SYNTHESIS OF DINUCLEOSIDE TETRAPHOSPHATES AND THEIR ANALOGS

by

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and approved by

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ABSTRACT OF THE DISSERTATION

SYNTHESIS OF DINUCLEOSIDE TETRAPHOSPHATES

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Dinucleoside polyphosphates, Np_nN' (n = 2 – 7), are observed in many biological processes and are recognized as playing important regulatory functions. Ap_4A is a particularly important signaling molecule. $AZTp_4A$ is the product of excision of AZT by HIV-1 RT from a growing chain of viral DNA, and can be used to study details of the excision process. Its hydrolysis-resistant analogs can be used to study the inhibition of AZT excision. Current methods to prepare dinucleoside tetraphosphates, enzymatic or chemical, are not satisfactory.

A convenient and high-yield method was developed to prepare dinucleoside tetra- and pentaphosphates. The method was based on a mixture of P(III) and P(V) chemistry and involved a trimetaphosphate intermediate. Six dinucleoside tetraphosphates and two pentaphosphates were prepared. In addition, the method was modified to prepare thio, seleno, borano, methylene and difluoromethylene analogs of Ap_4A and $AZTp_4A$. These analogs are good model compounds for their unmodified versions. Ap_4A analogs can be

used to study the mechanisms of its molecular signaling processes. The AZTp_4A analogs are currently being used in HIV-1 RT drug resistance studies. The configurations of the diastereomers of the thio, seleno and borano analogs were assigned based on the elution order on reverse-phase HPLC. Enzymatic degradation of the Ap_4A analogs was done with snake venom phosphodiesterase in order to confirm the configuration assignments.

Furthermore, this method was extended to prepare multimodified hydrolysis-resistant AZT$p_S p_{CX2} p p_S$A and AZT$p_S p_{CX2} p p_S$AZT (X = H or F) as potential inhibitors of the AZT excision reaction by HIV-1 RT. The reactions were carried out by the coupling of adenosine or AZT H-phosphonate with trimetaphosphate analogs, taking advantage of the differential power of elemental sulfur to sulfurize H-phosphonate diesters but not monoesters. Enzymatic degradation was performed in order to assign configurations of the diastereomers and compare their enzymatic stabilities.

DEDICATION

This Work is dedicated to my parents, Yongzhen Han and Aiyun Shi, my wife, Xuejun Sun, and my son, Eric Bolin Han.

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LIST OF ABBREVIATIONS

A	adenosine
aaRS	aminoacyl-tRNA synthetase
Ac	acetyl
ADP	adenosine diphosphate
AIDS	acquired immune deficiency syndrome
Ap_4A	diadenosine 5′,5‴-P^1,P^4-tetraphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AZT	3′-azido-3′-deoxythymidine
Bu	butyl
calcd.	calculated
CBC	cap binding complex
CDI	1,1′-carbonyldiimidazole
DCC	dicyclohexyl carbodiimide
DCU	dicyclohexylurea
BH_3-DIPEA	borane-*N*,*N*-diisopropylethylamine complex
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
DMT	dimethoxytrityl
DNA	deoxyribonucleic acid
dT	thymidine

equiv.	equivalent
ESI/MS	electrospray ionization/mass spectrometry
Fhit	fragile histidine triad
G	guanosine
GMP	guanosine monophosphate
HIV	human immunodeficiency virus
HMPA	hexamethylphosphoramide
HPLC	high performance liquid chromatography
I	inosine
iPr	isopropyl
LC/MS	liquid chromatography/mass spectrometry
m/z	mass/charge
NMP	*N*-methylpyrrolidinone
NMR	nuclear magnetic resonance
NPC	nuclear pore complex
NRTI	nucleoside reverse transcriptase inhibitor
RNA	ribonucleic acid
RP	reverse phase
RT	reverse transcriptase
TEA	triethylamine
TEAA	triethylammonium acetate

Chapter 1: Introduction

1. Biological Background

Dinucleoside-5′, 5′′′-P^1, P^n-polyphosphates (Np_nN') are a family of compounds of great biological interest. They are made up of two nucleosides connected by a two-to-seven phosphodiester linkage. As a typical dinucleoside polyphosphate, the structure of A(5′)p_4(5′)A is shown in **Figure 1-1**. In 1953, A(5′)p_2(5′)A and U(5′)p_2(5′)U were detected by Lord Alexander R. Todd's group as side-products of the synthesis of A(5′)p(5′)U.[1] This was the first observed existence of any dinucleoside polyphosphate. About ten years later, natural versions were first observed in the eggs of the brine shrimp *Artemia*.[2,3] However, there was little further progress reported until the 1980's, when it was realized that dinucleoside polyphosphates play important signaling and regulatory roles in the cell metabolism of prokaryotes[4-7] and eukaryotes.[8-10] In 2001, it was proposed that a dinucleoside tetraphosphate AZT(5′)p_4(5′)A is involved in HIV drug resistance.[11-15] Current study of Np_nN's mainly focuses on three aspects: HIV drug resistance, molecular signaling and the RNA cap. Both natural and modified Np_nN's have been used widely to study their functions in various biological processes. They can be synthesized *in vitro* by different enzymes, and this approach has been used to prepare Np_nN's on small scales. However, due to the limits of enzymatic methods, much effort has been devoted to develop efficient chemical methods.

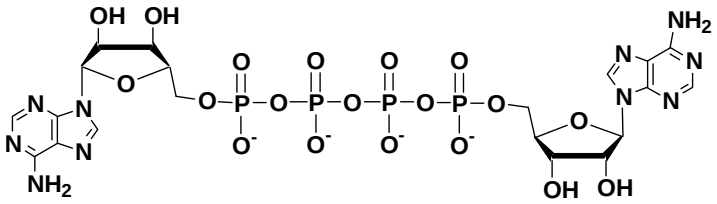


Figure 1-1. The Structure of A(5′)p_4(5′)A

1.1. HIV Drug Resistance

1.1.1. AIDS and AZT

Acquired Immune Deficiency Syndrome (AIDS) is a lethal disease caused by a RNA virus, Human Immunodeficiency Virus (HIV).[16-20] It was first reported on June 5, 1981 in five homosexual men in Los Angeles. According to reports in 2006 by the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO), AIDS has killed over 25 million people since its first report, and over 30 million live with HIV globally, including over 2 million children. December 1 has been named as World AIDS Day since 1988. HIV attacks the human immune system by destroying CD4+ T cells, a white cell that activates the immune system against infections. Without protection by the immune system, the body can develop serious and often deadly infections, called opportunistic infections. It is these various opportunistic infections that kill AIDS patients. Even though currently available treatments might slow the progress of AIDS, there is no vaccine or cure for HIV. **Figure 1-2** shows a typical life cycle of HIV and how it destroys a CD4+ cell (http://www.aidsmeds.com/lessons/LifeCycle2.htm).

1: Binding to a CD4+ cell and fusion inside
2: Reverse transcription of viral RNA to viral DNA
3: Integration of viral DNA into cell DNA
4: Transcription of viral DNA to mRNA with cell DNA
5: Translation of mRNA into viral proteins
6: Assembly and release of new HIV

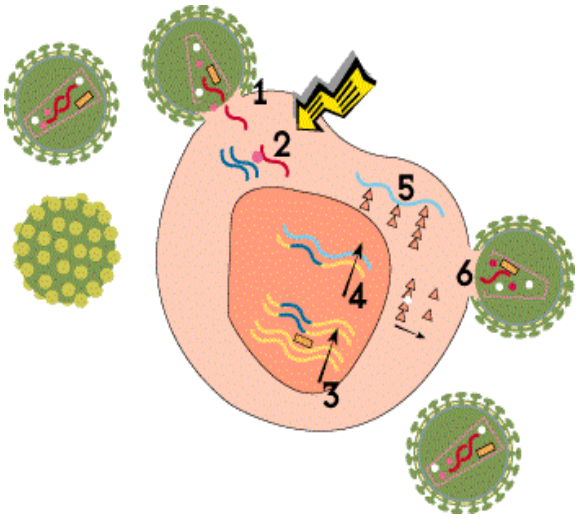


Figure 1-2. Life Cycle of HIV
(Arrow shows where AZT works)

AZT (3′-azido-3′-deoxythymidine, zidovudine) was the first nucleoside analog drug used to treat AIDS, and its structure is shown in **Figure 1-3**. As a nucleoside reverse transcriptase inhibitor (NRTI), AZT prevents the reverse transcription of viral RNA to viral DNA by selectively inhibiting the enzyme HIV reverse transcriptase (RT).[21-23] Thus it works at the reverse transcription step of the HIV life cycle (see **Figure 1-2**). After AZT enters the host cell, it is phosphorylated to a triphosphate (AZT-TP) by the same enzymes from host cells that convert thymidine (dT) to dT-TP. When HIV fuses to the host cell and starts reverse transcription, it cannot distinguish AZT-TP from dTTP, and AZT is incorporated into the viral DNA.[21,22] Since AZT has no 3′-OH, viral DNA chain elongation is terminated and the infection cannot continue (see **Figure 1-3**). AZT was approved to treat AIDS by the FDA on March 19, 1987.

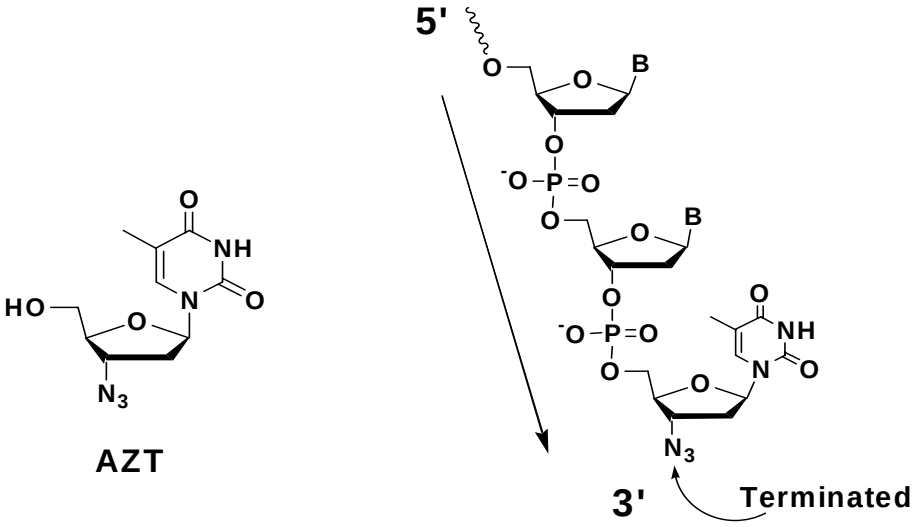


Figure 1-3. AZT Inhibits HIV RT by Terminating Chain Elongation

1.1.2. AZT Drug Resistance

Unfortunately, after the use of AZT by patients for six months or more, HIV develops high-level drug resistance against it by mutations in the HIV RT gene.[24] The mutations

involved include M41L, D67N, K70R, T215Y/F, and K219E/Q.[11,25] Generally there are two possibilities for the cause of AZT resistance of mutated HIV RT. One is decreased AZT-TP incorporation, and the other is increased AZT excision at the terminated viral DNA. Even though some mutations can selectively interfere with the incorporation of AZT, more and more evidence suggests that excision of AZT after its incorporation is the predominant reason for AZT drug resistance. Further investigation revealed that only the terminus at the N-site (incoming dNTP site) of the catalytic center can undergo the excision reaction.[12-15] Standard deoxynucleosides move from the N-site to the P-site (primer site) after being incorporated into the viral DNA, and the next incoming dNTP will occupy the N-site and block excision of the previous nucleotide. However AZT cannot shift to the P-site due to the steric clash of its azido with Asp185 (see **Figure 1-4**).[11] Thus AZT remains at the N-site where it can be excised.

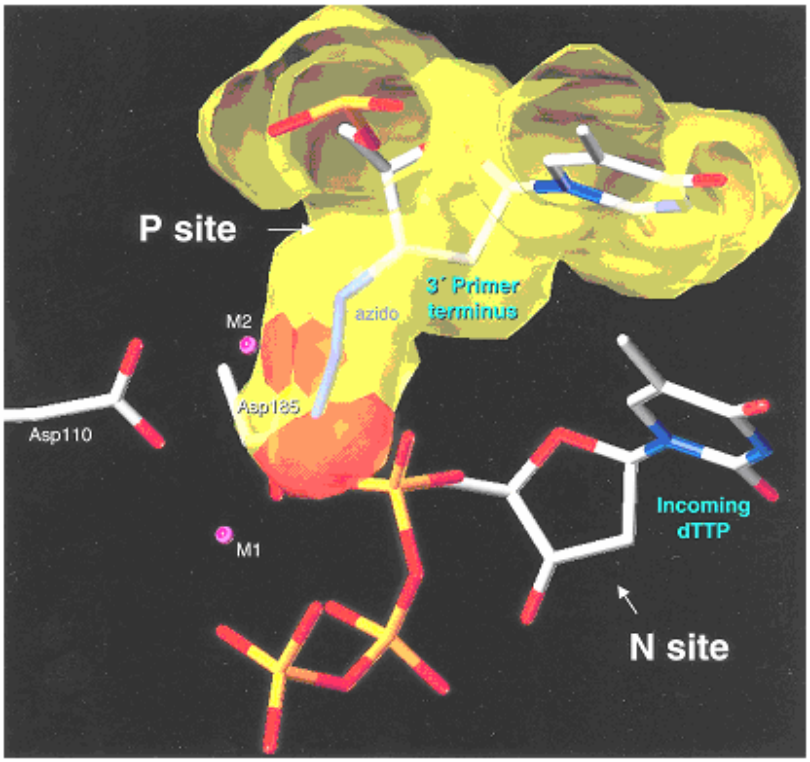
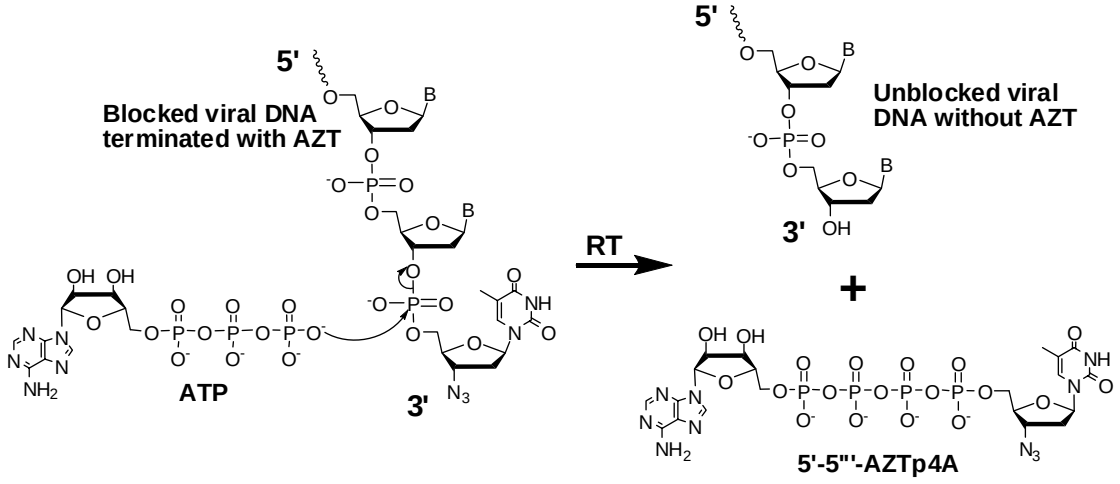


Figure 1-4. Steric Hindrance of End-AZT Translocation From N-site to P-site[11]

The excision of AZT is analogous to a pyrophosphorolysis reaction, with ATP being the pyrophosphate donor. ATP is required for the polymerization step, so is easily available for the excision reaction. The excision process is briefly shown in **Scheme 1-1**. At the HIV RT catalytic site (N site), the terminal phosphate of a nearby ATP attacks the end AZT phosphate. The nucleophilic reaction removes the chain-terminating AZT, and viral DNA elongation resumes. At the same time one molecule of AZT(5′)p_4(5′)A forms.[12,13] During the process, enhanced interaction of ATP with the mutant HIV RT is critical.[11] AZT(5′)p_4(5′)A and its analogs made by chemical synthesis can be used to study details of the excision process. Interactions of AZT(5′)p_4(5′)A with wild type and mutant HIV RT will help to clarify the drug resistance mechanism and provide valuable clues for drug design. Further, its hydrolysis-resistant analogs have been proposed to be potential inhibitors of AZT drug resistance.[15,26-28]



Scheme 1-1. Interaction of ATP with a T215Y AZT-resistant HIV RT

1.2. Molecular Signaling

Naturally occurring Np_nN's are ubiquitously distributed in both cytosol (intracellular Np_nN's) and some secretory granules (extracellular Np_nN's). It has been suggested that they play important signaling and regulatory functions in various tissues.[8,10,29] Their concentrations increase dramatically under cellular stress (such as oxidative stress and heat shock) and they may therefore serve as alarmones.[30-32] However, other studies have suggested that they might simply be toxic byproducts that affect essential enzyme activities.[10,33] Although many natural Np_nN's have been identified, diadenosine polyphosphates are the most abundant and widely studied, and of these, $A(5')p_4(5')A$ and $A(5')p_3(5')A$ are the most important.

1.2.1. Diadenosine Tetraphosphate (Ap_4A)

Paul Zamecnik and his collaborators discovered Ap_4A as a byproduct of a reaction catalyzed by lysyl-tRNA synthetase in 1966.[34,35] About 10 years later they found that the concentration of Ap_4A was directly related to the proliferative state of the cells,[36] and this discovery stimulated study of the signaling and regulatory functions of Ap_4A.[37-41]

The back reaction of aminoacyladenylate with ATP catalyzed by aminoacyl-tRNA synthetases (aaRS) is the main source of intracellular Ap_4A, and in vitro experiments indicated it can be formed through similar reactions catalyzed by firefly luciferase and RNA ligases. Many nonspecific enzymes as well as specific enzymes, such as Ap_4A hydrolase (a MutT motif enzyme), degrade Ap_4A either symmetrically or asymmetrically. It is now recognized that Ap_4A is an important component of the S-phase checkpoint system for DNA damage in the cell cycle (for cell cycle, see **Figure 1-5**[42]). According to

a proposed mechanism, when damage is detected during DNA synthesis, the relevant repair enzymes, including poly(ADP-ribose) polymerase and DNA ligase III, are activated near the damage site. DNA ligase III then synthesizes Ap_4A, which is modified to ADP-ribosylated Ap_4A by poly(ADP-ribose) polymerase. The modified Ap_4A then binds to and inhibits DNA polymerase-α by temporarily forcing polymerase-α away from the DNA by means of the strong negative charge of Ap_4A.[43] Thus the DNA synthesis slows down or stops long enough to allow damage repair. This model of cell growth arrest is shown in **Figure 1-6**.[10] If the damage is too serious and needs a long time to repair, DNA ligase III will keep synthesizing Ap_4A to a level that induces apoptosis (cell self-destruction). Thus Ap_4A helps to avoid production of abnormal cells with damaged genes by regulating the S-phase DNA damage checkpoint. Other studies have suggested that intracellular Ap_4A might also play the role of a secondary messenger to regulate the β-cell ATP-sensitive K^+ channels.[44,45]

M-phase: Mitosis (nuclear division) and cytokinesis (cell division)

G1-phase: Gap between M-pahse and S-phase, cell grows but no DNA replication

S-phase: DNA synthesis phase

G2-phase: Gap between S-phase and M-phase, cell grows to prepare for mitosis

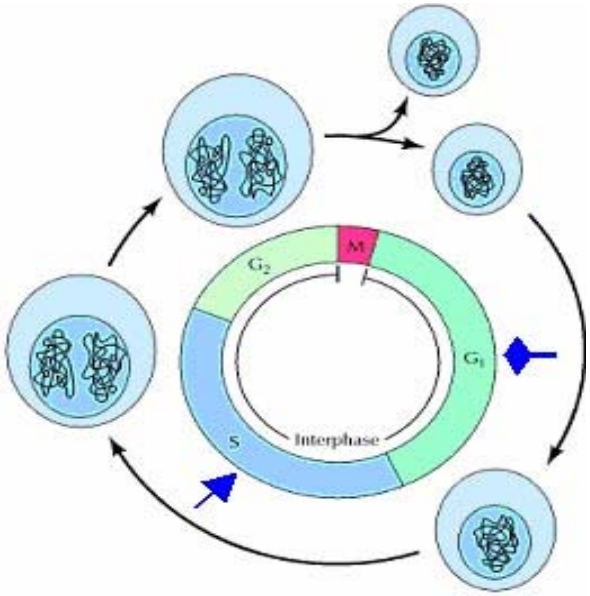


Figure 1-5. The Life Cycle of an Eukaryotic Cell[42]
Arrow shows where Ap_4A works
Diamond arrow shows where Ap_3A works

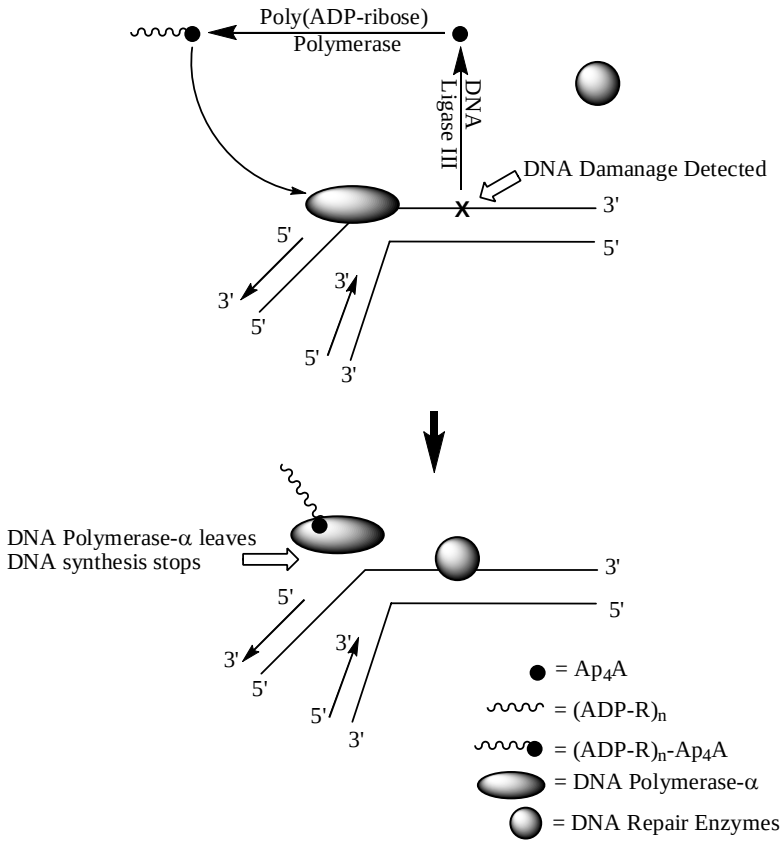


Figure 1-6. Arrest of Cell Cycle at S-phase Checkpoint by Ap_4A[10]

Extracellular Ap_4A has been observed in secretory granules, including adrenal chromaffin granules,[46,47] cholinergic synaptic vesicles,[48] and blood platelet dense granules.[49] The nucleotide receptors on cell surfaces (P2Y and P2X), which are activated by extracellular nucleotides to change the cells' functional state, are sensitive to Ap_4A and other dinucleoside polyphosphates.[50-53] Dinucleotide receptor, also named P4 receptor, is specifically stimulated by dinucleoside polyphosphates, but is not sensitive to mononucleotides.[53,54] Even though there is still controversy about the origin of these dinucleoside polyphosphates,[10,55,56] they do play regulatory functions outside of cells, such as vascular tone,[57,58] platelet disaggregation,[59,60] neurotransmission,[53,61-63] renal Na^+ excretion, and urine flow.[64,65] Since various specific and nonspecific enzymes can hydrolyze Ap_4A,[66,67] its hydrolysis-resistant analogs have been used to study their

potential medical uses. Zamecnik reported $App_{CHCl}ppA$ and $Ap_Sp_{CHCl}pp_SA$ as anti-thrombotic agents.[60,68] Recently Andrew D. Miller's group found that $App_{CH2}ppA$ could control postsynaptic excitation via a NO-dependent mechanism and may be a potent neuroprotective agent.[69]

Divalent metal cations play important roles in regulation of the concentration of dinucleoside polyphosphates, including Ap_4A.[70,71] Mg^{2+} and Zn^{2+} have been shown to be essential for the synthesis of Ap_4A by some aminoacyl-tRNA synthetases.[6] Release of Zn^{2+} might increase the concentration of Ap_4A by activating relevant synthetases and inhibiting hydrolases.[35,70] Divalent metal cations work as enzyme cofactors, or by modulating the conformation of Ap_4A to fit in the active site of relevant enzymes. Ap_4A in solution may exist in three conformations, "open", "folded unstacked" and "folded stacked".[71,72] Zn^{2+} prefers to bind Ap_4A at the N-7 site of adenine and leads to ring-destacking,[70,73] while Mg^{2+} prefers to bind at the phosphate chain and stabilizes a ring-stacking conformation.[74] Miller and coworkers proposed a modulation mechanism involving Zn^{2+} and diadenosine polyphosphates in cells under heat shock stress.[70] The heat shock induces the release of Zn^{2+} stored in metallothioneins,[75,76] which results in the increase of Ap_4A levels through activation of Ap_4A synthetases and inhibition of Ap_4A hydrolases. The subsequent binding of Ap_4A with relevant heat shock stress proteins, such as GroEL and DnaK, activates the proteins to perform protective adjustments.[31]

1.2.2. Diadenosine Triphosphate (Ap_3A)

Ap_3A occurs at high levels in many cells, with 5-10 times higher intracellular concentrations than Ap_4A.[10] However little effort was devoted to its study until the

1990's, when it was demonstrated to play an important role in the tumor suppression process.[29,77,78] Mammalian tryptophanyl-tRNA synthetase specifically synthesizes Ap_3A,[79] and this process can be stimulated by antiproliferative interferons α or γ.[80] Only the specific enzyme Ap_3A hydrolase can degrade Ap_3A efficiently.[81]

Ap_3A hydrolase is a member of the fragile histidine triad hydrolase family (Fhit), which is encoded by the tumor suppression gene FHIT.[82] Tumor cells and tissues have lower levels of Fhit expression and therefore much higher Ap_3A concentrations than normal cells.[77,78] Introduction of Fhit into Fhit-negative tumor cells leads to G_0 or G_1 arrest of cell growth (see **Figure 1-5**), and cells accumulate in the S-phase. This allows enough time for other factors to induce apoptosis. However introduction of Fhit into Fhit-positive normal cells does not affect them.[83,84] The concentration increase of Ap_3A in the Fhit-positive cells also leads to cell growth arrest. Further investigation has revealed that Ap_3A suppresses tumor cells by an Ap_3A•Fhit complex.[66,85-87] In one proposed model, two Ap_3A molecules bind to a pFhit dimer, and the complex initiates the tumor cell suppression signaling process by activating other signaling molecules and preventing tumor cell proliferation.[85,86] However the Ap_3A•Fhit complex has poor stability due to hydrolysis of Ap_3A. Another model was proposed in which 2′,5′-oligoadenylate synthetase was activated together with tryptophanyl-tRNA synthetase by the interferons. After synthesis of Ap_3A, it serves as a primer to form 2′,5′-oligoadenylated-Ap_3A. It is the 2′,5′-oligoadenylated-Ap_3A complex with Fhit (but not Ap_3A•Fhit alone) that inhibits tumor cell growth.[66,87] **Figure 1-7** shows these models for tumor suppression.[9]

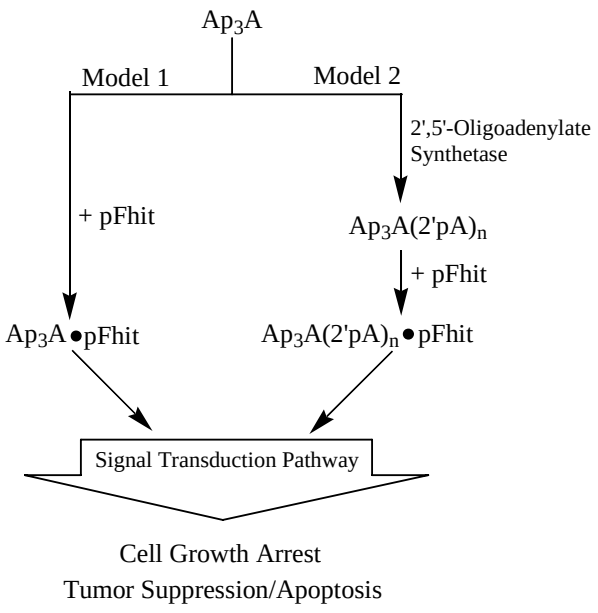


Figure 1-7. Models of Tumor Suppression by Ap_3A•pFhit Complex[9]

In recent work, the very conserved small regulatory protein, ubiquitin conjugating enzyme 9 (Ubc9), was shown to selectively bind the Ap_3A•Fhit complex but not free Fhit.[88,89] The binding interaction was shown to indirectly affect the post-translational modification of Fhit and its translocation to the nucleus. As a result, the Ap_3A•Fhit-Ubc9 interaction stabilized the Ap_3A•Fhit complex and consequently increased antitumor activity.[90-92] Recently Barnes and coworkers found that Y214 in Fhit could be phosphorylated, thereby increasing the stability of the Ap_3A•Fhit complex.[93,94] Due to a competition between Ap_3A and Ap_4A in their interactions with many relevant enzymes, the ratio of Ap_3A/Ap_4A may affect cell growth, proliferation, and apoptosis considerably.[95]

1.2.3. Other Dinucleoside Polyphosphates

Ap_5A and Ap_6A have been detected in isolated perfused rat kidney and possibly play a part in the regulation of blood pressure.[96] Ap_2A and Ap_3A were found in human cardiac

tissue and play a role as endogenous modulators of myocardial function and coronary perfusion.[97] A study of isolated perfused rat mesenteric arterial bed indicated that Ap_5A and Ap_6A elicit vasoconstriction while Ap_2A and Ap_3A elicit vasodilatation.[57] As a good inhibitor of adenylate kinase, Ap_5A might play an important role in the bioenergetics and metabolism of cardiac muscle.[98] Dramatic increases of Ap_5A levels have been seen with ischemia, involving the ATP-sensitive K^+ channel.[99,100] Dinucleotide receptors are specifically sensitive to dinucleoside polyphosphates, and Ap_5A was shown to be the most efficient antagonist.[101]

Although Gp_4G and Gp_3G were the first natural versions of dinucleoside polyphosphates identified, they were only observed in brine shrimp (*Artemia*) eggs[2,3] and the encysted embryos of the related crustacean, *Dophnia magna*. Gp_4G is formed by GTP:GTP guanylyltransferase[10,102-104] and degraded by specific and nonspecific hydrolases.[3] Gp_nG and Ap_nG (n = 3 - 6) were later found in blood platelet dense granules, and Gp_5G and Gp_4G are thought to effectively stimulate P4 receptors.[101] Recently Gp_5G was detected in human plasma at a level of 9 ± 5 nmol/L, which is high enough to modulate vascular tone. Study of the isolated perfused rat kidney has shown that Gp_5G is involved in blood pressure regulation.[105]

1.3. RNA Cap

During transcription of DNA to RNA that is catalyzed by RNA polymerase II in eukaryotes, several important modifications take place on the nascent RNA strand. One of them is the addition of a “cap” at the 5′-end of the mRNA precursor which consists of a 7-methylated guanosine whose 5′-hydroxyl is connected to the 5′-end of the RNA

through a triphosphate chain (**Figure 1-8**).[106,107] The cap structure was first identified in 1974.[108] Since its discovery its formation and important roles in various biological processes have been extensively studied.[106,109-115] Capping of RNA occurs at an early stage of transcription through a three-step reaction, starting with RNA with a triphosphate at its 5′-end as shown in **Scheme 1-2**.[116] The terminal phosphate is removed and the resulting diphosphate reacts with GTP to form a new triphosphate linkage in which the α- and β-phosphates come from the 5′-end of RNA, and γ-phosphate comes from the added capping guanosine triphosphate. In the last step the capping guanosine is methylated at N7. In addition to the N7-methylation of the capping guanosine, the 2′-OH groups of the first several nucleotides of RNA are also methylated in some animal cells.[117] The enzymes that catalyze cap formation are attached to RNA polymerase II before transcription starts. Many different cap analogs have been used to study its biological interactions.[118-120]

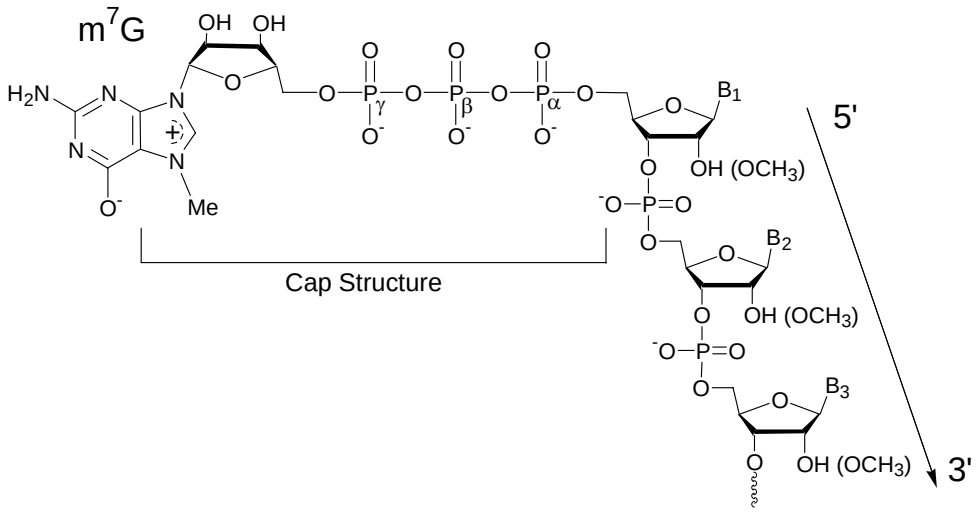
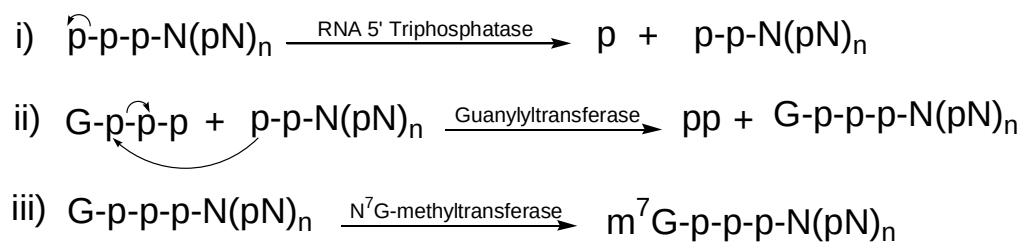


Figure 1-8. The Structure of a Cap on a RNA Precursor



Scheme 1-2. Capping Reaction of the 5′-end of a RNA

The 5′-end cap of mRNA plays important roles in the entire transcription and translation process. In the nucleus, the cap protects the nascent mRNA from nucleases that hydrolyze nucleic acids.[116,121] Following transcription, the cap interacts with cap binding proteins, which activate splicing of pre-mRNA and promote addition of a poly A tail at the 3′-end.[122,123] During RNA export from the nucleus to the cytoplasm, capped RNA is the exclusive substrate of the cap binding complex (CBC), which interacts with the nuclear pore complex (NPC) and facilitate export.[124] After export, the cap structure protects mRNA from degradation by various mRNA hydrolysis enzymes.[125,126] During initiation of translation, the N7-methylated guanosine interacts with the translation initiation factor eIF-4F (Eukaryotic Initiation Factor 4F), and ribosomes gather and attach to the mRNA, allowing the translation process to start.[127-129]

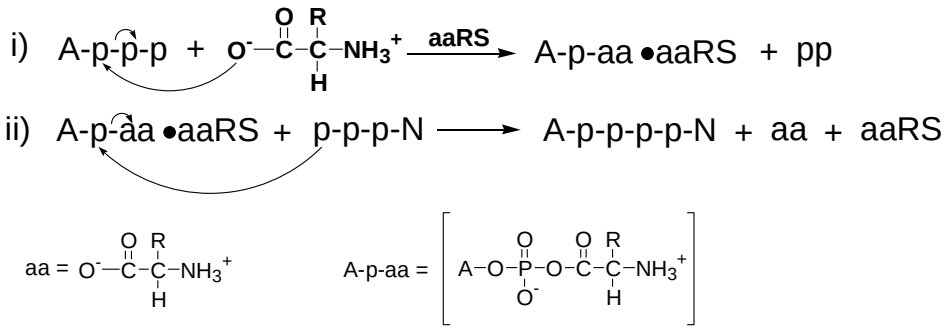
2. Synthetic Background

Even though Np_3N's and Np_4N's are very important compounds, existing methods to synthesize them have serious drawbacks. Current methods involve either enzymatic synthesis[35,130-133] or chemical synthesis.[134-136]

2.1. Enzymatic Methods

In 1966 Ap_4A was found to be formed in a reaction of aminoacyl adenylate with ATP and lysyl-tRNA synthetase.[35] This was the first time that Ap_4A was prepared by enzymatic methods *in vitro*. Later more and more enzymes were reported to catalyze the synthesis of dinucleoside polyphosphates and even some of their analogs *in vitro* and *in vivo*.[10,71,137-141] Now *in vitro* enzymatic methods are widely used in many labs to prepare dinucleoside polyphosphates and their analogs in small amounts.

Several classes of enzymes have been reported to synthesize Np_3N's and Np_4N's with different substrate-specificity and efficiency. The first is the family of aminoacyl-tRNA synthetases (aaRSs).[10,35,140-142] They are the first enzymes discovered that can catalyze the synthesis and are the most studied. Usually aaRSs catalyze the aminoacylation of tRNA through a two-step reaction. ATP first reacts with an amino acid to form an aminoacyl-adenylate, which then reacts with tRNA to give an aminoacyl-tRNA.[143] However, when NTPs or NDPs are available during the second step, they will react with the adenylate phosphate of the aminoacyl-adenylate still bound to the synthetase to form Ap_4N.[35,130-133] The process is known as a “back-reaction” and is shown in **Scheme 1-3**. Divalent metal cations are required by many aaRSs, and Zn^{2+} activates some aaRSs very efficiently.[71,141] Amino acids that have been used include Trp, Leu, Ala, Lys, Val, Phe and Met, with their corresponding aaRSs. It is noteworthy that aaRSs have different specificities, and tryptophanyl-tRNA synthetase only catalyzes the synthesis of Ap_3A.[79]



Scheme 1-3. Enzymatic Synthesis of Ap_4N

A recently-discovered type of enzyme that catalyzes formation of dinucleoside polyphosphates is firefly luciferase, which is an oxidoreductase with similar properties as a ligase.[138,144] Firefly luciferase normally catalyzes a light emission reaction with luciferase•luciferin•AMP as an intermediate. When NTP or NDP is available, the luciferase•luciferin•AMP complex will convert to luciferase•dehydroluciferin•AMP and then react with NTP or NDP to give Ap_4N or Ap_3N. Firefly luciferase has a broad substrate specificity, and in forcing conditions, it can catalyze the synthesis of almost any Np_4N', even some analogs. A third class of enzymes are Ap_4A phosphorylases, which were first found to hydrolyze Ap_4A to ADP and ATP by a phosphorolytic reaction. Since the reaction is reversible, these enzymes can catalyze the reaction of ADP with ATP to give Ap_4A.[145,146] Later research showed they can produce a variety of Np_4N's.[146,147] The fourth class are guanylyltransferases from several sources.[148-150] They can synthesize Gp_4N and Gp_3N specifically. Finally, several ligases, such as T4 DNA ligase and T4 RNA ligase, have also been reported to synthesize Np_4N's.[138] Acetyl-CoA synthetase and acyl-CoA synthetase are also thought to be involved in Np_4N' synthesis.[138]

Some enzymes have loose specificity and can be employed to prepare Np_4N' analogs.[137,138] These analogs may be resistant to hydrolysis or phosphorolysis and have

an advantage over natural Np_4N's with shorter lifetimes in various biological systems.[140] One type of modification is at a bridging oxygen. Most aaRSs and firefly luciferase can accomodate such a modified NTP as the adenylate acceptor. For example, $AppCH_2p$ and $ApCH_2pp$ react with aminoacyl adenylate to form $AppCH_2ppA$ and $ApCH_2pppA$ respectively.[137,151] A second type of modification is at a non-bridging oxygen, such as the α-monothiophosphate, Ap_SpppA. Lysyl-tRNA synthetase, yeast Np_4N' phosphorylase and firefly luciferase have been reported to be able to use adenosine α-thiotriphosphate (Ap_Spp) as the adenylate acceptor.[138,152] [S_P]-Ap_Spp reacts faster than [R_P]-Ap_Spp for lysyl-tRNA synthetase, and [R_P]-Ap_Spp reacts faster than [S_P]-Ap_Spp for Np_4N' phosphorylase. A third category are modified at the nucleosides. When 1,N^6-ethenoadenosine triphosphate (εAppp), dAppp, ddAppp and araAppp are used as acceptors (**Figure 1-9**), εAppppA (fluorescent), dAppppA, ddAppppA and araAppppA are formed, respectively.[35,153-155] If a given modified nucleoside triphosphate is the sole NTP, the double modified nucleoside tetraphosphate forms, for example εAppppεA.[138,153,156] Recently 2′-*O*-[*N*-methylanthraniloyl]adenosine triphosphate (mant-Appp) and 8-bromo-2′-*O*-[*N*-methylanthraniloyl]adenosine triphosphate (Br-mant-Appp) were used to make the corresponding fluorescent-labeled Ap_4A analogs with lysyl-tRNA synthetase.[157,158] Finally, additional groups can be added to the dinucleoside tetraphospahtes post-synthetically. For example 2′,5′-poly(A) synthetase was reported to incorporate poly-dA residues at the 2′-position of Ap_4A,[66] and poly(A) polymerase incorporated poly-A residues at the 3′-position.[159] Adenosine-phosphate deaminase was used to produce Ip_nN from the corresponding Ap_nN.[160]

Nevertheless, work on these enzymatic syntheses has usually been done to investigate the activities of the relevant enzymes, rather than to develop synthetic methods. Furthermore, since the catalyzing enzymes are substrate-specific, only limited nucleotides can be synthesized.[140,141] Enzymatic synthesis can only be done on extremely small scales, and the products are usually complicated and hard to separate. Therefore, dinucleoside polyphosphates are usually very expensive, and an efficient and general chemical method is highly desired.

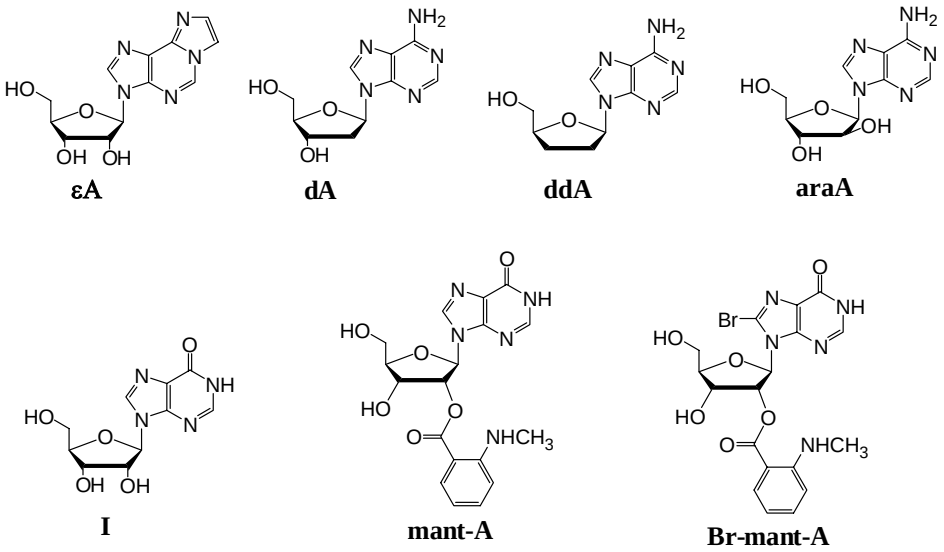
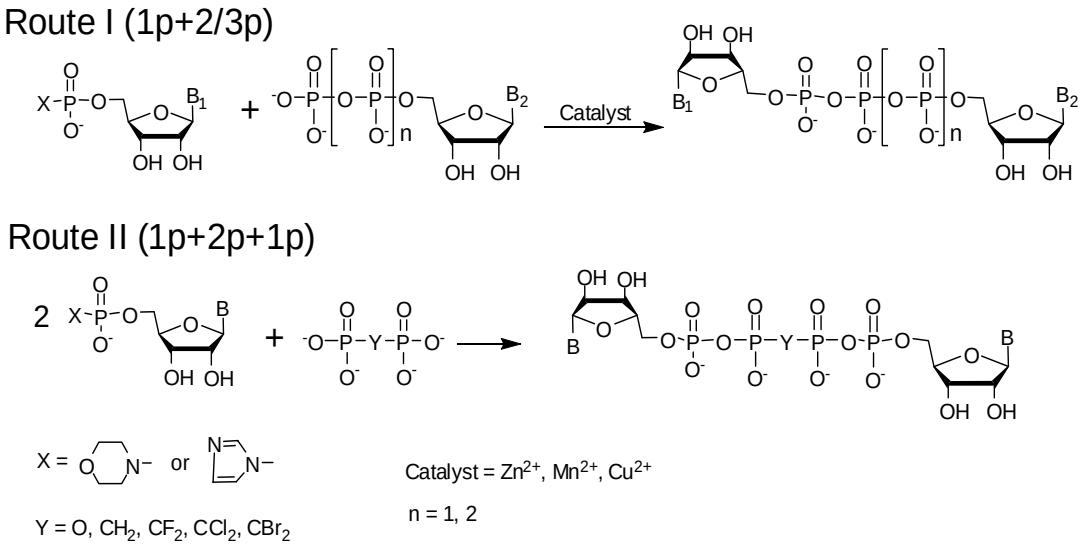


Figure 1-9. Structures of *ε*A, dA, ddA, araA, I, mant-A and Br-mant-A

2.2. Chemical Methods

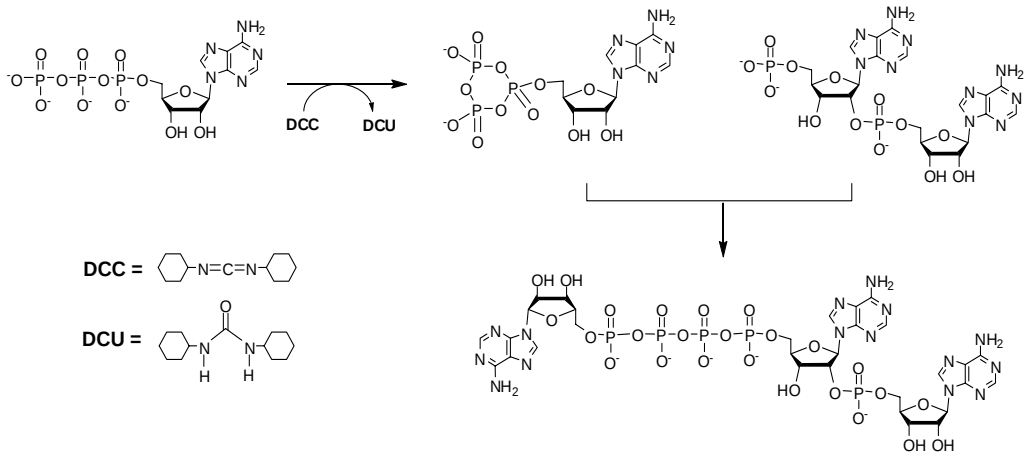
The first chemical synthesis of dinucleoside polyphosphates was reported in 1965, when J. G. Moffatt's group made dinucleoside tri and tetraphosphates in the dismutation reactions of nucleoside polyphosphates.[134,135] As more and more regulatory and signaling functions of Np_4N's were discovered, much effort has been devoted to developing an efficient chemical method to prepare Np_4N's and their analogs.[71,137,140,158,161]

The current methods for chemical synthesis of Np_4N's all involve P(V) chemistry and the coupling of a nucleoside phosphate with an activated nucleoside phosphate. The first method uses a phosphoromorpholidate or phosphorimidazolidate.[135,136,162-165] (see **Scheme 1-4**) In 1965 Moffatt coupled pyrophosphate with two equivalents of a nucleoside phosphoromorpholidate to prepare symmetrical dinucleoside tetraphosphates (**Route II**).[135] During the long reaction, pyrophosphate reacted with one nucleoside phosphoromorpholidate at each end to give the product in low yield. This approach was later used by Blackburn to synthesize various nucleoside triphosphates and dinucleoside tetraphosphates modified at the bridging oxygen with a methylene or halomethylene group.[136,166,167] Nucleoside phosphorimidazolidates were first used in 1965 by Hoard and Ott to couple with a nucleoside monophosphate to make dinucleoside diphosphates (**Route I**).[162] The nucleoside phosphorimidazolidate was generated *in situ* by the reaction of 1,1′-carbonyldiimidazole (CDI) and a nucleoside monophosphate. This method was later employed to prepare dinucleoside tri- to hexaphosphates.[168,169] Carbonyldiazoles and carbonyldibenzimidazole have been used to replace CDI in the reaction.[170,171] The coupling of a pyrophosphate or diphosphonate with two equivalents of nucleoside phosphorimidazolidate have been reported.[26,172] Although the yields were poor (~20%) in the earliest reports, Sawai has improved the reaction with the use of metal catalysts and polar organic solvents, usually DMF,[163,165] and the nucleoside phosphorimidazolidate was obtained through the condensation of imidazole with nucleoside monophosphate activated by dithiopyridine and triphenylphosphine.[173]



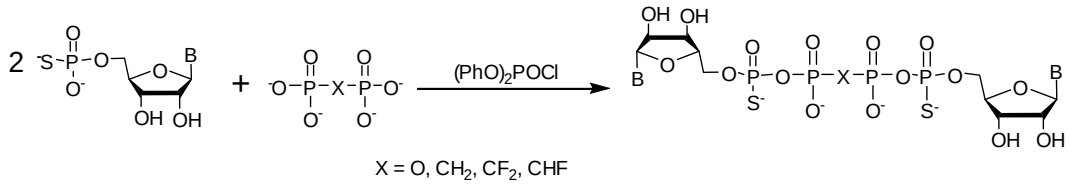
Scheme 1-4. Synthesis of Standard and Modified Dinucleoside Polyphosphates Using Phosphoromorpholidate or Phosphorimidazolidate

A second method involves activation of a nucleotide by a carbodiimide. Ng and Org employed 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 1-cyclohexyl-3-(β-[*N*-methylmorpholino]ethyl)-carbodiimide *p*-toluenesulfonate to synthesize a full range of diadenosine polyphosphates, and an isourea was suggested to be the critical intermediate.[164] Another coupling reagent is dicyclohexylcarbodiimide (DCC), which was used to prepare Ap_3G with a poor yield.[174,175] DCC was also found to be able to promote the cyclization of a linear nucleoside triphosphate. As a result, a cyclic trimetaphosphate was formed and DCC was converted to dicyclohexylurea (DCU).[176,177] A subsequent reaction with nucleoside mono and diphosphate produced dinucleoside tetra and pentaphosphates, respectively.[178,179] (see **Scheme 1-5**) Recently trimetaphosphates have been used in the chemical synthesis of several standard and modified mono and dinucleoside polyphosphates.[180-183] Even though better procedures have been developed to make trimetaphosphates,[180,183,184] the DCC method is still in use.



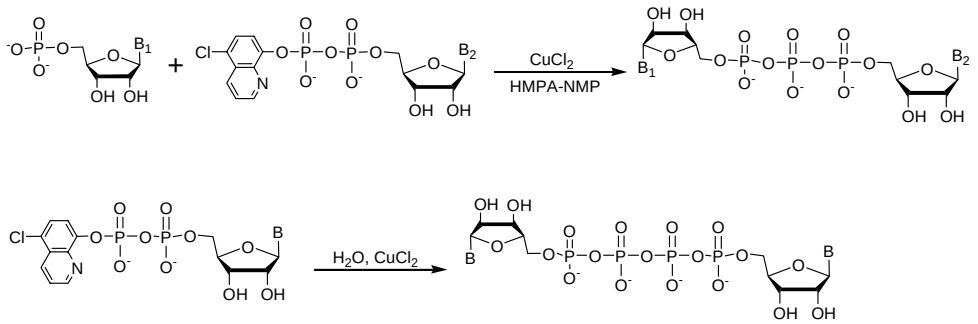
Scheme 1-5. Synthesis of Ap_4ApA Using DCC and Trimetaphosphate[178]

A third method uses diphenyl phosphorochloridate to activate a nucleotide by the Michelson procedure.[185,186] Even though mononucleoside and dinucleoside polyphosphates have been produced by this method, it was mainly employed to make thiophosphates. Imbach prepared various dinucleoside monothiopolyphosphates, such as Ap_SpppA, by the phosphorochloridate-activated coupling of a nucleoside thiophosphate with mononucleoside polyphosphate.[187,188] They found that the [R_P]-diastereomer of the dinucleoside monothiopolyphosphates always had a longer retention time on reverse phase HPLC than the [S_P]-diastereomer. Using the same approach, Blackburn and coworkers focused on dinucleoside dithiopolyphosphates.[189,190] With diphenyl phosphorochloridate as activator, a phosphate, pyrophosphate or diphosphonate was reacted with two equivalents of nucleoside thiophosphate to produce symmetrical hydrolysis-resistant dithio-analogs of Np_nNs, including Ap_Spp_SA, Ap_Sppp_SA and Ap_SpXpp_SA (X = CH_2, CF_2, CHF) respectively (see **Scheme 1-6**). The [S_P,S_P]-diastereomer was found to be very hydrolysis resistant to snake venom phosphodiesterase and had the shortest retention time on reverse phase HPLC. Related work was reported involving the coupling of two ^{17}O and ^{18}O-labeled adenosine monothiodiphosphates.[191]



Scheme 1-6. Synthesis of Dithio-Analogs of Dinucleoside Tetraphosphates

In a fourth method, Hata introduced a route involving the quinolinide of a nucleoside diphosphate.[192,193] Its coupling with a nucleoside monophosphate using $CuCl_2$ as a catalyst in a mixture of hexamethylphosphoramide (HMPA) and *N*-methylpyrrolidinone (NMP) forms Np_3N'.[192] Symmetrical Np_4Ns can be made by the *in situ* dimerization of the partially hydrolyzed diphosphate quinolinide in the presence of $CuCl_2$ in an aqueous solution.[193] (see **Scheme 1-7**) Using a related method, in 1987 Schwartz synthesized a dinucleoside diphosphate by the condensation of a nucleoside *S*-4-methylphenyl-phosphorothioate and a nucleoside monophosphate.[194]



Scheme 1-7. Synthesis of Np_3N' and Np_4N Using a Quinolinide

Synthesis of Np_nN' analogs is of great interest and almost every part of Np_nN's can now be modified. Many analogs with one or more modifications were made by the methods mentioned above using modified subunits, including those with isotope labels or fluorescent probes.[137,140,158] In addition to the methods already discussed, Np_nN's and their analogs have been modified post-synthetically to produce new analogs for different

purposes. By the reaction of Ap_4A with chloroacetaldehyde, fluorescent $\varepsilon Ap_4\varepsilon A$ was prepared.[195-197] Recently Miller's group developed a procedure called "tandem synthetic biosynthetic", in which Np_nN's and their analogs were synthesized enzymatically and then modified further chemically.[158] Sodium periodate was used to oxidize mant-Ap_4A in order to generate a dialdehyde group, giving dial-mant-Ap_4A (**Figure 1-10**).[198,199] In addition, the enzymatic product, Br-mant-Ap_4A, was treated with triethylammonium azide to incorporate an azide group, producing N_3-mant-Ap_4A.[158,200]

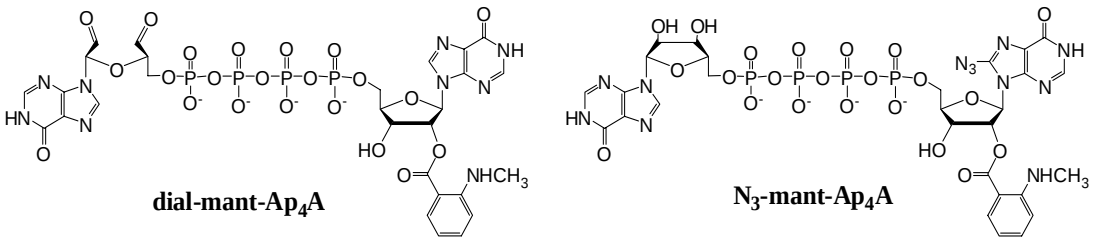


Figure 1-10. Structures of dial-mant-Ap_4A and N_3-mant-Ap_4A

Although much effort has been made to develop efficient chemical methods to prepare dinucleoside tetraphosphates and their analogs, the current methods are not satisfactory. They are usually multi-step reactions, and the necessary reagents are unstable and hard to prepare. The reactions produce complicated mixtures and the purification is difficult. The yields are always fairly low. Furthermore, the current methods are hard to adapt to make these important molecules on large scales. A new method is necessary to prepare these compounds efficiently and in large amounts.

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Chapter 2: Synthesis of Dinucleoside Tetra- and Pentaphosphates, and Thio, Seleno, Borano, Methylene and Difluoromethylene Analogs of Ap_4A and $AZTp_4A$

1. Introduction

Dinucleoside tetra- and pentaphosphates are very important biological molecules.[1-5] Enzymatic methods are not appropriate for their preparation on large scales. The current chemical methods also have serious drawbacks. Among them, trimetaphosphates are important intermediates. Some time ago, nucleoside trimetaphosphates were prepared by the treatment of nucleoside triphosphates with DCC.[6-8] Then the trimetaphosphate was reacted with a nucleophile to form a linear polyphosphate chain, with the nucleophile attacking the β- or γ-phosphate.[9] The method is shown in **Figure 2-1**, and has been used to make Ap_4ApA, Ap_5A and nucleoside dye polyphosphates.[10-12]

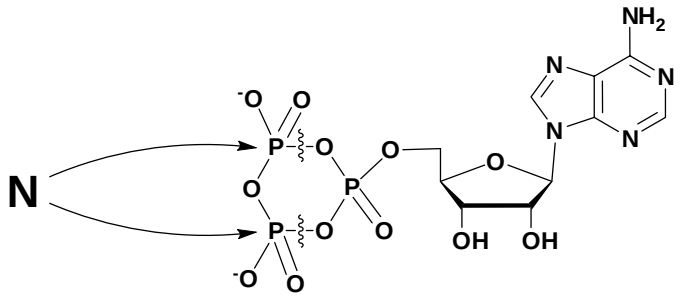


Figure 2-1. Nucleophilic Attack on a Trimetaphosphate

New methods were later developed to form trimetaphosphate intermediates. In 1989 Eckstein reacted 2-chloro-4*H*-l,3,2-benzo-dioxaphosphorin-4-one with protected nucleosides followed by addition of pyrophosphate. Subsequent reaction with an oxidant formed trimetaphosphates.[13] Eckstein then hydrolyzed the trimetaphosphates with

aqueous ammonia to prepare nucleoside triphosphates. His procedure was later adapted to make various modified nucleoside di- and triphosphates very efficiently, including nucleoside boranophosphates by Shaw's group.[14-21] Other phosphitylating reagents have also been used to make trimetaphosphates as intermediates for the preparation of modified nucleoside polyphosphate.[22-26]

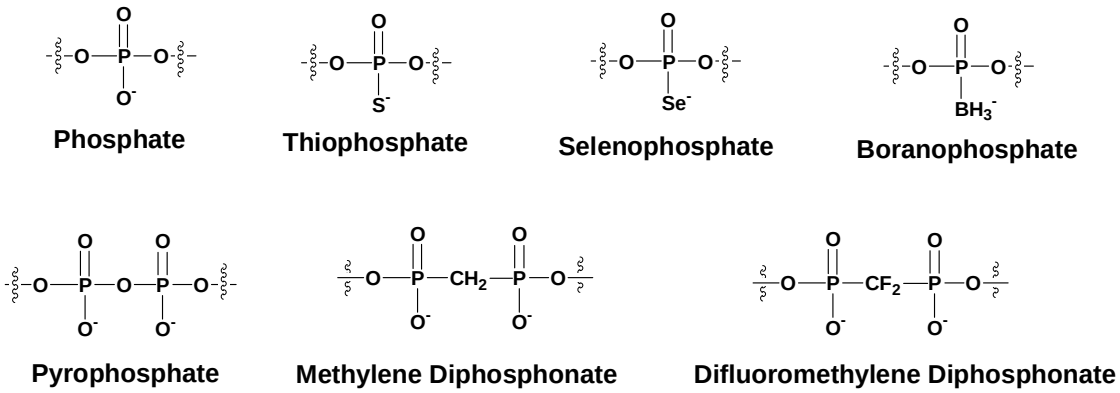


Figure 2-2. Structures of Phosphate and Its Analogs

Nucleotide analogs with modifications in the phosphate chain have been widely used in the study of enzyme kinetics and substrate properties. As potential diagnostic and therapeutic agents, they have been applied in many biochemical and pharmacological processes. One modification is the replacement of a nonbridging oxygen with a sulfur, selenium or borane group. The structures of several such analogs are shown in **Figure 2-2**. In some cases the incorporated group introduces a chiral center. The modifications bestow chemical and metabolic stability, resulting in resistance to hydrolysis. These analogs therefore have special advantages over regular phosphates, which are prone to degradation by various specific and nonspecific enzymes. The study of thiophosphates was pioneered by Eckstein with his classic work on synthesis and enzyme study.[27] Thiophosphates are the most studied polyphosphate analogs and have been employed to investigate enzyme function and properties. Experimental and theoretical results

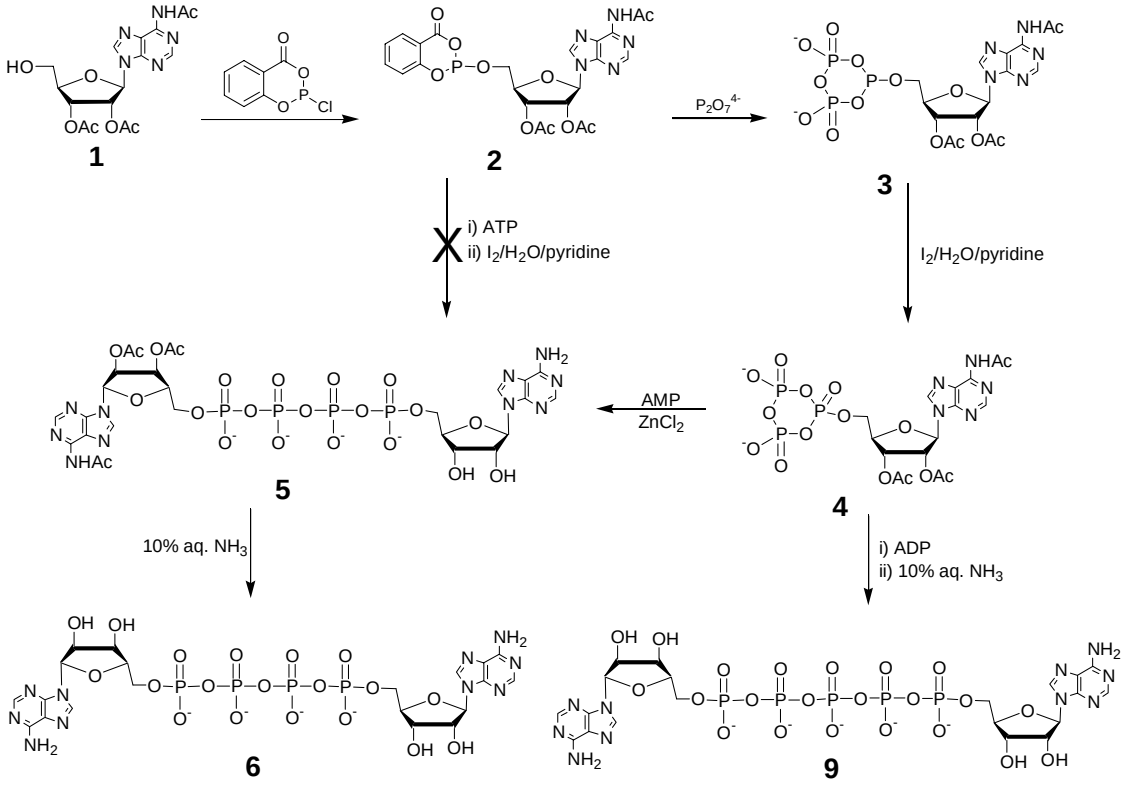
suggested that the charge is mainly located at the sulfur and the bond order of P-S is close to one.[28-30] The study of thiophosphates has been well reviewed.[29,31-34] Synthesis and properties of dinucleoside polyphosphorothioates have also been studied.[22,35-39] The study of boranophosphates was pioneered by Shaw's group from Duke University.[14-18,24,40] The borane group is isoelectronic and isosteric with the nonbridging oxygen while having a different charge distribution and polarity. While maintaining high aqueous solubility, the borane group imparts lipophilicity to boranophosphates which cannot form hydrogen bonds or coordinate with metals through the borane.[23,25,41] Boranophosphates were indicated as potential antiviral drugs with low toxicity.[19,24,25,42-45] Selonophosphates are less well-known but are attracting more and more attention due to the significant role of selenium in biological systems. They have also been used in nucleic acids structure determination.[18,21,46-50]

Other modifications can be made by substitution of the bridging oxygen a with methylene or halogen methylene group. The halogen methylene groups are especially interesting because they are isoelectronic and isosteric with the bridging oxygen.[51-53] This maintains important interactions and function while providing additional stability, especially the CF_2 group that is isopolar with oxygen.[51] Ap_4A analogs with methylene or halogen methylene groups have been synthesized in modest yields.[54-56] They have been shown to be potential antithrombotic agents.[57,58]

2. Results and Discussions

2.1. Dinucleoside Tetra- and Pentaphosphates

An efficient one-flask method was developed to synthesize dinucleoside tetra- and pentaphosphates.[59] The reactions are shown in **Scheme 2-1**. The method starts with the Eckstein procedure for the preparation of trimetaphosphate, **4**.[13] To synthesize Ap_4A, **6**, 2′,3′-*O*-6-*N*-triacetyl protected adenosine, **1**, was phosphitylated by 2-chloro-4*H*-l,3,2-benzo-dioxaphosphorin-4-one followed by reaction with inorganic pyrophosphate in the tributylammonium form, and subsequent oxidation using I_2/H_2O/pyridine to give trimetaphosphate, **4**. A supply of ATP was obtained by hydrolysis of this intermediate.[13,16,20,24] We first tried to couple adenosine 5′-triphosphate (ATP) with intermediate **2**, followed by oxidation and hydrolysis, but this method gave complicated mixtures, and only traces of Ap_4A were detected. This result is consistent with the low yields obtained in a synthesis of dinucleoside thiotetraphosphates by a similar approach.[22]



Scheme 2-1. Synthesis of Ap_4A and Ap_5A

However we found that the reaction of trimetaphosphate with adenosine 5′-monophosphate (AMP), under conditions that do not bring about hydrolysis, produces partially protected Ap_4A, **5**, in a clean conversion. The reaction was effectively catalyzed by $ZnCl_2$, but only moderately catalyzed by $MgCl_2$. After a mild ammonia treatment to remove the protecting acetyl groups, Zn^{2+} was removed as a soluble ammonium complex by cation (Na^+) exchange resin. The final Ap_4A, **6**, was then purified by reverse-phase HPLC in a yield of 85%. By similar methods Ap_4G (**7**) and Gp_4G (**8**) were prepared. Furthermore Ap_5A (**9**) was prepared by the reaction of trimetaphosphate with adenosine 5′-diphosphate (ADP) (see **Scheme 2-1**). In the above reactions, AMP was in the proton form, and GMP and ADP were in the sodium form. Because they all gave efficient coupling, the cation form is apparently not critical for the reaction. In the cation exchange of Gp_4G, Li^+ cation exchange resin was used instead of Na^+ to avoid forming G-quadruplex.[60-62]

$AZTp_4A$ (**10**) is the product of the AZT drug excision process. To investigate further details of the process, $AZTp_4A$ was prepared for a collaboration with Dr. Edward Arnold's group at the Center for Advanced Biotechnology and Medicine. In addition, $AZTp_4AZT$ (**11**), dTp_4A (**12**) and $AZTp_5A$ (**13**) were synthesized as controls. The azido group of AZT plays an important role in the excision process by keeping the terminating AZT at the N-site through steric hindrance. dTp_4A has a dT-3′-OH instead of an azido and might serve as a useful control. The $AZTp_4A$ prepared by the method described here was used by Dr. Arnold's group to study crystal structures with wild and mutant HIV-1 RT. Some important interactions of $AZTp_4A$ with HIV-1 RT were identified. $AZTp_4AZT$ was also synthesized by a different procedure described in the next chapter, and both

detailed procedures are included together in the **Experimental Procedures** section of this chapter for convenience.

All the above dinucleoside tetra- and pentaphosphates were characterized by NMR (^{1}H, ^{13}C and ^{31}P), UV and MS. Although the ^{31}P NMR spectra showed complex second order spectra, the resonance envelopes of the terminal phosphates are well separated from those of the central ones. Spectra for Ap_4A agree well with literature results.[63] For Ap_5A, the second and third phosphates are not resolved and show up as a broad singlet. This is consistent with the ^{31}P NMR data for sodium pentaphospate ($Na_7P_5O_{16}$) in a study of a series of inorganic polyphosphates, in which the difference in the second and third chemical shifts are smaller than their coupling constant.[64] All the synthesized dinucleoside tetra- and pentaphosphates are listed in **Table 2-1**, along with their yields.

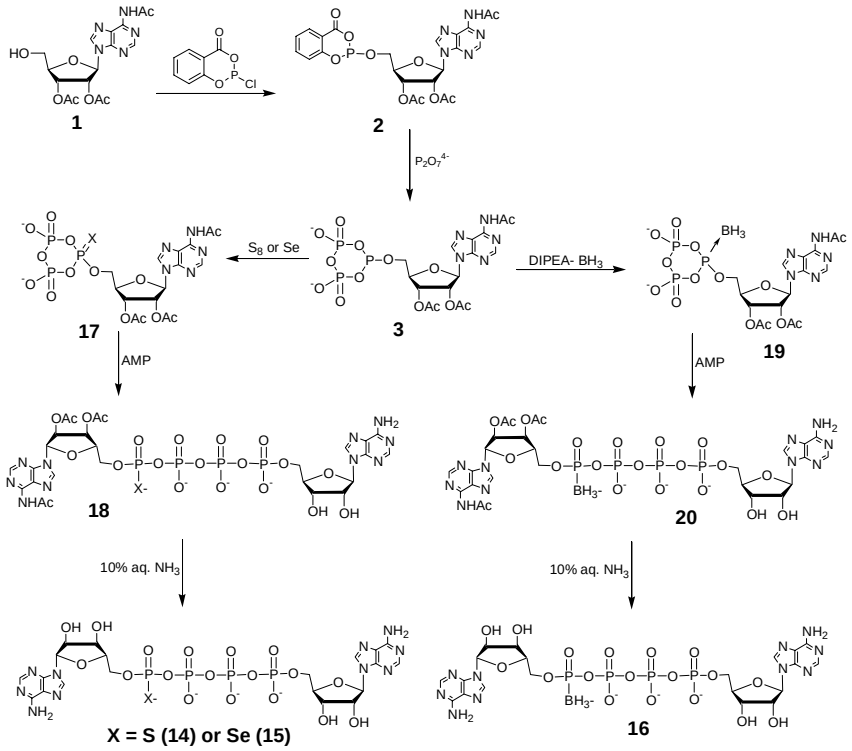
Table 2-1. Dinucleoside Tetra- and Pentaphosphates

Dinucleoside Tetra- and Pentaphosphates	Yields
$A^{5'}pppp^{5'}A$ (**6**)	85%
$A^{5'}pppp^{5'}G$ (**7**)	65%
$G^{5'}pppp^{5'}G$ (**8**)	47%
$A^{5'}p_5{}^{5'}A$ (**9**)	48%
$AZT^{5'}pppp^{5'}A$ (**10**)	68%
$AZT^{5'}pppp^{5'}AZT$ (**11**)	77%
$dT^{5'}pppp^{5'}A$ (**12**)	66%
$AZT^{5'}p_5{}^{5'}A$ (**13**)	38%

2.2. Thio, Seleno, Borano, Methylene and Difluoromethylene Analogs of Ap_4A

The above method was modified to synthesize the thio, seleno and borano analogs of Ap_4A and the reactions are shown in **Scheme 2-2**. For the synthesis of Ap_SpppA (**14**),

elemental sulfur (S_8) was used to sulfurize intermediate **3**, and then the same procedure used for Ap_4A was followed. The sulfurization reaction was complete after 30 min. Two diastereomers of **14** were purified and separated by reverse-phase HPLC with approximately equal yields. For the synthesis of $Ap_{Se}pppA$ (**15**), elemental selenium in toluene was employed to selenize intermediate **3**. This step was much slower than sulfurization and required at least 7 hr. Toluene was used as the solvent since selenium is highly soluble in it. The syntheses of both thio and seleno analogs were conducted with $ZnCl_2$ as the catalyst. In the synthesis of $Ap_{BH3}pppA$ (**16**), borane-*N*,*N*-diisopropylethylamine complex was used for the boranation of intermediate **3**. For the coupling of AMP with the boranotrimetaphosphate, **19**, $MgCl_2$ was used as a catalyst because it showed better efficiency than $ZnCl_2$. The two diastereomers were purified and separated with approximately equal yields.



Scheme 2-2. Synthesis of Thio, Seleno and Borano Ap_4A

The configurations of the diastereomers were assigned based on the elution order from reverse-phase HPLC. For thiophosphates, Eckstein first reported that the [*R*]-diastereomer of adenosine α-thiotriphosphate (Ap_Spp) was degraded much faster than the [*S*]-diastereomer by snake venom phosphodiesterase, and the [*R*]-diastereomer had a longer retention time than the [*S*]-diastereomer on reverse-phase HPLC.[65] Later Blackburn and McLennan used this enzymatic degradation method to assign configurations of the three diastereomers of $Ap_Sp_{CH2}pp_SA$.[37] Imbach assigned configurations of the two diastereomers of Ap_SpppA based on ^1H and ^{31}P NMR, and found that retention times of Ap_SpppA from reverse-phase HPLC were correlated in the same way with configuration assignments.[35,36,53] For boranophosphates, Canard determined the absolute configuration of α-boranothymidine diphosphate using X-ray crystallography,[42] and later Shaw correlated this assignment with the retention times of the diastereomers on reverse-phase HPLC.[17,18,24,25] Because the group priorities around the chiral phosphorus of a boranophosphate are opposite of those around thiophosphates, the designation of [*R*] and [*S*] are reversed for a given absolute configuration in these two families of analogs. Thus the [*R*]-diastereomer of α-boranophosphate has a shorter retention time than the [*S*]-diastereomer. Shaw and a Biota Inc. group have both used this correlation of retention time and stereochemistry to make strong arguments for assignments of other boranoate and selenoate analogs.[18,20,21,25,66]

Based on these correlations, for thio- and selenotetraphosphate, **14** and **15**, the first eluting diastereomer was assigned as [*S*] and the second as [*R*]. Since the borane group has the opposite priority order as sulfur and selenium, the configuration assignment for **17** was opposite, and the first eluting isomer was assigned as [*R*] and the second as [*S*].

The configuration assignments were confirmed by their enzymatic degradation with snake venom phosphodiesterase. This phosphodiesterase was previously used in the determination of configurations of mononucleoside thiopolyphosphates.[65] The [*R*]-diastereomer is digested faster by the phosphodiesterase than the corresponding [*S*]-diastereomer. But it has not previously been employed to determine the configuration of borano and seleno phosphate diastereomers, although it is known that borano phosphates are resistant to snake venom phosphodiesterase and nuclease digestion.[67-69] The enzyme degradations of **14**, **15** and **16** were carried out at conditions under which they were hydrolyzed to corresponding triphosphate analogs fairly quickly (<15min). The further digestion of the resulting triphosphates was followed by LC-MS over a longer time (~ 12hr). For Ap_SpppA (**14**), the [*R*]-diastereomer was digested faster than the [*S*]-diastereomer, which is consistent with previously reported correlations and therefore confirmed the configuration assignment.[65] A plot of the disappearance of Ap_Spp against time is shown in **Figure 2-3**. For both diastereomers the second points are higher than the first since the formation of Ap_Spp is faster than its digestion at first. Subsequently Ap_Spp is digested slowly and the degradation of the [*R*]-diastereomer is faster than of the [*S*]-diastereomer. For Ap_{Se}pppA (**15**), the [*R*]-diastereomer was also digested faster by the phosphodiesterase than the corresponding [*S*]-diastereomer, whereas the opposite is true for Ap_{BH3}pppA (**16**) due to the opposite priority of the borane group. The corresponding plots of the disappearance of Ap_{Se}pp and Ap_{BH3}pp against time are shown in **Figure 2-4** and **Figure 2-5**. These results imply that snake venom digestion can be used to differentiate [*R*] and [*S*] diastereomers of seleno- and boranophosphates. For all three

analogs, the diastereomers eluting faster on reverse-phase chromatography are always digested more slowly by snake venom phosphodiesterase.

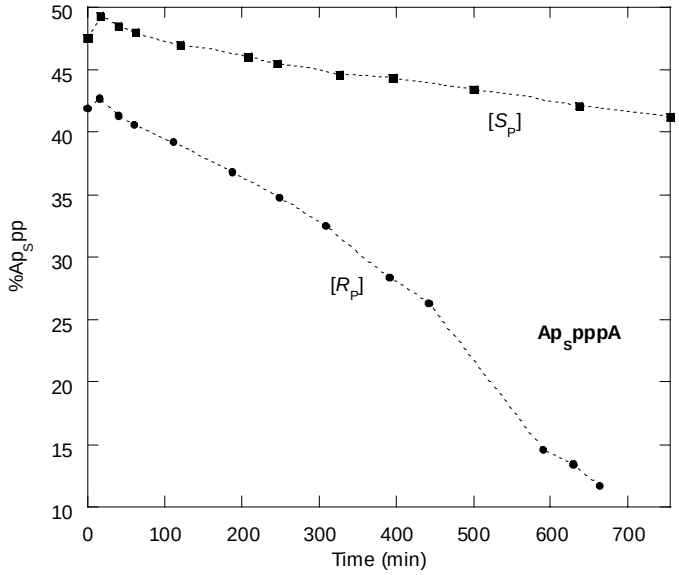


Figure 2-3. Disappearance of Ap$_S$pp with Enzymatic Degradation of Ap$_S$pppA (14)

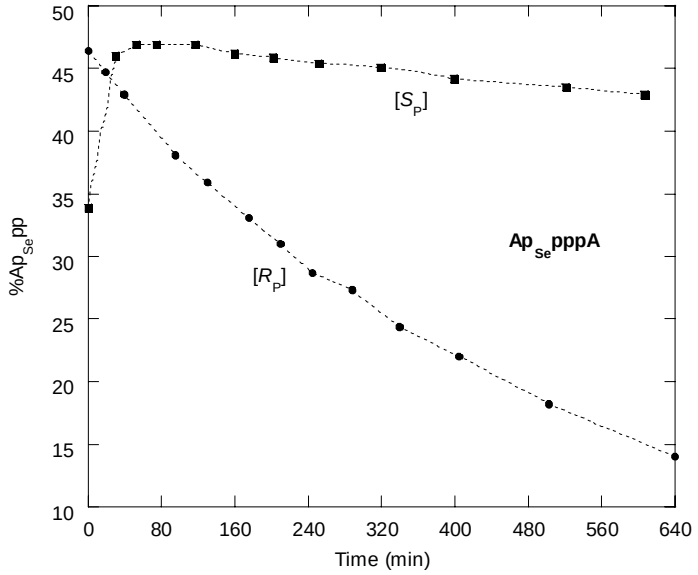


Figure 2-4. Disappearance of Ap$_{Se}$pp with Enzymatic Degradation of Ap$_{Se}$pppA (15)

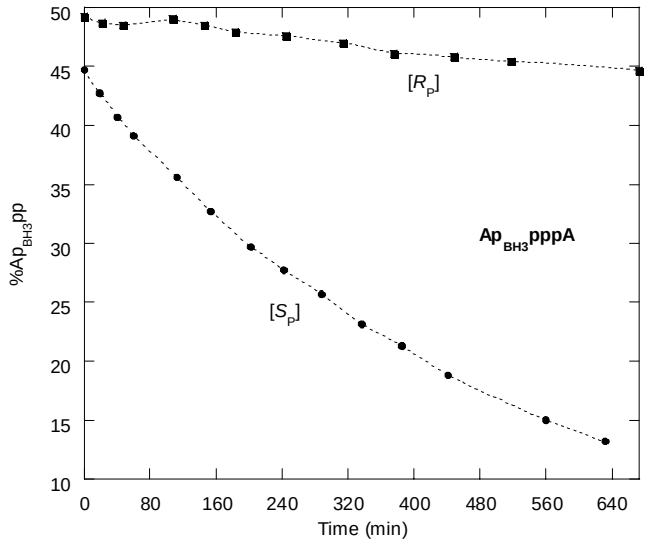
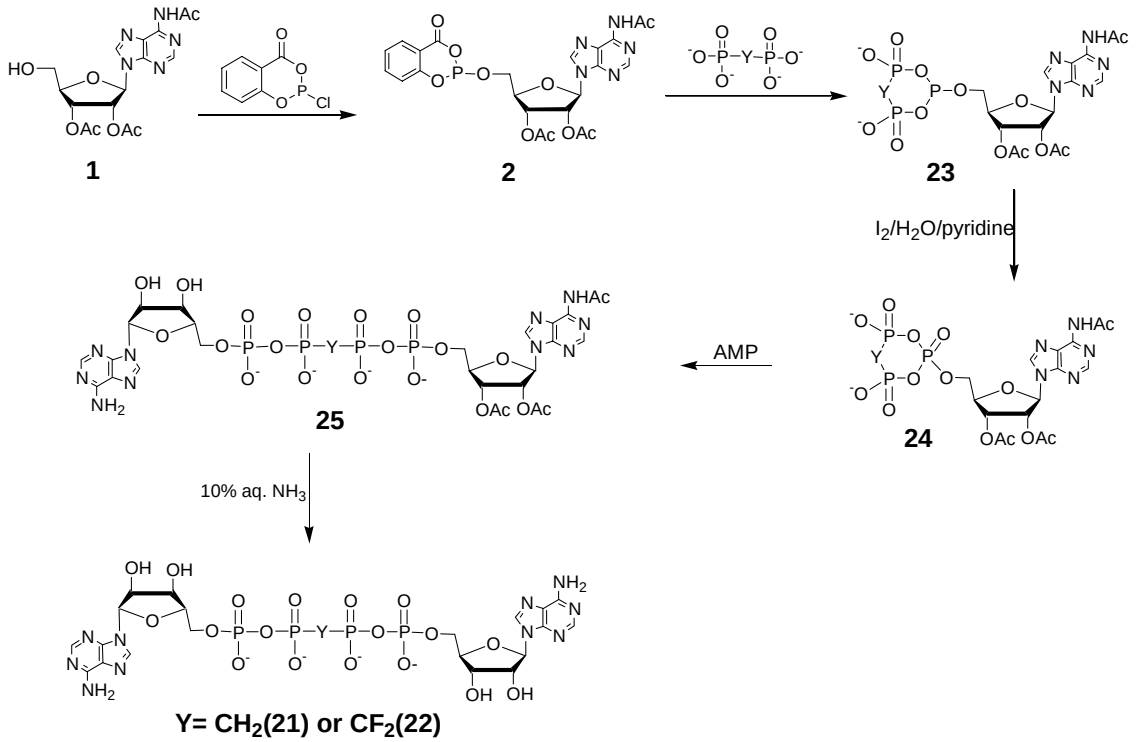


Figure 2-5. Disappearance of $Ap_{BH3}pp$ with Enzymatic Degradation of $Ap_{BH3}pppA$ (16)

The trimetaphosphate method was also modified to make the diphosphonate analogs of Ap_4A with comparable yields, in which the central bridging oxygen was substituted with a methylene or difluoromethylene group, giving $App_{CH2}ppA$ (**21**) and $App_{CF2}ppA$ (**22**). Because difluoromethylene is isosteric and isopolar with the bridging oxygen, it might provide particular advantages for biological study.[51-53] As shown in **Scheme 2-3**, methylene diphosphonic acid or difluoromethylene diphosphonate was used to react with intermediate **2** instead of inorganic pyrophosphate. After oxidation by I_2/H_2O/pyridine and coupling with AMP, treatment with mild ammonia gave the methylene or difluoromethylene analogs of Ap_4A. Methylene diphosphonic acid is commercially available, and difluoromethylene diphosphonate was prepared by a procedure described by Boyle.[70]



Scheme 2-3. Synthesis of Methylene and Difluoromethylene Analogs of Ap_4A

2.3. Thio, Seleno, Borano, Methylene and Difluoromethylene Analogs of $AZTp_4A$

Since $AZTp_4A$ is the excision product of HIV-1 AZT drug resistance, its analogs can be used to study the inhibition of AZT excision and may inhibit the AZT excision reaction.[71,72] The thio ($AZTp_SpppA$, **26**), seleno ($AZTp_{Se}pppA$, **27**), borano ($AZTp_{BH3}pppA$, **28**), methylene ($AZTpp_{CH2}ppA$, **29**) and difluoromethylene ($AZTpp_{CH2}ppA$, **30**) analogs of $AZTp_4A$ were synthesized by similar procedures as those described for Ap_4A analogs. The configurations were assigned based on the elution order from reverse-phase HPLC. These $AZTp_4A$ analogs are listed together with Ap_4A analogs in **Table 2-2**. Also listed in **Table 2-2** is $AZTpppp_SA$ (**31**), which was synthesized in 76% yield using a different method that is described in the next chapter. Some of these compounds have shown very good inhibition properties in a collaboration with Dr.

Michael A. Parniak's group at the University of Pittsburgh.

Table 2-2. Monomodified Ap_4A and $AZTp_4A$ analogs

AppppA Analogs	Yields	AZTppppA Analogs	Yields
$A^{5'}ppCH_2pp^{5'}A$ (**21**)	80%	$AZT^{5'}ppCH_2pp^{5'}A$ (**29**)	61
$A^{5'}ppCF_2pp^{5'}A$ (**22**)	82%	$AZT^{5'}ppCF_2pp^{5'}A$ (**30**)	65
$A^{5'}$ $p(S^-)ppp$ $^{5'}A$ (**14**)	[S_P]: 36% [R_P]:30%	$AZT^{5'}$ $p(S^-)ppp$ $^{5'}A$ (**26**)	[S_P]:39% [R_P]:36%
		$AZT^{5'}$ $ppp(S^-)p$ $^{5'}A$ (**31**)	[S_P]: 33% [R_P]:43%
$A^{5'}$ $p(Se^-)ppp$ $^{5'}A$ (**15**)	[S_P]: 21% [R_P]:17%	$AZT^{5'}$ $p(Se^-)ppp$ $^{5'}A$ (**27**)	[S_P]: 29% [R_P]:28%
$A^{5'}$ $p(BH_3^-)ppp$ $^{5'}A$ (**16**)	[R_P]:40% [S_P]:26%	$AZT^{5'}$ $p(BH_3^-)ppp$ $^{5'}A$ (**28**)	[R_P]: 36% [S_P]:30%

3. Conclusions

An efficient chemical method was developed to synthesize dinucleoside tetraphosphates with yields up to 85% by coupling a nucleoside monophosphate with a trimetaphosphate derivative of a nucleoside. This one-flask method was employed to synthesize Ap_4A, Ap_4G, Gp_4G, Ap_5A, $AZTp_4A$, $AZTp_4AZT$ and dTp_4A. Furthermore the method was modified to synthesize thio, seleno, borano, methylene and difluoromethylene analogs of Ap_4A and $AZTp_4A$. The configurations of the diastereomers of the thio, seleno and borano analogs were assigned based on the elution order on reverse-phase HPLC. Enzymatic degradation of the Ap_4A analogs was done with snake venom phosphodiesterase in order to confirm the configuration assignments.

The [*R*]-diastereomer was digested faster than the [*S*]-diastereomer for the thio and seleno analogs, while the opposite was true for the borano analogs.

4. Experimental Procedures

General Procedures. Methylenediphosphonic acid, 2-chloro-4*H*-l,3,2-benzo-dioxaphosphorin-4-one, tri-*n*-butylamine, AMP monohydrate, ADP, guanosine monophosphate (GMP), elemental selenium and borane-*N*,*N*-diisopropylethylamine complex were purchased from Aldrich. Elemental sulfur was purchased from J.T.Baker. AZT was obtained from Transgenomic. Bis(tri-*n*-butylammonium) pyrophosphate was prepared according to the procedure described by Eckstein,[13] AZT monophosphate (AZT-MP) was prepared by the Yoshikawa procedure,[73,74] and bis(tri-*n*-butylammonium) difluoromethylenediphosphonate was prepared by the procedure reported by Boyle.[70] The snake venom phosphodiesterase (Type IV from *Crotalus atrox*) was purchased from Sigma. The preparative reverse-phase HPLC was performed on a PrepPak system with three Waters Delta-Pak PrepPak® cartridges (C18, 300Å, 40 mm × 100 mm, 15 μm) using acetonitrile and 0.1 M ammonium bicarbonate (pH 7.5) or water. The analytical samples were analyzed on a Waters Atlantis™ analytical column (dC18 column, 100Å, 4.6 mm × 50 mm, 3.0 μm) using acetonitrile and 0.1 M triethylammonium acetate (TEAA) buffer (pH 6.8), and the flow rate was 1.0 mL/min.

Preparation of diadenosine 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate (6). To a solution of 2′,3′-*O*-6-*N*-triacetyladenosine (0.13 g, 0.33 mmol) in 2 mL of anhydrous *N*,*N*-dimethylformamide (DMF) was added 2-chloro-4*H*-l,3,2-benzo-dioxaphosphorin-4-one (0.13 g, 0.64 mmol,

1.9 equiv). The solution was stirred for 15 min at room temperature under N_2. A 0.5 M solution of bis(tri-*n*-butylammonium) pyrophosphate in anhydrous DMF (1.3 mL, 0.65 mmol, 2.0 equiv) was vortexed with tri-*n*-butylamine (0.60 mL, 2.5 mmol, 7.6 equiv) and immediately added to the reaction mixture. After 20 min, a solution of iodine (0.12 g, 0.47 mmol, 1.4 equiv) in 1.5 mL of pyridine and 0.01 mL of water was added. After 15 min, a mixture of AMP, in the proton form (0.45 g, 1.2 mmol, 3.6 equiv), and zinc chloride (0.42 g, 3.1 mmol, 9.4 equiv) that had been dried together by evaporation of pyridine and DMF was added with stirring. After 16 h, 10% aqueous ammonia (20 mL, 118 mmol, 358 equiv) was added, and the deprotection was complete after 1 h. The dilute basic solution was applied to a column of sodium cation-exchange resin (50WX2, 10 mL, 18 equiv) to remove Zn^{2+}. The product was concentrated and purified by preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.25 g of **6** in the ammonium form (0.28 mmol, 85%): UV λ_{max} 260 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.40 (s, 2H), 8.15 (s, 2H), 6.01 (d, *J* = 5.7 Hz, 2H), 4.69 (t, *J* = 5.4 Hz, 2H), 4.54 (t, *J* = 4.3 Hz, 2H), 4.39-4.34 (m, 2H), 4.33-4.21 (m, 4H); ^{13}C NMR (D_2O, 400 MHz): δ 157.1, 154.4, 151.2, 142.6, 120.7, 89.6, 86.5 (d, J_{CP} = 9.1 Hz), 77.3, 73.0, 67.9 (d, *J* = 5.3 Hz); ^{31}P NMR (D_2O, 400 MHz): δ (-)9.90-(-)10.50 (m, 2P), (-)21.72-(-)22.19 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m*/*z* (M-1) 835.33 (calculated for $C_{20}H_{27}N_{10}O_{19}P_4^-$, 835.04).

Preparation of adenosine guanosine 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate (7). Starting with 2′,3′-*O*-2-*N*-triacetylguanosine (0.14 g, 0.34 mmol), **7** was prepared by the same procedure described for **6**. The product was purified by preparative reverse-phase HPLC

using 0.1 M ammonium bicarbonate in acetonitrile to give 0.20 g of **7** in the ammonium form (0.22 mmol, 65%): UV λ_{max} 256 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.40 (s, 1H), 8.12 (s, 1H), 8.00 (s, 1H), 6.04 (d, *J* = 5.5 Hz, 1H), 5.80 (d, *J* = 5.9 Hz, 1H), 4.71 (t, *J* = 5.4 Hz, 2H), 4.58-4.50 (m, 2H), 4.39-4.18 (m, 6H); ^{13}C NMR (D_2O, 400 MHz): δ 161.2, 157.9, 156.3, 155.4, 154.1, 151.5, 142.4, 140.1, 120.9, 118.5, 89.6, 89.5, 86.5, 86.4, 77.2, 76.5, 73.0, 72.9, 67.94 (d, *J* = 5.3 Hz), 67.89 (d, J_{CP} = 5.3 Hz); ^{31}P NMR (D_2O, 400 MHz): δ (-)9.82-(-)10.41 (m, 2P), (-)21.65-(-)22.28 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m*/*z* (M-1) 851.22 (calculated for $C_{20}H_{27}N_{10}O_{20}P_4^-$, 851.04).

Preparation of diguanosine 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate (8). Starting with 2′,3′-*O*-2-*N*-triacetylguanosine (0.13 g, 0.32 mmol), **8** was prepared by the same procedure described for **6**, except that 1.1 mmol of GMP, in the tri-*n*-butylammonium form (3.4equiv) was used instead of AMP, and the cation-exchange resin was in the Li^+ form rather than in the Na+ form to minimize aggregation of the product. The product was purified by preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.14 g of **8** in the ammonium form (0.15 mmol, 47%): UV λ_{max} 253 nm with the shoulder at 275 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.04 (s, 2H), 5.84 (d, *J* = 5.9 Hz, 2H), 4.74 (t, *J* = 5.8 Hz, 2H), 4.54 (t, *J* = 4.1 Hz, 2H), 4.35-4.30 (m, 2H), 4.30-4.20 (m, 4H); ^{13}C NMR (D_2O, 400 MHz): δ 161.4, 156.4, 154.3, 140.3, 118.7, 89.6, 86.5 (d, J_{CP} = 9.1 Hz), 76.4, 73.0, 67.9 (d, *J* = 5.5 Hz); ^{31}P NMR (D_2O, 400 MHz): δ (-)8.89-(-)9.47 (m, 2P), (-)20.56-(-)21.29 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m*/*z* (M-1) 867.37 (calculated for $C_{20}H_{27}N_{10}O_{21}P_4^-$, 867.03).

Preparation of diadenosine 5′,5‴-*P*^{1},*P*^{5}-pentaphosphate (9). Starting with 2′,3′-*O*-6-*N*-triacetyladenosine (0.12 g, 0.31 mmol), **9** was prepared by the same procedure described for **6**, except that ADP, in the proton form (0.29 g, 0.68 mmol, 2.2 equiv) was used in the coupling instead of AMP. The product was purified by preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.15 g of **9** in the ammonium form (0.15 mmol, 48%): UV λ_{max} 259 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.45 (s, 2H), 8.16 (s, 2H), 6.02 (d, *J* = 5.8 Hz, 2H), 4.70 (t, *J* = 5.4 Hz, 2H), 4.56 (t, *J* = 4.2 Hz, 2H), 4.43-4.35 (m, 2H), 4.34-4.20 (m, 4H); ^{13}C NMR (D_2O, 400 MHz): δ 156.5, 153.6, 151.1, 143.0, 120.7, 89.7, 86.7 (d, J_{CP} = 8.9 Hz), 77.3, 73.0, 68.1 (d, *J* = 4.8 Hz); ^{31}P NMR (D_2O, 400 MHz): δ (-)9.71-(-)10.37 (m, 2P), (-)21.12-(-)22.36 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m*/*z* (M-1) 915.17 (calculated for $C_{20}H_{28}N_{10}O_{22}P_5^-$, 915.01).

Preparation of adenosine 3′-azido-3′-deoxythymidine 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate (10). Starting with AZT (0.084 g, 0.31 mmol), **10** was prepared by the same procedure described for **6**. The product was purified by preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.19 g of **10** in the ammonium form (0.21 mmol, 68%): UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.52 (s, 1H), 8.22 (s, 1H), 7.61 (s, 1H), 6.14-6.06 (m, 2H), 4.60-4.48 (m, water suppression reduces intensity), 4.42-4.35 (m, 1H), 4.33-4.15 (m, 4H), 4.14-4.09 (m, 1H), 2.45-2.29 (m, 2H), 1.86 (s, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.1, 158.1, 155.3, 154.2, 151.8, 142.7, 139.8, 121.2, 114.3, 89.3, 87.5, 86.8 (d, J_{CP} = 9.2 Hz), 85.7 (d, J_{CP} = 9.2 Hz), 77.1, 73.2, 68.5 (d, J_{CP} = 5.4 Hz), 68.0 (d, J_{CP} = 5.5 Hz), 63.7, 39.1, 14.4; ^{31}P NMR (D_2O, 300 MHz): δ (-)10.56-(-

)11.69 (m, 2P), (-)21.78-(-)23.18 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 835.52 (calculated for $C_{20}H_{27}N_{10}O_{19}P_4^-$: 835.04).

Preparation of di-3′-azido-3′-deoxythymidine 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate (11). **Method A:** Starting with AZT (0.084 g, 0.31 mmol), **11** was prepared by the same procedure described for **6**, except that AZT-MP, in the ammonium form (0.51 g, 1.3 mmol, 4.2 equiv) instead of AMP was used to react with the trimetaphosphate intermediate. The product was purified by preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.22 g of **11** (0.24 mmol, 77%) in the ammonium form. **Method B:** Starting with AZT (0.085 g, 0.32 mmol), **11** was prepared by the same procedure described for **6**, except that more iodine (0.55 g, 2.2 mmol, 6.9 equiv) was used and 3′-azido-3′-deoxythymidine 5′-H-phosphonate, in the pyridinium form (0.39 g, 0.95 mmol, 3.0 equiv) instead of AMP was used to react with the trimetaphosphate intermediate. The product was purified by preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.22 g of **7** (0.24 mmol, 75%) in the ammonium form: UV λ_{max} 267 nm; ^{1}H NMR (D_2O, 400 MHz): δ 7.75 (d, *J* = 1.2 Hz, 2H), 6.23 (t, *J* = 6.9 Hz, 2H), 4.61-4.53 (m, water suppression reduces intensity), 4.26-4.15 (m, 6H), 2.50-2.40 (m, 4H), 1.92 (d, *J* = 1.1 Hz, 6H); ^{13}C NMR (D_2O, 400 MHz): δ 169.3, 154.4, 140.1, 114.6, 87.5, 85.8 (d, J_{CP} = 9.3 Hz), 68.5 (d, J_{CP} = 5.6 Hz), 63.8, 39.1, 14.4; ^{31}P NMR (D_2O, 300 MHz): δ (-)10.91-(-)11.63 (m, 2P), (-)22.21-(-)23.00 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 835.39 (calculated for $C_{20}H_{27}N_{10}O_{19}P_4^-$: 835.04).

Preparation of adenosine thymidine 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate (12). Starting with 3′-*O*-acetylthymidine (0.093 g, 0.33 mmol), **12** was prepared by the same procedure described for **6**. The product was purified by preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.19 g of **12** in the ammonium form (0.22 mmol, 67%): UV λ_{max} 261 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.51 (s, 1H), 8.20 (s, 1H), 7.59 (d, *J* = 1.0 Hz, 1H), 6.21 (t, *J* = 6.9 Hz, 1H), 6.09 (d, *J* = 6.2 Hz, 1H), 4.63-4.55 (m, water suppression reduces intensity), 4.42-4.36 (m, 1H), 4.33-4.08 (m, 4H), 4.14-4.09 (m, 1H), 2.33-2.19 (m, 2H), 1.84 (d, *J* = 0.6 Hz, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.1, 158.3, 155.6, 154.3, 151.8, 142.6, 139.8, 121.2, 114.3, 89.3, 88.2 (d, J_{CP} = 9.1 Hz), 87.6, 86.8 (d, J_{CP} = 9.3 Hz), 77.1, 73.5, 73.2, 68.2 (d, J_{CP} = 5.8 Hz), 68.1 (d, J_{CP} = 5.5 Hz), 41.3, 14.4; ^{31}P NMR (D_2O, 300 MHz): δ (-)10.87-(-)11.82 (m, 2P), (-)22.69-(-)23.58 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 810.00 (calculated for $C_{20}H_{28}N_7O_{20}P_4^-$: 810.37).

Preparation of adenosine 3′-azido-3′-deoxythymidine 5′,5‴-*P*^{1},*P*^{5}-pentaphosphate (13). Starting with AZT (0.069 g, 0.26 mmol), **13** was prepared by the same procedure described for **9**. The product was purified by preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.10 g of **13** in the ammonium form (0.10 mmol, 38%): UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.53 (s, 1H), 8.23 (s, 1H), 7.67 (s, 1H), 6.18 (t, *J* = 6.8 Hz, 1H), 6.10 (d, *J* = 6.2 Hz, 1H), 4.65-4.48 (m, water suppression reduces intensity), 4.45-4.35 (m, 1H), 4.34-4.08 (m, 5H), 2.47-2.34 (m, 2H), 1.87 (s, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.2, 158.4, 155.6, 154.3, 152.0, 142.7, 139.9, 121.3, 114.4, 89.2, 87.5 (d, J_{CP} = 3.0 Hz), 87.0 (d, J_{CP} = 9.2 Hz), 85.8 (d, J_{CP} = 9.3

Hz), 77.0, 73.3, 68.6 (d, J_{CP} = 5.8 Hz), 68.1 (d, J_{CP} = 5.4 Hz), 63.8, 39.1, 14.4; ^{31}P NMR (D_2O, 300 MHz): δ (-)10.18-(-)11.15 (m, 2P), (-)21.60-(-)22.41 (m, 3P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 915.30 (calculated for $C_{20}H_{28}N_{10}O_{22}P_5^-$, 915.01).

Preparation of [S_P] and [R_P] diadenosine-5′,5‴-P^1,P^4-(P^1-thio)-tetraphosphate (14a,b). Starting with 2′,3′-*O*-6-*N*-triacetyladenosine (0.13 g, 0.33 mmol), **14a,b** was prepared by the same procedure described for **6**, except that S_8 (0.032 g, 1.0 mmol, 3.0 equiv) was used to sulfurize **3** for 30 min. The two diastereomers were purified and separated by reverse-phase HPLC to give 0.11 g of **[S_P]-14a** (0.12 mmol, 36%) and 0.094 g of **[R_P]-14b** (0.10 mmol, 30%) in the ammonium form. The diastereomers eluted in the order listed. **[S_P]-14a**: UV λ_{max} 260 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.53 (s, 1H), 8.36 (s, 1H), 8.11 (s, 2H), 6.02 (d, *J* = 5.9 Hz, 1H), 6.01 (d, *J* = 5.7 Hz, 1H), 4.76-4.70 (m, water suppression reduces intensity), 4.61-4.54 (m, 2H), 4.41-4.21 (m, 6H); ^{13}C NMR (D_2O, 400 MHz): δ 157.7, 157.67, 155.2, 151.4, 151.3, 142.8, 142.4, 120.8, 120.7, 89.5, 89.4, 86.6 (d, J_{CP} = 9.7 Hz), 86.5 (d, J_{CP} = 9.1 Hz), 77.3, 77.2, 73.3, 73.0, 68.00 (d, J_{CP} = 5.6 Hz), 67.9 (d, J_{CP} = 6.4 Hz); ^{31}P NMR (D_2O, 300 MHz): δ 43.67 (d, *J* = 25.3 Hz, 1P), -10.85 (d, *J* = 17.8 Hz, 1P), -22.60 (t, *J* = 17.1 Hz, 1P), -23.62 (dd, *J* = 25.3 Hz, *J* = 16.5 Hz, 1P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 851.48 (calculated for $C_{20}H_{27}N_{10}O_{18}P_4S^-$: 851.02). **[$R_P$]-14b**: UV λ_{max} 260 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.42 (s, 1H), 8.35 (s, 1H), 8.10 (s, 2H), 6.01 (d, *J* = 5.7 Hz, 2H), 4.75-4.68 (m, water suppression reduces intensity), 4.55 (t, *J* = 4.3 Hz, 2H), 4.44-4.20 (m, 6H); ^{13}C NMR (D_2O, 400 MHz): δ 157.8, 155.4, 151.4, 142.4, 142.3, 120.79, 120.77, 89.5, 86.48

(d, J_{CP} = 9.2 Hz), 86.46 (d, J_{CP} = 10.0 Hz), 77.3, 77.2, 73.1, 73.0, 68.4 (d, J_{CP} = 5.9 Hz), 67.9 (d, J_{CP} = 5.5 Hz); ^{31}P NMR (D_2O, 300 MHz): δ 43.51 (d, *J* = 25.1 Hz, 1P), -10.97 (d, *J* = 18.1 Hz, 1P), -22.74 (t, *J* = 17.4 Hz, 1P), -23.67 (dd, *J* = 25.0 Hz, *J* = 16.7 Hz, 1P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 851.41 (calculated for $C_{20}H_{27}N_{10}O_{18}P_4S^-$: 851.02).

Preparation of [*S*$_P$] and [*R*$_P$] diadenosine-5′,5‴-*P*^{1},*P*^{4}-(*P*^{1}-seleno)-tetraphosphate (15a,b). Starting with 2′,3′-*O*-6-*N*-triacetyladenosine (0.12 g, 0.30 mmol), **15a,b** was prepared by the same procedure described for **6**, except that a dry solution of selenium (0.073 g, 0.92 mmol, 3.1 equiv) in 30 mL of toluene was used to oxidize **3** (7 hr) instead of iodine. The reaction solution was concentrated to remove toluene before the ammonia treatment (20 mL, 118 mmol, 393 eq) and was filtered to remove excess Se after the ammonia treatment. The mixture of two diastereomers was concentrated, purified and separated by reverse-phase HPLC to give 0.060 g of **[*S*$_P$]-15a** (0.062 mmol, 21%) and 0.050 g of **[*R*$_P$]-15b** (0.052 mmol, 17%) in the ammonium form. The diastereomers eluted in the order listed. **[*S*$_P$]-15a**: UV λ_{max} 259 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.56 (s, 1H), 8.35 (s, 1H), 8.12 (s, 1H), 8.11 (s, 1H), 6.02 (d, *J* = 6.1 Hz, 1H), 6.01 (d, *J* = 5.7 Hz, 1H), 4.75-4.72 (m, water suppression reduces intensity), 4.63-4.52 (m, 2H), 4.45-4.17 (m, 6H); ^{13}C NMR (D_2O, 400 MHz): δ 157.9, 157.8, 155.4, 151.5, 151.4, 142.9, 142.4, 120.81, 120.76, 89.5, 89.3, 86.6 (d, J_{CP} = 8.9 Hz), 86.5 (d, J_{CP} = 9.8 Hz), 77.3, 77.1, 73.4, 73.0, 68.00 (d, J_{CP} = 5.4 Hz), 67.96 (d, J_{CP} = 6.1 Hz); ^{31}P NMR (D_2O, 300 MHz): δ 33.92 (d, *J* = 30.0 Hz, 1P), -10.93 (d, *J* = 18.0 Hz, 1P), -22.80 (t, *J* = 17.4 Hz, 1P), -24.31 (dd, *J* = 30.1 Hz, *J* = 16.8 Hz, 1P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 899.28 (calculated for $C_{20}H_{30}BN_{10}O_{18}P_4^-$: 898.96). **[*R*$_P$]-15b**:

UV λ_{max} 259 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.44 (s, 1H), 8.36 (s, 1H), 8.12 (s, 2H), 6.01 (d, *J* = 5.7 Hz, 2H), 4.73-4.68 (m, water suppression reduces intensity), 4.59-4.51 (m, 2H), 4.43-4.15 (m, 6H); ^{13}C NMR (D_2O, 400 MHz): δ 157.88, 157.87, 155.4, 151.41, 151.39, 142.5, 142.3, 120.82, 120.79, 89.5, 89.4, 86.5 (d, J_{CP} = 9.1 Hz), 86.3 (d, J_{CP} = 10.0 Hz), 77.2, 73.2, 73.0, 68.6 (d, J_{CP} = 5.6 Hz), 67.9 (d, J_{CP} = 5.6 Hz); ^{31}P NMR (D_2O, 300 MHz): δ 33.90 (d, *J* = 30.4 Hz, 1P), -11.00 (d, *J* = 18.2 Hz, 1P), -22.54 (t, *J* = 17.5 Hz, 1P), -24.34 (dd, *J* = 30.4 Hz, *J* = 16.9 Hz, 1P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 899.60 (calculated for $C_{20}H_{30}BN_{10}O_{18}P_4^-$: 898.96).

Preparation of [S_P] and [R_P] diadenosine-5′,5‴-P^1,P^4-(P^1-borano)-tetraphosphate (16a,b). Starting with 2′,3′-*O*-6-*N*-triacetyladenosine (0.12 g, 0.30 mmol), intermediate **3** was prepared by the same procedure described for **6**. After 20 min, borane-*N*,*N*-diisopropylethylamine complex (1.0 mL, 5.8 mmol, 19 equiv) was added. After 16 hr, AMP monohydrate, in the proton form (0.50 g, 1.4 mmol, 4.7 equiv), was dissolved in a 40% tetrabutylammonium hydroxide aqueous solution (1.8 mL, 2.7 mmol, 9.0 equiv), dried by evaporation of pyridine and DMF and added to the reaction with stirring. Immediately $MgCl_2(H_2O)_6$ (0.19 g, 0.93 mmol, 3.1 equiv) that had been dried by evaporation of pyridine and DMF was added. The reaction was stirred for 3 hr and then 20 mL of water was added. The resulting precipitate was filtered and the filtrate was washed with 3 × 30 mL of CH_2Cl_2. The aqueous layer was concentrated and applied to a column of sodium cation exchange resin (50WX2, 10 mL, 6.0 mmol, 20 equiv). The eluate was concentrated and the two diastereomers were purified and separated by reverse-phase HPLC to give 0.11 g of **[R_P]-16a** (0.12 mmol, 40%) and 0.070 g of **[S_P]-16b**(0.078 mmol, 26%) in the ammonium form. The diastereomers eluted in the order

listed. **[R_P]-16a**: UV λ_{max} 260 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.45 (s, 1H), 8.35 (s, 1H), 8.14 (s, 1H), 8.12 (s, 1H), 6.05 (t, J = 5.5 Hz, 2H), 4.77-4.72 (m, water suppression reduces intensity), 4.65-4.54 (m, 2H), 4.40-4.11 (m, 6H), (+)0.99-(-)0.03 (br, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 157.85, 157.83, 155.41, 155.36, 151.5, 151.4, 142.7, 142.4, 120.8, 120.7, 89.6, 89.5, 87.0 (d, J_{CP} = 7.4 Hz), 86.5 (d, J_{CP} = 9.4 Hz), 77.4, 77.3, 73.5, 73.0, 67.9 (d, J_{CP} = 5.5 Hz), 64.4 (d, J_{CP} = 7.3 Hz); ^{31}P NMR (D_2O, 300 MHz): δ 86.03-80.61 (br, 1P), (-)10.54-(-)11.16 (m, 1P), (-)22.24-(-)23.15 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 833.37 (calculated for $C_{20}H_{30}BN_{10}O_{18}P_4^-$: 833.08). **[$S_P$]-16b**: UV λ_{max} 260 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.41 (s, 1H), 8.35 (s, 1H), 8.10 (s, 2H), 5.98 (d, J = 5.7 Hz, 2H), 4.70-4.63 (m, water suppression reduces intensity), 4.56-4.46 (m, 2H), 4.39-4.19 (m, 6H), (+)1.04-(-)0.03 (br, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 157.8, 155.3, 151.34, 151.28, 142.5, 142.4, 120.8, 89.6, 89.4, 86.8 (d, J_{CP} = 7.3 Hz), 86.6 (d, J_{CP} = 9.1 Hz), 77.5, 77.4, 73.2, 73.1, 68.0 (d, J_{CP} = 5.5 Hz), 65.3 (d, J_{CP} = 5.5 Hz); ^{31}P NMR (D_2O, 300 MHz): δ 86.54-81.39 (br, 1P), (-)10.61-(-)11.26 (m, 1P), (-)22.30-(-)23.10 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 833.31 (calculated for $C_{20}H_{30}BN_{10}O_{18}P_4^-$: 833.08).

Preparation of diadenosine-5′,5‴-*P*^{1},*P*^{4}-(*P*^{2},*P*^{3}-methylene)-tetraphosphate (21). To a solution of 2′,3′-*O*-6-*N*-triacetyladenosine (0.12 g, 0.30 mmol) in 2 mL of anhydrous DMF was added 2-chloro-4*H*-l,3,2-benzo-dioxaphosphorin-4-one (0.12 g, 0.59 mmol, 2.0 equiv). The solution was stirred for 15 min at room temperature under N_2. Tri-*n*-butylamine (0.60 mL, 2.5 mmol, 8.3 equiv) and methylenediphosphonic acid (0.073 g, 0.41 mmol, 1.4 equiv) were added. Following the same procedure described for **6**, the

intermediate was oxidized, reacted with AMP (0.39 g, 1.1 mmol, 3.7 equiv) and $ZnCl_2$ (0.44 g, 3.2 mmol, 11 equiv), treated with ammonia. The product was purified by reverse-phase HPLC to give 0.22 g of **21** in the ammonium form (0.24 mmol, 80%): UV λ_{max} 259 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.47 (s, 2H), 8.19 (s, 2H), 5.99 (d, *J* = 5.4 Hz, 2H), 4.61-4.51 (m, water suppression reduces intensity), 4.47 (t, *J* = 4.4 Hz, 2H), 4.44-4.36 (m, 2H), 4.35-4.19 (m, 4H), 2.61 (t, *J* = 21.0 Hz, 2H); ^{13}C NMR (D_2O, 400 MHz): δ 155.9, 153.4, 150.6, 143.1, 120.4, 89.8 (d, J_{CP} = 3.5 Hz), 86.4 (d, J_{CP} = 9.7 Hz), 77.7, 72.8, 67.9, 33.0 (t, J_{CP} = 133.1 Hz); ^{31}P NMR (D_2O, 300 MHz): δ 8.33 (d, *J* = 25.6 Hz, 2P), -10.41 (d, *J* = 25.5 Hz, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 833.24 (calculated for $C_{21}H_{29}N_{10}O_{18}P_4^-$: 833.06).

Preparation of diadenosine-5′,5‴-*P*^{1},*P*^{4}-(*P*^{2},*P*^{3}-difluoromethylene)-tetraphosphate (22). Starting with 2′,3′-*O*-6-*N*-triacetyladenosine (0.13 g, 0.33 mmol), **22** was prepared by the same procedure described for **6**, except that a 0.2 M solution of bis(tri-*n*-butylammonium) difluoromethylenediphosphonate in anhydrous DMF (3.0 mL, 0.60 mmol, 1.8 equiv) instead of the pyrophosphate was vortexed with tri-*n*-butylamine (0.60 mL, 2.5 mmol, 7.6 equiv). The product was purified by reverse-phase HPLC to give 0.25 g of **22** in the ammonium form (0.27 mmol, 82%): UV λ_{max} 259 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.40 (s, 2H), 8.16 (s, 2H), 6.00 (d, *J* = 5.8 Hz, 2H), 4.67-4.58 (m, water suppression reduces intensity), 4.50 (t, *J* = 5.7 Hz, 2H), 4.42-4.34 (m, 2H), 4.31-4.19 (m, 4H); ^{13}C NMR (D_2O, 400 MHz): δ 156.6, 154.0, 150.9, 142.8, 120.5, 89.6 (d, J_{CP} = 1.4 Hz), 86.6 (d, J_{CP} = 9.9 Hz), 77.6, 73.1, 68.0; ^{31}P NMR (D_2O, 300 MHz): δ -5.90 (tm, *J* = 82.8 Hz, 2P), (-)10.12-(-)11.02 (m, 2P); ^{19}F NMR (D_2O, 400 MHz): δ -119.78 (t, *J* = 83.7

Hz, 2F). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 869.65 (calculated for $C_{21}H_{27}F_2N_{10}O_{18}P_4^-$: 869.04).

Preparation of [S_P] and [R_P] adenosine 3′-azido-3′-deoxythymidine 5′,5‴-P^1,P^4-(P^4-thio)-tetraphosphate (26a,b). Starting with AZT (0.087 g, 0.33 mmol), **26a,b** was prepared by the same procedure described for **14a,b**. The two diastereomers were purified and separated by reverse-phase HPLC to give 0.12 g of **[S_P]-26a** (0.13 mmol, 39%) and 0.11 g of **[R_P]-26b** (0.12 mmol, 36%) in the ammonium form. The diastereomers eluted in the order listed. **[S_P]-26a**: UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.52 (s, 1H), 8.22 (s, 1H), 7.66 (s, 1H), 6.14-6.06 (m, 2H), 4.60-4.50 (m, water suppression reduces intensity), 4.40-4.35 (m, 1H), 4.33-4.16 (m, 4H), 4.15-4.09 (m, 1H), 2.40-2.33 (m, 2H), 1.88 (s, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.2, 158.3, 155.6, 154.2, 151.9, 142.7, 139.9, 121.3, 114.4, 89.3, 87.5, 86.8 (d, J_{CP} = 9.1 Hz), 85.6 (d, J_{CP} = 10.0 Hz), 77.1, 73.1, 68.6 (d, J_{CP} = 6.2 Hz), 68.1 (d, J_{CP} = 5.5 Hz), 63.1, 39.1, 14.5; ^{31}P NMR (D_2O, 300 MHz): δ 43.29 (d, *J* = 24.33 Hz, 1P), -10.98 (d, *J* = 18.07 Hz, 1P), -22.74 (t, *J* = 17.35 Hz, 1P), -23.68 (dd, *J* = 24.32 Hz, *J* = 16.44 Hz, 1P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 851.54 amu (calculated for $C_{20}H_{27}N_{10}O_{18}P_4S^-$: 851.02). **[$R_P$]-26b**: UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.52 (s, 1H), 8.22 (s, 1H), 7.65 (d, *J* = 1.11 Hz, 1H), 6.15-6.06 (m, 2H), 4.60-4.50 (m, water suppression reduces intensity), 4.42-4.36 (m, 1H), 4.33-4.18 (m, 4H), 4.15-4.09 (m, 1H), 2.40-2.33 (m, 2H), 1.88 (s, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.1, 158.3, 155.6, 154.2, 151.9, 142.7, 139.8 (d, J_{CP} = 3.3 Hz), 121.3, 114.4, 89.3 (d, J_{CP} = 3.8 Hz), 87.4 (d, J_{CP} = 3.6 Hz), 86.8 (d, J_{CP} = 9.1 Hz), 85.7 (d, J_{CP} = 8.0 Hz), 77.1, 73.2, 68.9, 68.1, 63.9, 39.1, 14.5; ^{31}P

NMR (D_2O, 300 MHz): δ 43.20 (d, *J* = 25.87 Hz, 1P), -10.97 (d, *J* = 18.07 Hz, 1P), -22.73 (t, *J* = 17.33 Hz, 1P), -23.71 (dd, *J* = 25.80 Hz, *J* = 16.45 Hz, 1P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 851.54 amu (calculated for $C_{20}H_{27}N_{10}O_{18}P_4S^-$: 851.02).

Preparation of [S_P] and [R_P] adenosine 3′-azido-3′-deoxythymidine 5′,5‴-P^1,P^4-(P^4-seleno)-tetraphosphate (27a,b). Starting with AZT (0.081 g, 0.30 mmol), **27a,b** was prepared by the same procedure described for **15a,b**. The two diastereomers were purified and separated by reverse-phase HPLC to give 0.085 g of **[S_P]-27a** (0.088 mmol, 29%) and 0.082 g of **[R_P]-27b** (0.085 mmol, 28%) in the ammonium form. The diastereomers eluted in the order listed. **[S_P]-27a**: UV λ_{max} 261 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.52 (s, 1H), 8.22 (s, 1H), 7.66 (s, 1H), 6.14-6.06 (m, 2H), 4.80-4.74 (m, water suppression reduces intensity), 4.60-4.50 (m, 2H), 4.42-4.35 (m, 1H), 4.35-4.17 (m, 4H), 4.16-4.10 (m, 1H), 2.41-2.34 (m, 2H), 1.89 (d, *J* = 1.69 Hz, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.2, 158.3, 155.6, 154.2, 151.9, 142.7, 139.9, 121.3, 114.4, 89.3, 87.5, 86.7 (d, J_{CP} = 9.1 Hz), 85.4 (d, J_{CP} = 10.0 Hz), 77.1, 73.1, 68.6 (d, J_{CP} = 6.3 Hz), 68.1 (d, J_{CP} = 5.5 Hz), 63.6, 39.0, 14.5; ^{31}P NMR (D_2O, 300 MHz): δ 33.73 (d, *J* = 28.70 Hz, 1P), -10.98 (d, *J* = 18.27 Hz, 1P), -22.73 (t, *J* = 17.08 Hz, 1P), -24.18 (dd, *J* = 29.07 Hz, *J* = 16.32 Hz, 1P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 899.34 amu (calculated for $C_{20}H_{27}N_{10}O_{18}P_4Se^-$: 898.96). **[$R_P$]-27b**: UV λ_{max} 261 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.52 (s, 1H), 8.22 (s, 1H), 7.65 (s, 1H), 6.14-6.06 (m, 2H), 4.83-4.78 (m, water suppression reduces intensity), 4.62-4.51 (m, 2H), 4.43-4.36 (m, 1H), 4.35-4.17 (m, 4H), 4.16-4.10 (m, 1H), 2.41-2.31 (m, 2H), 1.89 (s, 3H); ^{13}C NMR (D_2O,

400 MHz): δ 169.2, 158.3, 155.5, 154.2, 151.9, 142.7, 139.8, 121.3, 114.4, 89.3, 87.4, 86.8 (d, J_{CP} = 9.1 Hz), 85.5 (d, J_{CP} = 9.7 Hz), 77.1, 73.2, 69.1 (d, J_{CP} = 5.9 Hz), 68.1 (d, J_{CP} = 5.1 Hz), 64.0, 39.1, 14.6; ^{31}P NMR (D_2O, 300 MHz): δ 33.64 (d, *J* = 31.09 Hz, 1P), -10.98 (d, *J* = 18.19 Hz, 1P), -22.77 (t, *J* = 17.31 Hz, 1P), -24.25 (dd, *J* = 31.08 Hz, *J* = 16.57 Hz, 1P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 899.47 amu (calculated for $C_{20}H_{27}N_{10}O_{18}P_4Se^-$: 898.96).

Preparation of [S_P] and [R_P] adenosine 3′-azido-3′-deoxythymidine 5′,5‴-P^1,P^4-(P^4-borano)-tetraphosphate (28a,b). Starting with AZT (0.087 g, 0.33 mmol), **28a,b** was prepared by the same procedure described for **16a,b**. The two diastereomers were purified and separated by reverse-phase HPLC to give 0.11 g of **[R_P]-28a** (0.12 mmol, 36%) and 0.088 g of **[S_P]-28b** (0.98 mmol, 30%) in the ammonium form. The diastereomers eluted in the order listed. **[R_P]-28a**: UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.49 (s, 1H), 8.20 (s, 1H), 7.55 (s, 1H), 6.13 (dd, *J* = 7.76 Hz, *J* = 6.27 Hz, 1H), 6.09 (d, *J* = 5.82 Hz, 1H), 4.77-4.73 (m, water suppression reduces intensity), 4.56 (dd, *J* = 4.92 Hz, *J* = 3.89 Hz, 1H), 4.54-4.47 (m, 1H), 4.42-4.35 (m, 1H), 4.33-4.21 (m, 2H), 4.18-4.12 (m, 2H), 4.11-4.06 (m, 1H), 2.39-2.20 (m, 2H), 1.86 (s, 3H), (+)0.95-(-)0.05 (br, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.2, 158.3, 155.6, 154.3, 151.9, 142.7, 139.7, 121.3, 114.4, 89.3, 87.6, 86.7 (d, J_{CP} = 9.2 Hz), 86.0 (d, J_{CP} = 7.4 Hz), 77.0, 73.1, 68.0 (d, J_{CP} = 5.3 Hz), 65.4 (d, J_{CP} = 6.9 Hz), 64.2, 39.2, 14.5; ^{31}P NMR (D_2O, 300 MHz): δ 86.00-81.00 (br, 1P), (-)10.40-(-)11.07 (m, 1P), (-)21.75-(-)22.81 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 833.37 amu (calculated for $C_{20}H_{30}BN_{10}O_{18}P_4^-$: 833.08). **[$S_P$]-28b**: UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ

8.51 (s, 1H), 8.21 (s, 1H), 7.61 (s, 1H), 6.09-6.04 (m, 2H), 4.77-4.72 (m, water suppression reduces intensity), 4.56 (dd, J = 5.12 Hz, J = 3.12 Hz, 1H), 4.48-4.43 (m, 1H), 4.41-4.35 (m, 1H), 4.33-4.19 (m, 3H), 4.17-4.06 (m, 2H), 2.42-2.26 (m, 2H), 1.87 (d, J = 0.94 Hz, 3H), (+)0.87-(-)0.14 (br, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.2, 158.3, 155.5, 154.2, 151.9, 142.7, 139.8, 121.3, 114.3, 89.3, 87.5, 86.8 (d, J_{CP} = 9.2 Hz), 86.0 (d, J_{CP} = 7.2 Hz), 77.2, 73.2, 68.1 (d, J_{CP} = 4.9 Hz), 65.6 (d, J_{CP} = 7.0 Hz), 63.9, 39.3, 14.5; ^{31}P NMR (D_2O, 300 MHz): δ 86.75-79.77 (br, 1P), (-)10.78-(-)11.15 (m, 1P), (-)22.36-(-)23.13 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 833.31 amu (calculated for $C_{20}H_{30}BN_{10}O_{18}P_4^-$: 833.08).

Preparation of adenosine 3′-azido-3′-deoxythymidine 5′,5‴-*P*^{1},*P*^{4}-(*P*^{2},*P*^{3}-methylene)-tetraphosphate (29). Starting with AZT (0.084 g, 0.31 mmol), 29 was prepared by the same procedure described for **8**. The product was purified by preparative reverse-phase HPLC to give 0.17 g of **29** in the ammonium form (0.19 mmol, 61%): UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.52 (s, 1H), 8.21 (s, 1H), 7.64 (d, J = 0.9 Hz, 1H), 6.16 (t, J = 6.9 Hz, 1H), 6.10 (d, J = 5.8 Hz, 1H), 4.76-4.70 (m, water suppression reduces intensity), 4.60-4.48 (m, 2H), 4.42-4.35 (m, 1H), 4.31-4.11 (m, 5H), 2.51 (t, $J_{C\text{-}H}$ = 21.0 Hz, 2H), 2.42-2.33 (m, 2H), 1.86 (s, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.1, 158.2, 155.5, 154.2, 151.8, 142.6, 139.8 (d, J_{CP} = 2.3 Hz), 121.3, 114.4, 89.6 (d, J_{CP} = 2.8 Hz), 87.5 (d, J_{CP} = 3.2 Hz), 86.6 (d, J_{CP} = 9.1 Hz), 85.7 (d, J_{CP} = 9.0 Hz), 77.2, 73.1, 68.2 (d, J_{CP} = 4.6 Hz), 67.7 (d, J_{CP} = 5.3 Hz), 63.7, 39.0, 32.7 (t, J_{CP} = 129.9 Hz), 14.4; ^{31}P NMR (D_2O, 300 MHz): δ 9.25-7.50 (m, 2P), (-)10.00-(-)11.33 (m, 2P). The mass was

confirmed by ESI-MS in negative mode as *m/z* (M-1) 833.37 (calculated for $C_{21}H_{29}N_{10}O_{18}P_4^-$: 833.06).

Preparation of adenosine 3′-azido-3′-deoxythymidine 5′,5‴-*P*^{1},*P*^{4}-(*P*^{2},*P*^{3}-difluoromethylene)-tetraphosphate (30). Starting with 3′-azido-3′-deoxythymidine (0.084 g, 0.31 mmol), **30** was prepared by the same procedure described for **22**. The product was purified by preparative reverse-phase HPLC to give 0.19 g of **30** in the ammonium form (0.20 mmol, 65%): UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400M Hz): δ 8.49 (s, 1H), 8.21 (s, 1H), 7.59 (s, 1H), 6.14-6.05 (m, 2H), 4.73 (t, *J* = 5.5 Hz, water suppression reduces intensity), 4.60-4.46 (m, 2H), 4.40-4.34 (m, 1H), 4.30-4.13 (m, 4H), 4.13-4.07 (m, 1H), 2.43-2.25 (m, 2H), 1.84 (s, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.0, 158.2, 155.5, 154.2, 151.8, 142.6, 139.7, 121.2, 114.4, 89.5, 87.5, 86.8 (d, J_{CP} = 9.6 Hz), 85.7 (d, J_{CP} = 9.5 Hz), 77.4, 73.2, 68.5 (d, J_{CP} = 5.7 Hz), 68.0 (d, J_{CP} = 5.5 Hz), 63.8, 39.0, 14.4; ^{31}P NMR (D_2O, 300 MHz): δ -6.16 (tm, *J* = 83.2 Hz, 2P), (-)10.53-(-)11.63 (m, 2P); ^{19}F NMR (D_2O, 400 MHz): δ -119.7 (t, *J* = 83.4 Hz). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 868.87 (calculated for $C_{21}H_{27}F_2N_{10}O_{18}P_4^-$: 869.04).

Identification of Configurations of the Diastereomers of Ap$_S$pppA (14a,b), Ap$_{Se}$pppA (15,b) and Ap$_{BH3}$pppA (16a,b) To a solution of 0.32 μmol of one diastereomer from each pair in 500 μL of 100 mM Tris•HCl buffer (pH = 8.7), 2 mM $MgCl_2$, was added 4 μL of snake venom phosphodiesterase in water (50 μg/μL). The solution was maintained at 37 ^{o}C and analyzed by LC-MS (20 μL each time, λ = 280 nm) after various time intervals. The percentages of Ap$_X$pp (X = S, Se or BH_3) (relative to the

total nucleoside-containing peaks) were monitored and plotted against time. The other diastereomer was assayed following the same procedure.

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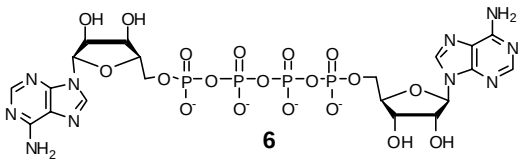
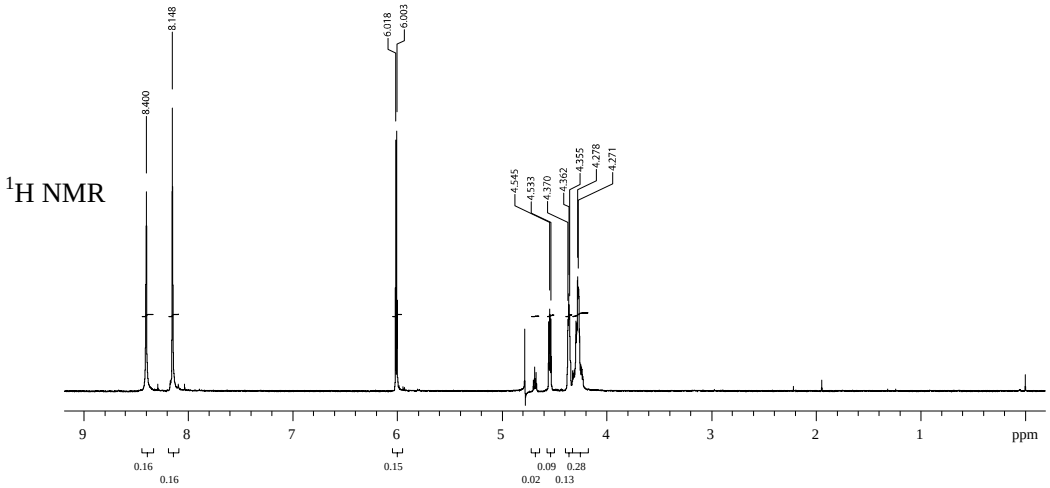
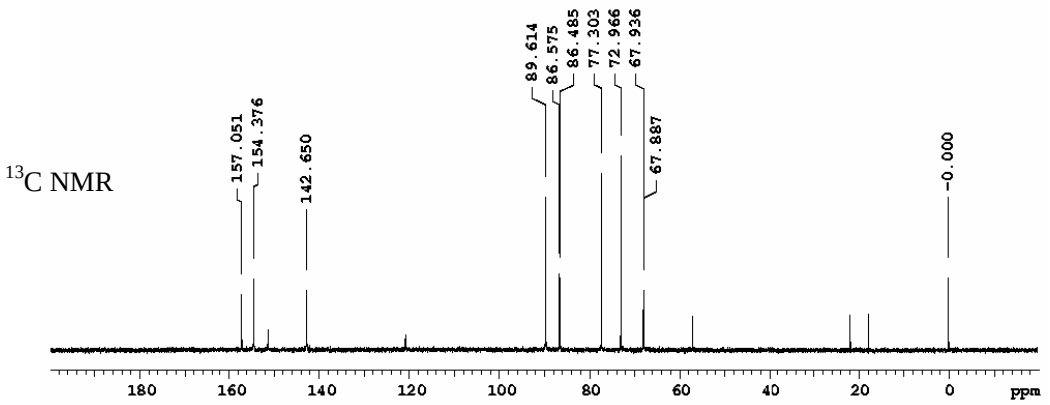
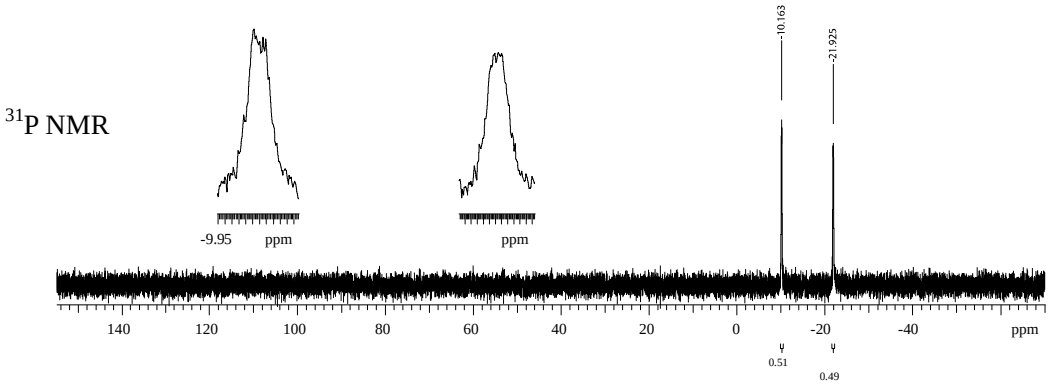
6. Appendix

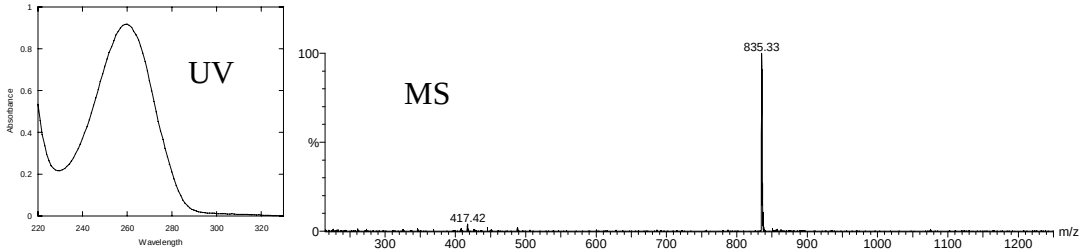
The ^{31}P NMR spectra were acquired on a Varian Unity 300 MHz spectrometer. All other NMR spectra were acquired on a Varian Unity 400 MHz spectrometer. The samples were all converted to the sodium form and dissolved in D_2O. The ^{1}H and ^{13}C spectra were referenced to 3-(trimethylsilyl)-1-propane-sulfonic acid, sodium salt. Signals in the ^{13}C spectra at 57.1, 21.8, 17.7 and 0.0 are from this standard. The ^{31}P NMR spectra were referenced to 10% phosphoric acid in D_2O. The ^{19}F spectra were referenced to hexafluorobenzene. In the ^{1}H NMR spectra, water suppression diminishes the adjacent resonances. UV spectra were acquired on a Varian Cary 4000 or an Aviv 14 UV spectrophotometer, and the MS acquired on a Waters/Micromass Platform LCZ mass spectrometer.



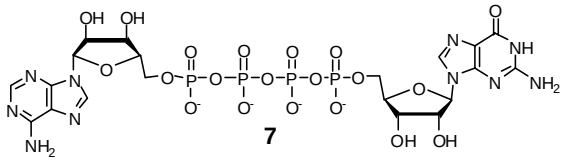


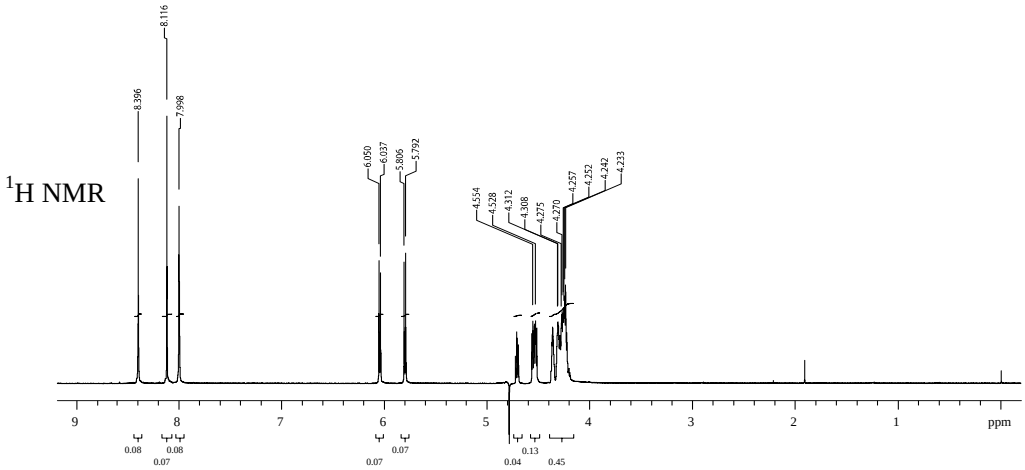


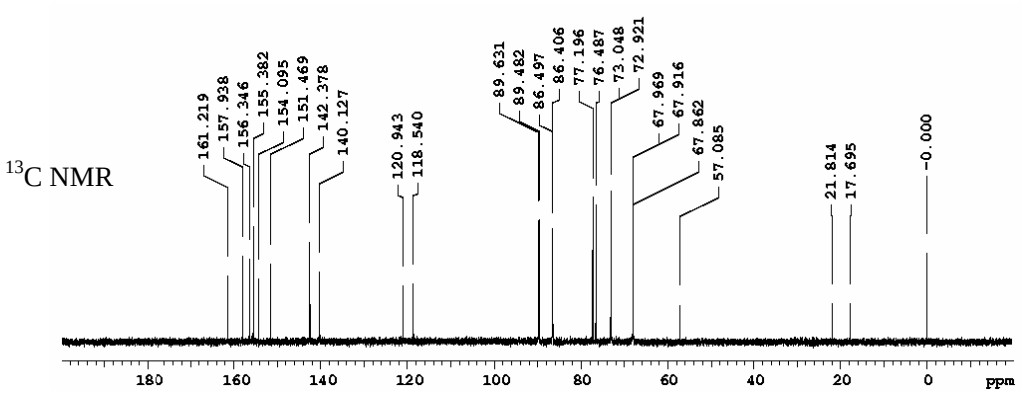


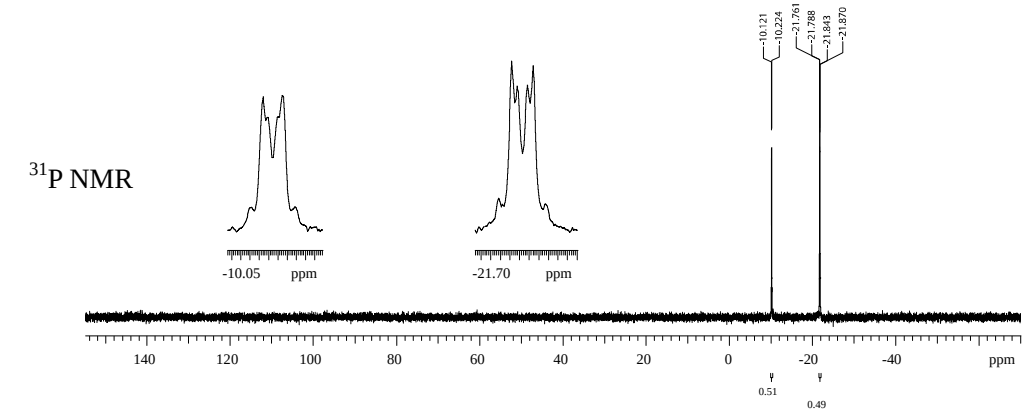


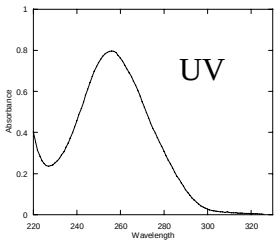
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for Ap_4A (6)

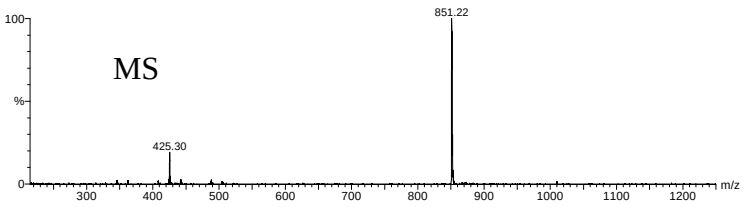




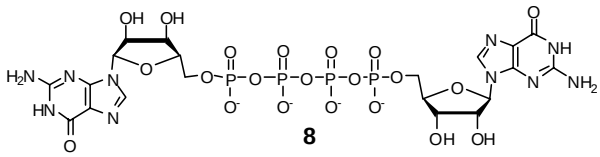


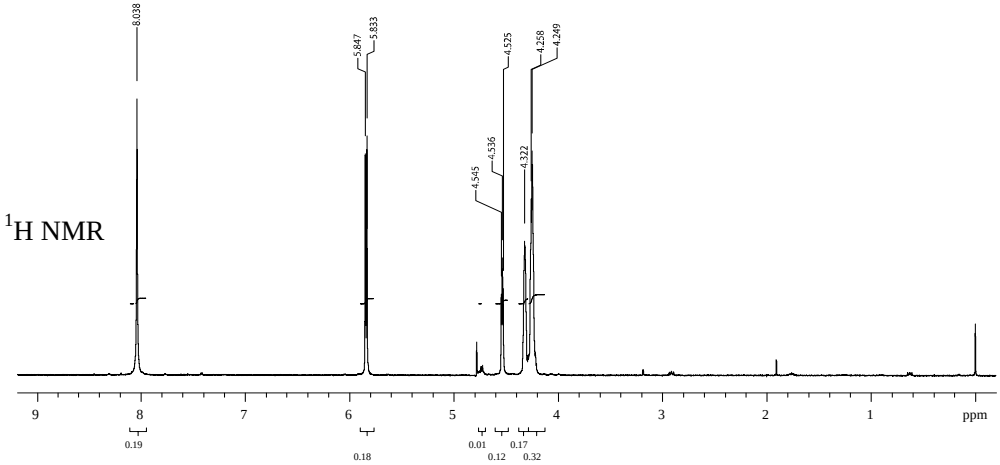


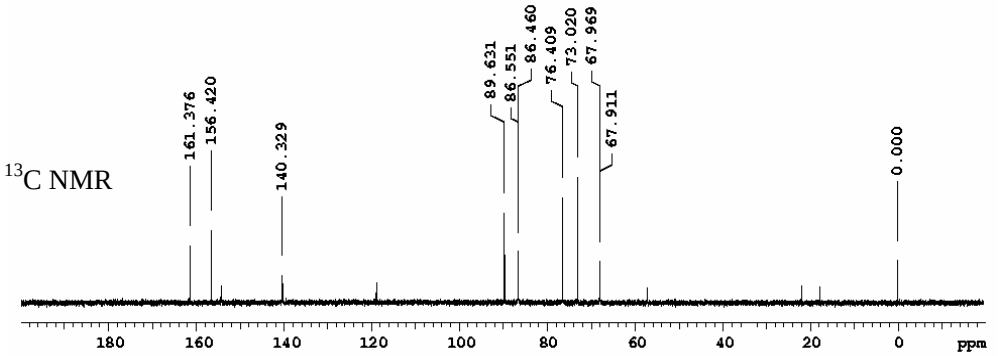


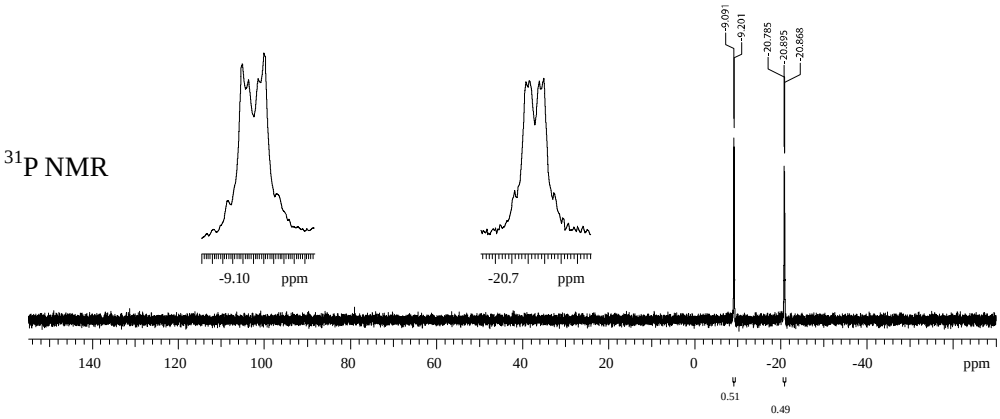


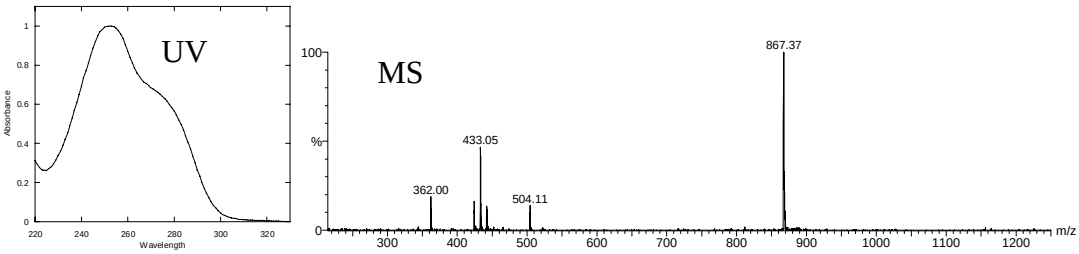
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for Ap$_4$G (7)



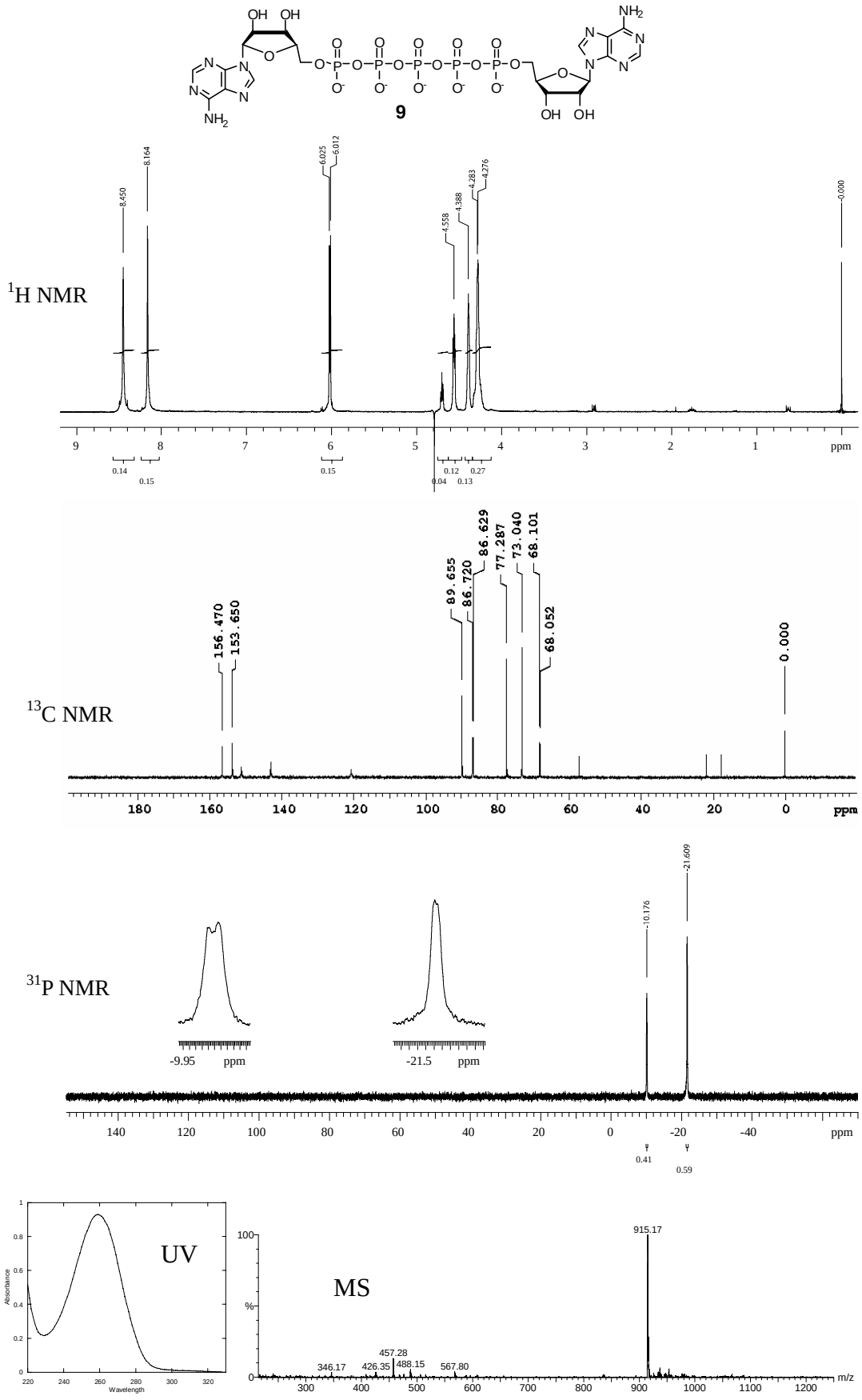




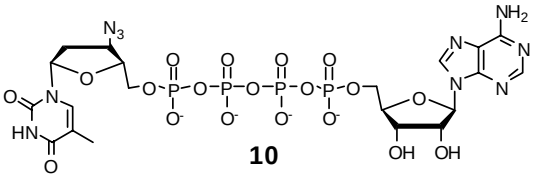


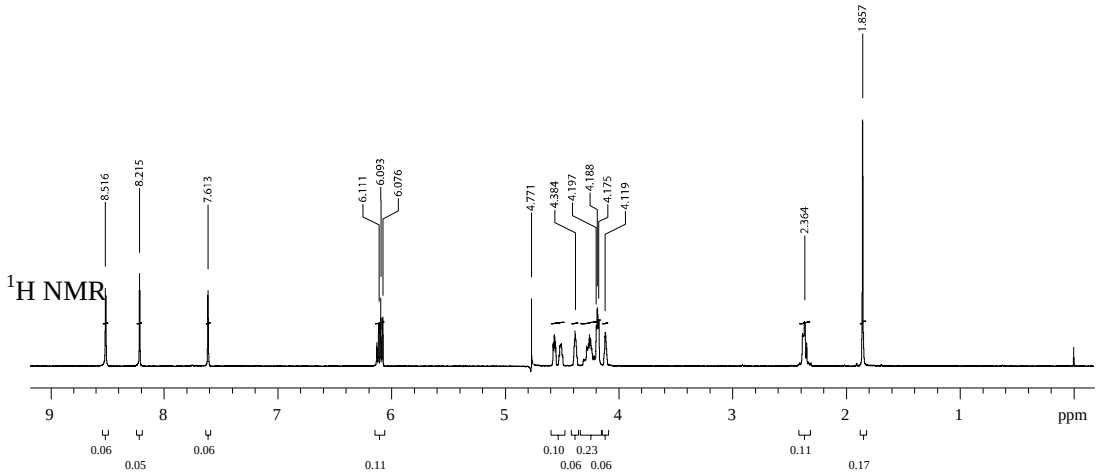


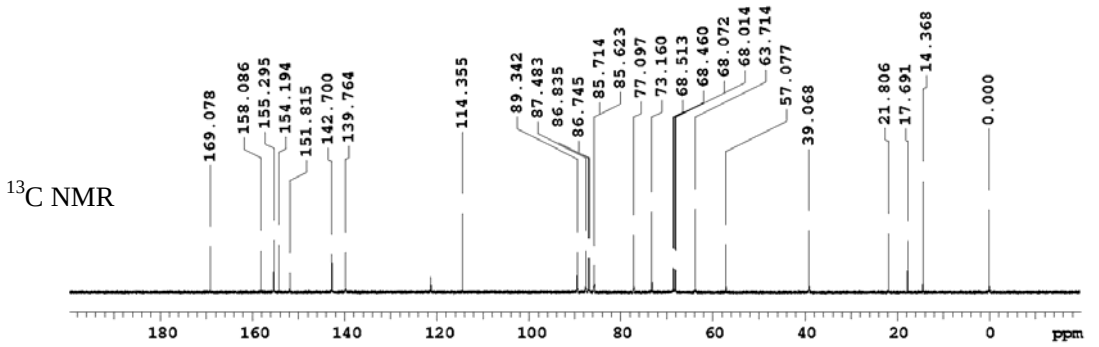
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for Gp_4G (8)

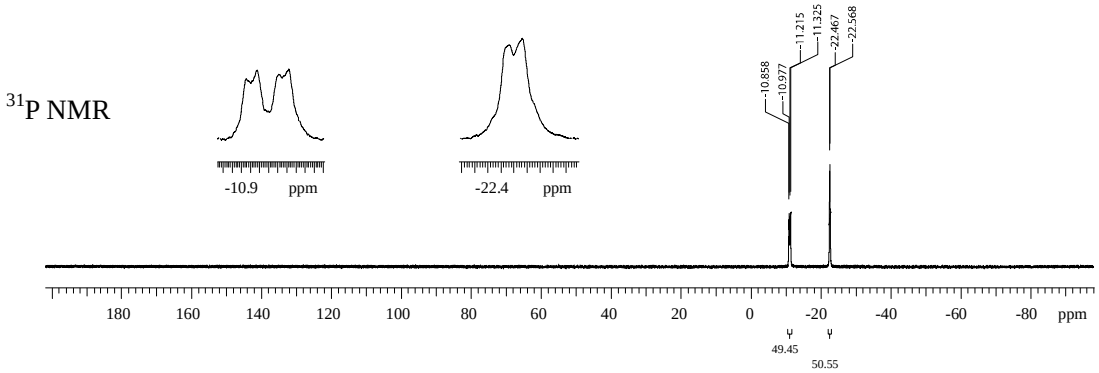


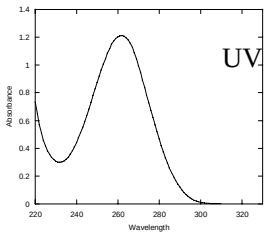
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for Ap_5A (9)

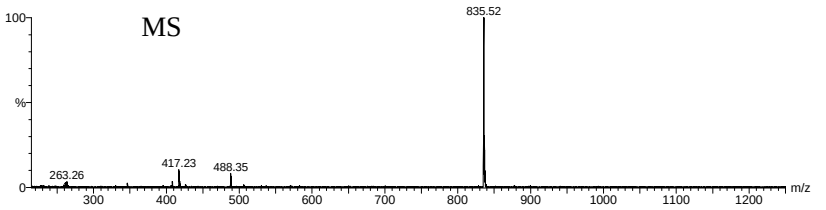




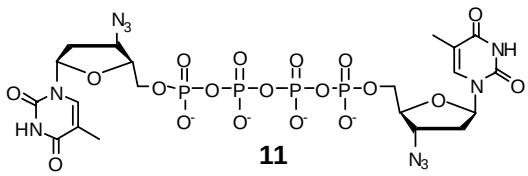


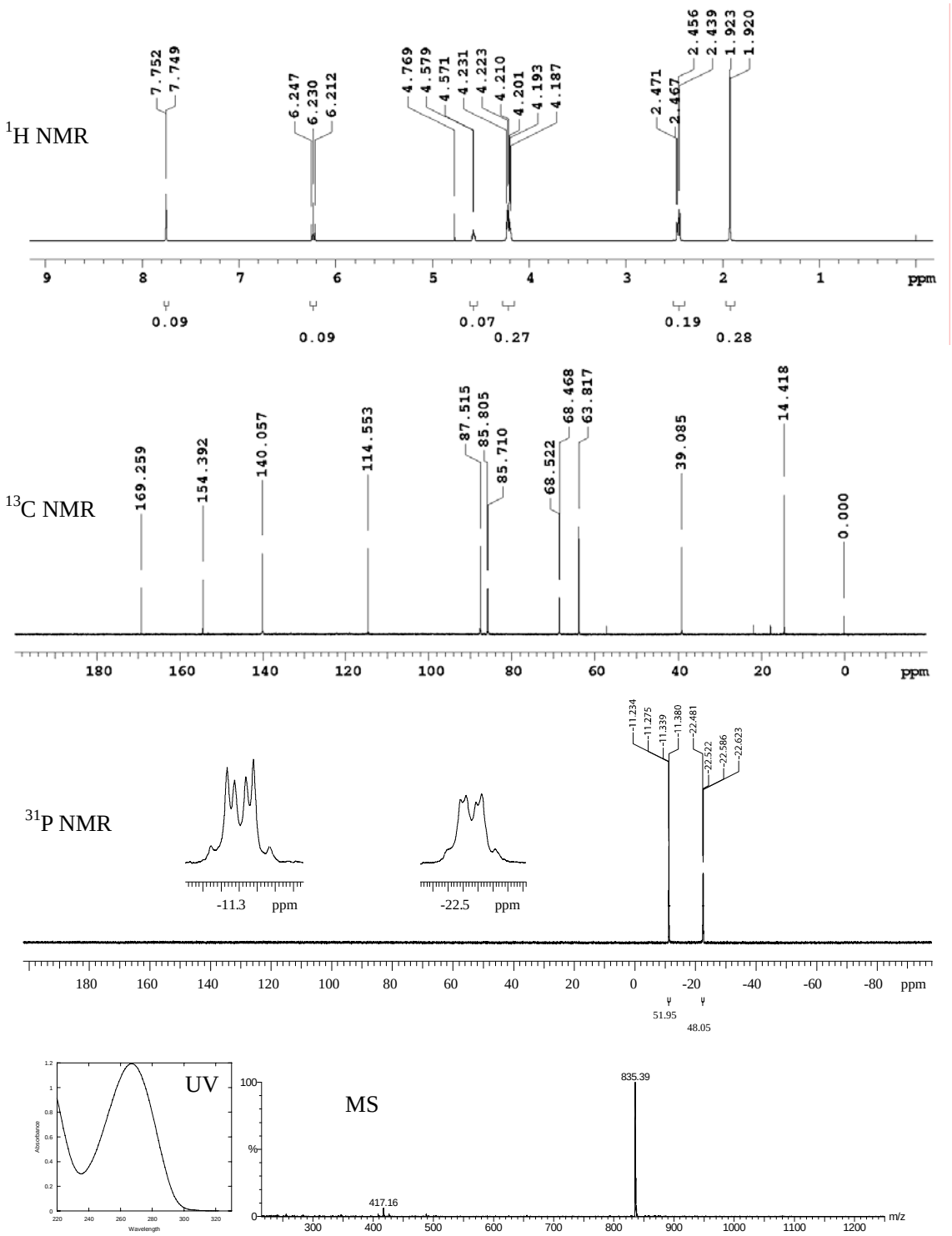




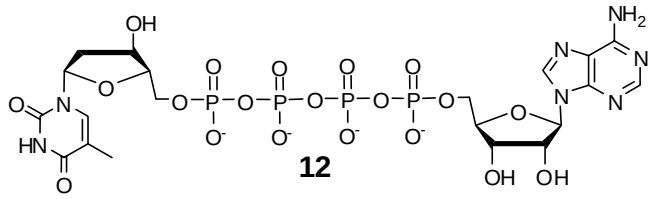


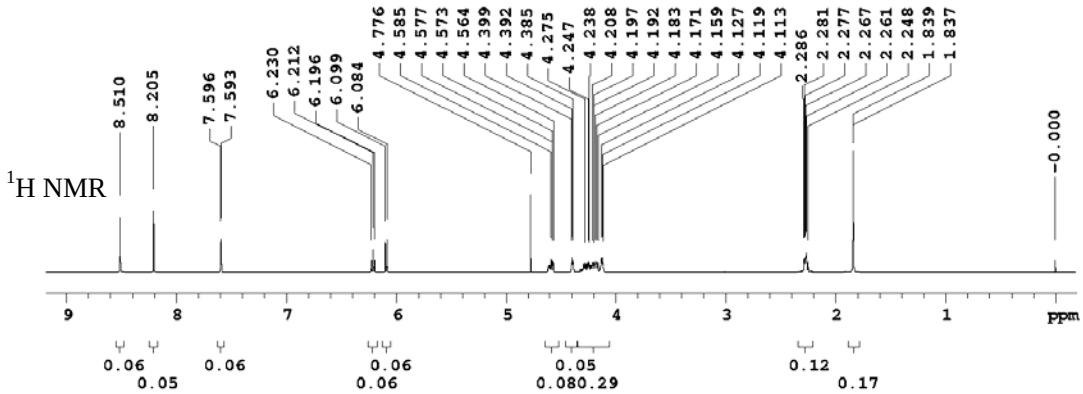
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for AZTp$_4$A (10)

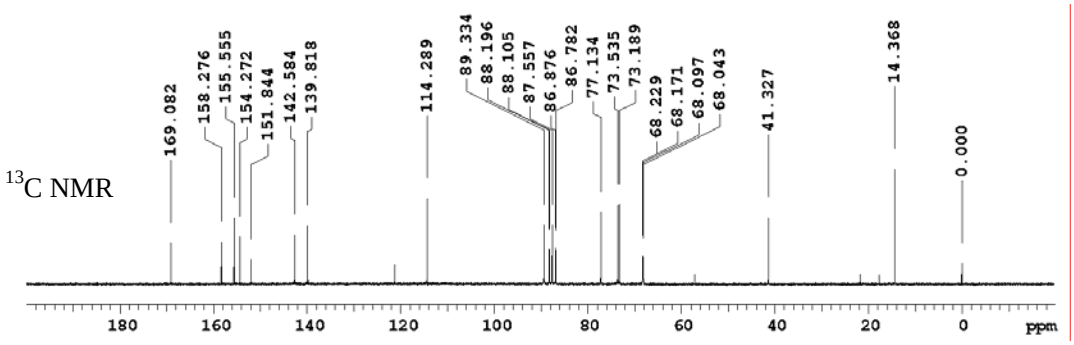


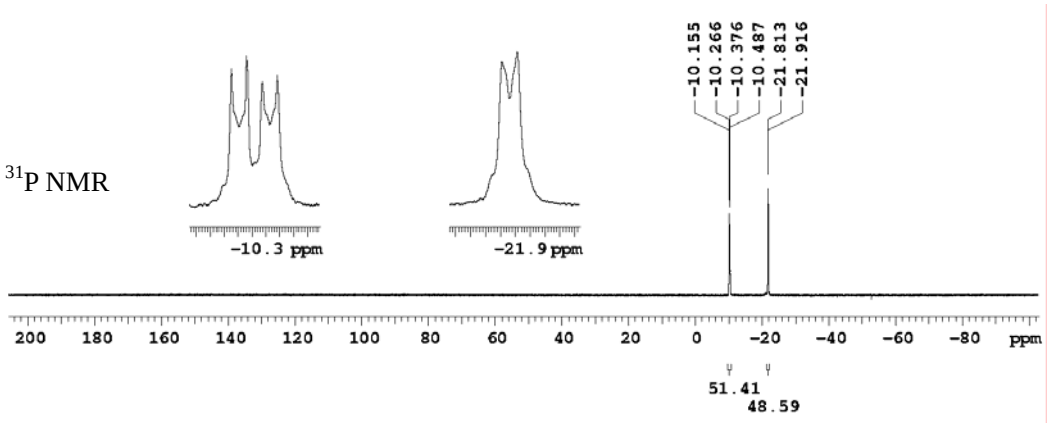


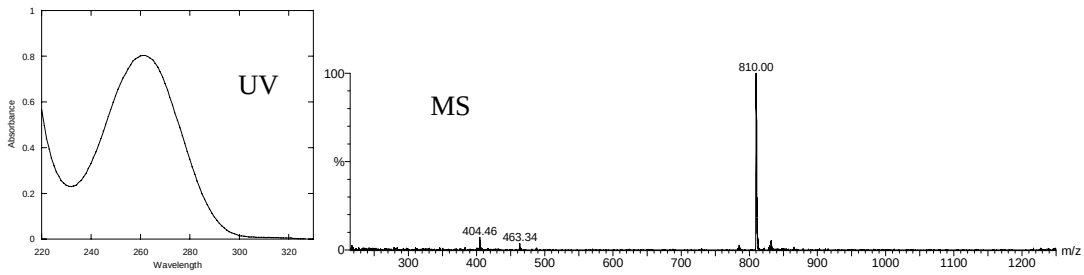
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for AZTp$_4$AZT (11)



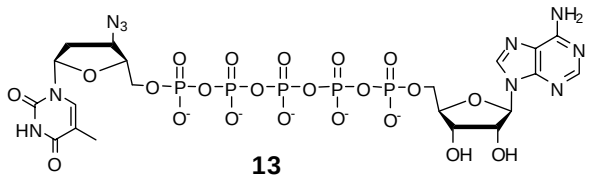


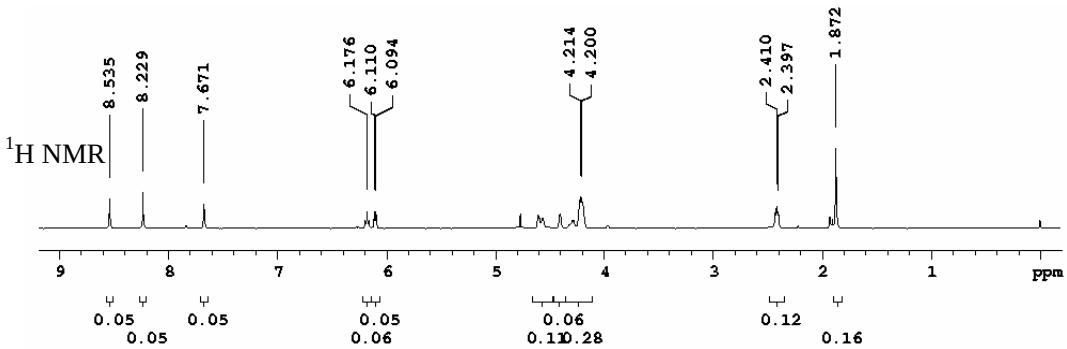


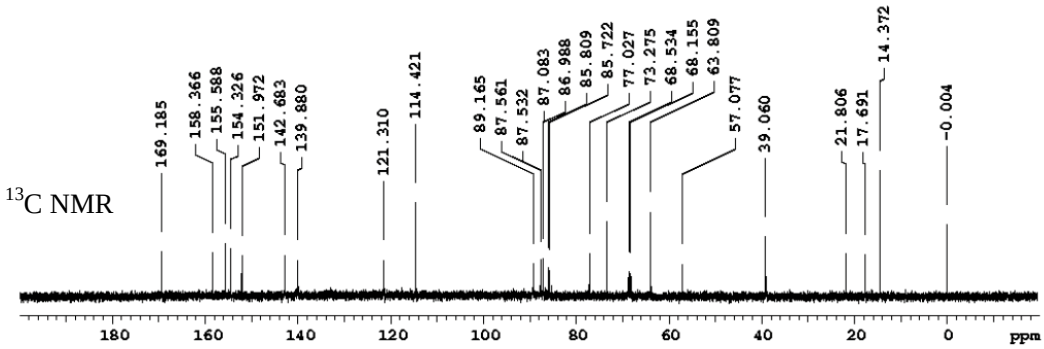


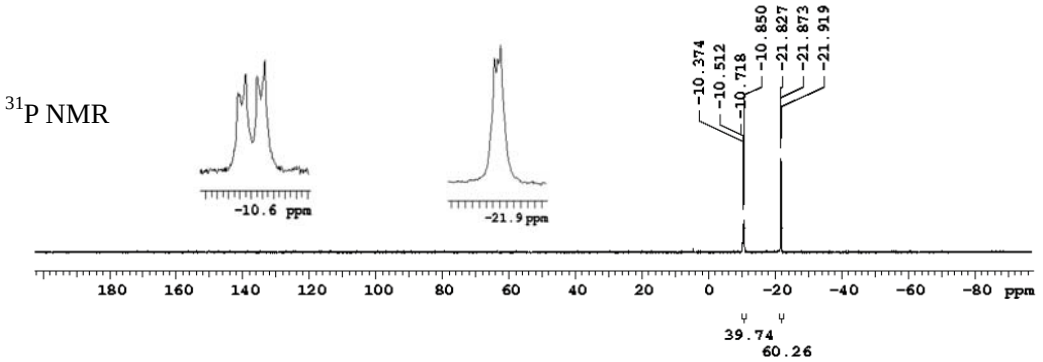


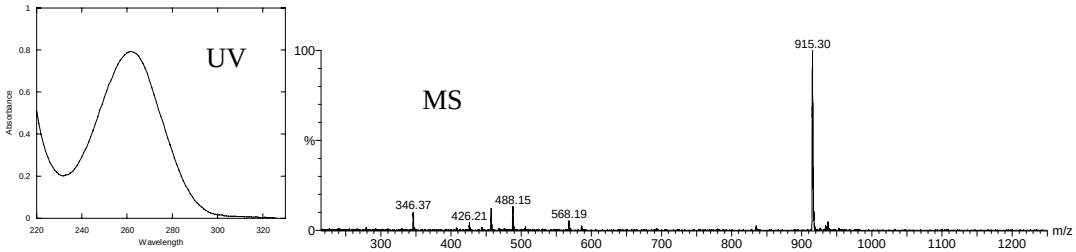
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for dTp4A (12)



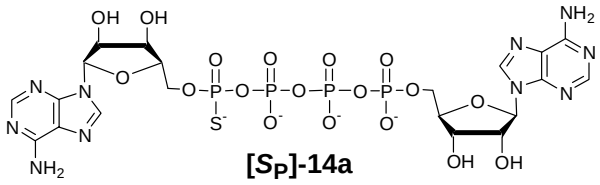


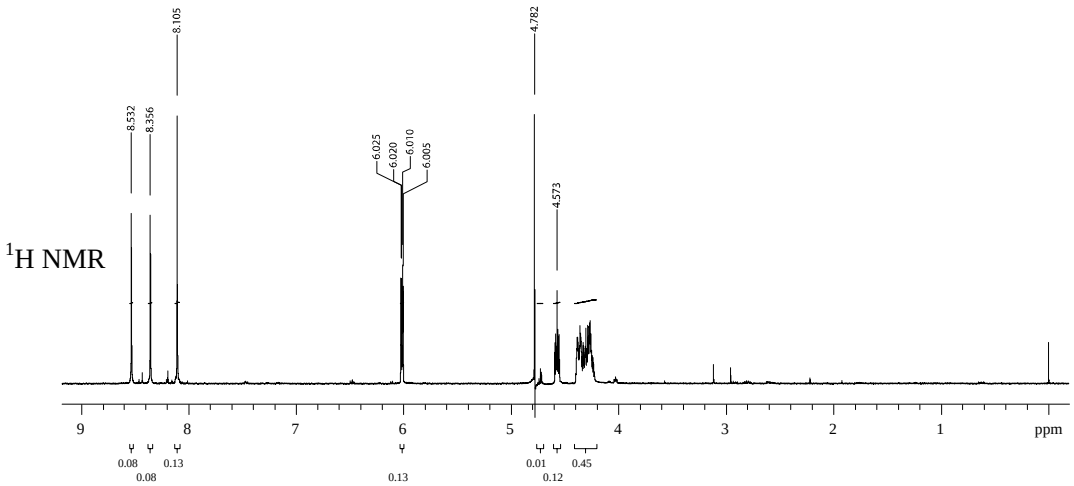


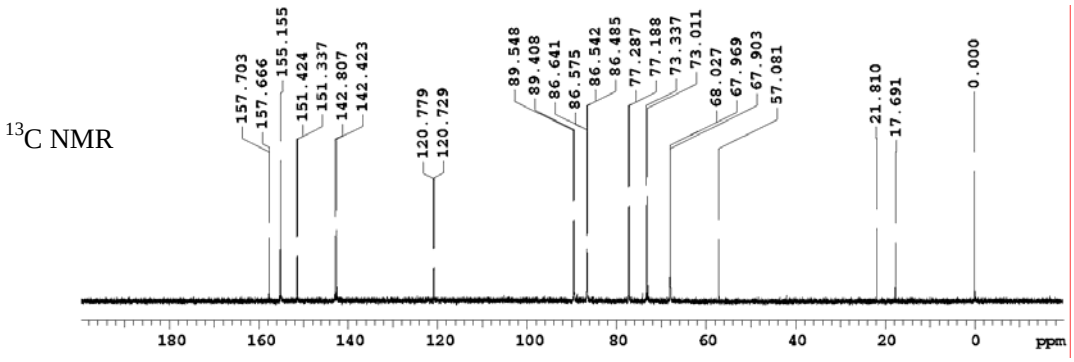


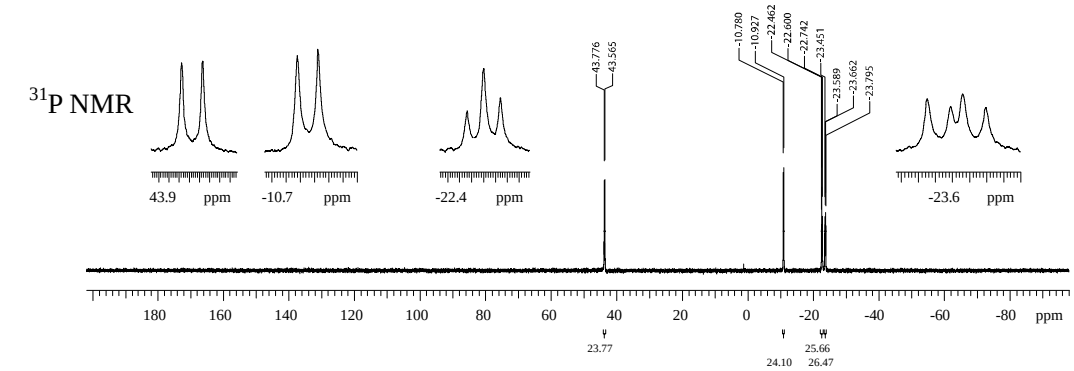


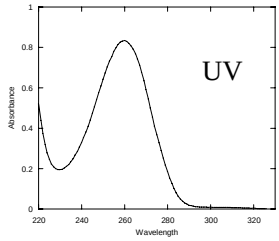
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for AZTp5A (13)

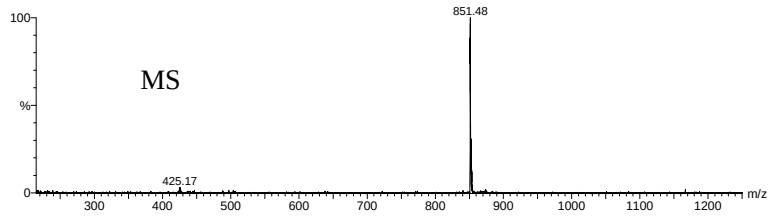




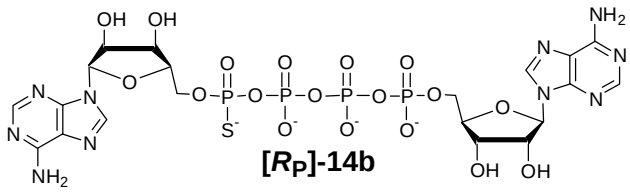


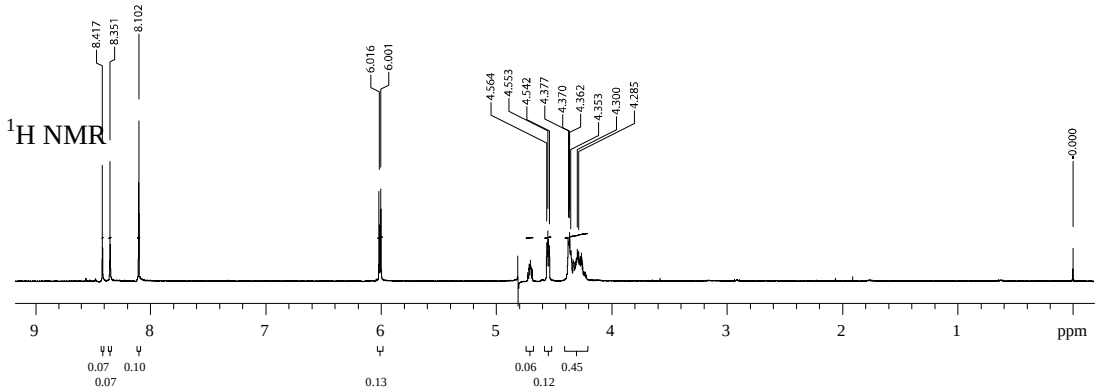


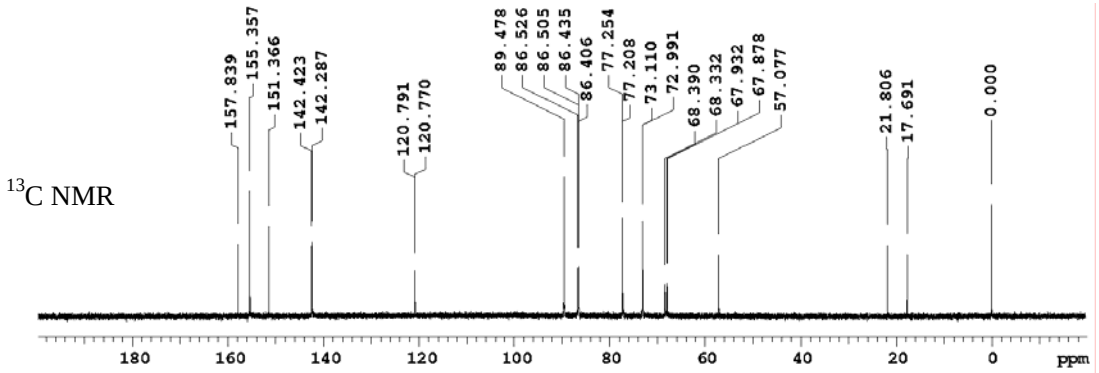


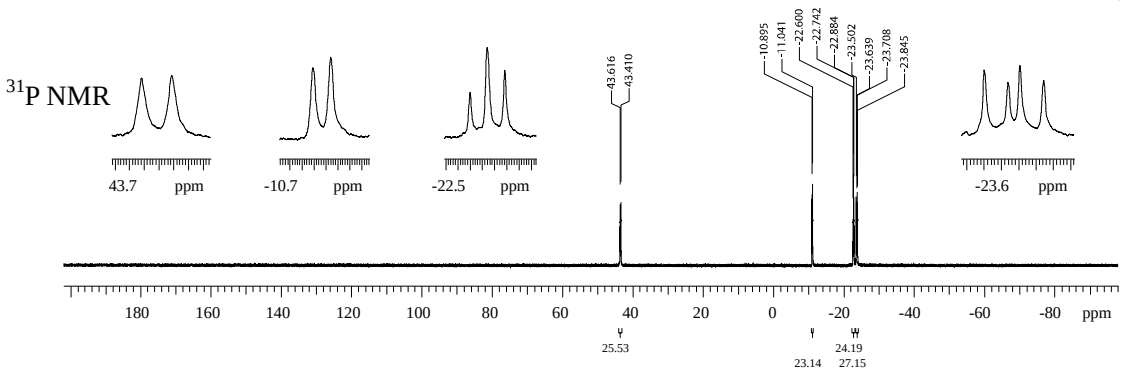


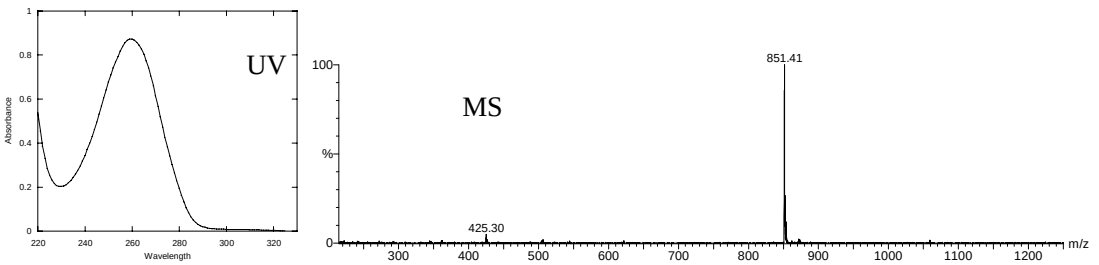
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [S_P]-Ap$_S$pppA (14a)



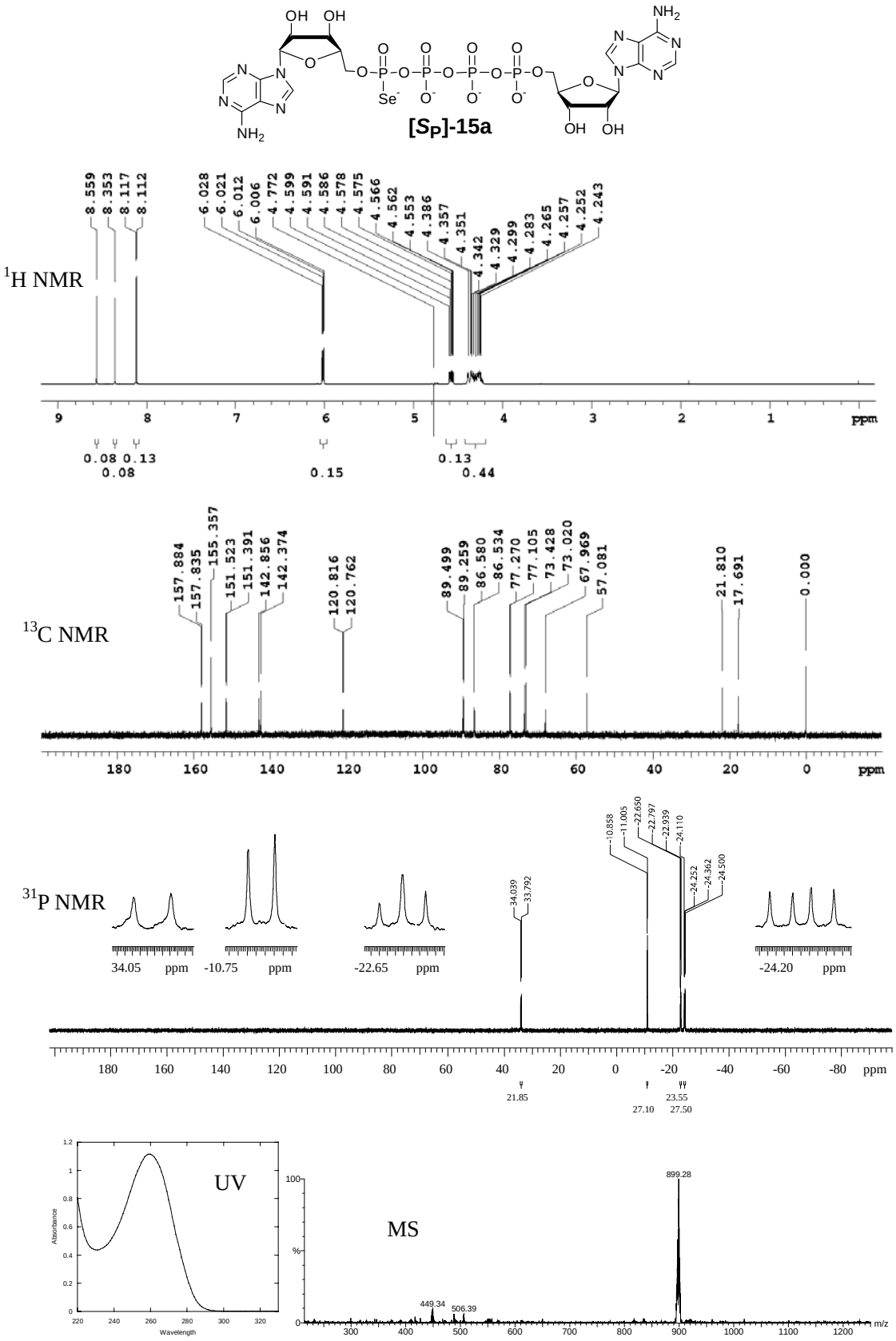




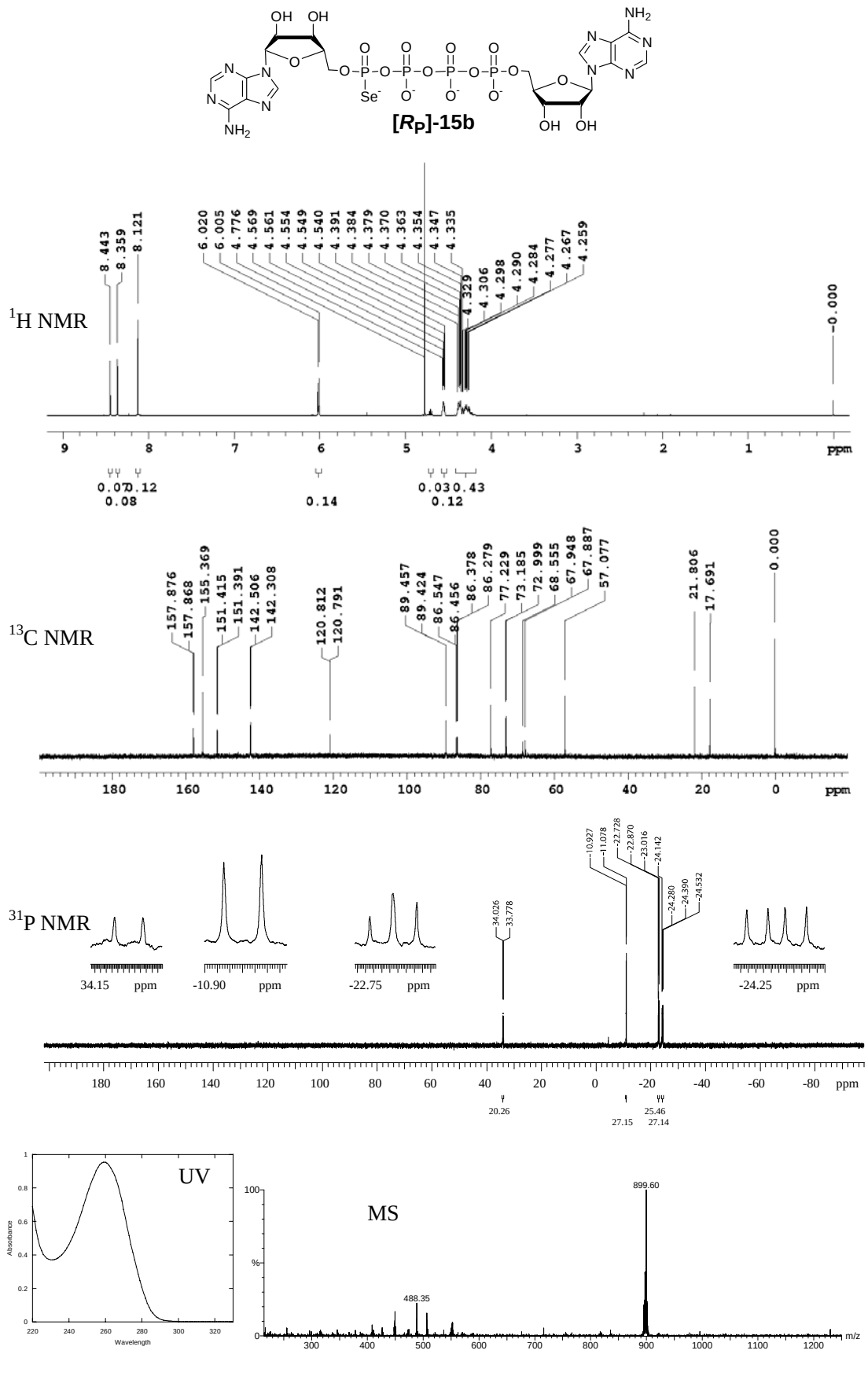




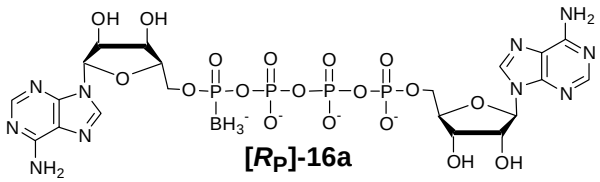
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [R_P]-Ap$_S$pppA (14b)

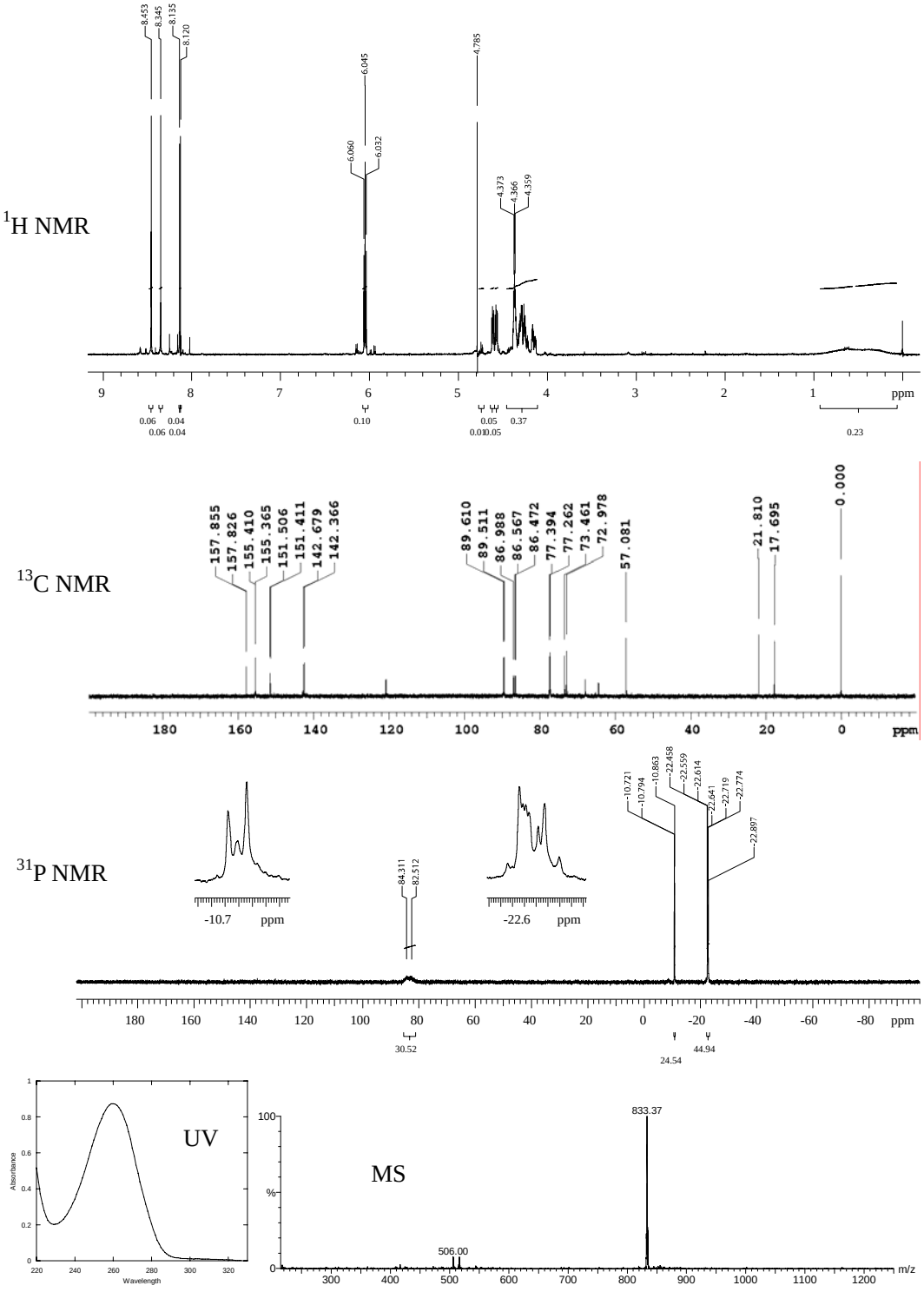


^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [S_P]-Ap$_{Se}$pppA (15a)

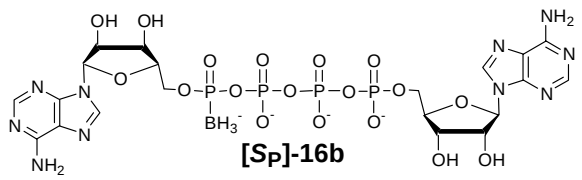


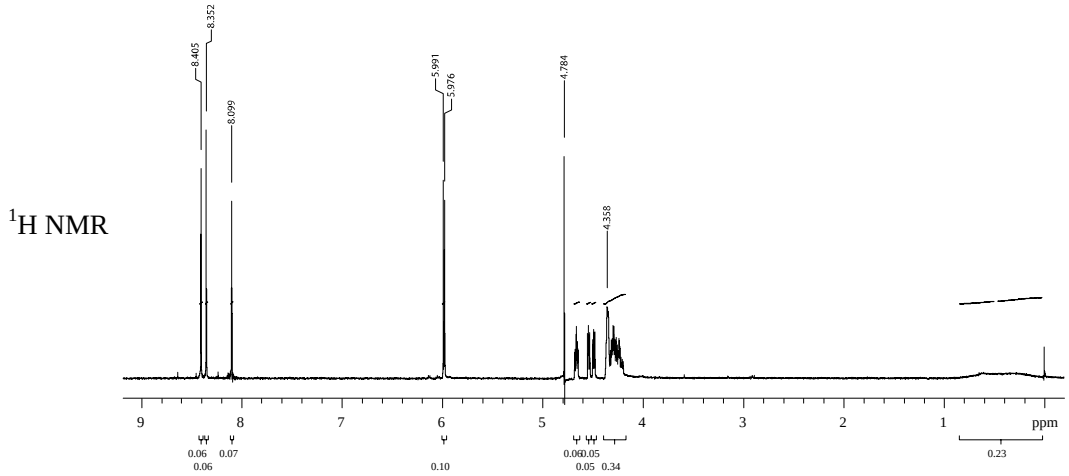
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [R_P]-Ap$_{Se}$pppA (15b)

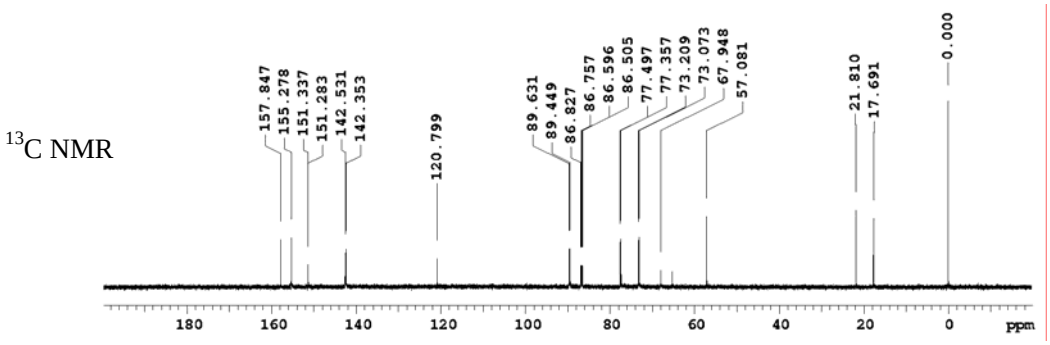


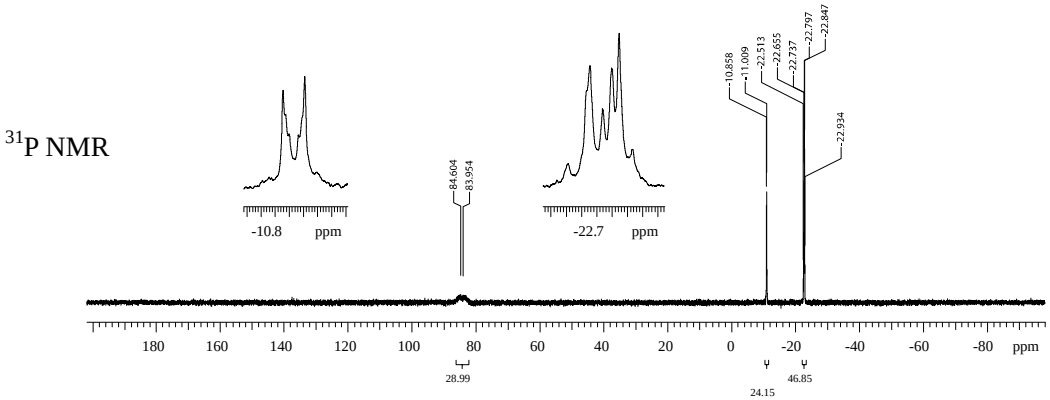


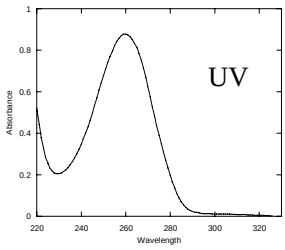
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [R_P]-Ap$_{BH3}$pppA (16a)

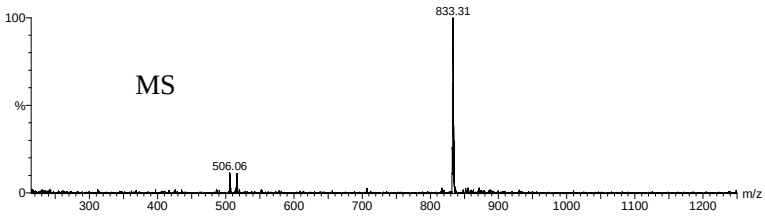




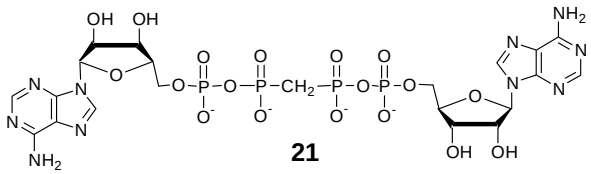


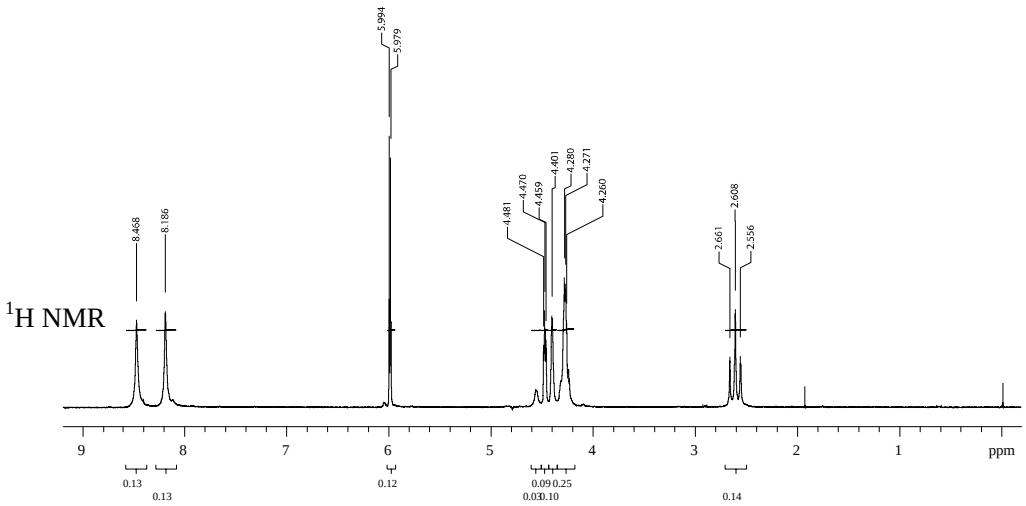


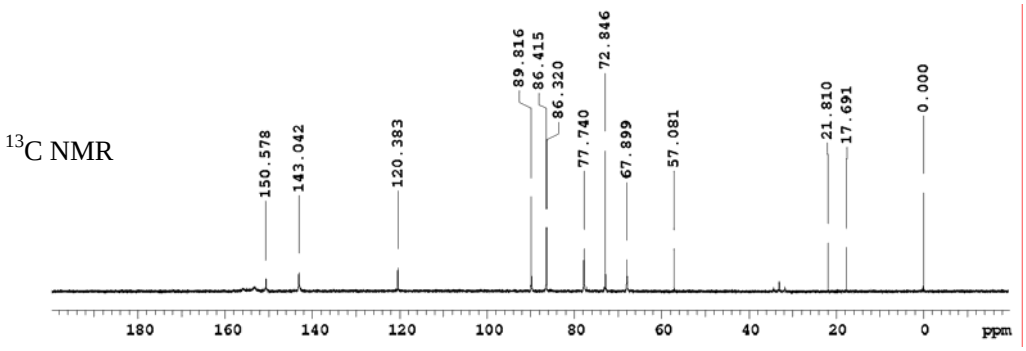


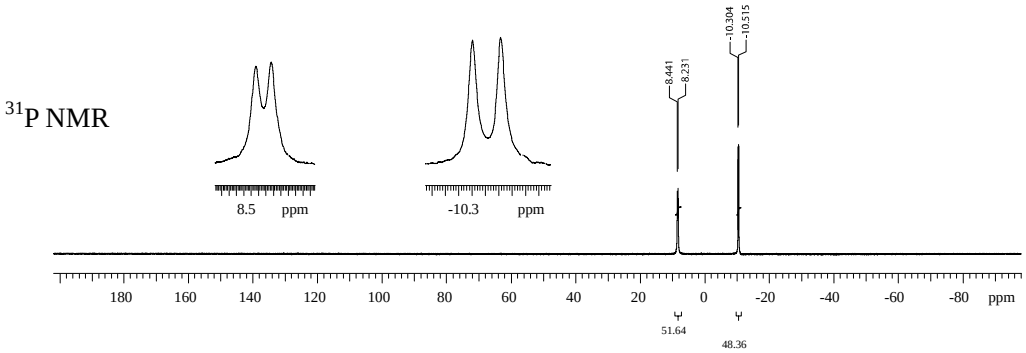


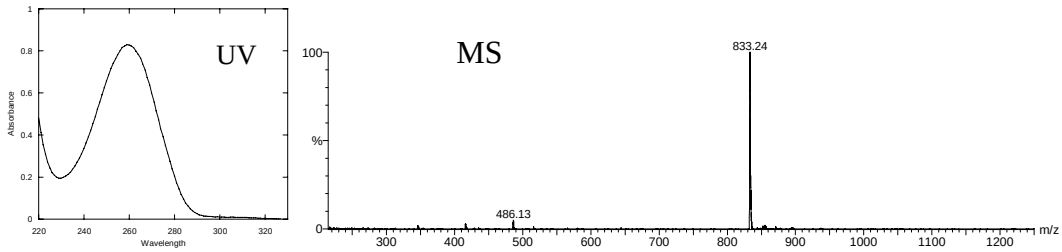
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [*S*$_P$]-Ap$_{BH3}$pppA (16b)



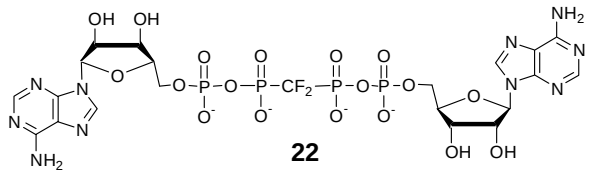


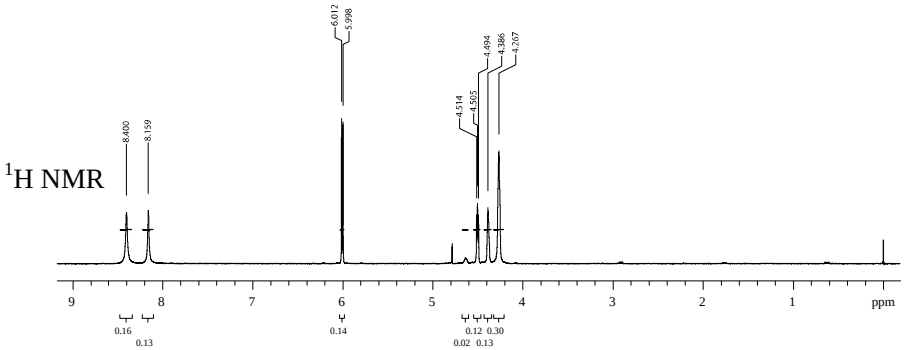


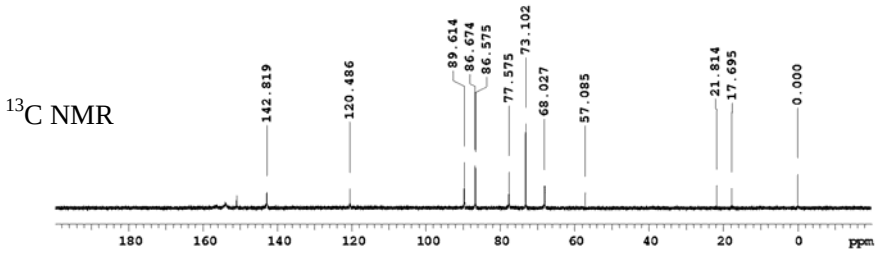


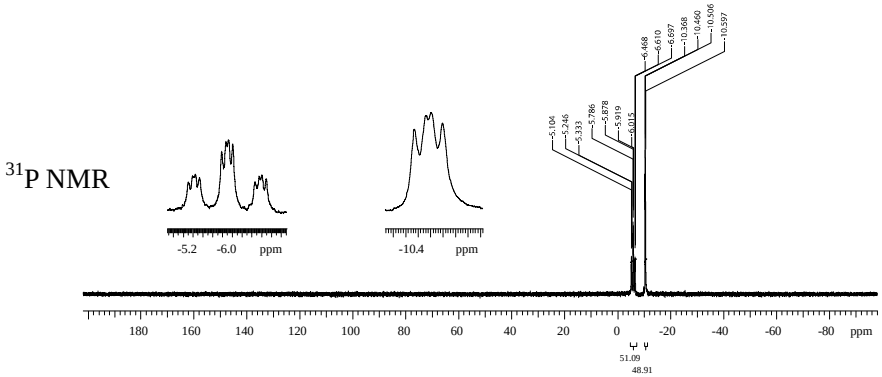


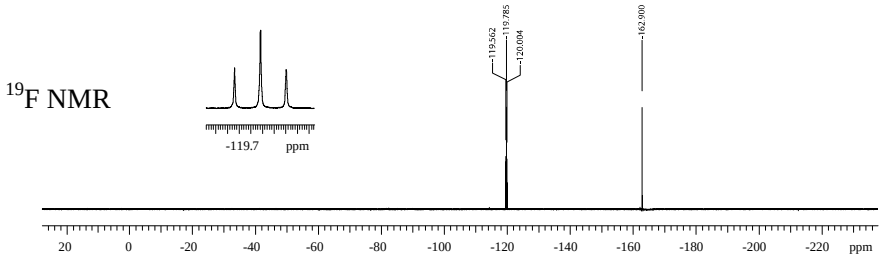
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for App$_{CH2}$ppA (21)

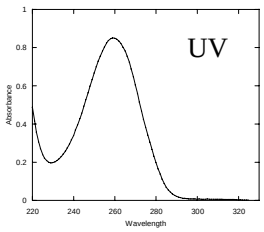


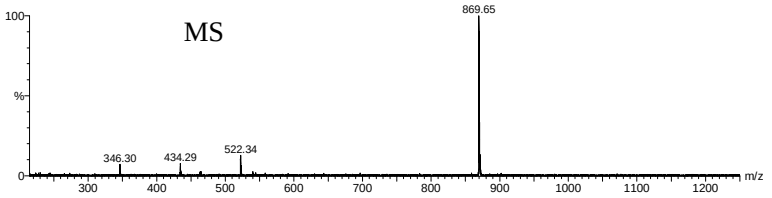




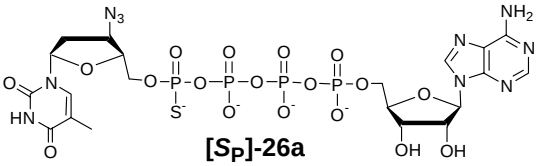




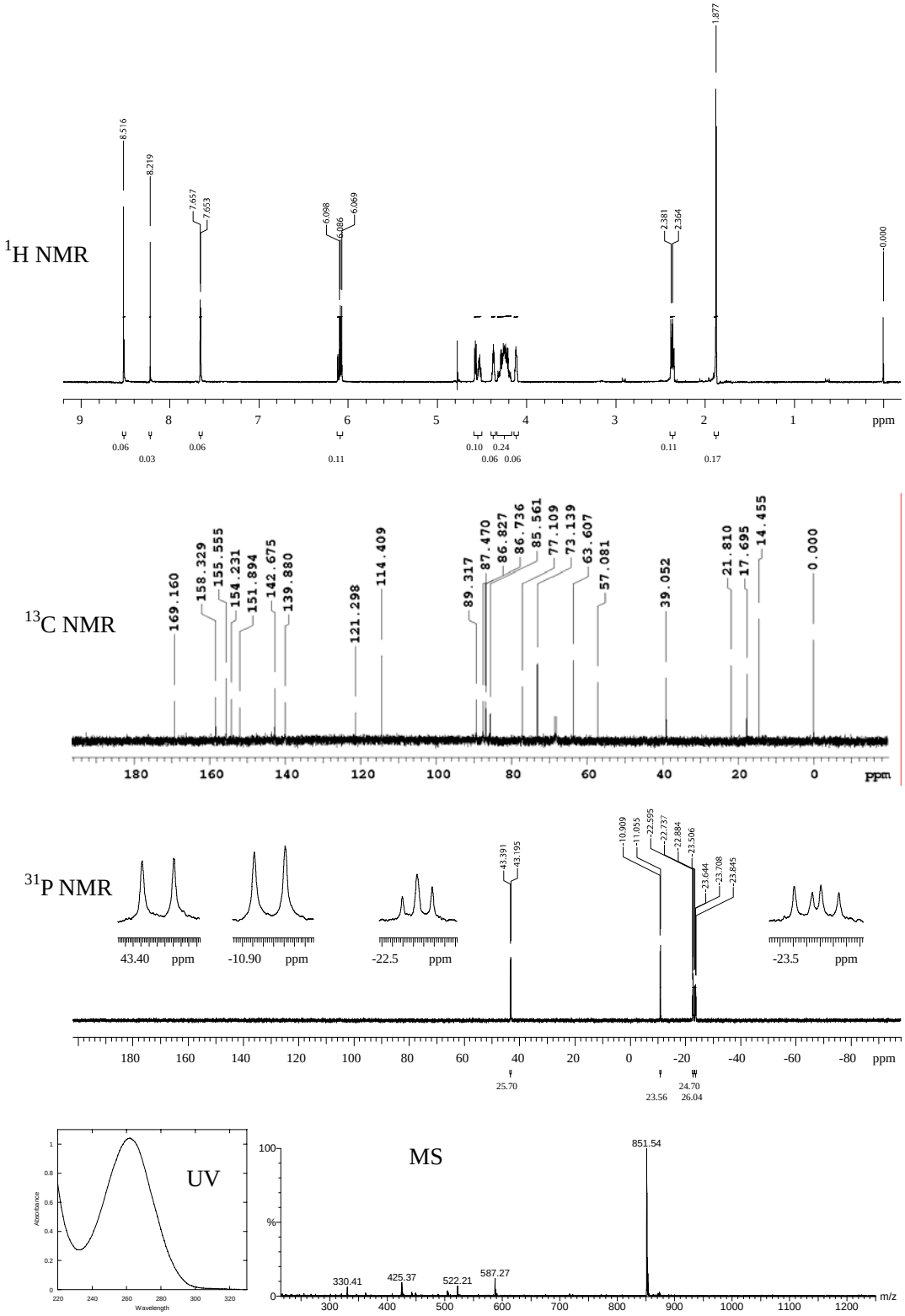




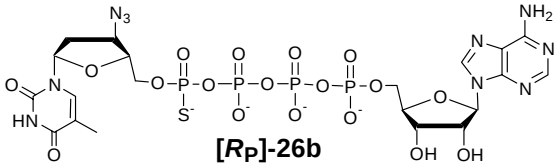
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ^{19}F NMR, UV and Mass Spectra for App$_{CF2}$ppA (22)

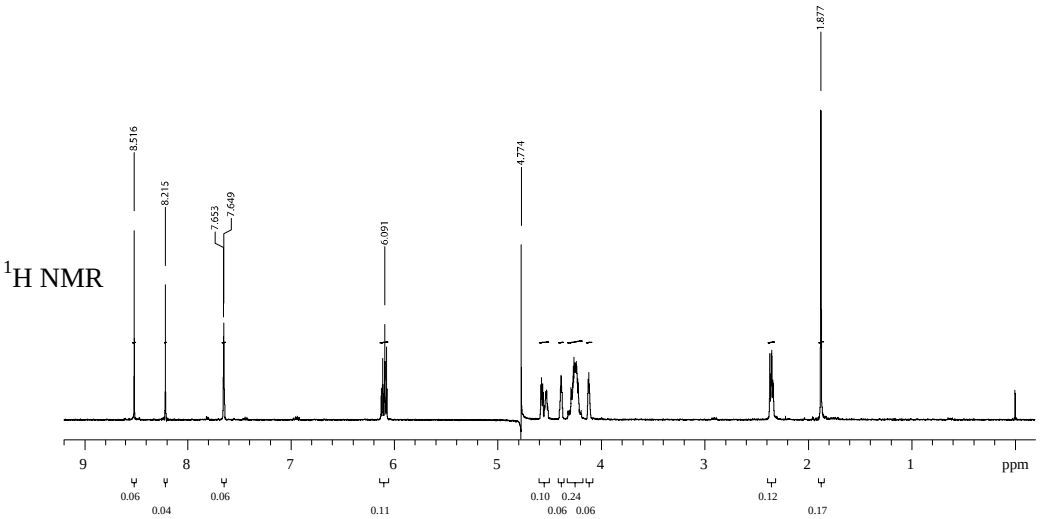


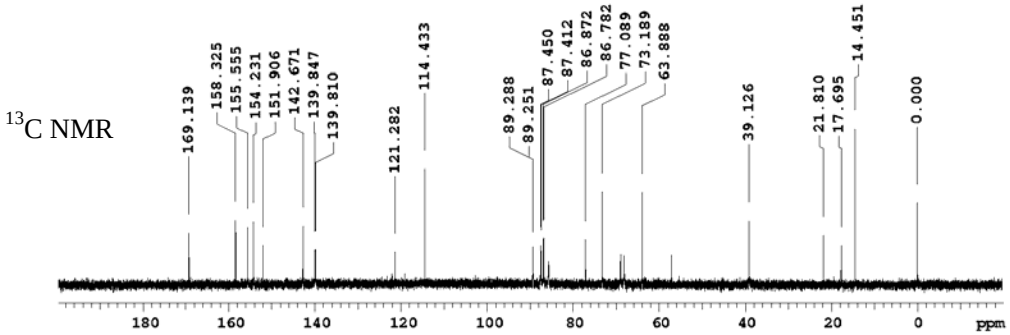
[S_P]-26a

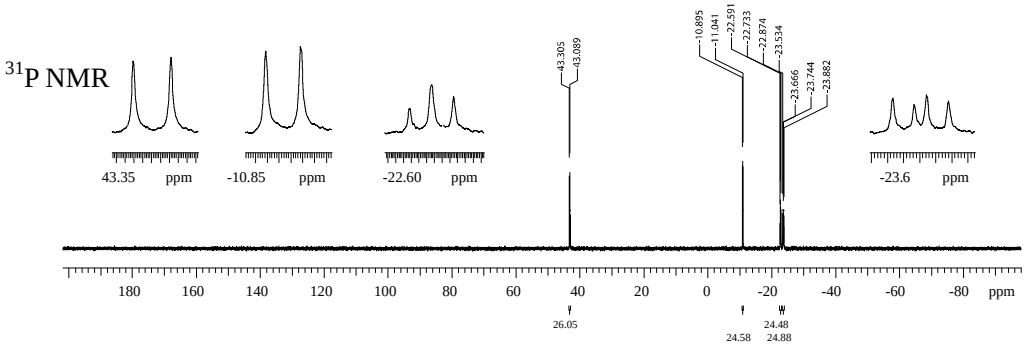


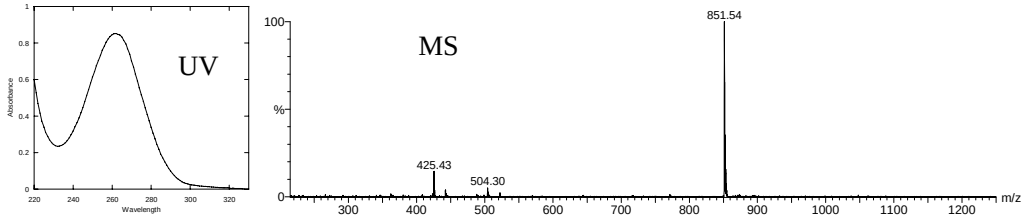
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [S_P]-AZTp$_S$pppA (26a)



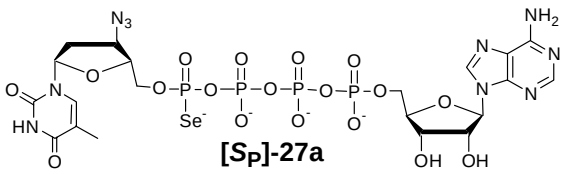


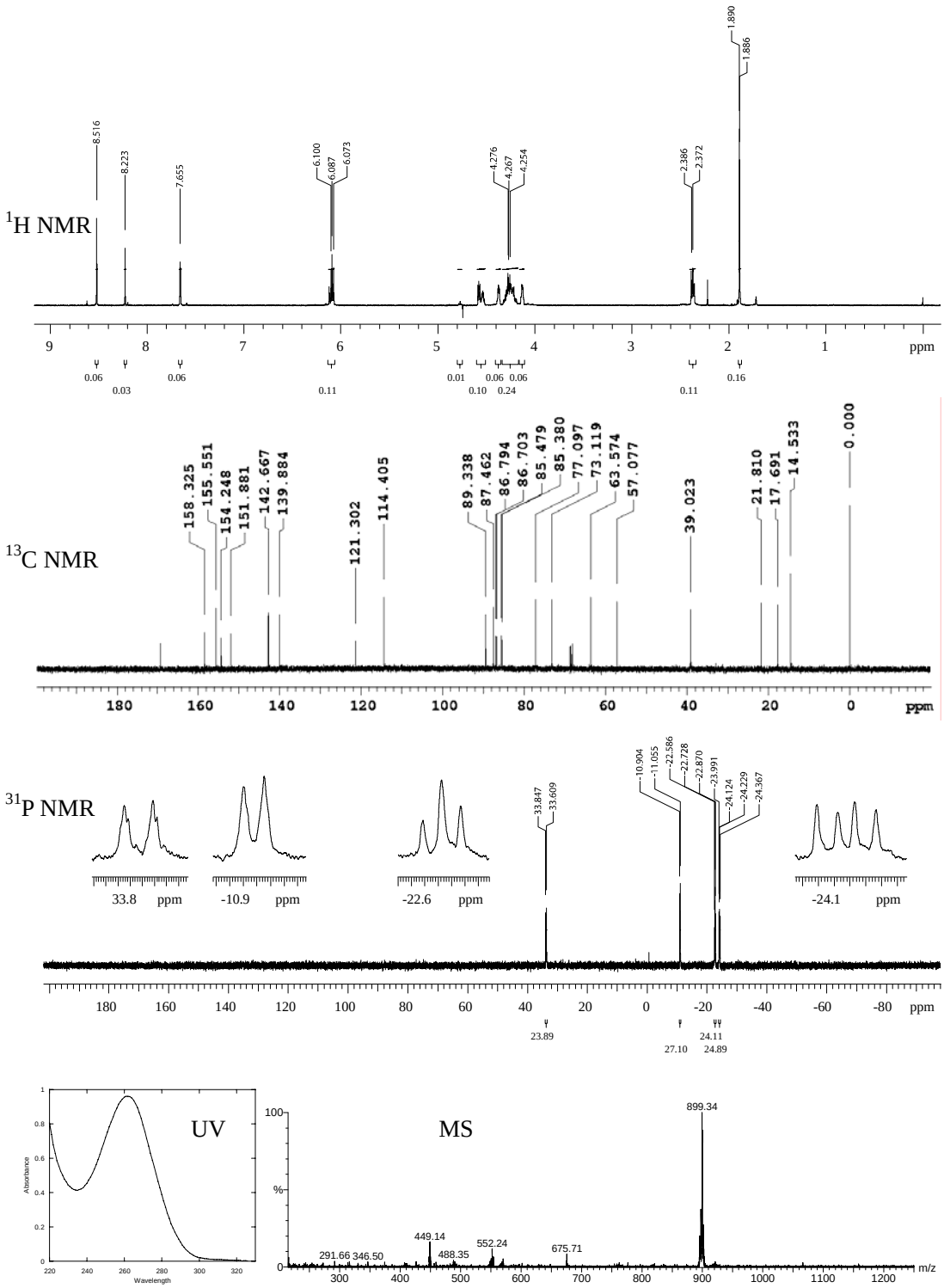




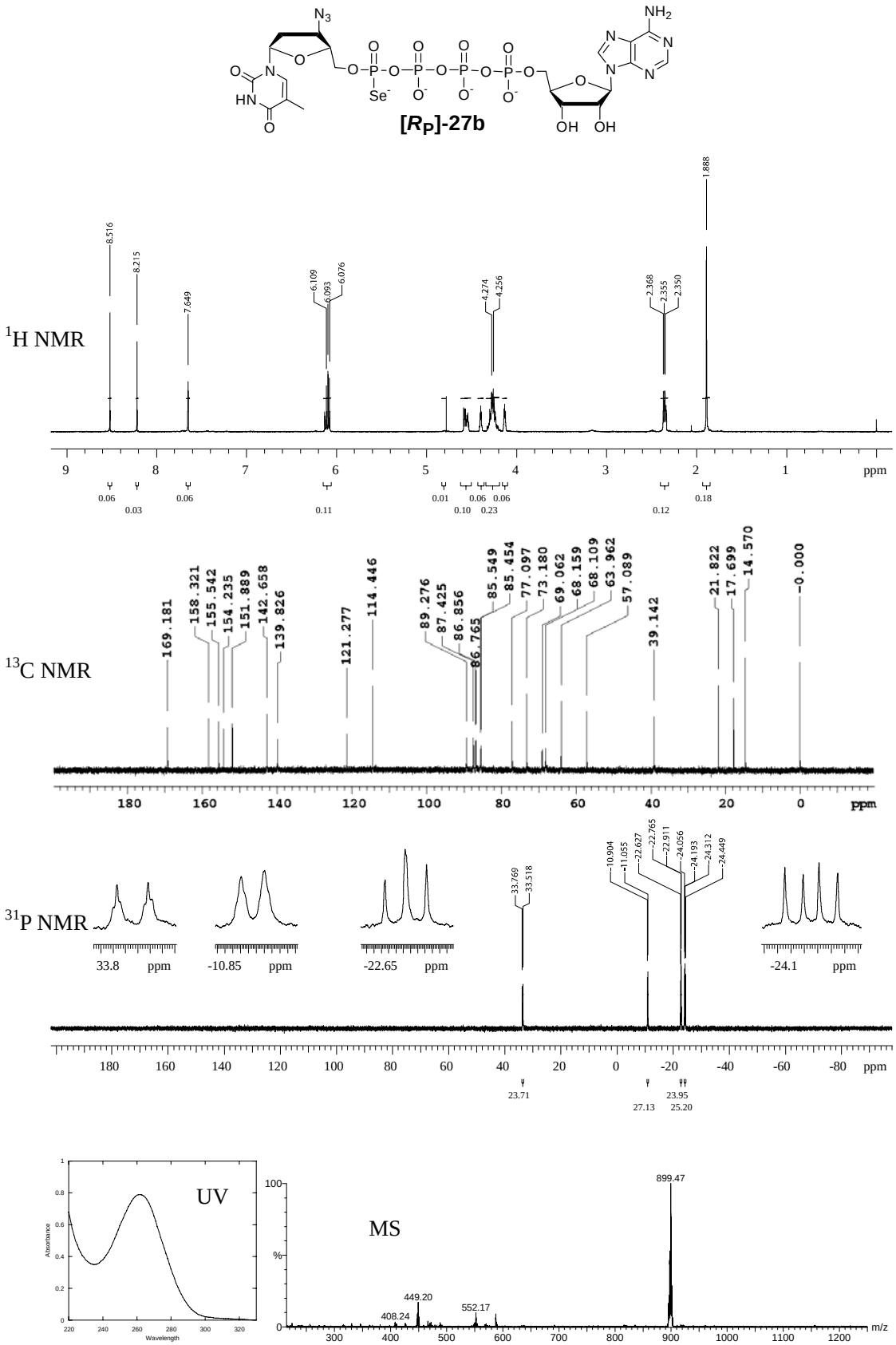


^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [R_P]-AZTp$_S$pppA (26b)

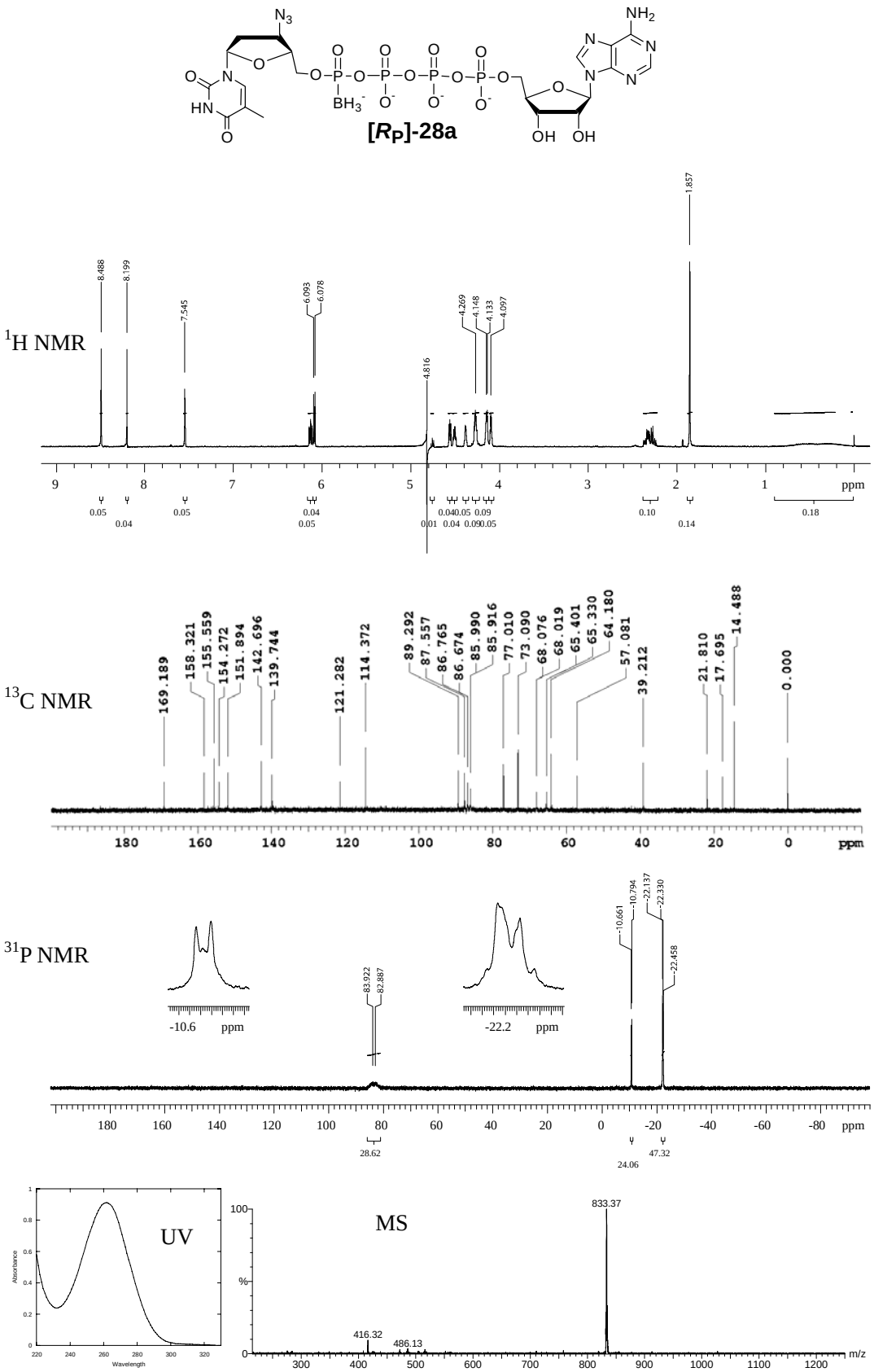




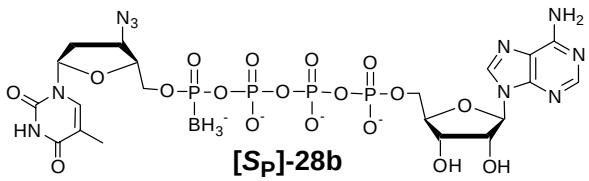
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [S_P]-AZTp$_{Se}$pppA (27a)

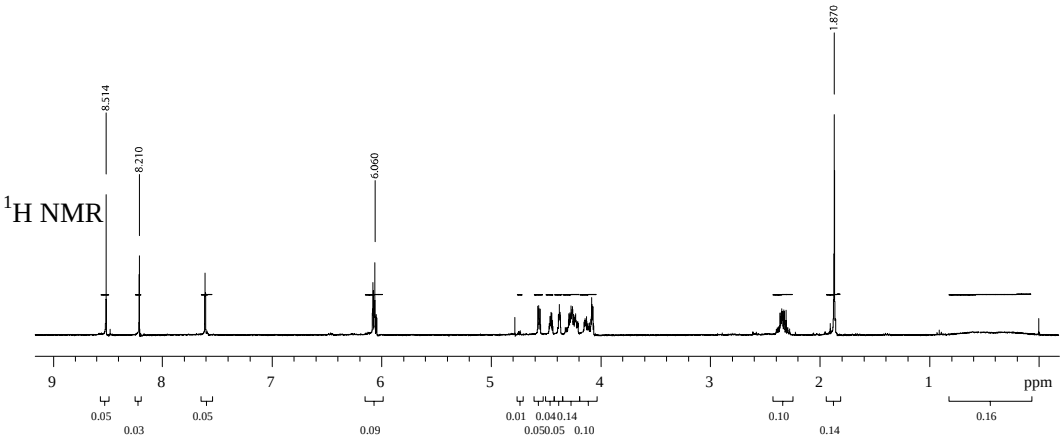


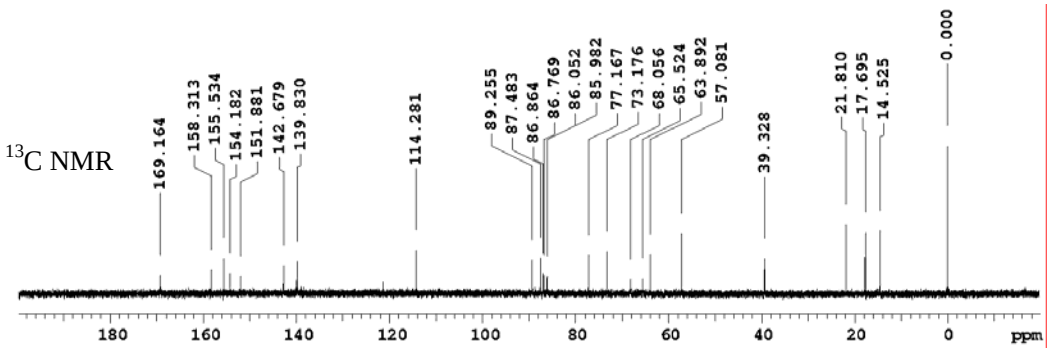
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [R_P]-AZTp$_{Se}$pppA (27b)

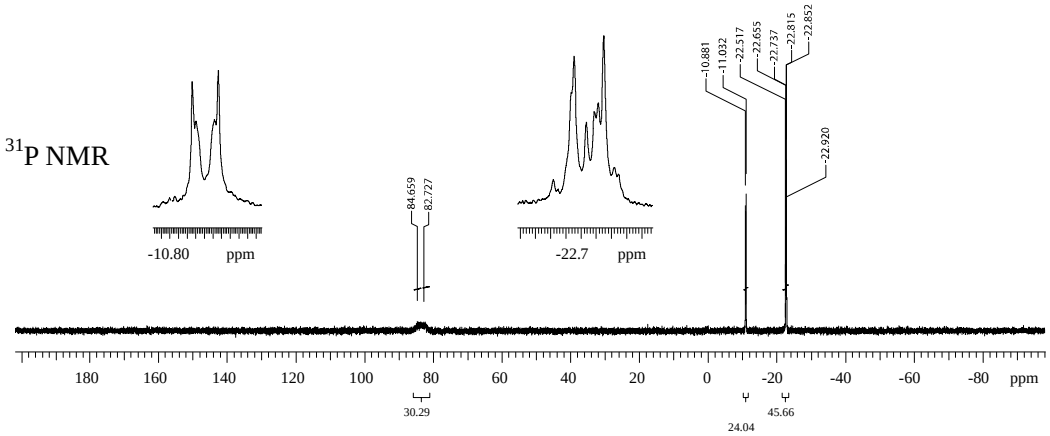


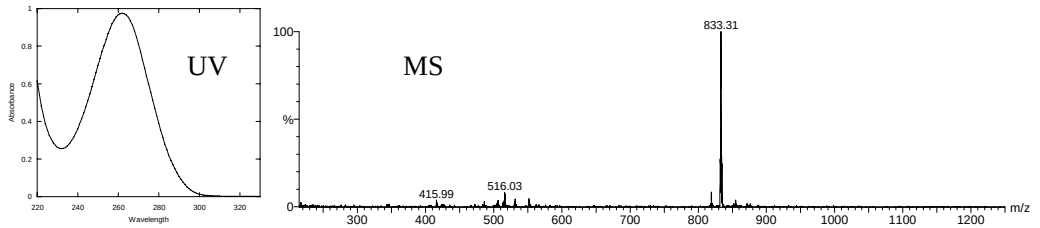
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [R_P]-AZTp$_{BH3}$pppA (28a)



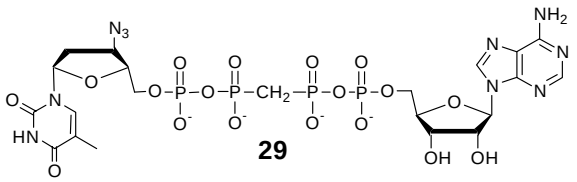


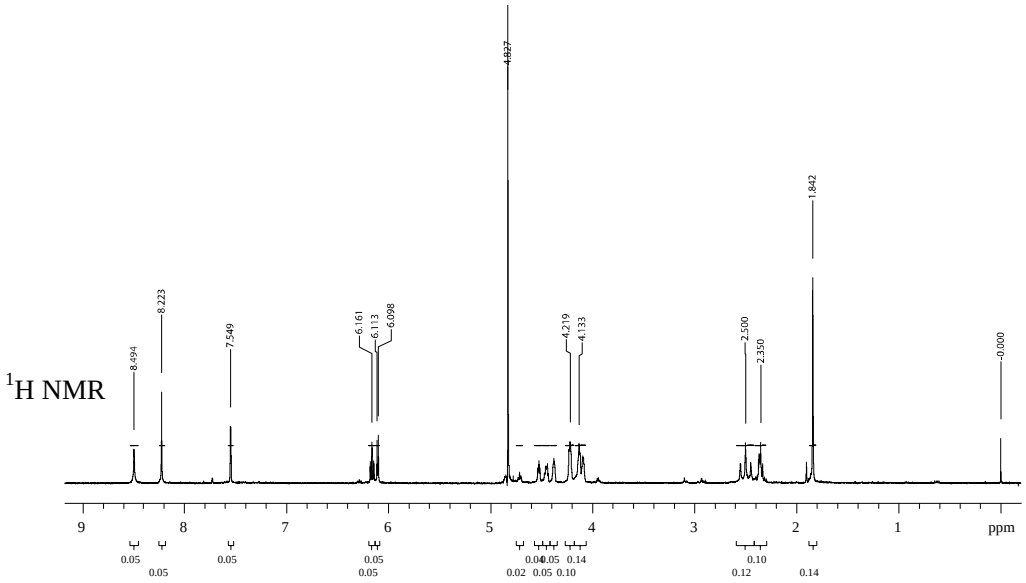


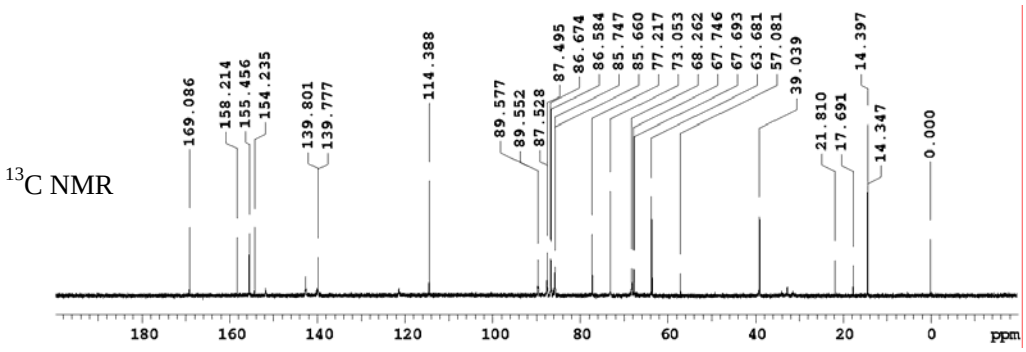


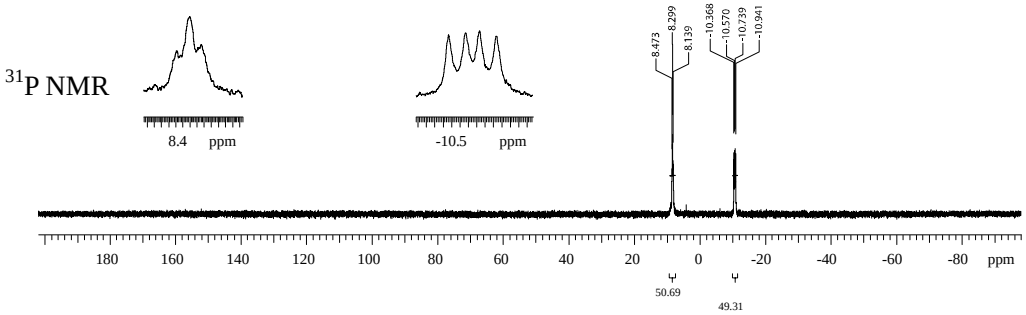


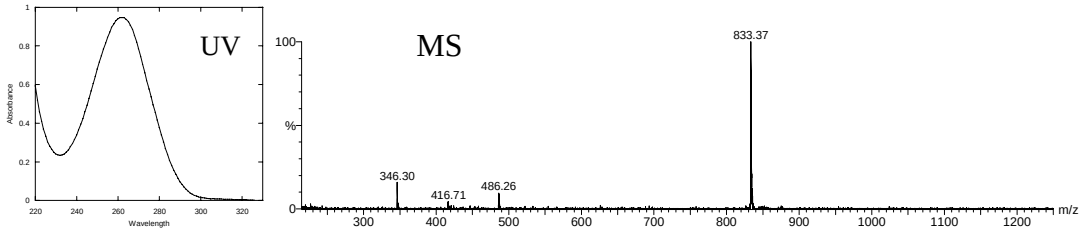
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [S_P]-AZTp$_{BH3}$pppA (28b)



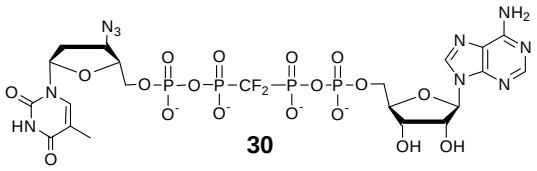


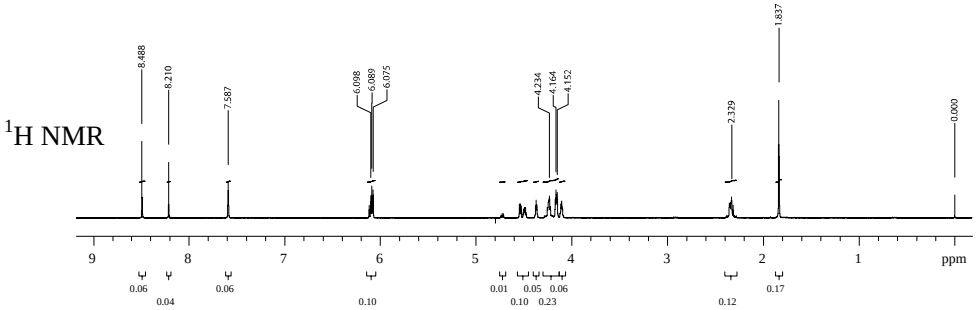


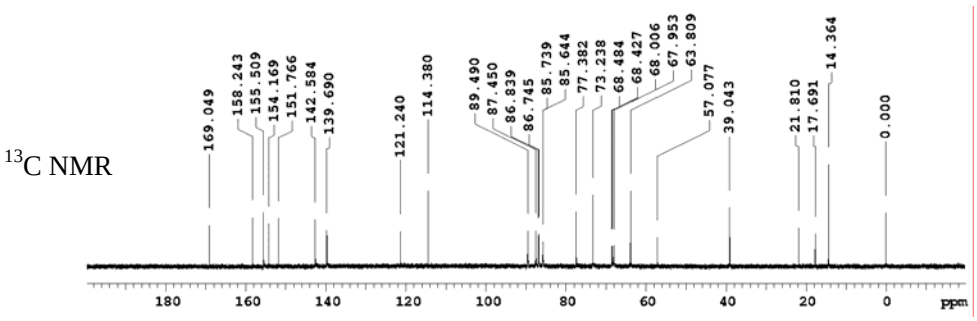


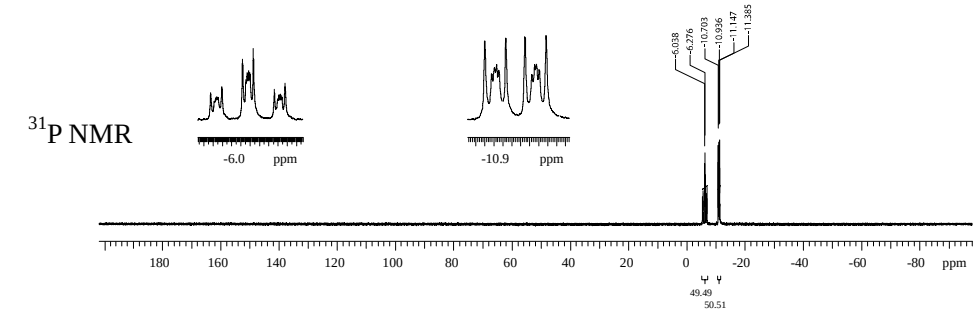


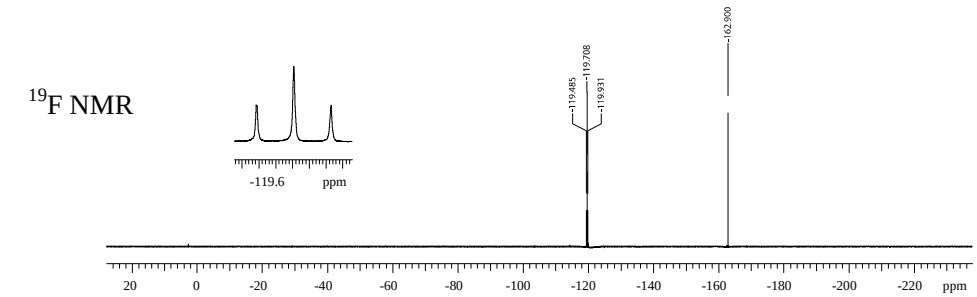
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for AZTpp$_{CH2}$ppA (29)

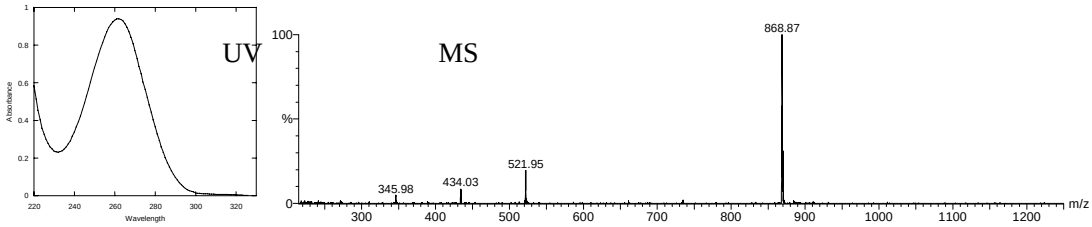












^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ^{19}F NMR, UV and Mass Spectra for AZTpp$_{CF2}$ppA (30)

Chapter 3: Synthesis of Hydrolysis-Resistant $AZTp_Sp_{CX2}pp_SA$ and $AZTp_Sp_{CX2}pp_SAZT$ (X = H, F) as Potential Inhibitors of the AZT Excision Reaction by HIV-1 RT

1. Introduction

Drug resistance is a serious and ubiquitous problem in the current therapies for AIDS. In the case of AZT drug resistance, more and more evidence suggests that AZT excision is the predominant reason.[1,2] The excision process is a phosphorolysis reaction. At the catalytic center, the terminal phosphate of a nearby ATP attacks the terminating AZT phosphate, thereby unblocking the terminated viral DNA. The efficient binding of ATP is important for the excision process.[3] The excision product, $AZTp_4A$, was shown to have a higher affinity for TAM-RT (Thymidine Analogue Mutations RT) than for wt RT (Wild Type RT).[4] It has been proposed that hydrolysis resistant analogs of dinucleoside tetraphosphates might be efficient inhibitors of drug excision by binding at both the N-site and the ATP-binding site of HIV-1 RT.[5,6] Dinucleoside tetraphosphates were shown to be very good substrate of HIV-1 RT.[7] Their analogs might therefore be incorporated very efficiently and inhibit both the viral DNA chain elongation and the AZT excision.[5]

Hydrolysis resistant analogs of Ap_4A have been synthesized with multiple modifications in the tetraphosphate chain. Blackburn made $Ap_Sp_Xpp_SA$ (X = CH_2, CF_2, CHF) by the coupling of diphosphonate with thiomonophosphate activated by diphenyl chlorophosphate.[8-10] The central methylene group resists symmetrical hydrolysis, and the dithiophosphates inhibit asymmetrical hydrolysis. Shirokova made $A_{CH2}pp_{CH2}pp_{CH2}A$ and $G_{CH2}pp_{CH2}pp_{CH2}G$ by coupling of triphosphonate with the imidazolide of a

monophosphonate.[6] $Ap_{CH2}pp_{CH2}pA$ was prepared by self condensation of adenosine diphosphonate with dicyclohexylcarbodiimide (DCC) as the coupling reagent.[11-13] All modified phosphates in the above analogs were resistant to degradation by various enzymes. Blackburn also made $Ap_{S}ppp_{S}A$ by coupling of pyrophosphate with thiomonophosphate activated by diphenyl chlorophosphate and tested its stability to snake venom.[8] The same compound was made by self condensation of thiodiphosphate with diphenyl chlorophosphate as the activator in a study of ^{17}O and ^{18}O labeled dinucleoside tetraphosphates.[14] All the above methods gave only modest yields and a better method is highly desirable.

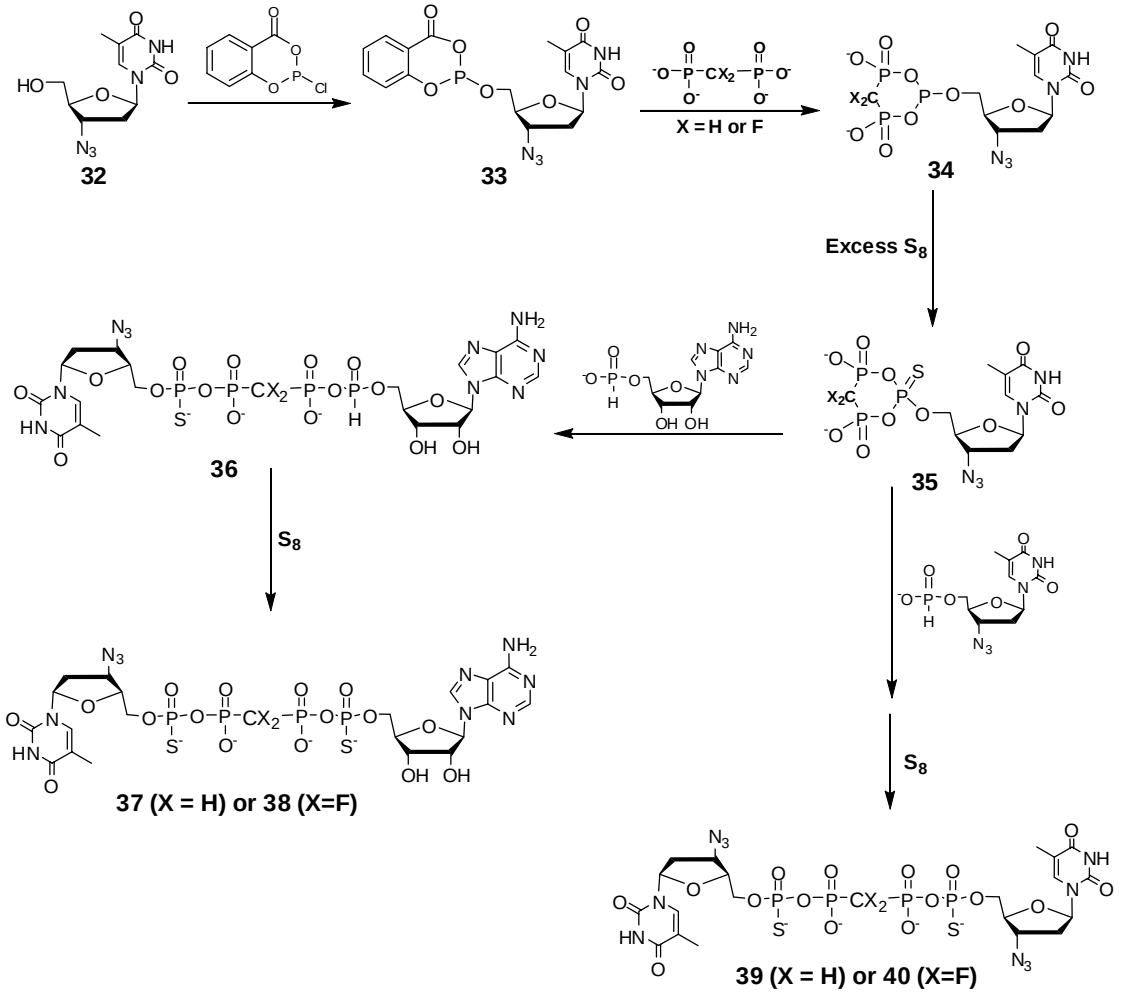
2. Results and Discussion

2.1 Synthesis of $AZTp_{S}p_{CX2}pp_{S}A$ and $AZTp_{S}p_{CX2}pp_{S}AZT$ (X = H, F)

The $AZTp_4A$ analogs, $AZTp_{S}p_{CX2}pp_{S}A$ (X = H or F), were designed to be particularly hydrolysis resistant since all four phosphates are modified. The resulting differences in steric and electronic properties of the analogs prevent tight binding in the active site of some enzymes. However, to retain some interaction with reverse transcriptase, we used thioates at the terminal phosphates at which a charge may be important. In addition, the adenosine might help bind to the mutant HIV-1 RT. The analogs with AZT at both ends, $AZTp_{S}p_{CX2}pp_{S}AZT$ (X = H or F), were also synthesized. These compounds might bind well to wild type HIV-1 RT.

The method described in Chapter 2 is efficient for preparation of dinucleoside tetraphosphates and their analogs with sulfur at only one terminal phosphate, but it cannot be used to prepare analogs with sulfur at both terminal phosphates, P_1,P_4-dithiotetraphosphates. To solve this problem, an H-phosphonate was used to couple with

the trimetaphosphate derivatives and the subsequent sulfurization produced the dithio-analogs. This reaction took advantage of the fact that elemental sulfur can efficiently sulfurize H-phosphonate diesters but not monoesters.



Scheme 3-1. Synthesis of AZTp$_S$p$_{CX2}$pp$_S$A and AZTp$_S$p$_{CX2}$pp$_S$AZT

Reactions for the synthesis of AZTp$_S$p$_{CX2}$pp$_S$A, **37** (X = H) and **38** (X = F), and AZTp$_S$p$_{CX2}$pp$_S$AZT, **39** (X = H) and **40** (X = F), are shown in **Scheme 3-1**. The trimetaphosphate analog, **35**, was made by the procedure described in Chapter 2.[15,16] AZT was reacted with 2-chloro-4*H*-l,3,2-benzo-dioxaphosphorin-4-one and then with methylene or difluoromethylene diphosphonate. The subsequent reaction with excess

elemental sulfur produced intermediate **35**. To make AZTp$_S$p$_{CX2}$pp$_S$A, adenosine-H-phosphonate was added and the nucleophilic reaction formed intermediate **36**. The excess elemental sulfur from the first sulfurization efficiently sulfurized intermediate **36**, which is an H-phosphonate diester, while adenosine-H-phosphonate is a monoester and cannot be sulfurized under these conditions. With two chiral centers, four diasetereomers of these asymmetrical products (**37** and **38**) were obtained.

To make AZTp$_S$p$_{CX2}$pp$_S$AZT, AZT-H-phosphonate was used instead of adenosine-H-phosphonate. Since the products have AZT at both ends, AZTp$_S$p$_{CX2}$pp$_S$AZT has only three diastereomers, with [*RS*] and [*SR*] being identical meso compounds. All the diasetereomers, four each for asymmetrical AZTp$_S$p$_{CX2}$pp$_S$A and three each for symmetrical AZTp$_S$p$_{CX2}$pp$_S$AZT, were separated and purified by reverse-phase HPLC, with the total isolated yields ranging from 57% to 82%. The yields for all diastereomers are listed in **Table 3-1**. All the above reactions were conducted in the presence of $ZnCl_2$ as a catalyst.

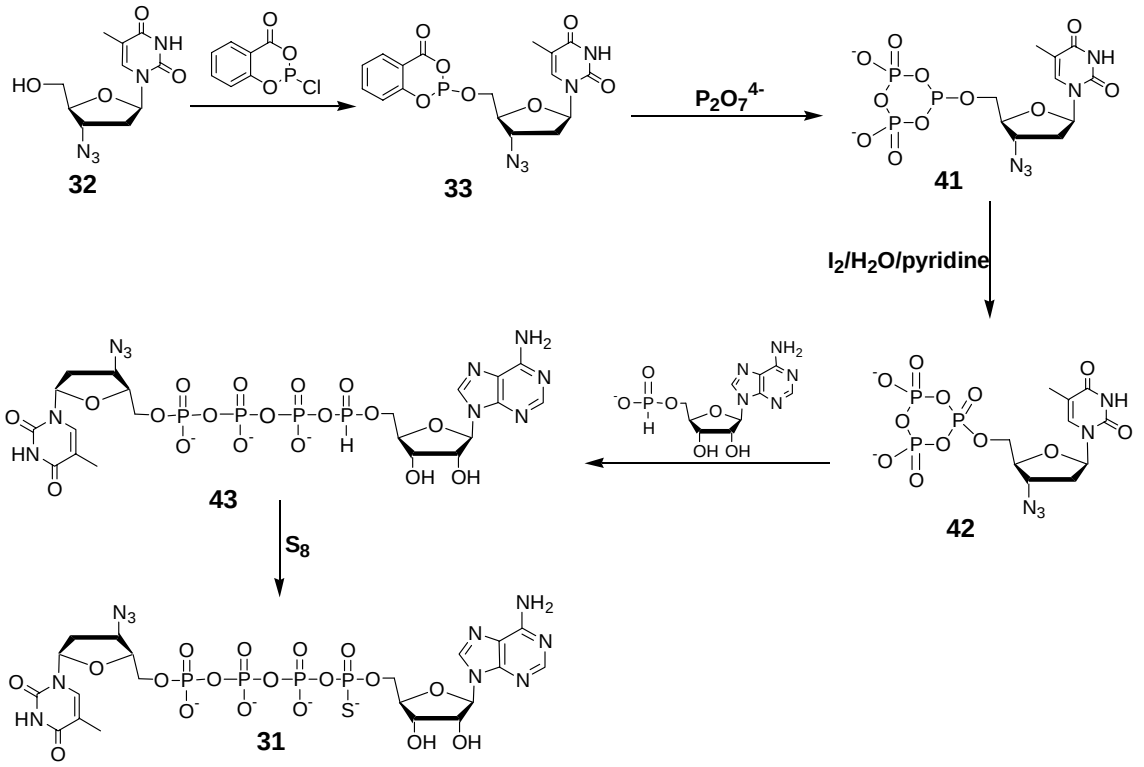
Table 3-1. Yields of AZTp$_S$p$_{CX2}$pp$_S$A and AZTp$_S$p$_{CX2}$pp$_S$AZT

AZTp$_S$p$_{CH2}$pp$_S$A	**Yields**	**AZTp$_S$p$_{CH2}$pp$_S$AZT**	**Yields**
AZT$^{5'}$ppCH$_2$pp$^{5'}$A (**37**)	[S_{P1},S_{P4}]: 9% [S_{P1},R_{P4}]: 11% [R_{P1},S_{P4}]: 17% [R_{P1},R_{P4}]: 20%	AZT$^{5'}$ppCH$_2$pp$^{5'}$AZT (**39**)	[S_{P1},S_{P4}]: 17% [S_{P1},R_{P4}]: 27% [R_{P1},R_{P4}]: 31%
AZT$^{5'}$ppCF$_2$pp$^{5'}$A (**38**)	[S_{P1},S_{P4}]: 19% [S_{P1},R_{P4}]: 14% [R_{P1},S_{P4}]: 20% [R_{P1},R_{P4}]: 29%	AZT$^{5'}$ppCF$_2$pp$^{5'}$AZT (**40**)	[S_{P1},S_{P4}]: 15% [S_{P1},R_{P4}]: 25% [R_{P1},R_{P4}]: 26%

The coupling of an H-phosphonate with a trimetaphosphate is a very efficient way to form the linear tetraphosphate chain. AZTp$_4$AZT (**11**) was made in 75% yield through the

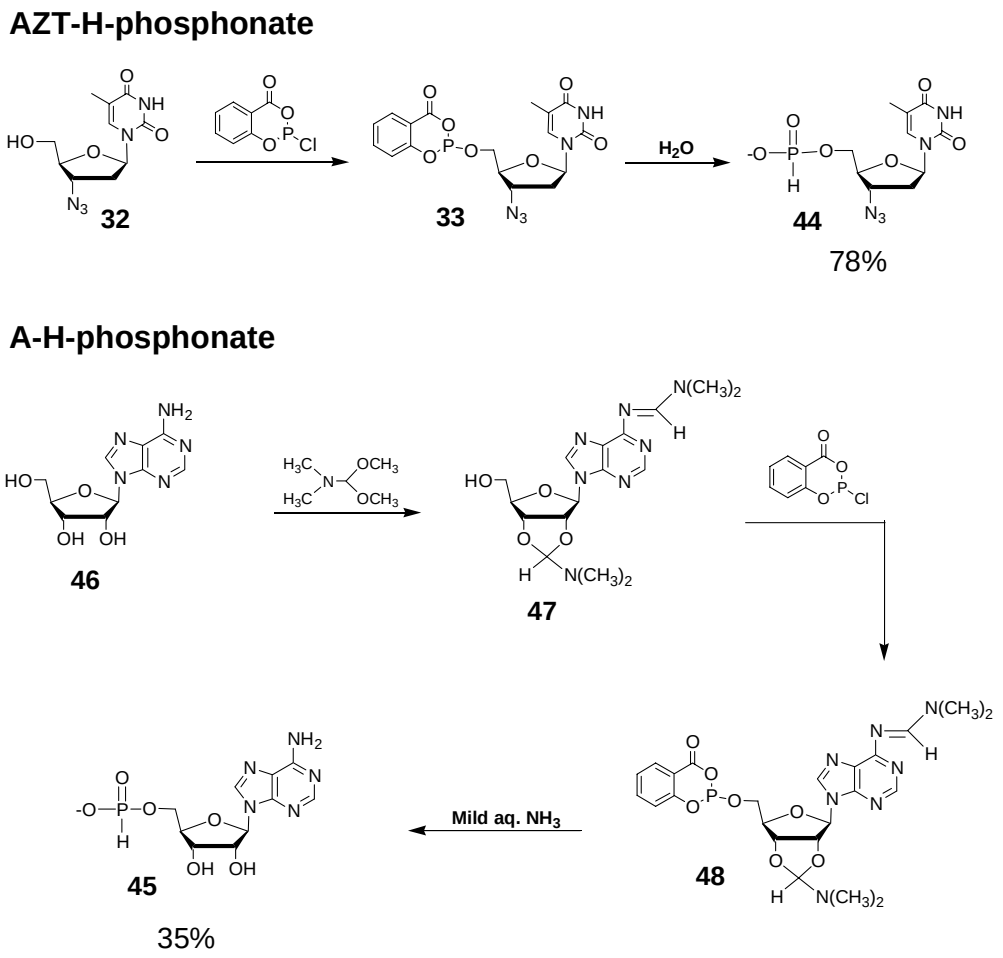
coupling of AZT-H-phosphonate with AZT trimetaphosphate followed by oxidation using I_2/H_2O/pyridine. The same compound was also made by the reaction of AZT monophosophate with trimetaphosphate with a comparable yield (77%) as discussed in Chapter 2. AZTp$_S$p$_{CH2}$pp$_S$AZT (**39**) was also made by the direct reaction of an AZT monophosphothioate with the intermediate **35** with very poor yield (25%). Therefore for the preparation of the modified compounds AZTp$_S$p$_{CX2}$pp$_S$A (**37** + **38**) and AZTp$_S$p$_{CX2}$pp$_S$AZT (**39** + **40**), the H-phosphonate method described in this chapter has obvious advantages over the phosphate method described in chapter 2.

The monomodified AZTpppp$_S$A, **31**, with the thioate adjacent to the adenosine was also prepared by the H-phosphonate method. The reactions are shown in **Scheme 3-2**. Starting with AZT, intermediate **41** was prepared by the same procedure described in Chapter 2. The subsequent careful oxidation formed the trimetaphosphate derivative of AZT, **42**. Then adenosine-H-phosphonate and excess elemental sulfur were added together. Once the intermediate **43** was produced, it was immediately sulfurized to give AZTpppp$_S$A (**31**). This procedure can be employed to make dinucleoside tetraphosphate analogs with different modifications at two terminal phosphates. Starting with protected adenosine, AZTpppp$_S$A (**31**) could also be synthesized by the phosphate method described for AZTp$_S$pppA in Chapter 2.



Scheme 3-2. Synthesis of AZTpppp$_S$A (31)

Syntheses of the H-phosphonates are shown in **Scheme 3-3**. The AZT-H-phosphonate, **44**, was prepared very conveniently by the reaction of AZT with 2-chloro-4*H*-l,3,2-benzo-dioxaphosphorin-4-one.[17] The preparation of adenosine H-phosphonate, **45**, needed prior protection. The reagent *N*,*N*-dimethylformamide dimethyl acetal (DMF-DMA) was employed to transiently protect the 2′,3′-hydroxyl and 6-amino groups.[18,19] After phosphitylation of the 5′-hydroxyl group, the protecting groups were removed by treatment with mild aqueous ammonia to produce adenosine H-phosphonate, **45**. In order to increase its solubility in DMF, the adenosine H-phosphonate was converted to its tributylammonium form through cation exchange before its use, while the AZT H-phosphonate could be directly used without cation conversion.



Scheme 3-3. Syntheses of H-phosphonates

2.2 Configurations and Stabilities of AZTp$_S$p$_{CX2}$pp$_S$A and AZTp$_S$p$_{CX2}$pp$_S$AZT

The configurations of the diastereomers of AZTp$_S$p$_{CH2}$pp$_S$A, **37**, were assigned by comparison of their enzyme degradation rates using snake venom phosphodiesterase, a method developed by Eckstein to determine the configurations of mononucleoside polyphosphates.[20] Blackburn used this method to assign configurations of diadenosine tetraphosphate analogs.[8] Each diastereomer of AZTp$_S$p$_{CH2}$pp$_S$A was digested by snake venom phosphodiesterase, and the results were followed by LC-MS. The degradation products were identified by MS, UV and retention time. Hydrolysis of the

phosphorothioate adjacent to AZT (P4 in our nomenclature) produced AZT monophosphothioate, and hydrolysis of the thioate adjacent to adenosine (P1) produced adenosine + adenosine monophosphate. The percent of these degradation products relative to total nucleoside derivatives were plotted against time in **Figure 3-1** and **Figure 3-2**. The isomer eluting first, **I**, was poorly hydrolyzed at either end, which indicated an [*S*]-configuration at both phosphorothioates. The isomer eluting second, **II**, was readily hydrolyzed at the thioate near AZT, forming AZT monophosphothioate, which indicated an [*R*]-configuration of the thioate near AZT. The third isomer, **III**, predominately formed adenosine + adenosine monophosphate, which indicated an [*R*]-configuration of the thioate near adenosine. The fourth isomer, **IV**, was degraded at both thioates, which indicated [*R*]-configurations at both positions. These results are summarized in **Table 3-2**.

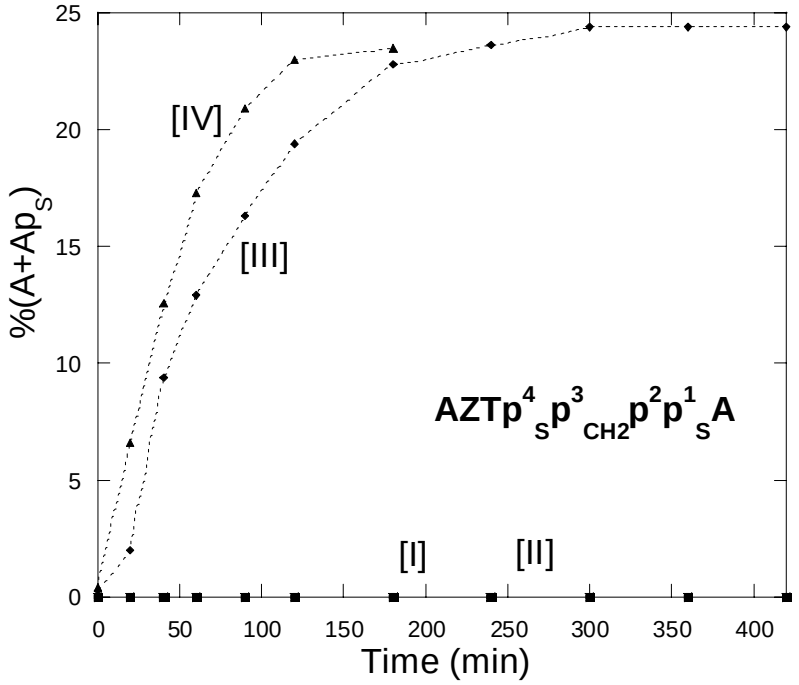


Figure 3-1. Appearance of (A + Ap$_S$) from Enzymatic Degradation of AZTp^4_Sp$^3_{CH2}$p^{2}p^1_SA (37)

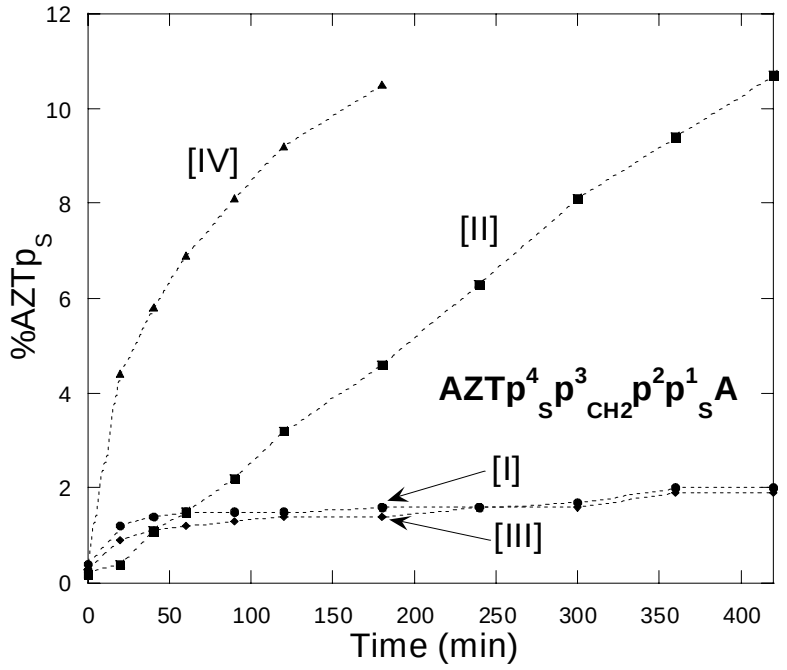


Figure 3-2. Appearance of AZTp$_S$ from Enzymatic Degradation of AZTp^4_Sp$^3_{CH2}$p^{2}p^1_SA (37)

Table 3-2. Configuration Identification of AZTp$_S$p$_{CH2}$pp$_S$A

	A-(P1)	AZT-(P4)	Configuration
I	×	×	**[S_{P1},S_{P4}]**
II	×	√	**[S_{P1},R_{P4}]**
III	√	×	**[R_{P1},S_{P4}]**
IV	√	√	**[R_{P1},R_{P4}]**

The percent of remaining AZTp$_S$p$_{CH2}$pp$_S$A during the enzyme degradation was also monitored by LC-MS and plotted against time for all four isomers (see **Figure 3-3**). The elution order of the diastereomers on reverse-phase HPLC was found to correlate with their digestion rates by snake venom, with the isomer eluting first being digested most slowly. The first isomer to elute was [S_{P1},S_{P4}] and was very resistant to degradation at

both ends, since it was digested the most slowly. The fourth isomer to elute was [R_{P1},R_{P4}] and was digested at both ends the fastest. The two intermediate diastereomers were [S_{P1},R_{P4}] and [R_{P1},S_{P4}], and each was digested at only one end, so their degradation rates were between the others. Thus, the digestion results for all diastereomers were consistent with the configuration assignments. The configurations of the four diastereomers of AZTp$_S$p$_{CF2}$pp$_S$A (**38**) were assigned similarly (data shown in **Figures 4-6**). AZTp$_S$p$_{CH2}$pp$_S$AZT (**39**) and AZTp$_S$p$_{CF2}$pp$_S$AZT (**40**) each have three diastereomers. The isomers eluting first were very hydrolysis-resistant and were assigned as [S_{P1},S_{P4}]. The isomers eluting third were digested fastest and were assigned as [R_{P1},R_{P4}]. The isomers eluting second were assigned as [S_{P1},R_{P4}] (data shown in **Figure 3-7** and **Figure 3-8**). This correlation of digestion rates with configuration assignments for all four analogs was consistent with Blackburn's published results described above.[8]

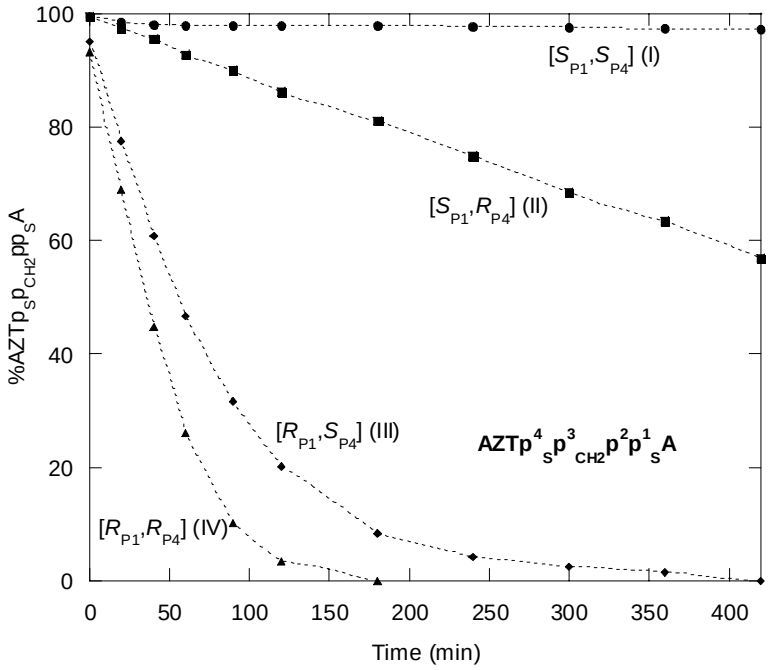


Figure 3-3. Disappearance of AZTp^4_Sp$^3_{CH2}$p^{2}p^1_SA (37) with Enzymatic Degradation

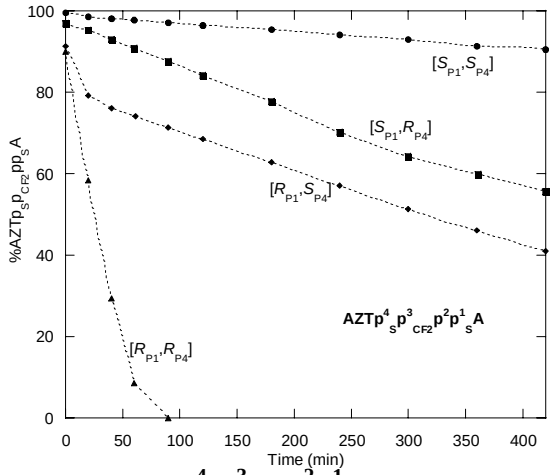


Figure 3-4. Disappearance of AZTp^4_Sp$^3_{CF2}$p^{2}p^1_SA (38) with Enzymatic Degradation

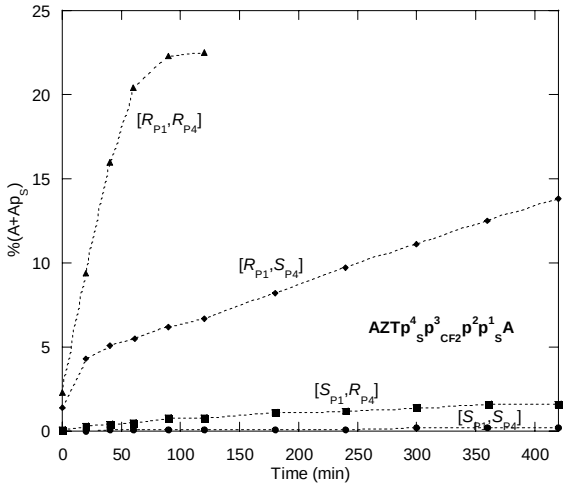


Figure 3-5. Appearance of (A + Ap$_S$) from Enzymatic Degradation of AZTp^4_Sp$^3_{CF2}$p^{2}p^1_SA (38)

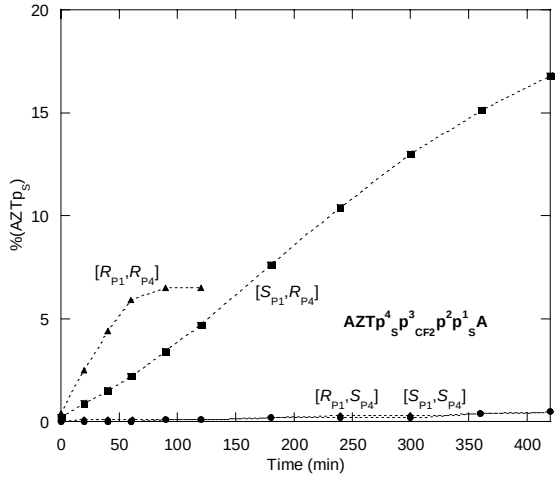


Figure 3-6. Appearance of AZTp$_S$ from Enzymatic Degradation of AZTp^4_Sp$^3_{CF2}$p^{2}p^1_SA (38)

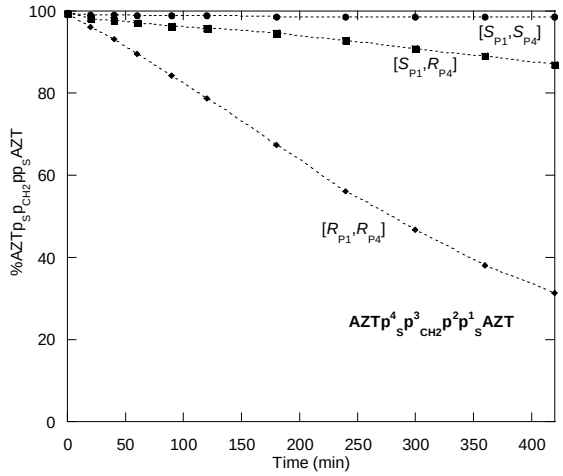


Figure 3-7. Disappearance of AZTp^4_Sp$^3_{CH2}$p^{2}p^1_SAZT (39) with Enzymatic Degradation

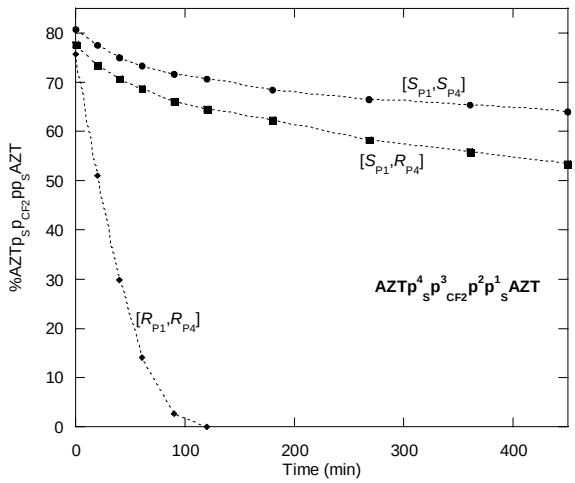


Figure 3-8. Disappearance of AZTp^4_Sp$^3_{CF2}$p^{2}p^1_SAZT (40) with Enzymatic Degradation

The configuration assignments described above were supported by the NMR data. For AZTp$_S$p$_{CH2}$pp$_S$A and AZTp$_S$p$_{CH2}$pp$_S$AZT, the ^{1}H NMR resonances of the central -CH$_2$- are simple triplets when the two terminal phosphothioates have the same configuration, ([S_{P1},S_{P4}] or [R_{P1},R_{P4}]). In contrast they display complex second order peaks when the two terminal phosphothioates have different configurations ([S_{P1},R_{P4}] or [R_{P1},S_{P4}]). These ^{1}H NMR resonances are shown in **Figure 3-9**. For AZTp$_S$p$_{CH2}$pp$_S$AZT and AZTp$_S$p$_{CF2}$pp$_S$AZT, the ^{13}C NMR resonances of the two AZT units are exactly

overlapped when the two terminal phosphothioates have the same configuration, ([S_{P1},S_{P4}] or [R_{P1},R_{P4}]). In contrast, the 5′-C and other ^{13}C NMR resonances of the two AZT units are more complicated when the two terminal phosphothioates have different configurations, ([S_{P1},R_{P4}]). These spectra are shown in the Appendix. The range of chemical shifts in the ^{13}P NMR is very small, and there is no obvious correlation of patterns with configuration assignments.

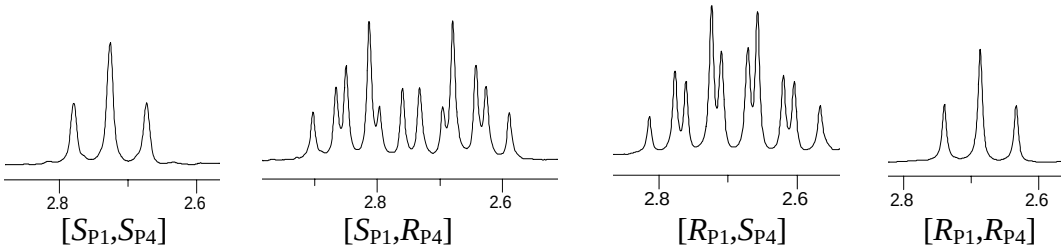


Figure 3-9. ^{1}H NMR Resonances of the Central -CH_2- in the Four AZTp$_S$p$_{CH2}$pp$_S$A Diastereomers

The stabilities toward enzymatic degradation of AZTp$_S$p$_{CX2}$pp$_S$A (**37** and **38**) and AZTp$_S$p$_{CX2}$pp$_S$AZT (**39** and **40**) were compared with the control compounds AZTp$_4$A, **10**, AZTp$_4$AZT, **11**, AZTpp$_{CH2}$ppA, **29**, and AZTpp$_{CF2}$ppA, **30**. The enzyme degradation data of these controls are shown in **Figure 3-10**. All four control compounds were hydrolyzed more than 95% by snake venom phosphodiesterase by 2 min, while less than 3% of [S_{P1},S_{P4}]-AZTp$_S$p$_{CH2}$pp$_S$A was hydrolyzed even after 7 hr under the same conditions. Thus the hydrolysis-resistant analogs described in this chapter, especially the [S_{P1},S_{P4}]-diastereomers, are very stable and might persist in biological systems long enough to be very efficient inhibitors.

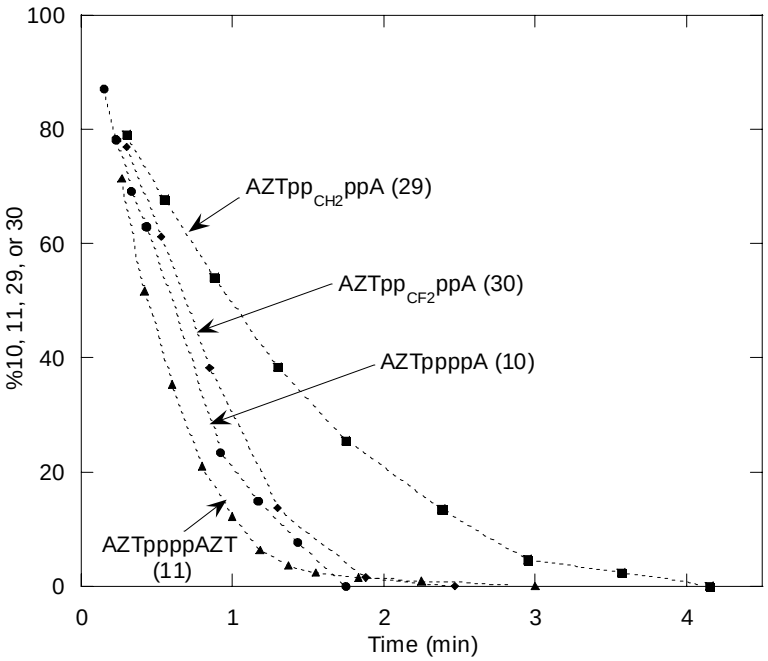


Figure 3-10. Disappearance of 10, 11, 29 and 30

The configurations of the diastereomers of AZTpppp$_S$A, **31**, were assigned based on the elution order from reverse-phase HPLC. The diastereomer eluting first was assigned as the [S_P]-configuration and the second was assigned as the [R_P]-configuration. The assignment was confirmed by enzymatic degradation data. The diastereomers were digested by snake venom using conditions at which they were converted to Ap$_S$pp within 15 min, and the disappearance of Ap$_S$pp was then monitored. The percent of remaining Ap$_S$pp was plotted against time as shown in **Figure 3-11**. The [S_P]-diastereomer was degraded more slowly than the [R_P]-diastereomer, which is consistent with results published by Eckstein for Ap$_S$pp.[20]

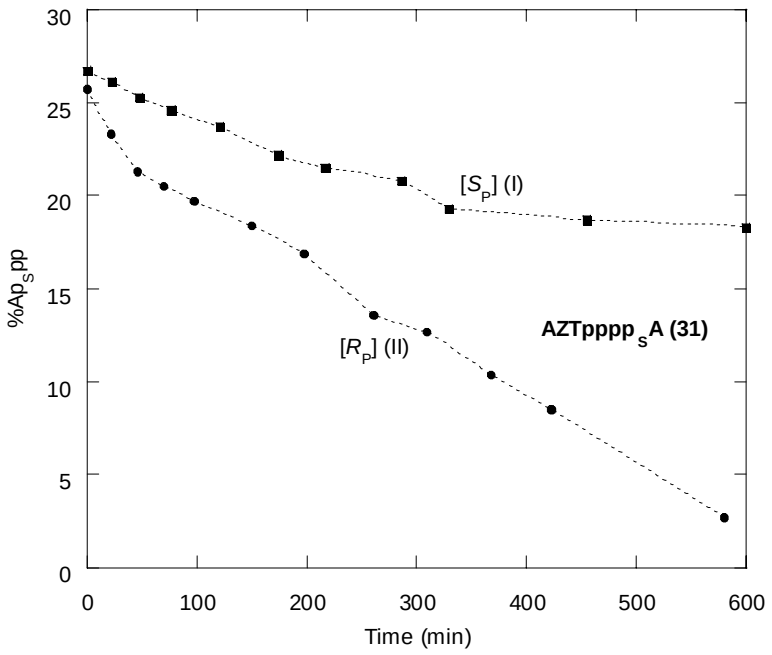


Figure 3-11. Enzyme Degradation of AZTpppp$_S$A (31)

3. Conclusions

Hydrolysis-resistant AZTp$_S$p$_{CX2}$pp$_S$A and AZTp$_S$p$_{CX2}$pp$_S$AZT (X = H or F) were synthesized as potential inhibitors of the AZT excision reaction by HIV-1 RT. The reactions were carried out by the coupling of adenosine or AZT H-phosphonate with trimetaphosphate analogs, taking advantage of the differential power of elemental sulfur to sulfurize H-phosphonate diesters but not monoesters. The configurations of their diastereomers were assigned by comparing rates of degradation using snake venom phosphodiesterase. Their stabilities to this phosphodiesterase were compared to AZTp$_4$A, AZTp$_4$AZT, AZTpp$_{CH2}$ppA and AZTpp$_{CF2}$ppA. AZTpppp$_S$A was also prepared and the configurations of its diastereomers were assigned by elution order on reverse-phase HPLC and snake venom digestion.

4. Experimental Procedures

General Procedures. Methylenediphosphonic acid, 2-chloro-4*H*-l,3,2-benzo-dioxaphosphorin-4-one, tri-*n*-butylamine and AMP monohydrate were purchased from Aldrich. Elemental sulfur was purchased from J.T.Baker. AZT was obtained from Transgenomic. Bis(tri-*n*-butylammonium) pyrophosphate was prepared according to the procedure described by Eckstein,[15] and bis(tri-*n*-butylammonium) difluoromethylenediphosphonate was prepared by the procedure reported by Boyle.[21] The snake venom phosphodiesterase (Type IV from *Crotalus atrox*) was purchased from Sigma. The preparative reverse-phase HPLC was performed on a PrepPak system with three Waters Delta-Pak PrepPak® cartridges (C18, 300Å, 40 mm × 100 mm, 15 μm) using acetonitrile and 0.1 M ammonium bicarbonate (pH 7.5) or water. The analytical samples were analyzed on a Waters Atlantis™ analytical column (dC18 column, 100Å, 4.6 mm × 50 mm, 3.0 μm) using acetonitrile and 0.1 M triethylammonium acetate (TEAA) buffer (pH 6.8), and the flow rate was 1.0 mL/min.

Preparation of [S_P] and [R_P] adenosine 3′-azido-3′-deoxythymidine 5′,5‴-P^1,P^4-(P^1-thio)-tetraphosphate (31a,b). To a solution of 3′-azido-3′-deoxythymidine (0.081 g, 0.30 mmol) in 2 mL of anhydrous DMF was added 2-chloro-4*H*-l,3,2-benzo-dioxaphosphorin-4-one (0.078 g, 0.39 mmol, 1.3 equiv). The solution was stirred for 15 min at room temperature under nitrogen. A 0.5 M solution of bis(tri-*n*-butylammonium) pyrophosphate in anhydrous DMF (1.3 mL, 0.65 mmol, 2.2 equiv) was vortexed with tri-*n*-butylamine (0.60 mL, 2.5 mmol, 8.3 equiv) and immediately added to the reaction mixture. After 20 min a solution of iodine (0.092 g, 0.36 mmol, 1.2 equiv) in 2.0 mL of

pyridine and 0.01 mL of water was added. After 15 min, a mixture of 0.16 M of adenosine 5′-H-phosphonate, **45**, in the tri-*n*-butylammonium form (6.0 mL, 0.96 mmol, 3.2 equiv), S_8 (0.056 g, 1.7 mmol, 5.7 equiv) and zinc chloride (0.36 g, 2.64 mmol, 8.8 equiv) that had been dried together by evaporation of pyridine and DMF was added with stirring. After 21 hr, 10% aqueous ammonia (20 mL, 118 mmol) was added to keep the zinc in solution, and the dilute basic solution was washed with methylene chloride to remove the excess sulfur and DMF, and then applied to a column of sodium cation exchange resin (50WX2, 10 mL, 6.0 mmol, 20 equiv) to remove Zn^{2+}. The product was concentrated and purified by preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.094 g of **[*S*$_P$]-31a** (0.10 mmol, 33%) and 0.12 g of **[*R*$_P$]-31b** (0.13 mmol, 43%) in the ammonium form. **[*S*$_P$]-31a**: UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.67 (s, 1H), 8.21 (s, 1H), 7.59 (d, *J* = 1.0 Hz, 1H), 6.15-6.05 (m, 2H), 4.62-4.50 (m, water suppression reduces intensity), 4.44-4.31 (m, 2H), 4.29-4.06 (m, 4H), 2.43-2.26 (m, 2H), 1.83 (d, *J* = 0.8 Hz, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.0, 158.3, 155.6, 154.2, 151.9, 143.0, 139.7, 121.2, 114.4, 89.3, 87.4, 86.9 (d, J_{CP} = 9.8 Hz), 85.7 (d, J_{CP} = 9.2 Hz), 77.2, 73.5, 68.6 (d, J_{CP} = 5.7 Hz), 68.1 (d, J_{CP} = 6.5 Hz), 63.8, 39.2, 14.4; ^{31}P NMR (D_2O, 400 MHz): δ 44.32 (d, *J* = 25.7 Hz, 1P), -10.57 (d, *J* = 17.6 Hz, 1P), -21.94 (t, *J* = 16.8 Hz, 1P), -23.00 (dd, *J* = 25.7 Hz, *J* = 16.1 Hz, 1P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 851.40 (calculated for $C_{20}H_{27}N_{10}O_{18}P_4S^-$: 851.02). **[*R*$_P$]-31b**: UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.60 (s, 1H), 8.22 (s, 1H), 7.62 (d, *J* = 1.1 Hz, 1H), 6.14-6.05 (m, 2H), 4.61-4.48 (m, water suppression reduces intensity), 4.43-4.08 (m, 6H), 2.43-2.28 (m, 2H), 1.86 (d, *J* = 0.7 Hz, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.1, 158.3, 155.6, 154.2, 151.9, 142.8,

139.8, 121.3, 114.4, 89.3, 87.5, 86.8 (d, J_{CP} = 9.7 Hz), 85.7 (d, J_{CP} = 9.2 Hz), 77.1, 73.4, 68.6 (d, J_{CP} = 5.8 Hz), 68.4 (d, J_{CP} = 6.2 Hz), 63.8, 39.1, 14.4; ^{31}P NMR (D_2O, 400 MHz): δ 44.18 (d, *J* = 26.0 Hz, 1P), -10.59 (d, *J* = 17.9 Hz, 1P), -22.00 (t, *J* = 17.3 Hz, 1P), -23.00 (dd, *J* = 25.9 Hz, *J* = 16.4 Hz, 1P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 851.46 (calculated for $C_{20}H_{27}N_{10}O_{18}P_4S^-$: 851.02).

Preparation of [S_{P1},S_{P4}], [S_{P1},R_{P4}], [R_{P1},S_{P4}] and [R_{P1},R_{P4}] adenosine 3′-azido-3′-deoxythymidine 5′,5‴-P^1,P^4-(P^1,P^4-dithio-P^2,P^3-methylene)-tetraphosphate (37a-d). To a solution of AZT (0.13 g, 0.49 mmol) in 3 mL of anhydrous DMF was added 2-chloro-4*H*-l,3,2-benzo-dioxaphosphorin-4-one (0.20 g, 0.99 mmol, 2.0 equiv). The solution was stirred for 15 min at room temperature under N_2. Tri-*n*-butylamine (1.0 mL, 4.2 mmol, 8.6 equiv) and methylenediphosphonic acid (0.16 g, 0.91 mmol, 1.9 equiv) were added to the reaction mixture. After 20 min excess S_8 (0.16 g, 5.0 mmol, 10 equiv) dried by evaporation of pyridine and DMF was added. After 30 min, a mixture of 0.17 M adenosine 5′-H-phosphonate, **45**, in the tri-*n*-butylammonium form (10 mL, 1.7 mmol, 3.5 equiv) in DMF and $ZnCl_2$ (0.59 g, 4.3 mmol, 8.8 equiv) that had been dried together by evaporation of pyridine and DMF was added with stirring. After 19 hr, 10% aqueous ammonia (30 mL, 177 mmol) was added and the precipitated S_8 was removed by filtration. The basic filtrate was applied to a column of sodium cation exchange resin (50WX2, 10 mL, 6.0 mmol) to remove Zn^{2+}. The eluate was concentrated and the four diastereomers were purified and separated by preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.043 g of **[S_{P1},S_{P4}]-37a** (0.046 mmol, 9.4%), 0.050 g of **[S_{P1},R_{P4}]-37b** (0.053 mmol, 11%), 0.077 g of **[R_{P1},S_{P4}]-37c** (0.082

mmol, 17%) and 0.095 g of **[R_{P1},R_{P4}]-37d** (0.10 mmol, 20%) in the ammonium form. The products eluted in the order listed. **[S_{P1},S_{P4}]-37a**: UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.67 (s, 1H), 8.22 (s, 1H), 7.67 (s, 1H), 6.16 (t, *J* = 6.9 Hz, 1H), 6.10 (d, *J* = 5.3 Hz, 1H), 4.74-4.69 (m, water suppression reduces intensity), 4.63-4.51 (m, 2H), 4.43-4.10 (m, 6H), 2.73 (t, $J_{C\text{-}H}$ = 21.2 Hz, 2H), 2.43-2.32 (m, 2H), 1.84 (s, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.1, 158.3, 155.5 (d, *J* = 3.2 Hz), 154.3, 151.7, 142.9 (d, *J* = 3.6 Hz), 139.8 (d, *J* = 7.9 Hz), 121.3, 114.5, 89.8 (d, J_{CP} = 7.9 Hz), 87.4 (d, J_{CP} = 9.6 Hz), 86.5 (d, J_{CP} = 8.1 Hz), 85.6 (d, J_{CP} = 6.6 Hz), 77.5, 73.1, 68.3, 67.6, 63.8, 39.1, 33.7 (t, J_{CP} = 131.3 Hz), 14.5; ^{31}P NMR (D_2O, 400 MHz): δ 42.64-41.78 (m, 2P), 7.39-6.83 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 865.53 (calculated for $C_{21}H_{29}N_{10}O_{16}P_4S_2^-$: 865.02). **[$S_{P1}$,$R_{P4}$]-37b**: UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.73 (s, 1H), 8.20 (s, 1H), 7.56 (d, *J* = 1.0 Hz, 1H), 6.18 (dd, *J* = 5.9 Hz, *J* = 8.2 Hz, 1H), 6.10 (d, *J* = 6.1 Hz, 1H), 4.76-4.71 (m, water suppression reduces intensity), 4.63-4.53 (m, 2H), 4.43-4.10 (m, 6H), 2.92-2.56 (m, 2H), 2.42-2.20 (m, 2H), 1.81 (s, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 168.9, 158.3, 155.6 (d, *J* = 2.2 Hz), 154.2, 151.8, 142.9 (d, *J* = 2.4 Hz), 139.4 (d, *J* = 4.9 Hz), 121.1, 114.5, 89.4 (d, J_{CP} = 5.5 Hz), 87.1 (d, J_{CP} = 6.4 Hz), 87.0 (d, J_{CP} = 9.8 Hz), 85.6 (d, J_{CP} = 7.5 Hz), 77.7, 73.8, 69.0, 67.7, 64.3, 39.4, 33.8 (t, J_{CP} = 130.1 Hz), 14.5; ^{31}P NMR (D_2O, 400 MHz): δ 41.98 (d, *J* = 32.5 Hz, 2P), 7.27-6.68 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 865.59 (calculated for $C_{21}H_{29}N_{10}O_{16}P_4S_2^-$: 865.02). **[$R_{P1}$,$S_{P4}$]-37c**: UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.60 (s, 1H), 8.22 (d, *J* = 0.8 Hz, 1H), 7.69 (d, *J* = 1.1 Hz, 1H), 6.18 (t, *J* = 6.9 Hz, 1H), 6.11 (d, *J* = 5.5 Hz, 1H), 4.76-4.72 (m, water suppression reduces intensity), 4.61-4.50 (m, 2H), 4.42-4.14 (m, 6H), 2.83-2.54 (m, 2H),

2.44-2.36 (m, 2H), 1.88 (s, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.2, 158.3, 155.5, 154.3, 151.7, 142.7, 139.9 (d, J = 2.5 Hz), 121.3, 114.5, 89.8 (d, J_{CP} = 3.1 Hz), 87.5 (d, J_{CP} = 3.6 Hz), 86.4 (d, J_{CP} = 9.7 Hz), 85.6 (d, J_{CP} = 9.4 Hz), 77.3, 73.1, 68.3 (d, J_{CP} = 6.4 Hz), 67.8 (d, J_{CP} = 5.1 Hz), 63.7, 39.1, 33.7 (t, J_{CP} = 131.1 Hz), 14.5; ^{31}P NMR (D_2O, 400 MHz): δ 42.64-42.00 (m, 2P), 7.60-6.94 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 865.53 (calculated for $C_{21}H_{29}N_{10}O_{16}P_4S_2^-$: 865.02). **[$R_{P1}$,$R_{P4}$]-37d**: UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.61 (s, 1H), 8.22 (s, 1H), 7.69 (d, J = 1.1 Hz, 1H), 6.17 (t, J = 6.9 Hz, 1H), 6.11 (d, J = 5.8 Hz, 1H), 4.76-4.72 (m, water suppression reduces intensity), 4.62-4.51 (m, 2H), 4.43-4.14 (m, 6H), 2.69 (t, $J_{C\text{-}H}$ = 21.2 Hz, 2H), 2.41-2.33 (m, 2H), 1.89 (d, J = 1.0 Hz, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.2, 158.3, 155.5, 154.3, 151.8, 142.8 (d, J = 1.5 Hz), 139.9 (d, J = 4.5 Hz), 121.3, 114.5, 89.6 (d, J_{CP} = 5.0 Hz), 87.5 (d, J_{CP} = 5.4 Hz), 86.6 (d, J_{CP} = 9.8 Hz), 85.7 (d, J_{CP} = 7.8 Hz), 77.3, 73.2, 68.6, 68.0, 63.9, 39.2, 33.7 (t, J_{CP} = 131.0 Hz), 14.5; ^{31}P NMR (D_2O, 400 MHz): δ 42.46-41.89 (m, 2P), 7.50-6.89 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 865.46 (calculated for $C_{21}H_{29}N_{10}O_{16}P_4S_2^-$: 865.02).

Preparation of [S_{P1},S_{P4}], [S_{P1},R_{P4}], [R_{P1},S_{P4}] and [R_{P1},R_{P4}] adenosine 3′-azido-3′-deoxythymidine 5′,5‴-P^1,P^4-(P^1,P^4-dithio-P^2,P^3-difluoromethylene)-tetraphosphate (38a-d). Starting with AZT (0.13 g, 0.49 mmol), **38a-d** was prepared by the same procedure described for **37a-d**, except that a 0.2 M solution of bis(tri-*n*-butylammonium) difluoromethylenediphosphonate in anhydrous DMF (4.0 mL, 0.80 mmol, 1.6 equiv) was vortexed with tri-*n*-butylamine (1.0 mL, 4.2 mmol, 8.6 equiv) and added instead of methylenediphosphonic acid. The four diastereomers were purified and separated by

preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.090 g of **[*S*$_{P1}$,*S*$_{P4}$]-38a** (0.093 mmol, 19%), 0.069 g of **[*S*$_{P1}$,*R*$_{P4}$]-38b** (0.071 mmol, 14%), 0.096 g of **[*R*$_{P1}$,*S*$_{P4}$]-38c** (0.099 mmol, 20%) and 0.14 g of **[*R*$_{P1}$,*R*$_{P4}$]-38d** (0.14 mmol, 29%) in the ammonium form. The products eluted in the order listed. **[*S*$_{P1}$,*S*$_{P4}$]-38a**: UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.60 (s, 1H), 8.21 (s, 1H), 7.61 (d, *J* = 1.1 Hz, 1H), 6.10-6.02 (m, 2H), 4.76-4.69 (m, water suppression reduces intensity), 4.58-4.46 (m, 2H), 4.42-4.06 (m, 6H), 2.40-2.26 (m, 2H), 1.84 (s, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.1, 158.2, 155.5 (d, *J* = 3.4 Hz), 154.2, 151.7, 142.9, 139.7 (d, *J* = 8.6 Hz), 121.2, 114.4, 89.6 (d, J_{CP} = 7.9 Hz), 87.4 (d, J_{CP} = 7.8 Hz), 86.8 (d, J_{CP} = 10.4 Hz), 85.6, 77.6 (d, J_{CP} = 5.3 Hz), 73.4, 68.6 (d, J_{CP} = 6.0 Hz), 68.3 (d, J_{CP} = 4.5 Hz), 63.8, 39.1, 14.4; ^{31}P NMR (D_2O, 400 MHz): δ 43.14-42.18 (m, 2P), (-)6.26-(-)7.80 (tm, *J* = 83.1 Hz, 2P); ^{19}F NMR (D_2O, 400 MHz): δ -119.3 (t, *J* = 83.3 Hz). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 901.27 (calculated for $C_{21}H_{27}F_2N_{10}O_{16}P_4S_2^-$: 901.00). **[*S*$_{P1}$,*R*$_{P4}$]-38b**: UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.64 (s, 1H), 8.21 (d, *J* = 0.6 Hz, 1H), 7.61 (d, *J* = 1.0 Hz, 1H), 7.61 (t, *J* = 7.0 Hz, 1H), 7.61 (d, *J* = 6.3 Hz, 1H), 4.76-4.71 (m, water suppression reduces intensity), 4.60-4.48 (m, 2H), 4.44-4.08 (m, 6H), 2.40-2.26 (m, 2H), 1.85 (s, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.0, 158.3, 155.5 (d, *J* = 3.4 Hz), 154.2, 151.8, 142.9, 139.7 (d, *J* = 8.7 Hz), 121.2, 114.5, 89.4 (d, J_{CP} = 8.1 Hz), 87.3 (d, J_{CP} = 9.2 Hz), 86.9 (d, J_{CP} = 10.9 Hz), 85.7, 77.5 (d, J_{CP} = 7.2 Hz), 73.5, 68.9 (d, J_{CP} = 5.6 Hz), 68.2 (d, J_{CP} = 4.6 Hz), 64.1, 39.0, 14.4; ^{31}P NMR (D_2O, 400 MHz): δ 43.30-42.12 (m, 2P), (-)6.25-(-)7.81 (tm, *J* = 83.6 Hz, 2P); ^{19}F NMR (D_2O, 400 MHz): δ -119.2 (t, *J* = 83.6 Hz). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 901.33 (calculated for

$C_{21}H_{27}F_2N_{10}O_{16}P_4S_2^-$: 901.00). **[*R*$_{P1}$,*S*$_{P4}$]-38c**: UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.57 (s, 1H), 8.22 (s, 1H), 7.64 (d, *J* = 1.2 Hz, 1H), 6.12-6.07 (m, 2H), 4.76-4.71 (m, water suppression reduces intensity), 4.59-4.45 (m, 2H), 4.42-4.04 (m, 6H), 2.40-2.29 (m, 2H), 1.86 (d, *J* = 1.0 Hz, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.1, 158.3, 155.5 (d, *J* = 1.8 Hz), 154.2, 151.8, 142.8, 139.8 (d, *J* = 4.2 Hz), 121.2, 114.4, 89.5 (d, J_{CP} = 4.8 Hz), 87.5 (d, J_{CP} = 4.5 Hz), 86.8 (d, J_{CP} = 10.2 Hz), 85.6 (d, J_{CP} = 10.6 Hz), 77.4 (d, J_{CP} = 3.2 Hz), 73.4, 68.6 (d, J_{CP} = 5.7 Hz), 68.2 (d, J_{CP} = 6.3 Hz), 63.8, 39.1, 14.5; ^{31}P NMR (D_2O, 400 MHz): δ 43.18-42.26 (m, 2P), (-)6.21-(-)7.77 (tm, *J* = 84.0 Hz, 2P); ^{19}F NMR (D_2O, 400 MHz): δ -119.3 (t, *J* = 83.4 Hz). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 901.33 (calculated for $C_{21}H_{27}F_2N_{10}O_{16}P_4S_2^-$: 901.00). **[*R*$_{P1}$,*R*$_{P4}$]-38d**: UV λ_{max} 262 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.57 (s, 1H), 8.22 (s, 1H), 7.64 (d, *J* = 1.2 Hz, 1H), 6.13 (t, *J* = 7.0 Hz, 1H), 6.09 (d, *J* = 6.2 Hz, 1H), 4.76-4.73 (m, water suppression reduces intensity), 4.60-4.48 (m, 2H), 4.43-4.09 (m, 6H), 2.38-2.28 (m, 2H), 1.87 (d, *J* = 1.0 Hz, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.1, 158.3, 155.6 (d, *J* = 2.1 Hz), 154.2, 151.9, 142.8, 139.8 (d, *J* = 5.2 Hz), 121.2, 114.5, 89.4 (d, J_{CP} = 5.5 Hz), 87.4 (d, J_{CP} = 5.9 Hz), 86.8 (d, J_{CP} = 10.0 Hz), 85.6 (d, J_{CP} = 8.4 Hz), 77.3 (d, J_{CP} = 3.9 Hz), 73.4, 68.9 (d, J_{CP} = 5.6 Hz), 68.3 (d, J_{CP} = 5.7 Hz), 64.0, 39.0, 14.5; ^{31}P NMR (D_2O, 400 MHz): δ 43.18-42.41 (m, 2P), (-)6.20-(-)7.67 (tm, *J* = 83.6 Hz, 2P); ^{19}F NMR (D_2O, 400 MHz): δ -119.4 (t, *J* = 83.7 Hz). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 901.40 (calculated for $C_{21}H_{27}F_2N_{10}O_{16}P_4S_2^-$: 901.00).

Preparation of [*S*$_{P1}$,*S*$_{P4}$], [*S*$_{P1}$,*R*$_{P4}$] and [*R*$_{P1}$,*R*$_{P4}$] di-3′-azido-3′-deoxythymidine 5′,5‴-*P*^{1},*P*^{4}-(*P*^{1},*P*^{4}-dithio-*P*^{2},*P*^{3}-methylene)-tetraphosphate (39a-c). Starting with AZT (0.084

g, 0.31 mmol), **39a-c** was prepared by the same procedure described for **37a-d**, except that 3′-azido-3′-deoxythymidine 5′-H-phosphonate, **44**, in the pyridinium form (0.34 g, 0.83 mmol, 2.7 equiv) was dried together with $ZnCl_2$ (0.40 g, 2.9 mmol, 9.4 equiv) and added to the reaction solution instead of adenosine 5′-H-phosphonate. The three diastereomers were purified and separated by preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.049 g of **[S_{P1},S_{P4}]-39a** (0.052 mmol, 17%), 0.079 g of **[S_{P1},R_{P4}]-39b** (0.085 mmol, 27%) and 0.089 g of **[R_{P1},R_{P4}]-39c** (0.95 mmol, 31%) in the ammonium form. The products eluted in the order listed. **[S_{P1},S_{P4}]-39a**: UV λ_{max} 267 nm; ^{1}H NMR (D_2O, 400 MHz): δ 7.81 (d, *J* = 1.1 Hz, 2H), 6.25 (t, *J* = 6.9 Hz, 2H), 4.64-4.56 (m, water suppression reduces intensity), 4.32-4.16 (m, 6H), 2.72 (t, $J_{C\text{-}H}$ = 21.3 Hz, 2H), 2.51-2.41 (m, 4H), 1.95 (d, *J* = 1.1 Hz, 6H); ^{13}C NMR (D_2O, 400 MHz): δ 169.2, 154.3, 140.0, 114.6, 87.5, 85.7 (d, J_{CP} = 9.7 Hz), 68.3 (d, J_{CP} = 6.4 Hz), 63.8, 39.2, 33.7 (t, J_{CP} = 130.6 Hz), 14.6; ^{31}P NMR (D_2O, 300 MHz): δ 42.23 (d, *J* = 30.9 Hz, 2P), 7.38 (d, *J* = 30.9 Hz, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 865.48 (calculated for $C_{21}H_{29}N_{10}O_{16}P_4S_2^-$: 865.02). **[$S_{P1}$,$R_{P4}$]-39b**: UV λ_{max} 267 nm; ^{1}H NMR (D_2O, 400 MHz): δ 7.80 (d, *J* = 1.1 Hz, 2H), 6.25 (t, *J* = 6.9 Hz, 2H), 4.63-4.53 (m, water suppression reduces intensity), 4.33-4.16 (m, 6H), 2.83-2.53 (m, 2H), 2.52-2.38 (m, 4H), 1.95 (d, *J* = 0.9 Hz, 6H); ^{13}C NMR (D_2O, 400 MHz): δ 169.3, 154.4, 140.0, 114.61, 114.59, 87.5, 85.74 (d, J_{CP} = 9.8 Hz), 85.70 (d, J_{CP} = 9.7 Hz), 68.5 (d, J_{CP} = 6.1 Hz), 68.3 (d, J_{CP} = 6.1 Hz), 63.9, 63.6, 39.2, 39.1, 33.7 (t, J_{CP} = 130.7 Hz), 14.56, 14.55; ^{31}P NMR (D_2O, 300 MHz): δ 42.85-41.90 (m, 2P), 7.79-7.10 (m, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 865.48 (calculated for $C_{21}H_{29}N_{10}O_{16}P_4S_2^-$: 865.02). **[$R_{P1}$,$R_{P4}$]-39c**: UV λ_{max} 267 nm; ^{1}H NMR (D_2O, 400 MHz):

δ 7.80 (d, J = 1.2 Hz, 2H), 6.26 (t, J = 6.9 Hz, 2H), 4.62-4.56 (m, water suppression reduces intensity), 4.31-4.19 (m, 6H), 2.68 (t, $J_{C\text{-}H}$ = 21.2 Hz, 2H), 2.54-2.40 (m, 4H), 1.95 (d, J = 1.0 Hz, 6H); ^{13}C NMR (D_2O, 400 MHz): δ 169.3, 154.4, 140.1, 114.6, 87.6, 85.7 (d, J_{CP} = 9.8 Hz), 68.6 (d, J_{CP} = 6.0 Hz), 63.9, 39.1, 33.7 (t, J_{CP} = 130.8 Hz), 14.5; ^{31}P NMR (D_2O, 300 MHz): δ 42.27 (d, J = 31.4 Hz, 2P), 7.50 (d, J = 31.2 Hz, 2P). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 865.35 (calculated for $C_{21}H_{29}N_{10}O_{16}P_4S_2^-$: 865.02).

Preparation of [S_{P1},S_{P4}], [S_{P1},R_{P4}] and [R_{P1},R_{P4}] di-3′-azido-3′-deoxythymidine 5′,5‴-P^1,P^4-(P^1,P^4-dithio-P^2,P^3-difluoromethylene)-tetraphosphate (40a-c). Starting with AZT (0.094 g, 0.35 mmol), **40a-c** was prepared by the same procedure described for **38a-d**, except that 3′-azido-3′-deoxythymidine 5′-H-phosphonate, **44**, in the pyridinium form (0.43 g, 1.0 mmol, 2.9 equiv) was dried together with $ZnCl_2$ (0.41 g, 3.0 mmol, 8.6 equiv) and added to the reaction solution instead of adenosine 5′-H-phosphonate. The three diastereomers were purified and separated by preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.052 g of **[S_{P1},S_{P4}]-40a** (0.054 mmol, 15%), 0.083 g of **[S_{P1},R_{P4}]-40b** (0.086 mmol, 25%) and 0.089 g of **[R_{P1},R_{P4}]-40c** (0.092 mmol, 26%) in the ammonium form. The products eluted in the order listed. **[S_{P1},S_{P4}]-40a**: UV λ_{max} 267 nm; ^{1}H NMR (D_2O, 400 MHz): δ 7.794 (d, J = 1.0 Hz, 1H), 7.791 (d, J = 1.0 Hz, 1H), 6.22 (t, J = 6.8 Hz, 2H), 4.62-4.54 (m, water suppression reduces intensity), 4.36-4.17 (m, 6H), 2.43 (t, J = 5.8 Hz, 4H), 1.93 (s, 6H); ^{13}C NMR (D_2O, 400 MHz): δ 169.2, 154.3, 140.0, 114.6, 87.5, 85.8 (d, J_{CP} = 10.2 Hz), 68.7 (d, J_{CP} = 6.3 Hz), 63.8, 39.2, 14.5; ^{31}P NMR (D_2O, 300 MHz): δ 43.07-42.33 (m, 2P), (-)5.71-(-)7.89 (tm, J

= 82. 8 Hz, 2P); ^{19}F NMR (D_2O, 400 MHz): δ -119.3 (t, *J* = 83.0 Hz). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 901.56 (calculated for $C_{21}H_{27}F_2N_{10}O_{16}P_4S_2^-$: 901.00). **[*S*$_{P1}$,*R*$_{P4}$]-40b**: UV λ_{max} 267 nm; ^{1}H NMR (D_2O, 400 MHz): δ 7.794 (d, *J* = 0.9 Hz, 1H), 7.781 (d, *J* = 0.8 Hz, 1H), 6.24 (t, *J* = 7.0 Hz, 1H), 6.23 (t, *J* = 6.8 Hz, 1H), 4.63-4.53 (m, water suppression reduces intensity), 4.35-4.13 (m, 6H), 2.53-2.35 (m, 4H), 1.94 (s, 6H); ^{13}C NMR (D_2O, 400 MHz): δ 169.24, 169.23, 154.4, 140.02, 139.99, 114.66, 114.62, 87.52, 87.50, 85.77 (d, J_{CP} = 10.0 Hz), 85.75 (d, J_{CP} = 10.0 Hz), 68.8 (d, J_{CP} = 6.5 Hz), 68.7 (d, J_{CP} = 6.0 Hz), 64.1, 63.8, 39.1, 14.5; ^{31}P NMR (D_2O, 300 MHz): δ 43.19-42.48 (m, 2P), (-)5.66-(-)7.83 (tm, *J* = 83.2 Hz, 2P); ^{19}F NMR (D_2O, 400 MHz): δ -119.3 (t, *J* = 83.4 Hz). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 901.62 (calculated for $C_{21}H_{27}F_2N_{10}O_{16}P_4S_2^-$: 901.00). **[*R*$_{P1}$,*R*$_{P4}$]-40c**: UV λ_{max} 267 nm; ^{1}H NMR (D_2O, 400 MHz): δ 7.79 (d, *J* = 1.2 Hz, 2H), 6.25 (t, *J* = 7.0 Hz, 2H), 4.63-4.57 (m, water suppression reduces intensity), 4.33-4.19 (m, 6H), 2.52-2.38 (m, 4H), 1.94 (d, *J* = 1.2 Hz, 6H); ^{13}C NMR (D_2O, 400 MHz): δ 169.3, 154.4, 140.0, 114.7, 87.5, 85.8 (d, J_{CP} = 10.0 Hz), 68.9 (d, J_{CP} = 6.3 Hz), 64.1, 39.1, 14.5; ^{31}P NMR (D_2O, 300 MHz): δ 43.23-42.52 (m, 2P), (-)5.49-(-)7.80 (tm, *J* = 83.6 Hz, 2P); ^{19}F NMR (D_2O, 400 MHz): δ -119.4 (t, *J* = 83.9 Hz). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 901.36 (calculated for $C_{21}H_{27}F_2N_{10}O_{16}P_4S_2^-$: 901.00).

Preparation of 3′-azido-3′-deoxythymidine 5′-H-phosphonate (44). To a solution of AZT (2.7 g, 10 mmol) in 30 mL of anhydrous 1,4-dioxane was added a solution of 2-chloro-4*H*-l,3,2-benzo-dioxaphosphorin-4-one (2.5 g, 12 mmol, 1.2 equiv) in 30 mL of

anhydrous 1,4-dioxane and 10 mL of anhydrous pyridine. The reaction was stirred at room temperature under N_2. After 20 min, 40 mL of water was added to the reaction mixture. After 2 hr, the solution was washed with methylene chloride and concentrated to 5 mL. The product was purified by preparative reverse-phase HPLC using water in acetonitrile to give 3.2 g of **44** (7.8 mmol, 78%) in the pyridinium form: UV λ_{max} 267 nm; ^{1}H NMR (D_2O, 400 MHz): δ 7.69 (s, 1H), 6.77 (d, J_{HP} = 638.2 Hz, 1H), 6.26 (t, J = 6.3 Hz, 1H), 4.58-4.41 (m, water suppression reduces intensity), 4.30-3.98 (m, 3H), 2.50 (t, J = 5.9 Hz, 2H), 1.91 (s, 3H); ^{13}C NMR (D_2O, 400 MHz): δ 169.1, 154.2, 139.9 (d, J_{CP} = 4.2 Hz), 114.3, 87.6 (d, J_{CP} = 6.0 Hz), 85.7 (d, J_{CP} = 6.9 Hz), 65.8(d, J_{CP} = 4.1 Hz), 63.2, 39.0, 14.3; ^{31}P NMR (D_2O, 400 MHz): δ 6.37 (dt, $^1J_{PH}$ = 638.4 Hz, $^3J_{PH}$ = 6.3 Hz). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 330.25 (calculated for $C_{10}H_{13}N_5O_6P^-$: 330.06).

Preparation of adenosine 5′-H-phosphonate (45). Adenosine (1.4 g, 5.2 mmol) was dried three times by evaporation of pyridine, the last time leaving 50 mL. To the suspension was added *N*,*N*-dimethylformamide dimethyl acetal (2.7 mL, 20 mmol, 3.8 equiv), and the reaction was stirred at room temperature under nitrogen. After 16 hr, the reaction mixture was dried three times by evaporation of pyridine, the last time leaving 50 mL. To this mixture was added 2-chloro-4*H*-l,3,2-benzo-dioxaphosphorin-4-one (1.3 g, 6.4 mmol, 1.2 equiv). The solution was stirred for 30 min under N_2 at room temperature and then transferred to an ice bath. After addition of 20 mL of water, the solution was washed with methylene chloride, concentrated to 15 mL, and 15 mL of concentrated aqueous ammonia was added. The mixture was sealed and left at room

temperature. After 10 hr, the mixture was concentrated and purified by preparative reverse-phase HPLC using 0.1 M ammonium bicarbonate in acetonitrile to give 0.62 g of **45** (1.8 mmol, 35%) in the ammonium form. The product was applied to a column of proton form cation exchange resin (50WX2, 50 mL, 30 mmol, 17 equiv) and eluted into a solution of tri-*n*-butylamine (0.45 mL, 1.8 mmol, 1.0 equiv) in 20 mL of ethanol. The solution was concentrated to an oil and 10 mL of DMF was added to make a 0.17 M solution of **45**, in the tri-*n*-butylammonium form: UV λ_{max} 259 nm; ^{1}H NMR (D_2O, 400 MHz): δ 8.36 (s, 1H), 8.11 (s, 1H), 6.77 (d, J_{HP} = 639.4 Hz, 1H), 6.08 (d, J = 5.4 Hz, 1H), 4.75 (t, J = 5.3 Hz, water suppression reduces intensity), 4.52 (t, J = 4.7 Hz, 1H), 4.44-4.36 (m, 1H), 4.24-4.10 (m, 2H); ^{13}C NMR (D_2O, 400 MHz): δ 158.0, 155.4, 151.4, 142.2, 121.0, 89.8, 86.3 (d, J_{CP} = 8.0 Hz), 76.9, 73.0, 65.6 (d, J_{CP} = 4.1 Hz); ^{31}P NMR (D_2O, 400 MHz): δ 6.68 (dt, $^{1}J_{PH}$ = 639.5 Hz, $^{3}J_{PH}$ = 6.3 Hz). The mass was confirmed by ESI-MS in negative mode as *m/z* (M-1) 330.31 (calculated for $C_{10}H_{13}N_5O_6P^-$: 330.06).

Identification of Configuration of AZTpppp$_S$A (31a,b) To a solution of 0.38 μmol of one diastereomer of **31** in 500 μL of 100 mM Tris•HCl buffer (pH = 8.7), 2 mM $MgCl_2$, was added 4 μL of snake venom phosphodiesterase in water (50 μg/μL). The solution was maintained at 37°C and analyzed by LC-MS (20 μL each time, λ = 280 nm) after various time intervals. The percentages of Ap$_S$pp (relative to the total nucleoside-containing peaks) were monitored and plotted against time. The other diastereomer was assayed following the same procedure.

Identification of Configurations of the Diastereomers of AZTp$_S$p$_{CH2}$pp$_S$A (37a-d) To a solution of 0.47 μmol of one diastereomer of **37** in 490 μL of 100 mM Tris•HCl buffer (pH = 8.7), 2 mM $MgCl_2$, was added 10 μL of snake venom phosphodiesterase in water (20 μg/μL). The solution was maintained at 37 ^oC and analyzed by LC-MS (20 μL each time, λ = 280 nm) after various time intervals. The percentages of undegraded AZTp$_S$p$_{CH2}$pp$_S$A, the percentages of (Ap$_S$ + A) formed, and the percentages of AZTp$_S$ formed (relative to the total nucleoside-containing peaks) were monitored and plotted against time. The other three diastereomers were assayed following the same procedure.

Identification of Configurations of the Diastereomers of AZTp$_S$p$_{CF2}$pp$_S$A (38a-d) All four diastereomers of **38a-d** were assayed following the same procedure described for **37a-d**, except that the concentration of the snake venom phosphodiesterase in water was 50 μg/μL.

Identification of Configurations of the Diastereomers of AZTp$_S$p$_{CH2}$pp$_S$AZT (39a-c) All three diastereomers of **39a-c** were assayed following the same procedure described for **37a-d**, except that only the percentages of undegraded **39** were plotted.

Identification of Configurations of the Diastereomers of AZTp$_S$p$_{CF2}$pp$_S$AZT (40a-c) To a solution of 0.47 μmol of one diastereomer of **40** in 400 μL of 100 mM Tris•HCl buffer (pH = 8.7), 2 mM $MgCl_2$ was added 100 μL of snake venom phosphodiesterase in water (50 μg/μL, Type IV from *Crotalus atrox*). The same procedure described for **37a-d**

was then followed for all three diastereomers, except that only the percentages of undegraded **40** were plotted.

Degradation of AZTppppA (10), AZTppppAZT (11), AZTpp$_{CH2}$ppA (30) and AZTpp$_{CF2}$ppA (31) All four samples were assayed following the same procedure described for **37a-d**, except that only the percentages of undegraded starting material were plotted.

5. References

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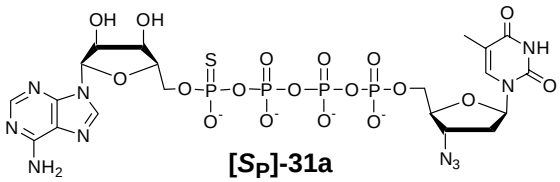
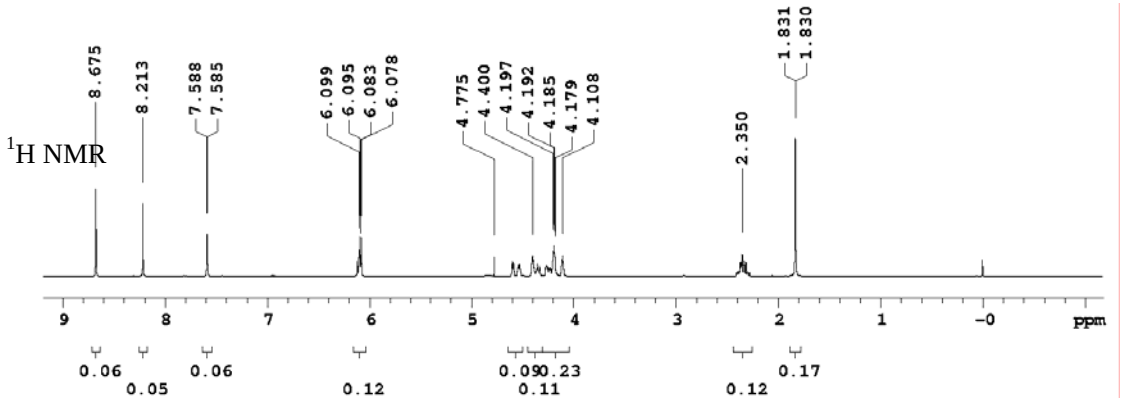
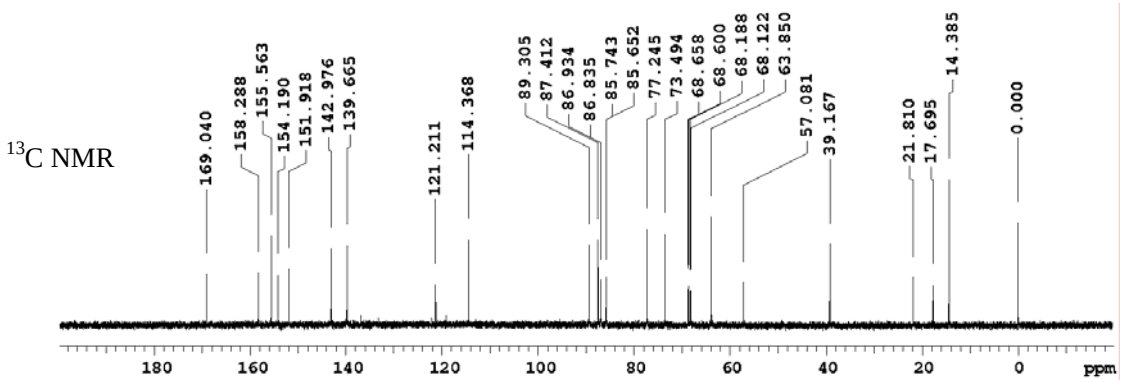
6. Appendix

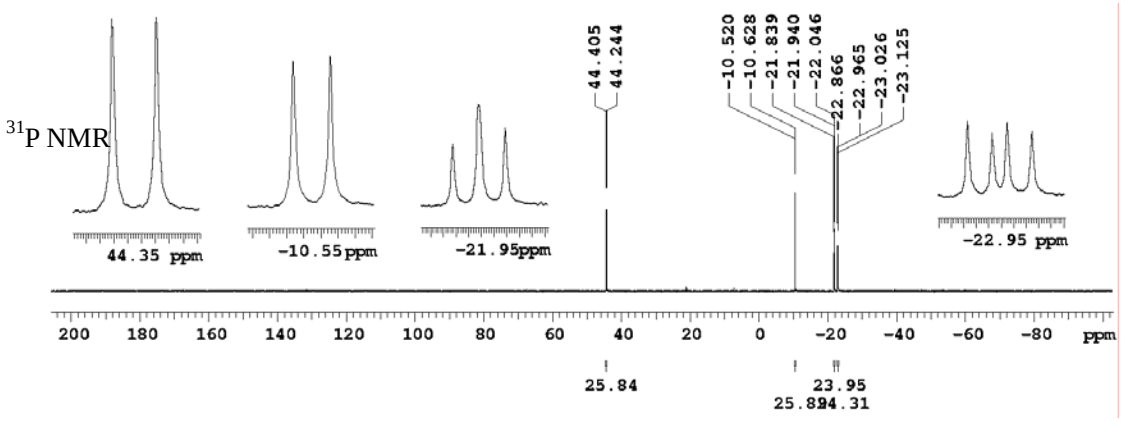
The ^{31}P NMR spectra for **39** and **40** were acquired on a Varian Unity 300 MHz spectrometer. All other NMR spectra were acquired on a Varian Unity 400 MHz spectrometer. The samples were all converted to the sodium form and dissolved in D_2O. The ^{1}H and ^{13}C spectra were referenced to 3-(trimethylsilyl)-1-propane-sulfonic acid, sodium salt. Signals in the ^{13}C spectra at 57.1, 21.8, 17.7 and 0.0 are from this standard. The ^{31}P spectra were referenced to 10% phosphoric acid in D_2O. The ^{19}F spectra were referenced to hexafluorobenzene. In the ^{1}H NMR spectra, water suppression diminishes the adjacent resonances. UV spectra were acquired on a Varian Cary 4000 or an Aviv 14 UV spectrophotometer, and the MS acquired on a Waters/Micromass Platform LCZ mass spectrometer.

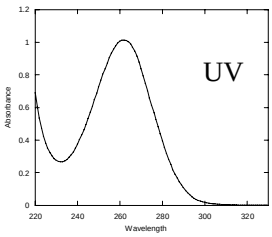


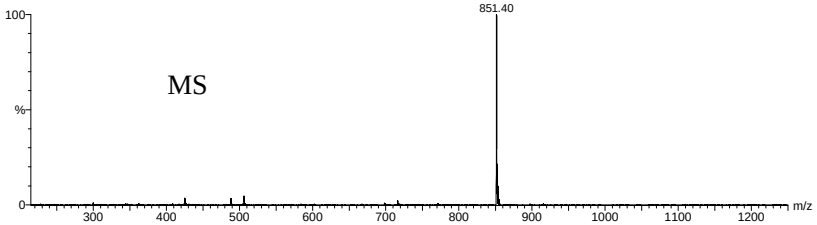
[S_P]-31a



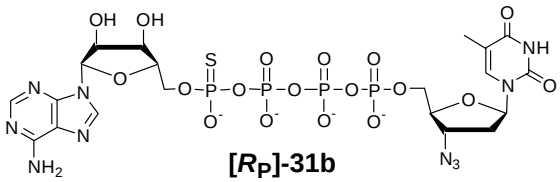


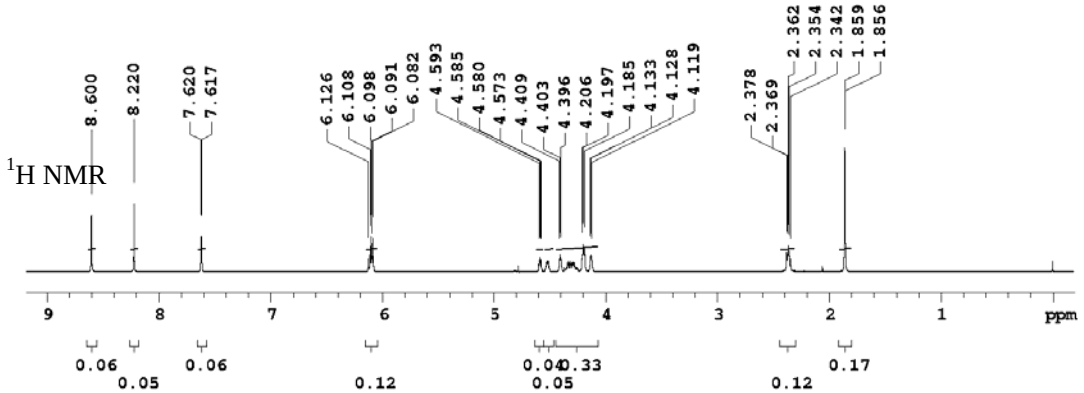


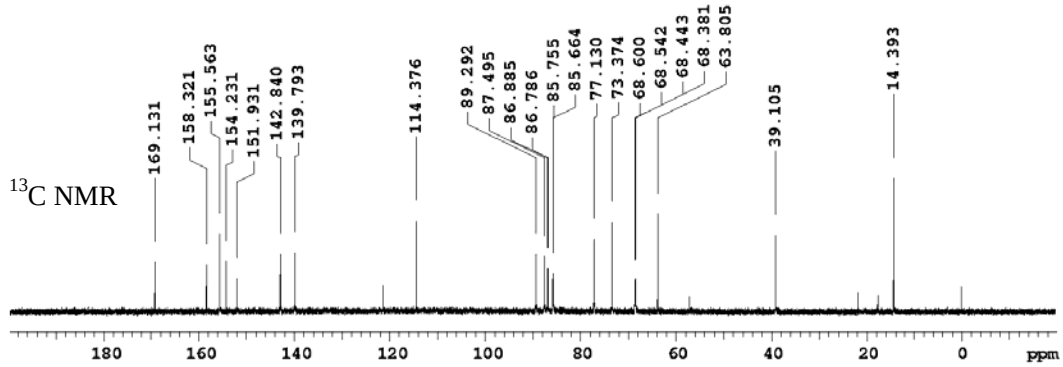


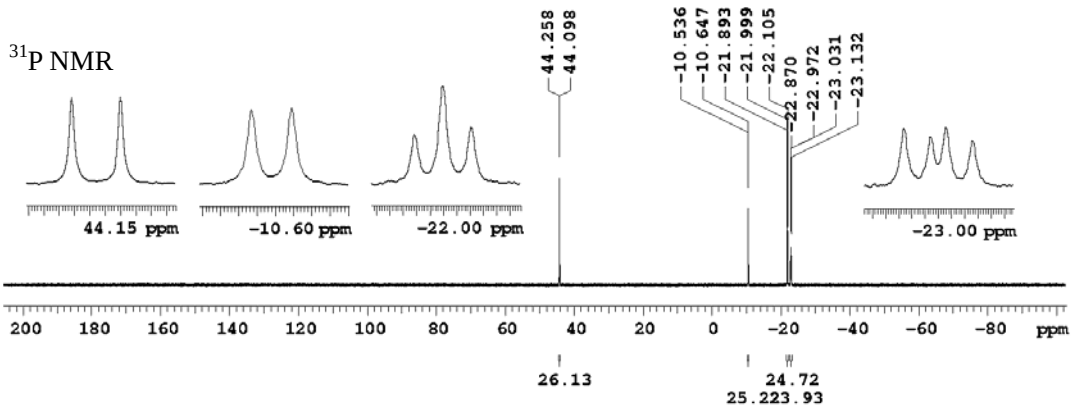


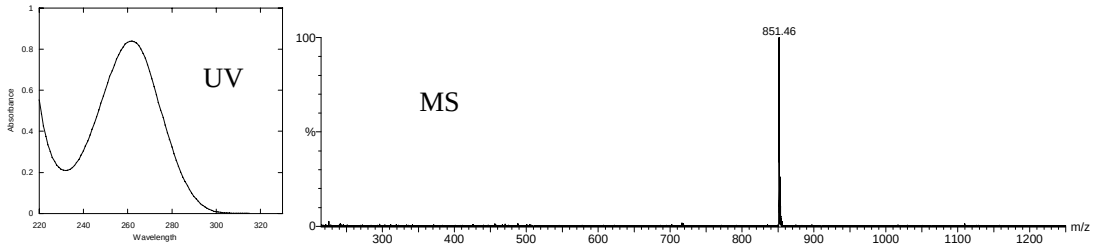
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [S_P]-AZTpppp$_S$A (31a)



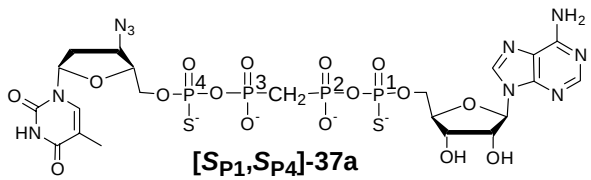






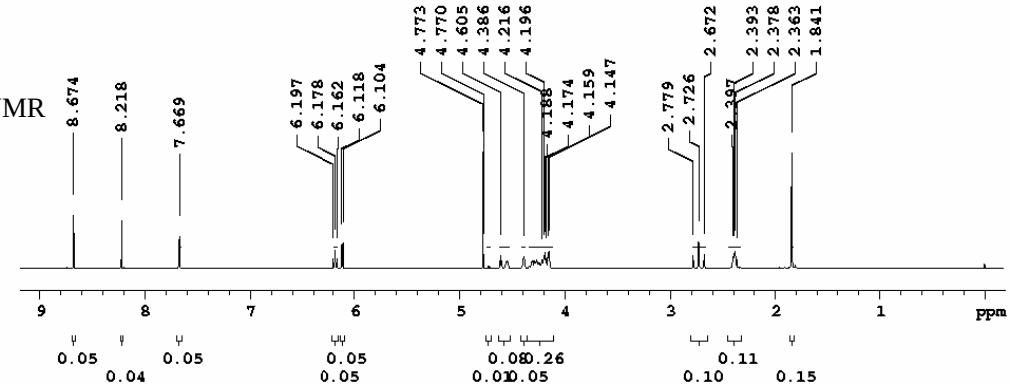


^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [R_P]-AZTpppp$_S$A (31b)

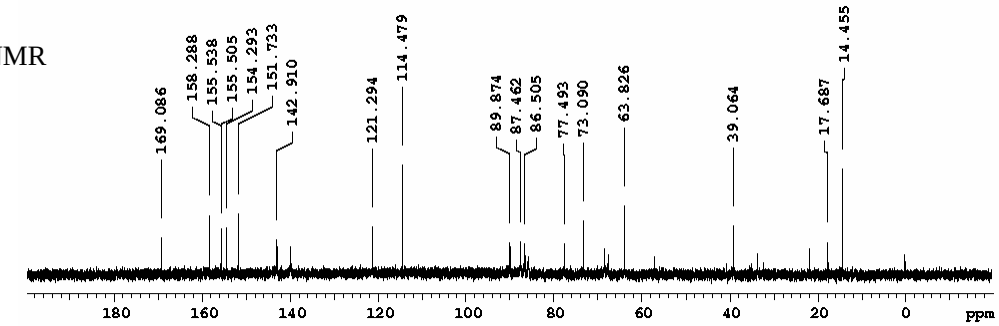


[S_{P1},S_{P4}]-37a

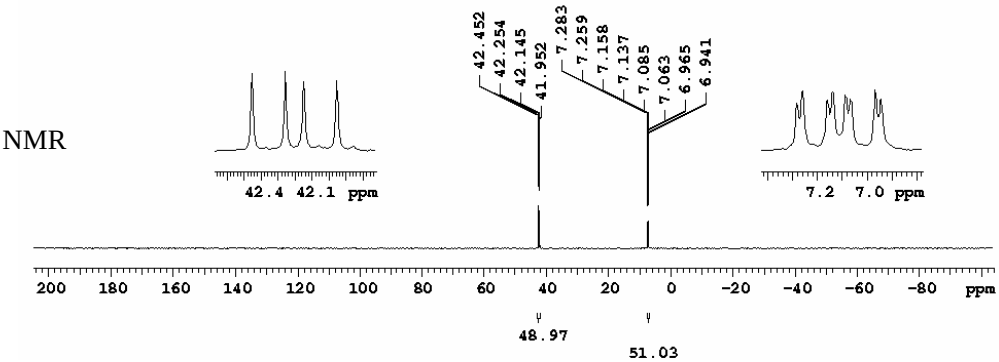
^{1}H NMR

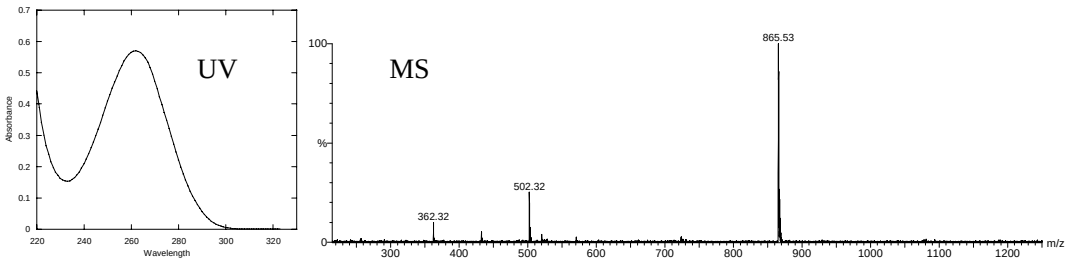


^{13}C NMR

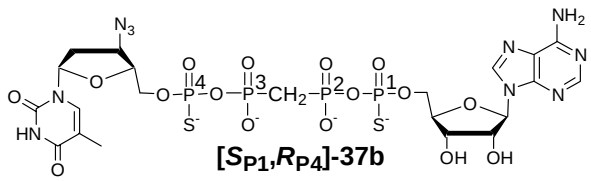


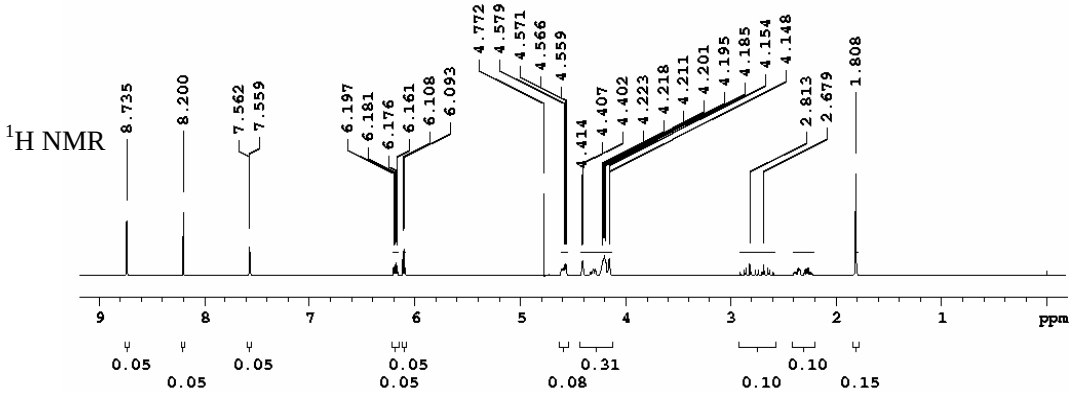
^{31}P NMR

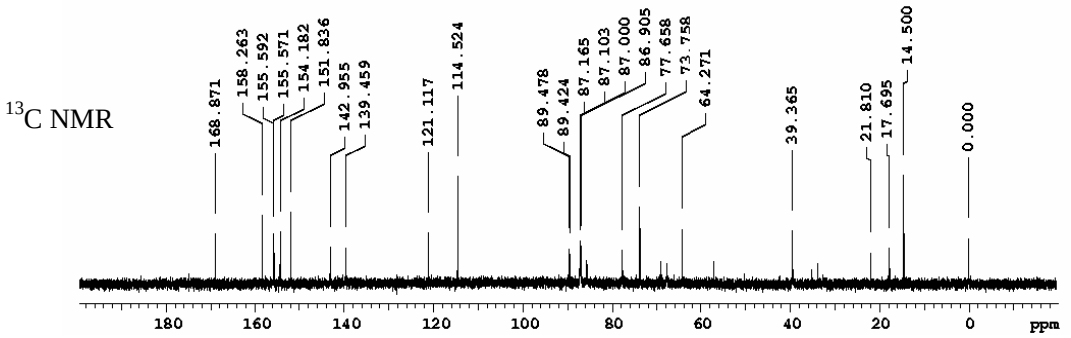


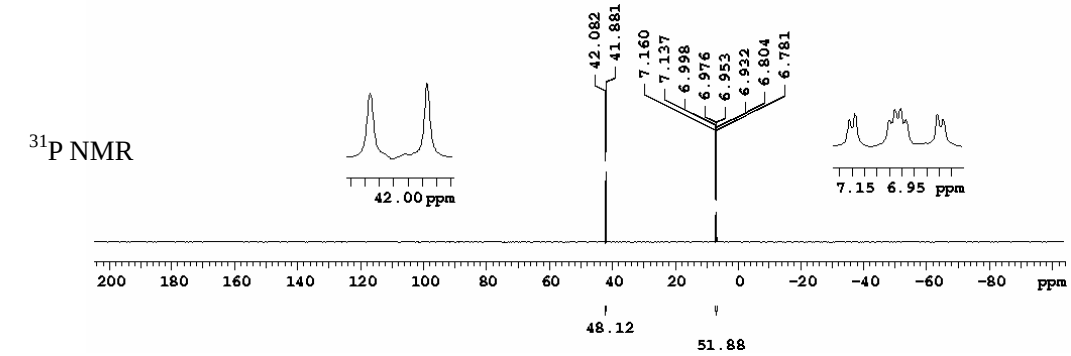


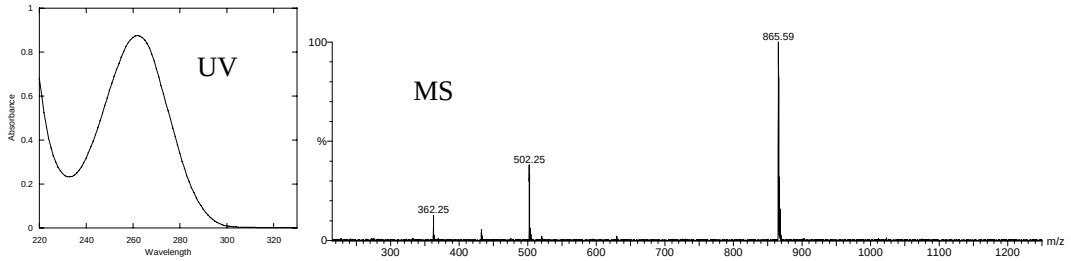
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [S_{P1},S_{P4}]-AZTp^{4}$_{S}$p^{3}$_{CH2}$p^{2}p^{1}$_{S}$A (37a)



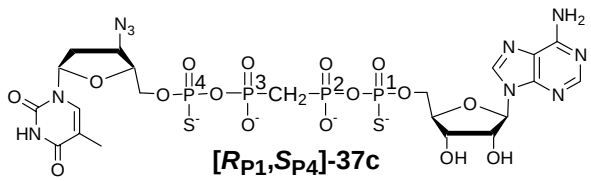


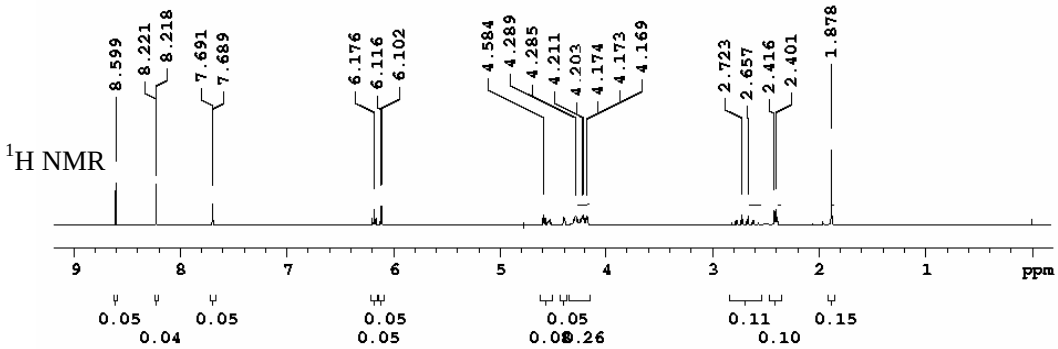


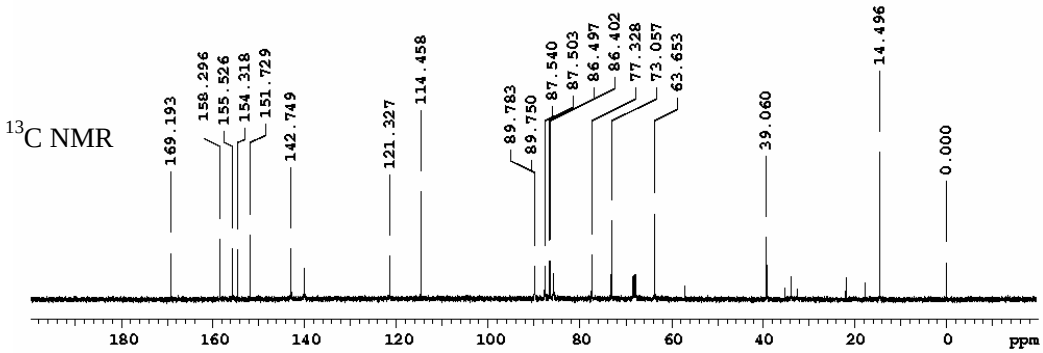


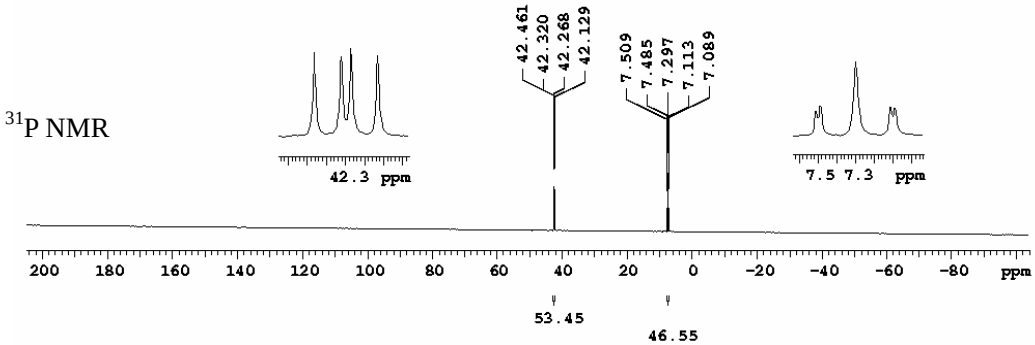


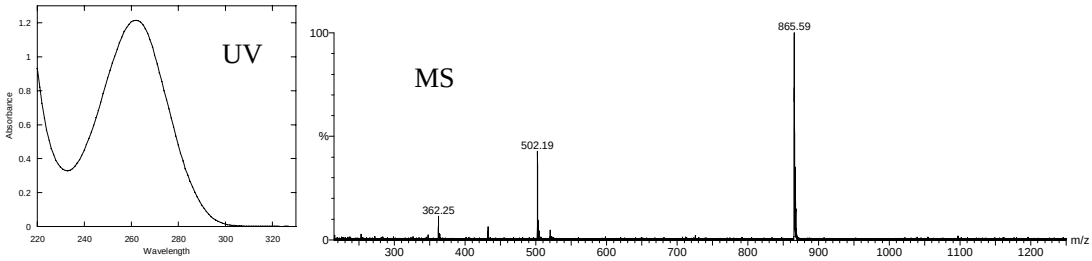
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [S_{P1},R_{P4}]-AZTp^4$_S$p^3$_{CH2}$p^{2}p^1$_S$A (37b)



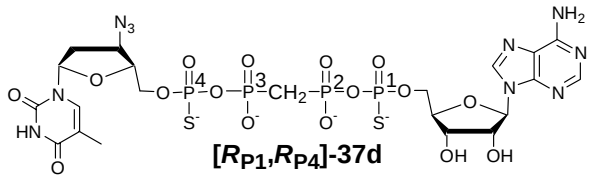


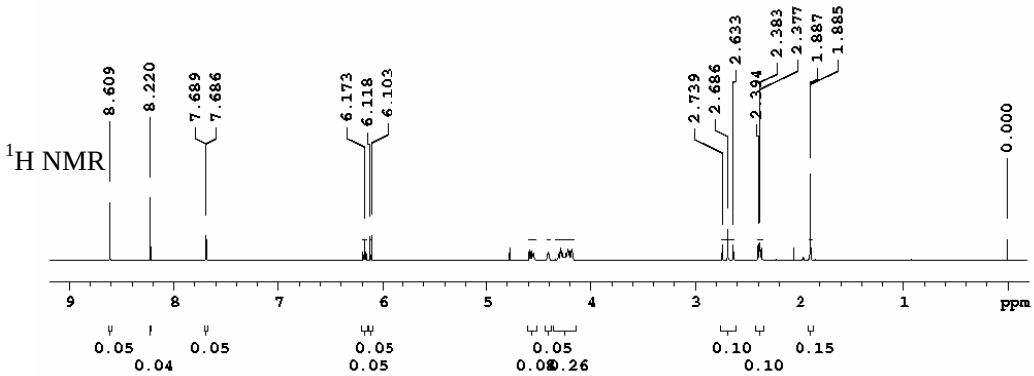


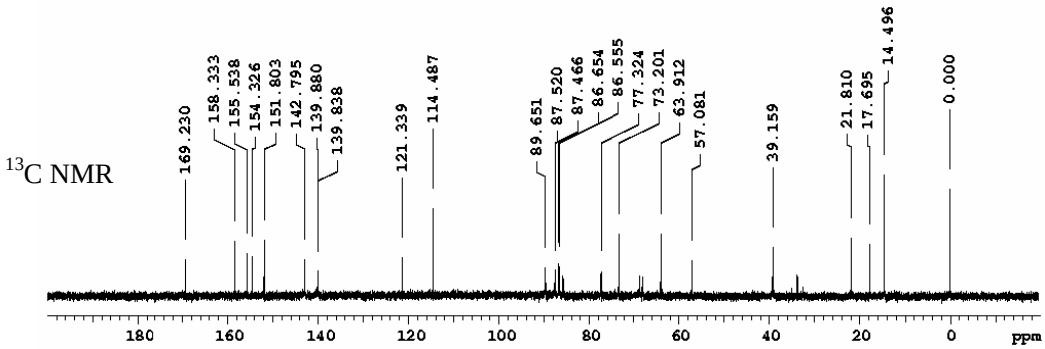


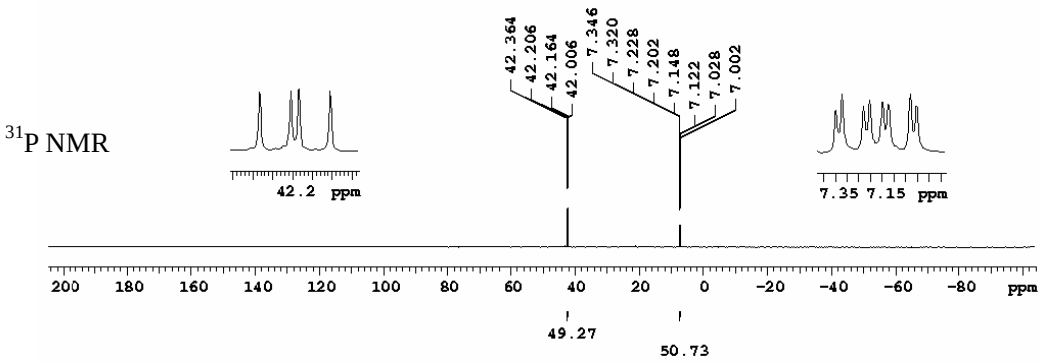


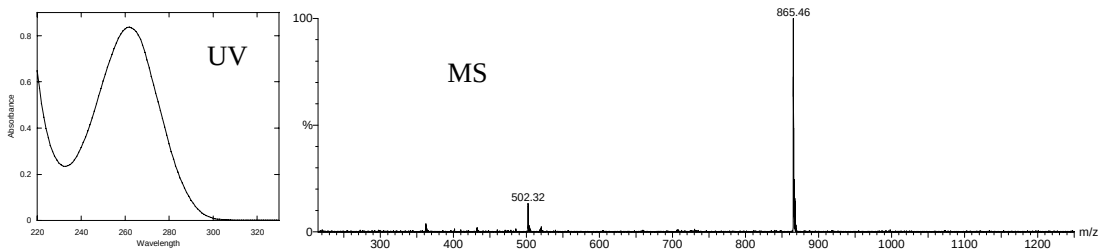
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [R_{P1},S_{P4}]-AZTp^{4}$_{S}$p^{3}$_{CH2}$p^{2}p^{1}$_{S}$A (37c)



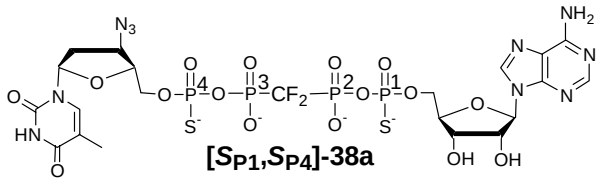


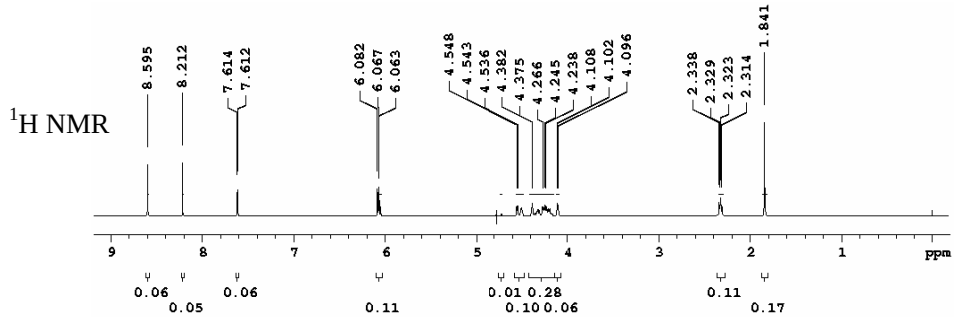


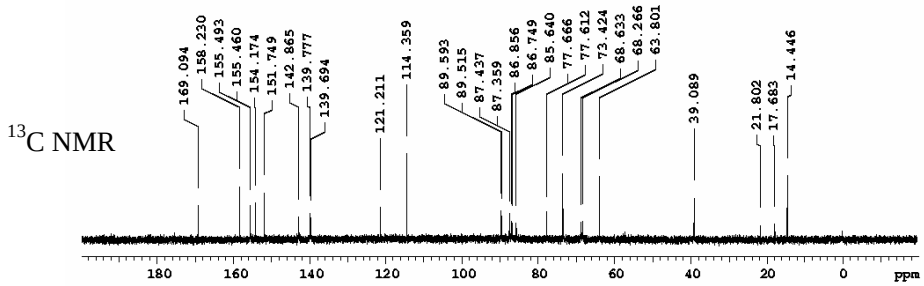


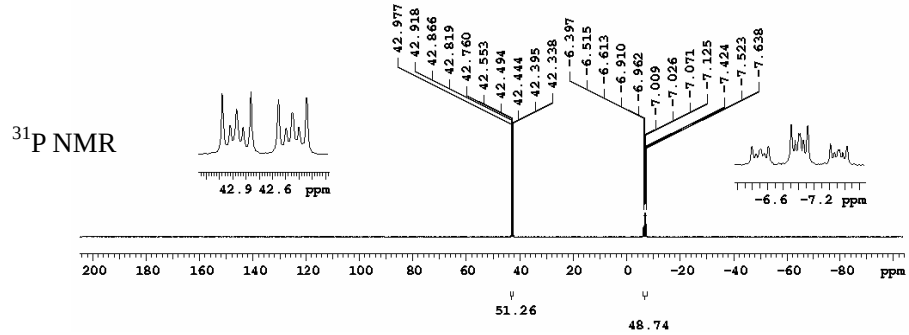


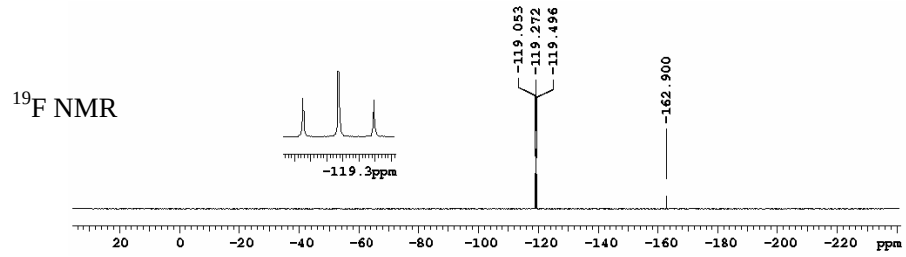
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [R_{P1},R_{P4}]-AZTp^4$_S$p^3$_{CH2}$p^{2}p^1$_S$A (37d)

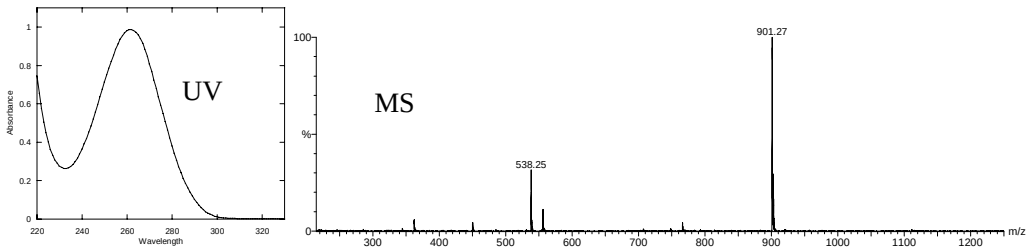




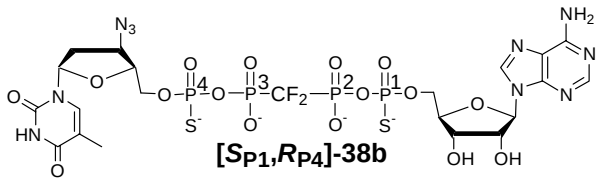






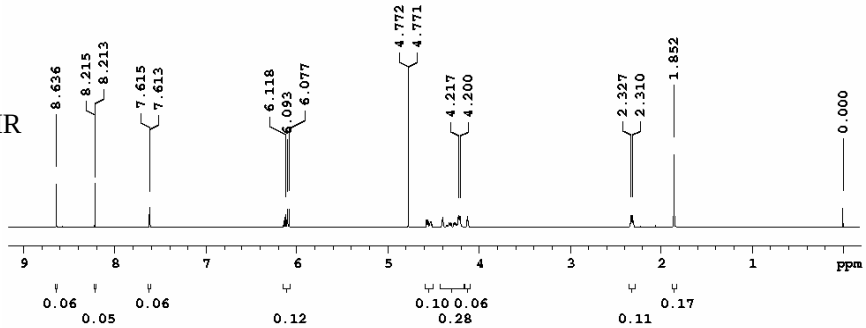


^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ^{19}F NMR, UV and Mass Spectra for [S_{P1},S_{P4}]-AZTp^{4}$_{S}$p^{3}$_{CF2}$p^{2}p^{1}$_{S}$A (38a)

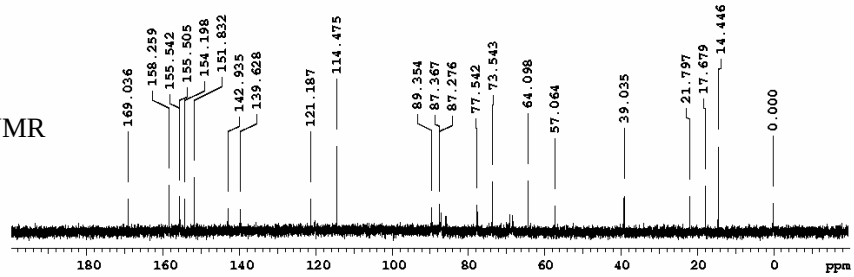


[S_{P1},R_{P4}]-38b

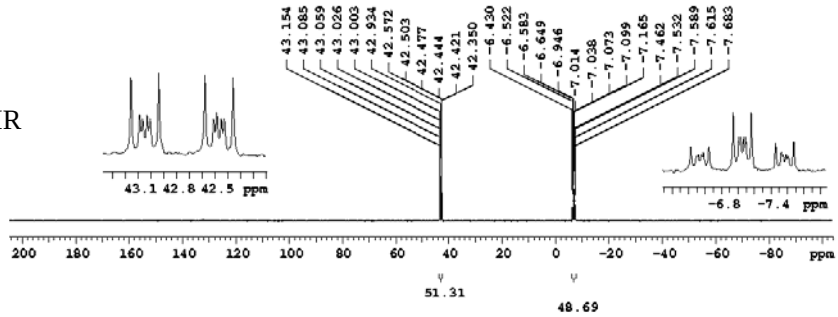
^{1}H NMR



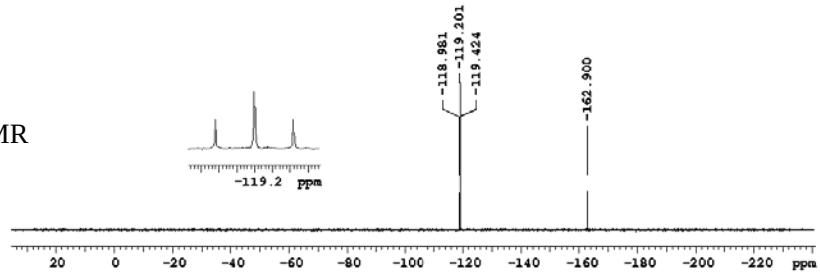
^{13}C NMR

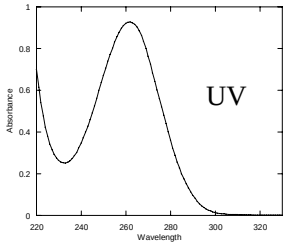


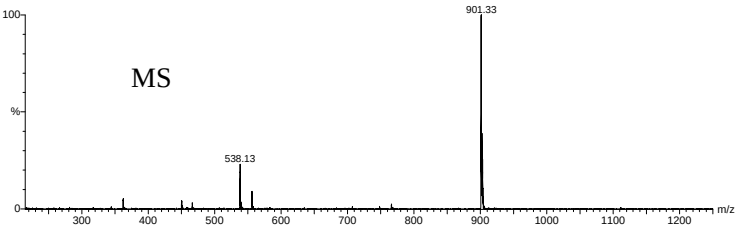
^{31}P NMR



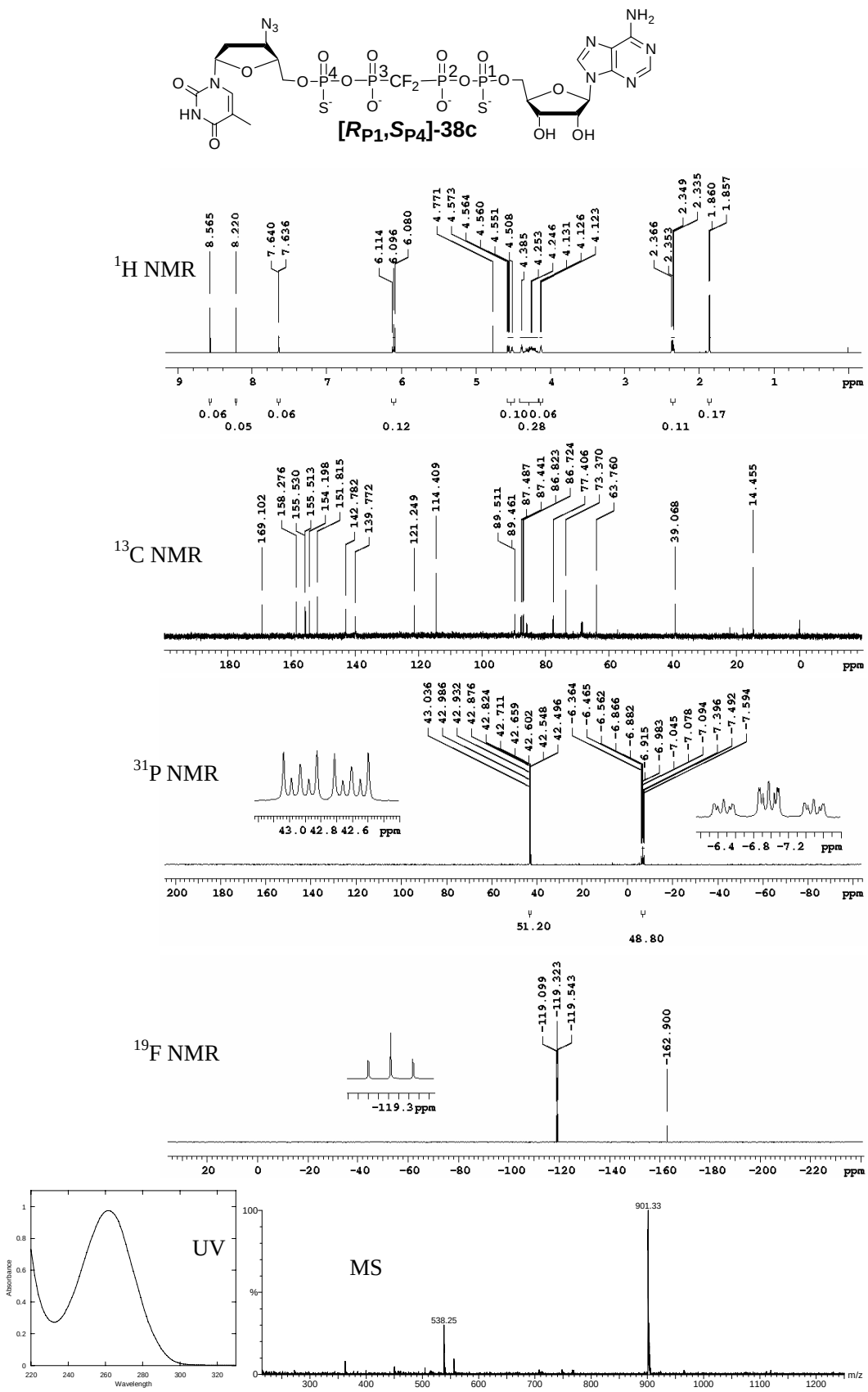
^{19}F NMR



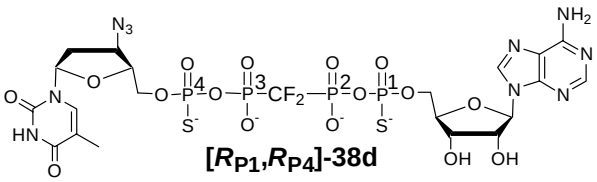




^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ^{19}F NMR, UV and Mass Spectra for [S_{P1},R_{P4}]-AZTp^{4}$_{S}$p^{3}$_{CF2}$p^{2}p^{1}$_{S}$A (38b)

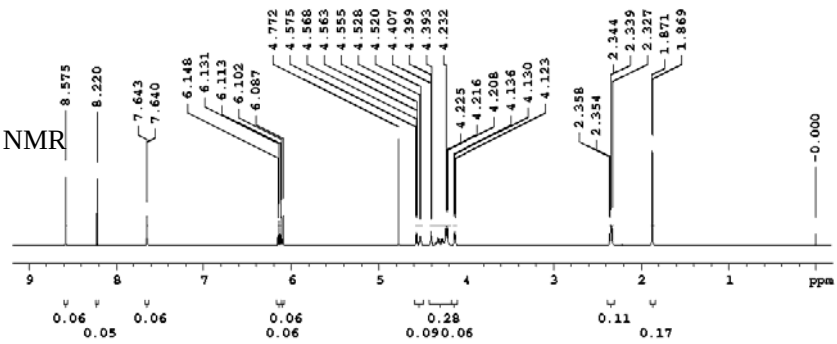


^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ^{19}F NMR, UV and Mass Spectra for [R_{P1},S_{P4}]-AZTp^{4}$_{S}$p^{3}$_{CF2}$p^{2}p^{1}$_{S}$A (38c)

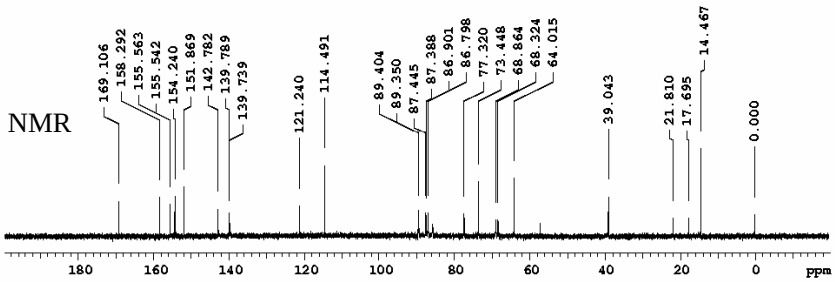


[R_{P1},R_{P4}]-38d

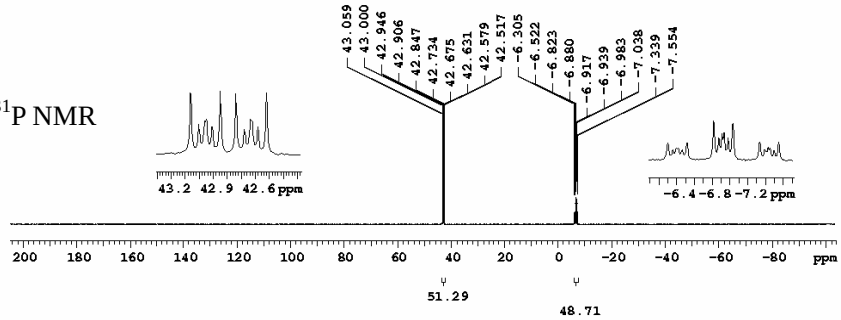
^{1}H NMR



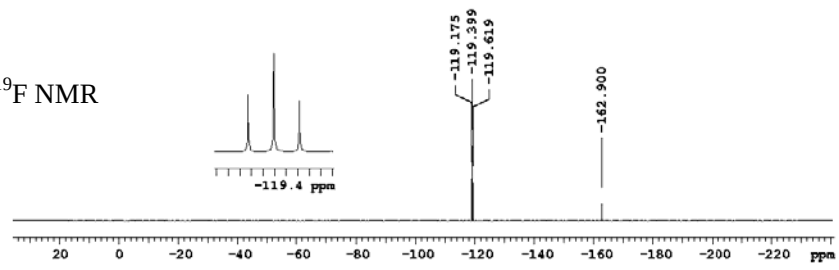
^{13}C NMR

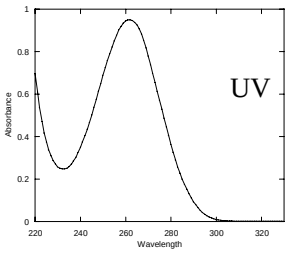


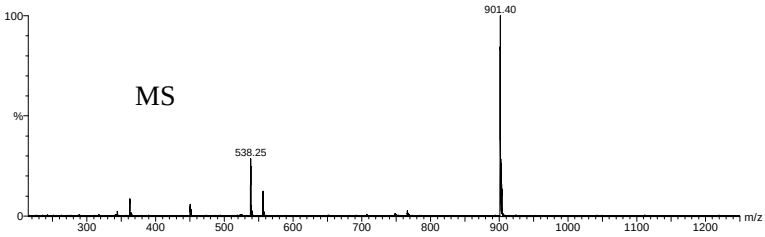
^{31}P NMR



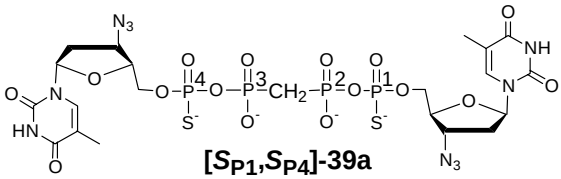
^{19}F NMR

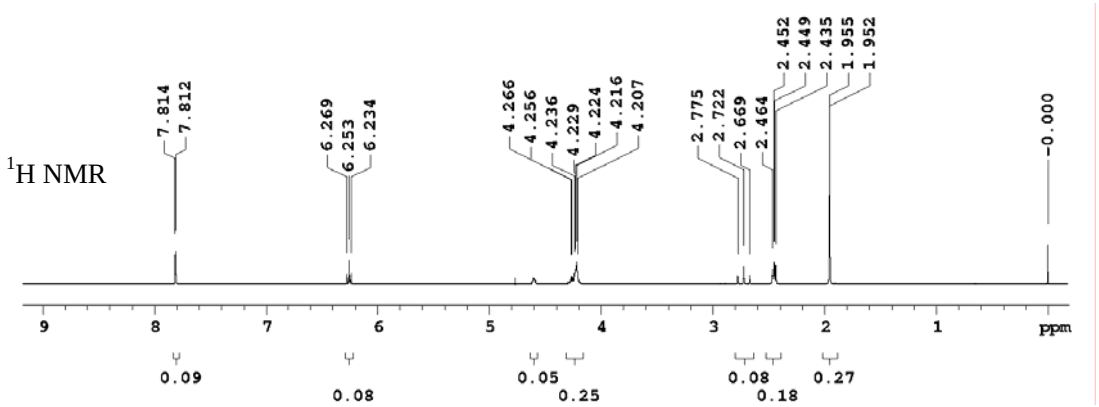


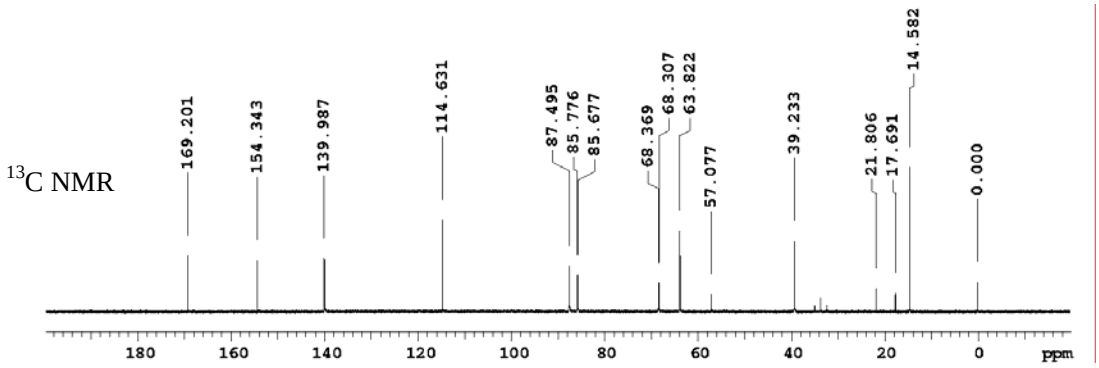


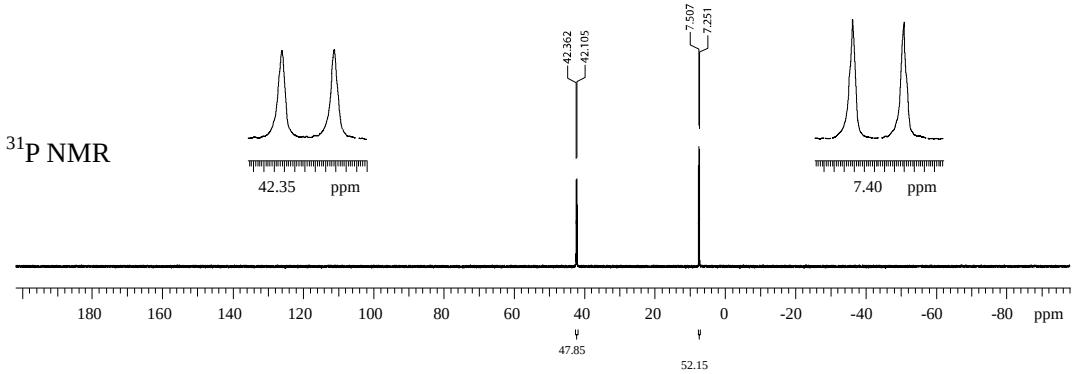


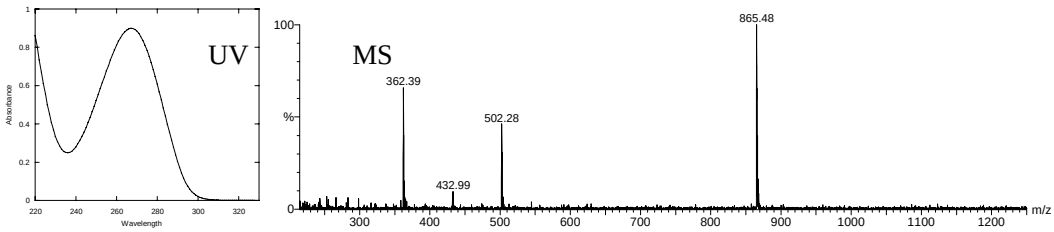
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ^{19}F NMR, UV and Mass Spectra for [R_{P1},R_{P4}]-AZTp^{4}$_{S}$p^{3}$_{CF2}$p^{2}p^{1}$_{S}$A (38d)



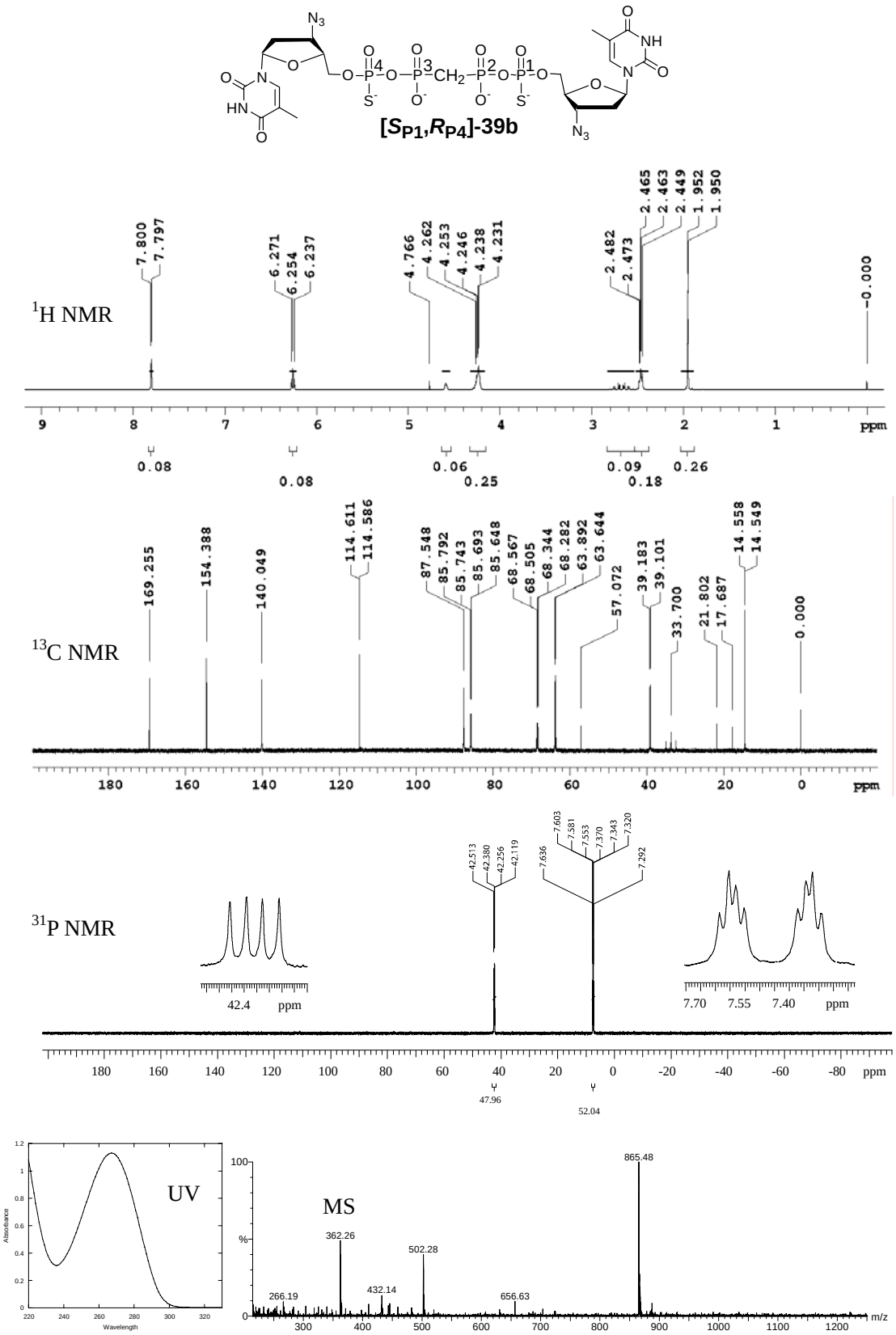




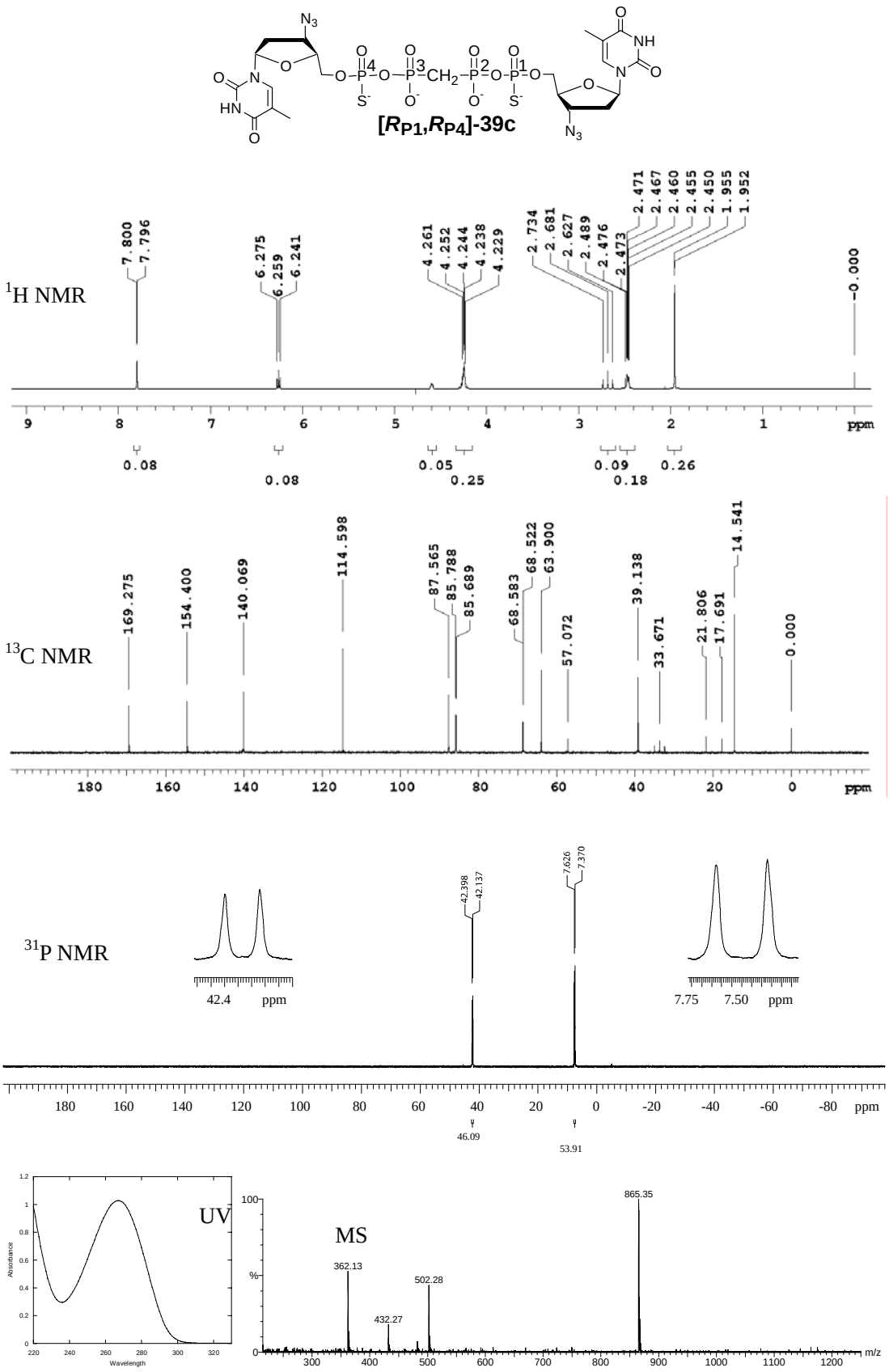




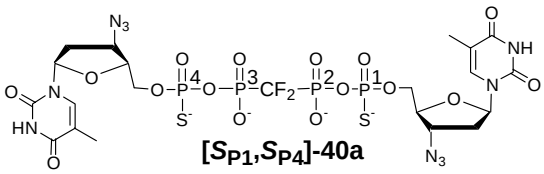
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [S_{P1},S_{P4}]-AZTp^4$_S$p^3$_{CH2}$p^{2}p^1$_S$AZT (39a)

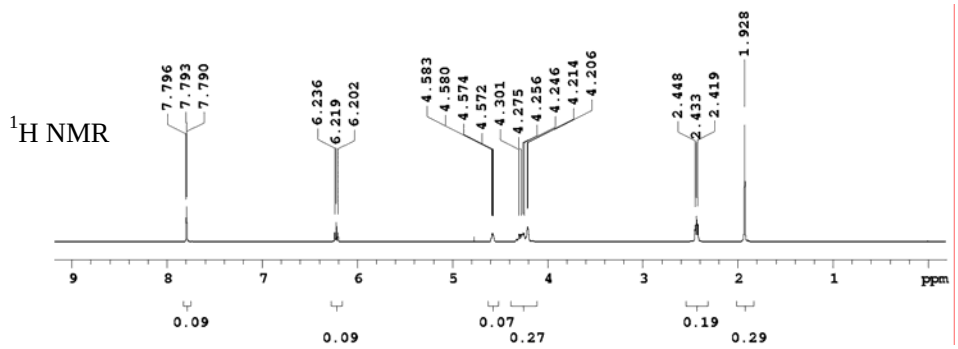


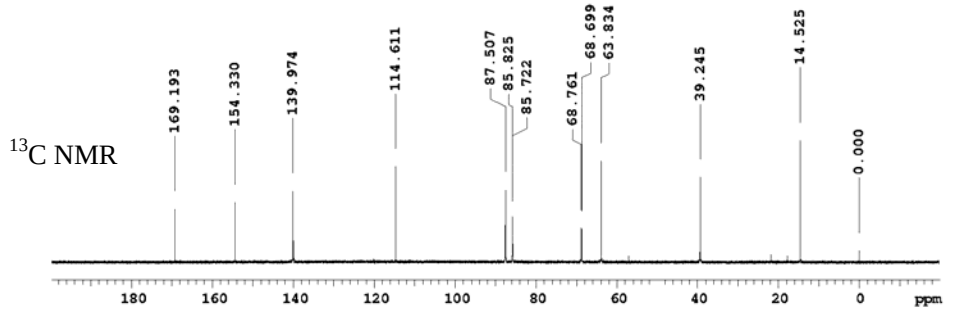
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [S_{P1},R_{P4}]-AZTp^4$_S$p^3$_{CH2}$p^{2}p^1$_S$AZT (39b)

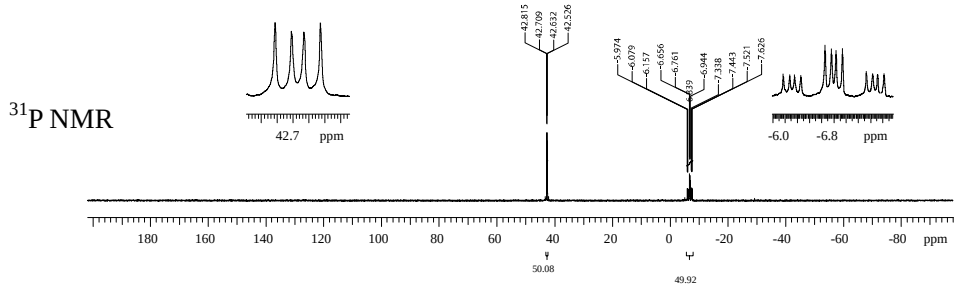


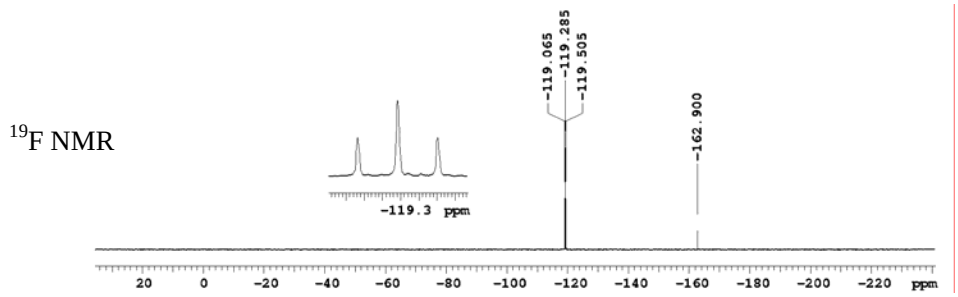
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for [R_{P1},R_{P4}]-AZTp$^{4}_{S}$p$^{3}_{CH2}$p^{2}p$^{1}_{S}$AZT (39c)

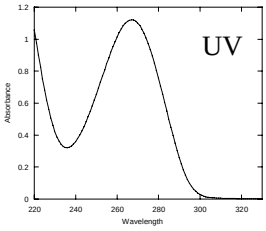


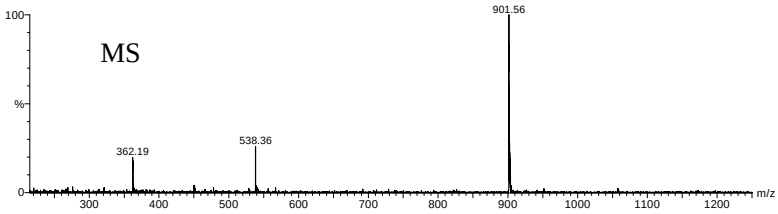




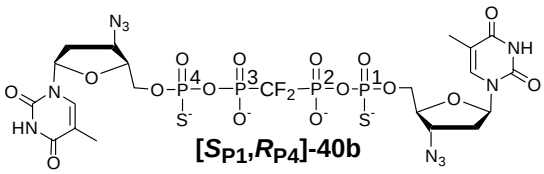


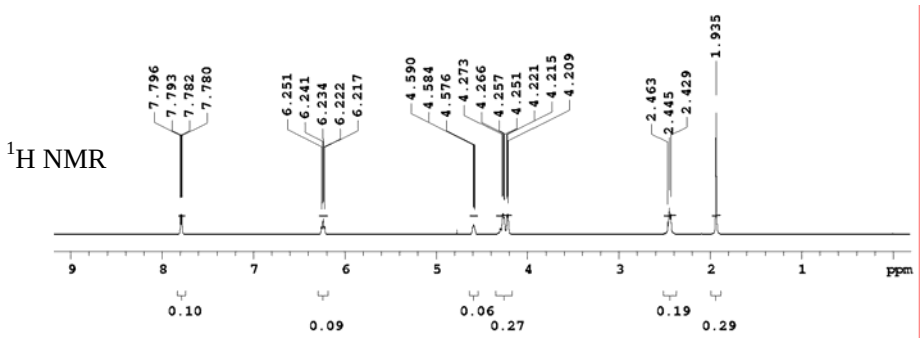


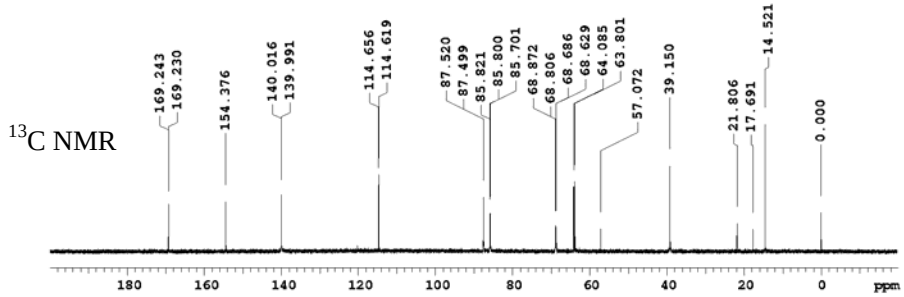


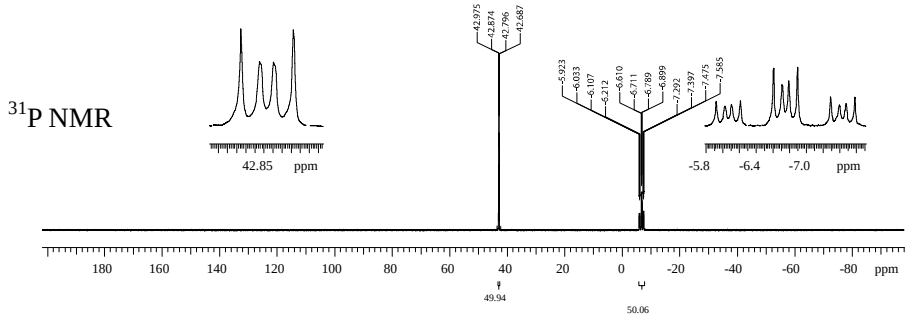


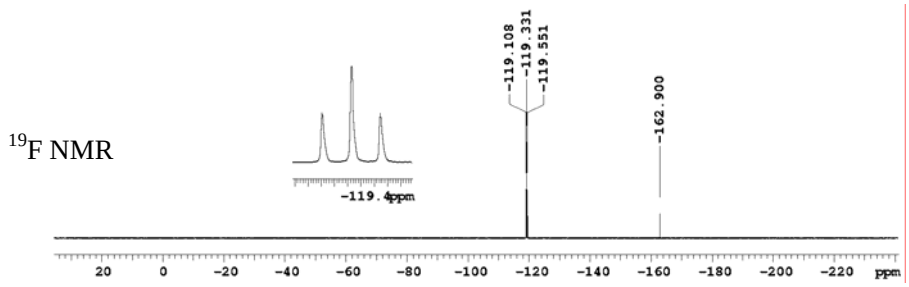
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ^{19}F NMR, UV and Mass Spectra for [S_{P1},S_{P4}]-AZTp^4$_S$p^3$_{CF2}$p^{2}p^1$_S$AZT (40a)

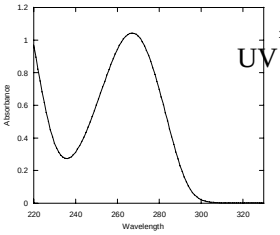


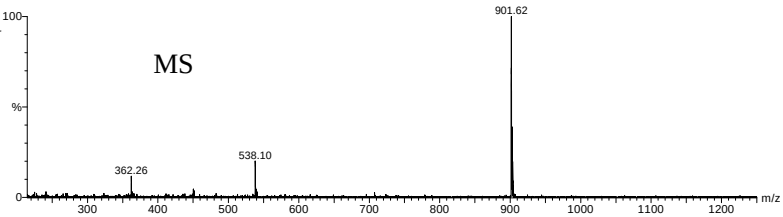




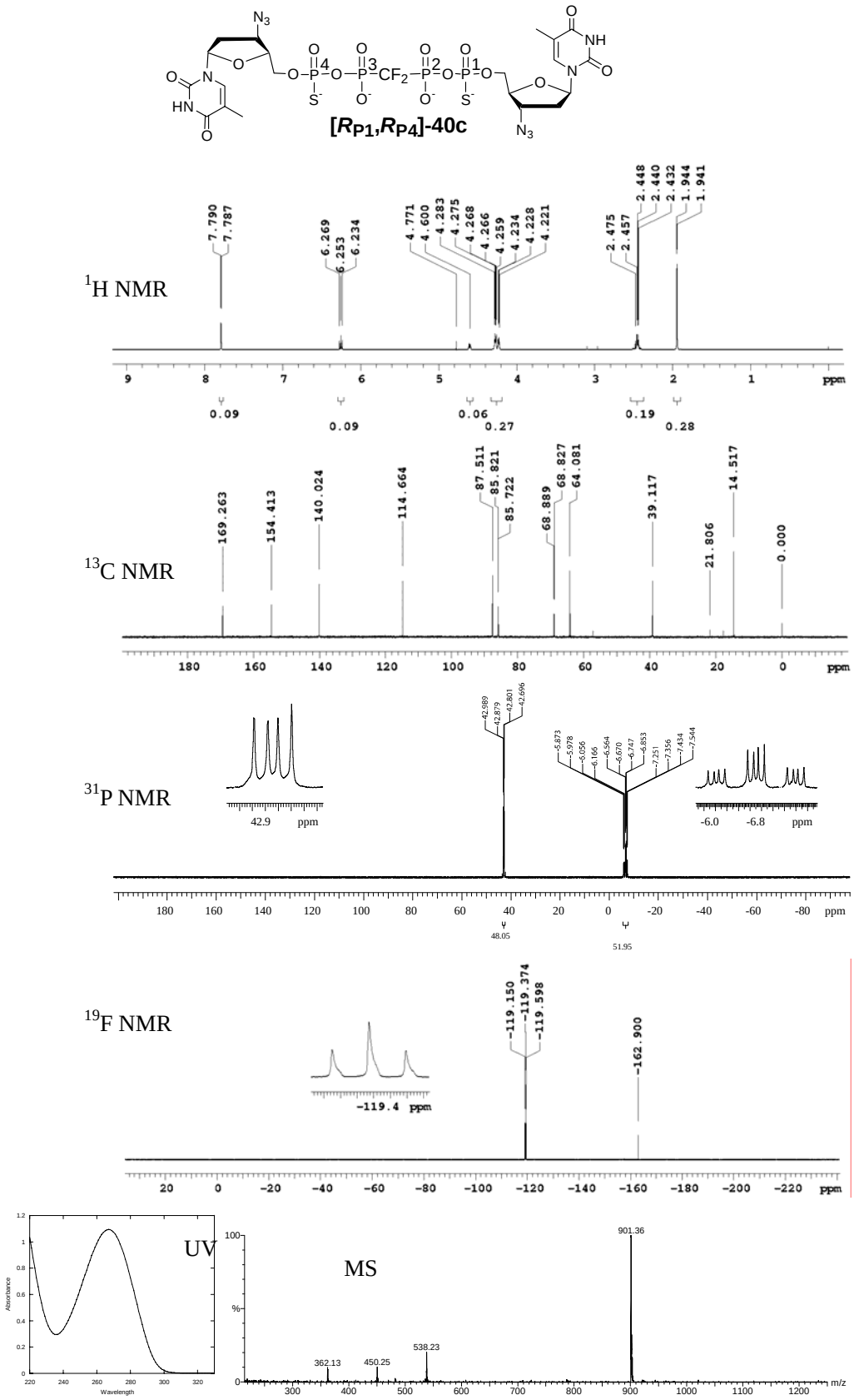




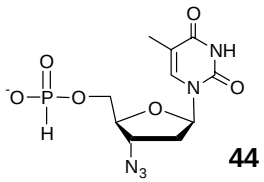




^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ^{19}F NMR, UV and Mass Spectra for [S_{P1},R_{P4}]-AZTp^4$_S$p^3$_{CF2}$p^{2}p^1$_S$AZT (40b)

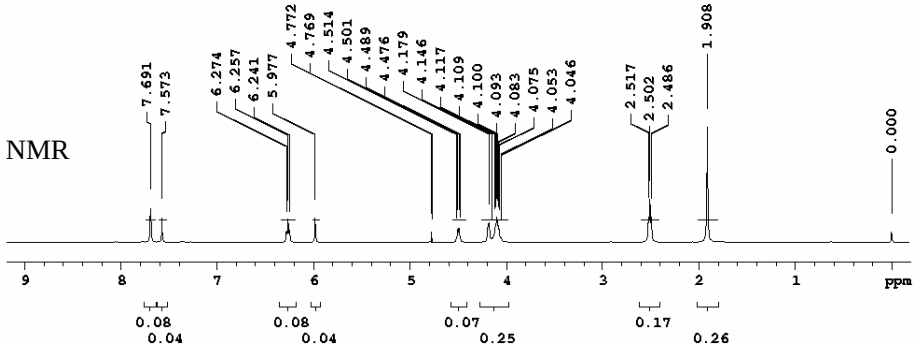


^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ^{19}F NMR, UV and Mass Spectra for [*R*$_{P1}$,*R*$_{P4}$]-AZTp^{4}$_{S}$p^{3}$_{CF2}$p^{2}p^{1}$_{S}$AZT (40c)

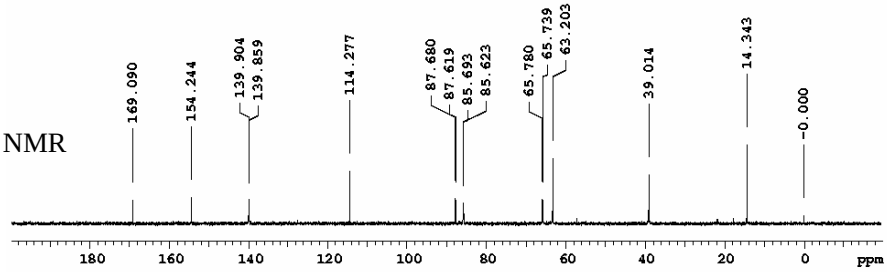


44

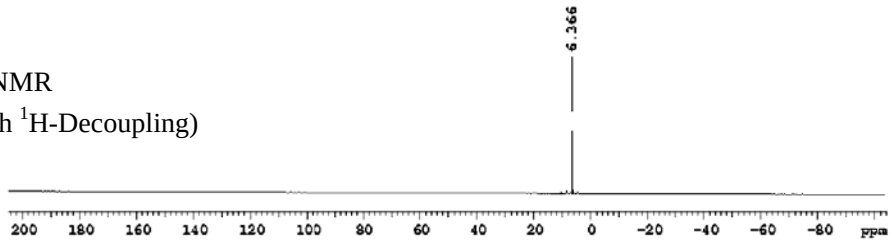
^{1}H NMR



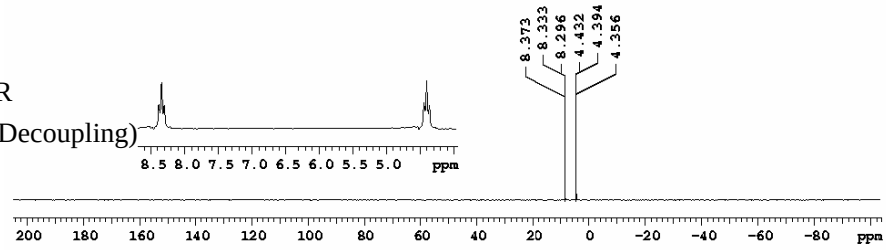
^{13}C NMR

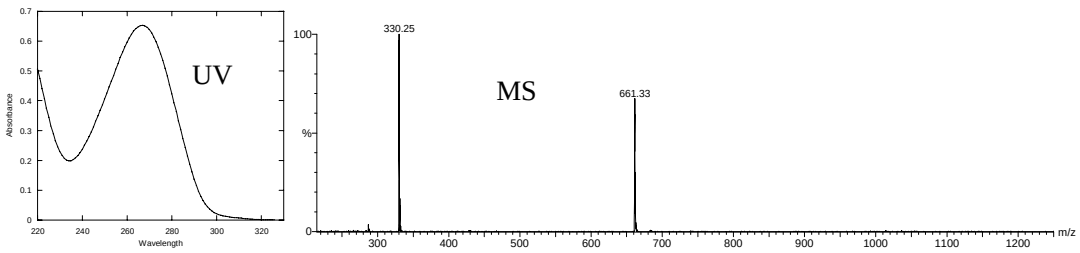


^{31}P NMR
(With ^{1}H-Decoupling)

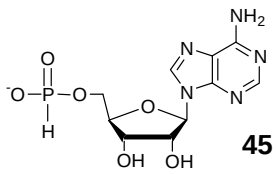


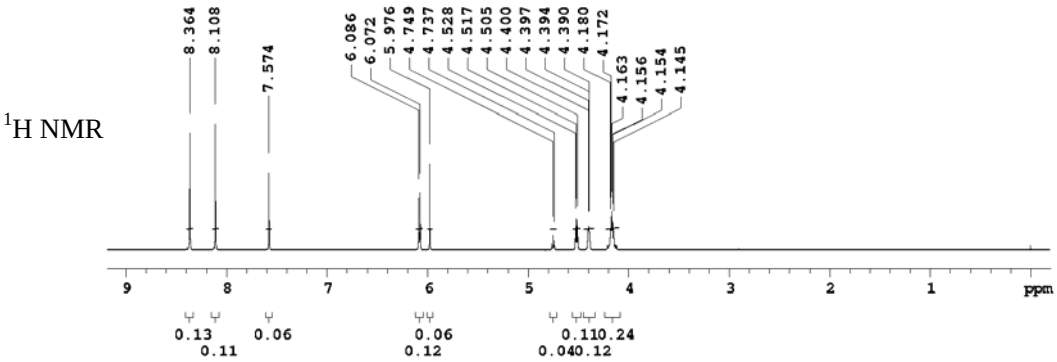
^{31}P NMR
(No ^{1}H-Decoupling)

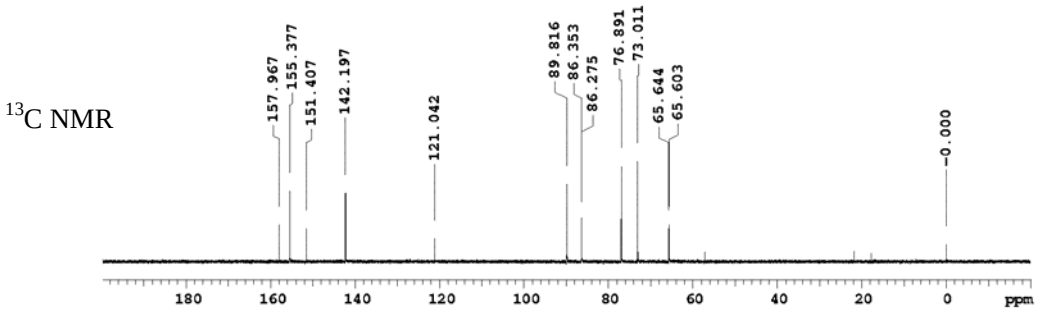


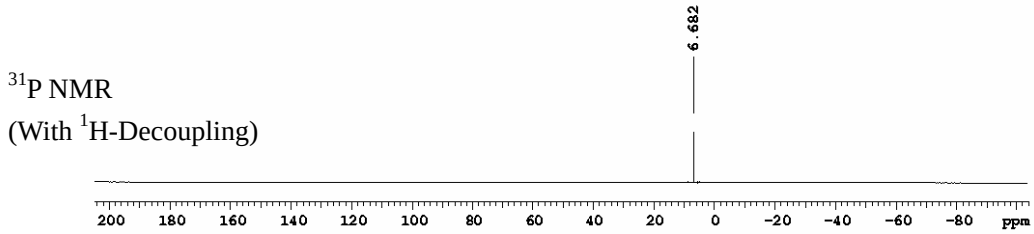


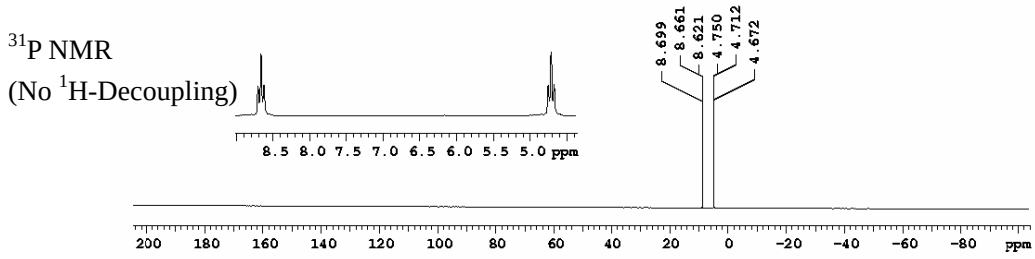
^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for AZT-5′-H-phosphonate (44)

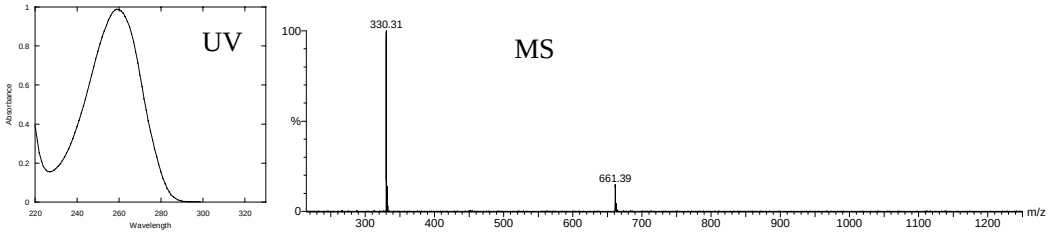












^{1}H NMR, ^{13}C NMR, ^{31}P NMR, UV and Mass Spectra for A-5′-H-phosphonate (45)

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Qianwei Han, Barbara L. Gaffney, and Roger A. Jones; "One-Flask Synthesis of Dinucleoside Tetra- and Pentaphosphates"; *Organic Letters*; **2006**; 8(10) pp 2075 – 2077.

Qianwei Han, Xiaoqing Zhu, Xubo Hu, Jinpei Cheng; "Synthesis of a Family of Ferrocene-bearing Cyclo-pseudo-peptides by Liquid Method and Molecular Recognition"; *Chemical Journal of Chinese Universities*; **2002**; 23(11) pp 2076 – 2079.