Journal of Medicinal Chemistry

Article

Chemoenzymatic synthesis and pharmacological characterization of functionalized aspartate analogues as novel excitatory amino acid transporter inhibitors

Haigen Fu, Jielin Zhang, Pieter Tepper, Lennart Bunch, Anders Jensen, and Gerrit J. Poelarends

J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.8b00700 • Publication Date (Web): 16 Jul 2018

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Chemoenzymatic synthesis and pharmacological characterization of functionalized aspartate analogues as novel excitatory amino acid transporter inhibitors

Haigen Fu,[a] Jielin Zhang,[a] Pieter G. Tepper,[a] Lennart Bunch,[b]

Anders A. Jensen,[b,*] and Gerrit J. Poelarends[a,*]

[a]Department of Chemical and Pharmaceutical Biology, Groningen Research Institute of Pharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

[b]Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, DK-2100 Copenhagen OE, Denmark

[*]Address correspondence to Prof. Dr. Gerrit J. Poelarends. Tel: +31-503633354; E-mail: g.j.poelarends@rug.nl.

[*]Address correspondence to Dr. Anders A. Jensen. Tel: +45-35333260; E-mail: aaj@sund.ku.dk.

ABSTRACT

Aspartate (Asp) derivatives are privileged compounds for investigating the roles governed by excitatory amino acid transporters (EAATs) in glutamatergic neurotransmission. Here, we report the synthesis of various Asp derivatives with (cyclo)alkyloxy and (hetero)aryloxy substituents at C-3. Their pharmacological properties were characterized at the EAAT1-4 subtypes. The L-*threo*-3-substituted Asp derivatives **13a-e** and **13g-k** were non-substrate inhibitors, exhibiting pan activity at EAAT1-4 with IC_{50} values ranging from 0.49 to 15 μM. Comparisons between (DL-*threo*)-**19a-c** and (DL-*erythro*)-**19a-c** Asp analogues confirmed that the *threo* configuration is crucial for the EAAT1-4 inhibitory activities. Analogues (**3b-e**) of L-TFB-TBOA (**3a**) were shown to be potent EAAT1-4 inhibitors, with IC_{50} values ranging from 5-530 nM. Hybridization of the nonselective EAAT inhibitor L-TBOA with EAAT2-selective inhibitor WAY-213613 or EAAT3-preferring inhibitor NBI-59159 yielded compounds **8** and **9**, respectively, which were non-selective EAAT inhibitors displaying considerably lower IC_{50} values at EAAT1-4 (11-140 nM) than those displayed by the respective parent molecules.

KEYWORDS:

Asymmetric synthesis, biocatalysis, aspartate derivative, EAATs, inhibitor.

INTRODUCTION

L-Glutamate (Glu) is the major excitatory neurotransmitter in the mammalian central nervous system (CNS), where it mediates numerous physiological and pathophysiological processes.[1-5] However, accumulation of high levels of extracellular Glu may lead to hyper-activity in the glutamatergic system and neuronal injury.[6] Five subtypes of excitatory amino acid transporters (termed EAAT1-5 in humans) have been identified in glial cells (predominantly EAAT1,2) and neurons (predominantly EAAT3-5), where they are key players in the regulation of glutamatergic transmission.[7] EAAT2 is the major contributor to this, as it is estimated to be responsible for over 90% of total extracellular Glu uptake in the brain, while EAAT1 and EAAT3 are also widely expressed in the CNS. Notably, EAAT4 and EAAT5 are almost specifically located in cerebellum and the retina, respectively. Malfunction of EAAT function has been implicated in many neurological disorders, such as Alzheimer's disease, epilepsy, amyotrophic lateral sclerosis, and Huntington's disease.[8] However, in contrast to the considerable medicinal chemistry efforts in the fields of ionotropic and metabotropic Glu receptors, the EAATs have received much less attention as putative drug targets.[4]

One of the most important scaffolds for the development of EAAT ligands is the endogenous substrate L-aspartate (**1**, L-Asp, Figure 1B). Represented by L-*threo*-3-benzyloxyaspartate (**2**, L-TBOA, Figure 1B), 3-aryloxy substituted Asp analogues were identified as the first class of non-transportable EAAT inhibitors by Shimamoto and coworkers.[9-11] None of these analogues exhibited substantial selectivity or preference towards

different EAAT subtypes. Further development of the 3-aryloxy substituted Asp analogues as pharmacological tools in neurobiological research led to the identification of (L-*threo*)-3-[3-[4-(trifluoromethyl)benzoylamino]benzyloxy]aspartate (**3a**, L-TFB-TBOA, Figure 1B), the most potent EAAT inhibitor reported to date.[12-14] Interestingly, elimination of the ether oxygen in L-TBOA (**2**) gives the analogue L-*threo*-3-benzylaspartate (**4**, L-3-BA, Figure 1B), which displayed a 10-fold preference as an inhibitor for EAAT3 over EAAT1 and EAAT2.[15] Most recently, [3-(trifluoromethyl)phenyl]sulfonamide-L-aspartate (**5**, Figure 1B) was found to be a potent EAAT2 inhibitor exhibiting over 30-fold selectivity for EAAT2 over EAAT1 and EAAT3.[16] In addition, other medicinal chemistry efforts have focused on 4-carboxylate-modified Asp analogues, which resulted in the discovery of N^4-[4-(2-bromo-4,5-difluorophenoxy)phenyl]-L-asparagine (**6**, WAY-213613) as a EAAT2-selective nonsubstrate inhibitor[17-19] and N^4-(9*H*-fluoren-2-yl)-L-asparagine (**7**, NBI-59159) as an EAAT3-preferring inhibitor (Figure 1B).[19,20]

The fact that derivatization of the Asp structure has yielded several important classes of EAAT inhibitors (Figure 1B) encouraged us to further explore the potential of this scaffold in this field (Figure 1A). Here, we describe the chemoenzymatic asymmetric synthesis of a series of novel Asp derivatives comprising (cyclo)alkyloxy and (hetero)aryloxy substituents at the C-3 position, using an engineered variant of methylaspartate ammonia lyase (MAL-L384A) as the biocatalyst.[21,22] In addition, we report the synthesis of two Asp derivatives by hybridization of the nonselective EAAT inhibitor L-TBOA (**2**) with the EAAT2-selective inhibitor WAY-213613 (**6**) or the EAAT3-preferring inhibitor NBI-59159 (**7**). The pharmacological properties of the newly synthesized Asp derivatives, as well as those of

previously synthesized analogues and homologues of L-TFB-TBOA, have been characterized at stable cell lines expressing the human EAAT1-3 and rat EAAT4 and the structure-activity relationships of these analogues elucidated.

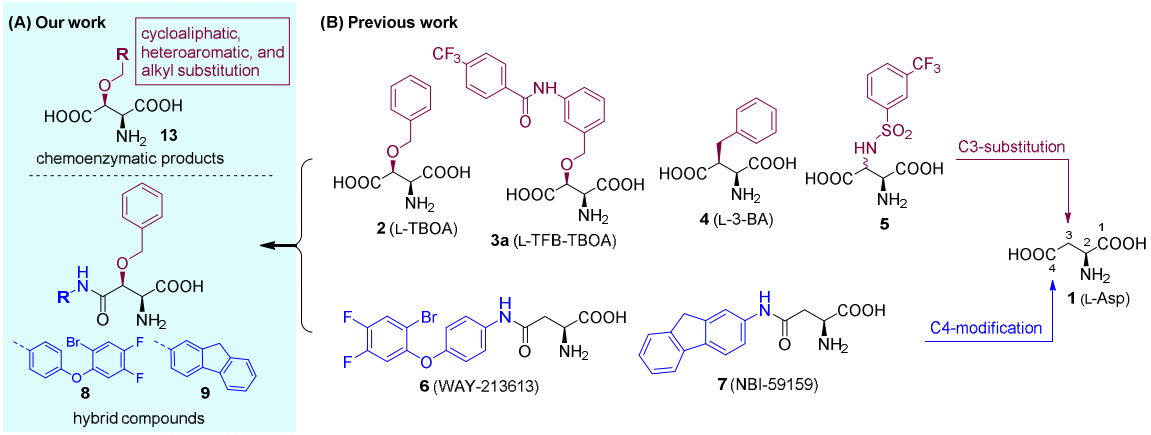


Figure 1. EAAT inhibitors derived from the L-Asp scaffold. (A) Structures of chemoenzymatic products and designed hybrid compounds. (B) Structures of previously developed EAAT inhibitors.

RESULTS AND DISCUSSION

Chemoenzymatic synthesis of 3-substituted Asp analogues

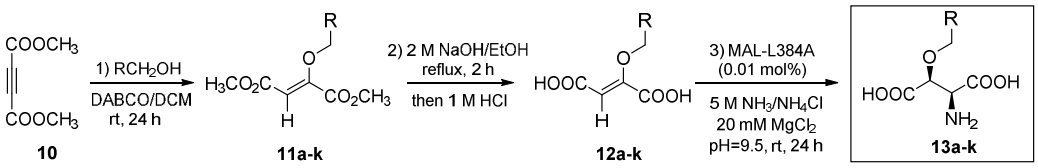
The asymmetric synthesis of Asp derivatives with alkyloxy or aryloxy substituents at the C-3 position has proven to be challenging. Shimamoto and coworkers firstly reported the synthesis of L-TBOA (**2**) through an elaborate 11-step procedure.[11] A rapid chemoenzymatic methodology for asymmetric synthesis of **2** and its analogues by using MAL-L384A as biocatalyst has been developed in our group.[21,22] In order to further explore the substrate

scope and synthetic usefulness of MAL-L384A, a series of 2-substituted fumarate derivatives with various cycloalkyloxy (**12a-f**), heteroaryloxy (**12g-j**) or alkyloxy (**12k**) substituents were prepared through the addition of appropriate alcohols to dimethyl acetylenedicarboxylate (**10**), followed by hydrolysis of the methyl esters (Table 1). Interestingly, compounds **12a-e** and **12g-k** were accepted as substrates by MAL-L384A, giving excellent conversions (60-98%) and yielding the corresponding amino acid products in 26-58% isolated yield (Table 1). Hence, MAL-L384A has a remarkably broad substrate scope, allowing the addition of ammonia to a wide variety of fumarate derivatives, providing a powerful synthetic tool for the preparation of valuable 3-substituted aspartic acids. The most bulky compound, 2-cyclooctylmethoxyfumarate (**12f**), was not accepted as substrate by MAL-L384A.

The amino acid product **13a**, which is representative for the series of chemoenzymatically prepared 3-cycloalkyloxy substituted aspartic acids, was identified as the desired *threo* diastereomer (*de* = 97%, Figure S1) by comparison of its ^{1}H-NMR signals and *J*-coupling values to those of an authentic standard with known L-*threo* configuration and chemically synthesized DL-*threo* and DL-*erythro* stereoisomers (Scheme 1). To determine the absolute configuration of product **13a**, HPLC analysis on a chiral stationary phase was conducted by using corresponding reference molecules with known L-*threo* or DL-*threo* configuration. This analysis revealed that chemoenzymatically produced **13a** was present as a single enantiomer with exclusively the L-*threo* configuration (*ee* >99%, Figure S62). Similarly, the representative chemoenzymatic products **13g** and **13k** were also identified as the desired L-*threo* isomers with excellent *de* (>98%) and *ee* (>99%) values (Table 1, Figures S2, S3, S63 and S64). Although the relative configurations of products **13b-e** and **13h-j** were not

determined by comparison to authentic standards, we assume the relative configurations to be *threo* for all enzymatic products on the basis of analogy.

Table 1. Three-step chemoenzymatic synthesis of 3-substituted Asp analogues.



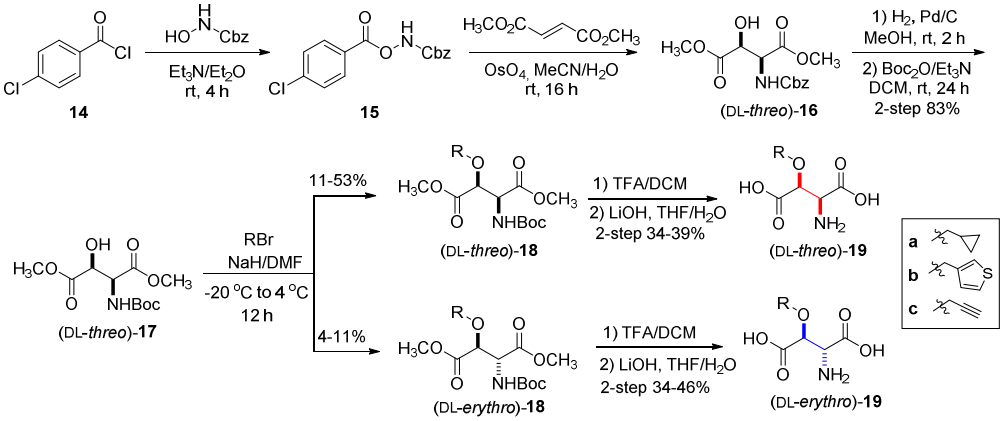
Entry	R	Substrate	Product	Conv.[a] (yield[b]) [%]	*de*[c] [%]	*ee*[e] [%]
1		**12a**	**13a**	88 (31)	97 (*threo*)	>99 (L-*threo*)[f]
2		**12b**	**13b**	89 (58)	>98 (*threo*)[d]	n.d.[g]
3		**12c**	**13c**	85 (39)	>98 (*threo*)[d]	n.d.
4		**12d**	**13d**	90 (56)	>98 (*threo*)[d]	n.d.
5		**12e**	**13e**	60 (26)	>98 (*threo*)[d]	n.d.
6		**12f**	**13f**	0	---	---
7		**12g**	**13g**	98 (47)	>98 (*threo*)	>99 (L-*threo*)[f]
8		**12h**	**13h**	98 (48)	>98 (*threo*)[d]	n.d.
9		**12i**	**13i**	98 (35)	>98 (*threo*)[d]	n.d.
10		**12j**	**13j**	98 (39)	>98 (*threo*)[d]	n.d.
11		**12k**	**13k**	83 (47)	>98 (*threo*)	>99 (L-*threo*)[f]

[a]Conversions were determined by comparing ^{1}H NMR signals of substrates and corresponding products. [b]Yield of isolated product after cation exchange chromatography. [c]The diastereomeric excess (*de*) was determined by ^{1}H NMR. [d]The purified products were tentatively assigned the *threo* configuration on the basis of analogy. [e]The enantiomeric excess (*ee*) was determined by HPLC on a

chiral stationary phase using authentic standards with known L-*threo* and DL-*threo* configuration (Figures S62-S64). [f]Absolute configuration of products **13a**, **13g** and **13k** were determined unambiguously by comparison of ^{1}H NMR and chiral HPLC data to those of authentic samples with known DL-*erythro*, DL-*threo*, and L-*threo* configuration. [g]n.d., not determined.

Synthesis of (DL-*threo*)- and (DL-*erythro*)-3-substituted Asp derivatives

To confirm the importance of the relative configuration of 3-substituted Asp derivatives for inhibition of EAATs, representative compounds with DL-*threo* and DL-*erythro* configurations were synthesized according to the route given in Scheme 1. The building block (DL-*threo*)**-16** was prepared based on the Sharpless aminohydroxylation procedure as previously reported.[23] Compound (DL-*threo*)**-17**, with the *N*-protective group transferred from Cbz to Boc, was achieved by hydrogenolysis of (DL-*threo*)**-16** using H_2/Pd/C followed by Boc-protection in an excellent yield (2 steps, 83%). To facilitate *O*-alkylation of **17** with the proper RBr, the strong base NaH was used to deprotonate the hydroxyl group of **17** which provided the desired (DL-*threo*)**-18** with an isolated yield of 11-53%.[24] In addition, a small amount of epimerized product (DL-*erythro*)**-18** was observed and separated by flash column chromatography in 4-11% isolated yield. Subsequently, global deprotection of (DL-*threo*)**-18** was conducted *via* treatment with trifluoroacetic acid followed by hydrolysis of the methyl esters with LiOH, providing the desired final product (DL-*threo*)**-19** with 34-39% isolated yield over two steps. Following the same procedure, compound (DL-*erythro*)**-19** was obtained in 34-46% yield. Notably, (DL-*threo*)**-19** and (DL-*erythro*)**-19** are not only valuable molecules for exploring the stereochemistry-activity relationship of 3-substituted Asp analogues as EAAT inhibitors, but also for determining the stereochemistry of the chemoenzymatically prepared products.

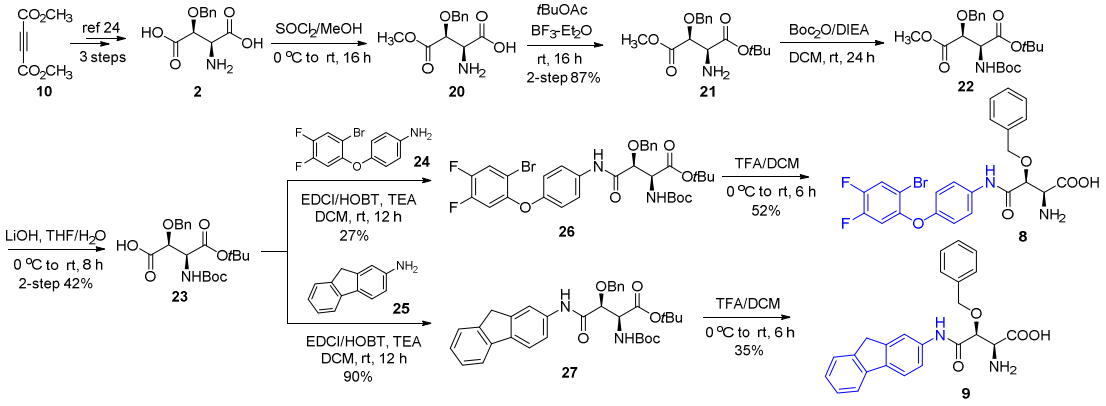


Scheme 1. Synthesis of (DL-*threo*)- and (DL-*erythro*)-3-substituted Asp analogues.

Design and synthesis of hybrid compounds 8 and 9

L-TBOA (**2**) is a potent nonselective EAAT inhibitor, while WAY-213613 (**6**) and NBI-59159 (**7**) are EAAT2-selective and EAAT3-preferring inhibitors, respectively (Figure 1B).[17-20] We envisioned that hybridization of L-TBOA (**2**) with WAY-213613 (**6**) or NBI-59159 (**7**) would result in a synergistic effect on inhibitory activity at the EAATs. Thus, two hybrid compounds **8** and **9** were designed by integrating **2** with **6** or **7** (Figure 1A), and their synthesis was achieved according to the route presented in Scheme 2. The multi-gram scale chemoenzymatic synthesis of **2** (L-TBOA, *de* >98%, *ee* >99%), starting from commercially available dimethyl acetylenedicarboxylate **10** and using MAL-L384A as biocatalyst, has been previously reported.[24] Selective mono-esterification at the 4-carboxylate of **2**, which was accomplished under ambient condition using one equivalent of $SOCl_2$ in dry methanol, delivered intermediate **20** without the need for purification. The 1-carboxylate of compound **20** was subsequently protected by a *tert*-butyl group *via* treatment with BF_3-Et_2O/*t*BuOAc,

providing compound **21** with 87% isolated yield over two steps. Starting from **21**, the chiral building block **23** was achieved in two successive reactions including Boc-protection and selective hydrolysis of the 4-methyl ester with LiOH (2 steps, 42%). The desired Asp derivatives **8** and **9** were obtained *via* amidation of **23** with appropriate amine **24** or **25** by using EDCI/HOBT as condensation reagents followed by global deprotection in TFA/DCM with 14-32% isolated yield over two steps.



Scheme 2. Synthesis of hybrid compounds **8** and **9**. The parent compounds for the blue and black parts of compound **8** are WAY-213613 (**6**) and L-TBOA (**2**), respectively, whereas the parent compounds for the blue and black parts of compound **9** are NBI-59159 (**7**) and L-TBOA (**2**), respectively.

Pharmacological characterization of the Asp derivatives at EAAT1-4

The pharmacological properties of the aspartate derivatives **13a-e**, **13g-k**, (DL-*threo*)-**19a-c** and (DL-*erythro*)-**19a-c**, the hybrid molecules **8** and **9**, and selected EAAT reference ligands were determined at HEK293 cell lines stably expressing human EAAT1, EAAT2 and EAAT3

(hEAAT1, hEAAT2 and hEAAT3, respectively) and at a tsA201 cell line stably expressing rat EAAT4 (rEAAT4) in a conventional [^{3}H]-D-Asp uptake assay.[25] The rank order and absolute values of the IC_{50} values exhibited by the reference EAAT substrates L-Glu, L-Asp and (L-*threo*)-3-hydroxyaspartate (L-THA) and the reference EAAT non-substrate inhibitors DL-TBOA and WAY-213613 at the four EAATs in the [^{3}H]-D-Asp uptake assay were in good agreement with previously reported pharmacological properties (Table 2).[10,11,17,25-27]

Overall, the 3-substituted Asp derivatives **13a-e** and **13g-k** were all relatively potent inhibitors of the four EAATs, and even though most of them displayed slightly lower IC_{50} values at hEAAT1 and hEAAT2 compared to hEAAT3 and rEAAT4, they were essentially non-selective inhibitors (Table 2). The Asp derivatives displayed comparable high-nanomolar/low-micromolar IC_{50} values at the four transporter subtypes, the differences between the highest and lowest IC_{50} values in the compound series being 5.5-, 4.9-, 13- and 9.4-fold at hEAAT1, hEAAT2, hEAAT3 and rEAAT4, respectively. Interestingly, the (L-*threo*)-3-substituted Asp derivatives **13a-e** and **13g-k** and (DL-*threo*)-3-substituted Asp derivatives **19a-c** were equipotent with DL-TBOA (Table 2). Based on direct comparisons between racemic Asp analogues (DL-*threo*)-**19a-c** and (DL-*erythro*)-**19a-c**, it is clear that the (DL-*threo*)-Asp analogues were significantly more potent inhibitors than the corresponding (DL-*erythro*)-Asp analogues at all four EAAT subtypes (Table 2). Notably, the enantiopure Asp derivatives (L-*threo*)-**13a**,**g,k** only displayed slightly lower IC_{50} values than their racemic counterparts (DL-*threo*)-**19a-c** (Table 2). These results are in line with previous observations that L-TBOA and L-TFB-TBOA were more potent inhibitors of EAATs than their corresponding (L-*erythro*)-diastereomers.[11,28] These structure-activity relationships thus

demonstrate that essentially every (cyclo)alkyloxy and (hetero)aryloxy substituent in the 3-position of Asp yields a potent EAAT ligand and also confirm that the relative *threo* configuration of 3-substituted Asp analogues is crucial for their inhibitory activities at EAATs.

The retained EAAT inhibitory activity in the Asp analogues **13a-e** and **13g-k** regardless of the size of their respective 3-substituent contrasts the substantial gradual reduction in EAAT activity observed upon the introduction of 4-substituents of increasing size into Glu.[29,30] It seems reasonable to ascribe these SAR differences to the substituents in the 3-substituted Asp analogue and the 4-substituted Glu analogue projecting out into different regions of the EAAT substrate binding pocket. Perhaps more interesting, the complete lack of subtype-selectivity or –preference exhibited by all of the analogues in this study (**13a-e** and **13g-k**) differs remarkably from our recent findings for a series of sulfonamido functionalized 3-substituted aspartate analogues that comprises both EAAT1-preferring and EAAT2-selective inhibitors.[16] It seems that even when comparing different 3-substituted Asp analogues, different functionalities (an ether vs a sulfonamide group) in this position of the Asp molecule will result in the substituents being projected out into different binding pocket regions, which again is reflected in markedly different subtype-selectivity profiles across the EAATs.

Whereas Asp (both L-Asp and D-Asp) and its derivative (L-*threo*)-3-hydroxy Asp (L-THA) are substrates for EAAT1-4, the (DL-*threo*)-3-benzyloxy Asp (DL-TBOA) analogue is a non-substrate inhibitor of all four EAAT subtypes.[10,11,17,25-27] Since the series of Asp analogues in the present study comprised analogues with 3-substituents covering a considerable spectrum in terms of sizes, we decided to test these analogues for both substrate

and non-substrate inhibitor activity at the hEAAT1-3 cell lines in the fluorescence-based FLIPR Membrane Potential Blue (FMP) assay. This assay measures the membrane potential changes induced in the cell lines by the substrate translocation and the concomitant co-transport of Na^+, H^+ and the counter-transport of K^+ mediated by the EAAT. In a previous study we have found the assay to be capable of distinguishing substrates from non-substrate inhibitors within a series of reference EAAT ligands.[25] None of the 3-substituted Asp analogues (**13a-e** and **13g-k**) were substrates at hEAAT1-3, as they did not evoke a significant increase in the fluorescence levels in the assay when applied on their own (all at concentrations of 300 μM) at hEAAT1-, hEAAT2- and hEAAT3-HEK293 cell lines (data not shown). In contrast, preincubation and co-application of the analogues (all at concentrations of 300 μM) together with L-Glu EC_{80} completely eliminated the Glu-induced response at all three transporters (data not shown), and thus all 3-substituted Asp analogues were non-substrate inhibitors of the EAATs. Given that L-THA is a substrate for EAAT1-4, these findings clearly demonstrate that the borderline between when the Asp analogue can be transported by the EAAT or not lies right between the 3-hydroxy substituent (L-THA) and 3-substituents such as cyclopropyl (**13a**), prop-2-yn (**13k**) and larger groups in the series of Asp analogues tested here (Figure 1, Table 2).

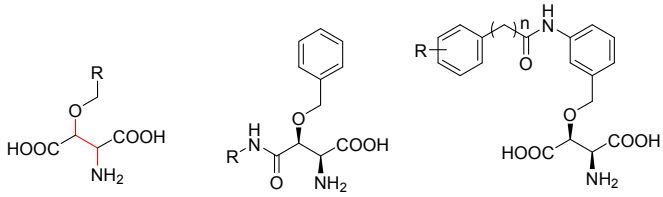
The IC_{50} values displayed by the TBOA/WAY-213613 hybrid, compound **8**, at hEAAT1, hEAAT2, hEAAT3 and rEAAT4 in the [^{3}H]-D-Asp uptake assay were 190-, 53-, 40- and 16-fold lower than those of DL-TBOA and 78-, 2.0-, 20- and 11-fold lower than those of WAY-213613 (**6**), respectively (Table 2, Figure 2). Thus, introduction of a benzyloxy group into the 3-position of WAY213613 converts this EAAT2-selective inhibitor into a

non-selective but much more potent EAAT inhibitor. Hybrid compound **9**, derived from TBOA (**2**) and NBI-59159 (**7**), displayed IC_{50} values of 12, 47, 55 and 80 nM and was thus 180-, 40-, 69- and 29-fold more potent as inhibitor at hEAAT1, hEAAT2, hEAAT3 and rEAAT4, respectively, than DL-TBOA. Since we did not test NBI-59159 (**7**) in the [^{3}H]-D-Asp uptake assay, we cannot make a direct comparison between the inhibitory potencies displayed by **9** and those of this other parent compound. However, compared to the reported inhibitory data of NBI-59159 (**7**),[19] the hybrid analogue **9** displayed 83-, 30- and 1.6-fold increased inhibitory potencies at EAAT1, EAAT2 and EAAT3, respectively (Table 2, Figure 2). Analogously to the observation for the hybrid analogue **8** and its parent compound WAY-213613, introducing a benzyloxy group at the 3-position of NBI-59159 converted this EAAT3-preferring inhibitor into a non-selective but considerably more potent EAAT inhibitor.

The chemoenzymatic synthesis of L-TFB-TBOA (**3a**) and its four derivatives **3b-e** was reported elsewhere.[24] In agreement with the original study of L-TFB-TBOA,[12] the chemoenzymatically prepared compound **3a** was found to be a potent EAAT inhibitor displaying IC_{50} values of 3.6, 10, 120 and 40 nM at hEAAT1, hEAAT2, hEAAT3 and rEAAT4, respectively, thus displaying some preference for hEAAT1,2 over hEAAT3 (Table 2). Whereas substitution of the *p*-CF_3 group in L-TFB-TBOA with *o*-CF_3 (**3b**) resulted in significantly decreased inhibitory potencies at the EAATs, the *m*-CF_3 analogue (**3c**) displayed comparable IC_{50} values as L-TFB-TBOA (**3a**) at all four transporter subtypes. While extension of the functional group at the C-3 position in L-TFB-TBOA with one carbon (**3d**) resulted in 5-10-fold increased IC_{50} values at hEAAT1, hEAAT2 and rEAAT4, the homologue retained the inhibitory potency of L-TFB-TBOA at hEAAT3. Interestingly, extension of the C-3

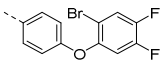
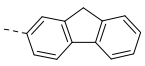
functional group with two carbons resulted in a homologue (**3e**) with similar inhibitory properties at the four EAATs as L-TFB-TBOA (Table 2).

Table 2. Pharmacological properties of EAAT reference ligands, 3-substituted aspartate analogues and hybrid compounds at hEAAT1, hEAAT2, hEAAT3 and rEAAT4 in the [^{3}H]-D-aspartate uptake assay. IC_{50} values are given in μM with pIC_{50} ± S.E.M. in brackets, and the number of independent experiments (n) are given in superscript behind each pIC_{50} ± S.E.M. value.



3-substituted Asp analogues **13a-k** and **19a-c** | Hybrid compounds **8** and **9** | L-TFB-TBOA analogues **3a-e**

No.	R	hEAAT1 IC_{50} (μM) [pIC_{50} ± S.E.M.]$^{(n)}$	hEAAT2 IC_{50} (μM) [pIC_{50} ± S.E.M.]$^{(n)}$	hEAAT3 IC_{50} (μM) [pIC_{50} ± S.E.M.]$^{(n)}$	rEAAT4 IC_{50} (μM) [pIC_{50} ± S.E.M.]$^{(n)}$
Reference EAAT ligands					
L-Glu		11 [4.94 ± 0.07]$^{(4)}$	52 [4.28 ± 0.02]$^{(4)}$	32 [4.49 ± 0.07]$^{(4)}$	13 [4.88 ± 0.06]$^{(3)}$
L-Asp		9.3 [5.03 ± 0.11]$^{(3)}$	32 [4.48 ± 0.09]$^{(3)}$	20 [4.71 ± 0.13]$^{(3)}$	11 [4.96 ± 0.07]$^{(3)}$
L-THA		3.9 [5.41 ± 0.15]$^{(3)}$	9.6 [5.02 ± 0.03]$^{(3)}$	9.6 [5.02 ± 0.08]$^{(3)}$	2.8 [5.55 ± 0.09]$^{(3)}$
DL-TBOA		2.1 [5.68 ± 0.09]$^{(4)}$	1.9 [5.72 ± 0.08]$^{(4)}$	3.8 [5.42 ± 0.06]$^{(4)}$	2.3 [5.64 ± 0.07]$^{(3)}$
WAY-213613 (**6**)		0.86 [6.07 ± 0.01]$^{(3)}$	0.071 [7.15±0.05]$^{(3)}$	1.9 [5.73 ± 0.05]$^{(3)}$	1.5 [5.97 ± 0.10]$^{(3)}$
L-threo-3-substituted Asp analogues					
13a		3.4 [5.46 ± 0.07]$^{(3)}$	1.4 [5.86 ± 0.02]$^{(3)}$	3.1 [5.51 ± 0.05]$^{(3)}$	8.3 [5.08 ± 0.02]$^{(3)}$
13b		1.7 [5.78 ± 0.06]$^{(3)}$	0.69 [6.16 ± 0.07]$^{(3)}$	2.1 [5.67 ± 0.04]$^{(3)}$	3.0 [5.52 ± 0.01]$^{(3)}$
13c		1.4 [5.86 ± 0.06]$^{(4)}$	0.94 [6.03 ± 0.07]$^{(4)}$	2.7 [5.57 ± 0.04]$^{(4)}$	2.4 [5.61 ± 0.07]$^{(3)}$
13d		0.62 [6.20 ± 0.05]$^{(4)}$	0.49 [6.31 ± 0.06]$^{(4)}$	2.6 [5.59 ± 0.05]$^{(4)}$	1.6 [5.79 ± 0.02]$^{(3)}$
13e		2.2 [5.65 ± 0.11]$^{(3)}$	2.4 [5.61 ± 0.04]$^{(3)}$	11 [4.98 ± 0.06]$^{(3)}$	7.8 [5.11 ± 0.01]$^{(3)}$

13g		1.6 [5.79 ± 0.03](3)	0.92 [6.03 ± 0.04](3)	0.86 [6.06 ± 0.04](3)	5.0 [5.31 ± 0.06](3)
13h		1.2 [5.91 ± 0.06](3)	0.77 [6.11 ± 0.04](3)	1.2 [5.93 ± 0.02](3)	3.3 [5.48 ± 0.07](3)
13i		1.3 [5.90 ± 0.06](3)	0.76 [6.12 ± 0.04](3)	1.2 [5.92 ± 0.03](3)	3.1 [5.51 ± 0.07](3)
13j		3.0 [5.52 ± 0.05](3)	0.83 [6.08 ± 0.05](3)	1.6 [5.79 ± 0.02](3)	7.8 [5.11 ± 0.05](3)
13k		2.7 [5.57 ± 0.06](3)	1.6 [5.79 ± 0.02](3)	0.84 [6.07 ± 0.04](3)	15 [4.82 ± 0.07](3)
Racemic DL-threo- and DL-erythro-3-substituted Asp analogues					
DL-*threo*-**19a**		5.8 [5.24 ± 0.03](3)	3.0 [5.53 ± 0.04](3)	3.7 [5.43 ± 0.04](3)	16 [4.80 ± 0.07](3)
DL-*erythro*-**19a**		87 [4.06 ± 0.12](3)	75 [4.13 ± 0.10](3)	97 [4.01 ± 0.12](3)	~300 [~3.5](3)
DL-*threo*-**19b**		2.4 [5.61 ± 0.04](3)	1.6 [5.80 ± 0.05](3)	1.6 [5.80 ± 0.07](3)	4.3 [5.37 ± 0.07](3)
DL-*erythro*-**19b**		29 [4.53 ± 0.06](3)	33 [4.48 ± 0.10](3)	31 [4.51 ± 0.04](3)	~100 [~4.0](3)
DL-*threo*-**19c**		6.9 [5.16 ± 0.11](4)	2.8 [5.56 ± 0.04](4)	1.6 [5.81 ± 0.05](4)	13 [4.90 ± 0.07](3)
DL-*erythro*-**19c**		99 [4.00 ± 0.05](3)	82 [4.09 ± 0.04](3)	28 [4.55 ± 0.06](3)	~100 [~4.0](3)
Hybrid analogues					
8		0.011 [7.97 ± 0.10](4)	0.036 [7.4 ±0.11](4)	0.094 [7.03 ± 0.08](4)	0.14 [6.86 ± 0.09](4)
9		0.012 [7.93 ± 0.05](3)	0.047 [7.33±0.12](4)	0.055 [7.26 ± 0.09](4)	0.080 [7.10 ±0.10](4)
L-TFB-TBOA analogues					
3a	n=0, *p*-CF_3	0.0036 [8.45 ± 0.09](3)	0.010 [8.00±0.14](3)	0.12 [6.93 ± 0.11](3)	0.040 [7.40±0.11](3)
3b	n=0, *o*-CF_3	0.031 [7.51 ± 0.13](4)	0.084 [7.07±0.06](4)	0.53 [6.28 ± 0.08](4)	0.17 [6.77 ± 0.09](4)
3c	n=0, *m*-CF_3	0.0061 [8.21 ± 0.03](4)	0.017 [7.78±0.07](4)	0.29 [6.54 ± 0.03](4)	0.071 [7.15±0.05](4)
3d	n=1, *p*-CF_3	0.021 [7.68 ± 0.02](4)	0.11 [6.97 ± 0.03](4)	0.13 [6.89 ± 0.03](4)	0.19 [6.71 ± 0.03](4)
3e	n=2, *p*-CF_3	0.0051 [8.9 ± 0.07](4)	0.018 [7.74±0.04](4)	0.11 [6.98 ± 0.04](4)	0.044 [7.35±0.05](4)

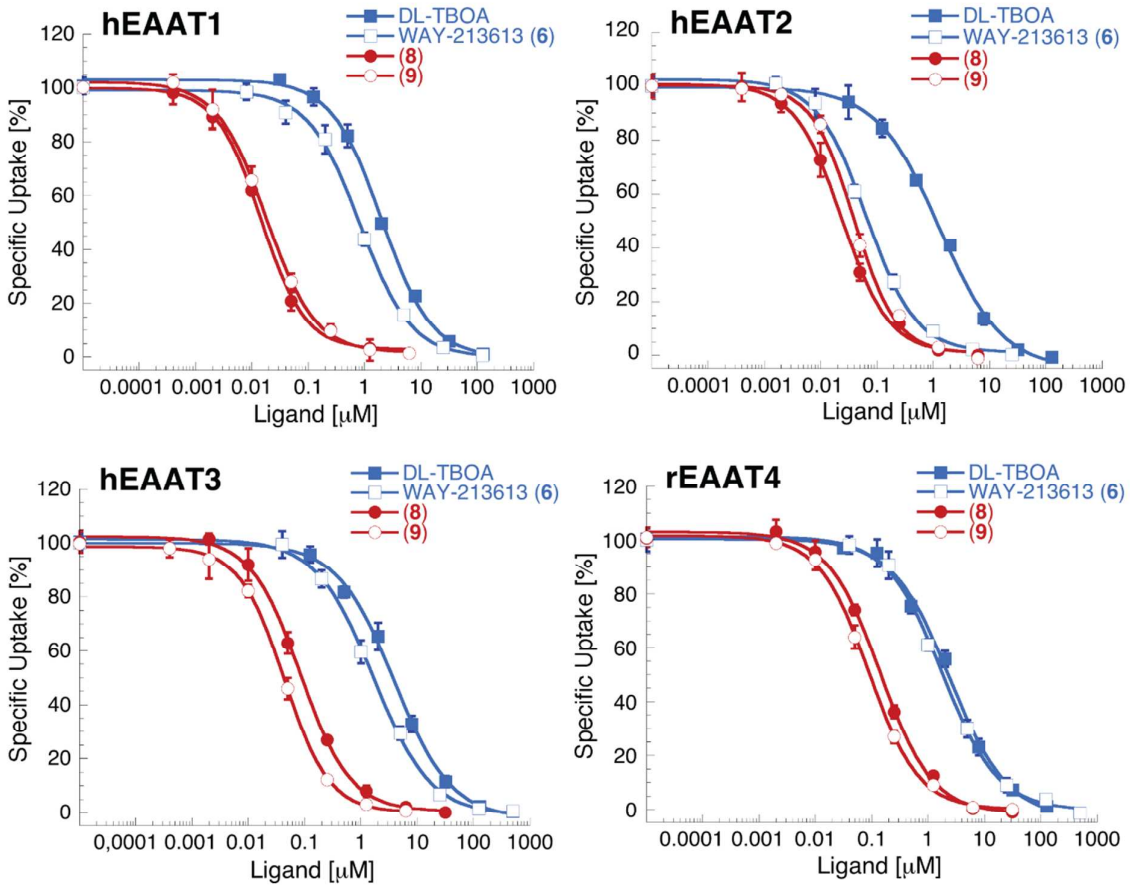


Figure 2. The functional properties of the hybrid analogues **8** and **9** as EAAT inhibitors. Representative concentration-inhibition curves for **8**, **9,** DL-TBOA (parent compound of both **8** and **9**) and WAY-213613 (**6**, parent compound of **8**) at hEAAT1-HEK293, hEAAT2-HEK293, hEAAT3-HEK293 and rEAAT4-tsA201 cells in the [^{3}H]-D-Asp uptake assay. Data are from a representative specific experiment and given as mean ± S.D. values (based on duplicate determinations).

CONCLUSION

We have presented the design, synthesis and pharmacological characterization of an elaborate series of 3-substituted Asp analogues as inhibitors of hEAAT1, hEAAT2, hEAAT3 and rEAAT4. Inspired by the potential of the Asp scaffold for the development of EAAT

inhibitors, various new Asp derivatives with (cyclo)alkyloxy and (hetero)aryloxy substituents at the C-3 position were synthesized using a key stereoselective enzymatic step. Remarkably, all these Asp derivatives were found to be potent non-substrate pan inhibitors of the EAATs. The functional properties exhibited by the Asp analogues also provide insight into the relation between ligand structure and EAAT transport. Whereas Asp and (L-*threo*)-3-hydroxy Asp (L-THA) are substrates of EAATs, it is clear from this series that basically any Asp derivative with a substituent at C-3 larger than a hydroxyl group will be a non-substrate inhibitor. Since all these compounds are Asp derivatives, it is reasonable to assume that they function as competitive inhibitors, like DL-TBOA,[25] and act through the substrate binding site in the transporters. On the other hand, judging from the fact that all 3-substituted Asp analogues comprising a wide range of substituents displayed comparable inhibitory potencies, the 3-substituent either does not contribute substantially to this inhibitory potency by forming interactions with residues in the substrate binding site of the transporter or alternatively all of the 3-substituents in the tested analogues are able to form similar interactions. Finally, our results confirm that the *threo* configuration of the 3-substituted Asp analogue is crucial for its high inhibitory activities at EAATs.

Unique EAAT inhibitors were developed by hybridization of the non-selective EAAT inhibitor L-TBOA (**2**) with the EAAT2-selective inhibitor WAY-213613 or the EAAT3-preferring inhibitor NBI-59159 to yield hybrid analogues **8** and **9**, respectively. Compounds **8** and **9** displayed significantly higher inhibitory activities at EAATs than both of their respective parent structures, although the hybridization in both cases leads to pan inhibitors of EAAT1-4. Thus, while the additional interactions with the transporter formed by

the benzyloxy group at the 3-position in **8**/**9** in both cases leads to increased inhibitory potency, it also seems to induce a binding conformation of the Asp analogue that dictates a different spatial orientation of the 4-(2-bromo-4,5-difluorophenoxy)phenyl/(9H-fluoren-2-yl) substituent than in WAY-213613/NBI-59159. We propose that this and other hybridization strategies could advance future design and development of more potent EAAT inhibitors.

We recently reported the synthesis and evaluation of L-TFB-TBOA-based inhibitors of the prokaryotic aspartate transporter Glt_{Tk} with photo-controlled activity, allowing the remote, reversible and spatiotemporally resolved regulation of transport.[31] Work is in progress to convert the hybrid compounds **8** and **9**, which were shown here to be highly potent non-selective EAAT inhibitors, into light-controlled glutamate transporter inhibitors by introducing a photoswitchable azobenzene moiety.

EXPERIMENTAL SECTION

Chemicals and general methods. All chemicals were purchased from commercial sources unless otherwise specified. All the solvents were of analytical reagent grade and were used without further purification. ^{1}H NMR, ^{13}C NMR and ^{19}F NMR analysis were performed on a Bruker Avance III 400/500 MHz spectrometer. High resolution mass spectra (HRMS) were recorded on a LTQ Orbitrap XL. Flash chromatography was performed on a GRACE X2 system with silica gel (200−300 mesh) purchased from Merck. High performance liquid chromatography (HPLC) analysis was performed with a Shimadzu LC-10AT HPLC with a

Shimadzu SP-M10A ELSD detector and a Shimadzu SPD-M10A photodiode array detector. Analytical HPLC was performed using a Kinetex C18 column (150 x 4.6 mm, 5 μm) with 5-95% MeCN gradient in H_2O (0.5% TFA) as mobile phase. The purity of all the target compounds was determined to be >95%. Chiral HPLC analysis for compound **13g** was perfomed using Nucleosil chiral-1 column (250 x 4 mm, 5 μm) with 0.5 mM aqueous $CuSO_4$ as mobile phase. Chiral HPLC analysis for compounds **13a** and **13k** was perfomed using CROWNPAK CR-I (+) column (150 x 3 mm, 5 μm) with isocratic MeCN/H_2O (98%, v/v, 0.5% TFA) as mobile phase.

Synthesis of hybrid compounds 8 and 9

(L-threo)-2-Amino-3-(benzyloxy)-4-[[4-(2-bromo-4,5-difluorophenoxy)phenyl]amino]-4-oxobutanoic acid (8)

To a stirred solution of **26** (68 mg, 0.1 mmol, see below) in dry DCM (2 mL), in an ice-bath, was added trifluoroacetic acid (2 mL) dropwise. After the complete addition of trifluoroacetic acid, the ice-bath was removed and the reaction was allowed to proceed at room temperature for further 6 h. After completion of the starting material, the solvent was removed *in vacuo* to provide pure **8** as a trifluoroacetate salt (brown solid, 34 mg, yield 52%). ^{1}H NMR (500 MHz, D_2O/DMSO-d_6): δ 7.74 (dd, J = 9.8, 8.3 Hz, 1H), 7.43 – 7.41 (m, 2H), 7.32 – 7.25 (m, 5H), 7.10 – 7.02 (m, 1H), 6.89 – 6.88 (m, 2H), 4.62 – 4.56 (m, 2H), 4.46 (d, J = 4.0 Hz, 1H), 4.16 (d, J = 3.9 Hz, 1H); ^{13}C NMR (126 MHz, DMSO-d_6): δ 168.1, 166.4, 152.4, 149.5 (dd, J = 8.2, 3.8 Hz), 148.5 (dd, J = 374.9, 13.9 Hz), 148.6 (dd, J = 372.3, 13.9 Hz), 136.9, 134.2, 128.2

(2C), 128.0 (2C), 127.9, 122.0, 121.9, 117.8 (2C), 115.3 (d, J = 21.4 Hz), 110.2 (d, J = 20.2 Hz), 108.6 (dd, J = 7.6, 3.8 Hz), 76.8, 72.1, 54.1; ^{19}F NMR (376 MHz, DMSO-d_6): δ -73.51 (s), -134.99 (dt, J = 20.6, 10.1 Hz), -141.25 (dt, J = 23.2, 9.0 Hz). HRMS: calcd. for $C_{23}H_{20}BrF_2N_2O_5$ $[M+H]^+$: 521.0518, found: 521.0520. HPLC: purity 98%, retention time 9.0 min.

(L-threo)-4-[(9H-fluoren-2-yl)amino]-2-amino-3-(benzyloxy)-4-oxobutanoic acid (9)

Hybrid compound **9** was prepared from **27** (100 mg, 0.18 mmol, see below) following a procedure similar to that used for compound **8**. The title product was obtained as a white solid (32 mg, 35%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.35 (s, 1H), 7.99 (s, 1H), 7.84 (t, J = 7.8 Hz, 2H), 7.58 (dd, J = 13.4, 7.9 Hz, 2H), 7.42 (d, J = 7.3 Hz, 2H), 7.38 – 7.34 (m, 3H), 7.32 – 7.27 (m, 2H), 4.70 (d, J = 11.7 Hz, 1H), 4.63 (d, J = 4.1 Hz, 1H), 4.59 (d, J = 11.7 Hz, 1H), 4.06 (bs, 1H), 3.92 (s, 2H); ^{13}C NMR (126 MHz, Methanol-d_4): δ 169.5, 168.4, 145.4, 144.5, 142.4, 140.0, 138.7, 137.7, 137.4, 129.7 (2C), 129.6, 127.9, 127.7, 126.0, 121.0, 120.6, 120.5, 118.6, 107.3, 77.7, 74.6, 56.1, 37.7. HRMS: calcd. for $C_{24}H_{23}N_2O_4$ $[M+H]^+$: 403.1652, found: 403.1653. HPLC: purity 98%, retention time 8.4 min.

Synthesis of dimethyl 2-substituted fumarate derivatives 11a-k. The synthesis of the dimethyl 2-substituted fumarate derivatives **11a-k** was based on a previously published procedure.[22,32] Briefly, to a stirred solution of dimethyl acetylenedicarboxylate (**10**, 568 mg, 4 mmol) in dichloromethane (30 mL) was added DABCO (45 mg, 0.4 mmol) followed by the appropriate alcohol (5 mmol). The reaction mixture was stirred at room temperature for 12 h.

The solvent was removed under reduced pressure to give the crude product as a dark brown oil. The *trans/cis* isomers of the products were separated by flash chromatography (silica gel) using 5% EtOAc/Petroleum ether (boiling point 40 to 60 °C) as eluent. The preferred *trans*-isomers **11a-k** (i.e., the fumarate derivatives) were used in further experiments.

Dimethyl 2-(cyclopropylmethoxy)fumarate (11a)

Clear oil. 230 mg (27% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.11 (s, 1H), 3.84 (d, J = 7.2 Hz, 2H), 3.72 (s, 3H), 3.63 (s, 3H), 1.16 – 1.08 (m, 1H), 0.49 – 0.45 (m, 2H), 0.21 – 0.18 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 164.5, 163.4, 154.1, 109.6, 78.4, 52.6, 51.4, 10.6, 3.0 (2C).

Dimethyl 2-(cyclobutylmethoxy)fumarate (11b)

Clear oil. 164 mg (18% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.11 (s, 1H), 4.00 (d, J = 6.7 Hz, 2H), 3.77 (s, 3H), 3.68 (s, 3H), 2.69 – 2.61 (m, 1H), 2.04 – 1.97 (m, 2H), 1.87 – 1.76 (m, 4H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 164.7, 163.4, 154.5, 108.9, 77.6, 52.7, 51.5, 35.1, 24.5 (2C), 18.3.

Dimethyl 2-(cyclopentylmethoxy)fumarate (11c)

Clear oil. 136 mg (14% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.15 (s, 1H), 3.96 (d, J = 6.9 Hz, 2H), 3.82 (s, 3H), 3.74 (s, 3H), 2.34 – 2.25 (m, 1H), 1.81 – 1.75 (m, 2H), 1.62 – 1.53 (m, 4H), 1.35 – 1.29 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 164.9, 163.7, 154.7, 108.6, 78.0, 52.9, 51.8, 39.8, 29.2 (2C), 25.6 (2C).

Dimethyl 2-(cyclohexylmethoxy)fumarate (11d)

Clear oil. 130 mg (13% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.13 (s, 1H), 3.88 (d, J = 6.4 Hz, 2H), 3.82 (s, 3H), 3.74 (s, 3H), 1.84 – 1.66 (m, 6H), 1.30 – 1.14 (m, 3H), 1.05 – 0.97 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 165.0, 163.7, 154.9, 108.1, 79.4, 52.9, 51.8, 38.5, 29.6 (2C), 26.6, 25.9 (2C).

Dimethyl 2-(cycloheptylmethoxy)fumarate (11e)

Clear oil. 83 mg (8% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.12 (s, 1H), 3.85 (d, J = 6.6 Hz, 2H), 3.81 (s, 3H), 3.72 (s, 3H), 1.93 – 1.86 (m, 1H), 1.83 – 1.77 (m, 2H), 1.69 – 1.63 (m, 2H), 1.58 – 1.54 (m, 2H), 1.51 – 1.39 (m, 4H), 1.27 – 1.20 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 164.9, 163.6, 154.7, 108.3, 79.2, 52.9, 51.7, 39.9, 30.7 (2C), 28.6 (2C), 26.5 (2C).

Dimethyl 2-(cyclooctylmethoxy)fumarate (11f)

Clear oil. 56 mg (5% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.12 (s, 1H), 3.85 (d, J = 6.6 Hz, 2H), 3.82 (s, 3H), 3.73 (s, 3H), 1.97 – 1.91 (m, 1H), 1.74 – 1.65 (m, 4H), 1.62 – 1.46 (m, 8H), 1.36 – 1.28 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 164.9, 163.7, 154.8, 108.2, 79.6, 52.9, 51.7, 38.2, 29.1 (2C), 27.0 (2C), 26.5, 25.5 (2C).

Dimethyl 2-(thiophen-3-ylmethoxy)fumarate (11g)

Light yellow solid. 275 mg (27% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.35 – 7.34 (m, 1H), 7.30 (dd, J = 5.0, 3.0 Hz, 1H), 7.16 (dd, J = 5.0, 1.3 Hz, 1H), 6.23 (s, 1H), 5.22 (s, 2H), 3.82 (s, 3H), 3.74 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 164.7, 163.4, 153.6, 137.2, 127.6, 126.3, 124.6, 110.4, 70.2, 53.0, 51.8.

Dimethyl 2-(thiophen-2-ylmethoxy)fumarate (11h)

Light yellow solid. 310 mg (38% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.33 (dd, J = 5.1, 1.2 Hz, 1H), 7.08 (d, J = 3.4 Hz, 1H), 6.98 (dd, J = 5.1, 3.5 Hz, 1H), 6.28 (s, 1H), 5.37 (s, 2H), 3.83 (s, 3H), 3.75 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 164.7, 163.4, 153.0, 138.1, 128.53, 127.4, 126.9, 111.5, 69.2, 53.0, 51.9.

Dimethyl 2-(furan-3-ylmethoxy)fumarate (11i)

Light yellow solid. 345 mg (36% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.46 (dd, J = 1.7, 0.9 Hz, 1H), 7.39 (t, J = 1.7 Hz, 1H), 6.49 (d, J = 1.2 Hz, 1H), 6.24 (s, 1H), 5.10 (s, 2H), 3.82 (s, 3H), 3.74 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 164.7, 163.5, 153.5, 143.6, 141.8, 120.8, 110.6 (2C), 66.6, 53.0, 51.8.

Dimethyl 2-(furan-2-ylmethoxy)fumarate (11j)

Light yellow solid. 382 mg (40% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.42 (dd, J = 1.9, 0.9 Hz, 1H), 6.41 (d, J = 3.3 Hz, 1H), 6.35 (dd, J = 3.3, 1.8 Hz, 1H), 6.29 (s, 1H), 5.17 (s, 2H), 3.83 (s, 3H), 3.73 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 164.6, 163.4, 153.0, 149.7, 143.7, 111.4, 111.3, 110.6, 66.7, 53.0, 51.7.

Dimethyl 2-(prop-2-yn-1-yloxy)fumarate (11k)

Clear oil. 295 mg (37% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.35 (s, 1H), 4.90 (d, J = 2.5 Hz, 2H), 3.84 (s, 3H), 3.75 (s, 3H), 2.55 (t, J = 2.5 Hz, 1H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 164.5, 163.2, 152.0, 111.6, 82.9, 77.1, 60.4, 53.1, 52.0.

Synthesis of 2-substituted fumaric acid derivatives 12a-k. To a stirred solution of dimethyl 2-substituted fumarate **11** (**11a**, 214 mg, 1.0 mmol; **11b**, 150 mg, 0.66 mmol; **11c**, 121 mg,

0.50 mmol; **11d**, 115 mg, 0.45 mmol; **11e**, 70 mg, 0.26 mmol; **11f**, 56 mg, 0.20 mmol; **11g**, 112 mg, 0.44 mmol; **11h**, 87 mg, 0.34 mmol; **11i**, 62 mg, 0.26 mmol; **11j**, 70 mg, 0.30 mmol; **11k**, 198 mg, 1.0 mmol; respectively) in EtOH (2 mL) was added 2 M NaOH (2 mL), and the reaction mixture was heated to reflux for 2 h. After completion of the hydrolysis, the reaction mixture was allowed to cool to room temperature followed by removing the EtOH under vacuum. For compounds **11a-f** and **11k**, the resulting aqueous layer was acidified with HCl (1 M) until white precipitates appeared (pH 1). The precipitates were filtered and dried under vacuum to provide pure products **12a-f** and **12k** as white solids. For compounds **12g-j**, the resulting aqueous solutions were directly used for the next enzymatic step after adjusting the pH of the solution to pH 9.5 with 1 M HCl.

2-(Cyclopropylmethoxy)fumaric acid (12a)

White solid. 150 mg (81% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ 6.02 (s, 1H), 3.85 (d, J = 7.1 Hz, 2H), 1.13 – 1.07 (m, 1H), 0.51 – 0.47 (m, 2H), 0.25 – 0.22 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6): δ 165.4, 164.2, 153.8, 109.8, 76.8, 10.6, 3.0 (2C).

2-(Cyclobutylmethoxy)fumaric acid (12b)

White solid. 110 mg (84% yield). ^{1}H NMR (500 MHz, Methanol-d_4): δ 6.07 (s, 1H), 4.02 (d, J = 5.6 Hz, 2H), 2.68 – 2.62 (m, 1H), 2.04 – 2.00 (m, 2H), 1.91 – 1.83 (m, 4H); ^{13}C NMR (126 MHz, Methanol-d_4): δ 167.9, 165.8, 156.5, 110.0, 78.2, 36.6, 25.5 (2C), 19.2.

2-(Cyclopentylmethoxy)fumaric acid (12c)

White solid. 77 mg (72% yield). ^{1}H NMR (500 MHz, Methanol-d_4): δ 6.00 (s, 1H), 3.90 (d, J

= 7.0 Hz, 2H), 2.24 – 2.15 (m, 1H), 1.72 – 1.66 (m, 2H), 1.57 – 1.47 (m, 4H), 1.31 – 1.23 (m, 2H); ^{13}C NMR (126 MHz, Methanol-d_4): δ 168.0, 165.8, 156.7, 109.3, 78.4, 41.0, 30.1 (2C), 26.4 (2C).

2-(Cyclohexylmethoxy)fumaric acid (12d)

White solid. 78 mg (76% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ 5.93 (s, 1H), 3.81 (d, J = 6.4 Hz, 2H), 1.74 – 1.56 (m, 6H), 1.24 – 1.10 (m, 3H), 1.00 – 0.92 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6): δ 165.8, 164.6, 154.8, 108.5, 78.2, 38.3, 29.3 (2C), 26.3, 25.6 (2C).

2-(Cycloheptylmethoxy)fumaric acid (12e)

White solid. 42 mg (67% yield). ^{1}H NMR (500 MHz, Methanol-d_4): δ 6.05 (s, 1H), 3.86 (d, J = 6.4 Hz, 2H), 1.88 – 1.78 (m, 3H), 1.70 – 1.65 (m, 2H), 1.60 – 1.43 (m, 6H), 1.29– 1.23 (m, 2H); ^{13}C NMR (126 MHz, Methanol-d_4): δ 168.0, 165.8, 156.5, 109.1, 79.6, 41.1, 31.7 (2C), 29.7 (2C), 27.5 (2C).

2-(Cyclooctylmethoxy)fumaric acid (12f)

White solid. 20 mg (39% yield). ^{1}H NMR (500 MHz, Methanol-d_4): δ 6.03 (s, 1H), 3.82 (d, J = 6.7 Hz, 2H), 1.93 – 1.86 (m, 1H), 1.72 – 1.64 (m, 4H), 1.59 – 1.44 (m, 8H), 1.33 – 1.27 (m, 2H); ^{13}C NMR (126 MHz, Methanol-d_4): δ 168.0, 165.8, 156.6, 109.0, 80.1, 39.3, 30.2 (2C), 28.0 (2C), 27.7, 26.5 (2C).

Disodium 2-(thiophen-3-ylmethoxy)fumarate (12g)

^{1}H NMR (500 MHz, DMSO-d_6): δ 7.53 – 7.50 (m, 2H), 7.15 (dd, J = 4.9, 1.3 Hz, 1H), 6.05 (s,

1H), 5.10 (s, 2H); ^{13}C NMR (126 MHz, DMSO-d_6): δ 165.3, 164.0, 153.5, 137.7, 127.6, 126.6, 124.2, 110.2, 69.1.

Disodium 2-(thiophen-2-ylmethoxy)fumarate (12h)

^{1}H NMR (500 MHz, D_2O): δ 7.42 (dd, J = 5.0, 1.3 Hz, 1H), 7.08 – 7.05 (m, 1H), 7.00 (dd, J = 5.1, 3.5 Hz, 1H), 5.91 (s, 1H), 5.14 (s, 2H); ^{13}C NMR (126 MHz, D_2O): δ 173.9, 170.9, 152.4, 138.7, 128.6, 127.5, 126.9, 114.4, 66.6.

Disodium 2-(furan-3-ylmethoxy)fumarate (12i)

^{1}H NMR (500 MHz, D_2O): δ 7.50 (dd, J = 1.6, 0.8 Hz, 1H), 7.44 (t, J = 1.9 Hz, 1H), 6.49 (s, 1H), 5.89 (s, 1H), 4.85 (s, 2H); ^{13}C NMR (126 MHz, D_2O): δ 174.0, 171.0, 152.7, 143.6, 142.0, 120.8, 113.8, 110.6, 63.8.

Disodium 2-(furan-2-ylmethoxy)fumarate (12j)

^{1}H NMR (500 MHz, D_2O): δ 7.45 (dd, J = 1.9, 0.8 Hz, 1H), 6.38 (dd, J = 3.3, 0.8 Hz, 1H), 6.36 (dd, J = 3.3, 1.9 Hz, 1H), 5.91 (s, 1H), 4.89 (s, 2H); ^{13}C NMR (126 MHz, D_2O): δ 173.8, 170.8, 152.2, 150.0, 143.8, 114.6, 111.2, 110.5, 64.2.

2-(Prop-2-yn-1-yloxy)fumaric acid (12k)

White solid. 142 mg (83% yield). ^{1}H NMR (500 MHz, DMSO-d_6): δ 6.14 (s, 1H), 4.82 (d, J = 2.5 Hz, 2H), 3.63 (t, J = 2.5 Hz, 1H); ^{13}C NMR (126 MHz, DMSO-d_6): δ 165.1, 163.7, 151.8, 111.7, 79.4, 78.4, 59.1.

Enzymatic synthesis of 3-substituted aspartic acid derivatives 13a-k. Reaction mixtures

consisted of 2-substituted fumaric acid derivatives **12** (**12a**, 21 mg, 0.11 mmol; **12b**, 40 mg, 0.20 mmol; **12c**, 42 mg, 0.20 mmol; **12d**, 28 mg, 0.12 mmol; **12e**, 23 mg, 0.095 mmol; **12f**, 12 mg, 0.047 mmol; **12g**, 0.44 mmol; **12h**, 0.34 mmol; **12i**, 0.26 mmol; **12j**, 0.30 mmol; **12k**, 9.7 mg, 0.056 mmol; respectively) in buffer (5 M NH_3/NH_4Cl and 20 mM $MgCl_2$, pH adjusted to pH 9.5). The reaction was started by addition of freshly purified MAL-L384A mutant (0.01 mol%),[21,22] and the reaction mixture was incubated at room temperature for 24 h. After completion of the reaction, the enzyme was inactivated by heating to 70 °C for 10 min. The progress and conversion yield of the enzymatic reaction was monitored by ^{1}H NMR spectroscopy by comparing signals of substrates and corresponding products. For a typical purification procedure, the precipitated enzyme was removed by filtration. The filtrate was acidified with 1 M HCl to pH 1 and loaded onto a column packed with cation-exchange resin (10 g of Dowex 50W X8, 50-100 mesh), which was pre-treated with 2 M aqueous ammonia (4 column volumes), 1 M HCl (2 column volumes) and distilled water (4 column volumes). The column was washed with distilled water (2 column volumes) and the desired product was eluted with 2 M aqueous ammonia (4 column volumes). The ninhydrin-positive fractions were collected and lyophilized to yield the product as ammonium salt.

(L-threo)-3-(Cyclopropylmethoxy)aspartate (13a)

White solid. 7 mg (conversion 88%, isolated yield 31%). ^{1}H NMR (500 MHz, D_2O): δ 4.38 (d, J = 2.5 Hz, 1H), 3.98 (d, J = 2.5 Hz, 1H), 3.45 (dd, J = 10.7, 7.0 Hz, 1H), 3.28 (dd, J = 10.7, 7.2 Hz, 1H), 1.05 – 0.97 (m, 1H), 0.53 – 0.46 (m, 2H), 0.20 – 0.15 (m, 2H); ^{13}C NMR (126 MHz, D_2O): δ 176.1, 171.6, 77.4, 75.7, 56.6, 9.5, 2.6, 2.1. HRMS: calcd. for $C_8H_{14}NO_5$

$[M+H]^+$: 204.0866, found: 204.0861. HPLC: purity 96%, retention time 4.2 min. Comparison of the ^1H NMR data of **13a** with the ^1H NMR data of chemically prepared racemic (DL-*threo*)-**19a** and (DL-*erythro*)-**19a** showed that the *de* of product **13a** was 97% (Figure S1). Chiral HPLC analysis: CROWNPAK CR-I (+) 150 x 3 mm. Phase A: ACN+0.5% TFA, phase B: H_2O+0.5% TFA, A/B = 98:2. Flow rate 0.4 mL/min, column temperature 25 °C, detected by ELSD at 35 °C, t_R = 2.8 min, *ee* >99% (Figure S62).

3-(Cyclobutylmethoxy)aspartate (13b)

White solid. 25 mg (conversion 89%, isolated yield 58%). ^1H NMR (500 MHz, D_2O): δ 4.24 (d, J = 2.4 Hz, 1H), 3.95 (d, J = 2.4 Hz, 1H), 3.60 (dd, J = 9.8, 6.8 Hz, 1H), 3.39 (dd, J = 9.8, 7.3 Hz, 1H), 2.56 – 2.47 (m, 1H), 2.01 – 1.95 (m, 2H), 1.86 – 1.77 (m, 2H), 1.68 – 1.61 (m, 2H); ^{13}C NMR (126 MHz, D_2O): δ 176.4, 171.7, 78.3, 75.6, 56.5, 34.1, 24.5, 24.2, 18.0. HRMS: calcd. for $C_9H_{16}NO_5$ $[M+H]^+$: 218.1023, found: 218.1022. HPLC: purity 98%, retention time 6.5 min.

3-(Cyclopentylmethoxy)aspartate (13c)

White solid. 19 mg (conversion 85%, isolated yield 39%). ^1H NMR (500 MHz, D_2O): δ 4.28 (d, J = 2.5 Hz, 1H), 3.98 (d, J = 2.5 Hz, 1H), 3.54 (dd, J = 9.4, 7.0 Hz, 1H), 3.25 (dd, J = 9.4, 7.7 Hz, 1H), 2.17 – 2.11 (m, 1H), 1.70 – 1.63 (m, 2H), 1.56 – 1.47 (m, 4H), 1.22 – 1.13 (m, 2H); ^{13}C NMR (126 MHz, D_2O): δ 176.2, 171.5, 78.2, 75.7, 56.4, 38.5, 28.9, 28.7, 24.9 (2C). HRMS: calcd. for $C_{10}H_{18}NO_5$ $[M+H]^+$: 232.1180, found: 232.1180. HPLC: purity 97%, retention time 9.3 min.

3-(Cyclohexylmethoxy)aspartate (13d)

White solid. 17 mg (conversion 90%, isolated yield 56%). ^{1}H NMR (500 MHz, D_2O): δ 4.28 (d, J = 2.6 Hz, 1H), 3.99 (d, J = 2.5 Hz, 1H), 3.48 (dd, J = 9.6, 6.3 Hz, 1H), 3.20 (dd, J = 9.6, 6.9 Hz, 1H), 1.69 – 1.55 (m, 6H), 1.25 – 1.11 (m, 3H), 0.91 – 0.85 (m, 2H); ^{13}C NMR (126 MHz, D_2O): δ 175.9, 171.3, 78.1, 76.9, 56.3, 37.0, 29.3, 29.1, 26.1, 25.2 (2C). HRMS: calcd. for $C_{11}H_{20}NO_5$ $[M+H]^+$: 246.1336, found: 246.1336. HPLC: purity 98%, retention time 7.8 min.

3-(Cycloheptylmethoxy)aspartate (13e)

White solid. 7 mg (conversion 60%, isolated yield 26%). ^{1}H NMR (500 MHz, D_2O): δ 4.27 (d, J = 2.1 Hz, 1H), 3.97 (d, J = 2.2 Hz, 1H), 3.45 (dd, J = 9.5, 6.6 Hz, 1H), 3.17 (dd, J = 9.5, 7.2 Hz, 1H), 1.79 – 1.72 (m, 1H), 1.69 – 1.58 (m, 4H), 1.55 – 1.52 (m, 2H), 1.49 – 1.37 (m, 4H), 1.17 – 1.09 (m, 2H); ^{13}C NMR (126 MHz, D_2O): δ 176.2, 171.4, 78.2, 76.5, 56.4, 38.3, 30.3 (2C), 28.2 (2C), 25.9 (2C). HRMS: calcd. for $C_{12}H_{22}NO_5$ $[M+H]^+$: 260.1492, found: 260.1494. HPLC: purity 95%, retention time 10.0 min.

(L-threo)-3-(Thiophen-3-ylmethoxy)aspartate (13g)

Light yellow solid. 47 mg (conversion 98%, isolated yield 47%). ^{1}H NMR (500 MHz, D_2O): δ 7.43 – 7.42 (m, 1H), 7.39 – 7.38 (m, 1H), 7.11 (dd, J = 4.9, 1.3 Hz, 1H), 4.70 (d, J = 11.9 Hz, 1H), 4.50 (d, J = 11.9 Hz, 1H), 4.29 (d, J = 2.2 Hz, 1H), 3.96 (d, J = 2.2 Hz, 1H); ^{13}C NMR (126 MHz, D_2O): δ 175.8, 171.4, 137.7, 127.8, 126.6, 124.7, 77.0, 67.2, 56.5. HRMS: calcd. for $C_9H_{12}NO_5$ $[M+H]^+$: 246.0431, found: 246.0430. HPLC: purity 99%, retention time 5.2 min.

Comparison of the ^{1}H NMR data of **13g** with the ^{1}H NMR data of chemically prepared racemic racemic (DL-*threo*)-**19b** and (DL-*erythr*o)-**19b** showed that the *de* of product **13g** was >98% (Figure S2). Chiral HPLC conditions: Nucleosil chiral-1 column with 0.5 mM aqueous $CuSO_4$ solution as mobile phase with a flow rate of 1.0 mL/min at 60 °C, UV detection at 254 nm, t_R = 7.2 min, *ee* >99% (Figure S63).

3-(Thiophen-2-ylmethoxy)aspartate (13h)

Light yellow solid. 40 mg (conversion 98%, isolated yield 48%). ^{1}H NMR (500 MHz, D_2O): δ 7.46 (dd, J = 5.0, 1.3 Hz, 1H), 7.11 (dd, J = 3.4, 1.1 Hz, 1H), 7.04 (dd, J = 5.0, 3.4 Hz, 1H), 4.88 (d, J = 12.6 Hz, 1H), 4.69 (d, J = 12.6 Hz, 1H), 4.35 (d, J = 2.3 Hz, 1H), 3.99 (d, J = 2.3 Hz, 1H); ^{13}C NMR (126 MHz, D_2O): δ 175.5, 171.3, 139.0, 128.2, 127.3, 127.0, 76.5, 66.3, 56.4. HRMS: calcd. for $C_9H_{12}NO_5$ $[M+H]^+$: 246.0431, found: 246.0430. HPLC: purity 98%, retention time 5.1 min.

3-(Furan-3-ylmethoxy)aspartate (13i)

Light yellow solid. 21 mg (conversion 98%, isolated yield 35%). ^{1}H NMR (500 MHz, D_2O): δ 7.54 – 7.53 (m, 1H), 7.49 (t, J = 1.7 Hz, 1H), 6.51 – 6.48 (m, 1H), 4.59 (d, J = 12.1 Hz, 1H), 4.40 (d, J = 12.1 Hz, 1H), 4.31 (d, J = 2.3 Hz, 1H), 3.98 (d, J = 2.3 Hz, 1H); ^{13}C NMR (126 MHz, D_2O): δ 175.8, 171.4, 143.8, 141.9, 120.8, 110.5, 76.6, 63.4, 56.5. HRMS: calcd. for $C_9H_{12}NO_6$ $[M+H]^+$: 230.0659, found: 230.0659. HPLC: purity 96%, retention time 4.4 min.

3-(Furan-2-ylmethoxy)aspartate (13j)

Light yellow solid. 26 mg (conversion 98%, isolated yield 39%). ^{1}H NMR (500 MHz, D_2O): δ

7.51 (dd, *J* = 1.9, 0.9 Hz, 1H), 6.47 (d, *J* = 3.2 Hz, 1H), 6.42 (dd, *J* = 3.2, 1.9 Hz, 1H), 4.62 (d, *J* = 13.2 Hz, 1H), 4.50 (d, *J* = 13.2 Hz, 1H), 4.32 (d, *J* = 2.4 Hz, 1H), 3.97 (d, *J* = 2.4 Hz, 1H); ^{13}C NMR (126 MHz, D_2O): δ 175.4, 171.3, 150.1, 143.8, 111.0, 110.4, 76.6, 63.9, 56.4. HRMS: calcd. for $C_9H_{12}NO_6$ $[M+H]^+$: 230.0659, found: 230.0659. HPLC: purity 98%, retention time 4.4 min.

(L-threo)-3-(Prop-2-yn-1-yloxy)aspartate (13k)

White solid. 5 mg (conversion 83%, isolated yield 47%). ^{1}H NMR (500 MHz, D_2O): δ 4.49 (d, *J* = 2.3 Hz, 1H), 4.30 (dd, *J* = 16.0, 2.4 Hz, 1H), 4.20 (dd, *J* = 16.0, 2.4 Hz, 1H), 4.02 (d, *J* = 2.3 Hz, 1H), 2.83 (t, *J* = 2.4 Hz, 1H); ^{13}C NMR (126 MHz, D_2O): δ 175.3, 171.4, 76.8, 76.3, 76.3, 57.7, 56.5. HRMS: calcd. for $C_7H_{10}NO_5$ $[M+H]^+$: 188.0554, found: 188.0550. HPLC: purity 97%, retention time 2.0 min. Comparison of the ^{1}H NMR data of **13k** with the ^{1}H NMR data of chemically prepared racemic (DL-*threo*)-**19c** and (DL-*erythr*o)-**19c** showed that the *de* of product **13k** was >98% (Figure S3). Chiral HPLC analysis: CROWNPAK CR-I (+) 150 x 3 mm. Phase A: ACN+0.5% TFA, phase B: H_2O+0.5% TFA, A/B = 98:2. Flow rate 0.4 mL/min, column temperature 25 °C, detected by ELSD at 35 °C, t_R = 3.2 min, *ee* >99% (Figure S64).

Synthesis of (DL-*threo*)- and (DL-*erythro*)-3-substituted Asp derivatives

(DL-threo)-Dimethyl 2-[(tert-butoxycarbonyl)amino]-3-hydroxy succinate (17)

The chemical synthesis of compound (DL-*threo*)-**16** has been described elsewhere.[23] To a stirred solution of **16** (622 mg, 2 mmol) in THF/MeOH (1:1, 30 mL) was added Pd/C 10% (50.0 mg) under nitrogen atmosphere. The reaction was stirred under H_2 atmosphere (balloon)

for 2 h at room temperature. After completion of the reaction, the reaction mixture was filtered through Celite and washed with MeOH (5 mL). The filtrate was concentrated under vacuum to provide a colorless oil which was directly used for the next step without purification. To a stirred solution of the colorless oil in dry DCM (20 mL) was added TEA (300 mg, 3 mmol) and Boc_2O (654 mg, 3 mmol) under cooling in an ice-bath. After 10 minutes, the cooling was removed and the reaction mixture was stirred at room temperature for further 24 h. After completion of the reaction, the reaction mixture was diluted with DCM (20 mL), and washed with 0.5 M HCl (50 mL), saturated $NaHCO_3$ solution (50 mL) and brine (50 mL). The organic layer was dried over Na_2SO_4 and concentrated under vacuum to give crude product **17**, which was purified *via* flash chromatography (EtOAc/Petroleum ether, 15%, v/v) to provide pure **17** as a white solid (460 mg, two-step yield 83%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.29 (d, J = 9.0 Hz, 1H), 4.78 (d, J = 9.3 Hz, 1H), 4.69 (dd, J = 5.7, 2.0 Hz, 1H), 3.82 (s, 3H), 3.80 (s, 3H), 3.24 (d, J = 5.5 Hz, 1H), 1.42 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 172.4, 170.0, 155.4, 80.4, 71.2, 56.2, 53.2, 53.0, 28.2 (3C). HRMS: calcd. for $C_{11}H_{19}NO_7Na$ $[M+Na]^+$: 300.1054, found: 300.1052.

Dimethyl 2-(cyclopropylmethoxy)-3-[(tert-butoxycarbonyl)amino]succinate (18a)

To a stirred solution of compound **17** (195 mg, 0.70 mmol) in dry DMF (3 mL) was added bromomethylcyclopropane (191 mg, 1.4 mmol) at -20 °C. After 10 min, NaH (60% in mineral oil, 28 mg, 0.70 mmol) was added to the reaction mixture. The reaction mixture was stirred at -20 °C for 4 h and stirred at 4 °C for further 8 h. After completion of the reaction, the reaction mixture was quenched with cold water, extracted with EtOAc (30 mL x 3), washed with brine

(50 mL x 3), and dried over Na_2SO_4. The solvent was evaporated to provide crude product, which was purified *via* flash chromatography (EtOAc/Petroleum ether, 10%, v/v) to give pure (DL-*threo*)-**18a** (26 mg, 11%) and (DL-*erythro*)-**18a** (9 mg, 4%) as clear oil.

(DL-*threo*)-**18a:** ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.33 (d, J = 9.9 Hz, 1H), 4.80 (dd, J = 10.0, 2.4 Hz, 1H), 4.53 (d, J = 2.1 Hz, 1H), 3.77 (s, 3H), 3.74 (s, 3H), 3.50 (dd, J = 10.6, 6.9 Hz, 1H), 3.21 (dd, J = 10.5, 7.0 Hz, 1H), 1.41 (s, 9H), 1.02 – 0.95 (m, 1H), 0.54 – 0.46 (m, 2H), 0.19 – 0.11 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 170.1, 170.0, 155.6, 80.3, 77.6, 76.0, 56.2, 52.8, 52.5, 28.3 (3C), 10.2, 3.4, 2.7. HRMS: calcd. for $C_{15}H_{25}NO_7Na$ $[M+Na]^+$: 354.1523, found: 354.1522.

(DL-*erythro*)-**18a:** ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.43 (d, J = 8.9 Hz, 1H), 4.85 (dd, J = 8.9, 3.4 Hz, 1H), 4.35 (d, J = 3.4 Hz, 1H), 3.77 (s, 3H), 3.73 (s, 3H), 3.56 (dd, J = 10.7, 6.9 Hz, 1H), 3.34 (dd, J = 10.7, 7.0 Hz, 1H), 1.45 (s, 9H), 1.06 – 1.00 (m, 1H), 0.56 – 0.49 (m, 2H), 0.24 – 0.17 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 170.1, 169.5, 155.3, 80.4, 78.3, 76.2, 55.7, 52.8, 52.4, 28.4 (3C), 10.2, 3.5, 2.7. HRMS: calcd. for $C_{15}H_{25}NO_7Na$ $[M+Na]^+$: 354.1523, found: 354.1523.

Dimethyl 2-(thiophen-3-ylmethoxy)-3-[(tert-butoxycarbonyl)amino]succinate (18b)

To a stirred solution of compound **17** (197 mg, 0.71 mmol) in dry DMF (3 mL) was added 3-(bromomethyl)thiophene (251 mg, 1.42 mmol) at -20 °C. After 10 min, NaH (60% in mineral oil, 28.4 mg, 0.71 mmol) was added to the reaction mixture. The reaction mixture was stirred at -20 °C for 4 h and stirred at 4 °C for further 8 h. After completion of the reaction, the reaction mixture was quenched with cold water, extracted with EtOAc (30 mL x 3),

washed with brine (50 mL x 3), and dried over Na_2SO_4. The solvent was evaporated to provide crude product, which was purified *via* flash chromatography (EtOAc/Petroleum ether, 10%, v/v) to give (DL-*threo*)-**18b** (140 mg, 53%) and (DL-*erythro*)-**18b** (27 mg, 10%) as clear oil.

(DL-*threo*)-**18b:** ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.30 (dd, J = 5.0, 2.9 Hz, 1H), 7.18 (dd, J = 3.0, 1.1 Hz, 1H), 6.98 (dd, J = 4.9, 1.3 Hz, 1H), 5.34 (d, J = 10.0 Hz, 1H), 4.82 – 4.77 (m, 2H), 4.47 (d, J = 2.3 Hz, 1H), 4.43 (d, J = 12.3 Hz, 1H), 3.77 (s, 3H), 3.65 (s, 3H), 1.42 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 169.9, 169.8, 155.6, 137.7, 127.7, 126.4, 124.2, 80.4, 76.6, 67.8, 56.1, 52.8, 52.6, 28.3 (3C). HRMS: calcd. for $C_{16}H_{24}NO_7S$ $[M+H]^+$: 374.1268, found: 374.1267.

(DL-*erythro*)-**18b:** ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.30 (dd, J = 4.9, 2.9 Hz, 1H), 7.22 (d, J = 2.8 Hz, 1H), 7.07 (dd, J = 5.0, 1.2 Hz, 1H), 5.33 (d, J = 8.7 Hz, 1H), 4.87 (dd, J = 8.6, 3.0 Hz, 1H), 4.84 (d, J = 12.3 Hz, 1H), 4.57 (d, J = 12.3 Hz, 1H), 4.27 (d, J = 3.1 Hz, 1H), 3.79 (s, 3H), 3.73 (s, 3H), 1.43 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 169.7, 169.2, 155.0, 137.8, 127.3, 126.3, 123.8, 80.3, 77.4, 68.2, 55.6, 52.7, 52.3, 28.3 (3C). HRMS: calcd. for $C_{16}H_{24}NO_7S$ $[M+H]^+$: 374.1268, found: 374.1266.

Dimethyl 2-(prop-2-yn-1-yloxy)-3-[(tert-butoxycarbonyl)amino]succinate (18c)

To a stirred solution of compound **17** (200 mg, 0.72 mmol) in dry DMF (3 mL) was added propargyl bromide (80% in toluene, 156 µL, 1.44 mmol) at -20 °C. After 10 min, NaH (60% in mineral oil, 29 mg, 0.72 mmol) was added to the reaction mixture. The reaction mixture was stirred at -20 °C for 4 h and stirred at 4 °C for further 8 h. After completion of the

reaction, the reaction mixture was quenched with cold water, extracted with EtOAc (30 mL x 3), washed with brine (50 mL x 3), and dried over Na_2SO_4. The solvent was evaporated to provide crude product, which was purified *via* flash chromatography (EtOAc/Petroleum ether, 10%, v/v) to give (DL-*threo*)-**18c** (54 mg, 24%) and (DL-*erythro*)-**18c** (25 mg, 11%) as clear oil.

(DL-*threo*)-**18c:** ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.29 (d, J = 9.8 Hz, 1H), 4.87 (dd, J = 9.9, 2.4 Hz, 1H), 4.78 (d, J = 2.3 Hz, 1H), 4.37 (dd, J = 16.3, 2.4 Hz, 1H), 4.20 (dd, J = 16.2, 2.4 Hz, 1H), 3.78 (s, 3H), 3.77 (s, 3H), 2.45 (t, J = 2.4 Hz, 1H), 1.41 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 169.7, 169.4, 155.5, 80.4, 78.1, 76.0, 75.8, 57.9, 56.0, 52.9, 52.7, 28.3 (3C). HRMS: calcd. for $C_{14}H_{21}NO_7Na$ $[M+Na]^+$: 338.1210, found: 338.1211.

(DL-*erythro*)-**18c:** ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.44 (d, J = 8.7 Hz, 1H), 4.92 (dd, J = 8.7, 2.9 Hz, 1H), 4.58 (d, J = 2.9 Hz, 1H), 4.43 (dd, J = 16.3, 2.4 Hz, 1H), 4.31 (dd, J = 16.3, 2.4 Hz, 1H), 3.79 (s, 3H), 3.72 (s, 3H), 2.45 (t, J = 2.4 Hz, 1H), 1.45 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 169.4, 169.0, 155.3, 80.4, 78.3, 76.6, 76.2, 58.4, 55.7, 52.9, 52.5, 28.4 (3C). HRMS: calcd. for $C_{14}H_{21}NO_7Na$ $[M+Na]^+$: 338.1210, found: 338.1211.

(DL-threo)-3-(Cyclopropylmethoxy)aspartate [(DL-threo)-19a]

To a stirred solution of (DL-*threo*)-**18a** (26 mg, 0.08 mmol) in dry DCM (2 mL) was added trifluoroacetic acid (0.8 mL) dropwise under cooling in an ice-bath. After the complete addition of trifluoroacetic acid, the ice-bath was removed and the reaction was allowed to proceed at room temperature for further 1.5 h. After completion of the starting material, the solvent was removed in vacuo to provide deBoc product quantitatively as a clear oil, which

was directly used for the next step without purification.

To a stirred solution of the clear oil in THF/H_2O (1:1, each 1 mL) was added LiOH (9.6 mg, 0.40 mmol), and the reaction mixture was stirred at room temperature for 2 h. Volatiles were removed *in vacuo*, and the residue was washed with EtOAc (1 mL). The aqueous layer was acidified with 1 M HCl (until pH=1) and loaded onto a column packed with cation-exchange resin (10 g of Dowex 50W X8, 50-100 mesh), which was pre-treated with 2 M aqueous ammonia (4 column volumes), 1 M HCl (2 column volumes) and distilled water (4 column volumes). The column was washed with distilled water (2 column volumes) and the product was eluted with 2 M aqueous ammonia (2 column volumes). The ninhydrin-positive fractions were collected and lyophilized to yield the desired product (DL-*threo*)-**19a** as ammonium salt (white solid, 7 mg, two-step yield of 37%). ^{1}H NMR (500 MHz, D_2O): δ 4.40 (d, J = 2.5 Hz, 1H), 4.00 (d, J = 2.5 Hz, 1H), 3.47 (dd, J = 10.7, 7.0 Hz, 1H), 3.30 (dd, J = 10.7, 7.3 Hz, 1H), 1.06 – 0.98 (m, 1H), 0.57 – 0.45 (m, 2H), 0.22 – 0.16 (m, 2H); ^{13}C NMR (126 MHz, D_2O): δ 176.0, 171.5, 77.4, 75.8, 56.4, 9.5, 2.7, 2.1. HRMS: calcd. for $C_8H_{14}NO_5$ $[M+H]^+$: 204.0866, found: 204.0866. HPLC: purity 97%, retention time 4.2 min. Chiral HPLC analysis: CROWNPAK CR-I (+) 150 x 3 mm. Phase A: ACN+0.5%TFA, phase B: H_2O+0.5% TFA, A/B = 98:2. Flow rate 0.4 mL/min, column temperature 25 °C, detected by ELSD at 35 °C, t_R (D-*threo*) = 2.3 min, t_R (L-*threo*) = 2.8 min (Figure S62).

(DL-erythro)-3-(Cyclopropylmethoxy)aspartate [(DL-erythro)-19a]

Compound (DL-*erythro*)-**19a** was prepared from (DL-*erythro*)-**18a** (9 mg, 0.027 mmol) following a procedure similar to that used for (DL-*threo*)-**19a**. The title compound was

obtained as a white solid (3 mg, two-step yield of 46%). ^{1}H NMR (500 MHz, D_2O): δ 4.29 (d, J = 3.8 Hz, 1H), 4.08 (d, J = 3.8 Hz, 1H), 3.50 (dd, J = 10.6, 7.1 Hz, 1H), 3.37 (dd, J = 10.6, 7.3 Hz, 1H), 1.12 – 1.04 (m, 1H), 0.58 – 0.51 (m, 2H), 0.27 – 0.18 (m, 2H); ^{13}C NMR (126 MHz, D_2O/DMSO-d_6, 1:1): δ 179.8, 179.0, 99.0, 76.5, 60.2, 11.4, 4.5, 3.9. HRMS: calcd. for $C_8H_{14}NO_5$ $[M+H]^+$: 204.0866, found: 204.0867.

(DL-threo)-3-(Thiophen-3-ylmethoxy)aspartate [(DL-threo)-19b]

Compound (DL-*threo*)-**19b** was prepared from (DL-*threo*)-**18b** (140 mg, 0.37 mmol) following a procedure similar to that used for (DL-*threo*)-**19a**. The title compound was obtained as a white solid (40 mg, two-step yield of 39%). ^{1}H NMR (500 MHz, D_2O): δ 7.43 (dd, J = 4.9, 2.9 Hz, 1H), 7.39 (dd, J = 2.9, 1.3 Hz, 1H), 7.12 (dd, J = 4.9, 1.4 Hz, 1H), 4.71 (d, J = 12.1 Hz, 1H), 4.50 (d, J = 11.9 Hz, 1H), 4.30 (d, J = 2.2 Hz, 1H), 3.97 (d, J = 2.3 Hz, 1H); ^{13}C NMR (126 MHz, D_2O): δ 175.8, 171.4, 137.8, 127.8, 126.6, 124.7, 76.9, 67.2, 56.4. HRMS: calcd. for $C_9H_{12}NO_5S$ $[M+H]^+$: 246.0431, found 246.0430. HPLC: purity 98%, retention time 5.2 min. Chiral HPLC conditions: Nucleosil chiral-1 column with 0.5 mM aqueous $CuSO_4$ solution as mobile phase with a flow rate of 1.0 mL/min at 60 °C, UV detection at 254 nm, t_R (L-*threo*) = 7.3 min, t_R (D-*threo*) = 8.3 min (Figure S63).

(DL-erythro)-3-(Thiophen-3-ylmethoxy)aspartate [(DL-erythro)-19b]

Compound (DL-*erythro*)-**19b** was prepared from (DL-*erythro*)-**18b** (27 mg, 0.072 mmol) following a procedure similar to that used for (DL-*threo*)-**19a**. The title compound was obtained as a white solid (7 mg, two-step yield of 35%). ^{1}H NMR (500 MHz, D_2O): δ 7.47 – 7.45 (m, 2H), 7.19 (dd, J = 4.8, 1.5 Hz, 1H), 4.74 (d, J = 11.9 Hz, 1H), 4.56 (d, J = 11.9 Hz,

1H), 4.16 (d, J = 4.1 Hz, 1H), 3.93 (d, J = 4.1 Hz, 1H); ^{13}C NMR (126 MHz, D_2O): δ 175.1, 171.6, 137.8, 127.9, 126.9, 124.9, 78.1, 66.8, 56.6. HRMS: calcd. for $C_9H_{12}NO_5S$ $[M+H]^+$: 246.0431, found: 246.0430. HPLC: purity 96%, retention time 4.9 min.

(DL-threo)-3-(Prop-2-yn-1-yloxy)aspartate [(DL-threo)-19c]

Compound (DL-*threo*)-**19c** was prepared from (DL-*threo*)-**18c** (54 mg, 0.17 mmol) following a procedure similar to that used for (DL-*threo*)-**19a**. The title compound was obtained as a white solid (13 mg, two-step yield of 34%). ^{1}H NMR (500 MHz, D_2O): δ 4.50 (d, J = 2.4 Hz, 1H), 4.31 (dd, J = 16.0, 2.5 Hz, 1H), 4.21 (dd, J = 16.0, 2.4 Hz, 1H), 4.03 (d, J = 2.4 Hz, 1H), 2.85 (t, J = 2.4 Hz, 1H); ^{13}C NMR (126 MHz, D_2O): δ 175.3, 171.4, 78.6, 76.9, 76.4, 57.7, 56.5. HRMS: calcd. for $C_7H_{10}NO_5$ $[M+H]^+$: 188.0554, found: 188.0554. HPLC: purity 95%, retention time 2.0 min. Chiral HPLC analysis: CROWNPAK CR-I (+) 150 x 3 mm. Phase A: ACN+0.5%TFA, phase B: H_2O+0.5% TFA, A/B = 98:2. Flow rate 0.4 mL/min, column temperature 25 °C, detected by ELSD at 35 °C, t_R (D-*threo*) = 2.4 min, t_R (L-*threo*) = 3.0 min (Figure S64).

(DL-erythro)-3-(Prop-2-yn-1-yloxy)aspartate [(DL-erythro)-19c]

Compound (DL-*erythro*)-**19c** was prepared from (DL-*erythro*)-**18c** (25 mg, 0.08 mmol) following a procedure similar to that used for (DL-*threo*)-**19a**. The title compound was obtained as a white solid (6 mg, two-step yield of 34%). ^{1}H NMR (500 MHz, D_2O): δ 4.37 (dd, J = 16.1, 2.2 Hz, 1H), 4.31 (d, J = 3.8 Hz, 1H), 4.22 (dd, J = 16.1, 2.1 Hz, 1H), 4.01 (d, J = 3.8 Hz, 1H), 2.87 (t, J = 2.4 Hz, 1H); ^{13}C NMR (126 MHz, D_2O): δ 174.7, 171.7, 78.2, 78.1 (2C), 57.4, 56.5. HRMS: calcd. for $C_7H_{10}NO_5$ $[M+H]^+$: 188.0554, found: 188.0553.

Synthesis of precursors for hybrid compounds

(L-threo)-1-(Tert-butyl)-4-methyl-2-amino-3-(benzyloxy)succinate (21)

The multi-gram scale synthesis of L-TBOA (**2**) was based on a previously published procedure.[24] To a stirred suspension of **2** (1.20 g, 5 mmol) in dry MeOH (20 mL) was added $SOCl_2$ (0.45 mL, 5 mmol) dropwise (in an ice-bath). After 20 minutes, the cooling system was removed and the reaction mixture was stirred for further 16 h. After completion of the reaction, the solvent was removed to provide crude product **20** as a white solid (1.4 g, 96%). No purification was needed and the crude product **20** was directly used for the next step. To a stirred suspension of compound **20** (1.3 g, 4.5 mmol) in *t*-BuOAc (20 mL) was added BF_3/Et_2O (3 mL). The reaction mixture was stirred at room temperature for 16 h. After completion of the reaction, 2 M NaOH (20 mL) was added to the reaction mixture and stirred for another 10 min. The reaction mixture was extracted with EtOAc (30 mL x 3), washed with brine (50 mL x 3), and dried over Na_2SO_4. The organic solvent was evaporated to provide pure product **21** as a clear oil (1.25 g, 91%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.36 – 7.28 (m, 5H), 4.82 (d, J = 11.3 Hz, 1H), 4.48 (d, J = 3.1 Hz, 1H), 4.45 (d, J = 11.3 Hz, 1H), 3.83 (s, 3H), 3.80 (d, J = 3.1 Hz, 1H), 1.76 (brs, 2H), 1.43 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 171.7, 171.0, 137.1, 128.4 (2C), 128.2 (2C), 128.0, 81.9, 79.3, 73.3, 57.9, 52.2, 28.0 (3C). HRMS: calcd. for $C_{16}H_{24}NO_5$ $[M+H]^+$: 310.1649, found: 310.1651.

(L-threo)-2-(Benzyloxy)-4-(tert-butoxy)-3-[(tert-butoxycarbonyl)amino]-4-oxobutanoic acid (23)

To a stirred solution of **21** (1.2 g, 3.9 mmol) in dry DCM (25 mL) was added DIEA (1.3 mL, 8

mmol) and Boc_2O (1.7 g, 7.8 mmol) under cooling in an ice-bath. After 10 minutes, the cooling system was removed and the reaction mixture was stirred at room temperature for further 24 h. After completion of the reaction, the reaction mixture was diluted with DCM (20 mL), and washed with 0.5 M HCl (50 mL), saturated $NaHCO_3$ solution (50 mL) and brine (50 mL). The organic layer was dried over Na_2SO_4 (s) and concentrated under vacuum to give product **22** as a clear oil (1.35 g, 85%). No purification was needed and compound **22** was directly used for the next step. To a stirred solution of **22** (1.35 g, 3.3 mmol) in THF/H_2O (1:1, each 5 mL) was added LiOH (240 mg, 10 mmol) under cooling in an ice-bath. After 10 minutes, the cooling system was removed and the reaction mixture was stirred at room temperature for further 8 h. After completion of the starting material, THF was removed *in vacuo* and the resulting aqueous solution was acidified with 1 M HCl to pH=1. The reaction mixture was extracted with EtOAc (50 mL x 3), washed with brine (50 mL x 3), and dried over Na_2SO_4. The organic solvent was evaporated to provide crude product, which was purified *via* flash chromatography (MeOH/DCM 10%, v/v) to give **23** as a clear oil (640 mg, 49%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.10 (brs, 1H), 7.36 – 7.29 (m, 5H), 6.45 (d, J = 9.6 Hz, 1H), 4.70 (d, J = 11.6 Hz, 1H), 4.46 – 4.37 (m, 3H), 1.37 (s, 9H), 1.35 (s, 9H); ^{13}C NMR (126 MHz, DMSO-d_6): δ 170.6, 168.3, 155.4, 137.5, 128.1 (2C), 127.7 (2C), 127.6, 81.5, 78.7, 77.4, 72.0, 56.2, 28.1 (3C), 27.5 (3C). HRMS: calcd. for $C_{20}H_{30}NO_7$ $[M+H]^+$: 396.2017, found: 396.2011.

(L-threo)-Tert-butyl-3-(benzyloxy)-4-[[4-(2-bromo-4,5-difluorophenoxy)phenyl]amino]-2-[(tert-butoxycarbonyl)amino]-4-oxobutanoate (26)

To a stirred solution of **23** (150 mg, 0.38 mmol) in dry DCM (3 mL) was added EDCI (96 mg, 0.5 mmol), HOBT (67 mg, 0.5 mmol) and TEA (50 mg, 0.5 mmol) under cooling in an ice-bath. After 30 min, the cooling system was removed and a solution of amine **24** (100 mg, 0.33 mmol) in DCM (1 mL) was added to the reaction mixture. The reaction mixture was stirred for 12 h at room temperature. After completion of the reaction, the solvent was removed *in vacuo*. The reaction mixture was extracted with EtOAc (20 mL x 3), washed with brine (50 mL), and dried over Na_2SO_4. The organic solvent was evaporated to provide crude product, which was purified *via* flash chromatography (EtOAc/Petroleum ether 5%, v/v) to give **26** as a white solid (70 mg, 27%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.19 (s, 1H), 7.50 – 7.46 (m, 3H), 7.41 – 7.35 (m, 5H), 6.93 (dd, J = 9.0, 3.2 Hz, 2H), 6.73 – 6.70 (m, 1H), 5.34 (d, J = 9.4 Hz, 1H), 4.83 (d, J = 9.4 Hz, 1H), 4.72 (d, J = 11.2 Hz, 1H), 4.62 (dd, J = 11.3, 3.2 Hz, 1H), 4.51 (s, 1H), 1.48 (s, 9H), 1.33 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 168.9, 167.4, 155.3, 152.7, 149.1 (dd, J = 409.5 Hz, 13.9 Hz), 150.5 (dd, J = 7.6, 3.8 Hz), 147.0 (dd, J = 408.2 Hz, 13.9 Hz), 136.2, 133.5, 129.0 (2C), 128.8, 128.5 (2C), 121.8, 121.7, 121.6, 119.2, 115.6 (d, J = 22.7 Hz), 108.7 (d, J = 21.4 Hz), 107.8 (dd, J = 6.3 Hz, 3.8 Hz), 83.0, 80.8, 80.2, 74.4, 56.0, 28.2 (3C), 28.1 (3C). HRMS: calcd. for $C_{32}H_{36}BrF_2N_2O_7$ $[M+H]^+$: 677.1668, found: 677.1675.

(L-threo)-Tert-butyl-4-[(9H-fluoren-2-yl)amino]-3-(benzyloxy)-2-[(tert-butoxycarbonyl) amino]-4-oxobutanoate (27)

Compound **27** was prepared from **23** (100 mg, 0.25 mmol) and (9*H*)-fluoren-2-amine (**25**, 58 mg, 0.32 mmol), using EDCI (61 mg, 0.32 mmol), HOBT (43 mg, 0.32 mmol) and TEA (32

mg, 0.32 mmol) and following a procedure similar to that used for **26**. The title product was obtained as a white solid (128 mg, 90%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.36 (s, 1H), 7.95 (s, 1H), 7.73 (dd, J = 17.7, 7.9 Hz, 2H), 7.55 (d, J = 7.5 Hz, 1H), 7.46 – 7.37 (m, 6H), 7.34 – 7.29 (m, 2H), 5.43 (d, J = 9.5 Hz, 1H), 4.91 (dd, J = 9.4, 2.3 Hz, 1H), 4.77 (d, J = 11.1 Hz, 1H), 4.69 (d, J = 11.0 Hz, 1H), 4.57 (d, J = 2.3 Hz, 1H), 3.90 (s, 2H), 1.52 (s, 9H), 1.35 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 169.0, 167.3, 155.3, 144.4, 143.3, 141.3, 138.4, 136.3, 135.8, 129.0 (2C), 128.8, 128.5 (2C), 126.8, 126.5, 125.1, 120.1, 119.6, 118.6, 116.9, 83.0, 80.9, 80.2, 74.3, 56.0, 37.1, 28.2 (3C), 28.1 (3C). HRMS: calcd. for $C_{33}H_{39}N_2O_6$ $[M+H]^+$: 559.2803, found: 559.2808.

Cell culture. All cell lines were cultured at 37 ºC in a humidified 5% CO_2 atmosphere. The hEAAT1-, hEAAT2- and hEAAT3-HEK293 cell lines were maintained in *Culture Medium I* [Dulbecco's Modified Eagle Medium Glutamax™-I (DMEM) supplemented with 5% dialyzed fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 1 mg/mL G-418], and the stable rEAAT4-tsA201 cell line was maintained in *Culture Medium II* (DMEM supplemented with 5% tetracycline-free fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.2 mg/mL hygromycin B and 10 μg/mL blasticidin).

[^{3}H]-D-Asp uptake assay. The pharmacological characterization of various reference EAAT ligands and test compounds in the [^{3}H]-D-Asp uptake assay was performed essentially as described previously.[25] The day before the assay, cells were split into poly-D-lysine-coated white 96-well plates (PerkinElmer, Boston, MA) in *Culture Medium I* (hEAAT1-, hEAAT2- and hEAAT3-HEK293) or in *Culture Medium II* supplemented with 1 μg/mL tetracycline

(rEAAT4-tsA201). After 16−24 h, the culture medium was aspirated and cells were washed twice with 100 μL of assay buffer (Hank's Buffered Saline Solution supplemented with 20 mM HEPES, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4). Then 50 μL of assay buffer supplemented with 100 nM [^{3}H]-D-Asp (PerkinElmer, Boston, MA) and various concentrations of test compounds were added to the wells, and the plate was incubated at 37 °C for 4 min. Notably, aspartate uptake is still in the linear range at 4 min after initiation of uptake (for all four cell lines). Nonspecific [^{3}H]-D-Asp uptake/binding in the cells was determined in the presence of 3 mM L-Glu. The assay mixtures were quickly removed from the wells, and the cells were washed with 3 x 100 μL ice-cold assay buffer, after which 150 μL of Microscint[20] scintillation fluid (PerkinElmer, Boston, MA) was added to each well. Then the plate was shaken for at least 1 h and counted in a TopCounter (PerkinElmer, Boston, MA). For each compound, three or four independent assays were performed in duplicate (the specific numbers are stated for each compound at the four different transporters in Table 2).

The FLIPR Membrane Potential Blue (FMP) assay. The substrate or non-substrate inhibitor properties of all 3-substituted Asp derivatives were tested at hEAAT1-, hEAAT2- and hEAAT3-HEK293 cells in the FMP assay essentially as previously described, using L-Glu and L-THA as reference substrates and DL-TBOA as a reference non-substrate inhibitor at the EAATs.[25] Briefly, the cells were split into poly-D-lysine-coated black 96-well plates (BD Biosciences, Palo Alto, CA) with a clear bottom. The following day, the culture medium was aspirated, and the cells were washed once with 100 μL assay buffer (140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11 mM HEPES, 10 mM D-Glucose, pH 7.4), after which the cells were incubated in 100 μL assay buffer supplemented with FMP dye (0.5

mg/mL) at 37 °C for 30 min. In the inhibitor experiments, the test compound (300 μM) was added to the assay buffer at this point. The 96-well plate was assayed in a FLEXStation Benchtop Multi-Mode Microplate Reader (Molecular Devices) measuring emission [in fluorescence units (FU)] at 565 nm caused by excitation at 525 nm before and up to 90 s after addition of 33.3 μL assay buffer supplemented with test compound in the substrate experiments (giving rise to an assay concentration of 300 μM of the test compound) or with L-Glu in the inhibitor experiments (giving rise to an assay concentration of L-Glu EC_{80} for the specific EAAT). For each compound, three independent experiments were done in triplicate.

Data Analysis. Data from the [^{3}H]-D-Asp uptake assay were fitted to the equation %Uptake = 100%Uptake/[1+([L]/IC_{50})^{nH}], and IC_{50} values for the test compounds were derived from this equation. Concentration-inhibition curves were generated by nonweighted least-squares fits using the program KaleidaGraph 3.08 (Synergy Software, Reading, PA).

Acknowledgments

This research was financially supported by the Netherlands Organization for Scientific Research (KIEM grants 731.013.110 and 731.015.108 to G.J.P). Haigen Fu and Jielin Zhang acknowledge funding from the China Scholarship Council. Lennart Bunch and Anders A. Jensen acknowledge funding from the Danish Medical Research Council. Drs. Peter Kovermann and Christoph Fahlke are thanked for their generous gift of the stable rEAAT4-tsA201 cell line.

Abbreviations

EAAT, excitatory amino acid transporter; DABCO, 1,4-diazabicyclo[2.2.2]octane; Boc, *t*-butoxycarbonyl; DIEA, *N*,*N*-Diisopropylethylamine; TEA, triethylamine; TFA, trifluoroacetic acid; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBT, 1-hydroxybenzotriazole; THF, tetrahydrofuran; DCM, dichloromethane.

Supporting Information

Synthetic procedures for chiral reference compounds, ^{1}H NMR and ^{13}C NMR spectra illustrating chemical structures (Figures S1-S61), and chiral HPLC analysis for enzymatic products **13a**, **13g** and **13k** (Figures S62-S64) (PDF).

Molecular formula strings and the associated biological data (CSV).

Corresponding Author

*For G. J. P.: E-mail: g.j.poelarends@rug.nl

*For A. A. J.: E-mail: aaj@sund.ku.dk

Notes

The authors declare no competing financial interest.

References

(1) Danbolt, N. C. Glutamate uptake. *Prog. Neurobiol.* **2001**, *65*, 1-105.

(2) Beart, P.; O'shea, R. Transporters for L-glutamate: An update on their molecular pharmacology and pathological involvement. *Br. J. Pharmacol.* **2007**, *150*, 5-17.

(3) Vandenberg, R. J.; Ryan, R. M. Mechanisms of glutamate transport. *Physiol. Rev.* **2013**, *93*, 1621-1657.

(4) Jensen, A. A.; Fahlke, C.; Bjørn-Yoshimoto, W. E.; Bunch, L. Excitatory amino acid transporters: recent insights into molecular mechanisms, novel modes of modulation and new therapeutic possibilities. *Curr. Opin. Pharmacol.* **2015**, *20*, 116-123.

(5) Jiang, J.; Amara, S. G. New views of glutamate transporter structure and function: advances and challenges. *Neuropharmacology* **2011**, *60*, 172-181.

(6) Benarroch, E. E. Glutamate transporters diversity, function, and involvement in neurologic disease. *Neurology* **2010**, *74*, 259-264.

(7) Tzingounis, A. V.; Wadiche, J. I. Glutamate transporters: confining runaway excitation by shaping synaptic transmission. *Nat. Rev. Neurosci.* **2007**, *8*, 935-947.

(8) Bunch, L.; Erichsen, M. N.; Jensen, A. A. Excitatory amino acid transporters as potential drug targets. *Expert. Opin. Ther. Targets.* **2009**, *13*, 719-731.

(9) Lebrun, B.; Sakaitani, M.; Shimamoto, K.; Yasuda-Kamatani, Y.; Nakajima, T. New β-hydroxyaspartate derivatives are competitive blockers for the bovine glutamate/aspartate transporter. *J. Biol. Chem.* **1997**, *272*, 20336-20339.

(10)Shimamoto, K.; Lebrun, B.; Yasuda-Kamatani, Y.; Sakaitani, M.; Shigeri, Y.; Yumoto, N.; Nakajima, T. DL-threo-β-benzyloxyaspartate, a potent blocker of excitatory amino acid

transporters. *Mol. Pharmacol.* **1998**, *53*, 195-201.

(11) Shimamoto, K.; Shigeri, Y.; Yasuda-Kamatani, Y.; Lebrun, B.; Yumoto, N.; Nakajima, T. Syntheses of optically pure β-hydroxyaspartate derivatives as glutamate transporter blockers. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2407-2410.

(12) Shimamoto, K.; Sakai, R.; Takaoka, K.; Yumoto, N.; Nakajima, T.; Amara, S. G.; Shigeri, Y. Characterization of novel L-threo-β-benzyloxyaspartate derivatives, potent blockers of the glutamate transporters. *Mol. Pharmacol.* **2004**, *65*, 1008-1015.

(13) Tsukada, S.; Iino, M.; Takayasu, Y.; Shimamoto, K.; Ozawa, S. Effects of a novel glutamate transporter blocker, (2S, 3S)-3-{3-[4-(trifluoromethyl) benzoylamino] benzyloxy} aspartate (TFB-TBOA), on activities of hippocampal neurons. *Neuropharmacology* **2005**, *48*, 479-491.

(14) Shimamoto, K. Glutamate transporter blockers for elucidation of the function of excitatory neurotransmission systems. *Chem. Rec.* **2008**, *8*, 182-199.

(15) Esslinger, C. S.; Agarwal, S.; Gerdes, J.; Wilson, P. A.; Davis, E. S.; Awes, A. N.; O'brien, E.; Mavencamp, T.; Koch, H. P.; Poulsen, D. J. The substituted aspartate analogue l-β-threo-benzyl-aspartate preferentially inhibits the neuronal excitatory amino acid transporter EAAT3. *Neuropharmacology* **2005**, *49*, 850-861.

(16) Hansen, J. C.; Bjørn-Yoshimoto, W. E.; Bisballe, N.; Nielsen, B.; Jensen, A. A.; Bunch, L. β-Sulfonamido functionalized aspartate analogues as excitatory amino acid transporter inhibitors: distinct subtype selectivity profiles arising from subtle structural differences. *J. Med. Chem.* **2016**, *59*, 8771-8786.

(17) Dunlop, J.; McIlvain, H. B.; Carrick, T. A.; Jow, B.; Lu, Q.; Kowal, D.; Lin, S.;

Greenfield, A.; Grosanu, C.; Fan, K. Characterization of novel aryl-ether, biaryl, and fluorene aspartic acid and diaminopropionic acid analogs as potent inhibitors of the high-affinity glutamate transporter EAAT2. *Mol. Pharmacol.* **2005**, *68*, 974-982.

(18) Greenfield, A.; Grosanu, C.; Dunlop, J.; McIlvain, B.; Carrick, T.; Jow, B.; Lu, Q.; Kowal, D.; Williams, J.; Butera, J. Synthesis and biological activities of aryl-ether-, biaryl-, and fluorene-aspartic acid and diaminopropionic acid analogs as potent inhibitors of the high-affinity glutamate transporter EAAT-2. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4985-4988.

(19) Dunlop, J.; Marquis, K. Glutamate transport inhibitors as targets for treating psychosis. *Drug Discov. Today Ther. Strateg.* **2007**, *3*, 533-537.

(20) Dunlop, J.; Butera, J. A. Ligands targeting the excitatory amino acid transporters (EAATs). *Curr. Top. Med. Chem.* **2006**, *6*, 1897-1906.

(21) Raj, H.; Szymański, W.; de Villiers, J.; Rozeboom, H. J.; Veetil, V. P.; Reis, C. R.; de Villiers, M.; Dekker, F. J.; de Wildeman, S.; Quax, W. J.; Thunnissen, A.-M. W. H.; Feringa, B. L.; Janssen, D. B.; Poelarends, G. J. Engineering methylaspartate ammonia lyase for the asymmetric synthesis of unnatural amino acids. *Nat. Chem.* **2012**, *4*, 478-484.

(22) de Villiers, J.; de Villiers, M.; Geertsema, E. M.; Raj, H.; Poelarends, G. J. Chemoenzymatic synthesis of ortho-, meta-, and para-substituted derivatives of L-threo-3-benzyloxyaspartate, an important glutamate transporter blocker. *ChemCatChem* **2015**, *7*, 1931-1934.

(23) Harris, L.; Mee, S. P.; Furneaux, R. H.; Gainsford, G. J.; Luxenburger, A. Alkyl 4-chlorobenzoyloxycarbamates as highly effective nitrogen source reagents for the base-free, intermolecular aminohydroxylation reaction. *J. Org. Chem.* **2010**, *76*, 358-372.

(24) Fu, H.; Younes, S. H.; Saifuddin, M.; Tepper, P. G.; Zhang, J.; Keller, E.; Heeres, A.; Szymanski, W.; Poelarends, G. J. Rapid chemoenzymatic route to glutamate transporter inhibitor L-TFB-TBOA and related amino acids. *Org. Biomol. Chem.* **2017**, *15*, 2341-2344.

(25) Jensen, A. A.; Bräuner-Osborne, H. Pharmacological characterization of human excitatory amino acid transporters EAAT1, EAAT2 and EAAT3 in a fluorescence-based membrane potential assay. *Biochem. Pharmacol.* **2004**, *67*, 2115-2127.

(26) Arriza, J. L.; Fairman, W. A.; Wadiche, J. I.; Murdoch, G. H.; Kavanaugh, M. P.; Amara, S. G. Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. *J. Neurosci.* **1994**, *14*, 5559-5569.

(27) Shigeri, Y.; Shimamoto, K.; Yasuda-Kamatani, Y.; Seal, R. P.; Yumoto, N.; Nakajima, T.; Amara, S. G. Effects of threo-beta-hydroxyaspartate derivatives on excitatory amino acid transporters (EAAT4 and EAAT5). *J. Neurochem.* **2001**, *79*, 297-302.

(28) Leuenberger, M.; Ritler, A.; Simonin, A.; Hediger, M. A.; Lochner, M. Concise asymmetric synthesis and pharmacological characterization of all stereoisomers of glutamate transporter inhibitor TFB-TBOA and synthesis of EAAT photoaffinity probes. *ACS Chem. Neurosci.* **2016**, *7*, 534-539.

(29) Alaux, S.; Kusk, M.; Sagot, E.; Bolte, J.; Jensen, A. A.; Bräuner-Osborne, H.; Gefflaut, T.; Bunch, L. Chemoenzymatic synthesis of a series of 4-substituted glutamate analogues and pharmacological characterization at human glutamate transporters subtypes 1−3. *J. Med. Chem.* **2005**, *48*, 7980-7992.

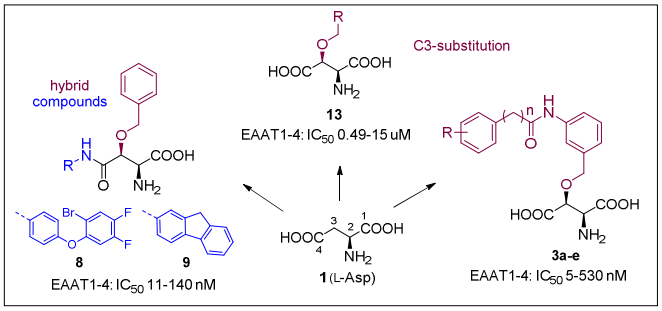
(30) Sagot, E; Pickering, D. S.; Pu, X.; Umberti, M.; Stensbøl, T. B.; Nielsen, B.; Chapelet, M.; Bolte, J.; Gefflaut, T.; Bunch L. Chemo-enzymatic synthesis of a series of

2,4-syn-functionalized (S)-glutamate analogues: new insight into the structure-activity relation of ionotropic glutamate receptor subtypes 5, 6, and 7. *J. Med. Chem.* **2008**, *51*, 4093-4103.

(31)Hoorens, M.W.H.; Fu, H..; Duurkens, R.H.; Trinco, G.; Arkhipova, V.; Feringa, B.L.; Poelarends, G.J.; Slotboom, D.J.; Szymanski, W. Glutamate transporter inhibitors with photo-controlled activity. *Adv. Therap.* **2018**, doi:10.1002/adtp.201800028.

(32)Fan, M.-J.; Li, G.-Q.; Liang, Y.-M. DABCO catalyzed reaction of various nucleophiles with activated alkynes leading to the formation of alkenoic acid esters, 1, 4-dioxane, morpholine, and piperazinone derivatives. *Tetrahedron* **2006**, *62*, 6782-6791.

TOC graphic


hybrid compounds
C3-substitution
13
EAAT1-4: IC50 0.49-15 uM
8
9
EAAT1-4: IC50 11-140 nM
1 (L-Asp)
3a-e
EAAT1-4: IC50 5-530 nM