Published in final edited form as:
Bioorg Med Chem Lett. 2010 April 15; 20(8): 2680–2683. doi:10.1016/j.bmcl.2009.10.020.

Conformationally-Restricted Amino Acid Analogues Bearing a Distal Sulfonic Acid Show Selective Inhibition of System X_c^- over the Vesicular Glutamate Transporter

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Abstract

A panel of amino acid analogs and conformationally-restricted amino acids bearing a sulfonic acid were synthesized and tested for their ability to preferentially inhibit the obligate cysteine-glutamate transporter system x_c^- versus the vesicular glutamate transporter (VGLUT). Several promising candidate molecules were identified: R/S-4-[4′-carboxyphenyl]-phenylglycine, a biphenyl substituted analog of 4-carboxyphenylglycine and 2-thiopheneglycine-5-sulfonic acid both of which reduced glutamate uptake at system x_c^- by 70–75% while having modest to no effect on glutamate uptake at VGLUT.

Keywords

glutamate; amino acid analog; inhibitor; VGLUT; system X_c^-; sulfonic acid

L-Glutamate (**1**) is a key neurotransmitter responsible for the vast majority of the fast excitatory synaptic communication in the mammalian CNS. L-Glutamate acts at ionotropic glutamate receptors to mediate ligand gated ion channels and at metabotropic glutamate receptors to couple intracellular second messenger systems via G-proteins.[1–5] The importance of L-glutamate as a contributor to higher order processing required in development, plasticity, learning, and memory is well established.[4,6,7] However, glutamatergic excitotoxicity can result when an excess of L-glutamate occurs and continually activates glutamate receptors.[2,5,8] To maintain the proper titer of L-glutamate there is a network of strategically positioned transporters that shuttle L-glutamate in and out of cells and organelles. Most notable among these transporters are the excitatory amino acid transporters (EAATs) that facilitate the uptake of L-glutamate into neurons.[8]

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In addition to EAATs, other transporters maintain intra- and extra-cellular levels of glutamate including; system x_c^-, a chloride-dependent, sodium-independent obligate exchanger that couples the export of intracellular L-glutamate with the import of extracellular L-cystine[9–12] and the vesicular glutamate transporter (VGLUT) that mediates the uptake of intracellular glutamate into synaptic vesicles.[6,13,14]

System x_c^- and VGLUT are structurally and functionally distinct from the EAATs and also from each other. Although system x_c^- and VGLUT differ pharmacologically,[5,8,15,16] both transporters remove L-glutamate from the cytosol. In principle, therefore, intracellular L-glutamate levels could be regulated by modulating one or both of these transporters.[16] As such, the development of inhibitors that selectively block system x_c^- and/or VGLUT represents an interesting pharmacologic challenge. Some inhibitors[9,16,17] have been reported for system x_c^- and for VGLUT[6,15,18–26] (Fig. 1). Two interesting features common to several system x_c^- and VGLUT inhibitors are the use of aromatic rings to conformationally lock[27] the acid groups (e.g., CPG) and sulfonic acid isosteres [24,25,28] in place of a carboxylic acid. Seeing these as opportunities to explore similarities and differences in the specificities of system x_c^- and VGLUT, we prepared a number of conformationally-restricted glutamate analogs bearing a sulfonic acid group in place of the distal (γ) carboxylic acid of glutamate.

The target compounds were synthesized as shown in Schemes 1 and 2. Simple amino acid analogs (**3a–e**) of phenylglycine were synthesized via hydrolysis of the corresponding hydantoin intermediates (**2a–e**).[29–32] The preparation of sulfonic acid analogs **5a–i** was carried out by reaction of commercially available amino acids with fuming sulfuric acid to afford monosulfonic acid analogs.[33] To explore the relative contribution of the amino acid center to inhibition two additional targets, compounds **7a–b**, were synthesized by hydrolysis of the commercially available structures **6a–b** using 2N NaOH. Each synthesized compound was characterized by ^{1}H NMR, IR and mass spectral analysis[34] prior to testing at the two transporter systems (Table 1). Activity was assessed by quantifying the ability of the compounds to inhibit the specific uptake of ^{3}H-L-gluamate by either system xc- or VGLUT. System x_c^- mediated uptake of L-glutamate (100 μM) was measured in SNB19 glioma cells under Na-free conditions, corrected for non-specific uptake, and normalized to protein content.[9] VGLUT mediated uptake of L-glutamate (250 μM) was measured in synaptic vesicles isolated from rat brain, corrected for non-specific uptake, and normalized to protein content.[20]

The rationale for testing compounds **3a–f** was based on the fact that a thienylglycine heterocycle contains an embedded cysteine. The imidazole structure was prepared as a control analog. Structure **3e** was built as a chain extended homologue of 4-CPG, which was found to be a good inhibitor of system x_c^- but a poor inhibitor of VGLUT. The activity of **3e** also suggests the likelihood that the compounds are interacting with lipophilic domains associated with the transporter, as has also been shown to occur with EAAT inhibitors.[12] Interestingly, all the thiophene-containing structures showed inhibition of system x_c^- and VGLUT with the 5-bromo thienylglycine **3d** and benzothienylglycine **3f** blocking about 60% and 70% of VGLUT uptake, respectively (Table 1). The imidazo analog **3c** was completely inactive indicating the importance of the thiophene ring and/or possibly contribution by the sulfur atom. Most surprising in this initial screen was the finding that the biphenyl analog of CPG **3e** blocked 73% of glutamate uptake at system x_c^- but was a poor inhibitor of VGLUT.

Sulfonic acid analogs of the amino acids phenylglycine, phenylalanine and thienylglycine **5a–i** were prepared to determine the role of stereochemistry, isostere contribution and limitations of the γ-carboxylic acid group. We rationalized that CPG and cysteate are

inhibitors of system x_c^- and therefore, CPG analogs bearing a γ-sulfonic acid would be more potent, and potentially highly selective inhibitors when compared as inhibitors of VGLUT. Neither D- or L-4-sulfophenylalanine **5ab** nor α-methyl 4-sulfophenylalanine **5d** blocked glutamate uptake at either transporter (Table 1). Sulfonation of 4-bromophenylalanine was conducted to afford the phenylalanine-2-sulfonic acid analog **5c** to reduce the distance between the two acid groups, and render it a conformationally-restricted analog of homocysteate.

However, compound **5c** was a poor inhibitor of both transporters. (R)-4-sulfo-phenylglycine **5e** did not block glutamate uptake at either transporter, yet **5f** was a selective inhibitor of system x_c^-. We attribute this selectivity to the fact that system x_c^- generally requires an S-configured amino acid center for inhibitors whereas VGLUT shows no need for this center and, in fact, does not require a basic amine.

Using (S)-4-sulfophenylglycine as a new lead, we prepared the thiophene analogs that position the sulfonic acid and amino acid groups at a distance midway between 4-sulfophenylglycine and homocysteic acid. Both (R)-**5h** and (S)-4-sulfothienylglycine **5g** blocked uptake of glutamate at system x_c^-, 45% and 70%, respectively (Table 1). The latter compound proved as potent as the endogenous substrate L-cystine. Unlike system x_c^- both were less potent at VGLUT.

The last set of analogs we prepared to test specificity differences between system x_c^- and VGLUT were aminothiophenecarboxylic acids **7ab**. Since the thiophene scaffold showed promise in system x_c^- inhibitors, we next queried whether or not replacement of the α-amino acid group with an aniline-type amine and carboxylic acid would preferentially block VGLUT. In both instances, glutamate uptake was blocked at system x_c^- and not VGLUT indicating that the presence of an α-amino acid group is not a requirement for system x_c^- inhibitor structure. This is also consistent with the activity of sulfazaline, an inhibitor of system x_c^-, which lacks the free α-amino acid head group that typifies the majority of known inhibitors. Sulfasalazine is of particular interest because it suggests that system x_c^- may represent a viable point of therapeutic intervention in the treatment of glial brain tumors.[35]

Overall, the only sulfonic acid analog of phenylglycine or phenylalanine that showed activity was **5f** that selectively blocked 45% transport at system x_c^-. Substituted thienyl- and benzthienylglycines blocked VGLUT with marginal selectivity over system x_c^-, however, the presence of a sulfonic acid group on the thiophene (**5fgh**) afforded selective system x_c^- transport inhibitors. We are currently developing a system x_c^- pharmacophore model using the thiophene template to produce better inhibitors. One inhibitor that effectively reduced the uptake of glutamate at both transporters was R/S-benzothiophene-3-glycine. In summary, we have identified new system x_c^- inhibitors that we envision will become important pharmacologic tools, but additional work is needed to identify more effective dual inhibitors.

Acknowledgments

The authors thank J. Gerdes and N. Natale for advice. This research was supported in part by NIH NS38248 (CMT), SBIR grants to ATERIS Technologies LLC (R43 ES016392 U44 NS058229), NS30570 (RJB) and NS42077 (RJB), as well as by the Core Laboratory for Neuromolecular Production (NIH P30-NS055022) and the COBRE Center for Structural and Functional Neuroscience (NIH P20-RR015583).

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29. *Synthesis of hydantoins **2a–e***. Compounds **2a–e** were synthesized from aldehydes (1a–e). Compounds **1a–e** (1.0 g; 5.40 mmol) were dissolved in 1:1 CH_3OH/H_2O and $(NH_4)_2CO_3$ (4.5 g; 47.4 mmol) and KCN (1.3 g, 20 mmol) were added. The mixture was heated (58–60°C; 3h), concentrated to two-thirds, and chilled to 0°C. Crystalline products were filtered, washed with

water, dried and characterized by ^{1}H-NMR and MS. The resultant hydantoins were hydrolyzed directly.

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34. *Spectral data for selected compounds*. Compound **2c:** Yield 75%; mp 258–261°C; ^{1}H NMR (400MHz, DMSO- *d6*): δ 12.02 (br s, 1H), 10.59 (br s, 1H), 8.06 (s, 1H), 7.59 (s, 1H), 7.09 (bs, 1H), 4.99 (s, 1H); ESI MS *m/z* = 167 (M+1); IR (KBr) (υ_{max}/Cm^{-1}): 3414, 3241, 2700, 1729, 1456. Anal. Calcd for $C_6H_6N_4O_2$: C, 43.38; H, 3.64; N, 33.72. Found: C, 43.33; H, 3.59; N, 33.62. Compound **3c:** Yield 55%; mp >300 °C; ^{1}H NMR (400MHz, D_2O): δ 7.51 (s, 1H), 6.88 (s, 1H), 4.91 (s, 1H); ESI MS *m/z* = 142 (M+1); IR (KBr) (υ_{max}/Cm^{-1}): 3250, 2348, 2287, 1593, 1462, 1377. Anal. Calcd for $C_5H_7N_3O_2$: C, 42.55; H, 5.00; N, 29.77. Found: C, 41.88; H, 5.20; N, 29.64. Compound **5h:** Yield 52%; mp > 300 °C; ^{1}H NMR (400MHz, D_2O): δ 7.32 (d, *J* = 7.35 Hz, 1H), 7.11 (d, *J* = 7.35 Hz, 1H), 5.07 (s, 1H); ^{13}C: δ 173.5, 148.3, 141.2, 131.8, 131.5, 55.7; HRMS *m/z* = 237.9833 (M+1); IR (KBr) (υ_{max}/Cm^{-1}): 2634, 1746, 1613, 1527, 1214, 1165. Anal. Calcd for $C_6H_7NO_5S_2$: C, 30.37, H, 2.97, N, 5.90. Found: C, 30.51, H, 2.88, N, 5.94.
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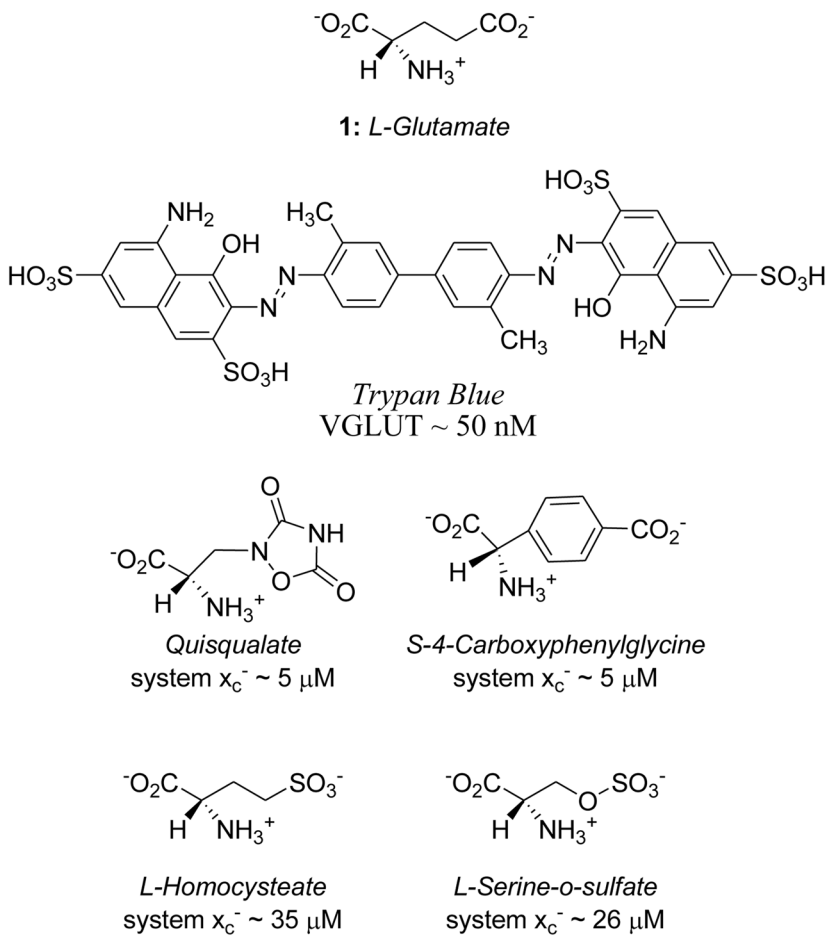
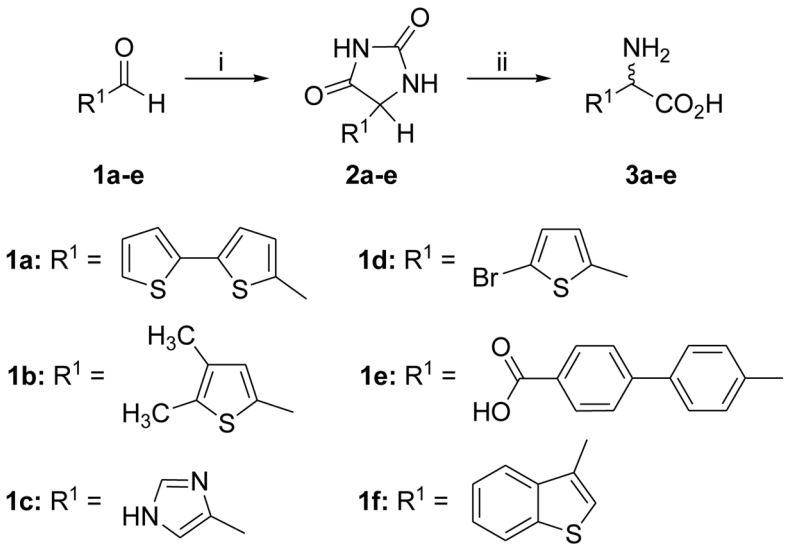
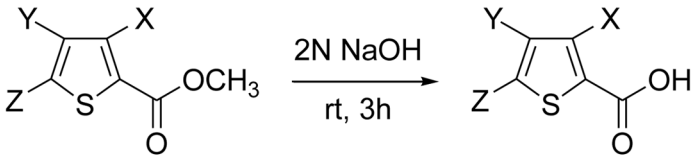


Figure 1.
Structures of glutamate, VGLUT and system x_c^- inhibitors and their corresponding IC_{50} values.



Scheme 1.

Synthesis of amino acid analogs **3a–f**. Reagents and conditions: (i) $(NH_4)_2CO_3$, KCN, 1:1 MeOH, H_2O, 50–60 °C, 3 h; (ii) $Ba(CO_3)_2$, H_2O, 100 °C, 72 h.



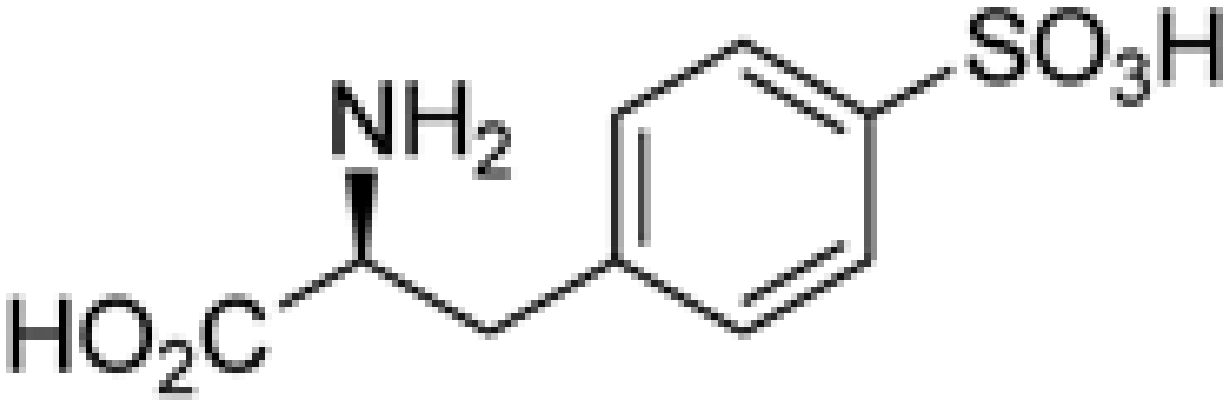
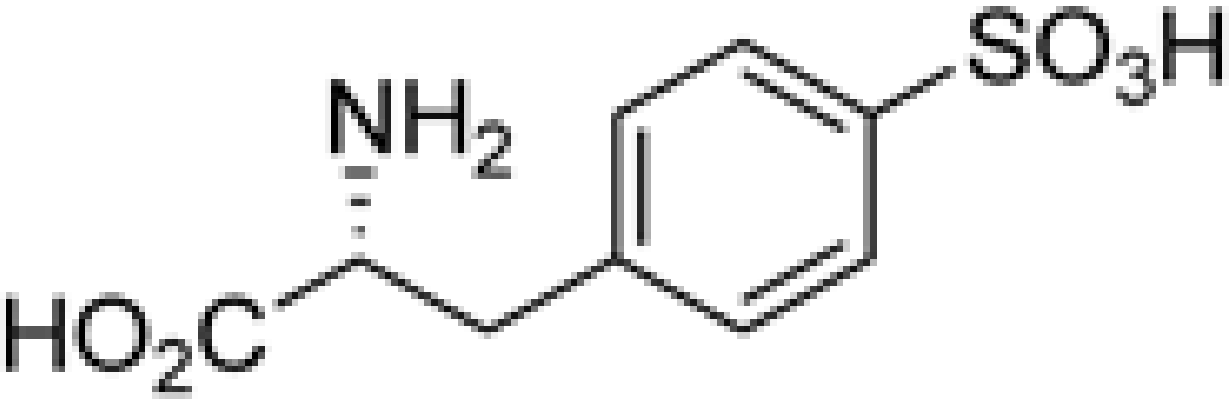
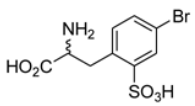
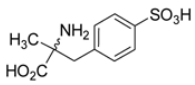
6a/6b **7a/7b**

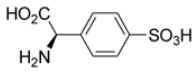
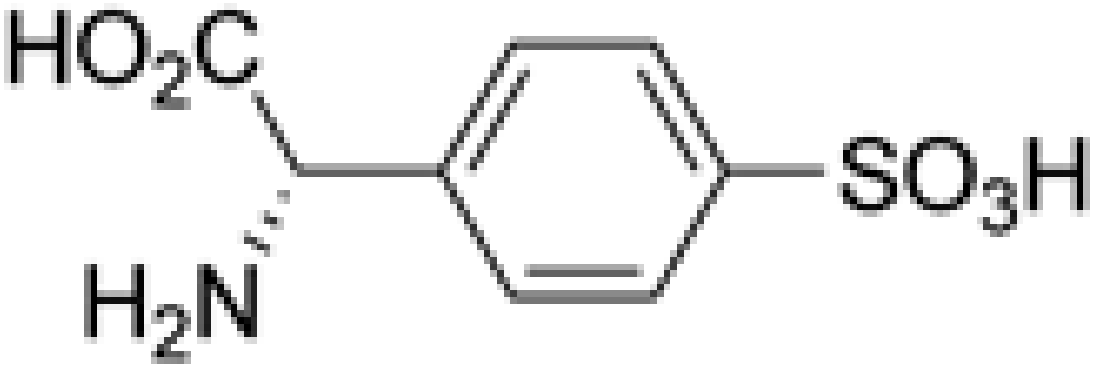
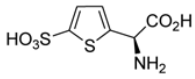
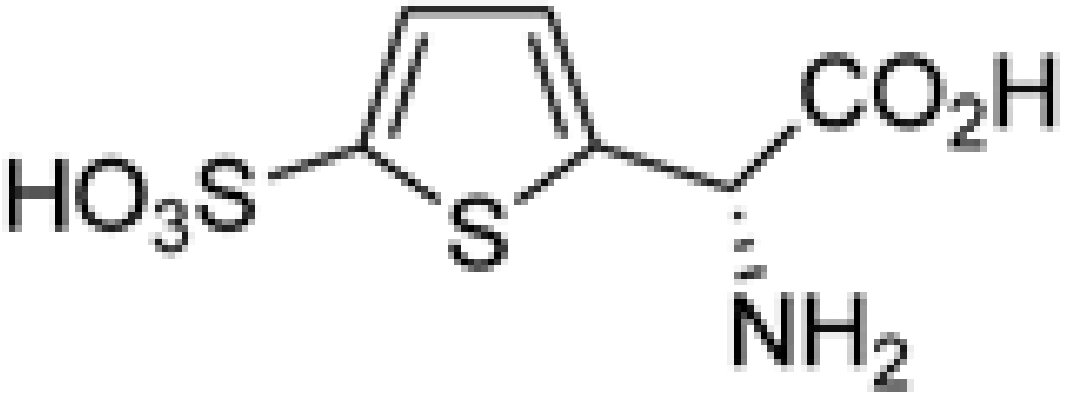
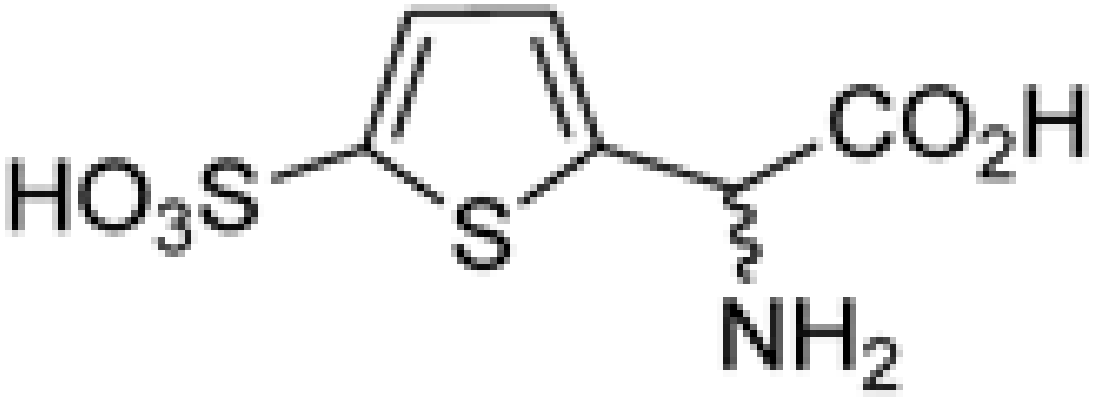
a: X = NH_2, Y = CH_3, Z = H;
b: X = CH_3, Y = CO_2H, Z = NH_2

Scheme 2.
Preparation of thiophene analogs **7a/7b**.

Table 1

Percent of L-glutamate uptake in system x_c^- and VGLUT for compounds **3a–f**, **5a–i** and **7ab**. System x_c^- assay: 100 uM L-glutamate and 500 uM of inhibitor. VGLUT assay: 250 uM L-glutamate and 5 mM of inhibitor.

Entry	Structure or name	sys x_c^- upta
3a	R/S-5-(2-thienyl)-2- thienylglycine	69
3b	R/S-4,5-dimethyl-2- thienylglycine	73
3c	R/S-5-imidazoylglycine	96
3d	R/S-5-bromo-2-thienylglycine	83
3e	R/S-4-[4′-carboxyphenyl]-phenylglycine	27
3f	R/S-benzothiophene-3-glycine	53
5a		90
5b		10
5c		83
5d		106

Entry	Structure or name	sys x_c^- upta
5e		98
5f		55
5g		32
5h		55
5i		26

Entry	Structure or name	sys x_c^- upta
7a	3-amino, 4-methylthiophene-2- carboxylic acid	83
7b	3-methyl, 5-aminothiophene-2,4-dicarboxylic acid	57
Cntrls	L-cystine Congo Red	22 n