for all fractions. The fractions were combined, and the solvent was removed in vacuo. Water was added several times and reevaporated to remove excess ammonium bicarbonate. The residue was dissolved in water and freeze-dried, giving 0.102 g, 79% yield, of the title compound: ^{1}H-NMR (DMSO) 7.40 (s, 1 H, H6), 6.05 (dd, $J_{1',2'H_a}$ = 2.6 Hz, $J_{1',2'H_b}$ = 6.6 Hz, 1 H, H1'), 4.3–4.15 (m, 1 H, H3'), 3.8–3.6 (m, 1 H, H4'), 2.45–2.15 (m, 4 H, H2' and H6'), 1.78 (s, 3 H, 5-CH_3), 1.6–1.25 (m, 2 H, H5'). Anal. ($C_{11}H_{16}N_5O_6P$) C, H, N.

(2*R*,4*S*,5*R*)-1-[4-Azidotetrahydro-5-(2-triphosphonoethyl)-2-furyl]thymine (7). Compound **6** (0.071 g, 0.17 mmol) was converted to the triethylammonium salt by evaporation from 35 mL of 100 mM triethylammonium bicarbonate two times. Residual water was removed by coevaporation with acetonitrile. The triethylammonium salt was dissolved in 3.5 mL of 1,3-dimethyl-3,4,5,6-tetrahydropyrimidin-2(1*H*)-one that had been dried over calcium hydride. 1,1'-Carbonyldiimidazole (0.143, 0.88 mmol) was added, and the reaction was stirred at room temperature for 45 min. Methanol (0.06 mL, 1.5 mmol) was added, and stirring was continued for 35 min. Tributylammonium pyrophosphate (0.404 g, 0.87 mmol) was added and stirring continued for an additional 65 min. The reaction was terminated by the addition of 30 mL of cold water. The entire mixture was diluted to 100 mL with water and applied to a 2.5 × 15 cm column of DEAE Sephadex A-25 that had been equilibrated in 50 mM ammonium bicarbonate. The column was eluted with 300 mL of 50 mM ammonium bicarbonate followed by 1100 mL of 100 mM ammonium bicarbonate. A linear gradient of 1 L of ammonium bicarbonate (200–600 mM) was used to elute compound **7**. The fractions containing **7** were combined, dried in vacuo, redissolved in water, and then dried and dissolved twice more to give 0.125 mmol (73% yield) as the tetraammonium salt. Ammonia was determined by a previously published method.[13] The purity of **7** was determined to be 99.7% by analytical HPLC (strong anion-exchange column eluted with a gradient of 10 mM to 1 M ammonium phosphate, pH 5.5) monitored at 266 nm: UV pH1 HCl λ_{max} 266 nm, λ_{min} 234 nm, pH 7.0 potassium phosphate λ_{max} 266 nm, λ_{min} 234 nm, pH 13 NaOH λ_{max} 266 nm, λ_{min} 244 nm; ^{1}H NMR (D_2O) δ 7.4 (1 H, s, H6), 6.1 (1 H, t, H1), 4.1 (1 H, m, H3'), 3.8 (1 H, m, H4'), 2.4 (2 H, t, H6'), 1.8 (5 H, m, H2', H5', CH_3); ^{31}P NMR (D_2O, 1 mM EDTA) δ 18.5 (d, α-P), −21.9 (dd, β-P), −6.3 (d, λ-P).

An aliquot of **7** (12 mM) was treated with alkaline phosphatase (30 IU/mL) or snake venom phosphodiesterase I (30 IU/mL), and 33 mM 2-amino-2-methylpropanol (pH 9.5) at room temperature. Samples were withdrawn at various times and chromatography was performed on thin-layer PEI-cellulose (Brinkman) in 0.8 M LiCl/0.8 M formic acid 1:1 (R_f = 0.06 for **7**, 0.37 for the diphosphate analog, and 0.76 for **6**). Over a 4-h period, sequential conversion of triphosphate to diphosphate to monophosphonate was observed in the alkaline phosphatase-treated samples. In the phosphodiesterase-treated samples, the triphosphate was cleaved directly to the monophosphonate.

(*E*,2*R*,4*S*,5*R*)-1-[4-Azidotetrahydro-5-[2-(*O*-phenylphosphono)vinyl]-2-furyl]thymine (8). Compound **3** (0.25 g, 0.51 mmol) was converted to the title compound in a manner analogous to the conversion of compound **4** to compound **5** (60% yield, 0.140 g): ^{1}H-NMR (DMSO) 7.38 (s, 1 H, H6), 7.2–6.85 (m, 5 H, phenyl), 6.5–6.2 (m, 1 H, H5'), 6.1–5.85 (m, 2 H, H6' and H1'), 4.3–4.15 (m, 1 H, H3'), 4.15–4.05 (m, 1 H, H4'), 2.4–2.2 (m, 2 H, H2'), 1.75 (s, 3 H, 5-CH_3). Anal. ($C_{17}H_{18}N_5O_6P$) C, H, N.

(*E*,2*R*,4*S*,5*R*)-1-[4-Azidotetrahydro-5-[2-phosphonovinyl]-2-furyl]thymine (9). Compound **8** was converted to the title compound in a manner analogous to the conversion of compound **5** to compound **6** (79% yield): ^{1}H-NMR (DMSO) 7.48 (s, 1 H, H6), 6.2–5.9 (m, 3 H, H1', H6', and H5'), 4.5–4.3 (m, 1 H, H3'), 4.2–4.1 (m, 1 H, H4'), 2.4–2.2 (m, 2 H, H2'), 1.8 (s, 3 H, 5-CH_3). Anal. ($C_{11}H_{14}N_5O_6P$) C, H, N.

Acknowledgment. The authors thank H.M. McGuire for naming compounds, M. St. Clair for antiviral screening, R.C. Crouch for the NMR spectra, and S.R. Shaver and J. Kelly-Hart for technical assistance.

(13) Chaney, A. L.; Marbach, E. P. Modified Reagents for Determination of Urea and Ammonia. *Clin. Chem.* **1962**, *8*, 130–132.

S-(5'-Deoxy-5'-adenosyl)-1-ammonio-4-(methylsulfonio)-2-cyclopentene: A Potent, Enzyme-Activated Irreversible Inhibitor of *S*-Adenosylmethionine Decarboxylase[1]

Yongqian Wu and Patrick M. Woster*

Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, Wayne State University, Detroit, Michigan 48202. *Received April 3, 1992*

The compound *S*-(5'-deoxy-5'-adenosyl)-1-ammonio-4-(methylsulfonio)-2-cyclopentene (AdoMac) was prepared and evaluated as an irreversible inhibitor of *S*-adenosylmethionine decarboxylase (AdoMet-DC). AdoMac was shown to inhibit AdoMet-DC in a time-dependent manner with a K_I of 18.3 μM and a k_{inact} of 0.133 min^{-1}. In addition, AdoMet-DC activity could not be restored following extensive dialysis of the enzyme–inhibitor complex, and the enzyme was protected from irreversible inactivation by the known competitive inhibitor methylglyoxal bis(guanylhydrazone). HPLC analysis of the enzymatic reaction products revealed a time-dependent decrease in the peak coeluting with AdoMac, and a corresponding increase in the peak coeluting with (methylthio)adenosine (MTA), a byproduct of the irreversible binding of AdoMac to the enzyme. Thus, AdoMac appears to function as an enzyme-activated, irreversible inhibitor of AdoMet-DC.

Introduction

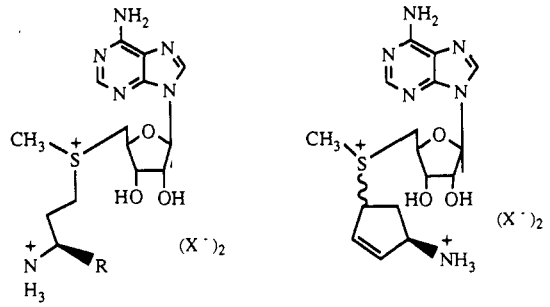
The polyamine pathway represents a logical target for chemotherapeutic intervention, since depletion of polyamines results in the disruption of a variety of cellular functions, and ultimately in cell death.[2] Inhibitors of the polyamine pathway, therefore, have traditionally been developed as potential antitumor or antiparasitic agents. Such inhibitors also play a critical role as research tools

(1) Presented in part at the 203rd National Meeting of the American Chemical Society, April 5–10, 1992, San Francisco, CA (Abstract #MEDI 24).

(2) Sunkara, P. S.; Baylin, S. B.; Luk, G. D. Inhibitors of Polyamine Biosynthesis: Cellular and *in Vivo* Effects on Tumor Proliferation. In *Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies* McCann, P. P., Pegg, A. E., Sjoerdsma, A., Eds.; Academic Press: New York, 1987; pp 121–140.

0022-2623/92/1835-3196$03.00/0

Chart I

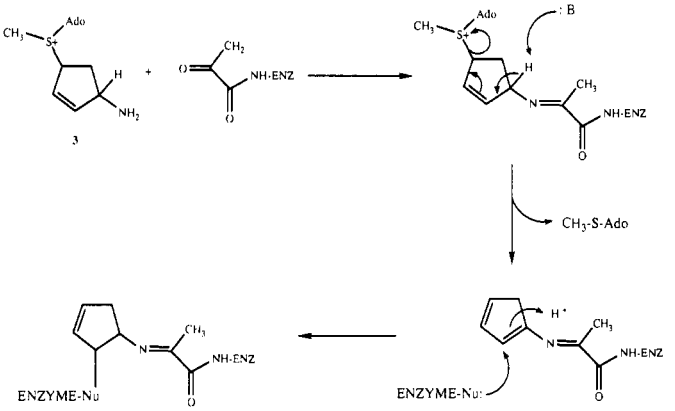


1 R = COOH
2 R = H

3

to elucidate the cellular functions of the naturally occurring polyamines, especially if these agents are specific for a single enzyme in the pathway. However, a lack of information concerning the catalytic sites of the enzymes involved in polyamine biosynthesis, coupled with an incomplete understanding of the metabolism and cellular functions of the individual polyamines, presents a formidable problem in terms of the rational design of novel inhibitor molecules. *S*-Adenosylmethionine decarboxylase (AdoMet-DC) is a rate-controlling enzyme in polyamine biosynthesis.[3] It catalyzes the decarboxylation of *S*-(5′-deoxy-5′-adenosyl)methionine, **1** (AdoMet, Chart I), to give decarboxylated AdoMet, **2** (dc-AdoMet), which is the aminopropyl donor for the aminopropyltransferases spermidine synthase an spermine synthase. AdoMet-DC contains a covalently linked pyruvate which is essential for activity and participates in catalysis by formation of a Schiff's base with the substrate.[4] Danzin and co-workers have taken advantage of this mechanistic feature to synthesize a potent, enzyme-activated irreversible inhibitor of AdoMet-DC.[5] As part of an ongoing program to develop restricted-rotation inhibitors of the polyamine biosynthetic pathway, we have designed the putative irreversible inhibitor **3** (Chart I), a conformationally restricted analog of dc-AdoMet. Such an analog, if active, could prove to be of considerable value as a conformational active site probe for AdoMet-DC. It was proposed that compound **3** would bind to the enzyme-linked pyruvoyl residue of AdoMet-DC in the same manner as the natural substrate, and would then form a highly reactive Michael acceptor at the active site by general-base catalysis, as shown in Scheme I. Attack by a nucleophile located on the surface of the enzyme would then lead to an irreversible binding of the inhibitor to the active site, resulting in a nonfunctional enzyme. In order to determine the feasibility of using compound **3** as an irreversible inhibitor of AdoMet-DC, we have developed a synthetic route which affords the target molecule in good overall yield, and which is adaptable to the ultimate production of each of the four possible diastereoisomers of **3** in pure form. We now report the synthesis and enzymatic characterization of *S*-(5′-deoxy-5′-adenosyl)-1-ammonio-4-(methylsulfonio)-2-cyclopentene (AdoMac), **3**, an enzyme-activated, irreversible inhibitor of AdoMet-DC.

Scheme I



Chemistry

The synthetic route leading to compound **3** is outlined in Scheme II. (+)-(1*R*,4*S*)-*cis*-1-Acetoxy-4-hydroxy-2-cyclopentene (**6**) was synthesized from the corresponding *meso*-diacetate[6,7] by lipase-mediated cleavage of the *pro*-(*S*)-acetate.[8] The desired enantiomer of **6** is determined to be present in 97% enantiomeric excess by optical rotation, and by NMR analysis of the corresponding Mosher ester.[9] Substitution of phthalimide at the free hydroxyl under Mitsunobu conditions[10] produces the *N*-phthalimidoamino ester **7** with 100% inversion of configuration. Compound **7** is then subjected to hydrazinolysis to afford the amino ester **8**. The amine is Boc protected (di-*tert*-butyl dicarbonate[11]) to yield **9**, followed by hydrolysis of the acetate (LiOH) to afford the free alcohol **10**. The desired enantiomer of **10** is found to be present in 97% ee by NMR analysis of the Mosher ester,[9] confirming that no racemization has occurred during the synthesis. The *N*-Boc-protected amino alcohol **10** is then converted to the corresponding chloride **11** (MsCl, LiCl[12]). During this conversion of an allylic alcohol to the corresponding chloride, the stereochemical control is lost at position 1, but retained at position 4 of compound **11**. Thus the cyclopentenyl synthon **11** is isolated as a 64:36 mixture of the cis and trans diastereoisomers, respectively, as determined by NMR analysis. Our attempts to separate

(3) Pegg, A. E. Recent Advances in the Biochemistry of Polyamines in Eukaryotes. *Biochem. J.*, **1986**, *234*, 249–262.

(4) Wickner, R. B.; Tabor, C. W.; Tabor, H. Purification of Adenosylmethionine Decarboxylase from *Escherichia coli* W: Evidence for Covalently Bound Pyruvate. *J. Biol. Chem.* **1970**, *245*, 2132–2139. (b) Recsei, P. A.; Snell, E. E. Pyruvoyl Enzymes. *Annu. Rev. Biochem.* **1984**, *53*, 357–387.

(5) Casara, P.; Marchal, P., Wagner, J.; Danzin, C. 5′-{[(Z)-4-Amino-2-butenyl]methylamino}-5′-deoxyadenosine: A Potent Enzyme Activated Irreversible Inhibitor of S-Adenosyl-L-methionine Decarboxylase from *Escherichia coli*. *J. Am. Chem. Soc.* **1989**, *111*, 9111–9113.

(6) Kaneko, C., Sugimoto, A.; Tanaka, S. A Facile One-Step Synthesis of *cis*-2-Cyclopentene and *cis*-2-Cyclohexene-1,4-diols from the Corresponding Cyclodienes. *Synthesis* **1974**, 876–877.

(7) Johnson, C. R.; Penning, T. D. Triply Convergent Synthesis of (−)-Prostaglandin E_2 Methyl Ester. *J. Am. Chem. Soc.* **1988**, *110*, 4726–4735.

(8) Laumen, K.; Schneider, M. P. A Facile Chemoenzymatic Route to Optically Pure Building Blocks for Cyclopentanoid Natural Products. *J. Chem. Soc. Chem. Commun.* **1986**, 1298–1299.

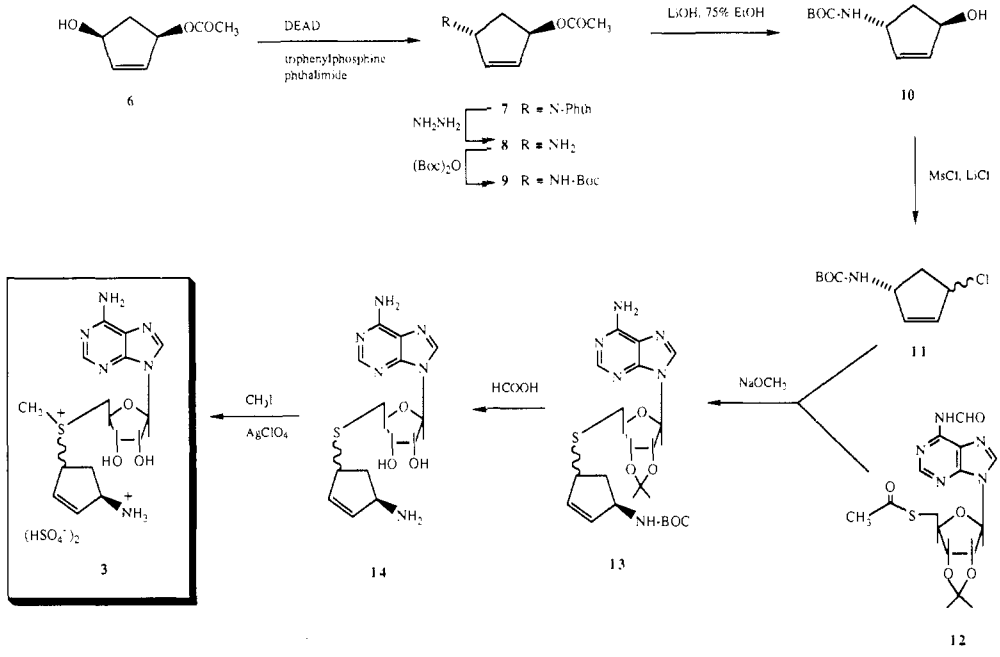
(9) Dale, J. A., Dull, D. L.; Mosher, H. S. α-Methoxy-α-trifluoromethylphenylacetic Acid, a Versatile Reagent for the Determination of Enantiomeric Composition of Alcohols and Amines. *J. Org. Chem.* **1969**, *34*, 2543–2549.

(10) Mitsunobu, O. The Use of Diethyl Azodicarboxylate and Triphenylphosphine in Synthesis and Transformation of Natural Products. *Synthesis* **1981**, 1–28.

(11) Keller, O.; Keller, W. E.; van Look, G.; Wersin, G. *tert*-Butoxycarbonylation of Amino Acids and their Derivatives: N-*tert*-Butoxycarbonyl-L-phenylalanine. *Org. Synth.* **1985**, *63*, 160–171.

(12) Collington, E. W.; Meyers, A. I. A Facile and Specific Conversion of Allylic Alcohols to Allylic Chlorides Without Rearrangement. *J. Org. Chem.* **1971**, *36*, 3044–3045.

Scheme II



this mixture by careful flash column chromatography have been unsuccessful.

Coupling of the substituted cyclopentenyl ring **11** to 5′-(thioacetyl)-2′,3′-isopropylideneadenosine **12** (synthesized by a literature procedure[13,14]) is accomplished in a 50:50 mixture of methanol and DMF in the presence of sodium methoxide[15] (Scheme II), resulting in the formation of the fully protected thionucleoside **13**. Rigorous exclusion of oxygen is ensured during this procedure by freezing and thawing the mixture five times under a vigorous stream of argon, since even a trace of oxygen catalyzes the rapid dimerization of the thiolate derived from **12** in the presence of sodium methoxide. As was the case with compound **11**, we have been unable to resolve the mixture of diastereomers of compound **13** on a preparative scale by either flash chromatography or HPLC. Thus, subsequent steps in the synthesis are carried out on the diastereomeric mixture. Simultaneous removal of both the *N*-Boc and 2′,3′-isopropylidene protecting groups in **13** (88% formic acid) yields thioether **14**, which is methylated using a modification of the method of Samejima[16] (CH_3I, $AgClO_4$, AcOH/HCOOH) to provide the desired target compound **3**.

Biological Results

A series of experiments were conducted to determine whether compound **3** produces time-dependent inactivation of AdoMet-DC. The time-dependent decrease in enzyme activity was monitored at 7, 10, 20, and 30 μM concentrations of **3** over a period of 20 min. In each case, the decrease in activity is linear (Figure 1), and a rate constant was derived from each line. The resulting data was then replotted using the Kitz–Wilson method[17] (Figure 2). This plot indicates that compound **3** has a K_I value of 18.3 μM for AdoMet-DC and exhibits a k_{inact} value of

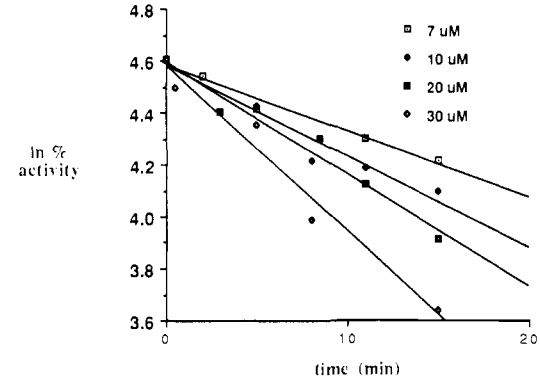


Figure 1. Time-dependent inactivation of AdoMet-DC by **3** at concentrations of 7, 10, 20, and 30 μM.

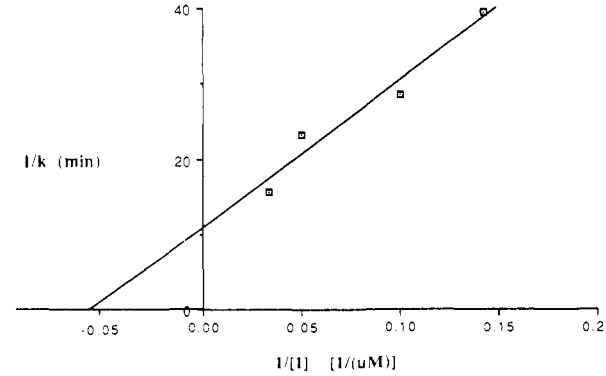


Figure 2. Results of Kitz–Wilson analysis for compound **3**.

(13) Coward, J. K.; Anderson, G. L.; Tang, K.-C. Aminopropyltransferase Substrates and Inhibitors. *Methods Enzymol.* **1984**, *94*, 286–294.

(14) Woster, P. M.; Black, A. Y.; Duff, K. J.; Coward, J. K.; Pegg, A. E. Synthesis and Biological Evaluation of S-Adenosyl-1,12-diamino-3-thio-9-azadodecane, a Multisubstrate Adduct Inhibitor of Spermine Synthase. *J. Med. Chem.* **1989**, *32*, 1300–1307.

(15) Douglas, K. A.; Zormeier, M. M.; Marcolina, L. M.; Woster, P. M. Restricted Rotation Analogs of Decarboxylated S-Adenosylmethionine as Inhibitors of Polyamine Biosynthesis. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 267–270.

(16) Samejima, K.; Nakazawa, Y.; Matsunaga, I. Improved Synthesis of Decarboxylated S-Adenosylmethionine and Related Sulfonium Compounds. *Chem. Pharm. Bull.* **1978**, *26*, 1480–1485.

(17) Kitz, R.; Wilson, I. B. Esters of Methanesulfonic Acid as Irreversible Inhibitors of Acetylcholinesterase. *J. Biol. Chem.* **1962**, *237*, 3245.

0.133 min^{-1}. Two additional studies suggest that **3** is an active-site-directed, irreversible inhibitor of AdoMet-DC. When the enzyme is incubated with 100 μM MGBG (a known inhibitor of AdoMet-DC) for 10 min and then exposed to **3** for 10 min and assayed, 84.6% of the AdoMet-DC activity is retained. Under the same reaction conditions (except for the exclusion of MGBG), only 20% of the activity remains after 10 min, suggesting that **3** is an active-site-directed agent. In addition, compound **14**, the thioether precursor to target compound **3**, was evaluated as an inhibitor of AdoMet-DC and found to be inactive. Presumably, the lack of a charged sulfonium center in **14** diminishes its affinity for the active site of AdoMet-DC, and thus it is unable to interact with the terminal pyruvate of the catalytic subunit. The irreversibility of **3** is demonstrated by the inability to dialyze away the inhibitor following binding to AdoMet-DC. AdoMet-DC is incubated with 50 μM **3** for 30 min and then assayed (22% activity remaining). The sample is then dialyzed overnight (62.5 mM Tris-HCl/100 mM $MgSO_4$, pH 7.4, four buffer changes) and reassayed. There is no change in the activity level of AdoMet-DC following dialysis, suggesting that the binding of **3** to AdoMet-DC is irreversible.

HPLC analysis of the product mixture after quenching the enzymatic reaction at various times is accomplished using the reverse-phase, ion pairing assay system to Danzin.[18] This experiment reveals the time-dependent appearance of a peak which coeluted with MTA (t_R = 18.27 min), and the corresponding disappearance of the peak coeluting with **3** (t_R = 23.26 min, data not shown), suggesting that MTA is generated from **3** in the enzymatic reaction as predicted.

Discussion

The purpose of this preliminary study was 2-fold: (1) to determine whether a cyclopentenyl restricted-rotation analog of AdoMet such as **3** would serve as an enzyme-activated, irreversible inhibitor of AdoMet-DC and (2) to develop a synthetic route which would be adaptable to the production of all of the possible pure diastereoisomers. The data presented indicates that compound **3** is indeed a potent, irreversible, enzyme-activated inhibitor of AdoMet-DC. In addition, the synthetic route leading to **3** can be readily adapted to the production of all four possible diastereomers of **3**.

Although the primary amino acid sequence of AdoMet-DC is known, there is little information available regarding the three-dimensional structure of the enzyme, and in particular, the amino acid residues present in the catalytic site. Diaz and Anton[19] have recently proposed the existence of an active site cysteinyl residue which is alkylated during the substrate-dependent inactivation of the enzyme. This cysteine residue is proposed to react with an acrolein-like substance which is generated once every 6000–7000 turnovers as a result of transamination of the terminal pyruvate. The driving force for transamination of the terminal pyruvate is enzyme-assisted decarboxylation, followed by incorrect protonation of the enolate intermediate and then hydrolysis of the resulting imine. Although the proposed adduct generated from imine formation between AdoMac and the terminal pyruvate of AdoMet-DC does not possess a carboxylate moiety (see Scheme I), the elimination of MTA is a sufficient driving force for generation of the proposed latent electrophile. A nucleophilic amino acid residue may then be envisioned as adding to this conjugated system by either a 1,4- or 1,6-addition mechanism. A mechanism for transamination of the terminal pyruvate of AdoMet-DC by **3** following formation of the imine linkage may also be envisioned, although it is probably less likely than elimination of MTA and conjugate addition of a nucleophilic residue. Since MTA generation is observed during inactivation of AdoMet-DC by **3**, it is reasonable to assume that the latent electrophile shown in Scheme I is formed and that inactivation of the enzyme is most likely the result of cross-link formation with a neighboring nucleophilic residue. Additional experiments are required to support this hypothesis and to rule out the possibility of inactivation of AdoMet-DC by transamination in the presence of **3**. Due to the similarity between the latent electrophile produced during substrate-dependent inactivation and that produced by interaction of AdoMet-DC with **3**, we postulate that AdoMac could react with a nucleophilic residue such as the cysteine proposed by Diaz and Anton, resulting in the formation of a modified amino acid residue in the catalytic site. Treatment of the alkylated enzyme adduct shown in Scheme I with $NaBH_4$ would then trap this cross-linked enzyme species, resulting in a stable adduct which could be analyzed by sequencing the modified protein. Information of this type would be valuable in terms of elucidating specific active-site amino acid residues, and these experiments are currently in progress.

As stated above, one purpose of this preliminary study was to demonstrate the feasibility of using a restricted-rotation analog of AdoMet such as **3** as an irreversible inhibitor of AdoMet-DC. Although it is beyond the scope of this preliminary investigation, it may prove valuable to isolate each of the four possible diastereomeric forms of **3** for use as conformational active-site probes. A procedure is available to synthesize (−)-(1*R*,4*S*)-*cis*-1-hydroxy-4-acetoxy-2-cyclopentene, the enantiomer of compound **6**, via selective cleavage of the *pro*-(*R*)-acetate of *meso*-diacetate **5**.[20] Elaboration of this intermediate via the synthetic pathway outlined in Scheme II should then yield a mixture of the other two diastereomers of inhibitor **3**. Adaptation of the synthesis as described above, as well as development of a method for the resolution of the pure diastereomers of **3**, are ongoing concerns in our laboratories.

Experimental Section

cis-2-Cyclopentene-1,4-diol (**4**) and *cis*-1,4-diacetoxy-2-cyclopentene (**5**) were synthesized according to the procedure of Kaneko[6] as modified by Johnson.[7] Compound **6** was synthesized from *cis*-1,4-diacetoxy-2-cyclopentene (**5**) as described by Laumen and Schneider.[8] 5′-(Thioacetyl)-2′,3′-isopropylideneadenosine **12** was synthesized from 2′,3′-isopropylideneadenosine in two steps as previously described.[13,14] Porcine lipase (Type II, EC 3.1.1.3) was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. and were used without further purification except as noted below. Pyridine was dried by passing it through an aluminum oxide column and then stored over KOH. Triethylamine was distilled from potassium hydroxide and stored under a nitrogen atmosphere. Methanol was distilled from magnesium and iodine under a nitrogen atmosphere and

(18) Wagner, J.; Danzin, C.; Mamont, P. Reversed-Phase Ion Pair Liquid Chromatographic Procedure for the Simultaneous Analysis of S-Adenosylmethionine, Its Metabolites and the Natural Polyamines. *J. Chromatogr.* **1982**, *227*, 349–368.

(19) Diaz, E.; Anton, D. L. Alkylation of an Active-Site Cysteinyl Residue during Substrate-Dependent Inactivation of *Escherichia coli* S-Adenosylmethionine Decarboxylase. *Biochemistry* **1991**, *30*, 4078–4081.

(20) Laumen, K.; Schneider, M. Enzymatic Hydrolysis of Prochiral *cis*-1,4-Diacyl-2-cyclopentenediols: Separation of (1S,4R) and (1R,4S)-4-Hydroxy-2-cyclopentnyl Derivatives, Versatile Building Blocks for Cyclopentanoid Natural Products. *Tetrahedron Lett.* **1984**, *25*, 5875–5878.

stored over molecular sieves. Methylene chloride was distilled from phosphorus pentoxide, and chloroform was distilled from calcium sulfate. Tetrahydrofuran was purified by distillation from sodium and benzophenone. Dimethyl formamide was dried by distillation from anhydrous calcium sulfate and was stored under nitrogen. Preparative-scale chromatographic procedures were carried out using E. Merck silica gel 60, 230–440 mesh. Thin-layer chromatography was conducted on Merck precoated silica gel 60 F-254.

All ^{1}H- and ^{13}C-NMR spectra were recorded on a General Electric QE-300 spectrometer, and all chemical shifts are reported as δ values referenced to TMS. Infrared spectra were recorded on a Nicolet 5DXB-FT-IR spectrophotometer and are referenced to polystyrene. Ultraviolet spectra were recorded on a Beckman DU-8B spectrophotometer. In all cases, ^{1}H-NMR, ^{13}C-NMR, IR, and UV spectra were consistent with assigned structures. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN, and are within 0.4% of the calculated values.

(1*R*,4*R*)-*trans*-1-Acetoxy-4-(*N*-phthalimidoamino)-2-cyclopentene (7). A 2.0-g portion (0.014 mol) of **6** was dissolved in 14 mL of dry THF under a nitrogen atmosphere along with 3.69 g (0.014 mol) of triphenylphosphine and 2.07 g (0.014 mol) of phthalimide. Diethyl azodicarboxylate (2.45 g, 0.014 mol) dissolved in 6 mL of THF was added slowly via syringe, and the reaction was allowed to stir at room temperature overnight under nitrogen. A 10-mL portion of ether was added to precipitate the triphenylphosphine oxide, the mixture was filtered, and the filtrate was concentrated in vacuo. The residue was purified on silica gel (hexane/ethyl acetate 6:4) to afford 3.16 g (83.2% yield) of **7** as a white solid: ^{1}H NMR ($CDCl_3$) δ 7.82 (m, 2 H, aromatic H-3 and H-6), 7.73 (m, 2 H, aromatic H-4 and H-5), 6.15 (m, 1 H, H-3), 6.05 (m, 1 H, H-1), 5.98 (m, 1 H, H-2), 5.56 (m, 1 H, H-4), 2.55 (m, 1 H, H-5 cis to OH), 2.27 (m, 1 H, H-5 trans to OH), 2.07 (s, 3 H, CH_3); ^{13}C NMR ($CDCl_3$) δ 170.9 (ester C=O), 167.9 (phthalimide C=O), 134.8 (C-2), 134.1 (aromatic C-1 and C-2), 133.6 (aromatic C-3 and C-6), 131.9 (C-4), 123.2 (aromatic C-4 and C-5), 79.6 (C-1), 54.3 (C-4), 35.5 (C-5), 21.2 (CH_3); IR (KBr) 2944 (aliphatic C—H), 1728 (phthalimido C=O), 1709 (ester C=O), 1470, 1383 (C=H) cm^{-1}. Anal. ($C_{15}H_{13}NO_4$), C, H, N.

(1*R*,4*R*)-*trans*-1-Acetoxy-4-amino-2-cyclopentene (8). Compound **7** (0.856 g, 0.0032 mol) was dissolved in 20 mL of 0.2 M methanolic hydrazine and allowed to reflux for 7 h. After cooling, a white precipitate was filtered off, and the solvent was removed in vacuo. The solid, light yellow residue was dissolved in 25 mL of dichloromethane, refiltered, and washed with two 25-mL portions of water. The organic layer was then evaporated to afford relatively pure **8** (estimated by NMR) as a yellow oil (0.375 g, 82.9%) which was immediately used in the subsequent step without further purification: ^{1}H NMR ($CDCl_3$) δ 6.04 (m, 1 H, H-2), 5.87 (m, 1 H, H-3), 5.73 (m, 1 H, H-1), 4.16 (m, 1 H, H-4), 2.25 (m, 1 H, H-5 cis to OH), 2.00 (s, 3 H, CH_3), 1.83 (m, 1 H, H-5 trans to OH), 1.62 (broad s, 2 H, NH_2); ^{13}C NMR δ ($CDCl_3$) 170.9 (C=O), 142.8 (C-2), 130.0, (C-3), 79.4 (C-1), 56.6 (C-4), 41.4 (C-5), 21.2 (CH_3); IR (KBr) 3340 (N—H), 1713 (ester C=O), 1460, 1371 (C=H) cm^{-1}.

(1*R*,4*R*)-*trans*-1-Acetoxy-4-[[(*tert*-butyloxy)carbonyl]amino]-2-cyclopentene (9). To a solution of $NaHCO_3$ (0.361 g, 0.0043 mol) and NaCl (0.643 g, 0.011 mol) in 10 mL of water was added 0.600 g (0.0043 mol) of compound **8** in 10 mL of $CHCl_3$, and the reaction mixture was cooled to 0 °C in an ice bath. A 0.938-g portion (0.0043 mol) of di-*tert*-butyl dicarbonate was added slowly, and the two-phase mixture was allowed to warm to room temperature. The reaction mixture was then refluxed for 2.5 h, cooled, and the chloroform was removed with a rotary evaporator. The aqueous layer was diluted to 25 mL and washed with three 25-mL portions of $CHCl_3$. The combined organic layers were then washed with 25 mL each of 5% $NaHSO_4$, water, and saturated NaCl and then dried over anhydrous $MgSO_4$. Filtration and removal of the solvent in vacuo afforded a clear oil which was chromatographed on silica gel (hexane/ethyl acetate 6:4) to give 1.01 g (96.5%) of **9** as a white solid: ^{1}H NMR ($CDCl_3$) δ 6.07 (m, 1 H, H-2), 5.98 (m, 1 H, H-3), 5.77 (m, 1 H, H-1), 4.94 (m, 1 H, H-4), 4.53 (m, 1 H, NH), 2.35 (m, 1 H, H-5 cis to OH), 2.05 (s, 3 H, CH_3), 1.94 (m, 1 H, H-5 trans to OH), 1.47 (s, 9 H, C-CH_3); ^{13}C NMR ($CDCl_3$) δ 173.2 (ester C=O), 155.3 (carbamate C=O), 138.5 (C-2), 132.2 (C-3), 79.6 (C-1), 55.6 (C-4), 38.8 (C-5), 27.1 (C-CH_3), 21.1 (ester CH_3); IR (KBr) 3367 (N—H), 2924 (aliphatic C—H), 1728 (ester C=O), 1679 (carbamate C=O), 1461, 1377 (C=C) cm^{-1}. Anal. ($C_{12}H_{19}NO_4$) C, H, N.

(1*R*,4*R*)-*trans*-1-Hydroxy-4-[[(*tert*-butyloxy)carbonyl]amino]-2-cyclopentene (10). A 0.500-g portion of compound **9** (0.0021 mol) was dissolved in 25 mL of 75% ethanol along with 0.130 g (0.0031 mol) of $LiOH\cdot H_2O$, and the mixture was allowed to stir at room temperature overnight. The reaction mixture was then evaporated, and the residue was partitioned between 25 mL each of water and ethyl acetate. The aqueous layer was washed with two additional 25-mL portions of ethyl acetate, and the combined organic layers were washed with 25 mL each of 5% $NaHSO_4$, water, and saturated NaCl and then dried over anhydrous $MgSO_4$. Filtration and removal of the ethyl acetate in vacuo then afforded the crude product, which was purified on silica gel (hexane ethyl acetate 4:6) to yield pure **10** (0.403 g, 96.3%) as a white solid {$[\alpha]^{20}_D$ 182.1° (*c* 10.0, $CHCl_3$ + 1% EtOH}. Analysis of the product by the Mosher procedure[9] confirmed an enantiomeric excess of >99%: ^{1}H NMR ($CDCl_3$) δ 5.97 (complex m, 2 H, H-2 and H-3), 4.96 (complex m, 2 H, H-1 and H-4), 4.43 (m, 1 H, NH), 2.18 (m, 1 H, H-5 cis to OH), 1.94 (m, 1 H, H-5 trans to OH), 1.45 (s, 9 H, CCH_3); ^{13}C NMR ($CDCl_3$) δ 155.5 (C=O), 136.4 (C-2), 136.2 (C-3), 76.2 (C-1), 56.0 (C-4), 42.4 (C-5), 28.6 (C-CH_3); IR (KBr) 3339, 3186 (N—H, O—H), 2959 (aliphatic C—H), 1695 (C=O), 1461, 1380 (C=C) cm^{-1}. Anal. ($C_{10}H_{17}NO_3$) C, H, N.

(4*R*)-1-Chloro-4-[[(*tert*-butyloxy)carbonyl]amino]-2-cyclopentene (11). A 0.500-g portion of compound **10** (0.0025 mol) was dissolved in 10 mL of dry DMF along with 0.293 g (0.0029 mol, 0.404 mL) of triethylamine and 0.123 g (0.0029 mol) of LiCl under a nitrogen atmosphere. The reaction was cooled to 0 °C and methanesulfonyl chloride (0.332 g, 0.0029 mol, 0.225 mL) was added dropwise. The reaction was allowed to stir at 0 °C overnight. The DMF was removed (rotary evaporator, 0.2 mmHg) and the dark residue was dissolved in 25 mL of chloroform. The chloroform solution was washed with two 25-mL portions of cold 1 N HCl, 25 mL of cold water, and 25 mL of cold saturated NaCl and then dried over anhydrous $MgSO_4$. Filtration, removal of the solvent in vacuo, and chromatography on silica gel (hexane/ethyl acetate 4:6) then afforded pure **11** (64:36 ratio of cis to trans isomers as determined by ^{1}H NMR) as a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 6.01 (complex m, 2 H, H-2 and H-3), 5.05 (m, 0.5 H, H-1), 4.95 (m, 0.5 H, H-4), 4.85 (m, 0.5 H, H-1), 4.75 (m, 0.5 H, H-4), 4.43 (m, 1 H, NH), 2.93 (m, 0.5 H, H-5 of cis isomer cis to Cl), 2.64 (m, 0.5 H, H-5 of trans isomer cis to Cl), 2.05 (m, 0.5 H, H-5 of trans isomer trans to Cl), 1.97 (m, 0.5 H, H-5 of cis isomer trans to Cl), 1.45 (s, 9 H, CH_3); ^{13}C NMR ($CDCl_3$) δ 137.0, 135.1, 134.9, 131.3 (C-2 and C-3), 62.2 and 61.4 (C-1), 55.1 (C-4), 43.1 and 42.7 (C-5), 28.4 (C-CH_3); IR (neat) 2944 (aliphatic C—H), 1675 (C=O), 1456, 1370 (C=C), 666 (C—Cl) cm^{-1}. Although reasonably stable for short periods at low temperature, compound **11** proved to be too unstable to obtain a suitable combustion analysis, and was used immediately in the subsequent step.

***S*-(2′,3′-Isopropylidene-5′-deoxy-5′-adenosyl)-1-[[(*tert*-butyloxy)carbonyl]amino]-4-mercapto-2-cyclopentene (13).** A 0.280-g portion (0.0013 mol) of compound **11** and 0.759 g (0.0019 mol) of 5′-deoxy-N^6-formyl-2′,3′-isopropylidene-5′-(thioacetyl)-adenosine (**12**) were dissolved in 20 mL of a 1:1 mixture of dry DMF and dry methanol under an argon atmosphere. The solution was then frozen and thawed five times in succession with liquid nitrogen while a vigorous stream of argon bubbled through the solution, and then 0.208 g (0.0039 mol) of sodium methoxide powder was added through the condenser. The reaction was then allowed to stir under argon, protected from light, for 3 days. The solvent was then removed (rotary evaporator, 0.2 mmHg), and the residue was partitioned between 50 mL each of water and chloroform. The aqueous layer was washed with two additional 50-mL portions of $CHCl_3$, and the combined organic layers were washed with 50 mL of saturated sodium chloride and dried over anhydrous $MgSO_4$. Filtration, removal of the chloroform, and chromatography on silica gel (ethyl acetate/2-propanol 24:1) afforded **13** as a pale yellow foam (0.356 g, 54.3% yield): ^{1}H NMR ($CDCl_3$) δ 8.36 (s, 1 H, H-2), 7.92 (s, 1 H, H-8), 6.07 (d, 1 H, H-1′), 5.95–5.45 (m, 3 H, H-2′, H-2″, H-3″), 5.66 (br s, 2 H, NH_2), 5.10 (m, 1 H, H-3′), 4.78 (m, 0.5 H, H-1″), 4.73 (m, 0.5 H, H-4″), 4.55–4.30 (m, 2 H, H-4′, NH-Boc), 3.84 (m, 2 H, H-5′), 3.74 (m,

0.5 H, H-4″), 3.70 (m, 0.5 H, H-1″), 3.00–2.50 (complex m, 1 H, H-5″ of cis isomer cis to S and H-5″ of trans isomer cis to S), 2.29 (m, 0.5 H, H-5″ of trans isomer trans to S), 1.93 (m, 0.5 H, H-5″ of cis isomer trans to S), 1.61 (acetonide CH_3), 1.44 (s, 9 H, Boc-CH_3), 1.41 (s, 3 H, acetonide CH_3); IR (neat) 3329 (N—H), 2888 (aliphatic C—H), 1679 (C=O), 1463, 1385 (C=C) cm^{-1}. Anal. ($C_{23}H_{32}N_6O_5S$) C, H, N.

***S*-(5′-Deoxy-5′-adenosyl)-1-amino-4-mercapto-2-cyclopentene (14).** A 0.150-g portion of **13** was dissolved in 3 mL of 88% formic acid and allowed to stir at room temperature for 2 days. The reaction mixture was then diluted to 25 mL with water, and the aqueous layer was extracted with three 25-mL portions of ether and lyophilized to yield a pale yellow solid. The solid was then purified by chromatography on silica gel ($CHCl_3$/MeOH/NH_4OH 9:2:1); fractions containing the product (R_f = 0.52) were pooled, 50 mL of water was added, and the mixture was concentrated on a rotary evaporator until the pH of the aqueous layer was neutral. The aqueous solution was lyophilized to afford pure **14** (0.093 g, 86.2% yield) as a white solid: ^1H NMR (CD_3OD) δ 8.31 (s, 1 H, H-2), 8.21 (s, 1 H, H-8), 6.17 (d, 1 H, H-1′), 5.97 (m, 1 H, H-2″), 5.80 (m, 1 H, H-3″), 5.02 (m, 1 H, H-2′), 4.79 (m, 1 H, H-3′), 4.32 (m, 1 H, H-4′), 4.18 (m, 1 H, H-1″), 4.06 (m, 1 H, H-4″), 3.30 (m, 2 H, H-5′), 2.97 (complex m, 2 H, H-5″); IR (KBr) 3336 (N—H), 3192 (O—H), 2917 (aliphatic C—H) cm^{-1}. Anal. ($C_{15}H_{20}N_6O_3S$) C, H, N.

***S*-(5′-Deoxy-5′-adenosyl)-1-ammonio-4-(methylsulfonio)-2-cyclopentene Disulfate (3).** Compound **14** (0.093 g, 0.00026 mol) was dissolved in 2 mL of a 50:50 mixture of formic acid and acetic acid along with 0.148 g (0.065 mL, 0.001 mol) of iodomethane. A 0.108-g (0.00052 mol) portion of silver perchlorate dissolved in 1.08 mL of 50:50 formic acid/acetic acid (10% w/v) was then added. The reaction mixture was allowed to stir at room temperature overnight, after which the yellow precipitate was removed by centrifugation. The clear solution was diluted to 50 mL with water and extracted with three 25-mL portions of ether, and the aqueous layer was lyophilized to afford a light yellow solid. The solid residue was chromatographed on silica gel (butanol/acetic acid/water 1:1:1), and the product-containing fractions were pooled and diluted to 50 mL with water. The aqueous layer was washed with 25 mL of ether and lyophilized to give a white solid. The solid was dissolved in 0.1 N H_2SO_4, and ethanol was added to precipitate the product as a white solid (0.107 g, 71.6%): ^1H NMR (CD_3OD) δ 8.49 (s, 1 H, H-2), 8.47 (s, 1 H, H-8), 6.46 (m, 1 H, H-2″), 6.40 (m, 1 H, H-3″), 6.12 (d, 1 H, H-1′), 5.08 (m, 1 H, H-2′), 4.75 (m, 1 H, H-3′), 4.58 (m, 1 H, H-4′), 3.91 (complex m, 2 H, H-1″ and H-4″), 2.90 (complex m, 2 H, H-5″), 2.82 (s, 3 H, CH_3); IR (KBr) 3536, 3403 (N—H, O—H), 2935 (aliphatic C—H) cm^{-1}. Anal. ($C_{16}H_{24}N_6O_3S\cdot 2HSO_4\cdot 0.5EtOH$) C, H, N.

Enzyme Purification. AdoMet-DC is isolated from *Escherichia coli* using a modification of the methylglyoxal bis(guanylhydrazone) (MGBG) Sepharose affinity column procedure of Anton and Kutny.[21] The column is prepared by incubating MGBG with epoxy-activated Sepharose 4B at pH 11 as described. *E. coli* (3/4 log phase, Grain Processing Co., Ames, IA) are lysed in 5 volumes of 10 mM Tris-HCl, 0.5 mM EDTA, and 0.5 mM dithiothreitol, pH 8.0, by a single pass through a French press. A 5% solution of streptomycin sulfate is then added to give a final concentration of 1%, and the cell debris is removed by centrifugation at 20000*g* for 2 h. AdoMet-DC is allowed to adsorb to the gel by stirring the gel and the lysate supernatant together for 1 h after bringing the $MgCl_2$ concentration to 10 mM. Binding is considered complete when residual AdoMet-DC activity in the supernatant is determined to be 1–3% of the original value. The gel is then packed into a column and washed (20 mM Tris-HCl, 10 mM $MgCl_2$, 0.6 M KCl, 0.5 mM EDTA, and 0.5 mM dithiothreitol, pH 8.0) until UV absorption reaches baseline. AdoMet-DC is then eluted using 20 mM potassium phosphate, 0.6 M KCl, 0.5 mM EDTA, and 0.5 mM dithiothreitol, pH 7.0, and the fractions of highest activity are pooled and concentrated (Amicon ultrafiltration cell, PM-30 membrane). Protein is measured by the method of Bradford.[22] With this method enzyme purity is greater than 90%, and the specific activity is determined to be 0.80 μmol/min per mg of protein at 37 °C.

Enzyme Assay. AdoMet-DC activity is monitored by following the evolution of $[^{14}C]CO_2$ from *S*-adenosyl-L-[carboxy-^{14}C]-methionine using a modification of the method of Markham.[23] Each reaction mixture contains 50 μg of AdoMet-DC, 40 μL of *S*-adenosyl-L-[carboxy-^{14}C]methionine (0.9 mCi/mmol, 20 μM final concentration) in 62.5 mM Tris-HCl/100 mM $MgSO_4$, pH 7.4, with a final volume of 2 mL. Radiolabeled CO_2 is trapped on a filter disk in a vial cap soaked with 40 μL of hyamine. After 15 min the reaction is quenched, and the disk is placed in a scintillation vial with 10 mL of scintillation cocktail and counted (counting efficiency 95% or greater). Each data point represents the average of two determinations, which in each case differ by less than 5%.

Acknowledgment. Financial support through a research grant from the Elsa U. Pardee Foundation is gratefully acknowledged. We are also indebted to Mr. Kirk A. Douglas and Mr. Kelvin Grant for excellent technical assistance.

(21) Anton, D. L.; Kutny, R. Escherichia coli S-Adenosylmethionine Decarboxylase: Subunit Structure, Reductive Amination and NH_2-Terminal Sequences. *J. Biol. Chem.* **1987**, *262*, 2817–2822.

(22) Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(23) Markham, G. D.; Tabor, C. W.; Tabor, H. S-Adenosylmethionine Decarboxylase of *Escherichia coli*. *J. Biol. Chem.* **1982**, *257*, 12063–12068.

Application of Neural Networks: Quantitative Structure–Activity Relationships of the Derivatives of 2,4-Diamino-5-(substituted-benzyl)pyrimidines as DHFR Inhibitors

Sung-Sau So and W. Graham Richards*

Physical Chemistry Laboratory, South Parks Road, Oxford OX1 3QZ, United Kingdom. *Received March 5, 1992*

A comparative study of quantitative structure–activity relationships involving diaminopyrimidines as DHFR inhibitors using regression analysis and the neural-network approach suggests that the neural network can outperform traditional methods. The technique permits the highlighting the functional form of those parameters which have an influence on the biological activity.

Introduction

The formulation of quantitative structure–activity relationships (QSAR) has had a momentous impact upon medicinal chemistry for the past 30 years. Hansch demonstrated that the biological activities of drug molecules can be correlated by a linear combination of the physicochemical parameters of the corresponding drug. Since then there have been many attempts to include cross-product terms in the regression analysis, but this only added complexity to the study and resulted in no significant im-

0022-2623/92/1835-3201$03.00/0