Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 3509–3515

BIOORGANIC & MEDICINAL CHEMISTRY

Methylation of L-*trans*-2,4-Pyrrolidine Dicarboxylate Converts the Glutamate Transport Inhibitor from a Substrate to a Non-substrate Inhibitor

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Received 2 May 2002; accepted 13 June 2002

Abstract—The 4-methyl analogue of the potent inhibitor of CNS L-glutamate neurotransmitter transporters, L-*trans*-2,4-PDC, was synthesized via a 1,3-dipolar cycloaddition reaction sequence. The bioassays performed not only exhibit increased potency of the methylated derivative over L-*trans*-2,4-PDC, but also exhibit non-substrate properties at the rat forebrain synaptosomal glutamate transporter while the parent L-*trans*-2,4-PDC exhibits substrate properties. These results support two hypotheses developed for distinguishing the physiological properties of transport inhibitors based on molecular modeling studies, and are reported here.

Introduction

L-Glutamate is recognized as the major excitatory neurotransmitter in the mammalian central nervous system (CNS). Through the activation of a variety of ionotropic and metabotropic excitatory amino acid (EAA) receptors, L-glutamate participates not only in fast excitatory transmission, but also in the higher order signal processing required in development, learning, memory and synaptic plasticity (for review see: refs 1 and 2). Of equal interest, over activation of EAA receptors by excessive levels of L-glutamate can also induce excitotoxic-mediated neuronal pathology.[3,4] High-affinity transporter proteins that translocate L-glutamate across the cellular membranes of neurons and glia are thought to play a significant role in regulating extracellular levels of L-glutamate and maintaining the balance between its physiological and pathological actions. As a consequence of the ability to efficiently clear L-glutamate from the synaptic spaces surrounding EAA receptors, these excitatory amino acid transporters (EAATs) are postulated to participate not only in the maintenance of glutamate concentrations below those which are excitotoxic, but also in signal termination and neurotransmitter recycling.[5–8] Of the five sodium-dependent high-affinity EAATs identified thus far, the EAAT2-subtype (rat homologue: GLT1) present on glial cells throughout the forebrain is thought to be the dominant transporter responsible for these functions.[9] Recently, a splice variant of EAAT2/GLT1 has also been reported to be present on neurons.[10,11]

Previous studies identified L-*trans*-2,4-pyrrolidine dicarboxylate (L-*trans*-2,4-PDC) as a potent competitive inhibitor of EAAT2 (as well as all of the other EAATs) that can also act as a substrate of this transporter (Fig. 1).[12,13] While more conformationally constrained than L-glutamate, L-*trans*-2,4-PDC can, owing to conformational inter-conversions of the ring, mimic more than one conformation of L-glutamate. Based upon modeling comparisons of glutamate with L-*trans*-2,4-PDC (Fig. 2 A and B, R = H), we initially postulated that **A** is the bioactive conformation for binding to the substrate site on the transporter protein. This hypothesis was predicated simply upon binding data garnered from competitive inhibition studies; the issue of whether a given

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PII: S0968-0896(02)00250-X

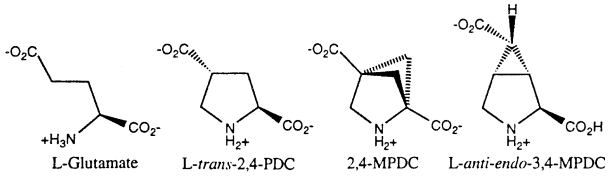


Figure 1. L-Glutamate and conformationally constrained competitive inhibitors of EAAT2.

ligand was transported (i.e., translocated across the membrane) or not was ignored. To further test this model and ascertain if **A** might indeed be the active form, conformationally locked mimics of the two 'extreme' conformations of L-*trans*-2,4-PDC (there are many closely related conformers as well) were prepared: L-*anti,endo*-3,4 - methanopyrrolidine - 3,4 - dicarboxylate (L-*anti,endo*-3,4-MPDC, **A′**) representing the 'folded' (pseudo-axial) conformation (**A**) and 2,4-methanopyrrolidine-2,4-dicarboxylate (2,4-MPDC, **B′**) which closely aligns with the extended form **B** (psedo-equatorial).[14–16] Our criterion for good alignment was an RMS deviation of less than 0.1 Å. for atoms of the carboxylate carbons and ammonium nitrogen atom, and the two derivatives shown gave the best fits of all compounds inspected.

Interestingly, both analogues were able to effectively bind to the transporter, as indicated by the potency with which they competitively blocked the synaptosomal uptake of D-[^{3}H]aspartate (e.g., $K_i \approx 5$ and 7 μM, respectively, Table 1). These values are within experimental error for the activity of the endogenous substrate, L-glutamate ($K_i \approx 5$ μM). It was suspected, however, that these results reflected more than just a binding domain with a broader than expected specificity range. Indeed, more recent experiments demonstrated that there is a significant functional difference between **A′** and **B′** that inhibition-based assays cannot distinguish: **B′** is very effectively translocated across the membrane (i.e., is a substrate comparable to glutamate itself), while **A′** is not.[13] These findings support the hypothesis that **B′** and **B** define the substrate pharmacophore (i.e., good binding, efficient transport), while **A′** and **A** represent the equivalent of an non-substrate inhibitor pharmacophore (good binding, poor transport). A functional analogy may be drawn between substrates and non-substrate inhibitors of neurotransmitter transporters and the respective actions of agonists and antagonists at neurotransmitter receptors. This conclusion is consistent with known properties of L-*trans*-2,4-PDC (Fig. 2 A and B: R = H), which is readily transported because it can achieve the 'transportable' conformation represented by **B/B′**.[13,17] This clearly suggests that substituents on L-*trans*-2,4-PDC that would favor the folded conformer **A** should decrease transport rates without necessarily decreasing binding affinity. However, it is possible that such structural modifications, including those present on L-*anti,endo*-3,4-MPDC or 2,4-MPDC, may independently influence activity as a consequence of the introduction of steric bulk. Thus, similar structure/activity studies carried out with a larger library of compounds, the majority of which were less conformationally constrained than either L-*anti,endo*-3,4-MPDC or 2,4-MPDC, also suggested that steric arguments can be made to differentiate the pharmacophores of substrates and non-substrate inhibitors.[13]

As a further test of the these relationships, we have synthesized 2*S*,4*R*-4-methyl-pyrrolidine-2,4-dicarboxylate (4-Me-L-*trans*-2,4-PDC) **6** as a conformationally biased analogue of L-*trans*-2,4-PDC and used it as a probe with which to further delineate the chemical constraints that differentially govern the ability of analogues to bind to and then be (or not be) translocated by the EAAT2/GLT1 subtype of glutamate transporter. Specifically, 4-Me-L-*trans*-2,4-PDC was found to competitively inhibit the uptake of D-[^{3}H]aspartate in rat forebrain synaptosomes, but did not exchange with radiolabeled substrate in complementary efflux assays (Fig. 3, Table 1). Significantly, these results suggest that the addition of the 4-methyl group essentially converted the parent compound L-*trans*-2,4-PDC from an

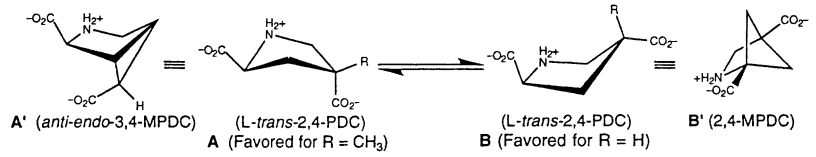


Figure 2. Pseudo-axial, 'folded' (A) and pseudo-equitorial, 'extended' (B) conformations of L-*trans*-2,4-PDC.

Table 1. Glutamate and related pyrrolidine dicarboxylates as transporter inhibitors and substrates

Compd	K_i (μM)	Concd (μM)	Exchange rate (pmol/mg pro/2 min)	Substrate activity (% of Glu)
L-GIu	4.9±1.0	50	344±16	100
2(*S*),4(*R*)-4-Me-2,4-PDC	**3.1±0.5**	**30**	**0±8**	**0**
(2*S*,4*R*)-4-Me-Glu	4.5±0.9	45	194±5	56
(2*S*,4*S*)-4-Me-Glu	>50	—	—	—
L-trans-2,4-PDC[a]	1.5±0.5	15	208±28	60
2,4-MPDC[a]	6.8±3.0	75	280±30	81
L-anti-endo-3,4-MPDC	4.9±2.0	50	120±18	35

[a]As reported in Koch et al.[13]

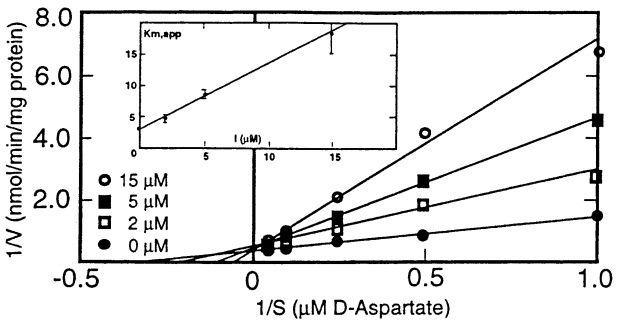


Figure 3. Representative Lineweaver–Burk plot demonstrating competitive inhibition of D-[^{3}H]aspartate uptake into rat forebrain synaptosomes by 4-Me-L-*trans*-2,4-PDC. The inset shows a replot of $K_{m,app}$ versus [inhibitor]. The plots shown were generated using the k_{cat} kinetic program (BioMetallics Inc., Princeton, NJ, USA) with weighting based on constant relative error, and yielded values of: $K_m = 2.8$ μM (D aspartate), $K_i = 3.6$ μM, and a V_{max} of 2.6 nmol/min/mg protein. Three such analyses yielded an average $K_i = 3.1 \pm 0.5$ μM.

alternative substrate into a non-substrate inhibitor. The development of such analogues, as well as the elucidation of the pharmacological relationships controlling substrate translocation, are key to understanding the structural and functional properties of the glutamate transporter proteins.

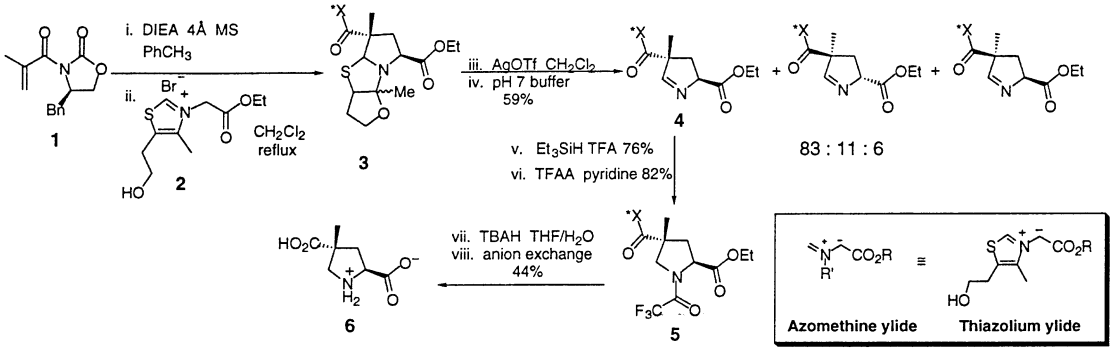
Chemistry

We first prepared 2(*S*),4(*R*)-4-Methyl-2,4-PDC (**6**) from a protected form of 2,4-PDC (which is turn prepared from L-hydroxyproline[12]) via methylation of the selectively-formed γ-enolate.[18] That route was rather lengthy, however, and we therefore investigated an alternative strategy employing a chiral auxiliary-directed 1,3-dipolar cycloaddition (Scheme 1).[19,20] In principle, an acrylate and a simple azomethine ylide might be employed,[21–23] but these reactions proved to be generally unacceptable, at least with the requisite mono-activated dipolarophiles. The use of a synthetic equivalent of the azomethine ylide was therefore required. The thiazolium ylide shown is a known synthon for the simple azomethine dipole and, fortuitously, undergoes cycloadditions with higher regioselectivity than the parent ylide.[24,25] In order to control the enantioselectivity of the reaction, an Evans' oxazolidinone was incorporated into the acrylate dipolarophile, as in **1**.[26] Despite the additional complexities introduced by utilizing this thiazolium ylide, including the production of 'temporary' *N*,*O*-acetal diastereomers and the need to cleave the C–S and C–N bonds, this strategy promised to provide a very concise means of producing the target compound (Scheme 1).

The key cycloaddition was conducted by reaction of four equivalents of the acrylate **1**[28] with the thiazolium salt **2**[25] to give the diastereomeric tricyclic adducts **3**. Since the separation of the minor isomers was problematic (most likely due to the labile *N*,*O*-acetal), the crude mixture was treated with AgOTf[29,30] to generate the corresponding imine intermediate **4** and two minor diastereomers in a ratio of 83:11:6. The desired major product **4** is consistent with regioselective, auxiliary-directed '*exo*' attack of the chiral acrylate on the conformation of the dipole shown, all in accord with literature precedent. These imines, however, were also inseparable on a preparative scale. They were therefore reduced as a mixture to the corresponding pyrrolidines and then protected to give a separable mixture of trifluoroacetamides, from which **5** could be obtained in pure form. Finally, the trifluoroacetamide **5** was deprotected and purified to give the target compound **6**, which was identical to material prepared by the more circuitous route from L-hydroxyproline via L-*trans*-2,4-PDC (data not shown). This regio-, stereo-, and enantioselective route provides the highly substituted (2*S*, 4*R*)-4-methyl-*trans*-2,4-PDC in reasonable yield in only five steps.

Results and Discussion

The inhibitory activity of 4-Me-L-*trans*-2,4-PDC was assessed by quantifying its ability to block the uptake of D-[^{3}H]aspartate in rat forebrain synaptosomes, a preparation that exhibits a pharmacological profile most closely aligned with EAAT2.[13] As illustrated in the Lineweaver–Burk plot depicted in Figure 3, the analogue blocked uptake in a manner consistent with that of a competitive inhibitor and exhibited a K_i value similar to that of L-glutamate (e.g., 3.1 μM vs 4.9 μM). To determine whether or not 4-Me-L-*trans*-2,4-PDC could also act as a substrate, the analogue was tested for its ability to exchange with D-[^{3}H]aspartate that had been pre-equilibrated into the synaptosomes.[13,31] Thus, synaptosomes were first incubated with D-[^{3}H]aspartate, reisolated, resuspended (D-[^{3}H]aspartate content = 1152 ± 65 pmol/mg protein), and then diluted 30-fold into assay buffer (37 °C) containing potential substrates. To insure



Scheme 1.

comparable levels of binding, the analogues were included in the efflux assay at a concentration approximately 10-fold greater than the K_i values with which they inhibited uptake. The extent of the efflux was measured over a two min time course and corrected for the efflux that occurred in the absence of inhibitor (228 ± 8 pmol/mg protein/2 min). As summarized in Table 1, known substrates, such as L-glutamate, L-*trans*-2,4-PDC and 2,4-MPDC all produced substantial increases in the synaptosomal efflux of D-[^{3}H]aspartate that are indicative of the process of heteroexchange.[13,31] As previously reported, L-*anti-endo*-3,4-MPDC proved to be a poor substrate, producing only about one third of the amount of exchange observed with L-glutamate.[13] Significantly, inclusion of the 4-Me-L-*trans*-2,4-PDC did not stimulate an efflux of D-[^{3}H]aspartate, consistent with the action of a non-substrate inhibitor. In contrast, its acyclic counterpart (2*S*,4*R*)-4-Me-glutamate did act as a partial substrate in the synaptosomal preparation, exhibiting about half the activity of L-glutamate. Considering that (2*S*,4*R*)-4-Me-Glu has previously been shown to be a non-substrate inhibitor of EAAT2 expressed in *Xenopus* oocytes (Vandenberg, 1997 #1271), as has L-*anti-endo*-3,4-MPDC (M. Kavanaugh, personal communication), it would suggest that the synaptosomal preparation likely contains other transporters, possibly EAAT3/EAAC1, at which the compounds act as substrates. The fact that no heteroexchange was observed in the presence of 4-Me-L-*trans*-2,4-PDC indicates that it is not only a non-substrate inhibitor of EAAT2/GLT1, but also of any other EAATs present in the synaptosomes.

These results indicate that the specific addition of a methyl group to the 4-position of L-*trans*-2,4-PDC does not diminish its ability to bind the transporter, yet it substantially alters its ability to serve as a substrate and be translocated by the uptake system. While this structure–activity relationship is consistent with the proposed conformational hypothesis discussed above, the addition of any new substituent introduces another variable, that is steric bulk. Accordingly, modeling studies (see Experimental) were conducted to assess the potential steric role of the added methyl group in binding and translocation. Two sets of comparisons were made using 4-Me-L-*trans*-2,4-PDC and the known inhibitors L-*trans*-2,4-PDC, L-*anti-endo*-3,4-MPDC, and 2,4-MPDC. As depicted in Figure 4, the minimized pseudo-axial conformation of 4-Me-L-*trans*-2,4-PDC (the more stable conformation by $\sim$1.5 kcal/mol, or approx. a 14:1 ratio, induced by the addition of the methyl group) is overlaid with the non-transportable inhibitor L-*anti-endo*-3,4-MPDC and the pseudo-axial conformation of L-*trans*-2,4-PDC, giving a close fit of overlaid atoms (Panel A, hydrogens are omitted for clarity,), RMS deviation (both carboxyl C's and the ammonium N) of 0.043 Å. When the same conformation of 4-Me-L-*trans*-2,4-PDC is similarly overlaid with the alternative substrate inhibitor 2,4-MPDC and the pseudo-equatorial conformation of L-*trans*-2,4-PDC, the result is a poor fit, RMS deviation of 0.331 Å. This suggests the more stable conformation of 4-Me-L-*trans*-2,4-PDC more closely resembles the non-transportable orientation. In a second modeling exercise (lower panels of Fig. 4), the molecular volumes of the 'folded' and 'extended' conformations of 4-Me-L-*trans*-2,4-PDC are compared with a composite substrate volume (depicted as the yellow mesh) generated from the additive molecular volumes of the known transportable (alternative substrates) inhibitors: L-glutamate, 2,4-MPDC, L-*trans*-2,4-PDC (axial, **A**/**A**′), and 1-aminocyclobutane-1,3-dicarboxylate (ACBD).[13] Previous modeling studies revealed that several known non-substrate inhibitors of EAAT2, dihydrokainate (DHK) and β-*threo*-benzyloxyaspartate (TBOA), exhibited substantial deviations from this volume.[13] In panels C and D of Figure 4, regions of the volumes of 4-Me-L-*trans*-2,4-PDC that protrude beyond of the composite substrate volume are represented by the green mesh. When the more stable pseudo-axial conformation of 4-Me-L-*trans*-2,4-PDC is overlaid onto the analogous conformation of L-*trans*-2,4-PDC within the confines of composite substrate volume, steric differences between the two appear to be primarily limited to the region occupied by the 4-methyl group (panel C). Interestingly, this region is in the same vicinity as the larger volumetric deviations observed with the other two non-substrate inhibitors, DHK and TBOA.[13] When the less favored conformation of 4-Me-L-*trans*-2,4-PDC is similarly compared with the pseudo-equatorial (**B**/**B**′) conformation of L-*trans*-2,4-PDC, there are a number of regions that fall outside the composite substrate volume (panel D). As there are presently no other analogues available with which to independently assess whether or not the observed volumetric differences produced by the methyl group are sufficient to alter substrate activity, we conclude that both the original conformational hypothesis and the steric refinement lead to the same result. Biasing the conformational preferences of the pyrrolidine ring away from extended towards folded conformation disfavors the requirement for efficient transport of the molecule, as does adding steric bulk in the 4-position. Thus, either of these factors, or both acting in concert, appears to significantly influence the ability of these analogues to act as substrates of EAAT2/GLT1.

Experimental

Thiazolium salt (2). The salt was prepared according to the procedure used by Monn and Valli[41] except for the use of acetone as a recrystallization solvent which afforded a white solid: mp 97–98 °C; IR ($CDCl_3$) υ_{max} 3330, 2986, 1748, 1589, 1469, 1396, 1375, 1347, 1225, 1096, 1068; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.12 (s, 1H), 5.65 (s, 2H), 5.2 (br s, 1H), 4.25 (q, 2H, $J = 7.2$ Hz), 3.65 (t, 2H, $J = 5.4$ Hz), 3.06 (t, 2H, $J = 5.4$ Hz), 2.39 (s, 3H), 1.26 (t, 3H, $J = 7.2$ Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.1, 159.1, 142.0, 135.3, 62.3, 59.6, 53.0, 29.4, 13.9, 11.1; HRMS $C_{10}H_{16}N_1S_1$-Br, calcd 230.0852 found 230.0851.

Imine diastereomers (4–6). (4*R*)-3-(2-Methyl-2-propenoyl)-4-(phenylmethyl)-2-oxazolidinone[27] (0.9877 g, 4.03 mmol) was added to 10 mL toluene and 4 Å

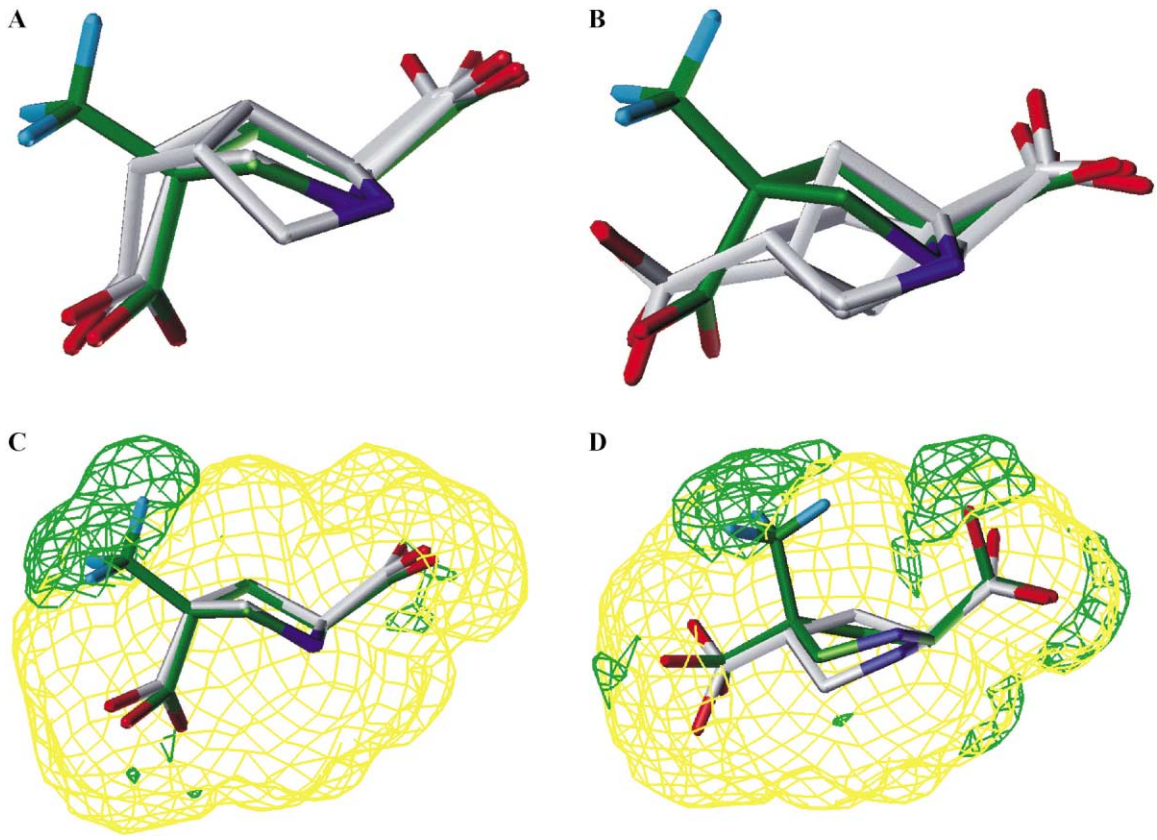


Figure 4. Overlay of: (A) the pseudo-axial, 'folded' conformation of 4-Me-L-*trans*-2,4-PDC (green) with pseudo-axial conformation of L-*trans*-2,4-PDC and L-*anti,endo*-3,4-MPDC yields a RMS = 0.043 Å; and (B) the pseudo-axial, 'folded' conformation of 4-Me-L-*trans*-2,4-PDC (green) with pseudo-equatorial, 'extended' conformation of L-*trans*-2,4-PDC and 2,4-MPDC yields an RMS = 0.331 Å. Overlay of: (C) the pseudo-axial, 'folded' conformations of 4-Me-L-*trans*-2,4-PDC and L-*trans*-2,4-PDC within the confines of a composite substrate shell (yellow mesh) representing added molecular volumes (see text); and (D) the pseudo-equatorial 'extended' conformations of 4-Me-L-*trans*-2,4-PDC and L-*trans*-2,4-PDC within the same composite substrate shell. Regions of the volumes of 4-Me-L-*trans*-2,4-PDC that protrude beyond of the composite substrate volume are represented by the green mesh. Modeling studies were carried out as described in the Experimental.

molecular sieves heated to reflux. Diisopropylethylamine (0.35 mL, 2.0 mmol) was added next followed by the slow dropwise addition of thiazolium salt **2** (0.3076 g, 0.992 mmol) in 4 mL CH_2Cl_2. An additional 0.6 mL CH_2Cl_2 was used to quantititatively transfer the solution, and the reaction mixture was heated at relux for 5 h. After cooling to room temperature the reaction mixture was diluted with Et_2O and partitioned with 0.1 N HCl. The organic layer was then washed with H_2O followed by brine and dried over $MgSO_4$. The crude cycloadducts were isolated in vacuo and then dissolved in 20 mL of CH_2Cl_2. The reaction flask was covered with aluminum foil, AgOTf (0.3757 g, 1.46 mmol) was added, and the reaction mixture was stirred for 1 h. Next, 40 mL of pH 7 phosphate buffer was added and the reaction mixture allowed to stir vigourously for 1 h. The solution was then washed with H_2O followed by brine. The H_2O layers were combined and back extracted with CH_2Cl_2. The combined CH_2Cl_2 layers were then dried over $MgSO_4$ and purified by flash chromatography (2:1 hexanes/ethyl acetate gradient eluted to 1:1 hexanes/ethyl acetate) to remove excess (4*R*)-3-(2-methyl-2-propenoyl)-4-(phenylmethyl)-2-oxazolidinone, giving an otherwise pure mixture of imine diastereomers in 59% combined yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (d, 0.11H, J = 2.4 Hz), 7.91 (d, 0.06H, J = 2.4 Hz), 7.89 (d, 0.83H, J = 2.4 Hz), 7.54–7.32 (m, 3H), 7.23–7.02 (m, 2H), 4.78 (ddd, 1H, J = 8.8, 6.0, 2.4 Hz), 4.74–4.69 (m, 1H), 4.35–4.13 (m, 4H), 3.27 (dd, 0.2H, J = 13.6, 2.8 Hz), 3.21 (dd, 0.8H, J = 13.4, 3.2 Hz), 2.87 (dd, 1H, J = 13.6, 9.2 Hz), 2.73 (dd, 1H, J = 14.4, 8.8 Hz), 2.28 (dd, 1H, J = 14, 6), 1.65 (s, 3H), 1.33 (t, 3H, J = 7.2 Hz); HRMS $C_{19}H_{22}N_2O_5$ + H calcd 359.1608, found 359.1607.

Major trifluoroacetamide (7). The imine mixture (0.0897 g, 0.250 mmol) was transferred to a 15 mL round bottomed flask, concentrated in vacuo, and redissolved in 5 mL CH_2Cl_2. Next, TFA (0.09 mL, 1.03 mmol) and Et_3SiH (0.16 mL, 1.00 mmol) were added to the reaction mixture. After stirring for 24 h, the reaction mixture was diluted with 70 mL of Et_2O and washed 3 × 20 mL H_2O. The aqueous layers were combined, concentrated in vacuo and azeotroped with $PhCH_3$ to give a 76% combined yield of the TFA salts, which (0.0896 g, 0.189 mmol) were transferred to a 10 mL round bottomed flask, concentrated in vacuo, redissolved in 2 mL of CH_2Cl_2 and cooled to 0 °C. Next, TFAA (0.03 mL, 0.212 mmol) and pyridine (0.03 mL, 0.371 mmol) were added to 2 mL CH_2Cl_2 at 0 °C, into which was cannulated the TFA salt mixture.The reaction mixture stirred for 1.5 h, and then diluted with 70 mL Et_2O, washed with 2 × 20 mL H_2O followed by brine, and dried over $MgSO_4$. After concentration in vacuo the crude trifluoroacetamides were separated by flash chromatography (1:1 Et_2O/hexanes),

providing an 83:11:6 ratio of the three diastereomers in a combined yield of 79%. **Major trifluoroacetamide** (**7**, 66% yield): IR (neat) 3081–2852, 1786, 1681, 1452, 1386, 1352, 1324, 1214, 1105, 1048, 981, 914, 757, 700 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, major rotamer) δ 7.72–7.34 (m, 3H), 7.25–7.23 (m, 2H), 4.79–4.70 (m, 2H), 4.51 (app t, 1H, $J=17.2$ Hz), 4.39–4.26 (m, 4H), 3.64 (d, 1H, $J=12.1$ Hz), 3.32–3.24 (m, 2H), 2.87 (dd, 1H, 1, $J=13.4$, 9.4 Hz), 2.00 (dd, 1H, $J=13.7$, 8.8 Hz), 1.64 (s, 3H), 1.33 (t, 3H, $J=11.0$ Hz); ^1H NMR (400 MHz, DMSO-d_6, 438K) δ 7.35–7.22 (m, 5H), 4.85–4.79 (m, 1H),

(2*R*,4*S*)-4-Methyl-2,4-PDC (8). The major trifluoroacetamide (**7**) (0.1391 g, 0.305 mmol) was dissolved in 6 mL of THF and cooled to 0 °C. TBAH (0.5 mL, 1.91 mmol) was added dropwise, and the reaction mixture was allowed to warm to room temperature and stirred for 40 h. The volatiles were evaporated, H_2O was used to transfer the residue to a separatory funnel, and the aqueous layer was extracted three times with CH_2Cl_2. The aqueous layer was then concentrated in vacuo and chased three times with toluene. The resultant residue was dissolved in a minimal amount of H_2O and loaded onto a column of 3.6 g of AG1X2 acetate resin. The column was washed with eight column volumes of H_2O, and then the product was eluted with 1 N HOAc to give a 61% crude yield. The product-containing fractions were concentrated in vacuo, chased with H_2O followed by toluene, and dried under high vacuum. The white solid obtained was then triturated with MeOH to give 44% of the title compound: mp (dec) 255–256 °C; $[\alpha]_D^{25}$ −54.3° (*c* 0.49, H_2O), lit.[18] −54.8); IR (KBr) br 3422, 3146, 3055, br 1933, 1711, 1617, 1456, 1400, 1344, 1272, 1228, 1156, 1044, 911 cm^{-1}; ^1H NMR (400 MHz, D_2O calibrated to internal HOD at 4.80) δ 4.29 (app t, 1H, $J=8.8$ Hz), 3.82 (d, 1H, $J=12.0$ Hz), 3.26 (d, 1H, $J=12.0$ Hz), 2.83 (dd, 1H, $J=13.6$, 8.4 Hz), 2.07 (dd, 1H, $J=13.6$, 9.2 Hz), 1.39 (s, 3H); ^{13}C NMR (125 MHz, D_2O/CD_3CN calibrated to internal $CHCD_2CN$ at 118.20) δ 177.4, 172.5, 60.0, 52.5, 48.6, 38.7, 19.9; HRMS C7H11N1O4+H calcd 174.0766 found 174.0762.

Synaptosomal transport and exchange

Rat cortical synaptosomes were prepared essentially by the procedure of Booth and Clark,[32] using a discontinuous Ficoll/sucrose gradient as described previously.[13] Uptake of D-[^{3}H]aspartate was measured essentially by the method of Kuhar and Zarbin.[33] Synaptosomes were suspended in assay buffer (10 mM Tris–acetate, 128 mM NaCl, 10 mM D-glucose, 5 mM KCl, 1.5 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.4) at a concentration of 0.2 mg of protein/mL. After a 5-min preincubation at 25 °C, uptake assays were initiated by the addition of D-[^{3}H]aspartate (1–20 μM) to the synaptosomes. In the inhibition experiments, D-[^{3}H]aspartate and inhibitor were added simultaneously. After a 2-min incubation at 25 °C, the assay was rapidly quenched by the addition of 4 mL of ice-cold assay buffer. The suspension was quickly filtered through Whatman GF/F micro-fiber filters and rinsed with an additional 4 mL of ice-cold assay buffer. Filters were transferred to scintillation fluid (National Diagnostics, Atlanta, GA, USA) and the retained radioactivity was quantified by liquid scintillation counting. Within each experiment, uptake rates were determined in duplicate. Nonspecific uptake and/or binding was corrected for by subtracting the amount of D-[^{3}H]aspartate accumulated at 4 °C. Previous studies demonstrated that under these conditions, uptake was linear with respect to both time and protein content (data not shown). Protein levels were determined by the bicinchoninic acid assay (Pierce, Rockford, IL, USA).

Heteroexchange-mediated release of D-[^{3}H]aspartate from synaptosomes was quantified as described by Koch.[13] Essentially, synaptosomes were suspended in assay buffer at a concentration of 0.45 mg of protein/mL. Aliquots (10 mL) of this suspension were allowed to incubate with 2.5 μM D-[^{3}H]aspartate for 15 min at 25 °C. Synaptosomes containing the [^{3}H]-substrates were reisolated by centrifugation (28,150*g*, 20 min, 4 °C), rinsed, resuspended to 1 mg of protein/mL of ice-cold assay buffer, and maintained on ice. The total content of radiolabel in the synaptosomes was determined by adding 100 μL of the suspension to 2.9 mL of ice-cold assay buffer and immediately vacuum filtering as described above. Radioactivity present in the synaptosomes was quantified by liquid scintillation counting. This value was determined at the beginning, middle, and end of each experiment to ensure that the synaptosomal content of D-[^{3}H]aspartate did not change during maintenance on ice and that different preparations contained similar levels of each of the radiolabeled substrates. Assays quantifying the efflux of D-[^{3}H]aspartate were initiated by adding a 100-μL aliquot of synaptosomes containing the radiolabeled compounds to 2.9 mL of assay buffer pre-equilibrated and maintained at 37 °C. In those experiments in which heteroexchange was examined, the indicated compounds were included in the assay buffer. Assays were terminated 1–5 min later by the addition of ice-cold assay buffer. The radioactivity remaining in the synaptosomes was determined as described above.

Computational analysis

The molecular modeling was performed on a Silicon Graphics Iris computer using the Sybyl 6.2 software by Tripos Inc. Minimization parameters used were: Tripos force field, dielectric constant = 80, Pullman charge calculation, and Powell minimization method. The minimized (local minima) structures were overlaid using a three-point comparison: the two carboxylate carbons and the nitrogen. The volume comparisons (green and yellow mesh) are representations of the additive Van der Waals radii of the substrate molecules (yellow) subtracted from the 2(S),4(*R*)-4-Me-2,4-PDC Van der Waals radius (green).

Acknowledgements

The authors gratefully acknowledge that this work was supported in part by the NIH: NINDS NS30570

(R.J.B.), NS27600 (A.R.C.), NS10156 (C.S.E.) and NCRR RR15583 (R.J.B., C.S.E.). The authors also wish to thank M. P. Kavanaugh, C. M. Thompson, and J. M. Gerdes for their insightful discussions.

References and Notes

1. Monaghan, D. T.; Bridges, R. J.; Cotman, C. W. *Annu. Rev. Pharmacol. Toxicol.* **1989**, *29*, 365.
2. Monaghan, D. T.; Howson, P. A.; Jane, D. E.; Bridges, R. J. In *Biological Psychiatry*; D'haenen, H., den Boer, J. A., Westenberg, H., Willner, P., Eds.; John Wiley and Sons: Chichester, UK. In press.
3. Meldrum, B. S. *J. Nutr.* **2000**, *130*, 1007S.
4. Zipfel, G. J.; Babcock, D. J.; Choi, D. W. *J. Neurotrauma* **2000**, *17*, 857.
5. Gegelashvili, G.; Schousboe, A. *Mol. Pharmacol.* **1997**, *52*, 6.
6. Seal, R. P.; Amara, S. G. *Annu. Rev. Pharmacol. Toxicol.* **1999**, *39*, 431.
7. Takahashi, M.; Billups, B.; Rossi, D.; Sarantis, M.; Hamann, M.; Atwell, D. *J. Exp. Biol.* **1997**, *200*, 401.
8. Danbolt, N. C. *Prog. Neurobiol.* **2001**, *65*, 1.
9. Robinson, M. B. *Neurochem. Int.* **1998**, *33*, 479.
10. Chen, W.; Aoki, C.; Mahadomrongkul, V.; Gruber, C. E.; Wang, G. J.; Blitzbau, R.; Irwin, N.; Rosenberg, P. A. *J. Neurosci.* **2002**, *22*, 2142.
11. Schmitt, A.; Asan, E.; Lesch, K. P.; Kugler, P. *Neuroscience* **2002**, *109*, 45.
12. Bridges, R. J.; Stanley, M. S.; Anderson, M. W.; Cotman, C. W.; Chamberlin, A. R. *J. Med. Chem.* **1991**, *34*, 717.
13. Koch, H. P.; Kavanaugh, M. P.; Esslinger, C. S.; Zerangue, N.; Humphrey, J. M.; Amara, S. G.; Chamberlin, A. R.; Bridges, R. J. *Mol. Pharmacol.* **1999**, *56*, 1095.
14. Bridges, R. J.; Lovering, F. E.; Humphrey, J. M.; Stanley, M. S.; Blakely, T. N.; Cristofaro, M. F.; Chamberlin, A. R. *Bioorg. Med. Clem. Lett.* **1993**, *3*, 115.
15. Bridges, R. J.; Lovering, F. E.; Koch, H.; Cotman, C. W.; Chamberlin, A. R. *Neurosci. Lett.* **1994**, *174*, 193.
16. Esslinger, C.; Koch, H.; Kavanaugh, M.; Phillips, D.; Chamberlin, A.; Thompson, C.; Bridges, R. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3101.
17. Arriza, J. L.; Fairman, W. A.; Wadiche, J. I. G. H. M.; Kavanaugh, M. P.; Amara, S. G. *J. Neurosci.* **1994**, *14*, 5559.
18. Hull, C. E. Master's Thesis, Department of Chemistry, University of California Irvine, Irvine, CA, 1996.
19. In *1,3-Dipolar Cycloaddition Chemistry*; Padwa, A., Ed.; Wiley-Interscience: City 1984; Vols. 1 and 2.
20. Tsuge, O.; Kanemasa, S. In *Advances in Heterocyclic Chemistry*; Academic: Wiley-Interscience, New York, 1989; Vol. 49, p 231.
21. DeSchong, P.; Kell, D. A.; Sidler, D. R. *J. Org. Chem.* **1985**, *50*, 2309.
22. Husinec, S.; Porter, A. E.; Roberts, J. S.; Strachan, C. H. *J. Chem. Soc., Perkins Trans 1* **1984**, 2517.
23. Tsuge, O.; Kanemasa, S.; Masayuki, O.; Yorozu, K.; Takenaka, S.; Ueno, K. *Bull. Chem. Soc. Jpn.* **1987**, *60*, 4067.
24. Collado, I.; Ezquerra, J.; Mateo, A. I. C. P.; Rubio, A. *J. Org. Chem.* **1999**, *64*, 4304.
25. Monn, J. A.; Valli, M. J. *J. Org. Chem.* **1994**, *59*, 2773.
26. Desimoni, G.; Faita, G.; Mella, M.; Righetti, P.; Zema, M. *Tetrahedron* **1999**, *55*, 8509.
27. Karlsson, S.; Han, F.; Hogberg, H. E.; Caldirola, P. *Tetrahedron: Asymmetry* **1999**, *10*, 2605.
28. Hosokawa, T.; Yamanaka, T.; Itotani, M.; Murahashi, F. *J. Org. Chem.* **1995**, *60*, 6159.
29. Craig, D.; Munasinghe, R. N. *Tet. Lett.* **1992**, *33*, 663.
30. Luhowy, R. R.; Cieciuch, R. F.; Meneghini, F. *Tetrahedron Lett.* **1973**, 1285.
31. Koch, H. P.; Chamberlin, A. R.; Bridges, R. J. *Mol. Pharmacol.* **1999**, *55*, 1044.
32. Booth, A. G.; Kenny, A. J. *Biochem. J.* **1974**, *142*, 575.
33. Kuhar, M. J.; Zarbin, M. *J. Neurochem.* **1978**, *31*, 251.