Structure−Activity Study of New Inhibitors of Human Betaine-Homocysteine *S*-Methyltransferase

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Betaine-homocysteine *S*-methyltransferase (BHMT) catalyzes the transfer of a methyl group from betaine to L-homocysteine, yielding dimethylglycine and L-methionine. In this study, we prepared a new series of BHMT inhibitors. The inhibitors were designed to mimic the hypothetical transition state of BHMT substrates and consisted of analogues with NH, N(CH$_3$), or N(CH$_3$)$_2$ groups separated from the homocysteine sulfur atom by a methylene, ethylene, or a propylene spacer. Only the inhibitor with the N(CH$_3$) moiety and ethylene spacer gave moderate inhibition. This result led us to prepare two inhibitors lacking a nitrogen atom in the *S*-linked alkyl chain: (*RS,RS*)-5-(3-amino-3-carboxypropylthio)-3-methylpentanoic acid and (*RS*)-5-(3-amino-3-carboxypropylthio)-3,3-dimethylpentanoic acid. Both of these compounds were highly potent inhibitors of BHMT. The finding that BHMT does not tolerate a true betaine mimic within these inhibitors, especially the nitrogen atom, is surprising and evokes questions about putative conformational changes of BHMT upon the binding of the substrates/products and inhibitors.

Introduction

Betaine-homocysteine *S*-methyltransferase[1] (BHMT; EC 2.1.1.5) catalyzes a methyl transfer from betaine to L-homocysteine to form dimethylglycine and L-methionine, respectively. This enzyme is abundant in mammals but only in the liver and kidney.[2] It has been predicted that BHMT is responsible for up to half of the homocysteine remethylation capacity in liver.[3] BHMT follows an ordered Bi−Bi reaction mechanism with homocysteine being the first substrate to bind and methionine being the last product off.[4] Cloning of human BHMT cDNA, overexpression of the enzyme in *E. coli*, as well as a purification protocol for preparation of homogeneous enzyme have been previously described.[5] BHMT was shown to be a Zn-metal-loenzyme. The metal ion is essential for its catalytic activity. Site-directed mutagenesis studies have shown that the Zn^{2+} ion is coordinated by three cysteine ligands.[6] BHMT is a member of a family (Pfam 02574) of zinc and thiol/selenol-dependent methyltransferases.[7]

Our laboratory is particularly interested in the synthesis and biological evaluation of inhibitors of BHMT. During the past several years, we developed potent and selective inhibitors of human recombinant BHMT: phosphinic pseudopeptides[8–14] and *S*-alkylated derivatives of homocysteine.[15] Among the series of *S*-alkylated homocysteines, we identified compounds **1−3** as highly potent inhibitors of BHMT. They were believed to mimic the structure of the hypothetical transition state **4** of BHMT substrates, betaine and L-homocysteine, upon binding to the active site of the enzyme. The competitive inhibitor **1** (*K*$_i^{app}$

toward betaine of 12 nM)[15,16] was cocrystallized with BHMT, and the structure of the complex was determined by X-ray crystallography.[7] Inhibitor **1** was also used in a study of the intrinsic fluorescence of BHMT.[17] This study helped to provide a better understanding of the catalytic mechanism of the enzyme and confirmed the ordered Bi−Bi reaction. Recently, inhibitor **1** was used in mice to study the role of BHMT in sulfur metabolism in vivo.[18] This pioneering pharmacological study confirmed the role of BHMT in the synthesis of methionine from homocysteine in vivo.

Other identified and possible physiological functions of BHMT require further characterization. Betaine has been shown to be an osmolyte in rat liver cells.[19] It is likely that BHMT helps to maintain the cellular osmolytic equilibrium by maintaining the correct betaine concentration.[20] Potent, stable, and selective BHMT inhibitors would be useful in studies of the role of the enzyme in liver and kidney osmolytic balance. BHMT activity was shown to be increased in type 2 diabetes.[21] A role for BHMT in lipid and phospholipid metabolism is highly probable, but it remains unclear and demands further investigation.[22,23] It is clear that many questions about the physiological role of BHMT in mammalian organisms exist and that potent, selective, and stable inhibitors of this enzyme will have an important role in future studies, especially if BHMT-deficient mice remain unavailable.[24]

We are particularly interested in the structural requirements for ligand binding to the active site of BHMT. The design and synthesis of compounds that closely resemble the structure of substrates upon binding to BHMT, especially the structure of the hypothetical transition state (**4**), should yield highly potent inhibitors.[25–27] The structures of inhibitors **1−3**, which we developed previously,[15] are considerably different from the structure of the transition state (**4**). The main difference is in the "betaine" part of their molecules. Therefore, we decided to design and synthesize new compounds that would better mimic the structure of the transition state (**4**), which should yield highly

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potent inhibitors of BHMT that could be useful in future in vivo pharmacological studies.
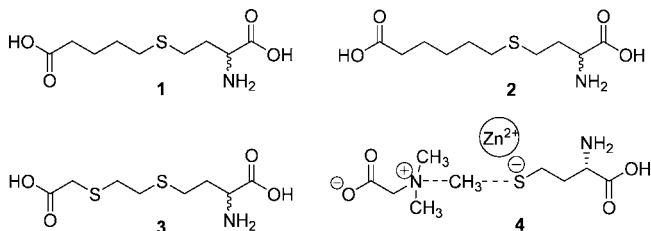
Results and Discussion

We prepared two series of BHMT inhibitors (Table 1). Compounds **5−10** in series 1 were designed using the scaffold of the previously published potent inhibitor **1** to investigate the role of the number and position of sulfur atom(s) in the inhibitors. Compounds **11−21** from series 2 were designed to mimic the hypothetical transition state (**4**) of betaine and homocysteine substrates upon binding to the active site of BHMT.

Chemistry. Compounds **5** and **6** were synthesized directly by recombination of free unprotected acids (Scheme 1). Acidic ring-opening of γ-aminobutyrolactone in an aqueous solution containing DL-homocysteine followed by oxidation with hydrogen peroxide afforded disulfide **5** (accompanied by disulfide **22** and DL-homocystine as byproduct). Similarly, compound **6** bearing the thioformyl moiety was obtained by the phase-transfer catalyzed reaction[28] of 3-mercaptopropionic acid and DL-homocysteine in dichloromethane, followed by HPLC resolution of the resulting mixture.

The treatment of diethyl acetamidomalonate (DEAM[a]) with NaH followed by addition of 1-bromo-3-chloropropane yielded a product that is considered in the literature[29,30] to be chloride **23a**. However, our analysis revealed that the product was a mixture of chloride **23a** and bromide **23b** at a ratio of 59:41 (as determined by NMR). The coupling of a mixture of the compounds **23a** and **23b** with sodium 4-mercaptoethylbutanoate produced the corresponding protected sulfide **24** in good yield. The target compound **7** was obtained after treatment with 3 N HCl under reflux.

Synthesis of intermediate **29** (including its precursors **27** and **28**), which was proposed as a precursor for the synthesis of compounds **8** and **9**, is described in detail in Scheme S1, Supporting Information.

Attempts to prepare the oxygen-containing analogue (**8**) of sulfide **1** by standard Williamson ether synthesis employing alcohol **28**, bromide **29**, or several similar precursors were unsuccessful. Therefore, an alternative approach (Scheme 2) was used starting from DEAM, which was alkylated with 2,2′-dichloro-diethylether.[31] The resulting chloride **30** was converted into iodide **31** and reacted with methylacrylate in the presence of the Zn(Cu) pair,[32] which gave the desired structure **32** in high yield. The final analogue **8** was obtained after acidic deprotection.

The selenium analogue (**9**) of sulfide **1** was synthesized from **29** in three steps (Scheme 3). The reaction with in situ generated disodium diselenide[33] afforded diselenide **33**, which was then treated with NaBH$_4$ and ethyl 5-bromovalerate to generate

selenide **34**. Deprotection of the latter compound yielded the desired selenide **9**.

Amino acid **10** was prepared according to the method of Barraclough et al.[30] from DEAM and ethyl 8-bromooctanoate, followed by standard acidic decarboxylation and deprotection.

For the synthesis of the transition state analogues bearing the *N*-CH$_2$-S motif (Scheme 4), a protected homocysteine derivative **36** was prepared in high yield following the synthetic method described by Zhu et al.[34] Alkylating agent **37** was obtained by a phase-catalyzed reaction[35] of **36** with bromochloromethane in the presence of crushed solid KOH. We did not succeed in the isolation of this compound due to its high reactivity (e.g., rapid decomposition on a silica column); however, the CH$_2$Cl$_2$/CH$_2$BrCl solution of compound **37** proved to be sufficiently stable for subsequent synthetic use. During the preparation of **37**, formation of the djenkolic acid[36] homologue **38** was invariably observed. After standard acidic deprotection, free amino acid **39** was obtained.

The alkylation of dimethylglycine *tert*-butyl ester with chloromethylsulfide **37** in the presence of one equivalent of DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) at room temperature resulted in the formation of quaternary compound **40**, which gave the desired amino acid **11** after deprotection.

While alkylation of the tertiary amines proceeded smoothly at room temperature, alkylation of primary and secondary amines demanded prolonged heating of the reaction mixture. Surprisingly, when 2 equiv of DBU were used in the reaction of **37** with *tert*-butyl-2-(methylamino)acetate hydrochloride, formation of an unknown compound was observed and **43** was not detected in the reaction mixture. Analysis of the product revealed *N*-alkylated DBU (**41**), which gave the corresponding compound **42** after deprotection. In addition, whereas the alkylation of the above amine in the presence of 1 equiv of DBU gave the expected compound **43**, an attempt to prepare the respective secondary amine from β-alanine *tert*-butyl ester (as well as from glycine and alanine *tert*-butyl esters) failed, giving only bis-alkylated product **44** and, despite our best efforts, no trace of the expected secondary amine. This observation indicates a strong preference of **37** for secondary and tertiary amines over primary amines. Moreover, the acidic deprotection of **43** and **44** caused their complete decomposition, and no traces of the desired free compounds were detected.

The series of compounds bearing the ethylene linker *N*-CH$_2$-CH$_2$-S and propylene linker *N*-(CH$_2$)$_3$-S was prepared from halogenides **45** and **53**, respectively (Schemes 5 and 6). Unlike **37**, both **45** and **53** are stable compounds, with much weaker reactivity toward amines than **37**; therefore, harsh reaction conditions[37,38] were required. The alkylation of dimethylglycine *tert*-butyl ester to quaternary amines was not feasible. This problem was successfully bypassed by the alkylation of **46** and **54** by methyl iodide. During alkylation of β-alanine *tert*-butyl ester, a bis-alkylated compound **52** was isolated as a byproduct.
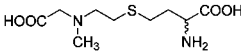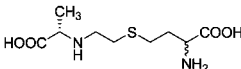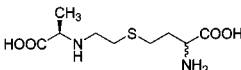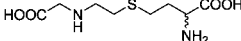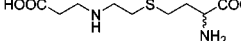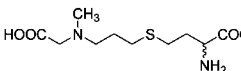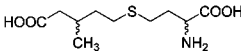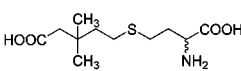
Finally, inhibitors **20** and **21**, with branched alkyl side chains, were synthesized starting from 3-methyl- and 3,3-dimethylglutaranhydride, respectively, which were converted in two steps[39–41] into bromide esters **58** and **59**, respectively. After coupling with thiol **36**, protected sulfides **60** and **61** were obtained in good yields, which, after deprotection, afforded the target compounds **20** and **21** (Scheme 7).

Inhibition Experiments. First, we determined the percent inhibition of BHMT using the test compounds at 20 μM. For the most potent compounds, we then determined the percent inhibition at 1 μM and their IC$_{50}$ values. The percentages of inhibition were measured at relatively low concentrations of

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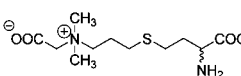[a] Abbreviations: ACES, *N*-(2-acetamido)-2-aminoethanesulfonic acid; DEAM, diethyl acetamidomalonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ITC, isothermal titration calorimetry; TEBAC, benzyl triethylammonium chloride; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

Table 1. Relative Inhibition of Human BHMT by Compounds **1** and **5−21**[a]

| Compound	% Inhibition[b] (average of triplicates) (0.25 mM betaine, 0.1 mM DL-homocysteine) 20 μM	1 μM	IC$_{50}$ (μM) ± SEM[c] (n = 3) (2 mM betaine, 1 mM DL-homocysteine)
1	100.0	97.6	0.138 ± 0.019
Series 1			
5	19.1	Nd	Nd
6	97.6	26.3	3.26 ± 0.59
7	5.4	Nd	Nd
8	9.8	Nd	Nd
9	100.0	94.3	0.649 ± 0.103
10	2.11	Nd	Nd
Series 2			
11	0.0	0.0	Nd
12	23.8	5.1	Nd
Series 2 (Continued)			
13	98.5	58.9	2.50 ± 0.19
14	19.8	Nd	Nd
15	15.5	Nd	Nd
16	37.1	Nd	Nd
17	0.0	Nd	Nd
18	3.3	0.0	Nd
19	79.01	22.4	Nd
20	100.0	97.8	0.139 ± 0.012
21	100.0	98.1	0.084 ± 0.004

[a] The percent inhibition of each compound was determined at 20 and 1 μM. See the Experimental Section for details. [b] All assays were done in triplicate, and the obtained data were reproducible within ±15%. [c] All data points for IC$_{50}$ values were derived from assays performed in duplicate, and the values were obtained from 3 different assays. Nd means not determined.

substrates, 0.25 mM betaine and 100 μM DL-homocysteine (K_m values of BHMT for betaine and DL-homocysteine are 2 mM and 8 μM, respectively), in order to maximize our ability to detect inhibition. In contrast, we determined IC$_{50}$ values of the most potent inhibitors at higher substrate concentrations (2 mM betaine and 1 mM DL-homocysteine) so that we could determine IC$_{50}$ values in measurable concentrations more accurately. The results of the inhibition experiments are summarized in Table 1. For a clearer description of inhibitor structures, we will use the numbering of atoms as shown in a simple inhibitor scaffold (**62**, below).

R is COOH or CH$_2$-COOH

62
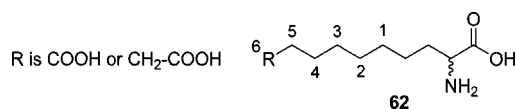
The inhibitory potencies of all tested compounds were compared to the "reference" inhibitor **1**. In our previous study,[15] we determined a K_i^{app} of 12 nM with respect to betaine for this compound. In the present series of experiments, we determined an IC$_{50}$ of 0.138 μM for this compound.

Among the inhibitors **5−10**, only compound **9** with a selenium atom at position 1 in scaffold **62** and with four CH$_2$ groups in the alkyl chain strongly inhibited BHMT. Nevertheless, replacement of sulfur with a selenium atom yields an inhibitor that is 5-fold weaker than compound **1**. If position 1 is occupied by an oxygen or CH$_2$, the respective compounds **8** and **10** are almost inactive. The low inhibitory potency of compound **7**, having a sulfur atom at position 2, reveals the high specificity of BHMT for the "homocysteine" moiety in inhibitors. Previously reported[15] inhibitor **3**, which has a second sulfur atom at position 4, displayed very strong potency toward BHMT. In this study, we prepared and tested compounds **5** and **6**, which have a second sulfur atom at positions 2 and 3, respectively. Disulfide compound **5** is a very weak inhibitor of BHMT, and compound **6**, which has a thioformyl moiety, displays only moderate inhibitory potency with an IC$_{50}$ of 3.26 μM. The data on compounds **5−10** reveal the crucial role of a sulfur atom at position 1, which provides a good balance of nucleophilicity, electronegativity, and atom size for the interaction between inhibitors and the catalytic Zn^{2+} in the active site of BHMT. Hence, the potential replacement of the "homocysteine" moiety in inhibitors with a suitable mimetic remains a challenge.

Scheme 1[a]

[a] Reagents and conditions: (i) (a) HCl (20%), 60 h, rt, Ar; (b) H₂O₂ (30%), rt, 1 d. (ii) NaOH, CH₂Cl₂, H₂O, benzyl triethylammonium chloride (TEBAC), rt, Ar, 1 d. (iii) (a) NaH, DMF, rt; (b) Br(CH₂)₃Cl, 30 °C, 1 d. (iv) Sodium 4-mercaptoethylbutanoate, EtOH, rt, 1 d. (v) 3 M HCl, reflux, 3 h.

Scheme 2[a]

[a] Reagents and conditions: (i) (a) NaH, DMF, rt; (b) (ClCH₂CH₂)₂O. (ii) NaI, Me₂CO, 65 °C. (iii) Methyl acrylate, Zn(Cu), EtOH-H₂O, ultrasound, rt. (iv) 3 M HCl, reflux.

Scheme 3[a]

[a] Reagents and conditions: (i) Se, NaOH, N₂H₄·H₂O, DMF, Ar, 60 °C. (ii) NaBH₄, ethyl 5-bromovalerate, EtOH, Ar, 0 °C. (iii) (a) aq NaOH, rt, 1 h; (b) TFA/CH₂Cl₂/thioanisole, rt, 0.5 h. (iv) (a) EtONa, DMF, rt; (b) Br(CH₂)₇COOEt, reflux, 10 h. (v) 3 M HCl, reflux, 3 h.

Concerning the inhibitors of series 2, we first prepared compounds **11** and **12**, which have a dimethylamino moiety in positions 3 and 4, respectively. These structures should ideally mimic the transition state **4**; however, compound **11** was completely inactive at 20 μM, and inhibitor **12** gave only very weak inhibition at this concentration. Therefore, we prepared compounds **13−17**, all of which have a nitrogen atom at position 4, not as quaternary amines, but as tertiary or secondary amines. We found that all secondary amines **14−17** were very poor

inhibitors of BHMT. Only tertiary amine **13** gave promising, but still moderate, inhibition of BHMT, with an IC₅₀ value of about 2.5 μM. Given this finding, we prepared compounds **18** and **19**, both with a nitrogen atoms at position 5, with a quaternary amine in **18**, and a tertiary amine in **19**. The idea was to investigate if the propylene spacer placed between sulfur and nitrogen is better suited for optimal inhibition of BHMT than the ethylene spacer. The propylene spacer should provide sufficient flexibility to the inhibitors for better adaptation to

Scheme 4[a]

[a] Reagents and conditions: (i) NaOH, CH₂Cl₂, H₂O, TEBAC, rt, Ar, 1 d. (ii) TFA/CH₂Cl₂/thioanisole, rt, 0.5 h. (iii) DBU, CH₂Cl₂, rt, 1 h. (iv) DBU, CH₂Cl₂, 50 °C, 6 h.
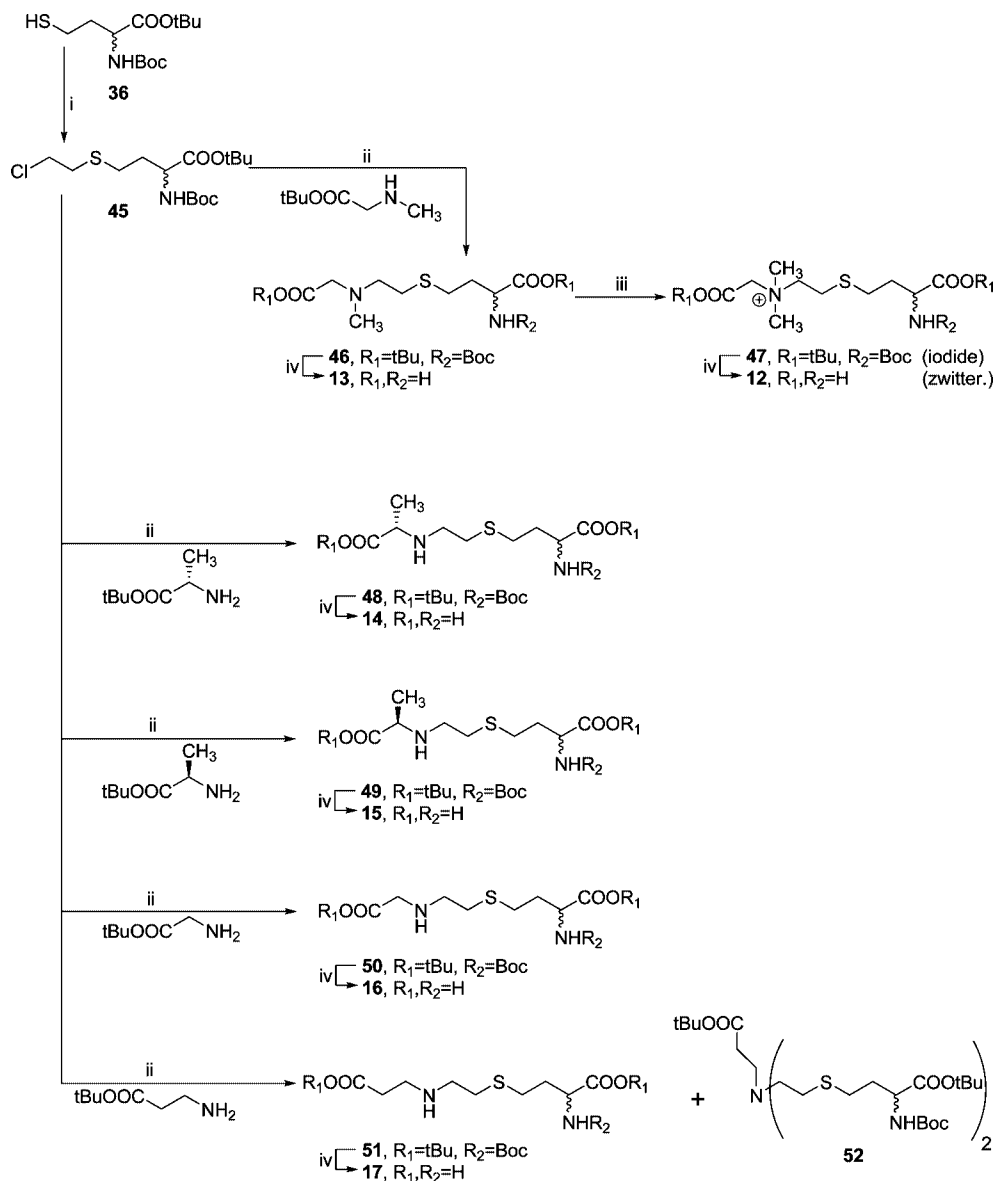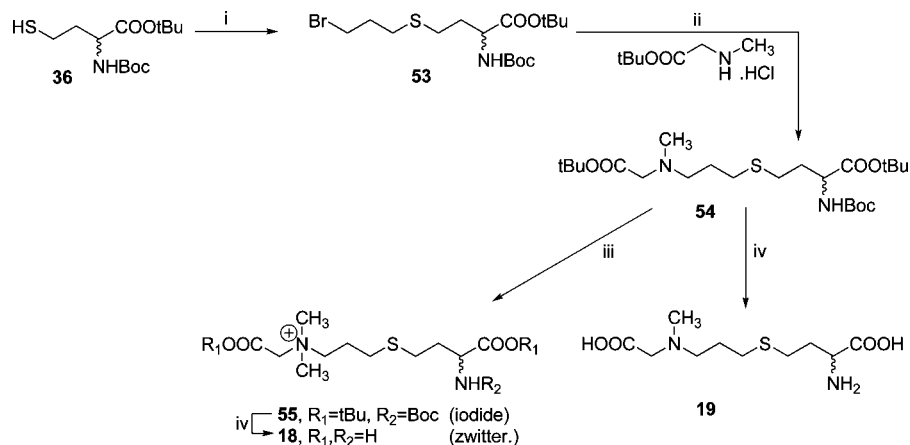
the active site. However, compound **18** did not inhibit BHMT at all, and compound **19**, with a tertiary amine at position 5, was only a modest inhibitor of BHMT, as it was significantly weaker than its homologue **13**, which has a tertiary amine at position 4.
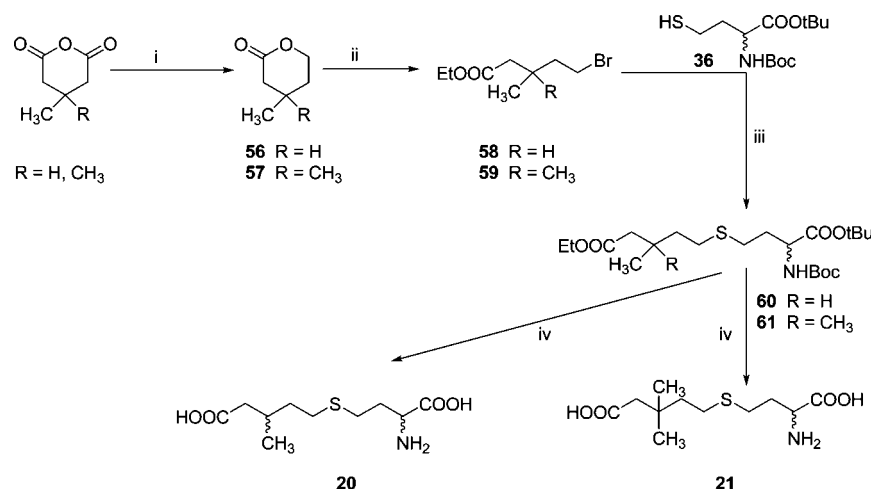
The above-described results indicate the following: (i) position 4 of scaffold **62** is the best suited for substitutions in the alkyl chain of inhibitors, (ii) a tertiary amine is better accommodated than a quaternary amine or secondary amine at position 4, but (iii) the BHMT enzyme does not accept a nitrogen atom at positions 3−5 of the scaffold **62** very well.

Given these data, we decided to synthesize and test compounds **20** and **21**, for which position 4 of the scaffold is branched with one or two methyl groups, respectively. We found that both compounds are highly potent inhibitors of BHMT; inhibitor **20** is equipotent to the "reference" compound **1**, and we found that compound **21** has an IC₅₀ value of 84 nM toward BHMT. This value is lower than the IC₅₀ that we determined for inhibitor **1** of about 138 nM. In a previous study,[15] we determined the K_i^{app} of inhibitor **1** for betaine to be about 12 nM. We can competently assume that inhibitor **21** will also have a significantly stronger K_i^{app} than inhibitor **1**. However, in this study, we did not repeat the extremely time-consuming enzyme activity assays required to determine the K_i^{app}, which also require high levels of radioactive betaine. Instead, we investigated the interaction of BHMT with inhibitor **21** in more detail using isothermal titration calorimetry. This technique allows for the precise determination of K_d together with the determination of changes in the total Gibbs free energy upon binding (decomposed to enthalpic and entropic contributions).

Isothermal Titration Calorimetry (ITC). Isothermal titration calorimetry (ITC) was used to monitor the binding of inhibitor **21** to human recombinant BHMT. The experiments were performed without the presence of BHMT substrates. Three independent titrations of compound **21** in a solution of BHMT were done separately in three buffers with different enthalpies of ionization (HEPES, ΔH_{ion} = 5.03 kcal·mol⁻¹; ACES, ΔH_{ion} = 7.51 kcal·mol⁻¹; Tris, ΔH_{ion} = 11.3 kcal·mol⁻¹). These experiments indicated the same binding enthalpies, as can be seen from the superposition of the individual titration curves (Figure S1, Supporting Information), which suggests that there is no net proton transfer associated with ligand binding.[42,43] The titration performed in HEPES buffer is shown in Figure 1. Compound **21** is a mixture of two enantiomers. The crystal structure of inhibitor **1** complexed with BHMT revealed that only the *S*-enantiomer binds to the active site of the enzyme.[7] The Glu159 of BHMT forms a hydrogen bond with the amino group of the L-homocysteine moiety of inhibitor **1**. Models of D-homocysteine in the active site place the amino group more than 4.0 Å away from the carboxylate of Glu159. Glu159 therefore establishes stereospecificity for the L-form of homocysteine. From these results, we presumed that only the *S*-enantiomer of compound **21** will interact with BHMT. Later, during the manuscript revision process, we confirmed this

Scheme 5[a]

[a] Reagents and conditions: (i) ClCH₂CH₂Cl, DBU 1 equiv, rt, Ar, 3 h. (ii) NaI, Na₂CO₃, Bu₄NBr, dioxane, Ar, reflux, 40 h. (iii) CH₃I, dioxane, rt, 1 d. (iv) TFA/CH₂Cl₂/thioanisole, rt, 0.5 h.

Scheme 6[a]

[a] Reagents and conditions: (i) Br(CH₂)₃Br, DBU 1 equiv, rt, 1 h. (ii) NaI, Na₂CO₃, Bu₄NBr, dioxane, Ar, reflux, 16 h. (iii) CH₃I, dioxane, rt, 1 d. (iv) TFA/CH₂Cl₂/thioanisole, rt, 0.5 h.

Scheme 7[a]

[a] Reagents and conditions: (i) (a) NaBH$_4$, THF, rt, 3 d (b) H$^+$. (ii) HBr/EtOH, rt, 3 d. (iii) NaH, THF, rt, 1 d. (iv) (a) 1 M NaOH, H$_2$O-Dx, 60 °C, 5 h; (b) TFA/CH$_2$Cl$_2$/thioanisole, rt, 0.5 h.
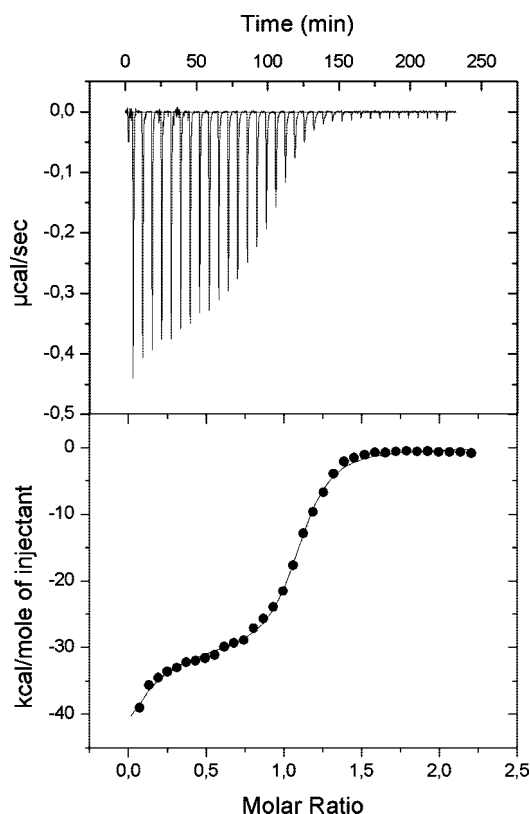
Figure 1. Isothermal titration of inhibitor **21** with BHMT. Titration was performed at 25 °C in 50 mM HEPES/NaOH, pH 7.5, containing 5 mM 2-mercaptoethanol. Upper graph: experimental data. Lower graph: fit (line) to the integrated heats (full circles) from each injection of compound **21**, corrected for the heat of dilution of this inhibitor. For details, see Experimental Section.

presumption by the synthesis and characterization of pure *S*- and *R*- enantiomers of compound **21** (compounds **63** and **64**, Supporting Information). Therefore, in our calculation of enthalpy change, we considered the concentration of compound **21** to be half of the total concentration of the *R,S*-compound. After addition of a small amount of ligand to the protein, an unknown exothermic process was detected from the titration curve (Figure 1). The other main exothermic response was considered to be due to the interaction of the reactants. Using a model for two sets of sites using the Origin software, it was

possible to separate the interaction step from the unknown exothermic process. The stoichiometry of the main interaction was estimated to be 1.1 ± 0.1, which is in a good agreement with the previous finding that one molecule of the similar inhibitor **1** binds to one BHMT monomer subunit (four inhibitor molecules per BHMT tetramer).[7] Inhibitor **21** (*S*-enantiomer) binds to BHMT with a dissociation constant (K_d) of about 51 nM (calculated from an association constant $K_a = (2.0 ± 0.6) × 10^7 \text{ M}^{-1}$), which corresponds to a total Gibbs free energy change $\Delta G = -10.0 ± 0.2 \text{ kcal·mol}^{-1}$. It is decomposed into enthalpic ($\Delta H = -29.5 ± 1.2 \text{ kcal·mol}^{-1}$) and entropic ($-T\Delta S = 19.6 ± 1.3 \text{ kcal·mol}^{-1}$) contributions. This considerably large and favorable enthalpic contribution suggests a strong and direct interaction of the inhibitor with the enzyme via hydrogen bonds or ionic interactions. On the other hand, a large and positive entropic contribution is unfavorable and may reflect possible conformational changes of the enzyme upon binding of the inhibitor. Recently, we studied the binding mechanism of BHMT using its intrinsic fluorescence.[17] This study confirmed the previously proposed ordered Bi−Bi mechanism of BHMT. It was shown that homocysteine is the first substrate to bind and that this binding probably induces a conformational change of the enzyme, which allows the binding of betaine, the second substrate. In 2004, it was shown that dimerization of BHMT might be required for substrate binding.[44] His338 of the BHMT "dimerization arm" (residues 319−371) contributes to betaine binding at the active site of the other monomer, indicating that the complete active site is formed upon dimerization. A more recent study[45] suggested that loop L2 (residues 74−79) is involved in the conformational change associated with occupancy at the betaine-binding site. Gonzalez et al.[46] proposed that, in ligand-free BHMT, L2 is open, which allows ligands access to the active site, but L2 closes after formation of the ternary complex (binding of betaine). Phe76 and Tyr77 are important betaine-binding determinants in this process. It is probable that inhibitor **21** interacts with BHMT in a similar manner; first, the "homocysteine" part of the inhibitor binds, and after an induced conformational change of BHMT, the *S*-linked alkyl chain of the inhibitor binds. These conformational changes could explain the high and positive entropic contribution that we observed. In the above-mentioned fluorescence study,[17] we determined the K_d of compound **1** (mixture of *R*- and *S*-enantiomers) for the enzyme to be about 280 nM. The K_d of

inhibitor **21** for BHMT as determined in this study using ITC is significantly tighter (51 nM for *S*-enantiomer) and confirms the status of compound **21** as the most potent inhibitor of BMHT ever reported.

Considerations Regarding the Mode of Interaction between Inhibitors and BHMT. The higher inhibitory potency of inhibitor **21** suggests that this compound might represent a better mimic of transition state **4** than the previously reported inhibitor **1**. Nevertheless, a number of questions arise from the inhibitory activities of our new compounds.

Molecular modeling of the BHMT−inhibitor **21** complex (see Supporting Information, Figure S2) suggests that compound **21** might bind BHMT in a similar manner as compound **1**. However, the calculation of binding energies for compounds **1** and **21** (see Supporting Information) indicated that inhibitor **21** should be a weaker binder than inhibitor **1** in this type of interaction. We predicted binding energies of −8.5 and −5.9 kcal·mol^{-1} for compounds **1** and **21**, respectively. This observation contradicts our inhibition experiments, which revealed that compound **21** was a tighter inhibitor than **1** (IC$_{50}$ values of 84 and 138 nM, respectively), and it also contradicts an experimental value of ΔG of about −10.0 kcal.mol^{-1} for compound **21** as determined by ITC. These findings also suggest that inhibitor **21** is a stronger binder than **1**. We suppose that inhibitor **21** binds to BHMT in a similar but not identical manner as inhibitor **1**. This slightly different mode of interaction results in a higher binding affinity of compound **21** compared to **1**.

The finding that BHMT does not tolerate betaine mimics in our inhibitors, especially the presence of the nitrogen atom, is surprising. However, the structure[7] of the complex of human BHMT with inhibitor **1** reveals that the inhibitor butyl chain is surrounded by a series of aromatic residues (Phe76, Tyr77, Tyr160, Phe261, and Phe 267) and that the carboxyl group interacts with Trp44 and Tyr77. The positive charge of the quaternary amine in compounds **11**, **12**, and **18** would be rather disadvantageous for binding to the hydrophobic pocket of BHMT surrounding the butyl chain of inhibitor **1**. It is probable that compounds with secondary (**14−17**) and tertiary (**13** and **19**) amines have protonated nitrogen atoms, which are responsible for their low inhibitory potency compared to inhibitors **1**, **20**, and **21**. We evaluated this hypothesis by NMR spectroscopy of compounds **13** and **16** over a broad pH range. The NMR spectra of quaternary amine **12** were measured for comparison. The ^{1}H and ^{13}C NMR titration experiments (see Supporting Information, Figure S3) revealed a p$K_2 \sim 9.5$ for the secondary and tertiary amino groups in both compounds **13** and **16**. These values indicate that the equilibrium between protonated and deprotonated forms of the nitrogen atoms at position 4 at pH 7.5, the pH used in our enzymatic assay, is shifted strongly to the protonated form, which may explain why they were much weaker inhibitors compared to compounds **20** and **21**. This observation implies that the deprotonated forms of compounds **13** and **16** likely bind to BHMT and are responsible for the observed moderate inhibition. The higher inhibitory potency of inhibitor **13** compared to **16** could be attributed to a positive contribution of the *N*-linked methyl group.

All of the above reasoning leads us to the conclusion that compound **1**, and probably also compounds **20** and **21**, do not correctly mimic the 3D structure of transition state **4** and that the substrates betaine and homocysteine bind BHMT differently than our inhibitors. The inhibitor-binding cavity of BHMT is relatively narrow and is buried deeply in the structure of the BMHT monomer.[7] It was clearly shown that the sulfur atom of inhibitor **1** directly interacts with the Zn^{2+} atom in the active
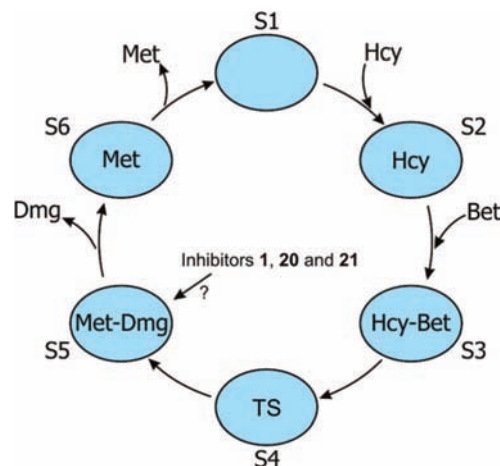
Figure 2. Schematic representation of proposed structural changes in BHMT during binding of substrates and products. Blue ovals designated as S1−S6 represent different structural states of the BMHT monomer. Hcy is homocysteine, Bet is betaine, Met is methionine, Dmg is dimethylglycine, and TS is the putative transition state of the substrates. For details, see the text.

site of BHMT,[7] and it was recently shown that the binding of ligands (substrates or inhibitor) replaces Tyr160, which is the fourth, displaceable ligand of zinc in BHMT.[46,47] These facts do not support the hypothesis that our inhibitors bind outside of the active site of BHMT, but rather evoke questions about putative conformational changes of BHMT upon binding of substrates/products or our inhibitors to the active site. Interestingly, Awad et al.[16,48] proposed a similar hypothesis 25 years ago. They suggested that the binding site for betaine might exist in two states, one permitting the binding of a positively charged group and the other a neutral group. It is possible that our potent inhibitors (e.g., **1**, **20**, and **21**) bind to some transient structure of BHMT, which may be similar to a structure of the enzyme that exists when it binds methionine (Met) or dimethylglycine (Dmg), for example, but relatively different from the structure of BHMT in a complex with homocysteine (Hcy) or betaine (Bet). Along this line of reasoning, it is possible that the 3D structure of the hypothetical transition state **4** is different from the structures presented by inhibitors **1**, **20**, and **21**. We attempted to express this hypothesis in Figure 2. We suppose that the structure of the active site of BHMT may exist in several different conformations (S1−S6 in Figure 2). Therefore, due to local rearrangements of its active site, BHMT can adapt its structure to different binders, substrates, and products (Hcy, Bet, Met, and Dmg). Structure S1 is the structure of BHMT without substrates. After binding of the first substrate, Hcy, the BHMT structure is modified such that it opens the binding site for the second substrate, Bet, and then adopts the structure S2. The transfer of a methyl group from Bet to Hcy proceeds via the structure of transition state when BHMT passes through structure S4. This process results in the formation of products Met, Dmg, and the respective BHMT structure S5. Finally, Dmg and then Met leave the active site and the BHMT structure changes from S5 to S6 and then back to the basic structure S1. It is not excluded that, for example, structures S2 and S6 are the same or that S3 does not exist because S4 is formed immediately after Bet binds. Such relatively complicated structural rearrangements of BHMT during a catalytic cycle would be in agreement with a very slow turnover rate of the enzyme.[5,6,49] We propose that our strong inhibitors **1**, **20**, and **21** fit well into the active site of structure S5. We suppose that Dmg bound to BMHT (in S5) has a deprotonated tertiary amine

nitrogen atom, probably because it is formed in the active site. On the other hand, our inhibitors that have a nitrogen atom at position 4 exist as mainly protonated molecules. For this reason, they bind weakly but their carbon-containing counterparts (**20** and **21**) bind strongly.

Conclusions

We prepared two series of new inhibitors of human recombinant BHMT and performed a systematic structure−activity study with the aim of investigating the structural requirements of the active site of the enzyme. The first series of inhibitors varies with regard to the position and number of sulfur atoms in the compounds, which are derived from the structure of the previously published inhibitor **1**. We found that the presence of a well-positioned sulfur atom in this type of compound is necessary for potent inhibition of BHMT. The second series of inhibitors was designed to mimic the hypothetical transition state (**4**) of the BHMT substrates betaine and homocysteine upon binding to the active site. We synthesized and tested analogues with NH, $N(CH_3)$, or $N(CH_3)_2$ groups separated from the homocysteine sulfur atom by methylene, ethylene, or propylene spacers. Only the inhibitor with the $N(CH_3)$ moiety and an ethylene spacer between nitrogen and sulfur gave moderate inhibition. These results led us to prepare two inhibitors lacking a nitrogen atom in the S-linked alkyl chain, inhibitor **20** and inhibitor **21**, which are both highly potent inhibitors of BHMT. We also investigated the interaction of inhibitor **21** with BHMT using isothermal titration microcalorimetry and found a highly enthalpic interaction and K_d of about 51 nM for the *S*-enantiomer of this inhibitor. Compound **21** is the most potent inhibitor of BHMT reported to date. The finding that BHMT does not tolerate betaine mimics in inhibitors, especially the presence of the nitrogen atom, is surprising and evokes questions about the catalytic mechanism of the reaction and about putative conformational changes of BHMT upon the binding of substrates/ products in the active site. It seems reasonable to assume that the predominant effect driving the binding of inhibitors or substrates to BHMT is an electrostatic effect. However, the work reported here shows that potent inhibitors of BHMT require that the central part of the compounds be without a positive charge. We interpret these results to indicate that our inhibitors bind to the enzyme in a mode similar to the products Met and Dmg. On the other hand, good mimics of the 3D structure of transition state **4** should demand the presence of a positive charge in the central part of the molecule, however, the structure and composition of true transition state **4** mimics have thus far eluded us. Additional information about the structure of BHMT complexed with varying substrate(s)−product(s) combinations, or complexed with the new inhibitory ligands described here (e.g., inhibitors **20** and **21**), will lead our future synthetic efforts to prepare BHMT ligands that deepen our understanding of the chemical events and structural changes that occur during BHMT catalysis.

Experimental Section

Chemistry: General. Unless otherwise stated, materials 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. The course of the reactions was checked on TLC plates (Fluka, Merck). More specifically, the products were detected by UV monitoring after spraying with ninhydrin (dark-blue color of amines), a 1% aqueous solution of $KMnO_4$ (sulfur compounds), and a 1% ethanolic solution of 4-(4-nitrobenzyl)pyridine, followed by heating and treating with gaseous ammonia (blue

color of esters). For flash column chromatography, silica gel 40−60 μm (Fluka) was used. TLC and preparative silica gel chromatography were carried out in the following solvent 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−conc aqueous ammonia−water 7:1:2 (IPAV), 50% EtOAc−toluene (T1), 20% ethyl acetate−toluene (T2). For gradient RP-HPLC purification and analysis, a Waters LC 625 System (Milford, MA) was used. Different gradients of acetonitrile (1−80%) in water containing 0.1% (v/v) of TFA were used for the elution of compounds. Preparative RP-HPLC of the target compounds was performed using a Phenomenex (Luna C-18, 5 μm, 25 cm × 2.12 cm, Torrance, CA) column. Purified compounds were coevaporated several times with water at 75 °C to remove TFA and then lyophilized from the water. Analytical RP-HPLC was performed using a Watrex (Nucleosil 120, 5 μm, C18, 25 cm × 0.46 cm; Prague, Czech Republic) column. Anion-exchange analytical HPLC was performed using an AminoPac PA10 (0.4 cm × 25 cm, Dionex Corporation, Sunnyvale, CA) column with a BioLC system (GP50 gradient pump, ED50 electrochemical detector) from Dionex Corporation (Sunnyvale, CA). Details of the HPLC conditions are given in the Supporting Information. HRMS mass spectra were obtained on a FTMS mass spectrometer LTQ-orbitrap XL (Thermo Fisher, Bremen, Germany) in electrospray ionization mode. ^1H and ^{13}C NMR spectra were recorded on a Bruker AVANCE-500 (^1H at 500.13 MHz, ^{13}C at 125.7 MHz) and an AVANCE-600 spectrometer (^1H at 600.13 MHz, ^{13}C at 150.9 MHz) in $CDCl_3$, DMSO-d_6 or D_2O solution. The 2D-H,H-COSY, 2D-H,C-HSQC, and 2D-H,C-HMBC spectra were used for the structural assignment of proton and carbon signals.

Deprotection: General Procedure A. A solution of the protected compound in aqueous HCl (4 M, 10 mL/mmol) was heated under reflux for 3 h and then evaporated in vacuo. The residue was dissolved in water and purified by the standard procedure (see General part).

Deprotection: General Procedure B. A solution of the protected compound in a mixture of dioxane (5 mL/mmol) and EtOH (2 mL/ mmol) was treated with aqueous NaOH (5 M, 2 mL/mmol), and the reaction mixture was stirred for 4 h at 80 °C (TLC in T1). The solution was acidified with saturated aq citric acid to pH 3 before being extracted with EtOAc (2 × 50 mL). The organic layer was washed with water (50 mL) and evaporated in vacuo. The residue was dissolved in a mixture of TFA−CH_2Cl_2−thioanisole−H_2O (47: 47:3:3 v/v, 5 mL/mmol), and the solution was stirred at rt for 30 min. Then, H_2O (10 mL/mmol) was added, and the aqueous phase was washed with Et_2O (5 × 10 mL). The final product was obtained after standard purification (see General part).

Deprotection: General Procedure C. The protected compound was dissolved in a mixture of TFA−CH_2Cl_2−thioanisole−H_2O (47: 47:3:3 v/v, 5 mL/mmol), and the solution was stirred at rt for 30 min. Then, H_2O (10 mL/mmol) was added, and the aqueous phase was washed with Et_2O (5 × 10 mL). The final product was obtained after standard purification (see General part).

Synthesis of compounds **27−29**, **63**, and **64** and spectral data for nontarget compounds **23a**, **23b**, **24**, **30−35**, **38−55**, and **58−61** are given in Supporting Information.

(*RS*)-2-Amino-4-[3-carboxypropyl)disulfanyl]butanoic acid (5) and 4,4′-disulfanediyldibutanoic acid (22). A solution of DL-homocysteine (0.300 g, 2.22 mmol) and γ-thiobutyrolactone (0.576 mL, 6.66 mmol, 3 equiv) in 5 M hydrochloric acid (20 mL) was stirred at rt for 60 h under an argon atmosphere, and then hydrogen peroxide (30% aq solution, 0.454 mL, 2 equiv) was added. Formation of a white precipitate was observed after 10 min, and the reaction was allowed to proceed overnight (TLC in IPAV). The reaction mixture was then coevaporated three times with water. The residue was treated with water (20 mL), and the insoluble solid was filtered off and dried over P_2O_5 to give **22** as a white solid (0.523 g, 66% from the total γ-thiobutyrolactone). ^1H NMR (500 MHz, D_2O) 1.80 (m, 4H), 2.15 (m, 4H), 2.61 (m, 4H). ^{13}C NMR (125.7 MHz, D_2O) 25.45 (2 × C), 36.20 (2 × C), 37.86 (2 × C),

182.72 (2 × C). HRMS (ESI) calcd for $C_8H_{15}O_4S_2$ [M + H]$^+$ 239.0412; found 239.0404.

The aqueous filtrate was concentrated in vacuo, and the crude product was purified by the standard procedure (see General part), giving **5** as a colorless hygroscopic semisolid (0.134 g, 24% from DL-homocysteine). ^{1}H NMR (500 MHz, D$_2$O) 1.89 (m, 2H), 2.20 (m, 1H), 2.28 (m, 1H), 2.40 (m, 2H), 2.67 (t, 2H), 2.74 (t, 2H), 4.03 (dd, 1H). ^{13}C NMR (125.7 MHz, D$_2$O) 23.61, 29.29, 32.22, 32.53, 36.76, 51.95, 178.05 (2 × C). HRMS (ESI) calcd for $C_8H_{16}NO_4S_2$ [M + H]$^+$ 254.0521; found 254.0514.

(RS)-2-Amino-4-[(2-carboxyethylthio)methylthio]butanoic Acid (6). 3-Mercaptopropionic acid (386 μL, 4.44 mmol, 3 equiv) and TEBAC (0.017 g, 0.075 mmol 0.05 equiv) were added to a stirred solution of DL-homocysteine (0.200 g, 1.48 mmol) and NaOH (1.2 g, 30 mmol, 20 equiv) in H$_2$O (10 mL). Then, dichloromethane (10 mL) was added and the reaction mixture was vigorously stirred at rt for 1 d (TLC in IPAV). The aqueous layer was passed through a column of Dowex 50 (Et$_3$N-cycle) and evaporated to dryness. The residue was dissolved in water and purified by the standard procedure to give **6** as a white solid (0.102 g, 27%). ^{1}H NMR (500 MHz, D$_2$O + NaOD) 1.81 (m, 1H), 1.93 (m, 1H), 2.49 (t, 2H), 2.70 (m, 2H), 2.85 (t, 2H), 3.33 (dd, 1H), 3.80 (s, 2H). ^{13}C NMR (125.7 MHz, D$_2$O + NaOD) 29.83, 29.97, 36.60, 36.93, 39.77, 57.95, 183.50, 185.25. HRMS (ESI) calcd for $C_8H_{14}NO_4S_2$ [M + H]$^+$ 252.0364; found 252.0360.

Diethyl 2-(3-Chloropropyl)-2-acetamidomalonate (23a) and Diethyl 2-(3-Bromopropyl)-2-acetamidomalonate (23b). Diethyl acetamidomalonate (4.88 g, 22.5 mmol, 1.00 equiv) was added in five portions to an ice-cooled suspension of NaH (60% suspension in oil, 1.04 g, 26.0 mmol, 1.17 equiv) in anhydrous DMF (12.5 mL) in a flask equipped with a calcium dichloride tube, and the mixture was left to stand at rt for 1 h. Then, 1-bromo-3-chloropropane (2.46 mL, 25.0 mmol, 1.12 equiv) was added dropwise over 10 min, and the reaction was allowed to proceed at 30 °C for 24 h (TLC in T1). After removal of the solvent in vacuo, the crude product was suspended in Et$_2$O, and the inorganic solids were filtered off. The organic phase was washed with H$_2$O twice before being dried over Na$_2$SO$_4$ and evaporated in vacuo. The resulting residue was purified by flash chromatography on silica (elution with a linear gradient of EtOAc in toluene) to yield 3.70 g of a mixture of **23a** and **23b** as colorless needles. The mixture was used directly in the next step without further resolution.

Diethyl 2-[3-(3-Ethoxycarbonylpropylthio)propyl]-2-acetamidomalonate (24). Sodium 4-mercaptoethylbutanoate[50] (0.40 g, 2.20 mmol, 1.5 equiv) was added to a mixture of **23a** and **23b** (0.50 g, ca. 1.57 mmol in total) in anhydrous EtOH (10 mL), and the reaction mixture was stirred overnight (TLC in T1). The resultant white precipitate was filtered off, and the solution was evaporated in vacuo. The crude product was purified by flash chromatography on silica (elution with a linear gradient of EtOAc in toluene) to give compound **24** as a colorless solid (0.317 g, 50%).

(RS)-2-Amino-5-(3-carboxypropylthio)pentanoic Acid (7). Compound **24** (0.310 g, 0.76 mmol) was deprotected according to general procedure A, affording **7** (0.147 g, 82%) as a white solid. ^{1}H NMR (600 MHz, D$_2$O) 1.70 (m, 1H), 1.77 (m, 1H), 1.90 (m, 2H), 2.02 (m, 1H), 2.06 (m, 1H), 2.50 (m, 2H), 2.62 (m, 2H), 2.64 (m, 2H), 4.04 (dd, 1H). ^{13}C NMR (150.9 MHz, D$_2$O) 26.77, 26.97, 31.68, 32.83, 32.90, 35.39, 55.68, 172.66, 181.00. HRMS (ESI) calcd for $C_9H_{19}N_2O_4S$ [M + H]$^+$ 251.1066; found 251.1062.

Diethyl 2-[2-(2-Chloroethoxy)ethyl]-2-acetamidomalonate (30). Diethyl acetamidomalonate (5 g, 23 mmol, 1 equiv) was slowly added to an intensively stirred suspension of NaH (60% dispersion in oil; 0.92 g, 23 mmol, 1 equiv) in DMF (40 mL) at 0 °C. After 20 min, sodium iodide (0.345 g, 2.3 mmol, 0.1 equiv) and bis(2-chlorethyl)ether (13.5 mL, 115 mmol, 5 equiv) were added, and the reaction mixture was stirred for 24 h at 60 °C (TLC in T1). The mixture was then concentrated in vacuo, and the dark-orange residue was partitioned between water (250 mL) and Et$_2$O (2 × 250 mL). The organic layer was separated, washed with water (2 × 100 mL), dried over Na$_2$SO$_4$, 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 **30** as a pale-yellow viscous oil (4.24 g, 57%).

Diethyl 2-[2-(2-Iodoethoxy)ethyl]-2-acetamidomalonate (31). Anhydrous sodium iodide (2.78 g, 18.5 mmol, 3 equiv) was added to a solution of chloride **30** (2.0 g, 6.2 mmol) in dry acetone (20 mL), and the reaction mixture was heated to 65 °C in a sealed flask for 24 h with stirring. The mixture was then diluted with Et$_2$O (50 mL), and the precipitated inorganic salts were removed by filtration. Evaporation of the filtrate under reduced pressure gave a crude product, which was purified by flash chromatography on silica (elution with a linear gradient of EtOAc in toluene) to give compound **31** as a colorless viscous oil (2.43 g, 95%).

Diethyl 2-{2-[4-(Methoxycarbonyl)butoxy]ethyl}-2-acetamidomalonate (32). The zinc−copper couple was prepared according to a previously described procedure[32] by the sonication of zinc dust (0.50 g, 8 mmol) and CuI (0.356 g, 2.4 mmol) in water (2 mL) under argon; after 2 min, the mixture turned to a black heavy suspension. Then, EtOH (2 mL) and methyl acrylate (0.90 mL, 10 mmol, 10 equiv) were added through the septum via syringe, and finally a solution of **31** (0.415 g, 1 mmol) in iPrOH (1.5 mL) was added over a period of 1 h under sonication; the temperature was not allowed to rise above 20 °C. Sonication was continued for 1 h, and then saturated aq NaCl (5 mL) was added. The resulting precipitate was filtered over celite. The solid was extracted with Et$_2$O (100 mL), and the filtrate was partitioned between water and Et$_2$O, dried over Na$_2$SO$_4$, and concentrated in vacuo. The residue was purified by flash chromatography on silica (elution with a linear gradient of EtOAc in toluene) to give compound **32** as a colorless viscous oil (0.195 g, 52%).

(RS)-5-(3-Amino-3-carboxypropoxy)pentanoic Acid (8). Compound **32** (0.180 g, 0.82 mmol) was deprotected according to general procedure A, yielding **8** as a white solid (0.098 g, 93%). ^{1}H NMR (600 MHz, D$_2$O) 1.62 (m, 4H), 2.23 (m, 2H), 2.41 (t, 2H), 2.52 (m, 2H), 3.68 (m, 2H), 4.16 (dd, 1H). ^{13}C NMR (150.9 MHz, D$_2$O) 23.64, 30.65, 32.10, 36.10, 54.16, 68.97, 73.22, 174.63, 181.56. HRMS (ESI) calcd for $C_9H_{18}NO_5$ [M + H]$^+$ 220.1185; found 220.1183.

(RS,RS)-Di-tert-butyl-4,4′-diselanediylbis[2-(tert-butoxycarbonylamino)butanoate] (33). N$_2$H$_4$·H$_2$O (0.062 mL, 1.27 mmol, 1.1 equiv) was added to a suspension of Se (0.100 g, 1.27 mmol, 1.1 equiv) and NaOH (0.138 g, 3.45 mmol, 3 equiv) in DMF (5 mL), and the mixture was stirred at 60 °C under argon for 3 h. Then, a solution of bromide **29** (0.390 g, 1.15 mmol, 1 equiv) in DMF (3 mL) was added to the flask through the septum by syringe, and stirring was continued for a further 3 h at 60 °C (TLC in T2). The reaction mixture was concentrated in vacuo, and the residue was partitioned between Et$_2$O and H$_2$O. The organic phase was separated, washed with brine, and dried over Na$_2$SO$_4$. After evaporation, the crude product was purified by flash chromatography on silica (elution with a linear gradient of EtOAc in toluene) to give compound **33** as a yellow viscous oil (0.295 g, 76%).

(RS)-Ethyl 5-[3-tert-butoxycarbonyl-3-(tert-butoxycarbonylamino)propylselanyl]pentanoate (34). NaBH$_4$ (0.048 g, 1.26 mmol, 3 equiv) was added to a solution of diselenide **33** (0.285 g, 0.42 mmol) and ethyl 5-bromovalerate (0.150 mL, 0.93 mmol, 2.2 equiv) in abs EtOH (5 mL) under an argon atmosphere at 0 °C, and the mixture was stirred until the yellow color of the diselenide disappeared (ca. 5 min). The reaction was allowed to proceed at rt overnight (TLC in T2). The mixture was then partitioned between Et$_2$O and H$_2$O. The organic layer was washed with brine, dried over Na$_2$SO$_4$, 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 **34** as a colorless viscous oil (0.344 g, 87%).

(RS)-5-(3-Amino-3-carboxypropylselanyl)pentanoic Acid (9). Compound **34** (0.320 g, 0.69 mmol) was deprotected according to general procedure B, yielding **9** as a white solid (0.122 g, 63%). ^{1}H NMR (600 MHz, D$_2$O + NaOD) 1.63 (m, 2H), 1.67 (m, 2H), 1.87 (m, 1H), 1.97 (m, 1H), 2.19 (t, 2H), 2.60 (m, 2H), 2.64 (m, 2H), 3.30 (dd, 1H). ^{13}C NMR (150.9 MHz, D$_2$O + NaOD) 22.02,

25.97, 28.89, 32.37, 38.53, 39.76, 58.96, 185.56, 186.44. HRMS (ESI) calcd for $C_9H_{18}O_4NSe$ [M + H]$^+$ 284.0396; found 284.0395.

Diethyl 2-[(7-Ethoxycarbonyl)heptyl]-2-acetamidomalonate (35). Sodium ethanolate was generated in situ by dissolving sodium (0.072 g, 3.13 mmol, 1.2 equiv) in abs EtOH (2.5 mL) at rt in a flask equipped with a reflux condenser fitted with a calcium chloride tube. DEAM (0.571 g, 2.63 mmol, 1.1 equiv) was added, and after 15 min, a solution of ethyl 8-bromooctanoate (0.600 g, 2.39 mmol, 1 equiv) in EtOH (3 mL) was introduced, and the reaction was heated to reflux for 10 h. The mixture was evaporated, 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 **35** as a colorless viscous oil (0.547 g, 51%).

(RS)-2-Aminodecanedioic Acid (10). Compound **35** (0.320 g, 0.69 mmol) was deprotected according to general procedure A, yielding **10** as a white solid (0.156 g, 87%). ^{1}H NMR (600 MHz, D_2O + NaOD) 1.30 (m, 2H), 1.32 (m, 4H), 1.34 (m, 2H), 1.54 (m, 2H), 1.74 (m, 1H), 1.80 (m, 1H), 2.16 (t, 2H), 3.59 (dd, 1H). ^{13}C NMR (150.9 MHz, D_2O + NaOD) 27.09, 28.54, 30.89, 31.04, 31.25, 34.16, 40.34, 57.86, 180.04, 187.08. HRMS (ESI) calcd for $C_{10}H_{20}NO_4$ [M + H]$^+$ 218,1392; found 218,1382.

(RS,RS)-Di-*tert*-butyl 4,4′-(methylenedithio)-2,2′-di(*tert*-butoxycarbonylamino)dibutanoate (38) and (RS)-1-[3-(*tert*-butoxycarbonylamino)-3-(*tert*-butoxycarbonyl)propylthio]-N-(*tert*-butoxycarbonylmethyl)-N,N-dimethylmethanammonium chloride (40). A solution of chloromethylsulfide **37**, which is necessary for the amine alkylation, was obtained by intensive stirring of thiol **36** (0.345 g, 1.18 mmol),[34] crushed solid KOH (0.067 g, mmol, 1 equiv), and TEBAC (0.027 g, 0.12 mmol, 0.1 equiv) in a mixture of CH_2BrCl (2.5 mL) and CH_2Cl_2 (2.5 mL) for 1 h at rt. The solid precipitate was filtered off, and the solution was directly used in subsequent reaction. A solution of **37** was added to a solution of *tert*-butyl 2-(dimethylamino)acetate hydrochloride (0.160 g, 0.82 mmol, 1 equiv) and DBU (0.135 mL, 0.90 mmol, 1.1 equiv) in CH_2Cl_2 (4 mL), and the reaction was allowed to proceed at rt for 1 h. The mixture was evaporated in vacuo, and the residue was purified by flash chromatography on silica. Elution with a linear gradient of EtOAc in toluene gave byproduct **38** as a colorless viscous oil (0.065 g, 19% from thiol **36**). Further elution with a linear gradient of H1 in EtOAc yielded the desired ammonium salt **40** (0.125 g, 31% from the amine hydrochloride). Isolation of the byproduct **38** in the following procedures that employ a solution of **37** was not performed.

(RS,RS)-4,4′-(Methylenedithio)-2,2′-(diamino)dibutanoic Acid (39). Compound **38** (0.110 g, 0.18 mmol) was deprotected according to the general procedure C, affording **39** as a white solid (0.045 g, 86%).

(RS)-2-{[(3-Amino-3-carboxypropylthio)methyl]dimethylammonium}acetate (11). Compound **40** (0.065 g, 0.13 mmol) was deprotected according to the general procedure C, affording **11** as a white solid (0.020 g, 63%). ^{1}H NMR (500 MHz, D_2O) 2.22 (m, 1H), 2.33 (m, 1H), 2.97 (m, 1H), 3.02 (m, 1H), 3.26 (s, 6H), 4.14 (dd, 1H), 4.17 (s, 2H), 4.91 (s, 2H). ^{13}C NMR (125.7 MHz, D_2O) 32.59, 32.66, 53.20 (2 × C), 54.42, 63.54, 70.92, 170.74, 174.09. HRMS (ESI) calcd for $C_9H_{19}N_2O_4S$ [M]$^+$ 251.1066; found 251.1077.

(RS)-1-{[3-(*tert*-Butoxycarbonyl)-3-(*tert*-butoxycarbonylamino)-propylthio]methyl}-2,3,4,6,7,8,9,10-octahydropyrimido[1,2-a]azepin-1-ium Chloride (41). The solution of **37**, prepared as above, was added to a solution of *tert*-butyl 2-aminoacetate hydrochloride (0.200 g, 1.19 mmol, 1 equiv) and DBU (0.381 mL, 2.50 mmol, 2.1 equiv) in CH_2Cl_2 (4 mL), and the reaction was allowed to proceed in a sealed flask under argon at 50 °C for 6 h. The mixture was evaporated in vacuo, and the residue was purified by flash chromatography on silica. Elution with a linear gradient of H1 in EtOAc gave **41** as a white foam (0.180 g, 0.37 mmol).

(RS)-2-Amino-4-[(2,3,4,6,7,8,9,10-octahydropyrimido[1,2-a]azepin-1-ium-1-yl)methylthio]butanoate (42). Compound **41** (0.172 g, 0.35 mmol) was deprotected according to the general procedure C, affording **42** as a white solid (0.062 g, 59%).

(RS)-*tert*-Butyl 2-(*tert*-butoxycarbonylamino)-4-{[(*tert*-butoxycarbonylmethyl)(methyl)amino]methylthio}butanoate (43). The solution of **37**, prepared as above, was added to a solution of *tert*-butyl 2-(methylamino)aminoacetate hydrochloride (0.216 g, 1.19 mmol, 1 equiv) and DBU (0.181 g, 1.19 mmol, 1 equiv) in CH_2Cl_2 (4 mL), and the reaction was allowed to proceed in a sealed flask under argon at 50 °C for 6 h. Then second portion of DBU (0.181 mL, 1.19 mmol, 1 equiv) was added, the mixture evaporated in vacuo, and the residue was purified by flash chromatography on silica. Elution with a linear gradient of EtOAc in toluene gave **43** as a colorless glassy solid (0.250 g, 31%).

Di-*tert*-butyl (RS,RS)-4,4′-(*tert*-Butoxycarbonylmethylamino)bis-(methylene)bis(sulfanediyl)bis[(2-*tert*-butoxycarbonylamino)butanoate] (44). The solution of **37**, prepared as above, was added to a solution of *tert*-butyl 2-aminoacetate hydrochloride (0.200 g, 1.19 mmol, 1 equiv) and DBU (0.181 mL, 1.19 mmol, 1 equiv) in CH_2Cl_2 (4 mL), and the reaction was allowed to proceed in a sealed flask under argon at 50 °C for 24 h. Then second portion of DBU (0.181 mL, 1.19 mmol, 1 equiv) was added, the mixture was evaporated in vacuo, and the residue was purified by flash chromatography on silica. Elution with a linear gradient of EtOAc in toluene gave **44** as a colorless glassy solid (0.143 g, 11%).

(RS)-*tert*-Butyl 2-(*tert*-butoxycarbonylamino)-4-(2-chloroethylthio)butanoate (45). DBU (1.353 mL, 8.89 mmol, 1.1 equiv) was added to a solution of thiol **36** (2.355 g, 8.08 mmol) in 1,2-dichloroethane (20 mL), and the reaction mixture was heated under argon at 80 °C for 3 h (TLC in T2). Excessive solvent was evaporated in vacuo, and the crude product was purified by flash chromatography on silica (elution with a linear gradient of EtOAc in toluene) to give compound **45** as a white crystalline solid (2.456 g, 86%).

(RS)-*tert*-Butyl 2-(*tert*-butoxycarbonylamino)-4-{2-[(*tert*-butoxycarbonylmethyl)(methyl)amino]ethylthio}butanoate (46). Chloride **45** (0.366 g, 1.03 mmol, 1 equiv), *tert*-butyl 2-(methylamino)acetate (0.150 g, 1.03 mmol, 1 equiv), sodium carbonate (0.165 g, 1.55 mmol, 1.5 equiv), sodium iodide (0.155 g, 1.03 mmol., 1 equiv), and tetrabutylammonium bromide (0.166 g, 0.52 mmol, 0.5 equiv) were placed in a flask, and the mixture was codistilled twice with anhydrous CH_2Cl_2 (10 mL) to remove traces of water. Dioxane (15 mL) was added, and the flask was equipped with a reflux condenser and heated to reflux under argon for 40 h (TLC in T2). After cooling, the mixture was filtered over celite, the solid extracted with EtOAc (50 mL), and the filtrate evaporated in vacuo. The crude product was purified by flash chromatography on silica (elution with a linear gradient of EtOAc in toluene) to give compound **46** as a colorless solid (0.298 g, 62%).

(RS)-2-[3-(*tert*-Butoxycarbonylamino)-3-(*tert*-butoxycarbonyl)-propylthio]-N-(*tert*-butoxycarbonylmethyl)-N,N-dimethylethanammonium Iodide (47). A solution of **46** (0.196 g, 0.42 mmol) in dioxane (5 mL) was treated with methyl iodide (0.066 mL, 0.84 mmol, 2.5 equiv), and the reaction mixture was left to react at rt for 4 d (TLC in C1). The solution was then concentrated in vacuo, and the residue was purified by flash chromatography on silica (elution with a linear gradient of H3 in EtOAc) to give compound **47** as a dark-yellow solid (0.196 g, 89%).

(RS)-2-{[2-(3-Amino-3-carboxypropylthio)ethyl]dimethylammonium}acetate (12). Compound **47** (0.188 g, 0.31 mmol) was deprotected according to the general procedure C, affording **12** as a white solid (0.035 g, 42%). ^{1}H NMR (500 MHz, D_2O) 2.18 (m, 1H), 2.28 (m, 1H), 2.78 (m, 2H), 2.99 (m, 2H), 3.27 (s, 6H), 3.83 (m, 2H), 4.10 (s, 2H), 4.15 (t, 1H). ^{13}C NMR (125.7 MHz, D_2O) 26.03, 29.44, 32.36, 54.54 (2xC), 54.68, 65.11, 65.82, 170.67, 174.80. HRMS (ESI) calcd for $C_{10}H_{21}N_2O_4S$ [M]$^+$ 265.1222; found 265.1236.

(RS)-2-Amino-4-{2-[(carboxymethyl)(methyl)amino]ethylthio}-butanoic Acid (13). Compound **46** (0.181 g, 0.39 mmol) was deprotected according to the general procedure C, affording **13** as a white solid (0.71 g, 73%). ^{1}H NMR (600 MHz, D_2O) 2.20 (m, 1H), 2.31 (m, 1H), 2.80 (m, 2H), 2.99 (s, 3H), 3.00 (m, 2H), 3.50 (m, 2H), 4.08 (m, 2H), 4.21 (t, 1H). ^{13}C NMR (150.9 MHz, D_2O)

27.62, 28.90, 32.05, 43.82, 54.41, 58.00, 59.37, 171.27, 174.54. HRMS (ESI) calcd for $C_9H_{19}N_2O_4S$ $[M + H]^+$ 251.1066; found 251.1070.

***tert*-Butyl 2-(*RS*)-(*tert*-Butoxycarbonylamino)-4-[2-(*S*)-(1-*tert*-butoxycarbonylethylamino)ethylthio]butanoate (48).** Using the procedure outlined for **46**, compound **48** was prepared from **45** (0.377 g, 1.06 mmol), (*S*)-*tert*-butyl 2-aminopropanoate (0.170 g, 1.17 mmol, 1.1 equiv), sodium carbonate (0.372 g, 3.51 mmol, 3.0 equiv), sodium iodide (0.175 g, 1.17 mmol, 1 equiv), and tetrabutylammonium bromide (0.190 g, 0.59 mmol, 0.5 equiv) as a colorless solid (0.234 g, 47%).

2-(*RS*)-Amino-4-[2-(*S*)-(1-carboxyethylamino)ethylthio]butanoic Acid (14). Compound **48** (0.220 g, 0.48 mmol) was deprotected according to the general procedure C, affording **14** as a white solid (0.36 g, 30%).

***tert*-Butyl 2-(*RS*)-(*tert*-Butoxycarbonylamino)-4-[2-(*R*)-(1-*tert*-butoxycarbonylethylamino)ethylthio]butanoate (49).** Using the procedure outlined for **46**, compound **49** was prepared from **45** (0.354 g, 1.00 mmol), (*R*)-*tert*-butyl 2-aminopropanoate (0.160 g, 1.10 mmol, 1.1 equiv), sodium carbonate (0.318 g, 3.00 mmol, 3.0 equiv), sodium iodide (0.150 g, 1.00 mmol, 1 equiv), and tetrabutylammonium bromide (0.161 g, 0.50 mmol, 0.5 equiv) as a colorless solid (0.171 g, 37%).

2-(*RS*)-Amino-4-[2-(*R*)-(1-carboxyethylamino)ethylthio]butanoic Acid (15). Compound **49** (0.154 g, 0.33 mmol) was deprotected according to the general procedure C, affording **15** as a white solid (0.022 g, 27%). NMR: Mixture of diastereoisomers ca. 1:1; doubling of carbon signals observed. ^{1}H NMR (600 MHz, D_2O) 1.58 (d, 3H), 2.19 (m, 1H), 2.29 (m, 1H), 2.78 (m, 2H), 2.93 (m, 2H), 3.34 (m, 2H), 4.06 (q, 1H), 4.18 (t, 1H). ^{13}C NMR (150.9 MHz, D_2O) 17.01 + 17.05, 28.82 + 28.86, 29.53, 32.11, 47.41 + 47.45, 54.58 + 54.59, 58.78 + 58.82, 174.75, 175.22 + 175.24. HRMS (ESI) calcd for $C_9H_{19}N_2O_4S$ $[M + H]^+$ 251.1066; found 251.1057.

(*RS*)-*tert*-Butyl 2-(*tert*-butoxycarbonylamino)-4-[2-(*tert*-butoxycarbonylmethylamino)ethylthio]butanoate (50). Using the procedure outlined for **46**, compound **50** was prepared from **45** (0.403 g, 1.14 mmol), *tert*-butyl 2-aminoacetate (0.164 g, 1.25 mmol, 1.1 equiv), sodium carbonate (0.397 g, 3.75 mmol, 3.0 equiv), sodium iodide (0.171 g, 1.14 mmol, 1 equiv), and tetrabutylammonium bromide (0.184 g, 0.57 mmol, 0.5 equiv) as a pale-yellow thick oil (0.250 g, 45%).

(*RS*)-2-Amino-4-[2-(carboxymethylamino)ethylthio]butanoic Acid (16). Compound **50** (0.220 g, 0.49 mmol) was deprotected according to the general procedure C, affording **16** as a white solid (0.012 g, 10%). ^{1}H NMR (600 MHz, D_2O) 2.17 (m, 1H), 2.26 (m, 1H), 2.75 (m, 2H), 2.94 (m, 2H), 3.35 (t, 2H), 3.84 (s, 2H), 4.06 (t, 1H). ^{13}C NMR (150.9 MHz, D_2O) 28.88, 29.44, 32.36, 48.78, 50.75, 55.23, 172.63, 175.50. HRMS (ESI) calcd for $C_8H_{17}N_2O_4S$ $[M + H]^+$ 237.0909; found 237.0906.

(*RS*)-*tert*-Butyl 2-(*tert*-butoxycarbonylamino)-4-[2-(2-*tert*-butoxycarbonylethylamino)ethylthio]butanoate (51) and Di-*tert*-butyl (*RS*,*RS*)-4,4′-[2,2′-(2-*tert*-Butoxycarbonylethylaminodiyl)bis(ethane-2,1-diyl)bis(sulfanediyl)]bis[2-(*tert*-butoxycarbonylamino)butanoate] (52). The procedure outlined for **46** was followed using **45** (0.354 g, 1.00 mmol), *tert*-butyl 3-aminopropanoate (0.160 g, 1.10 mmol, 1.1 equiv), sodium carbonate (0.318 g, 3.00 mmol, 3.0 equiv), sodium iodide (0.150 g, 1.00 mmol, 1 equiv), and tetrabutylammonium bromide (0.161 g, 0.50 mmol, 0.5 equiv). Elution with a linear gradient of EtOAc in toluene gave **51** (0.377 g, 81%) and **52** (0.120 g, 15%) as colorless thick oils.

(*RS*)-2-Amino-4-[2-(2-carboxyethylamino)ethylthio]butanoic Acid (17). Compound **51** (0.370 g, 0.80 mmol) was deprotected according to the general procedure C, affording **17** as a white solid (0.087 g, 43%). ^{1}H NMR (600 MHz, D_2O) 2.18 (m, 1H), 2.26 (m, 1H), 2.76 (m, 2H), 2.86 (t, 2H), 2.94 (m, 2H), 3.34 (t, 2H), 3.38 (t, 2H), 4.09 (t, 1H). ^{13}C NMR (150.9 MHz, D_2O) 28.96, 29.31, 32.38, 32.71, 45.66, 49.05, 55.14, 175.40, 177.10. HRMS (ESI) calcd for $C_9H_{18}NO_4S$ $[M + H]^+$ 236.0957; found 236.0945.

(*RS*)-*tert*-Butyl 4-(3-Bromopropylthio)-2-(*tert*-butoxycarbonylamino)butanoate (53). DBU (0.598 mL, 4.32 mmol, 1.1 equiv) was added to a solution of thiol **36** (1.145 g, 3.93 mmol) in 1,3-dibromopropane (10 mL), and the reaction mixture was left to stand overnight at rt (TLC in T2). Excessive solvent was evaporated in vacuo, and the crude product was purified by flash chromatography on silica (elution with a linear gradient of EtOAc in toluene) to give compound **53** as a white crystalline solid (1.32 g, 81%).

(*RS*)-*tert*-Butyl 2-(*tert*-butoxycarbonylamino)-4-{3-[(*tert*-butoxycarbonylmethyl)(methyl)amino]propylthio}butanoate (54). Using the procedure outlined for **46**, compound **54** was prepared from **53** (0.300 g, 0.73 mmol), *tert*-butyl 2-(methylamino)acetate hydrochloride (0.145 g, 0.80 mmol, 1.1 equiv), sodium carbonate (0.231 g, 2.18 mmol, 3.0 equiv), sodium iodide (0.109 g, 0.73 mmol, 1 equiv), and tetrabutylammonium bromide (0.117 g, 0.36 mmol, 0.5 equiv) as a colorless solid (0.290 g, 82%).

(*RS*)-2-Amino-4-{3-[(carboxymethyl)(methyl)amino]propylthio}butanoic Acid (19). Compound **54** (0.225 g, 0.47 mmol) was deprotected according to the general procedure C, affording **19** as a white solid (0.69 g, 55%). ^{1}H NMR (600 MHz, D_2O) 2.06 (m, 2H), 2.18 (m, 1H), 2.28 (m, 1H), 2.68 (m, 2H), 2.74 (t, 2H), 2.97 (s, 3H), 3.28 (m, 1H), 3.41 (m, 1H), 3.96 (bd, 1H), 4.05 (bd, 1H), 4.18 (t, 1H). ^{13}C NMR (150.9 MHz, D_2O) 26.16, 29.07, 30.13, 32.21, 44.10, 54.65, 58.67, 59.49, 171.57, 174.79. HRMS (ESI) calcd for $C_{10}H_{21}N_2O_4S$ $[M + H]^+$ 265.1222; found 265.1219.

(*RS*)-3-[3-(*tert*-Butoxycarbonylamino)-3-(*tert*-butoxycarbonyl)propylthio]-*N*-(carboxymethyl)-*N*,*N*-dimethylpropan-1-ammonium iodide (55). The procedure outlined for **47** was followed using amine **54** (0.222 g, 0.47 mmol) and methyl iodide (0.072 mL, 1.16 mmol, 2.5 equiv). The reaction was complete after 2 d, and compound **55** was isolated as a dark-yellow solid (0.153 g, 53%).

(*RS*)-2-{[3-(3-Amino-3-carboxypropylthio)propyl]dimethylammonio}acetate (18). Compound **55** (0.140 g, 0.23 mmol) was deprotected according to the general procedure C, affording **18** as a white solid (0.059 g, 94%). ^{1}H NMR (600 MHz, D_2O) 2.07 (m, 2H), 2.15 (m, 1H), 2.22 (m, 1H), 2.67 (m, 2H), 2.71 (t, 2H), 3.24 (s, 3H), 3.27 (s, 3H), 3.68 (m, 2H), 3.95 (s, 2H), 3.99 (t, 1H). ^{13}C NMR (150.9 MHz, D_2O) 24.77, 29.17, 30.06, 32.65, 54.33 (2 × C), 55.76, 66.00, 66.20, 171.50, 176.00. HRMS (ESI) calcd for $C_{11}H_{23}N_2O_4S$ $[M]^+$ 279.1379; found 279.1370.

(*RS*)-Ethyl 5-Bromo-3-methylpentanoate (58). (*RS*)-Tetrahydro-4-methylpyran-2-one[39] (0.410 g, 3.59 mmol) was treated with a saturated solution of anhydrous HBr in EtOH (15 mL), and the mixture was stirred at rt for 3 d (TLC in T2). The reaction was quenched by pouring it into 200 mL of water and extracting it with Et_2O (3 × 50 mL). The combined organic extracts were sequentially washed with water (100 mL), sat. aq $NaHCO_3$ (50 mL), and water (100 mL), dried over Na_2SO_4, and evaporated in vacuo. The crude product was purified by flash chromatography on silica (isocratic elution, toluene only) to give compound **58** as a colorless liquid with a camphor odor (0.660 g, 82%).

Ethyl 5-Bromo-3,3-dimethylpentanoate (59). The procedure outlined for **58** was followed using tetrahydro-4,4-dimethylpyran-2-one[39] (1.03 g, 8.00 mmol) and saturated solution of anhydrous HBr in EtOH (20 mL), giving compound **59** as a colorless liquid with a camphor odor (1.690 g, 89%).

Ethyl (*RS*,*RS*)-5-[3-(*tert*-Butoxycarbonylamino)-3-(*tert*-butoxycarbonyl)propylthio]-3-methylpentanoate (60). NaH (60% dispersion in oil; 0.061 g, 1.52 mmol, 1.2 equiv) was added to a solution of thiol **36** (0.369 g, 1.27 mmol, 1 equiv) in anhydrous THF (5 mL) in a flask equipped with a calcium chloride tube. Bromide **58** (0.282 g, 1.27 mmol, 1 equiv) was added after 10 min, and the reaction was allowed to proceed overnight (TLC in T2). The mixture was then concentrated in vacuo, and the residue was purified by flash chromatography on silica (elution with a linear gradient of EtOAc in toluene) to give compound **60** as a colorless thick oil (0.300 g, 55%). NMR: Mixture of diastereoisomers—some carbon signals are doubled.

Ethyl (*RS*)-5-[3-(*tert*-Butoxycarbonylamino)-3-(*tert*-butoxycarbonyl)propylthio]-3,3-dimethylpentanoate (61). The procedure outlined for **60** was followed using thiol **36** (0.369 g, 1.27 mmol, 1

equiv), NaH (60% dispersion in oil; 0.061 g, 1.52 mmol, 1.2 equiv), and bromide **59** (0.300 g, 1.27 mmol, 1 equiv), giving compound **61** as a colorless thick oil (0.430 g, 76%).

(RS,RS)-5-(3-Amino-3-carboxypropylthio)-3-methylpentanoic Acid (20). Compound **60** (0.290 g, 0.67 mmol) was deprotected according to the general procedure B, affording **20** as a white solid (0.106 g, 64%). NMR: Mixture of diastereoisomers ca. 1:1; doubling of the most of carbon signals observed. ^{1}H NMR (600 MHz, DMSO-d_6) 0.89 (d, 3H), 1.40 (m, 1H), 1.53 (m, 1H), 1.93 (m, 1H), 1.97 (m, 1H), 2.02 (dd, 1H), 2.03 (m, 1H), 2.24 (dd, 1H), 2.48 (m, 1H), 2.53 (m, 1H), 2.56 (m, 1H), 2.62 (m, 1H), 3.96 (dd, 1H), 8.25 (b, 2H), 12.10 (b, 2H). ^{13}C NMR (150.9 MHz, DMSO-d_6) 19.36 + 19.39, 26.50 + 26.54, 28.39 + 28.43, 29.20 + 29.22, 30.36 + 30.38, 35.84 + 35.88, 41.09 + 41.11, 51.38, 170.98, 174.05. HRMS (ESI) calcd for $C_{10}H_{20}NO_4S$ [M + H]$^+$ 250.1113; found 250.1105.

(RS)-5-(3-Amino-3-carboxypropylthio)-3,3-dimethylpentanoic Acid (21). Compound **61** (0.430 g, 0.96 mmol) was deprotected according to the general procedure B, affording **21** as a white solid (0.154 g, 61%). ^{1}H NMR (600 MHz, D$_2$O) 1.02 (s, 6H), 1.63 (m, 2H), 2.18 (m, 1H), 2.27 (m, 1H), 2.28 (s, 2H), 2.61 (m, 2H), 2.72 (t, 2H), 4.14 (dd, 1H). ^{13}C NMR (150.9 MHz, D$_2$O) 28.51, 28.97, 29.09, 29.10, 32.32, 35.63, 43.88, 48.10, 54.69, 175.04, 180.04. HRMS (ESI) calcd for $C_{11}H_{22}NO_4S$ [M + H]$^+$ 264.1270; found 264.1276.

BHMT Inhibition Assays. Human recombinant BHMT was prepared as described previously,[6] and N-methyl-^{14}C-betaine (57 mCi/mmol) was prepared and supplied by Moravek Biochemicals (Brea, CA). Compounds were tested for their ability to inhibit BHMT activity using an assay procedure that we have described previously in detail.[15,49] Briefly, DL-homocysteine was freshly prepared by dissolving DL-homocysteine thiolactone hydrochloride (15.4 mg) in 400 μL of 2 M NaOH. The solution was allowed to stand for 5 min at rt. The solution was then neutralized by the addition of 600 μL of a saturated solution of KH$_2$PO$_4$ and immediately used in the BHMT assay.

The standard BHMT assay mixture (500 μL) used to determine percent inhibition of activity contained 0.2 μM BHMT, different concentrations of inhibitor (20 or 1 μM), 100 μM DL-homocysteine, 250 μM betaine (0.05 μCi), 10 mM β-mercaptoethanol, and 50 mM K-phosphate buffer at pH 7.5. Human recombinant BHMT was first mixed with the inhibitor(s), and then the substrates were added and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by transfer of the reaction tubes to ice water and by the addition of 2.5 mL ice-cold water. The samples were applied to a Dowex 1 × 4 (200−400 mesh) column, and the nonreacted betaine was washed from the column with water. Dimethylglycine and methionine were eluted into scintillation vials with 1.5 mL of 1.5 M HCl, and then 10 mL of the scintillation mixture were added to each vial and counted. Blanks contained all of the reaction components except the enzyme; their values were subtracted from the sample values. All samples were assayed in triplicate, and the results (reproducible within ± 15%) are expressed relative (%) to a sample containing no inhibitor.

Inhibition curves for the determination of IC$_{50}$ values were measured using the conditions described above, except that the concentrations of substrates used were 1 mM DL-homocysteine and 2 mM betaine (0.15 μCi). The inhibition at 10 different inhibitor concentrations in duplicate was determined for each curve. The data were analyzed by use of nonlinear regression fit using the GraphPad Prism 5 program. The experiment was repeated three times, and the IC$_{50}$ data in Table 1 are presented as means of three independent experiments ± SEM.

Isothermal Titration Calorimetry (ITC). The binding of inhibitor **21** to BHMT was monitored using a VP-ITC microcalorimeter (MicroCal Inc., Northampton, MA) at 25 °C. The solutions of reactants were prepared in 50 mM HEPES/NaOH, ACES/NaOH, and Tris/HCl buffers, at pH 7.5, containing 5 mM β-mercaptoethanol. The concentration of BHMT protein (calculated for a BHMT monomer with a relative molecular weight of 45 kDa) was determined by amino acid analysis after thorough dialysis against the respective buffer. The concentration of inhibitor **21** was determined based on elemental analysis. Typically, 9 μL aliquots

of 50 μM compound **21** (calculated for the S-enantiomer) were stepwise injected into the sample cell containing 1.43 mL of 5.1 μM protein until saturation was achieved. The assay was accompanied by a control experiment in which compound **21** was injected into buffer alone. The thermodynamic parameters and association constants were estimated using MicroCal Origin software.

Supporting Information Available: Synthesis of compounds **27**−**29**, **63**, and **64**. Spectral data for nontarget compounds **23a**, **23b**, **24**, **30**−**35**, **38**−**55**, and **58**−**61**. HPLC purity data for target compounds. Isothermal titration calorimetry (ITC). Molecular modeling of inhibitor **21** into the active site of BHMT. ^{1}H and ^{13}C NMR spectra measurements for pH titration of compounds **12**, **13**, and **16**. Inhibition experiments with enantiomers **63** and **64**. This material is available free of charge via the Internet at http://pubs.acs.org.

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