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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

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Abstract—Excitatory amino acid transporters (EAATs) play a pivotal role in maintaining glutamate homeostasis in the mammalian central nervous system, with the EAAT-2 subtype thought to be responsible for the bulk of the glutamate uptake in forebrain regions. A complete elucidation of the functional role of EAAT-2 has been hampered by the lack of potent and selective pharmacological tools. In this study, we describe the synthesis and biological activities of novel aryl-ether, biaryl-, and fluorene-aspartic acid and diaminopropionic acid analogs as potent inhibitors of EAAT-2. Compound (**16**) represents one of the most potent (IC_{50} = 85 ± 5 nM) and selective inhibitors of EAAT-2 identified to date.

It is widely believed that the dysfunction of glutamate transmission participates in the etiology of a number of neurodegenerative and neuropsychiatric disorders and diseases. In the mammalian central nervous system, the excitatory amino acid transporter (EAAT) family of proteins is responsible for the high-affinity sodium-dependent uptake of glutamate into both astroglial cells and neurons. Normal EAAT function is required both for the efficient termination of glutamatergic neurotransmission and clearance of glutamate from the synaptic cleft, thereby preventing excitotoxicity. Among the five recently identified subtypes of excitatory aminoacid transporters (EAAT-1–5), three of them (EAAT-1/GLAST, EAAT-2/GLT-1, and EAAT-3/EAAC1) are involved in synaptic glutamate homeostasis. Further classification distinguishes neuronal transporter EAAT-3 from glial transporters EAAT-1 and EAAT-2, with the latter being the major contributor to glutamate uptake[1] from the synapse.

An ever-growing interest in the area led to the discovery of several classes of restricted glutamate analogs as inhibitors of EAATs (pyrolidine dicarboxylates,[2] aminocyclobutane dicarboxylates,[3] and carboxycyclopropyl glycines[4]). Many of these relatively compact molecules act as competitive (transportable) substrates inducing transport currents and heteroexchange. A close structural similarity of these earlier series with glutamate presents a plausible explanation for the poor selectivity across EAAT subtypes and substantial affinity to other glutamate receptors (mGluRs and iGluRs). Finally, their use as pharmacological tools is also affected by modest micromolar potency and poor physico-chemical properties. While more recently designed non-transportable ligands, such as dl-*threo*-β-hydroxyaspartate and its benzylated derivative (TBOA),[5] and the novel heptane dicarboxylate-3-amino-tricyclo[2.2.1.$0^{2,6}$]heptane-1,3-dicarboxylic acid[6] offered improved potency, they still fall short of being ideal tool molecules. The latest generation of TBOA-based analogs delivers nanomolar potent EAAT-2 inhibitors (measured in transfected MDCK cells), with the most selective agent PMB-TBOA ((2*S*, 3(S)-3-[3-(4-methoxybenzoylamino)benzyloxy]aspartate)[7] exhibiting a 39-fold selectivity over EAAT-3. Manifesting a notably better profile, this series still leaves room

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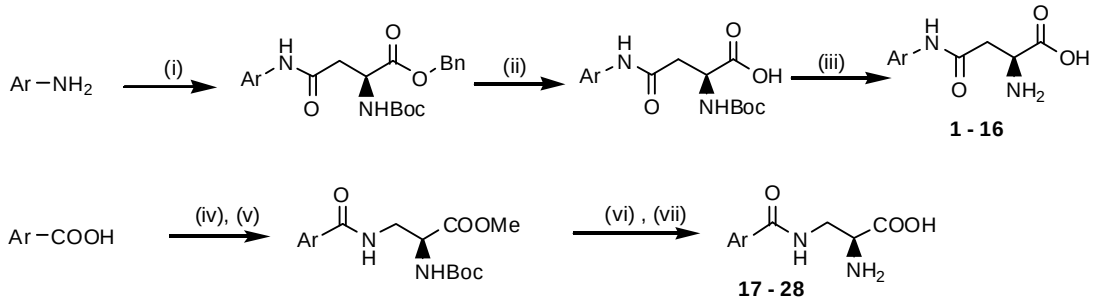


Scheme 1. Synthesis of aspartamides and diaminopropionic acid analogs. Reagents and conditions: (i) *N*-Boc-L-aspartic acid benzyl ester, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, CH_2Cl_2, (ii) H_2/Pd on carbon, 50 psi, (iii) trifluoroacetic acid (TFA), CH_2Cl_2, (iv) 1. *N*-hydroxysuccinimide, *N*,*N*′-diisopropylcarbodiimide, CH_2Cl_2. 2. diisopropylethylamine, *N*-Boc-3-amino-L-alanine, (v) TMS–diazomethane, (vi) NaOH, MeOH, THF, (vii) trifluoroacetic acid (TFA), CH_2Cl_2.

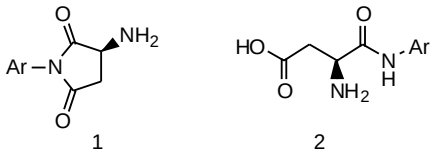


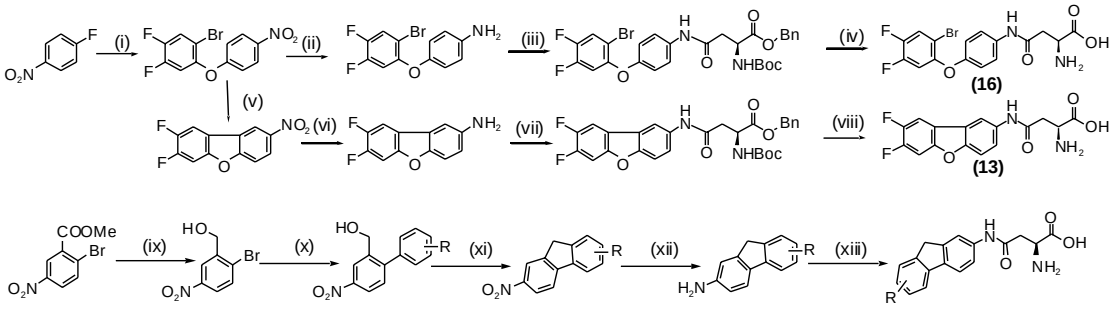
Figure 1. Typical side products of aspartamide synthesis.

for improvement in selectivity and physico-chemical properties.

In this study, we present the synthesis and structure–activity studies of a structurally distinct series possessing high potency and selectivity in HEK cell lines together with a potentially promising overall biopharmacological profile. Focusing on creating sufficient structural dissimilarity with glutamate and improvement of pharmacological characteristics, we constructed variable lipophilic structural fragments with the terminal carboxy- and amino-groups of aspartic and 2,3-diaminopropionic acid correspondingly. In comparison with TBOA series,[7] these inhibitors possess only one chiral center and a free carboxylic acid group, potentially simplifying the syntheses and improving physico-chemical properties.

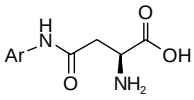
The general synthetic routes, leading to aspartate and 2,3-diaminopropionate analogs, are shown in Scheme 1. As outlined in Scheme 1, aspartic acid analogs **1**–**16** were synthesized through standard carbodiimide-mediated coupling between an amine and an appropriately protected aspartic acid. It is worth mentioning that step (ii) in Scheme 1 proved to be challenging in the synthesis of aspartamides. For example, basic hydrolysis or even exposure of the coupled protected material to elevated temperatures led to the contamination of the target materials with the product of intramolecular cyclization (1) and its subsequent non-selective opening (2) (Fig. 1). Hydrogenolysis with vigorous stirring at elevated pressure (50 psi) or Cu^{2+}-mediated debenzylation[8] (step (ii) in Scheme 1, and steps (iv) and (viii) in Scheme 2) allowed us to overcome these difficulties.

Diaminopropionic acid analogs were prepared via in situ formation of hydroxysuccinimide ester with the appropriate aryl carboxylic acid, followed by coupling with diaminopropionic acid.[9] To simplify isolation and purification of the intermediate, the crude material was subjected to esterification with trimethylsilyl diazomethane. Final hydrolysis of a methyl ester with diluted (1 N) sodium hydroxide and removal of Boc-group with TFA afforded analogs **17**–**28**.



Scheme 2. Syntheses of the analogs with highly functionalized cores. Reagents and conditions: (i) 2-bromo-4,5-difluorophenol, NaH, DMSO, (ii) $SnCl_2$, EtOH, 80 °C, (iii) *N*-Boc-L-aspartic acid-a-benzyl ester, 1-[3-dimethylamino)propyl]-3-ethylcarbodiimide, CH_2Cl_2, (iv) 1. trifluoroacetic acid (TFA), CH_2Cl_2, 2. $CuSO_4$, EtOH, 40 °C, (v) $Pd(OAc)_2/Na_2CO_3$/DMA, 120 °C, (vi) H_2/PtO_2, MeOH, THF, (vii) *N*-Boc-L-aspartic acid-a-benzyl ester, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, CH_2Cl_2, (viii) 1. H_2/Pd on carbon, 50 psi, 2. trifluoroacetic acid (TFA), CH_2Cl_2, (ix) $LiBH_4$, E_2O, (x) $Pd(OAc)_2$, Tol_3P, arylboronic acid, dioxane, (xi) polyphosphoric acid, 110 °C, (xii) H_2, PtO_2, MeH, THF, (xiii) *N*-Boc-L-aspartic acid-a-benzyl ester, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, CH_2Cl_2, 2. H_2/Pd on carbon, 50 psi, 3. trifluoroacetic acid (TFA), CH_2Cl_2.

Table 1. The inhibitory properties of aspartamides and diaminopropionic acid analogs



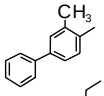
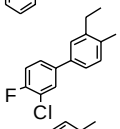
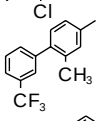
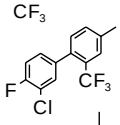
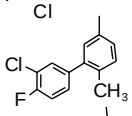
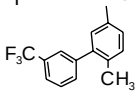
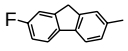
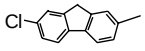
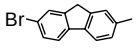
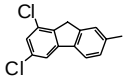
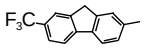
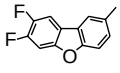
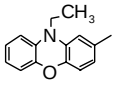
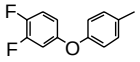
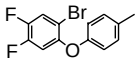
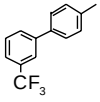
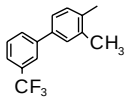
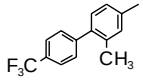
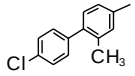
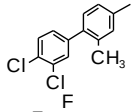
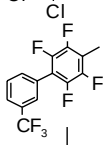
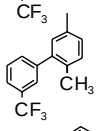
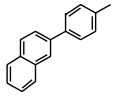
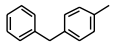
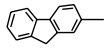
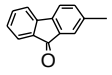
	Inhibition (IC_{50}, μM)[a]	EAAT-3	EAAT-1	EAAT-2
1		10	2	0.1
2		0.6	0.3	0.2
3		0.3	0.2	0.6
4		0.14	0.13	0.1
5		0.6	0.4	0.2
6		1	3	0.5
7		5.6	6	0.7
8		0.05	0.1	0.08
9		0.2	0.15	0.1
10		0.65	0.3	0.2
11		0.45	0.2	0.3
12		2.4	3.8	0.1
13		1.8	1	0.5
14		1	0.7	0.3
15		14.5	2.9	0.13
16		3.8	5	0.08
17		1	2	0.7
18		0.5	0.1	0.06*

Table 1 (*continued*)

	Inhibition (IC_{50}, μM)[a]	EAAT-3	EAAT-1	EAAT-2
19		0.6	1	0.4
20		3	20	0.3
21		1.5	2	0.2
22		0.85	0.7	0.1
23		5	6	1
24		9	63	67
25		3	100	0.3
26		9	22	1
27		0.42	0.3	0.7
28		2	3	13

[a] Concentration required to inhibit glutamate uptake in HEK cells by 50%. The error margin is ±10%.
* $IC_{50} \leqslant 10$ nM in MDCK cell line.

Examples of the synthesis of the targets with highly elaborated lipophilic cores are shown in Scheme 2. Yields usually ranged from good to excellent (40–90%). The structural integrity of the analogs was confirmed by traditional analytical techniques (NMR, MS, CHN, and IR).

It is noteworthy that among the plethora of nitro-group reduction methods, only $SnCl_2$ in ethanol[10] (step (ii) in Scheme 2) showed excellent regioselectivity preserving the bromine atom intact. A potentially problematic Pd-catalyzed formation of dibenzofuran[11] (v) and polyphosphoric acid mediated cyclization of biaryl methanols into corresponding fluorenes[12] (xi), both requiring strenuous conditions (110–120 °C), took place without major complications, routinely providing products with satisfactory yield (40–70%).

The EAAT inhibitory properties of compounds, prepared in the course of the study, are shown in Table 1.

The compounds under study were tested in a HEK cell line expressing each of the human transporter subtypes

Table 2. Pharmacological profile of bromo-ether **16**

System	HEK	Oocytes	Synaptosomes	mGluR	iGluR
Inhibition (IC_{50}, nM)	85 ± 5	130 ± 1	35 ± 7	No effect	No effect

EAAT-1–3.[13] Compound **18** was also tested for its EAAT-2 uptake inhibitory effect on MDCK cells in an effort to benchmark its potency with TBOA series.[7] It showed potent inhibition of EAAT-2 uptake with an $IC_{50} < 10$ nM, which is comparable with the best TBOA analogs reported to date.

Overall, both aspartamide and diaminopropionamide series routinely elicited high EAAT-2 inhibitory potency in HEK cells with an $IC_{50} < 100$ nM (**3**, **8**, **16**, and **18**). Linear arrangement of amino acid fragment with respect to distal aromatic ring is a prerequisite for potency in diaminopropionamide series (weakly active **24**). The selectivity proved to be an elusive and complex issue. As exemplified by compounds **26**, **27**, and **28**, breaking of aryl–aryl bond with concomitant loss of rigidity did not deteriorate EAAT-2 inhibition but gave rise to some selectivity (10- to 20-fold) versus EAAT-1 and EAAT-3. On the other hand, fluorenone **28**, not only lost EAAT-2 potency, but also reversed the sense of inhibitory preference. The best results achieved in diaminopropionamide series are represented by analogs **20** and **25**. Possessing significant EAAT-2 potency, they also showed excellent separation for EAAT-1 (60- to 300-fold) and a moderate one for EAAT-3 (~10-fold). Linear, in respect to amino acid residue, arrangement in biaryl analogs, while lacking selectivity, revealed a uniformly high blocking potency against EAAT-2 transporter in both series (aspartamides- and diaminopropionamides). Planarity derived from the conversion of biaryls into fluorenes (**8**–**12**, **27**) did not affect the properties, leaving them potent non-selective inhibitors (with the exception of **12**) of EAAT-2 transporter. On the other hand, perturbations in the proximity of aryl–aryl linkage in some cases (**1**, **20**–**22**) produced the desired selectivity trend. Our attempts to exploit these results led to the synthesis and evaluation of the ethers **13**–**16**. While potent EAAT-2 blockers dibenzofuran **13** and phenoxazine **14** showed no selectivity, less rigid ethers **15** and, especially, **16** showed both increased potency and desired inhibitory preference. In addition to being the most potent compound, bromo-ether **16** (EAAT-2 $IC_{50} \sim 85$ nM) was the most selective with 59- and 45-fold selectivity over EAAT-1 and EAAT-3, respectively. Because of superior combination of potency and selectivity, the compound **16** was fully characterized pharmacologically. It showed high potency in rat cortical synaptosomes and EAAT-2 expressing oocytes (Table 2). In addition, the compound did not show cross-receptor reactivity (failed to activate both ionotropic and metabotropic glutamate receptors) and proved to be a competitive non-substrate inhibitor of EAAT-2 (by failure to activate transporter-like current when applied to oocytes expressing EAAT-1–3 transporters[14]).

In conclusion, we have designed and characterized a novel series of EAAT-blockers, exemplified by **16** (N^4-[4-(2-bromo-4,5-difluorophenoxy)phenyl]-L-asparagine)—a potent, selective, competitive non-substrate inhibitor of EAAT-2. As one of the most potent and selective EAAT-2 inhibitors identified to date, compound **16** represents a unique addition to the arsenal of pharmacological tools which can be used to elucidate further the role of specific EAAT subtypes and to improve our understanding of hyperglutamatergic and neurodegenerative disorders.

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