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Letter

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2-Arylidene Hydrazinecarbodithioates as Potent, Selective Inhibitors of Cystathionine γ-Lyase (CSE)

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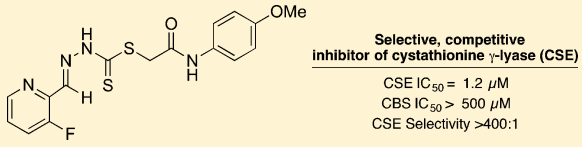
Supporting Information

ABSTRACT: Hydrogen sulfide is produced from L-cysteine by the action of both cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) and increasingly has been found to play a profound regulatory role in a range of physiological processes. Mounting evidence suggests that upregulation of hydrogen sulfide biosynthesis occurs in several disease states, including rheumatoid arthritis, hypertension, ischemic injury, and sleep-disordered breathing. In addition to being critical tools in our understanding of hydrogen sulfide biology, inhibitors of CSE hold therapeutic potential for the treatment of diseases in which increased levels of this gasotransmitter play a role. We describe the discovery and development of a novel series of potent CSE inhibitors that show increased activity over the benchmark inhibitor and, importantly, display high selectivity for CSE versus CBS.



KEYWORDS: *Cystathionine γ-lyase, cystathionine β-synthase, hydrogen sulfide, 2-pyridyl thiosemicarbazones*

In mammals, the pyridoxal-5′-phosphate (PLP)-dependent enzyme cystathionine γ-lyase (CSE) mediates the conversion of cystathionine to hydrogen sulfide (H_2S) through the intermediacy of L-cysteine (Cys).[1] Together with cystathionine β-synthase (CBS)[2] and, to a lesser extent, 3-mercaptopyruvate sulfurtransferase (3-MST),[3] CSE is the principle source of endogenous H_2S, which has been found to play an important regulatory role in a wide array of physiological processes such as inflammation,[4] blood pressure homeostasis,[5] neuromodulation,[6] cytoprotection,[7] and aging.[8]

Upregulation of CSE and increased H_2S biosynthesis has been implicated in several disease states, including inflammatory joint disease, chronic obstructive pulmonary disease, Alzheimer's disease, and endotoxemia.[9] Reducing H_2S levels through inhibition of the enzymes involved in its production has been found to hold promise as a therapeutic intervention.[10,11] Notably, the benchmark CSE inhibitor L-propargyl glycine (L-PAG, **1**) normalizes breathing and reduces hypoxia-induced hypertension in rodent models of sleep-disordered breathing, suggesting that inhibition of H_2S biosynthesis within the carotid body may be a new approach to treat hypertension in patients with sleep apnea (Figure 1).[12] The development of inhibitors that display selectivity between CSE and CBS is also of importance because expression of these enzymes is more widespread and less tissue specific than once thought. The future delineation of the roles individually played by CSE and CBS will be dependent on the availability of selective inhibitors.

Since the discovery of compound **1** as a mechanism-based inhibitor (IC_{50} = 40 μM)[13] in 1976,[14] few inhibitors of CSE have been identified, and only a limited number of these display selectivity over CBS. Furthermore, many of these molecules have significant drawbacks, e.g., compound **1** also acts as an inhibitor of alanine monotransferase and aspartate aminotransferase at the concentrations employed,[15] while β-cyanoalanine (BCA, **2**) is a neurotoxin.[16] The inherent polarity of these amino acids is an additional drawback to their use since it leads to poor cell permeability.

The paucity of selective CSE inhibitors is a significant impediment to the study of H_2S pathways and has prompted

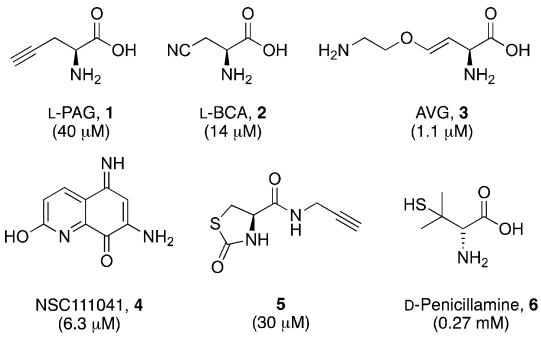


Figure 1. Chemical structure and IC_{50} values of selected inhibitors of cystathionine γ-lyase (CSE). Compounds **1**–**3** and **6** show some degree of selectivity for CSE over cystathionine β-synthase (CBS).

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considerable recent interest in this area. In 2016, the natural product L-α-(2-aminoethoxyvinyl)glycine (AVG, **3**)[17] was reported by Asimakopoulou and Panopoulos, to be a selective inhibitor of CSE (IC_{50} = 1.1 μM) versus CBS, although its selectivity against other PLP-dependent enzymes remains to be determined.[13] In 2013, high throughput screening (HTS) efforts by Zu identified iminoquinolinone derivative NSC111041 (**4**) as an inhibitor of CSE (IC_{50} = 6.3 μM),[18] while Pastore and Caliendo have recently reported the development of *N*-propargyl D-cysteine derivative **5** (IC_{50} = 30 μM).[19] Interestingly, D-penicillamine (**6**), which has previously been employed clinically to alleviate the symptoms of rheumatoid arthritis, has also been reported to selectively inhibit CSE, albeit weakly (IC_{50} = 0.27 mM).[20]

As part of a program to develop therapeutic agents for sleep-disordered breathing, we recently initiated a search for small-molecule inhibitors of CSE that display selectivity over CBS. In this letter, we report the discovery of a series of α-pyridyl alkylthio(thiocarbonyl)hydrazones that not only potently inhibit CSE but also display high selectivity over CBS. Thiosemicarbazones and their metal complexes display a range of biological activities, including antibacterial[21] and anticancer properties[22−24] for which they are the subject of considerable interest.

To identify inhibitors, recombinant human CSE was screened against >100,000 compounds from several commercial collections, including ChemDiv, Chembridge, Maybridge, and Prestwick. The high-throughput primary assay monitored CSE activity by detecting production of L-cysteine from the cleavage of L-cystathionine using the thiol-reactive fluorogenic probe, CPM (7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin). Compounds were screened in duplicate at a concentration of 40 μM, and those displaying >30% enzyme inhibition (243 compounds) were validated in secondary assays. Remaining positive hits (22 compounds) were repurchased and then assessed for inhibitory activity against CSE- and CBS-catalyzed H_2S production, using the hydrogen sulfide selective probe, 7-azido-4-methylcoumarin (AzMC) (see Supporting Information).[25] From this screening effort, one compound, initially identified as structure **7**, which inhibited CSE at 40 μM but showed weak activity against CBS, was selected for further investigation (Figure 2). This hit was reconfirmed and found to display dose-dependent inhibition of CSE, with an IC_{50} of 6 μM. We tested the reversibility of this compound by dialysis experiments (see Supporting Information) and found that compound **7** was a reversible inhibitor of CSE. Our independent synthesis of this compound (*vida infra*) indicates that its structure was incorrectly identified in the commercial library as a cyclic system, namely, 2,3-dihydro-1,3,4-thiadizole **7**, rather than Schiff base **9**.

Having identified **9** as a promising hit, we now sought to optimize the inhibitory activity of this compound, through chemical modification, and to examine the structure−activity relationships (SARs). Thus, a library of 32 analogues (**17**−**55**) were synthesized following the general strategies outlined in Scheme 1. Employing the one-pot method of Busch,[26] reaction of carbon disulfide, hydrazine hydrate, and alkyl halides **10** in the presence of base generated the corresponding *S*-alkyl dithiocarbazates **11**, which underwent condensation with aldehydes and ketones **12** to generate hydrazones **15** as single diastereomers. Alternatively, treatment of hydrazones **13** with carbon disulfide generated 1,3,4-thiadiazolidine-2-thiones **14**,[27]

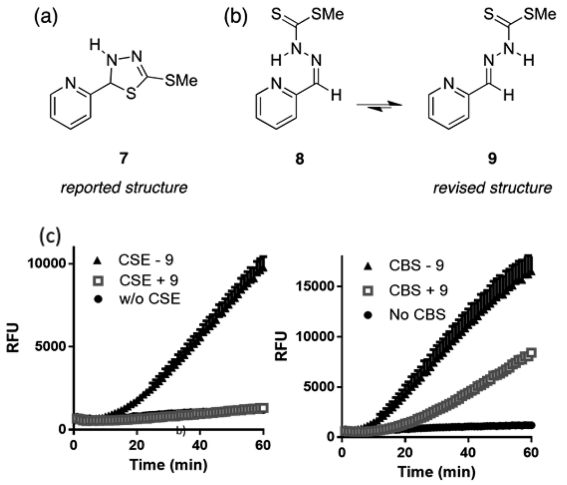
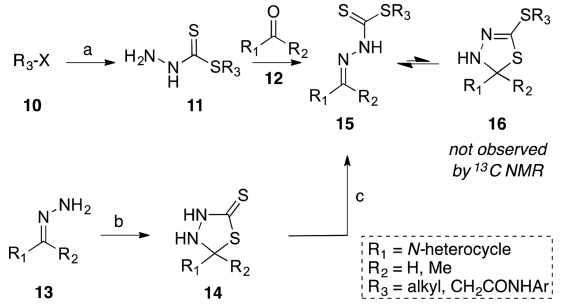


Figure 2. Structural identity of high-throughput screening (HTS) hit. (a) Reported chemical structure of library hit compound **7**. (b) Revised chemical structure **9**, established through synthesis, and associated data of this cystathionine γ-lyase (CSE) inhibitor. 2-Pyridyl thiosemicarbazones can exist in *syn*- (**8**) and *anti*-isomeric (**9**) forms, which interconvert in protic solvents. The *anti*-isomers are thermodynamically favored. (c) CSE and CBS activity assays, measuring H_2S production in the presence and absence of compound **9** (40 μM), showing selectivity for CSE over CBS. Using this AMC assay, IC_{50} = 1−2 μM for CSE.

Scheme 1. General Synthetic Scheme for the Preparation of 2-Arylidene Hydrazinecarbodithioates[a]



[a]Reagents and conditions: (a) R_3-X (**10**), CS_2, $NH_2NH_2{\cdot}H_2O$, KOH, EtOH, rt, 1−3 h; **12**, rt, 3 h (60−80%); (b) CS_2, DMF, reflux, 6 h 80−90%); (c) R_3-X (**10**), KOH, acetone, H_2O, rt, 1 h (60−80%).

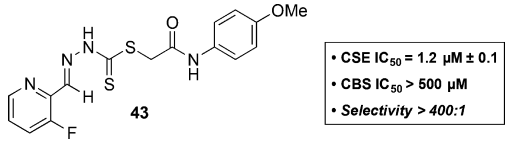


Figure 3. Profile of compound **43**, a potent, competitive inhibitor of cystathionine γ-lyase (CSE), which displays high selectivity versus cystathionine β-synthase (CBS).

which underwent ring-opening and exclusive *S*-alkylation to generate **15**. While alkylthio(thiocarbonyl)hydrazones can potentially exist in equilibrium with 2,3-dihydro-1,3,4-thiadiazoles **16**, no evidence for the presence of such *S*,*N*-acetals was found in the ^{13}C NMR spectra of these products, which display characteristic carbodithioate and hydrazone signals at approximately 200 and 150 ppm, respectively. In further regard to the structure of **15**, although 2-pyridyl thiosemicarbazones can exist

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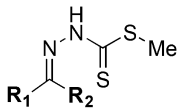
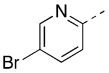
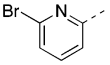
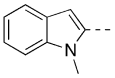
Table 1. Structure and Cystathionine γ-Lyase (CSE) Inhibitory Activity of Compounds 8 and 17−30[a]



Compd	R_1	R_2	IC_{50} (μM)	Compd	R_1	R_2	IC_{50} (μM)
8		H	6	24		H	5
17		H	50	25		H	6
18		H	40	26		H	10
19		Me	>100	27		H	12
20		H	15	28		H	5
21		H	7	29		H	>100
22		H	15	30		H	>100
23		H	6				

[a]IC_{50} values were obtained as described in the Supporting Information. The values shown are the mean of a single experiment conducted in triplicate.

as *syn*- and *anti*-isomers (**8** and **9**), detailed studies by Venkatachalam and co-workers have recently shown that, in protic solvents, interconversion takes place and favors the *anti*-isomers.[28] In all cases, the ^1H and ^{13}C NMR spectra of **15** displayed a set of signals corresponding to a single diastereomer.

The results of our initial SAR study are summarized in Table 1.[29] Retaining the methyl group at R_3, we first probed the importance of the 2-pyridyl ring at R_1 for activity, finding that substitution with 3- and 4-pyridyl systems led to an approximately 10-fold decrease in potency in both cases. Substitution with larger electron-rich *N*-heterocycles (**29** and **30**) at R_1 proved to be unproductive, resulting in complete loss of activity. That the introduction of 2-pyridyl isosteres, including piperazine (**20**), thiazoles (**21**), and pyrazoles (**22** and **23**), at the C-2 position maintains activity further validates the structural requirement of a π-deficient 2-azaheterocyclic group at R_1 that may act as a hydrogen bond acceptor. While the presence of the 2-pyridyl group is clearly of importance, the influence of substituents on this ring appears not to be dramatic, as indicated in the series **24**−**28**; compound **28**, derived from 3-fluoropicolinaldehyde proved to be the most potent member of this group. Interestingly, analogs such as **19**, derived from ketones and consequently bearing non-hydrogen groups at R_2, display no significant inhibitory effects against CSE at concentrations up to 100 μM.

The results of our second SAR study are summarized in Table 2. Initially retaining the 2-pyridyl group at R_1, the influence of the thioether substituent (R_3) was then explored. While replacement of the methyl group of initial hit **9** with simple alkyl, allyl and benzyl substituents ablated all CSE inhibitory activity (data not shown), introduction of an *N*-phenylacetamide group (**31**) proved to be more productive. Encouraged that this substituent resulted in equipotent activity with the parent system **9** and provided another point for introduction of structural diversity, we now varied aryl substitution. Introduction of alkyl (**32** and **33**) and alkoxy groups (**34**) at the C-4 position of the acetanilide proved to be the most productive, leading to a three-fold increase in potency in the case of 4-methoxyphenyl acetamide **34**. The poor aqueous solubility of several derivatives (**35**, **36**, **47**, and **51**) prevented analysis of SARs for these compounds. Prompted by the activity of 2-fluoropyridine derivative **28**, C-4 acetanilides derivatives **41**−**44** were prepared. Of this series, compound **43** proved to be the most potent CSE inhibitor found during our study with an IC_{50} of 1.2 μM. The beneficial influence of the C-4 methoxy group is also apparent with chloropyrazole **54** and thiazole **55**, which have potency close to that found with 2-pyridyl derivative **34**. Interestingly, introduction of *N*-arylacetamide units at R_2 did increase the activity of 3-pyridyl derivatives **48** and **49** relative to their *S*-Me congener **17**.

In conclusion, through high throughput screening of commercial libraries and structure-based optimization efforts, we have identified a series of potent, small molecule inhibitors of cystathionine γ-lyase (CSE), one of the principle sources of endogenous H_2S.

By way of example, compound **43** (Figure 3) has potency for CSE inhibition greater than that reported for L-PAG, the most widely used inhibitor of CSE, and displays at least 400-fold selectivity over inhibition of CBS. As such, it and other members of the series reported herein are potentially valuable pharmacological tools for the study of H_2S signaling.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00313.

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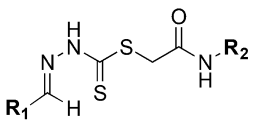
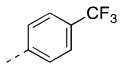
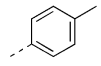
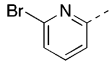
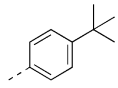
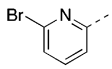
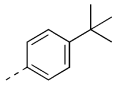
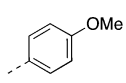
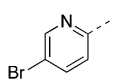
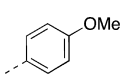
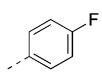
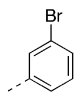
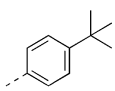
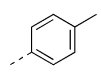
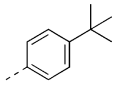
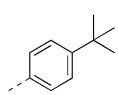
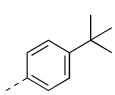
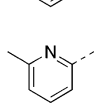
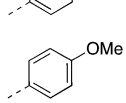
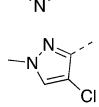
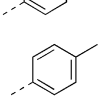
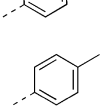
Table 2. Structure and Cystathionine γ-Lyase (CSE) Inhibitory Activity of Compounds 31−55[a]



Compd	R_1	R_2	IC_{50} (μM)	Compd	R_1	R_2	IC_{50} (μM)
31			6	44			6.0
32			2	45			10
33			3	46			15
34			2	47			>100[b]
35			>100[b]	48			25
36			>100[b]	49			12
37			3.5	50			40
38			6	51			>100[b]
39			1.9	52			3.0
40			2.5	53			6.0
41			2.0	54			2.7
42			2.0	55			2.1
43			1.2				

[a]IC_{50} values were obtained as described in the Supporting Information. The values shown are the mean of a single experiment conducted in triplicate. [b]Lack of activity may be due to low solubility.

Experimental details and characterization data for the reported compounds, NMR spectra, and biological data (PDF)

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‡These authors contributed equally. A.B, A.S., and D.J.W. designed and synthesized compounds; K.R., L.Y., and L.D.-R. performed bioassays. P.A.P. carried out the docking studies. D.J.W. wrote the paper with input from coauthors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AVG, aminoethoxyvinylglycine; CBS, cystathionine-β-synthase; CSE, cystathionine γ-lyase; CPM, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; HTS, high throughput screening; H_2S, hydrogen sulfide; L-PAG, L-propargyl glycine; PLP, pyridoxal-5′-phosphate; SAR, structure−activity relationship

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(29) For a docking study of **43** and other members of this series with the active site of cystathionine γ-lyase, see the Supporting Information.

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