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Inhibitors of ATP: L-methionine *S*-adenosyltransferase—L-Methionine analogues

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Methionine adenosyltransferase (ATP: L-methionine *S*-adenosyltransferase, EC 2.5.1.6) catalyzes the transfer of the adenosyl moiety of ATP to the sulfur atom of methionine, resulting in the formation of *S* adenosyl L-methionine, the primary biological methyl donor [1, 2]. *S*-Adenosyl-L-methionine (SAM) is involved in many important biological functions, including the methylation of nucleic acids, proteins, lipids and other metabolites, the synthesis of polyamines, and the regulation of various enzymatic reactions including its own synthesis [2].

The substrate specificity of rat liver methionine adenosyltransferase with respect to methionine has been well studied by various investigators [3–7]. From the inhibitory potency of a series of structural analogues of methionine, deductions have been made concerning the preferred conformation of methionine at the active site of methionine adenosyltransferase. A folded but planar conformation of methionine has been proposed in which the sulfur atom and the terminal methyl group are in a somewhat extended position rather than folded back on the rest of the molecule. The present paper describes the synthesis of additional structural analogues of methionine and the evaluation of these compounds as inhibitors of rat liver methionine adenosyltransferase in *in vitro* assays of the enzyme.

Materials

All solutions were prepared in glass distilled deionized water from chemicals of the best commercial grades available. 1,3-Butanediol, acrolein and the mercaptans were obtained from the Aldrich Chemical Co., Cedar Knoll, NJ. Bovine serum albumin and Trizma base were obtained from the Sigma Chemical Co., St. Louis, MO. L-Methionine and glutathione were purchased from the Schwarz/Mann Corp., Orangeburg, NY. Adenosine 5′triphosphate (disodium) was obtained from Boehringer Mannheim, Indianapolis, IN. Yeast inorganic pyrophosphatase was supplied by the ICN Nutritional Biochemicals Corp., Cleveland, OH. L-[Methyl-^{14}C]methionine (51.2 or 56.4 mCi/m-mole) and enzyme grade ammonium sulfate were products of the Schwarz/Mann Corp. Cellulose phosphate ion exchange discs (23 mm diameter, type P81) were purchased from Whatman-Reeve Angel Inc., Clifton, NJ. The primary and secondary scintillators, PPO (2,5-diphenyloxazole) and POPOP (*p*-bis-[2-(5-phenyloxazolyl)]-benzene), were products of New England Nuclear, Boston, MA. All i.r. spectra were done as KBr discs on a Beckman I.R. 20A or 18A. Nuclear magnetic resonance spectra were done on a Varian T-60 or Em-360, and the appropriate deuterated solvents were obtained from the Aldrich Chemical Co. Chemical shifts are reported relative to tetramethylsilane (TMS) or 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Melting points were taken on a Micro Melting Point apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Scintillation counting was done on a Packard Tri-Carb scintillation counter, model 3003 or 3380.

Synthetic methods

The amino acids used in this study were synthesized from the appropriate ketone or aldehyde according to a Bucherer synthesis [8]. After crystallization and drying, the hydantoins were subjected to either acid or base hydrolysis to yield the amino acids which were recrystallized from hot aqueous ethanol.

1-Aminocyclopentane-1-carboxylic acid (*cycloleucine*). Cyclopentanone was reacted in the Bucherer synthesis as described above and the hydantoin was hydrolyzed in 60% H_2SO_4 to the amino acid with 20 per cent yield, m.p. 300–301°d [9, 10].

4-Aminotetrahydrathiopyran-4-carboxylic acid. 4-Ketotetrahydrothiopyran was synthesized according to literature methods [11–13] and then reacted in the Bucherer synthesis. The hydantoin was hydrolyzed by heating a mixture of 0.01 mole of the hydantoin, 10 g (0.03 mole) of $Ba(OH)_2$, 110 ml of water and 10 ml of 95% ethanol in a pressurized reaction bomb for 4 hr at 165° [14]. The cloudy white reaction mixture was filtered and neutralized with excess ammonium carbonate. The amino acid (3 per cent yield) precipitated from the clear solution after reduction of the reaction volume on a rotary evaporator and was recrystallized from hot aqueous ethanol, m.p. 306–307°d. Anal. calc. for $C_6H_{11}NO_2S$: C, 44.88; H, 6.85. Found: C, 44.60; H, 6.62.

4-Aminotetrahydropyran-4-carboxylic acid. 4-Ketotetrahydropyran was prepared according to a modification of the method of Olsen *et al.* [15–17]. The 4-hydroxytetrahydropyran intermediate was oxidized to the ketone by the use of the Jones Reagent [18]. The ketone was used in the Bucherer synthesis and the resulting hydantoin was hydrolyzed with $Ba(OH)_2$, as described above to give the amino acid (5 per cent yield), m.p. 305–306°d. Anal. Calc. for $C_6H_{11}NO_3$: C, 49.82; H, 7.60. Found: C, 49.55; H, 7.67.

Para-substituted S-phenyl homocysteine derivatives (R = H, OCH_3, Br, Cl, CH_3). The corresponding *S*-phenyl substituted aldehydes were prepared according to the method of Pierson *et al.* [8]. In the case of solid mercaptans, the thiol was dissolved in 25–50 ml dioxane or benzene and the reaction carried out. The aldehyde product was separated from the reaction mixture by vacuum distillation or ether extraction. In some cases, extraction with diethyl ether achieved a good separation of the product, and no further purification was necessary. The aldehydes were then used in the Bucherer synthesis as described earlier. The hydantoins of this series of compounds were hydrolyzed by refluxing in sodium hydroxide [8].

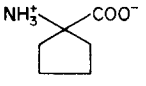
The para-substituted *S*-phenyl homocysteine derivatives were obtained in yields ranging from 10 to 30 per cent and all had λ_{max} values between 250 and 260 nm, where, R = H m.p. 226–229°d [19]; R = CH_3 m.p. 238–239.5°d [20], Anal. Calc. for $C_{11}H_{15}NO_2S$: C, 58,64: H, 6.70. Found: C, 58.76; H, 6.58; R = OCH_3 m.p. 229–231°d, Anal. Calc. for $C_{11}H_{15}NO_3S$; C, 54.75; H, 6.26. Found: C, 54.75; H, 6.38; R = Br m.p. 254–257°d [5], Anal. Calc. for $C_{10}H_{12}NO_2S$ Br: C, 41.39; H, 4.17. Found: C, 41.38; H, 4.18; R = Cl m.p. 239–240°d, Anal. Calc. for $C_{10}H_{12}NO_2S$ Cl: C, 48.88; H, 4.92. Found: C, 48.89; H, 4.77.

The amino acids synthesized are shown in Fig. 1 and fall into two structural classes: (a) carbocyclic and heterocyclic amino acids; and (b) para-substituted *S*-phenyl homocysteine derivatives. The structures of these amino acids are supported by their physical properties, i.r. and n.m.r. spectra, and elemental analysis.

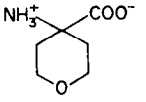
Enzymatic methods

Partial purification of methionine adenosyltransferase. The preparation of the enzyme followed essentially the proce-

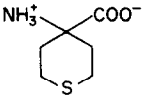
A. Carbocyclic and heterocyclic amino acids



1. 1-Aminocyclopentane-1-carboxylic acid (cycloleucine)

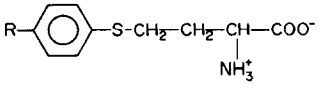


2. 2H-pyran-4-carboxylic acid-4-aminotetrahydro (4-amino-tetrahydropyran-4-carboxylic acid)



3. 2H-thiopyran-4-carboxylic acid-4-aminotetrahydro (4-amino-tetrahydrothiopyran-4-carboxylic acid)

B. Para-substituted *S*-phenyl homocysteine derivatives



1. R = H *S*-phenyl -DL- homocysteine
2. R = OCH_3 *S*-(*p*-methoxyphenyl)-DL-homocysteine
3. R = Br *S*-(*p*-bromophenyl)-DL-homocysteine
4. R = Cl *S*-(*p*-chlorophenyl)-DL-homocysteine
5. R = CH_3 *S*-(*p*-methylphenyl)-DL-homocysteine

dure of Lombardini *et al.* [3] with a few modifications as noted below. Liver tissue (84.50 g) from eight female rats (250–300 g average wt) was homogenized in 4 vol. (338 ml) of homogenization buffer (0.03 M phosphate buffer, pH 7.0, 5 mM β-mercaptoethanol and 0.2 mM EDTA) at 4°. The homogenate was centrifuged at 27,000 *g* for 30 min and 90,000 *g* for 90 min. The supernatant fluid was removed and fractionated with a saturated ammonium sulfate solution (pH 7.0, 5 mM β-mercapoethanol and 0.2 mM EDTA) at 0°. The pellet fractionating between 40 and 60% saturation was taken, and the remainder of the purification followed the previously published procedure [3]. Fractions containing high specific activity were pooled and frozen in small aliquots at −15°. Protein concentration was determined by trichloroacetic acid (TCA) precipitation followed by the Lowry method for the determination of protein, as described in Ref. 21. Absorbance at 650 nm was read on a Gilford 300N spectrophotometer.

Enzyme assays [22, 23]

The assays performed in this work are based on the separation of *S*-adenosyl-L-methionine from L-methionine on cellulose phosphate cation exchange discs. The assays were carried out as described previously with modifications noted. The standard assay system contained the following in a volume of 0.25 or 0.5 ml: 160 mM Tris–HCl (pH 7.6), 200 mM KCl, 300 or 60 mM $MgCl_2 \cdot MgCl_2 \cdot 6H_2O$ (300 mM for earlier assays, 60 mM for all assays subsequent to DEAE chromatography due to precipitate formation in the presence of excess $MgCl_2$), 8 mM glutathione (pH 7.0), 20 mM ATP (pH 7.0), 3 units inorganic pyrophosphatase, 10 mM L-methionine (pH 7.0) and L-[methyl-^{14}C]methionine (51.2 or 57.4 mCi/m-mole) in various amounts, as specified in each experiment and enzyme. The purity of the radioactive methionine used in the assays was assessed by ascending chromatography on cellulose plates (Mn-300 Analtech Inc., Newark, DE) or on Whatman No. 1 chromatography paper in *n*-butanol–glacial acetic acid—H_2O (60: 15: 25) or acetone–H_2O (4: 1). In both systems, 92–95 per cent of the applied radioactivity migrated with an authentic methionine standard.

All assays were done under conditions at which the reaction was linear with respect to time and protein concentration. After completion of the reaction, the discs were dried for 15 sec and then immersed in a large volume (900 ml) of ice-cold water. This method of terminating the assay was compared to a reported method [22] and found to give comparable results. In contrast to other reports [23], simple spotting on discs and drying does not terminate the reaction. The discs (approximately twenty samples) were batch washed on a large Buchner funnel with 1500 ml water. After drying they were counted in an eluting scintillation mixture as described in Ref. 22. Blanks, obtained for each sample by incubation of the complete reaction mixture minus ATP or enzyme (giving equivalent results), were routinely assayed and subtracted. Standards were prepared by adding a known amount of [$^{14}CH_3$]-*S*-adenosyl-L-methionine (51.8 mCi/mmole, ICN Nutritional Biochemicals) to the complete reaction mixture minus radioactive methionine. Inactive enzyme was added to the standards and a 50-μl aliquot was removed and spotted on two cellulose phosphate discs which were then washed and dried as described. The efficiency of counting calculated from these standards was either 78 or 88 per cent depending on the instrument used and the experiment.

The inhibitory potency of all the synthetic analogues was assessed by assaying various concentrations of each inhibitor in the standard reaction mixture (minus inorganic pyrophosphatase; volume = 0.25 ml) at a fixed concentration of L-methionine (26.3 or 8.8 μM, as specified). Addition of the highest concentration of each inhibitor tested to standards prepared as described previously did not alter the efficiency of counting.

Preparation of methionine adenosyltransferase and substrate kinetics

The elution pattern obtained for methionine adenosyltrans-

ferase during chromatography on DEAE-cellulose exhibited one major peak of enzyme activity at a phosphate buffer concentration of 0.14 M. The partially purified rat liver enzyme preparation showed a 34-fold increase in specific activity over the crude extract and represented a 12.3 per cent recovery of total enzyme activity. The preparation had a specific activity of 2.49 units/mg of protein where a unit of activity is defined as 1 μmole SAM formed/30 min.

A double reciprocal plot of the reaction velocity with respect to methionine concentration, at saturating levels of ATP, is shown in Fig. 2. The Lineweaver–Burk plot indicates two straight line components giving a biphasic appearance to the overall plot. The K_m (methionine) values were calculated from the two linear segments, as defined by linear regression analysis. At the lower range of methionine concentrations (17–98 μM), the K_m (met) = 28.6 μM and the V_{max} = 69 nmoles SAM formed/min/mg of protein. In the higher range of methionine concentrations (158 μM to 2.03 mM) the K_m (met) = 189.5 μM and the V_{max} = 121 nmoles SAM formed/min/mg of protein. The pattern of response with respect to methionine concentration agrees with that obtained by Liau *et al.* [24]. These investigators reported similar biphasic kinetics with regard to methionine concentration with low and high apparent K_m (methionine). In addition, a similar pattern of kinetic response to increasing methionine concentrations has been reported by Finkelstein *et al.* [25] for the human liver and rat liver enzymes. In contrast to the work of Lombardini *et al.* [26], no sigmoidal kinetics at low methionine concentrations were observed in this study.

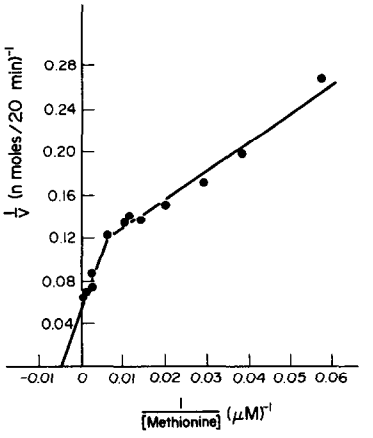


Fig. 2. Lineweaver–Burk plot of the activity of methionine adenosyltransferase at varying concentrations of L-methionine. Each point is the average of duplicate determinations. The reaction was initiated by the addition of 7.6 μg of enzyme protein to the standard assay mixture (volume = 0.25 ml; 60 mM $MgCl_2$) which contained varying concentrations of L-[methyl-^{14}C]-methionine (51.2 mCi/m-mole), or L-[methyl-^{14}C]methionine (0.122 or 0.336 μCi) plus various amounts of 0.01 M or 1.5 mM L-methionine to give the desired concentrations ranging from 8.8 μM to 2.03 mM L-methionine. Samples were processed as described previously and counted in the liquid scintillation system to a preset count. V is given in terms of nmoles SAM synthesized/20 min. All reaction rates were linear with respect to time and protein concentration.

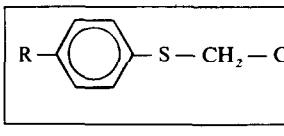
Inhibition studies

Para-substituted S-phenyl homocysteine derivatives. All of the inhibitors from this series, plus the heterocyclic sulfur amino acid, were found to be insoluble in water and approximately twenty other solvents tested. However, they were soluble in acid and base, and, therefore, were added to the assay as solutions of 0.3 N HCl. An equivalent amount of 0.3 N KOH was added to the assays to maintain the pH of the reaction. Addition of equivalent amounts of 0.3 N HCl and 0.3 N KOH to control tubes with each assay with done routinely and was found to influence neither the pH of the reaction nor the activity of the enzyme. The concentration ranges used for the *S*-phenyl substituted amino acids were verified by u.v. absorbance studies. In all cases, the concentration of inhibitor calculated from the absorbance was within 5 per cent of the expected concentration for the concentration ranges used in the inhibition studies.

Table 1 shows the highest concentration of each inhibitor tested in this series and the rate of the reaction in the presence and absence of each inhibitor. Comparison of the reaction velocity in the presence of each inhibitor in this series with the appropriate control experiments showed that none of the amino acids in this series was an inhibitor of methionine adenosyltransferase at a methionine concentration of 26.3 μM. No higher concentrations of inhibitors were tested due to the limits of solubility. In addition, at methionine concentrations of 8.8 μM there was essentially no inhibition of methionine adenosyltransferase by any of the amino acids in this series. With the large inhibitor to substrate ratios used in these experiments any significant inhibition of the enzyme should have been detected in this system.

Carbocyclic and heterocyclic amino acids. These inhibitors were tested at a series of concentrations in the presence of 26.3 μM L-methionine and the reaction rates were related, as a percentage, to those obtained in the absence of inhibitor. By plotting the activity or its reciprocal [27] against the inhibitor concentration, the I_{50} value could be determined graphically. This value is defined as the concentration of inhibitors giving 50 per cent of control activity at a specified substrate concentration, in this case 26.3 μM methionine. An I_{50} value of 2.67 mM was obtained for 1-aminocyclopentane-1-carboxylic acid which agrees well with literature reports [3]. Similar studies with the oxygen heterocyclic amino acid, 4-aminotetrahydropyran-4-carboxylic acid, resulted in an I_{50} value of 150 mM and hence, this compound is a much poorer inhibitor

Table 1. Summary of inhibition studies of para-substituted *S*-phenyl homocysteine derivatives at 26.3 μM methionine*



	I (mM)	V = nmoles SAM synth./10 min V + inhibitor	V Control	I/S
R = H	6.0	2.42 ± 0.13	2.61 ± 0.70	228
R = OCH_3	3.0	2.38 ± 0.67	2.52 ± 0.93	114
R = CH_3	1.80	2.56 ± 0.04	2.68 ± 0.38	68
R = Cl	0.97	2.71 ± 0.11	2.68 ± 0.38	37
R = Br	0.84	2.66 ± 0.09	2.68 ± 0.38	32

* The highest concentration of each inhibitor tested is shown with the rate of the reaction in the presence of inhibitor plus the rate of the appropriate control experiments at 26.3 μM L-[methyl-^{14}C]methionine. I/S values indicate the inhibitor/substrate for each compound tested. V is given in terms of nmoles SAM synthesized/10 min. Each value represents the average of triplicate determinations ± the standard error. The inhibitor concentration is given in terms of mM. All reactions were done under conditions which were linear with respect to time and protein concentration. The reaction was initiated by the addition of 7.6 μg of enzyme protein to the standard assay mixture (volume = 0.25 ml; 60 mM $MgCl_2$).

of the reaction as compared to the cyclopentane derivative. The heterocyclic sulfur amino acid, 4-aminotetrahydrothiopyran-4-carboxylic acid, was a better inhibitor of methionine adenosyltransferase than the oxygen analogue but a poorer inhibitor of the enzyme than cycloleucine. The I_{50} value obtained for the compound from the representative plot shown in Fig. 3 was 58 mM. In addition, it was noted that the activity of the enzyme was slightly stimulated at low concentrations of inhibitor for all three inhibitors tested. This has been noted for other inhibitors of rat and mouse liver methionine adenosyltransferase [26] and has been discussed in terms of the proposed regulatory properties of the rat liver enzyme.

The substrate kinetics with respect to methionine are basically in agreement with the kinetic patterns observed by Liau *et al.* [24] and Finkelstein *et al.* [25]. Both groups of investigators have reported a biphasic response in double reciprocal plots of reaction velocity versus methionine concentration. This biphasic response can be explained by negative cooperativity, the presence of isoenzymes, or isoenzymes exhibiting negative cooperativity. The presence of isoenzymes in our enzyme preparation is possible, since our purification method would not completely separate isoenzymes and, in addition, other investigators have suggested the presence of multiple enzyme forms in rat liver [24, 28]. Furthermore, Chiang and Cantoni [29] have reported the presence of two isoenzymes in yeast, each exhibiting negative cooperativity with respect to L-methionine.

S-Phenylhomocysteine and the para-substituted derivatives failed to inhibit the adenosyltransferase reaction at any of the concentrations tested. This was established by assaying various concentrations of each inhibitor in the presence of both 26.3 μM and 8.8 μM [methyl-^{14}C]-L-methionine. In this latter system, with inhibitor to substrate ratios ranging from 100 to 700, depending on the inhibitor used, any significant inhibition of the enzyme should have been detected. These results were surprising, since Coulter *et al.* [5] reported that *S*-phenyl-DL-homocysteine inhibited rat liver methionine adenosyltransferase by 50 per cent at a concentration of 11.5 mM in the presence of 37.5 μM [methyl-^{14}C]-L-methionine. The value of 11.5 mM was determined by graphical extrapolation from the maximal concentration tested (4 mM). However, in this system, we observed that concentrations of *S*-phenyl-DL-homocysteine up to 6.0 mM gave no inhibition of the enzyme. The concentrations of *S*-phenyl-DL-homocysteine and all the other aromatic derivatives used in the assays were verified by u.v. spectroscopy and, in all cases, the concentrations determined from the absorbances were in good agreement with the expected values. Therefore, it is possible that the inhibition of the rat liver enzyme reported previously for *S*-phenyl-DL-homocysteine is due to the fact that this compound was not in solution at the concentration tested.

To test this possibility, an assay was performed in exactly the same manner as that used by Coulter *et al.* [5]. Despite the fact that the amino acid was found to be water insoluble and addition of the stock solution to the assay mixture resulted in further precipitation of the amino acid, the assay was performed. Assay of the enzyme in this manner in the presence of 4.0 mM *S*-phenyl-DL-homocysteine resulted in an approximately 60 per cent decrease in enzyme activity. However, as mentioned previously, the amino acid precipitated from both the stock solution and the reaction mixture during the course of the experiment. These results suggest that the inhibition reported previously for this compound is due possibly to some type of solid adsorption and subsequent inactivation of the enzyme and not a true inhibition of methionine adenosyltransferase by *S*-phenyl-DL-homocysteine. It is also possible that the discrepancy in inhibitory potency observed for *S*-phenyl-DL-homocysteine might only reflect different isoenzyme compositions in various preparations of methionine adenosultransferase. However, the procedure used for the isolation of the enzyme was nearly the same as that used by Lombardini *et al.* [3]. In addition, the I_{50} value for cycloleucine, a compound that is not limited by its solubility, agrees well with the reported value [3].

The two heterocyclic compounds were found to be inhibitors of the enzyme but were inferior as compared to cycloleucine, which supports the observations made by Coulter *et al.* [7]. Both of these compounds are structural analogues of the substrate methionine and, as expected, display competitive inhibition patterns, as determined from the response of the enzyme to each inhibitor at different concentrations of methionine. However, these inhibition studies were hampered by the possible existence of multiple enzyme forms, as suggested by the substrate kinetics with respect to methionine, and must be interpreted with caution.

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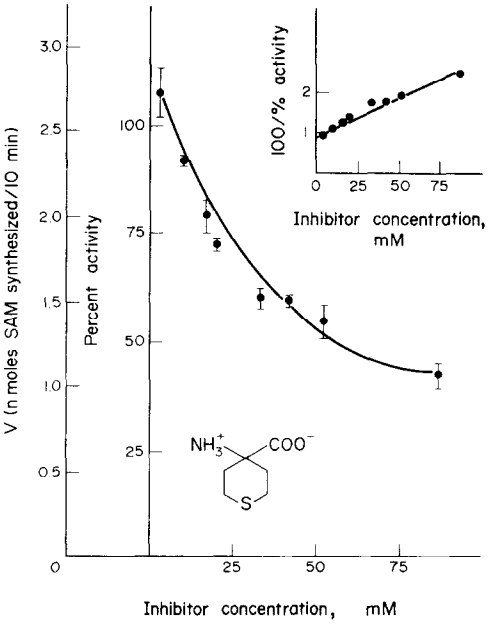

Fig. 3. Inhibition of methionine adenosyltransferase by 4-amino-tetrahydrothiopyran-4-carboxylic acid. Each point is the average of duplicate determinations ± the standard error shown by the vertical lines. The reaction was initiated by the addition of 7.6 μg of enzyme protein to the standard assay mixture [volume = 0.25 ml; 60 mM $MgCl_2$; 26.3 μM L-[methyl-^{14}C]methionine (51.2 mCi/m-mole)] with concentrations of inhibitor ranging from 3.34 to 86.5 mM. Samples were processed as described previously and assays were linear with respect to time and protein concentration. *V* is given in terms of nmoles SAM synthesized/10 min and is also expressed as per cent of control activity with the reciprocal plot in the inset. Control experiments contained the complete reaction mixture as described minus inhibitor.

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