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An approach for clarification of the mechanism of inactivation of ribonucleotide reductases with 3'<^{17}O>-labeled 2'-azido-2'-deoxynucleotides

Saiful Mahmud Chowdhury
Florida International University

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

AN APPROACH FOR CLARIFICATION OF THE MECHANISM OF INACTIVATION OF RIBONUCLEOTIDE REDUCTASES WITH 3'[^{17}O]-LABELED 2'-AZIDO-2'-DEOXYNUCLEOTIDES

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by

Saiful Mahmud Chowdhury

2001

To: Dean Arthur W. Herriott
College of Arts and Sciences

This thesis written by Saiful Mahmud Chowdhury, and entitled An Approach for Clarification of the Mechanism of Inactivation of Ribonucleotide Reductases with 3'[^{17}O]-Labeled 2'-Azido-2'-Deoxynucleotides, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

Dr. Kevin O' Shea

Dr. Leonard Keller

Dr. Stanislaw F. Wnuk, Major Professor

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The thesis of Saiful Mahmud Chowdhury is approved.

Dean Arthur W. Herriott
College of Arts and Sciences

Interim Dean Samuel S. Shapiro
Division of Graduate Studies

Florida International University, 2001

DEDICATION

TO MY PARENTS

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ABSTRACT OF THE THESIS

AN APPROACH FOR CLARIFICATION OF THE MECHANISM OF INACTIVATION OF RIBONUCLEOTIDE REDUCTASES WITH 3'[^{17}O]-LABELED 2'-AZIDO-2'-DEOXYNUCLEOTIDES

by

Saiful Mahmud Chowdhury

Florida International University, 2001

Miami, Florida

Professor Stanislaw F. Wnuk, Major Professor

Inactivation of ribonucleotide reductases by 2'-azido-2'-deoxynucleotides is accompanied by appearance of new EPR signals for a nitrogen-centered radical. The structure of this elusive nitrogen-centered radical has been studied extensively and shown to be derived from azide moiety. Synthesis of 3'[^{17}O]-labeled 2'-azido-2'-deoxyuridine-5'-diphosphate was targeted in this research. Such a labeled analogue should perturb the EPR spectrum in predictable fashion, and the hyperfine interaction between the free electron and the ^{17}O nucleus should allow the choice between the recently proposed structures of this elusive radical (Van Der Donk, W. A. *et al. J. Am. Chem. Soc.* **1995**, *117*, 8908-8916).

The labeled 2'-azido-2'-deoxynucleotides was prepared by thermolysis of O^2,3'-anhydrouridine to give the more stable O^2,2'-anhydrouridine derivatives with concomitant rearrangement of the 2'-*O*-benzoyl[^{17}O] group into the 3'-[^{17}O]-benzoyl

intermediate. Deprotection and ring opening with LiF/Me_3SiN_3 gave the 3'[^{17}O]-2'-azido 2'-deoxyuridine. Tosylation (O5') and displacement of the 5'-tosylate by treatment with tris(tetra-*n*-butylammonium) hydrogen pyrophosphate gave 3'[^{17}O]-2'-azido-2'-deoxyuridine 5'-diphosphate. All the intermediates and the products were characterized by ^{1}H-NMR, ^{13}C-NMR, ^{31}P-NMR and isotopic enhancement was confirmed by using mass spectrometry.

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1. INTRODUCTION

1.1 GENERAL INTRODUCTION

One of the major scientific achievements of the twentieth century has been the identification, at the molecular level, of the chemical interactions that are involved in the transfer of genetic information and the control of protein biosynthesis. The substances involved are biological macromolecules called nucleic acids. The history of the determination of the structure and function of nucleic acids began in 1868 when F. Miesher [1] isolated a material rich in phosphorus from the nuclei of pus cells and from the sperm of a salmon. Miesher later found that this material is a common constituent of yeast, kidney liver, testicular, and nucleated red blood cells. He named this material nuclein. It was later named nucleic acid by Altmann.[2]

The two major types of nucleic acids are named ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). DNA and some RNA are very large polymeric molecules of high molecular weight, which preserve hereditary information that is transcribed and translated in a way that allows the synthesis of all of the various proteins in the cell. The genetic message is transcribed from DNA onto a form of RNA called messenger RNA (mRNA). This messenger RNA acts as a template for protein synthesis.[3,4]

Nucleic acids are long, thread-like polymers made up of a linear array of monomers called nucleotides. Different nucleic acids can have from around 80 nucleotides, as in transfer RNA, to over 10^8 nucleotide pairs as in a single eukaryotic chromosome.[5] The unit size of a nucleic acid is called a base pair (for double-stranded

species) and a base (for single-stranded species). The genomic DNA of a single human cell has 3900 Mbp (million base pairs) and is 990 mm long.

Mild degradation of nucleic acids yields their monomeric units, a mixture of phosphate esters called nucleotides. These are components of both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). RNA is made up of ribonucleotides while the monomers of DNA are called 2'-deoxyribonucleotides. Removal of the phosphate group from nucleotides converts them into compounds known as nucleosides.

All nucleotides are constructed from three components: a nitrogen heterocyclic base, a pentose sugar, and a phosphate residue. The major bases are monocyclic pyrimidines or bicyclic purines. The major bases obtained from DNA are the purines, adenine (A) **1** and guanine (G) **2**, and the pyrimidines cytosine (C) **3**, thymine (T) **4**. RNA bases are mainly adenine, guanine, cytosine, and another pyrimidine base, uracil (U) **5** (**Figure 1**). In nucleosides and nucleotides, the purine or pyrimidine base is joined from the ring nitrogen to carbon-1 of a pentose sugar. The nucleosides, which are found in RNA are named adenosine **6**, guanosine **7**, cytidine **8**, and uridine **10**. In ribonucleic acid (RNA), the pentose is D-ribose which is locked into a five membered furanose ring by a bond from C-1 of the sugar to N-1 of C or U or to N-9 of A or G. In DNA, the pentose is 2-deoxy-D-ribose and the four nucleosides are 2'-deoxyadenosine **11**, 2'-deoxyguanosine **12**, 2'-deoxycytidine **13**, and 2'-deoxythymidine **9**.

The phosphate esters of nucleosides are called nucleotides, and the simplest of them have one of the hydroxyl groups of the pentose esterified by a single phosphate monoester function. Adenosine 5'-phosphate **14** is a 5'-ribonucleotide and is also called adenylic acid (**Figure 2**). Similarly, 2'-deoxycytidine 3'-phosphate **15** is a 3'-deoxy

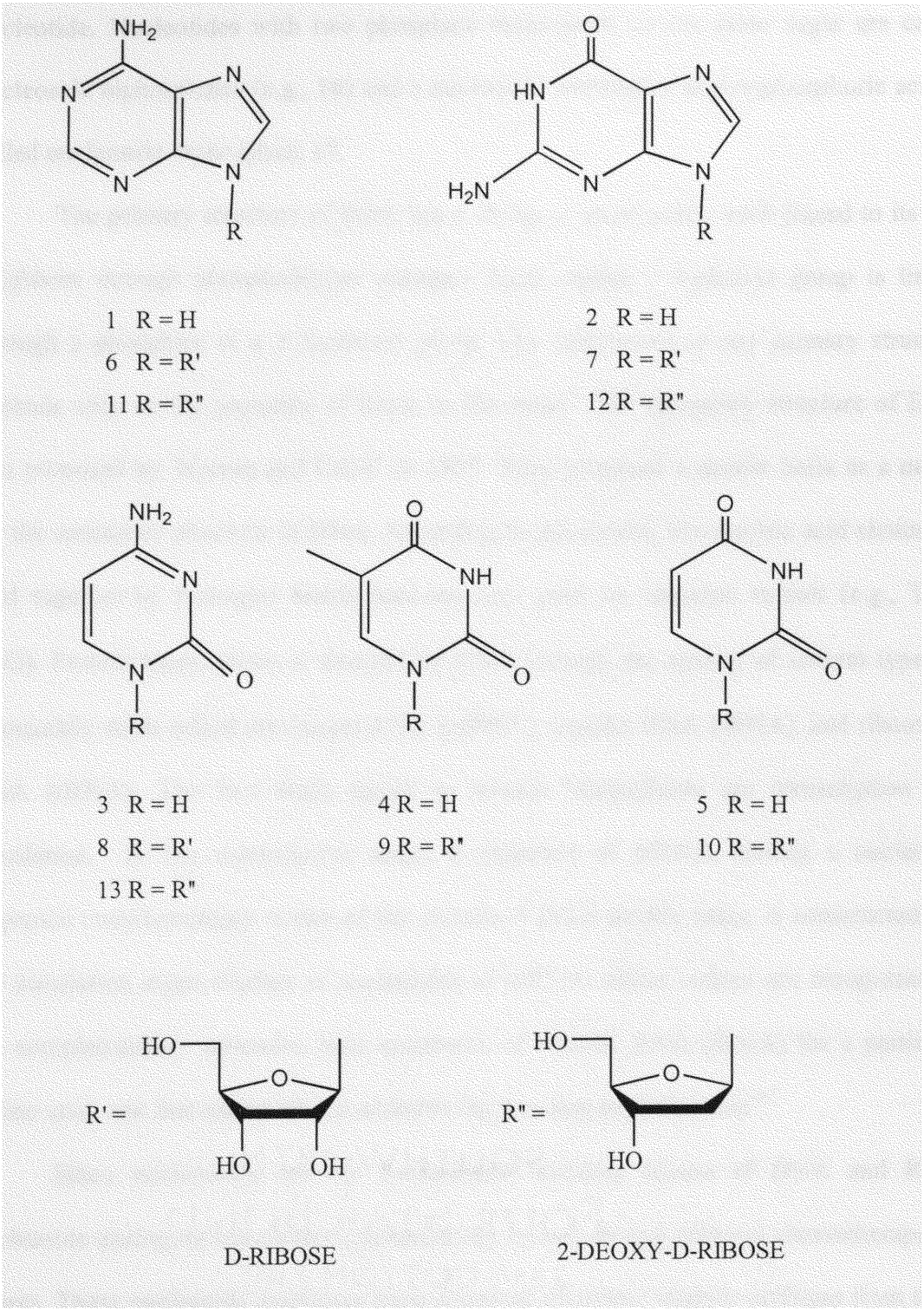


Figure 1: Different types of nucleosides

nucleotide. Nucleotides with two phosphate monoesters on the same sugar are called nucleoside biphosphates (e.g., **16**) and a nucleoside monoester of pyrophosphoric acid is called nucleoside diphosphate **17**.

The primary structure of DNA has a string of nucleosides, each joined to its two neighbors through phosphodiester linkages. Each regular 5'-hydroxyl group is linked through a phosphate to a 3'-hydroxyl group. The uniqueness of any primary structure depends only on the sequence of bases in the chain.[5] The secondary structure of DNA was proposed by Watson and Crick[6] in 1953. They proposed a double helix as a model for the secondary structure of DNA. According to this model, two nucleic acid chains are held together by hydrogen bonds between base pairs on opposite strands (e.g., T=A; C≡G). Protein biosynthesis is directed by DNA through the agency of several types of ribonucleic acids called messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). The two main stages in protein biosynthesis are transcription and translation. In the transcription stage, a molecule of mRNA, having a nucleotide sequence complementary to one of the strands of DNA double helix, is constructed. In the translation stage, triplets of nucleotides of mRNA, called codons are recognized by the complementary anticodon base sequences of transfer RNA (tRNA) for a particular amino acid, and that amino acid is added to the growing peptide chain.[3,7]

Since nucleosides are the fundamental building blocks of DNA and RNA, nucleoside analogues have been synthesized as anticancer and antiviral chemotherapeutic agents. These nucleoside analogues have chemical structures slightly different from those of natural nucleosides. If the "foreign" nucleoside is incorporated into the nucleic acid of a cancer cell, it could interfere with the cell metabolism and be fatal to the cell. Viral

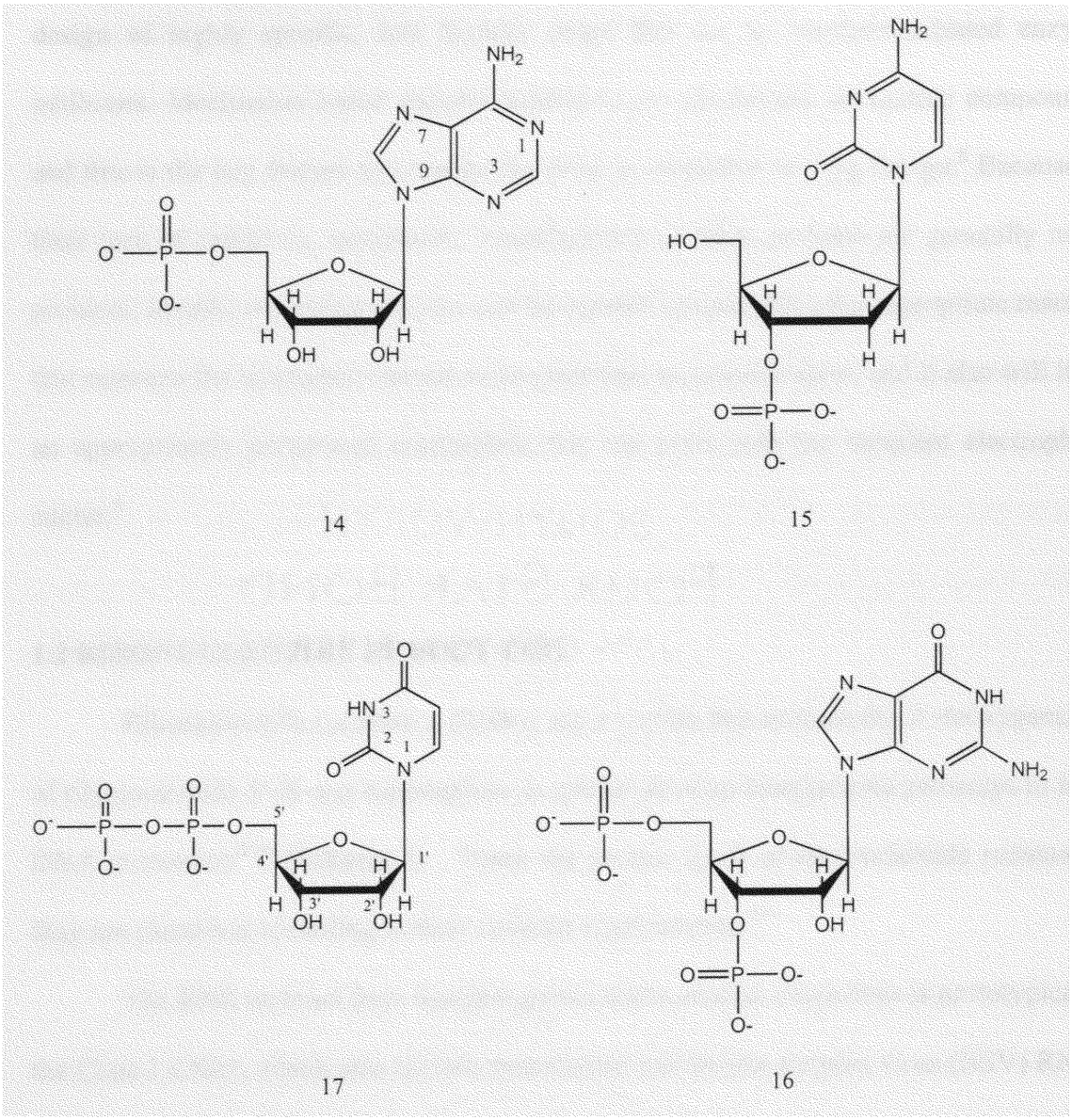


Figure 2: Different types of nucleotides

nucleic acids can be modified in the same way. This antimetabolite approach has stimulated a search for new types of this class of compounds. The second approach is the design of highly specific, low toxicity drugs that act as mechanism-based enzyme inhibitors. Mechanism-based enzyme inhibitors are chemically unreactive compounds, and this is the key feature that makes the drug so amenable to drug design.[8] Because of their lack of reactivity, nonspecific modifications of other proteins are generally not a problem. Ideally, one target enzyme will be capable of catalyzing the appropriate reaction that converts the mechanism-based inactivator into its reactive form, and it also will have an appropriately juxtaposed nucleophile that can react with the incipient electrophilic center.[8]

1.2 RIBONUCLEOTIDE REDUCTASES

Ribonucleotide reductases (RNRs) are enzymes that execute the 2'-deoxygenation of ribonucleotide 5'-di-and triphosphate in unique *de novo* biosynthesis pathways to form DNA monomers[9,10] (**Scheme I**). There are several types of ribonucleotide reductases; they are classified according to their cofactor requirements.[11]

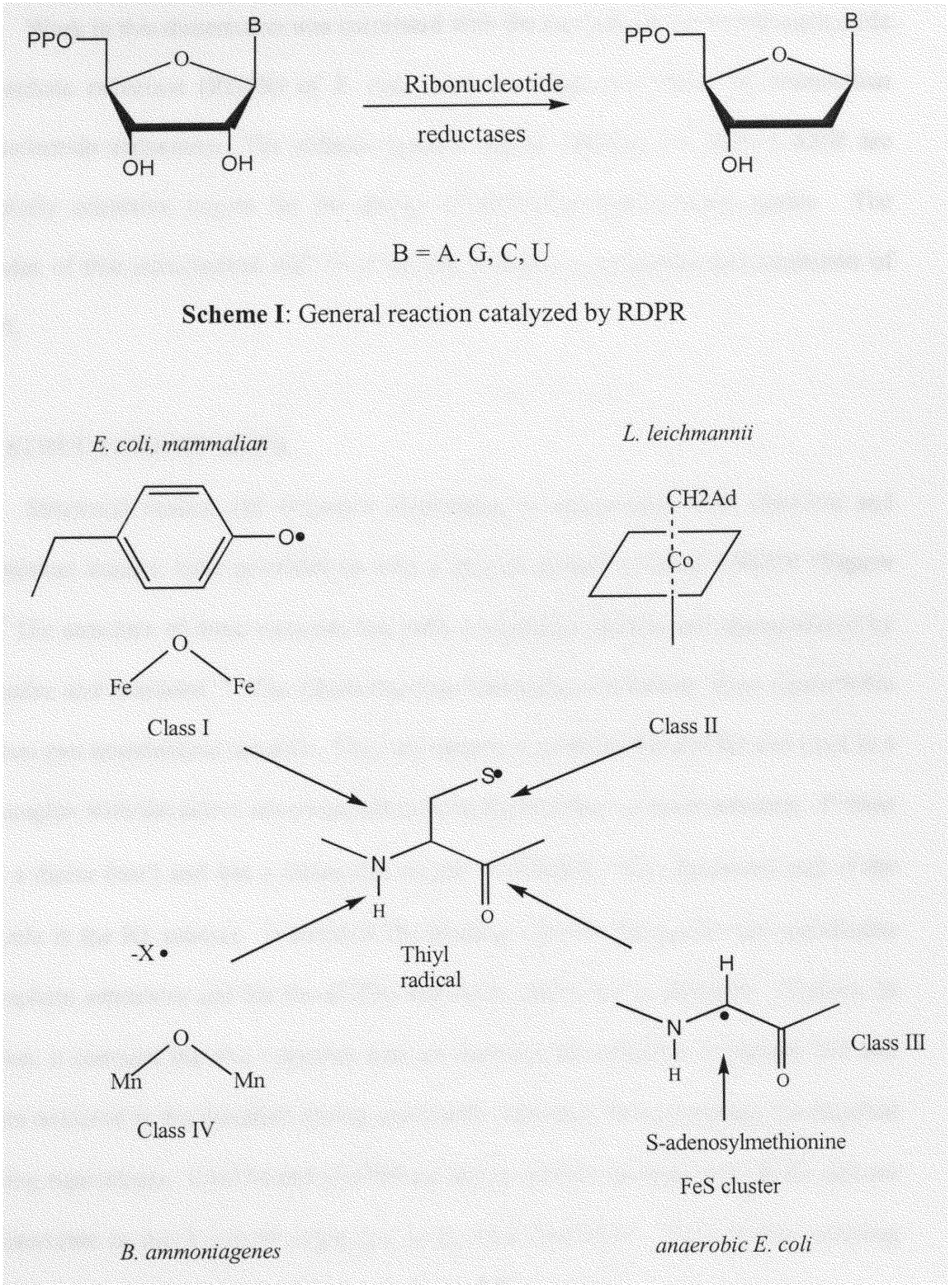
The RNR isolated from bacteria grown under aerobic conditions is prototypical of the Class I RNRs, which also include mammalian and herpes simplex virus (HSV) RNRs. This enzyme uses ribonucleotide diphosphate (RDPR) as the substrate. It is composed of two homodimeric subunits, R1 and R2. The R2 subunit contains a cofactor, which is composed of an unusual μ-oxo-bridged diferric cluster adjacent to a tyrosyl radical (•Tyr 122 in *Escherichia coli*). The tyrosyl radical is essential for catalysis and is generated by the diferrous form of R2 in the presence of molecular oxygen.

The RNR isolated from *Lactobacillus leichmannii* (class II) requires adenosyl cobalamin (Adocbl = coenzyme B12) as a radical generated cofactor. These enzymes use ribonucleotide triphosphate (RTPR) as substrate. RTPR is a single polypeptide (M_r = 76 kda), which catalyzes the conversion of nucleotide triphosphates (NTPs) to 2'-deoxynucleotide triphosphate (dNTPs) with concomitant oxidation of two active sites of thiols to disulfides.

The RNR isolated from *E. coli* grown under anaerobic conditions (class III) is distinct from the enzyme produced under aerobic conditions (class I). When isolated, it is proved to have an essential glycyl radical, which is generated by the second subunit of the enzyme that is equivalent to R2 in the aerobic *E. coli* RNR. The activating enzyme requires *S*-adenosylmethionine and an iron-sulfur cluster to generate the glycyl radical *via* a novel mechanism.

The RNR from *Brevibacterium ammoniagenes* (which is considered as class IV) has been recently characterized. It is thought to possess an $\alpha_2\beta$ subunit structure and a dinuclear Mn^{3+} cluster analogous to the diferric cluster of aerobic *E.coli* RNR. This class of enzymes is poorly understood.

Despite the dramatic differences in these reductases (**Figure 3**),[11] their mechanisms unusual, involving exquisitely controlled radical base chemistry. Each of the metallo-cofactors initiates these radical-dependent nucleotide reduction processes by generating a transient protein radical. The working model is based on the two best characterized RNRs, those from *E. coli* and *L. leichmannii*. The function of both the tyrosyl radical (in *E. coli*) and the adenosylcobalamin radical (in *L. leichmannii*) is to generate thiyl radical.[11,12]



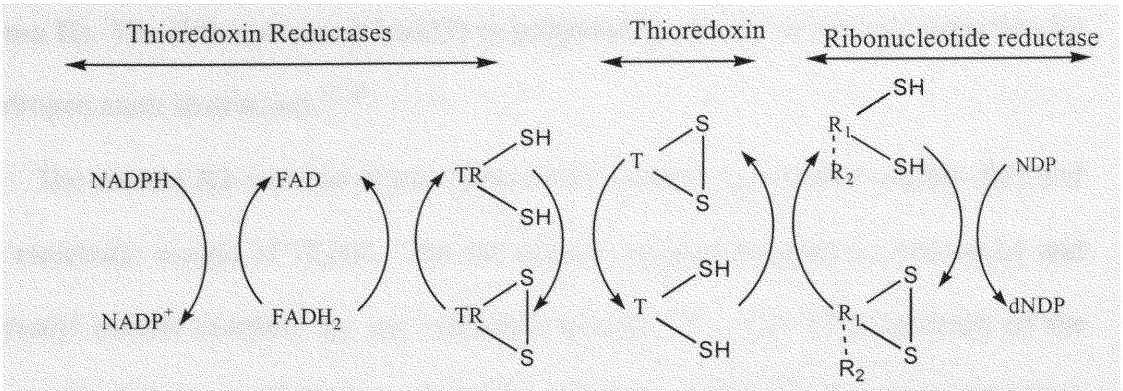
Scheme I: General reaction catalyzed by RDPR

Figure 3: The cofactors used by four major classes of RNRs

Work in this dissertation was correlated with the mechanism of the ribonucleoside diphosphate reductase (RDPR) of *E. coli* since it closely resembles the mammalian ribonucleotide reductases. The diferric–tyrosyl radical required by class I RNR are potentially attractive targets for the design of antitumor and antiviral agents. The reminder of this introduction will focus on the structure, mechanism and inhibition of RDPR.

1.2.1 STRUCTURE OF RDPR

Structural studies and sequence alignments, in conjunction with chemical and biochemical studies, have provided us with a generic picture of class I RDPR (**Figure 4**).[9-13] The structure of these enzymes has been extensively studied and characterized by Thelander and Reichard.[14] The ribonucleoside diphosphate reductase from *Escherichia coli* has two nonidentical subunits. They are known as proteins R1 and R2 and exist in a 1:1 complex with the active site proposed to be at the interface of these subunits. Protein R1 is a dimer ($\alpha\alpha'$) and has a molecular weight of 160,000. The functional end of the molecule is the R1 subunit. It contains the binding sites for the purine and pyridimine diphosphate substrates and for the dNTPs and ATP, which act as allosteric effectors. In addition, it contains the five cysteines that are essential for catalysis. Cysteines 225 and 462 are oxidized to the disulfide during nucleotide reduction, thus providing the required reducing equivalents. Cys754 and Cys759 are at the carboxy-terminal trail of R1 and are not detectable in the X-ray structure due to thermal flexibility. They shuttle reducing equivalents into and out of the active sites *via* disulfide interchange with thioredoxin



Scheme II: RDPR reduction systems

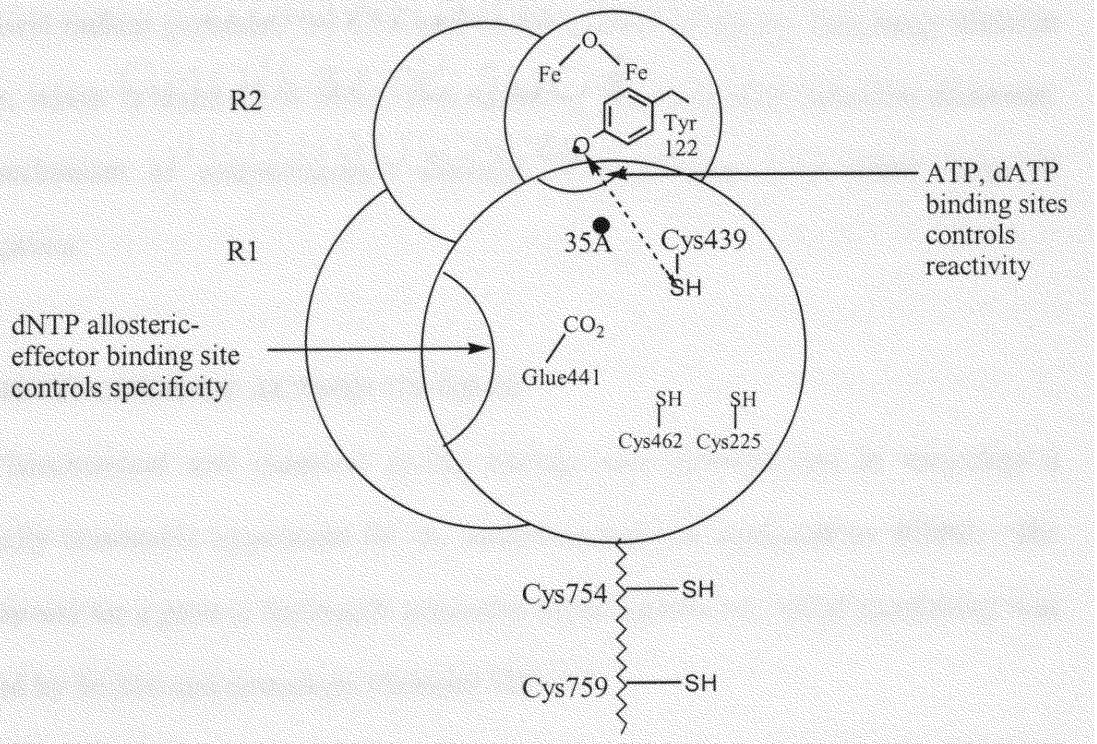


Figure 4: Generic picture of Class I RNR

Scheme II). The fifth cysteine (Cys439) is proposed to initiate nucleotide reduction by 3'-hydrogen atom abstraction.[11,15]

The protein R2 consists of two apparently-identical polypeptide chains (β2) and has a molecular weight of 78,000. The R2 subunit contains the diferric iron center and the tyrosyl radical essential for the reduction process.[16,17] The EPR spectrum of the tyrosyl radical shows a doublet (g = 2.0047), with super imposed hyperfine interaction. The UV-VIS spectrum possesses a sharp absorption at 410 nm (ε = 3250 $M^{-1}cm^{-1}$). This cofactor is buried in R2, lying 10Å from the nearest surface. It has been proposed that the tyrosyl radical generates the thiyl radical on Cys439 of R1 by long-range electron transfer, which is some 35 Å away, thus initiating the nucleotide reduction processes. The mechanism of communication between these subunits is a major focus of investigation.

1.2.2 MECHANISM OF ACTION OF RDPR

Biochemical and chemical model studies have allowed one to formulate a chemically reasonable hypothesis for the reduction reaction catalyzed by RDPR. The first proposal for a generic nucleotide reduction which involves a radical mechanism was proposed by Stubbe and coworkers (**Scheme III**).[18,19]

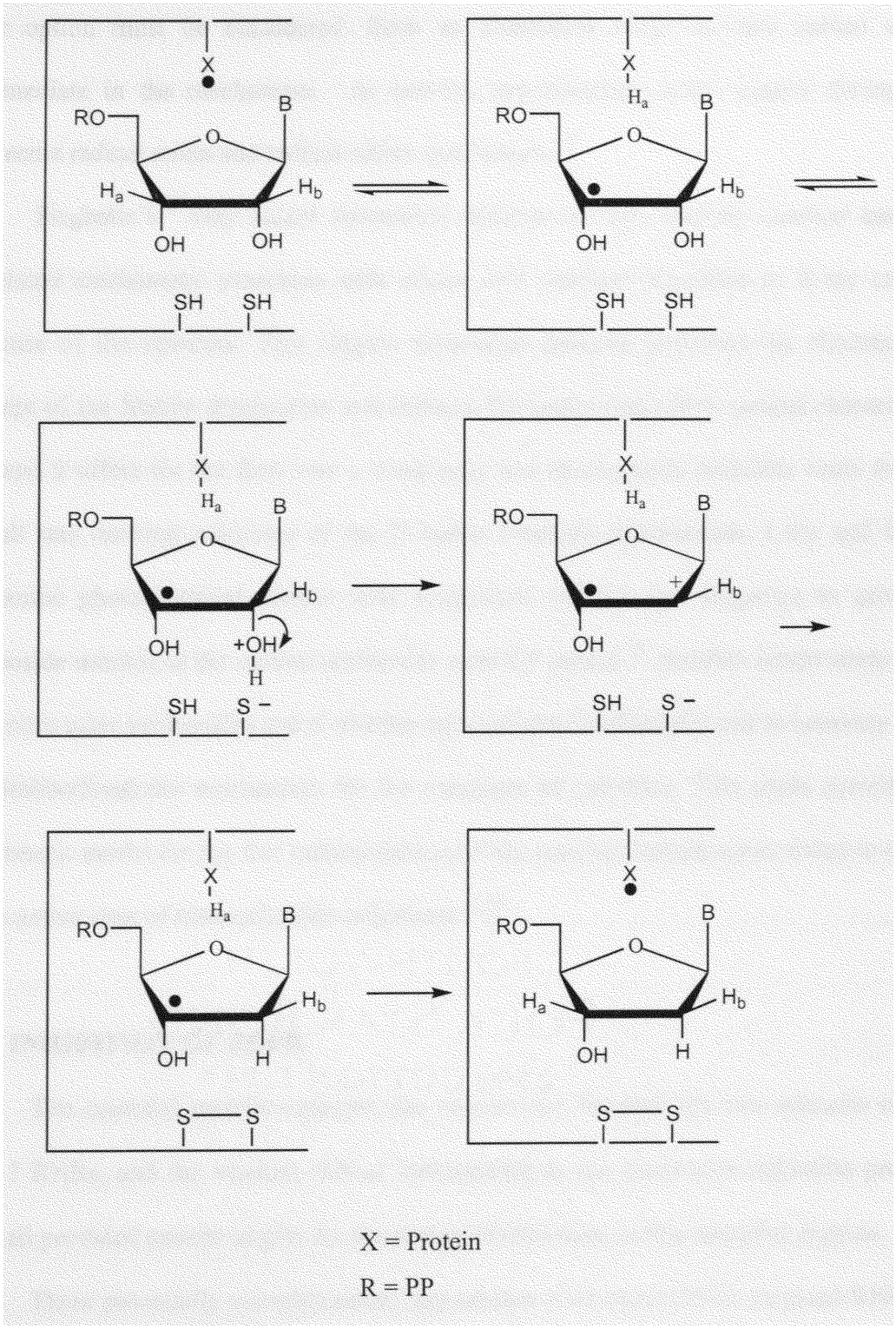
The first steps in this mechanism involve a protein-mediated hydrogen atom abstraction from the 3'-carbon of NDP to produce a 3'-nucleotide radical. In the second step, protonation of 2'-OH can be achieved by proton transfer from the active dithiols in the R1 subunit. This would facilitate the cleavage of the C2'-OH bond and result in formation of a radical cation. After loss of water, the two cysteines on the α face of the

nucleotides (Cys462, Cys225) deliver the required reducing equivalents, generating a 3'-radical 2'-deoxynucleotide intermediate. This intermediate is subsequently reduced to give dNDP and a disulfide with regeneration of the radical initiator for the next turnover of the enzyme.

Two important features of this mechanism are: (1) abstraction of the H3' atom which is necessary for the cleavage of the C2'-OH bond; (2) the hydrogen, which is abstracted from the 3' position in the starting materials, is returned to the 3' position in the products.

These two predictions have been experimentally established. The first prediction is that C3'-H bond of the NDP substrate is cleaved. Use of [3'-^{3}H] NDPs and measurement of isotope effects where N is either pyrimidine or purine and with the presence of differing allosteric effectors have unambiguously established that RDPR is capable of catalyzing cleavage of the C3'-H bonds. Control experiments using [1'-^{3}H] and [2'-^{3}H] NDPs indicate no observable selection effects on RDPR- catalyzed reductions. In addition, reduction of [3'-^{2}H] UDPs by RDPR shows positive isotopic effects in support of C3'-H cleavage. The second predictions, that hydrogen abstracted from the 3' position in the starting materials is returned to the 3' position in the dNDP products, were verified by the use of [3'-H] UDP and by analysis of the end-product by NMR.

The main feature of the proposed mechanism is the involvement of a radical intermediate. Experimental efforts to ascertain its intermediacy utilized stopped- flow techniques as well as the premise that X• (protein radical) is tyrosyl radical 122. Although the mechanism suggests the feasibility of a radical cation intermediate, no direct evidence for this intermediate exists, and model studies indicated that at least one



Scheme III: Postulated RDPR radical mechanisms

other option must be considered. Such an alternative might involve radical anion intermediate in the mechanism. At present, experimental results cannot distinguish between a radical anion and radical cation mechanism.[19]

Siegbahn's[20] very recent theoretical analysis of the substrate reaction cascade correlated mechanistic processes with amino acid residues identified in X-ray crystal structure of the subunits. This elegant theoretical analysis preserves the fundamental concept of the Stubbe mechanism and corrects the postulated cation radical character at C2', and it offers for the first time a chemically and theoretically plausible route for the overall rate limiting reduction of the 2'-deoxy-3'-ketone intermediate. Lenz and Giese performed photochemical studies with selenoester models that fragment to generate nucleoside mimics of the natural nucleoside with C3' radical.[21] Another recent study with 5'-*O*-nitro ester nucleosides and 6'-*O*-nitro ester of homonucleosides was in harmony with the Stubbe/Siegbahn mechanism for the reduction of substrate. This study provided a biomimetic model for the free radical-induced relay reaction cascades postulated to occur at the active sites of ribonucleotide reductases.[22,23]

1.2.3 INHIBITION OF RDPR

The essential metallo-cofactor, the interactions between the two subunits of the class I RNRs, and the unusual radical intermediate in the nucleotide reduction process have all provided proven targets for the design of inhibitors of this essential enzyme.

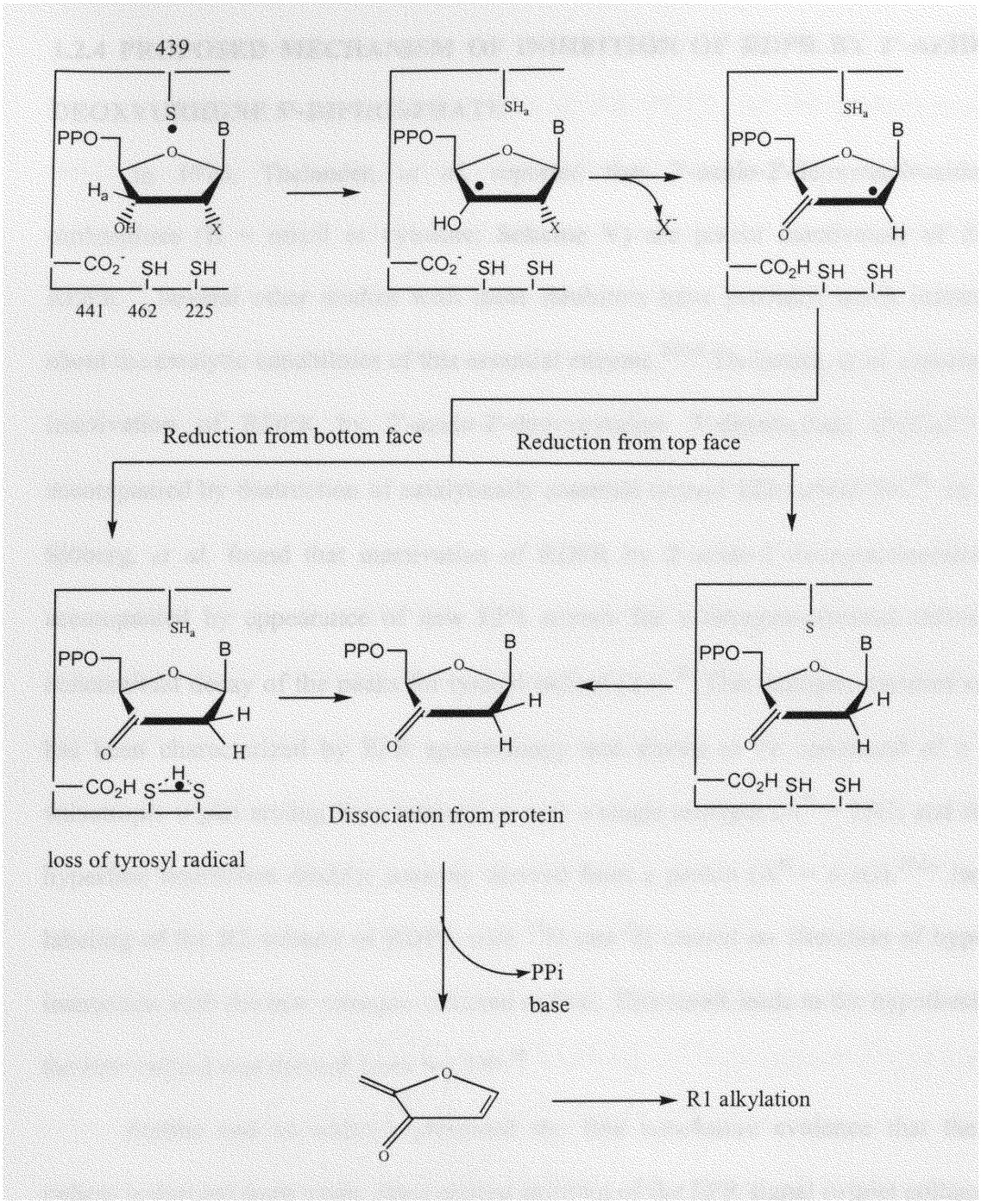
Three potentially complementary approaches have successfully targeted RNRs *in vitro* based on the detailed understanding of the biochemistry of this system and of nucleotide metabolism. One involves nucleotide analogs as mechanism-based inhibitors.

A second involves the reduction of tyrosyl radical required for initiation of nucleotide reduction. The third focuses on the inhibition of the interaction of R1 and R2 subunits, both of which are required for nucleotide reduction.

In 1976, Thelander and co-workers reported that 2'-chloro-2'-deoxynucleotides such as ClCDP (X = Cl, **Scheme IV**) and 2'-azido-2'-deoxunucleotides (X = N_3, **Scheme IV**) such as N_3UDP or N_3CDP are potent inactivators of RDPR.[24] A detailed understanding of the mechanism by which these compounds inactivate the enzyme has evolved since their discovery.[11]

A generalized view of the mechanism responsible for enzyme inactivation by nucleotide analogs is shown in **Scheme IV**.[11] The initial steps, as in the case of NDP reduction, is 3'-hydrogen atom abstraction by a thiyl radical at Cys439. Deprotonation of 3'-OH concomitant with removal of leaving group X results in the formation of a 2'-deoxy-3'-ketonucleotide radical which can then be reduced from the top face (β-face) or the bottom face (α-face) of the inhibitor. Reduction from the bottom face by dithiols results ultimately in the inability to regenerate the thiyl radical on Cys439 and hence in the case of *E. coli* RNR, the tyrosyl radical cannot be reformed. Since tyrosyl radical is essential for catalysis, inactivation of R2 is a major mechanism of RNR inhibition by many nucleotide mechanism based inhibitors.

Reduction from the β-face by Cys439 results also in the formation 3'-keto-2'-deoxynucleotides which dissociate from the active sites and decompose to yield the 2-methylene-3(*2H*)-furanone, which nonspecifically alkylates the R1 subunit of the enzyme, thus inactivating it. The factors that determine whether the reduction occurs from the top face or from the bottom face are not completely understood.



Scheme IV: A generalized view of mechanism of inhibition of RDPR by nucleotide analog

1.2.4 PROPOSED MECHANISM OF INHIBITION OF RDPR BY 2'-AZIDO-2'-DEOXYURIDINE 5'-DIPHOSPHATE

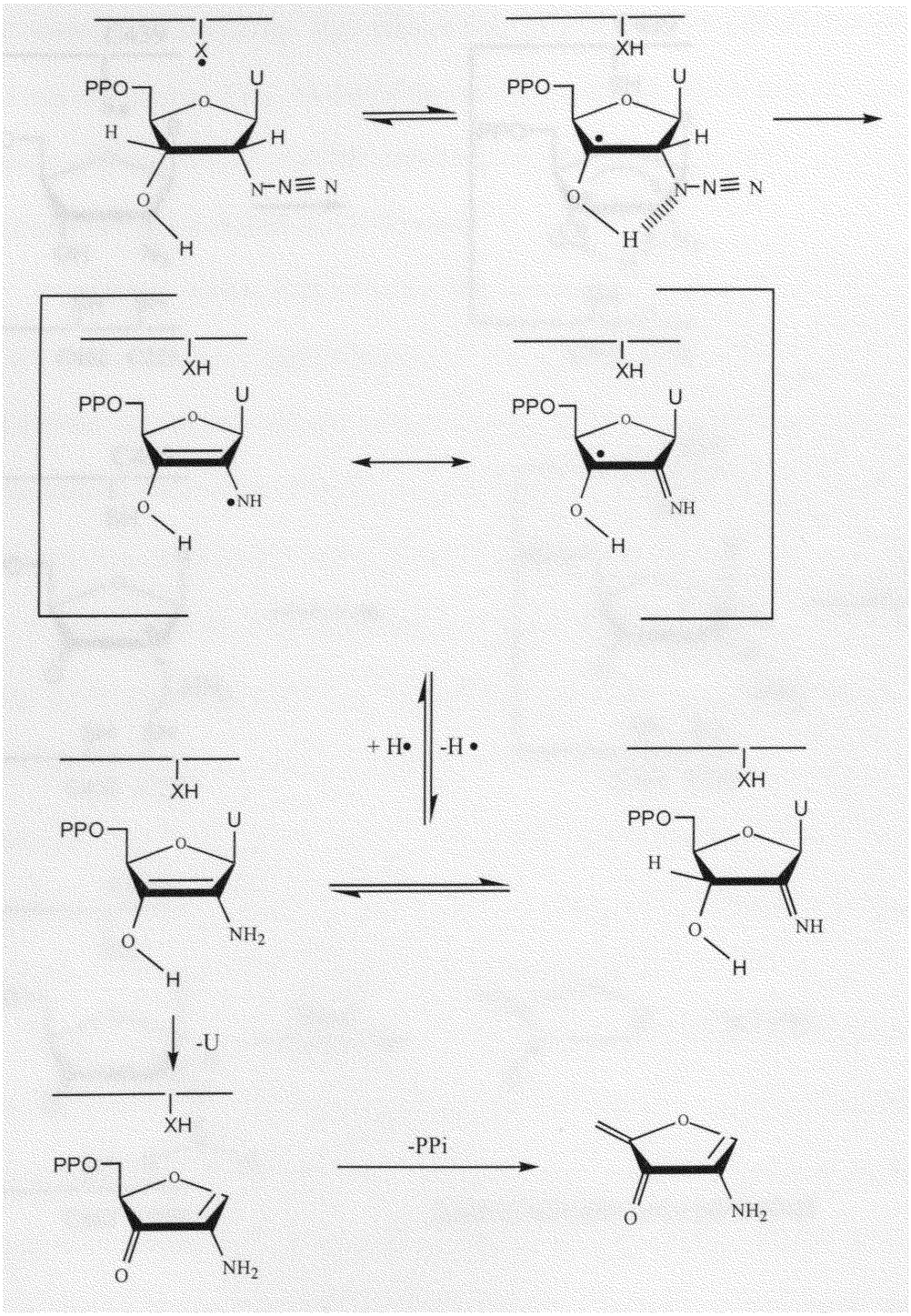
In 1976, Thelander, *et al.* reported that 2'-azido-2'-deoxynucleosides 5'-diphosphate (B = uracil or cytosine; **Scheme V**) are potent inactivators of *E. coli* RDPR.[24] Several other studies with these inhibitors have provided much information about the catalytic capabilities of this essential enzyme.[25-30] Thelander, *et al.* reported that inactivation of RDPR by 2'-azido-2'-deoxycytidine 5'-diphosphate (N_3CDP) was accompanied by destruction of catalytically essential tyrosyl 122 radical Y•.[24] In 1983, Sjöberg, *et al.* found that inactivation of RDPR by 2'-azido-2'-deoxynucleotides was accompanied by appearance of new EPR signals for a nitrogen-centered radical and concomitant decay of the peaks for tyrosyl radical (Y•).[25] This nitrogen-centered radical has been characterized by EPR spectroscopy and shown to be composed of a 1:1:1 anisotropic triplet arising from interaction with a single nitrogen (A^N = 25G) and second hyperfine interaction doublet possibly derived from a proton (A^H = 6.3G).[25,26] Isotopic labeling of the R2 subunit of RDPR with ^{15}N and ^{2}H caused no alteration of hyperfine interaction with the new nitrogen-centered radical. This result leads to the hypothesis that the new radical was derived from N_3CDP.[25]

Stubbe and co-workers presented the first conclusive evidence that the new radical is derived from azide, since altered splitting of the EPR signal (triplet collapsed to a doublet) was observed upon inactivation of RDPR with 2'-[^{15}N] N_3UDP. The observation of a substrate-derived radical provided the first direct evidence for free radical chemistry with RDPR.[26]

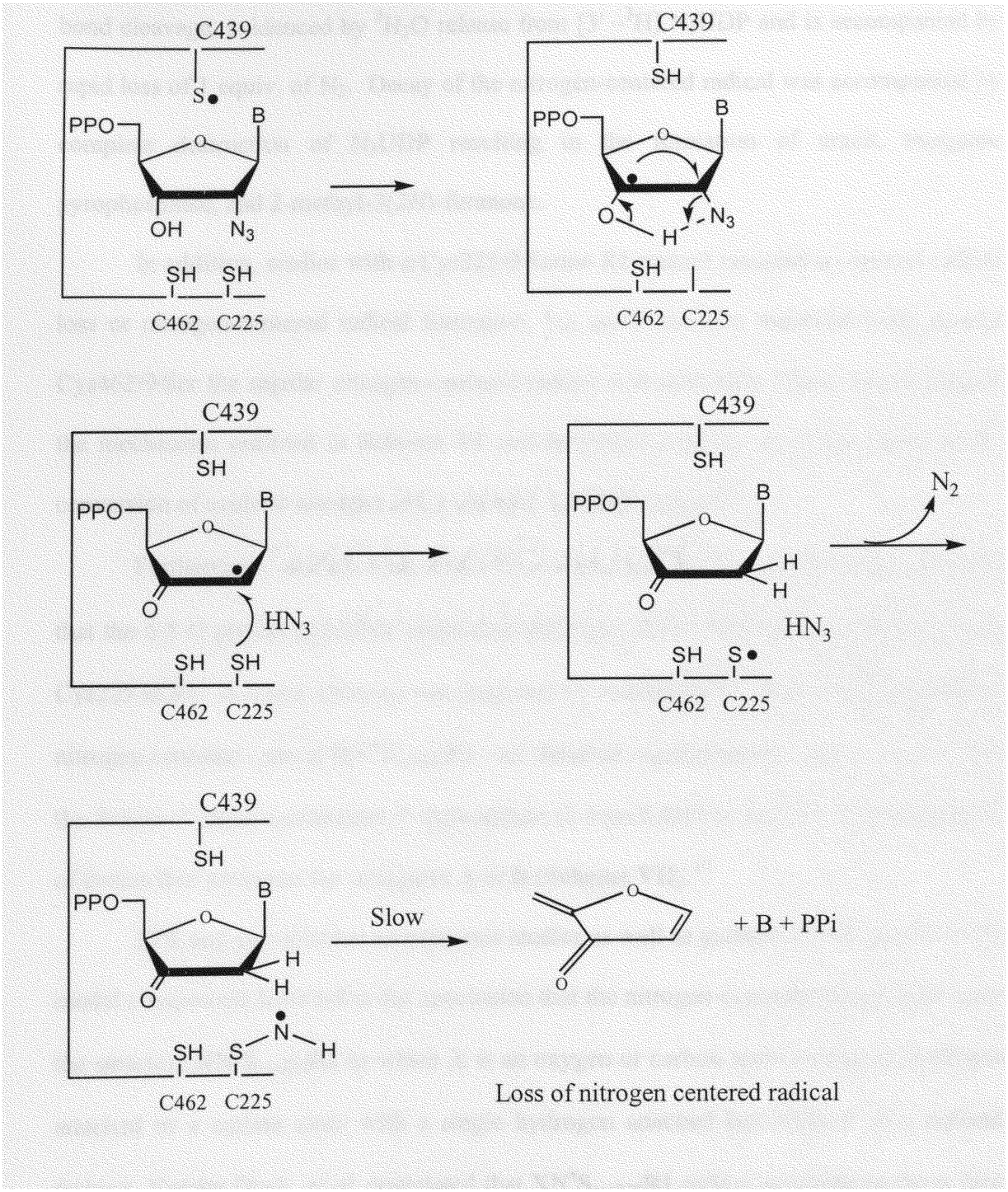
To understand the complex reaction catalyzed by RDPR, specific isotopically labeled N_3UDPs were prepared and incubated with RDPR, and the products and their stoichiometries were ascertained. It was found that one equivalent of N_3UDP inactivated one equivalent of RDPR. This inactivation was accompanied by cleavage of the 3' carbon-hydrogen bond (C3'-H) and production of one equivalent each of uracil, inorganic pyrophosphate, and molecular nitrogen was observed. No N_3^- was detected. Incubation of [5'-^{3}H] N_3UDP with the protein resulted in covalent modification of the R1 subunit with one equivalent of label, accompanied by a change in absorption of the protein at 320 nm.[27]

These results, in conjunction with EPR studies, allowed the formulation of a model (**Scheme V**)[27] proposed by Salowe, *et al.* in 1987. These studies, however, were unsuccessful in resolving some the aspects of the mechanism. The identity of the spin ½ nucleus responsible for the observed 6.5 G doublet hyperfine interaction with the nitrogen-centered radical (N•) has remained a mystery but is crucial to defining the structure of the N•. Deuterium labeling experiments, with [^{2}H]-labeled R2 and with N_3UDP ([^{2}H]- labeled at the 1', 2', 3', 4' positions) have failed to reveal the source of protein hyperfine interaction.[25,26,28] Doubly-labeled 2'[$^{15}N_3$]- azido-2'-[^{13}C]-UDP was prepared and incubated with RDPR.[28] The reaction product was analyzed by EPR and electron spin echo envelope modulation (ESEEM) spectroscopy. It was found that the spectrum of the resulting nitrogen-centered radical has no hyperfine interaction with the C13 nucleus, which requires cleavage of C2'-nitrogen bond.

Based on this experimental data, a new mechanism of inactivation was formulated (Scheme VI).[28] The inactivation process is assumed to be initiated by 3' carbon-hydrogen



Scheme V: Proposed mechanism of inactivation of RDPR by N_3UDP by Salowe, *et al.*[27]



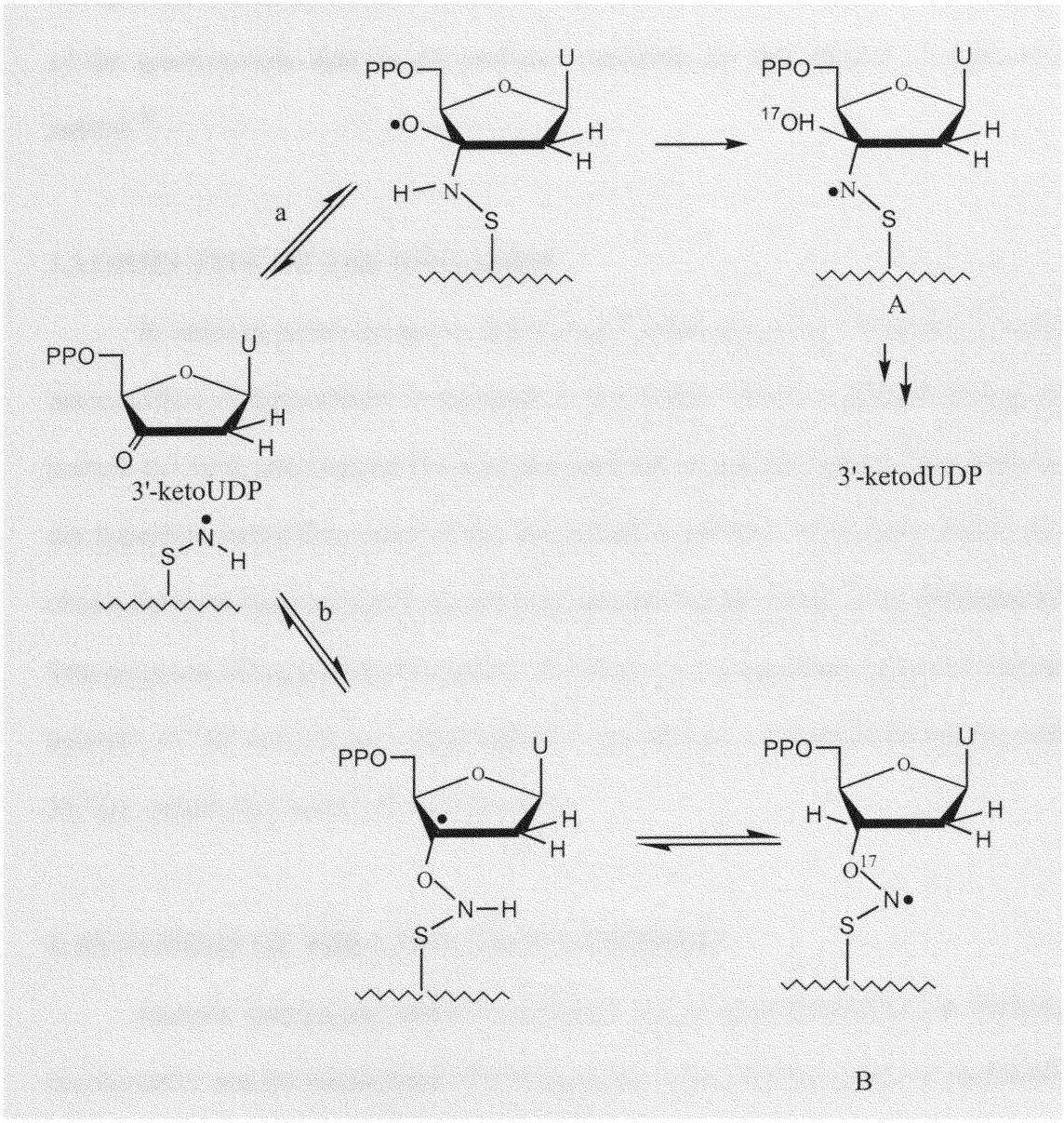
Scheme VI: Mechanism for mode of inactivation of RNR by N_3UDP

bond cleavage, evidenced by ^3H_2O release from [3' $-^3$H] N_3UDP and is accompanied by rapid loss of 1 equiv. of N_2. Decay of the nitrogen-centered radical was accompanied by complete destruction of N_3UDP resulting in the formation of uracil, inorganic pyrophosphate, and 2-methyl-3(*2H*)-furanone.

In addition, studies with a Cys225→Serine R1 mutant revealed no tyrosyl radical loss or nitrogen-centered radical formation, but azide ion was observed (with mutant Cys462→Ser the regular nitrogen-centered radical was detected). These results support the mechanism outlined in **Scheme VI** and implicates Cys225 as a key player in the conversion of azide to nitrogen and a nitrogen-centered radical.[30]

Furthermore, studies with a [β-^{2}H]-cysteine labeled R1 subunit have indicated that the 6.5 G proton hyperfine interaction observed results from a β-hydrogen from of Cys225 in R1. A novel structure was proposed by Vander Donk, *et al.* in which an initial nitrogen-centered radical $\mathbf{HN^{\bullet}S_{cys225}R1}$, not detected experimentally, reacts further with the 3'-keto-2'-deoxynucleotides 5'-diphosphate (3-ketodUDP) to produce a new sequence of events that generates the structures **A** or **B** (**Scheme VII**).[30]

EPR and site-directed mutagenesis studies as well as published EPR results of the model compounds have led to the conclusion that the nitrogen-centered radical must have the structure $\mathbf{XN^{\bullet}S_{cys225}R1}$ in which X is an oxygen or carbon atom having no hydrogen attached or a carbon atom with a single hydrogen attached but oriented in a defined fashion. Vander Donk, *et al.* postulated that $\mathbf{XN^{\bullet}S_{cys225}R1}$ radical is sitting on the α face of the carbonyl group of the 3'-ketoUDP and that it can interact with the carbonyl of this ketone to generate either compound **A** or compound **B** (**Scheme VII**). Molecular modeling studies indicate that the distance between the 2'-proton and nitrogen in structure



Scheme VII: Proposed structures of nitrogen-centered radical by Vander Donk, *et al.*[30]

A must be < 5Å. Electron spin echo envelope modulation (ESEEM) spectroscopy confirmed that structure **A,** while chemically appealing, is not a viable candidate for nitrogen-based radical. Vander Donk, *et al.* proposed that structure **B,** which explains all of the spectroscopic data, is the probable candidate for this elusive nitrogen-centered radical.[30]

1.3 OBJECTIVE OF THE RESEARCH

In order to probe the above hypothesis,[30] synthesis of 3'[^{17}O]-labeled 2'-azido-2'-deoxyuridine 5'-diphosphate is targeted in this study. Such a labeled analog should perturb the EPR spectrum of the nitrogen-centered radical in a predictable fashion, and the hyperfine interaction between the free electron and the ^{17}O nucleus should allow a choice between the recently proposed structures of Vander Donk, *et al.* (**Scheme VII**).[30] The proposed nitrogen-centered radical **B** would have a one bond hyperfine interaction between 3'[^{17}O] and nitrogen while radical **A** would have a two bond interaction with the 3'[^{17}O] , which also has a ^{17}O-^{1}H interaction.

2. SYNTHESIS OF THE LABELED NUCLEOSIDES

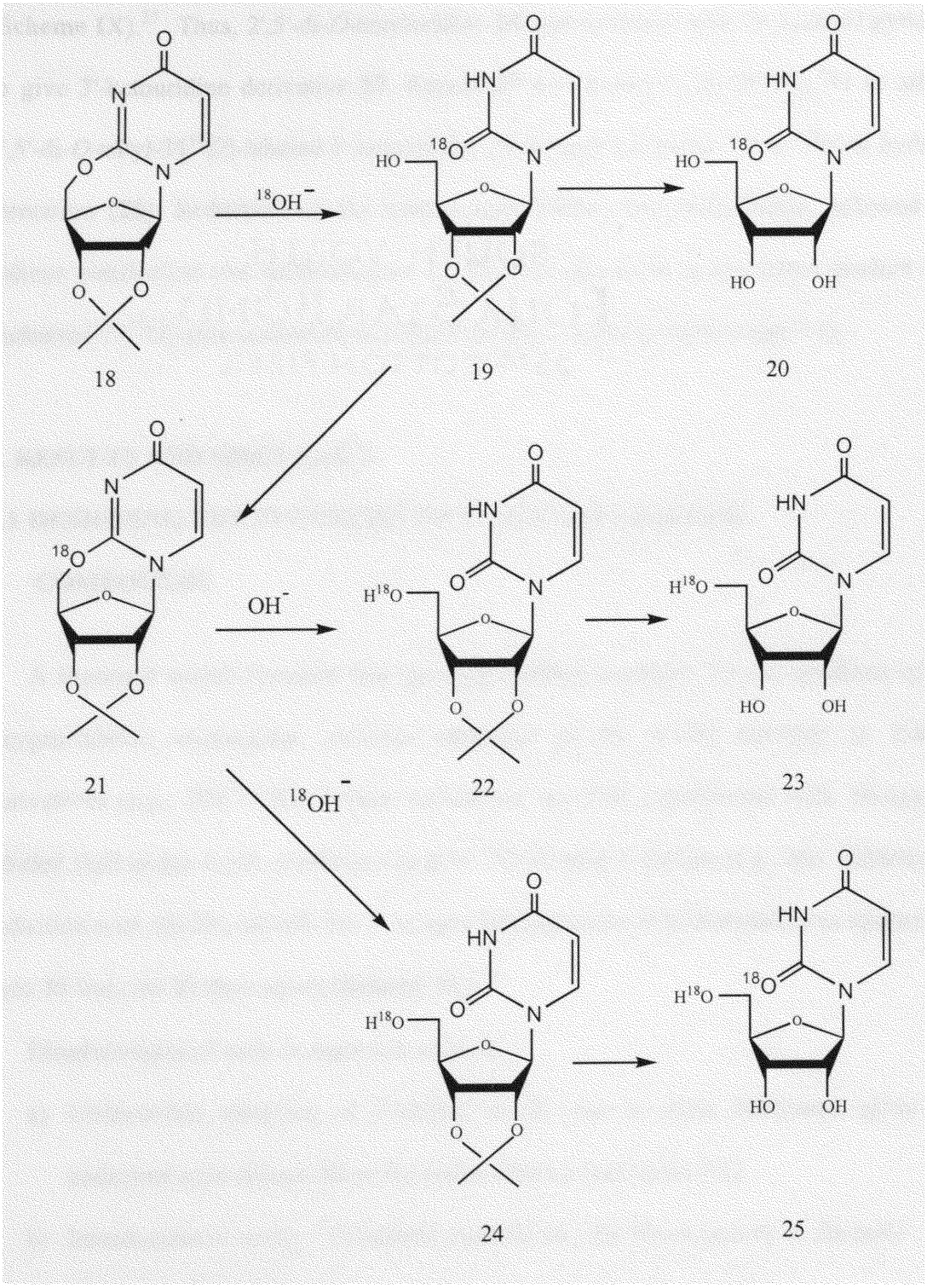
Specific isotopically labeled compounds are of great importance in studying the biochemistry and the metabolism of both naturally-occurring and synthetic nucleosides of clinical importance. A literature survey indicates that there is a lack of a general procedure for the incorporation of stable isotopes in nucleic acid components. Although synthetic methods describing the incorporation of 18-oxygen and deuterium labeled into the heterocyclic portion of nucleosides have been reported, procedures for labeling the

sugar moiety have been limited.[31,32] Anhydronucleosides have been shown to be versatile intermediates for the synthesis and the interconversion of pyrimidine nucleosides. Unfortunately, most ring opening reactions of anhydropyrimidine nucleosides result in the formation of pentofuranosides possessing the arabino or xylo configuration rather then the desired ribo isomer.[33]

The first general approach for the synthesis of specifically-labeled pyrimidine nucleosides was reported by Schram, *et al.*[34] A facile method for the synthesis of highly enriched ^{18}O-labeled pyridimine ribonucleotide was described using uridine as the precursor. The isotopic label was selectively incorporated into O^2 of the uracil moiety and ribo portion of the molecule at the 5'-position. This methodology is outlined in **Scheme VIII**.[34]

Basic hydrolysis of the O^2,5'-anhydro bridge in **18** with Na^{18}OH proceeds by nucleophilic attack specifically at the C2 position of the heterocyclic base to afford **19** which was then deprotected to give **20**.[34] Reformation of the O^2,5'-anhydro bridge by reaction of **19** with triphenylphosphine and diethyl azodicarboxylate (DEAD) provided the key intermediate compound **21**. Treatment of **21** with unlabeled NaOH resulted in cleavage of the anhydro linkage with the ^{18}O-label attached directly to the 5'-position to give **22**. Deprotection of **22** yielded 5'[^{18}O]-uridine **23**. Alternatively, the 2,5'-anhydro bond was cleaved with Na^{18}OH with the resultant incorporation of a second ^{18}O atom (structure **24**) which, after removal of isopropylidene group, afforded the di-O^2,5'[^{18}O]-labeled uridine **25**.

The nonstereoselective synthesis of 3'[^{18}O] uridine was reported by McCloskey, *et al.* using the moderately unstable 3'-keto derivative **27** as the critical intermediate



Scheme VIII: A general method for the synthesis of specifically labeled pyrimidine nucleosides by Schram, *et al.*[34]

(**Scheme IX**).[35] Thus, 2',5'-di-*O*-trityluridine **26** was oxidized with CrO_3/Ac_2O/pyridine to give 3'-ketouridine derivative **27**. Ketone **27** was treated with HCl-$H_2{}^{18}O$ to afford 2',5'-di-*O*-trityl-3'[^{18}O]-labeled 3'-ketouridine **29** through exchange ^{18}O to ^{16}O *via* hydrate formation (**28**). Reduction of the ketone with $NaBH_4$ and detritylation followed by tedious purification and isolation gave 3'[^{18}O]-ribo isomer **31** as the minor product and undesired 3'[^{18}O]-xylo isomer **30** as a major product (major to minor ratio 3:1).

3. RESULTS AND DISCUSSION

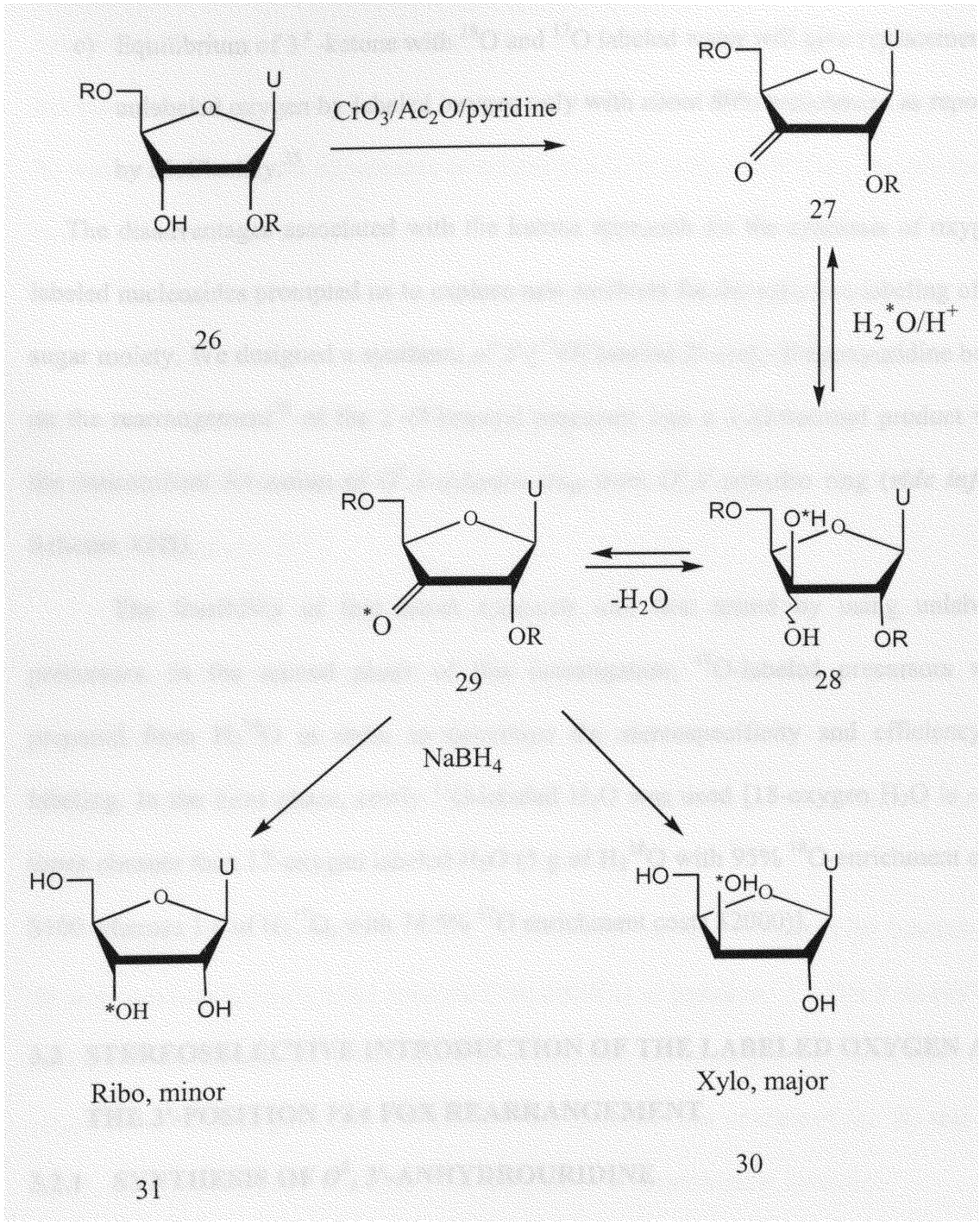
3.1 DESIGNING THE SYNTHESIS OF 3'-OXYGEN LABELED COMPOUNDS

A literature search revealed that the only method available for the synthesis of 3'-oxygen-labeled nucleosides involves oxidation of the 3'-OH function to 3'-keto derivatives (e.g., **27**).[35] The 3'-keto derivatives are then equilibrated with 18-oxygen labeled H_2O under acidic conditions to give ^{18}O-labeled 3'-ketone (e.g., **29**). Subsequent reduction with $NaBH_4$ affords the 3'-oxygen labeled nucleoside derivatives as epimers of xylo **30** and ribo **31** derivatives (**Scheme IX**).[35]

Disadvantages of such an approach include:

a) Unfavorable reduction of 3'-ketone which, due to steric hindrance, gives the undesired xylo epimer **30** as the major product (xylo/ribo 3:1).

b) Introduction of costly ^{17}O-labeled material into the ribose moiety in the early stage of synthesis of the targeted 2'-azido-2'-deoxyuridine inhibitor.



Scheme IX: Synthesis of 3'-oxygen labeled nucleosides by McCloskey, *et al.*[35]

c) Equilibrium of 3'-ketone with ^{18}O and ^{17}O labeled water will give replacement of unlabeled oxygen by labeled oxygen only with about 80% enrichment as reported by McCloskey.[35]

The disadvantages associated with the ketone approach for the synthesis of oxygen-labeled nucleosides prompted us to explore new methods for the selective labeling of the sugar moiety. We designed a synthesis of 3'-[^{17}O] labeled 2'-azido-2'-deoxyuridine based on the rearrangement[36] of the 2'-*O*-benzoyl precursor into a 3'-*O*-benzoyl product with the concomitant formation of O^2,2-anhydro ring from O^2,3'-anhydro ring (***vide infra*** ; **Scheme XIII**).

The feasibility of this novel approach was first tested by using unlabeled precursors. In the second phase of this investigation, ^{18}O-labeled precursors were prepared from $H_2{}^{18}O$ in order to determine the stereospecificity and efficiency of labeling. In the third phase, costly ^{17}O-labeled H_2O was used [18-oxygen H_2O is ~100 times cheaper than 17-oxygen labeled H_2O (5 g of $H_2{}^{18}O$ with 95% ^{18}O-enrichment costs $100 whereas 1 g of $H_2{}^{17}O$, with 74.5% ^{17}O enrichment costs $2000)].

3.2 STEREOSELECTIVE INTRODUCTION OF THE LABELED OXYGEN AT THE 3'-POSITION *VIA* FOX REARRANGEMENT

3.2.1 SYNTHESIS OF O^2, 3'-ANHYDROURIDINE

Treatment of uridine **32** with three equivalents of triphenylmethyl chloride (TrCl) gave 2',5'-di-*O*-trityluridine **33** as the only crystalline product in 45% yield as reported by Fox.[36] This reaction was repeated several times at various scales from (2 g to15 g), and no attempts were made to increase the yield by purification of the mother liquors by

column chromatography. Treatment of **33** with methanesulfonyl chloride (MsCl) and pyridine gave the corresponding 3'-*O*- mesyl derivative **34**. The structure of **34** (**Scheme X**) was confirmed by the appearance of singlet at δ 2.95 in the ^{1}H-NMR spectrum for the methyl protons of the CH_3SO_2 group.

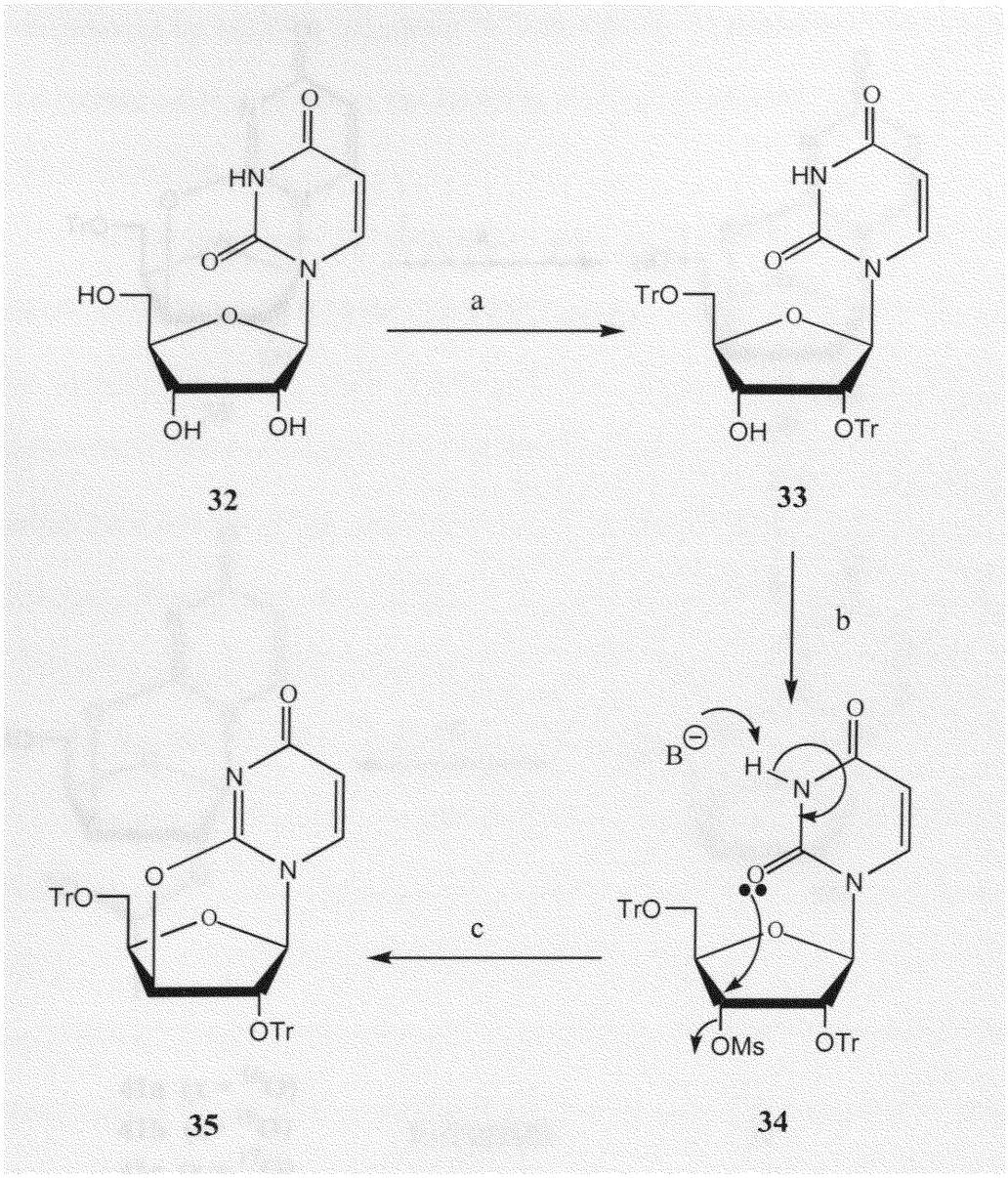
Treatment of **34** with sodium benzoate and *N*,*N*-dimethylformamide (DMF) gave O^2,3'-anhydro-2',5'-di-*O*-trityluridine **35** in 43 % yield. Abstraction of hydrogen from the uracil base by benzoate anion and attack at the basic C2-oxygen at the 3' position in ribose induces the displacement of the mesyl group to give the desired O^2,3'-anhydro-2',5'-di-*O*-trityluridne **35**.

Removal of the trityl protecting group from **35** was achieved with anhydrous HCl followed by treatment of the resulting reaction mixture with ethanol (**Scheme XI**). Crystallization of the crude material from methanol gave fully-deprotected O^2,3'-anhydro-1-(β-D-xylofyranosyl)uracil **36** (85%) as a highly polar compound in various TLC systems.[36]

Regioselective protection of the 5'-OH group was accomplished by treatment of **36** with *tert*-butyldimethylsilyl chloride (TBDMSiCl) in pyridine.[37,38] The 5'-*O*-silylated analog **37** was obtained in 80% yield after purification on silica gel. Crystallization from ethanol/ethyl acetate gave fine needles, and the structure was confirmed by ^{1}H- and ^{13}C-NMR, mass spectrometry and elemental analysis (see Experimental Section).

3.2.2 SYNTHESIS OF OXYGEN LABELED BENZOYL CHLORIDE

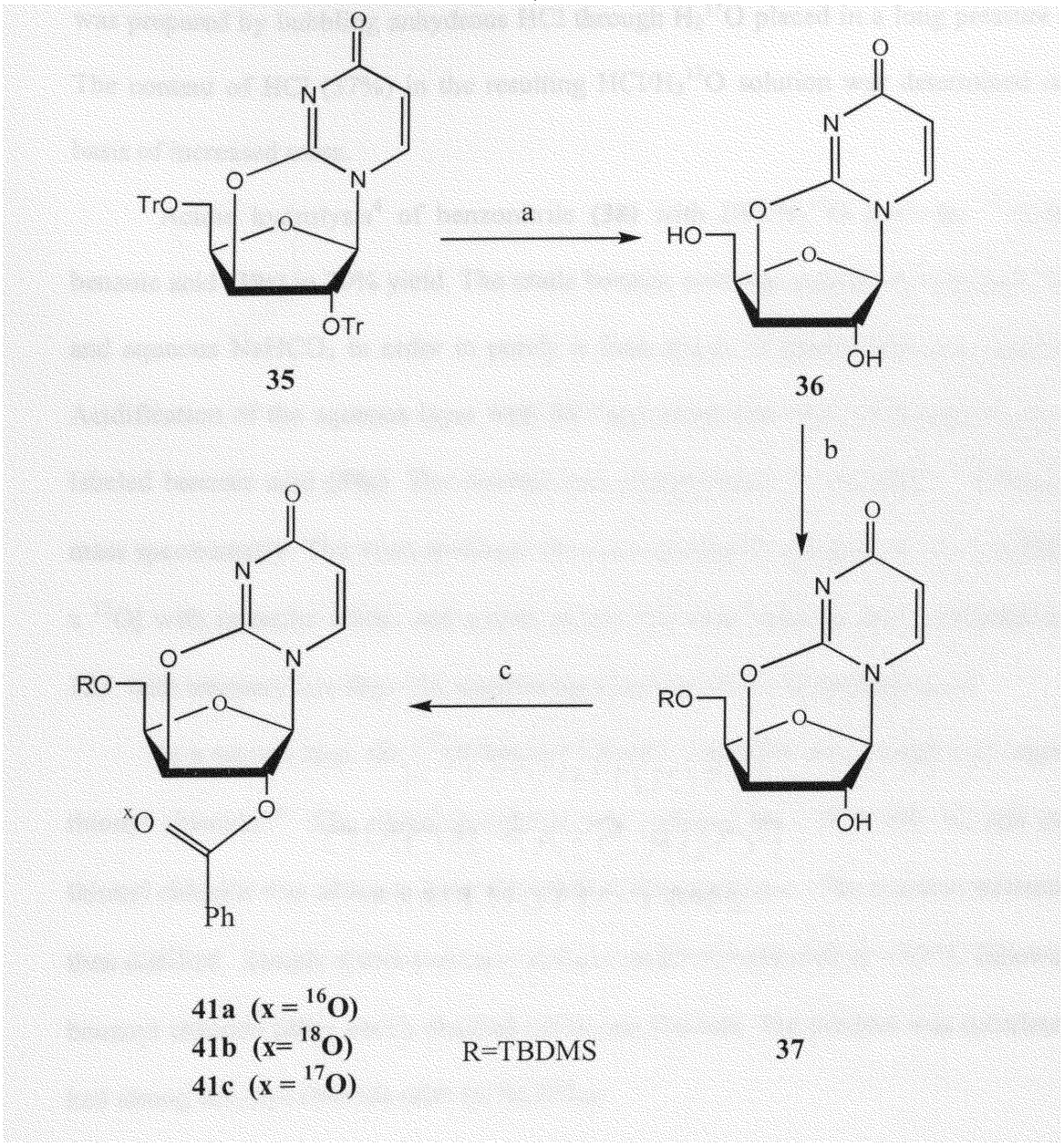
In order to prepare ^{17}O-labeled benzoyl chloride (**Scheme XII**), ^{17}O-labeled water (1g, 74.5% enrichment) was employed. First a solution of concentrated HCl/$H_2{}^{17}O$



(a) Triphenylmethyl chloride/pyridine (b) Methanesulfonyl chloride/pyridine

(c) Sodium benzoate/DMF

Scheme X: Synthesis of O^2,3'-anhydrouridine



35 **36** **37**

41a (x = ^{16}O)
41b (x= ^{18}O)
41c (x = ^{17}O)

(a) Anhydrous HCl / Ethanol / Methanolic ammonia (b) *Tert*-butyldimethylsilyl chloride Imidazole/ DMF (c) Benzoyl chloride (^{17}O & ^{18}O)

Scheme XI: Synthesis of 2'-*O*-benzoyl compounds

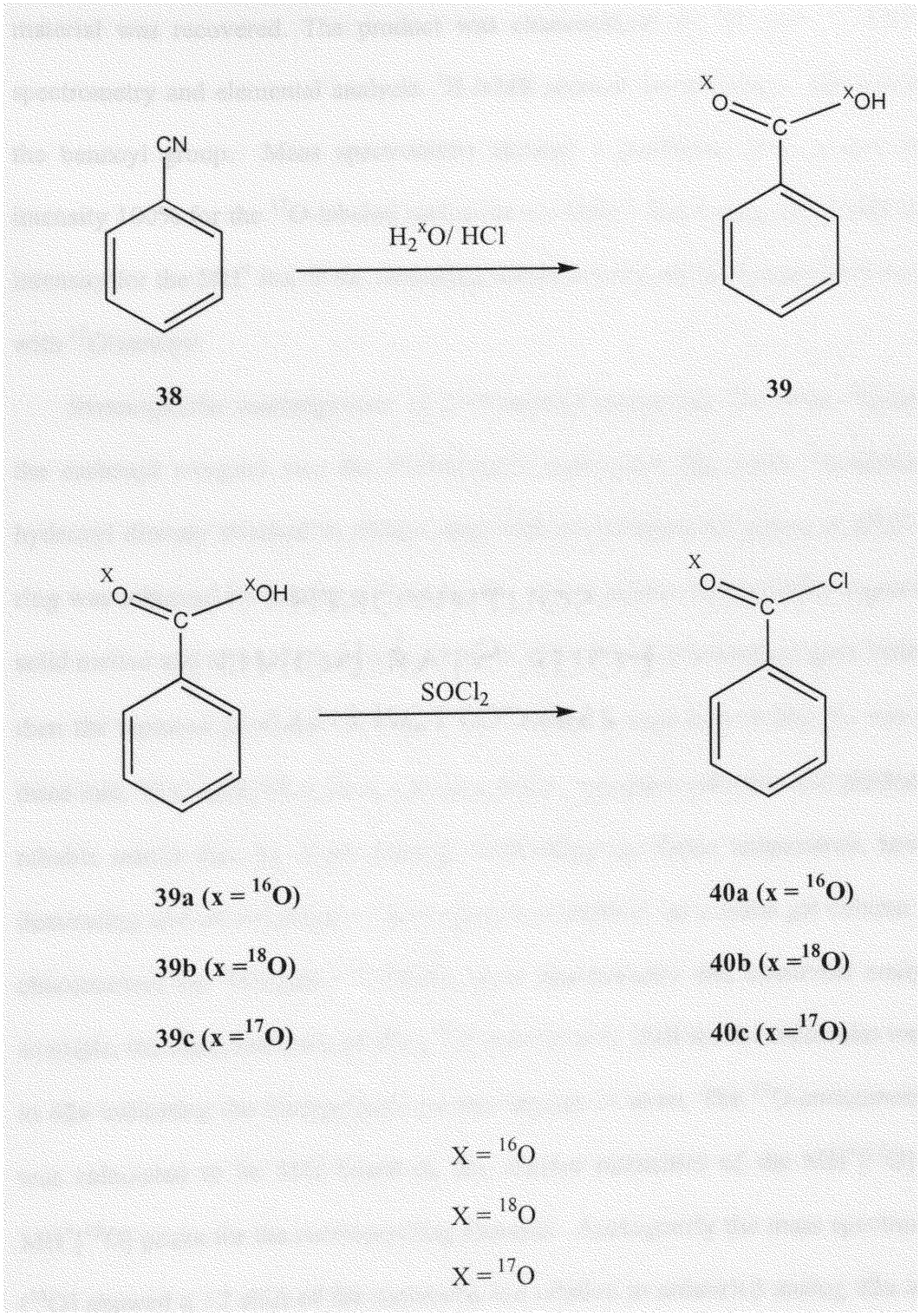
was prepared by bubbling anhydrous HCl through $H_2{}^{17}O$ placed in a long pressure tube. The content of HCl (37%) in the resulting $HCl/H_2{}^{17}O$ solution was determined on the basis of increased mass.

Acidic hydrolysis[4] of benzonitrile **(38)** with $HCl/H_2{}^{17}O$ gave the ^{17}O-labeled benzoic acid **(39c)** in 70% yield. The crude benzoic acid was partitioned between CH_2Cl_2 and aqueous $NaHCO_3$ in order to purify it from traces of benzonitrile and benzamide. Acidification of the aqueous layer with HCl and extraction with CH_2Cl_2 gave pure ^{17}O-labeled benzoic acid **(39c)**. The product was characterized by ^{1}H-NMR, ^{13}C-NMR and mass spectrometry. The mass spectrum showed a molecular ion peak at *m/z* 125 [$MH,^+$ 2 x ^{17}O] with intensity 100%, and a peak at *m/z* 124 with intensity 34%, and peak at *m/z* 123 with intensity less then 2%, confirming a high level of ^{17}O-incorporation.

In a second step, the [^{17}O]-labeled benzoic acid **(39c)** was treated 1.25 equiv. of thionyl chloride.[39] The resulting mixture was refluxed for 1 h at 100 °C, and excess thionyl chloride was added to keep the solution homogeneous. The reaction mixture was then distilled. Simple distillation removed excess thionyl chloride at ~7O °C followed by benzoyl chloride **(40c)** which distilled off in one fraction. The product was colorless and had strong benzoyl chloride odor (yield 85%).

3.2.3 REARRRAGEMENT OF 2'-*O*-BENZOYL COMPOUNDS

Treatment of 1-[2,3'-anhydro-5'-*O*-(*tert*-butyldimethylsilyl)-β-D-xylofuranosyl]uracil **(37)** (**Scheme XI**) with two equivalents of ^{17}O-benzoyl chloride gave 2'-*O*-benzoyl derivative **41c.** ^{17}O-Labeled was incorporated at the carbonyl oxygen in 50% yield. The product was purified on a silica gel column, and unreacted starting



Scheme XII: Synthesis of oxygen labeled benzoyl chloride

material was recovered. The product was characterized by ^{1}H- and ^{13}C-NMR, mass spectrometry and elemental analysis. ^{1}H-NMR showed characteristic chemical shifts for the benzoyl group. Mass spectrometry showed a prominent peak at *m/z* 446 with intensity 100% for the ^{17}O-labeled molecular ion (MH^+) and a peak at *m/z* 445 with 77% intensity for the MH^+ ion of the remaining unlabeled material indicating 56% enrichment with ^{17}O-isotope.

Stereospecific rearrangement of 2'-*O*-benzoyl compound **41c** (with ^{17}O-labeled on the carbonyl oxygen) into the 3'-*O*-benzoyl compound **42c** (with ^{17}O-labeled on 3' hydroxyl directly attached to ribosyl ring) with concomitant formation of O^2,2'-anhydro ring was achieved by heating compound **41c** with a Bunsen burner under argon until the solid melted and turned brown (about 1 min). This thermolysis method gave better results then the reported procedure of Fox, *et al.*[36] where a sand bath at 252 °C was used for three min. TLC analysis showed that conversion was more effective and produced more reliable results than by direct heating. Controlling the flame temperature, however, is demanding and inconvenient. The product was purified on a silica gel column and was characterized by ^{1}H-NMR, ^{13}C-NMR, mass spectrometry and elemental analysis. For example, the mass spectrum of **42c** [^{17}O] showed a +1 shift for the molecular ion relative to **42a** indicating the incorporation of one oxygen-17 atom. The ^{17}O-enrichment for **42c** was calculated to be 55% based on the relative intensities of the MH^+[^{17}O] and the MH^+[^{16}O] peaks for the corresponding isotopes. Analogously the mass spectrum of **42b** [^{18}O] showed a +2 shift of the molecular ion relative to unlabeled analog **42a** indicating the incorporation of one oxygen-18 atom.

3.3 SYNTHESIS OF 2'-AZIDO- 2'-DEOXYNUCLEOTIDES

Deprotection of the O^2,2'-anhydro compounds **42** was first attempted on a small scale with unlabeled analogue in order to find the best conditions for the removal of the 5'-*O*-silyl protecting group and the 3'-*O*-benzoyl group in the presence of the base-sensitive O^2,2'-anhydro bridge. Two approaches were investigated. In the first approach, removal of the 5'-*O*-silyl protecting group with NH_4F/MeOH[40] was followed by removal of the 3'-benzoyl group with NH_3/MeOH. TLC showed polar, lower moving compounds in low yields. In a second approach, benzoyl protection was removed by first adding NH_3/MeOH and stirring the compound for one hour. The 5'-*O*-silyl protecting group was then removed by adding NH_4F/MeOH.[40] TLC indicated formation of a more polar product as well as stability of O^2,2'-anhydro bridge.

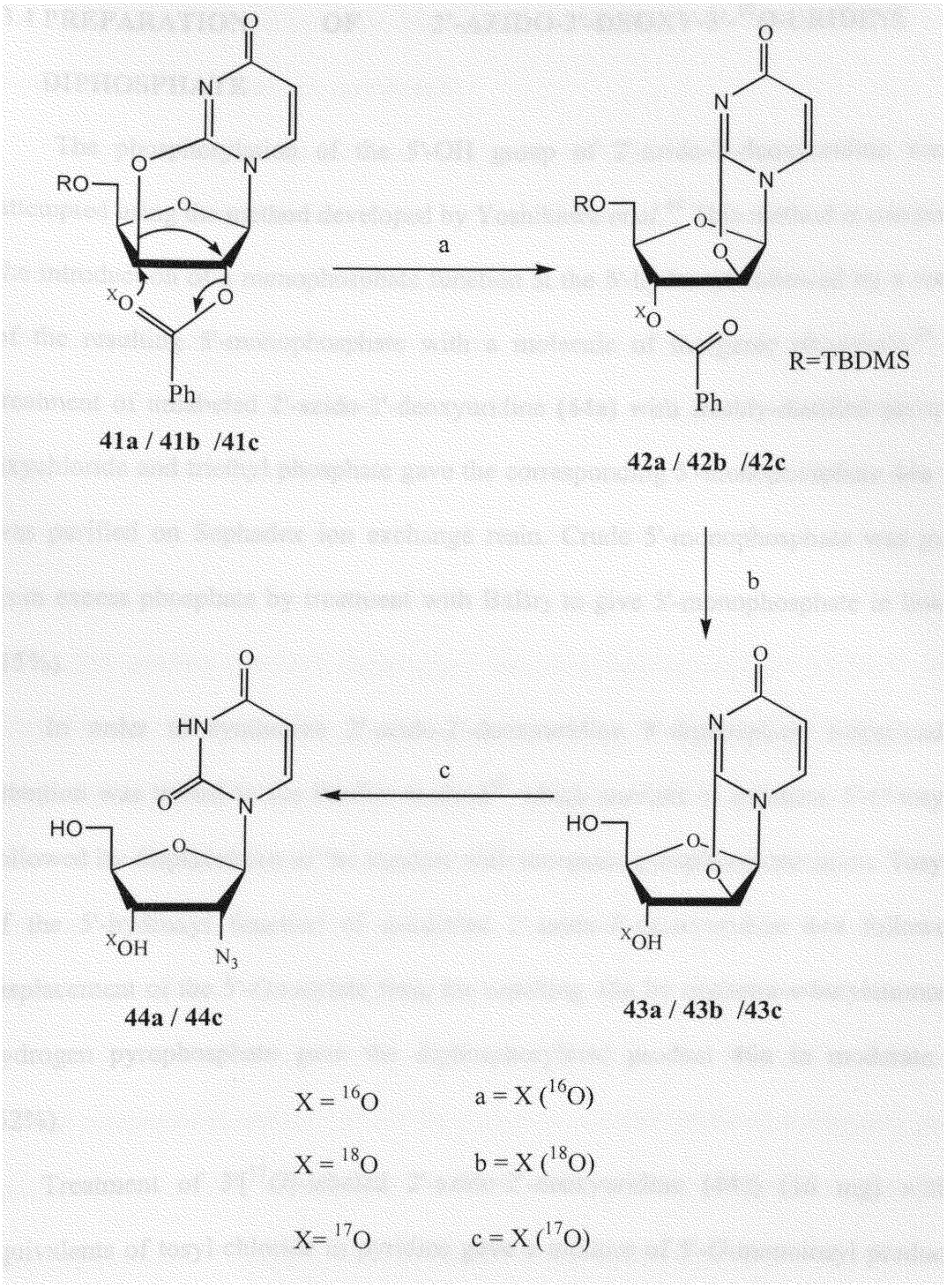
Labeled 5'-*O*-TBDMS-2'-^{17}*O*-benzoyl-O^2,2'-anhydrouridine **(42c)** was dissolved in MeOH/NH_3 and stirred for two hours. The reaction mixture was evaporated, and the residue was treated with excess NH_4F in MeOH and stirred at room temperature overnight. Purification by Reverse Phase-HPLC gave product **43c** as white crystals in 65% yield. The ^{1}H-NMR and ^{13}C-NMR gave identical chemical shifts for compound **43c** as those reported in the literature for the unlabeled analog **43a**.[41] The mass spectrum, using the APCI technique, showed a peak at *m/z* 227 with intensity 100% for $MH^+[^{16}O]$ and a peak at *m/z* 228 with intensity 98% for $MH^+[^{17}O]$ proving that ^{17}O-enrichment at ~50% level had occurred.

Several attempts were made to synthesize 3'[^{17}*O*]-labeled 2'-azido-2'-deoxyuridine. First, the Moffatt procedure[42] for the ring opening of O^2,2'-anhydrouridine **43a** with azide anion was tested on unlabeled compound. Treatment of **43a** with lithium azide and

hexamethylphosphoramide (HMPA) followed by purification on silica gel and Dowex as described by Moffat, *et al.*[41] gave desired **44a** in ~20% yield. Cleavage of glycosylic linkage with the formation of uracil had occurred. Other attempts to improve the yield for the conversion of **43a** into the 2'-azido-2'-deoxyuridine failed repeatedly giving poorer results than the 43% yield reported in the literature.[42]

In 1994 Kirschenbenter, *et al.*[43] reported an improved synthesis of the 2'-azido-2'-deoxyuridine employing generated *in situ* lithium azide. Treatment of **43a** with LiF, *N,N,N,N*-tetramethylethylenediamine (TMEDA) and azidotrimethylsilane (Me_3SiN_3) reagent gave **44a** in higher yield (83%) than the Moffatt procedure. A second approach employed LiN_3 which was complexed with 18-crown-6. While both sets of conditions gave 2'-azido-2'-deoxyuridine in higher yield (~ 60%), difficulties in purification of the final product from the 18-crown-6 ether was experienced in the second approach.

The 3'[^{17}O]-labeled O^2,2'-anhydrouridine **43c** was added to LiN_3 generated *in situ* from LiF and Me_3SiN_3. The reaction mixture was worked up with methanol and ethyl acetate. After purification on silica gel and RP-HPLC, the product **44c** was obtained in 27 % yield **(Scheme XIII)**. Proton NMR of **44c** showed the identical chemical shift as reported previously in the literature for the unlabeled analog.[42,43] Mass spectrometry confirmed the presence of isotopic labeling. MS using APCI technique showed a molecular ion peak at *m/z* 271 with 100% intensity for $MH^+[^{17}O]$ with respect to the peak at *m/z* 270 for $MH^+[^{16}O]$ isotope with 98% intensity, confirming 50% enrichment of 17-oxygen at 3'-hydroxyl group.



(a) 252 ^{0}C (b) Ammonia/MeOH and NH_4F/ MeOH (c) LiF/Me_3SiN_3 /TMEDA/ DMF

Scheme XIII: Synthesis of 2'-Azido-3'-^{17}O -2'-deoxyuridine

3.4 PREPARATION OF 2'-AZIDO-2'-DEOXY-3'-^{17}O-URIDINE 5'-DIPHOSPHATE

The phosphorylation of the 5'-OH group of 2'-azido-2'-deoxyuridine was first attempted using the method developed by Yoshikawa *et al.*[44] This method is composed of the introduction of a monophosphate function at the 5'-hydroxyl followed by a coupling of the resulting 5'-monophosphate with a molecule of inorganic phosphate.[28] Thus, treatment of unlabeled 2'-azido-2'-deoxyuridine **(44a)** with freshly-distilled phosphorus oxychloride and triethyl phosphate gave the corresponding 5'-monophosphate **44a** which was purified on Sephadex ion exchange resin. Crude 5'-monophosphate was purified from excess phosphate by treatment with $BaBr_2$ to give 5'-monophosphate in low yield (15%).

In order to synthesize 2'-azido-2'-deoxyuridine 5'-diphosphate compounds **46**, attention was turned to the Poulter method[45] which consists of selective 5'-*O*-tosylation followed by displacement of the tosylate with inorganic pyrophosphate anion. Tosylation of the 5'-hydroxyl function of unlabeled 2'-azido-2'-deoxyuridine **44a** followed by displacement of the 5'-*O*-tosylate from the resulting **45a** by tris(tetra-*n*-butylammonium) hydrogen pyrophosphate gave the diphosphorylated product **46a** in moderate yield (52%).

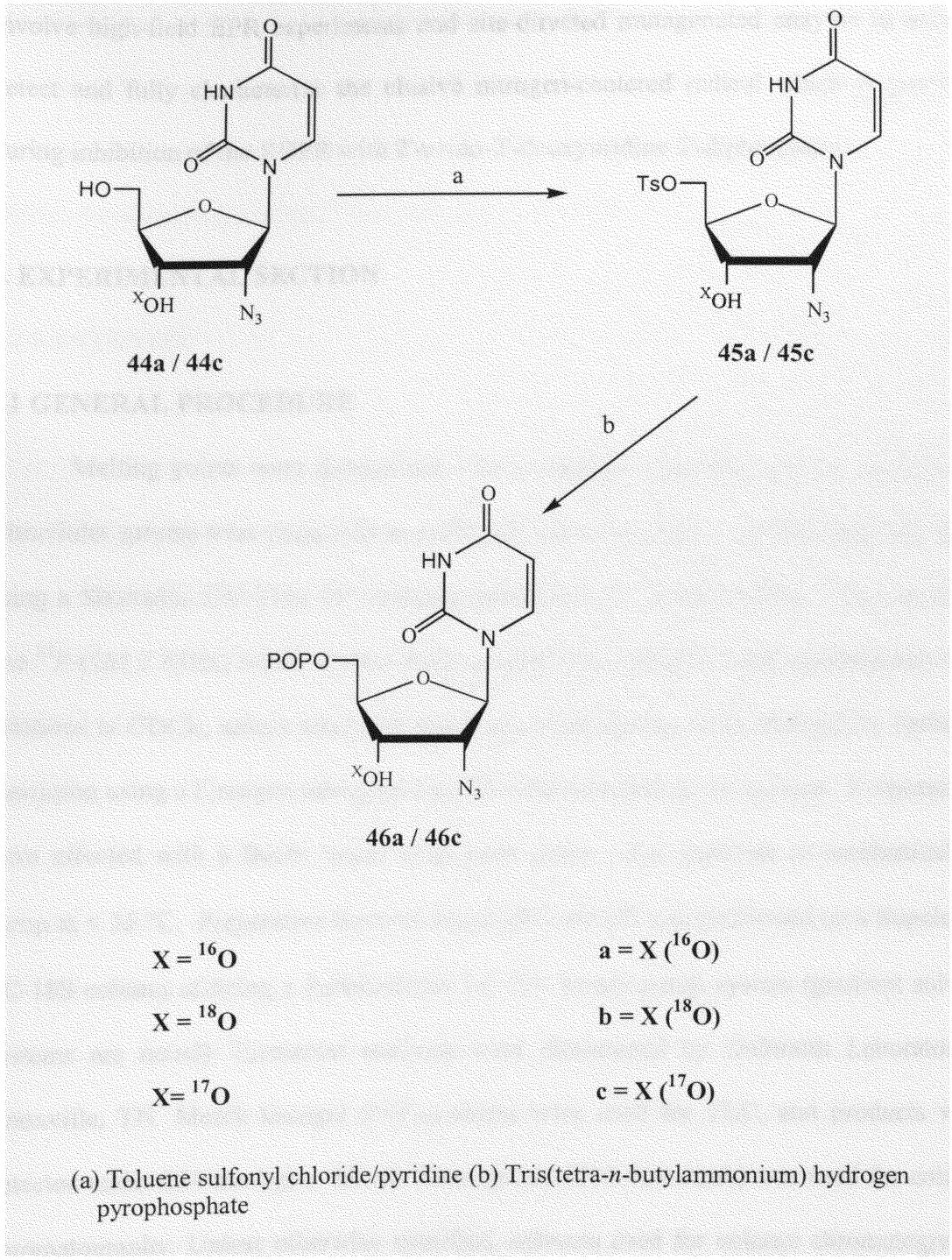
Treatment of 3'[^{17}O]-labeled 2'-azido-2'-deoxyuridine **(44c)** (16 mg) with 1.5 equivalents of tosyl chloride in pyridine gave a mixture of 5'-*O*-monotosyl product **45c** along with small quantities of the corresponding 3',5'-di-*O*-tosyluridine byproduct. The 5'-monotosylated compound **45c** was separated from ditosylated compound on a silica gel column. The product **45c** was obtained in 63% yield and was characterized by ^{1}H- and

^{13}C-NMR and mass spectrometry. Mass spectrometry showed ~ 50% of ^{17}O-enrichment based on the $^{17}O/^{16}O$ isotope distribution.

In order to synthesize the final compound, 3'[^{17}O]-labeled 2'-azido-2'-deoxyuridine 5'-diphosphate **(46c) (Scheme XIV)**, tosylate **45c** was treated with 1.5 equivalents of tris(tetra-*n*-butylammonium) hydrogen pyrophosphate in acetonitrile for 24 hour at ambient temperature. The reaction mixture was evaporated to remove acetonitrile, and the crude **46c** was diluted with water and purified on a Sephadex ion exchange column using triethylammonium bicarbonate as a gradient eluant. Proton NMR showed the presence of triethylammonium salt in the product. The product was converted to its sodium salt by passing it through a Dowex (Na^+) column that was prepared from Dowex (H^+). The product obtained, diphosphate **46c**, was characterized by ^{1}H-NMR and ^{31}P-NMR. Proton NMR showed no peaks for the TEA salt. Phosphorus-31 NMR showed that compound **46c** still contained 70% inorganic pyrophosphate as impurity.

The final purification of compound **46c** was achieved on RP-HPLC with a semi-preparative column (Supelcosil LC-18T). Elution with 3% H_2O/CH_3CN gave the ^{17}O-labeled diphosphate **46c** (4 mg, 22%) with a retention time of 5-6 minutes. The compound was characterized by ^{1}H-NMR and ^{31}P-NMR. Phosphorus-31 NMR showed that the compound contained 50% of the inorganic pyrophosphate as an impurity and also consist of two doublets of doublets at δ -10.17 and δ - 9.26 for phosphorus α and β with a coupling constant of Jp_α-p_β = 21.2 Hz diagnostic for diphosphate **46c**.

Biological studies with the 3'[^{17}O]-2'-azido-2'-deoxyuridine 5'-diphosphate **46c** are now being performed in Professor Stubbe's laboratory at the Massachusetts Institute of Technology. Enzymatic studies with ribonucleoside diphosphate reductase (RDPR)



Scheme XIV: Preparation of 2'-azido-2'-deoxy-3'-^{17}O-uridine 5'-diphosphate

involve high-field EPR experiments and site-directed mutagenated enzyme in order to detect and fully characterize the elusive nitrogen-centered radical which is generated during inhibition of the RDPR with 2'-azido-2'-deoxyuridine 5'-diphosphates.

4. EXPERIMENTAL SECTION

4.1 GENERAL PROCEDURE

Melting points were determined with a capillary apparatus and are uncorrected. Ultraviolet spectra were measured in methanol, absolute ethanol, 50% ethanol and water using a Shimadzu UV-2101 PC scanning spectrometer. ^{1}H-(400 MHz), ^{13}C-(100 MHz) and ^{31}P-(161.7 MHz) NMR spectra were recorded on a Bruker NMR spectrometer with solutions in $CDCl_3$, unless otherwise specified. Mass spectra were obtained by chemical ionization using a Finnigan navigator LC/MS with methanol as the solvent. Evaporations were effected with a Büchi rotary evaporator under water aspirator or mechanical oil pump at < 35 °C. Preparative Reverse-Phase (RP)-HPLC was performed on a Supelcosil LC-18S column utilizing a Perkin-Elmer LC 200 binary pump system (gradient solvent systems are noted). Elemental analyses were determined by Galbraith Laboratories, Knoxville, TN. Merck kiselgel 60-F_{254} sheets were used for TLC, and products were detected under 254 nm light. Merck Kieselgel 60 (230-400 mesh) was used for column chromatography. Unless otherwise specified, solvents used for column chromatography and TLC were as follows: **(S1)** EtOAc; **(S2)** SSE [the upper phase of EtOAc/*i*-PrOH/H_2O (4:1:2)]; **(S3)** EtOAc/SSE (90:10); **(S4)** EtOAc/SSE (75:25); **(S5)** DSSE (30% MeOH in SSE); **(S6)** $CHCl_3$/MeOH (95:5); **(S7)** *i*-PrOH/NH_4OH/H_2O (3:1:1). Reagent grade

chemicals were used, and solvents were dried by reflux over and distillation from calcium hydride under argon. Sonication was performed with a Branson 5200 ultrasonic bath. Ion exchange chromatography was performed with Sephadex-DEAE A-25, (40-120μ) and Dowex-50W-hydrogen foam, 2% cross linking, 100-200 mesh ion exchange resin.

4.2 SYNTHESIS

2',5'-Di-*O*-trityluridine (33)

Uridine **(32)** (11 g, 45 mmol), dissolved in anhydrous pyridine (95 mL), was treated with three equivalents of triphenylmethyl chloride (36 g, 129 mmol) and allowed to remain at room temperature overnight. The resulting red solution was heated at 110 °C for four hours after which it was cooled, poured into water, and stirred. After decantation, the gummy solid was again treated with water, stirred and the water decanted. This process was repeated several times after which the residue was dissolved in acetone and concentrated to dryness. The resulting viscous liquid was treated with 500 mL of hot water, stirred, and the water decanted. This process was repeated twice. The residue was dissolved in methylene chloride and dried over anhydrous sodium sulphate. After filtration, the solution was concentrated and treated with a minimum amount of hot benzene to give a homogeneous solution. Diethyl ether was added to the point of faint opalescence. After cooling (and scratching), a creamy solid precipitated that was filtered to give **33** (32g, 45%): mp 224-225 °C (lit.[46] mp 223-224 °C); UV max 264 nm, min 245 nm; ^{1}H NMR δ 2.30 (br s, 1, OH3'), 2.80 (d, $J_{3'\text{-}2'}$ = 4.6 Hz, 1, H3') 3.12 (br s, 2, H5',5''), 3.98 (br s, 1, H4'), 4.51 (t, $J_{2'\text{-}1'}$ = 4.7 Hz, 1, H2'), 5.11 (d, $J_{1'\text{-}2'}$ = 6.1 Hz, 1, H1'), 6.36 (d,

$J_{5\text{-}6}$ = 7.4 Hz, 1, H5), 7.00-7.60 (m, 15, H_{arom}), 7.70 (d, $J_{6\text{-}5}$ = 8.0 Hz, 1, H6), 8.50 (s, 1, NH).

3'-*O*-Methanesulfonyl-2'-5'-di-*O*-trityluridine (34)

Methanesulfonyl chloride (1.1 mL) was added dropwise to a cooled solution of **33** (8.8 g, 12 mmol) in anhydrous pyridine (100 mL), and the reaction mixture was stored at ~5 °C for 16 h. A crystalline product was formed during this time. The reaction mixture was treated with ethanol (2 mL) and allowed to stand for 2 h more. After concentration *in vacuo* to a red syrup, the residue was treated with ethanol (200 mL) whereupon a heavy white precipitate formed. After filtration and trituration of the precipitate with ethanol, a solid was obtained (7.3 g, 75%) which was washed repeatedly with ethanol and diethyl ether and was dried under vacuum. This crude product was recrystallized from ethanol to afford pure **34**: mp 224-226 °C (lit.[36] mp 225-226 °C); UV max 259 nm, min 242 nm; ^{1}H NMR δ 2.95 (s, 3, Me), 3.25 ("dd", $J_{5'\text{-}5''}$ = 10.8 Hz, 2, H5',5''), 4.25 (d, $J_{3'\text{-}2'}$ = 5.0 Hz, 1, H3'), 4.42 (br s 1, H4'), 4.66 (t, J = 7.6 Hz, 1, H2'), 5.14 (d, $J_{5\text{-}6}$ = 8.1 Hz, 1, H5), 6.99 (d, $J_{1'\text{-}2'}$ = 7.8 Hz, 1, H1'), 6.96 (d, $J_{6\text{-}5}$ = 7.4 Hz, 1, H6), 7.00-7.80 (m, 15, H_{arom}), 8.80 (s, 1, NH).

1-(2, 3'-Anhydro -2', 5'-di-*O*-trityl-β-D-xylofuranosyl)uracil (35)

Compound **34** (7.0 g, 8.7 mmol) was added to a suspension of sodium benzoate (14 g) in DMF (280 mL) and heated at 130-140 °C (internal temperature) for 18 h. The cooled mixture was treated with 1 L of water and stirred for 2 h. The precipitate was separated and washed well with water. After filtration, the precipitate was triturated with

ethanol (100 mL) whereupon a white granular precipitate slowly formed. After 2 h of stirring, the precipitate was filtered from the amber colored solution. The precipitate was directly chromatographed on a silica gel column. Gradient elution (80% EtOAc/hexane → EtOAc → 5% MeOH/EtOAc) and evaporation of the appropriate fractions (R_f in S1 0.8) gave **35** (2.6 g, 43%); mp 237-238 °C (lit.[36] 237 °C); UV max 248, 269 (shoulder) nm, min 239 nm; ^{1}H NMR δ 3.29 (dd, $J_{5'-5''}$ = 9.7 Hz, $J_{5'-4''}$ = 3.2 Hz, 1, H5''), 3.34 (dd, $J_{5'-4''}$ = 4.2 Hz, H5'), 4.03 (q, $J_{4'-3'}$ = 6.8 Hz, 1, H4'), 4.41 ("s", 1, H3'), 4.53 ("dt", $J_{2'-3'}$ = 2.1 Hz, 1, H2'), 4.59 (s, 1, H1'), 5.76 (d, J_{5-6} = 7.4 Hz, 1, H5), 6.28 (d, J_{6-5} = 7.4 Hz, 1, H6), 7.00-7.50 (m, 15, H_{arom}).

1-(2, 3'-Anhydro-β-D-xylofuranosyl)uracil (36)

Compound **35** (2.2g, 3.0 mmol) was placed in ethanol (50 mL) which had been saturated with anhydrous hydrogen chloride (0 °C for 10 minutes), and kept ~70 °C for 10 miniuets. The solution was concentrated *in vacuo* to a syrup and repeatedly evaporated with benzene. Absolute ethanol (10 mL) was added to the syrup and cooled to 0 °C. The resulting solution was neutralized to about pH 5 with methanolic ammonia whereupon crystallization took place. Recrystallization from 95% methanol gave pure **36** (0.6 g, 85%); mp. 227-228 °C (lit.[36] mp 225-227); UV max 231 nm, min 215 nm; ^{1}H NMR (D_2O) δ 3.61 (m, 2, H5', 5''), 4.45 (s, 1, H4'), 4.70 (br s, 1, H3'), 4.97 (s, 1, H2'), 5.55 (br s, 1, H1'), 5.91 (d, J_{5-6} = 7.3 Hz, 1, H5), 7.55 (d, J_{6-5} = 7.4 Hz, 1, H6).

1-[2, 3'-Anhydro-5'-*O*-(*tert*-butyldimethylsilyl)-β-D-xylofuranosyl]uracil (37)

To a suspension of 1-(2,3'-anhydro-β-D-xylofuranosyl)uracil **(36)** (1.13 g, 5 mmol) in anhydrous DMF (30 mL) were added imidazole (0.725 g, 12.5 mmol) and *tert*-butyldimethylsilyl chloride (0.903 g, 6.0 mmol). The reaction mixture was stirred overnight under argon at ambient temperature. Volatiles were evaporated under vacuum, and the residue was directly chromatographed on a silica gel column. Gradient elution [EtOAc → 50% S2/EtOAc → MeOH/S2/EtOAc (2:49:49)] and evaporation of the appropriate fractions (R_f 0.71 in S3; compound **36** had R_f 0.1) gave **37** (1.36 g, 80%). Crystallization from EtOH/EtOAc gave sharp needles: mp 246-247 °C; UV max 231 nm (ε 9800), min 216 nm (ε 6900); ^{1}H NMR (DMSO-d_6) δ 0.02 (s, 6, 2 x Me), 0.85 (s, 9, *t*-Bu), 3.71 (dd, $J_{5''\text{-}5'}$ = 11.0 Hz, $J_{5''\text{-}4'}$ = 6.4 Hz, 1, H5''), 3.81 (dd, $J_{5'\text{-}4'}$ = 6.0 Hz, 1, H5'), 4.41 ("dt", $J_{4\text{-}5',\,5''}$ = 6.2 Hz, $J_{4'\text{-}3'}$ = 2.5 Hz, 1, H4'), 4.74 (br s, 1, H3'), 4.92 (br s, 1, H2'), 5.64 (s, 1, H1'), 5.80 (d, $J_{5\text{-}6}$ = 7.4 Hz, 1, H5), 6.45 (br s, 1, OH2'), 7.68(d, 1, H6); ^{13}C NMR (DMSO-d_6) δ - 5.00 (2 x Me), 18.27 (*t*-Bu), 25.99 (*t*-Bu), 61.37 (C5'), 69.73 (C3'), 79.47 (C2'), 83.58 (C4'), 89.64 (C1'), 108.19 (C5), 141.33 (C6), 153.67 (C2), 170.59 (C4); MS (CI) *m/z* 341 (100, MH^+), 283 (30, M^+- *t*-Bu); HRMS (CI) calcd. for $C_{15}H_{24}N_2O_5Si$ + H. 341.1532. Found 341.1538. Anal. Calcd. for $C_{15}H_{24}N_2O_5Si$ (340.45): C, 52.92; H, 7.11; N, 8.23. Found: C, 52.43; H, 7.26; N, 8.11.

Preparation of Concentrated HCl in ^{18}O-Labeled H_2O

The Oxygen-18-labeled water (1.05 g, 52.5 mmol; 97.4 atom % ^{18}O from Isotec, Inc.) was placed in long pressure tube (Ace glass) and was cooled in an ice bath.

Anhydrous hydrogen chloride was bubbled in for 3 min, and the content of HCl (37%) was calculated on the basis of increased mass.

Preparation of ^{18}O-Labeled Benzoic Acid from Benzonitrile and $HCl/H_2{}^{18}O$ (39b)

Benzonitrile **(38)** (1.78 ml, 1.8 g, 17.5 mmol) was suspended in concentrated $HCl/H_2{}^{18}O$ (prepared as above) in a long pressure tube, and the reaction mixture was heated for 20 h at 100 °C with vigorous stirring. During that time the whole reaction mixture solidified. The reaction mixture was then partitioned between cold methylene chloride and ice-cold, saturated aqueous $NaHCO_3$ solution. The methylene chloride layer was then quickly extracted again with $NaHCO_3$ solution. The aqueous extracts were combined and water layer was extracted back with CH_2Cl_2 to remove traces of benzonitrile and benzamides. Fresh methylene chloride was then added to the water layer, and the mixture was acidified to pH ~3. The aqueous layer was separated, and the CH_2Cl_2 layer was extracted with brine. The methylene chloride layer was then dried (anhydrous Na_2SO_4) and evaporated to give **39b** (1.55 g, 70%) as a white solid. ^1H NMR δ 7.49 (t, J = 7.4 Hz, 2, H), 7.65 (t, J = 7.4 Hz, 1,H), 8.15 (d, J = 7.0 Hz, 2,H), 13.00 (s, 1, COOH); ^{13}C NMR δ 128.99 (C3), 129.81 (C1), 130.72 (C2), 134.34 (C4) 173.05 (COOH); MS (CI) *m/z* 127 (100, MH^+ [2 x ^{18}O]), 126 (60) 125 (20), 124 (19), 107 (65, $PhC^{18}O^+$), 105 (2, $PhCO^+$) and no peaks at *m/z* 122 and 123 were observed for unlabeled benzoic acid.

Preparation of Concentrated HCl in ^{17}O-Labeled H_2O

Oxygen-17 labeled water (1 .08 g, 57.7 mmol; 74.5 atom % ^{17}O from Isotec, Inc.) was placed in a long pressure tube (Ace glass) and was cooled in an ice bath. Anhydrous hydrogen chloride was bubbled in for 3 min, and the content of HCl (35.2%) was calculated on the basis of increased mass.

Preparation of ^{17}O- Labeled Benzoic Acid from Benzonitrile and $HCl/H_2{}^{17}O$ (39c)

Treatment of benzonitrile **(38)** (2.08 g, 2.06 ml, 20.2 mmol) with $HCl/H_2{}^{17}O$ (prepared as above) was effected as described for **39b** and afforded gave ^{17}O-labeled benzoic acid **39c** (1.54 g, 62%). MS (CI) *m/z* 125 (100, MH^+ [2 x ^{17}O]), 124 (34, $MH^+[^{17/16}O]$), 123 (21, MH^+ [2 x ^{16}O]).

Preparation of ^{18}O-Labeled Benzoyl Chloride (40b)

^{18}O-Labeled benzoic acid **(39b)** (0.88 g, 6.98 mmol) was placed in a 25 mL round-bottomed flask, and thionyl chloride (0.65 mL, 1.05 g, 8.8 mmol) was added. The reaction mixture was gently heated for 1 h at 100 °C (an additional 1 mL of thionyl chloride was added after 20 min. to minimize the effect of loss of thionyl chloride during heating and to keep the solution homogeneous). The reaction mixture was cooled after one hour and distilled. Simple distillation at atmosphere pressure removed excess $SOCl_2$ (~70 °C). Vacuum was then applied, and the temperature was increased to ~130 °C to distill most of the liquid in one fraction to give **40b** (0.85 g, 85%). The distilled liquid was colorless and had strong odor of benzoyl chloride.

Preparation of ^{17}O-Labeled Benzoyl Chloride (40c)

Treatment of the ^{17}O-labeled benzoic acid **(39c)** (1.4 g, 11.29 mmol) with thionyl chloride (1.67 g, 14.11 mmol) as described for **40b** gave [^{17}O] benzoyl chloride **(40c)** (1.3 g, 80%).

1-[2,3'-Anhydro-2'-*O*-benzoyl-5'-*O*-(*tert*-butyldimethylsilyl)-β-D-xylofuranosyl] uracil (41a)

To a solution of **37** (20 mg, 0.05 mmol) in anhydrous pyridine (1 mL) was added benzoyl chloride (0.05 mL), and stirring was continued overnight at ambient temperature and then for 2 h at 40 °C. Volatiles were removed *in vacuo,* and the residue was co-evaporated with toluene. The crude product was chromatographed on a silica gel column. Gradient elution [EtOAc/hexane (90:10)→ EtOAc → 5% S2/EtOAc → MeOH/S2/EtOAc (2:10:88)] and evaporation of the appropriate fractions (R_f 0.64 in S3; compound **37** had R_f 0.12) gave compound **41a** (10 mg, 50%) as a white solid: mp 238-240 °C; UV max 233 nm (ε 26 100), min 213 nm (ε 12 000); ^{1}H NMR δ 0.02 (s, 6, 2 x Me), 0.85 (s, 9, *t*-Bu), 3.89 (dd, $J_{5''\text{-}5'}$ = 10.7 Hz, $J_{5''\text{-}4'}$ = 7.3 Hz, 1, H5''), 3.93 (dd, $J_{5'\text{-}4'}$ = 6.0 Hz, 1, H5'), 4.70 ("dt", $J_{4\text{-}5',5''}$ = 7.4 Hz, $J_{4'\text{-}3'}$ = 2.5 Hz, 1, H4'), 5.22 (br s, 1, H3'), 5.55 (br s, 1, H1'), 5.70 (br s, 1, H2'), 6.13 (d, $J_{5\text{-}6}$ = 7.3 Hz, 1, H5), 7.21 (d, 1, H6) 7.52 (t, J = 7.9 Hz, 2, H_{arom}), 7.70 (t, J = 7.4 Hz, 1, H_{arom}), 8.06 (d, J = 7.1 Hz, 2, H_{arom}); ^{13}C NMR δ -0.50 (2 x Me), 18.76 (*t*-Bu), 26.24 (*t*-Bu), 61.09 (C5'), 72.63 (C2'), 77.61 (C3'), 85.22 (C4'), 88.82 (C1'), 110.53 (C5), 139.81(C6), 153.51 (C2), 171.35 (C4); MS (CI) *m/z* 445 (100, MH^+). Anal. Cald for $C_{22}H_{28}N_2O_6Si$ (444.56): C, 59.44; H, 6.35; N, 6.30. Found: C, 59.03; H, 6.57; N, 6.30.

1-[2,3'-Anhydro-2'-^{18}*O*-benzoyl-5'-*O*-(*tert*-butyldimethylsilyl)-β-D-xylofuranosyl]uracil (41b)

Treatment of **37** (200 mg, 0.59 mmol) with ^{18}O-labeled benzoyl chloride **(40b)** (0.14 mL) as described for **41a** gave compound **41b** (180 mg, 68%) with identical physical and spectroscopic properties except for the mass spectrum: MS (CI) *m/z* 447 (100, $MH^+[^{18}O]$), 445 (16, $MH^+[^{16}O]$).

1-[2,3'-Anhydro-2'-^{17}*O*-benzoyl-5'-*O*-(*tert*-butyldimethylsilyl)-β-D-xylofuranosyl]uracil (41c)

Treatment of **37** (1.15 g, 3.3 mmol) with ^{17}O-labeled benzoyl chloride **(40c)** (0.5 ml) as described for **41a** gave compound **41c** (0.45 g, 30%) with identical physical and spectroscopic properties except for the mass spectrum. MS (CI) *m/z* 446 (100, 446 $MH^+[^{17}O]$), 445 (77, $MH^+[^{16}O]$).

1-[2,2'-Anhydro-3'-*O*-benzoyl-5'-*O*-(*tert*-butyldimethylsilyl)-β-D-arabinofuranosyl]uracil (42a)

Compound **41a** (100 mg, 0.22 mmol) was placed in a 25 mL round bottomed flask and heated directly by Bunsen burner under argon for 1 min until the solid melted and turned brown. After cooling, the product was directly chromatographed on a silica gel column. Gradient elution [EtOAc → 10%/S2/EtOAc → MeOH/S2/EtOAc(2:10:88)] and evaporation of the appropriate fraction (R_f 0.75 in S4; and compound **41a** had R_f 0.37) gave compound **42a** (65 mg, 65%) as a white solid: mp 224-226 °C; UV max 231 nm (ε 20 100), min 210 nm (ε 9000); ^{1}H NMR δ 0.02 (s, 6, 2 x Me), 0.90 (s, 9, *t*-Bu),

3.67 (dd, $J_{5''\text{-}5'}$ = 11.1 Hz, $J_{5''\text{-}4'}$ = 4.4 Hz, 1, H5''), 3.81 (dd, $J_{5'\text{-}4'}$ = 5.9 Hz, 1, H5'), 4.50 ("dt", $J_{4\text{-}5',\,5''}$ = 4.4 Hz, $J_{4'\text{-}3'}$ = 2.6 Hz, 1, H4'), 5.60 (d, $J_{2'\text{-}1'}$ = 5.5 Hz, 1, H2'), 5.70 (br s, 1, H3'), 6.08 (d, $J_{5\text{-}6}$ = 7.4 Hz, 1, H5), 6.42 (d, $J_{1'\text{-}2'}$ = 5.7 Hz,1, H1') 7.42 (d, $J_{6\text{-}5}$ = 7.4 Hz, 1, H6) 7.50 (t, J = 7.9 Hz, 2, H_{arom}), 7.65 (t, J = 7.4 Hz, 1, H_{arom}), 8.05 (d, J = 7.2 Hz, 2, H_{arom}); ^{13}C NMR δ -0.50 (2 x Me), 19.01(*t*-Bu) , 26.35 (t-Bu), 63.58 (C5'), 78.34 (C4'), 87.38 (C3'), 88.77 (C2'), 91.18 (C1'), 110.59 (C5), 128.84-134.42 (C_{arom}), 160.09 (C2), 165.84 (C_{bz}), 170.42 (C4); MS (CI) *m/z* 445 (100, MH^+). Anal. Cald. for $C_{22}H_{28}N_2O_6Si$ (444.56): C, 59.44; H, 6.35; N, 6.30. Found: C, 59.80; H, 6.42; N, 6.32.

1-[2,2'-Anhydro-3'-^{18}*O*-benzoyl-5'-*O*-(*tert*-butyldimethylsilyl)-β-D-arabinofuranosyl]uracil (42b)

Treatment of compound **41b** (70 mg, 0.15 mmol) as described for **42a** gave **42b** (44 mg, 63%) with identical physical and spectroscopic properties except for the mass spectrum. MS (CI) *m/z* 447 (100, $MH^+[^{18}O]$), 445 (35, 445 $MH^+[^{17}O]$).

1-[2,2'-Anhydro-3'-^{17}*O*-benzoyl-5'-*O*-(*tert*-butyldimethylsilyl)-β-D-arabinofuranosyl]uracil (42c)

Treatment of compound **41c** (50 mg, 0.11 mmol) as described for **42a** gave compound **42c** (27 mg, 54%) with identical physical and spectroscopic properties except for the mass spectrum. MS (CI) *m/z* 446 (100, $MH^+[^{17}O]$), 445 (85, $MH^+[^{16}O]$).

1-(2,2'-Anhydro- β-D-arabinofuranosyl)uracil (43a)

Compound **42a** (22 mg, 0.5 mmol) was dissolved in a saturated solution of NH_3 in MeOH (5 mL) and was stirred at ~0 °C. After 2 h, the reaction mixture was evaporated, and to the white residue NH_4F (28 mg, 0.75 mmol) and anhydrous MeOH (5 mL) were added to the white residue. The resulting solution was stirred at room temperature overnight. TLC in S5 showed compound **43a** with R_f 0.4 (compound **42a** had R_f 0.75). Volatiles were evaporated, and the residue was purified on RP- HPLC (elution 8% CH_3CN/92% H_2O, retention time 25 min.) to give compound **43a** as off-white crystals (8 mg, 71%): mp 244-245 °C (lit.[46] mp 238-244°C); UV max 225, 251 nm (ε 7000, 5850), min 237, 212 nm (ε 5200, 5200); ^{1}H NMR (DMSO-d_6) δ 3.16-3.47 (m, 2, H5',5''), 4.07 (t, $J_{3'\text{-}2'}$ = 4.5 Hz, 1, H3'), 4.38 (s, 1, H4'), 4.99 (t, $J_{5'\text{OH-}\ 5'/5''}$ = 5.2 Hz, 1, OH5'), 5.20 (d, $J_{3'\text{-}2'}$ = 5.6 Hz, 1, H2'), 5.85 (d, $J_{5\text{-}6}$ = 7.4 Hz, 1, H5), 5.90 (d, $J_{3'\text{OH-}\ 3'}$ = 4.3 Hz, 1, OH3'), 6.31 (d, $J_{1'\text{-}2'}$ = 5.7 Hz,1, H1') 7.85 (d, $J_{6\text{-}5}$ = 7.4 Hz, 1, H6); ^{13}C NMR (DMSO-d_6) δ 61.68(C5'), 75.77(C4'), 89.60(C3'), 90.08(C2'), 90.88(C1'), 109.46(C5), 137.73(C6), 160.66(C2), 172.07(C4); MS (CI) *m/z* 227 (100, $MH^+[^{16}O]$).

1-(2,2'-Anhydro-3'-^{18}*O*-β-D-arabinofuranosyl)uracil (43b)

Deprotection of compound **42b** (8 mg, 0.034 mmol) as described for **42a** gave compound **43b** (3mg, 73%) with identical physical and spectroscopic properties except for the mass spectrum: MS (CI) *m/z* 229 (100, $MH^+[^{18}O]$), 227 (37, $MH^+[^{16}O]$).

1-(2,2'-Anhydro-3'-^{17}O-β-D-arabinofuranosyl)uracil (43c)

Deprotection of compound **42c** (22 mg, .05 mmol) as described for **42a** gave compound **43c** (7.4 mg, 65%) with identical physical and spectroscopic properties except for the mass spectrum: MS (CI) *m/z* 228 (98, $MH^+[^{17}O]$), 227 (100, $MH^+[^{16}O]$).

2'- Azido-2'-deoxyuridine (44a)

Lithium fluoride (47 mg, 1.8 mmol) was suspended in DMF (2 mL), and the resulting suspension was heated to 105 °C with stirring. Then *N,N,N,N* - tetramethylethylenediamine (2 mL) was added followed by azidotrymethylsilane (0.24 mL).[37] After stirring for 30 min, compound **43a** (0.226 g, 1.0 mmol) was added, and the reaction was allowed to proceed for 48 h at 110 °C (oil bath temperature). The volatiles were evaporated under vacuum, and the residue was co-evaporated three times with methanol. The residue was dissolved in methanol (10 mL), and ethyl acetate (40 mL) was added to precipitate most of the salts and residual starting materials. The filtered solution was applied to a silica gel column and eluted with methanol/ethyl acetate (20/80) to give **44a** (0.22 g, 83%) as a yellowish foam (0.22 g, 83%). This material was re-purified by RP-HPLC (elution, 15% CH_3CN/85% H_2O) to yield pure **44a** (0.14 g, 52%) as an off-white foam: UV max 261 nm, min 232 nm; ^{1}H NMR (DMSO-d_6) δ 3.62 (m, 2, H5'/5"), 3.89 (s, 1, H4'), 4.04 (d, $J_{3'\text{-}2'}$ = 5.3 Hz, 1, H3'), 4.29 ('q', $J_{2'\text{-}1'/3'}$ = 5.0 Hz, 1, H2'), 5.20 (t, $J_{5'\text{OH-}\ 5'/5''}$ = 5.0 Hz, 1, OH5'), 5.68 (d, $J_{5\text{-}6}$ = 8.0 Hz, 1, H5), 5.87 (d, $J_{3'\text{OH-}\ 3'}$ = 5.4 Hz, 1, OH3'), 5.90 (d, $J_{1'\text{-}2'}$ = 5.4 Hz, 1, H1') 7.67 (d, $J_{6\text{-}5}$ = 8.0 Hz, 1, H6), 11.4 (s, 1, NH); MS (CI) *m/z* 270 (100, $MH^+[^{16}O]$).

2'-Azido-3'-^{17}*O*-2'-deoxyuridine (44c)

Treatment of compound **43c** (50 mg, 0.22 mmol) as described for **44a** gave compound **44c** (27 mg, 45%) as a yellowish foam. RP-HPLC purification gave compound **44c** as an off-white foam (16 mg, 27%) with identical physical and spectroscopic properties except for the mass spectrum: MS (APCI) *m/z* 271 (100, $MH^+[^{17}O]$), 270 (98, $MH^+[^{16}O]$).

2'-Azido-2'-deoxy-5'-*O*-(*para*-toluenesufonyl)uridine (45a)

P-toluenesulfonyl chloride (21.08 mg, 0.11 mmol) was added to a stirred solution of compound **44a** (20 mg, 0.074 mmol) in anhydrous pyridine (1 mL) at ambient temperature. After 14 h the reaction mixture was evaporated, and the residue was partitioned (0.1 N $HCl/H_2O//CHCl_3$). The organic layer was washed ($NaHCO_3$, NaCl), dried ($MgSO_4$), and evaporated. TLC in S6 showed a small amount of upper moving 3',5'-*O*-ditosylated compound, but the lower moving 5'-*O*-monotosylated **45a** was the major product. This crude material was chromatographed on a silica gel column. Gradient elution ($CHCl_3$ → 5%MeOH/$CHCl_3$) and evaporation of the appropriate fractions (R_f for compound **45a** in S6 was 0.4 and 3',5'-*O*-ditosylated byproduct had R_f 0.6) gave compound **45a** (21 mg, 67 %): UV max 226, 262 nm; ^{1}H NMR δ 4.17 (s, 2, H5'/5"), 4.32 ("t", J = 11.1 Hz, 1, H4'), 4.36 (br s 1, H3') 4.40 ("d", J = 5.5 Hz, 1, H2'), 5.75 (d, $J_{5\text{-}6}$ = 8.0 Hz, 1, H5), 5.88 (d, $J_{1'\text{-}2'}$ = 2.8 Hz, 1, H1'), 7.41 (d, J = 7.6 Hz, 2, H_{arom}) 7.51 (d, $J_{6\text{-}5}$ = 7.7 Hz, 1, H6) 7.82 (d, J = 7.4 Hz, 2, H_{arom}) 9.24 (s, 1, NH); ^{13}C NMR δ 22.16 (Me), 66.32 (C5'), 67.90 (C3') 70.18 (C2'), 81.65(C4'), 88.72 (C1'), 103.31(C5), 128.31 ($C3_{arom}$) 130.65 ($C2_{arom}$) 132.43 ($C4_{arom}$) 139.68 (C6), 145.50 ($C1_{arom}$) 150.51 (C2),

163.49 (C4); MS (APCI) *m/z* 424 (100, $MH^+[^{16}O]$), 425 (17.5, $MH^+[^{16}O]$+1), 426 (8, $MH^+[^{16}O]$+2).

2'-Azido-2'-deoxy-3'-^{17}*O*-5'-*O*-(*para*-toluenesufonyl)uridine (45c)

Treatment of compound **44c** (17 mg, 0.063 mmol) with tosyl chloride as described for **45a** gave compound **45c** (17 mg, 63%) with identical physical and spectroscopic properties except for the mass spectrum: MS (APCI) *m/z* 424 (98, $MH^+[^{16}O]$), 425 (100, $MH^+[^{17}O]$), 426 (45, $MH^+[^{17}O]$+1).

Preparation of tris(Tetra-*n*-butylammonium) hydrogen Pyrophosphate

Method A. Tetrasodium pyrophosphate (1.11g, 2.5 mmol) was dissolved in deionised water (10 mL). The dissolved salt was applied to a column of Dowex 50W x 2-200 (H^+) ion exchange resin (2% cross linking, 100-200 mesh, hydrogen form) and eluted with deionised water (~30 mL). Acidic fractions (pH ~1.5) were collected, and the resulting solution was neutralized (pH ~7.3) by dropwise addition of a 40% (wt/wt) solution tetra-*n*-butylammonium hydroxide in water. The neutralized solution was evaporated to yield a viscous, clear syrup, which upon high vacuum drying gave a hygroscopic white solid (2.2 g, 98%). ^{1}H NMR (D_2O), 0.77 (t, J = 7.3 Hz, 3H), 1.20 (m, 4H), 3.02 (t, J = 8.3 Hz, 2H); ^{31}P NMR (D_2O) -6.67 (s).

Note: Tetrasodium pyrophosphate can be substituted by disodium hydrogen pyrophosphate to give tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (97% yield) with identical spectroscopic characteristics.

Method B. A 10% solution (v/v) of disodium hydrogenpyrophosphate (3.33g, 15.0 mmol) in aqueous ammonium hydroxide (15 mL) was passed through a 2.5 cm x 7.0 cm column of Dowex 50WX2-200 ion exchange resin (2% cross linking, 100-200-mesh, hydrogen form). The free acid was eluted with deionised water (100 mL), and the resulting solution (pH ~1.5) was immediately neutralized to pH 7.3 with aqueous tetra-*n*-butylammonium hydroxide. The resulting solution (approximately 150 ml total volume) was dried by lyophilization to yield 13.1 g (97%) of a hygroscopic white solid.

2'-Azido-2'-deoxyuridine 5'-diphosphate (46a)

Compound **45a** (7 mg, 0.017 mmol) was dissolved in acetonitrile (0.2 mL) and tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (25 mg, 0.028 mmol), prepared as above, was added in one portion. The reaction mixture was stirred at room temperature for 48 h. Volatiles were evaporated, and the residue was dissolved in water and purified on an ion exchange column (Sephadex-DEAE A-25, 40-120μ). Gradient elution with triethylammonium bicarbonate (0.05N → 0.5N), evaporation of the appropriate fractions, and co-evaporation with H_2O and MeOH (R_f for compound **46a** in S7 was 0.6 and compound **45a** had R_f 0.8) gave compound **46a** as the triethylammonium salt (4.2 mg, 52%): UV max 261 nm, min 227 nm; ^{1}H NMR (D_2O) δ 4.09 (m 2, H5'/5"), 4.14 ("t", J = 2.8 Hz, 1, H4'), 4.26 (t, $J_{3'-2'}$ = 5.3 Hz, 1, H3') 4.49 (t, $J_{2'-1'}$ = 5.0 Hz, 1, H2'), 5.84 (d, J_{5-6} = 8.1 Hz, 1, H5), 5.95 (d, $J_{1'-2'}$ = 5.2 Hz, 1, H1'), 7.85 (d, J_{6-5} = 8.0 Hz, 1, H6); ^{31}P NMR – 10.24 (d, J = 19.5 Hz), -9.57 (br s). ^{1}H NMR showed a triplet (1.2 ppm) and a quartet (3.4 ppm) for the triethylammonium counterions, and ^{31}P NMR showed a considerable amount of pyrophosphate impurity (-9.50 ppm) in the product.

2'-Azido-2'-deoxy-3'-^{17}O-uridine 5'-diphosphate (46c)

Treatment of compound **45c** (17 mg, 0.041 mmol) with tris(tetra-*n*-butylammonium) hydrogen pyrophosphate (54 mg, 0.06 mmol) as described for **46a** gave compound **46c.** Purification with ion exchange resin (Sephadex-DEAE A-25, 40-120μ) and co-evaporation with H_2O and MeOH gave **46c** (8 mg, 44%). This compound was dissolved in water and converted to the sodium salt by passing a solution through a DOWEX (Na^+) column [prepared from DOWEX-50W (H^+), 2%-cross linking, 100-200 mesh]. Final purification by RP-HPLC (gradient elution, 3% H_2O/CH_3CN, R_T-5.6 min., semi-prep column, supelcosil LC-18T) gave **46c** (4 mg, 22%) with 50% pyrophosphate as impurity: ^{1}H NMR (D_2O) δ 4.09 (m 2, H5'/5"), 4.14 ("t", J = 2.8 Hz 1, H4'), 4.26 (t, $J_{3'-2'}$ = 5.3Hz, 1, H3') 4.49 (t, $J_{2'-1'}$ = 5.0 Hz, 1, H2'), 5.84 (d, J_{5-6} = 8.1 Hz, 1, H5), 5.95 (d, $J_{1'-2'}$ = 5.2 Hz, 1, H1') 7.85 (d, J_{6-5} = 8.0 Hz, 1, H6); ^{31}P NMR –10.17 (d, J = 21.1Hz, P_α), -9.26 (d, J = 21.1 Hz, P_β).

CONCLUSION

Ribonucleotide reductases (RNRs) are enzymes that execute 2'-deoxygenation of ribonucleoside 5'-di and tri-phosphates in unique *de novo* biosynthetic pathways to DNA monomers. In 1976, it was reported that pyrimidine 2'-azido-2'-deoxynucleotide 5'-diphosphates were potent inactivators of ribonucleoside diphosphate reductase (RDPR). Inactivation of RDPR by 2'-azido 2'-deoxynucleosides was accompanied by appearance of a new EPR signal for a nitrogen-centered radical with concomitant decay of the peaks of tyrosyl radical. This nitrogen-centered radical has been characterized by EPR spectroscopy and shown to be composed of a 1:1:1 anisotropic triplet arising from interaction with a single nitrogen and a second hyperfine interaction possibly derived from a proton. The structure of this elusive nitrogen radical has been studied extensively and shown to be derived from the azide moiety. EPR and site-directed mutagenesis studies with the model compounds have led to the conclusion that the nitrogen-centered radical must have the structure $\mathbf{XN^{\bullet}S_{cys225}R1}$ in which X is an oxygen or carbon atom (presumably 3') having no hydrogen attached or with a single hydrogen attached.

The goal of this study was to synthesize 3'[^{17}O]-2'-azido-2'-deoxyuridine 5'-diphosphate. Such a labeled analogue should perturb the EPR spectrum of nitrogen centered radical in a predictable fashion, and the hyperfine interaction between the free electron and the ^{17}O nucleus should allow a choice between the recently proposed structures of nitrogen-centered radical by Vander Donk, *et al* (*J. Am. Chem. Soc.* **1995,** *117*,8908). In addition, this stereoselective ^{17}O-labeling methodology could be employed as a general procedure for the synthesis of 3'[^{17}O and ^{18}O] labeled pyrimidine nucleosides.

The labeled 2'-azido-2'-deoxynucleotides were prepared by thermolysis of O^2,3'-anhydrouridine to give the more stable O^2,2'-anhydrouridine derivatives with concomitant rearrangement of the 2'-*O*-benzoyl[^{17}O] group into the 3'-[^{17}O]-benzoyl intermediate. Deprotection and ring opening with LiF/Me_3SiN_3 gave the 3'[^{17}O]-2'-azido-2'-deoxyuridine. Tosylation (O5') and displacement of the 5'-tosylate by treatment with tris(tetra-*n*-butylammonium) hydrogen pyrophosphate gave 3'[^{17}O]-2'-azido-2'-deoxyuridine 5'-diphosphate. All intermediates and products were characterized by ^{1}H-NMR, ^{13}C-NMR, ^{31}P-NMR, and isotopic enhancement was confirmed by using mass spectrometry.

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