J. Enzyme Inhibition, 1998, Vol. 13, pp. 361–367
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A NEW SERIES OF S-ADENOSYL-L-METHIONINE SYNTHETASE INHIBITORS

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(Received 20 February 1998)

A new series of epithio and epoxy amino acid analogues of L-methionine or L-methoxinine were examined as potential inhibitors of the enzyme S-adenosylmethionine (AdoMet) synthetase. The kinetic behaviour of these compounds was studied using recombinant rat liver S-adenosyl-L-methionine sythetase (α-isoform) fractionated from *E. coli*, transformed with the plasmid pSSRL-T7N. All the compounds tested were competitive inhibitors with respect to L-methionine and the (2*S*, 4*S*)-2-amino-4,5-epoxy pentanoic acid was found to be a very potent inhibitor of the enzyme compared to those already reported for AdoMet synthetase from other mammalian tissues.

Keywords: S-Adenosyl-methionine synthetase; Inhibition; Epoxy- and epithio amino acids

INTRODUCTION

S-Adenosylmethionine synthetase (EC 2.5.1.6, ATP: L-methionine-S-adenosyl transferase) catalyses the reaction of ATP and L-methionine to yield S-adenosylmethionine (AdoMet), pyrophosphate and orthophosphate.[1]

AdoMet is utilised by methyltransferases for the methylation of RNA, DNA, histones, proteins, polysaccharides, steroids and numerous other metabolites.[2] S-Adenosylhomocysteine (AdoHcy) is the by-product of these AdoMet-dependent methyltransferases. The cellular concentration of S-adenosylmethionine, and hence the level of methylation activity, is

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controlled primarily by S-adenosylmethionine synthetase and by S-adenosylhomocysteine hydrolase which degrades adenosylhomocysteine, a potent product inhibitor of most S-adenosylmethionine utilising enzymes, to homocysteine and adenosine.[3]

The rational design of methylation inhibitors based on the inhibition of each of these enzymes involved in AdoMet metabolism has attracted the attention of medicinal chemists in the search for antitumour[4] and antiviral agents.[5] But, from recent reports, it appears that each of these two general approaches to the inhibition of cellular methylation has limitations in their therapeutic applications.[6,7]

However, it has been observed recently that combined modulation of S-adenosylmethionine biosynthesis and S-adenosylhomocysteine metabolism enhances the inhibition of nucleic acid methylation and L12.10 cell growth, in addition the antiviral activity of AdoHcy hydrolase inhibitors is potentiated by inhibitors of AdoMet synthetase.[5,8] This concept of altering cellular methylation by simultaneous use of inhibitors of AdoMet synthetase and AdoHcy hydrolase stimulated our effort in the search of a new series of potent inhibitors of AdoMet synthetase. Steric, electronic and conformational requirements have been described for analogues of L-methionine, essential to their function as substrates or inhibitors of AdoMet synthetase.[9]

These results led us to consider that analogues of L-methionine or L-methoxinine bearing an epithio or epoxy function could be good candidates as inhibitors of AdoMet synthetase. Moreover, these amino acids fulfil, in principle, the basic requirements of being active-site directed inhibitors as they represent substrate analogues containing a chemically reactive group.

We have recently synthesised two series of epoxy- and epithio amino acids **I(a)–(c)** and **II(a)–(c)**, the chemical structures of which are shown in Figure 1. The present study describes their kinetic properties towards the tetrameric isoform of recombinant rat liver S-adenosyl-L-methionine synthetase.

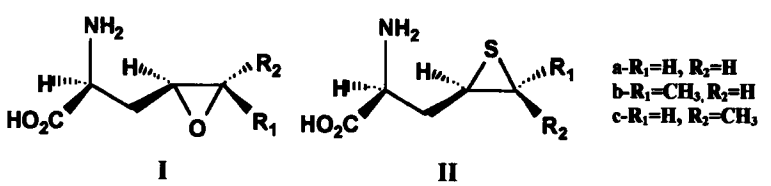


FIGURE 1 Epithio and epoxy amino acid analogues of L-methionine and methoxinine.

MATERIALS AND METHODS

General

All reagents used were of analytical grade. ($^{14}CH_3$)-L-Methionine (2.2 GBq/mmol) was purchased from NEN. A Packard TRICARB 1900 TR was used to measure radioactivity by scintillation counting. Protein was determined using bovine serum albumin (BSA) as a standard by the method of Bradford.[10] FPLC experiments were carried out on a Biorad Biologic chromatograph system. The *E. coli* BL 21 (DE3) strain transformed with the plasmid pSSRL-T7N was a gift from Dr Luis Alvares, Instituto de Investigationes Biomedicas C.S.I.C. (Madrid, Spain).

Inhibitors

The enantioselective synthesis of epoxy amino acids **I(a)–(c)** and the corresponding epithio derivatives **II(a)–(c)** have been already described by us[12,13] in four steps starting from the readily available (*S*)-*cis* and (*S*)-*trans* crotylglycines via halolactonisation of these γ,δ-unsaturated, suitably protected amino acids.[11,12] The chemical homogeneity of **I(a)–(c)** and **II(a)–(c)** and their stability in buffer assay were checked by HPLC prior to enzymic evaluation.

Purification of AdoMet Synthetase (α-isoform)

The tetramer (α-isoform) of recombinant rat liver AdoMet synthetase was fractionated from the cell-free extract of *E. coli* BL21(DE3) strain transformed with the plasmid pSSRL-T7N and grown in LB medium in the presence of isopropyl β-D-thiogalactopyranosin by a procedure reported by Alvarez *et al.*[13] The purification and separation of the two oligomeric forms of AdoMet synthetase were performed according to the method described.[13] This included $(NH_4)_2SO_4$ fractionation (33–55%) of the cell-free extract and gel filtration chromatography on sephacryl S-300. About 27 mg of enzyme (0.5 units/mg) was obtained from 24 g of IPTG induced cells (6 l cultures). The purification of the major tetrameric form of the recombinant enzyme was continued by FPLC using a Fractogel (EMD-TMAE-6505S°, 25–40 μm; 14 ml) Merck column equilibrated with 10 mM HEPES buffer, 10 mM $MgSO_4$ and 1 mM EDTA, pH 7.5. After loading the column with an aliquot of enzyme and washing with 50 ml of buffer, the column was eluted with a gradient of NaCl (0–1 M) at a flow rate of 4 ml/min.

Active fractions corresponding to the α-isoform of AdoMet were pooled, concentrated by ultrafiltration and deionised on an Econo-PacP6 (5 ml) BioRad column; 10 mg (2 U/mg) of purified tetrameric form of AdoMet synthetase was obtained by this procedure. An unit of enzyme activity was defined as the quantity of AdoMet synthetase that catalyses the formation of 1 μmol of AdoMet per minute under the conditions of the assay.

Assay of AdoMet Synthetase Activity

AdoMet synthetase activity was measured by a radioactive assay using (^{14}C-methyl)-L-methionine as described by Sullivan and Hoffman[14] with minor modifications to give better efficiency in the separation of labelled residual methionine and AdoMet formed. Assays were carried out at 37°C in a volume of 120 μl which contained in addition to enzyme (30 μU), the following final concentration of various components: 240 mM Hepes (pH 7.5), 700 mM KCl, 36 mM $MgSO_4$, 9.6 mM DTT, 5 mM ATP and 10–120 μM (^{14}C-methyl)-L-methionine (total activity 700 Bq). Reactions were initiated by addition of enzyme and stopped with 10 μl of 2 M $HClO_4$. After centrifugation 100 μl of the supernatent was spotted on a 2.5 × 2.5 cm square of Whatman P-81 phosphocellulose paper. Unreacted ^{14}C-methionine was removed by washing the paper three times in 100 mM ammonium formate pH-3, followed with subsequent washings on a Buchner funnel with 100 mM ammonium formate pH-3, ethanol and finally with ether (10 ml of each solution per filter). Filters were dried at 70°C for a few minutes. The ^{14}C-AdoMet formed and fixed on the filter was quantitated by liquid scintillation counting in 4 ml of Ultima Flow AP (Packard) scintillation fluid.

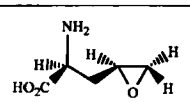
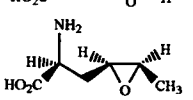
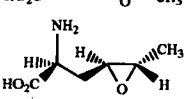
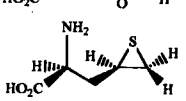
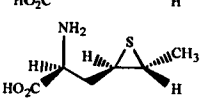
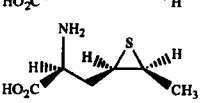
Inhibition Study

The same assay was used for evaluation of the inhibition effect of the amino acids **I(a)–(c)** and **II(a)–(c)**, with compounds tested being present in the incubation mixture at a final concentration varying from 5 to 250 μM. All kinetic constants were determined from Lineweaver–Burk plots. The computer program described by Cleland[15] (Ultra-fit software Macintosh) was used for data analysis. IC_{50} values (concentrations required for 50% inhibition) were determined according to the method of Dixon.[16] For inactivation assays of AdoMet synthetase by **I(a)–(c)** or **II(a)–(c)**, AdoMet synthetase (0.1 μg) was preincubated at 37°C in 50 μl buffer assay for 10–30 min in the presence of compounds tested (50–100 μM). Activity remaining was assayed every 10 min by transfer of a 10 μl portion of this incubation mixture to the standard assay mixture.

RESULTS AND DISCUSSION

The reaction rate of the recombinant rat liver AdoMet synthetase studied showed normal Michaelis–Menten kinetics with methionine as the variable substrate and ATP (5 mM) as the fixed substrate. A K_m value for methionine of 75 μM was calculated from a double-reciprocal plot. The enzyme inhibition by epoxy **I(a)–(c)** and epithioamino acids **II(a)–(c)** was analysed first by the preincubation method. **I(a)–(b)** and **II(a)–(c)** failed to irreversibly inactivate the enzyme, no loss of activity being observed in these experiments. Next, these amino acids were tested as competitive inhibitors with respect to methionine. Kinetic analysis of the inhibition exerted by **I(a)** and **II(a)** is shown, as examples, in Figure 2 where the double reciprocal plots are indicative of competitive inhibition. Similar experiments were carried out with the other derivatives **I(b)–(c)** and **II(b)–(c)** which also exhibit the same type of competitive inhibition pattern. The K_i values, thus obtained, are listed in Table I, as well as the IC_{50} values. From these results it appears that the inhibitory potency of epoxy amino acids is significantly higher than their corresponding epithio structural analogues. Similar increases in the binding capacity of inhibitors with replacement of a sulphur atom by an oxygen has already been observed.[17] It is difficult to rationalise this

TABLE I Kinetic constants of compounds **I(a)–(c)**, **II(a)–(c)** for α-isoenzyme from the recombinant rat liver AdoMet synthetase

	Compound	K_i (mM)	IC_{50} mM*
I(a)		0.007	0.05
I(b)		0.061	0.10
I(c)		0.105	0.11
II(a)		0.10	0.14
II(b)		0.14	0.3
II(a)		0.14	0.4

*The enzyme activity was measured at 40 μM L-methionine.

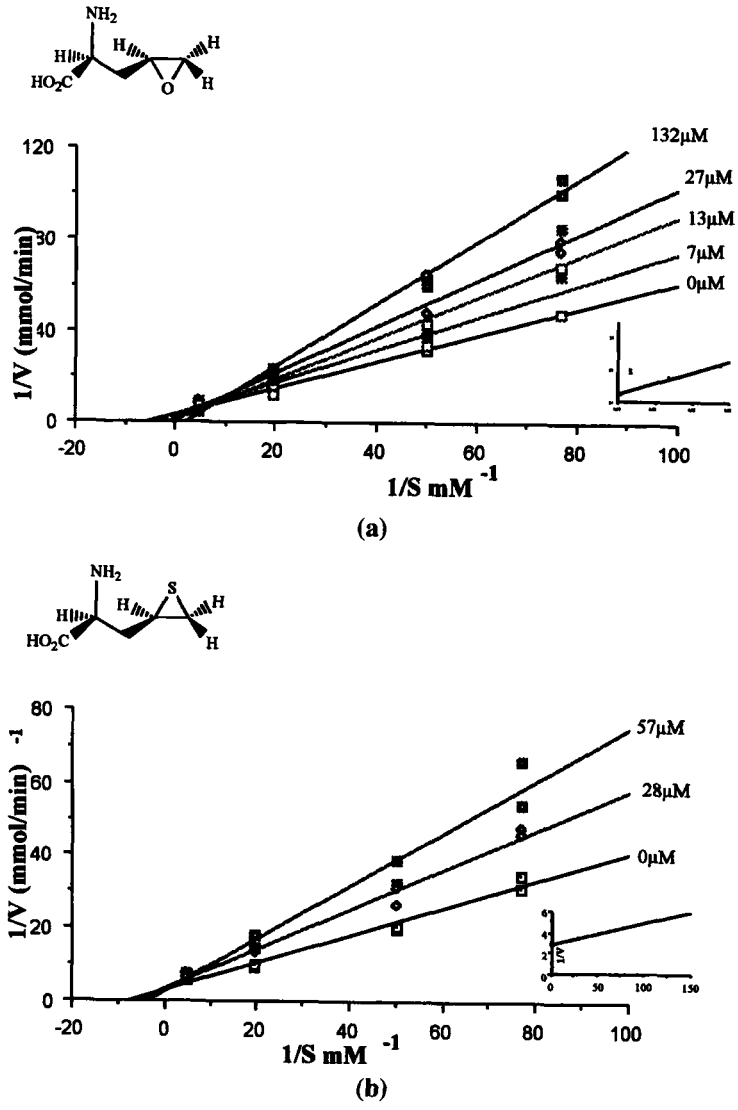


FIGURE 2 Lineweaver–Burk plots of AdoMet synthetase inhibition by **I(a)** and **II(a)**. Purified enzyme preparation was incubated in the standard mixture at a fixed 5 mM ATP concentration while the concentration of Met* was varied from 12 to 200 μM, in the absence or presence of varied concentrations of inhibitor as indicated. The inset shows Dixon plots for IC_{50} determinations, [Met] = 40 μM.

observation but a plausible hypothesis might be that a better coordination effect is developed by oxygen analogues of the substrate with the bivalent cations present at the active site of the enzyme.[18] When the oxirane ring in **I** or thiirane ring in **II** is substituted by a methyl group, irrespective of the

stereochemistry at C-5, steric hindrance is encountered, binding becomes more difficult and less inhibition is observed.

Among the compounds tested, the (2*S*, 4*S*)-2-amino-4,5-epoxypentanoic acid, with an apparent $K_i = 7\,\mu M$, is a potent inhibitor compared with other well studied AdoMet synthetase inhibitors, cycloleucine[19] or L-*cis*-AMB and L-*cis*-AMTB.[17] Therefore this epoxy amino acid is of interest for further combination drug studies using new mechanism based inhibitors of AdoHcy hydrolase that we have recently synthesised.[20]

Acknowledgements

The authors thank the CNRS and ZENECA Pharma (Reims) for financial support and the "Conseil Général de la Marne" for a fellowship (K.L.).

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