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Double-Headed Sulfur-Linked Amino Acids As First Inhibitors for Betaine-Homocysteine *S*-Methyltransferase 2

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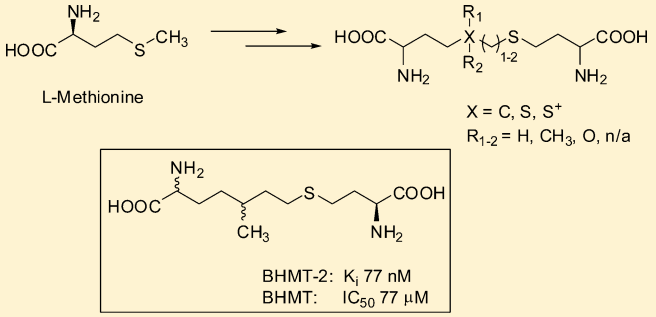
Supporting Information

ABSTRACT: Betaine-homocysteine *S*-methyltransferase 2 (BHMT-2) catalyzes the transfer of a methyl group from *S*-methylmethionine to L-homocysteine, yielding two molecules of L-methionine. It is one of three homocysteine methyltransferases in mammals, but its overall contribution to homocysteine remethylation and sulfur amino acid homeostasis is not known. Moreover, recombinant BHMT-2 is highly unstable, which has slowed research on its structural and catalytic properties. In this study, we have prepared the first series of BHMT-2 inhibitors to be described, and we have tested them with human recombinant BHMT-2 that has been stabilized by copurification with human recombinant BHMT. Among the compounds synthesized, (2*S*,8*RS*,11*RS*)-5-thia-2,11-diamino-8-methyldodecanedioic acid (**11**) was the most potent (K_i^{app} ~77 nM) and selective inhibitor of BHMT-2. Compound **11** only weakly inhibited human BHMT (IC_{50} about 77 μM). This compound (**11**) may be useful in future in vivo studies to probe the physiological significance of BHMT-2 in sulfur amino acid metabolism.



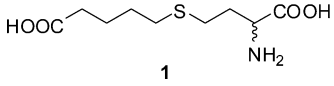
INTRODUCTION

For decades, only two mammalian enzymes were known to methylate homocysteine to yield methionine (i.e., remethylation): cobalamin-dependent methionine synthase and betaine-homocysteine *S*-methyltransferase (BHMT). Both are cytosolic enzymes belonging to Pfam02574, which are characterized as zinc-dependent methyltransferases that methylate selenols or thiols. Methionine synthase uses 5-methyltetrahydrofolate as the methyl donor[1] and is expressed in all tissues. BHMT uses betaine as the methyl donor, but its expression is limited to liver and kidney cortex. In liver, it has been estimated that both enzymes contribute equally to homocysteine (Hcy) remethylation.[2]

The structure, catalytic properties and physiological function of BHMT have been well-characterized. The crystal structures of the human enzyme complexed with a transition state inhibitor[3] and the ligand-free rat enzyme[4] have been determined. Pharmacological studies using a potent and specific inhibitor of BHMT (compound **1**, Chart 1) in mice[5] and rats[6,7] have confirmed the important role of BHMT in liver sulfur amino acids homeostasis. Recently, Teng et al.[8] demonstrated that the deletion of the *Bhmt* gene in mice, besides disrupting sulfur amino acid metabolism, broadly perturbs choline, phospholipid, and 1-carbon metabolism, resulting in fatty liver and hepatocellular carcinomas.

More recently, a third enzyme able to remethylate homocysteine has been described, BHMT-2. The human and mouse *Bhmt-2* genes were discovered and characterized in 2000 by Chadwick et al.,[9] although their enzymatic activities were not determined. The human *Bhmt-2* gene encodes a 363-residue protein (40.3 kDa) that has 73% amino acid identity to BHMT. Similar to BHMT, BHMT-2 is most abundantly expressed in liver and kidney.[9] The first attempts to express human BHMT-2 were done by Li et al.,[10] and they found that BHMT-2 was rapidly degraded in mammalian cells and aggregated after bacterial expression. In COS-1 cells, the yield of BHMT-2 protein was increased by cotransfection with BHMT. In addition, expressing BHMT-2 in HEK293T cells cultured in the presence of 1 mM Hcy increased the yield of BHMT-2 protein. However, no catalytic activity was measured when extracts were incubated in the presence of betaine and homocysteine.[10]

Chart 1. Previously Reported BHMT Inhibitor (1)



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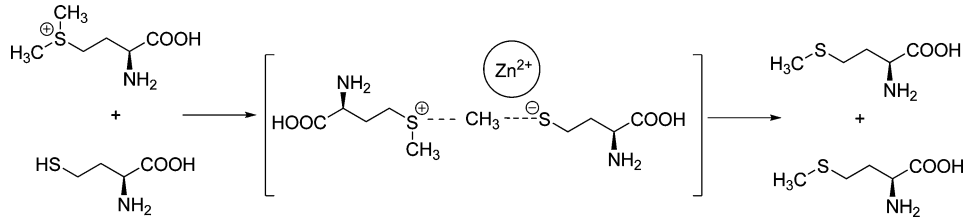
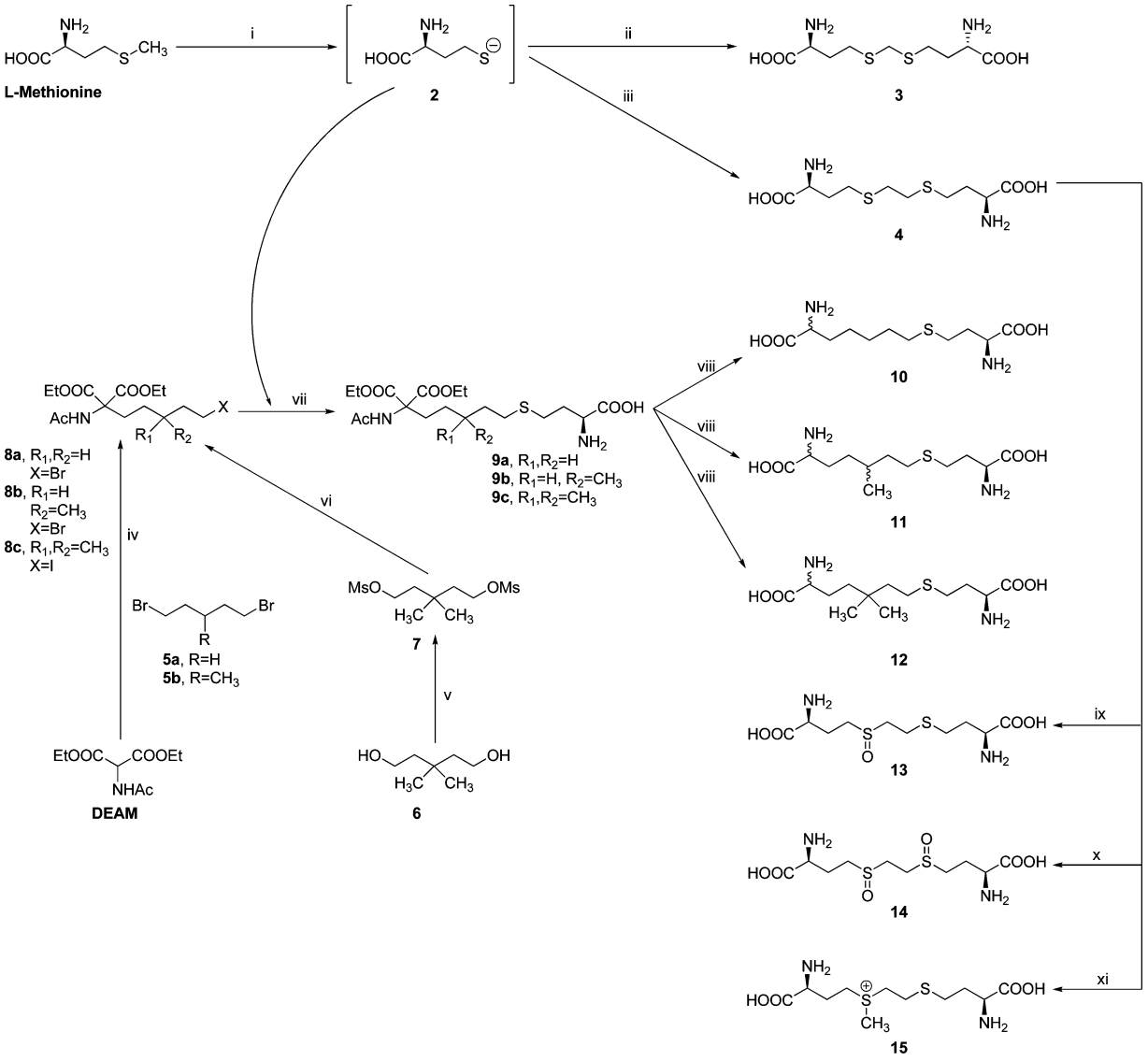
Scheme 1. Reaction Catalyzed by BHMT-2



Scheme 2[a]



[a]Reagents and conditions: (i) Na, NH_3(l), 1 h; (ii) CH_2Br_2, −33 °C, 1 h; (iii) $BrCH_2CH_2Br$, −33 °C, 1 h; (iv) EtONa, EtOH, reflux 3 h; (v) MsCl, TEA, THF, 0 °C, 12 h; (vi) DEAM, EtONa, NaI, EtOH, Ar, reflux 3 d; (vii) −33 °C, 2 h; (viii) 5 M HCl, reflux 2 h; (ix) $NaIO_4$ 1 equiv, H_2O, rt, 1 h; (x) $NaIO_4$ 2 equiv, H_2O, rt, 1 h; (xi) CH_3I, H_2O-MeOH, rt, 2d.

The breakthrough in BHMT-2 research came in 2008 when Szegedi et al.[11] expressed human BHMT-2 in *Echerichia coli* and prepared a sufficiently stable enzyme that enabled detailed biochemical and kinetic characterization of the enzyme. The authors demonstrated that BHMT-2 is a zinc metalloenzyme that methylates Hcy using *S*-methyl-L-methionine (SMM) as the methyl donor substrate. The reaction is shown in Scheme 1, including a proposed hypothetical transition state (in brackets). SMM is not synthesized in mammals but is a major sulfur-containing metabolite of many plants including cruciferous vegetables (e.g., cabbage, broccoli, asparagus, garlic, wheat germ, soybean meal, green tea, and peanuts) and fungi, and therefore it is a universal component of the mammalian diet.[12,13] SMM was originally called vitamin U by practitioners of naturopathic medicine. Szegedi et al.[11] have shown that BHMT activity is 20-fold greater than BHMT-2 activity in

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mouse liver extracts, but the activities of both enzymes are similar in kidney. They have also shown that BHMT-2 is not able to use betaine as a methyl donor and that it is only very weakly inhibited by a potent inhibitor of BHMT (compound **1** in this study, Chart 1).

At present, the significance of BHMT-2 in all of sulfur amino acid and one-carbon metabolism is not completely understood, but this enzyme was recently identified as a genetic risk factor for acetaminophen-induced liver toxicity.[14] Therefore, further studies for determining the physiological roles of BHMT-2 are needed.

During the past years, our laboratory has been interested in the synthesis and biological evaluation of inhibitors for BHMT. We have developed potent and selective inhibitors of this enzyme[15−17] that have been used for functional studies of BHMT.[5−7,18] In this study, we designed and synthesized a series of compounds intended to act as biproduct or transition state analogues of the BHMT-2 catalyzed reaction and tested their ability to inhibit the recombinant human enzyme. Our first series of inhibitors of BHMT-2 are reported herein, and at least one may be useful for pharmacological studies on the role of this enzyme in liver and kidney function.

RESULTS

Chemistry. All the inhibitors used in this study were prepared from L-methionine according to synthetic Scheme 2. In general, cleavage of the L-methionine methyl group by sodium in liquid ammonia generated a solution of L-homocysteine thiolate (**2**), which was alkylated in situ by appropriate halogenide.

For the synthesis of the symmetrical compounds **3** and **4**, alkylation with dibromoalkanes was employed. Thus, reaction of thiolate **2** with dibromomethane afforded homologue of djenkolic acid **3**,[17] while reaction with 1,2-dibromoethane gave compound **4**.[19−21]

The precursors **8a** and **8b** necessary for the synthesis of amino acids **10** and **11** were prepared starting from either commercial 1,5-dibromopentane **5a** or dibromide **5b**, which was obtained by bromination of corresponding commercial diol by CBr_4/PPh_3. Alkylation of diethylacetamidomalonate (DEAM) with **5a** or **5b** gave bromides **8a** and **8b**, respectively, which were then reacted with the solution of L-homocysteine (**2**) to furnish intermediates **9a** or **9b** (not isolated). Finally, refluxing in 5 M hydrochloric acid yielded the desired amino acids **10** or **11**, respectively.

Because isolation of 1,5-dibromo-3,3-dimethylpentane proved to be somewhat inconvenient, the diol **6** (obtained by reduction of 3,3-dimethylglutaric acid using $LiAlH_4$) was converted smoothly to dimesylate **7**, which was then reacted with DEAM and NaI to form iodide **8c**. The final compound **12** was obtained from **8c** via **9c** by a series of steps described above for target compounds **10**−**11**.

Compounds **13**−**15** were all synthesized by derivatization of amino acid **4**; its treatment with one equiv of $NaIO_4$ in aqueous solution gave sulfoxide **13**, while reaction with 2 equiv of periodate lead to the formation of disulfoxide **14**. At last, methylation of **4** by excess of methyl iodide in aqueous methanol afforded sulfonium salt **15**.

The cold substrate for BHMT-2, *S*-methyl-L-methionine (SMM), was prepared by direct methylation of L-methionine with an excess of methyl iodide.

S-[Methyl-^{14}C]methyl-L-methionine ([methyl-^{14}C]SMM) was prepared by the methylation of L-methionine by [^{14}C]-methyl iodide in the aqueous methanol in 79% radiochemical yield. Its specific activity was assayed using ^{13}C NMR as 54.2 mCi/mmol, and its radiochemical purity (>99%) was conserved during one year storage of aqueous aliquots in liquid nitrogen.

Human BHMT-2 and BHMT Overexpression and Purification. Whereas purifying human recombinant BHMT was successful from the beginning and gave sufficient amounts of stable enzyme, the first attempts at purifying human recombinant BHMT-2 were not successful. Neither the addition of stabilizing agents, such as glycerol, BSA, or octyl β-D-glucopyranoside, nor concentrating the enzyme by ultrafiltration helped preserve enzyme activity. These initial efforts resulted in low yields of BHMT-2 protein that had insignificant levels of activity despite being highly pure enzyme preparations (Figure S1, Supporting Information). Moreover, any activity measured in these preparations quickly disappeared within one or two days. Li et al.[10] suggested that 1 mM Hcy can at least partially protect BHMT-2 protein expressed in HEK293T cells. However, in our hands, growing *E. coli* transformants harboring pTYB3-hBHMT-2 in the presence of 1 mM Hcy resulted in extremely low yield of BHMT-2 protein without any sign of significant enzyme activity.

Earlier studies[10,11] proposed the idea that BHMT and BMHT-2 might co-oligomerize, and if so, their interaction could stabilize the BHMT-2 enzyme. We decided to investigate this hypothesis by growing and lysing BHMT- and BHMT-2-expressing cells together, followed by their copurification on the chitin affinity column. The idea was that once both proteins were released from their fusion tags they would then associate with each other as they eluted from the column. The SDS-PAGE combined with MS analyses of tryptic digests confirmed the presence and highly purified BHMT and BHMT-2 proteins (Figure S1, Supporting Information). BHMT (Supporting Information Figure S1, line C, band 1) was identified with 58.1% sequence coverage and 35 peptide hits (Table S1, Supporting Information). BHMT-2 (Supporting Information Figure S1, line C, band 2) was identified with 34.4% sequence coverage and 14 peptide hits (Supporting Information Table S1). The experimentally determined relative molecular masses of recombinant BHMT and BHMT-2 were similar to their predicted masses of 45 and 40 kDa, respectively, which were estimated from their position in the gel. Despite using the same amount of BHMT- and BHMT-2-expressing transformants during the coculturing process, the yield of human BHMT protein was much higher than that of BHMT-2. The protein ratio in the mixture was approximately 30:1 in favor to BHMT (as determined by densitometry of 1-D SDS gel, data not shown). Nevertheless, BHMT-2 specific activity was significant, and it was stable for a minimum of one month at 4 °C, which allowed further studies.

BHMT-2 Activity Measurements. After solving the problem of stabilizing BHMT-2 activity, we determined that recombinant BHMT in 50 mM Tris/HCl buffer (pH 7.5) does not metabolize SMM (Figure S2, Supporting Information) and hence does not interfere with BHMT-2 activity assay. This important finding allowed us perform a series of kinetic and inhibition experiments with BHMT-2 even though it was present in a mixture with BHMT protein. Next, we determined that the K_m^{app} of BHMT-2 toward D,L-Hcy is about 107 ± 10 μM (Figure 1).

Inhibition Studies. We tested the inhibition potencies of new compounds **3**, **4**, and **10**−**15** toward BHMT-2 (Figure 2A) and toward pure BHMT (Figure 2B). The inhibition data are

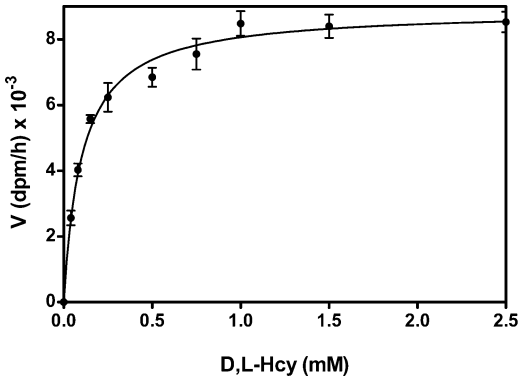

Figure 1. The determination of K_m^{app} (107 ± 10 μM) of BHMT-2 for D,L-Hcy. For details, see Experimental Section.

displayed as percentage of inhibition at 20 μM concentration of the inhibitors. We compared the inhibition data of new compounds for both enzymes with the inhibition potency of compound **1** (shown in Chart 1), a previously reported potent inhibitor of BHMT.[16,17]

Compound **3** does not inhibit BHMT-2 at 20 μM concentration at all (Figure 2A). On the other hand, another analogue of djenkolic acid, compound **4**, gives 60% inhibition as well as the derivative **10**, in which the sulfur atom of the "methionine part" of the inhibitors is replaced by methylene. The oxidation of one or two sulfur atoms in the inhibitor **4**, yielding compounds **13** and **14**, respectively, reduces the inhibition potency. Compound **15** is only a very weak inhibitor of the enzyme at 20 μM. Compounds **11** and **12** displayed the best inhibition potency among the tested compounds, giving more than 90% inhibition at 20 μM concentration. Compound **1**, a potent inhibitor of BHMT,[17] inhibited BHMT-2 only very weakly, which is consistent with previous findings of Szegedi et al.[11]

All compounds were also tested at 20 μM concentration for their inhibition of BHMT (Figure 2B) to decipher their selectivity among both closely related enzymes. Among all compounds tested, only the previously reported inhibitor **1** and new compound **12** strongly inhibited BHMT. Compound **11** gave a relatively modest inhibition of about 28%. Therefore, we decided to characterize the inhibition properties of compound **11** toward both enzymes in more detail. We determined that the IC_{50} of compound **11** toward BHMT-2 is about 3.6 μM (Figure 3A) and toward BHMT about 77 μM (Figure 3B). Finally, we decided to determine the K_i^{app} (in respect to D,L-Hcy) of inhibitor **11** for BHMT-2. From the Dixon plot shown in Figure 4, the K_i^{app} was determined to be about 77 nM, which confirms that compound **11** strongly inhibits BHMT-2. The intersection point of lines appears in the left upper quadrant of the plot, which denotes a competitive mechanism for the inhibition of BHMT-2 by compound **11**.

DISCUSSION AND CONCLUSIONS

In this study, we found that copurifying BHMT and BHMT-2 stabilizes BHMT-2 enzyme activity. This finding was crucial for the advancement of our kinetic inhibition experiments with BHMT-2 because all our previous attempts to stabilize BHMT-2, when purified without BHMT, including the addition of BSA, glycerol, or β-D-glucopyranoside, were unsuccessful. These data strongly suggest that, in our expression system, BHMT-2 must have specifically interacted with BHMT to stabilize the BHMT-2 protein and therefore its activity.[22]

A major difference between the primary sequences of BHMT-2 and BHMT is the lack of 34 residues at the C-terminus (residues 373−406) of BHMT-2 relative to BHMT.[11] The crystal structure of BHMT shows that these residues have an important role in oligomerization, which include a terminal α-helix that is required for BHMT dimers to dimerize into a tetramer.[4] Truncating the C-terminus of BHMT to encode a protein that is the same size as BHMT-2 results in an unstable protein.[22] Although BHMT-2 retains key residues found to be important in the oligomerization of BHMT, including those that are clearly required for dimerization (residues 264, 266, 268, 269, 275, 279, 346, 352, 358, and 361) and tetramerization (residues 316, 321, 329, 330, 349, and 387),[11] it appears likely that the lack of the terminal α-helix prevents BHMT-2 from forming homo-oligomers and so it must oligomerize with BHMT to stabilize its structure and therefore preserve its activity. We propose, therefore, that BHMT-2 forms hetero-dimers and/or heterotetramers with BHMT in vitro and in vivo and that these similar enzymes work in a close structural cooperativity.

We must note that mixing pure BHMT and BHMT-2 immediately after their elution from the affinity column does not rescue or stabilize BHMT-2 activity (data not shown). Only the simultaneous lysis of both transformed cell lines and the subsequent simultaneous affinity purification and elution of

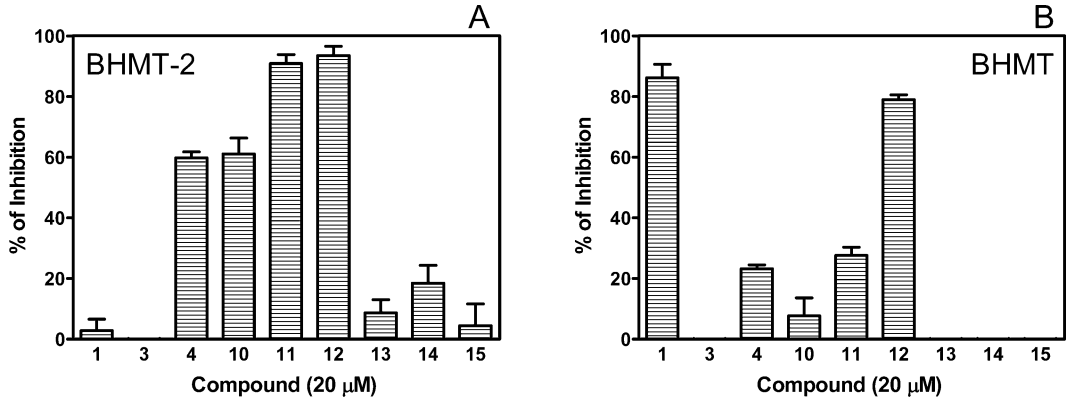

Figure 2. Relative inhibition of human BHMT-2 (A) and of human BHMT (B) by compounds **1**, **3**, **4**, and **10**−**15**. The percent of inhibition by compounds was determined at 20 μM concentration. The points were measured in tetraplicates, and the data are displayed as means ± SEM. See Experimental Section for details.

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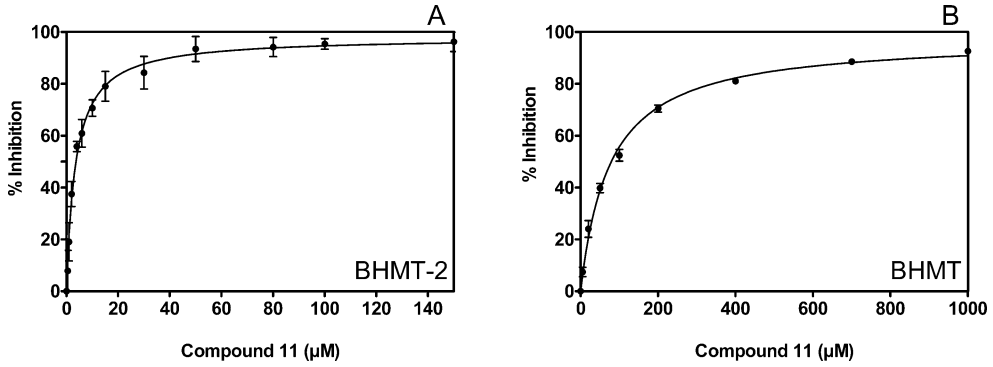

Figure 3. (A) The representative curve for the determination of the IC_{50} (3.6 ± 0.5 μM) of compound **11** toward BHMT-2. (B) The representative curve for the determination of the IC_{50} (76.6 ± 9 μM) of compound **11** toward BHMT. All points were measured in duplicates, and the IC_{50}s (±SEM) were calculated from three independent measurements. See Experimental Section for details.

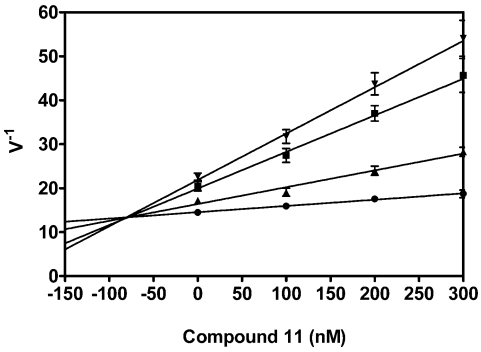

Figure 4. Determination of K_i^{app} of compound **11** for human BHMT-2 toward D,L-Hcy. The curves were measured at fixed concentration of SMM (250 μM, 0.3 μCi) and four different concentrations of D,L-Hcy: (▼) 75 μM, (■) 100 μM, (▲) 150 μM, and (●) 250 μM. All points were measured in triplicates and in four separate measurements. The data are displayed as means ± SEM. The intersection point of the lines gives K_i^{app} of about 77 ± 7 nM. For details, see Experimental Section.

BHMT and BHMT-2 proteins results in stable BHMT-2 activity. These results may signify that BHMT-2 needs to oligomerize with BHMT immediately after its cleavage from the affinity column and that similar processes may occur in mammalian cells after the natural expression of the enzymes.

Another important finding, which allowed us the further work with BHMT-2, was that BHMT is not able to use SMM as a methyl donor in the Tris/HCl buffer. This observation seems to be contradictory to the findings of Szegedi et al.,[11] who detected specific BHMT activity for SMM. However, we found that BHMT substrate specificity is dependent on the presence or absence of potassium ions. Indeed, in the presence of potassium ions, BHMT is able to use SMM as a substrate, but it is not able when the buffer is completely void of potassium (data not shown, manuscript in preparation).

We found that K_m^{app} of BHMT-2 toward D,L-Hcy is about 107 ± 10 μM. Szegedi et al.[11] have previously determined the K_m^{app} of BHMT-2 for SMM to be about 940 μM. It suggests that BHMT-2 binds Hcy more tightly than its methyl donor SMM.

The majority of the new inhibitors for BHMT-2 are not charged in the "central part" of their molecules (compounds **3**, **4**, and **10**–**12**). These compounds resemble the structures of products (two methionines) separated by different linkers and, therefore, might be considered as biproduct analogues.[23,24] We previously used a similar strategy for the development of inhibitors for BHMT.[16,17] The only compound that might be considered a possible transition state analogue[25,26] is the charged inhibitor **15**.

The preference of BHMT-2 for compounds **11** and **12** altogether with its weak affinity for the compound **1**, a strong inhibitor of BHMT that does not contain "methionine" moiety in its structure, confirms the findings of Szegedi et al.[11] that BHMT-2 uses SMM and not betaine as a methyl group donor in vivo. The similar affinity of compounds **11** and **12** for BHMT-2 shows that both configurations, *R* and *S*, are allowed for the methyl group at the position 8 of the inhibitor **11**. The stronger inhibition of BHMT by compound **12** compared to compound **11** (Figure 2B) is in agreement with results of our previous study[17] when (*RS*)-5-(3-amino-3-carboxypropylthio)-3,3-dimethylpentanoic acid was a stronger inhibitor of BHMT than (*RS*,*RS*)-5-(3-amino-3-carboxypropylthio)-3-methylpentanoic acid. Moreover, the preference of BHMT-2 for the ethylene moiety (compound **4**) and not for methylene (compound **3**), separating "homocysteine" and "methionine" parts of inhibitors and its intolerance for charged atoms in the central part of inhibitors (compounds **13**–**15**) but tolerance for sulfur or alkyl groups in this position (compounds **4** and **10**–**12**), suggest that both enzymes, BHMT-2 and BHMT, are similar in their catalytic mechanisms. It appears that the most potent BHMT-2 inhibitors from this study are more likely biproduct analogues than transition state analogues. The same rules governing the affinity for inhibitors we recently discovered for BHMT.[17]

Compound **11** is a mixture of four diastereomers, and compound **12** is a mixture of two diastereomers. It would have been interesting to separate and characterize the diastereomers and to determine which diastereomer (11*S* or 11*R*) is most active. However, the synthesis of all four diastereomers of **11** or both two of **12** would substantially extend the scale of synthetic work, which we considered unnecessary at this stage. We plan to develop a synthetic strategy for the most potent compounds as pure distereomers in the next phase of the project.

The finding that compound **11** is a strong and competitive inhibitor of BHMT-2 (K_i^{app} is about 77 nM) but only moderately inhibits BHMT (IC_{50} about 77 μM) is important for future in vivo studies of the physiological functions of BHMT-2.

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EXPERIMENTAL SECTION

Unless otherwise stated, reagents and materials were obtained from commercial suppliers (Sigma-Aldrich, Fluka, Merck) and used without purification.

Synthesis of *S*-[Methyl-^{14}C]methyl-L-methionine Chloride. L-Methionine (15.7 mg, 105 μmoles) was dissolved in 300 μL of water and 42 μL of methanol in a 25 mL flask equipped with a magnetic stirrer and adapter for connection to a vacuum manifold with stopcock. The [^{14}C]methyl iodide (5 mCi, 87 μmol, ARC, Inc.) was transferred to the reaction mixture from the break-seal ampule using vacuum manifold. The pressure in the reaction flask was brought to atmospheric by argon via the adapter stopcock. The reaction flask was covered by aluminum foil, and the reaction mixture was stirred at room temperature for 48 h. The product was isolated using two ion-exchange resin columns arranged in tandem. The reaction mixture was transferred on the first column containing 3 g of DOWEX 1 × 4 100–200 mesh in Cl^- form. The second column contained 3 g of BioRex 70 in H^+ form. The columns in tandem were washed by 30 mL of water. Then the second column with BioRex was washed with another 25 mL of water. The product was washed out from the BioRex 70 column by 20 mL of 1N HCl. The eluate was evaporated to dryness on vacuum line, and the residue was dissolved in 5 mL of water. The yield of *S*-[methyl-^{14}C]methyl-L-methionine hydrochloride (SMM) was 3.96 mCi (79% on [^{14}C]methyl iodide), the radiochemical purity was >99% (radio-HPLC; Luna 3 μm C18 100 Å, 150 mm × 4.6 mm column from Phenomenex; solvents: A = 50 mM KH_2PO_4 and 5 mM sodium 1-heptanesulfonate in water, B = 50 mM KH_2PO_4 and 5 mM sodium 1-heptanesulfonate in 50% aqueous acetonitrile; flow 1 mL/min; temperature 25 °C; gradient elution: t = 0 min (0% B), t = 5 min (0% B), t = 10 min (100% B); radiochromatographic detector Ramona with LSC cell from Raytest, Germany). As anticipated, in the ^{13}C NMR of [methyl-^{14}C]SMM, the intensity of CH_3S doublet at 27.4 ppm was significantly lower than intensity of this doublet in the cold standard. From this decrease of intensity, the specific activity of [methyl-^{14}C]-SMM was calculated as equal to 54.2 mCi/mmol. The activity concentration was adjusted to 0.1 mCi/mL, and the aliquots were stored in liquid nitrogen.

Overexpression and Purification of Human BHMT and Human BHMT-2. BHMT and BHMT-2 proteins were purified as described.[11,27] In brief, 30 mL of 2xYT media containing 0.1 mg/mL ampicillin was inoculated with a culture of *Escherichia coli* BL31(DE3) cells transformed with pTYB4-hBHMT or pTYB3-hBHMT-2 plasmids, respectively. After growing the cells (37 °C) to A_{595} = 0.6, the cells were collected by centrifugation (1500*g*, 10 min) and used to inoculate 1 L of 2xYT media containing 250 μM zinc chloride and 0.1 mg/mL ampicillin. When BHMT and BHMT-2 were copurified, the corresponding transformants were separately grown in 30 mL of 2xYT media, but following centrifugation, then combined and used to inoculate the larger flask containing 1 L of 2xYT media. The 1 L cultures were grown (37 °C) until A_{595} = 1 and then induced with IPTG (0.3 mM, Duchefa Biochemie BV) and incubated for a minimum of 16 h. Following the induction period, the cells were collected by centrifugation at 4500*g* for 10 min (4 °C) and resuspended in 20 mL of cold 20 mM Tris/HCl buffer (pH 8.0) containing 0.5 M NaCl, 0.1 mM EDTA, and 0.1% Triton X-100 (w/v). Then, Protease Inhibitor Cocktail Set I (Calbiochem) and TCEP (Sigma) and DNase I (Sigma) (about 5 mg of each) were added to the cells, which were then lysed using a French press. The extract was then sonicated to shear the cellular DNA. The lysate was centrifuged (22500*g*, 90 min, 4 °C) and the enzyme(s) purified at 4 °C using chitin affinity chromatography (Chitin Beads, New England Biolabs). The chitin affinity column (20 mL) was washed with 200 mL of Milli-Q water and then equilibrated with 200 mL of 20 mM Tris/HCl (pH 8.0) buffer containing 0.5 M NaCl, 0.1 mM EDTA, 0.1% Triton X-100 (w/v), and 0.2 mM TCEP. The lysate was supplemented with a pinch of DNase I and then applied to the column (0.5 mL/min). The column was washed (1 mL/min) with 600 mL of the same buffer and then 100 mL of buffer containing 20 mM Tris/HCl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA, 0.2 mM TCEP, and 10% glycerol (w/v), and finally rapidly (5 mL/min) with 30 mL of the same buffer supplemented with 30 mM β-mercaptoethanol. The column was then allowed to stand for a minimum of 16 h at 4 °C. Afterward, proteins were eluted using 20 mM Tris/HCl (pH 8.0) buffer containing 50 mM NaCl, 0.1 mM EDTA, 0.2 mM TCEP, 10% glycerol (w/v), and 30 mM β-mercaptoethanol. The protein concentration was determined using the Bradford assay.[28]

Protein identity and purity was confirmed by SDS-PAGE and MS analysis of tryptic digests. Selected bands were excised and the following procedures were performed as previously described[29] with slight modifications. Briefly, after dehydration, the dry gel pieces were reduced for 30 min at 65 °C using 50 mM amonium bicarbonate and 20 mM DTT and then alkylated at rt in the dark for 30 min with 1 μL of 18 M 2-iodoacetamide. Dehydration was repeated with 200 μL of CH_3CN for 5 min at 30 °C. The peptides were extracted from the gel with sonication (Elma, Singen, Germany), and the extracted peptides were then concentrated in a SpeedVac (Thermo Fisher Scientific, USA). The resulting peptides were analyzed by LC-MS/MS on an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Germany) coupled to a Rheos 2000 2-D capillary chromatograph (Flux Instruments, Switzerland). The first dimension column was a PepSwift Monolithic PS-DVB, 200 μm × 5 mm (Dionex, USA), and the second dimension column was a PepMap C18 (3 μm), 75 μm × 150 mm (Dionex, USA). The linear gradient was formed by 0.1% formic acid in H_2O (mobile phase A) and 99.9% CH_3CN, 0.1% formic acid (mobile phase B), from 2% to 95% of mobile phase B in 85 min at a flow rate of 200–300 nL/min, and total length of the LC separation was 140 min.

A data-dependent scan composed of one full MS scan (resolution of 60000) and three CID MS/MS scans (resolution of 7500, normalized collision energy 35 V) was used as the mass spectrometry method. The mass tolerance for peptide identification was 2 ppm. Electrospray ionization parameters were as follows: capillary temperature, 200 °C; capillary voltage, 40 V; tube lens, 100 V; ion spray voltage, 1.5 kV.

The collected MS data from LTQ-XL Orbitrap were searched in the Uniprot-SwissProt protein database (version accessed at March, 2011) by Bioworks Browser 3.3.1 SP1 and Sequest 2.0 software (Thermo Fisher Scientific, USA) with carbamidomethylation of cysteine and oxidation of methionine specified as variable modification and without restriction on the taxonomy.

BHMT Activity Assay. BHMT activity was assayed as previously described[17] with several modifications. *N*-Methyl-^{14}C-betaine (57 mCi/mmol) was prepared and supplied by Moravek Biochemicals (Brea, CA). The standard reaction mixture (0.5 mL) contained 2 mM betaine (0.15 μCi), 1 mM D,L-Hcy, 50 mM Tris/HCl (pH 7.5), 0.07% (w/v) β-mercaptoethanol, and different concentrations of BHMT (0.2 or 0.4 μM). D,L-Hcy was freshly dissolved in 50 mM Tris/HCl (pH 7.5) and immediately used in the assay. Reaction tubes were kept in ice-cold water until the assay was started by transferring the tubes into a 37 °C water bath. After 30 min, the reaction was stopped by transferring the tubes into ice-cold water, and 1 mL of ice-cold water was added to each reaction tube. The samples were then applied to a Dowex 1 × 4 (OH^-, 200–400 mesh) column (1 mL of resin). Unreacted betaine was washed with water (3 × 5 mL) and dimethylglycine and methionine products were eluted into vials with 1.5 M HCl (1.5 mL). The scintillation coctail (10 mL) was added to each vial, and the samples were counted. Blank tubes contained all of the reaction components except enzyme, and their values were subtracted from the sample values. All samples were assayed at least in triplicate.

BHMT-2 Activity Assay. The standard reaction mixture (0.2 mL) contained 0.25 mM SMM (0.3 μCi), 0.2 mM zinc chloride, 0.1% (w/v) β-mercaptoethanol, 50 mM Tris/HCl (pH 7.5), and 2.5 mM D,L-Hcy (freshly prepared in the buffer). The amount of the enzyme was calculated based on the total protein concentration[28] (BHMT-2 plus BHMT) and varied in different experiments (0.2–1 μM). Reaction tubes were kept in ice-cold water until the assay was started by transferring the tubes into a 37 °C water bath. After 60 min, samples were transferred into ice-cold water, methanol (1.5 mL) was added to each reaction tube, and the tubes were centrifuged (16000*g*, 5 min, 4

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°C). The supernatants were applied to a Dowex 1 × 4 (OH^-; 200–400 mesh) columns (1 mL of resin), and the following procedures were the same as for BHMT assay. All samples were assayed at least in triplicates.

The apparent K_m^{app} of BHMT-2 for D,L-Hcy was determined at 0.25 mM SMM (0.3 μCi), while the concentration of D,L-Hcy varied from 40 μM to 2.5 mM. The data were analyzed using the GraphPad Prism 5 program by use of nonlinear regression fit and K_m^{app} (±SEM) calculated from three independent measurements with all points in tetraplicates.

Inhibition Assays. For both enzymatic activities (BHMT and BHMT-2) the conditions for the determination of percent of inhibition at fixed concentration (20 μM) of compounds **1**, **3**, **4**, and **10**–**15** and for IC_{50} values of inhibitors **11** and **12** were the same as described above. The curves for the determination of IC_{50} values consisted of at least nine different inhibitor concentrations. All points were measured in duplicates and IC_{50}s (±SEM) calculated from three independent measurements. The data were analyzed using the GraphPad Prism 5 program by use of nonlinear regression fit.

The apparent inhibition constant (K_i^{app}) of compound **11** toward D,L-Hcy was measured at fixed concentration of SMM (0.25 mM, 0.3 μCi) and variable concentrations of D,L-Hcy (from 75 to 250 μM) and inhibitor **11** (from 0 to 300 nM). All points were measured in triplicates and in four separate measurements. The data were analyzed by Dixon plot[30] as described previously.[16]

Chemistry: General. Unless otherwise stated, reagents and solvents used in this study were obtained from commercial suppliers (Sigma-Aldrich, Fluka, Merck) and used without purification. The solvents were evaporated at 40 °C and 2 kPa, and the products were dried over phosphorus pentoxide at rt and 13 Pa. TLC on silica gel coated aluminum plates (Fluka) was performed in the following systems (v/v): chloroform–ethanol 9:1 (C1), chloroform–ethanol 19:1 (C2), ethyl acetate–acetone–ethanol–water 4:1:1:1 (H1), ethyl acetate–acetone–ethanol–water 6:1:1:0.5 (H3), 2-propanol–concd aqueous ammonia–water 7:1:2 (IPAV), 50% ethyl acetate–toluene (T1), 20% EtOAc–toluene (T2), 5% EtOAc–toluene (T3). The compounds were visualized by exposure to UV light at 254 nm by ninhydrin spraying (dark blue color of amines), by 1% $KMnO_4$ spraying (yellow color of sulphides), and by spraying with a 1% (v/v) ethanolic solution of 4-(4-nitrobenzyl)pyridine followed by heating and treating with gaseous ammonia (blue color of esters). Flash chromatography purifications were carried out on silica gel (40–63 μm, Fluka). Preparative RP-HPLC chromatography of the target compounds was carried out on a C18 Luna column (Phenomenex, 250 mm × 21.2 mm, 10 μm) at a flow rate 9 mL/min. Solvent A: 0.1% TFA in H_2O. Solvent B: 80% CH_3CN, 0.1% TFA, H_2O. The following gradient was used: t = 0 min (0% B), t = 10 min (0% B), t = 20 min (20% B), t = 25 min (40% B), t = 30 min (80% B), t = 31 min (0% B). Purified compounds were lyophilized from a water/acetonitrile/TFA mixture. Analytical RP-HPLC chromatography was carried out at a flow rate of 1 mL/min on a C18 Nucleosil column (250 mm × 4 mm, 5 μm) from Watrex (Praha) and on C18 Luna column (150 mm × 4.6 mm, 5 μm) from Phenomenex using the same gradients and solvents. Eluted compounds were detected at 210 and 218 nm. ^1H and ^{13}C NMR spectra were measured on a Bruker AVANCE-600 spectrometer (^1H at 600.13 MHz, ^{13}C at 150.9 MHz) in $CDCl_3$, DMSO-d_6, CD_3OD, or D_2O solution at 300 K. The 2D-H,H-COSY, 2D-H,C-HSQC, and 2D-H,C-HMBC spectra were recorded and used for the structural assignment of proton and carbon signals. HRMS spectra were obtained on a FTMS mass spectrometer LTQ-orbitrap XL (Thermo Fisher, Bremen, Germany) in electrospray ionization mode or in the case of HRMS (EI) on GCT Premier (Waters). Purity of target compounds was confirmed by elemental analysis (C, H, N, S, F), agreeing with calculated values within 0.4% and by analytical RP-HPLC chromatography as described above confirming ≥95% purity.

The determination of C, H, and N in solid samples was performed using an automatic PE 2400 series II CHNS/O analyzer. For analysis of F, the samples were combusted by Schöniger method (i.e., in quartz Erlenmeyer flask in oxygen atmosphere). Formed F^- was determined potentiometrically by using ion-selective electrode. For analysis of S, the sample was combusted by Schöniger method and formed SO_4^{2-} ions were titrated in the presence of 2-propanol by a standard solution of $Ba(ClO_4)_2$ with thorin as an indicator.

Alkylation of Homocysteine: General Procedure. L-Methionine (1 equiv) was dissolved in an intensively stirred liquid ammonia (cca 6 mL/mmol, −33 °C) in a three-necked flask equipped with potassium hydroxide tube on inlet and outlet. Sodium (3.1 equiv) was slowly added in small pieces until the blue color of the reaction mixture persisted. Appropriate alkyl halogenide (1.1 equiv) or dihalogenide (0.5 equiv) was then added (in the case of solid bromides, their solution in minimal amount of anhydrous THF was used), and the stirring was continued without cooling until the ammonia had evaporated completely (1–2 h). Specific workup of the solid residue is given for the each compound separately.

5,7-Dithia-(2*S*,10*S*)-diaminoundecanedioic Acid (3). Following the general procedure, the target compound was prepared from L-methionine (3.0 g, 20 mmol, 1 equiv), sodium (1.44 g, 62 mmol, 3.1 equiv), and dibromomethane (0.726 mL, 10 mmol, 0.5 equiv). The crude product was purified by HPLC and lyophilized, giving **3** as di(trifluoroacetate) (white solid, 0.570 g, 20%).

^1H NMR (600 MHz, D_2O): 2.19 (m, 2H) and 2.27 (m, 2H, C(3)H_2, and C(9)H_2), 2.82 (m, 4H, C(4)H_2, and C(8)H_2), 3.83 (s, 2H, C(6)H_2), 4.07 (dd, 2H, J = 6.8 and 5.9, C(2)H and C(10)H).

^{13}C NMR (150.9 MHz, D_2O): 28.50 (C-4 and C-8), 32.37 (C-3 and C-9), 36.36 (C-6), 55.37 (C-2 and C-10), 175.47 (C-1 and C-11).

HRMS (ESI) calculated for $C_9H_{17}O_4N_2S_2$, m/z 281.0635, found 281.0634 $(M - H)^-$.

(2*S*,11*S*)-5,8-Dithia-2,11-diaminododecanedioic Acid (4). Following the general procedure, the target compound was prepared from L-methionine (3.0 g, 20 mmol, 1 equiv), sodium (1.44 g, 62 mmol, 3.1 equiv), and dibromoethane (0.866 mL, 10 mmol, 0.5 equiv). The crude product was purified by HPLC and lyophilized, giving **4** as di(trifluoroacetate) (white solid, 1.24 g, 27%).

^1H NMR (600 MHz, D_2O): 2.19 (m, 2H) and 2.29 (m, 2H, C(3)H_2, and C(10)H_2), 2.76 (m, 4H, C(4)H_2, and C(9)H_2), 2.85 (s, 4H, C(6)H_2 and C(7)H_2), 4.18 (dd, 2H, J = 6.7 and 6.1, C(2)H and C(11)H).

^{13}C NMR (150.9 MHz, D_2O): 29.13 (C-4 and C-9), 32.36 (C-3 and C-10), 33.29 (C-6 and C-7), 54.69 (C-2 and C-11), 174.77 (C-1 and C-12).

HRMS (ESI) calculated for $C_{10}H_{19}O_4N_2S_2$, m/z 295.0792, found 295.0791 $(M - H)^-$.

1,5-Dibromo-3-methylpentane (5b). A solution of triphenylphosphine (8.88 g, 33.8 mmol, 4 equiv) in dichloromethane (15 mL) was slowly added to an intensively stirred solution of 3-methyl-1,5-pentanediol (1.0 g, 8.46 mmol, 1 equiv) and CBr_4 (11.23 g, 33.9 mmol, 4 equiv) in dichloromethane (20 mL) at 0 °C, and the reaction was allowed to proceed for 1 h. The reaction mixture was then concentrated in vacuo, the solid residue was extracted with hexane, the solution was evaporated, and the crude product was purified by flash chromatography on silica (elution with a linear gradient of hexane in toluene) to give compound **5b** as a colorless oil (1.76 g, 85%).

^1H NMR (600 MHz, $CDCl_3$): 0.94 (d, J = 6.8, 3H, CH_3), 1.72 and 1.97 m (4H, C(2)H_2 and C(4)H_2), 3.41 and 3.47 m (4H, C(1)H_2 and C(5)H_2).

^{13}C NMR (150.9 MHz, $CDCl_3$): 18.14 (CH_3), 30.55 (C-3), 31.24 (C-1 and C-5), 39.37 (C-2 and C4).

HRMS (EI) calculated for $C_6H_{12}Br_2$, m/z 241.9306, found 241.9313 $(M)^+$.

1,5-Bis(mesyloxy)-3,3-dimethylpentane (7). A solution of mesyl chloride (5.65 mL, 73.0 mmol, 3 equiv) in THF (20 mL) was added dropwise to a solution of 3,3-dimethylpentane-1,5-diol[31] (**6**, 3.22 g, 24.3 mmol, 1 equiv) and triethylamine (20.3 mL, 146.1 mmol, 6 equiv) in THF (80 mL) at 0 °C, and the reaction was stirred overnight at rt. The mixture was then evaporated and partitioned between Et_2O and H_2O, and the organic layer was dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by flash chromatography on silica (elution with a linear gradient of EtOAc in toluene) to give compound **7** as pale-yellow crystals (6.20 g, 88%).

^{1}H NMR (600 MHz, $CDCl_3$): 1.02 (s, 6H, $C(CH_3)_2$), 1.76 (t, J = 7.2, 4H, 2× CH_2), 4.30 (t, J = 7.2, 4H, 2× O−CH_2), 3.03 (s, 6H, 2× S-CH_3).

^{13}C NMR (150.9 MHz, $CDCl_3$): 27.40 (2× CH_3 ($C(CH_3)_2$), 31.79 (>C<), 37.49 (2× S-CH_3), 40.20 (2× CH_2), 66.64 (2× O−CH_2).

HRMS (EI) calculated for $C_9H_{20}O_6NaS_2$, m/z 311.05935, found 311.05924 $(M + Na)^+$.

2-Carboxyethyl-2-acetamido-7-bromoheptanoic Acid (8a). Sodium ethanolate was generated in situ by dissolving sodium (0.318 g, 13.83 mmol, 1.0 equiv) in abs EtOH (20 mL) at rt in a flask equipped with a reflux condenser fitted with a calcium chloride tube. Diethyl acetamidomalonate (DEAM, 3.0 g, 13.81 mmol, 1.0 equiv) was added, and after 30 min, 1,5-dibromopentane (5.64 mL, 41.43 mmol, 3 equiv) was introduced, and the reaction was heated to reflux for 5 h. The mixture was evaporated and partitioned between Et_2O and H_2O, and the organic layer was dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by flash chromatography on silica (elution with a linear gradient of EtOAc in toluene) to give compound **8a** as pale- yellow crystals (2.96 g, 58%).

^{1}H NMR (600 MHz, $CDCl_3$): 1.14 (m, 2H, $C(4)H_2$), 1.26 (t, J = 7.1, 6H, 2× CH_3 (OEt)), 1.45 (m, 2H, $C(5)H_2$), 1.84 (m, 2H, $C(6)H_2$), 2.04 (s, 3H, CH_3 (NHAc)), 2.33 (m, 2H, $C(3)H_2$); 3.37 (t, J = 6.8, 2H, $C(7)H_2$), 4.25 (q, J = 7.1, 4H, 2× CH_2 (OEt)), 6.78 (b, 1H, NH).

^{13}C NMR (150.9 MHz, $CDCl_3$): 13.98 (2× CH_3 (OEt)), 22.78 (C-4), 23.07 (CH_3 (NHAc)), 27.72 (C-5), 31.87 (C-3), 32.33 (C-6), 33.50 (C-7), 62.54 (2× CH_2 (OEt)), 66.42 (C-2), 168.12 (2× C-1), 168.97 (CO (NHAc)).

HRMS (ESI) calculated for $C_{14}H_{25}O_5NBr$, m/z 366.0911, found 366.0911 $(M + H)^+$.

(*RS*)-2-Carboxyethyl-2-acetamido-5-methyl-7-bromoheptanoic Acid (8b). Using the procedure outlined for **8a**, compound **8b** was prepared from **5b** (2.246 g, 9.21 mmol), sodium (0.106 g, 4.60 mmol), and DEAM (1.00 g, 4.60 mmol) as pale-yellow crystals (0.544 g, 31%).

^{1}H NMR (600 MHz, $CDCl_3$): 0.89 (d, J = 6.8, 3H, C(5)-CH_3), 0.97, and 1.13 m (2H, $C(4)H_2$), 1.26 (t, J = 7.1, 6H, 2× CH_3 (OEt)), 1.65 (m, 1H, C(5)H), 1.66 m and 1.84 (2H, $C(6)H_2$), 2.04 (s, 3H, CH_3 (NHAc)), 2.36 and 2.42 m (2H, $C(3)H_2$), 3.36 and 3.42 m (2H, $C(7)H_2$), 4.25 (q, J = 7.1, 4H, 2× CH_2 (OEt)), 6.77 (b, 1H, NH).

^{13}C NMR (150.9 MHz, $CDCl_3$): 14.00 (2× CH_3 (OEt)), 18.70 (CH_3), 23.06 (CH_3 (NHAc)), 29.33 (C-3), 29.97 (C-4), 31.28 (C-5), 31.64 (C-7), 38.46 (C-6), 62.54 and 62.57 (2× CH_2 (OEt)), 66.48 (C-2), 168.10 and 168.15 (2× C-1), 168.94 (CO (NHAc)).

HRMS (ESI) calculated for $C_{15}H_{26}O_5NBrNa$, m/z 402.0887, found 402.0878 $(M + Na)^+$.

2-Carboxyethyl-2-acetamido-5,5-dimethyl-7-iodoheptanoic Acid (8c). Sodium ethanolate was generated in situ by dissolving sodium (0.640 g, 27.8 mmol, 1.3 equiv) in abs EtOH (40 mL) at rt in a flask equipped with a reflux condenser fitted with a calcium chloride tube. Diethyl acetamidomalonate (DEAM, 5.58 g, 25.7 mmol, 1.2 equiv) was added; after 30 min, mesylate **7** (6.17 g, 21.4 mmol, 1 equiv; as solution in 15 mL of THF) was introduced, followed by addition of NaI (6.41 g, 42.8 mmol, 2 equiv), and the reaction was heated to reflux under argon for 3 days. The reaction mixture was then partitioned between Et_2O and 10% aqueous citric acid, and the organic layer was dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by flash chromatography on silica (elution with a linear gradient of EtOAc in toluene) to give compound **8c** as dark-orange crystals (2.16 g, 23%).

^{1}H NMR (600 MHz, $CDCl_3$): 0.85 (s, 6H, $C(CH_3)_2$), 0.98 (m, 2H, $C(4)H_2$), 1.26 (t, J = 7.1, 6H, 2× CH_3 (OEt)), 1.88 (m, 2H, $C(6)H_2$), 2.04 (s, 3H, CH_3 (NHAc)), 2.30 (m, 2H, $C(3)H_2$), 3.07 (m, 2H, $C(7)H_2$), 4.25 (q, J = 7.1, 4H, 2× CH_2 (OEt)), 6.73 (b, 1H, NH).

^{13}C NMR (150.9 MHz, $CDCl_3$): 0.24 (C-7), 14.02 (2× CH_3 (OEt)), 23.02 (CH_3 (NHAc)), 26.20 (2× CH_3 ($C\underline{C}H_3)_2$), 26.85 (C-3), 34.83 (C-4), 35.32 (C-5), 47.09 (C-6), 62.57 (2× CH_2 (OEt)), 66.54 (C-2), 168.13 (2× C-1), 168.83 (CO (NHAc)).

HRMS (ESI) calculated for $C_{16}H_{28}O_5NINa$, m/z 464.09044, found 464.09039 $(M + Na)^+$.

(2*S*,11*RS*)-5-Thia-2,11-diaminododecanedioic Acid (10). Following the general procedure, the intermediate **9a** was prepared from L-methionine (0.50 g, 3.35 mmol, 1 equiv), sodium (0.239 g, 10.4 mmol, 3.1 equiv), and **8a** (1.35 g, 3.69 mmol, 1.1 equiv; added as a solution in 3 mL of THF).

After the ammonia had evaporated, the residue (crude **9a**) was dissolved in aqueous HCl (5 M, 30 mL) and heated under reflux for 2 h. The chilled solution was then passed through a column of Dowex 50 (H^+-cycle) and washed successively with water, methanol, and again water. The crude product was eluted with 10% aqueous NH_3, and the solution was evaporated to dryness. The residue was purified by HPLC and lyophilized, giving **10** as di(trifluoroacetate) (white solid, 0.70 g, 37% overall yield from **8a**).

^{1}H NMR (600 MHz, D_2O): 1.41 (m, 1H) and 1.46 (m, 1H, $C(9)H_2$), 1.46 (m, 2H, $C(8)H_2$), 1.62 (m, 2H, $C(7)H_2$), 1.90 (m, 1H) and 1.96 (m, 1H, $C(10)H_2$), 2.16 (m, 1H) and 2.24 (m, 1H, $C(3)H_2$), 2.60 (t, 2H, $C(6)H_2$), 2.70 (t, 2H, $C(4)H_2$), 4.01 (dd, J = 6.8 and 5.7, C(11)H), 4.12 (dd, 1H, J = 6.9 and 5.8, C(2)H).

^{13}C NMR (150.9 MHz, D_2O): 26.39 (C-9), 29.06 (C-4), 30.09 (C-8), 30.80 (C-7), 32.39 (C-3), 32.40 (C-10), 33.25 (C-6), 55.04 (C-2), 56.90 (C-11), 175.11 (C-12), 175.52 (C-1).

HRMS (ESI) calculated for $C_{11}H_{21}O_4N_2S$, m/z 277.1228, found 277.1228 $(M - H)^-$.

(2*S*,8*RS*,11*RS*)-5-Thia-2,11-diamino-8-methyldodecanedioic Acid (11). Following the general procedure, the intermediate **9b** was prepared from L-methionine (0.185 g, 1.24 mmol, 1 equiv), sodium (0.089 g, 3.86 mmol, 3.1 equiv), and **8b** (0.52 g, 1.37 mmol, 1.1 equiv; added as a solution in 3 mL of THF).

After the ammonia had evaporated, the residue (crude **9b**) was dissolved in aqueous HCl (5 M, 30 mL) and heated under reflux for 2 h. The chilled solution was then passed through a column of Dowex 50 (H^+-cycle) and washed successively with water, methanol, and again water. The crude product was eluted with 10% aqueous NH_3, and the solution was evaporated to dryness. The residue was purified by HPLC and lyophilized, giving **11** as di(trifluoroacetate) (white solid, 0.104 g, 15% overall yield from **8b**).

^{1}H NMR (600 MHz, D_2O), 4 diastereoisomers, only some signals resolved: 0.91 (d, 3H, J = 6.6, CH_3), 1.23 m, 1.31 m, 1.39 and 1.47 m (2H, $C(9)H_2$), 1.47 and 1.61 m (2H, $C(7)H_2$), 1.61 (m, 1H, C(8)H), 1.90−2.00 (m, 2H, $C(10)H_2$), 2.17 and 2.26 m (2H, $C(3)H_2$), 2.60 and 2.65 m (2H, $C(6)H_2$), 2.71 (m, 2H, $C(4)H_2$), 4.02 (t, J = 6.1, 1H, H-11), 4.14 (t, J = 6.1, 1H, H-2).

^{13}C NMR (150.9 MHz, D_2O): 26.39 (C-9), 29.06 (C-4), 30.09 (C-8), 30.80 (C-7), 32.39 (C-3), 32.40 (C-10), 33.25 (C-6), 55.04 (C-2), 56.90 (C-11), 175.11 (C-12), 175.52 (C-1).

HRMS (ESI) calculated for $C_{12}H_{23}O_4N_2S$, m/z 291.1384, found 291.1384 $(M - H)^-$.

(2*S*,11*RS*)-5-Thia-2,11-diamino-8,8-dimethyldodecanedioic Acid (12). Following the general procedure, the intermediate **9c** was prepared from L-methionine (0.717 g, 4.8 mmol, 1 equiv), sodium (0.343 g, 14.9 mmol, 3.1 equiv), and **8c** (2.12 g, 4.8 mmol, 1 equiv; added as a solution in 3 mL of THF).

After the ammonia had evaporated, the residue (crude **9c**) was dissolved in aqueous HCl (5 M, 50 mL) and heated under reflux for 2 h. The chilled solution was then passed through a column of Dowex 50 (H^+-cycle) and washed successively with water, methanol, and again water. The crude product was eluted with 10% aqueous NH_3, and the solution was evaporated to dryness. The residue was purified by HPLC and lyophilized, giving **12** as di(trifluoroacetate) (white solid, 0.177 g, 7% overall yield from **8c**).

^{1}H NMR (600 MHz, D_2O), 2 diastereoisomers, only some carbon signals resolved: 0.90 (s, 6H, $C(CH_3)_2$), 1.26 and 1.36 m (2H, $C(9)H_2$), 1.51 (m, 2H, $C(7)H_2$), 1.92 (m, 2H, $C(10)H_2$), 2.18 and 2.26 m (2H, $C(3)H_2$), 2.55 (m, 2H, $C(6)H_2$), 2.72 (t, J = 7.6, 2H, $C(4)H_2$), 4.02 (m, 1H, H-11), 4.14 (dd, J = 6.7 and 6.0, 1H, H-2).

^{13}C NMR (150.9 MHz, D_2O): 27.50 (C-10); 28.45, 28.46, and 28.48 (2× CH_3 ($C\underline{C}H_3)_2$); 28.56 (C-6); 29.05 (C-4); 32.34 (C-3); 34.96 (C-8); 38.15 and 38.17 (C-9); 43.25 and 43.26 (C-7); 54.94 (C-2); 56.32 (C-11); 175.04 (C-1); 175.37 (C-12).

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HRMS (ESI) calculated for $C_{13}H_{25}O_4N_2S$, m/z 305.15405, found 305.15411 $(M - H)^-$.

(2*S*,5*RS*,11*S*)-5,8-Dithia-2,11-diaminododecanedioic Acid 5-Oxide (13). To a suspension of crude **4** (0.24 g, content ca. 50%, thus 0.40 mmol, 1 equiv) in water (4 mL) solid $NaIO_4$ (0.085 g, 0.40 mmol, 1 equiv) was added. A slightly exothermic reaction occurred, and the mixture turned brown (ca. 5 min). After 30 min, the reaction was quenched by dilution with MeOH (20 mL), precipitated salts were filtered off, and the solution was concentrated in vacuo. The residue was purified by HPLC and lyophilized, giving **13** as di(trifluoroacetate) (white solid, 0.061 g, ca. 23%).

^{1}H NMR (600 MHz, D_2O), 2 diastereoisomers, ca. 3:1, only some signals resolved: 2.20 and 2.29 m (2H, C(10)H_2), 2.41 (m, 2H, C(3)H_2), 2.79 (m, 2H, C(9)H_2), 2.97 and 3.05 m (2H (C(7)H_2), 3.12 (m, 2H, C(4)H_2), 3.22 (m, 2H, C(6)H_2), 4.14 m (1H, C(2)H), 4.15 (m, 1H, C(11)H).

^{13}C NMR (150.9 MHz, D_2O): 26.27 (C-3), 26.48 and 26.55 (C-7), 29.30 and 29.40 (C-9), 32.20 and 32.23 (C-10), 48.96 and 49.04 (C-4), 53.23 and 53.28 (C-6), 54.77 and 54.79 (C-2 and C-11), 174.35 and 174.94 (C-1 and C-12).

HRMS (ESI) calculated for $C_{10}H_{21}N_2O_5S_2$, m/z 313.0892, found 313.0891 $(M + H)^+$.

(2*S*,5*RS*,8*RS*,11*S*)-5,8-Dithia-2,11-diaminododecanedioic Acid 5,8-Dioxide (14). Using the procedure outlined for **13**, compound **14** was prepared from **4** (0.24 g, content ca. 50%, thus 0.40 mmol, 1 equiv) and solid $NaIO_4$ (0.170 g, 0.80 mmol, 2 equiv). The residue was purified by HPLC and lyophilized, giving **14** as di(trifluoroacetate) (white solid, 0.065 g, ca. 28%).

^{1}H NMR (600 MHz, D_2O), 4 diastereoisomers, only some signals resolved: 2.43 (m (4H, C(3)H_2 and C(10)H_2), 3.10−3.26 (m, 4H, C(4)H_2 and C(9)H_2), 3.33 and 3.43 m (4H, C(6)H_2 and C(7)H_2), 4.17 m (2H, C(2)H and C(11)H).

^{13}C NMR (150.9 MHz, D_2O): 26.11, 26.14, 26.34, and 26.37 (C-3 and C-10); 46.20, 46.22, 46.46, and 46.50 (C-6 and C-7); 49.30, 49.41, 49.48, and 49.59 (C-4 and C-9); 54.68 and 54.86 (C2 and C-11); 174.17 (C-1 and C-12).

HRMS (ESI) calculated for $C_{10}H_{21}O_6N_2S_2$, m/z 329.08355, found 329.08374 $(M + H)^+$.

((*S*)-3-Amino-3-carboxypropyl)[2-((*S*)-3-amino-3-carboxypropylthio)ethyl](methyl)sulfonium Di(trifluoroacetate) (15). Suspension of crude **4** (0.25 g, content ca. 50%, thus 0.42 mmol) in a mixture of water (2 mL) and MeOH (4 mL) was treated with excess of methyl iodide (4 mL). The reaction was allowed to proceed under exclusion of light for 2 days at rt. The reaction mixture was then partitioned between water (100 mL) and Et_2O (100 mL), and the aqueous phase was concentrated in vacuo. The residue was purified by HPLC and lyophilized, giving **15** as di(trifluoroacetate) (white solid, 0.118 g, ca. 52%).

^{1}H NMR (600 MHz, D_2O), 2 diastereoisomers, only some signals resolved (main chain numbered as in previous compounds): 2.20 and 2.29 m (2H, C(10)H_2), 2.45 (m, 2H, C(3)H_2), 2.69 and 2.82 m (2H, C(9)H_2), 3.01 (s, 3H, CH_3), 3.09 (m, 2H (C(7)H_2), 3.49 m, 3.60 and 3.69 m (2H, C(4)H_2), 3.67 and 3.73 m (2H, C(6)H_2), 4.09 (t, J = 6.5, 1H, C(2)H), 4.14 (t, J = 6.5, 1H, C(11)H).

^{13}C NMR (150.9 MHz, D_2O): 25.16 and 25.22 (CH_3), 27.57 and 27.64 (C-3), 31.26 and 31.63 (C-9), 32.22 and 32.38 (C-10), 40.52 and 40.54 (C-4), 44.78 and 44.86 (C-6), 54.43 and 54.53 (C-2), 54.68 C-11), 173.97 and 174.89 (C-1), 174.91 (C-12).

HRMS (ESI) calculated for $C_{11}H_{23}N_2O_4S_2^+$, m/z 311.1094, found 311.1096 $(M)^+$.

Cold *S*-Methyl-L-methionine Chloride (SMM). A solution of L-methionine (3.0 g, mmol) in a mixture of water (56 mL) and MeOH (8 mL) was treated with excess of methyl iodide (3 mL). The reaction was allowed to proceed under exclusion of light for 2 days at rt.[32] The reaction mixture was then concentrated in vacuo, giving white crystalline solid, which was then dissolved in water and passed through a column of Dowex 1 × 4 (Cl^--cycle). The solution was evaporated, and the residue was dissolved in MeOH (250 mL) at 60 °C. After cooling, Et_2O (100 mL) was added, and the solution was left at −20 °C overnight. Precipitated product was filtered off and dried in vacuo over P_2O_5. Fine white crystals (2.83 g, 70%).

^{1}H NMR (600 MHz, D_2O): 2.38 (m, 2H, C(3)H_2), 2.96 (s, 6H, 2× SCH_3), 3.43 (m, 1H) and 3.53 (m, 1H, C(4)H_2), 3.88 (t, 1H, J = 6.5, C(2)H).

^{13}C NMR (150.9 MHz, D_2O): 27.38 and 27.44 (2× SCH_3), 27.75 (C-3), 42.20 (C-4), 55.48 (C-2), 175.24 (C-1).

HRMS (ESI) calculated for $C_8H_{14}O_2NS$, m/z 164.0740, found 164.0740 $(M)^+$.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Electrophoretic analysis of purified BHMT and BHMT-2, BHMT activities in 50 mM Tris/HCl buffer (pH 7.5), and results of LC-MS/MS analyses of tryptic digests of purified BHMT and BHMT-2, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

1-D, one-dimensional; 2-D, two-dimensional; app, apparent; arb, arbitrary unit; BHMT, betaine-homocysteine *S*-methyltransferase BHMT-2 betaine-homocysteine *S*-methyltransferase 2; CID, collision-induced dissociation; DEAM, diethyl acetamidomalonate; IPTG, isopropyl β-D-1-thiogalactopyranoside; Hcy, homocysteine; MsCl, methanesulfonyl chloride; rt, room temperature; SMM, *S*-methyl-L-methionine; TCEP, tris(2-carboxyethyl)phosphine; TEA, triethylamine

■ REFERENCES

(1) Elmore, C. L.; Matthews, R. G. The many flavors of hyperhomocyst(e)inemia: insights from transgenic and inhibitor-based mouse models of disrupted one-carbon metabolism. *Antioxidants Redox Signaling* **2007**, *9*, 1911−1921.

(2) Finkelstein, J. D.; Martin, J. J. Methionine metabolism in mammals. Distribution of homocysteine between competing pathways. *J. Biol. Chem.* **1984**, *259*, 9508−9513.

(3) Evans, J. C.; Huddler, D. P.; Jiracek, J.; Castro, C.; Millian, N. S.; Garrow, T. A.; Ludwig, M. L. Betaine-homocysteine methyltransferase. Zinc in a distorted barrel. *Structure* **2002**, *10*, 1159−1071.

(4) Gonzalez, B.; Pajares, M. A.; Martinez-Ripoll, M.; Blundell, T. L.; Sanz-Aparicio, J. Crystal structure of rat liver betaine homocysteine *S*-methyltransferase reveals new oligomerization features and conformational changes upon substrate binding. *J. Mol. Biol.* **2004**, *338*, 771−782.

(5) Collinsova, M.; Strakova, J.; Jiracek, J.; Garrow, T. A. Inhibition of betaine-homocysteine *S*-methyltransferase causes hyperhomocysteinemia in mice. *J. Nutr.* **2006**, *136*, 1493−1497.

(6) Strakova, J.; Williams, K. T.; Gupta, S.; Schalinske, K. L.; Kruger, W. D.; Rozen, R.; Jiracek, J.; Li, L.; Garrow, T. A. Dietary intake of *S*-(alpha-carboxybuty1)-DL-homocysteine induces hyperhomocysteinemia in rats. *Nutr. Res. (N.Y.)* **2010**, *30*, 492−500.

(7) Strakova, J.; Gupta, S.; Kruger, W. D.; Dilger, R. N.; Tryon, K.; Li, L.; Garrow, T. A. Inhibition of betaine-homocysteine *S*-methyltransferase in rats causes hyperhomocysteinemia and reduces liver cystathionine beta-synthase activity and methylation capacity. *Nutr. Res. (N.Y.)* **2011**, *31*, 563−571.
(8) Teng, Y. W.; Mehedint, M. G.; Garrow, T. A.; Zeisel, S. H. Deletion of betaine-homocysteine *S*-methyltransferase in mice perturbs choline and 1-carbon metabolism, resulting in fatty liver and hepatocellular carcinomas. *J. Biol. Chem.* **2011**, *286*, 36258−36267.
(9) Chadwick, L. H.; McCandless, S. E.; Silverman, G. L.; Schwartz, S.; Westaway, D.; Nadeau, J. H. Betaine-homocysteine methyltransferase-2: cDNA cloning, gene sequence, physical mapping, and expression of the human and mouse genes. *Genomics* **2000**, *70*, 66−73.
(10) Li, F.; Feng, Q. P.; Lee, C.; Wang, S. Z.; Pelleymounter, L. L.; Moon, I.; Eckloff, B. W.; Wieben, E. D.; Schaid, D. J.; Yee, V.; Weinshilboum, R. M. Human betaine-homocysteine methyltransferase (BHMT) and BHMT2: common gene sequence variation and functional characterization. *Mol. Genet. Metab.* **2008**, *94*, 326−335.
(11) Szegedi, S. S.; Castro, C.; Koutmos, M.; Garrow, T. A. Betaine-homocysteine *S*-methyltransferase-2 is an *S*-methylmethionine-homocysteine methyltransferase. *J. Biol. Chem.* **2008**, *283*, 8939−8945.
(12) Mudd, S. H.; Datko, A. H. The *S*-Methylmethionine cycle in *Lemna paucicostata*. *Plant Physiol.* **1990**, *93*, 623−630.
(13) Augspurger, N. R.; Scherer, C. S.; Garrow, T. A.; Baker, D. H. Dietary *S*-methylmethionine, a component of foods, has choline-sparing activity in chickens. *J. Nutr.* **2005**, *135*, 1712−1717.
(14) Liu, H. H.; Lu, P.; Guo, Y. Y.; Farrell, E.; Zhang, X.; Zheng, M.; Bosano, B.; Zhang, Z. M.; Allard, J.; Liao, G. C.; Fu, S. Y.; Chen, J. Z.; Dolim, K.; Kuroda, A.; Usuka, J.; Cheng, J.; Tao, W.; Welch, K.; Liu, Y. Z.; Pease, J.; de Keczer, S. A.; Masjedizadeh, M.; Hu, J. S.; Weller, P.; Garrow, T.; Peltz, G. An integrative genomic analysis identifies Bhmt2 as a diet-dependent genetic factor protecting against acetaminophen-induced liver toxicity. *Genome Res.* **2010**, *20*, 28−35.
(15) Collinsova, M.; Castro, C.; Garrow, T. A.; Yiotakis, A.; Dive, V.; Jiracek, J. Combining Combinatorial Chemistry and Affinity Chromatography: Highly Selective Inhibitors of Human Betaine:Homocysteine *S*-Methyltransferase. *Chem. Biol.* **2003**, *10*, 113−122.
(16) Jiracek, J.; Collinsova, M.; Rosenberg, I.; Budesinsky, M.; Protivinska, E.; Netusilova, H.; Garrow, T. A. *S*-Alkylated homocysteine derivatives: new inhibitors of human betaine-homocysteine *S*-methyltransferase. *J. Med. Chem.* **2006**, *49*, 3982−3989.
(17) Vanek, V.; Budesinsky, M.; Kabeleova, P.; Sanda, M.; Kozisek, M.; Hanclova, I.; Mladkova, J.; Brynda, J.; Rosenberg, I.; Koutmos, M.; Garrow, T. A.; Jiracek, J. Structure−activity study of new inhibitors of human betaine-homocysteine *S*-methyltransferase. *J. Med. Chem.* **2009**, *52*, 3652−3665.
(18) Castro, C.; Gratson, A. A.; Evans, J. C.; Jiracek, J.; Collinsova, M.; Ludwig, M. L.; Garrow, T. A. Dissecting the catalytic mechanism of betaine-homocysteine *S*-methyltransferase by use of intrinsic tryptophan fluorescence and site-directed mutagenesis. *Biochemistry* **2004**, *43*, 5341−5351.
(19) Snyder, H. R.; Howe., E. E.; Cannon, G. W.; Nyman, M. A. ω,ω'-Bimethionine. *J. Am. Chem. Soc.* **1943**, *65*, 2211−2214.
(20) Frankel, M.; Gertner, D. Syntheses of Homologues of djenkolic Acid. *J. Chem. Soc.* **1960**, 898−899.
(21) Magee, R. J.; Mazurek, W.; O'Conner, M. J.; Phillip, A. T. Spectroscopic studies on some sulphur-containing amino acid complexes of cobalt, nickel and copper. *Aust. J. Chem.* **1974**, *27*, 1629−1637.
(22) Szegedi, S. S.; Garrow, T. A. Oligomerization is required for betaine-homocysteine *S*-methyltransferase function. *Arch. Biochem. Biophys.* **2004**, *426*, 32−42.
(23) Byers, L. D.; Wolfenden, R. Potent reversible inhibitor of carboxypeptidase A. *J. Biol. Chem.* **1972**, *247*, 606−608.
(24) Byers, L. D.; Wolfenden, R. Binding of by-product analog benzylsuccinic acid by carboxypeptidase A. *Biochemistry* **1973**, *12*, 2070−2078.
(25) Schramm, V. L. Enzymatic transition-state analysis and transition-state analogs. *Methods Enzymol.* **1999**, *308*, 301−355.
(26) Schramm, V. L. Enzymatic transition states, transition-state analogs, dynamics, thermodynamics, and lifetimes. *Annu. Rev. Biochem.* **2011**, *80*, 703−732.
(27) Breksa, A. P., III; Garrow, T. A. Recombinant human liver betaine-homocysteine *S*-methyltransferase: identification of three cysteine residues critical for zinc binding. *Biochemistry* **1999**, *38*, 13991−13998.
(28) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(29) Selicharova, I.; Sanda, M.; Mladkova, J.; Ohri, S. S.; Vashishta, A.; Fusek, M.; Jiracek, J.; Vetvicka, V. 2-DE analysis of breast cancer cell lines 1833 and 4175 with distinct metastatic organ-specific potentials: comparison with parental cell line MDA-MB-231. *Oncol. Rep.* **2008**, *19*, 1237−1244.
(30) Dixon, M. The graphical determination of K_m and K_i. *Biochem. J.* **1972**, *129*, 197−202.
(31) Kelly, B. D.; Lambert, T. H. Cyclopropenium-activated cyclodehydration of diols. *Org. Lett.* **2011**, *13*, 740−743.
(32) Koch, T.; Buchardt, O. Synthesis of L-(+)-Selenomethionine. *Synthesis* **1993**, 1065−1067.