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Tetrahedron: Asymmetry
Tetrahedron: *Asymmetry* 19 (2008) 867–875

Synthesis of enantiomerically pure HIP-A and HIP-B and investigation of their activity as inhibitors of excitatory amino acid transporters

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Received 15 February 2008; accepted 3 March 2008

Abstract—The present report deals with the synthesis of the two couples of enantiomers (+)-(3a*S*,4*R*,6a*S*)-/(−)-(3a*R*,4*S*,6a*R*)-3-hydroxy-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]isoxazole-4-carboxylic acid [(+)-**HIP-A** and (−)-**HIP-A**] and (+)-(3a*S*,6*S*,6a*S*)-/(−)-(3a*R*,6*R*,6a*R*)-3-hydroxy-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]isoxazole-6-carboxylic acid [(+)-**HIP-B** and (−)-**HIP-B**], and the investigation of their inhibitory activity at EAATs. When tested in a [^{3}H]D-aspartate uptake assay on native EAATs present in rat brain synaptosomal fractions, (−)-**HIP-A** and (+)-**HIP-B** turned out to be considerably more potent than their enantiomers; in particular, (−)-**HIP-A** showed an eudismic ratio (ER) higher than 100. Molecular modeling investigations gave clues with regard to the key amino acid residues involved in the binding with our inhibitors and provided explanations for the observed stereoselectivity.

1. Introduction

L-Glutamate (Glu) is the most abundant excitatory neurotransmitter in the brain; it is involved in physiological functions of outmost importance, such as synaptic development and plasticity, learning and memory, cognition, pain, and nociception.[1,2] An abnormal increase in the extracellular concentration of Glu induces toxicity (excitotoxicity) leading to neuronal cell death.

Under physiological conditions, the concentration of Glu in the synaptic cleft is kept at a low level by a number of plasma membrane proteins acting as EAATs.[3] So far, five different human EAATs (EAAT1-5) have been cloned. Subtypes EAAT1, EAAT2, and EAAT3 have been identified in the human motor cortex[4] and correspond to the rat homologues GLAST, [5] GLT1,[6] and EAAC1,[7] respectively, whereas subtypes EAAT4 and EAAT5 are expressed almost exclusively in cerebellar Purkinje cells[8,9] and retina,[10] respectively. The available data suggest that the removal of Glu from the synaptic cleft is mainly performed by astroglial GLT1/EAAT2 and GLAST/EAAT1 transporters, which are the most abundant EAAT subtypes in the forebrain and cerebellum, respectively.[11,12]

The transport of Glu and/or of glutamine, its precursor,[13] across plasma membranes, is crucial for both silencing the glutamatergic synapses and in the process of neurotransmitter recycling.[14,15] Since glutamatergic synapses are involved in a large number of brain functions, it is not surprising that an imbalance of EAATs could cause serious neurological and mental disorders (e.g., amyotrophic lateral sclerosis, schizophrenia).[12,16–22] It is worth mentioning that under pathological conditions in which energy levels fall and the transmembrane gradient of Na^+ collapses (e.g., ischemia, neurotrauma), EAATs release additional Glu through the reversed mode of operation, thus contributing to neuronal cell death.[23,24] This scenario supports the idea that the blockade of EAATs by non-transportable inhibitors (blockers) could be a useful therapeutic approach to prevent glutamate release and neuronal death after cerebral ischemia.[25–27] Unfortunately, EAAT blockers are likely to be neurotoxic under physiological conditions.[28,29]

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doi:10.1016/j.tetasy.2008.03.001

In a previous investigation,[30] we characterized the pharmacological properties of two conformationally constrained aspartate and glutamate analogues (Fig. 1), (±)-3-hydroxy-4,5,6,6a-tetrahydro-3a*H*-pyrrolo[3,4-*d*]isoxazole-4-carboxylic acid (±)-**HIP-A** and (±)-3-hydroxy-4,5,6,6a-tetrahydro-3a*H*-pyrrolo[3,4-*d*]isoxazole-6-carboxylic acid (±)-**HIP-B**, at the native EAATs present in a crude synaptosomal fraction of the rat brain cortex, which behave as a GLT1-like subtype.[31,32] The amino acids (±)-**HIP-A** and (±)-**HIP-B** turned out to be potent blockers of Glu uptake with IC_{50} values (17–18 μM) very similar to those of **TBOA**, a reference compound of the EAATs non-transportable inhibitors.[30] Unexpectedly, (±)-**HIP-A** and (±)-**HIP-B**, in contrast to **TBOA**, inhibited Glu-induced [^{3}H]D-aspartate release at a concentration lower than that required to block [^{3}H]Glu uptake. Such a peculiar feature identified (±)-**HIP-A** and (±)-**HIP-B** as lead compounds in the search for drugs capable of counteracting ischemia-induced neuronal degeneration.

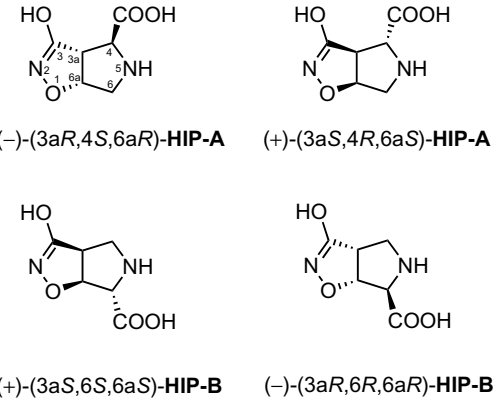


Figure 1. Target compounds.

Since EAATs show stereoselective anomalies, that is, they transport both L- and D-aspartate and solely L-glutamate,[33,34] we planned to test the single enantiomers of **HIP-A** and **HIP-B**, which are structurally related to aspartate and glutamate, respectively.

Herein, we report the synthesis of the four stereoisomers (+)-**HIP-A**, (−)-**HIP-A**, (+)-**HIP-B**, and (−)-**HIP-B** (Fig. 1) and their effectiveness in inhibiting the uptake mediated by native rat EAATs.

Molecular modeling studies were carried out in order to single out the amino acid residues involved in the interaction with our ligands and to rationalize the biological results.

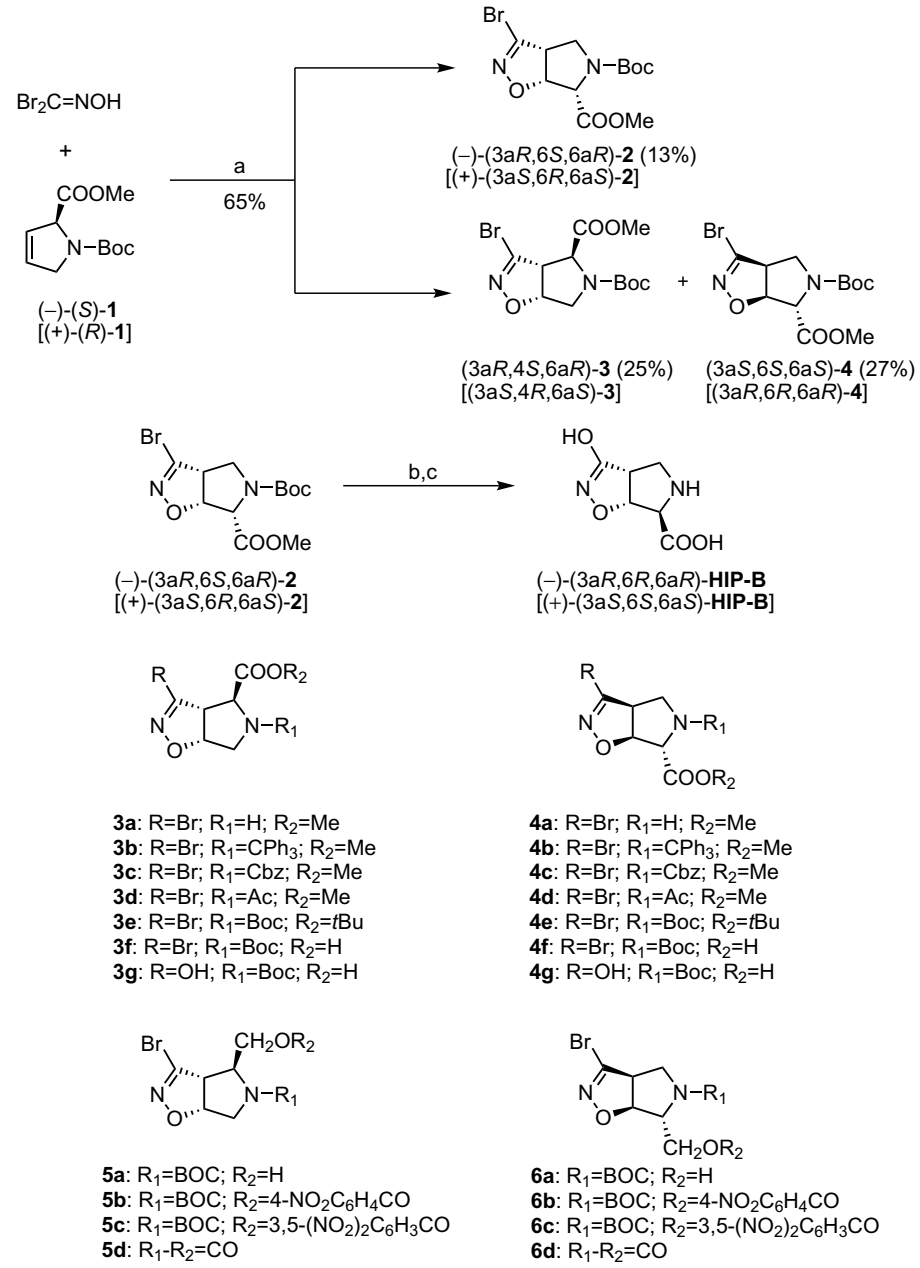
2. Chemistry

In the first attempt to prepare target enantiomeric pairs (+)-**HIP-A**/(−)-**HIP-A** and (+)-**HIP-B**/(−)-**HIP-B**, we used the strategy applied to the synthesis of the corresponding racemates.[35] Bromonitrile oxide was reacted with (*S*)-*N*-Boc-3,4-didehydroproline methyl ester **1**[36] [or its enantiomer (*R*)[37]] to yield three out of the four possible stereoisomers in comparable amounts (Scheme 1). Column chromatography of the reaction mixture gave two fractions containing pure **2** and a mixture of **3** and **4**. Unfortunately, at variance with the mixture of racemates, the fraction containing **3** and **4** turned out to be a viscous oil and, consequently, attempts to split the mixture by fractional crystallization failed. As shown in Scheme 1, we also performed several modifications at the nitrogen, at the 3-bromo group, and at the ester function. At first, we modified the substituent at the nitrogen. The Boc moiety of **3** and **4** was removed and then replaced by trityl, benzyloxycarbonyl, and acetyl groups. All of the modifications invariably gave a single spot by TLC. Subsequently, we turned our attention to the ester moiety. The methyl ester of **3** and **4** was either transformed into the *tert*-butyl esters **3e** and **4e** or hydrolyzed to the corresponding acids **3f** and **4f**, and **3g** and **4g**. These modifications were also unsuccessful. The ester moiety was then reduced to the corresponding alcohols **5a** and **6a** and transformed into either benzoates **5b** and **6b**, and **5c** and **6c** or cyclic carbamates **5d** and **6d**. Among the modifications described, none turned out to be productive.

To sum up, all the performed experiments allowed the isolation of the minor stereoisomer (−)-**2** [or (+)-**2**] only, which could be transformed into the final amino acid (−)-**HIP-B** [or (+)-**HIP-B**] in 6% overall yield, following the reaction sequence reported in Scheme 1. Conversely, we were unable to isolate regioisomer **3** and, consequently, we could not prepare the couple of enantiomers of **HIP-A**. It is worth pointing out that by using (*S*)-*N*-Boc-3,4-didehydroproline methyl ester (*S*)-**1** as the dipolarophile, we obtained (−)-**HIP-B**, which is characterized by the (*R*)-configuration at the corresponding stereocenter; a complete inversion of configuration occurred when treating (−)-**2** or (+)-**2** with sodium hydroxide at 60 °C. Since we aimed at preparing the pair of enantiomers (−)-**HIP-A**/(+)-**HIP-A** and also at improving the yield of (−)-**HIP-B** and (+)-**HIP-B**, we considered the opportunity to increase the difference among diastereoisomers **3** and **4** by adding a chiral auxiliary. For such a purpose, the mixture of acids (3a*R*,4*S*,6a*R*)-**3f** and (3a*S*,6*S*,6a*S*)-**4f** was reacted with (1*S*)-(−)-2,10-camphorsultam[38] in the presence of dimethylaminopyridine (DMAP) and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), to give a mixture of amides (−)-(3a*R*,4*S*,6a*R*)-**7** and (−)-(3a*S*,6*S*,6a*S*)-**8** in 80% yield. Finally, these derivatives could be separated by flash chromatography and then transformed into amino acids (−)-**HIP-A** (11% overall yield) and (+)-**HIP-B** (13% overall yield), respectively, by treatment with a sodium hydroxide solution followed by removal of the *N*-Boc protecting group (Scheme 2). In this case, treatment with alkali did not affect the stereochemical integrity. The same procedure, applied to the mixture of (3a*S*,4*R*,6a*S*)-**3f** and (3a*R*,6*R*,6a*R*)-**4f**, gave final amino acids (+)-**HIP-A** and (−)-**HIP-B** in comparable yield.

3. Results and discussion

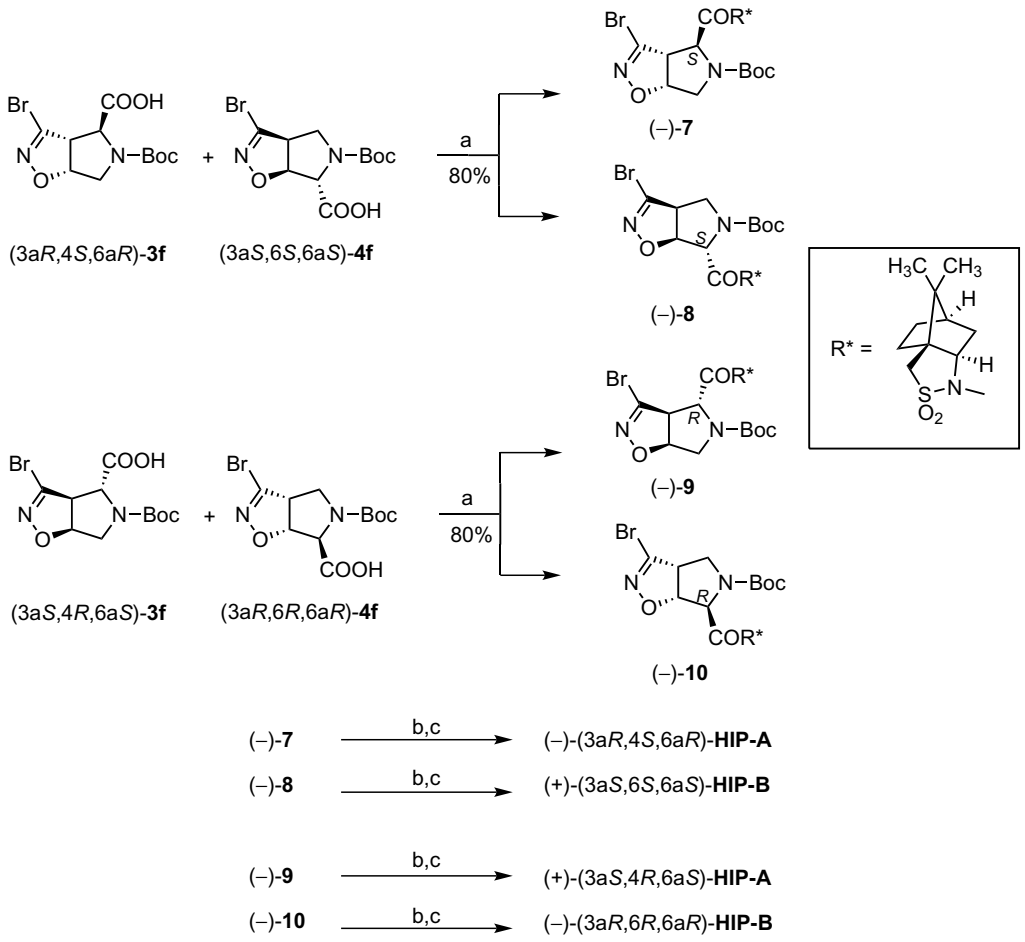
The two pairs of enantiomers (−)-**HIP-A**/(+)-**HIP-A** and (−)-**HIP-B**/(+)-**HIP-B** were investigated for their ability



Scheme 1. Reagents and conditions: (a) $NaHCO_3$/AcOEt; (b) 1 M NaOH/H_2O–dioxane, 60 °C; (c) 30% CF_3COOH/CH_2Cl_2.

to inhibit [^{3}H]D-aspartate uptake in a crude synaptosomal fraction (P2) of the rat brain cortex. As shown in Table 1, amino acid (−)-**HIP-A** was found to be the most potent uptake inhibitor followed by (+)-**HIP-B**. These two diastereoisomers share the same (*S*)-configuration at the amino acidic stereogenic center, thus evidencing the relevance of stereochemical requirements in the interaction with the EAAT binding site. This aspect is particularly evident for the pair of enantiomers (−)-**HIP-A**/(+)-**HIP-A**, whose eudismic ratio (ER) was >100. A lower ER value was obtained for the pair of enantiomers (+)-**HIP-B**/(−)-**HIP-B** (ER = 10).

To describe the molecular interactions of **HIP-A** and **HIP-B** with the amino acid residues located in the transporter binding pocket, we built up a model derived from the recently published crystal structure of the glutamate transporter from *Pyrococcus horikoshii* (Glt_{Ph}) co-crystallized with TBOA.[39] In spite of its bacterial origin, Glt_{Ph} shares 37% amino acid identity and about 56% similarity with human and rat excitatory amino acid transporters EAAT1-3, as reported by Yernool et al.[40] Moreover, the sequence differences mainly concern residues not engaged in the binding, or amino acids that interact with the ligand through their backbone, regardless of the side chain, as in the case of R276 of Glt_{Ph}, which is replaced by serine or alanine in human transporters (Fig. 2). Consequently, the binding mode statistically preferred by our ligands to Glt_{Ph} can reasonably be extended to human and rat transporters. This hypothesis is further supported by the fact that



Scheme 2. Reagents and conditions: (a) (1*S*)-(−)-2,10-camphorsultam/DMAP–HBTU–CH_2Cl_2; (b) 1 M NaOH/H_2O–dioxane, 60 °C; (c) 30% CF_3COOH/CH_2Cl_2.

Table 1. IC_{50} values (μM) of the enantiomers of HIP-A and HIP-B as inhibitors of EAAT-mediated effects[a]

	[^{3}H]D-aspartate uptake in the synaptosomal fraction
(±)-**HIP-A**	19[b]
(−)-**HIP-A**	10 (*5.0 ± 0.04*)
(+)-**HIP-A**	1500 (*2.8 ± 0.04*)
ER[c]	150
(±)-**HIP-B**	19[b]
(−)-**HIP-B**	78 (*4.1 ± 0.06*)
(+)-**HIP-B**	8.0 (*5.1 ± 0.06*)
ER[c]	10
(±)-**TBOA**	19[b]

[a] The IC_{50} values are given in μM (with $pIC_{50} \pm$ S.E.M values in brackets).
[b] Values taken from Ref. 30.
[c] Eudismic ratio.

(±)-**HIP-B** was shown to be an inhibitor of the Glu-induced currents mediated by Glt_{Ph} reconstituted in proteoliposomes.[41]

The analysis of the Glt_{Ph}-TBOA crystal complex[39] reveals that the distal carboxylic group of **TBOA** ties up with the side chain of the highly conserved R397 and T314 by hydrogen bonds, while the amino group interacts with the side chain of T398, D394 and R276. The backbone of S278 and the side chain of N401 create a supplementary hydrogen bond network capable of linking the α-carboxylate moiety (Fig. 3A). Since our compounds behave as non-transportable inhibitors, similar to **TBOA**, we performed the docking experiments using the 'open' form of the transporter, according to the mechanism proposed by Boudker et al.[39,40] The results obtained indicate that the binding mode of (−)-**HIP-A** and (+)-**HIP-B** to Glt_{Ph} is similar to that described in the literature[39] for **TBOA**. The only differ-

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                 275       310             354       390
GLt_Ph   ---TRSSS---NMDGTALYQ----GVPG----DAILDMGRTMVNVTG---
hEAAT1   ---TSSSS---NMDGTALYE----GIPQ----DWFLDRLRTTTNVLG---
hEAAT2   ---TASSA---NMDGTALYE----SIPS----DWLLDRMRTSVNVVG---
hEAAT3   ---ISSSS---NMDGTALYE----GVPQ----DWLLDRFRTMVNVLG---
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Figure 2. Partial alignment of the Glt_{Ph} and human EAAT residues involved in glutamate transport (highlighted in red).

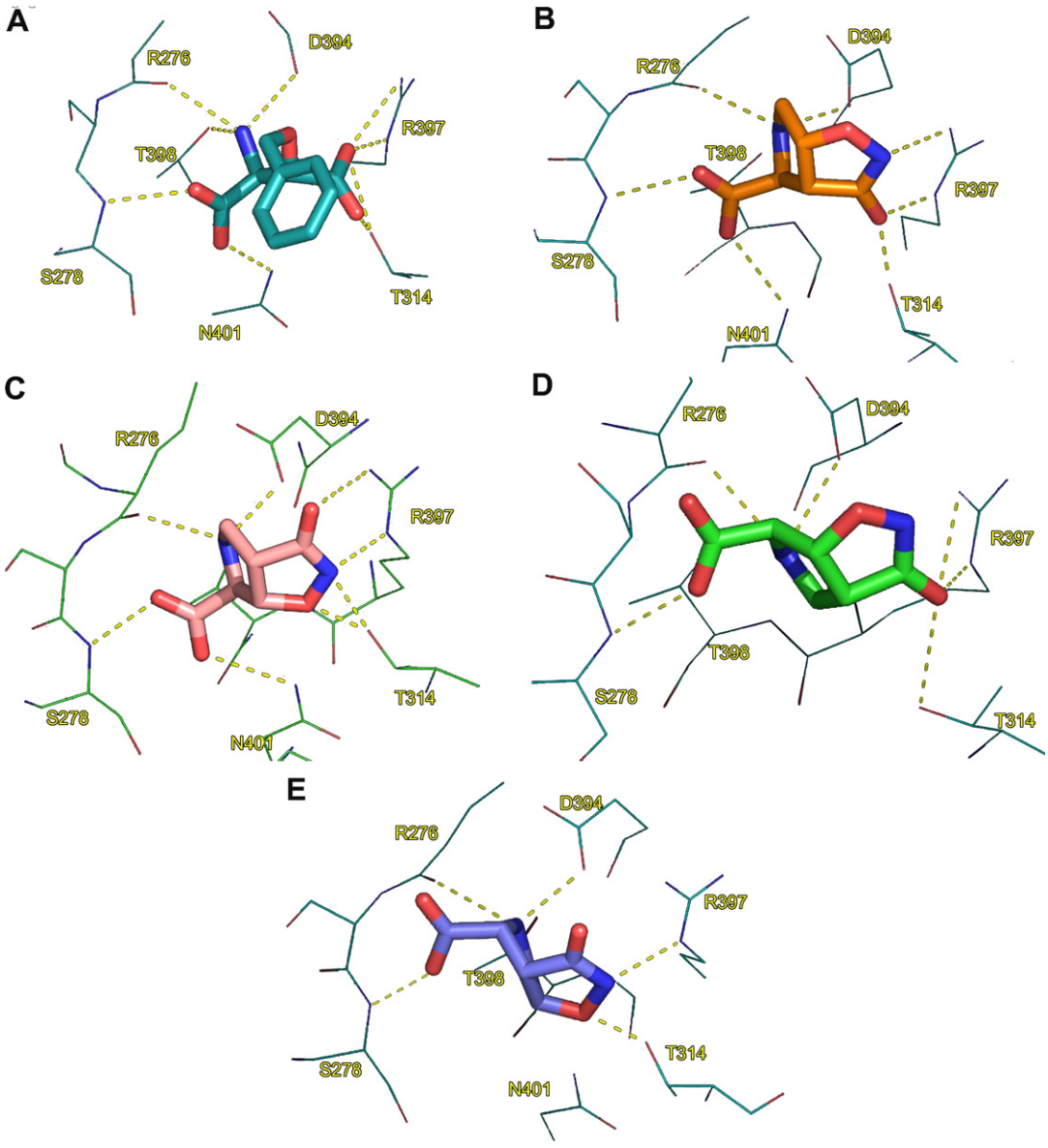

Figure 3. Schematic representations of the Glt_{Ph} binding pocket (cyan lines) with ligands (colored sticks): **TBOA**, carbon atoms colored in cyan (**A**), (−)-**HIP-A** carbon atoms colored in orange (**B**), (+)-**HIP-B** carbon atoms colored in pink (**C**). (−)-**HIP-B** carbon atoms colored in green (**D**) and (+)-**HIP-A** carbon atoms colored in violet (**E**).

ence is the lack of interaction with T398 due to the presence of a secondary amino group (Fig. 3B and C). The lower affinity displayed by diastereomers (−)-**HIP-B** and (+)-**HIP-A** (Fig. 3D and E, respectively) could be due to the inability of their α-carboxylate group to bind to residue N401. Moreover, while (−)-**HIP-B** interacts with the inner binding region formed by the R397 and the T314 side chains creating an H-bond network by means of its distal charged oxygen atom, (+)-**HIP-A** is not able to bind efficiently to those residues; in fact only a weaker interaction

with the heteroatoms of the isoxazoline ring can be observed (Fig. 3E). Such a difference correlates well to the residual affinity for the EAATs observed for (−)-**HIP-B** versus the complete inactivity of (+)-**HIP-A**.

4. Conclusion

In conclusion, we have prepared the four enantiomerically pure amino acids (+)-**HIP-A**, (−)-**HIP-A**, (+)-**HIP-B**, (−)-**HIP-B** and evaluated their ability to interact with rat glutamate transporters. The biological results, as well as docking experiments, clearly show that the absolute configuration of the stereogenic centers plays a pivotal role in the interactions with the target proteins: in both cases, the eutomer is characterized by the (*S*)-configuration at the α-amino acidic carbon, that is, (−)-**HIP-A** and (+)-**HIP-B**. It is worth noting that in a parallel investigation,[42] (−)-**HIP-A** was found to be able to block Glu-induced [^{3}H]D-aspartate release in the synaptosomal fraction at concentrations significantly lower than those required to inhibit the re-uptake; a preliminary investigation on the in vitro neuroprotective activity of (−)-**HIP-A** has provided evidence of a potential therapeutic application for the treatment of cerebral ischemia.

5. Experimental

5.1. Material and methods

L-Glutamic acid was obtained from Sigma (St Louis, MO). [^{3}H]Glu and [^{3}H]D-aspartate were purchased from Amersham Biosciences (Buckinghamshire, UK). Compound **TBOA** was obtained from Tocris (Ellisville, MO). Dibromoformaldoxime,[43] methyl (*S*)-*N*-Boc-3,4-didehydroproline methyl ester (*S*)-**1**,[36] and (*R*)-*N*-Boc-3,4-didehydroproline methyl ester (*R*)-**1**[37] were prepared according to literature procedures.

^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hertz. Chiral HPLC analyses were performed with a Jasco PU-980 pump equipped with a UV–vis detector Jasco UV-975. Enantiomers (−)-**HIP-A**/(+)-**HIP-A** and (−)-**HIP-B**/(+)-**HIP-B** were separated under the following conditions: column, CHIROBIOTIC TAG, Astec; eluent, 50 mM NH_4OAc in (EtOH–MeOH 3:2)/water 4:1 (v/v) with 0.1% acetic acid; flow rate, 1.00 mL/min. Rotary power determinations were carried out with a Jasco J-810 spectropolarimeter coupled with a Haake N3-B thermostat. TLC analyses were performed on commercial Silica Gel 60 F_{254} aluminum sheets; spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution or with ninhydrin. Melting points were determined on a model B 540 Büchi apparatus and are uncorrected. Microanalyses (C, H, N) of new compounds agreed with the theoretical value within ±0.3%.

5.1.1. 1,3-Dipolar cycloaddition of bromonitrile oxide to (−)-(*S*)-1 and (+)-(*R*)-1. To a solution of (*S*)-*N*-Boc-3,4-didehydroproline methyl ester (*S*)-**1** (4.54 g, 20 mmol) in ethyl acetate (90 mL) was added dibromoformaldoxime (12.2 g, 60 mmol) and $NaHCO_3$ (20 g). The mixture was vigorously stirred for 3 days, then another 1.5 equiv (6.1 g, 30 mmol) of dibromoformaldoxime was added and the mixture was stirred for an additional 3 days. The progress of the reaction was monitored by TLC (petroleum ether/ethyl acetate 7:3). Water was added to the reaction mixture and the organic layer was separated and dried over anhydrous sodium sulfate. The crude material, obtained after evaporation of the solvent, was chromatographed on silica gel (eluent: petroleum ether/ethyl acetate 7:3) to give 0.91 g of (−)-(3a*R*,6*S*,6a*R*)-**2** and 3.62 g of a mixture of cycloadducts (3a*R*,4*S*,6a*R*)-**3** and (3a*S*,6*S*,6a*S*)-**4**. Overall yield: 65%.

The same procedure was applied to the cycloaddition of bromonitrile oxide to (*R*)-(+)-**1** to yield the mixture of cycloadducts (3a*S*,6*R*,6a*S*)-(+)-**2**, (3a*S*,4*R*,6a*S*)-**3**, and (3a*R*,6*R*,6a*R*)-**4**, which were separated into two fractions by column chromatography as described above.

Mixtures **3** and **4**: R_f (cyclohexane/ethyl acetate 7:3) 0.35.

5.1.1.1. (−)-(3a*R*,6*S*,6a*R*)-3-Bromo-5-*tert*-butoxycarbonyl-6-methoxycarbonyl-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]-isoxazole (−)-2. Colorless oil; R_f (cyclohexane/ethyl acetate 7:3) 0.28; ^{1}H NMR ($CDCl_3$): 1.43 (s, 9H); 3.76 (s, 3H); 3.78 (dd, $J = 9.0$, 11.5, 1H); 3.84 (dd, $J = 2.4$, 11.5, 1H); 4.13 (ddd, $J = 2.4$, 9.0, 10.2, 1H); 4.70 (d, $J = 8.5$, 1H); 5.43 (dd, $J = 8.5$, 10.2, 1H); ^{13}C NMR ($CDCl_3$): 29.2, 49.7, 53.4, 57.2, 66.1, 82.3, 85.1, 141.4, 154.4, 169.5; $[\alpha]_D^{20} = -105.4$ (c 1.00, $CHCl_3$). Anal. Calcd for $C_{12}H_{17}BrN_2O_5$ (349.18): C, 41.28; H, 4.91; N, 8.02. Found: C, 41.55; H, 5.09; N, 7.85.

5.1.1.2. (+)-(3a*S*,6*R*,6a*S*)-3-Bromo-5-*tert*-butoxycarbonyl-6-methoxycarbonyl-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]-isoxazole (+)-2. $[\alpha]_D^{20} = 102.6$ (c 1.00, $CHCl_3$). Anal. Calcd for $C_{12}H_{17}BrN_2O_5$ (349.18): C, 41.28; H, 4.91; N, 8.02. Found: C, 41.56; H, 5.00; N, 7.90.

5.1.2. Synthesis of (3a*R*,4*S*,6a*R*)-3-bromo-5-*tert*-butoxycarbonyl-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]isoxazole-4-carboxylic acid (3a*R*,4*S*,6a*R*)-3f and (3a*S*,6*S*,6a*S*)-3-bromo-5-*tert*-butoxycarbonyl-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]isoxazole-6-carboxylic acid (3a*S*,6*S*,6a*S*)-4f. A mixture of (3a*R*,4*S*,6a*R*)-**3** and (3a*S*,6*S*,6a*S*)-**4** (3.62 g, 10.4 mmol) was dissolved in dioxane (35 mL) and treated with a 1 M NaOH aqueous solution (10 mL) until disappearance of the starting material (2 h). The mixture was extracted with ethyl acetate (2 × 20 mL) and the aqueous layer made acidic with 2 M HCl and newly extracted with ethyl acetate (4 × 10 mL). The organic extracts were pooled and dried over anhydrous sodium sulfate. The solvent was removed under vacuum to give 2.95 g (88% yield) of a mixture of acids (3a*R*,4*S*,6a*R*)-**3f** and (3a*S*,6*S*,6a*S*)-**4f** as a white solid.

5.1.3. Synthesis of (3a*S*,4*R*,6a*S*)-3-bromo-5-*tert*-butoxycarbonyl-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]isoxazole-4-carboxylic acid (3a*S*,4*R*,6a*S*)-3f and (3a*R*,6*R*,6a*R*)-3-bromo-5-*tert*-butoxycarbonyl-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]isoxazole-6-carboxylic acid (3a*R*,6*R*,6a*R*)-4f. The above-reported procedure carried out on a mixture of cyclo-

adducts (3a*S*,4*R*,6a*S*)-**3** and (3a*R*,6*R*,6a*R*)-**4** and gave a mixture of acids (3a*S*,4*R*,6a*S*)-**3f** and (3a*R*,6*R*,6a*R*)-**4f**.

5.1.4. Synthesis of (3a*R*,4*S*,6a*R*)-3-bromo-5-*tert*-butoxycarbonyl-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]isoxazole-4-carbonyl-(*S*)-sultam (−)-7 and (3a*S*,6*S*,6a*S*)-3-bromo-5-*tert*-butoxycarbonyl-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]isoxazole-6-carbonyl-(*S*)-sultam (−)-8. A mixture of (3a*R*,4*S*,6a*R*)-**3f** and (3a*S*,6*S*,6a*S*)-**4f**(2.95 g, 8.8 mmol) was dissolved in dry CH_2Cl_2 (90 mL); (1*S*)-(−)-2,10-camphorsultam (2.28 g, 10.6 mmol), HBTU (4.0 g, 10.6 mmol), and DMAP (4.3 g, 35.2 mmol) were sequentially added and the reaction mixture was stirred under an N_2 atmosphere overnight. A white precipitate was formed, which was filtered off and the organic layer was washed with 1 M HCl (3 × 30 mL), dried over anhydrous Na_2SO_4 and evaporated. The crude material was chromatographed on silica gel (eluent: petroleum ether/AcOEt 4:1) to give 1.71 g of (−)-**7** and 1.73 g of (−)-**8**. Overall yield: 80%.

5.1.4.1. Compound (−)-7. Crystallized from diisopropyl ether as colorless prisms, mp 180 °C dec.; R_f (petroleum ether/ethyl acetate 7:3) 0.45; $[\alpha]_D^{20} = -56.4$ (*c* 1.07, $CHCl_3$); ^{1}H NMR (DMSO-d_6, $T = 100$ °C): 0.97 (s, 3H); 1.10 (s, 3H); 1.22–1.52 (m, 11H); 1.75–2.18 (m, 5H); 3.58–3.70 (m, 2H); 3.72–3.88 (m, 2H); 3.93 (dd, $J = 5.1$, 7.3, 1H); 4.22 (d, $J = 9.2$, 1H); 5.11 (s, 1H); 5.35 (ddd, $J = 1.1$, 6.2, 9.2, 1H). ^{13}C NMR (DMSO-d_6, $T = 100$ °C): 20.4, 21.8, 26.6, 28.8, 33.0, 38.6, 45.5, 48.3, 49.9, 53.0, 55.9, 61.4, 63.3, 65.2, 81.2, 83.9, 138.8, 153.9, 169.2. Anal. Calcd for $C_{21}H_{30}BrN_3O_6S$ (532.45): C, 47.37; H, 5.68; N, 7.89. Found: C, 47.52; H, 5.74; N, 7.80.

5.1.4.2. Compound (−)-8. Crystallized from diisopropyl ether as colorless prisms, mp 183 °C dec.; R_f (petroleum ether/ethyl acetate 7:3) 0.40; $[\alpha]_D^{20} = -33.4$ (*c* 1.022, $CHCl_3$); ^{1}H NMR (DMSO-d_6, $T = 100$ °C): 0.97 (s, 3H); 1.10 (s, 3H); 1.24–1.56 (m, 11H); 1.75–2.00 (m, 4H); 2.02–2.14 (m, 1H), 3.56–3.70 (m, 2H); 3.74–3.84 (m, 2H); 3.87 (dd, $J = 4.7$, 7.7, 1H); 4.23 (ddd, $J = 1.8$, 7.7, 9.1, 1H); 5.08 (s, 1H); 5.23 (d, $J = 9.1$, 1H). ^{13}C NMR (DMSO-d_6, $T = 100$ °C): 20.4, 21.5, 26.7, 28.8, 32.9, 38.4, 45.2, 48.2, 49.8, 50.4, 53.0, 56.9, 65.3, 69.1, 81.1, 88.2, 140.9, 153.9, 167.8. Anal. Calcd for $C_{21}H_{30}BrN_3O_6S$ (532.45): C, 47.37; H, 5.68; N, 7.89. Found: C, 47.64; H, 5.79; N, 7.82.

5.1.5. Synthesis of (3a*S*,4*R*,6a*S*)-3-bromo-5-*tert*-butoxycarbonyl-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]isoxazole-4-carbonyl-(*S*)-sultam (−)-9 and (3a*R*,6*R*,6a*R*)-3-bromo-5-*tert*-butoxycarbonyl-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]isoxazole-6-carbonyl-(*S*)-sultam and (−)-10. The above-reported procedure was carried out on a mixture of acids (3a*S*, 4*R*,6a*S*)-**3f** and (3a*R*,6*R*,6a*R*)-**4f** gave derivatives (−)-**9** and (−)-**10**.

5.1.5.1. Compound (−)-9. Crystallized from diisopropyl ether as colorless prisms, mp 183 °C dec.; R_f (petroleum ether/ethyl acetate 7:3) 0.48; $[\alpha]_D^{20} = -43.0$ (*c* 0.99, $CHCl_3$); ^{1}H NMR (DMSO-d_6, $T = 100$ °C): 0.95 (s, 3H); 1.18 (s, 3H); 1.22–1.52 (m, 11H); 1.75–2.05 (m, 5H); 3.58–3.72 (m, 2H); 3.78–3.96 (m, 3H); 4.33 (d, $J = 9.1$, 1H); 5.22 (s, 1H); 5.34 (ddd, $J = 1.5$, 6.7, 9.1, 1H). ^{13}C NMR (DMSO-d_6, $T = 100$ °C): 20.3, 21.6, 26.7, 28.7, 33.0, 38.9, 45.4, 48.2, 49.9, 53.3, 56.4, 60.9, 62.3, 65.6, 81.4, 84.1, 138.9, 153.8, 170.0. Anal. Calcd for $C_{21}H_{30}BrN_3O_6S$ (532.45): C, 47.37; H, 5.68; N, 7.89. Found: C, 47.65; H, 5.85; N, 7.72.

5.1.5.2. Compound (−)-10. Crystallized from diisopropyl ether as colorless prisms, mp 184 °C dec.; R_f (petroleum ether/ethyl acetate 7:3) 0.25; $[\alpha]_D^{20} = -96.0$ (*c* 1.04, $CHCl_3$); ^{1}H NMR (DMSO-d_6, $T = 100$ °C): 0.95 (s, 3H); 1.18 (s, 3H); 1.20–1.50 (m, 11H); 1.75–2.10 (m, 5H); 3.65 (d, $J = 14.1$, 1H); 3.72–3.86 (m, 3H); 3.92 (dd, $J = 5.3$, 7.1, 1H); 4.23 (ddd, $J = 3.2$, 7.1, 9.1, 1H); 5.03 (s, 1H); 5.25 (d, $J = 9.1$, 1H). ^{13}C NMR (DMSO-d_6, $T = 100$ °C): 20.3, 21.6, 26.6, 28.7, 33.0, 38.8, 45.4, 48.2, 49.9, 51.2, 53.1, 56.8, 65.6, 68.0, 81.4, 87.8, 140.9, 153.8, 169.3. Anal. Calcd for $C_{21}H_{30}BrN_3O_6S$ (532.45): C, 47.37; H, 5.68; N, 7.89. Found: C, 47.60; H, 5.82; N, 7.76.

5.1.6. Synthesis of (3a*R*,4*S*,6a*R*)-3-hydroxy-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]isoxazole-6-carboxylic acid (−)-HIP-A. (a) Compound (−)-**7** (1.71 g, 3.2 mmol) was suspended in 1 M NaOH (32 mL). The mixture was stirred and heated at 60 °C for 4 h. After cooling at room temperature, the solution was extracted with AcOEt (3 × 10 mL), and the aqueous layer made acidic with 2 M HCl and newly extracted with AcOEt (4 × 10 mL). The organic extracts were pooled and dried over anhydrous sodium sulfate. The solvent was removed under vacuum to give 0.85 g (98% yield) of (3a*R*,4*S*,6a*R*)-**3g** as a white solid, which was directly submitted to the next step.

(b) Compound (3a*R*,4*S*,6a*R*)-**3g** (0.85 g, 3.12 mmol) was treated with a 30% dichloromethane solution of trifluoroacetic acid (8 mL) at 0 °C. The reaction mixture was stirred at room temperature until disappearance of the starting material (2 h). The volatiles were removed under vacuum and the residue was taken up with methanol and filtered under vacuum to give 0.370 g (69% yield) of (−)-**HIP-A**.

5.1.6.1. (−)-HIP-A. Colorless prisms; mp > 190 °C dec.; $[\alpha]_D^{20} = -9.0$ (*c* 0.14, H_2O). R_f (butanol/H_2O/acetic acid 60:25:15) 0.34. Chiral HPLC, retention time: 15.67 min; ee >99.5%; ^{1}H NMR (D_2O): 3.56 (dd, $J = 4.7$, 13.9, 1H); 3.65 (d, $J = 13.9$, 1H); 3.85 (dd, $J = 2.6$, 8.1, 1H); 4.41 (d, $J = 2.6$, 1H); 5.34 (dd, $J = 4.7$, 8.1, 1H); ^{13}C NMR (D_2O): 51.4, 51.7, 63.3, 82.1, 170.2, 170.5. Anal. Calcd for $C_6H_8N_2O_4$(172.14): C, 41.86; H, 4.68; N, 16.27. Found: C, 41.60; H, 4.80; N, 16.03.

5.1.7. Synthesis of (3a*S*,4*R*,6a*S*)-3-hydroxy-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]isoxazole-6-carboxylic acid (+)-HIP-A. The above-reported procedure carried out on (−)-**9** gave amino acid (+)-**HIP-A**.

5.1.7.1. (+)-HIP-A. Mp > 180 °C dec.; $[\alpha]_D^{20} = +8.0$ (*c* 0.10, H_2O). Chiral HPLC, retention time: 37.22 min; ee >99.5%. Anal. Calcd for $C_6H_8N_2O_4$(172.14): C, 41.86; H, 4.68; N, 16.27. Found: C, 41.67; H, 4.77; N, 16.06.

5.1.8. Synthesis of (3a*S*,6*S*,6a*S*)-3-hydroxy-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]isoxazole-6-carboxylic acid (+)-HIP-B. The above-reported procedure carried out on (−)-**8** gave amino acid (+)-**HIP-B** in comparable yield.

5.1.8.1. (+)-HIP-B. Mp > 170 °C dec.; $[\alpha]_D^{20} = +66.6$ (*c* 0.105, H_2O). Chiral HPLC, retention time: 15.39 min; ee >99.5%. Anal. Calcd for $C_6H_8N_2O_4$ (172.14): C, 41.86; H, 4.68; N, 16.27. Found: C, 41.61; H, 4.78; N, 16.13.

5.1.9. Synthesis of (3a*R*,6*R*,6a*R*)-3-hydroxy-3a,4,6,6a-tetrahydro-pyrrolo[3,4-*d*]isoxazole-6-carboxylic acid (−)-HIP-B. The above-reported procedure carried out on (−)-**10** gave amino acid (−)-**HIP-B** in comparable yield.

5.1.9.1. (−)-HIP-B. Colorless prisms; mp > 170 °C dec.; $[\alpha]_D^{20} = -73.0$ (*c* 0.112, H_2O); R_f (butanol/H_2O/acetic acid 60:25:15) 0.37. Chiral HPLC, retention time: 21.12 min; ee >99.5%; ^{1}H NMR (D_2O): 3.54 (dd, $J = 8.4$, 12.5, 1H); 3.62-3.76 (m, 2H); 4.31 (d, $J = 2.2$, 1H); 5.34 (dd, $J = 2.2$, 8.4, 1H); ^{13}C NMR (D_2O): 46.7, 47.3, 67.7, 85.5, 169.5, 170.4. Anal. Calcd for $C_6H_8N_2O_4$ (172.14): C, 41.86; H, 4.68; N, 16.27. Found: C, 41.72; H, 4.89; N, 16.00.

5.1.10. An alternative synthesis of (−)-HIP-B. (a) To a solution of cycloadduct (−)-(3a*R*,6*S*,6a*R*)-**2** (0.9 g, 2.6 mmol) in dioxane (18 mL) was added an aqueous solution of 1 M sodium hydroxide (6.0 mL) and the mixture was magnetically stirred at 60 °C until the disappearance of the starting material (3 h). The mixture was extracted with ethyl acetate (2 × 10 mL) and the aqueous layer made acidic with 2 M HCl and newly extracted with ethyl acetate (4 × 10 mL). The organic extracts were combined and dried over anhydrous sodium sulfate. The solvent was removed under vacuum to give 0.5 g (71% yield) of a colorless solid.

(b) The above-prepared intermediate (0.5 g, 1.8 mmol) was treated with a 30% dichloromethane solution of trifluoroacetic acid (4.7 mL) at 0 °C. The reaction mixture was stirred at room temperature until disappearance of the starting material (2 h). The volatiles were removed under vacuum and the residue was taken up with methanol and filtered under vacuum to give 0.205 g (65% yield) of (−)-**HIP-B**. The analytical data of the sample obtained in this way matched those reported above.

5.1.11. An alternative synthesis of (+)-HIP-B. The above-reported procedure carried out on cycloadduct (+)-(3a*S*,6*R*,6a*S*)-**2** gave final derivative (+)-**HIP-B** in 67% yield. The analytical data of the sample obtained in this way matched those reported above.

5.2. Biological assays

5.2.1. Synaptosomal [^{3}H]D-aspartate in the crude synaptosomal fraction of rat brain cortex.[30] Rats (adult male CRL:CD(SD)BR, Charles River, Calco, Italy) were killed by decapitation and their brain cortex was rapidly dissected out and homogenized in 40 volumes of ice-chilled 0.32 M sucrose, pH 7.4, with 10 mM phosphate buffer, in a glass homogenizer with a Teflon pestle. The homogenates were centrifuged at 1000*g* for 5 min (4 °C), and the supernatants centrifuged again at 12000*g* for 20 min. The pellets constituted the crude synaptosomal fraction (P2).[31] The P2 pellets were diluted to a concentration of about 3 mg of tissue/mL, in physiological buffer [128 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1.5 mM NaH_2PO_4, 10 mM D-Glucose, 10 mM Tris–acetate, pH 7.4].[31] Samples of 0.5 mL were preincubated for 7 min at 35 °C in a water bath, in the absence and in the presence of different concentrations of the compounds to be tested. The non-specific uptake was determined in the presence of 300 μM L-Glu.[30] Uptake was started by adding 10 nM [^{3}H]D-aspartate (13 Ci/mmol) and was stopped 4 min later by adding 2 mL of ice-chilled assay buffer. Uptake was linear over this period of time.[32] Samples were immediately filtered through cellulose mixed ester filters (0.65 μm pore size; Millipore Corporation, Milano, Italy) and washed with 2 mL of assay buffer. The radioactivity trapped on the filters was counted in 4 mL of Ultima Gold MV (PerkinElmer Life and Analytical Sciences, Groningen, the Netherlands) in a Wallac 1409 rack-beta liquid scintillation counter with a counting efficiency of about 50%. The experiments were performed in triplicate, in at least three different experiments.

5.2.2. Data analysis. The inhibition curves were fitted using the 'one-site competition' equation built into Prism version 4.0 for Windows (**graphpad** Software, San Diego, CA) This analysis gives the pIC_{50} (i.e., the drug concentration inhibiting the specific uptake by 50%), with its S.E.M.

5.3. Molecular modeling

Ligands docked into the transporter binding cleft were built by SYBYL7.3 (Tripos Inc., 1699 South Hanley Rd., St. Louis, MO 63144) and preliminarily minimized by the DFT/B3LYP/6-31g(d) level as implemented in Gaussian03.[44] The amino- and the two acidic groups were considered in the ionized form to better simulate the physiological conditions.

The crystal structure of the Glt_{Ph}-**TBOA** complex was retrieved from the Protein Data Bank (code 2NWW).[39] Docking experiments with our novel ligands, performed with the GOLD 3.1program,[45] were carried out on the Glt_{Ph} binding cleft deprived of **TBOA** (open form). The goldscore fitness function and the distribution of torsion angles were chosen as indicators of the quality of the docking results. Van der Waals and hydrogen bonding radii were set at 4.0 and 3.0 Å, respectively; genetic algorithm parameters were kept at the default value. The resulting complexes were then optimized by means of molecular mechanic method implemented in SYBYL7.3 software. Figures were acquired by PYMOL software.[46]

Acknowledgments

This work was financially supported by MIUR (PRIN 2005, Rome) and Università degli Studi di Milano (FIRST).

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