Published in final edited form as:
Bioorg Med Chem Lett. 2011 July 15; 21(14): 4358–4362. doi:10.1016/j.bmcl.2011.05.018.

Use of the Hydantoin Isostere to Produce Inhibitors Showing Selectivity Toward the Vesicular Glutamate Transporter versus the Obligate Exchange Transporter System x_c^-

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Abstract

Evidence was acquired prior to suggest that the vesicular glutamate transporter (VGLUT) but not other glutamate transporters were inhibited by structures containing a weakly basic α-amino group. To test this hypothesis, a series of analogs using a hydantoin (pKa ~ 9.1) isostere were synthesized and analyzed as inhibitors of VGLUT and the obligate cystine-glutamate transporter (system x_c^-). Of the hydantoin analogs tested, a thiophene-5-carboxaldehyde analog **2l** and a bis-hydantoin **4b** were relatively strong inhibitors of VGLUT reducing uptake to less than 6% of control at 5 mM but few inhibited system x_c^- greater than 50% of control. The benzene-2,4-disulfonic acid analog **2b** and *p*-diaminobenzene analog **2e** were also good hydantoin-based inhibitors of VGLUT reducing uptake by 11% and 23% of control, respectively, but neither analog was effective as a system x_c^- inhibitor. In sum, a hydantoin isostere adds the requisite chemical properties needed to produce selective inhibitors of VGLUT.

Keywords

Hydantoin scaffolds; Glutamate; System x_c^-; Inhibitor; VGLUT

L-Glutamate (*L*-Glu, **1**), the primary excitatory neurotransmitter in the synaptic pathways of the mammalian central nervous system (CNS), plays an important role in many integrative brain functions[1–7] through its activation of a variety of ionotropic and metabotropic receptors. To maintain the proper levels of the transmitter and generate the desired receptor responses without risking the excitotoxic consequences of excessive activation, *L*-Glu concentrations are controlled by an array of transporters that regulate uptake and release of

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L-Glu in and out of cells and organelles (e.g., astrocytes, synaptic terminals, and synaptic vesicles).[8, 9] For example, the excitatory amino acid transporters (EAATs) facilitate the uptake of *L*-Glu into neurons and astrocytes whereas the vesicular glutamate transporter (VGLUT) regulates uptake between cytosolic and luminal compartments.[4, 10, 11] System x_c^- (Sx_c^-), a chloride-dependent, sodium-independent obligate exchanger, couples the export of intracellular *L*-Glu with the import of extracellular *L*-cystine that helps maintain intracellular levels of glutathione.[12, 13] To decipher the individual and collective contributions that these transporters have on intra- and extracellular concentrations and CNS signaling, selective inhibitors are needed. However, since each transporter recognizes *L*-Glu, as well as a subset of structurally-similar inhibitors, a challenge exists to develop inhibitors that exploit individual elements of each transporter pharmacophore and thereby achieve selectivity amongst them. A number of successful approaches have been undertaken to create selective inhibitors for glutamate receptors and transporters including conformational restriction, isostere substitution, and combinations of these approaches.[14, 15]

The goal of this work was to identify a new class of structures that could discriminate between Sx_c^- and VGLUT, two transporters that share sensitivity to inhibitors possessing sulfonate groups (Fig. 1). Aryl- and heteroaryl sulfonic acid analogs, however, yielded only a few blockers with the desired selectivity.[16] Further examination of Sx_c^- specificity indicated that substituting a heterocyclic carboxylic acid isostere in place of the γ-carboxylate (ibotenate, quisqualate) yielded potent inhibitors.[17] Likewise, the inhibition of VGLUT may be accomplished with structures that incorporate a non-basic nitrogen (e.g., aniline, quinoline, etc.)[18–20] in place of the α-amino group. Therefore, the substitution of a heterocyclic [carboxylic acid] isostere that contains a weakly basic α-amine group should confer the desired inhibitory effect at VGLUT, whereas placement of the same heterocycles at the γ-position would lead to improved inhibition of Sx_c^-.

Hydantoins are readily accessible α-amino acid derivatives[21–24] that are weak carboxylic acid isosteres by virtue of the imide-NH (pKa ~ 9.1). However, few if any reports utilize the hydantoin group as an isostere substituting for both the α-carboxylate and the weakly basic α-amine moiety (requisite for VGLUT inhibitors). Thus, the goal of this study was to prepare α-hydantoin-based inhibitors that would show selectivity toward VGLUT over Sx_c^-. Likewise, bis-hydantoins resembling cystine would be expected to show selectivity activity toward Sx_c^- in comparison to VGLUT.

Herein, the syntheses of C-5 substituted hydantoin and bis-hydantoin compounds were conducted including substituted aromatic and heteroaromatic rings, and arylsulfonic acids; the latter as known inhibitors of VGLUT (Schemes 1 and 2). Commercially available aldehydes (**1a–q**) were subjected to the Bucherer-Berg reaction[25] to afford the hydantoin compounds **2a–q** in 35–82% yield. The wide variation in yield (Table 1) is likely due to the difference in electron donating (lower yield) and withdrawing (higher yields) groups on the aryl aldehydes. The preparation of **4a–b** was conducted similarly by reaction of commercially available dials (**3a** and **3b**) in 59% and 68% yield, respectively.[26] Each compound was fully characterized by ^{1}H, ^{13}C NMR, IR, and mass spectral analysis.[27]

Target compounds **5a–b** were prepared by reaction of **2d** with 1,3 or 1,4 dibromoalkane in the presence of K_2CO_3 (Scheme 3). Compounds **5a–b** lack the more acidic imide NH group thereby reducing interaction of this bioisostere with the transporters via the α-amide NH and H-bonding acceptor carbonyls. Compounds **5a–b** are also highly lipophilic, which has been shown to improve inhibitory action at VGLUT.

The inhibition of VGLUT and Sx_c^- by the hydantoins were assessed by quantifying the ability of the compounds to block the specific uptake of ^{3}H-*L*-glutamate by either transporter

(Note: Due to well to well variability in the assay, the rate may be higher than 100% in wells where the inhibitor has no activity). Sx_c^- mediated uptake of *L*-glutamate (100 μM) was quantified in *SNB19* glioma cells under Na-free conditions, corrected for non-specific uptake, and normalized to protein content.[28] 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.[29] The ability of the hydantoins **2a–q**, **4a–b** and **5a–b** to block uptake by the two transporters as compared to known inhibitors (Table 1).

Following the hypothesis that the hydantoins serve as a bioisostere with both acidic (imide proton) and non-basic amine (amide) moieties, we found that hydantoin-containing analogues failed as Sx_c^- inhibitors, but a number of structures were identified that blocked VGLUT at a level comparable to that observed with the prototypical inhibitor Congo Red (Fig. 1; standard). This observation is consistent with work demonstrating that VGLUT interacts more strongly with glutamate analogs that contain non-basic amines.[16]

Among those tested, compounds **2b**, **2e**, **2l**, and **4b** were the most effective at blocking the VGLUT-mediated accumulation of glutamate into the synaptic vesicles, allowing only 5–23% to be transported versus control (Congo Red; ~100% uptake blocked at 500 μM). The structures of these inhibitors each contain the hydantoin bioisostere but were attached to either and phenyl or thiophene ring. Inhibitor **2b** contains a disulfonic acid motif that is present in many azo dye inhibitors[20] of VGLUT and more recently shown to improve VGLUT inhibition, for example, sulfophenylglycine and sulfophenylalanine analogs.[16]

The finding that compound **2e** blocked the uptake of glutamate at VGLUT by 77% was unexpected. Although less potent than **2b**, the electron-donating p-dimethylamino substituent improves the inhibition and is more potent than the inactive phenylhydantoin (100% of control) and 1,4-bishydantoin (**4a**; 78% of control) - dimethylamino replaced with a hydantoin moiety. Since compounds **2l** and **4b** nearly blocked all glutamate uptake by VGLUT, more detailed inhibition assays were conducted. Compounds **2l** and **4b** exhibited a typical dose-curve with IC_{50}'s of 355 μM and 511 μM, respectively (Figure 2). To further confirm the selectivity of compounds **2l** and **4b** toward VGLUT each was tested at EAATs 1–3,[30] however, neither reduced uptake by greater than 12% at 100 μM (25 μM D-aspartate as substrate).

Compound **2l** is a rare example of an aldehyde-containing VGLUT inhibitor and as such, we suspected that the aldehyde may have converted to the carboxylic acid during the assay. To test this possibility, the 5-COOH analog **7** corresponding to the oxidized form of aldehyde **2l** was prepared (Scheme 3). Starting thiophene ester **6** was converted to the known 5-formylated intermediate[31–33] using the Duff reaction – the first report using this substrate. The aldehyde was converted to the hydantoin and the ester hydrolyzed to obtain target compound **7** (Scheme 3).[34] Carboxylic acid **7** also proved to be a good inhibitor of VGLUT, reducing uptake level to 7% of control. This suggests that all or part conversion of the aldehyde to the corresponding carboxylic acid could account for the observed activity.

The large difference in VGLUT inhibitory activity between compound **4b** and inactive bis-hydantoins **4a** and **5a/5b** suggests that the thiophene sulfur atom and/or the angular difference imposed on the bis-hydantoin substituents by the five-membered ring may play a role in blocking uptake at VGLUT. Further, compounds **5a/5b** lack the acidic imide protons that may be needed for effective binding to VGLUT. Molecular modeling using previously defined pharmacophore models[20] provided no clear insight for hydantoin-containing VGLUT inhibitors although the addition of lipophilic groups has not yet been thoroughly explored.

As noted, the majority of the hydantoins tested were essentially inactive as inhibitors of Sx_c^-, with none exhibiting inhibitory activity comparable to cystine, although, compounds **2k**, **2l**, **2o** and **4b** blocked glutamate uptake from 34–51% of control at 500 μM. Interestingly, each of these compounds contains a thiophene-linked hydantoin. The only other structure shown to block uptake at Sx_c^- to an appreciable amount was benzylhydantoin **2f** at 47% of control. This lack of activity was somewhat surprising, given the structural similarities between the hydantoins and numerous isoxazole-based inhibitors.[15,26] It remains to be determined if this reflects an unfavorable interaction directly between the hydantoin group and the Sx_c^- binding site or the moiety's influence on the R-group position (or a combination of both). Owing to poor inhibition of the hydantoins at Sx_c^-, compounds were not tested as individual enantiomers although further studies are underway with stereoisomers to refine and improve the potency as VGLUT inhibitors.

In sum, the hydantoin group has been shown to be an effective carboxylic acid isostere in the design of new inhibitors of the vesicular glutamate transporter (VGLUT), but one of questionable value in the further development of blockers of the obligate exchange transporter, Sx_c^-.

Acknowledgments

This research was supported by NS38248 (CMT), the Core Laboratory for Neuromolecular Production P30 NS055022 (CMT), NS058229 (ATERIS Technologies LLC), NS30570 (RJB), NS42077 (RJB) and the COBRE Center for Structural and Functional Neuroscience (NIH P20-RR015583).

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26. Synthesis of compounds 4a–b. To approx 2.0 g of carbonyl compound (0.02 mole) dissolved in 50% methanol (50 mL) were added ammonium carbonate (9.1 g; 0.08 mole) and potassium cyanide (2.6 g; 0.04 mole). The mixture was warmed to 58–60 °C for 3 h, concentrated to 15 mL, and chilled to 0 °C to produce white-off yellow crystals.
27. Spectral data for selected compounds. Compound 2b: Yield 35%; mp > 300 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ (s, 1H), 7.73 (d, *J* = 8.25 Hz, 1H), 7.50 (d, *J* = 8.25 Hz, 1H), 5.77 (s, 1H); ^{13}C: δ 169.5, 158.7, 143.4, 140.5, 136.8, 134.4, 129.7, 128.6, 54.2; ESI MS m/z = 336 (M +1); Anal. Calcd For $C_9H_8N_2O_8S_2$: C, 32.14; H, 2.40; N, 8.33. Found: C, 32.33; H, 2.22; N, 8.62. Compound 2l : ^{1}H NMR (400 MHz, acetone-d_6). δ. 10.9 (bs, 1H), 9.81 (s, 1H), 7.81 (d, J = 3.9 Hz 1H), 7.71 (d, J = 3.9 Hz, 1H), 5.91 (s, 1H); ^{13}C: δ 182.1, 164.6, 164.1, 148.2, 141.4, 138.4, 129.5, 64.2; ESI MS m/z = 211 (M+1); Anal. Calcd For $C_8H_6N_2O_3S$: C, 45.71; H, 2.88; N, 13.33. Found: C, 45.33; H, 2.91; N, 13.67. Compound 2m : Yield 75%; mp 258–261 °C; ^{1}H NMR (400 MHz, DMSO- d_6): 12.02 (bs, 1H), 10.58 (bs, 1H), 8.06 (bs, 1H), 7.59 (s, 1H), 7.09 (s, 1H), 4.99 (s, 1H); ESI MS m/z = 167 (M+1); (n max/cm^{-1}): 3414, 3241, 2700, 1729, 1456. Anal. Calcd For $C_6H_6N_4O_4$: C, 43.38; H, 3.64; N, 33.72. Found: C, 43.33; H, 3.59; N, 33.62. Compound 4a: Yield 59%; mp >300 °C; 1H NMR (400 MHz, DMSO- d_6): 10.78 (bs, 1H), 8.39 (bs, 1H), 7.33 (s, 4H), 5.15 (s, 2H); ESI MS m/z = 275 (M+1); (n max/cm^{-1}): 3237, 2925, 2721, 1701, 1458, 1377, 722. Anal. Calcd For $C_{12}H_{10}N_4O_4$: C, 52.56; H, 3.68; N, 20.43. Found: C, 52.33; H, 3.44; N, 20.62.
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34. Compound 7. Ethyl thiophene-2-carboxylate (0.43 mL, 3.2 mmol) was dissolved in TFA (5 mL) and to this solution was added hexamethylenetetramine (1.34 g, 9.6 mmol) at 90 °C for 3 h. After cooling to rt, the solution was concentrated, 3 mL of water added, and the solution brought to pH 8 with Na_2CO_3. The solution was extracted extracted with $CHCl_3$ (2×250 mL), dried over Na_2SO_4, concentrated and purified by column chromatography (EtOAc:hex, 1:4) to affford ethyl 5-formylthiophene-2-carboxylate as an off-white solid (548.4 mg, 93%). The aldehyde was converted to the hydantoin per Scheme 1. Hydantoin (0.100 g; 0.39 mmol) was placed in 1:1 MeOH/H_2O (4 mL) and LiOH (19.71 mg, 0.82 mmol) for 48 h, the reaction mixture concentrated, exrtacted with EtOAc (100 mL), and the organic phase concentrated and purified by chromatography (EtOAc:hexane, 4:1) to afford 5-(2,5-dioxoimidazolidine-4-yl)thiophene-2-carboxylic acid 7 (47.1 mg, 53%). ^{1}H NMR (400 MHz, acetone-d6). δ. 13.21(bs, 1H), 8.22 (bs, 1H), 7.55(d, J = 4.2 Hz 1H), 7.19 (d, J = 4.2 Hz, 1H), 5.88 (s, 1H); ^{13}C: δ 163.5, 163.1, 162.2, 147.1, 139.4, 134.2, 129.5, 68.2; ESI MS m/z = 227 (M+1); Anal. Calcd For $C_8H_6N_2O_4S$: C, 42.48; H, 2.27; N, 12.38. Found: C, 42.11; H, 2.32; N, 12.51.

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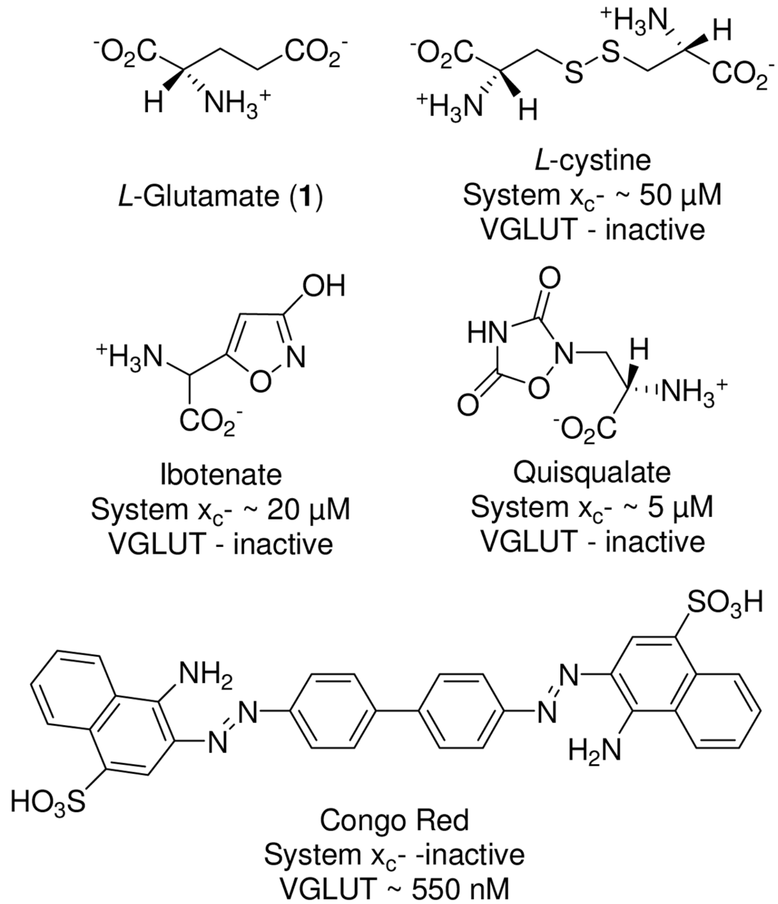

Figure 1.
Structures of system x_c^- and VGLUT inhibitors and the corresponding IC_{50} values.

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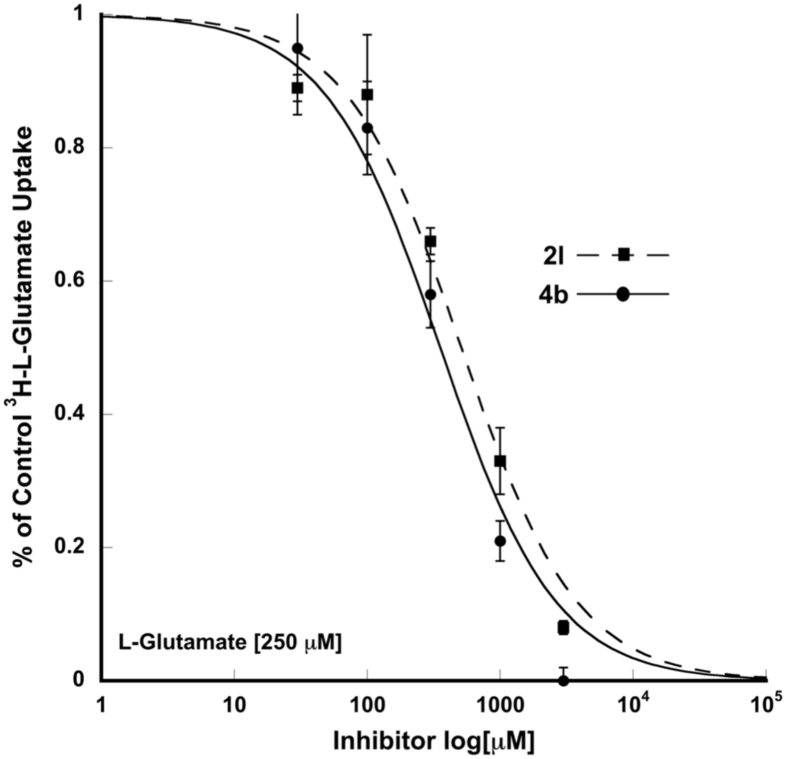
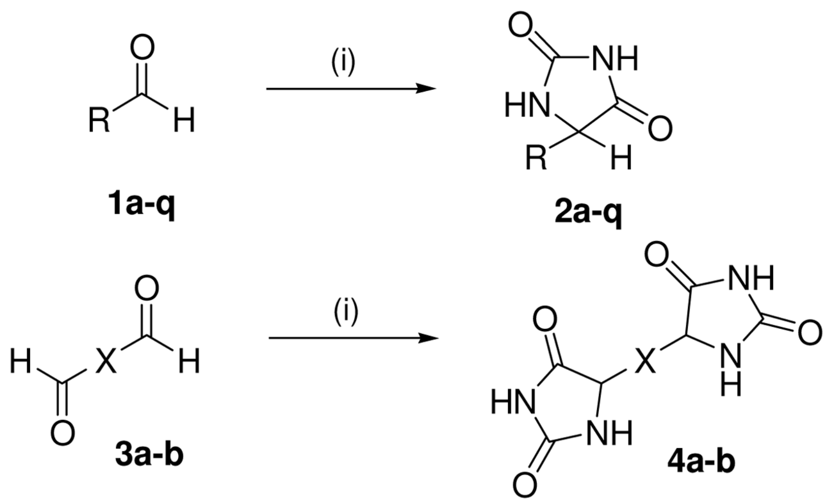


Figure 2.

Inhibition of VGLUT by **2l** and **4b**.



Scheme 1.

Synthesis of hydantoin analogs **2a–q** and **4ab** (X = thiophene, phenyl). Reagents and conditions: (i) $(NH_4)_2CO_3$, KCN, 1:1 MeOH, H_2O, 50–60 °C, 3 h.

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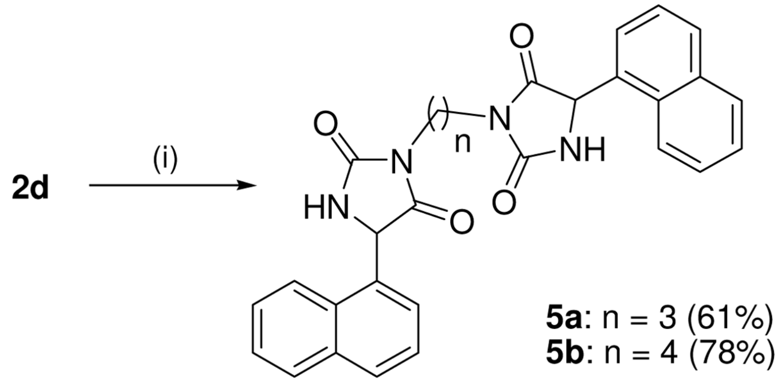

Scheme 2.

Synthesis of bis(5-(naphthalene-1-yl) hydantoins **5a–b**.

Reagents and conditions: (i) $Br(CH_2)_nBr$, K_2CO_3 MeCN, reflux, 24 h.

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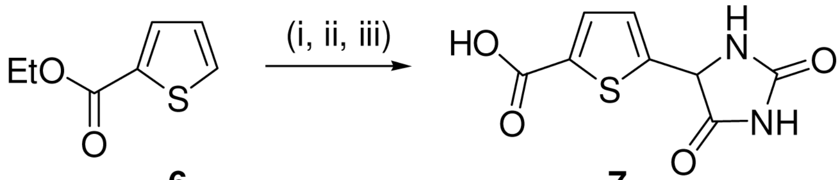

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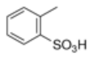
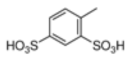
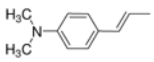
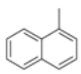
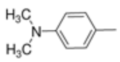
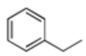
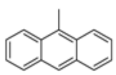
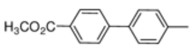
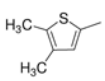
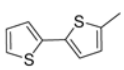
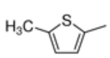
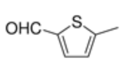
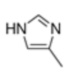
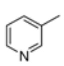
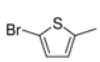
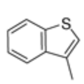
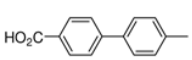
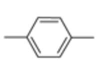
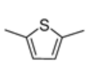
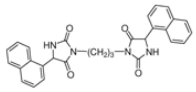
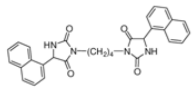
Scheme 3.

Synthesis of 5-(2,5-dioxoimidazolidin-4-yl)thiophene-2-carboxylic acid. Reagents: (i) hexamethylenetetramine, CF_3CO_2H, 90 °C, 3 h, 93%; (ii) $(NH_4)_2CO_3$, KCN, 1:1 EtOH, H_2O, 50–60 °C, 3 h, 67%; (iii) LiOH, THF/MeOH (1:1), RT, 48 h, 53%.

Table 1

Structures, yields and percent of *L*-glutamate uptake in system x_c^- and VGLUT following inhibition by compounds **2a–q**, **4a–b** and **5a–b**

Entry	R	Yield	Uptake[a] Sx_c^-	Uptake[a] VGLUT
2a		42	100±11	121±12
2b		35	100±1	11±4
2c		67	90±10	63±4
2d		77	83±17	91±14
2e		69	87±4	23±4
2f		81	53±2	80±28
2g		78	81±3	81±4
2h		71	93±6	95±7
2i		68	87±8	93±2
2j		56	85±3	87±7
2k		76	66±3	76±4
2l		73	49±3	5±1
2m		75	82±11	103±4
2n		78	95±3	111±6
2o		61	65±2	75±4
2p		66	118±5	79±10
2q		58	104±7	37±8
4a		59	85±3	78±12
4b		68	51±5	6±3

Entry	R	Yield	Uptake[a] Sx_c^-	Uptake[a] VGLUT
5a		61	80±7	97±5
5b		78	92±13	74±5
	L-Cystine	-	22±3	90±19
	Congo Red	-	82±5	31±2[b] 0±2[c]

[a]Percent of *L*-glutamate uptake by system x_c^- and VGLUT inhibited by **2a–q**, **4a–b** and **5a–b**. System x_c^- assay: 100 μM *L*-glutamate and 500 μM of inhibitor. VGLUT assay: 250 μM *L*-glutamate and 5 mM of inhibitor.

[b][I] = 500 μM.

[c][I] = 5 mM.