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Syntheses of Optically Pure β-Hydroxyaspartate Derivatives as Glutamate Transporter Blockers

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Abstract—DL-*threo*-β-Benzyloxyaspartate (DL-TBOA) is a non-transportable blocker of the glutamate transporters that serves as an indispensable tool for the investigation of the physiological roles of the transporters. To examine the precise interaction between a blocker and the transporters, we synthesized the optically pure isomers (L- and D-TBOA) and its *erythro*-isomers. L-TBOA is the most potent blocker for the human excitatory amino acid transporters (EAAT1–3), while D-TBOA revealed a difference in the pharmacophores between EAAT1 and EAAT3. We also synthesized the substituent variants (methyl or naphthylmethyl derivatives) of L-TBOA. The results obtained here suggest that bulky substituents are crucial for non-transportable blockers.

L-Glutamate acts as the principal excitatory neurotransmitter in the mammalian central nervous systems (CNS). The extracellular glutamate concentrations are tightly controlled by a transport system that limits the activation of receptors during signalling and maintains below excitotoxic level.[1] It has been reported that glutamate transporters transport Na^+ ions and one proton with glutamate while one K^+ ion is counter-transported.[2] Therefore, net inward movement of positive charges into the cell is observed as a transport current. A number of pharmacological agents have been shown to inhibit glutamate transport but most of them indeed act as competitive substrates, which was demonstrated by their ability to induce transport currents and heteroexchange with intracellular glutamate.[3] Therefore, non-transportable blockers are indispensable tools for the investigation of the physiological roles of glutamate transporters.

Recently, we reported that some derivatives of DL-*threo*-β-hydroxyaspartate (DL-THA) acted as blockers.[4,5] Among them, DL-*threo*-β-benzyloxyaspartate (DL-TBOA) was the most potent blocker for the human excitatory amino acid transporters, EAAT1 and EAAT2.[5] In the previous paper, however, we have characterized a racemic mixture of TBOA. Optically pure compounds are needed for the precise discussion of structure–activity relationships. L-Glutamate is a high affinity substrate, whereas D-glutamate is poorly transported. On the other hand, both L- and D-aspartate are excellent substrates. It would be interesting to know whether TBOA shows such stereoselectivity. We describe here the syntheses and characterization of the optically pure TBOA and its *erythro*-derivatives and some new substituent variants.

Chemistry

We synthesized optically pure hydroxyaspartate derivatives by the route shown in Scheme 1. In accordance with the literature precedence,[6] addition of vinyl magnesiumbromide to (*R*)-Garner aldehyde (**1**)[7] gave *erythro* (*anti*)-**2** with a moderate selectivity (*threo*:*erythro* = 1:3) whereas reaction with vinylzinc chloride showed the opposite diastereoselectivity (*threo*:*erythro* = 6:1). A diastereomixture of methyl ester **4** was prepared by the following steps; (1) benzylation of **2**, (2) oxidative cleavage of the vinyl group of **3**, (3) further oxidation to carboxylic acid, and (4) esterification. Separation of diastereomers by a flash column chromatography followed by removal of the acetonide group provided pure *threo*-**5** and *erythro*-**5**. Jones oxidation of the hydroxyl group of *threo*-**5** gave monomethyl ester **6**. Deprotection of *threo*-**6** was accomplished without epimerization to give L-

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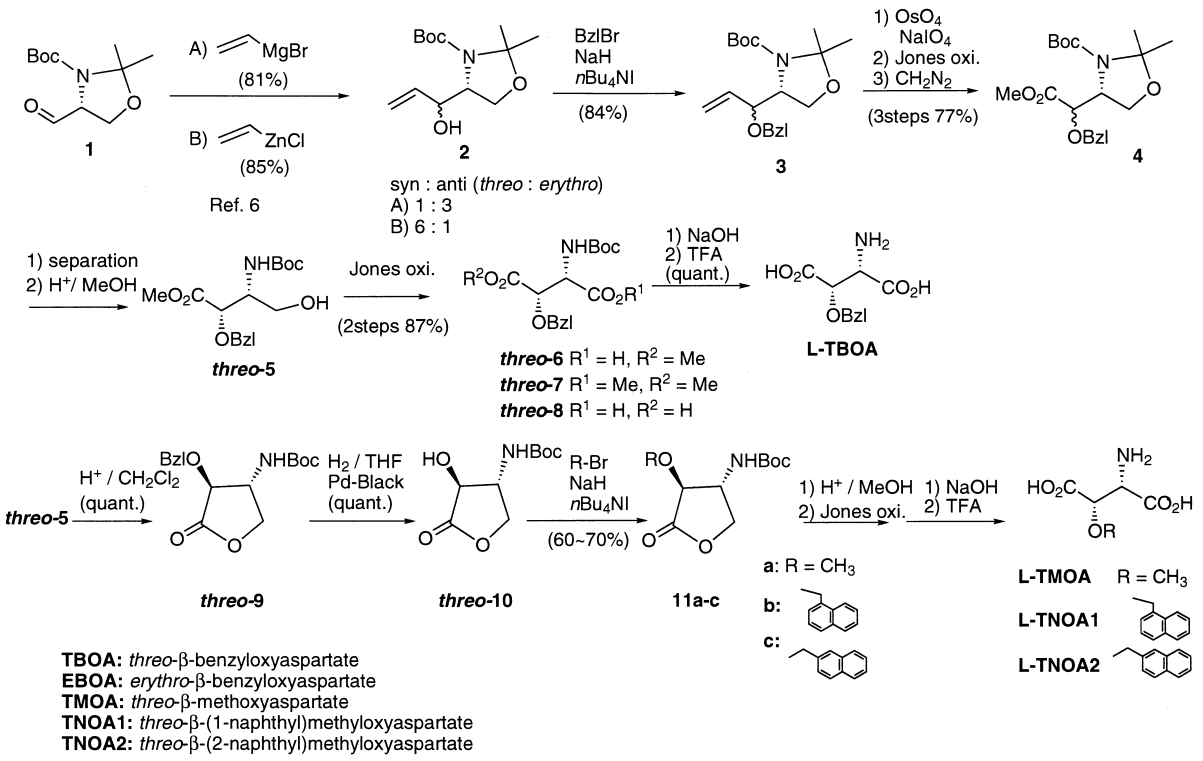

Scheme 1.

TBOA.[8–10] L-*erythro*-β-Benzyloxyaspartate (L-EBOA) was prepared from *erythro*-**5** in a similar manner. D-TBOA and D-EBOA were synthesized from (*S*)-**1**. Introduction of other substituents was performed from the lactone **10** because it would be a useful common intermediate for various analogues. The benzyl group of *threo*-**9** was removed by hydrogenation and the hydroxyl group of *threo*-**10** was substituted by methyl, 1-, or 2-naphthylmethyl group (**11a**–**c**). Since *trans*-substituted lactone was thermodynamically more stable than *cis*-substituted lactone, etherification of *erythro*-**10** also gave *trans*-products (**11a**–**c**) predominantly (3:1–6:1). Ring opening of lactones followed by oxidation and deprotection provided the substituent variants of L-TBOA.

Inhibition of Glutamate Uptake

Inhibition of radiolabeled glutamate uptake was measured in COS-1 cells transiently expressing EAAT1–3.[5] EAAT-transfected cells accumulated five to ninefold more [^{14}C] glutamate than vector-transfected cells. All four isomers of benzyloxyaspartate markedly inhibited [^{14}C]glutamate uptake in COS-1 cells (Fig. 1). Among them, L-TBOA showed the most potent inhibitory action for each EAAT subtype. *threo*-Isomers were more potent than *erythro*-isomers and L-isomers were more potent than D-isomers. The IC_{50} values are summarized in Table 1. Concerning the substituent variants, L-TMOA, L-TNOA1, and L-TNOA2 showed inhibitory activities almost comparable to that of L-TBOA (Table 1).

Discrimination Between Blockers and Substrates

We performed electrophysiological analyses on *Xenopus* oocytes expressing EAAT1–3 to determine whether each compound acts as a blocker or a substrate.[4,5] None of the four benzyl derivatives elicited a detectable current in voltage-clamped oocytes but all of them did reduce

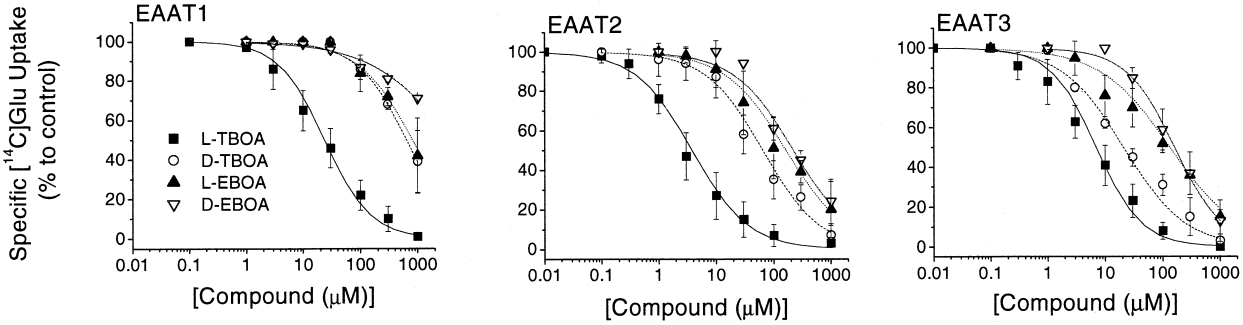

Figure 1. All isomers of benzyloxyaspartate inhibited uptake of [^{14}C]glutamate in COS-1 cells expressing EAAT1–3. L-TBOA is the most potent for all EAAT subtypes. Values are presented as mean ± S.E.M. of at least three determinations.

Table 1. IC_{50} values of THA derivatives for blocking [^{14}C]glutamate uptake in COS-1 cells expressing EAAT1–3

	EAAT1 (IC_{50} μM)	EAAT2 (IC_{50} μM)	EAAT3 (IC_{50} μM)
DL-TBOA	48±4.1	7.0±1.1	8.0±1.9
L-TBOA	23±4.4	3.8±1.0	7.0±1.5
D-TBOA	637±175	64±14	21±2.3
L-EBOA	817±455	164±42	134±14
D-EBOA	>1000	221±39	161±41
L-TMOA	69±13	2.0±0.34	8.4±1.9
L-TNOA1	15±2.4	1.3±0.44	4.8±0.79
L-TNOA2	16±3.4	2.2±0.45	6.5±1.6

the glutamate (100 μM)-induced inward currents, confirming they act as blockers for EAAT1–3. Among the substituent variants, L-TMOA proved to be a substrate for EAAT1 and EAAT3, while being a blocker for EAAT2. The other substituent variants are blockers for EAAT1–3. Relative transport currents obtained for each compound (100 μM) in the absence or presence of 100 μM glutamate are shown in Table 2.

Effects on Glutamate Receptors

Next, we analyzed the activities of our compounds on the ionotropic glutamate receptors by binding assay using rat brain synaptic membranes. *N*-Methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (KA) receptors were labeled with [^{3}H]CGP 39653 [DL-(*E*)-2-amino-4-propyl-5-phosphono-3-pentenoic acid], [^{3}H]AMPA, and [^{3}H]KA, respectively.[5] L-TBOA showed a low affinity for AMPA and KA-type receptors (IC_{50} >1 mM). However, its affinity for NMDA receptors was relatively high (IC_{50} = 65±5.4 μM).[11] In order to examine whether L-TBOA activates ionotropic receptors, we measured the inward currents in oocytes injected with rat whole brain mRNA. At 100 μM, none of the compounds elicited inward currents nor did they antagonize NMDA-evoked inward currents. In addition, none of the compounds at 1 mM showed agonist or antagonist activity on the metabotropic glutamate receptors (mGluRs) stably expressed in CHO cells.[5]

Discussion

L-*threo*-β-Hydroxyaspartate derivatives possessing bulky substituents, L-TBOA, L-TNOA1 and L-TNOA2, are found to be potent blockers for all transporter subtypes. On the other hand, L-TMOA acts as a substrate for EAAT1 and EAAT3, but a blocker for EAAT2, like (2*S*,4*R*)-4-methylglutamate (SYM 2081)[12] and L-*anti-endo*-3,4-methanopyrrolidine dicarboxylate (MPDC).[13,14] According to our [^{14}C]Glu uptake assays, L-TMOA seems to have almost the same affinity for the binding site of transporters as that of L-TBOA. Based on our results, it is likely that the bulky substituent in L-TBOA inhibits the translocation step of the transporters, whereas a smaller substituent would be sufficient for such an inhibition on EAAT2.

The synthesis of optically pure L-TBOA isomers allowed us to investigate the importance of the bulky substituent's spatial orientation. It has been proposed that the active conformation of glutamate to transporters is the folded form and that of aspartate is the extended form.[5,15] Recently Bridges et al. proposed a pharmacophore model of blockers using the structure of MPDC as a template.[13] L-TBOA appears to fit with this pharmacophore model, in which the aspartate backbone of L-TBOA takes an extended form. Inspection of molecular models suggested that the bulky substituents of L-TBOA and dihydrokainate (DHKA), a selective EAAT2 blocker, have large overlaps (Fig. 2).[16] When

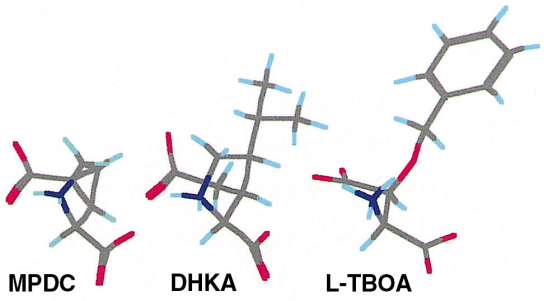

Figure 2. Molecular models of MPDC, DHKA, and L-TBOA.[16]

Table 2. Potency of the hydroxyaspartate derivatives to induce a transport current in the absence or presence of L-Glu (100 μM) in oocytes expressing EAAT1-3. The results are expressed relative to the current obtained with 100 μM L-Glu in the same cells (mean ± S.D., n = 3)

	EAAT1 I/Icont (%)		EAAT2 I/Icont (%)		EAAT3 I/Icont (%)	
	Compound (100 μM)	+ Glu (100 μM)	Compound (100 μM)	+ Glu (100 μM)	Compound (100 μM)	+ Glu (100 μM)
DL-TBOA	0	30.1±3.9	0	0	0	12.7±1.5
L-TBOA	0	14.5±0	0	0	0	8.0±2.7
D-TBOA	0	84.7±0.7	0	0	0	27.6±1.5
L-EBOA	0	77.0±4.0	0	0	0	64.4±2.7
D-EBOA	0	99.6±5.1	0	60.7±3.6	0	83.1±4.2
L-TMOA	4.7±1.3	33.3±4.2	0	0	5.9±0.1	25.4±2.0
L-TNOA1	0	10.0±2.0	0	0	0	13.0±0.1
L-TNOA2	0	18.1±3.6	0	0	0	29.8±4.1

the functional groups of the other stereoisomers (D- or *erythro*-isomers) fit with the pharmacophore, their substituents can not occupy the same space as that of the bulky substituent of DHKA or L-TBOA.

So far, it is well known that the pharmacological profiles of EAAT1 and EAAT3 are very similar while EAAT2 shows different selectivity from these subtypes. Interestingly, D-TBOA shows a major loss of activity on EAAT1 (30-fold less potent than L-TBOA), while it remains substantially active on EAAT3 (only threefold less potent than L-TBOA). EAAT3 is more tolerant to the D-isomers.

In summary, the optically pure isomers provided precise information on the structure–activity relationship of glutamate transporter blockers. These compounds can be used as lead compounds to design new blockers with greater subtype selectivity.

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8. Purity of each isomer of **6** was confirmed by conversion to dimethyl ester **7**. No peaks corresponding to methyl esters of the other isomer were detected by 400 MHz NMR (3.61 and 3.77 ppm for *threo*-**7**; 3.73 and 3.79 ppm for *erythro*-**7**). However, deprotection of the dimethyl ester *threo*-**7** was accompanied with the epimerization (~30%).
9. Enantiomeric purity (>95%) of each compound was confirmed by chiral HPLC [column; CROWNPAK CR(+) or CR(−), Daicel Chem. Ind. Ltd (Japan), eluent; 1% aqueous $HClO_4$].
10. Analytical data for L-TBOA: mp 172–175 °C (dec.); $[\alpha]_D$ −473.6° (*c* 0.68, 1 N HCl); ^1H NMR (1 N DCl/D_2O, 400 MHz): δ 4.57 (d, 1H, $J=2.5$ Hz), 4.62 (d, 1H, $J=11.5$ Hz), 4.71 (d, 1H, $J=2.5$ Hz), 4.87 (d, 1H, $J=11.5$ Hz), 7.45 (m, 5H); ^{13}C NMR (1 N DCl/D_2O, 100 MHz): δ 55.4, 74.5, 75.1, 129.6, 129.7, 129.8, 136.8, 169.4, 172.5; HRMS (FAB) *m/z* calcd for $C_{11}H_{14}O_5N$ $(M+H)^+$ 240.0871, found 240.0863.
11. The IC_{50} values of both L- and D-TBOA were lower than the reported value for DL-TBOA (ref 5), since [^{3}H]CGP 39653 (2 nM) was used as a radioligand instead of [^{3}H]CGS 19755. IC_{50} values for [^{3}H]CGP 39653 binding were as follows (μM); D-TBOA 181±23, L-EBOA 680±58, D-EBOA 26±1.3, L-TMOA 511±17, L-TNOA1 151±20, L-TNOA2 194±7.0. L-TMOA inhibited [^{3}H]KA binding (48±5.6 μM) and [^{3}H]AMPA binding (222±42 μM). The other derivatives showed very low affinity (>500 μM) for KA and AMPA receptors.
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14. In our study, MPDC (100 μM) also evoked the transport currents in EAAT1 (16% to control) and EAAT3 (8%) although it blocked EAAT2 as shown in ref 13b.
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16. Molecular modelings were performed by using QUANTA/CHARMm system (Molecular Simulations Inc.). The CHARMm energy minimization with distance-dependent dielectric term (ε=80) was applied. See Shimamoto, K.; Ohfune, Y. *J. Med. Chem.* **1996**, *39*, 407. In this reference we have also reported the preparation of MPDC as CMP-III.