Synthesis of Nucleoside Polyphosphates and their Conjugates

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Nucleoside polyphosphates and their conjugates, such as nucleoside triphosphates, nucleoside tetraphosphates, sugar nucleotides, dinucleoside pyro- and higher order polyphosphates, 2',3'-cyclic nucleoside monophosphates, and 2´-deoxynucleoside-5´-tetraphosphates in which a fluorescent label is attached to the terminal phosphate have many biological roles and have been developed into drugs. However, their synthesis remains a challenge. Several novel and efficient approaches to the synthesis of nucleoside polyphosphates and their conjugates were developed. In the first approach dinucleoside polyphosphates (Np_nN's where n = 2-4) are prepared via in situ trifluoroacetate protection and imidazolium activation of nucleoside 5'-monophosphates. This methodology was also used to prepare a substrate-intermediate analog of the reaction catalyzed by cytidine triphosphate synthase (CTPS) a recognized target for the development of antineoplastic, antiviral and antiprotozoal agents. The second approach uses sulfonylimidazolium salts as key reagents for generating highly reactive nucleotide donors. The procedure is rapid, produces a wide variety of nucleoside polyphosphates and their conjugates in high yield, does not require protection and subsequent deprotection of the nucleotide donors or acceptors and can be used to activate nucleoside mono-, di-, and triphosphates and a wide variety of acceptors. Finally an entirely new approach to the synthesis of nucleoside tetraphosphates (Np_4's), dinucleoside pentaphosphates (Np_5N's) and nucleoside tetraphosphates in which a fluorescent dye is attached to the terminal phosphate is described employing an activated form of cyclic trimetaphosphate as a novel phosphorylating agent. Attempts to prepare nucleoside triphosphates by subjecting unprotected ribonucleosides and 2'-deoxyribonucleosides to activated cyclic trimetaphosphate failed. Instead nucleoside 2',3'-cyclic

phosphates were obtained in good yield with the ribonucleoside substrates. This represents a new and convenient approach to the synthesis of this class of compounds.

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Finally, I want to express my special acknowledgement to my wife Alaa who has been supporting me with love, encouragement and dedication.

This work is dedicated to my wife Alaa and my mother

Table of Contents

List of Figures

List of Tables

List of Abbreviations

3-DaU	3-Deazauridine 5’-triphosphate
7mGDP	7-methylguanosine 5’-diphosphate
A	Adenine
aaRSs	Aminoacyl-tRNA synthetases
ADP	Adenosine 5’-diphosphate
Alase	Amino ligase
AMP	Adenosine 5’-monophosphate
Ap_2A	Diadenosine-5’,5’-diphosphate
Ap_2G	P^1-Adenosine, P^2-guanosine-5’-diphosphate
Ap_2U	P^1-Adenosine, P^2-uridine-5’-diphosphate
Ap_3A	Diadenosine-5’,5’-triphosphate
Ap_3U	P^1-Adenosine, P^3-uridine-5’-triphosphate
Ap_4	Adenosine 5’-tetraphosphate
Ap_4A	Diadenosine-5’,5’-tetraphosphate
Ap_4T	P^1-Adenosine, P^4-thymidine-5’-tetraphosphate
Ap_5	Adenosine 5’-pentaphospahate
Ap_6A	Diadenosine-5’,5’-hexaphosphate
ATP	Adenosine 5’-triphosphate
AZT	Azidothymidine
Bu	Butyl
C	Cytosine
CDI	Carbonyldiimidazole
CMP	Cytidine 5’-monophosphate
CPEC	Cyclopentenyl cytosine
CTP	Cytidine 5’-triphosphate
CTPS	Cytidine triphosphate synthase
DABCO	1,4-diazabicyclo[2.2.2]octane
dAp_4G	P^1-2’-deoxy-Adenosine, P^4-guanosine-5’-tetraphosphate

DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	Dicyclohexylcarbodiimide
DDAO	1,3-Dichloro-7-hydroxy-9,9-dimethylacridin-2(9*H*)-one
DIPEA	Diisopropylethylamine
DMA	*N*,*N*-dimethylaniline
DMAP	4-*N*,*N*-dimethylaminopyridine
DMBA	3,5-Dimethoxybenzyl alcohol
DMF	Dimethylformamide
DMI	1,2-Dimethylimidazole
DMSO	Dimethylsulfoxide
Deoxyribonucleic acid	Deoxyribonucleic acid
dNDP	2'-Deoxynucleoside 5'-diphosphate
dNMP	2'-Deoxynucleoside 5'-monophosphate
dNTP	2'-Deoxynucleoside-5'-triphosphate
dTDP	2'-Deoxythymidine 5'-diphosphate
dTp_5A	P^1-2-Deoxythymidine, P-adenosine-5'-pentaphosphate
dTTP-β-L-rhamnose	2'-Deoxythymidinediphosphate-β-L-rhamnose
EcCTPS	*Escherichia coli* cytidine triphosphate synthetase
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDT	Ethanedithiol
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
G	Guanine
GATase	Glutamine amidotransferase
GlfT2	galactofuranosyl transferase
GMP	Guanosine 5'-monphosphate
Gp_3G	Diguanosine-5',5'-triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMC	7-Hydroxy-4-methylcoumarin
HMPA	Hexamethylphosphoramide

HPLC	High performance liquid chromatography
ImpA	Adenosine 5’-phosphoimidazolidate
ImpN	Nucleoside 5’-phosphoimidazolidate
Impp7mG	7-methylguanosine 5’-diphosphoimidazolidate
ImppA	Adenosine 5’-diphosphoimidazolidate
ImppN	Nucleoside 5’-diphosphoimidazolidate
Imp**U**	Uridine 5’-phosphoimidazolidate
Ip_2I	Diinosine-5’,5’-diphosphate
iP_3	Inorganic triphosphate
iP_4	Inorganic tetraphosphate
iPP	Inorganic pyrophosphate
KDa	Kilodalton
L-Up_4U	L-Diuridine-5’,5’-tetraphosphate
MPD	1-Methylpyrrolidone
NAD	Nicotinamide adenine dinucleotide
NDP	Nucleoside 5’-diphosphate
NMI	*N*-methylimidazole
NMP	Nucleoside-5’-monophosphate
Np_2N	Dinucleoside-5’,5’-diphosphates
Np_3N	Dinucleoside-5’,5’-triphosphates
Np_4	Nucleoside-5’-tetraphosphate
Np_5N	Dinucleoside-5’,5’-pentaphosphate
Np_nN	Dinucleoside-5’,5’-polyphosphate
NTP	Nucleoside 5’-triphosphates
Np_4	Nucleoside 5’-tetraphosphate
Ph	Phenyl
PPi	Inorganic pyrophosphate
RNA	Ribonucleic acid
RP-HPLC	Reversed-phase high performance liquid chromatography
T	Thymine
TBAA	Tributylammonium acetate

TBAH	Tetrabutylammonium hydroxide
^{t}Bu	*tert*-Butyl
TCE	Trichloroethyl
TEA	Triethylamine
TEAA	Triethylammonium acetate
TFA	Trifluoroacetic acid
TFAA	Trifluoroacetic anhydride
TFE	Trifluoroethyl
TMP	Thymidine-5'-monphosphate
TMS	Tetramethylsilane
Tp_2T	Dithymidine-5',5'-diphosphates
TriMP	Trimetaphosphate
U	Uracil
UDP	Uridine 5'-diphosphate
UMP	Uridine 5'-monophosphate
Up_2T	P^1-Uridine, P^2-thymidine-5'-diphosphate
Up_2U	Diuridine 5',5'-diphosphate
Up_4A	P^1-Uridine, P^4-adenosine-5'-tetraphosphate
Up_4U	Diuridine 5',5'-tetraphosphate
UTP	Uridine 5'-triphosphate
UTP-4-P	UTP-4-phosphate

Chapter 1

Thesis Overview

1.1 Nucleoside polyphosphates and their conjugates

Nucleoside polyphosphates (p_nN's, where n = 2-5) and their conjugates (Xp_nN, where n = 2-7 and X = nucleoside or other chemical entity) are ubiquitous in nature. This class of compounds includes (amongst many others) such well-known chemical entities as nucleoside triphosphates (NTP's), sugar nucleotides (sugar-p_nN), and dinucleoside pyro- and higher order polyphosphates (Np_nN, n = 2-6). Their biological roles are numerous. For example, NTP's are the precursors to the building blocks of DNA and RNA, they initiate signaling pathways (ie. GTP), and ATP (Figure 1.1) is a key energy source in many living systems. Sugar nucleotides act as glycosyl donors in the enzymatic synthesis of polysaccharides and some sugar nucleotides, such as 2'-deoxythymidinediphosphate-β-L-rhamnose (dTTP-β-L-rhamnose, Figure 1.1), are essential for the synthesis of bacterial cell walls. Dinucleoside pyrophosphates (Np_2N's) such as flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD) (Figure 1.1) function as cofactors in enzyme-catalyzed reactions. Higher order dinucleoside polyphosphates (Np_nN's where n = 3-7) such as Ap_nA's (Figure 1.1) act as signaling molecules and have vasodilatory effects. In addition to their natural biological roles, nucleoside polyphosphates and their conjugates have been widely used as inhibitors and probes of therapeutically significant enzymes. A dinucleoside tetraphosphate, Up_4U (Figure 1.1) has recently been approved as a drug for treating dry eye syndrome.[1,2] Very recently, novel and rapid methods for sequencing DNA have been reported using nucleoside tetraphosphates δ-labeled with fluorogenic dyes (Figure 1.1).[3-7]

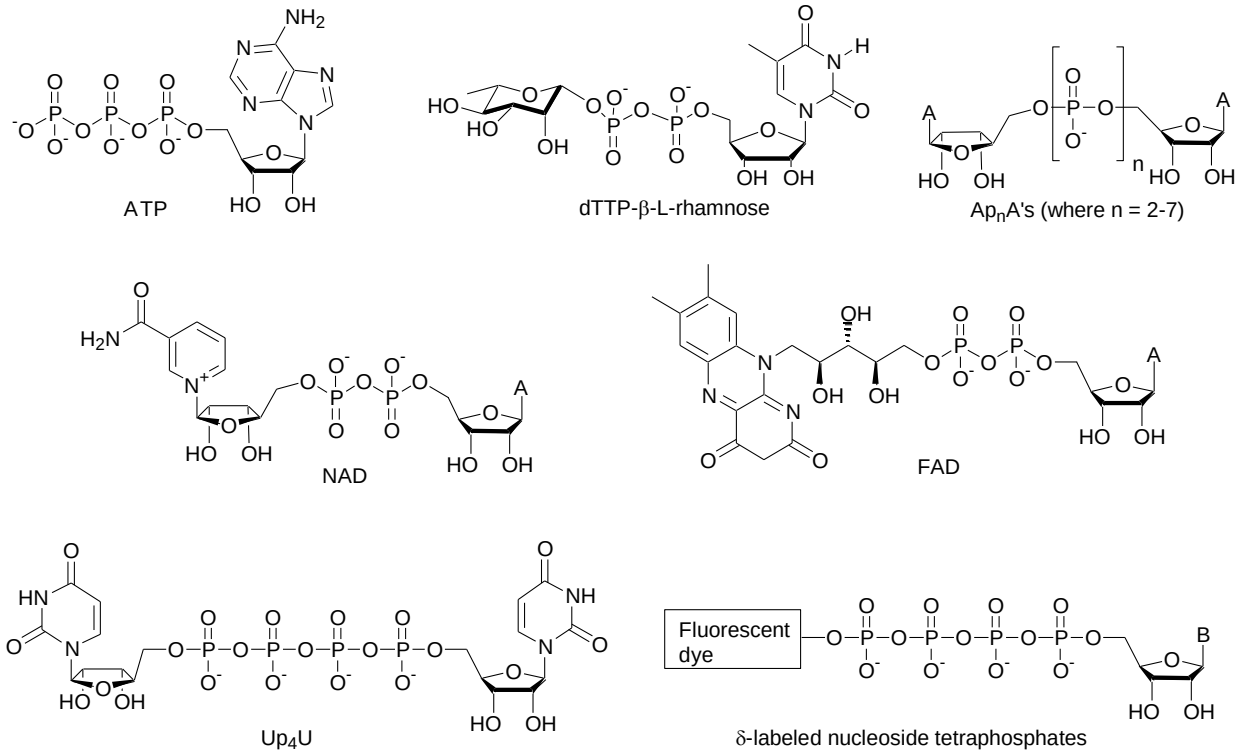


Figure 1.1. Examples of nucleoside polyphosphates and their conjugates.

1.2 Challenges in synthesising nucleoside polyphosphates and their conjugates

In 1957, Sir Alexander Todd was awarded the Nobel prize for his pioneering work on the structure and synthesis of nucleotides, nucleosides, and nucleotide coenzymes. In his Nobel prize speech he made the following statement: "we are thus still seeking an ideal method for unsymmetrical pyrophosphate synthesis". Although he was referring to the challenges of constructing a pyrophosphate linkage between two different nucleotide 5'-monophosphates, his statement would have applied equally well to the synthesis of almost any polyphosphorylated nucleoside or conjugate with the challenge being the introduction of the polyphosphate moiety or the coupling together of two phosphate groups. Todd did not define what he meant by an "ideal method". Nevertheless, we can suggest what an ideal method should be for the polyphosphorylation of a nucleoside or for the coupling of two phosphates to give a nucleoside polyphosphate conjugate. First and foremost, it should permit the synthesis of any nucleoside

polyphosphate or nucleoside polyphosphate conjugate in good yield. A good yield implies that the reaction must be clean (form as few byproducts as possible) which should aid in the purification. Purification is a key issue in the synthesis of these types of compounds. The products are highly polar and usually insoluble in organic solvents. This means conventional normal-phase silica gel flash chromatography that is widely used to purify organic compounds is not an option here. Reversed-phase or ion exchange media are usually required to purify these compounds. Moreover, the substrates and impurities are usually phosphorylated or polyphosphorylated and so can be difficult to remove from the desired product unless preparative high pressure liquid chromatography (HPLC) is used and, even with HPLC, purification can still be challenging. In order to obtain good yields the process should also be regio- and chemoselective. The use of protecting groups in the nucleoside, nucleotide and other types of substrates should be kept to a minimum. This is not just for reasons of atom economy and synthetic efficiency. If protecting groups are present after the polyphosphorylation/coupling event then they must be removed to get the final product. This can potentially reduce the overall yield as polyphosphate groups in polyphosphorylated nucleosides are often unstable to extremes of pH and, to some extent, nucleophiles, which can make the removal of protecting groups problematic. Finally, it would be convenient if the polyphosphorylation reactions could be conducted in water. Most of the substrates that are encountered during nucleoside polyphosphate syntheses are highly polar because they often contain one or more phosphate groups. However, it is usually not practical to perform the reactions in water since at least one of the substrates has to be converted into a highly reactive form in order to get the reactions to proceed at a reasonable rate (if at all). These activated forms of the substrates are usually unstable in water and so the reactions must be conducted in strictly anhydrous polar, aprotic organic solvents such as dry

DMF or acetonitrile. However, in order for the substrates to be soluble in organic solvents their phosphate moieties first must be converted into the corresponding alkyl ammonium salts (e.g. tetrabutylammonium salts) and be kept absolutely dry. It is time consuming to prepare these salts (they are usually not commercially available) and dry the organic solvents or it is expensive to purchase such solvents. Hence it would be very convenient if the reaction could be done in aqueous solution. This is a particularly difficult issue to overcome since it requires the development of activated substrates that are water stable.

Due to their importance in many biological processes the synthesis of nucleoside polyphosphates and their conjugates has been the subject of intensive research for over 60 years; however, although it has been 56 years since Lord Todd's Nobel prize, an ideal method for introducing polyphosphates into nucleosides has yet to be realized. This is hardly surprising as the chemical properties of nucleosides, nucleoside analogs and the compounds to which they may be conjugated are very diverse. It is possible that an ideal polyphosphorylation method may never be realized. Indeed, for many polyphosphorylation reactions it is still challenging just to meet the first criterion mentioned above which is to obtain the products in good yield: poor to moderate yields are often still the norm for many polyphosphorylation reactions.

1.3 Global Objective

The global objective of this thesis was to develop new and improved methods for preparing nucleoside polyphosphates and their conjugates. We do not claim to have developed an ideal method. Our concern was mainly with the issues of yield, purity (or purification), regio- and chemoselectivity. The issue of performing the reactions in aqueous solution was not tackled here. Nevertheless, we believe that the work presented in this thesis represents a significant step forward in the development of polyphosphorylation methodology.

1.4 Scope of the Thesis.

Chapter 2 focuses on the synthesis of dinucleoside polyphosphates (Np_nN, where n = 2-4). We describe a new approach to the synthesis of these compounds which is based upon methodology developed by Bogachev for the synthesis of nucleoside 5'-triphosphates.[8] We used this methodology to prepare a unique dinucleoside polyphosphate that is designed to inhibit the enzyme cytidine triphosphate synthase (CTPS) by acting as substrate-intermediate analog of the CTPS-catalyzed reaction. As CTPS is a recognized target for the development of antineoplastic, antiviral and antiprotozoal agents it is anticipated that these studies may prove to be useful in developing compounds that can be used as leads for drug development.

Chapter 3 describes a powerful new approach to synthesising a wide variety of nucleoside polyphosphates and their conjugates. Key to this methodology is the use of a class of compounds called sulfonyl imidazolium salts as activating agents. Using these reagents, it is possible to prepare nucleoside polyphosphates and their conjugates such as symmetrical and unsymmetrical dinucleoside polyphosphate, sugar nucleotides and nucleotide triphosphates in almost unprecedented yields.

In chapter 4 we describe an entirely new approach to the synthesis of nucleoside tetra-(Np_4's) and dinucleoside pentaphosphates (Np_5N's). This methodology employs an activated form of cyclic trimetaphosphate as a novel phosphorylating agent. We then show that this methodology can be used to prepare nucleoside tetraphosphates in which the terminal phosphate is labelled with a fluorescent dye. As mentioned in section 1.1, such compounds are important as they are currently being used by others as reagents for the high throughput sequencing of DNA. The potential applications of this methodology goes beyond the synthesis of just nucleoside tetra- and pentaphosphates and their conjugates as it also suggests a direct approach

to the synthesis of nucleoside triphosphates directly from nonphosphorylated nucleoside precursors. Although attempts to prepare nucleoside triphosphates by subjecting unprotected ribonucleosides and 2'-deoxyribonucleosides to activated cyclic trimetaphosphate failed, nucleoside 2',3'-cyclic phosphates were obtained in good yield with the ribonucleoside substrates. This represents a new and convenient approach to the synthesis of this class of compounds.

A brief word about abbreviations. Whenever we refer to nucleoside mono- (NMP), di- (NDP) or triphosphates (NTP) we are referring to nucleotides in which the phosphate group is attached to the 5'-OH of the nucleoside unless stated otherwise. For example, adenosine 5'-mono-, di- and tri phosphate are abbreviated AMP, ADP and ATP respectively. 2'-deoxynucleoside 5'-mono-, di-, or triphosphates are abbreviated dNMP, dNDP or dNTP respectively.

Chapter 2.

Synthesis of Dinucleoside Polyphosphates via in situ Trifluoroacetate Protection and Imidazolium Activation

2.1 Introduction

2.1.1 Biological and pharmaceutical significance of dinucleoside polyphosphates.

Dinucleoside 5',5'-polyphosphates (Figure 2.1), commonly abbreviated as Np_nN, comprise a group of compounds with two nucleosides linked by their 5'-OH's to a variable number of phosphate groups. They have been shown to play key roles in various biological processes. The most well-studied and biologically significant Np_nN's are the diadenosine polyphosphates (Ap_nA, n = 2-7). Ap_nA's are released into the circulation from several cell types, including platelets, chromaffin cells of the adrenal glands, tubular cells, or from synaptic vesicles. Ap_nA's are important neurotransmitters in the nervous system.[9] Ap_2A, Ap_3A, and Ap_4A have vasodilatory effects.[10,11] Ap_nA's (n = 2-5), as well as Ap_2G and Gp_nG (n = 2-6), cause vascular smooth muscle cell proliferation.[12-14] Ap_nA's are potent antagonists of ADP-induced platelet aggregation, where Ap_5A is the most potent of this series.[15] The actions of Ap_nA's can be attributed to the interaction of these compounds with ATP receptors. These receptors, called the P2 receptor superfamily, are divided into two classes, P2X and P2Y, each having multiple receptor subtypes. The P2X subfamily, composed of ligand-gated cationic channels has seven subtypes, ($P2X_1$-$P2X_7$). The P2Y subfamily are G-protein coupled receptors has eight subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$).[16]

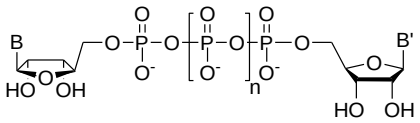


Figure 2.1. General structure of the Np_nN's. n = 0-5, B, B' represent bases, natural (purine and/ or pyrimidine bases, adenine, guanine, cytosine, and uridine) or unnatural.

Np_nN's have also been shown to act as substrates for a variety of DNA polymerases and function as inhibitors of kinases, endonucleases, inosine monophosphate dehydrogenase, adenylosuccinate synthetase, and poly(ADP-ribose) polymerase.[17-24]

Np_nN's such as diadenosine and diuridine polyphosphates, have been shown to possess beneficial properties in the treatment of some diseases such as chronic obstructive pulmonary disease.[25] Np_nN's facilitate the clearance of mucus secretions from the lungs of mammals, including humans being treated for cystic fibrosis. They act through an agonistic effect on the $P2Y_2$ receptors of the lung tissue leading to increased secretion of chloride and water, increased cilia beat frequency and increased mucin release.[26] INS37217 [P^1-(uridine 5')-P^4-(2' deoxycytidine 5') tetraphosphate tetrasodium salt **2-1** is considered as next-generation $P2Y_2$ receptor agonist for the treatment of cystic fibrosis (Figure 2.2).[27] Dinucleoside 5',5'-polyphosphates, such as compound **2-2**, have been developed as selective $P2Y_6$ agonists.[28] As mentioned in chapter 1 (section 1.1) Up_4U (Figure 1.1) marketed as Diquas, has been approved in Japan as a drug for the treatment of dry eye syndrome.[1,2]

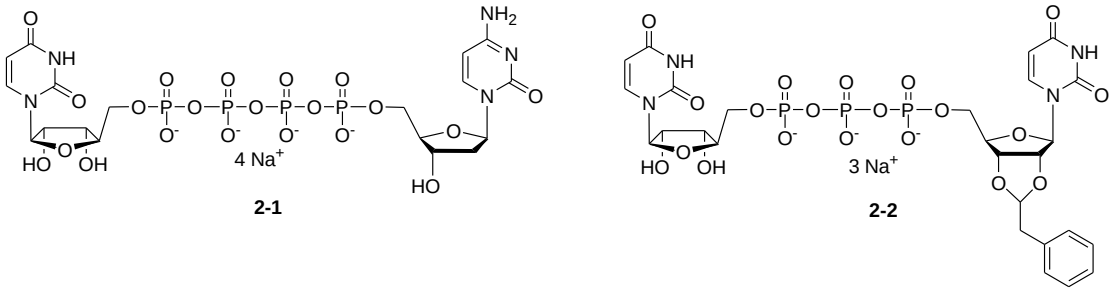
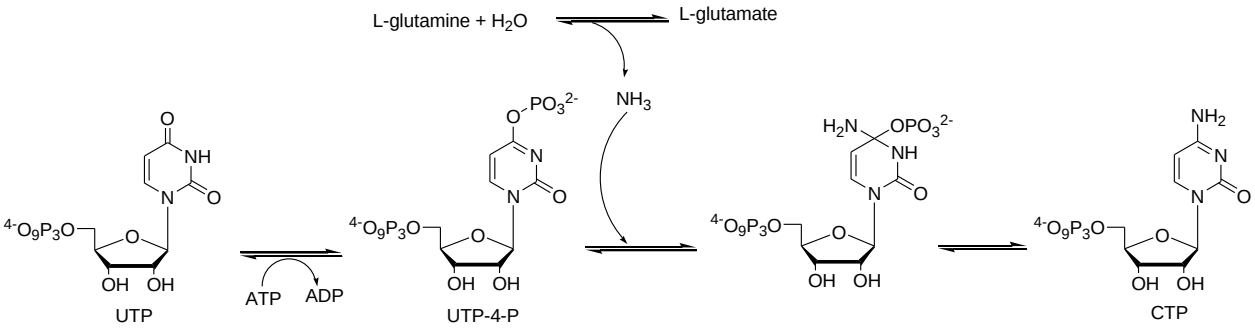


Figure 2.2. Structures of some synthetic, pharmaceutically active dinucleoside polyphosphates.

2.1.2 Inhibitors of cytidine 5'-triphosphate synthase.

Our interest in the synthesis of Np_nN's began as a result of our desire to prepare inhibitors of cytidine 5'-triphosphate synthase (CTPS, EC 6.3.4.2). CTPS catalyzes the ATP-dependent synthesis of cytidine 5'-triphosphate (CTP) from uridine 5'-triphosphate (UTP) using either ammonia or L-glutamine as nitrogen source (Scheme 2.1). The hydrolysis of glutamine to give ammonia occurs in the C-terminal glutamine amide transfer domain (GATase domain). The resulting ammonia is transferred via an ammonia tunnel to the N-terminal kinase amino ligase domain (Alase) where all other chemistry occurs. The currently accepted mechanism has CTPS performing its action by transferring the γ-phosphate from ATP to the oxygen at position 4 of UTP to form UTP-4-phosphate (UTP-4-P) as a reactive intermediate and forming adenosine 5'-diphosphate (ADP). This step requires magnesium ions in the active site of the enzyme. The ammonia then displaces the phosphate at position 4 of UTP-4-P to give CTP.[29-35]



Scheme 2.1. The reaction catalyzed by CTPS.

In solution, CTPS exists as a homotetramer in equilibrium with two identical dimers. UTP and ATP binding induce tetramerization of the inactive CTPS dimers to the active tetramer leading to positive cooperative behaviour at physiological enzyme concentrations (Figure 2.3).[29,36,37] The product, CTP, provides negative feedback inhibition by acting as a competitive inhibitor of the substrate UTP.[31]

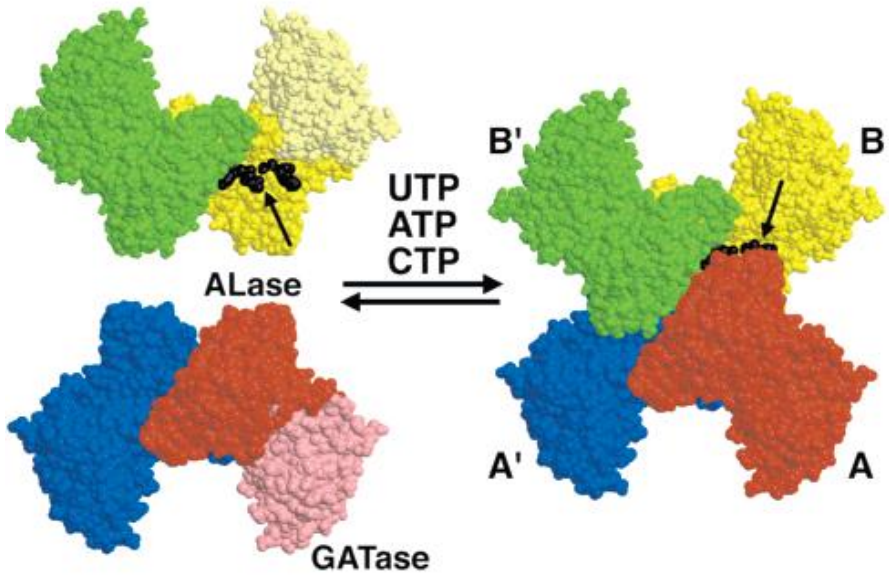


Figure 2.3. Space-filling representation of the *Escherichia coli* CTPS (EcCTPS) tetramer of nearly identical subunits. kinase ammonia ligase (ALase) domain (saturated yellow and red) mediates tetramerization of A-A' (red and blue) and B-B' (yellow and green) dimers. The glutamine amidotransferase (GATase) domain (light yellow and red) catalyzes the GTP-activated glutamine hydrolysis. ATP, UTP, and CTP promote tetramerization by binding in the CTP synthesis active site at the tetramer interfaces (black arrows). Bound ADP and CTP ligands at the B-A interface are also indicated (black). (reprinted with permission from Endrizzi et. al. *Biochemistry*,**2005**, 44, 13491. Copyright 2005 American chemical society).

Figure 2.4 shows the crystal structure of the active site of EcCTPS with the products ADP and CTP bound.[38] UTP has been modelled into the active site.[38] Although CTP acts as a negative feedback inhibitor by competing with UTP, it has been proposed, based upon the crystal structure shown in Figure 2.4, that only the triphosphate portions of CTP and UTP share common binding sites.[38,39]

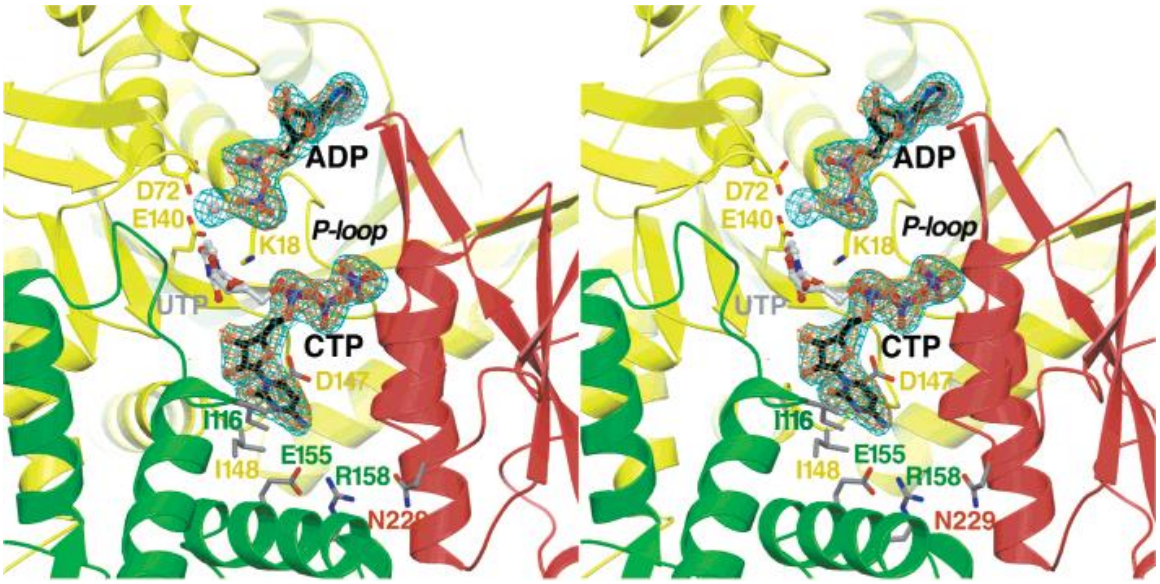


Figure 2.4. UTP and CTP binding sites of EcCTPS. Ribbons indicate secondary structure for the A (red), B (yellow), and B' (green) EcCTPS subunits. The CTP and ADP positions are enclosed by the electron density (black carbons). The hypothetical UTP positioning is also shown. UTP and CTP share the same binding site for their 5'-triphosphate moieties. (reprinted with permission from Endrizzi et. al (*Biochemistry*,**2005**, 44, 13491. Copyright 2005 American chemical society).

CTP plays an important role in the biosynthesis of nucleic acids, phospholipids[40,41] and sialic acid[42] Consequently, CTPS is a recognized target for the development of antineoplastic,[43] antiviral,[44] and antiprotozoal[45-47] agents. Surprisingly, very few studies have focused on the development of nucleotide analogues as inhibitors of this enzyme.[48,49] Cyclopentenyl cytosine (CPEC) 5'-triphosphate (IC_{50} ~6 μM)[50] and 3-deazauridine 5'-triphosphate (3-DaU) (IC_{50} ~18 μM)[51] are substrate and product analogues, respectively, that are the most studied. (Figure 2.5) These inhibitors lacked specificity which led to side effects in clinical trials and CTPS rapidly developed resistance mutations.[51-59] Therefore, the development of new, potent, and selective CTPS inhibitors is required.

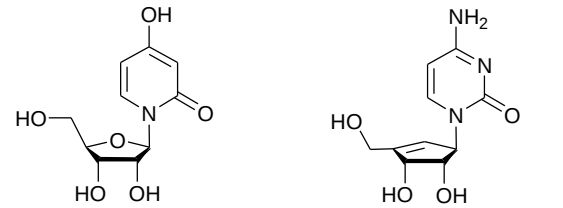


Figure 2.5. Structures of CPEC and 3-DaU.

Enzymes catalyze reactions by specifically binding and stabilizing high energy intermediates and transition states formed along a reaction pathway. Hence it is not surprising that compounds that mimic the transitions states or intermediates formed during enzyme-catalyzed reactions are often highly potent enzyme inhibitors. Such inhibitors are called intermediate or transition state analogs. It is this approach that the Taylor group has taken to develop inhibitors of CTPS.

Two of the inhibitors designed in the Taylor group are compounds **2-4** and **2-5** (Figure 2.6). These compounds were designed to act as either an intermediate analog (compound **2-5**) or as a substrate-intermediate analog (compound **2-4**) of the CTPS-catalyzed reaction. One of the components of **2-4** and **2-5**, compound **2-3,** had been previously prepared in the Taylor group;[60] however, no attempts have been made to convert it into **2-4** and **2-5**. The work described in this chapter focuses on the synthesis of compound **2-4** (which is a dinucleoside triphosphate or Np_3N) and other Np_nN's. A discussion of literature methods for preparing Np_nN's is given below.

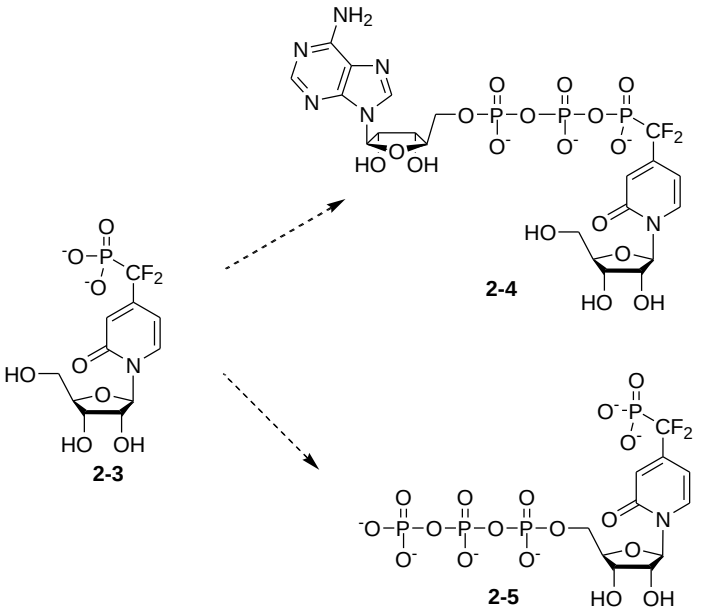


Figure 2.6. Structures of compounds **2-3 – 2-5**.

2.1.3 Synthesis of dinucleoside 5', 5'-polyphosphates

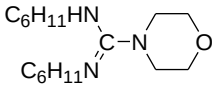
As a result of their importance in biological processes and their potential as pharmaceutical agents considerable effort has gone into developing both chemical and enzymatic routes to Np_nN's. A discussion of the most common and effective approaches to these compounds is given below. As we will see in later chapters in this thesis, many of the methodologies that have been developed for preparing Np_nN's have also been used to prepare other types of nucleoside polyphosphates and their conjugates.

2.1.3.1 Early studies - Synthesis of dinucleoside 5', 5'-diphosphates

Early workers in this field were concerned mainly with the synthesis of dinucleoside 5', 5'-diphosphates (Np_2N's). The methods that were developed were simply dimerization reactions conducted as one-pot reactions. The intermediates were neither isolated nor identified by any means during the reaction course. Early methods used either the free acid or the sodium salt

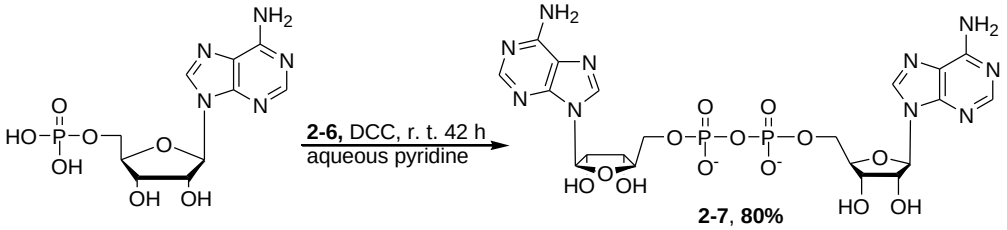
forms of the nucleoside 5'-monophosphates but more recent methods use the organic salt forms that are soluble in anhydrous organic solvents.

Todd and coworkers were the first to prepare Np_2N's. They prepared Ap_2A and Up_2U in a 17% yield by reacting AMP or UMP with trifluoroacetic anhydride overnight and evaporating to dryness.[61] Shortly thereafter Ap_2A was synthesized by Khorana and coworkers from AMP as the free acid form using a very large excess of dicyclohexylcarbodiimide (DCC) as a coupling agent in aqueous pyridine with mechanical stirring at room temperature for 42 hours.[62] In his attempt to reduce the amount of DCC used in the reaction and to use anhydrous reaction conditions, Khorana tried the use of various bases as solubilizers for AMP in anhydrous pyridine. Tri-*n*-butylamine and tri-*n*-octylamine were unsatisfactory for this purpose, but 4-morpholine-*N*,*N*'-dicyclohexylcarboxamidine **2-6** (Figure 2.7) as a base allowed the dissolution of AMP in anhydrous pyridine and so only 3 equivalents of DCC gave exclusively Ap_2A in an 80 % yield (Scheme 2.2).[63]



2-6

Figure 2.7. Structure of 4-morpholine-*N*,*N*'-dicyclohexylcarbodiimide.



AMP

Scheme 2.2. Khorana's synthesis of Ap_2A.

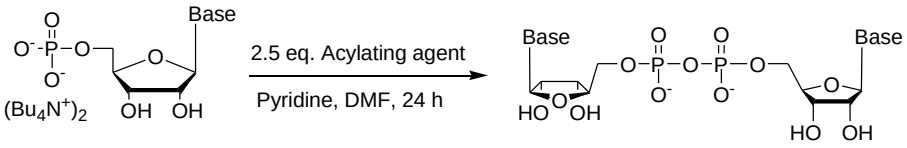
Using Khorana's methodology, Lord Todd and coworkers prepared Ap_2U in a 25 % yield by subjecting a mixture of AMP and UMP to DCC in 90 % pyridine/10% water for 40 h. These

coworkers also prepared Ap_2U in a 17% yield by reacting AMP with UMP in water containing 5% of pyridine in the presence of an excess of dimethylcyanamide at 92 ^oC for 13 hr. In all instances Up_2U and Ap_2A were also formed in significant quantities.[64]

Ng and Orgel later found that it is possible to perform the dimerization of AMP in an aqueous reaction medium by stirring the disodium salt form of AMP and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in aq. HEPES buffer at 37^oC for 2 hours. The yield is highly dependent on the presence of magnesium ions, where in presence of magnesium ions in the reaction mixture the yield of Ap_2A is 80% while in the absence of magnesium ion the yield drops to 19%.[65]

More recently, Kim and Behrman have synthesized the symmetrical Np_2N's by reaction of TMP, CMP, UMP, and AMP in excellent yields with a variety of common acylating reagents. The nucleotides were converted to their tetrabutylammonium salts and then treated with the reagent in anhydrous DMF in the presence of pyridine.[66] Among the reagents examined (Table 2.1), tosyl chloride and p-nitrophenyl chloroformate gave the highest yields.

Table 2.1. Dimerization of NMP's using four different acylating agents.

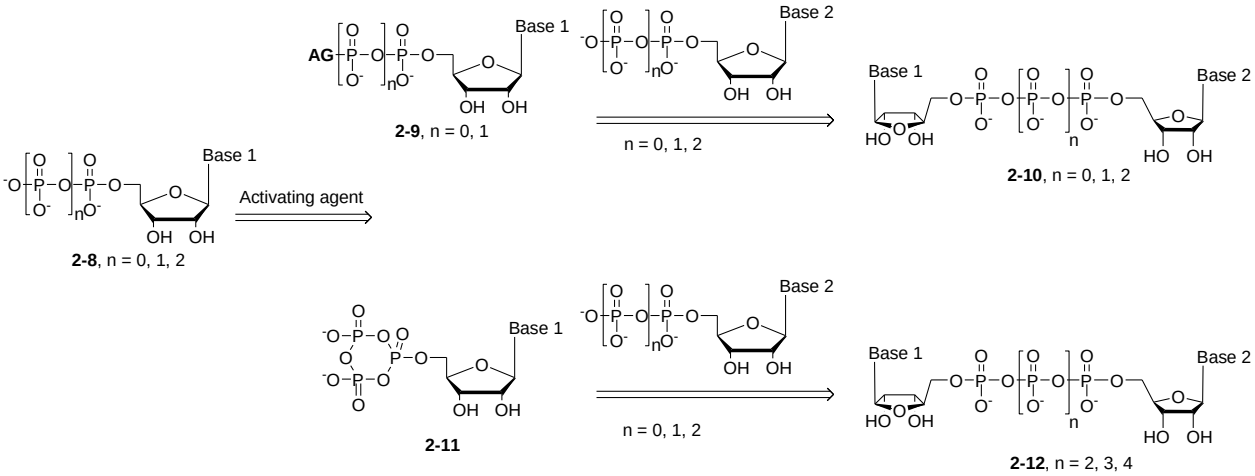


Base	Reagent	Product	Yield (%)
Uracil	Benzoyl Chloride	Up_2U	75
	Bis-(p-nitrophenyl)carbonate		80
	p-Nitrophenyl chloroformate		93
	Tosyl chloride		90
Cytosine	Benzoyl chloride	Cp_2C	50
	Bis-(p-nitrophenyl)carbonate		45
	p-Nitrophenyl chloroformate		50
	Tosyl chloride		55
Thymine	Benzoyl chloride	Tp_2T	75
	Bis-(p-nitrophenyl)carbonate		80
	p-Nitrophenyl chloroformate		90
	Tosyl chloride		90
Adenine	Benzoyl chloride	Monobenzoylated Ap_2A	50
	Bis-(p-nitrophenyl)carbonate	Ap_2A	40
	p-Nitrophenyl chloroformate		60
	Tosyl chloride	Monotosylated Ap_2A	55

2.1.3.2 Chemical synthesis of dinucleoside 5',5'-polyphosphates using activated nucleotide intermediates.

Most methods utilized today for the synthesis of unsymmetrical and symmetrical Np_nN's rely on initial activation of one of the nucleotides by attaching an activating group to the terminal phosphate group. Once activated, this nucleotide acts as an electrophile or donor for the subsequent reaction with the second nucleotide which acts as the nucleophile or acceptor

(Scheme 2.3). The activated electrophilic nucleotide donors can sometimes be isolated but this is usually not necessary and so they are often employed directly in the next step. The activation of NTP donors usually results in the formation of a cyclic nucleoside 5'-trimetaphosphate intermediate **2-11** which undergoes a nucleophilic ring opening reaction with the acceptor nucleotide. In general, these coupling reactions require anhydrous conditions. Both the donor and the acceptor nucleotides usually have to be initially converted from their corresponding free acid or sodium or potassium salts into a form that is soluble in organic solvents such as acetonitrile, DMF, or DMSO. Usually, nucleotides are converted into their corresponding trialkyl- or tetralkylammonium salts to dissolve in these solvents. Below we discuss the most common and/or effective types of activated nucleotides that are used for Np_nN syntheses.

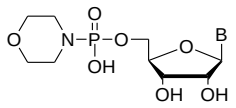


Scheme 2.3. General approach for the synthesis of symmetric and asymmetric dinucleoside Np_nN's, AG refers to activating group.

2.1.3.2.1 Nucleoside 5'-phosphomorpholidates

Nucleoside 5'-phosphomorpholidates as activated forms of nucleotides, compounds of type **2-13**, were first introduced by Moffatt and Khorana (Figure 2.8).[67] These compounds were prepared by adding a mixture of the free acid of NMP and morpholine in water to a boiling *t*-

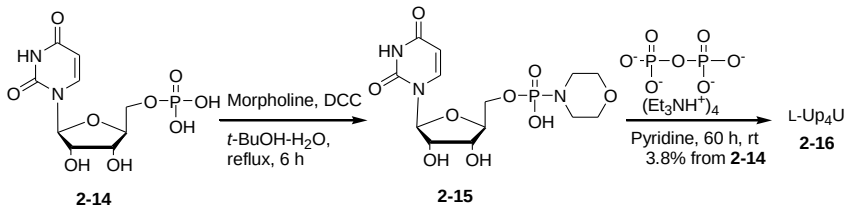
butanol-water mixture containing four equivalents of DCC and continue refluxing for 6 h. The isolated nucleoside 5'-phosphomorpholidates were purified by precipitation from a concentrated solution in methyl alcohol by addition of ether[68] Reactions of morpholidates with a tri- or tetraalkylamommium salts of NMP's usually gives Np_2N's in good yields though the reactions are often sluggish. For example Ap_2A has been prepared in 95% yield by reacting the morpholidate of AMP and the tributylammonium salt of AMP in anhydrous pyridine for 72 h.[69] Nucleoside 5'-phosphomorpholidates are still used today for the synthesis of both symmetrical and unsymmetrical Np_2N's often in high yield.



2-13

Figure 2.8. General structure of nucleoside 5'-phosphomorpholidates (**2-13**).

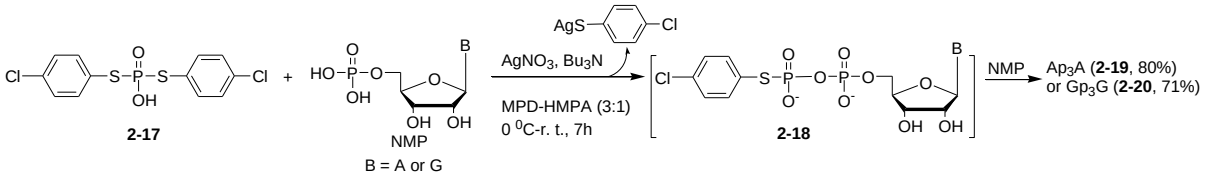
The morpholidate methodology has been used to prepare higher order Np_nN's where n = 3-4; however, it is not widely used for preparing these compounds as the yields tend to be low to moderate. For example, Ap_3U has been prepared in a 22 % yield by reacting the bis-tri-*n*-butylammonium salt of UDP with the morpholidate of AMP in anhydrous pyridine in the presence of 1H-tetrazole at 50 ^{o}C for 24 h.[70] Another example is the synthesis of the enantiomeric analogue of Up_4U namely L-diuridine 5',5'-tetraphosphate (L-Up_4U, **2-16**) as a metabolically more stable analogue of Up_4U, (Scheme 2.4). An excess of the phosphomorpholidate of L-UMP was reacted with the triethylammonium salt of pyrophosphate for 60 h which gave L-Up_4U in a 3.8 % yield. Unlike Up_4U, L-Up_4U was stable to the hydrolysis by both 5'-phosphodiesterase and acid phosphatase.[71]



Scheme 2.4. Synthesis of L-Up$_4$U via the phosphomorpholidate approach.

2.1.3.2.2 *S,S*'-bis (4-Chlorophenyl) phosphorodithioate and *O*-8-(5-chloroquinolyl) *S*-phenylphosphorothioate as activating agents

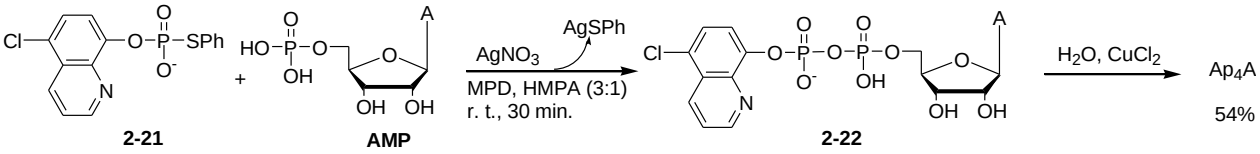
S,S'-bis (4-Chlorophenyl) phosphorodithioate (**2-17**) has been used as an activating agent for the one-pot synthesis of symmetrical dinucleoside Np$_3$N's. The reaction of **2-17** with 3 equivalents of the free acid forms of AMP and GMP in HMPA/1-methylpyrrolidone (MPD) in the presence of 4 eq. $AgNO_3$ and 3 eq. butyl amine afforded Ap$_3$A and Gp$_3$G in good yield (Scheme 2.5). The reaction is believed to proceed through the formation of the intermediate **2-18** which is then reacted with more NMP in presence of silver ions to afford the Np$_3$N's in good yield.[72] The drawback to this procedure is the use of toxic HMPA as solvent and compound **2-17** is not commercially available.



Scheme 2.5. Phosphorodithioate approach to the synthesis of symmetrical Np$_3$N's **2-19** and **2-20.**

In a related synthesis, *O*-8-(5-chloroquinolyl) *S*-phenylphosphorothioate **2-21** (not commercially available) has been employed for the synthesis of Ap$_4$A from AMP (Scheme 2.6). The free acid form of AMP was allowed to react with **2-21** in presence of silver nitrate to give the chloroquinolyl pyrophosphate intermediate **2-22**. Addition of water hydrolyses some of compound **2-22** producing ADP. Subsequent reaction between ADP and **2-22** affords Ap$_4$A in

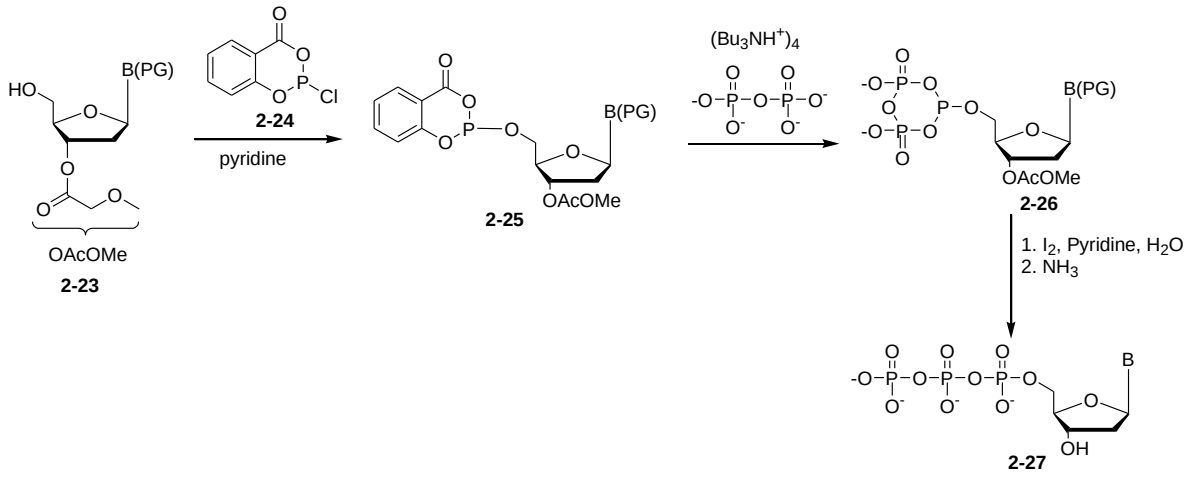
54% yield.[73] The chloroquinolyl pyrophosphates of guanosine and 7-methylguanosine have been isolated in 30 and 56% yield respectively and used to prepare capped mRNA.[74,75]



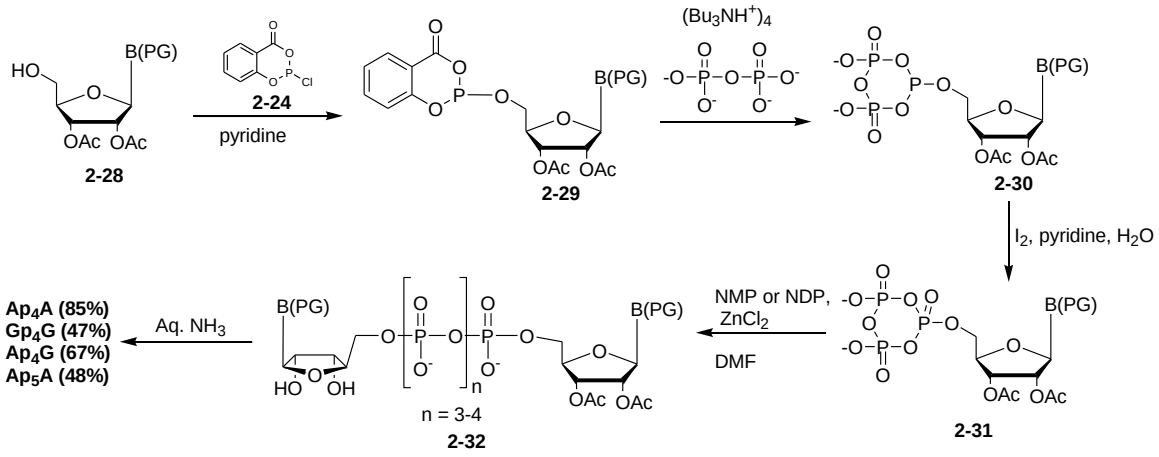
Scheme 2.6. Synthesis of Ap_4A using compound **2-21** as activating agent.

2.1.3.2.3 Salicylphosphites and *cyclo*Salphosphates

2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (salicylchlorophosphite, **2-24**) has been used by Ludwig and Eckstein for the synthesis of dNTP's from their protected 2'-deoxynucleoside precursors (Scheme 2.7). Reactions of protected nucleosides of type **2-23** with **2-24** yields compounds of type **2-25** as a mixture of diastereomers. Reaction of **2-25** with the tri-*n*-butyl ammonium salt of inorganic pyrophosphate affords the cyclic derivative **2-26**. Oxidation and hydrolysis of **2-26**, followed by deprotection affords dNTP's.[76] Jones and coworkers have modified this approach and used it for the synthesis of Ap_4A, Gp_4G, Ap_4G and Ap_5A in moderate to good yields (Scheme 2.8). Their route begins with the Ludwig and Eckstein procedure for the preparation of triphosphate by phosphitylation of protected nucleosides **2-28** with **2-24** followed by reaction with inorganic pyrophosphate to give the cyclic derivatives **2-30**. In the original Eckstein procedure oxidation and hydrolysis of this type of intermediate are concomitant. Jones and coworkers found that careful oxidation of **2-30** under conditions that do not bring about hydrolysis affords the 5'-trimetaphosphate derivative **2-31** which then reacts with an NMP or NDP to afford the partially protected Np_4N or Np_5N **2-32**. Deprotection of **2-32** afforded the Np_nN's.[77]



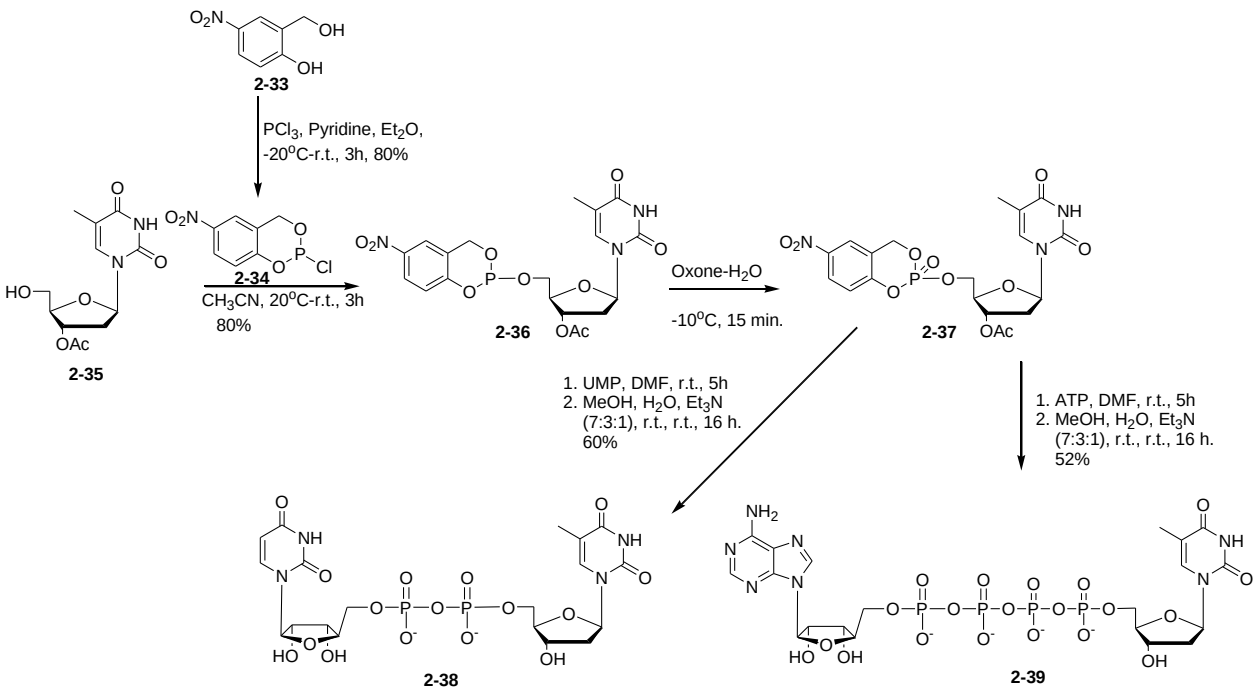
Scheme 2.7. An example of the Ludwig and Eckstein's approach to the synthesis of 2'-deoxynucleoside 5'-triphosphates.



Scheme 2.8. Jones and coworkers synthesis of Np_4N's and an Np_5N.

Warnecke and Meier used the protected *cyclo*Sal nucleotides as activated nucleotides for the synthesis of Np_nN's (Scheme 2.9). For example, reaction of 5-nitrosalicyl alcohol **2-33** with phosphorus trichloride gives 5-nitro-*cyclo*Sal-phosphochloridite **2-34**. Reaction of 3'-acetylthymidine **2-35** with **2-34** and subsequent oxidation using Oxone provided **2-37** in excellent yield. Reaction of **2-37** with the tetrabutylammonium salts of either UMP or ATP

provided Up_2T (compound **2-38**) and Ap_4T (compound **2-39**) in 60% and 52% yields respectively.[78,79]



Scheme 2.9. Warnecke and Meier's synthesis[79] of an unsymmetrical Np_2N and an Np_4N using the *cyclo*Sal approach.

2.1.3.2.4 Nucleoside 5'-phosphoimidazolidates

Nucleoside 5'-phosphoimidazolidates (ImpN's, Figure 2.9) are the most widely used donors for phosphate coupling reactions. They can be isolated or they can be reacted in situ with acceptors. They were first synthesized by Cramer and coworkers.[80-82] They isolated ImpA as its sodium salt in yields of 43-67% by treating the tri-*n*-butylammonium salt of AMP with carbonyldiimidazole (CDI) in trichloroacetonitrile or DMF followed by precipitation of the product in an anhydrous solution of $NaClO_4$ in acetone and ether. They prepared FAD in 92% yield by reacting ImpA with flavin mononucleotide (FMN) in DMF/piperidine followed by precipitation of the product in an anhydrous solution of $NaClO_4$ in acetone and ether.[82]

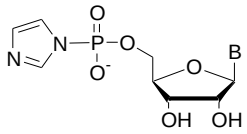
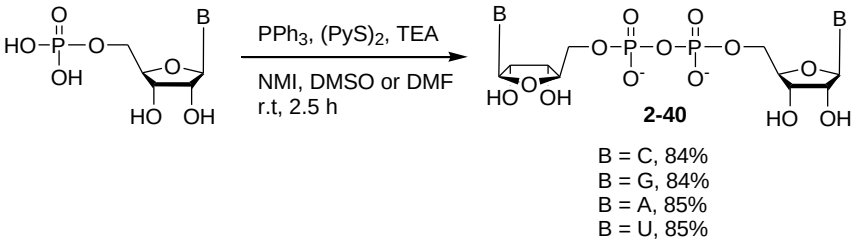


Figure 2.9. General structure of nucleoside 5'-phosphoimidazolidates (Im_pN's)

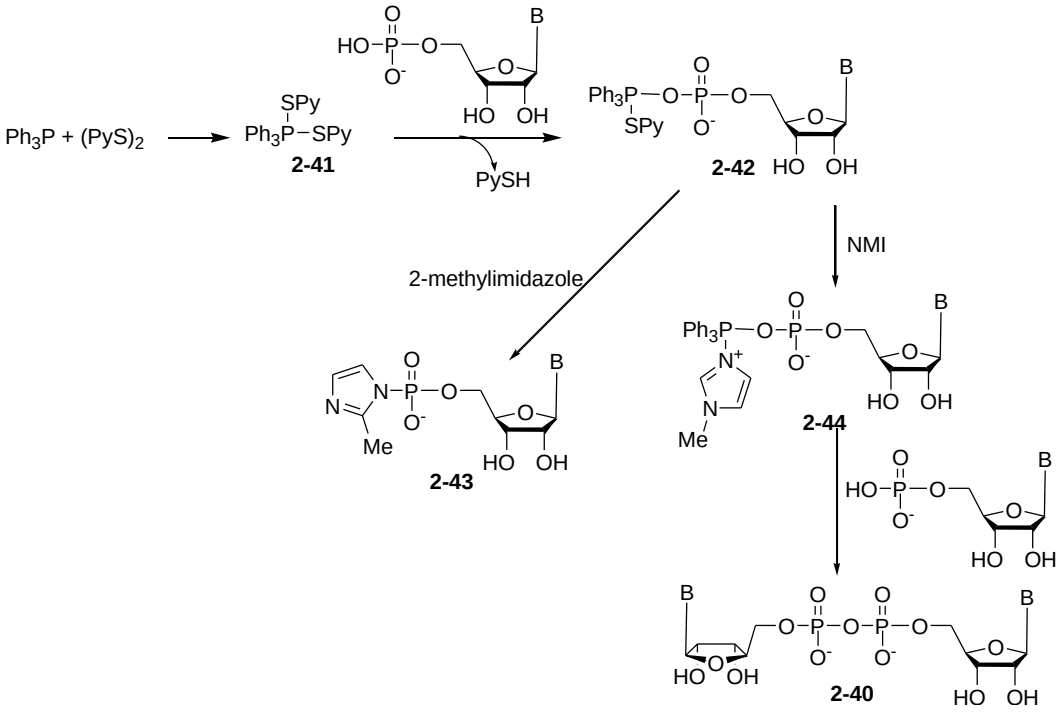
Mukaiyama and Hashimoto[83] and later Lohrman and Orgel[84] demonstrated that ImpA and ImpU could be isolated as their sodium salts in almost quantitative yields by reacting the triethylammonium salt of UMP or AMP with an excess of imidazole, triphenylphosphine and 2,2'-dipyridyldisulfide in DMF at rt for 20 min to 1 h followed by precipitation of the imidazolidates in an anhydrous solution of $NaClO_4$ in acetone and ether. Mukaiyama and Hashimoto also reported the synthesis of adenosine 5'-phosphomorpholidate in quantitative yield using this procedure.[83]

Hurly and coworkers found that when the free acids of NMP's were subjected to 3 equiv of 2,2'-dipyridyldisufide and 3 equiv of triphenylphosphine in the presence of 20-fold excess of *N*-methylimidazole (NMI) in either DMF or DMSO rapid dimerization occurred and Np_2N's were produced in high yields (Scheme 2.10).[85]



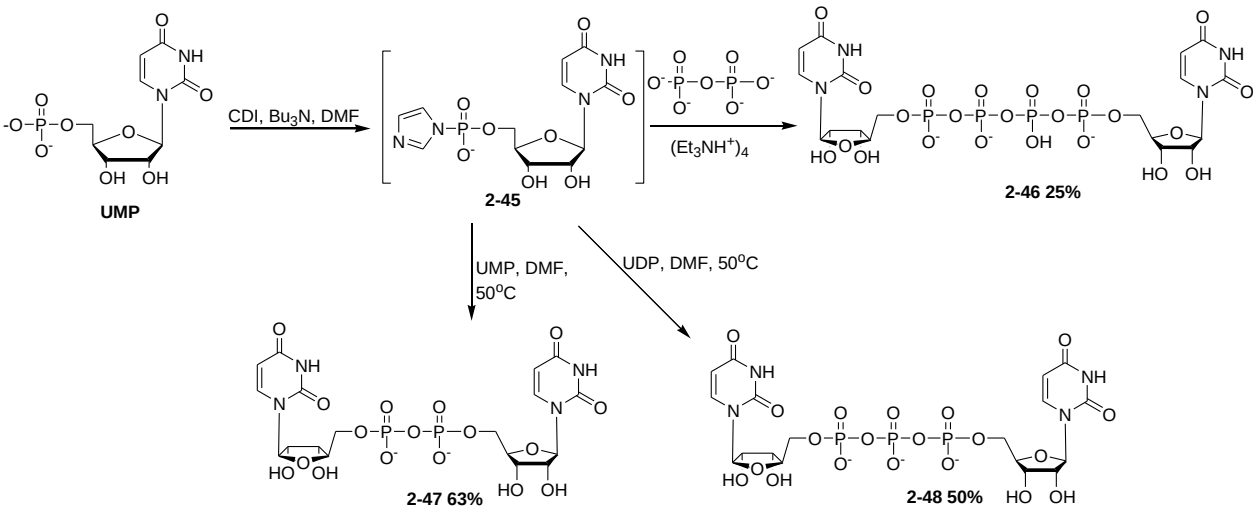
Scheme 2.10. Hurly et al. dimerization of NMP's.

The proposed mechanism of this reaction is shown in Scheme 2.11. [85] Intermediates **2-41** and **2-42** have been postulated by Mukaiyama and Hashimoto.[86,87] The absence of significant amounts of Np_2N's in the presence of imidazole is a consequence of the low nucleophilic reactivity of nucleoside 5'-phosphoric acids compared to that of imidazole and **2-43** is only formed. When NMI is used instead of imidazole the formation of **2-44** was proposed which is strongly activated towards nucleophilic attack by the NMP to form **2-40**.



Scheme 2.11. Proposed mechanism for the Hurly et al. synthesis of symmetrical Np_2N's.

ImpN's can be reacted with other nucleotides/phosphates to give higher order Np_nN's (i.e. where n = 3-4). For example, treatment of the tri-*n*-butylammonium salt of UMP (Scheme 2.12) with carbonyl diimidazole (CDI) in DMF produces ImpU (**2-45)** as the activated nucleotide donor. This was not isolated but was reacted in situ with the tri-*n*-butylammonium salts of either UMP, uridine 5'-diphosphate (UDP), or inorganic pyrophosphate in DMF to produce respectively Up_2U, Up_3U and Up_4U in low to modest yields.[70,88]

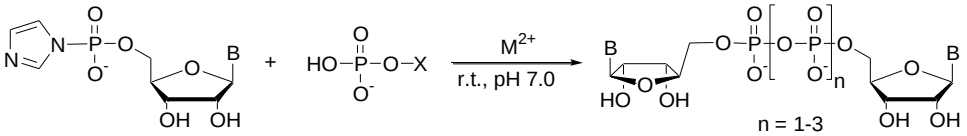


Scheme 2.12. Synthesis of Up_2U, Up_3U, and Up_4U, starting from UMP via the UMP-imidazolidate intermediate.

Skoblov et al. have prepared a wide variety of 2'-deoxy Np_4N's (d(Np_4N's)) by reacting the tri-*n*-butyl ammonium salt of NMPs with CDI for 1 h followed by the addition of the tri-*n*-butylammonium salt of the corresponding NTP and stirring for 24 h. Yields ranged for 46-70%. They also prepared dTp_5A in 36 % yield by reacting the tri-*n*-butyl ammonium salt of dTDP with CDI for 1 h followed by the addition of the tri-*n*-butylammonium salt of ATP.[89]

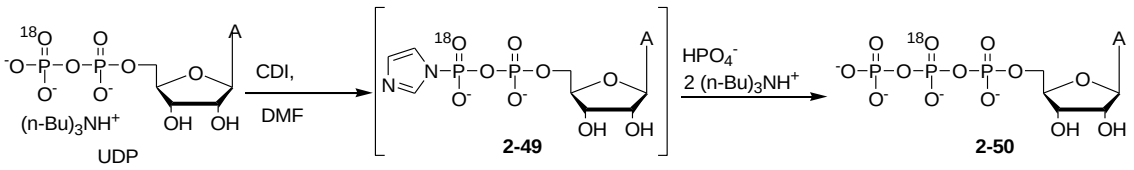
ImpN's hydrolyse in aqueous media at neutral pH. However, Sawai and coworkers demonstrated that in presence of some divalent cations, they can couple with nucleotide acceptors forming Np_nN's in aq. 0.25 M *N*-ethylmorpholine-HC1 buffer at pH 7.0 though the reactions are very slow (Table 2.2).[90,91] The significance of this work is that the coupling were done in aqueous solution and, in some instances, reasonable yields were obtained (entries 1-6).

Table 2.2. Divalent cation-catalyzed reaction of nucleotide imidazolidates in aqueous medium.



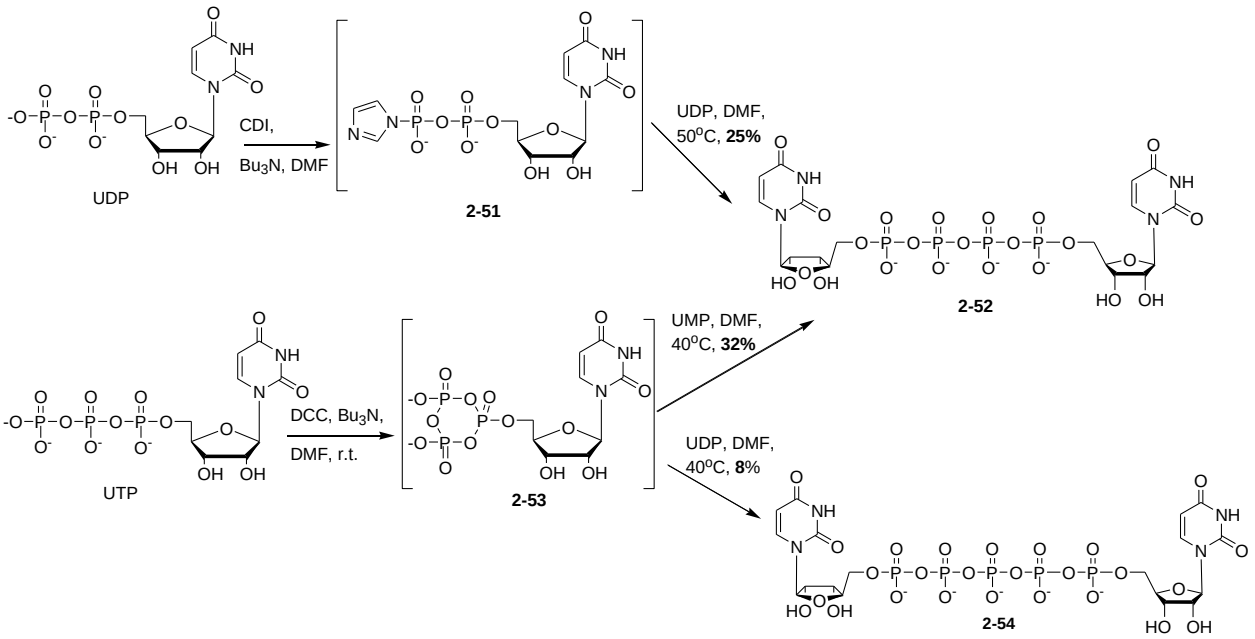
Entry	B	X	Product	M^{+2}	time (days)	Yield (%)
1	Adenine	Adenosine	Ap_2A	Cd^{2+}	8	41
2	Adenine	Adenosine	Ap_2A	Mn^{2+}	8	44
3	Adenine	AMP	Ap_3A	Cd^{2+}	4	59
4	Adenine	AMP	Ap_3A	Mn^{2+}	4	63
5	Adenine	ATP	Ap_4A	Cd^{2+}	4	59
6	Adenine	ATP	Ap_4A	Mn^{2+}	4	54
7	Hypoxanthine	Inosine	Ip_2I	Cd^{2+}	4	28
8	Hypoxanthine	Inosine	Ip_2I	Mn^{2+}	4	15

Imidazolides of nucleoside 5'-diphosphates (ImppN's) are also used to prepare nucleoside polyphosphates. Lowe and Sproat were the first to report the formation of such an imidazolide. They reacted the tri-*n*-butylammonium salt of β-^{18}O-labeled ADP with CDI in DMF to generate the imidazolide **2-49**. **2-49** was not isolated (Scheme 2.13). It was reacted with mono(tri-*n*-butylammonium) phosphate in DMF for 28 h which gave adenosine 5'-[β-^{18}0]triphosphate in 53 % yield.[92]



Scheme 2.13. Lowe and Sproat's synthesis of ^{18}O-labeled Imp_2A and the synthesis of adenosine 5'-[β-^{l8}0,]triphosphate.

ImppN's are now widely used to prepare Np_nN's. For example, reaction of UDP with CDI gives ImppU (**2-51** in Scheme 2.14). **2-51** was not isolated. It was reacted with UDP which gave Up_4U (**2-52**) in a 25 % yield. Alternatively **2-52** was formed from cyclization of UTP using DCC. The resultant cyclic uridine 5'-trimetaphosphate intermediate **2-53** was reacted with either UMP to produce **2-52** in a 32 % yield or with UDP to produce Up_5U (**2-54**) in an 8 % yield (Scheme 2.14).[89] It should be pointed out that the reaction of a cyclic nucleoside 5'-trimetaphosphate intermediate with a NDP to give Np_5N's does not always proceed in such poor yields. For example, reaction of the cyclic nucleoside 5'-trimetaphosphate of ATP, formed by the reaction of ATP with DCC in DMSO, with tris(tri-*n*-buty1ammonium)-ADP in DMF at 35 ^{o}C for 18 h gave Ap_5A in a 54% yield.[21]

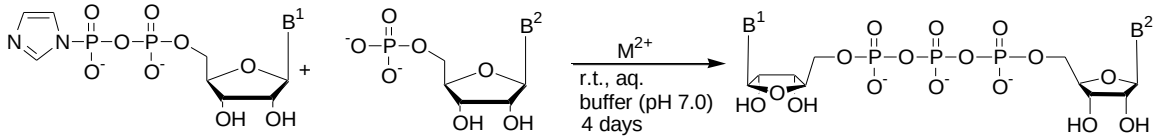


Scheme 2.14. Synthesis of Up_4U from UDP via ImppU (**2-51**). Synthesis of Up_4U and Up_5U via uridine 5'-trimetaphosphate **2-53**.

Sawai et al. reported that the imidazolide of NDP's (ImppN's) can be isolated.[93] These workers reacted the triethylammonium salt of ADP or 7-methylguanosine diphosphate (7mGDP) with a 10-fold molar excess each of imidazole, di-2-pyridyldisulfide and triphenylphosphine in dry DMF containing triethylamine and tri-*n*-octylamine for 24 h at rt. The reaction mixture was poured into a solution containing dry acetone and dry ether saturated with $NaClO_4$ and triethylamine which resulted in the precipitation of the sodium salts of ImppA and Impp7mG in 82 and 71% yield respectively.[93] Sawai's procedure is now the most common procedure used for preparing and isolating ImppN's. Sawai demonstrated that these ImppN's could be coupled to acceptors in aq. solution. For example, the reaction of the 1 equiv of ImppA with 1 equiv of AMP in 0.2 M aq. *N*-ethylmorpholine buffer (pH 7.0) for 4 days in the presence of 1 equiv $MnCl_2$ or $CdCl_2$ at 30 ^oC gave Ap_3A in 37-48% yield. Reaction of the 5 equiv of Impp7mG with 1 equiv of GMP in 0.2 M aq. *N*-ethylmorpholine buffer (pH 7.0) for 4 days in the presence of 5

equiv $MnCl_2$ or $CdCl_2$ at 30 ^oC gave $Gp_3$7mG in an impressive 67-77% yield (Table 2.3). Studies on the stability of ImppA revealed that it is more stable in aq. solution compared ImpA.[93]

Table 2.3. Synthesis of Np_3N's in aq. buffered solution via ImppN's

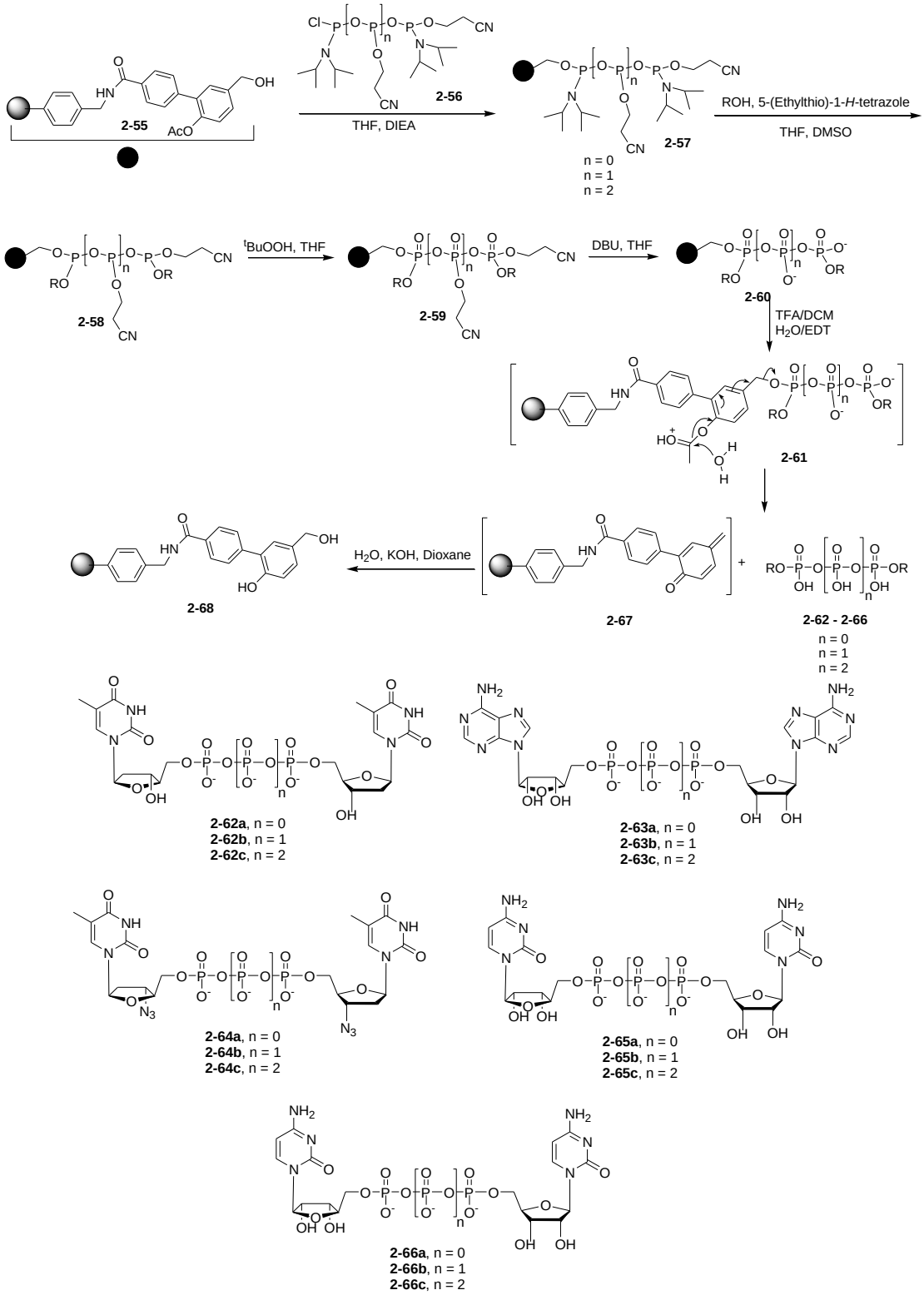


Entry	B^1	B^2	Compound	M^+	Yield (%)
1	A	A	Ap_3A	Cd^{2+}	37
2	A	A	Ap_3A	Mn^{2+}	48
3	7-mG	G	$7mGp_3G$	Cd^{2+}	67
4	7-mG	G	$7mGp_3G$	Mn^{2+}	77

2.1.3.2.5 Solid-phase synthesis of dinucleoside 5',5'-polyphosphates

Ahmadibini and Parang have developed a solid-phase synthesis suitable for making symmetric Np_nN's. The trifunctional phosphitylating reagent **2-56** (Scheme 2.15) contains one chlorine group and two isopropylamino groups that are replaced after the attachment of the reagent to the polymer-bound linker and after coupling reactions with two unprotected nucleosides respectively. The steric bulkiness of **2-57** allowed sufficient steric hindrance for only the 5'-OH of the unprotected nucleosides to react. The trivalent phosphite groups of compound **2-58** were oxidized into phosphate groups using ^{t}BuOOH, cyanoethyl groups were removed by DBU, followed by cleavage of the product from the polymer support using dichloromethane/ trifluoroacetic acid (TFA)/Water/ ethanedithiol (EDT) mixture. After cleavage and filtration, the crude products were further purified by reversed-phase HPLC. Yields range from 59-78%.[94] We

do not think that this is a very practical approach to Np_nN's as it requires the multistep synthesis of a polymer with a unique linker and multistep syntheses of polyphosphites **2-56** prior to the solid phase chemistry, the products still require HPLC purification and the yields are not significantly better than what has been reported by other groups using solution phase methodologies.

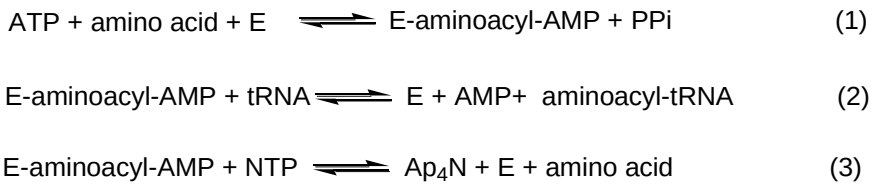


Scheme 2.15. Solid-phase synthesis of symmetric dinucleoside 5',5'-polyphosphates, ROH= thymidine, adenosine, azidothymidine (AZT), cytidine, inosine.

2.1.3.2.6 Enzymatic methods for the synthesis of Np_nN's.

The first *in vitro* enzymatic synthesis of an Np_nN was reported by Zamecnik and Stephenson in 1966.[95] They found that Ap_4A can be formed by a reaction of aminoacyladenylate with ATP catalyzed by lysyl-tRNA synthetase. Since that time, several enzyme classes have been reported for the synthesis of Np_nN. These enzymes differ mainly in their substrate-selectivity and catalytic efficiency.

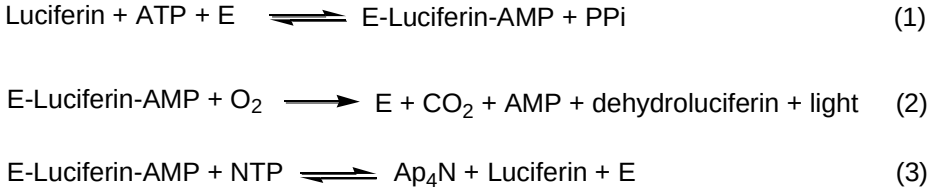
The first class of enzymes is the aminoacyl-tRNA synthetases (aaRSs). They synthesize Ap_4N's by catalyzing what is known as the "back-reaction" of an aminoacyladenylate, with an acceptor nucleotide.[96] These enzymes catalyze the activation of an amino acid with ATP to form an enzyme-bound aminoacyladenylate and pyrophosphate (Scheme 2.16, equation 1). In the presence of tRNA, only aminoacyl-tRNA is produced (Scheme 2.16, equation 2). In the absence of tRNA, attack of aminoacyladenylate by ATP (or in some cases other NTP's) produces Ap_4A (or generally Ap_4N) (Scheme 2.16, equation 3).[97-100]



Scheme 2.16. Synthesis of Ap_4N's catalyzed by aminoacyl-tRNA synthetases, E = enzyme, PPi = inorganic pyrophosphate. Yields have not been reported.

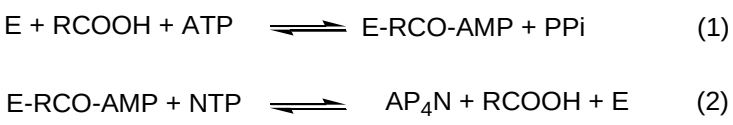
Firefly luciferase is another enzyme that is capable of making Ap_4N's. The enzyme forms E-Luciferin-AMP (Scheme 2.17, equation 1) and, in the presence of oxygen, is responsible for light emission (Scheme 2.17, equation 2). Although luciferase is classified as an oxidoreductase, it has molecular properties shared by ligases. When ATP (or other NTP's) is available the E-Luciferin-AMP intermediate can react with these NTP's to give Ap_4N's (Scheme

2.17, equation 3). Divalent cations are important for Ap_4N formation and the yield is greatly enhanced by pyrophosphatase that hydrolyses inorganic pyrophosphate (PPi) into organic monophosphates which prevents the reverse reaction.[101]



Scheme 2.17. Reactions catalyzed by firefly luciferase. E = enzyme. Yields have not been reported.

A third class is the acyl-CoA synthetase. It catalyzes the synthesis of Ap_4N's through reactions (1) and (2) in Scheme 2.18. While the first step of this reaction is very specific to ATP substrate, the second step is not selective as any NTP, and inorganic tri- and tetraphosphates (iP_3, iP_4) can act as acceptor substrates. In case of iP_3, iP_4, adenosine 5'-tetraphosphate (Ap_4) and adenosine 5'-pentaphospahate (Ap_5) are formed instead of Ap_4N. This enzyme can make diadenosine 5',5'-hexaphosphate (Ap_6A) by using ATP and iP_4 and allowing more reaction time for the enzyme than that required for the synthesis of Ap_5. In this case first makes Ap_5 and then utilizes it as an acceptor for AMP from E-RCO-AMP intermediate. In the same way Ap_5A could by synthesized by the enzyme from ATP and iP_3.[105,106]



Scheme 2.18. Synthesis of NTP catalyzed by acyl-CoA synthetase. E = enzyme. Yields have not been reported.

A fourth class of enzymes are Ap_nA phosphorylases. Ap_4A hydrolase hydrolyzes Ap_4A in the presence of inorganic phosphate to ATP and ADP. Since the reaction is reversible, these enzymes can catalyze the reaction of ATP and ADP to give Ap_4A.[107-109] Human Fhit is an Ap_3A

hydrolase that catalyzes the Mg^{2+}-dependent hydrolysis of Ap_3A into AMP and ADP (Scheme 2.19, equations 1 and 2). Mutation of His96 into glycine gives H96G-Fhit which catalyzes the reaction of ImpN's with nucleoside di- and triphosphates to give Ap_3A, Ap_3N's and some Ap_4N's in yields ranging from 63-75% (Scheme 2.19, equation 3).[110] The enzyme is also capable of accepting the imidazolates of CMP and UMP which enabled the synthesis of Tp_3U and Cp_3U in yields of 69 and 71% respectively.

$$E^{Fhit}\text{-}His^{96} + MgAp_3A \rightleftharpoons E^{Fhit}\text{-}His^{96}\text{-}AMP + MgADP \quad (1)$$

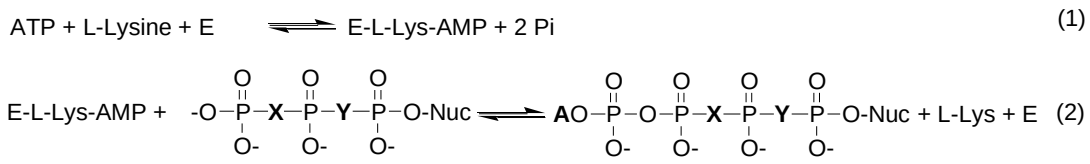
$$E^{Fhit}\text{-}His^{96}\text{-}AMP + H_2O \longrightarrow E^{Fhit}\text{-}His^{96} + AMP \quad (2)$$

$$E^{H96G\text{-}Fhit}\text{▪}ImpA + MgAp_xN \longrightarrow E^{H96G\text{-}Fhit} + Ap_nN \quad (3)$$

Scheme 2.19. Hydrolysis of Ap_3A by natural human Fhit and synthesis of Ap_nN's by engineered human Fhit (E^{Fhit}). x = 1, 2, n = 3, 4.

Finally, T4DNA ligase can transfer AMP from the E-AMP complex to in organic triphosphate, ADP, ATP, GTP, or dATP producing adenosine tetraphosphate (Ap_4), Ap_3A, Ap_4A, Ap_4G, and dAp_4G, respectively.[101,111,112] GTP:GTP guanylyl transferase (Gp_4G synthetase) catalyzes the conversion of two molecules of GTP to Gp_4G and pyrophosphate.[113,114]

Non-natural dinucleoside polyphosphate analogs where one of the bridging oxygens of the polyphosphate group is replaced with a chemically stable methylene group, such as $NpCH_2pppN$', have been widely used as inhibitors and probes of enzymatic reactions. By a mechanism similar to aminoacyl-tRNA synthetases, stress protein LysU can synthesize these analogs (Scheme 2.20).[115,116]



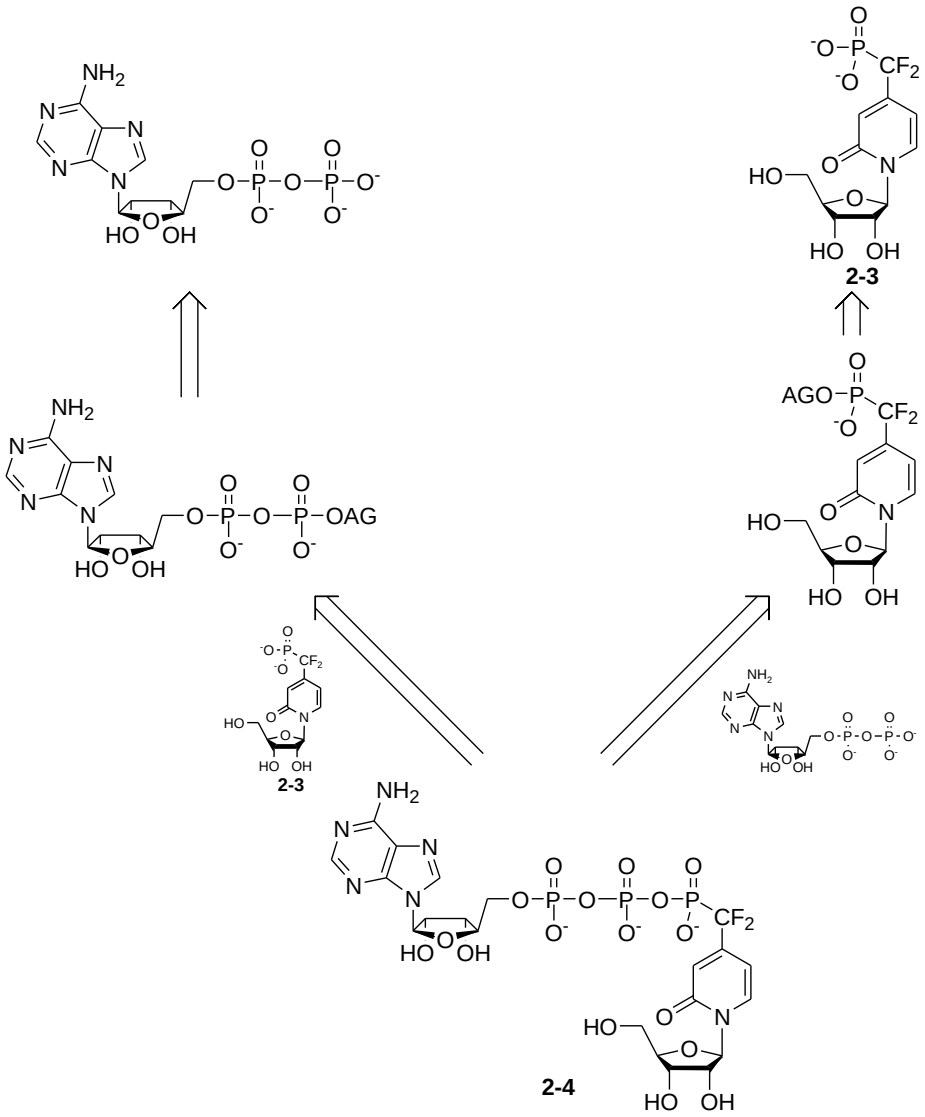
Scheme 2.20. Synthesis of dinucleoside tetraphosphate analogs by stress protein LysU. A = adenosine, N = nucleoside analog, x,y = O or CH_2, Pi = inorganic monophosphate.

Although the enzymatic approach is used for Np_nN syntheses, it suffers from limited substrate specificity and scalability.

2.1.4 Approaches to the synthesis of compound 2-4

After analyzing the different approaches to Np_nN's it was clear that there were two potential routes to the synthesis of compound **2-4** (Scheme 2.21). One was to activate compound **2-3** and then react it with ADP. The other was to activate ADP and then react it with compound **2-3**. The next issue was to decide what reagent should be used to activate **2-3** or ADP. As the imidazolides (ImpN's or ImppN's) are the most widely used donors and tend to give the best yields we focussed our attention on these species. ImppA has been isolated in good yield and used as a donor.[93] However, reactions of ImppN's with phosphate acceptors are slow and so we were concerned that the reaction of **2-3** with ImppA would be particularly slow as the two fluorines reduce the nucleophilicity of the phosphonate oxygens (pK_{a2} of an α,α–difluoromethylenephosphonate is 5.5 which is approximately 2 pKa units lower than the pKa_2 of a typical phosphate group). The other approach was to convert **2-3** to its imidazolide; however, it was found that the reaction of the tetrabutylammonium salt of **2-3** with CDI was very slow which was again probably due to the relatively low nucleophilicity of the phosphonate oxygens. Consequently, we decided to examine an alternative procedure that had never before been used for preparing Np_nN's, but had found considerable success in preparing other types of

nucleoside polyphosphates and their conjugates. This was a procedure developed by Bogachev for the preparation of NTP's.[8,117]

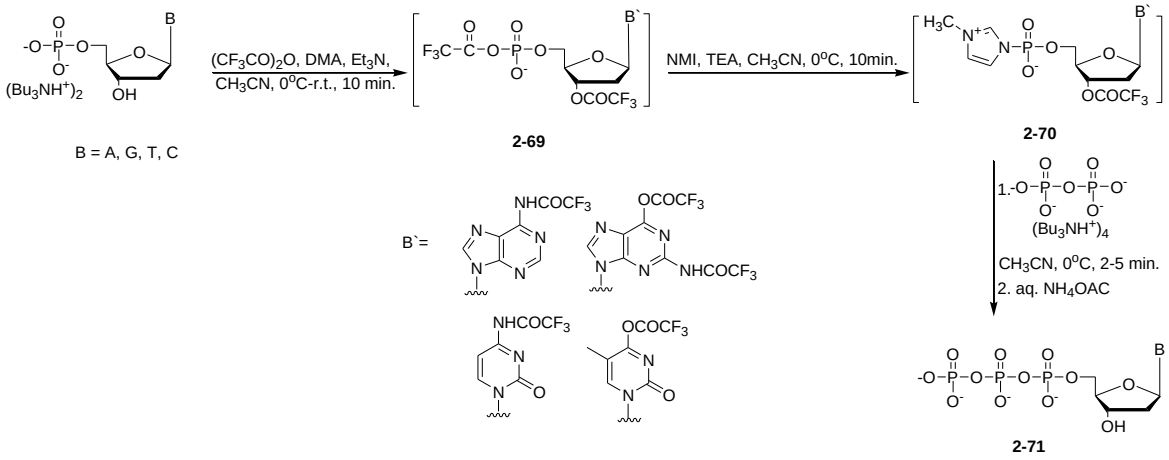


Scheme 2.21. Potential routes to the synthesis of compound **2-4.** AG stands for activating group.

2.1.5 Bogachev's approach to the synthesis of nucleoside polyphosphates and their conjugates

Bogachev demonstrated that anhydrides of strong carboxylic acids are useful intermediates in dNTP synthesis. He showed that in the reaction of trifluoroacetic anhydride (TFAA) with the tri-*n*-butylammonium salts of dNMP's in the presence of triethylamine (TEA)

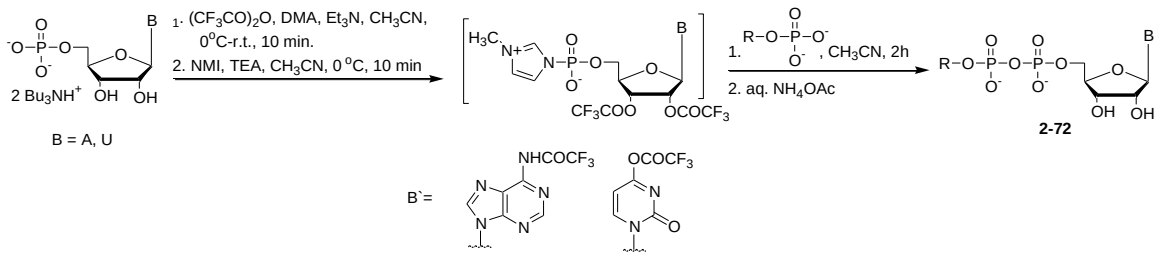
and *N*,*N*-dimethylaniline (DMA) in acetonitrile, acylphosphates of type **2-69** are formed (Scheme 2.22) along with temporary protection of the nucleotide hydroxyl and amino groups.[117] Like most acylphosphates, they display acylating rather than phosphorylating properties.[118-120] However, after their treatment with nucleophilic catalysts like NMI or *N*,*N*-dimethylaminopyridine (DMAP), the trifluoroacetyl containing acylphosphates are converted into nucleotide imidazolium zwitter ions (**2-70**, Scheme 2.22). Reaction of **2-70** with the tri-*n*-butylammonium salt of inorganic pyrophosphate (Scheme 2.22) provided nucleoside dNTP's in 89-92% yields. The reaction times do not exceed 10 minutes in any synthetic step.[8]

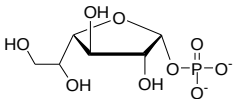
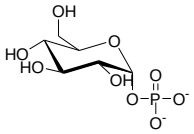
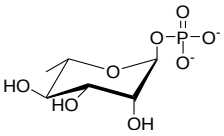
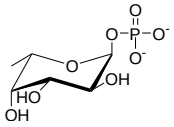
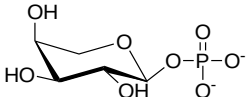


Scheme 2.22. Bogachev's synthesis of dNTP's (B = A, G, T, C).

Bogachev's procedure has been applied to the synthesis of NDP-sugars. Marlow and Kiessling have applied Bogachev's procedure to the synthesis of UDP-α-D-galactofuranose (Table 2-1 entry 1)[121] The same procedure has been also used by Timmons and Jakeman for the synthesis of some other NDP-sugars (Table 2.4 entries 2-9).[122] Although the reaction yields are low, the coupling reaction time has greatly reduced (2h) compared to other procedures that utilize imidazolates or morpholidates as donors.[123-126]

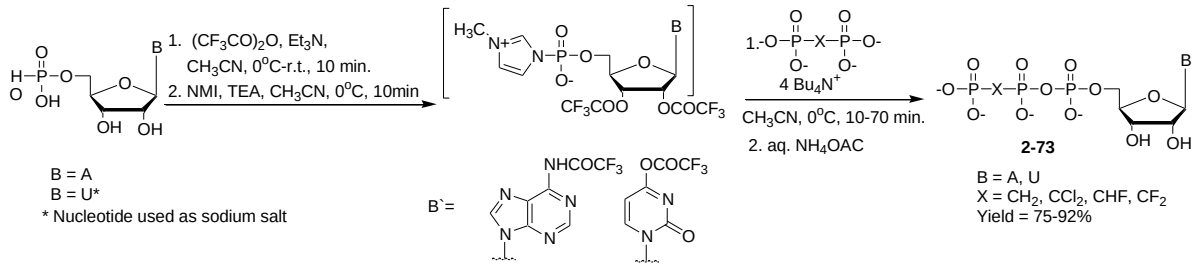
Table 2.4. Synthesis of some NDP-sugars using Bogachev's method.



Entry	Sugar-1-phosphate$^-$	NMP	Product	Yield (%)
1		UMP	UDP-α-D-galactofuranose	35
2		AMP	ADP-α-D-glucose	48
3		UMP	UDP-α-D-glucose	35
4		AMP	ADP-α-L-rhamnose	25
5		UMP	UDP-α-L-rhamnose	30
6		AMP	ADP-β-L-fucose	28
7		UMP	UDP-β-L-fucose	26
8		AMP	ADP-α-L-arabinose	35
9		UMP	UDP-α-L-arabinose	32

NMP's are usually commercially available as either the free acid or sodium salt forms. These forms are not soluble in acetonitrile and possess water of crystallization (usually in the form of hydrates) so in the typical Bogachev procedure these nucleotides have been converted to their corresponding tri-*n*-butylammonium salts. Jakeman and Mohamady have found that there is no need to convert these starting nucleotides from their commercially available forms to the corresponding tri-*n*-butylammonium salts if TFAA was used in larger excess, as the TFAA used

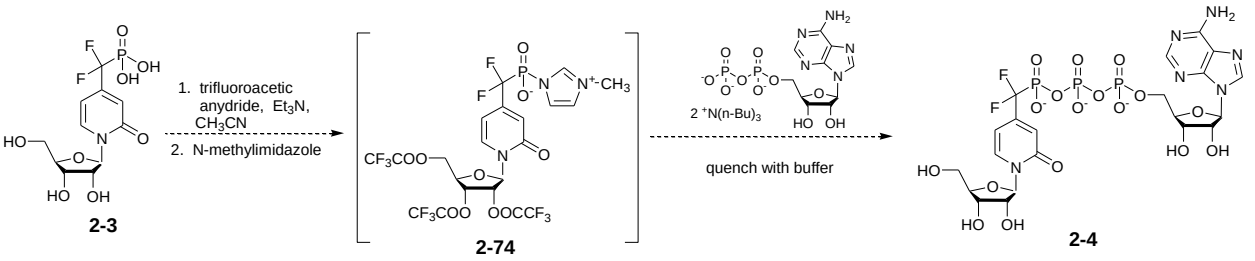
can act as solubilizing and sequestering agent in addition to the formation of the mixed anhydrides, thus eliminates the long time spent in conversion of the salt forms and drying of nucleotides. They also omitted DMA from the first step. This modification has been used in the synthesis of some NTP analogs (Scheme 2.23).[127]



Scheme 2.23. Jakeman and Mohamady's synthesis of NTP analogs.

2.2 Objectives

Although Bogachev's procedure had never been used to prepare Np_nN's it was anticipated that it would be suitable for this purpose and particularly for the synthesis of compound **2-4**. TFAA is very reactive and so we anticipated that we could form intermediate **2-74** readily in spite of the fluorines that are adjacent to the phosphonate group (Scheme 2.24). Reaction of **2-74** with ADP would give compound **2-4**. However, before applying this approach to the synthesis of compound **2-4** we thought it prudent to first determine if this procedure would work using more conventional, commercially available nucleotide substrates. Hence, the objectives of this work were: (1) to determine if Bogachev's procedure can be used to prepare Np_nN's and, if successful, (2) use this procedure for preparing compound **2-4**.



Scheme 2.24. Proposed route to compound **2-4**.

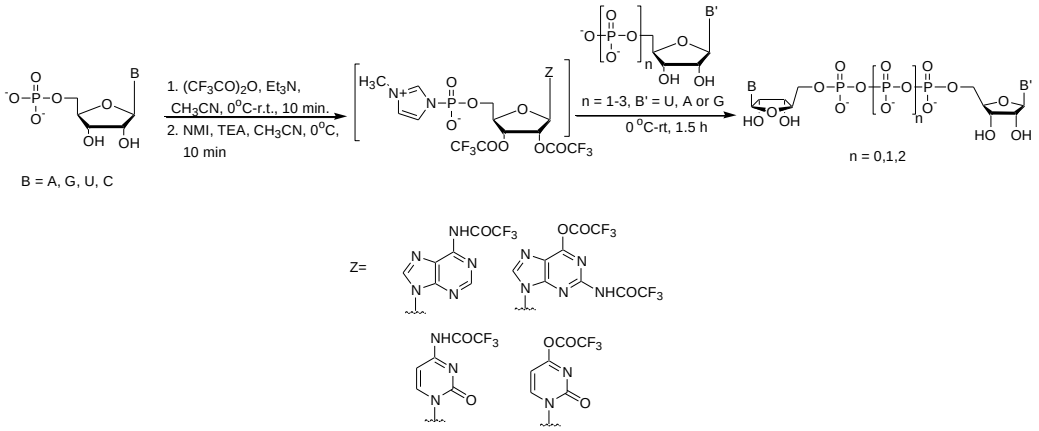
2.3 Results and discussion

Bogachev prepared deoxynucleoside 5'-monophosphate-*N*-methylimidazolium salt donors by first reacting tri-*n*-butylammonium salts of dNMP's with a 5-fold excess of TFAA in acetonitrile in the presence of TEA and DMA and stirring for 10 min. This results in temporary protection of the hydroxyl and amino (if present) groups and formation of the mixed anhydride. These solutions are concentrated to remove volatiles and then treated with excess NMI in the presence of TEA in acetonitrile to give the highly reactive 5'-*N*-methylimidazolium salt donors. For the synthesis of the Np_nN's, we prepared these donors in a similar manner except we employed the modifications that Jakeman and Mohamady developed for the synthesis of nonhydrolyzable NTP analogs.[127] These modifications consist of using commercially available sodium salts rather than the tri-*n*-butylammonium salts nucleotides and a considerable excess (16 equiv) of TFAA and TEA. In Bogachev's original procedure, the tri-*n*-butylammonium salts of the nucleotide donor precursors were used and they had to be vigorously dried before reaction with TFAA. By using a larger excess of TFAA, the commercial sodium salts, which are sold as hydrates, can be used without vigorous drying, as the water is removed by reacting with the excess of TFAA.

This procedure was applied to the synthesis of Np_nN's where n = 2-4 (Table 2.5). Yields ranging from 51-68% were obtained. However, for the synthesis of Up_4A (**2-85**), the sodium salt

of UMP gave a low yield (not shown) of the tetraphosphate product. Moreover, for compounds containing guanosine (compounds **2-77** and **2-82**) using the sodium salt of GMP and large excess of TFAA resulted in complete decomposition of the nucleotide. Consequently, for compounds **2-77**, **2-82**, and **2-85** we found that the best yields were obtained using predried tri-*n*-butylammonium salts of UMP or GMP and reducing the equivalents of TFAA to five (for GMP) or eight (for UMP). For UMP, AMP, CMP, and GMP, ^{31}P NMR analyses of the reaction mixtures indicated that the mixed anhydrides, appearing at approximately δ_p 2.0 ppm, were formed within a few minutes.

Table 2.5. Yields of Np_nN's using the modified Bogachev procedure.



Entry	Donor precursor[a]	Acceptor[b]	Product	Yield (%)
1	UMP	UMP	B = B' = U, n = 0 (**2.75**)	64
2	AMP	AMP	B = B' = A, n = 0 (**2-76**)	61
3	GMP[b]	GMP	B = B' = G, n = 0 (**2-77**)	68
4	UMP	AMP	B = U, B' = A, n = 0 (**2-78**)	66
5	CMP	AMP	B = C, B' = A, n = 0 (**2-79**)	60
6	CMP	UMP	B = C, B' = U, n = 0 (**2-80**)	67
7	AMP	ADP	B = B' = A, n = 1 (**2-81**)	51
8	GMP[b]	GDP	B = B' = G, n = 1 (**2-82**)	53
9	UMP	ADP	B = U, B' = A, n = 1 (**2-83**)	56
10	CMP	UDP	B = C, B' = U, n = 1 (**2-84**)	58
11	UMP[b]	ATP	B = U, B' = A, n = 2 (**2-85**)	60

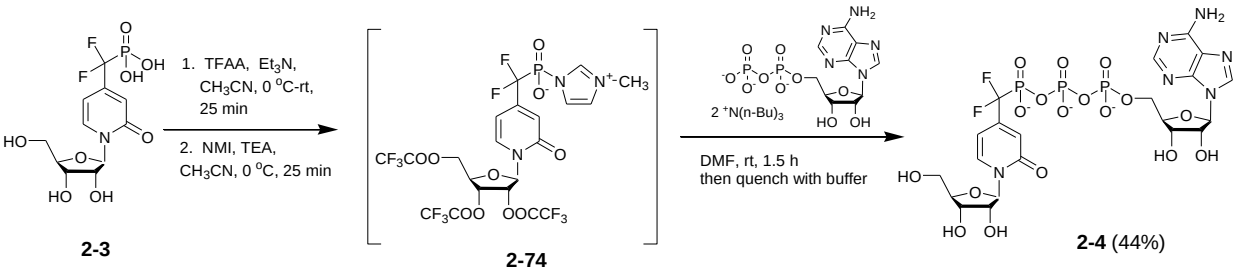
[a] Employed as hydrates of the sodium salts or free acids. [b] Tri-*n*-butylammonium salt used.

Formation of the *N*-methylimidazolium salts could also be followed by ^{31}P NMR as these species exhibited chemical shifts at approximately δ_p -9 to -10 ppm. In all cases the formation of *N*-methylimidazolium salts was complete within 10 min and the crude donor acetonitrile solutions could be used directly for the coupling reactions. Addition of the donor solutions to

solutions of the bis(tri-*n*-butylammonium) salts of the acceptors in acetonitrile resulted in the precipitation of the acceptors. However, this problem was eliminated by using a DMF solution of the acceptor.

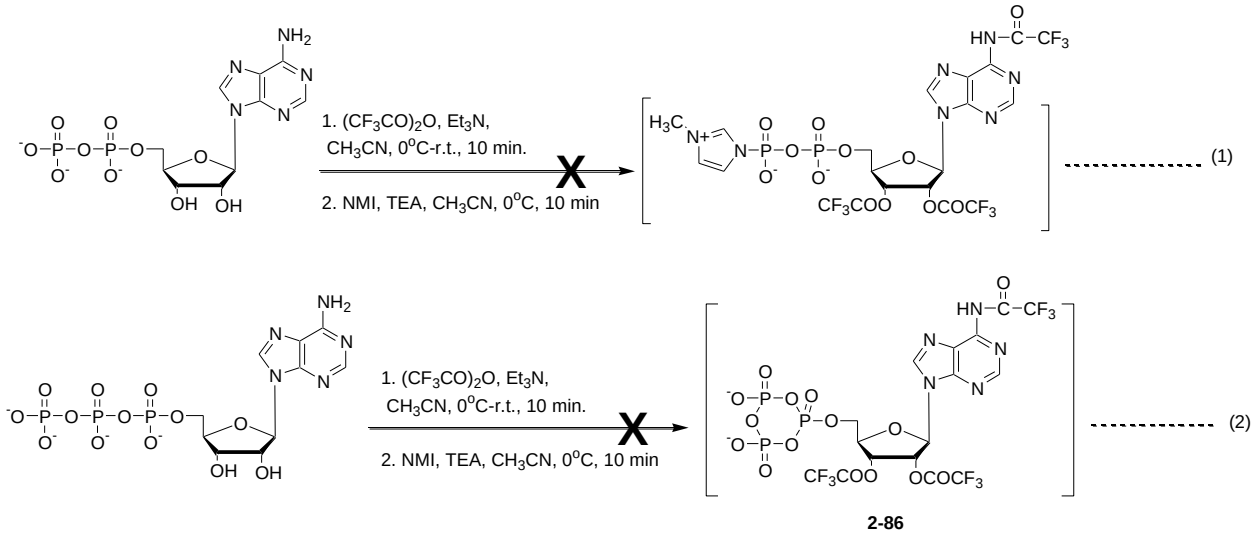
The reactions could be performed at 0 ^{o}C, as typical for the reaction of 5'-monophosphate –*N*-methylimidazolium salts with nucleophiles.[8,120,121,126] However, it was found that performing the reaction at room temperature led to a reduction of reaction times without affecting the yields. It was previously reported by Bogachev that for phosphate couplings the reactions were enhanced by the addition of *N*,*N*-dimethylaniline (DMA).[8] For most of the compounds we did not find DMA to be necessary, as did Mohamady and Jakeman,[127] though for couplings involving guanosine the addition of DMA was found to be beneficial. ^{31}P NMR analysis of the reaction mixture revealed that the reaction was usually complete within 1.5 h. Dimerized donor was often formed as a minor by-product for compounds **2-78** to **2-85.** When the reaction was complete it was quenched by the addition of aq. ammonium acetate, and the resulting solution was washed with chloroform, freeze-dried, and purified by RP-HPLC.

Having demonstrated that Bogachev's procedure worked for synthesisizing Np_nN's, the method was then applied it to the synthesis of compound **2-4** (Scheme 2.25). The formation of the mixed anhydride of compound **2-3** was considerably slower than with the above mentioned NMP's, requiring 25 min as determined by ^{31}P NMR with the starting triplet appearing at δ_p 2.5 ppm and the mixed anhydride intermediate appearing at δ_p -1.62 ppm. The slower reaction with compound **2-3** is most likely due to the electron-withdrawing effect of the fluorines making the phosphonyl anion a much poorer nucleophile. The *N*-methylimidazolium salt, **2-74**, which had chemical shift of δ_p -4.0 ppm, formed readily. Reaction of **2-74** with ADP gave compound **2-4** in a 44 % yield.



Scheme 2.25. Synthesis of compound **2-4.**

We also attempted the formation of the imidazolium salts of ADP under the same conditions used for activation of NMP's but the reaction did not work and decomposition of the ADP occurred (Scheme 2.26, equation 1). The same thing happened with ATP, in that we did not get the 5'-adenosine trimetaphosphate **2-86** but we had decomposition of the ATP (Scheme 2.26, equation 2).



Scheme 2.26. Attempted activation of ADP and ATP.

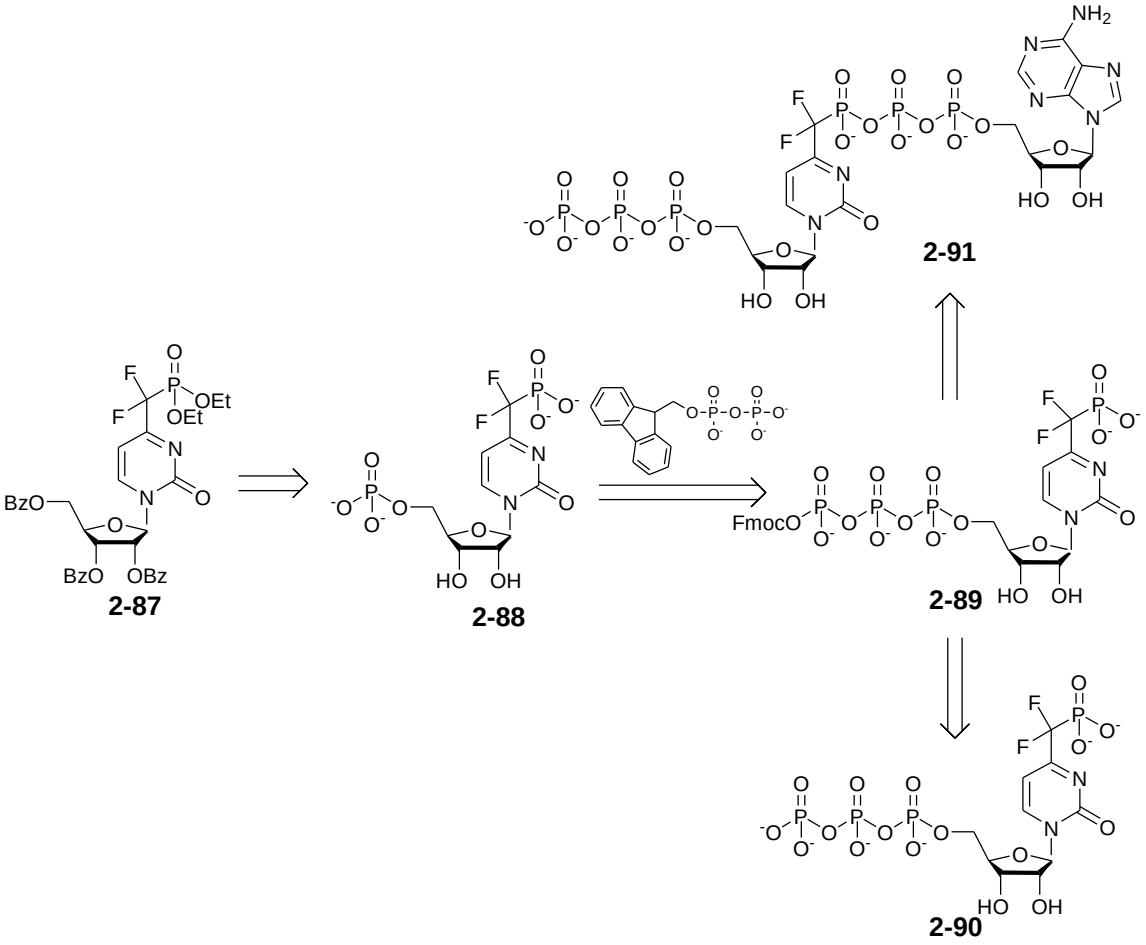
2.4 Conclusions and future work.

In this study, it has been demonstrated that Bogachev's method for nucleoside polyphosphate synthesis can be applied to the synthesis of Np_nN's, where n = 2-4, as well as compound **2-4**. The yields obtained for symmetrical and unsymmetrical Np_2N's (Table 2.5,

entries 1-6) are greater than 60% which is reasonable; however, as shown in section 2.1.3, there are literature methods that produce these compounds in better yields though the method reported here is faster than most. Moreover, symmetrical Np_2N's cannot be produced via one-pot dimerization of their NMP's using this procedure. The yields of the Np_3N's and Np_4N's using this procedure (Table 2.5, entries 7-11) are comparable to the best chemical methods reported in the literature and the process is considerably faster. Hence, this is a good approach to these compounds. Attempts to prepare the activated forms of ADP's and ATP's led to decomposition of these nucleotides, hence the method is limited to a maximum of Np_4N's.

Compound **2-4** was evaluated for its ability to inhibit CTPS in the laboratory of Prof. Stephen Bearne at Dalhousie University, who is an expert on CTPS and CTPS inhibitors. Unfortunately, this compound was a weak CTPS inhibitor with an IC_{50} of only 400 μM. One possible reason for this is that the compound does not have a triphosphate moiety at the 5'-position of the "uridine" portion. It has been shown that the triphosphate group in UTP is essential for UTP binding to CTPS. Another possible reason for this is the lack of a nitrogen at the 3-position of the uracil ring.[128] Compound **2-91** (Scheme 2.27) may prove to be a much more potent inhibitor. A possible retrosynthetic route to compound **2-91**, as well as compound **2-90**, is shown in Scheme 2.27. This route takes advantage of the differences in reactivity between the 5'-phosphate group and fluorophosphonate group in **2-90**. A phosphate group will need to be introduced into the 5'-position of compound **2-87** and the resulting product deprotected to give **2-88**.[60] Coupling Fmoc-protected pyrophosphate[129] to **2-88** should occur much faster at the more reactive 5'-phosphate group to give **2-89** as the dominant product. Deprotection of **2-89** would give **2-90**. Coupling of **2-89** to ADP followed by deprotection would give **2-91**. Should this approach prove successful and compounds **2-90** and/or **2-91** prove to be good CTPS inhibitors,

then the crystal structure of them bound to CTPS will be obtained by another collaborator, crystallographer Enoch Baldwin (UC Davis), who determined the structure of E.coli CTPS.[38,39] These studies will provide a wealth of information about the interactions that occur between CTPS and its substrates and intermediates. This information will be invaluable for future drug development.



Scheme 2.27. Proposed route for the synthesis of compound **2-91**.

2.5 Experimental

2.5.1 General information

All reagents and starting nucleotides were obtained from commercial sources unless stated otherwise. Before using the commercial sodium salts or free acids of the nucleotides, they were suspended in dry acetonitrile, the acetonitrile was removed by rotary evaporation, and the resulting residue was subjected to high vacuum for 3 h. Acetonitrile and DMF were distilled from calcium hydride. Triethylamine was distilled from sodium hydroxide. NMI was stored over 4 Å molecular sieves. TFAA (Aldrich Chemical Company) was used without distillation. All reactions were monitored by ^{31}P-NMR and carried out under argon. All NMR spectra were recorded using deuterium oxide as solvent. For ^{1}H NMR, chemical shifts are reported in ppm relative to the solvent residual peak (δ 4.79). For proton-decoupled ^{13}C NMR spectra chemical shifts are reported in ppm relative to CH_3OH in D_2O (δ 49.5, external standard). For proton-decoupled ^{31}P-NMR chemical shifts are reported in ppm relative to aqueous 85% H_3PO_4 (δ 0 ppm, external standard). For proton-decoupled ^{19}F-NMR, chemical shifts are reported in ppm relative to $CFCl_3$ (δ 0 ppm, external standard). Preparative HPLC was performed using a C-18 reverse phase (250 x 20 mm) semipreparative column.

2.5.2 Preparation of alkyl ammonium salts of nucleotides

Tri-*n*-butylammonium salts of UMP and GMP. These were prepared from UMP disodium salt dihydrate (110 mg, 0.27 mmol) and GMP disodium salt hydrate (54 mg, 0.1335 mmol) by dissolving them in water (5 mL) and passing the resulting solutions through Dowex-50-W (H^+ form) into a flask containing 10 mL of ethanol and 0.5 mL of tri-*n*-butylamine. The resin was washed with water (3 x 5 mL). The resulting solutions were stirred for few minutes and

then concentrated by high vacuum rotary evaporation. Residual water was removed by coevaporation with toluene (3 x 2 mL).

Tri-*n*-butylammonium salt of AMP. This was prepared from AMP free acid monohydrate (100 mg, 0.27 mmol) by the addition of tri-*n*-butylamine (0.25 mL) in 1 mL of DMF, and the mixture was stirred over 4 Å molecular sieves under argon overnight.

Trialkylammonium salts of ADP and GDP. These were prepared by dissolving ADP disodium salt dehydrate (100 mg, 0.20 mmol) and GDP disodium salt (97 mg, 0.20 mmol) in a few milliliters of water and passed through Dowex-50-W H^+ form into a flask containing 10 mL of ethanol and 0.30 mL of tri-*n*-butylamine. The resin was washed with water (3 x 5 mL). the resultant solution was stirred for a few minutes and then concentrated by high vacuum rotary evaporation. Residual water was removed by coevaporation with toluene (3 x 2 mL). The residue was dissolved in dry DMF (0.75 mL). Triethylamine (0.10 mL) was added which resulted in the formation of a precipitate. A few 4 Å molecular sieves (beads) were added, and the resulting suspension was stirred under argon for 16 h whereby the initially formed precipitate dissolved. This solution was used directly for the coupling reactions.

Triethylammonium salt of UDP. This salt was prepared by reacting the *N*-methylimidazolium salt of UMP (prepared using the general procedure described under 2.3.2) with the tetrabutylammonium salt of inorganic phosphate (3 equiv.) in dry acetonitrile at 0 ^oC for 1.5 h. The reaction was quenched by the addition of pH 7 aqueous 250 mM ammonium acetate. The resulting solution was then washed with 10 mL water, and centrifuged and the supernatant was purified by RP-HPLC (C18 column) using 9% CH_3CN-91% 100 mM triethylammonium acetate (pH 7) as eluent. This material was freeze-dried several times until ^1H NMR showed disappearance of the acetate CH_3 singlet at 1.78 ppm.

Tri-*n*-butylammonium salt of ATP. Was prepared by dissolving ATP trisodium salt (126 mg, 0.2 mmol) in a few milliliters of water and passed through freshly prepared Dowex-50-W (pyridinium) form into a flask containing 10 mL of ethanol and 0.3 mL of tri-*n*-butylamine. The resin was washed with water (3 x 5 mL). The resultant solution was stirred for a few minutes and then concentrated by high vacuum rotary evaporation. Residual water was removed by coevaporation with toluene (3 x 2 mL) and subjected to high vacuum overnight. The residue was then dissolved in 1.5 mL of dry DMF and 4 Å molecular sieves.

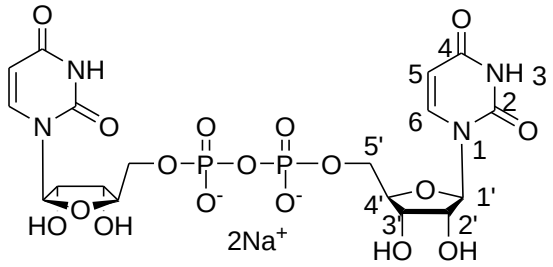
2.5.3 General procedure for the preparation of Np_nN's

NMP (1 equiv.), as its free acid hydrate, disodium salt hydrate, or tri-*n*-butylammonium salt, was suspended in a mixture of acetonitrile (1 mL of CH_3CN/100 mg of NMP) containing triethylamine (16.1 equiv. for all compounds except GMP, where 5 equiv. is used). When GMP was used, 3 equiv. of DMA was added. The mixture was cooled to 0 ^oC using an ice-bath and stirred under argon. Trifluoroacetic anhydride (16 equiv. for the sodium salts, 8 equiv. for the tri-*n*-butylammonium salt of UMP, and 5 equiv. for the tri-*n*-butylammonium salt of GMP) was added dropwise over a period of 1 min. The mixture was stirred for 10 min at room temperature (25 min for compound **2-14**) and then concentrated using a rotary evaporator (aspirator pressure). The residue was cooled to 0 ^oC using an ice-bath and dissolved in dry acetonitrile (0.4 mL/100 mg NMP) under an argon atmosphere. Triethylamine (10.6 equiv. for all compounds except GMP, where 3 equiv. was used) was added followed by *N*-methylimidazole (5.5 equiv.). The reaction was allowed to stir for 10 min at 0 ^oC, after which time a bright yellow solution was obtained. This solution was added over 1 min to a flask containing the tri-*n*-butylammonium or triethylammonium salt of nucleoside mono-, -di-, or –triphosphate acceptor (1.2-2 equiv.) in dry DMF (1-1.5 mL/100 mg acceptor) and 4 Å molecular sieves. The mixture was stirred for 1.5-2 h

under argon and then quenched with aqueous ammonium acetate (250 mM, pH 7, 6 mL/100 mg 5'-NMP). The solution was washed with chloroform and freeze-dried. The resulting yellowish powder was diluted with water (6 mL/100 mg NMP) and centrifuged using a desktop microcentrifuge for a few minutes. The supernatant was subjected to RP-HPLC using a semipreparative C18 column and a gradient of acetonitrile and buffer (100 mM triethylammonium acetate, pH 7.0 or pH 9.0) at 6 mL/min and monitored at 255 and 280 nm. Fractions containing the desired product were pooled, concentrated by high vacuum rotary evaporation, and the residue was dissolved in water and repeatedly freeze-dried until the ^{1}H NMR spectrum indicated that no residual buffer was present. The resulting white powder was converted to sodium salt using a Dowex-50-W ion-exchange resin in Na^{+} form.

2.5.4 Characterization data

P^{1}, P^{2}-Diuridine-5`-diphosphate, disodium salt (2-75).

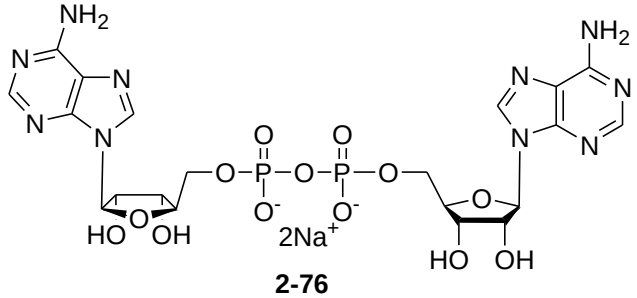


2-75

UMP disodium salt hydrate (55 mg, 0.135 mmol) was used as the donor precursor. The tri-*n*-butylammonium salt of UMP was used as the acceptor. Pure **2-75** was obtained in 64% yield (58 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 8% CH_3CN-92% buffer over 35 min, t_r = 29 min] followed by passage through a Dowex-50-W-Na^{+} ion exchange column. ^{1}H NMR (D_2O, 300 MHz): δ 4.00-4.24 (m, 10H, $H_{2',3',4',5'}$), 5.81 (d, J = 7.8 Hz, 2H, H_5), 5.83 (s, 2H, $H_{1'}$), 7.78 (d, *J* = 7.8 Hz, 2H, H_6). ^{13}C NMR (D_2O, 75 MHz): δ 64.8, 69.5, 73.7, 83.1 (t, *J* = 4.5 Hz), 88.3, 102.6, 141.5, 151.7, 166.10. ^{31}P NMR (D_2O, 121

MHz): δ -9.78 (s). TOF MS ES-, *m/z* 629.05 $[M-H]^-$, HRMS (TOF MS ES-) *m/z* = 629.0521, $C_{18}H_{23}N_4O_{17}P_2$ $[M-H]^-$ requires 629.0533.

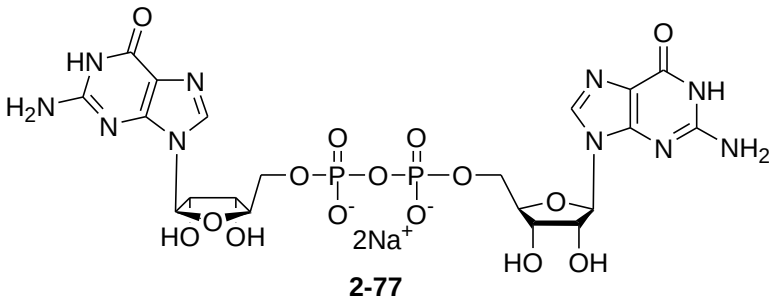
P^1, P^2-Diadenosine-5`-diphosphate, disodium salt (2-76).



2-76

AMP free acid monohydrate (50 mg, 0.135 mmol) was used as the donor precursor. The tri-*n*-butylammonium salt of AMP (2 equiv.) was used as the acceptor. Pure **2-76** was obtained in 61% yield (59 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 10% CH_3CN-90% buffer over 45 min. and then a linear gradient to 20% CH_3CN-80% buffer over 5 min, t_r = 43 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^1H NMR (D_2O, 300 MHz): δ 4.05-4.15 (m, 2H, $H_{4`}$), 4.15-4.25 (m, 4H, $H_{5`}$), 4.25-4.35 (m, 2H, $H_{3`}$), 4.40-4.50 (m, 2H, $H_{2`}$) 5.80 (two overlapping sinlglets, 2H, $H_{1`}$), 7.9 (s, 2H, H_2), 8.1 (s, 2H, H_8). ^{13}C NMR (D_2O, 75 MHz): δ 65.0, 69.8, 74.5, 83.4 (t, *J* = 4.5 Hz), 87.1, 117.5, 139.6, 147.8, 150.7, 153.5. ^{31}P NMR (D_2O, 121 MHz): δ -9.62 (s). TOF MS ES+, *m/z* 677.12 $[M+H]^+$, HRMS (TOF MS ES+) *m/z* = 677.1224, $C_{20}H_{27}N_{10}O_{13}P_2$ $[M+H]^+$ requires 677.1234.

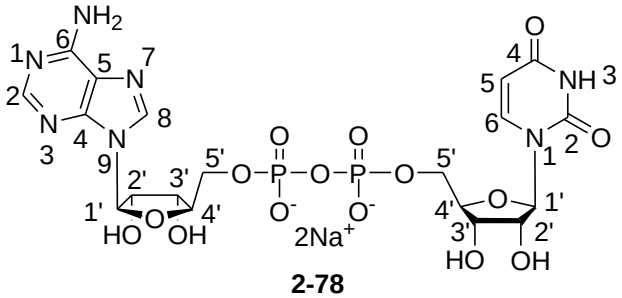
P^1, P^2-Diguanosine-5`-diphosphate, disodium salt (2-77).



2-77

GMP disodium salt hydrate (54 mg, 0.135 mmol) was converted into its tri-*n*-butylammonium salt and used as the donor precursor and acceptor (1.5 equiv.). Pure **2-77** was obtained in 68% yield (69 mg) after purification by RP-HPLC [linear gradient of 2% CH_3CN-98% buffer (pH 7.0) to 10% CH_3CN-90% buffer over 50 min, t_r = 40 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^1H NMR (D_2O, 300 MHz): δ 4.06 (s, 2H, $H_{4'}$), 4.15 (s, 4H, $H_{5'}$), 4.29 (t, 2H, *J* = 4.7 Hz, $H_{3'}$), 4.47 (t, 2H, *J* = 5.1 Hz, $H_{2'}$), 5.63 (d, *J* = 5.0 Hz (m, 2H, $H_{1'}$), 7.79 (s, 2H, H_8). ^{13}C NMR (D_2O, 75 MHz): δ 64.9, 69.9, 73.9, 83.1 (t, *J* = 4.4 Hz), 87.2, 115.9, 151.1, 153.6, 158.5. ^{31}P NMR (D_2O, 121 MHz): δ -9.69 (s). TOF MS ES-, *m/z* 707.08 $[M-H]^-$, HRMS (TOF MS ES-) *m/z* = 707.0983, $C_{20}H_{25}N_{10}O_{15}P_2$, $[M-H]^-$, requires 707.0976.

P^1-Adenosine, P^2-Uridine-5`-diphosphate, disodium salt (2-78).

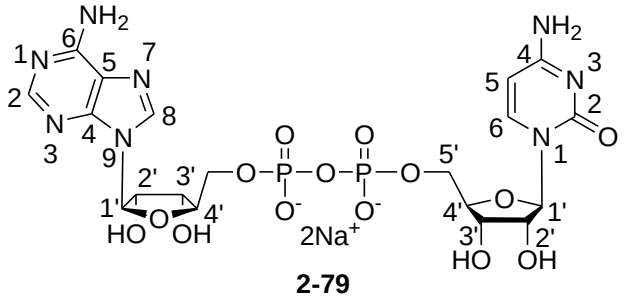


2-78

UMP disodium salt dihydrate (55 mg, 0.135 mmol) was used as the donor precursor. The tri-*n*-butylammonium salt of AMP (2 equiv.) was used as the acceptor. Pure **2-78** was obtained in 66% yield (62 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 9% CH_3CN-91% buffer over 55 min then a linear gradient to 20% CH_3CN-80% buffer over 5 min. t_r = 42 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^1H NMR (D_2O, 300 MHz): δ 3.95-4.20 (m, 7H, $H_{2'U,3'U,4'U,5'U,5'A}$), 4.24 (bs, 1H, $H_{4'A}$), 4.38 (bs, 1H, $H_{3'A}$), 5.52 (d, *J* = 7.8 Hz, 1H, H_{5U}), 5.66 (d, *J* = 3.9 Hz, 1H, $H_{1'U}$), 5.92 (d, *J* = 5.9 Hz, 1H,

$H_{1'A}$), 7.49 (d, *J* = 7.8 Hz, 1H, H_{6U}), 8.02 (s, 1H, H_{2A}), 8.28 (s, 1H, H_{8A}). ^{13}C NMR (D_2O, 75 MHz): δ 64.8, 65.2, 69.4, 70.3, 73.9, 74.1, 82.9, 83.7, 86.7, 88.2, 102.1, 118.3, 139.5, 140.8, 148.9, 151.3, 152.7, 155.3. 165.6. ^{31}P NMR (D_2O, 121 MHz): δ – 9.71. TOF MS ES-, *m/z* 652.06 $[M-H]^-$, HRMS (TOF MS ES-) *m/z* = 652.0788, $C_{19}H_{24}N_7O_{15}P_2$ $[M-H]^-$ requires 652.0806.

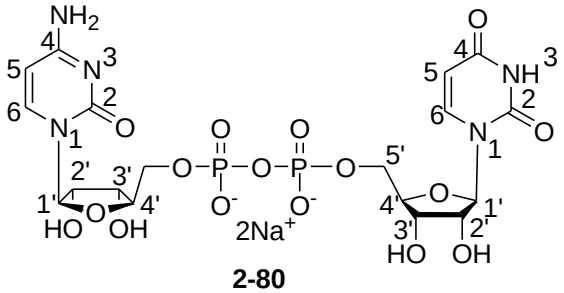
P^1-Adenosine, P^2-Cytidine-5`-diphosphate, disodium salt (2-79)



2-79

CMP disodium salt dihydrate (55 mg, 0.135 mmol) was used as the donor precursor. The tri-*n*-butylammonium salt of AMP (2 equiv.) was used as the acceptor. Pure **2-79** was obtained in 60% yield (56 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 9.0) to 5% CH_3CN-95% buffer over 55 min, t_r = 34 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^1H NMR (D_2O, 300 MHz): δ 3.85-4.20 (m, 7H, $H_{2'C,3'C,4'C5'C,5'A}$), 4.24 (bs, 1H, $H_{4'A}$), 4.35-4.42 (m, 1H, $H_{3'A}$), 4.65 (m, 1H, $H_{2'A}$), 5.60-5.73 (m, 2H, $H_{1'C,5C}$), 5.88 (d, *J* = 5.6 Hz, 1H, $H_{1'A}$), 7.54 (d, *J* = 7.8 Hz, 1H, H_{6C}), 7.94 (s, 1H, H_{2A}), 8.23 (s, 1H, H_{8A}). ^{13}C NMR (D_2O, 75 MHz): δ 64.4, 65.2, 68.8, 70.3, 74.1, 74.3, 82.4 (t, *J* = 4.0 Hz), 83.7 (t, *J* = 3.5 Hz), 86.8, 89.2, 118.1, 139.7, 141.3, 148.7, 151.9, 154.6, 154.7, 163.5. ^{31}P NMR (D_2O, 121 MHz): δ – 9.66 (s). TOF MS ES-, *m/z* 651.09 $[M-H]^-$, HRMS (TOF MS ES-) m/z = 651.0955, $C_{19}H_{25}N_8O_{14}P_2$ $[M-H]^-$ requires 651.0965.

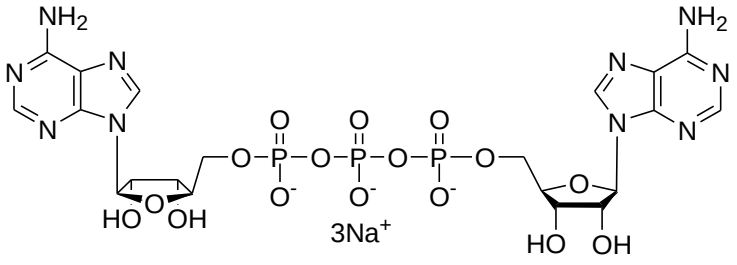
P[1]-Cytidine, P[2]-Uridine-5`-diphosphate, disodium salt (2-80)



2-80

CMP disodium salt dihydrate (55 mg, 0.135 mmol) was used as the donor precursor. The tri-*n*-butylammonium salt of UMP (2 equiv.) was used as the acceptor. Pure **2-80** was obtained in 67% yield (61 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 8% CH_3CN-92% buffer over 45 min, t_r = 33 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^1H NMR (D_2O, 300 MHz): δ 4.04 (m, 10 H, $H_{2',3',4',5}$), 5.77-5.88 (m, 3H, $H_{1',5C}$), 5.94 (d, *J* = 7.4 Hz, 1H, H_{5U}), 7.87 (t, *J* = 7.7 Hz, 2H, H_6). ^{13}C NMR (D_2O, 75 MHz): δ 64.5, 64.8, 69.1, 69.5, 73.7, 74.2, 82.4 (t, *J* = 4.0 Hz), 83.0 (t, *J* = 4.0 Hz), 88.3, 89.2, 96.4, 102.53, 141.3, 141.4, 151.6, 157.3, 165.8, 165.9. ^{31}P NMR (D_2O, 121 MHz): δ -9.72 (s). TOF MS ES-, *m/z* 628.04 $[M-H]^-$, HRMS (TOF MS ES-) *m/z* = 628.0681, $C_{18}H_{24}N_5O_{16}P_2$ $[M-H]^-$ requires 628.0693.

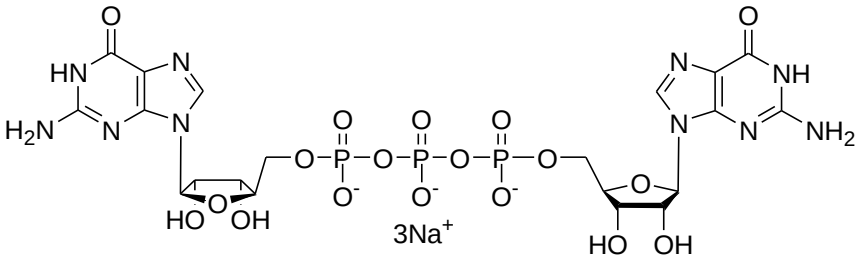
P[1], P[3]-Diadenosine-5`-triphosphate, trisodium salt (2-81)



2-81

AMP free acid monohydrate (50 mg, 0.135 mmol) was used as the donor precursor. The trialkylammonium salt of ADP (1.5 equiv.) was used as the acceptor. Pure **2-81** was obtained in 51% yield (57 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 9% CH_3CN-91% buffer over 45 min. then a linear gradient to 20% CH_3CN-80% buffer over 5 min, t_r = 47 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^1H NMR (D_2O, 300 MHz): δ 4.16-4.24 (m, 6H, $H_{4',5'}$), 4.30 (t, *J* = 4.4 Hz, 2H, $H_{3'}$), 4.38 (t, *J* = 4.9 Hz, 2H, $H_{2'}$) 5.77 (d, *J* = 3.9 Hz, 2H, $H_{1'}$), 7.80 (s, 2H, H_2), 8.07 (s, 2H, H_8). ^{13}C NMR (D_2O, 75 MHz): δ 64.5, 64.6, 69.4, 74.7, 82.8, 82.9, 87.3, 117.5, 139.5, 147.9, 152.1, 154.4. ^{31}P NMR (D_2O, 121 MHz): δ -9.79 (d, *J* = 17.8 Hz), -21.02 (t, *J* = 17.8 Hz). TOF MS ES-, *m/z* 755.03 $[M-H]^-$, HRMS (TOF MS ES-) *m/z* = 755.0748, $C_{20}H_{26}N_{10}O_{16}P_3$ $[M-H]^-$ requires 755.0741.

P^1, P^3-Diguanosine-5`-triphosphate, trisodium salt (2-82).

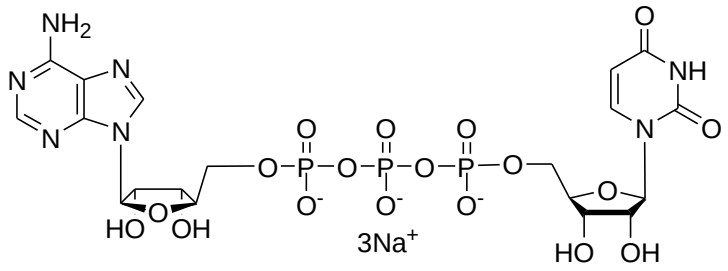


2-82

GMP disodium salt hydrate (54 mg, 0.135 mmol) was converted into its tri-*n*-butylammonium salt and used as the donor precursor. The trialkylammonium salt of GDP (1.5 equiv.) was used as the acceptor. Pure **2-82** was obtained in 53% yield (61 mg) after purification by RP-HPLC [linear gradient of 1% CH_3CN-99% buffer (pH 7.0) to 8% CH_3CN-92% buffer over 60 min then a linear gradient to 100% CH_3CN over 10 min, t_r = 55 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^1H NMR (D_2O, 300 MHz): δ 4.11 (m, 6H, $H_{4',5'}$), 4.32 (t, 2H, *J* = 4.7 Hz, $H_{3'}$), 4.48 (t, 2H, *J* = 5.2 Hz, $H_{2'}$), 5.66 (d, 2H, *J* = 5.2 Hz, $H_{1'}$), 7.84 (s, 2H, H_8). ^{13}C NMR (D_2O, 75 MHz): δ 64.68, 64.74, 69.8, 73.8, 83.2, 87.2, 115.8,

151.2, 153.6, 158.5. ^{31}P NMR (D_2O, 121 MHz): δ -10.77 (d, *J* = 18 Hz), -22.18 (t, *J* = 18 Hz). TOF MS ES-, *m/z* 787.14 $[M-H]^-$, HRMS (TOF MS ES-) *m/z* = 787.0631, $C_{20}H_{26}N_{10}O_{18}P_3$, $[M-H]^-$, requires 787.0639.

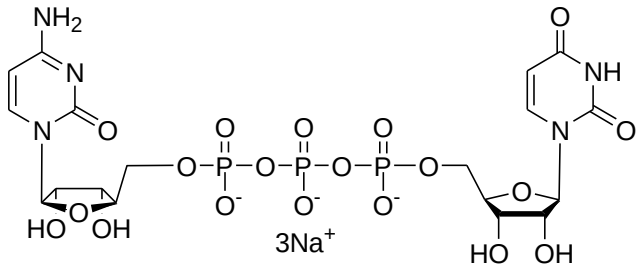
P^1-Adenosine, P^3-Uridine-5`-triphosphate, trisodium salt (2-83).



2-83

UMP disodium salt dihydrate (55 mg, 0.135 mmol) was used as the donor precursor. The trialkylammonium salt of ADP (1.2 equiv.) was used as the acceptor. Pure **2-83** was obtained in 56% yield (61 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 8% CH_3CN-92% buffer over 45 min then a linear gradient to 10% CH_3CN-90% buffer over 10 min. t_r = 48 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^{1}H NMR (D_2O, 300 MHz): δ 4.07-4.20 (m, 7H, $H_{2'U,3'U,4'U,5'U,5'A}$), 4.25 (bs, 1H, $H_{4'A}$), 4.40 (brt, *J* = 3.9 Hz,1H, $H_{3'A}$), 5.57 (d, *J* = 7.8 Hz, 1H, H_{5U}), 5.68 (d, *J* = 3.9 Hz, 1H, $H_{1'U}$), 5.92 (d, *J* = 5.4 Hz, 1H, $H_{1'A}$), 7.62 (d, *J* = 8.3 Hz, 1H, H_{6U}), 8.00 (s, 1H, H_{2A}), 8.32 (s, 1H, H_{8A}). ^{13}C NMR (D_2O, 75 MHz): δ 64.49 (d, *J* = 5.1 Hz), 65.2(d, *J* = 5.1 Hz), 69.1, 70.3, 73.9, 74.6, 82.7 (d, *J* = 9.2 Hz), 83.6 (d., *J* = 8.6 Hz), 86.7, 88.3, 102.1, 118.2, 139.5, 140.9, 148.7, 151.3, 152.6, 155.1, 165.6. ^{31}P NMR (D_2O, 121 MHz): δ -9.88 (d, *J* = 17.8 Hz), -21.46 (t, *J* = 17.8 Hz). TOF MS ES-, *m/z* 732.03 $[M-H]^-$, HRMS (TOF MS ES-) *m/z* = 732.0440, $C_{19}H_{25}N_7O_{18}P_3$ $[M-H]^-$ requires 732.0469.

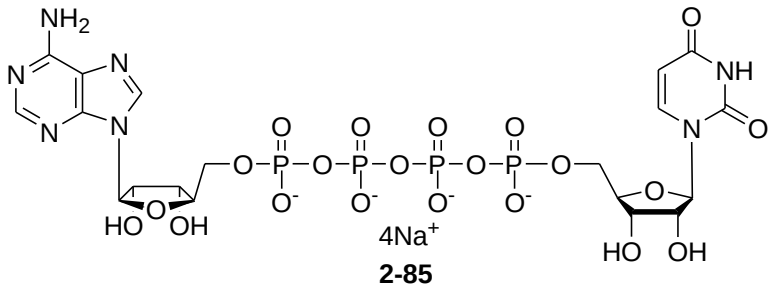
P^1-Cytidine, P^3-Uridine-5`-triphosphate, trisodium salt (2-84).



2-84

CMP disodium salt dihydrate (55 mg, 0.135 mmol) was used as the donor precursor. The triethylammonium salt of UDP (1.5 equiv.) was used as the acceptor. Pure **2-84** was obtained in 62% yield (64 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 10% CH_3CN-90% buffer over 45 min, t_r = 35 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^{1}H NMR (D_2O, 300 MHz): δ 4.13 (br, 10 H, $H_{2',3',4',5}$), 5.81 (d, J = 4.4 Hz, 3H, $H_{1',5C}$), 6.03 (d, *J* = 7.6 Hz, 1H, H_{5U}), 7.80 (t, *J* = 8.3 Hz, 1H, H_{6C}). 7.89 (d, J = 7.5 Hz, 1H, H_{6U}). ^{13}C NMR (D_2O, 75 MHz): δ 64.4 (d, *J* = 5.1 Hz), 64.8 (d, *J* = 5.6 Hz), 69.0, 69.5, 73.8, 74.3, 82.6 (d, *J* = 9.1 Hz), 83.1 (d, *J* = 9.1 Hz), 89.2, 96.1, 102.6, 141.5, 142.1, 151.7, 155.1, 164.1, 166.0. ^{31}P NMR (D_2O, 121 MHz): δ -9.73 (d, *J* = 22.2 Hz), -21.30 (t, *J* = 17.8 Hz). TOF MS ES-, *m/z* 708.03 $[M-H]^-$, HRMS (TOF MS ES-) *m/z* = 708.0367, $C_{18}H_{25}N_5O_{19}P_3$ $[M-H]^-$ requires 708.0357.

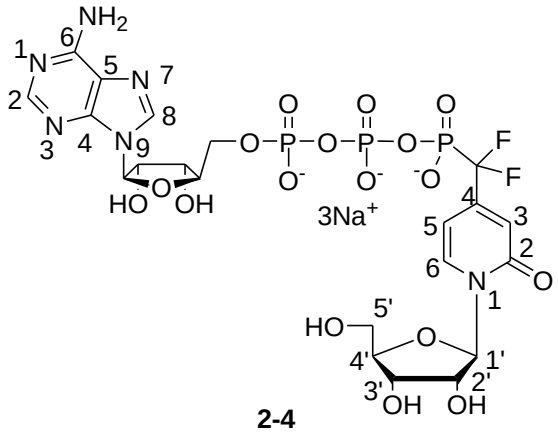
P^1-Adenosine, P^4-Uridine-5`-tetraphosphate, tetrasodium salt (2-85).



2-85

UMP disodium salt dihydrate (55 mg, 0.135 mmol) was used as the donor precursor. The tri-*n*-butylammonium salt of ATP (1.5 equiv.) was used as the acceptor. Pure **2-85** was obtained in 60% yield (73 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 10% CH_3CN-90% buffer over 45 min then a linear gradient to 20% CH_3CN-80% buffer over 10 min. t_r = 43 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^1H NMR (D_2O, 300 MHz): δ 4.05-4.30 (m, 8H, $H_{4'A,\ 5'A,2'U,3'U,4'U,5'U}$), 4.43 (t, *J* = 4.0 Hz,1H, $H_{3'A}$), 4.62 (d, *J* = 5.6 Hz, 1H, $H_{2'A}$), 5.68 (d, *J* = 8.0 Hz, 1H, H_{6U}), 5.75 (d, *J* = 4.9 Hz, 1H, $H_{1'U}$), 5.94 (d, *J* = 5.9 Hz, 1H, $H_{1'A}$), 7.69 (d, *J* = 8.0 Hz, 1H, H_{6U}), 8.00 (s, 1H, H_{2A}), 8.37 (s, 1H, H_{8A}). ^{13}C NMR (D_2O, 75 MHz): δ 64.9 (d, *J* = 5.1 Hz), 65.2 (d, *J* = 5.1 Hz), 69.5, 70.4, 74.0, 74.3, 83.1 (d, *J* = 8.6 Hz), 84.0 (d., *J* = 8.6 Hz), 86.6, 88.0, 102.4, 118.4, 140.0, 141.3, 148.9, 151.6, 152.0, 154.9, 165.8. ^{31}P NMR (D_2O, 121 MHz): δ -9.68 (d, *J* = 17.8 Hz), -21.32 (d, *J* = 13.7 Hz). TOF MS ES-, *m/z* 812.02 $[M-H]^-$, HRMS (TOF MS ES-) *m/z* = 812.0127, $C_{19}H_{26}N_7O_{21}P_4$ $[M-H]^-$ requires 812.0132.

2.5.5 Synthesis of 5'-Adenosinyl, [3-Deaza, 4-deoxy-uridine-4-[difluoromethyl]-phosphonyl]-diphosphate, trisodium salt (2-4)



2-4

3-Deaza-4-deoxyuridine-4-[difluoromethyl]phosphonic acid (40 mg, 0.1 mmol) was used as the donor precursor. The trialkylammonium salt of ADP (1.9 equiv.) was used as the acceptor.

Pure **2-4** was obtained in 44% yield (37 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 9% CH_3CN-91% buffer over 55 min then a linear gradient to 20% CH_3CN-80% buffer over 19 min. t_r = 54 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^1H NMR (D_2O, 300 MHz): δ 3.72 (d, *J* = 12.7 Hz, 1H, $H_{5'U}$), 3.8 (d, J = 12. 7.0 Hz, 1$H_{5'U'}$), 4.10 (m, 5H, $H_{5'A,2'U',3'U',4'U'}$), 4.24 (s, 1H, $H_{4'A}$), 4.41 (t, *J* = 4.2 Hz, 1H, $H_{3'A}$), 5.92 (s, 1H, $H_{1'U'}$), 5.95 (d, *J* = 5.9 Hz, 1H, $H_{1'A}$), 6.46 (d, *J* = 7.3 Hz, 1H, $H_{5'U'}$), 6.55 (s, 1H, $H_{4U'}$), 7.78 (d, *J* = 7.3 Hz, 1H, $H_{6U'}$), 8.07 (s, 1H, H_{2A}), 8.31 (s, 1H, H_{8A}). ^{13}C NMR (D_2O, 75 MHz): δ 60. 5, 65.3 (d, *J* = 5.5 Hz), 68.8, 70.2, 74.0, 74.8, 83.7 (d, *J* = 9.9 Hz), 86.9, 90.6, 105.5, 116.7, 118.5, 133.5, 139.9, 147.5 (m) 148.9, 152.7, 155.4, 162.9. ^{31}P NMR (D_2O, 121 MHz): δ -5.72 (td, *J* = 102.1 Hz, 26.7 Hz), -10.04 (d, *J* = 22.3). -22.00 (dd, *J* = 31.8 Hz, 18.5 Hz). ^{31}F NMR (D_2O, 121 MHz): δ -112.38 (dd, *J* = 101.2, 12.1 Hz). TOF MS ES-, *m/z* 765.01 $[M-H]^-$, HRMS (TOF MS ES-) *m/z* = 765.0511, $C_{21}H_{26}N_6O_{17}F_2P_3$ $[M-H]^-$ requires 765.0535.

Chapter 3

Sulfonyl Imidazolium Salts as Reagents for the Rapid and Efficient Synthesis of Nucleoside Polyphosphates and Their Conjugates

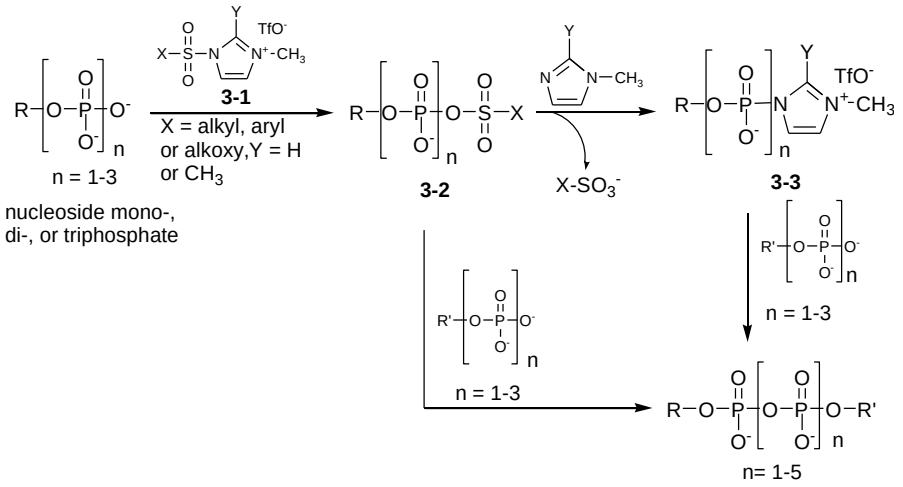
3.0 Introduction

3.1 Sulfuryl and sulfonyl imidazolium salts as activating agents for the synthesis of nucleoside polyphosphates and their conjugates

In chapter 2 a new method for preparing Np_nN's (n = 2-4) based on Bogachev's approach to dNTP synthesis was presented. Although it provided the desired compounds in reasonable yields it had its limitations. First, the yields of symmetrical Np_2N's were modest compared to the best literature methods. Although the yields of the Np_3N's and Np_4N's using this procedure were comparable to the best reported chemical methods, the yields were still modest (50-60%). Moreover, since this route could not be used to activate NDP's and NTP's the method cannot be used to prepare Np_nN's where n > 4. Also other groups reported that this method gives sugar nucleotides in low to modest yields (chapter 2, section 2.1.5). We wished to develop a more efficient route to not just Np_nN's but to other nucleoside polyphosphates and their conjugates as well.

In Bogachev's method the donor is a highly reactive phosphoryl imidazolium species. We wished to use this species as a donor but generate it without temporary protection of the hydroxyl or amino groups on the nucleoside donor which was accomplished in the Bogachev method by using an excess amount of TFAA which we believed was problematic when activating NDP's or NTP's. We reasoned that this could be achieved by using a sulfonyl- or sulfurylimidazolium salt of type **3-1** (Scheme 3.1) as an activating agent. The general approach would involve reacting a nucleoside mono-, di-, or triphosphate with a sulfonyl- or

sulfurylimidazolium salt of type **3-1** (Scheme 3.1). This would initially produce mixed anhydride **3-2**. This could potentially act as a donor and react with a phosphorylated acceptor to give the desired nucleoside polyphosphates and their conjugates. Alternatively, the released NMI could react with **3-2** to produce a highly reactive imidazolium salt of type **3-3** which could also act as a donor and react with acceptors to give the desired products. If NMI is added to the reaction mixture then it is possible that the reaction would proceed entirely via intermediate **3-3**.



Scheme 3.1. General procedure for preparing nucleoside polyphosphates and their conjugates using a sulfonyl- or sulfurylimidazolium salt of type **3-1.**

Compounds of type **3-1**, like sulfonyl chlorides, have been used as reagents for the sulfonation or sulfation of hydroxyl and amino groups.[130-132] Sulfonyl chlorides readily sulfonate the hydroxyl groups of carbohydrates and the hydroxyl and amino groups of nucleosides and nucleotides.[133,134] This suggests that imidazolium salts of type **3-1** might be problematic for the synthesis of nucleoside polyphosphates using unprotected substrates. However, we reasoned that the reaction between the negatively charged phosphate moiety and the positively charged sulfonyl imidazolium salt would be much faster than the reaction between the salt and neutral hydroxyl and amino groups of the nucleoside substrate producing the intermediate **3-2** in high

yield. We also expected that subsequent reaction between the charged donor and phosphate group of the acceptor would also be relatively rapid as was the case when using Bogachev's procedure.

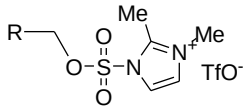
3.2 Objectives

The objective of the work presented in this chapter was to determine if compounds of type **3-1** can be used as activating agents for the efficient (rapid and high yielding) synthesis of nucleoside polyphosphates and their conjugates.

3.3. Results and discussion

3.3.1 Preliminary investigations using sulfuryl imidazolium salts as activating agents

We initially examined trichloroethyl (TCE) and trifluoroethyl (TFE) sulfurylimidazolium salts **3-4** and **3-5** as activating agents (Figure 3.1). These compounds were chosen because they were already available in the Taylor group having been prepared and used by the Taylor group as reagents for introducing protected sulfates into carbohydrates.[131]



3-4, R = CCl_3
3-5, R = CF_3

Figure 3.1. Structure of sulfurylimidazolium salts **3-4** and **3-5**.

We started our investigations by examining the one pot dimerization of NMP's (Scheme 3.2) using reagent **3-4** and UMP as a model nucleotide substrate. The reaction was performed by adding 1.0 equiv of **3-4** to 2.0 equiv of the tri-*n*-butylammonium salt of UMP in DMF in the presence of 3 equiv of NMI at 0 ^{o}C (ice-bath) then allowing the reaction to stir at room temperature. The reaction was monitored by ^{31}P NMR (Figure 3.2). The first spectrum, recorded 10 minutes into the reaction, showed a peak at -9.9 ppm which corresponded to the desired

product, Up_2U (**2-75**), along with peaks corresponding to unreacted **3-7** (-9.9 ppm) and UMP (2.0 ppm) and a small unidentified peak at -8.8 ppm. After 40 minutes the reaction was essentially complete in that almost all of the imidazolium intermediate was consumed. The reaction was then cooled to 0 ^{o}C, diluted with aq. ammonium acetate, washed with chloroform to remove excess base and purified by RP-HPLC which gave **2-75** in an 86% yield. Applying this procedure to the dimerization of AMP gave Ap_2A (**2.76**) in an equally good yield (Scheme 3.2). The isolated yields were considerably higher than those obtained using the Bogachev method discussed in chapter 2. Moreover, the reaction time was considerably shorter than the Bogachev method for preparing Np_2N's (90 min) (see section 2.4.3). This reduction in reaction time suggests that some of the mixed anhydride **3-6** might be reacting directly with the acceptor nucleotide. No sulphated products were observed.

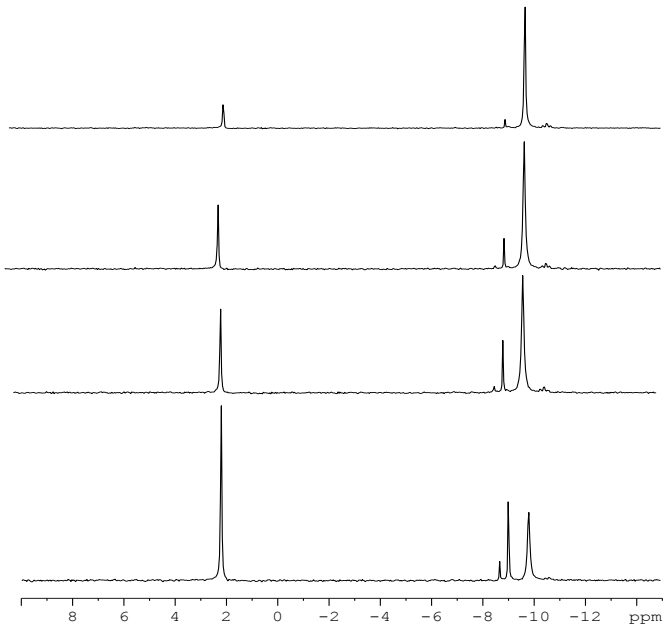
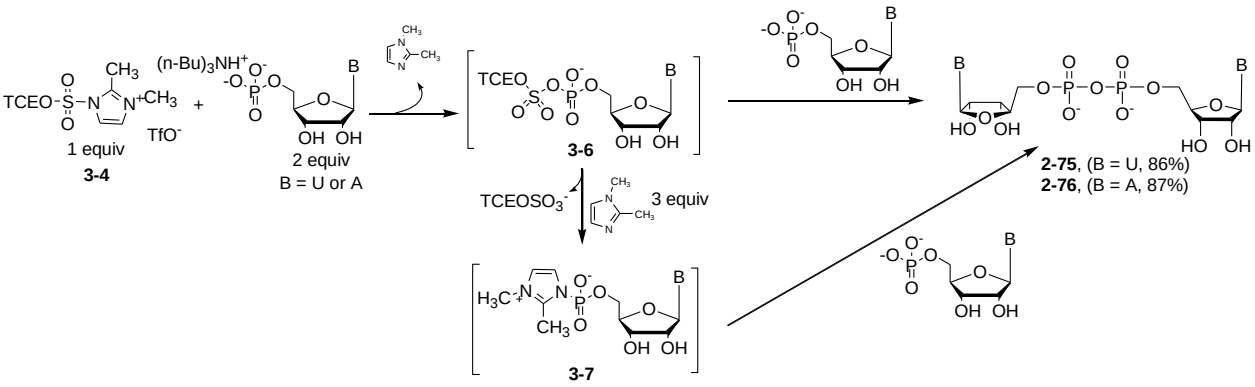


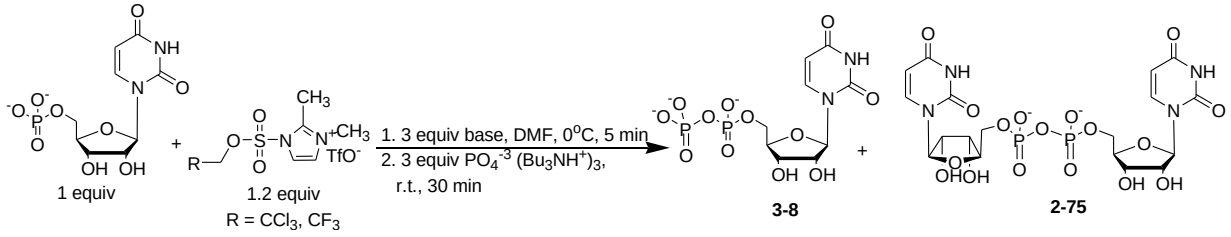
Figure 3.2. ^{31}P-NMR spectra of the dimerization reaction of UMP using **3-4**. Spectra were recorded at 10, 20, 30, and 40 minutes (from bottom to top). Peaks at 2.0, -9.0 and -9.9 ppm correspond to UMP, imidazolium intermediate **3-7** and, Up_2U respectively.



Scheme 3.2. Proposed mechanism for the dimerization of UMP and AMP using **3-4** as an activating agent and in the presence excess NMI.

To study the reaction in which the donor and acceptor were different we examined the synthesis of UDP from UMP and inorganic phosphate. The reaction was done in two steps. The first step was the addition of 1.2 equiv of **3-4** or **3-5** to the tri-*n*-butylammonium salt of UMP and a base in DMF at 0 ^{o}C. The solution was stirred for 5 min and then added to a stirred solution of tri-*n*-butylammonium salt of inorganic phosphate (Pi) and the reaction was stirred at room temperature and monitored by ^{31}P NMR. The results are shown in Table 3.1. The dimerized NMP was always formed as a by-product and with reagent **3-5** it was the dominant product. So in the 5 minutes before the Pi is added some of the unreacted UMP reacts with the mixed anhydride intermediate or imidazolium intermediate to give dimer. We hypothesized that if we could increase the rate of formation of the reactive intermediate(s) in comparison to the rate at which the intermediate(s) reacts with unreacted UMP then it might be possible to reduce the amount of dimer formed (since the UMP would be rapidly sequestered as the mixed anhydride intermediate or imidazolium intermediate) and increase the amount of UDP formed. This led us to the conclusion that the structure assigned to the sulfurylimidazolium salt had to be changed.

Table 3.1. Attempted synthesis of UDP using sulfurylimdazolium salts **3-4** and **3-5**.



entry	R	Base	Yield[a] of **3-8** (%)	Yield[a] of **2-75** (%)
1	CCl_3	NMI	52	48
2	CF_3	NMI	10	90
3	CCl_3	2,6-Lutidine	72	27
4	CF_3	2,6-Lutidine	20	80

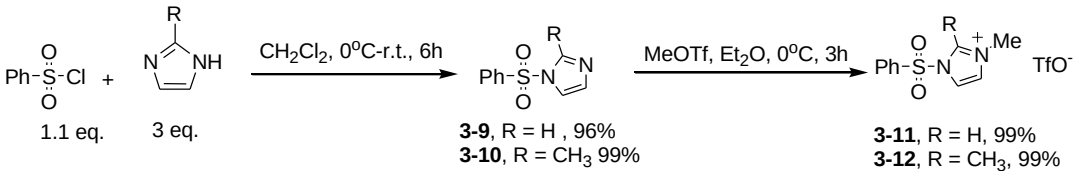
[a]Yields are calculated by integration of the ^{31}P-NMR peaks.

3.3.2 Preliminary investigations using sulfonylimidazolium salts as activating agents

We focussed our attention on using phenylsulfonyl imidazolium salts **3-11** and **3-12** (Scheme 3.3). Our previous studies on the reactivity of sulfonyl and sulfuryl imidazolium salts with carbohydrates indicated that sulfonyl imidazolium salts, such as **3-11**, were more reactive than sulfuryl imidazolium salts **3-4** and **3-5.**[135] The reasons for this are not entirely clear. Although sulfuryl imidazolium salts **3-4** and **3-5** have electron withdrawing TCE and TFE groups the ester oxygen can still donate electrons into the S=O by resonance and so perhaps making the sulfur atom less electrophilic in comparison to the sulfur atom in **3-11** (a benzene group is not considered to be highly electron donating (σ_p = -0.01)). There might also be some steric hindrance from the fluorines or chlorines in **3-4** and **3-5**. We also decided to examine the phenyl group simply because benzenesulfonyl chloride is inexpensive and readily available.

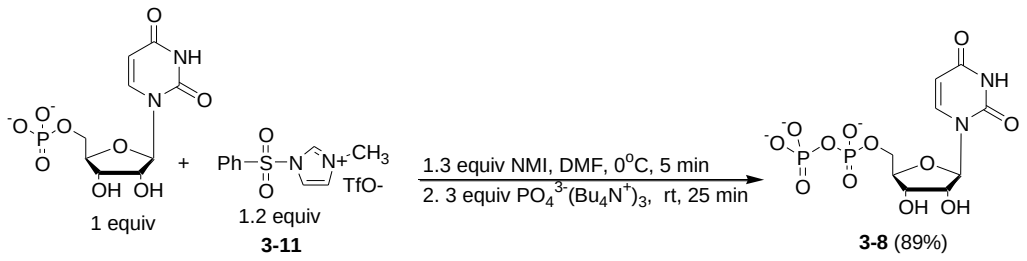
Benzenesulfonyl chloride was reacted with imidazole or 2-methylimidazole to give **3-9** and **3-10** in 96% and 99% yield respectively. Compounds **3-9** and **3-10** were reacted with

methyl triflate in anhydrous ether at rt which results in the precipitation of compound **3-11** and **3-12** as white powders. Filtration of the reaction mixture gave **3-11** and **3-12** in quantitative yield and no further purification was necessary. Compounds **3-11** and **3-12** can be stored under Ar or N_2 at -20 ^oC for months without any detectable decomposition. Compound **3-12** exhibited slightly better solubility properties in organic solvents, such as DMF, than compound **3-11** though both slowly decomposed when stored as solutions in organic solvents.



Scheme 3.3. Synthesis of sulfonyl imidazolium salts **3-11** and **3-12**.

The synthesis of UDP was attempted as before using compound **3-11** as the activating agent (Scheme 3.4). In this case, we did not observe any dimerization of UMP and obtained almost complete conversion to UDP as judged by ^{31}P NMR of the reaction mixture (Figure 3.3). After RP-HPLC purification **3-8** was obtained in 89% yield. The entire process took just 30 min.



Scheme 3.4. Formation of UDP using reagent **3-11**.

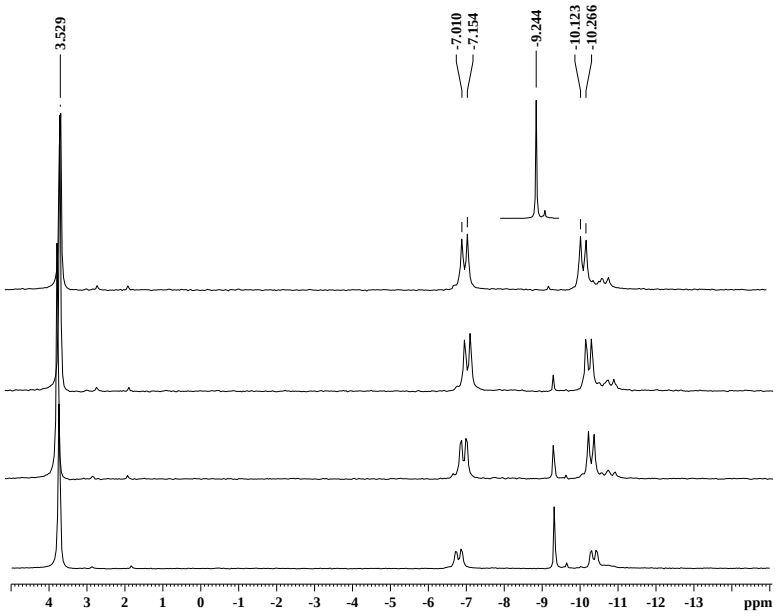
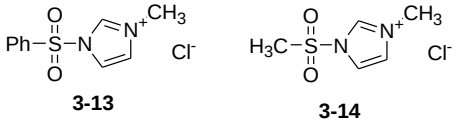


Figure 3.3. ^{31}P-NMR spectra of the reaction mixture of UMP and inorganic phosphate recoreded at 5, 8, 15, 25 minutes (bottom to top) offset shows UMP-imidazolium intermediate. Inorganic phosphate appears at δ_p 3.5, UDP appears as two doublets at δ_p -7.0 and -10.1.

The dimerization of NMP's using **3-11** and **3-12** was examined. We also examined this reaction with two other salts, **3-13** and **3-14** (Figure 3.4), in which the triflate anion was replaced with a chloride ion as these two salts did not require the use of methyl triflate in their preparation.

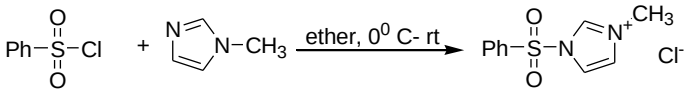


3-13 **3-14**

Figure 3.4. Structures of salts **3-13** and **3-14**.

Salt **3-13** was prepared in one step by reacting benzenesulfonyl chloride with NMI in anhydrous ether. **3-13** precipitated out of solution and was collected by suction filtration in quantitative yield (Scheme 3.5). **3-13** was found to be somewhat unstable in that it could only be stored for only 1-2 weeks under argon at -20 ^{o}C before significant decomposition occurred. We attempted the preparation of **3-14**, by reacting methanesulfonyl chloride with NMI in anhydrous

ether but the compound was found to be extremely unstable and could not be isolated and stored for any appreciable amount of time.

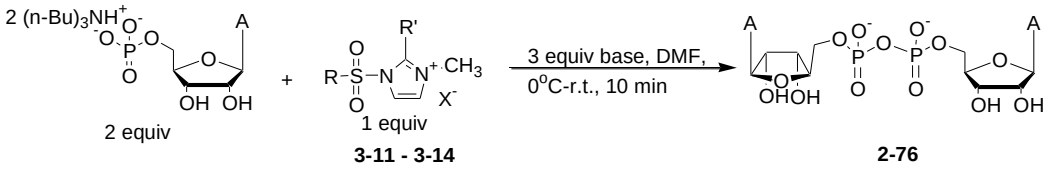


3-13

Scheme 3.5. Synthesis of **3-13.**

The dimerization of AMP was examined using the conditions described above for the dimerization of UMP. Salts **3-11** and **3-12** both gave Ap_2A (**2-76**) in excellent yields (Table 3.2, entries 1 and 2). The yield of Ap_2A using salt **3-13** was considerably lower (entry 3). We also tried generating the salt **3-13** in situ by adding NMI to benzenesulfonyl chloride in DMF, stirring for 5 min and then this mixture was added to AMP in DMF but the yield was only marginally better (entry 4). Since salt **3-14** could not be isolated we attempted the reaction by mixing methanesulfonyl chloride with NMI in DMF a separate flask, stirring for 5 min and then this mixture was added to solution of AMP in DMF; however, this gave none of the desired product. When the methanesulfonyl chloride was added directly to a cold mixture of AMP and 3 equiv NMI Ap_2A was formed in a 64% yield (entry 5).

Table 3.2. Synthesis of Ap_2A using sulfonyl imidazolium salts **3-11 - 3-14**.



Entry	Salt	Base	Yield (%)[a]
1	**3-11** (R =Ph, R' = H, X = TfO^-)	NMI	93
2	**3-12** (R =Ph, R' = CH_3, X = TfO^-)	1,2-DMI	96
3	**3-13** (R =Ph, R' = H, X = Cl^-)	NMI	55
4	**3-13** (R =Ph, R' = H, X = Cl^-)	NMI	45
5	**3-14** (R =CH_3, R' = H, X = Cl^-)	NMI	64

[a]Yields are calculated by integration of ^{31}P-NMR peaks.

We observed that all of these coupling reactions were faster (in less than 10 min.) than the corresponding reactions done using sulfurylimidazolium salts (about 40 min). The ^{31}P-NMR spectra of the reactions did not show any intermediates. This suggests that with these salts the intermediate reacting with the acceptor nucleotide might be the mixed sulfonic-phosphoric anhydride which might have reacted very rapidly and consumed totally before recording the ^{31}P-NMR.

3.3.3 Synthesis of symmetrical Np_2N's and Np_4N's using sulfonylimidazolium salts 3-11 and 3-12 as activating agents

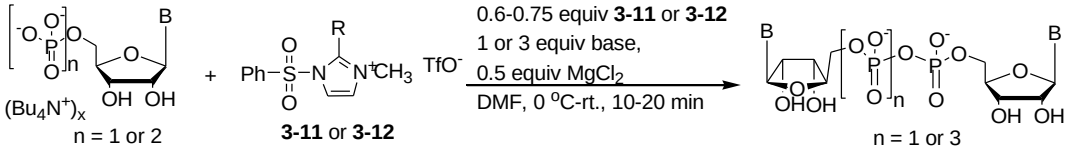
The dimerization of AMP using reagents **3-11** and **3-12** was examined in more detail. We first determined if addition of NMI (or 1,2-DMI) was required to obtain high yields. Compound **3-11**(0.6 equiv, a slight excess was used since compounds **3-11** and **3-12** decompose in DMF) was added to a solution of the tetrabutylammonium salt of AMP (the number of tetrabutylammonium ions per molecule of nucleotide was determined by ^{1}H-NMR, see

experimental section for details) in DMF at 0 ^{o}C. The cooling bath was removed and the reaction was followed by ^{31}P NMR. After 10 min no further reaction occurred and Ap_2A was formed in a 67% yield as determined by ^{31}P-NMR (Table 3.3, entry 1). Performing the reaction in the presence of one equiv of NMI gave Ap_2A in a ^{31}P-NMR yield of 94 % (entry 2) indicating that the presence of NMI or perhaps just a base is required for good yields.

As mentioned in chapter 2, the presence of divalent cations, such as Mg^{+2}, often increases the yields of polyphosphorylation/phosphate-phosphate coupling reactions. Performing this reaction in the presence of 0.5 equiv of $MgCl_2$ gave Ap_2A in a 99% yield by ^{31}P-NMR (entry 3). After quenching this reaction with triethylammonium acetate (pH 7.0), extraction with $CHCl_3$ to remove the tetrabutylammonium salt of phenylsulfonate and NMI, and purification by reversed-phase HPLC, Ap_2A was obtained in 93% yield. No products resulting from sulfonation of the hydroxyl or amino groups were detected. Salt **3-12** gave similar results (entries 4 and 5). Surprisingly, we found that it was not necessary to use anhydrous $MgCl_2$ as the more economical and easily handled trihydrate form gave equally good results.

The optimal conditions described above (2 equiv NMP, 0.6 equiv salt, 0.5 equiv $MgCl_2$, 1 equiv NMI) were applied to the synthesis of other symmetrical Np_2N's. Gp_2G and Up_2U were obtained in excellent yields with either salt **3-11** or **3-12** (entries 9-11). Cp_2C was obtained in a 70 % yield. The effect of magnesium ions on the dimerization of CMP and GMP was more pronounced than on the dimerization of other NMP's (compare entries 6 and 8 to entries 7 and 9). We do not know why this is the case.

Table 3.3. Synthesis of symmetrical Np_2N's and Np_4N's using salts **3-11** and **3-12**.

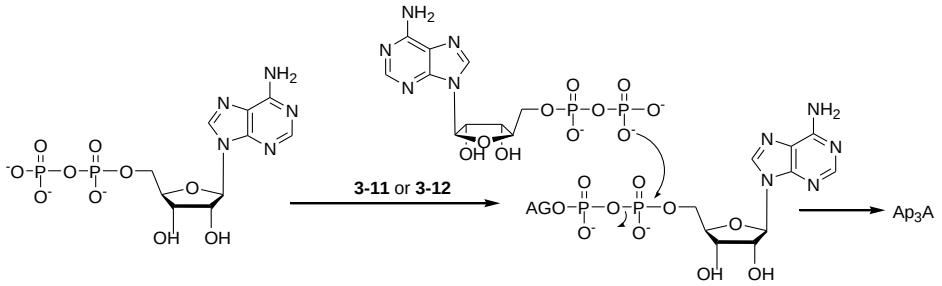


Entry	NMP	R	Base	$MgCl_2$	Product[c]	Yield (%)[a]	Yield (%)[b]
1	AMP	H	-	-	Ap_2A (**2-76**)	67	-
2	AMP	H	NMI	-	Ap_2A (**2-76**)	94	-
3	AMP	H	NMI	Present	Ap_2A (**2-76**)	99	93
4	AMP	CH_3	1,2-DMI	-	Ap_2A (**2-76**)	96	-
5	AMP	CH_3	1,2-DMI	Present	Ap_2A (**2-76**)	99	93
6	CMP	CH_3	1,2-DMI	-	Cp_2C (**3-15**)	52	-
7	CMP	CH_3	1,2-DMI	Present	Cp_2C (**3-15**)	70	-
8	GMP	CH_3	1,2-DMI	-	Gp_2G (**2-77**)	45	-
9	GMP	CH_3	1,2-DMI	Present	Gp_2G (**2-77**)	100	94
10	GMP	H	NMI	Present	Gp_2G (**2-77**)	100	94
11	UMP	H	NMI	Present	Up_2U (**2-75**)	100	93
12	GDP	H	NMI	Present	Gp_4G (**3-16**)	55	-
13	GDP	H	NMI[c]	Present	Gp_4G (**3-16**)[d]	92	84
14	UDP	H	NMI[c]	Present	Up_4U (**3-17**)[d]	88	81

[a]Yields calculated by integration of ^{31}P-NMR peaks. [b]Isolated yields. [c]0.6 equiv of base and 10 min reaction times employed unless stated otherwise. [d]0.75 equiv **3-11**, 3 equiv of NMI, 20 min reaction time.

To determine if NDP's can also act as donors we examined the dimerization of GDP and UDP using the above conditions. The reactions were slower and the symmetrical tetraphosphates were obtained in moderate yields by ^{31}P NMR; however, by using 0.75 equiv of the coupling agent, 3 equiv of base, and a 20 min reaction time, Gp_4G and Up_4U were obtained

in excellent isolated yield (Table 3.3, entries 13 and 14). The dimerization of ADP to Ap_4A using these procedure gave Ap_4A contaminated with about 30% Ap_3A which was difficult to remove. This result suggests that upon activaton of ADP by **3-11** or **3-12**, the resulting activating intermediate can be attacked at the α-phosphorus by ADP to give Ap_3A (Scheme 3.6). This has been noted before by Ng and Orgel who found that the reaction of ADP with EDC in the presence of $MgCl_2$ in aq. HEPES buffer at 37°C resulted in the formation of both Ap_4A and Ap_3A.[65] We do not know why this occurs more readily with activated ADP than with other activated NDP's.



Scheme 3.6. Proposed mechanism for the formation of Ap_3A.

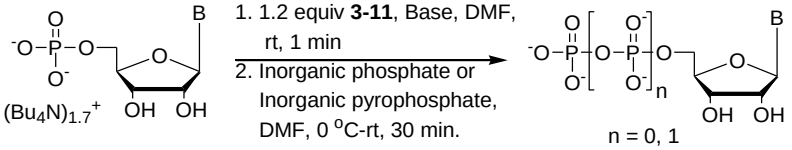
3.3.4. Synthesis of NDP's and NTP's and unsymmetric Np_2N's and Np_4N's using reagents 3-11 as activating agent

Next we examined the synthesis of NDP's and NTP's using reagent **3-11**. Reaction of the tetrabutylammonium salts of AMP and UMP with 1.2 equiv of **3-11** and 1 equiv NMI for 1 min at room temperature followed by reaction with 2 equiv of the bis-tetrabutylammonium salt of inorganic pyrophosphate (PPi) for 30 min gave ATP and UTP in excellent isolated yields after the usual workup and HPLC purification (Table 3.4, entries 1 and 2). The addition of $MgCl_2$ did not significantly improve yields.

When GMP was the substrate, the yield of GTP was low due to competing dimerization (Table 3.4, entry 3). This was also the case when we attempted the synthesis of GDP and CDP

using the bis-tetrabutylammonium salt of inorganic phosphate (Pi) as acceptor (entries 4 and 7). Switching the base to TEA did not prevent dimerization (entry 5); however, when using diisopropylethylamine (DIPEA) instead of NMI as the base the dimerization reaction was suppressed and CDP and GDP were obtained in good to high yields as determined by ^{31}P-NMR (entries 6 and 8). The suppression of dimerization by DIPEA may be due to the complete deprotonation of the donor precursors by the sterically hindered DIPEA thus increasing the rate of formation of activated intermediate.

Table 3.4. Synthesis of NTP's and NDP's using salt **3-11**.

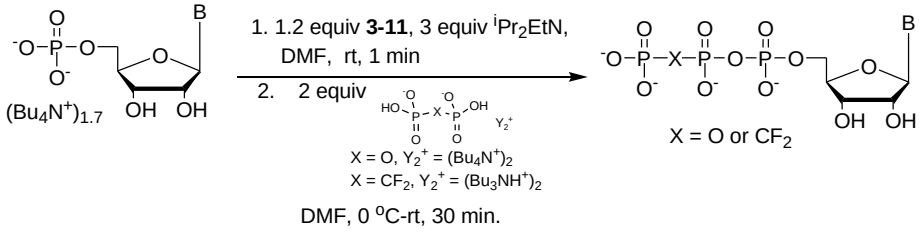


entry	Donor	Base	Acceptor	Product	Yield (%)[a]	Yield (%)[b]
1	AMP	NMI	PPi	ATP (**3-18**)	93	85
2	UMP	NMI	PPi	UTP (**3-19**)	98	88
3	GMP	NMI	PPi	GTP/Gp_2G	40/60	-
4	GMP	NMI	Pi	GDP/Gp_2G	40/60	-
5	GMP	TEA	Pi	GDP/Gp_2G	33/67	-
6	GMP	$^{i}Pr_2EtN$	Pi	GDP	100	-
7	CMP	NMI	Pi	CDP/Cp_2C	35/65	-
8	CMP	$^{i}Pr_2EtN$	Pi	CDP	75	-

[a]Yields calculated from integration of ^{31}P-NMR peaks. [b]Isolated yields

DIPEA could also be used as a base for the activation of AMP and UMP. This optimized procedure was used for the synthesis of the four natural NTP's and the analog **3-22** in excellent isolated yields (Table 3.5).

Table 3.5. Synthesis of NTP's and NTP analog **3-22** using salt **3-11** and DIPEA as base.



Entry	Base	X	Product	Yield (%)
1	A	O	ATP (**3-18**)	86
2	U	O	UTP (**3-19**)	90
3	G	O	GTP (**3-20**)	88
4	C	O	CTP (**3-21**)	84
5	A	CF_2	AppCF_2p (**3-22**)	89

^{31}P NMR of the reaction between AMP and **3-11** in presence of 3 equiv. of DIPEA showed rapid formation of an imidazolium salt **3-23** (Figure 3.5) by ^{31}P NMR indicating that excess NMI was not required for imidazolium salt formation though the presence of DIPEA appears to promote its formation presumably by increasing the extent of deprotonation of the donor precursors.

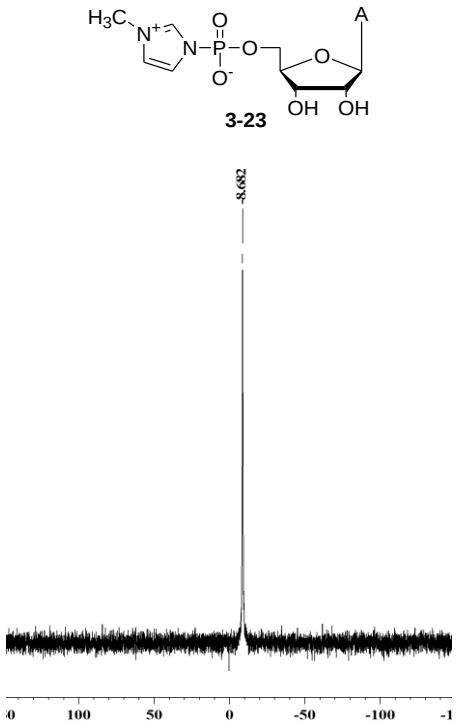
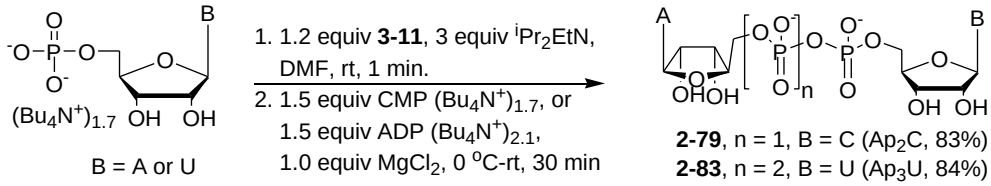


Figure 3.5. ^{31}P-NMR spectrum of a mixture of the tetrabutylammonium salt of AMP, 1.2 equiv salt **3-11** and 3 equiv of DIPEA in DMF after 5 min.

Perhaps the best method for preparing NTP's prior to this work was Bogachev's which was discussed in detail in chapter 2 in section 2.1.5. The yields of NTP's reported by Bogachev were excellent (89-92%). Although our yields of NTP's are just slightly lower there are several advantages to using our procedure as opposed to Bogachev's. Our method uses a one-step one-min. activation of NMP's. Bogachev's procedure requires two activation steps. The first step is the addition of excess TFAA (5 eq.) which has to be completely removed by rotary evaporation before the next activation step with NMI. In our method after quenching the reaction and washing with chloroform we purify the reaction mixture directly by HPLC while in the Bogachev method, one needs to deprotect the product, which is achieved by hydrolysis with water followed by freeze drying. The only advantage of Bogachev's method over ours is that his method uses commercially available reagents.

The procedure we developed for the synthesis of NTP's also works very well for the construction of unsymmetrical Np_2N's and Np_3N's as demonstrated by the synthesis of Ap_2C and Ap_3U in very good yields starting from AMP or UMP and using CMP or ADP as acceptors (Scheme 3.7). Ap_3U has been prepared in a 73% yield using an engineered diadenosine triphosphate hydrolase.[110] We are not aware of any other chemical synthesis of this compound or any previous syntheses of Ap_2C.

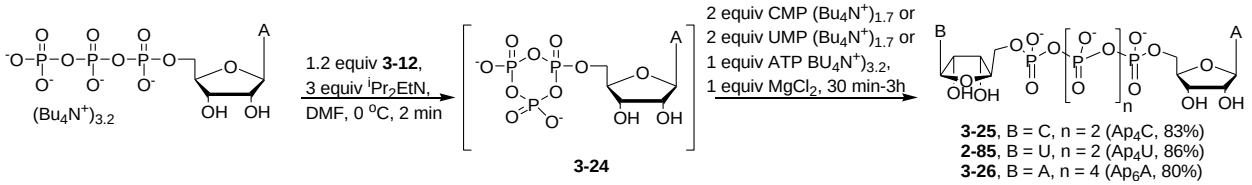


Scheme 3.7. Synthesis of Ap_2C and Ap_3U.

3.3.5. Synthesis of unsymmetrical Np_4N's and a symmetrical Np_6N using reagent 3-11 or 3-12 as activating agents

ATP was used as a model substrate for preparation of unsymmetrical Np_4N's and symmetrical Np_6N's via activation of NTP's. Adding 1.2 equiv of reagent **3-11** or **3-12** to the tetrabutylammonium salt of ATP in the presence of DIPEA in DMF resulted in the formation of cyclic adenosine trimetaphosphate **3-24** (Scheme 3.8) as determined by ^{31}P NMR (Figure 3.6).[136] We found that this reaction was essentially quantitative within 2 min at 0 ^oC using reagent **3-12**. Reagent **3-11** gave similar results except it is slightly less soluble in DMF at 0 ^oC resulting slightly longer reaction times. Treating **3-24** with a solution of the tetrabutylammonium salts of UMP or CMP and 1 equiv of $MgCl_2$ in DMF and stirring for 30 min at room temperature gave Ap_4U and Ap_4C, in excellent isolated yields. This procedure compared very favorably with literature methods. Ap_4U has been prepared in 73% yield using an engineered diadenosine

triphosphate hydrolase.[110] Ap_4C has been prepared in 63% yield using lysyl tRNA synthetase.[100] We are not aware of a chemical synthesis of this compound.



Scheme 3.8. Synthesis of Ap_4C, Ap_4U, and Ap_6A from ATP using reagent **3-12**.

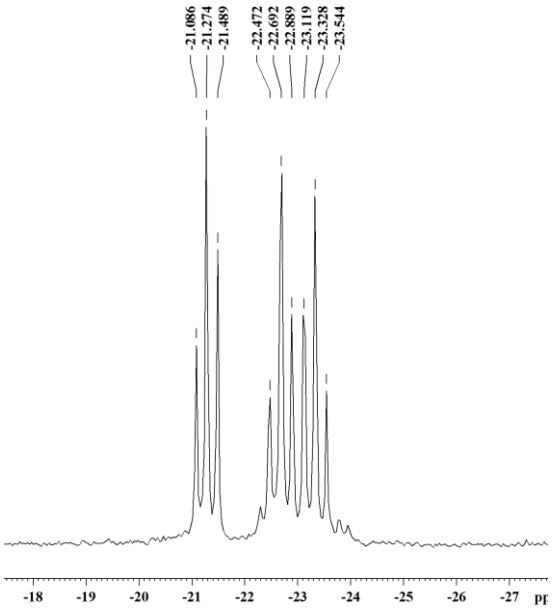


Figure 3.6. ^{31}P-NMR of adenosine 5'-trimetaphosphate **3-24** formed from the reaction 1.2 equiv. of reagent **3-12** with the tetrabutylammonium salt of ATP in the presence of DIPEA in DMF.

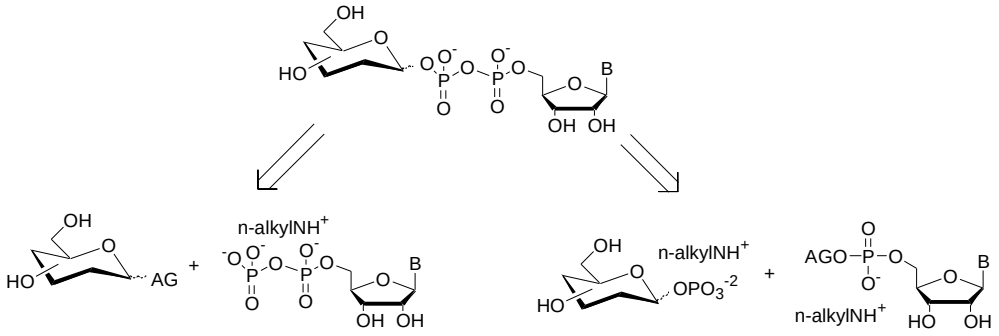
Ap_6A was obtained in an 80% yield by adding 0.6 equiv of **3-12** to a solution of ATP, 1 equiv of $MgCl_2$, and 3 equiv of DIPEA at 0 ^oC and then stirring for 3 h at room temperature. Ap_6A, which exerts vasoconstrictive effects,[137] has been prepared by Ng and Orgel in aq. solution by treating ATP with a 5-fold excess of EDC and $MgCl_2$ in Hepes buffer at pH 6.5 for 2 h at 37 ^oC. The compound was purified by paper electrophoresis and each UV absorbing spot

was eluted from the chromatogram and the optical density was read at 259 nm. The reported yield, expressed as the percentage of the total absorbancy of all the material on the paper, was a remarkable 90% though they also reported that it was contaminated with a small amount of Ap_5A which they could not remove.[65]

3.3.6 Synthesis of nucleoside 5'-diphosphate sugars (NDP-sugars) using reagent 3-11 as activating agent

The two most common approaches to NDP sugars are shown in Scheme 3.9. One is to react a sugar-1-phosphate acceptor with an activated nucleotide donor. The other is to react an NDP acceptor with a glycosyl donor such as a glycosyl halide, a 2-(α–D-glycosyloxy)-3-methoxypyridine (MOP glycoside) or triethylsilylated and benzylated epoxide derived from a glycal.[138-141] (Scheme 3.9). The glycosyl donor method usually produces the NDP-sugars in lower yields than the corresponding reaction involving nucleotide imidazolate or morpholidate donors.[142] On the other hand, these reactions usually don't take as long and the glycosyl donors are sometimes easier to prepare than the glycosyl-1-phosphates though they sometimes require protecting groups on the donor which must be removed at the end of the synthesis. Hence, the approach using activated nucleotide donors is by far the most common approach with imidazolates and, more often, morpholidates of NMP's used as donors (see chapter 2, section 2.1.3.2.1 and 2.1.3.2.4 for a discussion on morpholidate and imidazolate donors for Np_nN syntheses).[143-148] With the morpholidates the yields tend to be low to moderate (rarely exceeding 70%) and the coupling reactions very sluggish often taking days (reaction times of 5 days are not unusual). In some instances reasonable yields (i.e. 75%) have been reported using imidazolate donors and the reaction tend to be faster with these donors but still take 1-2 days.[142] It has been reported that the addition of 3 equiv tetrazole to coupling reactions involving the morpholidate donors can sometimes increase the yield of the NDP-sugar products (in one case up to 91%)

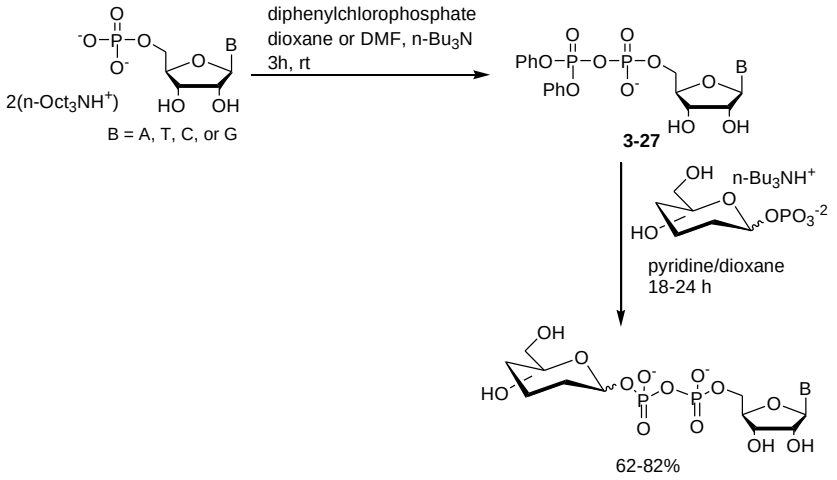
though the reaction still takes 24-48 h.[149]; however, this approach does not appear to work any better, in terms of yields and reaction times, than when using imidazolate donors.[142] Bogachev's method (discussed in Chapter 2, section 2.1.5), which used nucleotide imidazolium donors, provides the NDP-sugars faster than the morpholidate or imidazolate donors but the yields are quite low.[121, 122]



Scheme 3.9. Chemical approaches to NDP-sugars. AG stands for activating group.

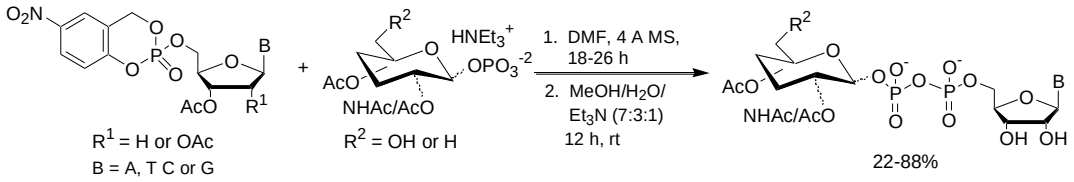
In addition to the morpholidates and imidazolates, other donors have been developed but are not as widely used. For example, Michelson reported the synthesis of a wide variety of NDP-sugars by reacting the tri-*n*-octylammonium salts of NMP's with diphenyl chlorophosphate in dioxane or DMF in the presence of tri-*n*-butylamine for 3 h to give a mixed anhydride **3-27** (Scheme 3.10) which was isolated in almost quantitative yield by precipitation in ether. A solution of the tri-*n*-butylammonium salt of a sugar-1-phosphate in pyridine is added to a solution of **3-27** in dioxane and the mixture stirred overnight. The product is precipitated in ether and then purified by ion exchange chromatography. Using this procedure Michelson reported the synthesis of a wide variety of NDP-sugars in 62-82% yield. Michelson also used this approach to prepare NTP's (from the mixed anhydride of NMP's and the tri-*n*-octylammonium salt of iPP) in yields ranging from 75-85% and FAD (from the mixed anhydride

of AMP and the tri-*n*-octylammonium salt of flavin mononucleotide) in 75% yield. Surprisingly, this approach has not been widely adopted.[150]



Scheme 3.10. Michelson's approach to NDP-sugars.

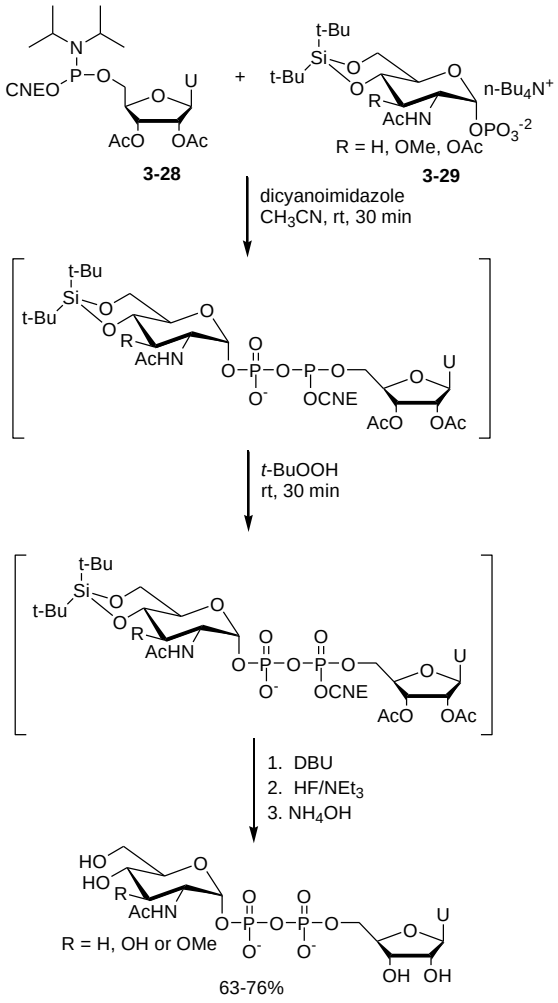
More recently, Meier and coworkers have used their *cyclo*Sal approach (see Chapter 2, section 2.1.3.2.3 for a discussion of the *cyclo*Sal approach to Np_nN's) to prepare a large number of NDP-sugars (Scheme 3.11).[151] There are drawbacks to this approach. The *cyclo*Sal nucleoside substrates must be synthesized and the yields of these compounds tends to be low (around 30%). The substrates must be protected and the products deprotected. Finally, the yield of NDP-sugar is highly variable (22-88%). We do not anticipate that this procedure will be widely adopted.



Scheme 3.11. Meier and coworkers cycloSal approach to NDP-sugars.

Gold et al., have recently reported a method using phosphoramidite donors of type **3-28** (Scheme 3.12).[152] Although the yields of the NDP-sugars were fairly good the process requires

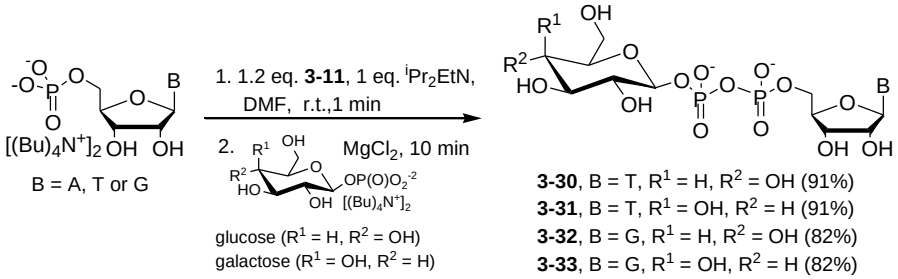
multistep syntheses of protected phosphoramidite donors and protected glycosylphosphate acceptors (**3-29**). Deprotection of the coupled/oxidized product is required at the end of the synthesis. We do not anticipate that this procedure will be widely adopted.



Scheme 3.12. Gold et al.'s approach to the synthesis of NDP-sugars.

The preparation of sugar nucleotides using enzymes (nucleotidyltransferases) is emerging as an alternative to chemical synthesis.[153-160] However, substrate specificity and substrate inhibition is a limiting factor in this approach.[160-162]

To demonstrate that our procedure is a powerful approach to the synthesis of NDP-sugars) we prepared compounds **3-30 - 3-33** using the same procedure employed for preparing **2-79** and **2-83** (Scheme 3.13). These reactions proceeded well; however, we found that small amounts of unreacted NMP were time-consuming to remove. This is a common issue when preparing NDP-sugars using activated NMPs as donors.[139,151] This problem is usually dealt with by removing the contaminant with alkaline phosphatase.[139,151] We also found this to be a good way to remove the unreacted NMP: by treating the quenched reaction with a small amount of alkaline phosphatase for 16 h, the unreacted NMP was converted into a nucleoside and inorganic phosphate and the purification became straightforward. Excellent yields were obtained for all four NDP-sugars including **3-32** and **3-33** which had been previously prepared in low yields by the *cyclo*Sal approach (22% for **3-32** and 34% for **3-33**).[151] The entire reaction time was less than 15 minutes.



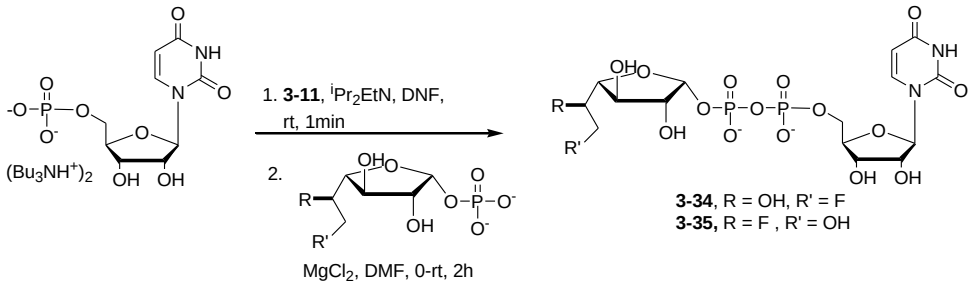
Scheme 3.13. Synthesis of NDP-sugars using **3-11** as activating agent

3.4 Conclusion and future work

We have described a novel and broadly applicable procedure for preparing nucleoside polyphosphates and their conjugates. The procedure is rapid and high yielding, does not require prior protection and subsequent deprotection of the donors or acceptors, and can be used to

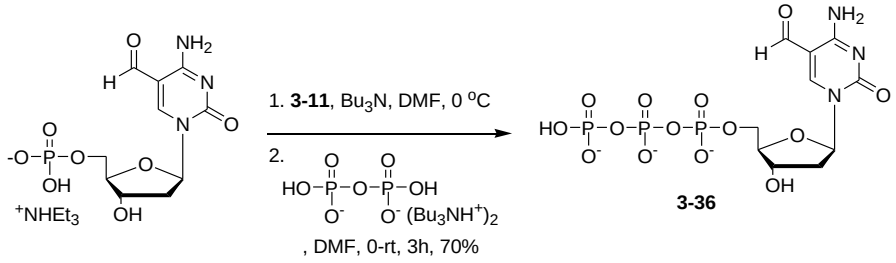
activate nucleoside 5'-mono-, di-, and triphosphates, and a wide variety of acceptors and donors can be used.[163,164]

The methodology described in this chapter was rapidly adopted by others. Just 2 months after our work was published on-line in *Org. Lett.*[163] Kiessling and coworkers used it for the synthesis of uridine diphosphate-6-deoxy-6-fluoro-α-D-galactofuranose **3-34** and uridine diphosphate-5-deoxy-5-fluoro-α-D-galactofuranose **3-35** (Scheme 3.14).[165,166] These compounds were used as chain terminating substrates for some glycosyl transferases in particular galactofuranosyl transferase (GlfT2) from *m. tuberculosis* which mediate the cell wall galactan production in *m. tuberculosis.*[166]



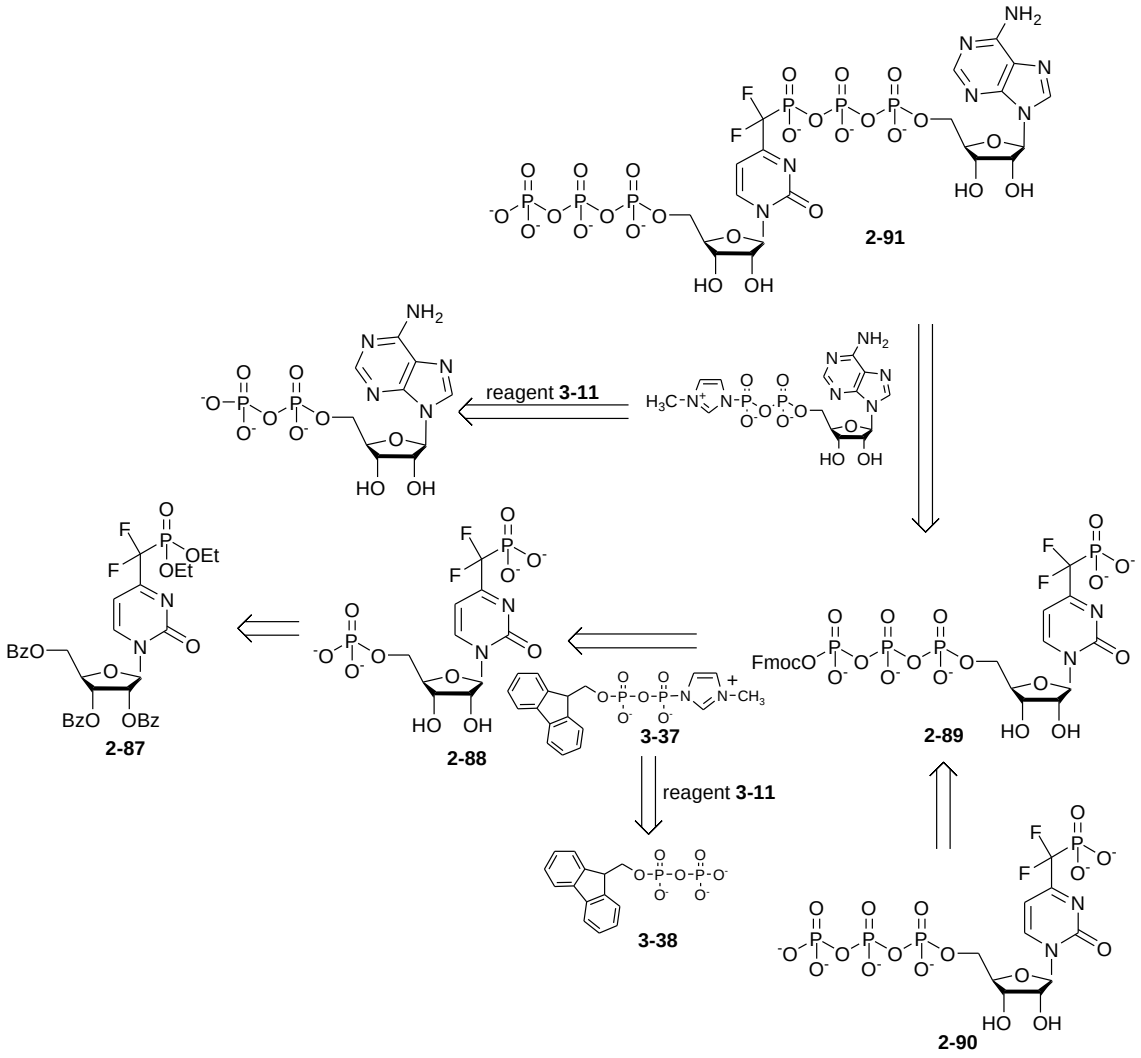
Scheme 3.14. Kiessling synthesis of compounds **3-34** and **3-35** using salt **3-11**.

This method has been also used by Carell and coworkers for the synthesis of 5-formyl-2-deoxycytidine 5'-triphosphate **3-36** (Scheme 3.15).[167]



Scheme 3.15. Carell et al.'s synthesis of **3-36** using salt **3-11**.

Future studies will involve the application of this methodology to the synthesis of compounds **2-90** and **2-91** (Scheme 3.16). Reagent **3-11** (or **3-12**) will be used to activate substrate **3-38** and ADP for the key coupling reactions.



Scheme 3.16. Proposed route to compounds **2-90** and **2-92** using reagent **3-11**.

3.5 Experimental

3.5.1 General Information.

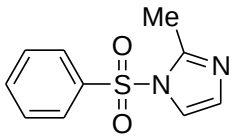
All reagents and starting nucleotides were obtained from commercial sources unless stated otherwise. $MgCl_2$ was obtained and used as its trihydrate. Alkaline phosphatase (Calf intestinal mucosa, 69 KDa, 10,000 U/mL) was obtained from Sigma-Aldrich Corp (St. Louis, MO, USA). Acetonitrile and DMF were distilled from calcium hydride. NMI and 1,2-dimethylimidazole (DMI) were distilled from sodium hydroxide. Diethyl ether was distilled from sodium metal. For ^{1}H-NMR spectra run in $CDCl_3$, chemical shifts are reported in ppm relative to tetramethylsilane (external standard). For proton-decoupled ^{13}C-NMR spectra run in $CDCl_3$, chemical shifts are reported in ppm relative to the solvent residual peak (δ 77.0, central peak). For ^{1}H-NMR and proton-decoupled ^{13}C-NMR spectra run in acetone-d_6, chemical shifts are reported in ppm relative to TMS. For ^{1}H-NMR spectra run in D_2O, chemical shifts are reported in ppm relative to the solvent residual peak (δ 4.79). For proton-decoupled ^{13}C-NMR spectra run in D_2O, chemical shifts are reported in ppm relative to CH_3OH in D_2O (δ 49.5, external standard). For proton-decoupled ^{31}P-NMR, chemical shifts are reported in ppm relative to aqueous 85% H_3PO_4 (δ 0 ppm, external standard). For proton-decoupled ^{19}F NMR, chemical shifts are reported in ppm relative to $CFCl_3$ (δ 0 ppm, external standard). Preparative HPLC was performed using a C-18 reverse phase semipreparative column.

3.5.2 Synthesis of salts 3-11 and 3-12

Imidazole (0.47 mol, 31 g), or 2-methylimidazole (0.47 mol, 38.55 g) was suspended in dry dichloromethane (400 mL) and cooled to 0 ^{o}C in an ice bath. Benzenesulfonyl chloride (0.157 mol, 27.64 g, 20 mL) was added dropwise via a syringe over 15 min. during the addition

all the imidazole dissolves and in the case of compound **3-9** a white precipitate starts to appear. The reaction mixture was stirred at room temperature for 5 hours. For compound **3-9**, the mixture was filtered and the filter cake was washed with dichloromethane. The filtrate was washed with brine (2x) and water (1x). In the case of compound **3-10**, the 2-methylimidazolium hydrochloride by-product did not precipitate out during the reaction so the filtration step was omitted. The organic layer was concentrated and the crude product recrystallized from ethyl acetate-hexane to give a white crystals. Compound **3-9** was obtained in a 96% yield (31.4 g). Its ^{1}H-NMR was identical to that previously reported.[168]

1-Benzenesulfonyl-2-methylimidazole (3-10)

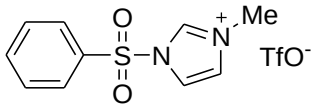


3-10

Compound **3-10** was obtained in a 99% yield (34.7 g). Mp = 47-48 ^{o}C. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 2.47 (s, 3H, H_2), 6.86 (s, 1H, H_4), 7.38 (s, 1H, H_5), 7.52 (t, 2H, J = 7 Hz, $H_{3',5'}$), 7.63 (t, 1H, J = 7 Hz, $H_{4'}$), 7.84 (d, 2 H, J = 7 Hz, $H_{2',6'}$). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 15.1, 119.3, 127.2, 128.1, 129.7, 134.6, 137.9, 145.8. LRMS (ESI+): *m/z* = 222.14.

Compound **3-9** (11.3 mmol, 2.35 g) or **3-10** (11.3 mmol, 2.5 g) was dissolved in dry ether (100 mL), then methyl triflate (0.118 mole, 1.94 g, 1.3 mL) was added dropwise over 15 min via syringe at room temperature. A white precipitate rapidly appeared during the addition. The reaction mixture was stirred for 3 h, suction filtered, washed with dry ether (3 x 25 mL) and dried under vacuum.

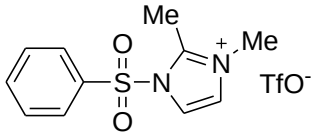
1-Methyl-3-benzenesulfonylimidazolium triflate (**3-11**).



3-11

Yield = 4.16 g (99 %, white powder). ^{1}H-NMR (acetone-d_6, 300 MHz): δ 4.11 (s, 3H, H_1), 7.77 (t, J = 8 Hz, 2H, $H_{3',5'}$), 7.89-7.96 (m, 2H, $H_{4',5}$), 8.21 (d, J = 1.7 Hz, 1H, H_4), 8.28 (dd, J = 8 Hz, 1.1 Hz, 2H, $H_{2',6'}$), 9.81 (s, 1H, H_2). ^{13}C-NMR (acetone-d_6, 75 MHz): δ 119.0, 123.3 (CF_3, J = 319 Hz), 120.2, 126.4, 129.2, 130.7, 134.5, 137.3, 138.5. ^{19}F NMR (acetone-d_6, 282 MHz): δ -78.6. TOF MS ES+; m/z 223.02; HRMS (ESI+): *m/z* = 223.0539, $C_{10}H_{11}N_2O_2S$, [M^+] requires 223.0541.

1,2-Dimethyl-3-benzenesulfonylimidazolium triflate (**3-12**).



3-12

Yield = 4.32 g (99 %, white powder) ^{1}H-NMR (acetone-d_6, 300 MHz): δ 2.97 (s, 3H, H_2), 3.95 (s, 3H, H_1), 7.78 (m, 3H, $H_{3',5',5}$), 7.96 (td, 1H, J = 7.7, 1.1 Hz, $H_{4'}$), 8.15 (d, 1H, J = 2.3 Hz, H_4), 8.26 (dd, 2H, J = 7.5,1.1 Hz $H_{2',6'}$). ^{13}CNMR (acetone-d_6, 75 MHz): δ 10.9, 35.5, 119.8, 124.0, 128.9, 130.7, 134.8, 137.1, 205.3. ^{19}F NMR (acetone-d_6, 282 MHz): δ -78.8. LRMS (ESI+): *m/z* = 237.06. HRMS (ESI+): *m/z* = 237.0691, $C_{11}H_{13}N_2O_2S$, [M^+] requires 237.0698.

3.5.3 Preparation of the tetrabutylammonium salts of NMP's, NDP's and NTP's and Pi.

The sodium salts of the nucleotides were converted into their free acids using a Dowex-50W ion exchange column (H^+ form) then titrated to pH 7.0 with a dilute solution of

tetrabutylammonium hydroxide then concentrated by high vacuum rotary evaporation to approximately one seventh the original volume and lyophilized. The lyophilized powder was dried by dissolving it in acetonitrile, an equal amount of dry toluene was added and the solution concentrated by rotary evaporation to dryness (3x). The ^{1}HNMR spectra of the residue indicated that there was 1.7 tetrabutylammonium ions per nucleoside monophosphate. The residue was subjected to high vacuum for 1 h. The flask was removed under Ar then dissolved in dry DMF in the presence of 4Å molecular sieves. This solution was allowed to stand for at least one hour prior to the coupling reactions.

The sodium salts of inorganic pyrophosphate and the nucleoside di- and triphosphates were converted into their pyridinium salts using a Dowex-50W ion exchange column (pyridinium form). The resulting solution was titrated to pH 7.0 with a dilute solution of tetrabutylammonium hydroxide then concentrated by high vacuum rotary evaporation to approximately one-seventh the original volume then lyophilized. The lyophilized powder was dried by dissolving it in acetonitrile, an equal amount of dry toluene was added and the solution concentrated by rotary evaporation to dryness (3x). The ^{1}H-NMR spectra of the nucleoside di- and triphosphates revealed that there was 2.1 and 3.2 tetrabutylammonium ions per each molecule of nucleoside di- and triphosphate respectively. The pyrophosphate was presumed to be the bis(tetrabutylammonium) salt. The residue was subjected to high vacuum for 1 h. The flask was removed under Ar then dissolved in dry DMF in the presence of 4Å molecular sieves. This solution was allowed to stand for at least one hour prior to the coupling reactions.

3.5.4. General procedure for preparation of symmetric nucleoside diphosphates 2-75, 2-76, and 2-77.

To a solution of NMP $(Bu_4N^+)_{1.7}$ (0.27 mmol) in dry DMF (4 mL) was added NMI (0.27 mmol, 22 µL) and magnesium chloride (0.135 mmol, 13 mg). The mixture was stirred at room temperature for 2 min. and then cooled to 0 ^oC using an ice-bath. Reagent **3-11** (0.16 mmol, 60 mg) was added, the ice bath was removed and the reaction mixture was stirred at room temperature for 10 min. The mixture was cooled in an ice bath for 5 min then quenched with 50 mM triethylammonium acetate buffer (5 mL, pH 7.0) and washed with chloroform (3 x 10 mL). Chelex resin (ca. 0.2 g) was added and the mixture stirred for 1 min then filtered through a cotton plug. For Gp_2G (**2-77**) the reaction was quenched with water to avoid precipitation of the product and the Chelex is added before the chloroform wash. The filtrate was purified by RP-HPLC using a semipreparative C18 column and a gradient of acetonitrile and buffer (50 mM triethylammonium acetate, pH 7) at 6 mL/min and monitored at 255 and 280 nm. Fractions containing the desired product were pooled, concentrated by high vacuum rotary evaporation, and the residue was dissolved in water and repeatedly freeze-dried until the ^1H-NMR spectrum indicated that no residual buffer was present. The resulting white powder was converted to its sodium salt using a Dowex-50-W ion-exchange resin in Na^+ form.

3.5.5 General procedure for preparation of symmetric dinucleoside tetraphosphates 3-16 and 3-17.

To a solution of NDP $(Bu_4N^+)_{2.1}$ (0.27 mmol) in dry DMF (4 mL) was added NMI (0.81 mmol, 65 µL) and magnesium chloride (0.135 mmol, 0.5 eq. 13 mg). The mixture was stirred at room temperature for 2 min. and then cooled to 0 ^oC using an ice-bath. Reagent **3-11** (0.135 mmol, 0.5 eq., 50 mg) was added, the ice bath was removed and the reaction mixture was then stirred at room temperature for 10 min. The mixture was cooled using an ice bath and another

portion of **3-11** (0.067 mmol, 25 mg) was added and the reaction mixture was kept stirring at 0 ^{o}C for another 10 min. The reaction was quenched with 50 mM triethylammonium acetate buffer (5 mL, pH 7.0) and washed with chloroform (3 x 10 mL). Chelex resin (ca. 0.2 g) was added and the mixture stirred for 1 min then filtered through a cotton plug. The filtrate was purified by RP-HPLC using a semipreparative C18 column and a gradient of acetonitrile and buffer (50 mM triethylammonium acetate, pH 7) at 6 mL/min and monitored at 255 and 280 nm. Fractions containing the desired product were pooled, concentrated by high vacuum rotary evaporation, and the residue was dissolved in water and repeatedly freeze-dried until the ^{1}H-NMR spectrum indicated that no residual buffer was present. The resulting white powder was converted to its sodium salt using a Dowex-50-W ion-exchange resin in Na^{+} form.

3.5.6 General procedure for the synthesis of 3-18-3-22, 2-79, 2-83 and 3-31-3-34.

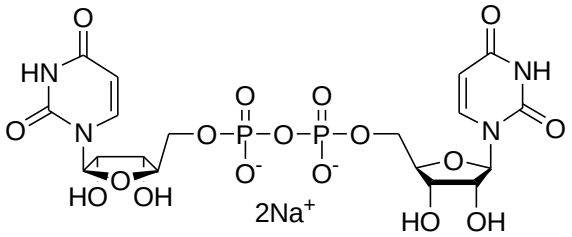
To a solution of NMP $(Bu_4N^{+})_{1.7}$ (0.27 mmol) or NDP $(Bu_4N^{+})_{2.1}$ (0.27 mmol) in dry DMF (4 mL) was added diisopropylethylamine (0.81 mmol, 141 µL) followed by reagent **3-11** (0.32 mmol, 120 mg) and the reaction mixture stirred at room temperature for 1 min. This solution was added over 30 seconds to a solution of the acceptor (0.405 mmol for nucleoside mono- and diphosphates and sugar phosphates, 0.54 mmol for PPi and difluoromethylenebisphosphonate)[169] and magnesium chloride (0.27 mmol, 26 mg, for **2-79**, **2-83,** and **3-30**-**3-33**) in dry DMF (4 mL) at 0 ^{o}C (ice bath). The ice bath was removed and the reaction mixture stirred at room temperature for 30 min. The mixture was cooled to 0 ^{o}C (ice bath) and quenched with 50 mM triethylammonium acetate buffer (5 mL, pH 7.0) and washed with chloroform (3 x 10 mL). For reactions containing magnesium chloride, Chelex resin (ca. 0.2) was added and the mixture was stirred for 1 min and filtered through a cotton plug. For sugar nucleotides **3-30**-**3-33**, alkaline phosphatase (10 µL, 40 units) was added after the Chelex

treatment and left at room temperature for 16 hours. Purification was achieved by RP-HPLC using a semipreparative C18 column and a gradient of acetonitrile and buffer (50 mM triethylammonium acetate, pH 7) at 6 mL/min and monitored at 255 and 280 nm. Fractions containing the desired product were pooled, concentrated by high vacuum rotary evaporation, and the residue was dissolved in water and repeatedly freeze-dried until the ^{1}H-NMR spectrum indicated that no residual buffer was present. The resulting white powder was converted to its sodium salt using a Dowex-50-W ion-exchange resin in the Na^{+} or NH_4^{+} form.

3.5.7 General procedure for preparation of 2-85 and 3-25.

To a solution of ATP $(Bu_4N^{+})_{2.3}$ (0.27 mmol) in dry DMF (4 mL) was added diisopropylethylamine (0.81 mmol, 141 µL) and the mixture cooled to 0 ^{o}C (ice bath). Reagent **3-12** was added (0.32 mmol, 125 mg) and the mixture stirred for 2 min at 0 ^{o}C. A solution of NMP $(Bu_4N^{+})_{1.75}$ (0.54 mmol) in dry DMF (4 ml) was added followed by magnesium chloride (0.27 mmol, 26 mg). The mixture was stirred at room temperature for 30 min, cooled to 0 ^{o}C (ice bath) and quenched with 50 mM triethylammonium acetate buffer (5 mL, pH 7.0), washed with chloroform (3 x 10 mL). Chelex resin (ca. 0.2 g) was added and the mixture stirred for 1 min then filtered through a cotton plug. Purification was achieved by RP-HPLC using a (250 x 20 mm) semipreparative C18 column and a gradient of acetonitrile and buffer (50 mM triethylammonium acetate, pH 7) at 6 mL/min and monitored at 255 and 280 nm. Fractions containing the desired product were pooled, concentrated by high vacuum rotary evaporation, and the residue was dissolved in water and repeatedly freeze-dried until the ^{1}HNMR spectrum indicated that no residual buffer was present. The resulting white powder was converted to its sodium salt using a Dowex-50-W ion-exchange resin in Na^{+} form.

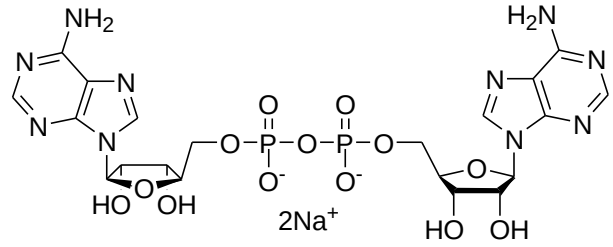
3.5.8 Characterization data for nucleoside polyphosphates and conjugates P1,P2-Diuridine-5'-diphosphate (2-75).



2-75

Obtained in 93% yield as its disodium salt (84 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 8% CH_3CN-92% buffer over 35 min, t_r = 29 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.01-4.21 (m, 10H, $H_{2',3',4',5'}$), 5.79 (two overlapping doublets, *J* = 7.9 Hz, 4H, $H_{1',5}$), 7.77 (d, *J* = 8.1 Hz, 2H, H_6). ^{13}C-NMR (D_2O, 75 MHz): δ 64.8, 69.5, 73.7, 83.1 (t, *J* = 4.5 Hz), 88.3, 102.6, 141.5, 151.7, 166.1. ^{31}P-NMR (D_2O, 121 MHz): δ -9.78 (s). MS (ESI-): *m/z* = 629.16 $[M-H]^-$

P1,P2-Diadenosine-5'-diphosphate (2-76).

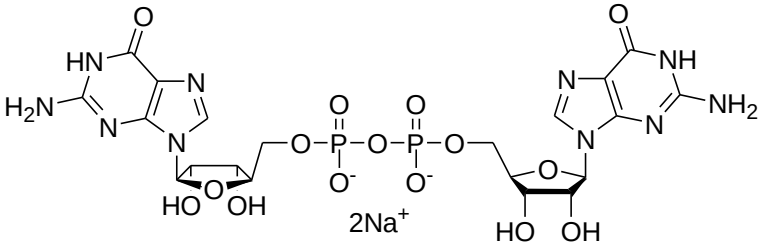


2-76

Obtained in 93% yield as its disodium salt (90 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 10% CH_3CN-90% buffer over 45 min and then a linear gradient to 20% CH_3CN-80% buffer over 5 min, t_r = 43 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.11-4.31 (m, 8H, $H_{5',4',3'}$), 4.42-4.45 (m, 2H, $H_{2'}$) 5.80 (d, *J* = 4.8, 2H, $H_{1'}$), 7.89 (s, 2H, H_2), 8.07 (s, 2H, H_8). ^{13}C-NMR

(D_2O, 75 MHz): δ 65.1, 69.9, 74.6, 83.4, 87.1, 117.6, 139.6, 147.9, 150.9, 153.7. ^{31}P-NMR (D_2O, 121 MHz): δ -9.64 (s). MS (ESI-): *m/z* = 675.23 $[M - H]^-$.

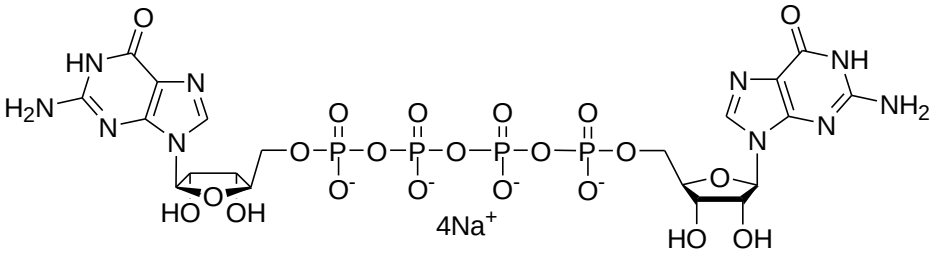
P1,P2-Diguanosine-5'-diphosphate (2-77).



2-77

Obtained in 94% yield as its disodium salt (90 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 10% CH_3CN-90% buffer over 45 min, t_r = 34 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.09 (br, 2H, $H_{4'}$), 4.20 (br, 4H, $H_{5'}$),4.32 (t, *J* = 4.7, 2H, $H_{3'}$), 4.49 (m 2H, $H_{2'}$), 5.68 (d, *J* = 5.0 Hz, 2H, $H_{1'}$), 7.94 (s, 2H, H_8). ^{13}C-NMR (D_2O, 75 MHz): δ 64.9, 69.7, 74.0, 83.2, 87.4, 114.7, 136.9, 150.8, 153.8, 157.9. ^{31}P-NMR (D_2O, 121 MHz): δ -9.72 (s). MS (ESI-): *m/z* = 707.21 $[M-H]^-$.

P1,P4-Digauanosine-5'-tetraphosphate (3-16).

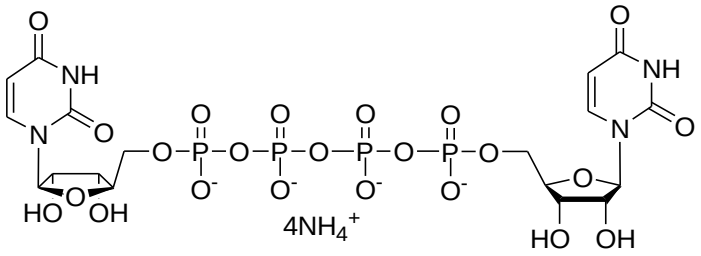


3-16

Obtained in 84% yield as its tetrasodium salt (108 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 12% CH_3CN-88% buffer over 45 min, t_r = 37 min]

followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.11-4.18 (m, 6H, $H_{4',5'}$), 4.38 (s, 2H, $H_{3'}$), 4.55 (t, *J* = 5.2, 2H, $H_{2'}$), 5.68 (d, *J* = 5.6 Hz, 2H, $H_{1'}$), 7.78 (s, 2H, H_8). ^{13}C-NMR (D_2O, 75 MHz): δ 65.0 (d, *J* = 5.2 Hz), 70.1, 73.7, 83.5 (d, *J* = 8.5), 86.8, 115.8, 137.3, 151.3, 153.5, 158.4. ^{31}P-NMR (D_2O, 121 MHz): δ -9.58 (d, *J* = 14.6 Hz), -21.22 (d, *J* = 12.9 Hz). MS (ESI-): *m/z* = 867.11 $[M-H]^-$.

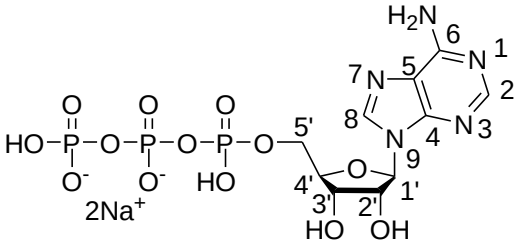
P1,P4-Diuridine-5'-tetraphosphate (3-17).



3-17

Obtained in 81% yield as its tetraammonium salt (94 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 7% CH_3CN-93% buffer over 50 min, t_r = 41 min] followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.09-4.11 (m, 6H, $H_{4',5'}$), 4.22 (m, 4H, $H_{2',3'}$), 5.82 (m, 4H, $H_{1',5}$), 7.79 (d, *J* = 8.5 Hz, 2H, H_6). ^{13}C-NMR (D_2O, 75 MHz): δ 64.9 (d, *J* = 5.3 Hz), 69.6, 73.7, 83.3 (d, *J* = 9 Hz), 88.0, 102.6, 141.6, 151.8, 166.1. ^{31}P-NMR (D_2O, 121 MHz): δ -9.88 (d, *J* = 14.7 Hz), -21.65 (d, *J* = 14.2 Hz). MS (ESI-): *m/z* = 789.12 $[M-H]^-$.

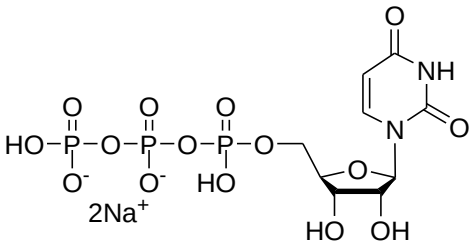
Adenosine-5'-triphosphate disodium salt (3-18).



3-18

Obtained in 86% yield as its disodium salt (129 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 10% CH_3CN-90% buffer over 45 min., t_r = 32 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.12-4.22 (m, 3H, $H_{4'}$,$_{5'}$), 4.33 (t, *J* = 4.1, 1H, $H_{3'}$), 4.45 (t, *J* = 4.8, 1H, $H_{2'}$) 5.88 (d, *J* = 4.8, 1H, $H_{1'}$), 8.2 (s, 1H, H_2), 8.37 (s, 1H, H_8). ^{13}C-NMR (D_2O, 75 MHz): δ 65.0 (d, *J* = 5.1 Hz), 69.9, 74.8, 83.8 (d, *J* = 8.9 Hz), 87.5, 117.8, 141.8, 145.2, 147.7, 149.6. ^{31}P-NMR (D_2O, 121 MHz): δ -8.99 (d, *J* = 17.8 Hz), -9.57 (d, *J* = 18.4 Hz), -21.11 (overlapping dd, *J* = 17.8, 17.8 Hz). MS (ESI-): *m/z* = 506.06 [M- H]$^-$.

Uridine-5'-triphosphate disodium salt (3-19).

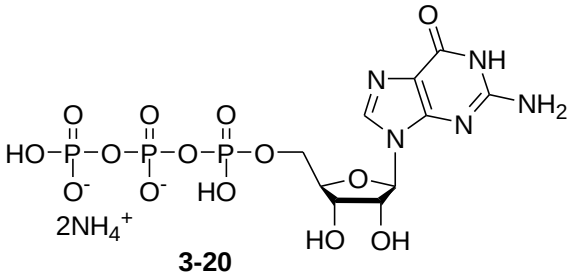


3-19

Obtained in 90% yield as its disodium salt (130 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 5% CH_3CN-95% buffer over 40 min., t_r = 28 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^{1}H-NMR (D_2O, 300

MHz): δ 3.87 (m, 2H, $H_{5'}$), 4.14 (s, 1H, $H_{4'}$), 4.23 (s, 1H, $H_{3'}$), 4.28 (t, 1H, *J* = 4.9), 5.87 (m, 2H, $H_{1',5}$), 7.98 (d, *J* = 8.0 Hz, 1H, H_6). ^{13}C-NMR (D_2O, 75 MHz): δ 63.1 (d, *J* = 4.4 Hz), 70.0, 73.9, 84.1 (d, *J* = 8.7 Hz), 88.2, 102.6, 142.0, 152.0, 166.5. ^{31}P-NMR (D_2O, 121 MHz): δ -9.02 (d, *J* = 19.6 Hz), -9.83 (d, *J* = 22.0 Hz), -21.60 (overlapping dd, *J* = 17.8, 17.8 Hz). MS (ESI-): *m/z* = 483.04 $[M-H]^-$.

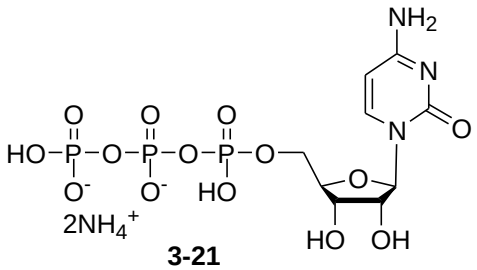
Guanosine-5'-triphosphate diammonium salt (3-20).



3-20

Obtained in 88% yield as its diammonium salt (133 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 5% CH_3CN-95% buffer over 50 min., t_r = 40 min] followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 3.82 (m, 2H, $H_{5'}$), 4.15 (d, *J* = 2.4 Hz, 1 H, $H_{4'}$), 4.33 (t, *J* = 4.6 Hz, 1H, $H_{3'}$), 4.55 (t, *J* = 5.1, 1H, $H_{2'}$), 5.73 (d, *J* = 5.1 Hz, 2H, $H_{1'}$), 8.02 (s, 1H, H_8). ^{13}C-NMR (D_2O, 75 MHz): δ 63.3 (d, *J* = 4.5 Hz), 70.5, 74.0, 84.4 (d, *J* = 8.3 Hz), 86.6, 115.9, 151.5, 153.9, 158.9. ^{31}P-NMR (D_2O, 121 MHz): δ -9.12 (d, *J* = 18.4 Hz), -9.65 (d, *J* = 21.1 Hz), -21.51 (overlapping dd, *J* = 19.5, 22.3 Hz). MS (ESI-): *m/z* = 522.05 $[M-H]^-$

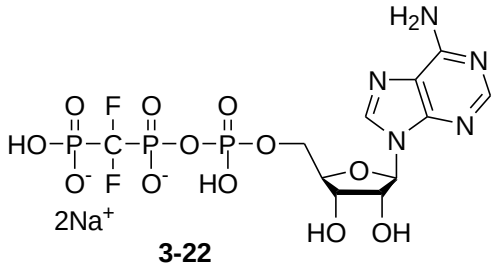
Cytosine-5'-triphosphate diammonium salt (3-21).



3-21

Obtained in 89% yield as its diammonium salt (125 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 6% CH_3CN-94% buffer over 40 min., t_r = 28 min] followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.03-4.12 (m, 5H, $H_{2',3',4',5'}$), 5.72 (s, 1H, $H_{1'}$), 5.88 (d, *J* = 7.0 Hz, 1H, H_5),7.71 (d, *J* = 7.3 Hz, 1H, H_6). ^{13}C-NMR (D_2O, 75 MHz): δ 64.5 (d, *J* = 5.3 Hz), 69.0, 74.0, 82.4 (d, *J* = 9 Hz), 89.1, 96.2, 141.6, 156.1, 164.8. ^{31}P-NMR (D_2O, 121 MHz): δ -7.46 (d, *J* = 19.4 Hz), -9.71 (d, *J* = 18.3 Hz), -21.05 (overlapping dd, *J* = 17.8, 17.8 Hz). MS (ESI-): *m/z* = 482.04 [M-H]$^-$

Adenosine 5'-β,γ-difluoromethylenetriphosphate disodium salt (3-22).

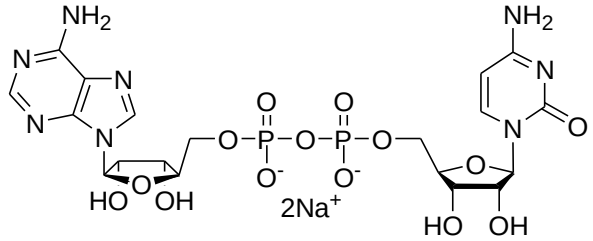


3-22

Obtained in 89% yield as its disodium salt (142 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 6% CH_3CN-94% buffer over 50 min., t_r = 40 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.11 (s, 2H, $H_{5'}$), 4.25 (t, *J* = 2.5 Hz, $H_{4'}$), 4.40 (t, *J* = 3.7, 1H, $H_{3'}$), 4.57 (t, *J* = 5.4, 1H,

$H_{2'}$) 5.92 (d, *J* = 5.8, 1H, $H_{1'}$), 8.00 (s, 1H, H_2), 8.31 (s, 1H, H_8). ^{13}C-NMR (D_2O, 75 MHz): δ 65.0 (d, *J* = 5.3 Hz), 70.2, 74.3, 83.8 (d, *J* = 9.2 Hz), 86.8, 118.2, 139.9, 148.6, 151.5, 154.4. ^{31}P-NMR (D_2O, 121 MHz): δ 2.78-5.13 (m), -2.77-4.46 (m), -9.76 (d, *J* = 30.7 Hz). ^{19}F NMR (D_2O, 282 MHz): δ -120.25 (t, *J* = 85.4 Hz). MS (ESI-): *m/z* = 540.02 $[M- H]^-$.

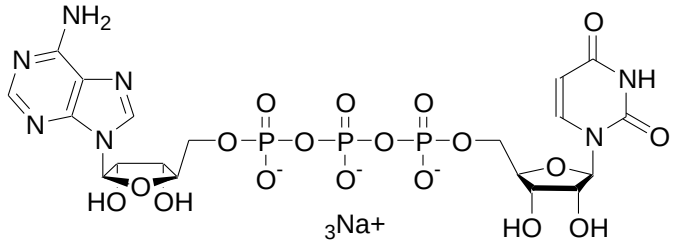
P1-Adenosine-P2-cytidine-5'-diphosphate (2-79).



2-79

Obtained in 83% yield as its disodium salt (156 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 5% CH_3CN-95% buffer over 55 min, t_r = 34 min] followed by passage through a Dowex-50-W-Na^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.01-4.19 (m, 7H, $H_{3'A,4'A,4'C,5'C,5'A}$), 4.27 (s, 1H, $H_{4'A}$), 4.39 (t, *J* = 4.0 Hz,1H,$H_{3'A}$), 5.64 (d, *J* = 7.6 Hz, 1H, H_{5C}), 5.70 (d, *J* = 3.8 Hz, $H_{1'A}$), 5.96 (d, *J* = 5.8 Hz, 1H, $H_{1'C}$), 7.49 (d, *J* = 7.6 Hz, 1H, H_{6C}), 8.07 (s, 1H,H_{2A}), 8.30 (s, 1H, H_{8A}). ^{13}C-NMR (D_2O, 75 MHz): δ 64.4, 65.2, 68.8, 70.3, 74.1, 74.3, 82.3, 83.7, 86.7, 89.1, 95.6, 118.1,139.5, 141.0, 148.6, 152.1, 154.8, 155.3, 164.1. ^{31}P-NMR (D_2O, 121 MHz): δ -9.66 (s). MS (ESI-): *m/z* = 651.21 $[M – H]^-$.

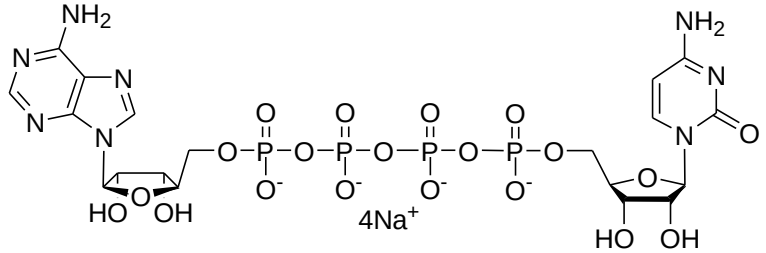
P1-Adenosine-P3-uridine-5'-triphosphate (2-83).



2-83

Obtained in 84% yield as its trisodium salt (181 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 8% CH_3CN-92% buffer over 45 min then a linear gradient to 10% CH_3CN-90% buffer over 10 min, t_r = 48 min] followed by passage through a Dowex-50-W Na^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.14-4.17 (m, 7H, $H_{5'A,2'U,3'U,4'U,5'U}$), 4.25 (bs, 1H, $H_{4'A}$), 4.42 (brt, *J* = 3.9 Hz, 1H, $H_{3'A}$), 4.60 (brt, *J* = 4.9 Hz, 1H, $H_{2'A}$), 5.60 (d, *J* = 8.0 Hz, 1H, H_{5U}), 5.71 (d, *J* = 3.8 Hz, 1H, $H_{1'U}$), 5.96 (d, *J* = 5.6 Hz, 1H, $H_{1'A}$), 7.65 (d, *J* = 8.6 Hz, 1H, H_{6U}), 8.05 (s, 1H, H_{2A}), 8.36 (s, 1H, H_{8A}). ^{13}C-NMR (D_2O, 75 MHz): δ 64.5 (d, *J* = 4.8 Hz), 65.1 (d, *J* = 5.0 Hz), 69.1, 70.3, 73.9, 74.6, 82.7 (d, *J* = 9.2 Hz), 83.6 (d, *J* = 8.9 Hz), 86.7, 88.3, 102.1, 118.2, 139.5, 140.9, 148.7, 151.3, 152.6, 155.1, 165.6. ^{31}P-NMR (D_2O, 121 MHz): δ -9.80 (d, *J* = 19.5 Hz), -21.30 (t, *J* = 18.3 Hz). MS (ESI-): *m/z* = 732.17 $[M - H]^-$.

P1-Adenosine-P4-cytidine-5'-tetraphosphate (3-25).

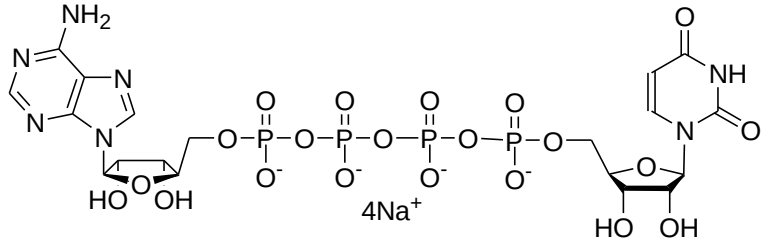


3-25

Obtained in 83% yield as its tetrasodium salt (201 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 7% CH_3CN-93% buffer over 55 min, t_r = 42 min]

followed by passage through a DOWEX-50-W-Na^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 3.99-4.16 (m, 9H, $H_{3'A,\ 3'C\ 4'A,4'C,5'C,5'A}$), 4.45 (s, 1H, $H_{2'A}$), 5.77 (d, *J* = 4.1 Hz, 1H, $H_{1'C}$), 5.87 (d, *J* = 7.6 1H, H_{5C}), 5.97 (d, *J* = 6.1Hz, 1H, $H_{1'A}$), 7.74 (d, *J* = 7.6 Hz, 1H, H_{6C}), 8.09 (s, 1H,H_{2A}), 8.38 (s, 1H, H_{8A}). ^{13}C-NMR (D_2O, 75 MHz): δ 64.5 (d, *J* = 5.1), 65.2 (d, *J* = 5.6 Hz), 69.1, 70.4, 74.2, 74.3, 82.5 (d, *J* = 9.1 Hz), 84.0 (d, *J* = 9.4 Hz), 86.5, 89.0, 96.1, 118.4, 139.8, 141.4, 148.9, 152.3, 155.0, 155.9, 164.6. ^{31}P-NMR (D_2O, 121 MHz): δ -9.63 (d, *J* = 14.1 Hz), 21.24 (d, *J* = 10.1Hz). MS (ESI-), *m/z* 811.11 $[M - H]^-$.

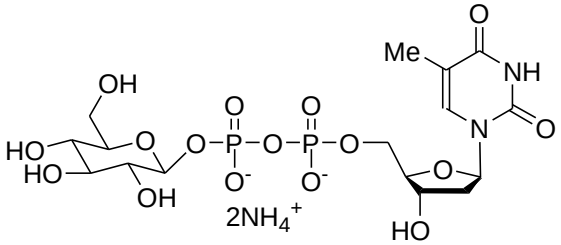
P1-Adenosine-P4-uridine-5'-tetraphosphate (2-85).



2-85

Obtained in 86% yield as its tetrasodium salt (209 mg) after purification by RP-HPLC [linear gradient of 100% buffer (pH 7.0) to 9% CH_3CN-91% buffer over 60 min, t_r = 47 min] followed by passage through a DOWEX-50-W-Na^+ ion exchange column. ^{1}H NMR (D_2O, 300 MHz): δ 4.12-4.29 (m, 8H, $H_{4'A,5'A,2'U,3'U,4'U}$), 4.46 (t, *J* = 4.0 Hz,1H, $H_{3'A}$), 5.72 (d, *J* = 8.1 Hz, 1H, H_{6U}), 5.78 (d, *J* = 4.9 Hz, 1H, $H_{1'U}$), 5.97 (d, *J* = 5.9 Hz, 1H, $H_{1'A}$), 7.73 (d, *J* = 8.0 Hz, 1H, H_{5U}), 8.06 (s, 1H,H_{2A}), 8.37 (s, 1H,H_{8A}). ^{13}CNMR (D_2O, 75 MHz): δ 64.9 (d, *J* = 5.1 Hz), 65.2,(d, *J* = 5.1 Hz), 69.5, 70.3, 73.7,74.4, 83.1 (d, *J* = 8.6 Hz), 83.9 (d, *J* = 8.6 Hz), 86.7, 88.1, 102.4, 118.3, 139.9, 141.3, 148.8, 151.6, 152.0, 154.8, 165.8. ^{31}P NMR (D_2O, 121 MHz): δ -9.60, -21.03 (d, *J* = 9.9 Hz). MS (ESI-): *m/z* 812.16 $[M - H]^-$.

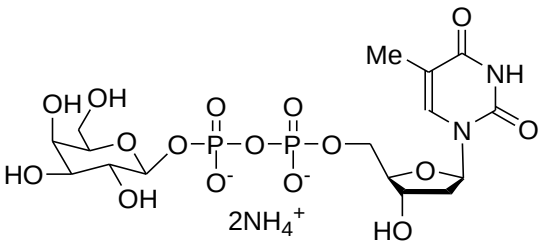
β-D-glucose-thymidine-5'-diphosphate (3-30).



3-30

The acceptor, β-D-glucose-1-phosphate was prepared using a procedure similar to that used by Binch et al. for the synthesis of β-L-galactose-1-phosphate.[170] Obtained in 91% yield as its diammonium salt (150 mg) after purification by RP-HPLC [linear gradient of 0% CH_3CN-100% buffer (pH 7.0) to 6% CH_3CN-94% buffer over 40 min then a linear gradient to 100% CH_3CN over10 min, t_r = 34 min] followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 1.80 (s, 3H,H_{methyl}), 2.25 (m, 2H, $H_{2'}$),3.22-3.80 (m, 6 H, $H_{2glu,3glu,4glu,5glu,6glu}$), 4.06 (br, 3H, $H_{4',5'}$), 4.50 (br, 1 H, $H_{3'}$), 4.88 (t, *J* = 7.8 Hz, 1H, H_{1glu}), 6.22 (t, *J* = 7.2, 1H, $H_{1'}$), 7.62 (s, 1H, H_6). ^{13}C-NMR (D_2O, 75 MHz): δ 11.6, 38.5, 60.7, 62.4, 65.4 (d, *J* = 5.3 Hz), 69.4, 71.0, 73.4, 73.5, 73.6, 75.1, 76.4, 84.9, 85.3 (d, *J* = 9 Hz), 97.7 (d, *J* = 7.5 Hz), 111.7, 137.3, 151.7, 166.5. ^{31}P-NMR (D_2O, 121 MHz): δ-9.93 (d, *J* = 19.8 Hz),-11.51 (d, *J* = 19.7 Hz). MS (ESI-): m/z 563.15$[M - H]^-$.

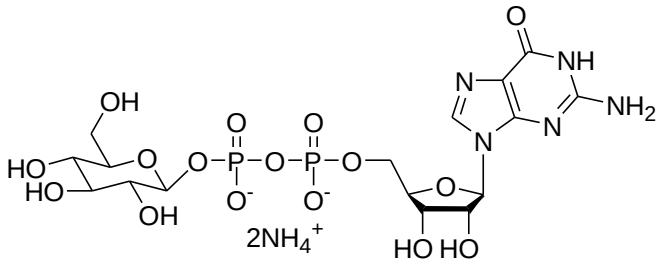
β-D-galactose-thymidine-5'-diphosphate (3-31).



3-31

The acceptor, β-D-glalactose-1-phosphate was prepared using a procedure similar to that used by Binch et al for the synthesis of β-L-galactose-1-phosphate.[170] Obtained in 91% yield as its diammonium salt (150 mg) after purification by RP-HPLC [linear gradient of 0% CH_3CN-100% buffer (pH 7.0) to 6% CH_3CN-94% buffer over 40 min then a linear gradient to 100% CH_3CN over10 min, t_r = 32 min] followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 1.77 (s, 3H, H_{methyl}), 2.25 (m, 2H, $H_{2'}$), 3.08-3.64 (m, 5H, $H_{2gal,3gal,5gal,6gal}$), 3.78 (d, *J* = 3.2, 1H, H_{4gal}), 4.04 (br, 3H, $H_{4',5'}$), 4.53 (br, 1H, $H_{3'}$), 4.82 (t, *J* = 7.8 Hz, 1H, H_{1gal}), 6.22 (t, *J* = 7.2, 1H, $H_{1'}$), 7.62 (s, 1H, H_6). ^{13}C-NMR (D_2O, 75 MHz): δ 11.6, 38.5, 61.1, 62.4, 65.4 (d, *J* = 5.3 Hz), 68.5, 71.0, 71.1, 71.9, 72.2, 75.8, 84.9, 85.3 (d, *J* = 9 Hz), 98.4 (d, *J* = 6 Hz), 111.7, 137.3, 151.7, 166.5. ^{31}P-NMR (D_2O, 121 MHz): δ-9.67 (d, *J* = 18.9 Hz),-11.11 (d, *J* = 21.9 Hz). MS (ESI+): *m/z* = 565.06 $[M + H]^+$.

β-D-glucose-guanosine-5'-diphosphate (3-32).

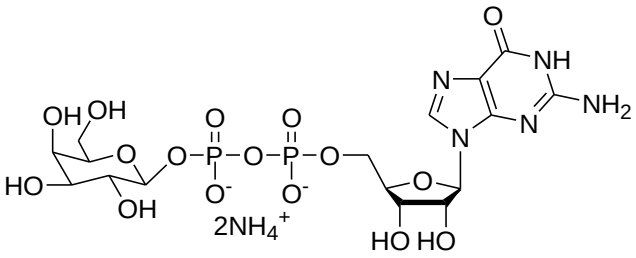


3-32

Obtained in 82% yield as its diammonium salt (141 mg) after purification by RP-HPLC [linear gradient of 0% CH_3CN-100% buffer (pH 7.0) to 4% CH_3CN-96% buffer over 45 min

then a linear gradient to 100% CH_3CN over10 min, t_r = 38 min] followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 3.19 (m, 2H, $H_{2glu,4glu}$), 3.32-3.38 (m, 2H, $H_{3glu,5glu}$), 3.50, 3.7 (AB system, 2H, *J* = 12 Hz, H_{6glu}), 4.04 (s, 2H, $H_{5'}$), 4.17 (s, 1H, $H_{4'}$), 4.34 (s, 1H, $H_{3'}$), 4.57 (t, *J* = 5.6 Hz, 1H, $H_{2'}$), 4.82 (t, *J* = 7.8 Hz, 1H, H_{1glu}), 5.73 (d, *J* = 5.8, 1H, $H_{1'}$), 7.95 (s, 1H, H_8). ^{13}C-NMR (D_2O, 75 MHz): δ 60.6, 65.14 (d, *J* = 5.3 Hz), 69.3, 70.3, 73.4, 73.5, 73.6, 75.0, 76.4, 83.6 (d, *J* = 7.5 Hz), 86.8, 97.7 (d, *J* = 6 Hz), 115.8, 137.4, 151.5, 153.8, 158.6. ^{31}P-NMR (D_2O, 121 MHz): δ-9.60 (d, *J* = 18.7 Hz),-11.34 (d, *J* = 20.3 Hz). MS (ESI-): *m/z* = 604.17 [M - H]$^-$.

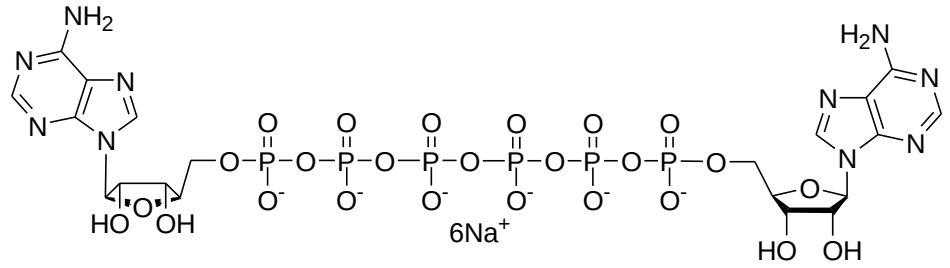
β-D-galactose-guanosine-5'-diphosphate (3-33).



3-33

Obtained in 82% yield as its diammonium salt (141 mg) after purification by RP-HPLC [linear gradient of 0% CH_3CN-100% buffer (pH 7.0) to 5% CH_3CN-96% buffer over 40 min then a linear gradient to 100% CH_3CN over10 min, t_r = 28 min] followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 3.44- 3.64 (m, 5H, $H_{2gal,3gal,5gal,6gal}$), 3.72 (d, *J* = 3.0 Hz, H_{4gal}), 4.05 (br, 2H, $H_{5'}$), 4.18 (br, 1H, $H_{4'}$), 4.35 (t, *J* = 3.5, 1H, $H_{3'}$), 4.58 (T, *J* = 5.6, 1H, $H_{2'}$), 4.78 (t, *J* = 7.7 , 1H, H_{1gal}), 5.74 (d, 1H, $H_{1'}$), 7.95 (s, 1H, H_8). ^{13}C-NMR (D_2O, 75 MHz): δ 61.2, 65.3 (d, *J* = 6 Hz), 68.6, 70.4, 71.2, 71.3, 72.3, 73.8, 75.8, 83.7 (d, *J* = 9 Hz), 86.9, 98.5 (d, *J* = 6 Hz), 115.8, 137.4 151.6, 153.9, 158.7. ^{31}P-NMR (D_2O, 121 MHz): δ-9.61 (d, *J* = 19.7 Hz),-11.28 (d, *J* = 19.1 Hz). MS (ESI-): *m/z* 604.16 [M - H]$^-$.

3.5.9 Synthesis of P1,P6-diadenosine hexaphosphate (3-26).



3-26

To a solution of 5´-ATP $(Bu_4N^+)_{3.2}$ (0.27 mmol) in dry DMF (4 mL) was added magnesium chloride (0.32 mmol, 26 mg) and the mixture was stirred for 2 min. until magnesium chloride dissolved. Diisopropylethylamine (0.81 mmol, 141 µL) was added and the reaction mixture was cooled to 0 ^oC (ice bath). Reagent **3-12** (0.16 mmol, 63 mg) was added, the ice bath was removed and the mixture was stirred at room temperature for 3 hours. The mixture was cooled to 0 ^oC (ice bath) and quenched with 50 mM triethylammonium acetate buffer (5 mL, pH 7.0), washed with chloroform (3 x 10 mL). Chelex resin (ca. 0.2 g) was added and the mixture stirred for 1 min then filtered through a cotton plug. Purification was achieved by RP-HPLC using a semipreparative C18 column and a gradient of acetonitrile and buffer (50 mM triethylammonium acetate, pH 7) at 6 mL/min and monitored at 255 and 280 nm with linear gradient of 100% buffer (pH 7.0) to 20% CH_3CN-80% buffer over 55 min (t_r = 40 min). Fractions containing the desired product were pooled, concentrated by high vacuum rotary evaporation, and the residue was dissolved in water and repeatedly freeze-dried until the ^{1}HNMR

spectrum indicated that no residual buffer was present. The resulting white powder was passed through a Dowex-50-W ion-exchange resin in Na^+ form and then lyophilized which gave 121 mg of **3.35** as its hexasodium salt (80%). ^{1}H-NMR (D_2O, 300 MHz): δ 4.13 (br, 4H, $H_{5'}$), 4.27 (br, 2H, $H_{4'}$), 4.46 (br, 2H, $H_{3'}$), 5.92 (d, *J* = 5.8 Hz, 2H, $H_{1'}$), 8.01 (s, 2H, H_2), 8.33 (s, 2H, H_8). ^{13}C-NMR (D_2O, 75 MHz): δ 65.0 (d, *J* = 5.2 Hz), 70.4, 74.2, 84.0 (d, *J* = 49.2 Hz), 86.6, 118.1, 140.0, 148.7, 151.8, 154.6. ^{31}P-NMR (D_2O, 121 MHz): δ -9.88 (d, *J* = 15.2 Hz), -21.6 (s). MS (ES-): *m/z* 995.11 [M - H]$^-$, HRMS (ESI-): *m/z* = 994.9750, $C_{20}H_{29}N_{10}O_{25}P_6$ [M - H]$^-$, requires 994.9731.

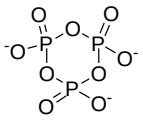
Chapter 4

Activated Cyclic Trimetaphosphate as a Reagent for the Synthesis of Nucleoside Polyphosphates and their Conjugates

4.1 Introduction

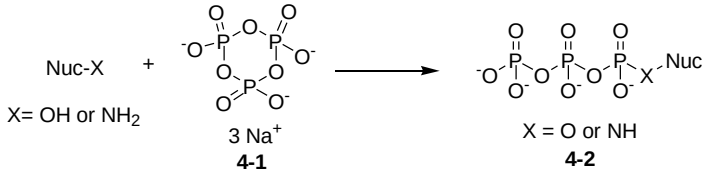
4.1.1 Trimetaphosphate as a phosphorylating agent

Trimetaphosphate (TriMP, **4-1**) is a six-membered ring, cyclic triphosphate (Figure 4.1). It is commercially available as its trisodium salt and is very inexpensive ($33 for 500 g from Sigma-Aldrich). It has been examined as a triphosphorylating agent (Scheme 4.1) since the 1960's.[172,174] The use of TriMP as a triphosphorylating agent is very appealing because the triphosphate product can potentially be produced in one step as opposed to other triphosphorylating methodologies that are multistep processes such as Bogachev's (see chapter 2, section 2.1.5) and Eckstein's procedure (see chapter 2, section 2.1.3.2.3).[171] In spite of this potential advantage only a few examples of the triphosphorylation of hydroxyl groups, on nucleosides or other types of OH-bearing compounds, have been reported using TriMP. One of the reasons for this is that TriMP is not a potent electrophile. Modest hydroxyl nucleophiles (alcohols and phenols) react extremely slowly with it. For example, the reaction of a five-fold excess of phenol at pH 12 at rt with TriMP produces the triphosphorylated product **4-3** in a 67% yield after 11 days (Scheme 4.2).[172,173] The rate of reaction increases with increasing pH (no reaction occurs at all at pH 7). This is because the concentration of phenoxide nucleophile increases with increasing pH (pK_a of phenol = 10). At 70 ^{o}C after 2 h **4-3** was produced in a 36% yield. However, decomposition of the TriMp also occurred at the higher temperature.

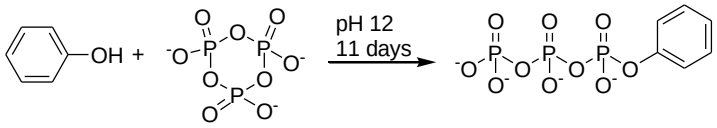


4-1

Figure 4.1. Structure of trisodium TriMP.

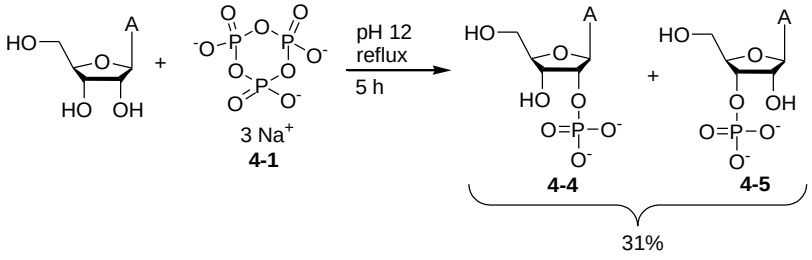


Scheme 4.1. TriMP as a triphosphorylating agent.



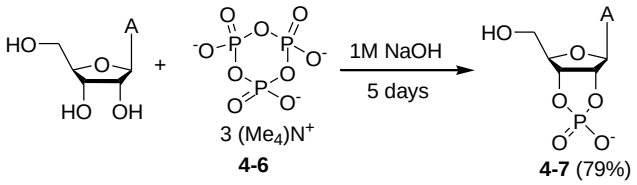
Scheme 4.2. Synthesis of triphosphate **4-3** using TriMP as a triphosphorylating agent.

The first attempt to phosphorylate a nucleoside using TriMP was reported by Schwartz who found that refluxing an aq. alkaline (pH 12) solution of TriMP and adenosine for 5 h gave a mixture of adenosine 2'- and 3'-monophosphates **4-4** and **4-5** in 31% overall yield (Scheme 4.3).[174] No triphosphorylated product was produced.



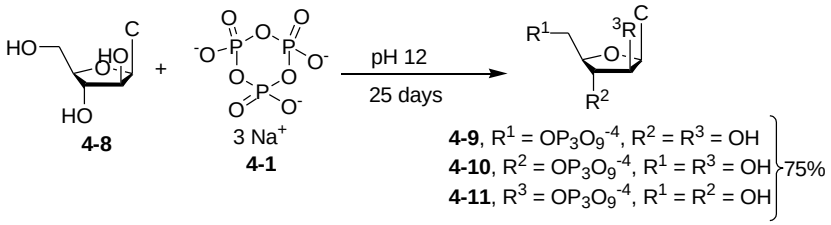
Scheme 4.3. Preparation of **4-4** and **4-5** using TriMP.

Saffhil found that by subjecting a mixture of the tri(tetramethyammonium) salt of TriMP and adenosine to aq. 1 M NaOH for 5 days at rt that adenosine 2',3'-cyclic phosphate could be isolated in a 79% yield (Scheme 4.4).[175] Yamagata et al[176] reported that the reaction of TriMP with adenosine could occur at neutral conditions (pH 7, 41 ^{o}C, 1 day) in the presence of a cat. amount of Mg^{+2} to afford **4-7** in a 9% yield.



Scheme 4.4. Synthesis of **4-7** using TriMP.

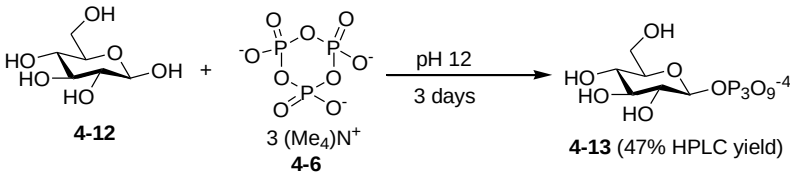
Kusuhara et al[177] performed the triphosphorylation of cytarabine (Scheme 4.5) using a 10-fold excess of **4-1** in aqueous solutions at pH 12 and room temperature for 25 days and obtained a 75% yield of a mixture of cytarabine 5'-triphosphate, cytarabine 3'-triphosphate, and cytarabine 2'-triphosphate.



Scheme 4.5. Triphosphorylation of cytarabine using TriMP.

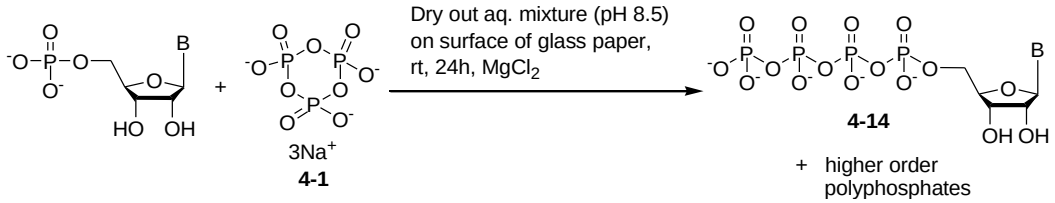
Although the triphosphorylation of nucleosides using TriMP has not been very successful, the triphosphorylation of certain other hydroxyl-bearing compounds has been achieved more successfully with this reagent. For example, the reaction of glucose with a five-fold excess of **4-1** at pH 12 for 3 days at room temperature gave mainly β-D-glucopyranosyl-1-triphosphate in an HPLC yield of 47 % (Scheme 4.6).[178,179] The phosphorylation of the

disaccharides cellobiose, lactose, and α,α-trehalose under the same conditions gave β-D-glucopyransosyl-(1-4)-β-D-glucopyranosyl-1-triphosphate, β-D-galactopyransosyl-(1-4)-β-D-glucopyranosyl-1-triphosphate, and 3-*O*-triphospho-α-D-glucopyranosyl (1-1)-α-D-galactopyranoside with maximum yields of 28%, 35%, and 20% respectively.[180]



Scheme 4.6. Triphosphorylation of glucose using TriMP.

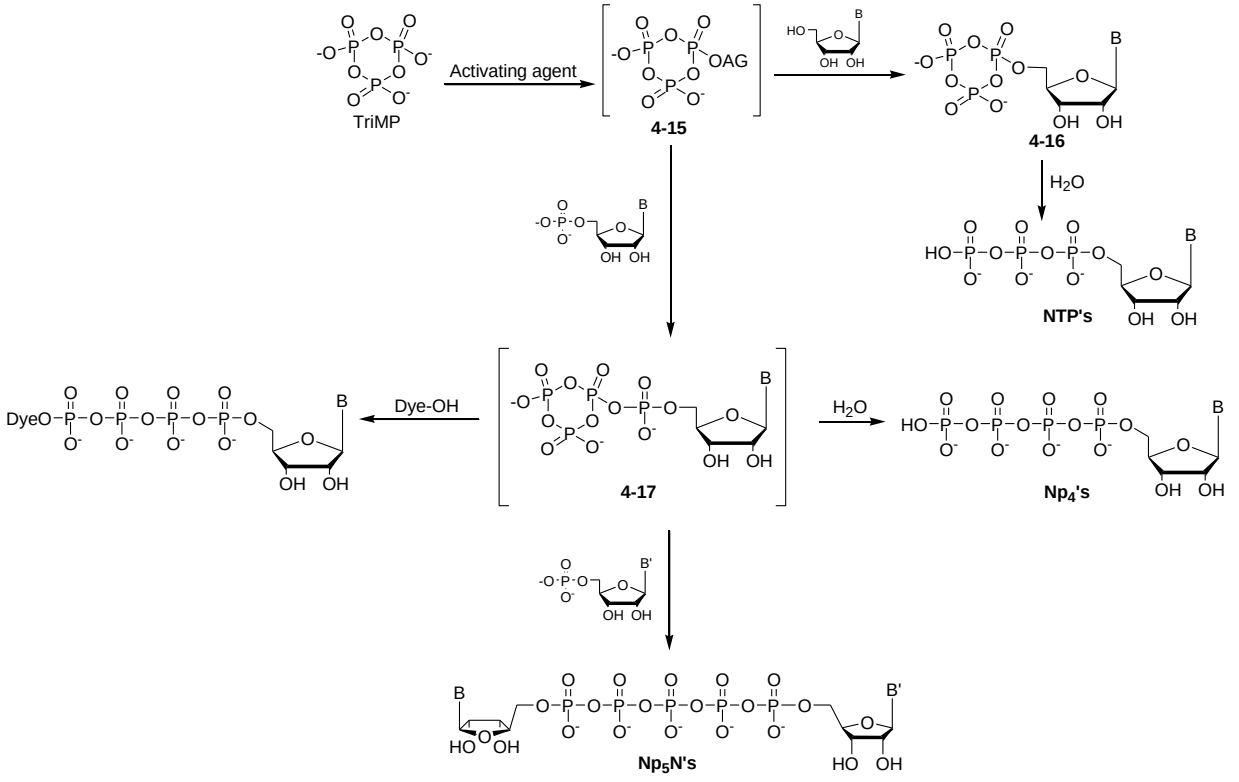
It appears that TriMP can phosphorylate the phosphate groups of nucleotides. Lohrmann showed that when solutions of NMPs and TriMP are dried out on the surface of glass paper at ambient temperature over 24 h a mixture of mainly nucleoside-5'-tetraphosphates (Np_4's) and higher order nucleoside 5'-polyphosphates are formed (Scheme 4.7). The reaction is catalyzed by magnesium ions. These compounds were never separated and isolated and yields of isolated products were not given.[181]



Scheme 4.7. Reaction of TriMp with NMP's to give Np4's and higher order nucleoside 5'-polyphosphates.

As stated above, the problem with TriMP as a triphosphorylating agent of hydroxyl groups stems from its rather poor electrophilicity/reactivity. One approach by which TriMP could be made more electrophilic is to react it with an activating species thus converting one of

the phosphoryl oxygens into a good leaving group (Scheme 4.8). Reaction of this activated species with a nucleoside could, in theory, produce intermediate **4-16**. Hydrolysis of intermediate **4-16** would give an NTP. Besides acting as a triphosphorylating agent activated TriMP could, in theory, also be used for preparing higher order nucleoside polyphosphates and their conjugates. For example, an activated form of TriMP could react with an NMP to give intermediate **4-17** which could then be reacted with nucleophiles such as NMP's to give an Np_5N's, or with water to give Np_4's, or with a fluorescent dye to give Np_4's where the fluorescent tag is attached to the terminal phosphate group (Scheme 4.8). As much of the work described in this chapter focusses on the synthesis of Np_5N's and Np_4's a discussion of their biological roles, applications and synthesis is given below.



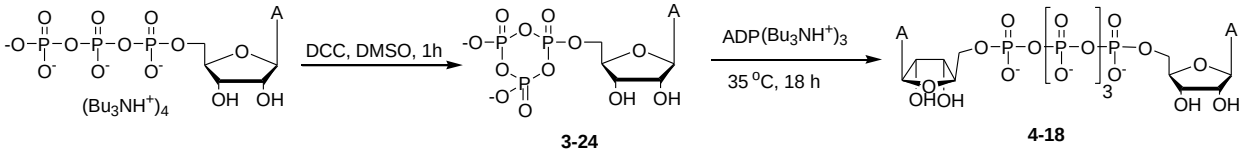
Scheme 4.8. Synthesis of NTP's, Np_4s', fluorescently tagged Np_4's and Np_5N's via activated TriMP. AG = activating group.

4.1.2. Biological roles, applications and synthesis of dinucleoside 5',5'-pentaphosphates (Np_5N) and nucleoside 5'-tetraphosphates (Np_4's).

Dinucleoside 5',5'-pentaphosphates (Np_5N's) and nucleoside 5'-tetraphosphates (Np_4's) play important roles in biological systems, have potential as drugs and have been used as tools and reagents in biochemistry and biotechnology. Ap_5A in nanomolar concentrations is found to significantly stimulate the proliferation of vascular smooth muscle cells by interacting with P2Y receptors.[182] AP_5A is also a P2X receptor agonist. P2X receptors are a family of cation-permeable ligand-gated ion channels that open in response to the binding of extracellular adenosine 5'-triphosphate.[182] Ap_5A is a specific adenylate kinase inhibitor in the hippocampus.[17] It decreases the rate of decomposition of ADP and the formation of ATP which influences the availability of purines in the central nervous system. Ap_5A, along with Ap_5T, are potent inhibitors of other enzymes such as thymidine kinase, thymidylate kinase and ribonucleotide reductase.[17-24] The crystal structure of *Aquifex aeolicus* adenylate kinase complexed with Ap_5A was used to determine how the enzyme achieves a catalytically competent state.[23] Np_5N's can also be enzyme substrates For example, Ap_5A is a good substrate for *Humulus lupulus* adenylate isopentyltransferase exhibiting a higher affinity than AMP, ADP and ATP.[24] Recently, considerable attention has also been directed towards Np_4's as these species have been shown to be selective agonists of the $P2Y_4$ receptor.[183] They are also used as synthons to prepare 2'-deoxynucleoside-5'-tetraphosphates containing terminal fluorescent labels which, as a result of being excellent substrates of DNA polymerase, have found use in various applications in DNA sequencing and labeling.[4]

Various approaches have been reported for the preparation of Np_5N and Np_4's; however, for the most part, these procedures are either lacking in scope and/or do not produce the desired products in good yield. For example, Ap_5A has been prepared by: (1) reaction of tri-*n*-

butylammonium pyrophosphate with adenosine 5´-phosphoromorpholidate (4% yield),[19] (2) conversion of ATP to Ap_4 followed by condensation of the latter with P^1-(adenosine-5')-P^2,P^2-diphenyl pyrophosphate (AMP-P(O)$(OPh)_2$) (10% yield),[184] (3) condensation of ADP with P^1-(adenosine-5´)-P^4,P^4-diphenyl tetraphosphate (ATP-P(O)$(OPh)_2$) (25% yield),[185] (4) reaction of 2,3-*O*-6-*N*-triacetyladenosine with salicylchlorophosphite followed by sequential reaction with the tri-*n*-butylammonium salts of inorganic pyrophosphate and ADP and then removal of the protecting groups (48% yield, see Chapter 2, section 2.1.3.2.3, Scheme 2.8)[77] and, (5) treating ATP with DCC to give adenosine 5´-trimetaphosphate (**3-24**) and then reacting the tri-*n*-butyl ammonium salt of this compound with the tri-*n*-butyl ammonium salt of ADP. This gave Ap_5A in 52% yield which is the highest reported yield of this compound to date (Scheme 4.9).[21]

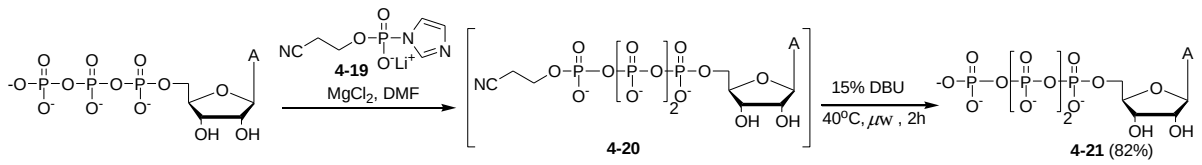


Scheme 4.9. Synthesis of Ap_5A through formation of adenosine trimetaphosphate followed by reaction with ADP.

Up_5U (**2-54,** chapter 2) has been obtained in 8% yield by cyclization of UTP with DCC followed by reaction of the resultant uridine 5'-cyclic trimetaphosphate with UDP (see Chapter 2 section 2.1.3.2.4**,** Scheme 2.14).[88,186] Ap_5T has been obtained in 22% yield by reaction of ATP-morpholidate with the bis(tri-*n*-octylammonium salt) of thymidine 5'-diphosphate.[22]

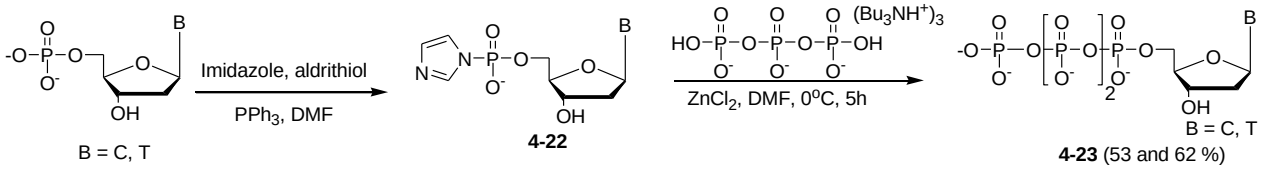
Kumar et al. reported the synthesis of Np_4's by reaction of nucleoside 5'-trimetaphosphates with orthophosphate though no yields or experimental details were provided.[4] Ko et al. reported the synthesis of 5'-tetraphosphates of thiouridine derivatives in yields of less than 8% by reaction of the nucleosides with phosphorus oxychloride followed by the addition of

tri-*n*-butylammonium pyrophosphate.[187] Gp_4 has been prepared by Zuberek et al in 82% yield by the reaction of GDP-imidazolidate with triethylammonium salt of pyrophosphate. Employing a similar procedure Skoblov et al prepared dTp_4 in 49% yield. [89,188] Very recently new approaches to the synthesis of Np_4's have appeared in the literature. Strenkowska et al reported the synthesis of Ap_4 in an 82% yield by reacting ATP with cyanoethylphosphoimdazolidate **4-19** under microwave irradiation followed by phosphate deprotection with DBU (Scheme 4.10).[189] Although this method provided Ap_4 in good yield it requires the synthesis of **4-19**, microwave radiation and a deprotection step at the end of the synthesis.

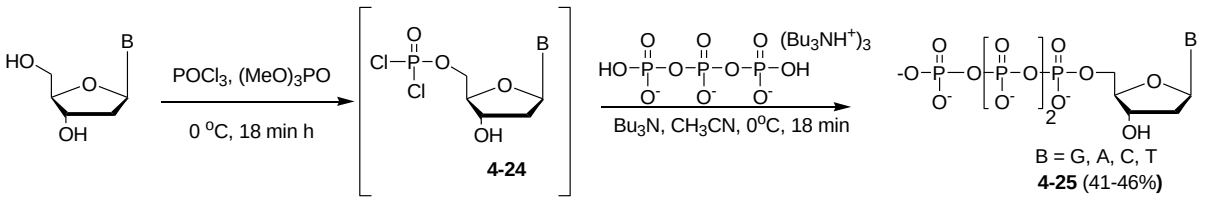


Scheme 4.10. Synthesis of Ap_4 by Strenkowska et al.

Kore et al prepared dCp_4 and Tp_4 (Scheme 4.11) in yields of 62% and 53% respectively by coupling tris(tri-*n*-butylammonium) triphosphate with the isolated imidazolidates of dCMP and TMP.[190] Shortly thereafter, the same group reported a one-pot synthesis of dCp_4, dGp_4, dAp_4, and dTp_4 in yields of 41-46% by reacting nucleosides with $POCl_3$ followed by reaction of the resulting nucleoside dichlorophosphoridate **4-24** with tris(tri-*n*-butylammonium) triphosphate (Scheme 4.12).[190]



Scheme 4.11. Kore et al. synthesis of dCP_4 and Tp_4 via imidazolate **4-22**.

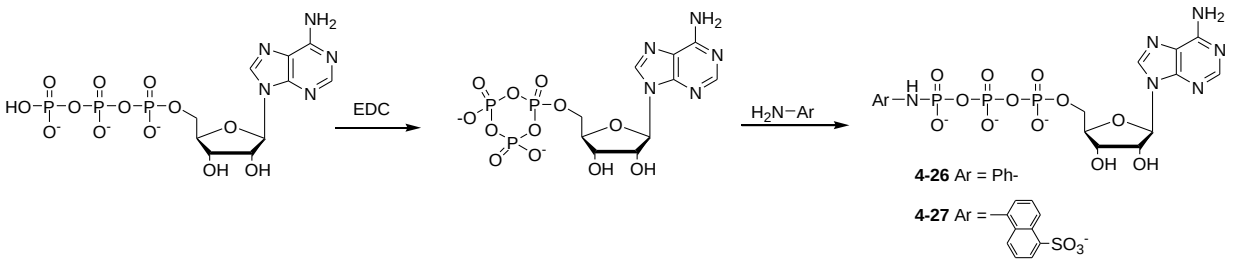


Scheme 4.12. Kore et al synthesis of dCP_4 and Tp_4 via nucleoside dichlorophosphoridate **4-22.**

Ap_4 has been prepared using enzymes; however, this approach to Np_4 synthesis is limited by scale and the substrate specificity of the enzymes.[116]

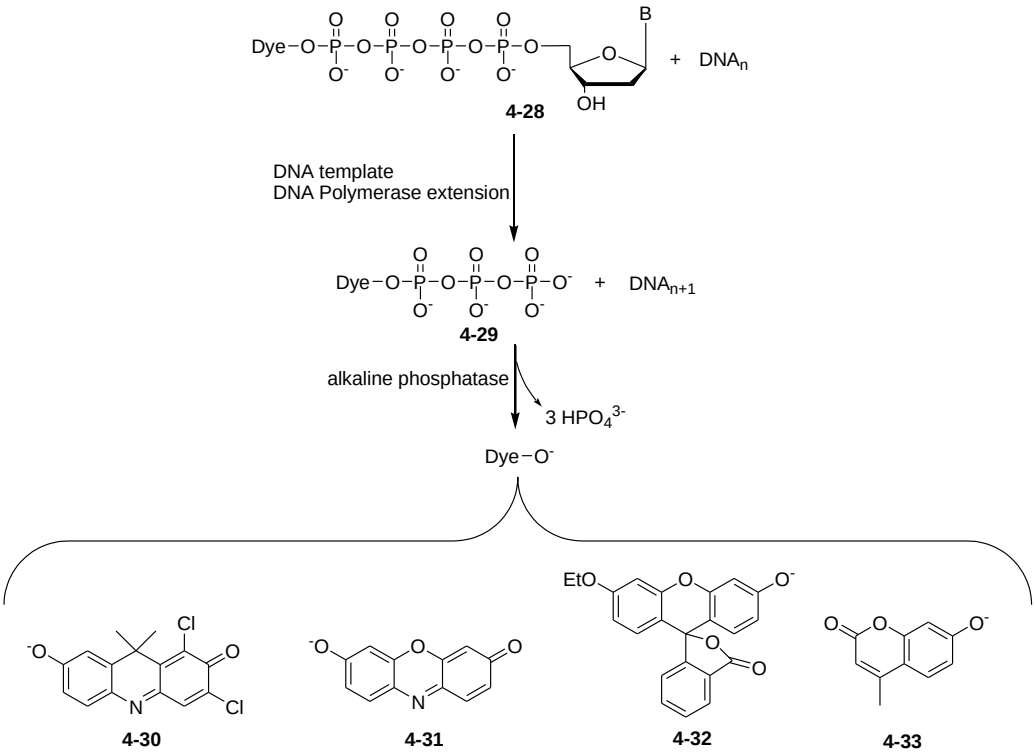
4.1.3 Applications and synthesis of Np_4's containing a terminal fluorescent label

Terminal phosphate-labelled nucleotides have been used as tools and probes in biochemistry and biotechnology for many years.[192-194] Grachev and Zaychikov reported the synthesis of an analog of ATP containing aniline bound to the γ-phosphate via a phosphoamidate linkage (**4-26** in Scheme 4.13) through cyclization of ATP using EDC to form cyclic adenosine trimetaphosphate followed by ring opening with aniline to afford ATP-γ-anilidate **4-26** which was found to be a good substrate for DNA-dependent RNA polymerase of *E. Coli.*[194] Yarbrough has prepared compound **4-27** which contains the fluorophore, 1-aminonaphthalene-5-sulfonate, attached via a γ-phosphoamidate bond via the same route. This analog is strongly fluorescent and is substrate for DNA-dependent RNA polymerase of *E. Coli* and wheat germ RNA polymerase II. Cleavage of the of the α-β phosphoryl bond as a result of RNA synthesis produces a shift in the fluorescence emission spectrum making **4-27** a useful compound for mechanistic studies of these enzymes.[193]



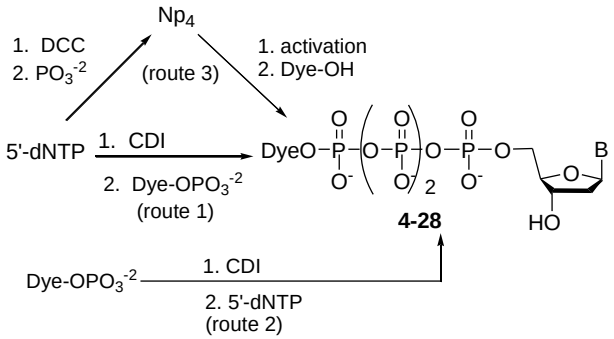
Scheme 4.13. Synthesis of γ-labeled ATP analogs.

Very recently there has been considerable interest in 2′-deoxynucleoside 5′-polyphosphates in which a fluorescent label is attached to the terminal phosphate. Such nucleotides are used as key reagents in high-throughput DNA sequencing techniques and in single nucleotide polymorphism typing assays.[3-7] Central to the success of these methodologies is the ability of the terminally-labeled nucleotides to as act as substrates for DNA polymerase. Nucleotides bearing more than three linear 5′-phosphates, such as δ-labeled nucleoside 5′-tetraphosphates (general structure **4-28** in Scheme 4.14), are used as it has been shown that such nucleotides are much better substrates for DNA polymerases than the corresponding γ-labeled 5′-triphosphates.[3] Once the nucleotide is incorporated into a DNA template using DNA polymerase, a triphosphorylated dye (**4-29**) is released (Scheme 4.14). This is rapidly hydrolyzed by alkaline phosphatase to release the free dye anion which is strongly fluorescent. The dye selected for these assays must be able to change color or fluorescence when converted from an alcohol (-OH) form to phosphate ester form. 1,3-Dichloro-7-hydroxy-9,9-dimethylacridin-2(9*H*)-one (**4-30**, DDAO), resorufin (**4-31**), ethylfluorescein (**4-32**), 7-hydroxy-4-methylcoumarin (**4-33)** are some examples of compounds used dye labels (Scheme 4.14).



Scheme 4.14. The use of phosphate-labelled nucleotidetetraphosphates for DNA sequencing.

The synthesis of δ-labeled nucleoside 5´-tetraphosphates has been achieved by several routes (Scheme 4.15). In one approach (route 1) a dNTP is reacted with CDI followed by reacting the resulting imidizolidate with a phosphorylated fluorescent dye.[7] Alternatively, a phosphorylated dye is activated with CDI followed by reaction with a dNTP (route 2).[3] In another approach (route 3) a dNTP is reacted with DCC to give a cyclic trimetaphosphate nucleotide derivative which is ring opened with orthophosphate to give a nucleoside 5´-tetraphosphates (Np_4).[4] This species is activated and then reacted with a dye. Specific yields for these routes were not reported. All of these approaches require expensive dNTP's as substrates and, in the case of routes 1 and 2, the synthesis or purchase of expensive phosphorylated dyes.



Scheme 4.15. Literature routes to nucleoside 5´-tetraphosphates bearing terminal fluorescent dyes.

4.2 Objectives

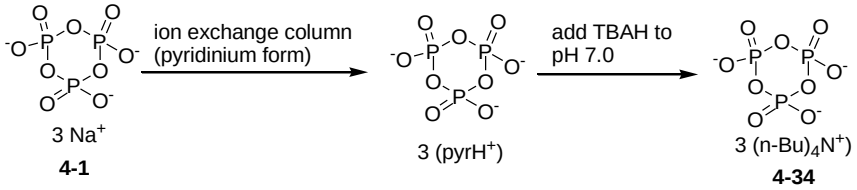
The objective of the work presented in this chapter is to develop improved methods for preparing NTP's, Np_5N's, Np_4's and fluorescent conjugates of Np_4's using activated TriMP as the key reagent as outlined in Scheme 4.8.

4.3 Results and discussion

4.3.1 Preparation of the tris(tetrabutylammonium) salt of TriMP

It was expected that an activated form of TriMP would be sensitive to moisture and so would have to be made under anhydrous conditions in dry organic solvents. Moreover, all subsequent reactions involving the activated TriMP would also have to be conducted under anhydrous conditions in organic solvents. The trisodium salt of TriMP, the commercially available form, is completely insoluble in polar aprotic solvents such as pyridine, acetonitrile or DMF. Hence before our studies could begin it was necessary to convert the trisodium salt into a tri- or tetraalkylammonium salt. We first converted TriMP into its tris(triethylammonium) salt; however, we found that this form is not soluble in pyridine, DMF, or acetonitrile. Therefore we decided to prepare the tris(tetrabutylammonium salt) of TriMP (compound **4-34**). Besecker et al. prepared **4-34** in 72% yield by passing the trisodium salt of TriMP through a cation exchange

resin in the tetrabutylammonium form followed by concentration of the effluent followed by a series of washing and filtering steps.[195] We found that this procedure consumes a lot of expensive tetrabutylammonium hydroxide (TBAH) in conversion of the resin from its H^+ form to its tetrabutylammonium form and so we examined other approaches to this compound. Glonek et al prepared **4-34** in an unspecified yield by passing a solution TriMP through a cation exchange resin in H^+ form followed by titration of the resulting solution with TBAH.[196] When we used this procedure we found that some decomposition occurred as determined by the ^{31}P-NMR of the freeze dried material. This probably occurred during its conversion to the free acid form. Nevertheless, we found that we could prepare **4-34** in almost quantitative yield and free of decomposition products by first converting TriMP to its pyridinium form using an ion exchange column followed by titrating the eluent solution to pH 7 with a dilute solution of TBAH followed by freeze drying to a white powder (Scheme 4.16). The ^{31}P-NMR of **4-34** in D_2O prepared in this manner exhibited a singlet at δ -19.2. No pyridinium species were evident by ^{1}H-NMR. Elemental analysis revealed that it was obtained in its tris(tetrabutylammonium) form. **4-34** was found to be soluble in pyridine, acetonitrile and DMF.

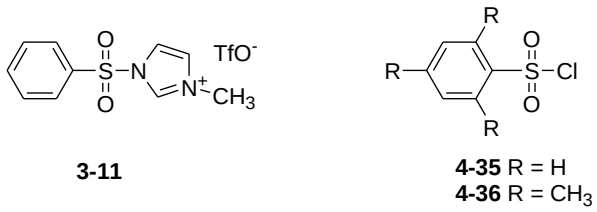


Scheme 4.16. Preparation of the tris(tetrabutylammonium) salt of TriMP.

4.3.2 Synthesis of Np_4's

Given the success that we had with activating NMP's, NDP's and NTP's with sulfonylimidazolium salts such as **3-11** we decided to use this salt to activate **4-34**. We also

decided to investigate benzenesulfonyl chloride (**4-35**) and mesitylene chloride (**4-36**), in combination with NMI, as activating agents as compounds **4-35** and **4-36**, unlike reagent **3-11**, are commercially available, relatively inexpensive and compound **4-36** in combination with NMI has been used as a coupling agent for phosphate ester bond formation (Figure 4.2).[197]



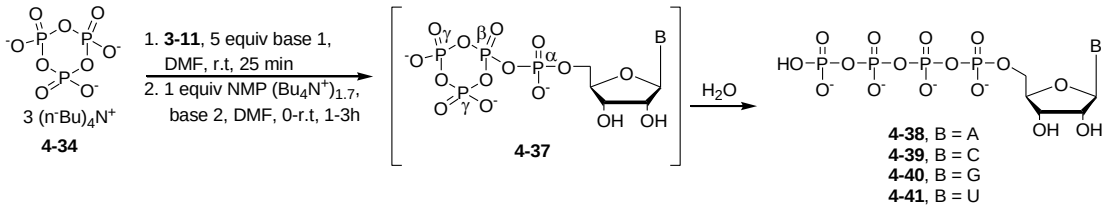
3-11

4-35 R = H
4-36 R = CH_3

Figure 4.2. Agents examined for activating TriMP.

Compound **3-11** was examined first as an activating agent for the preparation of Np_4's. Upon adding 1 equiv of **3-11** (Table 4.1 entry 1) to an acetonitrile solution of 1.0 equiv **4-34** in the presence of 5 equiv of NMI at room temperature, the reaction mixture became slightly turbid after about a minute.

Table 4.1. Synthesis of Np_4's using **4-34** and **3-11** as activating agent.



Entry	Solvent	Equiv **3-11**	Equiv **4-34**	Base 1	NMP	Base 2	Time (h)	Product	Yield[a]	Yield[b]
1	CH_3CN	1.0	1.0	NMI	AMP	TEA	3	Ap_4 (**4-38**)	-	-
2	DMF	1.0	1.0	NMI	AMP	TEA	1	Ap_4 (**4-38**)	75	62
3	DMF	1.0	1.0	DMAP	AMP	TEA	1	Ap_4 (**4-38**)	75	-
4	DMF	1.0	1.0	NMI	AMP	-	1	Ap_4 (**4-38**)	75	-
5	DMF	1.3	1.3	NMI	AMP	-	3	Ap_4 (**4-38**)	90	-
6	DMF	1.35	1. 5	NMI	AMP	-	3	Ap_4 (**4-38**)	93	83
7	DMF	1.35	1. 5	NMI	CMP	-	3	Cp_4 (**4-39**)	-	84
8	DMF	1.35	1. 5	NMI	GMP	-	3	Gp_4 (**4-40**)	-	84
9	DMF	1.35	1. 5	NMI	UMP	-	3	Up_4 (**4-41**)	-	84

[a]Yields calculated by integration of ^{31}P-NMR peaks. [b]Isolated yields.

The mixture was allowed to stir for further 25 min. and then added to a cooled (ice bath) solution of 1 equiv AMP and 3 equiv TEA in CH_3CN. A thick precipitate formed. The reaction mixture was allowed to stir at room temperature. After 3 h an aliquot was removed and quenched with 100 mM triethylammonium acetate buffer. The ^{31}P NMR spectrum of this sample exhibited two singlets: one at 6.0 ppm which is characteristic of AMP and the other at -19.3 ppm for TriMP which means that the reaction did not work using these conditions. On switching the solvent to DMF instead of acetonitrile (Table 4.1 entry 2) it was found that upon addition of **3-11** to **4-34** there was still some turbidity but it rapidly disappeared upon addition to the flask containing the tetrabutylammonium salt of the nucleotide in DMF. The ^{31}P-NMR spectra of the reaction mixture at 15 min, 30 min, and 1.5 h are shown in Figures 4.3, 4.4, and 4.5 respectively. These

spectra showed no further change after 1.5 h. Since these spectra were run for the unquenched reaction mixture they allowed us to speculate about the intermediates formed during the reaction. The ^{31}P NMR after 15 min (Figure 4.3) spectrum shows unreacted AMP (peak at δ 5.8), some remaining TriMP at δ -19.6, and what appeared to be a doublet at δ -22.0 and a triplet at δ -23.0. These two latter sets of peaks disappeared with time (see Figure 4.4 and 4.5) which indicated that they could be an activated form of trimetaphosphate. In Figures 4.4 and 4.5 two doublets at approximately δ -11.3, δ -22.7, and one quartet at δ -32.6 are evident. We assigned the doublet at δ -11.3 to the α-phosphorus atom of intermediate **4-37**, the quartet at δ -32.6 to the β-phosphorus of **4-37** if we assume it is an overlapping doublet of triplets and, the large doublet at δ -22.7 to the two γ-phosphorus atoms of **4-37**. No other significant peaks were seen in the spectrum in Figure 4.5, which suggested that the reactive trimetaphosphate intermediate **4-15** had been almost completely consumed.

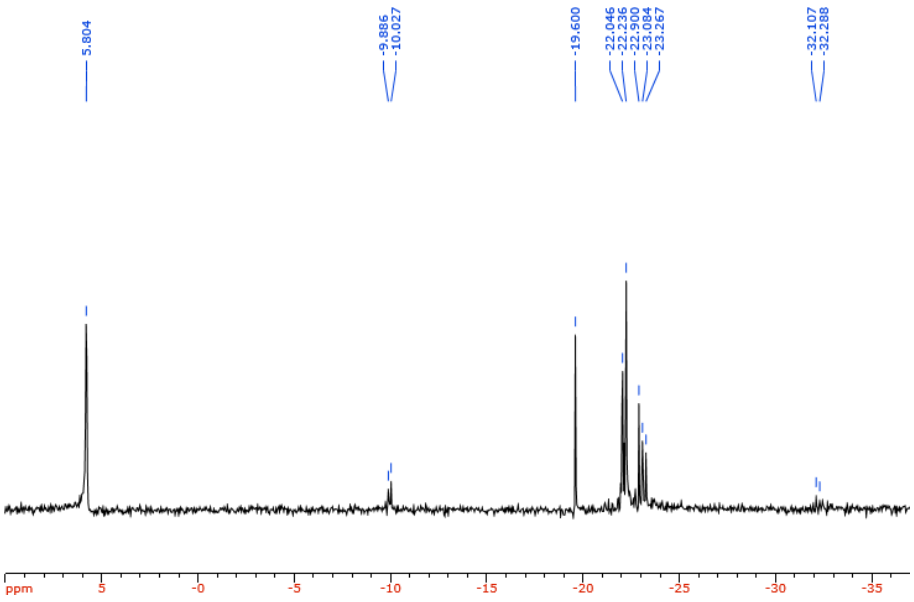


Figure 4.3. ^{31}P-NMR spectrum of the reaction of 1 equiv AMP with a mixture of 1 equiv **3-11** and **4-34** in the presence of 5 equiv NMI and 3 equiv TEA after 15 min.

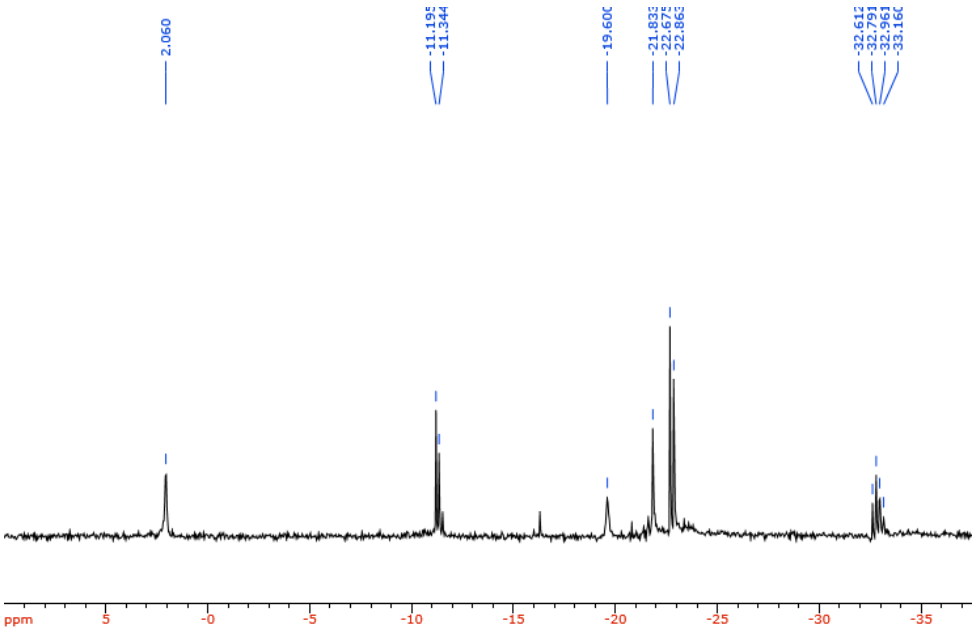


Figure 4.4. ^{31}P-NMR spectrum of the reaction of 1 equiv AMP with a mixture of 1 equiv **3-11** and **4-34** in the presence of 5 equiv NMI and 3 equiv TEA after 30 min.

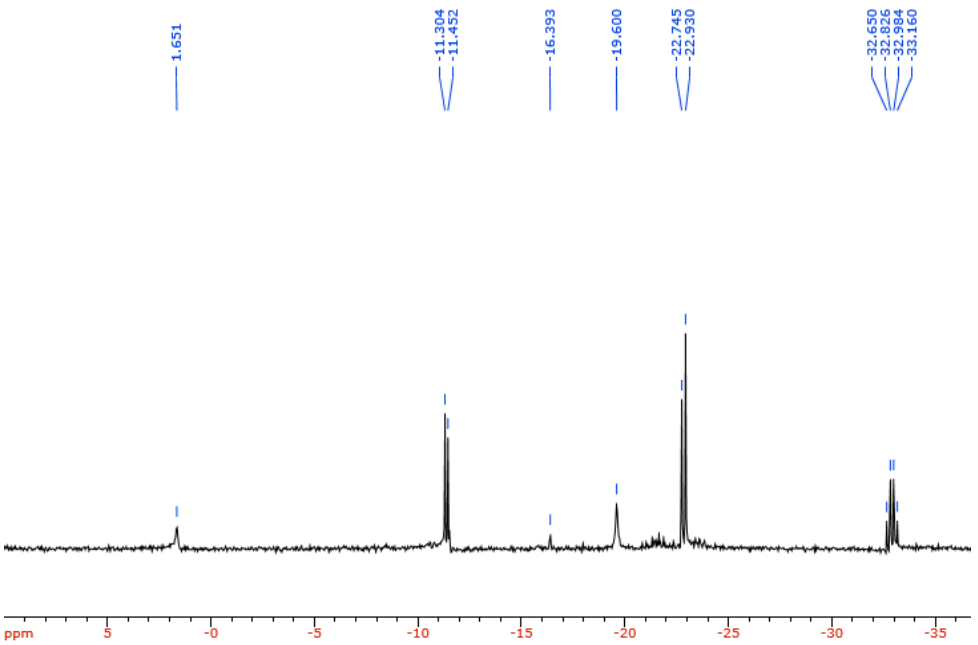


Figure 4.5. ^{31}P-NMR spectrum of the reaction of 1 equiv AMP with a mixture of 1 equiv **3-11** and **4-34** in the presence of 5 equiv NMI and 3 equiv TEA after 90 min.

The reaction mixture was cooled to 0 ^{o}C and quenched with 100 mM triethylammonium acetate buffer, washed with chloroform to remove excess base and then allowed to stand at room temperature with concomitant monitoring by ^{31}P NMR. The spectra kept changing with time and after 2 h it gave a spectrum shown in Figure 4.6 which shows peaks at δ -7.8, δ -9.4, δ -21.0 suggesting the formation of Ap_4 though the broad peaks made it difficult to interpret. Nevertheless, after subjecting the mixture to semi-preparative RP-HPLC Ap_4 was isolated in a 62% yield after freeze drying and conversion to ammonium salt.

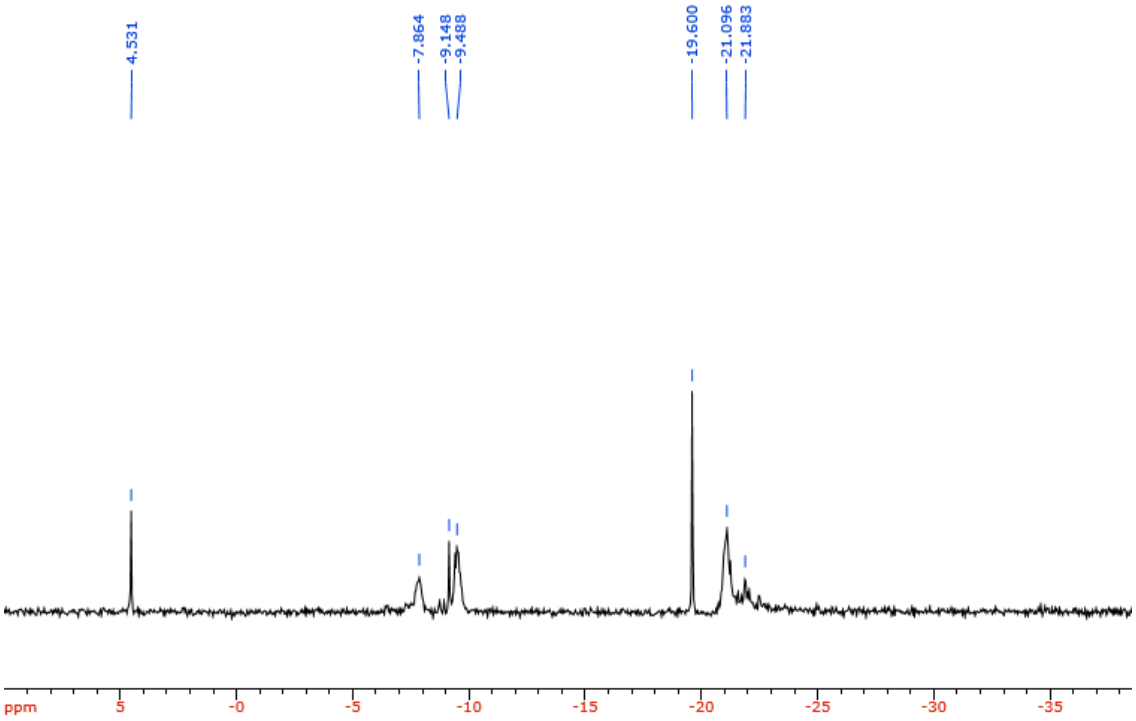


Figure 4.6. ^{31}P-NMR spectrum of the reaction of 1 equiv AMP with a mixture of 1 equiv **3-11** and **4-34** in the presence of 5 equiv NMI and 3 equiv TEA after 120 min followed by quenching with TEAA and stirring for 2 h.

Further optimization studies were done. Addition of triethylamine to the AMP solution was found to be unnecessary so it was omitted (Table 4.1, entry 4). Upon increasing the amount of activated TriMP, achieved by increasing the amount of both TriMP **4-34** and reagent **3-11** in

the activation step and, allowing the coupling reaction to run for 3 h, the ^{31}P-NMR yield increased considerably (entries 5 and 6, and Figure 4.7). The best isolated yield of Ap_4 (83%) was obtained using 1.5 equiv **4-34** and 1.35 equiv of **3-11** (entry 6). These optimized conditions were applied to the synthesis of other Np_4's and excellent yields were obtained in all cases (entries 7-9).

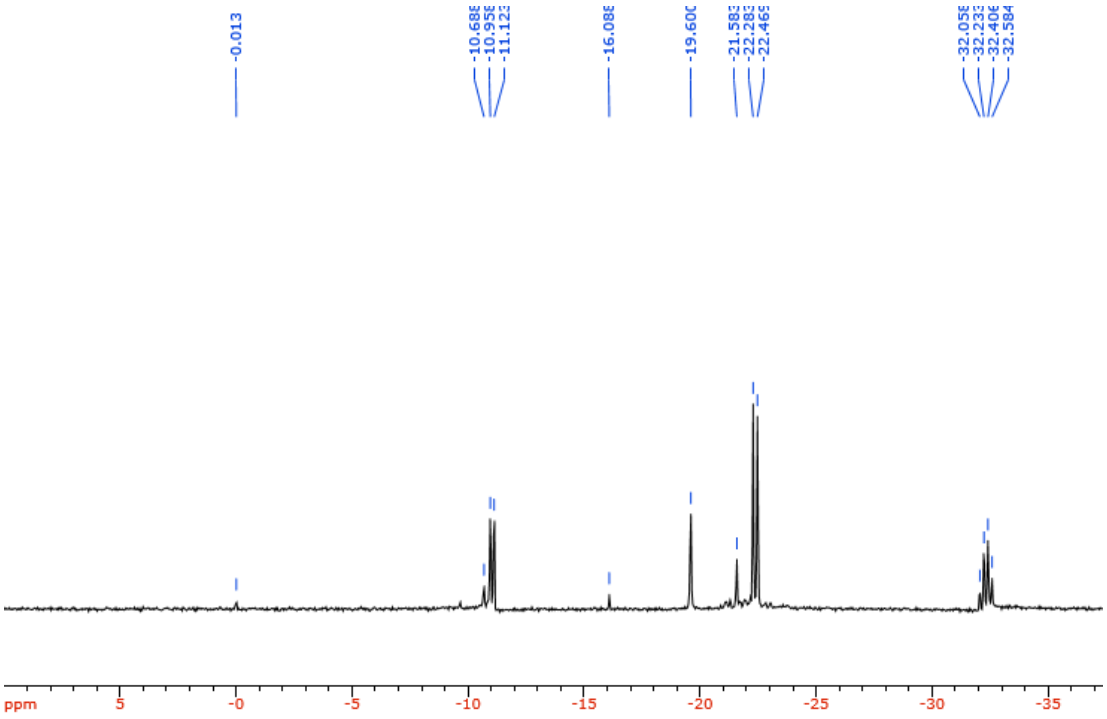


Figure 4.7. ^{31}P-NMR spectrum of the reaction of 1 equiv AMP with a mixture of 1.5 equiv **4-34** and 1.35 equiv **3-11** and in the presence of 5 equiv NMI after 3 h.

Being commercially available, arenesulfonyl chlorides are attractive alternatives to reagent **3-11** as activating agents. We examined benzenesulfonyl chloride as an activating agent using the optimized condition developed above. Complete consumption of starting NMP occurred after 3 h. However, owing to the presence of several byproducts, the purification was very difficult. Using mesitylenesulfonyl chloride (**4-36)** as activating agent complete consumption of the NMP's occurred but without the formation of significant amounts of

byproducts and so the purification by RP-HPLC was straightforward. Figure 4.8 shows the unquenched reaction mixture for the synthesis of Up_4 after 3 h. Excellent yields of **4-38** – **4-41** were obtained (Table 4.2, entries 3-6)

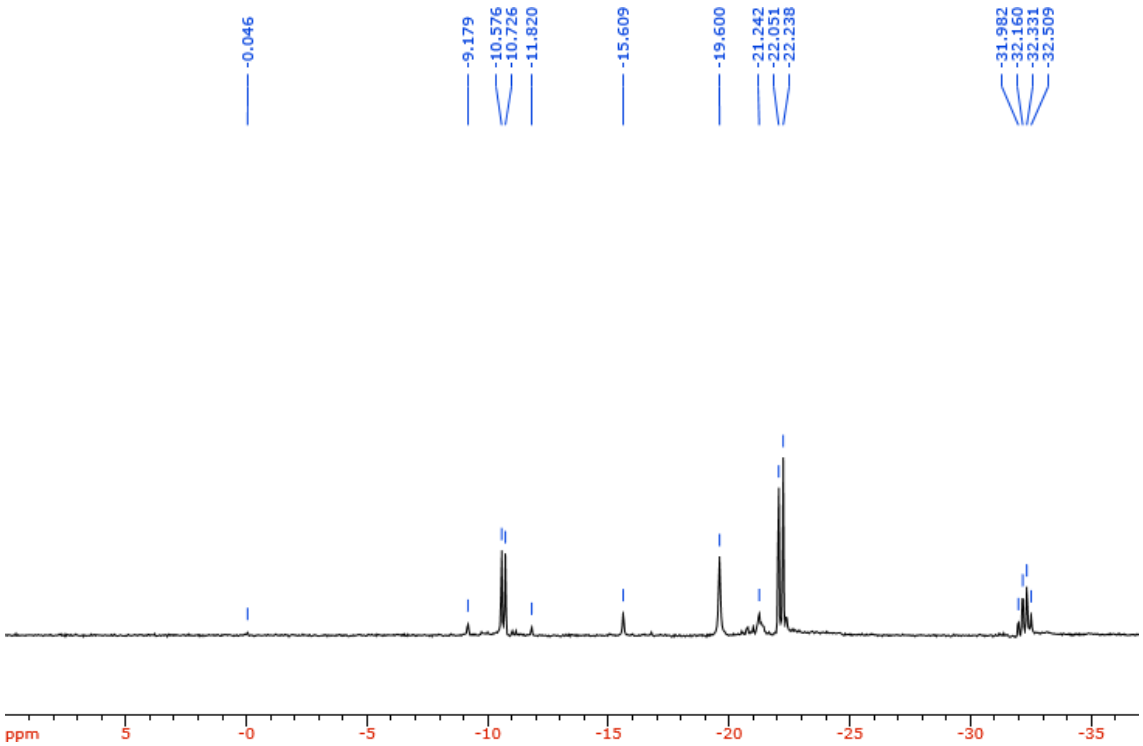
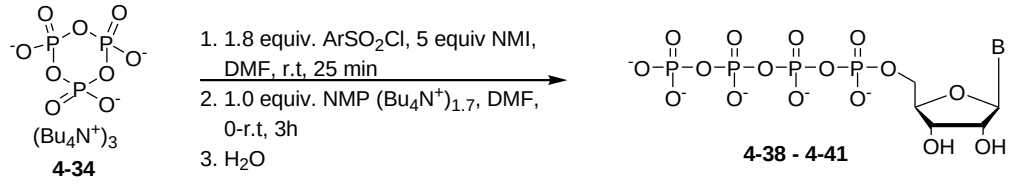


Figure 4.8. ^{31}P-NMR spectrum of the reaction mixture of activated **4-34** and UMP after 3 h.

Table 4.2. Synthesis of Np_4's using arenesulfonyl chlorides.



Entry	Ar	NMP	Product	Yield (%)
1	Ph	UMP	Up_4 (**4-41**)	-
2	Ph	GMP	Gp_4 (**4-40**)	-
3	2,4,6-Trimethylbenzene	AMP	Ap_4 (**4-38**)	84

4	2,4,6-Trimethylbenzene	CMP	Cp_4 (**4-39**)	86
5	2,4,6-Trimethylbenzene	GMP	Gp_4 (**4-40**)	84
6	2,4,6-Trimethylbenzene	UMP	Up_4 (**4-41**)	86

It is interesting to note that Ng and Orgel reported that AMP was the major product when Ap_4 was treated with EDC in HEPES buffer at pH 6.5. They postulated an intermediate of type **4-37** forming first followed by hydrolysis of **4-37** to give AMP.[65] ^{31}P-NMR of the reaction mixture of activated **4-34** and AMP (Figure 4.9) and after addition of TEAA buffer followed by chloroform wash and standing for 2 h (Figure 4.10) indicated that only ~ 5% of **4-37** was hydrolyzed to AMP.

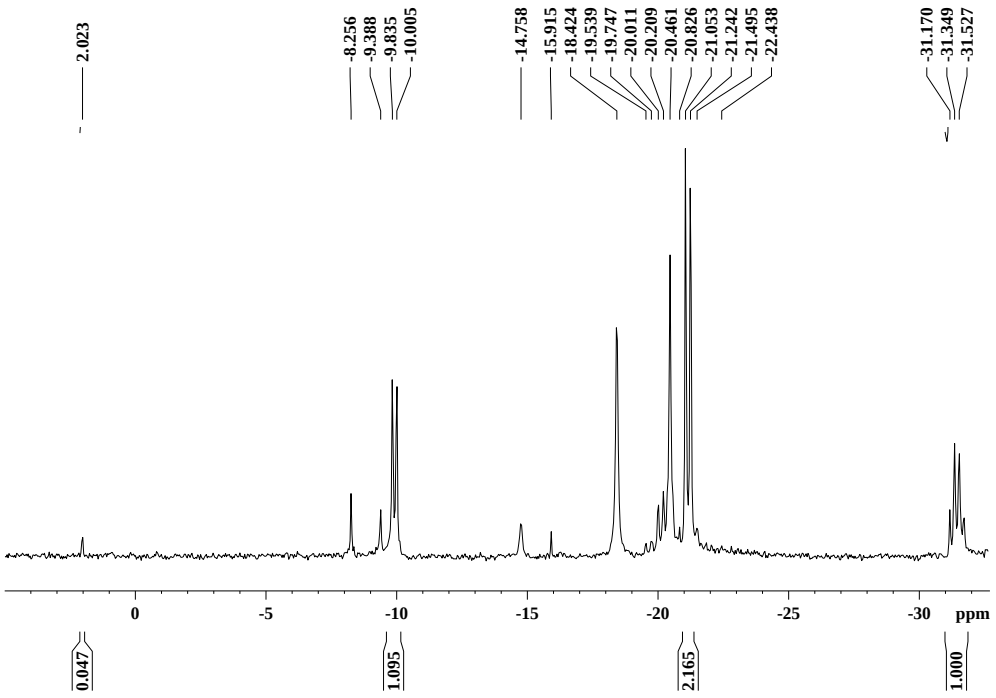


Figure 4.9. ^{31}P-NMR spectrum of the reaction mixture of activated **4-34** and AMP after 2.5 hours. Peaks corresponding to the proposed intermediate **4-37** appear at δ -31.4 (q), -21.1 (d) and -9.8 (d). Unreacted TriMP and AMP appear at δ 2.03 and δ -18.4 respectively.

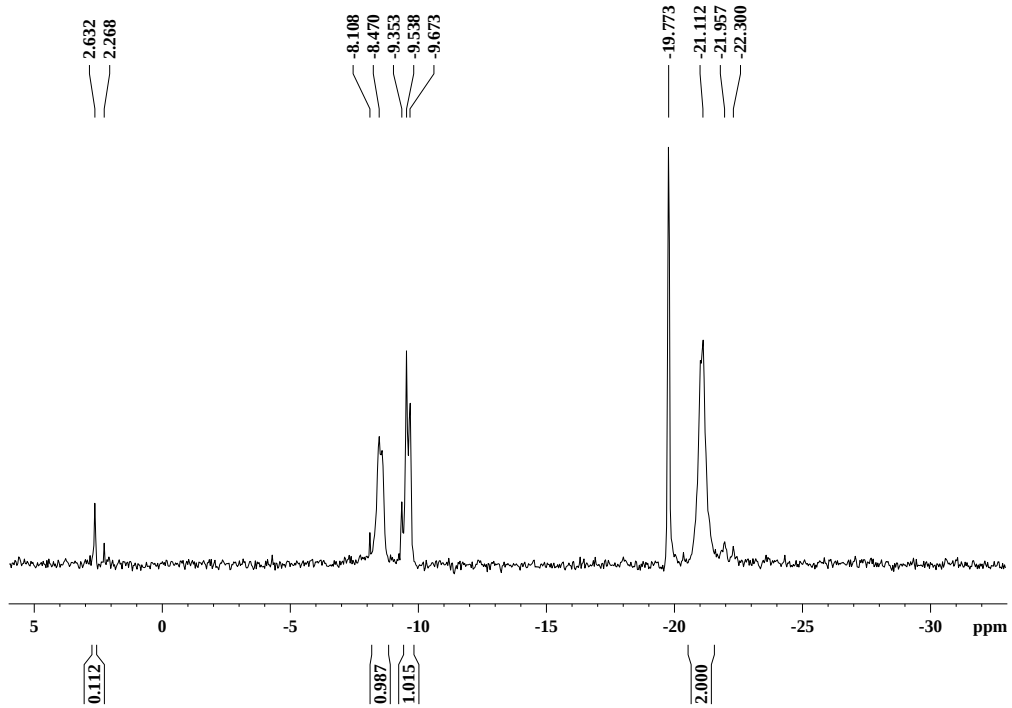
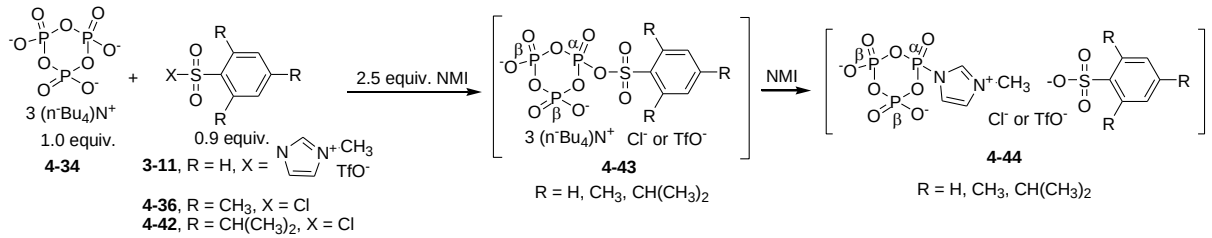


Figure 4.10. ^{31}P-NMR spectrum of the reaction mixture after quenching of proposed intermediate **4-37** with TEAA, washing with $CHCl_3$ and stirring the aq. layer for 2 h. Peaks corresponding to Ap_4 appear at δ -8.1, -9.5 (d) and -21.1 AMP appears at δ 2.63. TriMP appears at δ -19.7.

4.3.3 The activated form of 4-34

There are two possible activated forms of **4-34** that can be produced during the reaction. One is the mixed anhydride **4-43** and the other is the imidazolium species **4-44** formed via reaction of NMI with **4-43** (Scheme 4.17). To determine if just **4-43** is formed or both are formed during the reaction and to determine which one (or both) is the species that reacts with the NMP to give **4-37** we reacted **4-34** with reagents **3-11**, **4-36** and triisopropylbenzenesulfonyl chloride (**4-42**) (Scheme 4.17). We reasoned the reactivity of intermediate **4-43** should decrease as the steric bulk on the benzene ring increased and that we would be able to detect it and its possible conversion to **4-44** by ^{31}P-NMR.



Scheme 4.17. Activation of trimetaphosphate with reagents **3-11**, **4-36** and **4-42**.

Reagent **3-11**, **4-36**, or **4-42** (0.9 equiv) was added to a solution of 1.0 equiv of **4-34** and 2.5 equiv NMI in DMF. The reaction was followed by ^{31}P-NMR. With reagent **4-36** after 10 min much of **4-34**, which appears at approximately δ -19.0, was consumed and the ^{31}P-NMR spectrum, in addition to a few very minor peaks, consisted of a relatively small triplet at δ -31.4, a doublet at δ -20.7 and a triplet at δ -21.5 (see Figure 4.11 A). After 30 minutes the triplet at δ -31.4 had disappeared leaving the doublet at δ – 20.7 and the triplet at δ -21.5 though the relative peak heights within the triplet at δ -21.5 had changed (Figure 4-11 B). No further change occurred after 30 minutes. We propose that these signals can be accounted for by a reaction between **4-34** and **4-36** to give mixed anhydride **4-43** which reacts relatively rapidly with NMI to give imidazolium intermediate **4-44** (Scheme 4.17). The triplet at δ -31.4 corresponds to the α-phosphorus atom of **4-43**. The doublet that should be associated with the two β-phosphorus atoms of **4-43** coincides with the central and right side peaks of the triplet that is due to the α-phosphorus atom in compound **4-44** which appears at δ -21.5. The doublet at δ -20.7 is due to the two β-phosphorus atoms of **4-44**. The α- and β-phosphorus atoms in **4-44** have vey similar chemical shifts and are strongly coupled to one another which results in the doublet at δ -20.7 and triplet at δ -21.5 to be highly skewed towards one another. The relative simplicity of the spectra and the fact that some unreacted TriMP remained suggested that the formation of dimers or higher order oligomers is not readily occurring.

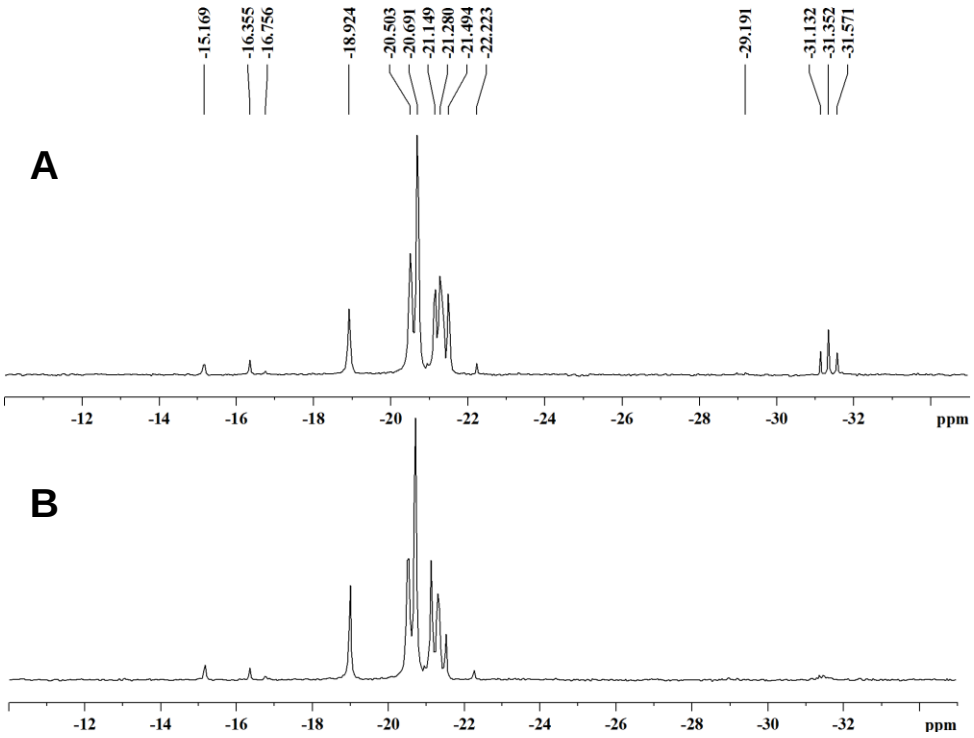


Figure 4.11. ^{31}P-NMR spectra of the reaction of 0.9 equiv reagent **4-36** with 1 equiv compound **4-34** in the presence of 2.5 equiv NMI in DMF. (A): Spectrum recorded after 10 minutes. (B): Spectrum recorded after 30 minutes. See the text for details.

The above peak assignments are supported by the ^{31}P-NMR spectra of the reactions of reagents **3-11** and **4-42** with **4-34**. The reaction of the more sterically hindered reagent **4-42** after 10 min is shown in Figure 4.12. As expected peaks corresponding to intermediate **4-43**, such as the triplet at -31 ppm and doublet at -21.3 ppm are more intense than the corresponding peaks of intermediate **4-43** formed when using reagent **4-36**. This can be explained by the fact that the greater steric hindrance in **4-42** makes nucleophilic attack by NMI slower and so **4-43** converts more slowly to **4-44**. It is worthy of note that it took 45 min of this particular intermediate to fully convert to **4-44** and a ^{31}P-NMR spectrum similar to the one in Figure 4-11 B.

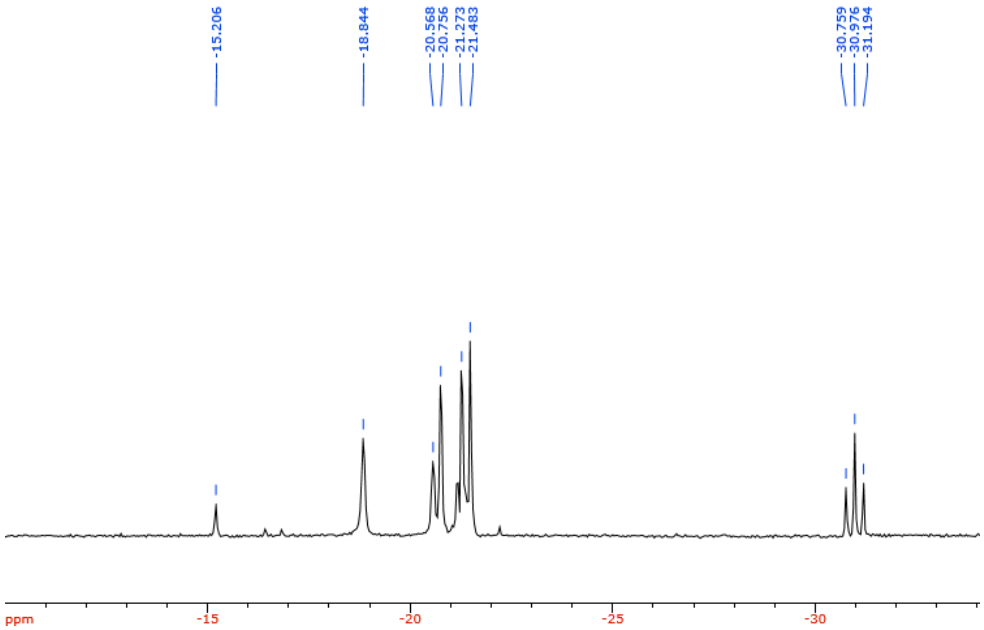


Figure 4.12. ^{31}P-NMR spectrum of the reaction of 0.9 equiv reagent **4-42** with 1 equiv compound **4-34** in the presence of 2.5 equiv NMI in DMF after 10 minutes.

The ^{31}P-NMR of the reaction between reagent **3-11** and compound **4-34** after 10 min was almost identical to the ^{31}P-NMR of the reaction between **4-36** and **4-34** after 30 min and the reaction of **4-42** and **4-34** after 45 min. NMR spectra taken after 20 and 30 min. showed no change. No triplet at approximately δ -31 was evident in any of the spectra suggesting that the reaction between reagent **3-11** and compound **4-34** and the subsequent reaction of the mixed anhydride to give the intermediate imidazolium salt of type **4-44** is very fast possibly because the mixed anhydride formed with reagent **3-11** is less sterically hindered than the one formed with reagents **4-36** or **4-42**. To confirm this, the reaction between reagent **3-11** and compound **4-34** was repeated in presence of 3 equiv diisopropylethylamine (DIPEA) instead of 2.5 equiv NMI. The ^{13}P-NMR spectrum (Figure 4-13) shows a triplet at δ -31.0 and doublet at δ -22.4 that corresponds to proposed intermediate **4-43**. We propose that, in this case, since only a limited amount of NMI is present during the reaction (only that produced from the release of NMI upon

reaction of **3-11**) the reaction between NMI and the mixed anhydride **4-43** is slow and so we are able to detect the triplet at δ -31.0 and a doublet at δ -21.4 corresponding to **4-43**.

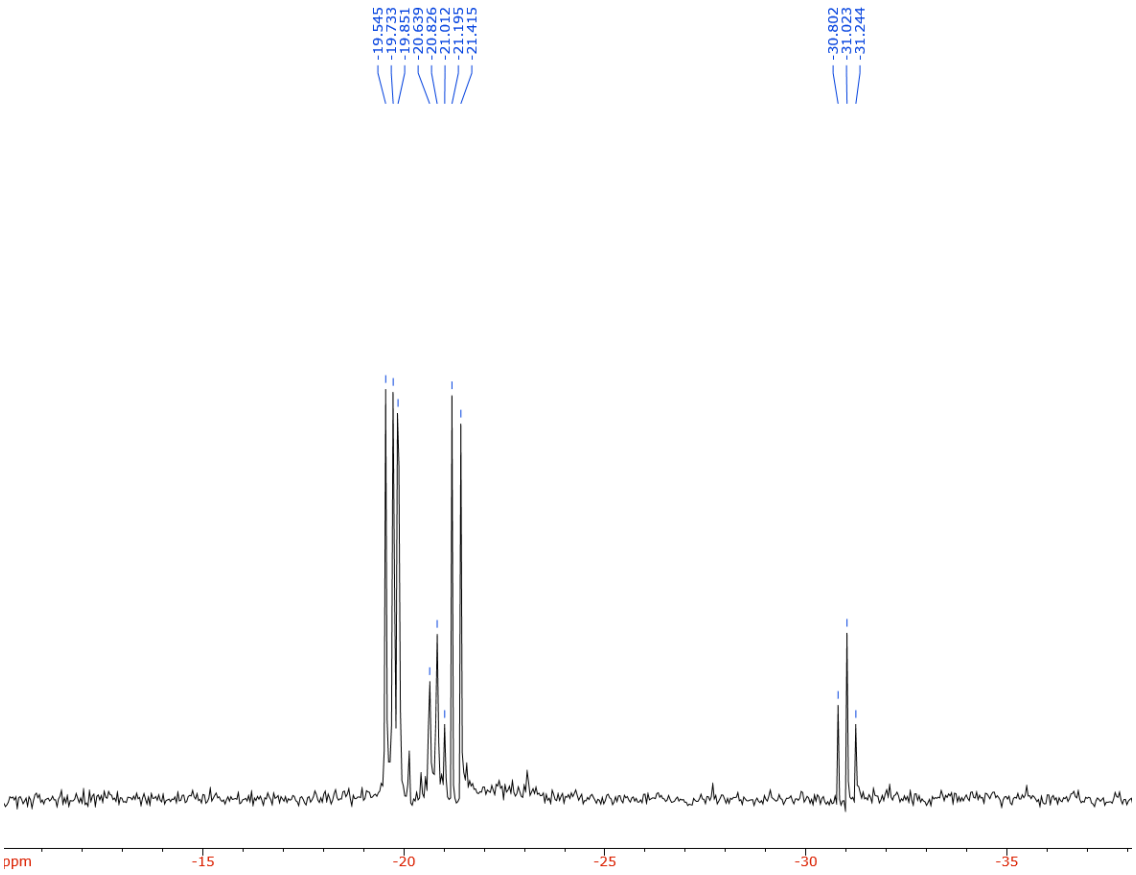


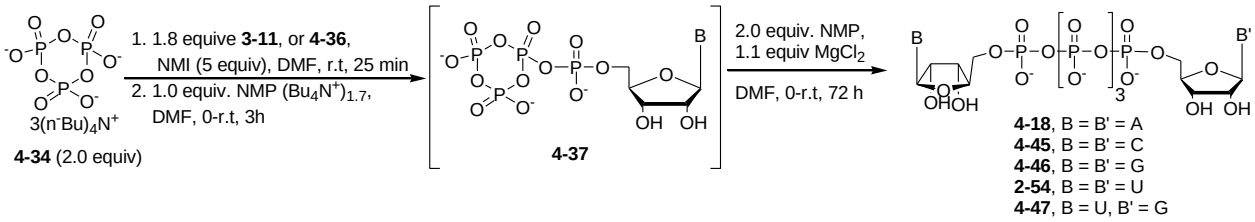
Figure 4.13. ^{31}P-NMR spectrum of the reaction of 1.8 equiv reagent **3-11** with 2 equiv compound **4-34** in the presence of 3 equiv DIPEA in DMF. Spectrum recorded after 10 minutes

4.3.4 Synthesis of Np_5N's

Np_5N's were synthesized by first preparing intermediate **4-37** as described above. Instead of quenching intermediate **4-37** with buffer, 2.0 equiv of the tetrabutylammonium salt of NMP in DMF and 1.1 equiv anhydrous $MgCl_2$ were added. ^{31}P-NMR analysis of the mixture indicated the reactions were complete after 3 days. Quenching with 100 mM TEAA buffer followed by washing with chloroform, purification by RP-HPLC then ion exchange chromatography gave

Np_5N's as their ammonium salts in very good yield using either reagent **3-11** or **4-36** (Table 4.3). The reaction was extremely slow in the absence of magnesium ions. We also found that unsymmetrical Np_5N can be produced using this procedure (Table 4.3, entry 5).

Table 4.3. Synthesis of Np_5N's via activated **4-34**.



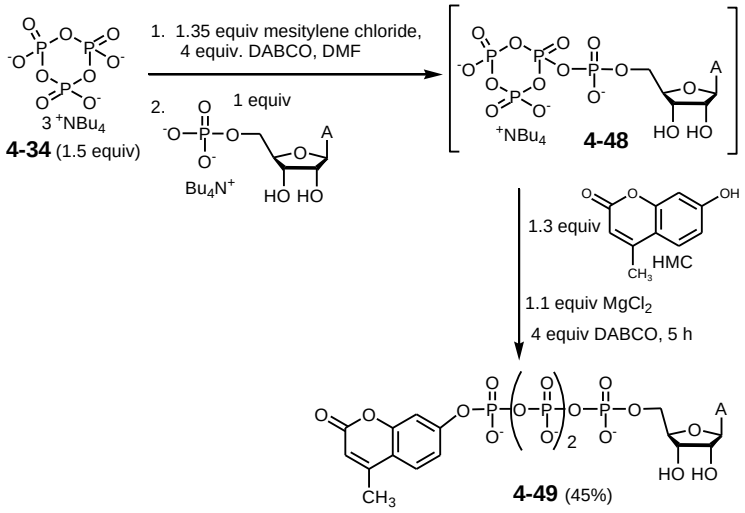
entry	Product	Yield[a](%)	Yield[b](%)
1	Ap_5A (**4-18**)	85	81
2	Cp_5C (**4-45**)	82	84
3	Gp_5G (**4-46**)	85	84
4	Up_5U (**2-54**)	80	84
5	Up_5G (**4-47**)	-	77

[a]Isolated yields using reagent **3-11**. [b]Isolated yields using reagent **4-36**.

4.3.5 Synthesis of Np_4's containing a terminal fluorescent label using activated TriMP

7-Hydroxy-4-methylcoumarin (HMC, Scheme 4.18) was chosen as a model fluorescent dye as it is relatively inexpensive and readily available. We initially attempted to prepare the adenosine derivative **4-49** by reacting intermediate **4-48**, prepared using our previously reported procedure (section 4.3.2) with HMC in the presence of $MgCl_2$ (Scheme 4.18) This did not result in consumption of the HMC as determined by TLC. However, when an excess of 1,4-diazabicyclo[2.2.2]octane (DABCO) was present the coumarin was consumed and the formation of **4-49** was evident by ^{31}P-NMR (Figure 4.14, characteristic peaks: -9.1 ppm (d), -15.0 ppm (d)

and two apparent triplets at approximately -20.0 - -20.6 ppm) along with other unidentified impurities. Optimal conditions were developed which consisted of 1.3 equiv HMC, 4 equiv DABCO and 1.1 equiv $MgCl_2$. No more consumption of HMC was observed after about 5 h. After quenching with TEAA and purification by reversed-phase column chromatography, compound **6** was obtained in a 45% yield.



Scheme 4.18. Synthesis of compound **4-49** via intermediate **4-48**.

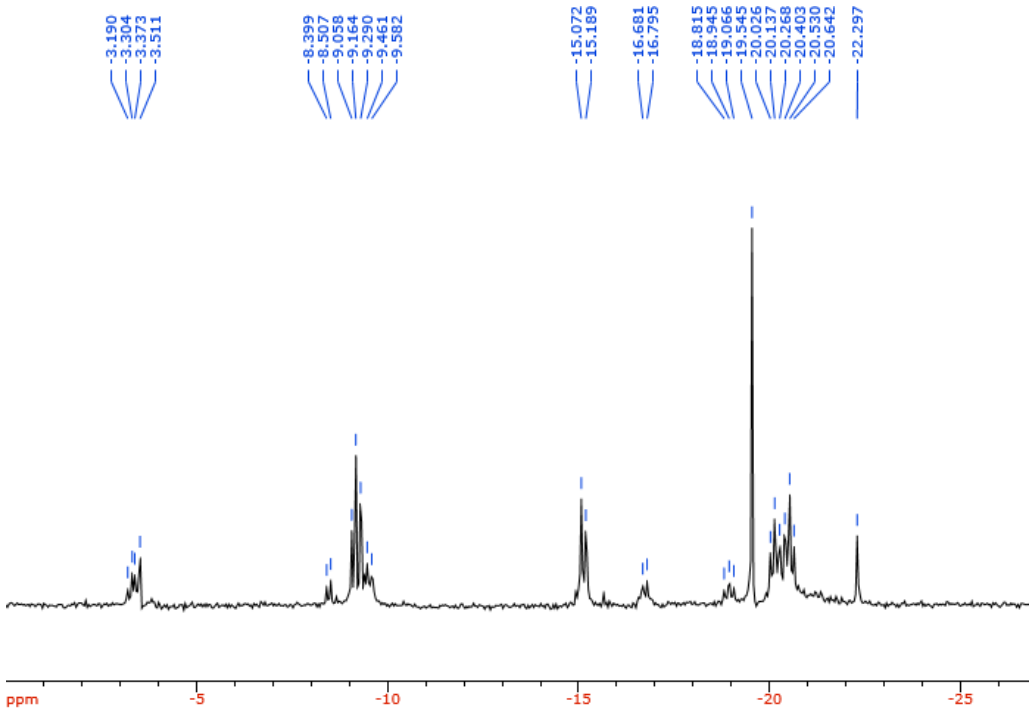
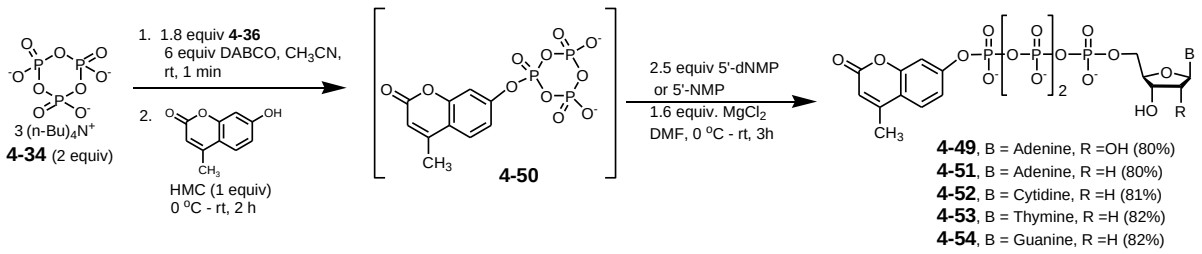


Figure 4.14. ^{31}P-NMR spectrum 5 h after the addition of HMC and magnesium chloride to a reaction mixture containing proposed intermediate **4-48** (Scheme 4.18). The reaction was quenched with TEAA buffer (pH 7.0) and magnesium ions were removed with Chelex resin prior to obtaining the spectrum. Peaks corresponding to product appear at -9.1 ppm (d), -15.0 ppm (d) and two apparent triplets at approximately -20.0 - -20.6 ppm. The singlet at -19.5 ppm is TriMP. Other unidentified impurities at -3.1 ppm (m), -8.3 (d), -9.0 (s), -9.4 (d), -16.6 (d), -18.8 (t), and -22.2 (s).

The modest yield obtained with the approach outlined in Scheme 4.18 prompted us to examine the reverse procedure in which the coumarin is reacted with activated TriMP followed by the addition of NMP (Scheme 4.19). To ensure complete phosphorylation of the OH group of HMC and to prevent formation of the dicoumarin triphosphate we employed a 1.8-fold excess of activated trimetaphosphate. Hence, 1.8 equiv mesitylene chloride was added to a solution of 2.0 equiv trimetaphosphate (**4-34)** and 6 equiv DABCO and the mixture stirred for 1 min and then 1.0 equiv of HMC was added. The reaction turns from a clear solution to a white turbid mixture after about 45 minutes. All of the coumarin was consumed in about 2 h as determined by TLC. No reaction occurred in the absence of DABCO and it was found that NMI was not necessary for

reaction to occur. This mixture was then added dropwise to a cooled (ice bath) solution of 1.6 equiv $MgCl_2$ and 2.5 equiv of 5´-AMP as its tetrabutylammonium salt in DMF. The ice bath was removed and the progress of the reaction was monitored by withdrawing samples, quenching them with a 5 % solution of EDTA in triethylammonium acetate buffer (pH 7.0) and then analyzing them by ^{31}P-NMR. Peaks corresponding to **4-49** were clearly evident. After 3 h peaks corresponding to **4-49** no longer increased in intensity and **4-49** was the dominant product. The reaction was quenched with TEAA buffer (pH 7.0) and the resulting solution washed with chloroform and magnesium ions were removed by Chelex resin. The ^{31}P-NMR of the crude material exhibited peaks corresponding to product as well as peaks corresponding to trimetaphosphate, unreacted 5´-AMP and minor peaks which we attributed to Ap_5A (for example see Figure 4.15). With the activated trimetaphosphate and AMP being in considerable excess to HMC it was expected that a considerable amount of Ap_5A byproduct to have been produced and little or no unreacted AMP or TriMP to be present. Since this was not the case, we suggest that the reaction of the AMP with any *activated* trimetaphosphate intermediates that might be formed during the reaction (*vide infra*) is slower than the reaction of AMP with intermediate **4-50** in the absence of NMI and/or in the presence of DABCO. Compound **4-49** was obtained in an 80% yield after purification by reversed-phase chromatography using tributylammonium acetate (TBAA) buffer in methanol/water and conversion to its tetraammonium salt using Dowex 50W resin (NH_4^+ form). It is worthy of note that the Ap_5A byproduct exhibited a very different retention time from **4-49** and so was easily removed. This procedure was then applied to the four common deoxynucleotides which gave the labelled products **4-51 – 4-54** in yields of 80-82%. Purification of these compounds was equally straightforward.



Scheme 4.19. Synthesis of δ-labeled nucleoside 5´-tetraphosphates via intermediate **4-50.**

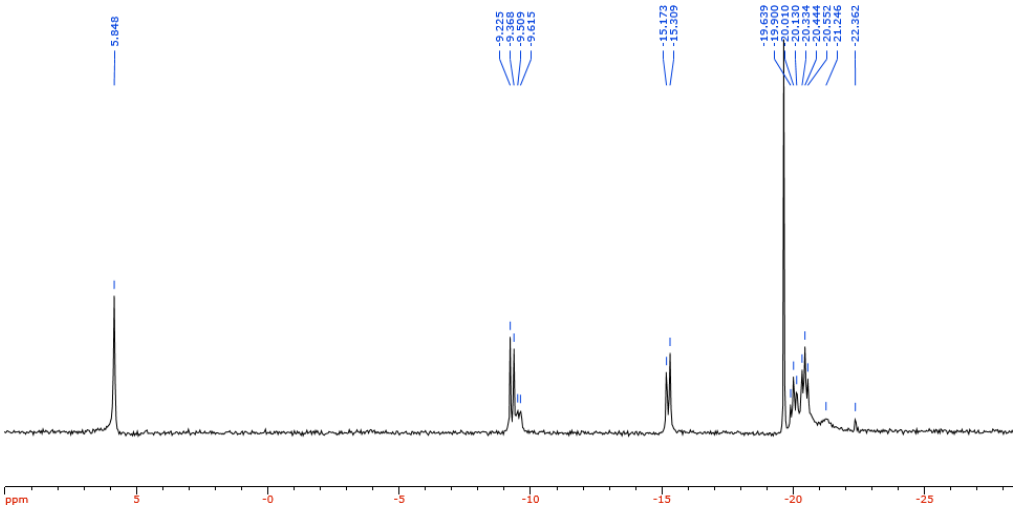


Figure 4.15. ^{31}P-NMR spectrum 3 h after the addition of a solution of proposed intermediate **4-50** to a solution of AMP (Scheme 4.19). The reaction was quenched with TEAA buffer (pH 7.0) and magnesium ions were removed with Chelex resin prior to obtaining the spectrum. Peaks corresponding to product appear at -9.3 ppm (d), -15.2 ppm (d) and two apparent triplets at approximately -20.0 - -20.3 ppm. The singlet at -19.5 ppm is trimetaphosphate and the singlet at 5.8 ppm is AMP. The small broad doublet at -9.5 ppm and the broad singlet at -21.2 ppm are attributed to Ap_5A.

^{31}P-NMR data suggests that intermediate **4-50** is indeed formed during the reaction. The ^{31}P-NMR spectrum of a mixture of HMC, trimetaphosphate, mesitylenesulfonyl chloride and DABCO (approximately 1:1.3:1.3:4) in CH_3CN after 2 h showed mainly a doublet at -20.7 ppm and a triplet at -24 ppm (Figure 4.16). The ^{31}P-NMR spectrum of this mixture after having been quenched with water showed doublets at -4.0 and -14.5 ppm and a triplet at -20 ppm (see Figure

4.17) and these peaks can be attributed to compound **4-55** formed by hydrolysis of intermediate **4-50** (Scheme 4.20).[173]

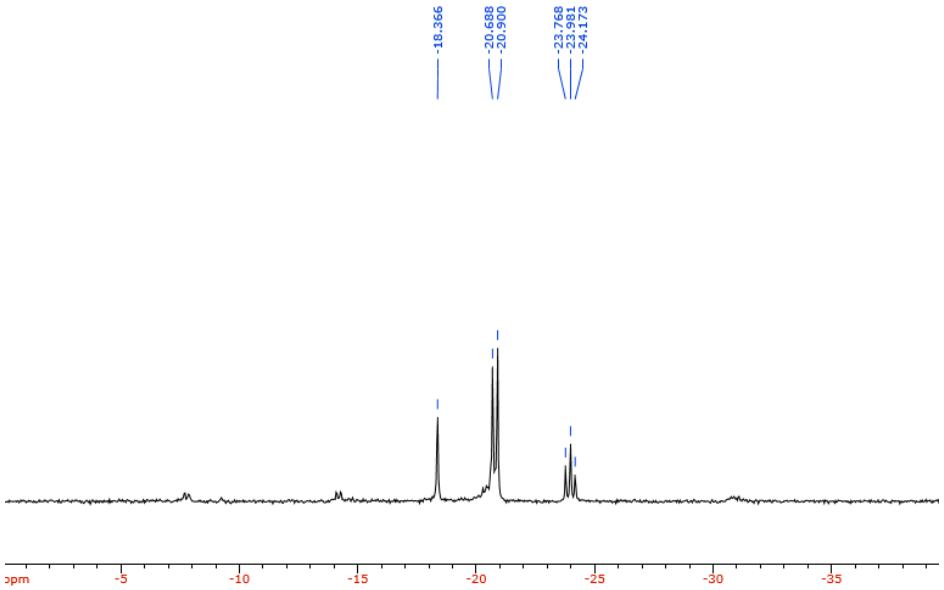


Figure 4.16. The ^{31}P-NMR spectrum of a mixture of HMC, trimetaphosphate, mesitylenesulfonyl chloride and DABCO (app. 1:1.3:1.3:4) in CH_3CN after 2 h. The peak at -18.6 ppm is TriMP.

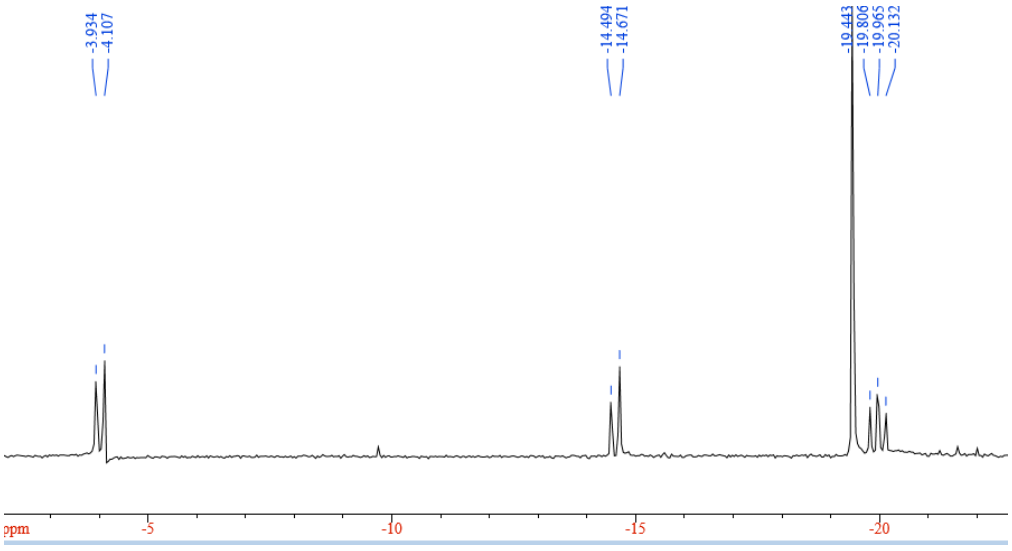
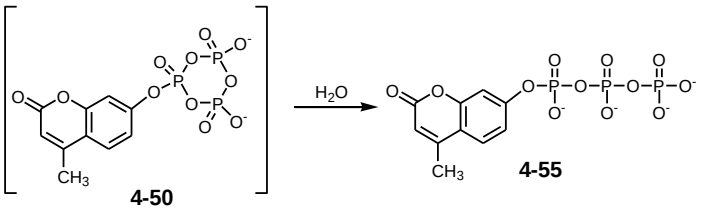


Figure 4.17. The ^{31}P-NMR spectrum of a mixture of HMC, trimetaphosphate, mesitylenesulfonyl chloride and DABCO (app. 1:1.3:1.3:4) after 2 h and quenching with water. The singlet at -19.4 ppm is TriMP.



4-50 4-55

Scheme 4.20. Formation of triphosphate **4-55** by hydrolysis of intermediate **4-50**.

Although we believe that the reaction proceeds via intermediate **4-50** the route by which this intermediate is formed is not clear. The ^{31}P-NMR spectrum of a mixture of TriMP, DABCO and **4-36** (1:3:0.9) in acetonitrile after 5 min shows a doublet at -6.3 ppm and a triplet at -20.6 ppm (see Figure 4.18). However, after about one hour these peaks are no longer present. The mixture becomes turbid and the ^{31}P-NMR spectrum shows a triplet at -31 ppm and a multiplet at around -20 ppm possibly consisting of overlapping doublets and triplets (see Figure 4.19). Since it takes 2 h for the coumarin to be completely consumed this data suggests that intermediate **4-50** might be formed by HMC reacting with more than one intermediate. It is possible that the initially formed mixed anhydride **4-43**[198] reacts rapidly with DABCO to give intermediate **4-56** and this species may react with HMC to give intermediate **4-50** (Scheme 4.21). Moreover, dimeric or higher order polyphosphates might be formed and these species may also be capable of reacting with HMC to somehow give intermediate **4-50**. In an attempt to isolate and identify the white precipitate formed during the reaction, the same experiment was repeated in absence of HMC (2 equiv **4-34**, 1.8 equiv **4-36** and 6 equiv. DABCO). In this case the reaction mixture was stirred only for 1 min and was then left undisturbed for 24 h, precipitate started forming after about 1h. This precipitate was collected by suction filtration and dried. This precipitate was soluble in water with concomitant hydrolysis to TriMP. Its' ^{31}P-NMR spectrum in D_2O shows only a singlet at -19.6 characteristic of TriMP. Its ^{1}H-NMR shows only a singlet 3.25 ppm

proton peaks characteristic of DABCO. This result indicates that this white precipitate consists of TriMP and DABCO but does not confirm its exact structure.

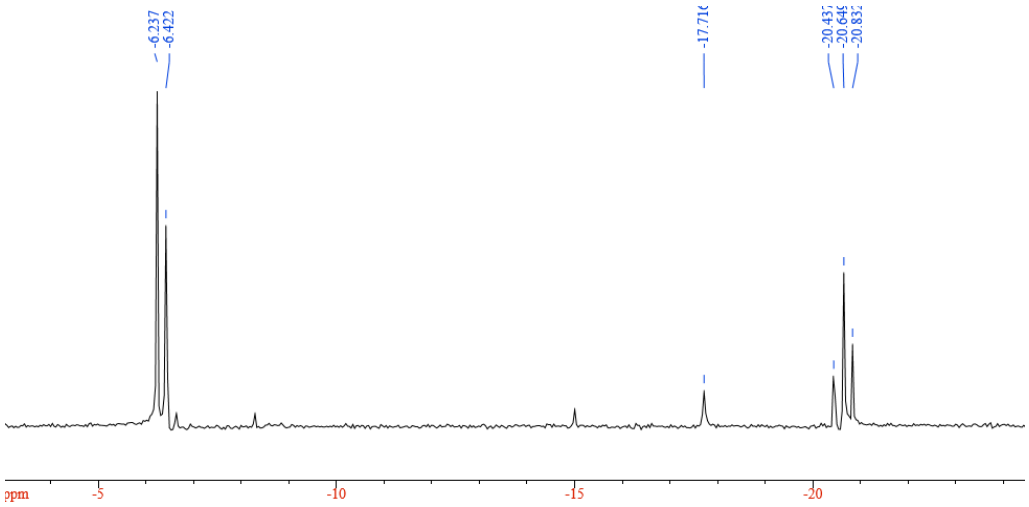


Figure 4.18. The ^{31}P-NMR spectrum of a mixture of TriMP, DABCO and **4-36** (1:3:0.9) in acetonitrile after 5 min.

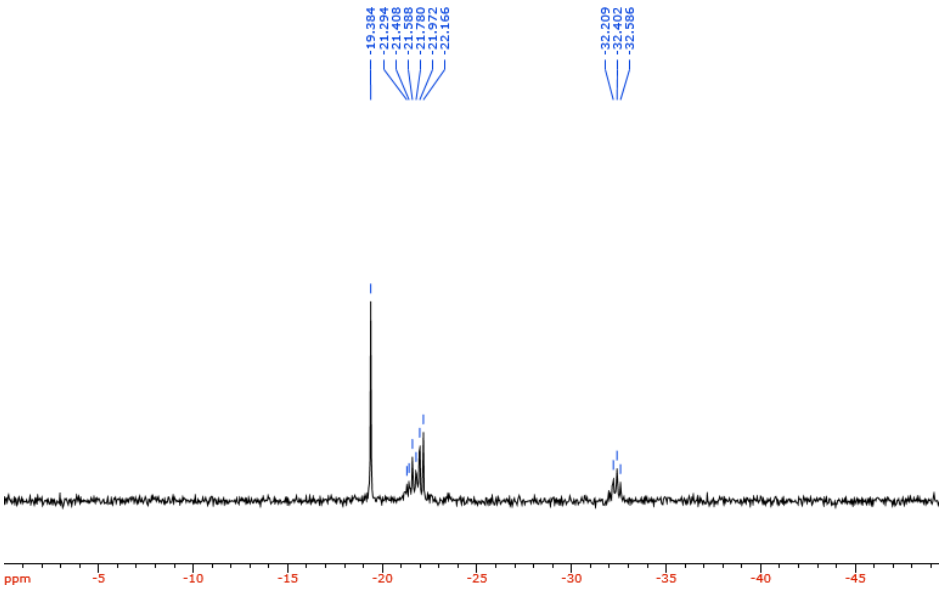
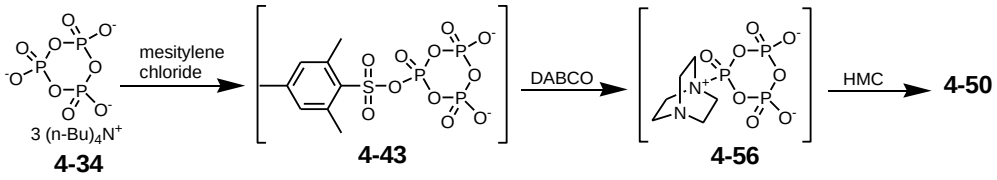


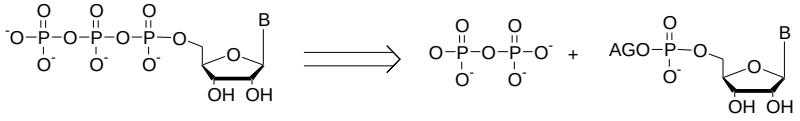
Figure 4.19. The ^{31}P-NMR spectrum of a mixture of TriMP, DABCO and **4-36** (1:3:0.9) in acetonitrile after 1 h.



Scheme 4.21. A possible pathway for the formation of intermediate **4-50.**

4.3.6 Attempted synthesis of NTP's using activated TriMP.

One of the most widely used approaches to the synthesis of NTP's involves the disconnection shown in Scheme 4.22. In this approach pyrophosphate reacts with an activated NMP donor. Many activated NMP's have been used for this purpose some of which have already been described in Chapter 2. Some activated NMP's (Figure 4.20) such as phosphoramidates (**4-57**),[199] phosphomorpholidates (**4-58**),[67] 2,2,2-tribromoethylphosphomorpholidates (**4-59**),[200] phosphoimidazolidates (**4-60**),[201] and phosphoimidazolium salts (**4-61**)[8] and the method that we developed in Chapter 3, section 3.3.4, do not require protection of the 2',3'-OH's or other nucleophilic groups.[8,163] Some others do require protection of 2',3'-OH's such as the *cyclo*Sal-nucleotides (**4-62**),[79] nucleoside 5`-*H*-phosphonates (**4-63)**[202,203] and phosphopiperidates (**4-64**).[204] In all of the approaches described above initial phosphorylation or phosphitylation of the nucleoside is required.



Scheme 4.22. A common disconnection used for the synthesis of NTPs. AG stands for activating group.

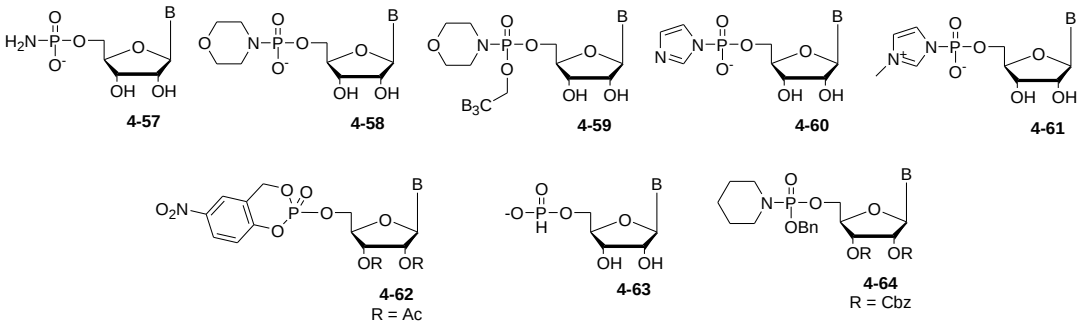
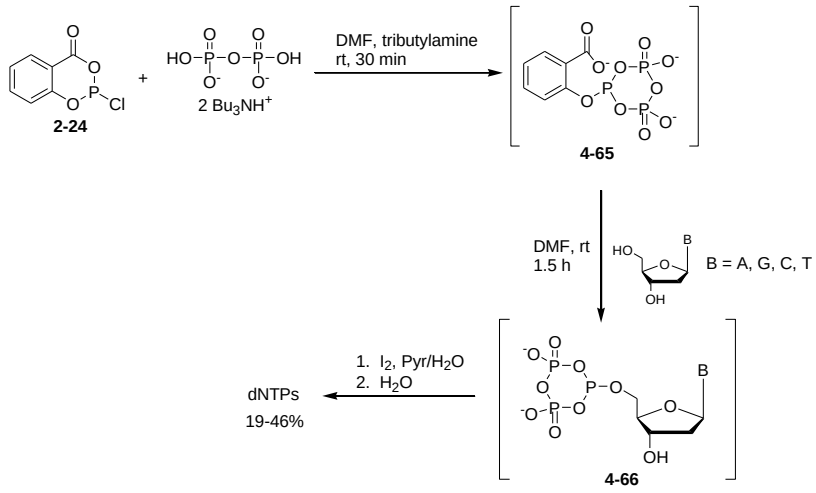


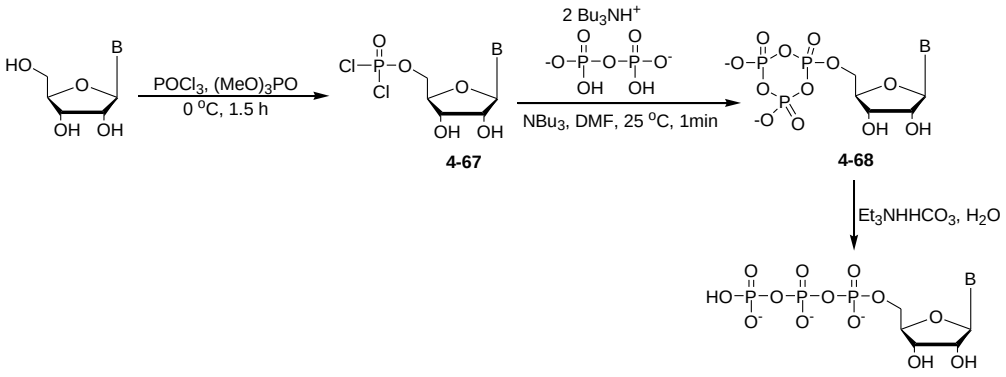
Figure 4.20. Structures of activated NMP's used for the synthesis of NTP's.

Sometimes the activated NMP donor can be prepared in situ such as in Ludwig and Eckstein's approach (see Chapter 2, section 2.1.3.2.3, Scheme 2.7).[76] However, their route uses protected nucleosides. Very recently, Huang and coworkers reported an interesting modification of Ludwig's and Eckstein's procedure (Scheme 4.23). These workers reacted reagent **2-24** with PPi to give intermediate **4-65**. Reaction of **4-65** with an *unprotected* deoxynucleoside gives intermediate **4-66** which is then oxidized and then hydrolyzed to give NTPs in 19-46% yields. To maintain the high regioselectivity of 5'-cyclic triphosphite intermediates the reactions between intermediate **4-65** and unprotected nucleosides were allowed to run to only 70% completion which may, in part, account for the rather low yields.[205]



Scheme 4.23. Huang and coworkers' approach to dNTP's.

One of the most widely used approaches to NTP syntheses, a "one-pot, three-step" route developed by Ludwig in 1981,[206] involves the generation of nucleoside dichlorophosphoridate **4-67**[207] followed by reaction with tri-*n*-butylammonium pyrophosphate to give nucleoside 5'-trimetaphosphate **4-68** which upon hydrolysis yields NTP's (Scheme 4.24). Although this procedure is widely used the yields are often low because of the formation of various byproducts such as nucleoside 3'-triphosphates, nucleoside 5'-monophosphates, 2',3'-cyclophosphate-5'-triphosphates, and TriMP.[208-210] By making some minor modifications to Ludwig's procedure, Kore et al. very recently reported the synthesis of dATP, dGTP, dCTP, dTTP in 65-70% yields.[211]

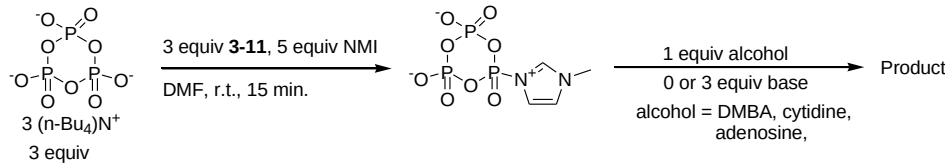


Scheme 4.24. Ludwig's synthesis of NTPs.

To the best of our knowledge, a regioselective (5'-position), one-step triphosphorylation of nucleosides has not been reported in the literature. Our strategy, as outlined in Scheme 4.8, relies on the direct triphosphorylation of nucleosides using an activated form of TriMP followed by hydrolysis of the resultant nucleoside 5'-trimetaphosphate. In our initial trials we used 3,5-dimethoxybenzyl alcohol (DMBA) as a model substrate because it has a single 1^{o} alcohol group and its disappearance could be conveniently followed by TLC. Reagent **3-11** was added to a mixture of TriMP and NMI in DMF and stirred at room temperature for 15 min followed by the

addition of DMBA. No reaction occurred even after 72 h (Table 4.4). The addition of 3 equiv DMAP and performing the reaction at 45 ^{o}C did not help. Cytidine and adenosine were later examined as substrates but again no reaction occurred even when 3 equiv of TEA was added as determined by ^{31}P-NMR of the reaction mixtures.

Table 4.4. Attempted triphosphorylations using activated TriMP**.**



Entry	R-OH	Base	Temp (^{o}C)	Time (h)	Product
1	DMBA	-	r.t	5	-
2	DMBA	-	r.t	24	-
3	DMBA	-	r.t	72	-
4	DMBA	DMAP	45	72	-
5	cytidine	-	r.t	72	-
6	cytidine	DMAP	45	72	-
7	cytidine	DMAP	70	24	-
8	cytidine	TEA	45	24	-
9	adenosine	-	-	45	-

We decided to change the reaction solvent to acetonitrile and use a stronger base such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). 3 equiv **4-34** was added to a solution of 3 equiv TriMP and 3 equiv NMI in acetonitrile and stirred for 15 min at r.t. A white suspension developed in about 3 min. DMBA was added followed by 5 equiv DBU. The white turbidity rapidly dissolved upon addition of the DBU. The reaction mixture was stirred at room

temperature and monitored by TLC. The DMBA was consumed very slowly under these conditions.

The reaction was repeated except then 3 equiv 4-nitroimidazole was added after the first step followed by 5 equiv DBU. The white precipitate dissolved. Then DMBA was added and the reaction was left stirring at room temperature overnight. TLC showed complete consumption of DMBA after 24 h. Doubling the amount of 4-nitroimidazole increased the rate of the reaction and reduced the time for consumption of DMBA to 10 h. However, the ^{31}P-NMR of the reaction mixture was complex (Figure 4.21) and it was difficult to tell if the product was indeed formed.

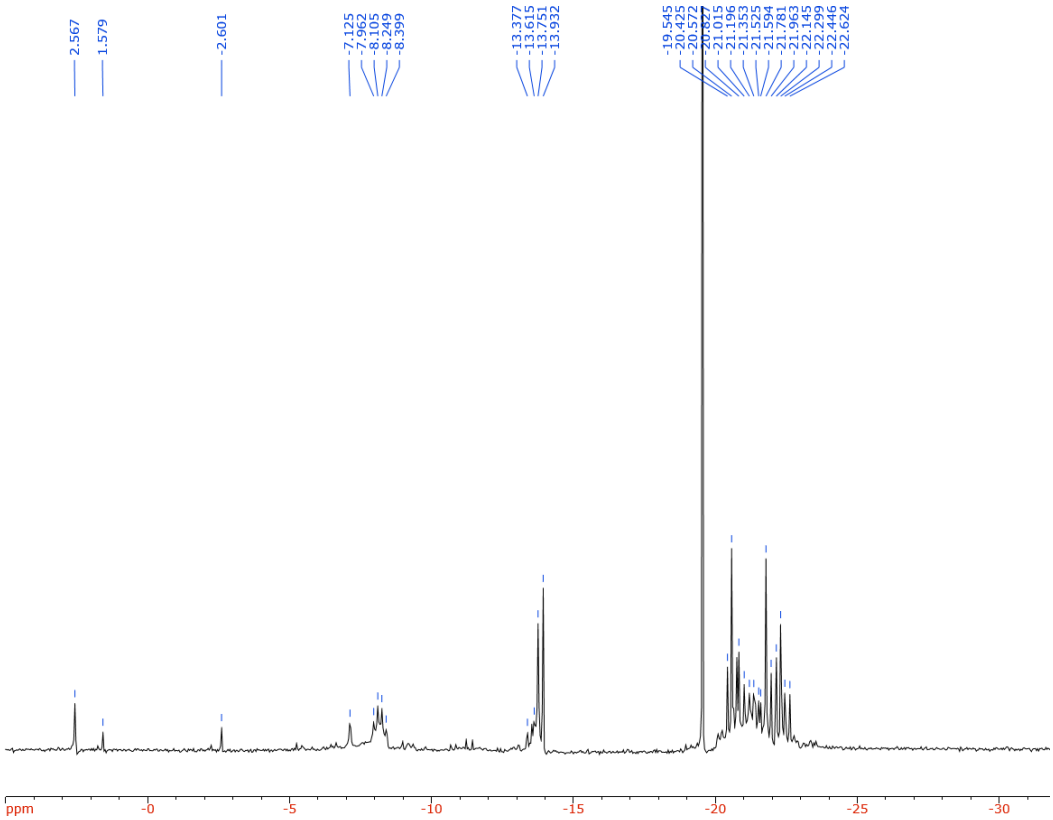
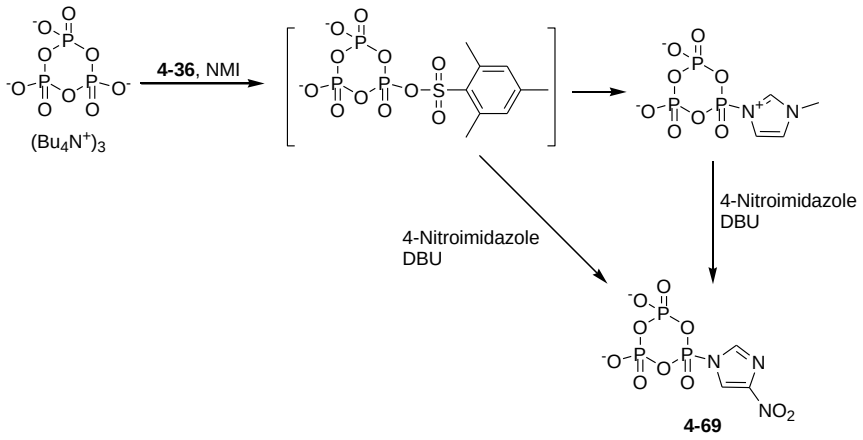


Figure 4.21. ^{31}P-NMR of the crude reaction mixture of 3 equiv **4-34**, 3 equiv **4-36**, 5 equiv NMI, 3 equiv 4-nitroimidazole, 5 equiv DBU and 1 equiv DMBA reaction was stirred at r.t for 24h and was quenched with TEAA

4-Nitroimidazole was used in an attempt to produce intermediate **4-69** (Scheme 4.25) which we hoped would be more reactive towards nucleophiles. We attempted to determine if a new intermediate is indeed formed by repeating the reaction but without the addition of the DMBA and recording the ^{31}P-NMR spectrum of the mixture after 10 min. Although the spectrum, which is shown in Figure 4.22, is different from those seen for either the mixed anhydride (**4-43**) or imidazolium-trimetaphosphate (**4-44**) (see Figures 4.11 and 4.12) we could not tell if the multiplet at approximately 21 ppm corresponded to intermediate **4-69**. Nevertheless, this result suggests that a new intermediate is indeed formed.



Scheme 4.25. Formation of proposed intermediate **4-69**.

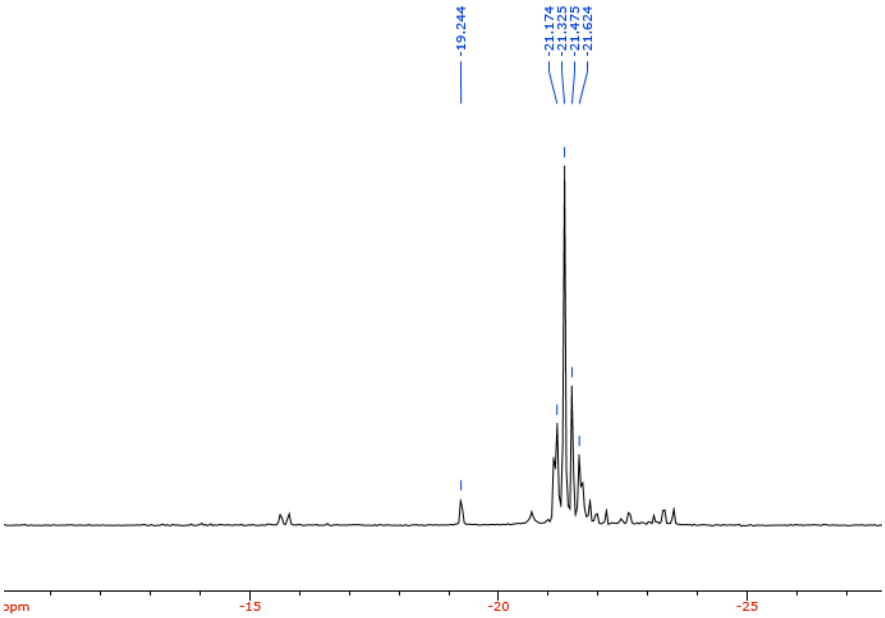
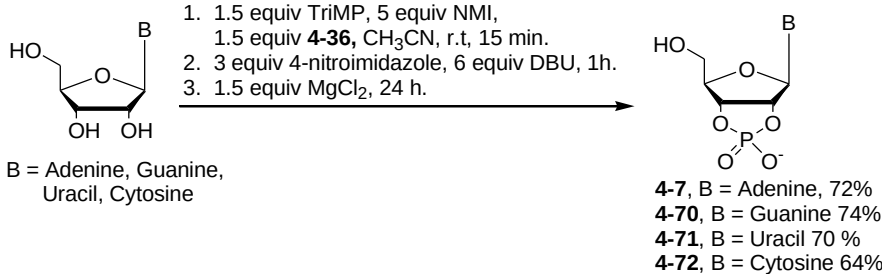


Figure 4.22. ^{31}P-NMR spectrum of a mixture of 3 equiv **4-36**, 3 equiv TriMP and 3 equiv NMI which was stirred for 15 min at room temperature. 3 Equiv 4-nitroimidazole was added followed by 5 equiv DBU and the spectrum was recorded 10 minutes later.

We applied the above conditions to nucleosides. The amount of activated TriMP that was generated was reduced to 1.5 equiv because of the presence of more than one hydroxyl groups. The reactions were performed by adding 1.5 equiv **4-36** to a mixture of 1.5 equiv **4-34** and 5 equiv NMI in acetonitrile. A white suspension is formed immediately after the addition of **4-36**. The mixture was then left stirring at room temperature for 15 min. 4-Nitroimidazole (3 equiv) followed by 6 equiv DBU were added and the solution turned clear yellow. The nucleoside (1 equiv) was added to this mixture and the reaction mixture stirred at room temperature until the nucleoside dissolves (takes approximately 1 h). Anhydrous magnesium chloride (1.5 equiv) was added and the reaction was stirred for 24 hours. A white precipitate appeared after a few minutes and increased with time (only in case of using acetonitrile as solvent). The reaction was monitored by ^{31}P-NMR by taking a100 µL aliquots of the reaction mixture quenched with 5% EDTA in 100 mM ammonium acetate buffer. Surprisingly, the dominant peak in the ^{31}P-NMR

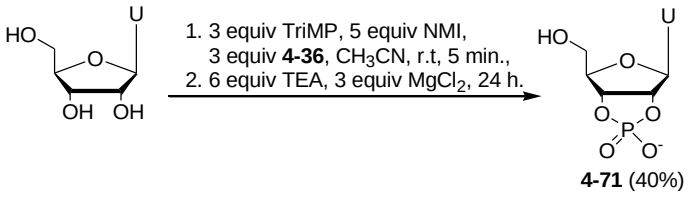
spectra was at approximately 22 ppm. This is characteristic of 2',3'-cyclic nucleoside monophosphates. The reaction mixture was then cooled to 0^oC in an ice bath and quenched with 500 mM ammonium acetate, washed with chloroform and purified by reversed phase column chromatography using tri-*n*-butylammonium acetate-methanol and a Biotage purification system. Fractions containing the desired product were pooled together and freeze dried. Conversion into the corresponding ammonium salts using a Dowex -50 W NH_4^+ column afforded 2',3'-cyclic nucleoside monophosphates **4-7** and **4-70** – **4-72** (Scheme 4.26) as ammonium salts in 64-74% yields.



Scheme 4.26. Synthesis of 2',3'-cyclic nucleoside monophosphates.

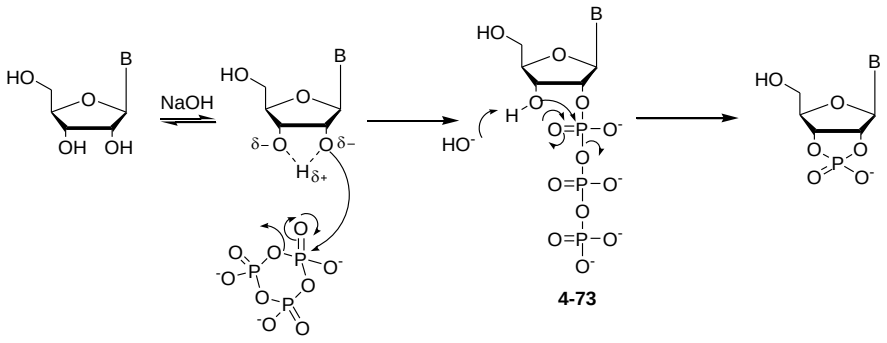
The reaction can be performed in DMF but in this case the reaction time is longer (about 48 h). We observed that no precipitation occurs after addition of the anhydrous magnesium chloride when DMF is the solvent. Although the reaction can proceed without anhydrous magnesium chloride its presence increases the yield of the reaction (yield for **4-71** without $MgCl_2$ is 48% as compared to 70% in case of $MgCl_2$). 3-Nitrotriazole was also tried but found to be less effective than 4-nitroimidazole (**4-71**, 60% as compared to 70% in case of 4-nitroimidazole). Low yields are obtained in the absence of any nitroimidazole (less than 10 % for **4-71**) of product. A relatively strong organic base such as DBU was found to be necessary. It probably deprotonates the hydroxyl groups (partially) of the nucleoside and the 4-nitroimidazole making them more nucleophilic. The reaction will proceed with triethylamine in

place of DBU without nitroimidazole in which case a moderate yield of **4-71** was obtained (Scheme 4.27).



Scheme 4.27. Formation of 2',3'-cyclic UMP using TEA as base.

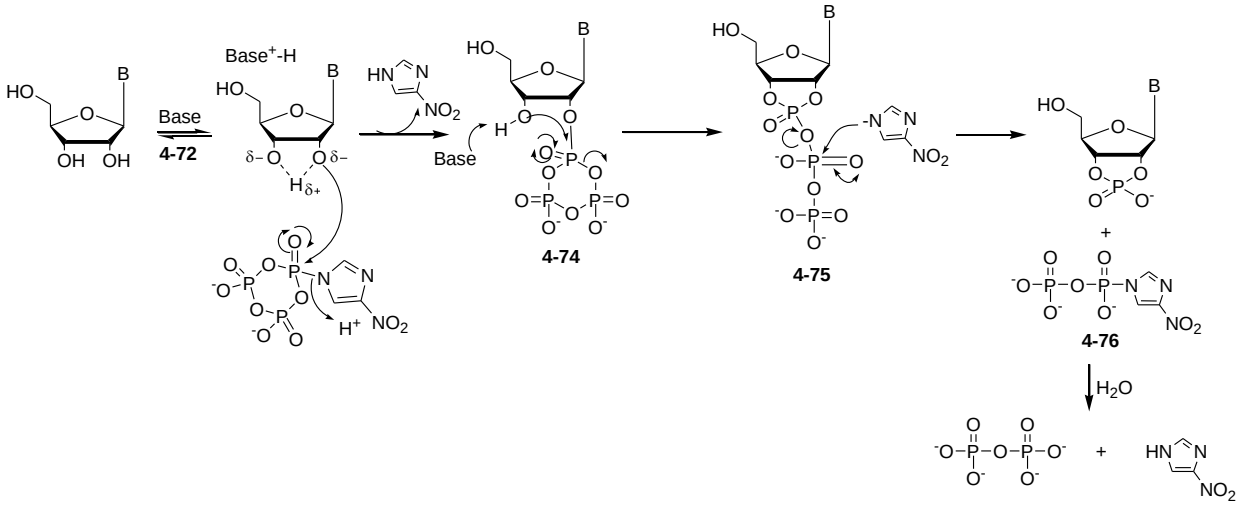
Compound **4-7** has been previously made by Saffhil using trisodium trimetaphosphate and adenosine (see Scheme 4.4).[175] The proposed mechanism is straightforward: attack of the 2'-OH or 3'-OH on the TriMP to give triphosphate **4-73** followed by cyclization to give product (Scheme 4.28). Such a mechanism has been proposed by Inoue et al. for the phosphorylation of catechol with sodium TriMP.[173]



Scheme 4.28. Proposed mechanism of formation 2',3'-cyclic-NMP's using TriMP.

When using activated triMP the mechanism is not so straightforward. One can envisage an attack of the 2'-OH on the activated TriMP to give **4-74** followed by cyclization and ring opening to give **4-75** (Scheme 4.29). ^{31}P-NMR of the reaction mixture indicates that the product is formed *before* quenching of the reaction with water. Perhaps 4-nitroimidazole attacks the central phosphorus of **4-75** to give product. However, one might argue that the 4-nitroimidazole

is more likely to attack the ring phosphorus as this ring is highly stained.[212] More studies will be necessary to elucidate the mechanism of this reaction.



Scheme 4.29. Proposed mechanism of formation 2',3'-cyclic-NMP's using activated TriMP.

To determine what products would be formed using a 2'-deoxynucleoside as substrate we reacted 2'-deoxycytidine under the same conditions (adding 1.5 equiv **4-36** to a mixture of 1.5 equiv **4-34** and 5 equiv NMI in acetonitrile. The mixture was then left stirring at room temperature for 15 min. 3 equiv 4-nitroimidazole followed by 6 equiv DBU were added followed by 1 equiv of the nucleoside, the mixture was stirred for 1 h followed by adding 1.5 equiv $MgCl_2$); however, this failed to produce a triphosphorylated product or 3',5'-cyclic cytidine monophosphate, as evidenced by the absence of characteristic ^{31}P-NMR signals in the quenched crude reaction mixture (Figure 4.23). We were unable to determine what was being formed from this NMR spectrum. Surprisingly, an attempt to purifiy product(s) from this reaction by HPLC resulted only in isolation of a significant amount of deoxycytidine.

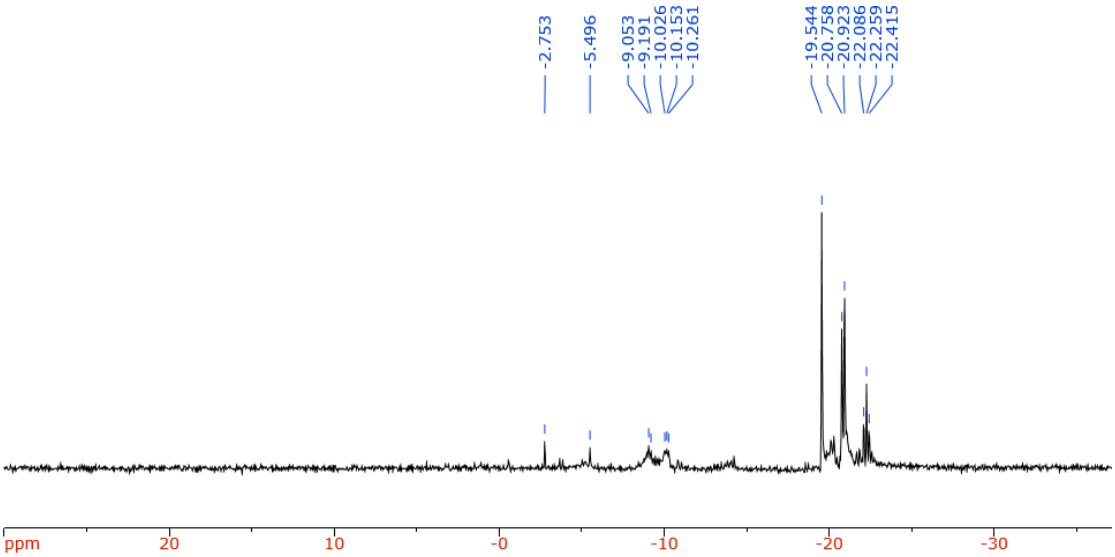
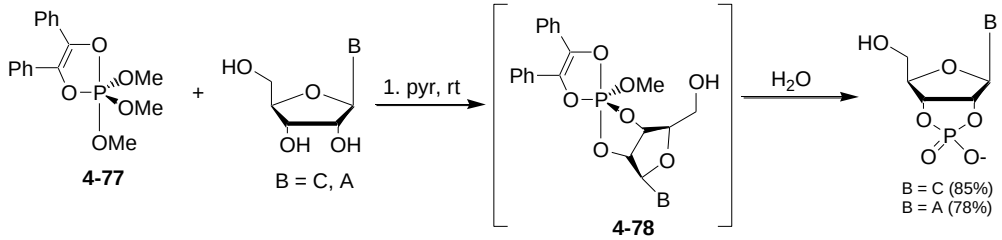


Figure 4.23. ^{31}P-NMR spectrum of the reaction of 1.5 equiv **4-36** to a mixture of 1.5 equiv **4-34** and 5 equiv NMI in acetonitrile. The mixture was then left stirring at room temperature for 15 min. 3 equiv 4-nitroimidazole followed by 6 equiv DBU were added followed by 1 equiv of the 2-deoxycytidine, the mixture was left stirring at room temperature for 1 h then 1.5 equiv $MgCl_2$ were added and the reaction was left stirring for 24 h at r.t. ^{31}P-NMR taken for the quenched reaction mixture.

It is difficult to rationalize why no phosphorylated product was isolated from the reaction with the deoxycytidine. The 5'-OH group of a nucleoside should be the most reactive hydroxyl group based on sterics. However, with ribonucleosides, under basic conditions, electrophiles often preferentially react at the 2'-OH or 3'-OH rather than at the 5'-OH unless the electrophile is very sterically hindered.[213,214] This may be because there is a higher concentration of the conjugate base of the 2'-OH or 3'-OH group compared to that of the 5'-OH group as the pK_a of the 2'-OH or 3'-OH group is app. 12.5[215,216] which is about 2.5-3 pK_a units lower than that of the 5'-OH.[217] This lower pK_a is mainly due to intramolecular H-bonding interactions and inductive effects.[216] The 2'-OH group is generally more reactive than the 3'-OH group though the reasons for this are not very well understood. It may be that the 5'-OH of the deoxycytidine is not nucleophilic enough for reaction with activated TriMP to occur. Perhaps the activated TriMP

reacted with the amino group and this product slowly hydrolyzed back to deoxycytidine upon quenching of the reaction with buffer.

Although we have not yet been able to obtain the NTP's using activated TriMP we have developed a new approach to nucleoside 2'-3'cyclic phosphates. These compounds are of interest as they have been shown to be intermediates during the enzymatic and non-enzymatic hydrolysis of RNA. Although this method is similar to that outlined in section 4.1.1 (see Scheme 4.4) it is considerably faster (1 day versus 5 days). Other methods for preparing these types of compounds have been developed. Holy et al. reacted ribonucleosides with triethyl phosphite to give the corresponding ribonucleoside phosphites. These were hydrolyzed to a mixture of 2'- and 3'-phosphites and then oxidized to give the corresponding 2',3'-cyclic phosphates in 57-70% yields. The preparation procedure involved two purification steps with DEAE cellulose column chromatography.[218] Perhaps the most widely used approach to these compounds is that of Chen et al. These workers reacted unprotected ribonucleosides with oxyphosphorane **4-77** (Scheme 4.30). This intermediate is hydrolyzed to give the desired cyclic phosphate products in good yield after purification by ion exchange chromatography. The only drawback to this method is that phosphorane **4-77** is not commercially available.[219]



Scheme 4.30. Synthesis of nucleoside 2'-3'cyclic phosphates by Chen et al.

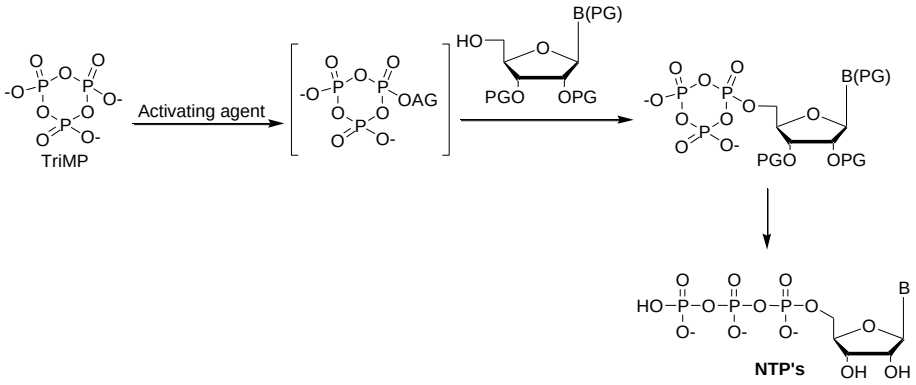
4.4. Conclusions and future work

In summary, a novel approach to the synthesis of nucleoside 5'-tetraphosphates and dinucleoside 5',5'-pentaphosphates via activation of cyclic trimetaphosphate has been reported.[220] This procedure has several advantages over current methods. Unlike some procedures,[88,189] it does not require protection of the nucleoside or phosphate groups or special conditions (i.e. microwaves). Readily available and inexpensive mesitylenesulfonyl chloride/NMI can be used as activating agent. It utilizes very inexpensive sodium trimetaphosphate as a substrate which is easily converted into its tetrabutylammonium salt in almost quantitative yields. It employs NMP's which are considerably less expensive or easier to prepare than nucleoside 5'-di- or triphosphates. The products are relatively easy to purify by RP-HPLC. Most significantly, the products are produced in excellent yield.

This study has led to a novel and efficient approach to the synthesis of δ-labeled nucleoside 5´-tetraphosphates in which the terminal phosphate is labelled with a fluorescent dye. The procedure is more rapid than previously reported methods, it utilizes relatively inexpensive or easily prepared NMP's as opposed to more expensive NTP's and the target compounds are obtained in excellent yields.

Finally, although it was not possible to develop a one-step synthesis of NTP's from nucleosides, a new route to nucleoside 2'-3'cyclic phosphates was developed. The yields are comparable to the best literature methods and this approach uses commercially available reagents and substrates.

Future work in this area should focus on developing a one-step synthesis of NTP's and dNTPs from nucleosides as outlined in Scheme 4.8; however, it looks like we will have to use protected nucleosides as substrates (Scheme 4.30).



Scheme 4.30. Proposed route to NTP's using activated TriMP and protected nucleosides.

4.5. Experimental

4.5.1 General Information.

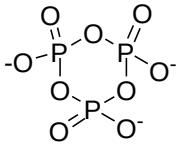
All reagents and starting nucleotides were obtained from commercial sources unless stated otherwise. Strictly anhydrous conditions were found to be essential for obtaining the reported yields. Acetonitrile and DMF were distilled from calcium hydride. NMI was distilled from sodium hydroxide and stored over 4Å molecular sieves. All reactions were conducted under an inert atmosphere of Ar. All NMR spectra were recorded using D_2O as solvent. For ^{1}H-NMR spectra, chemical shifts are reported in ppm relative to the solvent residual peak (δ 4.79). For proton-decoupled ^{13}C-NMR spectra, chemical shifts are reported in ppm relative to CH_3OH in D_2O (δ 49.5, external standard). For proton-decoupled ^{31}P-NMR, chemical shifts are reported in ppm relative to aqueous 85% H_3PO_4 (δ 0 ppm, external standard). Preparative reversed phase chromatography was performed using a Biotage Isolera One Flash purification system equipped

with a C-18 reverse phase preparative Biotage 30 g column (used for compounds **4-7**, **4-49** – **4-54**, and **4-70** – **4-72.** RP-HPLC was used for the other compounds). Negative ion high resolution electrospray mass spectra were obtained using a high resolution, accurate mass Thermo Scientific Q-exactive Orbitrap mass spectrometer.

4.5.2 Preparation of the tetrabutylammonium salts of NMP's

The sodium salts of the nucleotides were converted into their free acids using a Dowex-50W ion exchange column (H^+ form) column then titrated to pH 7.0 with a dilute solution of tetrabutylammonium hydroxide then concentrated by high vacuum rotary evaporation to approximately one seventh the original volume and lyophilized. The lyophilized powder was dried by dissolving it in acetonitrile, an equal amount of dry toluene was added and the solution concentrated by rotary evaporation to dryness (3x). The ^{1}H NMR spectra of the residue indicated that there were 1.7 tetrabutylammonium ions per nucleoside monophosphate. The residue was subjected to high vacuum for 1 h. The flask was removed under Ar then dissolved in dry DMF in the presence of 4Å molecular sieves. This solution was allowed to stand for at least one hour prior to the coupling reactions.

4.5.3 Preparation of the tris(tetrabutylammonium) trimetaphosphate (4-34).



$(Bu_4N^+)_3$

4-34

The trisodium salt of trimetaphosphate was converted into its pyridinium salt using a Dowex-50W ion exchange column (pyridinium form). Fractions containing the desired

pyridinium salt were pooled and then a dilute solution of tetrabutylammonium hydroxide was added with stirring until pH 7.0 was reached. The solution was concentrated by high vacuum rotary evaporation to approximately one-seventh the original volume then lyophilized. The lyophilized powder was dried by dissolving it in acetonitrile, an equal amount of dry toluene was added and the solution concentrated by rotary evaporation to dryness (3x). The residue was subjected to high vacuum for 1 h. The flask was removed under Ar then dissolved in dry DMF in the presence of 4Å molecular sieves. This solution was allowed to stand for at least one hour prior to the coupling reactions. ^{31}P-NMR (D_2O, 121 MHz): δ -19.16.

4.5.4 General method for the synthesis of nucleoside-5'-tetraphosphates (4-38 – 4-41).

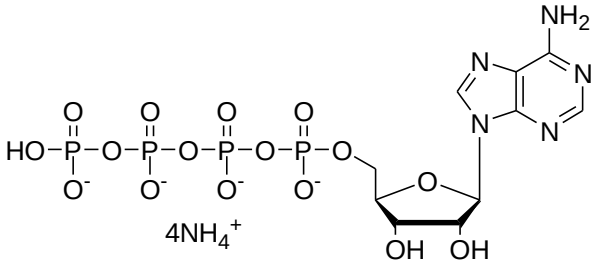
Method A: To a solution of **4-34** (195 mg, 0.21 mmol, 2 equiv) in dry DMF (2.5 mL) was added NMI (55 mg, 54µL, 0.68 mmol., 5.0 equiv) and mesitylenesulfonyl chloride (reagent **4-36**, 40 mg, 0.18 mmol, 1.8 equiv). The mixture was stirred at room temperature for 25 min then added dropwise with stirring over a period of 30 seconds into a cooled flask (ice bath) containing a tetrabutylammonium salt of a nucleoside-5'-monophosphate (0.135 mmol, 1.0 equiv) in dry DMF (1.5 mL). The ice-bath was removed and the mixture was stirred at room temperature for 3h. The reaction was cooled to 0^{o}C (ice-bath) and quenched with 100 mM triethylammonium acetate buffer (4 mL, pH 7). The resulting solution was washed with chloroform (3 x 10 mL) then left to stand at room temperature for 2 hours. The desired product was purified by RP-HPLC using a semipreparative C18 column and a gradient of acetonitrile and buffer (50 mM triethylammonium acetate, pH 7) at 6 mL/min and monitored at 265 and 280 nm. Fractions containing the desired product were pooled, concentrated by high vacuum rotary evaporation, the residue was dissolved in water and repeatedly freeze-dried until the ^{1}H-NMR

spectrum indicated that no residual buffer was present. The resulting white powders were converted into their ammonium salts using a Dowex-50-W ion-exchange resin in NH_4^+ form.

Method B: The same as method A except 1-benzenesulfonyl-3-methyl-imidazolium triflate (reagent **3-11**, 68 mg, 0.18 mmol, 1.8 equiv) was used instead of reagent **4-36**.

4.5.5 Characterization data of nucleoside-5'-tetraphosphates (4-38 – 4-41).

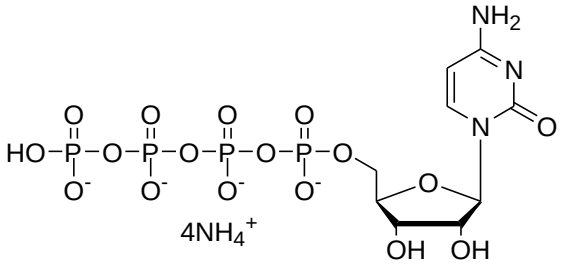
Adenosine-5'-tetraphosphate, tetraammonium salt (4-38).



4-38

Obtained in 85% yield (75 mg) using method A and in 84% yield (74 mg) using method B after purification by RP-HPLC (linear gradient of 100% buffer (pH 7.0) to 9% CH_3CN-90% buffer over 45 min., t_r = 40 min) followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.07 (s, 2H, $H_{5'}$), 4.22 (s, 1H, $H_{4'}$), 4.39 (s, 1H, $H_{3'}$), 5.89 (s, 1H, $H_{1'}$), 7.93 (s, 1H, H_2), 8.29 (s, 1H, H_8). ^{13}C-NMR (D_2O, 75 MHz): δ 65.1 (d, *J* = 5.3 Hz), 70.2, 74.1, 83.8, 86.6, 139.7, 152.3, 155.1. ^{31}P-NMR (D_2O, 121 MHz): δ -5.2, -9.0, -20.4. TOF MS ES-, *m/z* = 585.95 $[M - H]^-$. HRMS (ESI-): *m/z* = 585.9545, $C_{10}H_{16}N_5O_{16}P_4$, $[M-H]^-$ requires 585.9543.

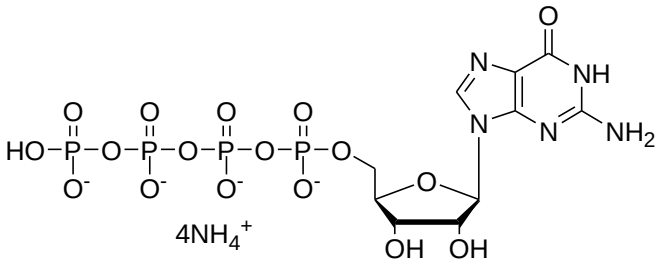
Cytosine-5'-tetraphosphate, tetraammonium salt (4-39).



4-39

Obtained in 87% yield (74 mg) using method A and 85 % yield (72 mg) using method B after purification by RP-HPLC (linear gradient of 100% buffer (pH 7.0) to 6% CH_3CN-94% buffer over 45 min., t_r = 32 min) followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.10-4.21 (m, 5H, $H_{2',3',4',5'}$), 5.80 (d, *J* = 4.1 Hz 1H, $H_{1'}$), 5.99 (d, *J* = 6.5 Hz, 1H, H_5),7.79 (d, *J* = 7.5 Hz, 1H, H_6). ^{13}C-NMR (D_2O, 75 MHz): δ 64.6 (d, *J* = 5.3 Hz), 69.1, 74.0, 82.6 (d, *J* = 9 Hz), 89.0, 96.2, 141.7, 155.6, 164.4. ^{31}P-NMR (D_2O, 121 MHz): δ -8.12, -9.06 (d, *J* = 15.3 Hz), -20.72. TOF MS ES-, *m/z* = 561.90 $[M-H]^-$. HRMS (ESI-): *m/z* = 561.9430, $C_9H_{16}N_3O_{17}P_4$, $[M-H]^-$ requires 561.9430.

Guanosine-5'-tetraphosphate, tetraammonium salt (4-40).

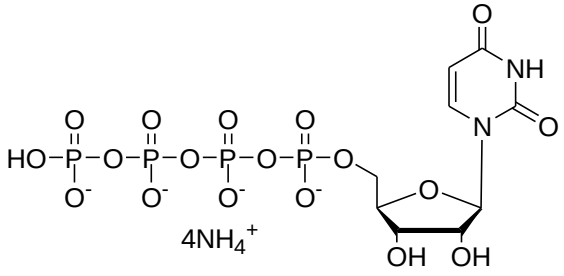


4-40

Obtained in 84% yield (77 mg) using method A and 84 % yield (77 mg) using method B after purification by RP-HPLC (linear gradient of 100% buffer (pH 7.0) to 6% CH_3CN over 50

min., t_r = 41 min) followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.10 (br, 2H, $H_{5'}$), 4.20 (s, 1 H, $H_{4'}$), 4.41 (t, *J* = 4.6 Hz, 1H, $H_{3'}$), 5.76 (d, *J* = 5.9 Hz, 1H, $H_{1'}$), 7.97 (s, 1H, H_8). ^{13}C-NMR (D_2O, 75 MHz): δ 65.2 (d, *J* = 5.5 Hz), 70.2, 73.5, 83.6 (d, *J* = 9 Hz), 86.6, 116.0, 137.4, 151.5, 153.7, 158.7. ^{31}P-NMR (D_2O, 121 MHz): δ -6.87, -9.16 (d, *J* = 15.7 Hz), -20.61. TOF MS ES-, *m/z* = 601.89 $[M-H]^-$. HRMS (ESI-): *m/z* = 601.9487, $C_{10}H_{16}N_5O_{17}P_4$, $[M-H]^-$ requires 601.9492.

Uridine-5'-tetraphosphate, tetraammonium salt (4-41).



4-41

Obtained in 86% yield (74 mg) using method A and 84 % yield (72 mg) using method B after purification by RP-HPLC (linear gradient of 100% buffer (pH 7.0) to 7% CH_3CN-95% buffer over 45 min., t_r = 33 min) followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.07-4.12 (m, 3H, $H_{5',4'}$), 4.22-4.23 (br, 2H, $H_{2',3'}$), 5.81 (m, 2H, $H_{1',5}$), 7.76 (d, *J* = 8.0 Hz, 1H, H_6). ^{13}C-NMR (D_2O, 75 MHz): δ 64.9 (d, *J* = 5.2 Hz), 69.5, 73.5, 83.1 (d, *J* = 9.0 Hz), 88.1, 102.5, 141.5, 151.7, 166.0. ^{31}P-NMR (D_2O, 121 MHz): δ -7.89, -8.92, -20.51. TOF MS ES-, *m/z* = 562.89 $[M-H]^-$. HRMS (ESI-): *m/z* = 562.9273, $C_9H_{15}N_2O_{18}P_4$, $[M-H]^-$ requires 562.9270.

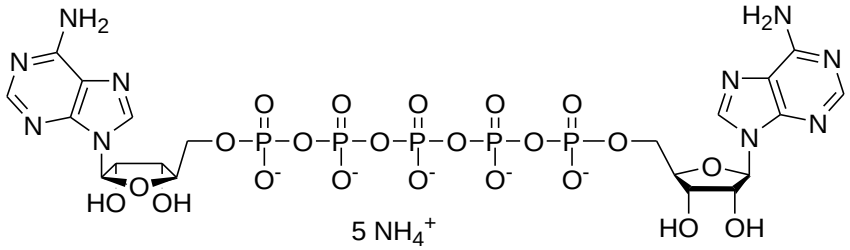
4.5.6 General method for the synthesis of dinucleoside-5',5'-pentaphosphates (2-54, 4-18, 4-21, and 4-45 - 4-47).

Method A: To a solution of **4-34** in (195 mg, 0.21 mmol, 2 equiv) in dry DMF (2.5 mL) was added NMI (55 mg, 54µL, 0.68 mmol., 5 equiv) and mesitylene chloride (reagent **4-36**, 40 mg, 0.18 mmol, 1.8 equiv). The mixture was stirred at room temperature for 25 min then added dropwise with stirring over a period of 30 seconds into a cooled flask (ice bath) containing a tetrabutylammonium salt of a nucleoside-5'-monophosphate (0.135 mmol, 0.64 equiv) in dry DMF (1.5 mL). The ice-bath was then removed and the reaction mixture was stirred at room temperature for 3 h. A solution of a tetrabutylammonium salt of a nucleoside-5'-monophosphate (0.27 mmol, 2 equiv) in dry DMF (3 mL) was added dropwise into the reaction mixture followed by the addition of anhydrous magnesium chloride (14 mg, 0.15 mmol, 0.7 equiv). The mixture was then stirred at room temperature for 72 h, cooled to 0^{o}C (ice-bath) and quenched by the addition of 100 mM triethylammonium acetate buffer (pH 7, 6 mL) containing EDTA disodium salt (60 mg, 0.16 mmol, 1.2 equiv). The resulting solution was washed with chloroform (3 x 10 mL), then purified by RP-HPLC using a semipreparative C18 column and a gradient of acetonitrile and buffer (50 mM triethylammonium acetate, pH 7) at 6 mL/min and monitored at 255 and 280 nm. Fractions containing the desired product were pooled, concentrated by high vacuum rotary evaporation, and the residue was dissolved in water and repeatedly freeze-dried until the ^{1}H-NMR spectrum indicated that no residual buffer was present. The resulting white powder was converted to its ammonium salt using a Dowex-50-W ion-exchange resin in NH_4^+ form.

Method B: The same as method A except reagent **3-11** (68 mg, 0.18 mmol, 1.8 equiv) was used in place of reagent **4-36**.

4.5.7 Characterization data of dinucleoside-5',5'-pentaphosphates (2-54, 4-18, 4-21, and 4-45 - 4-47)

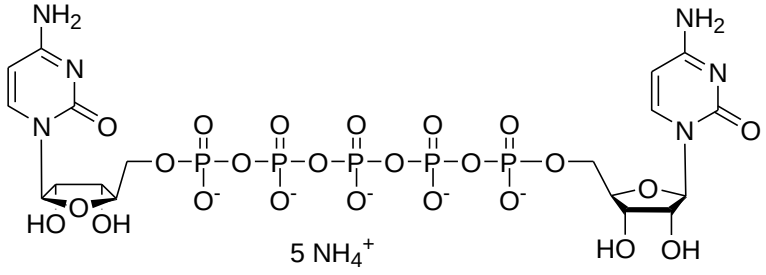
P1,P5-diadenosine pentaphosphate, pentaammonium salt (4-18).



4-18

Obtained in 81% yield (110 mg) using method A and 85 % yield (116 mg) using method B after purification by RP-HPLC (linear gradient of 100% buffer (pH 7.0) to 12% CH_3CN over 50 min., t_r = 43 min) followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.15 (br, 4H, $H_{5'}$), 4.24 (br, 2H, $H_{4'}$), 4.39 (br, 2H, $H_{3'}$), 4.50 (d, *J* = 4.8 Hz 5.82 (d, *J* = 5.4 Hz, 2H, $H_{1'}$), 7.96 (s, 2H, H_2), 8.27 (s, 2H, H_8). ^{13}C-NMR (D_2O, 75 MHz): δ 65.2 (d, *J* = 5.0 Hz), 70.1, 74.4, 83.7 (d, *J* = 9.0 Hz), 86.9, 117.7, 140.2, 148.1, 150.2, 153.1. ^{31}P-NMR (D_2O, 121 MHz): δ -8.88, -20.60. TOF MS ES-, m/z 914.95 $[M - H]^-$, HRMS (ESI-): *m/z* = 915.0077, $C_{20}H_{28}N_{10}O_{22}P_5$ $[M - H]^-$, requires 915.0068.

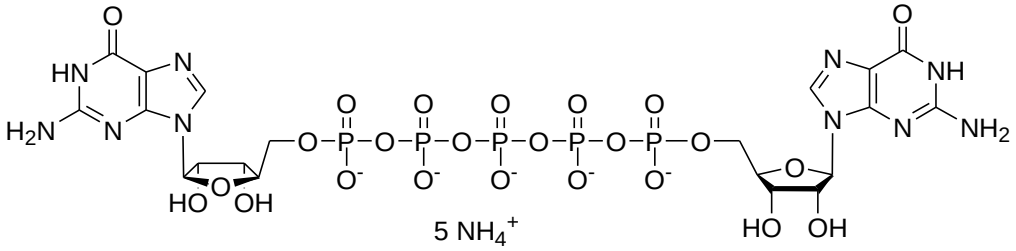
P1,P5-Dicytidine-5'-pentaphosphate, pentaammonium salt (4-45).



4-45

Obtained in 84% yield (108 mg) using method A and 82 % yield (106 mg) using method B after purification by RP-HPLC (linear gradient of 100% buffer (pH 7.0) to 10% CH_3CN-94% buffer over 45 min., t_r = 35 min) followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.15-4.24 (m, 10H, $H_{2',3',4',5'}$), 5.82 (s, 2H, $H_{1'}$), 6.12 (d, *J* = 7.1 Hz, 2H, H_5), 7.92 (d, *J* = 7.4 Hz, 2H, H_6). ^{13}C-NMR (D_2O, 75 MHz): δ 64.5 (d, *J* = 4.8 Hz), 69.2, 74.3, 83.0 (d, *J* = 9.2 Hz), 89.1, 96.0, 142.6, 152.9, 162.3. ^{31}P-NMR (D_2O, 121 MHz): δ -8.70 (d, *J* = 12.4 Hz), -20.2. TOF MS ES-, m/z 866.95 $[M - H]^-$, HRMS (ESI-): *m/z* = 866.9838, $C_{18}H_{28}N_6O_{24}P_5$ $[M - H]^-$, requires 866.9843.

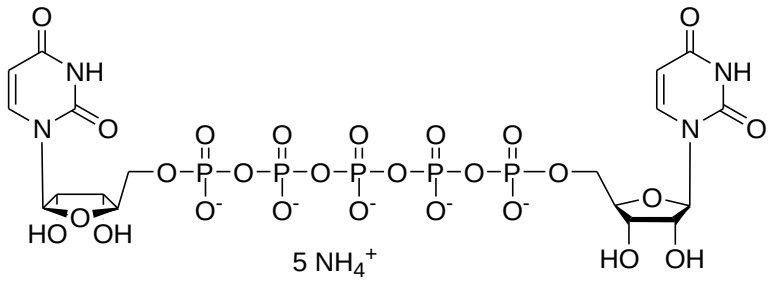
P1,P5-Diguanosine-5'-pentaphosphate, pentaammonium salt (4-46).



4-46

Obtained in 85% yield (118 mg) using method A and 86 % yield (119 mg) using method B after purification by RP-HPLC (linear gradient of 100% buffer (pH 7.0) to 10% CH_3CN over 50 min., t_r = 42 min) followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.16 (br, 4H, $H_{5'}$), 4.22 (br, 2H, $H_{4'}$),4.41 (br, 2H, $H_{3'}$), 4.50 (br, 2H, $H_{2'}$), 5.73 (d, *J* = 4.8 Hz, 2H, $H_{1'}$), 8.16 (s, 2H, H_8). ^{13}C-NMR (D_2O, 75 MHz): δ 64.9, 69.8, 74.0, 83.5 (d, *J* = 9.0 Hz) , 87.4, 113.8, 136.9, 150.7, 153.8, 157.4. ^{31}P-NMR (D_2O, 121 MHz): δ -9.45 (d, *J* = 15.2 Hz), -21.3(d, *J* = 16.9 Hz) . TOF MS ES-, m/z 946.97 $[M - H]^-$, HRMS (ESI-): *m/z* = 946.9948, $C_{20}H_{28}N_{10}O_{24}P_5$ $[M - H]^-$, requires 946.9966.

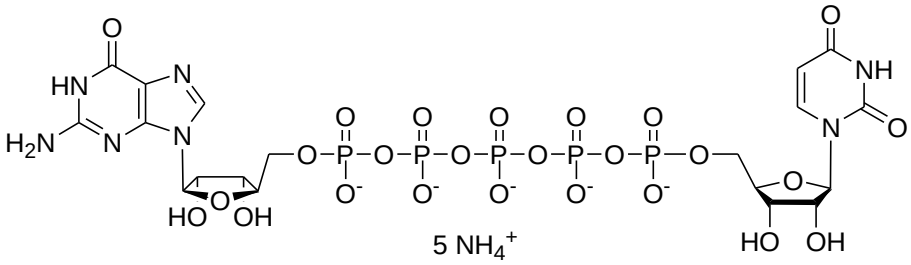
P1,P5-Diuridine-5'-pentaphosphate, pentaammonium salt (2-54).



2-54

Obtained in 84% yield (109 mg) using method A and 80 % yield (104 mg) using method B after purification by RP-HPLC (linear gradient of 100% buffer (pH 7.0) to 9% CH_3CN over 45 min., t_r = 39 min) followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H-NMR (D_2O, 300 MHz): δ 4.10-4.15 (m, 6H, $H_{4',5'}$), 4.22-4.27 (m, 4H, $H_{2',3'}$), 5.83 (m, 4H, $H_{1',5}$), 7.79 (d, *J* = 8.1 Hz, 2H, H_6). ^{13}C-NMR (D_2O, 75 MHz): δ 64.9 (d, *J* = 5.5 Hz), 69.6, 73.6, 83.3 (d, *J* = 9 Hz), 88.0, 102.5, 141.5, 151.7, 166.0. ^{31}P-NMR (D_2O, 121 MHz): δ -9.88 (d, *J* = 14.6 Hz), -21.21. TOF MS ES-, m/z 868.92 $[M - H]^-$, HRMS (ESI-): *m/z* = 868.9528, $C_{18}H_{26}N_4O_{26}P_5$ $[M - H]^-$, requires 868.9523.

P1-Uridine-P5-guanosine-5'-pentaphosphate, pentaammonium salt (4-47).



4-47

Obtained in 77% yield (104 mg) using method B as its pentaammonium salt after purification by RP-HPLC (linear gradient of 100% buffer (pH 7.0) to 8% CH_3CN over 50 min., t_r

= 43 min) followed by passage through a Dowex-50-W-NH_4^+ ion exchange column. ^{1}H NMR (D_2O, 300 MHz): δ 4.12-4.29 (br, 5H, $H_{5'G,5'U,3'U}$), 4.26-4.28 (m, 3H, $H_{3'G,4'G,4'U}$) 4.41 (br, 1H, $H_{3'U}$), 5.73 -5.83 (m, 3H, $H_{1'G,}$ $H_{1'U,}$ H_{5U}), 7.73 (d, *J* = 7.9 Hz, 1H, H_{6U}), 8.03 (s, 1H, H_{8G}). ^{13}C NMR (D_2O, 75 MHz): δ 64.9 (d, *J* = 5.6 Hz), 65.2,(d, *J* = 5.2 Hz), 69.5, 70.1, 73.6, 83.2 (d, *J* = 9.1 Hz), 83.7 (d, *J* = 9.0 Hz), 86.7, 88.0, 102.4, 115.6, 137.4, 141.4, 151.6, 153.8, 158.5, 165.9. ^{31}P NMR (D_2O, 121 MHz): δ -8.91, -20.67 (d, *J* = 9.9 Hz). TOF MS ES-, m/z 907.93 $[M - H]^-$, HRMS (ESI-): *m/z* = 907.9745, $C_{19}H_{27}N_7O_{25}P_5$ $[M - H]^-$, requires 907.9745

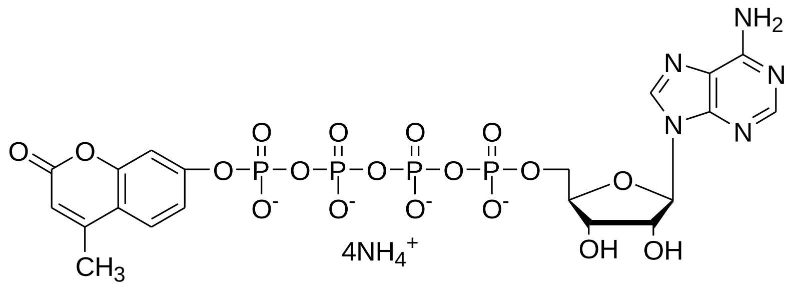
4.5.8 Preparation of the tetrabutylammonium salts of 5´-AMP, 5´-dAMP, 5´-dTMP and 5´-dCMP.

The free acids or free acid monohydrates of 5´-AMP, 5´-dAMP, 5´-dTMP and 5´-dCMP (0.5 g) were dissolved in distilled deionized water (50 mL) and titrated to pH 7.0 with a dilute solution of tetrabutylammonium hydroxide.[221] The solutions were concentrated by high vacuum rotary evaporation to approximately one seventh the original volume and lyophilized. The lyophilized powders were stored in the freezer. An equal amount of dry acetonitrile and dry toluene was added to round bottom flasks containing 0.675 mmol (519 mg of AMP, 508 mg of 2´-dAMP, 502 mg 5´-dTMP and 492 mg of 5´-dCMP) of these nucleotides and the solutions concentrated by rotary evaporation to dryness. This was repeated two more times. The ^{1}H-NMR spectra of these nucleotides indicated that there was 1.7 tetrabutylammonium ions per nucleoside monophosphate. The nucleotides were subjected to high vacuum for 3 h. The flasks were removed under Ar, the nucleotides dissolved in dry DMF and 4Å molecular sieves were added. These solutions were allowed to stand for at least 3 h hour prior to the coupling reactions.

4.5.9 Preparation of the tri-*n*-butylammonium salt of 2´-dGMP.

Using the above procedure to prepare the tetrabutylammonium salt of 2´-dGMP from commercially available 2´-dGMP disodium salt dihydrate resulted in its decomposition upon conversion to its free acid and titration with tetrabutylammonium hydroxide. Hence the tri-*n*-butylammonium salt was prepared using a procedure similar to that developed by Gibson and Leonard.[222] An aqueous solution of 2´-dGMP disodium salt dihydrate (0.5 g in 20 mL distilled deionized water) was applied to a Dowex-50-W column (8-10 grams) in its pyridinium form. The eluate was collected in a flask containing 20 mL of ethanol and 1.0 mL of tributylamine. The column was washed with water (3 x 30 mL) while collecting the eluate in the above-mentioned flask. The resulting solution was stirred for 10 minutes and then concentrated by high vacuum rotary evaporation to about one seventh the original volume and then lyophilized. The lyophilized powder was stored in the freezer. An equal amount of dry acetonitrile and dry toluene was added to a round bottom flask containing 0.675 mmol (396 mg) of the tributylammonium salt and the solution concentrated by rotary evaporation to dryness. This was repeated two more times. The ^{1}H-NMR spectrum of the residue indicated that there were 1.3 tributylammonium ions per nucleoside monophosphate. The nucleotide was subjected to high vacuum for 3 h. The flask was removed under Ar, the nucleotide dissolved in dry DMF and 4Å molecular sieves were added. This solution was allowed to stand for at least 3 h hour prior to the coupling reaction.

4.5.10 Preparation of δ-(4-methyl-7-coumarinyl) adenosine 5'-tetraphosphate tetraammonium salt (4-49) via intermediate 4-48 (**Scheme 4.18**).



4-49

To a mixture of **4-34** (0.405 mmol, 390 mg) in acetonitrile (4.5 mL) and *N*-methylimidazole (0.12 mmol, 97 μL) was added mesitylene chloride (0.36 mmol, 80 mg) at room temperature, and the reaction mixture was stirred for 15 min. This solution was withdrawn via syringe and injected dropwise over 1 min into a cooled flask (ice bath) containing tetrabutylammonium salt of AMP (0.27 mmol, 206 mg) in DMF (3 mL). 7-Hydroxy-4-methylcoumarin (0.351 mmol, 62.0 mg) and DABCO (0.81 mmol, 91 mg) were added to the reaction flask followed by anhydrous magnesium chloride (0.315 mmol, 30 mg). The ice bath was then removed and the reaction mixture was stirred at room temperature for 5 h. The reaction mixture was then cooled in ice and quenched with 100 mM triethylammonium acetate buffer (pH 7.0, 6 mL) then washed with chloroform (3 x 10 mL). Chelex resin (ca. 0.1 g) was added and the mixture stirred for 1 min then filtered through a cotton plug. The filtrate was purified by reversed-phase column chromatography using a gradient of 100% buffer A (10 mM tributylamine-30 mM acetic acid), 0% Buffer B (methanol, 15 mM tributylamine) to 40% buffer A, 60% buffer B over 55 min. The flow rate was 25 mL/min and monitored at 265 and 280 nm (t_r = 40 CV). Fractions containing the desired product were pooled, concentrated by high vacuum rotary evaporation, and the residue was dissolved in water and repeatedly freeze-dried until the ^{1}H-NMR spectrum indicated that no residual buffer was present (four times). The resulting white

powder was converted to its ammonium salt using a Dowex-50-W ion-exchange resin in NH_4^+ form to afford after lyophilization, 99 mg (45%) of the tetraammonium salt of compound **4-49**. ^{1}H-NMR (D_2O, 300 MHz): δ 2.10 (s, 3H, CH_3), 4.14 (s, 2H), 4.19 (s, 1H),4.35 (s, 1H),4.46 (t, *J* = 4.5 Hz, 1H), 5.75 (d, *J* = 4.3 Hz, 1H), 5.89 (s, 1H), 6.8 (s, 1H), 6.92 (d, *J* = 8.6 Hz, 1H), 7.27 (d, *J* = 8.6 Hz, 1H), 7.88 (s, 1H), 8.15 (s, 1H). ^{13}C-NMR (D_2O, 75 MHz): δ 17.8, 65.0 (d, *J* = 5.2 Hz), 70.1, 74.5, 83.5 (d, *J* = 9.2 Hz), 86.9, 107.9, 108.0, 111.5, 115.6, 117.4 (d, *J* = 4.5 Hz), 117.6, 125.9, 140.0, 147.8, 149.8, 152.8, 154.3 (d, *J* = 6.6 Hz), 155.5, 163.7. ^{31}P-NMR (D_2O, 121 MHz): δ -9.02 (d, *J* = 16.5 Hz), -14.52 (d, *J* = 14.4 Hz), -21.02 (m). HRMS (ESI-): *m/z* = 743.99319, $C_{20}H_{22}N_5O_{18}P_4$ $[M - H]^-$, requires 743.99158.

4.5.11 General method for the preparation of δ-(4-methyl-7-coumarinyl) nucleoside 5´-tetraphosphate tetraammonium salts (4-49, and 4-51 – 4-54) via intermediate 4-50 (Scheme 4.19).

To a mixture of **4-34** (0.54 mmol, 520 mg) and DABCO (1.62 mmol, 180 mg) in dry acetonitrile (6 mL) was added mesitylenesulfonyl chloride (0.48 mmol, 106 mg). The mixture was stirred at room temperature for one minute then 7-hydroxy-4-methylcoumarin (HMC, 0.27 mmol, 48 mg) was added and the reaction mixture was allowed to stir at room temperature for 2 h. A white turbidity appears after about 45 min. The reaction mixture was then withdrawn by a syringe and injected dropwise over one min into a cooled (ice bath) solution of NMP or 2´-dNMP (0.675 mmol) and anhydrous magnesium chloride (0.42 mmol, 40 mg) in DMF (7.5 mL). The ice bath was removed and the reaction mixture was allowed to stir at room temperature for 3 h. The turbidity disappears except in the case of 2´-dCMP or 2´-dGMP. The reaction mixture was then cooled in an ice bath and quenched by adding triethylammonium acetate buffer (pH 7.0, 8 mL). The solution was washed with chloroform (3 x 10 mL). Chelex resin (ca. 0.1 g) was added and the mixture stirred for 1 min then filtered through a cotton plug. The filtrate was

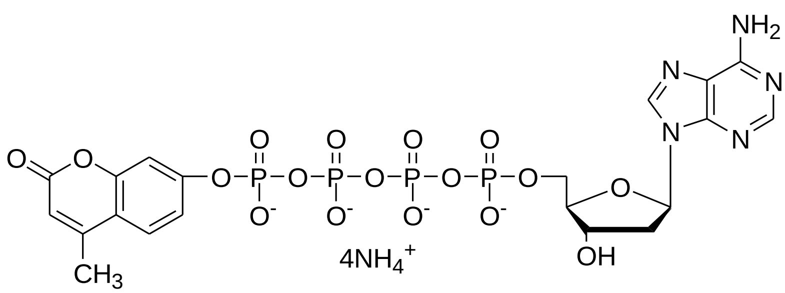
purified by reversed-phase chromatography using a linear gradient of 100% buffer A (10 mM tributylamine-30 mM acetic acid), 0% Buffer B (15 mM tributylamine in MeOH) to 40% buffer A, 60% buffer B over 55 min. The flow rate was 25 mL/min and eluate was monitored at 265 and 280 nm. Fractions containing the desired product were pooled, concentrated by high vacuum rotary evaporation, and the residue was dissolved in water and repeatedly freeze-dried until the ^{1}H-NMR spectrum indicated that no residual buffer was present (four times). The resulting white powder was converted to its ammonium salt using a Dowex-50-W ion-exchange resin in NH_4^+ form.

4.5.12 Characterization data for δ-(4-methyl-7-coumarinyl) nucleoside 5´-tetraphosphates 4-49 and 4-51 – 4-54.

δ-(4-methyl-7-coumarinyl) adenosine 5´-tetraphosphate, tetraammonium salt (4-49).

Compound **4-49** was obtained in 80% yield as its tetraammonium salt (176 mg). Characterization data were identical to that reported above when prepared via intermediate **4-48**.

δ-(4-methyl-7-coumarinyl) 2´-deoxyadenosine 5´-tetraphosphate, tetraammonium salt (4-51).

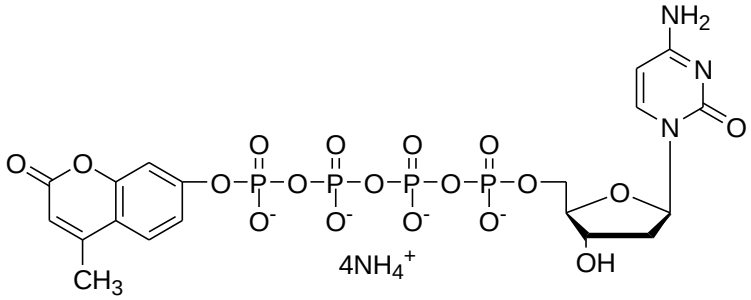


4-51

Compound **4-51** was obtained in 80% (172 mg) yield as its tetraammonium salt. t_r = 32 min. ^{1}H-NMR (D_2O, 300 MHz): δ 2.05 (s, 3H), 2.31-2.51 (m, 2H), 4.01-4.08 (m, 3H), 4.56 (s, 1H), 5.83 (s, 1H), 6.05 (t, *J* = 6.8 Hz, 1H), 6.82 (s, 1H), 6.94 (dd, *J* = 9.0, 1.8 Hz, 1H), 7.25 (d, *J*

= 9.0 Hz, 1H),7.79 (s, 1H), 8.07 (s, 1H). ^{13}C-NMR (D_2O, 75 MHz): δ 17.8, 39.2, 65.3 (d, *J* = 5.4 Hz), 71.0, 83.5, 85.4 (d, *J* = 8.9 Hz), 107. 8, 107.9, 111.4, 115.5, 117.4 (d, *J* = 4.5 Hz), 125.9, 139.6, 147.4, 150.5, 152.6, 153.4, 154.2 (d, *J* = 6.8 Hz), 155.3, 163.5. ^{31}P-NMR (D_2O, 121 MHz): δ -9.06 (d, *J* = 15.7 Hz), -14.77 (d, *J* = 12.8 Hz), -21.02 (s). HRMS (ESI-): *m/z* = 727.99745, $C_{20}H_{22}N_5O_{17}P_4$ $[M - H]^-$, requires 727.99666.

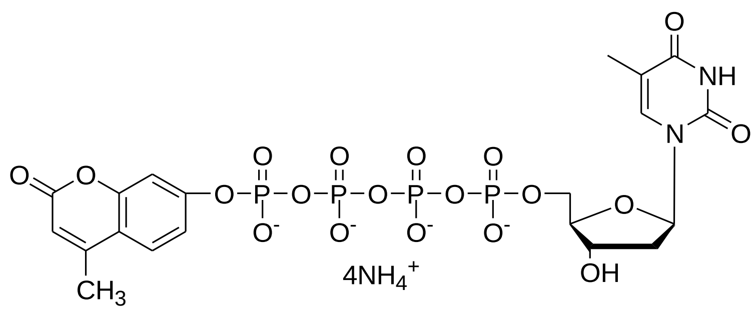
δ-(4-methyl-7-coumarinyl) 2´-deoxy-cytosine 5´-tetraphosphate, tetraammonium´ salt (4-52).



4-52

Compound **4-52** was obtained in 81% (169 mg) yield as its tetraammonium salt. t_r = 31 min. ^{1}H-NMR (D_2O, 300 MHz): δ 2.01 (m, 1H), 2.18 (m, 1H), 2.24 (s, 3H), 3.98 (br, 3H), 4.38 (s, 1H), 5.88 (br, 2H), 6.06 (s, 1H), 7.06 (br, 2H), 7.52 (d, *J* = 7.6 Hz, 1H), 7.70 (d, *J* = 6.0 Hz, 1H). ^{13}C-NMR (D_2O, 75 MHz): δ 17.9, 39.4, 65.1 (d, *J* = 5.2 Hz), 70.5, 85.6 (d, *J* = 9.3 Hz), 86.0, 95.4, 108.2 (d, *J* = 5.1 Hz), 111.8, 116.1, 117.6 (d, *J* = 5.0 Hz), 126.4, 142.8, 150.6, 153.2, 154.6 (d, *J* = 6.8 Hz), 155.8, 160.6, 164.0. ^{31}P-NMR (D_2O, 121 MHz): δ -8.96 (d, *J* = 9.8 Hz), -14.77 (d, *J* = 10.6 Hz), -20.94 (s). HRMS (ESI-): *m/z* = 703.98597, $C_{19}H_{22}N_3O_{18}P_4$ $[M - H]^-$, requires 703.98543.

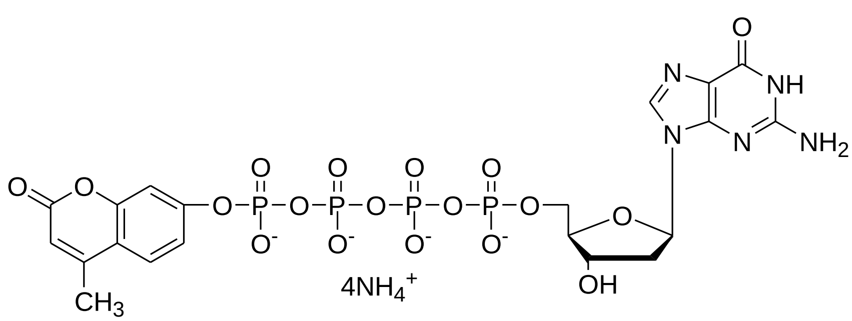
δ-(4-methyl-7-coumarinyl) 2´-deoxythymidine 5´-tetraphosphate, tetraammonium salt (4-53).



5-53

Compound **5-53** was obtained in 82% (174 mg) yield as its tetraammonium salt. t_r = 34 min. ^{1}H-NMR (D_2O, 300 MHz): δ 1.64 (s, 3H), 2.07-2.13 (m, 2H), 2.27 (s, 3H), 3.96 (s, 1H), 4.04 (brs, 2H), 4.43 (brs, 1H), 6.01 (t, 1H), 6.09 (s, 1H), 7.12 (br, 2H), 7.38 (s, 1H), 7.55 (d, *J* = 6.0 Hz, 1H). ^{13}C-NMR (D_2O, 75 MHz): δ 11.5, 17.8, 39.5, 65.3 (d, *J* = 5.5 Hz), 70.7, 84.6 85.0 (d, *J* = 9.2 Hz), 108.1 (d, *J* = 5.2 Hz), 111.2, 111.7, 116.0, 117.6 (d, *J* = 5.0 Hz), 126.3, 136.8, 151.1, 153.2, 154.6 (d, *J* = 6.9 Hz), 155.8, 163.9, 165.8. ^{31}P-NMR (D_2O, 121 MHz): δ -9.26 (d, *J* = 15.9 Hz), -14.78 (d, *J* = 16.3 Hz), -21.09 (s). HRMS (ESI-): *m/z* = 718.98522, $C_{20}H_{23}N_2\ O_{19}P_4$ $[M - H]^-$, requires 718.98510.

δ-(4-methyl-7-coumarinyl) 2´-deoxy-guanosine 5´-tetraphosphate tetraammonium salt (4-54)



4-54

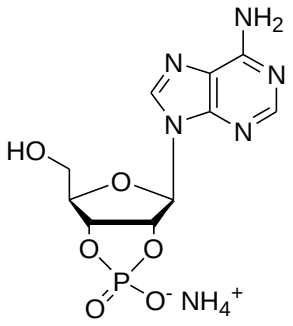
Compound **4-54** was obtained in 82% (180 mg) yield as its tetraammonium salt. t_r = 36 min. ^{1}H-NMR (D_2O, 300 MHz): δ 2.10 (s, 3H), 2.34 (m, 1H), 2.42 (m, 1H), 4.06 (m, 3H), 4.54 (s, 1H), 5.90 (brs, 2H), 6.90 (s, 1H), 7.00 (d, *J* = 8.6, 1H), 7.33 (d, *J* = 8.6 Hz, 1H), 7.99 (s, 1H). ^{13}C-NMR (D_2O, 75 MHz): δ 17.7, 39.0, 65.2 (d, *J* = 5.4 Hz), 70.8, 83.9, 85.4 (d, *J* = 9.0 Hz), 107. 8 (d, *J* = 5.5 Hz), 111.4, 113.0, 117.3 (d, *J* = 4.5 Hz), 126.0, 136.3, 149.7, 152.8, 153.5, 154.3 (d, *J* = 6.8 Hz), 155.4, 156.6, 163.5. ^{31}P-NMR (D_2O, 121 MHz): δ -9.05 (d, *J* = 15.3 Hz), -14.77 (d, *J* = 13.9 Hz), -21.03 (s). HRMS (ESI-): *m/z* = 743.99260, $C_{20}H_{22}N_5O_{18}P_4$ $[M - H]^-$, requires 743.99158.

4.5.13 General procedure for the synthesis of 2',3'-cyclic nucleoside monophosphates (4-7 and 4-70 – 4-72)

To a mixture of **4-34** (0.405 mmol, 390 mg) and NMI (1.35 mmol, 108 µL) in 4.5 mL acetonitrile added mesitylenesulfonyl chloride (0.405 mmol, 88 mg) at room temperature. White turbidity appears after ~2 min. The mixture was stirred at room temperature for 15 min. then 4-nitroimidazole (0.81 mmol, 92 mg was added followed by DBU (1.62 mmol, 240 µL) added dropwise over 2 min. the solution become clear yellowish. Nucleoside (0.27 mmol) was added and the reaction mixture was stirred for further 1-2 h where the nucleoside dissolves. Anhydrous magnesium chloride (0.405 mmol, 38 mg) was then added to the stirring reaction mixture where a white turbidity starts appearing and increased by time. The reaction mixture was allowed to stand for 24 h. The reaction mixture was cooled in ice-bath and quenched with 6 mL 500 mM ammonium acetate buffer (pH 7.0), washed with chloroform (3 x 10 mL) and filtered through cotton and purified by RP-Biotage 12 g column at flow rate of 12 mL per min. using a gradient of buffer A (10 mM tributylamine-30 mM acetic acid in ddH_2O) and buffer B (15 mM tributylamine in methanol). Fractions were monitored under *uv* at 265 and 280 nm. Fractions containing the desired compound were pooled and, concentrated by high vacuum rotary

evaporation, and the residue was dissolved in water and repeatedly freeze-dried until the ^{1}H-NMR spectrum indicated that no residual buffer was present (four times). The resulting white powder was converted to its ammonium salt using a Dowex-50-W ion-exchange resin in NH_4^+ form.

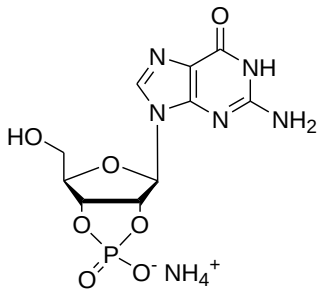
2',3'-cyclic adenosine monophosphate ammonium salt (4-7)



4-7

Compound **4-7** was obtained in 72% yield as ammonium salt (67 mg) using a gradient of 100 Buffer A to 60% Buffer A and 40% Buffer B over 30 min, t_r = 15 min. ^{1}H-NMR (D_2O, 300 MHz): δ 3.73-3.77 (m, 2H, $H_{5'}$), 4.32 (d, 1H, *J* = 3.3 Hz, $H_{4'}$), 4.99 (m, 1H, $H_{3'}$), 5.26 (m, 2H, $H_{2'}$), 6.10 (d, *J* = 3.9 Hz, $H_{1'}$), 8.04 (s, 1H, H_2), 8.16 (s, 1H, H_8). ^{13}C NMR (D_2O, 75 MHz): δ 60.9, 77.3, 80.3, 85.1 (d, *J* = 3.9 Hz), 89.2 (d, *J* = 3.5 Hz), 118.3, 140.9, 147.7, 150.4, 153.6. ^{31}P NMR (D_2O, 121 MHz): δ 22.92 (s). HRMS (ESI-): *m/z* = 328.04554, $C_{10}H_{11}N_5O_6P$ $[M - H]^-$, requires 328.04524.

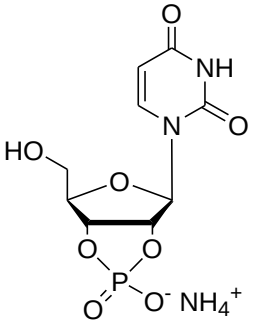
2',3'-cyclic guanosine monophosphate ammonium salt (4-70)



4-70

Compound **4-70** was obtained in 74% yield as ammonium salt (72 mg) using a gradient of 100 Buffer A to 65% Buffer A and 35% Buffer B over 30 min, t_r = 12 min. ^{1}H-NMR (D_2O, 300 MHz): δ 3.70-3.74 (m, 2H, $H_{5'}$), 4.26 (brd, 1H, *J* = 4.4 Hz, $H_{4'}$), 4.97 (m, 1H, $H_{3'}$), 5.24 (m, 2H, $H_{2'}$), 5.95 (d, *J* = 3.5 Hz, $H_{1'}$), 7.86 (s, 1H, H_8). ^{13}C NMR (D_2O, 75 MHz): δ 60.8, 77.3, 80.3 (d, *J* = 1.8 Hz), 85.0 (d, *J* = 3.1 Hz), 88.7 (d, *J* = 4.6 Hz), 116.2, 138.0, 150.9, 153.5, 158.6. ^{31}P NMR (D_2O, 121 MHz): δ 22.47 (s). HRMS (ESI-): *m/z* = 344.04040, $C_{10}H_{11}N_5O_7P$ $[M - H]^-$, requires 344.04016.

2',3'-cyclic uridine monophosphate ammonium salt (4-71)

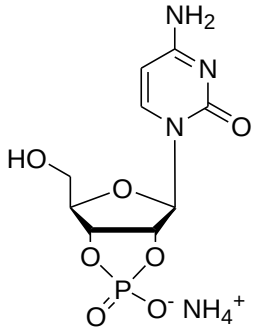


4-71

Compound **4-71** was obtained in 70% yield as ammonium salt (61 mg) using a gradient of 100 Buffer A to 90% Buffer A and 10% Buffer B over 20 min, t_r = 16 min. ^{1}H-NMR (D_2O, 300 MHz): δ 3.67-3.80 (m, 2H, $H_{5'}$), 4.16 (brd, 1H, *J* = 4.1 Hz, $H_{4'}$), 4.76 (m, 1H, $H_{3'}$), 5.00 (m,

2H, $H_{2'}$), 5.71 (d, *J* = 8.0 Hz, H_5), 5.76 (d, *J* = 2.1 Hz, $H_{1'}$), 7.57 (d, *J* = 8.0 Hz, H_6). ^{13}C NMR (D_2O, 75 MHz): δ 60.7, 76.9, 80.4 (d, *J* = 2.2 Hz), 84.9 (d, *J* = 2.0 Hz), 92.5 (d, *J* = 6.0 Hz), 101.9, 143.6, 151.0, 166.1. ^{31}P NMR (D_2O, 121 MHz): δ 22.54 (s). HRMS (ESI-): *m/z* = 305.01827, $C_9H_{10}N_2O_8P$ [M - H]$^-$, requires 305.01802.

2',3'-cyclic cytosine monophosphate ammonium salt (4-72)



4-72

Compound **4-72** was obtained in 64% yield as ammonium salt (56 mg) using a gradient of 100 Buffer A to 95% Buffer A and 5% Buffer B over 20 min, t_r = 10 min. ^{1}H-NMR (D_2O, 300 MHz): δ 3.72-3.79 (m, 2H, $H_{5'}$), 4.22 (brd, 1H, *J* = 3.0 Hz, $H_{4'}$), 4.82 (m, 1H, $H_{3'}$), 5.02 (m, 2H, $H_{2'}$), 5.76 (d, *J* = 2.0 Hz, $H_{1'}$), 5.98 (d, *J* = 8.0 Hz, H_5), 7.68 (d, *J* = 8.0 Hz, H_6). ^{13}C NMR (D_2O, 75 MHz): δ 60.8, 77.1, 80.8 (d, *J* = 2.3 Hz), 85.3 (d, *J* = 2.0 Hz), 93.6 (d, *J* = 6.0 Hz), 144.7, 152.9, 163.2. ^{31}P NMR (D_2O, 121 MHz): δ 22.53 (s). HRMS (ESI-): *m/z* = 304.03421, $C_9H_{11}N_3O_7P$ [M - H]$^-$, requires 304.03401.

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