AN ABSTRACT OF THE THESIS

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Title : Preparation of ^{13}C And ^{15}N Modified Derivatives of Adenosine and 2′-Deoxyadenosine.

Abstract approved : Eric L. Ingram

This project deals with synthesis of modified nucleosides and their incorporation into 10-base sequence DNA and RNA strands (oligomers). These DNA and RNA fragments possess the proper polarity to easily enter cells through the cell membranes. Enzymes within the cell cleave these molecules, releasing the modified nucleosides. In the present investigation, ^{13}C And ^{15}N modified derivatives of adenosine and 2′-deoxyadenosine were prepared. These derivatives are prepared to see the bond weakening between base pairs, adenine and 5-fluoro-2′-deoxyuridine , which are incorporated into two respective strands of DNA or RNA. This would inhibit DNA replication resulting in the death of cancer cells. The studies are being conducted in Eppley Cancer Institute in Omaha, Nebraska to see their effect against various cancer cells and to study base pairing by Nuclear Magnetic Resonance (NMR) spectroscopy. The results of the present research showed the successful formation of unlabeled adenosine by a new synthetic methodology, which will be used to prepare labeled adenosine and ^{13}C enrichment at C8 of 2′ deoxyadenosine.

Preparation of ^{13}C And ^{15}N Modified Derivatives of Adenosine and 2′-Deoxyadenosine.

A Thesis

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by

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PREFACE

This thesis was written in the style required by the ACS Style Guide: A Manual for Authors and Editors (2nd edition).

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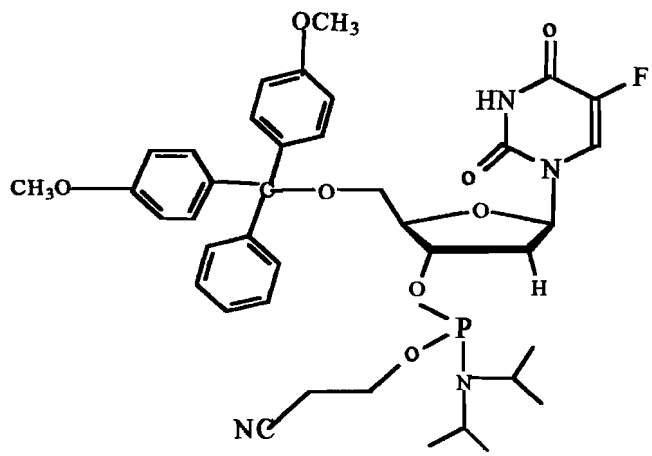
INTRODUCTION

Anti-metabolites are useful chemotherapeutic agents for treatment of cancer. These compounds interfere with the nucleic acid synthesis either by inhibiting production of deoxyribonucleoside triphosphates, the immediate precursors for DNA synthesis, or by substituting for triphosphates in the synthesis of DNA, in which case replicated DNA differs from the template (Gmeiner, 1995). The oligodeoxy-nucleotides composed solely of nucleoside analogues represent a new strategy for the delivery of the anti-metabolites. Anti-metabolites must be activated to their monophosphate or triphosphate forms to interact with their molecular targets (Srinivasan *et al.*, 1995). Activated forms of anti-metabolites can be produced in two ways; 1) Oligodeoxynucleotides can be degraded intracellularly by exonucleases to release their nucleotide monophosphate components, and 2) Oligodeoxynucleotides, with nucleoside analogue such as 5-fluorouridine (FUrd) incorporated in place of natural nucleosides can be prepared which on enzymatic degradation yields only 2′-deoxy-5-fluorouridylate (FdUMP), the fully activated form of the anti-cancer drug 5FU. The mechanism by which anti-metabolites cause cell death is not clear but they are the most potent anti-cancer drugs (Gmeiner, 1995).

Oligomeric forms of nucleoside anti-metabolites have advantages over monomeric forms. The nucleoside monophosphates can be produced from oligomeric forms by simple exonuclease activity, requiring no further metabolic activity while the monomeric forms require glycosylation and phosphorylation to reach the same level of activity. Oligomeric forms of drugs may be chemically modified to alter their bio-distribution and cellular uptake in ways that monomeric forms may not. Oligomers have larger size and are cleared from body

more slowly, thus requiring lower overall dosages for the same therapeutic effect. The oligomers possess the proper polarity to easily enter cells through the cell membranes while monomers cannot.

Many of the drugs used for anti-cancer chemotherapy have been in use for several decades. 5-fluorouracil (FUra) is an antineoplastic agent used clinically in the treatment of solid tumors, particularly for malignancies of the gastrointestinal tract, breast, neck and head. It is administered into the cell in the 5 fluorouridine (FUrd) form, which is shown below.



Monomeric forms of 5-fluorouracil produce cardiotoxic 2-fluoro- β-alanine (FBAL) and are rapidly cleared from the body. Studies are being conducted to minimize the production of FBAL (Srinivasan *et al.*, 1995). The anticancer activity of 5FUra is believed to result primarily from its metabolism to 2′-deoxy-5-fluorouridylate (FdUMP), which forms an extremely stable complex with thymidylate synthase (TS), the enzyme required for 2′-deoxythymidine triphosphate (dTMP) production, thereby inhibiting the replication of DNA (Santi *et al.*,1974; Weck becker,1991). 5FUra may also exert anti-cancer and cytotoxic activities as a consequence of its metabolism to 5-fluorouridine triphosphate (FUTP) and the

incorporation of FUrd into cellular RNA (Doong and Dolnick, 1988) and also by the incorporation of 5-fluoro-2′-deoxyuridine triphosphate (FdUTP) into genomic DNA that can result in single and double-strand breaks (Willmore and Durkacz, 1993). The effects of FUra metabolites on RNA and DNA biochemistry are referred to as the RNA- and DNA-mediated effects of FUra.

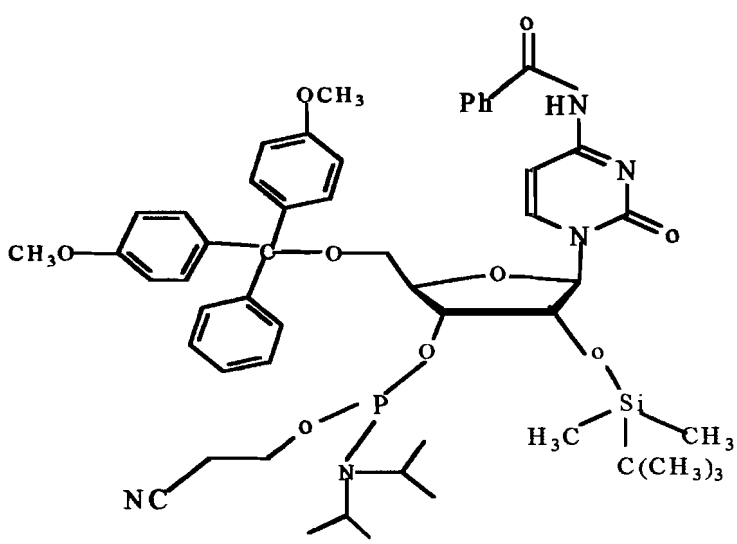
The effects of FUrd substitution on the structure of duplex RNA are less well studied than corresponding effects of FUrd substitution in duplex DNA. It has been reported that FUrd incorporation into both pre-mRNA and small nuclear (sn) RNA reduce the efficiency of pre-mRNA splicing (Schmittgen et al., 1994; Doong and Dolnick, 1998). RNA-mediated effects of 5FU mainly arise from FUrd substitution in non duplex regions of RNA or from disruption of RNA-protein recognition processes. Few data have become available that directly assess the effects of FUrd substitution on RNA structure and function. It has been reported that single FUrd substitutions in the RNA duplex are stabilizing because of the increase thermal stability arising from the greater base overlap of 5-FU to base pair by using C-4 carbonyl (Sahasrabudhe *et al.*,1995).

FUrd incorporation has been shown to affect the structure of duplex DNA near the site of substitution but otherwise it has little structural effect (Stolarski *et al.*, 1992). FdUrd incorporation into duplex DNA may lead to base pair mismatches during transcription that result in the synthesis of mutant, dysfunctional proteins. FdUrd substitution could also affect the recognition of duplex DNA by transcription factor complexes and interfere with gene regulation. Kremer and co-workers conducted NMR studies and reported FdUrd substitution destabilized DNA duplex due to ionization of FdUrd imino proton (Kremer *et al.*, 1987)

The importance of FdUTP incorporation into genomic DNA for the activity of FUra is demonstrated by numerous reports indicating that DNA isolated from cells treated with FdUrd had undergone strand scission (Willmore and Durkacz, 1993). Exposure of cells in culture to FUra results in a cytostatic condition referred to as "thymidine cell death" (Weckbecker, 1991). Resistance of a human ovarian cancer cell line to FUra has also been associated with decreased levels of FdUrd in DNA (Doong and Dolnick, 1998).

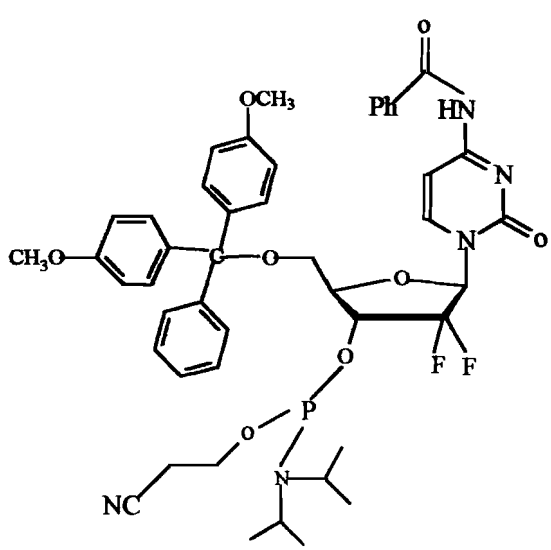
The evidence that nucleic acid mediated processes are altered by incorporation of FUra metabolites into both DNA and RNA is both compelling and long standing. The extent to which the pertubation of nucleic acid mediated processes happens due to alteration of structure of duplex RNA and DNA is not presently known. 5-FU was incorporated in the 10-base sequence DNA and RNA strands (oligomers) and had undergone cell culture screening at the National Cancer Institute (NCI). It was effective against lung and ovarian cancer cells. Animal studies with the compound are currently in progress at the NCI.

Cytrabine (1-β-d-arabino furanosylcytosine), another anti-cancer drug is used against leukemia and viral infections and is administered into the cell as phosphoramidite.



Cytrabine substitution for deoxycytidine inhibits the extension of Okazaki fragments, that are intermediates during DNA replication and reduces the stability and structure of Okazaki fragments (Gmeiner et al., 1998). When cytrabine is taken in by cells, it is metabolized to nucleoside triphosphate interfering with elongation of the lagging strand of the replication fork during DNA synthesis and resulting in structural perturbations in DNA: DNA duplex portion of Okazaki fragment. It does not however, terminate chain elongation but leads to production of very small nascent DNA fragments and causes localized unfolding at the site of substitution in the nascent DNA from the Okazaki fragment, inhibiting the completion of replication synthesis. Understanding the structural and thermodynamic consequences of cytrabine substitution will allow more accurate and detailed rationalizations of its biological properties to be developed. The Studies on cytrabine's effect on cancer cells are in progress.

2′, 2′- difluoro- 2′- deoxycytidine (dFdC) (Gemcitabine) phosphoramidite, (shown below), is an anti-metabolite that is effective against leukemia and has shown therapeutic response in adenocarcinomas of lung and colon.



The cytotoxic action of dFdC appears to be dependent on phosphorylation by deoxycytidine kinase to dFdCTP which inhibits DNA synthesis by inhibiting the activity of ribonucleotide reductase causing a lowering of intracellular deoxynucleotide pool concentrations (Richardson *et al.*, 1992). The tetramer of gemcitabine (2′, 2′- difluoro- 2′- deoxycytidine) monophosphate has been prepared and submitted to the NCI and to the University of Frankfurt in Germany for cell culture screening and results are pending.

Analogs of 2′-deoxyadenosine are of considerable interest because of their potent growth inhibitory effects on some cancer cells (Huang *et al.*,1981) . Although millimolar concentrations of deoxyadenosine are required in the medium to inhibit cell growth and intracellular DNA synthesis, high concentrations are used due to the rapid destruction of deoxyadenosine by adenosine deaminase and the subsequent action of purine nucleoside phosphorylase. It was reported that if adenosine deaminase inhibitors are used, cell growth is inhibited by deoxyadenosine at concentration in the range 1-100 mM. It therefore seems possible that analogs of 2′- deoxyadenosine, which are poor substrates for deaminase, might be powerful inhibitors of cell replication even in the absence of deaminase inhibitor. 2-chloro and 2-bromo analogs of 2′-deoxyadenosine are potent growth inhibitors for E.Coli (K_{12}) and KB cells (Huang *et al.*, 1981).

^{13}C enriched modified derivatives of adenosine and 2′ deoxyadenosine and ^{15}N adenosine were prepared to study the mechanism of antimetabolite drugs such as 5-fluoro-2′ deoxyadenosine. The derivatives of isotopically enriched adenosine and 2′deoxyadenosine will be prepared and incorporated into dacamer DNA and RNA strands and the effect of fluorine substitution on base pairing will be studied through cell culture screening at the National

Cancer Institute (NCI). Labeled isotopes have greater advantage over the conventional methods of analysis of radio-chemical isotopes because site specific incorporation of magnetically active nuclei especially ^{13}C and ^{15}N provides a simplified nuclear magnetic resonance (NMR) spectra since proton signals not bonded to labeled nuclei are suppressed. The measurement of ^{13}C NMR spectra of nucleic acid model before and after treatment by carcinogen identifies the site of action(Sharma *et al.*, 1983).

MATERIALS AND METHODS

Materials:

^{13}C Sodium formate, adenine, 4,5,6-Triamino-pyrimidine sulfate hydrate, HMDS (hexamethyl disilazane), TCS (trimethylchlorosilane), benzoyl chloride, trimethylsilyltriflate, ABR (β-D-Ribofuranose 1-acetate 2, 3, 5-tribenzoate), $SnCl_4$, 4, 4-dimethoxtrityl chloride, imidazole, tert-butyldimethylsilyl chloride, 6-chloropurine riboside were provided by the Eppley Institute for Cancer Research, University of Nebraska Medical Center. Thymidine, and the enzymes, nucleoside phosphorylase and thymidine phosphorylase were purchased from Sigma Chemicals. The nuclear Magnetic Resonance spectroscopy data was made using 500MHz Gemini Varian spectrometer. Additional background articles are included in the reference section.

Preparation of [8- ^{13}C] protected Adenosine :

Preparation of Morpholinium ^{13}C formate:

Sodium formate, ^{13}C enriched (3.02g, 36mmol) was dissolved in 2.78mL of water and 4.44mL of 12M hydrochloric acid. Freshly distilled morpholine (5.37mL, 5mmol) was added drop wise to the acid solution at 0^0C with stirring. The solution was then evaporated to dryness under a rotary evaporator, and the solid residue obtained was dissolved in absolute ethanol and filtered to separate NaCl. The residue was washed with absolute ethanol. The filtrate and the washings were combined and evaporated under a rotary evaporator and a colorless solid residue was obtained.

Preparation of [8-^{13} C] Adenine:

The morpholinium salt obtained above was heated to 95 ^{0} C under nitrogen for 45min.

4,5,6-Triamino-pyrimidine sulfate hydrate (3.5g, 14.5 mmol) was dissolved in 10mL of 1N HCl with gentle heating and added to the morpholinium salt. The resulting mixture was stirred under nitrogen at 95^{0}C. After 36 hours the reaction was cooled to room temperature, neutralized with 6N NaOH, and refrigerated for 24 hours. The crystals were collected on a glass frit, washed with water, and dried over P_2O_5 in a desiccator. The mother liquor was lyophilized. The residue was recrystallized from hot water and dried over P_2O_5.

Preparation of [8-^{13} C] Benzoyl Adenine:

[8-^{13}C] adenine (6.48g, 48mmol) was dried three times by evaporation of pyridine using a rotary evaporator and was suspended in 50mL of dry pyridine. Trimethylchlorosilane (5.4mL, 50mmol) was added and the mixture was stirred for 15min. Benzoyl chloride (5.8mL, 50mmol) was then added and the reaction was maintained at room temperature for 2 hours. The mixture was then cooled and 10mL of water was added. After 5 min 20mL of 29% aqueous ammonia was added and mixture was stirred at room temperature for 0.5 hours. The reaction mixture was then evaporated to dryness and the residue was dissolved in 150mL of water. The solution was washed once with a 50mL portion of ethyl acetate. The layers were separated and the organic layer was retained and evaporation of the solvent yielded a white solid.

Preparation of [8-^{13}C] Adenosine using trimethylsilyltriflate as a catalyst:

Benzoyl adenine (2.40g,10mmol) was refluxed with 35mL HMDS (1, 1, 1, 3, 3, 3-Hexamethyl disilazane) and 0.5mL TCS (Trimethylchlorosilane) for 7 hours, and the solvents were evaporated under a rotary evaporator. The yellowish, oily silyl compound obtained was mixed with (5.04g, 10mmol) of ABR (β-D-Ribofuranose 1-acetate 2, 3, 5-tribenzoate) and

dissolved in 25mL of purified 1,2-dichloroethane (100mL of 1,2-dichloroethane was extracted with concentrated sulfuric acid, water, dilute potassium hydroxide and water followed by drying with calcium hydride and distillation) and refluxed for 12 hours with 0.2mmol trimethylsilyltriflate (2.2mL standard solution in purified benzene (benzene was purified by extracting 100mL of benzene with concentrated sulfuric acid, water, dilute sodium hydroxide and then followed by drying with calcium hydride and distillation). The mixture was diluted with methylene chloride and the organic phase was extracted with ice-cold saturated $NaHCO_3$ solution. The organic phase was then dried with sodium sulfate and evaporated to give protected adenosine, which was dissolved in 250mL methanolic ammonia and the solution was kept for 16 hours at 24°C. The methanolic ammonia was evaporated under vacuum, and the residue was dissolved in 75mL water and extracted several times with ether and chloroform to remove benzamide and methyl benzoate as well as other material. The aqueous phase was evaporated under a rotatory evaporator and crude adenosine was obtained.

Preparation of [8-^{13}C] Adenosine using $SnCl_4$ as a catalyst:

Benzoyl adenine (1.71g, 7.1mmol) was suspended in 6mL of HMDS (hexamethyldisilazane) and 3 drops of TCS (trimethylchlorosilane). The mixture was reflexed for 3 hours under argon atmosphere and 5mL of 1, 2-dichloroethane was added. A solution of (2.17g, 6.8mmol) of ABR (1, 2, 3, 5-tetra-O-acetyl-β-D-ribofuranose), dissolved in 40mL of dichloroethane was added to the preceding solution. The resulting mixture was stirred in an ice-bath and then (0.59mL, 5.12mmol) of redistilled $SnCl_4$ was added. The pale yellow solution was allowed to stand at room temperature for 18 hours and volatiles were evaporated .The residue was dissolved in chloroform and extracted with saturated solutions

of $NaHCO_3$ and NaCl and dried over $MgSO_4$. The solution was filtered and evaporated under a rotatory evaporator. The residue was dissolved in 20mL NH_3/MeOH and stirred for 24 hours at 5^0C. The solution was evaporated to obtain the product.

Preparation of [8-^{13}C] Benzoyl adenosine:

Adenosine (1.25g, 4.8mmol) was dried three times by evaporation of pyridine using a rotary evaporator and was suspended in 50mL of dry pyridine. Trimethylchlorosilane (3.2mL, 25mmol) was added and the mixture was stirred for 15min. Benzoyl chloride (2.9mL, 25mmol) was then added and the reaction was maintained at room temperature for 2 hours. The mixture was then cooled in ice-bath and 5mL of water was added. After 5 min 10mL of 29% aqueous ammonia was added and the mixture was stirred at room temperature for 0.5 hours. The reaction mixture was then evaporated to dryness and the residue was dissolved in 75mL of water. The solution was washed once with a 50mL portion of ethyl acetate. The layers were separated and organic layer was kept in the hood and benzoyl adenosine was obtained and impurities were removed using charcoal.

Preparation of 5$^{/}$ -O- (4, 4$^{/}$ -dimethoxy trityl) [8-^{13}C] Adenosine:

Benzoyl adenosine (1.358g, 3.57mmol) was dissolved in 10mL pyridine under argon atmosphere to which 4, 4-dimethoxtrityl chloride (1.45g, 4.28mmol) was added in four equal fractions over 2 hours. The reaction mixture was stirred at room temperature overnight. The mixture was then diluted with 10mL of dichloromethane and the organic solution was washed with three 25mL aliquots of saturated sodium bicarbonate and sodium chloride solutions. The organic layers were separated, dried over anhydrous sodium sulfate and the solvent was removed under vacuum using a rotatory evaporator and a yellow foam product was obtained.

Preparation of 5′-O-(4,4′-dimethoxy trityl)-2′-O-(tert-butyldimethylsilyl)[8-^{13}C] Adenosine:

The above prepared compound was dissolved in 40mL of pyridine under argon atmosphere to which imidazole (5.0g, 72mmol) was added. The solution was stirred for one hour at which time tert-butyldimethylsilyl chloride (3.0g, 20mmol) was added as a single portion. The reaction mixture was kept at room temperature for 5 hours and then diluted with 25mL of dichloroethane. The solution was extracted with saturated solution of $NaHCO_3$ and NaCl. The organic layer was dried over anhydrous sodium sulfate and the solvent was removed under vacuum using a rotatory evaporator to give the crude product.

Preparation of [8-^{13}C] protected deoxy adenosine:

Preparation of [8-^{13} C] deoxyadenosine:

[8-^{13} C] Adenine (0.582g, 4mmol), Thymidine (2.91g, 13mmol), and 23.28mL of 10mM KH_2PO_4 (pH=7.2) were added to a 500mL flask and the mixture was stirred for 5 min after which the pH of the solution was adjusted to 7.3 with 1N KOH. Nucleoside phosphorylase (67.52 units, 5.67mg) and thymidine phosphorylase (37.40 units, 0.03mL) were then added. The mixture was kept in incubator at 37°C for 72 hours. The solution was purified by column chromatography using BioRad AG1-X8 (200 mesh) in the hydroxide form as stationary phase and 0-30% methanol/water step gradient was applied. The appropriate fractions were pooled followed by solvent removal to give the product.

Preparation of [8-^{13} C] Benzoyl deoxy-adenosine:

Adenosine (1.25g, 4.8mmol) was dried three times by evaporation of pyridine using a rotary evaporator and was suspended in 50mL of dry pyridine. Trimethylchlorosilane (3.2mL, 25mmol) was added and the mixture was stirred for 15min. Benzoyl chloride (2.9mL,

25mmol) was then added and the reaction was maintained at room temperature for 2 hours. The mixture was then cooled in ice-bath and 5mL of water was added. After 5 min 10mL of 29% aqueous ammonia was added and the mixture was stirred at room temperature for 0.5 hours. The reaction mixture was then evaporated to dryness and the residue was dissolved in 75mL of water. The solution was washed once with a 50mL portion of ethyl acetate. The layers were separated and organic layer was kept in the hood and benzoyl adenosine was obtained and purified using charcoal.

Preparation of 5′ -O- (4, 4′ -dimethoxy trityl) [8-^{13} C] deoxy adenosine:

Benzoyl adenosine (1.36g, 3.57mmol) was dissolved in 10mL pyridine under argon atmosphere to which 4, 4-dimethoxtrityl chloride (1.45g, 4.28mmol) was added in four equal fractions over 2 hours. The reaction mixture was stirred at room temperature overnight. The mixture was then diluted with 10mL of dichloromethane and the organic solution was washed with three 25mL aliquots of saturated sodium bicarbonate and sodium chloride solutions. The organic layers were then separated and dried over anhydrous sodium sulfate and the solvent was removed under vacuum using a rotatory evaporator and a yellow foam product was obtained.

Preparation of 5′ -O- (4, 4′ -dimethoxy trityl)-2 ′-O- (tert-butyldimethylsilyl) 3 ′- (cyanoethyl N, N-diisopropyl phosphoramidite) [8-^{13} C] deoxy-adenosine:

Diisopropylethylamine (1.5mL, 11.6mmol), Cyanoethyl N, N-diisopropyl phosphonamidic chloride (1.03mL, 4.4mmol) and DMAP (4-dimethyl aminopyridine) (0.09g, 0.7mmol) was dissolved in 10mL of THF (tetrahydrofuran). The compound prepared in the above step was dissolved in 5mL of THF and the above prepared solution was added to it.

The resulting mixture was stirred for 3 hours, diluted with 20mL of ethyl acetate and extracted with a saturated solution of NaCl. The organic layer was kept in the hood to remove the solvent to obtain the crude product.

Preparation of unlabeled adenosine:

Adenine (135.1mg, 1mmol) was suspended in a solution of (0.5g, 1mmol) of ABR (β-D-Ribofuranose 1-acetate 2, 3, 5-tribenzoate) in 50mL of anhydrous acetonitrile. Stannic chloride (2mmol) was dissolved in 20mL of acetonitrile and added to the above mixture and stirred overnight. The reaction mixture was concentrated to a small volume and (600mg) of sodium bicarbonate and 2mL of distilled water were added. The reaction mixture was evaporated under a rotary evaporator. The residue obtained was extracted three times with hot chloroform and organic layers were collected and evaporated down. The product obtained was purified by column chromatography using silica gel as a stationary phase and chloroform- methanol (9:1, v/v) as the eluent. The eluate was concentrated under reduced pressure and the product obtained was dissolved in 50mL of methanolic ammonia at 0^{0}C and heated at 50^{0} C for 10 hours. The solvent was then evaporated off and the residue was mixed with 10mL of water and extracted with chloroform several times. The aqueous layer was evaporated and the residue was dissolved in water and kept in the refrigerator overnight to give the final product.

Preparation of ^{15}N adenosine:

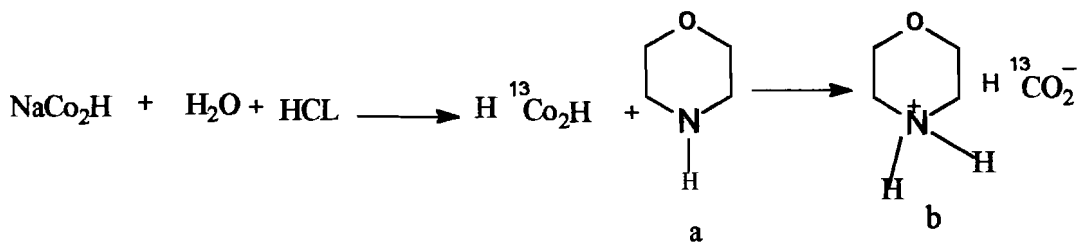
6-chloropurine riboside (0.6g, 14mmol) was suspended in a round bottom flask to which 2-propanol (PrOH) (25mL) was added. The flask was connected to a claisen adapter and a break seal flask containing ammonia gas. Ammonia gas was allowed to react with the

reaction mixture by breaking the seal with the weight and the reaction was heated for five days. Thin layer chromatography of the resulting compound and the starting material (6-chloropurine riboside) were compared.

RESULTS

Preparation of protected ^{13}C Adenosine:

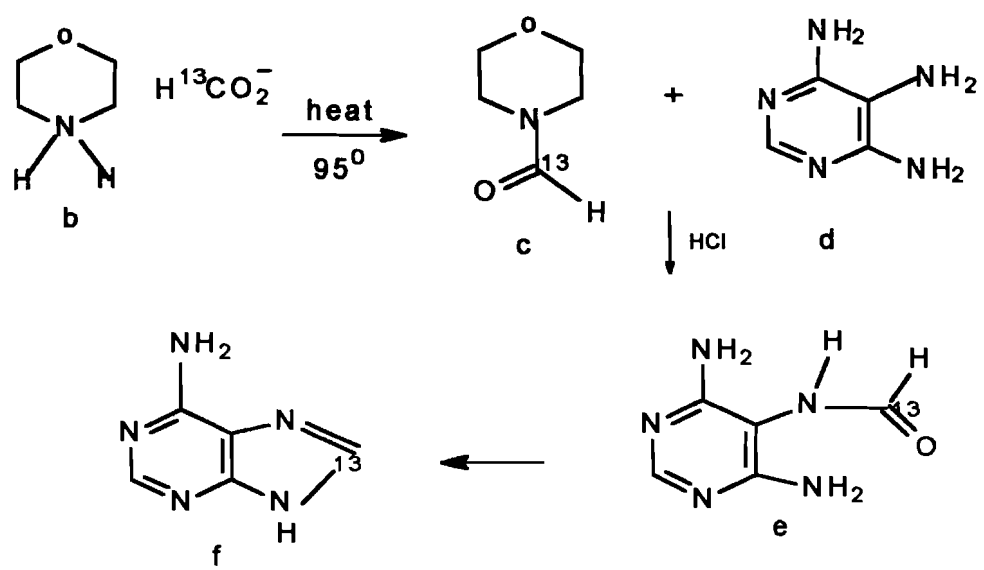
Synthesis of ^{13}C Morpholinium sulfate:



Scheme 1.

^{13}C Sodium formate and hydrochloric acid were mixed with freshly distilled morpholine (a) and colorless viscous solution was obtained, which was crystallized to obtain a solid, morpholinium ^{13}C formate (b). The weight of the product was 2.80g. The percent yield was 58 %. The reaction was performed under nitrogen atmosphere as the reaction was moisture sensitive.

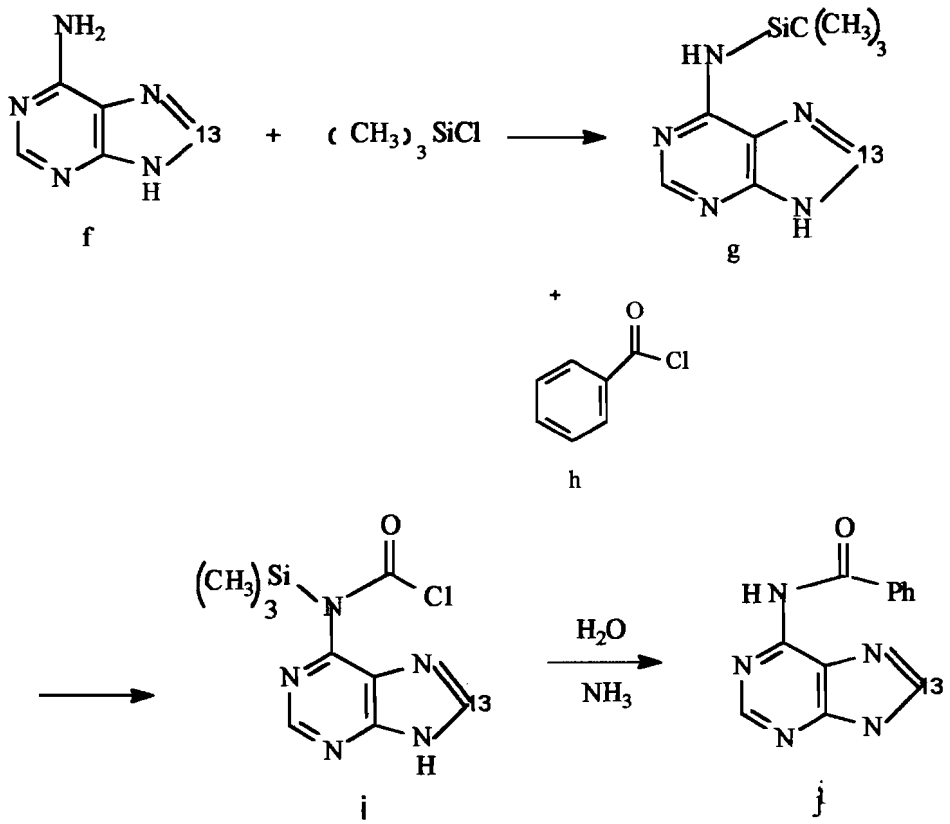
Synthesis of ^{13}C Adenine:



Scheme 2.

The morpholinium salt (b) was heated in a flask on an oil bath under nitrogen atmosphere to produce N formylmorpholine (c) which was then reacted with 4, 5, 6-triaminopyrimidine (d), dissolved in HCl to produce (e), where upon ring closure yielded adenine (f). The weight of the product was 1.46g. The percent yield was 74.5 %. Thin layer chromatography performed on the resulting product (f), 4, 5, 6-triaminopyrimidine and unlabeled adenine in 7:2:1 isopropanol: water: ammonia mixture, showed some traces of unreacted starting material (d).

Synthesis of [8-^{13}C] Benzoyl Adenine:

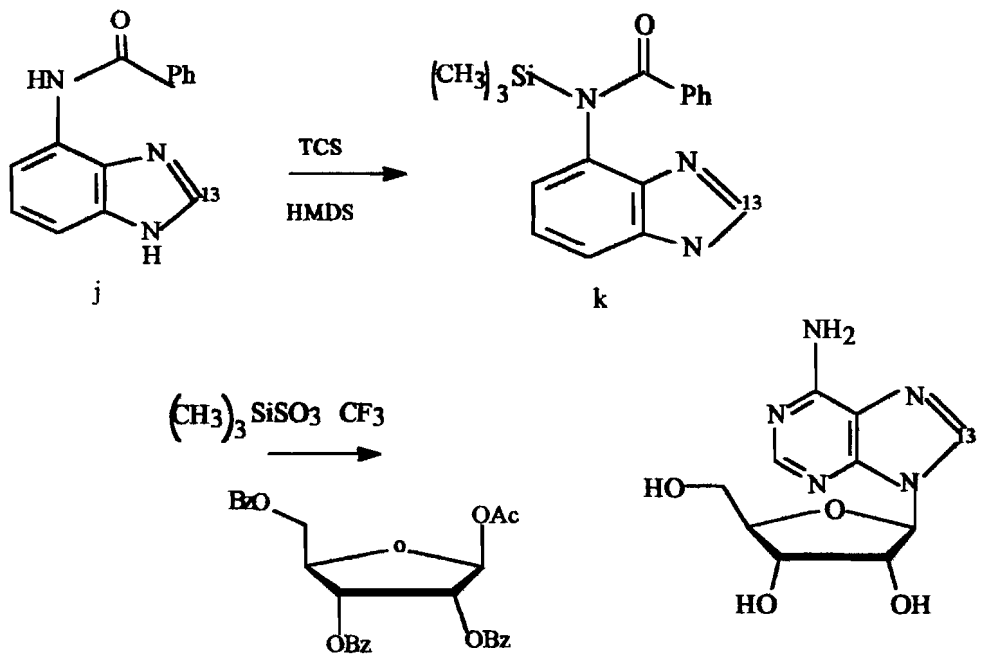


Scheme 3.

Adenine (f) was dissolved in pyridine and dried under vacuum to remove the water of crystallization. The removal of water was necessary, as water can react with benzoyl

chloride and trimethyl chlorosilane and inhibit the progress of the reaction. Dried adenine was dissolved in trimethyl chlorosilane and (g) was obtained, which was mixed with benzoyl chloride (h) to produce an orange solution containing (i). After maintaining the reaction mixture at room temperature for 2 hours, the product was combined with aqueous ammonia and the resulting solution was evaporated. The residue was dissolved in water and on extraction with ethyl acetate yielded (j). The weight of the product was 6.42g. The percent yield was 59.4 %.

Synthesis of [8-^{13}C] adenosine with trimethyl silyl triflate $(CH_3)_3SiSO_3CF_3$ as catalyst:



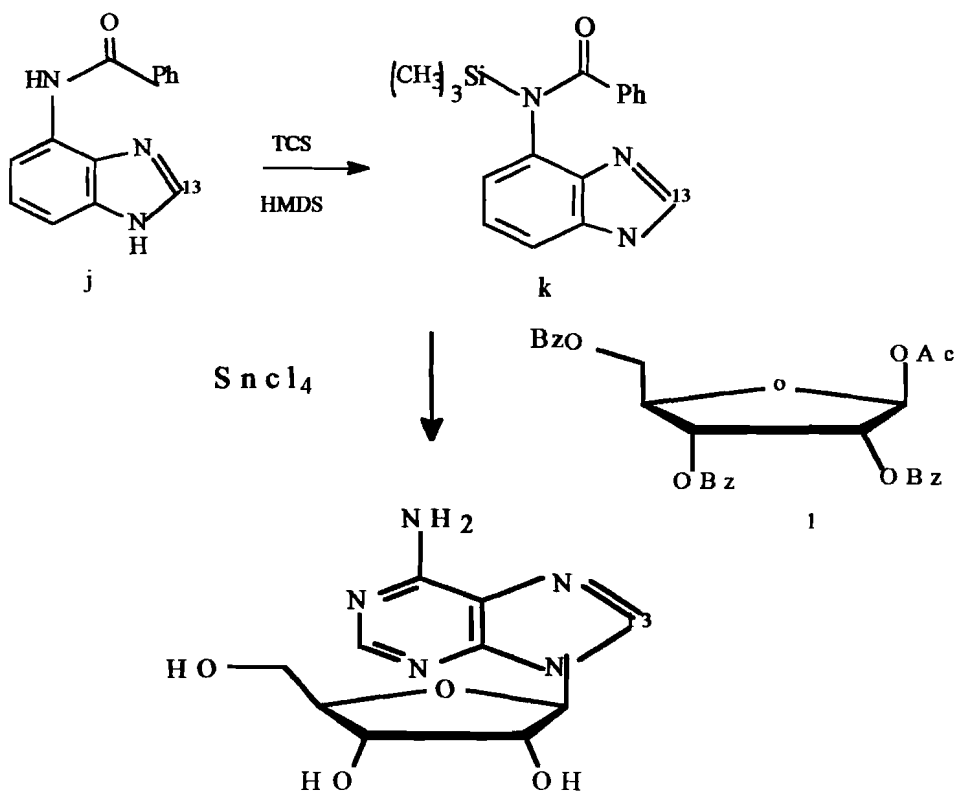
j k l m

Scheme 4.

A dark, brown, oily product (k) was obtained when benzoylated adenine (j) was refluxed with trimethyl chlorosilane (TCS) and hexamethyl disilazane (HMDS) for 7 hours. Trimethyl silyl triflate, $(CH_3)_3SiSO_3\ CF_3$, the catalyst was mixed with purified benzene in a glove bag, since it is moisture sensitive. The product (k), 1- O-acetyl-2, 3, 5-tri- O-benzoyl-

β-D-ribofuranose (ABR) (l) and the catalyst, were mixed resulting in a yellow solution. After extracting the yellow solution with $NaHCO_3$, the organic layer was evaporated to give crude protected adenosine which, when dissolved in methanolic ammonia, yielded the debenzoylated product (m). The solvent was evaporated to give white colored crystals of compound (m). These crystals were dissolved in water and extractions with ether and chloroform were performed to remove the side products, benzamide and methyl benzoate. The aqueous phase collected from the extractions was concentrated, and the crude product was recrystallized from water. The weight of the product was 1.32g. The percent yield was 52 %.

Synthesis of [8-^{13}C] adenosine with tin chloride $SnCl_4$ as catalyst:

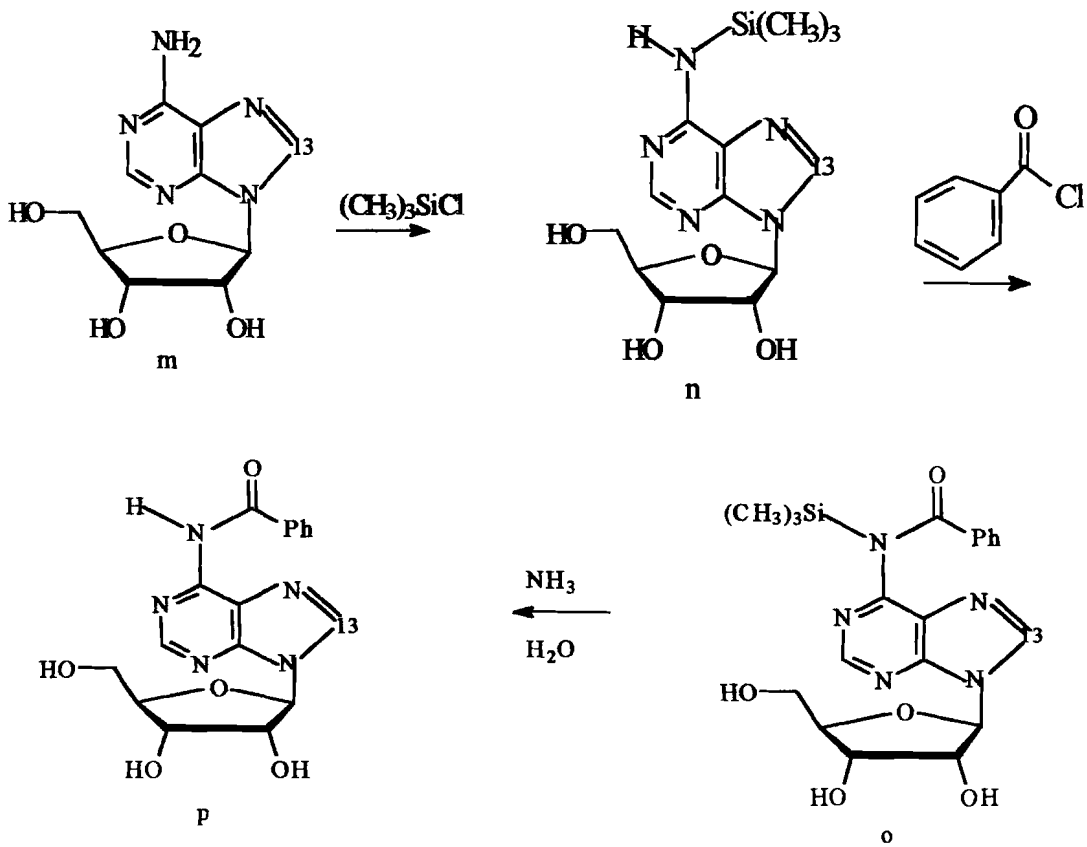


Scheme 5.

A solid, yellow, silyl compound (k) was obtained when 6-N benzoylated adenine (j) was refluxed with trimethyl chlorosilane (TCS) and hexamethyl disilazane (HMDS). The

compound (k) was reacted with 1-O-acetyl-2, 3, 5-tri-O-benzoyl-β-D-ribofuranose (ABR) (l) and $SnCl_4$ in a glove bag to prevent the violent reaction of $SnCl_4$ with the moisture in the air and to prevent unwanted side products. The yellow solution obtained was allowed to stand for 18 hours and later was evaporated to produce a white paste which was diluted with chloroform and extracted with sodium bicarbonate. The organic layer was reacted with aqueous ammonia to yield the crude product (m). The weight of the product was 0.86g. The percent yield was 48%.

Synthesis of [8-^{13}C] Benzoyl Adenosine:

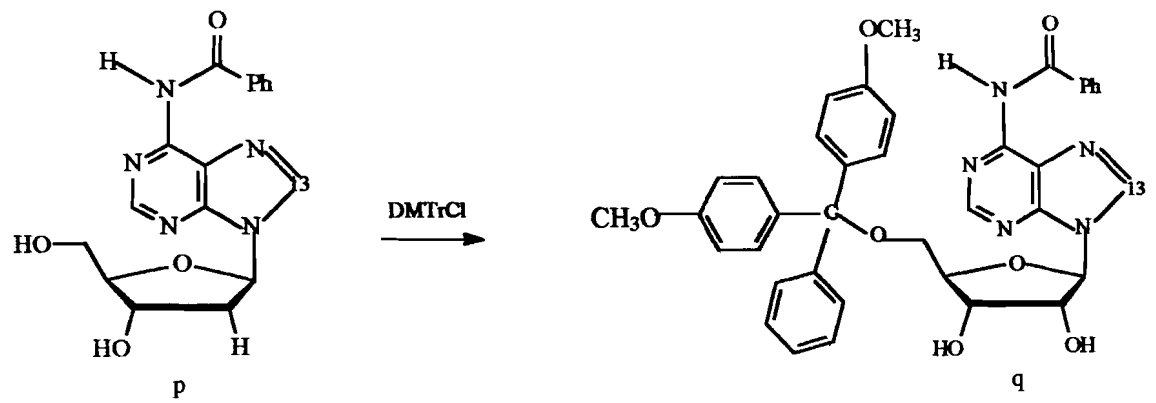


Scheme 6.

The raw product obtained by the reaction of adenosine (m) with trimethyl chlorosilane was (n). The resulting compound was dissolved in benzoyl chloride to obtain an

orange colored solution containing compound (o). After 2 hours, the solution was treated with methanolic ammonia and stirred for 0.5 hours. The reaction mixture was evaporated to produce white colored crystals. The crystals were dissolved in water and extracted with ethyl acetate. The top layer was evaporated and a thick paste of 6-N benzoyl adenosine (p) was obtained. The weight of the product was 1.02g. The percent yield was 69%.

Synthesis of 5′ -O- (4, 4′ -dimethoxy trityl) [8-^{13}C] deoxy adenosine:



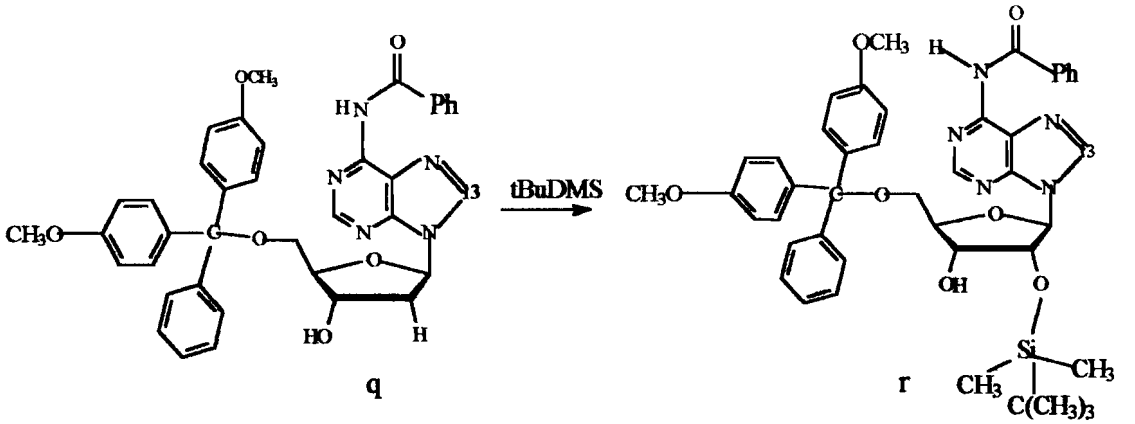
p q

Scheme 7.

Benzoyl adenosine (p) was dissolved in pyridine and 4, 4 dimethoxytrityl chloride (dMTrCl) was added over a period of 2 hours to the above solution under argon atmosphere. The light yellow solution obtained was stirred overnight. Later, the solution was diluted with dichloromethane and extracted with $NaHCO_3$. The organic layer was separated and evaporated under vacuum to produce the product (q). Thin layer chromatography on the product (q) showed an orange spot when HCl acid (0.3M) was squirted on the plate. The weight of the product was 1.70g. The percent yield was 73.4%.

Synthesis of 5′ -O- (4, 4′ -dimethoxy trityl)-2′ -O- (tert-butyldimethylsilyl) [8-^{13}C] adenosine:

adenosine:

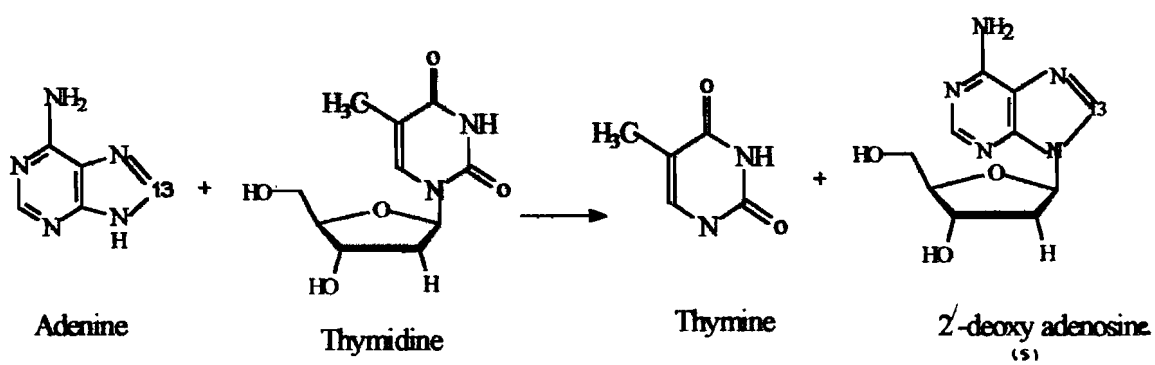


Scheme 8.

The compound (q) was reacted with imidazole and tertbutyl dimethylsilyl chloride (tBuDMS) resulting in a yellow solution. The solution was extracted with $NaHCO_3$ and NaCl. The organic layer obtained from the extraction was evaporated to obtain the crude product (r). The weight of the product was 0.66g. The percent yield was 65.4%.

Preparation of [8-^{13}C] protected 2′-deoxy adenosine:

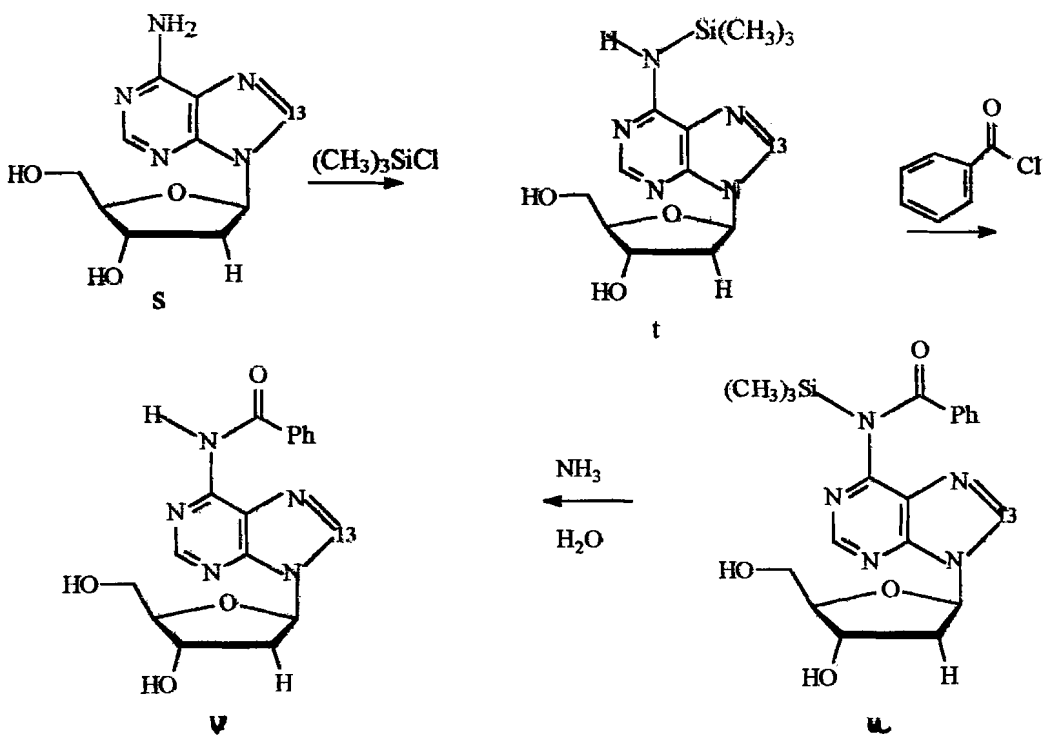
Synthesis of [8-^{13} C] deoxyadenosine:



Scheme 9.

For the above reaction, Lactobacillus bacteria was grown in glucose medium to isolate the enzyme, deoxyribosyl transferase, but the bacterial growth did not occur. Therefore, commercially available enzymes (nucleoside phosphorylase and thymidine phosphorylase) were used for the preparation of 2′-deoxyadenosine (s).

Synthesis of [8-^{13} C] Benzoyl deoxy adenosine:



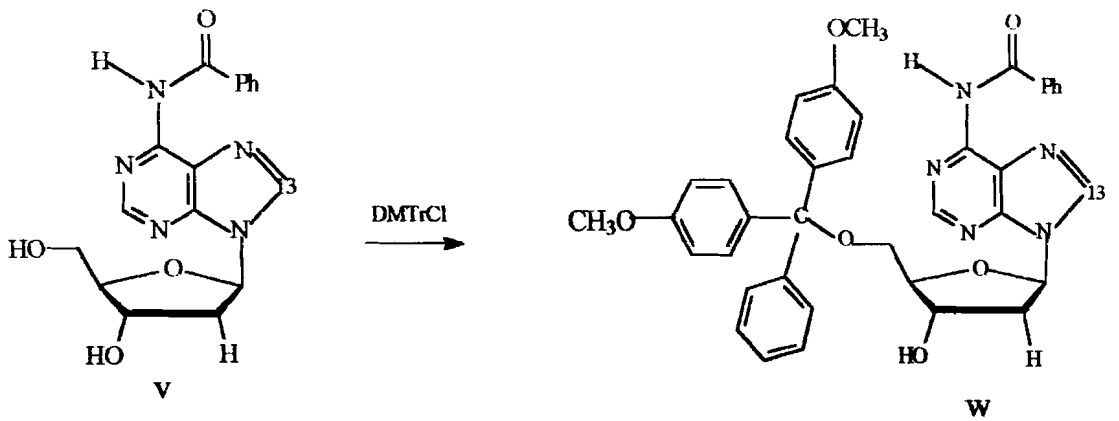
s t u v

Scheme 10.

Deoxy adenosine (s) was reacted with trimethyl chlorosilane producing (t). The product (t) was dissolved in benzoyl chloride to obtain a light, orange, colored solution containind compound (u). The solution was treated with methanolic ammonia for 0.5 hours. The reaction mixture was evaporated to produce a white paste which was dissolved in water and extracted with ethyl acetate. The top layer was evaporated and benzoyl deoxyadenosine

(v) was obtained. The weight of the product was 0.98g. The percent yield was 63.4%.

Synthesis of 5′ -O- (4, 4′ -dimethoxy trityl) [8-^{13} C] deoxy adenosine:

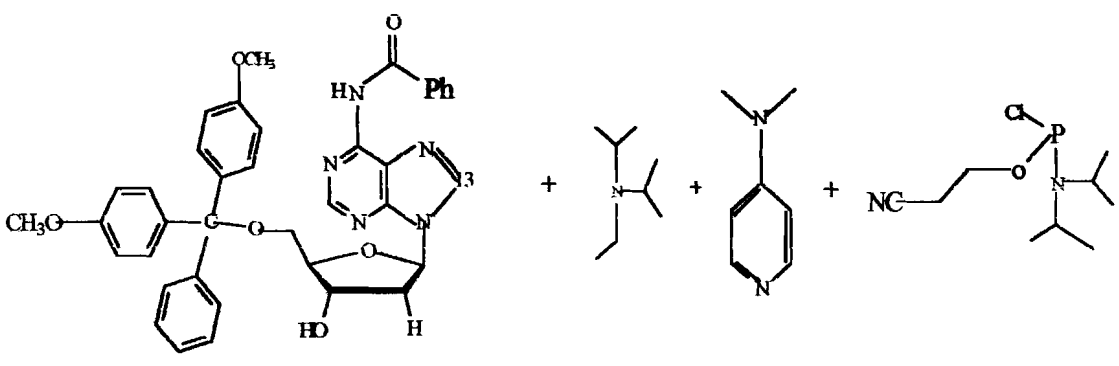


V W

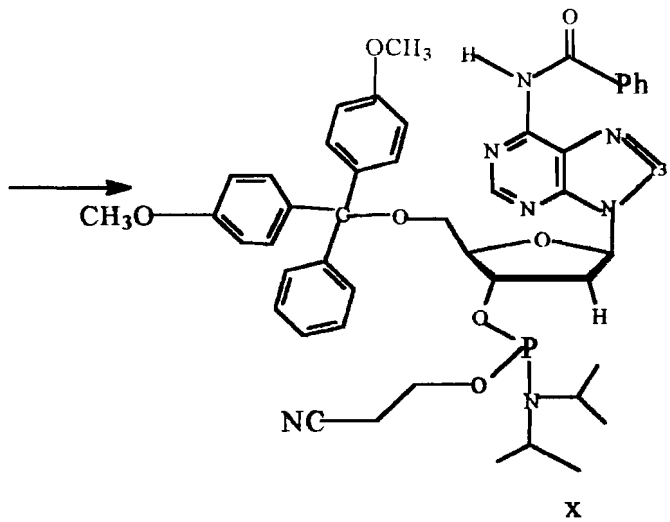
Scheme 11.

Benzoyl deoxy adenosine (v) was reacted with 4, 4 dimethoxytrityl chloride (dMTrCl) producing a light, yellow, colored solution which was stirred overnight. The solution was diluted with dichloromethane and extracted with $NaHCO_3$. The top layer was evaporated under vacuum to produce the product (w). The weight of the product was 1.24g. The percent yield was 52 %.

Synthesis of 5′ -O- (4, 4′ -dimethoxy trityl)-2′ -O- (tert-butyldimethylsilyl) 3′ - (cyanoethyl N, N–diisopropyl phosphoramidite) [8-^{13} C] deoxy-adenosine:



W

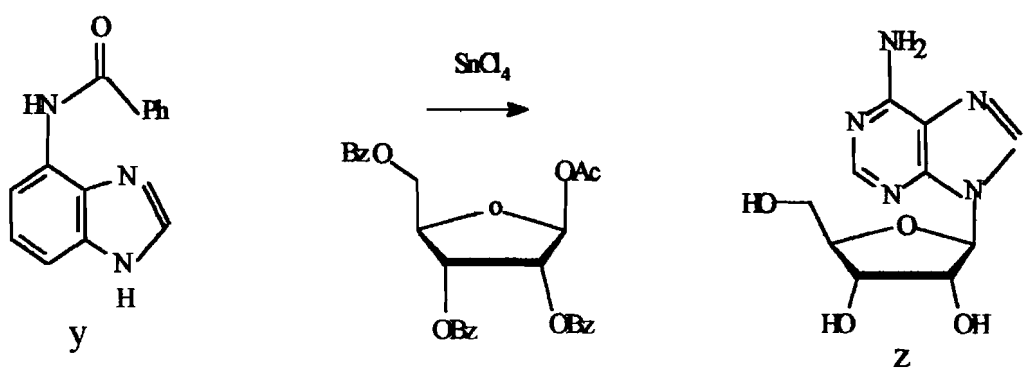


x

Scheme 12.

(W) was reacted with diisopropyl ethylamine, DMAP (4-dimethylamino pyridine) and Cyanoethyl N, N - diisopropyl phosphonamidic chloride producing a colorless solution. The solution was extracted with saturated NaCl solution and the organic layer was evaporated to yield the crude product (x). The weight of the product was 0.42g. The percent yield was 48 %.

Synthesis of adenosine:

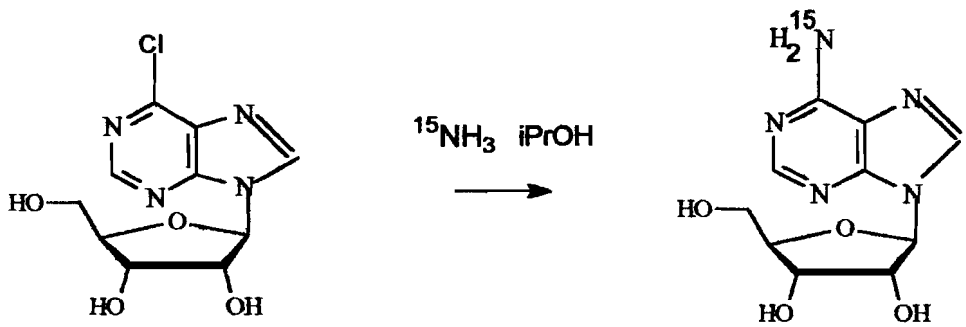


y z

Scheme 13.

Adenine (y) was stirred with $SnCl_4$ and 1- O-acetyl-2, 3, 5-tri- O-benzoyl- β-D-ribofuranose (ABR) overnight. Thin layer chromatography in chloroform-methanol, 9:1 showed three spots after 30 min, corresponding to adenine, benzoylated adenosine and ABR. TLC spots corresponding to sugar and adenine faded on stirring the reaction mixture overnight. The solution was evaporated under vacuum and a pink colored paste was deposited on the sides of the flask. The resulting product was treated with $NaHCO_3$ and extracted with hot chloroform. The solution was loaded on the silica column and five fractions were collected. Thin layer chromatography confirmed the presence of the product in fifth fraction. The fraction was evaporated down and heated with methanolic ammonia for 10 hours to yield (z). Thin layer chromatography was performed on the compound (z) and adenosine sample provided by Nebraska medical institute which confirmed the formation of adenosine.

Synthesis of [6- ^{15}N] adenosine:



z1 z2

Scheme 14.

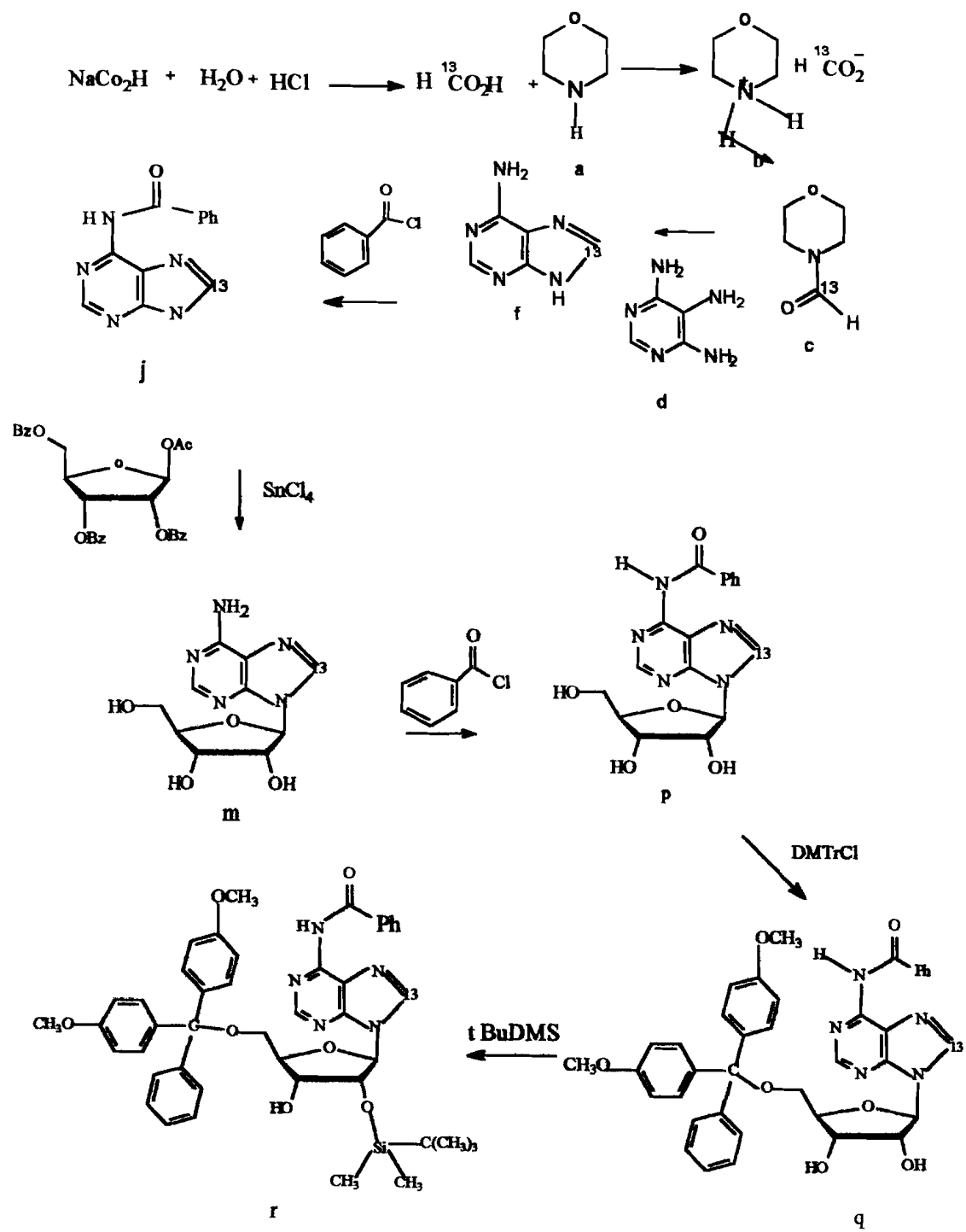
6-chloropurine riboside (z1) was added to 2-propanol (iPrOH) and ^{15}N ammonia gas was passed through the reaction mixture. The solution was heated for five days resulting in

a cloudy, white colored solution containing compound (z2). Thin layer chromatography of the resulting solution and the starting material (6-chloropurine riboside) in hexane: ethyl acetate showed that both the spots moved the same distance which confirmed that the reaction did not occur.

DISCUSSION

In the first part of the project, derivatives of adenosine and deoxyadenosine were prepared with 4,4-dimethoxytrityl, tertiary butyl dimethylsilyl and cyanoethyl N, N-diisopropyl phosphoramidite, respectively as protecting groups, on 5′, 2′, 3′ positions of the riboside with ^{13}C incorporated at 8-position of adenine to give 5′-O-(4,4-dimethoxytrityl)-2′-O-(tertbutyldimethylsilyl)-3′-(cyanoethyl N, N-diisopropyl phosphoramidite) 5-adenosine and 4,4-dimethoxytrityl, cyanoethyl N, N-diisopropyl phosphoramidite, respectively as protecting groups, on 5′, 3′ positions of deoxyriboside to give 5′-O-(4,4-dimethoxytrityl)-3′-(cyanoethyl N, N-diisopropyl phosphoramidite)-5-deoxy adenosine. The protecting groups mentioned above are used because of their utility in automated synthesis. These protecting groups were used previously to prepare the analogous 5-fluorouridine compound (Srinivasan *et al.*, 1995), which has long been thought to be effective against lung and ovarian cancer cells. These derivatives of adenosine and deoxyadenosine will be incorporated into DNA and RNA fragments, and oligomers will be prepared which will be studied to monitor the effect of fluorine substitution on base pairing by Nuclear Magnetic Resonance spectroscopy. In the second part of the experiment, an attempt was made to prepare ^{15}N enriched adenosine, which will also be studied for the probing of local interaction in polynucleotides by multidimensional Nuclear Magnetic Resonance (NMR) spectroscopy.

The reaction scheme for the preparation of 5′-O-(4,4-dimethoxytrityl)-2′-O-(tertbutyldimethylsilyl)--3′-(cyanoethyl N, N-diisopropyl phosphoramidite) 5-adenosine is shown in Scheme 15.


NaCo2H + H2O + HCl
H 13CO2H
H 13CO2−
a
b
c
d
f
j
m
p
q
r
SnCl4
DMTrCl
t BuDMS

Scheme 15.

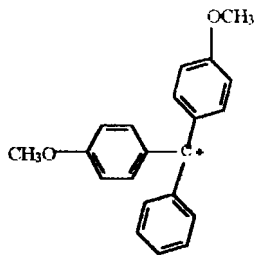
The first step involved the formation of morpholinium ^{13}C formate (b), which was then heated at 95^0C to obtain N-formylmorpholine (c). The latter compound was reacted with (d) to yield the intermediate (e), which undergoes ring closure to yield adenine (f). The product, adenine was characterized by nuclear magnetic resonance spectroscopy (NMR). The data showed three peaks at 9.5, 10 and 10.84 ppm and a broad band at 10.36 ppm which corelates with the structure of adenine. For the next reaction, adenine was dried by dissolving in pyridine, since pyridine and water (present in adenine) form an azeotrope and will evaporate at low temperature, hence water will be removed from the sample. The removal of water from adenine was important because water can react with benzoyl chloride in reaction number 3 and inhibit the progress of the reaction. Adenine (f) was then reacted with $(CH_3)_3SiCl$ to protect hydrogen at N6 of adenine (f) to yield the product (g). The purpose of $(CH_3)_3SiCl$ was to prevent the formation of N, N-dibenzoyl adenine. The product (g) was reacted with benzoyl chloride and treated with aqueous ammonia to give 8-^{13}C benzoyl adenine (j). Ammonia was used to remove trimethyl silyl group. The product (j) was characterized by ^1H NMR spectroscopy which showed three large peaks at 7.5, 7.85 and 8.1 ppm which revealed the formation of benzamide instead of benzoyl adenine. The reason for this could be attributed to the loss of the product in aqueous layer during the extractions with ethyl acetate.

Benzoyl adenine prepared in the above step was used for the preparation of adenosine. 8-^{13}C benzoyl adenine (j) was reacted with trimethyl silyl chloride (TCS) to protect the hydrogen at N6 position of (j) from being pulled away by Lewis acid trimethyl silyl triflate ($CH_3SiSO_3CF_3$). Hexamethyl disilazane (HMDS), was used as an aprotic solvent (with no acidic hydrogen) in the reaction, since protic solvent can protonate Si in

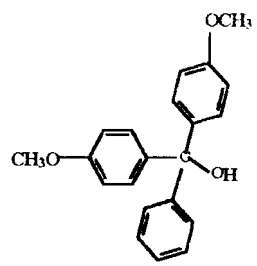
Lewis acid and destroy its efficiency to produce (k). The product (k) was reacted with 1-O-acetyl-2, 3, 5-tri- O-benzoyl-β -D-ribofuranose (l) and trimethyl silyl triflate ($CH_3SiSO_3CF_3$), the catalyst, to yield a yellow solution, which was extracted with sodium bicarbonate to remove the $Si(CH_3)$ group at 6N position of (k) and treated with aqueous ammonia to debenzoylate the hydroxyl group at $2'$, $3'$ and $5'$ yielding the product (m). The side product in the reactions can be $CH_3SiOCOCH_3$. Lewis acids, like $(CH_3)_3SiOSO_2C_4F_9$ and $(CH_3)_3SiClO_4$ can also be used as catalyst for this reaction. These catalysts are highly selective and efficient lewis acids and give higher yields of N-1 nucleosides. The product (m) was characterized by NMR but no peaks confirming the formation of adenosine were observed.

Adenosine was then prepared using $SnCl_4$ as a catalyst. The procedure similar to that of the reaction of adenosine with trimethyl silyltriflate was used. In this case during the extraction of the yellow solution with $NaHCO_3$/ CH_2Cl_2 , emulsions were formed. Several extractions were performed to get clear layers. NMR results did not show the desired product (m). The catalysts mentioned above are superior to $SnCl_4$, as the latter produce unwanted N-3 nucleosides. $SnCl_4$was used as it is easier to work with rather than ($CH_3SiSO_3CF_3$), which reacts violently with the moisture in the air.

Adenosine (m) was then benzoylated according to the similar procedure as adenine, to produce 6-N benzoylated adenine (p). The product (p) was then dissolved in distilled pyridine and reacted with 4, 4-dimethoxytrityl chloride (dMTrCl) to introduce trityl group at $5'$-OH of riboside in (p) to produce (q). Tritylation occur regiospecifically. Thin layer chromatography of (q) showed an orange spot on TLC plate when HCl acid (0.3M)was squirted on the plate. This can be due to carbocation, (a) or trityl alcohol, (b)

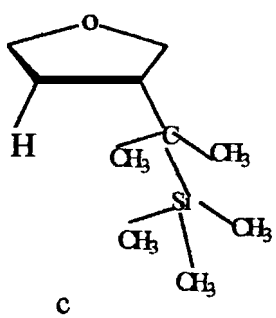


a

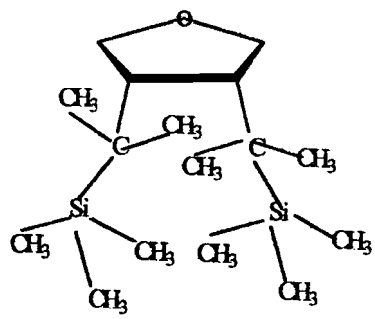


b

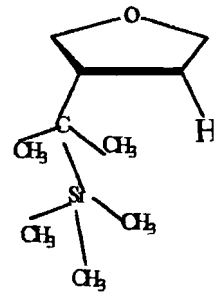
The formation of only one spot signified no chromatography separation. The product (q) was characterized by NMR spectroscopy, showing (b) was formed. The product (q) was then silylated by reacting with imidazole and tertiary butyl dimethyl silyl chloride (tBuDMSCl) to introduce tBuDMS group at 2′-OH position of riboside. Silyation may produce three different products, (c), (d), and (e).



c



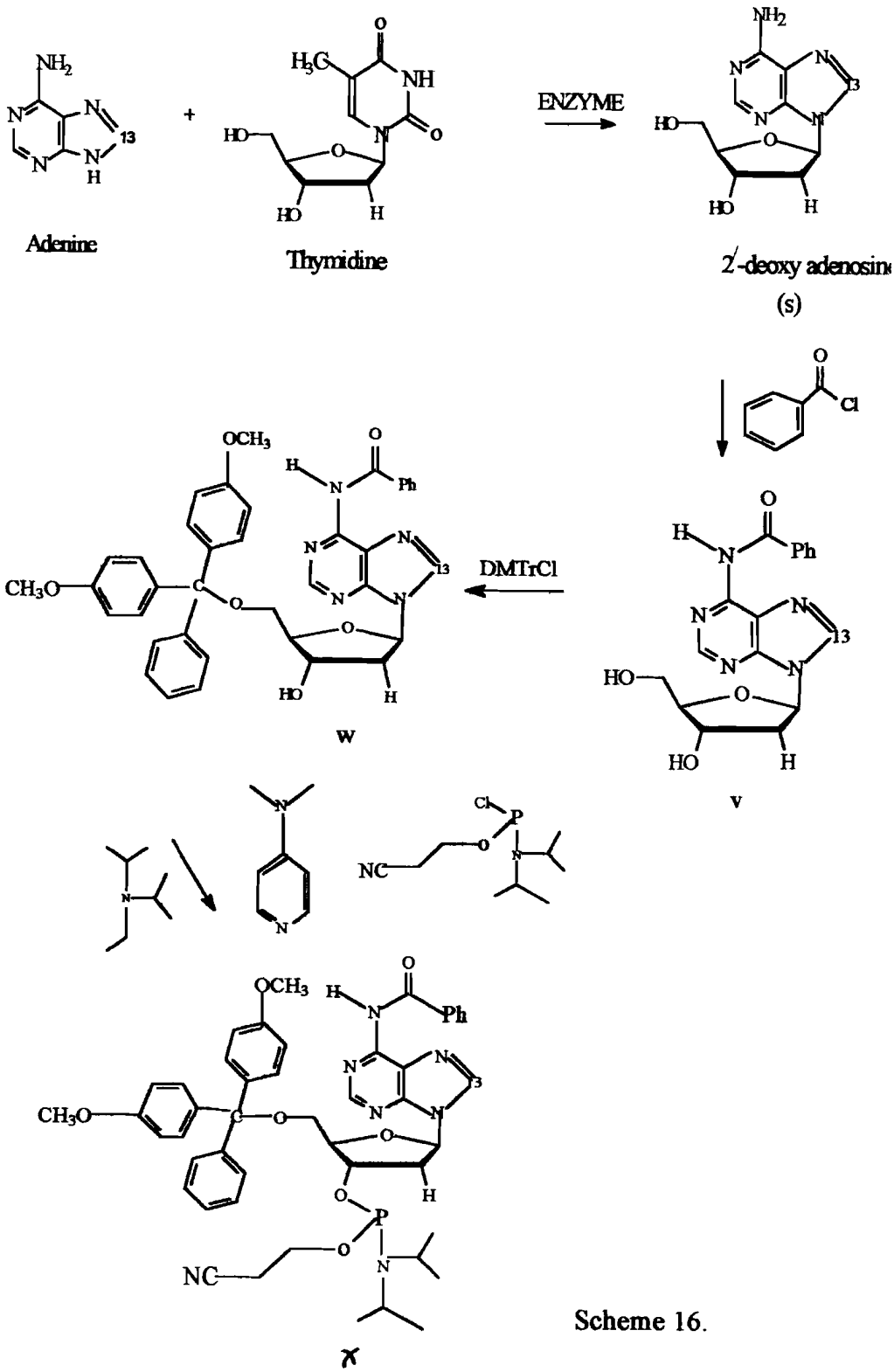
d



e

These products can be separated by chromatographic methods. The main product in this reaction is (a). Thin layer chromatography could not confirm the product. The introduction of cyanoethyl N, N-diisopropyl phosphoramidite at 3′-OH position of the riboside was not performed since the results of the previous step could not be confirmed.

The reaction scheme for the preparation of 5′-O-(4,4-dimethoxytrityl)-3′-(cyanoethyl N, N-diisopropyl phosphoramidite)-5-deoxy adenosine is shown in scheme 16.



Scheme 16.

Labeled adenine (f) prepared in the second reaction was reacted with thymidine and nucleoside phosphorylase and thymidine phosphorylase which were used as catalysts to produce 2′ deoxyadenosine (s). The product (s) was purified by column chromatography. A number of fractions were collected and were spotted on TLC plate. The fractions were visualized by ultraviolet light, and fractions which showed a spots on TLC plate were collected and evaporated down to give the product (s). Benzoylation of 2′ deoxy adenosine was then performed in a manner similar to that of benzoylation of adenosine to give the product (v). The product (v) was then reacted with dMTrCl to introduce trityl group at 5′-OH position of deoxyriboside to produce (w). Thin layer chromatography on the product (w) showed one orange spot which can correspond to carbocation (a) or trityl alcohol, (b). The product was not characterized by NMR spectroscopy. Cyanoethyl N, N-diisopropyl phosphoramidites was introduced at 3′- OH position of deoxyriboside. The product was characterized by nuclear magnetic resonance (NMR) spectroscopy which showed the formation of product (x) but the major product was benzamide. NMR showed that it is ^{13}C enriched. No protecting group was introduced at 2′-OH position since there is only -H present on the 2′ position of deoxyriboside.

The reaction of $SnCl_4$ and 1- O-acetyl-2, 3, 5-tri- O-benzoyl- β-D-ribofuranose (ABR) was then performed with unlabeled adenine without any protecting groups to confirm the formation of adenosine. Thin layer chromatography performed on the reaction mixture after thirty minutes of stirring in chloroform : methanol, (9:1) showed three spots corresponding to adenine, benzoylated adenosine and ABR. TLC was then performed on the reaction mixture after it was stirred overnight and spots corresponding to sugar and adenine faded which showed adenosine being formed.. The reaction mixture was treated

which showed adenosine being formed.. The reaction mixture was treated with ammonia and then loaded on column for purification. Several fractions were collected and spotted on TLC. The fifth fraction showed two spots on TLC plate which may be the mixture of α and β anomers of adenosine. The fraction was evaporated down to give (z). Thin layer chromatography was performed on the compound (z) and adenosine sample provided by Eppley Cancer Institute, which confirmed the formation of adenosine.

^{15}N enriched adenosine was prepared by reacting 6-chloropurine riboside with iPrOH and ^{15}N ammonia in a special break glass joint with a weight attached to it as shown in the figure 1.

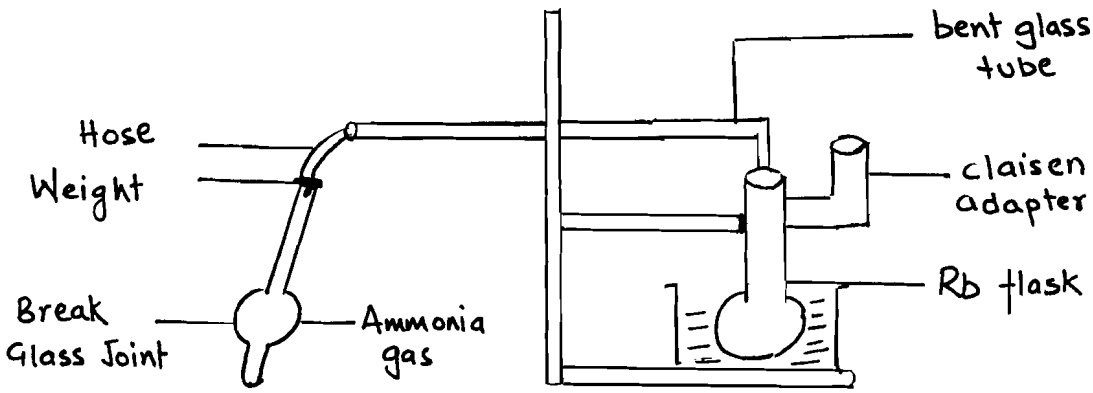


Figure 1. The apparatus for the preparation of ^{15}N adenosine

Ammonia gas was passed through the reaction mixture and heated for 5 days to produce adenosine (z2). After 5 days thin layer chromatography (TLC) of 6-chloropurine riboside and ^{15}N adenosine was performed but both the starting material and the product travelled the same distance on the plate so nothing could be concluded from the TLC. The probable reason may be that the starting nucleoside, i.e 6-chloropurine riboside, was not completely soluble in iso-propanol and ammonia gas may have leaked since the reaction

In this study, modified nucleosides, 5′-O-(4,4-dimethoxy-trityl)-2′-O-(tertbutyl-dimethylsilyl)-3′-(cyanoethyl N, N-diisopropyl phosphoramidite) 5-adenosine and 5′-O-(4,4-dimethoxytrityl)-3′-(cyanoethyl N, N-diisopropyl phosphoramidite)-5-deoxy adenosine are prepared to study the base pairing of adenine with flurouracil in 5-fluoro 2′-deoxyuridine. These modified nucleosides will be incorporated into DNA and RNA fragments (oligomers). The present study requires the preparation of oligomers due to their distinct advantage over the corresponding monomers. The oligomers possess the required polarity to easily enter cells through cell membranes and the possess the cytotoxicity in their own right by interfering with RNA translation. These oligomers will undergo cell culture screening against cancer cells. The main consideration of our research is to observe the bond weakening between base pairs. This would inhibit DNA replication thereby resulting in the death of the cancer cells.

Analogs of 2′-deoxyadenosine have considerable interest in cancer research. The effects of various analogs of 2′-deoxyadenosine have been reported (Huang et al., 1981). It is seen that 2-fluoro and 2-chloro analogs of deoxyadenosine are strongly inhibitory of CCRF-CEM and KB cells in culture. Analogs of 2′-deoxyadenosine are mostly prepared enzymatically. The enzymatic method offers the advantage that α -anomer is not formed and the difficult task of separating α and β anomers is avoided. Hence , they produce better yields than the chemical synthetic methods.

There is an intense interest in the function of the bacterial enzymes which catalyze the transfer of deoxyribosyl group from one purine or pyrimidine to another and produce deoxyribosides (nucleoside phosphorylase and thymidine phosphorylase) used in this study for the reaction

Adenine + thymidine $\rightleftharpoons$ 2′-deoxyadenosine + thymine

It is speculated that these bacterial enzymes may be the components of bacterial nucleic acid, or they serve as intermediates in the synthesis of bacterial nucleic acid.

The main accomplishments of the present research are the preparation of 5′-O-(4,4-dimethoxytrityl)-2′-O-(tertbutyldimethylsilyl)-3′-(cyanoethyl N, N-diisopropyl phosphoramidite) 5-adenosine and 5′-O-(4,4-dimethoxytrityl)-3′-(cyanoethyl N, N-diisopropyl phosphoramidite)-5-deoxy adenosine. The NMR study of the first compound could not confirm its formation. Hence, unlabeled adenine was used to prepare adenosine using $SnCl_4$ with no protecting groups (reaction number 13). The preceding reaction was studied by NMR and showed the presence of adenosine. Future research will emphasize the preparation of labeled compounds with the above mentioned method. The NMR study of the latter compound showed the formation of the desired product, the major product being benzamide.

The results of this study can be improved by starting with commercially available ^{13}C adenine avoiding the time consuming production method, although it is considerably more expensive than sodium formate. The efficiency of the ^{15}N adenosine preparation could be enhanced by using a reaction vessel fitted with a pressure guage and internal thermocouple. Perhaps the reaction could have been improved by esterifying the -OH groups in sugar to render the compound more soluble in 2-propanol.

A greater understanding of the molecular basis for anti cancer drugs that are currently useful clinically may lead to the design and development of more potent and selective inhibitors in the future. The isotopically enriched compounds being studied as a potent drugs for cancer research have made Nuclear magnetic resonance spectroscopy

(NMR) very useful in probing structure and function of these drugs since all the proton signals not corresponding to the labeled signal are suppressed.

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Preparation of ^{13}C And ^{15}N modified derivatives of Adenosine and 2′-deoxyadenosine.

Title of the Thesis Report

Doug Cooper

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December 22, 1999

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