SYNTHESIS OF C-2 AND C-6 FUNCTIONALIZED RIBOFURANOSYLPURINE ANALOGUES AS POTENTIAL ANTIVIRAL AGENTS TARGETING INHIBITION OF INOSINE MONOPHOSPHATE DEHYDROGENASE.

by

Eric Osei-Tutu Bonsu

(Under the Direction of Vasu Nair)

ABSTRACT

IMPDH is a key enzyme in the *de novo* biosynthesis of purine nucleotides. It catalyzes the conversion of inosine monophosphate (IMP) to xanthosine monophosphate (XMP) using NAD as a cofactor. XMP is successively converted to deoxyguanosine triphosphate (substrate for DNA synthesis) by GMP synthetase, phosphorylating enzymes and ribonucleotide reductase. IMPDH exists in two isoforms, type I and type II. These isoforms have the same size and share 84% homology. The type I isoform is expressed in both normal and rapidly proliferating cells, whereas type II is preferentially upregulated in proliferating cells.

Inhibition of IMPDH has anticancer, antiviral, antibacterial and immunosuppressive effects. Three inhibitors of IMPDH are currently in clinical use: ribavirin (a broad spectrum antiviral), mizoribine (immunosuppressant used in Japan) and mycophenolic mofetil (prodrug of mycophenolic acid, US approved immunosuppressant). None of these inhibitors possess significant selectivity against the type II isoform over the type I, hence there are severe side effects. The quest for specific isozyme inhibitors led to the discovery of the potential antiviral activity of certain C-2 functionalized hypoxanthine and C-2, C-6 modified purine systems against HSV1, HSV2, VV, VSV and RSV.

Nair and coworkers have synthesized similar congeners, including 2-vinylinosine (broad spectrum antiviral), which is active due to its C-2 vinyl moiety acting as a Michael Acceptor. This dissertation elucidates the design and synthesis of new Michael Acceptor-type nucleosides. These novel compounds were designed mechanistically as transition-state analogues similar to mizoribine 5′-monophosphate and "Fat base" nucleotide (two potent IMPDH inhibitors). The structural design was fashioned to mimic the hydrogen bonding interactions observed in the crystal structure of the E-XMP* adduct of human IMPDH II and IMP.

The C-2 functionalized hypoxanthine nucleosides were synthesized *via* a newly developed reproducible, efficient and high-yielding non-enzymatic methodology, whereas the C-2 and C-6 functionalized ribofuranosylpurine nucleosides were synthesized by Stille coupling from either the 2-I, 6-Cl or 2-I, 6-I ribofuranosylpurine intermediate. Both methodologies represent a significant improvement over previous methodologies. The modifications involved moities such as 2-furyl, 2-thienyl, vinyl, thio, methoxy and thiomethyl. These compounds were designed and synthesized as potential antiviral compounds targeting IMPDH inhibition.

INDEX WORDS: IMPDH, Stille Coupling, Michael Acceptors, Antiviral.

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May 2005

DEDICATION

This work is sincerely dedicated to the three special ladies in my life. To my daughter Chanel thanks for being born. You have always and will dearly remain a source of inspiration and love to “Daddee”. To my best and most beautiful friend and wife Akyere, words can not justify how much you mean to me. Thanks for your love, support and patience throughout the years. It is comforting knowing I can always count on you. To my mother, “Omaa”, this will be non-existent had it not been for you. I love you dearly. Thanks for your unmatched motherly love, encouragement and prayers.

Special gratitude goes to my father for his love and proper upbringing. Love and miss you so much.

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LIST OF ABBREVIATIONS

Ac_2O	acetic anhydride
ACV	acyclovir
ADA	adenosine deaminase
ADP	adenosine 5′-diphosphate
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AIDS	acquired immunodeficiency syndrome
AIR	5-Aminoimidazole ribotide
AK	adenosine kinase
AMP	adenosine 5′-monophosphate
APRT	adenine phosphoribosyl transferase
Arg	arginine
Asp	aspartic acid
ATCase	aspartate transcarbamoylase
ATP	adenosine 5′-triphosphate
AZT	3′-azido thymidine
$Bu_3SnCH{=}CH_2$	tributyl(vinyl) tin
$Bu_3Sn(C_4H_3O)$	tributyl(furan) tin
$Bu_3Sn(C_4H_3S)$	tributyl(thiophene) tin
BVDU	(E)-5-(2-bromovinyl)-2′-deoxyuridine
BVisoDU	(E)-5-(2-bromovinyl)-2′-isodeoxyuridine
C	cytosine
CAIR	carboxyaminoimidazole ribotide

Calcd.	calculated
cAMP	cyclic adenosine monophosphate
CCl_4	tetrachloromethane
$(CF_3SO_2)_2O$	triflic anhydride
$CHBr_3$	bromoform
CH_3CN	acetonitrile
CH_3SiI	trimethylsilyl iodide
CH_2I_2	diiodomethane
CHI_3	iodoform
CMP	cytidine 5′-monophosphate
CMV	cytomegalovirus
CPE	cytopathic effects
CPS-I	carbmoyl phosphate synthetase I
CPS-II	carbmoyl phosphate synthetase II
CTP	cytidine 5′-triphosphate
CI_4	tetraiodomethane
dCK	2′-deoxycytidine kinase
dCMP	2′-deoxycytidine 5′-monophosphate
ddC	2′,3′-dideoxycytidine
ddI	2′,3′-dideoxyinosine
DEA	N, N-diethylaniline
decomp.	decomposition
dGK	2′-deoxyguanosine kinase

DMAP	4-(dimethylamino) pyridine
DMF	N, N-Dimethylformamide
dNDP	deoxynucleoside diphosphate
dNTP	deoxynucleoside triphosphate
dTDP	2′-deoxythymidine 5′-diphosphate
dTMP	2′-deoxythymidine 5′-monophosphate
dTTP	2′-deoxythymidine 5′-triphosphate
dUDP	2′-deoxyuridine 5′-diphosphate
dUTP	2′-deoxyuridine 5′-triphosphate
EICAR	5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
Et_3N	triethylamine
Et_4NF	tetraethylammonium fluoride
$Et_3N.3HF$	triethylamine trihydrofluoride
FAB	fast atomic bombardment
FAD	flavin adenine dinucleotide
FAICAR	formamidoimidazole carboxamide ribonucleotide
FGAM	formylglycinamidine ribonucleotide
FGAR	formylglycinamide ribonucleotide
GAR	glycinamide ribonucleotide
Gln	glutamine

Glu	glutamic acid
Gly	glycine
GMP	guanosine 5′-monophosphate
GTP	guanosine 5′-triphosphate
HBV	hepatitis B virus
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
HHV	human herpes virus
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HPV	human papilloma virus
HRMS	high resolution mass spectrometry
HSV	herpes simplex virus
HTLV-1	human T-lymphotropic virus type 1
ICTV	international committee on viral taxonomy
IMP	inosine 5′-monophosphate
IMPDH	inosine 5′-monophosphate dehydrogenase
IsoddA	(S,S)-2′,3′-isodideoxyadenosine
JEV	Japanese encephalitis virus (alphavirus)
K_2CO_3	potassium carbonate
kDA	kilodalton
KF	potassium fluoride

MAD	mycophenolic adenine dinucleotide
MeOH	methanol
$MgSO_4$	magnesium sulfate
MMF	mycophenolic mofetil
MPA	mycophenolic acid
mp	melting point
mRNA	messenger ribonucleic acid
NA	nucleoside analogues
NAD^+	nicotinamide adenine dinucleotide
NDP	nucleoside diphosphate
NH_4F	ammonium fluoride
NMP	nucleoside monophosphate
NMR	nuclear magnetic resonance
NNA	non-nucleoside analogue
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
NTP	nucleoside triphosphate
OMP	orotate 5′-monophosphate
$Pd(CH_3CN)_2Cl_2$	bis(acetonitrile) dichloropalladium
$Pd(PPh_3)_4$	tetrakis(triphenylphosphine) palladium
Pi	phosphate ion
PIC	pichinde (arenavirus)
PNP	purine nucleoside phosphorylase

$POCl_3$	phosphorous oxychloride
PRPP	5-phospho-α- D-ribosyl-1-pyrophosphate
PT	punta toro (phlebovirus)
Pur	purine
PVAS	polyvinylalcohol sulphate
PVS	polyvinylsulphonate
Pyr	pyridine
RNA	ribonucleic acid
RR	ribonucleotide reductase
RMP	ribavirin 5′-monophosphate
rRNA	ribosomal ribonucleic acid
RSV	respiratory syncitial virus
RT	reverse transcriptase
RTP	ribavirin 5′-triphosphate
RVF	rift valley fever (phlebovirus)
SAD	selenazofurin adenine dinucleotide
SAH	S-adenosylhomocysteine hydrolase
SACAIR	5-Aminoimidazole-4-(N-succinylo-carboxamide) ribonucleotide
SAMP	adenylosuccinate monophosphate
SCID	severe combined immunodeficiency syndrome
Ser	serine
$Sn(CH=CH_2)_4$	tetravinyl tin
T	thymine

TAD	tiazofurin adenine dinucleotide
TBAF	tetrabutylammonium fluoride
TBDMSCl	tert-butyldimethylsilyl chloride
tBuONO	tert-butylnitrite
TCA	citric acid cycle
THF	tetrahydrofolate
Thr	threonine
Thy	thymine
TMSCCH	trimethylsilylacetylene
tRNA	transfer ribonucleic acid
TS	thymidylate synthase
Tyr	tyrosine
U	uracil
UDP	uridine 5′-diphosphate
UMP	uridine 5′-monophosphate
UTP	uridine 5′-triphosphate
UV	ultraviolet
VEE	Venezuelan equine encephalomyelitis (alphavirus)
VSV	vesicular stomatitis virus (rhabdovirus)
VV	vaccinia virus
VZV	varicella zoster virus
XMP	xanthosine 5′-monophosphate
XO	xanthine oxidase

YF	yellow fever (flavivirus)
2-FVIMP	2-[2-(*Z*)-fluorovinyl]inosine-5′-monophosphate
2-VIMP	2-vinylinosine-5′-monophosphate

CHAPTER 1

NUCLEOSIDES AND NUCLEOTIDES

NUCLEIC ACIDS

The period, 1869 through to 1930, saw the discovery and isolation of "impure" nucleic acids (DNA-protein complex) by Friedrich Miescher, to the first purified protein-free samples of nucleic acids by Richard Altman and the determination of the composition of nucleic acids as well as the discovery of deoxyribonucleic acid (DNA).[1-5] Since then, tremendous achievement has been made in understanding the structure, composition and biochemistry of nucleic acids as it relates to their structure and function as the genetic material. Nucleic acids are made up of nucleotides; each nucleotide is composed of a nitrogenous base linked to a sugar to which at least one phosphate group is attached. The bases are planar, aromatic, heterocyclic and are structural derivatives of either purine or pyrimidine.[6]

PYRIMIDINES

Pyrimidine is an aromatic, six-membered ring that contains two nitrogens at positions 1 and 3 (Figure 1.1). There are two numbering systems: the IUPAC and the Beilstein (Figure 1.1).[6] The IUPAC system will be used throughout this dissertation.

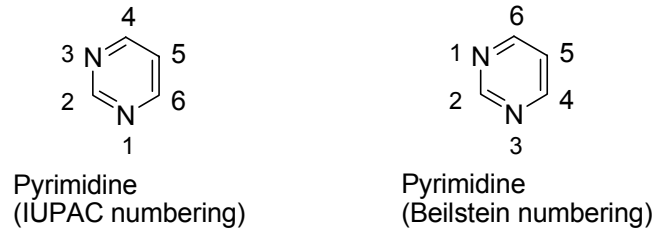


Figure 1.1. Numbering Systems of Pyrimidine Ring.

The most abundant pyrimidines found in nature are uracil (U, in RNA), cytosine (C, RNA and DNA) and thymine (T, DNA). However, certain modified forms such as 5-methylcytosine and 5-hydroxymethylcytosine (Figure 1.2) are sometimes found in DNA and certain viruses, respectively. Transfer RNAs are also known to contain small quantities of other methylated pyrimidines.[7]

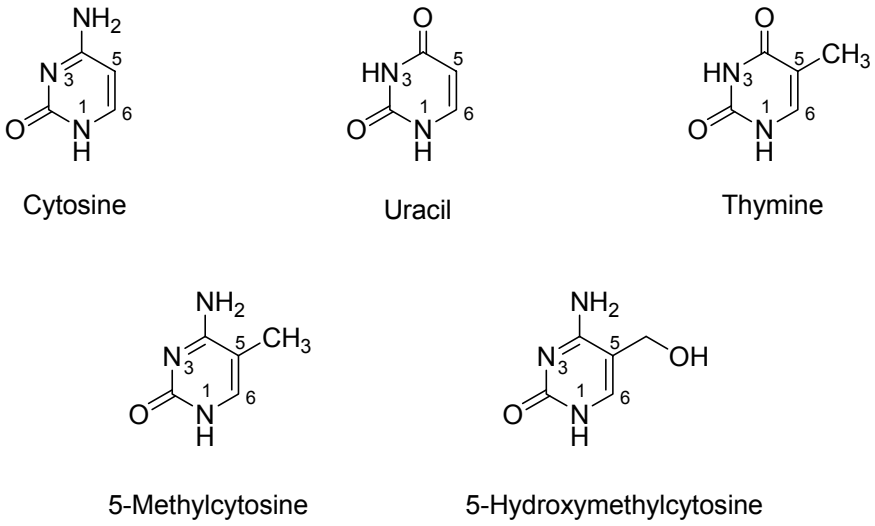


Figure 1.2. Major and Minor Pyrimidines.

There are two well-known processes that occur with pyrimidines that relate to their structure and function: tautomerization and photodimerization. Due to tautomerization, pyrimidines can exist in equilibrium either in the cyclic amide form (lactam) or in the lactim form. This equilibrium leans heavily towards the lactam with an equilibrium constant in the range of 10^{-4}. In Figure 1.3, the lactim forms 3 and 4 are possible for non-bonded thymine, but in nucleic acids N-1 is bonded to C-1 of a ribose or deoxyribose, so that forms 3 and 4 are impossible.[6]

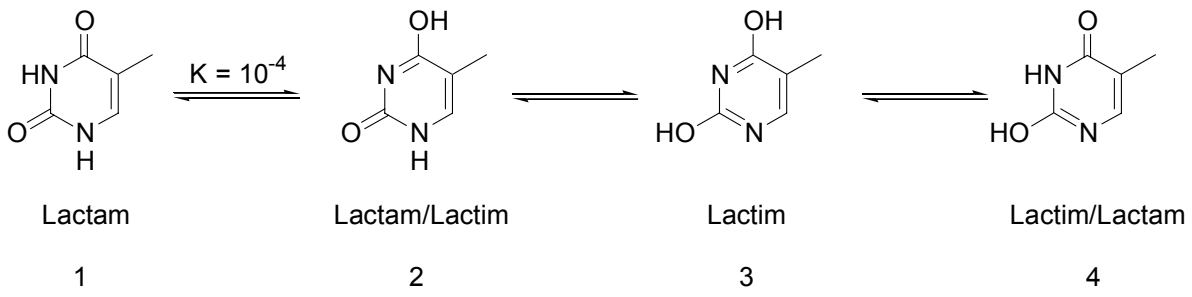


Figure 1.3. Tautomerization of Thymine.

Photodimerization is of particular biological importance, because exposure of solutions of thymine to ultraviolet radiation (UV) (210 nm < λ < 280 nm) results in dimerization of thymine. This phenomenon can cause the inactivation of DNA as adjacent thymines in a strand of DNA can photodimerize and disrupt DNA replication. Although other pyrimidines can also dimerize, thymine dimers are the most important biologically. This inactivation of DNA by UV radiation is the basis for germicidal lights used in hospitals.[6, 8]

PURINES

Purine derivatives will be dealt with in depth because most of this dissertation centers on chemical modifications of purine nucleosides.

The purine ring can be seen as an annulation of a pyrimidine and an imidazole ring. This tetraaza bicyclic molecule is aromatic with its π-electrons extensively delocalized over both ring systems.[9] The numbering system of purines follows the Beilstein convention with regard to the pyrimidine ring (Figure 1.4).

The effect of four electronegative nitrogen atoms in the purine ring causes an overall π-electron deficiency. This deficiency dictates to a large extent the chemistry and reactivity of

purines, such that nucleophilic substitution reactions are the predominant reaction types on the purine ring, particularly on the carbon atoms. Electrophilic reactions (except at nitrogen) are relatively unusual.

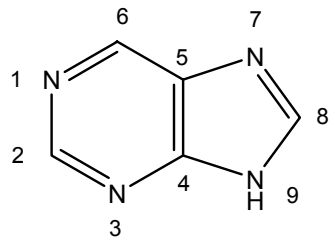


Figure 1.4. Numbering System of Purine Ring.

The presence of either electron-donating or electron-withdrawing groups on the purine ring influences the regioselectivity (point of attack) of nucleophilic or electrophilic agents. Also, whether or not the ring is in the ionized form, and the kind as well as degree of substitution, determines the relative reactivity of the various sites in purines to reactions of any given type.

To better understand the chemistry at the individual atom sites around the purine ring, comparison will be made with pyrimidine (resonance forms), imidazole, pyridine and nitrobenzene. Also a look at the calculated (approximate) π-electron densities at the individual atom sites (Table 1.1) puts the reasoning into perspective.[10]

Table 1.1. Electron Densities Around Purine and Pyrimidine.

Position	1	2	3	4	5	6	7	8	9
Purine	1.195	0.902	1.216			0.907	1.308	0.895	1.592
Pyrimidine	1.095	0.925	1.095	0.946	0.993	0.925			

Compared to the imidazole part, the pyrimidine part of the purine ring is π-electron deficient. The chemistry at N-1 through to C-6 in the purine system is comparable to that of the corresponding positions in unfused pyrimidines (the C-4 and C-5 positions of pyrimidine correspond to the purine ring annulation site, which is not commonly available for chemical reactions). Because of the effects of the electronegative nitrogens at N-1 and N-3 of pyrimidine (and also purines), the reactivity of C-2 and C-6 (of purines) has been likened to that of C-2 and C-6 of unfused pyrimidines, the C-2 and C-4 in pyridine, or the ortho and para sites in nitrobenzene. At these loci nucleophilic attack is facile. Resonance forms of pyrimidine illustrating this phenomenon are shown in figure 1.5.[11]

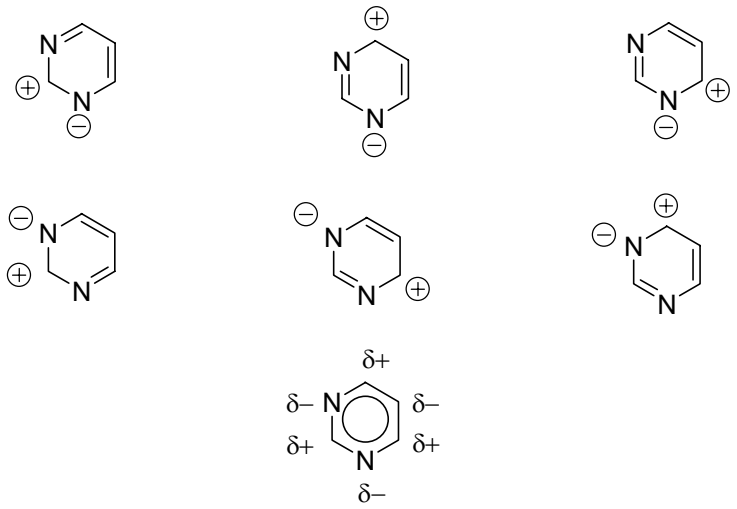
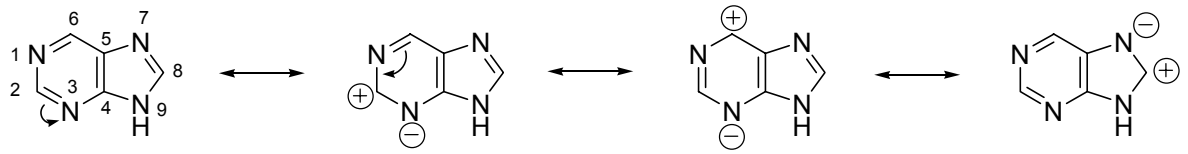


Figure 1.5. Resonance Forms of Pyrimidine Ring.

Considering the purine ring as a whole, it is evident that the principal resonance structures show that positions 2, 6 and 8 are susceptible to nucleophilic attack, whereas positions 3 and 7 being electron rich are susceptible to electrophilic attack (Scheme 1.1).



Scheme 1.1. Principal Purine Resonance Structures.

Electron-donating groups on the purine ring tend to offset the π-electron deficiency, by increasing the electron-density at each ring carbon, thus making the purine ring more aromatic, comparable to benzene. This allows electrophilic substitution to occur more readily and results in diminished susceptibility of positions C-2 and C-6 to nucleophilic attack.

On the other hand, electron-withdrawing groups on the pyrimidine moiety of the purine ring makes C-2 and C-6 increasingly susceptible to nucleophilic attack. Addition reactions are characteristic of diminished aromatic delocalization and are also more favorable in the presence of electron-withdrawing substituents.

Imidazole provides a good model for the reactivity at C-7 and C-9 in purines. The labile imidazole -NH- confers acidicity on purines. Carbon-8 of purines resembles the C-2 position of free imidazole. The C-8 position of purine is therefore relatively susceptible to electrophilic attack. The deficiency of the adjacent pyrimidine ring, however, draws some electron density away from the imidazole fragment, making it less electron-rich than imidazole itself.

Neutral (unionized) purines are susceptible to nucleophilic attack in the order of reactivity C-8 > C-6 > C-2.[11, 12, 13] If the purines are converted to an anionic form by loss of the imidazole proton, the resulting order becomes C-6 > C-2 > C-8.[12]

The purine ring is acidic in property because of the labile imidazole proton. N-alkylation and other N-substitutions destroy the acidic properties of the ring system by displacement of the dissociable proton.

The purine ring is also basic in nature. This is due to the presence of nitrogens (N-1, N-3 and N-7) that have lone pairs of electrons that are non-participating in the delocalized π-system. These lone pairs of electrons are available for donation and like the nitrogen lone pair electrons of pyridine, accounts for the basicity of the purine system. Electron-donating substituents increase the basicity of purines. Electron-withdrawing substituents decrease basicity and, if a dissociable proton is present, increase the acidity of the purine system. pKa values (for dissociation of a proton from a neutral species) of 6-chloro, 6-unsubstituted, 6-amino and 2,6-diaminopurine are 7.7, 8.9, 9.8 and 10.8 respectively, as might be expected.[14-17]

Adenine and guanine are the most common purines. Xanthine, hypoxanthine and uric acid are also important purines, although they are far less abundant than adenine and guanine. (Figure 1.6).

CARBOHYDRATES

This section on carbohydrates is not intended to be a complete review, but rather a summary of the pertinent issues relating to nucleosides and nucleotides and the biology and chemistry of DNA and RNA.

D-Ribose and D-2-deoxyribose form the two very important five-membered sugars: β-D-ribofuranose and β-D-2-deoxyribofuranose. These sugars are predominant in natural nucleosides, nucleotides and nucleic acids as seen in DNA and RNA units. Their formation is the result of C-4 hydroxyl attack on the C-1 aldehyde of either D-ribose or D-2-deoxyribose in their open-chain

forms. This attack leads to the formation of the α and β anomers of D-ribofuranose and D-2-deoxyribofuranose.

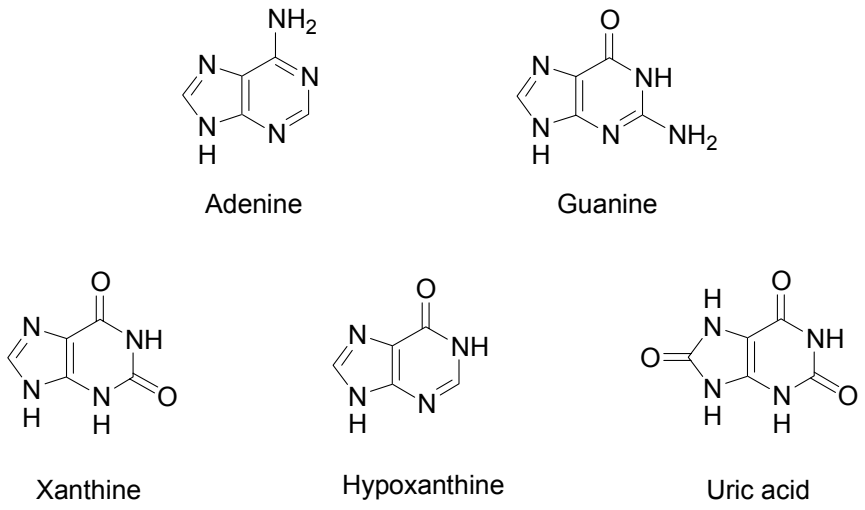
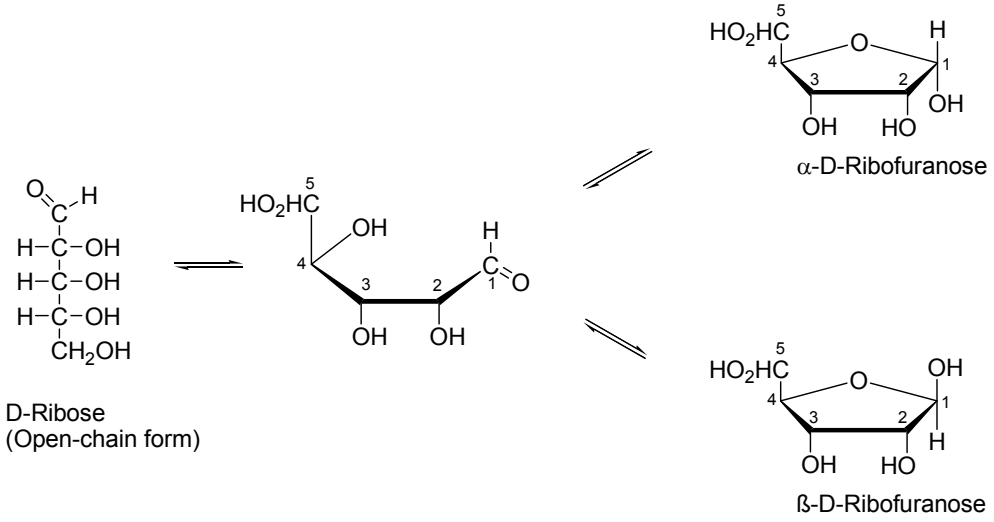


Figure 1.6. Purine Bases.

Scheme 1.2 shows the case for α and β-D-ribofuranose. The replacement of the C-2 hydroxyl with hydrogen is the corresponding case for α and β-D-2-deoxyribofuranose.[18]

GLYCOSIDIC BONDS

Carbohydrates can be joined to alcohols or amines by glycosidic bonds. For virtually all naturally-occurring biomolecules including nucleosides, nucleotides, RNA and DNA, the N-glycosidic bonds have the β-configuration. Generally, glycosidic bonds are stable in basic solutions, because they are acetals. However, in acidic solutions, they undergo hydrolysis to form the corresponding carbohydrate and alcohol or amine depending on the starting compound.[6, 18] The hydrolysis of glycosidic bonds will be discussed later.



Scheme 1.2. Cyclization of D-ribose.

NUCLEOSIDES

Nucleosides are made up of heterocyclic bases of two classes: the pyrimidines and the purines, connected through a β-N-glycosidic bond to a carbohydrate. The carbohydrate is almost always either β-D-ribofuranose or 2-deoxy-β-D-ribofuranose as shown earlier in Scheme 1.2. Figure 1.7 shows the structures of some common nucleosides. Their corresponding 2′-deoxyribonucleosides are formed when the 2′-hydroxyl group is replaced by a hydrogen atom.

The numbering system for the nucleobase (purine or pyrimidine) follows the same convention for the free bases, whereas the carbon atoms of the carbohydrate are numbered with primes to distinguish them from the nucleobase numbers (Figure 1.7).

The glycosidic bond in nucleosides is formed between the C-1′ of the carbohydrate and the N-1 of the pyrimidine base or the N-9 of the purine base to give the pyrimidine and purine nucleosides respectively. The plane of the purine or pyrimidine ring bisects the plane of the carbohydrate ring almost perpendicularly.[6, 19]

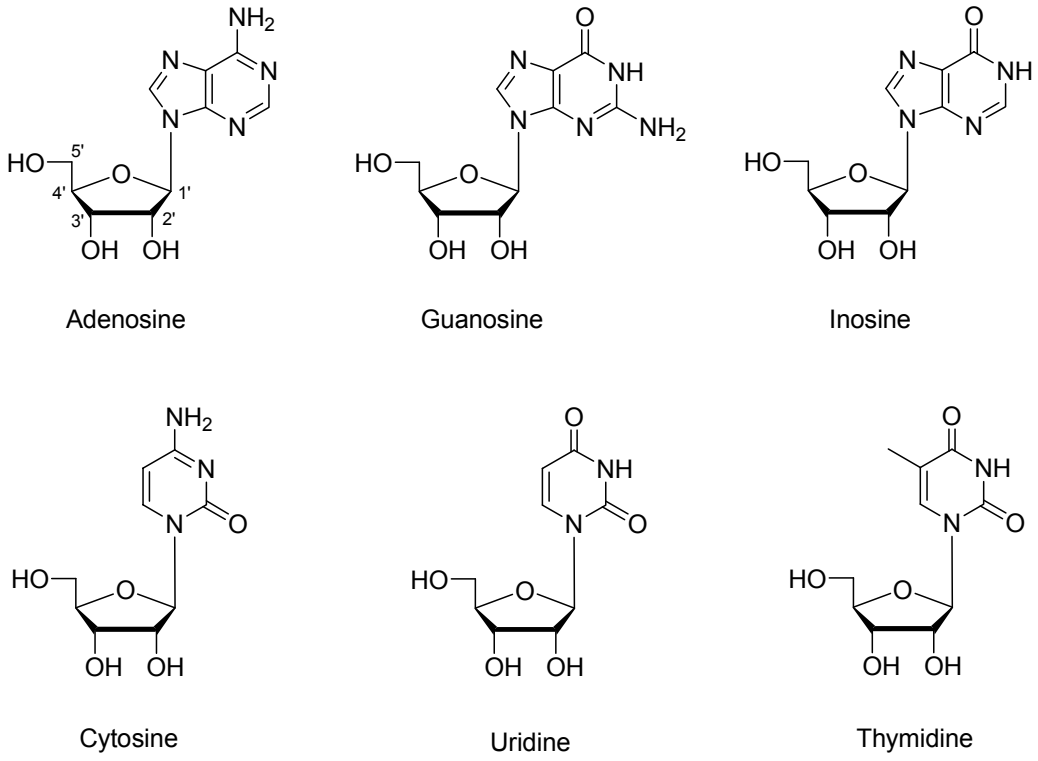


Figure 1.7. Structures of Common Nucleosides.

The furanose sugar rings in nucleosides are not planar due to the tetrahedral geometry of their saturated carbon atoms. They assume conformations that are either half-chair (T) or an envelope (E). In the half-chair conformation, C-2′ and C-3′ are displaced above or below a plane defined by C-4′, O-1′ and C-1′, whereas in the envelope conformation, they are puckered such that a single atom, either C-2′ or C-3′ is displaced about 5 Å above or below a plane defined by the respective remaining atoms (Figure 1.8). In both cases, if the displaced atom lies above the defined plane (i.e., toward the ring or on the same side of the plane as the C-5′ atom); the ring conformation is known as endo. If the displaced atom lies below the defined plane (i.e., on the opposite side of the C-5′ atom), the ring conformation is known as exo. In free nucleosides, the C-2′ endo and C-3′ endo conformations are in about equal proportions. The C-3′ exo

conformation is found in deoxyribonucleosides, the C-2′ exo conformation has not been found in nucleosides, nucleotides, or in nucleic acids.[20-22] In naturally occurring biomolecules (RNA, DNA), the ribofuranose has either C-2′ or C-3′ out of plane and on the same side as C-5′. These are the C-2′ endo and C-3′ endo conformers. The sugars found in RNA are the C-3′endo conformer whereas the C-2′ endo conformer is found in DNA. The furanose ring is highly flexible and can interconvert rapidly between different conformational states. This flexibility may be why they are naturally selected as components of RNA and DNA.[6, 20]

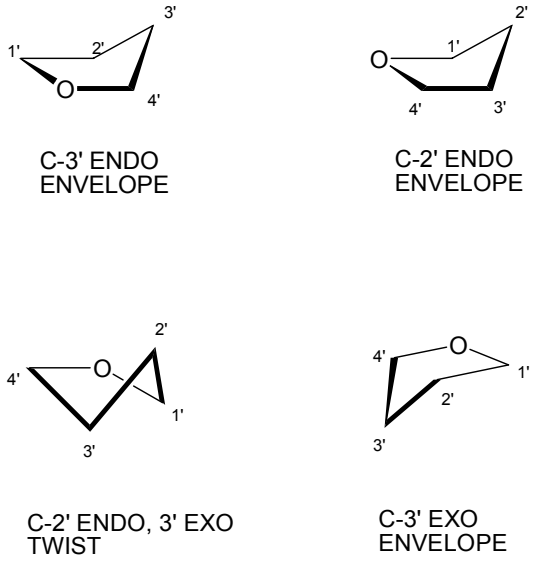


Figure 1.8. Envelope and Twist Conformations of Furanose.

Another aspect of nucleosides where conformations are important is the glycosidic bond. Being a single bond, there is rotation about it to yield different conformers. This rotation however is sterically hindered by a proton at the C-2′ endo position. Thus, nucleosides and nucleotides can exist in an entire range of conformations between an anti and syn conformation.

In the anti conformation, the O-2 in pyrimidine nucleosides, or the N-3 in purine nucleosides points away (i.e., further away) from the C-5′ atom of the carbohydrate, whereas in the syn conformation, the O-2 in pyrimidine nucleosides, or the N-3 in purine nucleosides points toward (i.e., closer to) the C-5′ atom of the carbohydrate. In both cases the plane of the nucleobase is at roughly right angles to the plane of the sugar. The anti conformation is more stable for both purine and pyrimidine nucleosides.[6, 22] It must be noted that the above findings are for natural unsubstituted nucleobases. For purine nucleosides, it is observed that bulky substitutions such as bromine or iodine at the C-8 position of the base warrants the syn conformation which prevents severe steric hindrance between the C-8 bromine or iodine and the O-1′ sugar atom or the C-2′ endo hydrogen. Figure 1.9 shows the syn and anti configurations of guanosine, 8-bromoguanosine and cytidine.

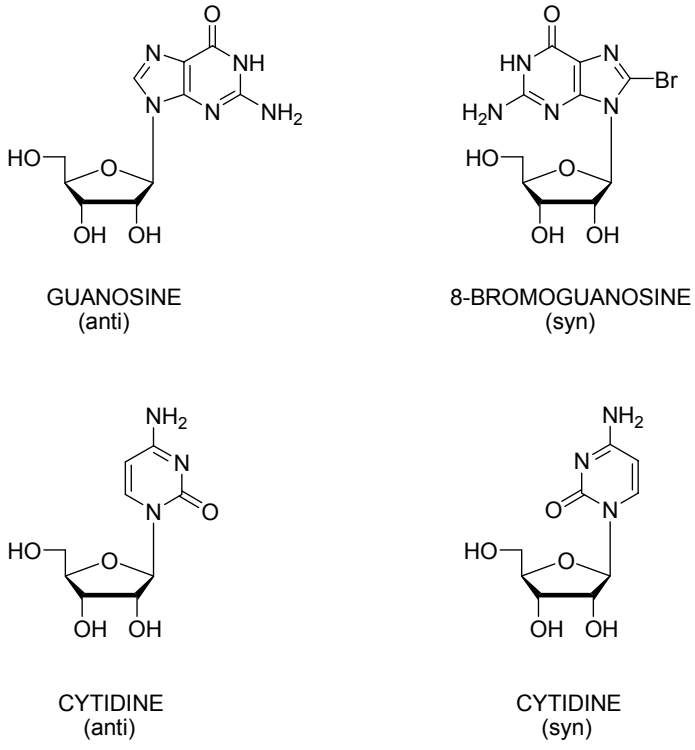


Figure 1.9. Syn and Anti Conformations of Nucleosides.

Nucleoside conformations around C-4′ and C-5′ of the sugar moiety also exist. The rotation about the C-4′-C-5′ bond leads to three different conformations which have about equal stabilities in solutions of the free nucleosides.[22]

ACID / BASE STABILITY OF NUCLEOSIDES

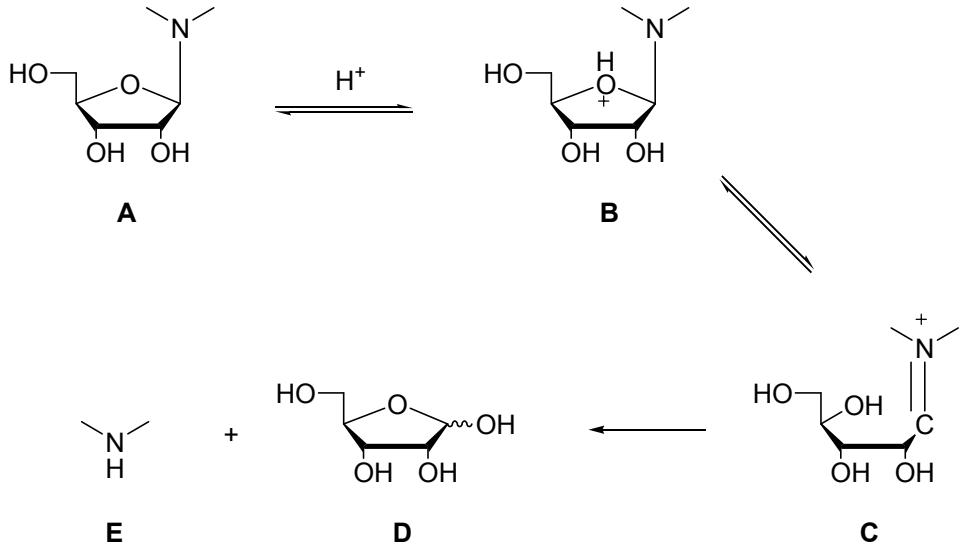
Knowledge of the acid/base stability of nucleosides is of great importance. It helps in determining what kinds of reactions, conditions and work-up methodologies are feasible with nucleosides. Hydrolytic studies provide information about how stable a particular nucleoside or family of nucleosides is in varying strengths of acid or base.[23-26]

It also establishes the mechanism by which the hydrolysis is occurring. As a general rule, nucleosides are stable in neutral media, but are hydrolyzed in acidic or alkaline media. The hydrolysis in alkaline media occurs at elevated temperatures (70˚C – 100˚C). This hydrolysis leads to cleavage of the N-glycosidic bond with subsequent formation of a base and the corresponding sugar. Hydrolysis of the N-glycosidic bond is much slower compared to that of an O-glycosidic bond. It is also observed that hydrolysis of purine nucleosides is faster than that of pyrimidine nucleosides. This is because the purine base is more basic and thus forms a more stable conjugate acid upon protonation. Also, since the purine base can better delocalize the positive charge over its bicyclic ring system, thus forming more resonance structures, purine nucleoside are better hydrolyzed than their pyrimidine counterparts.[6, 22]

Acid-Catalyzed Hydrolysis

Two possible mechanisms have been proposed for the acid-catalyzed hydrolysis of the glycosidic bond of nucleosides.

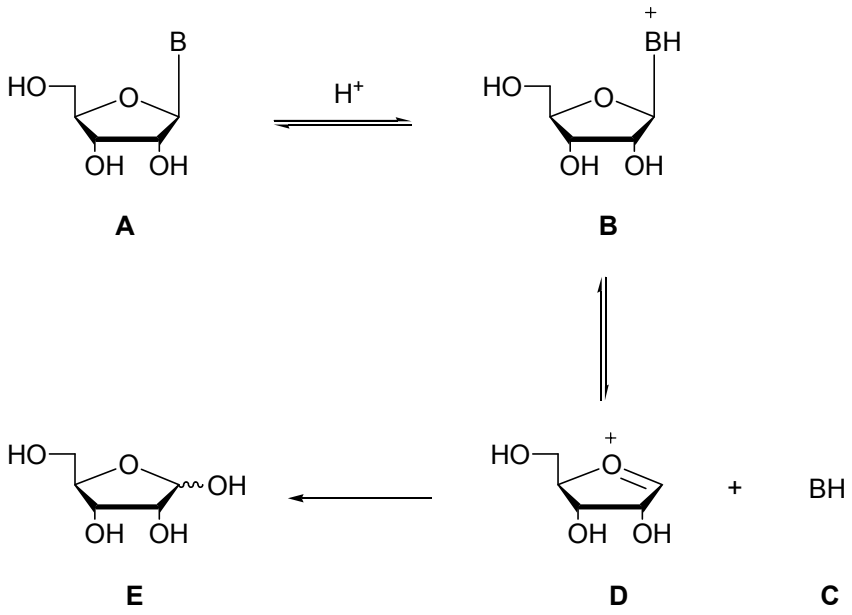
The first mechanism considered is the Schiff base mechanism (Scheme 1.3).[27-30] This mechanism proposes initial protonation of the sugar ring oxygen in acidic solution to give the intermediate **B**. Subsequent opening of the sugar and attack of water on the intermediate Schiff base **C** affords the base **E** and sugar **D**. This mechanism, based on the hydrolytic studies of simple glycosylamines, [31, 32] has not found much experimental support. However some pyrimidine nucleosides are reported to undergo hydrolysis by this mechanism.[33]



Scheme 1.3. Acid-Catalyzed Hydrolysis (Schiff Base Mechanism).

The A 1 mechanism is the second possible mechanism (Scheme 1.4). This mechanism for the acid-catalyzed hydrolysis is supported by experimental evidence. It involves initial protonation of the base followed by the rate-limiting dissociation of the protonated nucleoside **B** to the free base **C** and glycosyl carbonium ion (acylliuim **D**). Water then reacts with **D** to form the sugar **E**. This mechanism is dependent on the concentration of the protonated nucleoside. Experimental data lend support to the A1 mechanism.[34-38] The data suggest that the transition

state of the hydrolysis has considerable carbonium ion character with the glycosidic bond largely or entirely cleaved. Linear pH rate profiles found for the hydrolysis of nucleosides are consistent with the cleavage of the C-N glycosyl bond.[39-41] In contrast, the Schiff base model gave a bell-shaped pH-rate profile instead of the linear profile observed.[42, 43]



Scheme 1.4. A 1 Mechanism for Acid-Catalyzed Hydrolysis.

The major difference between the A 1 and Schiff base mechanisms is the point of initial protonation on the nucleoside. Unlike the Schiff base mechanism where initial protonation is specifically on the sugar ring oxygen, the A 1 mechanism has initial protonation on an unspecified atom of the base. It is therefore very important to know about the pKa's of nucleosides and their nucleobases to better understand the mechanism of hydrolysis, especially where the initial protonation occurs. A number of experimental techniques have been used to determine these pKa values (ionization constants). Also, ^{1}H and ^{13}C NMR and UV spectroscopy,

as well as potentiometric and calorimetric procedures are utilized for the determination of the protonation sites on the base moiety of nucleosides.[44-47] Table 1.2 shows the pKa's for some nucleosides and their bases.[35, 37, 41, 43, 48, 49]

Table 1.2. pKa Values of Some Nucleosides and Nucleobases.

COMPOUND	pKa
Purine	-1.66, 2.39, 8.9
Adenine	-0.43, 4.15, 9.8
Guanine	3.3, 9.2, 12.3
Hypoxanthine	1.9, 8.94, 12.1
Pyrimidine	1.3
Cytosine	4.45, 12.2
Thymine	0.5, 9.9, >13
Uracil	0, 9.5, 13
Nebularine	2.1, 10
6-Methoxynebularine	0.89
6-Chloronebularine	-0.60
Adenosine	-1.48, 3.63, 12.5
8-Bromoadenosine	4.02
8-Methoxyadenosine	3.85
2′-Deoxyadenosine	3.80
3′-Deoxyadenosine	3.71
2′, 3′-Dideoxyadenosine	3.77
Guanosine	1.6, 9.2, 12.5
8-Aminoguanosine	4.66
8-Bromoguanosine	0.2
2′-Deoxyguanosine	1.6-2.2, 9.16-9.5
Inosine	1.2, 8.8, 12.5
Cytidine	4.22, 12.5
2′-Deoxycytidine	4.25, 13
Thymidine	9.8
Uridine	9.17, 12.5
2′-Deoxyuridine	9.3, >13

The values in the table show ionization constants (pKa's) for the removal of protons from the sugar moiety (2′, 3′ and 5′ OH's), the removal of protons from the base moiety (i.e., –NH protons) and lastly the protonation of the base moiety (i.e., -N-).

The sugar hydroxyl groups of ribonucleosides and 2′-deoxyribonucleosides possess ionization constants in the range of 12-13. Thus, at basic solution (pH >12) the hydroxyl groups will exist in the anionic form.

The pKa values in the 8-10 range correspond to the removal of a proton on the heterocyclic base. For adenine, the N-9 proton has a pKa value of 9.8. The N-1 positions of guanosine and inosine become ionized at pH values of 9.2 and 8.8, respectively. Ionization of the N-3 position of thymidine occurs at pH 9.8.

The lone pair of electrons on nitrogens in the base moiety of nucleosides can be protonated under acidic conditions. The site of protonation depends on the structure of the nucleoside. For example, adenosine is initially protonated at N-1 (pKa 3.63) and then at N-7 (pKa -1.48). Protonation of guanosine occurs at N-7 (pKa 1.6). The pyrimidine nucleoside, cytidine, undergoes protonation at N-3 (pKa 4.22).

Sugar and/or base structural modifications in the nucleoside can affect pKa values, inductive effects and thus the rate of hydrolysis of the glycosidic bond. For example, comparison of adenosine and its deoxygenated analogues (Table 1.2) shows that the removal of hydroxyl groups from adenosine does not significantly alter the pKa values. In contrast to this observation, changes in the base moiety can have profound effects on the pKa values. For example, comparison of the pKa values of adenosine (3.63), nebularine (2.1) and 6-methoxynebularine (0.89).

The effects of structural modifications on the hydrolysis of nucleoside analogues have been investigated. It was found that sugar modifications seem to have a greater effect than changes in the base moiety on the rate of glycosidic bond cleavage. In general, deoxygenated nucleosides are less stable than their corresponding ribonucleosides.[37, 38] The removal of the 2′-hydroxyl group of adenosine caused a 1200-fold increase in the rate of hydrolysis (Table 1.3).[38] Similar results were observed with 2′-deoxyguanosine and guanosine,[39] and 2′-deoxyuridine and uridine.[50] The loss of the inductively electron-withdrawing hydroxyl group, which retards formation of the carbonium ion intermediate during the hydrolysis through a –I effect, caused the rate increase.[38, 51, 52] A smaller change in the rate of hydrolysis was noted with 3′-deoxyadenosine. The greater distance between the 3′-position and the anomeric center and also the stabilizing effect of the 2′-OH result in only a 15-fold increase in the rate of hydrolysis for 3′-deoxyadenosine.[38] When both 2′- and 3′-OH's of adenosine were removed (i.e., 2′,3′-dideoxyadenosine), the glycosidic bond was hydrolyzed approximately 40,000 times faster than that of ribonucleoside. This illustrates the importance of the hydroxyl groups, especially the 2′-OH, in stabilizing the glycosidic bond of nucleosides.

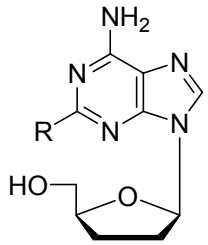
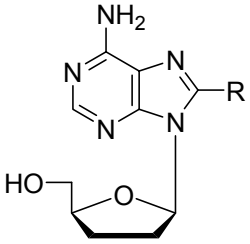
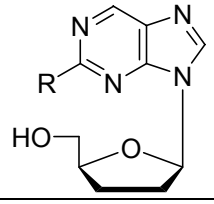
Table 1.3. Relative Rates of Hydrolysis of Adenosine Nucleosides.

NUCLEOSIDE	RELATIVE RATE
ADENOSINE	1
2′-DEOXYADENOSINE	1200
3′-DEOXYADENOSINE	15
2′, 3′-DIDEOXYADENOSINE	40,000
8-BROMOADENOSINE	3
8-METHOXYADENOSINE	0.4
2-CHLOROADENOSINE	1.2
2-METHYLADENOSINE	0.95

Electron-withdrawing groups introduced at the 2′-position of the sugar moiety exert effects similar to the 2′-OH i.e., they stabilize the glycosidic bond and retard hydrolysis. For example, 2′-O-(p-nitrobenzenesulfonyl) adenosine and 2′,3′-dideoxy-2′-fluoroadenosine are very stable nucleosides under acidic conditions.[37, 53]

The dideoxynucleosides are a very important class of antiviral compounds, however, they are very unstable with respect to hydrolytic cleavage of their glycosidic bond. Work has been done on the effects of base modifications on the rate of glycosidic bond hydrolysis of dideoxyadenosine (ddA) analogues (Table 1.4).[54]

Table 1.4: Relative Rates of Hydrolysis of 2′,3′-Dideoxyadenosine Analogues at pH 3 and 22˚C.

	R	REL. RATE AT pH 3
	H	100
	NH_2	20
	CN	47
	I	55
	SCH_3	64
	CH_2CH_3	75
	CF_3	79
	OH	0
	OCH_2Ph	39
	SCH_3	40
	OCH_3	61
	NH_2	2050
	H	177
	NH_2	110

The results indicate that even though the effects of base modifications are not as prominent as sugar modifications, they are nevertheless significant. In general C-2 modifications of ddA result in decreased rates of hydrolysis, whereas removal of the C-6 amino group coupled with C-2 modification resulted in increased hydrolysis rates. The most dramatic effects were observed with appropriate substitution at the C-8 position, particularly with -OH and $-NH_2$. The C-8-OH analogue was totally resistant to hydrolysis even at pH 1, whereas the C-8-NH_2 analogue showed the most increase in hydrolysis rate.[54] In all cases the rate of hydrolysis was compared with that of 2′,3′-dideoxyadenosine (ddA, rate = 100%).

The *cis* or *trans* orientation between the base and 2′-OH along the C-1′-C-2′ bond of the sugar affects the stability of the glycosidic bond i.e., the α or β orientation of the 2′-OH and the base. Nucleosides with a *trans* relationship (i.e., 2′-OH is α and base is β orientation) between the base and the 2′-OH are more stable than those with a *cis* configuration. Thus, nucleosides with ribose and xylose moieties are hydrolyzed more slowly than those with arabinose and lyxose.[38] The steric strain of the *cis*-nucleoside was suggested to be responsible for the faster hydrolysis rates.

The position of attachment of the sugar moiety to the purine base also affects the hydrolysis rate (i.e., the N-7 versus N-9 glycosylation). For example, 7-(β-D-ribofuranosyl)adenine is hydrolyzed 34 times faster than adenosine.[35] A more elaborate comparison of a series of N-7 and N-9 glycosylated purine nucleosides revealed that the N-7 isomers were hydrolyzed 2 – 34 times faster than the corresponding N-9 isomers. Also, the anomeric orientation of the base was found to influence the hydrolytic stability of the nucleoside as well. The beta-anomer was about six times more stable then the alpha-anomer due to a reverse anomeric effect.[38, 55]

The effects of purine ring substituents are generally smaller than changes in the sugar moiety. Considering the mechanism of hydrolytic cleavage, substituents that increase electron density at N-9 would tend to retard the transfer of electrons to N-9 from the C-N glycosidic bond. Thus, electron-donating groups would be expected to decrease the rate of hydrolysis, whereas electron-withdrawing substituents would be expected to accelerate the hydrolysis rate due to a lower electron density at N-9, which facilitates the cleavage of the C-N bond. Also, due to the proximity of the C-8 position to N-9, C-8 modifications have more influence on hydrolytic stability than C-2 or C-6 modifications. The hydrolytic rates of a series of nucleosides with both electron-withdrawing and donating groups at the C-8 position have been compared. In the adenosine series the relative order of hydrolysis was 8-Br > 8-H > 8-OCH_3 (Table 1.3).[37] The same inductive effects were found in the guanosine,[43] cytidine,[56] 2′-deoxycytidine,[50] and 2′-deoxyadenosine series.[37] The inductive effects of substituents at the C-2 position have a much smaller effect on the hydrolysis presumably due to the greater distance from the glycosidic bond.[37] Groups at the 6-position also influence the hydrolytic cleavage of the C-N bond through inductive effects. For example 6-dimethylaminonebularine was hydrolyzed 6 times slower than nebularine.[35]

Additionally, substituents at C-2 and C-6 may also influence the hydrolytic stability of nucleosides by changing the pKa of the site of initial protonation. Since protonation of the purine base is an essential step in hydrolytic cleavage, any change in the ease of protonation would be expected to change the concentration of protonated nucleoside and hence the hydrolysis rate. Protonation of purines is considered to take place at N-1. The proximity of substituents at C-2 or C-6 to N-1 would therefore modify the electron density at N-1 and give rise to different pKa values. Electron withdrawing groups will retard protonation of N-1 while electron donating

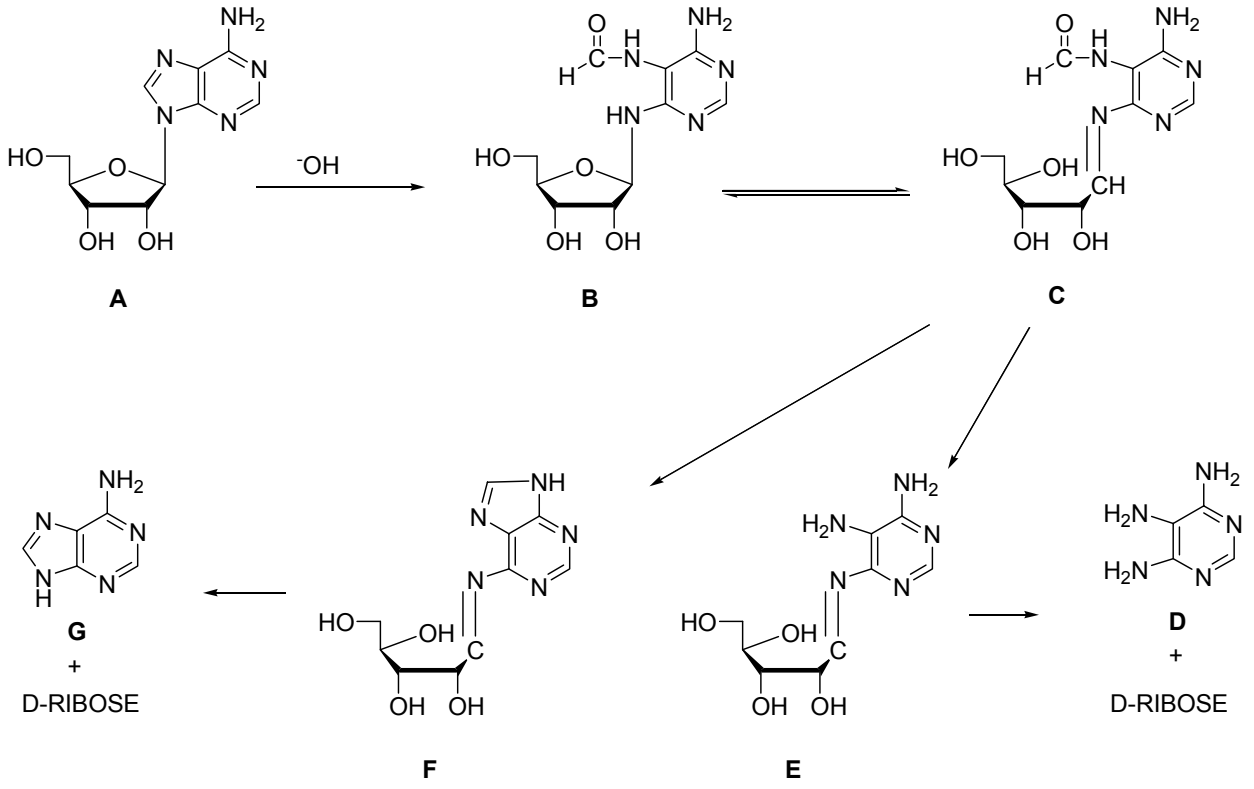
groups will make N-1 more basic. This effect on the ease of protonation competes with the inductive effects of the base substituent on the N-9 nitrogen and may largely cancel each other out, resulting in similar rates of hydrolysis for both electron withdrawing and electron donating substituents. This has been observed in the hydrolysis of 6-substituted nebularines and 2′-deoxynebularines.[41, 57, 58]

Base-Catalyzed Hydrolysis

The base-catalyzed hydrolysis of glycosidic bonds is less studied than the acid-catalyzed hydrolysis. An understanding of how nucleosides behave in basic solutions helps in determining reaction types, work-up methodology and mechanisms involved in synthetic nucleoside transformations.[59-63] As stated earlier, purine nucleosides, are in general, stable in dilute alkaline media at room temperature. However, at elevated temperatures (70˚C-100˚C) they undergo hydrolysis. This was reported by Gordon *et al* in 1957,[64] when they observed that nebularine, upon treatment in alkaline solution, decomposed to form 5-formamido-4-ribosylamino and 5-amino-4-ribosylaminopyridine. Since then, the base-catalyzed hydrolysis of nebularine and adenosine has been studied by NMR spectroscopy and liquid chromatography.[65, 66]

Scheme 1.5 shows the mechanism for the hydrolysis of adenosine.[67] Initial nucleophilic attack of a hydroxyl at C-8 opens the imidazole ring to form the 5-formamido intermediate **B** which isomerizes to the Schiff base **C**. Cyclization of **C** forms compound **F**, which hydrolyzes to adenine and D-ribose. A hydroxyl ion attack on the formamide group in **C** followed by the loss of formate ion yields **E**, which decomposes rapidly to form **D** and D-ribose. Nebularine was found to decompose by a similar mechanism.[65] 6-Chloronebularine also undergoes attack at C-8 by hydroxyl ion.[67] Inosine on the other hand is decomposed by -OH attack on the C-2 carbon of

the hypoxanthine base. This happens under alkaline conditions at high temperatures and results in the formation of the ring-opened product, 5-aminoimidazole-4-carboxamide-1-B-D-ribofuranoside, in low yields.[68]



Scheme 1.5. Mechanism of Base-Catalyzed Hydrolysis.

Structural modifications of the nucleoside base affect the rate of alkaline hydrolysis significantly, whereas sugar modifications do not.[69] For example adenosine and 2′-deoxyadenosine were both hydrolyzed at similar rates. However 8-bromoadenosine was hydrolyzed 17 times faster than adenosine, whereas 6-dimethylaminonebularine was hydrolyzed at a decreased rate. These findings are consistent with the proposed mechanism.

NUCLEOTIDES

Phosphoric acid esterification of the free hydroxyl groups of nucleoside sugars leads to the formation of nucleotides. A ribose sugar yields the ribonucleotides, whereas the deoxyribose sugar yields the deoxyribonucleotides. Phosphate esters of 2′, 3′ and 5′ ribonucleosides are all known. Even cyclic 2′- 3′ and cyclic 3′-5′ nucleotides are known and found in trace amounts in cells. The 5′-triphosphates of ribonucleosides and deoxyribonucleotides are known and they form the backbone of DNA and RNA. Some common guanosine nucleotides are shown in Figure 1.10.

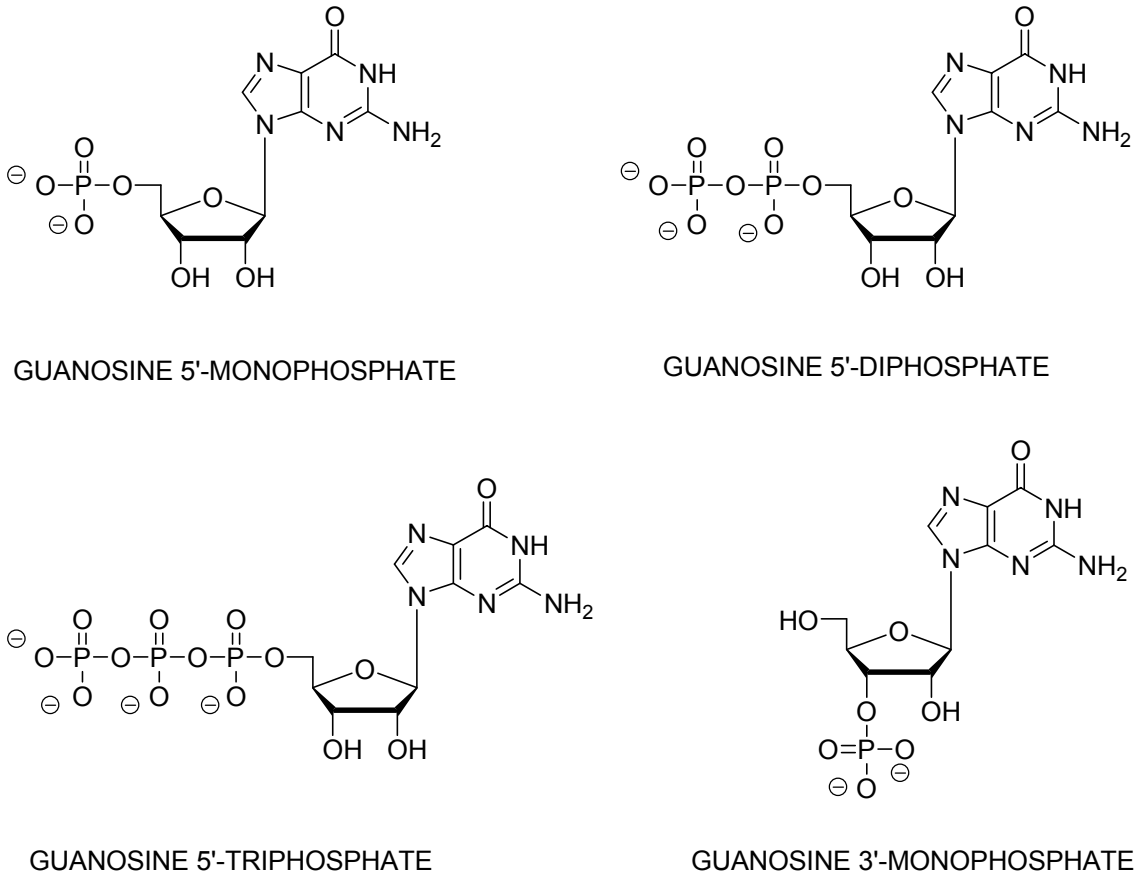


Figure 1.10. Different Forms of Guanosine Nucleotides.

The mono-, di- and tri-phosphate esters are strong acids and they are extensively hydrolyzed at physiological pH. The mono-phosphate esters have two ionizable protons with pKa values of approximately 1.0 and 6.0, hence at pH 7.0 they exist predominantly as di-anions.

The di-phosphate esters have three ionizable protons and they exist in either the -3 (fully ionized) or -2 (partially ionized) form; e.g., adenosine diphosphate (ADP) has three ionizing protons, the pKa of the first two are less than 5; the pKa of the third is 6.7. At pH 7, 67% of ADP is in the fully ionized form (ADP^{3-}) and 33% is in the partially ionized form (ADP^{2-}).

The tri-phosphate esters have four ionizable protons and they exist in either the -4 (fully ionized) or -3 (partially ionized) form. Using ATP as an example, the pKa values of the first three protons are less than 5 and the pKa of the fourth for the equilibrium between ADP^{4-} and ADP^{3-} is 6.95. Therefore at pH 7, 53% of ATP is in the fully ionized form (ATP^{4-}) and 47% is in the partially ionized form (ATP^{3-}).[22] The pKa values of the ionizable protons of the nucleobases are also important; they are all neutral at pH 7.

Nucleotides are very essential in life. Not only do they serve as building blocks of DNA and RNA, they are also substrates and intermediates of many metabolic pathways. They also serve as energy sources and are components of certain coenzymes such as coenzyme A, NADP and NAD.

NUCLEOTIDE METABOLISM

Nucleotides are the building blocks of nucleic acids. They perform a wide range of biochemical functions. For example, ATP is a high energy compound that provides the free energy that makes various biochemical reactions thermodynamically favorable.[70] The biosynthetic pathway of purine and pyrimidine nucleotides produce ATP, GTP, UTP and CTP

(building blocks of RNA), activated metabolites such as UDP-glucose and CDP-choline and enzyme co-factors of metabolism including FAD, NAD^+ and coenzyme A. Nucleoside diphosphates are converted into dATP, dGTP, dTTP and dCTP which are precursors of DNA.[71] Nearly all cells can synthesize nucleotides from *de novo* or salvage pathways. The ability of cells to salvage nucleotides nullifies the need of nucleotides as a dietary supplement.[19]

Enzymes such as endonucleases, phosphodiesterases and nucleoside phosphorylases play very critical roles in nucleotide metabolism. Endonucleases degrade DNA and RNA at internal sites to form oligonucleotides. After this degradation, phosphodiesterases break down oligonucleotides to free nucleosides. The nucleosides are then hydrolyzed by the action of phosphorylases to their constituent bases and ribose-1-phosphate. The nucleosides and/or bases can either be salvaged back to nucleotides as regulated by the cell or the purine bases are degraded to uric acid and the pyrimidines to β-alanine and β-aminoisobutyrate, NH_3 and CO_2. Nucleoside-5′-phosphates are the final products of both the salvage and *de novo* biosynthetic pathways. The starting compound is the activated sugar intermediate 5-phospho-α- D-ribosyl-1-pyrophosphate (PRPP), which is the product of the enzymatic reaction of PRPP synthetase on α-D-ribose-5-phosphate and requires energy in the form of ATP.[18, 19]

Here, we will discuss nucleotide (purine and pyrimidine) biosynthesis (*de novo* and salvage), their regulation and the consequences of their blockade, both genetic and chemotherapeutic.

Purine Nucleotide Biosynthesis

In 1948, John Buchanan determined the origins of the component parts of purine nucleotides by feeding pigeons isotopically labeled compounds and chemically determining their

positions in their excreted uric acid (a purine). His findings (Figure 1.11), concluded that purines' N-1 is from aspartate's amino group; C-2 and C-8 from formate; N-3 and N-9 from glutamine's amide group; C-4, C-5 and N-7 from glycine (this strongly suggests that glycine is wholly incorporated into the purine ring); and C-6 comes from HCO_3^-. Buchanan's work assisted in elucidating how these origins are incorporated into the actual purine nucleotide biosynthesis.[19]

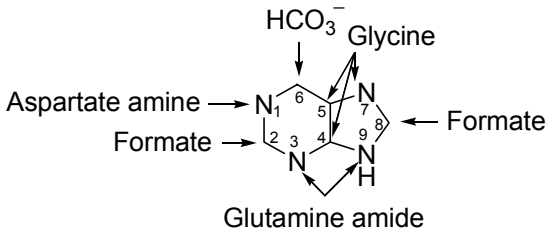
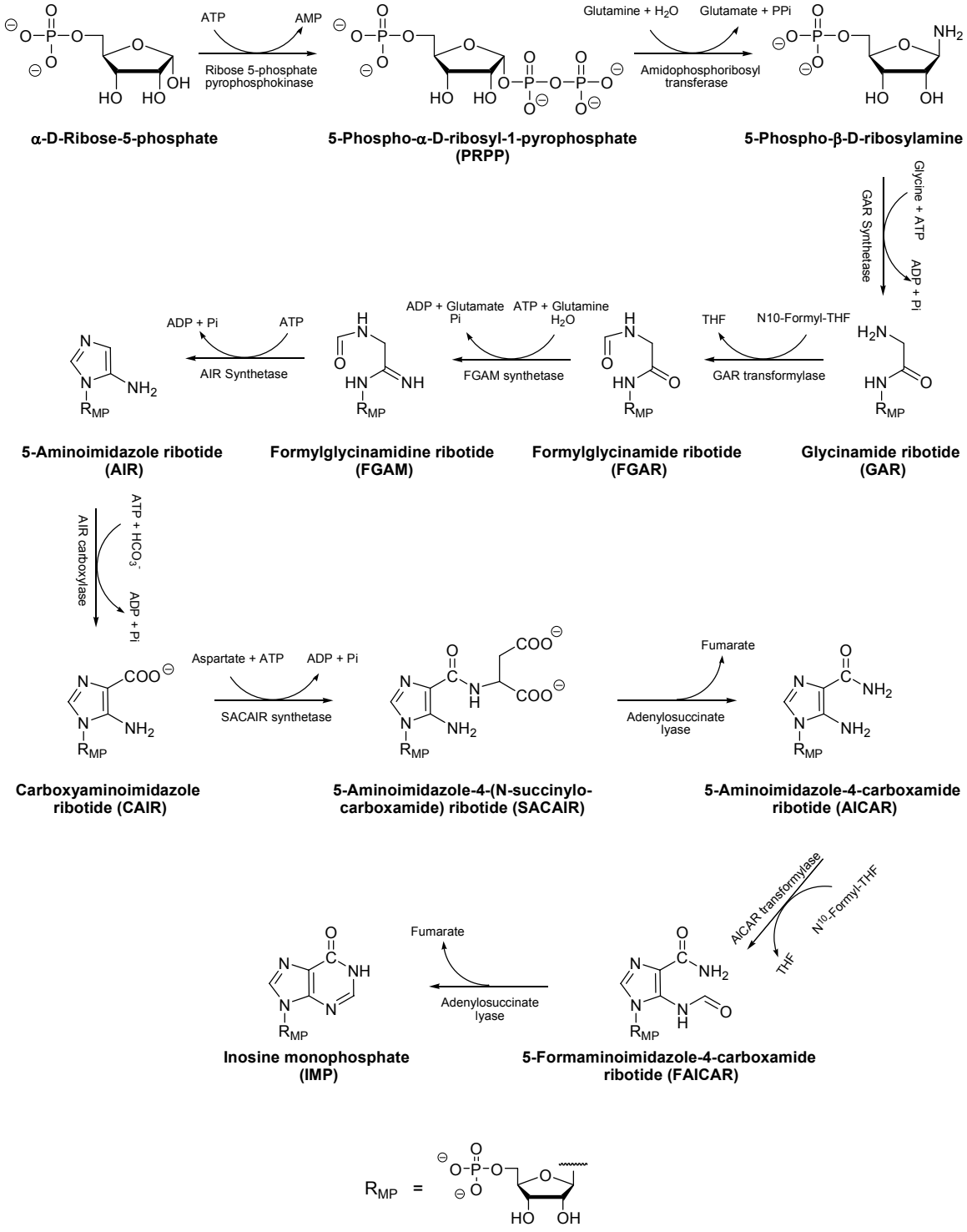


Figure 1.11. Origins of Purine Components.

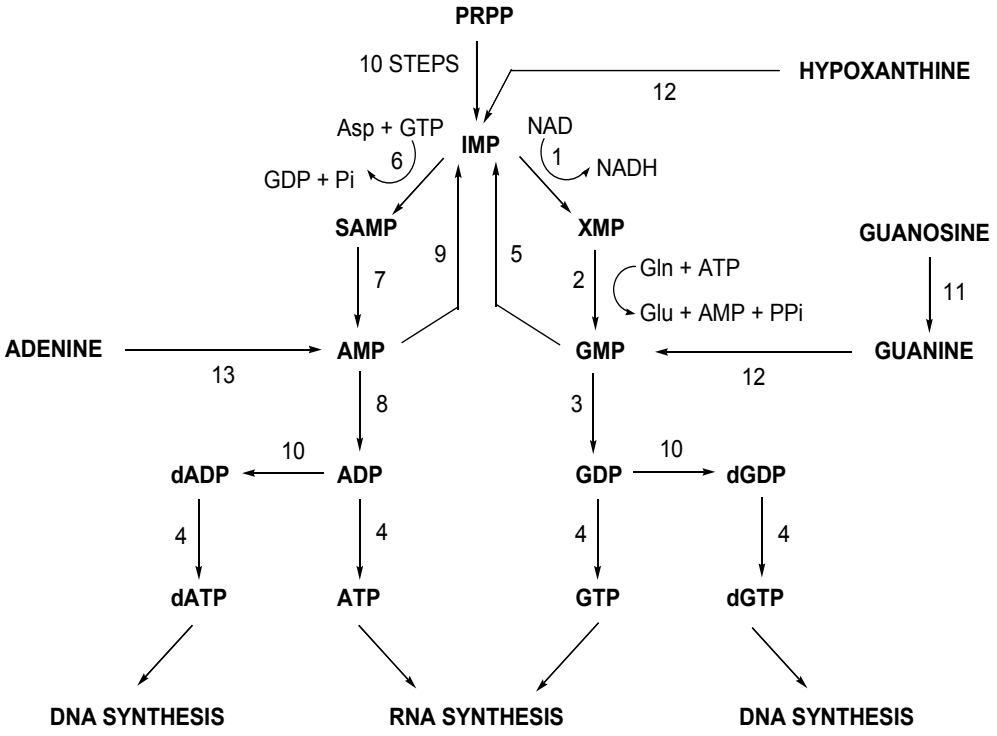
Even though nearly all cells can synthesize nucleotides, the major site of purine synthesis is in the liver. Purine nucleotide synthesis begins with α-D-ribose-5-phosphate (a product of the pentose phosphate pathway) and ends with the first fully synthesized purine nucleotide, inosine 5′-monophosphate (IMP) (Scheme 1.6).

This *de novo* biosynthesis is an 11-step pathway. The purine hypoxanthine base is built upon α-D-ribose-5-phosphate by a series of enzymes. In all, the pathway requires six moles of ATP, two moles of glutamine, one mole of glycine, one mole of HCO_3^-, one mole of aspartate and two moles of formate. The formyl moieties are carried on tetrahydrofolate (THF) in the form of N^{10}-formyl-THF.



Scheme 1.6. *De Novo* Biosynthesis of IMP.

IMP sits at a branch point for purine biosynthesis. IMP does not accumulate in the cell, but is rapidly converted to adenosine monophosphate (AMP) and guanosine monophosphate (GMP) through two distinct reaction pathways (Scheme 1.7). AMP and GMP are successively converted into ATP and dATP and GTP and dGTP respectively.[72]



1. IMPDH (Inosine Monophosphate Dehydrogenase)
2. GMP SYNTHASE (GMP: Guanosine Monophosphate)
3. GMP KINASE
4. NUCLEOSIDE DIPHOSPHATE KINASE
5. GMP REDUCTASE
6. ADENYLOSUCCINATE SYNTHETASE
7. ADENYLOSUCCINATE LYASE
8. AMP KINASE (AMP: Adenosine Monophosphate)
9. AMP DEAMINASE
10 RIBONUCLEOTIDE REDUCTASE
11. PURINE NUCLEOSIDE PHOSPHORYLASE
12. HGPRT (Hypoxanthine-Guanine Phosphoribosyl Transferase)
13. APRT (Adenine Phosphoribosyl Transferase)

SAMP: Adenylosuccinate
XMP: Xanthosine Monophosphate
PRPP: 5-Phosphoribosyl-α–pyrophosphate

Scheme 1.7. *De Novo* and Salvage Pathway of Purine Nucleotides Biosynthesis.

The first reaction of the pathway leading to AMP involves the amino group of aspartate attacking the carbonyl group of IMP to form adenylosuccinate monophosphate (SAMP). This reaction is catalyzed by adenylosuccinate synthetase and requires energy in the form of GTP. In the second reaction, adenylosuccinate lyase eliminates fumarate from adenylosuccinate monophosphate to form AMP (Scheme 1.7).

On the other hand, IMP is converted to GMP in two steps. This pathway leading to GMP, initially requires the oxidation of IMP to xanthosine monophosphate (XMP) using the very important enzyme, inosine monophosphate dehydrogenase (IMPDH), and NAD^+ as a cofactor. NAD^+ is reduced in the process to NADH. In the second half, the enzyme guanosine monophosphate synthase converts XMP to GMP by transferring the glutamine amide nitrogen to the C-2 position of XMP in an ATP-hydrolysis driven reaction.

To become nucleic acid subunits, GMP and AMP are further modified into their diphosphates by base-specific kinases, then to their triphosphates by nucleoside diphosphate kinases and also to their deoxyribonucleotides by the appropriate ribonucleotide reductases.

GMP kinase using ATP as a cofactor converts GMP to GDP and ADP, whereas AMP kinase also using ATP as a cofactor converts AMP into two ADP molecules. There is no catalytic discrimination between ribose and deoxyribose in the substrate with these nucleoside monophosphate kinases. Further enzymatic processing converts GDP to GTP and ADP to ATP using ATP as the phosphoryl donor, in both cases ADP is a byproduct. The nucleoside diphosphate kinases like their monophosphate counterparts do not discriminate between ribose and deoxyribose in the substrate (Scheme 1.7). The GTP and ATP formed are then used for RNA synthesis.

Besides their conversion to GTP and ATP, GDP and ADP can be deoxygenated at the C-2′ of the ribose sugar to their respective dGDP and dADP forms. Ribonucleotide reductases are responsible for this conversion; they replace the C-2′-OH of ribose with "H" *via* an effective free radical mechanism. The dinucleotides, dGDP and dADP, are subsequently transformed into dGTP and dATP (DNA-synthesis precursors) *via* the same nucleoside diphosphate kinases that act on GDP and ADP.

Regulation of Purine Nucleotide Biosynthesis

Since the concentration of purine nucleotides in the cell is tightly regulated there are checks and balances within both the *de novo* and salvage pathways to ensure this tight regulation.[72] For this purpose IMP, ATP and GTP are individually regulated (Figure 1.12).

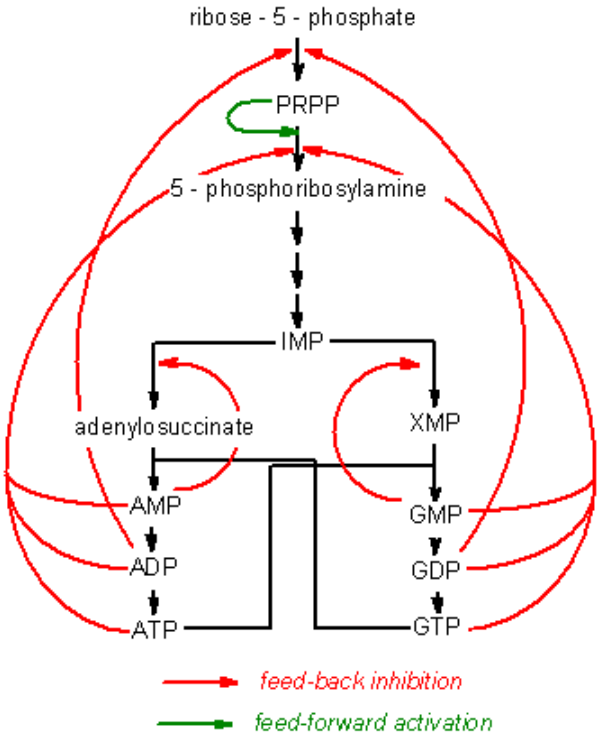


Figure 1.12. Regulation of Purine Nucleotide Synthesis.

For the IMP pathway, the essential rate limiting steps occur at the first two steps of the pathway i.e., the catalytic synthesis of PRPP and 5-phosphoribosylamine. The synthesis of PRPP from ribose-5-phosphate by ribose phosphate pyrophosphokinase is feed-back inhibited by both ADP and GDP. Likewise the enzyme amidophosphoribosyl transferase which converts PRPP into 5-phosphoribosylamine is also feed-back inhibited by binding ATP, ADP and AMP at one inhibitory site and GTP, GDP and GMP at another. PRPP also allosterically stimulates amidophosphoribosyl transferase by a feed forward mechanism. Thus, the rate of IMP synthesis is independently yet synergistically controlled by the concentrations of adenine and guanine nucleotides in the cell.

The two distinctive reaction pathways where IMP is converted into either GMP or AMP are also regulated. The excessive buildup of AMP and GMP from IMP is prevented because both AMP and GMP are feed-back competitive inhibitors of IMP. Also the rates of synthesis of AMP (eventually converted to ATP) and GMP (eventually converted to GTP) are coordinated. The pathway from IMP to AMP uses GTP for energy, whereas that from IMP to GMP uses ATP for energy. This reciprocity of utilization of energy sources allows the cell to control the proportions of AMP and GMP to near equivalence (which is required for nucleic acid biosynthesis). Thus, accumulation of excess GTP will lead to accelerated AMP synthesis from IMP, at the expense of GMP synthesis. Conversely, since the conversion of IMP to GMP requires ATP, the accumulation of excess ATP leads to accelerated synthesis of GMP over that of AMP. Another means of regulation of IMP concentration in the cell is by conversion of AMP and GMP to IMP by AMP deaminase and GMP reductase, respectively, when levels are low. All the above mentioned mechanisms work independently yet synergistically to maintain a physiological production rate of IMP.

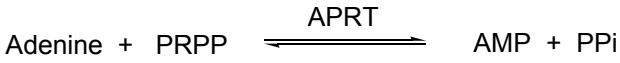
Purine Salvage Pathway

The metabolism of nucleic acids results in the release of the free nucleobases adenine, guanine and hypoxanthine as well as free nucleosides.

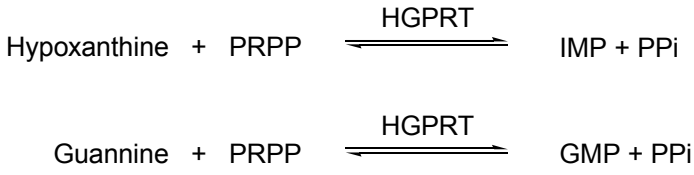
The synthesis of nucleotides from these purine bases and nucleosides takes place in a series of steps known as the salvage pathway.

The free purine bases: adenine, guanine and hypoxanthine can be reconverted to their corresponding nucleotides by phosphoribosylation using the two key transferase enzymes, adenine phosphoribosyltransferase(APRT) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT).

APRT catalyzes AMP re-formation using PRPP as shown below:



HGPRT catalyzes the respective re-formation reactions for both hypoxanthine and guanine as shown below:



The free nucleosides, on the other hand, are salvaged back to their respective nucleotides by phosphorylation of their 5′-OH group. For adenosine, this phosphorylation is undertaken by adenosine kinase (AK; the only purine kinase in humans). For the deoxyribonucleosides, 2′-deoxyguanosine kinase (dGK) and 2′-deoxycytidine kinase (dCK) are responsible for the

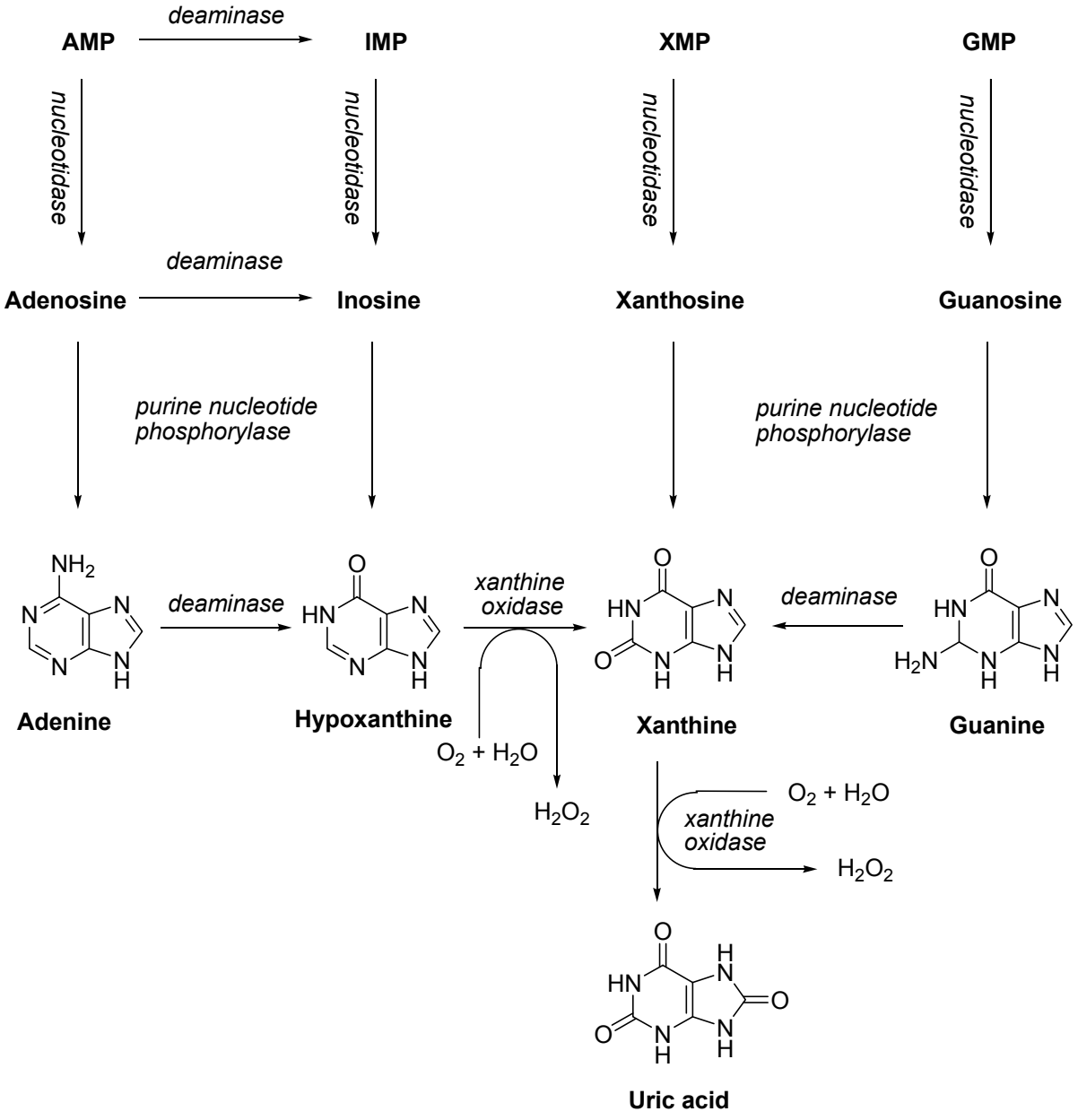
phosphorylation. dGK phosphorylates deoxyguanosine, deoxyadenosine and deoxyinosine whereas dCK phosphorylates deoxycytidine, as well as deoxyguanosine and deoxyadenosine to their respective monophosphates.[2, 70]

Purine Nucleotide Catabolism

Degradation of dietary and cellular nucleic acids produces free nucleosides, nucleobases, ribose and ribose-1-phosphate. Since the *de novo* pathway to a large extent meets an organism's need for nucleotides, these degradation products are either salvaged or further degraded and excreted. In humans and other primates, this catabolism leads ultimately to the production of uric acid which is excreted in urine as sodium urate crystals, since it is only sparingly soluble in water.[19] The major catabolic pathways of purine nucleotides and deoxynucleotides are shown in Scheme 1.8.

Initially the nucleotides AMP (dAMP), GMP (dGMP), IMP and XMP are dephosphorylated by 5′-nucleotidase to their respective nucleosides (deoxynucleosides). Alongside this initial step, AMP deaminase converts AMP into IMP; the analogous reaction occurs for XMP to GMP on the right side of the scheme. Purine nucleoside phosphorylase (PNP) hydrolyzes the glycosidic bonds of the nucleosides (deoxynucleosides) to form free purine bases and ribose-1-phosphate (a precursor of PRPP) and deoxyribose-1-phosphate (from deoxynucleosides). The rate of adenosine hydrolysis is very slow (~350,000 times slower than that of inosine). To circumvent this problem, adenosine deaminase converts adenosine into inosine to capitalize on its efficient hydrolysis. An analogous conversion occurs on the right hand side of the Scheme. Adenine and guanine are converted to hypoxanthine and xanthine

respectively by deaminases. Xanthine oxidase converts hypoxanthine to xanthine and finally oxidizes xanthine to uric acid for excretion.[19, 69]



Scheme 1.8. Catabolism of Purine Nucleotides.

Purine Nucleotide Cycle

The conversion of IMP to AMP, the biosynthesis of IMP and the salvage of IMP *via* AMP catabolism have the net effect of deaminating aspartate to fumarate. This process is referred to as the purine nucleotide cycle (Figure 1.13) This cycle is very important in skeletal muscle cells. Increase in muscle activity creates a demand for an increase in the citric acid cycle, in order to generate more NADH for the production of ATP. However, muscles lack most of the enzymes of the major anaplerotic (filling up) reactions, so the muscles replenish their citric acid cycle intermediates with fumarate generated in the purine nucleotide cycle.[18, 19] Additionally, the cycle is an effective means of regulating the levels of IMP in the cell, as it converts of IMP to AMP, which can be converted to the very important nucleotides ATP or dATP as need be.

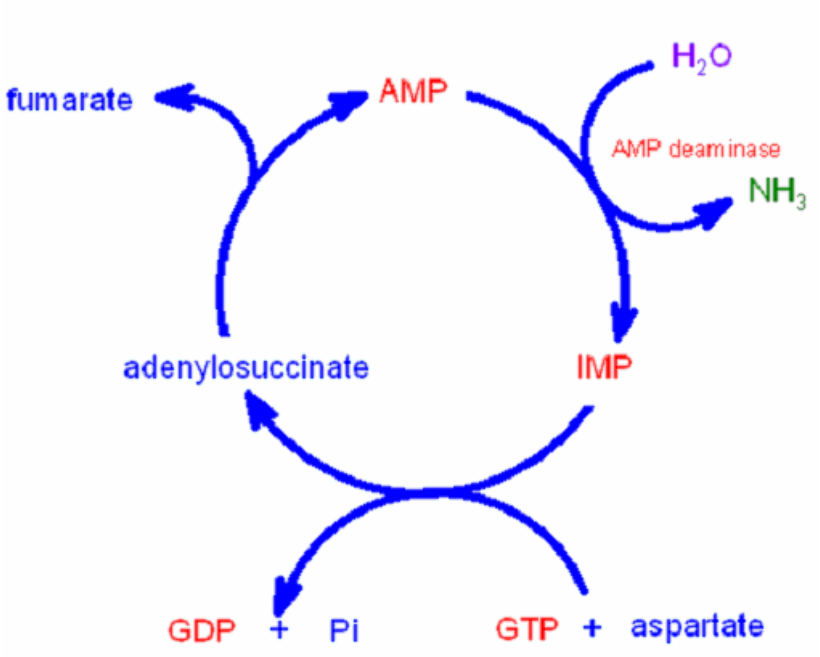


Figure 1.13. Purine Nucleotide Cycle.

Clinical Significances of Purine Metabolism

In humans, diseases and disorders arise from abnormal purine nucleotide metabolism. The effects of these diseases and disorders may be mild, severe or even fatal. The accumulation of insoluble uric acid (final degradation byproduct) is almost solely responsible for all the adverse clinical manifestations of abnormal purine metabolism (Table 1.5).

Table 1.5. Diseases and Disorders of Abnormal Purine Metabolism.

Disorder	Defect	Nature of Defect	Comments
Gout	PRPP synthetase	Increased enzyme activity due to elevated Vmax	Hyperuricemia
Gout	PRPP synthetase	Enzyme is resistant to feed-back inhibition	Hyperuricemia
Gout	PRPP synthetase	Enzyme has increased affinity for ribose-5-phosphate (lowered K_m)	Hyperuricemia
Gout	PRPP amidotransferase	Loss of feed-back inhibition of enzyme	Hyperuricemia
Gout	HGPRT	Partially defective enzyme	Hyperuricemia
Lesch-Nyhan syndrome	HGPRT	Lack of enzyme	
SCID	ADA	Lack of enzyme	
Immunodeficiency	PNP	Lack of enzyme	
Renal lithiasis	APRT	Lack of enzyme	2,8-dihydroxyadenine renal lithiasis
Xanthinuria	Xanthine oxidase	Lack of enzyme	Hypouricemia and xanthine renal lithiasis
von Gierke's disease	Glucose-6-phosphatase	enzyme deficiency	

Pyrimidine Nucleotide Biosynthesis

Just as with purines, isotopic labeling experiments enabled the discovery of the origins of the atoms in the pyrimidine ring (Figure 1.14). N-1, C-4 and C-6 originate from aspartic acid, C-2 is from HCO_3 and N-3 arises from glutamine.

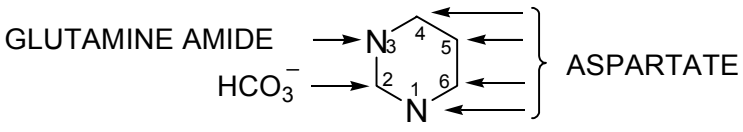
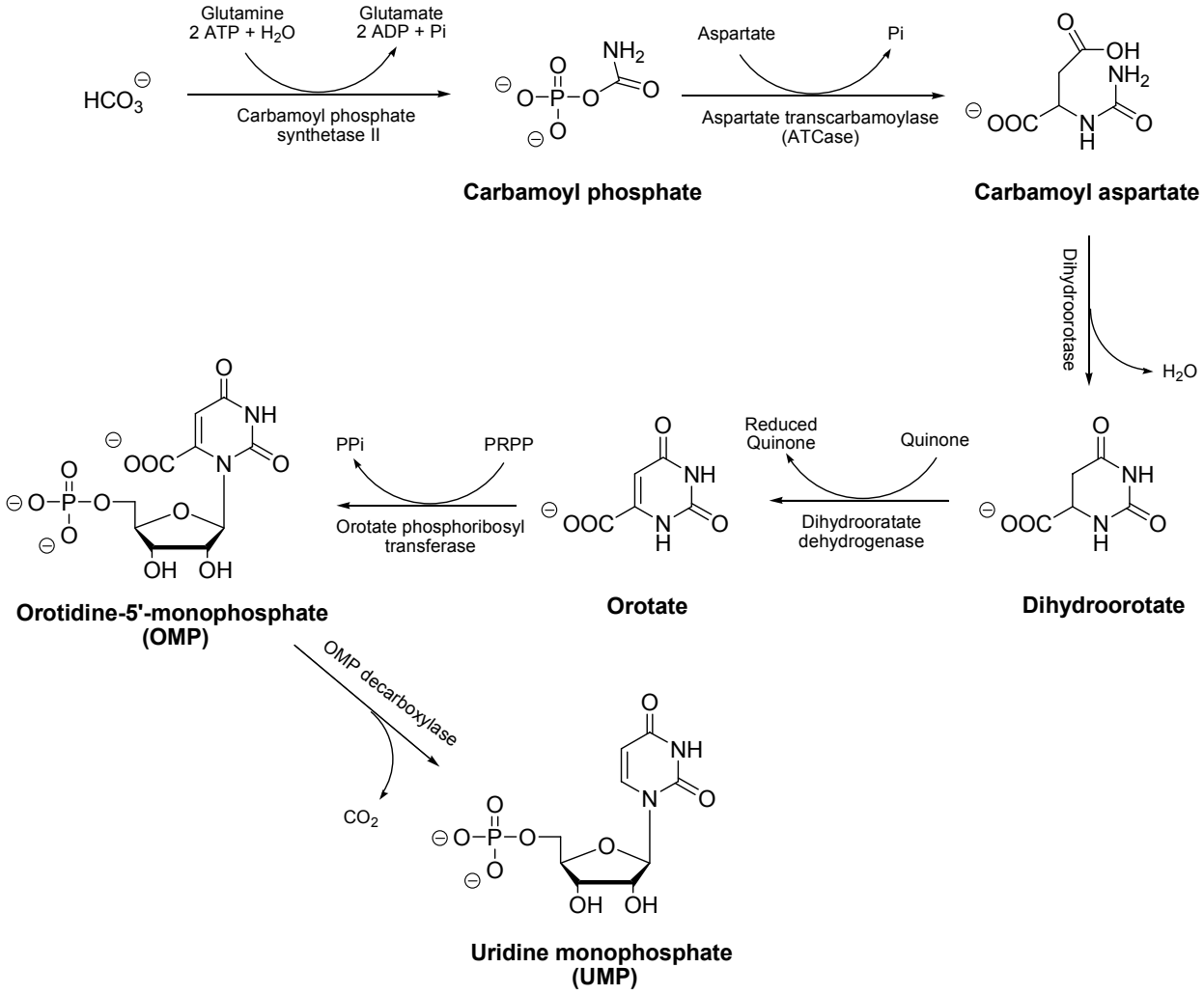


Figure 1.14. Origins of Pyrimidine Ring Atoms.

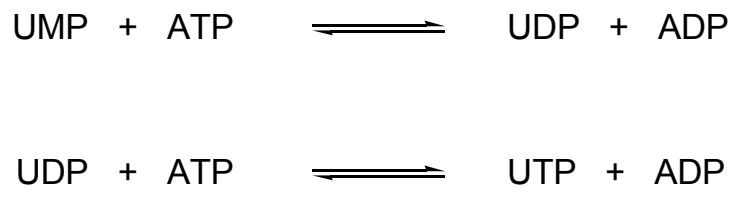
The *de novo* biosynthesis of pyrimidine nucleotides is a metabolic pathway consisting of six enzyme-catalyzed reactions (Scheme 1.9). The first fully synthesized pyrimidine nucleotide is uridine monophosphate (UMP), which is afterwards converted to cytidine monophosphate (CMP) and thymidine monophosphate (TMP).

Further conversions to the diphosphate and triphosphate are also effected, as well as the 2′-deoxyribonucleotides counterparts. Pyrimidine nucleotide synthesis is less complicated than that of the purines, since the base is much simpler. There are two significant differences between them. First, in the pyrimidine case, the ring structure is assembled as a free base, not built upon PRPP. PRPP is then coupled to the first fully formed pyrimidine base (orotate), forming orotate monophosphate (OMP), which is subsequently decarboxylated to UMP. Secondly, there is no branch point intermediate (like IMP in the purine case) in the pyrimidine biosynthetic pathway.[18, 19]

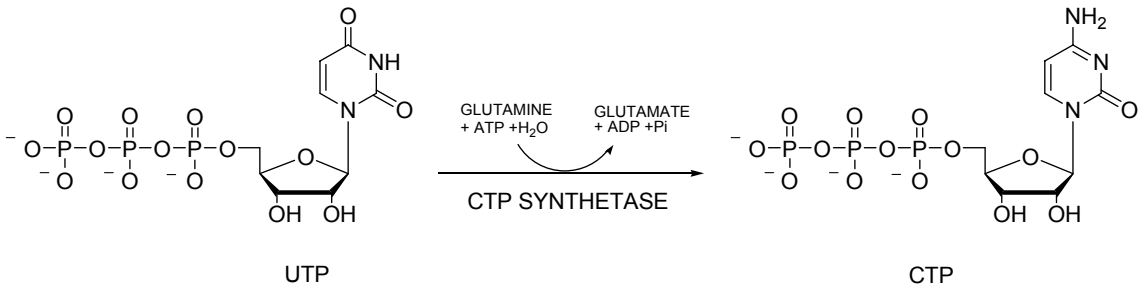


Scheme 1.9. *De Novo* Biosynthesis of UMP.

Following completion of UMP synthesis, it is phosphorylated twice to yield UTP (ATP is the phosphate donor, as well as the energy source). The first phosphorylation is catalyzed by uridylate kinase and the second by the ubiquitous nucleoside diphosphate kinase. Both conversions are shown below:



UTP's 6-oxo group is replaced with an amino group by the action of CTP synthetase to form CTP (Scheme 1.10). The source of the amino group is glutamine in animals whereas in bacteria it's supplied directly by ammonia.

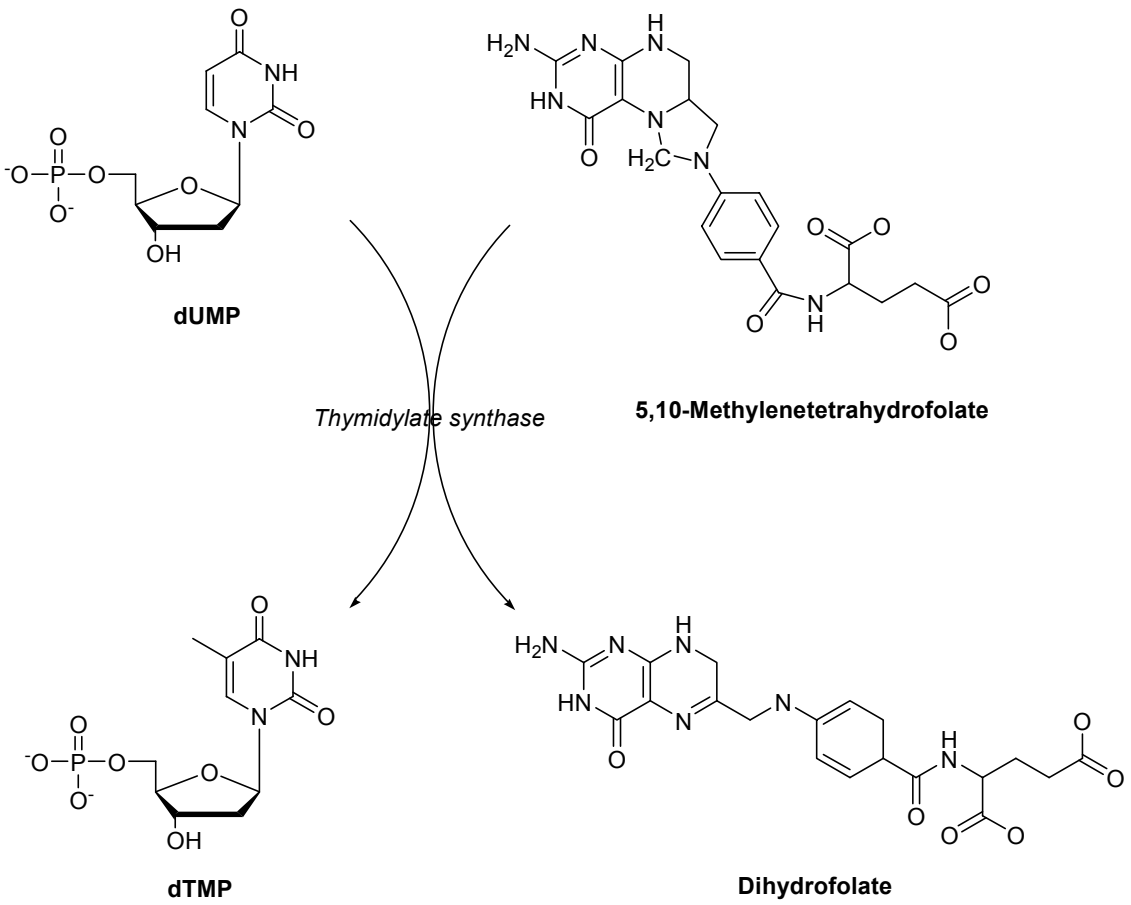


Scheme 1.10. Synthesis of CTP from UTP in Animals.

Thymine nucleotides are derived from *de novo* synthesis using dUMP as a precursor, or by salvage pathways with deoxyuridine or deoxythymidine as precursors.

The dTTP component utilized in DNA synthesis arises from sequential steps which begin with hydrolysis of dUTP to form dUMP by the enzyme dUTP diphosphohydrolase (dUTPase). dUMP is then methylated at C-5 of the pyrimidine ring to form dTMP by the enzyme thymidylate synthase using N^5,N^{10}-methylenetetrahydrofolate as the methyl donor (Scheme 1.11). The enzyme thymidylate synthase is very important because its inhibition has anticancer and antiviral chemotherapeutic implications.

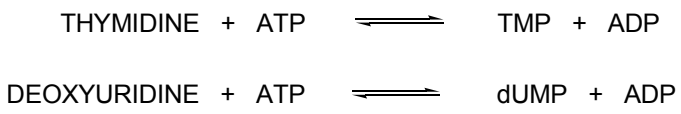
It seems possible that dUMP can be directly methylated to form dTMP, rather than the cells undertaking the energy-expensive route of dephosphorylating dUTP to dUMP then methylating and rephosphorylating to yield dTMP.



Scheme 1.11. Synthesis of dTMP from dUMP.

The latter route is preferred because dUTP levels need to be minimized in cells to prevent the incorporation of dUTP into DNA since the enzyme system that synthesizes DNA from dNTP's can not efficiently discriminate between dUTP and dTTP.

The salvage pathway leading to thymine nucleotides or its precursors, requires the enzyme thymidine kinase which uses either thymidine or deoxyuridine as a substrate, as shown below.



Regulation of Pyrimidine Nucleotide Biosynthesis

Bacteria regulate pyrimidine nucleotide biosynthesis by ATP-induced allosteric stimulation and feedback inhibition of the enzyme ATCase by CTP (minor) and UTP (major). Unlike bacteria, the regulation in mammals is effected at the first step which is catalyzed by carbamoyl phosphate synthetase II. Carbamoyl phosphate synthetase II is feedback inhibited by UDP and UTP and feed forward activated by ATP and PRPP. Another regulatory enzyme is OMP decarboxylase, which is competitively inhibited by UMP and CMP (lesser extent). Also, the rate of OMP production depends on the concentration of its precursor PRPP, which requires ATP for production. Thus ATP stimulates the production of PRPP, which in turn activates the production of OMP in a feed forward fashion (Figure 1.15).

Catabolism of Pyrimidine Nucleotides

DNA and RNA are digested by nucleases to produce their constituent pyrimidine nucleotides CMP, UMP, dCMP and dTMP (Scheme 1.12). Deamination, dephosphorylation and glycosidic bond hydrolysis breaks down these nucleotides into their component nucleobases uracil and thymine. Uracil and thymine are further broken down in the liver by reduction to

ultimately yield the amino acids β-alanine (when CMP and UMP are degraded) and/or β-aminoisobutyrate and NH_3 and CO_2 (when dTMP is degraded).

The resulting amino acids β-alanine and β-aminoisobutyrate are metabolized as they serve as $-NH_2$ donors in transamination of α-ketoglutarate to glutamate (Scheme 1.13); subsequent activation reaction converts the products to malonyl-CoA (precursor of fatty acid synthesis) and/or methylmalonyl-CoA (which is converted to succinyl-CoA and can be shunted to the TCA cycle). Thus, pyrimidine catabolism contributes to energy production within the cell.[19]

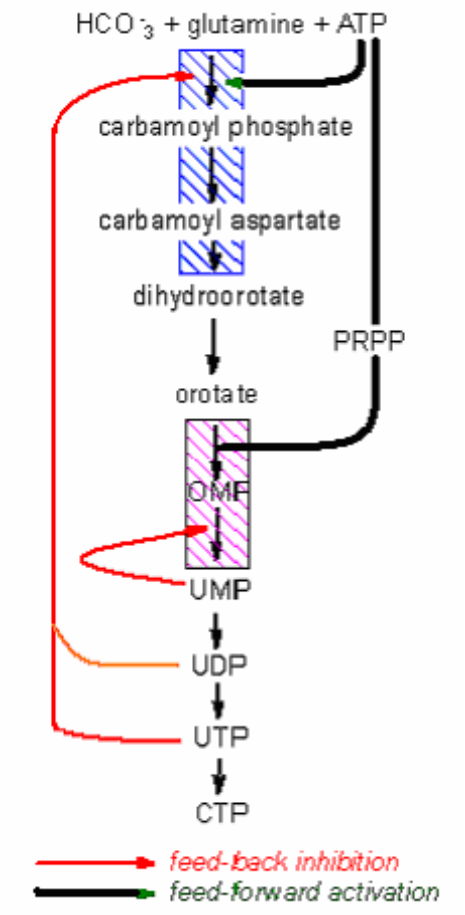
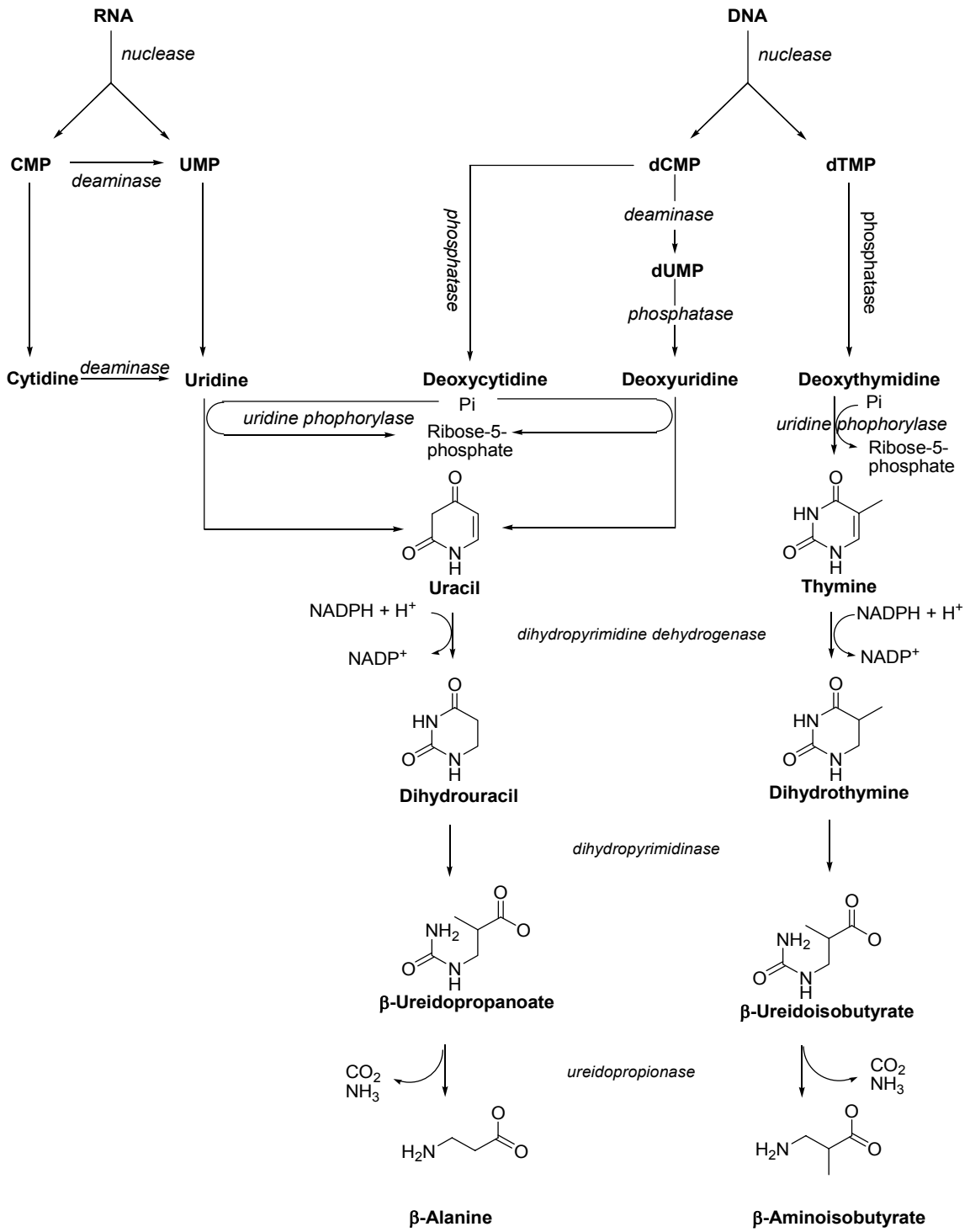
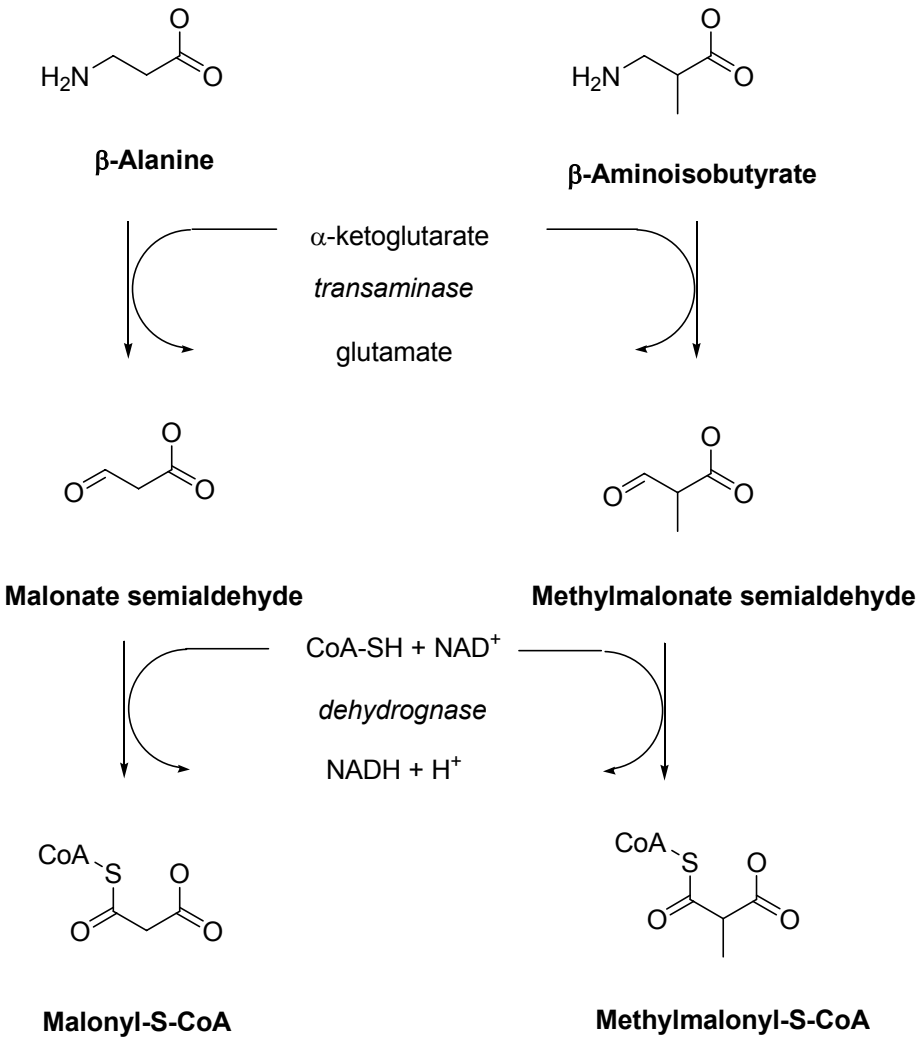


Figure 1.15. Mammalian Pyrimidine Biosynthesis Regulation.



Scheme 1.12. Pyrimidine Nucleotide Catabolism.

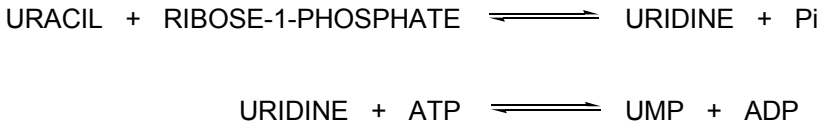


Scheme 1.13. Final Catabolism of Pyrimidine Nucleotides.

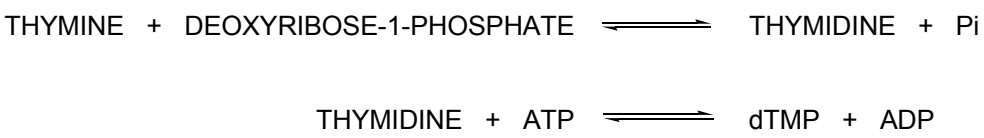
Salvage Pathway of Pyrimidine Nucleotides

As described earlier nucleic acid turnover as well as catabolism produces free pyrimidine bases (uracil and thymine) as well as free nucleosides. As needed by the cells these free bases and nucleosides can be salvaged back to nucleotides.

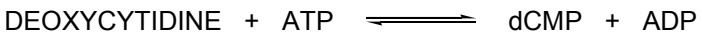
Uracil can be salvaged to form UMP through the concerted action of uridine phosphorylase and uridine kinase as shown below. Deoxyuridine is also a substrate for uridine kinase.



Formation of dTMP is by salvage of thymine and requires the enzymes thymine phosphorylase and thymidine kinase as shown below:



The salvage of deoxycytidine is catalyzed by deoxycytidine kinase as follows:



The major function of the pyrimidine nucleoside kinases is to maintain a cellular balance between the level of pyrimidine nucleosides and their monophosphates. However, since the overall cellular and plasma concentrations of the pyrimidine nucleosides, as well as those of ribose-1-phosphate, are low, the salvage of pyrimidines by these kinases is relatively inefficient.[18, 19]

Clinical Significances of Pyrimidine Metabolism

Abnormal pyrimidine metabolism has fewer clinical consequences compared to the purines, due to the good solubility of the by-products of pyrimidine catabolism. The salvage pathway leading to thymidine nucleotide synthesis is especially important in the preparation for cell division.

Table 1.6 shows some of the disorders and the enzymes involved. The major disorder is orotic aciduria, which is an inherited disorder resulting from the enzymes involved in the last two steps of UMP synthesis: orotate phosphoribosyl transferase and OMP decarboxylase. Orotic aciduria causes retarded growth and severe anemia, as well as leucopenia. The disorder can be treated with uridine and/or cytidine, which increases UMP production. UMP then inhibits CPS-II thereby controlling orotic acid production.

Table 1.6. Disorders of Abnormal Pyrimidine Metabolism.

Disorder	Defective Enzyme	Comments
Orotic aciduria, Type I	Orotate phosphoribosyl transferase and OMP decarboxylase	
Orotic aciduria, Type II	OMP decarboxylase	
Orotic aciduria (mild, no hematologic component)	The urea cycle enzyme, ornithine transcarbamoylase, is deficient	Increased mitochondrial carbamoyl phosphate exits and augments pyrimidine biosynthesis; hepatic encephalopathy
β-aminoisobutyric aciduria	Transaminase, affects urea cycle function during deamination of a-amino acids to of α-keto acids	Benign, frequent in Orientals
Drug induced orotic aciduria	OMP decarboxylase	Allopurinol and 6-azauridine treatments cause orotic acidurias without a hematologic component; their catabolic by-products inhibit OMP decarboxylase

Formation of Deoxyribonucleotides

The typical cell contains 5 to10 times as much RNA (mRNAs, rRNAs and tRNAs) as DNA. This means that both purine and pyrimidine nucleotide biosynthesis lead to an excess production of nucleoside triphosphates (NTPs) over their counterpart deoxynucleoside triphosphates (dNTPs). However, dNTPs are very important since they are the precursors of DNA synthesis and as such are needed by proliferating cells to replicate their genomes.

dNTP formation starts with the reduction of NDPs by the enzyme ribonucleotide reductase (RR) (to yield dNDPs), followed by phosphorylation by nucleoside diphosphate kinase (to yield the dNTPs), rather than by *de novo* biosynthesis from deoxyribose-containing precursors.

Ribonucleotide reductase (RR) works by replacing the 2′-OH of ribose with "H" *via* a free radical mechanism. This multifunctional enzyme contains a redox-active sulfhydryl pair for the transfer of electrons during the reduction reactions. In the process of reducing NDPs to dNDPs, RR becomes oxidized (disulfide form). RR is reactivated by disulfide bond interchange with one of its physiological reducing agents thioredoxin or glutaredoxin (Figure 1.16). The ultimate source of the electrons for this reduction is NADPH. The electrons are shuttled through a complex series of steps involving enzymes that regenerate the reduced forms of thioredoxin or glutaredoxin. These enzymes are thioredoxin reductase and glutathione reductase, respectively.

Regulation of dNTP Formation

Ribonucleotide reductase (RR) is solely responsible for the formation of all deoxyribonucleotides. As such it must be very well regulated to ensure that all four dNTPs are synthesized to maintain the proper intracellular ratios needed for DNA synthesis.

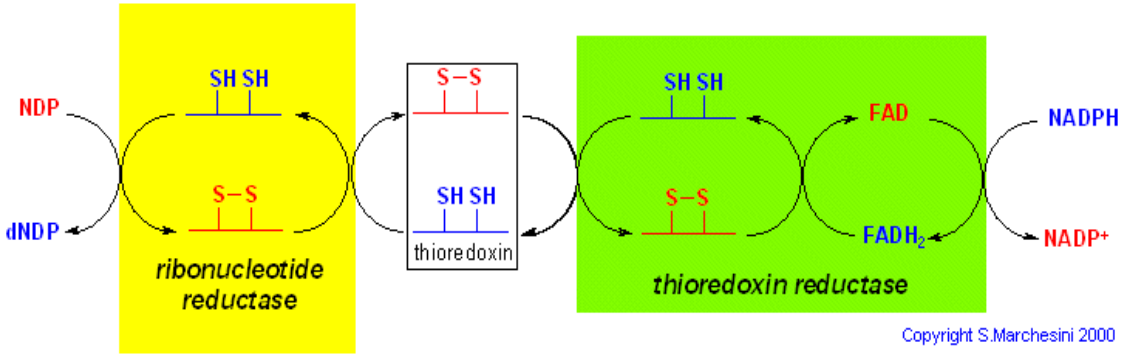


Figure 1.16. Formation of Deoxyribonucleotides.

This is very important because a shortage of any dNTP is lethal, whereas an excess of any dNTP is mutagenic because of its increased probability of being erroneously incorporated into a growing DNA strand. The activity of RR is regulated by the binding of nucleoside triphosphate effectors to either the activity sites or the specificity sites of the enzyme complex. Either ATP or dATP binds at the activity sites with low affinity, whereas ATP, dATP, dGTP, or dTTP bind with high affinity at the specificity sites. The binding of ATP at activity sites leads to increased enzyme activity, while the binding of dATP inhibits the enzyme toward all substrates. The binding of nucleotides at specificity sites effectively allows the enzyme to detect the relative abundance of the four dNTPs and to adjust its affinity for the less abundant dNTPs (or its precursors) in order to achieve a balance of production. Thus ATP or dATP binding stimulates the reduction of CDP and UDP (eventually leading to dCTP and dTTP), dTTP binding leads to GDP reduction (eventually forming dGTP) and inhibition of CDP and UDP reduction (i.e., stopping production of dTTP eventually). dGTP binding stimulates ADP reduction (eventual formation of dATP), but inhibits CDP, UDP and GDP reduction (i.e., prevents formation of dCTP, dTTP and dGTP).[19, 22]

Interconversion of Nucleotides

The nucleoside mono- and diphosphates released during the catabolism of nucleic acids do not accumulate in the cells just as the nucleosides and free nucleobases. These mono- and diphosphates are re-phosphorylated by nucleoside monophosphate (NMP) kinases and nucleoside diphosphate (NDP) kinases respectively to meet the cells need for energy and metabolic intermediates.

NMP kinases catalyze the ATP-dependent conversion of NMP to NDP; they do not discriminate between the ribose or deoxyribose sugar moiety of the nucleotide. There are four classes of NMP kinases that catalyze, respectively, the phosphorylation of:

1. AMP and dAMP
2. GMP and dGMP.
3. CMP, UMP and dCMP.
4. dTMP.

NDP kinases catalyze the conversion of NDPs to NTPs. It can use either ATP or any NTP or dNTP as a phosphate donor. The activity of the NDP kinases can range from 10 to 100 times higher than that of the NMP kinases. This difference in activity maintains a relatively high intracellular level of (d)NTPs relative to that of (d)NDPs. Unlike the substrate specificity seen for the NMP kinases, the NDP kinases recognize a wide spectrum of (d)NDPs and (d)NTPs.

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CHAPTER 2

VIRUSES

INTRODUCTION

Viruses (Latin word for 'poison') are non-living entities that have been implicated as etiological agents in many animal and plant diseases.[1] Their effects can be devastating, such as the early 1918 influenza plague that killed up to an estimated 20 million people.[2] Among the many common diseases caused by viruses are hepatitis B, herpes, yellow fever, viral meningitis, chicken pox, colds, cold sores, mononucleosis, mumps, rabies, polio, shingles, smallpox, warts, viral pneumonia, AIDS and some cancers. Additionally, residual viral proteins are capable of influencing the immune system to attack the body, resulting in auto-immune diseases such as lupus erythematosus, multiple sclerosis and rheumatoid arthritis. In Hepatitis B infections for example, viral DNA and proteins left in the host cells are carried unto daughter cells, these unique viral markings mislead the host immune system to attack liver cells causing more liver damage than by the actual virus itself.[1]

The origin of viruses is unascertained. Some evolutionists hypothesize that viruses 'evolved' from bacteria by natural selection. Others believe that viruses were the first form of "life", and that bacteria evolved from them (as did all other life). Yet still, other scientists speculate that a reverse symbiosis occurred, and that viruses arose out of cell parts such as bacterial plasmids and other organelles, and eventually evolved into their current forms.[3] So far evidence is lacking for each of these theories. Viruses date back several million years; it is believed that the herpes viruses have existed and co-evolved with mammals and other species for over 200 million years, whilst others such as HIV and the measles virus have entered human populations recently due to changes in agriculture (use of domestic animals), population

dynamics such as urbanization, migration of populations, commerce and changes in the environment.[4]

The notion exists that viruses serve no useful purpose whatsoever, except to infect other species and cause diseases.[5] A contrary perception argues that normally viruses are non-harmful, and viral pathogenesis only occurs when something goes wrong, such as a mutation or accidental inappropriate movement of genes.[1] HIV for example, infects some primates without causing illness or death, and has probably lived in them in a commensal relationship for generations. However, inappropriate human actions caused the transfer of the virus from monkeys to humans. As long as the HIV lentivirus lived in monkeys, it was not a threat for humans. As a matter of fact, HIV in monkeys (called SIV) is harmless to the monkeys,[6] and deliberate infections of baboons and certain animals with HIV have resulted in non-virulence.

Unarguably, viruses are known to be critical for medical and molecular research as noted by Zimmerman and Zimmerman: "today is the day of the virus' and 'nothing being studied in medicine, nothing in biology, is more important".[2] With more and more microbes developing resistance to antibiotic drugs, medicine is resorting to bacteriophages to help control bacterial growth and spread.[7] Bacteriophage therapy is very promising because almost all known bacteria have a specific predatory phage.[1] Unlike antibiotics that are non-specific (kill both harmful and even useful bacteria that aid in digestion), bacteriophages are highly specific. Antibiotics also have issues with resistant bacteria, intestinal disorders, yeast infections, and in some cases violent allergic reactions, none of which apply to bacteriophages.[7] Even though bacteriophage therapy is not perfect yet, it is very promising and has achieved some alleged success which includes the treatment of dysentery, typhoid fever, food and blood poisoning, as well as skin, throat, and urinary tract infections.[7]

Another medical use of viruses is the use of a modified virus to kill another virus. For example, a benign virus coated with CD4 and CCR5 (receptors on immune system cells) succeeded in locking specifically onto HIV-infected macrophages, whereas when coated with CD4 and CXCR4, the benign virus locked specifically into HIV-infected T-cells. This approach is being developed to specifically deliver antiviral genes to HIV-infected cells *in vivo*.[8]

Viruses also serve as gene-carrier vectors in gene therapy; viruses are the ideal carriers to transport genes as they naturally infect cells to deposit genetic material.[9] The present goal of gene therapy is to load virus-carriers with healthy genes and then infect the relevant tissues so that the cell incorporates the new virus-carried genes into its own DNA. These viral-vectors are designed to commit suicide after delivering the therapeutic gene.[10] Gene therapy is especially promising for some neurological disorders such as Alzheimer's and Parkinson's and many inherited disorders and types of brain tumors. This is so because certain modified viruses can pass through the rather impenetrable blood/brain barrier and deliver therapeutic genes to the brain, to not only produce needed neuro-transmitters, but also to treat brain tumors.[9] More than a hundred different clinical trials are under way to develop this very promising therapy.[9]

The science of virology as a comprehensive discipline is only about 70 years old (since 1930s), and scientist believe that pathogenic viruses are only the 'tip of the iceberg' of virus types, and the more that is learned about the biological world the more scientists realize the critical role (both harmful and medicinal) that viruses play in life.[2]

THE DISCOVERY OF VIRUSES

Researchers observed that fluid extracts from certain diseased animals and plants were capable of causing disease even after filtration through the finest filters known then (unglazed

ceramic plates).[11] This included the observation by the Russian Scientist Dmitri Ivanovski, who found that the filtrate from a section of a tobacco mosaic disease infected leaf would infect healthy plants.[12] Martinus Bijerinck (a Dutch botanist) disproved the assumption that toxins must have caused the disease when he showed that the sap could successively transmit the fully virulent disease through a large number of plant generations. This indicated that the disease agent was multiplying in the plant, otherwise it would have become successively weaker as it spread to each new plant generation. Some scientists speculated that bacterial spores far smaller than those that had been discovered were the cause of the disease. It was eventually realized that the culprit was a non-cellular agent that could diffuse through the cell walls and membranes into the cell's protoplasm. This lead to the discovery in the 1890s that the cause of tobacco mosaic disease was a filterable agent smaller than bacteria; this agent became known as a virus.[1] By the early 1900s, additional viruses had been identified, including viruses that caused tumors in chickens, as well as the first discovered human virus in 1901 (yellow fever virus).[4] It was not until the development of the electron microscope and improved filtering technology in the1930s, that scientists could actually visualize viruses and appreciate them for their extremely small size and complex structures.[13]

GENERAL CHARACTERISTICS OF VIRUSES

Viruses are generally characterized as follows:

1. They are obligate intracellular parasites. Viruses are acellular (contain no cytoplasm or cellular organelles), and as such are totally dependent on a host cell for replication.
2. They are the smallest organisms with diameters ranging from 5 nm - 300 nm. They are actually filterable through bacteriological filters.

3. They have distinct morphological shapes.
4. Their genome consists of a single type of nucleic acid, either DNA or RNA, never both. This genome is a single or segmented, circular or linear molecule of nucleic acid and it is either single-stranded or double-stranded DNA or RNA, which codes for the synthesis of viral components and viral enzymes for replication.
5. They possess a protein coat (capsid) consisting of individual protein units (capsomeres). The capsid houses the nucleic acid and other structures that facilitate preserving the genes they contain.[3, 14] The capsid may be surrounded by a lipid bilayer and glycoprotein envelope, which is derived from the host cell membrane during the process of viral budding. These viral envelopes contain virally-encoded proteins, (often glycoproteins), which play a role in the process of virus attachment, entry and uptake. Envelopes are not present on all viruses and viruses which contain envelopes are usually less stable than those that do not; for example, herpes viruses (enveloped) are less stable than polio and human papillomaviruses (non-enveloped).[4]
6. They have proteins (antigens) extending from their surface that facilitate specific binding with specific receptors on the host cell.
7. They multiply inside living cells by using the biosynthetic machinery of the host.

MORPHOLOGY OF VIRUSES

Viruses are the smallest infectious agents known and range from 200 nm for vaccinia to 20 nm for parvovirus.[1] The general range in size is considered to be from 5 nm to 300 nm, even though some paramyxoviruses can be up to 14,000 nm long. Viruses are submicroscopic and

require an electron microscope for detailed viewing. Figure 2.1 shows size comparisons of viruses, proteins and atoms, and the instruments used for their detection.[4]

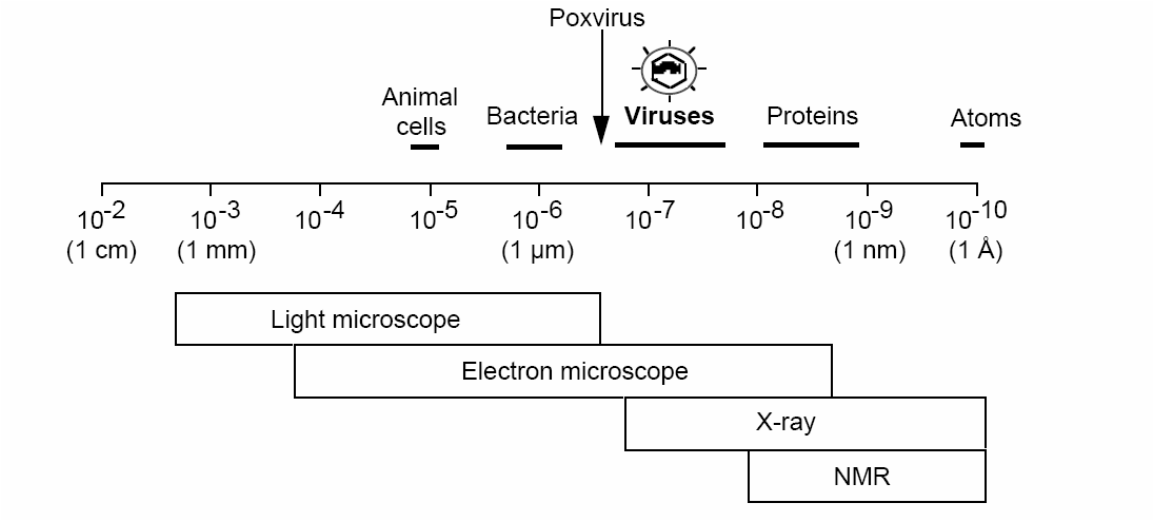


Figure 2.1. Size of Viruses.

Compared to animal cells, viruses are extremely small. To help put the size of viruses into perspective, the following comparisons have been drawn; comparing a virus to an animal cell is like comparing a basketball to the New York World Trade Center.[1] It takes about 50 million polioviruses to fully fit into the average human cell.[15] For comparison to a typical bacterium, which is 1micron in diameter, a bacteriophage is one-fortieth of a micron long. Likewise, in a major discovery by marine biologists, that a major proportion of the sea's biomass is microscopic and is comprised of viruses, bacteria, algae and protozoa, it was concluded that a teaspoon of seawater may contain more than a billion viruses.[16] Viruses come in different sizes and shapes (Figures 2.2, 2.3, 2.4).[17]

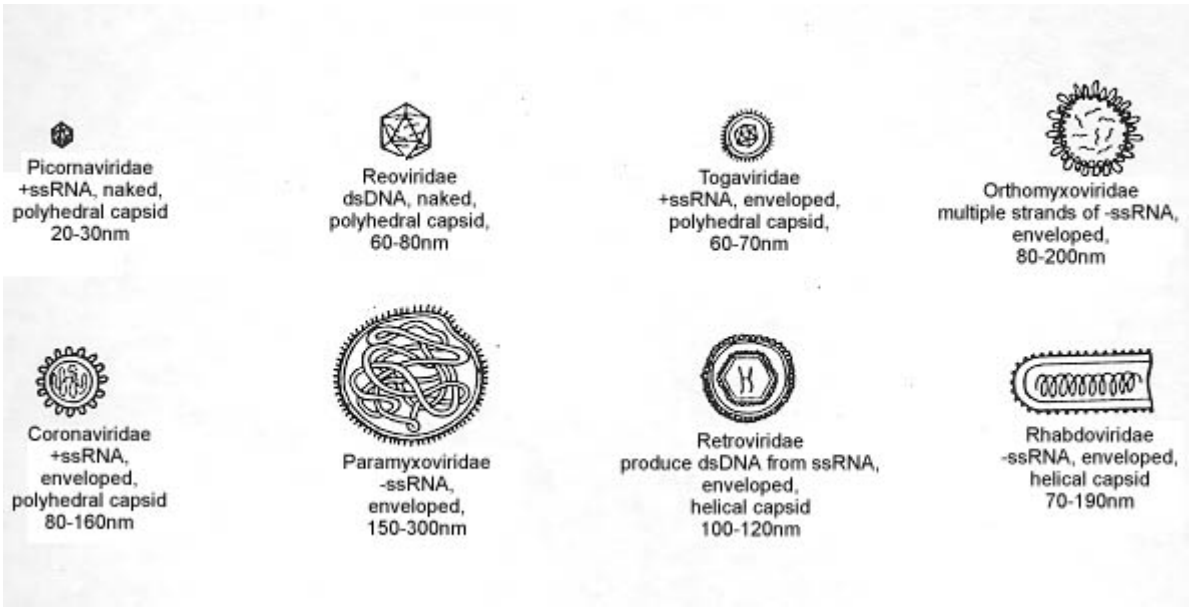


Figure 2.2. Sizes and Shapes of Animal RNA Viruses.[18]

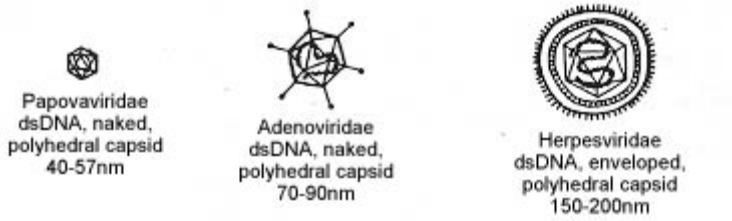


Figure 2.3. Sizes and Shapes of Animal DNA Viruses.[18]

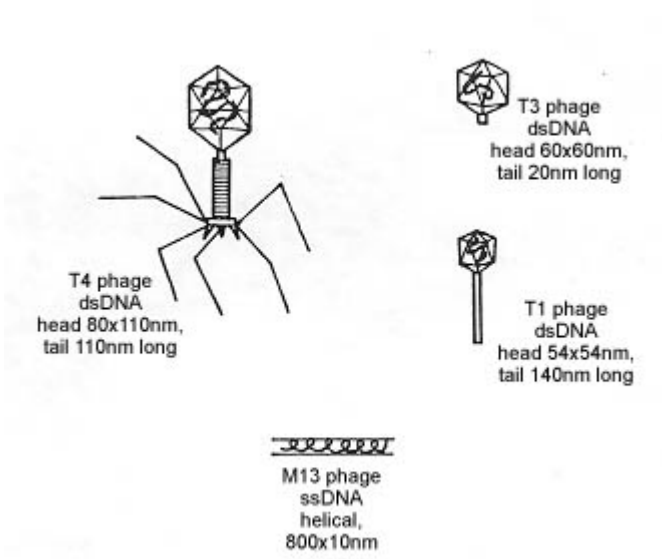


Figure 2.4. Sizes and Shapes of Viruses (Bacteriophages).[18]

Viruses can be classified into four morphological types depending on the symmetry of their capsid or envelop.

1. Helical (Rod-like): Viruses having this shape have their genome surrounded by a hollow protein cylinder or capsid and they possess a rod-like, helical structure e.g., bacteriophage M13 (Figure 2.5).
2. Polyhedral (Cubic): The viral genome is encapsulated by a polyhedral (many-sided) shell or capsid (Figure 2.6). The major form here is an icosahedron, which has 20 identical equilateral triangular faces, 12 vertices and 30 edges e.g., poliovirus.[19, 20]
3. Enveloped: The viral genome is covered by either a helical capsid (e.g., influenza virus) or a polyhedral capsid (e.g., herpes simplex) and is surrounded by a loose membranous envelope. The envelope is roughly spherical but makes varying shapes because it is not rigid (Figures 2.7 and 2.8).

4. Binal (Complex): This is where the genome encapsulation is neither helical nor polyhedral, but rather irregularly shaped (pleomorphic) and complex i.e., they do not possess clearly defined capsids, but have several coats around the nucleic acid core e.g., poxviruses (Figure 2.9).

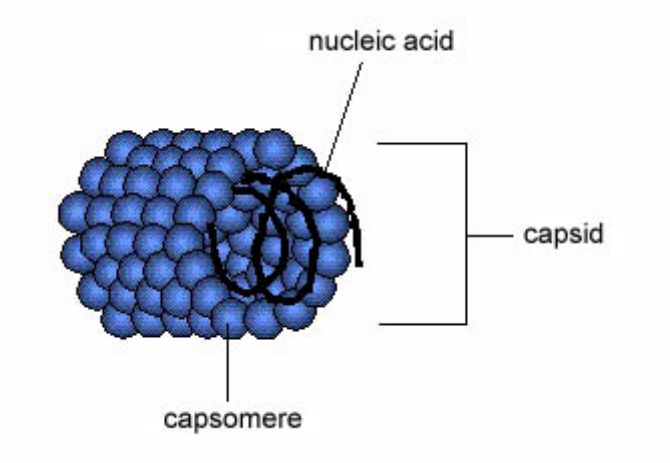


Figure 2.5. Viral Structure (Helical Virus).[18]

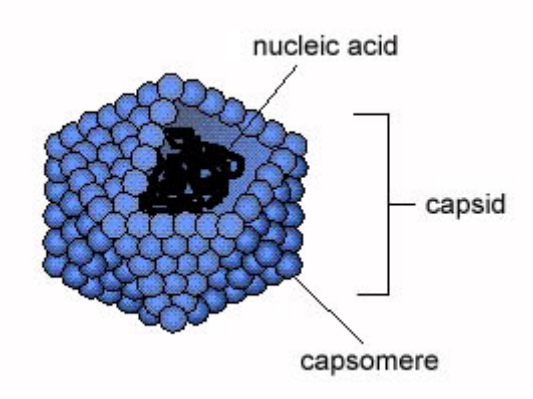


Figure 2.6. Viral Structure (Polyhedral Virus).[18]

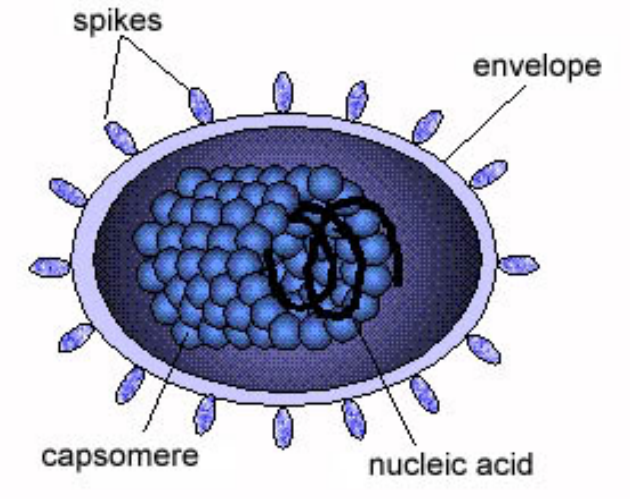


Figure 2.7. Viral Structure (Enveloped Helical Virus).[18]

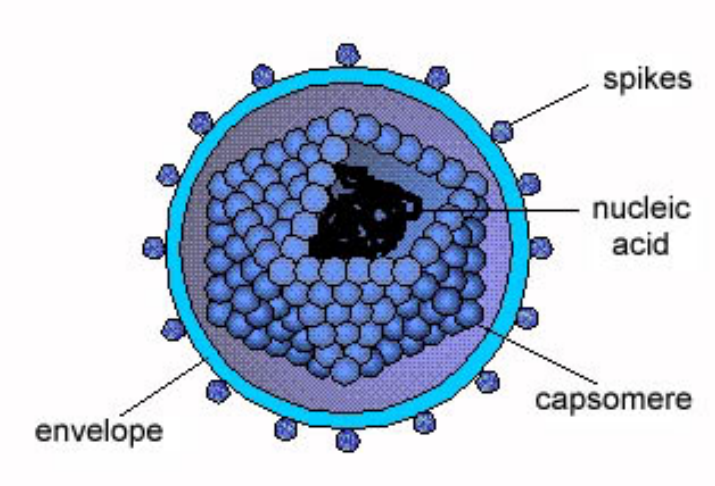


Figure 2.8. Viral Structure (Enveloped Polyhedral Virus).[18]

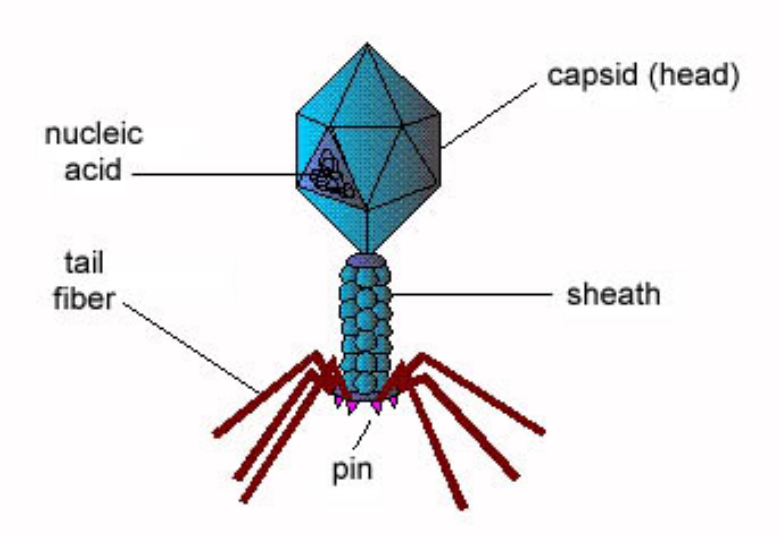
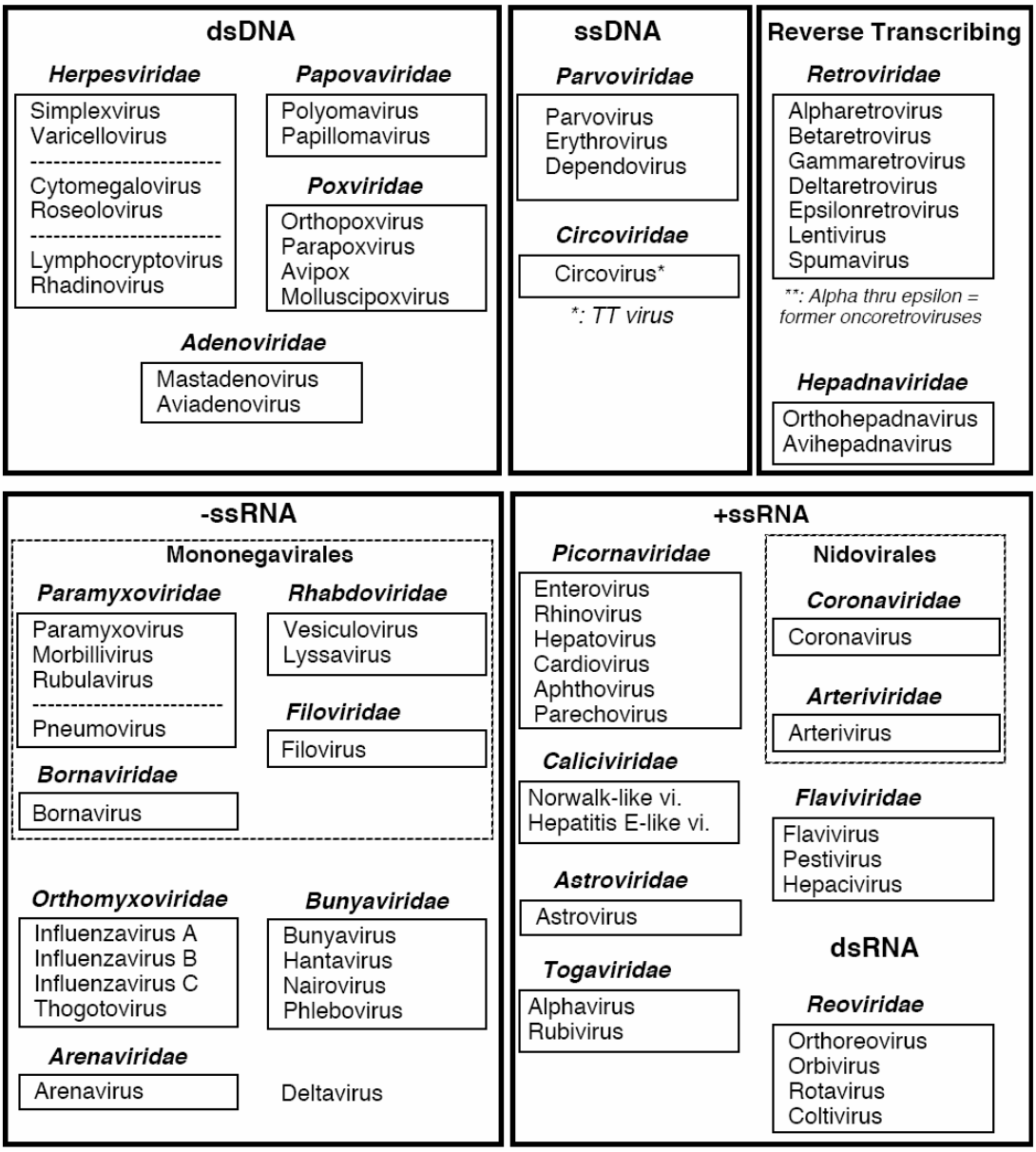


Figure 2.9. Viral Structure (Binal).[18]

CLASSIFICATION OF VIRUSES

Viruses are often classified by the type of nucleic acid they have for their genome, the shape and symmetry of their capsid (helical or polyhedral etc), the presence or absence of an envelope, and the size of the virus particle. The International Committee on Viral Taxonomy (ICTV) emphasizes the viral genome as the primary determinant for their viral taxonomy (Table 2.1).[4, 21] Other systems such as the more comprehensive Baltimore Classification (Table 2.2), which compliments the ICTV's, is based on genetic content and replication strategies of viruses. According to the Baltimore classification, viruses can be grouped into the following seven classes: dsDNA, ssDNA, dsRNA, (+)-sense ssRNA, (-)-sense ssRNA, RNA reverse transcribing and DNA reverse transcribing viruses (Table 2.2). Currently, there is a trend towards the use of genomics for virus classification, that is, sequence analysis of the viral genome and comparison to other known virus sequences.[4]

Table 2.1. International Committee on Viral Taxonomy.[21]



ss = single strand; ds = double strand;
(+) RNA is one which can function as mRNA for the synthesis of proteins.
(-) RNA cannot function as mRNA.

Table 2.2. Baltimore Classification of Viruses.

CLASS	NUCLEIC ACID	EXAMPLES	ENVELOPE	GENOME SIZE (kb)
I	dsDNA	Herpes virus Poxvirus Adenovirus Papillomavirus	Yes Yes No No	120-220 130-375 3.0-4.2 5.3-8.0
II	ssDNA	Adeno-associated virus	No	5.0
III	dsRNA	Reovirus	No	18-31
IV	(+) ssRNA	Togavirus Poliovirus Foot-and-Mouth disease virus Hepatitis A virus Hepatitis C virus	Yes No No No Yes	9.7-11.8 7.4 7.5 7.5 10.5
V	(-) ssRNA	Influenza virus	Yes	12-15
VI	(Reverse) RNA	HIV	Yes	9.7
VII	(Reverse) DNA	Hepatitis B virus	Yes	3.1

ss = single strand; ds = double strand;
(+) RNA is one which can function as mRNA for the synthesis of proteins.
(-) RNA cannot function as mRNA.

As noted earlier, the viral genome is strictly either DNA or RNA and never both. Generalizations concerning the classification and genome of viruses can be made, but with a few exceptions. For example, almost all DNA viruses that infect animals contain double-stranded DNA, except Parvoviridae (e.g., parvovirus B19, adeno-associated virus) and Circoviridae (e.g., recently discovered TT virus, which may be related to the development of some cases of hepatitis), which are single stranded. With the exception of the Reoviridae (e.g., rotaviruses) that contain double-stranded RNA, all RNA viruses contain single-stranded RNA; they are broadly subdivided into positive strand (+) RNA genomes and negative strand (-) RNA genomes. (+) RNA genome has the same polarity as mRNA. They include picornaviridae and caliciviridae. (-)

RNA genome has opposite polarity to mRNA. Viruses in this category all have helical capsids. Some viruses have segmented genomes e.g., orthomyxoviruses have eight, whereas the arenaviruses and bunyaviruses have either two or three. The arenaviruses and some bunyaviruses are also unique in that they possess ambisense genomes (i.e., contain both (+) and (-) strand RNAs).[4]

VIRAL REPLICATION CYCLE

All known life forms can be infected by viruses, however some are more prone than others.[22] The means of multiplication of a virus in its host cell is referred to as its replication cycle.[17] Even though there are variations among viruses, a generalized viral replication cycle consists of some basic steps. Also, the replication cycle of an enveloped virus is slightly different from that of a non-enveloped (naked) virus; the differences will be pointed out during the following discussion of the replication cycle of an enveloped virus. It should be noted that most viruses are highly specific in their infectivity. This specificity is not only for a particular host, but also for organ and cell types within the host.[23]

Attachment. All the different animal cells possess specific membrane receptors, just as viruses do. These receptors facilitate specific attachment between viruses and animal cells if compatibility exists (Figure 2.10). For example, the human rhinovirus (causative agent of the common cold) attaches specifically to intercellular adhesion molecules (ICAM-1) found on nasal epithelial cells, whereas the HIV (causative agent of the AIDS) attaches specifically to CD4 molecules and chemokine receptors on human T4-lymphocytes and macrophages. Thus, the attachment is usually species-specific, with a specific virus type infecting only a specific animal or plant cell or type. There are however some virus types such as rabies and influenza that have a

wide range of hosts. For enveloped and naked viruses, the mechanism of attachment is the same, except that enveloped viruses use receptors on their envelope, whereas naked viruses use receptors on their capsid.[1, 17]

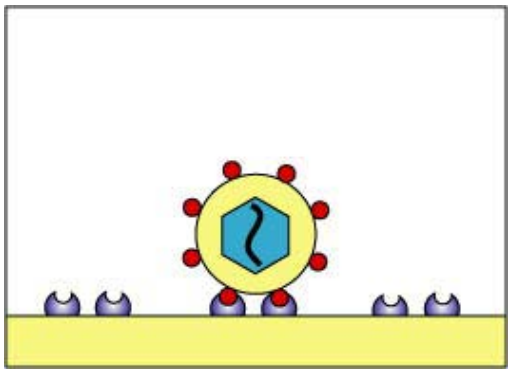


Figure 2.10. Attachment of Viral Receptors to Host Cell Receptors.[18]

Penetration. This is the means by which the virus (either whole, or just its genome) enters the host cell. After attachment, penetration can occur in one of two ways: 1) by endocytosis (the same process the cell uses to take in nutrients) where the host cell membrane invaginates and inwardly pinches off to introduce the whole virus as an endocytic vesicle into the cytoplasm (Figure 2.11). For both enveloped and naked viruses, this process is similar; 2) the bond between the receptors opens up channels in the host cell membrane allowing the viral genome to flow into the cytoplasm (Figure 2.12). It is relevant to point out that for enveloped viruses, the genome enters the cytoplasm surrounded by the capsid (Figure 2.12), whereas for naked viruses the genome enters as a 'neat' nucleic acid (Figure 2.13).[17] There are also some virus types that can directly pass through the pores in the host cell membrane and enter the cytoplasm.[1]

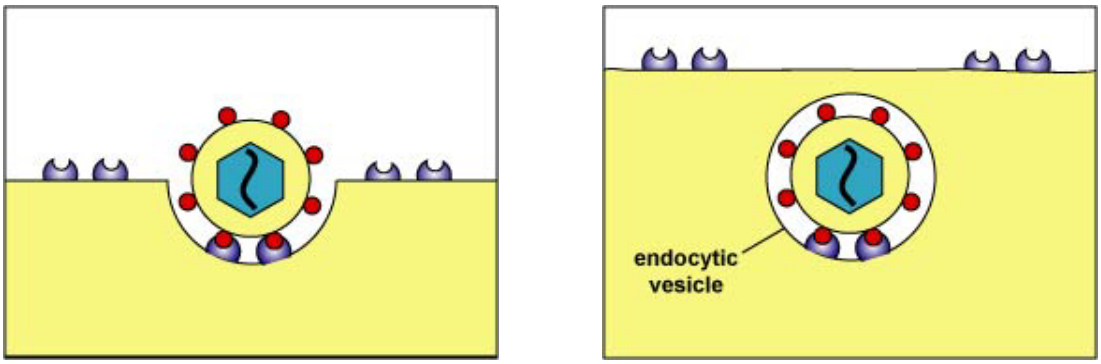


Figure 2.11. The Process of Viral Endocytosis.[18]

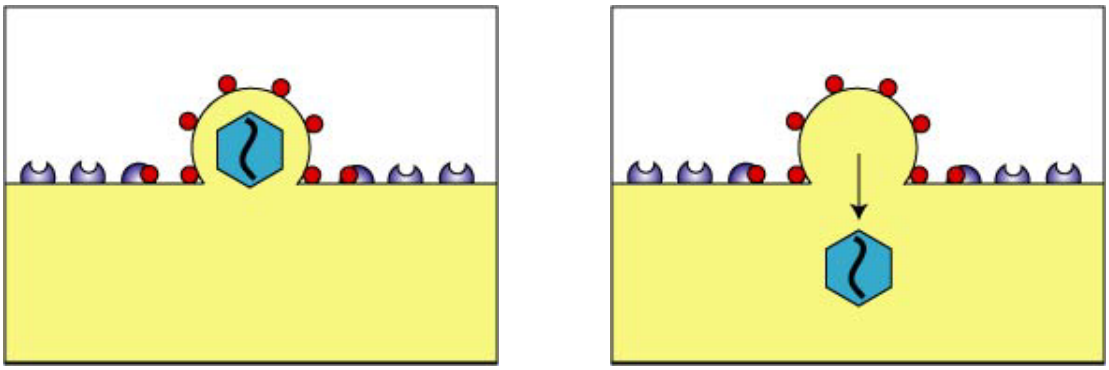


Figure 2.12. Introduction of Viral Genome into Host Cell (Enveloped Virus).[18]

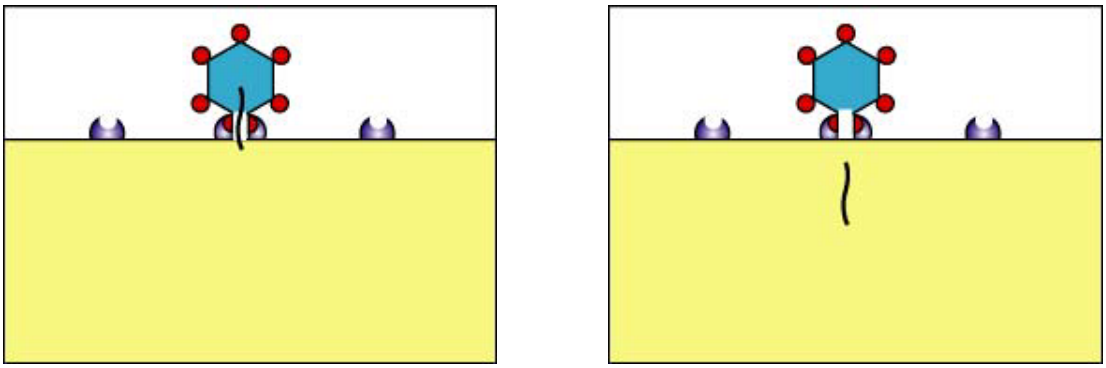


Figure 2.13. Introduction of Viral Genome into Host Cell (Naked Virus).[18]

Uncoating. This is the process by which the viral genome ('neat' nucleic acid) is released from the remainder of the virus in the cytoplasm. This is necessary for endocytic vesicles resulting from endocytosis of both enveloped and naked viruses, as well as the capsid of enveloped viruses in the cytoplasm. For enveloped viruses entering by endocytosis, the viral envelope is removed by fusion with the membrane of the endocytic vesicle. The viral capsid is then removed enzymatically to release the viral genome in the cytoplasm.

For naked viruses that entered the cytoplasm by endocytosis, the endocytic vesicle and the viral capsid are removed enzymatically to expose the viral genome. Uncoating ushers in the eclipse period during which no intact virions can be detected within the cell.[17]

Replication. This is the process where the viral genome uses the host cell's metabolic machinery (enzymes, nutrients, energy, ribosomes, tRNA, etc.) to synthesize viral nucleic acids, enzymes and viral parts. The different forms of viral genome need to be transcribed into viral mRNA (Figure 2.14), which then goes to the host cell's ribosomes for translation into viral genome, viral enzymes, and viral structural proteins. For example, for retroviruses where the viral genome is RNA, it is reverse-transcribed into DNA by the enzyme, reverse transcriptase. The viral DNA is then integrated into a specific site on the host DNA by integrase. Integrase cuts the circular plasmid DNA, then splices in the viral DNA and repairs the two splice sites. The incorporated viral DNA is then transcribed into viral mRNA by the host cell's RNA polymerase, and further processing is undertaken. Viral genome replication is very high in the early replication stage, whereas during the late stage, concentration is on viral structural proteins, envelope glycoproteins and maturation enzymes. At this point also, viral-genome-encoded proteins and glycoproteins are incorporated into the host cell's membrane. Up to this point the virus is not infectious.[17]

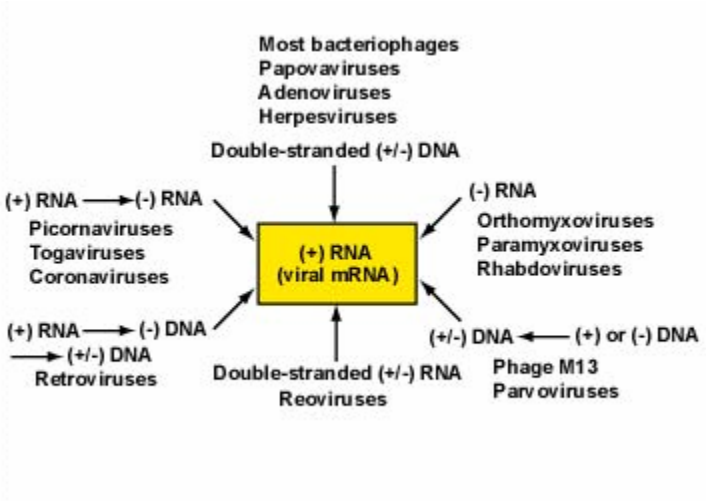


Figure 2.14. Transcription of Different Viral Genome to Viral mRNA.[18]

Maturation. During this stage, the newly formed viral components are assembled into individual viral particles. This also involves the process where viral structural proteins are assembled into a capsid to enclose the newly synthesized viral genome. Some viral structural proteins possess the ability to self assemble into a capsid without the assistance of enzymes or other outside influence.[1, 17]

Release. Naked viruses and enveloped viruses are released differently. With naked viruses, the host cell lyses and releases progeny viruses which infect other cells. With enveloped viruses, the host cell may not be lysed. The newly formed viruses bud out. This budding process involves the evagination and pinching off to form the viral envelope and it can occur either at the outer cytoplasmic membrane, the nuclear membrane, or at the Golgi apparatus membrane. At the cytoplasmic membrane, the viruses evaginate off and are ready to infect other cells. On the other hand, viruses that get their envelopes from the nuclear membrane or Golgi apparatus, leave the cell by exocytosis *via* transport vesicle. It is known that as many as 10,000 to 50,000 new viruses

can result from a single infected host cell.[17] By this means the new viruses are released from the cell to infect other cells, spreading even more genes to other cells.[24] Some newly-formed viruses are not released but transported by cell-to-cell contact from one cell to another by fusion.

VIRAL INFECTIONS

Even though it is well understood how a virus replicates to form progeny viruses, which successively infect other cells, not all viral infections have the same outcome. A viral infection could lead to one or a combination of four possible outcomes: cytopathic effects (CPE), persistent infection, latent infection and/or tumor production (Figure 2.15).

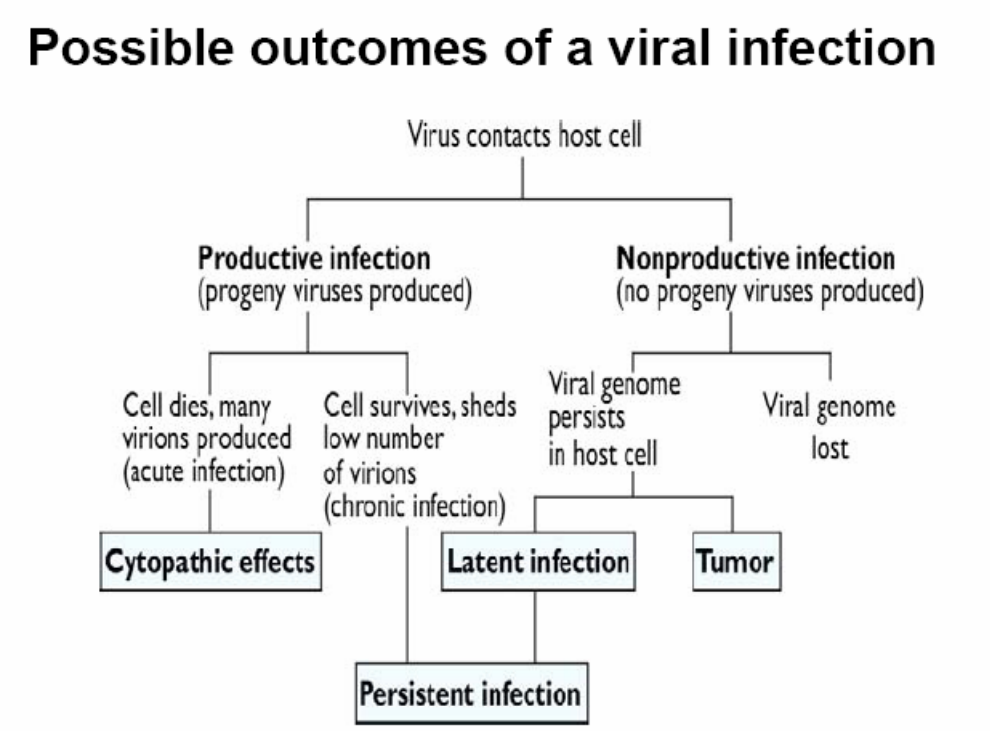


Figure 2.15. Possible Outcomes of a Viral Infection.[25]

In one case of infection, productive infection occurs. This could lead to efficient production of several thousands of progeny viruses, which kill the cell as they break out. Reoccurring and subsequent infection causes a lot of cells to be killed, which over a period of time can lead to severe cytopathic effects and even death. On the other hand, the productive infection may not be as efficient and produce a low viral count which is continually present in the body.

The cell survives as it only sheds a low number of progeny viruses. This case leads to a persistent (chronic) infection where there is no death, but the organism suffers the effects of the virus' pathogenicity.

In another case, the viral infectivity is non-productive and no progeny viruses are produced. The viral genome could be lost as it is destroyed by the host immune system or just remain latent in the host cell, e.g., HSV-1 (fever blister), HSV-2 (genital herpes), VZV (chickenpox, shingles) and HIV. In one case this latency can flair-up into a persistent infection when the host's immune system is compromised, or when other conditions favorable to the virus presents itself. Some viruses such as human papilloma virus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV), Epstein-Barr virus (EBV) and human T-lymphotropic virus type 1 (HTLV-1) are capable of viral transformation, i.e., they transform normal cells into malignant cells. They contribute to over 15% of the world's cancer. Up to 80% of these human viral-associated cancers are cervical cancers (associated with HPV) and liver cancer (associated with HBV and HCV).[1, 17, 26] It is believed that the presence of the viral nucleic acid within the cell disturbs cellular metabolism and causes the formation of cancer cells or increases the risk.[1]

THE MAJOR VIRUSES AFFECTING MAN

Table 2.3. Viruses Causing Human Diseases.[23]

VIRUS	SIZE (nm)	FAMILY	GENOME	ROUTE OF INFECTION	DISEASE	INCUBATION PERIOD	CURRENT LICENSED/EXPT. THERAPIES
Vaccinia	250	Poxviridae	DNA	Skin abrasion	Small pox		Eliminated
Rotavirus	70	Reoviridae	RNA(-)	Ingestion	Diarrhea		Rehydration
Polio	25	Picornaviridae	RNA(+)	Ingestion	Poliomyelitis	7-14 days	Vaccination
Rhinovirus	25	Picornaviridae	RNA(+)	Respiratory	Colds	1-3 days	Self-limiting
HAV	25	Picornaviridae	RNA(+)	Ingestion	Liver disease	3-5 weeks	Self-limiting/Vaccination
Rubella	80	Togaviridae	RNA(+)	Respiratory	German measles	14-16 days	Vaccination
Yellow fever	30	Flaviviridae	RNA(+)	Insect bite	Yellow fever	3-6 days	Vaccination
HCV	30	Flaviviridae	RNA(+)	Body fluids	Liver disease		Interferons
Bunya	100	Bunyaviridae	RNA(-)		Tropical fevers		None
HSV	150	Herpesviridae	DNA	Skin abrasion Sexual contact	Cold sores Genital sores		Nucleosides
VZV	150	Herpesviridae	DNA	Respiratory Reactivation	Chicken pox Shingles	13-17 days Years	Nucleosides Nucleosides
HCMV	150	Herpesviridae	DNA	Respiratory	Various		Nucleosides
EBV	150	Herpesviridae	DNA	Respiratory	Mononucleosis	4-6 weeks	Nucleosides
Adeno	80	Adenoviridae	DNA	Direct contact Respiratory	Eye infections Cold symptoms		None used None used

HPV	50	Papovaviridae	DNA	Skin abrasion Sexual contact	Warts Genital warts		None None
HBV	40	Hepadnaviridae	DNA	Body fluids	Liver disease	10-12 weeks	Vaccination Nucleosides, Interferons
Corona	200	Coronaviridae	RNA(+)	Respiratory	Cold symptoms Bronchitis		None
RSV	150	Paramyxoviridae	RNA(-)	Respiratory	Bronchitis		Nucleosides
Measles	150	Paramyxoviridae	RNA(-)	Respiratory	Measles	13-14 days	Vaccination
Mumps	150	Paramyxoviridae	RNA(-)	Respiratory	Mumps	14-18 days	Vaccination
Influenza	100	Orthomyxoviridae	RNA(-)	Respiratory	Flu	1-3 days	Vaccination Amantadine, Neuraminidase inhibitors
Ebola	800x40	Filoviridae	RNA(-)	Body contact	Haemorrhagic fever		None
HIV	100	Retroviridae	RNA(+)	Body fluids	AIDS	Long	Nucleosides, reverse transcriptase/protease inhibitors

The viral replication cycle leads to the production of several thousands of new progeny viruses, which intercellularly infect other cells of the host over and over again, thereby exerting its cytopathic effects or any one of the possible outcomes shown earlier. Besides the spread of viruses within a host, viruses can also be transmitted from one host to another, and even spread across populations. The epidemiology of viruses indicates that the route of infection varies for different viruses and it could range from mere body contact for the Ebola virus to respiratory and sexual contact for influenza and HIV respectively (Table 2.3).[23]

CURRENT ANTIVIRAL CHEMOTHERAPY

It is believed that the first incidence of antiviral chemotherapy was in 1796, when Jener demonstrated scientifically the protection against smallpox by inoculation with cowpox. The implications were not well understood then, since viruses were discovered some 134 years later in 1930.[23] Success in antiviral chemotherapy, when compared to bacteria and fungal chemotherapy is relatively young and more difficult. This difficulty is because most viruses replicate very quickly (some species have a two-hour replication cycle) and common transcription mistakes made in their genome are not edited out by their polymerases, thereby leading to frequent mutated resistant viruses.

The saying is true that 'prevention is better than cure'. Even though prevention by way of vaccination has effectively controlled viral diseases such as polio, measles, mumps, rubella, yellow fever, hepatitis B, rabies and has completely eradicated smallpox, it cannot control all viral diseases. Vaccines are effective for viruses with a limited propensity for mutation, sadly, for an individual who has already contracted a virus, prevention is too late and treatment and control are the only options. Viral infections cannot be treated with antibiotics, however it is not uncommon that secondary opportunistic bacterial and fungal infections can occur following a viral disease which may require their own independent treatment.

The discovery and development of effective antiviral drugs seemed implausible until the discovery of methisazone (Figure 2.16), which showed efficacy against smallpox. Following this discovery, a tremendous effort in medicinal chemistry was focused on the design and synthesis of several nucleosides as antivirals. This effort led to the discovery of acyclovir (ACV, Figure 2.16), which is the first truly effective and selective antiviral agent. Acyclovir owed its discovery to the understanding of the fundamental biochemistry of viral replication and also to the field of

nucleic acid synthesis in the 1950s. It is estimated that in 1994, the market for antiviral chemotherapeutics was worth about £1.8 billion and about half of this revenue was attributed to acyclovir. This made antiviral chemotherapy one of the major success stories of modern medicinal research, and nucleic acids, particularly nucleosides, the most important antiviral chemotherapeutic targets from the point of view of medicinal chemistry.[23]

Methisazone Acyclovir

Figure 2.16. Structures of Methiazone and Acyclovir.

Unfortunately, viral diseases that have gained most funding and research for potential chemotherapy are those afflicting the developed world.[23] Current strategies employed in the development of antiviral compounds are based on a thorough understanding of the viral replication cycle. Figure 2.17 shows a representative (generic) scheme of the viral replication cycle and highlights the possible target areas of antiviral chemotherapy.

Although the detailed mechanism by which each virus type undergoes replication is specific, there are some general similarities in the way they replicate which can be exploited for antiviral chemotherapy. The individual stages of viral replication (discussed earlier) that continue to be targeted for antiviral chemotherapy include:

Attachment/Penetration into the host.

Uncoating of virus to release genome

Transcription / Translation of viral genome

Maturation / Release of progeny viruses.

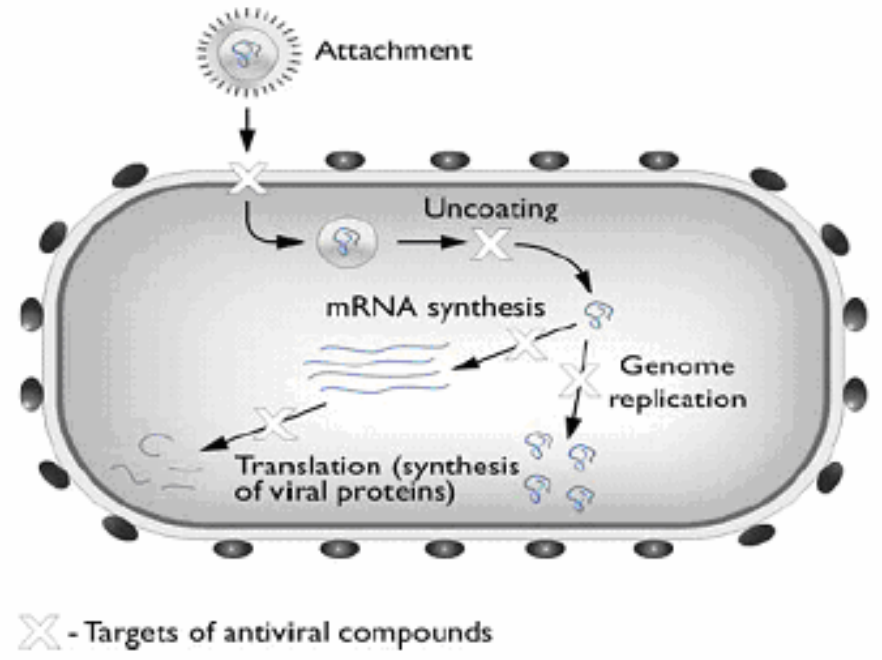


Figure 2.17. Viral Replication Cycle.[25]

An antiviral repertoire featuring clinically-approved compounds, their targeted viruses and enzymes, as well as the stage of the replication cycle they target is shown in Table 2.4.[27, 28] Table 2.4 shows two very important cellular enzymes namely, inosine monophosphate dehydrogenase (IMPDH) and S-adenosylhomocysteine hydrolase (SAH), whose inhibition offer remarkable antiviral chemotherapy.[27] IMPDH is the focus of this dissertation and will be discuss in-depth hereafter.

Table 2.4. The Antiviral Repertoire.

Approach	Target virus(es)	Compounds approved	Selected compounds in development for the indicated target virus
Virus adsorption inhibitors	HIV, HSV, CMV, RSV and other enveloped viruses		Polysulphates,polysulphonates, polycarboxylates,polyoxometalates, chicoric acid, zintevir, cosalane derivatives, negatively charged albumins
Virus–cell fusion inhibitors	HIV, RSV and other paramyxoviruses		HIV: AMD3100, TAK779 and T20 derivatives
Viral DNA polymerase inhibitors	Herpes viruses (HSV-1, -2, VZV, CMV, EBV, HHV-6, -7, -8)	Acyclovir, valaciclovir, ganciclovir,valganciclovir, penciclovir, famciclovir, brivudin*, foscarnet	Bicyclic furopyrimidine nucleoside analogues, A5021, cyclohexenylguanine
Reverse transcriptase inhibitors	HIV	NRTIs: zidovudine, didanosine,zalcitabine, stavudine, lamivudine‡, abacavir NNRTIs: nevirapine, delavirdine, efavirenz	Emtricitabine, amdoxovir Emivirine, UC781, DPC083, TMC125 (R165335)
Acyclic nucleoside phosphonates	DNAviruses(polyoma-,papilloma-, herpes ,adeno-and poxviruses), HIV, HBV	CMV: cidofovir HIV: tenofovir	HBV: adefovir
Inhibitors of processes associated with viral RNA synthesis.	HIV, HCV		
Viral protease inhibitors	HIV, herpesviruses, Rhinoviruses, HCV	HIV: saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir	HIV: atazanavir, mozenavir, tipranavir Human rhinovirus: AG7088
Viral neuraminidase inhibitors	Influenza A and B virus	Zanamivir, oseltamivir§	RWJ270201
IMP dehydrogenase inhibitors	HCV, RSV	Ribavirin‖	Mycophenolic acid, EICAR, VX497
S-adenosyl homocysteine hydrolase inhibitors	(–)RNA hemorrhagic fever viruses (for example, Ebola)		

(*Brivudin is approved in some countries; for example, Germany. ‡ Lamivudine is also approved for the treatment of HBV. § In addition to zanamivir and oseltamivir, amantadine and rimantadine have been approved as anti-influenza drugs, but these compounds are targeted at the viral uncoating process, not the viral neuraminidase. || Ribavirin is used in combination with interferon-α for HCV.[27]

Figures 2.18 and 2.19 give a visual appreciation of some of the approved antiviral drugs from Table 2.4, as well as some of the basic pharmacophores of antiviral agents.

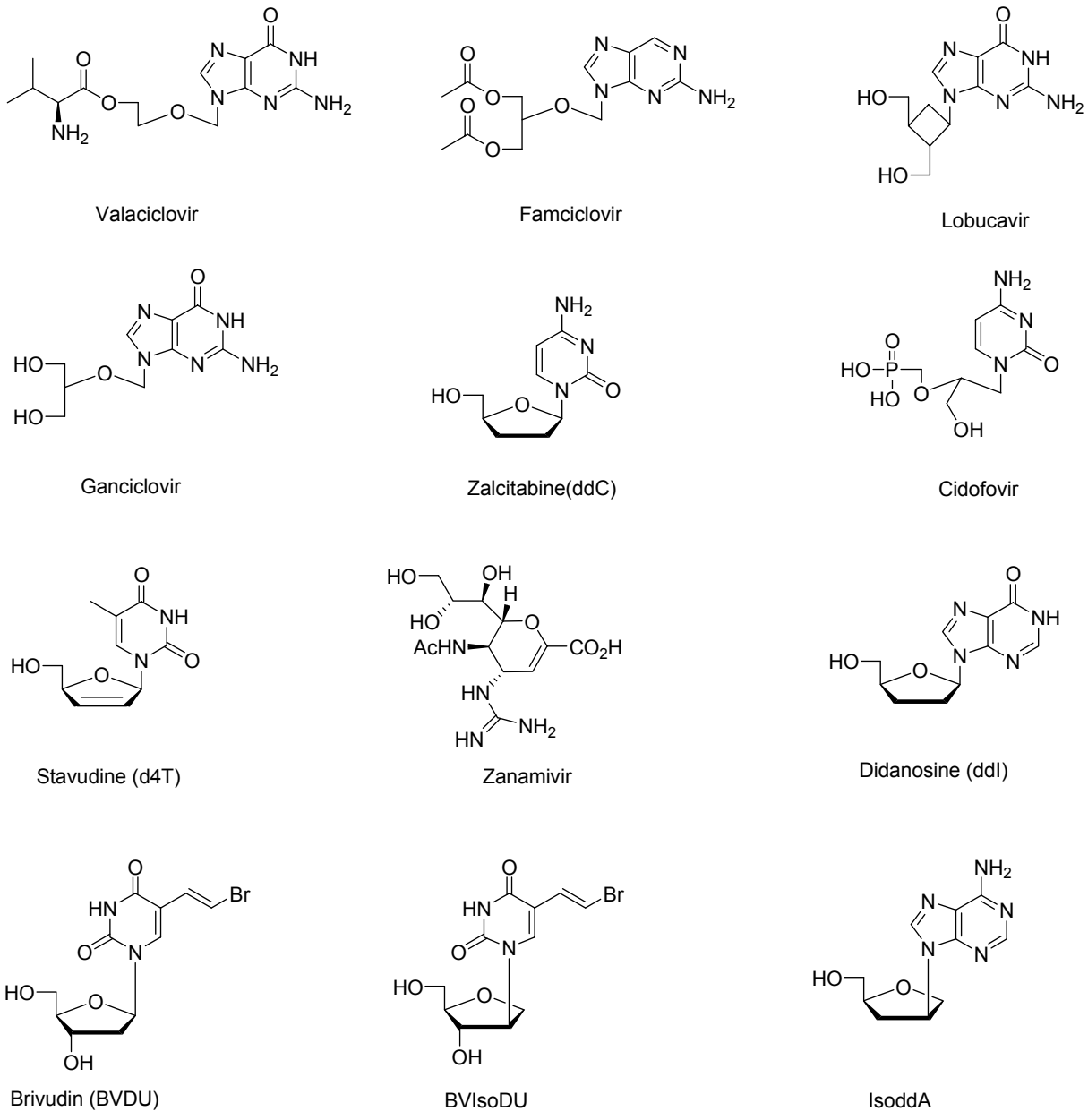


Figure 2.18. Structure of some Antiviral Compounds.

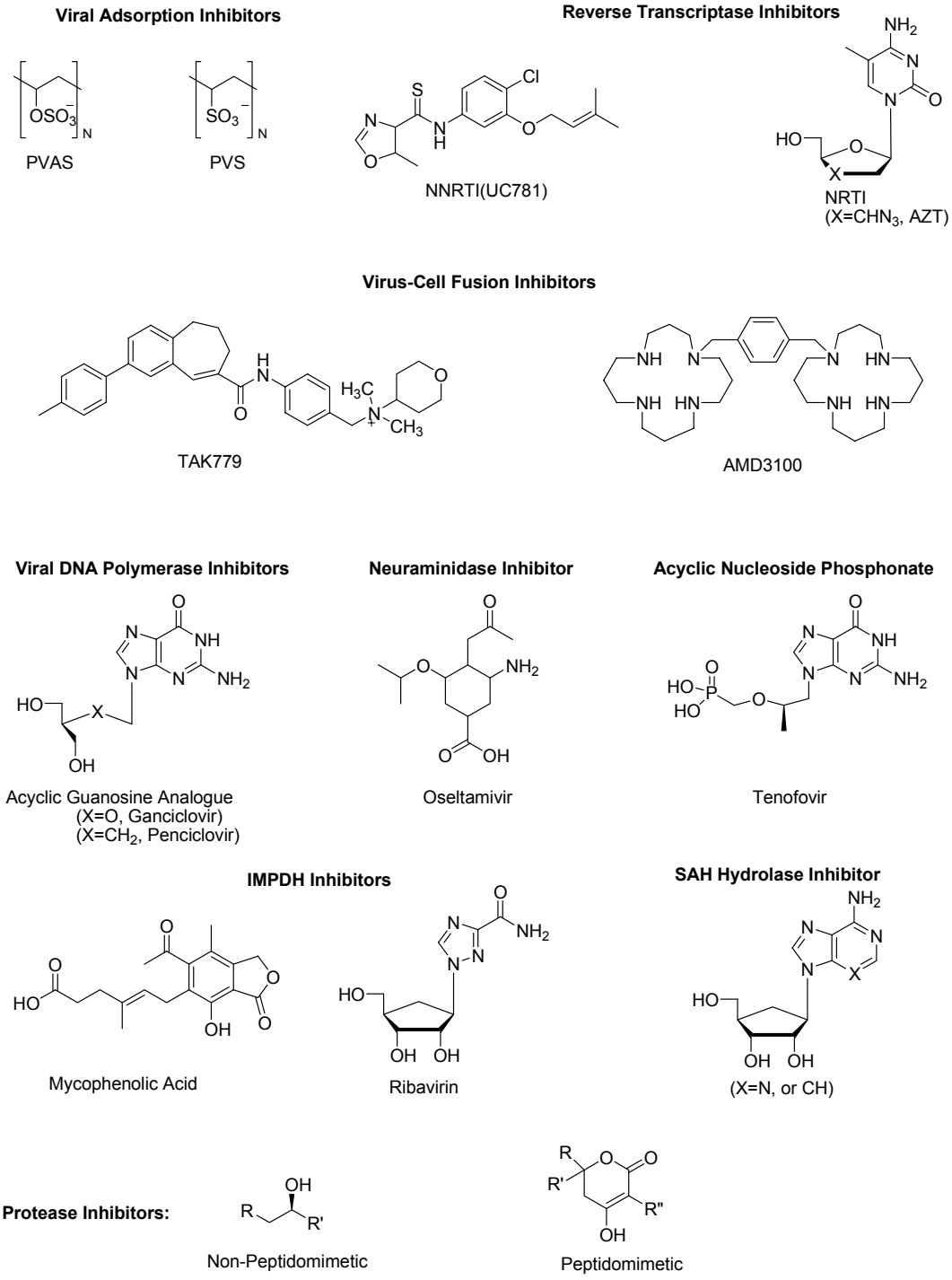


Figure 2.19. Basic Pharmacophores of Classes of Antiviral Agents.
AZT (azidothymidine); IMPDH (inosine monophosphate dehydrogenase); NNRTI (non-nucleoside reverse transcriptase inhibitor); NRTI (nucleoside reverse transcriptase inhibitor); PVAS (polyvinylalcohol sulphate); PVS (polyvinylsulphonate); SAH (*S*-adenosylhomocysteine).

It can be readily appreciated how nucleosides and their analogues are very important in antiviral chemotherapy (Figures 2.18 and 2.19).

Some antivirals worth mentioning include BVDU, BVIsoDU and IsoddA (Figure 2.18). BVDU (brivudin) is potent and selective against HSV-1, HSV-2 and VZV.[29] Studies show that it is significantly more efficacious than acyclovir.[30] The more stable BVIsoDU (discovered by Nair and coworkers), also shows significant and selective activity against HSV-1.[31, 32]

IsoddATP, the activated form of IsoddA (also discovered by Nair and coworkers) is one of the most potent inhibitors of HIV reverse transcriptase known in the field of antiviral chemotherapy.[33, 34]

Despite the development of the prior mentioned antivirals as well as several others that have been approved in the field of antiviral chemotherapy, there is still need for improvement. This is because these compounds are not always efficacious or well tolerated.[27] Additionally, there is the problem of continual emergence of viral resistance to these antiviral compounds as well as their intolerable side effects. These problems need to be addressed by the refinement of the antiviral drug design and development process.

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CHAPTER 3

INOSINE MONOPHOSPHATE DEHYDROGENASE (IMPDH)

OVERVIEW

The enzymatic conversion of inosine 5′-monophosphate (IMP) to xanthosine monophosphate (XMP) was discovered in the early 1950s by Carter *et al*. Further work, done several years later, identified the enzyme inosine monophosphate dehydrogenase (IMPDH) to be responsible for this conversion.[1]

IMPDH was first purified from extracts of *Aerobacter aerogenes* in 1957.[2] Since then, IMPDH has been isolated from mammalian,[3, 4, 5] bacterial,[6, 7, 8] parasitic,[9, 10] and plant sources.[11, 12] The purified enzymes enabled remarkable research that unfolded the characteristics, mechanism, and biological functions of IMPDH. The role of IMPDH as a target for antiviral, anticancer, immunosuppressive and antibacterial chemotherapy is especially interesting in the field of medicinal chemistry.

Experiments conducted by Weber *et al* into IMP metabolism revealed that IMPDH is the rate-limiting enzyme, showing the lowest activity of all the purine biosynthetic and degradative enzymes. However, in rapidly proliferating hepatoma, IMPDH shows the highest increase in activity of all the enzymes involved (Table 3.1).[13, 14] Furthermore, the specific activity of IMPDH is markedly increased in all animal and human cancer cells (Table 3.2),[3, 14, 15] with up to 2768 % increase in activity over the control in some cases. A rat model shows that IMPDH is well distributed in mammalian tissue; some of the most rapidly growing cells (testis, ovary and bone marrow) have high concentrations of the enzyme (Table 3.3). Despite these high relative amounts in normal cells, IMPDH amounts can reach 13 times higher during proliferation.[15]

Table 3.1. Activities of Enzymes of the Anabolic and Catabolic Pathways of Purine Metabolism.[13, 14]

Enzymes	EC No.	Normal Liver nmol/hr/mg/protein	Rapid Hepatoma 3683F (% of liver)
Synthesis			
IMPDH	1.2.1.14	2	1350
GMP synthase	6.3.4.1	33	548
Adenylosuccinate Synthase	6.3.4.4	36	308
Amidophosphoribosyl transferase	2.4.2.14	60	280
Adenylosuccinase	4.3.2.2	288	175
AMP deaminase	2.5.4.6	1730	433
GMP kinase	2.7.4.8	6090	121
Catabolism			
Xanthosine oxidase	1.2.3.2	100	10
Uricase	1.7.3.3	645	4
Inosine phosphorylase	2.4.2.1	28800	19

Table 3.2. Increased IMPDH Activity in Cancer Cells.[1]
[+]Clinical samples.

Samples	IMPDH Activity	
	nmol/hr/mg protein	% of control
Rat		
Liver	2.1 ± 0.2	100
Hepatoma 3924A	27.3 ± 1.5	1300
Human		
Normal leukocytes[+]	3.1 ± 0.5	100
CGL in blast crisis[+]	85.8 ± 23.6	2768
K562 cells	44.6 ± 1.6	1439
HL-60 cells	31.7 ± 2.2	1023
Myeloma 8226 cells	44.5 ± 3.5	1435
Normal breast HMEC cells	8.9 ± 0.2	100
MDA-MB-435 cells	29.5 ± 1.2	331
MDA-MB-435 tumors	25.0 ± 4.0	281
MCF-7 cells	26.0 ± 1.3	292
Normal Ovarian[+]	2.9 ± 0.9	100
Ovarian carcinoma[+]	19.6 ± 3.8	676
OVCAR-5 cells	61.2 ± 3.0	2110

Table 3.3. Rat Tissue Distribution of IMPDH (Activity and Amounts).[16]

TISSUE	IMPDH (μU/mg)	RELATIVE AMOUNT
Thymus	347	15.5
Spleen	264	11.8
Testis	181	8.1
Ovary	159	7.1
Bone Marrow	127	5.7
Lung	111	5.0
Adipose Tissue	78	3.5
Brain	73	3.3
Intestine	67	3.0
Liver	46	2.0
Kidney	44	2.0
Heart	44	2.0
Peripheral Leukocytes	23	1.0
Skeletal Muscle	22	1.0

In 1989, Natsumeda *et al*. discovered that human IMPDH exists as two isoforms; type I and type II.[17] The isoforms are of identical size and share 84% sequence identity.[18] The type I isoform is expressed constitutively in normal cells, whereas type II expression is preferentially up-regulated in rapidly proliferating cells and activated T and B-lymphocytes. The type II enzyme is, however, down-regulated during the differentiation of proliferating cells.[19-25] The two isoforms are encoded by two separate genes. The type I IMPDH gene is located on chromosome 7,[26] whereas the type II is on chromosome 3.[27] This observed disproportionate increase in type II IMPDH activity during cell proliferation has made it a major target for the development of anticancer[28, 29] and immunosuppressive drugs.[22, 23] IMPDH seems to be well distributed in the mammalian system; its activity in the various organs bears a slight correlation with the frequency and types of cancer in mammals.

IMPDH catalyzes the first committed step (rate-limiting) of a multi-step biosynthesis that starts with IMP and ends in either GTP or dGTP (Figure 3.1). Interestingly, this same GTP is an

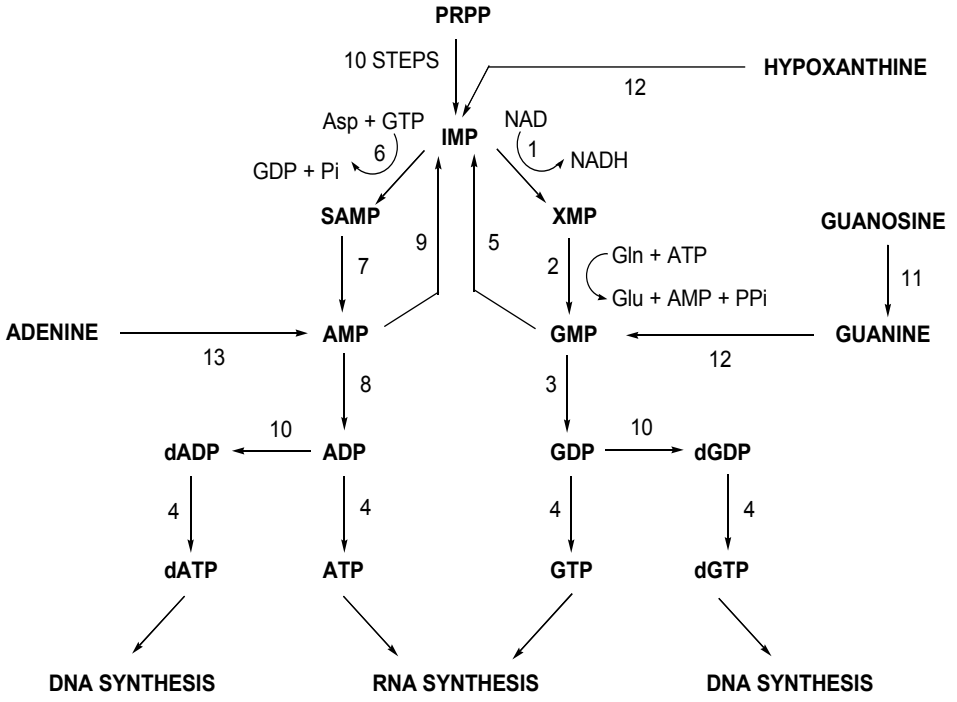
essential cofactor in the first step of a multi-step biosynthesis of ATP and dATP. This places IMP at a very important branch point. As such, inhibition of IMPDH has a variety of biological effects. This includes the proven potent anti-proliferative effect against various tumor cells,[30-43] and lymphocytes.[44-46] The anti-proliferative mechanism is attributed to the reduction in intracellular concentrations of guanine nucleotides, especially GTP and dGTP.[47]

Reduction in GTP and dGTP interrupts DNA and RNA synthesis[48] and suppresses both T and B-lymphocyte proliferation since growth and differentiation of human lymphocytes are highly dependent upon the IMPDH-catalyzed *de novo* pathway for purine nucleotide synthesis.[16, 29, 49] IMPDH inhibition also causes a decline in intracellular signaling[50-54] and down-regulation of c-myc and Ki-ras oncogenes *in vitro*.[28, 55, 56] IMPDH inhibition can also lead to antiviral activity since there is an increased demand for purine nucleotides for RNA and DNA synthesis in virus-infected cells during the viral genome replication stage. Furthermore, antiviral activity results from the inhibition of a virus' enzyme or interruption of the replication cycle with an appropriate inhibitor. For example, ribavirin (an antiviral agent) may induce error-prone replication by the incorporation of ribavirin triphosphate into the viral genome. However, even this mechanism appears to be dependent on adequate guanine nucleotide levels.[57]

IMPDH inhibition has also been exploited for antiparasitic chemotherapy, mainly because bacterial and other parasitic sources of IMPDH differ significantly from mammalian sources.

These species differences are manifested in terms of substrate, cofactor and ion binding, as well as differences in the biochemistry of purine metabolism. Furthermore, the IMPDH crystal structure of a number of microbial enzymes show that bacterial and human IMPDH share only 20-30% amino acid sequences.[59] Their active site residues (especially in the cofactor-binding

site) are significantly different. These differences are targeted in the development of clinically useful antibiotics against *Haemophilus influenza*, *Tritrichomonas foetus* (infectious protozoan), *Mycobacterium tuberculosis*, *Pneumocystis carinii* (opportunistic pathogen in AIDS patients) and *Borrelia burgdorferi* (causative agent for Lyme disease).[1, 12, 60, 61] This approach has been very promising since the emergence of bacterial resistance to known antibiotics is on the rise.



1. IMPDH (Inosine Monophosphate Dehydrogenase)
2. GMP SYNTHASE (GMP: Guanosine Monophosphate)
3. GMP KINASE
4. NUCLEOSIDE DIPHOSPHATE KINASE
5. GMP REDUCTASE
6. ADENYLOSUCCINATE SYNTHETASE
7. ADENYLOSUCCINATE LYASE
8. AMP KINASE (AMP: Adenosine Monophosphate)
9. AMP DEAMINASE
10 RIBONUCLEOTIDE REDUCTASE
11. PURINE NUCLEOSIDE PHOSPHORYLASE
12. HGPRT (Hypoxanthine-Guanine Phosphoribosyl Transferase)
13. APRT (Adenine Phosphoribosyl Transferase)

SAMP: Adenylosuccinate
XMP: Xanthosine Monophosphate
PRPP: 5-Phosphoribosyl-α–pyrophosphate

Figure 3.1. Adenine and Guanine Nucleotide Biosynthesis and Salvage Pathways.[58]

A secondary yet very important benefit of IMPDH inhibition is the fact that it leads to increased intracellular levels of IMP, which can serve as phosphate donors for the phosphorylation of 5′-nucleotides of 2′,3′-dideoxynucleosides such as ddI, thus the ability to potentiate the anti-HIV activity of this type of nucleosides.[62, 63]

A closer look at Figure 3.1 shows that purine nucleotides are not only synthesized by the *de novo* pathway, but also by salvage pathways. These two pathways control tightly the concentration of purine nucleotides in cells.[64, 65] In the *de novo* pathway, IMP is synthesized in ten steps from the high energy intermediate, PRPP; adenine and guanine nucleotides are subsequently synthesized from the branch-point compound, IMP. On the other hand, the salvage pathway recycles existing purines and their nucleosides and nucleotides.[58] It seems plausible that contributions from the salvage pathway could nullify the well sought-after inhibition of IMPDH. For example, HGPRT catalyzes the one step production of IMP and GMP from hypoxanthine and guanine respectively with the co-substrate, 5-phosphoribosyl 1-pyrophosphate (PRPP).[66] In the case of the salvage formation of GMP, unlike IMP, the essence of IMPDH is rendered completely useless and even less efficient since HGPRT makes GMP in one step as compared to the two-step *de novo* synthesis of GMP. Studies have also shown that the salvage pathway's contribution is much higher than that of the *de novo* pathway in all tissues and cells;[67-69] also, the affinity of HGPRT for its substrates is orders of magnitude higher, thus explaining why the effects of IMPDH inhibition are quickly reversed upon addition of the salvage precursors, guanine and hypoxanthine.[39, 43, 44, 46, 70] Nevertheless, since the salvage precursors, guanine and guanosine, are not fully available in plasma, the salvage pathway of guanine nucleotides is very limited in humans.[70] Unlike guanine and guanosine, hypoxanthine is fully available in plasma,

and it is converted to IMP (*via* the salvage pathway) by HGPRT; IMPDH inhibition will still block conversion of this salvage IMP into guanine nucleotides.

CRYSTAL STRUCTURE AND LIGAND-BINDING INTERACTIONS

A number of crystal structures of IMPDH have been solved. These include human type I and type II isoforms,[71] hamster,[71, 72] *Streptococcus pyogenes*,[59] *Borrelia burgdorferi*,[73] and *Tritrichomonas foetus*.[74, 75] These crystal structures were obtained at different resolutions (1.9Å-2.9Å) as either binary or ternary complexes with a number of ligands. The ligands include the substrate IMP, substrate analogues 6-chloro-IMP, ribavirin 5′-monophosphate (RMP), the cofactor NAD analogues SAD, β-TAD, C-2-MAD and MPA. These structures have provided very useful information about IMPDH mechanism, ligand binding specificity, hydrogen bonding and amino acid residue interactions within the active site, as well as drug design tips. Of all the IMPDH structures known, the mouse and Chinese hamster bear the closest similarity (in primary structure) to the human type II IMPDH, differing in only 6 and 7 amino acids out of 514, respectively.[17, 18, 76] The prior mentioned similarity is unusual given that not even the human type I and type II isoforms show such similarity. The difference between the human type I and type II IMPDH sequences is much more extensive; 84 out of 514 amino acids differ.[17] Among these changes, 52 are conservative amino acid substitutions and 32 diverge with respect to their chemical properties. There is a 25-30% sequence identity between IMPDHs from T. foetus and other known mammals.

All IMPDH structures are homotetrameric (Figure 3.2) with monomers generally containing about 500 amino acid residues (B. burgdorferi being an exception with 400 residues). A few higher order aggregates (octamers) are known.[8, 74]

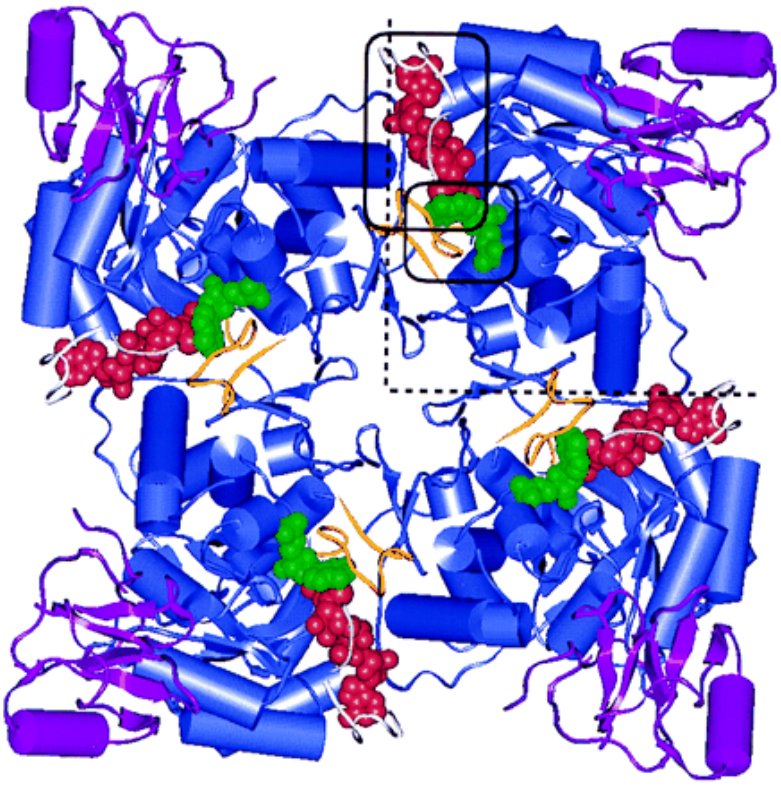


Figure 3.2. Crystal Structure of Human Type II IMPDH Tetramer [with bound dinucleotide analogue SAD (circled, red) and substrate analogue 6-Cl-IMP (circled, green).The dinucleotide binds at the monomer-monomer interface (dotted lines). The following structures are illustrated: catalytic β-barrel domain (blue), flanking domain (magenta), active site loop (yellow) and active site flap fragments (white)].[71]

Each monomer is approximately 55kDa in molecular weight, and can be categorized into four components:

1) Active Site loop

2) Active Site flap

3) Small flanking domain

4) Large catalytic domain

The 15-residue active site loop (residues 325-340 in human and hamster) is a highly mobile and flexible element containing the catalytically critical residue Cys 331. Cys 331 attacks

the C-2 position of the physiological substrate IMP in the first step of IMPDH's mechanism. During binding in both the IMP and NAD-binding sites, the loop engages in several hydrogen bonding (polar interactions) that not only anchors ligands in place, but also closes off one side of the IMP-binding site, and the nicotinamide end of the NAD-binding site. The loop's high degree of flexibility and mobility, as well as its function as the enzyme's catalytic arm, confers some regioselective promiscuity on IMPDH. For example, the crystal structure of human type II IMPDH, 6-Cl IMP and SAD complex shows that a covalent linkage is formed between the C-6 position on the purine ring and Cys 331,[71] rather than the usual C-2 position. This confirms that 6-Cl-IMP undergoes IMPDH-catalyzed dehalogenation *via* nucleophilic attack by the thiol group of Cys-331.[71, 77] There are other incidences where it is asserted that the nucleophilic attack occurs at C-8 of the purine ring. These are possible due to the re-orientation of the loop, rather than the ligands, since the ligands are anchored in place during catalysis (Figure 3.3).[1]

The 50-residue active site flap (residues 400-450 in human and hamster) is also highly mobile, and covers the active site cleft. It protects the binding of both substrate and cofactor through polar interactions. The proximal part of the flap contributes conserved Try 411, Met 414, Gly 415, and Gln 441 which interact with bound IMP.[59, 72] The terminal end of the flap interacts with the phosphate and adenosine portions of the dinucleotide site. The flap contains four residues not conserved between types I and II isoforms of human IMPDH, thus providing room for isoform-specific drug design (Figure 3.3).[1] Studies suggest that IMP binding protects the active site flap against proteolysis, and that the entire flap serves to stabilize both substrate and dinucleotide binding.[75]

The 120-residue small flanking domain (residues 113-232 in human and hamster) lies adjacent to the catalytic domain (Figure 3.2). Its function is unknown; however, it is known that

it is not required for IMPDH activity.[72, 78, 79] This domain displays varying degrees of disorder among species,[59, 74] and the linkages between this domain and the large catalytic domain are highly flexible.[71]

The large catalytic domain (394 residues in the human and hamster IMPDHs) consists of an eight-stranded parallel α,β barrel of about 40 x 40 x 50Å in dimensions (Figure 3.2). The active site (part of the large catalytic domain) is a long continuous cleft that has an IMP-binding site and an NAD cofactor-binding site in continuum on the C-terminal face of the α,β barrel (Figure 3.3). The active site is located close to the monomer-monomer interface and some of its residues (those of the cofactor-binding site) are from adjacent catalytic monomers (in the case of the human enzyme).

As mentioned earlier, several crystal structures involving different substrates and species have been solved and characterized. Here, only the details of the observed and deduced hydrogen bonding interactions of the physiological substrate, IMP and cofactor, NAD complex of human type II IMPDH will be discussed. Although the binding of NAD has not been solved crystallographically, the binding of SAD and other NAD-analogues in both human and hamster IMPDHs have helped in deducing and identifying enzyme-cofactor interactions.[80]

When IMP binds in the IMP-binding site, its phosphate group, ribose and purine moieties participate in numerous polar interactions that appear to be conserved across species.[80] These polar interactions involve both stationary residues (forms stationary back 'wall' of the IMP-binding pocket) and the mobile residues of the active-site loop and flap. Upon the binding of IMP, the loop contributes two very important residues which are conserved across species: Ser 329 and Cys 331. Ser 329 forms a hydrogen bond with the IMP phosphate group, and the catalytic Cys 331 attacks C-2 of the inosine ring.

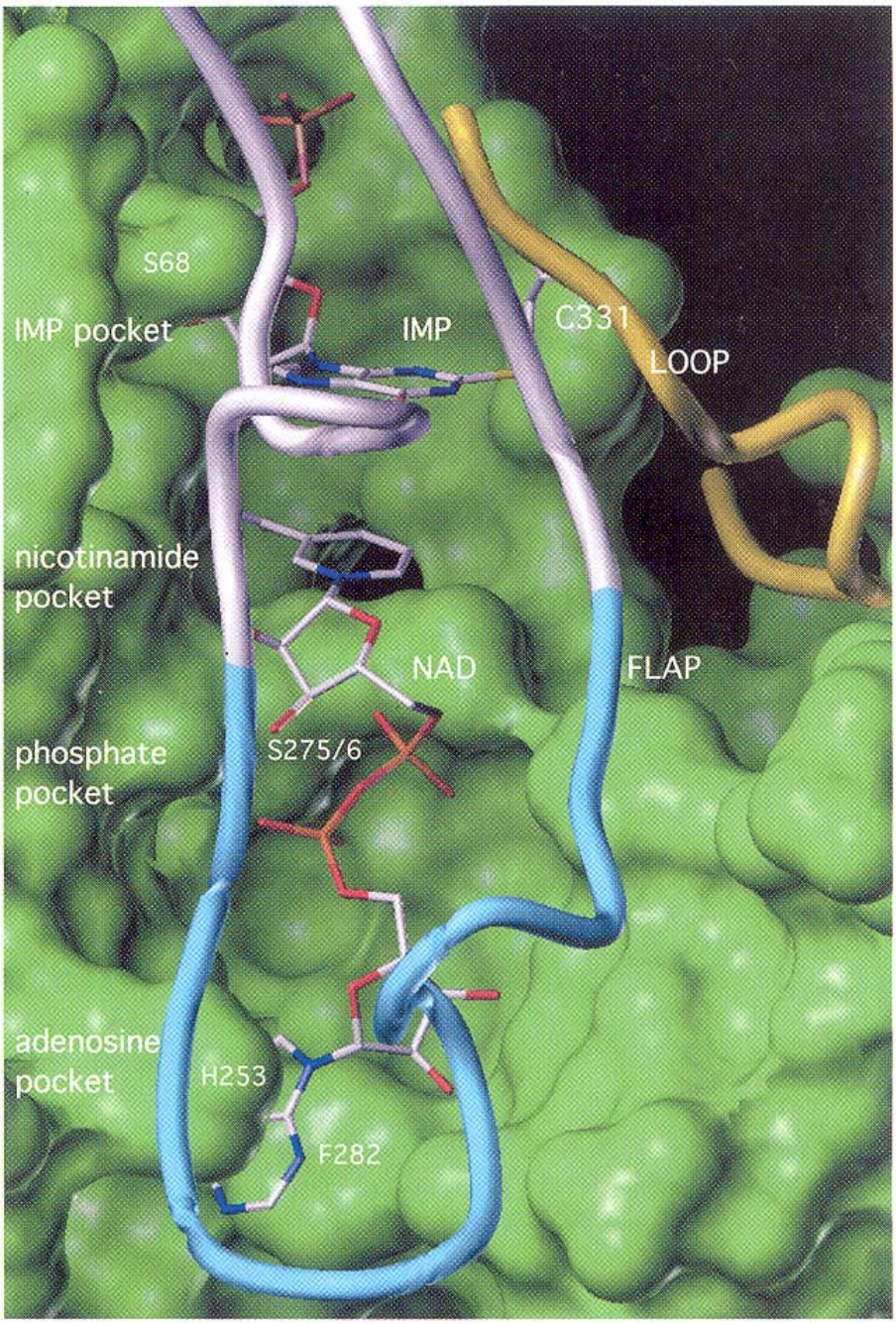


Figure 3.3. Enhanced Image of IMPDH Active Site.[1]

[Shows IMP and NAD binding sites; the active site loop (yellow), and flap model (white and cyan) are from the human type II RMP-C-2-MAD complex. The terminal (cyan) end of the flap contains four residues not conserved between types I and II isoforms. The NAD ligand is from the human type II-6-Cl IMP-NAD complex. IMP is from the hamster complex. Ligand atoms are standard color-coded; the sub-components of the NAD site and selected residues are labeled for reference. Residues of the barrel domains are illustrated as a solvent accessible surface].

The 'back' wall of the IMP site provides a number of stationary residues that further stabilize IMP binding; the phosphate group is locked into position by polar interactions with the main chain nitrogens of Gly 366, Gly 387, and Ser 388, whereas the ribose hydroxyls form hydrogen bonds with the side chains of Ser 68 and Asp 364. Four residues of the active site flap engage in polar interactions with IMP;[59, 74] the hydroxyl of conserved Tyr 411 stabilizes the phosphate group, whiles the main chain atoms of Met 414, Gly 415, and Gln 441 hydrogen bond with N-7, C-6 carbonyl, and N-1 of the inosine base respectively (Figure 3.4).[80] These stabilizing polar interactions are conserved fully or in part with other IMP-analogue substrates.

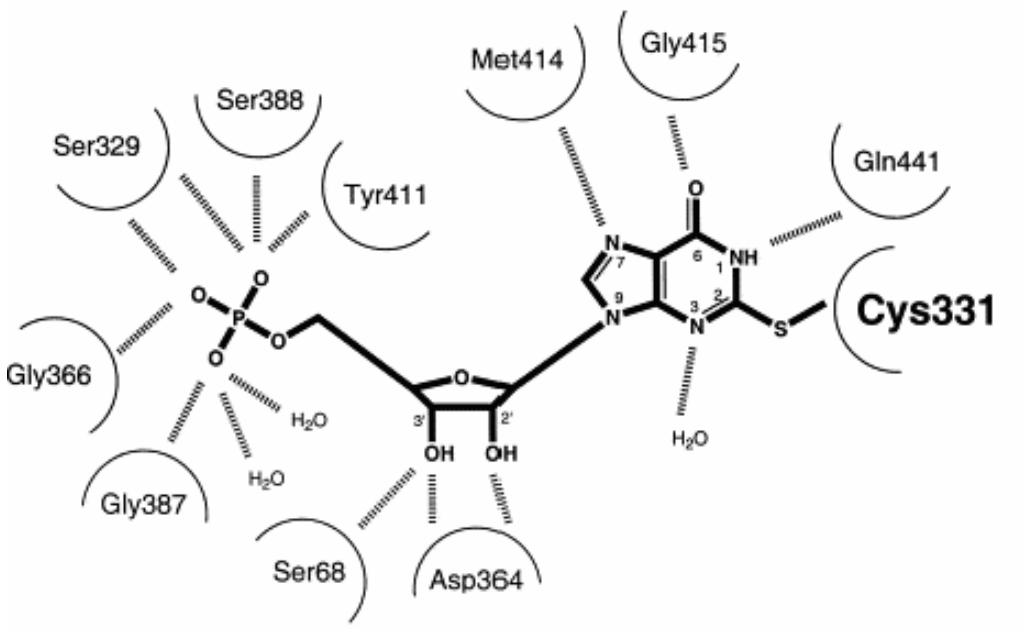


Figure 3.4. Schematic Representation of XMP*-IMPDH Interactions.

The NAD cofactor-binding site is also well characterized. It occupies a deep pocket in the active site capped on the top by the binding location of the IMP hypoxanthine ring; it then

extends downwards almost perpendicularly reaching the monomer-monomer interface. Similar to the IMP-binding site, when NAD binds, it is held in the active site by a series of polar interactions involving conserved residues on the walls of the active site cleft as well as residues on the active site loop and the highly mobile active site flap. NAD binds adjacent to the IMP subsite with its nicotinamide ring stacked against the IMP inosine base. The nicotinamide ring has an anti-conformation relative to its ribose sugar;[81] this conformation facilitates an efficient hydride transfer between the two rings during catalysis.[82] Three well-conserved stationary residues: Asn 303, Arg 322, and Asp 274, anchor the nicotinamide carboxamide group through polar interactions. The adjoining nicotinamide ribose and two phosphate groups bind perpendicularly downwards from the nicotinamide ring in the active site groove. The nicotinamide ribose hydrogens bond with Asp 274 (the same conserved residue that interacts with the nicotinamide carboxamide group) and the phosphate groups form hydrogen bonds with successive serines 275 and 276 (conserved in all eukaryotic IMPDH). The adenosine end of NAD lies in the remainder of the active site groove where it forms an unusual number of inter-monomer polar interactions.[71, 72] The adjacent monomer contributes Gln 469 and Ala 46 which form hydrogen bonds with the adenosine ribose hydroxyl groups. Finally, the adenine ring is bound into place by four surrounding residues; Phe 282 and His 253 on opposite sides, and Thr 252 and Thr 45 also on opposite sides (Figure 3.5). Specifically, the adenine amino group forms a hydrogen bond with Thr 252, and on the opposite side, its N-3 forms a hydrogen bond with Thr 45 (on the adjacent monomer). Just like the IMP binding-site, these NAD binding-site interactions are conserved fully or in part with other NAD-analogue substrates.

Figure 3.5 gives a spatial perspective of how IMP and NAD and its analogues are oriented in the active site of IMPDH during binding. It also shows the pertinent residues.

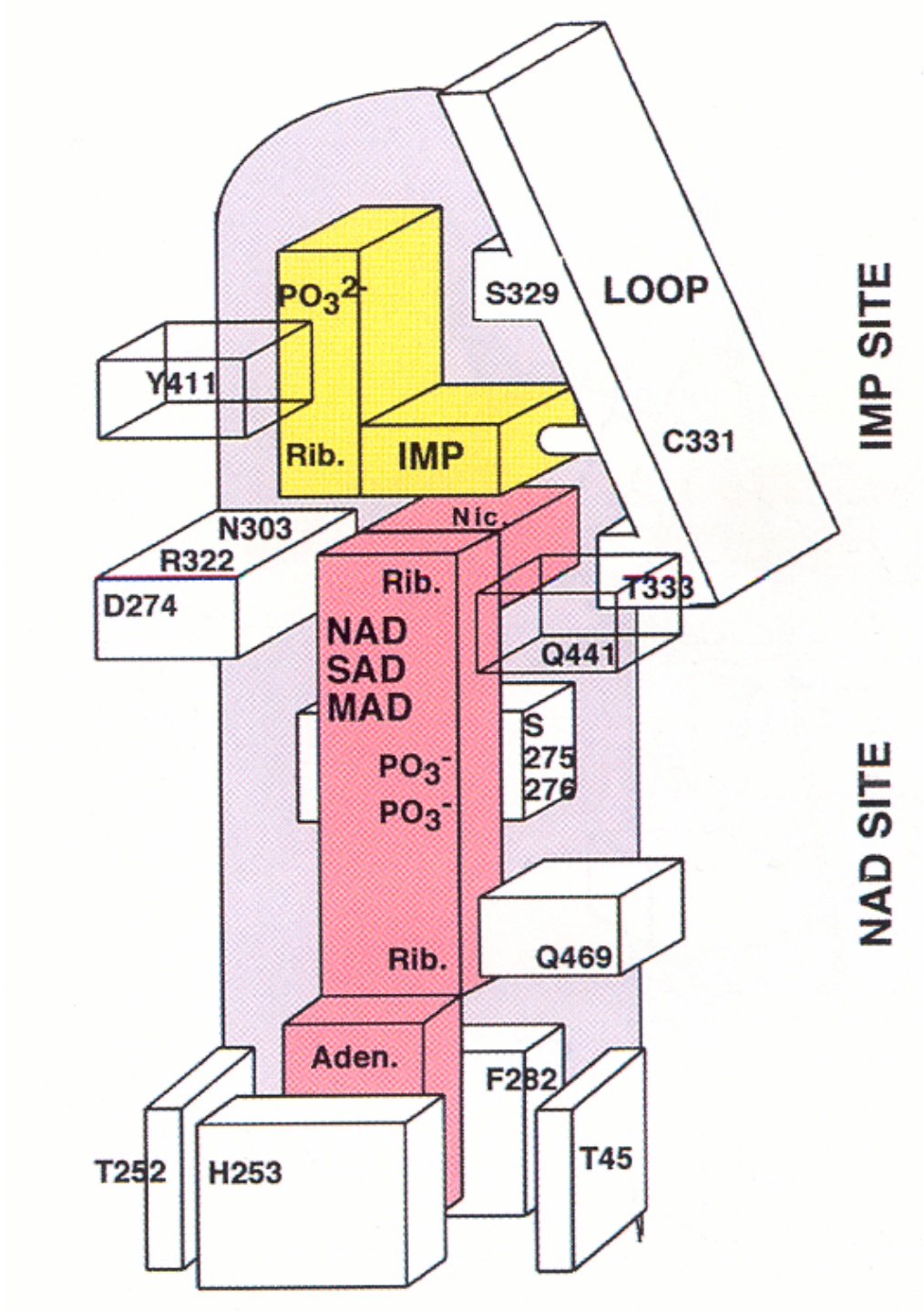


Figure 3.5. Spatial Orientation of Bound IMP (yellow) and NAD (red).[1]
Pertinent residues of IMP and NAD binding are schematically shown. The flap is omitted for clarity; its residues are shown as transparent boxes.

MECHANISM OF ACTION OF IMPDH

IMPDH catalyzes the conversion of the physiological substrate, IMP, to XMP using NAD as a cofactor. NAD is concomitantly reduced to NADH (Figure 3.6), and the process is apparently irreversible.[58]

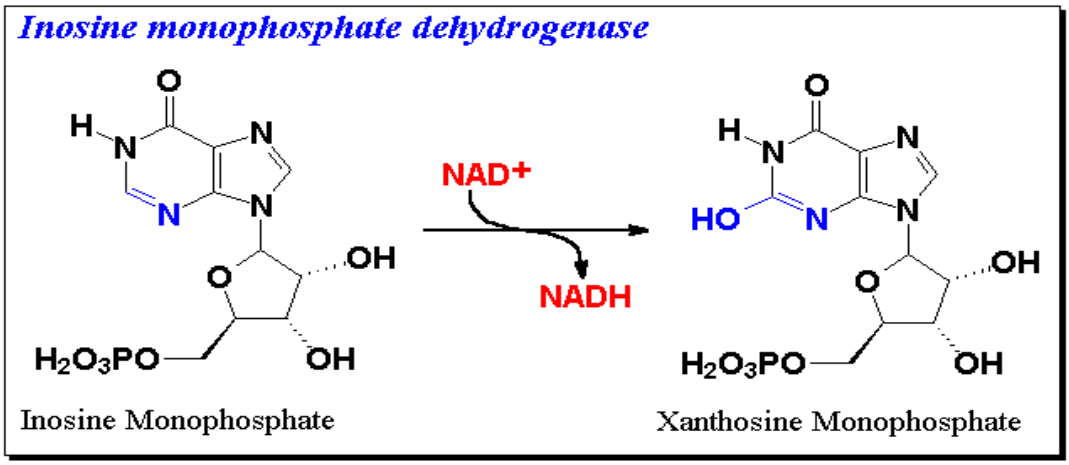
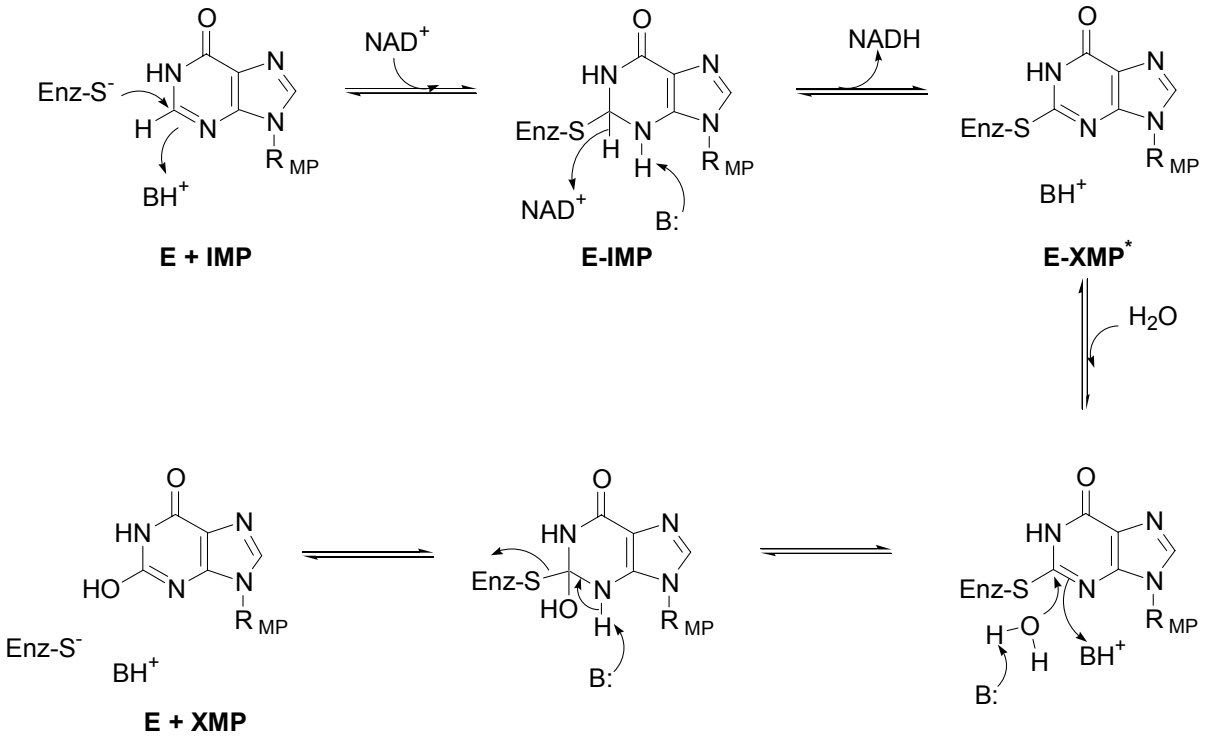


Figure 3.6. IMPDH-Catalyzed Conversion of IMP to XMP.

The reaction involves the nucleophilic attack of the thiol group of Cys-331 on the C-2 of the IMP base, which leads to formation of a tetrahedral E-IMP covalent adduct.[72, 83] IMP is bound in the anti-conformation of the glycosidic bond placing C-2 away from the sugar ring. This position facilitates a feasible nucleophilic attack of Cys-331.[84] Subsequently the C-2 hydride is transferred to the favorably positioned nicotinamide ring of NAD (pro-S face) to give the intermediate thioimidate E-XMP* (after NADH release) covalently bound to the enzyme. Finally, water attacks at C-2 to regenerate free Cys-331 and the product XMP (Scheme 3.1). These final steps of the reaction, hydrolysis of the enzyme-bound intermediate (E-XMP*) and

release of the final product XMP, are rate limiting for the human enzyme.[85] The enzyme activity is optimal at pH 8.



Scheme 3.1. Proposed Mechanism of IMPDH.

CHARACTERISTICS AND KINETIC MECHANISM OF IMPDH CATALYSIS

IMPDH does not have any allosteric effectors. Earlier erroneous reports of allosteric effects were due to proteolytic degradation and impure enzymes.[86] The kinetic mechanism of IMPDH appears to follow simple Michaelis-Menten kinetics. [87-89] Binding of IMP and NAD is random, unlike the traditionally thought, ordered bi-bi mechanism.[82, 85, 90] IMP or NAD can bind to the enzyme in the presence of K^+, but only IMP binds in the absence of K^+.[91, 92] Hydride transfer to NAD is fast and reversible, and NADH release precedes hydrolysis of E-XMP*. It has

been observed that NADH release is partially rate-limiting.[85, 87, 93, 94] A conformational change occurs when IMP binds. This conformational change could be the ordering of the active site flap and/or loop. Unlike IMP binding, binding of NAD does not induce conformational changes. Also the presence of NAD has no effect on IMP binding, whereas the presence of IMP facilitates NAD binding.[90] The final step of the IMPDH mechanism, hydrolysis of E-XMP*, is partially rate-limiting thus causing an accumulation of E-XMP*. High concentrations of NAD inhibits IMPDH by trapping E-XMP*; this NAD inhibition (same means of inhibition by NAD-analogue inhibitors) is a means of regulating IMPDH activity *in vivo*.[9, 90] These features and kinetic mechanism were delineated using *T. foetus* and *A. aerogenes*; however it appears (from preliminary experiments with human type II and *E. coli*) that this mechanism is applicable to all IMPDH reactions.

In the mechanism of IMPDH, attack of Cys 331 and hydride transfer could either be stepwise or concerted (Scheme 3.1). However, isolation, characterization and crystal structures have well established the existence of E-XMP*,[72, 85, 93, 95-97] unlike E-IMP and E-XMP. Both E-IMP and E-XMP intermediates are expected to be unstable outside of the enzyme active site. Although enzyme synergism is widespread in enzymology, at present there is no proof it exists with the IMPDH mechanism, i.e., to prove that maybe E-IMP or E-XMP is formed in the presence of NAD(H). In fact, NAD does not regulate the activity of Cys 331, and IMPDH activation or inactivation is not affected by the presence or absence of NAD.[98, 99] These facts suggest that addition of Cys 331 to IMP is concerted with hydride transfer (formation of E-XMP*), and hydrolysis of E-XMP* is concerted with elimination of Cys 331 to form product XMP and regeneration of the enzyme (Scheme 3.1).[90]

For the step-wise consideration, negative charges result at N-3 and/or O-6 (by resonance) of IMP from the nucleophilic attack of Cys 331 on C-2. Similarly, negative charges result from attack on C-2 of E-XMP* by water. It is asserted that these negative charges are stabilized through hydrogen bonding with an amino acid residue or a general acid (BH^+). Also a general base catalyst (B:) activates water for the hydrolysis of E-XMP*. These residues have not been identified.[90, 100]

IMP and NAD are the respective physiological substrate and cofactor of IMPDH; however the enzyme shows broad substrate specificity for both IMP and NAD. The following IMP analogues are also recognized as substrates by IMPDH: 2′-deoxy-IMP, ara-IMP, 8-aza-IMP, 6-thio-IMP, inosine-5′-phosphorothiolate, 5′-mercapto-5′-deoxyinosine-5′-S-phosphate, and 5′-amino-5′-deoxyinosine-5′-N-phosphate.[101-104] The kinetic parameters for the reaction of these substrates are similar to those of IMP. Some analogues of NAD that are also substrates for IMPDH (although less efficient) include: acetylpyridine adenine dinucleotide, thionicotinamide adenine dinucleotide, 3-pyridinealdehyde adenine dinucleotide, nicotinamide hypoxanthine dinucleotide and nicotinamide guanine dinucleotide.[10, 85, 105] Also IMPDH catalyzes the hydrolysis of 2-Cl, and 2-F-IMP even in the absence of NAD with similar kinetic parameters as IMP.[99] This broad spectrum of substrates makes IMPDH a rather versatile yet promiscuous enzyme.

IMPDHs are activated by K^+ and other monovalent cations. K^+ increases the activity of IMPDHs by at least 100-fold and it appears to be linked with IMP binding in human type II IMPDH.[91, 106] The human type II isoform is activated, not only by K^+, but also by NH_4^+, Na^+, Ti^+, and Rb^+. It is, however, unaffected by Li^+.[106] Even though a K^+-binding site has been identified in IMPDH, the mechanism by which it activates IMPDH is still not clear.[72, 75]

IMPDH INHIBITION AND CHEMOTHERAPEUTIC IMPLICATIONS

As noted earlier, IMPDH inhibition blocks the conversion of IMP to XMP. This blockade leads to depletion of the guanylate pools (GMP, GDP, GTP and dGTP), and since GTP is a cofactor in the conversion of IMP to AMP (*via* succinyl AMP), ATP and dATP pools are also depleted.[30, 62, 63] The subsequent biological effects are anticancer, antiviral, immunosuppressive and antiparasitic chemotherapy.

The anticancer effect is due to the fact that inhibition of IMPDH causes a reduction in guanine nucleotide pools (most importantly GTP, and dGTP) as well as ATP and dATP pools. Rapidly proliferating cells need an adequate supply of GTP and ATP for RNA, and dGTP and dATP for DNA synthesis; IMPDH inhibition causes a decline in supply of GTP, dGTP, ATP and dATP which terminates RNA and DNA synthesis and leads to cell death.

The antiviral effects of IMPDH inhibition can be multi-faceted and unclear in some cases. On the one hand, virus-infected cells need GTP, dGTP, ATP and dATP for RNA and DNA synthesis during their replication cycle. IMPDH inhibition therefore provides antiviral effects through depletion of GTP, dGTP, ATP, and dATP, thus preventing viral replication. In other cases, as exemplified by ribavirin (commonly used antiviral agent), antiviral activity derives from one or more of three possible modes, depending on its phosphorylation state in a particular cell type.[58, 107] As shown in Figure 3.7, RTP (activated form of ribavirin) inhibits RNA polymerase, thus preventing viral RNA transcription and ultimately viral replication. RTP is also known to inhibit viral mRNA guanylyl transferase and mRNA-guanine-N^7-methyl transferase, thus disrupting guanine pyrophosphate capping of the 5′ end of viral mRNA and final formation of capped viral mRNA.[58] This prevents translation of viral transcripts, thereby hampering viral replication. Furthermore, RMP is well characterized as an inhibitor of IMPDH, depleting GTP

and dGTP pools (Ki = 250 nM).[108] It is also believed that incorporation of RTP into the viral genome induces errors during replication leading to the formation of non-virulent proteins.

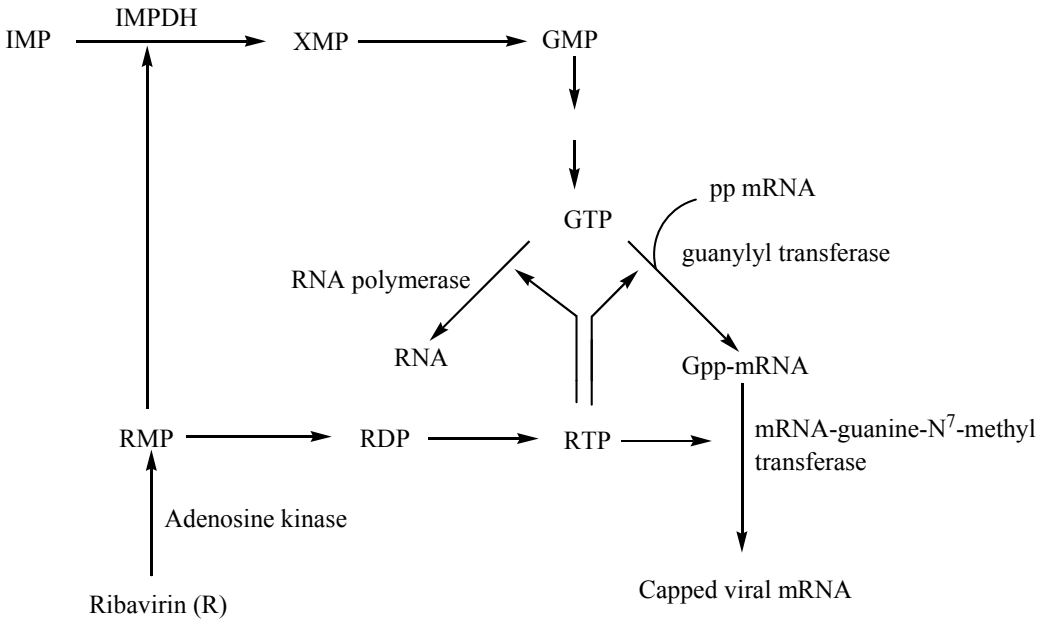


Figure 3.7. Target Viral Enzymes for Ribavirin Triphosphate Inhibiton.[109]

The immunosuppressive effect of IMPDH inhibition is owed to the fact that proliferation and differentiation of human lymphocytes (both T and B-lymphocytes) are highly dependent upon the IMPDH-catalyzed *de novo* pathway rather than the salvage pathway to generate sufficient levels of nucleotides necessary to initiate a proliferative response to mitogen or antigen.[16, 29, 49, 110] Without this proliferation and differentiation of lymphocytes, a person's immune system is lowered to accommodate transplantation. The detailed biochemistry mechanism of immunosuppression will not be discussed.

MODES OF INHIBITION

Although both IMP and especially NAD are substrates in many enzymatic reactions, specific and reversible inhibition of IMPDH is possible.[90] Effective inhibition demands a thorough understanding of IMPDH's mechanism of action, which will enable targeting strategic points for inhibitory drug design (mechanism-based drug design). Based on this, three modes of inhibition are readily identifiable: IMP-site inhibition, NAD-site inhibition, and E-XMP* trapping.

IMP-site inhibition seeks to design molecules that mimic IMP and will bind to the IMP-binding site to act as competitive inhibitors. Such competitive inhibitors of IMPDH are well known and are primarily nucleoside analogues.[111] To meet this criteria, such nucleosides must at least be capable of cellular conversion to their mono-phosphorylated forms, mainly through the action of adenosine kinase or other cellular kinases.[58] These monophosphates can parallel the metabolic fate of IMP either completely or partially, leading to some exotic compounds other than the needed XMP, GMP, GDP, GTP, or dGTP. They could also serve as substrates for NAD phosphorylase, becoming NAD mimics and exerting inhibition as such. Just as with ribavirin and EICAR, this can lead to multiple inhibitor species whose specific line of action is indeterminable. Although this type of inhibition is not mechanistically clean, such compounds have proven to be efficacious and are currently in clinical use.

The NAD-site inhibition involves NAD mimics which bind in the NAD-binding pocket. They can be modified nucleosides which are designed to be incapable of facilitating the critical mechanistic step of hydride transfer. They are either synthesized as modified adenine dinucleotide or as nucleoside analogues with the propensity to undergo cellular conversions (adenosine kinase and NAD phosphorylase) to become NAD analogues.

As mentioned earlier in the kinetic mechanism of IMPDH, the hydrolysis of E-XMP* is a rate-limiting step. This leads to a significant accumulation of E-XMP*. Certain non-nucleoside, NAD-site binding uncompetitive inhibitors are known to trap this intermediate and prevent hydrolysis to yield the product XMP.

KNOWN IMPDH INHIBITORS

Based on the modes of inhibition, known inhibitors of IMPDH (whether in clinical use, in development, or future directions) are best divided into three major categories: nucleoside analogues (NAs), NAD analogues, and non-nucleoside analogues.

Nucleoside Analogues (NAs)

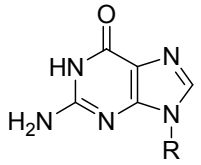
Historically, the most sought-after species of IMPDH inhibition were nucleoside analogues, primarily those of guanosine. As noted, NAs must minimally be converted to their monophosphates for activity. They are primarily competitive inhibitors that bind in the IMP-binding site and participate as substrates and/or inhibitors in multiple enzymatic pathways. Their mechanistic mode of inhibition (even though efficacious) is not clear, nor specific.[58] Starting from the fact that the product of an enzyme-catalyzed reaction may act as an inhibitor of the enzyme, GMP and six of its analogues were synthesized and found to inhibit IMPDH;[103, 109, 111] so did several purine analogues including 3-deazaguanine, its nucleoside and 5′-nucleotide. They showed broad antiviral activity against a variety of DNA and RNA viruses, as well as inhibitory action against several tumor cells.[109] Following this, a host of NAs have been synthesized or isolated from natural sources and tested. NAs that have received much attention because of their remarkable activity include ribavirin, mizoribine, EICAR, and a ‘fat base nucleotide’ (Figure

3.8). A similarity shared among these compounds, except 'fat base', is the possession of a five-membered heterocyclic ring with modified substituents.

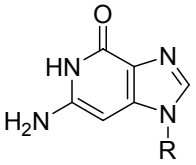
Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, Virazole, Rebetol, ICN Pharmaceuticals) shows broad-spectrum *in vitro* and *in vivo* antiviral activity. It is active against a variety of DNA and RNA viruses, including HBV, HCMV, HSV1, HSV2, HVV, Influenza A, Parainfluenza-3, as well as HIV. It was approved in the U.S. in 1986 as Virazole® as an antiviral for RSV, and in 1999 as Rebetol® for use in combination with interferon-alpha2b as an anti-HCV agent.[58, 112] The mode of inhibition of ribavirin is multi-faceted and efficacious (IMPDH inhibition: Ki = 250 nM). A crystal structure of RMP-human type II IMPDH complex shows that RMP binds indistinguishably in the IMP-binding site making the same binding interactions with IMPDH as IMP.[42, 83, 113] Even though approved, ribavirin shows severe side effects (that have restricted its use) including hemolytic anemia and teratogenicity.[58]

Derivatives of ribavirin have been synthesized, they include ribamidine (3-carboxamidine derivative), the thiocarboxamide derivative, and the L-enantiomer. Ribamidine possibly acts as a prodrug which is slowly converted to ribavirin *in vivo*; it is highly active against Punta Toro virus in mice,[114] and Pichinde virus in hamsters.[115] The thiocarboxamide derivative is only active against DNA viruses,[116] whereas the L-enantiomer of ribavirin is inactive against a panel of DNA and RNA viruses.[117]

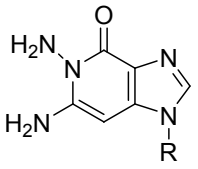
Mizoribine (Bredinin) was discovered in 1971 in Japan as a fungal metabolite based on its antibiotic activity. It also showed remarkable antiviral, anticancer and immunosuppressive activity.[118] After *in vivo* phosphorylation to mizoribine 5′-monophosphate (MZMP), it is an effective competitive inhibitor of IMPDH and GMP synthetase with Ki's of 10 nM and 10 μM respectively.[100]



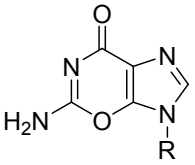
R = H: **GUANINE**
R = Rib: **GUANOSINE**
R = RibMP: **GMP**



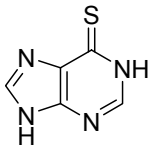
R = H: **3-DEAZAGUANINE**
R = Rib: **3-DEAZAGUANOSINE**
R = RibMP: **3-DEAZAGUANOSINE MP**
BROAD-SPECTRUM ANTIVIRAL(DNA/RNA)
L1210, HeLa, HL-60.
HUMAN KB, EMT-625.



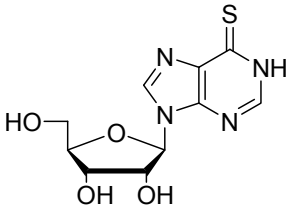
1 - **AMINOGUANOSINE**
IC_{50} (IMPDH) = 0.1uM
L1210



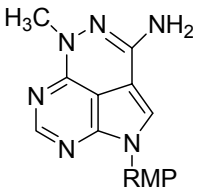
OXANOSINE
HeLa, L1210



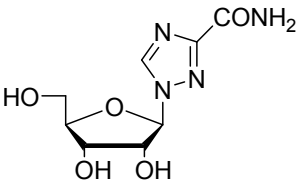
6-MERCAPTOPURINE
INFANTILE LEUKEMIA



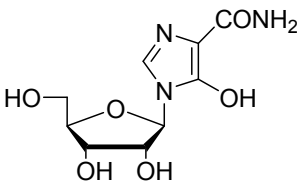
6-THIOINOSINE



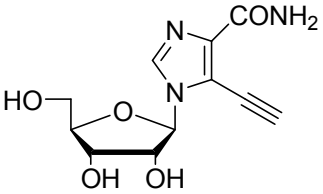
TRICRIBINE PHOSPHATE
66% @ 1.2uM/ 8uM IMP



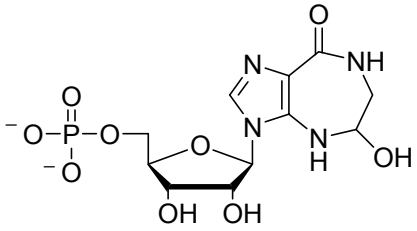
RIBAVIRIN
BROAD SPECTRUM ANTIVIRAL
Ki (IMPDH) = 250nM



MIZORIBINE
Ki (IMPDH) = 10nM



EICAR
IC_{50} (IMPDH) = 0.80 - 1.40uM
L1210



"FAT BASE"
Ki (IMPDH, HUMAN) = 1.4nM
Ki (IMPDH, E.Coli) = 53nM

Figure 3.8. Nucleoside-Analogue IMPDH Inhibitors.

MZMP inhibits both types I and II isoforms of IMPDH (Ki = 4 nM and 8 nM respectively) and is thought to act as a transition state analogue. The formal negative charge located on the 5-oxygen of the MZMP aglycone ring mimics the negative charge which develops on N-3 of IMP during the reaction with Cys-331.[90] Even though mizoribine is approved in Japan for prevention of rejection following renal transplantation and for treatment of lupus nephritis, rheumatoid arthritis and nephrotic syndrome,[119] it is not approved in the U.S. clinically. Side effects of its use include GI toxicity.[58]

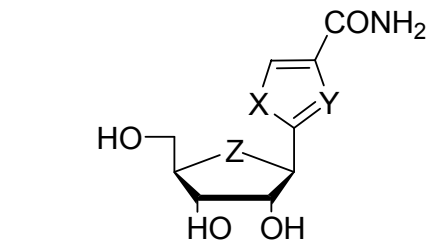
EICAR (5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide) is a novel mechanism-based inhibitor of IMPDH. It shows potent cytostatic activity against a number of human solid tumors as well as broad-spectrum antiviral activity.[118] EICAR acts through multiple modes; however, its primary mode of action is through its monophosphate (EICAR-MP), which competitively inhibits IMPDH by binding at the IMP-binding site. Its IMPDH inhibition has an IC_{50} range of 0.80-1.40 μM.[120] Mechanistically, it is believed that the active site sulfhydryl group of Cys-331 engages in an irreversible nucleophilic attack on the acetylenic moiety, which has been shown to be necessary for activity. The mode of action of EICAR is rather complex; its broad biological activity is apparently due to the formation of several anabolites, including di- and triphosphates, as well as its NAD analogue (EAD).[118]

'Fat Base' (Imidazo[4,5-e][1,4]diazapine nucleotide) is a potent reversible IMP-analogue inhibitor of IMPDH. Its dehydro form reacts with IMPDH to form a transition state analogue (tetrahedral adduct) resembling E-IMP and E-XMP. The bigger ring system makes its tetrahedral adduct more stable. Fat base nucleotide is a potent time-dependent inhibitor of both human and *E. coli* type II IMPDH with Ki values of 1.4 nM and 53 nM, respectively.[90, 121]

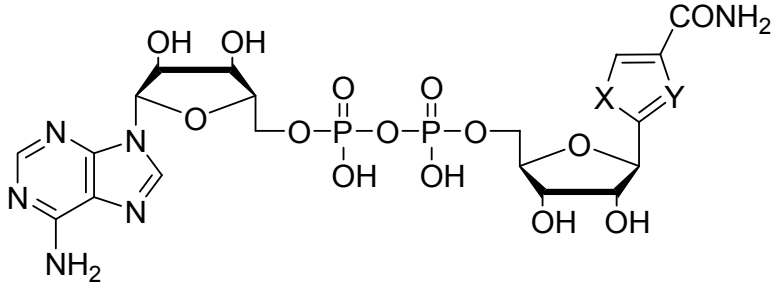
NAD Analogues

These compounds are designed to bind in the NAD-binding site and be incapable of facilitating the critical mechanistic step of hydride transfer, thus inhibiting IMPDH. NAD analogues are either synthesized as adenine dinucleotides or as nucleoside analogues (NAs) which undergo cellular conversions to their NAD analogues *in vivo*. This metabolic fate of NAs has been exploited with a number of compounds including, but not limited to, tiazofurin and selenazofurin. Figure 3.9 shows a host of NAs and some of the corresponding NAD-analogues into which they are converted. As of the year 2000, TAD (adenine dinucleotide form of tiazofurin), was the sole member of this class of compounds to advance to clinical trials and, while showing dose-limiting toxicities in phase I, it demonstrated mixed efficacy in an assortment of antineoplastic phase II/III clinical trials.[28]

Tiazofurin adenine dinucleotide (TAD) is the active form of tiazofurin (TR, 1- β-D-ribofuranosylthiazole-4-carboxamide). It is a potent and specific inhibitor of IMPDH formed from TR undergoing a unique cellular metabolic activation. TR is phosphorylated by adenosine kinase and/or 5′-nucleotidase to its mononucleotide, which is coupled to AMP by NMN adenylyltransferase producing the active metabolite TAD.[122, 123] In TAD, the nicotinamide riboside moiety of the normal cofactor, NAD, is replaced by tiazofurin, which cannot participate in hydride transfer. This results in potent noncompetitive inhibition of IMPDH (Ki = 0.1 μM). TAD (like the other NAD analogues) is metabolically unstable as it can be degraded back to its components tiazofurin and adenosine by the action of cellular phosphodiesterases and phosphatases. To circumvent the metabolic instability as well as the poor metabolic conversion of some of these NAs to the corresponding NAD analogue, synthetic NAD analogues that are stable and do not require metabolic activation were synthesized.



1. TIAZOFURIN X = S, Y = N, Z = O
2. SELENAZOFURIN X = Se, Y = N, Z = O
3. OXAZOFURIN X = O, Y = N, Z = O
4. THIOPHENFURIN X = S, Y = CH, Z = O
5. FURANFURIN X = O, Y = CH, Z = O
6. SELENOPHENFURIN X = Se, Y = CH, Z = O
7. FURANTHIOFURIN X = O, Y = CH, Z = S
8. THIOPHENTHIOFURIN X = S, Y = CH, Z = S
9. IMIDAZOFURIN X = NH, Y = N, Z = O

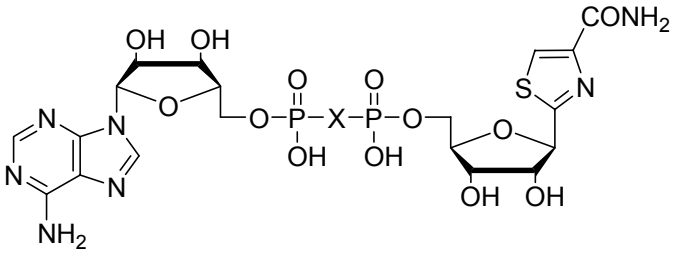


10. TAD (X = S, Y = N)
11. SAD (X = Se, Y = N)
12. TFAD (X = O, Y = CH)
13. FFAD (X = O, Y = CH)
14. SFAD (X = Se, Y = CH)

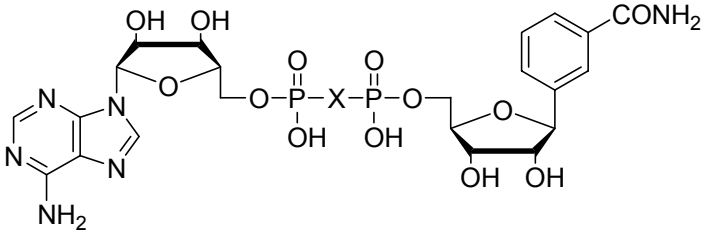
Figure 3.9. NAD-Analogue IMPDH Inhibitors.

These included the methylenebis(phosphonate) and difloromethylenebis(phosphonate) analogues of TAD and BAD (Figure 3.10), which are just as active as their parent compounds, and even active in tiazofurin-resistant cell lines.

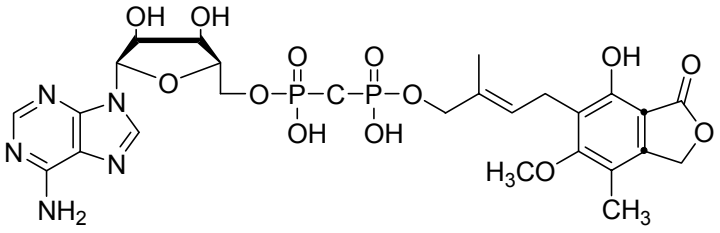
Also bis(phosphonate) analogues of mycophenolic adenine dinucleotide (MAD) have been made as mimics of NAD (Figure 3.10). These compounds, C-2-MAD and C-4-MAD, are stable, do not require activation, and are potent and specific inhibitors of IMPDH. They are an order of magnitude more potent than tiazofurin in K562 cells,[124] and are resistant to glucuronidation.[125]



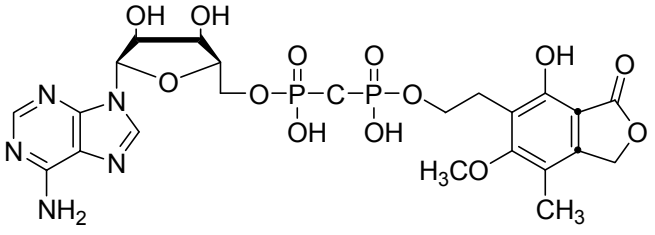
Bis(phosphonate)analogue of TAD
X = CH_2 or CF_2



Bis(phosphonate)analogue of BAD
X = CH_2 or CF_2



C-4-MAD



C-2-MAD

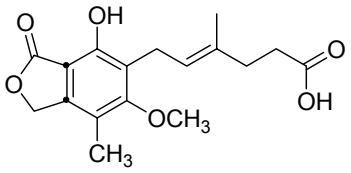
Figure 3.10. Stable NAD-Analogue IMPDH Inhibitors.

Non-Nucleoside Analogues (NNAs)

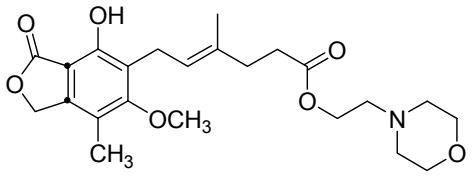
This class of compounds shares a common mechanism of inhibition: acting as uncompetitive inhibitors that trap the E-XMP* intermediate before hydrolysis and release of product. They bind at the NAD-binding site. Being non-nucleosides, they are not susceptible to the barrage of *in vivo* modifications suffered by NAs, thus affording room for selective IMPDH inhibitors. Three different types of compounds have been identified in this class of compounds: mycophenolate mofetil, VX-497, and the pyridazines. These compounds share structural features such as an aromatic ring system and strategically placed heteroatoms that form hydrogen bonds with the residues in the NAD-binding site. Their aromatic rings bind tightly to the purine ring of the E-XMP* intermediate through π-stacking thereby 'trapping' it.

This class of compounds boasts of some highly potent uncompetitive inhibitors of IMPDH with Ki = 50 nM.[58] Some of the compounds in this class that have attracted attention include mycophenolic mofetil (MMF), VX-497 and its analogues, pyridazines (Figure 3.11) and the derivatives of 1,5-diazabicyclo-[3.1.0]hexane-2,4-dione (Figure 3.12).

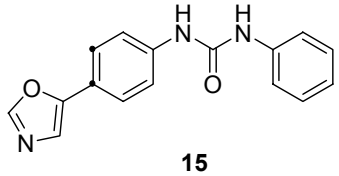
Mycophenolate mofetil (MMF, Cell-Cept, Roche) is a prodrug of the natural product mycophenolic acid (MPA). MPA is potent, uncompetitive, reversible inhibitor of IMPDH.[58, 126] MPA binds to human IMPDH with Ki ranging in the literature from 11 nM to 33-37 nM for type I and 7-10 nM for type II.[105, 127] MPA remains the NAD-site binding inhibitor with the highest affinity and specificity for type II IMPDH.[29] MPA inhibition of type II IMPDH is 4.8-fold lower in Ki value than the type I. Such selective inhibition may limit toxicities caused by the concomitant inhibition of the constitutively expressed type I isoform.[105] MMF is approved in the US and Europe as an immunosuppressant for the treatment of acute rejection in renal and heart transplantations.[128, 129]



MYCOPHENOLIC ACID
Ki (IMPDH II) = 7 - 10nM
Ki (IMPDH I) = 11 - 37nM

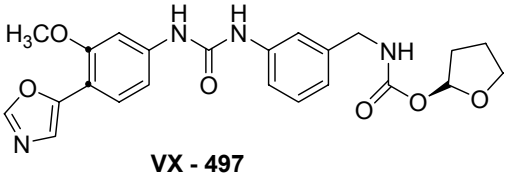


MYCOPHENOLATE MOFETIL (MMF)
MPA PRODRUG

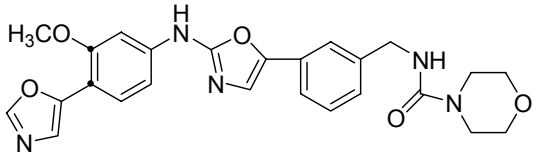


15

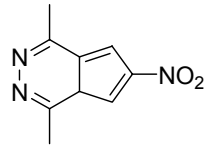
Ki = 3.5uM



VX - 497
VERTEX PHARM.
Ki (IMPDH II) = 7 - 10nM
BROAD SPECTRUM ANTIVIRAL
10 - 100 FOLD > RIBAVIRIN
PHASE 2 TRIALS (HCV)

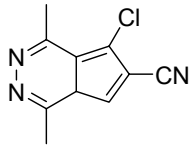


BMS-337197



16

IC_{50} (IMPDH) = 1.93uM
IC_{50} (EMT6) = 39.7uM



17

IC_{50} (IMPDH) = 0.67uM
BETTER THAN MPA

Figure 3.11. Non-Nucleoside Analogues IMPDH Inhibitors.

It has also been used successfully to treat small numbers of patients with other immune disorders.[130] *In vivo*, MMF is metabolized to MPA, which exerts its immunosuppressive activity by directly reducing both T and B-lymphocyte proliferation *via* inhibition of IMPDH.[29] The

efficacy of MPA is limited by its rapid metabolic conversion to the inactive MPA-7-O-glucuronide.[131, 132] About 90% of the drug circulates in the glucuronide form which results in gastro intestinal (GI) toxicity.[1]

A high-throughput screening of databases for IMPDH inhibitors, led to the discovery of the commercially available compound **15** (Figure 3.11), which inhibits IMPDH with Ki = 3.5 μM.[58]

Synthesis of analogues of **15** by Vertex yielded VX-497, which is a reversible, uncompetitive inhibitor of human type II IMPDH with Ki of 7-10 nM. It binds at the nicotinamide end of the NAD-binding site. VX-497 acts as an immunosuppressant by its cellular antiproliferative activity against T and B lymphocytes. It also showed broad spectrum *in vitro* antiviral activity, exhibiting 10 to 100-fold greater potency than ribavirin against HBV, HCMV, RSV, HSV-1, parainfluenza 3 virus, EMCV, and VEE viral infections in cell culture. Unlike MPA, VX-497 cannot be glucuronidated (due to lack of a phenolic group) and thus does not show the same clinical limitations of GI toxicity.[58, 133]

Structurally similar to VX-497, BMS-337197, synthesized by Bristol-Myers Squib (Figure 3.11), also inhibits IMPDH in the nanomolar range.[134, 135]

After a high throughput screening of 80,000 compounds against IMPDH (Zeneca), the pyridazine derivative **16** (Figure 3.11) with immunosuppressive activity was discovered.[136] This pyridazine is an uncompetitive inhibitor of IMPDH with an IC_{50} of 1.93 μM and shows an IC_{50} of 39.7 μM against the proliferation of EMT6 cells. Upon modifications of **16**, the derivative **17** with an improved potency of 0.76 μM against IMPDH was discovered. Compound **17** functions *via* the same mechanism as MPA (traps the covalent intermediate E-XMP*) and it showed

significant immunosuppressive activity over MPA in the inhibition of delayed type hypersensitivity in mouse models.

A miscellaneous class of IMPDH inhibitors that do not fit into any of the above mentioned categories is a series of three derivatives of 1,5-diazabicyclo-[3.1.0]hexane-2,4-dione (**18, 19** and **20,** Figure 3.12). They have shown specific inhibition of the type II IMPDH isoform. Surprisingly, these non-nucleosides bind at the IMP-binding site and act as competitive inhibitors with Ki values in the 5 - 44 μM range. Although their Ki values are not very low, they provide an excellent lead for isoform-specific chemotherapy.[137]

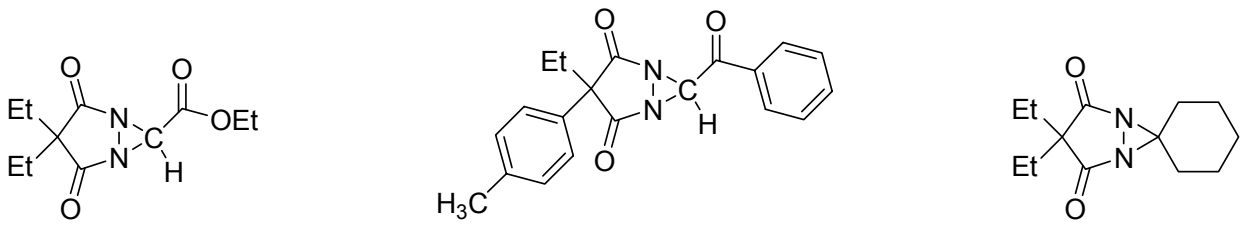


Figure 3.12. Specific Inhibitors of IMPDH Type II.

FUTURE DIRECTIONS

Remarkable research on IMPDH has provided a comprehensive understanding of the structure, mechanism and role of IMPDH both as a key enzyme in purine metabolism and as a target for anticancer, antiviral, immunosuppressive and antibacterial chemotherapy. However, further work is needed in the chemotherapeutic area. Of the three approved IMPDH inhibitors (ribavirin, mizoribine and mycophenolic mofetil), none shows significant selectivity against IMPDH type II, and they are all limited by toxicity and/or metabolic inactivation. Even though

structure-based design of isoform-specific agents remain hampered by the 84% sequence identity between the type I and type II, the available IMPDH crystal structure data and known inhibitors provide in-depth information about specific residue interactions in both IMP-binding site and the NAD-binding site. This information can be explored in the design of isoform-specific inhibitors.

The IMP-binding site is well conserved for both isoforms across all species. This poses difficulties for isoform-specific inhibition. However, the discovery of derivatives of 1,5-diazabicyclo-[3.1.0]hexane-2,4-dione that specifically inhibit the type II isoform needs to be explored further to circumvent this problem.

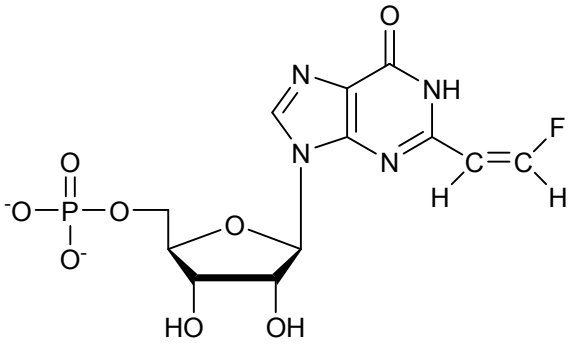
In contrast to the IMP-binding site, the NAD-binding site is not conserved. The crystal structure of the type II IMPDH shows that three of the four residues that interact with the cofactor adenine moiety differ between the type I and type II isoforms,[71] suggesting that modifications of the adenine moiety of cofactor-type inhibitors may be exploited in the design of isoform-specific agents.

The cofactor adenine binding region of IMPDH is also a source of species-specific differences. The importance of the NAD site in the development of species-specific inhibitors is thus very feasible and requires further exploration.[60]

Furthermore, in the wake of terrorism and bioterrorism threats, it is tremendously imperative for continued work in the discovery of novel antiviral agents against existing and emerging viral diseases that can be used as weapons. In this respect, the ingenius design and synthesis of nucleoside analogues as antiviral compounds inhibiting IMPDH has proven to be a promising means.[138]

INHIBITION OF IMPDH BY 2-FLUOROVINYL INOSINE MONOPHOSPHATE

In an ongoing antiviral drug discovery program, the Nair and coworkers designed and synthesized the novel compound, 2-[2-(*Z*)-fluorovinyl]inosine-5′-monophosphate (2-FVIMP, **21**, Figure 3.13). Compound **21** is related to 2-vinylinosine and its monophosphate; both compounds have been previously investigated in our laboratory and also by others.[139-143]



21

Figure 3.13. Structure of 2-[2-(Z)-Fluorovinyl]Inosine-5′-Monophosphate.

Enzymology studies on the inhibition of IMPDH (isolated from *E. coli* B3 cells) by 2-FVIMP has been conducted in our laboratory.[144] According to the results, incubation of IMPDH with 2-FVIMP exhibited a time-dependent decrease in V_t/V_o (Figure 3.14). This indicates that 2-FVIMP inactivates the enzyme.

The activity of inhibited/inactivated enzyme could not be restored by dialysis. Urea denaturation and renaturation of the 2-FVIMP-inactivated IMPDH also did not restore enzyme activity. In a control experiment containing untreated enzyme there was 70 % recovery of

activity after urea treatment. These results imply that a covalent bond is formed between 2-FVIMP and IMPDH and during the process, the enzyme is irreversibly inactivated.

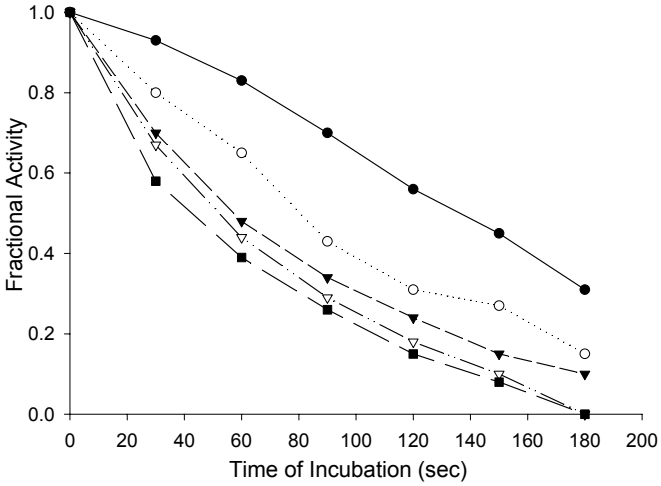


Figure 3.14. Inhibition of E. Coli IMPDH by 2-FVIMP with Respect to Time.
(●, 0.25μM; ○, 0.50μM; ◄, 0.75μM; Δ, 1.0μM; ■, 1.5μM).

To determine the mechanism of inhibition, the k_{obs} values obtained by using the equation ($\ln(V_t/V_o) = -k_{obs}\, t$); where Vt is the activity at time t, and Vo is the activity at time t = 0, were plotted against inhibitor concentration. The k_{obs} values displayed a hyperbolic relationship with 2-FVIMP concentration (Figure 3.15).

This indicates that 2-FVIMP reacts with IMPDH through a two-step mechanism as follows:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.I \xrightarrow{k_{inact}} E\text{-}I$$

where $K_i = k_1/k_{-1}$ is the dissociation constant, k_{inact} is rate constant of inactivation, E is IMPDH, E.I is the reversibly bound enzyme-inhibitor complex and E-I is the irreversibly inactivated enzyme. Thus, the mechanism of inactivation of IMPDH by 2-FVIMP involves the initial

reversible formation of an E.I complex followed by the inactivation step. The values of k_{inact} and K_i were determined using the equation ($k_{obs} = k_{inact}[I]/(K_i+[I])$ and plotting the reciprocal of k_{obs} versus the reciprocal of inhibitor concentration.

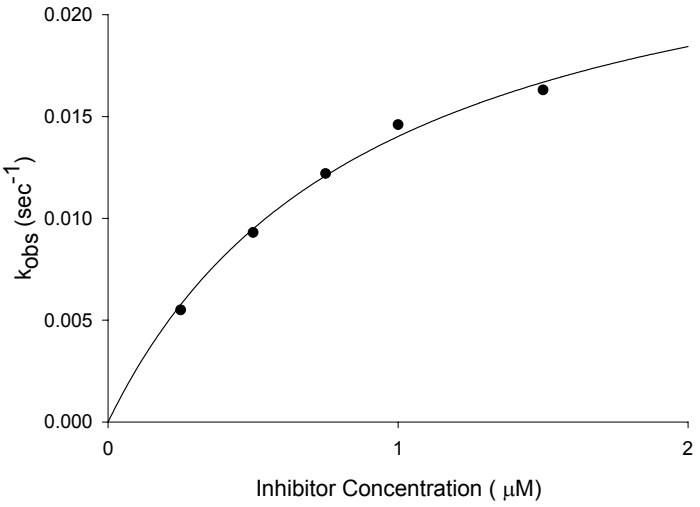


Figure 3.15. Relationship of k_{obs} Values with the Inhibitor (2-FVIMP) Conc.

The values of k_{inact} and K_i were 0.0269 s^{-1} and 1.11 μM, respectively, whereas the well-known IMPDH inhibitor, 6-chloropurine ribonucleoside monophosphate, in these studies gave values of 0.076 min^{-1} and 62.0 μM. The type of inactivation of IMPDH shown by 2-FVIMP is related to that exhibited by ethynylimidazole carboxamide riboside monophosphate (EICARMP).[98] These results conclude that 2-FVIMP is a potent inhibitor of IMPDH. The inactivation is time-dependent and follows a two-step mechanism. Antiviral screening of 2-FV (the nucleoside form of **21**) against the vaccinia virus (HFF cell line) showed moderate activity

(T.I. < 4). The mechanism of this antiviral activity is likely associated with the ability of the cellularly produced monophosphate, **21**, to be an inhibitor of IMPDH.[144]

This enzymology data on 2-FVIMP and that of 2-VIMP lends reason to the fact that similarly designed compounds could also potentially be IMPDH inhibitors. The design rational and synthesis of such compounds are discussed in detail in the next chapter.

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CHAPTER 4

RESULTS AND DISCUSSION

RATIONAL DRUG DESIGN

Currently, there is growing interest in the synthesis of novel C-2 and C-6 modified purine nucleosides. This pertains to the fact that this class of compounds has shown potent antiviral activity as well as exhibiting enzymological usefulness as biological probes for the study of key mammalian and viral-encoded enzymes.[1-7, 8-12] The Nair research program, being one of the pioneers in this field, continues in its search of novel antiviral nucleosides with focused attention on functionalized C-2 hypoxanthine and C-2, C-6 modified purine systems.[1, 8, 13, 14, 15]

In the past, most C-2 substituted hypoxanthine nucleosides were synthesized from imidazole nucleosides through ring closure reactions,[16-18] or by initially masking the C-6 carbonyl functionality in protected guanosine with either chloro or methoxy while making modifications at the C-2 position. To revert to the hypoxanthine system, the 6-chloro or 6-methoxy is unmasked either chemically (using trimethylsilyl iodide) or chemoenzymatically using ADA. This masking and unmasking of the C-6 position gives variable results and the procedure is lengthy with a low overall yield.[1, 8, 14, 15, 19]

This chapter reports the development of a new reproducible, efficient and high-yielding general methodology for the non-enzymatic synthesis of novel C-2 functionalized hypoxanthine nucleosides as well as C-2, C-6 modified ribofuranosylpurine nucleosides. We also expound on already established methodologies in our group and their usage in the synthesis of our new target compounds. The results, rational drug design and the application of new as well as old methodologies in the synthesis of our novel targets are discussed in this chapter. Since several compounds with antiviral activity (e.g., ribavirin, 3-deazaguanosine, EICAR, mizoribine, etc.)

have been found to be inhibitors of IMPDH, our novel nucleoside analogues were designed as potential antiviral agents targeting inhibition of IMPDH. Since IMPDH has been discussed in detail in Chapter 3, pertinent facts and references will be re-called into the discussion here.

The two major drug design strategies employed in our work are mechanism-based drug design and structure-based drug design. Mechanism-based drug design of IMPDH inhibition has helped identify three modes of inhibition, IMP-binding site inhibition being our group's focus is one of them. In this mode, nucleoside analogues mimicking IMP are synthesized as potential inhibitors. Structure-based drug design on the other hand, relies on the structure of existing active compounds and establishes a structure-activity relationship. In this respect, it is very advantageous that several crystal structures of IMPDH with different ligands (known inhibitors) have been fully characterized, thus providing indispensable information on hydrogen bonding interactions between active-site amino acid residues and ligand atoms. Our work focused on structures of known inhibitors such as 6-mercaptopurine, 6-thioinosine, 6-chloropurine, and 2-vinylinosine (a compound with broad-spectrum antiviral activity developed by Nair and coworkers), as well as the suspected pharmacophores of other compounds that have expressed either antiviral or antiparasitic properties by means of IMPDH inhibition. This chapter also justifies our concepts for the mechanism and structure-based design of our proposed IMPDH inhibitors, as well as the possible means by which they could exert their inhibitory effect.

The key intermediates in the synthesis were 6-chloro-2-iodo-9-β-(2′,3′,5′-tri-O-acetyl)-D-ribofuranosylpurine **1** and 2-iodo-9-β-(2′,3′,5′-tri-O-acetyl)-D-ribofuranosylhypoxanthine **13**, which were developed and synthesized by us. Finally, our novel target compounds were realized by further elaboration of these intermediates using among other reaction types, a crucial step

involving a palladium-catalyzed Stille cross-coupling reaction of the key intermediates with organostannanes containing the desired synthons.

STRUCTURE-BASED DRUG DESIGN

Structure-based drug design is essential in the quest for more potent and isoform-specific inhibitors of IMPDH type II. In this respect, it is a tremendous advantage to have a number of crystal structures of IMPDH showing in detail how active site residues interact with known inhibitors. The schematic representation of hydrogen bonding interactions of XMP*-IMPDH (Figure 3.4) shows the involvement of eleven active site residues. Of the eleven residues, five are stationary residues (Ser 68, Asp 364, Gly 366, Gly 387, and Ser 388), four are contributed by the active site flap (Tyr 411, Met 414, Gly 415, and Gln 441), and the remaining two are from the active site loop (Ser 329 and Cys 331). Through hydrogen bonding, Ser 68 and Asp 364 stabilize the 2′ and 3′ hydroxyl groups of the sugar, respectively. Ser 329, Gly 366, Gly 387, Ser 388, and Tyr 411 stabilize the 5′-phosphate moiety. Met 414, Gly 415, and Gln 441 stabilize the purine ring at N-7, C-6-O and N-1-H, respectively, and Cys 331 covalently binds at C-2 of the purine ring. Since the residues of the IMP-binding site appear to be well conserved among IMPDHs (even across different species),[20, 21] our drug design targeted these residues to hold our novel inhibitors in the active site.

As discussed earlier, nucleosides minimally need to be converted to their 5′-monophosphates (i.e., nucleotide form) to exert their activity. It is therefore not surprising that all nucleoside analogue inhibitors that have been crystallized with IMPDH for X-ray crystallographic purposes have been in the 5′-monophosphate form. Unfortunately, these nucleotides are polyanionic compounds which do not cross easily through cellular membranes to

assume their therapeutic role. To circumvent this problem, inhibitors are synthesized in the nucleoside form since nucleosides and their analogues (unlike nucleotides) are neutral molecules which can enter cells easily. However, upon entry into the cells, their biological activity depends on their subsequent intracellular phosphorylation by adenosine kinase, 5′-nucleotidase or other kinases.[22, 23] In some cases, as exemplified by 2-vinylinosine, the means of phosphorylation is not as described above. 2-Vinylinosine is not a substrate for adenosine kinase (AK), thus it does not get phosphorylated to its 5′monophosphate by AK. Rather, it is cleaved slowly by purine nucleoside phosphorylase (PNP),[24] and the resulting base, being a substrate for hypoxanthine-guanine phosphoribosyl transferase (HGPRT),[25] is attached to a 5′-phosphoribosyl moiety to yield the active therapeutic form 2-vinylinosine monophosphate.

Based on the above discussion, our novel targets were synthesized in the nucleoside form, relying on intracellular enzymes to activate them into their 5′-monophosphates for antiviral activity. Furthermore, based on the hydrogen bonding interactions of XMP* and IMP with IMPDH active site residues derived from X-ray crystallography, our novel targets were designed to have a similar architecture as IMP.

MECHANISM-BASED DRUG DESIGN

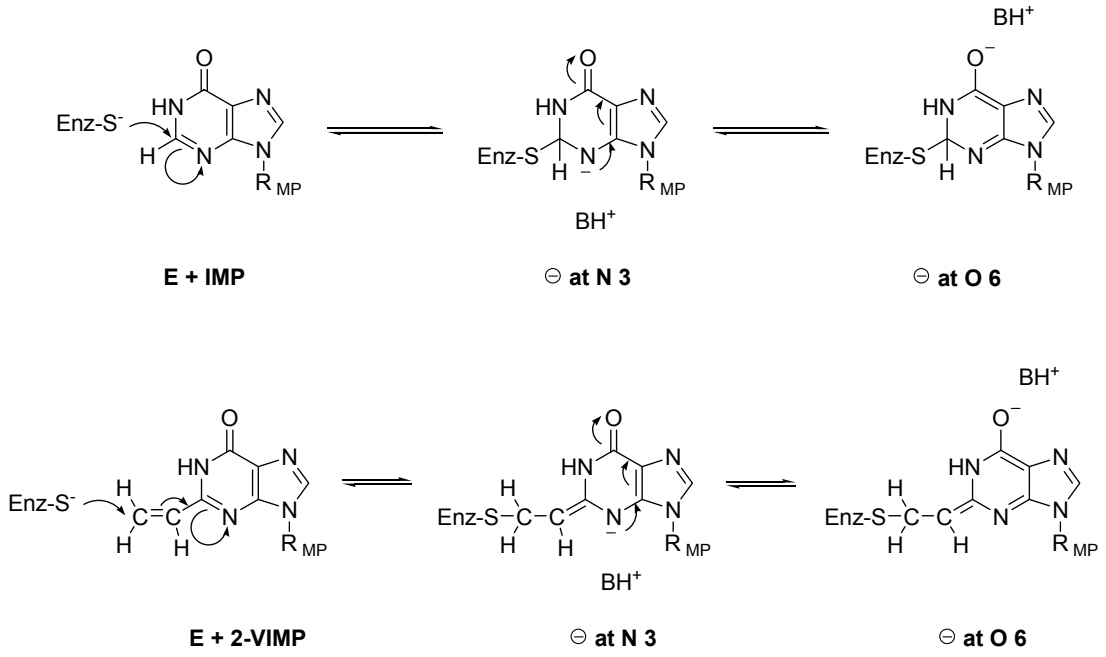
A thorough understanding of the mechanism of action of IMPDH is required in mechanism-based drug design of IMPDH inhibitors. As noted earlier in the mechanism of IMPDH, the initial step involves the nucleophilic attack of the thiol group of Cys-331 on the C-2 of the IMP base to form the tetrahedral adduct E-IMP.[20, 26] Based on this proposed IMPDH mechanism, it has been plausibly conceptualized that any inosine nucleoside with a substituent at the C-2 position which is reactive to the sulfhydryl group of Cys-331 may be a

potential inhibitor of IMPDH.[27] In support of this concept is the observation that nucleosides with unsaturated moieties conjugated to the purine ring at C-2 have shown antiviral activity by virtue of IMPDH inhibition. One such compound is 2-vinylinosine (2-VI) synthesized by Nair and coworkers [1, 14] which shows broad-spectrum activity against exotic RNA viruses.[28]

Enzymology and kinetic studies conducted on 2-vinylinosine-5′-monophosphate (2-VIMP, the active intracellular form of 2-VI) indicates that it may be exerting its inhibition of IMPDH by the formation of an apparently irreversible covalent bond with IMPDH, where the vinyl moiety conjugated to the purine ring of 2-VIMP acts as a Michael acceptor presumably for the sulfhydryl group of Cys-331.[29]

Clarification of how this is actually happening can be inferred from the characteristics and kinetic mechanism of the IMPDH reaction discussed earlier. In the mechanism of IMPDH, the initial attack of the sulfhydryl group of Cys-331 on C-2 of IMP and the subsequent hydride transfer to NAD, could either be stepwise or concerted.[20, 30-34] In the stepwise consideration (Scheme 4.1), negative charges result at N-3 and/or O-6 of IMP (by resonance) from the nucleophilic attack of Cys-331 on C-2, these negative charges are stabilized through hydrogen bonding with an amino acid residue or by a general acid (BH^+).[35, 36]

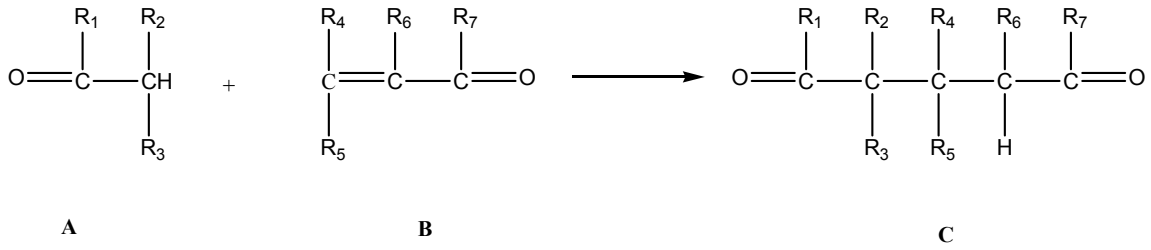
Similarly, nucleophilic attack of Cys-331 on the conjugated vinyl group (Michael acceptor) of 2-VIMP results in the formation of negative charges at N-3 and/or O-6 of 2-VIMP (by resonance) (Scheme 4.1). These negative charges are expected to be stabilized through hydrogen bonding just as for IMP. In this fashion, 2-VIMP mechanistically mimics IMP as a transition-state analogue and thus exerts its antiviral activity by IMPDH inhibition. Our novel targets were designed to mimic IMP and 2-VIMP in this respect.



Scheme 4.1. Stepwise Consideration of IMPDH Mechanism.

THE MICHAEL REACTION

Originally, the Michael reaction (Scheme 4.2) represents a 1, 4 attack of the α-carbanion (nucleophile) of a donor **A** on the α,β unsaturation of an acceptor **B**. The α-carbanion of the donor is generated by the use of a base such as sodium ethoxide.



Scheme 4.2. The Michael Reaction.

Comparing the Michael reaction to the mechanism by which 2-VIMP inhibits IMPDH, it can be seen that the sulfhydryl group of Cys-331 (a nucleophile just like the α-carbanion of the donor **A**) attacks the conjugated vinyl group in 2-VIMP (an acceptor similar to the α,β unsaturation of the acceptor **B**) in a 1, 8-fashion as opposed to the usual 1, 4-attack in the Michael reaction (Scheme 4.2). The range of Michael acceptors is very broad; they include α,β-ethylenic aldehydes, aliphatic α,β-ethylenic ketones, α,β-acetylenic ketones, aromatic α,β-ethylenic ketones, heterocyclic α,β-ethylenic ketones, cycloalkenones and acyl cycloalkenes, α,β-unsaturated nitriles and their amides, α,β-ethylenic aliphatic esters, olefins with substituents, 2- and 4-vinylpyrdines, fulvenes, etc. Following the discovery that a Michael acceptor (unsaturated moieties) conjugated to C-2 of the purine ring leads to antiviral activity, there is a possibility that any of the aforementioned Michael acceptors similarly conjugated to C-2 of the purine ring could be a potential antiviral compound. In this quest, 2-ethynyl and substituted ethynyl groups have been investigated as unsaturated analogs of 2-vinylinosine. Also 2-(2-fluorovinyl) analogs have been designed to increase the electrophilic character of the double bond of 2-vinylinosine for enhanced nucleophilic addition of the sulfhydryl group of Cys-331 at the fluorovinyl group. In addition, since the strained ring of a cyclopropyl group undergoes addition reactions similar to alkenes,[37] it has been introduced at the C-2 position for investigation. Figure 4.1 shows the compounds synthesized and Table 4.1 shows the antiviral evaluations of selected compounds against vaccinia virus (*in vitro*).[19, 28] In this series of compounds, 6-chloro-2-ethynylpurine ribonucleoside **I** was the most active but most toxic. 6-Chloro-2-(3-fluoro-propynyl) purine riboside **IV** and 2-(3-fluoro-propynyl) inosine **X** had comparable activity to 2-ethynylinosine **VII**.

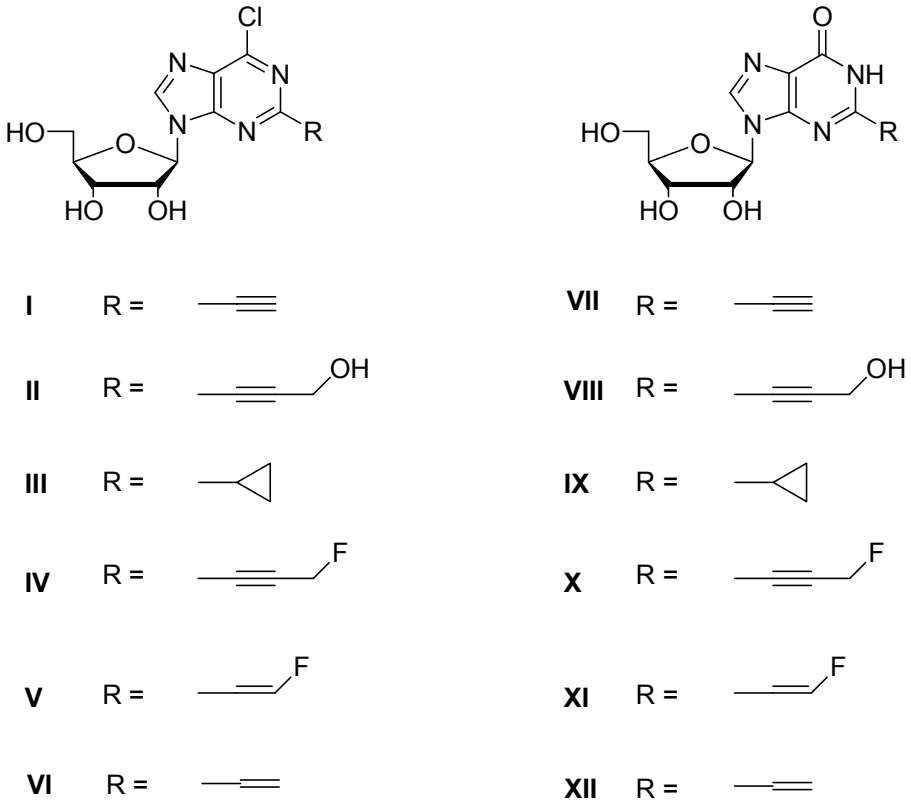


Figure 4.1. Nucleosides with C-2 Unsaturated Moieties.[19]

Table 4.1. Antiviral Evaluation of Selected Nucleoside Analogues.[19]

COMPOUND	VACCINIA VIRUS (IC_{50})	VACCINIA VIRUS (CC_{50})
6 -Cl, 2-ETHYNYL **I**	> 0.8μg/mL	3.0 μg/mL
2-ETHYNYL INOSINE **VII**	> 20μg/mL	66.5 μg/mL
2 – VINYL INOSINE **XII**	13 μg/mL	~ 100μg/mL

Both 2-(2-fluorovinyl) inosine **XI** and 6-chloro-2-(2-fluorovinyl) purine **V** were not as active as 2-vinylinosine **XII**, but showed some activity towards the vaccinia virus in HFF cells. The other compounds in the series were not active.[19] In addition to the vaccinia virus, compound **XII** showed broad-spectrum antiviral activity (IC_{50} in Vero Cells): IC_{50} 13μg/mL; CC_{50} 100ug/mL; JEV (3.2μg/mL); PIC (2.5μg/mL); PT (2.7μg/mL); RVF (24μg/mL);VEE (7.7μg/ml); YF (7.7μg/mL).[28]

From the above findings, it is clear that the vinyl group supercedes the ethynyl, and modifications made to the vinyl group only results in decreased activity. Since 6-chloro-2-ethynylpurine ribonucleoside **I** is more active than 2-ethynylinosine **VII**, and the only difference between them is the substituent at C-6, it can be inferred that appropriate substitution at C-6 can improve activity; however the C-6-chloro may not be desirable since it conferred the worst cytotoxicity (CC_{50} = 3.0 μg/mL) on 6-chloro-2-ethynylpurine ribonucleoside **I**. Substitutions at C-6 and their effects are discussed below.

SIGNIFICANCE OF C-6 MODIFICATIONS OF PURINE NUCLEOSIDES

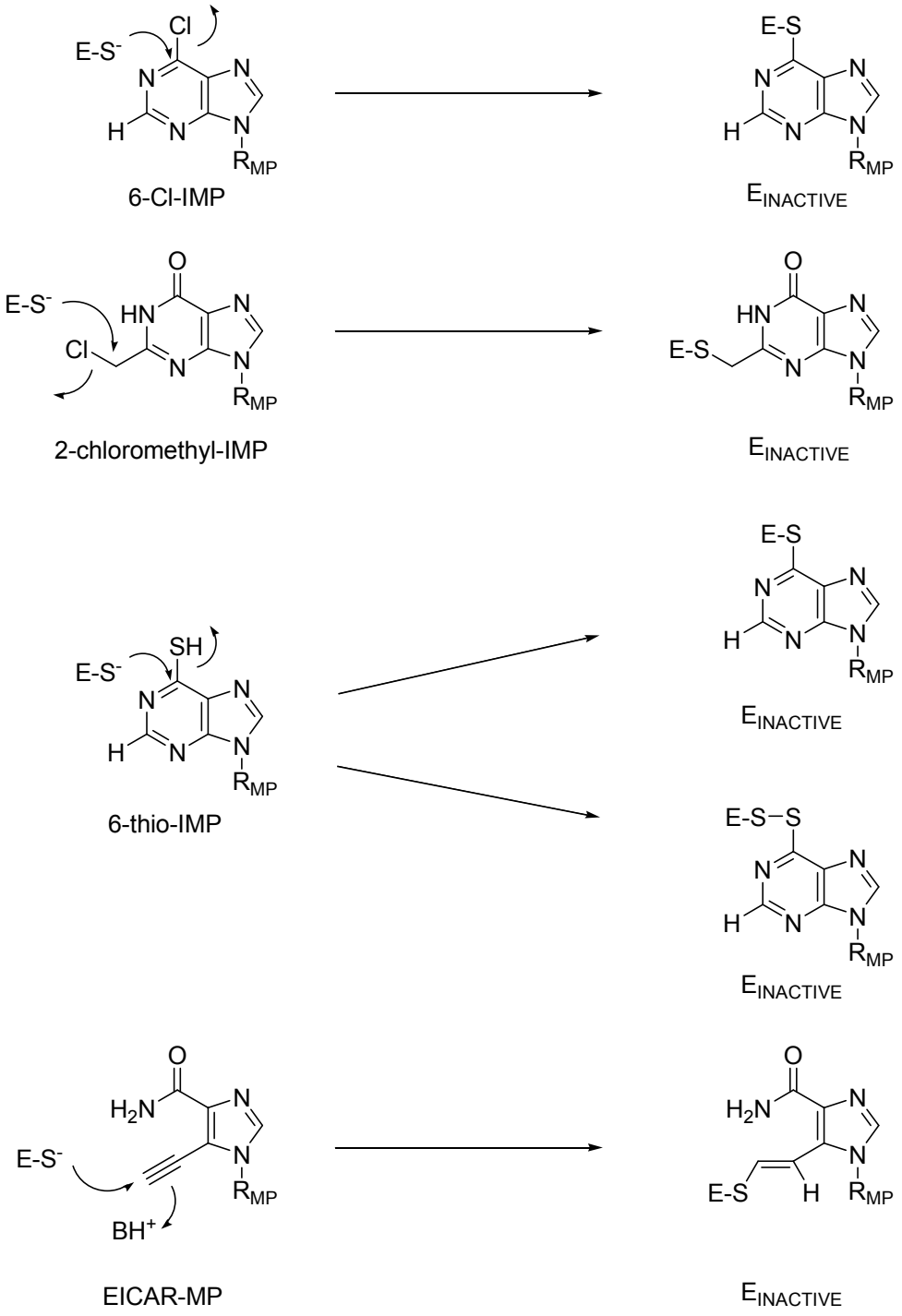
As discussed earlier, the high mobility and flexibility of the active site loop (with Cys 331) and flap is particularly intriguing. For instance, it is well established from the mechanism of IMPDH that Cys-331 attacks the C-2 position of IMP. Additionally, the crystal structure complex of human type II IMPDH, 6-Cl-IMP, and SAD shows a covalent linkage between the C-6 position of the purine ring and Cys-331 (rather than the usual C-2 position).[21, 38] This undoubtedly proves that Cys-331 can attack the C-2 as well as the C-6 position of the purine ring. The crystal structures of E-XMP* and the 6-Cl-IMP adduct further confirms that the

nucleotides are immobilized in the active site, while the Cys-331 loop is re-oriented to the site of attack on the purine ring.[38-41]

Further work has been done with several IMP analogs designed as affinity agents and mechanism-based inactivators of IMPDH targeting Cys-331.These analogues include the already mentioned 6-Cl-IMP, EICARMP, 2-chloromethyl-IMP and 6-thio-IMP (Scheme 4.3).[42-45] All of these compounds are time-dependent irreversible inactivators of IMPDH and the point where they are attacked exemplifies the flexibility of IMPDH. The mechanism of inactivation of 6-Cl-IMP, EICARMP, and 2-chloromethyl-IMP is through the formation of covalent adducts with Cys-331. Unfortunately, the mechanism of inactivation by 6-thio-IMP has not been elucidated yet. It seems two pathways are likely; in one case the sulfhydryl group of Cys-331 may displace the 6-thio group in a reaction analogous to that of 6-Cl-IMP (addition followed by elimination), and in a second case a disulfide bond may form between the sulfhydryl group of Cys-331 and the 6-thio-group.[30, 38, 39, 45] The nucleophilic displacement of 6-SH and the possible formation of disulfide bonds at C-6 gives a hint for potential IMPDH inactivation by the introduction of sulfur containing moieties at the C-6 position of the purine ring. The C-6 thiocarbonyl and C-6 thiomethyl functionalities have been explored in this dissertation.

Also, 8-(2-Cl-4-N_2O-PhCH_2S)-IMP has been found to be a time-dependent inactivator of IMPDH just as 6-Cl-IMP, EICARMP, 2-chloromethyl-IMP and 6-thio-IMP. Although its mechanism of inactivation has not been elucidated, it is suspected that it forms a covalent adduct with Cys-331 at the C-8 position on the purine ring,[41] lending reason to believe that IMPDH can attack the C-8-position as well as the C-2 and C-6 positions of the purine ring.

Further search of the literature for other C-6 substituted moieties that could potentially confer antiviral activity led to some C-6 alkenyl and C-6 alkynyl purine analogues.



Scheme 4.3. Time-Dependent Irreversible Inactivators of IMPDH.[36]

These compounds showed therapeutic potential against a host of plant and human enzymes as well as against human chronic myelogenous leukemia cell line K-562. Most of them displayed cytotoxicity comparable to or better than the known anticancer drug 6-mercaptopurine (which is also an IMPDH inhibitor as its ribonucleoside).[46-58] The 6-vinyl purines in this family of compounds show high cytotoxicity, and it has been proposed that the way they exert their activity is through the attack of nucleophilic species on their C-6 alkenyl moiety.[59] It is also reported that 6-vinylpurines have potential as cross-linking agents as they form adducts with guanosine and cytidine at the N-7 and N-4 positions respectively.[60-69] These C-6 alkenyl purine nucleosides have attracted considerable attention as potential anticancer compounds. Their mechanism of action (nucleophilic attack on their C-6 alkenyl moiety) is similar to how Cys-331 of IMPDH attacks compounds such as 2-vinylinosine and 6-Cl-IMP. Based on these similarities, it was worth investigating the effects of C-6 modifications (e.g., C-6-vinyl) in our class of novel IMPDH inhibitors.

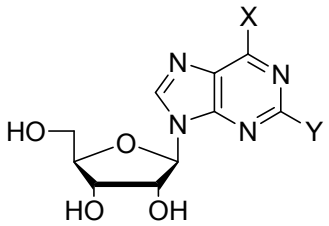
THIENYL AND FURYL MOIETIES

There is precedence in the literature of the strategic employment of the thienyl and furyl moieties in nucleoside analogues that exhibit anticancer, and antibacterial (particularly *M .tuberculosis*) chemotherapy.[46, 53, 54, 59, 70-74] Even though the mechanism of action of these compounds have not been ascertained, their activity and suspected mechanism of action have been compared with existing and known inhibitors of IMPDH such as 6-mercaptopurine and 3-deazaguanosine.[54, 59] It is well known that IMPDH inhibition has anticancer and antibacterial as well as antiviral chemotherapy; also IMPDH from *M. tuberculosis* shows remarkable conservation of amino acid sequences in the nucleoside analogue-binding site compared with the

human IMPDH type II. This is in agreement with the fact that the nucleoside analogue binding-site is well conserved among IMPDHs and even across all species. One implication or hypothesis that can be drawn from this conservation of amino acid sequence is that nucleoside analogues active against *M. tuberculosis* IMPDH may well be active against human IMPDH type II.

Work done by Gundersen *et al*. investigating the antimycobacterium activity of C-2 and C-6-arylpurines, found that C-6-(2-furyl), C-6-(2-thienyl), and to a certain extent C-6-phenyl-9-benzylpurines showed high antimycobacterium activity.[57] They discovered that 9-benzyl-2-chloro-6-(2-furyl)purine had the lowest minimum inhibitory concentration value of 0.78 μg/ml (comparable to the 0.8 μg/ml antiviral activity of our 6-chloro-2-ethynylpurine ribonucleoside **I**); it also exhibited relatively low cytotoxicity and was even active against several singly drug-resistant strains of M. tuberculosis.[57, 70, 75-77] This means that the 2-furyl and 2-thienyl moieties may be conferring antimycobacterium activity by IMPDH inhibition.This observation lends reason for the investigation of the thienyl and furyl moieties for antiviral activity by IMPDH inhibition. Since this work is based on placement of potential antiviral-conferring moieties at positions on purine nucleosides that are susceptible to attack by the sulfhydryl group of Cys-331 (i.e., C-2 and C-6 positions), the thienyl and furan moieties were strategically placed at these positions. It is our anticipation that our design of having these unsaturated moieties (thienyl and furyl) conjugated to the purine ring would result in Michael acceptors which would exert inhibition of IMPDH just as in the case of 2-vinylinosine.

The above discussion of structure and mechanism-based drug design relating to C-2 and C-6 modifications of the ribopurine system, led us to the rational design of our target compounds. Our target compounds can formally be represented as the general structures shown in Figure 4.2.



X = O, S, SCH_3, OCH_3, NH_2, $CH=CH_2$, thienyl, furan

Y = $CH=CH_2$, ethynylcyclohexen, thienyl, furan

Figure 4.2. Representation of Target Compounds.

MECHANISTIC JUSTIFICATION OF TARGET COMPOUNDS

6-Thiomethyl and 6-Methoxy-2-Vinyl-9-(β-D-Ribofuranosyl)Purine

As discussed earlier, modifications (e.g., fluorination) made directly on the vinyl group (conjugated to the purine ring) to make it more electrophilic resulted in less activity. Perhaps, modifications made elsewhere on the purine ring could make the vinyl group more reactive towards Cys-331. Generally, alkoxyl groups activate aromatic rings by resonance effect, whereas halogens deactivate aromatic rings by their stronger inductive effects. The effect of the latter has been investigated for antiviral activity (IMPDH inhibition) in C-2 modified compounds such 6-chloro-2-ethynylpurine ribonucleoside, 6-chloro-2-(3-fluoro-propynyl) purine riboside, and 6-chloro-2-(2-fluorovinyl) purine. In all of these cases, the antiviral activity was either decreased or the cytotoxicity of the compound reached undesirable limits as in the case of 6-chloro-2-ethynylpurine ribonucleoside.[19]

To investigate the effect of a C-2 modified activated purine ring system on antiviral activity, 6-methoxy and 6-thiomethyl-2-vinylpurine ribonucleoside were synthesized. Antiviral

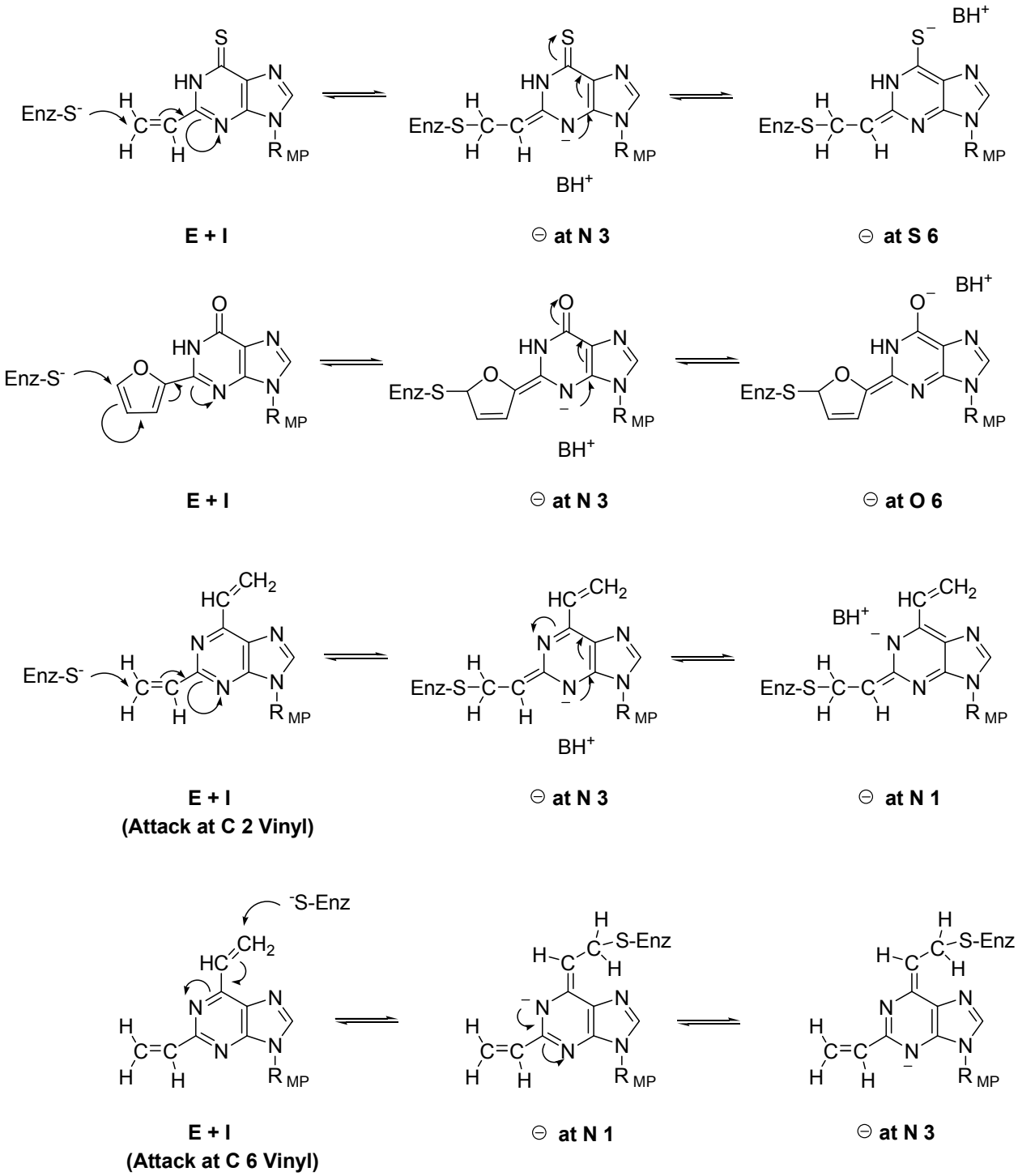
testing will provide comparative results and a lead into the possible mechanism of an activated ring system over an inactivated one.

6-Thio-2-Vinyl-9-(β-D-Ribofuranosyl)Hypoxanthine

For 6-thio-2-vinyl-9-(β-D-ribofuranosyl)hypoxanthine, the expected mechanism of IMPDH inhibition is analogous to that of 2-vinyl-9-(β-D-ribofuranosyl)hypoxanthine shown previously in Scheme 4.1. Here, the initial attack of Cys-331 sulfhyryl group on the vinyl group will result in negative charges at N-3 and/or C-6-S by resonance (Scheme 4.4). The difference between the 6-thio-2-vinyl-9-(β-D-ribofuranosyl)hypoxanthine and either IMP or 2-vinylinosine is the formation of a negative charge on C-6-S and not C-6-O. Similarly, the C-6-S negative charge is expected to be stabilized through hydrogen bonding or by a general acid (BH^+) in the active site. There is also a possibility that, rather than stabilization of C-6-S^- , it could enter into a disulfide bond with a nearby cysteine residue in IMPDH's active site.

2-(2-Furyl) and 2-(2-Thienyl)-9-(β-D-Ribofuranosyl)Hypoxanthine

The expected mechanism of action of 2-(2-furyl)-9-β-D-ribofuranosyl hypoxanthine and 2-(2-thienyl)-9-β-D-ribofuranosyl hypoxanthine will be identical since the only atom that is different (sulfur) between them is not expected to participate in the mechanism. Attack of Cys-331 sulfhyryl group on C-5 of the furan moiety will cause delocalization of electrons which will eventually result in the formation of negative charges at N-3 and/or C-6-O by resonance, just as described for the previous compounds (Scheme 4.4). These negative charges will be stabilized as described earlier.



Scheme 4.4. Mechanistic Justification of Target Compounds.

2,6-Divinyl, 2,6-Di-(2-furyl), and 2,6-Di-(2-Thienyl)-9-(β-D-ribofuranosyl)purine

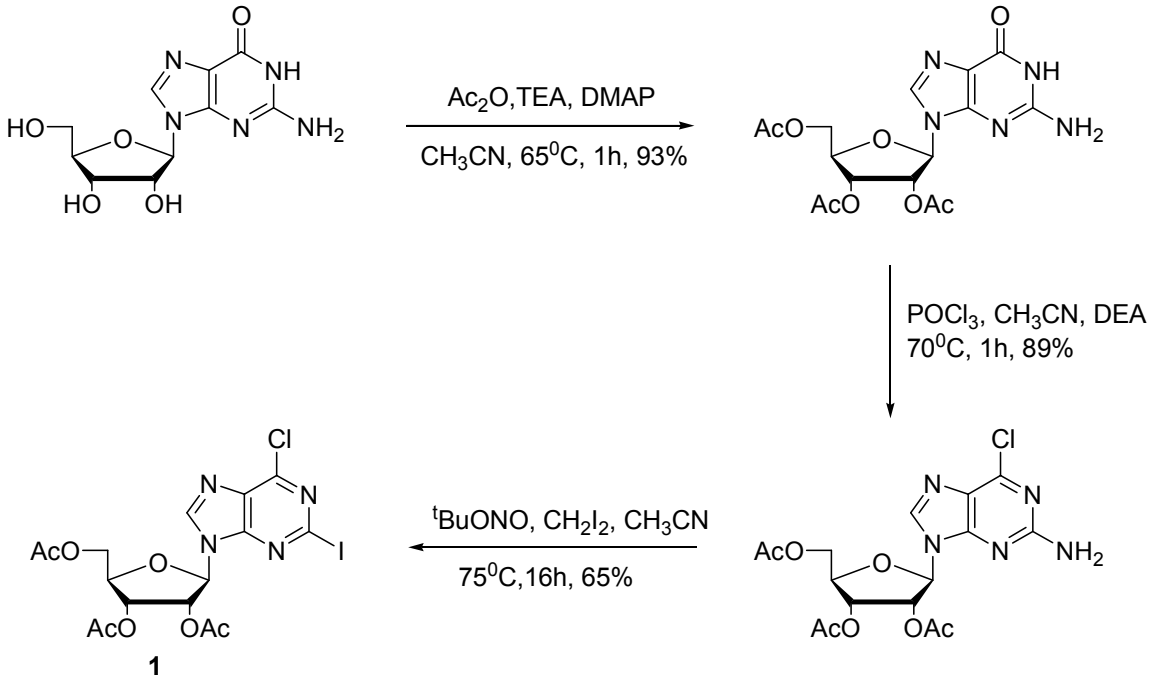
The three 2,6-disubstituted compounds 2,6-di-(2-furyl)-9-(β-D-ribofuranosyl)purine, 2,6-di-(2-thienyl)-9-(β-D-ribofuranosyl)purine, and 2,6-divinyl-9-(β-D-ribofuranosyl)purine are expected to have analogous mechanisms of action. Both the C-2 and C-6 positions of the purine ring are susceptible to attack by Cys-331 since this class of compounds present two similar moieties at these positions, that are susceptible to attack by Cys-331. These compounds will answer the unexplored question as to the regioselective preference of Cys-331 for the two positions.

Using 2,6-divinyl-9-(β-D-ribofuranosyl)purine as a model for this class, attack of Cys-331 sulfhyryl group on the C-2 vinyl group will cause delocalization of electrons which will eventually lead to the formation of negative charges at N-3 and/or N-1 by resonance (Scheme 4.4). The N-3 negative charge is not unusual in this case since it is similar to the case of IMP and 2-vinylinosine. The N-1 negative charge, on the other hand, has not been encountered in the proposed mechanism of IMPDH and needs justification. As discussed in chapter one, the calculated π-electron densities at the individual atom sites of the purine ring are 1.195 and 1.216 for N-1 and N-3, respectively. The difference of 0.021 is not expected to cause a significant change in the electronic and chemical properties between N-1 and N-3. It is reasonable therefore, that if N-3 can hold a negative charge which is stabilized by an amino acid residue or a general acid BH^+, then so can N-1, thus justifying the mechanism for this class of compounds.

The attack of Cys-331 sulfhyryl group could very well be on the C-6 vinyl group, in which case the same delocalization of electrons forms negative charges at N-1 and/or N-3 (Scheme 4.4).

SYNTHESIS OF KEY INTERMEDIATES

The key intermediates in the synthesis of our target compounds are 6-chloro-2-iodo-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl]purine **1** and 2-iodo-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl]hypoxanthine **13**.



Scheme 4.5. Synthesis of 6-Chloro-2-Iodo-9-[(2′,3′,5′-Tri-O-Acetyl)-β-D-ribofuranosyl] purine.

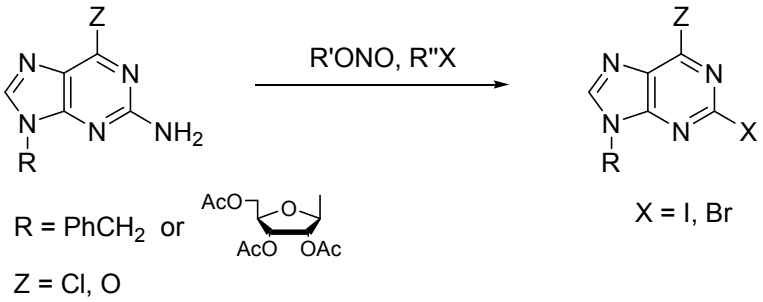
6-Chloro-2-iodo-9-[(2',3',5'-tri-O-acetyl)-β-D-ribofuranosyl]purine **1**

This intermediate was synthesized from commercially available guanosine using standard procedures previously described by Nair and coworkers (Scheme 4.5).[1, 14] The first 2 steps involved the selective acetylation of guanosine in 93% yield using acetic anhydride, triethylamine, and 4-(dimethylamino) pyridine in acetonitrile,[1, 78] followed by treatment of the

triacetylated guanosine with phosphorous oxychloride and N,N-diethylaniline at 70 ^{o}C for 1 hour to give the masked 6-Cl compound in about 89% yield.[1, 13, 79] Conversion of the 6-chloro-2-amino compound to 6-chloro-2-iodo ribofuranosylpurine involved a radical deamination-halogenation reaction using tert-butylnitrite, and diiodomethane in acetonitrile to afford the key intermediate, **1**, in 65% yield (Scheme 4.5).[13, 80] Compound **1** was characterized by its high-field ^{1}H and ^{13}C NMR spectra, which were consistent with data previously reported by us.[1, 14, 80]

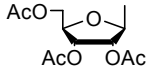
2-iodo-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl]hypoxanthine **13**

The key reaction in the synthesis of this intermediate was a radical deamination-halogenation reaction developed by Nair and coworkers.[81] This reaction has been used to convert 9-substituted 6-aminopurines to the corresponding 6-chloro, 6-bromo, and 6-iodopurines using dry CCl_4, $CHBr_3$, and CH_2I_2 respectively with the radical generator *n*-pentyl nitrite. The yields ranged from 21% - 72.7%, reaction times ranged from 2 - 23 hours at temperatures between 60 ^{o}C - 120 ^{o}C.[81] The highest yield of 72.7% was achieved with the reaction of 2′,3′,5′-tri-O-acetyladenosine with 20 equivalents of *n*-pentyl nitrite, 171.75 equivalents of bromoform at 80 ^{o}C for 3.5 hours to obtain 6-bromo-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl]purine. Later on, this reaction was employed by Nair and coworkers in the conversion of 2-amino-6-chloro-2′,3′,5′-triacetylribofuranosylpurine to the corresponding 2-chloro, 2-bromo and 2-iodo derivatives. The yields ranged from 55% - 83%, and reaction times spanned 1 to 20 hours at reflux temperatures (Scheme 4.6) (Table 4.2).[1, 13, 80-83] It should be noted that the conversions here have only been employed on 2-amino-6-chloropurine systems (i.e., a masked C-6 purine system). Co-reagents such as trimethylsilyl iodide in hexane,[13] CuI and I_2, and CuBr and Br_2 have been used as modifications of this procedure.[71]



Scheme 4.6. Modifications of Radical Deamination-Halogenation Reaction.

Table 4.2. Data for Radical Deamination-Halogenation of Some 2-Aminopurines.
Data Compiled From References: * = 71, ** = 84, † = 80, †† = 1.

R	Z	R′ONO	R″X	Co-Reagents	Temp	Time	Yield
$PhCH_2$	Cl*	*n*-pentylONO	CH_2I_2		85		35
$PhCH_2$	Cl*	*i*-pentylONO	CH_2I_2	CuI, I_2	Reflux	75min	67
$PhCH_2$	Cl*	*n*-pentylONO	$CHBr_3$		85		28
$PhCH_2$	Cl*	*i*-pentylONO	$CHBr_3$		85		34
$PhCH_2$	Cl*	*i*-pentylONO	$CHBr_3$	CuBr, Br_2	50	20h	45
	Cl†	*n*-pentylONO	$CHBr_3$		85	8h	55
	Cl†	*n*-pentylONO	CH_2I_2		85	1h	83
	Cl††	*n*-pentylONO	CH_2I_2	CH_3CN(solvent)	Reflux	20h	55
	O**	*n*-pentylONO	$CHBr_3$		90	30min	35

Trivedi *et al* have used a modification of Nair's procedure to directly brominate 2′,3′,5′-triacetylguanosine using bromoform and *n*-pentyl nitrite at 90 ^{o}C for 30 minutes to afford 2-bromo-2′,3′,5′-triacetylinosine in a 35% yield (Scheme 4.6) (Table 4.2).[84] It is important to point

out here that the radical deamination-halogenation reaction was carried out directly on a lactam unprotected purine system.

Yet another modification of the reaction employed in this dissertation used *t*-butylnitrite (substitute for *n*-pentylnitrite), diiodomethane, and acetonitrile (solvent) in a 16-hour reaction to give a 65% yield. It is clear from the table and the later modifications that this procedure gives variable results, thus requiring improvement, particularly with the direct halogenation of the lactam unprotected purine system since it serves our scheme of synthesis better.

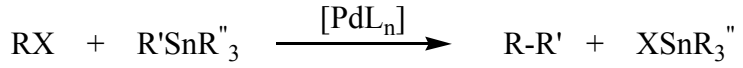
In most of our synthetic schemes the C-6 carbonyl functionality of 2′,3′,5′-tri-O-acetylguanosine is protected or masked with either chloro or a methoxy group to allow for further elaboration of the base moiety. Eventually the protected C-6 position (i.e., 6-Cl or 6-OCH_3) is unmasked either chemically using the low yielding CH_3SiI plus CH_3CN method or the expensive and lengthy chemoenzymatic reaction using adenosine deaminase (ADA) to revert to the desired C-6 carbonyl functionality. It became necessary to develop a high-yielding, fast, and less expensive methodology that would circumvent the C-6 masking step as well as the use of ADA, since in the event that commercial scale synthesis of these compounds is required, the use of ADA (expensive, variable results and time-consuming) may not be the route of choice. The best approach would be a direct iodination of 2′,3′,5′-tri-O-acetylguanosine using an improved procedure for the radical deamination-halogenation reaction. Such a procedure would shorten the synthesis and significantly improve the overall yield, as well as provide the best system (C-2-iodo) for the subsequent Stille coupling reactions.

In the first trial of such an approach, we treated 2′,3′,5′-triacetyl guanosine directly with CH_2I_2, and *n*-pentyl nitrite using acetonitrile as a solvent as detailed in the Nair reaction.[1] This reaction was carried out for different reaction times, different temperature ranges (50 ^oC - 90 ^oC),

varying equivalents of CH_2I_2 and *n*-pentyl nitrite, and even substitution of *i*-pentyl nitrite and *t*-butyl nitrite for *n*-pentyl nitrite. This series of reactions resulted in an inseparable multi-product mixture, including unreacted starting material. Since CH_2I_2 did not work well as the source for halogen abstraction by the purin-2-yl radical that is formed here,[80] other sources such as tetraiodomethane (CI_4) and iodoform (CHI_3) were explored. Repeating the above procedure using CI_4 in place of CH_2I_2 was even worse due to the insolubility of CI_4 under the reaction conditions. Variations in reactions conditions, just as above, but using iodoform as the source of the halogen proved to be a better route. The best result was obtained when 2′,3′,5′-triacetylguanosine (in acetonitrile) was treated with 4 equivalents of iodoform, and 10 equivalents of *i*-pentyl nitrite at 90˚C for 1 hour; the desired product 2-iodo-2′,3′,5′-triacetylinosine, **13**, was produced in 50% yield as the sole product. This methodology represents a new reproducible and efficient use of the radical deamination-halogenation reaction for the synthesis of the key intermediate 2-iodo-2′,3′,5′-triacetylinosine.

STILLE COUPLING REACTION

Another very important reaction used in this work is the Stille coupling reaction, which involves the palladium-catalyzed cross-coupling of organotin reagents with organic halides (Scheme 4.7).[14, 85] This reaction is one of the best synthetic methodologies available for the formation of carbon-carbon bonds;[14, 86-90] it is high yielding, mild, versatile, regioselective and stereospecific, as well as tolerant to a wide variety of functional groups,[1] thus making it appropriate for nucleosides. The order of reactivity of the organic halides is generally found to be R - I > R - Br >> R - Cl.[80, 91] The choices of R′ (the desired synthon to be transferred) are very broad and depends on what target compounds are to be synthesized.



X = Cl, Br, I

R = Alkyl, Alkenyl, Alkynyl, Aryl, Benzyl, Allyl, etc.

R' = Alkyl, Alkenyl, Alkynyl, Aryl, Benzyl, Allyl, etc.

R" = Alkyl (Methyl or Butyl)

Scheme 4.7. The Stille Cross Coupling Reaction.

For our target compounds, our choices of organic halides (RX) were the key intermediates, 2-iodo-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl]hypoxanthine **13** and 6-Chloro-2-iodo-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl]purine **1**. The organotin reagents were required to have transferable synthons (R′) (i.e., vinyl, furyl and thienyl). R″ was the butyl group. Either $Pd(PPh_3)_4$ or $Pd(CH_3CN)_2Cl_2$ was chosen for the catalyst.

To synthesize of our novel targets, we used this crucial Stille reaction for the carbon-carbon bond formation between our key intermediates and organotin reagents containing our desired synthons. The desired synthons are transferred onto the organic halides to complete the coupling reaction. A plausible mechanism for this transfer involves the initial oxidative insertion of palladium into the carbon-halogen bond at C-2 and/or C-6 of the key intermediates. This is followed by cross-coupling of the derived Pd(II) complex with the transferable desired synthon, then *trans-cis* isomerization, and finally reductive elimination (*via* the *cis*-intermediate) to give the desired product, with concomitant regeneration of the Pd(O) catalyst to initiate another reaction cycle (Figure 4.3). [14, 80, 92]

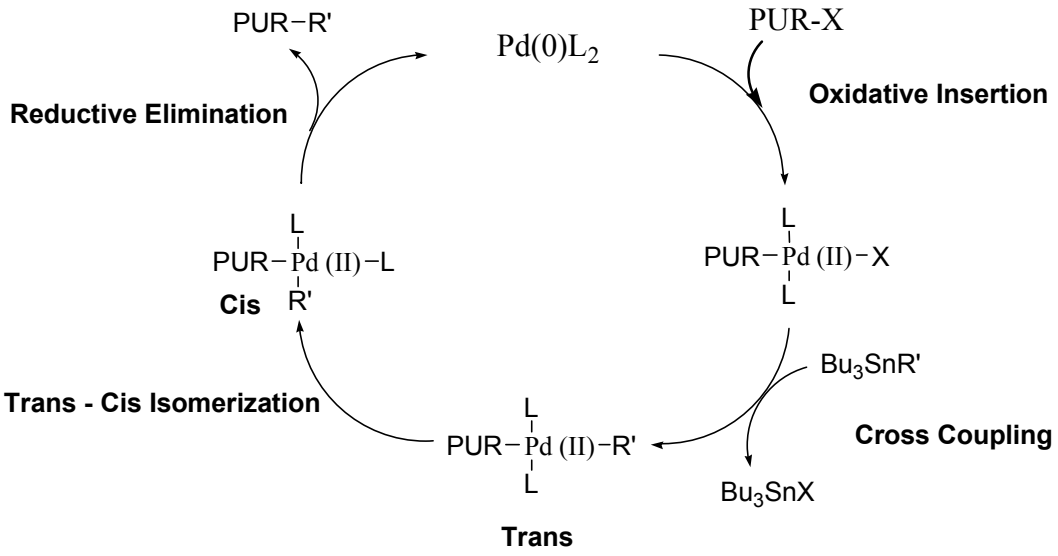


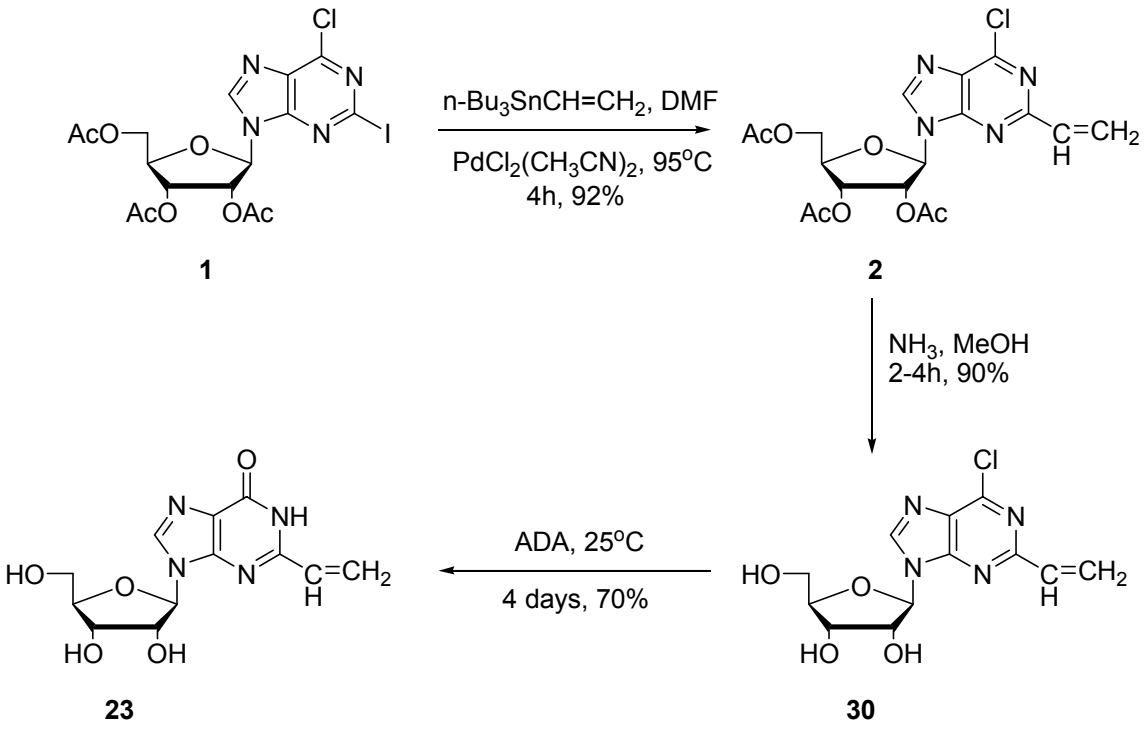
Figure 4.3. Mechanism of Stille Coupling Reaction.

For our key intermediate 2-iodo-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl] hypoxanthine **13**, this mechanism occurs at C-2-I, whereas for the intermediate, 6-chloro-2-iodo-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl]purine, **1**, it occurs at both C-2-I and C-6-Cl. There is a difference between the ease of coupling for C-I and C-Cl of **1**. This is expected because of the differences in bond energies between C-I (220 kJ/mol) and C-Cl (330 kJ/mol).[80, 93] This difference is discussed in detail later.

PREVIOUS SYNTHESIS OF 2-VINYLINOSINE

2-Vinylinosine has been synthesized in two major ways in our group; a chemoenzymatic method where one of the key reactions is the hydrolytic dechlorination of a C-6-chloropurine nucleoside (using ADA), and a non-enzymatic (chemical) synthesis. In the chemoenzymatic method (Scheme 4.8), 6-chloro-2-vinyl-9-(β-D-ribofuranosyl)purine **30** was synthesized from

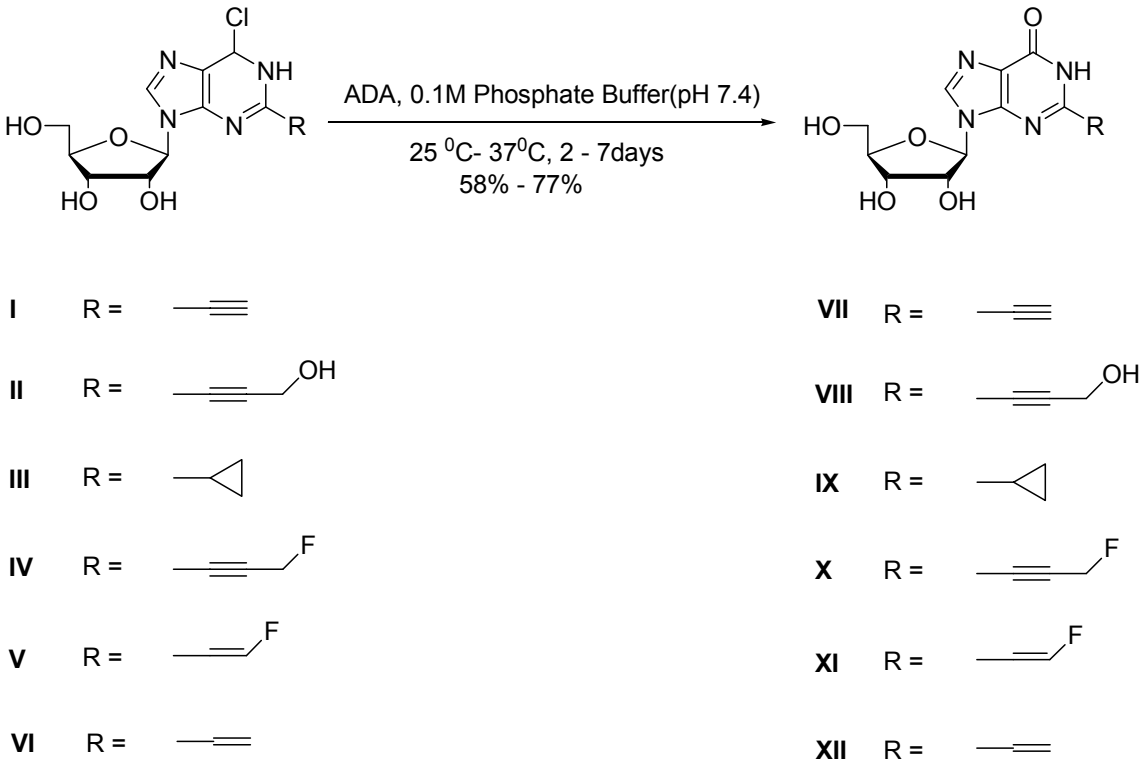
intermediate **1** by cross-coupling with $Bu_3Sn(CH=CH_2)$ and $PdCl_2(CH_3CN)_2$ in DMF, followed by careful deprotection of the acetate groups with methanolic ammonia.[1, 29]



Scheme 4.8. Chemoenzymatic Synthesis of 2-Vinylinosine.

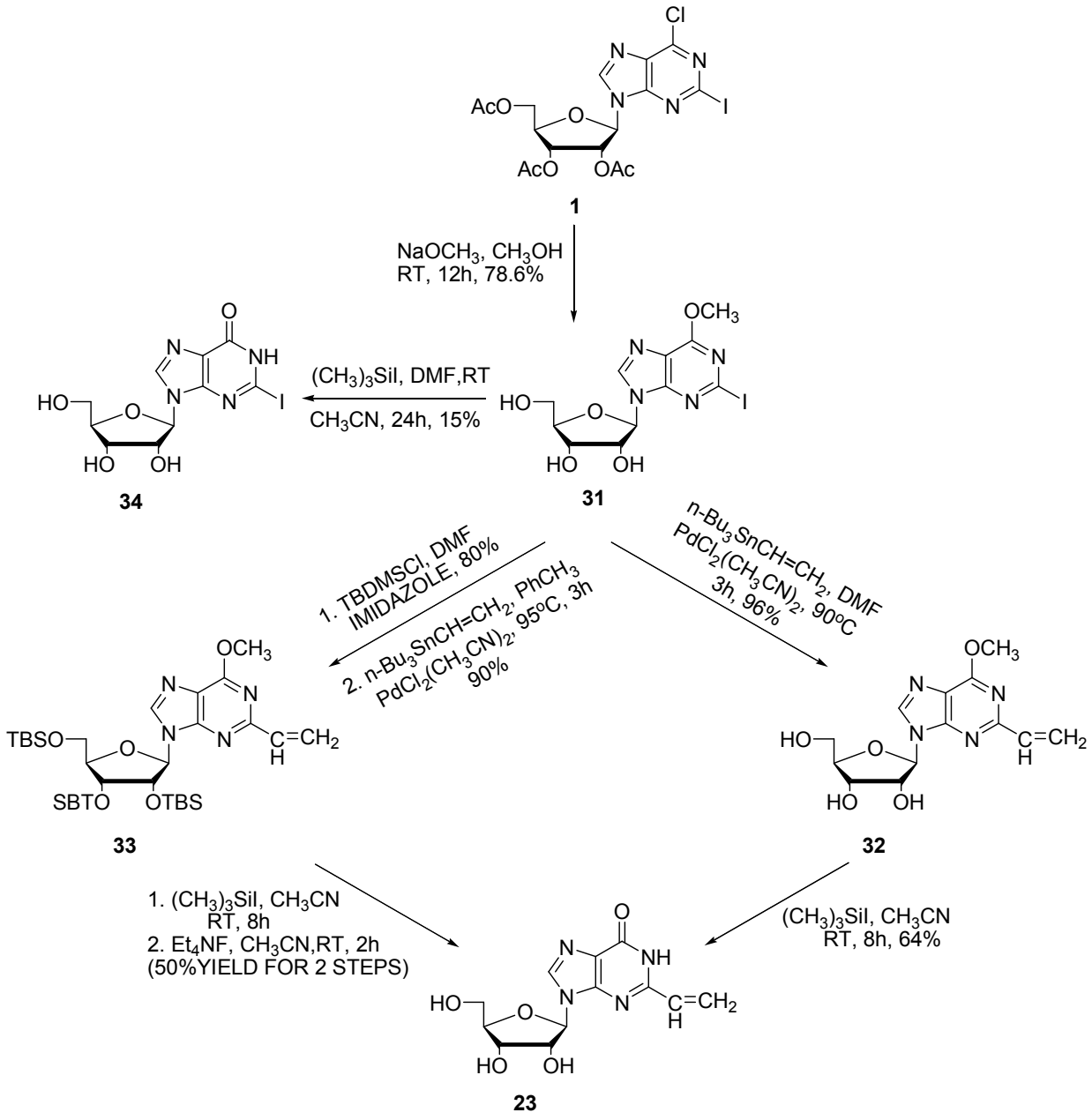
Hydrolytic dechlorination of **30** using ADA afforded 2-vinylinosine **23** in 70% yield.[19] Calculations for this chemoenzymatic method indicates that using 1 gram of guanosine as starting material yields 324 mg of the final compound, 2-vinylinosine. This translates to an overall yield of 31%, for this chemoenzymatic method. The use of ADA has proved to be efficient and mild where other deprotection methods such as 2N NaOH in dioxane,[94] and thioethanol and sodium methoxide in methanol have failed.[95] Physiologically, ADA catalyzes the hydrolytic deamination of adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine,

respectively.[96, 97] Consequently, ADA has been frequently used in our group as well as in the literature.[39] Scheme 4.9 shows an extensive use of ADA in our group for the synthesis of target compounds. The data shows that the chemoenzymatic conversion requires temperatures between 25 ˚C to 37 ˚C, reaction times ranging from 2 - 7 days, and gives yields ranging from 58% to 77%.[19, 29] Robins *et al* have also used ADA for the hydrolytic deamination of adenosine 1-N-oxide to 1-hydroxyinosine and this conversion took 3 days and gave 88% yield.[98] As evident from the examples above, the use of ADA although efficient and mild is rather lengthy and expensive, and as mentioned earlier may not be the route of choice for commercial scale synthesis. This necessitates a reproducible, efficient, and economic method for the synthesis of 2-vinylinosine (a patented antiviral compound).[99]



Scheme 4.9. Hydrolytic Dechlorination of Some Nucleosides Using ADA.

In the non-enzymatic method (Scheme 4.10), nucleophilic displacement of C-6-chloro in the key intermediate **1** with methoxide ion also deprotected the acetate groups to afford the branch point compound, 6-methoxy-2-iodo ribonucleoside **31**, in 78.6% yield.



Scheme 4.10. Non-Enzymatic Synthesis of 2-Vinylinosine.

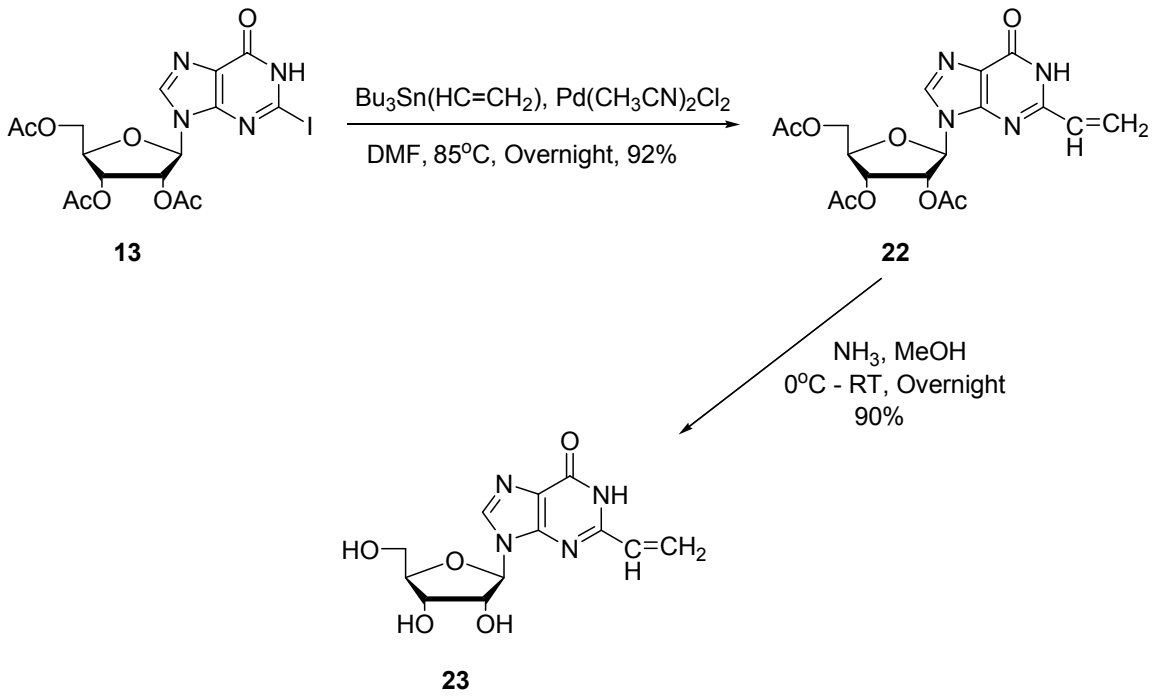
Reprotection of **31** with TBDMSCl, followed by C-2 vinyllation using Stille coupling reaction gave the vinyl compound **33**, which upon a double deprotection of C-6-methoxy and OTBDMS using CH_3SiI/CH_3CN and Et_4NF, respectively, afforded 2-vinylinosine **23**.[1] In a shorter and better route, **31** was directly vinylated to give **32**, which upon deprotection also gave 2-vinylinosine **23**.[1]

With 1 gram of guanosine as the starting material, the latter route yields 270 mg of final compound **23** (26% overall yield), involves 6 steps, and a total reaction time of 41 hours. Interestingly, deprotection of **31** gave 2-iodoinosine **34** in 15% yield (Scheme 4.10). For comparison, it is worthy to mention that work done in this dissertation has synthesized 2-iodo-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl]hypoxanthine **13** (the sugar-protected form of 2-iodoinosine) from triacetylated guanosine (an intermediate three steps prior to **31**) in 50% yield.

NEWLY DEVELOPED NON-ENZYMATIC SYNTHESIS OF 2-VINYLINOSINE

Our newly-developed methodology synthesized 2-vinylinosine **23** in 2 steps starting from the key intermediate **13** (Scheme 4.11). C-2-vinylation of **13** using $n\text{-}Bu_3SnCH{=}CH_2$ and $Pd(CH_3CN)_2Cl_2$ gave the vinyl compound **22** in 92% yield; subsequent deprotection with methanolic ammonia gave the target **23** in 90% yield. Starting from 1gram guanosine, this 4-step 18-hour synthesis yields 400 mg of final compound. This represents an overall yield of 39%, compared with the 26% overall yield for the previous 6-step 41-hour non-enzymatic method and the 31% overall yield for the 6-step 120-hour previous chemoenzymatic method. Thus for a 1 kg scale synthesis of 2-vinylinosine **23**, the newly developed method, which is shorter and inexpensive, will have an excess of 76 and 130 grams more than the previous methods,

respectively (assuming no unpredicted problems in the scale up). This represents a significant improvement over the previous methods.



Scheme 4.11. Newly-Developed Non-Enzymatic Synthesis of 2-Vinylinosine.

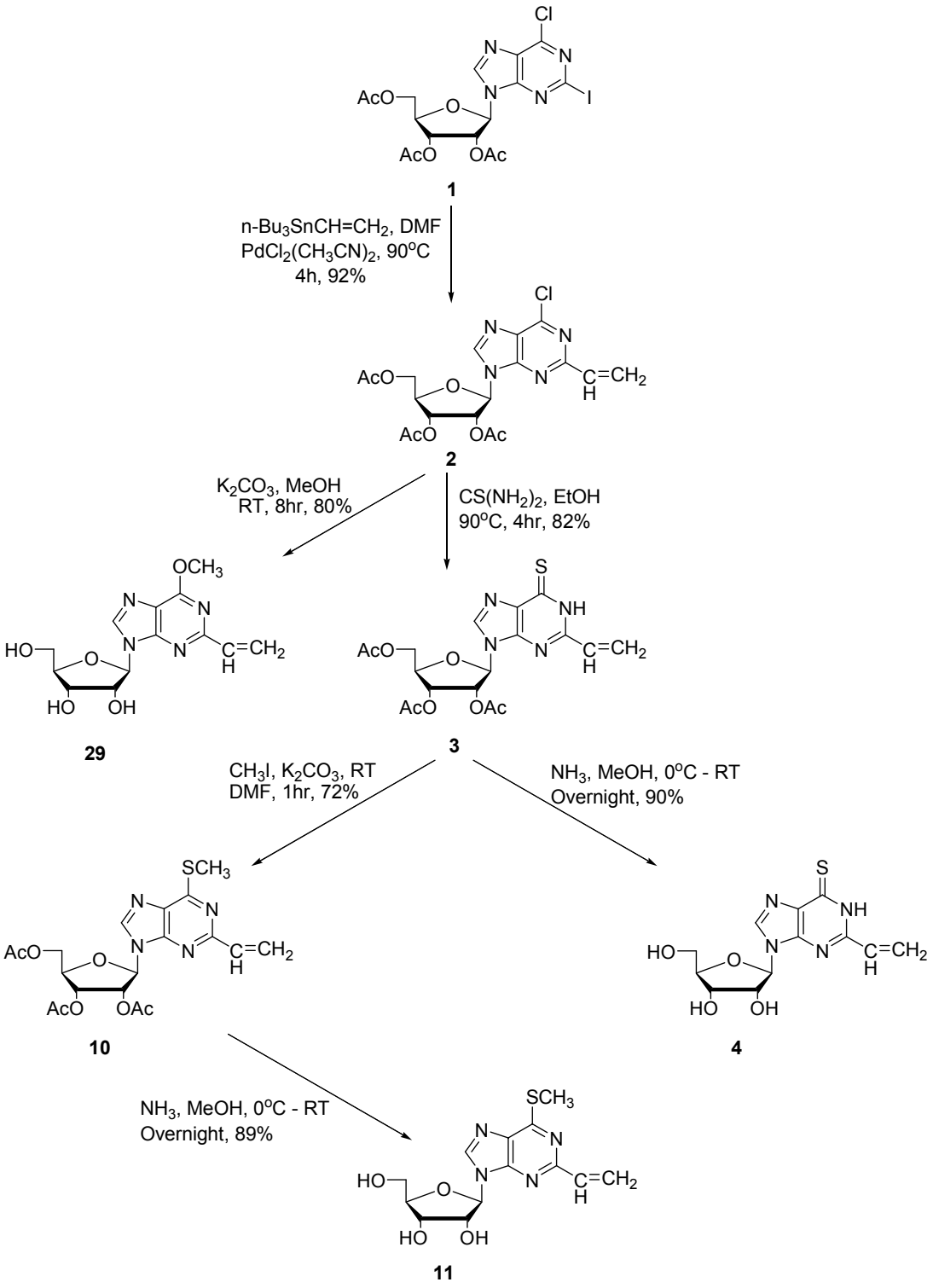
DISCUSSION OF THE SYNTHESIS OF OTHER TARGET COMPOUNDS

For identification purposes, target compounds (new molecular entities) have been fully characterized by ^{1}H and ^{13}C NMR, UV and HRMS (FAB). On the other hand, precursors (known or novel) were identified by differences between the ^{1}H and/or ^{13}C NMR of the starting material and product of each individual reaction. Since our target compounds and precursors involve C-2 functionalized hypoxanthine (and 6-thio) systems as well as C-2, C-6 functionalized ribofuranosyl systems, the literature-reported ^{13}C NMR spectra of inosine, 6-thioinosine, guanosine, 6-thioguanosine and 6-Cl nebularine will be used for comparison and validation.[100]

^{1}H NMR data (among other uses) were used to justify the absence and/or presence of pertinent ^{1}Hs.

Generally, the ^{13}C NMR spectra of normal and deoxy nucleosides and nucleotides show peaks between 40 and 100 ppm (pyranoses and furanose) and between 90 and 170 ppm (heterocyclic base).[101] In our case, the values were consistently 60 – 90 ppm (ribofuranose) and 115 – 165 ppm (purine base). The peaks are in the order C-5′ < C-3′ < C-2′ < C-4′ < C-1′ and C-5 < C-8 < C-4 < C-2 < C-6, however, certain C-2 and C-6 modifications are known to reverse the order of C-2 and C-6,[65] as well as cause significant changes (5 – 20 ppm) to the position of C-2, C-4, C-5 and C-6.[65] These changes have been used to unequivocally identify new and shifted ^{13}C peaks after a reaction.

6-thio-2-vinyl-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl]purine **3** was a branch point compound for the synthesis of two target compounds **4** and **11** (Scheme 4.12). Compound **3** was synthesized by C-2-vinylation of the key intermediate, 6-chloro-2-iodo-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl]purine **1**, by a cross-coupling reaction using n-$Bu_3SnCH{=}CH_2$ and $Pd(CH_3CN)_2Cl_2$ in DMF, followed by conversion of the C-6-chloro to the thiocarbonyl using thiourea in ethanol. ^{1}H NMR ($CDCl_3$) of **3** showed a broad singlet at 11.41 ppm, which is indicative of the N-1 proton of the thio-lactam. This proton is absent from the ^{1}H NMR ($CDCl_3$) of the 6-Cl precursor, **2**.[19] Deprotection of **3** using saturated methanolic ammonia in an overnight reaction yielded the target compound 6-thio-2-vinyl-9-(β-D-ribofuranosyl)hypoxanthine **4**, in 90% yield. The ^{1}H NMR of **4** (Figure 4.4) confirms the N-1 proton of the thio-lactam, whereas the ^{13}C NMR (Figure 4.5) shows all pertinent peaks, particularly, the C-6 thiocarbonyl peak at 178.2 ppm (comparable to that of 6-thioguanosine and 6-thioinosine). Also, HRMS (FAB) calculated for **4** ($C_{12}H_{15}N_4O_4S$ $[M + H]^+$) is 311.0814, 311.0810 was found.



Scheme 4.12. Synthesis of Some Target Compounds.

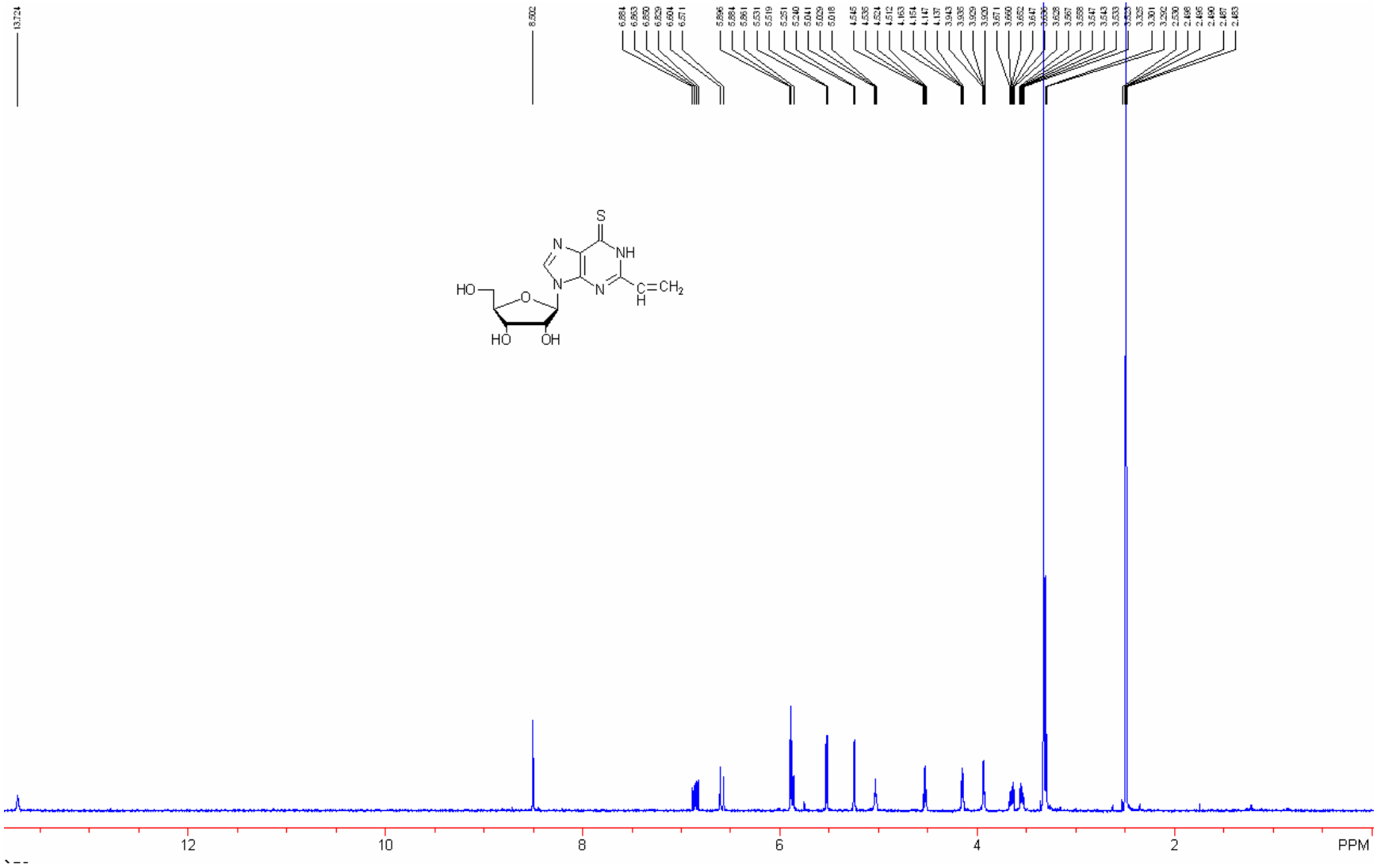


Figure 4.4. 500 MHz ^{1}H NMR of 6-thio-2-vinyl-9-(β-D-ribofuranosyl)hypoxanthine.

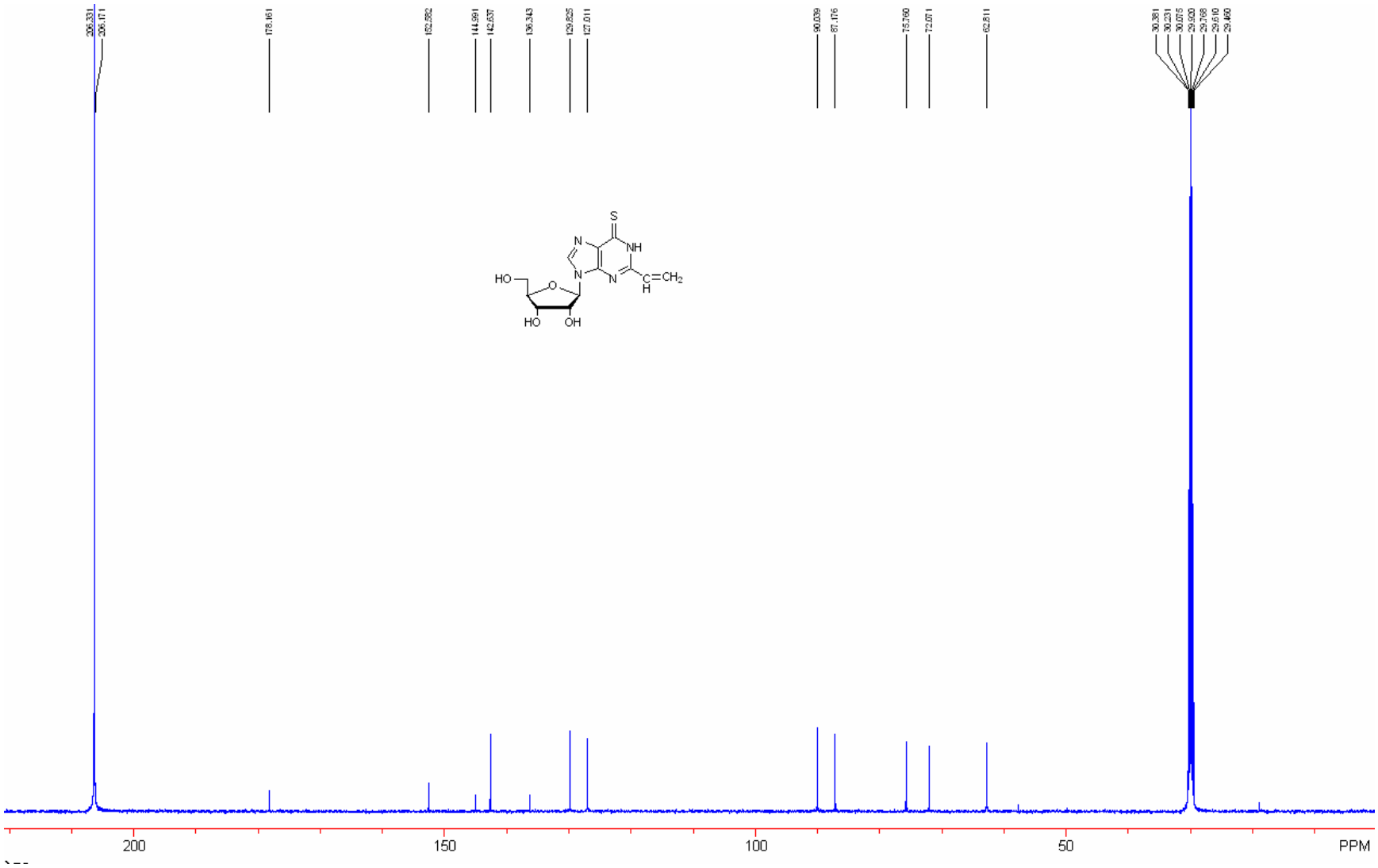
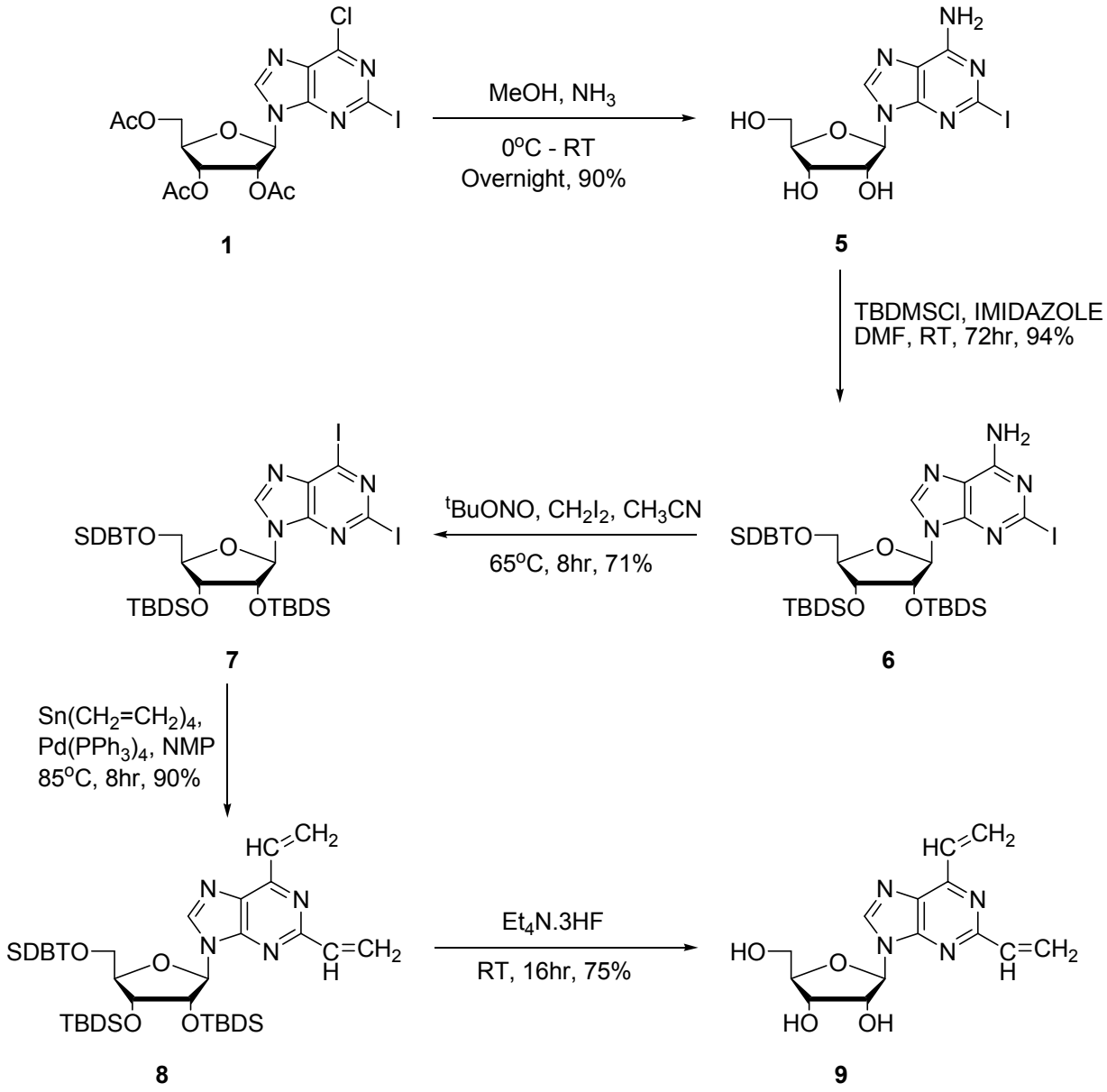


Figure 4.5. 125 MHz ^{13}C NMR of 6-thio-2-vinyl-9-(β-D-ribofuranosyl)hypoxanthine.

Methylation of **3** using iodomethane and potassium carbonate, yielded the thiomethyl compound **10,** which after a similar deprotection, afforded the second target compound, 6 thiomethyl-2-vinyl-9-β-D-ribofuranosyl purine **11,** in 89% yield. The peaks δ (s, 2.71, 3H) and δ (11.6, S-CH_3) in the respective ^{1}H and ^{13}C NMR of **11** indicates the presence of a thiomethyl group. Also, the disappearance of the ^{13}C peak at 178.2, ascertains methylation of the thiocarbonyl functionality. Other characterizations (UV and HRMS) are given in the experimental section.

Scheme 4.13 represents the successful methodology of the two that were tried in the synthesis of 2,6-divinyl-9-(β-D-ribofuranosyl)purine, **9**. The presence of the electron-withdrawing group (iodine) at the C-2 position of **1** makes the C-6-position very susceptible to nucleophilic displacement.[1] Thus nucleophilic displacement of 6-Cl with ammonia occurred with ease with concomitant deprotection of the acetyl groups to give the 6-amino-2-iodo ribonucleoside **5** in 90% yield. It must be noted here that for this reaction, the absence of 2-iodo prevents nucleophilic displacement of 6-Cl, and only deprotection of the sugar moiety is effected. This was realized when this same reaction was run on the 6-Cl-2-NH_2 derivative of **1** and the sole product was the 6-Cl-2-NH_2 ribonucleoside. Protecting the carbohydrate moiety of **5** with TBDMSCl gave compound **6** in 94% yield. The silyl protecting groups are more synthetically appropriate, solubility-wise, in this case as will be explained later. The 6-NH_2 in **6** was converted to 6-I using *t*-butylnitrite and diiodomethane to give the diiodo compound **7**. Several attempts at a one-pot divinylation reaction of **7** using $Bu_3SnCH{=}CH_2$, $Pd(CH_3CN)_2Cl_2$ in DMF at 85˚C - 90˚C, only resulted in monovinylation,[73, 74, 102, 103] even when excess $Bu_3SnCH{=}CH_2$ was used with prolonged reaction times. Changing the organotin reagent, palladium catalyst and solvent to the more reactive $Sn(CH{=}CH_2)_4$, $Pd(PPh_3)_4$ and N-

methylpyrolidinone, respectively effected the one-pot divinylation to yield the new divinyl compound **8** in 90% yield. Switching of the protecting groups from acetyl to silyl was because the acetyl-protected derivative of **8** was very insoluble and could not be purified for complete characterization.



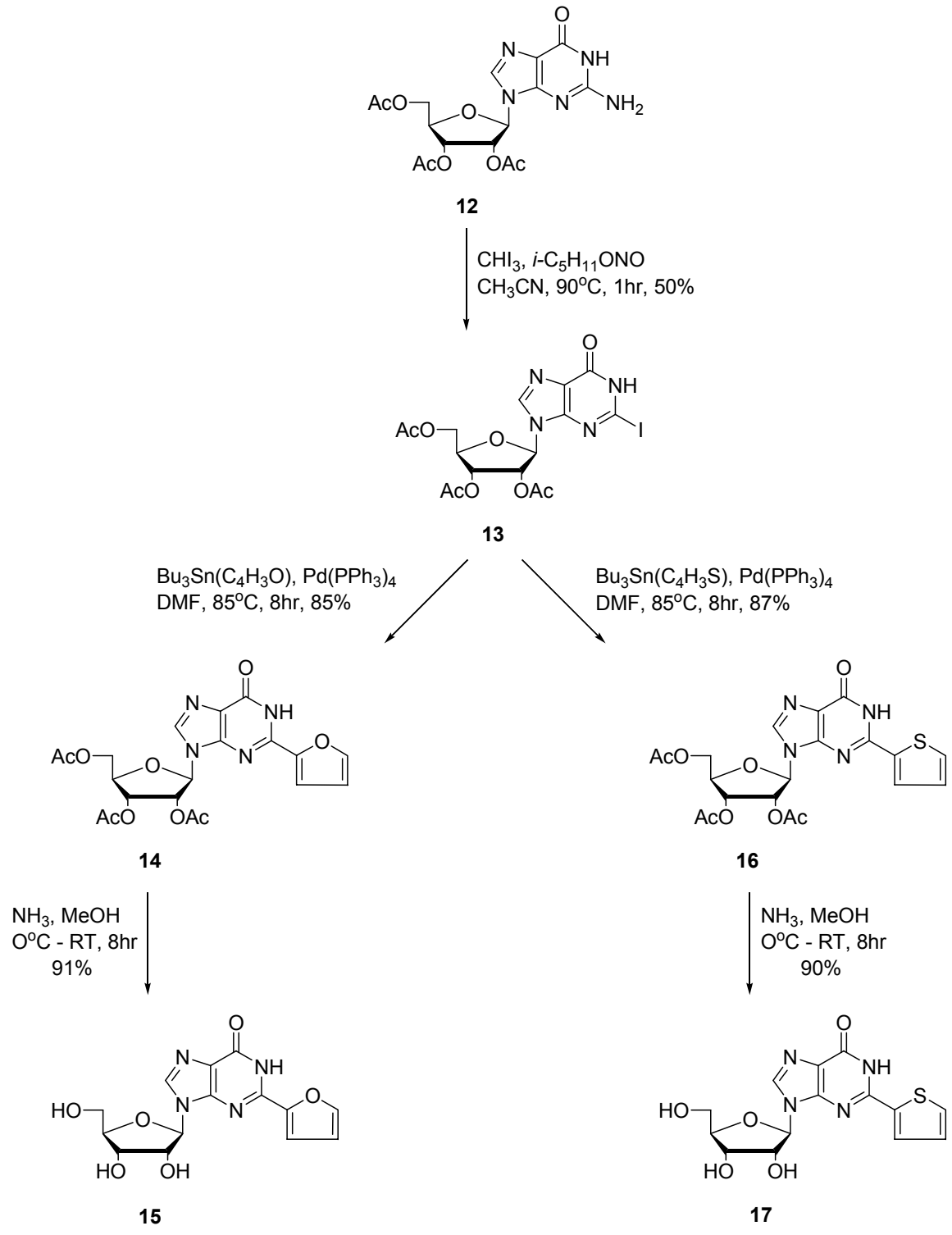
Scheme 4.13. Synthesis of 2,6-Divinyl-9-(β-D-ribofuranosyl)purine

Desilylation of **8** using the conventional sources of fluoride ions (TBAF, NH_4F, Et_4NF),[104-108] resulted in inseparable impurities in the target compound[109-113] after column chromatography and even after HPLC purification. Other unconventional means such as CH_3OH/CCl_4,[114] $Pd(CH_3CN)_2Cl_2$ in DMF/Acetone/H_2O,[115] KF/MeOH,[57] and, even the modification, KF/MeOH/18-crown-6, could not remove the impurities. Using the commercially available triethylamine trihydrofluoride, deemed the best for nucleosides/nucleotides,[116, 117] in a 16-hour room temperature reaction gave pure compound **9** in 75% yield after column chromatography. ^{13}C NMR of **9** (Figure 4.7) shows the vinylic carbons at C-6 (126.6 and 132.2 ppm) and C-2 (122.7 and 136.6 ppm) which are consistent with this class of compounds.[65] The presence of the C-6 vinyl group causes a deshielding effect on C-5, thus causing its signal to down-shift from the regular 115 – 118 ppm to 129.9 ppm. Also, the ^1H NMR (Figure 4.6) shows six protons in the range 5.5 – 7.5 ppm, consistent with vinylic protons. Furthermore, HRMS (FAB) calculated for **9** ($C_{14}H_{17}N_4O_4$ $[M + H]^+$) is 305.1250, 305.1239 was found.

Employing our newly developed method, triacetylated guanosine was directly halogenated to the key intermediate, 2-iodo-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl] hypoxanthine **13,** in a reproducible 50% yield. Direct cross-coupling of **13** with tributylfurantin and tributylthiophenetin gave 2-(2-furyl)-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl] hypoxanthine, **14**, and 2-(2-thienyl)-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl]hypoxanthine, **16**, respectively, which, upon careful deprotection, afforded the target compounds **15** and **17** (Scheme 4.14). Compounds **15** and **17** being almost identical showed similar features in their ^1H and ^{13}C NMR. For example, ^1H and ^{13}C NMR of **17** (Figures 4.8 and 4.9) showed the N-1 proton (δ 12.67) as well as the C-6 peak (δ 157.4) characteristic of the lactam nucleosides, inosine and guanosine.[100] UV data comparison of **15** (UV (MeOH), 314nm (ε 10,063), 260nm (ε 7022),

205nm (ε 8726) and **17** (UV (MeOH), 323nm (ε 14,029), 259nm (ε 11,838), 202nm (ε 19,712) with guanosine (UV (H_2O), 252 nm (ε 13,700), 188 nm (ε 26,800)[100] and 2-vinylinosine (UV (H_2O), 292 nm (ε 6486), 260 nm (ε 7850), 207 nm (ε 19,891)[1], indicates the presence of more conjugation in **15** and **17** since they absorbed at lower energies (higher wavelength). This difference is attributed to the furan and thiophene moieties respectively. Furthermore, HRMS of **15** (calcd for $C_{14}H_{15}N_4O_6$ $[M + H]^+$ 335.0992, found 335.0998) and **17** (calcd for $C_{14}H_{15}N_4O_5S$ $[M + H]^+$ 351.0763, found 351.0758) unequivocally validated the two compounds.

It should be pointed out here that if **15** and **17** had been synthesized using previous literature methods starting from guanosine, it would have required: guanosine protection (1 hour, 93% yield), C-6-chlorination (1 hour, 89% yield), C-2-iodonation (16 hours, 65% yield), Stille cross- coupling (8 hours, 85% - 90% yield), triacetyl deproctection (2 - 4 hours, 90% yield), and finally hydrolytic dechlorination using ADA (2 - 7 day, 58% - 77% yield). Thus, at least 76 hours of reaction time with yields ranging from 58% to 93% would be required. The limiting step in this procedure is the chemoenzymatic step, taking 2 -7 days for completion and using rather expensive reagents. On the contrary, this new method required: guanosine protection (1 hour, 93% yield), direct C-2-iodonation (1 hour, 50% yield), Stille cross-coupling (8 hours, 85% - 90% yield), and finally triacetyl deproctection (8 hours, 90% yield). Thus requiring 18 hours reaction time with yields ranging from 50% to 93%. The strongest step in this procedure is the C-2 iodonation using rather inexpensive reagents such as iodoform and *i*-pentyl nitrite. This procedure cuts down the number of steps from 6 to 4 and avoids the limiting step in the previous methods.



Scheme 4.14. Synthesis of 2-(2-Furyl) and 2-(2-Thienyl)-9-(β-D-ribofuranosyl)hypoxanthine.

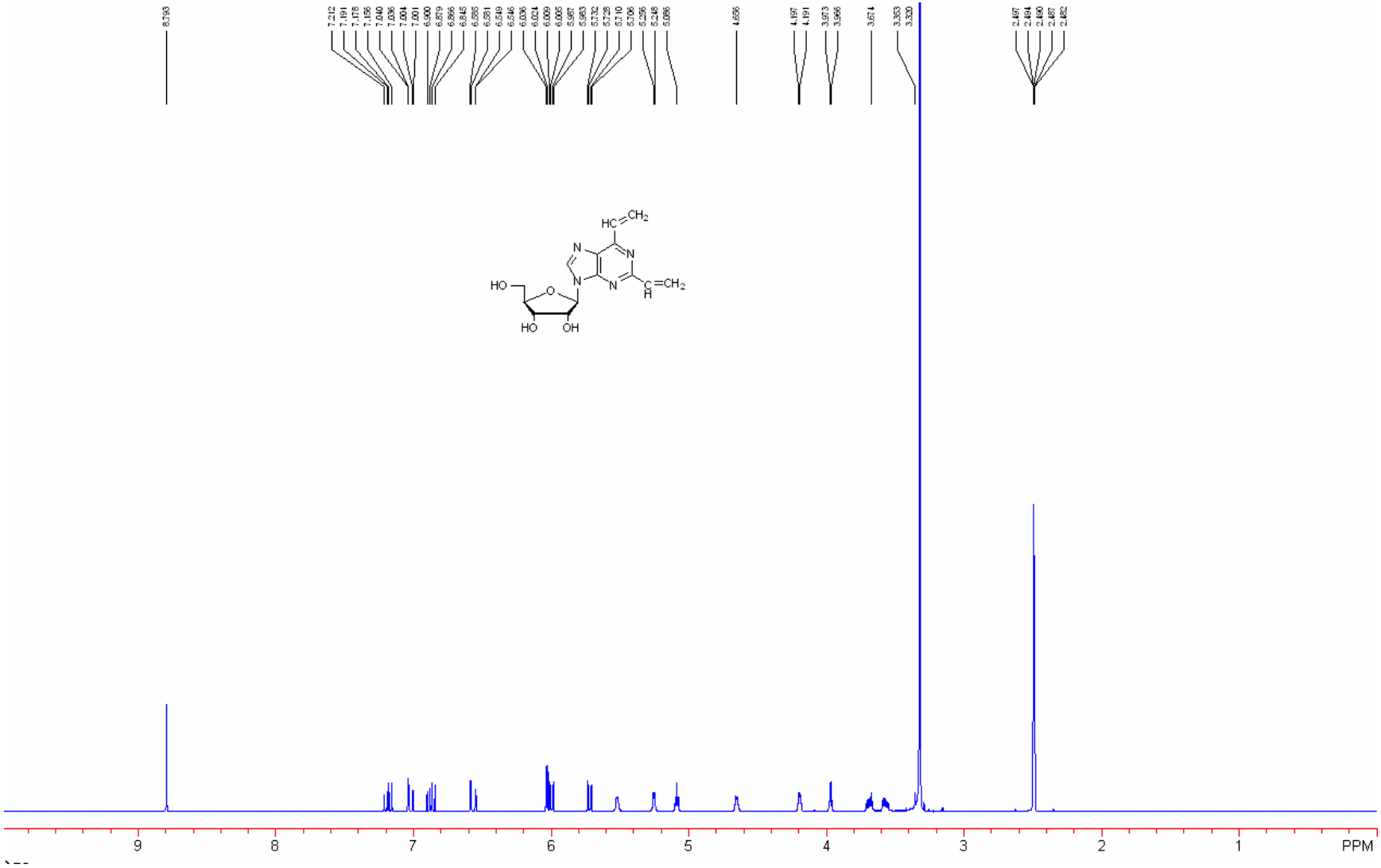


Figure 4.6. 500 MHz ^{1}H NMR of 2,6-divinyl-9-(β-D-ribofuranosyl)purine.

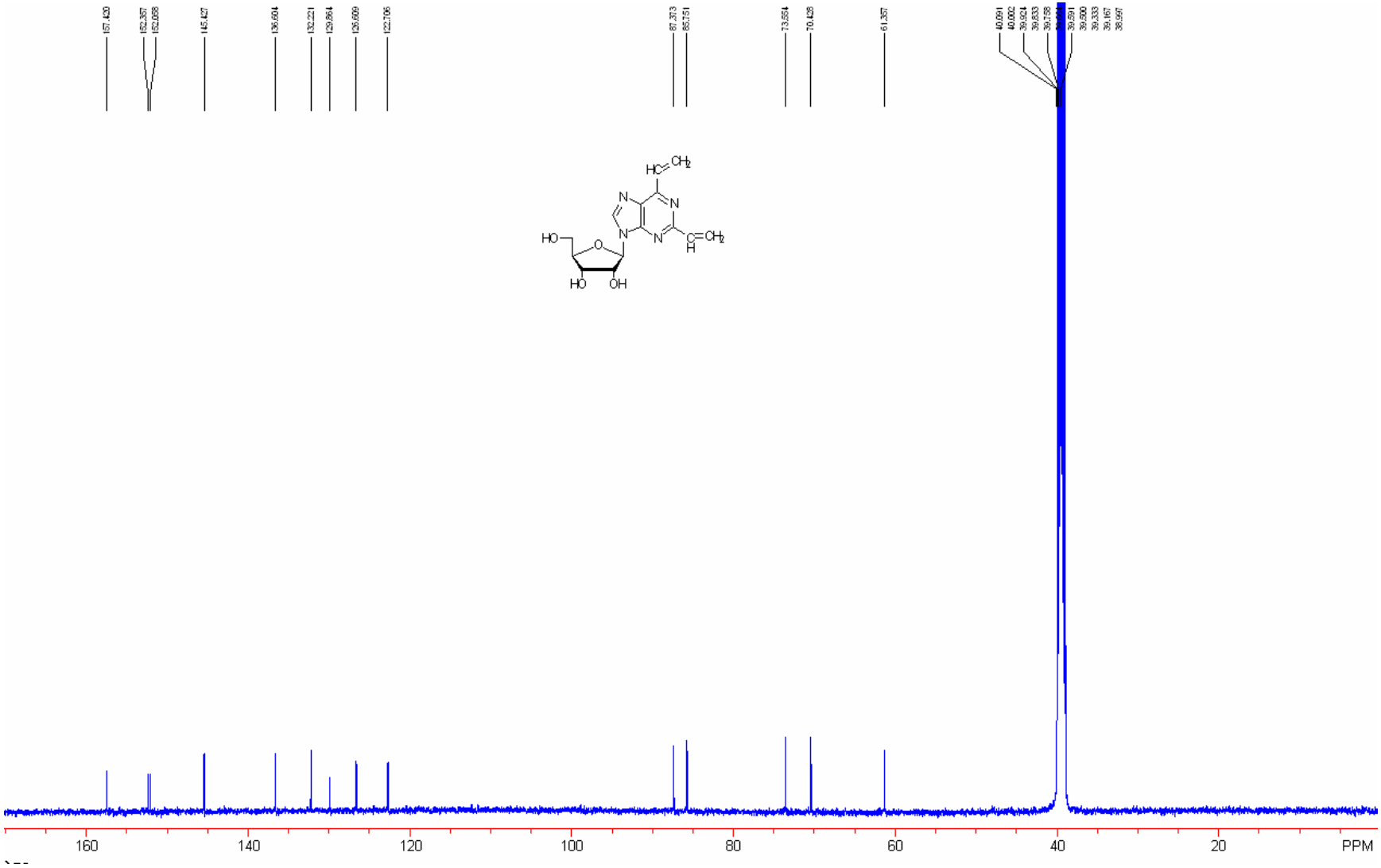


Figure 4.7. 125 MHz ^{13}C NMR of 2,6-divinyl-9-(β-D-ribofuranosyl)purine.

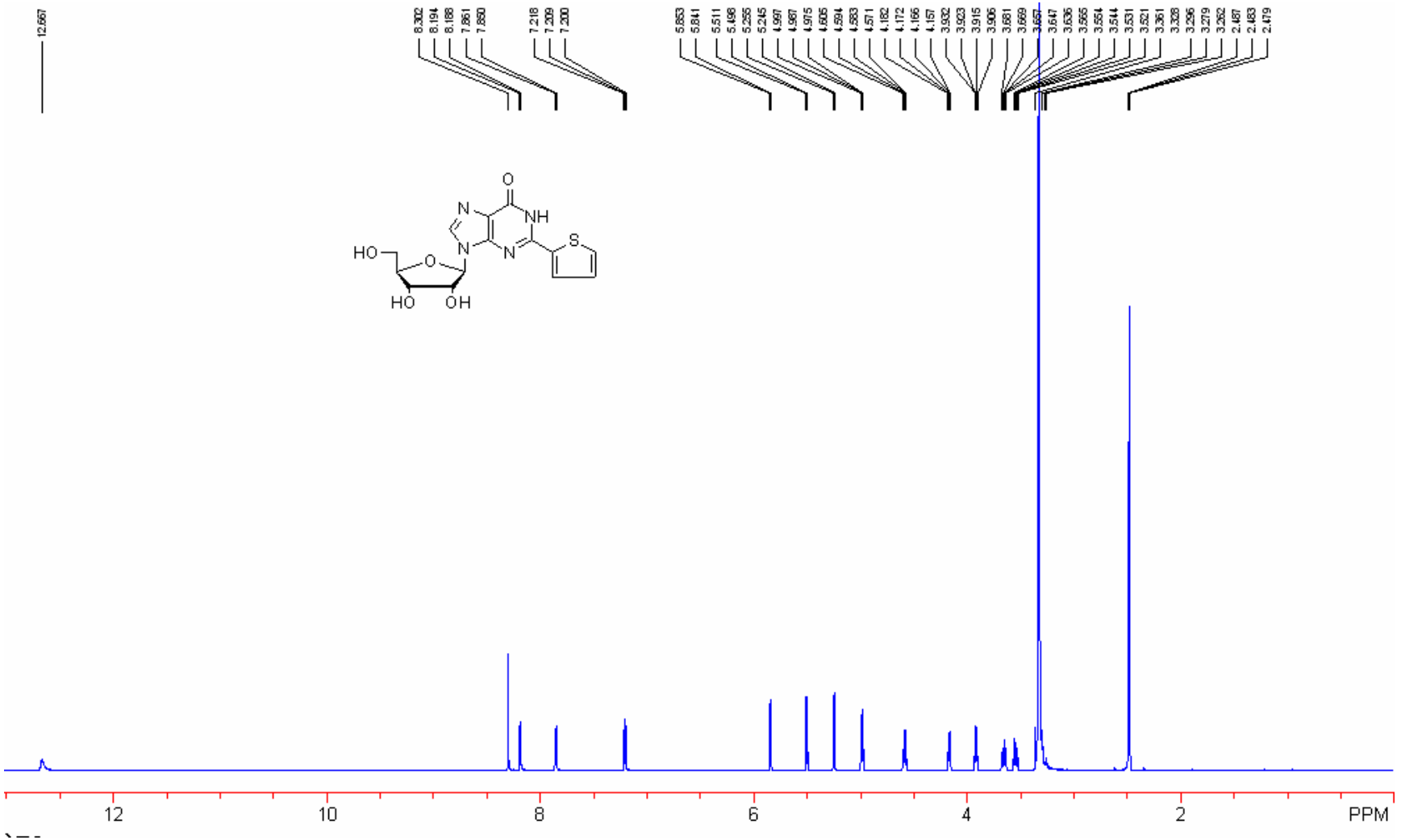


Figure 4.8. 500 MHz ^{1}H NMR of 2-(2-thienyl)-9-(β-D-ribofuranosyl)hypoxanthine.

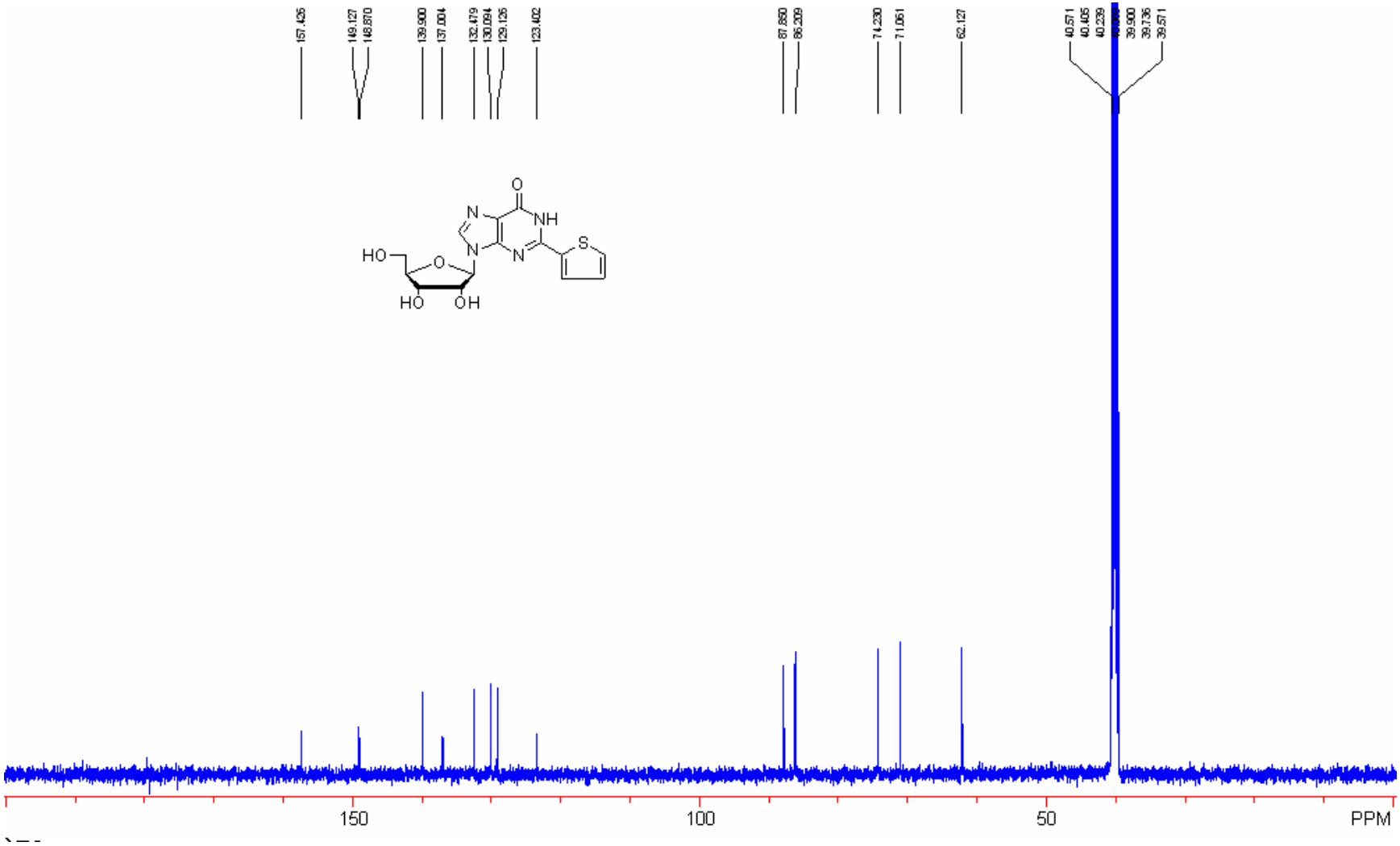
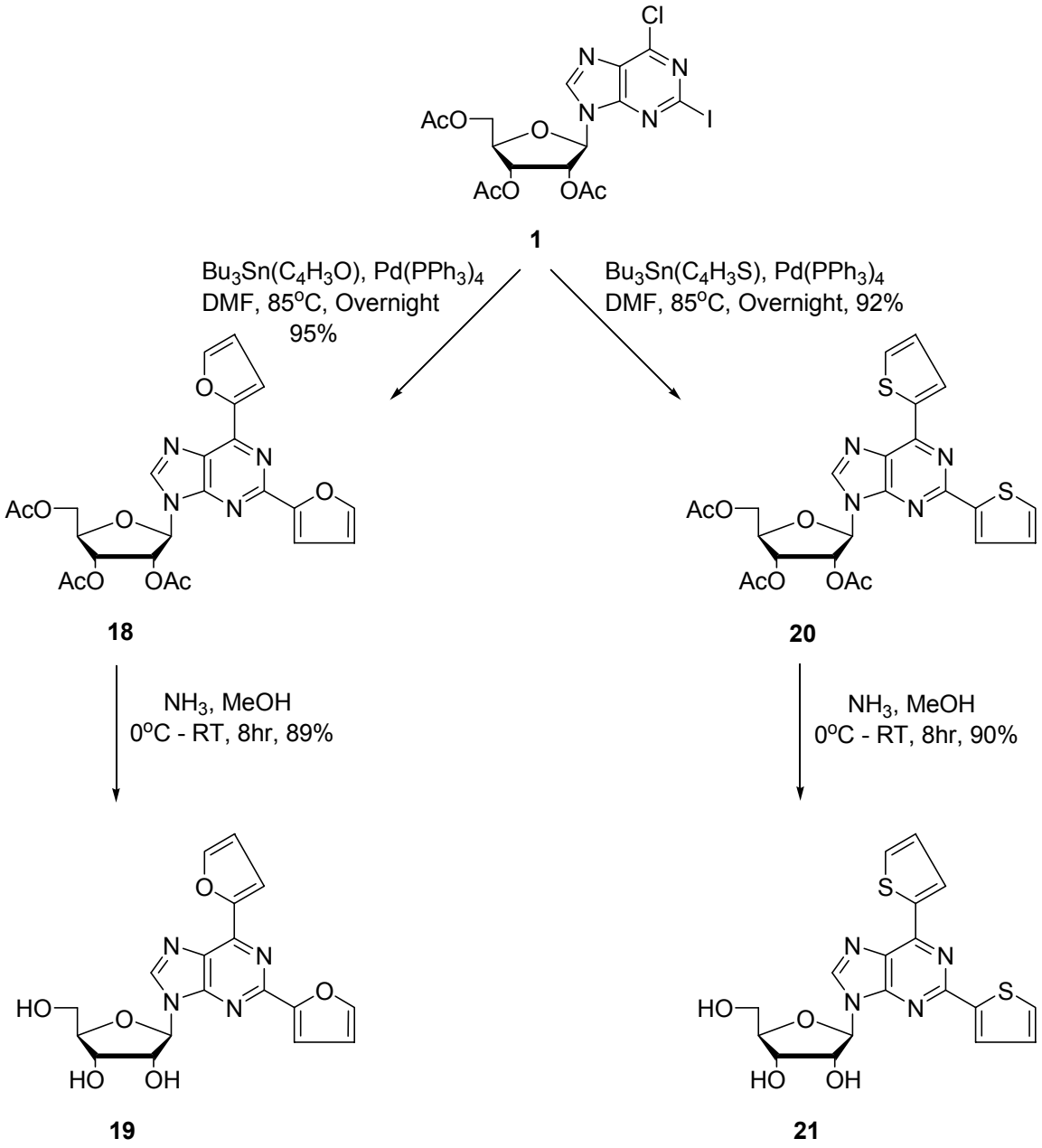


Figure 4.9. 125 MHz ^{13}C NMR of 2-(2-thienyl)-9-(β-D-ribofuranosyl)hypoxanthine.



Scheme 4.15. Synthesis of 2,6-di-(2-furyl) and 2,6-di-(2-thienyl)-9-β-D-ribofuranosylpurine.

The 2,6-disubstituted target compounds 2,6-di-(2-furyl)-9-β-D-ribofuranosyl purine **19** and 2,6-di-(2-thienyl)-9-β-D-ribofuranosyl purine **21** were each synthesized in a two-step

procedure starting from the key intermediate, 6-chloro-2-iodo-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl]purine, **1** (Scheme 4.15).

Regioselectivity in Stille cross-coupling reactions of 2,6-dihalopurines is dictated to a large extent by the identity of the substituent halogens; the order of reactivity and selectivity being R-I > R-Br >> R-Cl. 2,6-Dichloropurines couple preferentially at the 6-Cl whereas the 2-iodo-6-chloro derivatives couple at the 2-I position.[1, 14, 71, 118, 119] This trend in the ease of coupling follows the trend in bond dissociation energies of C-I (220 kJ/mol) < C-Br (275 kJ/mol) < C-Cl (330 kJ/mol).[80, 93] As discussed earlier, during oxidative insertion (first step in the coupling reaction), the palladium catalyst breaks and inserts itself in between the C-X bond. Thus, C-I having the lowest bond dissociation energy in the series facilitates this process readily compared to C-Br and C-Cl. Therefore, if the 6-Cl-2-I intermediate **1** is subjected to the regular coupling reaction time of 4 – 8 hours with stoichiometric amounts of organotin reagent, the coupling will prefer the C-2-I position. To avoid this situation and achieve the disubstituted compounds, **1** was treated with an excess of tributylfurantin and tributylthiophenetin in overnight reactions to afford the disubstituted compounds, **18** and **20,** respectively, in over 90% yield. Deprotection gave the target compounds, 2,6-di-(2-furyl)-9-β-D-ribofuranosylpurine **19** and 2,6-di-(2-thienyl)-9-β-D-ribofuranosylpurine **21,** in 90% yield. Just as with compounds **15** and **17**, **19** and **21** showed similar ^{1}H and ^{13}C NMR spectra. For example, **21** showed six peaks δ (7.23, 1H), (7.36, 1H), (7.75, 1H), (7.95, 1H), (8.0, 1H) and (8.64, 1H) indicative of the two thienyl groups, whereas the ^{13}C spectrum accounted for all eighteen carbon atoms (Figures 4.10 and 4.11). HRMS calculated for **21** ($C_{18}H_{17}N_4O_4S_2$ $[M + H]^+$) is 417.0691, 417.0689 was found.

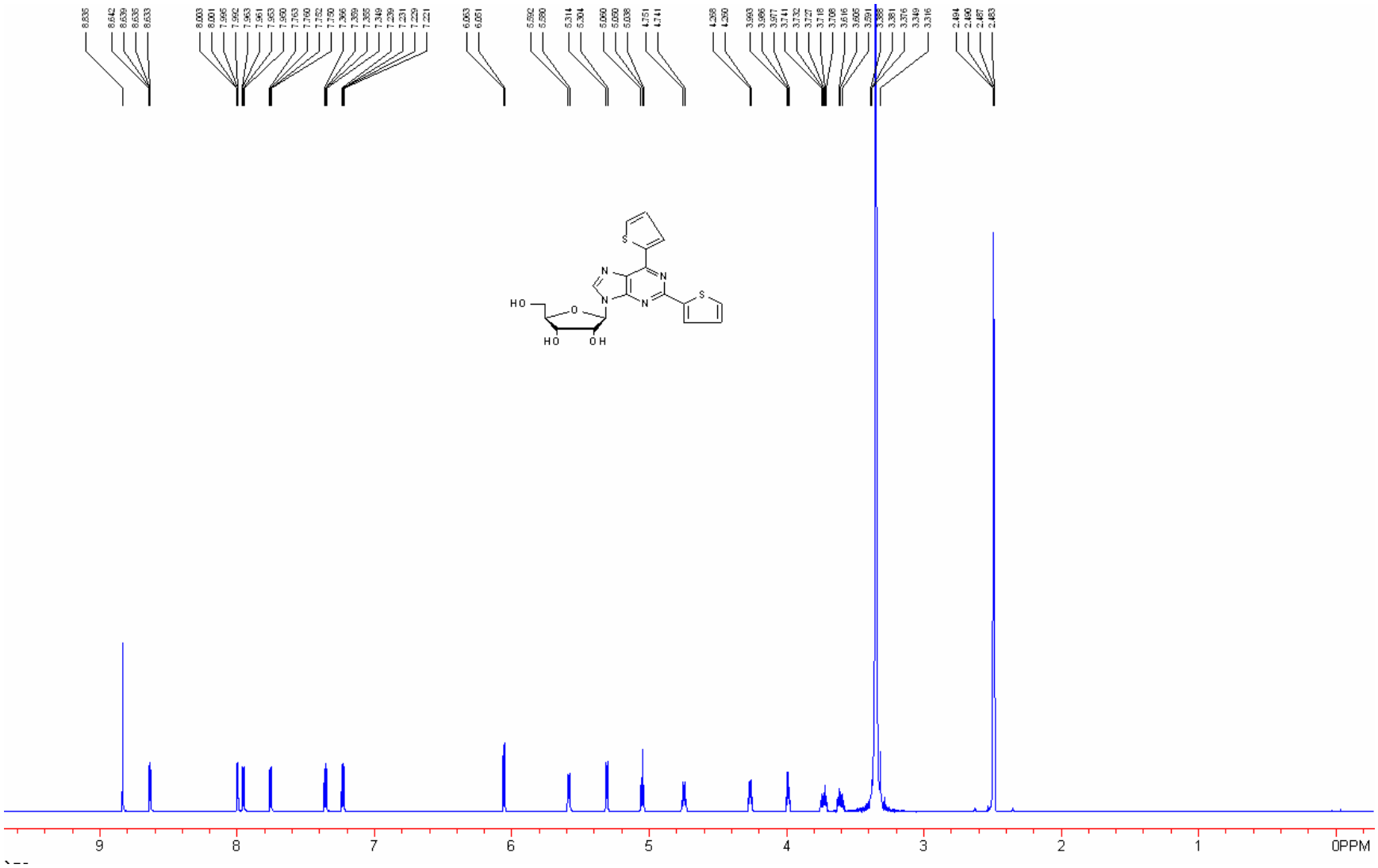


Figure 4.10. 500 MHz ^{1}H NMR of 2,6-di-(2-thienyl)-9-β-D-ribofuranosylpurine.

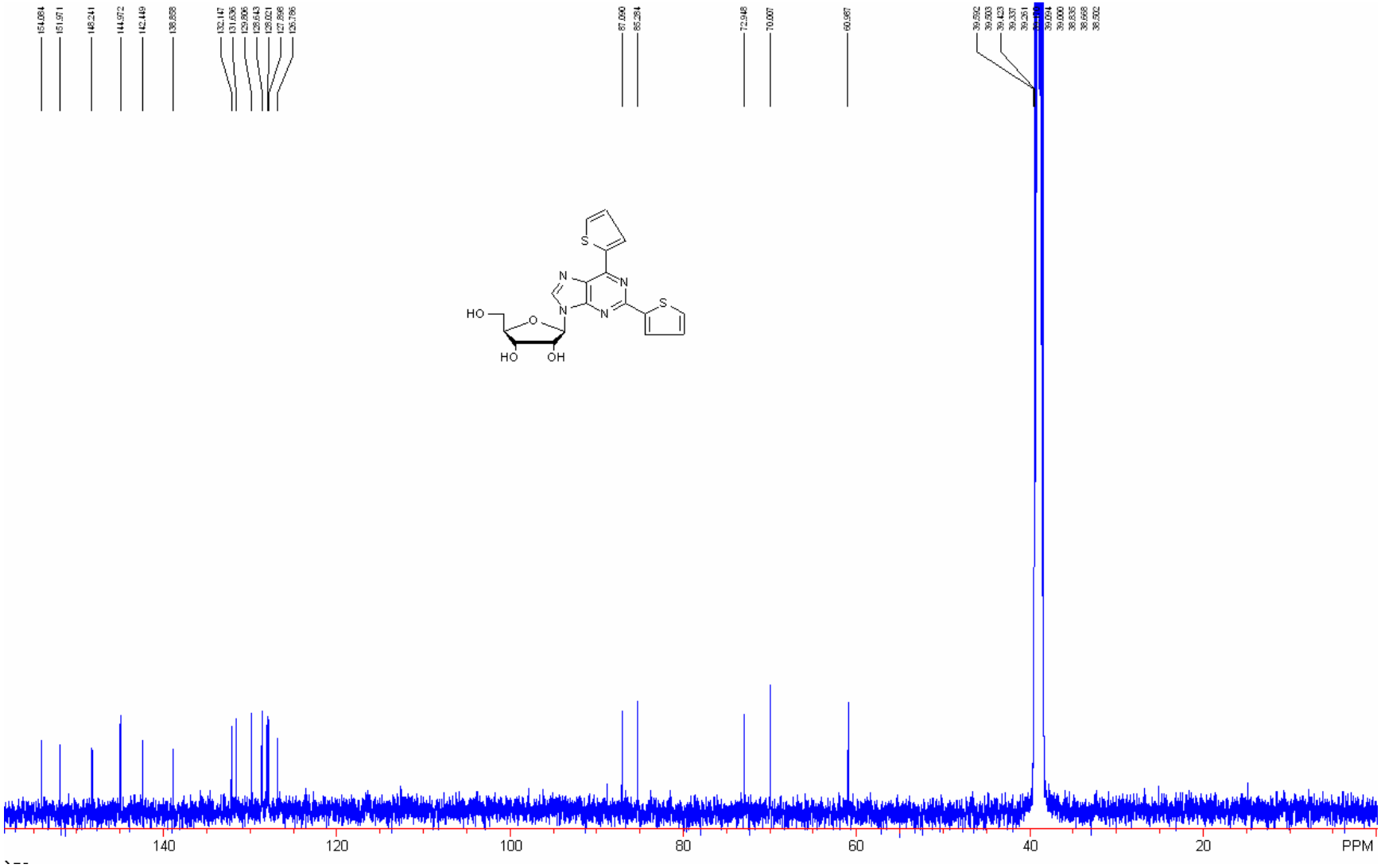
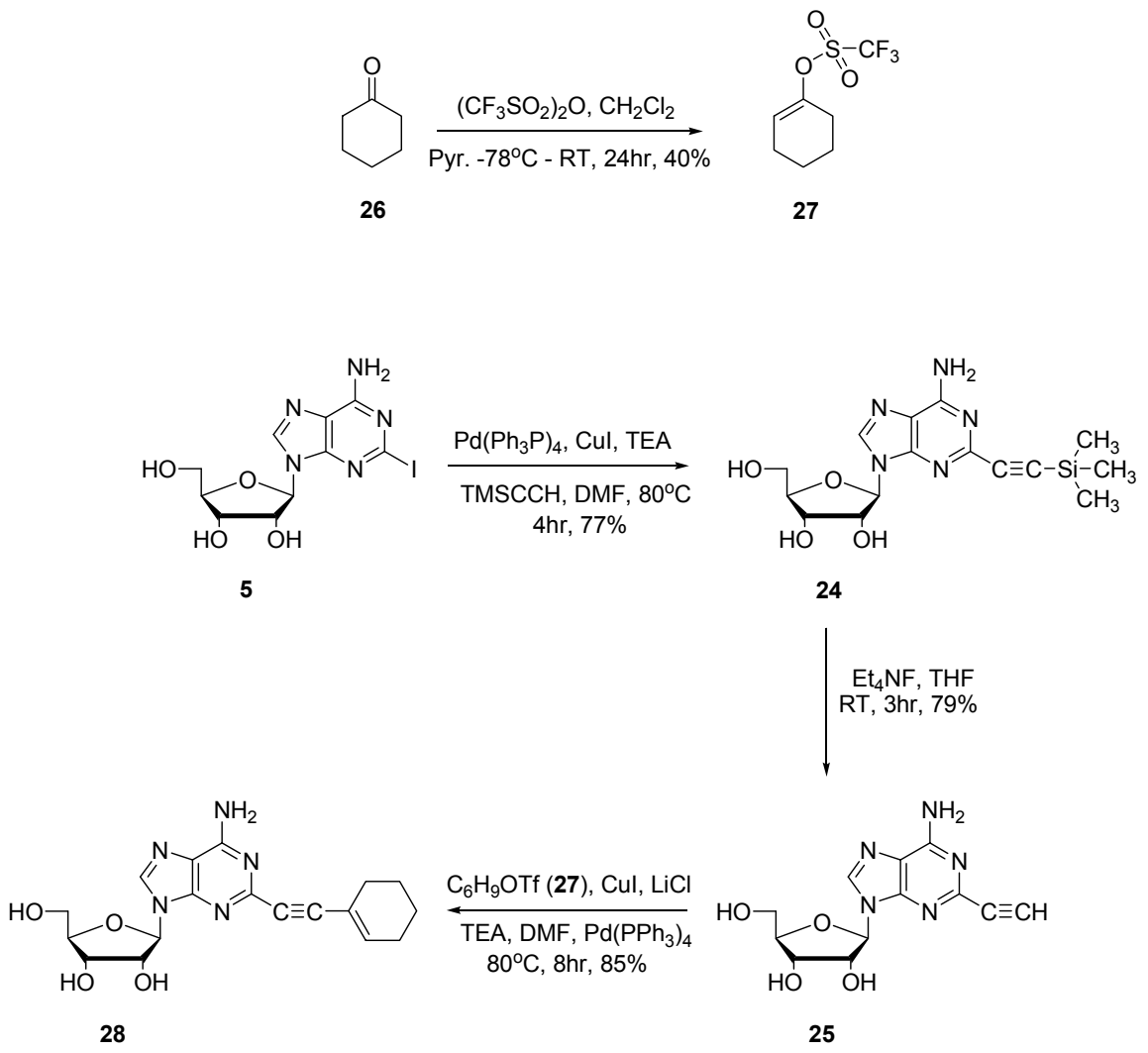


Figure 4.11. 125 MHz ^{13}C NMR of 2,6-di-(2-thienyl)-9-β-D-ribofuranosylpurine.



Scheme 4.16. Synthesis of 2-[2-(1-Cyclohexenyl)ethynyl]adenosine.

Synthesis of 2-[2-(1-cyclohexenyl)ethynyl]adenosine **28** started from the intermediate 2-iodoadenosine **5** and involved 2 separate applications of the palladium-catalyzed cross-coupling reaction (Scheme 4.16). The first coupling was with **5**, trimethylsilylacetylene, CuI, triethylamine, and $Pd(PPh_3)_4$ in DMF at 80˚C for 4 hours to give the 2-trimethylsilylethynyladenosine, **24**. Deprotection of **24** was carried out using Et_4NF in THF to afford the ethynyladenosine, **25**, in 79% yield. The second coupling reaction was similar to the

first except for the addition of LiCl [85] (necessary for transmetalation) and the use of the organic triflate 1-cyclohexenyl-1-yl triflate **27** (synthesized from cyclohexanone **26**).[120] Besides organotin and organozinc reagents, organotriflates are also known to be well suited for palladium-catalyzed cross coupling reactions of nucleosides.[121-123] This efficient reaction afforded the target compound **28** in 85% yield. Characterization of **28** (experimental section) was consistent with data previously reported by us.[123] The final goal of this project was the conversion of **28** into the inosine derivative using ADA; unfortunately, multiple trials at this chemoenzymatic conversion failed in my hands. This failure was the initial motivation to develop a non-enzymatic method for future use.

EXPERIMENTAL SECTION

All chemicals were purchased from Aldrich or Sigma Chemical Companies. Solvents were distilled before use according to standard methods when required. Melting points reported are uncorrected and were determined on an Electrothermal Engineering Ltd. Melting point apparatus. Nuclear magnetic resonance spectra were recorded on a Varian 500 MHz NMR instrument. Ultraviolet spectra were recorded on a Varian Cary Model 3 UV spectrophotometer. Column chromatography was carried out using glass columns packed with 230-400 mesh silica gel. High performance liquid chromatography (HPLC) purifications were carried out on a Beckman-Coulter instrument with C-18 reversed-phase columns. Purity criteria came from HPLC, ^{13}C NMR spectra, and quantitative UV data (ε values). Overnight reactions imply reactions run for 8 – 12 hours.

6-Chloro-2-vinyl-9-[(2′, 3′, 5′-tri-O-acetyl)-β-D-ribofuranosyl]purine (2).

A mixture of compound **1**(0.50g, 0.93mmols), $Pd(CH_3CN)_2Cl_2$ (0.01g, 0.05mmols), and tributyl(vinyl)tin (1.4ml, 4.64mmols) in 5ml of anhydrous DMF was heated at 90˚C for 4 hrs. After the reaction, DMF was removed under reduced pressure, chloroform (50ml) was added and palladium filtered off on celite. The product mixture was washed with water (3 x 20ml), dried over anhydrous Na_2SO_4, and adsorbed unto silica gel. The product was purified on a column using 1% methanol/chloroform to afford compound **2** (0.20g, 92% yield) as clear oil. ^{1}H NMR ($CDCl_3$) δ 2.06 (s, 3H), 2.12 (s, 3H), 2.17 (s, 3H), 4.35 (m, 1H), 4.47 (m, 2H), 5.81 (m, 2H), 6.02 (t, J = 5.0 Hz, 1H), 6.16 (d, J = 4.5 Hz, 1H), 6.72 (q, 1H), 6.90 (q, 1H), 8.20 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 20.4, 20.5, 20.7, 62.7, 70.2, 73.2, 80.1, 87.2, 125.0, 130.8, 135.2, 143.7, 151.3, 151.6, 159.5, 169.3, 169.5, 170.3.

6-Thio-2-vinyl-9-[(2′, 3′, 5′-tri-O-acetyl)-β-D-ribofuranosyl]purine (3).

A mixture of compound **2** (0.23g, 0.52mmols) and thiourea (0.3g, 3.90mmols) was dried on a vacuum pump for 3 hours to remove any traces of water. Absolute ethanol (20ml) was added under vacuum with vigorous stirring. The vacuum was broken with a nitrogen balloon, a condenser affixed and reaction mixture put into 90^o C oil bath. The reaction mixture was refluxed for 3 hours. After the reaction, ethanol was removed under reduced pressure, and product mixture adsorbed unto silica gel. The product was purified by column chromatography using 1% methanol/chloroform. This yielded compound **3** (0.19g, 0.43mmols, 82%) as a light yellow powder. ^{1}H NMR ($CDCl_3$) δ 2.07 (s, 3H), 2.08 (s, 3H), 2.15 (s, 3H), 4.34 (m, 1H), 4.46 (m, 2H), 5.69 (t, J = 5.0 Hz, 1H), 5.94 (m, 2H), 6.16 (d, J = 4.3 Hz, 1H), 6.70 (m, 2H), 8.06 (s, 1H), 11.41 (br, s 1H); ^{13}C NMR ($CDCl_3$) δ 20.4, 20.5, 20.7, 62.7, 70.1, 73.3, 80.0, 87.1, 127.1, 128.5, 128.8, 130.9, 141.1, 144.0, 151.5, 169.3, 169.5, 170.3.

6-Thio-2-vinyl-9-(β-D-ribofuranosyl)hypoxanthine (4).

Compound **3** (0.11g, 0.25mmols) was dried under vacuum for 3 hours. Anhydrous methanol (15ml) was added under vacuum with stirring. This solution was saturated with ammonia gas for 1 hour at 0^{o} C. The reaction was run at room temperature overnight. After the reaction, the product mixture was stirred for 30 minutes to expel the residual ammonia gas. The mixture was then adsorbed unto silica gel and purified by column chromatography using 8% methanol/chloroform as the solvent system. The product **4** (0.07g, 0.23mmols, 90% yield) was further purified by reverse phase HPLC (methanol/water) for testing purposes. ^{1}H NMR (Me_2SO-d_6) δ 3.55 (m, 1H), 3.65 (m, 1H), 3.94 (q, 1H), 4.15 (q, 1H), 4.52 (q, 1H), 5.03 (t, J = 6 Hz, 1H), 5.25 (d, J = 5.5 Hz, 1H), 5.53 (d, J = 6 Hz, 1H), 5.88 (m, 2H), 6.59 (d, J = 16.5 Hz, 1H), 6.86 (q, 1H), 8.50 (s, 1H), 13.72 (br, s 1H); ^{13}C NMR (Me_2CO-d_6) δ 62.8, 72.1, 75.8, 87.2, 90.0, 127.0, 129.8, 136.3, 142.6, 145.0, 152.6, 178.2; decomp. 171˚ C - 173˚ C; UV (MeOH), 205nm (ε 15,430), 250nm (ε 9204), 326nm (ε 12963); HRMS (FAB) calcd for $C_{12}H_{15}N_4O_4S$ [M + H]$^{+}$ 311.0814, found 311.0810.

6-Amino-2-iodo-9-(β-D-ribofuranosyl)purine (5).

Compound **1** (2.40g, 4.45mmols) was dried on a vacuum pump for 3 hours and then taken up in anhydrous methanol (35ml). This solution was saturated with ammonia gas for 1 hour at 0^{o} C. The reaction was stirred at room temperature overnight. After the reaction, the product mixture was stirred for 30 minutes to expel the residual ammonia gas. The crude product was then adsorbed unto silica gel and purified by column chromatography starting with 1:10 methanol/chloroform and increasing the polarity to 1.5:10 methanol/chloroform. The column fractions were concentrated to give compound **5** (1.57g, 4.01mmols, 90 %). ^{1}H NMR (Me_2SO-d_6) δ 3.54 (m, 1H), 3.60 (m, 1H), 3.93 (q, IH), 4.11 (q, IH), 4.51 (q, 1H), 5.05 (t, 1H), 5.23 (d,

1H), 5.48 (d, 1H), 5.81 (d, IH), 7.76 (br s, 2H), 8.30 (s, 1H); ^{13}C NMR (Me_2SO-d_6) δ 61.4, 70.5, 73.6, 85.8, 87.2, 119.0, 120.9, 139.4, 149.7, 155.9.

6-Amino-2-iodo-9-[2′,3′,5′-O-tri-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]purine (6).

A 100ml round-bottom flask charged with compound **5** (2.33g, 5.97mmols) and imidazole (3.25g, 47.76mmols) was dried on a vacuum pump. TBDMSCl (3.6g, 23.88mmols) dissolved in anhydrous DMF (15ml) was added under vacuum with vigorous stirring. The reaction mixture was flushed with nitrogen and run for 3 days at room temperature. After the reaction, DMF was removed under reduced pressure leaving thick yellow syrup. Chloroform was added and adsorption unto silica gel facilitated by a vacuum pump. The crude product was then purified by column chromatography using 100% hexanes, 10% ethyl acetate/hexanes followed by 30% ethyl acetate/hexanes. The column fractions were concentrated to give compound **6** (4.11g, 5.61mmols, 94%) as clear oil. ^{1}H NMR ($CDCl_3$) δ 0.05 – 0.16 (m, 18H), 0.84 – 0.95 (m, 27H), 3.78 (m, 1H), 4.03 (m, 1H), 4.12 (d, J = 3.5 Hz, 1H), 4.29 (t, J = 3.5 Hz, 1H), 4.67 (t, J = 4.5 Hz, 1H), 5.90 (d, J = 4.5 Hz, 1H), 6.64 (br, s, 2H), 8.06 (s, 1H); ^{13}C NMR ($CDCl_3$) δ -4.9, -4.8, -4.5, -4.3, -4.2, -3.9, 18.4, 18.5, 18.9, 26.2, 26.3, 26.6, 62.5, 71.9, 75.7, 85.5, 89.6, 119.8, 120.4, 140.0, 150.1, 155.9.

2,6-Diiodo-9-[2′,3′,5′-O-tri-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]purine (7).

The protected nucleoside **6** (2.66g, 3.63mmols) was dissolved in anhydrous acetonitrile (10ml). Diiodomethane (1.2ml, 14.52mmols) and tert-butylnitrite (2.20ml, 18.18mmols) were added. The reaction mixture was purged with nitrogen for 30 minutes. A drying tube was attached to the condenser and the reaction was run in a 65^{o}C oil bath for 8 hours. After the reaction, all solvent was striped off in vacuo, and chloroform (50ml) added. The chloroform portion was washed with Na_2SO_3 solution (2 x 30ml) to remove residual diiodomethane, and

then dried over Na_2SO_4 granules. The crude product was adsorbed unto silica gel and purified by column chromatography using 5% ethyl acetate/hexanes. This afforded compound **7** (2.17g, 2.57mmols, 71%) as a light viscous oil. ^{1}H NMR ($CDCl_3$) δ -0.24 – 0.13 (m, 18H), 0.79 – 0.92 (m, 27H), 3.77 (m, 1H), 3.98 (m, 1H), 4.11 (m, 1H), 4.24 (t, J = 3.5 Hz, 1H), 4.56 (t, J = 4.5 Hz, 1H), 5.95 (d, J = 4.5 Hz, 1H), 8.38 (s, 1H); ^{13}C NMR ($CDCl_3$) δ -4.9, -4.8, -4.6, -4.3, -4.2, -3.9, 18.3, 18.5, 19.0, 26.0, 26.1, 26.2, 26.3, 26.4, 26.5, 26.6, 62.5, 71.9, 76.3, 86.0, 89.7, 117.0, 122.1, 139.9, 143.9, 148.6.

2,6-Divinyl-9-[2′,3′,5′-O-tri-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]purine(8).

A mixture of **7** (1.58g, 1.87mmols) and $Pd(PPh_3)_4$ (0.6g, 0.56mmols) was dissolved in 10ml of NMP. Tetravinyltin (1.4ml, 7.48mmols) was added to the solution and the reaction mixture was purged with nitrogen for 20 minutes. The reaction was run at 80^{o}C overnight. Upon completion, NMP was evaporated under reduced pressure at 80^{o}C with the assistance of a vacuum pump. Ethyl acetate was added and the Pd was filtered off on celite. The crude product was adsorbed unto silica gel and purified by column chromatography using ethyl acetate/ hexanes system. The 5% ethyl acetate/ hexanes eluent was concentrated to give compound **8** (1.08g, 1.68mmols, 90%) as a colorless viscous oil. ^{1}H NMR ($CDCl_3$) δ 0.00 – 0.33 (m, 18H), 0.99 – 1.14 (m, 27H), 3.98 (m, 1H), 4.24 (m, 1H), 4.32 (m, 1H), 4.51 (t, J = 3.5 Hz, 1H), 4.87 (t, J = 4.5 Hz, 1H), 5.84 (q, J = 1.5 Hz, 1H), 6.11 (q, J = 1.5 Hz, 1H), 6.28 (d, J = 4 Hz, 1H), 6.83 (q, J = 1.5 Hz, 1H), 7.12 (m, 1H), 7.24 (q, J = 1.5 Hz, 1H), 7.48 (m, 1H), 8.59 (s, 1H); ^{13}C NMR ($CDCl_3$) δ -5.0, -4.9, -4.5, -4.4, -4.3, -3.9, 18.3, 18.5, 18.9, 26.1, 26.2, 26.5, 62.7, 72.0, 75.9, 85.4, 88.9, 122.6, 126.3, 130.8, 132.5, 137.3, 144.1, 152.6, 153.5, 158.9.

2,6-Divinyl-9-(β-D-ribofuranosyl)purine(9).

A 50ml round bottom flask was charged with the protected nucleoside **8** (0.13g, 0.21mmols) and dried on a vacuum pump for 3 hours. THF (3ml) was added under vacuum with stirring to completely dissolve starting material. The vacuum was broken with a nitrogen balloon and $Et_3N.3HF$ was carefully added to the reaction mixture. The reaction was run for 16 hours at room temperature. After reaction, product mixture was cooled to room temperature, silica gel was directly added for adsorption. The crude compound was purified by column chromatography using 3% methanol/chloroform as the eluting solvent. The combined fractions containing the product was stripped off solvent to a volume just when the product started to precipitate (~5ml), 50% methanol/water (10ml) was added to redissolve the product. This solution was then purified by reverse phase HPLC using methanol/water on a Waters Deltapak 300 x 50mm I.D. C18, 15μm, 100Å column. The solution containing pure compound was lyophilized to afford the divinyl compound **9** (0.05g, 0.16mmols, 75%) as off-white powder. ^{1}H NMR (Me_2SO-d_6) δ 3.56 (m, 1H), 3.66 (m, 1H), 3.97 (q, 1H), 4.19 (q, 1H), 4.65 (d, 1H), 5.09 (t, J = 5 Hz, 1H), 5.25 (d, 1H), 5.51 (d, 1H), 5.71 (q, 1H), 6.01 (m, 2H), 6.58 (q, 1H), 6.88 (q, 1H), 7.04 (q, 1H), 7.19 (q, 1H), 8.80 (s, 1H); ^{13}C NMR (Me_2SO-d_6) δ 61.4, 70.4, 73.6, 85.8, 87.4, 122.7, 126.6, 129.9, 132.2, 136.6, 145.4, 152.1, 152.4, 157.4; decomp. 120˚ C - 122˚ C; UV (MeOH), 240nm (ε 27,435), 273nm (ε 10,250); HRMS (FAB) calcd for $C_{14}H_{17}N_4O_4$ $[M + H]^+$ 305.1250, found 305.1239.

6-Thiomethyl-2-vinyl-9-[(2′, 3′, 5′-tri-O-acetyl)-β-D-ribofuranosyl]purine (10).

Compound **3** (0.15g, 0.34mmols) and K_2CO_3 (0.05g, 0.37mmols) were dried on a vacuum pump at 50^{o}C for 3 hours. DMF (2ml) was added under vacuum with stirring. The vacuum was broken with a nitrogen balloon and CH_3I (0.03ml, 0.44mmols) was carefully added. The reaction

mixture was flushed with nitrogen for 15 minutes and the reaction run at room temperature for 1 hour. After the reaction, DMF was removed under reduced pressure. The crude product was re-dissolved in chloroform and adsorbed unto silica gel. Purification by column chromatography eluted pure compound in 100% chloroform to give compound **10** (0.11g, 0.25mmols, 72%) as light yellow crystals. ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 3H), 2.09 (s, 3H), 2.13 (s, 3H), 2.77 (s, 3H), 4.36 (m, 1H), 4.47 (m, 2H), 5.73 (q, 1H), 5.90 (t, J = 5.5 Hz, 1H), 6.06 (t, J = 5.0 Hz, 1H), 6.17 (d, J = 4 Hz, 1H), 6.72 (q, 1H), 6.93 (q, 1H), 8.06 (s, 1H).

6-Thiomethyl-2-vinyl-9-(β-D-ribofuranosyl)purine (11).

Dried compound **10** (0.08g, 0.17mmols) was up taken up in anhydrous methanol (20ml). This solution was saturated with ammonia gas for 1 hour at 0^o C. The reaction was stirred at room temperature overnight. After the reaction, the product mixture was stirred for 30 minutes to expel the residual ammonia gas. The crude compound was purified by column chromatography using 3% - 5% methanol/chloroform as the eluting solvent. The combined fractions containing the product were striped down to 3ml; 10ml of 50:50 methanol/water was added to redissolve the product. This solution was then purified by reverse phase HPLC using methanol/water on a Waters Deltapak 300 x 50mm I.D. C18, 15μm, 100Å column. The solution containing pure compound was lyophilized to afford compound **11** (0.05g, 0.15mmols, 89%) as light yellow powder. ^{1}H NMR (MeOH-d_4) δ 2.71 (s, 3H), 3.76 (q, 1H), 3.90 (q, 1H), 4.16 (d, J = 2.5 Hz, 1H), 4.36 (q, 1H), 4.76 (t, J = 5 Hz, 1H), 5.69 (q, 1H), 6.05 (d, J = 5.5 Hz, 1H), 6.67 (q, 1H), 6.82 (q, 1H), 8.49 (s, 1H); ^{13}C NMR (MeOH-d_4) δ 11.6, 63.2, 72.4, 75.5, 87.8, 91.0, 123.9, 131.5, 137.2, 144.5, 149.4, 159.3, 162.6; decomp. 180˚ C - 183˚ C; UV (MeOH), 210nm (ε 11,058), 252nm (ε 19,638), 290nm (ε 13,850); HRMS (FAB) calcd for $C_{13}H_{17}N_4O_4S$ $[M + H]^+$ 325.0971, found 325.0976.

2-Iodo-9-[(2′, 3′, 5′-tri-O-acetyl)-β-D-ribofuranosyl]hypoxanthine (13).

A three neck 1000ml round bottom flask was charged with tri-acetylated guanosine **12** (21.54g, 52.62mmols) and CHI_3 (82.87g, 210mmols) and dried on a vacuum pump for 3 hours. Anhydrous acetonitrile (500ml) and isoamylnitrite (70.37ml, 526.2mmols) was added quickly with vigorous stirring. The reaction suspension was flushed with nitrogen for 20 minutes and put in 90^{o}C oil bath. The reaction was refluxed for 1 hour, after which time TLC showed total conversion of starting material to product. The product mixture was cooled and stripped of solvent. Chloroform and silica gel were added, the solvent was then removed with the aid of a vacuum pump for adsorption. The crude product was purified by column chromatography using initially 1L chloroform to flush out excess unreacted CHI_3, and then gradually increasing the polarity to 5% methanol/chloroform. The combined fractions containing the product was stripped of solvent to give the protected 2-iodoinosine **13** (10.32g, 19.84mmols, 50%) as yellow crystals. ^{1}H NMR (Me_2SO-d_6) δ 2.08 (s, 3H), 2.10 (s, 3H), 2.11 (s, 3H), 4.27 (m, 1H), 4.39 (m, 2H), 5.58 (t, J = 4.5 Hz, 1H), 5.82 (t, J = 5.5 Hz, 1H), 6.15 (d, J = 5.0 Hz, 1H), 8.27 (s, 1H), 13.14 (br, s 1H); ^{13}C NMR (Me_2SO-d_6) δ 21.1, 21.3, 22.6, 63.2, 69.0, 72.9, 80.1, 86.0, 110.8, 124.8, 139.4, 149.5, 157.3, 169.7, 169.8, 170.4.

2-(2-Furyl)-9-[(2′, 3′, 5′-tri-O-acetyl)-β-D-ribofuranosyl]hypoxanthine (14).

Anhydrous DMF was added to a very dry sample of **13** (0.52g, 1mmol) under argon with stirring. $Pd(PPh_3)_4$ (0.12g, 0.1mmols) dissolved in 1ml of DMF was then added followed by the addition of tributylfurantin (1.6ml, 5mmols) both under argon. A condenser was affixed with argon flush and the reaction mixture put in an 85^{o}C oil bath and run overnight. After the reaction, DMF was removed under vacuum; chloroform was added and Pd removed by filtration through celite. Chloroform was striped off and 100ml acetonitrile was added and washed with hexanes (3

x 50ml) to remove the excess unreacted tin reagent. The acetonitrile portion was rotovaped down and adsorbed unto silica gel for column purification. A 1.5% methanol/chloroform solvent system eluded pure compound **14** (0.39g, 0.85mmols, 85%) as a light oil. ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 3H), 2.06 (s, 3H), 2.14 (s, 3H), 4.38 (m, 1H), 4.48 (m, 2H), 5.91 (t, J = 5.5 Hz, 1H), 5.99 (q, J = 1.5 Hz, 1H), 6.14 (d, J = 4.5 Hz, 1H), 6.67 (q, J = 1.5 Hz, 1H), 7.72 (d, J = 1.0 Hz, 1H), 7.77 (d, J = 3.5 Hz, 1H), 7.94 (s, 1H), 11.89 (br, s, 1H); ^{13}C NMR ($CDCl_3$) δ 20.5, 20.6, 20.7, 62.9, 70.2, 73.5, 80.0, 87.3, 113.3, 115.4, 124.0, 139.0, 145.5, 145.6, 146.1, 148.8, 157.8, 169.4, 169.6, 170.4.

2-(2-Furyl)-9-(β-D-ribofuranosyl)hypoxanthine (15).

Dried compound **14** (0.39g, 0.85mmols) was up taken up in anhydrous methanol (20ml). This solution was saturated with ammonia gas for 1 hour at 0^{o} C. The reaction was stirred at room temperature overnight. After the reaction, the product mixture was stirred for 30 minutes to expel the residual ammonia gas. The crude compound was adsorbed unto silica gel and purified by column chromatography using 8% - 10% methanol/chloroform as the eluting solvent. The combined fractions containing the product were striped down to 3ml; 10ml of 50:50 methanol/water was added to redissolve the product with the aid of sonication. This solution was then purified by reverse phase HPLC using methanol/water on a Waters Deltapak 300 x 50mm I.D. C18, 15μm, 100Å column. The solution containing pure compound was lyophilized to afford compound **15** (0.05g, 0.15mmols, 91%) as light yellow powder. ^{1}H NMR (Me_2SO-d_6) δ 3.63 (m, 1H), 3.70 (m, 1H), 3.98 (d, J = 3.0 Hz, 1H), 4.19 (t, J = 1.5 Hz, 1H), 4.58 (d, J = 5 Hz, 1H), 5.04 (t, J = 5.5 Hz, 1H), 5.27 (q, J = 3.0 Hz, 1H), 5.52 (q, J = 4.0 Hz, 1H), 5.95 (t, J = 4.5 Hz, 1H), 6.78 (m, 1H), 7.65 (d, J = 3.5 Hz, 1H), 8.02 (s, 1H), 8.38 (d, J = 2.0 Hz, 1H), 12.59 (s, 1H); ^{13}C NMR (Me_2SO-d_6) δ 62.1, 71.1, 74.2, 86.2, 87.8, 123.4, 129.1, 130.1, 132.5, 137.0,

139.9, 148.9, 149.1, 157.4; m.p. 185˚ C - 187˚ C; UV (MeOH), 314nm (ε 10,063), 260nm (ε 7022), 205nm (ε 8726); HRMS (FAB) calcd for $C_{14}H_{15}N_4O_6$ $[M + H]^+$ 335.0992, found 335.0998.

2-(2-Thienyl)-9-[(2′, 3′, 5′-tri-O-acetyl)-β-D-ribofuranosyl]hypoxanthine (16).

Anhydrous DMF was added to a very dry sample of **13** (0.26g, 0.50mmol) under argon with stirring. $Pd(PPh_3)_4$ (0.06g, 0.05mmols) dissolved in 1ml of DMF was then added followed by the addition of tributylthiophenetin (0.8ml, 2.5mmols) both under argon. A condenser was affixed with argon flush and the reaction mixture put in an 85^{o}C oil bath and run overnight. After the reaction, DMF was removed under vacuum; chloroform was added and Pd removed by filtration through celite. Chloroform was striped off and 100ml acetonitrile was added and washed with hexanes (3 x 50ml) to remove the excess unreacted tin reagent. The acetonitrile portion was adsorbed unto silica gel for column purification. A 1.5% methanol/chloroform solvent system eluded Pure compound **16** (0.18g, 0.85mmols, 85%) as a light oil. ^{1}H NMR (MeOH-d_4) δ 2.08 (s, 3H), 2.09 (s, 3H), 2.13 (s, 3H), 4.39 (m, 1H), 4.48 (m, 2H), 5.83 (t, J = 5.5 Hz, 1H), 6.02 (t, J = 5.5 Hz, 1H), 6.22 (d, J = 4.5 Hz, 1H), 7.18 (q, J = 0.5 Hz, 1H), 7.74 (d, J = 5.0 Hz, 1H), 8.07 (t, J = 1.0 Hz, 1H), 8.18 (s, 1H); ^{13}C NMR (MeOH-d_4) δ 19.0, 19.1, 19.2, 62.8, 70.4, 73.4, 79.9, 87.7, 128.2, 129.3, 131.6, 136.1, 138.5, 139.9, 148.4, 149.6, 157.6, 169.9, 170.0, 170.8.

2-(2-Thienyl)-9-(β-D-ribofuranosyl)hypoxanthine (17).

Dried compound **16** (0.17g, 0.36mmols) was up taken up in anhydrous methanol (20ml). This solution was saturated with ammonia gas for 1 hour at 0^o C. The reaction was stirred at room temperature overnight. After the reaction, the product mixture was stirred for 30 minutes to expel the residual ammonia gas. The crude compound was adsorbed unto silica gel and purified

by column chromatography using 12% methanol/chloroform as the eluting solvent. The combined fractions containing the product was striped down to 3ml; 10ml of 50:50 methanol/water was added to redissolve the product with the aid of sonication. 2 X 6.5ml of this solution was then purified by reverse phase HPLC using methanol/water on a Waters Deltapak 300 x 50mm I.D. C18, 15µm, 100Å column. The solution containing pure compound was lyophilized to afford compound **17** (0.11g, 0.32mmols, 90%) as a white powder. ^{1}H NMR (Me_2SO-d_6) δ 3.54 (m, 1H), 3.66 (m, 1H), 3.91 (q, J = 4.0 Hz, 1H), 4.17 (q, J = 3.0 Hz, 1H), 4.58 (q, J = 5.5 Hz, 1H), 4.99 (t, J = 5.5 Hz, 1H), 5.25 (d, J = 5.0 Hz, 1H), 5.50 (d, J = 1.5 Hz, 1H), 5.84 (d, J = 6.0 Hz, 1H), 7.21 (t, J = 4.5 Hz, 1H), 7.85 (d, J = 5.5 Hz, 1H), 8.19 (d, J = 3.0 Hz, 1H), 8.30 (s, 1H), 12.67 (s, 1H); ^{13}C NMR (Me_2SO-d_6) δ 62.1, 71.1, 74.2, 86.2, 87.8, 123.4, 129.1, 130.1, 132.5, 137.0, 139.9, 148.9, 149.1, 157.4; m.p. 190˚ C - 192˚ C; UV (MeOH), 323nm (ε 14,029), 259nm (ε 11,838), 202nm (ε 19,712); HRMS (FAB) calcd for $C_{14}H_{15}N_4O_5S$ $[M + H]^+$ 351.0763, found 351.0758.

2,6-Di-(2-furyl)-9-[(2′, 3′, 5′-tri-O-acetyl)-β-D-ribofuranosyl]purine (18).

Anhydrous DMF was added to a very dry sample of **1** (0.57g, 1.1mmol) under argon with stirring. $Pd(PPh_3)_4$ (0.13g, 0.11mmols) dissolved in 1ml of DMF was then added followed by the addition of tributylfurantin (1.7ml, 5.5mmols) both under argon. A condenser was affixed with argon flush and the reaction mixture put in an 85^{o}C oil bath and run overnight. After the reaction, DMF was removed under vacuum; 100ml of ethyl acetate was added and filtered over a celite plug to remove palladium. Ethyl acetate was striped off and 100ml acetonitrile was added, the acetonitrile portion was washed with hexanes (3 x 50ml) to remove the excess unreacted tin reagent. The acetonitrile portion was adsorbed unto silica gel for column purification. 100% chloroform eluted pure compound **18** (0.50g, 1.04mmols, 95%) as a light colorless oil. ^{1}H NMR

($CDCl_3$) δ 2.05 (s, 3H), 2.06 (s, 3H), 2.14 (s, 3H), 4.44 (m, 1H), 4.52 (m, 2H), 5.99 (t, J = 5.5 Hz, 1H), 6.10 (q, J = 1.5 Hz, 1H), 6.27 (d, J = 4.5 Hz, 1H), 6.63 (q, J = 1.5 Hz, 1H), 6.71 (q, J = 2.0 Hz, 1H), 7.52 (d, J = 3.5 Hz, 1H), 7.70 (t, J = 1.5 Hz, 1H), 7.84 (d, J = 1.5 Hz, 1H), 7.92 (d, J = 3.5 Hz, 1H), 8.21 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 20.4, 20.6, 20.7, 62.8, 70.3, 73.4, 80.1, 87.0, 112.2, 112.6, 113.3, 117.8, 127.4, 143.2, 144.9, 146.2, 146.5, 149.5, 152.0, 152.2, 152.6, 169.4, 169.6, 170.4.

2,6-Di-(2-furyl)-9-(β-D-ribofuranosyl)purine (19).

Compound **18** (0.45g, 0.94mmols) was dried in a 50 ml round bottom flask on a vacuum pump overnight. It was then taken up in anhydrous methanol (20ml). A copper wire was tightly tied around the septum affixed on the round bottom flask to prevent popping off. The reaction mixture was saturated with ammonia gas for 1 hour at 0^o C. The reaction was stirred at room temperature overnight. After the reaction, the product mixture was stirred for 30 minutes to expel the residual ammonia gas. The crude compound was adsorbed unto silica gel and purified by column chromatography. The UV active compound eluting in 5% methanol/chloroform was collected in vials. The combined fractions containing the product was striped down to 3ml; 10ml of 50:50 methanol/water was added to redissolve the product with the aid of sonication. 2 X 6.5ml of this solution was then purified by reverse phase HPLC using methanol/water on a Waters Deltapak 300 x 50mm I.D. C18, 15μm, 100Å column. The solution containing pure compound was reduced in volume on a Rotovap and lyophilized to afford compound **19** (0.30g, 0.84mmols, 89%) as a white powder. ^1H NMR (Me_2SO-d_6) δ 3.63 (m, 1H), 3.74 (m, 1H), 4.01 (q, J = 3.5 Hz, 1H), 4.25 (q, J = 4.0 Hz, 1H), 4.72 (q, J = 5 Hz, 1H), 5.08 (t, J = 5.5 Hz, 1H), 5.31 (d, J = 5.0 Hz, 1H), 5.59 (d, J = 6.0 Hz, 1H), 6.10 (d, J = 6.0 Hz, 1H), 6.74 (q, 1H), 6.86 (q, 1H), 7.36 (d, J = 3.0 Hz, 1H), 7.92 (d, J = 3.5 Hz, 1H), 7.95 (d, J = 1.5 Hz, 1H), 8.13 (t, J = 1.0 Hz,

1H), 8.86 (s, 1H); ^{13}C NMR (Me_2SO-d_6) δ 61.8, 70.9, 74.1, 86.3, 87.8, 112.9, 113.3, 113.4, 118.4, 127.3, 145.5, 145.7, 145.8, 147.1, 149.2, 151.7, 152.5, 152.7; m.p. 178˚ C - 180˚ C; decomp. 173˚ C - 175˚ C; UV (MeOH), 207nm (ε 13,500), 295nm (ε 45,277); HRMS (FAB) calcd for $C_{18}H_{17}N_4O_6$ $[M + H]^+$ 385.1148, found 385.1139.

2,6-Di-(2-thienyl)-9-[(2′, 3′, 5′-tri-O-acetyl)-β-D-ribofuranosyl]purine (20).

Anhydrous DMF was added to a very dry sample of **1** (0.26g, 0.48mmol) under argon with stirring. $Pd(PPh_3)_4$ (0.06g, 0.05mmols) dissolved in 1ml of DMF was then added followed by the addition of tributylthiophenetin (0.8ml, 2.4mmols) both under argon. A condenser was affixed with argon flush and the reaction mixture put in an 85^{o}C oil bath and run overnight. After the reaction, DMF was removed under vacuum; 100ml of ethyl acetate was added and filtered over a celite plug to remove palladium. Ethyl acetate was then striped off and 100ml acetonitrile was added, the acetonitrile portion was washed with hexanes (3 x 50ml) to remove the excess unreacted tin reagent. The acetonitrile portion was adsorbed unto silica gel for column purification. Pure compound **20** (0.24g, 0.44mmols, 92%) elude in 100% chloroform and was striped of solvent to give a light colorless oil. ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 3H), 2.16 (s, 3H), 2.22 (s, 3H), 4.42 (m, 1H), 4.53 (m, 2H), 5.99 (t, J = 5.5 Hz, 1H), 6.15 (q, J = 1.0 Hz, 1H), 6.21 (d, J = 4.0 Hz, 1H), 7.20 (q, J = 1.5 Hz, 1H), 7.30 (q, J = 2.0 Hz, 1H), 7.51 (q, J = 4.0 Hz, 1H), 7.66 (q, J = 1.0 Hz, 1H), 8.18 (m, 2H), 8.69 (q, J = 2.5 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 20.5, 20.6, 20.7, 62.9, 70.3, 73.4, 80.1, 87.4, 127.8, 128.2, 128.7, 128.9, 129.5, 131.2, 132.8, 139.9, 142.9, 143.5, 150.4, 152.0, 156.1, 169.4, 169.5, 170.5.

2,6-Di-(2-thienyl)-9-(β-D-ribofuranosyl)purine (21).

Compound **20** (0.27g, 0.49mmols) was dried in a 50 ml round bottom flask on a vacuum pump overnight. It was then taken up in anhydrous methanol (20ml). A copper wire was tightly

tied around the septum affixed on the round bottom flask to withstand the pressure of the ammonium gas during the course of the reaction. The reaction mixture was saturated with ammonia gas for 1 hour at 0^{o} C. The reaction was stirred at room temperature overnight. After the reaction, the product mixture was stirred for 30 minutes to expel the residual ammonia gas. The crude compound was adsorbed unto silica gel and purified by column chromatography. The UV active compound eluting in 3% methanol/chloroform was collected in vials. The combined fractions containing the product was striped down to 3ml; 10ml of 50:50 methanol/water was added to redissolve the product with the aid of sonication. 2 X 6.5ml of this solution was then purified by reverse phase HPLC using methanol/water on a Waters Deltapak 300 x 50mm I.D. C18, 15μm, 100Å column. The solution containing pure compound was reduced in volume on a Rotovap and lyophilized to afford compound **21** (0.18g, 0.44mmols, 90%) as a white powder. ^{1}H NMR (Me_2SO-d_6) δ 3.61 (m, 1H), 3.73 (m, 1H), 3.99 (q, 1H), 4.27 (q, 1H), 4.75 (q, 1H), 5.05 (t, J = 5 Hz, 1H), 5.30 (d, J = 5 Hz, 1H), 5.58 (d, J = 6 Hz, 1H), 6.05 (d, J = 6 Hz, 1H), 7.23 (q, J = 4.0 Hz, 1H), 7.36 (q, J = 3 Hz, 1H), 7.75 (q, J = 1.5 Hz, 1H), 7.95 (q, J = 1.5 Hz, 1H), 8.0 (q, J = 1.5 Hz, 1H), 8.64 (q, J = 1.5 Hz, 1H), 8.84 (s, 1H); ^{13}C NMR (Me_2SO-d_6) δ 61.5, 70.5, 73.4, 85.8, 87.6, 127.3, 128.4, 128.5, 129.1, 130.6, 132.1, 132.6, 139.4, 142.9, 145.5, 148.7, 152.5, 154.6; m.p. 202˚ C - 204˚ C; UV (MeOH), 205nm (ε 15,737), 262nm (ε 13,447), 304nm (ε 27,237); HRMS (FAB) calcd for $C_{18}H_{17}N_4O_4S_2$ $[M + H]^+$ 417.0691, found 417.0689.

2-Vinyl-9-[(2′, 3′, 5′-tri-O-acetyl)-β-D-ribofuranosyl]hypoxanthine (22).

A mixture of dry compound **13** (2.40g, 4.61mmols), $Pd(CH_3CN)_2Cl_2$ (0.06g, 0.23mmols), and tributyl(vinyl)tin (6.7ml, 23.05mmols) in 15ml of anhydrous DMF was heated at 90˚C for 4 hrs. After the reaction, DMF was removed under reduced pressure, chloroform (150ml) was added and palladium filtered off on celite column. The product mixture was washed with water

(3 x 50ml), dried over anhydrous Na_2SO_4, and adsorbed unto silica gel. The product was purified on a column using an ethyl acetate/hexanes solvent system. Pure product eluted in 90% ethyl acetate/hexanes to afford the 2-vinyl compound **22** (1.78g, 4.24mmols, 92% yield) as clear oil with a tint of yellow. ^{1}H NMR ($CDCl_3$) δ 2.10 (s, 3H), 2.14 (s, 3H), 2.17 (s, 3H), 4.35 (m, 1H), 4.47 (m, 2H), 5.81 (t, J = 5.0 Hz, 1H), 5.91 (q, J = 2.0 Hz, 1H), 5.99 (t, J = 4.5 Hz, 1H), 6.13 (d, J = 4.5 Hz, 1H), 6.81 (m, 2H), 8.0 (s 1H), 13.21 (br, s, 1H); ^{13}C NMR ($CDCl_3$) δ 20.5, 20.6, 20.8, 62.8, 70.1, 73.4, 79.9, 87.1, 124.0, 126.7, 129.6, 139.2, 149.2, 152.9, 159.2, 169.4, 169.6, 170.4.

2-Vinyl-9-(β-D-ribofuranosyl)hypoxanthine (23).

Compound **22** (0.39g, 0.92mmols) was dried in a 50 ml round bottom flask on a vacuum pump overnight. It was then taken up in anhydrous methanol (20ml). A copper wire was tightly tied around the septum affixed on the round bottom flask to withstand the pressure of the ammonium gas during the course of the reaction. The reaction mixture was saturated with ammonia gas for 1 hour at 0^o C. The reaction was stirred at room temperature overnight. After the reaction, the product mixture was stirred for 30 minutes to expel the residual ammonia gas. The crude compound was adsorbed unto silica gel and purified by column chromatography. The UV active compound eluting in 8% methanol/chloroform was collected. The combined fractions containing the product was pure and did not need any further purification. This afforded the deprotected nucleoside **23** (0.19g, 0.63mmols, 70%) as a white powder. ^{1}H NMR (Me_2SO-d_6) δ 3.54 (m, 1H), 3.64 (m, 1H), 3.92 (q, 1H), 4.13 (q, 1H), 4.52 (q, 1H), 5.03 (t, J = 5.5 Hz, 1H), 5.23 (d, J = 5 Hz, 1H), 5.48 (d, J = 6.0 Hz, 1H), 5.80 (q, J = 1.5 Hz, 1H), 5.86 (d, J = 6.0 Hz, 1H), 6.56 (m, 2H), 8.32 (s, 1H), 12.41 (s, 1H); ^{13}C NMR (Me_2SO-d_6) δ 61.4, 70.5, 73.9, 85.7, 87.2, 123.4, 125.4, 129.6, 139.4, 148.9, 151.8, 156.9.

2-(2-Trimethylsilylethynyl)-6-amino-9-β-D-ribofuranosyl)purine (24).

Anhydrous DMF (8ml) was added to a very dry sample of **5** (0.55g, 1.39mmol), and CuI (0.02g, 0.11mmols) under argon with stirring. $Pd(PPh_3)_4$ (0.08g, 0.05mmols) dissolved in 1ml of DMF, and triethylamine (0.2ml, 1.53mmols) were then added followed by the addition of trimethylsilylacetylene (0.8ml, 5.56mmols) all under argon. A condenser was affixed with argon flush and the reaction mixture put in an 80^0C oil bath and run for 4 hours. After the reaction, DMF was removed under vacuum and the product mixture was taken up in a chloroform/methanol system and adsorbed unto silica gel for column purification. Pure compound **24** (0.40g, 1.08mmols, 77%) elude in 5% methanol/chloroform and was striped of solvent to give a light brown powder. ^{1}H NMR (MeOH-d_4) δ 0.21 – 0.26 (m, 9H), 3.79 (m, 2H), 4.17 (s, 1H), 4.34 (d, 1H), 4.69 (t, 1H), 5.98 (d, 1H), 8.40 (s, 1H).

2-Ethynyl-6-amino-9-(β-D-ribofuranosyl)purine (25).

Compound **24** (0.33g, 0.90mmols) and Et_4NF (0.18g, 1.17mmols) were dried on a vacuum pump overnight. Anhydrous THF (10ml) was added under nitrogen and the reaction was run for 3hrs at room temperature under a nitrogen atmosphere. After the reaction, the product mixture was adsorbed unto silica gel and the crude product purified by column chromatography. Pure compound **25** (0.21g, 0.71mmols, 79%) eluded in 5% methanol/chloroform as a white powder. ^{1}H NMR (Me_2CO-d_6) δ 3.13 (m, 1H), 3.22 (m, 1H), 3.51 (q, 1H), 3.59 (s, 1H), 3.68 (q, 1H), 4.10 (q, 1H), 4.76 (t, J = 8.5 Hz, 2H), 5.03 (d, J = 10.5 Hz, 1H), 5.40 (d, J = 10 Hz, 1H), 7.10 (br s, 2H), 7.99 (s, 1H).

1-Cyclohexenyl-1-yl triflate (27).

A mixture of cyclohexanone (3.30ml, 32.13mmols) and anhydrous pyridine (3ml, 35.34mmols) in 50ml CH_2Cl_2 was stirred at -78^0C for 30 minutes. Triflic anhydride (5ml,

35.34mmols) in 20ml of CH_2Cl_2 was added over a 1 hour period under a nitrogen atmosphere. The reaction mixture was allowed to warm to RT and run for 24 hours. After the reaction, the solvents were removed under reduced pressure and the residue taken up in pentane and filtered. The filtrate was dried over $MgSO_4$ and concentrated to afford 1-cyclohexenyl-1-yl triflate (2.96g, 12.85mmols, 40%) as clear oil. ^{1}H NMR ($CDCI_3$) δ 1.59 (m, 2H), 1.75 (m, 2H), 2.16 (m, 2H), 2.30 (m, 2H), 5.73 (m, 1H).

2-[2-(1-Cyclohexenyl)ethynyl]adenosine (28).

Compound **25** (0.14g, 0.47mmols), CuI (0.007g, 0.038mmols), and LiCl (0.002g, 0.05mmols) was dried on a vacuum pump overnight. $Pd(PPh_3)_4$ (0.027g, 0.023mmols) in 1ml DMF, triethylamine (0.07ml, 0.52mmols), and compound **27** (0.43g,1.88mmols) were added under a nitrogen atmosphere with stirring. The reaction mixture was then put in an 80^{o}C oil bath and the reaction run for 8 hours. After the reaction, DMF was removed under reduced pressure and the residue taken up in 100ml EtOAc. The organic layer was washed with 3 x 30ml of Na EDTA solution, dried over $MgSO_4$ and adsorbed unto silica gel. Pure compound **28** (0.14g, 0.4mmols, 85%) eluted in 10% methanol/chloroform solvent system. ^{1}H NMR (Me_2SO-d_6) δ 1.61 (m, 4H), 2.30 (m, 4H), 3.66 (m, 2H), 3.96 (q, 1H), 4.15 (q, 1H), 4.52 (q, 1H), 5.08 (t, 1H), 5.22 (d, 1H), 5.51 (d, 1H), 5.95 (d, 1H), 6.40 (br s, 1H), 8.67 (s, 1H); m.p. 119˚ C - 120˚ C; UV (EtOH), 297nm (ε 17,300), 311nm (sh) (ε 13,100); HRMS (FAB) calcd for $C_{18}H_{22}N_5O_4$ $[M + H]^+$ 372.1672, found 372.1671.

6-Methoxy-2-vinyl-9-[(2′,3′,5′-tri-O-acetyl)-β-D-ribofuranosyl]purine (29)

A 50ml round bottom flask was charged with compound **2** (0.11g, 0.26mmols) and 50mg of K_2CO_3 and dried on a vacuum pump overnight. 10ml anhydrous methanol was added and the reaction was stirred for 8 hours at room temperature. After the reaction, the crude compound was

adsorbed unto silica gel and purified by column chromatography. The combined fractions that eluted in 8% methanol/chloroform were striped of solvent and dried to afford the pure nucleoside **29** (0.06g, 0.21mmols, 80% yield) as a white powder. ^{1}H NMR (Me_2SO-d_6) δ 3.57 (m, 1H), 3.69 (m, 1H), 3.97 (d, 1H), 4.13 (s, 3H), 4.17 (d, 1H), 4.61 (d, 1H), 5.16 (s, 1H), 5.27 (d, 1H), 5.52 (d, 1H), 5.74 (d, 1H), 5.98 (d, J = 6.0 Hz, 1H), 6.57 (d, 1H), 6.78 (q, 1H), 8.59 (s, 1H); ^{13}C NMR (Me_2SO-d_6) δ 54.4, 63.6, 73.2, 73.5, 88.0, 92.6, 120.7, 125.4, 134.9, 142.2, 150.0, 158.8, 159.4; HRMS (FAB) calcd for $C_{13}H_{17}N_4O_5$ $[M + H]^+$ 309.1199, found 309.1200.

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