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Synthesis of nitrogen analogues of S-adenosylmethionine

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SYNTHESIS OF NITROGEN ANALOGUES OF S-ADENOSYLMETHIONINE

by

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DISSERTATION

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To my parents

Acknowledgments

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Synthesis of Nitrogen Analogues of S-Adenosylmethionine

Abstract

Albert A. Minnick, Jr.

S-Adenosyl-L-methionine (SAM) is well known as a biochemical alkylating agent. The sulfonium group of SAM, which is the source of its unique properties, is also responsible for its inherent instability. Substituting a nitrogen atom for the sulfur atom in SAM would provide a stable tertiary amino analogue of SAM, which will retain the positive charge by protonation at physiological pH. Replacing the methyl group with larger saturated and unsaturated alkyl groups would provide a series of SAM analogues which would be useful in studying the active sites of methyltransferases and other SAM-utilizing enzymes.

The original synthesis of the nitrogen analogue of SAM [Davis, M., Dudman, N. P. B., White, H.F., *Aust. J. Chem.*, 36, 1623-1627 (1983)] employed a catalytic hydrogenation step which prohibits the incorporation of unsaturated alkyl groups into these compounds. We have developed a more general synthetic approach to these analogues which does not employ catalytic hydrogenation. The key step is the alkylation of a 5'-alkylamino-5'-deoxy-2',3'-O-isopropylideneadenosine with methyl 2(R,S)-trifluoroacetamido-4-iodo-butyrate. Subsequent removal of the protecting groups from the trialkylamine intermediate by alkaline and then acid hydrolysis provided the final compound. We have used this procedure to prepare the methyl, ethyl, n-propyl, allyl, n-butyl, n-pentyl, and n-octyl nitrogen analogues of SAM.

The inherent instability of SAM precludes its immobilization on affinity adsorbents which might help purify any methyltransferase or other SAM-binding protein. Replacing the methylsulfonium moiety of SAM with 1,6-diaminohexane would permit immobilization through the 6-amino-1-hexyl group. In this way, no

potential enzyme-ligand binding interactions involving the amino acid moiety are compromised, as occurs with S-adenosyl-L-homocysteine affinity adsorbents. The SAM affinity ligand was prepared by alkylation of 5'-[6-(benzyloxycarbonylamino)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine with methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate. Deprotection of the trialkylamine intermediate was accomplished by alkaline hydrolysis, catalytic hydrogenation, and finally acid hydrolysis. The final product, N^4-(5'-adenosyl)-N^4-(6-amino-1-hexyl)-2(R,S),4-diaminobutyric acid, was immobilized on epoxy-activated Sepharose 6B and activated CH-Sepharose 4B.

5'-Tritioadenosine was prepared by the reduction of ethyl 2',3'-O-isopropylideneadenosine 5'-carboxylate with *in situ* generated lithium borotritide, followed by acid hydrolysis of the isopropylidene ketal.

Finally, an efficient modern synthesis of D,L-glyceramide has been developed, which involves the esterification of D,L-glyceric acid with diazomethane followed by amination in liquid ammonia.

George L. Kenyon

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Chapter 1

General Synthesis of Stable Nitrogen Alkyl Analogues of S-Adenosylmethionine

Introduction

S-Adenosyl-L-methionine (SAM) is one of the most versatile compounds ever discovered in biological systems. Early studies established that both methionine and adenosine 5'-triphosphate (ATP) were necessary for biochemical transmethylation.[1-3] The structure of the postulated transmethylation "active methionine" intermediate was established in 1953 by Cantoni[4] (Figure 1). Since that time, much has been learned about the formation of SAM and the roles it plays in biological processes.

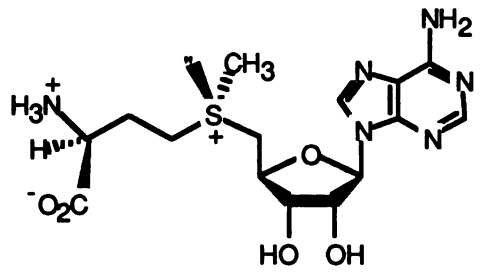


Figure 1. The structure of S-adenosyl-L-methionine (SAM).

SAM is special among biochemicals in that it contains a sulfonium group, a chemically reactive group which is ultimately responsible for all of the biochemistry in which SAM is involved. The sulfonium group arises from the displacement of the triphosphate group of ATP by the nucleophilic sulfur atom of methionine (Figure 2). The enzyme which catalyzes this reaction is called S-adenosylmethionine synthetase, or SAM synthetase.[5] Decarboxylation of SAM by the pyruvate-requiring enzyme SAM decarboxylase[5] provides a second

sulfonium compound, decarboxylated SAM (Figure 2). The sulfonium group is the key feature in both SAM and decarboxylated SAM which gives them their utility as biochemical alkylating agents.

An alkylating agent, in a purely chemical sense, must possess an excellent leaving group which activates the adjacent carbon atom toward nucleophilic attack. The prototypal chemical alkylating agent is methyl iodide. This compound has been widely used for decades in organic chemistry laboratories to methylate nucleophilic groups in a wide variety of compounds. In fact, the ability of methyl iodide to methylate DNA randomly and thereby disrupt normal base pairing makes it a potential carcinogen. The release of iodide ion, which is the conjugate base of the strong acid, hydriodic acid (HI), is responsible for the high reactivity of methyl iodide.

The fundamental principles of organic chemistry apply to biochemistry as well and not surprisingly, a biochemical alkylating agent must also possess an excellent leaving group to make the alkylation chemically feasible. The release of a stable dialkyl sulfide as the leaving group from a nucleophilic attack on the "high energy" sulfonium center of either SAM or decarboxylated SAM is consistent with the thermodynamic principle that reactions will proceed in the direction leading to lower free energy of the system[6]. Unlike chemical alkylations, however, biochemical alkylations must be highly specific and are mediated by enzymes. This specificity arises when a compound has several points of recognition to ensure proper binding only to the enzymes catalyzing the alkylation. With the adenine amino group, the ribose hydroxyl groups, the sulfonium group, and primary amino group in both SAM and decarboxylated SAM and the additional carboxylate group in SAM, a large number of possible binding arrangements to specific complementary proteins can be envisioned. In these ways, both SAM and decarboxylated SAM fulfill the requirements

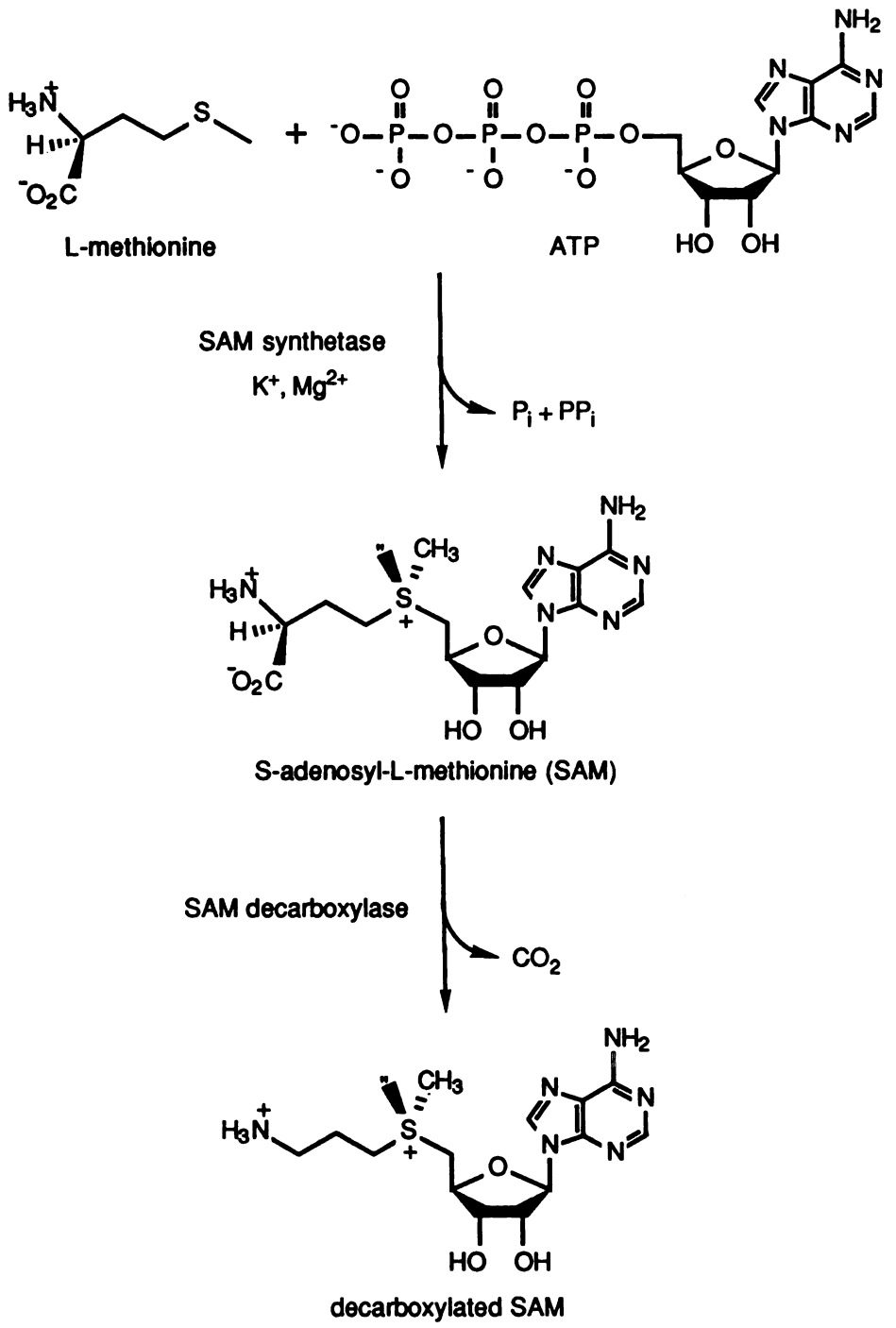


Figure 2. The formation of SAM by SAM synthetase and its decarboxylation by SAM decarboxylase. (NOTE: The stereochemistry at the sulfur atom in decarboxylated SAM is assumed to be identical to the proven stereochemistry at the sulfonium center of SAM itself.)

expected of a biochemical alkylating agent.

An examination of the chemical structures of both SAM and decarboxylated SAM would indicate that each compound could provide any of three alkyl groups to an attacking nucleophile. Just which alkyl group is transferred would depend on how the enzyme would bind its sulfonium and nucleophilic substrates and brings them together in the actual catalytic event. SAM could potentially donate its methyl, 5'-adenosyl, or 3-amino-3-carboxypropyl group to an attacking nucleophile (Figure 3). Decarboxylated SAM could similarly donate its methyl, 5'-adenosyl, or 3-aminopropyl group (Figure 4).

The most important transalkylation reactions in which decarboxylated SAM is involved are those observed in polyamine biosynthesis. The starting material in polyamine biosynthesis is the amino acid L-ornithine, which is decarboxylated by ornithine decarboxylase to give putrescine, or 1,4-diaminobutane. Then, in two successive reactions, each amino group of putrescine receives a 3-aminopropyl group from decarboxylated SAM to give first spermidine and finally spermine (Figure 5). Putrescine, spermidine, and spermine are highly basic compounds and at physiological pH exist as multiply-charged cations. For this reason, polyamines are often found in association with and help stabilize the structures of highly acidic nucleic acids such as DNA and are essential for the growth of both prokaryotes and eukaryotes.[7]

SAM is considerably more versatile than decarboxylated SAM with respect to the variety of transalkylation reactions in which it participates. Although both reactions are rare, numerous examples can be found for the transfer of the 5'-adenosyl and 3-amino-3-carboxypropyl groups of SAM to an attacking nucleophile. In the most unusual and least understood of SAM's transalkylation reactions, the activation of the enzyme pyruvate-formate lyase is

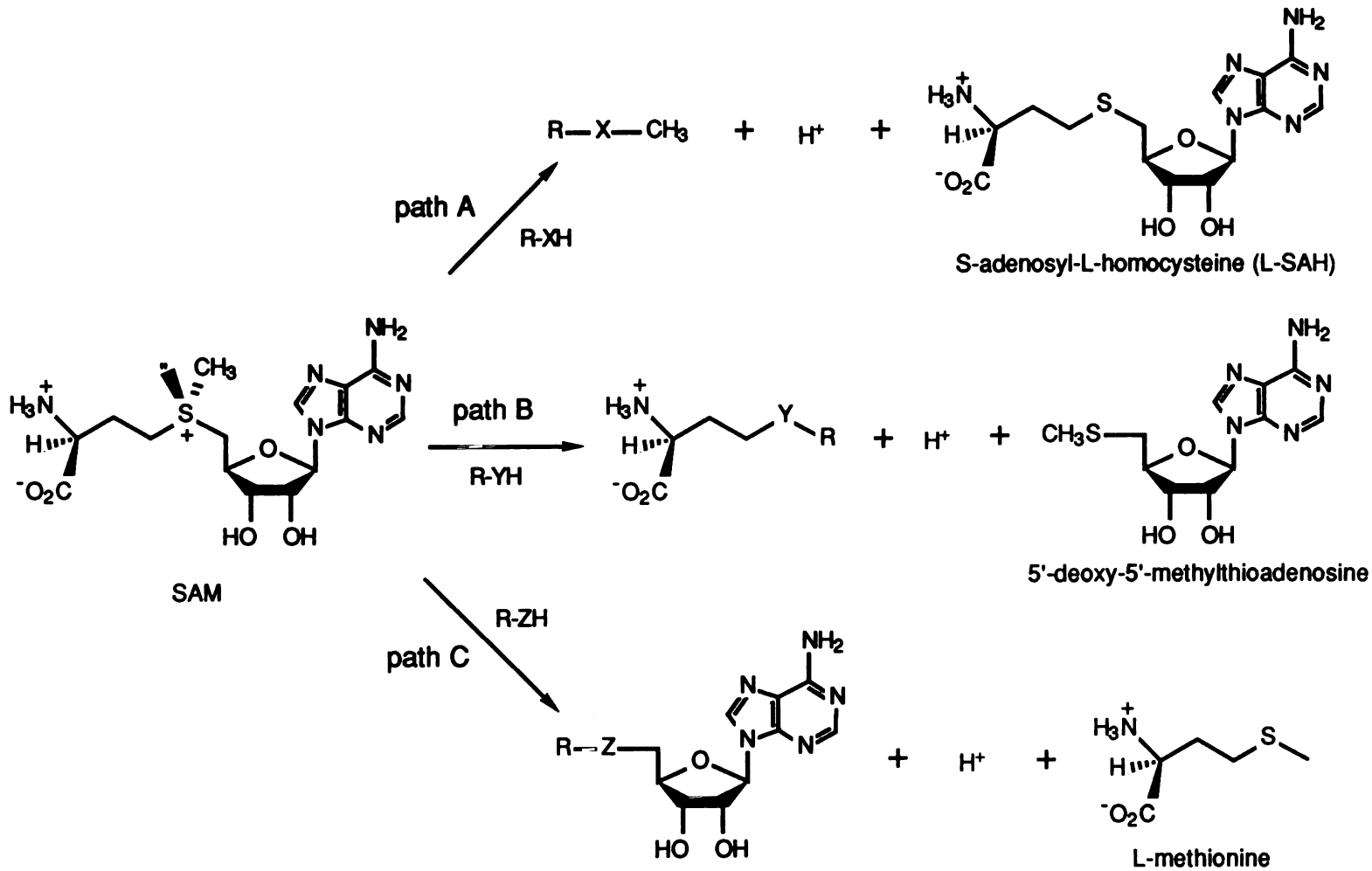


Figure 3. Potential alkylation reactions involving SAM: path A, methyl transfer; path B, 3-amino-3-carboxypropyl transfer; path C, 5'-adenosyl transfer.

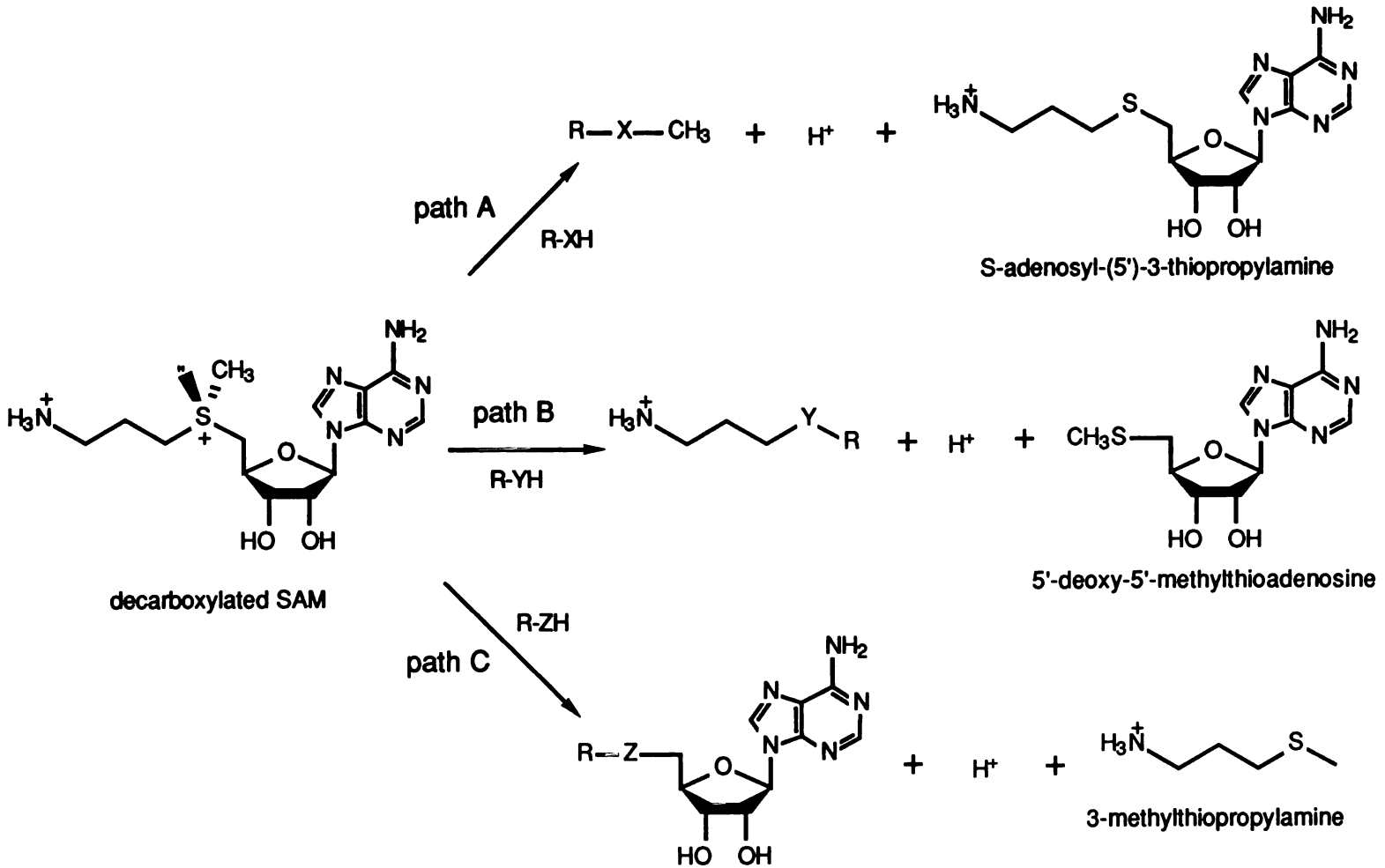


Figure 4. Potential alkylation reactions involving decarboxylated SAM: path A, methyl transfer; path B, 3-aminopropyl transfer; path C, 5'-adenosyl transfer.

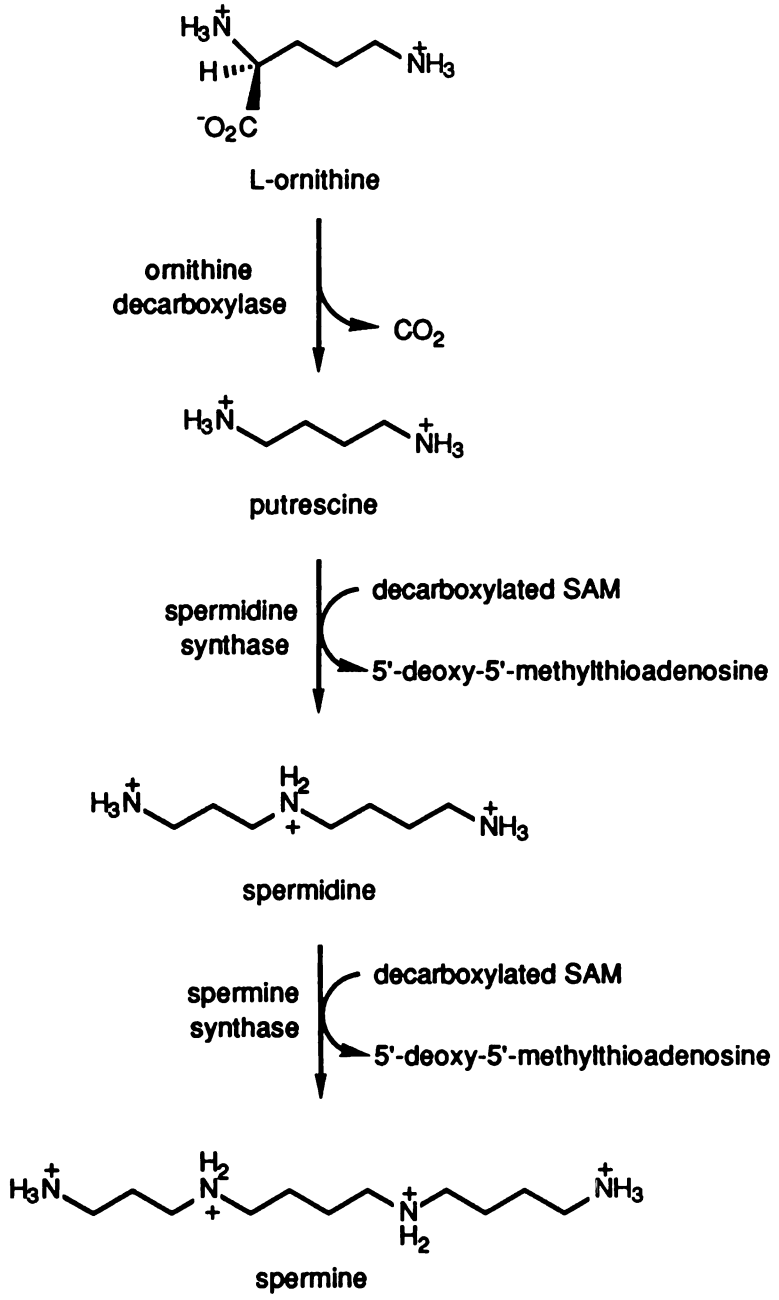
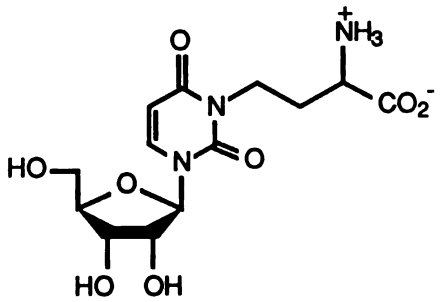


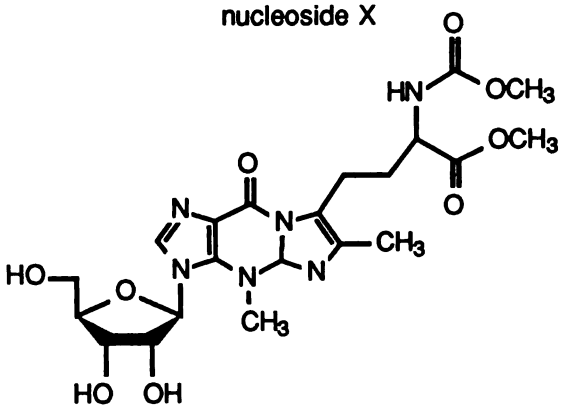
Figure 5. The reactions involved in polyamine biosynthesis, including the donation of two 3-aminopropyl groups from two decarboxylated SAM molecules.

believed to occur by the transfer of the 5'-adenosyl group from SAM to the Fe(II) atom in the inactive form of the enzyme. This enzyme, which is involved in the anaerobic metabolism of bacteria, becomes active after the 5'-adenosyl group is reduced to 5'-deoxyadenosine.[8,9] Transalkylations involving the 3-amino-3-carboxypropyl group of SAM have been implicated in the formation of modified transfer RNA nucleotides in bacteria and yeast. Nucleoside X, which is isolated only after degradation of the transfer RNA of *E. coli* , is formed during the transfer of a 3-amino-3-carboxypropyl group from SAM to a uridine residue in the intact transfer RNA.[10,11] The unusual nucleoside Y in yeast transfer RNA also contains the 3-amino-3-carboxypropyl group obtained from SAM.[12-14] This group is also found in discadenine, a 3-amino-3-carboxypropyl N^6-isopentenyladenine derivative, which is produced by certain slime molds to inhibit the germination of their own spores.[15,16] Finally, the 3-amino-3-carboxypropyl group of SAM is believed to be transferred to a phenolic hydroxyl group followed by an inversion of configuration at the α-carbon atom during the biosynthesis of nocardicin A, a monocyclic β-lactam produced by the microorganism *Nocardia uniformis*.[17] The structures of nucleosides X and Y, discadenine, and nocardicin A are shown in Figure 6.

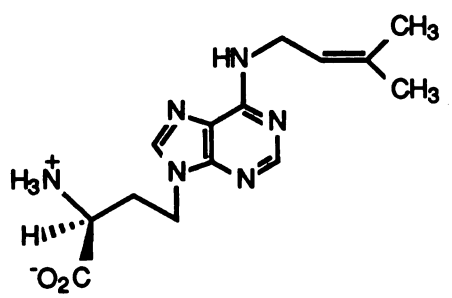
SAM finds its greatest role in biochemical systems as a donor of methyl groups to a wide variety of molecules of all sizes. SAM differs from tetrahydrofolate, which shuttles one-carbon equivalents of methanol, formaldehyde, and formate among important biochemicals, in that the transfer of one-carbon methyl cation equivalents from SAM to acceptors is not a reversible process. Even so, the scope of this irreversible methyl transfer is so great that SAM can be considered to be biochemistry's answer to organic chemistry's methyl iodide. A few examples can be given to illustrate the utility of SAM in this respect.



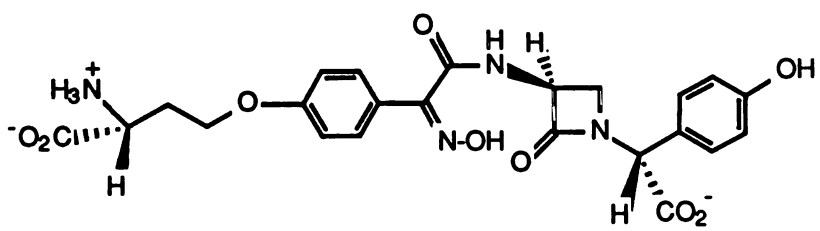
nucleoside X



nucleoside Y



discadenine



nocardicin A

Figure 6. Compounds which have been found to contain a 3-amino-3-carboxypropyl group derived from SAM.

The enzyme DNA (cytosine-5-)-methyltransferase catalyzes the transfer of the methyl group of SAM to the carbon in position 5 of certain cytosines in DNA. This enzyme, presumably acting in a manner similar to that of thymidylate synthetase,[18] generates a nucleophile at position 5 which ultimately attacks SAM only after an enzyme-bound nucleophile, possibly a cysteine thiolate, covalently adds at position 6 of the cytosine. After the methyl transfer, the loss of the hydrogen at position 5 as a proton and the accompanying expulsion of the postulated thiolate from position 6 provide the 5-methylcytosine derivative (Figure 7). The methylation of certain cytosines in DNA is particularly noteworthy because it is associated with a deactivation of the gene containing them.[19,20] Recent studies with DNA containing 5-azacytidine in place of the normal cytidines have revealed that the methylation is inhibited and that normal gene function is restored in the replicated DNA.[21,22] The gross effect of 5-azacytidine on a cellular level is a promotion of cellular differentiation. In short, the methylation of DNA serves as as on-off switch for gene function.

Messenger RNA is another macromolecular acceptor of the methyl group of SAM. This ribonucleic acid carries the protein amino acid sequence message encoded on the DNA inside the nucleus to the protein-synthesizing machinery of the ribosomes in the outlying cytoplasm. An unusual cap structure is found at the 5'-terminus of the messenger RNA. This cap structure is believed to protect the messenger RNA from 5'-exonuclease hydrolysis as well as to provide a recognition point facilitating binding of the messenger RNA to the protein-synthesizing enzymes in the ribosomes.[23] The key feature in the cap structure is the 5'-5'-linkage of an added guanosine with the messenger RNA terminus through an intervening triphosphate bridge. Methylations at the N-7 position of the added guanosine residue, the 2'-hydroxyl groups of the ultimate and penultimate bases, and the adenine amino group if the ultimate base is an

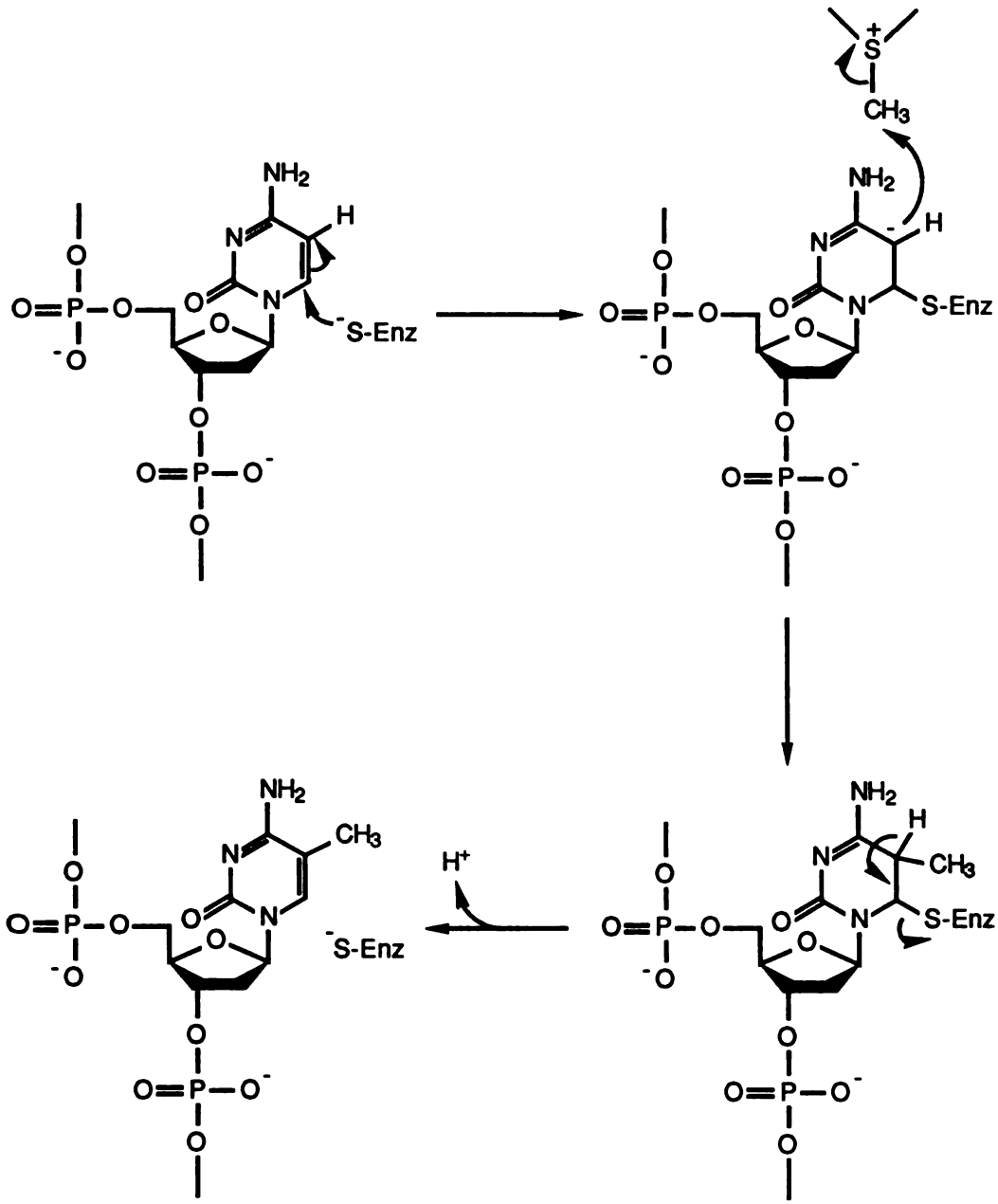


Figure 7. The mechanism of action of DNA (cytosine-5-)-methyltransferase proposed by Santi et. al. (see reference 18).

adenine complete the messenger RNA 5'-terminus cap structure (Figure 8). Each of these methylations uses SAM as the methyl group donor and is carried out by a different enzyme. The viral messenger RNA (guanine-7-)- and (nucleoside-2'-)-methyltransferases are well-characterized enzymes[24-27] and their activity is essential for stabilization of the RNA from RNA viruses which invade cells and redirect the normal cellular metabolism toward their own multiplication. Such a "takeover" on a large scale leads to the clinical appearance of a viral infection.

SAM is also involved in the post-translational modification of proteins involved in bacterial chemotaxis.[28-30] In the highly regulated chemotactic system, SAM is used to methylate glutamate residues in certain proteins to give labile glutamate methyl esters. These proteins, called methyl-accepting chemotaxis proteins (MCPs), have a role in regulating the movements of the flagellum, the whip-like structure which controls the direction in which the bacterium "swims." The methylations and demethylations of the glutamate residues in the MCPs are ongoing simultaneous processes. Whether a bacterium swims toward an attractant such as an essential amino acid or away from a repellent toxic substance is determined by tipping the chemotactic system in favor of a net methylation or demethylation of the glutamates in the MCPs. Firm rules regarding the identities of attractants and repellents as well as a correlation of the level of methylation with attraction or repulsion do not exist and vary from species to species.

A wide variety of small molecules can be enzymatically methylated with SAM. Included in this group are histamine,[31] guanidinoacetate (whose methylation gives creatine),[32] 5-hydroxyindoles,[33] indoleethylamines,[34] and phosphatidylcholine[35,36](Figure 9). Perhaps the best known of these small molecules is norepinephrine, which is a substrate for both catechol O-

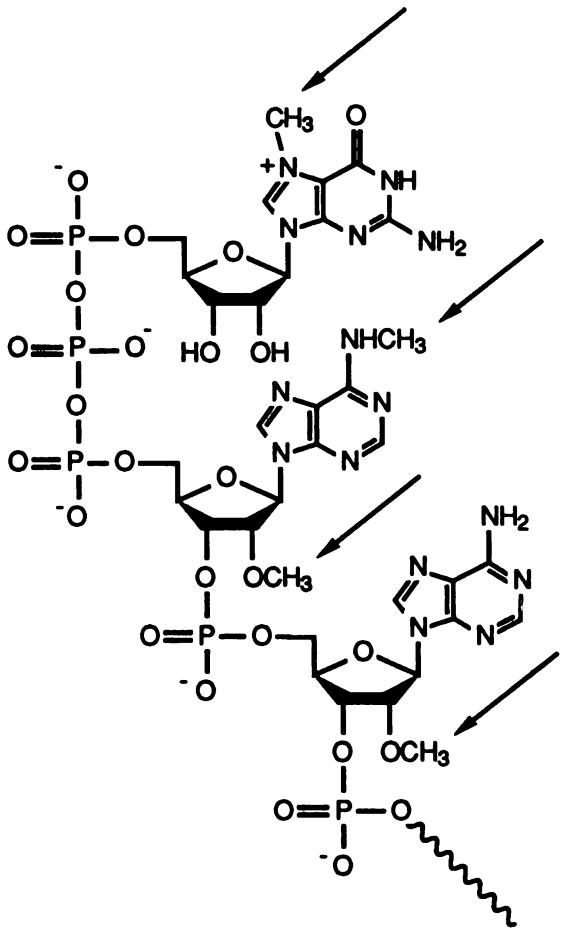


Figure 8. The structure of the 5'-terminal cap of messenger RNA, including the methyl groups which are donated by SAM.

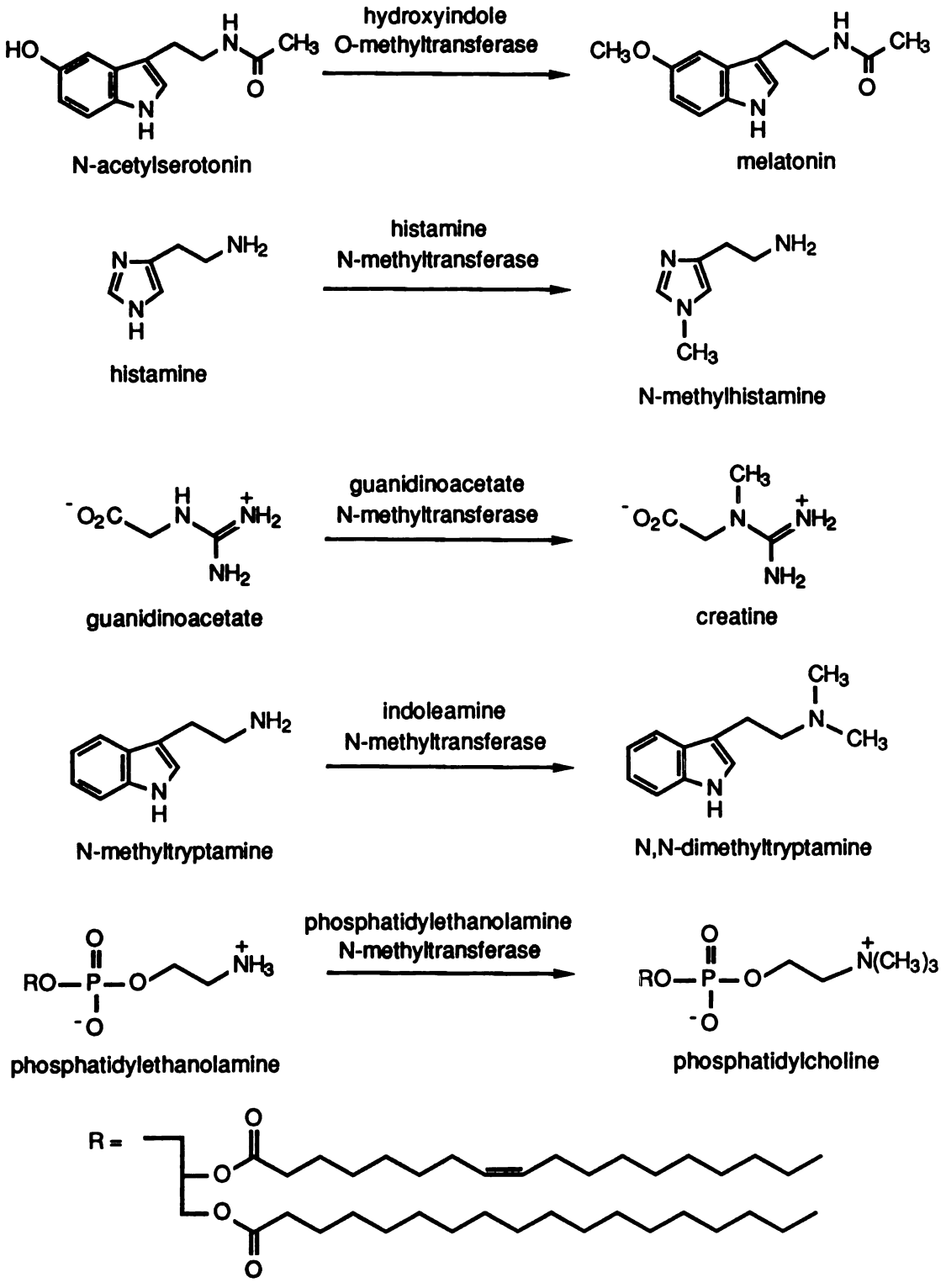


Figure 9. Some representative small molecules which are enzymatically methylated one or more times by SAM.

methyltransferase (COMT) and phenylethanolamine N-methyltransferase (PNMT). Norepinephrine is a catecholamine neurotransmitter released by postganglionic neurons in the sympathetic nervous system. Stimulation of this part of the nervous system (including the adrenal medulla) by norepinephrine results in pupil dilation, increased cardiac function and blood pressure, increased blood glucose levels, and inhibition of digestive processes and cutaneous blood flow.[37] The norepinephrine released into the synaptic space is removed to a large extent by re-uptake into the presynaptic neuron. Residual norepinephrine in the synaptic space is "removed" through metabolic inactivation by COMT. This enzyme uses SAM to methylate the 3-hydroxyl group of norepinephrine to give the pharmacologically inactive normetanephrine derivative (Figure 10). In the adrenal medulla, PNMT uses SAM to methylate the amino group of norepinephrine to provide epinephrine (Figure 10). The release of epinephrine into the bloodstream elicits many of the same responses as does norepinephrine in the sympathetic nervous system in addition to an increase in both muscular circulation and general metabolism.[38] Epinephrine and norepinephrine circulating in the bloodstream are metabolically inactivated by COMT present in the liver in a fashion similar to that observed in the synaptic space outlined above, as well as by the enzyme monoamine oxidase.

Apart from the numerous enzyme-catalyzed methylations in which it participates, SAM has also been found to play an important regulatory role in the enzymatic reduction of N^5,N^{10}-methylene tetrahydrofolate to N^5-methyltetrahydrofolate[39] (Figure 11). The enzyme methylenetetrahydrofolate reductase carries out this NADPH-dependent reduction. The methyl group in N^5-methyltetrahydrofolate is subsequently transferred to the thiol group of L-homocysteine by the vitamin B_{12}-dependent enzyme homocysteine S-

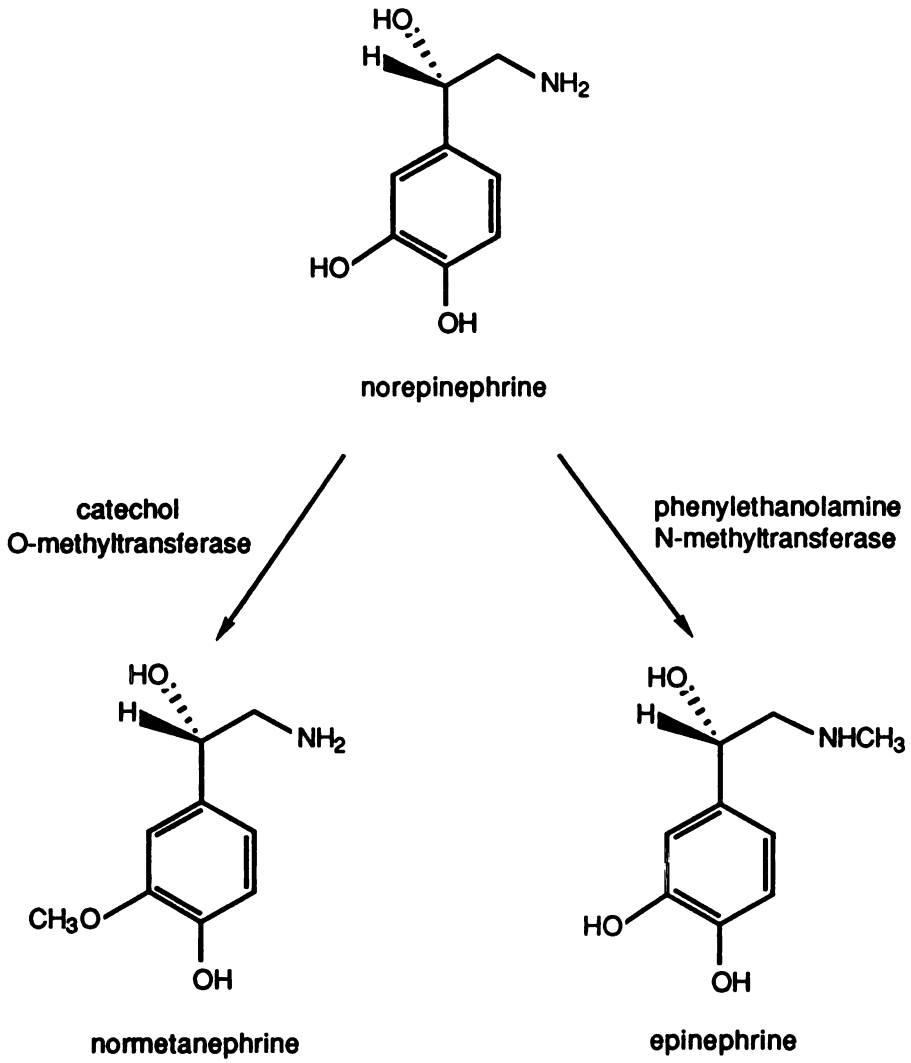


Figure 10. Two different enzyme-catalyzed methylations of the catecholamine norepinephrine, using SAM as the methyl group donor.

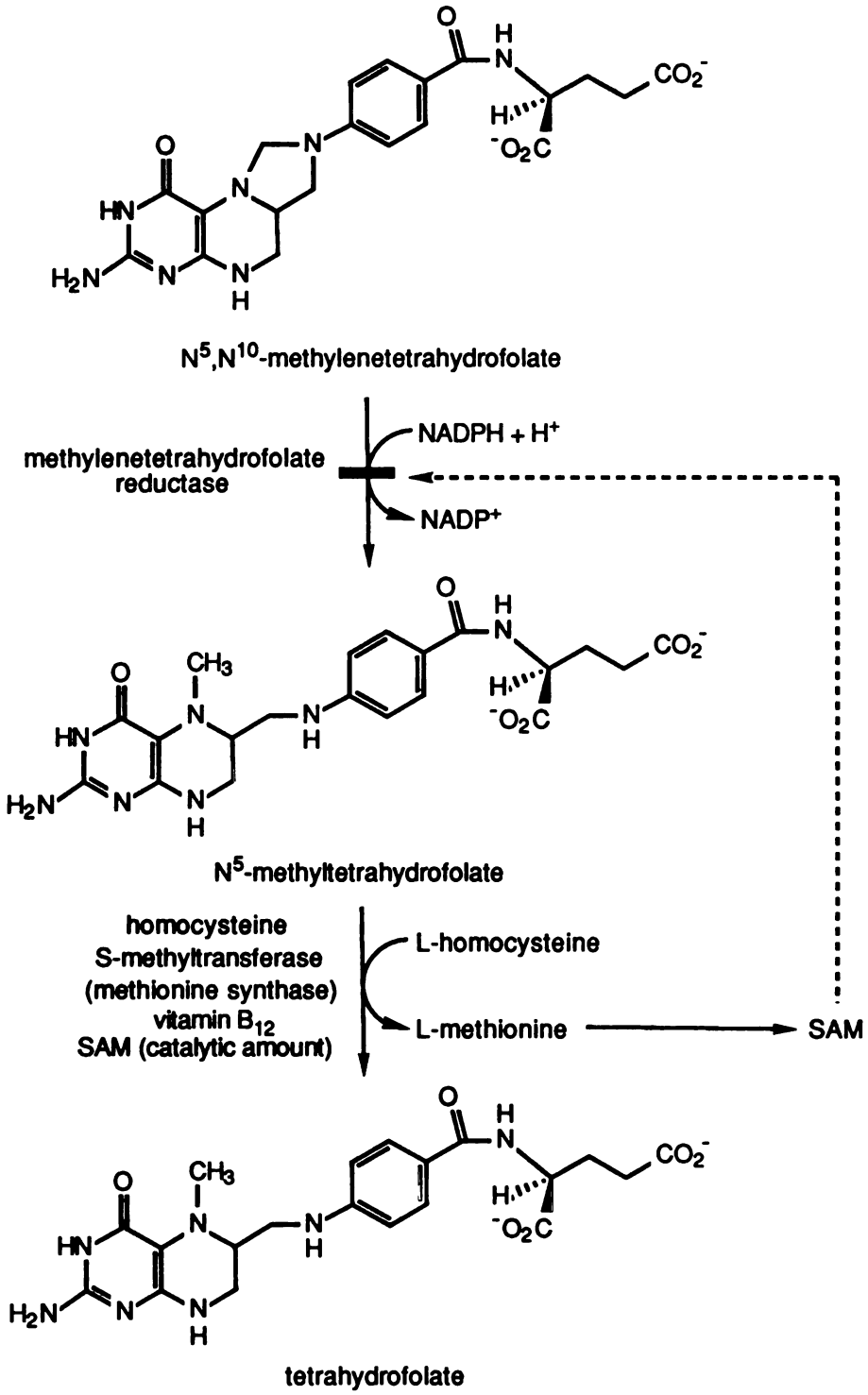


Figure 11. The reduction of N^5,N^{10}-methylenetetrahydrofolate by methylenetetrahydrofolate reductase and subsequent transfer of the N^5-methyl group to L-homocysteine to form L-methionine. SAM synthesized from L-methionine is an allosteric inhibitor of methylenetetrahydrofolate reductase (see reference 39).

methyltransferase (methionine synthase) to form L-methionine (Figure 11). The resulting methionine can then be assimilated into proteins or incorporated into SAM by SAM synthetase (Figure 2). Some mechanism must exist to prevent an unnecessary diversion of the biochemical machinery from methionine synthesis when dietary methionine alone suffices to meet the demand (e.g., protein synthesis) for this amino acid. Since much of the N^5-methyltetrahydrofolate synthesized by methylenetetrahydrofolate reductase is linked through methionine to the biosynthesis of SAM, SAM could in turn be used to regulate the biosynthesis of N^5-methyltetrahydrofolate. SAM, not surprisingly, has been found to be an allosteric inhibitor of methylenetetrahydrofolate reductase.[39] The binding of SAM to its allosteric site on methylenetetrahydrofolate reductase has been proposed to induce a conformational change in the enzyme which prevents the normal redox chemistry in the active site some distance away from occurring.[39] High levels of SAM ensure that most of the methylene-tetrahydrofolate reductase molecules are inhibited and limit the production of more SAM. When the level of SAM falls as a consequence of high methyltransferase activity, the allosteric inhibition of methylenetetrahydrofolate reductase is lifted and the biosynthesis of N^5-methyltetrahydrofolate, methionine, and, ultimately, SAM is restored. This highly tuned feedback mechanism allows SAM to regulate its own synthesis.

From a pharmacological standpoint, inhibition of many of these processes might be clinically fruitful. For instance, a compound which inhibits the activity of viral methyltransferases *in vitro* might exhibit antiviral activity in the whole organism. A compound which could inhibit the metabolic inactivation of catecholamines by COMT present in the liver and the central and peripheral nervous systems might elicit a general physiologic stimulation. From a biochemical standpoint, increased basic knowledge of methyltransferases

would be useful in any program aimed at developing new chemotherapeutic agents. The drugs used to treat many disease or conditions are often enzyme inhibitors. Basic knowledge of the active site geometry and mechanism of action of the target enzyme was essential to the development of new antihypertensive drugs which function as inhibitors of angiotensin converting enzyme.[40] For these reasons, it was quite natural that bioorganic chemists would take an interest in synthesizing and evaluating potential inhibitors of methyltransferases.

Two approaches are available for the development of inhibitors of methyltransferases. These enzymes require two substrates, SAM and a methyl group acceptor, and could theoretically be inhibited by eliminating the reactive nucleophile in the methyl acceptor and/or the chemically labile methyl group in SAM. The first approach, involving chemical tinkering with the methyl acceptor, offers the best opportunities for selectively inhibiting one particular methyltransferase in the presence of many others. Such a task would be relatively easy for the small molecule methyltransferases where substitution of the normal methyl acceptor substrate with a compound of similar structure is trivial. Many examples of such a substitution resulting in inhibition of enzyme activity are known, particularly with COMT.[41-53] Problems quickly arise when the methyl acceptor is a macromolecule, such as a protein or nucleic acid. Replacing one protein with another analogous protein, which would have to be genetically engineered specifically for the inhibition of methylation, is clearly impractical. One important exception to this generalization is DNA containing 5-azacytidine in place of cytidine. As already discussed, DNA so substituted inhibits DNA (cytosine-5-)-methyltransferase.

The common link between all methyltransferases is the requirement for SAM as the methyl group donor. Thus, an attempt to inhibit one particular

methyltransferase in the presence of others by replacing SAM with another compound might not be successful and would more often than not affect the other enzymes to various degrees as well. The clinical manifestation of such a situation, which is commonly observed with anti-cancer drugs, would be a wide variety of side effects resulting from the concomitant inhibition of other transmethylation processes. Despite this worst case scenario, each methyltransferase is different and one might exhibit a higher affinity for SAM or one of its analogues than another enzyme. This situation would be evidence of the complementarity of the active site geometry with the structure of SAM or its inhibitor analogue. Thus, in principle, one methyltransferase could be inhibited in the presence of other enzymes by finding a compound which binds better to it than to others.

Nature provides the lead compound for the development of SAM analogue inhibitors of methyltransferases. The dialkyl sulfide leaving group in the transmethylation reaction, analogous to the iodide in methyl iodide methylations, is S-adenosyl-L-homocysteine (L-SAH). This compound is well known as a potent competitive product inhibitor of methyltransferases (Figure 12). However, without some mechanism for the removal of L-SAH from the biochemical gemisch of a living organism, soon all methyltransferases will be inhibited and death will soon result. Because the transmethylation reaction is not reversible, the rapidly accumulating L-SAH must be somehow degraded, and, fortunately, two major pathways for its removal are available.

In the first pathway, which is present in eukaryotes, L-SAH is reversibly hydrolyzed to L-homocysteine and adenosine by SAH hydrolase[54,55](Figure 12). The equilibrium in this reversible hydrolysis greatly favors the synthesis of L-SAH. Therefore, both L-homocysteine and adenosine must be removed so that their accumulation does not tip the equilibrium back in favor of the synthesis

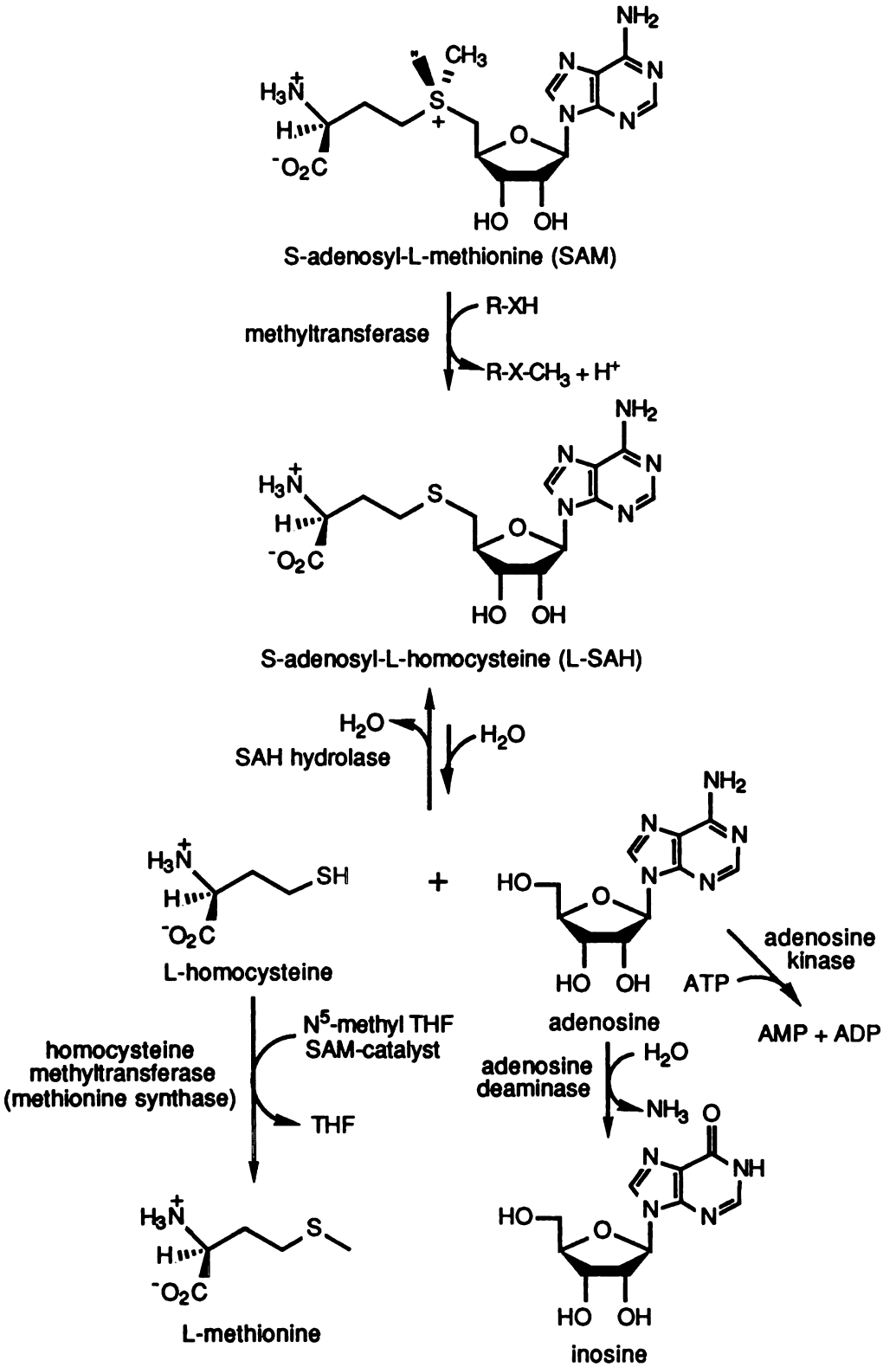


Figure 12. The formation of S-adenosyl-L-homocysteine (L-SAH) from SAM and its eukaryotic degradation pathways.

of L-SAH and the consequent inhibition of methylation. Removal of L-homocysteine is made possible by homocysteine methyltransferase, also known as methionine synthase. In the presence of a catalytic amount of SAM, the enzyme catalyzes the transfer of the methyl group of N^5-methyltetrahydrofolate to the homocysteine thiolate group (sometimes using the cobalt atom of the cobalamine coenzyme as an intermediary carrier) to form L-methionine.[56,57] Adenosine is removed by deamination to inosine by adenosine deaminase or returned to the nucleotide pool by adenosine kinase (Figure 12).

A second mechanism for L-SAH removal, present only in bacteria, involves the cleavage of the glycoside bond to give S-ribosyl-L-methionine and adenine. The enzyme catalyzing this hydrolysis reaction is called SAH nucleosidase[58] (Figure 13). Other minor pathways for the removal of L-SAH include deamination by adenosine deaminase in fungi to S-inosinyl-L-homocysteine[59] and oxidative deamination by L-amino acid oxidase in rats to give the urinary metabolite S-adenosyl-4-thio-2-ketobutyrate[60] (Figure 13). In yeast, the remethylation of L-SAH to SAM is possible.[61]

This analysis of SAM biochemistry would indicate that enzyme-catalyzed transmethylations could be inhibited by a number of methods. First, analogues of L-SAH could be tested for inhibitory activity against methyltranferases with the aim of finding one or more which could bind more tightly than L-SAH itself. Even if the analogue did not bind well, an analysis of why not would help generate a picture of the active site so that better inhibitors could be developed. Particularly noteworthy in this regard are the research efforts in the laboratories of Coward[62-66] and Borchardt[67-72]. Out of these laboratories have come dozens of analogues of L-SAH which have been tested as potential inhibitors of the activity of a wide variety of methyltransferases. These analogues have

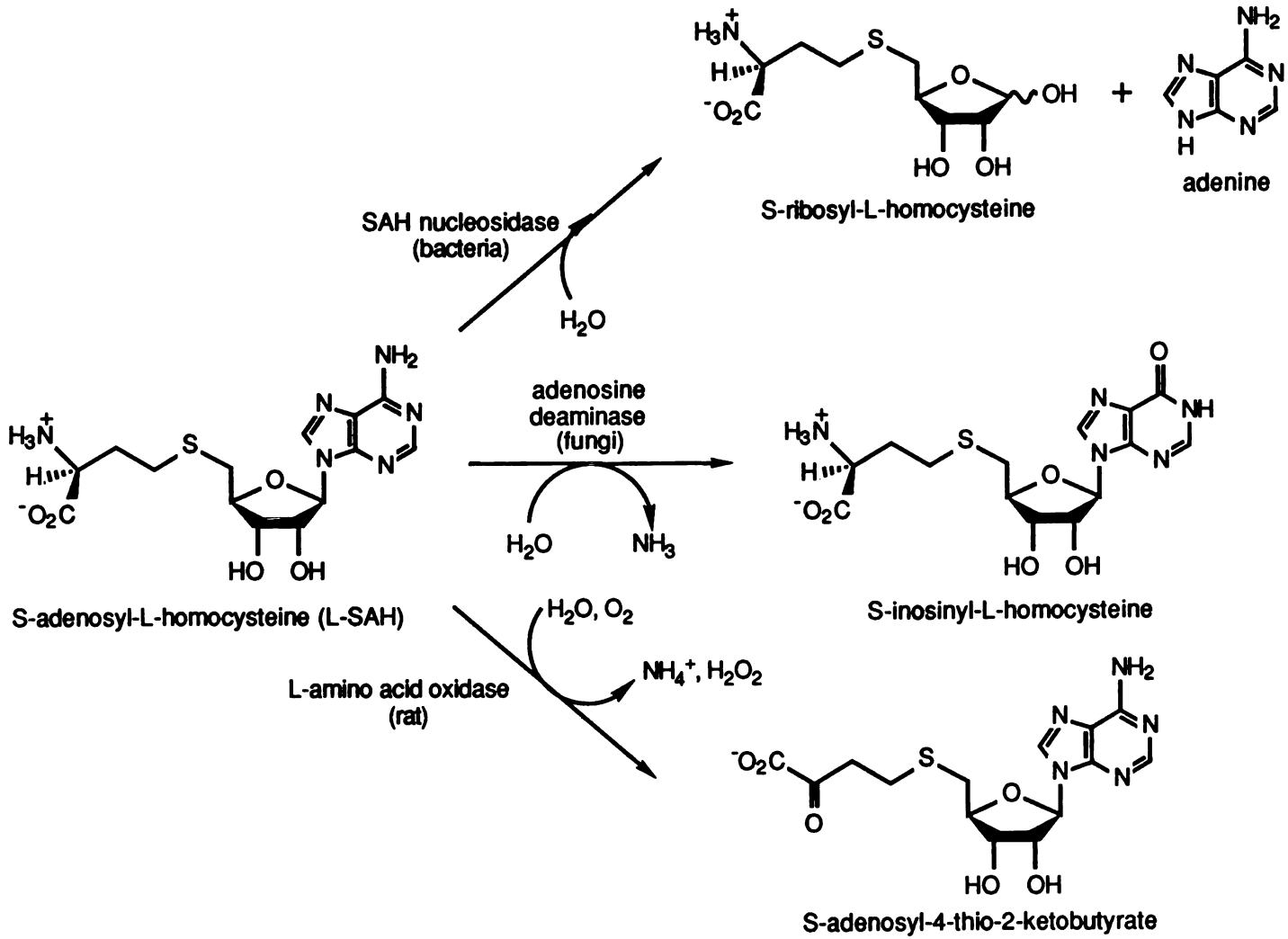
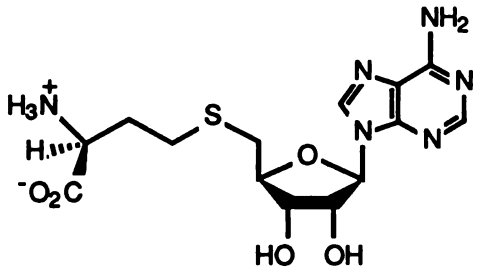
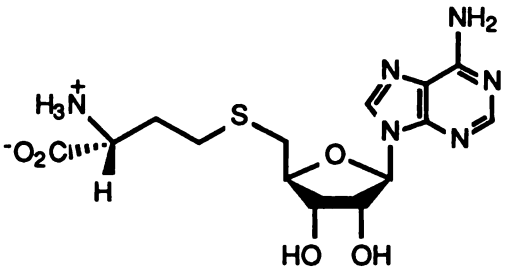


Figure 13. Minor pathways for the removal of S-adenosyl-L-homocysteine (L-SAH) in a variety of organisms.

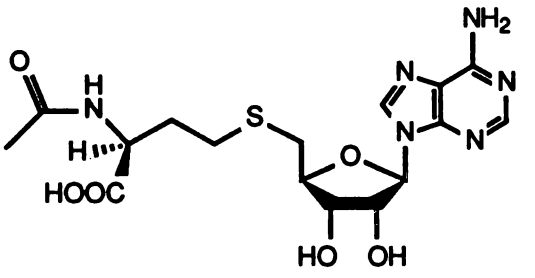
included some (Figure 14) which have been modified in the amino acid moiety (e.g., D-SAH, N-acetyl, and sulfoxide derivatives), the sugar ring (e.g., S-arabinosyl-L-homocysteine), and the aromatic ring system (e.g., S-tubercidinyl-L-homocysteine). Not surprisingly, the inhibitory potency of any given compound was found to depend on the particular enzyme being tested. Such an observation is a reflection of the fact that the active sites of different enzymes have different structures. An interesting example in this regard can be found with S-N^6-methyladenosyl-L-homocysteine[70] (Figure 15). This compound is as good an inhibitor (K_i = 17.4 ± 5.7μM) of indoleethylamine N-methyltransferase (INMT) as is L-SAH (K_i = 8.65 ± 0.71μM). However, the same compound exerts comparatively little inhibition (K_i = 1541 ± 205μM) of histamine N-methyltransferase (HMT) activity (Figure 9). Presumably, these results indicate that a pocket large enough to accommodate the extra methyl group on the adenine ring is present in INMT but is not present in HMT. Interestingly, S-N^6-dimethyladenosyl-L-homocysteine (Figure 15), which has two methyl groups on the adenine ring instead of one, is not an inhibitor of either INMT or HMT. The space in INMT that could accommodate one methyl group is apparently not big enough for two methyl groups. Thus, in a hypothetical two enzyme system, S-N^6-methyladenosyl-L-homocysteine would selectively inhibit INMT in the presence of HMT. In contrast, HMT can be inhibited irreversibly by S-adenosyl-L-homocysteine 2',3'-dialdehyde (Figure 15) in a manner which fulfills all of the requirements expected of an affinity labeling reagent,[72] whereas other methyltransferases, such as COMT, PNMT, and HIOMT, are not inhibited by this compound. Structure-activity studies such as these have provided much information about the functional groups on L-SAH which are important for maximal binding to a variety of methyltransferases. Unfortunately, these studies did not yield a compound which was a significantly better inhibitor of a



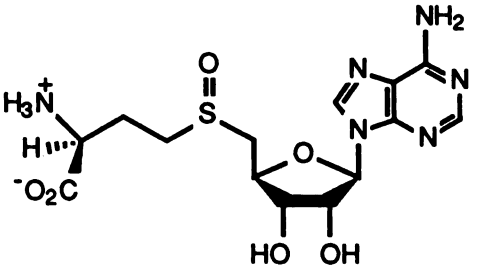
S-adenosyl-L-homocysteine (L-SAH)



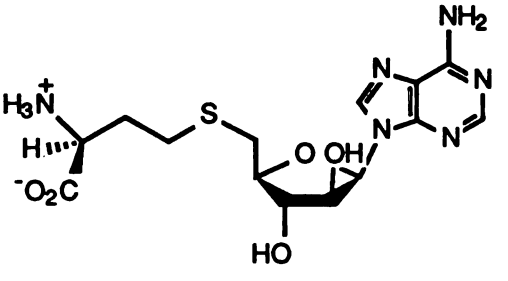
S-adenosyl-D-homocysteine (D-SAH)



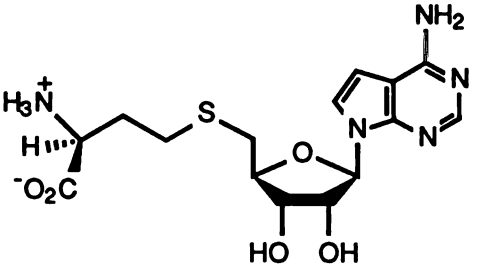
N-acetyl-L-SAH



L-SAH sulfoxide

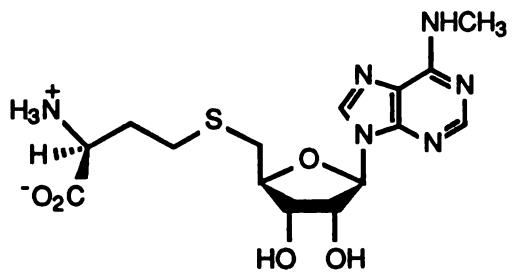


S-arabinosyl-L-homocysteine

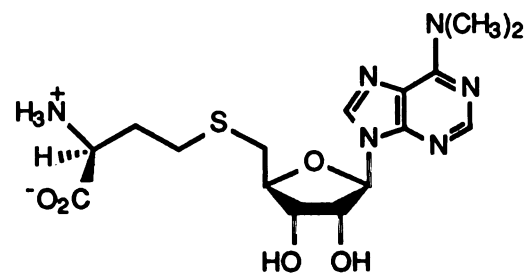


S-tubercidinyl-L-homocysteine

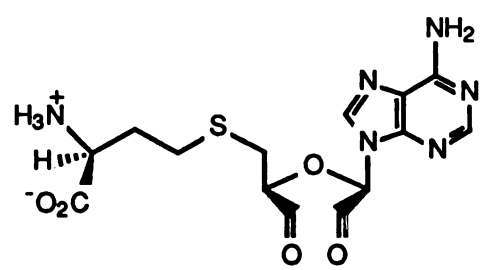
Figure 14. Some of the analogues of S-adenosyl-L-homocysteine which have been synthesized and tested as potential inhibitors of methyltransferases (L-SAH is at the top left for reference).



S-N^6-methyladenosyl-L-homocysteine



S-N^6-dimethyladenosyl-L-homocysteine



S-adenosyl-L-homocysteine 2',3'-dialdehyde

Figure 15. Additional analogues of S-adenosyl-L-homocysteine (L-SAH) which have been synthesized and tested as inhibitors of methyltransferases.

methyltransferase than L-SAH itself.

An indirect approach to the inhibition of methyltransferases involves the inhibition of the hydrolysis of L-SAH by SAH hydrolase. In the presence of such an inhibitor, L-SAH would rapidly accumulate and eventually inhibit the activity of all such methyltransferases. The mechanism of action of SAH hydrolase is known to involve an oxidation at C-3' in L-SAH by an enzyme-bound NAD^+.[73,74] Abstraction of the slightly acidic C-4' proton adjacent to the intermediate 3'-keto group by an enzyme-bound nucleophile followed by elimination of the thiolate of L-homocysteine from the C-5' position leaves 3'-keto-4',5'-dehydroadenosine bound to the enzyme. A Michael addition of water across the 4',5'-bond in this adenosine derivative followed by reduction of the 3'-keto group by the enzyme-bound NADH gives adenosine which is released from the enzyme[73,74] (Figure 16). An understanding of this mechanism helps to explain how the enzyme is irreversibly inactivated by 2'-deoxy-adenosine.[75,76] After oxidation to 3'-keto-2'-deoxyadenosine in the enzyme active site, an increase in the acidity of the C-2' protons leads to a facile elimination of adenine and the formation of a 3'-keto-1',2'-dehydroribose. This ribose derivative is not reduced by the enzyme-bound NADH although it might be susceptible to a Michael addition by an enzyme-bound nucleophile. The enzyme is inactivated because the tightly bound NADH cannot be reoxidized to NAD^+ which in turn is needed to initiate the forward and backward steps of normal catalysis. Thus, by effectively inducing SAH hydrolase to commit suicide, 2'-deoxyadenosine, and arabinosyladenine and the carbocyclic analogue of adenosine as well[75,76] (Figure 17), could indirectly inhibit biochemical transmethylations.

Irreversible inhibition of SAH hydrolase has been also demonstrated with carbocyclic nucleoside 2',3'-dialdehydes.[77] The most interesting of these

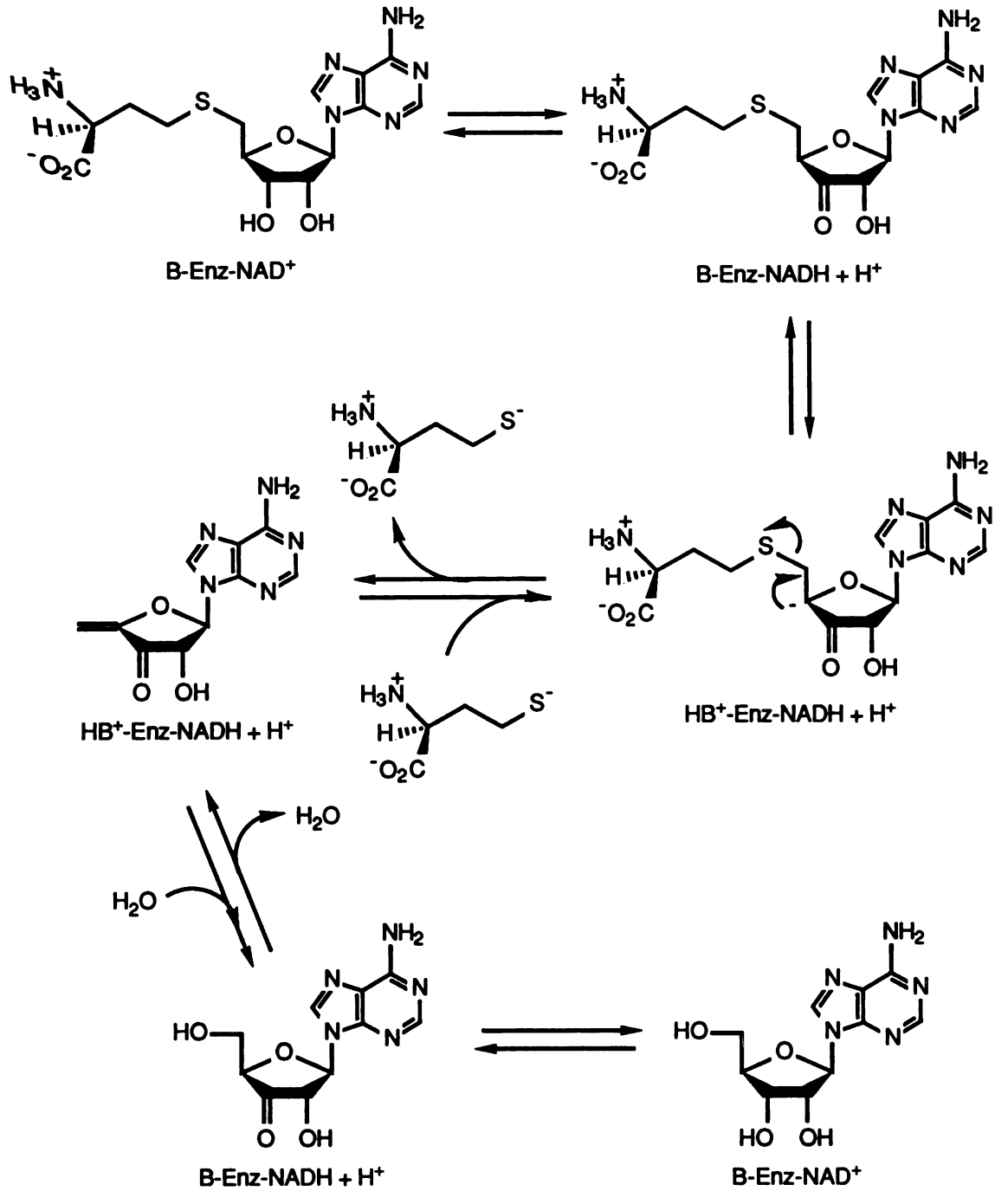


Figure 16. The mechanism of action of S-adenosyl-L-homocysteine hydrolase proposed by Palmer and Abeles (see references 73 and 74).

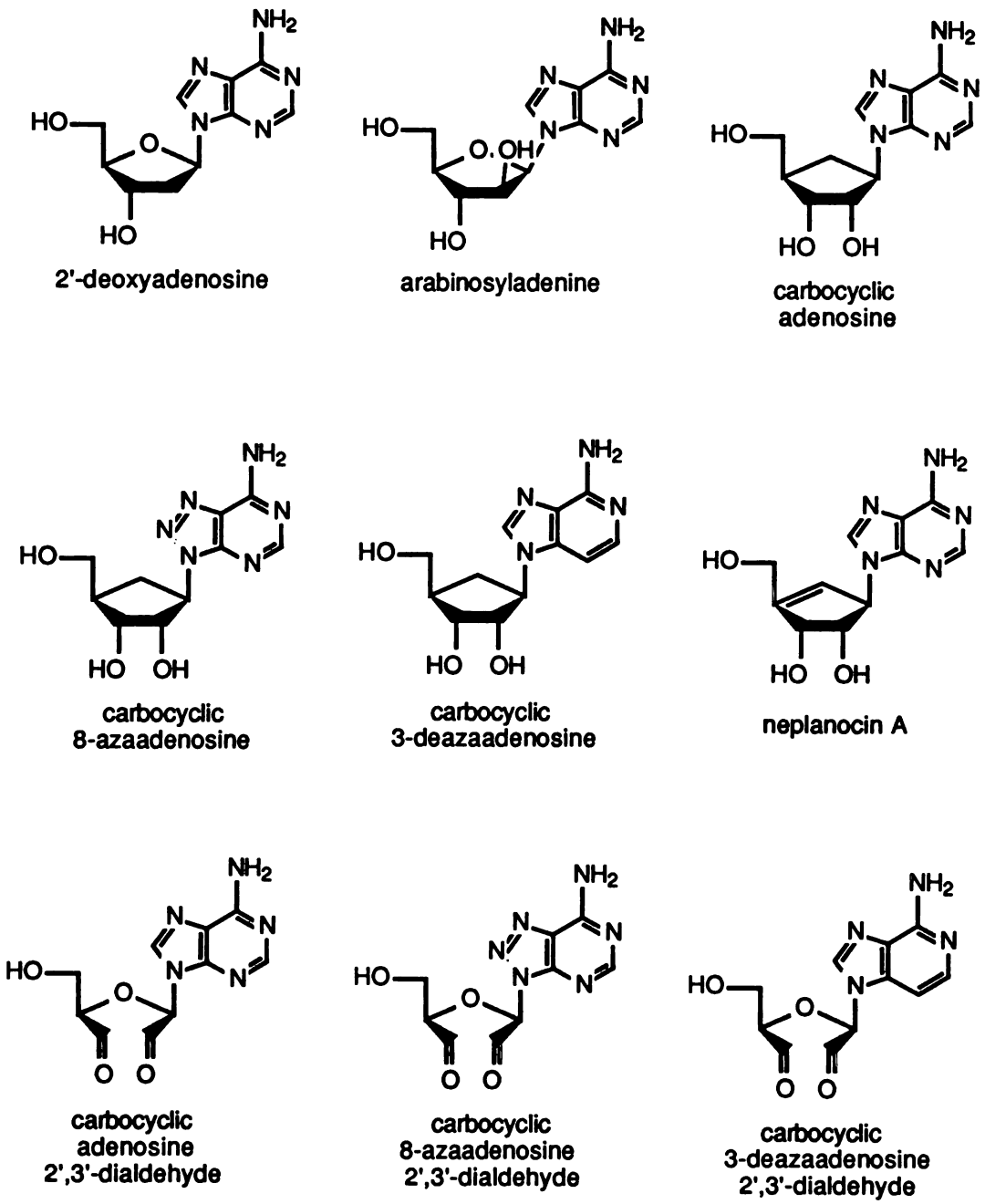
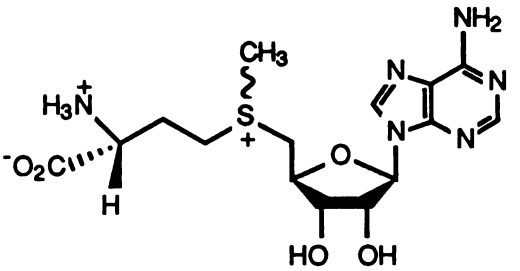


Figure 17. Potent reversible and irreversible inhibitors of S-adenosyl-L-homocysteine hydrolase.

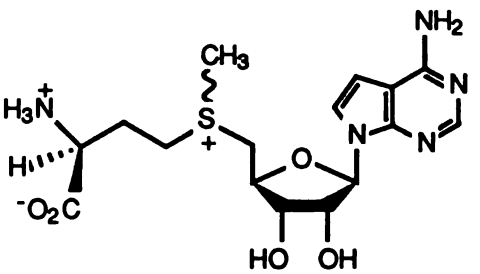
compounds include the carbocyclic adenosine, 8-azaadenosine, and 3-deazaadenosine 2',3'-dialdehydes (Figure 17). The corresponding carbocyclic nucleosides were also potent competitve, reversible inhibitors (nanomolar K_i values) but the corresponding carbocyclic nucleoside 2',3'-diols were only very weak inhibitors. The proposed link between the inhibition of SAH hydrolase, the accumulation of L-SAH, and the concomitant inhibition of methyltransferases received valuable support when some of these compounds were found to exert potent antiviral activity. The most effective inhibitors of vaccinia virus replication in cultured mouse cells were carbocyclic 3-deazaadenosine and carbocyclic 3-deazaadenosine 2',3'-dialdehyde[77] (Figure 17). Neplanocin A, a cyclopentenyl adenosine analogue (Figure 17), is another potent inhibitor of SAH hydrolase (K_i = 8.39nM) that has been found to inhibit vaccinia virus replication in cultured mouse cells.[78] The antiviral effects of neplanocin A, carbocyclic 3-deazaadenosine, and carbocyclic 3-deazaadenosine 2',3'-dialdehyde probably result from the inhibition of the messenger RNA (guanine-7-)- and (nucleoside-2'-)-methyltransferases involved in the obligatory "capping" of the 5'-terminus of viral messenger RNA (Figure 8) by the accumulated L-SAH.

A more direct approach to the inhibition of methyltransferase activity would involve the development of a series of analogues of SAM itself. These sulfonium compounds would be expected to compete with SAM for its place in the methyltransferase active site. Several of these analogues have been synthesized and tested both as inhibitors of methyltransferase activity and, as a consequence of their sulfonium nature, as potential substrates in methyltransferase catalyzed reactions.[79,80] Three of the most interesting compounds developed for this purpose include (±)-D-SAM, (±)-S-tubercidinyl-L-methionine, and (±)-3-deaza-SAM, each as a pair of diastereomers differing

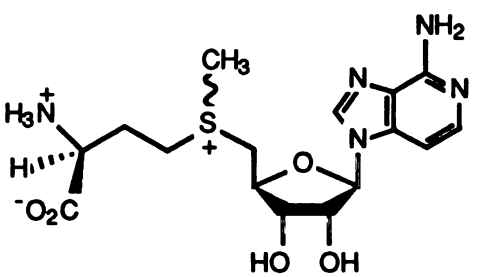
only in the configuration of the substituents around the sulfonium center (Figure 18). An inversion of the stereochemistry at the α-carbon atom in the amino acid moiety from the L(S)-configuration to the D(R)-configuration does not prevent tight binding of (±)-D-SAM to the active site of HMT (Figure 9). Consequently, this compound is a fairly potent competitive inhibitor of HMT activity (K_i = 92.2 ± 15.2μM),[80] although not so potent as either D-SAH (K_i = 10.5 ± 1.3μM) or L-SAH (K_i = 18.1 ± 2.9μM).[67] On the other hand, the SAM amino acid moiety binding site in COMT is less tolerant of deviations from the L(S)-configuration around the α-carbon and consequently (±)-D-SAM is only a poor inhibitor (K_i = 810 ± 169μM)[80] of COMT activity. The same situation is true for (±)-3-deaza-SAM, which is a potent competitive inhibitor of HMT activity (K_i = 93 ± 13μM) but not of COMT activity (K_i = 491 ± 62.7μM).[80] (±)-S-Tubercidinyl-L-methionine, on the other hand, is a potent competitive inhibitor of the activities of both COMT (K_i = 17 ± 1.7μM) and HMT (K_i = 28 ± 4μM).[80] Interestingly, only the (-)-sulfonium diastereomer of each compound was found to be a substrate for HMT. Both (-)-3-deaza-SAM and (-)-S-tubercidinyl-L-methionine were substrates for COMT, whereas the weakly binding (-)-D-SAM was not a substrate. The (+)-diastereomers of each compound are probably competitive inhibitors as is known to be the case with (+)-L-SAM (Figure 18).[81] Even though they are substrates for HMT and COMT, none of the (-)-diastereomers was as efficiently utilized as the natural (-)-L-SAM substrate (Figure 1). These SAM analogue structure-activity relationship studies complement the L-SAH analogue studies and provide further evidence that the active sites of a class of enzymes carrying out a particular type of reaction are quite different in structure. A more complete picture of the active sites of these enzymes might permit the development of compounds which could selectively inhibit one enzyme in the presence of many others.



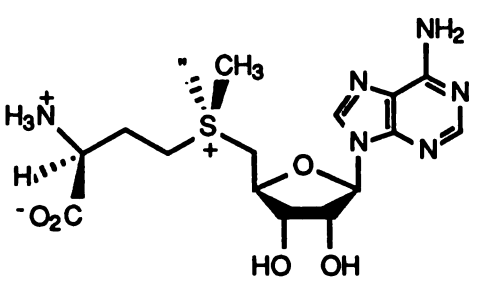
(±)-S-adenosyl-D-methionine



(±)-S-tubercidinyl-L-methionine



(±)-3-deaza-S-adenosyl-L-methionine



(+)-S-adenosyl-L-methionine

Figure 18. Analogues of S-adenosyl-L-methionine (SAM) which have been tested as inhibitors of methyltransferase activity and as potential substrates in methyltransferase-catalyzed reactions.

One problem which would seriously impede efforts to develop SAM analogue inhibitors of methyltransferases is the inherent chemical instability imparted on the molecule by the sulfonium center itself. This instability takes the form of racemization at the sulfonium group and sulfonium group-promoted degradation reactions. The chirality of the sulfonium group, which has three ligands and a free electron pair on the sulfur atom, has been known since the early 1900's.[82,83] Sulfonium compounds are known to racemize by pyramidal inversion[84,85] and SAM, which is known to exist in two diastereomeric forms differing in the configuration at the sulfur atom,[86] may racemize by a similar mechanism. The diastereomer of SAM with the S-configuration at the sulfonium center has been found to be the active substrate (-) form of SAM[87]; the other (+) diastereomer possessing the R-configuration at the sulfonium center is known to be an inhibitor of methyltransferases.[81,86,88] Borchardt[89] and Hoffman[90] have demonstrated that SAM can racemize between the inactive inhibitory R(+)-form and the active substrate S(-)-form (Figure 19, path **A**), although the rate of racemization is quite slow ($K_r = 1.8 \times 10^{-6}$ sec^{-1})[90] at pH 7.5 for this first-order process.

Whereas racemization at the sulfonium center in SAM is a reversible process, the intramolecular cyclization and acid/base-catalyzed hydrolysis reactions of both diastereomers of SAM are irreversible processes. In the former case, the carboxylate group of SAM can initiate a nucleophilic attack on the γ-carbon atom of the amino acid moiety, activated toward such an attack by the adjacent sulfonium group and the presence of an excellent dialkylsulfide leaving group. Under neutral and slightly acidic conditions at elevated temperatures, this intramolecular cyclization reaction rapidly proceeds to yield 5'-deoxy-5'-methylthioadenosine and L-α-amino-γ-butyrolactone which undergoes facile hydrolysis to give L-homoserine as the isolated product[86]

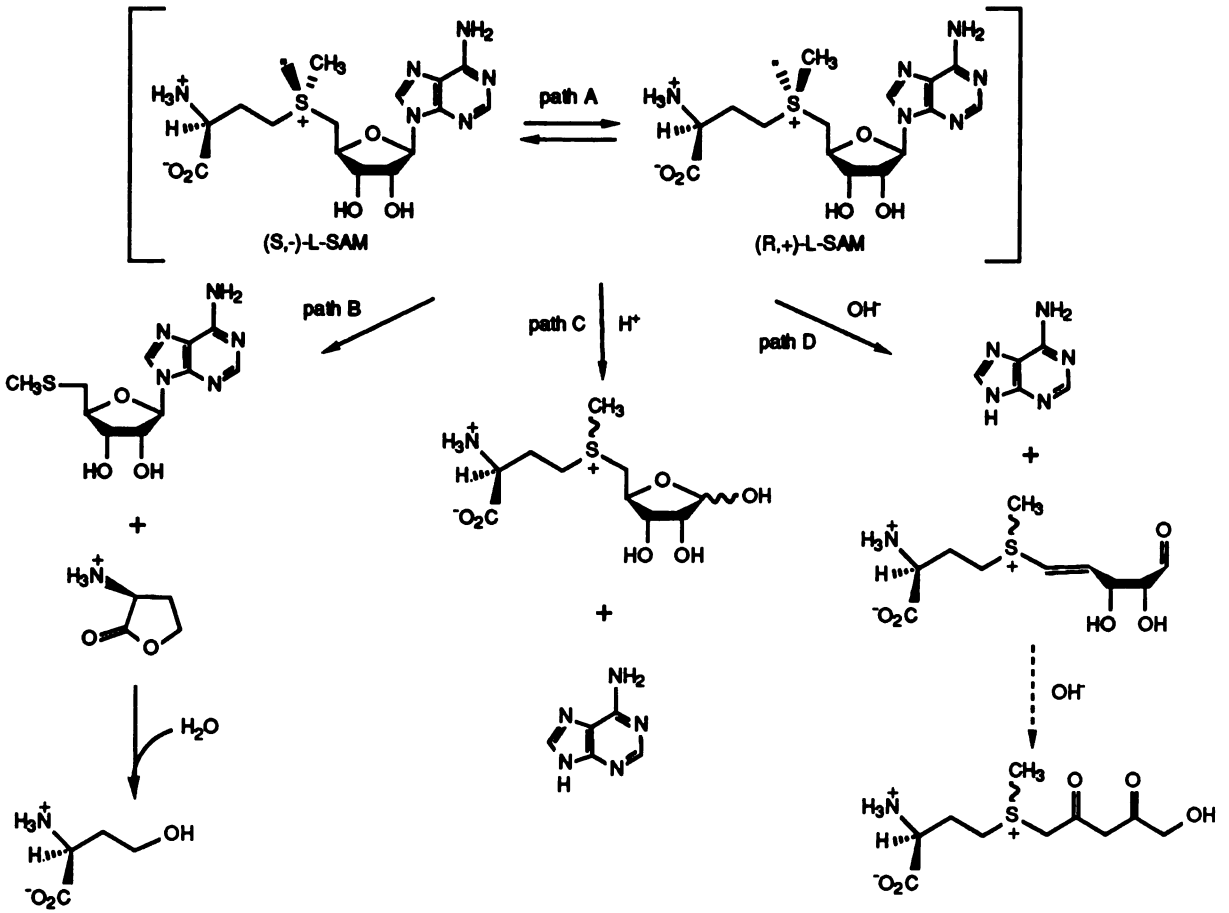


Figure 19. Reversible and irreversible decompositions of S-adenosyl-L-methionine: path A, racemization; path B, intramolecular cyclization; path C, acid-catalyzed hydrolysis of the glycoside bond; and path D, base-catalyzed hydrolysis of the glycoside bond.

(Figure 19, path **B**). The formation of five-membered rings is highly favored from an entropic point of view and SAM, with suitably situated nucleophilic and leaving groups, is particularly prone to this mode of decomposition.

Under more acidic conditions, below pH 2, the carboxylate group responsible for the intramolecular cyclization will be protonated and thus will be unable to function as a nucleophile. These acidic conditions are ideal for the cleavage of the ribose-adenine glycoside bond. The products of this cleavage reaction are adenine and S-ribosyl-L-methionine[91] (Figure 19, path **C**).

The glycoside bond of SAM is also cleaved under mildly basic conditions (pH 12-13) although the mechanism is quite different from that encountered in the acid hydrolysis and requires the presence of the sulfonium group several bonds distant.[87,92,93] The sulfonium group, in general, is known to increase the acidity of any α-hydrogens so that under basic conditions a sulfur ylid will form.[94] In dilute base, abstraction of one of the slightly acidic C-5' protons of SAM will produce a transient sulfur ylid which quickly eliminates adenine to leave a vinyl sulfonium intermediate which undergoes a series of base-catalyzed enolizations to yield a 1,3-diketone sulfonium compound (Figure 19, path **D**, and Figure 20).

Certainly, the acidic and basic hydrolyses of the glycoside bond of SAM described above would not be expected to plague studies of SAM or its analogues at physiological pH and 37°C.. In fact, Borchardt[93] has found that S-aristeromycinyl-L-methionine, which is a carbocyclic analogue of SAM (Figure 20) and is a reasonably good substrate for many small molecule methyltransferases (particularly PNMT),[80] is completely resistant to mild base hydrolysis. However, this compound would be expected to be susceptible to the same intramolecular cyclization reaction that SAM experiences. The sulfonium group which is so important in SAM biochemistry is thus a serious

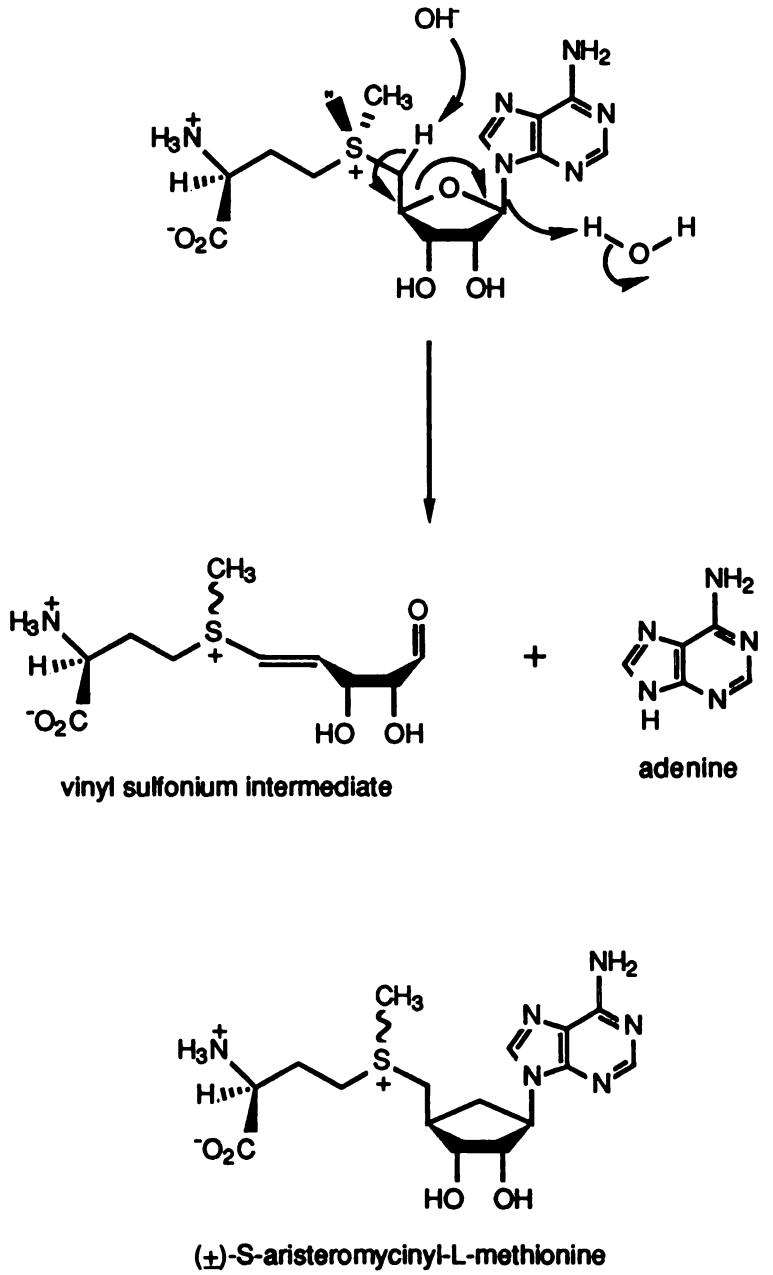


Figure 20. The initial steps in the base-catalyzed hydrolysis of S-adenosyl-L-methionine (above), and (±)-S-aristeromycinyl-L-methionine (below), a SAM analogue which is resistant to mild base hydrolysis.

obstacle in the development of potential methyltransferase inhibitors bearing a structural resemblance to SAM. The inherent instability imparted to the molecule by the sulfonium group would be alleviated by replacing that functionality with a tertiary amine.

Tertiary amines are quite stable under physiological conditions. In fact, the analogue of SAM in which the sulfur atom is replaced by a nitrogen atom (Figure 21) has been recently reported.[95] Such a compound would be stable under all conditions which are conducive to SAM degradation with the exception of those of low pH which hydrolyze the glycoside bond. At physiological pH, the inherent basicity of the tertiary amine functionality in the nitrogen SAM analogue would lead to its protonation and thus conserve the positive charge characteristic of the sulfonium group. The tertiary amine, despite its protonation under these conditions, will not undergo the intramolecular cyclization so characteristic of SAM (compare Figure 19, path B, and Figure 21, path A). The reason for this structural stabilization in the nitrogen methyl SAM analogue is that the potential leaving group is a dialkylamine in contrast to a dialkylsulfide as is the case with SAM. A dialkylamine is not nearly so good a leaving group as is a dialkylsulfide. According to its strict definition, an excellent leaving group is one in which the pKa of its conjugate acid is very low, which implies a great stability of the free conjugate base form. The pK_a of the conjugate acid of dimethylamine is approximately 10, whereas the pK_a of the conjugate acid of dimethylsulfide is about -5.4.[96] Thus, it would require a nucleophile much stronger than a carboxylate group to displace a dialkylamine rather than a dialkylsulfide. This argument would also suggest that the nitrogen methyl SAM analogue would not be a methyl group donor. Nucleophilic displacement of the methyl group could potentially release the nitrogen analogue of S-adenosylhomocysteine[66] as the

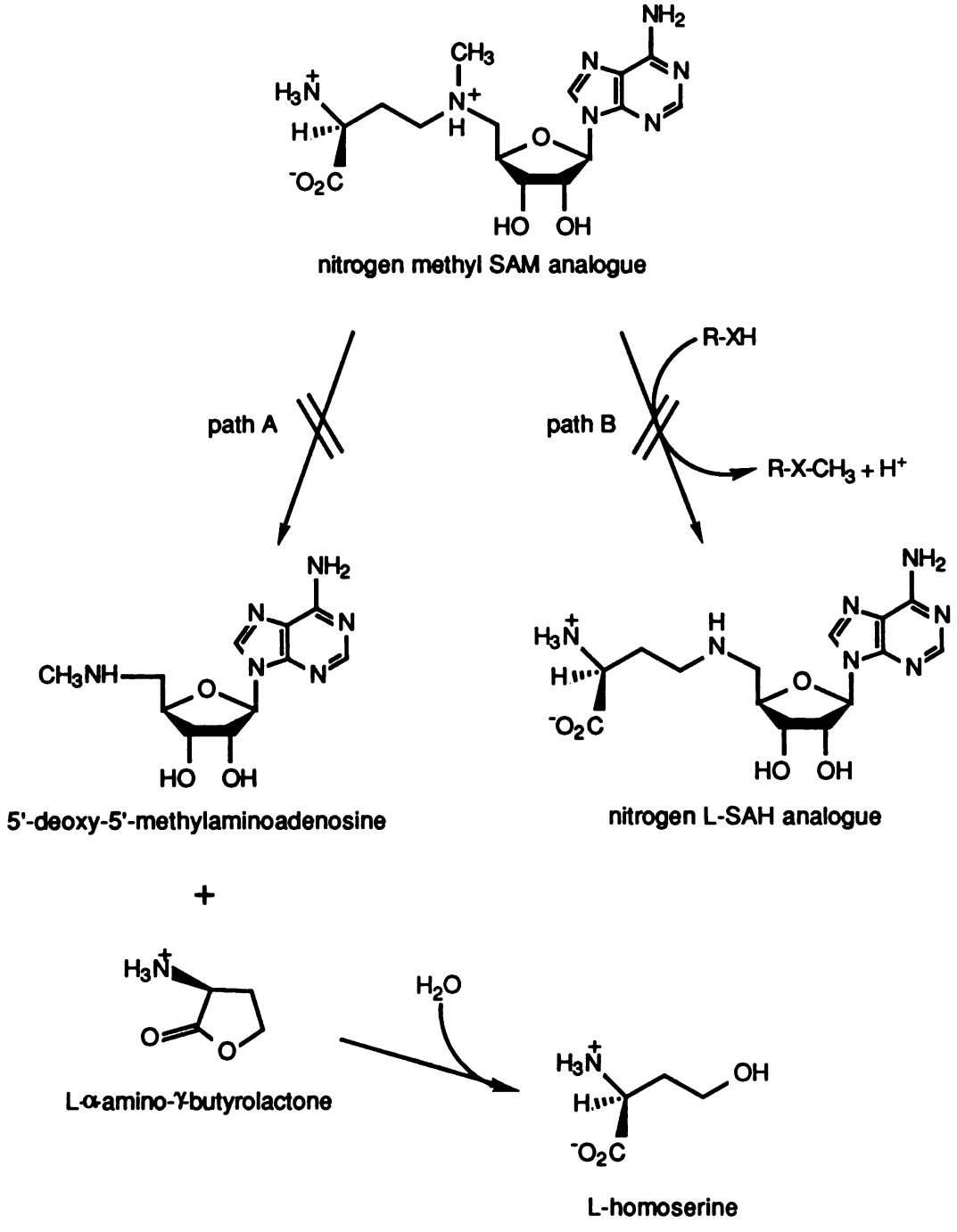


Figure 21. The nitrogen analogue of S-adenosyl-L-methionine, and some of the reactions of SAM to which it should be resistant.

leaving group (Figure 21, path B) but this is not likely to occur. Thus, the nitrogen analogue of SAM should exhibit a chemical stability not characteristic of SAM.

The racemization at the sulfonium center in SAM, possibly by a pyramidal inversion mechanism, would assume a different form in the nitrogen methyl SAM analogue. The protonation of the tertiary amine is fast and reversible. At neutral pH, roughly 0.1% of the tertiary amines exist in a free base form which can undergo pyramidal inversion much faster than that in sulfonium compounds.[97] A rapid equilibration among the amine pyramid forms followed by protonation ensures that interconversions between the two tertiary ammonium diastereomers will be quite rapid. Thus, it will be impossible to separate the rapidly interconverting tertiary ammonium diastereomers.

Other differences between SAM and its nitrogen methyl analogue can be attributed to the inherent nature of the sulfur and nitrogen atoms. The nitrogen atom is smaller than the sulfur atom and, as a consequence, carbon-nitrogen bonds are shorter than carbon-sulfur bonds. The average C-N bond length in trialkylammonium compounds is approximately 1.45 Angstroms,[98] whereas the average C-S bond length in trialkylsulfonium compounds is approximately 1.80 Angstroms.[99] As far as the bond angles are concerned, the C-S-C bond angles in trialkylsulfonium compounds are approximately 102°,[99] somewhat smaller than the tetrahedral angle of 109.5°. The approximate 111° C-N-C bond angles in trialkylammonium compounds[98] are only slightly larger than the tetrahedral angle. Apparently, the lone electron pair in the trialkylsulfonium compounds occupies a larger volume of space than the electron pairs involved in the covalent C-S bonds. Thus, the nitrogen SAM analogue would have three alkyl groups and a proton all projecting a comparatively short distance from the nitrogen atom in an almost perfect tetrahedral orientation; SAM, on the other

hand, has a dominant lone electron pair pushing the three more distant alkyl groups closer together than a normal tetrahedral structure would require (Figure 22).

As a very close structural analogue of SAM, the nitrogen methyl SAM analogue might be expected to occupy the SAM binding site in the active site of methyltransferases. The catalytic mechanism of COMT has received extensive study[100-103] and, not surprisingly, appears to involve an S_N2-like process. Consonant with this mechanism, COMT is believed to position the attacking nucleophile of the methyl acceptor directly behind the carbon end of the methylsulfonium C-S bond. The enzyme then pushes the two groups together to compress the trigonal-bipyramidal S_N2 transition state[100,103] leading to a very rapid transfer of the methyl group. Additional support for this mechanism comes from the observed inversion of configuration of the chiral methyl group donated by SAM.[104] This S_N2 mechanism is probably general for all methyltransferases.

Obviously, each methyltransferase must reserve space in its active site for the methyl acceptor substrate. Binding of the methyl acceptor might not be hindered by the nitrogen SAM analogue situated in the SAM binding site due to their comparable sizes. However, the transfer of the methyl group in this case would most likely not occur. Thus, an inhibition of methyltransferase activity would be observed. Substitution of the methyl group of the nitrogen methyl SAM analogue with larger alkyl groups (e.g., ethyl, propyl) might give a compound which could bind to the active site and partially occupy the space normally reserved for the methyl acceptor. Partial or complete prevention of binding by the methyl acceptor substrate would also inhibit enzyme activity. Increasing the size of the alkyl group might lead to a greater occupation of the remainder of the active site and lead to tighter binding by the SAM analogue

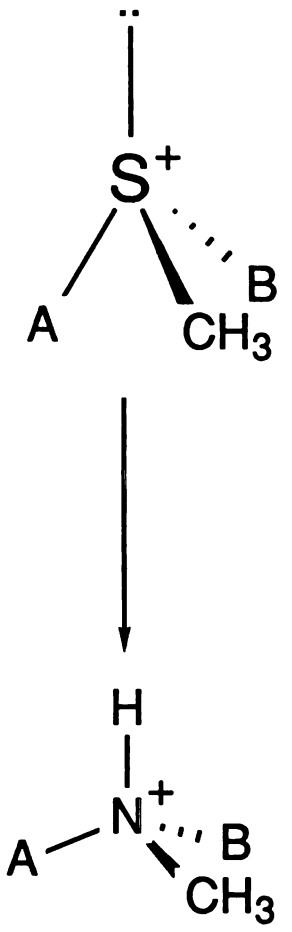


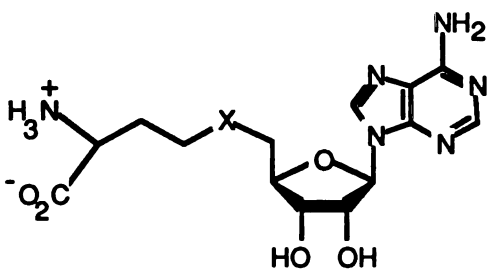
Figure 22. Restoration of tetrahedral geometry around the central atom upon substitution of nitrogen for sulfur in SAM (exaggeration): A, 5'-adenosyl; B, 3-amino-3-carboxypropyl.

and more potent inhibition of methyltransferase activity, particularly if the alkyl group fills a hydrophobic pocket. These ethyl, propyl, and higher alkyl nitrogen SAM analogues would be much more stable than the S-ethyl and S-propyl analogues of SAM, which inhibit transmethylations[105] yet suffer from the same decomposition reactions which are widely seen with SAM (Figure 19).

The availability of a wide variety of structurally stable nitrogen alkyl SAM analogues would be very useful to enzymologists studying methyltransferases. Consequently, a general procedure for their synthesis should be available so that any desired compound could be readily prepared. The procedure used by Davis *et al.*[95] for the synthesis of the nitrogen methyl SAM analogue employed a catalytic hydrogenation step which consequently precludes the replacement of the methyl group with an unsaturated alkyl group. For this reason, a research program aimed at developing a general synthesis of such analogues, compatible with both saturated and unsaturated alkyl groups, was initiated.

Results and Discussion

The general strategy followed in the synthesis of a series of nitrogen alkyl SAM analogues involved two successive monoalkylations of the primary alkylamine replacing the methylsulfonium moiety of SAM (**1**). The 5'-adenosyl group was linked to the primary alkylamine in the first alkylation and a homoserine equivalent was coupled to this intermediate dialkylamine in the second alkylation. The resulting trialkylamine, bearing three protecting groups, was the immediate precursor of the nitrogen alkyl SAM analogues. The amino acid and ribose 2',3'-diol protecting groups were removed by base and acid hydrolysis, respectively, to provide the final deprotected compounds (**2a-g**) shown below:



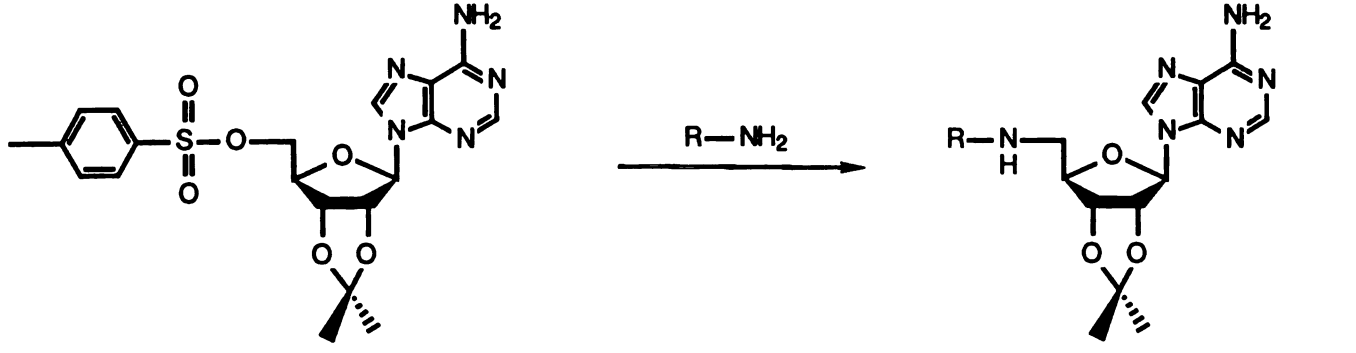
1,2a-g

1, X = Me-S^+
2a, X = Me-NH^+
b, X = Et-NH^+
c, X = n-Pr-NH^+
d, X = CH_2=CH-CH_2-NH^+
e, X = n-Bu-NH^+
f, X = n-Pe-NH^+
g, X = n-Oc-NH^+

Maintaining a capability for replacing the methylsulfonium moiety of SAM with either saturated or unsaturated primary alkylamines provided the major challenge in developing the general synthetic scheme described below. The synthesis of the nitrogen methyl SAM analogue (**2a**) by Davis *et al.*,[95] using many of the techniques originally developed by Chang and Coward,[66] included a step in which an azide group is catalytically hydrogenated to an amino group. Such hydrogenation-type reaction conditions were to be avoided in a more general synthetic scheme so that the incorporation of unsaturated primary

alkylamino moieties into the final products would not be compromised. Although the original scheme is somewhat more efficient in terms of the yields of some of the individual reactions, the method described forthwith provides access to a larger variety of nitrogen alkyl SAM analogues. Such compounds could be studied as potential inhibitors of methyltransferases and other SAM-utilizing enzymes.

A derivative of adenosine, activated toward nucleophilic attack at the 5'-position, was needed to conduct the first monoalkylation of the primary alkylamine being incorporated into the general SAM structure. Tosylation of the 5'-hydroxyl group of 2',3'-O-isopropylideneadenosine with p-toluenesulfonyl chloride in pyridine has been shown to activate that position toward attack by a wide variety of nitrogen, sulfur, selenium, and halogen nucleophiles.[106,107] By dissolving 5'-O-tosyl-2',3'-O-isopropylideneadenosine (**3**) in a large excess of a primary alkylamine, a series of 5'-alkylamino-5'-deoxy-2',3'-O-isopropylideneadenosine homologues (**4a-g**) could be readily obtained in 60-75% yield (Scheme I). (Monoalkylation of the primary alkylamine is favored over di- and trialkylation only when the amine is used in large excess.) Those primary alkylamines which have been used to displace this 5'-O-tosyl group included methylamine, ethylamine, n-propylamine, allylamine, n-butylamine, n-pentylamine, and n-octylamine. The 5'-allylamino-5'-deoxy-2',3'-O-isopropylideneadenosine homologue (**4d**) is of particular interest because it would ultimately be hydrogenated to the corresponding propylamino compound if there were no alternative to the established method of synthesizing nitrogen alkyl SAM analogues. This procedure has been used by Murayama *et al.*[108] to prepare a series of 5'-alkyl-amino-5'-deoxyadenosine compounds directly from 5'-O-tosyladenosine. This method was used to prepare 5'-deoxy-5'-



3

4a , R = Me
b , R = Et
c , R = n-Pr
d , R = CH_2=$CHCH_2$
e , R = n-Bu
f , R = n-Pe
g , R = n-Oc

Scheme I. Preparation of 5'-alkylamino-5'-deoxy-2',3'-O-isopropylideneadenosine homologues (**4a-g**).

methylamino-2',3'-O-isopropylidene-adenosine (**4a**), one of the important intermediates used in the synthesis of the nitrogen methyl SAM analogue.[95]

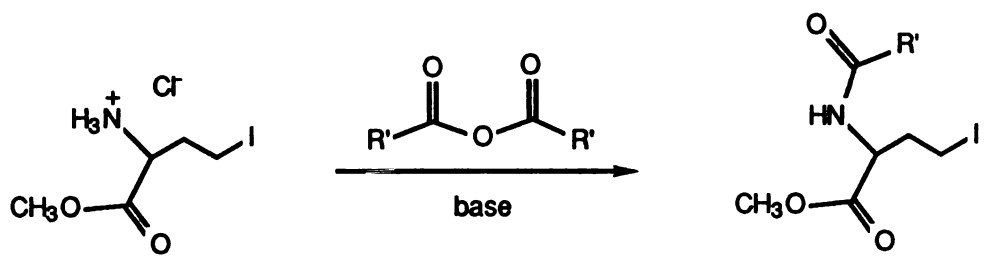
Alkylation of the these 5'-alkylamino-5'-deoxy-2',3'-O-isopropylidene-adenosine intermediates (**4a-g**) with a suitably protected homoserine derivative, activated toward nucleophilic attack at the 4-position, was expected to provide the desired product as a protected trialkylamine. Benzyl 2(R,S)-azido-4-iodobutyrate was used to alkylate the 5'-benzylamino-5'-deoxy-[66] and 5'-deoxy-5'-methylamino-2',3'-O-isopropylideneadenosines (**4a**)[95] in the syntheses of the nitrogen analogues of SAH and SAM, respectively. The catalytic reduction of the azido group to an amino group was an obligatory step in both syntheses and, as discussed above, would preclude the incorporation of unsaturated alkyl groups elsewhere in the final product. For this reason, another homoserine derivative, activated toward nucleophilic attack at the 4-position, was needed. Ideally, this compound should possess a suitably protected intact α-amino group, rather than a direct precursor thereof.

Some years ago, Frankel and Knobler[109] developed a chemical synthesis of D,L-homoserine. In the course of this work, one of the key intermediates, 2(R,S)-amino-4-iodobutyrate hydroiodide, was shown to be readily converted, under Fischer esterification conditions, to the corresponding methyl ester hydrochloride. Esterification of the carboxylic acid prevents the facile lactonization by nucleophilic attack of the carboxylate group on the activated 4-position from occurring under neutral and alkaline conditions. This ester provided an excellent starting point in the search for a suitable homoserine equivalent for the alkylation of the 5'-alkylamino-5'-deoxy-2',3'-O-isopropylideneadenosine homologues (**4a-g**).

More recently, the formylation of potentially reactive amino acid esters with formic anhydride has been described.[110,111] An excess of this reagent,

prepared *in situ* by a carbodiimide-mediated condensation of formic acid, is used to formylate an amino acid ester hydrochloride in the presence of two equivalents of a tertiary amine. The formylation of methyl 2(R,S)-amino-4-iodobutyrate hydrochloride (**5**), prepared in two steps from α-amino-γ-butyrolactone hydrobromide,[109] was carried out using this method to give methyl 2(R,S)-formamido-4-iodobutyrate (**6a**, Scheme II) in 36% yield. Any of a number of other anhydrides could replace formic anhydride in the general procedure to similarly protect the amino group. Of particular interest is commercially available trifluoroacetic anhydride. This highly reactive acylating agent was used to prepare methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) in excellent (90%) yield. These procedures complement those which have been used to protect the amino group of this ester as a benzyl[112-115] or methyl[13,14] carbamate. These successful acylations of methyl 2(R,S)-amino-4-iodobutyrate (**5**) clearly establish that its amino group can be easily protected under mild conditions without significant polymerization of the free base form.

Methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) was considered to be more suitable than methyl 2(R,S)-formamido-4-iodobutyrate(**6a**) for use in the alkylation of the 5'-alkylamino-5'-deoxy-2',3'-O-isopropylideneadenosine homologues (**4a-g**). Once coupled to the nucleoside, initial deprotection of the resulting trialkylamine was anticipated to proceed more readily under milder conditions when the amino acid methyl ester moiety was trifluoroacetylated rather than formylated. The trifluoroacetyl amino protecting group can be readily removed under moderately alkaline conditions, such as either sodium carbonate[116] or potassium carbonate[117,118] in aqueous methanol. Increasing the reaction time and/or the reaction temperature would be expected to completely hydrolyze the alkaline-labile methyl ester as well. As a result, both amino acid protecting groups could probably be removed simultaneously in a



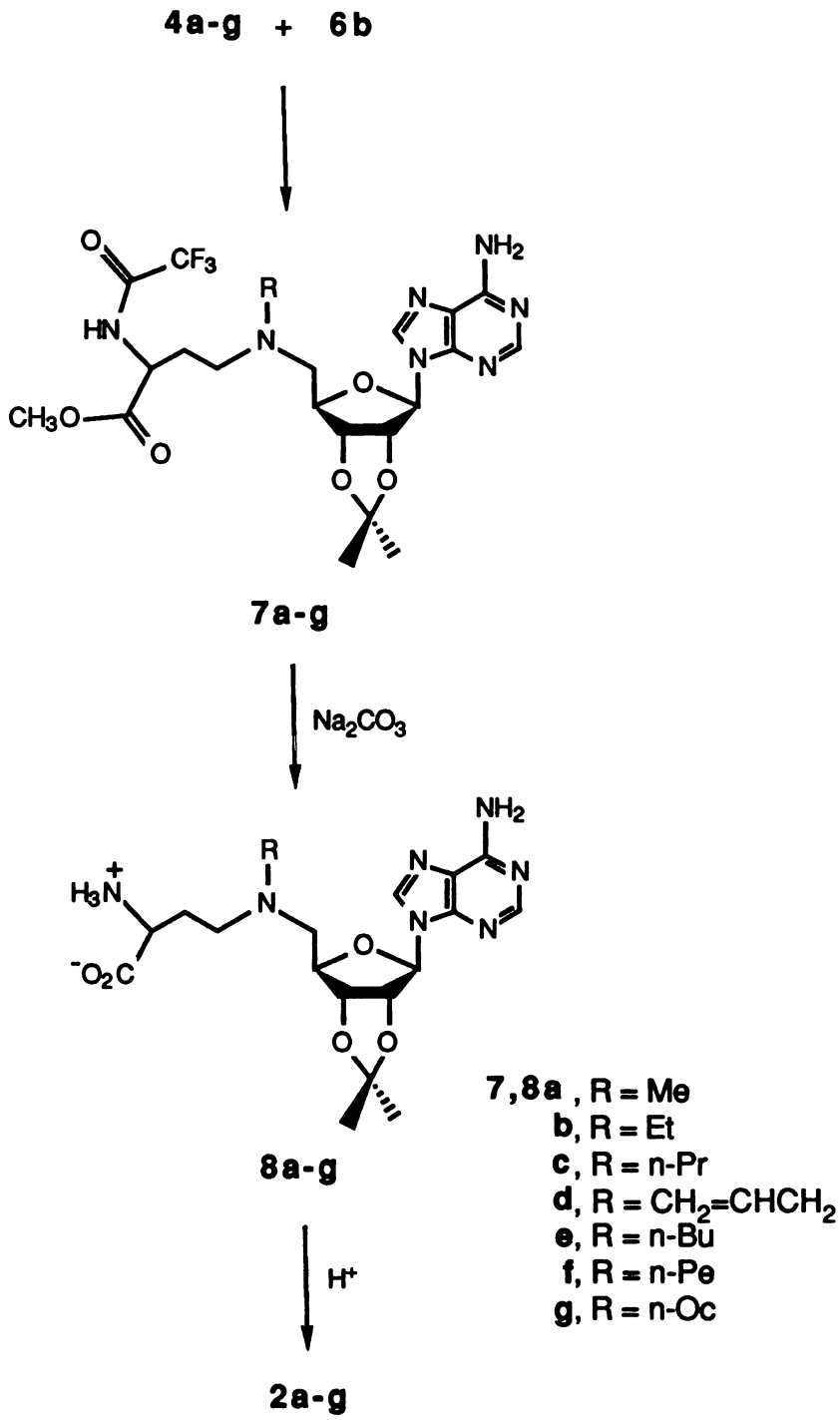
5

6 a, R' = H
b, R' = CF_3

Scheme II. Acylation of methyl 2(R,S)-amino-4-iodobutyrate hydrochloride (**5**).

one-pot reaction under mild conditions. [Two reports[119,120] substantiating the plausibility of this synthetic approach appeared during the course of this work. Novel chemical syntheses of SAH and its analogues were described using N,N-bis(trifluoroacetyl)-L-homocystine dimethyl ester to introduce the amino acid moiety into the final compounds. Complete deprotection of the trifluoroacetylated SAH/SAH analogue methyl esters in a one-pot reaction was readily achieved using barium hydroxide in aqueous methanol.]

A small excess (25%) of methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) was used to alkylate each 5'-alkylamino-5'-deoxy-2',3'-O-isopropylideneadenosine homologue (**4a-g**). This reaction, which according to the general synthetic strategy was the second alkylation of the original primary alkylamine (Scheme III), was carried out in acetonitrile at 70°C. in the presence of 1.1 equivalents of N,N-diisopropylethylamine. The fully protected trialkylamine products (**7a-g**), isolated from the crude reaction mixtures as their respective monohydrates by a combination of flash and low pressure liquid chromatography, were obtained in low to moderate yields. For example, the alkylation of 5'-deoxy-5'-methylamino-2',3'-O-isopropylideneadenosine (**4a**) with methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) provided the fully protected trialkylamine product (**7a**) in 42% yield. In contrast, Davis *et al.*[95] alkylated the same nucleoside with benzyl 2(R,S)-azido-4-iodobutyrate to give the corresponding trialkylamine product in 93% yield. Increasing the size of the 5'-alkylamino group in the new alkylation procedure resulted in a decrease in the yield of the product to approximately 20-25% regardless of the alkyl group involved. Attempts to optimize the reaction conditions, so as to improve the yield by including a large excess of N,N-diisopropylethylamine (5.0 equivalents) in the reaction mixture, failed. The discrepancy in the efficiencies of these analogous final coupling reactions must somehow be linked to the methyl



Scheme III. Synthesis and two-step deprotection of the fully protected nitrogen alkyl SAM analogues (**7a-g**).

2(R,S)-trifluoroacetamido-4-iodobutyrate alkylating reagent (**6b**). Although the exact nature of this problem is not clear, it can be easily circumvented by increasing the scale of the reaction.

Analysis by high resolution (500 MHz) proton NMR indicated, as expected, that each fully protected nitrogen alkyl SAM analogue was actually present as a 50:50 mixture of two diastereomers. Each pair of diastereomers differs in the stereochemistry around the α-carbon atom in the amino acid moiety. Particularly diagnostic of the presence of diastereomers were the resonances of both isopropylidene methyl groups, the methyl ester methyl group, the amino acid α-carbon hydrogen, the ribose C-2' and C-3' hydrogens, and the trifluoroacetamide hydrogen. These resonances, centered at 1.4, 1.6, 3.7, 4.5, 5.0, 5.5, and 9.5 ppm, respectively, were split into two groups of equal intensity, one group for each diastereomer. While many of the other hydrogens elsewhere in these compounds showed similar evidence of diastereomeric nonequivalence, these cases were not so dramatic as that exhibited by those listed above. For example, the ribose C-2' hydrogen resonances (Figure 23) of each diastereomer of the nitrogen propyl SAM analogue (**7c**) can be clearly distinguished as two doublets of doublets of equal intensity centered at 5.49 and 5.52 ppm. The distinctive splitting of each C-2' hydrogen resonance into a doublet of doublets is a consequence of each proton being coupled to the adjacent C-1' and C-3' hydrogens [coupling constants are listed in the Materials and Methods section, page 74]. Subtle variations in the shielding of each nucleus account for the small difference (0.03 ppm) in chemical shift between the two diastereomeric ribose C-2' hydrogens. The resonances of the α-carbon hydrogen (4.42 and 4.49 ppm), the C-3' hydrogen (4.96 and 4.98 ppm), and the adenine amino group hydrogens (5.81 and 5.82 ppm) of each diastereomer can be similarly distinguished. Such is not the case, however, with the C-1' and C-

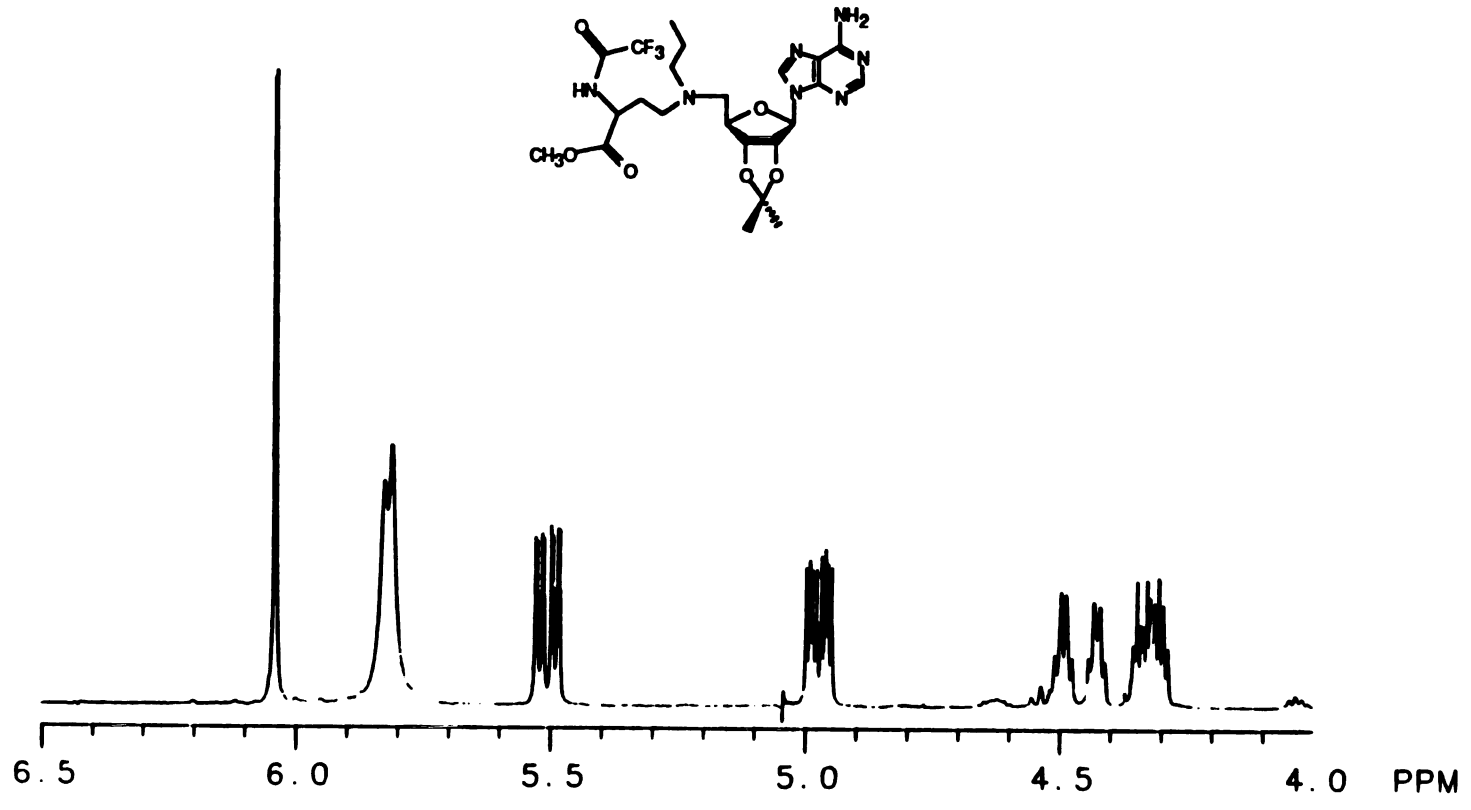


Figure 23. A portion of the 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of the protected nitrogen propyl SAM analogue (**7c**), providing evidence for the presence of an equimolar mixture of diastereomers.

4' resonances at 6.04 and 4.34 ppm, respectively. Thus, the NMR results clearly indicate that the alkylation of each chiral 5'-alkylamino-5'-deoxy-2',3'-O-isopropylideneadenosine homologue (**4a-g**) with a racemic mixture of the enantiomers of methyl 2-trifluoroacetamido-4-iodobutyrate (**6b**) proceeded without evidence of significant asymmetric induction.

The diastereomers of each methyl N^4-alkyl-N^4-(2',3'-O-isopropylidene-5'-adenosyl)-2(R,S)-trifluoroacetamido-4-aminobutyrate homologue (**4a-g**) were not readily separable by either flash or low pressure liquid silica gel chromatography. A potentially difficult and time-consuming resolution did not appear to be necessary at this time due to a lack of knowledge regarding which diastereomer has the R-configuration and which diastereomer has the S-configuration at the α-carbon atom, as well as preliminary enzyme inhibition data suggesting that one diastereomer (once completely deprotected) would be much more potent than the other. Considering these points, this 50:50 mixture of fully protected product diastereomers was suitable as such for deprotection.

As expected, both amino acid protecting groups of the fully protected nitrogen alkyl SAM analogues could be removed simultaneously by treatment with sodium carbonate in hot 50% aqueous methanol (Scheme III). The loss of both protecting groups was readily monitored by thin layer chromatography of aliquots of the reaction mixture on silica gel, using methanol as the developing solvent. The chromatogram (Figure 24) revealed that, after 15 minutes, the gradual fading of the starting material spot at approximately R_f 0.70 (UV, I_2) was accompanied by the appearance of an intense spot and another fainter spot at about R_f 0.60 (UV, I_2) and R_f 0.45 (UV, I_2, ninhydrin), respectively. The disappearance of the starting material spot (**A** in Figure 24) after 30 minutes coincided closely with the time required for complete dissolution of this compound in the reaction mixture. Also, by this time, the intensities of the two

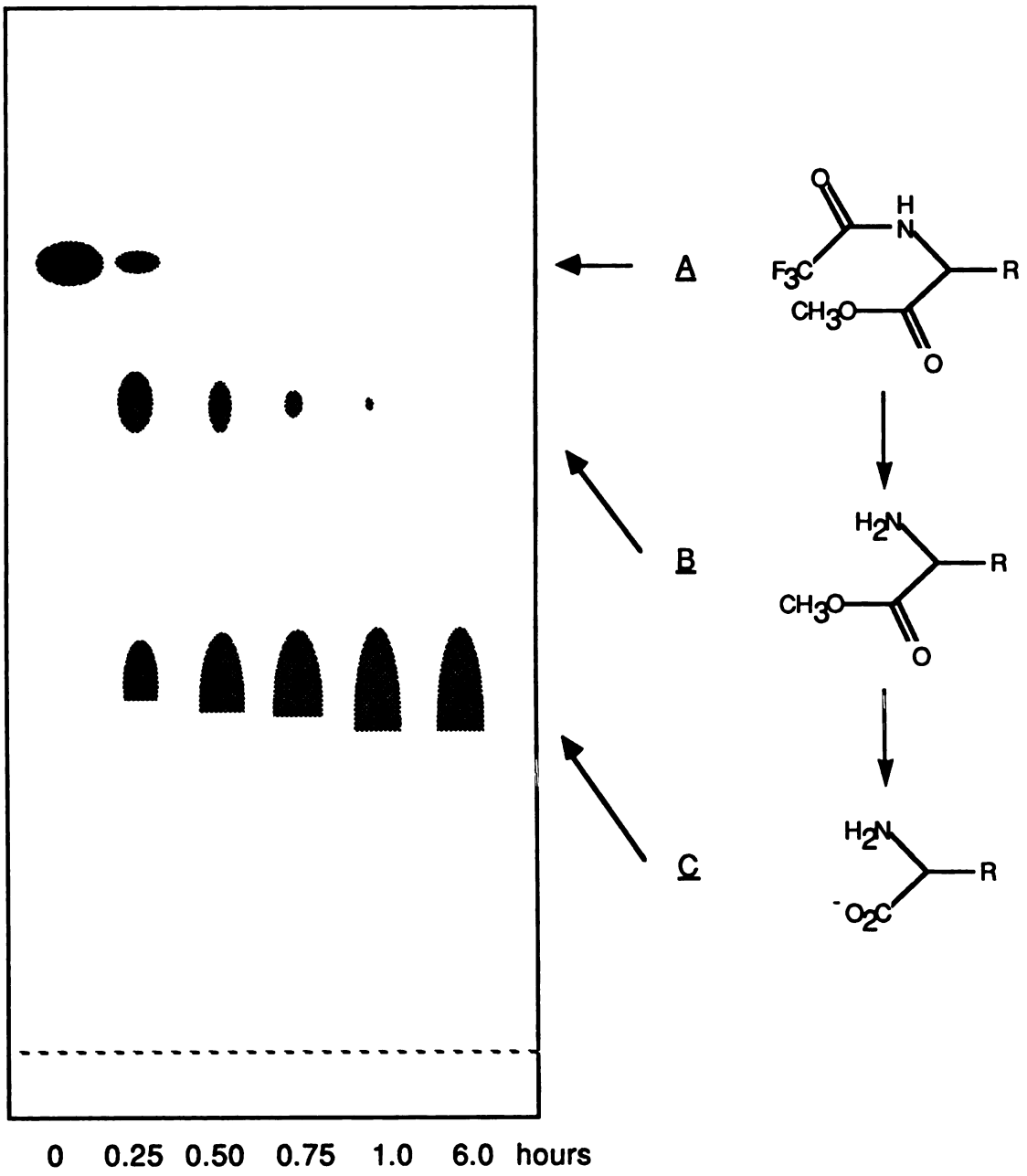


Figure 24. The general appearance of the silica gel chromatogram used to monitor the removal of both amino acid protecting groups from the fully protected nitrogen alkyl SAM analogues with sodium carbonate in hot 50% aqueous methanol.

slower-moving spots had become nearly equal. Presumably, the faster of these two spots corresponded to the detrifluoroacetylated methyl ester intermediate (**B** in Figure 24) since the trifluoroacetamide group is more alkaline-labile than the methyl ester and would, in most cases, be hydrolyzed first. The other spot corresponds to the completely deprotected amino acid product (**C** in Figure 24). As the reaction time was extended to two hours, the methyl ester was more extensively hydrolyzed so that the lower spot became more and more intense at the expense of the other spot until only the lower one remained. The reaction time was extended to six hours to ensure that the hydrolysis of all base-sensitive protecting groups was indeed complete. Each N^4-alkyl-N^4-(2',3'-O-isopropylidene-5'-adenosyl)-2(R,S),4-diaminobutyric acid homologue (**8a-g**) in a methanol extract of each neutralized reaction mixture was purified by flash chromatography in high yield. The recovered colorless solids, which were sufficiently pure for characterization by mass spectrometry and high resolution proton NMR, were used directly in the final deprotection step.

The removal of the final protecting group, the acid-labile isopropylidene ketal blocking the ribose 2',3'-diol, was carried out in dilute (0.1M) aqueous sulfuric acid (Scheme III). The progress of this reaction was readily followed by thin layer chromatography on silica gel using a mixture of ethanol (90%) and concentrated aqueous ammonium hydroxide (10%) as the eluting solvent. The starting material, appearing at approximately R_f 0.55 (UV, I_2, ninhydrin), was observed to disappear gradually, giving rise to the deprotected product, showing up at approximately R_f 0.20 (UV, I_2, ninhydrin). Although the reaction was somewhat slow (requiring about 11 days to complete), the conditions were sufficiently mild to avoid any concomitant hydroysis of the glycoside bond. The presence of adenine in the reaction mixture is indicative of this side reaction. Although the above TLC system was capable of detecting adenine as an

ultraviolet-absorbing spot at R_f 0.70, none was ever observed under the reaction conditions described above.

All N^4-(5'-adenosyl)-N^4-alkyl-2(R,S),4-diaminobutyric acid homologues (**2a-g**) were purified by cation exchange chromatography and characterized by microanalysis, mass spectrometry, and high resolution proton NMR. An interesting, but not surprising, correlation was observed between the length (i.e., hydrophobicity) of the N^4-alkyl group and the buffer concentration needed to elute the compound from the cation exchange column. As the size of this alkyl group increased, the hydrophobic interactions between the molecule and the polystyrene matrix of the Dowex 50X4-400 cation exchange resin also increased. Consequently, a higher concentration of ammonium bicarbonate buffer was required to elute the adsorbed compound. For example, the elution peak of the nitrogen ethyl SAM analogue (**2b**) occurred at an ammonium bicarbonate concentration of 0.86M. Extending the length of the alkyl group by three carbons, to give the nitrogen pentyl SAM analogue (**2f**), caused the elution peak to occur at a buffer concentration of 1.11M. Increasing the length by three more carbons gave a compound, the nitrogen octyl SAM analogue (**2g**), which could not be eluted from the column with an almost saturated (1.7M) solution of ammonium bicarbonate. Aqueous ammonium hydroxide (1M) was required to bring about its elution. These observations suggest that those analogues with large N^4-alkyl groups [i.e., butyl(**2e**), pentyl(**2f**), and octyl(**2g**)] could bind tightly to the active sites of methyltransferases catalyzing the methylation of hydrophobic compounds. Such compounds (e.g., sterols) would be expected to fit into a hydrophobic pocket, created by leucine, isoleucine, valine, and other amino acids, in the active sites of these enzymes (e.g., yeast sterol methyltransferase[121]). Hydrophobic interactions between the large N^4-

alkyl group and these amino acids could make a significant contribution to the binding energy between the inhibitor and the enzyme.

Enzyme studies in other laboratories have, thus far, been made with only the nitrogen methyl SAM analogue (**2a**), and preliminary results indicate that this compound does exhibit some interesting properties. Matthews and Frasca (unpublished results) at the University of Michigan have tested this compound as a potential inhibitor of methionine synthase activity. This bacterial enzyme (Figure 11) catalyzes the transfer of the methyl group of N^5-methyl-tetrahydrofolate to homocysteine to form methionine. SAM is required in only catalytic amounts to activate or "prime" the enzyme by methylation at an unknown site.[56,57] The incubation of methionine synthase with the nitrogen methyl SAM analogue led to an inhibition of its normal activity. This inhibition (K_i approximately 9 μM) is competitive with respect to SAM. The simplest interpretation of this result is that the analogue competes with SAM for its binding site on the enzyme. Because the analogue cannot donate its methyl group, the enzyme cannot be "primed" and consequently fails to exhibit catalytic activity.

Hardy and Santi at the University of California, San Francisco have tested this compound with E. coli tRNA (uracil-5-)-methyltransferase.[122] This enzyme, probably employing a mechanism of action similar to that described for DNA (cytosine-5-)-methyltransferase (Figure7) and thymidylate synthetase, replaces the hydrogen at position 5 of a tRNA uridine with the methyl group of SAM to give a thymidine. In the absence of SAM, the enzyme will catalyze the release of tritium at the 5 position into water at a rate 0.4% of that observed in the presence of SAM under normal turnover conditions. In the presence of the nitrogen methyl SAM analogue and in the absence of SAM, the rate of tritium release from the 5 position is essentially the same as that seen under normal

turnover conditions when SAM is present, but no product is formed. This result probably indicates that both SAM and the nitrogen methyl SAM analogue (**2a**) may induce conformational changes somehow facilitating tritium release. Whereas the presence of only SAM can lead to the formation of thymidine, the presence of only its nitrogen analogue does not lead to product formation.

In conclusion, a new synthetic route to nitrogen alkyl SAM analogues (**2a-g**) has been developed. This procedure avoids the hydrogenation-type conditions of an earlier procedure[95] which would preclude the replacement of the methylsulfonium moiety of SAM with an unsaturated alkylamine. The key step involves the alkylation of a 5'-alkylamino-5'-deoxy-2',3'-O-isopropylideneadenosine (**4a-g**) with methyl 2(R,S)-trifluoroacetamido-4-iodo-butyrate (**6b**), a derivative of homoserine. Deprotection of the resulting trialkylamine diastereomers by base and then acid hydrolysis afforded the final products in moderate yield. The previously unattainable nitrogen allyl SAM analogue (**2d**) was prepared by this method. Preliminary enzyme testing has revealed that the nitrogen methyl SAM analogue (**2a**) does indeed serve as an excellent mimic of SAM in important biochemical processes.

Materials and Methods

2',3'-O-Isopropylideneadenosine, p-toluenesulfonyl chloride, all alkylamines, α-amino-γ-butyrolactone hydrobromide, 4-methylmorpholine, formic acid, trifluoroacetic anhydride, flash silica gel (230-400 mesh), and Dowex 50X4-400 were purchased from Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was obtained from Sigma. Precoated Baker 1B2-F silica gel sheets, purchased from VWR, were used for analytical thin layer chromatography. Low pressure liquid chromatography, using an Altex 100 HPLC system and an Altex Model 153 Analytical UV detector (280 nm), was carried out on a Lobar Size B (310-25) Lichroprep^{TM} Si60 silica gel column obtained from VWR. All melting points were taken on a Thomas-Hoover melting point apparatus and are uncorrected. Ultraviolet spectra were taken on a Hitachi 100-80 UV-VIS spectrophotometer equipped with an automatic six-cell changer and a Peltier temperature controller. Low resolution EI mass spectra were obtained on a Kratos MS-25 mass spectrometer using a heated direct insertion probe. Samples for positive liquid secondary ion mass spectrometry (LSIMS) were run in a matrix of glycerol and 1M hydrochloric acid. The positive LSIMS spectra were acquired on a Kratos MS-50 mass spectrometer equipped with a 23-kG magnet, a postacceleration detector operated at -10kV, and a source built by the UCSF Mass Spectrometry Facility. Proton NMR spectra were acquired with either a General Electric GN-500 NMR spectrometer or a home-built 240 MHz NMR spectrometer interfaced with a Nicolet 1180 computer and a 293-B pulse programmer. NMR samples were dissolved in either D_2O or deuteriochloroform, and the acquired spectra referenced to the HDO (4.80 ppm) or tetramethylsilane peaks, respectively. Microanalyses were performed by the Microanalytical Laboratory in the Department of Chemistry at the University of California, Berkeley.

5'-O-Tosyl-2',3'-O-Isopropylideneadenosine (3) was prepared according to the procedure of Jahn[106] with slight modifications. 2',3'-O-Isopropylideneadenosine (25.12 g, 81.8 mmol) was dissolved with stirring in dry pyridine (140 mL). After cooling in a carbon tetrachloride-dry ice bath, p-toluenesulfonyl chloride (25.12 g, 132.2 mmol) was added and the mixture was stirred at -22°C. for 18 hours. The reaction was quenched with ice cold water (300 mL) and the product extracted into cold chloroform (3 x 175 mL). The combined chloroform extracts were washed with 1M sulfuric acid (4 x 200 mL), water (2 x 200 mL), 1M sulfuric acid again (3 x 200 mL), water (3 x 200 mL), saturated aqueous sodium bicarbonate (3 x 200 mL), and water (3 x 200 mL), respectively, all at 0°C.. Evaporation of the chloroform provided pure product as a bright yellow foam (36.45 g, 96.7%): mp 50-53°C.; 500 MHz ^{1}H NMR ($CDCl_3$), δ 1.37 (s, 3H, isopropylidene CH_3), 1.59 (s, 3H, isopropylidene CH_3), 2.39 (s, 3H, tosyl CH_3), 4.23 (dd, 1H, J = 6.2Hz, 10.6Hz, H-5'), 4.30 (dd, 1H, J = 4.4Hz, 10.6Hz, H-5'), 4.48 (m, 1H, J = 3.0Hz, 4.4Hz, 6.2Hz, H-4'), 5.05 (dd, 1H, J = 3.0Hz, 6.2Hz, H-3'), 5.34 (d, 1H, J = 6.2Hz, H-2'), 6.00 (br s, 2H, NH_2), 6.06 (s, 1H, H-1'), 7.17 (d, 2H, J = 7.7Hz, tosyl H-3 and H-5), 7.62 (d, 2H, J = 7.7Hz, tosyl H-2 and H-6), 7.85 (s, 1H, adenine H-2 or H-8), 8.23 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 260 nm (ε 11.5 x 10^3); MS (positive LSIMS), m/e 462 $(M + H)^+$, 290 $(M - \text{tosylate})^+$.

Anal. Calculated for $C_{20}H_{23}N_5O_6S$: C, 52.05; H, 5.02; N, 15.18; S, 6.95. Found: C, 52.07; H, 5.00; N, 15.08; S, 6.73.

General Preparation of 5'-Alkylamino-5'-Deoxy-2',3'-O-Isopropylideneadenosine Homologues(**4a-g**). 5'-O-Tosyl-2',3'-O-isopropylideneadenosine (10.00 g, 21.7 mmol) was dissolved in freshly distilled alkylamine (100 mL, large excess) and stirred at room temperature for three days. After evaporation to dryness, the residue was partitioned between methylene chloride (220 mL)

and 0.1M sodium hydroxide (220 mL). The aqueous layer was washed with methylene chloride (2 x 200 mL). All methylene chloride solutions were combined, washed with saturated aqueous sodium chloride (2 x 200 mL), dried over anhydrous magnesium sulfate, and evaporated. The residue was dissolved in chloroform (100 mL), concentrated to approximately 5 mL, and loaded onto a wet-packed flash silica gel column (44 x 3.5 cm, 200 g). The column was eluted with methanol at a flow rate of 10 mL/min and 28 mL fractions were collected. Pure product (as judged by TLC) generally appeared in fractions 15-80 depending on the alkylamino substituent. The appropriate fractions were pooled, evaporated to dryness, twice redissolved in chloroform (100 mL), and evaporated to give a colorless hygroscopic foam. Yields ranged from 65-80%.

5'-Deoxy-5'-Methylamino-2',3'-O-Isopropylideneadenosine[95] (**4a**) was prepared according to the above general procedure with only minor differences. 5'-O-Tosyl-2',3'-O-isopropylideneadenosine (**3**) was dissolved in anhydrous methylamine (approximately 75 mL, preparation described below) and sealed in a bomb for three days. The bomb was cooled in dry ice, opened, and the methylamine was allowed to boil off at room temperature overnight. Treatment of the residue as described in the general procedure above followed by purification on a slightly smaller silica gel column (40 x 3.5 cm, 180 g) provided 5.02 g (70.3%) of pure product in fractions 25-80: mp 54-56°C.; TLC, methanol, R_f 0.26 (UV, I_2); 500 MHz ^{1}H NMR ($CDCl_3$), δ 1.39 (s, 3H, isopropylidene CH_3), 1.62 (s, 3H, isopropylidene CH_3), 2.44 (s, 3H, CH_3NH), 2.85 (dd, 1H, J = 6.2Hz, 12.5 Hz, H-5'), 2.89 (dd, 1H, J = 4.4Hz, 12.5Hz, H-5'), 4.38 (m, 1H, J = 3.3Hz, 4.4Hz, 6.2Hz, H-4'), 5.02 (dd, 1H, J = 3.3Hz, 6.4Hz, H-3'), 5.48 (dd, 1H, J = 3.2Hz, 6.4Hz, H-2'), 6.01 (d, 1H, J = 3.2Hz, H-1'), 6.19 (br s, 2H, adenine NH_2), 7.92 (s, 1H, adenine H-2 or H-8), 8.36 (s, 1H, adenine H-2 or H-8); UV λ_{max}

(MeOH) 259 nm (ε 12.5 x 10^3); MS (EI), m/e 305 (M - isopropylidene CH_3)$^+$, 185 (M - adenine)$^+$, 136 (adenine + H)$^+$.

Anal. Calculated for $C_{14}H_{20}N_6O_3 \cdot 0.5H_2O$: C, 51.05; H, 6.42; N, 25.52. Found: C, 50.86; H, 6.19; N, 25.52.

Preparation of Anhydrous Liquid Methylamine. An aqueous solution (150 mL) of methylamine hydrochloride (153 g, 2.0 mol) was added over 30 minutes to potassium hydroxide pellets (168 g, 3.0 mol) under nitrogen in a three-neck flask (1.0 L) at room temperature. The wet methylamine (bp -6.3°C.) gas generated by the vigorous exothermic reaction was dried by passage through two small (250 mL) Erlenmeyer flasks containing potassium hydroxide pellets. The dried gas was then led to a condenser containing a chloroform/dry ice slush bath open to but protected from the atmosphere by a drying tube containing anhydrous calcium sulfate (Drierite). The liquified methylamine was collected in a three-neck flask (100 mL) sitting in a chloroform/dry ice slush bath beneath the condenser. Manual agitation of the large three-neck flask and the addition of more potassium hydroxide pellets (approximately 50 g) to its warm semi-solid contents were often necessary to release more methylamine gas. The anhydrous liquid methylamine (approximately 75 mL) so prepared was used immediately.

5'-Deoxy-5'-Ethylamino-2',3'-O-Isopropylideneadenosine (**4b**) was prepared according to the above general procedure with only slight variations. 5'-O-Tosyl-2',3'-O-isopropylideneadenosine (**3**) was dissolved in anhydrous ethylamine (approximately 75 mL, preparation described below) and sealed in a bomb for three days. The bomb was cooled in dry ice, opened, and the ethylamine was allowed to boil off at room temperature overnight. Treatment of the residue and purification as described in the general procedure above gave 5.46 g (73.4%) of pure product in fractions 24-70: mp 51-53°C.; TLC, methanol,

R_f 0.30 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.09 (t, 3H, J = 7.1Hz, CH_3CH_2NH), 1.39 (s, 3H, isopropylidene CH_3), 1.62 (s, 3H, isopropylidene CH_3), 2.65 (m, 2H, CH_3CH_2NH), 2.88 (dd, 1H, J = 6.4Hz, 12.5Hz, H-5'), 2.93 (dd, 1H, J = 4.3Hz, 12.5Hz, H-5'), 4.38 (m, 1H, J = 3.4Hz, 4.3Hz, 6.4Hz, H-4'), 5.03 (dd, 1H, J = 3.4Hz, 6.4Hz, H-3'), 5.47 (dd, 1H, J = 3.1Hz, 6.4Hz, H-2'), 5.80 (br s, 2H, adenine NH_2), 6.02 (d, 1H, J = 3.1Hz, H-1'), 7.92 (s, 1H, adenine H-2 or H-8), 8.35 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 12.3 x 10^3); MS (EI), m/e 334 (M^+), 319 (M - isopropylidene $CH_3)^+$, 199 (M - adenine)$^+$, 136 (adenine + H)$^+$.

Anal. Calculated for $C_{15}H_{22}N_6O_3 \cdot 0.5H_2O$: C, 52.47; H, 6.75; N, 24.47. Found: C, 52.56; H, 6.56; N, 24.67.

Preparation of Anhydrous Liquid Ethylamine. In a manner nearly identical to that described for the preparation of anhydrous liquid methylamine, anhydrous liquid ethylamine was prepared from an aqueous solution (150 mL) of ethylamine hydrochloride (122 g, 1.5 mol) and potassium hydroxide pellets (225 g, 2.0 mol). The only difference, arising from the higher boiling point (16.6°C.) of ethylamine, was that the large (1.0 L) three-neck flask containing the reaction mixture was heated in a water bath at approximately 40°C.. The anhydrous liquid ethylamine (approximately 75 mL) prepared in this manner was used immediately.

5'-(1-Propyl)Amino-5'-Deoxy-2',3'-O-Isopropylideneadenosine (**4c**) was prepared according to the general procedure above and, following flash silica gel chromatography, 5.24 g (69.4%) of pure product was recovered from fractions 18-39: mp 50-53°C.; TLC, methanol, R_f 0.31 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 0.88 (t, 3H, J = 7.4Hz, $CH_3CH_2CH_2NH$), 1.39 (s, 3H, isopropylidene CH_3), 1.46 (m, 2H, $CH_3CH_2CH_2NH$), 1.62 (s, 3H, isopropylidene CH_3), 2.56 (m, 2H, $CH_3CH_2CH_2NH$), 2.86 (dd, 1H, J = 6.5Hz,

12.6Hz, H-5'), 2.92 (dd, 1H, J = 4.3Hz, 12.6Hz, H-5'), 4.37 (m, 1H, J = 3.4Hz, 4.3Hz, 6.5Hz, H-4'), 5.02 (dd, 1H, J = 3.4Hz, 6.4Hz, H-3'), 5.47 (dd, 1H, J = 3.1Hz, 6.4Hz, H-2'), 5.61 (br s, 2H, adenine NH_2), 6.02 (d, 1H, J = 3.1Hz, H-1'), 7.93 (s, 1H, adenine H-2 or H-8), 8.35 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 13.6 x 10^3); MS (EI), m/e 348 $(M)^+$, 333 (M - isopropylidene $CH_3)^+$, 213 (M - adenine)$^+$, 136 (adenine + H)$^+$.

Anal. Calculated for $C_{16}H_{24}N_6O_3 \cdot 0.5H_2O$: C, 53.77; H, 7.05; N, 23.51. Found: C, 53.41; H, 6.85; N, 23.14.

5'-[1-(2-Propenyl)]Amino-5'-Deoxy-2',3'-O-Isopropylideneadenosine (**4d**) was synthesized according to the general procedure outlined above. Following purification by flash chromatography, 5.02 g (66.8%) was recovered from fractions 15-41: mp 45-47°C.; TLC, methanol, R_f 0.48 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.39 (s, 3H, isopropylidene CH_3), 1.62 (s, 3H, isopropylidene CH_3), 2.86 (dd, 1H, J = 6.1Hz, 12.6Hz, H-5'), 2.92 (dd, 1H, J = 4.1Hz, 12.6Hz, H-5'), 3.24 (m, 2H, CH_2=CHCH_2), 4.38 (m, 1H, J = 3.3Hz, 4.1Hz, 6.1Hz, H-4'), 5.04 (dd, 1H, J = 3.3Hz, 6.5Hz, H-3'), 5.10 (m, 2H, CH_2=CHCH_2), 5.47 (dd, 1H, J = 3.3Hz, 6.5Hz, H-2'), 5.74 (br s, 2H, adenine NH_2), 5.83 (m, 1H, CH_2=CHCH_2), 5.99 (d, 1H, J = 3.3Hz, H-1'), 7.93 (s, 1H, adenine H-2 or H-8), 8.33 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 13.1 x 10^3); MS (EI), m/e 331 (M - isopropylidene $CH_3)^+$, 211 (M - adenine)$^+$, 136 (adenine + H)$^+$.

Anal. Calculated for $C_{16}H_{22}N_6O_3$: C, 55.48; H, 6.40; N, 24.26. Found: C, 55.14; H, 6.36; N, 24.54.

5'-(1-Butyl)Amino-5'-Deoxy-2',3'-O-Isopropylideneadenosine (**4e**) was prepared and purified on a slightly smaller scale than that described in the general procedure. 5'-O-Tosyl-2',3'-O-isopropylideneadenosine (**3**) (9.39 g, 20.4 mmol) was dissolved in freshly distilled n-butylamine (100 mL) and after three days the mixture was treated as outlined above. Flash silica gel

chromatography provided 4.97 g (65.7%) of pure product in fractions 19-34: mp 46-49°C.; TLC, methanol, R_f 0.38 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 0.88 (t, 3H, J = 7.3Hz, $CH_3CH_2CH_2CH_2NH$), 1.31 (m, 2H, $CH_3CH_2CH_2CH_2NH$), 1.39 (s, 3H, isopro-pylidene CH_3), 1.42 (m, 2H, $CH_3CH_2CH_2CH_2NH$), 1.62 (s, 3H, isopropylidene CH_3), 2.58 (m, 2H, $CH_3CH_2CH_2CH_2NH$), 2.86 (dd, 1H, J = 6.5Hz, 12.5Hz, H-5'), 2.91 (dd, 1H, J = 4.3Hz, 12.5Hz, H-5'), 4.37 (m, 1H, J = 3.4Hz, 4.3Hz, 6.5Hz, H-4'), 5.02 (dd, 1H, J = 3.4Hz, 6.4Hz, H-3'), 5.48 (dd, 1H, J = 3.0Hz, 6.4Hz, H-2'), 6.03 (d, 1H, J = 3.0Hz, H-1'), 6.05 (br s, 2H, adenine NH_2), 7.93 (s, 1H, adenine H-2 or H-8), 8.34 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 13.5 x 10^3); MS (EI), m/e 347 (M - isopropylidene CH_3)$^+$, 227 (M - adenine)$^+$, 136 (adenine + H)$^+$.

Anal. Calculated for $C_{17}H_{26}N_6O_3 \cdot 0.5H_2O$: C, 54.97; H, 7.32; N, 22.63. Found: C, 55.43; H, 7.22; N, 22.66.

5'-(1-Pentyl)Amino-5'-Deoxy-2',3'-O-Isopropylideneadenosine (**4f**) was prepared and purified according to the general procedure with only one minor difference. Purification on a slightly larger flash silica gel column (48 x 3.5 cm, 225 g) gave 6.31 g (75.6%) of pure product in fractions 20-39: mp 45-47°C.; TLC, methanol, R_f 0.44 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 0.87 (t, 3H, J = 7.0Hz, $CH_3CH_2CH_2CH_2CH_2NH$), 1.27 (m, 4H, $CH_3CH_2CH_2CH_2CH_2NH$), 1.39 (s, 3H, isopropylidene CH_3), 1.44 (m, 2H, $CH_3CH_2CH_2CH_2CH_2NH$), 1.62 (s, 3H, isopropylidene CH_3), 2.58 (m, 2H, $CH_3CH_2CH_2CH_2CH_2NH$), 2.86 (dd, 1H, J = 6.5Hz, 12.5Hz, H-5'), 2.91 (dd, 1H, J = 4.3Hz, 12.5Hz, H-5'), 4.38 (m, 1H, J = 3.4Hz, 4.3Hz, 6.5Hz, H-4'), 5.02 (dd, 1H, J = 3.4Hz, 6.4Hz, H-3'), 5.48 (dd, 1H, J = 3.0Hz, 6.4Hz, H-2'),6.03 (d, 1H, J = 3.0Hz, H-1'), 6.15 (br s, 2H, adenine NH_2), 7.93 (s, 1H, adenine H-2 or H-8), 8.34 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 12.5 x 10^3); MS (EI), m/e 361 (M - isopropylidene CH_3)$^+$, 241 (M - adenine)$^+$, 136 (adenine + H)$^+$.

Anal. Calculated for $C_{18}H_{28}N_6O_3 \cdot 0.5H_2O$: C, 56.09; H, 7.58; N, 21.80. Found: C, 55.83; H, 7.47; N, 21.55.

5'-(1-Octyl)Amino-5'-Deoxy-2',3'-O-Isopropylideneadenosine (**4g**) was prepared and purified according to the general procedure with only minor differences. 5'-O-Tosyl-2',3'-O-isopropylideneadenosine (**3**) (10.00 g, 21.7 mmol) was dissolved in freshly distilled n-octylamine (125 mL) and, after stirring for three days at room temperature, the mixture was treated as described above. Purification on a slightly larger flash silica gel column (48 x 3.5 cm, 225 g) provided 6.75 g (72.8%) of pure product in fractions 19-38: mp 40-42°C.; TLC, methanol, R_f 0.47 (UV, I_2); 500 MHz ^{1}H NMR ($CDCl_3$), δ 0.87 (t, 3H, J = 7.0Hz, octyl CH_3), 1.25 (m, 10H, $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2NH$), 1.39 (s, 3H, isopropylidene CH_3), 1.44 (m, 2H, CH_2CH_2NH), 1.62 (s, 3H, isopropylidene CH_3), 2.58 (m, 2H, CH_2CH_2NH), 2.86 (dd, 1H, J = 6.5Hz, 12.5Hz, H-5'), 2.91 (dd, 1H, J = 4.3Hz, 12.5Hz, H-5'), 4.38 (m, 1H, J = 3.4Hz, 4.3Hz, 6.5Hz, H-4'), 5.02 (dd, 1H, J = 3.4Hz, 6.4Hz, H-3'), 5.47 (dd, 1H, J = 3.1Hz, 6.4Hz, H-2'), 5.93 (br s, 2H, adenine NH_2), 6.02 (d, 1H, J = 3.1Hz, H-1'), 7.92 (s, 1H, adenine H-2 or H-8), 8.35 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 11.9 x 10^3); MS (EI), m/e 403 (M - isopropylidene CH_3)$^+$, 283 (M - adenine)$^+$, 136 (adenine + H)$^+$.

Anal. Calculated for $C_{21}H_{34}N_6O_3 \cdot 0.5H_2O$: C, 58.99; H, 8.25; N, 19.66. Found: C, 58.98; H, 8.10; N, 19.32.

2(R,S)-Amino-4-Iodobutyric Acid Hydriodide was prepared according to the procedure of Frankel and Knobler[109] with slight modifications. 2(R,S)-Amino-4-butyrolactone hydrobromide (78.111 g, 0.429 mol) was dissolved in concentrated hydriodic acid (180 mL). Following the addition of toluene (180 mL) to this solution, the stirred two-phase mixture was heated at reflux for six hours. After the removal of all solvent by distillation, as reported in the

literature,[109] the cooled residue was triturated with hot chloroform (500 mL) for 30 minutes. Upon cooling, the dark solid material was filtered from the chloroform on a sintered glass filter and washed with diethyl ether (1.5 L) in 100 mL portions to remove most of the residual dark hydriodic acid. The final traces of hydriodic acid were removed from the yellow-brown crystals by continuous extraction with diethyl ether (500 mL) using a Soxhlet extractor. The recovered material was sufficiently pure (> 95%) for direct use in the preparation of the corresponding methyl ester. The yellow crystals of product (118.30 g, 77.2%) gradually darkened upon prolonged storage at -20°C.: mp 186-192°C. dec. (lit.[109] 195-200°C.); 240 MHz ^{1}H NMR (D_2O), δ 2.26-2.59 (m, 2H, CHC*H*$_2$CH$_2$), 3.32 (m, 2H, CHCH$_2$C*H*$_2$), 4.14 (t, 1H, J = 6.5Hz, C*H*CH$_2$CH$_2$); MS (positive LSIMS), m/e 230 $(M + H)^+$.

Methyl 2(R,S)-Amino-4-Iodobutyrate Hydrochloride (5) was prepared following the procedure of Frankel and Knobler[109] with slight modifications. 2(R,S)-Amino-4-iodobutyric acid hydroiodide (50.000 g, 0.140 mol) was dissolved in anhydrous methanol (1.25 L) and stirred at 35-40°C.. Hydrogen chloride, dried by passage through concentrated sulfuric acid, was bubbled through this solution for five hours, during which its color changed from yellow to deep purple. The solution was then evaporated almost to dryness and redissolved in anhydrous methanol (1.25 L). Dry hydrogen chloride was bubbled through the stirred solution at 35-40°C. for an additional five hours, after which it was evaporated nearly to dryness. The dark residue was dissolved in absolute ethanol (200 mL), divided into two equal portions, and each portion concentrated to approximately 25 mL. Diethyl ether (approximately 750 mL) was added to each portion to triturate the product. Crystallization of the methyl ester, which initially was an amorphous solid, was facilitated by storage overnight at -20°C.. The product in each portion was

filtered, redissolved in absolute ethanol (200 mL), concentrated to approximately 25 mL, and triturated with diethyl ether (approximately 750 mL) four more times. The color of the original ethanol solution of product gradually paled from deep purple to yellow by the fifth cycle. The final ethanolic solutions were filtered to remove some insoluble material after which the final triturations of each portion were carried out. The colorless crystals (29.54 g, 75.5%) were filtered and dried *in vacuo* overnight and used directly without further purification: mp 106-108°C. (lit.[109] 125°C.); 240 MHz ^{1}H NMR (D_2O), δ 2.25-2.58 (m, 2H, $CHCH_2CH_2$), 3.31 (t, 2H, J = 7.1Hz, $CHCH_2CH_2$), 3.82 (s, 3H, CH_3), 4.22 (t, 1H, J = 6.6Hz, $CHCH_2CH_2$); MS (positive LSIMS), m/e 244 $(M + H)^+$.

Methyl 2(R,S)-Formamido-4-Iodobutyrate (**6a**) was synthesized according to the general procedure described by Chen and Benoiten.[110] 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (679 mg, 3.57 mmol) was added to an ice-cold mixture of formic acid (2.59 mL, 7.14 mmol) and methylene chloride (20 mL, freshly distilled from P_2O_5). Simultaneously, 4-methylmorpholine (0.39 mL, 3.57 mmol) was added to a suspension of methyl 2(R,S)-amino-4-iodobutyrate hydrochloride (**5**) (500 mg, 1.79 mmol) in ice-cold methylene chloride (20 mL, freshly distilled from P_2O_5). After both solutions were stirred for 15 minutes, they were combined and stirred at 4°C. for 23 hours. The crude reaction mixture was washed successively with ice-cold solutions of 1M aqueous hydrochloric acid (2 x 40 mL), saturated aqueous sodium bicarbonate (2 x 40 mL), and saturated aqueous sodium chloride (2 x 40 mL). The methylene chloride solution was dried over anhydrous magnesium sulfate overnight, filtered, and evaporated to give 174 mg (35.8%) of the product as a pale yellow solid. An analytical sample was prepared by dissolving this solid in a minimal amount of ethyl acetate and triturating with hexanes. The resulting

colorless crystals were dried overnight *in vacuo*: mp 67-69°C.; 240 MHz ^{1}H NMR ($CDCl_3$), δ 2.40 (m, 2H, C*H*$_2$CH$_2$I), 3.16 (t, 2H, J = 7.2Hz, CH_2I), 3.80 (s, 3H, CH_3), 4.74 (q, 1H, CH), 6.21 (br s, 1H, NH), 8.25 (s, 1H, CHO); MS (EI), m/e 212 (M - CO_2CH_3), 144 (M - I)$^+$.

Anal. Calculated for $C_6H_{12}INO_3$: C, 26.59; H, 3.72; N, 5.17; I, 46.82. Found: C, 26.77; H, 3.70; N, 5.06; I, 46.58.

Methyl 2(R,S)-Trifluoroacetamido-4-Iodobutyrate (**6b**) was prepared by an extension of the formylation procedure originally described by Chen and Benoiten.[110] 4-Methylmorpholine (9.56 mL, 86.0 mmol) was added to an ice-cold stirred suspension of methyl 2(R,S)-amino-4-iodobutyrate hydrochloride (**5**) (12.000 g, 43.0 mmol) in methylene chloride (1.0 L, freshly distilled from P_2O_5). Trifluoroacetic anhydride (30.54 mL, 215.4 mmol) was added approximately 15 minutes later, and the resulting solution stirred at 4°C. for 18 hours. The crude reaction mixture was washed with 1M aqueous hydrochloric acid (5 x 700 mL), saturated aqueous sodium bicarbonate (4 x 700 mL), and saturated aqueous sodium chloride (4 x 700 mL). The methylene chloride solution was dried over anhydrous magnesium sulfate for 30 minutes, filtered, and then evaporated. The residual oil was dissolved in chloroform (100 mL), and the resulting solution concentrated to 20 mL and triturated with ice-cold hexanes (200 mL). All solvent was evaporated to give 13.19 g (90.5%) of almost pure pale brown product. A colorless analytical sample was obtained by filtering the triturated chloroform-hexanes mixture prior to evaporation: mp 54-58°C.; 240 MHz ^{1}H NMR ($CDCl_3$), δ 2.44 (m, 2H, C*H*$_2$CH$_2$I), 3.14 (t, 2H, J = 7.6Hz, CH_2I), 3.84 (s, 3H, CH_3), 4.69 (m, 1H, CH), 7.00 (br s, 1H, NH); MS (EI), m/e 280 (M - CO_2CH_3)$^+$, 212 (M - I)$^+$.

Anal. Calculated for $C_7H_9F_3INO_3$: C, 24.80; H, 2.72; N, 4.13; I, 37.43. Found: C, 24.88; H, 2.72; N, 4.22; I, 37.24.

General Preparation of Methyl N^4-Alkyl-N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-2(R,S)-Trifluoroacetamido-4-Aminobutyrate Homologues (**7a-g**). The 5'-alkylamino-5'-deoxy-2',3'-O-isopropylideneadenosine (**4a-g**) (15.0 mmol) and N,N-diisopropylethylamine (2.87 mL, 16.5 mmol, 10% excess) were dissolved in acetonitrile (250 mL) and heated with stirring to 70°C.. A solution of methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) (18.8 mmol, 25% excess) in acetonitrile (150 mL) was added over approximately one hour. The mixture was stirred at 70°C. for three days, during which time its initial pale brown color progressively deepened. After cooling to room temperature, the reaction mixture was evaporated to dryness, redissolved in chloroform (100 mL), concentrated to 5 mL, and loaded onto a wet-packed flash silica gel column (44 x 3.5 cm, 200 g). The column was eluted with chloroform:isopropanol (80:20 or 90:10) at a flow rate of 10 mL/min and 28 mL fractions were collected. Partially purified product (as judged by TLC) generally appeared in fractions 12-50, and the appropriate fractions were pooled and concentrated to approximately 15 mL. Further purification was achieved by low pressure liquid chromatography using a Lobar Size B (310-25) LiChroprep™ Si60 silica gel column. The column was eluted with chloroform:isopropanol (70:30 or 80:20) at a flow rate of 4.0 mL/min. Injection of 0.70 mL aliquots onto the column under these conditions generally led to the appearance (as detected by absorbance at 280 nm) of the product some 30-65 minutes later. Column eluate containing the product was collected, evaporated, redissolved in a minimal volume (5-10 mL) of the eluting solvent, and reinjected onto the same column until all impurities were removed. Any residual amber color in a chloroform solution (5 mL volume) of the product was removed by filtration through a wet-packed silica gel column (40 x 3.5 cm, 180 g). Elution with chloroform:isopropanol (92:8 or 95:5) at a flow rate of approximately 10 mL/min

and collection of 28 mL fractions generally led to the appearance of the product in fractions 19-62. The appropriate fractions were pooled, evaporated to dryness, twice dissolved in chloroform (100 mL), and evaporated to give a colorless foam. Yields ranged from 20-45%.

Methyl N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-N^4-Methyl-2(R,S)-Trifluoroacetamido-4-Aminobutyrate (Protected Nitrogen Methyl SAM Analogue, **7a**) was prepared according to the general procedure described above with only minor differences. 5'-Deoxy-5'-methylamino-2',3'-O-isopropylidene-adenosine hemihydrate (**4a**) (5.47 g, 16.6 mmol) and N,N-diisopropylethylamine (3.27 mL, 18.8 mmol) were dissolved in acetonitrile (350 mL). After heating to 70°C., a slightly smaller excess (10%) of methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) (6.37 g, 18.8 mmol) in acetonitrile (250 mL) was added over one hour. Initial silica gel chromatography (43 x 3.5 cm, 190 g) of the three-day-old reaction mixture using chloroform:isopropanol (90:10) as the eluting solvent provided crude product in fractions 27-50. Low pressure liquid chromatography (3.5 mL/min flow rate) using chloroform:isopropanol (70:30) as the eluting solvent provided product in the eluate collected 41-69 minutes after injection. Decolorization by filtration through silica gel using chloroform:isopropanol (92:8) as the eluting solvent provided 3.83 g (42.1%) of pure product as a colorless foam in fractions 31-62: mp 59-61°C.; TLC, chloroform:isopropanol (90:10), R_f 0.27 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.39 (s, 1.5H, isopropylidene CH_3), 1.40 (s, 1.5H, isopropylideneCH_3), 1.62 (s, 3H, isopropylidene CH_3), 1.87 (m, 2H, CH_2CH_2N), 2.23 (s, 1.5H, CH_3N), 2.24 (s, 1.5H, CH_3N), 2.43-2.61 (m, 2H, CH_2CH_2N), 2.71 (m, 2H, H-5'), 3.64 (s, 1.5H, CO_2CH_3), 3.77 (s, 1.5H, CO_2CH_3), 4.33 (m, 1H, H-4'), 4.54 (m, 1H, $CHCH_2CH_2N$), 4.93 (dd, 0.5H, J = 3.9Hz, 6.4Hz, H-3'), 5.00 (dd, 0.5H, J = 3.7Hz, 6.4Hz, H-3'), 5.46 (dd, 0.5H, J = 1.8Hz, 6.4Hz, H-2'), 5.51

(dd, 0.5H, J = 1.8Hz, 6.4Hz, H-2'), 5.77 (br s, 1H, adenine NH_2), 5.78 (br s, 1H, adenine NH_2), 6.04 (d, 0.5H, J = 1.8Hz, H-1'), 6.05 (d, 0.5H, J = 1.8Hz, H-1'), 7.85 (s, 0.5H, adenine H-2 or H-8), 7.91 (s, 0.5H, adenine H-2 or H-8), 8.33 (s, 0.5H, adenine H-2 or H-8), 8.34 (s, 0.5H, adenine H-2 or H-8), 9.46 (br d, 0.5H, J = 6.7Hz, $NHCOCF_3$), 9.49 (br d, 0.5H, J = 6.8Hz, $NHCOCF_3$); UV λ_{max} (MeOH) 259 nm (ε 14.9 x 10^3); MS (positive LSIMS), m/e 532 $(M + H)^+$, 397 (M + H - adenine)$^+$.

Anal. Calculated for $C_{21}H_{28}F_3N_7O_6 \cdot H_2O$: C, 45.90; H, 5.50; N, 17.84. Found: C, 45.62; H, 5.25; N, 17.56.

Methyl N^4-Ethyl-N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-2(R,S)-Trifluoroacetamido-4-Aminobutyrate (Protected Nitrogen Ethyl SAM Analogue, **7b**) was prepared according to the general procedure described above. 5'-Deoxy-5'-ethylamino-2',3'-O-isopropylideneadenosine hemihydrate (**4b**) (5.46 g, 15.9 mmol) and N,N-diisopropylethylamine (3.12 mL, 17.9 mmol) were dissolved in acetonitrile (300 mL). After heating to 70°C., a solution of methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) (6.91 g, 20.4 mmol) in acetonitrile (200 mL) was added over one hour. Initial silica gel chromatography of the three-day-old reaction mixture using chloroform:isopropanol (80:20) as the eluting solvent provided crude product in fractions 16-34. Low pressure liquid chromatography using the same eluting solvent yielded product in the eluate collected 38-76 minutes after injection. Decolorization by filtration through silica gel using chloroform:isopropanol (92:8) as the eluting solvent provided 1.95 g (21.8%) of pure product as a colorless foam in fractions 23-40: mp 51-54°C.; TLC, chloroform:isopropanol (80:20), R_f 0.52 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 0.86 (t, 1.5H, J = 7.1Hz, CH_3CH_2N), 0.95 (t, 1.5H, J = 7.1Hz, CH_3CH_2N), 1.38 (s, 1.5H, isopropylidene CH_3), 1.40 (s, 1.5H, isopropylidene CH_3), 1.61 (s, 1.5H, isopropylidene CH_3), 1.62 (s, 1.5H, isopropylidene CH_3),

1.81 (m, 2H, CHCH_2CH$_2$N), 2.35-2.46 (m, 2H, CH$_3$CH_2N), 2.52-2.61 (m, 2H, CHCH$_2$CH_2N), 2.63-2.86 (m, 2H, H-5'), 3.66 (s, 1.5H, CO_2CH_3), 3.75 (s, 1.5H, CO_2CH_3), 4.32 (m, 1H, H-4'), 4.43 (q, 0.5H, CHCH$_2$CH$_2$N), 4.51 (q, 0.5H, CHCH$_2$CH$_2$N), 4.96 (dd, 0.5H, J = 4.0Hz, 6.4Hz, H-3'), 5.00 (dd, 0.5H, J = 1.9Hz, 6.4Hz, H-3'), 5.48 (dd, 0.5H, J = 1.9Hz, 6.4Hz, H-2'), 5.51 (dd, 0.5H, J = 1.9Hz, 6.4Hz, H-2'), 5.77 (br s, 1H, adenine NH_2), 5.78 (br s, 1H, adenine NH_2), 6.04 (d, 1H, J = 1.9Hz, H-1'), 7.82 (s, 0.5H, adenine H-2 or H-8), 7.88 (s, 0.5H, adenine H-2 or H-8), 8.32 (s, 0.5H, adenine H-2 or H-8), 8.33 (s, 0.5H, adenine H-2 or H-8), 9.64 (br d, 0.5H, J = 5.9Hz, NHCOCF_3), 9.76 (br d, 0.5H, J = 6.2Hz, NHCOCF_3); UV λ_{max} (MeOH) 259 nm (ε 13.4 x 10^3); MS (positive LSIMS), m/e 546 $(M + H)^+$, 411 $(M + H - adenine)^+$.

Anal. Calculated for $C_{22}H_{30}F_3N_7O_6 \cdot H_2O$: C, 46.89; H, 5.72; N, 17.40. Found: C, 46.60; H, 5.53; N, 17.03.

Methyl N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-N^4-(1-Propyl)-2(R,S)-Trifluoroacetamido-4-Aminobutyrate (Protected Nitrogen Propyl SAM Analogue, **7c**) was prepared according to the above general procedure. 5'-(1-Propyl)amino-5'-deoxy-2',3'-O-isopropylideneadenosine hemihydrate (**4c**) (4.93 g, 13.8 mmol) and N,N-diisopropylethylamine (2.71 mL, 15.6 mmol) were dissolved in acetonitrile (250 mL), and after heating to 70°C., a solution of methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) (6.03 g, 17.8 mmol) in acetonitrile (150 mL) was added over one hour. Initial silica gel chromatography of the three-day-old reaction mixture using chloroform:isopropanol (80:20) as the eluting solvent provided partially purified product in fractions 15-23. Low pressure liqud chromatography using the same eluting solvent provided product in the eluate collected 40-68 minutes after injection. Decolorization of the product by filtration through silica gel using chloroform:isopropanol (95:5) as the eluting solvent provided 1.93 g (24.3%) of

pure product as a colorless foam in fractions 32-52: mp 52-60°C.; TLC, chloroform:isopropanol (80:20), R_f 0.60 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 0.75 (t, 1.5H, J = 7.3Hz, $CH_3CH_2CH_2N$), 0.81 (t, 1.5H, J = 7.3Hz, $CH_3CH_2CH_2N$), 1.25 (m, 1H, $CH_3CH_2CH_2N$), 1.36 (m, 1H, $CH_3CH_2CH_2N$), 1.38 (s, 1.5H, isopropylidene CH_3), 1.40 (s, 1.5H, isopropylidene CH_3), 1.62 (s, 1.5H, isopropylidene CH_3), 1.63 (s, 1.5H, isopropylidene CH_3), 1.82 (m, 2H, $CHCH_2CH_2N$), 2.20-2.33 (m, 2H, $CH_3CH_2CH_2N$), 2.38-2.60 (m, 2H, $CHCH_2CH_2N$), 2.67-2.87 (m, 2H, H-5'), 3.65 (s, 1.5H, CO_2CH_3), 3.74 (s, 1.5H, CO_2CH_3), 4.34 (m, 1H, H-4'), 4.42 (q, 0.5H, $CHCH_2CH_2N$), 4.49 (q, 0.5H, $CHCH_2CH_2N$), 4.96 (dd, 0.5H, J = 3.8Hz, 6.4Hz, H-3'), 4.98 (dd, 0.5H, J = 3.7Hz, 6.4Hz, H-3'), 5.49 (dd, 0.5H, J = 2.0Hz, 6.4Hz, H-2'), 5.52 (dd, 0.5H, J = 1.8Hz, 6.4Hz, H-2'), 5.81 (br s, 1H, adenine NH_2), 5.82 (br s, 1H, adenine NH_2), 6.04 (d, 1H, J = 1.9Hz, H-1'), 7.82 (s, 0.5H, adenine H-2 or H-8), 7.88 (s, 0.5H, adenine H-2 or H-8), 8.32 (s, 0.5H, adenine H-2 or H-8), 8.33 (s, 0.5H, adenine H-2 or H-8), 9.44 (br d, 0.5H, J = 6.2Hz, $NHCOCF_3$), 9.57 (br d, 0.5H, J = 6.4Hz, $NHCOCF_3$); UV λ_{max} (MeOH) 259 nm (ε 14.0 x 10^3); MS (positive LSIMS), m/e 560 $(M + H)^+$, 425 $(M + H - adenine)^+$.

Anal. Calculated for $C_{23}H_{32}F_3N_7O_6 \cdot H_2O$: C, 47.83; H, 5.93; N, 16.98. Found: C, 47.71; H, 5.70; N, 16.79.

Methyl N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-N^4-[1-(2-Propenyl)]-2(R,S)-Trifluoroacetamido-4-Aminobutyrate (Protected Nitrogen Allyl SAM Analogue, **7d**) was prepared according to the general procedure described above. 5'-[1-(2-Propenyl)]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**4d**) (9.46 g, 27.3 mmol) and N,N-diisopropylethylamine (5.22 mL, 30.0 mmol) were dissolved in acetonitrile (500 mL) and heated to 70°C.. A solution of methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) (11.56 g, 34.1 mmol) in acetonitrile (300 mL) was added over one hour. Initial silica gel

chromatography of the four-day-old reaction mixture using chloroform:isopropanol (90:10) as the eluting solvent provided partially purified product in fractions 20-42. Low pressure liquid chromatography using chloroform:isopropanol (80:20) as the eluting solvent provided purified product in the eluate collected 36-64 minutes after injection. Decolorization of the product by filtration through silica gel using chloroform:isopropanol (95:5) as the eluting solvent provided 3.34 g (21.3%) of pure product as a colorless foam in fractions 27-51: mp 53-57°C.; TLC, chloroform:isopropanol (80:20), R_f 0.53 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.38 (s, 1.5H, isopropylidene CH_3), 1.40 (s, 1.5H, isopropylidene CH_3), 1.62 (s, 1.5H, isopropylidene CH_3), 1.63 (s, 1.5H, isopropylidene CH_3), 1.85 (m, 2H, $CHCH_2CH_2N$), 2.56 (m, 2H, $CHCH_2CH_2N$), 2.69-2.90 (m, 2H, H-5'), 2.99-3.18 (m, 2H, CH_2=$CHCH_2N$), 3.64 (s, 1.5H, CO_2CH_3), 3.75 (s, 1.5H, CO_2CH_3), 4.35 (m, 1H, H-4'), 4.51 (m, 1H, $CHCH_2CH_2N$), 4.96 (dd, 0.5H, J = 3.9Hz, 6.4Hz, H-3'), 4.97-5.09 (m, 2H, CH_2=$CHCH_2N$), 5.01 (dd, 0.5H, J = 3.7Hz, 6.4Hz, H-3'), 5.47 (dd, 0.5H, J = 1.9Hz, 6.4Hz, H-2'), 5.51 (dd, 0.5H, J = 1.7Hz, 6.4Hz, H-2'), 5.82 (br s, 2H, adenine NH_2), 6.05 (d, 0.5H, J = 1.8Hz, H-1'), 6.06 (d, 0.5H, J = 1.8Hz, H-1'), 7.83 (s, 0.5H, adenine H-2 or H-8), 7.89 (s, 0.5H, adenine H-2 or H-8), 8.31 (s, 1H, adenine H-2 or H-8), 9.33 (br d, 0.5H, J = 6.2Hz, $NHCOCF_3$), 9.48 (br d, 0.5H, J = 6.7Hz, $NHCOCF_3$); UV λ_{max} (MeOH) 259 nm (ε 13.9 x 10^3); MS (positive LSIMS), m/e 558 $(M + H)^+$, 423 $(M + H - adenine)^+$.

Anal. Calculated for $C_{23}H_{30}F_3N_7O_6 \cdot H_2O$: C, 48.00; H, 5.60; N, 17.04. Found: C, 48.23; H, 5.39; N, 16.94.

Methyl N^4-(1-Butyl)-N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-2(R,S)-Trifluoroacetamido-4-Aminobutyrate (Protected Nitrogen Butyl SAM Analogue, **7e**) was prepared according to the general procedure described above. 5'-(1-Butyl)amino-5'-deoxy-2',3'-O-isopropylideneadenosine hemihydrate (**4e**) (4.68

g, 12.6 mmol) and N,N-diisopropylethylamine (2.47 mL, 14.2 mmol) were dissolved in acetonitrile (250 mL). After heating this mixture to 70°C., a solution of methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) (5.47 g, 16.1 mmol) in acetonitrile (150 mL) was added over one hour. The product was partially purified from the three-day-old reaction mixture by silica gel chromatography. Product appeared in fractions 14-23 when chloroform:isopropanol (80:20) was used as the eluting solvent. Low pressure liquid chromatography using the same eluting solvent provided product in the eluate collected 38-69 minutes after injection onto the column. Decolorization by filtration through silica gel using chloroform:isopropanol (95:5) as the eluting solvent provided 1.70 g (22.8%) of pure product as a colorless foam in fractions 29-49: mp 55-61°C.; TLC, chloroform:isopropanol (80:20), R_f 0.61 (UV, I_2); 500 MHz ^{1}H NMR ($CDCl_3$), δ 0.77 (t, 1.5H, J = 6.9Hz, C*H*$_3$CH$_2$CH$_2$CH$_2$N), 0.85 (t, 1.5H, J = 7.2Hz, C*H*$_3$CH$_2$CH$_2$CH$_2$N), 1.14 (m, 2H, CH$_3$C*H*$_2$CH$_2$CH$_2$N), 1.20 (m, 1H, CH$_3$CH$_2$C*H*$_2$CH$_2$N), 1.28 (m, 1H, CH$_3$CH$_2$C*H*$_2$CH$_2$N), 1.38 (s, 1.5H, isopropylidene CH$_3$), 1.40 (s, 1.5H, isopropylidene CH$_3$), 1.62 (s, 1.5H, isopropylidene CH$_3$), 1.63 (s, 1.5H, isopropylidene CH$_3$), 1.81 (m, 2H, CHC*H*$_2$CH$_2$N), 2.22-2.38 (m, 2H, CH$_3$CH$_2$CH$_2$C*H*$_2$N), 2.43-2.59 (m, 2H, CHCH$_2$C*H*$_2$N), 2.78-2.89 (m, 2H, H-5'), 3.66 (s, 1.5H, CO_2CH_3), 3.75 (s, 1.5H, CO_2CH_3), 4.33 (m, 1H, H-4'), 4.45 (q, 0.5H, C*H*CH$_2$CH$_2$N), 4.49 (q, 0.5H, C*H*CH$_2$CH$_2$N), 4.96 (dd, 0.5H, J = 3.8Hz, 6.4Hz, H-3'), 5.00 (dd, 0.5H, J = 3.7Hz, 6.4Hz, H-3'), 5.49 (dd, 0.5H, J = 2.0Hz, 6.5Hz, H-2'), 5.52 (dd, 0.5H, J = 1.8Hz, 6.4Hz, H-2'), 5.75 (br s, 1H, adenine NH_2), 5.76 (br s, 1H, adenine NH_2), 6.03 (d, 0.5H, J = 2.0Hz, H-1'), 6.04 (d, 0.5H, J = 1.8Hz, H-1'), 7.82 (s, 0.5H, adenine H-2 or H-8), 7.88 (s, 0.5H, adenine H-2 or H-8), 8.32 (s, 0.5H, adenine H-2 or H-8), 8.34 (s, 0.5H, adenine H-2 or H-8), 9.43 (br d, 0.5H, J = 6.1Hz, NHCOCF_3), 9.55 (br d, 0.5H, J = 6.4Hz,

NHCOCF$_3$); UV λ_{max} (MeOH) 259 nm (ε 13.8 x 10^3); MS (positive LSIMS), m/e 574 $(M + H)^+$, 439 $(M + H - adenine)^+$.

Anal. Calculated for $C_{24}H_{34}F_3N_7O_6 \cdot H_2O$: C, 48.73; H, 6.13; N, 16.57. Found: C, 48.86; H, 5.99; N, 16.71.

Methyl N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-N^4-(1-Pentyl)-2(R,S)-Trifluoroacetamido-4-Aminobutyrate (Protected Nitrogen Pentyl SAM Analogue, **7f**) was prepared according to the general procedure described above. 5'-(1-Pentyl)amino-5'-deoxy-2',3'-O-isopropylideneadenosine hemihydrate (**4f**) (6.02 g, 15.6 mmol) and N,N-diisopropylethylamine (3.06 mL, 17.6 mmol) were dissolved in acetonitrile (300 mL) and heated to 70°C.. A solution of methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) (6.78 g, 20.0 mmol) in acetonitrile (200 mL) was then added over one hour. Initial silica gel chromatography of the three-day-old reaction mixture using chloroform:isopropanol (80:20) as the eluting solvent provided partially purified product in fractions 13-29. Further purification by low pressure liquid chromatography using the same eluting solvent provided product in the eluate collected 35-52 minutes after injection onto the column. Decolorization by filtration through silica gel using chloroform:isopropanol (95:5) as the eluting solvent provided 2.09 g (22.1%) of pure product as a colorless foam in fractions 26-47: mp 50-57°C.; TLC, chloroform:isopropanol (80:20), R_f 0.65 (UV, I_2); 500 MHz ^{1}H NMR ($CDCl_3$), δ 0.80 (t, 1.5H, J = 7.3Hz, C*H*$_3$CH$_2$CH$_2$CH$_2$CH$_2$N), 0.85 (t, 1.5H, J = 7.1Hz, C*H*$_3$CH$_2$CH$_2$CH$_2$CH$_2$N), 1.16 (m, 4H, CH$_3$C*H*$_2$C*H*$_2$CH$_2$CH$_2$N), 1.24 (m, 1H, CH$_3$CH$_2$CH$_2$C*H*$_2$CH$_2$N), 1.31 (m, 1H, CH$_3$CH$_2$CH$_2$C*H*$_2$CH$_2$N), 1.39 (s, 1.5H, isopropylidene CH$_3$), 1.40 (s, 1.5H, isopropylidene CH$_3$), 1.62 (s, 1.5H, isopropylidene CH$_3$), 1.63 (s, 1.5H, isopropylidene CH$_3$), 1.81 (m, 2H, CHC*H*$_2$CH$_2$N), 2.22-2.36 (m, 2H, CH$_3$CH$_2$CH$_2$CH$_2$C*H*$_2$N), 2.41-2.59 (m, 2H, CHCH$_2$C*H*$_2$N), 2.68-2.89 (m, 2H, H-5'), 3.66 (s, 1.5H, CO$_2$CH$_3$), 3.75 (s, 1.5H,

CO_2CH_3), 4.33 (m, 1H, H-4'), 4.45 (q, 0.5H, C*H*CH_2CH_2N), 4.49 (m, 0.5H, C*H*CH_2CH_2N), 4.96 (dd, 0.5H, J = 3.7Hz, 6.4Hz, H-3'), 5.00 (dd, 0.5H, J = 3.7Hz, 6.4Hz, H-3'),5.49 (dd, 0.5H, J = 1.8Hz, 6.4Hz, H-2'), 5.52 (dd, 0.5H, J = 1.7Hz, 6.4Hz, H-2'), 5.72 (br s, 1H, adenine NH_2), 5.73 (br s, 1H, adenine NH_2), 6.04 (d, 1H, J = 1.8Hz, H-1'), 7.82 (s, 0.5H, adenine H-2 or H-8), 7.88 (s, 0.5H, adenine H-2 or H-8), 8.32 (s, 0.5H, adenine H-2 or H-8), 8.33 (s, 0.5H, adenine H-2 or H-8), 9.46 (br d, 0.5H, J = 5.8Hz, $NHCOCF_3$), 9.58 (br d, 0.5H, J = 6.3Hz, $NHCOCF_3$); UV λ_{max} (MeOH) 259 nm (ε 13.4 x 10^3); MS (positive LSIMS), m/e 588 $(M + H)^+$, 453 $(M + H - adenine)^+$.

Anal. Calculated for $C_{25}H_{36}F_3N_7O_6 \cdot H_2O$: C, 49.58; H, 6.32; N, 16.19. Found: C, 49.35; H, 6.03; N, 15.92.

Methyl N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-N^4-(1-Octyl)-2(R,S)-Trifluoroacetamido-4-Aminobutyrate (Protected Nitrogen Octyl SAM Analogue, **7g**) was prepared according to the general procedure outlined above. 5'-(1-Octyl)amino-5'-deoxy-2',3'-O-isopropylideneadenosine hemihydrate (**4g**) (6.25 g, 14.6 mmol) and N,N-diisopropylethylamine (2.87 mL, 16.5 mmol) were dissolved in acetonitrile (300 mL) and heated to 70°C., followed by the addition of a solution of methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) (6.36 g, 18.8 mmol) in acetonitrile (200 mL) over one hour. Initial silica gel chromatography of the three-day-old reaction mixture using chloroform:isopropanol (90:10) as the eluting solvent provided partially purified product in fractions 15-33. Further purification by low pressure liquid chromatography using chloroform:isopropanol (80:20) as the eluting solvent provided product in the eluate collected 27-43 minutes after injection onto the column. Decolorization by filtration through silica gel using chloroform:isopropanol (95:5) as the eluting solvent provided 2.41 g (25.5%) of pure product in fractions 19-36: mp 46-52°C.; TLC, chloroform:isopropanol

(80:20), R_f 0.63 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 0.87 (t, 1.5H, J = 7.1 Hz, octyl CH_3), 0.88 (t, 1.5H, J = 1.5Hz, octyl CH_3), 1.11-1.30 (m, 12H, $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2N$), 1.38 (s, 1.5H, isopropylidene CH_3), 1.40 (s, 1.5H, isopropylidene CH_3), 1.62 (s, 1.5H, isopropylidene CH_3), 1.63 (s, 1.5H, isopropylidene CH_3), 1.81 (m, 2H, $CHCH_2CH_2N$), 2.21-2.37 (m, 2H, $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2N$), 2.41-2.57 (m, 2H, $CHCH_2CH_2N$), 2.68-2.91 (m, 2H, H-5'), 3.66 (s, 1.5H, CO_2CH_3), 3.75 (s, 1.5H, CO_2CH_3), 4.33 (m, 1H, H-4'), 4.44 (q, 0.5H, $CHCH_2CH_2N$), 4.49 (q, 0.5H, $CHCH_2CH_2N$), 4.96 (dd, 0.5H, J = 3.8Hz, 6.4Hz, H-3'), 5.00 (dd, 0.5H, J = 3.7Hz, 6.4Hz, H-3'), 5.49 (dd, 0.5H, J = 1.9Hz, 6.4Hz, H-2'), 5.51 (dd, 0.5H, J = 1.8Hz, 6.4Hz, H-2'), 5.82 (br s, 1H, adenine NH_2), 5.83 (br s, 1H, adenine NH_2), 6.04 (d, 1H, J = 1.8Hz, H-1'), 7.83 (s, 0.5H, adenine H-2 or H-8), 7.88 (s, 0.5H, adenine H-2 or H-8), 8.32 (s, 0.5H, adenine H-2 or H-8), 8.33 (s, 0.5H, adenine H-2 or H-8), 9.49 (br d, 0.5H, J = 6.2Hz, $NHCOCF_3$), 9.62 (br d, 0.5H, J = 6.4Hz, $NHCOCF_3$); UV λ_{max} (MeOH) 259 nm (ε 14.2 x 10^3); MS (positive LSIMS), m/e 630 $(M + H)^+$, 495 $(M + H - adenine)^+$.

Anal. Calculated for $C_{28}H_{42}F_3N_7O_6 \cdot H_2O$: C, 51.92; H, 6.85; N, 15.14. Found: C, 52.13; H, 6.71; N, 15.17.

General Preparation of N^4-(5'-Adenosyl)-N^4-Alkyl-2(R,S),4-Diaminobutyric Acid Homologues (**2a-g**). A solution of sodium carbonate (663 mg, 6.25 mmol) in 50% aqueous methanol (30 mL) was added to the methyl N^4-alkyl-N^4-(2',3'-O-isopropylidene-5'-adenosyl)-2(R,S)-trifluoroacetamido-4-aminobutyrate monohydrate (**7a-g**) (2.50 mmol). This mixture was stirred at 60°C. for six hours, after which time it was neutralized with 1M aqueous hydrochloric acid and evaporated to dryness. The residue was extracted with methanol (4 x 25 mL) and the combined extracts concentrated to approximately 5 mL before being loaded onto a wet-packed flash silica gel column (44 x 3.5 cm, 200 g).

The column was eluted first with chloroform:methanol (70:30 or 50:50, 2.0 L) followed by 100% methanol (1.0 L) at a flow rate of approximately 10 mL/min and 28 mL fractions were collected. Those fractions containing the ultraviolet-absorbing product, which generally appeared in fractions 55-110, were pooled and evaporated to dryness. The intermediate N^4-alkyl-N^4-(2',3'-O-isopropylidene-5'-adenosyl)-2(R,S),4-diaminobutyric acid (**8a-g**) was generally obtained in very high yields and was sufficiently pure to be used directly in the next step.

The crude intermediate (**8a-g**) was dissolved in 0.1M aqueous sulfuric acid (75 mL) and the mixture stirred at room temperature for 11 days. The acid was neutralized by the addition of an equivalent amount of barium hydroxide octahydrate and the barium sulfate precipitate removed by centrifugation. The aqueous solution was filtered and evaporated to dryness. The residue was redissolved in water (300 mL) and loaded at 4°C. onto a Dowex 50X4-400 (NH_4^+ form) cation exchange column (44 x 3.5 cm, 450 g) at a flow rate of 2 mL/min and 28 mL fractions were collected. After washing with water (700 mL), the column was eluted with a gradient of aqueous ammonium bicarbonate (5.0 L, 0-1.3M) beginning with fraction 31 at a flow rate of 3 mL/min. The ultraviolet-absorbing product was eluted from the column at buffer concentrations ranging from 0.70-1.20M depending on the length (i. e., hydrophobicity) of the N^4-alkyl group. [The only exception to this generalization was the N^4-(1-octyl) homologue (**2g**), which could only be eluted from the column with 1M aqueous ammonium hydroxide]. The appropriate fractions were pooled in preparation for desalting and reloaded onto the Dowex 50X4-400 column (44 x 3.5 cm, 450 g, freshly regenerated into the NH_4^+ form after washing with 1M aqueous hydrochloric acid and 1M aqueous ammonium chloride) at a flow rate of 2 mL/min and 28 mL fractions were collected. The column was washed with

water (2.0 L) at a flow rate of 3 mL/min and the adsorbed product eluted with 1M aqueous ammonium hydroxide. Those fractions containing the desalted product were pooled and evaporated to dryness. Product not intially adsorbed onto the Dowex due to overloading was reloaded onto the same column (after washing with water) and washed, eluted, and evaporated as described above. The product was redissolved in water (10 mL) and lyophilized to give a colorless powder. Overall yields were between 60-80%.

N^4-(5'-Adenosyl)-N^4-Methyl-2(R,S),4-Diaminobutyric Acid (Nitrogen Methyl SAM Analogue, **2a**) was prepared according to the general procedure summarized above. A solution of sodium carbonate (2.123 g, 20.03 mmol) in 50% aqueous methanol (80 mL) was added to methyl N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-methyl-2(R,S)-trifluoroacetamido-4-aminobutyrate monohydrate (**7a**) (4.395 g, 8.01 mmol). This mixture was heated with stirring to 60°C. for six hours after which it was neutralized and evaporated. Flash silica gel chromatography (47 x 3.5 cm, 230 g) was used to purify the 2',3'-O-isopropylidene intermediate (**8a**) present in the concentrated (5 mL) methanol extract. All 125 fractions were eluted with chloroform:methanol (50:50). The crude N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-methyl-2(R,S),4-diaminobutyric acid intermediate (**8a**) (3.83 g, 97.4%) appeared in fractions 63-125: mp 120-123°C.; TLC, methanol, R_f 0.29 (UV, I_2, ninhydrin); 500 MHz ^{1}H NMR (D_2O), δ 1.45 (s, 3H, isopropylidene CH_3), 1.66 (s, 3H, isopropylidene CH_3), 1.79-1.98 (m, 2H, CHCH_2CH_2N), 2.22 (s, 1.5H, CH_3N), 2.23 (s, 1.5H, CH_3N), 2.52-2.77 (m, 4H, overlapping H-5' and CHCH_2CH_2N), 3.65 (m, 1H, CHCH_2CH_2N), 4.49 (m, 1H, H-4'), 5.03 (m, 1H, H-3'), 5.56 (m, 1H, H-2'), 6.25 (d, 0.5H, J = 2.5Hz, H-1'), 6.26 (d, 0.5H, J = 2.6Hz, H-1'), 8.24 (s, 1H, adenine H-2 or H-8), 8.29 (s, 1H, adenine H-2 or H-8); MS (positive LSIMS), m/e 422 (M + H)$^+$.

The crude intermediate (**8a**) was dissolved in 0.1M aqueous sulfuric acid (250 mL) and stirred at room temperature for 11 days. Following neutralization and filtration of the reaction mixture, purification by cation exchange chromatography resulted in the elution of the ultraviolet-absorbing product in fractions 114-149, with the peak occurring in fraction 132 ([NH_4HCO_3] = 0.76M). These fractions were pooled, desalted, and lyophilized to give 2.17 g (66.5%, based on the fully protected starting material) of analytically pure product as a colorless solid: mp 173-176°C.; TLC, ethanol:conc. NH_4OH (90:10), R_f 0.37 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 1.95 (m, 1H, CHC*H*$_2$CH_2N), 2.04 (m, 1H, CHC*H*$_2$CH_2N), 2.35 (s, 1.5H, CH_3N), 2.36 (s, 1.5H, CH_3N), 2.67-2.80 (m, 2H, CHCH_2C*H*$_2$N), 2.84-2.93 (m, 2H, H-5'), 3.71 (m, 1H, C*H*CH_2CH_2N), 4.22 (q, 1H, H-3'), 4.31 (m, 1H, H-4'), 4.75 (m, 1H, H-2'), 5.97 (d, 0.5H, J = 2.4Hz, H-1'), 5.98 (d, 0.5H, J = 2.5Hz, H-1'), 8.05 (s, 0.5H, adenine H-2 or H-8), 8.06 (s, 0.5H, adenine H-2 or H-8), 8.18 (s, 0.5H, adenine H-2 or H-8), 8.19 (s, 0.5H, adenine H-2 or H-8); UV λ_{max} (H_2O) 259 nm (ε 14.2 x 10^3); MS (positive LSIMS), m/e 382 $(M + H)^+$.

Anal. Calculated for $C_{15}H_{23}N_7O_5 \cdot 1.5H_2O$: C, 44.11; H, 6.42; N, 24.01. Found: C, 44.46; H, 6.23; N, 24.34.

N^4-(5'-Adenosyl)-N^4-Ethyl-2(R,S),4-Diaminobutyric Acid (Nitrogen Ethyl SAM Analogue, **2b**) was prepared according to the general procedure described above. A solution of sodium carbonate (769 mg, 7.25 mmol) in 50% aqueous methanol (30 mL) was added to methyl N^4-ethyl-N^4-(2',3'-O-isopropylidene-5'-adenosyl)-2(R,S)-trifluoroacetamido-4-aminobutyrate monohydrate (**7b**) (1.619 g, 2.90 mmol) and the resulting mixture heated with stirring to 60°C. for six hours. Following neutralization and evaporation of the reaction mixture, the 2',3'-O-isopropylidene intermediate (**8b**) was purified from the concentrated (5 mL) methanol extract by flash silica gel chromatography. The

first 58 fractions were eluted with chloroform:methanol (50:50) and an additional 34 fractions were eluted with 100% methanol. The crude N^4-ethyl-N^4-(2',3'-O-isopropylidene-5'-adenosyl)-2(R,S),4-diaminobutyric acid intermediate (**8b**) (1.07 g, 84.9%) appeared in fractions 58-92: mp 112-115°C.; TLC, methanol, R_f 0.31 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 0.67 (t, 1.5H, J = 7.1Hz, C*H*$_3$CH$_2$N), 0.76 (t, 1.5H, J = 7.1Hz, C*H*$_3$CH$_2$N), 1.41 (s, 3H, isopropylidene CH_3), 1.61 (s, 3H, isopropylidene CH_3), 1.62-1.84 (m, 2H, CHC*H*$_2$CH$_2$N), 2.45 (m, 2H, CH$_3$C*H*$_2$N), 2.51-2.66 (m, 4H, overlapping H-5' and CHCH$_2$C*H*$_2$N), 3.46 (m, 1H, C*H*CH$_2$CH$_2$N), 4.41 (m, 1H, H-4'), 4.97 (m, 1H, H-3'), 5.47 (dd, 0.5H, J = 2.2Hz, 6.4Hz, H-2'), 5.51 (dd, 0.5H, J = 2.0Hz, 6.3Hz, H-2'), 6.14 (d, 0.5H, J = 2.2Hz, H-1'), 6.16 (d, 0.5H, J = 2.0Hz, H-1'), 8.11 (s, 0.5H, adenine H-2 or H-8), 8.12 (s, 0.5H, adenine H-2 or H-8), 8.19 (s, 0.5H, adenine H-2 or H-8), 8.20 (s, 0.5H, adenineH-2 or H-8); MS (positive LSIMS), m/e 436 $(M + H)^+$.

The crude intermediate (**8b**) was dissolved in 0.1M aqueous sulfuric acid (75 mL) and stirred at room temperature for 11 days. After neutralization and filtration of the reaction mixture, purification by cation exchange chromatography led to the appearance of the ultraviolet-absorbing product in fractions 136-158. The peak appeared in fraction 146 ([NH_4HCO_3] = 0.86M). These fractions were pooled, desalted, and lyophilized to give 847 mg (69.3%, based on the fully protected starting material) of analytically pure product as a colorless solid: mp 160-170°C.; TLC, ethanol:conc. NH_4OH (90:10), R_f 0.19 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 1.04 (t, 1.5H, J = 7.1Hz, C*H*$_3$CH$_2$N), 1.05 (t, 1.5H, C*H*$_3$CH$_2$N), 1.96 (m, 1H, CHC*H*$_2$CH$_2$N), 2.05 (m, 1H, CHC*H*$_2$CH$_2$N), 2.70-2.84 (m, 2H, CH$_3$C*H*$_2$N), 2.89 (m, 2H, CHCH$_2$C*H*$_2$N), 2.99 (m, 2H, H-5'), 3.70 (m, 1H, C*H*CH$_2$CH$_2$N), 4.29 (m, 1H, H-3'), 4.34 (m, 1H, H-4'), 4.73 (m, 1H, H-2'), 6.04 (d, 0.5H, J = 1.9Hz, H-1'), 6.05 (d, 0.5H, J = 2.0Hz, H-1'),

8.17 (s, 1H, adenine H-2 or H-8), 8.25 (s, 1H, adenine H-2 or H-8); UV λ_{max} (H_2O) 259 nm (ε 13.7 x 10^3); MS (positive LSIMS), m/e 396 $(M + H)^+$.

Anal. Calculated for $C_{16}H_{25}N_7O_5 \cdot 1.5H_2O$: C, 45.49; H, 6.68; N, 23.21. Found: C, 45.60; H, 6.47; N, 23.11.

N^4-(5'-Adenosyl)-N^4-(1-Propyl)-2(R,S),4-Diaminobutyric Acid (Nitrogen Propyl SAM Analogue, **2c**) was prepared following the general procedure summarized above. A solution of sodium carbonate (774 mg) in 50% aqueous methanol (40 mL) was added to methyl N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-(1-propyl)-2(R,S)-trifluoroacetamido-4-aminobutyrate monohydrate (**7c**) (1.684 g, 2.92 mmol). The stirred mixture was heated to 60°C. for six hours. The 2',3'-O-isopropylidene intermediate (**8c**) in a concentrated (5 mL) methanol extract of the neutralized and evaporated reaction mixture was purified by flash silica gel chromatography. The first 75 fractions were eluted with chloroform:methanol (70:30) and an additional 30 fractions were eluted with 100% methanol. Fractions 88-103 contained the crude N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-(1-propyl)-2(R,S),4-diaminobutyric acid intermediate (**8c**) (1.49 g, > 100% due to the presence of an impurity): mp 132-137°C.; TLC, methanol, R_f 0.38 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 0.49 (t, 1.5H, J = 7.2Hz, $CH_3CH_2CH_2N$), 0.53 (t, 1.5H, J = 7.3Hz, $CH_3CH_2CH_2N$), 0.78-1.02 (m, 2H, $CH_3CH_2CH_2N$), 1.40 (s, 3H, isopropylidene CH_3), 1.59 (s, 3H, isopropylidene CH_3), 1.62-1.84 (m, 2H, $CHCH_2CH_2N$), 2.20 (m, 2H, $CH_3CH_2CH_2N$), 2.49-2.65 (m, 4H, overlapping H-5' and $CHCH_2CH_2N$), 3.48 (m, 1H, $CHCH_2CH_2N$), 4.42 (m, 1H, H-4'), 4.97 (m, 1H, H-3'), 5.53 (dd, 0.5H, J = 1.7Hz, 5.3Hz, H-2'), 5.57 (dd, 0.5H, J = 1.3Hz, 5.5Hz, H-2'), 6.17 (d, 0.5H, J = 1.8Hz, H-1'), 6.18 (d, 0.5H, J = 1.3Hz, H-1'), 8.11 (s, 0.5H, adenine H-2 or H-8), 8.13 (s, 0.5H, adenine H-2 or H-8), 8.20 (s, 1H, adenine H-2 or H-8); MS (positive LSIMS), m/e 450 $(M + H)^+$.

The crude intermediate (**8c**) was dissolved in 0.1M aqueous sulfuric acid (75 mL) and stirred at room temperature for 11 days. Following neutralization and filtration of the reaction mixture, purification by cation exchange chromatography led to the appearance of the ultraviolet-absorbing product in fractions 146-170, with the peak appearing in fraction 157 ([NH_4HCO_3] = 0.92M). These fractions were pooled, desalted, and lyophilized to afford 946 mg (76.0%, based on the fully protected starting material) of analytically pure product as a colorless solid: mp 150-157°C.; TLC, ethanol:conc. NH_4OH (90:10), R_f 0.19 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 0.78 (t, 3H, J = 7.3Hz, $CH_3CH_2CH_2N$), 1.43 (m, 2H, $CH_3CH_2CH_2N$), 1.97 (m, 1H, $CHCH_2CH_2N$), 2.03 (m, 1H, $CHCH_2CH_2N$), 2.50-2.65 (m, 2H, $CH_3CH_2CH_2N$), 2.74-2.91 (m, 2H, $CHCH_2CH_2N$), 2.94 (m, 2H, H-5'), 3.70 (m, 1H, $CHCH_2CH_2N$), 4.30 (m, 1H, H-3'), 4.33 (m, 1H, H-4'), 4.72 (m, 1H, H-2'), 6.06 (d, 1H, J = 4.1Hz, H-1'), 8.21 (s, 1H, adenine H-2 or H-8), 8.27 (s, 1H, adenine H-2 or H-8); UV λ_{max} (H_2O) 259 nm (ε 14.6 x 10^3); MS (positive LSIMS), m/e 410 $(M + H)^+$.

Anal. Calculated for $C_{17}H_{27}N_7O_5 \cdot H_2O$: C, 47.77; H, 6.84; N, 22.94. Found: C, 47.79; H, 6.68; N, 22.80.

N^4-(5'-Adenosyl)-N^4-[1-(2-Propenyl)]-2(R,S),4-Diaminobutyric Acid (Nitrogen Allyl SAM Analogue, **2d**) was prepared according to the general procedure described above. A solution of sodium carbonate (2.626 g, 24.8 mmol) in 50% aqueous methanol (90 mL) was added to methyl N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-[1-(2-propenyl)]-2(R,S)-trifluoroacetamido-4-aminobutyrate monohydrate (**7d**) (5.720 g, 9.91 mmol) and the mixture heated with stirring to 60°C. for six hours. The neutralized and evaporated reaction mixture was extracted with methanol (4 x 75 mL) and after the combined extracts were concentrated to 5 mL, the 2',3'-O-isopropylidene intermediate

(**8d**) was purified by flash silica gel chromatography. The first 70 fractions were eluted with chloroform:methanol (70:30) and another 50 fractions were eluted with 100% methanol. The ultraviolet-absorbing intermediate appeared in fractions 62-115. These fractions were pooled and evaporated to give 5.44 g (> 100% yield, due to the presence of an unidentified impurity) of crude N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-[1-(2-propenyl)]-2(R,S),4-diaminobutyric acid (**8d**): mp 133-137°C.; TLC, methanol, R_f 0.45 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 1.39 (s, 1.5H, isopropylidene CH_3), 1.59 (s, 3H, isopropylidene CH_3), 1.66-1.86 (m, 2H, CHC*H*$_2$CH_2N), 2.45-2.67 (m, 4H, overlapping H-5' and CHCH_2C*H*$_2$N), 2.77-3.00 (m, 2H, CH_2=CHC*H*$_2$N), 3.50 (m, 1H, C*H*CH_2CH_2N), 4.40 (m, 0.5H, H-4'), 4.44 (m, 0.5H, H-4'), 4.88-4.94 (m, 2H, C*H*$_2$=CHCH_2N), 4.97 (m, 1H, H-3'), 5.20 (m, 0.5H, CH_2=C*H*CH_2N), 5.39 (m, 0.5H, CH_2=C*H*CH_2N), 5.50 (dd, 0.5H, J = 2.1Hz, 6.5Hz, H-2'), 5.54 (dd, 0.5H, J = 1.9Hz, 6.3Hz, H-2'), 6.15 (d, 0.5H, J = 2.1Hz, H-1'), 6.17 (d, 0.5H, J = 1.9Hz, H-1'), 8.09 (s, 0.5H, adenine H-2 or H-8), 8.10 (s, 0.5H, adenine H-2 or H-8), 8.17 (s, 1H, adenine H-2 or H-8); MS (positive LSIMS), m/e 448 $(M + H)^+$.

The crude intermediate (**8d**) was dissolved in 0.1M aqueous sulfuric acid (300 mL) and stirred at room temperature for 11 days. After the reaction mixture was neutralized, filtered, and evaporated, the impure product was redissolved in water (500 mL) and purified by cation exchange chromatography. The column was washed with water (1.5 L) and the ammonium bicarbonate gradient started at fraction 61. The product was eluted in fractions 130-186, with the peak occurring in fraction 160 ([NH_4HCO_3)] = 0.81M). These fractions were pooled, desalted, and lyophilized to give 2.80 g (66.5%, based on the fully protected starting material) of analytically pure product as a colorless solid: mp 165-168°C.; TLC, ethanol:conc. NH_4OH (90:10), R_f, 0.18 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 1.92 (m, 1H, CHC*H*$_2$CH_2N), 2.00 (m, 1H, CHC*H*$_2$CH_2N),

2.64-2.86 (m, 4H, overlapping H-5' and $CHCH_2CH_2N$), 3.06-3.26 (m, 2H, CH_2=$CHCH_2N$), 3.68 (m, 1H, $CHCH_2CH_2N$), 4.22 (m, 1H, H-3'), 4.29 (m, 1H, H-4'), 4.73 (m, 1H, H-2'), 5.16 (m, 2H, CH_2=$CHCH_2N$), 5.69-5.78 (m, 1H, CH_2=$CHCH_2N$), 6.01 (d, 1H, J = 2.9Hz, H-1'), 8.16 (s, 1H, adenine H-2 or H-8), 8.21 (s, 0.5H, adenine H-2 or H-8), 8.22 (s, 0.5H, adenine H-2 or H-8); UV λ_{max} (H_2O) 259 nm (ε 15.3 x 10^3); MS (positive LSIMS), m/e 408 $(M + H)^+$.

Anal. Calculated for $C_{17}H_{25}N_7O_5 \cdot H_2O$: C, 47.99; H, 6.40; N, 23.05. Found: C, 48.03; H, 6.22; N, 22.95.

N^4-(5'-Adenosyl)-N^4-(1-Butyl)-2(R,S),4-Diaminobutyric Acid (Nitrogen Butyl SAM Analogue, **2e**) was prepared according to the general procedure outlined above. A solution of sodium carbonate (668 mg, 6.30 mmol) in 50% aqueous methanol (30 mL) was added to methyl N^4-(1-butyl)-N^4-(2',3'-O-isopropylidene-5'-adenosyl)-2(R,S)-trifluoroacetamido-4-aminobutyrate monohydrate (**7e**) (1.487 g, 2.52 mmol). The mixture was heated with stirring to 60°C. for six hours. The 2',3'-O-isopropylidene intermediate (**8e**) in a concentrated (5 mL) methanol extract of the neutralized and evaporated reaction mixture was purified by flash silica gel chromatography. Chloroform:methanol (70:30) was used to elute the first 75 fractions followed by 100% methanol. The partially purified N^4-(1-butyl)-N^4-(2',3'-O-isopropylidene-5'-adenosyl)- 2(R,S),4-diaminobutyric acid intermediate (**8e**) (1.41 g, > 100% due to the presence of an unidentified impurity) appeared in fractions 87-102: mp 134-138°C.; TLC, methanol, R_f 0.42 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 0.61 (m, 3H, $CH_3CH_2CH_2CH_2N$), 0.64-0.95 (m, 4H, $CH_3CH_2CH_2CH_2N$), 1.41 (s, 1.5H, isopropylidene CH_3), 1.42 (s, 1.5H, isopropylidene CH_3), 1.60 (s, 1.5H, isopropylidene CH_3), 1.61 (s, 1.5H, isopropylidene CH_3), 1.69-1.85 (m, 2H, $CHCH_2CH_2N$), 2.15-2.28 (m, 2H, $CH_3CH_2CH_2CH_2N$), 2.50-2.70 (m, 4H, overlapping H-5' and $CHCH_2CH_2N$),

3.52 (m, 1H, C*H*CH_2CH_2N), 4.44 (m, 0.5H, H-4'), 4.49 (m, 0.5H, H-4'), 5.02 (dd, 0.5H, J = 3.0Hz, 6.3Hz, H-3'), 5.05 (dd, 0.5H, J = 2.6Hz, 6.2Hz, H-3'), 5.61 (dd, 0.5H, J = 1.9Hz, 6.3Hz, H-2'), 5.65 (dd, 0.5H, J = 1.6Hz, 6.2Hz, H-2'), 6.23 (d, 0.5H, J = 1.9Hz, H-1'), 6.25 (d, 0.5H, J = 1.6Hz, H-1'), 8.18 (s, 0.5H, adenine H-2 or H-8), 8.19 (s, 0.5H, adenine H-2 or H-8), 8.24 (s, 1H, adenine H-2 or H-8); MS (positive LSIMS), m/e 464 $(M + H)^+$.

The crude intermediate (**8e**) was dissolved in 0.1M aqueous sulfuric acid (75 mL) and stirred at room temperature for 11 days. Cation exchange chromatography of the neutralized and filtered reaction mixture led to the elution of the ultraviolet-absorbing product in fractions 163-190, with the peak occurring in fraction 174 ($[NH_4HCO_3]$ = 1.03M). These fractions were pooled, desalted, and lyophilized to give 844 mg (75.8%) of analytically pure product as a colorless solid: mp 145-152°C.; TLC, ethanol:conc. NH_4OH (90:10), R_f 0.25 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 0.76 (t, 3H, J = 7.3Hz, $CH_3CH_2CH_2CH_2N$), 1.14 (m, 2H, $CH_3CH_2CH_2CH_2N$), 1.32 (m, 2H, $CH_3CH_2CH_2CH_2N$), 1.93 (m, 1H, $CHCH_2CH_2N$), 2.04 (m, 1H, $CHCH_2CH_2N$), 2.51-2.58 (m, 1H, $CH_3CH_2CH_2CH_2N$), 2.61-2.68 (m, 1H, $CH_3CH_2CH_2CH_2N$), 2.74-2.90 (m, 2H, $CHCH_2CH_2N$), 2.94 (m, 2H, H-5'), 4.33 (m, 2H, H-3' and H-4'), 4.76 (m, 1H, H-2'), 6.07 (d, 1H, J = 4.2Hz, H-1'), 8.22 (s, 1H, adenine H-2 or H-8), 8.28 (s, 0.5H, adenine H-2 or H-8), 8.29 (s, 0.5H, adenine H-2 or H-8); UV λ_{max} (H_2O) 259 nm (ε 14.9 x 10^3); MS (positive LSIMS), m/e 424 $(M + H)^+$.

Anal. Calculated for $C_{18}H_{29}N_7O_5 \cdot H_2O$: C, 48.97; H, 7.08; N, 22.21. Found: C, 49.06; H, 6.95; N, 22.25.

N^4-(5'-Adenosyl)-N^4-(1-Pentyl)-2(R,S),4-Diaminobutyric Acid (Nitrogen Pentyl SAM Analogue, **2f**) was prepared according to the general procedure summarized above. A solution of sodium carbonate (784 mg, 7.40 mmol) in 50% aqueous methanol (30 mL) was added to methyl N^4-(2',3'-O-isopropyli-

dene-5'-adenosyl)-N^4-(1-pentyl)-2(R,S)-trifluoroacetamido-4-aminobutyrate monohydrate (**7f**) (1.792 g, 2.96 mmol). The mixture was stirred at 60°C. for six hours, after which it was neutralized and evaporated to dryness. The 2',3'-O-isopropylidene intermediate (**8f**) in a concentrated (5 mL) methanol extract was purified by flash silica gel chromatography. Chloroform:methanol (70:30) was used to elute the first 75 fractions and 100% methanol was used to elute an additional 25 fractions. Fractions 71-97 contained 1.90 g (> 100% yield, due to the presence of one or more minor impurities) of the crude N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-(1-pentyl)- 2(R,S),4-diaminobutyric acid intermediate (**8f**): mp 118-122°C.; TLC, methanol, R_f 0.44 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 0.64 (t, 3H, J = 7.1Hz, C*H*$_3$CH$_2$CH$_2$CH$_2$CH$_2$N), 0.75 (m, 2H, CH$_3$C*H*$_2$CH$_2$CH$_2$CH$_2$N), 0.82-0.98 (m, 4H, CH$_3$CH$_2$C*H*$_2$C*H*$_2$CH$_2$N), 1.41 (s, 1.5H, isopropylidene CH$_3$), 1.42 (s, 1.5H, isopropylidene CH$_3$), 1.60 (s, 1.5H, isopropylidene CH$_3$), 1.61 (s, 1.5H, isopropylidene CH$_3$), 1.69-1.87 (m, 2H, CHC*H*$_2$CH$_2$N), 2.13-2.25 (m, 2H, CH$_3$CH$_2$CH$_2$CH$_2$C*H*$_2$N), 2.50-2.69 (m, 4H, overlapping H-5' and CHCH$_2$C*H*$_2$N), 3.52 (m, 1H, C*H*CH$_2$CH$_2$N), 4.42-4.49 (m, 1H, H-4'), 5.01 (dd, 0.5H, J = 2.9Hz, 6.3Hz, H-3'), 5.05 (dd, 0.5H, J = 2.9Hz, 6.3Hz, H-3'), 5.60 (dd, 0.5H, J = 1.9Hz, 6.3Hz, H-2'), 5.64 (dd, 0.5H, J = 1.7Hz, 6.3Hz, H-2'), 6.22 (d, 0.5H, J = 1.9Hz, H-1'), 6.24 (d, 0.5H, J = 1.7Hz, H-1'), 8.16 (s, 0.5H, adenine H-2 or H-8), 8.17 (s, 0.5H, adenine H-2 or H-8), 8.23 (s, 1H, adenine H-2 or H-8); MS (positive LSIMS), m/e 478 $(M + H)^+$.

The crude intermediate (**8f**) was dissolved in 0.1M aqueous sulfuric acid (75 mL) and stirred at room temperature for 11 days. After the reaction mixture was neutralized and filtered, the product was purified by cation exchange chromatography using a gradient of aqueous ammonium bicarbonate (0-1.5M, 6.2 L total volume). Fractions 172-207 contained the ultraviolet-absorbing product, with the peak appearing in fraction 186 ([NH_4HCO_3] = 1.11M). These

fractions were pooled, desalted, and lyophilized to give 1.02 g (75.3%, based on the fully protected starting material) of analytically pure product as a colorless solid: mp 146-155°C.; TLC, ethanol:conc. NH_4OH (90:10), R_f 0.28 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 0.62 (t, 3H, J = 5.5Hz, $CH_3CH_2CH_2CH_2CH_2N$), 0.93-1.01 (m, 4H, $CH_3CH_2CH_2CH_2CH_2N$), 1.21 (m, 2H, $CH_3CH_2CH_2CH_2CH_2N$), 1.87 (m, 1H, $CHCH_2CH_2N$), 1.97 (m, 1H, $CHCH_2CH_2N$), 2.42 (m, 1H, $CH_3CH_2CH_2CH_2CH_2N$), 2.53 (m, 1H, $CH_3CH_2CH_2CH_2CH_2N$), 2.64-2.88 (m, 4H, overlapping H-5' and $CHCH_2CH_2N$), 3.63 (m, 1H, $CHCH_2CH_2N$), 4.25 (m, 2H, overlapping H-3' and H-4'), 4.91 (m, 1H, H-2'), 5.98 (d, 1H, J = 4.8Hz, H-1'), 8.12 (s, 1H, adenine H-2 or H-8), 8.21 (s, 1H, adenine H-2 or H-8); UV λ_{max} (H_2O) 259 nm (ε 14.6 x 10^3); MS (positive LSIMS), m/e 438 $(M + H)^+$.

Anal. Calculated for $C_{19}H_{31}N_7O_5 \cdot H_2O$: C, 50.10; H, 7.30; N, 21.52. Found: C, 49.88; H,7.13; N, 21.37.

N^4-(5'-Adenosyl)-N^4-(1-Octyl)-2(R,S),4-Diaminobutyric Acid (Nitrogen Octyl SAM Analogue, **2g**) was prepared following the general procedure outlined above. A solution of sodium carbonate (880 mg, 8.30 mmol) in 50% aqueous methanol (40 mL) was added to methyl N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-(1-octyl)-2(R,S)-trifluoroacetamido-4-aminobutyrate monohydrate (**7g**) (2.148 g, 3.32 mmol). The stirred mixture was heated to 60°C. for six hours, after which time it was neutralized and evaporated. The 2',3'-O-isopropylidene intermediate (**8g**) present in a concentrated (5 mL) methanol extract of the crude reaction mixture was purified by flash chromatography. The first 62 fractions were eluted with chloroform:methanol (70:30) and an additional 23 fractions were eluted with 100% methanol. The crude N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-(1-octyl)-2(R,S),4-diaminobutyric acid (**8g**) (2.11 g, > 100% yield due to the presence of an unidentified impurity) appeared

in fractions 54-81: mp 113-117°C.; TLC, methanol, R_f 0.57 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 0.80 (m, 7H $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2N$), 0.91 (m, 2H, $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2N$), 0.98 (m, 2H, $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2N$), 1.07 (m,2H, $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2N$), 1.17 (m, 2H, $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2N$), 1.42 (s. 3H, isopropylidene CH_3), 1.62 (s, 1.5H, isopropylidene CH_3), 1.63 (s, 1.5H, isopropylidene CH_3), 1.73-1.91 (m, 2H, $CHCH_2CH_2N$), 2.20-2.35 (m, 2H, $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2N$), 2.54-2.77 (m, 4H, overlapping H-5' and $CHCH_2CH_2N$), 3.55 (m, 1H, $CHCH_2CH_2N$), 4.45-4.52 (m, 1H, H-4'), 5.04 (dd, 0.5H, J = 3.4Hz, 6.3Hz, H-3'), 5.08 (dd, 0.5H, J = 2.7Hz, 6.4Hz, H-3'), 5.62 (dd, 0.5H, J = 2.0Hz, 6.3Hz, H-2'), 5.65 (dd, 0.5H, J = 1.7Hz, 6.4Hz, H-2'), 6.27 (d, 0.5H, J = 2.0Hz, H-1'), 6.28 (d, 0.5H, J = 1.7Hz, H-1'), 8.23 (s, 0.5H, adenine H-2 or H-8), 8.24 (s, 0.5H, adenine H-2 or H-8), 8.27 (s, 0.5H, adenine H-2 or H-8), 8.28 (s, 0.5H, adenine H-2 or H-8); MS (positive LSIMS), m/e 520 $(M + H)^+$.

The crude intermediate (**8g**) was dissolved in 0.1M aqueous sulfuric acid (75 mL) and stirred at room temperature for 11 days. Following neutralization and filtration of the reaction mixture, the product was purified by cation exchange chromatography using a gradient of aqueous ammonium bicarbonate (0-1.7M, 6.5 L total volume) in fractions 31-260. This gradient failed to elute the hydrophobic product, which was subsequently eluted with 1M aqueous ammonium hydroxide and appeared in fractions 276-288. These fractions were pooled, desalted (to remove any residual ammonium bicarbonate), and lyophilized to give 1.10 g (66.9%, based on the fully protected starting material) of analytically pure product as a colorless solid: mp 135-152°C.; TLC, ethanol:conc. NH_4OH (90:10), R_f 0.32 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 0.77 (t,3H, J = 7.0Hz, $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2N$),

0.85-1.06 (m, 8H, $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2N$), 1.12 (m, 2H, $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2N$), 1.30 (m, 2H, $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2N$), 1.93 (m, 1H, $CHCH_2CH_2N$), 2.05 (m, 1H, $CHCH_2CH_2N$), 2.55 (m, 1H, $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2N$), 2.67 (m, 1H, $CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2N$), 2.75-3.06 (m, 4H, overlapping H-5' and $CHCH_2CH_2N$), 3.72 (m, 1H, $CHCH_2CH_2N$), 4.33 (m, 4H, overlapping H-3' and H-4'), 4.84 (m, 1H, H-2'), 6.06 (d, 1H, J = 3.1Hz, H-1'), 8.26 (s, 1H, adenine H-2 or H-8), 8.30 (s, 0.5H, adenine H-2 or H-8), 8.31 (s, 0.5H, adenine H-2 or H-8); UV λ_{max} (H_2O) 259 nm (ε 14.7 x 10^3); MS (positive LSIMS), m/e 480 $(M + H)^+$.

Anal. Calculated for $C_{22}H_{37}N_7O_5 \cdot H_2O$: C, 53.10; H,7.90; N, 19.70. Found: C, 52.83; H, 7.75; N, 19.47.

Preparation of the Ammonium (NH_4^+) Form of Dowex 50X4-400 Cation Exchange Resin. Dowex 50X4-400 cation exchange resin (450 g, total capacity approximately 2.3 equiv) was first converted to the acid (H^+) form by washing with 200 mL volumes of 1M aqueous hydrochloric acid (4.0 L) in a fritted glass filter. Residual acid was removed from the resin matrix by washing with 200 mL volumes of water (approximately 4.0 L) until the filtrate was neutral to pH paper. The resin was converted from the acid (H^+) form to the ammonium (NH_4^+) form by washing with 200 mL volumes of 1M aqueous ammonium chloride (4.0 L). Excess ammonium chloride was removed from the cation exchange resin by washing with 200 mL volumes of water (approximately 4.0 L) until no chloride ion could be detected in the filtrate. The presence of chloride ion, and by implication, ammonium ion as well, in the filtrate is detected by the formation of a white precipitate of silver chloride upon addition of a small aliquot (1 mL) of 1M aqueous silver nitrate. The cation exchange resin (approximately 450 mL in water) is then ready for use.

Chapter 2
Synthesis and Immobilization of a Potential SAM Affinity Ligand

Introduction

One of the most fundamental tasks encountered in biochemistry is protein purification. Proteins have many important roles in biochemical systems, such as providing structural support (e.g., collagen) and participating in muscular contraction (e.g., actin and myosin). Other proteins, called enzymes, function as biochemical catalysts by accelerating the rates at which otherwise slowly progressing reactions occur. In order to study any of these proteins, particularly the extremely important enzymes, the protein of interest must be separated from the hundreds or thousands of other proteins which, along with the nucleic acids, sugars and carbohydrates, lipids, salts, and other miscellaneous compounds, are part of the gemisch that is a living organism. In its pure form, the protein of interest can be studied reliably so that its structure can be determined and its mechanism of action elucidated.

The schemes followed in the purification of proteins are generally empirical in nature, devised largely through a trial and error approach. Usually, in the purification of a previously uncharacterized protein, the process begins with a consideration of one or more of the classical techniques[123] which separate proteins on the basis of their inherently different physical characteristics. For instance, some proteins are large and other proteins are small. Gel filtration techniques have been developed to exploit this basic physical difference among proteins and can easily separate large proteins from smaller proteins. The cross-linked dextran, agarose, and polyacrylamide beads used in gel filtration possess pores of varying sizes. Some proteins are too large to enter these pores, whereas others are small enough to enter the pores

and will remain there temporarily. Therefore, the passage of smaller proteins through such material is retarded and results in a crude separation. When a set of different proteins of known size are added to a sample of the protein of interest, gel filtration will permit an estimation of the size of the protein to be made.

Other separation techniques have been developed to separate proteins on the basis of their net ionic charge, which can vary from protein to protein and is in turn a function of the pH of the solution in which the protein is dissolved. In electrophoresis, proteins either in free solution or in a gel are separated on the basis of their varying mobilities in an applied electric field. The mobility of a protein is determined by its ionic charge, size, and the viscosity of the medium through which it moves. Positively charged proteins move toward the cathode and negatively charged proteins move toward the anode. In isoelectric focusing, a technique related to electrophoresis, proteins move through a pH gradient under the influence of an applied electric field until they reach their isoelectric point. The isoelectric point, or pI, is the pH at which the protein has a net charge of zero and thus can no longer move in such a gradient. Electrophoresis and isoelectric focusing are often considered to be analytical techniques but have been used preparatively to purify some proteins.

A more commonly used technique for the preparative purification of proteins is ion exchange chromatography. In this technique, proteins electrostatically bound to either an anion or cation exchanger can be eluted by gradually increasing the ionic strength of the buffer solution passing through the column. The same general elution system can be used to separate proteins adsorbed to calcium phosphate gels. Proteins are more or less tightly bound to these adsorbents through electrostatic interactions. When the ionic strength of the eluting buffer is gradually increased, these electrostatic interactions are

gradually weakened at different rates for different proteins. Eventually, such interactions become so weak that the protein spends most of its time in the solution phase and is ultimately eluted from the column.

Other techniques for purifying proteins include the "salting out" of either the protein of interest or other undesired proteins as the salt concentration of its aqueous solution is progressively increased. For any protein to be soluble in an aqueous solution (and some, such as the hydrophobic proteins present in membranes, are not water soluble), the amino acid residues interacting with the water must be predominantly hydrophilic in nature. In this way, the ionized or polar groups of such amino acid residues can be solvated by electrostatic interactions with the solvent water and thus pull the entire protein into solution. Hydrophobic amino acid residues at the surface cause an entropically unfavorable ordering of the water molecules facing them. When these "ordered" water molecules are removed to solvate the ions of an added salt, these hydrophobic amino acid residues are free to participate in hydrophobic interactions with similar amino acid residues in other proteins. The hydrophobic interactions gradually increase as the salt concentration increases and eventually overwhelm the solvating electrostatic interactions with the solvent water. At this point, individual protein molecules begin to aggregate in larger and larger numbers and finally precipitate from solution. The magnitude of the salt concentration that will cause a particular protein to precipitate depends on the proportion of hydrophilic to hydrophobic amino acid residues at the protein-water interface. Proteins with a largely hydrophobic surface will precipitate at low salt concentrations. Other proteins with a predominantly hydrophilic surface may resist precipitation until only the highest salt concentrations are attained, and even then they may still be soluble. The salt most commonly used in this protein purification technique is ammonium sulfate.

The addition of water-miscible solvents such as either ethanol or acetone to an aqueous solution of a protein can lead to the selective precipitation of either the protein of interest or any contaminating proteins. This technique is routinely used in the purification of compounds in organic chemistry laboratories. The ability of water to attenuate electrostatic interactions between different molecules in solution and thus prevent them from aggregating and precipitating is limited by its dielectric constant. When this attenuation is diminished by the gradual addition of an organic solvent, causing a decrease in the dielectric constant, stable electrostatic interactions among oppositely charged regions of different protein molecules can arise. Eventually, with addition of enough organic solvent, the precipitation of proteins will commence. Large proteins will tend to be the first to precipitate because they can participate in the largest number of possible electrostatic interactions. Conversely, smaller proteins will generally precipitate at higher organic solvent concentrations.

Usually, in enzyme purification, several of these techniques must be used in succession to obtain a homogeneous sample, or at least a sample with small amounts of one or two contaminating proteins. The typical purification scheme for a given enzyme has anywhere from four to seven empirically derived steps, all commonly done at 4°C. to minimize denaturation of the enzyme and the consequent loss of activity. The specific activity of the enzyme, defined as mg of protein per unit volume (mL), is measured after each step and should increase steadily with each successive step in the purification.

The isolation of two isozymes of catechol O-methyltransferase (COMT) from rat liver has been reported.[124] The scheme used in the isolation of one of these isozymes, COMT I, illustrates quite well how the above techniques can be used to purify an enzyme. Homogenization of fresh rat liver in Tris buffer[124] followed by removal of particulate matter by centrifugation at 30000 x g

provided a crude extract of the isozyme in the supernatant. Soluble ribosomes, involved in protein synthesis in the hepatocytes, were precipitated upon addition of neutralized streptomycin sulfate and removed by centrifugation at 30000 x g, again leaving the isozyme in the supernatant. Ammonium sulfate was added to the supernatant to 35% saturation and the precipitated proteins centrifuged and discarded. Increasing the ammonium sulfate saturation in the supernatant to 50% led to the precipitation of additional protein bearing the desired enzyme activity which was centrifuged to a pellet, redissolved in a buffer, and dialyzed against the same buffer to remove any residual ammonium sulfate.[124] Any nucleic acids present in the enzyme solution were precipitated by addition of neutralized protamine sulfate and removed by centrifugation at 30000 x g. The supernatant solution bearing the enzyme activity was subjected to gel filtration by Sephadex G-100 chromatography, which separated COMT I from higher molecular weight proteins as well as from the other isozyme COMT II. Those fractions containing COMT I were applied to a column of DEAE-cellulose for ion exchange purification using a gradient of potassium chloride and all fractions bearing COMT I activity were pooled.[124] In the final purification step, ammonium sulfate was added to the COMT I solution to 90% saturation and then the precipitated protein bearing the desired activity was centrifuged, redissolved in a buffer, dialyzed against this buffer, and stored at approximately -20°C.. The final protein solution, purified some 450 times, but with a yield of less than 10%, contained less than 2 mg of total protein starting from 75 g of fresh rat liver. Polyacrylamide gel electrophoresis of an analytical sample indicated the presence of three minor contaminants.[124]

Clearly, as illustrated above, the purification of an enzyme can be quite tedious. The reason for the large number of purification steps is that hundreds or even thousands of proteins may be present in the original crude homogenate

of liver, bacteria, etc.. The probability that at least a few of these proteins may have almost the same physical properties as the enzyme of interest is relatively high. Thus, it is not surprising that such proteins might be co-purified. In addition, degradation of the desired enzyme by air oxidation, extraneous protease activity, or any of a host of other factors during the purification scheme would lead to either a diminution or even total loss of active enzyme. What is needed to reduce the number of purification steps and minimize exposure to degradation agents is a more efficient method of separation, which in the ideal case, could selectively separate the protein of interest from all other extraneous proteins in a single step. One technique, still being refined ever since its formal introduction in 1968 by Cuatrecasus, Wilchek, and Anfinsen,[125] which holds promise for such purifications is affinity chromatography.

Affinity chromatography has been used to purify enzymes,[126-138] hormone receptors,[139-141] antibodies,[142-150] cell membrane proteins,[151-157] and even viruses and intact cells.[158-165] Each of these purifications is made possible by exploiting the highly specific and reversible binding interactions exhibited between the subject of interest and certain other small or large molecules. For instance, the extremely specific and tight binding interaction between an antibody and the antigen which elicited its formation is one of the cornerstones of the immune systems of higher organisms. Similarly, some drugs exert their pharmacological effects by serving as tight binding inhibitors of certain enzymes.

This simple and easily understood enzyme-inhibitor interaction can be used to illustrate the basic principles of affinity chromatography (Figure 25). In the biochemical milieu of a living cell, an inhibitor (I) will, in the ideal case, bind to only one particular enzyme (E_1) and not to any other enzymes (E_2) or miscellaneous proteins (P). The inhibitor can be covalently bound to an

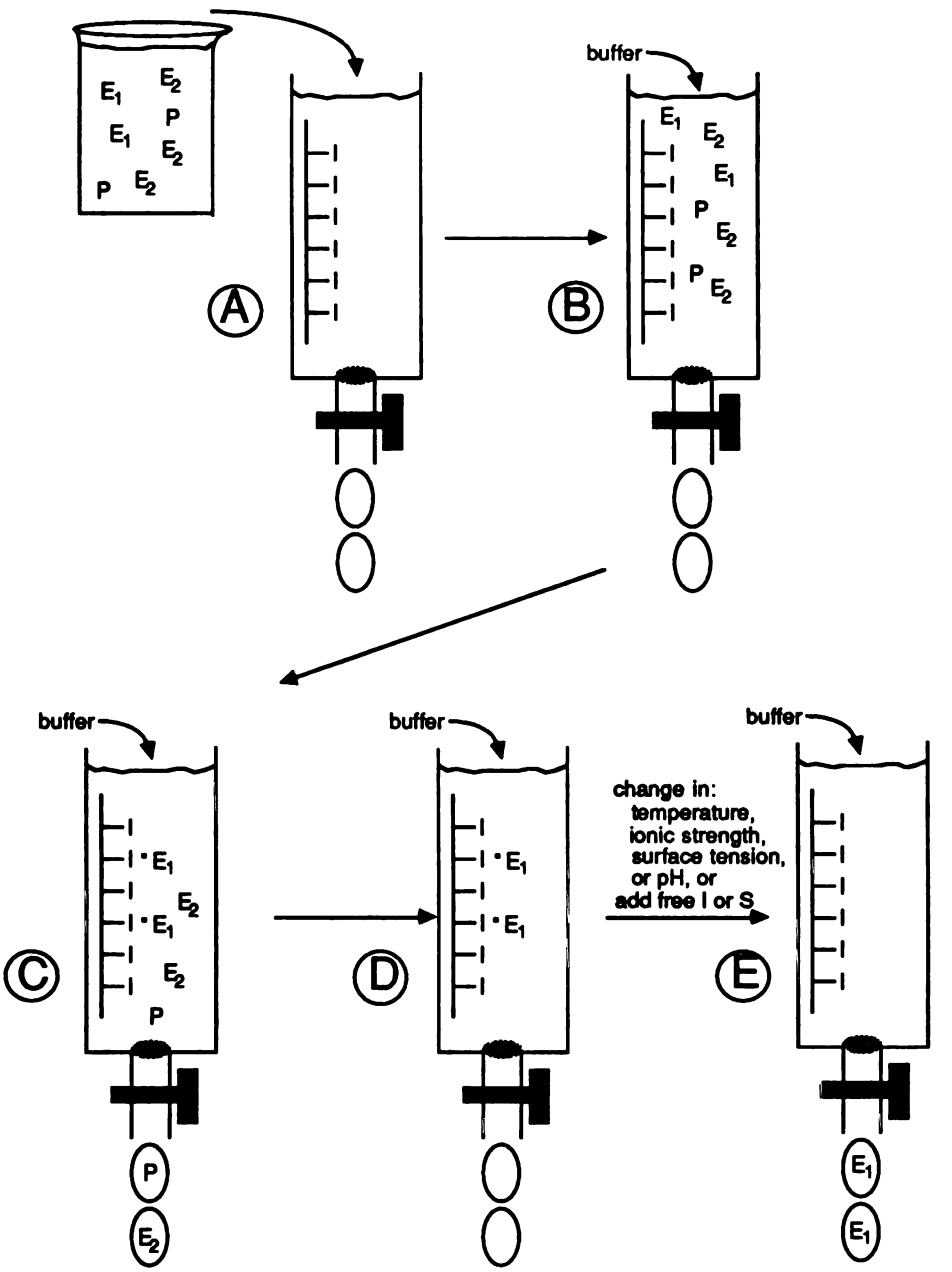


Figure 25. The basic principles of affinity chromatography demonstrated in enzyme purification. Temporary immobilization of an enzyme (E_1) on an affinity adsorbent possessing one of its bound inhibitors (I) leads to the elution of another enzyme (E_2) and a miscellaneous protein (P). Changes in the eluting solvent or addition of free substrate or inhibitor re-solubilizes the enzyme and leads to its elution in pure form.

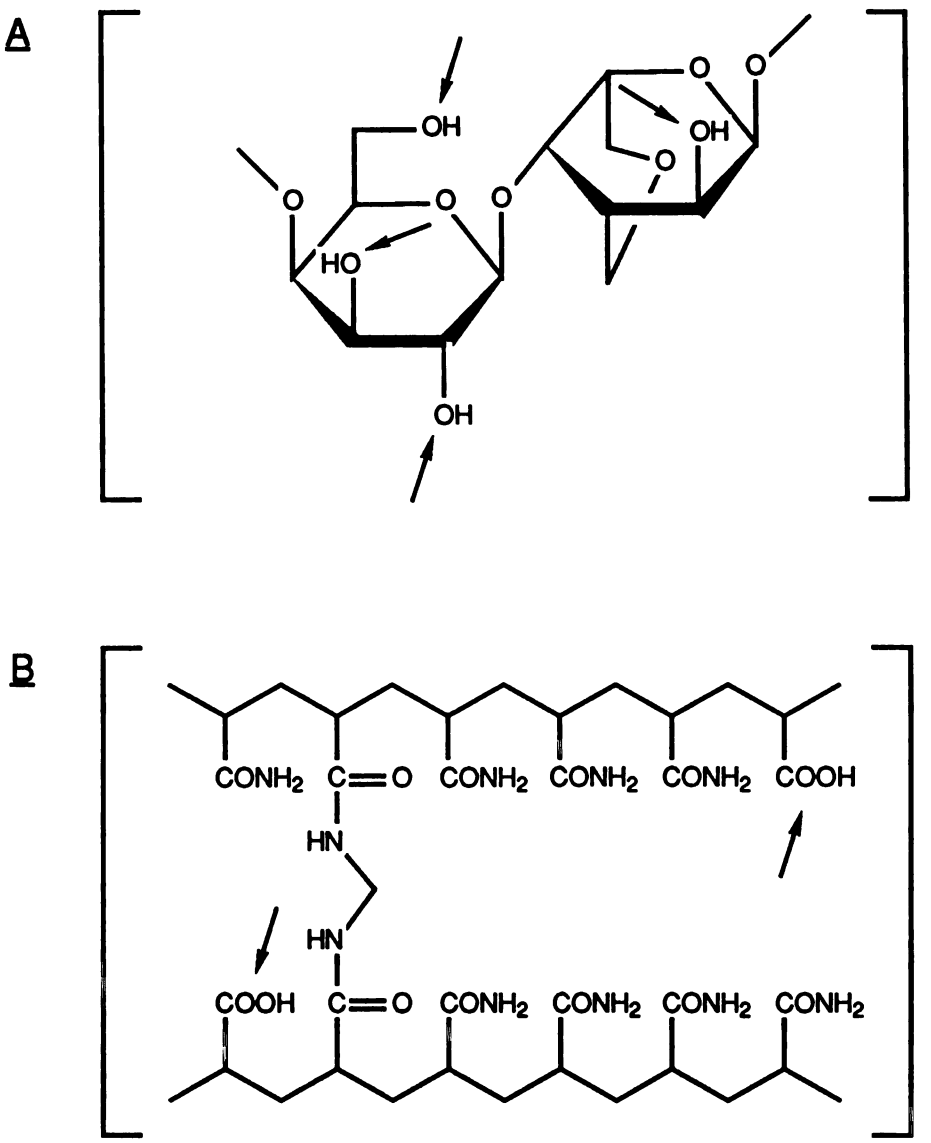


Figure 26. The basic D-galactose/3,6-anhydro-L-galactose repeating unit comprising agarose and its cross-linked, more mechanically stable Sepharose derivative (above, A), and the structure of a typical polyacrylamide gel including the N,N'-methylenebisacrylamide cross-link (below, B). The arrows indicate which groups can be derivatized to facilitate the immobilization of an affinity ligand such as an enzyme inhibitor.

insoluble polymer which can be packed into a column. This polymer is usually agarose or the cross-linked bead form of agarose known commercially as Sepharose, or a cross-linked polyacrylamide gel (Figure 26).[166] Passage of a buffered cellular extract containing the enzyme of interest (E_1) through the column will result in the immobilization of that enzyme on the polymer (Figure 25, parts A and B). This immobilization is brought about by a tight binding interaction between the enzyme of interest and its inhibitor attached to the insoluble polymer. Other enzymes (E_2) and miscellaneous proteins (P) will remain free in solution and can be eluted from the column by continued washing with an appropriate buffer and discarded (Figure 25, parts C and D). The enzyme (E_1) immobilized on the insoluble polymer can be re-solubilized by altering the conditions of the surrounding solution so as to substantially weaken the binding interaction between the enzyme and its immobilized inhibitor. Changes in temperature, ionic strength, surface tension, or pH, or the addition of the freely soluble form of the inhibitor or normal substrate have all been employed in one case or another to weaken this binding interaction and lead to the elution of the purified enzyme from the affinity column (Figure 25, part E).

The major challenge in developing an affinity adsorbent to implement a specific purification is the immobilization of one of the participants in the binding interaction. When enzymes are purified by affinity chromatography as already described, one of its more tightly binding inhibitors is immobilized on the insoluble polymer.[126,127] (Substrates can also be immobilized on the affinity adsorbent if the binding of the enzyme will not elicit a catalytic event altering the structure of that substrate. Only in such a case can the affinity adsorbent be easily reused.) The purification of solubilized hormone receptors by affinity chromatography is made possible by the immobilization of the hormone which binds to the receptor.[139-141] In a similar manner, the immobilization on

insoluble polymers of antibodies specific for viral proteins provides a system useful in the purification of viruses.[158,164] Despite the successes which have been achieved with affinity chromatography, this protein purification technique remains highly empirical. The success or failure of a particular purification often depends on how the irreversible immobilization of one of the participants in the binding interaction on the insoluble polymer is made. Trial and error with affinity chromatography techniques has established that direct attachment of the enzyme inhibitor, as a typical example, to the insoluble polymer often leads to either weak or no binding by the enzyme being purified. Steric interactions between the insoluble polymeric support and the enzyme, in its attempt to "engulf" the immobilized inhibitor, are often too great to permit strong reversible binding to the affinity adsorbent and will consequently lead to little or no purification of the enzyme of interest (Figure 27).

The solution to this problem is the indirect attachment of the inhibitor to the insoluble polymer via a spacer bridge. In this way, the immobilized enzyme inhibitor, tethered to the polymeric support, can reach further out into solution. Provided that the tether, or spacer, does not itself compromise any of the binding interactions with the soluble enzyme, steric interactions between the enzyme and the polymer should no longer be a limiting factor in the use of the affinity adsorbent (Figure 27). Commonly utilized spacers are bifunctional linear molecules such as 1,6-diaminohexane.

The length and hydrophobicity of the spacer are important considerations in the development of an affinity adsorbent. An optimum must be found through trial and error with respect to both variables due to the possibility that the spacer may fold back on itself through hydrophobic van der Waals interactions between its two ends. The net result would be a shorter spacer bridge, a weaker binding interaction between the enzyme and its immobilized inhibitor,

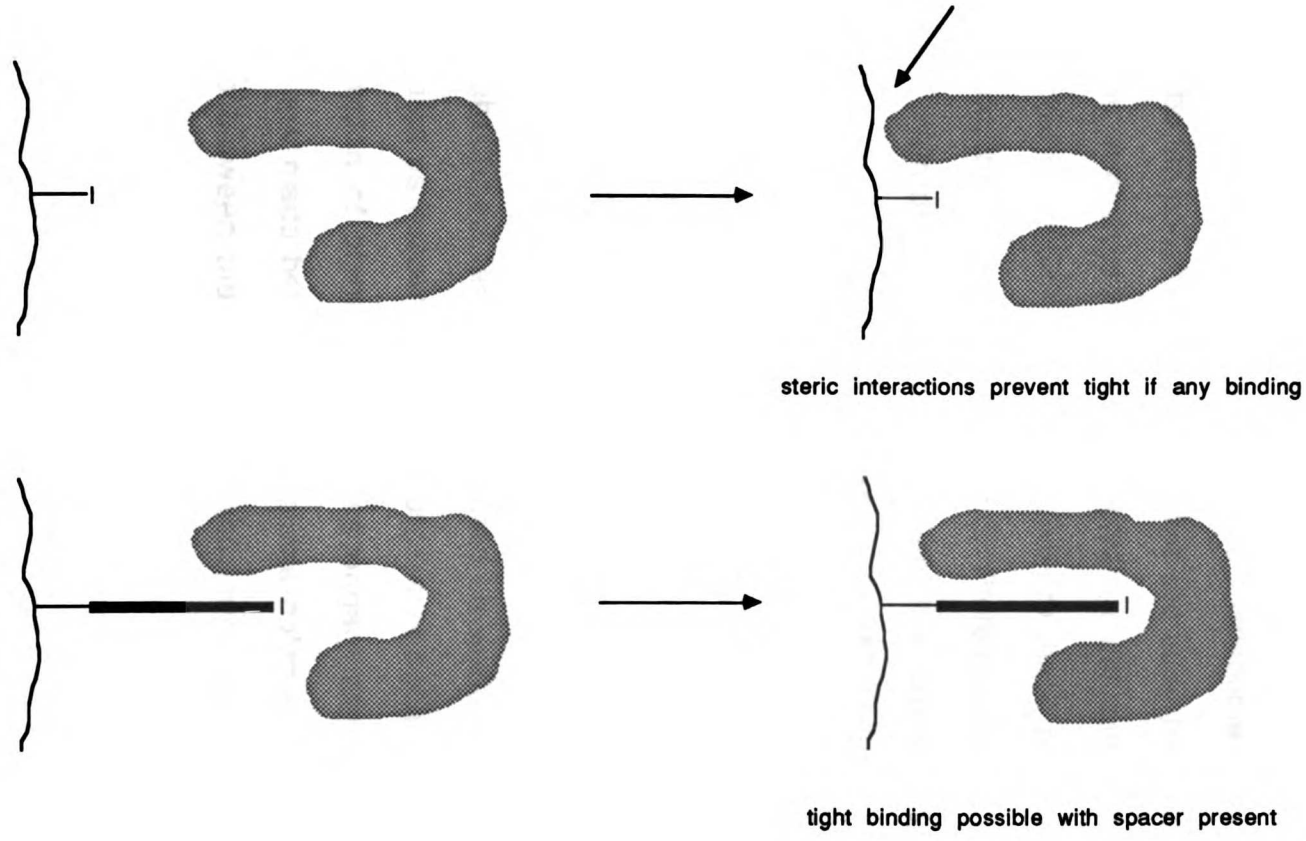


Figure 27. The steric interactions between the enzyme and the insoluble polymer in the affinity adsorbent will often prevent tight if any binding when the inhibitor is immobilized directly on the polymer (top). Tethering the inhibitor to the polymer by means of a spacer will permit tight binding by the enzyme (bottom).

and, ultimately, a much less efficient purification. A spacer which would be particularly susceptible to such a problem is 1,12-diaminododecane. Sometimes, the large hydrophobic region in such a spacer can enter into van der Waals interactions with hydrophobic regions of the proteins passing through the adsorbent. Some proteins are more susceptible to this type of interaction and can be temporarily immobilized while the other proteins are eluted from the adsorbent. A change in the eluting solvent, conducive to a weakening of these hydrophobic interactions, would re-solubilize these proteins and result in their elution from the column in purified form. Proteins are thus purified on the basis of their hydrophobic interactions with the adsorbent, and hydrophobic chromatography of this nature is well known.[167] However, the unique "affinity" interaction is lost. When the hydrophobic nature of a spacer as large as 1,12-diaminododecane, or even smaller spacers such a 1,6-diaminohexane, becomes a serious problem in affinity chromatography, a hydrophilic spacer such as 1,3-diamino-2-propanol should instead be incorporated into the affinity adsorbent.

Substituted and unsubstituted α,ω-diaminoalkanes are often incorporated as spacers in affinity adsorbents because they can participate in the formation of stable amide or secondary alkylamine linkages. These linkages are made between the insoluble polymeric support and the spacer, and also between the spacer and the immobilized enzyme inhibitor. The formation of amide bonds with such spacers requires the participation of carboxylic acids on both the insoluble polymer and the enzyme inhibitor being immobilized. Prior activation of the carboxylic acid with either water-soluble carbodiimides or N-hydroxysuccinimide ester intermediates followed by reaction with the primary aliphatic amine is the method generally followed in the introduction of the amide linkage. Replacing the α,ω-diaminoalkane spacer

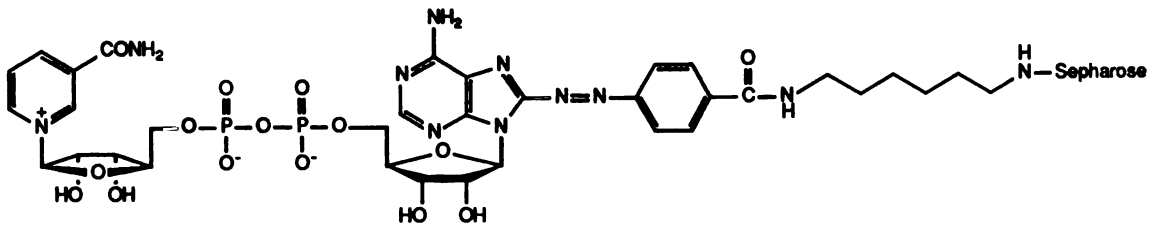
with an α,ω-alkyldicarboxylic acid could also provide a stable affinity adsorbent, provided that the coupling is again through amide bonds to amino groups on both the insoluble polymer and the enzyme inhibitor being immobilized. Ester linkages are not usually employed in the construction of affinity adsorbents due to their lower stability over time in aqueous environments. Affinity adsorbents possessing such ester linkages would gradually exhibit a decreasing capacity and progressively poorer purifications with repeated use. Secondary alkylamine linkages coupling the support to the spacer, or the spacer to the ligand, are formed by the reaction of the primary aliphatic amino group of the spacer with an epoxide present either in the support or in the ligand. These linkages are even more stable than amide bonds and have the added feature that they would be resistant to the action of any amidase also present in the enzyme solution.

The purification of many enzymes has been greatly improved and simplified since the advent of affinity chromatography. Applications of this technique generally provide the best results when a majority of the miscellaneous proteins are removed by one or two preliminary steps. The precipitation of large quantities of proteins upon the addition of ammonium sulfate is one of the classical purification techniques used in this respect. Among those proteins removed in these preliminary steps are those which may show a similar affinity for either the immobilized enzyme substrate or inhibitor. Thus, the potential for overloading an affinity adsorbent of limited capacity is reduced. At the same time, the probability that the enzyme of interest will indeed be immobilized is increased. Continued washing of the adsorbent with an appropriate buffer solution will remove any nonbinding proteins. Almost all proteins, by virtue of their tryptophan and tyrosine residues, absorb at 280 nm and, thus, when the buffer washing the affinity adsorbent no longer absorbs at

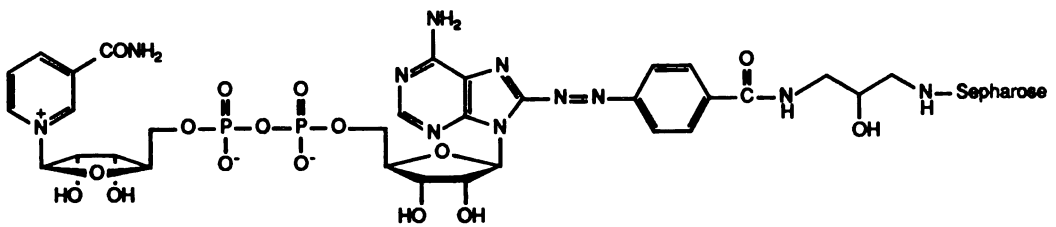
this wavelength, it can be safely assumed that only the enzyme of interest remains immobilized. Using one of the procedures previously described (Figure 25, parts D and E), this enzyme can be re-solubilized and obtained in a highly purified form. The appearance of the enzyme of interest can be determined by again monitoring the 280 nm absorbance of the effluent solution, as well as by assaying for its activity.

For instance, the immobilization of nicotinamide adenine dinucleotide cofactors on agarose and Sepharose has been instrumental in the purification of NAD^+ and $NADP^+$-dependent dehydrogenases.[168-175] These cofactors bind tightly to dehydrogenases and play important roles in biological redox chemistry. The immobilization of NAD^+ and $NADP^+$ to the insoluble polymeric matrix is most often accomplished through an azo linkage to the adenine C-8 position employing either the hydrophobic 1,6-diaminohexane or the hydrophilic 1,3-diamino-2-propanol spacer (Figure 28). Sturgeon muscle glyceraldehyde 3-phosphate dehydrogenase has been purified[175] using NAD^+ bound to Sepharose through an 8-azo linkage to both spacers. After miscellaneous dehydrogenases and other proteins were removed by preliminary ammonium sulfate precipitation and ion exchange chromatography steps, the enzyme solution was loaded onto 8-azo-NAD^+-Sepharose. Exhaustive washing with Tris buffer removed all nonbinding proteins. Elution of the temporarily immobilized enzyme was accomplished by washing with a linear gradient of buffered NAD^+. The affinity chromatography step improved the purification of the glyceraldehyde 3-phosphate dehydrogenase approximately 15-fold.[175]

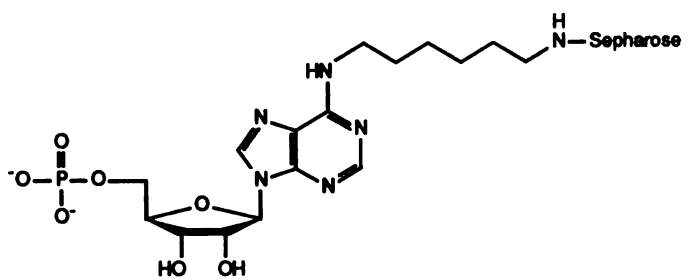
The adenosine 5'-monophosphate moiety in both NAD^+ and $NADP^+$ plays a major role in the binding of these cofactors to dehydrogenases. Not surprisingly, these enzymes have been purified using only 5'-AMP immobilized



p-(8-diazo-NAD$^+$)-N-(6-aminohexyl)benzamido-Sepharose



p-(8-diazo-NAD$^+$)-N-(3-amino-2-hydroxypropyl)benzamido-Sepharose

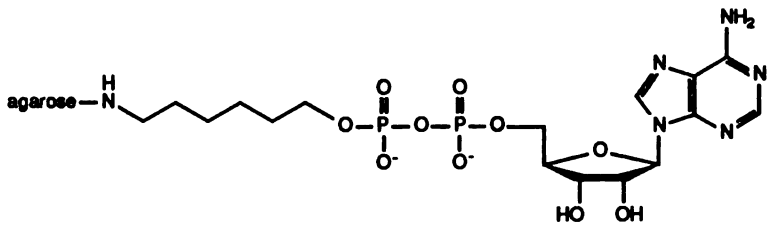


N^6-(6-aminohexyl)AMP-Sepharose

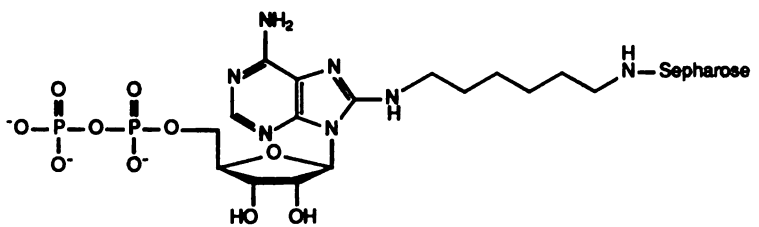
Figure 28. Three affinity adsorbents which have been used to purify dehydrogenases (see references 175 and 176).

on agarose and Sepharose. Only after the 1,6-diaminohexane spacer has been incorporated into the 5'-AMP molecule to give, for example, N^6-(6-aminohexyl)-5'-AMP, is the immobilization reaction performed. N^6-(6-Aminohexyl)-5'-AMP-agarose (Figure 28) prepared in this manner has been used to purify many dehydrogenases. For example, the various isozymes of horse liver alcohol dehydrogenase have been separated and purified using this affinity adsorbent.[176] The buffered extract containing the horse liver alcohol dehydrogenase isozymes was loaded onto the N^6-(6-aminohexyl)-5'-AMP-agarose affinity adsorbent only after the vast majority of other dehydrogenases and other 5'-AMP-binding proteins were first removed by ion exchange chromatography and an ammonium sulfate precipitation. Following exhaustive washing with buffer, the isozymes were eluted one after another by washing with a gradient of NAD^+ in 1.5 mM cholic acid.[176]

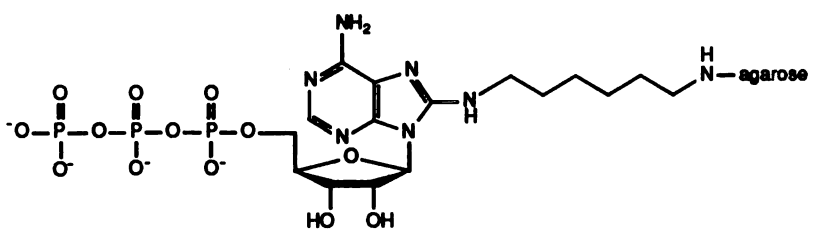
Interestingly, the purification of kinases using N^6-(6-aminohexyl)-5'-AMP-agarose has not met with much success. Presumably, this observation is a reflection of the importance of the unsubstituted adenine amino group in the binding interactions with kinases in general, which is something that does not seem to prevent tight binding to dehydrogenases. Kinase purification by affinity techniques has been aided by the immobilization of adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP) on agarose and Sepharose.[177-180] These compounds are respectively the by-product and substrate of most kinase reactions. The linkage of the 1,6-diaminohexane spacer to these nucleotides is made either to the phosphate groups or to the adenine C-8 position to give, for example, P^1-(6-aminohex-1-yl)-P^2-5'-(adenosyl)pyrophosphate-agarose, 8-(6-aminohexyl)-ADP-Sepharose, and 8-(6-aminohexyl)-ATP-agarose (Figure 29). For example, hexokinase and glucokinase are two enzymes which, in the first step of glycolysis, use ATP to phosphorylate α-D-glucose to give α-D-glucose-



P^1-(6-aminohexyl)-P^2-5'-(adenosyl)pyrophosphate-agarose



8-(6-aminohexyl)-ADP-Sepharose



8-(6-aminohexyl)-ATP-agarose

Figure 29. Three affinity adsorbents which have been used to purify kinases (see references 178 and 181).

6-phosphate. The observation that, unlike rat liver hexokinase, the corresponding glucokinase can be temporarily immobilized on 8-(6-aminohexyl)-ADP-Sepharose can in turn be utilized to separate these two otherwise similar enzyme activities. A crude rat liver extract containing both enzymes was loaded onto 8-(6-aminohexyl)-ADP-Sepharose. After exhaustive washing of hexokinase and other proteins from the affinity adsorbent, the remaining glucokinase was eluted with 10 mM ATP in a highly purified form.[178]

In one particularly interesting application of affinity chromatography, both agarose and Sepharose derivatized with various glycosides have been used to purify certain proteins which recognize and bind very tightly to the sugar groups present in glycoproteins. Each of these proteins, or lectins, is characterized by the specific sugar it recognizes. The most thoroughly studied of these lectins is concanavalin A which binds tightly to the α-D-mannopyranose and α-D-glucopyranose sugars present in some glycoproteins. Because glycoproteins are integral constituents of cell membranes and cell surfaces, lectins possessing two or more binding sites can cause cells to "clump together" or agglutinate in numbers large enough to be visible to the naked eye. The agglutination of certain red blood cells and not others upon the addition of a specific lectin provides the basis for the classification of blood into certain blood types.

The synthesis and immobilization of 6-aminoglycosides have been extensively studied[174,182-188] in order to simplify the purification of lectins. Prior to the introduction of affinity chromatography to lectin biochemistry, the separation of glycoproteins from other proteins was very difficult. The incorporation of 6-aminohexyl 2-acetamido-β-D-glucopyranoside (Figure 30) into a polyacrylamide gel has provided an affinity adsorbent which is useful in the isolation of wheat germ lectin.[174] The purification of this lectin first involves

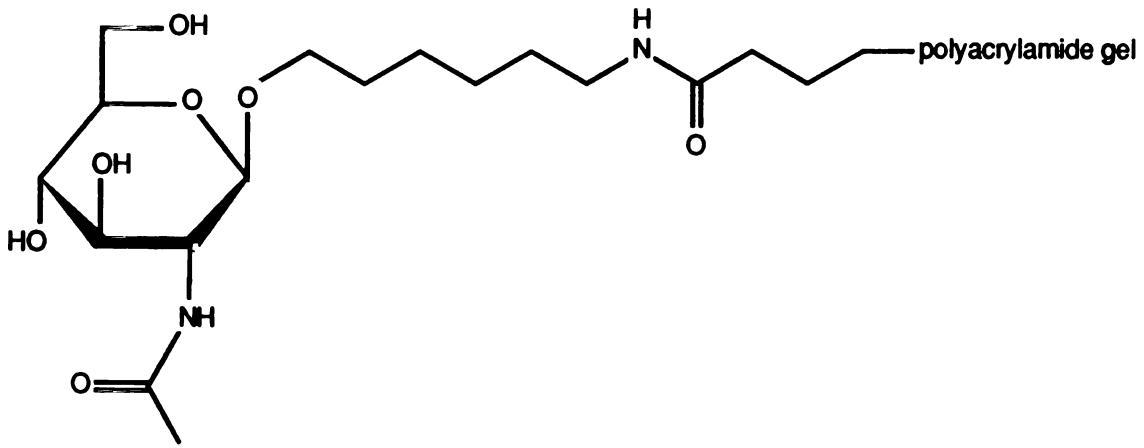


Figure 30. The structure of the 6-aminohexyl 2-acetamido-2-deoxy-β-D-glucopyranoside-polyacrylamide affinity adsorbent which has been used to carry out the purification of wheat germ lectin (see reference 174).

the removal of most extraneous proteins in an ammonium sulfate precipitation step. Then, after immobilization on the above affinity adsorbent and removal of extraneous proteins by exhaustive washing, the lectin was re-solubilized with 20 mM HCl. After neutralization of the recovered protein solution, the 130-fold purified wheat germ lectin crystallized.[174]

Purified wheat germ and other lectins have in turn been immobilized to take advantage of their highly specific interactions with cell membrane glycoproteins. The use of such affinity adsorbents has permitted the rapid and efficient separation of subpopulations of certain types of cells, a feat which would be extremely difficult to duplicate utilizing other techniques. The separation of lymphocytes into different B and T cell subpopulations, each of which plays a key role in the immune systems of higher organisms, has been accomplished using lectins isolated from wheat germ[189] and the snail, *Helix pomatia*,[190,191] immobilized on various affinity adsorbents. These lectins bind specifically to the N-acetyl-D-glucosamine and N-acetyl-D-galactosamine residues in the cell membrane glycoproteins, respectively.

Affinity chromatography techniques similar to those outlined above have been directed toward the purification of a variety of methyltransferases. S-Adenosyl-L-methionine (SAM), the methyl donor in methyltransferase-catalyzed reactions, cannot be immobilized due to its instability in aqueous solution over long periods of time (Figure 19 and 20). Instead, S-adenosyl-L-homocysteine (L-SAH) and various analogues of the normal methyl acceptor substrates or products have been immobilized on agarose and Sepharose for this purpose.

The isolation of one methyltransferase in the presence of many others is facilitated by the immobilization of one of that enzyme's more potent methyl acceptor substrate or product inhibitors. This approach has been quite useful in the purification of rat liver catechol O-methyltransferase (COMT).[192] The affinity

adsorbent developed for this purpose consists of 3,4-dimethoxy-5-hydroxyphenethylamine, a potent dead-end product inhibitor of COMT, indirectly linked to agarose through a spacer consisting of two diaminodipropylamine and two succinate groups (Figure 31). The most efficient purifications of COMT using this affinity adsorbent were obtained when other classical protein purification methods (e.g., ammonium sulfate precipitation, gel filtration, and adsorption to calcium phosphate) were also used to help remove other miscellaneous proteins from the initial rat liver homogenate. COMT was purified over 900-fold using the total purification scheme, which is significantly better than the 450-fold purification of one of the isozymes of COMT already outlined[124] which did not use affinity chromatography. The inclusion of the affinity chromatography step near the end of the isolation procedure allowed the purification of the COMT by another 10-fold to an overall 500-fold purification. Half of the remaining six or seven contaminants could be removed by a final gel filtration step.[192] Interestingly, passage of the crude rat liver homogenate in 5 mM phosphate buffer through the 3,4-dimethoxy-5-hydroxyphenethylamine-agarose affinity adsorbent followed by exhaustive washing and elution with 60 mM (i.e., higher ionic strength) phosphate buffer provided COMT purified some 40-fold.[192] COMT purified in this manner is still contaminated with many other proteins, but even so, the fact that such a purification can be achieved in one step directly from the crude rat liver homogenate clearly demonstrates the potential of affinity chromatography.

The observation that COMT can be temporarily immobilized on 3,4-dimethoxy-5-hydroxyphenethylamine-agarose in the absence of either its SAM substrate or its L-SAH product inhibitor offers clues to its kinetic mechanism. The ability of this enzyme to bind its substrates in any order is the reason for this observation and helps to confirm the bi bi random mechanism already

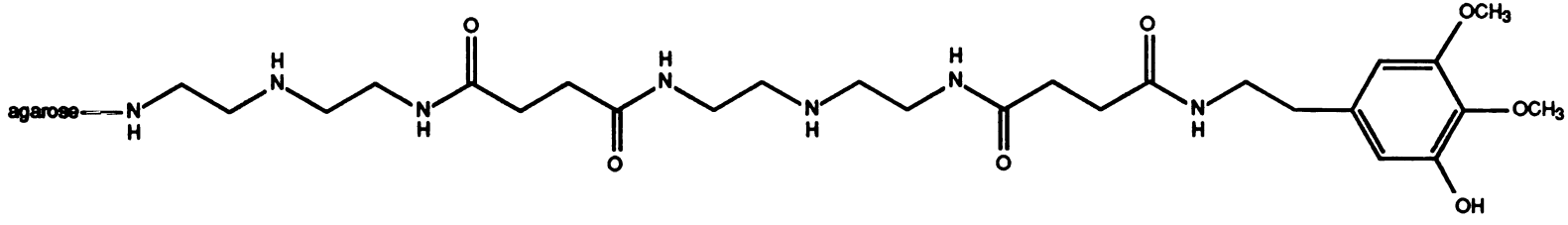


Figure 31. 3,4-Dimethoxy-5-hydroxyphenethylamine-agarose (shown with its accompanying diamino-dipropylamine-succinate spacer), which has been used to improve the purification of rat liver catechol O-methyltransferase (see reference 192).

proposed[193] for rat liver COMT. Not all enzymes have such a simple kinetic mechanism and a careful consideration of other possible mechanisms is sometimes required to bring about a purification on an affinity adsorbent.

For instance, the purification of the furanocoumarin 5- and 8-O-methyltransferases involved in the biosynthesis of furanocoumarins (Figure 32) in plants has been investigated.[194-196] An affinity adsorbent specifically developed to purify both enzymes in the presence of other methyltransferases has the monosuccinamide of 5-aminoxanthotoxin, a substrate analogue of both enzymes, linked to Sepharose through a 1,6-diaminohexane spacer (Figure 32). After a preliminary gel filtration purification of the two enzymes originally obtained from an extract of the plant, *Ruta graveolens* L., attempts were made to further purify them using 5-aminoxanthotoxin-Sepharose. Only when SAM or L-SAH was included in the Tris buffer containing both enzymes could they be immobilized on the affinity adsorbent. The removal of SAM or L-SAH from the Tris buffer led to the simultaneous elution of both enzymes in purified form.[196] These observations are probably indicative of a bi bi ordered mechanism for both furanocoumarin O-methyltransferases. In the normal catalytic cycle for these enzymes, SAM must bind first followed by the furanocoumarin. After the transfer of the methyl group, the O-methylated furanocoumarin leaves the enzyme first followed by the L-SAH. Thus, it is not surprising that these enzymes would not bind to the immobilized substrate analogue, 5-aminoxanthotoxin, in the absence of SAM or L-SAH. In this way, affinity chromatography can be helpful in providing information about the kinetic mechanism of an enzyme as well as in assisting in the purification of that enzyme.

L-SAH immobilized on either agarose or Sepharose would provide an affinity adsorbent which could be used to help purify a greater variety of

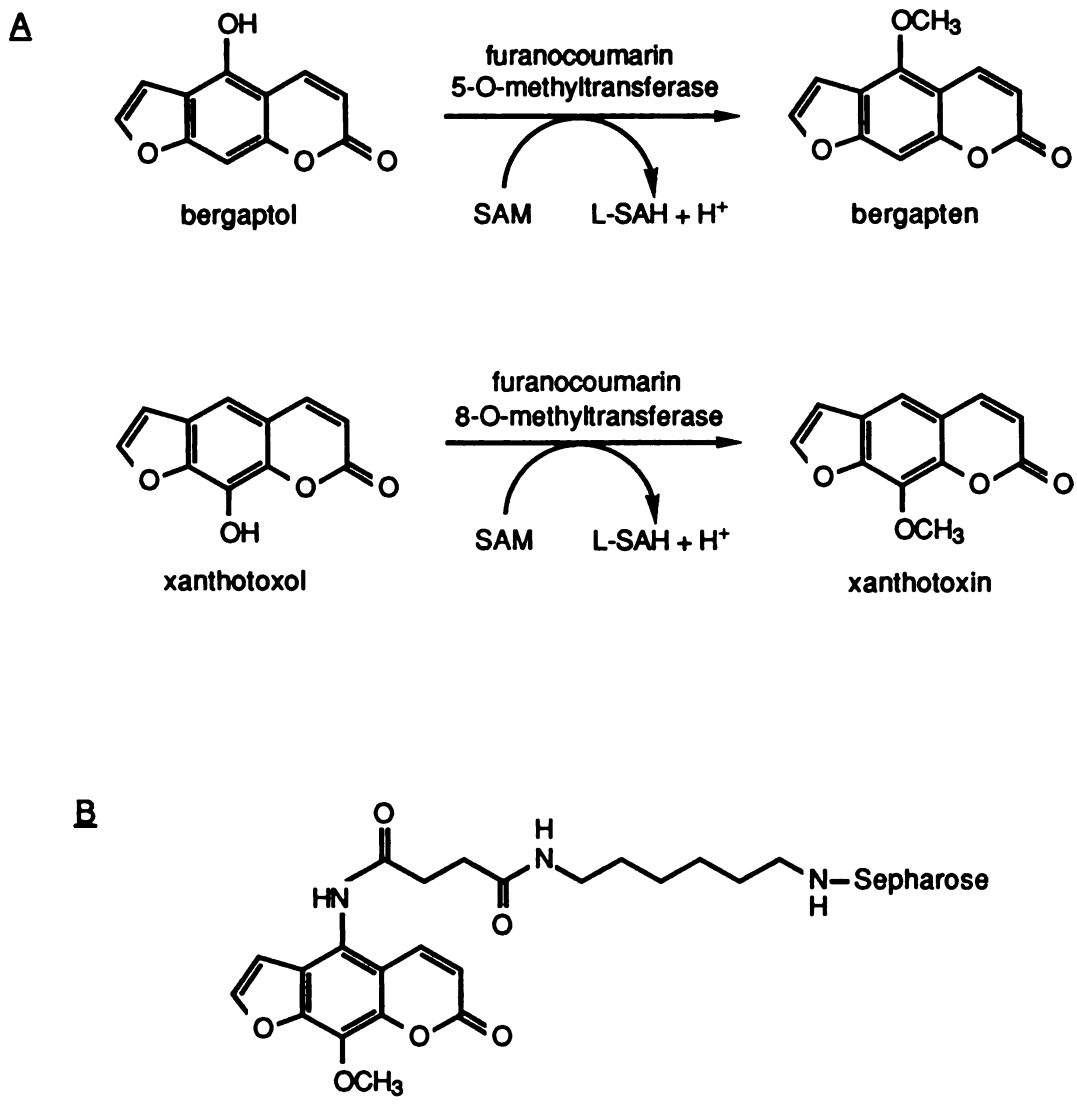


Figure 32. The methylations of the furanocoumarins, bergaptol and xanthotoxol, catalyzed by the furanocoumarin 5- and 8-O-methyltransferases, respectively (above, A), and the 5-aminoxanthotoxin-Sepharose affinity adsorbent (below, B) developed for their purification from *Ruta graveolens* L. (see references 194 and 196).

methyltransferases than the two adsorbents described above. This increased versatility arises from the fact that L-SAH is a potent product inhibitor of all methyltransferases. Whereas L-SAH binds tightly to all methyltransferases, 3,4-dimethoxy-5-hydroxyphenethylamine and 5-aminoxanthotoxin would be expected to bind tightly only to COMT and the furanocoumarin O-methyl-transferases, respectively. Thus, such an affinity adsorbent might be useful in purifying any one of the dozens of known methyltransferases. The immobilization of L-SAH is accomplished by means of an amide linkage between the carboxylate group of the amino acid moiety and the free primary amino group of 1,6-diaminohexane already bound to Sepharose (Figure 33). Generally, because one or more other methyltransferases may be co-purified along with the methyltransferase of interest, the use of this affinity adsorbent must be preceded by a number of classical protein purification techniques designed to remove these contaminating enzymes.

The furanocoumarin 5- and 8-O-methyltransferases described above (Figure 32) have also been purified using this L-SAH-Sepharose affinity adsorbent.[194-196] Before loading onto the affinity adsorbent as was done in the above purifications, the crude extract of these enzymes from *Ruta graveolens* L. was first subjected to gel filtration to assist in the removal of miscellaneous methyltransferases. As might be expected from a careful consideration of the implications of the bi bi ordered mechanism for each enzyme, their immobilization on the affinity adsorbent was not conditional on the presence of SAM or L-SAH in their Tris buffer solution. In fact, the addition of SAM to the Tris buffer used to wash the affinity adsorbent re-solubilized both enzymes and led to their co-elution in purified form. The addition of bergaptol or xanthotoxol to the Tris buffer washing the L-SAH-Sepharose failed to re-solubilize either enzyme.[196] Bergaptol and xanthotoxol (Figure 32) are the respective

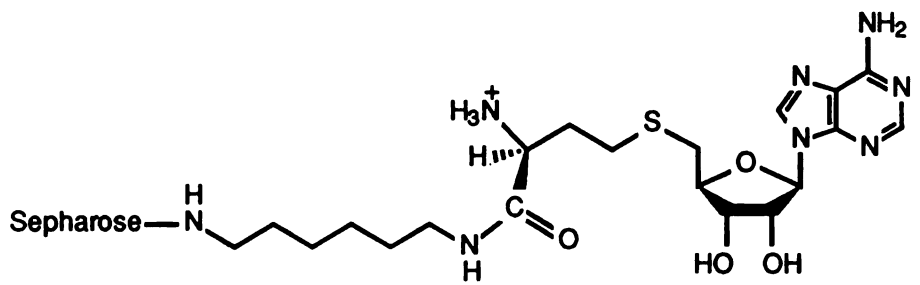


Figure 33. The structure of the L-SAH-Sepharose affinity adsorbent used to purify the furanocoumarin 5- and 8-O-methyltransferases (see references 194-196) and protein methylase II (see reference 197).

substrates of the furanocoumarin 5- and 8-O-methyltransferases. These results should not be surprising because the binding of bergaptol and xanthotoxol to these enzymes is conditional on the prior binding of either SAM or L-SAH. SAM or L-SAH in the Tris buffer can more effectively compete with the immobilized L-SAH for the active sites of these enzymes, and ultimately, lead to their re-solubilization. Bergaptol and xanthotoxol cannot displace the immobilized L-SAH from the active site and thus fail to bring about the elution of either enzyme from the affinity adsorbent.

One other procedure which can be used to re-solubilize the immobilized furanocoumarin O-methyltransferases involves merely washing the affinity adsorbent with dilute acetic acid (pH 3.0). At this lower pH, the binding interactions between the affinity adsorbent and the immobilized enzymes are sufficiently weakened so that the enzymes may once again enter solution. Both enzymes and another methyltransferase from *Ruta graveolens* L. have been purified approximately 50-fold using this technique.[195]

The same L-SAH-Sepharose affinity adsorbent (Figure 33) has been used to purify protein-carboxyl-O-methyltransferase from calf brain.[197] This enzyme is also known as protein methylase II and uses SAM to form aspartate and glutamate methyl esters in a wide variety of polypeptides and proteins, some of which function in mammalian secretory processes[198] and others in bacterial chemotaxis.[28-30] After preliminary purification of the enzyme and the removal of other contaminating methyltransferases from the crude homogenate by mild acid and ammonium sulfate precipitations, the buffered extract was loaded onto the affinity adsorbent. As is standard operating procedure, the miscellaneous nonbinding proteins were removed by exhaustive washing with phosphate buffer. The immobilized enzyme was then re-solubilized and eluted from the affinity adsorbent, purified approximately 375-fold in this step alone, by

washing with 20 μM SAM in the original buffer.[197] Higher and lower molecular weight impurities were removed in a final gel filtration step. The entire procedure, from crude homogenate to final preparation, had purified the enzyme some 3000-fold.[197] The key step in the purification scheme was the affinity chromatography step which was responsible for the removal of the vast majority of miscellaneous proteins from the protein methylase II extract.

The purification of two indoleethylamine N-methyltransferases (Figure 34) from *Phalaris tuberosa*, an Australian grass, by affinity chromatography has been reported.[199] The affinity adsorbent developed for this purpose utilized a diaminodipropylamine spacer to immobilize L-SAH through an amide linkage to its carboxylate group (Figure 34). The spacer incorporated into the L-SAH affinity adsorbents used to purify the furanocoumarin O-methyltransferases and protein methylase II outlined above was 1,6-diaminohexane. Nevertheless, the indoleethylamine N-methyltransferases, which were already partially purified by ammonium sulfate precipitation and ion exchange chromatography, could be immobilized on both L-SAH-agarose and L-SAH-Sepharose. Both enzymes were co-eluted by lowering the pH of the sodium maleate buffer used to wash the adsorbent from 7.0 to 6.0. Unfortunately, the enzymes could not be separated and rapidly lost activity in the more purified form.[199]

Immobilization of L-SAH on Sepharose can also be achieved by an amide bond linking the carboxylate group of a 6-aminohexanoic acid spacer to the α-amino group of the ligand. This type of affinity adsorbent has been used in the purification of N^2-guanine RNA methyltransferase (Figure 35) from chicken embryos.[200] This enzyme is involved in the modification of transfer RNA either for the purposes of recognition by other proteins or for protection from nucleases. After a preliminary 39-fold purification of the enzyme in the supernatant of a crude homogenate by chromatography on phosphocellulose,

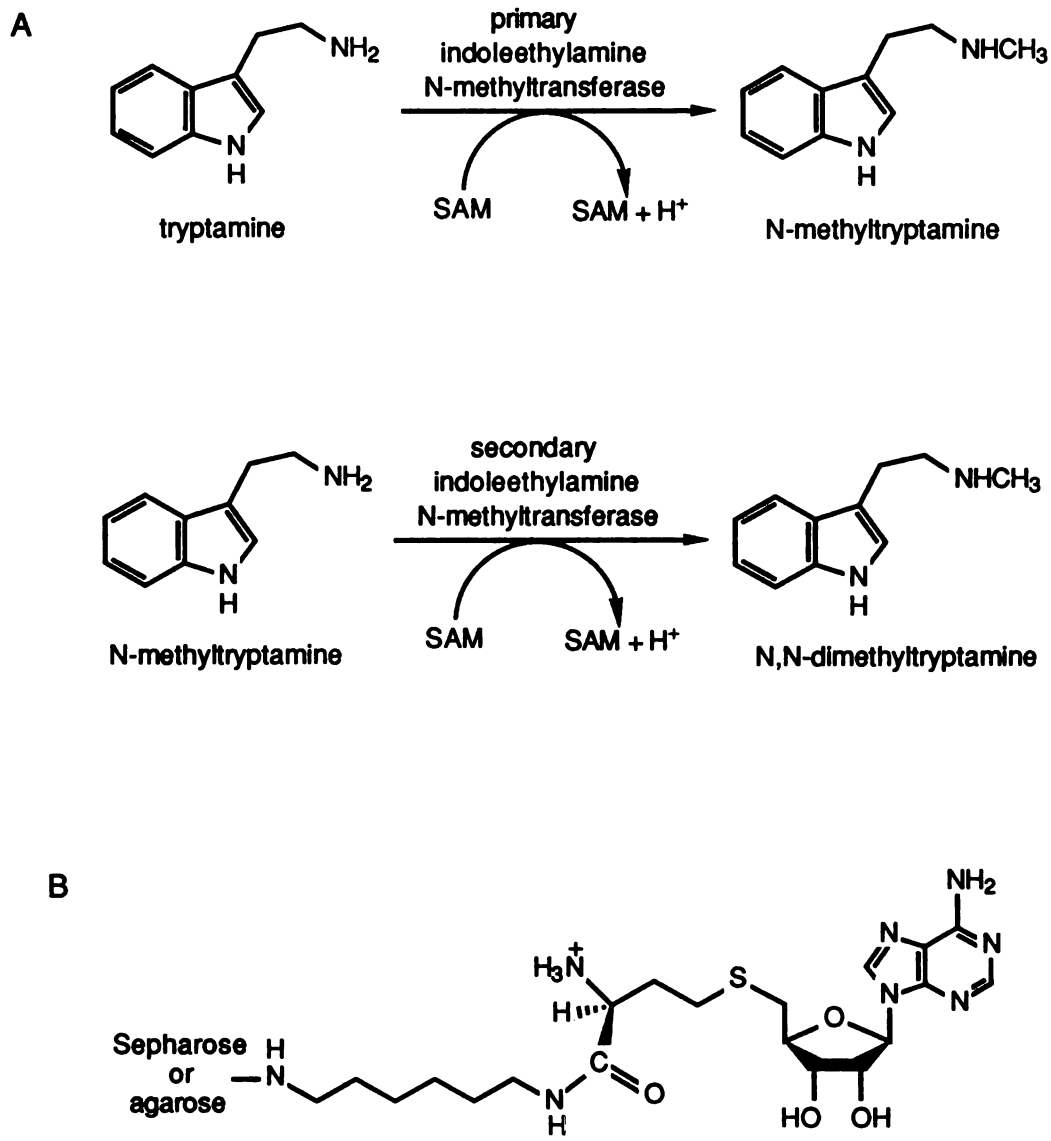


Figure 34. Examples of the methylations which can be catalyzed by the two indoleethylamine N-methyltransferases isolated from *Phalaris tuberosa* (above, A), and the L-SAH-Sepharose/agarose affinity adsorbent developed for their purification (below, B) (see reference 199).

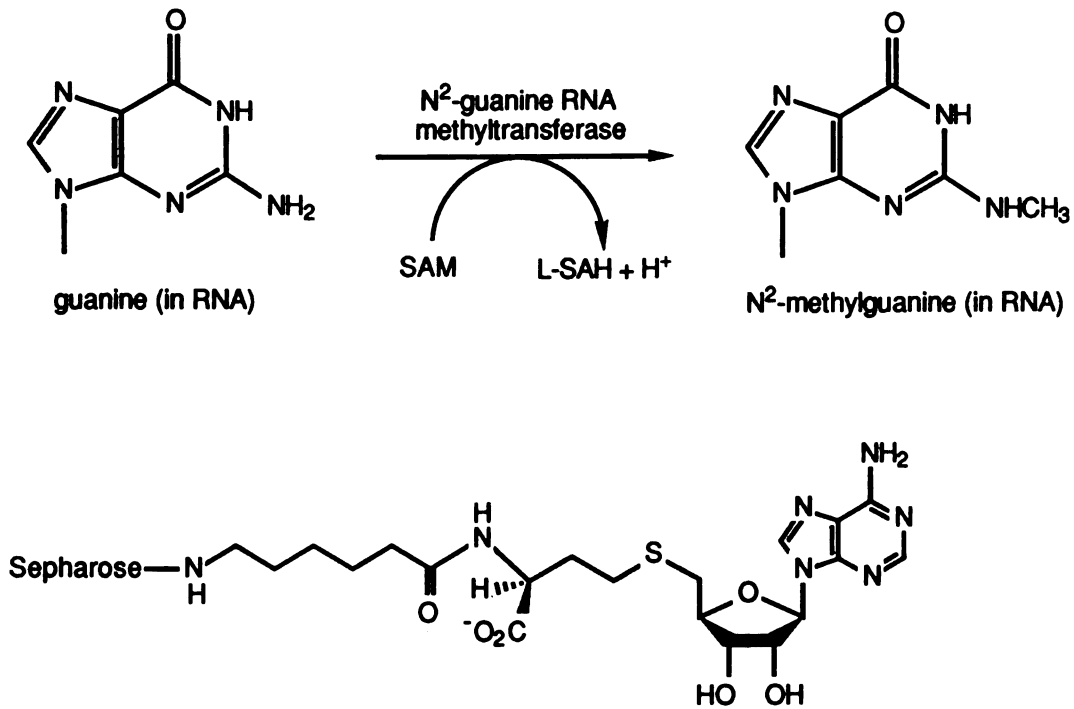
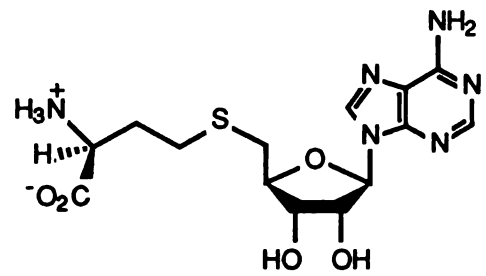


Figure 35. The methylation catalyzed by N^2-guanine RNA methyltransferase (above, A), and the L-SAH-Sepharose affinity adsorbent used in the purification of that enzyme (below, B) (see reference 200).

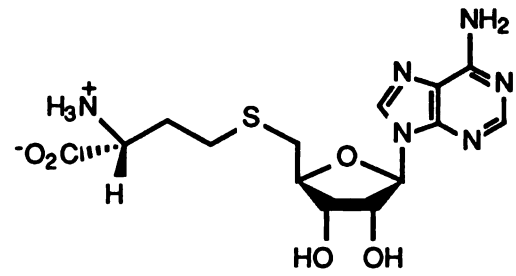
the methyltransferase was temporarily immobilized on the affinity adsorbent. By increasing the salt (NaCl) concentration of the eluting buffer, the enzyme was re-solubilized and eluted from the column purified another 26-fold.[200] This two-step procedure provided a more highly purified enzyme (1000-fold) than had been previously attained.

These examples clearly demonstrate that affinity chromatography can be of great value in the purification of methyltransferases. However, the development of an affinity adsorbent for the general purification of methyltransferases, analogous to the 8-azo-NAD^+-Sepharose commonly used to purify NAD^+-dependent dehydrogenases, must take into consideration the structure-activity relationships established for inhibitors of this class of enzymes. These structure-activity relationship studies have indicated that some of the binding energy between the enzyme and the potential inhibitor is lost when the amino acid portion of the L-SAH structure is modified. This loss of binding energy is reflected in an increase in the magnitude of the K_i value of the potential inhibitor.

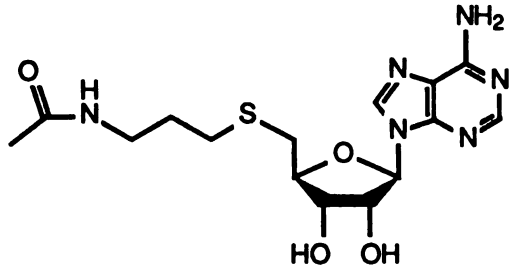
For instance, L-SAH is a potent inhibitor ($K_i = 36.2 \pm 2.20$ μM) of COMT, but D-SAH (Figure 36), which has an inverted stereochemistry at the α-carbon atom in the amino acid moiety, is a very poor inhibitor ($K_i = 1611 \pm 120$ μM) of COMT.[67] 5'-S-Adenosyl-3-N-acetylthiopropylamine, 5'-S-adenosyl-4-thio-butyric acid, methyl 5'-S-adenosyl-4-thiobutyrate, and 5'-S-adenosyl-L-N-acetylhomocysteine (Figure 36), which are all amino acid modified derivatives of L-SAH, have been shown to not be inhibitors of COMT activity.[67] Presumably these results are a manifestation of the importance of an electrostatic interaction and/or a steric limitation in the active site of this enzyme. Not surprisingly, these results are not unique to COMT. With the sole exception of D-SAH ($K_i = 10.5 \pm 1.3$ μM) being as good as inhibitor of histamine N-



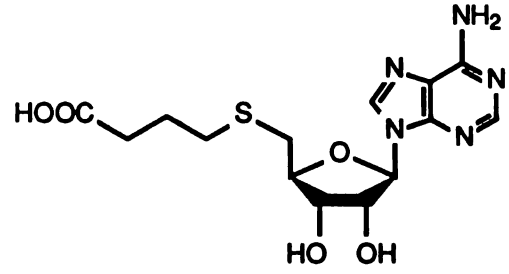
S-adenosyl-L-homocysteine (L-SAH)



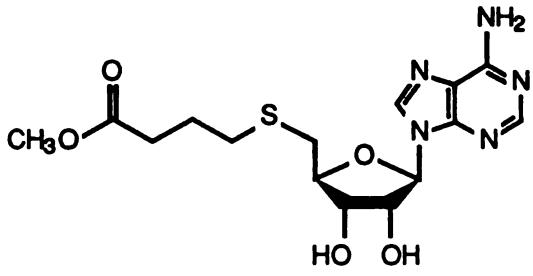
S-adenosyl-D-homocysteine (D-SAH)



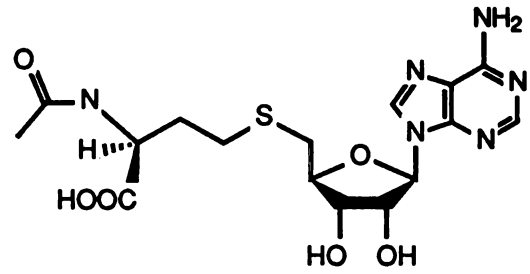
5'-S-adenosyl-3-N-acetylthiopropylamine



5'-S-adenosyl-4-thiobutyric acid



methyl 5'-S-adenosyl-4-thiobutyrate



5'-S-adenosyl-L-N-acetylhomocysteine

Figure 36. S-Adenosyl-L-homocysteine (L-SAH) and some of the amino acid modified derivatives which have been tested as potential inhibitors of some methyltransferases (see reference 67).

methyltransferase (HMT) as L-SAH (K_i = 18.1 ± 2.19 μM), each of the above listed derivatives of L-SAH shows either no or at best only very poor inhibition of the activities of phenylethanolamine N-methyltransferase (PNMT), hydroxyindole O-methyltransferase (HIOMT), and HMT (Figures 9 and 10).[67]

These results raise serious doubts as to how versatile a methyltransferase affinity adsorbent in which L-SAH is immobilized through an amide linkage to its carboxylate group could ever become. Because the natural unmodified configuration of substituents around the α-carbon atom of a potential inhibitor is important for tight binding to the above enzymes and probably many others, the compound immobilized on agarose, Sepharose, or polyacrylamide gels should have a similar unmodified structure. Perhaps, the furanocoumarin O-methyltransferases, protein methylase II, and the indoleethylamine N-methyltransferases are unique in their tolerance of the bulky N-alkyl amide group used to immobilize L-SAH in the affinity adsorbents used in their respective purifications. If this is true, then their purifications using this technique are the exceptions rather than the rule. This method of immobilizing L-SAH to the affinity adsorbent may eventually compromise the isolation of other methyltransferases, such as DNA (cytosine-5-)-methyltransferase. What is needed is an alternative means of linking L-SAH or one of its close analogues to the insoluble polymeric support in such a way as to leave unchanged any of the functional groups most likely to be involved in the binding interactions with the enzyme.

The central goal of this thesis is the development of a procedure for the synthesis of a series of analogues of SAM in which the inherently unstable sulfonium group is replaced by a much more stable tertiary amino group. By virtue of their protonation at physiological pH, these nitrogen SAM analogues will conserve the positive charge characteristic of the sulfonium group. A series

of compounds possessing either saturated or unsaturated alkyl groups bonded to the central nitrogen atom, in positions analogous to that occupied by the methyl group of SAM, has been synthesized. These nitrogen SAM analogues, with alkyl groups ranging in size from methyl to n-octyl, could then be used as probes of methyltransferase active site geometry.

The incorporation of a functional group into the alkyl group of one of these nitrogen SAM analogues might provide a "handle" through which the compound could be immobilized on agarose, Sepharose, or a polyacrylamide gel. The most obvious replacement for the unsubstituted alkyl group is the 6-amino-1-hexyl group. The 6-amino-1-hexyl nitrogen SAM analogue, which has the commonly used 1,6-diaminohexane spacer as a part of its structure, could be covalently bound by literature methods through a stable amide or secondary alkylamine linkage to a derivatized agarose, Sepharose, or polyacrylamide gel (Figure 37). The ribose hydroxyl groups, the adenine exocyclic amino group, and the amino acid moiety of this immobilized nitrogen SAM analogue are all unaltered and free to participate in the types of binding interactions in which SAM or L-SAH would participate with the soluble methyltransferases. The conservation of the positive charge in the tertiary ammonium group of the immobilized 6-aminohexyl nitrogen SAM analogue might provide an affinity adsorbent which would behave in ways similar to those which might be expected for a SAM affinity adsorbent. The inherent instability of SAM in aqueous solution would thwart any attempts to immobilize it. For this reason and also because the amino acid carboxylate group remains free and not involved in an amide linkage, this affinity adsorbent may prove to be superior to the L-SAH-Sepharose and L-SAH-agarose affinity adsorbents described above. The purification of a greater number of methyltransferases, including those which will not bind amino acid modified SAM or L-SAH analogues, might

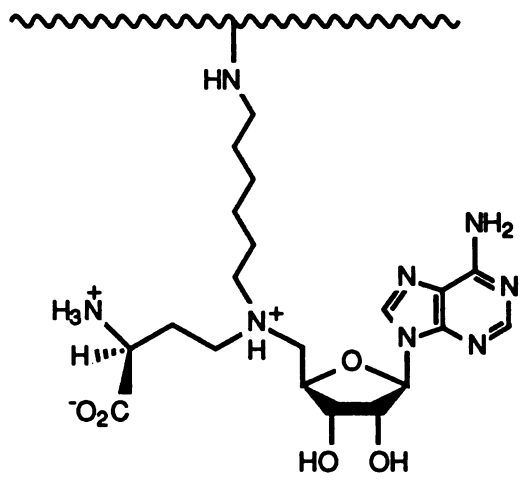
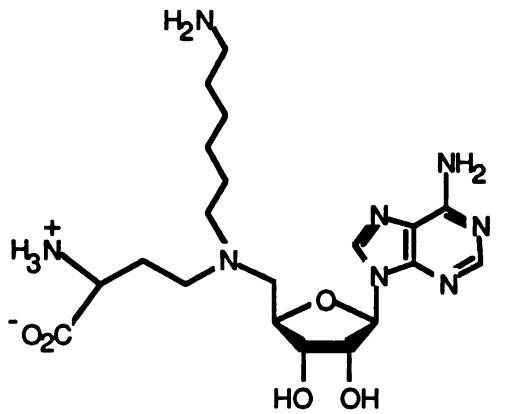


Figure 37. The immobilization of the 6-amino-1-hexyl nitrogen SAM analogue on an insoluble polymer such as agarose, Sepharose, or polyacrylamide gel to provide a novel methyltransferase affinity adsorbent.

be made possible with the proper use of this affinity adsorbent. After a careful consideration of the potential impact such an affinity adsorbent may have on methyltransferase biochemistry, the synthesis of the 6-amino-1-hexyl nitrogen SAM analogue, utilizing many of the same procedures already developed for the synthesis of the alkyl nitrogen SAM analogues described above, was begun.

Results and Discussion

The strategy followed in the synthesis of the potential SAM affinity ligand, N^4-(5'-adenosyl)-N^4-(6-amino-1-hexyl)-2(R,S),4-diaminobutyric acid (**9**), was to use as many of the techniques developed during the synthesis of the nitrogen alkyl SAM analogues (**2a-g**) (in Chapter 1) as possible. Thus, the alkylation of the secondary alkylamino group of a suitably protected derivative of 5'-(6-amino-1-hexyl)amino-5'-deoxy-2',3'-O-isopropylideneadenosine with methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) would provide the key fully protected trialkylamine intermediate. Step-by-step removal of each of the four protecting groups present in this intermediate would ultimately provide the final product (**9**) shown below:



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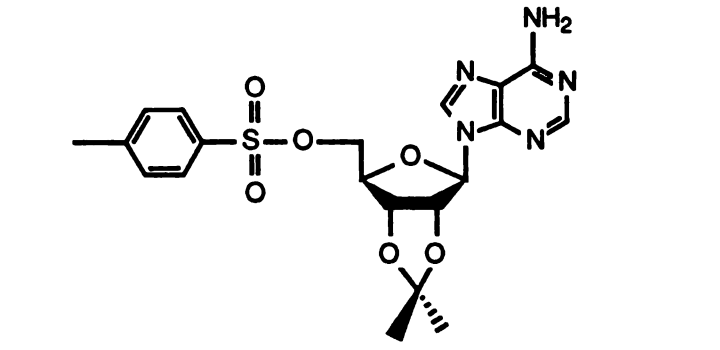
Immobilization of this compound, through its 6-amino-1-hexyl "tether," onto a suitably activated polymeric matrix would then be possible. The resulting affinity adsorbent, by having the potential to immobilize any SAM-binding protein temporarily, might eventually become a useful laboratory tool for the purification of methyltransferases.

The most challenging feature of the synthesis of this analogue of SAM was how to protect exclusively the primary alkylamino group of 5'-(6-amino-1-hexyl)amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**10**) in the presence of a secondary alkylamino group. The latter group must remain unprotected so that it can be selectively alkylated with methyl 2(R,S)-trifluoroacetamido-4-iodo-butyrate (**6b**) in a subsequent step. From a battery of commonly used amine protecting groups, one was eventually found to be suitable for protection of this primary alkylamino group, which ultimately made the synthesis of the SAM affinity ligand possible. The development of this synthetic procedure and the immobilization of this compound on two commonly used Sepharose coupling gels are described below.

Initial synthetic efforts were directed toward the preparation of 5'-[6-(trifluoroacetamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**11**). Alkylation of this compound with methyl 2(R,S)-trifluoroacetamido-4-iodo-butyrate (**6b**) was expected to provide the fully protected precursor of the final product. Concomitant removal of the trifluoroacetamide from the 6-amino-1-hexyl spacer and both amino acid protecting groups by mild alkaline hydrolysis, followed by acid hydrolysis of the isopropylidene ketal, would afford the final product. Thus, the possibility of removing three protecting groups in one step made trifluoroacetamide-protection of the 6-amino-1-hexyl moiety particularly attractive.

The first attempt to prepare 5'-[6-(trifluoroacetamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**11**) involved the direct trifluoro-acetylation of 5'-(6-amino-1-hexyl)amino-5'-deoxy-2',3'-O-isopropylidene-adenosine (**10**) (Scheme IV). The objective of this effort was to assess whether the less sterically hindered primary alkylamino group could be selectively protected in the presence of the secondary alkylamino group. The starting



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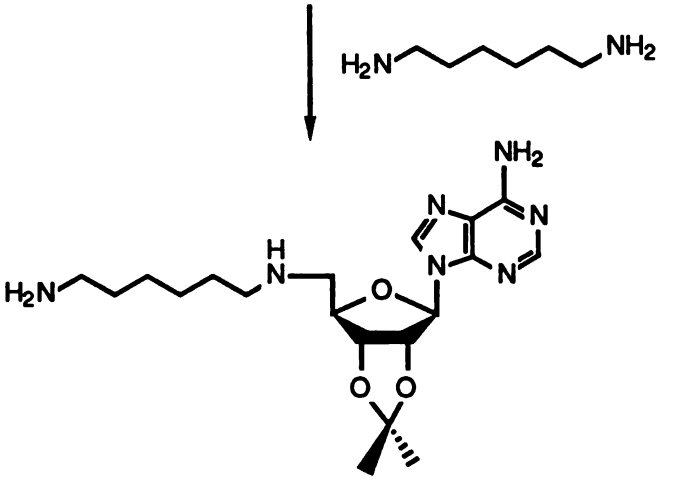

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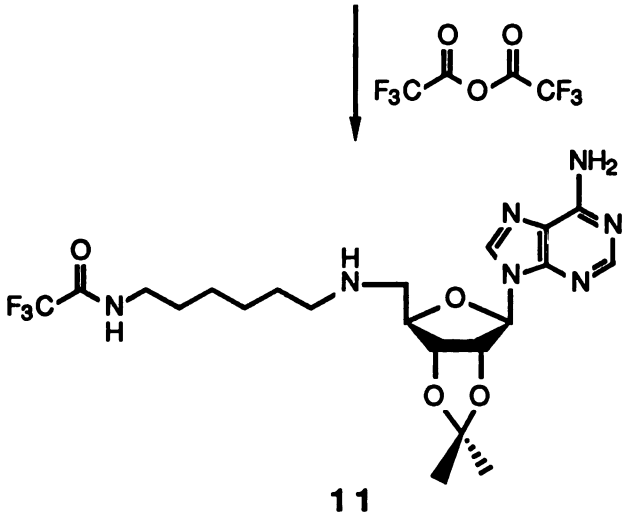

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Scheme IV. Initial low-yielding synthesis of 5'-[6-(trifluoroacetamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**11**).

material, 5'-(6-amino-1-hexyl)amino-5'-deoxy-2',3'-O-isopropylidene-adenosine(**10**), was prepared in a manner quite similar to that used to synthesize the 5'-alkylamino-5'-deoxy-2',3'-O-isopropylideneadenosine homologues (**4a-g**) described in Chapter 1. Following that general procedure, 1,6-diaminohexane was used to displace the tosyl group from 5'-O-tosyl-2',3'-O-isopropylideneadenosine (**3**). Because 1,6-diaminohexane is a solid at room temperature (mp 39-40°C.), this displacement reaction was carried out at elevated temperatures (100°C.). After the large excess of 1,6-diaminohexane was removed by distillation under reduced pressure, the product (**10**) was converted to its dihydrochloride salt. Only in this form could it be recovered partially pure in low yield (23%) by very tedious flash silica gel chromatography.

Trifluoroacetylation of the free base form of the recovered dihydrochloride salt of 5'-(6-amino-1-hexyl)amino-5'-deoxy-2',3'-O-isopropylidene-adenosine (**10**) with an equivalent of trifluoroacetic anhydride failed to provide a satisfactory yield of the expected product. Analysis by mass spectrometry indicated that most of the starting material had been bis-trifluoroacetylated (25%) rather than mono-trifluoroacetylated (5%). Using the positive LSIMS technique, the most prominent positive ion produced by the recovered material appeared at m/e 598 $(M + H)^+$, rather than at m/e 502 $(M + H)^+$, as would be expected for the mono-trifluoroacetylated compound. Further analysis by high resolution proton NMR suggested that the recovered bis-trifluoroacetylated material was actually a mixture of isomers. Aside from the trifluoroacetylation of both alkylamino groups, trifluoroacetylation of the adenine amino group and either one of the alkylamino groups had also occurred. No tris-trifluoroacetylated material was observed by either high resolution proton NMR or mass spectrometry. Precedents for this seemingly paradoxical observation

that bis-acylation of diamines sometimes occurs to a much greater extent than the expected mono-acylation are known.[201,202]

The recovered mono-trifluoroacetylated material was confirmed, by high resolution proton NMR spectroscopy in deuteriochloroform, to be exclusively the desired product, 5'-[6-(trifluoroacetamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**11**). This structural assignment was based on the appearance of the trifluoroacetamide hydrogen as a broad singlet at 6.77 ppm integrating to one proton. Exclusive trifluoroacetylation of the secondary alkylamino moiety would give a compound which does not possess a similar hydrogen atom. Because another broad singlet at 5.77 ppm integrating to two hydrogens was also present and could be assigned to the adenine amino group, the possibility that trifluoroacetylation had solely occurred on the adenine amino group could be dismissed. (The primary and secondary alkylamino protons were rarely if ever observed for any of the compounds described in this or the preceding chapter. In addition to normal exchange processes, the very short spin-spin relaxation time, T_2, of these hydrogens, bonded to the quadrupolar ^{14}N nucleus, causes their resonances to have very large line widths. The line widths are often so large that the "peaks" attributable to these hydrogens either appear as small humps or disappear altogether into the baseline. Why this effect is not so extreme for acylamido hydrogens such as that described above and others described later is beyond the scope of this thesis. In any event, these resonances can be used for structural assignments, even for those involving selective decoupling experiments.)

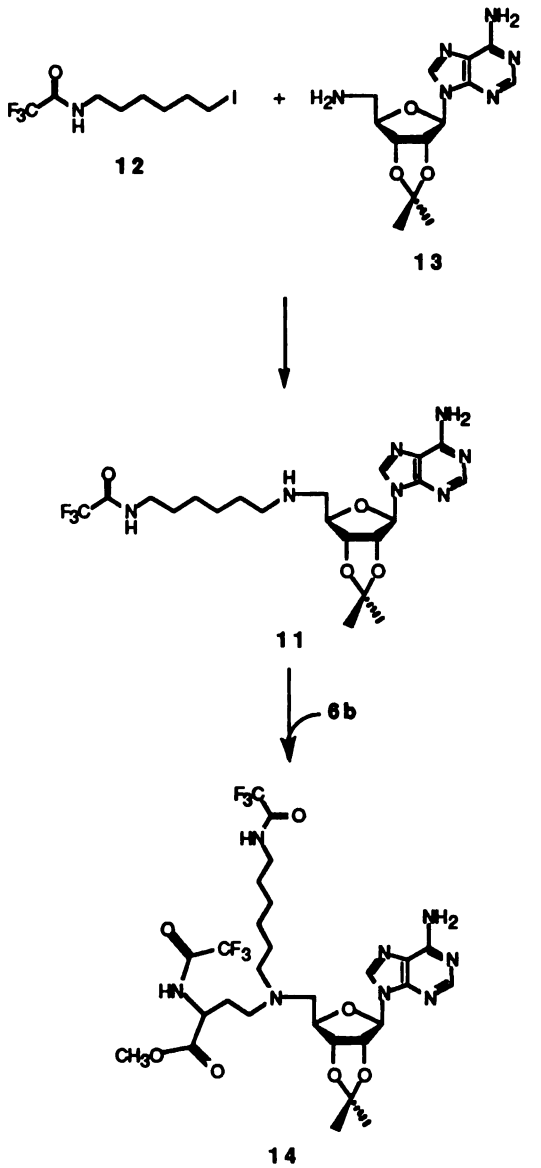
Thus, even though mono-trifluoroacetylation occurred to only a small degree, the original hypothesis that acylation would occur predominantly on the less sterically hindered primary alkylamino group of 5'-(6-amino-1-hexyl)amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**10**) proved to be correct. In

addition, the recovered product appeared to be quite stable with respect to a possible spontaneous disproportionation of the trifluoroacetyl group among the primary and secondary alkylamino moieties. Such stability was a necessary prerequisite for the eventual selective alkylation of the secondary alkylamino group with methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**). However, before this alkylation reaction could be carried out in its expected low yield (20-25%), a much more efficient synthesis of 5'-[6-(trifluoroacetamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**11**) had to be devised. The problem with the low yields encountered in the preparation of this compound had to be solved at this point in order to make the synthesis of the SAM affinity ligand (**9**) practical.

A review of the literature indicated that the immobilization of 1-thioglycosides onto solid matrices was possible through an intervening 6-amino-1-hexyl spacer group.[182,187] This spacer ligand was most often introduced by reaction of the thioglycoside with 1-iodo-6-(trifluoroacetamido)hexane (**12**). This compound, prepared in three steps from 6-amino-1-hexanol,[182,187] seemed to offer an ideal solution to the problems encountered in the synthesis of 5'-[6-(trifluoroacetamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**11**). Prior protection of the primary amino group, followed in a separate step by formation of the secondary amino group appeared to be a reasonable alternative to the low-yielding selective trifluoroacetylation procedure described above. A recent application of the Mitsunobu reaction[203] to nucleoside chemistry now permits 5'-amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**13**) to be prepared from commercially available 2',3'-O-isopropylideneadenosine in two high-yielding steps.[204] Thus, the alkylation of 5'-amino-5'-deoxy-2',3'-O-isopropylidene-adenosine (**13**) with 1-iodo-6-(trifluoroacetamido)hexane (**12**) would be expected to provide an ample supply

of 5'-[6-(trifluoroacetoamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylidene-adenosine (**11**) for eventual alkylation with methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**).

Indeed, in the presence of one equivalent of N,N-diisopropylethylamine, the alkylation of one equivalent of 5'-amino-5'-deoxy-2',3'-O-isopropylidene-adenosine (**13**) with one equivalent of 1-iodo-6-(trifluoroacetamido)hexane (**12**) did provide the desired product in moderate (46%) yield (Scheme V). Common side reactions encountered in the monoalkylation of primary amines include the di- and trialkylation of the starting material. So, not surprisingly, the dialkylation of 5'-amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**13**) was observed as well as the expected monoalkylation. Trialkylation probably also occurred to a limited extent, but the isolation of the resulting quaternary ammonium compound was never pursued. The desired monoalkylation product could be readily separated from the unreacted starting materials and the dialkylation side product by flash silica gel chromatography. Analysis of the recovered monoalkylation product by high resolution proton NMR indicated that it was indeed pure 5'-[6-(trifluoroacetamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**11**) and did not provide any evidence for a disproportionation of the trifluoroacetyl group among the primary and secondary alkylamino groups. This result erased any lingering doubts that this compound could not eventually be alkylated specifically and exclusively at the secondary alkylamino position. It was anticipated that subsequent alkylation of this compond with methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) would proceed in as low a yield (approximately 20-25%) as was the case with the protected nitrogen alkyl SAM analogues discussed earlier (Chapter 1). If this proved to be the case, the new and more efficient synthesis of gram quantities of 5'-[6-(trifluoroacetamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylidene-



Scheme V. Synthesis of the fully protected 5'-[6-(trifluoroacetamido)-1-hexyl] SAM affinity ligand (**14**), including the improved synthesis of 5'-[6-(trifluoroacetamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylidene-adenosine (**11**).

adenosine (**11**) would permit this alkylation reaction to be scaled up to provide adequate amounts of the fully protected trialkylamine intermediate.

As expected, this alkylation reaction (Scheme V) did proceed in low (23%) yield. As a consequence of the alkylation of a chiral compound (i.e., 5'-[6-(trifluoroacetamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**11**)) with a racemic mixture of enantiomers (i.e., methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**)), the recovered product was actually a mixture of diastereomers. Analysis of the purified fully protected 5'-[6-(trifluoroacetamido)-1-hexyl] SAM affinity ligand (**14**) by high resolution proton NMR indicated that the diastereomers were present in equal amounts. Thus, as was observed in the synthesis of the protected nitrogen alkyl SAM analogues (Chapter 1), this reaction had proceeded without any evidence of significant asymmetric induction.

One interesting feature of the diastereomers of this product, which was not seen with those of the protected nitrogen alkyl SAM analogues, was that they could be readily distinguished as two separate, albeit close, spots by thin layer chromatography. Flash silica gel chromatography could be used to actually separate the individual diastereomers of the fully protected 5'-[6-(trifluoroacetamido)-1-hexyl] SAM affinity ligand (**14**). Such a separation was possible when a nonpolar solvent system [i.e., chloroform:isopropanol (90:10)] was used which gave the diastereomers a mobility on silica gel characterized by an R_f value of approximately 0.25-0.35. Elution of a flash silica gel column with this type of solvent system resulted in the appearance of the fully protected 5'-[6-(trifluoroacetamido)-1-hexyl] SAM affinity ligand (**14**) in three distinct sets of fractions. The leading, intermediate, and final sets of fractions contained the pure faster-moving (higher R_f) diastereomer, a mixture of both diastereomers, and the pure slower-moving (lower R_f) diastereomer, respectively. About 75%

of the total loaded mixture of diastereomers was eluted in the intermediate set of fractions. Cycling this material through the same flash silica gel column three or four more times would probably have sufficed to separate the diastereomers on a 500-750 mg scale. For the purpose of developing a feasible synthesis of the SAM affinity ligand, though, the diastereomeric mixture was used. If, however, one completely deprotected diastereomer should appear to exhibit significantly better binding to a methyltransferase than the other diastereomer, and if their absolute configurations at the α-carbon atom could be readily determined, this separation could be pursued on a large scale. It is interesting, though, that these diastereomers could be readily separated, whereas the diastereomers of the fully protected nitrogen alkyl SAM analogues could not be easily separated. One explanation for this observation might be the presence of a hydrogen-bonding interaction between the protected amino acid moiety and the trifluoroacetamide group at the end of the hexane spacer. Diastereomers, unlike enantiomers, have different physical properties, and an interaction such as this, which would accentuate the inherent structural differences of these compounds, could ultimately be manifested in contrasting chromatographic mobilities.

The same conditions (i.e., sodium carbonate in 50% aqueous methanol) which were used to remove the amino acid protecting groups in the fully protected nitrogen alkyl SAM analogues described earlier, seemed ideal for the removal of the trifluoroacetamide group on the hexane spacer as well. Thus, three protecting groups could be removed in one step. The progress of this deprotection was monitored by thin layer chromatography on silica gel. By the time the starting material spot had completely disappeared (approximately 10 minutes), four slower-moving spots had appeared. The two intermediates and the expected final compound, resulting from the step-by-step hydrolysis of each

of the alkaline-labile protecting groups, could account for the three faster-moving of these four spots. Gradually, as the reaction time increased, these three spots faded and the fourth spot at the origin became more intense. After three or four hours, only the spot at the origin remained. In an attempt to determine what was responsible for the unexpected fourth spot, the residue of the neutralized and evaporated crude reaction mixture was analyzed by mass spectrometry using the positive LSIMS technique. This analysis indicated that none of the expected product, N^4-(6-amino-1-hexyl)-N^4-(2',3'-O-isopropylidene-5'-adenosyl)-2(R,S),4-diaminobutyric acid, could be detected as a peak at m/e 507 $(M + H)^+$.

This observation and a re-evaluation of the reaction conditions suggested that this fourth spot was a manifestation of a polymerization side reaction which rapidly consumed all of the starting material. The simplest mechanism by which polymerization could occur involves the formation of an amide linkage between the 6-amino-1-hexyl group of one deprotected molecule with the methyl ester of either the starting material or one of the detrifluoroacetylated intermediates (Figure 38). The alkaline reaction conditions ensured that this amino group would always be present in its free base, and thus, highly reactive form. Also, because the trifluoroacetamide group would hydrolyze at a much faster rate that the methyl ester, some of the ester would always be present for the amine to attack. The stable amide linkages of the resulting polymer would resist hydrolysis under the relatively mild alkaline reaction conditions. Thus, it would be unlikely that the monomeric form of the final product would eventually reappear. Attempts to minimize this polymerization side reaction by 100-fold dilution or neutralization after 25 minutes failed.

Although this apparent polymerization was foreseeable, the possibility of removing three protecting groups from the trialkylamine intermediate in one

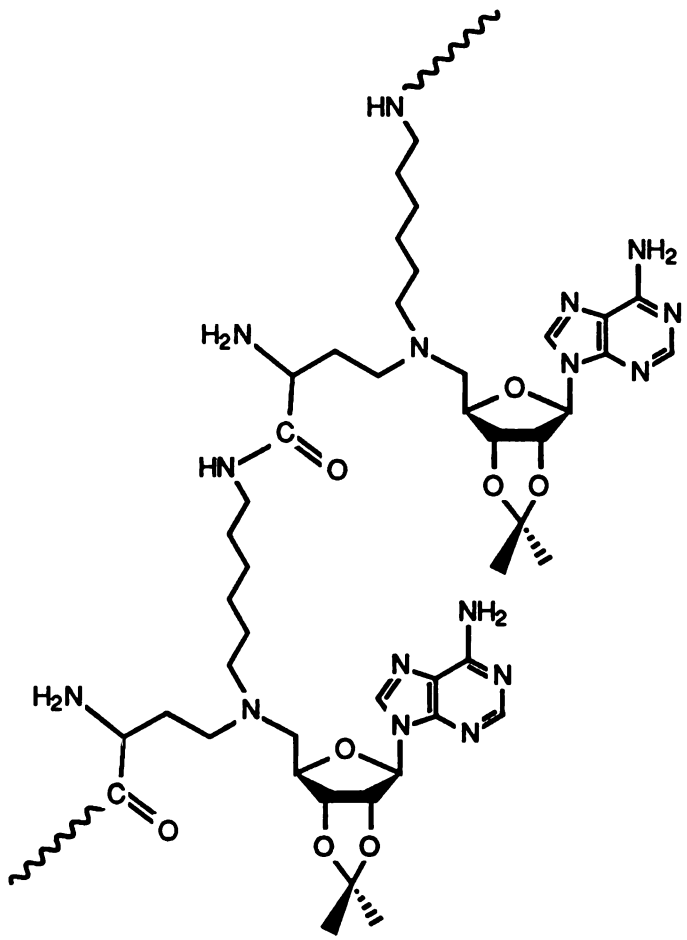


Figure 38. Suspected polymerization product arising from the deprotection of the fully protected 5'-[6-(trifluoroacetamido)-1-hexyl] SAM affinity ligand (14).

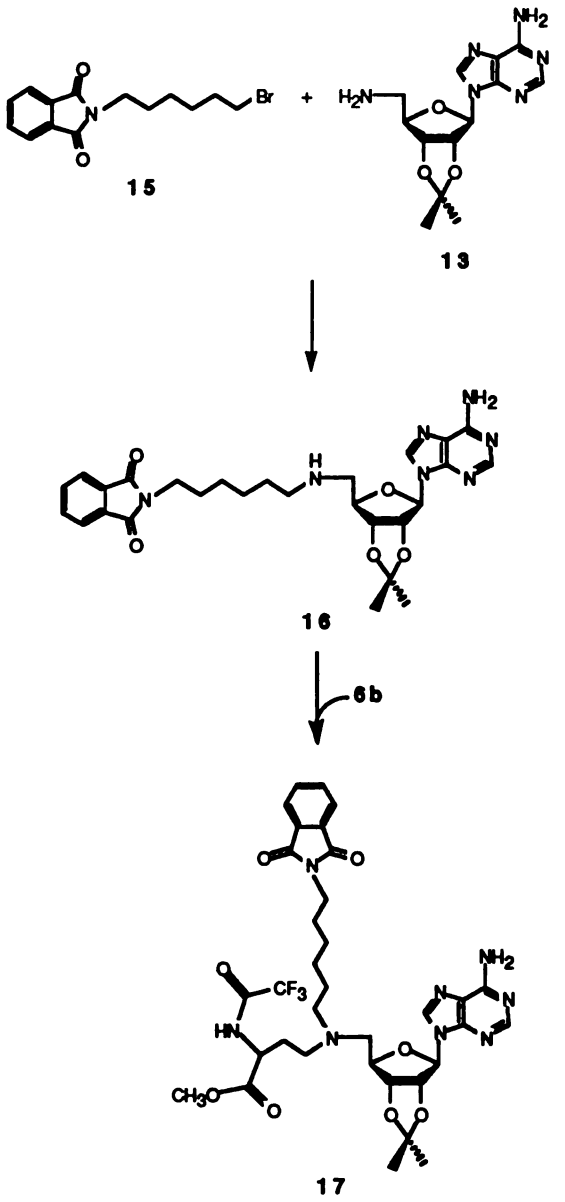
step was particularly attractive. The failure of the protected nitrogen alkyl SAM analogues (Chapter 1) to polymerize, once the amino acid trifluoroacetamide was hydrolyzed, would have suggested that this synthetic approach might have been fruitful. It was believed that the 6-amino-1-hexyl trifluoroacetamide protecting group would have been ideal because it is both easy to introduce and easy to remove under mild conditions. The fundamental flaw in the deprotection scheme, however, was the apparent unmasking of this reactive amino group in the presence of a methyl ester elsewhere in the molecule. Potential solutions to this problem involved either selective deprotection of the methyl ester in the presence of the other protecting groups, or protection of the 6-amino-1-hexyl moiety with another group that was not quite as reactive as the trifluoroacetamide group. Attempts to unmask the methyl ester in the presence of the two trifluoroacetamide groups by reaction with lithium iodide in 2,6-lutidine[205] or sodium cyanide in hexamethylphosphorus triamide (HMPA)[206] were considered. However, since the methods for complete deprotection of the amino acid moiety were already well-established (Chapter 1), the most rational solution to this synthetic problem was seen to involve another method of protecting the 6-amino-1-hexyl moiety.

A survey of commonly used amino protecting groups[207] suggested that the phthalimido group, unlike the trifluoroacetamido group discussed above, might adequately protect the 6-amino-1-hexyl spacer during the deprotection of the amino acid moiety. The phthalimido group is stable under mild alkaline conditions but is susceptible to hydrolysis under more vigorous alkaline conditions. Since the conditions for deprotection of the amino acid moiety (i.e., sodium carbonate in 50% aqueous methanol at 60°C. for six hours) were relatively mild, protection of the amino group in the spacer as a phthalimide was pursued. Once the amino acid moiety was completely deprotected, the intact

phthalimide group could be removed by treatment with hydrazine to leave the 2',3'-O-isopropylidene derivative of the SAM affinity ligand. Acid hydrolysis of the isopropylidene ketal would then afford the final compound (**9**).

Construction of the fully protected 5'-[6-(phthalimido)-1-hexyl] SAM affinity ligand (**17**) (Scheme VI) began with the preparation of N-(6-bromo-1-hexyl)phthalimide (**15**). This compound, synthesized from 1,6-dibromohexane and the potassium salt of phthalimide,[208] was the source of the protected 6-amino-1-hexyl spacer in the intermediate trialkylamine. The alkylation of 5'-amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**13**) with N-(6-bromo-1-hexyl)phthalimide (**15**) (Scheme VI) was modeled after the procedure used in the large scale preparation of 5'-[6-(trifluoroacetamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**11**) described above. As was observed in that synthesis, the desired monoalkylation of 5'-amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**13**) was accompanied by unavoidable di- (and probably tri-) alkylation side reactions. The monoalkylation product was readily separated from the dialkylation side product and unreacted N-(6-bromo-1-hexyl)phthalimide (**15**) by flash silica gel chromatography. A moderate (44%) yield of 5'-[6-(phthalimido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**16**) was obtained. Subsequent alkylation of this compound with methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) provided the fully protected 5'-[6-(phthalimido)-1-hexyl] SAM affinity ligand (**17**) (Scheme VI) in low (25%) yield. The diastereomers of this compound, like those of the fully protected 5'-[6-(trifluoroacetamido)-1-hexyl] SAM affinity ligand (**14**) discussed above, exhibited slightly different chromatographic mobilities, and could be separated, if so desired, by flash silica gel chromatography.

Deprotection of the amino acid moiety of the purified fully protected 5'-[6-(phthalimido)-1-hexyl] SAM affinity ligand (**17**) was carried out using the



Scheme VI. Synthesis of the fully protected 5'-[6-(phthalimido)-1-hexyl] SAM affinity ligand (17).

established mild alkaline hydrolysis procedure (i.e., sodium carbonate in warm 50% aqueous methanol). Thin layer chromatography on silica gel was used to monitor the hydroysis of the trifluoroacetamide and methyl ester groups. Unlike the analogous deprotection of the fully protected 5'-[6-(trifluoroacetamido)-1-hexyl] SAM affinity ligand (**14**) described previously, only two new spots were observed which corresponded to the loss of two protecting groups by the starting material. The absence of a third spot, particularly one at the origin, suggested that the fully protected 5'-[6-(phthalimido)-1-hexyl] SAM affinity ligand (**17**) had not polymerized.

Routine analysis of the recovered product by high resolution proton NMR, mass spectrometry, and UV spectroscopy had indicated that the phthalimido group had not survived the reaction intact. The phthalimido group had, apparently, been partially hydrolyzed to give the phthalamido ring-opened derivative. The most direct evidence for this side reaction was the nonequivalence of each phthalamido proton in the high resolution (500 MHz) proton NMR spectrum (Figure 39). Although each phthalamido proton resonance appeared as a complex multiplet integrating to one hydrogen, the general outlines of two sets of doublets and two sets of triplets (1:2:1) were readily apparent. This pattern would not be observed if the phthalimide group had remained intact and cannot be explained on the basis of diastereomeric nonequivalence. The phthalimide groups of each diastereomer of the starting material could not be readily distinguished from each other in the high resolution proton NMR spectrum. Observable coupling to the hydrogens in the ortho and meta positions, in conjunction with the symmetry of the phthalimide group, made the superimposed resonances of the isoindole hydrogens of each diastereomer appear as two sets of doublets of doublets. The proton resonances of an intact phthalimide group in the expected product, after

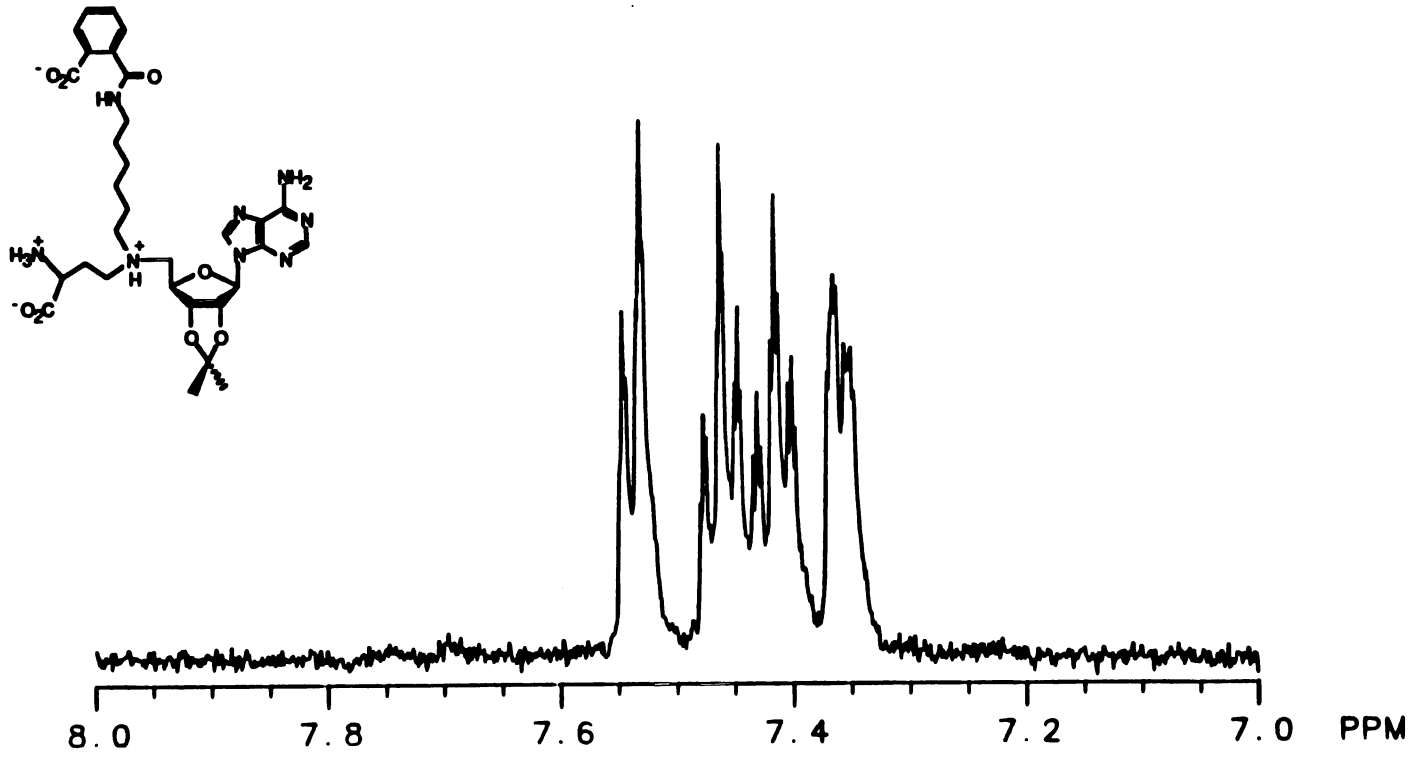


Figure 39. The phthalamido ring-opened product (**18**) formed during the deprotection of the fully protected 5'-[6-(phthalimido)-1-hexyl] SAM affinity ligand (**17**), and the 500 MHz ^{1}H NMR (D_2O) spectrum of the phthalamido protons.

deprotection of the amino acid moiety, should also appear as two sets of doublets of doublets (or four sets of doublets of doublets in the event of complete diastereomeric nonequivalence). The triplets (1:2:1) observed in the high resolution proton NMR spectrum of the product are more explainable on the basis of an asymmetric environment rather than diastereomeric nonequivalence. The latter phenomenon was generally less dramatic in the partially or completely deprotected nitrogen alkyl SAM analogues (Chapter 1) than it was in the fully protected compounds. The simplest explanation for each triplet (1:2:1) resonance involves the nearly identical coupling (very similar or identical coupling constants) of one hydrogen to two adjacent ortho position hydrogens. The doublets arise from the coupling of one hydrogen to another hydrogen in an ortho position. Long-range coupling to other hydrogens around the aromatic ring could give each doublet and triplet a more complex appearance. A loss of symmetry in the phthalimide group would be responsible for the observed chemical and magnetic nonequivalence of each of its four hydrogens. Partial hydrolysis of the phthalimide to a phthalamide (Figure 39) is most likely responsible for this loss of symmetry. Complete hydrolysis of the phthalimide to phthalate would restore symmetry to the aromatic ring. This event would have been characterized by two sets of doublets of doublets in the high resolution proton NMR spectrum.

Further evidence for the partial hydrolysis of the phthalimide group came from mass spectrometry. Using the positive LSIMS technique, the molecular ion of the expected product should have been observed at m/e 637 $(M + H)^+$. However, the most prominent peak in the positive LSIMS spectrum of the recovered material was observed at m/e 655 $(M + H)^+$. Nothing was present at m/e 637, although a small peak was seen at m/e 640. This smaller peak is probably evidence of fragmentation of the compound responsible for the peak

at m/e 655. The mass difference of 15 between these hypothesized parent and daughter ions could be attributed to the loss of an isopropylidene methyl group by the parent ion. (Such fragmentation has been commonly observed in the EI and positive LSIMS mass spectra of the 2',3'-O-isopropylidene nucleosides described in this thesis.) The mass difference of 18 between the expected and recovered products could be traced to the incorporation of a water molecule somewhere into the expected product. Hydrolysis is defined as the breaking of a bond by the insertion of the elements of water. The only bonds where such hydrolysis could occur, and not actually fragment the molecule into two smaller pieces, are the two amide bonds of the phthalimide group. Hydrolysis of either bond would convert the phthalimide group to a phthalamide group (Figure 39).

Finally, the ultraviolet spectra of all precursors of the recovered material which had contained an intact phthalimide group, including N-(6-bromo-1-hexyl)phthalimide, had a λ_{max} of 241 nm. This absorption peak was not seen in the ultraviolet spectrum of the recovered material. This observation implies that something had happened to the phthalimide group during the alkaline hydrolysis reaction. Thus, it is quite clear from this thorough analysis of the recovered material by high resolution proton NMR, mass spectrometry, and ultraviolet spectroscopy that the phthalimide group of the fully protected 5'-[6-(phthalimido)-1-hexyl] SAM affinity ligand (**17**) had indeed been partially hydrolyzed to the ring-opened phthalamide derivative (**18**) (Figure 39).

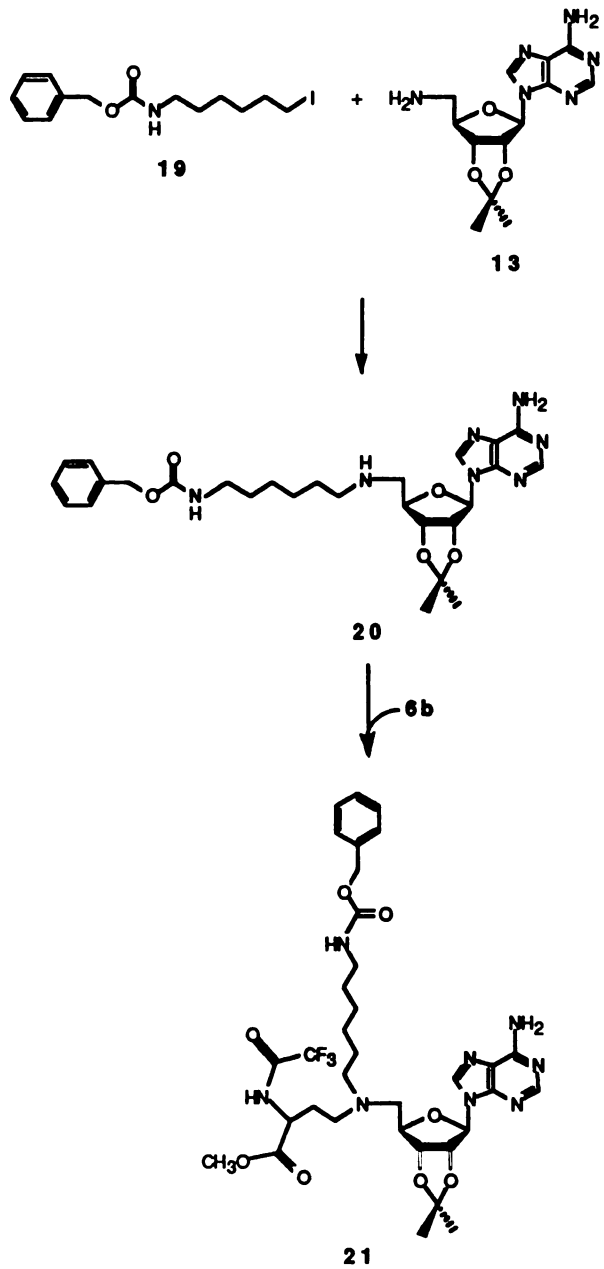
However, under more vigorous alkaline conditions, complete hydrolysis of the phthalamido group might be achieved to provide the 2',3'-O-isopropylidene derivative of the SAM affinity ligand. In a test reaction, a small amount of the recovered N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-[6-(1-phthalamoyl)-1-hexyl]-2(R,S),4-diaminobutyric acid (**18**) was dissolved in 1.0 M aqueous sodium hydroxide and heated to 100°C. for three hours. After the reaction

mixture was neutralized and evaporated, the residue was analyzed by high resolution proton NMR. Because the four nonequivalent phthalamide protons could be recognized by their distinctive pattern of two complex doublets and two complex triplets (1:2:1) (Figure 39), it was apparent that the phthalamido group had not been hydrolyzed. Free phthalate, the expected by-product of this reaction, by virtue of its inherent symmetry as discussed above, would have appeared as two doublets of doublets in the proton NMR spectrum. Further examination of the high resolution proton NMR spectrum revealed a complete absence of any of the expected resonances of the ribose hydrogens, including those of the isopropylidene methyl groups. This evidence clearly indicates that the sugar ring had been completely destroyed by the hot aqueous sodium hydroxide. Paradoxically, the reaction conditions which were not sufficiently vigorous to hydrolyze the obviously very stable amide bond of the phthalamido group were harsh enough to destroy the ribose moiety.

This result made it quite clear that the 6-amino-1-hexyl spacer could not be protected as a phthalimide during deprotection of the amino acid moiety and necessitated a complete re-evaluation of how adequate protection of this amino group might be achieved. The synthetic problems encountered up to this point could all be traced to amide amino protecting groups. Amino groups can also be protected as urethanes, or carbamates, as well as by other protecting groups.[207] A review of the literature indicated that benzyl carbamates, known to be resistant to mild alkaline hydrolysis,[207] have been used to protect 6-amino-1-hexyl spacers incorporated into glycosides for eventual immobilization onto affinity matrices.[182,209-212] For this reason, the protection of the 6-amino-1-hexyl spacer of the SAM affinity ligand as a benzyl carbamate was investigated.

This new approach to the synthesis of the SAM affinity ligand (**9**) commenced with the preparation of 6-(benzyloxycarbonyl)-1-iodohexane (**19**) from 6-amino-1-hexanol.[182] The alkylation of 5'-amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**13**) with this compound (Scheme VII) was conducted according to the previously described general procedure used in the large scale preparation of both 5'-[6-(trifluoroacetamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**11**) and 5'-[6-(phthalimido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**16**). Not surprisingly, side reactions involving the di- (and probably tri-) alkylation of 5'-amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**13**) occurred as well as the expected monoalkylation. Flash silica gel chromatography was again used to separate the desired monoalkylation product from unreacted starting materials and the dialkylation side product. Pure 5'-[6-(benzyloxycarbonylamino)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**20**) was obtained in moderate (48%) yield. The alkylation of this compound with methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) furnished the fully protected 5'-[6-(benzyloxycarbonylamino)-1-hexyl] SAM affinity ligand (**21**) in low (21%) yield (Scheme VII). As was previously observed with the fully protected 5'-[6-(trifluoroacetamido)-1-hexyl] (**14**) and 5'-[6-(phthalimido)-1-hexyl] SAM affinity ligands (**17**), both diastereomers of this trialkylamine intermediate displayed different chromatographic mobilities on silica gel. If necessary, repeated flash silica gel chromatography could be used to separate both diastereomers of the fully protected 5'-[6-(benzyloxycarbonylamino)-1-hexyl] SAM affinity ligand (**21**).

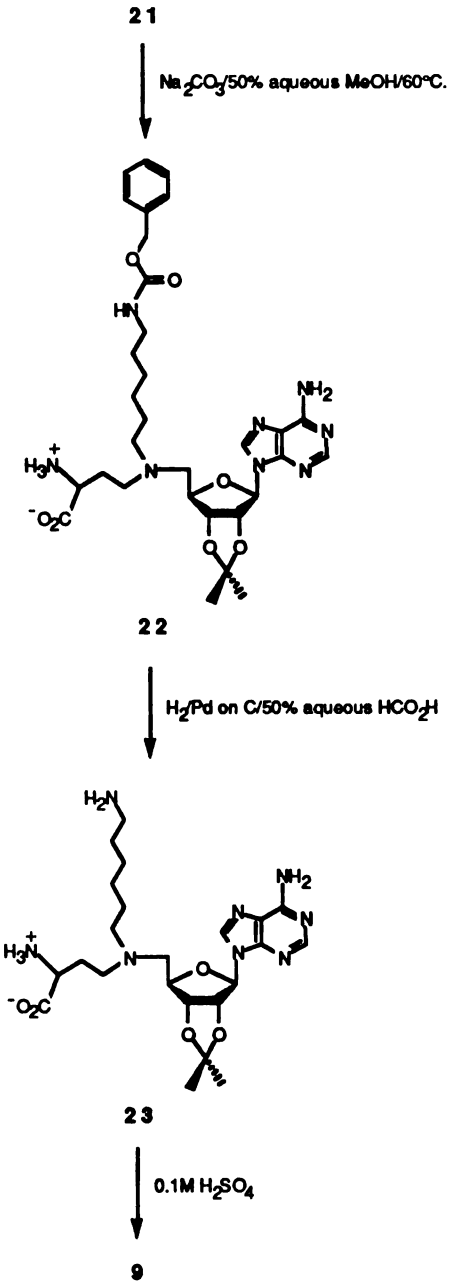
The deprotection of the amino acid moiety (Scheme VIII) of this compound by mild alkaline hydrolysis with sodium carbonate in warm 50% aqueous methanol proceeded smoothly, in contrast to the two previous attempts



Scheme VII. Synthesis of the fully protected 5'-[6-(benzyloxycarbonylamino)-1-hexyl] SAM affinity ligand (21).

described above. The hydrolysis of both the trifluoroacetamide and methyl ester protecting groups was easily followed by thin layer chromatography. Analysis of the recovered product by high resolution proton NMR and mass spectrometry clearly revealed that the benzyl carbamate protecting group had survived this deprotection reaction intact. The resonances of the aromatic and methylene hydrogens of the benzyl group, which integrated to five and two hydrogens, respectively, relative to the resonances of the other hydrogens of the product, could be plainly seen in the high resolution proton NMR spectrum. The molecular ion for the expected product, N^4-[6-(benzyloxycarbonylamino)-1-hexyl]-N^4-(2',3'-O-isopropylidene-5'-adenosyl)-2(R,S),4-diaminobutyric acid (**22**), was readily observed at m/e 641 $(M + H)^+$ using the positive LSIMS mass spectrometry technique. These results indicated that protection of the 6-amino-1-hexyl spacer as a benzyl carbamate was compatible with the deprotection of the amino acid moiety by alkaline hydrolysis.

Subsequent removal of the benzyl carbamate protecting group was readily accomplished by hydrogenation in 50% aqueous formic acid in the presence of a palladium catalyst (Scheme VIII). The acidic solvent, which was necessary for the rapid hydrogenolysis of the benzyl carbamate protecting group, was also capable of catalyzing the hydrolysis of the isopropylidene ketal and the glycoside bond. The failure to observe any free adenine in the filtered, neutralized, and desalted reaction mixture by thin layer chromatography indicated that hydrolysis of the glycoside bond had not occurred. However, the presence of a second slower-moving ultraviolet-absorbing spot, which like the spot produced by the 2',3'-O-isopropylidene SAM affinity ligand product (**23**), gave positive iodine and ninhydrin spot tests, provided evidence that partial (approximately 5%) hydrolysis of the isopropylidene ketal had occurred. Since this minor "contaminant" was probably the completely deprotected SAM affinity



Scheme VIII. Deprotection of the fully protected 5'-[6-(benzyloxycarbonyl-amino)-1-hexyl] SAM affinity ligand (**21**) to provide the potential SAM affinity ligand (**9**).

ligand and because the next and final step of the reaction sequence was the acid-catalyzed hydrolysis of the ribose 2',3'-diol isopropylidene ketal protecting group, the "impure" product was used without further purification.

Hydrolysis of the isopropylidene ketal was carried out in dilute aqueous sulfuric acid (0.1M) over eight days (Scheme VIII). As the progress of the reaction was monitored by thin layer chromatography, the starting material spot gradually faded and the original slower-moving spot gradually intensified until it was the only spot remaining. This observation clearly suggests that the starting material was indeed "contaminated" with some of the product. The completely deprotected SAM affinity ligand was isolated from the neutralized crude reaction mixture by cation exchange chromatography. Microanalysis, high resolution proton NMR, and mass spectrometry were used to confirm the identity of the recovered colorless solid as N^4-(5'-adenosyl)-N^4-(6-amino-1-hexyl)-2(R,S),4-diaminobutyric acid dihydrate (**9**).

The multi-step synthesis of the SAM affinity ligand (**9**) was facilitated by benzyl carbamate protection of the 6-amino-1-hexyl spacer. Functionalization of the N^4-alkyl group of the nitrogen SAM analogues (**2a-g**) (Chapter 1) was necessary in order to facilitate their eventual immobilization on Sepharose affinity matrices. Coupling to the matrix through the N^4-alkyl group would permit the immobilized ligand to serve as an analogue of SAM itself. Because no obvious potential binding interactions would be compromised, the resulting Sepharose/SAM affinity ligand conjugate could then be used to facilitate the purification of any enzyme which uses SAM as either a substrate or an allosteric effector.

The reactivity of the primary amino group of the spacer under alkaline conditions was evident when it was apparently responsible for the polymerization side reaction which plagued the amino acid deprotection of the fully

protected 5'-[6-(trifluoroacetamido)-1-hexyl] SAM affinity ligand (**14**) described earlier. This negative result demonstrates that this group might, as was originally expected, be sufficiently reactive to lead to covalent bond formation with a suitably activated polymeric matrix. Epoxy-activated and N-hydroxysuccinimide activated-ester Sepharose matrices are commercially available from Pharmacia. Coupling of the SAM affinity ligand (**9**) through the primary amino group of its spacer to such matrices would be through stable alkylamine and amide linkages, respectively. These Sepharose conjugates would be chemically stable under normal enzyme purification conditions and could conceivably be used repeatedly. Thus, coupling of the SAM affinity ligand (**9**) to both epoxy-activated Sepharose 6B and activated CH-Sepharose 4B was investigated.

According to the Pharmacia Affinity Chromatography-Principles and Methods handbook, the coupling of ligands to epoxy-activated Sepharose 6B is best carried out between pH 9-13. Assuming that the pK_a of the primary amino group of the 6-amino-1-hexyl spacer is approximately 10, about 95% of all such groups would exist in the reactive free base form at pH 11.5. Coupling at this pH would be expected to favor immobilization through the 6-amino-1-hexyl spacer rather than through the ribose 2',3'-diol (pK_a 12-12.5). Steric hindrance from the amino acid carboxylate group was expected to prevent or at least minimize immobilization through the α-amino group. Immobilization through the carboxylate group to form an ester with the Sepharose matrix was also conceivable. The adenine amino group is only a very weak base and thus would probably not play a role in the immobilization of the SAM affinity ligand. Thus, the primary amino group of the 6-amino-1-hexyl spacer was expected to be the most reactive nucleophile in the SAM affinity ligand (**9**) when the coupling reaction was conducted at pH 11.5.

The immobilization reaction was carried out at pH 11.5 according to the procedures outlined in the Pharmacia handbook. Any epoxide groups on the epoxy-activated Sepharose 6B matrix which had survived the coupling procedure were deactivated by treatment with ethanolamine. This treatment would have destroyed any ester linkages arising from an attack by the amino acid carboxylate group on an epoxide and thus re-solubilized the SAM affinity ligand (**9**) as its ethanolamido derivative. Because no ultraviolet-absorbing material could be washed from the Sepharose affinity matrix following overnight exposure to aqueous ethanolamine (1M), it was clear that immobilization of the ligand by an ester linkage to the carboxylate group of the ligand had not occurred. Immobilization had most likely occurred by attack on the epoxides on the Sepharose by the 6-amino-1-hexyl spacer, with minor contributions from attack by the α-amino group and/or the ribose 2',3'-diol anion.

The specific capacity of the coupled gel, defined as the quantity of bound ligand per mL of swollen adsorbent, was determined indirectly. Assuming that the SAM affinity ligand (**9**) had not decomposed during the coupling procedure, the amount of ligand which had been immobilized must be equal to the difference between the quantity originally present in the coupling solution and that which could be readily washed from the gel. Only the original coupling solution filtrate was observed to contain any ultraviolet-absorbing material. Because the only ultraviolet-absorbing compound which could be reisolated from this filtrate by cation exchange chromatography was the unmobilized SAM affinity ligand (**9**), the original assumption that this compound had not decomposed during the coupling procedure appeared to be correct. Thus, the difference between the quantity of the recovered SAM affinity ligand (**9**) and the amount originally present in the coupling solution was used to determine the specific capacity of the coupled gel to be 4.5 μequiv of bound ligand per mL.

This observation that the recovered quantity of the unmobilized SAM affinity ligand (**9**) was nearly identical to the quantity calculated to be present in the coupling solution filtrate using Beer's law indicates that no compound was lost during column operation. Consequently, the specific capacity of another batch of epoxy-activated Sepharose 6B/SAM affinity ligand conjugate (Figure 40) prepared in an identical manner could be readily determined from the absorbance of the coupling solution filtrate without reisolation of the unbound ligand (**9**).

Attempts to release adenine from the coupled gel by hydrolysis in aqueous hydrochloric acid, to provide direct evidence for the immobilization of the SAM affinity ligand, were inconclusive. The release of adenine would have been detected by thin layer chromatography on silica gel using ethanol:conc. NH_4OH (90:10) as the solvent system. Instead of observing adenine as an ultraviolet-absorbing spot at approximately R_f 0.70, another very intense ultraviolet-absorbing spot was seen at the origin. The identity of the material responsible for this spot was not determined but could have been furfural or a related furan compound. The acid-catalyzed hydrolysis of polymeric carbohydrates is a well-known method of preparing furfural.[213] Since Sepharose is actually a carbohydrate polymer, an analogous or identical reaction may have occurred in this case. Thus, any adenine which should have been released could have either reacted with the postulated furfural compounds and be present in spot at the origin or else be present in amounts too small to be seen even by thin layer chromatography.

In view of this result, probably the most direct and unambiguous method for directly demonstrating the presence of bound ligand in the coupled gel would involve the immobilization of a ^{3}H- or ^{14}C- labelled SAM affinity ligand (**9**). (Such a compound could conceivably be prepared from 5'-tritio-2',3'-O-

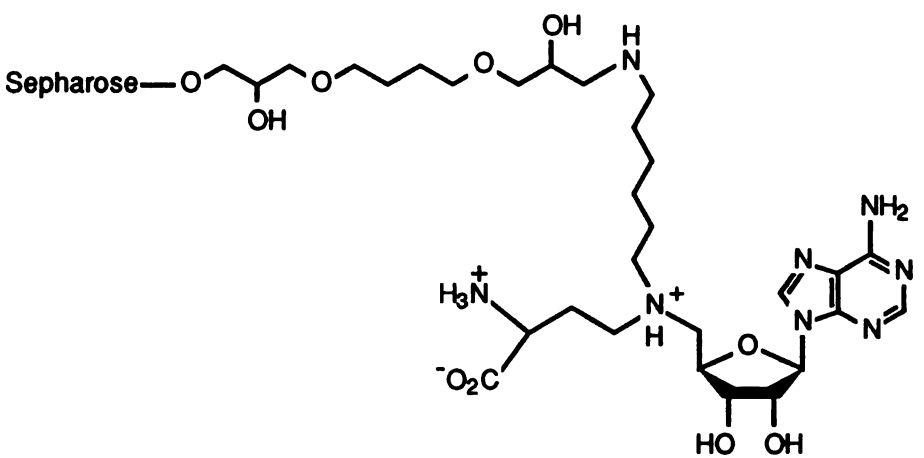


Figure 40. Postulated structure of the epoxy-activated Sepharose 6B/SAM affinity ligand conjugate.

isopropylideneadenosine, the synthesis of which is described in Chapter 3.) The production of a radioactive coupled gel would provide direct evidence for the immobilization of the SAM affinity ligand (**9**). The specific activity of the coupled gel would then be directly proportional to its specific capacity.

Without such a labelled compound, the method described above is the simplest and most practical for determining the specific capacity of the coupled gel. Because the commercially available Sepharose gel is specifically activated for this type of immobilization and because the SAM affinity ligand (**9**) is not decomposed during the coupling process, one can safely conclude that whatever quantity of this compound which "disappears" has indeed been immobilized.

Immobilization of the SAM affinity ligand (**9**) on activated CH-Sepharose 4B was also carried out following the procedures described in the Pharmacia Affinity Chromatography-Principles and Methods handbook. This Sepharose derivative contains N-hydroxysuccinimide ester groups, which will provide a very stable amide bond when attacked by a nucleophilic amine. According to the Pharmacia procedures, coupling to activated CH-Sepharose 4B should not be conducted above pH 10 to avoid excessive hydrolysis of the activated esters. At pH 10.0, about 50% of the primary amino groups (pK_a approximately 10) of the spacers will exist in the free base form, ready to attack an activated ester group. Whether the ribose 2',3'-diol (pK_a 12-12.5) would attack the N-hydroxy-succinimide ester as the neutral or anionic (less than 1%) species to form the corresponding ester is irrelevant because subsequent treatment with ethanol-amine would destroy the ester and re-solubilize the SAM affinity ligand (**9**). The same treatment would destroy any anhydride linkages arising from attack on the activated ester by the amino acid carboxylate group, provided that these groups had not been hydrolyzed first by water. An amide group formed by attack of the

adenine amino group would be labile and probably not survive the treatment with ethanolamine. The only serious competition to immobilization through the spacer would be the α-amino group. Coupling through this group is probably limited by steric hindrance from the adjacent carboxylate group. Thus, immobilization of the SAM affinity ligand (**9**) to the activated CH-Sepharose 4B was expected to occur predominantly through the spacer group, as it was originally intended.

The general coupling procedure described by Pharmacia was used to facilitate the immobilization of the SAM affinity ligand (**9**) on the activated CH-Sepharose 4B at pH 10.0. Any N-hydroxysuccinimide esters which had survived the coupling reaction were subsequently destroyed by treatment with aqueous ethanolamine (1 M, pH 9.0). The specific capacity of this coupled gel was again determined by the difference method assuming that the SAM affinity ligand (**9**) which had "disappeared" during the coupling procedure had been immobilized and had not somehow decomposed in the process. All of the ultraviolet-absorbing material which could be readily washed from the activated CH-Sepharose 4B appeared in the original coupling solution and aqueous ethanolamine filtrates. Cation exchange chromatography of these pooled filtrates was necessary to separate the unbound SAM affinity ligand from the ultraviolet-absorbing N-hydroxysuccinimide salts displaced from the Sepharose by the ligand or the ethanolamine. The unbound SAM affinity ligand (**9**) was the only adenine-containing compound which was isolated from the combined filtrates. The amount of the recovered SAM affinity ligand (**9**) was indeed less than that initially present in the coupling solution. The specific capacity of the activated CH-Sepharose 4B/SAM affinity ligand conjugate (Figure 41) was then readily determined by the difference method to be 5.1 μequiv of bound ligand per mL of coupled gel.

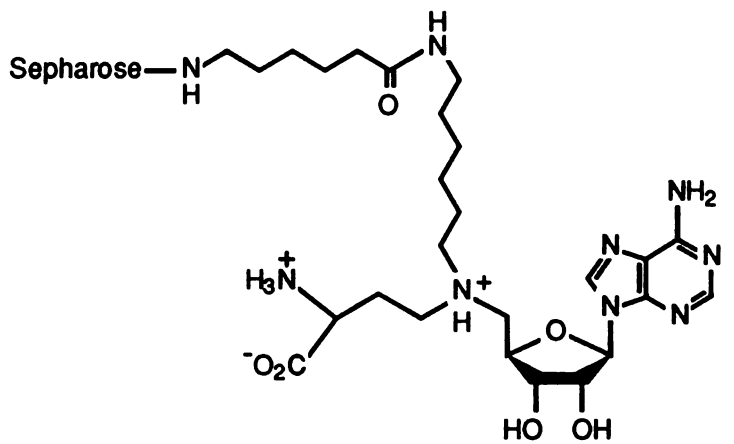


Figure 41. Postulated structure of the activated CH-Sepharose 4B/SAM affinity ligand conjugate.

An attempt to demonstrate directly that the immobilized SAM affinity ligand (Figure 41) could be re-solubilized by overnight treatment of the coupled gel with aqueous ammonia provided inconclusive results. Either the amount of re-solubilized SAM affinity ligand (**9**) was too small to be identified as such by thin layer chromatography or the amide bond linking it to the Sepharose affinity matrix was sufficiently stable to resist nucleophilic attack by ammonia. The stability of amide bonds has been already documented during the development of the SAM affinity ligand. During the deprotection of the 5'-[6-(1-phthalamoyl)-1-hexyl] SAM affinity ligand (**18**), hot aqueous sodium hydroxide (1.0 M) failed to hydrolyze an amide bond linking phthalate to the 6-amino-1-hexyl spacer. Also, the failure of sodium carbonate in warm 50% aqueous methanol to hydrolyze the amide bonds linking the monomers of the polymer generated during deprotection of the fully protected 5'-[6-(trifluoroacetamido)-1-hexyl] SAM affinity ligand (**14**) testifies to the stability of these bonds. Clearly, the most straightforward method of unambiguously demonstrating the presence of bound ligand in the coupled gel would again involve the immobilization of a ^{3}H- or ^{14}C-labelled SAM affinity ligand (**9**).

The difference method outlined above remains the most convenient procedure for determining the specific capacity of the coupled gel. Because the activated CH-Sepharose 4B has been specifically developed for this type of immobilization and because the SAM affinity ligand is otherwise stable during the coupling procedure, the compound which apparently has been "lost" has probably been immobilized on the gel.

Both Sepharose/SAM affinity ligand conjugates may exhibit some ion exchange character, which may or may not influence an enzyme purification. The deactivation of the excess epoxide groups present in the coupled epoxy-activated Sepharose 6B by treatment with ethanolamine results in the formation

of secondary amino groups. These amino groups will be protonated under normal enzyme purification conditions. The counterion associated with this protonated secondary amine will be exchangeable with any anion present in the buffer solution containing the enzyme being purified or with any anionic group present on the enzyme itself. Thus, the epoxy-activated Sepharose 6B/SAM affinity ligand conjugate (Figure 40) may possess some anion exchange properties. Similarly, in the preparation of the activated CH-Sepharose 4B/SAM affinity ligand conjugate (Figure 41), spontaneous hydrolysis of the N-hydroxysuccinimide esters before attack by the ligand or ethanolamine would provide free carboxylate groups. The counterions associated with these carboxylate groups would be similarly exchangeable with any buffer cations or cationic groups present in the enzyme being purified. Thus, any successful purification of an enzyme or other protein using these or other affinity matrices might ultimately be made possible by an interaction of the inherent cation exchange and affinity properties, rather than by the affinity properties alone.

The possibility that other SAM-binding proteins may also be temporarily immobilized on either affinity adsorbent necessitates that the enzyme of interest undergo preliminary purification using other techniques. The prior removal of such proteins ensures that they will not be co-purified with that enzyme. So, for best results, these affinity adsorbents should be used at the end of a purification scheme to remove the last 10 or 15 extraneous proteins. Once these contaminants have been washed from the matrix, a change in the buffer temperature, ionic strength, surface tension, or pH, or the addition of the unmobilized SAM affinity ligand or SAM itself to the buffer would re-solubilize the immobilized enzyme and lead to its elution in pure form.

In conclusion, the synthesis of N^4-(5'-adenosyl)-N^4-(6-amino-1-hexyl)-2(R,S),4-diaminobutyric acid (**9**), a structural analogue of SAM, has been achieved. The central feature of this compound is the 1,6-diaminohexane group which replaces the methylsulfonium moiety of SAM. Immobilization of this compound on an insoluble polymeric matrix is possible through the 6-amino-1-hexyl group. Protonation of the tertiary amino group at physiological pH will enable this immobilized compound to be a stable analogue of SAM. An affinity adsorbent containing the compound coupled in this fashion could be used to immobilize SAM-binding proteins temporarily. Because no obvious binding interactions with these proteins are compromised, this affinity adsorbent would provide a better alternative to others in which L-SAH is immobilized through its α-amino or carboxylate groups. The preparation and immobilization of this analogue of SAM should ultimately provide a new laboratory tool to facilitate the isolation of and improve the general understanding of methyltransferases and other SAM-binding proteins.

Materials and Methods

1,6-Diaminohexane, trifluoroacetic anhydride, 2',3'-O-isopropylidene-adenosine, triphenylphosphine, diethyl azodicarboxylate, phthalimide (and its potassium salt), 6-amino-1-hexanol, ethyl trifluoroacetate, p-toluenesulfonyl chloride, 1,6-dibromohexane, benzyl chloroformate, palladium (10%) on activated carbon, and flash silica gel (230-400 mesh) were obtained from Aldrich. The SP-Sephadex C-25 cation exchanger was purchased form Sigma. Epoxy-activated Sepharose 6B and activated CH-Sepharose 4B were obtained from Pharmacia. Methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) was prepared as reported previously (in Chapter 1). Analytical thin layer chromatography was carried out with precoated Baker 1B2-F silica gel sheets obtained from VWR. Low pressure liquid chromatography was conducted with a Lobar Size B (310-25) Lichroprep^{TM} Si60 silica gel column purchased from VWR. This column was integrated into an Altex 100 HPLC system equipped with an Altex Model 153 Analytical UV detector (280 nm). All melting points were taken on a Thomas-Hoover melting point apparatus and are uncorrected. A Kratos MS-50 mass spectrometer equipped with a 23-kG magnet, a postacceleration detector operated at -10kV, and a source constructed by the UCSF Mass Spectrometry Facility was used to obtain the positive LSIMS mass spectra. Samples for positive LSIMS were prepared in a matrix of glycerol and 1M hydrochloric acid. Low resolution EI mass spectra were obtained on a Kratos MS-25 mass spectrometer using a heated direct insertion probe. A Hitachi 100-80 UV-VIS spectrometer, equipped with an automatic six-cell changer and a Peltier temperature controller, was used to obtain the UV spectra. Proton NMR spectra were acquired with either a General Electric GN-500 NMR spectrometer or a home-built 240 MHz NMR spectrometer interfaced with a Nicolet 1180 computer and a 293-B pulse programmer. NMR samples were dissolved in either deuteriochlor-

oform or D_2O, and the acquired spectra referenced to the tetramethylsilane or HDO (4.80 ppm) peaks, respectively. The Microanalytical Laboratory in the Department of Chemistry at the University of California, Berkeley performed all microanalyses.

5'-(6-Amino-1-Hexyl)Amino-5'-Deoxy-2',3'-O-Isopropylideneadenosine (**10**). 5'-O-Tosyl-2',3'-O-isopropylideneadenosine (**3**) (15.00 g, 32.5 mmol) was dissolved in molten 1,6-diaminohexane (100 g, 862 mmol) at 100°C.. This mixture was heated with stirring at this temperature for six hours, after which the excess 1,6-diaminohexane was removed by vacuum distillation. The dark glassy residue was dissolved in hot ethanol (250 mL) and evaporated to a dark foam. This foam was partitioned between methylene chloride (300 mL) and 0.1N aqueous sodium hydroxide (325 mL), and the aqueous layer extracted with additional methylene chloride (2 x 200 mL). The pooled methylene chloride extracts were washed with saturated aqueous sodium chloride (8 x 150 mL) and evaporated to dryness. Water (200 mL) was added to the dark residue, followed by a minimal amount of 1M aqueous hydrochloric acid in 1-2 mL aliquots to effect complete dissolution and lower the pH to neutrality. The dark solution of the dihydrochloride salt of the product was then evaporated to dryness. The residue was redissolved in methanol (100 mL) and concentrated to approximately 5 mL before being loaded onto the top of a wet-packed flash silica gel column (46 x 3.5 cm, 230 g). This column was eluted with methanol:chloroform (25:75) at a flow rate of 10 mL/min and 28 mL fractions were collected. The dihydrochloride salt of the product was eluted with a faster-moving contaminant in fractions 42-63 and in pure form (as judged by TLC) in fractions 64-200. Each group of fractions was pooled and evaporated to dryness. The residue from pooled fractions 42-63 was dissolved in methanol (100 mL), concentrated to 5 mL, and loaded onto the top of another wet-packed

flash silica gel column (32 x 3.5 cm, 150 g) for repurification. The dihydrochloride salt of the product was eluted in pure form (as judged by TLC) in fractions 60-110, under conditions otherwise identical to those used in the initial purification. These fractions were pooled with the material obtained from fractions 64-200 from the initial purification and evaporated to dryness. The residue was partitioned between methylene chloride (200 mL) and 0.1N aqueous sodium hydroxide (200 mL), and the aqueous phase extracted with additional methylene chloride (2 x 200 mL). The combined methylene chloride extracts were washed with saturated aqueous sodium chloride (2 x 200 mL), dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. The residue was twice dissolved in chloroform (100 mL) and evaporated to give 3.01 g (22.9%) of product as a light brown foam. The recovered product contained a trace amount of an unidentified impurity and was used directly without further purification: mp 58-63°C.; TLC (dihydrochloride salt), methanol:chloroform (25:75), R_f 0.11 (UV, I_2); 500 MHz ^{1}H NMR ($CDCl_3$, free base), δ1.29 (m, 4H, $NH_2CH_2CH_2C\mathit{H}_2C\mathit{H}_2$), 1.39 (s, 3H, isopropylidene CH_3), 1.41 (m, 4H, $NH_2CH_2C\mathit{H}_2CH_2CH_2C\mathit{H}_2$), 1.62 (s, 3H, isopropylidene CH_3), 2.50-2.63 (m, 2H, $NH_2C\mathit{H}_2$ or $CH_2C\mathit{H}_2NHCH_2$), 2.66 (m, 2H, $CH_2C\mathit{H}_2NHCH_2$ or $NH_2C\mathit{H}_2$), 2.85 (dd, 1H, J = 6.6Hz, 12.5Hz, H-5'), 2.90 (dd, 1H, J = 4.2Hz, 12.5Hz, H-5'), 4.38 (m,1H, J = 3.3Hz, 4.2Hz, 6.6Hz, H-4'), 5.02 (dd, 1H, J = 3.3Hz, 6.2Hz, H-3'), 5.49 (dd, 1H, J = 3.0Hz, 6.2Hz, H-2'), 5.84 (br s, 2H, adenine NH_2), 6.03 (d, 1H, J = 3.0Hz, H-1'), 7.93 (s, 1H, adenine H-2 or H-8), 8.35 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 11.3 x 10^3); MS (positive LSIMS), m/e 406 $(M + H)^+$.

5'-[6-(Trifluoroacetamido)-1-Hexyl]Amino-5'-Deoxy-2',3'-O-Isopropylideneadenosine (Method A) (**11**). 5'-(6-Amino-1-hexyl)amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**10**) (1.66 g, 4.10 mmol) and N,N-diisopropyl-

ethylamine (0.78 mL, 4.51 mmol, 10% excess) were dissolved with stirring in methylene chloride (100 mL, freshly distilled from phosphorus pentoxide). A solution of trifluoroacetic anhydride (0.64 mL, 4.51 mmol, 10% excess) in methylene chloride (25 mL, freshly distilled from phosphorus pentoxide) was added and the mixture stirred at room temperature overnight. The evaporated reaction mixture was partitioned between methylene chloride (50 mL) and 0.1N aqueous sodium carbonate (50 mL), and the aqueous layer extracted with additional methylene chloride (2 x 50 mL). The pooled methylene chloride extracts were washed with saturated aqueous sodium chloride (2 x 50 mL), dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. The residue was dissolved in chloroform (100 mL), concentrated to approximately 3 mL, and loaded onto the top of a wet-packed flash silica gel column (42 x 3.5 cm, 200 g). This column was eluted with chloroform:isopropanol (70:30) at a flow rate of 10 mL/min and 28 mL fractions were collected. A mixture of bis-trifluoroacetylated side products was eluted in fractions 14-22, which were pooled and evaporated to dryness. The residue was twice dissolved in chloroform (100 mL) and evaporated to give 600 mg (24.6%) of a colorless foam [mp 49-58°C.; TLC, chloroform:isopropanol (70:30), R_f 0.70 (UV); MS (positive LSIMS), m/e 598 $(M + H)^+$]. The desired product was eluted in fractions 61-85, which were pooled and evaporated to dryness. The residue was twice dissolved in chloroform (50 mL) and evaporated to give 93 mg (4.5%) of product as a colorless thin film: TLC, chloroform:isopropanol (70:30), R_f 0.15 (UV,I_2); other characterization data are listed in Method B below.

5'-Deoxy-5'-Phthalimido-2',3'-O-Isopropylideneadenosine. According to the procedure of Kolb et al.,[204] 2',3'-O-isopropylideneadenosine (20.000 g, 65.4 mmol), phthalimide (9.614 g, 65.4 mmol), and triphenylphosphine (17.135

g, 65.4 mmol) were suspended with stirring in tetrahydrofuran (250 mL, freshly distilled from calcium hydride). Diethyl azodicarboxylate (10.28 mL, 65.4 mmol) was then added and the suspension stirred at room temperature for three hours. The reaction mixture was filtered and the recovered precipitate washed with diethyl ether (4 x 25 mL) and dried in vacuo overnight to give 17.31 g (61.1%) of product as a colorless solid: mp 147-150°C. (lit.[204] 198°C.); 500 MHz ^{1}H NMR ($CDCl_3$), δ 1.38 (s, 3H, isopropylidene CH_3), 1.59 (s, 3H, isopropylidene CH_3), 3.98 (dd, 1H, J = 6.0Hz, 13.4Hz, H-5'), 4.06 (dd, 1H, J = 6.0Hz, 13.4Hz, H-5'), 4.55 (m, 1H, H-4'), 5.26 (d, 1H, J = 8.9Hz, H-3'), 5.54 (d, 1H, J = 6.3Hz, H-2'), 5.78 (br s, 2H, adenine NH_2), 6.04 (s, 1H, H-1'), 7.71 (m, 2H, isoindole H-5 and H-6), 7.79 (m, 2H, isoindole H-4 and H-7), 7.87 (s, 1H, adenine H-2 or H-8), 8.06 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 241 nm (ε 14.0 x 10^3), 259 nm (ε 14.2 x 10^3); MS (positive LSIMS), m/e 437 $(M + H)^+$, 302 (M + H - adenine)$^+$.

5'-Amino-5'-Deoxy-2',3'-O-Isopropylideneadenosine (**13**). Following the procedure described by Kolb et al.,[204] 5'-deoxy-5'-phthalimido-2',3'-O-isopropylideneadenosine (10.509 g, 24.2 mmol) and anhydrous hydrazine (1.40 mL, 44.2 mmol) were dissolved in absolute ethanol (600 mL). The stirred solution was refluxed overnight, and after cooling to room temperature, was filtered to remove the phthalhydrazide precipitate and evaporated to dryness. The residue was dissolved in water (150 mL) and glacial acetic acid added in 1-2 mL aliquots to lower the pH to 4. The precipitate was removed by filtration and the pH of the solution increased to 10-11 by the addition of 4N aqueous sodium hydroxide. The alkaline solution was extracted with chloroform (4 x 100 mL) and the pooled chloroform extracts dried over anhydrous magnesium sulfate. The filtered chloroform solution was evaporated to give 6.35 g (86.0%) of product as a colorless solid: mp 198-200°C. (lit.[204] 203°C.); 500 MHz ^{1}H

NMR ($CDCl_3$), δ 1.40 (s, 3H, isopropylidene CH_3), 1.63 (s, 3H, isopropylidene CH_3), 2.96 (dd, 1H, J = 5.9Hz, 12.4Hz, H-5'), 3.04 (dd, 1H, J = 4.6Hz, 12.4Hz, H-5'), 4.26 (m, 1H, J = 3.5Hz, 5.9Hz, H-4'), 5.03 (dd, 1H, J = 3.5Hz, 6.5Hz, H-3'), 5.48 (dd, 1H, J = 3.0Hz, 6.5Hz, H-2'), 5.96 (br s, 2H, adenine NH_2), 6.04 (d, 1H, J = 3.0Hz, H-1'), 7.93 (s, 1H, adenine H-2 or H-8), 8.35 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 13.1 x 10^3); MS (positive LSIMS), m/e 307 $(M + H)^+$.

6-(Trifluoroacetamido)-1-Hexanol. Following the procedure of Connolly et al.,[187] ethyl trifluoroacetate (21.6 mL, 181.7 mmol) was added to 6-amino-1-hexanol (18.000 g, 153.8 mmol) and the mixture stirred at room temperature for approximately five hours. Water (300 mL) was then added and the mixture stirred at 4°C. for 16 hours during which the product crystallized. The crystals were filtered, washed with cold water (3 x 25 mL), and dried *in vacuo* to give 17.31 g (52.8%) of pure colorless product: mp 43-46°C. (lit.[187] mp 48-49°C., lit.[182] mp 52-53°C.); 500 MHz ^1H NMR ($CDCl_3$), δ 1.40 (m, 4H, $CH_2CH_2CH_2CH_2OH$), 1.60 (m, 4H, $CH_2CH_2CH_2CH_2CH_2OH$), 3.37 (q, 2H, J = 6.7Hz, $NHCH_2$), 3.66 (t, 2H, J = 6.3Hz, CH_2OH), 6.58 (br s, 1H, NH); MS (positive LSIMS), m/e 214 $(M + H)^+$.

1-(p-Tolylsulfonyloxy)-6-(Trifluoroacetamido)Hexane. The procedure of Chipowsky and Lee[182] was followed in the synthesis of this compound, although its purification was carried out by other means involving flash chromatography. 6-(Trifluoroacetamido)-1-hexanol (17.00 g, 79.8 mmol) was dissolved in anhydrous pyridine (150 mL). After cooling to 0°C., p-toluenesulfonyl chloride (16.682 g, 87.8 mmol) was added and the mixture stirred overnight at 4°C.. The mixture was then partitioned between water (500 mL) and chloroform (500 mL), and the aqueous layer was washed with chloroform (250 mL). The combined chloroform extracts were washed with 1M

aqueous sulfuric acid (4 x 750 mL), water (500 mL), and saturated aqueous sodium chloride (2 x 500 mL), and dried over anhydrous magnesium sulfate. The filtered chloroform solution was concentrated to 5-10 mL and loaded onto the top of a wet-packed flash silica gel column (49 x 3.5 cm, 230 g). The column was eluted with ethyl acetate:hexanes (30:70) at a flow rate of 10 mL/min and 28 mL fractions were collected. Pure product (as judged by TLC) appeared in fractions 40-80, which were pooled and evaporated to dryness. The residue was twice dissolved in chloroform (100 mL) and evaporated to dryness to give a colorless oil which gradually crystallized to give 18.59 g (63.4%) of pure product: mp 43-46°C. (lit.[182] 50-51°C.); TLC, ethyl acetate:hexanes (30:70), R_f 0.26 (UV,I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.35-1.48 (m, 4H, $NHCH_2CH_2C\mathit{H}_2C\mathit{H}_2$), 1.61 (m, 2H, $NHCH_2C\mathit{H}_2$), 1.83 (m, 2H, $C\mathit{H}_2CH_2O$), 2.46 (s, 3H, CH_3), 3.33 (q, 2H, J = 6.7Hz, $NHC\mathit{H}_2$), 4.02 (t, 2H, CH_2O), 6.58 (br s, 1H, NH), 7.36 (d, 2H, J = 8.2Hz, tosyl H-3 and H-5), 7.78 (d, 2H, J = 8.2Hz, tosyl H-2 and H-6); MS (EI), m/e 367 (M^+), 196 (M - tosylate)$^+$.

Anal. Calculated for $C_{15}H_{20}F_3NO_4S$: C, 49.04; H, 5.49; N, 3.81; S, 8.73. Found: C, 48.92; H, 5.49; N, 3.81; S, 8.59.

1-Iodo-6-(Trifluoroacetamido)Hexane (**12**). The synthesis of this compound was carried out according to the procedure of Chipowsky and Lee,[182] whereas its purification was achieved by other methods involving chromatography. A mixture of 1-(p-tolylsulfonyloxy)-6-(trifluoroacetamido)hexane (18.17 g, 49.5 mmol) and sodium iodide (11.15 g, 74.3 mmol, 50% excess) in 2-butanone (150 mL) was heated to 80°C. for three hours. After cooling to room temperature, all solvent was evaporated. Chloroform (150 mL) was added to the residue and the insoluble material was filtered and washed with chloroform (2 x 150 mL). The combined chloroform extracts were concentrated to approximately 10 mL and loaded onto the top of a wet-packed

flash silica gel column (50 x 3.5 cm, 230 g). The column was eluted with ethyl acetate:hexanes (20:80) at a flow rate of 10 mL/min and 28 mL fractions were collected. Pure product (as judged by TLC) appeared in fractions 22-36, which were pooled and evaporated to dryness. The residue was twice dissolved in chloroform (100 mL) and evaporated to dryness to give a colorless oil which crystallized to give 13.95 g (87.2%) of pure product: mp 40-42°C. (lit.[182] 47-47.5°C.); TLC, ethyl acetate:hexanes (20:80), R_f 0.34 (UV,I_2); 500 MHz ^{1}H NMR ($CDCl_3$), δ 1.37 (m, 2H, $NHCH_2CH_2CH_2$), 1.45 (m, 2H, $CH_2CH_2CH_2I$), 1.61 (m, 2H, $NHCH_2CH_2$), 1.83 (m, 2H, CH_2CH_2I), 3.19 (t, 2H, J = 6.9Hz, CH_2I), 3.37 (q, 2H, J = 6.8Hz, $NHCH_2$), 6.49 (br s, 1H, NH); MS (EI), m/e 323 (M^+), 196 $(M - I)^+$.

Anal. Calculated for $C_8H_{13}F_3INO$: C, 29.74; H, 4.06; N, 4.34; I, 39.28. Found: C, 29.79; H, 4.14; N, 4.32; I, 39.13.

5'-[6-(Trifluoroacetamido)-1-Hexyl]Amino-5'-Deoxy-2',3'-O-Isopropylideneadenosine (Method B) (**11**). 5'-Amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**13**) (3.060 g, 10.0 mmol) and N,N-diisopropylethylamine (1.74 mL, 10.0 mmol) were dissolved in acetonitrile (250 mL) and heated with stirring to 70°C.. 1-Iodo-6-(trifluoroacetamido)hexane (**12**) (3.230 g, 10.0 mmol) was then added to the hot solution in one portion. After stirring for 18 hours at 70°C., the mixture was cooled to room temperature and evaporated to dryness. The residue was partitioned between methylene chloride (200 mL) and 0.1N aqueous sodium carbonate (200 mL), and the aqueous layer washed with methylene chloride (2 x 200 mL). The combined methylene chloride extracts were washed with saturated aqueous sodium chloride (2 x 200 mL), dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. The residue was dissolved in chloroform (50 mL), concentrated to 5-10 mL, and loaded onto a wet-packed flash silica gel column (49 x 3.5 cm, 230 g). The column was eluted with chloroform:isopropanol (70:30) at a flow rate of 10 mL/min and 28

mL fractions were collected. Partially pure (as judged by TLC) dialkylated side product appeared in fractions 19-30, which were pooled and evaporated to dryness. The residue was twice dissolved in chloroform (100 mL) and evaporated to give 1.609 g (22.8%) of almost pure dialkylated material as a colorless foam. Fractions 35-85 contained the desired monoalkylated product in pure form (as judged by TLC). After evaporating these pooled fractions to dryness, the residue was twice dissolved in chloroform (100 mL) and evaporated to give 2.332 g (45.7%) of pure monoalkylated product as a colorless foam: mp 54-56°C.; TLC, chloroform:isopropanol (70:30), R_f 0.27 (UV,I_2); 500 MHz ^{1}H NMR ($CDCl_3$), δ 1.29 (m, 4H, $NHCH_2CH_2C\mathit{H}_2C\mathit{H}_2$), 1.39 (s, 3H, isopropylidene CH_3), 1.42 (m, 2H, $CH_2C\mathit{H}_2CH_2NHCH_2$), 1.54 (m, 2H, $CONHCH_2C\mathit{H}_2$), 1.62 (s, 3H, isopropylidene CH_3), 2.57 (m, 2H, $CH_2CH_2C\mathit{H}_2NHCH_2$), 2.89 (m, 2H, H-5'), 3.34 (m, 2H, $CONHC\mathit{H}_2$), 4.39 (m, 1H, H-4'), 5.04 (dd, 1H, J = 2.8Hz, 5.8Hz, H-3'), 5.50 (dd, 1H, J = 2.4Hz, 5.8Hz, H-2'), 5.77 (br s, 2H, adenine NH_2), 6.03 (d, 1H, J = 2.4Hz, H-1'), 6.77 (br s, 1H, CONH), 7.94 (s, 1H, adenine H-2 or H-8), 8.34 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 13.8 x 10^3); MS (positive LSIMS), m/e 502 $(M + H)^+$, 367 $(M + H - adenine)^+$.

Anal. Calculated for $C_{21}H_{30}F_3N_7O_4 \cdot 0.5H_2O$: C, 49.41; H, 6.12; N, 19.21. Found: C, 49.42; H, 5.90; N, 19.17.

An analytical sample of the dialkylated side product was prepared by low pressure liquid chromatography using a Lobar Size B (310-25) Lichroprep™ Si60 silica gel column. The column was eluted with chloroform:isopropanol (80:20) at a flow rate of 4.0 mL/min. Injection of 0.70 mL aliquots of the partially purified material, redissolved in the eluting solvent, onto the column led to the appearance (as detected by absorbance at 280 nm) of pure material 41-51 minutes and nearly pure material 51-71 minutes later. Both fractions were

collected and evaporated to dryness. The latter fraction was reinjected onto the column to remove a slower-moving impurity. The residual amber color in a chloroform solution (2 mL) of the combined purified fractions was removed by filtration through a wet-packed flash silica gel column (48 x 3.5 cm, 230 g). Elution with chloroform:isopropanol (85:15) at a flow rate of 10 mL/min and collection of 28 mL fractions led to the appearance of the dialkylated material in fractions 35-50. These fractions were pooled and evaporated to dryness. The residue was twice dissolved in chloroform (100 mL) and evaporated to give 244 mg of analytically pure 5'-deoxy-5'di[6-(trifluoroacetamido)-1-hexyl]amino-2',3'-O-isopropylideneadenosine as a hygroscopic colorless foam: mp 45-47°C.; TLC, chloroform:isopropanol (70:30), R_f 0.53 (UV,I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.19-1.31 (m, 12H, $NHCH_2CH_2CH_2CH_2CH_2$), 1.40 (s, 3H, isopropylidene CH_3), 1.51 (m, 4H, $NHCH_2CH_2$), 1.61 (s, 3H, isopropylidene CH_3), 2.38 (m, 4H, $CH_2CH_2NCH_2CH_2$), 2.62 (m, 2H, H-5'), 3.33 (m, 4H, $NHCH_2$), 4.35 (m, 1H, H-4'), 4.98 (dd,1H, J = 3.0Hz, 6.4Hz, H-3'), 5.57 (dd, 1H, J = 1.9Hz, 6.4Hz, H-2'), 5.73 (br s, 2H, adenine NH_2), 6.07 (d, 1H, J = 1.9Hz, H-1'), 6.83 (br s, 2H, CONH), 7.94 (s, 1H, adenine H-2 or H-8), 8.34 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 15.2 x 10^3); MS (positive LSIMS), m/e 697 $(M + H)^+$, 562 $(M + H - adenine)^+$.

Anal. Calculated for $C_{29}H_{42}F_6N_8O_5 \cdot 0.5H_2O$: C, 49.36; H, 6.14; N, 15.88. Found: C, 49.48; H, 6.12; N, 15.86.

Methyl N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-N^4-[6-(Trifluoroacetamido)-1-Hexyl]-2(R,S)-Trifluoroacetamido-4-Aminobutyrate [Protected 5'-[6-(Trifluoroacetamido)-1-Hexyl] SAM Affinity Ligand] (**14**). 5'-[6-(Trifluoroacetamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine hemihydrate (**11**) (4.500 g, 8.82 mmol) and N,N-diisopropylethylamine (1.72 mL, 9.88 mmol, 10% excess) were dissolved in acetonitrile (150 mL) and heated with stirring to

70°C.. A solution of methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) (3.805 g, 11.28 mmol, 25% excess) in acetonitrile (50 mL) was added over approximately 15 minutes. The mixture was stirred at 70°C. for three days, during which its initial pale brown color gradually deepened. The cooled reaction mixture was evaporated to dryness, redissolved in chloroform (100 mL), concentrated to 5 mL, and loaded onto the top of a wet-packed flash silica gel column (48 x 3.5 cm, 230 g). The column was eluted with chloroform:isopropanol (80:20) at a flow rate of 10 mL/min and 28 mL fractions were collected. Partially purified product (as judged by TLC) appeared in fractions 16-29, which were pooled and concentrated to approximately 15 mL. This material was further purified by low pressure liquid chromatography using a Lobar Size B (310-25) Lichroprep™ Si60 silica gel column. This column was eluted with chloroform:isopropanol (80:20) at a flow rate of 4.0 mL/min. The injection of 0.70 mL aliquots of partially purified product onto the column led to the appearance (as detected by absorbance at 280 nm) of product 29-46 minutes later. Column eluate bearing the product was collected, evaporated, redissolved in the eluting solvent (15 mL), and reinjected until all impurities were removed. The residual amber color in a chloroform solution (5 mL) of the product was removed by filtration through a wet-packed flash silica gel column (42 x 3.5 cm, 200 g). The column was eluted with chloroform:isopropanol (90:10) at a flow rate of 10 mL/min and 28 mL fractions were collected. Ultraviolet-absorbing material appeared in fractions 21-37, which were pooled and evaporated. The residue was twice dissolved in chloroform (50 mL) and evaporated to give 1.54 g (23.4%) of analytically pure product as a colorless foam: mp 48-56°C.; TLC, chloroform:isopropanol (80:20), individual diastereomers at R_f 0.53 and 0.58 (UV,I_2); 500 MHz ^{1}H NMR ($CDCl_3$), δ 1.03-1.14 (m, 4H, $CH_2CH_2CH_2CH_2N$), 1.19 (m, 2H, $CH_2CH_2CH_2NCH_2$), 1.39 (s,

1.5H, isopropylidene CH_3), 1.40 (s, 1.5H, isopropylidene CH_3), 1.47 (m, 2H, $CF_3CONHCH_2CH_2$), 1.61 (s, 1.5H, isopropylidene CH_3), 1.63 (s, 1.5H, isopropylidene CH_3), 1.85 (m, 2H, $CHCH_2CH_2N$), 2.30-2.45 (m, 2H, $CH_2CH_2CH_2NCH_2$), 2.54 (m, 2H, $CHCH_2CH_2N$), 2.69-2.95 (m, 2H, H-5'), 3.33 (m, 2H, $CF_3CONHCH_2$), 3.67 (s, 1.5H, CO_2CH_3), 3.76 (s, 1.5H, CO_2CH_3), 4.34 (m, 1H, H-4'), 4.51 (m, 1H, $CHCH_2CH_2N$), 5.00 (m, 1H, H-3'), 5.53 (m, 1H, H-2'), 5.80 (br s, 1H, adenine NH_2), 5.83 (br s, 1H, adenine NH_2), 6.06 (d, 1H, J = 6.6Hz, H-1'), 6.96 (br s, 0.5H, $CF_3CONHCH_2CH_2$), 7.15 (br s, 0.5H, $CF_3CONHCH_2CH_2$), 7.84 (s, 0.5H, adenine H-2 or H-8), 7.89 (s, 0.5H, adenine H-2 or H-8), 8.33 (s, 1H, adenine H-2 or H-8), 9.39 (br d, 0.5H, J = 5.7Hz, $CF_3CONHCH$), 9.54 (br d, 0.5H, J = 6.3Hz, $CF_3CONHCH$); UV λ_{max} (MeOH) 259 nm (ε 14.3 x 10^3); MS (positive LSIMS), m/e 713 $(M + H)^+$, 578 $(M + H - adenine)^+$.

Anal. Calculated for $C_{28}H_{38}F_6N_8O_7 \cdot H_2O$: C, 46.03; H, 5.52; N, 15.34. Found: C, 45.66; H, 5.30; N, 15.13.

Attempted Synthesis of N^4-(6-Amino-1-Hexyl)-N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-2(R,S),4-Diaminobutyric Acid. A solution of sodium carbonate (63 mg, 0.599 mmol) in 50% aqueous methanol (4 mL) was added to methyl N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-[6-(trifluoroacetamido)-1-hexyl]-2(R,S)-trifluoroacetamido-4-aminobutyrate monohydrate (**14**) (125 mg, 0.171 mmol). The mixture was heated with stirring to 60°C. for six hours. The progress of the reaction was followed by TLC (100% methanol solvent system) of aliquots (1 µL) removed every ten minutes over the first hour and one every hour afterwards. Starting material at R_f 0.81 (UV, I_2) was completely gone after 10 minutes and gave rise to four slower-moving spots at R_f 0.70 (UV, I_2), 0.36 (UV, I_2, ninhydrin), 0.19 (UV, I_2, ninhydrin), and 0.00 (UV, I_2, ninhydrin). After 3-4 hours, only the spot at the origin remained. The R_f 0.70 and 0.36 spots

presumably corresponded to the mono- and bis-detrifluoroacetylated intermediates, respectively. The third spot at R_f 0.19 was probably indicative of the further loss of the methyl ester protecting group giving rise to the desired 2',3'-O-isopropylidene intermediate. The fourth spot at the origin, indicative of a fourth reaction proceeding in the flask, probably corresponded to a polymerization side reaction occurring between the 6-amino-1-hexyl amino group of one molecule with the methyl ester of another molecule under the prevailing alkaline conditions. All attempts to minimize this presumed polymerization side reaction by either 100-fold dilution or neutralization after 25 minutes failed.

N-(6-Bromo-1-hexyl)Phthalimide (**15**). In a modification of the general procedure of Donahoe et. al.,[208] a mixture of potassium phthalimide (5.55 g, 30.0 mmol) and 1,6-dibromohexane (46.2 mL, 300.0 mmol) was heated with stirring at 150°C. for four hours. After cooling to room temperature, the mixture was diluted with chloroform (50 mL), filtered, and the colorless insoluble material washed with chloroform (2 x 50 mL). Removal of chloroform from the combined extracts by rotary evaporation and excess 1,6-dibromohexane by vacuum distillation left a yellow-brown oil. The oil was dissolved in chloroform (100 mL), concentrated to 5 mL, and loaded onto the top of a wet-packed flash silica gel column (48 x 3.5 cm, 230 g). The column was eluted with ethyl acetate:hexanes (25:75) at a flow rate of 10 mL/min and 28 mL fractions were collected. Pure product (as judged by TLC) appeared in fractions 27-43. These fractions were pooled and evaporated to dryness, then twice dissolved in chloroform (100 mL) and again evaporated to dryness to give 6.99 g (75.2%) of pure colorless product: mp 57-58°C.; TLC, ethyl acetate:hexanes (25:75), R_f 0.35 (UV, I_2); 500 MHz ^{1}H NMR ($CDCl_3$), δ 1.37 (m, 2H, $NCH_2CH_2CH_2$), 1.49 (m, 2H, $CH_2CH_2CH_2Br$), 1.70 (m, 2H, NCH_2CH_2), 1.86 (m, 2H, CH_2CH_2Br),

3.40 (t, 2H, J = 7.2Hz, CH_2Br), 3.69 (t, 2H, J = 7.2Hz, NCH_2), 7.72 (m, 2H, isoindole H-5 and H-6), 7.84 (m, 2H, isoindole H-4 and H-7); UV λ_{max} (MeOH) 241 nm (ε 10.2 x 10^3), 293 nm (1.8 x 10^3); MS (EI), m/e 309 (M^+, ^{79}Br), 311 (M^+, ^{81}Br).

Anal. Calculated for $C_{14}H_{16}BrNO_2$: C, 54.21; H, 5.20; N, 4.52; Br, 25.76. Found: C, 54.17; H, 5.20; N, 4.49; Br, 25.83.

5'-[6-(Phthalimido)-1-Hexyl]Amino-5'-Deoxy-2',3'-O-Isopropylidene-adenosine (**16**). 5'-Amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**13**) (3.825 g, 12.5 mmol) and N,N-diisopropylethylamine (2.17 mL, 12.5 mmol) were dissolved in acetonitrile (300 mL) and heated with stirring to 70°C.. N-(6-Bromo-1-hexyl)phthalimide (**15**) (3.875 g, 12.5 mmol) was then added in one portion and the mixture stirred at 70°C. for approximately 18 hours. After cooling, the mixture was evaporated to dryness. The residue was partitioned between methylene chloride (150 mL) and 0.1N aqueous sodium hydroxide (150 mL), and the aqueous layer was washed with methylene chloride (2 x 150 mL). The combined methylene chloride extracts were washed with saturated aqueous sodium chloride (2 x 150 mL), dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. The residue was dissolved in chloroform (50 mL), concentrated to approximately 5 mL, and loaded onto a wet-packed flash silica gel column (48 x 3.5 cm, 230 g). The column was eluted with chloroform:methanol (95:5) at a flow rate of 10 mL/min and 28 mL fractions were collected. Fractions 13-21 contained partially pure (as judged by TLC) dialkylated side product. These fractions were pooled and evaporated to dryness, and the residue twice dissolved in chloroform (50 mL) and evaporated to give 2.13 g (22.0%) of crude dialkylated material as a slightly yellow oil. The desired monoalkylated product appeared in pure form (as judged by TLC) in fractions 26-50, which were pooled and evaporated to dryness. The residue

was twice dissolved in chloroform (50 mL) and evaporated to give 2.96 g (43.5%) of pure monoalkylated product as a colorless foam: mp 53-56°C.; TLC,chloroform:methanol (95:5), R_f 0.16 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.30 (m, 4H, $CH_2CH_2CH_2CH_2NH$), 1.39 (s, 3H, isopropylidene CH_3), 1.43 (m, 2H, CH_2CH_2NH), 1.62 (s, 3H, isopropylidene CH_3), 1.65 (m, 2H, $=NCH_2CH_2$), 2.57 (m, 2H, CH_2CH_2NH), 2.88 (m, 2H, H-5'), 3.67 (t, 2H, J = 7.2Hz, $=NCH_2$), 4.37 (m, 1H, H-4'), 5.02 (dd, 1H, J = 3.3Hz, 6.4Hz, H-3'), 5.48 (dd, 1H, J = 2.5Hz, 6.4Hz, H-2'), 5.70 (br s, 2H, adenine NH_2), 6.02 (d, 1H, J = 2.5Hz, H-1'), 7.71 (dd, 2H, J = 2.9Hz, 5.7Hz, isoindole H-5 and H-6), 7.84 (dd, 2H, J = 2.9Hz, 5.7Hz, isoindole H-4 and H-7), 7.93 (s, 1H, adenine H-2 or H-8), 8.34 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 241 nm (ε 16.1 x 10^3), 259 nm (ε 14.6 x 10^3); MS (positive LSIMS), m/e 536 $(M + H)^+$, 401 $(M + H - adenine)^+$.

Anal. Calculated for $C_{27}H_{33}N_7O_5 \cdot 0.5H_2O$: C, 59.55; H, 6.29; N, 18.00. Found: C, 59.88; H, 6.05; N, 17.96.

Low pressure liquid chromatography with a Lobar Size B (310-25) Lichroprep™ Si60 silica gel column was used to prepare an analytical sample of the dialkylated side product. The column was eluted with chloroform:isopropanol (80:20) at a flow rate of 4.0 mL/min. The crude dialkylated material was redissolved in the eluting solvent (approximately 7 mL) and 0.15 mL aliquots were injected onto the column. The dialkylated side product was eluted from the column (as detected by absorbance at 280 nm) 26-37 minutes after injection, separated from unreacted and faster-moving N-(6-bromo-1-hexyl)phthalimide and other slower-moving contaminants. The last traces of these impurities were removed when the column eluate containing the dialkylated side product was evaporated, redissolved in the eluting solvent (5 mL), and reinjected onto the same column under the same conditions as described above. The residual amber color of a chloroform solution (2 mL) of

the purified dialkylated material was removed by filtration through a wet-packed flash silica gel column (42 x 3.5 cm, 200 g). The column was eluted with chloroform:isopropanol (92:8) at a flow rate of 10 mL/min and 28 mL fractions were collected. The dialkylated material appeared in fractions 19-24, which were pooled and evaporated to dryness. The residue was twice dissolved in chloroform (50 mL) and evaporated to give 175 mg of analytically pure 5'-deoxy-5'-di[6-(phthalimido)-1-hexyl]amino-2',3'-O-isopropylideneadenosine as a colorless oil: TLC, chloroform:methanol (95:5), R_f 0.26 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.16-1.29 (m, 12H, $CH_2CH_2CH_2CH_2NCH_2CH_2CH_2CH_2$), 1.39 (s, 3H, isopropylidene CH_3), 1.60 (s, 3H, isopropylidene CH_3), 1.63 (m, 4H, both $=NCH_2CH_2$), 2.37 (m, 4H, $CH_2CH_2NCH_2CH_2$), 2.61 (m, 2H, H-5'), 3.66 (t, 4H, J = 7.3Hz, both $=NCH_2$), 4.34 (m, 1H, H-4'), 4.95 (m, 1H, H-3'), 5.54 (m, 1H, H-2'), 5.80 (br s, 2H, adenine NH_2), 6.06 (d, 1H, J = 1.9Hz, H-1'), 7.70 (dd, 4H, J = 3.0Hz, 5.4Hz, both isoindole H-5 and H-6), 7.83 (dd, 4H, J = 3.0Hz, 5.4Hz, both isoindole H-4 and H-7), 7.94 (s, 1H, adenine H-2 or H-8), 8.33 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 241 nm (ε 23.3 x 10^3), 260 nm (ε 13.5 x 10^3); MS (positive LSIMS), m/e 765 $(M + H)^+$, 630 $(M + H - adenine)^+$.

Anal. Calculated for $C_{41}H_{48}N_8O_7 \cdot 0.5H_2O$: C, 63.63; H, 6.38; N, 14.48. Found: C, 63.96; H, 6.46; N, 14.42.

Methyl N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-N^4-[6-(Phthalimido)-1-Hexyl]-2(R,S)-Trifluoroacetamido-4-Aminobutyrate [Protected 5'-[6-(Phthalimido)-1-Hexyl] SAM Affinity Ligand] (**17**). 5'-[6-(Phthalimido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine hemihydrate (**16**) (5.014 g, 9.22 mmol) and N,N-diisopropylethylamine (1.79 mL, 10.31 mmol, 10% excess) were dissolved in acetonitrile (150 mL) and heated with stirring to 70°C.. A solution of methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) (3.971 g, 11.71 mmol, 25% excess) in acetonitrile (50 mL) was then added over about 15 minutes and

the mixture stirred at 70°C. for three days. Over this time, the initially pale brown color of this mixture gradually deepened. After cooling to room temperature, the reaction mixture was evaporated to dryness, redissolved in chloroform (200 mL), concentrated to 5-10 mL, and loaded onto a wet-packed flash silica gel column (48 x 3.5 cm, 230 g). The column was eluted with chloroform:isopropanol (80:20) at a flow rate of 10 mL/min and 28 mL fractions were collected. Fractions 14-30 contained the partially purified product (as judged by TLC). These fractions were pooled and concentrated to approximately 15 mL in preparation for further purification of the product by low pressure liquid chromatography using a Lobar Size B (310-25) Lichroprep™ Si60 silica gel column. This column was eluted with chloroform:isopropanol (80:20) at a flow rate of 4.0 mL/min. The injection of 0.50 mL aliquots of the crude material led to the elution of the product (as detected by absorbance at 280 nm) from the column 25-37 minutes later. The column eluate containing the product from this series of injections was pooled, evaporated to dryness, and redissolved in the eluting solvent (15 mL) for reinjection onto the same column under identical conditions to remove the last traces of any impurities. Filtration through a wet-packed flash silica gel column (48 x 3.5 cm, 230 g) removed the residual amber color in a chloroform solution (5 mL) of the product. This column was eluted with chloroform:isopropanol (95:5) at a flow rate of 10 mL/min and 28 mL fractions were collected. Fractions 26-49 contained the ultraviolet-absorbing product. These fractions were pooled and evaporated to dryness, and the residue was twice dissolved in chloroform (100 mL) and evaporated to give 1.74 g (24.7%) of analytically pure product as a colorless foam: mp 49-59°C.; TLC, chloroform:isopropanol (80:20), individual diastereomers at R_f 0.66 and 0.69 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.03-1.26 (m, 6H, $CH_2CH_2CH_2CH_2CH_2$-phthalimide), 1.38 (s, 1.5H, isopropylidene

CH_3), 1.39 (s, 1.5H, isopropylidene CH_3), 1.55 (m, 2H, C*H*$_2$$CH_2$-phthalimide), 1.61 (s, 1.5H, isopropylidene CH_3), 1.62 (s, 1.5H, isopropylidene CH_3), 1.75-1.94 (m, 2H, CHC*H*$_2$$CH_2$N), 2.31-2.49 (m, 2H, CH_2CH_2C*H*$_2$NCH_2), 2.52 (m, 2H, $CHCH_2$C*H*$_2$N), 2.71-2.89 (m, 2H, H-5'), 3.64 (m, 2H, CH_2-phthalimide), 3.65 (s, 1.5H, CO_2CH_3), 3.74 (s, 1.5H, CO_2CH_3), 4.29-4.35 (m, 1H, H-4'), 4.48 (m, 1H, C*H*CH_2CH_2N), 4.96 (dd, 0.5H, J = 3.7Hz, 6.4Hz, H-3'), 5.01 (dd, 0.5H, J = 3.4Hz, 6.4Hz, H-3'), 5.51 (dd, 0.5H, J = 1.8Hz, 6.4Hz, H-2'), 5.54 (dd, 0.5H, J = 1.5Hz, 6.4Hz, H-2'), 5.82 (br s, 1H, adenine NH_2), 5.89 (br s, 1H, adenine NH_2), 6.04 (d, 1H, J = 1.9Hz, H-1'), 7.71 (dd, 2H, J = 3.0Hz, 5.4Hz, isoindole H-5 and H-6), 7.81 (s, 0.5H, adenine H-2 or H-8), 7.84 (dd, 2H, J = 3.0Hz, 5.4Hz, isoindole H-4 and H-7), 7.88 (s, 0.5H, adenine H-2 or H-8), 8.31 (s, 1H, adenine H-2 or H-8), 9.46 (br d, 0.5H, J = 6.1Hz, CF_3CONH), 9.56 (br d, 0.5H, J = 6.5Hz, CF_3CONH); UV λ_{max} (MeOH) 241 nm (ε 15.5 x 10^3), 259 nm (ε 13.8 x 10^3); MS (positive LSIMS), m/e 747 $(M + H)^+$, 612 $(M + H - adenine)^+$.

Anal. Calculated for $C_{34}H_{41}F_3N_8O_8 \cdot H_2O$: C, 53.40; H, 5.67; N, 14.65. Found: 53.78; H, 5.49; N, 14.56.

Attempted Synthesis of N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-N^4-[6-(Phthalimido)-1-Hexyl]-2(R,S),4-Diaminobutyric Acid. A solution of sodium carbonate (159 mg, 1.500 mmol) in 50% aqueous methanol (15 mL) was added to methyl N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-[6-(phthalimido)-1-hexyl]-2(R,S)-trifluoroacetamido-4-aminobutyrate monohydrate (**17**) (382 mg, 0.500 mmol). This mixture was heated to 60°C. with stirring for six hours. Aliquots (1 µL) were removed every ten minutes for the first hour and once every hour afterwards to follow the course of the reaction by TLC (100% methanol solvent system). The starting material, which appeared at R_f 0.74 (UV, I_2), was completely gone after 10 minutes. Two slower-moving spots at R_f 0.63 (UV, I_2) and 0.48 (UV, I_2, ninhydrin) rapidly appeared and corresponded to the

intermediate methyl ester and the suspected product, respectively. By the end of two hours, only the slower-moving of these spots remained and no further changes were observed during the next four hours. The cooled reaction mixture was neutralized with 1M aqueous hydrochloric acid, evaporated to dryness, and extracted with methanol (4 x 10 mL). The pooled methanol extracts were concentrated to approximately 3 mL and loaded onto the top of a wet-packed flash silica gel column (25 x 3.0 cm, 80 g). The column was eluted with chloroform:methanol (40:60) at a flow rate of 10 mL/min and 28 mL fractions were collected. The ultraviolet-absorbing product appeared in fractions 20-45, which were pooled and evaporated to dryness. The residue was twice dissolved in water (50 mL) and evaporated to dryness, redissolved in water (5 mL) and lyophilized to give 164 mg (50.2%) of a colorless powder. This material was not the desired 5'-[6-(phthalimido)-1-hexyl] product but rather its 5'-[6-(1-phthalamoyl)-1-hexyl] ring-opened derivative (**18**): mp 170-173°C.; TLC, chloroform:methanol (40:60), R_f 0.19 (UV, I_2, ninhydrin); 500 MHz ^{1}H NMR (D_2O), δ 0.71-1.09 (m, 6H, C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$N), 1.35 (m, 2H, C*H*$_2$CH$_2$-phthalimide), 1.40 (s, 1.5H, isopropylidene CH_3), 1.41 (s, 1.5H, isopropylidene CH_3), 1.60 (s, 1.5H, isopropylidene CH_3), 1.61 (s, 1.5H, isopropylidene CH_3), 1.70-1.92 (m, 2H, CHC*H*$_2$CH$_2$N), 2.18 (m, 1H, CH$_2$CH$_2$C*H*$_2$N), 2.28 (m, 1H, CH$_2$CH$_2$C*H*$_2$N), 2.50-2.72 (m, 4H, overlapping H-5' and CHCH$_2$C*H*$_2$N), 3.20 (m, 2H, CH$_2$-phthalimide), 3.57 (m, 1H, C*H*CH$_2$CH$_2$N), 4.43-4.50 (m, 1H, H-4'), 5.02 (dd, 0.5H, J = 3.0Hz, 6.5Hz, H-3'), 5.05 (dd, 0.5H, J = 2.7Hz, 6.2Hz, H-3'), 5.60 (dd, 0.5H, J = 1.8Hz, 6.3Hz, H-2'),5.63 (dd, 0.5H, J = 1.7Hz, 6.2Hz, H-2'), 6.23 (d, 0.5H, J = 1.8Hz, H-1'), 6.24 (d, 0.5H, J = 1.5Hz, H-1'), 7.36 (m, 1H, phthalamido H-6), 7.41 (m, 1H, phthalamido H-5), 7.46 (m, 1H, phthalamido H-4), 7.53 (m, 1H, phthalamido H-3), 8.17 (s, 0.5H, adenine H-2 or H-8), 8.18 (s, 0.5H, adenine H-2 or H-8), 8.24 (s, 1H, adenine H-2 or H-8); UV λ_{max} (H_2O) 259

nm (ε 14.5 x 10^3); MS (positive LSIMS), m/e 655 $(M + H)^+$, 640 (M + H - isopropylidene $CH_3)^+$.

Attempted Hydrolysis of the Phthalamate Group of N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-N^4-[6-(1-Phthalamoyl)-1-Hexyl]-2(R,S),4-Diaminobutyric Acid (**18**). A solution of N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-[6-(1-phthalamoyl)-1-hexyl]-2(R,S),4-diaminobutyric acid (**18**) (45 mg, 0.069 mmol) in 1.0N aqueous sodium hydroxide (3.0 mL) was heated to 100°C. with stirring for three hours. After cooling to room temperature, the reaction mixture was neutralized with 1M aqueous hydrochloric acid and evaporated to dryness. Analysis of the residue by TLC (100% methanol solvent system) indicated that the starting material at R_f 0.24 (UV, I_2) had completely disappeared. Three new spots at R_f 0.48 (I_2), 0.31 (UV, I_2), and 0.00 (UV, I_2) had in the meantime appeared. Similar analysis by 500 MHz ^1H NMR (D_2O) revealed that the phthalamate group was intact due to the nonequivalence of its four aromatic protons: δ 7.33 (m, 1H, phthalamido H-6), 7.37 (m, 1H, phthalamido H-5), 7.41 (m, 1H, phthalamido H-4), 7.48 (m, 1H, phthalamido H-3). The failure to observe any of the 2',3'-O-isopropylidene ribose resonances (H-1', H-2', H-3', H-4', H-5', and isopropylidene CH_3) indicated that this moiety had been completely destroyed. The reaction conditions were apparently sufficiently drastic to destroy the sugar ring but not harsh enough to hydrolyze the phthalamate group.

6-(Benzyloxycarbonylamino)-1-Hexanol. According to the procedure of Chipowsky and Lee,[182] 6-amino-1-hexanol (30.00 g, 0.256 mol) was added in one portion to a stirred solution of sodium bicarbonate (70.90 g, 0.844 mol) in water (1000 mL). Benzyl chloroformate (54.8 mL, 0.384 mol) was added to this solution over 15 minutes and the resulting mixture stirred at room temperature for 6 hours and then at 4°C. for an additional 18 hours. During this time a

colorless precipitate formed, which was filtered and washed with water (3 x 100 mL). The crude product was redissolved in chloroform (400 mL) and any residual water removed with the aid of a separatory funnel. Following decolorization with charcoal, the chloroform solution was dried over anhydrous magnesium sulfate, filtered, and evaporated. The resulting syrup was redissolved in ethyl acetate (1000 mL), concentrated to 750 mL, and triturated with hexanes (750 mL). The precipitate was filtered and dried to give 23.84 g (37.1%) of pure colorless product: mp 79-81°C. (lit.[182] 83°C.); 500 MHz ^{1}H NMR ($CDCl_3$), δ 1.36 (m, 4H, $CH_2CH_2CH_2CH_2OH$), 1.51 (m, 4H, $CH_2CH_2CH_2CH_2CH_2OH$), 3.19 (m, 2H, $NHCH_2$), 3.62 (m, 2H, CH_2OH), 4.79 (br s, 1H, NH), 5.09 (s, 2H, benzyl CH_2), 7.36 (m, 5H, phenyl); MS (positive LSIMS), m/e 252 $(M + H)^+$.

6-(Benzyloxycarbonylamino)-1-(p-Tolylsulfonyloxy)Hexane. This compound was synthesized according to the procedure of Chipowsky and Lee[182] but was purified by other means involving chromatography. 6-(Benzyloxycarbonylamino)-1-hexanol (23.84 g, 95.0 mmol) was dissolved in dry pyridine (125 mL) and cooled to 0°C. in an ice bath. p-Toluenesulfonyl chloride (19.91 g, 104.5 mmol, 10% excess) was added in one portion and the mixture was stirred overnight at 4°C.. The mixture was evaporated to dryness and the residue partitioned between chloroform (450 mL) and 1M aqueous acetic acid (450 mL). The chloroform extract was washed with additional 1M aqueous acetic acid (450 mL) followed by water (2 x 450 mL) and saturated aqueous sodium chloride (2 x 450 mL), and was dried over anhydrous magnesium sulfate. After filtration and concentration to approximately 15 mL, the chloroform extract was loaded onto the top of a wet-packed flash silica gel column (48 x 3.5 cm, 230 g). The column was eluted with two mixtures of ethyl acetate:hexanes at a flow rate of 10 mL/min and 28 mL fractions were collected. The first 75

fractions were eluted with ethyl acetate:hexanes (20:80) and an additional 40 fractions were eluted with ethyl acetate:hexanes (50:50). Pure product (as judged by TLC) appeared in fractions 91-114, which were pooled and evaporated to dryness. The residue was twice dissolved in chloroform (100 mL) and evaporated to a colorless oil. After several days at -20°C., this oil crystallized to give 24.45 g (63.5%) of analytically pure product: mp 35-38°C. (lit.[182] 46-47°C.); TLC, ethyl acetate:hexanes (50:50), R_f 0.43 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.25 (m, 2H, $NHCH_2CH_2CH_2$), 1.32 (m, 2H, $CH_2CH_2CH_2O$), 1.45 (m, 2H, $NHCH_2CH_2$), 1.63 (m, 2H, CH_2CH_2O), 2.44 (s, 3H, CH_3), 3.14 (q, 2H, J = 6.5Hz, $NHCH_2$), 4.00 (t, 2H, J = 6.2Hz, CH_2CH_2O), 4.77 (br s, 1H, NH), 5.08 (s, 2H, benzyl CH_2), 7.35 (m, 7H, overlapping phenyl and tosyl H-3 and H-5), 7.78 (d, 2H, J = 7.4Hz, tosyl H-2 and H-6); MS (positive LSIMS), m/e 406 $(M + H)^+$.

Anal. Calculated for $C_{21}H_{27}NO_5S$: C, 62.20; H, 6.71; N, 3.45; S,7.91. Found: C, 62.13; H, 6.79; N, 3.42; S, 7.91.

6-(Benzyloxycarbonylamino)-1-iodohexane (19). The synthesis of this compound was conducted according to the procedure of Chipowsky and Lee,[182] although its purification was carried out by other means involving chromatography. 2-Butanone (200 mL) was added to a mixture of 6-(benzyloxycarbonylamino)-1-(p-tolylsulfonyloxy)hexane (24.45 g, 60.4 mmol) and sodium iodide (13.59 g, 90.6 mmol, 50% excess). This mixture was heated with stirring to 80°C. for three hours during which it assumed a milky appearance. After cooling to room temperature, the mixture was evaporated to dryness. Chloroform (100 mL) was added to the residue and the insoluble material removed by filtration and washed with chloroform (2 x 100 mL). The combined chloroform extracts were concentrated to approximately 10 mL and loaded onto the top of a wet-packed flash silica gel column (48 x 3.5 cm, 230 g).

The column was eluted with ethyl acetate:hexanes (20:80) at a flow rate of 10 mL/min and 28 mL fractions were collected. Pure product (as judged by TLC) appeared in fractions 23-54, which were pooled and evaporated to dryness. The residue was twice dissolved in chloroform (100 mL) and evaporated to a colorless oil. This oil crystallized upon cooling in dry ice to provide 20.63 g (94.6%) of analytically pure product as a colorless solid: mp 45-47°C. (lit.[182] 43-44.5°C.); TLC, ethyl acetate:hexanes (20:80), R_f 0.32 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.33 (m, 2H, $CH_2CH_2CH_2I$), 1.41 (m, 2H, $NHCH_2CH_2CH_2$), 1.51 (m, 2H, CH_2CH_2I), 1.81 (m, 2H, $NHCH_2CH_2$), 3.18 (m, 4H, overlapping $NHCH_2$ and CH_2I), 4.75 (br s, 1H, NH), 5.09 (s, 2H, benzyl CH_2), 7.36 (m, 5H, phenyl); MS (positive LSIMS), m/e 362 $(M + H)^+$.

Anal. Calculated for $C_{14}H_{20}INO_2$: C, 46.55; H, 5.58; I, 35.13; N, 3.88. Found: C, 46.49; H, 5.62; I, 35.28; N, 3.79.

5'-[6-(Benzyloxycarbonylamino)-1-Hexyl]Amino-5'-Deoxy-2',3'-O-Isopropylideneadenosine (**20**). 5'-Amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**13**) (3.06 g, 10.0 mmol) and N,N-diisopropylethylamine (1.74 mL, 10.0 mmol) were dissolved in acetonitrile (250 mL) and heated with stirring to 70°C.. 6-(Benzyloxycarbonylamino)-1-iodohexane (**19**) (3.61 g, 10.0 mmol) was added to this solution in one portion and the mixture heated with stirring to 70°C. for 18 hours. After cooling to room temperature and evaporation to dryness, the residue was partitioned between methylene chloride (200 mL) and 0.1N aqueous sodium carbonate (200 mL). The aqueous phase was washed with additional methylene chloride (2 x 200 mL) and the pooled methylene chloride extracts were washed with saturated aqueous sodium chloride (2 x 200 mL), dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. The residue was dissolved in chloroform (50 mL), concentrated to 5 mL, and loaded onto the top of a wet-packed flash silica gel column (48 x 3.5 cm, 230 g).

The column was eluted with chloroform:isopropanol (80:20) at a flow rate of approximately 10 mL/min and 28 mL fractions were collected. Partially pure (as judged by TLC) dialkylated side product appeared in fractions 14-24, which were pooled and evaporated to give 2.05 g (26.2%) of nearly pure dialkylated side product as a yellow oil. The desired monoalkylated product was eluted in pure form (as judged by TLC) in fractions 32-85, which were pooled and evaporated to dryness. The residue was twice dissolved in chloroform (100 mL) and evaporated to give 2.56 g (47.5%) of pure product as a colorless foam: mp 44-46°C.; TLC, chloroform:isopropanol (80:20), R_f 0.26 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.28 (m, 4H, $CH_2CH_2CH_2CH_2N$), 1.39 (m, 5H, overlapping isopropylidene CH_3 and $CH_2CH_2NHCH_2$), 1.47 (m, 2H, $CONHCH_2CH_2$), 1.61 (s, 3H, isopropylidene CH_3), 2.56 (m, 2H, $CH_2CH_2NHCH_2$), 2.87 (m, 2H, H-5'), 3.18 (q, 2H, J = 6.3Hz, $CONHCH_2$), 4.38 (m, 1H, H-4'), 5.02 (dd, 1H, J = 3.2Hz, 6.3Hz, H-3'), 5.10 (s, 2H, benzyl CH_2), 5.17 (br s, 1H, CONH), 5.47 (dd, 1H, J = 2.7Hz, 6.3Hz, H-2'), 5.69 (br s, 2H, adenine NH_2), 6.04 (d, 1H, J = 2.7Hz, H-1'), 7.35 (m, 5H, phenyl), 7.94 (s, 1H, adenine H-2 or H-8), 8.33 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 13.5 x 10^3); MS (positive LSIMS), m/e 540 $(M + H)^+$, 405 $(M + H - adenine)^+$.

Anal. Calculated for $C_{27}H_{37}N_7O_5$: C, 60.10; H, 6.91; N,18.17. Found: C, 59.89; H, 6.93; N, 17.94.

Low pressure liquid chromatography was used to prepare an analytical sample of the dialkylated side product. The Lobar SizeB (310-25) Lichroprep™ Si60 silica gel column used for this purpose was eluted with chloroform:isopropanol (80:20) at a flow rate of 4.0 mL/min. The dialkylated side product, partially purified by flash chromatography, was dissolved in approximately 10 mL of the eluting solvent. The injection of 0.70 mL aliquots of this solution onto the column led to the appearance (as detected by absorbance

at 280 nm) of the pure dialkylated side product 30-47 minutes later. Similar eluates from repeated injections were pooled and evaporated to dryness, redissolved in the eluting solvent (approximately 10 mL), and reinjected onto the same column under identical conditions to remove the last traces of any remaining impurities. The residual amber color of a chloroform solution (2 mL) of the purified compound was removed by filtration through a wet-packed flash silica gel column (44 x 3.5 cm, 200 g). The column was eluted with chloroform:isopropanol (90:10) at a flow rate of 10 mL/min and 28 mL fractions were collected. The dialkylated side product was eluted in fractions 16-25, which were pooled and evaporated to dryness. The residue was twice dissolved in chloroform (100 mL) and evaporated to give 1.252 g of 5'-deoxy-5'-di[6-(benzyloxycarbonylamino)-1-hexyl]amino-2',3'-O-isopropylideneadenosine as a colorless foam: mp 36-38°C.; TLC, chloroform:isopropanol (80:20), R_f 0.58 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.19 (m, 8H, $CH_2CH_2CH_2CH_2NCH_2CH_2CH_2CH_2$), 1.25 (m, 4H, $CH_2CH_2NCH_2CH_2$), 1.39 (s, 3H, isopropylidene CH_3), 1.43 (m, 4H, both $CONHCH_2CH_2$), 1.60 (s, 3H, isopropylidene CH_3), 2.38 (m, 4H, $CH_2CH_2NCH_2CH_2$), 2.60 (m, 2H, H-5'), 3.17 (q, 4H, J = 6.4Hz, both $CONHCH_2$), 4.37 (m, 1H, H-4'), 4.98 (m, 1H, H-3'), 5.11 (s, 4H, both benzyl CH_2), 5.30 (br s, 2H, both CONH), 5.56 (m, 1H, H-2'), 5.69 (br s, 2H, adenine NH_2), 6.07 (s, 1H, H-1'), 7.36 (m, 10H, both phenyl), 7.91 (s, 1H, adenine H-2 or H-8), 8.32 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 14.2 x 10^3); MS (positive LSIMS), m/e 773 $(M + H)^+$, 638 $(M + H - adenine)^+$.

Anal. Calculated for $C_{41}H_{56}N_8O_7 \cdot 0.5H_2O$: C, 62.98; H, 7.34; N, 14.33. Found: C, 63.24; H, 7.31; N, 14.48.

Methyl N^4-[6-(Benzyloxycarbonylamino)-1-Hexyl]-N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-2(R,S)-Trifluoroacetamido-4-Aminobutyrate [Protected 5'-[6-

(Benzyloxycarbonylamino)-1-Hexyl] SAM Affinity Ligand] (**21**). 5'-[6-(Benzyloxycarbonylamino)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylidene-adenosine (**20**) (4.753 g, 8.82 mmol) and N,N-diisopropylethylamine (1.69 mL, 9.70 mmol, 10% excess) were dissolved in acetonitrile (125 mL) and heated with stirring to 70°C.. Over approximately 15 minutes, a solution of methyl 2(R,S)-trifluoroacetamido-4-iodobutyrate (**6b**) (3.737 g, 11.03 mmol, 25% excess) in acetonitrile (50 mL) was added. The mixture was stirred at 70°C. for three days during which its initial pale brown color progressively deepened. After cooling to room temperature, the reaction mixture was evaporated to dryness, redissolved in chloroform (100 mL), concentrated to approximately 5 mL, and loaded onto the top of a wet-packed flash silica gel column (48 x 3.5 cm, 230 g). Elution of this column with chloroform:isopropanol (80:20) at a flow rate of approximately 10 mL/min and the collection of 28 mL fractions resulted in the appearance of the product (as judged by TLC) in fractions 14-29. These fractions were pooled and concentrated to approximately 15 mL in preparation for further purificatiion by low pressure liquid chromatography using a Lobar Size B (310-25) Lichroprep^{TM} Si60 silica gel column. This column was eluted with chloroform:isopropanol (80:20) at a flow rate of 4.0 mL/min. The injection of 0.70 mL aliquots of the crude product solution onto this column led to the appearance of purified product (as detected by absorbance at 280 nm) in the eluate 24-38 minutes later. Product-containing eluates from repeated injections were pooled and evaporated to dryness, redissolved in the eluting solvent (approximately 15 mL), and reinjected onto the same column under identical conditions to remove the last traces of any contaminants. The residual amber color of a chloroform solution (5 mL) of the column-purified product was removed by filtration through a wet-packed flash silica gel column (48 x 3.5 cm, 230 g). This column was eluted with chloroform:isopropanol (95:5) at a flow

rate of approximately 10 mL/min and 28 mL fractions were collected. Decolorized product appeared in fractions 29-53, which were pooled and evaporated to dryness. The residue was twice dissolved in chloroform (100 mL) and evaporated to provide 1.45 g (21.4%) of analytically pure product as a colorless foam: mp 50-57°C.; TLC, chloroform:isopropanol (80:20), individual diastereomers at R_f 0.57 and 0.63 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.00 (m, 4H, $CH_2CH_2CH_2CH_2N$), 1.12 (m, 2H, $CH_2CH_2CH_2CH_2N$), 1.31 (m, 2H, $CONHCH_2CH_2$), 1.39 (s, 1.5H, isopropylidene CH_3), 1.40 (s, 1.5H, isopropylidene CH_3), 1.61 (s, 1.5H, isopropylidene CH_3), 1.63 (s, 1.5H, isopropylidene CH_3), 1.83-1.95 (m, 2H, $CHCH_2CH_2CH_2N$), 2.30-2.42 (m, 2H, $CH_2CH_2CH_2CH_2N$), 2.48-2.60 (m, 2H, $CHCH_2CH_2N$), 2.63-2.92 (m, 2H, H-5'), 3.17 (m, 2H, $CONHCH_2$), 3.66 (s, 1.5H, CO_2CH_3), 3.75 (s, 1.5H, CO_2CH_3), 4.30-4.40 (m, 1H, H-4'), 4.51 (m, 1H, $CHCH_2CH_2CH_2N$), 4.99 (m, 0.5H, H-3'), 5.03 (m, 0.5H, H-3'), 5.12 (s, 1H, benzyl CH_2), 5.14 (s, 1H, benzyl CH_2), 5.43 (br s, 0.5H, $CONHCH_2$), 5.54 (d, 1H, J = 6.1Hz, H-2'), 5.61 (br s 0.5H, $CONHCH_2$), 5.69 (br s, 2H, adenine NH_2), 6.05 (d, 1H, J = 5.5Hz, H-1'), 7.29-7.40 (m, 5H, phenyl), 7.82 (s, 0.5H, adenine H-2 or H-8), 7.86 (s, 0.5H, adenine H-2 or H-8), 8.30 (s, 0.5H, adenine H-2 or H-8), 8.31 (s, 0.5H, adenine H-2 or H-8), 9.47 (br d, 0.5H, J = 4.8Hz, CF_3CONH), 9.57 (br d, 0.5H, J = 7.0Hz, CF_3CONH); UV λ_{max} (MeOH) 259 nm (ε 14.0 x 10^3); MS (positive LSIMS), 751 $(M + H)^+$, 616 (M + H - adenine)$^+$.

Anal. Calculated for $C_{34}H_{45}F_3N_8O_8 \cdot H_2O$: C, 53.12; H, 6.16; N, 14.58. Found: C, 52.92; H, 5.94; N, 14.38.

<u>N^4-[6-(Benzyloxycarbonylamino)-1-Hexyl]-N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-2(R,S),4-Diaminobutyric Acid, [Isopropylidene 5'-[6-(Benzyloxycarbonylamino)-1-Hexyl] SAM Affinity Ligand]</u> (**22**). A solution of sodium carbonate (1.074 g, 1.40 mmol) in 50% aqueous methanol (15 mL) was added

to methyl N^4-[6-(benzyloxycarbonylamino)-1-hexyl]-N^4-(2',3'-O-isopropylidene-5'-adenosyl)-2(R,S)-trifluoroacetamido-4-aminobutyrate monohydrate (**21**) (1.074 g, 1.40 mmol). This mixture was heated with stirring to 60°C. for six hours. After cooling to room temperature, the reaction mixture was neutralized with 1M aqueous hydrochloric acid, evaporated to dryness, and the residue extracted with methanol (4 x 25 mL). The combined methanol extracts were concentrated to approximately 5 mL and loaded onto the top of a wet-packed flash silica gel column (44 x 3.5 cm, 200 g). The column was eluted with two different mixtures of methanol:chloroform at a flow rate of 10 mL/min, and 28 mL fractions were collected. The first 100 fractions were eluted with methanol:chloroform (30:70) and an additional 32 fractions were eluted with 100% methanol. The ultraviolet-absorbing product was eluted in fractions 109-132, which were pooled and evaporated to dryness. The residue was twice dissolved in methanol (50 mL) and again evaporated to dryness to give 908 mg (> 100% yield due to the presence of an unidentified contaminant) of a colorless foam. The recovered material was used directly without further purification: mp 104-111°C.; TLC, methanol:chloroform (30:70), R_f 0.33 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 0.74-0.99 (m, 6H, $CH_2CH_2CH_2CH_2N$), 1.22 (m, 2H, $CONHCH_2CH_2$), 1.40 (s, 1.5H, isopropylidene CH_3), 1.41 (s, 1.5H, isopropylidene CH_3), 1.60 (s, 1.5H, isopropylidene CH_3), 1.61 (s, 1.5H, isopropylidene CH_3), 1.76-1.95 (m, 2H, $CHCH_2CH_2N$), 2.24-2.34 (m, 2H, $CH_2CH_2CH_2N$), 2.61 (m, 2H, $CHCH_2CH_2N$), 2.72 (m, 2H, H-5'), 2.97 (m, 2H, $CONHCH_2$), 3.65 (m, 1H, $CHCH_2CH_2N$), 4.46 (m, 1H, H-4'), 4.85 (m, 1H, H-3'), 5.05 (s, 2H, benzyl CH_2), 5.59 (m, 0.5H, H-2'), 5.62 (m, 0.5H, H-2'), 6.24 (d, 0.5H, J = 0.9Hz, H-1'), 6.26 (d, 0.5H, J = 1.5Hz, H-1'), 7.33 (m, 5H, phenyl), 8.19 (s, 0.5H, adenine H-2 or H-8), 8.20 (s, 0.5H, adenine H-2 or H-8), 8.23 (s, 1H,

adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 12.9 x 10^3); MS (positive LSIMS), m/e 641 $(M + H)^+$, 506 $(M + H$ - adenine$)^+$.

N^4-(6-Amino-1-Hexyl)-N^4-(2',3'-O-Isopropylidene-5'-Adenosyl)-2(R,S),4-Diaminobutyric Acid. [Isopropylidene 5'-(6-Amino-1-Hexyl) SAM Affinity Ligand] (**23**). N^4-[6-(Benzyloxycarbonylamino)-1-hexyl]-N^4-(2',3'-O-isopropylidene-5'-adenosyl)-2(R,S),4-diaminobutyric acid (**22**) (908 mg, 1.42 mmol) was dissolved in 50% aqueous formic acid (20 mL). 10% Palladium on charcoal (908 mg) was added and the mixture hydrogenated with shaking at 42 psi for five hours. The catalyst was removed by filtration through a glass fiber filter and the clear solution evaporated. The resulting syrup was redissolved in water (100 mL) and neutralized with 1M aqueous ammonium hydroxide. Ammonium formate present in this solution was removed by desalting on SP-Sephadex C-25 (34 x 2.5 cm, 30 g, NH_4^+ form, prepared by washing with 2 L of 1M aqueous ammonium chloride followed by 4 L of water). After the solution was loaded at a flow rate of 2 mL/min, the column was washed with water (1 L) and the adsorbed product eluted with 1M aqueous ammonium hydroxide. Column eluate containing the product was evaporated and the residue dissolved in water (100 mL) and again evaporated to dryness. The residue was redissolved in water (5 mL) and lyophilized to give 498 mg (69.3%) of product as a colorless solid. Due to the presence of a small amount of the fully deprotected derivative (approximately 5%), the recovered product was used directly without further purification: mp 101-109°C.; TLC, ethanol:conc. NH_4OH (90:10), R_f 0.17 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 0.80-1.09 (m, 6H, $CH_2CH_2CH_2CH_2N$), 1.41(s, 3H, isopropylidene CH_3), 1.43 (m, 2H, $NH_2CH_2CH_2$), 1.60 (s, 1.5H, isopropylidene CH_3), 1.61 (s, 1.5H, isopropylidene CH_3), 1.62-1.86 (m, 2H, $CHCH_2CH_2N$), 2.20 (m, 2H, $CH_2CH_2CH_2N$), 2.52 (m, 2H, $CHCH_2CH_2N$), 2.59 (m, 2H, H-5'), 2.85 (m, 2H, NH_2CH_2), 3.39 (m, 1H,

C*H*CH_2CH_2N), 4.46 (m, 1H, H-4'), 5.03 (dd, 0.5H, J = 2.9Hz, 6.3Hz, H-3'), 5.05 (dd, 0.5H, J = 2.6Hz, 6.3Hz, H-3'), 5.62 (dd, 0.5H, J = 1.8Hz, 6.3Hz, H-2'), 5.63 (dd, 0.5H, J = 1.6Hz, 6.3Hz, H-2'), 6.24 (d, 0.5H, J = 1.8Hz, H-1'), 6.25 (d, 0.5H, J = 1.6Hz, H-1'), 8.19 (s, 0.5H, adenine H-2 or H-8), 8.20 (s, 0.5H, adenine H-2 or H-8), 8.26 (s, 1H, adenine H-2 or H-8); UV λ_{max} (H_2O) 259 nm (ε 12.5 x 10^3); MS (positive LSIMS), m/e 507 $(M + H)^+$.

N^4-(5'-Adenosyl)-N^4-(6-Amino-1-Hexyl)-2(R,S),4-Diaminobutyric Acid. [5'-(6-Amino-1-Hexyl) SAM Affinity Ligand] (**9**). N^4-(6-Amino-1-hexyl)-N^4-(2',3'-O-isopropylidene-5'-adenosyl)-2(R,S),4-diaminobutyric acid (**23**) (498 mg, 0.98 mmol) was dissolved in 0.1M aqueous sulfuric acid (75 mL) and stirred at room temperature for eight days. The reaction mixture was neutralized with barium hydroxide octahydrate (2.366 g, 7.50 mmol) and 1M aqueous sulfuric acid, and the precipitated barium sulfate removed by centrifugation and filtration. The clear solution was evaporated to dryness, redissolved in water (300 mL), and loaded onto an SP-Sephadex C-25 column (71 x 2.5 cm, 60g, NH_4^+ form, prepared by washing with 4 L of 1M aqueous ammonium chloride and 6 L of water) at a flow rate of 2 mL/min and 28 mL fractions were collected. After washing the column with water (700 mL), a gradient of aqueous ammonium bicarbonate (0-0.7M, 4.3 L) was used to elute fractions 31-180. The ultraviolet-absorbing product appeared in fractions 86-100, with the peak occurring in fraction 93 ($[NH_4HCO_3]$ = 0.29M). These fractions were pooled and diluted with an equal volume of water. The same SP-Sephadex C-25 column, freshly regenerated in the NH_4^+ form, was used to desalt the recovered product solution. This solution was loaded at a flow rate of 2 mL/min and the column subsequently washed with water (3 L). The adsorbed product was eluted with 1M aqueous ammonium hydroxide. The column eluate containing the product was evaporated and the residue twice dissolved in water (100 mL) and again

evaporated to dryness. The residue was redissolved in water (5 mL) and lyophilized to give 434 mg (87.9% - based on the fully protected derivative, 61.4%) of analytically pure product as a colorless solid: mp 136-141°C.; TLC, ethanol:conc. NH_4OH (90:10), R_f 0.06 (UV, I_2, ninhydrin); 500 MHz ^1H NMR (D_2O), δ 1.05-1.15 (m, 4H, $CH_2CH_2CH_2CH_2N$), 1.28 (m, 2H, $CH_2CH_2CH_2CH_2N$), 1.47 (m, 2H, $NH_2CH_2CH_2CH_2$), 1.79 (m, 1H, $CHCH_2CH_2CH_2N$), 1.88 (m, 1H, $CHCH_2CH_2CH_2N$), 2.46 (m, 1H, $CH_2CH_2CH_2CH_2N$), 2.50 (m, 1H, $CH_2CH_2CH_2CH_2N$), 2.65 (m, 2H, $CHCH_2CH_2CH_2N$), 2.85 (m, 4H, overlapping H-5' and NH_2CH_2), 3.43 (m, 1H, $CHCH_2CH_2CH_2N$), 4.28 (m, 1H, overlapping H-3' and H-4'), 4.85 (m, 1H, H-2'), 6.03 (d, 1H, J = 4.4Hz, H-1'), 8.21 (s, 1H, adenine H-2 or H-8), 8.28 (s, 1H, adenine H-2 or H-8); UV λ_{max} (H_2O) 259 nm (ε 14.0 x 10^3); MS (positive LSIMS), m/e 467 $(M + H)^+$.

Anal. Calculated for $C_{20}H_{34}N_8O_5 \cdot 2H_2O$: C, 47.80; H, 7.62; N, 22.30. Found: C, 47.92; H, 7.43; N, 22.06.

Immobilization of N^4-(5'-Adenosyl)-N^4-(6-Amino-1-Hexyl)-2(R,S),4-Diaminobutyric Acid Dihydrate on Epoxy-Activated Sepharose 6B. The immobilization was modeled closely after the general procedure outlined in the Pharmacia Affinity Chromatography-Principles and Methods handbook. Epoxy-activated Sepharose 6B (7.50 g) was swelled in a minimal amount of water (approximately 20 mL) for 15 minutes and then washed extensively with water (750 mL) on a fritted glass filter. After most of the residual water was removed by suction, the moist Sepharose was transferred to a small (125 mL) Erlenmeyer flask. An aqueous solution (15 mL), with its pH adjusted to 11.5 by 1N aqueous sodium hydroxide, of N^4-(5'-adenosyl)-N^4-(6-amino-1-hexyl)-2(R,S),4-diaminobutyric acid dihydrate (9) (127.5 mg, 254.0 μmol) was then added to the epoxy-activated Sepharose 6B. The coupling solution and gel were mixed on a shaker (90 rpm) at room temperature for 26 hours. The

Sepharose was filtered on a glass frit and washed with water (4 x 25 mL). All filtrates were pooled and saved for reisolation of the ligand which had not been immobilized. Washing of the Sepharose was continued with 0.1M aqueous sodium bicarbonate, pH 8.0 (2 x 25 mL), water (2 x 25 mL), 0.1M aqueous sodium acetate, pH 4.0 (2 x 25 mL), and water (6 x 25 mL). The suction-dried Sepharose gel was resuspended in 1M aqueous ethanolamine (15 mL) in the same Erlenmeyer flask and mixed on a shaker (90 rpm) at room temperature for 20 hours. The gel was again filtered on a glass frit and washed successively with water (4 x 25 mL), 0.1M aqueous sodium bicarbonate, pH 8.0 (2 x 25 mL), water (2 x 25 mL), 0.1M aqueous sodium acetate, pH 4.0 (2 x 25 mL), water (2 x 25 mL), and 0.1M aqueous sodium bicarbonate (0.5M sodium chloride), pH7.0 (4 x 25 mL). The coupled gel (28.1 mL) was stored in 0.1M aqueous sodium bicarbonate (0.5M sodium chloride), pH7.0 until use.

The reisolation of the unbound ligand was carried out to determine the specific capacity of the coupled gel. The difference between the amount of ligand present in the original coupling solution and that which can be readily washed from the gel is equal to the amount of ligand which has been immobilized. The absorption at 260 nm of an aliquot (0.050 mL diluted 20-fold with water) of each solution used to wash the gel was determined. Only the pooled original coupling solution filtrate and the immediate water washings were found to contain ultraviolet-absorbing material. The application of Beer's law to this solution, using the extinction coefficient of the ligand dihydrate (14000 $M^{-1}cm^{-1}$), suggested that 67.7 mg of the ligand dihydrate had not been immobilized. That this ultraviolet-absorbing material was actually the ligand dihydrate was confirmed by its reisolation of SP-Sephadex C-25 (65 x 2.5 cm, 60 g, NH_4^+ form). The solution was neutralized with 1M aqueous hydrochloric acid, diluted to 250 mL, and loaded onto the column at a flow rate of 2 mL/min

and 28 mL fractions were collected. After washing the column with water (750 mL), a gradient of aqueous ammonium bicarbonate (0-0.7M, 4.3 L) was used to elute fractions 31-178. Ultraviolet-absorbing material was eluted only in fractions 85-92, with the peak occurring in fraction 89 at a buffer concentration (0.28M) almost identical to that needed to elute the ligand in a large scale purification (0.29M). These fractions were pooled and diluted with an equal volume of water, and then desalted on the same SP-Sephadex C-25 column (NH_4^+ form) under conditions identical to those described in the large scale preparation of the chemically synthesized ligand. The desalted material was dissolved in water (5 mL) and lyophilized to give 64.8 mg of reisolated compound. (This material was confirmed to be the ligand dihydrate by analysis using 500 MHz ^1H NMR.) This quantity is in excellent agreement with the amount determined to be present in the original coupling solution washings using Beer's law. (This observation indicates that the extent of immobilization of the ligand dihydrate on epoxy-activated Sepharose 6B could be readily determined by applying Beer's law to the pooled coupling solution washings. Reisolation of the unbound ligand would therefore not be necessary to calculate the specific capacity of the gel.) The recovery of 64.8 mg (129.1 μmol) of ligand dihydrate implies that 62.8 mg (125.1 μmol, 49.3%) had been immobilized on the Sepharose gel (28.1 mL). The specific capacity of the coupled gel is 4.5 μequiv of bound ligand per mL.

Immobilization of N^4-(5'-Adenosyl)-N^4-(6-Amino-1-Hexyl)-2(R,S),4-Diaminobutyric Acid Dihydrate on Activated CH-Sepharose 4B. The general procedure described in the Pharmacia Affinity Chromatography-Principles and Methods handbook was closely followed to implement this immobilization. Activated CH-Sepharose 4B (7.50 g) was placed in a fritted glass filter and swelled in a minimal amount of ice-cold 1mM aqueous hydrochloric acid

(approximately 20 mL). The gel was washed with ice-cold 1mM aqueous hydrochloric acid (1.5 L) and ice-cold water (500 mL), dried by suction, and transferred to a small (125 mL) Erlenmeyer flask. After adjusting the pH to 10.0 with 1M aqueous sodium hydroxide, the cold aqueous coupling solution (15 mL) of N^4-(5'-adenosyl)-N^4-(6-amino-1-hexyl)-2(R,S),4-diaminobutyric acid dihydrate (**9**) (79.3 mg, 158.0 μmol) was added to the moist gel. The resulting suspension was mixed on a shaker (90 rpm) for five hours at 4°C.. The Sepharose was filtered on a fritted glass filter and washed with water (4 x 25 mL), and all filtrates pooled and saved. The suction-dried gel was returned to the small Erlenmeyer flask, resuspended in 1M aqueous ethanolamine, pH 9.0, and mixed on a shaker (90 rpm) for 90 minutes at 4°C.. The gel was again filtered on a fritted glass filter and washed with water (4 x 25 mL). The ethanolamine-water filtrates were pooled with the coupling solution-water filtrates from above and saved for the reisolation of the unbound ligand. The coupled gel was then washed with 0.1M aqueous sodium bicarbonate, pH 8.0 (2 x 25 mL), water (2 x 25 mL), 0.1M aqueous sodium acetate, pH 4.0 (4 x 25 mL), water (4 x 25 mL), and 0.1M aqueous sodium bicarbonate (0.5M sodium chloride), pH 7.0. The activated CH-Sepharose 4B-ligand conjugate (17.5 mL) was stored in 0.1M aqueous sodium bicarbonate (0.5M sodium chloride), pH 7.0 until use.

The specific capacity of the coupled gel was determined by reisolating the unbound ligand. The amount of ligand dihydrate coupled to the activated CH-Sepharose 4B is equal to the difference between the quantity initially present in the coupling solution and that which can be readily washed from the gel. Aliquots (0.050 mL diluted 20-fold with water) from the coupling solution filtrate and all other solutions used to wash the gel were analyzed at 260 nm for the presence of ultraviolet-absorbing material. Only the coupling and

ethanolamine solution filtrates and their respective water washings were found to contain such material. The presence of unknown quantities of ultraviolet-absorbing salts of N-hydroxysuccinimide in these solutions precluded the use of Beer's law to determine how much unbound ligand was actually present. Therefore, these filtrates and washings were pooled and neutralized in preparation for the reisolation of the unbound ligand. The resulting solution was loaded onto an SP-Sephadex C-25 column (65 x 2.5 cm, 60 g , NH_4^+ form) at a flow rate of 2 mL/min and 28 mL fractions were collected. After the column was washed with water (600 mL), a gradient of aqueous ammonium bicarbonate (0-0.7M, 4.3 L) was used to elute fractions 31-180. Ultraviolet-absorbing material was eluted in fractions 10-26 and fractions 85-91. The earlier fractions were pooled and evaporated to a colorless solid residue, which was found by 240 MHz ^1H NMR (in D_2O, singlet at 2.83 ppm) to be either N-hydroxysuccinimide or one of its salts. The peak in the latter set of fractions of ultraviolet-absorbing material occurred in fraction 89 at a buffer concentration (0.28M) essentially identical to that necessary to elute the chemically synthesized ligand on a large scale (0.29M). These fractions were pooled and Beer's law used to determine that 37.8 mg of the ligand dihydrate was present. This quantity agrees quite well with the 34.8 mg of lyophilized material (identified as the pure ligand dihydrate by 500 MHz ^1H NMR) obtained when these pooled fractions were desalted on the original SP-Sephadex C-25 column. (This observation suggests that the desalting of the reisolated ligand might not be necessary to determine the specific capacity of the coupled gel.) The reisolation of 34.8 mg (69.3 μmol) of ligand dihydrate implies that 44.7 mg (89.0 μmol, 56.3%) of this compound in the original coupling solution had been immobilized on the Sepharose gel (17.5 mL). The specific capacity of the

activated CH-Sepharose 4B conjugate is thus 5.1 μequiv of coupled ligand per mL.

Chapter 3

Synthesis of 5'-Tritioadenosine

Introduction

Compounds specifically labeled with stable or radioactive isotopes are widely used in both organic chemistry and biochemistry. Such compounds are often used to help elucidate chemical and enzymatic reaction mechanisms. When an atom in a bond is replaced by one of its stable or radioactive isotopes, the resulting bond will have a slightly different length and strength when compared to the original bond. If that bond is broken or undergoes rehybridization during a chemical reaction, this difference in bond length and strength will often be manifested in a kinetic isotope effect. Consequently, the appearance and magnitude of a primary or secondary kinetic isotope effect in analogous reactions of labeled and unlabeled compounds can be used to develop a picture of the transition state of that reaction.

Other biochemical applications of such compounds, particularly radiolabeled compounds, involve their use as tracers in metabolic studies. In order to learn what happens to a particular molecule or part of that molecule in a complex biochemical system, it must be "tagged" somehow. Radioisotopic substitution of a stable atom in a particular compound will provide a radioactive derivative which should partake in all of the normal metabolic processes in which the unlabeled compound participates. Provided that the "tag" is an integral part of the molecule and cannot be lost by exchange with the solvent, the fate of the "tag" is the fate of either the molecule itself or the part of the molecule into which it was incorporated.

Some of the most commonly used isotopic labels for such mechanistic and metabolic studies include deuterium and tritium, which are the respective stable and radioactive isotopes of hydrogen. Such labels could be most

conveniently incorporated into a molecule using the same methods by which the protium isotope, or most abundant isotope of hydrogen, is introduced. Chemical reduction processes, as exemplified by the reduction of a ketone to a secondary alcohol, are among the most common methods of incorporating regular hydrogen into a compound. However, classical reduction methods employ reaction conditions which are often nonselective as well as too drastic for biologically important molecules to survive intact. For instance, not many of these compounds could withstand the sodium hydroxide and hydrazine in hot diethylene glycol during the Wolff-Kishner reduction of a carbonyl group to a methylene group. Such classical reduction methods are impractical for the labeling of a compound with deuterium and especially tritium. What is needed are methods for incorporating hydrogen into sensitive compounds under mild conditions.

Sodium borohydride is a chemical reducing agent which has had a major impact on both organic chemistry and biochemistry. Ever since its introduction in the 1940's and early 1950's,[214-216] reductions with sodium borohydride and its derivatives have largely superseded the classical methods for reducing organic compounds and have permitted the reduction of biologically important molecules, including enzymes. The major advantage borohydride reducing agents have over the classical reducing methods is that they can be used to selectively reduce one functional group in the presence of many others under mild conditions. Thus, sodium cyanoborohydride can be used selectively to reduce aldehydes in the presence of esters. Similarly, the selective reduction of an ester in the presence of an amide is possible using lithium borohydride. This selectivity is important because the label must often be incorporated into only one specific position in a molecule so that the results of an experiment can be reliably attributed to that particular label and not to another label elsewhere.

This is particularly true in radiochemical labeling for metabolic experiments since the fate of only one part of the "tagged" molecule may be of interest in a complex and dynamic biochemical system. The inevitable fragmentation of a molecule or any of its parts (e.g., the ribose group of adenosine) followed by reassembly into new compounds in normal ongoing metabolic processes requires that the labeling be specific for only one particular location. Thus, specific radiochemical labeling helps to simplify interpretations of the fate of the "tagged" molecule and avoids the chaos which might otherwise be encountered using a randomly radiolabeled tracer.

Nucleosides and nucleotides are important biological compounds which have received much attention from chemists and biochemists. These compounds are well known for their roles as energy carriers and for their assimilation into nucleic acids involved in information storage and protein synthesis. Adenosine and its phosphates are among the most versatile of these compounds. Adenosine itself is a potent vasodilator[217] and can be readily incorporated into S-adenosyl-L-homocysteine (Figure 12), which is an inhibitor of biological transmethylation processes. Aside from its role as an energy carrier, adenosine 5'-triphosphate (ATP) is used to make S-adenosyl-L-methionine (Figure 2), the biological equivalent of methyl iodide. Not surprisingly, in order to understand these processes more thoroughly, adenosine has been labeled with both deuterium and tritium, most often in the 8-position of the adenine ring, but also in the ribose moiety using a variety of enzymatic[74,76,218] and chemical methods, including selective hydride reductions.

Specific labeling of the ribose group permits the chemical and enzymatic processes affecting that part of adenosine to be studied. Thus, the hydrolysis of the glycosidic bond of adenosine 5'-monophosphate (AMP) and 2'-deoxyadenosine 5'-monophosphate (dAMP) was studied using analogues labeled

with tritium at the C-1' position.[219] The label was introduced by a sodium borotritide reduction of ribonolactone to give radiolabeled ribose, which was then assimilated into the nucleotide products enzymatically.[219] The preparation of adenosine and S-adenosyl-L-homocysteine, specifically deuterated in the ribose C-3' position by sodium borodeuteride reduction of a keto sugar precursor, has allowed the mechanism of action of S-adenosyl-L-homocysteine hydrolase (Figure 16) to be studied.[220] Deuterium and tritium have been incorporated into the 5'-position of the ribose moiety by the reduction of 2',3'-O-isopropylideneadenosine 5'-aldehyde with sodium borodeuteride[221] and sodium borotritide.[222,223] Stereospecific reductions of N^6-benzoyl-2',3'-O-isopropylideneadenosine 5'-aldehyde with chiral reducing agents have also been reported.[224-227] Bis-deuteration at the 5'-position has been achieved using sodium borodeuteride to reduce the *in situ* generated acid azide of 2',3'-O-isopropylideneadenosine 5'-carboxylic acid.[228] 5'-Deoxy-5'-dimethylthioadenosine-5',5'-d_2 iodide has been prepared in this manner to study the mechanism of alkaline hydrolysis of sulfonium nucleosides.[93] The reduction of the ethyl ester of adenosine 5'-carboxylic acid with sodium borodeuteride has been reported.[229] A similar reduction of an ester of adenosine 5'-carboxylic acid with sodium borotritide, followed by the incorporation of tritium into the adenine ring by catalytic tritiation, has been briefly mentioned.[230]

Lithium borohydride is usually a much more effective reagent than sodium borohydride in reducing esters to alcohols, particularly when diglyme is used as the solvent. This effect has been attributed to either an activation of the hydride by the more polarizing lithium ion or the ability of lithium ion to coordinate to one of the lone electron pairs of the carbonyl oxygen, thereby activating the carbonyl carbon toward attack by hydride ion.[231] In the presence of lithium salts, particularly lithium bromide, sodium borohydride can reduce

esters much more rapidly than usual.[232] Presumably, this observation is a manifestation of an *in situ* preparation of lithium borohydride.

The goal of the present work was to devise a method for incorporating tritium into the 5'-position of adenosine so that the product could be used as a tracer. A higher specific activity of the final product could be achieved by reducing an acid derivative with two hydride equivalents rather than by reducing an aldehyde with one hydride equivalent. Thus, the reduction of an ester of 2',3'-O-isopropylideneadenosine 5'-carboxylic acid with *in situ* generated lithium borotritide in diglyme, followed by acid hydrolysis of the ribose 2',3'-diol ketal protecting group, appeared to be a convenient route to the final compound. The reduction of the same ester with *in situ* generated lithium borodeuteride might also be a convenient and more efficient alternative to existing procedures for bis-deuteration at that position.[228,229] A series of alkyl and aryl esters of 2',3'-O-isopropylideneadenosine 5'-carboxylic acid was synthesized in order to find out which ester would give the best results in reductions with *in situ* generated lithium borohydride. This ester was then used to prepare samples of 5'-deuterated and 5'-tritiated adenosine.

Results and Discussion

The general strategy followed to prepare a sample of 5'-tritiated adenosine began with the synthesis of a series of alkyl and aryl esters of 2',3'-O-isopropylideneadenosine 5'-carboxylic acid (**24**). Each ester was subsequently reduced by *in situ* generated lithium borohydride to determine which one would provide the best overall yield of the 2',3'-O-isopropylideneadenosine intermediate. Acid hydrolysis of the isopropylidene ketal protecting group would then be carried out under mild conditions in high yield to give the final adenosine product. The original idea behind testing a variety of esters in the lithium borohydride reduction was that one, by virtue of the electron-withdrawing properties of its alkyl [e.g., 2,2,2-trifluoroethyl (**25d**)] or aryl [e.g., 4-bromophenyl (**25h**)] substituent, might prove to be more susceptible toward attack by hydride ion and give a significantly better overall yield of product. As it turned out, though, this hypothesis proved to be incorrect and the ultimate labeled reduction, after the general procedure was well established, was carried out using one of the simpler esters. The preparation and reduction of these esters with *in situ* generated lithium borohydride and the synthesis of 5'-deuterated (**27**) and 5'-tritiated adenosine (**29**) are described below.

The starting material for the preparation of these esters, 2',3'-O-isopropylideneadenosine 5'-carboxylic acid (**24**), was prepared using the known alkaline permanganate oxidation of 2',3'-O-isopropylideneadenosine.[228] The ethyl ester (**25b**) of this acid (**24**) was then prepared by two established routes[233] involving a Fischer esterification in ethanol and the ethanolysis of an intermediate acid chloride, prepared by reaction of the 5'-carboxylic acid (**24**) with thionyl chloride. Neither esterification procedure proved to be very efficient. An attempt to prepare the 2,2,2-trifluoroethyl ester (**25d**) by addition of 2,2,2-trifluoroethanol to the intermediate acid chloride also proved to be

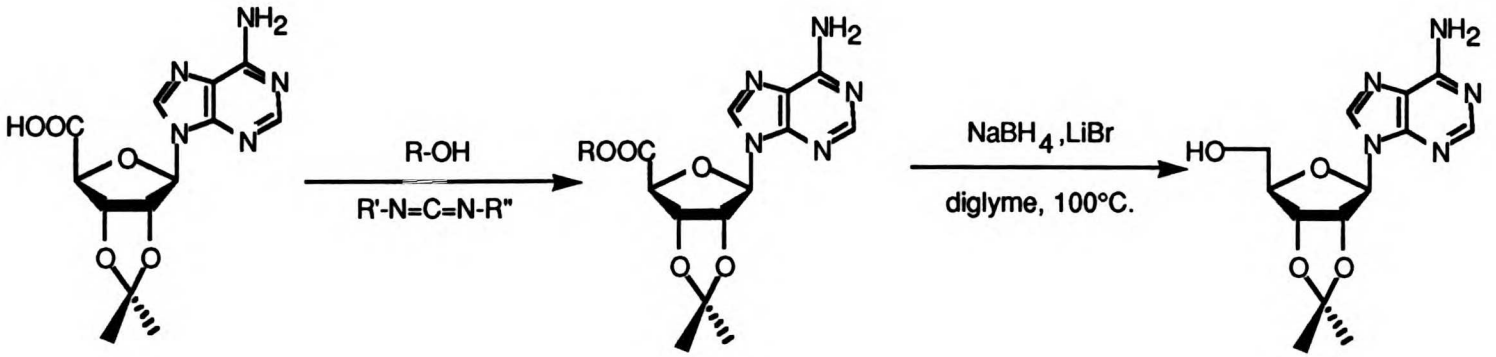
inefficient. In view of these results and the incompatibility of these methods with solid aryl alcohols (e.g., phenol and 4-bromophenol), an alternative method for the preparation of alkyl and aryl esters (**25a-h**) of 2',3'-O-isopropylidene-adenosine 5'-carboxylic acid (**24**) was investigated.

Carbodiimides are commonly used in peptide synthesis to activate amino acid carboxylate groups toward nucleophilic attack by the free base form of an amino acid ester. This activation process involves the formation of an intermediate O-acyl N,N'-disubstituted isourea. The amine then attacks this intermediate, or the symmetrical anhydride arising from a prior attack by another carboxylate group, to give a stable amide bond.[234] If the amine were replaced by an alcohol, an analogous attack on this intermediate would provide the corresponding ester. Thus, activation of the 5'-carboxylic acid (**24**) by a carbodiimide in the presence of an excess amount of an alkyl or aryl alcohol appeared to be a plausible route to the corresponding alkyl and aryl esters (**25a-h**). A similar activation of this same acid (**24**) with 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), another peptide coupling reagent has been used to carry out similar esterifications.[233]

Initial attempts to prepare the ethyl ester (**25b**) in acetonitrile using an excess of N,N-dicyclohexylcarbodiimide (DCC) to activate the acid (**24**) in the presence of an excess of ethanol had mixed results. The desired product could be observed in the crude reaction mixture by thin layer chromatography but could not be readily separated by flash silica gel chromatography or other means from the excess DCC or N,N-dicyclohexylurea (DCU) by-product of the coupling reaction. However, when the DCC was replaced with the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, a simple purification scheme was now possible. Thin layer chromatography of the crude reaction mixture on silica gel, using nonpolar

solvent systems such as chloroform:isopropanol (92:8) and chloroform:acetone (70:30), indicated that the positively-charged coupling reagent remained at the origin along with the unreacted 5'-carboxylic acid (**24**). In contrast, the product and a slower-moving ultraviolet-absorbing impurity (probably the symmetrical anhydride of the starting material) were mobile. Thus, flash silica gel chromatography of the concentrated crude reaction mixture could easily separate the ester product (**25b**) from the carbodiimide coupling reagent, the unreacted starting materials [i.e., 5'-carboxylic acid (**24**) and nonvolatile alcohol], and the suspected anhydride impurity. In this way, using acetonitrile as the reaction solvent, the methyl (**25a**), ethyl (**25b**), 2-chloroethyl (**25c**), 2,2,2-trifluoroethyl (**25d**), 2,2,2-trichloroethyl (**25e**), propargyl (**25f**), phenyl (**25g**), and 4-bromophenyl (**25h**) esters of 2',3'-O-isopropylideneadenosine 5'-carboxylic acid (**24**) were prepared (Scheme IX) in yields ranging from 47-86%.

The attempted reduction of each of these esters was carried out in hot anhydrous diglyme with *in situ* generated lithium borohydride. This reducing agent was prepared from equivalent amounts of sodium borohydride and lithium bromide.[232] Any unreacted borohydride at the conclusion of each reaction was destroyed by the addition of 50% aqueous acetone. As was expected, each ester was reduced by the *in situ* generated lithium borohydride. The 2',3'-O-isopropylideneadenosine product from each reduction (Scheme IX) was easily isolated in pure form from a chloroform extract of each evaporated reaction mixture by column silica gel chromatography. The yields of the recovered product ranged from 38% for the reduction of the 2-chloroethyl ester (**25c**) to 63% for the reduction of the ethyl ester (**25b**). Although the reductions of the propargyl (**25f**), 2,2,2-trichloroethyl (**25e**), 2,2,2-trifluoroethyl (**25d**), phenyl (**25g**), methyl (**25a**), and ethyl esters (**25b**) were somewhat more efficient (50-63%) than the reduction of the *in situ* generated 5'-acyl azide



24 **25a-h**

25a	, R = CH_3 (64.2%)	reduction of **25a**, 58.0%
b	, R = CH_3CH_2 (63.4%)	**b**, 63.4%
c	, R = $ClCH_2CH_2$ (47.4%)	**c**, 37.9%
d	, R = F_3CCH_2 (85.9%)	**d**, 56.0%
e	, R = Cl_3CCH_2 (65.2%)	**e**, 49.7%
f	, R = $HC\equiv CCH_2$ (82.3%)	**f**, 50.2%
g	, R = phenyl (85.1%)	**g**, 60.4%
h	, R = 4-bromophenyl (58.9%)	**h**, 43.1%

Scheme IX. Synthesis and reduction of the alkyl and aryl esters of 2',3'-O-isopropylideneadenosine 5'-carboxylic acid.

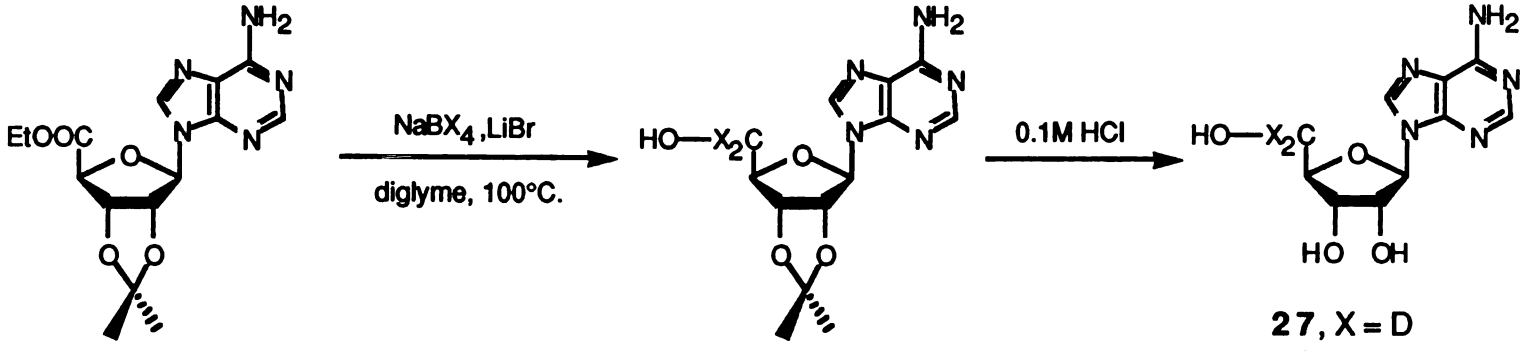
(48%),[228] no reduction was observed to be significantly more efficient than the others despite the diversity of the esters.

These reductions (Scheme IX) were, however, more efficient with *in situ* generated lithium borohydride than with either sodium borohydride or lithium borohydride alone. The most efficient reductions with *in situ* generated lithium borohydride were obtained with the ethyl (**25b**) (63%) and phenyl (**25g**) (60%) esters (Scheme IX). The reduction of the ethyl (**25b**) and phenyl (**25g**) esters with one equivalent of sodium borohydride alone provided significantly lower yields of product, 5% and 26%, respectively. These results show that the lithium ion was needed either to activate the hydride ion for attack or to coordinate to the carbonyl oxygen to activate the carbonyl carbon toward nucleophilic attack, or both.[231] The reduction of the ethyl ester (**25b**) with authentic lithium borohydride alone was not quite as efficient (40%) as that observed with *in situ* generated lithium borohydride (63%). Although the reason for this discrepancy was not immediately clear, the use of authentic lithium borohydride to reduce this ester was superior to the use of sodium borohydride alone.

Because the sodium borotritide which would be used to prepare a sample of tritiated adenosine was not carrier-free, an isotope effect would be expected during the reduction of the 2',3'-O-isopropylideneadenosine 5'-carboxylic acid ester. This reduction would ultimately be carried out with either the ethyl (**25b**) or the phenyl (**25g**) ester because these esters had been reduced most efficiently by *in situ* generated lithium borohydride. In order to determine if a significant isotope effect could be observed during simultaneous reduction by hydride and deuteride ions, each of these esters was reduced by a 50:50 mixture of *in situ* generated lithium borohydride and lithium borodeuteride. These very simple experiments were intended to simulate what might happen, although not to the same extent, during the simultaneous

reduction of either the ethyl (**25b**) or the phenyl (**25g**) ester by hydride and tritide ions in the final labeling reaction. The reduction of one ester with sodium borotritide might be characterized by a significantly larger isotope effect than the reduction of the other ester. Consequently, the product arising from the reduction of the first ester would have a lower specific activity than the product obtained by reduction of the other ester. Analysis of the recovered partially 5'-deuterated products from each mixed reduction by high-resolution proton NMR indicated that a somewhat smaller isotope effect might be expected during the reduction of the ethyl ester (**25b**) with *in situ* generated lithium borotritide than by a similar reduction of the phenyl ester (**25g**). The 5'-methylene protons in the products recovered from the mixed reductions of the ethyl (**25b**) and phenyl (**25g**) esters integrated to 1.13 and 1.21 protons, respectively. These results indicated that deuteride could compete with hydride somewhat more favorably during the reduction of the ethyl ester (**25b**) than during the reduction of the phenyl ester (**25g**). Extrapolating this information to the eventual borotritide reduction suggested that this reaction should be carried out with the ethyl ester (**25b**).

To firmly establish that the reduction of the ethyl ester (**25b**) would introduce the label exclusively into the 5'-position, as expected, complete deuteration of the 5'-position was accomplished by reduction of this ester (**25b**) with *in situ* generated lithium borodeuteride (Scheme X). This reduction provided a slightly smaller (41%) than expected yield of 5',5'-dideutero-2',3'-O-isopropylideneadenosine (**26**). Analysis of the recovered reduction product by high resolution proton NMR indicated that bis-deuteration at the 5'-position had indeed occurred, due to a lack of any proton resonances in the 3.75-4.05 ppm region where the 5'-methylene hydrogen resonances would normally appear with the unlabeled compound. All other resonances integrated to the expected



25b

26, X = D
28, X = T/H

27, X = D
29, X = T/H

Scheme X. Synthesis of 5',5'-dideuteroadenosine (**27**) and 5'-tritioadenosine (**29**).

number of hydrogens, confirming that the label was not somehow incorporated into any other positions. Similar analysis of this material by mass spectrometry indicated that the molecular weight had increased by two atomic mass units, which was to be expected with bis-deuteration. The 2',3'-O-isopropylidene ketal was then readily hydrolyzed in dilute aqueous hydrochloric acid (0.1 M) to afford 5',5'-dideuteroadenosine (**27**) in high yield (Scheme X). The overall bis-deuteration process was almost as efficient as the original method involving the reduction of the *in situ* generated acid azide of 2',3'-O-isopropylideneadenosine 5'-carboxylic acid (**24**) with sodium borodeuteride.[228] However, it was not so efficient as the direct reduction of ethyl adenosine 5'-carboxylate with sodium borodeuteride in ethanol described more recently.[229] The bis-deuteration method described above is probably best suited, therefore, for the preparation of 5',5'-dideutero-2',3'-O-isopropylideneadenosine (**27**).

The reduction of the ethyl ester (**25b**) with *in situ* generated lithium borotritide was readily carried out using the above well established procedure (Scheme X). The intermediate 5'-tritio-2',3'-O-isopropylideneadenosine (**28**) reduction product was readily isolated by column silica gel chromatography, although in a yield (7%) much lower than was originally expected. Autoradiography of a small aliquot of the recovered ultraviolet-absorbing material, which co-chromatographed with unlabeled 2',3'-O-isopropylideneadenosine on silica gel, indicated that it was indeed radioactive. The hydrolysis of the 2',3'-O-isopropylidene ketal protecting group was carried out in dilute aqueous hydrochloric acid (0.1 M) to provide a sample of 5'-tritiated adenosine (**29**) (Scheme X). The specific activity of the recovered material was 13.8 mCi/mmol. Adenosine tritiated in the 5'-position by this method should be suitable for use as a metabolic tracer because the label will not be lost through normal exchange processes with solvent. 5'-Tritiated adenosine (**29**) prepared

by an analogous sodium borotritide reduction of an ester of adenosine 5'-carboxylic acid has been briefly mentioned in the literature, but without a detailed discussion of the synthetic procedure.[230] The alternative method described above can be used to prepare 5'-tritiated adenosine (**29**) and its 2',3'-O-isopropylidene precursor (**28**) in high specific activity.

In conclusion, the synthesis of 5'-tritiated adenosine (**29**) has been achieved. The key step in its preparation was the reduction of ethyl 2',3'-O-isopropylideneadenosine 5'-carboxylate (**25b**) with lithium borotritide. This reducing agent was prepared *in situ* from sodium borotritide and lithium bromide. Preliminary screening of a series of alkyl and aryl esters of 2',3'-O-isopropylideneadenosine 5'-carboxylic acid (**25a-h**) had indicated that the ethyl ester (**25b**) would be best suited for this particular reduction. Acid hydrolysis of the 2',3'-O-isopropylidene ketal protecting group provided the final compound (**29**). An analogous reduction of this ester (**25b**) with *in situ* generated lithium borodeuteride, followed by deprotection, provided 5',5'-dideuteroadenosine (**27**). The 5'-tritiated adenosine (**29**), prepared by this procedure with high specific activity, could be subsequently used as a metabolic tracer.

Materials and Methods

2',3'-O-Isopropylideneadenosine, 2-chloroethanol, 2,2,2-trifluoroethanol, 2,2,2-trichloroethanol, propargyl alcohol, phenol, 4-bromophenol, thionyl chloride, sodium borodeuteride, lithium bromide, flash silica gel (230-400 mesh), and Dowex 50X8-100 cation exchange resin were obtained from Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and sodium borohydride were purchased from Sigma. Sodium borotritide (80 mCi) was obtained from Amersham. Precoated Baker 1B2-F silica gel sheets, obtained from VWR, were used for routine analytical thin layer chromatography. All melting points were taken on a Thomas-Hoover melting point apparatus and are uncorrected. Low resolution EI mass spectra were obtained on a Kratos MS-25 mass spectrometer using a heated direct insertion probe. A Kratos MS-50 mass spectrometer, which was equipped with a 23-kG magnet, a postacceleration detector operated at -10kV, and a source constructed by the UCSF Mass Spectrometry Facility, was used to obtain the positive LSIMS mass spectra. Samples for positive LSIMS mass spectra were prepared in a matrix of glycerol and 1M hydrochloric acid. Ultraviolet spectra were obtained on a Hitachi 100-80 UV-VIS spectrophotometer, which was equipped with an automatic six-cell changer and a Peltier temperature controller. High resolution proton NMR spectra were obtained with either a General Electric GN-500 NMR spectrometer or a home-built 240 MHz NMR spectrometer interfaced with a Nicolet 1180 computer and a 293-B pulse programmer. NMR samples were dissolved in either deuteriochloroform or D_2O, and the acquired spectra referenced to the tetramethylsilane or HDO (4.80 ppm) peaks, respectively. Microanalyses were performed by the Microanalytical Laboratory in the Department of Chemistry at the University of California, Berkeley. Kodak X-Omat Diagnostic Film, purchased from VWR Graphics, was used for

autoradiography. Radioactivity measurements were made on a Beckman LS 3801 scintillation counter using Ready-Solv™ CP scintillation cocktail purchased from Beckman.

2',3'-O-Isopropylideneadenosine 5'-Carboxylic Acid (**24**) was synthesized according to the procedure described by Schmidt et al..[228] 2',3'-O-Isopropylideneadenosine (6.000 g, 19.6 mmol) was dissolved in hot water (2.1 L) with stirring. After cooling to room temperature, potassium hydroxide (3.30 g, 76.9 mmol) was added to the solution in one portion, followed over approximately two hours by an aqueous solution (750 mL) of potassium permanganate (12.24 g, 78.6 mmol). The purple solution gradually became a brown suspension after stirring for three days at room temperature. Following the literature procedure,[228] 30% aqueous hydrogen peroxide (approximately 40 mL) was added to destroy the excess potassium permanganate and the insoluble manganese dioxide removed by filtration through a bed of Celite filter aid. The colorless filtrate was concentrated to approximately 300 mL and then cooled to 0°C.. Aliquots (1-2 mL) of 1M aqueous hydrochloric acid (total volume approximately 75 mL) were added to the stirred cold solution to lower the pH to about 4 to ultimately give a milky suspension. The precipitate was filtered and dried *in vacuo* at 50°C. to give 4.59 g (71.1%) of product as a colorless solid. An analytical sample (273 mg) was prepared from approximately 300 mg of the recovered product by recrystallization from water (2 L): mp 272-275°C., dec. (lit.[228] 276°C., dec.); TLC, acetone:conc. NH_4OH (10 mL:1 drop), R_f 0.22 (UV); 240 MHz ^1H NMR (4% NaOD in D_2O), δ 1.23 (s, 3H, isopropylidene CH_3), 1.39 (s, 3H, isopropylidene CH_3), 4.39 (d, 1H, J = 1.3Hz, H-4'), 5.21 (m, 2H, H-2' and H-3'), 5.97 (s, 1H, H-1'), 7.79 (s, 1H, adenine H-2 or H-8), 7.88 (s, 1H, adenine H-2 or H-8).

Anal. Calculated for $C_{13}H_{15}N_5O_5 \cdot 0.5H_2O$ (lit.,[228] anhydrous): C, 47.27; H, 4.88; N, 21.20. Found: C, 47.32; H, 4.57; N, 21.37.

Ethyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (Method A) (**25b**) was synthesized according to the general procedure of Prasad *et al.*,[233] although it was purified by other means involving chromatography. 2',3'-O-Isopropylideneadenosine 5'-carboxylic acid hemihydrate (**24**) (960 mg, 2.92 mmol) was suspended with stirring in ice-cold absolute ethanol (45 mL). Thionyl chloride (0.45 mL, 6.17 mmol, freshly distilled from triphenyl phosphite) was added, and the mixture stirred at room temperature for two days. The reaction was quenched by addition of an aqueous solution (60 mL) of sodium bicarbonate (2.016 g, 24.0 mmol). After the evaporation of all solvent, the residue was partitioned between chloroform (50 mL) and water (50 mL). The aqueous layer was washed with chloroform (3 x 50 mL), and the combined chloroform extracts dried over anhydrous magnesium sulfate. The filtered chloroform solution was concentrated to about 3 mL and loaded onto the top of a wet-packed flash silica gel column (14 x 3.0 cm, 40 g). The column was eluted with chloroform:isopropanol (75:25) at a flow rate of approximately 10 mL/min and 28 mL fractions were collected. The ultraviolet-absorbing product appeared in fractions 5-8, which were pooled and evaporated to give 426 mg (41.8%) of almost pure product as a colorless solid: TLC, chloroform:isopropanol (75:25), R_f 0.66 (UV, I_2); other characterization data reported in Method C below.

Ethyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (Method B) (**25b**) was prepared following the general procedure of Prasad *et al.*,[233] but was purified by other techniques involving chromatography. Dry N,N-dimethylformamide (1-2 drops) was added to an ice-cold stirred mixture of 2',3'-O-isopropylideneadenosine 5'-carboxylic acid hemihydrate (**24**) (960 mg, 2.92

mmol) in thionyl chloride (3.0 mL, 41.1 mmol, freshly distilled from triphenyl phosphite). After heating to 50°C. for 30 minutes, the mixture was evaporated to dryness, and the residue suspended three times in diethyl ether (10 mL) and evaporated to give a fine powder. Absolute ethanol (35.0 mL, 602 mmol) was added to the intermediate acid chloride, and the mixture stirred overnight at room temperature. The reaction was quenched with an aqueous solution (60 mL) of sodium bicarbonate (2.016 g, 24.0 mmol) and all solvent evaporated. The residue was partitioned between chloroform (50 mL) and water (50 mL), and the aqueous layer washed with chloroform (3 x 50 mL). The pooled chloroform extracts were dried over anhydrous magnesium sulfate. The filtered chloroform solution was concentrated to approximately 3 mL and loaded onto the top of a wet-packed flash silica gel column (17.5 x 3.0 cm, 50 g). The column was eluted with chloroform:isopropanol (75:25) at a flow rate of about 10 mL/min and 28 mL fractions were collected. Fractions 6-13 contained the ultraviolet-absorbing product and were pooled and evaporated to give 771 mg (25.7%) of pure product as a colorless solid: TLC, chloroform:isopropanol (75:25), R_f 0.69 (UV); other characterization data reported in Method C below.

2,2,2-Trifluoroethyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (Method B) (**25d**) was synthesized on the same scale and according to the same procedure (described immediately above) used to prepare the corresponding ethyl ester (**25b**). 2,2,2-Trifluoroethanol (20.0 mL, 274 mmol) was added to the intermediate acid chloride and the mixture stirred overnight at room temperature. The chloroform extract (approximately 3 mL) of the quenched reaction mixture was loaded onto the top of a wet-packed flash silica gel column (20.5 x 3.0 cm, 65 g). The column was eluted with chloroform:isopropanol (90:10) at a flow rate of about 10 mL/min and 28 mL fractions were collected. Ultraviolet-absorbing material appeared in fractions 9-

12, which were pooled and evaporated to give 190 mg (16.1%) of pure product as a colorless solid: TLC, chloroform:isopropanol (90:10), R_f 0.39 (UV); other characterization data reported in Method C below.

General Carbodiimide-Mediated Synthesis of Alkyl and Aryl 2',3'-O-Isopropylideneadenosine 5'-Carboxylates (Method C). 2',3'-O-Isopropylideneadenosine 5'-carboxylic acid hemihydrate (**24**) (987 mg, 3.0 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (2.873 g, 15.0 mmol) were suspended in dry acetonitrile (50 mL, freshly distilled from P_2O_5). The alkyl or aryl alcohol (15.0 mmol) was then added and the suspension stirred at room temperature for approximately 18 hours. After the almost clear reaction mixture was evaporated to dryness, the residue was redissolved in chloroform (100 mL) and concentrated to about 5 mL. The viscous liquid was loaded onto the top of a wet-packed flash silica gel column (for alkyl esters: 28 x 3.0 cm, 80 g; for aryl esters: 40 x 3.5 cm, 175 g). The column was eluted with mixtures of chloroform:isopropanol (for alkyl esters) or chloroform:acetone (for aryl esters) at a flow rate of approximately 10 mL/min and 28 mL fractions were collected. Those fractions containing the ultraviolet-absorbing product were pooled and evaporated to dryness, and the residue twice dissolved in chloroform (50 mL) and again evaporated to dryness. A final chloroform solution (50 mL) of the residue was concentrated to approximately 5 mL and slowly triturated with hexanes (100 mL). The precipitate was filtered and air-dried to provide the analytically pure product as a colorless powder in 47-86% yield.

Methyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (Method C) (**25a**) was prepared by condensation of the corresponding free acid (**24**) with methanol (0.61 mL) according to the general procedure described above. Flash silica gel chromatography of the crude reaction mixture using chloroform:isopropanol (92:8) as the eluting solvent provided 645 mg (64.2%)

of pure product in fractions 13-38: mp 239-240°C., dec. (lit.[237] 241-243°C.); TLC, chloroform:isopropanol (92:8), R_f 0.22 (UV, faint I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.42 (s, 3H, isopropylidene CH_3), 1.61 (s, 3H, isopropylidene CH_3), 3.40 (s, 3H, CO_2CH_3), 4.83 (d, 1H, J = 1.6Hz, H-4'), 5.52 (d, 1H, J = 6.0Hz, H-2'), 5.54 (br s, 2H, adenine NH_2), 5.70 (dd, 1H, J = 1.6Hz, 6.0Hz, H-3'), 6.18 (s, 1H, H-1'), 7.93 (s, 1H, adenine H-2 or H-8), 8.24 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 13.8 x 10^3); MS (EI), 335 $(M)^+$, 320 (M - isopropylidene $CH_3)^+$.

Anal. Calculated for $C_{14}H_{17}N_5O_5$: C, 50.15; H,5.11; N, 20.89. Found: C, 50.54; H, 5.23; N, 20.55.

Ethyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (Method C) (**25b**) was synthesized by condensation of the free acid (**24**) with ethanol (0.88 mL) according to the general procedure outlined above. Purification of the product by flash silica gel chromatography using chloroform:isopropanol (92:8) as the eluting solvent provided 664 mg (63.4%) of pure material in fractions 10-16: mp 169-171 °C. (lit.[233] 171-172°C.); TLC, chloroform:isopropanol (92:8), R_f 0.21 (UV, faint I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.00 (t, 3H, J = 7.2Hz, CH_3CH_2), 1.44 (s, 3H, isopropylidene CH_3), 1.61 (s, 3H, isopropylidene CH_3), 3.76 (m, 1H, CH_3CH_2), 3.95 (m, 1H, CH_3CH_2), 4.81 (d, 1H, J = 1.4Hz, H-4'), 5.55 (d, 1H, J = 6.0Hz, H-2'), 5.64 (br s, 2H, adenine NH_2), 5.70 (dd, 1H, J = 1.4Hz, 6.0Hz, H-3'), 6.20 (s, 1H, H-1), 7.95 (s, 1H, adenine H-2 or H-8), 8.24 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 13.0 x 10^3); MS (EI), m/e 349 $(M)^+$, 334 (M - isopropylidene $CH_3)^+$.

Anal. Calculated for $C_{15}H_{19}N_5O_5$: C, 51.57; H, 5.48; N, 20.05. Found: C, 51.62; H, 5.40; N, 19.98.

2-Chloroethyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (Method C) (**25c**) was prepared by the carbodiimide-mediated condensation of the free

acid (**24**) with 2-chloroethanol (1.01 mL) following the general procedure summarized above (Method C). Subjection of the crude reaction mixture to flash silica gel chromatography resulted in the elution of 545 mg (47.4%) of pure product in fractions 11-16: mp 82-85°C.; TLC, chloroform:isopropanol (92:8), R_f 0.22 (UV, faint I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.44 (s, 3H, isopropylidene CH_3), 1.61 (s, 3H, isopropylidene CH_3), 3.47 (m, 2H, $ClCH_2$), 3.95 (m, 1H, CH_2O), 4.21 (m, 1H, CH_2O), 4.88 (d, 1H, J = 1.4Hz, H-4'), 5.52 (d, 1H, J = 6.0Hz, H-2'), 5.77 (dd, 1H, J = 1.4Hz, 6.0Hz, H-3'), 6.06 (br s, 2H, adenine NH_2), 6.22 (s, 1H, H-1'), 7.95 (s, 1H, adenine H-2 or H-8), 8.24 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 13.1 x 10^3); MS(EI), m/e 383 (M, $^{35}Cl)^+$, 385 (M, $^{37}Cl)^+$, 368 [M(^{35}Cl) - isopropylidene $CH_3]^+$, 370 [M(^{37}Cl) - isopropylidene $CH_3]^+$.

Anal. Calculated for $C_{15}H_{18}ClN_5O_5$: C, 46.94; H, 4.73; N, 18.25; Cl, 9.24. Found: C, 46.64; H, 4.59; N, 18.03; Cl, 9.01.

2,2,2-Trifluoroethyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (Method C) (**25d**) was synthesized by the condensation of 2,2,2-trifluoroethanol (1.09 mL) and the free acid (**24**) according to the general procedure described above. Flash silica gel chromatography of the crude reaction mixture using chloroform:isopropanol (95:5) as the eluting solvent resulted in the appearance of 1.039 g (85.9%) of pure product in fractions 13-25: mp 98-100°C.; TLC, chloroform:isopropanol (95:5), R_f 0.13 (UV, faint I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.44 (s, 3H, isopropylidene CH_3), 1.62 (s, 3H, isopropylidene CH_3), 4.34 (m, 1H, CH_2O), 4.62 (m, 1H, CH_2O), 4.93 (d, 1H, J = 1.4Hz, H-4'), 5.49 (d, 1H, J = 6.0Hz, H-2"), 5.79 (dd, 1H, J = 1.4Hz, 6.0Hz, H-3'), 5.88 (br s, 2H, adenine NH_2), 6.20 (s, 1H, H-1'), 7.91 (s, 1H, adenine H-2 or H-8), 8.18 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 13.3 x 10^3); MS(EI), m/e 403 $(M)^+$, 388 (M - isopropylidene $CH_3)^+$.

Anal. Calculated for $C_{15}H_{16}F_3N_5O_5$: C, 44.67; H,4.00; N, 17.36. Found: C, 44.69; H, 4.14; N, 17.12.

2,2,2-Trichloroethyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (Method C) (**25e**) was prepared by the carbodiimide-mediated condensation of the free acid (**24**) and 2,2,2-trichloroethanol (1.56 mL) following the general procedure outlined above. Purification of the product by flash silica gel chromatography on a larger than usual column (38 x 3.5 cm, 175 g) utilizing chloroform:isopropanol (95:5) as the eluting solvent resulted in the appearance of 886 mg (65.2%) of pure material in fractions 34-54: mp 133-135°C.; TLC, chloroform:isopropanol (95:5), R_f 0.21 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.45 (s, 3H, isopropylidene CH_3), 1.62 (s, 3H, isopropylidene CH_3), 4.23 (d, 1H, J = 11.9Hz, CH_2O), 4.58 (d, 1H, J = 11.9Hz, CH_2O), 4.98 (d, 1H, J = 1.4Hz, H-4'), 5.52 (d, 1H, J = 6.0Hz, H-2'), 5.67 (br s, 2H, adenine NH_2), 5.83 (dd, 1H, J = 1.4Hz, 6.0Hz, H-3'), 6.21 (s, 1H, H-1'), 7.93 (s, 1H, adenine H-2 or H-8), 8.23 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 13.4 x 10^3); MS(EI), m/e 451 [M(all ^{35}Cl)]+, 453 [M (two ^{35}Cl, one ^{37}Cl)]+, 436 [M (all ^{35}Cl) - isopropylidene CH_3]+, 438 [M (two ^{35}Cl, one ^{37}Cl) - isopropylidene CH_3]+.

Anal. Calculated for $C_{15}H_{16}Cl_3N_5O_5$: C, 39.80; H, 3.56; N, 15.47; Cl, 23.50. Found: C, 39.64; H.3.50; N, 15.28; Cl, 23.28.

1-(2-Propynyl) 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (Method C) (**25f**) was synthesized on a slightly larger scale than that described in the general procedure summarized above. The free acid (**24**) (1.316 g, 4.0 mmol) and propargyl alcohol (1.16 mL, 20.0 mmol) were condensed in acetonitrile (70 mL) in the presence of the carbodiimide (3.820 g, 20.0 mmol). Flash silica gel chromatography (28 x 3.0 cm, 80 g) of the crude reaction mixture using chloroform:isopropanol (92:8) as the eluting solvent led to the appearance of 1.20 g (82.3%) of pure product in fractions 9-21: mp 174-176°C.; TLC,

chloroform:isopropanol (92:8), R_f 0.23 (UV, faint I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.44 (s, 3H, isopropylidene CH_3), 1.62 (s, 3H, isopropylidene CH_3), 2.47 (t, 1H, J = 2.5Hz, alkyne H), 4.40 (dd, 1H, J = 2.5Hz, 15.6Hz, CH_2O), 4.53 (dd, 1H, J = 2.5Hz, 15.6Hz, CH_2O), 4.89 (d, 1H, J = 1.4Hz, H-4'), 5.52 (d, 1H, J = 6.0Hz, H-2'), 5.80 (dd, 1H, J = 1.4Hz, 6.0Hz, H-3'), 6.24 (s, 1H, J-1'), 6.37 (br s, 2H, adenine NH_2), 7.96 (s, 1H, adenine H-2 or H-8), 8.23 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 14.0 x 10^3); MS(EI), m/e 359 $(M)^+$, 344 (M - isopropylidene $CH_3)^+$.

Anal. Calculated for $C_{16}H_{17}N_5O_5$: C, 53.48; H, 4.77; N, 19.49. Found: C, 53.31; H, 4.65; N, 19.32.

Phenyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (Method C) (**25g**) was prepared on a slightly larger scale than that described in the general procedure described above. The carbodiimide (3.820 g, 20.0 mmol) was used to promote the condensation of phenol (1.880 g, 20.0 mmol) and the free acid (**24**) (1.316 g, 4.0 mmol) in acetonitrile (70 mL). In the flash silica gel chromatographic purification of the product, the first 48 fractions were eluted with chloroform:acetone (70:30) and an additional 32 fractions were eluted with chloroform:acetone (20:80). Fractions 46-80 contained 1.351 g (85.1%) of pure product: mp 167-168°C.; TLC, chloroform:acetone (20:80), R_f 0.51 (UV, faint I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.46 (s, 3H, isopropylidene CH_3), 1.65 (s, 3H, isopropylidene CH_3), 5.07 (d, 1H, J = 1.6Hz, H-4'), 5.59 (d, 1H, J = 5.9Hz, H-2'), 5.74 (br s, 2H, adenine NH_2), 5.92 (dd, 1H, J = 1.6Hz, 5.9Hz, H-3'), 6.26 (s, 1H, H-1'), 6.79 (m, 2H, phenyl H-2 and H-6), 7.16 (m, 1H, phenyl H-4), 7.28 (m, 2H, phenyl H-3 and H-5), 7.95 (s, 1H, adenine H-2 or H-8), 8.24 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 12.4 x 10^3); MS(EI), m/e 382 (M - isopropylidene $CH_3)^+$, 276 (M - phenyl-OCO)$^+$.

Anal. Calculated for $C_{19}H_{19}N_5O_5$: C, 57.43; H, 4.82; N, 17.62. Found: C, 57.67; H, 4.98; N, 17.29.

4-Bromophenyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (Method C) (**25h**) was synthesized from the free acid (**24**) and 4-bromophenol (2.595 g) according to the general procedure outlined above. Purification of the product by flash silica gel chromatography using chloroform:acetone (70:30) to elute the first 48 fractions and chloroform:acetone (20:80) to elute an additional 32 fractions led to the appearance of 841 mg (58.9%) of pure material in fractions 44-77: mp 110-113°C.; TLC, chloroform:acetone (20:80), R_f 0.57 (UV, faint I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.47 (s, 3H, isopropylidene CH_3), 1.65 (s, 3H, isopropylidene CH_3), 5.09 (d, 1H, J = 1.1Hz, H-4'), 5.55 (d, 1H, J = 5.9Hz, H-2'), 5.98 (dd, 1H, J = 1.1Hz, 5.9Hz, H-3'), 6.09 (br s, 2H, adenine NH_2), 6.28 (s, 1H, H-1'), 6.72 (d, 2H, J = 8.8Hz, phenyl H-2 or H-6), 7.42 (d, 2H, J = 8.8Hz, phenyl H-3 and H-5), 7.97 (s, 1H, adenine H-2 or H-8), 8.21 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 13.2 x 10^3); MS(EI), m/e 460 [M(^{79}Br) - isopropylidene CH_3]$^+$, 462 [M(^{81}Br) - isopropylidene CH_3]$^+$.

Anal. Calculated for $C_{19}H_{18}BrN_5O_5$: C, 47.91; H, 3.81; N,14.70; Br, 16.78. Found: 47.70; H,3.72; N, 14.48; Br, 16.93.

General Procedure for the Borohydride Reduction of Alkyl and Aryl 2',3'-O-Isopropylideneadenosine 5'-Carboxylates to 2',3'-O-Isopropylideneadenosine. A mixture of sodium borohydride (19.0 mg, 0.5 mmol) and lithium bromide (43.5 mg, 0.5 mmol) in dry diglyme (7.5 mL, freshly distilled from calcium hydride) was added to the alkyl or aryl 2',3'-O-isopropylideneadenosine 5'-carboxylate (**25a-h**) (0.5 mmol). The cloudy mixture was heated with stirring to 100°C. for 6.5 hours. After cooling to room temperature, the reaction was quenched with 50% aqueous acetone (5.0 mL). The clear solution was evaporated to dryness, and the residue extracted with chloroform (4 x 25 mL).

The combined chloroform extracts were concentrated to approximately 2 mL and loaded onto the top of a wet-packed flash silica gel column (54 x 1.5 cm, 40 g). The column was eluted (by gravity) with chloroform:isopropanol (90:10) at a flow rate of 0.8 mL/min and 7 mL fractions were collected. The ultraviolet-absorbing product generally appeared in fractions 23-45, and the appropriate fractions were pooled and evaporated to dryness. The residue was twice dissolved in chloroform (25 mL) and evaporated to give variable yields of the pure product as a colorless solid. The recovered material was confirmed to be 2',3'-O-isopropylideneadenosine on the basis of (1) a melting point very close to or the same as the authentic compound (221-222°C.), (2) an identical mobility (R_f 0.18) on silica gel using chloroform:isopropanol (90:10) as the solvent system, and (3) an identical 240 MHz ^1H NMR spectrum.

Reduction of Methyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (**25a**). The reduction of this ester (**25a**) (168 mg) was carried out following the general procedure described above. Purification by column chromatography resulted in the appearance of 89 mg (58.0%) of pure product in fractions 25-38.

Reduction of Ethyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (Methods A-D) (**25b**).

Method A. This ester (**25b**) (175 mg) was reduced according to the above general procedure. Chromatography of the concentrated chloroform extract resulted in the elution of 97 mg (63.4%) of pure product in fractions 27-37.

Method B. Following a slight modification of the above general procedure, this ester (**25b**) (175 mg) was reduced by a mixture of sodium borohydride (9.5 mg, 0.25 mmol), sodium borodeuteride (10.5 mg, 0.25 mmol), and lithium bromide (43 mg, 0.5 mmol) in diglyme (7.5 mL). Purification by column chromatography led to the elution of 92 mg (59.7%) of the partially 5'-

deuterated product [isotope effect (H/D) = 1.3] in fractions 29-42: 240 MHz ^{1}H NMR ($CDCl_3$), δ 3.75-4.03 (m, 1.13H, H-5').

Method C. The ester (**25b**) (175 mg) was reduced with sodium borohydride (19.0 mg, 0.5 mmol) in the absence of lithium bromide under conditions otherwise identical to those described in the above general procedure. Column chromatography of the chloroform extract was used to isolate 7 mg (4.6%) of pure product in fractions 34-42.

Method D. The ester (**25b**) (175 mg) was reduced with only lithium borohydride (11.0 mg, 0.5 mmol) in dry diglyme (7.5 mL) under the conditions specified in the general procedure. Purification by column chromatography resulted in the elution of 61 mg (39.9%) of pure product in fractions 23-30.

Reduction of 2-Chloroethyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (**25c**). The general procedure described above was followed to reduce this ester (**25c**) (192 mg). Column chromatography of the chloroform extract resulted in the appearance of 58 mg (37.9%) of pure product in fractions 30-45.

Reduction of 2,2,2-Trifluoroethyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (**25d**). This ester (**25d**) (202 mg) was reduced to the corresponding alcohol according to the general procedure summarized above. Purification by silica gel chromatography led to the elution of 78 mg (56.0%) of pure product in fractions 28-40.

Reduction of 2,2,2-Trichloroethyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (**25e**). The reduction of this ester (**25e**) (226 mg) was carried out following the general procedure described above. Subjection of the chloroform extract to column chromatography provided 76 mg (49.7%) of pure product in fractions 28-40.

Reduction of 1-(2-Propynyl) 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (**25f**). This propargyl ester (**25f**) (178 mg) was reduced to the

corresponding alcohol following the above general procedure. Column chromatography of the crude chloroform extract resulted in the appearance of 77 mg (50.2%) of pure product in fractions 29-42.

Reduction of Phenyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (Methods A-C) (**25g**).

Method A. On a scale somewhat smaller than usual, this ester (**25g**) (125 mg, 0.33 mmol) was reduced by a mixture of sodium borohydride (13.3 mg, 0.33 mmol) and lithium bromide (30.5 mg, 0.33 mmol) in dry diglyme (5.0 mL) following the above general procedure. Purification of the chloroform extract using chloroform:isopropanol (80:20) as the eluting solvent provided 61 mg (60.4%) of pure product in fractions 17-38.

Method B. The general procedure was slightly modified to reduce the ester (**25g**) (189 mg) with a mixture of sodium borohydride (9.5 mg, 0.25 mmol), sodium borodeuteride (10.5 mg, 0.25 mmol), and lithium bromide (43 mg, 0.5 mmol) in dry diglyme (7.5 mL). Purification of the chloroform extract by column chromatography resulted in the appearance of 95 mg (61.9%) of partially 5'-deuterated product [isotope effect (H/D) = 1.5] in fractions 29-45: 240 MHz ^{1}H NMR ($CDCl_3$), δ 3.76-4.01 (m, 1.21H, H-5').

Method C. In the absence of lithium bromide, the ester (**25g**) (189 mg) was reduced with sodium borohydride (19.0 mg, 0.5 mmol) under conditions otherwise identical to those described in the general procedure. Purification by column chromatography led to the appearance of 40 mg (26.1%) of pure product in fractions 28-37.

Reduction of 4-Bromophenyl 2',3'-O-Isopropylideneadenosine 5'-Carboxylate (**25h**). The reduction of this ester (**25h**) (238 mg) to the corresponding alcohol was accomplished by the general procedure summarized above.

Column chromatography of the chloroform extract resulted in the elution of 66 mg (43.1%) of pure product in fractions 29-42.

5',5'-Dideutero-2',3'-O-Isopropylideneadenosine (**26**). A mixture of sodium borodeuteride (63 mg, 1.50 mmol, 98% atom D) and lithium bromide (131 mg, 1.5 mmol) in dry diglyme (22.5 mL, freshly distilled from calcium hydride) was added to ethyl 2',3'-O-isopropylideneadenosine 5'-carboxylate (**25b**) (524 mg, 1.50 mmol). The stirred suspension was heated to 100°C. for 6.5 hours. After cooling to room temperature, the reaction mixture was diluted with 50% aqueous acetone (15 mL) and evaporated to dryness. The residue was extracted with chloroform (4 x 25 mL). The combined chloroform extracts were concentrated to approximately 3 mL and loaded onto the top of a wet-packed flash silica gel column (54 x 1.5 cm, 40 g). The column was eluted (by gravity) with chloroform:isopropanol (90:10) at a flow rate of 1.0 mL/min and 7.5 mL fractions were collected. The ultraviolet-absorbing product was eluted in fractions 25-33, which were pooled and evaporated to dryness. The residue was twice dissolved in chloroform (100 mL) and evaporated to give 191 mg (41.2%) of pure product as a colorless solid: mp 219-221°C. (authentic 2',3'-O-isopropylideneadenosine, mp 221-222°C.); TLC, chloroform:isopropanol (90:10), Rf 0.18 (UV, I_2); 500 MHz ^1H NMR ($CDCl_3$), δ 1.38 (s, 3H, isopropylidene CH_3), 1.65 (s, 3H, isopropylidene CH_3), 4.54 (s, 1H, H-4'), 5.12 (d, 1H, J = 6.6Hz, H-3'), 5.21 (m, 1H, H-2'), 5.85 (br s, 2H, adenine NH_2), 5.86 (d, 1H, J = 4.3Hz, H-1'), 6.54 (s, 1H, CD_2OH), 7.84 (s, 1H, adenine H-2 or H-8), 8.32 (s, 1H, adenine H-2 or H-8); UV λ_{max} (MeOH) 259 nm (ε 13.5 x 10^3); MS(EI), m/e 309 $(M)^+$, 294 (M - isopropylidene $CH_3)^+$.

5',5'-Dideuteroadenosine (**27**). A solution of 5',5'-dideutero-2',3'-O-isopropylideneadenosine (**26**) (129 mg, 0.42 mmol) in 0.1M aqueous hydrochloric acid (7.5 mL) was stirred at room temperature for seven days. The

reaction mixture was then loaded directly onto a column of Dowex 50X8-100 (30 x 1.0 cm, 30 g, H^+ form). The column was washed with water (200 mL) at a flow rate of 2 mL/min and 7 mL fractions were collected. Beginning with fraction 26, the column was eluted with 1M aqueous ammonium hydroxide (250 mL). The ultraviolet-absorbing product appeared in fractions 34-50, which were pooled and evaporated to dryness. The residue was twice dissolved in water (50 mL) and evaporated, and then redissolved in water (5 mL) and lyophilized to give 109 mg (97.3%) of product as a colorless solid containing only a trace of adenine: mp 220-222°C. (authentic adenosine, mp 234-236°C.); TLC, acetone:conc. NH_4OH (10mL:5drops), R_f 0.33 (UV, I_2); 500 MHz ^1H NMR (D_2O), δ 4.25 (d, 1H, J = 3.3Hz, H-4'), 4.39 (m, 2H, overlapping H-2' and H-3'), 6.02 (d, 1H, J = 6.2Hz, H-1'), 8.19 (s, 1H, adenine H-2 or H-8), 8.29 (s, 1H, adenine H-2 or H-8); UV λ_{max} (H_2O) 259 nm (ε 13.7 x 10^3); MS (positive LSIMS), m/e 270 $(M + H)^+$.

5'-Tritio-2',3'-O-Isopropylideneadenosine (**28**). A mixture of sodium borotritide (17.42 mg, 0.46 mmol, 80 mCi) and lithium bromide (40.0 mg, 0.46 mmol) in dry diglyme (7.0 mL, freshly distilled from calcium hydride) was added to ethyl 2',3'-O-isopropylideneadenosine 5'-carboxylate (**25b**) (161 mg, 0.46 mmol). The suspension was stirred at 100°C. for 6.5 hours, and, after cooling to room temperature, was diluted with 50% aqueous acetone (5.0 mL). The clear solution was evaporated to dryness and the residue extracted with chloroform (4 x 25 mL). The pooled chloroform extracts were concentrated to approximately 2 mL and loaded onto the top of a wet-packed flash silica gel column. The column was eluted (by gravity) with chloroform:isopropanol (90:10) at a flow rate of 0.5 mL/min and 7.5 mL fractions were collected. Analysis of 1 μL aliquots of each fraction on silica gel by autoradiography indicated that most of the radioactivity had co-eluted with the ultraviolet-absorbing material appearing

in fractions 26-32. These fractions were pooled and evaporated. The residue was twice dissolved in chloroform (15 mL) and evaporated to give 9.2 mg (6.5%) of pure product as a colorless solid. The recovered material migrated on silica gel in a fashion identical to that of authentic unlabelled 2',3'-O-isopropylideneadenosine and was used directly in the next step: TLC, chloroform:isopropanol (90:10), R_f 0.15 (UV, I_2, radioactive).

5'-Tritioadenosine (**29**). A solution of 5'-tritio-2',3'-O-isopropylideneadenosine (**28**) (9.2 mg, 0.03 mmol) in 0.1M aqueous hydrochloric acid (0.5 mL) was stirred at room temperature for four days. The reaction mixture was loaded directly onto a column of Dowex 50X8-100 (8 x 0.5 cm, 1.0 g). The column was washed with water (160 mL) at a flow rate of 1.5 mL/min and 8 mL fractions were collected. Beginning with fraction 21, the column was washed with 1M aqueous ammonium hydroxide, which led to the elution of the product in fractions 22-26. Autoradiography of the material present in 1 μL aliquots of each of these fractions confirmed that the radioactivity applied to the column had been co-eluted with the ultraviolet-absorbing material. Fractions 22-26 were pooled and concentrated to approximately 2 mL, and subsequently lyophilized to give 7.5 mg (93.8%) of product as a colorless solid. This material migrated on silica gel in a manner similar to that of authentic unlabelled adenosine: TLC, acetone:conc. NH_4OH (10 mL:5 drops), R_f 0.17 (UV, I_2, radioactive). The total recovered radioactivity was 388 μCi, which corresponds to a specific activity of 13.8 mCi/mmol. The chemical and radiochemical yields, based on both the reduction and deprotection steps, were 6.1% and 0.5%, respectively.

Appendix A

An Efficient Modern Synthesis of D,L-Glyceramide

Two of the key intermediates in the glycolytic breakdown of α-D-glucose are 2-phospho-D-glycerate and 3-phospho-D-glycerate. The formation of 3-phospho-D-glycerate from 3-phosphoglyceroyl phosphate is catalyzed by phosphoglycerate kinase. After 3-phospho-D-glycerate is isomerized to 2-phospho-D-glycerate by phosphoglyceromutase, enolase catalyzes the subsequent dehydration of 2-phospho-D-glycerate to provide phosphoenolpyruvate. The carboxylate groups of 2-phospho-D-glycerate and 3-phospho-D-glycerate could be implicated as participants in their binding interactions with these glycolytic enzymes. Replacing the anionic carboxylate group in each compound with a neutral amide might be expected to somehow modify these interactions. Hypotheses regarding the nature of these interactions might then be developed from a careful analysis of the various kinetic parameters (i.e., V_{max}, K_m, and K_i) which may be extracted from the raw data accumulated during assays of the activity of the enzyme in the presence of the analogue.

Before all this can proceed though, an efficient method for introducing the amide functionality into these compounds must be developed. The simplest approach to the synthesis of 2-phospho-D-glyceramide and 3-phospho-D-glyceramide would involve the conversion of D-glycerate to D-glyceramide followed by chemical or enzymatic phosphorylation. The products might then be tested as potential substrates and/or inhibitors of phosphoglycerate kinase, phosphoglyceromutase, and enolase. The goal of the present work was the development of an efficient synthesis of D,L-glyceramide.

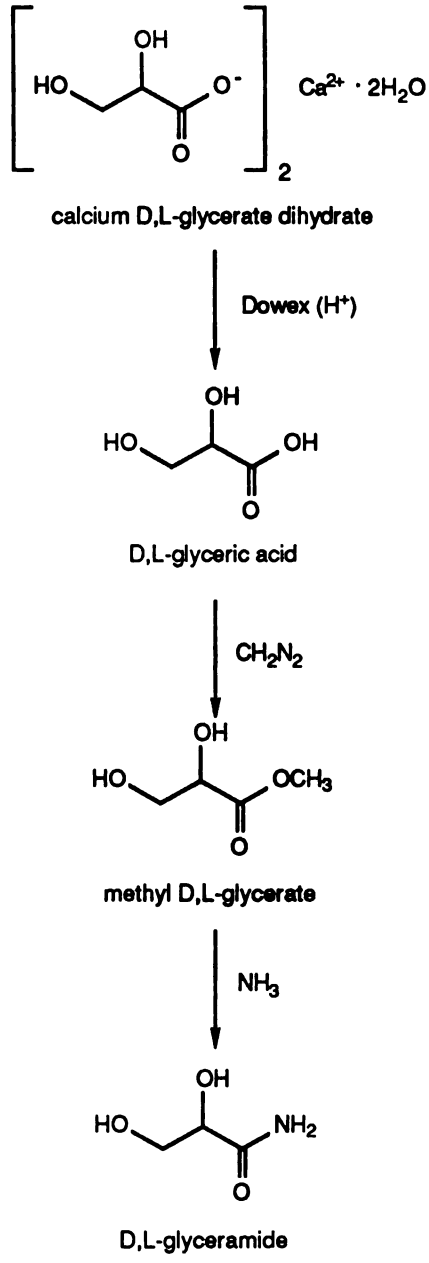
This compound has actually been known since the early 1900's when it was prepared by the amination of the corresponding methyl ester.[235] Attempts

to reproduce the original 1893 synthesis of methyl D,L-glycerate[236] have not been very successful using the same classical Fischer esterification methods. Therefore, the development of a modern synthesis of this ester was undertaken. Subsequent amination of methyl D,L-glycerate would then readily provide the desired D,L-glyceramide.

One modern reagent commonly used to prepare methyl esters is diazomethane (CH_2N_2), which is most often prepared from N-methyl-N-nitroso-p-toluenesulfonamide, or Diazald. Because this reagent only reacts with free acids, calcium D,L-glycerate was converted to D,L-glyceric acid by passage through a cation exchange column in the acid (H^+) form. Esterification of the free acid in ethanol solution using freshly prepared diazomethane was readily accomplished under very mild conditions. The methyl D,L-glycerate product was easily purified by vacuum distillation and characterized by high resolution proton NMR and mass spectrometry. Subsequent dissolution of the recovered methyl D,L-glycerate in anhydrous ammonia for several days ultimately provided the desired D,L-glyceramide as a colorless solid. The preparation of D,L-glyceramide using this procedure is shown in Scheme XI.

Materials and Methods

Calcium D,L-glycerate dihydrate, Dowex 50X8-100, and Diazald were purchased from Aldrich. High resolution proton NMR spectra were acquired with a home-built 240 MHz NMR spectrometer interfaced with a Nicolet 1180 computer and a 293-B pulse programmer. NMR samples were dissolved in deuterated DMSO and referenced to an internal tetramethylsilane standard. The mass spectrum of methyl D,L-glycerate was obtained with a Kratos AEI MS-902 mass spectrometer which had been specially equipped to do chemical ionization. The melting point of D,L-glyceramide was taken on a Thomas-



Scheme XI. The preparation of D,L-glyceramide from calcium D,L-glycerate dihydrate.

Hoover melting point apparatus and is uncorrected. The Microanalytical Laboratory in the Department of Chemistry at the University of California, Berkeley performed the microanalysis of D,L-glyceramide.

Methyl D,L-Glycerate. A mixture of calcium D,L-glycerate dihydrate (5.720 g, 20.0 mmol) and water (75 mL) was heated with a heat gun to dissolve most of the salt. The slightly cloudy solution was passed through a column of Dowex 50X8-100 (30 x 1.0 cm, 40 g, H^+ form) at a flow rate of approximately 5 mL/min. The acidic clear column eluate appearing at the void volume was collected and pooled with the water (300 mL) used to wash the ion exchange column. The aqueous solution of D,L-glyceric acid was evaporated to a syrup at room temperature. The syrup was redissolved in absolute ethanol (150 mL) and stirred in an open Erlenmeyer flask (1 L) at 0°C. in an ice bath. An ethereal solution of diazomethane (approximately 250 mL), prepared from Diazald (21.4 g, 100 mmol) according to the procedure outlined in the Aldrich Technical Bulletin No. AL-113, was carefully poured into the cold Erlenmeyer flask situated behind a blast shield. As the stirred solution was allowed to warm to room temperature overnight, its initial yellow color completely disappeared. The colorless solution was evaporated to leave a syrup from which the product (2.35 mL, 2.808 g, 58.2%) was vacuum distilled: bp 85-89°C. (4.5 mm Hg); 240 MHz ^1H NMR (d_6-DMSO), δ 3.57 (m, 2H, C*H*$_2$OH), 3.64 (s, 3H, CH_3), 4.07 (m, 1H, C*H*OH), 4.81 (t, 1H, J = 5.9Hz, CH_2O*H*), 5.35 (d, 1H, J = CHO*H*); MS (CI with isobutane), m/e 121 $(M + H)^+$.

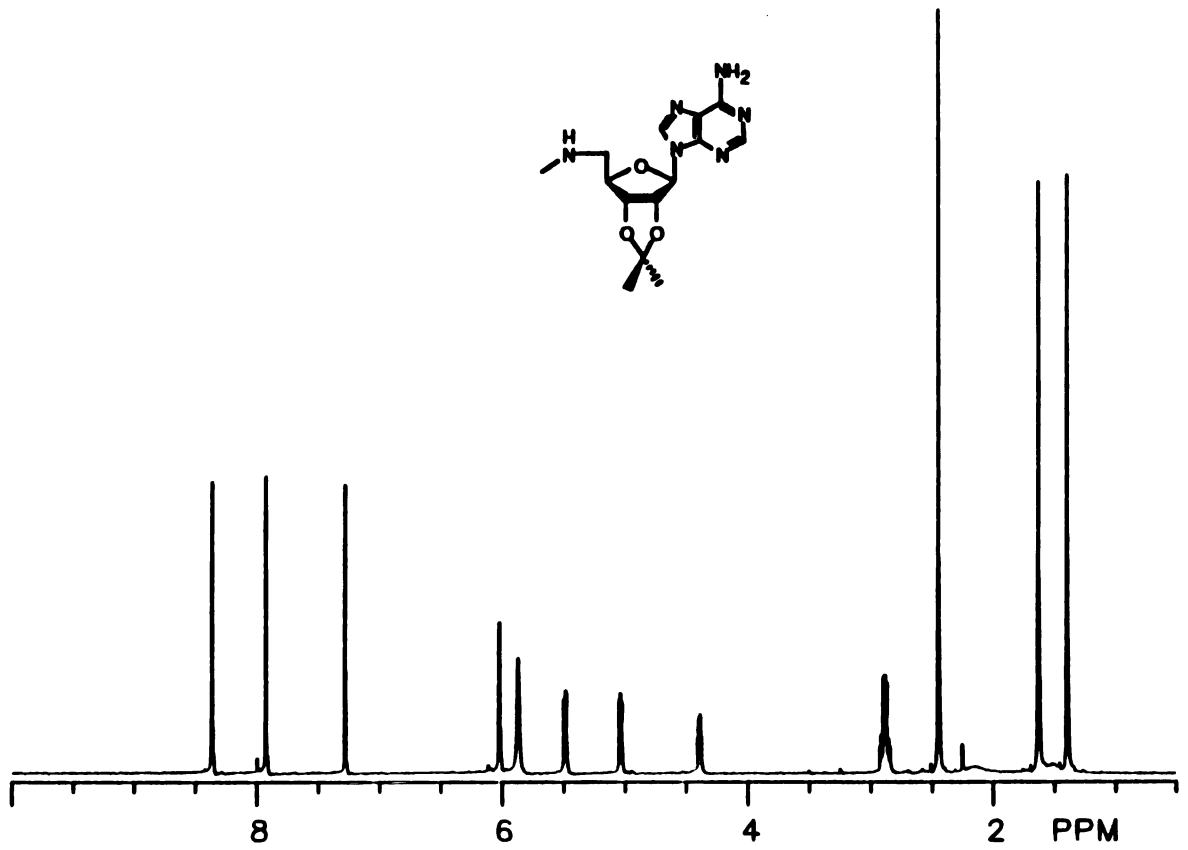
D,L-Glyceramide. Methyl D,L-glycerate (2.400 g, 20.0 mmol) was dissolved in anhydrous ammonia (100 mL, dried by passage through potassium hydroxide pellets) and the solution sealed in a bomb for seven days. After the bomb was cooled in dry ice and opened, the ammonia was allowed to boil off to leave a residue. The viscous material was dissolved in methanol (100 mL) and

evaporated, and redissolved in methanol (100 mL) and concentrated to approximately 20 mL. The methanolic solution was slowly diluted with diethyl ether (250 mL) and stored overnight at -20°C.. The resulting crystals were filtered and dried overnight at room temperature *in vacuo*. The mother liquors were concentrated to approximately 10 mL, slowly diluted with diethyl ether (250 mL), and stored at -20°C. overnight to provide a second crop of crystals. Recrystallization from a methanolic solution (100 mL) of the combined crops in an identical manner provided 1.724 g (82.1%) of analytically pure product as colorless crystals: mp 89.5-91.5°C. (lit.[235] 91.5-92.0°C.); 240 MHz ^{1}H NMR (d_6-DMSO), δ 3.40-3.61 (m, 2H, C*H*$_2$OH), 3.81 (m, 1H, C*H*OH), 4.65 (t, 1H, J = 5.8Hz, CH$_2$O*H*), 5.33 (d, 1H, J = 5.5Hz, CHO*H*), 7.14 (br s, 2H, NH$_2$).

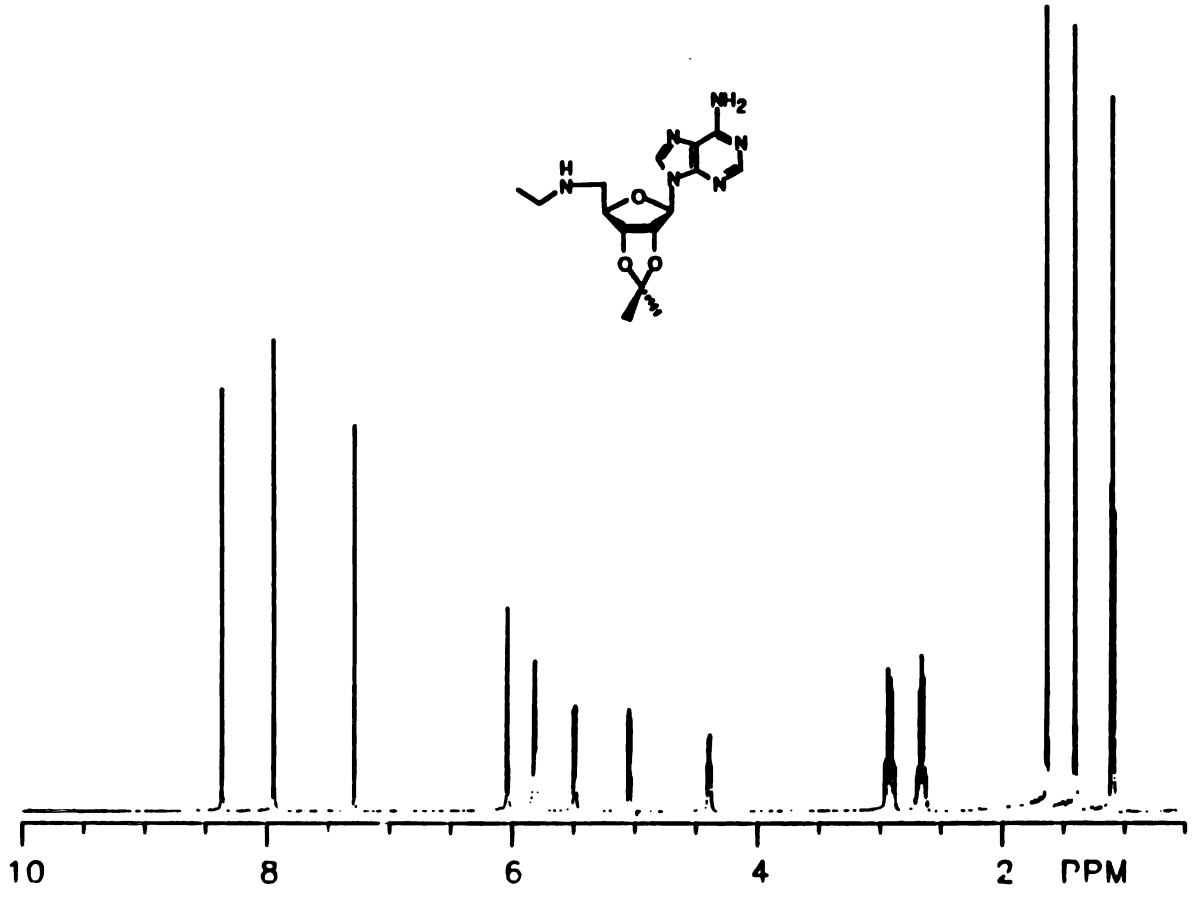
Anal. Calculated for $C_3H_7NO_3$: C, 34.29; H, 6.71; N, 13.33. Found: C, 34.35; H, 6.70; N, 13.40.

Appendix B

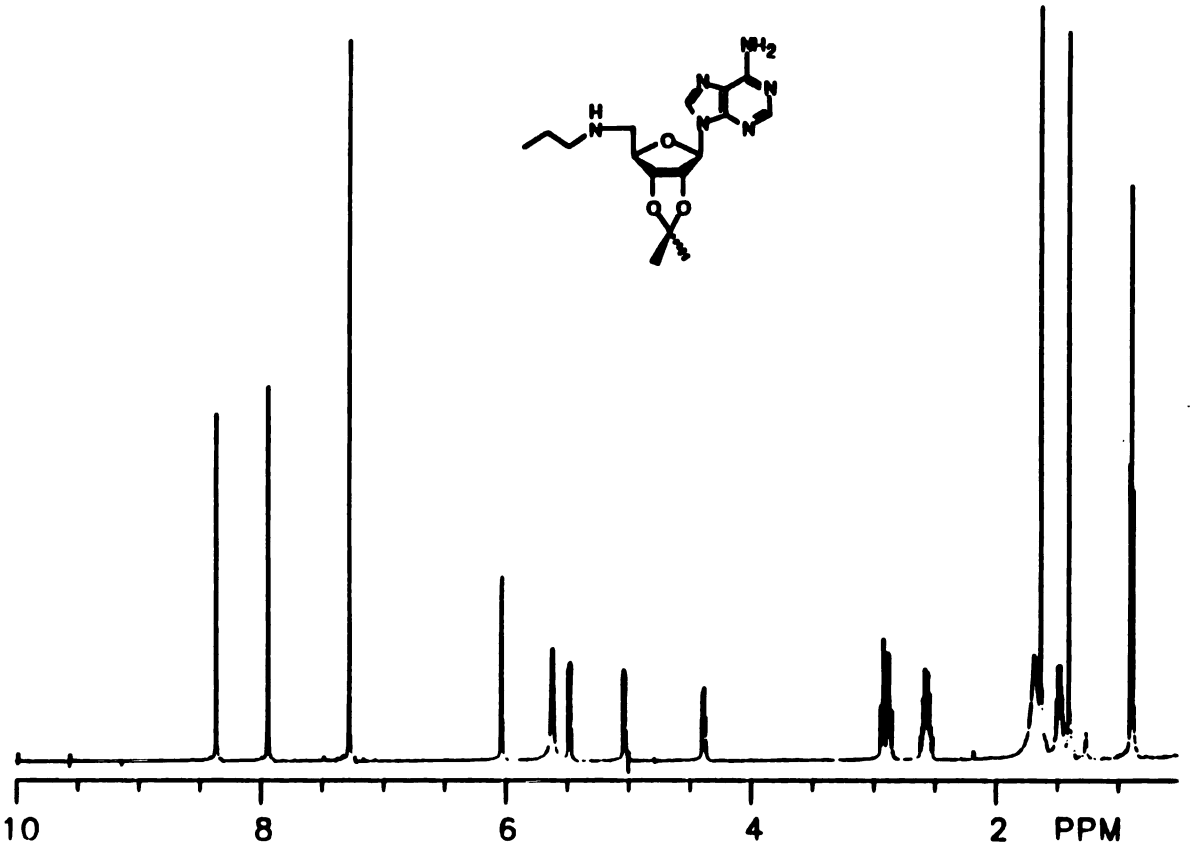
High Resolution ^{1}H NMR Spectra



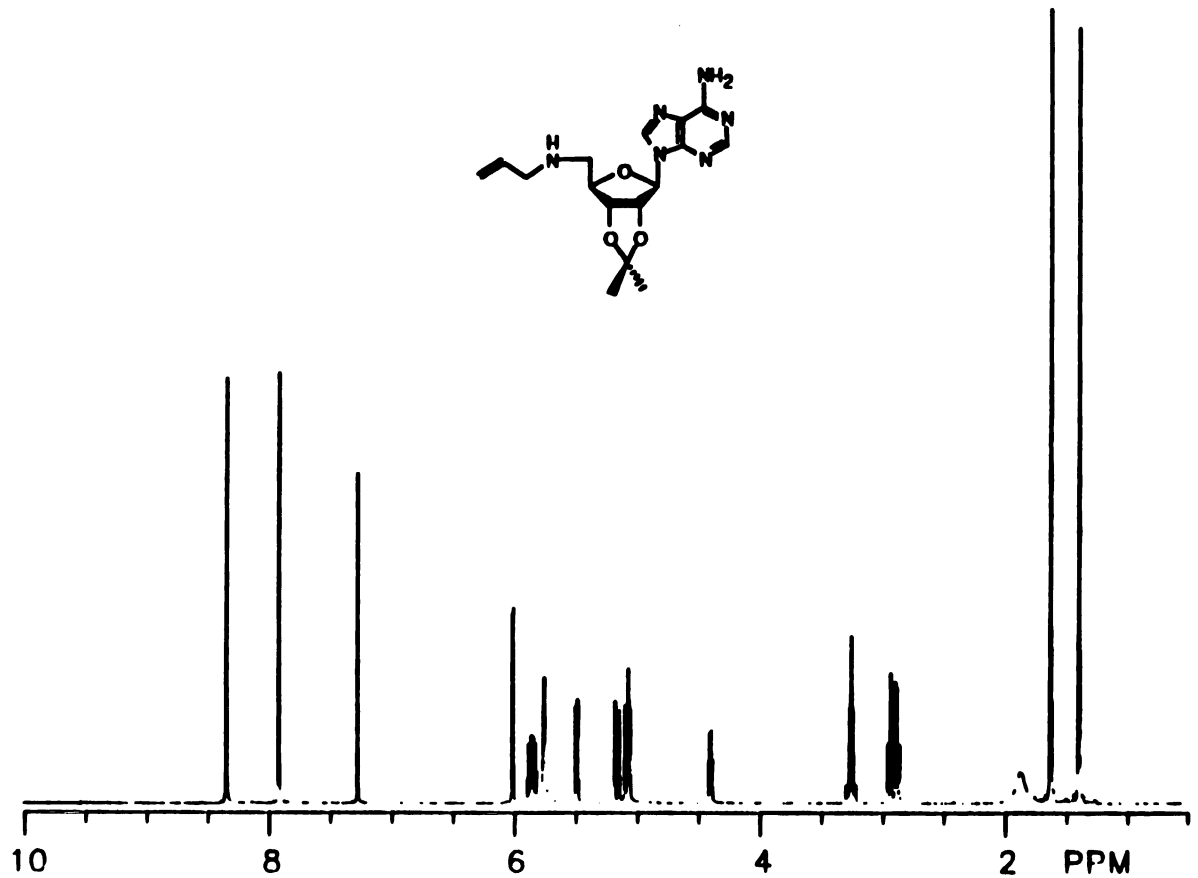
Spectrum 1. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of 5'-deoxy-5'-methylamino-2',3'-O-isopropylideneadenosine (4a).



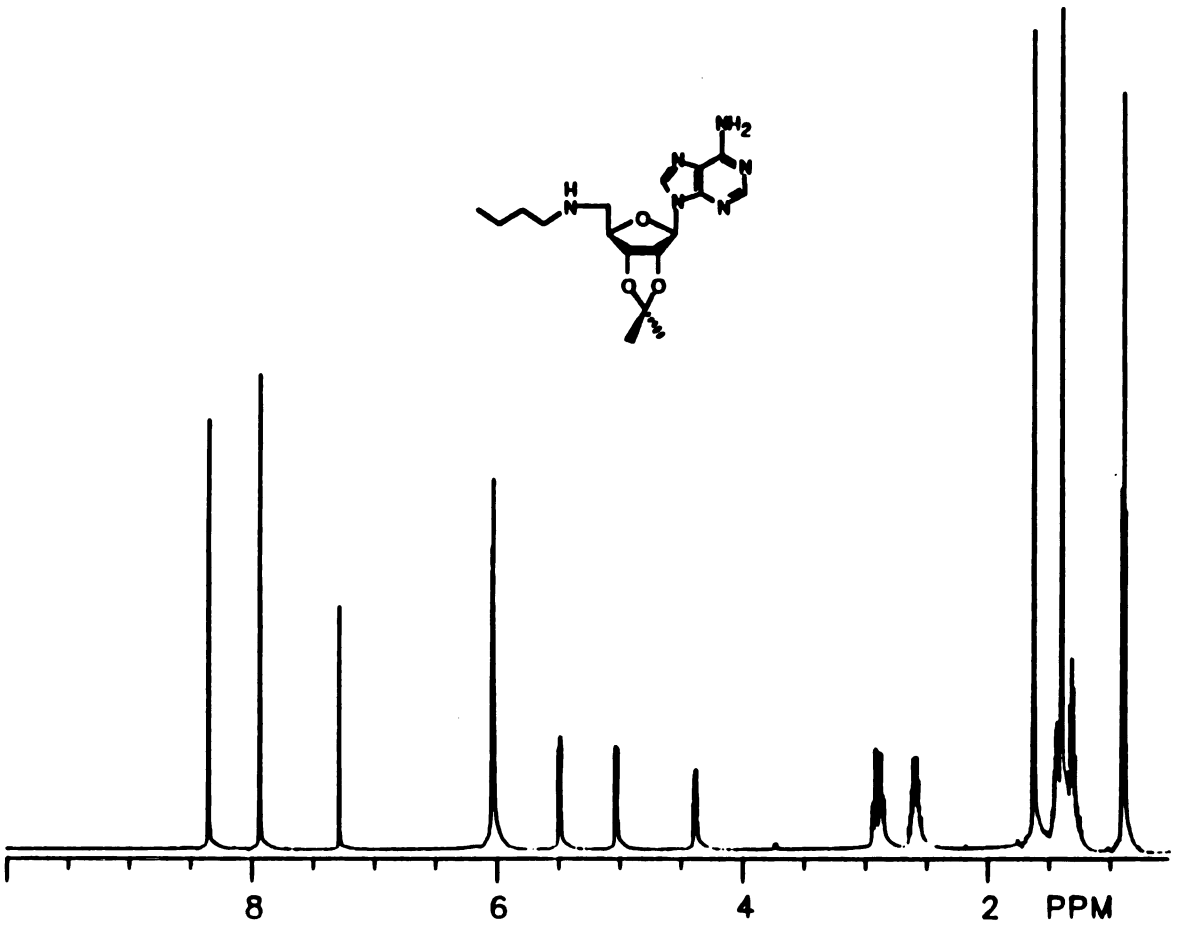
Spectrum 2. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of 5'-deoxy-5'-ethylamino-2',3'-O-isopropylideneadenosine (4b).



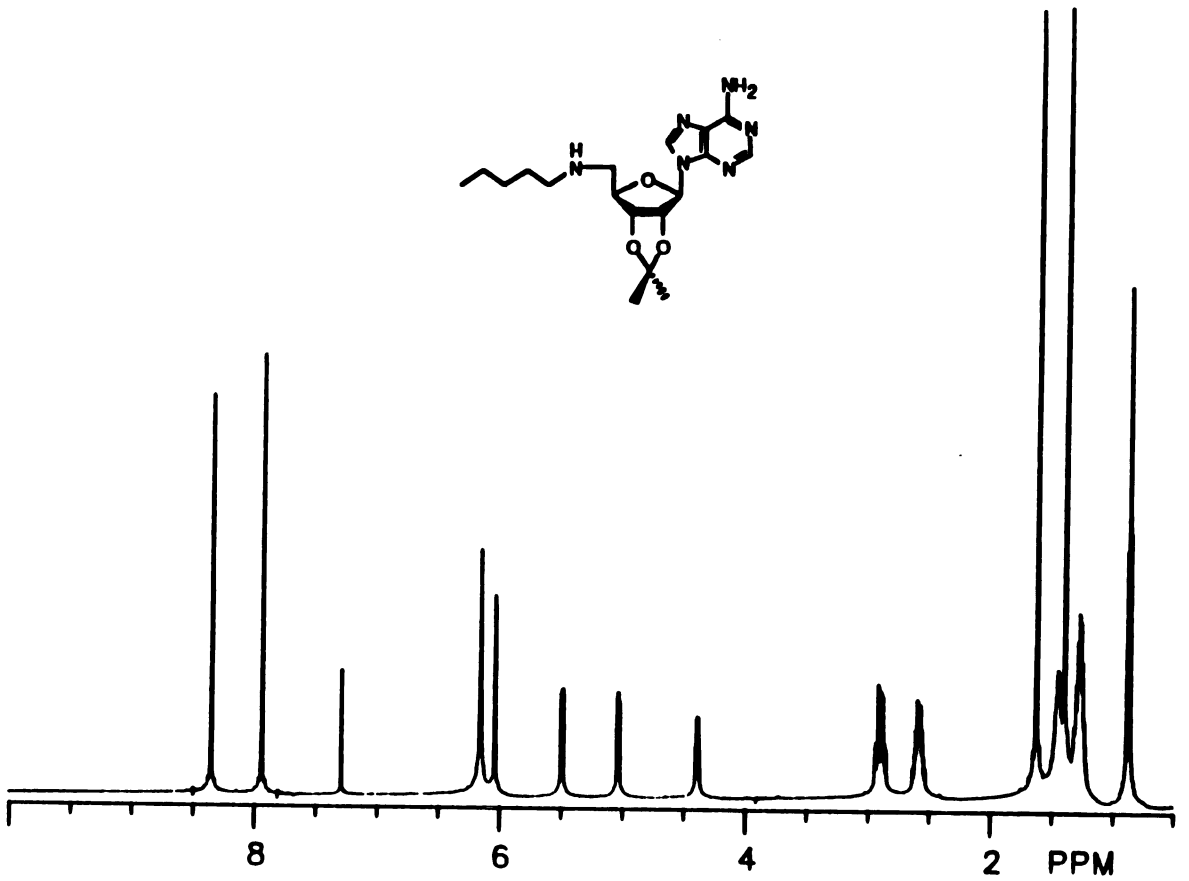
Spectrum 3. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of 5'-(1-propyl)amino-5'-deoxy-2',3'-O-isopropylideneadenosine (4c).



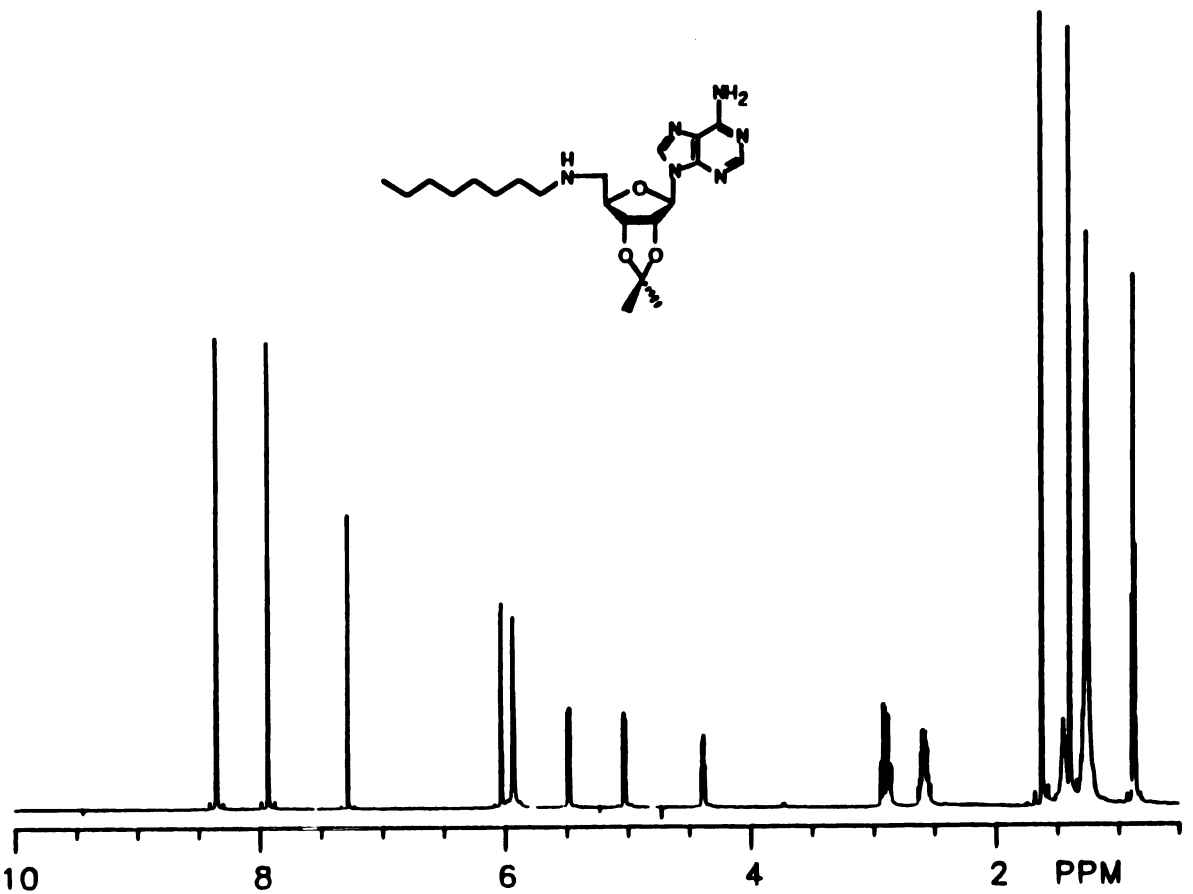
Spectrum 4. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of 5'-[1-(2-propenyl)]amino-5'-deoxy-2',3'-O-isopropylidene-adenosine (**4d**).



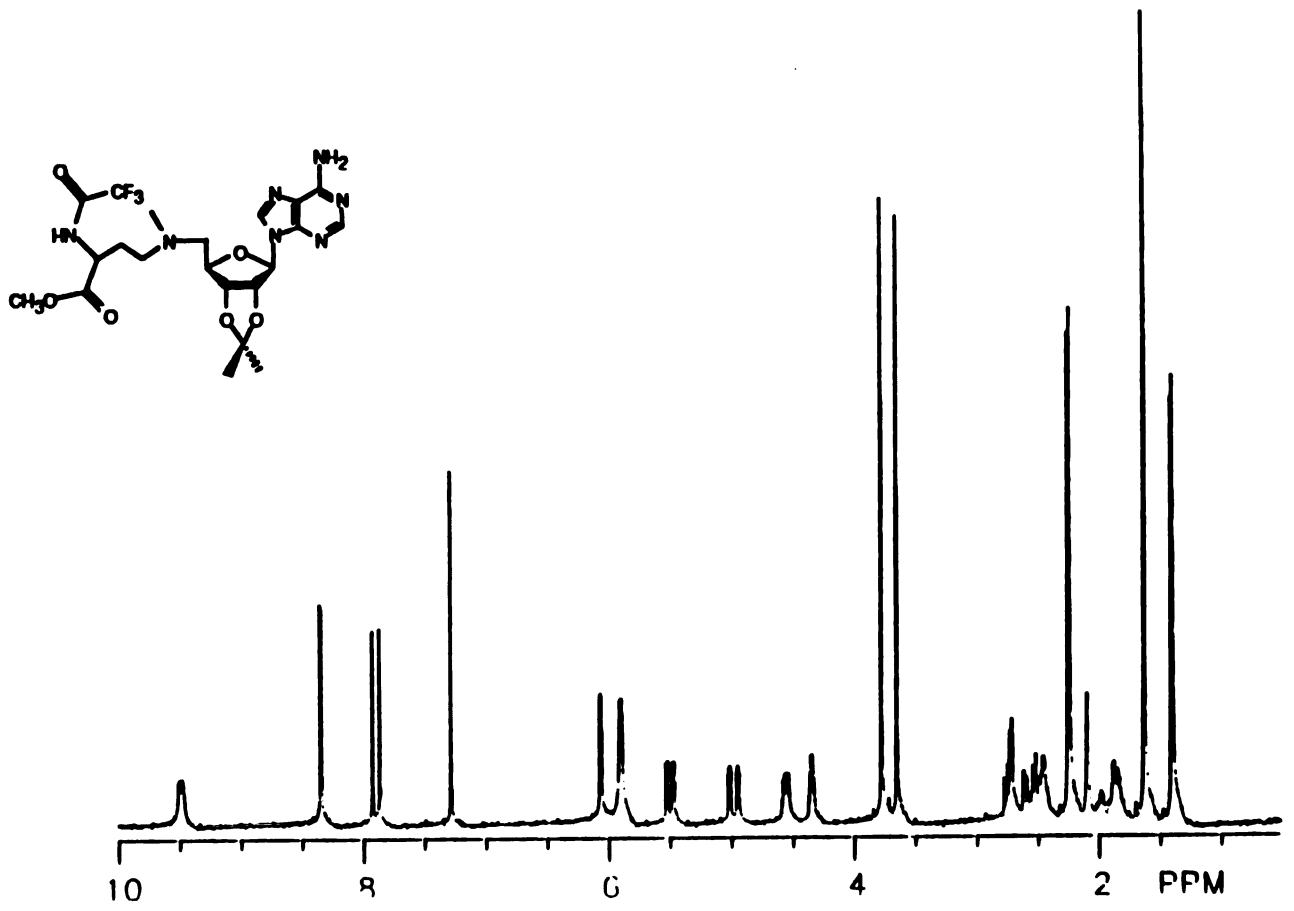
Spectrum 5. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of 5'-(1-butyl)amino-5'-deoxy-2',3'-O-isopropylideneadenosine (4e).



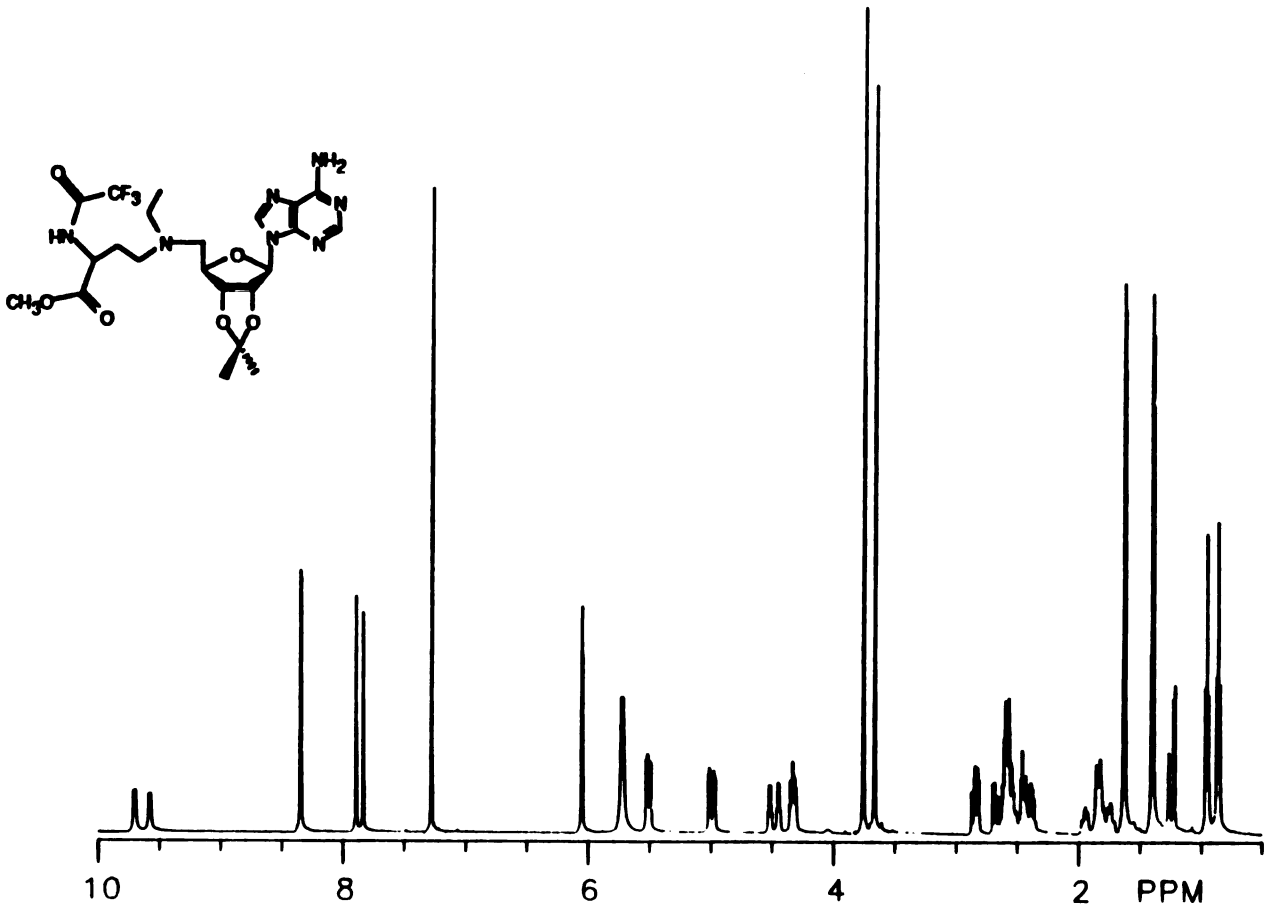
Spectrum 6. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of 5'-(1-pentyl)amino-5'-deoxy-2',3'-O-isopropylideneadenosine (4f).



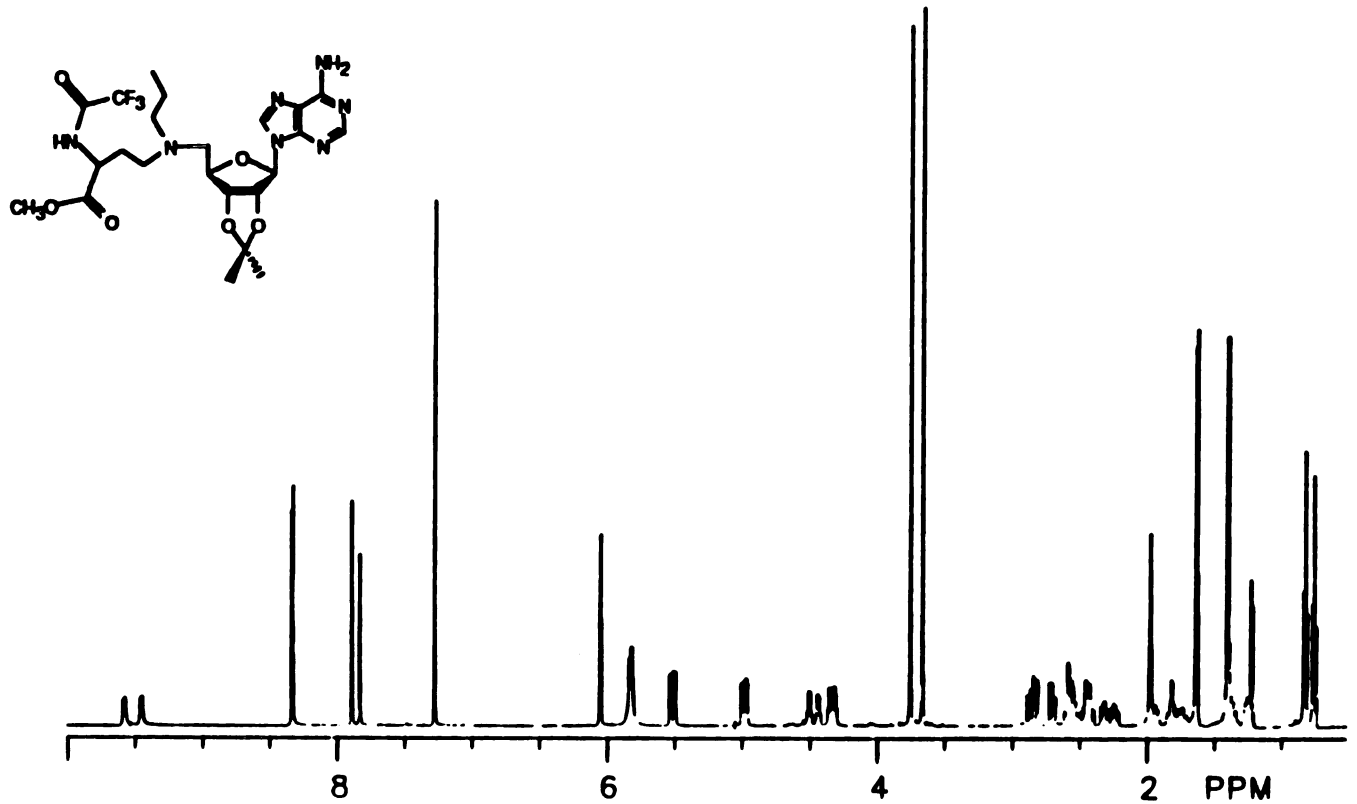
Spectrum 7. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of 5'-(1-octyl)amino-5'-deoxy-2',3'-O-isopropylideneadenosine (4g).



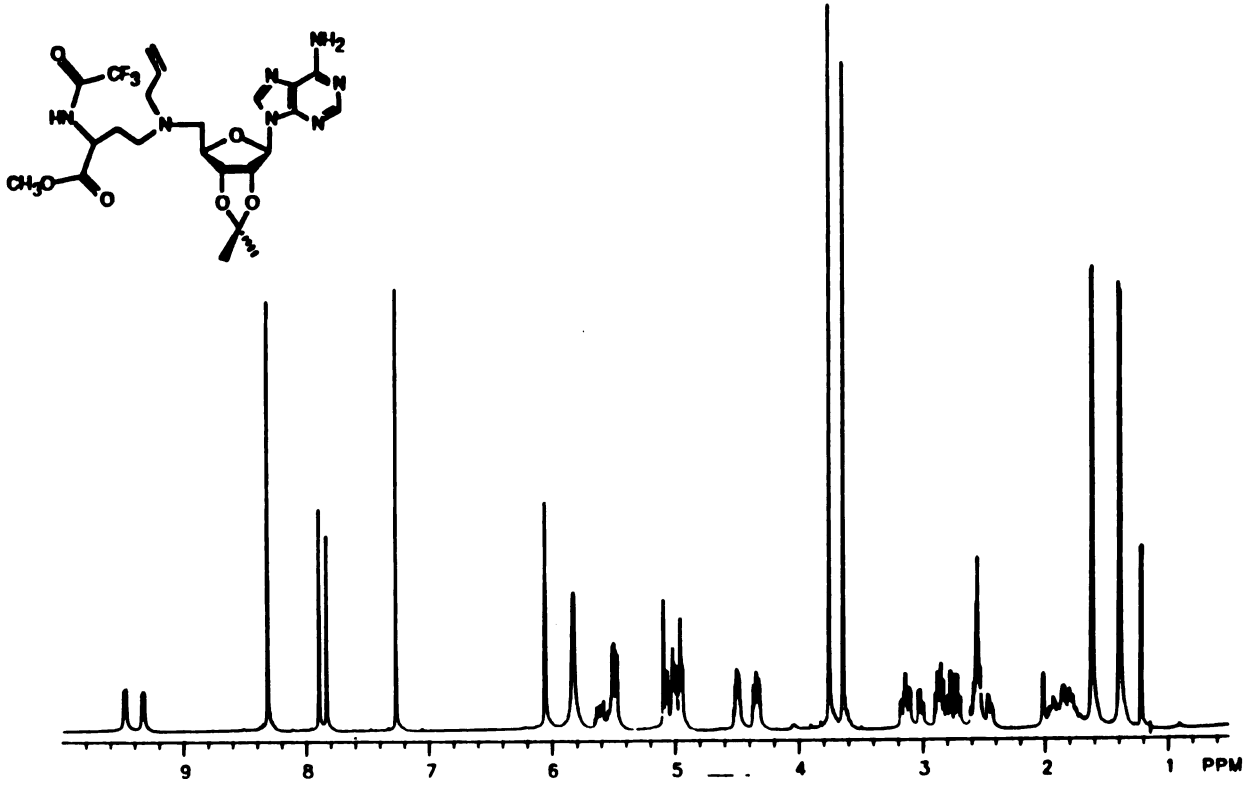
Spectrum 8. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of the protected nitrogen methyl SAM analogue (7a).



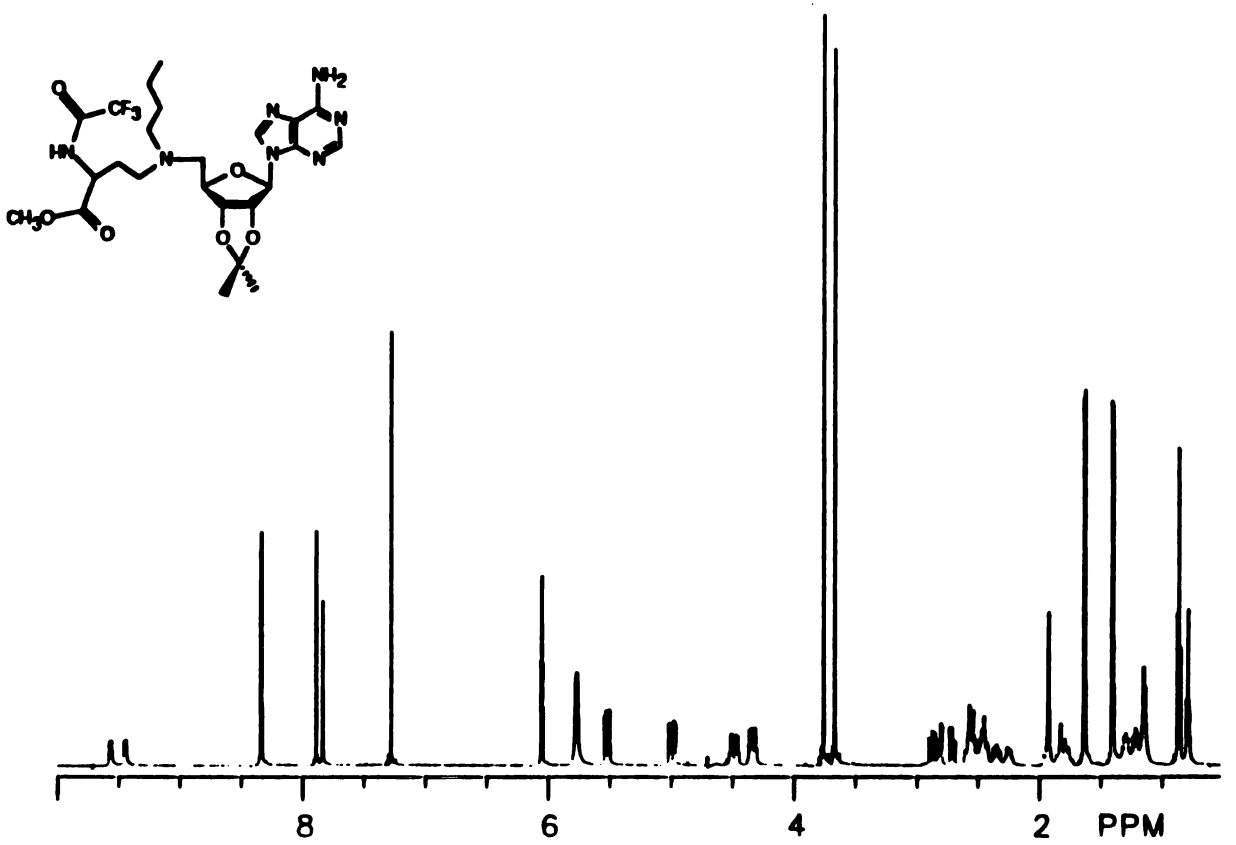
Spectrum 9. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of the protected nitrogen ethyl SAM analogue (7b).



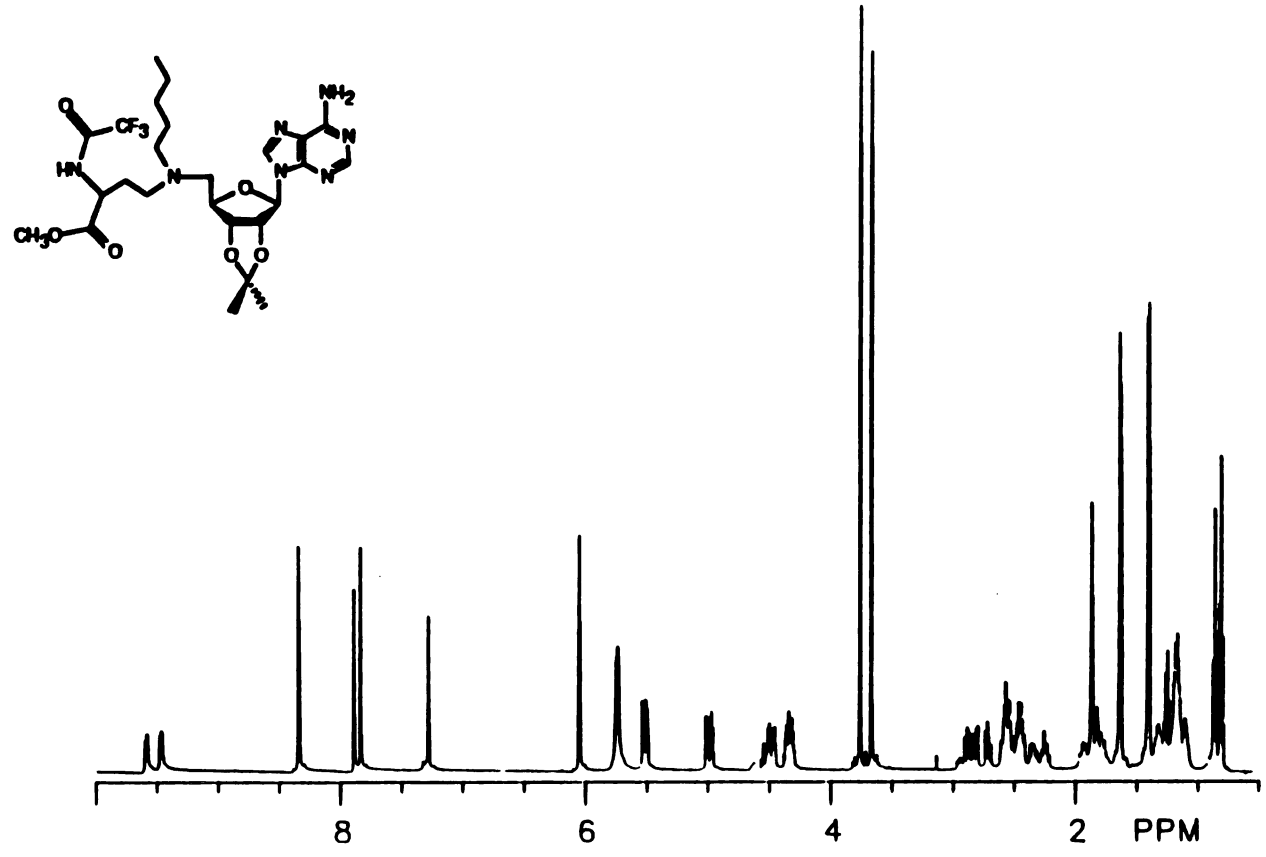
Spectrum 10. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of the protected nitrogen propyl SAM analogue (7c).



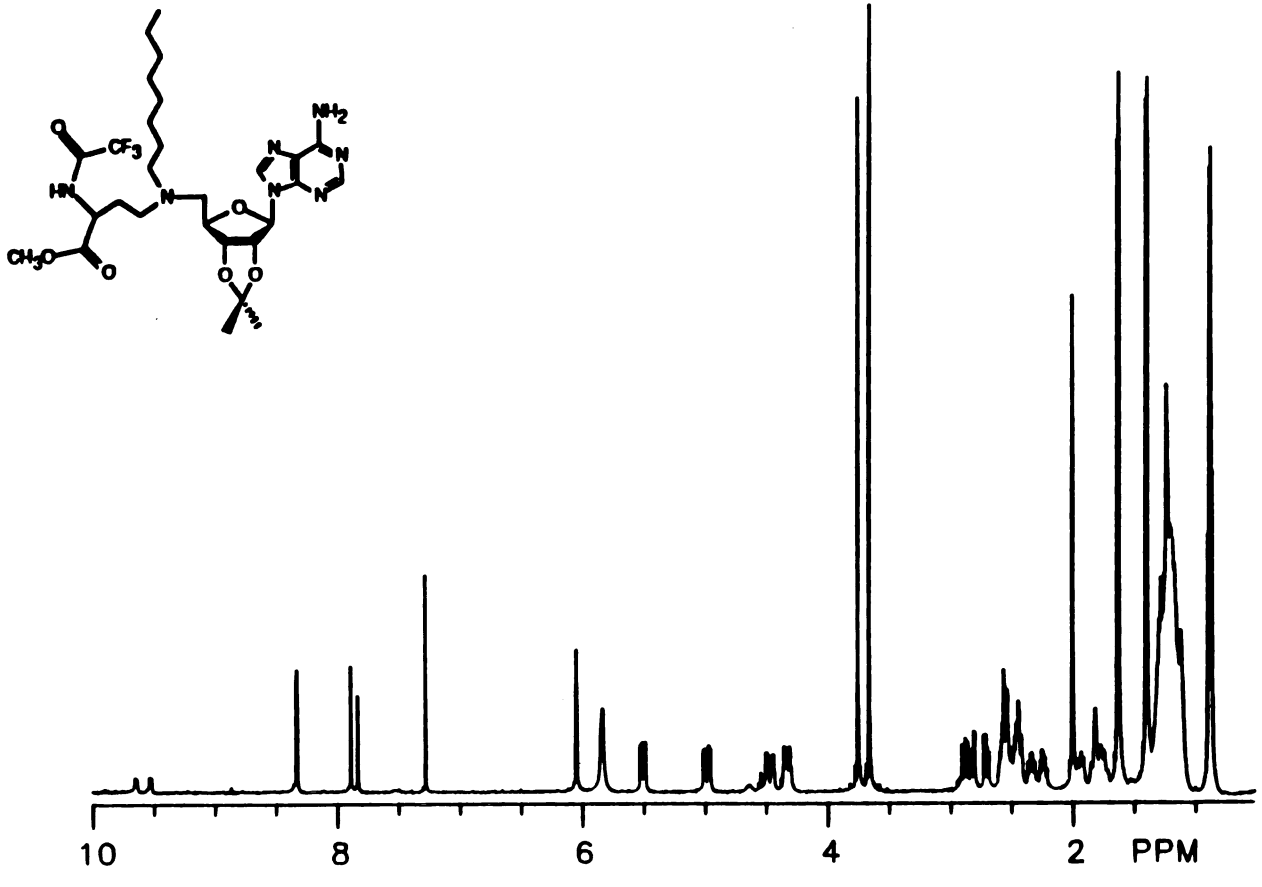
Spectrum 11. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of the protected nitrogen allyl SAM analogue (7d).



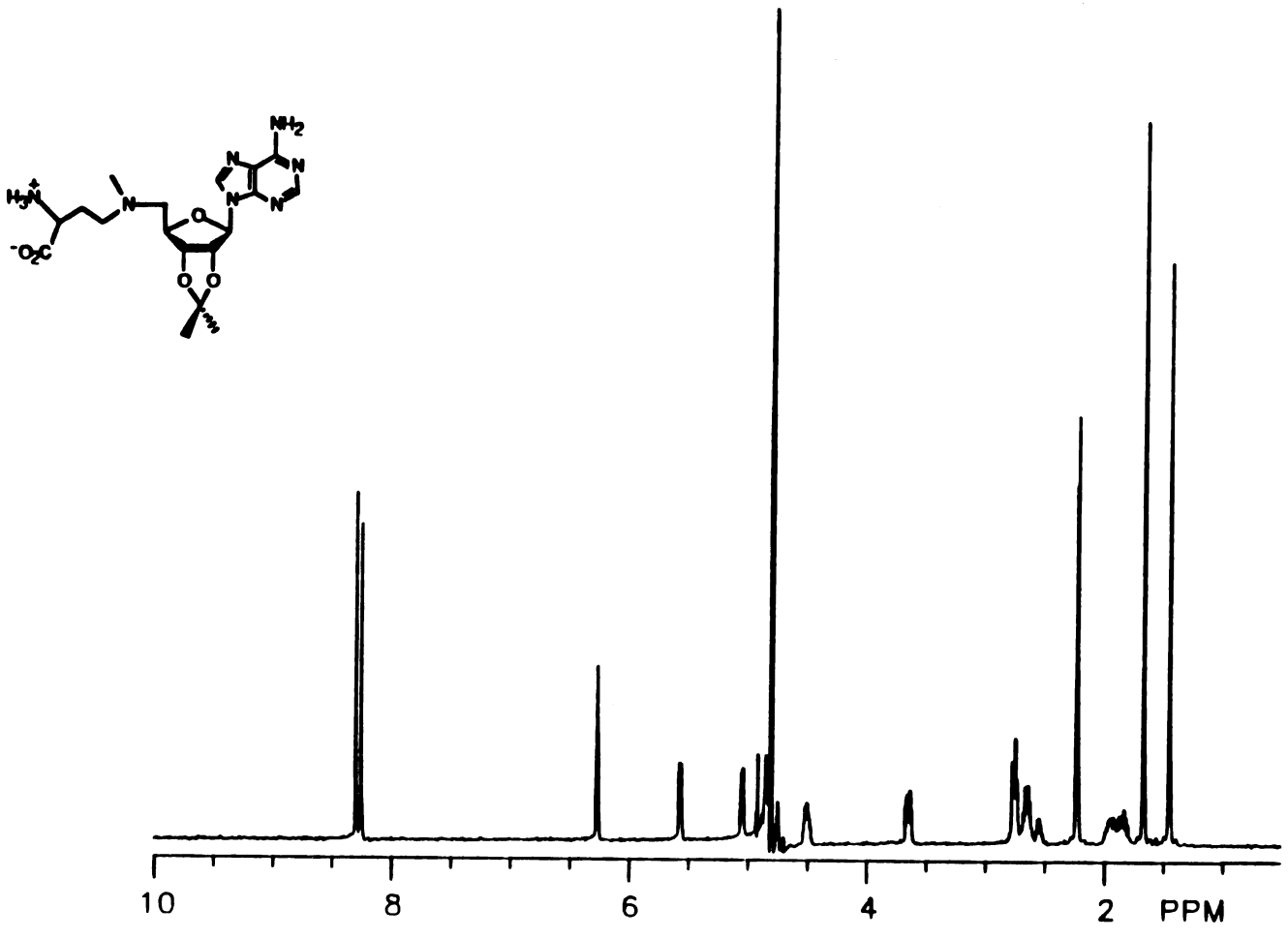
Spectrum 12. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of the protected nitrogen butyl SAM analogue (7e).



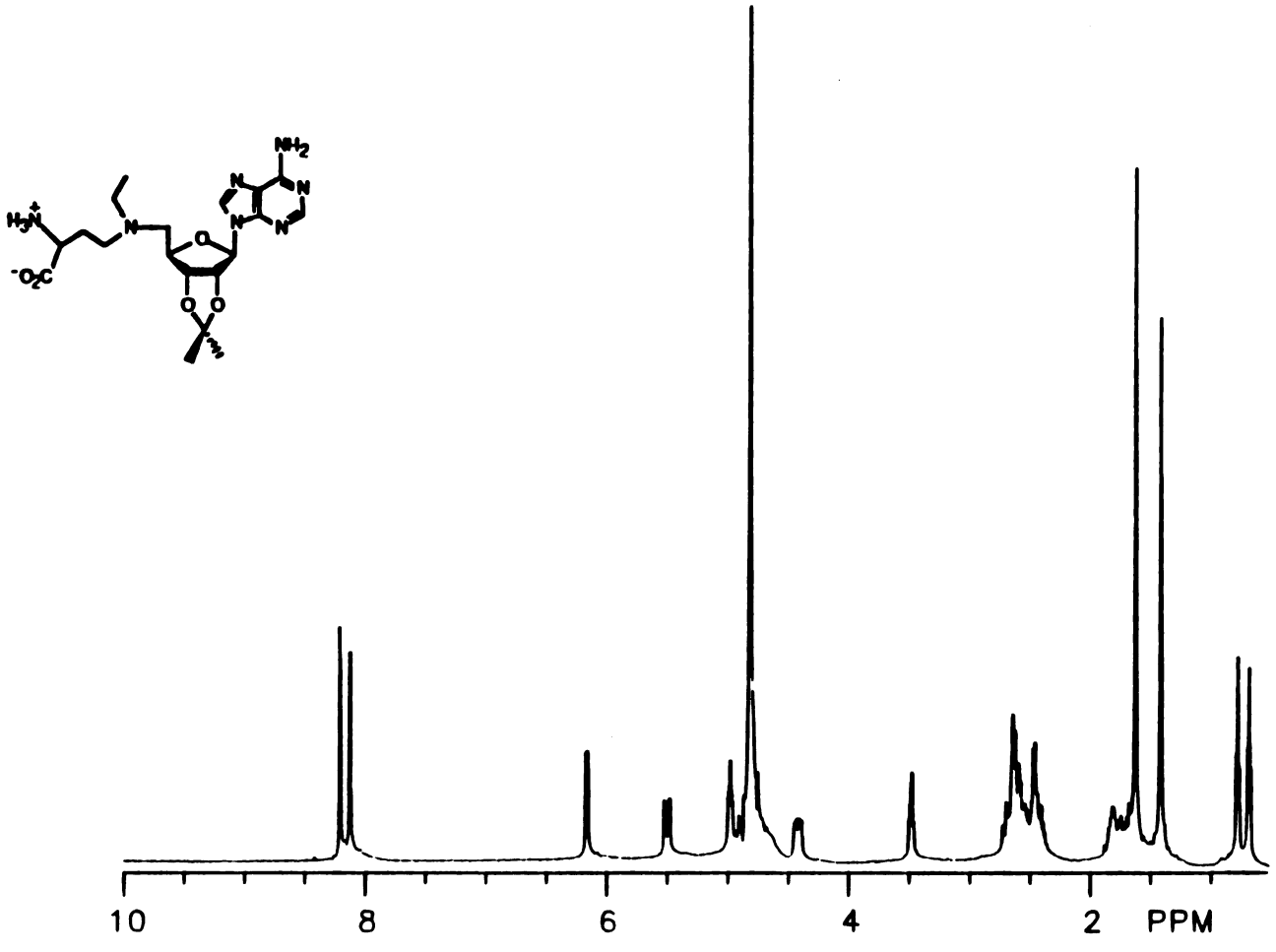
Spectrum 13. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of the protected nitrogen pentyl SAM analogue (7f).



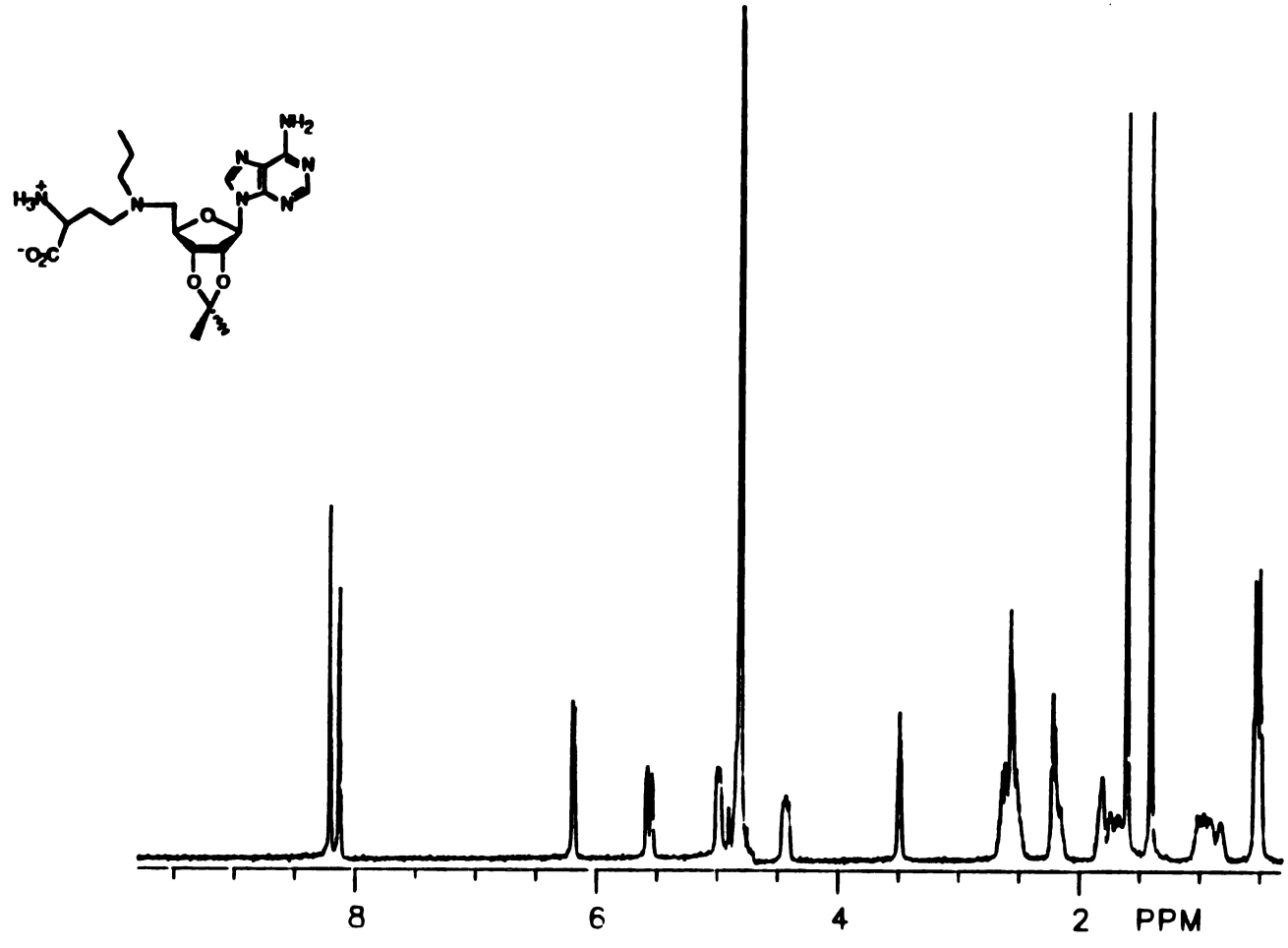
Spectrum 14. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of the protected nitrogen octyl SAM analogue (7g).



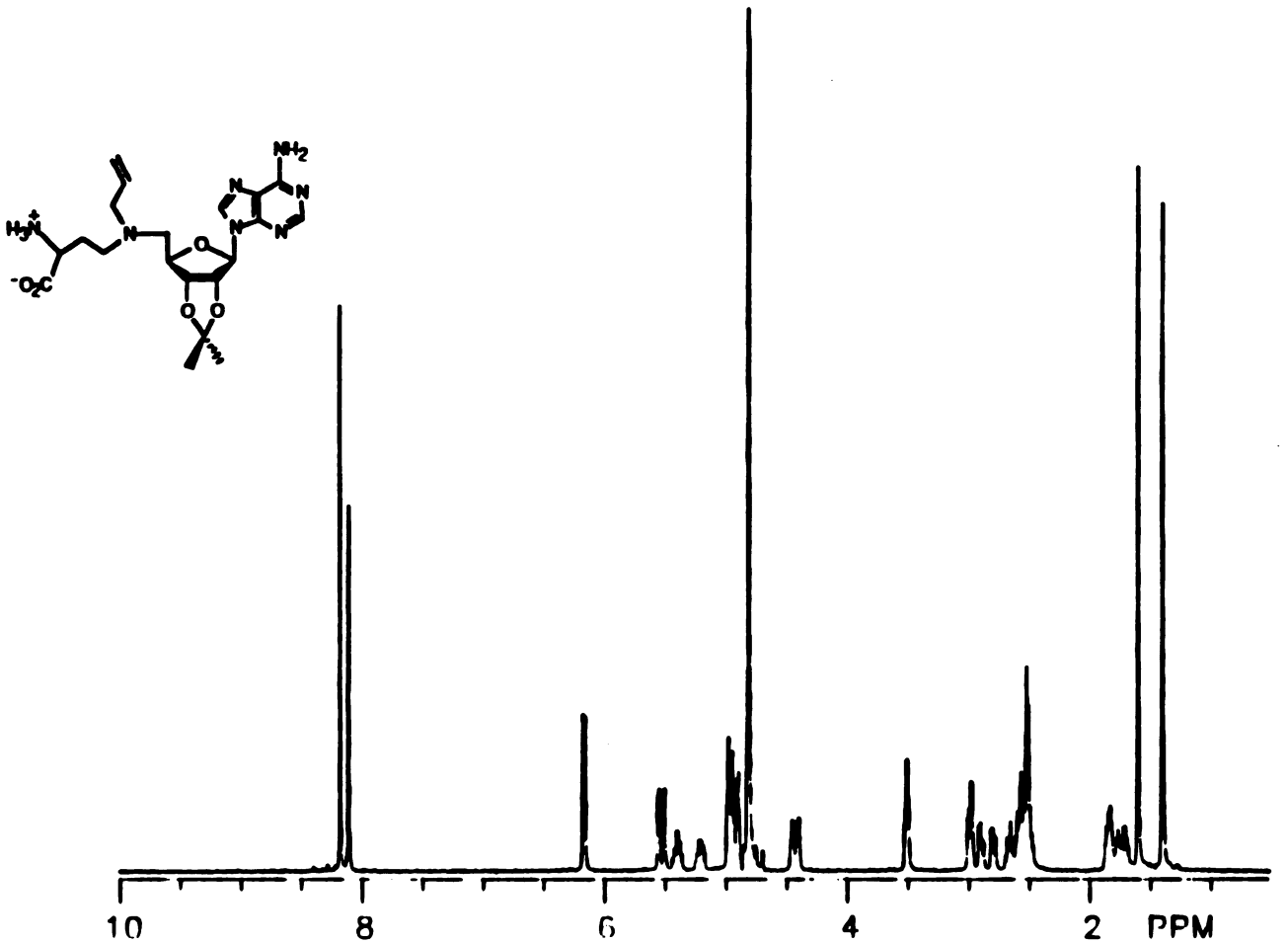
Spectrum 15. 500 MHz ^{1}H NMR (D_2O) spectrum of the 2',3'-O-isopropylidene nitrogen methyl SAM analogue (8a).



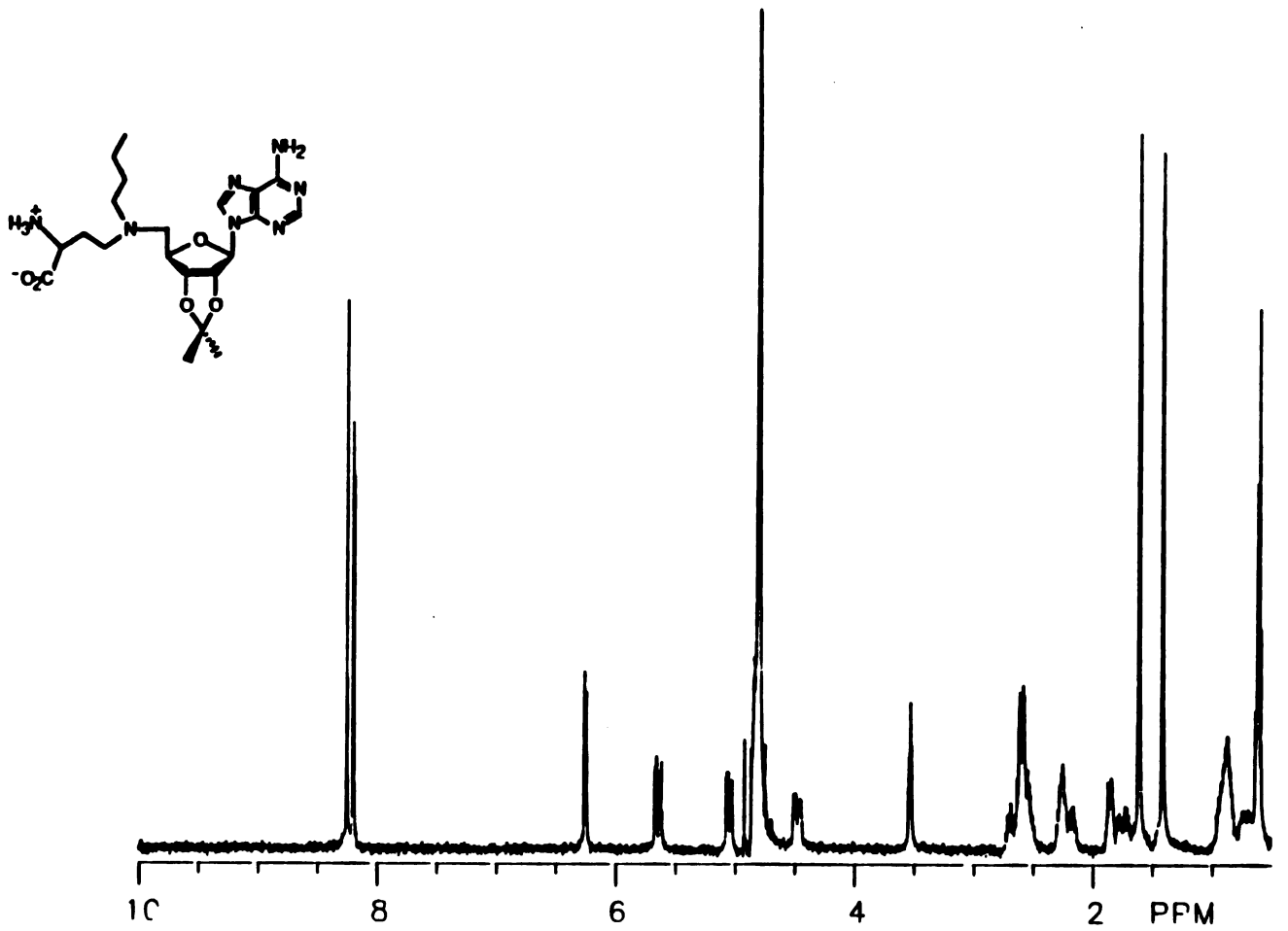
Spectrum 16. 500 MHz ^{1}H NMR (D_2O) spectrum of the 2',3'-O-isopropylidene nitrogen ethyl SAM analogue (8b).



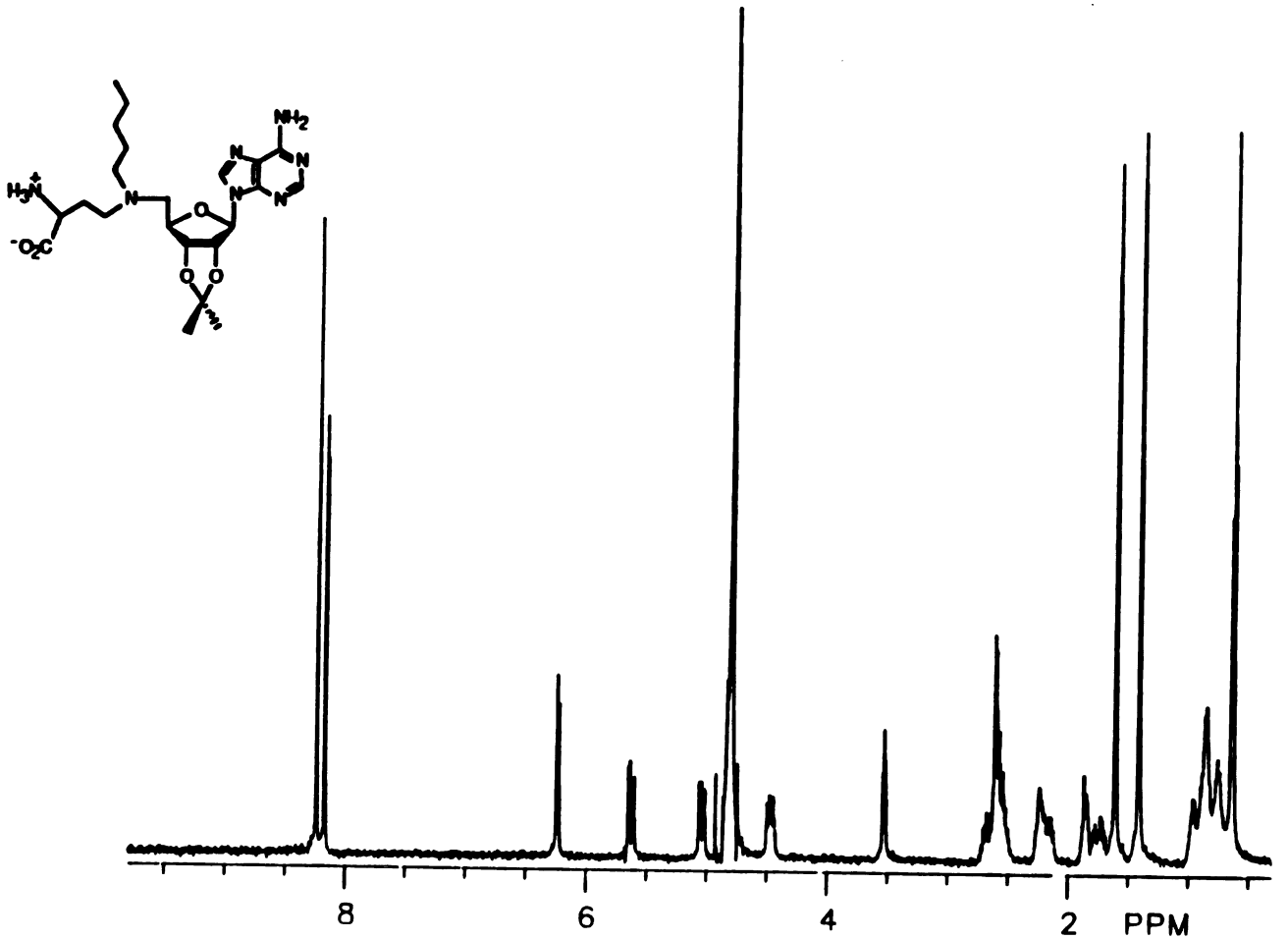
Spectrum 17. 500 MHz ^{1}H NMR (D_2O) spectrum of the 2',3'-O-isopropylidene nitrogen propyl SAM analogue (8c).



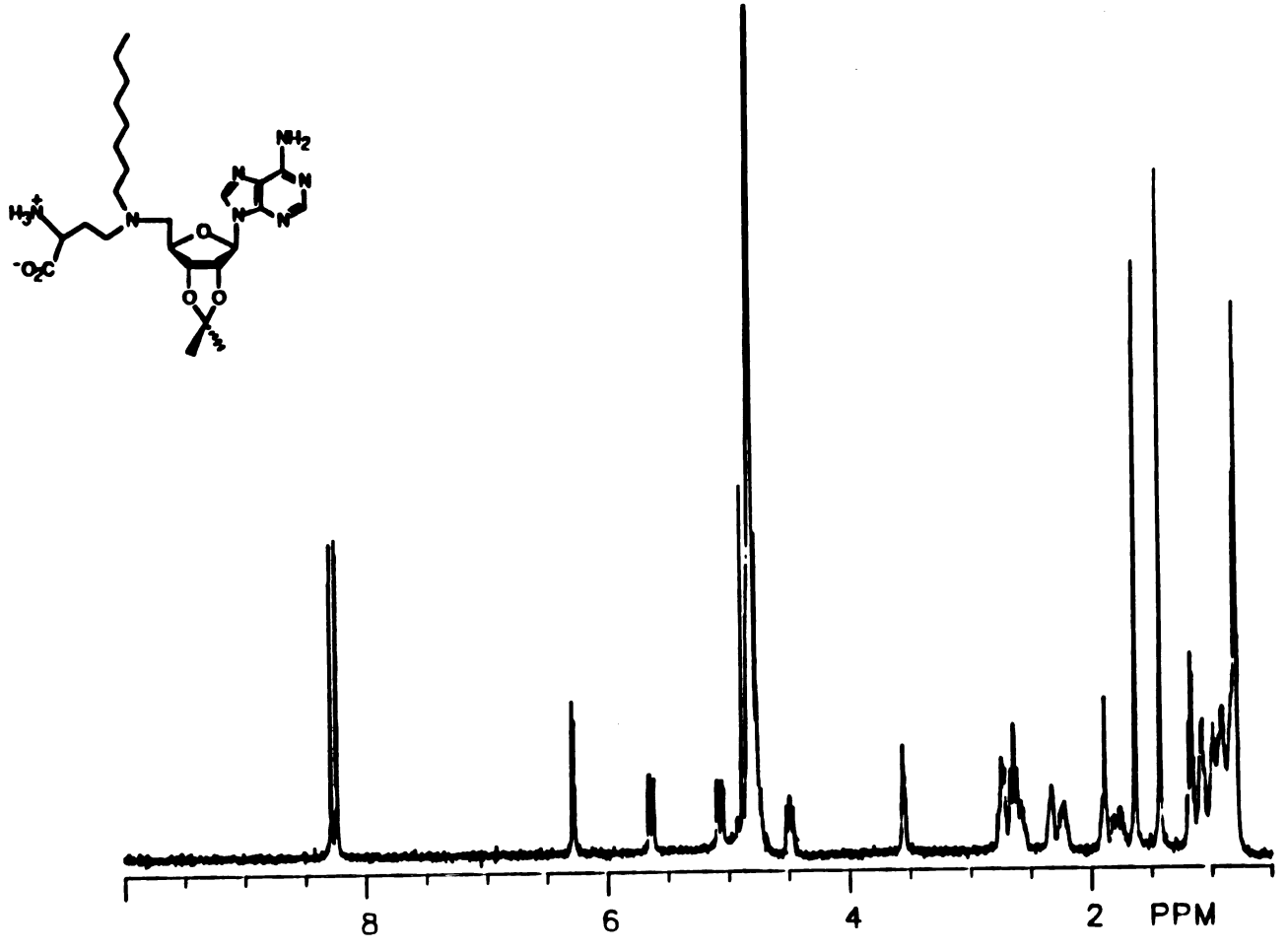
Spectrum 18. 500 MHz ^{1}H NMR (D_2O) spectrum of the 2',3'-O-isopropylidene nitrogen allyl SAM analogue (8d).



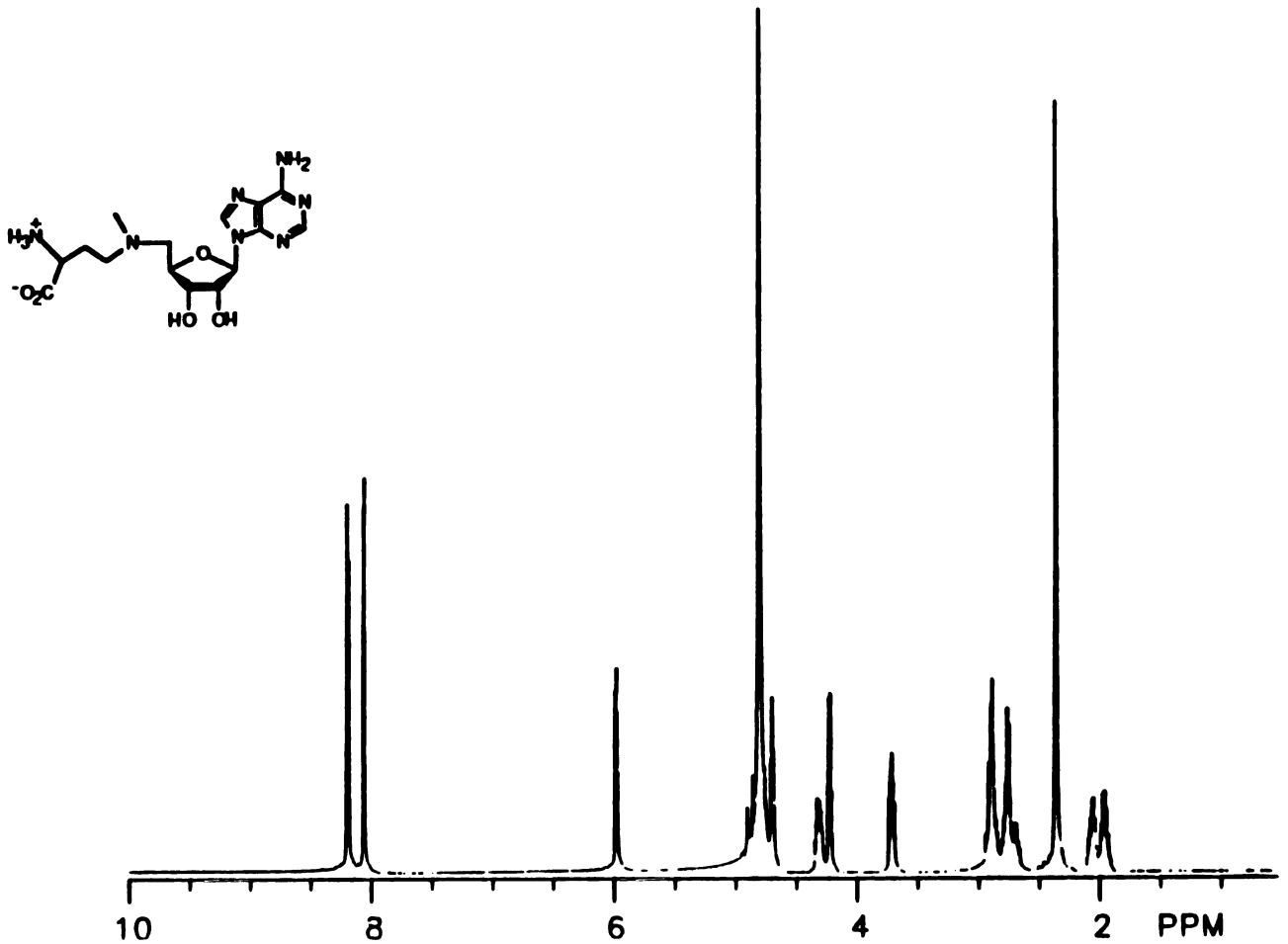
Spectrum 19. 500 MHz ^{1}H NMR (D_2O) spectrum of the 2',3'-O-isopropylidene nitrogen butyl SAM analogue (8e).



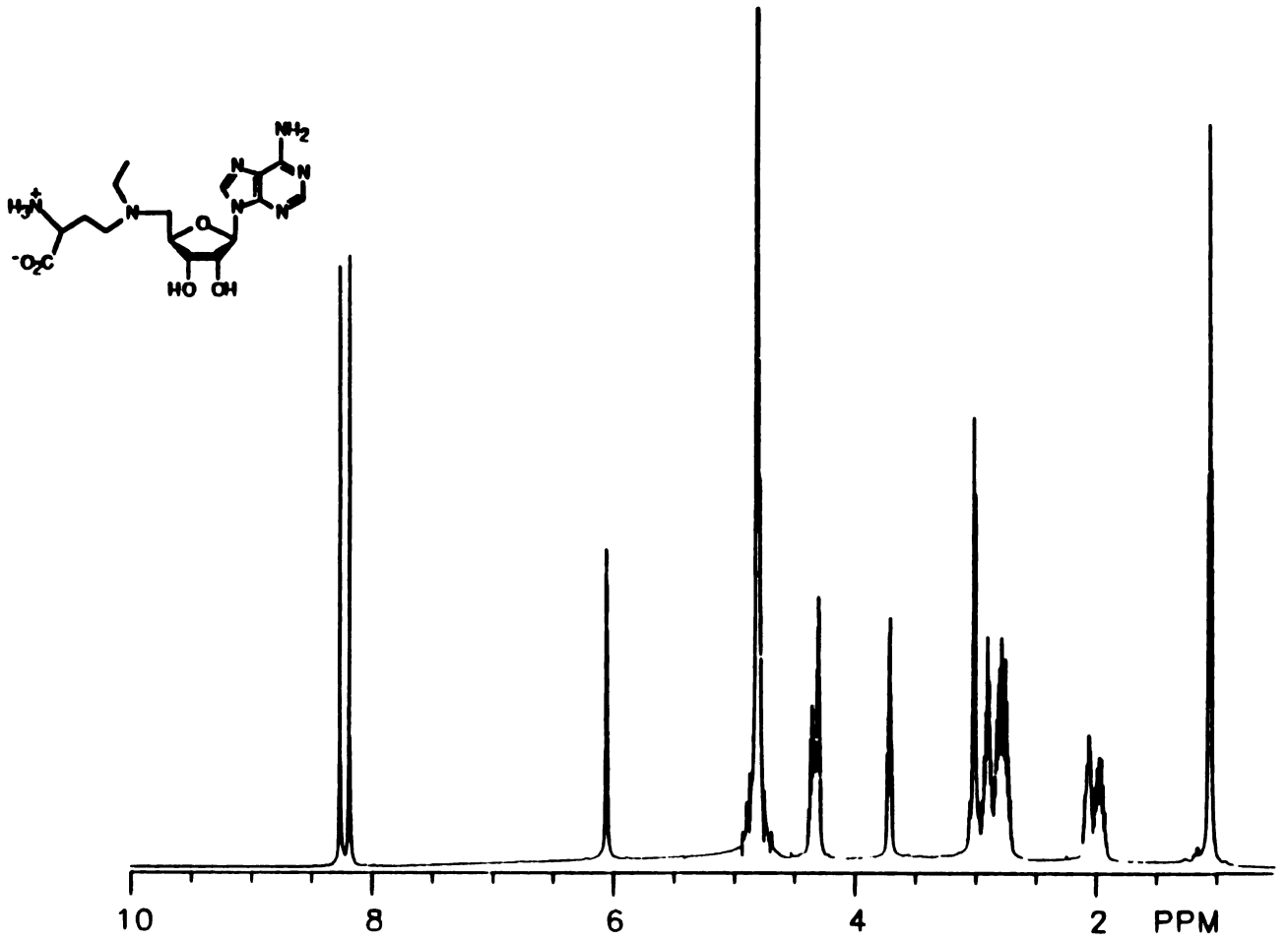
Spectrum 20. 500 MHz ^{1}H NMR (D_2O) spectrum of the 2',3'-O-isopropylidene nitrogen pentyl SAM analogue (8f).



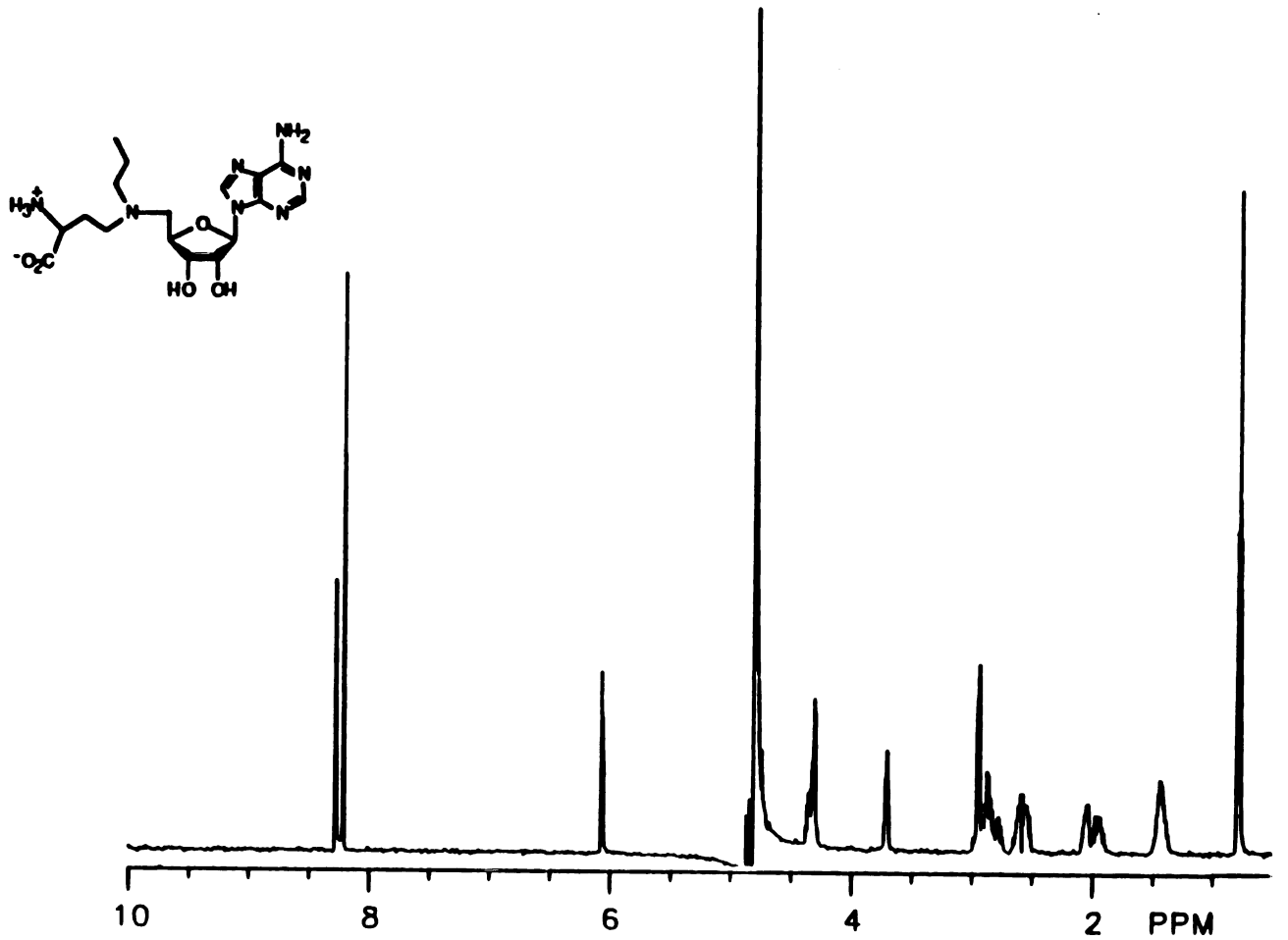
Spectrum 21. 500 MHz ^{1}H NMR (D_2O) spectrum of the 2',3'-O-isopropylidene nitrogen octyl SAM analogue (8g).



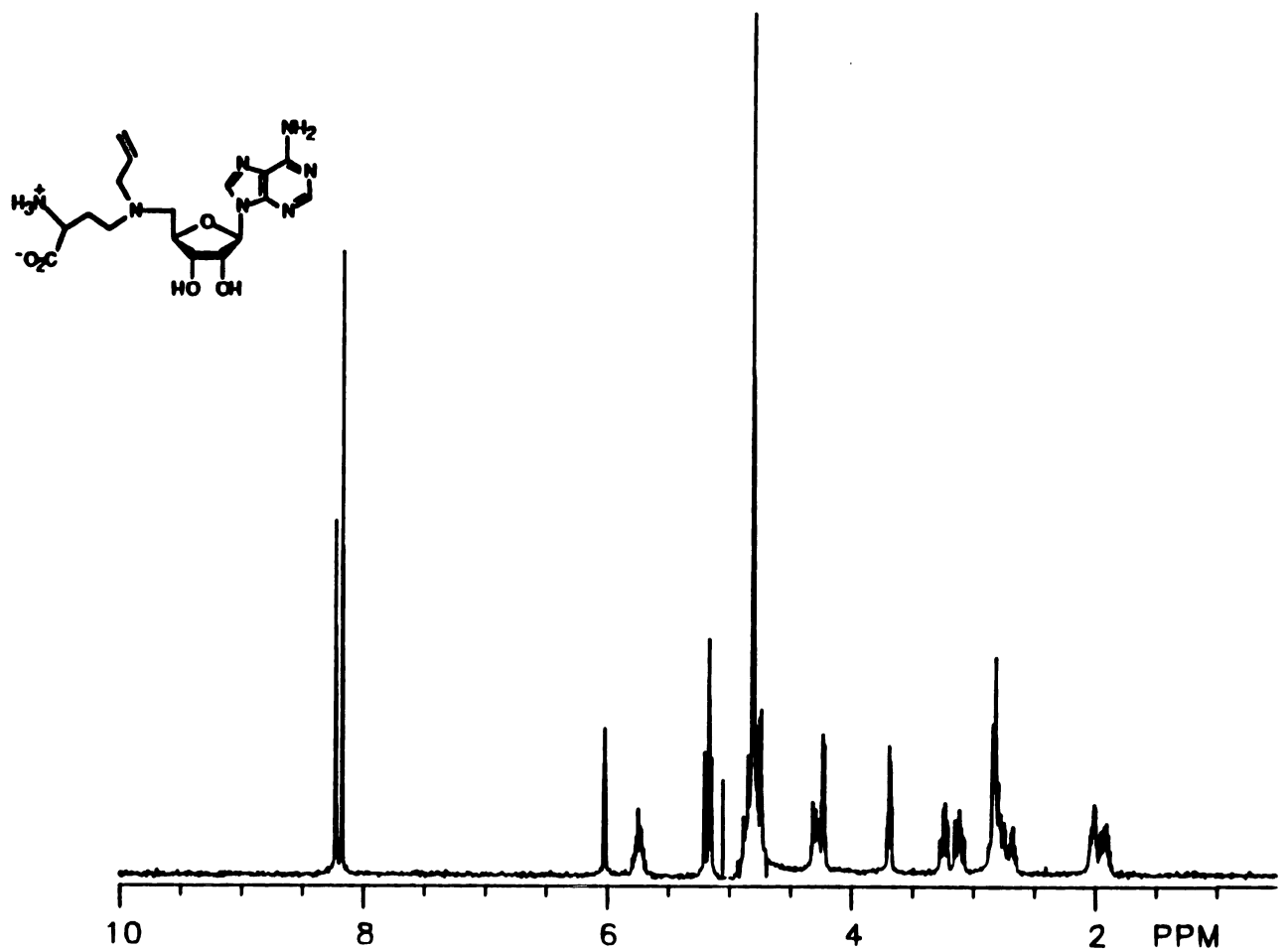
Spectrum 22. 500 MHz ^{1}H NMR (D_2O) spectrum of the nitrogen methyl SAM analogue (**2a**).



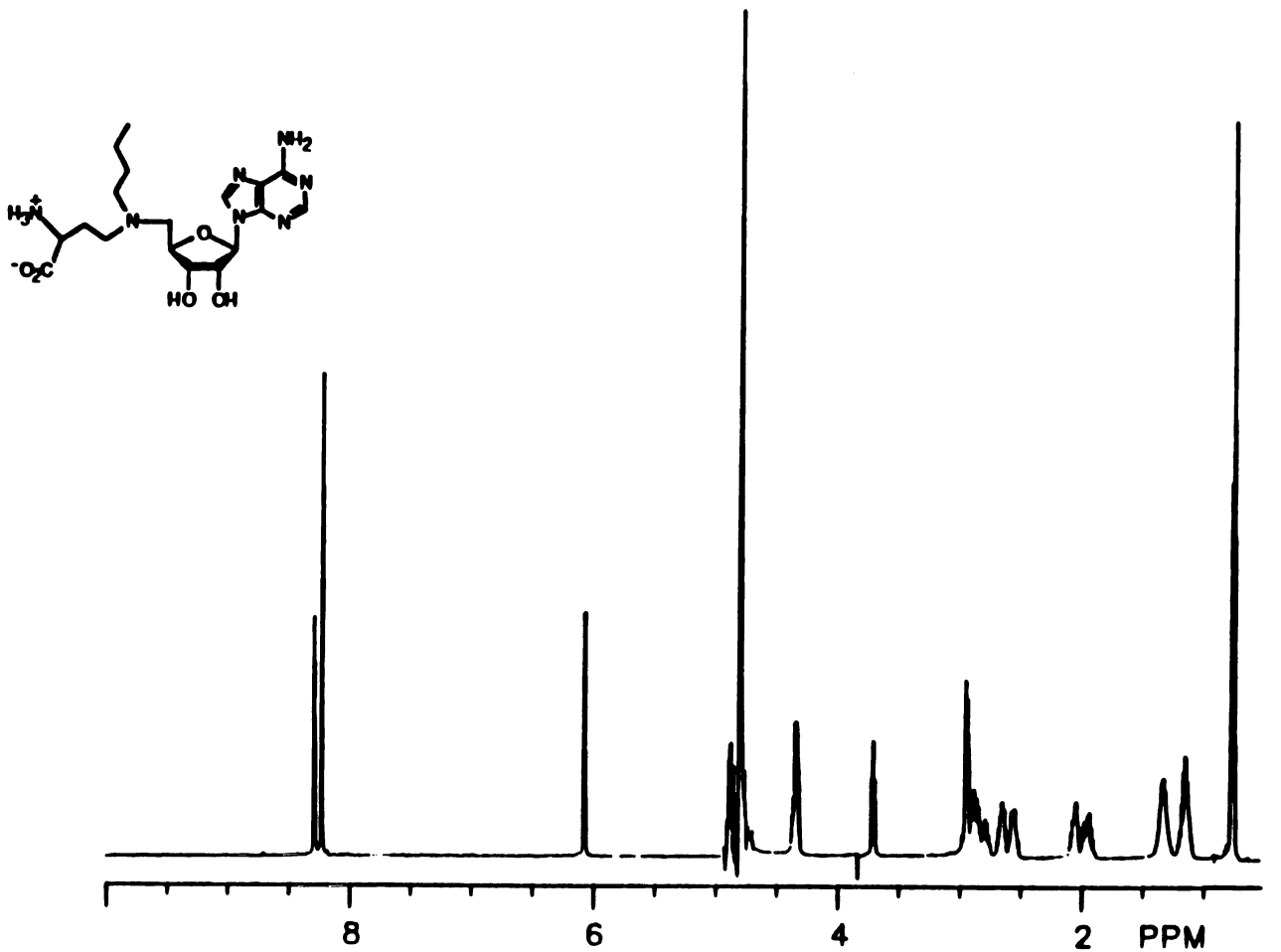
Spectrum 23. 500 MHz ^{1}H NMR (D_2O) spectrum of the nitrogen ethyl SAM analogue (2b).



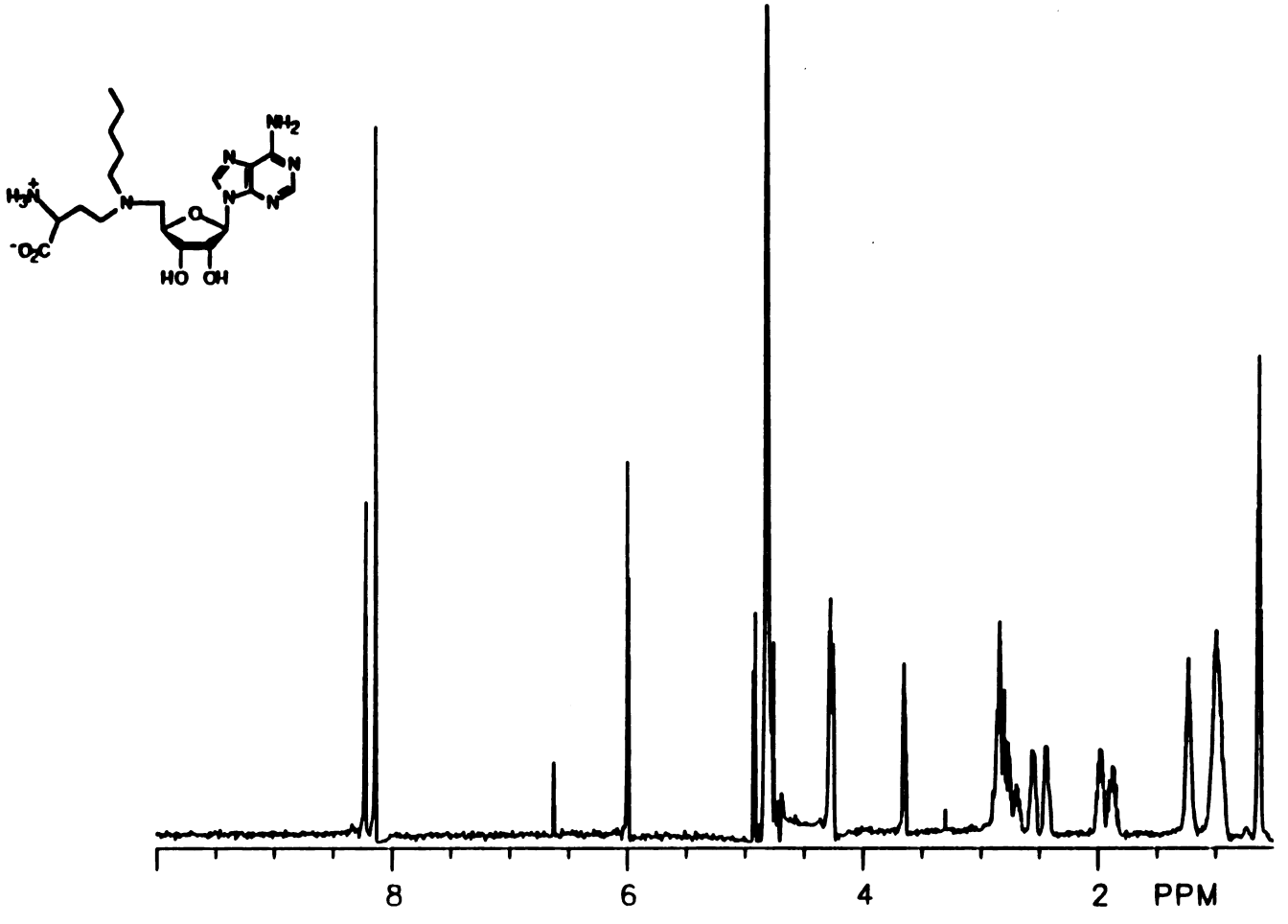
Spectrum 24. 500 MHz ^{1}H NMR (D_2O) spectrum of the nitrogen propyl SAM analogue (2c).



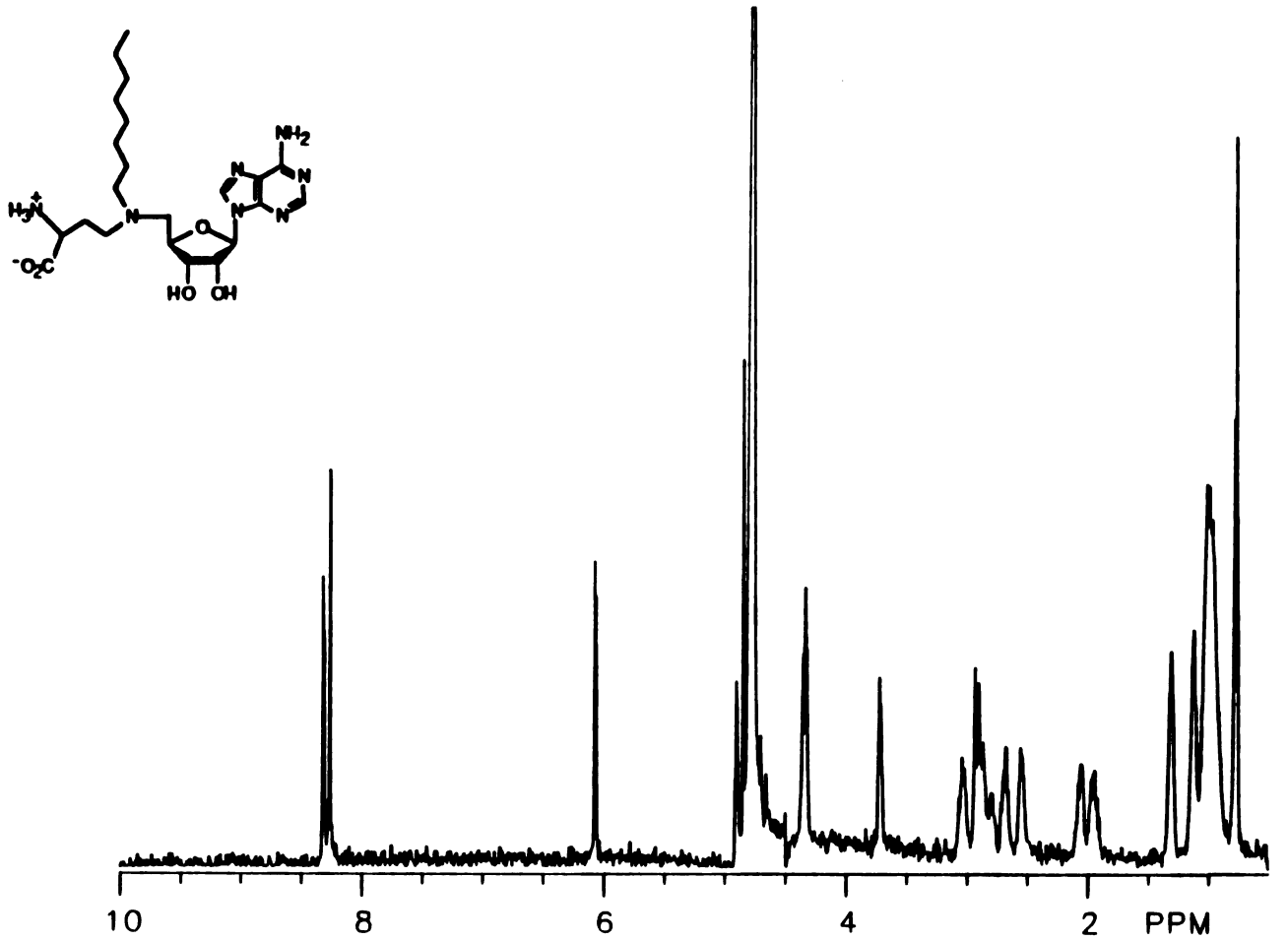
Spectrum 25. 500 MHz ^{1}H NMR (D_2O) spectrum of the nitrogen allyl SAM analogue (2d).



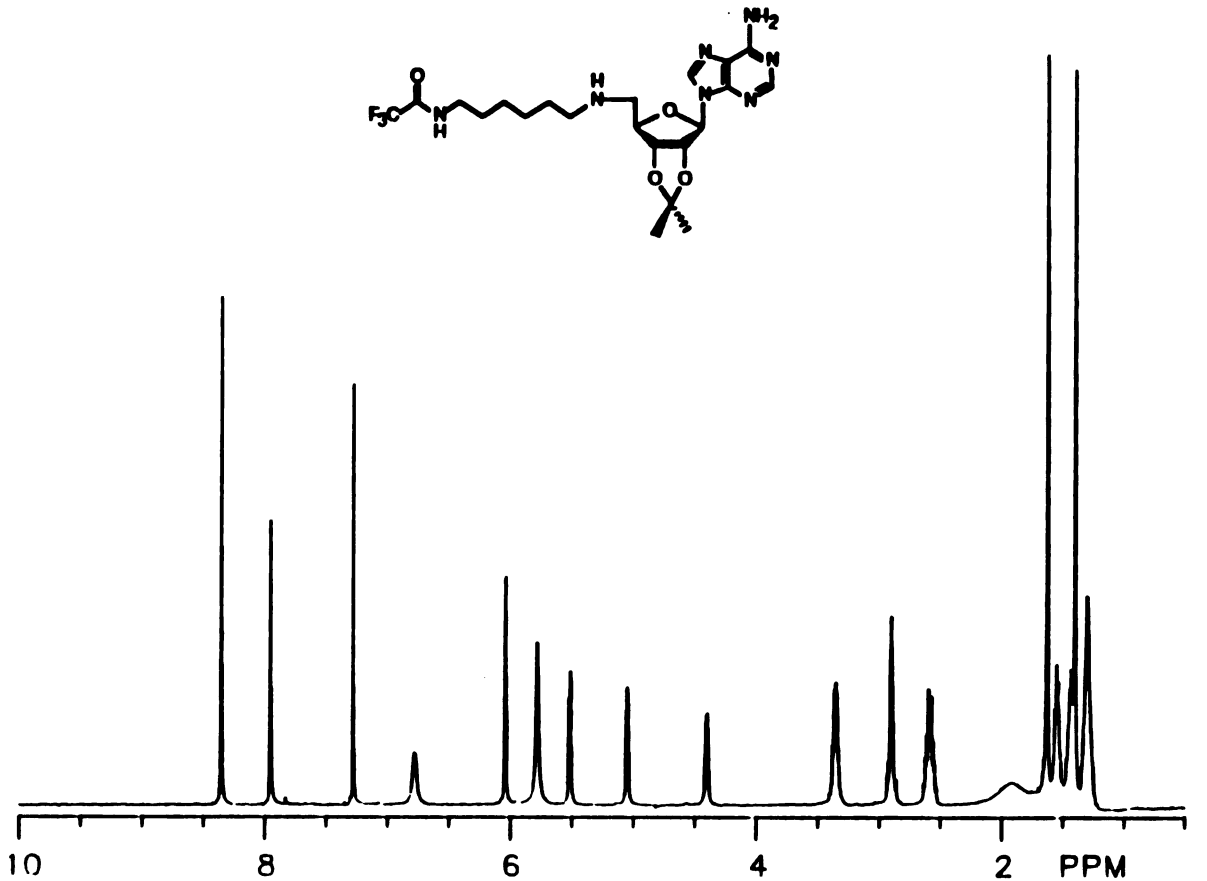
Spectrum 26. 500 MHz ^{1}H NMR (D_2O) spectrum of the nitrogen butyl SAM analogue (2e).



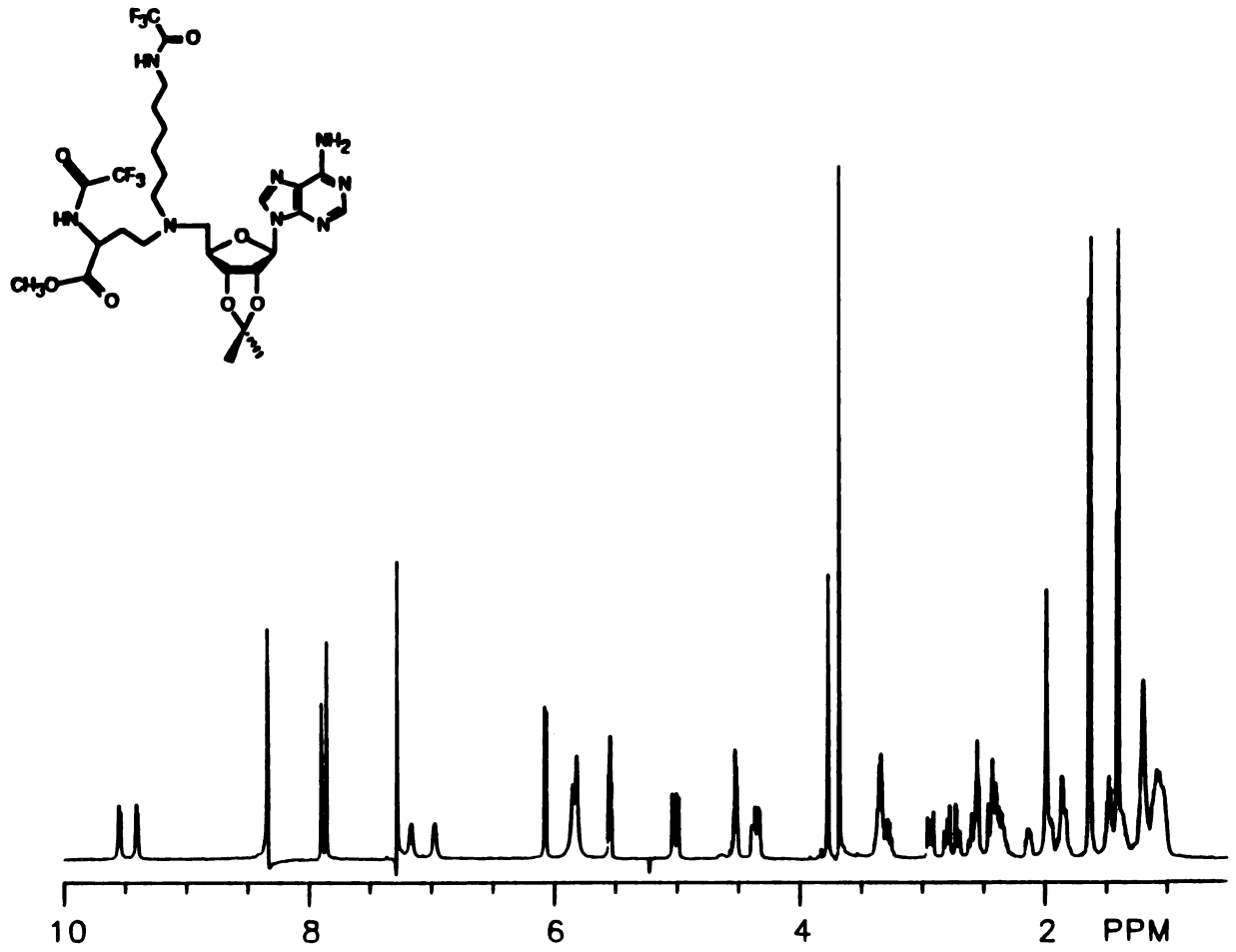
Spectrum 27. 500 MHz ^{1}H NMR (D_2O) spectrum of the nitrogen pentyl SAM analogue (**2f**).



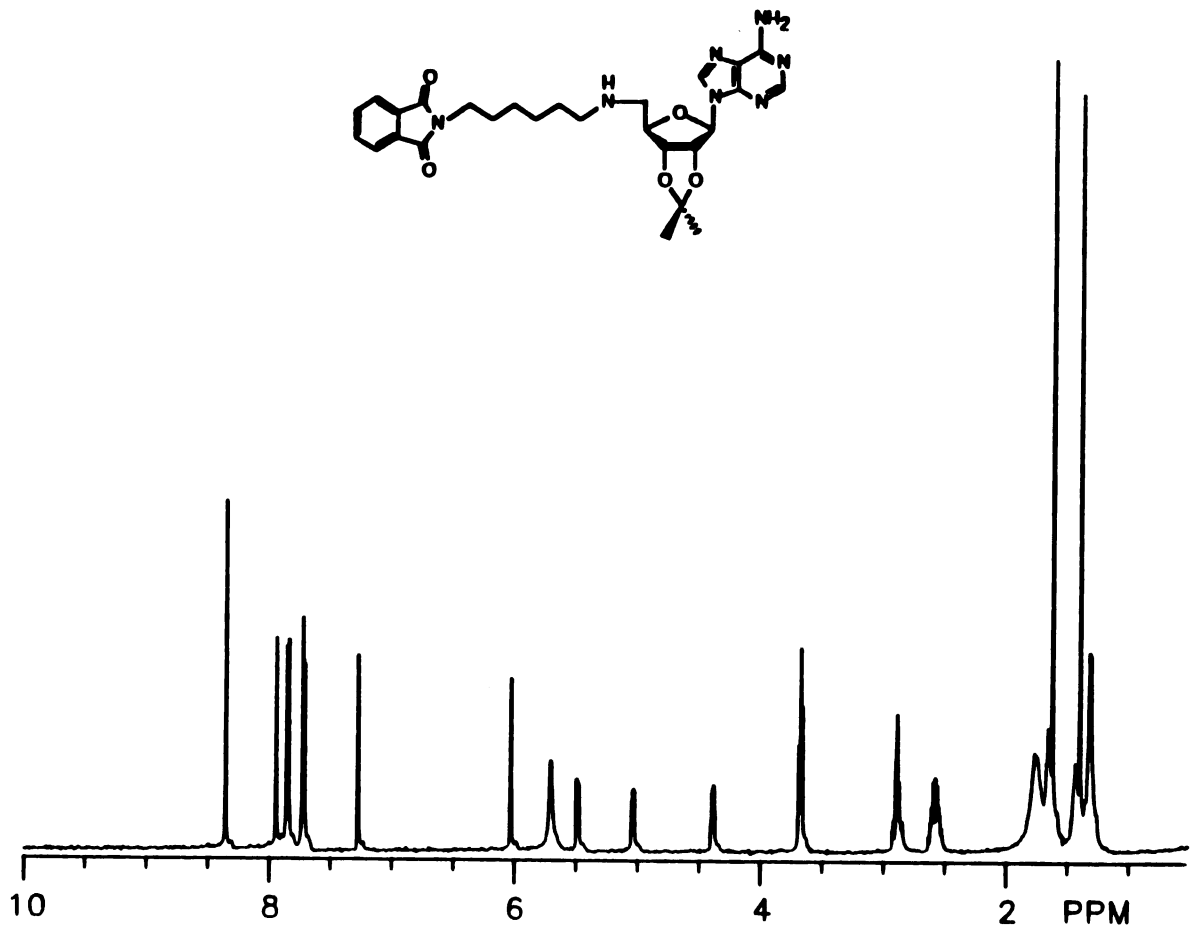
Spectrum 28. 500 MHz ^{1}H NMR (D_2O) spectrum of the nitrogen octyl SAM analogue (2g).



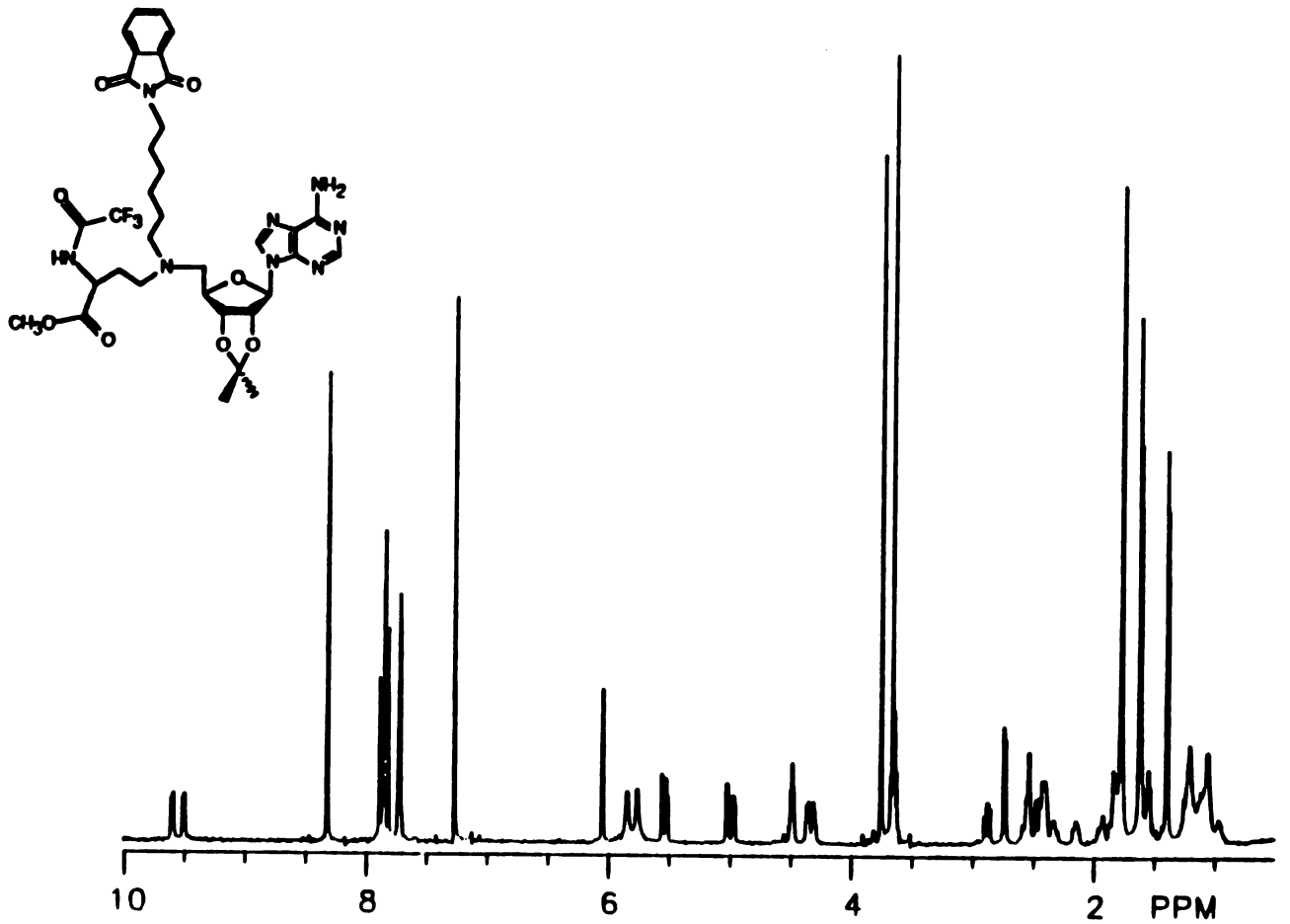
Spectrum 29. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of 5'-[6-(trifluoroacetamido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (11).



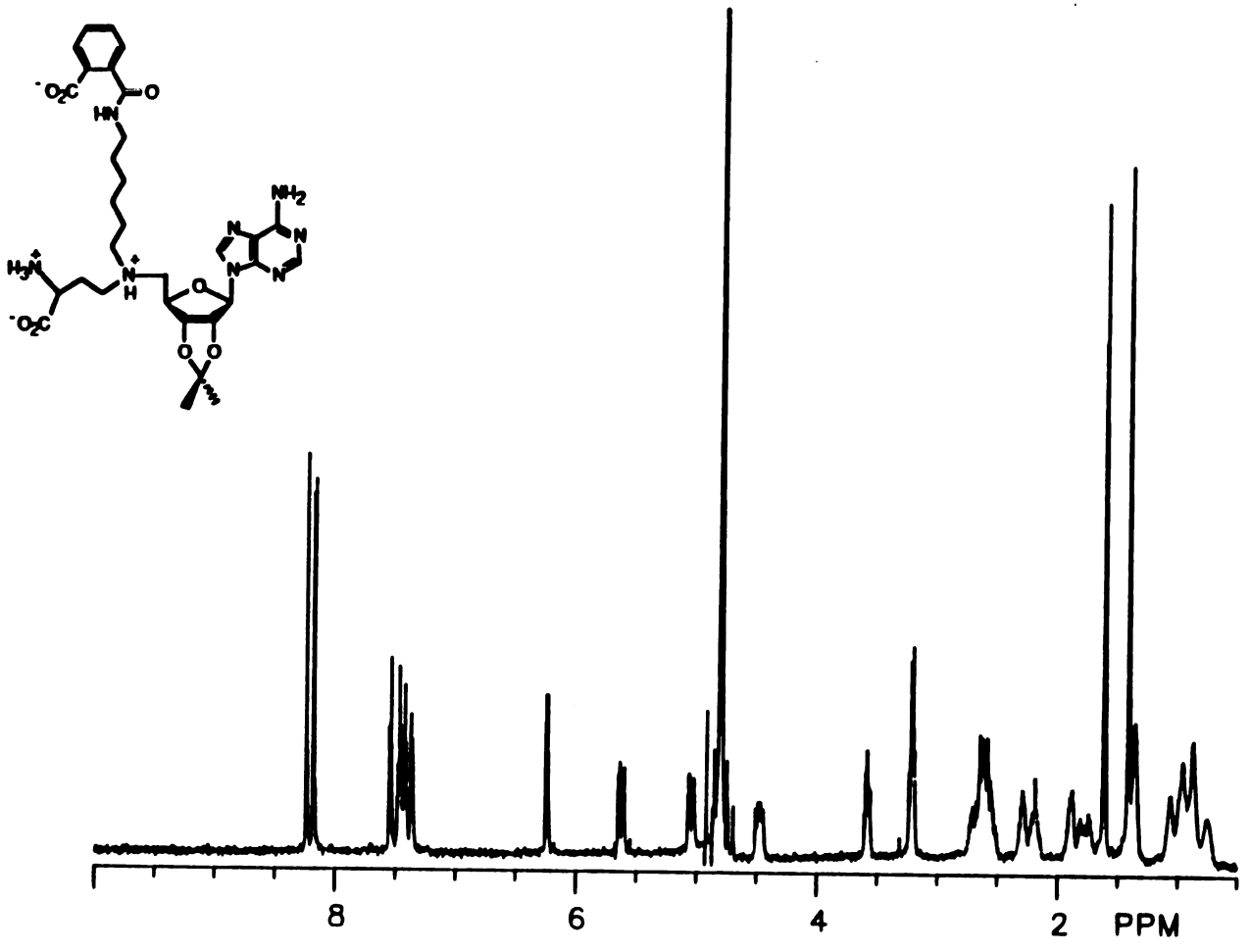
Spectrum 30. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of the protected 5'-[6-(trifluoroacetamido)-1-hexyl] SAM affinity ligand (14).



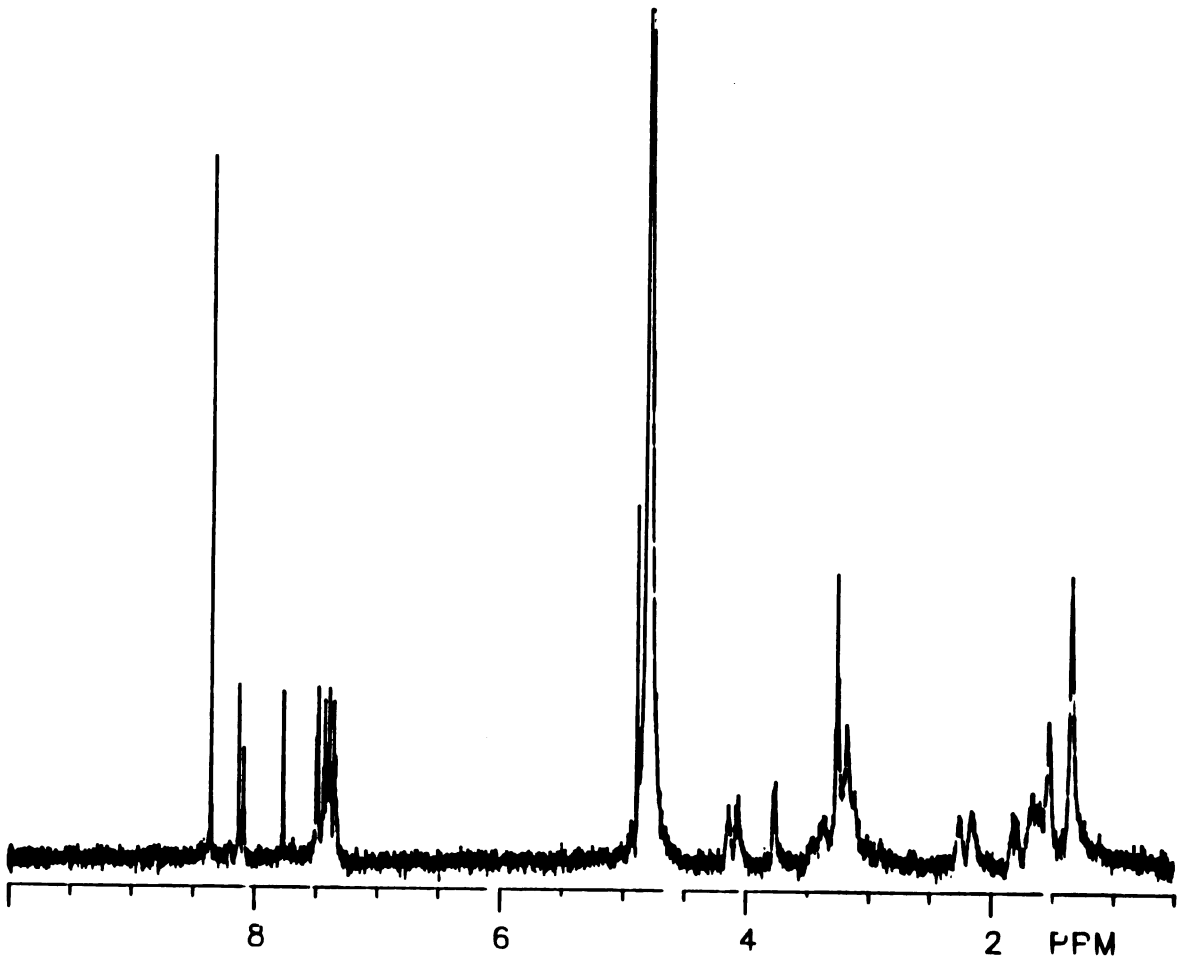
Spectrum 31. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of 5'-[6-(phthalimido)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (16).



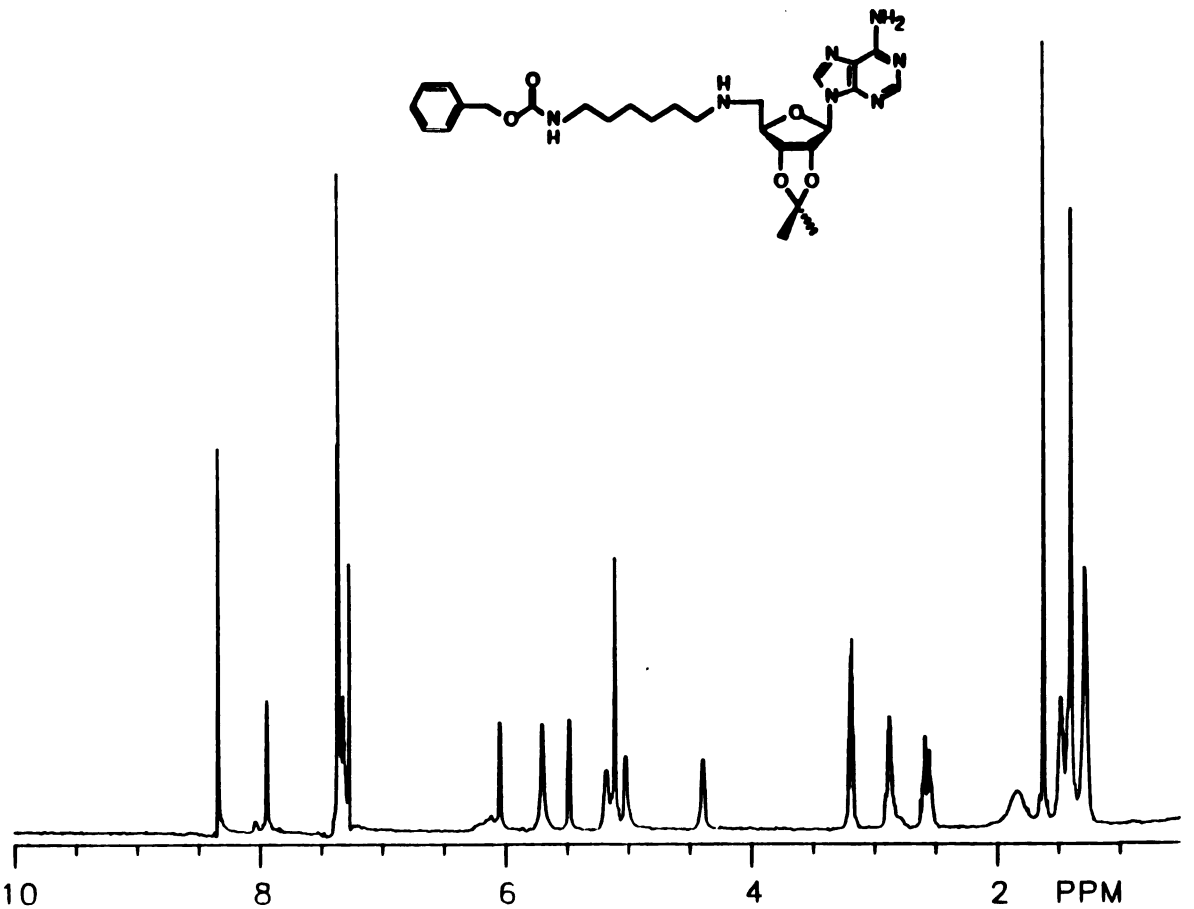
Spectrum 32. 500 MHz ^1H NMR ($CDCl_3$) spectrum of the protected 5'-[6-(phthalimido)-1-hexyl] SAM affinity ligand (17).



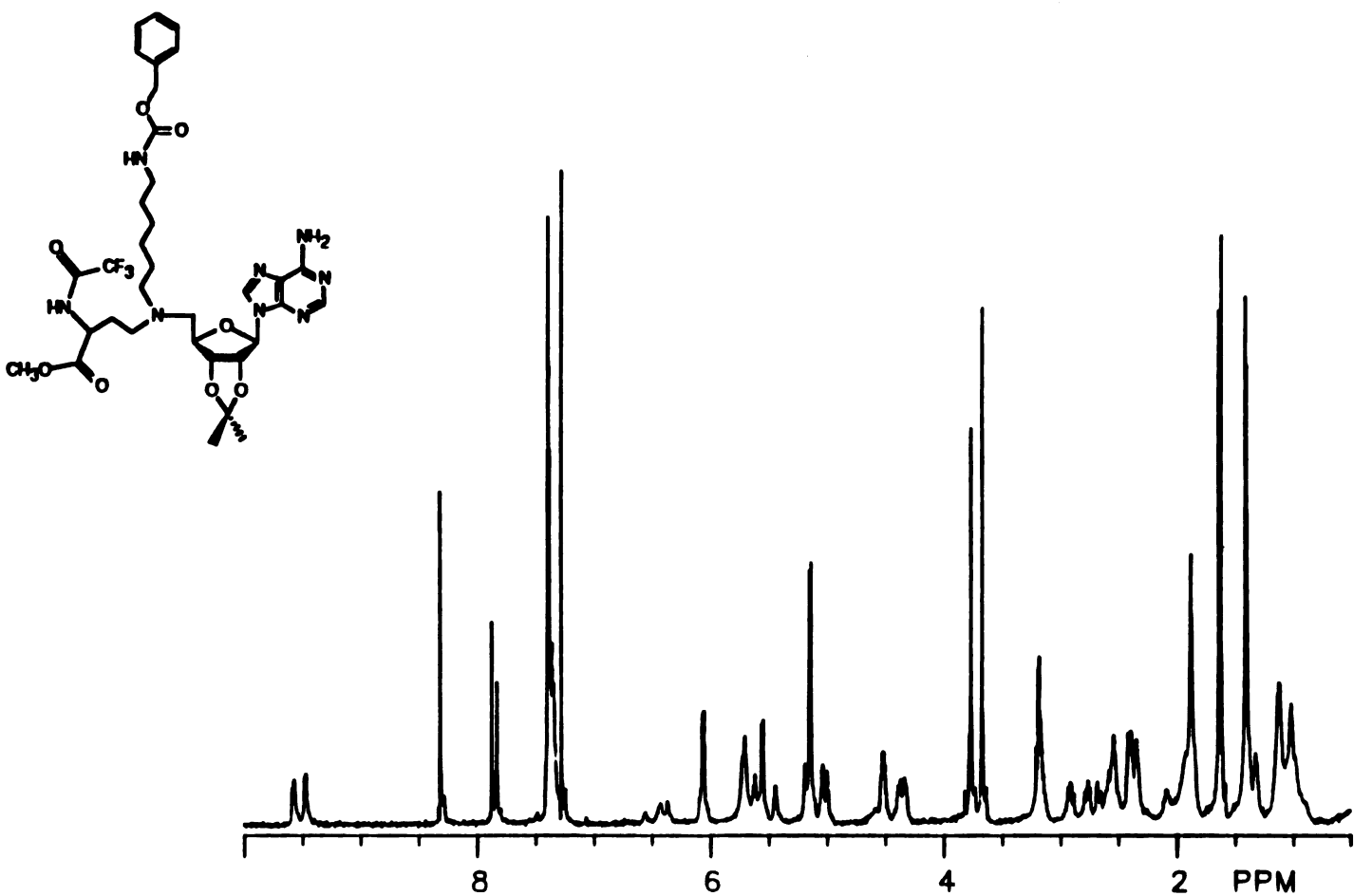
Spectrum 33. 500 MHz ^{1}H NMR (D_2O) spectrum of N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-[6-(1-phthalamoyl)-1-hexyl]-2(R,S),4-diaminobutyric acid (18).



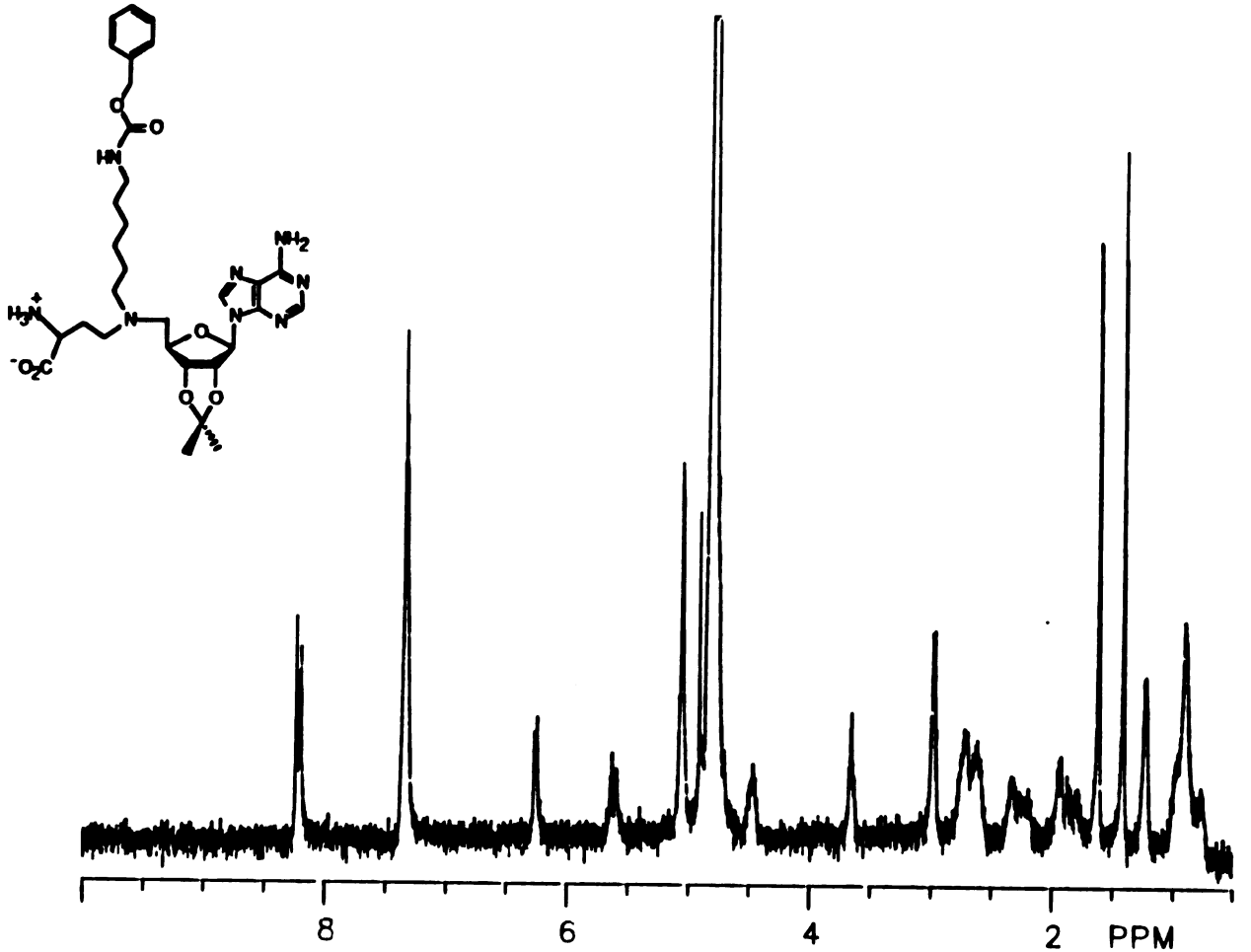
Spectrum 34. 500 MHz ^{1}H NMR (D_2O) spectrum of N^4-(2',3'-O-isopropylidene-5'-adenosyl)-N^4-[6-(1-phthalamoyl)-1-hexyl]-2(R,S),4-diaminobutyric acid (18), after treatment with 1.0 M aqueous sodium hydroxide at 100°C. for three hours.



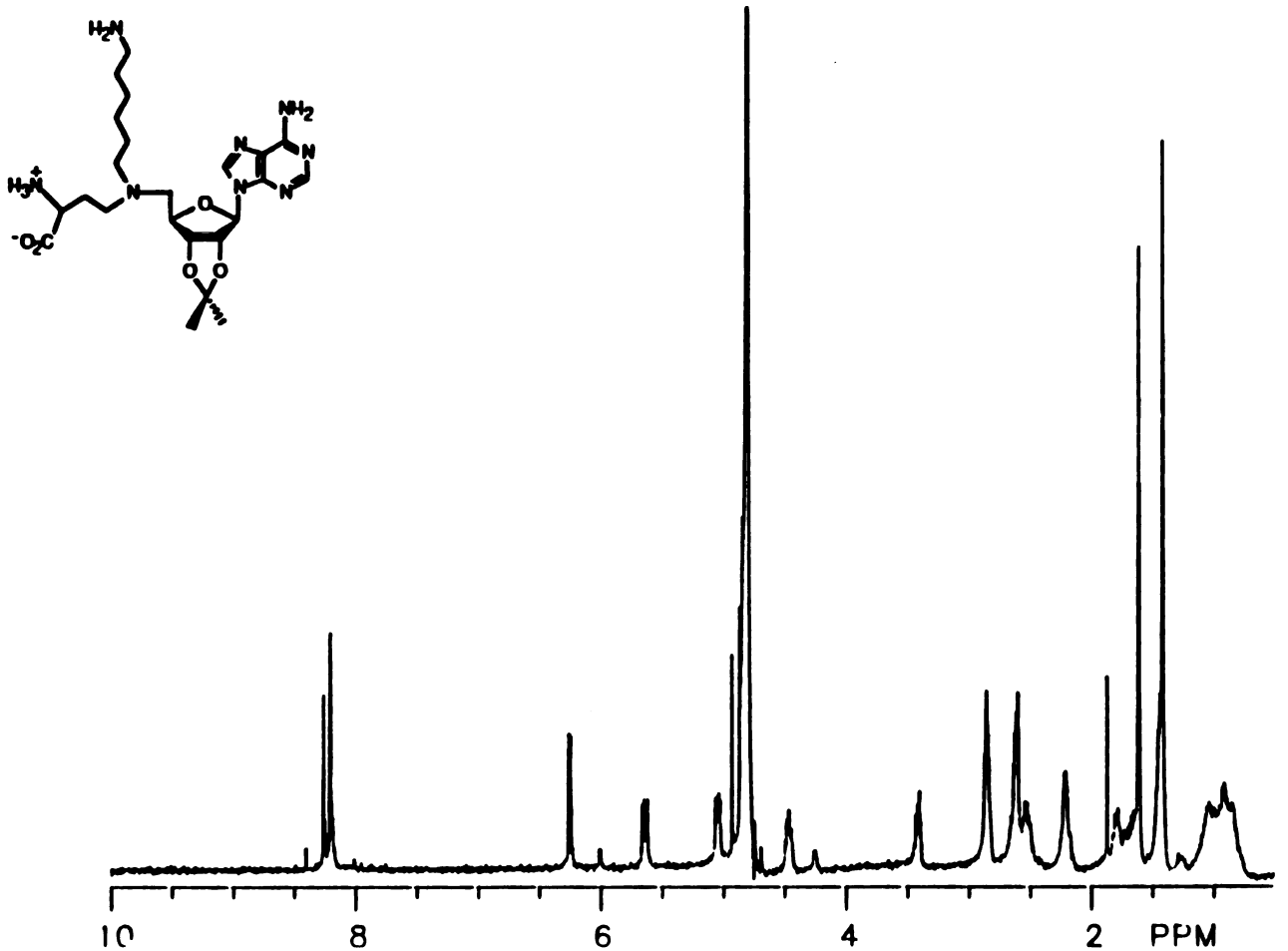
Spectrum 35. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of 5'-[6-(benzyloxycarbonylamino)-1-hexyl]amino-5'-deoxy-2',3'-O-isopropylideneadenosine (**20**).



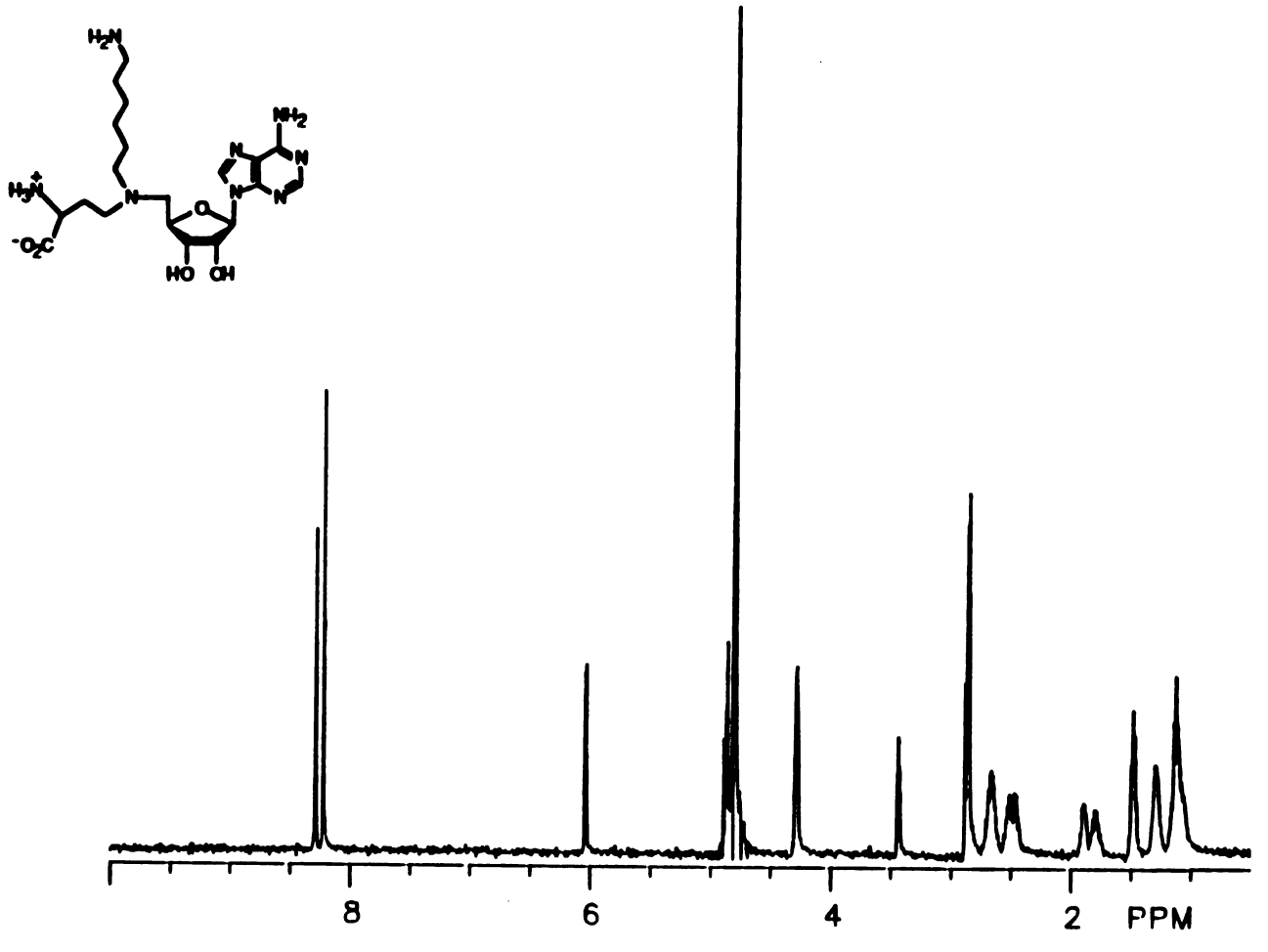
Spectrum 36. 500 MHz ^{1}H NMR ($CDCl_3$) spectrum of the protected 5'-[6-(benzyloxycarbonylamino)-1-hexyl] SAM affinity ligand (21).



Spectrum 37. 500 MHz ^{1}H NMR (D_2O) spectrum of the 2',3'-O-isopropylidene 5'-[6-(benzyloxycarbonylamino)-1-hexyl] SAM affinity ligand (22).



Spectrum 38. 500 MHz ^{1}H NMR (D_2O) spectrum of the 2',3'-O-isopropylidene 5'-(6-amino-1-hexyl) SAM affinity ligand (**23**).



Spectrum 39. 500 MHz ^{1}H NMR (D_2O) spectrum of the 5'-(6-amino-1-hexyl) SAM affinity ligand (9).

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