhibition of uptake. Transport was reduced to 38 ± 1% of Control (1.1 ± 0.2 nmol/min/mg protein, n = 16) when the level of

**Table 1.** Activity of 2,4-MPDC and related pyrrolidine dicarboxylates

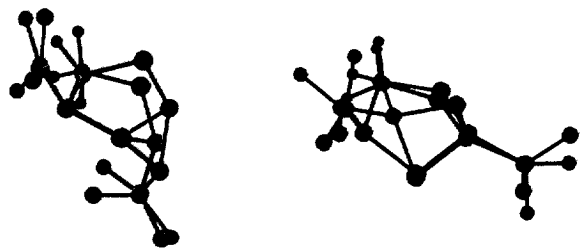
| COMPOUND | Inhibition of $^3$H-D-Aspartate (5 μM) Uptake in Rat Brain Synaptosomes | | Kinetic Parameters of Uptake in Oocytes Expressing EAAT2 | |
|---|---|---|---|---|
| | [Inhibitor] | % of Control | $K_m$ or $K_i$*(μM) | $I_{max}$ |
| Control | - | 100 | -- | -- |
| L-Glutamate | 25 μM | 38 ± 5 | 18 ± 3 | 100 |
| 2,4-MPDC | 25 μM | 38 ± 1 | 45 ± 3 | 115 ± 3 |
| | 250 μM | 4 ± 0.1 | -- | -- |
| L-*trans*-2,4-PDC | 25 μM | 18 ± 5 | 8 ± 1 | 32 ± 2 |
| L-*anti-endo*-3,4-MPDC | 25 μM | 26 ± 3 | 1.6* | 0 |
| Dihydrokainate (DHK) | 25 μM | 52 ± 4 | 9.2*[5] | 0 |

2,4-MPDC was decreased to 25 μM. This inhibition was comparable to that produced by similar amounts of L-glutamate, L-*anti-endo*-3,4-MPDC, or L-*trans*-2,4-PDC (Table 1). Using 2,4-MPDC as a pharmacophore

model, the results suggest that L-glutamate can also bind to this protein in an "extended" form. Given the distinct conformations of **B'** and **A'**, it was suspected that the similar activities did not simply reflect the non-selectivity of the EAAT binding site, but something more subtle. Specifically, it seemed plausible that both analogues might bind to the EAAT substrate site (i.e., act as competitive inhibitors) yet exhibit different abilities to be translocated across the plasma membrane. In this respect, L-*anti-endo*-3,4-MPDC and 2,4-MPDC might represent specific pharmacophores for nonsubstrates and substrates, respectively (Figure 2). This idea was examined in greater detail by quantifying uptake electrophysiologically in *Xenopus* oocytes expressing a cloned EAAT. Because this uptake is electrogenic, analogue-induced currents (reported as $I_{max}$ relative to L-glutamate) serve as direct indicators of substrate activity.[5] The human EAAT2 subtype was used in these studies, as it exhibits the greatest pharmacological similarity (e.g., sensitivity to DHK and all three PDCs[5]) to the tissue-derived synaptosomal system. Neither L-*anti-endo-3,4*-MPDC nor DHK produced an uptake-mediated current (Table 1), consistent with the action of nonsubstrate inhibitors. In these experiments, $K_i$ values were calculated by Schild analysis.[14] The $K_i$ of 1.6 μM for L-*anti-endo*-3,4-MPDC is similar to the value previously reported for synaptosomes, 5 ± 2 μM.[10] Unlike the nonsubstrates, L-*trans*-2,4-PDC produced an $I_{max}$ of about one third that of L-glutamate, while 2,4-MPDC yielded an $I_{max}$ value greater than L-glutamate itself (Table 1). The demonstration that 2,4-MPDC appears to be translocated at least as well as L-glutamate supports the conclusion that it embodies the substrate pharmacophore.

**Figure 2**

Overlay of folded conformer of L-*trans*-2,4-PDC (**A**) in blue and L-*anti-endo*-3,4-MPDC (**A'**) in green. Models were generated on an SGI work station using Macromodel v. 5.5.



Overlay of extended conformer of L-*trans*-2,4-PDC (**B**) in blue and 2,4-MPDC (**B'**) in green. Models were generated on an SGI work station using Macromodel v. 5.5.

These studies indicate that there are at least two distinct EAA pharmacophores that bind to this sodium-dependent, high affinity EAAT: one for substrates and the other for non-transportable inhibitors. As depicted above, the functional group positioning of the non-susbtrates is exemplified by a "folded" conformation of L-*trans*-2,4-PDC (**A**), while (**B**) represents a more extended conformer that typifies good substrates. It is of course possible that in addition to these functional group positional relationships, complementary variations in the carbon backbones contribute to the observed differences in activity between **A'** and **B'**. Further, it remains to be determined whether they bind to the transporter at two distinct sites or to two different states of a single site.

Toward the resolution of these issues these pharmacophore models should prove of considerable value in the development of more specific analogues with which to investigate this important transport system.

## Experimental

N-Benzoyl chloroalanine **3**. To D,L-serine (10.5 g, 100 mmol, 1.0 eq) in MeOH (100 mL) at 0 °C was added thionyl chloride (8.3 g, 5.1 mL, 70 mmol, 0.7 eq) over 5 min. The reaction was allowed to warm to room temperature and stir for 48 h, at which time the reaction was concentrated to a solid following repetitive evaporation with MeOH (3 x 20 mL), toluene (1 x 20 mL), and hexane (1 x 20 mL). The crude white solid was dissolved in acetyl chloride (120 mL), chilled to 0 °C, and phosphorus pentachloride (22.5 g, 107 mmol, 1.07 eq) was added. The reaction mixture was allowed to warm to room temperature and was stirred for an additional 9 h, at which time the mixture was cooled, and the solid precipitate was filtered and collected to yield 13.5 g of a light-yellow solid. This solid was suspended in THF (100 mL) and water (5 mL) at 0 °C and reacted with potassium carbonate (27 g, 195 mmol, 1.95 eq) and benzoyl chloride (26.6 g, 22 mL, 190 mmol, 1.9 eq) for 1 h at 0 °C. The reaction was stirred for 4 h at room temperature, then diluted with water (600 mL) and chilled to 0 °C overnight. The solid formed in the reaction mixture was filtered off and dried to yield the product **3** (10.1 g, 40%) as a white solid. $^1$H NMR (400 MHz, $CDCl_3$): δ 7.81 (m, 2H), 7.05 (m, 3H), 7.03 (br d, $J$ = 6.4 Hz, 1H), 5.17 (dt, $J$ = 6.7, 3.0 Hz, 1H), 4.08 (dd, $J$ = 3.3, 11.4 Hz, 1H), ), 4.05 (dd, $J$ = 3.3, 11.4 Hz, 1H), 3.82 (s, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 169.4, 167.0, 133.3, 132.0, 128.6, 127.1, 53.5, 53.1, 45.1. IR (NaCl, film) 3317, 3063, 2951, 1746, 1652 cm$^{-1}$.

Diene diester **4**. To **3** (1.78 g, 6.65 mmol, 1.0 eq) in dry THF (20 mL) at -78 °C under $N_2$ was added potassium *t*-butoxide (1.70 g, 15.18 mmol, 2.2 eq) in 20 mL dry THF, followed by the addition of ethyl 2-(bromomethyl)acrylate **2** (1.67 g, 8.65 mmol, 1.3 eq) via syringe. The reaction was then allowed to warm to room temperature over 30 min and stirred for an additional 18 h, whereupon saturated $NH_4Cl$ (aq) was added, the mixture was diluted with EtOAc and extracted, washed with water and brine, dried (sodium sulfate), concentrated and chromatographed on silica gel (30% EtOAc/70% hexanes) to yield **4** (2.02 g, 94%) as a pale-yellow oil. $^1$H NMR (400 MHz, $CDCl_3$): δ 7.48 (m, 2H), 7.34 (m, 3H), 6.40 (s, 1H), 6.01 (s, 1H), 5.96 (s, 1H), 5.49 (s, 1H), 4.59 (s, 2H), 4.20 (q, $J$ = 7.1 Hz, 2H), 3.60 (s, 3H), 1.29 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 171.0, 165.9, 164.3, 140.7, 135.5, 135.4, 130.3, 128.1, 127.9, 127.7, 127.1, 60.8, 52.3, 49.4, 14.0. IR (NaCl, film) 3057, 2984, 1726, 1662, 1631, cm$^{-1}$. HRMS calcd for $C_{12}H_{19}NO_5$ 317.1263, found 317.1263.

Diester **5**. Diene **4** (0.47 g, 1.48 mmol) was dissolved in 25 mL benzene (to give a 2% solution) and 0.05 g acetophenone was added (to give 0.2% solution of sensitizer) in a 50 mL Pyrex round bottom flask. The reaction solution was degassed and purged with nitrogen, stoppered, and irradiated with a medium pressure lamp (450 W Hanovia) for 65 h. The reaction mixture was concentrated and chromatographed on silica gel (30% EtOAc/70% hexanes) to yield **5** (0.163 g, 35%) as a colorless oil. $^1$H NMR (400 MHz, $CDCl_3$): δ 7.70 (m, 2H), 7.40 (m, 3H), 4.11 (q, $J$ = 7.1 Hz, 2H), 3.76 (s, 3H), 3.70 (s, 2H), 2.44 (d, $J$ = 4.8 Hz, 2H), 2.08 (d, $J$ = 4.8 Hz, 2H), 1.20 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 173.7, 168.9, 167.9, 133.6, 131.7, 128.4, 128.1, 67.4, 61.2, 55.2, 52.3, 48.0, 44.6, 31.4, 14.0. IR (NaCl, film) 2980, 1738, 1651cm$^{-1}$.

Diacid B'. To **5** (0.163 g, 0.51 mmol) was added 6 N HCl (5 mL) and heated to gentle reflux for 4.5 h. The reaction was cooled and concentrated and loaded on 2.4 g ion exchange resin (Bio-Rad®, Ag 1-2X, acetate form) and eluted with water (50 mL), 0.1 M HOAc (100 mL), and 0.2 M HOAc (200 mL). The ninhydrin positive fractions were combined and concentrated with consecutive water chases to yield **B'** (0.071 g, 82%) as a white solid. $^1$H NMR (400 MHz, $D_2O$): δ 3.49 (s, 2H), 2.55 (d, $J$ = 5.1 Hz, 2H), 2.03 (d, $J$ = 5.1 Hz, 2H). $^{13}$C NMR (100 MHz, $D_2O$): δ 171.3, 168.0, 68.8, 49.6, 49.3, 43.3. IR (KBr) 3476, 2975, 2692, 2540, 1718 cm$^{-1}$. Anal calcd for: $C_7H_9NO(H_2O)_{0.9}$ C 44.87%, H 5.81%, N 7.48%; found C 44.98%, H 5.66%, N 7.48%.

Synaptosomal transport. Synaptosomes were prepared from forebrains of male Sprague–Dawley rats (200–300 g) using a discontinuous Ficoll gradient.[15] The synaptosomal pellet was suspended in assay buffer (see below) at a final concentration of about 0.2 mg protein/mL. Uptake of $^3$H-D-aspartate (Dupont NEN, Boston, MA) through the high-affinity, sodium-dependent EAAT was quantified in assay buffer containing 128 mM NaCl, 10 mM glucose, 5 mM KCl, 1.5 mM $NaH_2PO_4$, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 10 mM Tris (pH 7.4).[16] Following a preincubation (5 min, 25 °C), uptake was initiated by the simultaneous addition of $^3$H-D-aspartate (final concentration 5 μM) and the EAA analogues. Uptake was allowed to proceed for 2 min, after which time the reaction was stopped by the addition of ice cold assay buffer (6 mL) and rapidly filtered on Whatman GF/F glass fiber filters. Following a rinse with ice cold buffer (6 mL), the radioactivity retained on the filters was quantified by liquid scintillation counting. All values were corrected for background by subtracting $^3$H-D-aspartate accumulated at 4°C and are reported as mean ± SEM (n = 3–9). Previous experiments have demonstrated that under these conditions, uptake is sodium-dependent and linear with respect to both protein content and time. Protein concentrations were determined by the Pierce BCA (bicinchoninic acid) assay.[17]

EAAT2 expression and transport. Capped RNA was transcribed from linearized plasmids containing the coding regions of EAAT2.[5] 50 ng RNA was injected into stage V oocytes and experiments were performed 2–6 days later. Current recordings were made with a two-microelectrode voltage clamp circuit. Electrodes contained 3 M KCl and had resistances of 100–500 kOhm. Oocytes were voltage clamped at -60 mV and continuously superfused with Ringer recording solution containing (in mM): NaCl 98.5; KCl 2; $CaCl_2$ 1.8; $MgCl_2$ 1; HEPES 5 (pH 7.5). Glutamate and analogs were bath applied by switching to a solution containing the compound at the indicated concentrations. The concentration-response of currents induced by the compounds were fitted by least squares to the equation $I = I_{max}$ ([compound] / ([compound] + Km). Km values are expressed as mean ± SEM from fits to individual oocytes. L-*anti-endo*-MPDC (3, 10, and 30 μM) was co-applied with varying concentrations of L-glutamate, and Schild analysis was performed to obtain estimates of antagonist equilibrium dissociation constants.[14] The values are reported in Table 1 as mean ± SEM (n ≥ 3 cells).

Conformational analysis: Computer-based molecular modeling studies were carried out on an SGI work station using Macromodel v. 5.5. Minimized conformations were first identified (Monte Carlo searches using AMBER* forcefield, dielectric = 80) and then spatially compared by superimposing their functional groups (i.e., α-amino, α-carboxyl, and distal carboxyl groups). Comparisons were also made with a library of stable solution conformers of L-glutamate.[18] A three point best fit analysis was then used to determine which conformers exhibited the greatest degree of overlap.[6] Our criterion for good alignment was an RMS deviation of atoms for the carboxylate carbons and ammonium nitrogen atom of less than 0.1 A.

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