Fluorescent Probes

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Identification of Cystathionine β-Synthase Inhibitors Using a Hydrogen Sulfide Selective Probe**

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Pyridoxal-5′-phosphate (PLP)-dependent enzymes catalyze several metabolically critical transformations including transaminations, amino-acid metabolism, and the metabolism of amino sugars.[1] All enzymes in this family share a common first step in their reaction mechanism: the formation of an aldimine intermediate between the PLP cofactor and the substrate.[2] After this initial step, the individual enzyme controls the reaction specificity, thus promoting the diversity of chemistry performed by this family of enzymes including decarboxylation, racemization, transamination, β- or γ-elimination, etc.[2] To date, over 140 PLP-dependent enzymes have been identified and several have been therapeutically targeted in humans. For example, DOPA decarboxylase inhibitors are routinely co-administered with L-DOPA for the treatment of Parkinson's disease and GABA aminotransferase inhibitors have found utility as antiepileptic drugs.[1] However, there are many PLP-dependent enzymes that are therapeutically attractive but have not yet been successfully targeted and even more whose therapeutic potential cannot be validated because of a lack of chemical tools for studying their biological activity.

Cystathionine β-synthase (CBS) is a PLP-dependent enzyme which plays a critical role in human sulfur metabolism. Deactivating mutations in the CBS gene are the primary cause of homocystinuria, a hereditary disease characterized biochemically by very high plasma levels of homocysteine and clinically by mental retardation, lens delocalization, skeletal abnormalities, and vascular disease.[3,4] In addition, CBS is responsible for 20–75% of total cellular production of the recently recognized cellular signaling gasotransmitter hydrogen sulfide (H_2S)[5,6] and up to 95% of the H_2S production in the brain.[7] Despite the importance of CBS activity in cellular signaling and human health, remarkably few chemical probes are available to study its activity. Currently, there are only two widely recognized CBS inhibitors, aminooxyacetate and hydroxylamine. Both have the ability to inhibit physiologically relevant CBS activity in the range of 1–10 mM, but both target the PLP cofactor and are therefore not selective for CBS.[8] Despite the poor potency and selectivity of these compounds, aminooxyacetate and hydroxylamine are routinely employed as CBS inhibitors in biological studies.[9,10] It is clear that the availability of probes with improved potency and selectivity would be of great value in understanding the biological roles of this important enzyme.

To obtain more potent and selective CBS inhibitors, an enzyme activity assay compatible with high-throughput screening was required. Given the recent explosion of interest in developing probes for H_2S,[11–18] we hypothesized that a fluorogenic probe capable of reacting selectively with hydrogen sulfide could be used to monitor CBS activity. For our purposes, we required a probe that would react rapidly and selectively with low levels of hydrogen sulfide to produce a robust fluorescent signal readily detectable by commercial plate readers without interference from much higher concentrations of biological thiolates, reducing agents, and other components of the enzyme assay buffer. In addition, a probe with a facile synthesis and purification protocol would make it easy to obtain sufficient quantities for medium- to high-throughput screening. To this end, we chose to use the azide derivative of 7-amino-4-methylcoumarin (AMC). AMC is commonly used in fluorogenic enzyme assays[19] and can easily be converted into a nonfluorescent azide using modifications of previously reported procedures.[13,20–22] The resulting 7-azido-4-methylcoumarin (AzMC) is now available commercially through Sigma–Aldrich and Echelon Biosciences. Aryl azides have been shown to react selectively with hydrogen sulfide in the presence of other reducing agents[18,22] and AzMC seemed an ideal probe for this assay.

Indeed, AzMC was readily synthesized as shown in Figure 1A. In the presence of NaHS, AzMC is converted into AMC with a concomitant increase in fluorescence which is linear for NaHS concentrations ranging from 100 nM to 100 μM (Figure 1B). This is an ideal dynamic range for enzyme assays, and is in line with that of other azide-based H_2S probes[23] including AzMC.[22] To use AzMC in a direct, continuous assay of CBS activity, we needed to ensure that the probe would not respond significantly to the components required in the assay buffer. As indicated in Figure 1C, there was no response to 10 mM cysteine or homocysteine, 1 mM PLP or 1 mM S-adenosyl-L-methionine (AdoMet), the allosteric activator of human CBS. The cofactor PLP does react with and reduce the fluorescence of AMC, so we optimized the assay to use a minimum amount of PLP while maximizing the enzyme activity. Finally, a reductant is commonly added for optimal CBS activity. We discovered that dithiothreitol

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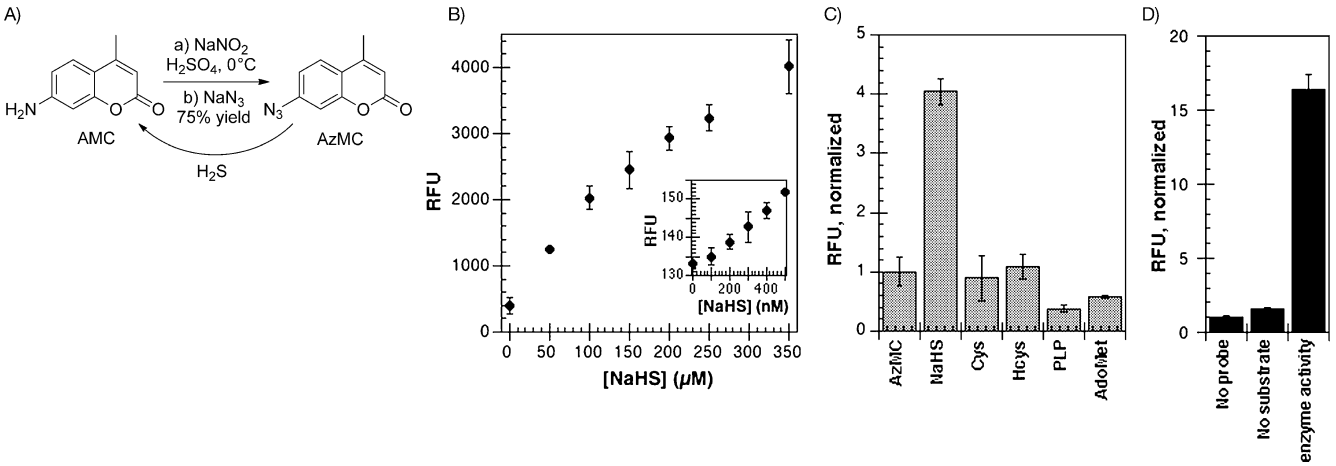

Figure 1. Utility of AzMC as an H_2S sensor and assay for CBS activity. A) Synthesis of AzMC from AMC. B) H_2S reduces AzMC (10 μM) to AMC with a concomitant linear increase in fluorescence. C) AzMC (10 μM) is selective for H_2S (100 μM) over Cys (10 mM), Hcys (10 mM), PLP (1 mM), and S-adenosyl-L-methionine (AdoMet, 1 mM). D) AzMC provides a sensitive assay for CBS activity in the presence of CBS (5 μg), AzMC (10 μM), Hcy (2.5 mM), Cys (2.5 mM), PLP (500 μM), TRIS·HCl pH 8.0 (200 mM), glutathione (10 mM).

and tris(2-carboxyethyl)phosphine (common reducing agents with large negative reduction potentials) were capable of reducing AzMC to AMC and interfering with the assay, while glutathione and β-mercaptoethanol (with smaller negative reduction potentials) did not interfere.

With a probe in hand that met all of the criteria required for a successful CBS assay, we attempted to monitor the CBS-catalyzed production of cystathionine and hydrogen sulfide from cysteine and homocysteine. For these studies, we used a truncated version of human CBS (Δ414-551), a version which does not contain the regulatory domain and has high constitutive activity in the absence of an activator.[24] As shown in Figure 1 D, significant signal over the background was recorded for CBS activity using AzMC. By comparing these results to the standard assay for measuring CBS-mediated H_2S production, the methylene blue assay,[25–27] it is clear that our assay provides a more stable signal with higher signal to noise ratio, as well as an improved dynamic range for detecting H_2S (see Figure S1 in the Supporting Information).

Considering our initial success in using AzMC to monitor H_2S-producing CBS activity, we wondered whether we might be able to use this probe to assay the activity of other PLP-dependent enzymes. First, we investigated the activity of cystathionine γ-lyase (CGL), another enzyme implicated in H_2S generation in vivo.[7] As shown in Figure 2 A, CGL-mediated production of H_2S was readily detected. Additionally, AzMC can provide a handle for monitoring the activity of other PLP-dependent enzymes.[3] As an example, tryptophan synthase (TS), the prototype type II fold PLP-dependent enzyme, catalyzes the synthesis of tryptophan from serine and indole in bacteria, yeasts, molds, and plants.[28] However, it had been shown that TS could also catalyze a β-elimination reaction on Cys to release H_2S.[27] Indeed, as shown in Figure 2 A, we are able to monitor TS activity using AzMC in the presence of Cys and HCys. In the presence of cysteine alone, the evolution of H_2S can be observed (not shown). The addition of homocysteine promotes the reaction, likely by reacting with the α-aminoacrylate intermediate formed upon release of H_2S to form cystathionine and thereby promoting catalytic turnover of the enzyme.

Based on the success of this novel enzyme assay, we carried out a screen for inhibitors of CBS activity using 1900 compounds from the Microsource Spectrum Collection. This commercially available library contains compounds selected to provide a wide range of biological activities and structural diversity including approximately 50% clinically used drugs, 30% natural products from sources worldwide, and 20% compounds with known biological activities. The library was screened in duplicate at a 50 μM compound concentration, thus resulting in the identification of 70 compounds for further investigation. The results from this initial screen are depicted schematically in Figure 2 B. The top hits were obtained in pure form and their ability to inhibit CBS activity was measured. After following up on the hits from our initial screen, we ended up with 12 compounds with significant inhibition at 150 μM (see Figure 2 C). Regarding these top hits, several interesting features should be noted. First, the fact that benserazide, a known inhibitor of the PLP-dependent enzyme DOPA decarboxylase used clinically in much of the world to treat Parkinson's disease,[1] came up as a top hit gives us confidence in the quality of our assay and screen. Second, some of the top inhibitors contain electrophilic moieties which could react directly with H_2S. For example, piperine contains a classical Michael acceptor.[29] Indeed, we discovered that piperine reacts directly with H_2S, and is a false positive from the initial screen. 1,4-Naphthoquinone, 12a-hydroxy-5-deoxydehydromunduserone, and α-mangostin also seem to react directly with H_2S as shown in Figure S2 of the Supporting Information. In contrast, dinitrophenol and dinitrocatechol can quench coumarin fluorescence (see Figure S3 in the Supporting Information), thus providing false positive signals through a different mechanism. Of the remaining hits, amiloride and benserazide are the only compounds that do not fall into the category of flavonoids and related compounds.

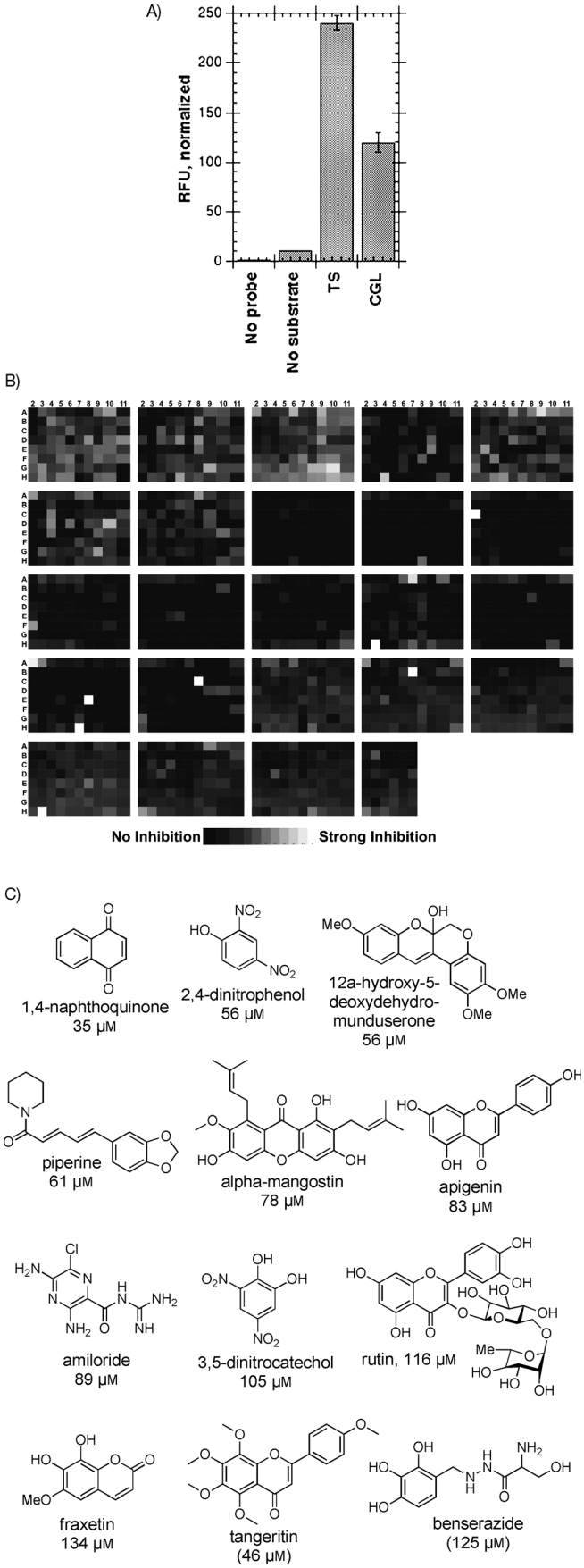

Figure 2. Application of AzMC assay to tryptophan synthase (TS) and cystathionine γ-lyase (CGL), and screening for CBS inhibitors. A) Application of the AzMC to assay for CGL and TS activity. Assays carried out in the presence of CGL or TS (5 μg), AzMC (10 μM), Hcys (2.5 mM), Cys (2.5 mM), PLP (5 μM), TRIS·HCl pH 8.0 (200 mM), glutathione (10 mM). B) Heat map showing results from the initial library screen. White boxes represent strong inhibition of CBS activity, black boxes represent no inhibition and grey boxes represent intermediate levels of inhibition. C) Top 12 hits from the CBS inhibitor screen, with IC_{50} values (or IC_{25} values in parentheses) in μM.

To investigate the selectivity of our hits, we used inhibition of CGL activity as a counterscreen. While most of the compounds showed similar activity against both CGL and CBS, benserazide and fraxetin were significantly more potent against CGL than CBS (with IC_{50} values against CGL of 102 μM and 73 μM, respectively) and tangeritin did not inhibit CGL activity at all (thus showing selectivity for CBS). Interestingly, 1,4-naphthoquinone also showed no inhibition of CGL activity. This is counterintuitive with the assumption that 1,4-naphthoquinone is a false positive and reacts directly with H_2S. Taken together, our initial screening of a small chemical library yielded two compounds, 1,4-naphthoquinone and tangeritin, which specifically inhibit H_2S production by CBS and do not affect CGL.

In conclusion, we have shown that AzMC, a fluorogenic probe selective for H_2S, provides a facile, sensitive, direct and continuous assay for monitoring the activity of PLP-dependent enzymes, specifically CBS, CGL, and TS. Using this assay, we have identified a series of novel inhibitors of CBS activity and they should be extremely useful in probing the roles of CBS in several human diseases because of their significant advantages over the nonselective, impotent CBS inhibitors currently available. Furthermore, this assay opens the door to future high-throughput screens for potent, selective CBS activators and inhibitors for use both as chemical probes and as potential therapeutics.

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