S-Alkylated Homocysteine Derivatives: New Inhibitors of Human Betaine-Homocysteine *S*-Methyltransferase

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A series of *S*-alkylated derivatives of homocysteine were synthesized and characterized as inhibitors of human recombinant betaine-homocysteine *S*-methyltransferase (BHMT). Some of these compounds inhibit BHMT with IC_{50} values in the nanomolar range. BHMT is very sensitive to the structure of substituents on the sulfur atom of homocysteine. The *S*-carboxybutyl and *S*-carboxypentyl derivatives make the most potent inhibitors, and an additional sulfur atom in the alkyl chain is well tolerated. The respective (*R*,*S*)-5-(3-amino-3-carboxy-propylsulfanyl)-pentanoic, (*R*,*S*)-6-(3-amino-3-carboxy-propylsulfanyl)-hexanoic, and (*R*,*S*)-2-amino-4-(2-carboxymethylsulfanyl-ethylsulfanyl)-butyric acids are very potent inhibitors and are the strongest ever reported. We determined that (*R*,*S*)-5-(3-amino-3-carboxy-propylsulfanyl)-pentanoic acid displays competitive inhibition with respect to betaine binding with a K_i^{app} of 12 nM. Some of these compounds are currently being tested in mice to study the influence of BHMT on the metabolism of sulfur amino acids in vivo.

Introduction

Betaine-homocysteine *S*-methyltransferase (BHMT, EC 2.1.1.5) is a cytosolic enzyme that catalyzes the transfer of a methyl group from betaine to L-homocysteine forming dimethylglycine and L-methionine (Met). The reaction follows an ordered bi−bi mechanism; homocysteine is the first substrate to bind, and Met is the last product off.[1] BHMT contains a zinc atom[2] that is tetrahedrally coordinated by three cysteines (Cys217, Cys299, and Cys300)[3,4] and one tyrosine (Tyr160).[5] The Zn^{2+} ion is absolutely essential for catalysis because it is required for the activation of the homocysteine thiol to the thiolate anion.[6,7] The crystal structures of BHMT indicate that it is a homotetramer.[4,5] The monomeric subunit has a molecular weight of 45 kDa, and oligomerization appears to be essential for activity.[8] The enzyme is abundant in human liver and kidney but absent from other major organs.[9]

BHMT probably has a critical role in betaine, homocysteine, methionine, and *S*-adenosylmethionine (AdoMet) homeostasis. Betaine is an intermediate of choline oxidation, and in addition to its role as a methyl donor it functions also as an organic osmolyte that is kept or released by the cell in response to osmotic changes in the kidney and liver.[10−12] It was recently shown that the expression of BHMT in liver and kidney is dramatically down-regulated in salt-loaded guinea pigs, suggesting that BHMT has a significant role modulating tissue betaine concentrations.[13]

An imbalance between homocysteine formation and catabolism can result in the elevation of plasma total homocysteine (tHcy), a condition known as hyperhomocysteinemia. The most common causes of hyperhomocysteinemia are suboptimal vitamin nutrition (folate, cobalamin, and/or vitamin B6) and/or genetic mutations that cause deficiencies of enzymes required for the synthesis of methylcobalaminn or deficiencies of methylenetetrahydrofolate reductase or cystathionine-β-synthase activities. During the last few decades many studies have shown that hyperhomocysteinemia represents a risk factor for the development of vascular diseases and thrombosis[14−16] and can result in pregnancy complications.[17,18] Homocysteine also has been reported to be neurotoxic[19] and to be associated with an increased risk for Alzheimer's disease.[20] High levels of tHcy are also found in connection with chronic renal failure.[21] Since an in vitro simulation of liver metabolism suggested that half of the conversion of homocysteine to methionine was BHMT-dependent,[22] it is certainly possible that a genetic defect that results in reduced BHMT activity could result in hyperhomocysteinemia and confer increased risk for homocysteine-related diseases.

If it is indeed true that half of the methionine produced in liver is BHMT-dependent,[22] and that the vast majority of AdoMet synthesis and utilization (perhaps ≥85%) occur in liver,[23] then it is reasonable to suggest that a dramatic reduction of BHMT activity also could result in reduced Met and AdoMet availability in liver and perhaps other organs as well. The consequences of reduced Met and AdoMet biosynthesis could be many, including a reduction in transmethylation reactions (e.g., reduced DNA methylation), but also a reduction in spermidine and spermine synthesis since the amino propyl moieties of these compounds are derived from AdoMet. Polyamines have a key role in cell growth and differentiation, and it is known that cancer cells have very high demands for AdoMet for both transmethylation reactions and polyamine synthesis.[24,25] Additionally, it is known that about half of the cysteine that is used for glutathione synthesis comes from AdoMet,[26] and since cysteine is a limiting reagent for glutathione synthesis, a significant reduction in BHMT activity could reduce tissue glutathione levels.

As discussed above, there is a lack of information regarding the physiological role BHMT has regulating betaine, homocysteine, methionine, and AdoMet metabolism. There has been no report of a human lacking BHMT activity, nor has a BHMT

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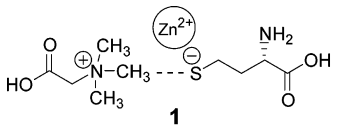
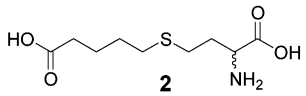
knockout mouse been generated to date. In addition, there has been no report describing the use of a BHMT inhibitor in vivo to investigate the biochemical and physiological consequences of such inhibition. To study BHMT function in vivo, it would be useful to have potent, selective, and metabolically stable inhibitors. Not only would such inhibitors be useful to determine whether a reduction in BHMT activity affects tHcy levels supporting or refuting whether a BHMT-related link to hyperhomocysteinemia exists, but they could be clinically useful as well. For example, it is possible that transiently inhibiting BHMT would reduce betaine degradation as a mechanism to restore osmotic balance during unwanted diuresis. Or, it is possible that a BHMT inhibitor could deplete the liver of methionine and AdoMet and be part of a combined strategy to treat some forms of cancer.

To date, only a few compounds have been synthesized that inhibit BHMT in vitro, and none have been tested in vivo. The first series of BHMT inhibitors were synthesized by Awad et al.[27] in 1983. These sulfur-containing compounds were designed as transition state **1** mimicking analogues. In Awad's study,[27] the most potent bisubstrate analogue (*R*,*S*)-5-(3-amino-3-carboxy-propylsulfanyl)-pentanoic acid **2** (or *S*-(4-carboxybutyl)-D,L-homocysteine, CBHcy) inhibited human liver BHMT with a K_i^{app} toward betaine of 6.5 μM. Recently, the crystal structure of human BHMT in complex with inhibitor **2** revealed[4] that the sulfur of compound **2** became the fourth ligand to the zinc atom, confirming the biochemical evidence of a homocysteine-S−Zn interaction. In 2004, using changes in intrinsic fluorescence of BHMT, we determined K_d of compound **2** toward the enzyme to be about 280 nM.[28] This high-affinity interaction was surprising considering the relatively high K_i (6.5 μM) reported by Awad et al.,[27] which will be discussed later in further detail. In 1992, a series of boronic acid based analogues of betaine was published.[29] The most potent compound, *N*,*N*,*N*-trimethylammonium-methylboronate **3**, was a competitive inhibitor at the betaine binding site of rat liver BHMT with K_i of about 45 μM. Recently, we reported[30−34] very selective affinity purification of rat BHMT using immobilized phosphinic pseudopeptide **4**. We determined K_i^{app}'s of compound **4** toward both substrates, D,L-homocysteine and betaine, to be about 10 μM, and we found that the type of inhibition toward both substrates is noncompetitive (Collinsova, M., Jiracek, J., unpublished results).



1



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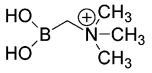

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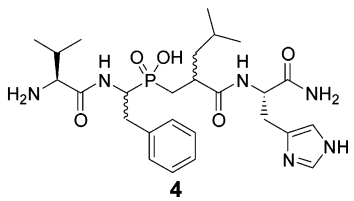


4

Results and Discussion

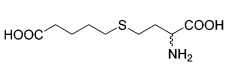
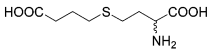
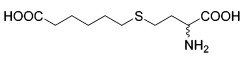
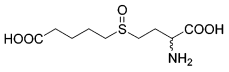
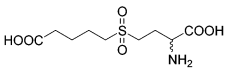
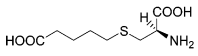
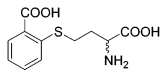
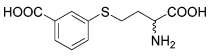
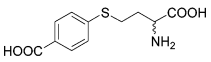
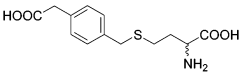
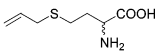
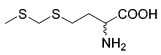
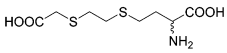
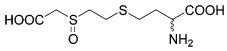
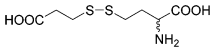
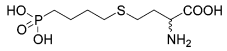
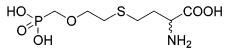
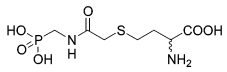
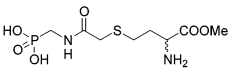
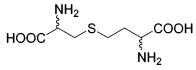
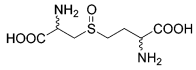
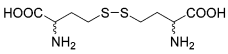
We synthesized a series of *S*-alkyl derivatives of homocysteine and one *S*-alkyl derivative of cysteine. These compounds are shown in Table 1. The syntheses were performed in aqueous solutions under alkaline conditions by (i) alkylation of unprotected D,L-homocysteine (**5**) or L-cysteine (**6**) with different alkylating agents (method A used for inhibitors **2**, **8**, **9**, **12**, **16**−**20**, **22**−**25**) and by (ii) alkylation of respective thiols with (*R*,*S*)-2-amino-4-bromo-butyrate (**7**) (method B used for inhibitors **13**−**15**). Compounds **10** and **11** were prepared by the oxidation of compound **2**. Compounds **26** and **28** (Table 1) are commercially available cystathionine and homocystine, respectively. Compound **27** was prepared by the oxidation of cystathionine. Table 2 shows the structures of the alkylating agents (**7**, **29**−**31**, and **35**−**42**) and thiols (**5**, **6**, **32**−**34**) used to produce each product and each reaction yield.

The average yields of methods A and B were around 30%. The lowest yield was 2% for compound **18**, probably due to the instability of its dithioformal moiety under acidic treatment, and the highest yield was 69% for compound **17**. The average yield was relatively low, even considering that the products were purified by RP-HPLC. To investigate the possibility that our low yields were due to the oxidation of homocysteine, we monitored the ratio of homocysteine/homocystine in a reaction mixture (method A) for 48 h using capillary electrophoresis (data not shown). We found that after 24 h the ratio was 89/11, and after 48 h it was 20/80. Since we used 3 equiv of homocysteine to 1 equiv of alkylating agent in our reactions, the consumption of the *S*-alkylating agent in aqueous alkaline media rather than the oxidation of homocysteine was responsible for the relatively low yields. In another experiment (method C), we prepared compound **2** by a reaction involving the reduction of homocystine by sodium in liquid ammonia. This procedure improved our yield of compound **2** to 76% and represents a suitable method for the preparation of our *S*-alkylated homocysteine derivatives in higher yields. However, we used methods A or B because they allowed us to rapidly prepare our target compounds in quantities sufficient for testing.

At first, we determined the percentage inhibition of BHMT using the test compounds at 20 μM. For the most potent compounds, we then determined the percentage inhibition at 1 μM and also their IC_{50} values. The percentages of inhibition were measured at relatively low concentrations of substrates, 0.25 mM betaine and 100 μM D,L-homocysteine (K_m of BHMT for betaine and D,L-homocysteine is 2 mM and 8 μM, respectively), to maximize our ability to detect inhibition. In contrast, we measured IC_{50} values of the most potent inhibitors at higher concentrations of substrates (2 mM betaine and 1 mM D,L-homocysteine) so that we could determine IC_{50} values in measurable concentrations and more accurately. The results of these inhibition experiments are summarized in Table 1.

Compound **2**, originally designed by Awad et al.,[27] inhibited BHMT very strongly ($IC_{50} \sim 0.087$ μM). This was surprising since Awad et al. reported a K_i value of only 6.5 μM. To investigate this discrepancy we decided to reevaluate the K_i^{app} of this compound, which proved to be difficult because of the low k_{cat} of the BHMT reaction and the high affinity of compound **2** for the enzyme. However, using a very low concentration of enzyme with high specific activity ^{14}C-betaine, we determined that inhibitor **2** shows competitive inhibition relative to betaine and that is has a K_i^{app} of 12 ± 0.9 nM (Figure 1). Although our estimate of K_i^{app} is much lower than the value reported by Awad et al.,[27] it is in better agreement with the K_d of the BHMT−compound **2** complex recently measured using intrinsic

Table 1. Inhibition (Relative) of Human BHMT by S-Substituted Derivatives of Homocysteine[a]

Compound	% of Inhibition[b] (0.25 mM betaine, 0.1 mM D,L-homocysteine) 20 μM	1 μM	IC_{50} (μM)[c] (2 mM betaine, 1 mM D,L-homocysteine)
2 	100	98.3	0.087
8 	20.1	nd	nd
9 	100	99.9	0.2
10 	97.3	67.8	5
11 	28.8	nd	nd
12 	0	nd	nd
13 	42.5	nd	nd
14 	12.8	nd	nd
15 	98.6	84.8	7
16 	10.1	nd	nd
17 	0	nd	nd
18 	20.5	nd	nd
19 	100	96.8	0.096
20 	89.8	55.3	nd
21 	30	0	nd
22 	97.8	74	5.7
23 	25.5	nd	nd
24 	8.8	nd	nd
25 	0	nd	nd
26 	18.8	nd	nd
27 	9.5	nd	nd
28 	0	nd	nd

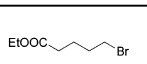
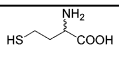
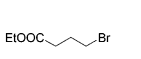
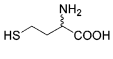
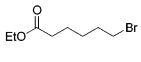
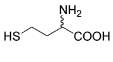
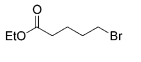
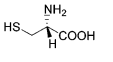
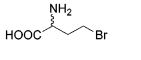
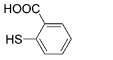
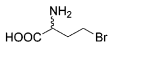
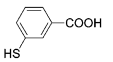
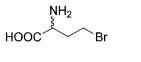
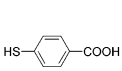
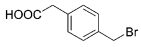
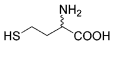
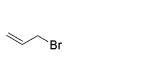
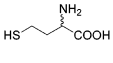
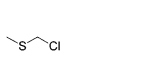
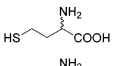
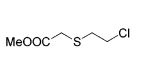
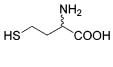
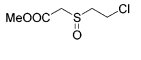
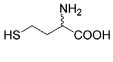
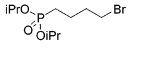
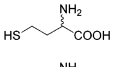
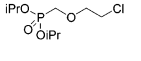
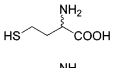
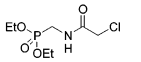
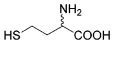
[a] The percentage inhibition of each compound was determined at 20 and 1 μM. See the Experimental Section for details. [b] All assays were done in triplicate, and the data obtained were reproducible within ±15%. [c] All data points for IC_{50} values were derived from assays performed in duplicate, and the values obtained from three different assays were reproducible within ±10%; nd means not determined.

fluorescence.[28] Why is compound **2** in our hands a much more potent inhibitor of BHMT than previously published by Awad et al.?[27] The discrepancy could be that our compound was more pure. We purified compound **2** by RP-HPLC, whereas they used a combination of DEAE−cellulose and Sephadex G-10 chromatography. The NMR and mass spectrometry data are not available. Therefore, it cannot be excluded that compound **2**, as prepared by Awad et al., was not pure despite analyses by TLC, electrophoresis, and amino acid analyzer. In addition, we used recombinant enzyme, whereas Awad et al. used enzyme isolated from human liver, and so it is possible that there are unknown differences in the kinetic properties of these enzymes.

The IC_{50} value (87 nM) obtained for compound **2** is less than half of the concentration of BHMT (200 nM) used in the assays. There is no evidence to suggest that this discrepancy can be explained by an allosteric interaction of the inhibitor with the enzyme. It is possible that the amount of active enzyme used in these reactions were overestimated since the Bradford[35] procedure cannot discern active from inactive protein. The loss of Zn^{2+} or enzyme denaturation could be factors that were not corrected for. Additionally, although it is accepted that BHMT is a tetramer composed of identical subunits best described as a dimer of dimers, it is not known whether a tetramer can catalyze four reactions simultaneously. Although kinetically there is no evidence of subunit interaction, it has been shown that residues from both monomers within a dimer pair are required to form an active site[8] and that some structural elements of one monomer undergo movement when the active site of its partner becomes occupied with ligand(s).[28] Hence, it is possible that only one of the two active sites that make up a dimer can be active at any given instant.

Compounds **8** and **9** are analogues of inhibitor **2** with shorter and longer alkyl chains, respectively. Compound **8** has been already prepared by Awad et al.,[27] and we confirmed that the shortening of carboxybutyl chain results in a drastic loss of affinity toward BHMT. On the other hand, compound **9** with a carboxypentyl chain is still an excellent inhibitor of BHMT with the IC_{50} value only slightly weaker that that of compound **2**. Compounds **10** and **11** are oxidation products of inhibitor **2**. Both these compounds are much weaker inhibitors than the parent compound **2**, decreasing in potency with increasing degree of oxidation at the sulfur atom. However, it is interesting

Table 2. Structures of Alkylating Agents and Thiols Used for the Synthesis of BHMT Inhibitors[a]

	Alkylating agent		Thiol	Reaction Product	Yield (%)
29		**5**		**2**	31[b], 76[d]
30		**5**		**8**	20[b]
31		**5**		**9**	27[b]
29		**6**		**12**	36[b]
7		**32**		**13**	36[c]
7		**33**		**14**	28[c]
7		**34**		**15**	19[c]
35		**5**		**16**	15[b]
36		**5**		**17**	69[b]
37		**5**		**18**	2[b]
38		**5**		**19**	53[b]
39		**5**		**20**	21[b]
40		**5**		**22**	46[b]
41		**5**		**23**	8[b]
42		**5**		**24**	52[b]

[a] The yields are reported after HPLC purification. For details see the Experimental Section. The structures of products after deprotection are shown in Table 1. [b] Product was prepared according to method A. [c] Product was prepared according to method B. [d] Product was prepared according to method C.

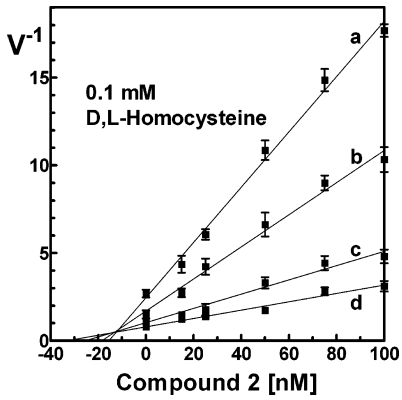


Figure 1. Determination of K_i^{app} of inhibitor **2** for human BHMT toward betaine. The curves were measured at a fixed concentration of D,L-homocysteine (100 μM) and four different concentrations of betaine: (a) 0.5 mM, (b) 1 mM, (c) 2 mM, and (d) 4 mM. The intersection point of the curves gives K_i^{app} of about 12.0 $\pm$ 0.9 nM. For details see the Experimental Section.

that sulfoxide **10** is still much better tolerated than the shortened compound **8**. We also synthesized compound **12**, which is the "cysteine" analogue of inhibitor **2**. The absolute lack of inhibition of BHMT by this compound, even at 20 μM concentration, underlines the selectivity of BHMT for homocysteine.

The crystal structure of BHMT in a complex with inhibitor **2** revealed that the carboxybutyl chain of inhibitor **2** is surrounded by a series of aromatic residues.[4] Therefore, we investigated compounds **13**−**15**, which have a 2-, 3-, or 4-carboxyphenyl group instead of the carboxybutyl moiety of inhibitor **2**. Only derivative **15** with the 4-carboxyphenyl group replacing the carboxybutyl group in **2** is a relatively potent BHMT inhibitor with an IC_{50} about 7 μM. Inhibitor **16**, with two extra methylene groups located both between the carboxyl group and phenyl ring and the sulfur atom and phenyl ring, exhibited a marginal affinity.

Compound **17**, which has the allyl group, is inactive. However, the inhibitor **18** of the same length as compound **17** but having dithioformal moiety in the chain still retains some activity. We hypothesize that the second sulfur atom of compound **18** could also interact with the Zn^{2+} of BHMT and that the analogue of **2**, *S*-(2-carboxyethylthiomethyl)homocysteine, containing a dithioformal moiety, could be a very potent inhibitor of BHMT. Unfortunately, our attempts to prepare this inhibitor failed, possibly due to the low stability of the S−CH_2−S moiety under acidic conditions. This hypothesis seems to be supported by the potency we observed for compound **19**, which is of the same length as compound **2** and includes the terminal carboxylate but contains the S−CH_2−CH_2−S moiety. Inhibitor **19** has about the same high affinity for BHMT as the "reference" compound **2**. Sulfoxide **20** is a much weaker inhibitor of BHMT. Compound **21** is the mixed disulfide of D,L-homocysteine and mercaptopropionic acid. This compound is slightly more active than inhibitor **8**, which has the same length.

According to Evans et al.,[4] the carboxyl group of the carboxybutyl moiety of inhibitor **2** forms two hydrogen bonds with side chains of Tyr77 and Trp44 of BHMT. We replaced the carboxyl by the more acidic phosphonate moiety and introduced two different structural alterations into the butyl chain. Of the phosphonate analogues **22**−**24**, only compound **22** retains significant affinity to BHMT, but it remains much weaker than compound **2**. Introduction of the oxygen atom or amide bond (analogues **23** and **24**) into the butyl chain of respective phosphonate analogues results only in further decrease of binding affinity. Compound **25** is the methyl ester of analogue **24**, and the lack of affinity confirms the crucial importance of free carboxyl of the homocysteine moiety of these inhibitors.

Derivatives **26**−**28** are naturally occurring compounds: cystathionine, its sulfoxide, and homocystine, respectively. All these compounds contain a homocysteine moiety but differ in substituents on the sulfur atom. Since these compounds participate in the metabolism of sulfur amino acids and could influence activity of BHMT in vivo, we decided to test them as inhibitors of BHMT. However, none of these compounds inhibit BHMT to any significant degree. We believe that cystathionine and homocystine are not inhibitors of BHMT in vivo.

Conclusions

We synthesized a series of S-substituted derivatives of homocysteine and evaluated them as potential inhibitors of human recombinant BHMT. Some of these compounds are very potent inhibitors, having IC_{50} values in the nanomolar range. We found that compound **2**, (*R*,*S*)-5-(3-amino-3-carboxy-propylsulfanyl)-pentanoic acid, is a much more potent inhibitor of BHMT than previously reported. We determined its K_i^{app} toward betaine to be about 12 nM. We found that BHMT is very sensitive to any modification in the structure of inhibitor

2 since most analogues were less active than this parent compound. Nevertheless, we found that elongating the alkyl chain by one methylene group leads to the very potent inhibitor **9** and that an additional sulfur atom in the otherwise alkyl chain is well tolerated (inhibitor **19**). Compounds **9** and **19** are of similar potency toward BHMT as inhibitor **2**. These compounds are the most potent inhibitors of BHMT prepared to date. All these compounds were prepared as mixtures of enantiomers. Evans et al.[4] found that only the *S*-enantiomer binds to the active site of BHMT. We assume that only *S*-enantiomers of our compounds inhibit BHMT and that respective IC_{50}'s or K_i^{app}'s will be lower than reported in this study. Compound **2** is also a very selective inhibitor of BHMT because it does not inhibit other enzymes involved in sulfur metabolism, such as methionine synthase, cystathionine-β-synthase, and cystathionase (data not shown). Our inhibitors are currently being tested in vivo in mice to study the influence of BHMT on the metabolism of sulfur amino acids (Collinsova, Strakova, Jiracek, and Garrow, *J. Nutr.* **2006**, in press).

Experimental Section

Chemistry. General. Unless otherwise stated, materials were obtained from commercial suppliers (Sigma-Aldrich, Fluka, Merck) and used without purification. 4-Mercaprobenzoic acid (**34**) was purchased from TCI America (Portland, OR). Compound **26**, (*R*,*S*,*R*,*S*)-2-amino-4-(2-amino-2-carboxy-ethylsulfanyl)-butyric acid (cystathionine) was purchased from Sigma-Aldrich. Compound **28**, (*R*,*S*,*R*,*S*)-2-amino-4-(3-amino-3-carboxy-propyldisulfanyl)-butyric acid (D,L-homocystine) was purchased from Fluka. Column chromatography was performed on silica gel 60 (70−230 mesh). Preparative RP-HPLC was performed using Vydac (218TP510, 25 cm × 1 cm; Columbia, MD) or Phenomenex (Luna C-18, 5μm, 25 cm × 2.12 cm, Torrance, CA) columns. Analytical RP-HPLC was performed using a Watrex (Nucleosil 120, 5μm, C18, 25 cm × 0.46 cm; Prague, Czech Republic) column. For gradient RP-HPLC 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. Anion-exchange analytical HPLC was performed using an AS11-HC column (0.2 cm × 25 cm, Dionex Corporation, Sunnyvale, CA) with a BioLC system (GP50 gradient pump, ED50 electrochemical detector) from Dionex Corporation (Sunnyvale, CA). Mass spectroscopy was performed using a ZAB-EQ spectrometer with BEQQ geometry (VG Analytical; Manchester, U.K.). NMR spectra were recorded on Bruker AVANCE-500 and Varian UNITY-500 (^{1}H at 500 MHz; ^{13}C at 125.7 MHz) in $CDCl_3$, DMSO-d_6, or D_2O solutions. Chemical shifts are given in ppm (referenced to tetramethylsilane) and coupling constants in Hz.

Method A for the Preparation of *S*-Alkylated Derivatives of Homocysteine (Used for Compounds 2, 8, 9, 12, 16−20, 22−25). The respective halogenated agent (1 mmol; compounds **29**−**31**, **35**−**39**, and **40**−**42** shown in Table 2) was added to D,L-homocysteine (**5**) or L-cysteine (**6**) (3 mmol) in 10% sodium carbonate in 50% aqueous ethanol (6 mL) and stirred under argon at room temperature. After 48 h, if needed, sodium hydroxide was added to 1 M concentration. After 1 h the reaction mixture was applied to Dowex 50W (H^+), the resin was washed with water, and the compound was eluted with 2.5% ammonia. After evaporation, the product was purified by RP-HPLC.

Method B for the Preparation of *S*-Alkylated Derivatives of Homocysteine (Used for Compounds 13−15). The respective mercapto-benzoic acid (1 mmol; compounds **32**−**34** shown in Table 2) was dissolved in 50% aqueous ethanol (10 mL) containing sodium carbonate (528 mg, 5 mmol). Then, the hydrobromide of compound **7** (210 mg, 0.8 mmol) was added. The reaction was stirred under argon at room temperature overnight. The reaction mixture was applied to Dowex 50W (H^+), the resin was washed with water, and the compound was eluted with 2.5% ammonia. After evaporation, the product was purified by RP-HPLC.

(*R*,*S*)-5-(3-Amino-3-carboxy-propylsulfanyl)-pentanoic acid (2) was first prepared according to method A using ethyl-5-bromo-pentanoate (**29**; 1.2 mmol, 251 mg). The yield was 88 mg (31%). ^{1}H NMR (DMSO): δ 1.55 (m, 4H), 1.80 (m, 1H), 1.96 (m, 1H), 2.22 (t, J = 7.0 Hz, 2H), 2.48 (t, J = 7.0 Hz, 1H), 2.56 (m, 2H), 3.27 (dd, J = 7.2 and 5.6 Hz, 1H). ^{13}C NMR (DMSO): δ 23.87, 27.42, 28.69, 30.47, 31.45, 33.53, 53.28, 174.64, 174.65. HR-MS (FAB) calculated for $C_9H_{18}NO_4S$ (MH^+) 236.0957, found 236.0961.

Modified Procedure Method C. Compound **2** was also prepared according to the modified procedure (method C) as follows. D,L-Homocystine (1 mmol, 0.236 g) was dissolved in liquid ammonia (about 30 mL) in a cooled flask, and sodium (4.3 mmol, 0.1 g) was slowly added in small pieces until the reaction mixture turned blue. Ethyl-5-bromo-pentanoate acid (**29**; 2.2 mmol, 0.46 g) was then added, and the reaction proceeded without cooling until the ammonia was completely evaporated. The dry residue was dissolved in 30 mL of water, and sodium hydroxide was added to 1 M (final). After 1 h, the reaction mixture was applied to Dowex 50W (H^+), the resin was washed with water, and the compound was eluted with 2.5% ammonia. After evaporation, the product was purified by RP-HPLC. The yield was 0.358 g (76%). The quality of the product was verified with MS and NMR.

Hydrobromide of (*R*,*S*)-2-Amino-4-bromo-butyric Acid (7). The title compound was prepared according to Farrington at al.[36] with several modifications. (*R*,*S*)-2-Amino-4-butyrolactone hydrobromide (1 g, 5.5 mmol) was heated to 60−65 °C for 48 h in a sealed tube with 20 mL of hydrobromic acid (33%) in acetic acid. The reaction mixture was evaporated to dryness, and the residue was treated with diethyl ether. White crystals of the hydrobromide of (*R*,*S*)-2-amino-4-bromo-butyric acid (**7**) were washed with diethyl ether. The yield was 1.42 g (99%). ^{1}H NMR (D_2O): δ 2.43 (m, J = 15.2, 7.3, 7.2, 6.1 Hz, 1H), 2.59 (m, J = 15.2, 7.3, 7.2, 6.1 Hz, 1H), 3.62 (ddd, J = 10.8, 7.2, 6.1 Hz, 1H), 3.67 (ddd, J = 10.8, 7.2, 6.1 Hz, 1H), 4.26 (t, J = 7.3, 7.3 Hz, 1H). ^{13}C NMR (D_2O): δ 29.92, 34.68, 53.44, 173.30. MS (FAB) calculated for $C_4H_9NO_2Br$ (MH^+) 183.9796 and 181.9817, found 183.9796 and 181.9818.

(*R*,*S*)-2-Amino-4-(3-carboxy-propylsulfanyl)-butyric Acid (8). The title compound was prepared from ethyl-4-bromo-butyrate (**30**; 195 mg, 1 mmol) according to method A. The yield was 49 mg (20%). ^{1}H NMR (D_2O): δ 1.90 (p, J = 7.5 Hz, 2H), 2.16 (m, J = 15.0, 7.2, 7.2 and 6.8 Hz, 1H), 2.26 (m, J = 15.0, 7.2, 7.2 and 5.9 Hz, 1H), 2.50 (t, J = 7.5 Hz, 2H), 2.63 (t, J = 7.5 Hz, 2H), 2.71 (t, J = 7.5 Hz, 2H), 4.13 (dd, J = 6.8 and 5.9 Hz, 1H). ^{13}C NMR (D_2O): δ 26.80, 28.94, 32.37, 32.66, 35.92, 55.03, 175.07, 180.90. HR-MS (FAB) calculated for $C_8H_{16}NO_4S$ (MH^+) 222.0800, found 222.0806.

(*R*,*S*)-6-(3-Amino-3-carboxy-propylsulfanyl)-hexanoic Acid (9). The title compound was prepared from ethyl-6-bromo-hexanoate (**31**; 223 mg, 1 mmol) according to method A. The yield was 68 mg (27%). ^{1}H NMR (DMSO): δ 1.34 (m, 2H), 1.51 (m, 4H), 1.90 (m, 1H), 2.00 (m, 1H), 2.20 (t, J = 7.4 Hz, 2H), 2.47 (t, J = 7.3 Hz, 2H), 2.57 (ddd, J = 13.5, 9.6 and 5.6 Hz, 1H), 2.60 (ddd, J = 13.5, 9.5 and 6.5 Hz, 1H), 3.63 (dd, J = 6.5 and 5.6 Hz, 1H). ^{13}C NMR (DMSO): δ 24.27, 26.91, 27.92, 28.90, 30.67, 31.01, 33.85, 52.34, 170.58, 174.66. HR-MS (FAB) calculated for $C_{10}H_{20}NO_4S$ (MH^+) 250.1035, found 250.1059.

(*R*,*S*)-5-(3-Amino-3-carboxy-propylsulfinyl)-pentanoic Acid (10). Compound **2** (10 mg, 42 μmoles) was suspended in 100 μL of water containing 46 μmoles of hydrochloric acid and 50 μmoles of hydrogen peroxide. The suspension was stirred at room temperature overnight. The product was purified by RP-HPLC. The yield was 9 mg (85%). ^{1}H NMR (D_2O): δ 1.70−1.80 (m, 4H), 2.25−2.37 (m, 4H), 2.90−3.15 (m, 4H). ^{13}C NMR (D_2O): mixture of diastereoisomers (1:1) leads to doubling of some carbon signals (shown in brackets), δ 23.94 (23.96), 26.10 (26.15), 26.79, 38.65, 48.38 (48.40), 52.44 (52.52), 55.56 (55.74), 175.40, 184.65. HR-MS (FAB) calculated for $C_9H_{18}NO_5S$ (MH^+) 252.0906, found 252.0895.

(*R*,*S*)-5-(3-Amino-3-carboxy-propylsulfonyl)-pentanoic Acid (11). Compound **2** (10 mg, 42 μmoles) was suspended in 100 μL of water containing 450 μmoles of hydrochloric acid and 200 μmoles of hydrogen peroxide. The suspension was stirred at room temperature overnight. The product was purified by RP-HPLC. The yield was 7.3 mg (65%). ^{1}H NMR (D_2O): δ 1.77 (m, 2H), 1.87

(m, 2H), 2.37 (m, 2H), 2.45 (t, J = 7.3 Hz, 2H), 3.32 (m, 2H), 3.36 (ddd, J = 14.0, 9.8 and 6.2 Hz, 1H), 3.44 (ddd, J = 14.0, 9.6 and 6.6 Hz, 1H), 3.89 (t, J = 6.2 Hz, 1H). ^{13}C NMR (D_2O): δ 22.27, 24.49, 24.80, 35.00, 49.94, 53.44, 54.65, 174.33, 179.86. HR-MS (FAB) calculated for $C_9H_{18}NO_6S$ (MH^+) 268.0840, found 268.0855.

(*R*)-5-(2-Amino-2-carboxy-ethylsulfanyl)-pentanoic Acid (12). The title compound was prepared from ethyl-6-bromo-pentanoate (**29**; 209 mg, 1 mmol) according to method A. The yield was 80 mg (36%). ^{1}H NMR (D_2O + NaOD): δ 1.58 (m, 2H), 1.62 (m, 2H), 2.19 (t, J = 7.2 Hz, 2H), 2.59 (t, J = 7.1 Hz, 2H), 2.76 (dd, J = 13.4 and 6.8 Hz, 1H), 2.85 (dd, J = 13.4 and 5.2 Hz, 1H), 3.40 (dd, J = 6.8 and 5.2 Hz, 1H). ^{13}C NMR (D_2O + NaOD): δ 27.85, 31.54, 34.14, 39.69, 39.86, 57.95, 183.91, 186.38. HR-MS (FAB) calculated for $C_8H_{16}NO_4S$ (MH^+) 222.0800, found 222.0804.

(*R*,*S*)-2-(3-Amino-3-carboxy-propylsulfanyl)-benzoic Acid (13). The title compound was prepared from 2-mercapto-benzoic acid (**32**; 106 mg, 0.69 mmol) according to the method B. The yield was 52 mg (36%). ^{1}H NMR (DMSO): δ 2.09 (m, 2H), 3.02 (ddd, J = 13.0, 9.3 and 5.8 Hz, 1H), 3.08 (ddd, J = 13.0, 9.6 and 6.4 Hz, 1H), 4.00 (t, J = 6.2 Hz, 2H), 7.24 (ddd, J = 7.8, 7.5 and 0.9 Hz, 1H), 7.40 (bd, J = 7.8 Hz, 1H), 7.54 (ddd, J = 7.8, 7.5 and 1.7 Hz, 1H), 7.89 (dd, J = 7.8 and 1.7 Hz, 1H), 8.30 (b, 2H), 13.20 (vb, 1H). ^{13}C NMR (DMSO): δ 26.64, 29.28, 51.74, 124.38, 125.55, 128.67, 131.26, 132.58, 139.86, 167.62, 170.69. HR-MS (FAB) calculated for $C_{11}H_{14}NO_4S$ (MH^+) 256.0644, found 256.0649.

(*R*,*S*)-3-(3-Amino-3-carboxy-propylsulfanyl)-benzoic Acid (14). The title compound was prepared from 3-mercapto-benzoic acid (**33**; 120 mg, 0.78 mmol) according to the method B. The yield was 46 mg (28%). ^{1}H NMR (DMSO): δ 2.04 (m, 2H), 3.14 (m, 2H), 3.93 (t, J = 6.4 Hz, 1H), 7.47 (t, J = 7.8 Hz, 1H), 7.59 (ddd, J = 7.8, 2.1 and 1.1 Hz, 1H), 7.77 (ddd, J = 7.8, 1.6 and 1.1 Hz, 1H), 7.85 (dd, J = 2.1 and 1.6 Hz, 1H); 8.30 (b, 2H), 13.20 (vb, 1H). ^{13}C NMR (DMSO): δ 28.06, 29.91, 51.57, 127.04, 128.91, 129.62, 131.96, 132.53, 136.23, 166.96, 170.56. HR-MS (FAB) calculated for $C_{11}H_{14}NO_4S$ (MH^+) 256.0644, found 256.0651.

(*R*,*S*)-4-(3-Amino-3-carboxy-propylsulfanyl)-benzoic Acid (15). The title compound was prepared from 4-mercapto-benzoic acid (**34**; 185 mg, 1.2 mmol) according to the method B. The yield was 47 mg (19%). ^{1}H NMR (DMSO + AcOD): δ 1.99 (m, 1H), 2.07 (m, 1H), 3.14 (ddd, J = 13.5, 9.7 and 5.7 Hz, 1H), 3.20 (ddd, J = 13.5, 9.7 and 6.0 Hz, 1H), 3.61 (t, J = 6.1 Hz, 1H), 7.39 (m, 2H), 7.85 (m, 2H). ^{13}C NMR (DMSO + AcOD): δ 27.17, 30.40, 52.62, 126.42 (2C), 127.59, 130.14 (2C), 142.83, 167.18, 170.52. HR-MS (FAB) calculated for $C_{11}H_{14}NO_4S$ (MH^+) 256.0644, found 256.0650.

(*R*,*S*)-2-Amino-4-(4-carboxymethyl-benzylsulfanyl)-butyric Acid (16). The title compound was prepared from (4-bromomethyl-phenyl)-acetic acid (**35**; 229 mg, 1 mmol) according to method A. The yield was 43 mg (15%). ^{1}H NMR (DMSO + D_2O): δ 1.93 (m, 1H), 2.03 (m, 1H), 2.48 (m, 2H), 3.52 (s, 2H), 3.58 (t, J = 6.0 Hz, 1H), 3.68 (s, 2H), 7.17 (m, 2H), 7.23 (m, 2H). ^{13}C NMR (DMSO + D_2O): δ 27.16, 30.80, 34.66, 40.51, 52.66, 128.77 (2C), 129.48 (2C), 133.68, 136.92, 170.93, 173.56. HR-MS (FAB) calculated for $C_{13}H_{18}NO_4S$ (MH^+) 284.0878, found 284.0899.

(*R*,*S*)-4-Allylsulfanyl-2-amino-butyric Acid (17). The title compound was prepared from 3-bromo-propene (**36**; 120 mg, 1 mmol) according to method A. The yield was 121 mg (69%). ^{1}H NMR (DMSO): δ 1.99 (m, 2H), 2.54 (m, 2H), 3.15 (ddd, J = 7.2, 1.3 and 0.9 Hz, 2H), 3.86 (t, J = 6.3 Hz, 1H), 5.08 (m, J = 10.0, 1.8, 0.9 and 0.9 Hz, 1H), 5.13 (m, J = 17.1, 1.8, 1.3 and 1.3 Hz, 1H), 5.76 (m, J = 17.1, 10.0, 7.2 and 7.2 Hz, 1H), 8.27 (bs, 1H). ^{13}C NMR (DMSO): δ 25.63, 30.27, 33.49, 51.59, 117.46, 134.51, 170.84. HR-MS (FAB) calculated for $C_7H_{14}NO_2S$ (MH^+) 176.0745, found 176.0749.

(*R*,*S*)-2-Amino-4-methylsulfanylmethylsulfanyl-butyric Acid (18). The title compound was prepared from chloro-methylsulfanyl-methane (**37**; 96 mg, 1 mmol) according to method A. The yield was 4 mg (2%). ^{1}H NMR (DMSO): δ 1.85 (m, J = 14.4, 7.8, 7.8 and 7.0 Hz, 1H), 2.01 (m, J = 14.4, 7.8, 7.8 and 5.4 Hz, 1H), 2.10 (s, 3H), 2.68 (t, J = 7.8 Hz, 2H), 3.36 (m, 1H), 3.73 (s, 2H), 7.86 (vb, 2H). ^{13}C NMR (DMSO): δ 14.19, 26.89, 30.93, 36.68, 52.99, 169.75, HR-MS (FAB) calculated for $C_6H_{14}NO_2S_2$ (MH^+) 196.0466, found 196.0429.

(*R*,*S*)-2-Amino-4-(2-carboxymethylsulfanyl-ethylsulfanyl)-butyric Acid (19). Methyl-mercapto-acetate (1.485 mg, 14 mmol) was added dropwise, on ice and under argon atmosphere, to triethylamine (1.413 g, 14 mmol) in dichloroethane (15 mL). After 18 h at room temperature, the reaction mixture was evaporated and the product, methyl-(2-chloro-ethylsulfanyl)-acetate (**38**), was purified on a column of silica gel using a linear gradient of ethyl acetate in toluene (0−5%) with the yield of 1.1 g (47%). The quality of the product was verified with MS and NMR. The title compound (**19**) was prepared from methyl (2-chloro-ethylsulfanyl)-acetate (**38**, prepared as described above; 150 mg, 0.89 mmol) according to method A. The yield was 120 mg (53%). ^{1}H NMR (DMSO): δ 1.89 (m, 1H), 2.01 (m, 1H), 2.60 (m, 2H), 2.70−2.80 (m, 4H), 3.25 (d, J = 14.5 Hz, 1H), 3.28 (d, J = 14.5 Hz, 1H), 3.58 (m, 1H). ^{13}C NMR (DMSO): δ 26.94, 30.55, 31.12, 32.11, 33.65, 52.65, 170.74, 172.14. HR-MS (FAB) calculated for $C_8H_{16}NO_4S_2$ (MH^+) 254.0521, found 254.0533.

(*R*,*S*)-2-Amino-4-(2-carboxymethylsulfinyl-ethylsulfanyl)-butyric Acid (20). Methyl-(2-chloro-ethylsulfanyl)-acetate (**38**, prepared as described above; 0.336 g, 2 mmol) was oxidized with hydrochloric acid and hydrogen peroxide as described for compound **10**: yield 0.206 g (56%). The resulting (2-chloro-ethanesulfinyl)-acetic acid methyl ester (**39**; 0.17 g, 0.92 mmol) was reacted with D,L-homocysteine according to method A. The yield was 53 mg (21%). ^{1}H NMR (D_2O): δ 2.21 (m, 1H), 2.30 (m, 1H), 2.80 (m, 2H), 2.98 (m, 1H), 3.08 (m, 1H), 3.30 (m, 2H), 3.92 (d, J = 15.0 Hz, 1H), 4.04 (d, J = 15.0 Hz, 1H), 4.14 (t, J = 6.4 Hz, 1H). ^{13}C NMR (D_2O): mixture of diastereoisomers (1:1) leads to doubling of some carbon signals (shown in brackets), δ 25.83 (25.91), 28.71 (28.82), 31.72 (31.75), 52.67 (52.70), 54.40 (54.42), 57.38, 171.60, 174.54. HR-MS (FAB) calculated for $C_8H_{16}NO_5S_2$ (MH^+) 270.0470, found 270.0466.

(*R*,*S*)-2-Amino-4-(2-carboxy-ethyldisulfanyl)-butyric Acid (21). D,L-Homocysteine (**5**; 202 mg, 1.5 mmol) and methyl-mercapto-acetate (178 mg, 1.5 mmol) were treated with hydrogen peroxide (169 μL, 1.65 mmol) dissolved in 20% aqueous ethanol (12 mL) at room temperature overnight. The reaction mixture was applied to Dowex 50W (H^+), the resin was washed with water, and the products was eluted with 2.5% ammonia. After evaporation, the product was purified with RP-HPLC. The yield was 42 mg (11%). ^{1}H NMR (D_2O): δ 2.32 (m, J = 14.8, 7.5, 7.0 and 6.9 Hz, 1H), 2.40 (m, J = 14.8, 7.5, 7.5 and 6.0 Hz, 1H), 2.83 (m, 2H), 2.87 (m, 2H), 2.98 (m, 2H), 4.16 (dd, J = 6.9 and 6.0 Hz, 1H). ^{13}C NMR (D_2O): δ 31.99, 35.13, 35.26, 36.29, 54.59, 174.74, 179.28. HR-MS (FAB) calculated for $C_7H_{14}NO_4S_2$ (MH^+) 240.0364, found 240.0370.

(*R*,*S*)-2-Amino-4-(4-phosphono-butylsulfanyl)-butyric Acid (22). The reaction of diisopropyl-(4-bromo-butyl)-phosphonate[37] (**40**; 0.3 g, 1 mmol) with D,L-homocysteine (**5**) according to method A afforded (*R*,*S*)-2-amino-4-[4-(diisopropoxy-phosphoryl)-butyl-sulfanyl]-butyrate in the yield of 0.164 g (46%). ^{1}H NMR (DMSO): δ 1.227 (d, J = 6.2 Hz, 6H), 1.230 (d, J = 6.2 Hz, 6H), 1.54 (m, 2H), 1.59 (m, 2H), 1.67 (m, 2H), 2.01 (m, 2H), 2.51 (t, J = 7.0 Hz, 2H), 2.55 (ddd, J = 13.5, 9.1 and 6.0 Hz, 1H), 2.62 (ddd, J = 13.5, 9.2 and 6.5 Hz, 1H), 3.99 (bt, $J \sim$ 6 Hz, 1H), 4.53 (dh, ^3J(H, P) = 8.0, J(H, H) = 6.2 Hz, 1H), 4.54 (h, J = 6.2 Hz, 1H), 8.28 (b, 2H). ^{13}C NMR (DMSO): δ 21.53 (d, ^3J(C, P) = 4.9 Hz), 23.98 (d, ^3J(C, P) = 4.4 Hz, 4C), 25.57 (d, ^1J(C, P) = 140.6 Hz), 26.40, 29.52, 30.19, 30.33, 51.24, 69.22 (d, ^2J(C, P) = 6.3 Hz), 170.90. HR-MS (FAB) calculated for $C_{14}H_{31}NO_5PS$ (MH^+) 356.1661, found 356.1651. This compound (0.156 g, 0.44 mmol) was treated with bromotrimethylsilane (0.456 g, 3 mmol) in dry DMF (3 mL) under argon atmosphere at 50 °C for 2 h. Then, 5 mL of methanol was added, and the reaction was warmed at 50 °C for an additional hour. The reaction mixture was evaporated, and the residue was partitioned between water and ethyl acetate. The water layer was evaporated to dryness, and the product, compound **22**, was purified by RP-HPLC. The yield was 76 mg (64%). ^{1}H NMR (D_2O): δ 1.61−1.89 (m, 6H), 2.18 (m, 1H), 2.28 (m, 1H), 2.62 (t, J = 7.0 Hz, 2H), 2.72 (t, J = 7.5 Hz, 2H), 4.16 (dd, J =

6.7 and 6.0 Hz, 1H). ^{13}C NMR (D_2O): δ 23.63 (d, ^3J(C, P) = 4.7 Hz), 28.21 (d, ^1J(C, P) = 134.2 Hz), 28.47, 31.59 (d, ^2J(C, P) = 16.6 Hz), 31.75, 32.31, 54.29, 174.30. HR-MS (FAB) calculated for $C_8H_{19}NO_5PS$ (MH^+) 272.0722, found 272.0734.

(*R*,*S*)-2-Amino-4-(2-phosphonomethoxy-ethylsulfanyl)-butyrate (23). The reaction of diisopropyl-(2-chloro-ethoxymethyl)-phosphonate[38] (**41**; 0.259 g, 1 mmol) with D,L-homocysteine (**5**) according to method A afforded (*R*,*S*)-2-amino-4-[2-(diisopropoxy-phosphorylmethoxy)-ethylsulfanyl]-butyrate in the yield of 21 mg (8%). ^{1}H NMR (DMSO): δ 1.97 (m, 1H), 2.04 (m, 1H), 2.64 (m, 2H), 2.68 (t, J = 6.6 Hz, 2H), 3.66 (t, J = 6.6 Hz, 2H), 3.76 (d, J(H, P) = 8.4 Hz, 2H), 3.92 (dd, J = 6.6 and 5.9 Hz, 1H), 4.60 (dh, J(H, P) = 7.7 and J(H, H) = 6.2 Hz, 2H), 8.22 (vb, 2H). ^{13}C NMR (DMSO): δ 23.93 (d, ^3J(C, P) = 4.5 Hz, 2C), 24.03 (d, ^3J(C, P) = 3.8 Hz, 2C), 27.04, 30.06, 30.52, 51.35, 64.77 (d, ^1J(C, P) = 164.9 Hz), 70.39 (d, ^2J(C, P) = 6.3 Hz), 72.08 (d, ^3J(C, P) = 12.1 Hz), 170.85. HR-MS (FAB) calculated for $C_{13}H_{29}NO_6PS$ (MH^+) 358.1453, found 358.1434. This compound (20 mg, 56 μmoles) was treated with bromotrimethylsilane (0.106 g, 0.7 mmol) in dry DMF (1 mL) under argon atmosphere at room temperature overnight. The reaction mixture was concentrated in vacuo, and the residue was coevaporated with 10% triethylamine in acetonitrile (3 × 1 mL) and then with water. The product **23** was purified by RP-HPLC. The yield was 15 mg (31%). ^{1}H NMR (D_2O): δ 2.21 (m, 1H), 2.30 (m, 1H), 2.78 (t, J = 7.5 Hz, 2H); 2.82 (t, J = 6.1 Hz, 2H), 3.70 (d, J(H, P) = 8.8 Hz, 2H), 3.79 (t, J = 6.0 Hz, 2H), 4.16 (dd, J = 6.6 and 6.0 Hz, 1H). ^{13}C NMR (D_2O): δ 27.60, 30.43, 31.03, 52.92, 67.08 (d, ^1J(C, P) = 167.1 Hz), 72.59 (d, ^3J(C, P) = 10.8 Hz), 172.94. HR-MS (FAB) calculated for $C_7H_{17}NO_6PS$ (MH^+) 274.0516, found 274.0523.

(*R*,*S*)-2-Amino-4-[(phosphonomethyl-carbamoyl)-methylsulfanyl]-butyrate (24). The reaction of diethyl-[(2-chloro-acetylamino)-methyl]-phosphonate[39] (**42**; 0.125 g, 0.51 mmol) with D,L-homocysteine (**5**) according to method A afforded (*R*,*S*)-2-amino-4-{[(diethoxy-phosphorylmethyl)-carbamoyl]-methylsulfanyl}-butyrate in the yield of 92 mg (52%). ^{1}H NMR (DMSO): δ 1.23 (t, J = 7.0 Hz, 6H), 2.00 (m, 1H), 2.08 (m, 1H), 2.68 (ddd, J = 13.5, 8.4 and 6.2 Hz, 1H), 2.73 (ddd, J = 13.5, 8.6 and 6.8 Hz, 1H), 3.19 (s, 2H), 3.58 (dd, J(H, P) = 11.6 and J(H, H) = 6.0 Hz, 2H), 3.96 (bdd, J = 6.8 and 6.2 Hz, 1H), 4.02 (m, 4H), 8.30 (vb, 2H), 8.43 (bt, J = 6.0 Hz, 1H). ^{13}C NMR (DMSO): δ 16.41 (d, ^3J(C, P) = 5.4 Hz, 2C), 27.37, 29.92, 33.68, 34.31 (d, ^1J(C, P) = 157.1 Hz), 51.20, 62.00 (d, ^2J(C, P) = 5.9 Hz, 2C), 169.17 (d, ^3J(C, P) = 4.3 Hz), 170.87. HR-MS (FAB) calculated for $C_{11}H_{24}N_2O_6PS$ (MH^+) 343.11093, found 343.1097. This compound (19 mg, 55 μmoles) was treated with bromotrimethylsilane (61 mg, 450 μmoles) in dry DMF (1 mL) under argon atmosphere at 50 °C overnight. The reaction mixture was concentrated in vacuo, and the residue was coevaporated with 10% triethylamine in acetonitrile (3 × 1 mL) and then with water. The product **24** was purified by RP-HPLC. The yield was 10 mg (62%). ^{1}H NMR (D_2O): δ 2.18 (m, 1H), 2.27 (m, 1H), 2.76 (m, 2H), 3.36 (d, J(H, P) = 0.6 Hz, 2H), 3.50 (d, J(H, P) = 12.4 Hz, 2H), 4.12 (t, J = 6.4 Hz, 1H). ^{13}C NMR (D_2O): δ 28.11, 30.23, 35.58, 38.26 (d, ^1J(C, P) = 171.5 Hz), 52.92, 172.58, 172.77. HR-MS (FAB) calculated for $C_7H_{16}N_2O_6PS$ (MH^+) 287.0467, found 287.0459.

In a parallel experiment, (*R*,*S*)-2-amino-4-{[(diethoxy-phosphorylmethyl)-carbamoyl]-methylsulfanyl}-butyrate (19 mg, 55 μmoles) was treated with bromotrimethylsilane by the same manner as described above for compound **24**. However, after completing of the deprotection, the reaction mixture was evaporated to dryness and then warmed at 50 °C for 1 h with methanol (5 mL). After evaporation, the product, methyl (*R*,*S*)-2-amino-4-[(phosphonomethyl-carbamoyl)-methylsulfanyl]-butyric acid ester (**25**), was purified by RP-HPLC. The yield was 5 mg (29%). ^{1}H NMR (D_2O): δ 2.21 (m, 1H), 2.31 (m, 1H), 2.78 (m, 2H), 3.36 (s, 2H), 3.51 (d, J(H, P) = 12.4 Hz, 2H), 3.86 (s, 3H), 4.30 (t, J = 6.5 Hz, 1H). ^{13}C NMR (D_2O): δ 29.52, 31.43, 37.05, 39.76 (d, ^1J(C, P) = 146.5 Hz), 53.76, 55.82, 172.60, 174.30. MS (FAB) calculated for $C_8H_{18}N_2O_6PS$ (MH^+) 301.05, found 301.00.

(*R*,*S*,*R*,*S*)-2-Amino-4-(2-amino-2-carboxy-ethylsulfinyl)-butyric Acid (27). This oxidized derivative was prepared starting from commercial cystathionine (**26**; 0.1 mg, 0.45 mmol) using the same procedure as for compound **10**. The yield was 67 mg (63%). The presence of four diastereoisomers leads to the observation of up to four signals for individual hydrogens and carbon atoms. ^{1}H NMR (D_2O): δ 2.35−2.51 (m, 2H), 3.09−3.33 (m, 2H), 3.51−4.03 (m, 2H), 4.15−4.20 (m, 1H), 4.43−4.47 (m, 1H). ^{13}C NMR (D_2O): δ 23.83, 23.89, 24.05 and 24.10 (CH_2), 47.66, 47.77, 48.28 and 48.38 (S−CH_2−), 50.83, 51.14, 51.18 and 52.61 (CH_2−S), 50.00 and 50.45 (CH−N), 52.61 and 52.76 (CH−N), 171.00, 171.99. HR-MS (FAB) calculated for $C_7H_{15}N_2O_5S$ (MH^+) 239.0702, found 239.0694.

BHMT Inhibition Assays. Human recombinant BHMT was prepared as described previously;[3] *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 we have described previously in detail[40] with only several modifications. Briefly, d,L-homocysteine was freshly prepared by dissolving D,L-homocysteine thiolactone hydrochloride (15.4 mg) in 400 μL of 2 M NaOH. The solution was allowed to stand for 5 min at room temperature. The solution was then neutralized by the addition of 600 μL of a saturated solution of KH_2PO_4 and immediately used in BHMT assay.

The standard BHMT assay (500 μL) used to determine the percent inhibition contained 0.2 μM BHMT, different concentrations of inhibitor (20 μM or 1 μM), 100 μM D,L-homocysteine, 250 μM betaine (0.05 μCi), 10 mM β-mercaptoethanol, and 50 mM potassium phosphate buffer pH 7.5. Human recombinant BHMT was first mixed with inhibitor(s), then the substrates were added and the mixture incubated at 37 °C for 30 min. The reaction was stopped by transferring the reaction tubes into ice water and by adding 2.5 mL of ice-cold water. The samples were applied to a Dowex 1 × 4 (200−400 mesh), 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 scintillation mixture was added into each vial and counted. Blanks contained all the reaction components except enzyme, and their values were subtracted from the sample values. All samples were assayed in triplicates and 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 the concentrations of substrates used were 1 mM D,L-homocysteine and 2 mM betaine (0.15 μCi). The inhibition at 10 different inhibitor concentrations was determined for each curve. The data were analyzed by nonlinear regression fit using program GraphPad Prism3.02.

The apparent inhibition constant (K_i^{app}, refs 41−43) of inhibitor **2** toward betaine was measured at fixed concentration of D,L-homocysteine (100 μM) and varied concentrations of betaine (0.5, 1, 2, and 4 mM) and inhibitor (0, 15, 25, 50, 75, and 100 nM). The reaction proceeded in a total volume of 250 μL and contained 15 nM BHMT and 1 μCi of ^{14}C-betaine. The enzyme was preincubated with inhibitor for 15 min at room temperature, and then the substrates were added, and the reaction was allowed to proceed for 1 h at 37 °C. The data were analyzed by Dixon plot.[44]

All points for determination of IC_{50}'s or K_i^{app}'s were measured in duplicate and the values from three different assays are reproducible within ±10%.

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Note Added after ASAP Publication. Data for Table 2 was revised since the original web posting date of May 27, 2006. The revised version appeared in the web posting of May 30, 2006.

Supporting Information Available: HPLC purity data and NMR spectra records for compounds **2**, **9**, and **19**. This material is available free of charge via the Internet at http://pubs.acs.org.

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