Applications of Ionic Liquids as Tags in Oligonucleotide Synthesis

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Dedicated to Annick, my wife, without you there is no meaning, and also to Gabriel, my son, you have shown me the future is still bright and full of discovery.

"…though we are not now that strength

which in old days moved earth and heaven;

that which we are, we are;

One equal temper of heroic hearts,

made weak by time and fate, but strong in will

To strive, to seek, to find, and not to yield."

Ulysses, *Alfred Lord Tennyson*, 1842

"Life is what happens to you while you're busy making

other plans."

Beautiful Boy, *John Lennon*, 1980

Copyright statement

Abstract

As oligonucleotide based therapeutics approach mainstream use, a need is being generated for alternative synthetic routes to that of the commonly used solid-phase synthesis approach. Solid-phase synthesis of oligonucleotides is unsurpassed in its ability to generate oligonucleotides simply and rapidly in small scale but the difficulty and cost using this technique to supply the potentially ton scale amounts that would be required may be prohibitive.

This thesis discusses the use of a soluble support based on ionic liquids to address this problem. To that effect, an ionic liquid supported synthesis (ILSS) methodology was developed and demonstrated to be applicable to both DNA and RNA oligonucleotides. Using the ILSS approach decamers of high quality and purity have been synthesized linearly in solution. It was found, however, that the coupling efficiency begins to decrease with increasing oligomer length.

An alternative approach to linear synthesis is the use of block condensations. To this end, a novel ionic tag was synthesized that incorporated a linker that was designed to be cleaved orthogonally to all other protecting groups used in oligonucleotide assembly. This tag was demonstrated to be easily derivatized to both DNA and RNA nucleosides and just as easily released upon treatment with buffered hydrazine.

In order to demonstrate the versatility of the ILSS approach, a series of analogues to RNA-X, a molecule having an unusually stable motif of a free 2'-hydroxyl vicinal to a phosphotriester, were synthesised. This synthesis was greatly simplified by the use of the ILSS technique.

Abrégé

Alors que les thérapeutiques basées sur les oligonucleotides frôlent l'utilisation courante, un besoin se crée pour des méthodes synthétiques alternatives afin de remplacer l'approche de synthèse en phase solide habituellement utilisée. La synthèse en phase solide des oligonucléotides n’est pas surpassée par sa capacité de produire des oligonucléotides simplement et rapidement dans des petites quantités, mais la difficulté et le coût d'utiliser cette technique pour fournir une quantité en tonnes qui serait potentiellement requise risque d'être inabordable.

Cette thèse discute de l'utilisation d'un support soluble basé sur des liquides ioniques pour adresser ce problème. À cet effet, une méthodologie de synthèse supportée par un liquide ionique (ILSS) a été développée et a démontré qu'elle pouvait s'appliquer aux oligonucléotides d'ADN et d'ARN. En utilisant l'approche ILSS, les decamers de haute qualité et de haute pureté ont été synthétisés en solution de façon linéaire. Il a cependant été constaté que l'efficacité d'accouplement diminue avec l'augmentation de la longueur de l'oligomère.

Une approche alternative à la synthèse linéaire est l'utilisation des condensations de blocs oligomeriques. À cet effet, un support ionique original a été synthétisé incorporant une attache qui a été conçue pour être enlevée orthogonalement à tous les autres groupes protecteurs utilisés pour l'assemblage d'oligonucléotides. Il a été démontré que ce support est facilement dérivatisé aux

nucléosides d'ADN et d'ARN ainsi que facilement enlevé par un traitement d'hydrazine tamponnée.

Afin de démontrer la polyvalence de l'approche ILSS, une série d'analogues à RNA-X, une molécule ayant un motif inhabituellement stable d'un 2’-hydroxyle libre vicinal à un phosphotriester, a été synthétisée. Cette synthèse a été considérablement simplifiée en utilisant la technique ILSS.

Acknowledgements

First, I would like to express my gratitude to Dr. Masad Damha for his patience with me and allowing me to do research in his lab. He has allowed me to grow as a scientist and develop a myriad of skills and expertise by allowing me the freedom to perform research at my own (sometimes slow) pace. He has always been a source of encouragement when I needed it the most. I would also like to thank Dr. Tak-Hang Chan, from whom the original ILSS project stems, for his help and support.

I have met and worked with a large group of very talented people in my years at McGill whom I would wish to thank: Kazim, Sandra, Mohamed, Katya, you guys got me started and were always helpful when I asked. Anne and Chris are great people to talk to about the early years of the Damha lab and always good for a laugh. Dilip is always helpful with complicated synthetic problems and a great source of commiseration about the vagaries of chemistry. Adam and Glen are both good sports, always eager to pitch in if needed, just don't get in their way if there's a fire alarm. Jeremy, David, and Alex were great to talk to about all thing hockey and Jer's a great sport about our teasing him about his team losing (constantly). Matt's always good for a chat about how republicans are destroying the world and I have never met anyone who is more cheerful, even in the face of impending doom. Julia and Nuria, our Catalan connection were great people to get to know, always with a great outlook on life and great for introducing new cuisine (at least new to me), which is always appreciated. Signe's a good friend who gave me a early glimpse into life as a parenthood,

though the glimpse still didn't prepare me for the experience. Jon and Siara are both good eggs, real friends who I wish were a lot closer so I could spend some more time with them. They were both always ready for the deeper questions into the meaning of life, the universe and everything, and were always good for a laugh, bad puns and rolled oats aside. Pascal and Richard are great guys to hang around with over a coffee and always good for extensive chemistry conversions, they are always appreciated, especially for editing this thesis and giving helpful suggestions to fix synthesis problems.

The people who keep the department running have my deep gratitude, Fred Kluck and Rick Rossi, who could fix any instrument, even after it's been nuked, and Nadim Saade and Fred Morin, who keep the specs running in spite of the carelessness of grad students hopped up on caffeine in the middle of the night. The ladies in the office, especially Colleen Macnamee (in our building), and Fay Nurse and Sandra Aerssen (in Otto Maass), who prevent implosion every day, without most of the rest of us even noticing; I hope you know how much we value you. And of course sincere thanks to Chantal Marrote, without whom most of us (graduate students) would be lost in wilderness somewhere whenever we have to deal with the rest of the university.

Finally, I need to thank my family, especially my Mom, for always encouraging me in the pursuit my career and for so much more that it would never be possible to write it all down. We are who we are mostly because of our families and I like who I am, by and large, so that's thanks to them. The last word I saved to thank Annick, to whom this thesis is dedicated, there are no words to

describe how much I love you and how lucky I feel that I get to grow old next to you. None of this would have been possible without you Minou.

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Abbreviations

% v/v	volume-volume percentage
2'F-RNA	2'-deoxy-2'-fluororibonucleic acid
A	adenosine
Å	Angstrom
Ac	acetyl
Ade	adenine
AIL	4-(1*H*-3-methylimidazol-1-yl-tetrafluoroboro)butanoic acid
Ar	aryl
B	base
BMT	5-benzylthio-1*H*-tetrazole
Bn	benzyl
br.	broad
Bz	benzoyl
C	cytidine
CE	2-cyanoethyl
CIGAR	constant time inverse-detected gradient accordion scaled long range heteronuclear multiple-bond correlation
COSY	correlation spectroscopy, homonuclear (NMR)
CPG	controlled pore glass
Cyt	cytosine
d	doublet
DCC	dicyclohexylcarbodiimide
DCI	4,5-dicyanoimidazole
DCU	dicyclohexylurea

dd	doublet of doublets
DIAD	diisopropylazodicarboxylate
DIPEA	*N,N*-diisopropylethylamine
DMAP	4-dimethylaminopyridine
dmf	dimethylformamidine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DMT	4,4'-dimethoxytrityl
DNA	2'-deoxyribonucleic acid
dt	doublet of triplets
E. coli	*Escherichia coli*
ESI-MS	electrospray ionization mass spectrometry
Et	ethyl
EtOAc	ethyl acetate
EtOH	ethanol
ETT	5-ethylthio-1*H*-tetrazole
FG	functional group
G	guanosine
Gua	guanine
HMQC	heteronuclear multiple quantum correlation spectroscopy (NMR)
HPLC	high performance (or high pressure) liquid chromatography
HR-MS	High resolution mass spectrometry
IL	ionic liquid
ILSS	ionic liquid supported synthesis
*i*Pr	2-propyl
IS	internal standard

J	scalar coupling constant (in Hz)
LC	liquid chromatography
LR-MS	low resolution mass spectrometry
Lv	levulinyl
M	molarity
m/z	mass to charge ratio
Me	methyl
MeCN	acetonitrile
MeOH	methanol
mmol	millimoles
MMT	4-monomethoxytrityl
mRNA	messenger RNA
mRNA	messenger RNA
MTBE	methyl-*t*-butyl ether
NMI	1-methyl-imidazole
NMR	nuclear magnetic resonance spectroscopy
NOE	nuclear Overhauser enhancement
NOESY	nuclear Overhauser effect spectroscopy (a 2D NOE experiment)
oNO_2Bn	2-nitrobenzyl
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
ppm	parts per million
*p*TSA	*p*-toluenesulfonic acid
Pu	purine
Py	pyrimidine
r.t.	room temperature

R_f	retention factor (in TLC, the ratio of the distance traveled by the center of a spot to the distance from the baseline to the solvent front)
RISC	RNA-induced silencing complex, the effector complex of RNA interference
rN	ribonucleoside
RNA	ribonucleic acid
RNAi	RNA interference
RNase H	ribonuclease H
RP	Reverse Phase
s	singlet
SELEX	systematic evolution of ligands by exponential enrichment
siRNA	small interfering RNA
snRNA	small nuclear ribonucleoprotein
ss	single-stranded
T	Thymidine
t	triplet
TBAF	tetrabutylammonium fluoride
TBDMS	*t*-butyldimethylsilyl
TBDPS	*t*-butyldiphenylsilyl
TBTU	*O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate
TCA	trichloroacetic acid
TEA	triethylamine
TEAA	triethylammonium acetate
TET	1*H*-tetrazole
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol

THF	tetrahydrofuran
Thy	thymine
TIPS	triisopropylsilyl
TLC	thin-layer chromatography
TREAT HF	triethylamine trihydrofluoride
tRNA	transfer RNA
U	uridine
Ura	uracil
UV	ultraviolet (spectroscopy)

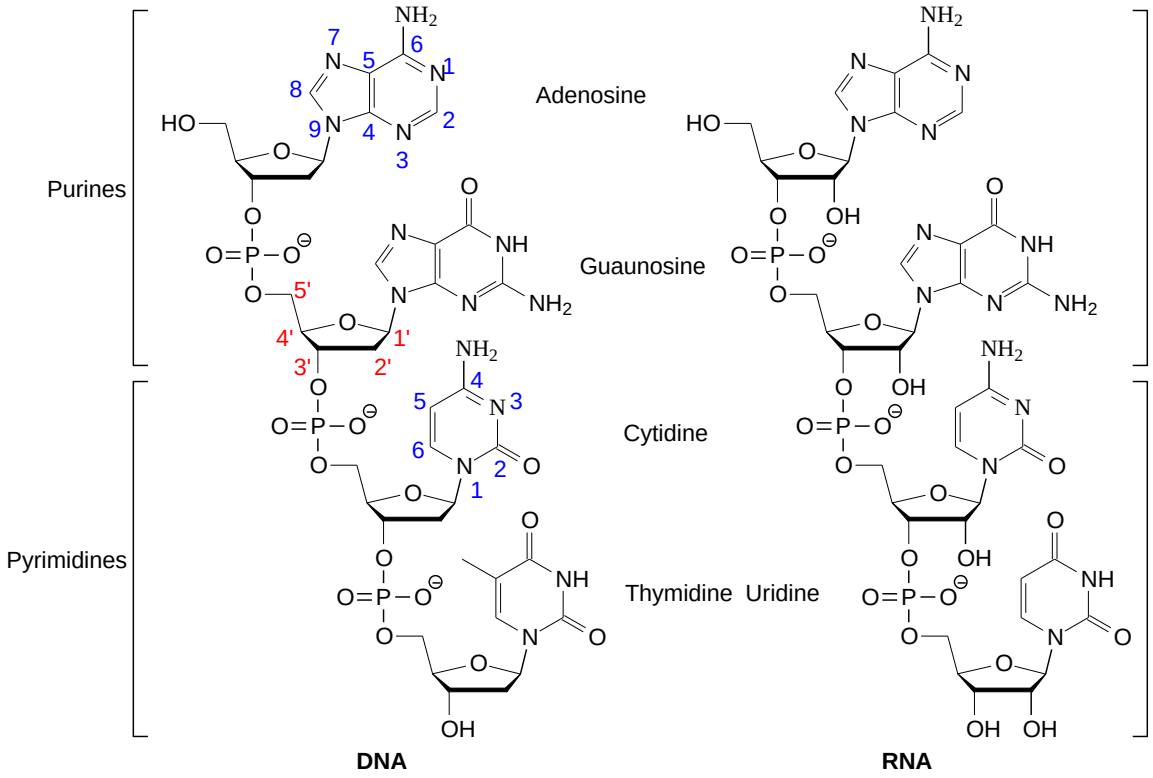
Chapter 1 Introduction

Since the days of Darwin (1809-1882) and Mendel (1822-1884), scientists have been trying to understand and manipulate hereditary traits in the offspring of living organisms. Mendel's original concept of a hereditary unit was eventually given a name in 1909 by the Danish botanist Wilhelm who coined the term 'gene', though an understanding of the chemical nature of genes would wait until Hershey and Chase showed the link between genes and DNA in their study of bacteriophage T4.[1] As it turned out, this link had been proposed but not proven by Avery, MacLeod and McCarty in 1944 in their study of *Streptococcus pneumonia*.[2] Once the chemical identity of genetic material was known and the physical structure of DNA was determined in 1952 by Watson, Crick, Wilkins, and Franklin[3], the study of the synthesis, uses and functions of nucleic acids began in earnest.

1.1 Nucleic acids

DNA and RNA units (nucleotides) are composed of three principle moieties, namely a nucleobase, a sugar, and a linking phosphate. The most common nucleobases (Figure 1-1) are the 5-member, 6-member fused ring heterocyclic purines, adenine and guanine, and the 6-member heterocyclic pyrimidine cytosine, which are all common to both DNA and RNA, while the remaining pyrimidines, thymine and uracil, also 6-member heterocycles, are specific to DNA

Figure 1-1: Structures of DNA and RNA



or RNA, respectively. The natural sugar moieties are the pentoses deoxyribose for DNA and ribose for RNA and the phosphate links the adjacent units through their 3' and 5'-oxygens as phosphodiesters. Figure 1-1 shows the names for the common RNA nucleotides, while the names of the DNA nucleotides are preceded by 2'-deoxy, as in 2'-deoxyadenosine, with the exception of thymidine, which stands alone. The position numbers for atoms within the sugar rings (indicated in red), by convention, are distinguished from the position numbers of the nucleobases (indicated in blue) by adding a prime symbol after them.

Individual nucleotides are linked together as repeating units to form nucleic acid polymers. Within biological systems, these polymers vary in size from a few units to several million units in length. DNA generally exists as pairs of polymers

held together by a series of hydrogen bonds between the individual strands. The pattern of hydrogen bonding is very specific, with the bases of each nucleotide unit associating almost exclusively with their complements, guanine with cytosine, and adenine with thymine. These pairs of strands arrange themselves in anti-parallel orientations, adopting one of several possible helical conformations, depending on hydration and salt concentration, which affects the conformation (pucker) of the individual sugar units within the strands as well as the distance between adjacent charges of the phosphate backbone, both inter- and intra-molecularly. The helical structure elucidated by Francis and Crick is termed the B-form and is the most common form of DNA-DNA duplexes. The characteristics of the various helices are summarized in Table 1-1.

Table 1-1: Features of major helix forms[4]

	A form	**B form**	**Z form**
Helical Sense	Right handed	Right handed	Left handed
Diameter	26 Å	20 Å	18 Å
Bases per turn	11	10	12
Rise per turn	28 Å	34 Å	45 Å
Base tilt (normal to axis)	20°	6°	7°
Major Groove	Narrow and deep	Wide and deep	Flat
Minor Groove	Wide and shallow	Narrow and deep	Narrow and deep
Sugar Pucker	C3'-*endo*	C2'-*endo*	C3'-*endo* for purines C2'-*endo for pyrimidines*

In addition to self-association, DNA is capable of forming a base-paired double helix with RNA, where uracil, within uridine, replaces thymine as the partner of adenine in base pairing. RNA is in turn capable of self-association,

though it is more often found as a single stranded species. The RNA-RNA double helix is generally of the A-form, while DNA-RNA duplexes locate somewhat between A-form and B-form.

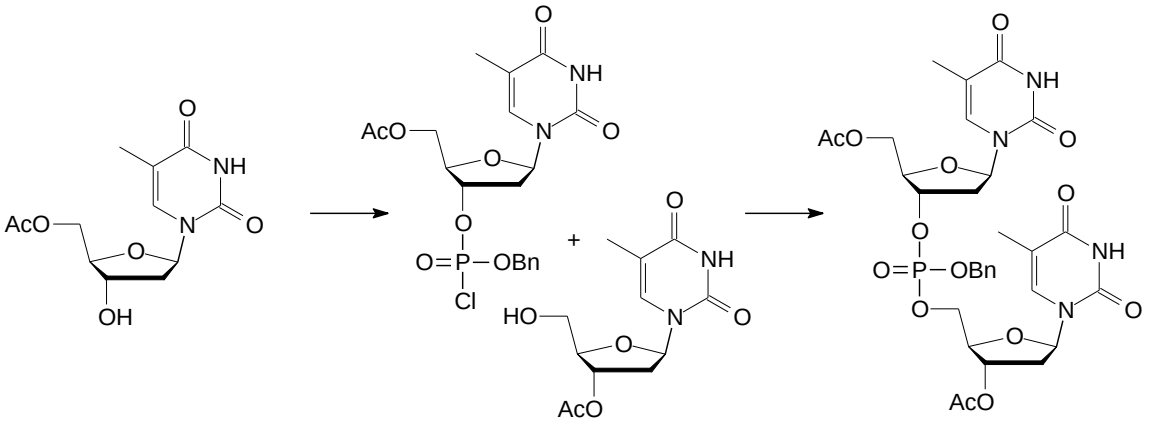
As with proteins, the order of constituent units in a sequence (nucleotides in DNA or RNA) is referred to as the primary structure while the local conformation assumed by these sequences due to base pairing is termed the secondary structure. Nucleic acids are capable of adopting larger defined global conformations as well, called tertiary structures, often in conjunction with proteins or in the presence of metal ions, which are capable of performing higher functions such as substrate recognition, as for tRNA, or the folding of DNA in chromatin to affect gene expression. This diversity of structure and function has engendered a great deal of interest into these molecules and their chemical synthesis has been a major undertaking, in order to study simplified systems.

1.2 Early years

Sir Alexander R. Todd, one of the pioneers in DNA and RNA synthesis, began his work in the late 1940s, his interest likely stemming from his work on the chemistry of group B vitamins.[5] In 1952 he demonstrated that alkaline hydrolysis of RNA resulted in a series of products and the nature of those products, in light of the data obtained to that point in various degradation studies using enzymes, could only be explained if the backbone of the oligonucleotides were composed exclusively of 2'-5' or 3'-5' linkages.[6]

Given that it had been shown that DNA could only be composed of exclusively 3'-5' linkages[7] and subsequent work performed in the Todd laboratory[8-10] it was further concluded that RNA oligomers were likewise linear molecules linked through 3'-5' phosphodiesters, without any phosphotriester structures present, thus supplying further evidence on which Watson and Crick could build their famous DNA model.

In 1955 the Todd group reported the first successful chemical synthesis of a DNA dimer, using a procedure that eventually became known as the phosphotriester method.[11] The work was undertaken to help provide more evidence of the proposed structures of DNA that had been determined from biological samples. By chemically synthesizing a dinucleotide in an unambiguous manner and demonstrating that it behaved in exactly the same way as the biologically obtained material, toward both chemical and enzymatic degradation, unequivocal evidence was provided for the proposed structures.

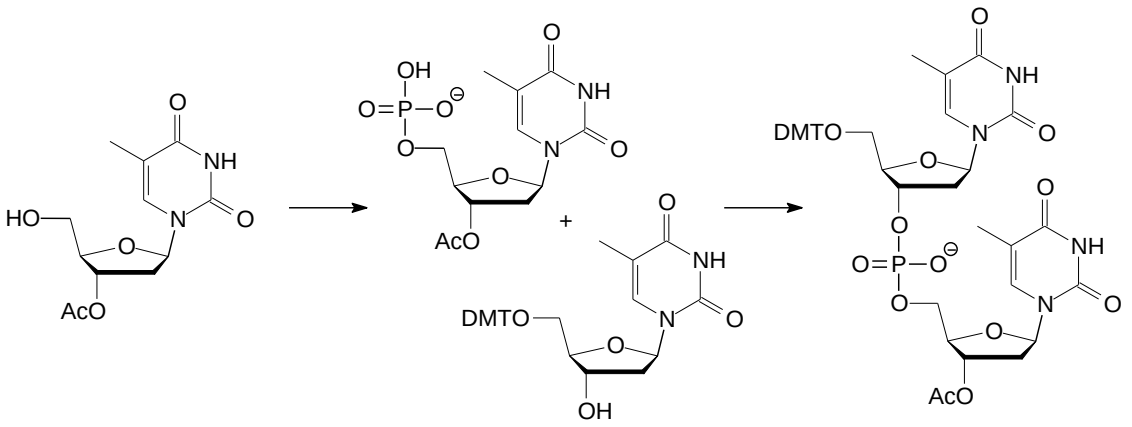


Scheme 1-1: First chemical synthesis of an oligonucleotide

The synthesis method (Scheme 1-1) involved the condensation of 5'-acetyl or 5'-phosphate (dibenzyl ester) thymidine with *O*-monobenzyl-*O*,*O*-diphenyl-pyrophosphate followed by *N*-chlorosuccinamide, to yield the corresponding 3'-phosphorochloridate, which was then further condensed with 3'-acetyl thymidine. The resulting dinucleotides were then deacetylated and, in the case of the 5'-dibenzyl phosphotriester (not shown), subjected to hydrogenolysis, to yield TpT and pTpT respectively. Alkaline and enzymatic treatments of the purified, deprotected dinucleotides behaved exactly the same as biologically derived materials, thus supporting their proposed structure. Though the authors proposed a way of using their method for synthesizing longer oligomers, it wouldn't be until the latter half of the 1960s that the phosphotriester approach would again be used.[5]

1.3 Phosphodiester approach

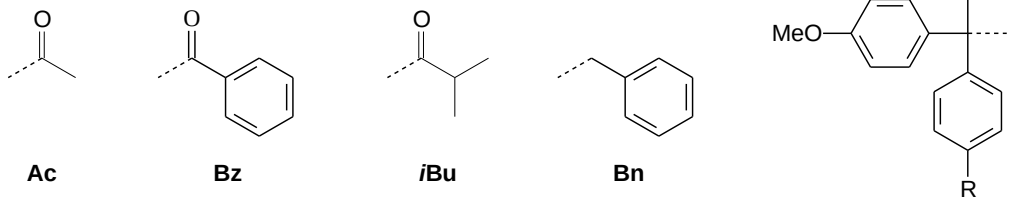
In the intervening time, the center of research moved from Sir Todd's laboratory to that of H.G. Khorana.[5] Khorana's main synthetic approach, known



Scheme 1-2: Phosphodiester approach

as the phosphodiester method, involved the condensation of an appropriately protected nucleoside with a nucleotide (or oligonucleotide) phosphomonoester to result in the formation of a new dinucleotide phosphodiester linkage (Scheme 1-2).[12,13]

Unlike Todd's approach, the resultant linkage was unprotected and this led to the formation of many side-products. Khorana felt, however, that the gains made in the presence of the charged phosphate, which could be used as a purification tool, outweighed the disadvantages of side-reaction and decreased yield, which could be mitigated through careful experimental design.[14] Indeed, Khorana showed the power of this synthetic approach by synthesizing two DNA duplexes entirely in solution, corresponding to the genes for yeast alanine transfer RNA (tRNA)[15] and tyrosine suppressor tRNA,[16] 77 and 126 base pairs in length, respectively, a huge achievement at the time (for further discussion see Section 3.1).



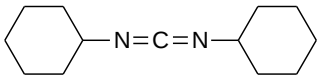
Ac **Bz** ***i*Bu** **Bn**

MMT: R = H
DMT: R = OMe

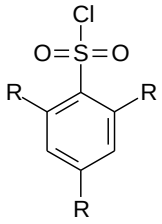
During this time, Khorana and co-workers began the development of protecting group strategies for the moieties not participating in the formation of the phosphate linkage, namely the exocyclic amines of the nucleobases and the

other hydroxyl functions of the sugars.[17,18] The base protecting groups employed at that time are still the most common in use today, acetyl or benzoyl amides (**Ac**, **Bz**) for adenosine and cytidine and isobutryl amide (***i*Bu**) for guanosine, while thymidine, and later uridine, were left unprotected at the nucleobase.

One of the most important innovations made was the introduction of the modified trityl ethers, monomethoxytrityl (**MMT**) and dimethoxytrityl (**DMT**) for the protection of the 5'-hydroxyl, replacing the often used acetyl (**Ac**) group.[18] These were an improvement over the parent trityl ether in that their rates of cleavage under acidic conditions were 10 times and 100 times faster, respectively, which was critical since purine containing deoxynucleotides, especially, undergo acid catalyzed cleavage of the glycosidic bond. The shorter treatments to remove the newer trityl ethers resulted in a large reduction in strand degradation, especially during the synthesis of longer sequences.



DCC

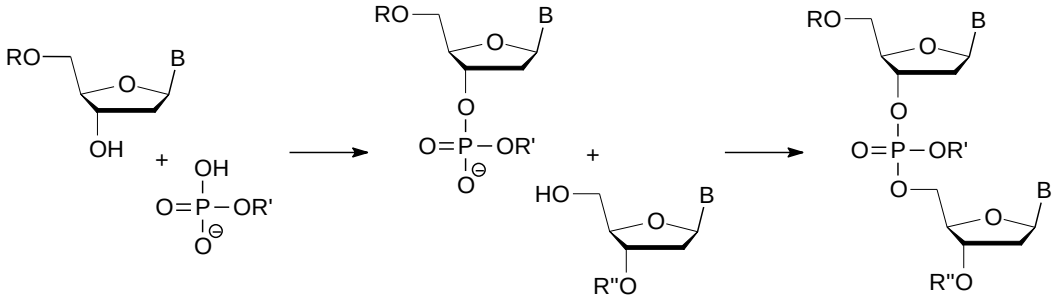


1.1
a: R = Me
b: R = *i*Pr

In addition to the protecting group strategies developed, new coupling agents were introduced to the field. Dicyclohexylcarbodiimide (**DCC**) was initially used as the coupling agent of choice but it often gave poor yields and side-products.[5,19] Newer coupling agents, based on aromatic sulphonyl chlorides (**1.1a**,**b**), resulted in faster couplings with higher yields and less side

products,[20,21] and were later employed when the field returned to Sir Todd's original methodology, the phosphotriester approach.

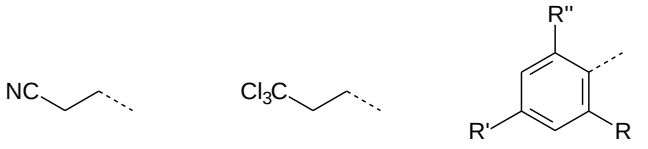
1.4 Phosphotriester approach



R = Ac, MMT, DMT
R' = CE, TCE, Ar
R" = Ac, Bz, solid support

Scheme 1-3: Phosphotriester approach

A major consideration for the phosphotriester approach, of course, was the nature of the phosphate protecting group. The benzyl protecting group (**Bn**) originally employed by Todd was too labile for the synthesis of long oligonucleotides therefore a great deal of effort was invested in finding alternatives.[5] Aryl based protecting groups (**Ar**) were attractive, since in theory their cleavage characteristics could be tuned by the substitution of functional



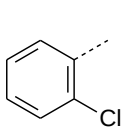
CE **TCE** **Ar**

R = H, F, Cl, NO_2
R' = H, F, Cl, NO_2
R" = H, F, Cl, NO_2

groups on the aromatic rings,[22,23] though in practice this proved to be difficult. This type of protecting group was dependant on P-O bond cleavage to generate the desired phosphodiester upon final deprotection of the oligonucleotide, often employing hydroxide ions as the nucleophiles. This often led to significant cleavage of the internucleotide linkages, however, until alternative reagents could be developed.

Another class of phosphate protecting groups, which employed C-O rather than P-O bond cleavage, were also explored. The 2-cyanoethyl protecting group (**CE**) employed by Letsinger and co-workers[24,25] as well as the 2,2,2-trichloroethyl protecting group (**TCE**) used by Eckstein and co-workers,[26,27] were initially more useful protecting groups than the aryl derivatives. The cyanoethyl group underwent facile cleavage by β-elimination under relatively mild basic conditions, while the 2,2,2-trichloroethyl group was cleaved using zinc dust in acetic acid. Another protecting group that went through C-O bond cleavage was a simple methyl group, cleavable by addition of a thiolate ion, as demonstrated by Daub *et*. *al*[28], but this protecting group did not become important until the development of the phosphite triester approaches. These protecting groups were very useful in the development of phosphotriester chemistry but were eventually replaced when the aryl-based deprotection strategies were perfected.[5]

The optimization of aryl-based protecting groups (**Ar**) initially focused on trying to decrease the p*Ka* of the phenolic unit by placing substituents in the *ortho* or *para*-positions of the ring, thus transforming the unit into a better leaving group.[23,29] This was achieved mostly through the use of fluoro and chloro



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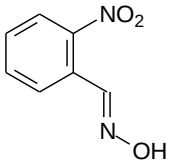

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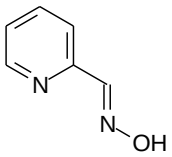


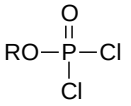
1.4

substituents but it was found that, if the p*Ka* of the phenoxy unit was decreased below approximately 8, the protecting group was likely to become unstable and thus no longer useful for longer syntheses.[29]

As an alternative, other nucleophiles were employed to carry out the P-O bond cleavage. One alternative, using fluoride ion as the nucleophile, usually in the form of the tetrabutylammonium salt (TBAF) was explored by several groups[30,31] but was finally found to induce an unacceptable amount of chain cleavage.[32] Ultimately it was in the search for antitoxins of cholinesterase inhibitors such as Sarin that a solution to this problem was found.[33] It was observed that the conjugate bases of oximes and hydroxamic acids were able to effectuate P-O bond cleavage selectively with little occurrence of chain cleavage. The most effective system, which became the standard for use in the phosphotriester approach, combined the 2-chlorophenyl protecting group, **1.2**, with the conjugate bases of 2-nitrobenzaldoxime (**1.3**) or pyridine-2-carboxaldoxime (**1.4**) used as the deprotecting agents.[34,35]

Another important consideration in using the phosphotriester approach was the phosphorylation method used to generate the necessary nucleotide building blocks. Earlier phosphorylating reagents, such as phosphorodichloridate monoesters (**1.5**),[22,27,36] where the ester moiety was the intended internucleotide

protecting group, resulted in the production of some 3'-3' or 5'-5' dimers, depending on the position being phosphorylated, during the phosphorylation step, reducing yields and increasing the difficulty of purification. This problem was eventually overcome by changing the phosphorylating agents to phosphorodiimidazolides (**1.6**),[37] or triazolides (**1.7**),[38] using them in up to three-fold excess and then performing aqueous alkaline work-ups to yield the desired phosphodiesters in high yield.



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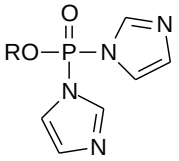

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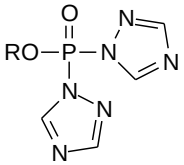
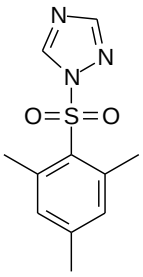


1.7

R = CE, TCE, Ar

The final consideration for the phosphotriester approach was the nature of the coupling agent used. The arylsulphonyl chlorides (**1.1**), introduced as an improvement over DCC in the phosphodiester approach, were initially used in the phosphotriester approach as well. The use of these agents often resulted in the darkening of the solution but Berlin and co-workers found that use of the



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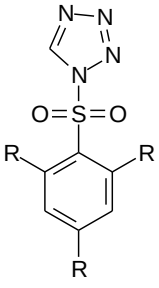

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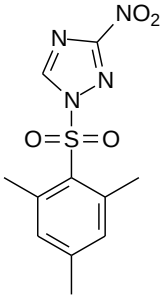


1.10

R = H, Me, *i*Pr

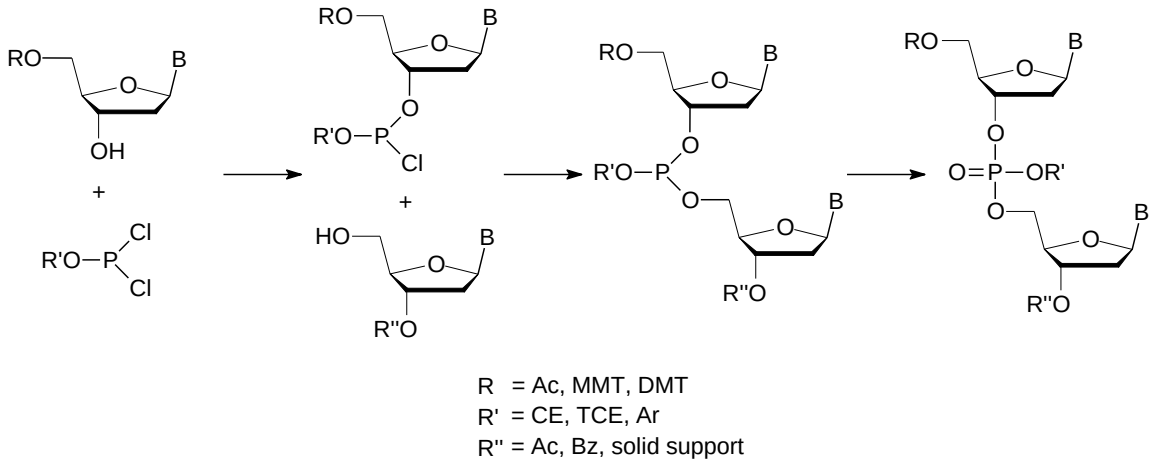
imidazolide analogues,[39] rather than the chlorides, eliminated this problem, though the coupling rate were slower. This prompted the investigation of other aryl sulphonyl derivatives, however, and both triazoles (**1.8**)[40] and tetrazoles (**1.9**)[41] were tested. The triazoles were found to give as fast couplings as the chloro analogues (**1.1**), with higher yields obtained, and the tetrazoles were even faster. Compound **1.10** eventually became the standard coupling agent for this approach.[34]

Though the phosphotriester approach saw a great deal of improvement from the system originally used by Todd, the coupling times were still long and yields were not sufficient to undertake the synthesis of long oligomers on a routine basis. For this to occur, a new approach was eventually introduced, the phosphite approach.

1.5 Phosphite and phosphoramidite approach

One of the biggest breakthroughs in oligonucleotide synthesis came when Letsinger and co-workers showed that phosphorylation and coupling reactions occurred much faster with P[III] species than P[V] species.[42] These researchers demonstrated that a nucleoside could be phosphorylated quickly at very low temperatures, using phosphorodichloridites, and then coupled directly with another nucleoside or oligonucleotide (Scheme 1-4). The resulting phosphite triester would then be oxidized *in situ*, prior to purification, to give a phosphotriester.

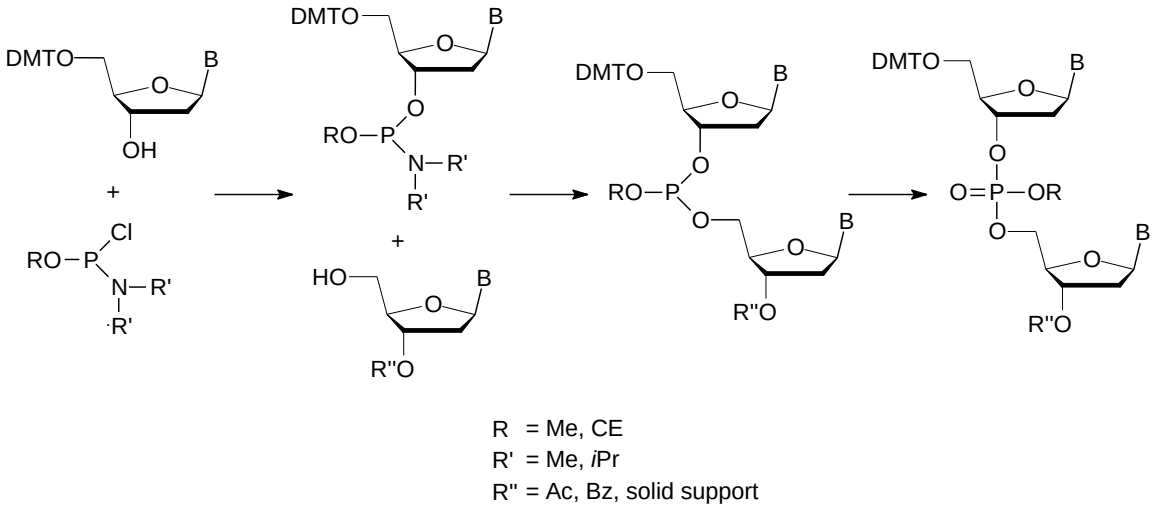
The main drawback of this approach was that the chlorophosphite nucleotide intermediate was unstable and could not be isolated. In addition, as in the phosphotriester approach when phosphorylation was performed using phosphorodichloridate, the formation of undesirable 3'-3' or 5'-5' dimers occurred, depending on the position being phosphorylated, reducing the yield and increasing the difficulty of eventual purification.



R = Ac, MMT, DMT
R' = CE, TCE, Ar
R'' = Ac, Bz, solid support

Scheme 1-4: Phosphite triester approach

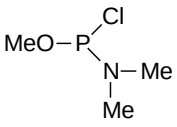
This problem was addressed when Beaucage and Caruthers discovered (Scheme 1-5) that phosphoramidites diesters, P[III] species bearing two alkoxyl groups and an amine, could be easily prepared and isolated in high yield and purity, without the concomitant formation of dimerized products.[43] These phosphoramidites could then be activated, in the presence of a nucleoside or oligonucleotide, by the addition of a weak acid, such as tetrazole, to achieve fast, highly efficient couplings.



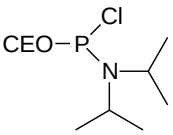
R = Me, CE
R' = Me, *i*Pr
R" = Ac, Bz, solid support

Scheme 1-5: Phosphoramidite approach

The initial system devised, achieved phosphorylation of the desired nucleoside using chloro(dimethylamino)methoxyphosphine (**1.11**). The resultant phosphoramidites could be purified and were reasonably stable upon storage. Later, the cyanoethyl protecting group replaced the methyl and the amine function was changed to the diisopropyl derivative, as in **1.12**, as this simplified deprotection and resulted in phosphoramidites with much longer shelf lives.[44,45] Indeed, this methodology has become the mainstay of oligonucleotide synthesis, especially after the development of solid-phase based synthesis (see Section 1.7).



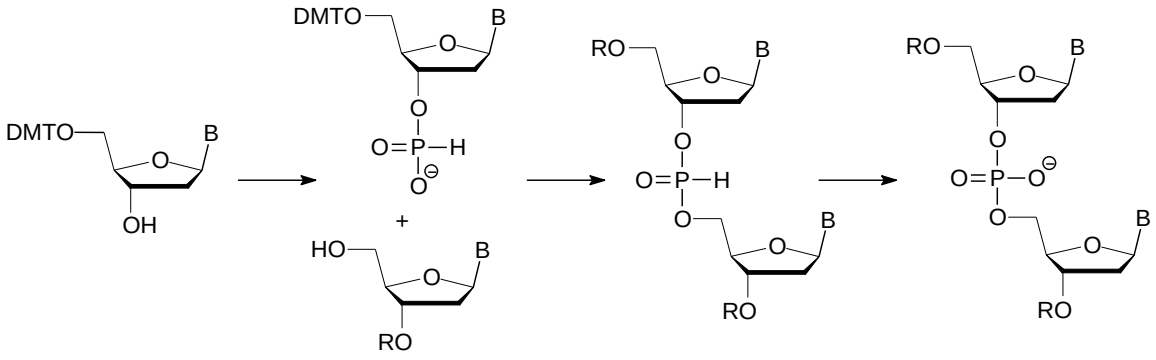
1.11



1.12

1.6 H-Phosphonate approach

Another innovation originating in the Todd laboratory was the H-phosphonate approach,[46] though it was not thoroughly explored until much later. This approach, outlined in Scheme 1-6, involved the condensation of a nucleotide H-phosphonate with a nucleoside, in the presence of an activating agent, such as pivaloyl chloride, to give an H-phosphonate diester. This approach was similar to the phosphotriester approach but occurred much faster and allowed for the synthesis of high molecular weight oligonucleotides on solid support. Unlike the intermediate phosphite triester obtained in the phosphoramidite approach, which was unstable and required oxidation prior to further chain extension, the intermediate H-phosphonate diester was reasonably stable, especially on solid support, and an oligomer assembled using this approach could be oxidized entirely in a single step at the end of synthesis. Solution phase use of this approach, however, usually involved the conversion of the H-phosphonate to an aryl protected phosphorothioate,[47] which could be deprotected in the final step if the phosphorothioate backbone was desired, or converted to the natural phosphate system, as needed.



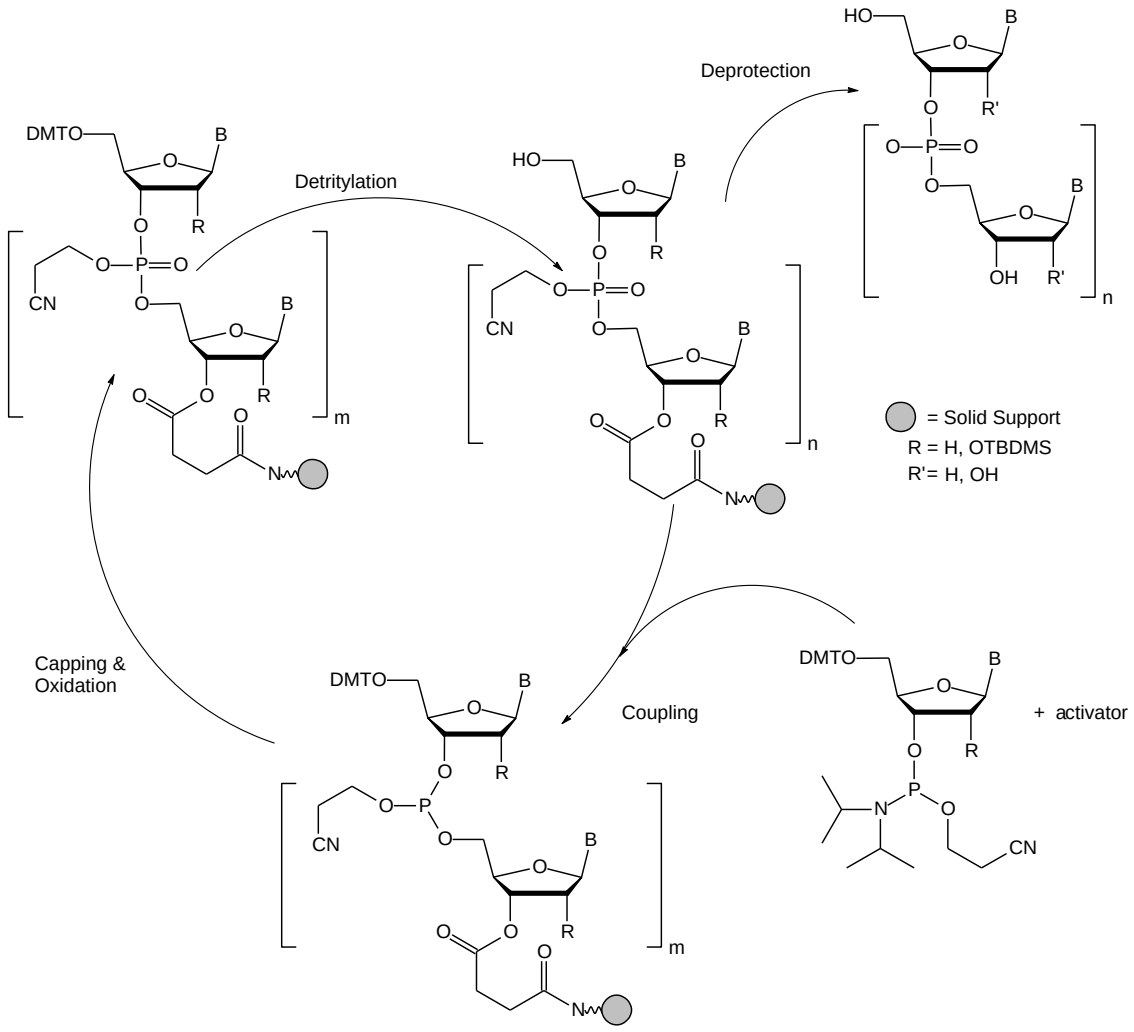
R = Ac, solid support

Scheme 1-6: H-Phosphonate approach

1.7 Solid-phase synthesis

One of the most revolutionary discoveries in the chemical synthesis of biopolymers was the ability to perform these syntheses on solid supports. Merrifield's initial disclosure of this approach to the synthesis of peptides[48] was followed quickly by that of Letsinger and co-workers that this approach could also be used for the synthesis of oligonucleotides.[24,49,50] With this discovery, many researchers began developing methods to link the first nucleoside to the solid support and adapting coupling methods originally designed for solution phase synthesis to this new medium. Initially yields were poor and the oligonucleotides generated were of limited length as the phosphodiester approach, which dominated the field at the time, was especially unsuited to this medium. As research shifted to the phosphotriester approach, however, yields began to improve, coupling times decreased, and sequences of useful lengths became available.[51] At the same time, the demand for oligonucleotides as probes and primers, in addition to synthetic genes for controlled mutagenesis experiments, began to grow significantly. Eventually, once the phosphoramidite approach was developed, solid-phase synthesis became routine and was finally automated.[52,53]

At its core, the most common solid-phase approach (Scheme 1-7) to oligonucleotide synthesis is an iterative process where a single nucleoside is first attached to a solid support and then a series of repeating steps is carried out to extend the chain. The first step unblocks the point where extension is to be achieved, the next step introduces a specific nucleotide building block in the presence of an activator to achieve coupling to the unblocked position, and then

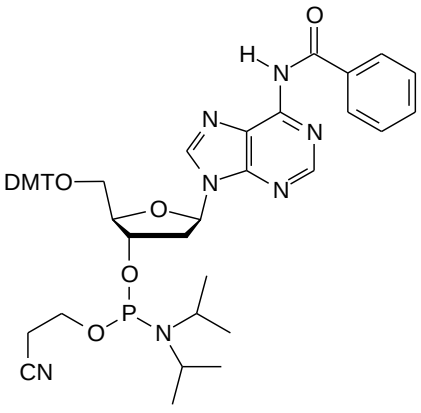


Scheme 1-7: Solid-phase synthesis cycle

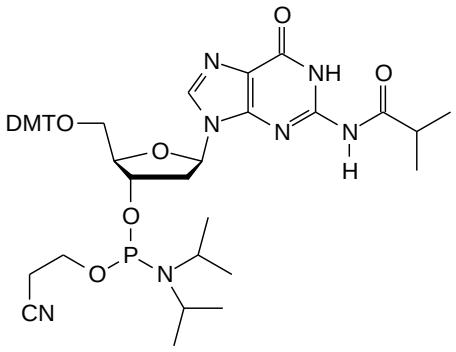
any unreacted sites are rendered inert by the addition of a cap. When the phosphoramidite approach is used, the initial linkage formed requires oxidation to render it stable enough for subsequent steps to be performed and then the entire cycle is repeated. In this way, the oligonucleotide chain is extended by one unit at a time until the desired length and sequence is obtained. No purification of the growing oligomer is performed and the excess reagents and by-products from each step are simply washed away, while the oligomer is retained on the solid support.

Several solid supports have been investigated for the solid-phase synthesis of oligonucleotides, with the two most common in current use being controlled-pore glass (CPG)[54] and a polystyrene resin that is highly crosslinked.[55] CPG is generally functionalized by attaching long chain alkyl groups, ending with amines, to the surface of the glass beads, whereas the polystyrene resin usually has aminomethyl groups at the surface. The first nucleoside is usually attached to the solid support by its 3'-hydroxyl through a linker moiety such as the commonly used succinyl linker, though many others are now commercially available.

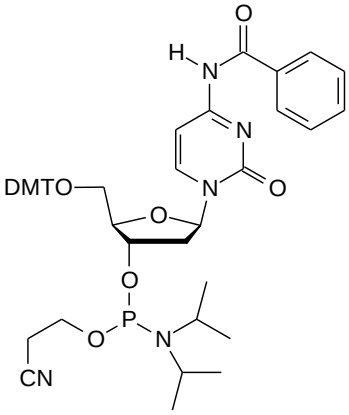
Once a nucleoside has been attached to the solid support through its 3'-oxygen, it is deblocked at the 5'-position to allow for chain extension. The most common protecting group strategy is to use the acid labile DMT protecting group



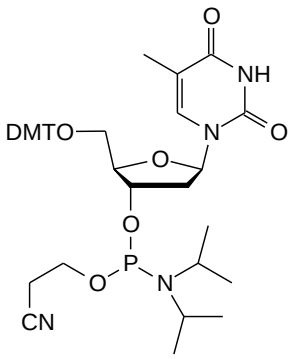
1.13a



1.13b



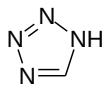
1.13c



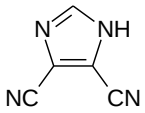
1.13d

for this position, both for the first nucleoside as well as for the phosphoramidite building blocks. An alternative approach, developed by Scaringe *et*. *al*, uses a fluoride labile silyl ether as the 5' protecting group,[56] though this approach has seen less general use due to proprietary and cost considerations.

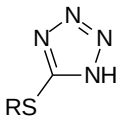
After the extension site is unblocked, the synthesis proceeds via the addition of a nucleotide building block, in the presence of an appropriate activator. The four common DNA phosphoramidite building blocks, **1.13a-d**, in the presence of any of the standard activators, such as 1*H*-tetrazole (**TET**), 4,5-dicyanoimidazole (**DCI**), 5-ethylthiotetrazole (**ETT**), and 5-benzylthiotetrazole (**BMT**), generally result in greater than 98% coupling yields per nucleotide addition, within coupling times of approximately 1-2 minutes.[52] This high coupling efficiency is critical, as for longer oligomers small reductions in the per step coupling yield lead to drastic reductions in the yields of the desired products and concomitant increases in the difficulty of purification.



TET



DCI



ETT R = Et

BMT R = CH_2Ph

Though the coupling yields are very high, they do not go to completion, therefore sites are still open that could grow further in subsequent coupling steps. It is important to avoid this as it greatly complicates the purification of the product. If no steps were taken to mitigate this, many ‘failure’ sequences would be generated, all with single deletions from the desired material but in different

positions within the sequences. These ‘n-1’ sequences are difficult to separate from the desired product, especially for long sequences, as the difference in mobility within a chromatographic system are very small between the full length product and the n-1 sequences. Contamination of even small amounts of these impurities within the desired material could lead to spurious results in biological studies they are used in or, in the case of the oligonucleotide being used as a therapeutic, undesirable off-target effects could be generated.

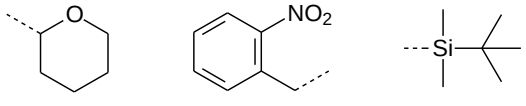
These n-1 sequences can be minimized by re-blocking any sites remaining unreacted after the coupling step with a group that will remain in place throughout the remainder of the synthesis, a process referred to as capping. The most commonly used group used for this purpose is an acetyl ester. In addition to reducing the amount of n-1 product generated during the synthesis cycle, capping with acetic anhydride solves another problem that had been observed in solid phase synthesis, the reaction of *O*6 in guanosine with phosphoramidite.[57] The resultant coupling at this position is reversed upon exposure to acetate anions if performed prior to oxidation, the oxidized product being stable and resulting in the generation of branched species.[58]

The phosphite triester present after coupling and capping is unstable and prone to cleavage in the acidic conditions required to remove the trityl protecting group that must be removed to continue with chain extension. This problem is solved by oxidizing to the more stable phosphate triester. Oxidation is generally achieved by exposure of the phosphite to a solution of iodine in water, with tetrahydrofuran (THF) added as a co-solvent, in the presence of a base, such as

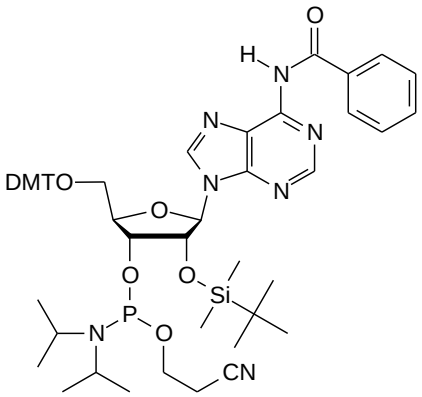
pyridine, to scavenge the acid generated in the reaction. The resultant P[V] species is stable to the conditions used in further synthesis and after this step the oligonucleotide is ready for further chain extension by repetition of all the steps or final deprotection if the desired length has been obtained.

The solid-phase approach works well for both DNA and RNA, though RNA requires more consideration, owing to the presence of the 2'-hydroxyl group. Protection of this position has been a major field of research, with many classes of protecting groups employed, from acid cleavable moieties such as tetrahydropyranyl acetals (**THP**),[59-61] alkaline labile groups such as acetyl and benzoyl esters (**Ac**, **Bz**),[62] light-sensitive groups such as *o*-nitrobenzyl (***o*-NO$_2$Bn**),[63] to the fluoride labile *t*-butyldimethylsilyl ether (**TBDMS**).[64]

The TBDMS ether is the most commonly used protecting group for a variety of reasons but some of the most important are its ease of use, not requiring any special precautions or alteration of other protecting groups employed in the molecule, its ease of synthesis, the TBDMS protected phosphoramidites (**1.13e-h**) are inexpensive to produce, obtainable in high yield and purity, and finally its orthogonal nature to the other protecting groups used in oligonucleotide synthesis, ensuring that strand cleavage due to premature deprotection of this position is minimal.



THP ***o*-NO$_2$Bn** **TBDMS**



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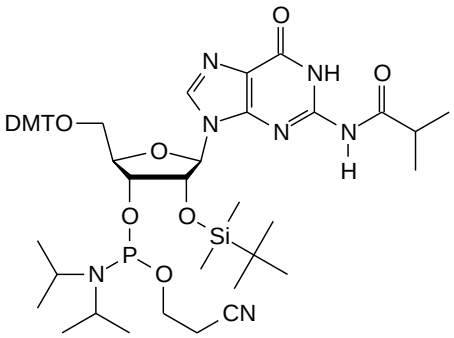

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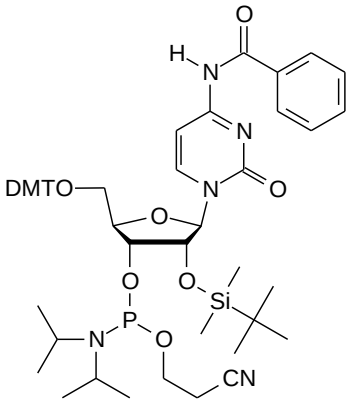
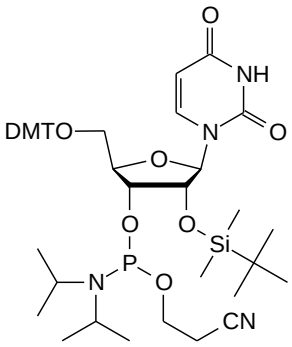


1.13g



1.13h

Solid-phase synthesis of both DNA and RNA has become so routine that sequences for biological studies are now obtainable for a few dollars each, in quantities and at purities that allow for new breakthroughs to be made at an ever increasing pace. Without this methodology, achievements such as the human genome project could never have been realized. Along with the crucial insights into basic biological function that have been realized using materials obtained from this approach, many practical applications have been discovered as well, one of the most critical being the advent of the therapeutic use of oligonucleotides.

1.8 Oligonucleotides as therapeutics

In biological systems, many disease states are caused by an untimely expression or over-expression of proteins. The traditional pharmaceutical approach has been to target these proteins with small molecules, which bind to active or allosteric sites to inhibit the function of the protein. Several discoveries, however, have shown that it is possible to target drugs further upstream, inhibiting the translation of mRNA into proteins. This achieves the same goal but is more efficient, since a single mRNA is translated multiple times, yielding many copies of a protein.

This approach was first undertaken by Zamecnik *et*. *al*, who used a synthetic oligodeoxyribonucleotide to inhibit the replication of a virus.[65] Initially it was believed that the nature of the inhibition was simple arrest of the translational machinery, that the introduced oligodeoxyribonucleotide bound tightly to the target mRNA and prevented the ribosome either from binding to the mRNA or from progressing further along the mRNA chain once the complementary site was reached. In either case, it was the physical blockage that would result in downregulation of protein expression. Later, however, it was discovered that an enzyme, RNase H, was capable of cleaving the RNA portion of an RNA-DNA duplex.[66,67] This indicated an even greater efficiency for inhibiting protein expression, since every oligodeoxyribonucleotide introduced resulted in the cleavage of multiple mRNA strands. This methodology became known as the antisense approach and eventually resulted in the regulatory approval of the first

oligonucleotide based therapeutic, Vitravene, for the treatment of cytomegalovirus retinitis.[68]

A further discovery, that of the RNA interference pathway,[69] led to another oligonucleotide based therapeutic approach, termed siRNA.[70-73] This approach takes advantage of a cellular process used for the regulation of gene expression, where a series of enzymes are able to cleave double stranded RNA. Introduction of an appropriately designed synthetic RNA duplex into a cell allows the duplex to be loaded into the RNA-induced silencing complex (RISC), where one of the strands, called the guide strand, is cleaved by an enzyme, Argonaut 2 in humans. The active complex thus formed is capable of cleaving mRNA sequences complementary to the remaining strand, in this way downregulating protein expression. This approach is routinely used for generating gene knockdown species in various organisms for study but many disease states may also be targeted with this approach and a great deal of research is currently invested in searching for suitable targets.

Another oligonucleotide based therapeutic approach that has been suggested is that of using oligonucleotides called aptamers to fill the role that small molecules currently play in the traditional pharmaceutical approach. A methodology has been developed where an oligonucleotide may be evolved such that it folds into conformation that can bind tightly to an active site or allosteric site on a protein.[74-76] The technique, referred to as systematic evolution of ligands by exponential enrichment, or SELEX, begins with a combinatorial library of oligonucleotides that are subjected to a selection process.[77] The approach

allows only the selected oligomers to be retained, which are then amplified using PCR and subjected to another round of selection. After several rounds, the lead candidates are identified and evaluated for their suitability as therapeutic agents. The advantage of this technique over that of small molecules is that the aptamers produced can have binding affinities rivaling those of antibodies,[78] and the selection process can be performed much more rapidly than the rational design and optimization of a small molecule to bind to the same target. That being said, the selection process used does not allow for the universal development of compounds for any target, only the subset adaptable to the technique.

Other approaches of the use of oligonucleotides as potential therapeutics are continually being discovered. Some of these approaches are less specific than those discussed above, resulting simply in an immunostimulatory response, but all these approaches bear in common the need for large scale synthesis of oligonucleotides to supply the necessary material for clinical trials and, after approval, to satisfy market demand. Currently, solid phase synthesis is being used to achieve this but at great expense. New solution phase approaches will be required to meet the growth in volume needed once oligonucleotide therapies become mainstream.

1.9 Thesis objectives

The main goal of this thesis, as the title implies, is to study the uses of ionic tags (see Section 2.1) in the synthesis of oligonucleotides, both as a means to

reduce the cost of large scale production of potential oligonucleotide based therapeutics and as an aid to synthesizing sensitive oligonucleotide structures.

Chapter 2 will outline the initial approach taken to exploit the solubility properties of ionic tags that allow for the synthesis of oligodeoxyribonucleotides in solution followed by their rapid and simple purification through precipitation from a solvent in high yields and purities. The chapter will go on to describe the evolution and optimization of the methodology, followed by the synthesis of oligoribonucleotides. Finally, the discussion will turn to the attempt at the linear solution-phase synthesis of an siRNA target molecule and the difficulties encountered.

Chapter 3 will discuss the development of an orthogonal linker, intended to facilitate the preparation of oligonucleotide block phosphoramidites. Various routes to this type of linker will be explored and several other routes will be proposed. The cleavage of one such linker, containing a *γ*-keto ester functionality susceptible to removal by treatment with hydrazine, will be discussed, its cleavage characteristics relative to the levulinyl ester protecting group will be evaluated and the sources of the differences will be examined. It will be shown that the tag can be removed easily from DNA and RNA nucleosides in a way similar to that which is used for the levulinyl protecting group, indicating that this approach will be successful in generating the desired block phosphoramidites.

Finally, Chapter 4 will discuss a novel oligonucleotide structure that has been found while exploring the possible catalytic role of RNA within the

splicesome. A series of analogues will be proposed to study this interesting structure and a route to their synthesis will be explored. It will be shown that this route is made easier by the use of ionic tags in their synthesis and the experiments proposed to study the analogues will be outlined.

Chapter 2 Tagged Oligonucleotide Synthesis

2.1 Introduction

The demands of the scientific community for synthetic oligonucleotides have grown exponentially over the past decades. The ready availability of DNA oligonucleotide primers has satisfied the tremendous needs of the genome sequencing efforts, research into functional genomics, and polymerase chain reaction (PCR)-based detection methods but significant advances in structural biology and biochemistry have only been achieved through concomitant advances in DNA and RNA chemical synthesis.[52,53,79-81] For instance, the current state of the art in ribozyme and siRNA research, including crystal structures, would not have been possible without the accompanying improvements in RNA synthesis.[79] Oligonucleotides have now begun to see widespread use in the development of therapeutics and in diagnostic applications and large quantities are now required.

Since the research groups of Merrifield[48] and Letsinger[24,82] introduced the use of polymer supports for the synthesis of oligopeptides and oligonucleotides, the use of insoluble supports has become an important tool for organic synthesis, especially in the synthesis of biopolymers such as oligonucleotides, peptides and more recently, carbohydrates.[83-85] Although extremely successful, because of the heterogeneous nature of the insoluble polymers, solid-phase synthesis has

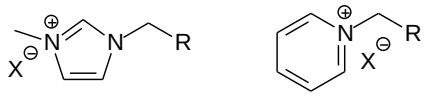
all the problems generally associated with heterogeneous reaction conditions in addition to the high cost of the supports themselves in oligonucleotide synthesis (30-40% of materials costs)[86,87] and the necessity of using excess phosphoramidites, making large scale synthesis of these compounds very expensive.

In recent years, the use of *soluble* polymer supports, supports that are soluble in the reaction medium but impart properties that allow for their isolation using methods other than chromatography, have received considerable attention because such “solution phase” syntheses retain many of the advantages of conventional solution phase chemistry, while still permitting facile purification of the product. Thus, soluble polyethylene glycol (PEG) and other polymers have been used for the synthesis of oligopeptides,[83] nucleotides,[84] saccharides,[85] and small molecules.[88,89] Some limitations experienced when using soluble polymer supports such as these include low loading capacity, limited solubility during the synthesis of longer peptides, and often low aqueous solubility [88,89] as well as an energy intensive cooling step required for purification.[84]

The use of a fluorous phase technique for organic synthesis has been advocated in recent years.[90-94] This technique is based on the concept that fluorinated reagents dissolve preferentially in perfluoroalkanes, the so-called fluorous phase. Purification can be achieved by a temperature switch that causes a phase separation between the previously miscible fluorous solvent and an organic co-solvent, thus facilitating separation since the desired product remains in one phase and the by-products and other reagents are retained in the

opposite phase. So far the use of fluorous phase synthesis has been demonstrated for small molecules and some biopolymers but the technique could be limited for large scale applications by the expense of perfluoroalkane solvents, the need for specialized fluorinated reagents, and the energy cost associated with the temperature switch.

Figure 2-1: Common ionic liquids



X = Cl^-, Br^-, I^-, BF_4^-, PF_6^-, etc.

Recently, there has been considerable interest in the use of ionic liquids as "green" alternatives to more traditional reaction media. A practical definition of an ionic liquid is that it is a salt with a melting temperature below, often much below, the boiling point of water.[95,96] A common feature of ionic liquids is that most have organic cations and inorganic anions. Commonly used species are the alkylimidazolium and pyridinium salts of halides, tetrafluoroborate and hexafluorophosphate (Figure 2-1), though many other cation and anion species are being studied.[97] Numerous chemical reactions, including some enzymatic reactions, have been carried out in ionic liquids.[98-100] Room temperature ionic liquids have also been widely explored as media for electrochemical technologies,[101,102] chemical extractions,[103,104] and other industrial processes.[105] This is due to several useful properties of ionic liquids, such as high thermal and chemical stability, insignificant vapour pressure, non-flammability, friction

reduction, anti-wear performance and high loading capacity and in many cases, the ionic liquids can be recycled easily.[96,100,106,107]

The idea of using ionic liquid based supports for organic synthesis [108,109] has been demonstrated for synthesis of small molecules,[110,111] oligopeptides,[112] and oligosaccharides.[113,114] This chapter describes the evolution of ionic liquid supported synthesis (ILSS) as applied to oligonucleotide synthesis, using simple precipitation and phase separation methods without the need for chromatography. This method provides high product purity at each step and, due to the homogeneous nature of solution phase chemistry, should be amenable to large scale manufacturing.

2.2 Oligodeoxyribonucleotide synthesis

2.2.1 Overview

As mentioned in Chapter 1, solid-phase synthesis of oligonucleotides using the phosphoramidite method[52,79,81,115,116] is an iterative process in which a solid support with an attached nucleoside is deblocked at the 5'-position by removing an acid labile protecting group, thus liberating a nucleophilic 5'-hydroxyl group. This terminal nucleophile is then allowed to couple to a suitably protected 3'-*O*-phosphoramidite monomer in the presence of an activator. The newly created phosphite triester 3', 5'-linkage is then oxidized to provide the desired and more stable phosphate triester. This process is repeated until an oligomer of the desired length and composition is obtained. The same iterative methodology may be used when soluble supports are employed.

The ionic tag must be stable under all conditions encountered during oligonucleotide synthesis including exposure to reasonably strong and weak acids as well as oxidative and acylating conditions. It must also be unreactive towards the reagents and intermediates encountered during chain elongation, such as the activated phosphoramidite and the capping reagents. Finally it must be attached to the first nucleoside of the desired sequence in a way that can be removed upon the completion of synthesis in a manner that does not degrade the desired oligonucleotide.

The ionic tag chosen for this work, **2.1**, consists of a methyl imidazolium moiety covalently linked to a two carbon chain, ending in a primary alcohol. The hydroxyl allows the tag to be linked to the 3'-OH of the first nucleoside through a linker in a manner analogous to solid-phase synthesis. Once attached through the linker, the tag is inert to all the reagents, intermediates and reaction conditions of oligonucleotide synthesis. The linker chosen was a succinyl group, one of the most stable systems used in solid-phase synthesis, which is easily cleaved under standard deprotection conditions once the oligomer of desired length and composition is obtained.

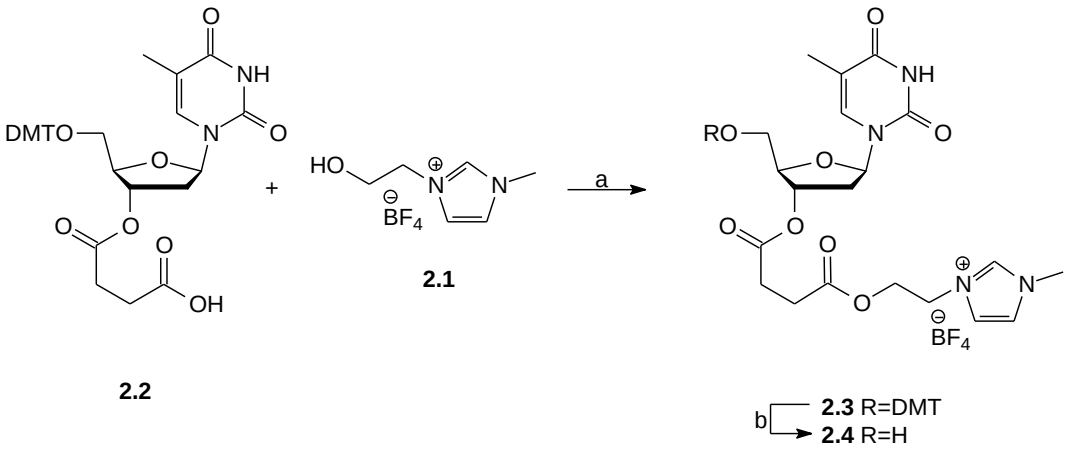
2.2.2 Component Synthesis

The synthesis of the ionic tag supported oligonucleotide began with the ionic liquid **2.1** (Scheme 2-1), which was readily prepared from the condensation of equimolar amounts of 1-methylimidazole (NMI) and 2-bromoethanol under solvent free, microwave assisted conditions. Unreacted starting materials were removed by triturating the resulting solid with diethyl ether and then anion

metathesis was performed by suspending the solid in acetone or acetonitrile and adding the sodium salt of a desirable anion with a diffuse charge, in this case tetrafluoroborate, to the suspension. The anion metathesis was necessary to obtain an acceptable solubility profile for the starting tagged oligonucleotide, i.e. soluble in the reaction medium but insoluble in the purification medium (anti-solvent). The imidazolium bromide salt was insoluble or poorly soluble in most organic solvents and thus not useful as a tag for solution phase synthesis, while the imidazolium tetrafluoroborate salt was readily soluble in many polar organic solvents but was completely insoluble in most ethers.

The metathesis progress was observed over time as the initially amorphous solid in the reaction mixture disappears and was replaced by crystals of sodium bromide. After stirring for several hours the remaining solid was filtered and the solvent was removed under vacuum yielding a viscous liquid. Upon standing for several days crystallization of residual sodium bromide may be observed. It was easily removed by filtration and has no effect on subsequent reaction steps.

The succinylated 5'-DMT-thymidine **2.2** was prepared by mixing commercially available 5'-DMT-thymidine with excess succinic anhydride in the presence of catalytic amounts of 4-dimethylaminopyridine (DMAP) in acetonitrile. Compound **2.2**[117] was then coupled to the ionic tag **2.1** as shown in Scheme 2-1 using DCC and a catalytic amount of DMAP in acetonitrile to give the ionic tag supported nucleoside **2.3**.



Scheme 2-1: Derivatization of nucleoside with ionic tag

Reagents and conditions: a) DCC, DMAP, CH_3CN 3 days rt, b) 3% TFA in CH_2Cl_2 or CH_3CN

Compound **2.3** was isolated and purified by first filtering off the resulting dicyclohexylurea (DCU) and then precipitating from an ethyl ether-ethyl acetate solution, the precipitate containing the desired tagged nucleoside and excess ionic tag while any unreacted succinylated nucleoside **2.2**, DCC and DMAP were removed with the ethereal layer. After filtration, the precipitate was taken up in chloroform and extracted with water. The excess ionic liquid **2.1** was removed in the aqueous phase while the desired product **2.3** remained in the organic phase due to the hydrophobicity of the dimethoxytrityl protecting group attached to the 5' hydroxyl of thymidine, yielding an off-white foam upon evaporation of the solvent. Exposure of **2.3** to acidic conditions followed by precipitation yielded compound **2.4** as a light brown foam in 96% yield. The structure and purity of **2.3** and **2.4** were verified by ^{1}H NMR and ESI-MS.

2.2.3 Dimer coupling

The dinucleoside phosphotriester dTpT, (**2.7**) was prepared at a 250 μmol scale by reacting the ionic tag supported nucleoside **2.4** with a 1.5-fold excess of the appropriate phosphoramidite (**1.13d**) using 4,5-dicyanoimidazole (DCI) as the activating agent in THF or acetonitrile and stirring for 1 to 2 h. This scale was 250 times that of a typical solid-phase synthesis and uses less than one tenth of the number of equivalents of amidite than would be used for a solid-phase synthesis of that scale.

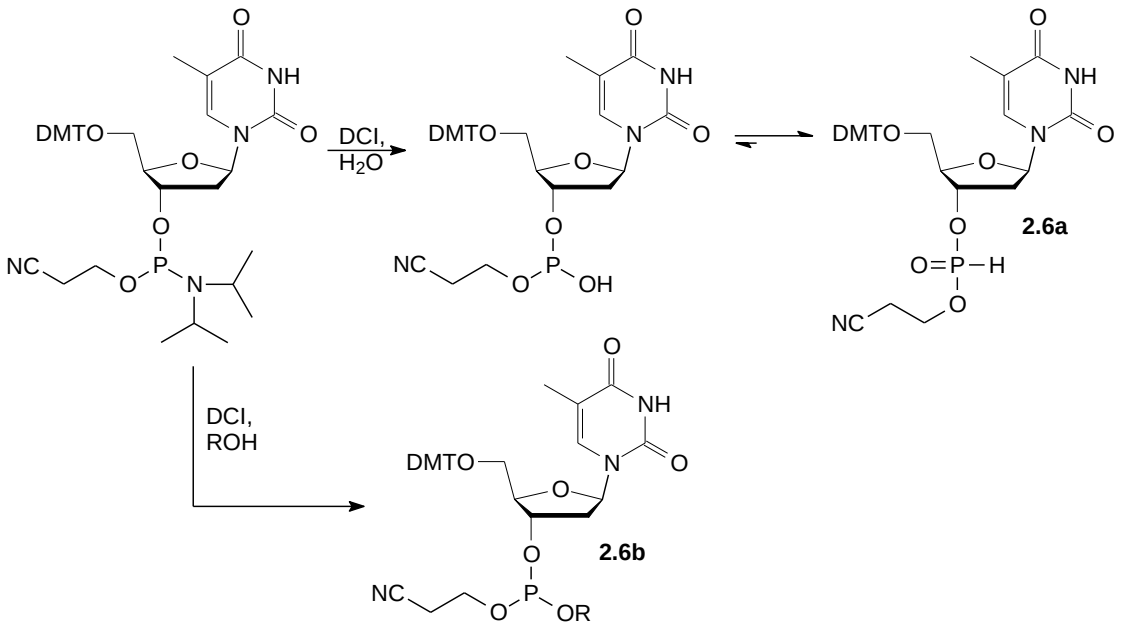
After the reaction has come to completion, the desired product must be isolated from the reaction mixture. The tag allows this to be achieved by precipitation rather than the chromatography that is typically required for solution phase reactions. Initially, a small amount of diethyl ether was added to the reaction mixture to achieve the purification. This caused the desired product to precipitate but it occurred so rapidly that unacceptably high levels of impurity were entrained within the resultant solid. The order of addition was then inverted and the reaction mixture was added dropwise to stirred diethyl ether. This resulted in an improved purity profile but both the excess amidite and the DCI activator were still present at unacceptable levels, even after a second precipitation was performed. The impurities were simply not soluble enough in the precipitation medium.

The solubility of these impurities could be increased by the addition of a co-solvent to the diethyl ether but the enhancement of solubility of the impurities must be balanced against the necessity of the tagged material remaining

insoluble. Chloroform as co-solvent was tested first due to its low polarity. Initially 10% v/v of chloroform was added to diethyl ether and to this was added the reaction mixture dropwise. There was a small improvement in the ability of the medium to purify the tagged material but a high level of impurity still remained. The concentration of chloroform was increased to 25% v/v but again this was found to be insufficient. An increase to 50% v/v resulted in the formation of an immiscible oil forming upon addition of the reaction mixture, instead of the desired finely dispersed solid normally obtained.

The co-solvent was changed to a slightly more polar solvent, ethyl acetate at 10% v/v. This solvent combination resulted in a marked improvement of the purity of the solid generated, with nearly all DCI removed in the first precipitation as well as the majority of the excess amidite. A second precipitation removed all but a trace of the DCI but not much of the excess amidite was removed.

The excess amidite present at this point was likely in the hydrolyzed H-phosphonate form (Scheme 2-2, **2.6a**). This molecule was highly polar and appeared to have a solubility profile similar to that of the tagged oligomer. The formation of a large amount of the H-phosphonate was avoided by the addition of a quenching agent, such as a primary alcohol, to give **2.6b**. Quenching the phosphoramidite with an alcohol facilitates its removal during purification since the phosphite triester **2.6b** is unable to undergo the tautomerization leading to the H-phosphonate, **2.6a**, is much less polar and much more soluble in the anti-solvent. Ethanol was initially chosen as the quenching agent, since it was readily available in an anhydrous form, but was later replaced (see Section 2.3.2).



Scheme 2-2: Phosphoramidite hydrolysis and quenching

Thus, the excess activated phosphoramidite was quenched after the coupling reaction had reached completion through the addition of anhydrous ethanol and a further 10 minutes of stirring. The desired dinucleoside phosphite triester intermediate was then isolated by simply precipitating from 1:9 v/v ethyl acetate/diethyl ether at room temperature, twice, and the purity, as determined by NMR and TLC, was deemed acceptable, with only trace amounts of impurities observed.

Precipitation of the dinucleoside phosphite triester at this stage, prior to oxidation, was critical however, as it was found that the quenched excess mononucleoside 3'-O-phosphite triester (**2.6b**) was much more soluble in ethyl acetate/ diethyl ether than the phosphate triester that results from oxidation, which has similar solubility properties to the H-phosphonate, thus making its

removal from the desired, tagged dinucleoside phosphite triester possible. Oxidation prior to purification made the purification of the desired product much more difficult, even with the addition of ethyl acetate as a co-solvent.

While it is known that dinucleoside phosphite triesters [P-(III)] are less stable than the corresponding phosphate triesters [P-(V)], no decomposition has ever been observed due to precipitation at this step.

2.2.4 Oxidation

Oxidation must be carried out prior to continuing with chain elongation of the oligomer because the phosphite triester was susceptible to cleavage during the highly acidic detritylation step that followed in the synthesis cycle. To carry out the oxidation of the phosphite triester intermediates, the collected precipitate was again dissolved in a small amount of acetonitrile, a small excess of pyridine and a large excess of a 0.1M solution of iodine in 2:1 v/v THF: water were added. Once the persistence of color due to iodine was established, 5% aqueous sodium bisulphite was added to reduce the excess iodine. This reaction mixture was then diluted with chloroform and extracted with water. The aqueous layer removed the resultant salts (NaI, Na_2SO_4, excess bisulphite, pyridinium iodide, etc) in addition to any uncoupled ionic tag supported nucleoside since it lacked a terminal trityl group, rendering it water soluble. The organic layer in principle contained only the desired product. Removal of the organic solvent under reduced pressure yielded the product (**2.7**) as a light brown foam in good yield (Table 2-1) and high purity, eliminating the need for a capping step in this instance. Capping is generally required when guanosine residues are present,

due to the possibility of coupling through *O*6, which is reversed under capping conditions only prior to oxidation and not after.[58]

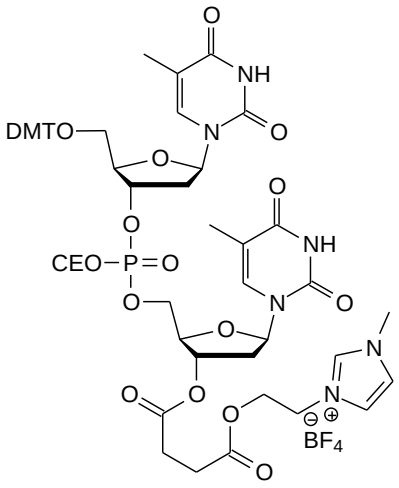
2.2.5 Detritylation

Detritylation of **2.7** was achieved by the addition of 3% trifluoroacetic acid (TFA) in dichloromethane or acetonitrile, stirring for 20 minutes and precipitation in 1:9 ethyl acetate/diethyl ether. The material obtained at this point often contains 5% or more of the tritylated starting material, which was initially explained as resulting from the equilibrium ($ROH + DMT^+ \rightarrow DMT\text{-}OR + H^+$) established during this step (see section 2.3.1 for further discussion of this issue). Quenching the trityl cation with ethanol to trap the DMT cation did not seem to eliminate this problem. However, purity was significantly enhanced (no tritylated product observed by TLC and ESI-MS in the bulk product), if the TFA treatment was repeated, (i.e. redissolution of the precipitate in 3% TFA solution followed with a second precipitation by dropwise addition to 1:9 ethyl acetate/diethyl ether). The purified products were simply filtered off, yielding off white to light yellow powdery solids in high yields (Table 2-1). The collected solid (**2.8**) was ready for further coupling or, in the case where the desired length of oligo had been achieved, deprotection.

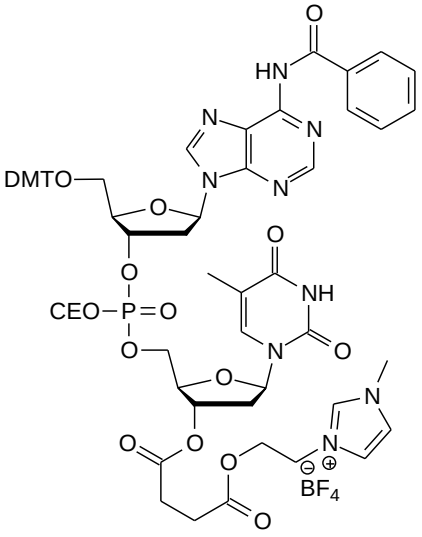
2.2.6 Mixed sequence

The sequences ^{DMT}dApT, ^{DMT}dCpT, and ^{DMT}dGpT (**2.9**, **2.10**, and **2.11** respectively) were synthesized using the same procedure as for **2.7**. Their coupling efficiency and recovery were similar to that of the thymidine dimer but it

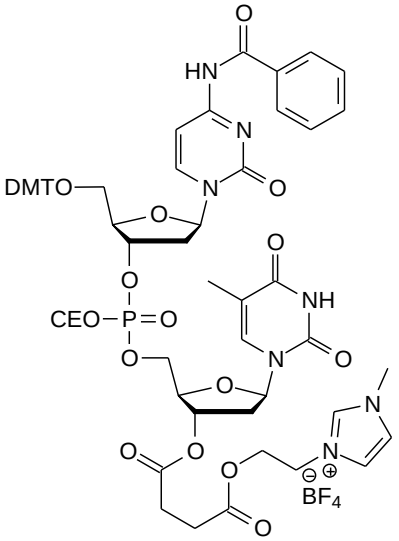
was observed during TLC monitoring of the purifications that the excess phosphoramidites of these nucleosides were all more soluble in the anti-solvent and thus more easily removed, likely owing to the presence of the protecting groups on the exocyclic amines. Indeed, the most soluble of the quenched amidites appeared to be that of guanosine, usually the most intransigent of the common nucleosides. The protecting group used for guanosine was an isobutyrl



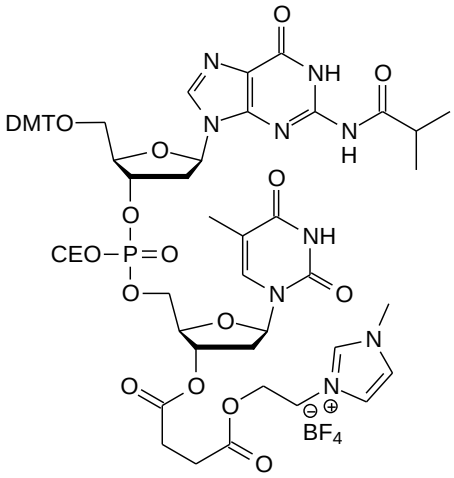
2.7



2.9



2.10



2.11

amide, which is more lipophilic than the benzoyl amides of cytosine and adenosine.

If one were to use this methodology for large scale synthesis, it might be advantageous to switch to the isobutryl protecting group for all amidites requiring protection, thought this needs to be determined.

The three dimers were also detritylated using the same procedure that yielded **2.8** and it was found that the levels of detritylation were only marginally better than for the thymidine dimer. It has been observed in the literature that the rate of detritylation for the thymidine nucleoside is slower than for the other three common nucleosides.[118] The recoveries for ^{HO}dApT, ^{HO}dCpT, and ^{HO}dGpT (**2.12**, **2.13**, and **2.14** respectively) were higher than for **2.8** but this was more likely due to improved technique on the part of the experimenter rather than a real difference in reactivity or solubility of the various species.

2.2.7 Longer deoxy oligos

In addition to the dimers described above, a thymidine trimer (**2.15**, **2.16**) and tetramer (**2.17**, **2.18**) were also synthesized at the 50-100 µmol scale. The identities of the ionic liquid supported compounds have been confirmed with both low and high-resolution ESI-MS (Table 2-1) as well as ^{31}P-NMR in conjunction with the use of a ^{31}P CIGAR experiment,[119] allowing the observation of the expected 3'-5' connectivity through the diastereomeric phosphotriester linkages. The yields are expressed as recoveries since traditional solution phase phosphoramidite chemistry is known to go to 98%-100% completion but for the

methodology employed, there are small losses associated with the manipulations involved in the purifications of the compounds. These losses are likely fixed and larger scale synthesis should show higher proportional recoveries.

Table 2-1: Recovery and physical data for tagged DNA oligomers[a]

Compound	Sequence	%Recovery	^{31}P NMR(ppm)	m/z (exp)	m/z (calc)
2.7	^{DMT}dTpT	91	-1.494, -1.584	1110.4	1110.4
2.9	^{DMT}dApT	89	-1.324, -1.477	1223.4	1223.4
2.10	^{DMT}dCpT	91	-1.545, -1.754	1199.4	1199.4
2.11	^{DMT}dGpT	90	-1.149, -1.194	1205.6	1205.4
2.8	^{HO}dTpT	78	-1.188, -1.284	808.3	808.3
2.12	^{HO}dApT	93	-1.176, -1.516	921.4	921.3
2.13	^{HO}dCpT	95	-1.381, -1.613	897.3	897.3
2.14	^{HO}dGpT	96	-1.047, -1.064	903.4	903.3
2.15	^{DMT}dTpTpT	92	-1.081 to -1.477	1467.5	1467.8
2.16	^{HO}dTpTpT	98	-1.157 to -1.531	1165.2	1165.3
2.17	^{DMT}dTpTpTpT	89	-1.169 to -1.859	1824.2	1824.5
2.18	^{HO}dTpTpTpT	100	-1.142 to -1.510	1522.4	1522.4

[a]Ade *N*6 and Cyt *N*4 benzoyl protected, Gua *N*2 isobutryl protected; 3' terminal thymidine linked through succinyl ester to **1** as in **3** of Scheme 2.

2.2.8 Deprotection and Comparison to Standards

The oligonucleotides synthesized above in solution have been compared to the same sequences synthesized on controlled pore glass (CPG; 1 μmol scale), a commonly used solid support, and the materials obtained by the two methodologies have been deprotected in parallel. Complete deprotection of the desired oligonucleotides was achieved by treating them with concentrated ammonium hydroxide/ethanol for 48 h at room temperature or 16 h at 60 ºC.

These conditions ensured complete cleavage of the cyanoethyl protecting group, the ionic liquid moiety, any protection of the exocyclic amines of the bases (Ade, Cyt and Gua), and the monosuccinate linker. The oligonucleotides were isolated by removal of the ethanol and ammonium hydroxide solution under vacuum, redissolution in water (the solid support was settled by centrifugation for the CPG supported oligomers) and then chromatographic purification by ion-pairing reverse phase HPLC, anion-exchange HPLC or polyacrylamide gel electrophoresis.

The products of the ILSS procedure have been compared via LCMS to those obtained through automated solid-phase synthesis techniques[52] (Table 2-2). In all cases, the retention times of the ILSS generated material correlate well with the CPG generated oligomers. The purities of the oligomers prepared by the two methods were comparable. For the dimers, the ILSS method gave products of better purities, whereas for the trimers and tetramers, the CPG method gave slightly purer products. The origin of impurities appears to be different in the two methods. In the ILSS generated trimer and tetramer the impurities are due to the presence of small amounts of “n-1” peaks which are likely due to the incomplete detritylation at the stage prior to coupling. This tritylated material would not be removed during the post-oxidation extraction and would be deblocked in the detritylation subsequent to coupling giving the n-1 oligomer. Thus it was extremely important to ensure complete detritylation at each step. On the other hand, thymidine nucleoside was visible in several of the HPLC traces of oligomers synthesized on CPG. Its presence arose from

incomplete coupling or partial detritylation of the solid support prior to the initial capping step. Though this is normally present in materials synthesized on CPG, the post-oxidation extraction largely removed this material from the ILSS mediated synthesis.

Table 2-2: HPLC/MS analysis of deprotected oligonucleotides[a]

Sequence	Synthetic Support	Retention Time (min)	% Total Area	Low Resolution MS (-ve mode)
dApT	IL	26.3	99.3	554.2
	CPG	26.3	96.1	554.2
dCpT	IL	16.7	93.3	530.2
	CPG	16.8	89.5	530.2
dGpT	IL	19.0	97.1	570.2
	CPG	18.9	95.1	570.2
dTpT	IL	25.3	91.7	545.2
	CPG	25.3	92.5	545.2
dTpTpT	IL	29.9	92.1	849.1
	CPG	29.5	94.0	849.2
dTpTpTpT	IL	32.4	87.3	1153.2
	CPG	31.9	90.5	1153.2

[a]IL =prepared with ionic-liquid support; CPG=prepared with controlled pore glass with gene machine; % Total Area for ILSS-based oligonucleotides excludes peak area due to the cleaved IL peaks.

2.3 Procedural Optimization

2.3.1 Detritylation

In the work outlined above the detritylation step was identified as a major issue for the proposed methodology. Subsequent work at larger scale has shown that the difficulty was mostly limited to the first nucleoside attached to the ionic moiety through the linker and that subsequent nucleotides underwent

detritylatation in a manner similar to that of the free nucleoside. The difficulty was initially thought to be due to the equilibrium (ROH + $DMT^+ \rightarrow$ DMT-OR + H^+) established upon addition of acid to the closed system of the reaction solution, a problem not encountered in solid-phase synthesis since the acid flows through the reaction, continuously removing the trityl cation generated. However, the lack of success in disrupting the equilibrium by adding simple alcohols such as methanol or ethanol to compete with the nucleoside for re-association with the trityl cation, thus driving the detritylation to completion indicated that the equilibrium was not the problem. Instead the problem was more likely to be a slower rate of trityl cleavage due to the close proximity of the positively charged imidazolium ion of the tag, which made protonation of the *O*5' rate limiting. A similar phenomenon is invoked to explain the slower rate of esterification of glycine (NH_3^+-CH_2-COOH) relative to propanoic acid under acidic conditions.[120] Consistent with this notion, the rate (and thus ease) of detritylation increased as the site of protonation was spatially removed from the positively charged ion tag, allowing the rate of cleavage to revert to the more acceptable level normally observed for free nucleosides and solid supported oligomers (*i.e*., rate of detritylation of trimer > monomer).

A potential method investigated to overcome the slow detritylation of the first nucleoside was to use a Lewis acid such as zinc bromide instead of a Brønsted acid to initiate the deprotection since weaker charge-charge repulsion would be expected between the ion tag and the zinc coordinated 5'-oxygen. In practice, the improvement was minor when zinc bromide was used alone. When

the Lewis acid was used in conjunction with a trityl scavenger such as pyrrole[121] however, a significant improvement was observed. Whereas upwards of twelve treatments with Brønsted acid were sometimes required for complete detritylation at larger scales, complete deprotection was achieved in three treatments using zinc bromide and pyrrole.

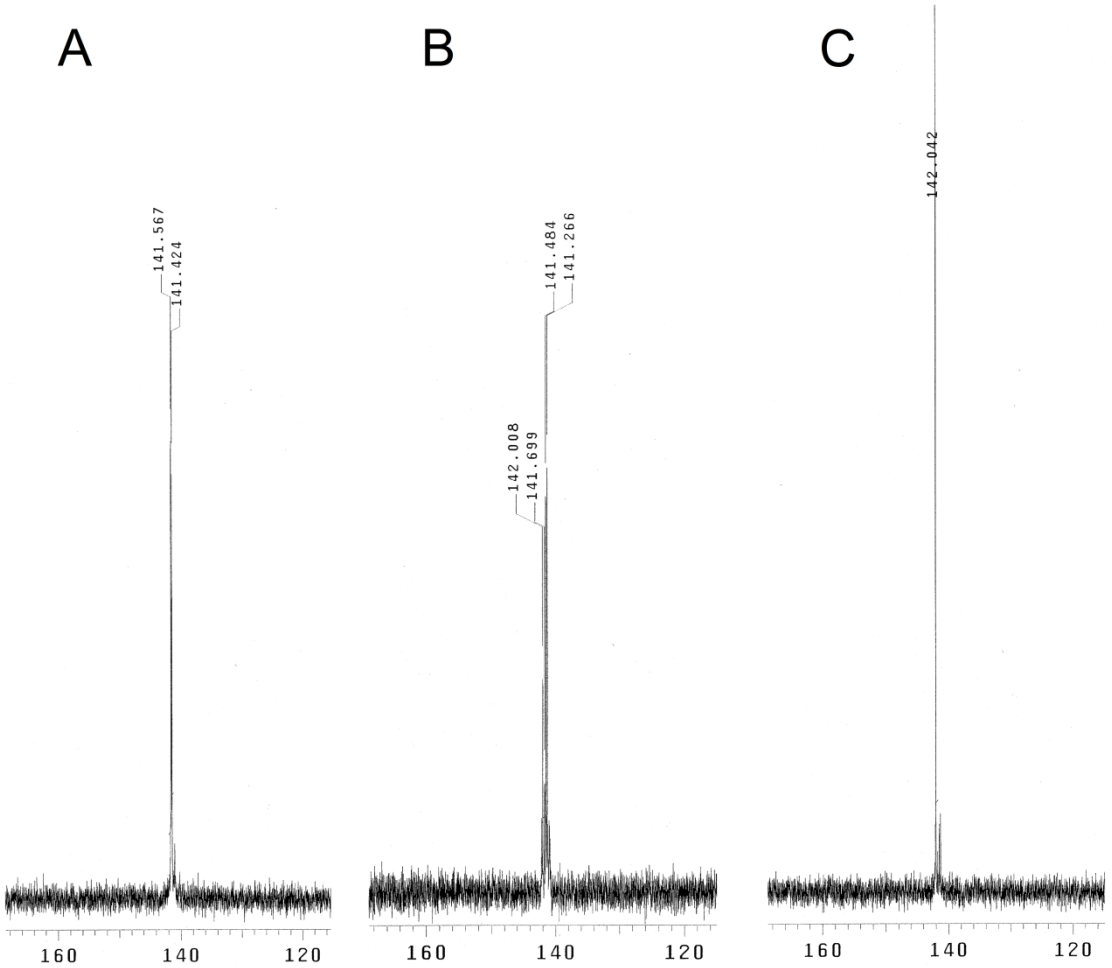
There were several drawbacks of this procedure, including the formation of an insoluble brown precipitate that entrained some of the desired product. In addition, Kierzek *et* al.[122] have shown that deacylation of the protected bases can occur when zinc bromide was used to detritylate nucleosides in the presence of methanol, which was frequently used as a solvent or co-solvent, and deacylation of both guanine and cytosine heterocycles have been observed experimentally. Finally, the high level of toxicity associated with pyrrole makes this reagent undesirable for large scale applications. These factors led to the reversion to using TFA for detritylation even though many treatments were sometimes required for the first nucleoside.

An alternate route to overcoming the detritylation problem would be to simply replace the trityl protecting group for the 5'-hydroxyl of first nucleoside. A protecting group that is not dependant on acid based cleavage, such as levulinyl, could be used for the first nucleoside, while the traditional protection strategy is maintained for the growth of the oligomer.

2.3.2 Quenching

It has been established earlier that in order to successfully separate the coupled oligomer from the excess phosphoramidite at each coupling step of the synthesis cycle it was necessary to quench the excess monomer with an alcohol prior to oxidation of the phosphite triester. This enhanced the excess reagents solubility in the precipitation medium, generally 10% v/v ethyl acetate in diethyl ether, allowing its removal.

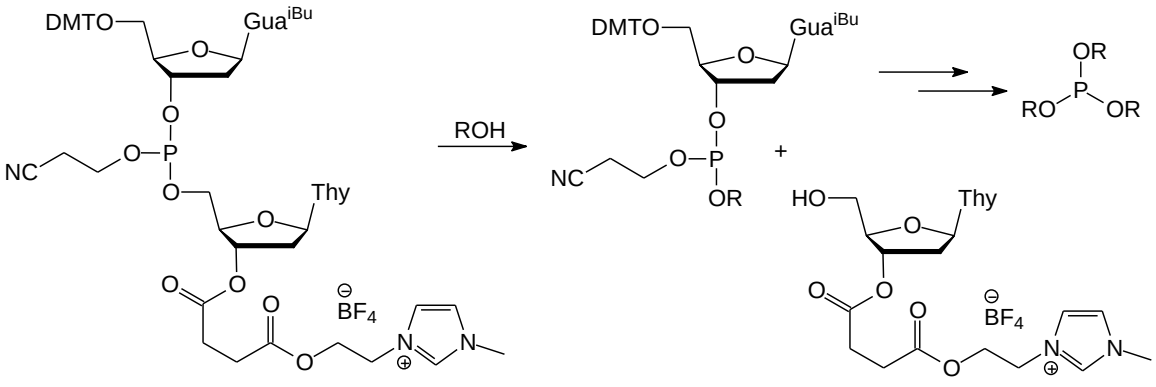
Figure 2-2: Degradation of phosphite triester by methanol upon quenching†



†**A**: post coupling, **B**: Quench at 5 min., **C**: Quench at 30 min.

Initially methanol or ethanol was used to quench the excess amidite but when ^{31}P-NMR and MS were used to characterize the reaction during the optimization process it was observed that even over short periods of time they can cause transesterification of the phosphite triester yielding undesirable compounds. Figure 2-2 shows that over time the two peaks due to the diastereomeric pair of the desired product are replaced by peaks due to transesterification products as illustrated in Scheme 2-3, which eventually coalesce into a single peak after 30 minutes, which did not undergo further change. The MS likewise shows the reappearance of the uncoupled, tagged nucleoside. This clearly shows that primary alcohols are not ideal quenching agents.

The secondary alcohol, 2-propanol was likewise found to cause transesterification, though at a much slower rate than methanol or ethanol. Finally, the tertiary alcohol, *tert*-butanol caused no observable transesterification after a ten hour exposure and was used as the quenching agent for all subsequent experiments.



Scheme 2-3: Transesterification of phosphotriesters by alcohols

2.3.3 Oxidation

The initial methodology employed the same iodine/water solution as the oxidant as is generally used for solid-phase synthesis, requiring an extraction step for purification. Extractions with the tagged oligomers were usually found to be difficult because they had the capacity to behave as strong emulsifiers and the emulsions they form were very slow to break. A non-aqueous based oxidant is preferable and indeed *tert*-butyl hydroperoxide has been demonstrated in solid-phase synthesis to be effective.[123] Used as a 5-6 M solution in decane, which was commercially available and added in five mole equivalents, it was found that the oxidation proceeded very rapidly (<5 minutes) and cleanly, with all the resulting components easily eliminated during the precipitation of the desired material.

2.4 Oligoribonucleotide Synthesis

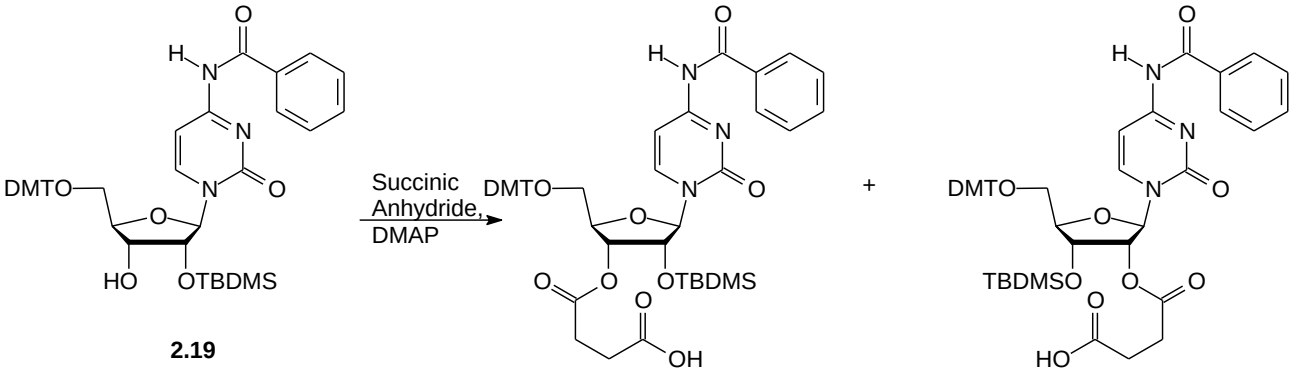
2.4.1 Overview

The sequence selected to test the tagged solution phase synthesis approach in RNA was that of the pentaribonucleotide representing base positions 45 to 49 of an unpaired coil region of *N*-formyl methionine tRNA from *E. coli*. It was selected primarily because it contains all four of the common ribonucleosides and it was also a sequence that has been previously synthesized in solution by Damha and Ogilvie using the phosphite triester approach, which required several weeks to achieve.[124] The latter compound served as a good

reference standard for the pentaribonucleotide made by the process described here.

2.4.2 Component Synthesis

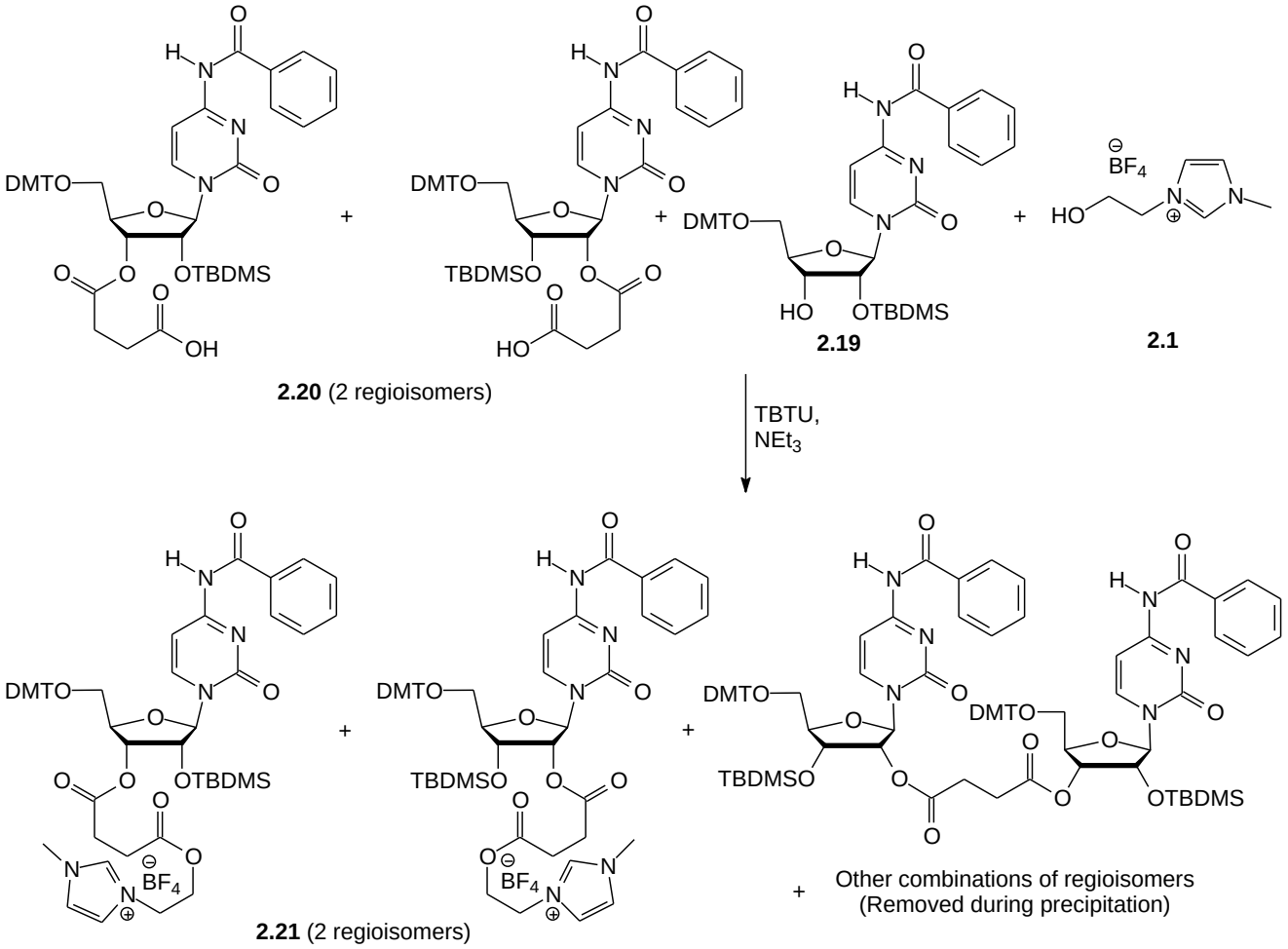
Synthesis began with the attachment of the 3'-hydroxyl of cytidine to the ionic ethyl-methyl-imidazolium tag through a succinyl linker. Thus, *N*-benzoyl-2'-*tert*-butyldimethylsilyl-5'-dimethoxytrityl-cytidine (**2.19**) was reacted with succinic anhydride in the presence of DMAP. The presence of the base, DMAP, caused the 2'-TBDMS protecting group to migrate, to the vicinal 3'-hydroxyl position, yielding a mixture of 2'-TBDMS and 3'-TBDMS protected cytidine with no alteration of the other protecting groups (Scheme 2-4). This 2'/3' isomerization was inconsequential, however, since the 2'/3'-silyl group and the 3'/2' succinyl linker of this terminal nucleoside will be removed to release the required 2' and 3'-OH groups during the final deprotection step of the desired full length pentamer. In addition, it would be expected that the solubility properties of the two regioisomers, an important consideration for the purification steps, would be very similar.



2.19

2.20 (2 regioisomers)

Scheme 2-4: Nucleoside succinylation



Scheme 2-5: Ribonucleoside derivatization with ionic tag

Succinylation at the free 3' or 2' hydroxyl group provided the 2'/3'-succinylated nucleoside along with some unreacted nucleoside. This crude mixture was then esterified with the free hydroxyl group of the ionic tag (Scheme 2-5).

In the earlier work described above, DCC was used as the condensing reagent but long reaction times were required (3-7 days) and the yield of desired succinate ester was only 65-85%. An alternative coupling reagent that is often used for peptides, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium

tetrafluoroborate (TBTU), proved to be more suitable for this reaction; in addition, its counter ion, tetrafluoroborate, matched that of the ionic liquid employed as the tag.

The succinylated nucleoside (**2.20**) and TBTU were first mixed in the solid state, followed by the addition of the ionic liquid [3-(2'-hydroxyethyl)-1-methylimidazolium tetrafluoroborate] (**2.1**), triethylamine and finally solvent. The reaction was complete in less than 90 minutes yielding the desired product (**2.21**) and a small amount of nucleoside dimer; the latter formed by the reaction of (**2.20**) with a small amount of 5'-DMT rC^{Bz} TBDMS that was carried forward from the succinylation step. At this point, a chloroform/water extraction was performed to remove any excess ionic liquid, the excess coupling reagent and the resulting tetramethylurea by-product of the reaction, leaving a mixture of nucleoside dimer and the desired product (**2.21**). The ion tagged nucleoside (**2.21**) was easily separated from the dimer and any other remaining impurities by two simple precipitations. In the DNA oligomer work,[125] the preferred precipitation medium consisted of a 1:9 v/v mixture of ethyl acetate and diethyl ether. This yielded purer precipitates than diethyl ether alone by effectively solubilizing undesired by-products, while generating the desired tagged oligomer as a finely divided precipitate. However, when this anti-solvent was used here, it was found that the tagged nucleoside (**2.21**) was slightly soluble, resulting in a small loss of the desired product and collapse of the precipitate into a slightly impure waxy solid that entrained impurities. This was due to the presence of the highly lipophilic 2'-*tert*-butyl-dimethylsilyl protecting group used for the RNA nucleosides.

Therefore, the anti-solvent was changed to neat diethyl ether as this was found to restore the desired properties of the precipitation medium with no appreciable loss of the tagged materials.

2.4.3 Detritylation

The next step in the synthesis cycle (Scheme 1-7) requires removal of the DMT group at the 5' terminus of the growing oligonucleotide chain. As described above for the work on DNA, this step was often problematic, especially for removing the DMT group from the nucleoside unit directly linked to the ionic tag. The alternate detritylation method employing zinc bromide was judged to be inappropriate for DNA synthesis due to the difficulties in removing it via precipitation and will be even more difficult in the case of RNA synthesis since the solubility of the Lewis acid is even lower in the anti-solvent used, i.e. neat diethyl ether. Though thymidine and cytidine, and to a greater extent adenosine and guanosine, are sensitive to acid mediated depyrimidation/depurination,[126] rendering the application of stronger acids troublesome in DNA synthesis, RNA is not as prone to acid-mediated depurination due to the inductive effects of 2' OH groups, therefore a stronger acid may be employed in the detritylation step. Thus, *p*-toluenesulphonic acid (*p*TSA) was selected as the detritylation reagent as it was found that a single treatment was sufficient to achieve complete detritylation of the first nucleoside. Weaker acids, such as trifluoroacetic acid, required multiple treatments and longer reaction times to achieve the same result. Thus the tagged nucleoside **2.21** was dissolved in a solution of 0.1M *p*TSA in acetonitrile and stirred for 10 minutes. Methanol was added to quench

the trityl cation and the resulting nucleoside was purified by precipitation to yield compound **2.22** in good yield.

2.4.4 Coupling

With the 5' position of the 3' nucleoside deblocked, the oligomerization cycle could begin (Scheme 1-1). The nucleoside (**2.22**) was mixed with 1.66 equivalents of uridine (2'-TBDMS protected) 3'-cyanoethylphosphoramidite (**1.13h**), using DCI as the activator (0.06 M in anhydrous acetonitrile). After 2 hours of stirring, excess *tert*-butanol (approximately 6 equivalents) was added to quench the excess phosphoramidite and to enhance the solubility of the resulting nucleoside 3'-(*tert*-butyl-2-cyanoethyl) phosphite triester by-product in the precipitation medium, as in the DNA synthesis method. The intermediate rUpC dimer (**2.23**) was isolated and purified by adding the reaction mixture dropwise to stirred diethyl ether. The resultant fine precipitate was filtered over celite and recovered from the filter by dissolving in acetone. The change in anti-solvent did not adversely affect the purity of the product since the quenched RNA phosphoramidites are more lipophilic than the corresponding DNA phosphoramidites due to the presence of the TBDMS protecting group and are thus more soluble in diethyl ether and do not require the addition of a co-solvent to solubilize them allowing for their removal during precipitation.

2.4.5 Capping and Oxidation

Once purified, the intermediate rUpC phosphite triester (**2.23**) was redissolved in anhydrous acetonitrile and a capping step was performed using

acetic anhydride and DMAP. In the initial DNA synthesis methodology (Section 2.2.4), oxidation of the intermediate phosphite triester to the phosphate was achieved using aqueous iodine as the oxidant. This procedure necessitated a difficult extraction to remove the salts generated but precluded the need for a capping step, since any unreacted oligomer would be removed with the aqueous phase. However for RNA, the difficulty of the extraction, due to the formation of strong emulsions, outweighed the advantage of the removal of the capping step, especially since the capping step overcomes the undesirable *O*6 extension sometimes observed for guanosine.[57,58] The use of *tert*-butyl hydroperoxide (5-6M) in decane was shown to be successful in the procedural optimization for DNA synthesis (Section 2.3.3) so it was attempted and found to function for RNA as well.

Since the post-oxidation extraction has been eliminated, the capping step was reinstated to eliminate the possibility of uncoupled oligomer from growing in subsequent coupling steps. The oxidation was performed immediately following capping in the same reaction mixture, without prior purification to yield compound **2.24**.

2.4.6 Anion Exchange

At this point, it was observed by mass spectroscopy that the original tetrafluoroborate counter anion of the tag had exchanged for dicyanoimidazolide, an effect that was not observed in the previous work on DNA synthesis. Indeed, anion exchange was observed to occur several times during subsequent steps of the synthesis cycle, alternating between dicyanoimidazolide and *p*-

toluenesulphonate, but this did not appear to adversely affect the solubility properties of the intermediate tagged di (rUpC, **2.25**), tri (rApUpC, **2.27**), tetra (rGpApUpC, **2.29**) and pentanucleotides (rApGpApUpC, **2.31**) isolated during and after each coupling step (see below). It has been previously observed that the solubility properties of the tagged molecules were adversely affected when the counter-ion exchanges for a halide, thus the identity of the counter ion was of importance since a change in solubility can have a dramatic impact on the quality and yield of the synthesis cycle. This must therefore be taken into account during the design of the oligomerization cycle such that if anion exchange occurs, the resulting ion paired molecule maintains an acceptable level of solubility in the solvents used for synthesis and recovery while also maintaining their insolubility in the anti-solvent.

Table 2-3: Recovery and physical data for tagged RNA oligomers

Compound	Sequence	Recovery (%)	^{31}P NMR (ppm)	m/z (calc)	m/z (exp)
2.21	5-DMT rC Succ-IL	73	-	972.4	972.3
2.22	5-HO rC Succ-IL	93	-	670.3	670.2
2.24	5-DMT rUC Succ-IL	93	-	1445.6	1446.0
2.25	5-HO rUC Succ-IL	>100	0.082 to -0.513	1143.4	1143.5
2.26	5-DMT rAUC Succ-IL	91	-	1034.4	1034.5
2.27	5-HO rAUC Succ-IL	>100	0.510 to -0.508	1743.6	1744.4
2.28	5-DMT rGAUC Succ-IL	99	-	1311.5	1311.7
2.29	5-HO rGAUC Succ-IL	91	0.637 to -0.466	1160.4	1161.0
2.30	5-DMT rAGAUC Succ-IL	95	-	1611.6	1611.8
2.31	5-HO rAGAUC Succ-IL	96	0.599 to -1.318	1460.5	1460.8

2.4.7 Oligomerization

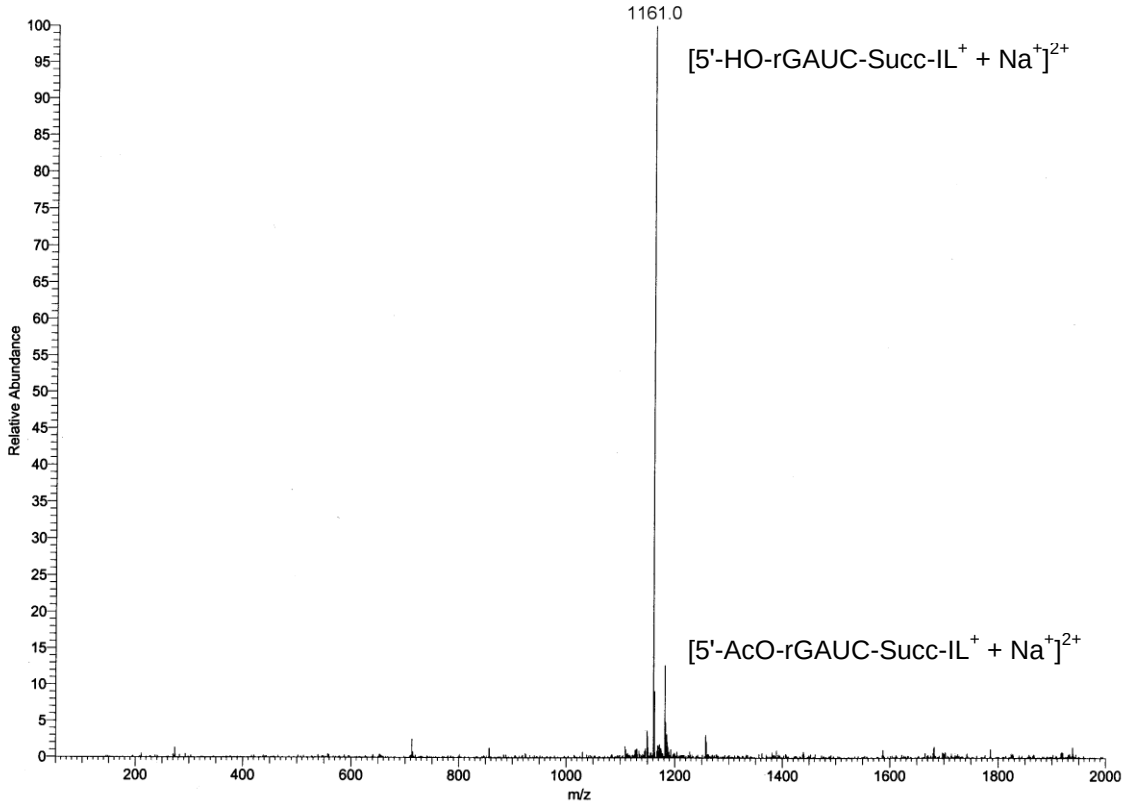
The chain extension proceeded as outlined above but it was found that the amount of material recovered exceeded 100% of the expected yield after the detritylation steps as determined by weight of recovered product (Table 2-3). This was determined to be due to the incomplete removal of *p*TSA, which subsequently interfered with the next coupling steps (e.g. premature detritylation following the next coupling step). Once this effect was realized, the detritylating reagent was substituted with the volatile trifluoroacetic acid, any residual amount of which that was remaining after the precipitation step could be readily removed by evaporation. This approach again necessitated two acid treatments per detritylation step but no further premature detritylation was observed at the coupling stage. Thus, while it has been found that weaker acids may be used for the detritylation step of the oligomerization cycle shortly after the initial nucleoside was deprotected, multiple treatments were still required, so it would be advantageous to use stronger acids if their complete removal could be assured, for instance if a solid supported strong acid were used.

2.4.8 Advantages of Tagged Synthesis of Oligonucleotides

One of the advantages of solution phase synthesis of oligomers over solid-phase was the capacity to directly monitor chain elongation by spectroscopic methods and mass spectrometry (MS). Indeed, ion tagged oligomers were especially amenable to this technique, since the molecules contained permanent positive charges. This meant that ion tagged molecules need only have their

solutions nebulized in an electrospray ionization (ESI) source and they gave very strong signals in the MS.

Figure 2-3: Low resolution ESI-MS of **2.29**



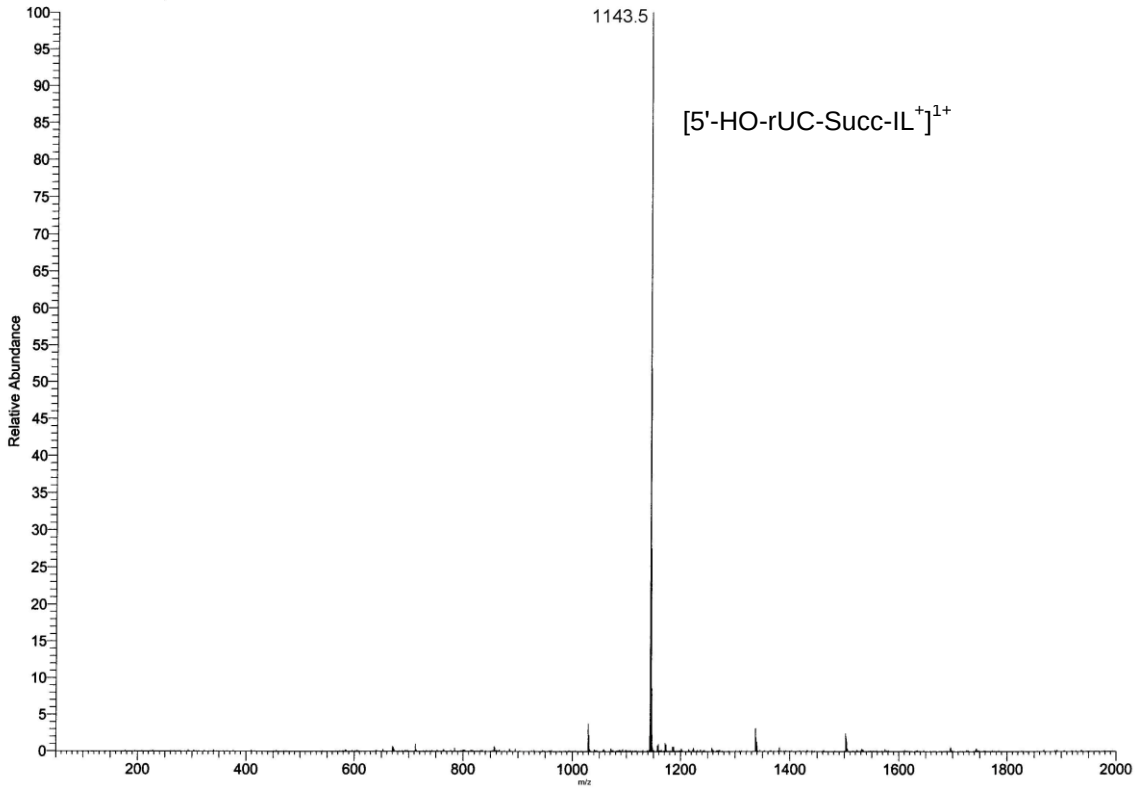
When using an octopole analyzer, short oligomers provided excellent results. Lengthening oligomers quickly exceed the upper limits of the mass detector; however in these cases multiple charging occurred more readily, pulling the analyte back into an observable window (Figure 2-3). This may be observed by comparing the MS for longer oligomers to that of rUpC (Figure 2-4). For the dimer, the ion tag resulted in a high energy barrier to protonation and likewise to

the formation of these adducts. As the chain length increased, the site of protonation was spatially removed from the positively charged ion tag and adducts were readily observed. Using this technique, the identities of all post-detritylation compounds were confirmed by high resolution ESI-MS (Table 2-4).

Table 2-4: High resolution ESI-MS of tagged RNA oligomers

Compound	Sequence	Formula*	m/z (calc)	m/z (exp)
2.22	5-HO rC Succ-IL	$C_{32}H_{44}N_5O_9Si^{1+}$	670.29069	670.29028
2.25	5-HO rUC Succ-IL	$C_{50}H_{73}N_8O_{17}Si_2P^{2+}$	572.21727	572.21795
2.27	5-HO rAUC Succ-IL	$C_{76}H_{106}N_{14}O_{24}Si_3P_2^{2+}$	872.31268	872.31383
2.29	5-HO rGAUC Succ-IL	$C_{97}H_{137}N_{20}O_{32}Si_4P_3^{2+}$	1149.39748	1149.39934
2.31	5-HO rAGAUC Succ-IL	$C_{123}H_{171}N_{26}O_{39}Si_5P_4^{3+}$	966.66590	966.66546

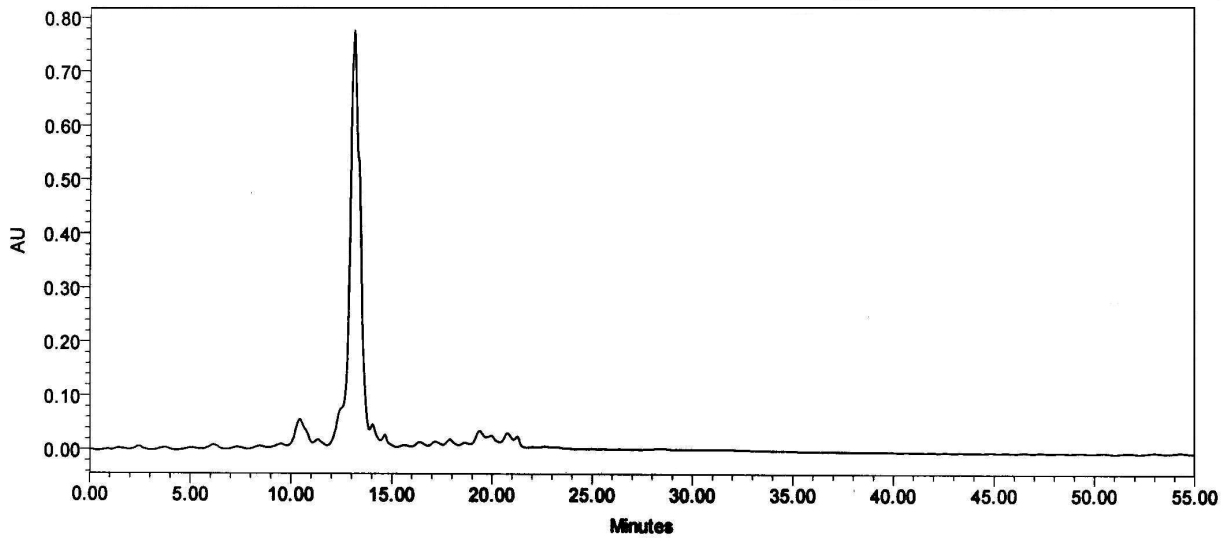
Figure 2-4: Low resolution ESI-MS of **2.25**



2.4.9 Deprotection and Comparison to Standards

Finally, the pentamer (**2.31**) was deprotected to the free oligonucleotide in parallel with a sample of the original sequence made by the original Damha and Ogilvie solution phase approach. This consisted of treatment of 1-2 milligrams of each detritylated (but otherwise fully protected oligomer) with 1 mL of 40% (v/v) aqueous methylamine and incubating at 60 °C for 20 minutes. The resultant solution was cooled to -78 °C and dried under vacuum. The resultant solid was treated with 300 µL of 1.5:0.75:1 (v/v/v) triethylamine trihydrofluoride-1-methyl-2-pyrrolidinone-triethylamine solution and incubated for 90 minutes at 60 °C.[127] The reaction mixture was then cooled to ambient temperature and 1 mL of anhydrous ethanol was added. The samples were mixed and cooled at -78 °C for 30 minutes resulting in precipitates. The samples were then centrifuged at 14000 rpm at 4 °C for 30 minutes and the supernatant was removed. The precipitates were washed with 200 µL of cold ethanol (-78 °C) and then the residual solvent was removed under vacuum. The precipitates were dissolved in 1 mL of autoclaved, deionized water and loaded onto Waters® Sep-Pak™ C_{18} cartridges for desalting. The cartridges were rinsed with water and then the desired products were eluted with 1:1:1 (v/v/v) water-methanol-acetonitrile. The solvent was removed under vacuum to yield the crude, deprotected AGAUC pentamers.

Figure 2-5: HPLC chromatogram of co-injection of deprotected pentamer **2.31** and a solution phase synthesized standard



The identities and purities of the pentamers were assessed using a triethylammonium acetate ion-pairing reverse phase HPLC analysis on a Phenomenex® Clarity™ semi-preparatory scale column. The samples were injected individually and the major peaks in the chromatograms were collected and analyzed by low resolution ESI-MS in the negative mode. The major ion found in both cases corresponded exactly to the expected mass of the fully deprotected AGAUC pentamers missing a single proton (1551 Da). The two samples were also co-injected at a 1:1 ratio on HPLC and found to overlap perfectly (Figure 2-5). As a final test to show that the deprotected compounds were identical, they were analyzed side by side and co-loaded on a 24% polyacrylamide gel and electrophoresed (data not shown). In all cases single bands with the same electrophoretic mobility were found, showing that indeed the

synthesis using the ionic tag yielded an identical compound after deprotection as that of the standard solution phase derived molecule.

2.5 Toward an siRNA in solution

2.5.1 Target

Once the utility of the methodology was demonstrated for both DNA and RNA systems, an attempt was made to synthesize a biologically relevant oligonucleotide. This work was done in collaboration with ScinoPharm Taiwan Ltd. The sequence used for the study (5'-UUAAUUAAAGACUUCAAGCgg-3')[128] corresponded to the antisense strand targeting the firefly luciferase gene. The 3'-end of the oligomer was composed of two deoxyguanosine residues (g) while the remainder was composed of ribonucleotides. The presence of the deoxynucleotides at the 3'-end of the oligomer imparts resistance to 3' exonucleases[128], the primary source of degradation in biological media, thereby increasing the stability of the oligomer, which is an important feature if the molecule is intended as a therapeutic agent, and was thus incorporated into the synthetic target.

2.5.2 Synthesis of oligomer

A new tag (**3.6**, p. 75) was developed for another project (see section 3.2 for discussion) that eliminated the need for a linker and was used here. The tag was condensed to a 5'-DMT, *N*-iBu deoxy-guanosine nucleoside at a 4 mmol scale using an excess of the coupling agent, TBTU, triethylamine and the tag in acetonitrile. The product was purified by first removing the solvent then

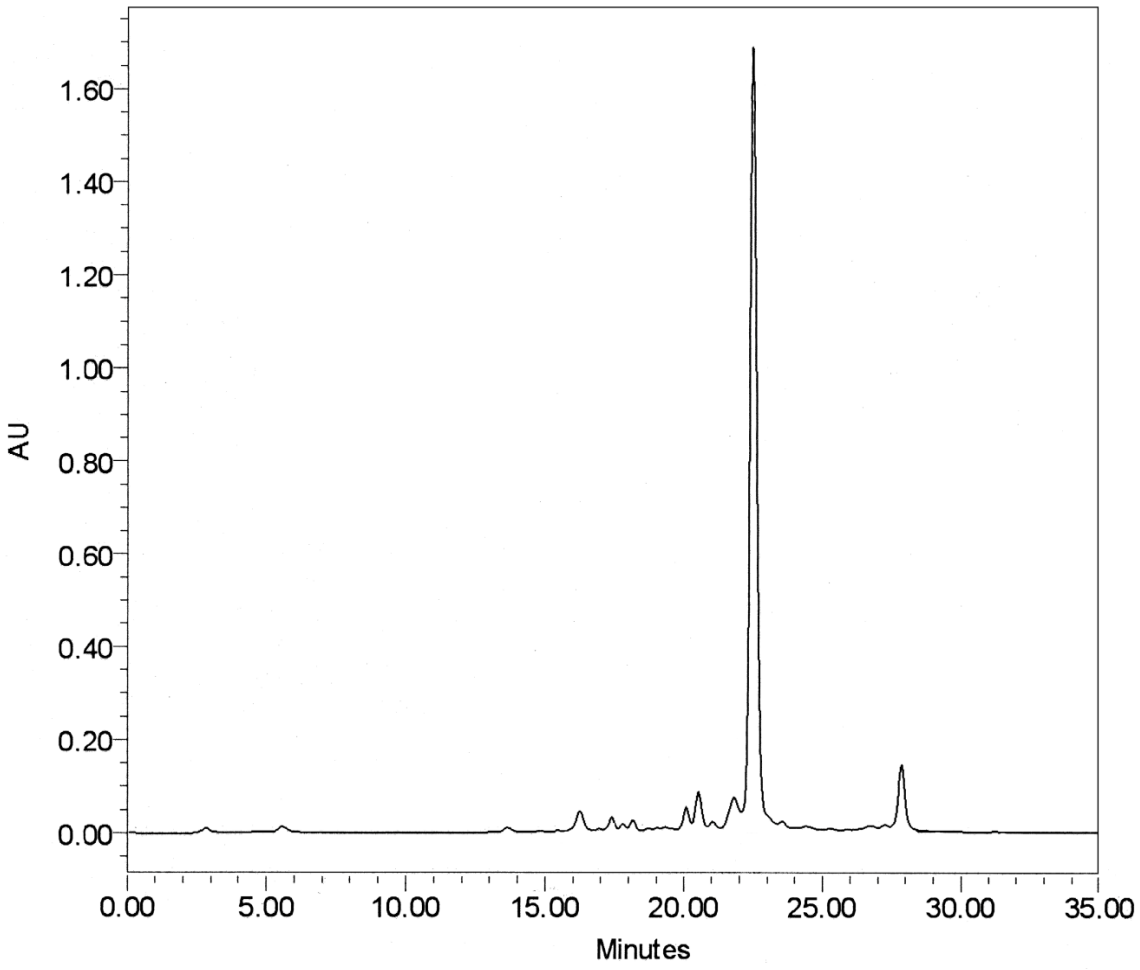
redissolving in dichloromethane and extracting several times with water. The organic phase was dried over magnesium sulphate and the condensed. This portion of the purification removed the excess tag and some of the tetramethylurea as well as hydrolysing the remaining TBTU. The partially purified material was then dissolved in acetone and precipitated from 10% v/v ethyl acetate in diethyl ether several times. The identity and purity of the product was confirmed by MS and NMR and was judged to be adequate.

The tagged nucleoside was detritylated using the procedure outlined previously, requiring a total of four acid treatments followed by a final precipitation without addition of acid but for all of these the anti-solvent was changed from the ethyl acetate/diethyl ether mixture normally used in the DNA work to MTBE (methyl-*t*-butyl ether, 2-methoxy-2-methylpropane). This is a more widely used ether in industrial processes due to its higher boiling point and lower propensity to form peroxides. Since this work was performed in conjunction with an industrial partner the change was necessary for comparability of the work but in all subsequent cases where MTBE was used as the anti-solvent the purity profiles obtained (as assessed by TLC, NMR and MS) were as good as or superior to those observed for the ethyl acetate/diethyl ether mixture.

The detritylated, tagged nucleotide was then condensed with the appropriate amidite to grow the desired sequence using the methodology previously established but using MTBE as the anti-solvent and the entire synthesis cycle was repeated until the desired pentamer (5'-AGCgg-3') was obtained. A small amount was deprotected and analysed by HPLC (Figure 2-6).

There was a concern moving forward however that solubility in the coupling reaction would become a problem so the scale of the synthesis was reduced in half but the volume of solvent was left the same.

Figure 2-6: Anion exchange HPLC of deprotected pentamer



Up to the pentamer stage, the reaction could be monitored by MS even though the molecular weight of the oligomer was above the upper limit of the detector (2000 m/z) due to the presence of multiply charged species, normally sodium ion adducts with the singly charged oligomer generating molecules with

an m/z within the detectable range but sequences longer than the pentamer did not give signals abundant enough to observe. Even the pentamer signal itself was only weakly observed by increasing the sample concentration. This fact precluded the ability to rapidly monitor the synthesis cycle beyond this point to verify that the desired products had been obtained at each stage. It was decided to pursue the synthesis and retain samples for later analysis.

The cycle was repeated as before up to a decamer at which point the scale was again reduced while maintaining the volume of solvent constant. The synthesis was continued and it appeared to progress as before until the twelfth coupling when, during the capping step for this cycle it was found that the oligomer was not being fully dissolved. A small amount of DMF was added to the reaction mixture to resolve the solubility problem and the synthesis was continued to the completion of the twentieth coupling. An increasing proportion of DMF was needed at each stage after the twelfth coupling to obtain a solution.

After completion of the twenty cycles selected intermediate products were deprotected and analysed by HPLC. It was found that the coupling efficiency after the pentamer had been reduced such that the product quality at the decamer was poor and beyond that length it continued to worsen. The presence of the decamer was confirmed by high resolution MS but none of the full length product was observed, though the HPLC analysis showed the presence of sequences longer than ten. It seemed likely that the dilution of the reaction mixture caused the poor coupling at each step given the progression of diminishing coupling.

The concern with solubility was found to be justified as even in the very dilute system (1/4 of the initial concentration) pure acetonitrile was unable to completely solvate the oligomer so the material obtained at the completion of the full synthesis cycle was used to determine that a 15% v/v DMF, acetonitrile mixture was sufficient to solvate a “long” oligomer (though of an indeterminate composition) at a concentration of approximately 0.06M. Using 2 mole equivalents of phosphoramidite with this amount of solvent would yield a concentration of 0.12M for that component, which was the concentration used at the initial scale of the reaction, where the coupling was found to be acceptable. The onset of poor coupling was observed at the hexamer stage, after the scale reduction without a corresponding decrease in solvent volume resulting in an amidite concentration of 0.06M, much lower that was normally used, and the loss in coupling efficiency intensified after the further scale reduction after the decamer. The hypothesis of poor coupling being due to low amidite concentration was supported by the results of ScinoPharm Taiwan Ltd.

2.5.3 Collaborators results

ScinoPharm Taiwan Ltd. undertook the synthesis of the sense strand of the firefly luciferase gene (5'-GCUUGAAGUCUUUAAUUAAtt-3') using the original tag **2.1** attached through a succinyl linker. The synthesis procedure followed was closer to that described in section 2.2 for oligodeoxyribonucleotide synthesis than the refined procedure used for the in-house attempt to synthesize the siRNA target except that MTBE was used as the anti-solvent.

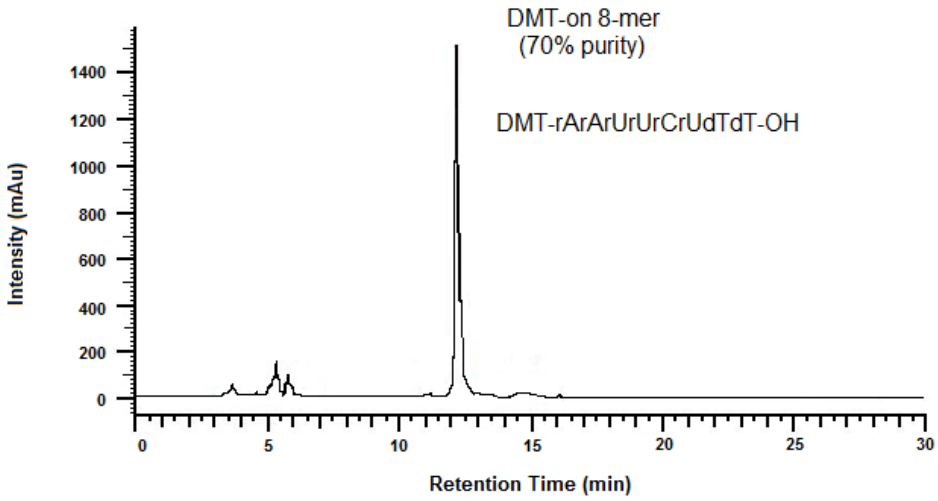
Scinopharm monitored the synthesis closely by removing small aliquots from each coupling step after precipitation but before capping and oxidation. The removed sample was oxidized and fully deprotected, then analyzed by LC-MS prior to proceeding to the next step. In many cases this revealed incomplete coupling, in which case the bulk was redissolved and more amidite was added until the coupling was found to have gone to completion. This work was performed concurrently with the work at McGill thus the concentration effect had not yet been realized and the couplings were sometimes performed at dilutions later found to be detrimental to adequate coupling. However the increased monitoring allowed the couplings to be repeated, sometimes several times, if they were indeed incomplete.

Using this method the heptamer was successfully synthesized but the subsequent coupling to the octamer could not be completed even after seven attempts. Several potential explanations were considered, one of which was the possibility of sequence specific problem. It has been shown by Sekine and co-workers[129] that protected oligomers can aggregate in solution, creating conditions that hinder chain extension. Another factor taken into account was water content of the reaction.

The first point was addressed by redesigning the sequence, the 3rd and 4th nucleotides were changed from AA to UC, respectively. The second point was addressed by adding molecular sieves to the reaction as well as co-evaporating the reagents with dry acetonitrile three times. Employing these changes, the octamer was achieved easily, though the amount of phosphoramidite had to be

increased at the synthesis of the heptamer and octamer. Figure 2-7 shows the purity of the successful revised octamer sequence. Subsequent to this a decamer was successfully completed, likewise with 70% purity using the same analysis.

Figure 2-7: Trityl on RP-HPLC of ScinoPharm generated octamer



2.5.4 Conclusion

A method has been developed capable of a more scalable synthesis of DNA and RNA oligomers in solution. The method has been modified and optimized for solution phase from that used in the conventional solid-phase synthesis in common use. The method has been shown to be effective up to the decamer stage but it can become more difficult past the heptamer, requiring longer coupling times and an increase in the amount of phosphoramidite needed to complete the step, though still much less than the molar excess used in solid-phase synthesis. Although the ultimate achievable length by stepwise synthesis

is yet unknown, the risk of failure prior to achieving the therapeutically relevant henicosamer (21-mer) grows with each step, making this approach less attractive. An alternative approach would be to produce phosphoramidite blocks that achieve longer chain extensions at each step, for instance a dimer or trimer block, which would cut down the total number of coupling steps during the synthesis of the target oligomer drastically. Indeed one could envision two decamer blocks being condensed to a tagged monomer, achieving the relevant henicosamer in two steps. Approaches to achieving these polymeric building blocks will be explored in Chapter 3.

Chapter 3 Approaches to Oligomeric Block Amidites

3.1 Introduction

The synthetic approach to preparing oligonucleotides by first synthesizing short sequences and then condensing those sequences as blocks to yield longer oligomers was first carried out by Khorana and co-workers[130] in the early days of research into the field of nucleic acid synthesis. Their initial approach[131-133] involved the chemical synthesis of dimer blocks of oligodeoxyribonucleotides using the phosphodiester approach and then phosphorylating and deprotecting the appropriate positions, 5' and 3', of the dimers. The individual dimers were then simply mixed with a coupling agent and the various polymer length products obtained were studied, generally consisting of mixtures ranging from tetramers to hexadecamers, depending on reaction time and concentration. As the researchers gained more experience, the condensations were performed in a controlled manner, exploiting orthogonal protecting groups to generate blocks of defined length and sequence, and assembling them as longer oligomers.[134-136] The longer oligomers still never exceeded hexadecamers, though, and were of limited sequence variability, relying on the synthesis of dimers that were coupled to form tetramers. These tetramers were in turn coupled to form octamers and finally the targeted hexadecamers, which were often obtained in poor yields. The methodology was also used by other researchers, with similar results. [137,138]

It was only with Khorana's seminal achievements of the total chemical synthesis of the double stranded genes of the major alanine yeast tRNA,[139,15,140-151] 77 nucleotides in length for each strand, and the precursor of a tyrosine suppressor tRNA from *E*. *Coli*,[16,152-161] 126 nucleotides in length for each strand, that complex sequences of nucleotides were assembled in a controlled manner. This was achieved by generating short blocks that were coupled together, again using the relatively primitive phosphodiester approach, to yield blocks up to icosamers with partial complementarity between the segments of the sense and antisense strands. This complementarity was exploited and used as templates to perform the final assembly of the full length oligomers, 77 or 126 nucleotides, using enzymatic ligation. Without the methodology to generate defined oligomeric blocks, though, this landmark achievement could not have been realized.

It was still later that researchers began to exploit alternative synthetic routes in nucleotide coupling for block condensation methodologies, such as the phosphotriester approach discussed in Chapter 1, with much of the work devoted to the nature of the phosphate protecting group and the nature of the coupling reagents, in an effort to improve yields.[162] Also researchers began to explore the possibility of generating oligoribonucleotide blocks, this being more difficult due to the presence of the 2'-hydroxyl group and the protection it required, thus this line of research lagged behind that of oligodeoxyribonucleotide blocks.[63,163-172]

Some effort was invested into using block condensation to grow oligodeoxyribonucleotides on solid support but, with the techniques used at the

time, monomer based coupling was seen as a better route to achieve the desired products.[173-175] Some research was also performed in generating oligomers on solid support, cleaving them and transforming the products into condensable blocks but by this time it was simply easier to synthesize the desired sequence linearly, entirely on a solid support.[176]

It was only when interest began to grow in performing random mutagenesis in proteins through the replacement of entire codons in a synthetic gene to avoid the generation of large numbers of non-coding segments that often resulted by performing single point mutations in the nucleotide sequence, that interest in the synthesis of block condensation came to the fore again.[177-182] The targets became condensable DNA and RNA trimer units, which were compatible with automated synthesizers, since codons are composed of trimeric nucleotide sequences. The new standard coupling approach had become the phosphoramidite method[43,52,183] and so researchers sought methods to synthesize block phosphoramidites in large quantities.[184-186]

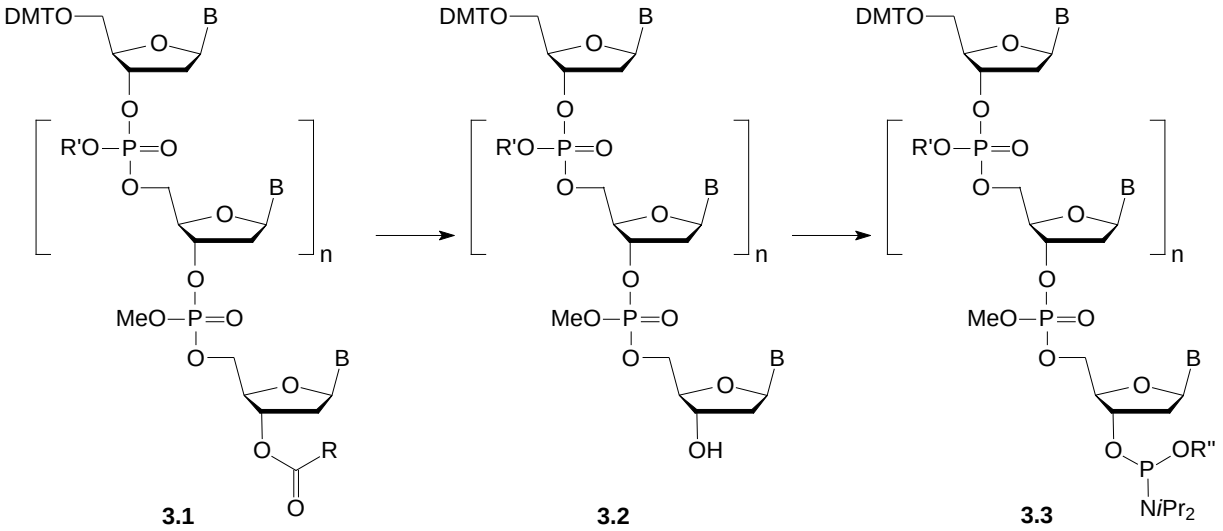
The final driving force behind research into the synthesis of oligonucleotide blocks in recent years has been the prospect of oligomers as therapeutic agents. Reese and Yan demonstrated, in their synthesis of Vitravene, that block condensation of trimer units in solution using the H-phosphonate method was an achievable goal.[68]

This chapter summarizes the work that has been performed and the future work that is proposed to achieve the goal of developing a methodology to synthesize reasonably large quantities of short oligonucleotides in solution using

the ILSS approach developed in Chapter 2 and then converting these tagged oligomers into 3'-phosphoramidite derivatives that could be used in block condensations.

3.2 AIL–Imidazolium butyric acid based tag

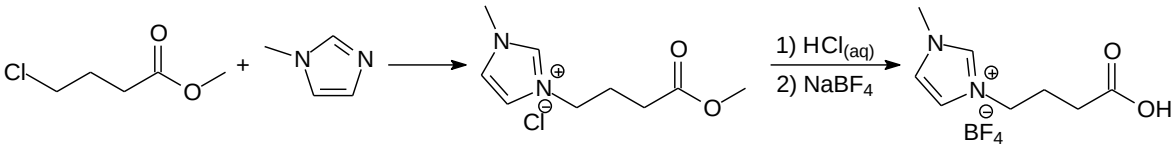
Previous reports had shown that it was possible to prepare oligomers containing methyl protected dinucleotide phosphotriesters, analogous to **3.1** (Scheme 3-1), and to partially deprotect them while leaving the internal phosphotriester linkages intact.[177,187-189] Some of these reports include the cleavage of ester protecting groups from the 3'-end of the oligomer to yield structures analogous to **3.2**,[177,187] as would be desired in an ILSS based approach, since the tag is attached to the nucleoside as an ester.



Scheme 3-1: Synthesis of oligomer block phosphoramidites

The reported deprotection conditions involved often protracted exposure times since the goal of those reports was the complete deprotection of the oligomer except for the phosphotriester, and the half-lives of the amide protecting groups of the exocyclic amines of the nucleobases were long. The half-lives of the esters used, however, were likely much shorter. It was hoped that the rate of cleavage of a 3'-ester, such as is used in the ILSS approach, would be different enough from the rate of cleavage for the amides that this difference could be exploited to effectuate the selective cleavage of the tag and generate the desired 3'-liberated oligomer.

To test this approach, a deoxynucleoside trimer was devised, 5'-CAG-3', which contained the three nucleobases that require amide protecting groups. The fourth common nucleoside, thymidine, was left out since it does not require protection.



3.4 **3.5** **3.6**

Scheme 3-2: Synthesis of acid containing ionic liquid (AIL)

Instead of continuing to use the original ILSS tag, a streamlined soluble support was envisioned that would eliminate the need for a separate linker, such as the succinate previously used, thus eliminating a step in the preparation of the 3'-nucleoside of the target oligomer and simplifying the cleavage from the support. The new tag would contain an imidazolium moiety attached to a short

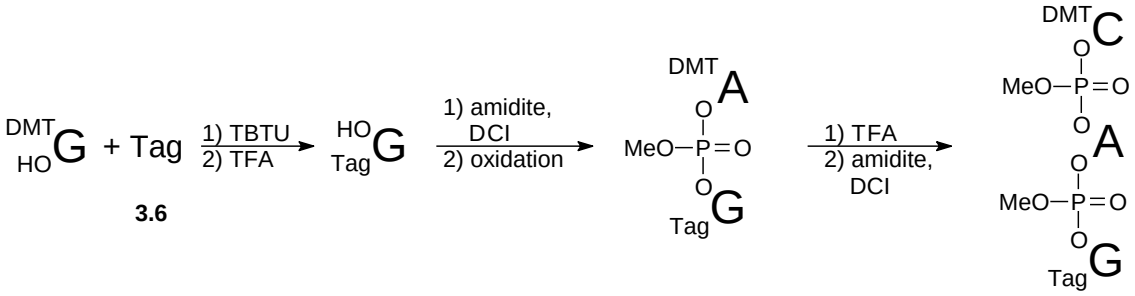
carbon chain that terminated in a carboxylic acid instead of the hydroxyl of the original tag. Such an entity was found in the literature[190] and was thought to be suitable (Scheme 3-2).

In order to obtain the desired molecule, **3.6**, NMI was condensed with methyl-4-chloro-butyrate, **3.4**, under solvent free conditions at 60 °C for 24 hours. The resultant reaction mixture was then rinsed several times with diethyl ether and subjected to reduced pressure to remove any excess reagent or residual solvent. The ester, **3.5**, was converted into the desired carboxylic acid by refluxing the compound in concentrated aqueous HCl for 12-18 hours. The reaction mixture was subsequently dried under reduced pressure for several days. The resultant solid was then suspended in acetone and mixed with sodium tetrafluoroborate to perform the necessary anion metathesis. After stirring for 18-24 hours, the undissolved material was removed by filtration and the organic layer was evaporated to dryness. The identity of the brown oil, **3.6**, thus obtained in 75-80% yield was confirmed by LR-MS and NMR.

The synthesis of the trimer began by coupling the acid containing ionic liquid (AIL), **3.6**, to the first nucleoside, *N*-Bz-5'-DMT-deoxyguanosine in anhydrous acetonitrile, using TBTU (the uronium salt of the same counter ion as the tag, i.e. tetrafluoroborate) in the presence of triethylamine. Initially, care was not taken as to the order of addition and AIL was added prior to triethylamine but when this was done, the solution quickly turned orange indicating that trityl cleavage was occurring due to the presence of the carboxylic acid. Subsequent to this, triethylamine and AIL were always premixed in acetonitrile prior to

addition to the nucleoside. When the proper order of addition was used, the desired tagged nucleoside was obtained in 95% yield after purification by aqueous extraction and precipitation from the anti-solvent, MTBE.

As with the original tag, the trityl cleavage of this first nucleoside required several acid treatments but was accomplished with 92-94% recovery. The complete removal of trityl was confirmed by TLC and LR-MS. Chain extension, as depicted in Scheme 3-3, was achieved as outlined in Chapter 2, the recovery of which were similar to those observed previously, all in the range of 94-97%.



3.6 **3.7**

Scheme 3-3: Trimer synthesis using AIL tag

With the desired trimer, **3.7**, in hand, attempts were then made to effectuate the selective removal of the tag. The cleavage reactions were monitored either by LR-MS and/or by TLC for possible products. Monitoring by MS in the positive mode could show the disappearance of the starting material, though not necessarily a strong signal for the appearance of the desired product, since the starting material displays an enhanced MS signal due to the permanent positive charge of the tag but the product is neutral and requires a proton, sodium ion or other ion to form an adduct and generate a signal. The negative mode was a better option for observing the products, given the basicity of the

deprotection solution, and especially for observing the undesirable cleavage of the methyl phosphotriesters since the product of that reaction would be an easily ionizable phosphodiester. Monitoring by TLC would be less precise, since both the tagged starting material and the undesirable methyl phosphotriester cleavage product would remain on the baseline and thus be indistinguishable. Only a positive result would be observable since the desired product would elute and a disappearance of material on the baseline could also be observed. In most cases MS was used as the primary indicator but TLC was employed if the deprotection condition was very similar to one already attempted. Results of all attempts made are summarized in Table 3-1.

The first approach attempted to selectively cleave the tag involved the use of methanolic potassium carbonate.[187,188] The procedure summarized in the literature was to mix the fully protected oligomer (or solid support attached to the oligomer) in a large molar excess of a solution of 0.05M K_2CO_3 in anhydrous methanol and this reaction was allowed to proceed for several hours in order to achieve the complete removal of all protecting groups except for the methyl phosphotriester.

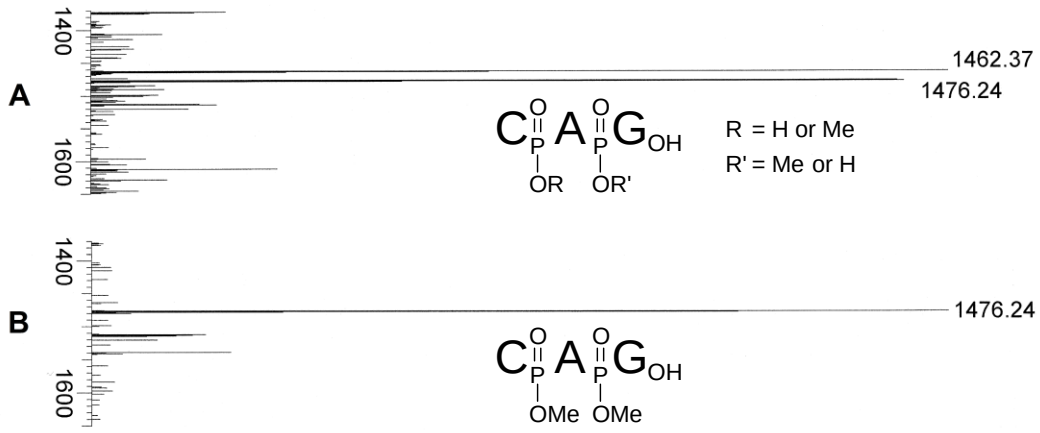
In order to adapt the methodology to this work, it was decided to begin with 5 mole equivalents of K_2CO_3 in methanol at the same concentration as reported. Indeed the initial result was promising, after 15 minutes of reaction time followed by a workup to quench the base and halt the reaction, the tag had been completely removed, no significant demethylation was observed but some cleavage of the nucleobase protecting groups had occurred.

Table 3-1: Results of AIL cleavage assays

Condition	**Result** (MS unless otherwise stated)
5 eq. 0.05M K_2CO_3	Complete removal of AIL, no demethylation, some base deprotection
2 eq. 0.05M K_2CO_3	Complete removal of AIL, significant demethylation
2 eq. 0.05M K_2CO_3, filtered through silica gel	Complete removal of AIL, recovered 30mg of desired material (<35% yield)
2 eq. 0.05M K_2CO_3, quenched with weak acid (DCl)	Complete removal of AIL, significant demethylation
2 eq. 0.05M K_2CO_3, quenched with glacial acetic acid, extracted in $CHCl_3$/PO_4 buffer	Complete removal of AIL, significant demethylation
Co-evaporated with pyridine (x3), 2 eq. 0.05M K_2CO_3, acetic acid quench, vacuum	Significant demethylation (by TLC)
2 eq. 0.05M K_2CO_3, -40 °C	Incomplete removal of AIL, significant demethylation
2 eq. 0.05M $KHCO_3$, 3 days	Complete removal of AIL, significant demethylation/base deprotection
2 eq. 0.05 KCH_3CO_2	Incomplete removal of AIL, significant demethylation
2 eq. 0.05M K_2CO_3, 0 °C	Incomplete removal of AIL, significant demethylation
2 eq. 0.05 KCH_3CO_2, 3 days	Incomplete removal of AIL, significant demethylation/base deprotection
5% NH_4OH in anhyd. MeOH	Incomplete removal of AIL, significant demethylation
1 eq. TBAF (2nd eq. @ 30 min)	Incomplete removal of AIL, no demethylation
10 eq. TBAF	Complete removal of AIL, competitive demethylation
2:1:1 MeOH/NEt_3/H_2O overnight	Complete removal of AIL & base protection, some demethylation
2:1:1 MeOH/NEt_3/H_2O, extracted @ 30 min	Incomplete removal of AIL, trimer degradation

A decrease in the number of equivalents of K_2CO_3 relative to substrate was thought to be a good approach to mitigate the cleavage of base but it also required a longer reaction time to completely cleave the AIL and this led to demethylation (Figure 3-1, **A**). Many variations were attempted in order to improve the result, including alteration of the workup in case that was the problem, but to no avail. The presence of water was an obvious concern since it would lead to the formation of hydroxide ions capable of cleaving the methyl phosphotriester so attempts were made to ensure that all possible sources of water were eliminated, even the starting material was rendered anhydrous by azeotroping off water using pyridine, with little effect in the cleavage results. The only method that generated the desired product reasonably cleanly was to quench the reaction on silica gel and elute the product immediately (Figure 3-1, **B**). This approach, however, yielded less than 35% of the desired material, too great a loss to be a viable procedure so alternate deprotection conditions were explored.

Figure 3-1: Representative LR-MS results for attempted AIL removal[†]



[†]Note: (-)-mode LR-MS representing: **A**) substantial demethylation; **B**) selective AIL removal

Other reports employed the use of ammonium hydroxide in methanol to achieve deprotection of oligomers in the presence of methyl protected phosphotriesters.[177,189] For the AIL tagged trimer, however, this approach proved unsuccessful. The 3' ester protecting groups cleaved in the literature reports were more labile than AIL proved to be. In some of the reports it was believed that the presence of mixed protection in the backbone of the substrates studied (i.e. some of the phosphates were protected by cyanoethyl groups, which cleaved very quickly) slowed the cleavage of the methyl phosphotriesters considerably, due to the build-up of negative charge, which was not the case in the AIL tagged trimer.

Finally, methods known to cleave esters in the presence of amides, such as TBAF[191] or a 2:1:1 v/v/v mixture of triethylamine/methanol/water, proved unsuccessful on the considered substrate, sometimes causing demethylation and sometimes nucleobase deprotection. A full summary of the cleavage methods attempted with pertinent observations is shown in Table 3-1.

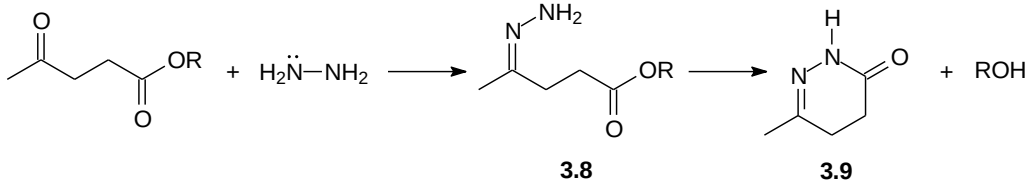
The AIL ester appeared to be too chemically similar to the methyl phosphotriester in terms of its cleavage characteristics and too close in its rate of cleavage to that of the nucleobase protection to be exploited as a selectively cleavable tag.

3.3 Approaches to an orthogonally cleavable tag

3.3.1 Rational and design

After the limited success of the selective AIL ester deprotection, another approach was needed to successfully achieve the objective of releasing an oligomer at the desired 3'-hydroxyl without concomitant removal of the other protecting groups. A tag capable of truly orthogonal cleavage was required. Given the sensitivity of the other protecting groups to acid (trityl group), base (amides of the exocyclic bases as well as the phosphate protecting group), and fluoride (2'-silyl ethers of RNA), an alternative set of reagents would have to be used.

Several moieties have been employed in the past as orthogonal protecting groups to block the 3'-hydroxyl of an oligomer of DNA or RNA, with the goal of generating polymeric building blocks as intended here.[169,172,177,180,186] One of these is the levulinyl ester, which employs one of the mildest set of cleavage conditions, simple treatment with hydrazine hydrate mixed with pyridine and acetic acid has been shown to be compatible with the protecting groups generally employed in oligonucleotide synthesis.



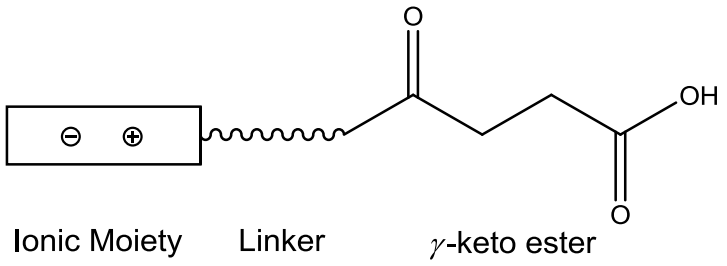
3.8 **3.9**

Scheme 3-4: Hydrazine cleavage of levulinyl esters

The pathway by which this cleavage occurs (Scheme 3-4) begins with the attack of hydrazine on the levulinyl methyl ketone to form a hydrazone intermediate, **3.8**, which then quickly cyclizes to give 6-methyl-4,5-dihydropyridazin-3(2H)-one, **3.9**, and the liberated alcohol.

The formation of hydrazones, however, is not limited to methyl ketones, thus the critical moiety in the protecting group is simply the *γ*-keto ester. The goal then becomes the synthesis of a molecule of the form shown in Figure 3-2, which should contain an ionic moiety for use in the precipitation based purification employed during the ILSS approach to form the oligomer block, a *γ*-keto acid moiety that can be esterified to the 3'-hydroxyl of the desired 3'-nucleoside for later cleavage with hydrazine, and a moiety to link them, most simply a short alkyl chain of one or more carbons.

Figure 3-2: Basic design of a hydrazine cleavable ionic tag

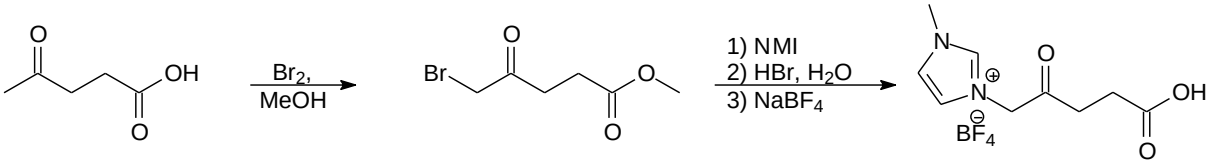


3.3.2 Levulinic acid derivative approach

The first approach used to generate a tag based on the levulinyl protecting group was to explore using levulinic acid itself as the precursor. It has been shown that levulinic acid is easily converted to methyl 5-bromo-4-oxopentanoate, **3.10** (Scheme 3-5).[192] Direct reaction with a suitable amine would yield a tag

attached to a *γ*-keto ester by a one carbon linker, the simplest form of the target molecule shown in Figure 3-2.

Freshly distilled levulinic acid was first dissolved in anhydrous methanol and then an excess of bromine was added slowly over 15 minutes. The reaction was stirred at room temperature for 30 minutes and was then refluxed for 3.5 hours. The solvent was subsequently removed; the product was dissolved in diethyl ether and then extracted with saturated aqueous sodium bicarbonate. The organic layer was dried over anhydrous magnesium sulphate and the solvent



3.10 **3.11**

Scheme 3-5: 5-Bromolevulinate approach to orthogonal tag

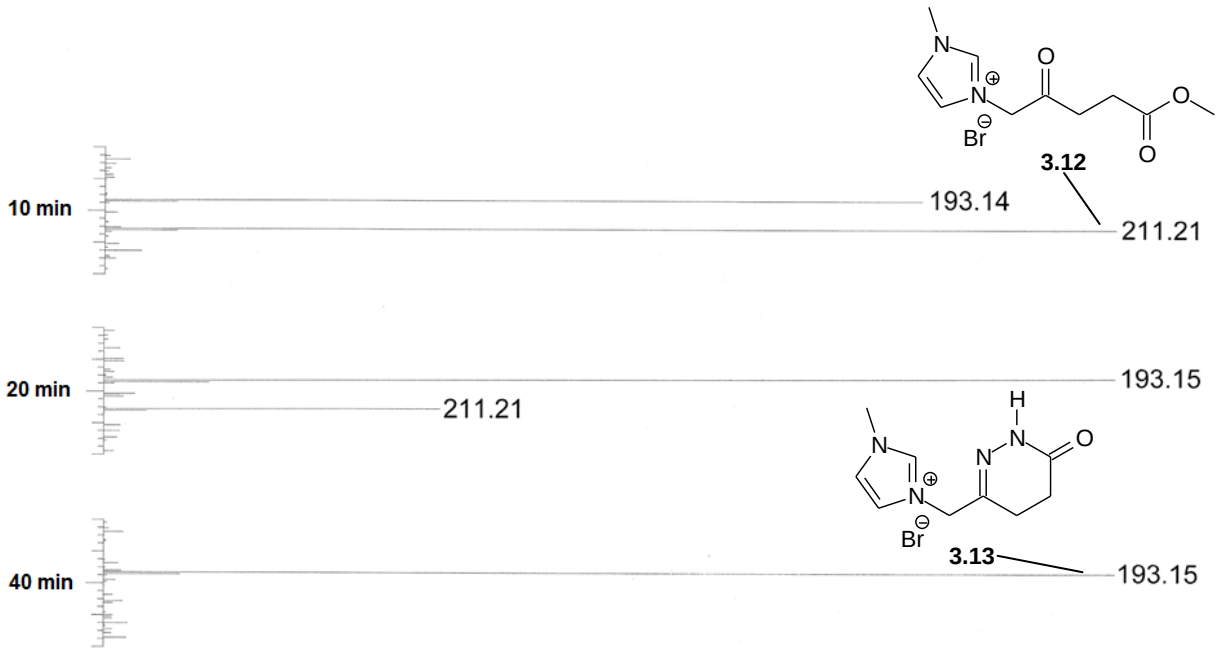
was again removed under reduced pressure. The desired product, **3.10**, was isolated by vacuum distillation in 58-60% yield. The major secondary product was the 3-bromo analogue, which accounted for approximately 25% of the remaining yield.

This *α*-bromo ketone (**3.10**) was first reacted with neat pyridine but the reaction was so exothermic that the pyridine flash boiled upon addition, therefore subsequent reactions were performed by slow additions of the amine to a cooled solution of **3.10** in acetonitrile. NMI was also condensed with the substrate, as shown in Scheme 3-5, in 96-98% yield, with the excess amine easily removed by multiple rinsings of the product with MTBE.

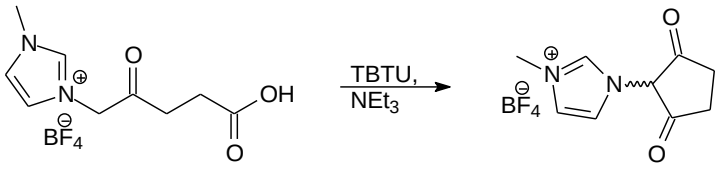
At this early point it was desirable to test the hypothesis that the *γ*-keto ester could be successfully cleaved with hydrazine hydrate, thus liberating the alcohol from the tag. A small amount of the imidazole based tag, **3.12** shown in Figure 3-3, was mixed with 5 equivalents of a 0.5M hydrazine hydrate solution in 3:2 v/v pyridine-acetic acid and the reaction was monitored over time by LR-MS.

Indeed, the reaction proceeded such that the starting material was entirely consumed within 40 minutes, yielding the cyclic hydrazone by-product **3.13** and presumably concomitant release of the alcohol, though methanol was too light to be observed by MS. The 40 minute timeframe is somewhat longer than was hoped for, since treatments to cleave levulinyl esters are usually performed for

Figure 3-3: LR-MS time-course of treatment of **3.12** with hydrazine



only 20 minutes, but would likely still be acceptable as the cleavage of the other protecting groups under these conditions requires much longer exposures.

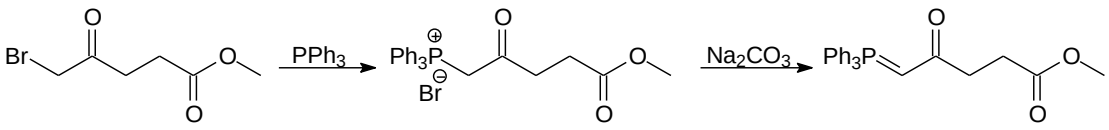


3.11 **3.14**

Scheme 3-6: Result of coupling reaction

In order to complete the tag synthesis (Scheme 3-5), the ester, **3.12**, was converted to a carboxylic acid by refluxing in aqueous hydrobromic acid overnight. LR-MS indicated complete hydrolysis of the ester therefore the excess acid and methanol generated in the reaction were removed *in vaccuo*. The anion was then exchanged by stirring this bromide salt of the tag in acetone with sodium tetrafluoroborate to give, **3.11**, giving the tag a more useful solubility profile as the tetrafluoroborate salt. The excess sodium tetrafluoroborate proved difficult to remove but its presence was unlikely to interfere with the subsequent coupling reaction so **3.11** was carried forward with this impurity present.

The coupling to a nucleoside was initially attempted using the same

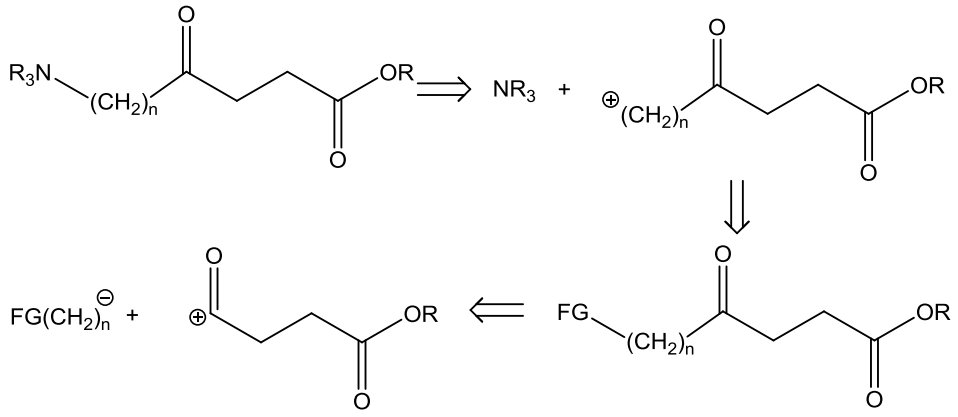


Scheme 3-7: Formation of the phosphonium ylid

procedures employed with the AIL tag, using TBTU as the coupling agent in the presence of either pyridine or triethylamine but none of the coupled product was observed. A more common coupling agent, DCC, was also used but did not produce better results. Upon re-examination of the LR-MS data for the TBTU/triethylamine based coupling, it was observed that none of the starting tag remained but a new species was generated corresponding to the loss of water. It was then realized that the protons on the carbon *α* to both the carbonyl and the imidazolide would be somewhat acidic and that internal cyclization to yield **3.14** (Scheme 3-6) was possible. A similar result was described by Ronald *et al*. in the formation of the ylid depicted in Scheme 3-7, which was achieved by treating the intermediate phosphonium bromide with sodium carbonate.[193] This result indicated that the linker must be longer than a single carbon in order to avoid this effect and that this particular approach using a levulinic acid derivative was not feasible; another route to the target was needed.

3.3.3 Grignard based approaches to orthogonally cleavable tag

The second retrosynthetic strategy explored (Scheme 3-8) in the synthesis of the *γ*-keto acid based tag was to generate a compound through carbon-carbon bond formation at the ketone of the already intact *γ*-keto acid moiety with a linker capable of further functionalization. This group (FG) would be amenable to subsequent condensation with an amine to yield the ionic portion of the molecule. The carbon-carbon bond forming reaction chosen to accomplish this was the Grignard reaction.

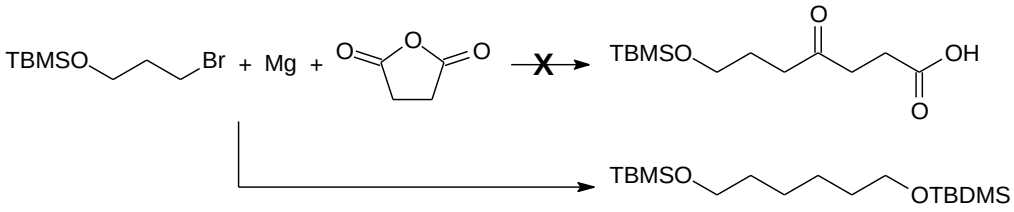


Scheme 3-8: Retrosynthetic plan for second approach

Grignard reagents are usually easily prepared from alkyl halides and have been shown to function in the presence of silyl ethers so the first nucleophile envisioned in this approach was the magnesium Grignard of a bromoalkylsilyl ether. Such a nucleophile would satisfy the later requirements of the linker to have some functionalization (FG), since cleavage of the silyl ether would yield a primary hydroxyl, capable of further reaction with an amine to form the ionic tag. Therefore, 3-bromopropan-1-ol was reacted with *t*-butyldimethylchlorosilane to give *t*-butyldimethylsiloxybromopropane. Upon reaction with magnesium, it was to be coupled to the *γ*-keto containing component chosen as the electrophile, succinic anhydride, but many attempts to generate the desired Grignard reagent were unsuccessful, either yielding no reaction with magnesium or rapid conversion to the Wurtz coupling product.

Attempts to generate the organolithium for use as the nucleophile instead of the organomagnesium halide via a lithium-halogen exchange using lithium wire

also did not provide the desired compound. It was presumed that the organolithium reagent was generated since the lithium wire mixed with the bromoalkane was consumed and a precipitate was observed, presumably the resultant lithium bromide salt, but addition to succinic anhydride did not result in any reaction. It was possible that Wurtz coupling was again responsible for the major product of this reaction but it was not confirmed.

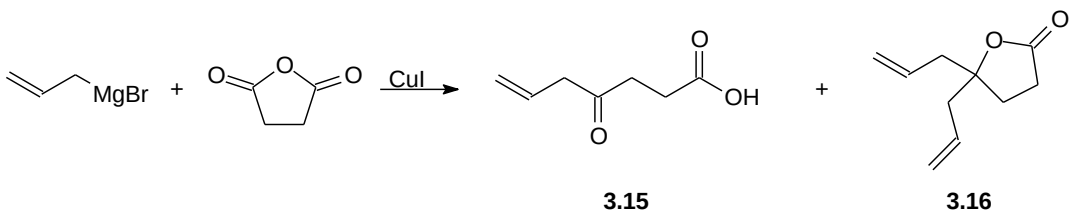


Scheme 3-9: Initial Grignard approach

Given the difficulties encountered in preparing the Grignard reagent from the bromoalkylsilyl ether, it was decided to use a commercially available Grignard reagent. Again the choice was limited by the requirement of functionalization required to later attach the tag. Of the choices available to serve this function, an alkene was thought to be suitable, thus allylmagnesium bromide was selected as the nucleophile. This approach, if successful, would yield a three carbon linker between the *γ*-keto acid moiety and the tag. Succinic anhydride was again intended as the electrophile.

Allylmagnesium bromide solution, 1.0M in diethyl ether, was added to a cooled (-15 °C) solution of succinic anhydride in THF containing a catalytic amount of copper iodide. A small amount of the desired product (**3.15**, Scheme 3-10) was obtained after purification (<15%) but the majority of the product was the lactone, **3.16,** that resulted from a second addition of allylmagnesium bromide

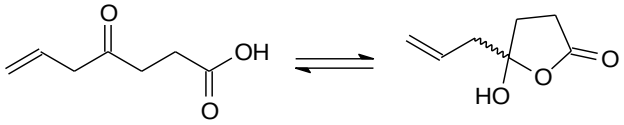
to **3.15** and subsequent lactonization of the tertiary hydroxyl thus generated, likely occurring during the acidic work-up.



3.15 **3.16**

Scheme 3-10: Condensation of Grignard with succinic anhydride

In addition to the problem of the undesired product of the reaction, the ^{1}H-NMR of the desired product is complicated by the equilibrium (Scheme 3-11) between the open chain and closed chain form of the molecule but the identity of the product was supported by LR-MS and confirmed upon further conversion of the acid to the ethyl ester, which is unable to form the cyclic product, thus having a more straightforward NMR.



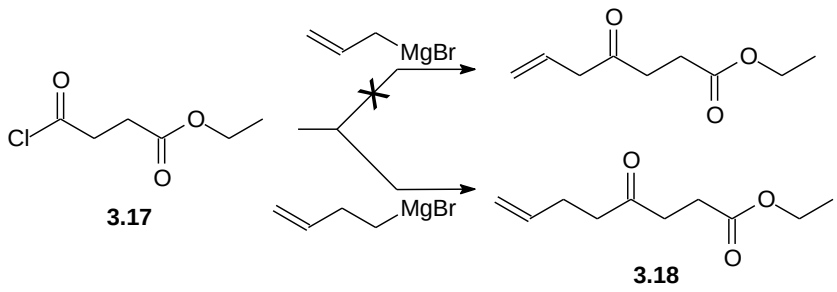
Scheme 3-11: Equilibrium structures of *γ*-keto acid **3.15**

In order to circumvent the over addition of the Grignard reagent to the product and thereby improve the overall yield, the reaction was repeated with a large excess (3 equivalents) of succinic anhydride but the results were similar to those obtained using the first condition. It appeared from these results that the rate of addition of the Grignard reagent to the ketone in the desired product was

faster than that of the addition to succinic anhydride. Another electrophile was needed to overcome this difficulty.

It has been previously shown that acylchlorides *γ* to esters are suitable substrates for regioselective additions to yield *γ*-keto esters as had been the goal here. Thus the electrophile was changed to ethyl 4-chloro-4-oxobutanoate (**3.17**).[194,195]

The reaction (Scheme 3-12) was performed by slow addition of the allylmagnesium bromide solution to a pre-cooled (-78 °C) solution containing an excess of the acylchloride as described by Sato *et. al*[194] but none of the desired product was obtained from the reaction. There was a concern that allylmagnesium bromide was too dissimilar from the Grignard reagents reported in the literature so the nucleophile was changed to butenylmagnesium bromide. Indeed when the reaction was repeated with this reagent some of the desired product, **3.18**, was obtained, though much less (<20%) than the amounts reported in the literature for similar substrates. Again some of the cyclized bis-addition product analogous to **3.16** was obtained, though this was not the major



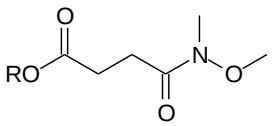
Scheme 3-12: Grignard additions to ethyl 4-chloro-4-oxobutanoate

product. The large number of products obtained and the difficulty encountered in purification led to the selection of yet another electrophile.

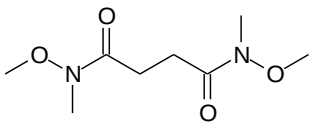
The major difficulty in this Grignard based approach to generate the desired product is the second addition of the Grignard reagent that occurs at the newly formed ketone. This problem has been addressed in the literature and may be circumvented by performing the additions on *N*-methoxy-*N*-methylamides. These derivatives form a chelated metal intermediate that is stable and not subject to further attack, only collapsing to the desired ketone upon acidic workup.[196] As such one possible electrophile that could be used would be 4-(methoxy(methyl)amino)-4-oxobutanoate (**3.19**) but there was the risk that the rate of addition to the ester would be competitive with that of addition to the amide, leading to undesirable side products. In order to avoid this complication, the new electrophile chosen was *N*1,*N*4-dimethoxy-*N*1,*N*4-dimethylsuccinamide, **3.20**.

It had previously been demonstrated that the amount of bis-addition to this symmetric bis-Weinreb amide could be tuned by adjusting the concentration of reagents and the rate of addition to generate an acceptable yield of desired mono-addition product.[197]

The needed *N*1,*N*4-dimethoxy-*N*1,*N*4-dimethylsuccinamide (**3.20**, Scheme 3-13) was therefore generated by mixing of *N*,*O*-dimethylhydroxylamine

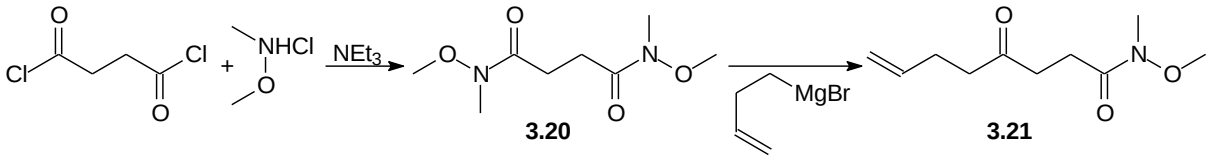


3.19



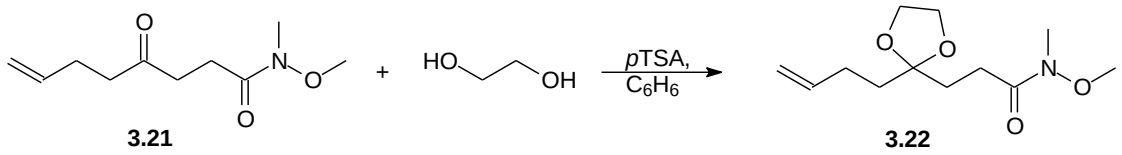
3.20

hydrochloride with triethylamine in dichloromethane, cooling to 0 °C, slowly adding a slightly limiting amount of a solution of succinyl chloride in dichloromethane dropwise and then warming to room temperature. The solution turned black immediately upon addition of the succinyl chloride and a tar was obtained after removal of the solvent. The desired product was isolated by mixing the black tar with boiling hexanes, decanting off the hexane layer and cooling, whereupon it crystallized. The tar was extracted in this fashion several times and the pooled solid was recrystallized from hexanes to give **3.20** in 53-65% yield.



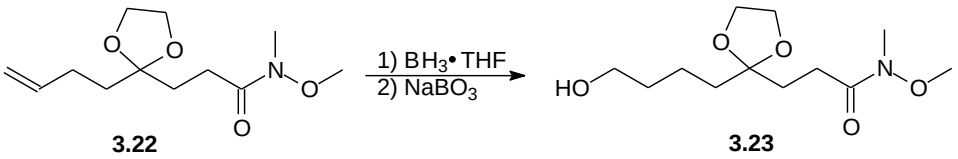
Scheme 3-13: Weinreb amide approach to the synthesis orthogonal tags

The next step in the synthesis was the addition of the Grignard reagent. A 20% excess of the butenylmagnesium bromide solution in THF was added slowly to a cooled (0 °C) dilute solution of the succinyl bis-Weinreb amide, **3.20**. After addition was complete the reaction was allowed to proceed for 4 hours, at which time an aqueous solution of ammonium chloride was added to quench the chelated intermediate and generate the desired ketone, **3.21**, in 47-50% isolated yield after flash column chromatography. Using a stoichiometric amount of the Grignard reagent decreased the amount of desired product obtained.



Scheme 3-14: Acetal protection of ketone **3.21**

At this point the ketone was protected as an acetal by mixing the *N*-methoxy-*N*-methyl-4-oxooct-7-enamide, **3.21**, with ethylene glycol in benzene, along with a catalytic amount of *p*TSA, attaching the reaction flask to a Dean-Stark apparatus and refluxing overnight. TLC showed complete conversion of the starting material to a single product, which was isolated in 92-95% yield and the identity was confirmed by NMR and LR-MS as that of the desired cyclic acetal, **3.22**.

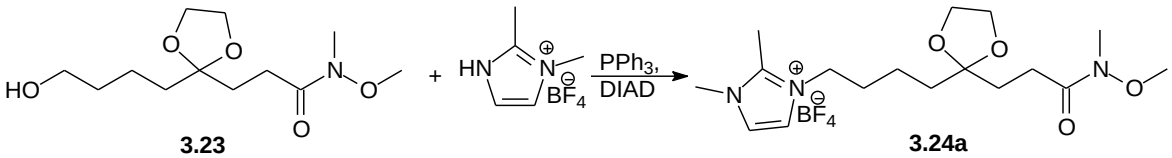


Scheme 3-15: Hydroboration of **3.22**

The next step in the synthesis was to convert the alkene moiety to a primary alcohol. Hydroboration was the simplest route to achieve this so the acetal protected *N*-methoxy-*N*-methyl-4-oxooct-7-enamide was mixed with 9-BBN in THF followed by oxidation with hydrogen peroxide in the presence of sodium hydroxide. The hydroborating reagent, 9-BBN, was chosen to limit the amount of secondary alcohol generated in this step as it was unknown if the separation of the primary and secondary alcohols would be feasible. Ironically the cyclooctane

diol by-product of the reagent itself proved to be difficult to remove from the product. Chromatography in several solvent systems was unable to separate the products satisfactorily so the hydroboration was instead performed using a borane tetrahydrofuran complex followed by oxidation with aqueous sodium perborate, yielding the desired 3-(2-(4-hydroxybutyl)-1,3-dioxolan-2-yl)-*N*-methoxy-*N*-methylpropanamide, **3.23**, in 51-52% yield. The undesirable secondary alcohol was not observed by TLC or NMR of the isolated product.

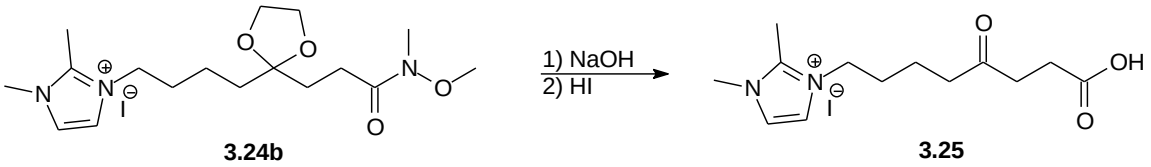
It has been shown that primary alcohols may be directly converted to their corresponding ionic liquids in the presence of the imidazolium salt of the desired anion using Mitsonobu type conditions.[198] Thus 3-(2-(4-hydroxybutyl)-1,3-dioxolan-2-yl)-*N*-methoxy-*N*-methylpropanamide, **3.23**, was mixed with 1,2-dimethylimidazolium tetrafluoroborate and 1,2-dimethylimidazole and dissolved in dry acetonitrile. Diisopropylazodicarboxylate (DIAD) and triphenylphosphine were mixed together and added as a solution to the other components and the reaction was stirred for several hours. The reaction mixture was then precipitated from MTBE and the resultant solid was removed by filtration. At this point the solid would have contained the desired product and unreacted imidazolium salt, which was much less soluble in acetone. Thus the salt was



Scheme 3-16: Mitsonobu style installation of tag

removed by adding a small amount of cold acetone to dissolve the product, **3.24a**, and filtering off the undissolved salt. After several repetitions NMR and TLC analysis indicated that the product was pure enough to continue, with an isolated yield of 45-48%.

The final step prior to coupling to a nucleoside was to cleave the ketal and convert the remaining Weinreb amide to a carboxylic acid suitable for derivatization to a nucleoside. The first approach attempted was using acid hydrolysis, which would accomplish both transformations simultaneously. In order to avoid an undesirable anion metathesis, aqueous HBF_4 was used as the acid. Upon heating however, a white, waxy solid formed, presumably from a reaction of water with tetrafluoroborate to produce borates that precipitate and also liberate fluoride anions. The resulting mixture, while containing the desired material as indicated by MS, was difficult to purify. Switching to a stepwise approach of treatment with sodium hydroxide to cleave the amide and acidification to a pH of 2-3 using HBF_4 to cleave the acetal and protonate the carboxylic acid yielded similar results.



3.24b **3.25**

Scheme 3-17: Deprotection to derivatizable tag **3.25**

The deprotection problem was solved by preparing the protected tag (**3.24b**) using the 1,2-dimethylimidazolium iodide salt instead of the 1,2-

dimethylimidazolium tetrafluoroborate salt and performing the deprotection using base hydrolysis, followed by acidification with hydroiodic acid and finally rinsing with MTBE to remove all impurities, yielding the derivatizable tag **3.25**.

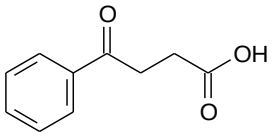
Low resolution MS indicated that the acetal and amide were both removed completely to yield the desired material but NMR revealed that some of the amine resulting from the amide cleavage still remained in the compound, likely in the form of a salt. All attempts to remove this impurity failed.

It was decided to proceed to the coupling step with the impure material, however, since the amine was present at less than stoichiometric amounts, so even if it consumed some of the tag the remainder would still be available to couple to the nucleoside. When the coupling was performed with TBTU and triethylamine very little of the tagged nucleoside was obtained but none of the expected coupling with the amine to re-form the Weinreb amide was observed either. Though the identity of the amide impurity is unknown, it clearly interferes with the reaction to derivatize the tag to a nucleoside so alternate routes were devised to generate an orthogonal tag of the desired form.

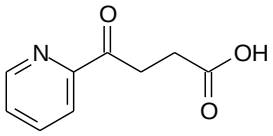
3.3.4 Stetter based approaches to orthogonally cleavable tag

Another method used in carbon-carbon bond formation resulting in ketones is the Stetter reaction (Scheme 3-18), which transforms aldehydes into nucleophiles using catalytic amounts of cyanide ions or thiazolium salts, such as **3.28**, in the presence of mild base.[199] The method has been used to generate both aromatic and alkyl *γ*-keto esters of the type required for the proposed tag by

performing Michael additions of the catalyst-activated aldehydes on acrylate esters.

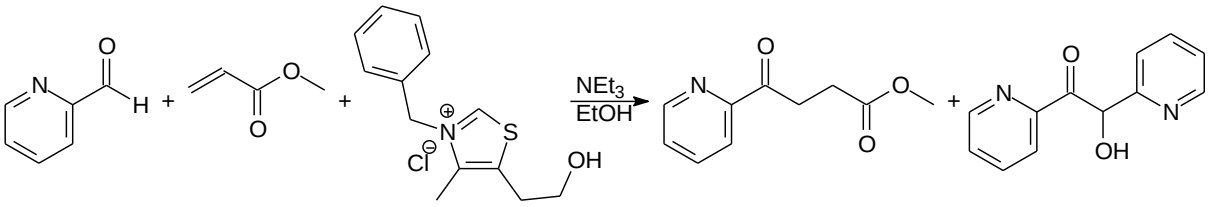


3.26



3.27

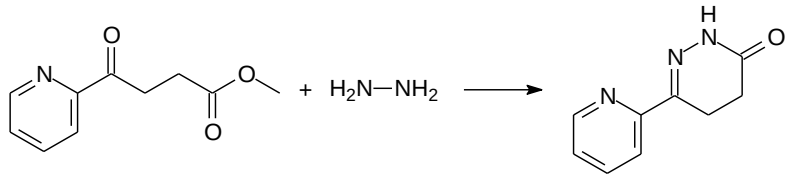
In the extensive research on oligonucleotide synthesis accomplished by Letsinger and co-workers it was shown that 4-oxo-4-phenylbutanoic acid, **3.26**, could be used effectively as a hydroxyl protecting group cleavable by exposure to hydrazine.[200-202] If the Stetter reaction could be used to generate the analogous 4-oxo-4-pyridinylbutanoic acid, **3.27**, it could quickly be transformed into the desired tag by reaction of the pyridinyl nitrogen with an alkyl halide. Therefore, the readily available picolinaldehyde was dissolved in ethanol, mixed with a slight excess of methyl acrylate, triethylamine and the catalyst, 3-benzyl-5-(2-hydroxyethyl)-4-methylthiazolium chloride, **3.28**. The reaction was refluxed gently for 16 hours and the solvent was then removed. The orange oil obtained was dissolved in dichloromethane and washed with water, the organic layer was dried and the solvent was again removed. The oil obtained was purified by flash chromatography to give a slightly yellow solid, **3.29**, in 47% yield.



3.28 **3.29** **3.30**

Scheme 3-18: Stetter approach to an aryl *γ*-keto ester tag

The bright orange colour that develops during the reaction is due to the formation of the major side product, the self-condensation of the aldehyde, in this case resulting in *α*-pyridoin, **3.30**. This kinetic self-condensation product is well known in Stetter reactions and can be minimized by altering the rate of addition of aldehyde, the specific catalyst and Michael acceptor used and the solvent chosen. In this case however, the scale was large enough that an acceptable amount of material was obtained in order to carry forward for proof of concept.



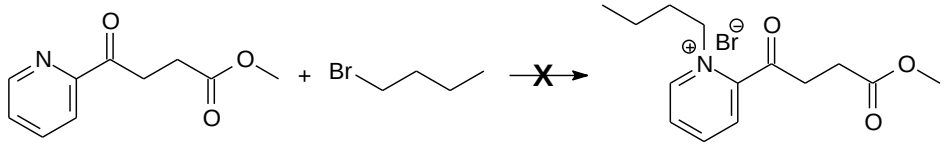
3.29

Scheme 3-19: Hydrazine cleavage of methyl ester

To show that a *γ*-keto ester of this nature could still be cleaved by hydrazine, a small amount of the purified methyl 4-oxo-4-(pyridin-3-yl)butanoate, **3.29**, was mixed with 10 mole equivalents of hydrazine hydrate in 3:2 v/v pyridine-acetic acid and the progress of the reaction was monitored by LR-MS. Over time the starting material disappeared and the cyclic hydrazone product formed. After 4.5 hours however, starting material was still present. This is consistent with the reported cleavage of the 4-oxo-4-phenylbutyrate protecting group, **3.26**, employed by Letsinger but the rapidity, and ease of generating this compound may still make it attractive.[200]

To generate the ionic moiety of the desired tag, the methyl 4-oxo-4-(pyridin-3-yl)butanoate, **3.29**, was mixed with a small excess of 1-bromobutane in

acetonitrile and the reaction was refluxed overnight. Analysis by TLC indicated no reaction had occurred and this was confirmed by LR-MS. The reaction was re-attempted with a larger excess of the alkyl halide and allowed to proceed for a longer period of time with no improvement. Changing the electrophile to the more reactive 1-iodobutane also resulted in no reaction. This result was somewhat surprising given the ease with which pyridine reacts with these substrates to yield the desired pyridinium salt. Given this result and the lengthy reaction time required for complete cleavage of the ester, it was unlikely that a pyridinyl *γ*-keto ester was a workable route to the desired tag.



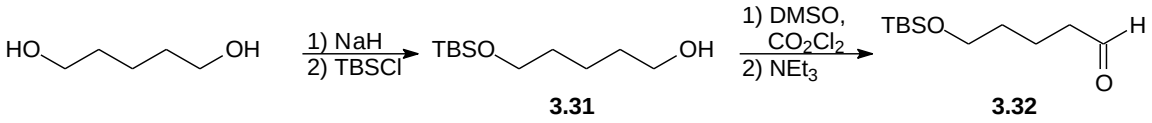
3.29

Scheme 3-20: Conversion of 3.29 to a protected ionic tag

It had been observed by van Boom and co-workers that alkyl *γ*-keto ester are cleaved much faster than the aryl analogues[203] and this is supported by other reports discussing the levulinyl protecting group[169,187] and the work described earlier on the ester of the 5-bromolevulinate derived tag, **3.12**. Though this presents a longer synthetic route to a product of the desired form, the work performed on the Weinreb amide approach indicates the synthesis would likely be viable, resulting in a similar molecule to the one that approach yielded, without the complications encountered due to the amide cleavage product.

The shortest route to the desired product would then be to condense an aldehyde attached to an aliphatic chain terminating a protected hydroxyl group.

The protecting group required would need to be cleavable under conditions that would not affect an ester or an acetal, thus a silyl ether was selected. The length of the alkyl chain was chosen to be the same as that used in the Weinreb amide approach (4 carbons). Therefore, 5-(*tert*-butyldimethylsilyloxy)pentan-1-ol was prepared by mixing 1,5-pentanediol with sodium hydride in THF at low temperature and then adding a limiting amount *t*-butyldimethylchlorosilane. After workup and flash chromatography, the product, **3.31**, was obtained as a colourless oil in 62% yield.

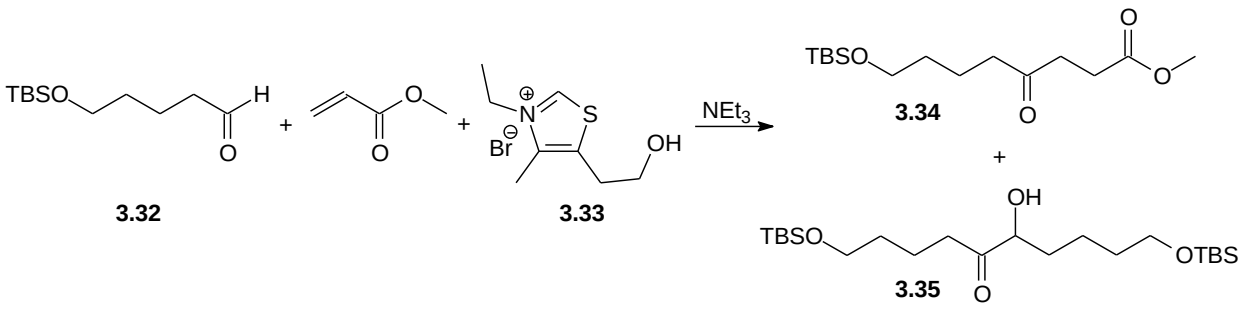


3.31 **3.32**

Scheme 3-21: Preparation of protected aldehyde **3.32**

The monoprotected diol, **3.31**, was then converted to the aldehyde by dissolving it in dichloromethane and adding it to a pre-mixed reaction solution (-78 °C) of oxalyl chloride and DMSO, likewise in dichloromethane. After a short reaction time, triethylamine was added to complete the oxidation, followed by an extraction workup with alternating aqueous acid and base. The solvent was removed under reduced pressure and the product, **3.32**, was characterized and used without further purification.

The Stetter condensation was performed as described previously using catalyst **3.28** but also with an alternative catalyst **3.33** (3-ethyl-5-(2-hydroxyethyl)-4-methylthiazolium bromide),[199] shown in Scheme 3-22, in an attempt to improve the yields for the aliphatic aldehydes. The product **3.34** was obtained in 30-33%



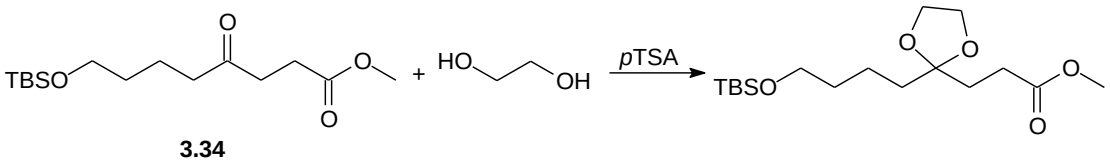
Scheme 3-22: Stetter condensation of silyl ether protected aldehyde

yield along with an even greater amount of the self-condensation product, **3.35**, than was obtained for the aryl aldehyde used in the previous case (Scheme 3-18), regardless of the catalyst used.

The self-condensation product is thought to be the kinetic product so an attempt was made to push the reaction toward the desired thermodynamic Michael addition product by mixing the isolated **3.35** with methyl acrylate in the presence of the catalyst. These conditions should exploit the equilibrium that should exist between **3.35** and the active intermediate complex of **3.32** and **3.33**. After refluxing for 18 hours, however, no change had occurred. Reports in the literature indicate that an optimized yield of 70-80% for the Stetter reaction are obtainable with similar substrates, so the yield could likely be improved upon optimization. Therefore the synthesis was continued, since even with poor yield a substantial amount of material (**3.34**) was obtained.

The next step in the synthesis was the protection of the ketone as a cyclic acetal (Scheme 3-23). Thus, **3.34** was mixed with ethylene glycol in benzene

and a catalytic amount of *p*TSA. The mixture was refluxed for 16-18 hours in a Dean-Stark apparatus and then subjected to alkaline workup. The product was characterized by TLC, LR-MS and NM and found to have been quantitatively converted from starting material and, ready for use without further purification.



3.34

Scheme 3-23: Protection of ketone in **3.34**

In order to proceed, it was necessary to cleave the silyl ether so that the resulting hydroxyl could be converted to the ionic moiety. The common reagent used for this cleavage, TBAF, is known to cleave esters so it was not considered usable here.[191] Instead an alternative desilylating reagent, triethylamine trihydrofluoride (TREAT-HF), was employed. Upon treatment with this reagent, the silyl ether was cleaved after 2 hours but the acetal was also partially cleaved. Since the silyl protecting group could not be removed cleanly another route was needed.

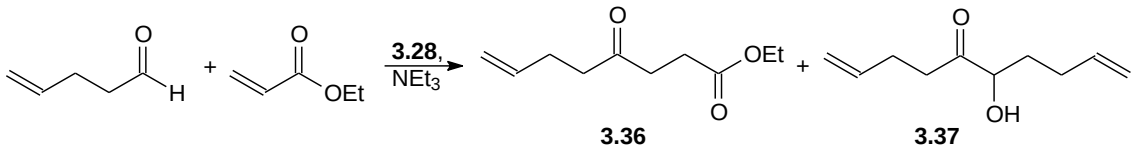
In the Weinreb amide approach described earlier, it was also not possible to incorporate a protected hydroxyl in the initially coupled substrates due to the difficulty encountered in preparing such a Grignard reagent. Therefore a terminal alkene was employed instead, and the subsequent transformations worked reasonably well. Though this strategy added more steps to the synthesis, the likelihood of generating the desired product was higher so the new aldehyde

substrate selected had an alkyl chain with a terminal alkene instead of a protected hydroxyl group.

In addition to the change of nucleophile, the electrophile was also changed to ethyl acrylate, since its boiling point was significantly higher than ethanol. One of the possible causes of the poor yield observed for the first two sets of Stetter substrates was that a portion of the methyl acrylate used in the reaction was in the gas phase or held up in the condenser. Since the boiling point of methyl acrylate was very close to that of ethanol, this led to a lower concentration of it in solution, allowing for an increase in formation of the undesired self condensation products, **3.30** and **3.35**. It was hoped that the change to ethyl acrylate would improve the yield.

The new substrates, 4-pentenal and ethyl acrylate were mixed with the thiazolium salt, **3.28**, and dissolved in ethanol (Scheme 3-24). The reaction was heated and once it was refluxing gently, the reaction was initiated by the addition of triethylamine. After 18 hours the solvent was removed and the material obtained was subjected to a dichloromethane/brine extraction followed by flash chromatography. Unfortunately the desired product, **3.36**, and the acyloin side-product, **3.37**, co-eluted in all the solvent system tested. The yield was also low, overall approximately 25% as estimated by NMR of the mixture. Another attempt to generate the desired product using the other catalyst, **3.33**, yielded similar results. Again, it was hoped that the reaction could be optimized later, since similar substrates described in the literature generated higher yields. For use as

a proof of concept, enough material was generated and it was hoped it would be separable from the side product at a subsequent synthetic step.



Scheme 3-24: Stetter approach to an alkene containing *γ*-keto ester

The next step in the planned synthetic route was to protect the ketone as an acetal. As before, ethylene glycol was employed in order to generate the cyclic acetal but in this case, the reaction did not show clean conversion to the desired product. Rather, a mixture of products was obtained, some of which seem to be due to transesterification with the ethylene glycol. It is unknown whether the problems encountered with this reaction were due to the nature of the substrate itself, which seems unlikely, or to the presence of the acyloin contaminant. It was then decided to use another approach to the acetal formation.

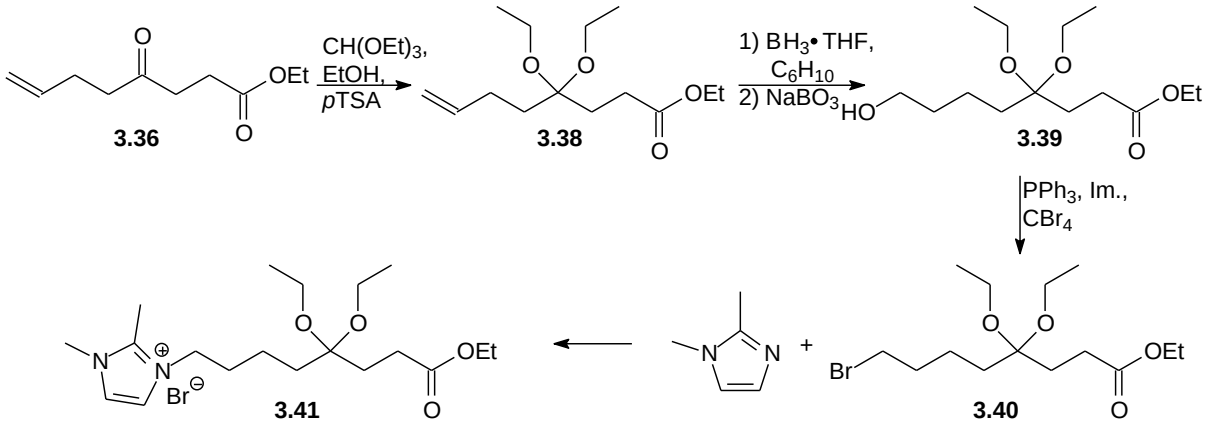
Since an ethyl ester was present in the molecule and scrambling of the ester seemed to be an issue with ethylene glycol installation, a diethyl acetal was envisioned, where transesterification would simply result in the same molecule (Scheme 3-25). Therefore, **3.36** was dissolved in ethanol with a catalytic amount of *p*TSA and ethyl orthoformate was employed as the dehydrating reagent, since its reaction with the water liberated during the acetal formation simply yielded more ethanol. Refluxing for 4 hours was found to be adequate for the reaction to reach completion and the desired product was obtained in 90-95% yield (based on the NMR estimation of the initial amount) and was easily separable from the

acyloin product, **3.37**, which did not appear to undergo any transformation. The identity and purity of the product, **3.38**, were confirmed by TLC, LR-MS and NMR.

With the *γ*-acetal-ester, **3.38**, in hand, the next step (Scheme 3-25) was the hydroboration of the terminal alkene. In the Weinreb amide approach described earlier, the analogous molecule was initially treated with 9-BBN to achieve this transformation in order to minimize the amount of secondary alcohol that could form. The product was found to co-elute with the octane diol that resulted from the oxidative cleavage of this reagent therefore borane-THF was finally employed. Though none of the undesired secondary alcohol was detected in that instance, it is generally accepted that up to 5% of this product forms and may be difficult to remove from the desired primary alcohol.

The substrate being considered here was an ester rather than an amide and was thus unlikely to co-elute with the 9-BBN by-product as had occurred before (Section 3.3.3). In order to ensure this was the case, however, an *in situ* generated dicyclohexyl borohydride reagent was used. An appropriate amount of cyclohexene was thus added to borane-THF at 0 °C. After being allowed to react for one hour, **3.38** was then added to the resultant slurry and the reaction was allowed to proceed for 2 hours at room temperature.

The oxidation was achieved by the addition of aqueous sodium perborate, since this was a mild enough system to leave the ester intact, and the reaction continued for another 2 hours. The reaction mixture was then extracted with ethyl acetate and the product, **3.39**, was purified by flash column chromatography



Scheme 3-25: Conversion of the alkenyl *γ*-keto ester to tag

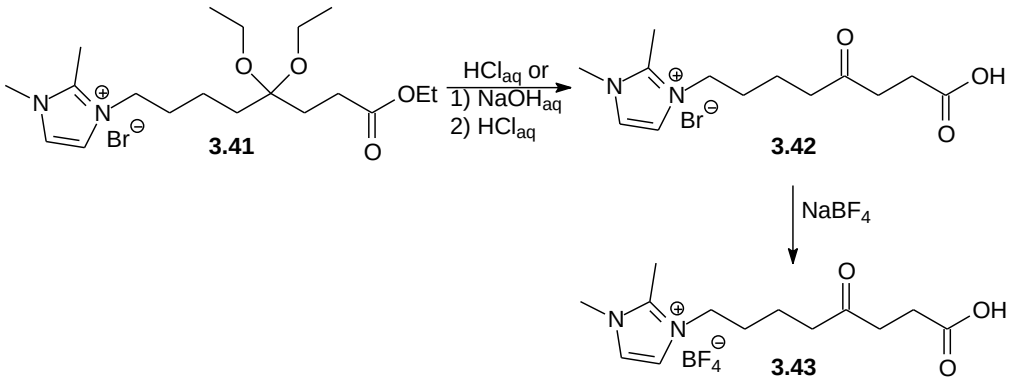
and was obtained as a colourless liquid in 80-85% yield with 15-20% of **3.38** recovered. This reaction was performed several times but it never proceeded further than 85% conversion, even with longer reaction times or an increase in the amount of the borohydride reagent generated relative to substrate.

In the Weinreb amide approach, the terminal alcohol was directly converted to the ionic tag but the yields obtained were lower than expected. It was decided to attempt the stepwise approach of conversion of the alcohol to a halide and then form the ionic tag to see if higher yields could be obtained. Therefore, the alcohol, **3.39**, was mixed with triphenylphosphine and tetrabromomethane in dichloromethane. The reaction appeared to proceed cleanly but upon aqueous workup, the acetal was cleaved, likely due to the production of hydrobromic acid generated from the hydrolysis of the excess reagents. The reaction was performed again in the presence of imidazole, which could neutralized any hydrobromic acid that might be generated, and thus the desired product, **3.40**, was obtained in 79-86% yield after flash chromatography. Subsequent

condensation with 1,2 dimethylimidazole resulted in the ionic tag, **3.41**, in 95% or greater yield. The two step conversion was indeed superior to the one step approach employed earlier.

The final steps in the synthesis (Scheme 3-26) involved cleavage of the acetal and the ester as well as anion metathesis and derivatization to a nucleoside. The most straightforward approach was to perform both cleavage reactions simultaneously using aqueous acid followed by anion metathesis and derivatization.

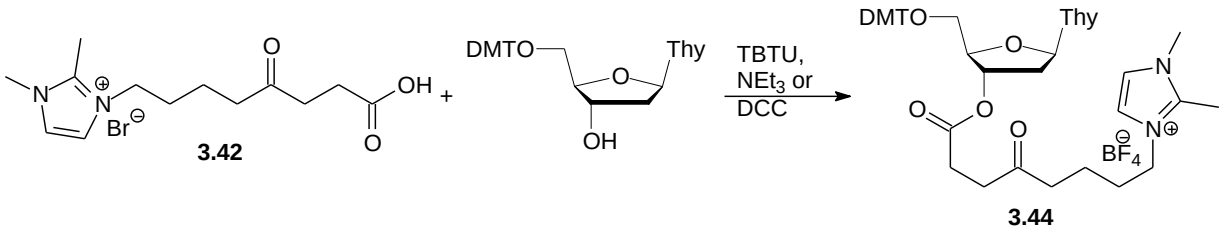
Refluxing **3.41** with aqueous hydrochloric acid was initially employed for the cleavage and resulted in complete conversion of **3.41** to the desired product, **3.42**, as evaluated by NMR and LR-MS, once the acid was removed under reduced pressure to yield an off white solid. Upon anion metathesis to yield the tetrafluoroborate salt **3.43**, however, the resultant pale brown oil quickly turned deep red and then black. Though no change was observed in the MS, subsequent attempts to couple this material to a nucleoside were unsuccessful.



Scheme 3-26: Final deprotection and anion metathesis

It was theorized that residual HCl was present and this decomposed the tetrafluoroborate in some manner.

A way to avoid having an excess of HCl present would be to perform a two step deprotection, where only a small amount of HCl would be added. Hence the protected material, **3.41**, was dissolved in aqueous sodium hydroxide and allowed to react for 16-18 hours followed by an acidification to approximately pH 1 with HCl and then drying under reduced pressure. The excess salt was removed by suspending the material in acetonitrile/dichloromethane and filtering off the insoluble material. The product obtained, **3.42**, was pure by LR-MS and NMR but likely still contained a small amount of sodium chloride.

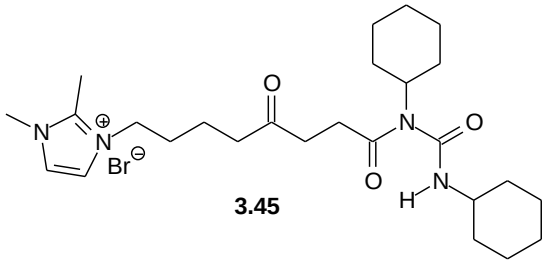


Scheme 3-27: Derivatization of tag to a deoxyribonucleoside

When stored as the bromide salt, the product remained an off white solid for a prolonged period of time but if anion metathesis was performed to generate **3.43**, the oil thus obtained again began to turn reddish brown within 24 hours and black within several days, even at -25 °C. The cause for the decomposition was likely not HCl but its source remains undetermined. The bromide salt, **3.42,**

appeared stable however, so an attempt was made to couple it to a nucleoside and then perform the anion metathesis afterwards.

Previously, whenever a nucleoside was coupled to an ionic tag, a large excess, 2 or more molar equivalents, of the tag was used and the yield was determined based on the amount of nucleoside that was added, with usually greater than 95% conversion. In this case however, it was decided that the tag would be used in limiting amounts so that the effectiveness of the coupling reagents TBTU and DCC could be compared. Since the bromide salt of the tag (**3.42**) was being used, instead of the tetrafluoroborate salt normally employed, DMF was added as a co-solvent (15% v/v) to acetonitrile, which would usually have been used neat. For both coupling agents 1.2 equivalents of 5'-DMT-thymidine was used as the nucleoside. For TBTU, 4 equivalents of the coupling agent were used along with 6 equivalents of triethylamine, while 1.5 equivalents of DCC were used with 0.5 equivalents of DMAP present. Both reactions were performed at a nucleoside concentration of 0.2M and were allowed to proceed overnight followed by the typical precipitation and extraction method normally used at this step.



3.45

The TBTU reaction resulted in the anion metathesis directly since the counter-ion for TBTU is the desired tetrafluoroborate whereas for the DCC coupled product, the metathesis was achieved during the extraction step by using a solution of sodium tetrafluoroborate as the aqueous phase. In both cases the exchange was confirmed by LR-MS as an absence of bromide ions and the presence of tetrafluoroborate in the negative mode. The DCC derived product was a white foam while the TBTU derived product was a brown foam.

The yields of **3.44** were similar for the two coupling agents, 79% for the TBTU reaction and 88% for the DCC reaction, by weight, but the DCC derived product contained the DCU-tag side-product, **3.45**, as a contaminant. This contaminant has the same solubility profile as the tagged nucleoside and could not be removed and affected the yield as determined by weight. When this was taken into account using NMR analysis to determine the molar ratio of the two components in the solid, the yields were nearly the same. While removing **3.45** from the desired product is problematic, a change to the similar coupling reagent, EDC, would likely eliminate this difficulty since EDC and presumably the EDC-tag side-product would be water soluble, and thus easily removable during extraction. In either case, the tag was shown to successfully couple to the nucleoside and the identity of the product was confirmed by NMR and HR-MS. The cleavage characteristics of the product will be discussed in the next section.

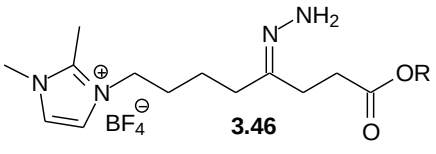
3.4 Cleavage characteristics of the ɣ-keto ester based tag

With the tagged nucleoside (**3.44**) in hand it was possible to study its cleavage characteristics in parallel with the same nucleoside protected as the levulinyl ester. The 3'-levulinyl ester group has been used in the past to generate oligomeric building blocks and is known to be easily removed without affecting any other protecting groups in the molecule.[169] If the tag is removable in a similar manner and timeframe then it is reasonable to assume that the tag is a viable route to generating oligomeric building blocks as well.

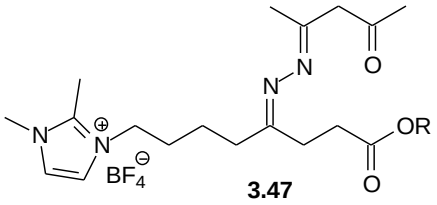
HPLC methods were developed using the starting materials, tagged or levulinated DMT-dT, as well as an internal standard (IS), acenapthene, which is inert to the cleavage reaction conditions and has a strong absorbance at the same wavelength as nucleosides. The protected nucleosides were mixed with an appropriate amount of the IS and a small amount of acetonitrile to aid in the dissolution once the deprotection solution was added. As before, the deprotection solution was composed of 0.5M hydrazine hydrate in 3:2 v/v pyridine/acetic acid and a volume corresponding to 10 mole equivalents of hydrazine was added to the nucleoside mixture. Samples were removed once every minute for the first 8 minutes and then every other minute until 20 minutes total reaction time had elapsed. Upon removal, the samples were quenched by the addition of 2,4-pentanedione, which reacts quickly with hydrazine, stopping the reaction. The samples were diluted and then analyzed by HPLC, standardizing the areas observed for each peak against the IS.

In both cases, an extra peak appeared which was due to species other than the starting materials or the final products. The new peak in each case was determined to be due to the formation of the hydrazone intermediate, **3.46** in the case of the tag, prior to cyclization and release of the nucleoside, though they were actually observed as the quenched product, **3.47**, in the HPLC study, as characterized by LR-MS. The amounts of these components with respect to reaction time may be seen in Figure 3-4 and Figure 3-5, for the levulinyl ester and tagged nucleoside respectively.

Overall, the rate of levulinyl ester cleavage was much faster than the tag, with half lives of approximately 0.76 and 2.82 minutes respectively, corresponding to complete cleavage of the levulinyl ester in just over 5 minutes while the tag required almost 20 minutes for full cleavage. The results also indicate that the rate limiting step in the cleavage was different for the two species.



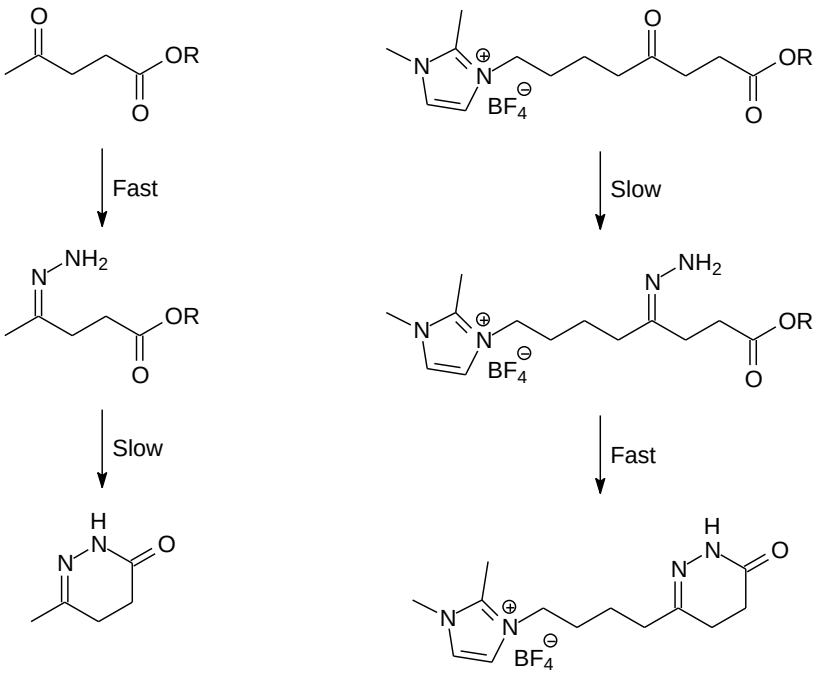
3.46



3.47

In the levulinyl ester, it was observed that the hydrazine addition was very rapid, leading to a build-up of the hydrazone intermediate prior to cyclization. In the case of the tagged nucleoside, disappearance of the starting material was the slower step, with the formation of the hydrazone intermediate held to a low, steady state amount indicating that the cyclization step was faster than the hydrazone formation (Scheme 3-28).

There are several possible explanations for this change in the overall rate, likely all of which play some role. Certainly, steric bulk around the ketone in the tagged nucleoside is higher than for the levulinyl ester but this seems unlikely to be the biggest contributor. More likely, the major cause of the rate change is due to charge repulsion.

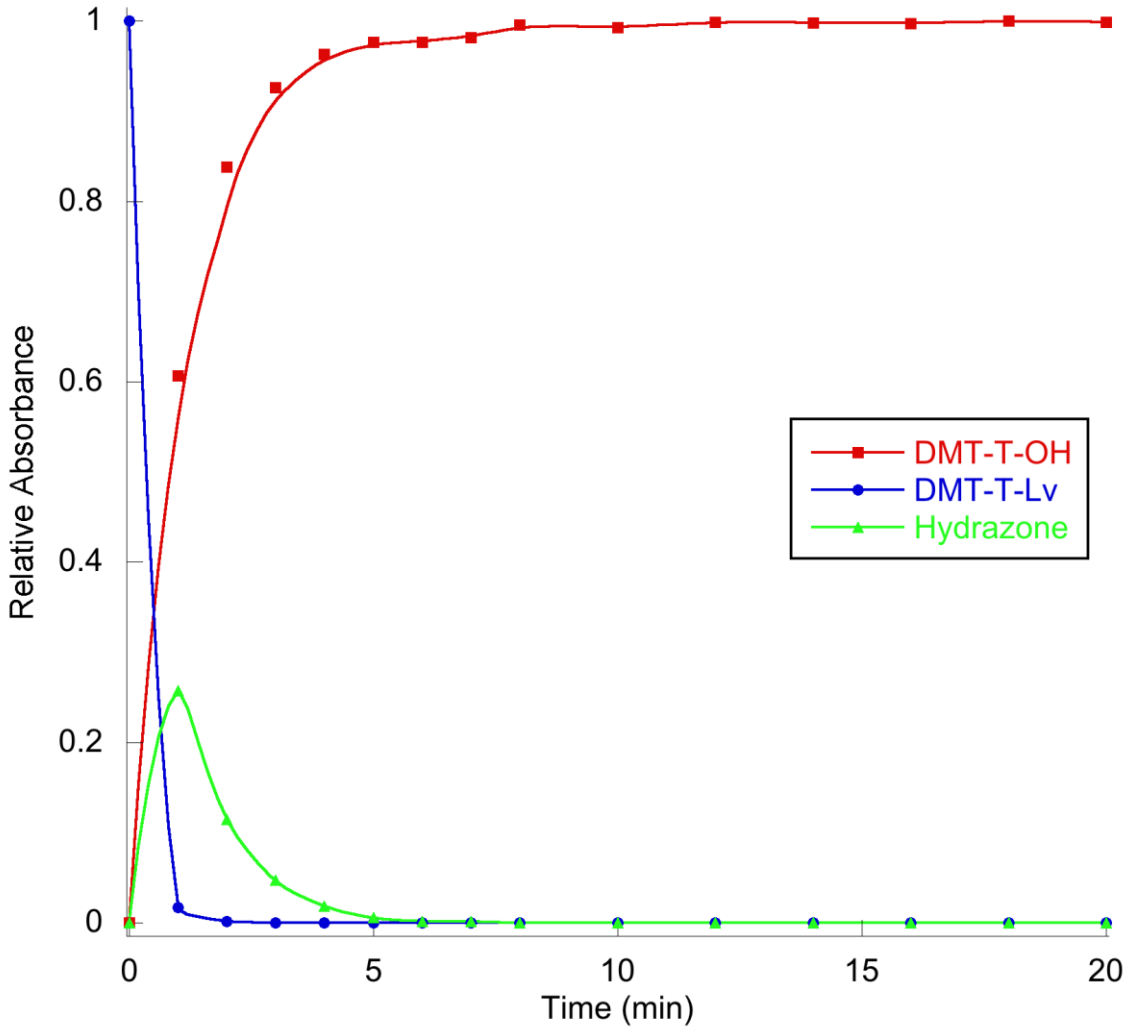


Scheme 3-28: Rate determining step for (**A**) levulinyl cleavage and (**B**) for tag cleavage

All of the key steps in the hydrazine cleavage reaction mechanism involve the development of positive charge and, as has been observed previously for tagged species, the formation of additional positive charge in a molecule that is already charged is difficult. This is illustrated in the difficulty in cleaving the trityl ether used to protect the 5'-hydroxyl of nucleosides and oligonucleotides using

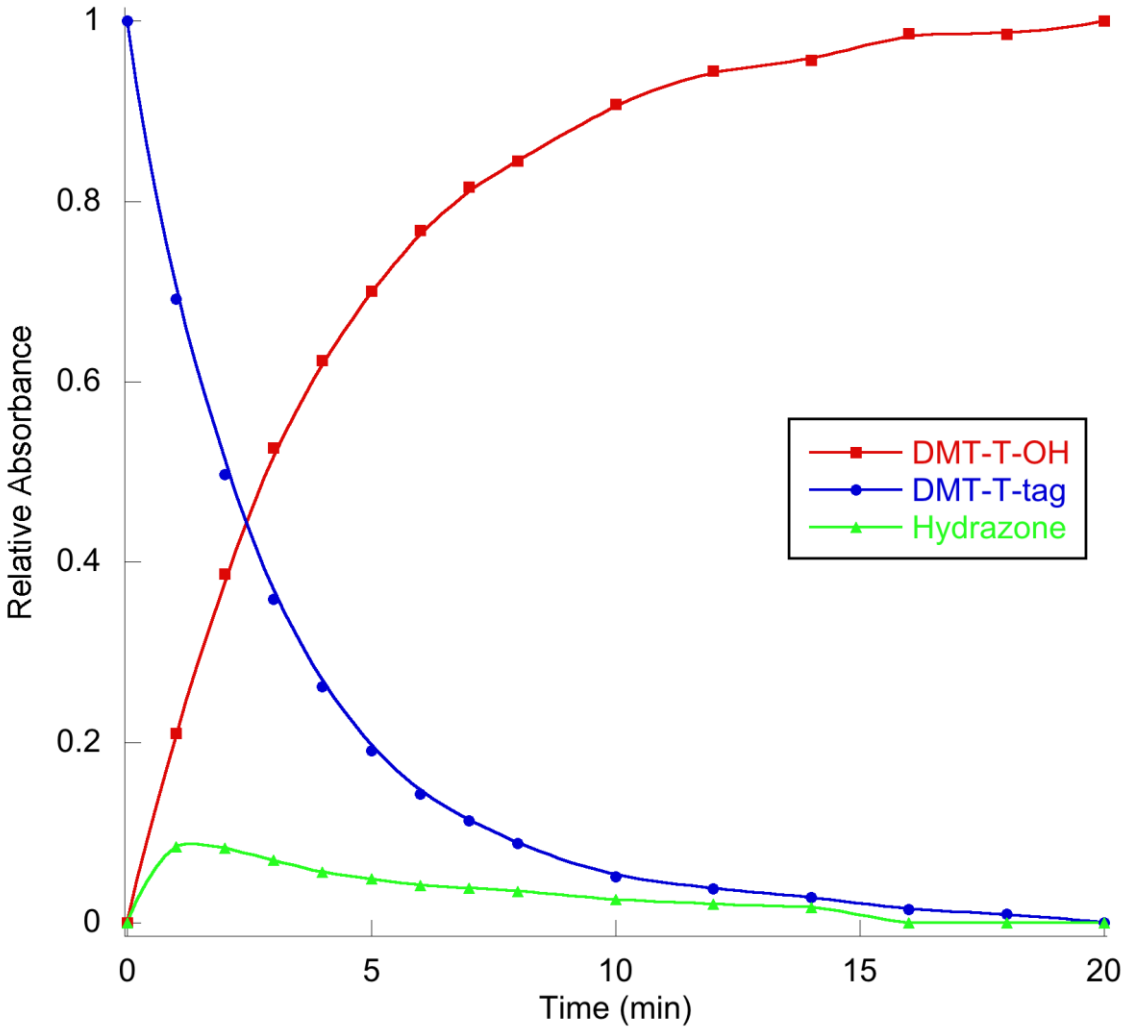
acid. This effect is at its strongest when the charges are close to each other within the molecule and in the tag being studied the charge is only a few bond lengths away from the site of first attack, namely the ketone. Looking back at the MS study performed on the 5-bromolevulinate derived tag, **3.12**, where the charge was even closer, the time to complete cleavage doubled to 40 minutes versus 20 minutes for **3.44**.

Figure 3-4: HPLC time-course of levulinyl cleavage from DMT-dT



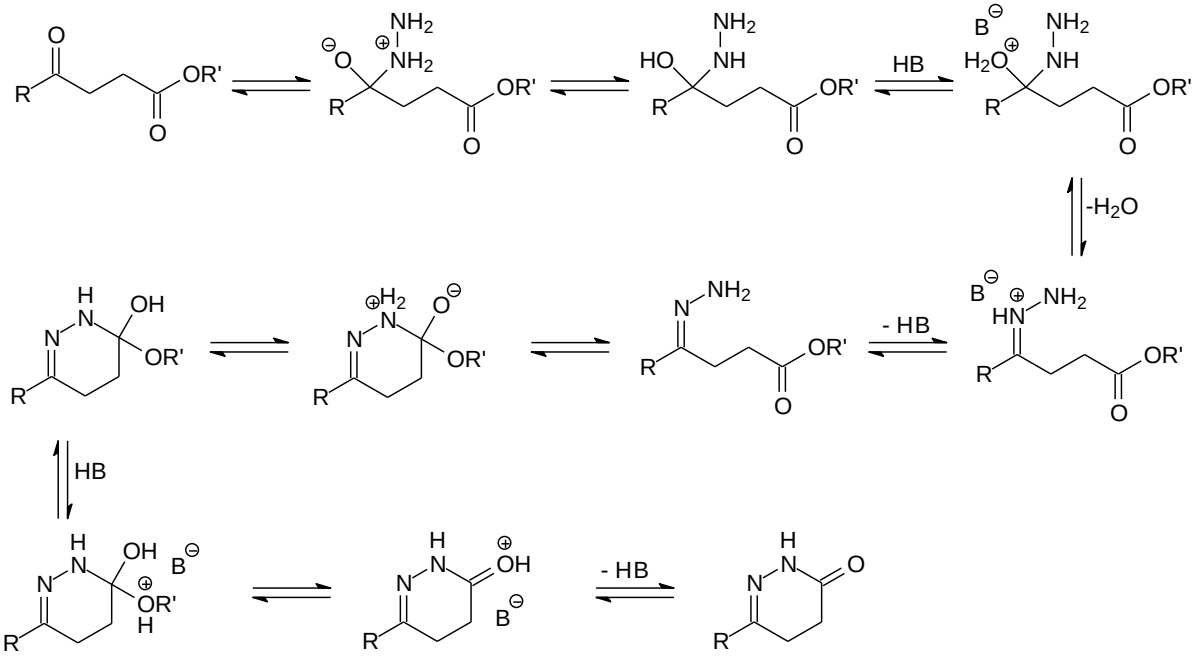
As shown in Figure 3-6 and Figure 3-7, the cleavage reaction requires charge build-up both in the formation of the hydrazone and in the *E*/*Z* isomerization that the hydrazone intermediate may be required to undergo to perform the cyclization. At the beginning of the reaction, the formation of the hydrazone intermediate involves attack of hydrazine on the ketone to yield a charged tetrahedral intermediate that quickly tautomerizes to the hemiaminal.

Figure 3-5: HPLC time-course of tag cleavage from DMT-dT



The collapse to the hydrazone likewise requires the development of positive charge as the hydroxyl must be protonated to generate water as the leaving group and the amine must be deprotonated to yield the hydrazone. For the hydrazone to cyclize, the *α*-nitrogen must attack the ester, again generating the charged tetrahedral intermediate prior to tautomerization and then the alcohol must protonate to leave as the amide forms.

Figure 3-6: Formation of charge during hydrazone cleavage of *γ*-keto esters



The geometry of the hydrazone double bond is important for the cyclization reaction since, in order for the *α*-nitrogen to attack the ester in the second half of the cleavage reaction it must be *syn* to the ester. The hydrazone may form in either the *syn* or the *anti* configuration with nearly equal ease and the *anti* geometry has a likely insurmountable path to attack the ester. If the hydrazone

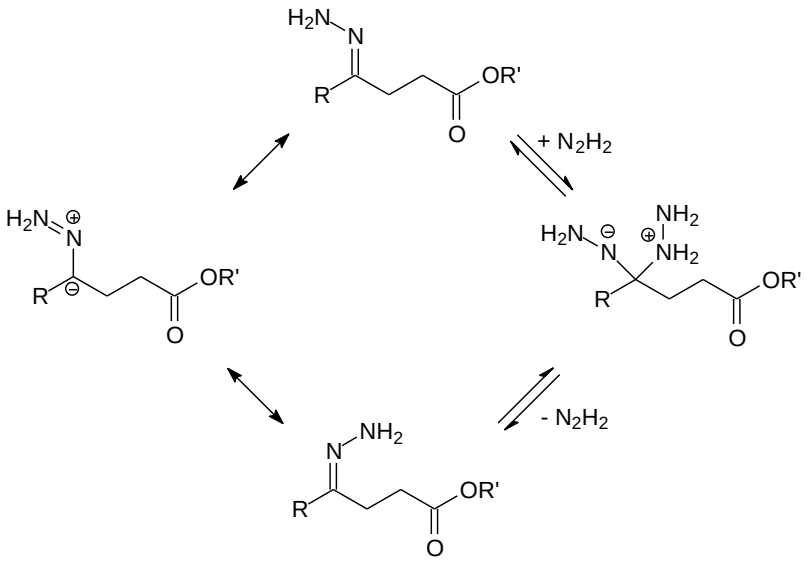
forms with the anti geometry it must first isomerize before the attack on the ester can occur.

Of the various mechanisms by which the hydrazone may undergo *E*/*Z* isomerization,[204] the two most likely, as shown in Figure 3-7 also require the formation of charge. Many of these mechanistic steps may be concerted and not result in the development of a full charge but charge is still developed and the energy cost is observed in the difference in the rates of cleavage between the tag and the levulinyl ester.

Likely the most important factor in the charge induced rate change is the simple fact that the vast majority of the hydrazine in solution is protonated. With a hydrazine pKa of 8.1 and a solution pH of 5.5-6.5, depending on the exact ratio of pyridine to acetic acid in the mixture, very few neutral hydrazine molecules are present in solution, thus the effective concentration of hydrazine capable of attacking the already charged tag is extremely low, whereas in the case of the levulinyl ester, the charged hydrazine may still attack, allowing the hydrazone intermediate to form much more quickly than for the tag.

Even though the tag cleaves more slowly than the levulinyl ester under these non-optimized conditions, the normal reaction time employed for the cleavage of the 3'-hydroxyl protected levulinyl ester by hydrazine is 20 minutes and no degradation is observed in any of the other protecting groups in that time. This amount of time has also been shown to be sufficient to cleave the tag and so this approach should be feasible to generate oligomers for block condensation.

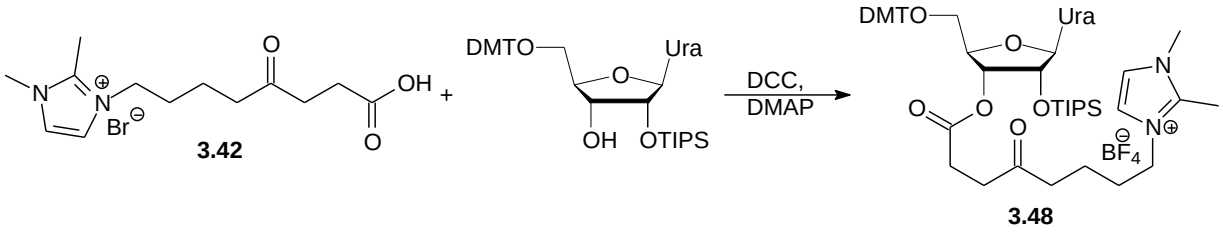
Figure 3-7: *E*/*Z* isomerization of hydrazone intermediate



The goal of this project was to synthesize RNA block amidites; the work thus far, however, has been on deoxynucleosides, which would not solve the problems encountered in Chapter 2, exploring the synthesis of siRNA therapeutics using the ILSS approach. Therefore, to meet the goal, the concept of an orthogonally cleavable tag must also be proven for ribonucleosides.

Synthesizing oligoribonucleotide blocks is made more difficult than synthesizing oligodeoxyribonucleotide blocks by the requirement that the group employed to protect the 2'-hydroxyl must not migrate upon deprotection of the 3'-hydroxyl or during the subsequent phosphitylation. Isomerization would result in the desired 3'-phosphoramidite being contaminated with some portion of 2'-phosphoramidite, which may not be separable and would yield 2'-5' linkages after the blocks were condensed. This could have significant consequence if this were intended as a therapeutic agent. It has been demonstrated, however, that the

selective release of the 3'-hydroxyl is achievable without isomerization when the 2'-hydroxyl is protected as a triisopropylsilyl ether (TIPS) and the 3'-hydroxyl is protected as a levulinyl ester.[205] Thus to demonstrate that the tag could also be useful in generating oligoribonucleotide blocks, it must simply be shown that the tag is cleavable from the 3'-hydroxyl of a nucleoside that has a TIPS protected 2'-hydroxyl under the same conditions as for the levulinyl ester.



Scheme 3-29: Derivatization of tag to ribonucleoside

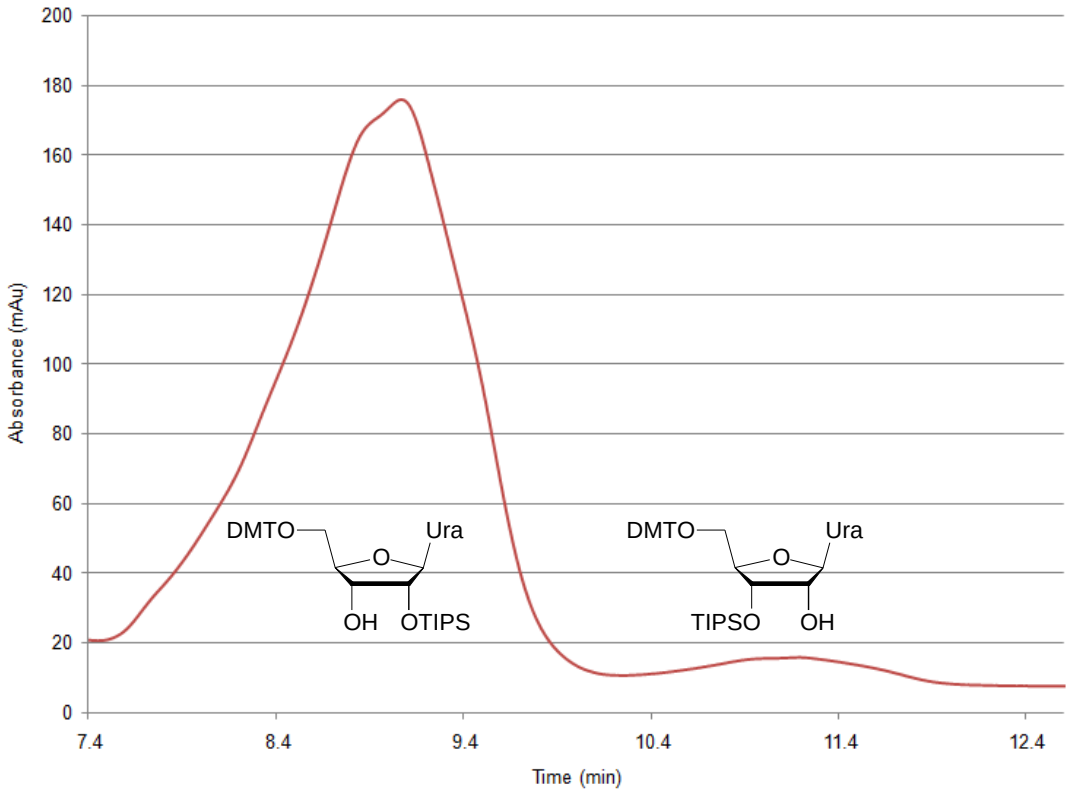
Condensation of the tag with 5'-*O*-DMT-2'-*O*-TIPS Uridine (Scheme 3-29) was accomplished using the same DCC condition employed for the derivatization of the deoxyribonucleoside, namely using a slight excess of nucleoside relative to tag. In retrospect this was a poor choice since, in the deoxy case, not all of the free tag derivatized to nucleoside, some of it formed the *N*-acyl urea product, **3.45** and indeed that was observed here as well. In fact there was more of it in the ribonucleoside case since the derivatization of the nucleoside was slower due to the steric bulk at the 2'-hydroxyl, allowing a longer residence time to undergo the side reaction. This longer residence time was significant as will become clear below.

The tagged nucleoside (**3.48**) was obtained in 62% yield, again contaminated by the *N*-acyl urea product, **3.45**, but the condensation consumed

the remainder of the available tag. The contaminated **3.48** was therefore used as such for the proof of concept.

The ^{1}H-NMR of the coupled nucleoside (**3.48**) did not appear to show any 2'-3' silyl isomerization but the contaminant made that assessment more difficult. The tagged ribonucleoside **3.48** was subjected to the same cleavage conditions as for the cleavage study of **3.44**, for the same 20 minute time period employed in the 3'-levulinyl ester oligoribonucleotide block alluded to earlier[205] and the products were analyzed by HPLC.

Figure 3-8: HPLC of tag cleavage of DMT-rU-2'-OTIPS-3'-O-tag



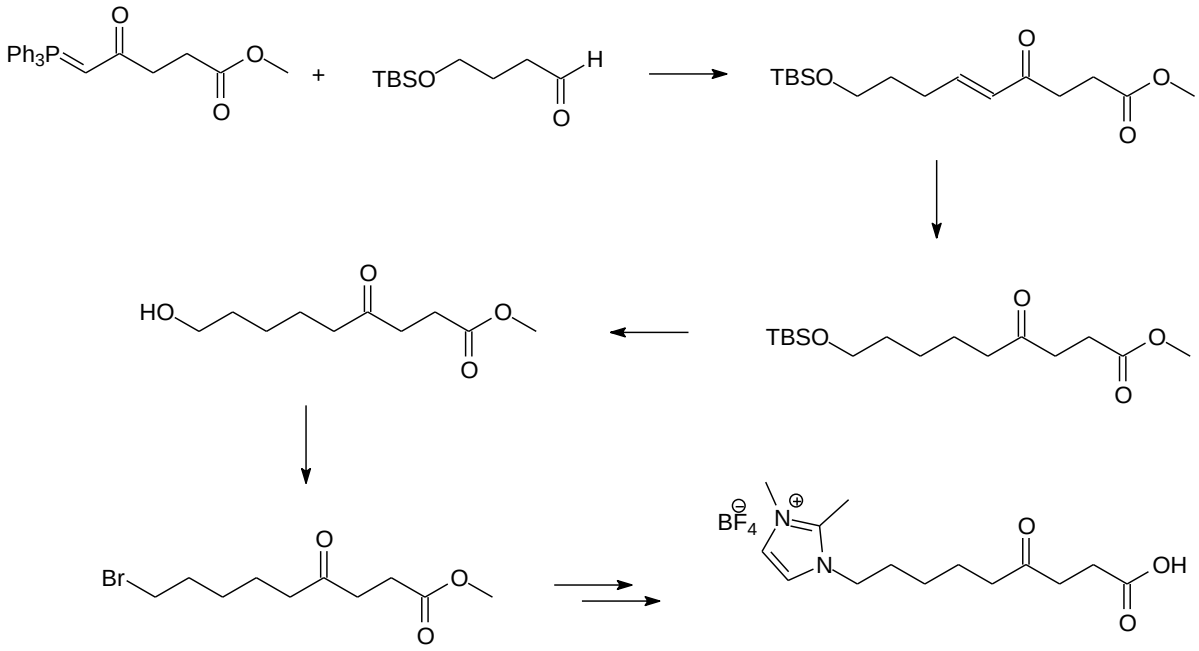
As can be seen in Figure 3-8, the vast majority of the product obtained was the desired 2'-*O*-TIPS ether but an amount corresponding to less than 4% of the 3'-*O*-TIPS isomer was observed. When the reaction time was doubled to 40 minutes, no change occurred in the amount of the 3'-*O*-TIPS isomer. The most likely explanation then for the 3'-*O*-TIPS isomer observed in the HPLC analysis, given that no isomerization was observed when the levulinyl ester was used and that no increase was observed upon prolonged exposure to the deprotection condition, is that the 3'-*O*-TIPS isomer was generated when the tag was installed and not during cleavage.

As mentioned earlier, the normal procedure for derivatizing the nucleoside with tag was to use an excess of tag relative to the nucleoside. This meant that the lifetime of the underivatized nucleoside in the reaction mixture would be shorter since, in terms of the nucleoside, the derivatization would occur more quickly. In this case, however, a limiting amount of tag was used and thus the lifetime of the underivatized nucleoside was relatively long, thus allowing for isomerization to occur prior to derivatization. When the typical conditions (i.e. excess acid) were used for the installation of the levulinyl ester, no isomerization was observed, tending to indicate that this would be achievable with the tag as well, if it were used in excess. That being true, use of the *γ*-keto ester based tag described here would be an excellent route to generate block amidites.

3.5 Future work

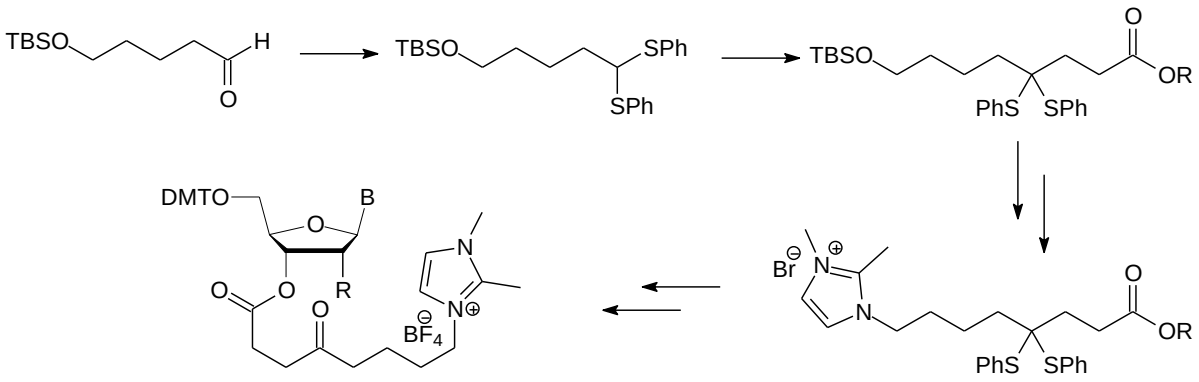
Several areas of improvement in the procedures described are possible, such as an alteration of the Stetter coupling step to attempt to improve the yield. Several solvents have been employed in the past, including DMF and dioxane, and altering the rate of addition of the aldehyde has also been shown to decrease the amount of self-condensation product and increase the yield of the *γ*-keto ester.[199] Alternatively, given that it has been shown that a tag containing *γ*-keto ester is orthogonally cleavable, several other routes to analogous molecules are also possible.

One approach to a similar molecule is shown in Scheme 3-30, exploiting the 5-bromolevulinyl derived ylid,[193] mentioned earlier, to attach the linker to the *γ*-



Scheme 3-30: 5-Bromolevulinyl derived phosphonium ylid approach

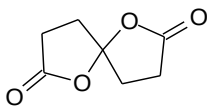
keto ester moiety. This would also utilize many of the transformations already demonstrated for the final Stetter approach used for the proof of concept. Indeed it may even be possible to link the tag in a single step with this approach, since compounds containing the ionic moiety and an aldehyde can be easily prepared. The hydrogenation of the resulting alkene would likely be necessary since it would be conjugated to the ketone and this conjugated system would likely react more slowly with hydrazine during the cleavage of the tag, similar to the slow rate observed for the aryl conjugated ketones discussed earlier, though this would need to be confirmed.



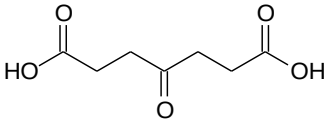
Scheme 3-31: Dithiane Umpolung approach

Another possible approach to the same substrate would be to use a more traditional Umpolung reaction than the Stetter approach, employing a dithiane derived from the same aldehydes as the nucleophile (Scheme 3-31). The same Michael acceptor could be used and this approach would eliminate the competing self-condensation reaction observed in the Stetter reaction. The dithiane would also serve as the ketone protecting group for the bulk of the reaction and would

likely be stable to the fluoride treatment employed for the silyl ether containing aldehyde approach originally outlined in Scheme 3-22. This approach would also use many of the transformations used in the current work, though the timing of the dithiane cleavage to liberate the ketone might be required prior to the installation of the ionic moiety, if the reagents used for this cleavage proved difficult to separate from the desired product. In that case, the dithiane could be cleaved immediately after the carbon-carbon bond formation and an acetal could then be installed.

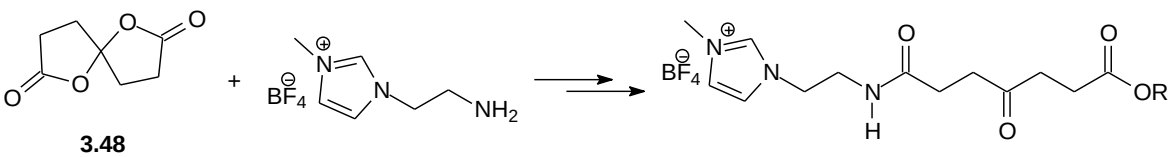


3.48



3.49

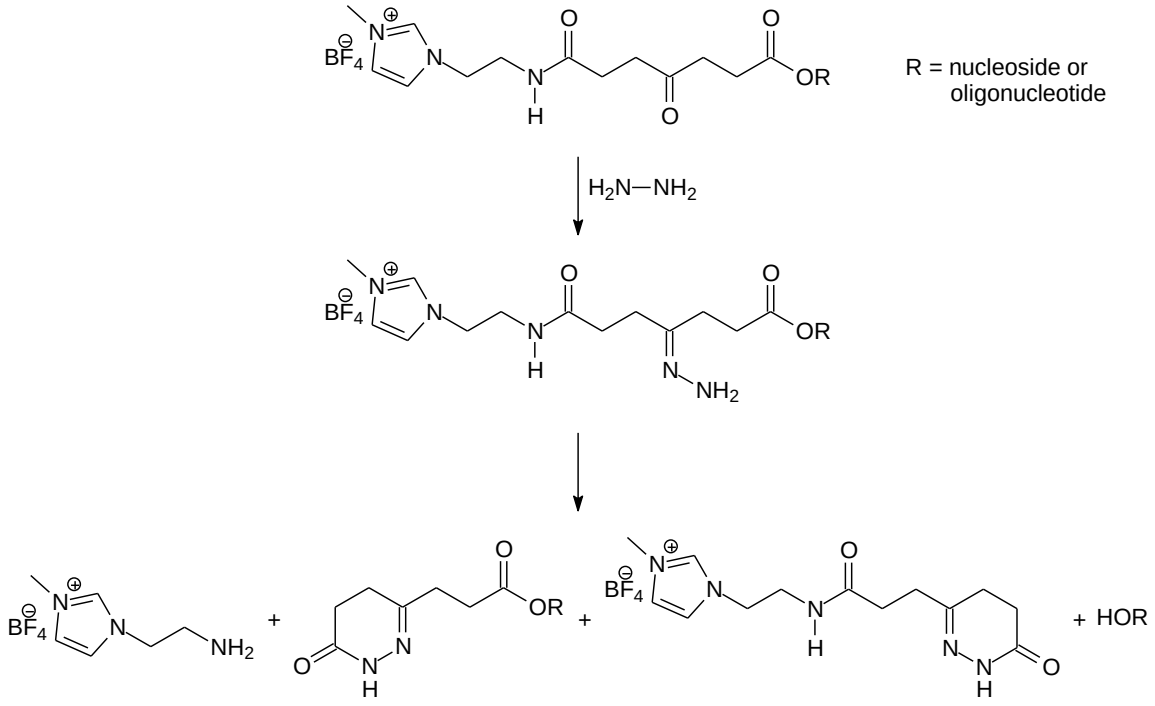
Recently, Lönnberg and co-workers demonstrated that the spiro compound 1,6-dioxaspiro[4,4]nonane-2,7-dione, **3.48**, can be used to easily tether nucleosides to solid support and rapidly cleaved using hydrazine.[206] The open chain form of the molecule, **3.49**, contains two *γ*-keto acid moieties, sharing the same ketone. In their work, one of these is esterified to a nucleoside by direct treatment of the spiro compound with the nucleoside in the presence of a base, while the other carboxylic acid is subsequently attached to the solid support as an amide.



3.48

Scheme 3-32: Spiro compound based approach

Cleavage of the linker occurs in the same manner as for the *γ*-keto esters discussed above. Upon treatment with hydrazine (Scheme 3-33), the central ketone would rapidly convert to the hydrazone. The subsequent attack of the *α*-nitrogen, however, could now proceed in one of two directions, either the cleavage of the ester and concomitant release of the nucleoside/oligonucleotide or cleavage of the amide and release of the support, leaving the linker attached to the nucleoside/oligonucleotide.

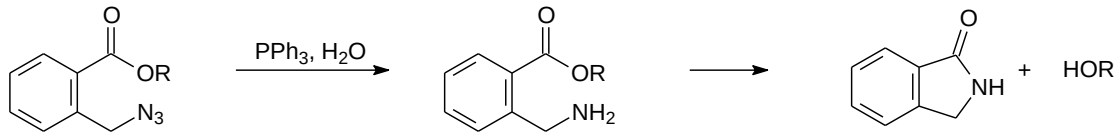


Scheme 3-33: Cleavage products of proposed Spiro based tag

Esters are usually much more labile than amides thus the desired nucleoside/oligonucleotide release due to cyclization is more likely to occur than cleavage of the support, as demonstrated by the authors, though only approximately 80% of the desired material is recovered from the solid support in their study. The nature of the remaining 20% of the material, however, is not

discussed and could be due to amide cleavage. Regardless, this is an interesting approach and should be easily adapted to use a solution phase tag (Scheme 3-32).

The final approach that will be suggested involves a very different system than those explored earlier. Yagodin *et al.* have demonstrated[186] that the 2-azidomethylbenzoyl protecting group (**3.49**), originally disclosed by Sekine and co-workers,[207] could be used to grow oligomers in solution and then be cleaved selectively (Scheme 3-34) by treatment with triphenylphosphine in dioxane/water to reduce the azide to an amine and subsequent cyclization resulting in ester cleavage and release of the oligomers. This allowed for the transformation of those oligomers into block phosphoramidites as is the goal of this research. A nicotinate analogue of this molecule could easily be utilized in an adaptation of this approach to yield an orthogonally cleavable tag (Scheme 3-35).

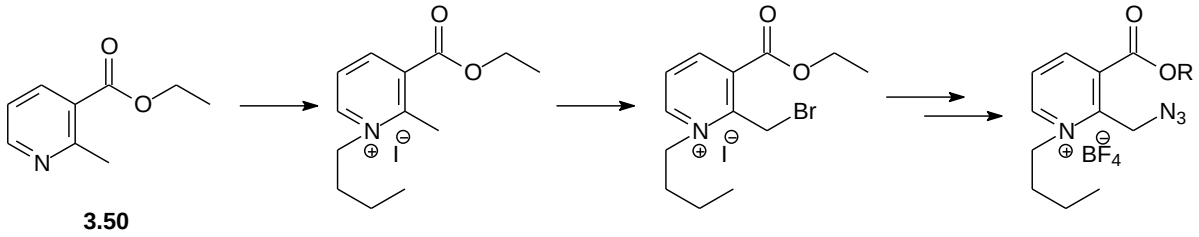


3.49

Scheme 3-34: Cleavage of azidomethylbenzoyl protecting group

Though difficulty was encountered in alkylating a similar molecule, **3.29** (Scheme 3-18), no such difficulty was encountered with ethyl 2-methylnicotinate, **3.50**, which was transformed quantitatively in the presence of excess iodobutane upon heating, the only step attempted by this researcher for this approach. The

subsequent steps in the synthesis would parallel those used to prepare **3.49** and should be easily accomplished for the desired tag as well.



3.50

Scheme 3-35: Nicotinate based approach

The proposed schemes in this section, along with the continuation of the previous work described above, should lead to an ILSS based approach to synthesizing block phosphoramidites, which will aid in the solution phase synthesis of an siRNA based therapeutic.

3.6 Conclusion

The goal of this project was to generate oligomeric block phosphoramidites using an ILSS based approach. Initial attempts to do this by using the simple tag, AIL, and to selectively cleave the ester attaching it to the oligomer were not successful enough to yield the desired quantities. A new generation of tags were proposed that would be orthogonally cleavable from the other protecting groups used in oligonucleotide synthesis, exploiting the *γ*-keto ester moiety's reactivity with hydrazine to achieve release. To this end, several approaches were explored to synthesize a molecule of this form with moderate success. The final synthesis approach discussed yielded a tag that was derivatizable to nucleosides and was likewise cleavable on the same time-scale as is used for the cleavage of

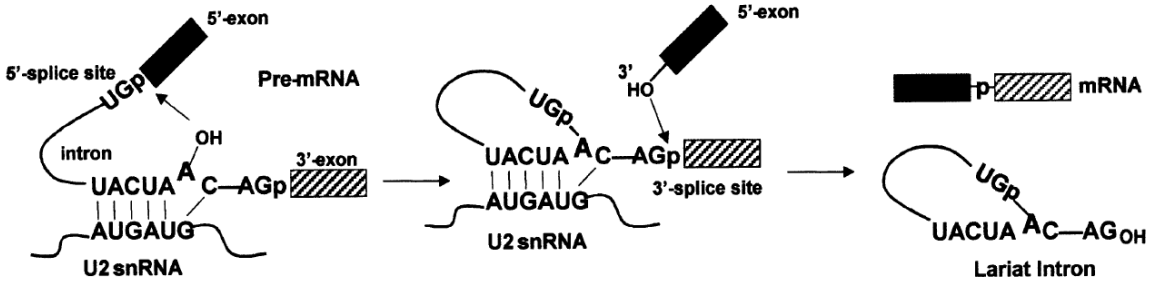
the common levulinyl ester protecting group. Though an oligomer has not been generated using this tag, the results obtained for the nucleoside derivatized substrates indicate that this should be achievable. In addition to the work performed, several new approaches have been proposed that may improve the synthesis and yield of the tag.

Chapter 4 RNA-X

4.1 Introduction

The central dogma of molecular biology, as first proposed by Francis Crick in 1958, states that DNA's genetic information is passed on to proteins through an RNA intermediate.[208,209] The mechanism by which this occurs is complex but may be viewed as a process where, when some function is required in a living organism, a triggering mechanism causes a length of DNA in the genome to be transcribed into a precursor mRNA (pre-mRNA). This pre-mRNA must then undergo a critical post-transcriptional processing step (Figure 4-1), termed splicing by Sharp and co-workers,[210] where non-coding segments, introns, are excised from the pre-mRNA and the coding segments, exons, are ligated to give a mature mRNA after capping and the addition of a polyadenylated tail. Cyclized RNA introns are formed along with the mRNA, structures that result from the

Figure 4-1: Spliceosome mediated splicing reaction[†]

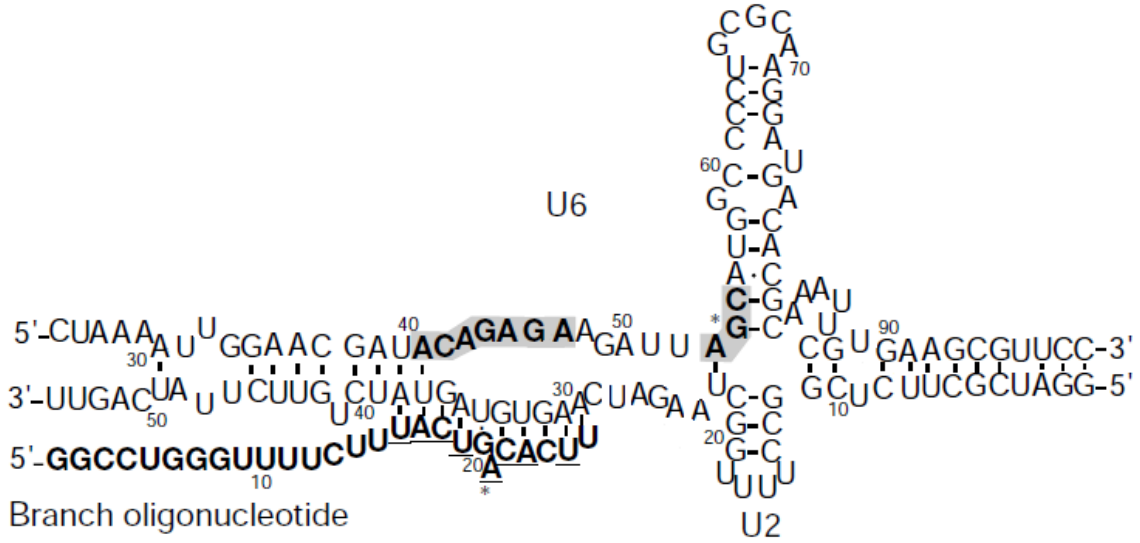


[†]Adapted from Carriero, S.; Damha, M. J. *Nucleic Acids Research* **2003,** *31*, 6157-67

formation of new phosphodiester bonds at internal positions, yielding lariat RNA structures. The mRNA is then translated into a protein, which folds into an active form that performs the necessary function.

In eukaryotic cells, splicing is mediated by the spliceosome, which is composed of five small nuclear RNA-protein complexes (snRNPs), U1, U2, U4, U5, and U6. The snRNPs assemble in a specific order around an mRNA to generate an active complex, which catalyzes the two transesterification reactions that occur for each intron excision and exon ligation. In prokaryotic cells, much of the splicing is achieved autocatalytically; the pre-mRNA performs a self-splicing reaction, without any proteins involved in the excision and ligation steps.[211-214] This catalytic function of RNA in prokaryotes hints at a similar role for the RNA found in eukaryotic spliceosomes as well.

Figure 4-2: Model system designed by Manley and co-workers[†]



[†]Adapted from Valadkhan, S.; Manley, J. L. *Nature* **2001,** *413*, 701-7

In exploring the possible catalytic function of RNA within eukaryotic splicing, Manley and co-workers designed a protein free system composed of the RNA segments found in the spliceosome.[215,216] In choosing the segments to include they noted that U1 and U4 were only involved in the assembly of the spliceosome, leaving the complex when it was prepared for splicing. As they were not involved in the catalytic activity that generates the spliced RNA transcript, they could be omitted from the model system. Likewise, U5 did not seem to be involved in the first steps of splicing and was also omitted for the sake of simplicity. It was observed that both U2 and U6 were highly conserved in their composition and heavily base-paired to both each other and to the pre-mRNA being spliced. Mutagenesis experiments and selective modification of the phosphate backbone to phosphorothioates identified two critical regions for the catalytic process, the ACAGAGA box and an AGC triad, both part of the U6 RNA sequence. Follow up experiments with their protein free system comprised minimal portions of U2 and U6 (complexed to each other) along with the critical sections thought to be involved in catalysis. These shortened U2 and U6 segments were mixed with a simulated substrate, a model pre-mRNA (Figure 4-2) that contained a single splice site, to see if catalytic activity could be observed. Indeed, a reaction did occur to generate a small amount of a species with different mobility from the initial components (PAGE analysis), confirming that the complex of these RNA can cause a reaction to occur. The product, however, was not the expected spliced compound but rather a much larger species.

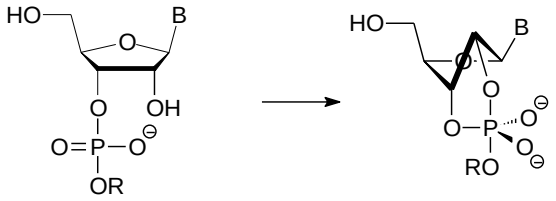
Further analysis of the obtained product revealed a novel structure, termed RNA-X, which contained the U6 and the model pre-mRNA substrate ligated together at internal sites to form an X-shaped molecule. The ligation occurred between the 2'-hydroxyl of a bulged adenosine unit in the pre-mRNA, indicated by the asterisk in Figure 4-2, and the phosphodiester bond between adenosine and guanosine in the AGC triad of the U6 segment. A bulged adenosine unit within the pre-mRNA substrate is a motif that exists in the natural spliceosome as well, caused by a single deletion mismatch in the base-pairing of the pre-mRNA to U2. This bulging of adenosine is thought to activate its 2'-hydroxyl as a nucleophile in the first transesterification reaction that occurs in splicing.

In the simulated system, the reaction between this 2'-hydroxyl and the phosphodiester bond within the U6 AGC triad does not result in a transesterification as normally occurs in the wild-type system but rather yields a phosphotriester bond, an uncommon moiety in biological systems. Even more remarkable was the fact that the ligation site phosphotriester was vicinal to a free 2'-hydroxyl, a species that was known to be very unstable.

The half-life of cleavage for a phosphodiester linkage of DNA in water at neutral pH has been estimated to be tens to hundreds of billions of years, whereas the half-life of RNA under the same conditions as roughly 110 years.[217] The vast difference in the relative stabilities is due to the presence of the 2'-hydroxyl in RNA, which can participate in and accelerate the cleavage of the charged phosphodiester vicinal to it. For phosphotriesters of RNA, however, when the 2'-hydroxyl is unprotected the half-life is reduced to a length of time

ranging from minutes to hours.[10,218-221] The attack of a 2'-hydroxyl on a vicinal phosphodiester linkage in RNA, and thus the rate of cleavage, is slow relative to that of an attack on a phosphotriester, due to the presence of the mono-anion in the phosphodiester. The intermediate phosphorane of the attack on the phosphodiester would be a di-anion, as shown in Figure 4-3, and the build-up of charge is energetically unfavourable. For the attack on the phosphotriester, however, the resultant phosphorane would be a mono-anion, much more prone to form than the di-anion, thus the attack is faster and the half-life of the molecule is shorter.

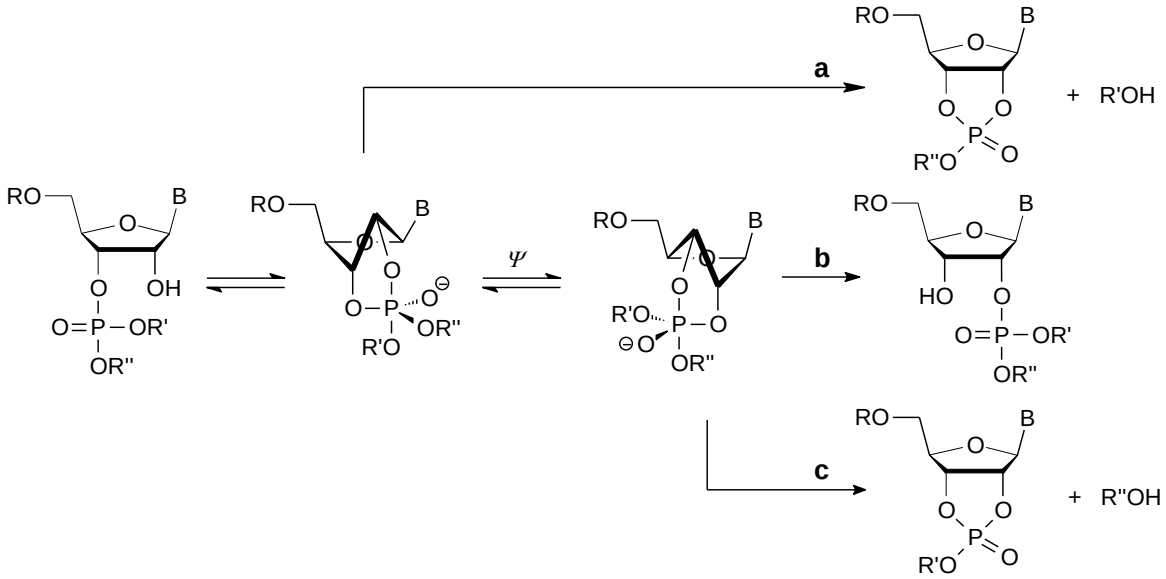
Figure 4-3: Phosphorane intermediate from a phosphodiester



The collapse of the phosphorane intermediate back to a tetrahedral ester requires a group to leave from an apical position. The initial phosphorane generated from an attack by the 2'-hydroxyl would have the 2'-oxygen occupying one apical position and one of the other alkoxyl groups (OR' or OR") occupying the other (Figure 4-4), in order to satisfy Westheimer's rules concerning the formation of phosphoranes.[222] This would allow cleavage of the molecule to occur if the alkoxyl group in the apical position leaves (route **a**) but the intermediate may also undergo pseudorotation (Ψ). If the 2'-oxygen leaves from the apical position, of course, it is simply a reversion to starting material.

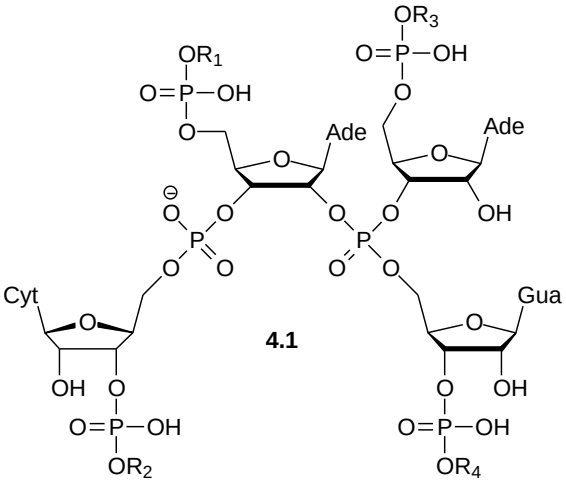
Pseudorotation allows other groups to move from equatorial positions to assume apical positions and act as leaving groups, while the original apical groups move to equatorial positions. For a cyclic system, as is being considered here, Westheimer noted that one oxygen member of the cycle must occupy an axial position while the other occupies an equatorial position.[222] If both were in equatorial positions, the ring strain would be tremendous, with one of the bond angles within the ring being 120°. Therefore pseudo-rotation may only result in the exchange the 2' and 3'-hydroxyls between apical and equatorial positions, with the other apical position exchanging between OR' and OR", since the non-bridging oxygen must remain in an equatorial position. If the 3'-hydroxyl enters an apical position and leaves, the result is a 2'-3' isomerization (route **b**), while if the other alkoxyl group leaves from the apical position (route **c**), the result is

Figure 4-4: Cleavage and isomerization of a phosphotriester



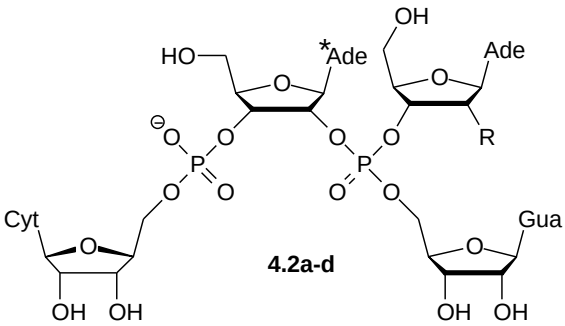
again cleavage of the molecule, with concomitant formation of a cyclic phosphotriester. Lönnberg and co-workers have determined that the rate of endocyclic cleavage, resulting in isomerization, is much faster than the exocyclic cleavage.[218-221,223]

The compound obtained by Manley and co-workers, **4.1**, showed extreme sensitivity to alkaline conditions, as would be expected since the hydroxide present, and not just the 2'-hydroxyl, could act as a nucleophile, but at neutral and acidic pH the compound was surprisingly stable, capable of being isolated and manipulated in various experiments that convincingly demonstrated the nature of the linkage was indeed a phosphotriester vicinal to an unprotected 2'-hydroxyl. The goal of this chapter is to discuss the synthesis of a series of analogues of the core structure of RNA-X (**4.2a-c)** that will be used to study the unusual motif observed in structure **4.1**.



4.1

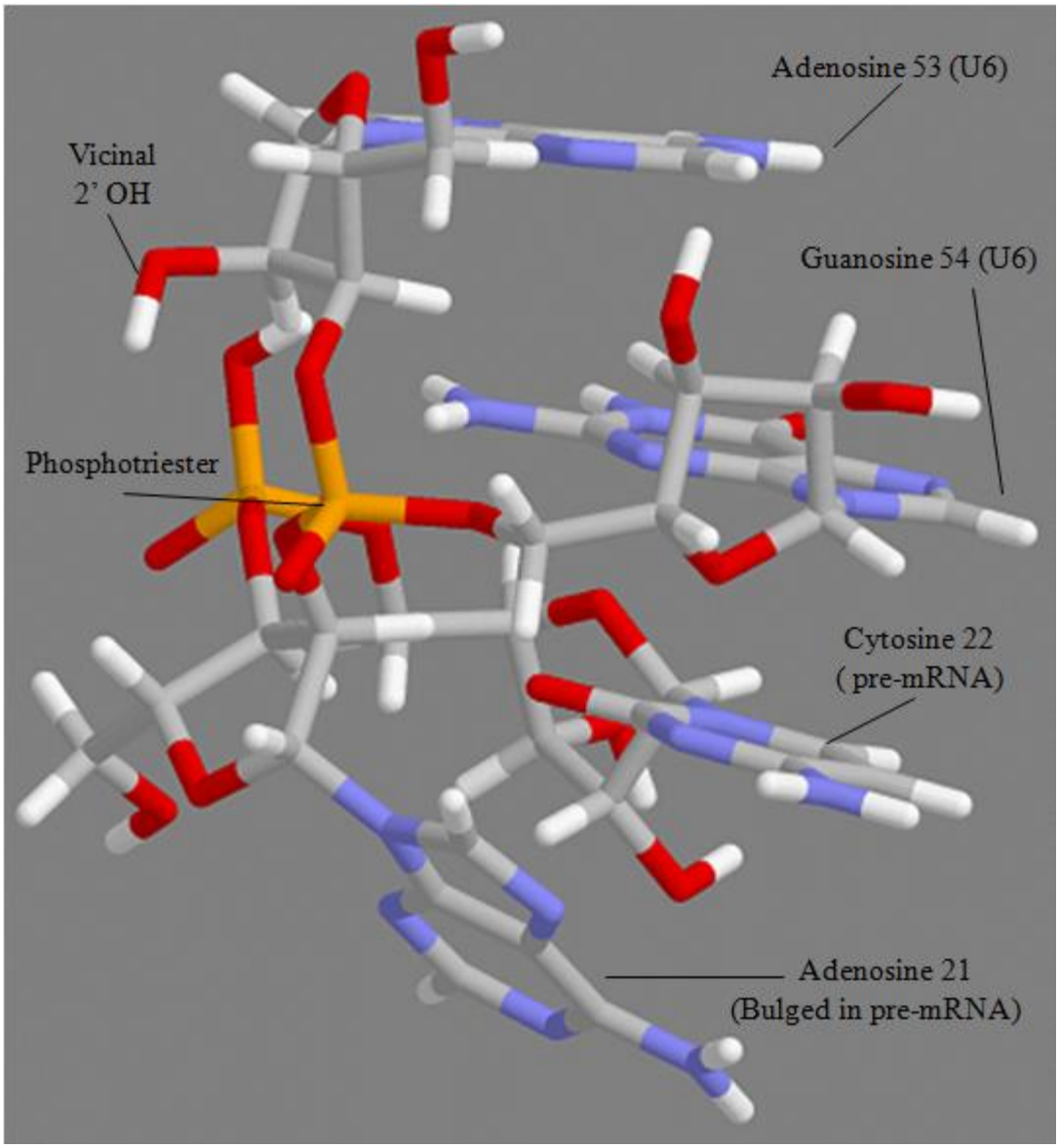
R_1: 5'-end of pre-mRNA
R_2: 3'-end of pre-mRNA
R_3: 5'-end of U6 segment
R_4: 3'-end of U6 segment



4.2a-d

R: **a**-OH, **b**-O-*o*-NO_2Bn, **c**-F

Figure 4-5: Preliminary model of RNA-X analogue **4.2a**



4.2 Preliminary modeling

In order to ascertain whether the 2'-OH analogue, **4.2a**, proposed to mimic the core of the RNA-X molecule could assume a conformation that might account for the unexpected stability of the structure, a preliminary *in silico* experiment was performed. A model of the S_p diastereomer of **4.2a** (2'-OH) was constructed in

HyperChem and a conformational search was performed using an Amber 3 force field to perform energy minimizations on the various conformers that arose as dihedral angles within the molecule were varied randomly. The search was continued until the ten lowest energy conformers were found at least three times each. The conformers were all similar and the lowest energy conformer is shown in Figure 4-5. Though not comprehensive in terms of modelling, the obtained structure represents a possible low energy conformation of the molecule, with intriguing indications as to the source of the stability.

It may be observed in the model that all four of the nucleotides are participating in base stacking, with exceptional π-overlap for Ade 53 and Gua 54, two of the members of the phosphotriester. The third member, Ade 21, is a branched nucleotide, analogous to the ligation point within the intron lariat derived from the splicing reaction. This branched adenosine has phosphate esters at both the 2' and 3' positions, the 2'-hydroxyl participating in the phosphotriester and the 3' linked to the 5' of Cyt 22 through a phosphodiester. Instead of being pushed out to one side, as was observed in structural studies of the lariat branch point,[224] Cyt 22 is folded across the molecule to participate in base stacking with the three members of the phosphotriester. This helps to dictate the orientation of the phosphotriester and likely rigidifies the molecule.

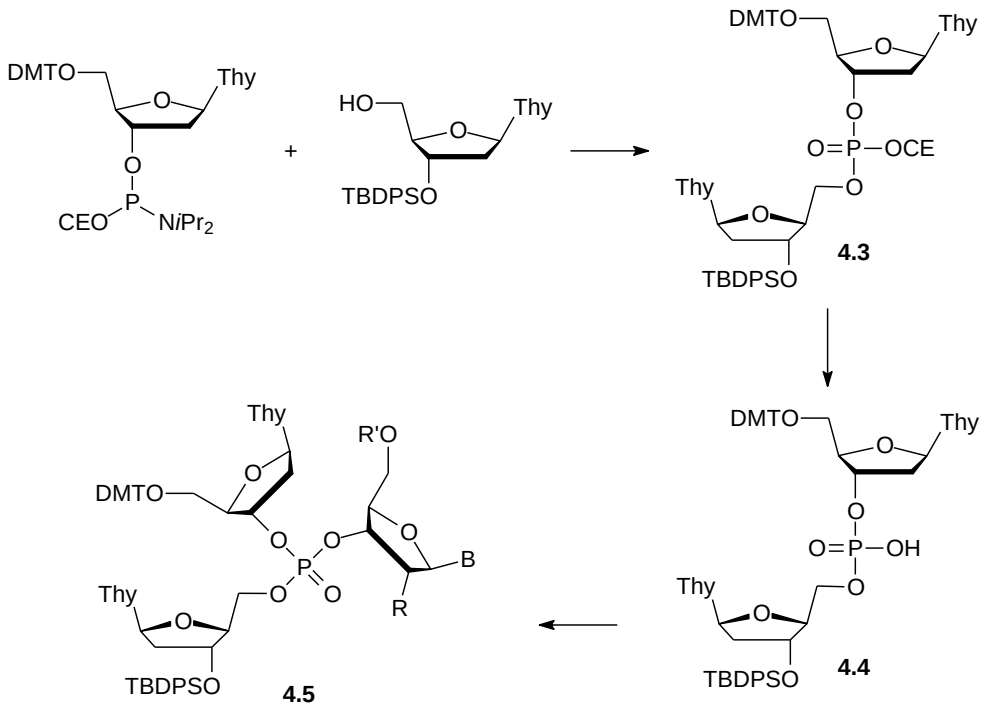
The critical nucleotide to consider of course is Ade 53, since it contains the phosphotriester bond and the vicinal 2'-hydroxyl that should render the molecule unstable. Examination of this nucleotide shows that, in order to minimize steric repulsion, the 3'-phosphotriester has assumed a pseudo-equatorial position

while, in order to maximize the π overlap in base stacking, the C1' has pitched upward slightly, resulting in a C3'-endo conformation for the ring, placing the critical 2'-hydroxyl in a pseudo-axial position. This is the expected conformation for RNA nucleotides within an oligomer and the pseudo-axial orientation of the 2'-hydroxyl also allows for favourable dipole-dipole cancellation with the glycosidic nitrogen bond.

The steric requirements around the phosphotriester moiety, along with the favourable energetics associated with the dipole cancellation and with the maximization of the π overlap in the base stack may result in an overall conformation where the 2'-hydroxyl is rigidly held in position, unable to approach the phosphotriester. This raises the intriguing possibility that the extra stability observed for the RNA-X phosphotriester is due to a conformational restriction of the nucleotide containing the 2'-hydroxyl, making its approach and attack on the phosphotriester difficult. Synthesis of the proposed analogues, **4.2b** and **4.2c**, would allow for further study of the core moiety of RNA-X. First, an NMR based model could be constructed using the 2'F analogue, **4.2c**, to confirm and/or refine the computationally generated model of **4.2a**, while the rate of cleavage of the model compound could be studied through controlled removal of the photolabile *o*-nitrobenzyl protecting group of analogue **4.2b** and monitoring the formation of products, to determine if the analogue behaves similarly to RNA-X.

4.3 Synthesis of model trinucleotide phosphotriesters

Preparation of the proposed analogues, **4.2b-c**, required the formation of trinucleotide phosphotriesters and the cleavage of the other protecting groups necessary for the synthesis without concomitant cleavage of those phosphotriesters. In order to develop a procedure for the synthesis of a trinucleotide phosphotriester and then to assess the phosphotriester's stability under the cleavage conditions necessary for deprotection, it was decided to build model compounds, prior to embarking on the full synthesis of the RNA-X analogues proposed. The first model compound, **4.5a**, was intended to perfect



a: R= H; R'=DMT; B: Thy
b: R= *o*-NO_2BnO; R'=MMT; B: N-Bz Ade
c: R= F; R'=DMT; B: N-Bz Ade

Scheme 4-1: Synthesis of model trinucleotide phosphotriesters

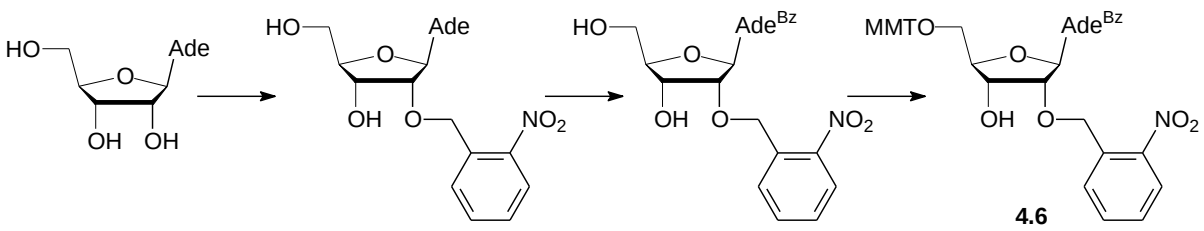
the phosphotriester formation reaction with the simplest and least expensive components possible. Thus the trinucleotide phosphotriester would be composed entirely of thymidine units. The subsequent models, **4.5b** and **4.5c**, would ensure that the variable adenosine unit of the intended analogues, **4.2b** and **4.2c**, could likewise be coupled using the developed methodology

The first reported synthesis of a trinucleotide phosphotriester was by Khorana and co-workers.[130] Its formation was as an unwanted side product during an attempt to synthesize a linear trimer using the phosphodiester approach with DCC as the coupling agent. Only a small amount was obtained and the authors determined that its formation could be avoided by slight alteration of ratio of the reaction components. Later oligonucleotide synthesis methodologies, such as the phosphotriester and phosphoramidite processes, avoided the formation of trinucleotide phosphotriesters entirely by using phosphate protecting groups. In this way, the products of the desired condensations were phosphotriesters but composed of only two nucleotides, with the third ester component being the protecting group itself.

The goal here, however, was to generate trinucleotide phosphotriesters in high yields. The methodology developed for this project accomplished this by using a combination of both the phosphoramidite and the phosphotriester methods (Scheme 4-1). A dinucleotide phosphotriester, **4.3**, could first be prepared very easily in solution using the phosphoramidite method. This dinucleotide phosphotriester could then be converted to a phosphodiester, **4.4**, by cleavage of the phosphate protecting group. The resultant phosphodiester

could then be converted to the desired trinucleotide phosphotriester, **4.5a-c**, using the phosphotriester approach.

Of the components required to synthesize the model compounds, **4.5a-c**, only the 5'-DMT-2'-*o*-NO_2Bn adenosine, **4.6**, was not commercially available. Its synthesis had previously been described by Chaulk *et al*.[225] and is summarized in Scheme 4-2. Briefly, adenosine was treated with sodium hydride in DMF and, after a short reaction period, *o*-nitrobenzylbromide was added, resulting in a mixture of the 2' and 3' benzyl ethers. The reaction mixture was poured into water and allowed to precipitate overnight. The precipitate was isolated and used without further purification. The authors had reported that the 3' isomer remains dissolved in the aqueous phase while the 2' isomer was insoluble and precipitates. The isolated precipitate was dried by co-evaporation with pyridine and then the exocyclic amine of the base was protected. This was achieved by transiently protecting the remaining free hydroxyl groups as trimethylsilyl ethers, treating this intermediate with benzoyl chloride and then cleaving the silyl ethers using a short treatment of cold ammonium hydroxide. Finally, the 5'-hydroxyl was protected as a trityl ether by treatment with monomethoxytrityl chloride, to give **4.6**.



Scheme 4-2: Synthesis of **4.6**

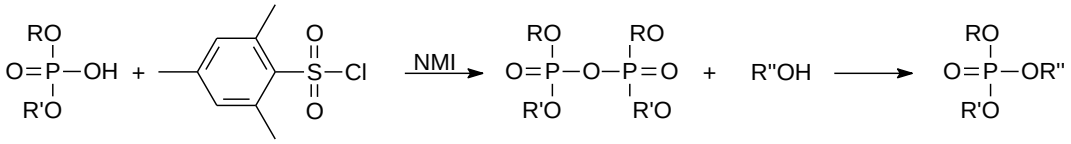
With all the required components in hand, the next step was to show that the proposed approach could be used to generate a trinucleotide phosphotriester in satisfactory yields. Again, the simplest trinucleotide phosphotriester, requiring no base protection and resulting in a single diastereomer, was **4.5a**, which contained three thymidine nucleotides, two attached to the phosphate through their 3'-hydroxyls and the third through its 5'-OH.

The first step in synthesizing **4.5a** was to generate the dinucleotide, **4.3**. Thus, 3'-*O*-TBDPS thymidine was mixed with a small excess of the 3'-*O*-phosphoramidite derivative of 5'-DMT thymidine in the presence of DCI as an activator. After 2 hours, the resultant phosphite triester was oxidized using a 0.1M solution of iodine in THF/water, 2:1 v/v, in the presence of collidine. The reaction mixture was diluted with dichloromethane; the organic layer was washed with aqueous sodium bisulphite to quench the excess iodine, and then with water, to remove any remaining salts. Finally, the organic layer was dried over sodium sulphate and the solvent was removed under reduced pressure.

The crude product thus obtained, **4.3**, was treated with a solution of 1:9 v/v triethylamine/acetonitrile[226] in order to cleave the cyanoethyl protecting group and after 2 hours of reaction time the solvent was again removed. The product was purified by flash chromatography to give **4.4** in 62% yield over the two steps.

The next step was to verify that the trinucleotide phosphotriester could be prepared with the intended method. To this end, the dinucleotide phosphodiester, **4.4**, was mixed with 2,4,6-triisopropylbenzenesulphonyl chloride,

NMI and a large excess of the nucleoside to be coupled, in this case 5'-DMT thymidine.



Scheme 4-3: Phosphotriester formation through pyrophosphate intermediate

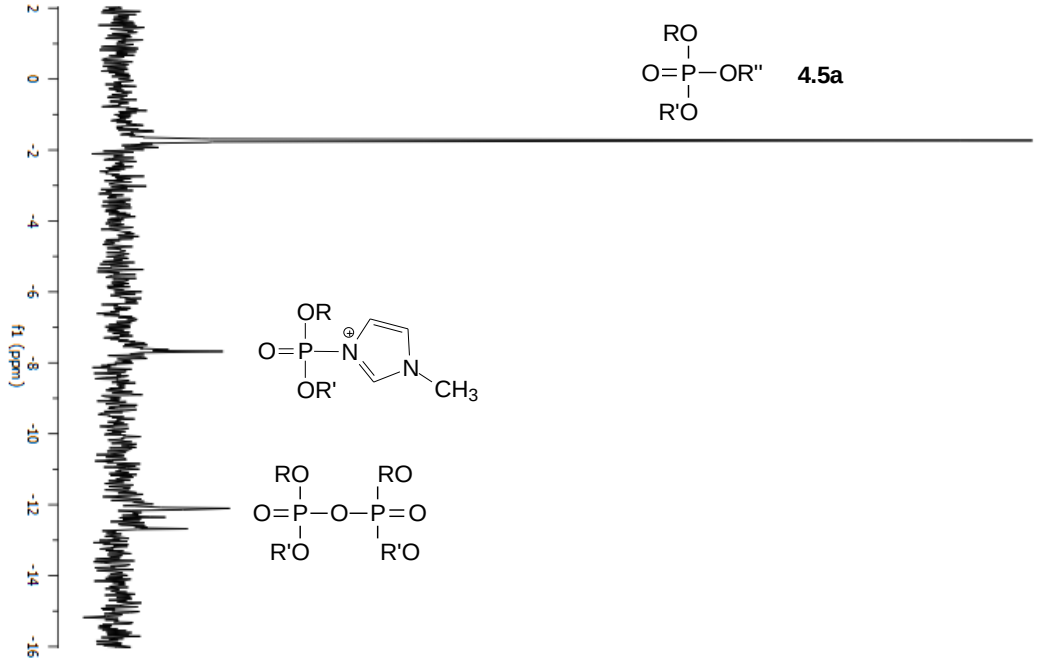
The phosphotriester reaction was known to go through a pyrophosphate intermediate (Scheme 4-3)[12,130,227] and indeed this was observed when the reaction progress was followed by ^{31}P-NMR (Figure 4-6). The signal due to the phosphodiester initially present in the reaction mixture disappeared before monitoring could even begin, while the resulting pyrophosphate and phosphoroimidazolide intermediates generated disappeared over time as they reacted with the nucleoside. The system being studied in this particular reaction resulted in an achiral phosphotriester, since two of the substituents were identical. The reaction was allowed to continue for 16-18 hours and analysis by LR-MS and ^{31}P-NMR showed complete disappearance of the starting phosphodiester and appearance of the desired trinucleotide phosphotriester in excellent yield, after purification.

The resultant compound, **4.5a**, allowed for the stability of the phosphotriester to be evaluated under the cleavage conditions required for removal of the trityl and silyl ethers. Indeed, treatment with 3% v/v trichloroacetic acid in 1,2-dichloroethane and, subsequent to purification, treatment with TREAT

HF in THF, allowed for the removal of the trityl and silyl ethers without any observed cleavage of the phosphotriester.

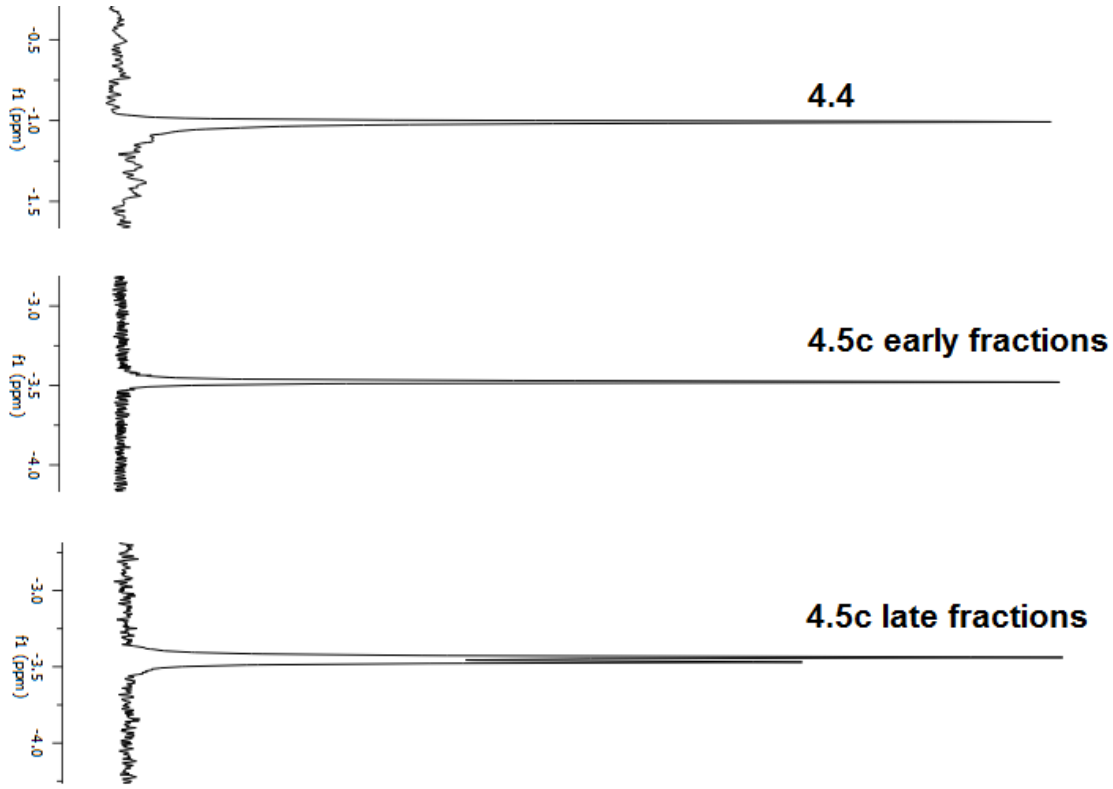
In order to verify that the other nucleosides that were to be coupled to form the phosphotriester in the RNA-X analogues **4.2b** and **4.2c** behaved in a similar manner, they too were coupled with **4.4** to yield the model trinucleotide phosphotriesters, **4.5b** and **4.5c**, and were obtained in similar yields to **4.5a**. These two phosphotriesters were isolated as a mixture of P-diastereomers, as verified by ^{31}P-NMR. In addition, the diastereomers were able to be somewhat separated using flash chromatography (Figure 4-7). The R_f values between each member of the diastereomeric pairs were so similar that no resolution could be observed by TLC but when early and late fractions of the eluting material were

Figure 4-6: ^{31}P NMR of the formation of **4.5a** after 30 minutes



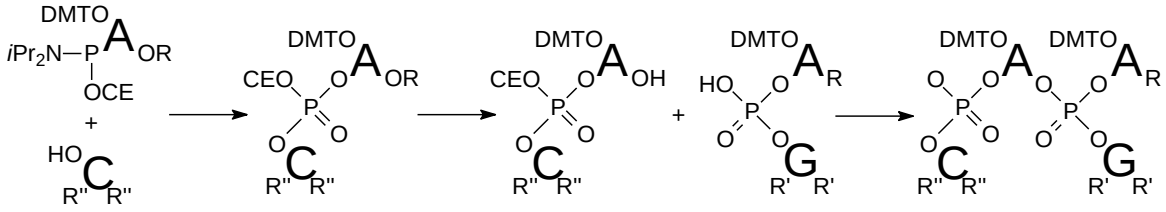
collected and analysed separately, ^{31}P-NMR signals could be observed for a single diastereomer in the early fractions. The majority of the eluted fractions, however, were mixtures. It was hoped that the more complex analogues, **4.2b** and **4.2c** would prove easier to separate but even if the small separation achieved for the simpler analogues **4.5b** and **4.5c** was all that was possible, enough material could be generated through multiple purifications to perform the desired studies.

Figure 4-7: ^{31}P NMR of partially separated phosphotriester diastereomers



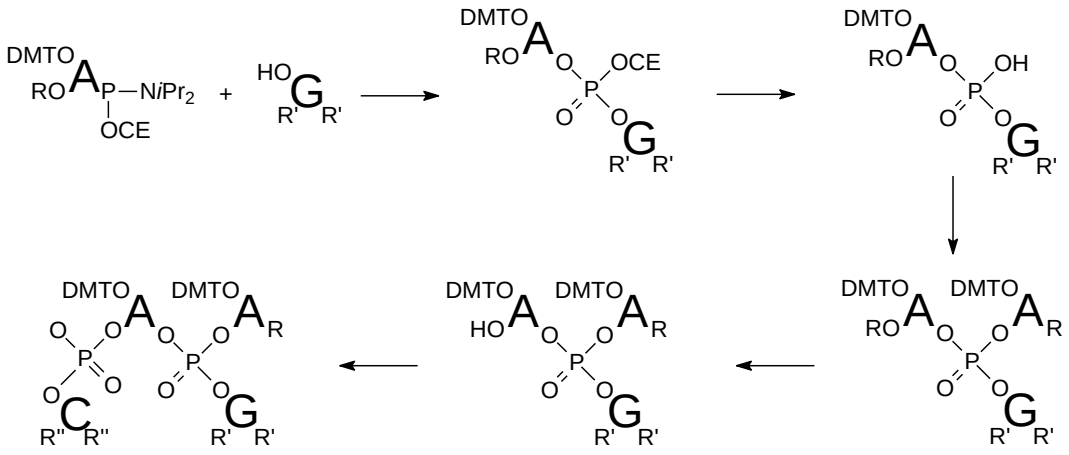
4.4 Synthesis of core RNA-X analogue

Though the chemical transformations necessary to synthesize the RNA-X analogues, **4.2b** and **4.2c**, were the same as for the model phosphotriesters, **4.5a-c**, the assembly of the RNA-X analogues was complicated by the presence of the branched adenosine (identified with an asterisk in structure **4.2**), which contained an extra phosphate linkage compared to the previous molecules synthesized. Careful consideration had to be given to the order of addition of the nucleotides that composed **4.2** in order to accommodate the necessity that both of the phosphate linkages had to be present in the final step, the formation of the trinucleotide phosphotriester, with one in the form of a protected dinucleotide phosphotriester and the other as an unprotected dinucleotide phosphodiester.



Scheme 4-4: RNA-X analogue synthetic route 1

The requirement of having both phosphate linkages present in the final step arises from the fact that the other possible synthetic routes using the intended coupling approaches would require a synthon having an unprotected hydroxyl vicinal to a phosphotriester at some point during the synthesis, as shown in the two routes described in Scheme 4-5 and Scheme 4-4, which is problematic since isomerization and/or cleavage of these synthons would reduce yields and make



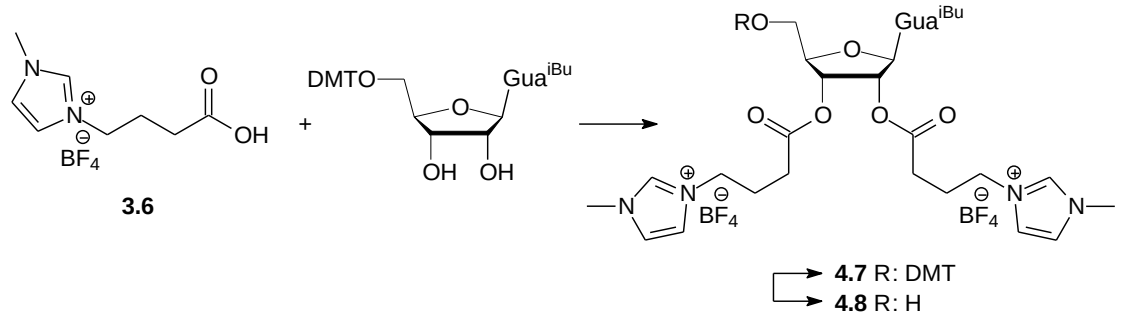
Scheme 4-5: RNA-X analogue synthetic route 2

isolation and characterization of the desired products much more difficult. In order to avoid this problem, Scheme 4-7 was devised.

The initial development of this project began prior to the work performed in Chapter 2 and Chapter 3 on the ILSS approach, with the initial intention of protecting the 2' and 3' hydroxyls of the guanosine nucleoside as benzoyl esters and performing column chromatography after each synthetic step to achieve purification. This was cumbersome and, in order to simplify the purification of the products, the ILSS methodology was eventually employed.

With this approach, the starting nucleoside, 5'-DMT riboguanosine required a tag. In order to overcome the difficulty of isomerization encountered in the ILSS approach explored in Chapter 2 (Scheme 2-4 and Scheme 2-5), when only one tag was used for RNA nucleosides, for this project both the 2' and the 3'-hydroxyls were derivatized with the AIL tag, **3.6**, to yield compound **4.7**. The coupling was performed using TBTU in the presence of triethylamine in 82-86%

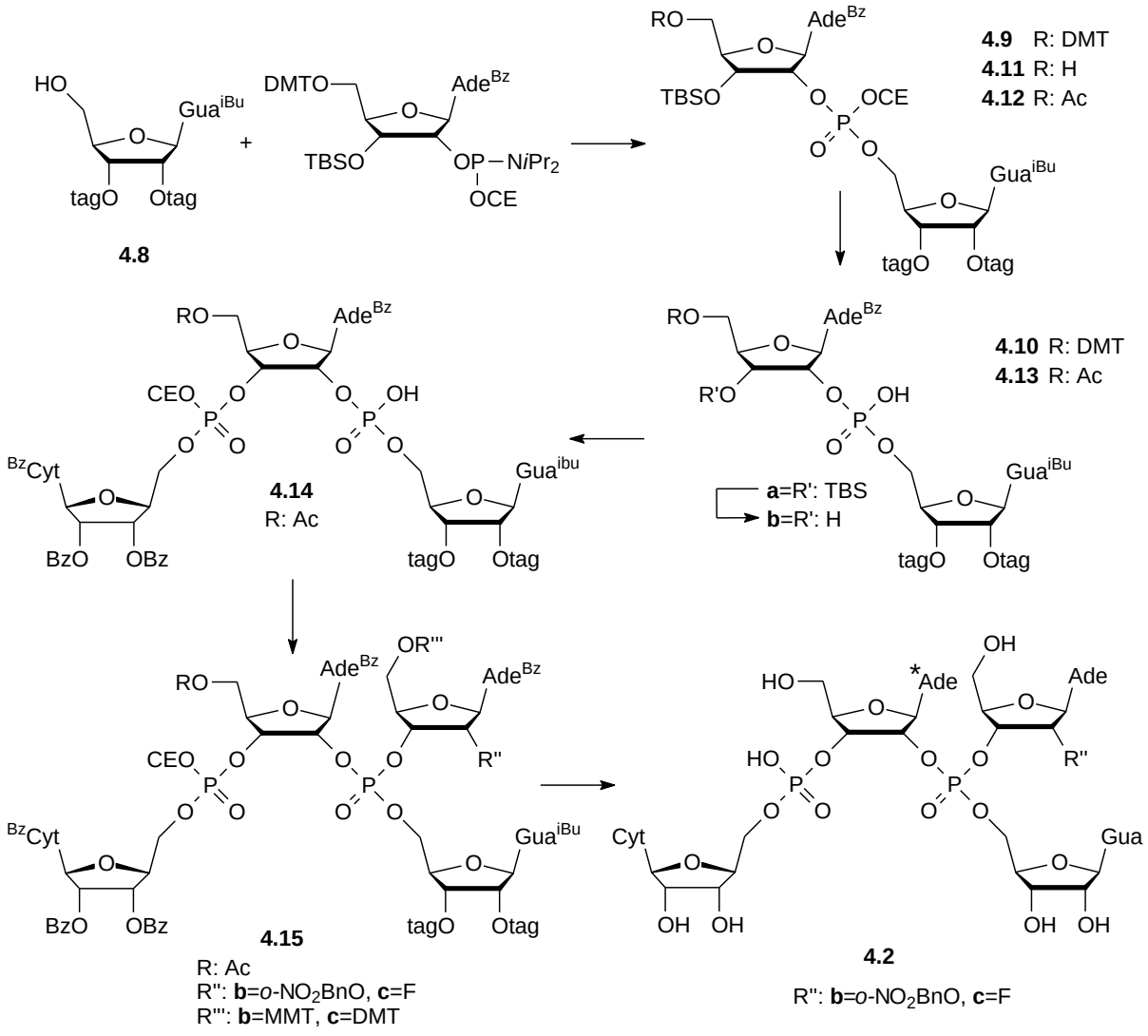
yield and then **4.7** was detritylated using 5% v/v TFA in dichloromethane to give **4.8** (Scheme 4-6). The detritylation did not require more treatments than would typically be used for a singly tagged nucleoside, despite the presence of two ionic moieties, and **4.8** was recovered in 95% yield.



Scheme 4-6: Synthesis of the bis-tagged guanosine **4.8**

The coupling of the first nucleotide, the 2' phosphoramidite of *N*-Bz,5'-DMT,3'-TBDMS adenosine (Scheme 4-7) was performed in the normal manner, with an excess of the phosphoramidite added to the tagged nucleoside, but the reaction only proceeded to approximately 75% completion to give **4.9**. The remaining unreacted tagged nucleoside, **4.8**, was acetylated at the 5' position during the capping step and could not be removed from the desired material. This is a drawback of the ILSS approach, where transformations that do not achieve completion result in the build-up of failure sequences that are carried forward along with the desired product. That being said, however, other than making the determination of yield more difficult in subsequent steps, these impurities are inert and do not interfere with the synthesis. The material was therefore carried forward with the impurity present.

The next step in the synthesis was to cleave the phosphotriester protecting group to yield the phosphodiester that would later be used in the formation of the trinucleotide phosphotriester. This also rendered the phosphate linkage stable to



Scheme 4-7: Synthetic route to RNA-X analogues **4.2b** and **4.2c**

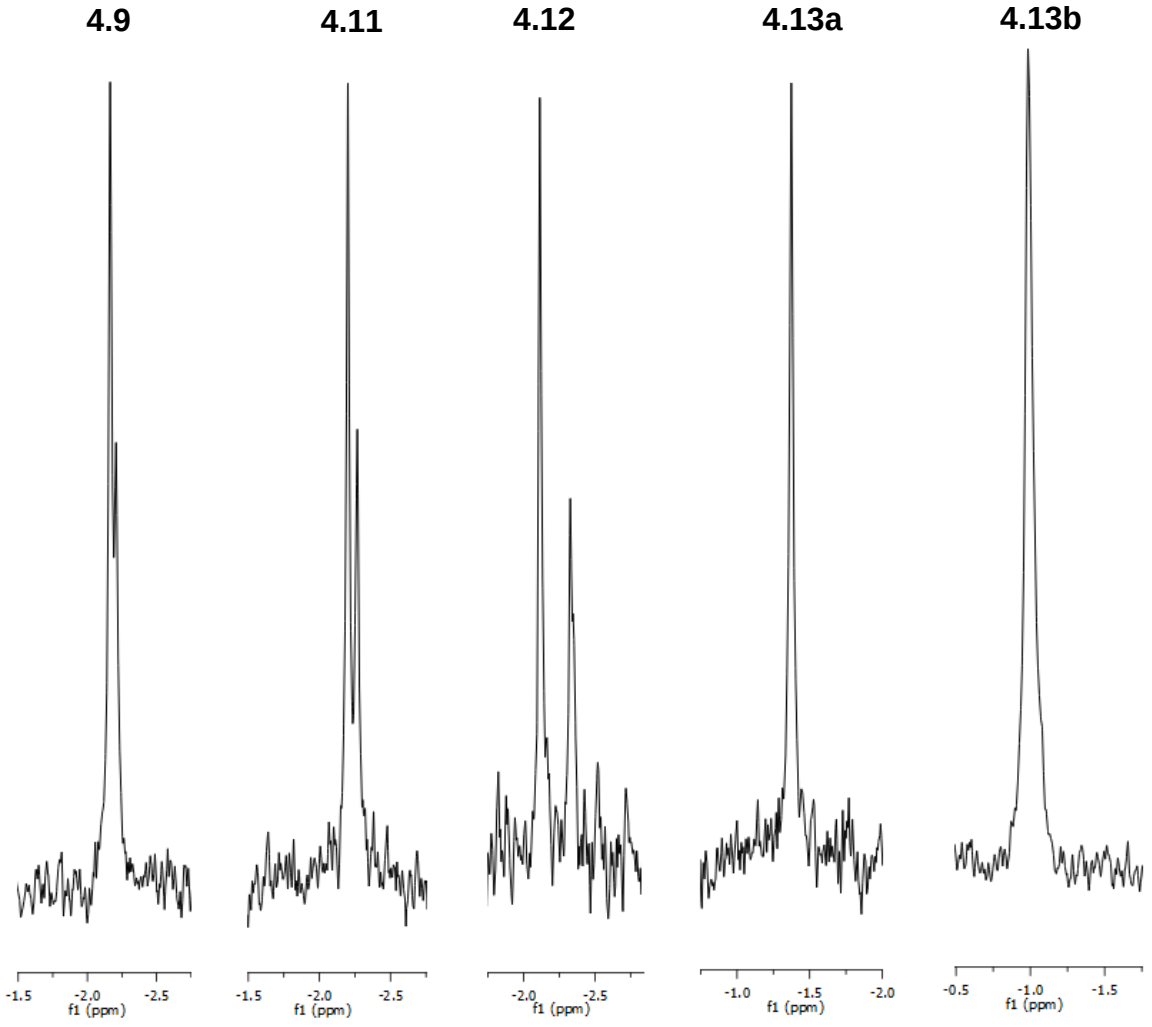
the presence of the free 3'-hydroxyl vicinal to it once that hydroxyl is liberated for further chain extension. Cleavage of the cyanoethyl protecting group to give **4.10a** was performed using anhydrous triethylamine in acetonitrile as was described earlier for the synthesis of **4.4**, and following the reaction, no starting material was observed by LR-MS and a only a single ^{31}P-NMR signal was observed, as expected.

Once the phosphate linkage was deprotected, it was possible to cleave the 3' silyl ether to progress towards the branch point synthesis, since phosphodiesters vicinal to free hydroxyls are significantly more stable. Use of TBAF as the fluoride source for this reaction resulted in significant removal of one of the two tags from the guanosine nucleotide, an observation that was made prior to the work in Chapter 3 and later used in Section 3.2 in an unsuccessful attempt to remove AIL selectively. This problematic cleavage was solved by using TREAT HF, which cleanly cleaved the silyl ether to give **4.10b**, with no isomerization or cleavage of the phosphate linkage detected by ^{31}P-NMR or LR-MS.

Extension could now be performed using *N*,2',3'-tribenzoyl cytidine 5'-phosphoramidite. Using the standard conditions employed previously, however, did not result in the formation of any product. Extension of the reaction time to 18 hours and an increase in the amidite concentration, from 1.5 mole equivalents to 4 mole equivalents, did not improve the result. This was puzzling, as branched oligonucleotides were well known compounds and their synthesis had previously been described.[226,228,229] These syntheses had always been performed through

a coupling at the 3' position first, however, followed by subsequent extension from the 2' position. It had been shown that for RNA nucleosides, the selective alkylation of the 2'-hydroxyl can be enhanced over the competing 3'-hydroxyl by increasing the steric bulk of the protecting group at the 5' position.[230] The possibility existed then that the 3'-hydroxyl of the adenosine unit of **4.10b** is too sterically crowded to allow for coupling when the 5' position is tritylated.

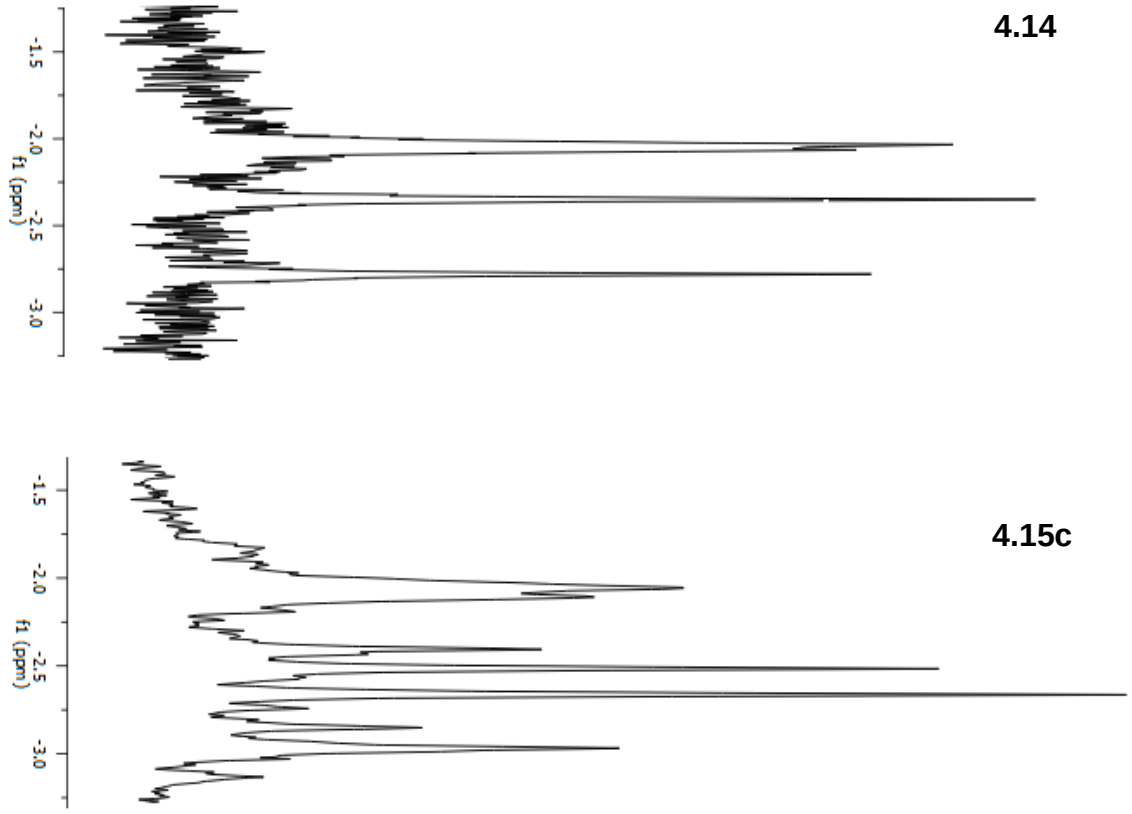
Figure 4-8: ^{31}P-NMR of 5' protecting group exchange, decyanoethylation and 3'-*O*-TBS cleavage



To reduce the steric crowding of the 3'-hydroxyl, the 5'-trityl group was replaced with a much less bulky acetyl group. This exchange of 5' protecting groups was performed after the first step of Scheme 4-7, once the initial coupling of the 2' phosphoramidite of *N*-Bz,5'-DMT,3'-TBDMS adenosine and the bis-tagged guanosine and subsequent oxidation, to yield **4.9**, had been achieved (Scheme 4-7). The trityl protecting group of **4.9** was removed quantitatively to give **4.11**. This was then subjected to the standard capping conditions to yield the 5'-*O*-acetylated material, **4.12**, again quantitatively.

The decyanoethylation and desilylation were performed on the 5' acetylated material as had been done to the 5' tritylated material, to give **4.13a** and **4.13b** respectively, with the similar results (Figure 4-8). The coupling with of *N*,2',3'-

Figure 4-9: ^{31}P-NMR of branched trimer **4.14** and protected analogue **4.15c**



tribenzoyl cytidine 5'-phosphoramidite was then reattempted, allowing for 18 hours of reaction time and using 4 mole equivalents of the phosphoramidite. Using these conditions, coupling went nearly to completion to give **4.14** (Figure 4-9), with less than 5% of the starting material remaining, as indicated by the fact that none of the starting material was visible in the ^{31}P-NMR, while it was observed by LR-MS. The free 3'-hydroxyls of the remaining unreacted starting material were then acetylated and the complete absence of **4.13b** (5'-*O*-Ac) was verified by LR-MS.

With the branched trinucleotide, **4.14**, in hand, the final step in the synthesis was the formation of the trinucleotide phosphotriester of a fully protected RNA-X core analogue. The phosphotriester approach to this synthesis step was used as outlined in Section 4.3. Using an excess of the commercially available *N*-Bz,5'-DMT, 2'-deoxy-2'-fluororiboadenosine in the presence of NMI and 2,4,6-triisopropylbenzenesulphonylchloride, **4.14** was allowed to react for 18 hours. The product of the reaction, **4.15c**, had an m/z value higher than the upper limit of the LR-MS instrument, so the reaction products were analyzed using an HR-MS instrument, which had a higher upper limit of detection. The analysis indicated that the desired product, **4.15c**, had been produced and none of the starting trinucleotide, **4.14**, remained. Likewise, none of the starting material was observed by ^{31}P NMR. Compound **4.15c** exhibited 8 signals as expected, due to the 4 possible diastereomers generated (Figure 4-9). In addition, HR-MS was used to confirm the composition of the molecule as being the fully protected RNA-X core analogue, **4.15c**, as shown in Table 4-1.

Though clearly more work is required to meet the goals of this project, the results obtained thus far demonstrate that the synthetic approach outlined is capable of generating the desired analogues for further study.

Table 4-1: HR-MS results for the RNA-X analogue **4.15c**

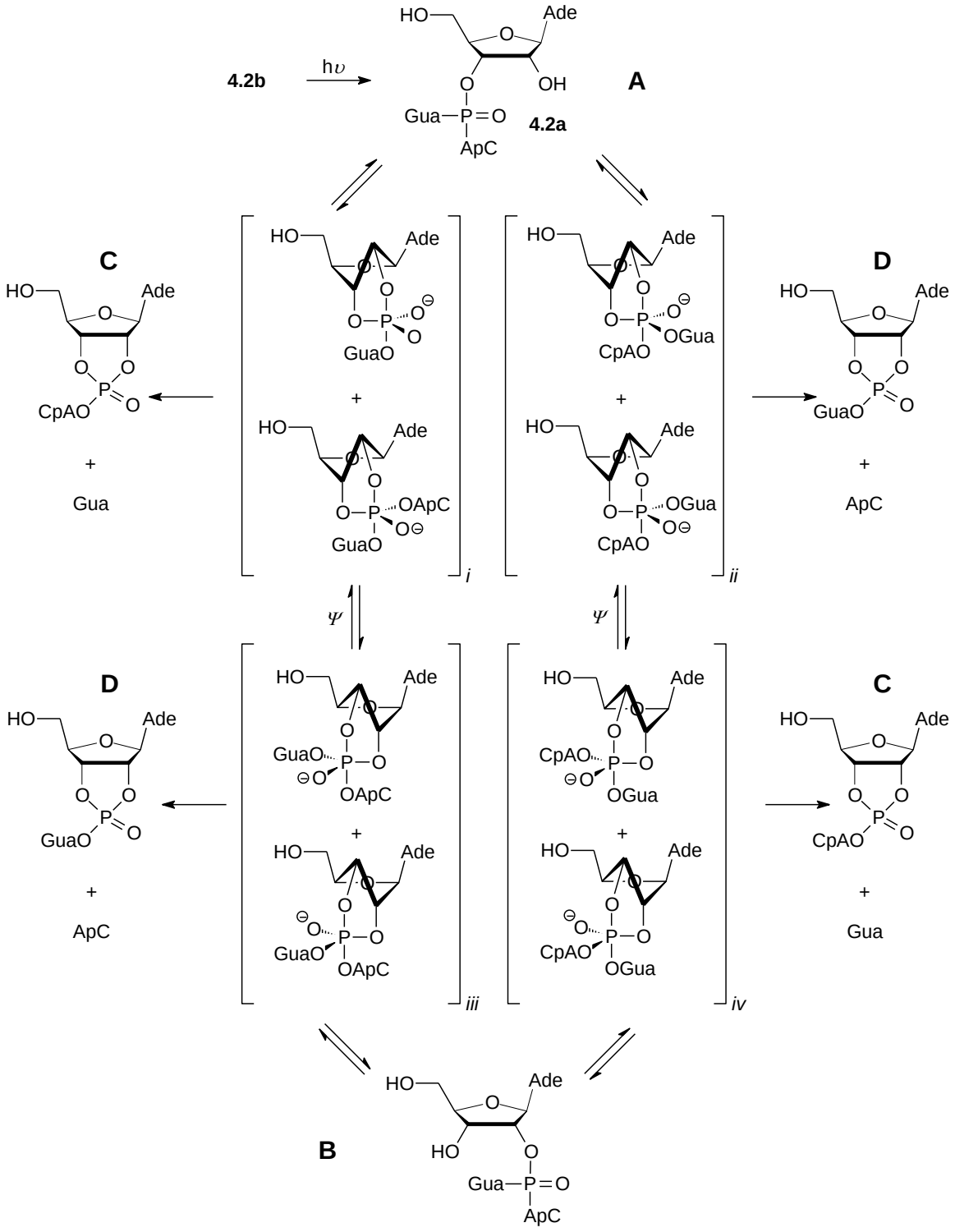
Species	Formula*	m/z (calc)	m/z (exp)	Error (ppm)
No counter ion	$C_{120}H_{118}FN_{23}O_{31}P_2^{2+}$	1228.89062	1228.88843	1.78
Loss of 1 proton	$C_{120}H_{117}FN_{23}O_{31}P_2^{1+}$	2456.77750	2456.77396	1.44
With 1 $Cl^{\ominus}$ counter ion	$C_{120}H_{118}ClFN_{23}O_{31}P_2^{1+}$	2492.75064	2492.75237	0.70
With 1 $BF_4^{\ominus}$ counter ion	$C_{120}H_{118}BF_5N_{23}O_{31}P_2^{1+}$	2544.78526	2544.79077	2.17

4.5 Future work

The next step in the project is to prepare analogue **4.15b** and then to deprotect the two analogues for study. The condensation of the *o*-nitrobenzyl protected nucleoside to form the desired trinucleotide phosphotriester should not pose any problems, given that its condensation with a dTpT dimer, **4.4**, in the model phosphotriester study was successful (**4.5b**). The detritylation reaction likewise was successful for the model phosphotriesters **4.5b** and **4.5c**, and should be successful for the fully protected RNA-X analogues, **4.15b** and **4.15c**, as well. The final deprotection of the molecules to remove the cyanoethyl phosphate protecting group, the benzoyl esters on the cytidine, the base protecting groups and the tags has not yet been demonstrated for these molecules but Lönnberg and co-workers had shown, in their study of the stability

of phosphotriesters with vicinal 2'-hydroxyls, that trinucleotide phosphotriesters may be treated with methanolic ammonia for prolonged periods in order to effectuate deprotection.[219-221] In addition, in the synthesis of oligomer block amidites, others have shown similar ability to deprotect various oligomers under study without concomitant cleavage of dinucleotide phosphotriesters, especially when deprotected phosphodiesters are also present in the molecule,[177,187-189] as discussed in Section 3.2, therefore this should be achievable for the deprotection of **4.15b** and **4.15c** as well.

If this deprotection approach does not prove successful, however, the protecting group strategy could be revised. It has been shown that hydrazine can be successfully used to fully deprotect an oligonucleotide that contains levulinyl protecting groups for the exocyclic amines of adenosine and cytidine along with dimethylformamidine (dmf) protection for guanosine.[231,232] For the RNA-X analogues, **4.15b** and **4.15c**, applying these changes in protecting groups, along with the replacement of the benzoyl esters used to protect the 2' and 3' hydroxyls of the cytidine unit with levulinyl esters and, finally, replacement of the AIL tags with the orthogonally cleavable tag developed in Chapter 3, would give molecules that could be completely deprotected by treatment with hydrazine. Hydrazine in pyridine-acetic acid has also been shown not to cleave phosphotriesters,[169,205] so this protecting group strategy is a viable alternative to the one originally used in the preparation of **4.15b** and **4.15c**, though this would require more time, as the required synthons are not commercially available.



Scheme 4-8: Routes of isomerization and decomposition of **4.2b** after deprotection

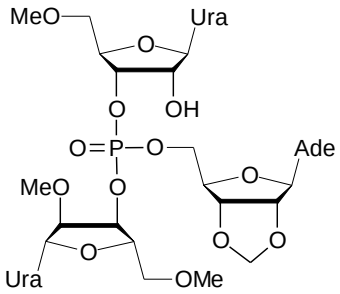
Once the deprotected analogues **4.2b** and **4.2c** are obtained and the diastereomers are separated, two studies will be performed. The first study will determine the rate of decomposition of the analogue **4.2a** by cleavage of the light labile *o*-nitrobenzyl protecting group of compound **4.2b**, to liberate the 2'-hydroxyl vicinal to the phosphotriester, as is thought to exist in RNA-X. The second study will be an NMR based elucidation of the overall conformation of the molecule.

For the first study, the possible routes of decomposition and isomerization of **4.2a,** through phosphorane intermediates, are shown in Scheme 2-1. Initial attack of the 2'-hydroxyl on the adjacent phosphotriester would result in the phosphorane intermediates grouped in brackets *i* or *ii*, depending on the diastereomer and the angle of attack. As stated earlier, the attacking group, in this case the 2'-hydroxyl, must initially occupy an apical position and the other apical position may not be occupied by the oxyanion or the other oxygen involved in the formation of the cyclic species, in this case from the 3' position, according to Westheimer's rules.[233] The other apical position must therefore be occupied by either the guanosine subunit or the branched adenosine, which is in turn linked to the cytidine subunit.

The phosphoranes in brackets *i* or *ii* may revert to the starting material, **A**, by the departure of the 2' oxygen, eliminate the alkoxyl from the other apical position, resulting in cleavage and formation of a cyclic phosphodiester, **C** or **D**, or undergo pseudo-rotation. Pseudo-rotation would exchange the 2' and 3' oxygens as well as the two other alkoxyl substituents, with the oxyanion acting as the pivot, resulting in the species grouped in brackets *iii* and *iv*. These species

could again eliminate the alkoxyl group in the apical position to lead to cleavage products, **C** or **D**, along with the cyclic phosphodiester, undergo pseudo-rotation to go back the grouped species, *i* and *ii*, or the 3' oxygen may leave to give **B**, a net 2'-3' isomerization from **A**.

Lönnberg has studied a model system for the cleavage of RNA-X, structure **4.16**, over a wide pH range and found that, under physiological conditions the molecule had a half-life of 100 seconds,[221] much shorter than was observed by Manley and co-workers for RNA-X.[215,216] Even under the best conditions, the half-life of the molecule studied by Lönnberg and co-workers was no longer than 1 hour, with isomerization proceeding even faster than cleavage.[221]



4.16

The authors noted that the steric bulk around the phosphotriester and the conformation of the oligonucleotide may play a role in the stabilization observed by Manley and co-workers. Though not noted in their study, the presence of an adjacent anionic phosphodiester may also play a role in slowing the attack of the vicinal hydroxyl on the phosphotriester, due to the extra energy required to develop a second negative charge so close to the first, as compared to **4.16**, where no other phosphate is present.

It is hoped that the proposed analogue, **4.2a**, to be derived from **4.2b**, will have cleavage characteristics more in line with those observed for RNA-X, **4.1**, than were observed for **4.13**. The analogue, **4.2a**, should be able to assume a conformation more similar to **4.1**, due to the use of the same purine nucleotides in the analogue as are present in that molecule, as the purines have larger π surfaces and are thus capable of stronger base stacking, as was observed in the modelling study of **4.2a**. In addition, the extra steric bulk of having a large nucleoside present at the branch point, along with the negative charge associated with it will hopefully result in a system more germane to the study of RNA-X.

The second proposed study will use NMR to analyze the structure of analogue **4.2c**. The conformations of each of the constituent nucleotides and the conformation of the overall molecule will be determined. The ^{3}J-coupling constants will be used along with the Karplus equation[234] and potentially a pseudo-rotational analysis program, such as PSEUROT,[235] to determine the puckers of the individual sugar moieties. These will then be combined with NOE coupling measurements between protons on the sugar rings, as well as on the nucleobases, to determine relative positions of the constituent members and construct a three-dimensional model of the compound. The 2'-deoxy-2'-fluororiboadenosine unit was chosen for our RNA-x analogue since the fluorine atom is isosteric to the 2'-oxygen present in the native RNA-X. It is hoped that the overall conformations of the core of the two molecules will be similar, allowing for some insight into the source of the extra stability observed in RNA-X.

4.6 Conclusion

In an attempt to study RNA-X, a molecule discovered by Manley and co-workers having an unusually stable motif of a free 2'-hydroxyl vicinal to a phosphotriester, several analogues have been proposed. The first corresponded directly the core structure of RNA-X, made up of a branched tetranucleotide, where one of the branches contained a trinucleotide phosphotriester, and was subjected to a brief modelling study. The lowest energy conformer found intriguingly suggested that the unusual stability might be due to conformational rigidity within the molecule, arising from favourable steric and electronic factors that hold the vicinal 2'-hydroxyl away from the phosphotriester, making the first step in the decomposition of the molecule, namely attack of the hydroxyl on the phosphotriester, difficult.

With this information in hand, two further analogues were proposed, one to study the rate of cleavage of the molecule under controlled conditions using a light labile protecting group for the 2'-hydroxyl and the other to study the overall conformation of the molecule using NMR analysis by replacing the 2'-hydroxyl vicinal to the phosphotriester with a nearly isosteric fluorine. To this end, model phosphotriesters were synthesized to develop a methodology that could be used to construct a trinucleotide phosphotriester and then these molecules were further used to demonstrate that the deprotection conditions intended did not cause unwanted cleavage of the phosphotriesters. With these studies successfully completed a route to the synthesis of the desired analogues was

devised and implemented successfully for the fully protected 2'-fluoroadenosine containing analogue.

With a route to the desired analogues successfully achieved, the two studies that are to be performed were outlined and discussed. It is believed that the results of this project will favourably enhance the knowledge in the field of RNA structure and stability.

Chapter 5 Experimental

5.1 General Experimental Methods

All reactions were carried out in oven-dried glassware under an argon atmosphere. Anhydrous acetonitrile, dichloromethane and THF were obtained from an MBraun solvent purification system. Anhydrous ethanol and methanol were obtained by serial treatments with 4 Å molecular sieves until the water content was below 100 ppm by Karl-Fisher titration. All other reagents, nucleosides and phosphoramidites were used as received from their suppliers without further purification.

Anion exchange HPLC was carried out using a Waters Protein-Pak DEAE 5PW column. A 30 min gradient from pure water to 0.20 M $LiClO_4$ was used. Reverse phase HPLC employed a Waters Symmetry C18 5 µm 4.6×150 mm column was used with a gradient of 25-100% v/v methanol in water, containing 100 mM triethylammonium acetate adjusted to pH 7.0, unless otherwise stated.

Thin-layer chromatography was used to confirm the purity of all compounds and was performed using Merck Kieselgel 60 F-254 aluminum-back analytical silica gel sheets (0.2 mm thickness, EM Science, Gibbstown, NJ) and visualized under UV light and/or with a solution of 10% H_2SO_4 in MeOH and/or Hanessian's Stain.

Conformational searching was performed using HyperChem 7.0. Conformations were generated by randomly altering the dihedral angles around the glycosyl bonds and around the sugar/phosphate bonds and then performing an energy minimization using an Amber 3 force field with the Polak-Ribiere conjugate gradient algorithm, with a root-mean-square gradient termination cut-off of 0.05 kcal $Å^{-1}mol^{-1}$. The search was continued until the ten lowest energy conformers were found at least three times each.

Low-resolution mass spectrometry measurements were performed on a ThermoQuest Finnigan LCQ Duo octopole mass spectrometer using electrospray ionization in either positive or negative mode.

High-resolution mass spectrometry measurements were made in positive ion electrospray mode with an IonSpec 7.0 tesla FTMS calibrated with polyethylene glycol 300, 600 and 1000.

Deprotection, purification and characterization of oligodeoxyribonucleotides

Removal of the cyanoethyl protecting group, the base protecting groups (for dA, dC, and dG) and the succinyl linker to the ionic tag or solid support was achieved by treatment with 3:1 v/v concentrated ammonium hydroxide/ absolute ethanol at 60 °C for 16 hours or at room temperature for 48 hours. After cooling the sample, the ammonia solution was evaporated to dryness and, for the solid support generated oligomers, were re-suspended in autoclaved, deionized water and centrifuged at 14000 rpm for 30 minutes to settle the support. The solution was then removed and subsequently evaporated to dryness. Oligonucleotides

were purified after cleavage from their respective supports by reverse phase HPLC. Separations were achieved using a Polymerex 10µ RP-1 column (Phenomenex, 10mm x 250mm, 10µ packing) heated to 60 °C, with a mobile phase flow rate of 1mL/min generated by a Waters 1525 binary HPLC pump. The initial mobile phase was comprised of an isocratic flow of 100mM triethylammonium acetate buffer (pH 7.0, 80:20 (v/v) water: methanol) for 2 minutes followed by a gradient shift to 100mM triethylammonium acetate buffer (pH 7.0, 70:30 (v/v) water: methanol) over a 35 minute period followed by 1 minute of isocratic flow before a 14 minute gradient switch back to initial condition and ending with a 5 minute period of isocratic flow. The elution was monitored on a Waters 2487 dual absorbance detector at 260nm and 217nm. Fractions of the eluting peaks were collected and the mobile phase was removed under vacuum. The samples were redissolved in 10% methanol-water and the molecular weights were subsequently determined by low-resolution electrospray ionization mass-spectrometry on a ThermoQuest Finnigan LCQ Duo mass spectrometer in the negative ion mode.

Deprotection of oligoribonucleotides

Removal of the cyanoethyl protecting group, the base protecting groups and the succinyl linker to the ionic liquid support was achieved by treatment of 1-2mg of each pentamer (**2.31** and solution phase sample) with 1 mL of 40% (v/v) aqueous methylamine and incubating at 60 °C for 20 minutes. The resultant solution was cooled to -78°C and dried under vacuum. The resultant solid was treated with 300 µL of 1.5:0.75:1 (v/v/v) triethylamine trihydrofluoride-1-methyl-2-

pyrrolidinone-triethylamine solution and incubated for 90 minutes at 60 °C. The reaction mixture was then cooled to ambient temperature and 1mL of anhydrous ethanol was added. The samples were vortexed and cooled at -78°C for 30 minutes resulting in precipitates. The samples were then centrifuged at 14000 rpm at 4°C for 30 minutes and the supernatant was removed. The precipitates were washed with 200 µL of -78°C ethanol and then the residual solvent was removed under vacuum. The precipitates were dissolved in 1 mL of autoclaved, deionized water and loaded onto Waters® Sep-Pak™ C_{18} cartridges for desalting. The cartridges were rinsed with water and then the desired products were eluted with 1:1:1 (v/v/v) water-methanol-acetonitrile. The solvent was removed under vacuum to yield the crude, deprotected AGAUC pentamers and the residues were redissolved in water and analyzed by ion pairing reverse phase HPLC and polyacrylamide gel electrophoresis (24% acrylamide gel).

General procedure for hydrazine hydrate cleavage studies

The required nucleoside (0.1 mmol) was mixed with the internal standard, acenapthene (0.03 mmol) and acetonitrile (0.1 mL). To this mixture was added 0.5M hydrazine hydrate in 3:2 v/v pyridine/acetic acid (2 mL, 1.0 mmol) and the reaction mixture was shaken quickly to dissolve all components. Samples (10 µL) were removed at timed intervals, generally 1,2,3,4,5,6,7,8,10,12,14,16,18, and 20 minutes after addition of hydrazine solution. Upon removal, the samples were added to a solution of 2,4-pentanedione (0.1 g/mL, 100 µL) in acetonitrile and diluted with acetonitrile (0.9 mL). Samples were then analyzed by RP-HPLC,

with re-injection of the first sample after the series was completed to confirm no change occurred in the samples over time.

5.2 Chapter 2

$^{DMT}T_{Succ\text{-}IL}$ (**2.3**)

Compound **2.2** (1.00 g, 1.63 mmol), substituted imidazolium tetrafluoroborate **2.1** (0.38 g, 1.76 mmol), and DMAP (0.05 g, 0.41 mmol) were placed in a dry, nitrogen-purged 100-mL round bottom flask. To this mixture dicyclohexylcarbodiimide (DCC) (0.68 g, 3.30 mmol) was added, followed by dry CH_3CN (20 mL). The reaction mixture was stirred for 3 days at room temperature. TLC analysis in 9:1 chloroform: methanol showed the formation of a more polar product. When the reaction was stopped, the insoluble DCU by-product was allowed to settle and the reaction mixture was filtered and washed with CH_3CN several times. The solvent was evaporated and the residue was again washed with ether to remove any unreacted DCC and finally collected and dried under vacuum. The product **2.3** was obtained as a light brown foam (1.1 g, 83% yield). ^{1}H NMR (Acetone-*d6*) δ 10.03 (1H, s, NH), 9.10 (1H, s, CH), 7.83 (1H, s, CH), 7.71 (1H, s, CH), 7.60 (1H, s, CH), 7.50-6.92 (14H, m, Ar-H), 6.33 (1H, t, *J* = 8.0 Hz, CH, H1'), 5.50 (1H, d, *J* = 6.0 Hz, CH, H3'), 4.55-4.51 (2H, m, CH_2), 4.70-4.66 (2H, m, CH_2), 4.15 (1H, broad s, CH), 4.05 (3, s, CH_3), 3.80 (3H, s, CH_3), 3.50-3.40 (2H, m, CH_2, H5'&5''), 2.70 (4H, broad s, 2 CH_2), 2.61-2.40 (2H, m, CH_2), 1.42 (3H, s, CH_3); $C_{40}H_{43}N_4O_9^{1+}$ ESI-MS calculated 753.3, found: 753.4.

$^{HO}T_{Succ\text{-}IL}$ (2.4)

To a solution of **2.3** (2.96g, 3.65 mmol) in dichloromethane (200 mL) was added 3% TFA in dichloromethane or CH_3CN (100 mL). During the addition of TFA, the solution became reddish orange and stirring was maintained for 20 min. The product was precipitated from 1:9 ethyl acetate: ethyl ether, filtered, redissolved in a minimum amount of the acid solution, precipitated again and filtered. The precipitate was rinsed with 1:9 ethyl acetate: ethyl ether, recovered from the filter by dissolving in CH_3CN and evaporated under reduced pressure, yielding compound **2.4** as a light brown foam (1.88 g, 96% yield). ^{1}H NMR (DMSO-*d6*) δ 11.40&11.39 (total of 1H, 2 s, NH), 9.10 (1H, s, CH), 7.76 (1H, s, CH), 7.72&7.45 (1H, s, CH), 7.70 (1H, s, CH), 6.15 (1H, m, CH, H1'), 5.27&5.19 (1H, m, CH, H3'), 4.60&3.60 (2H, m, CH_2, H5'&5''), 4.45 (2H, broad s, CH_2), 4.39 (2H, broad s, CH_2), 4.24-3.93 (1H, m, CH), 3.86 (3, s, CH_3), 2.61-2.60 (4H, unresolved m, 2 CH_2), 2.40-2.16 (2H, m, CH_2), 1.77 (3H, s, CH_3); $C_{20}H_{27}N_4O_8^{1+}$ ESI-HRMS required 451.18289, found 451.18234.

$^{DMT}TpT_{Succ\text{-}IL}$ (2.7)

Compound **2.4** (0.24g, 0.45 mmol), thymidine phosphoramidite **1.13d** (0.57g, 0.77 mmol), and DCI (0.66g, 5.6 mmol) were transferred to a 50 mL oven dried, nitrogen purged round bottom flask. To the mixture was added dry THF or CH_3CN (5 mL) to the flask and the resulting solution was stirred at room temperature for 1-2 h. At this point a small amount (1-2 eq) of anhydrous ethanol was added and stirring was continued for a further 10 min. The product was then

precipitated twice from 1:9 ethyl acetate: ethyl ether. At this point, the precipitate was redissolved in CH_3CN and 2,4,6-collidine or pyridine (approximately 300 μL) was added followed by addition of an aqueous iodine solution (0.1 M in THF/water 2:1, excess) to oxidize the phosphite triester intermediate. After 5 min the reaction mixture was quenched with an aqueous sodium bisulphite solution (9 mL), diluted with 90 mL $CHCl_3$ and extracted with 50 mL of water. The product was obtained in high yield as a foam of **2.7** (0.49 g, 91%). ^{31}P NMR (Acetonitrile-*d3*) δ -1.494, -1.584, $C_{54}H_{61}N_7O_{17}P^{1+}$ ESI-MS calculated 1110.4, found 1110.4.

^{HO}TpT$_{Succ\text{-}IL}$ (2.8)

To a solution of **2.7** (0.21g, 0.18 mmol) in CH_3CN (1-2 mL) was added 3% TFA in CH_3CN (2-3 mL). The reaction mixture was worked up following the same procedure as for **2.4** giving **2.8** as a foam in good yield (0.12 g, 78%). ^{1}H NMR (Acetonitrile-*d3*) δ 6.16-6.24 (5'-Thy and 3'-Thy H1'), 5.22-5.30 (3'-Thy H3'), 5.07-5.12 (5'-Thy H3'), 2.30-2.86 (3'-Thy H5'&5''), ^{31}P NMR (Acetonitrile-*d3*) δ -1.188, -1.284, $C_{33}H_{43}N_7O_{15}P^{1+}$ ESI-HRMS required 808.25548, found 808.25493.

^{DMT}TpTpT$_{Succ\text{-}IL}$ (2.15)

Compound **2.8** (0.065 g, 0.073 mmol) was mixed with 3′-phosphoramidite **1.13d** (0.23 g, 0.31 mmol) and DCI (0.31 g, 2.6 mmol) in dry CH_3CN (5 mL) at room temperature. After being stirred for 2 h and quenched with anhydrous ethanol for 10 min, the product was precipitated from 1:9 ethyl acetate: ethyl ether twice, oxidized and extracted in the same manner as for compound **2.7**, to give

compound **2.15** (0.10 g, 92% yield) in high purity. ^{31}P NMR (Acetonitrile-*d3*) -1.081, -1.194, -1.233, -1.262, -1.381, -1.403, -1.448, -1.477. $C_{67}H_{77}N_{10}O_{24}P_2^{1+}$ ESI-MS calculated 1467.5, found 1467.8.

^{HO}TpTpT$_{Succ\text{-}IL}$ (**2.16**)

To a solution of **8** (0.097 g, 0.06 mmol) in CH_3CN (1-2 mL) was added 3% TFA in CH_3CN (2-3 mL). The reaction mixture was worked up following the same procedure as for **2.4** giving **2.16** as a glassy solid (0.077 g, 98% yield). ^{31}P NMR (Acetonitrile-*d3*) -1.157 to -1.531 (broad overlap of peaks). $C_{46}H_{59}N_{10}O_{22}P_2^{1+}$ ESI-HRMS required 1165.32807, found 1165.32752.

^{DMT}TpTpTpT$_{Succ\text{-}IL}$ (**2.17**)

Compound **2.16** (0.17 g, 0.14 mmol) was mixed with 3′-phosphoramidite **1.13d** (0.16 g, 0.22 mmol) and DCI (0.21 g, 1.8 mmol) in dry CH_3CN (5 mL) at room temperature. After being stirred for 2 h and quenched with anhydrous ethanol for 10 min, the product was precipitated from 1:9 ethyl acetate: ethyl ether twice, oxidized and extracted in the same manner as for compound **2.7**, to give compound **2.17** (0.23 g, 89% yield) in high purity. ^{31}P NMR (Acetonitrile-*d3*) -1.169 to -1.859 (broad overlap of peaks). $C_{80}H_{93}N_{13}O_{31}P_3^{1+}$ ESI-MS calculated 1824.5, found 1824.2.

^{HO}TpTpTpT$_{Succ\text{-}IL}$ (2.18)

To a solution of **2.17** (0.19 g, 0.12 mmol) in CH_3CN (1-2 mL) was added 3% TFA in CH_3CN (2-3 mL). The reaction mixture was worked up as for **2.4** giving compound **2.18** (0.16 g, 99.9% yield). ^{31}P NMR (Acetonitrile-*d3*) -1.142 to -1.51 (broad overlap of peaks). $C_{59}H_{75}N_{13}O_{29}P_3^{1+}$ ESI-HRMS required 1522.40066, found 1522.40011.

N-benzoyl-2'/3'-*tert*-butyldimethylsilyl-3'/2'-succinyl-5'-dimethoxytrityl-cytidine (**2.20**)

N-benzoyl-2'-*tert*-butyldimethylsilyl-5'-dimethoxytrityl-cytidine (1.00 g, 1.31 mmol) was mixed with succinic anhydride (0.39 g, 3.93 mmol) and DMAP (0.16 g, 1.34 mmol) and dissolved in 30mL of dry acetonitrile. The reaction was stirred for 4 days at room temperature and the progress was determined by TLC (9:1 dichloromethane-methanol), which showed a disappearance of the starting material and the appearance of two products (2'-3' isomerization of the TBDMS protecting group). The solvent was removed under vacuum and the resulting solid was taken up in chloroform and extracted with water. The organic layer was dried with anhydrous sodium sulphate, filtered and the solvent was removed under vacuum. The resulting crude product (1.1 g, 97%) was used without further purification. $C_{47}H_{54}N_3O_{11}Si^{1+}$ low resolution ESI-MS calculated 864.3, found 863.9.

N-benzoyl-2'/3'-*tert*-butyldimethylsilyl-3'/2'-(1-succinyl-4-[3-(2"-oxyethyl)-1"-methylimidazolium tetrafluoroborate])-5'-dimethoxytrityl-cytidine (**2.21**)

Compound **2.20** (1.10 g, 1.31 mmol) was mixed with TBTU (0.50 g, 1.6 mmol), 3-(2'-hydroxyethyl)-1-methylimidazolium tetrafluoroborate (0.34 g, 1.6 mmol), and triethylamine (0.40 mL, 2.6 mmol). The mixture was dissolved in 20mL dry acetonitrile and stirred for 90 minutes. The solvent was then removed under vacuum and the resulting oil was taken up in chloroform and extracted with water. The organic layer was dried over sodium sulphate. The solvent was removed under vacuum and the resulting solid was dissolved in 5mL of acetone. The solution was added dropwise to stirred diethyl ether resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether, taken up in acetone and precipitated again. The solid was filtered off, rinsed and collected from the filter by dissolution in acetone (1.0 g, 73%) and was used without further purification. $C_{53}H_{62}N_5O_{11}Si^{1+}$ low resolution ESI-MS calculated 972.4, found 972.3, tetrafluoroborate counter ion by ESI-MS.

N-benzoyl-2'/3'-*tert*-butyldimethylsilyl-3'/2'-(1-succinyl-4-[3-(2"-oxyethyl)-1"-methylimidazolium *p*-toluenesulphonate])-cytidine (**2.22**)

Compound **2.21** (0.69 g, 0.65 mmol) was dissolved in 10 mL of 0.1M *p*-toluenesulphonic acid solution in acetonitrile and stirred for 10 minutes. Methanol (5 mL) was added to quench the trityl cation and the reaction mixture was added dropwise to stirred diethyl ether resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether, taken up in

acetone and precipitated again. The solid was filtered off, rinsed and collected from the filter by dissolution in acetone (0.51 g, 93%) and was used without further purification. $C_{32}H_{44}N_5O_9Si^{1+}$ high resolution ESI-MS required 670.29069; found 670.29028, *p*-toluenesulphonate counter ion by ESI-MS.

5'-DMT-rU-(2'-TBDMS)-3'-5'-rC (*N*-Bz)-2'/3'-TBDMS-Succ-IL

(as dicyanoimidazolide salt) (**2.24)**

Compound **2.22** (0.44 g, 0.53 mmol) was mixed with 2'-*tert*-butyldimethylsilyl-5'-dimethoxytrityl-uridine 3'-cyanoethylphosphoramidite (0.75 g, 0.88 mmol) and DCI (0.14 g, 1.2 mmol) and dissolved in 20 mL of dry acetonitrile. The reaction was stirred for 2 hours and then 0.5 mL of *tert*-butanol was added. The mixture was stirred for a further 15 minutes and was then precipitated from diethyl ether as with previous compounds. The precipitate was isolated from the filter by dissolving in acetone and then removing the solvent under vacuum. The resulting solid was dissolved in 5 mL of dry acetonitrile and 250 µL each of a 17.5% v/v solution of acetic anhydride in anhydrous acetonitrile and saturated DMAP in 25% v/v anhydrous pyridine-acetonitrile. This reaction was stirred for 10 minutes and then 0.5mL of 6M *tert*-butyl hydroperoxide in decane was added. After a further 10 minutes of stirring, the reaction mixture was added dropwise to stirred diethyl ether, resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether and collected from the filter by dissolution in acetone (0.77g, 93%) and was used without further purification.

$C_{71}H_{90}N_8O_{19}PSi_2^{1+}$ low resolution ESI-MS calculated 1445.6, found 1446.0, dicyanoimidazolide counter ion by ESI-MS.

5'-HO-rU (2'-TBDMS)-3'-5'-rC (*N*-Bz)-2'/3'-TBDMS-Succ-IL

(as *p*-toluenesulphonate salt) (**2.25**)

Compound **2.24** (0.77 g, 0.49 mmol) was dissolved in 10 mL of 0.1M *p*-toluenesulphonic acid solution in acetonitrile and stirred for 10 minutes. Methanol (5 mL) was added to quench the trityl cation and the reaction mixture was added dropwise to stirred diethyl ether resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether, taken up in acetone and precipitated again. The solid was filtered off, rinsed and collected from the filter by dissolution in acetone (0.68 g, 0.65 g theoretical yield, excess thought to be residual salts), dried under vacuum and was used without further purification. $C_{50}H_{73}N_8O_{17}Si_2P^{2+}$ high resolution ESI-MS required 572.21727, found 572.21795, *p*-toluenesulphonate counter ion by ESI-MS, ^{31}P NMR (ppm) 0.082 to -0.513.

5'-DMT-rA-(*N*-Bz, 2'-TBDMS)-3'-5'-rU-(2'-TBDMS)-3'-5'-rC-(*N*-Bz)-2'/3'-TBDMS-Succ-IL (as dicyanoimidazolide salt) (**2.26**)

Compound **2.25** (0.60 g, 0.46 mmol) was mixed with *N*-benzoyl-2'-*tert*-butyldimethylsilyl-5'-dimethoxytrityl-adenosine (1.65 g, 1.67 mmol) and DCI (0.12 g, 1.0 mmol) and dissolved in 10mL of dry acetonitrile. Upon addition of solvent,

the solution turned pale orange, indicating that the excess mass appreciated in the previous step was likely *p*-toluenesulphonic acid, causing some detritylation of the incoming phosphoramidite. The reaction was stirred for 1 hour and then 0.5mL of *tert*-butanol was added. The mixture was stirred a further 15 minutes and was then precipitated from diethyl ether as with previous compounds. The precipitate was isolated from the filter by dissolving in acetone and then removing the solvent under vacuum. The resulting solid was dissolved in 5mL of dry acetonitrile and 250 µL each of a 17.5% v/v solution of acetic anhydride in anhydrous acetonitrile and saturated DMAP in 25% v/v anhydrous pyridine-acetonitrile. This reaction was stirred for 10 minutes and then 0.5 mL of 6 M *tert*-butyl hydroperoxide in decane was added. After a further 10 minutes of stirring, the reaction mixture was added dropwise to stirred diethyl ether, resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether and collected from the filter by dissolution in acetone (0.89 g, 91%) and was used without further purification. $C_{97}H_{123}N_{14}O_{26}P_2Si_3Na^{2+}$ low resolution ESI-MS calculated 1034.4, found 1034.5, dicyanoimidazolide counter ion by ESI-MS.

5'-HO-rA-(*N*-Bz, 2'-TBDMS)-3'-5'-rU-(2'-TBDMS)-3'-5'-rC-(*N*-Bz)-2'/3'-TBDMS-Succ-IL (as *p*-toluenesulphonate salt) (**2.27**)

Compound **2.26** (0.89 g, 0.41mmol) was dissolved in 5 mL of 0.1M *p*-toluenesulphonic acid solution in acetonitrile and stirred for 10 minutes. Methanol (1 mL) was added to quench the trityl cation and the reaction mixture was added dropwise to stirred diethyl ether resulting in a finely divided

precipitate. The precipitate was filtered off, rinsed with diethyl ether, dissolved off the filter with acetone and dried under vacuum. TLC analysis indicated that detritylation was incomplete so the solid was dissolved in a further 5 mL of 0.1 M *p*-toluenesulphonic acid solution in acetonitrile and stirred for 10 minutes. Methanol (1 mL) was again added to quench the trityl cation and the reaction mixture was precipitated as before. The precipitate was filtered off, rinsed with diethyl ether, and collected off the filter by dissolution in acetone. The solution was concentrated under reduced pressure to an approximate volume of 5mL and was again precipitated from diethyl ether. The solid was filtered off, rinsed with diethyl ether and collected from the filter by dissolution in acetone (0.81 g, 0.79 g theoretical yield, excess thought to be residual salts), dried under vacuum and was used without further purification. $C_{76}H_{106}N_{14}O_{24}Si_3P_2^{2+}$ high resolution ESI-MS required 872.31268, found 872.31383, *p*-toluenesulphonate counter ion by ESI-MS, ^{31}P NMR (ppm) 0.510 to -0.508.

5'-DMT-rG-(*N*-Ac, 2'-TBDMS)-3'-5'-rA-(*N*-Bz, 2'-TBDMS)-3'-5'-rU-(2'-TBDMS)-3'-5'-rC (*N*-Bz)-2'/3'-TBDMS-Succ-IL (as *p*-toluenesulphonate salt) (**2.28**)

Compound **2.27** (0.71 g, 0.37 mmol) was mixed with *N*-acetyl-2'-*tert*-butyldimethylsilyl-5'-dimethoxytrityl-guanosine (0.72 g, 0.77 mmol) and DCI (0.10 g, 0.84 mmol) and dissolved in 7 mL of dry acetonitrile. Upon addition of solvent, the solution turned slightly orange, indicating that the excess mass appreciated in the previous step was likely *p*-toluene- sulphonic acid, causing some detritylation of the incoming phosphoramidite. The reaction was stirred for 1 hour and then

0.5mL of *tert*-butanol was added. The mixture was stirred a further 15 minutes and was then precipitated from diethyl ether as with previous compounds. The precipitate was isolated from the filter by dissolving in acetone and then removing the solvent under vacuum. The resulting solid was dissolved in 5mL of dry acetonitrile and 250 µL each of a 17.5% v/v solution of acetic anhydride in anhydrous acetonitrile and saturated DMAP in 25% v/v anhydrous pyridine-acetonitrile. This reaction was stirred for 10 minutes and then 0.5 mL of 6 M *tert*-butyl hydroperoxide in decane was added. After a further 10 minutes of stirring, the reaction mixture was added dropwise to stirred diethyl ether, resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether and collected from the filter by dissolution in acetone (1.02 g, 99%) and was used without further purification. Note: The low resolution MS indicated that no anion exchange occurred in this cycle (i.e. counter ion remained *p*-toluenesulphonate) $C_{118}H_{154}N_{20}O_{34}P_3Si_4Na^{2+}$ low resolution ESI-MS calculated 1311.5, found 1311.7, *p*-toluenesulphonate counter ion by ESI-MS.

5'-HO-rG-(*N*-Ac, 2'-TBDMS)-3'-5'-rA-(*N*-Bz, 2'-TBDMS)-3'-5'-rU-(2'-TBDMS)-3'-5'-rC-(*N*-Bz)-2'/3'-TBDMS-Succ-IL (as *p*-toluenesulphonate salt) (**2.29**)

Compound **2.28** (1.02 g, 0.37 mmol) was dissolved in 5 mL of 3% v/v trifluoroacetic acid in acetonitrile (app. 0.4 M) and stirred for 10 minutes. Acetone (1 mL) was added to facilitate precipitation and the reaction mixture was added dropwise to stirred diethyl ether resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether, dissolved off the filter with

acetone and dried under vacuum. The solid was redissolved in trifluoroacetic acid solution and stirred for 10 minutes. Acetone (1 mL) was again added and the reaction mixture was precipitated as before. The precipitate was filtered off, rinsed with diethyl ether and collected off the filter by dissolution in acetone. The solution was concentrated under reduced pressure to an approximate volume of 10mL and was again precipitated from diethyl ether. The solid was filtered off, rinsed with diethyl ether and collected from the filter by dissolution in acetone (0.82 g, 91%), dried under vacuum and was used without further purification. $C_{97}H_{137}N_{20}O_{32}Si_4P_3^{2+}$ high resolution ESI-MS required 1149.39748, found 1149.39934, *p*-toluenesulphonate counter ion by ESI-MS, ^{31}P NMR (ppm) 0.637 to -0.466.

5'-DMT-rA-(*N*-Bz, 2'-TBDMS)-3'-5'-rG-(*N*-Ac, 2'-TBDMS)-3'-5'-rA-(*N*-Bz, 2'-TBDMS)-3'-5'-rU-(2'-TBDMS)-3'-5'-rC-(*N*-Bz)-2'/3'-TBDMS-Succ-IL

(as *p*-toluenesulphonate salt) (**2.30**)

Compound **2.29** (0.71 g, 0.29 mmol) was mixed with *N*-benzoyl-2'-*tert*-butyldimethylsilyl-5'-dimethoxytrityl-adenosine (0.72 g, 0.77 mmol) and DCI (0.08 g, 0.65 mmol) and dissolved in 7 mL of dry acetonitrile. No colour change was observed upon solvent addition this time. The reaction was stirred for 2 hours and then 0.5 mL of *tert*-butanol was added. The mixture was stirred a further 15 minutes and was then precipitated from diethyl ether as with previous compounds. The precipitate was isolated from the filter by dissolving in acetone

and then removing the solvent under vacuum. The resulting solid was dissolved in 5 mL of dry acetonitrile and 250 µL each of a 17.5% v/v solution of acetic anhydride in anhydrous acetonitrile and saturated DMAP in 25% v/v anhydrous pyridine-acetonitrile. This reaction was stirred for 10 minutes and then 0.5 mL of 6 M *tert*-butyl hydroperoxide in decane was added. After a further 10 minutes of stirring 1mL of acetone was added and the reaction mixture was added dropwise to stirred diethyl ether, resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether and collected from the filter by dissolution in acetone (0.92 g, 95%) and was used without further purification. $C_{144}H_{187}N_{26}O_{41}P_4Si_5Na^{2+}$ low resolution ESI-MS calculated 1611.6, found 1611.8, *p*-toluenesulphonate counter ion by ESI-MS.

5'-HO-rA-(*N*-Bz, 2'-TBDMS)-3'-5'-rG-(*N*-Ac, 2'-TBDMS)-3'-5'-rA-(*N*-Bz, 2'-TBDMS)-3'-5'-rU-(2'-TBDMS)-3'-5'-rC-(*N*-Bz)-2'/3'-TBDMS-Succ-IL

(as *p*-toluenesulphonate salt) (**2.31**)

Compound **2.30** (0.92 g, 0.27 mmol) was dissolved in 5 mL of 3% v/v trifluoroacetic acid in acetonitrile (app. 0.4 M) and stirred for 10 minutes. Acetone (1 mL) was added to facilitate precipitation and the reaction mixture was added dropwise to stirred diethyl ether resulting in a finely divided precipitate. The precipitate was filtered off, rinsed with diethyl ether, dissolved off the filter with acetone and dried under vacuum. The solid was redissolved in trifluoroacetic acid solution and stirred for 10 minutes. Acetone (1 mL) was again added and the reaction mixture was precipitated as before. The precipitate was filtered off,

rinsed with diethyl ether and collected off the filter by dissolution in acetone. The solvent was removed under reduced pressure. TLC analysis indicated that detritylation was incomplete so a third acid treatment was performed following exactly the same procedure as before. After precipitation and redissolution off the filter with acetone, the solution was concentrated under reduced pressure to an approximate volume of 10mL and was again precipitated from diethyl ether. The solid was filtered off, rinsed with diethyl ether and collected from the filter by dissolution in acetone (0.81 g, 96%) and dried under vacuum. No further purification was performed. $C_{123}H_{171}O_{39}N_{26}P_4Si_5^{3+}$ high resolution ESI-MS required 966.66590, found 966.66546, *p*-toluene- sulphonate counter ion by low resolution ESI-MS, ^{31}P NMR (ppm) 0.599 to -1.318.

5.3 Chapter 3

4-(3-methyl-1*H*-imidazol-1-yl-tetrafluoroboro)butanoic acid (**3.6**)

1-Methyl imidazole (10.3 g, 126 mmol) was mixed with methyl-4-chlorobutyrate (17.2 g, 126 mmol) and warmed to 60 °C. The reaction was stirred overnight and then allowed to warm to room temperature. The oil was rinsed with diethyl ether to remove any unreacted starting material and then mixed with concentrated aqueous HCl (50 mL) and refluxed for 2 hours. The solvent was then removed under reduced pressure and then the oil was rinsed with ethyl acetate followed by diethyl ether. The oil was then mixed with acetone (50 mL) and sodium tetrafluoroborate (30 g, 273 mmol) and stirred overnight.

The remaining solid was filtered off and the solvent was removed under reduced pressure to give **3.6** (29.7 g, 92% yield) as a colourless liquid. ^{1}H NMR (400MHz, DMSO-*d6*) δ = 9.04 (s, 1 H, $CHCN_2$), 7.78 – 7.72 (d, 2 H, CH=CH), 4.44 (t, *J* = 7.6 Hz, 2 H, tag NC**H$_2$**CH_2CH_2-COO), 4.06 (s, 3 H, NCH_3), 2.44 (t, *J* = 7.7 Hz, 2 H, tag NCH_2CH_2C**H$_2$**-COO), 2.24 – 2.19 (m, 2 H, NCH_2C**H$_2$**CH_2-COO), $C_8H_{13}N_2O_2^{1+}$ low resolution ESI-MS calculated: 169.10, found: 169.15.

$^{DMT}CAG_{AIL}$ trimer (**3.7**)

Compound **3.6** (2.1 g, 8.0 mmol) was mixed with triethylamine (1.6 mL) and dissolved in acetonitrile (20 mL). To this solution was added DMT-dG nucleoside (2.5 g, 3.9 mmol) and TBTU (4.4 g, 13.6 mmol). The reaction was allowed to stir overnight and then approximately half of the solvent was removed under reduced pressure. The solution was then precipitated for 10% v/v ethyl acetate/diethyl ether, filtered and recovered from the filter in acetone. The solvent was removed under reduced pressure and the resulting solid was taken up in dichloromethane. The organic layer was washed with water and then dried over sodium sulphate. The solvent was removed under reduced pressure to give the tagged nucleoside, DMT-dG-AIL (3.3 g, 95% yield), as a white foam. The trityl group was then removed by treatment with 0.1M *p*TSA in acetonitrile followed by three precipitations from 10% v/v ethyl acetate/diethyl ether. No trityl was present in the final product as determined by TLC and low resolution by ESI-MS but the recovered yield was greater than 100%, likely due to anion exchange and residual salts being carried with the product through the precipitations. As

these do not interfere with subsequent reactions, the material was carried forward. The oligomerization was performed as previously described, with the 1.5 mole equivalents of amidite added to the tagged nucleoside/oligonucleotide along with 2 mole equivalents of DCI as the activator. The mixtures were dissolved in acetonitrile to give concentrations of approximately 0.10-0.15 M in terms of the amidite. After stirring for 45 minutes, the reactions were quenched with 10 mole equivalent of *t*-butanol and then the reaction products were precipitated twice from 10% v/v ethyl acetate/diethyl ether. The purified products were dissolved in acetonitrile at the same concentration as during the coupling and then capping was performed by the addition of Cap A (17.5% v/v acetic anhydride/ anhydrous acetonitrile) and Cap B (10% NMI in THF/pyridine) solutions followed by oxidation with 5 mole equivalents of *t*-butyl hydroperoxide (6 M in decane) and finally the products were purified by 2-3 precipitations from 10% v/v ethyl acetate/diethyl ether. The intermediate dimer, DMT-dApG-AIL, was detritylated in the same manner as the nucleoside, DMT-dG-AIL and the final product, DMT-dCAG-AIL, was left with the trityl group attached. All couplings and detritylations were accomplished in 94-97% yield, with the final product, **3.7**, obtained as a white foam. $C_{78}H_{84}N_{15}O_{21}P_2^{1+}$ low resolution ESI-MS calculated: 1628.54, found: 1628.42.

Methyl 5-bromo-4-oxopentanoate (**3.10**)

Levulinic acid (5.8 g, 50 mmol) was dissolved in methanol and to this solution was added bromine (8.0 g, 50 mmol) dropwise over 15 minutes. The reaction was then stirred at room temperature for 30 minutes followed by 3.5 hours at reflux. The majority of the solvent was then removed under reduced pressure and the remainder was mixed with diethyl ether and water and the mixture was brought to pH 8 by the addition of saturated aqueous sodium bicarbonate. The aqueous layer was then extracted with diethyl ether and the combined ethereal layers were washed with brine and dried over magnesium sulphate. The solvent was removed under reduced pressure and the product was isolated by vacuum distillation to give **3.10** (6.3 g, 60% yield) as a colourless liquid. ^{1}H NMR (400 MHz, Chloroform-*d*) d = 3.95 (s, 2 H, Br CH_2), 3.67 (s, 3 H, $COOCH_3$), 2.95 (t, *J* = 6.5 Hz, 2 H, C**H$_2$**CH_2COO), 2.64 (t, *J* = 6.5 Hz, 2 H, CH_2C**H$_2$**COO), $C_6H_9BrO_3Na^{1+}$ low resolution ESI-MS calculated: 230.96, found: 231.26.

5-(3-methyl-1*H*-imidazol-1-yl)-4-oxopentanoic acid (**3.11**)

Compound **3.10** (1.0 g, 4.8 mmol) was dissolved in 5 mL of acetonitrile and cooled to 0 °C. To this a solution of 1-methylimidazole (0.59 g, 7.2 mmol) in 5 mL of acetonitrile was added slowly. The reaction was allowed to warm to room temperature and was stirred for one hour. The solvent was then removed under reduced pressure and the resultant oil was rinsed 3 times with MTBE. Any residual solvent was then removed by again subjecting the oil to reduced

pressure, giving **3.12** (1.4 g, 96% yield). Compound **3.12** (1.4 g, 4.6 mmol) was then mixed with 25 mL of concentrated aqueous HBr and refluxed overnight. The solvent was then removed under reduced pressure. The resultant brown solid was suspended in 20 mL of acetonitrile, mixed with sodium tetrafluoroborate (1.48 g, 13.5 mmol) and stirred overnight. The undissolved solid was then removed by filtration and the solution was dried under reduced pressure to give **3.11** (1.4 g, >100% yield) as a pale brown solid, likely contaminated with sodium tetrafluoroborate. **3.12** ^{1}H NMR (400MHz, DMSO-d_6) d = 8.98 (s, 1 H, NCHN), 7.72 (s, 1 H, NCHCHN), 7.57 (s, 1 H, NCHCHN), 5.38 (s, 2 $HNCH_2CO$), 3.58 (s, 3 H, CH_3N), 2.86 (t, *J* = 6.5 Hz, 2 H, $C\mathbf{H_2}CH_2COO$), 2.56 (t, *J* = 6.3 Hz, 2 H, $CH_2C\mathbf{H_2}COO$), $C_{10}H_{15}N_2O_3^{1+}$ low resolution ESI-MS calculated: 211.11, found: 211.24 **3.11** $C_9H_{13}N_2O_3^{1+}$ low resolution ESI-MS calculated: 197.09, found: 197.23.

Ethyl 4-oxooct-7-enoate (**3.18**)

Ethyl 4-chloro-4-oxobutanoate (16.5 g, 100 mmol) was dissolved in 36 mL of THF and cooled to -78 °C. To this solution was added 100 mL of 0.5 M butenyl magnesium bromide in THF (50 mmol), dropwise over 30 minutes. The reaction mixture was then allowed to warm to room temperature and it was stirred for 1 hour. The reaction mixture was diluted with 100 mL of water and extracted with diethyl ether. The combined ethereal layers were then washed with 1 M aqueous sodium hydroxide followed by brine. The organic layer was dried over magnesium sulphate and the solvent was removed under reduced

pressure. The desired product was then isolated by vacuum distillation to give **3.18** (1.7 g 18% yield) as a colourless oil. ^{1}H NMR (300MHz, Acetonitrile-d_3) δ = 5.98 - 5.73 (m, 1 H, CH_2=C**H**), 5.13 - 5.06 (m, 2 H, C**H$_2$**=CH), 4.08 (q, *J* = 7.0 Hz, 2 H, COOC**H$_2$**), 2.74 - 2.68 (m, 2 H, C**H$_2$**CH_2COOEt), 2.55 - 2.48 (m, 2 H, CH_2C**H$_2$**COOEt), 2.35 - 2.26 (m, 2 H, CH_2=$CHCH_2$C**H$_2$**), 2.18 - 2.12 (m, 2 H, CH_2=CHC**H$_2$**CH_2), 1.21 (t, *J* = 7.0 Hz, 3 H, $COOCH_2$C**H$_3$**), $C_{10}H_{16}O_3Na^{1+}$ low resolution ESI-MS calculated: 207.10, found: 207.32.

*N*1,*N*4-dimethoxy-*N*1,*N*4-dimethylsuccinamide (**3.20**)

N,*O*-Dimethylhydroxylamine hydrochloride (11.2 g, 115 mmol) was dissolved in 167 mL of dichloromethane, mixed with 33 mL triethylamine and cooled to 0 °C. Another solution, composed of succinyl chloride (7.89 g, 51 mmol) in dichloromethane (10 mL), was then added slowly, dropwise over 20 minutes. The reaction was allowed to warm to room temperature and stirred overnight. The reaction mixture was then diluted with dichloromethane and washed with water, followed by brine. The organic layer was then dried over magnesium sulphate and the solvent was removed under reduced pressure. The resultant black tar was triturated with boiling hexanes several times. The collected hexanes layers were evaporated under reduced pressure and the resultant solid was recrystallized from more hexanes to give **3.20** (6.3 g, 60% yield) as pale yellow needles. ^{1}H NMR (400MHz, DMSO-d_6) d = 3.66 (s, 3 H, NOC**H$_3$**), 3.05 (s, 3 H, NC**H$_3$**), 2.59 (s, 2 H, COC**H$_2$**), $C_8H_{17}N_2O_4^{1+}$ low resolution ESI-MS calculated: 205.12, found: 205.32.

N-methoxy-*N*-methyl-4-oxooct-7-enamide (**3.21**)

4-Bromobutene (5.8 g, 43 mmol) was mixed with magnesium turnings (1.1 g, 43 mmol) in THF, along with a crystal of iodine. The reaction mixture was then heated to reflux for 1 hour, whereupon no residual magnesium was observed. The reaction mixture was then cooled to room temperature and added slowly, dropwise to a solution of **3.20** (6.9 g, 34 mmol) in 175 mL of THF that had been cooled to 0 °C. Upon completion of the addition, the reaction mixture was allowed to rise to room temperature and stirred for 4 hours. The reaction was then quenched by the addition of saturated aqueous ammonium chloride and extracted with diethyl ether. The combined organic layers were washed with brine and then dried over magnesium sulphate. The solvent was removed under reduced pressure and the product was purified by flash column chromatography using a hexanes/ethyl acetate gradient elution system to give **3.21** (3.4 g, 50% yield) as a colourless oil. ^{1}H NMR (500MHz, Chloroform-*d*) δ = 5.84 - 5.75 (m, 1 H, CH_2=C**H**), 5.04 - 4.95 (m, 2 H, C$\mathbf{H_2}$=CH), 3.72 (s, 3 H, NOC$\mathbf{H_3}$), 3.16 (s, 3 H, NC$\mathbf{H_3}$), 2.73 (br. s, 2 H, COC$\mathbf{H_2}$), 2.58 (t, *J* = 7.5 Hz, 2 H, CH_2=CHCH_2C$\mathbf{H_2}$), 2.35 - 2.31 (m, 2 H, CH_2=CHC$\mathbf{H_2}$CH_2), $C_{10}H_{17}NO_3Na^{1+}$ low resolution ESI-MS calculated: 222.11, found: 222.18.

3-(2-(but-3-en-1-yl)-1,3-dioxolan-2-yl)-*N*-methoxy-*N*-methylpropanamide (**3.22**)

Compound **3.21** (3.4 g, 17 mmol) was mixed with ethylene glycol (2.1 g, 34 mmol) and *p*TSA (0.1 g, 0.6 mmol) and dissolved in benzene (15 mL). The reaction mixture was attached to a Dean-Stark apparatus and refluxed overnight.

The mixture was then cooled to room temperature, diluted with diethyl ether and the organic layer was washed with saturated aqueous sodium bicarbonate followed by brine. The organic layer was then dried over magnesium sulphate and the solvent was removed under reduced pressure. The product was then purified using flash column chromatography with a hexanes/ethyl acetate gradient elution system to give **3.22** (3.8 g, 92% yield) as a colourless oil. ^{1}H NMR (500MHz, Chloroform-*d*) δ = 5.83 - 5.76 (m, 1 H, CH_2=C**H**), 5.03 - 4.92 (m, 2 H, C**H$_2$**=CH), 3.99 - 3.92 (m, 4 H, ethylene ketal), 3.68 (s, 3 H, NOC**H$_3$**), 3.17 (s, 3 H, NC**H$_3$**), 2.49 (t, *J* = 7.3 Hz, 2 H, ketal-CH_2C**H$_2$**), 2.17 - 2.03 (m, 2 H, CH_2=CHC**H$_2$**CH_2), 1.98 (t, *J* = 7.3 Hz, 2 H, ketal-C**H$_2$**CH_2), 1.72 (t, *J* = 8.3 Hz, 2 H, CH_2=$CHCH_2$C**H$_2$**), $C_{12}H_{21}NO_4Na^{1+}$ low resolution ESI-MS calculated: 266.14, found: 266.32.

3-(2-(4-hydroxybutyl)-1,3-dioxolan-2-yl)-*N*-methoxy-*N*-methylpropanamide **3.23**

Compound **3.22** (3.6 g, 14.8 mmol) was dissolved in 37 mL of THF and cooled to 0 °C. To this solution was added a 1 M solution of borane in THF (8.9 mL) and the reaction mixture was allowed to warm to room temperature. The reaction mixture was stirred for 2 hours and then water (7.4 mL) and sodium perborate tetrahydrate (2.73 g, 17.7 mmol) were added. The reaction was stirred for a further 2 hours and then cold brine solution (0 °C, 11 mL) was added to separate the organic and aqueous phases. The aqueous phase was extracted with ethyl acetate and combined with the THF layer. The combined organic layers were dried over magnesium sulphate and the solvent was removed under

reduced pressure. The crude product was purified by flash column chromatography using a hexanes/ethyl acetate gradient eluent to give compound **3.23** (1.97 g, 51% yield) as a colourless oil. ^{1}H NMR (400MHz, Chloroform-*d*) δ = 3.96 – 3.91 (m, 5 H, ethylene ketal and OH), 3.68 (s, 3 H, NOC**H$_3$**), 3.63 (m, 2 H, C**H$_2$**OH), 3.17 (s, 3 H, NC**H$_3$**), 2.46 (t, *J* = 7.6 Hz, 2 H, ketal-CH$_2$C**H$_2$**CON), 1.97 (t, *J* = 7.6 Hz, 2 H, ketal-C**H$_2$**CH$_2$CON), 1.64 (t, *J* = 8.0 Hz, 2 H, ketal-C**H$_2$**CH$_2$CH$_2$CH$_2$OH), 1.58 – 1.53 (m, 2 H, C**H$_2$**CH$_2$OH), 1.47 – 1.42 (m, 2 H, ketal-CH$_2$C**H$_2$**CH$_2$CH$_2$OH), $C_{12}H_{23}NO_5Na^{1+}$ low resolution ESI-MS calculated: 284.15, found: 284.37.

N-methoxy-*N*-methyl-3-(2-(4-(2,3-dimethyl-1*H*-imidazol-1-yl-tetrafluoroboro) butyl)-1,3-dioxolan-2-yl)propanamide (**3.24a**)

Compound **3.23** (0.21 g, 0.8 mmol) was mixed with 1,2-dimethylimidazolium tetrafluoroborate (0.16 g, 0.9 mmol),1,2-dimethylimidazole (0.08 g, 0.9 mmol) and dissolved in 0.47 mL of acetonitrile. This solution was mixed with another solution containing triphenylphosphine (0.4 g, 1.5 mmol) and DIAD (0.3 g, 1.5 mmol) in 0.3 mL acetonitrile and the resultant mixture was stirred for 2 hours at room temperature. The reaction mixture was then diluted by the addition of 1-2 mL of acetonitrile and precipitated from MTBE. The resultant solid was then filtered over celite and recovered from the filter by dissolution in acetonitrile. The solvent was removed under reduced pressure and the solid was then triturated with a minimal amount of cold acetone. The solid was removed by filtration and the recovered organic layer was dried under reduced pressure to give **3.24a**

(0.16 g, 46% yield) as a colourless oil. ^{1}H NMR (400MHz, DMSO-d_6) δ = 7.49 (d, J = 1.9 Hz, 1 H, CH_3-NCH=C**H**-N-CH_2), 7.42 (d, J = 1.9 Hz, 1 H, CH_3-NC**H**=CH-N-CH_2), 4.08 (t, J = 7.3 Hz, 2 H, N-C$\mathbf{H_2}$), 3.86 - 3.81 (m, 4 H, ethylene ketal), 3.72 (s, 3 H, C$\mathbf{H_3}$-NCH=CH-N-CH_2), 3.69 (s, 3 H, NOC$\mathbf{H_3}$), 3.63 (s, 3 H, NC$\mathbf{H_3}$), 2.56 (s, 3 H, C$\mathbf{H_3}CN_2$), 2.35 (t, J = 7.4 Hz, 2 H, ketal-CH_2C$\mathbf{H_2}$CON), 1.77 (t, J = 8.0 Hz, 2 H, ketal-C$\mathbf{H_2}CH_2$CON), 1.72 - 1.63 (m, 2 H, C$\mathbf{H_2}CH_2$N), 1.56 (t, J = 7.6 Hz, 2 H, ketal-C$\mathbf{H_2}CH_2CH_2CH_2$N), 1.35 - 1.25 (m, 2 H, ketal-CH_2C$\mathbf{H_2}CH_2CH_2$N), $C_{17}H_{30}N_3O_4^+$ low resolution ESI-MS calculated: 340.22, found: 340.30.

N-methoxy-*N*-methyl-3-(2-(4-(2,3-dimethyl-1*H*-imidazol-1-yl-iodo)butyl)-1,3-dioxolan-2-yl)propanamide (**3.24b**)

Compound **3.23** (1.56 g, 6.0 mmol) was mixed with 1,2-dimethylimidazolium iodide (2.0 g, 9.0 mmol) and dissolved in 8 mL of acetonitrile. This solution was mixed with another solution containing triphenylphosphine (3.14 g, 12.0 mmol) and DIAD (2.42 g, 12.0 mmol) in 8 mL acetonitrile and the resultant mixture was stirred for 2 hours at room temperature. The reaction mixture was then diluted by the addition of 5 mL of acetonitrile and precipitated from MTBE. The resultant solid was then filtered over celite and recovered from the filter by dissolution in acetonitrile. The solvent was removed under reduced pressure and the solid was then triturated with a minimal amount of cold acetone. The solid was removed by filtration and the recovered organic layer was dried under reduced pressure to give **3.24b** (1.3 g, 46% yield) as a colourless oil. ^{1}H NMR identical to **3.24a**, $C_{17}H_{30}N_3O_4^{1+}$ low resolution ESI-MS calculated: 340.22, found: 340.23.

8-(2,3-dimethyl-1*H*-imidazol-1-yl-iodo)-4-oxooctanoic acid (**3.25**)

Compound **3.24b** (2.80 g, 6.0 mmol) was mixed with 1 M aqueous sodium hydroxide (10 mL) and stirred overnight. The aqueous solution was washed with diethyl ether and then acidified to pH 1 by addition of 1.8-2 mL of concentrated aqueous HI. The aqueous solution was again rinsed with diethyl ether and then water was removed under reduced pressure. The resulting solid was triturated with a minimum of cold acetone and the solids left behind were removed by filtration. The acetone was removed under reduced pressure and the resultant solid was suspended in dichloromethane. The solid was removed by filtration and the recovered organic phase was dried under reduced pressure to yield **3.25** (2.0 g, 88% yield) as a colourless oil. ^{1}H NMR (400MHz, DMSO-d_6) δ = 7.52 (d, J = 1.8 Hz, 1 H, CH_3-NCH=C**H**-N-CH_2), 7.48 (d, J = 1.8 Hz, 1 H, CH_3-NC**H**=CH-N-CH_2), 4.07 (t, J = 7.0 Hz, 2 H, N-C$\mathbf{H_2}$), 3.76 (s, 3 H. C$\mathbf{H_3}$-NCH=CH-N-CH_2), 3.71 (s, Weinreb amide impurity), 2.85 (s, Weinreb amide impurity), 2.60 (t, J = 6.4 Hz, 2 H, C$\mathbf{H_2}CH_2$COOH), 2.54 (s, 3 H, C$\mathbf{H_3}CN_2$), 2.48 (d, J = 7.6 Hz, 2 H, COC$\mathbf{H_2}CH_2CH_2CH_2$N), 2.37 (t, J = 6.4 Hz, 2 H, CH_2C$\mathbf{H_2}$COOH), 1.70 - 1.60 (m, 2 H, C$\mathbf{H_2}CH_2$N), 1.48 - 1.38 (m, 2 H, CO-CH_2C$\mathbf{H_2}CH_2CH_2$N), ^{13}C NMR (126MHz, Acetone-d_6) δ = 122.5, 121.0, 65.9, 62.0, 59.8, 48.0, 46.4, 41.0, 36.6, 34.6, 20.0, 8.8, 8.2, $C_{13}H_{21}N_2O_3^{1+}$ low resolution ESI-MS calculated: 253.16, found: 253.22.

Methyl 4-oxo-4-(pyridin-2-yl)butanoate (**3.29**)

Picolinaldehyde (5.0 g, 58 mmol) was mixed with methyl acrylate (5.6 g, 53 mmol) and 3-benzyl-5-(2-hydroxyethyl)-4-methylthiazolium chloride (**3.28**, 1.42g,

5.3 mmol) and dissolved in 21 mL of anhydrous ethanol. The mixture was heated to reflux and then triethylamine was added to begin the reaction. The reaction was stirred at reflux overnight and then cooled to room temperature. The solvent was then removed under reduced pressure, the resulting slurry was re-suspended in dichloromethane and the organic layer was washed with water, then dried over magnesium sulphate. The product was purified by flash column chromatography using a hexanes/ethyl acetate gradient elution system to give **3.29** (4.8 g, 47% yield) as a yellow solid. ^{1}H NMR (500MHz, Chloroform-*d*) δ = 8.67 (d, *J* = 4.5 Hz, 1 H, H6), 8.02 (d, *J* = 8.0 Hz, 1 H, H3), 7.81 (m, 1 H, H4), 7.46 (m, 1 H, H5), 3.68 (s, 3 H, COOC**H$_3$**), 3.55 (t, *J* = 6.8 Hz, 2 H, C**H$_2$**CH$_2$COOCH$_3$), 2.75 (t, *J* = 6.4 Hz, 2 H, CH$_2$C**H$_2$**COOCH$_3$), $C_{10}H_{11}NO_3Na^{1+}$ low resolution ESI-MS calculated: 216.06, found: 216.20.

Methyl 8-((*tert*-butyldimethylsilyl)oxy)-4-oxooctanoate (**3.34**)

5-((*t*-Butyldimethylsilyl)oxy)pentanal (7.0 g, 32 mmol) was mixed with methyl acrylate (5.6 g, 65 mmol) and 3-ethyl-5-(2-hydroxyethyl)-4-methylthiazolium bromide (1.65 g, 6.5 mmol) and dissolved in 16 mL of anhydrous ethanol. The reaction mixture was heated to reflux and then triethylamine (5.4 mL) was added to begin the reaction. The reaction mixture was refluxed for 18 hours and was then cooled to room temperature. The ethanol was removed under reduced pressure and the reaction mixture was then suspended in dichloromethane and extracted with brine. The organic layer was dried over magnesium sulphate and the solvent was removed under reduced

pressure. The products were purified by flash column chromatography using a hexanes/ethyl acetate gradient elution system to give **3.34** (3.1 g, 32% yield) as a colourless oil. ^{1}H NMR (400MHz, Chloroform-*d*) δ = 3.68 (s, 3 H, COOC**H$_3$**), 3.66 (t, *J* = 6.1 Hz, 2 H, TBSOC**H$_2$**), 2.69 (t, *J* = 6.7 Hz, 2 H, COC**H$_2$**CH$_2$COOCH$_3$), 2.58 (t, *J* = 6.7 Hz, 2 H, COCH$_2$C**H$_2$**COOCH$_3$), 2.43 (t, *J* = 7.2 Hz, 2 H, TBS-O-CH$_2$CH$_2$CH$_2$C**H$_2$**-CO), 1.74 - 1.64 (m, 2 H, TBS-O-CH$_2$CH$_2$C**H$_2$**CH$_2$-CO), 1.61 - 1.51 (m, 2 H, TBS-O-CH$_2$C**H$_2$**CH$_2$CH$_2$-CO), 1.04 (s, 9 H, *t*-BuSi), 0.15 (s, 6 H, (C**H$_3$**)$_2$Si), $C_{15}H_{30}O_4SiNa^{1+}$ low resolution ESI-MS calculated: 325.18, found: 325.25.

Ethyl 4-oxooct-7-enoate (**3.36**)

4-Pentenal (3.8 g, 45 mmol) was mixed with 3-benzyl-5-(2-hydroxyethyl)-4-methylthiazolium chloride (**3.28**, 2.45 g, 9.1 mmol) and ethyl acrylate (9.0 g, 90 mmol) and dissolved in 22 mL of anhydrous ethanol. The reaction mixture was heated to reflux and then triethylamine (7.6 mL) was added to begin the reaction. The reaction mixture was refluxed for 18 hours and was then cooled to room temperature. The ethanol was removed under reduced pressure and the reaction mixture was then suspended in dichloromethane and extracted with brine. The organic layer was dried over magnesium sulphate and the solvent was removed under reduced pressure. The products were purified by flash column chromatography using a hexanes/ethyl acetate gradient elution system to give **3.36** (3.96 g, mixed with **3.37**, app. 25% yield) as a pale yellow oil. ^{1}H NMR (300MHz, Acetonitrile-d_3) δ = 5.99 - 5.73 (m, CH$_2$=C**H**, with acyloin impurity),

5.15 - 5.05 (m, 2 H, C**H$_2$**=CH), 5.04 - 4.93 (acyloin impurity), 4.15 - 4.06 (q, *J* = 7.0 Hz, 2 H, COOC**H$_2$**), 2.76 - 2.67 (m, 2 H, C**H$_2$**CH$_2$COOEt, with acyloin impurity), 2.67 - 2.60 (acyloin impurity), 2.55 - 2.48 (m, CH$_2$C**H$_2$**COOEt, with acyloin impurity), 2.35 - 2.26 (m, 2 H, CH$_2$=CHCH$_2$C**H$_2$**), 2.20 - 2.17 (acyloin impurity), 2.16 - 2.12 (m, 2 H, CH$_2$=CHC**H$_2$**CH$_2$), 1.99 - 1.94 (acyloin impurity), 1.21 (t, *J* = 7.0 Hz, 3 H, COO-CH$_2$C**H$_3$**), $C_{10}H_{16}O_3Na^{1+}$ low resolution ESI-MS calculated: 207.10, found: 207.31.

Ethyl 4,4-diethoxyoct-7-enoate (**3.38**)

Compound **3.36** (2.0 g, as mixture with **3.37**, app. 5.7 mmol) was dissolved in a 1:1 v/v mixture of ethyl orthoformate and ethanol (6 mL) and mixed with a catalytic amount of *p*TSA (0.1 g). The reaction mixture was then refluxed for 4 hours and it was then cooled to 0 °C. Saturated aqueous sodium bicarbonate (20 mL) was then added along with diethyl ether (20 mL). The aqueous phase was extracted several times with diethyl ether and then the combined organic layers were rinsed with aqueous brine and dried over magnesium sulphate. The solvent was removed under reduced pressure and the product was purified by flash column chromatography using a hexanes/ethyl acetate gradient system to give **3.38** (1.36 g, 92% yield) as a colourless oil. ^{1}H NMR (500MHz, DMSO-d_6) δ = 5.85 - 5.73 (m, 1 H, CH$_2$=C**H**), 5.05 - 4.88 (m, 2 H, C**H$_2$**=CH), 4.03 (q, *J* = 7.2 Hz, 2 H, COOC**H$_2$**), 3.32 (q, *J* = 7.0 Hz, 4 H, Ethyl acetal C**H$_2$**), 2.20 (t, *J* = 7.8 Hz, 2 H, CH$_2$C**H$_2$**COOEt), 1.97 - 1.88 (m, 2 H, CH$_2$=CHC**H$_2$**CH$_2$), 1.79 (t, *J* = 7.8 Hz, 2 H, C**H$_2$**CH$_2$COOEt), 1.53 (t, *J* = 8.4 Hz, 2 H, CH$_2$=CHCH$_2$C**H$_2$**), 1.16 (t, *J* =

7.2 Hz, 3 H, $COOCH_2C\mathbf{H_3}$), 1.06 (t, *J* = 7.0 Hz, 6 H, Ethyl acetal $C\mathbf{H_3}$), ^{13}C NMR (75MHz, Acetonitrile-d_3) δ = 173.0, 138.5, 113.8, 102.0, 60.1, 55.0, 32.3, 28.7, 28.3, 27.8, 14.6, 13.6, $C_{14}H_{26}O_4Na^{1+}$ low resolution ESI-MS calculated: 281.17, found: 281.13.

Ethyl 4,4-diethoxy-8-hydroxyoctanoate (**3.39**)

A 1 M solution of borane in THF (2.4 mL, 2.4 mmol) was cooled to 0 °C and to it was added, dropwise, 0.47 mL of cyclohexene (4.6 mmol). The reaction was stirred for 1 hour at 0 °C and to the resultant white slurry was added compound **3.38** (0.51 g, 2.0 mmol). The reaction was allowed to warm to room temperature and was stirred for 2 hours. After this time, sodium perborate tetrahydrate (1.07 g, 7.0 mmol) and 2.4 mL of water were added. The reaction was stirred for a further 2 hours and then the reaction mixture was extracted with ethyl acetate several times. The combine organic layers were dried of magnesium sulphate and the solvent was removed under reduced pressure. The products were purified using a dichloromethane/methanol gradient elution system to give **3.39** (0.45 g, 81% yield) as a colourless oil. Starting material (**3.38**) was also recovered. ^1H NMR (300MHz, DMSO-d_6) δ = 4.33 (t, *J* = 5.2 Hz, 1 H, O**H**), 4.02 (q, *J* = 7.1 Hz, 2 H, $COOC\mathbf{H_2}$), 3.36 (m, 2 H, $HOC\mathbf{H_2}$), 3.31 (q, *J* = 7.3 Hz, 4 H, Ethyl acetal $C\mathbf{H_2}$), 2.18 (t, *J* = 7.7 Hz, 2 H, $CH_2C\mathbf{H_2}COOEt$), 1.76 (t, *J* = 7.7 Hz, 2 H, $C\mathbf{H_2}CH_2COOEt$), 1.46 - 1.41 (m, 2 H, $HOCH_2CH_2CH_2C\mathbf{H_2}$), 1.40 - 1.31 (m, 2 H, $HOCH_2C\mathbf{H_2}CH_2CH_2$), 1.25 - 1.19 (m, 2 H, $HOCH_2CH_2C\mathbf{H_2}CH_2$), 1.16 (t, *J* = 7.1 Hz, 3 H, $COOCH_2C\mathbf{H_3}$), 1.05 (t, *J* = 7.3 Hz, 6 H, Ethyl acetal $C\mathbf{H_3}$), ^{13}C NMR

(75MHz, DMSO-d_6) δ = 173.1, 102.4, 61.0, 60.3, 55.0, 33.2, 33.0, 29.0, 28.5, 20.2, 15.7, 14.5, $C_{14}H_{28}O_5Na^{1+}$ low resolution ESI-MS calculated: 299.18, found: 299.19.

Ethyl 8-bromo-4,4-diethoxyoctanoate (**3.40**)

Compound **3.39** (0.50 g, 1.8 mmol) was mixed with triphenylphosphine (0.80 g, 3.0 mmol) and imidazole (0.20 g, 2.9 mmol) and dissolved in 18 mL of dichloromethane. The solution was cooled to 0 °C and a solution of carbon tetrabromide (0.85 g, 2.6 mmol) in dichloromethane (1.5 mL) was added slowly. The reaction mixture was allowed to warm to room temperature and was stirred for 1 hour. The reaction was then quenched by the addition of saturated aqueous sodium sulphite and extracted with dichloromethane. The combined organic layers were dried over sodium sulphate and the solvent was removed under reduced pressure. The product was purified by flash column chromatography with a hexanes/ethyl acetate gradient elution system to yield **3.40** (0.49 g, 81% yield) as a colourless oil. ^{1}H NMR (400MHz, DMSO-d_6) δ = 4.04 (q, *J* = 7.1 Hz, 2 H, COOC**H$_2$**), 3.54 (t, *J* = 6.5 Hz, 2 H, BrC**H$_2$**), 3.32 (q, *J* = 6.9 Hz, 4 H, Ethyl acetal C**H$_2$**), 2.22 (t, *J* = 7.8 Hz, 2 H, CH_2C**H$_2$**COOEt), 1.83 - 1.74 (m, 4 H, C**H$_2$**CH_2COOEt and BrCH_2C**H$_2$**CH_2CH_2), 1.47 (t, *J* = 7.2 Hz, 2 H, Br$CH_2CH_2CH_2$C**H$_2$**), 1.35 - 1.25 (m, 2 H, BrCH_2CH_2C**H$_2$**CH_2), 1.18 (t, *J* = 7.1 Hz, 3 H, COOCH_2C**H$_3$**), 1.07 (t, *J* = 6.9 Hz, 6 H, Ethyl acetal C**H$_3$**), ^{13}C NMR (126MHz ,Acetonitrile-d_3) d = 173.0, 102.1, 60.1, 55.0, 34.2, 32.5, 32.1, 28.8, 28.4, 22.0,

14.7, 13.5, $C_{14}H_{27}BrO_4Na^{1+}$ low resolution ESI-MS calculated: 361.10, found: 361.11.

Ethyl 4,4-diethoxy-8-(2,3-dimethyl-1*H*-imidazol-1-yl-bromo)octanoate (**3.41**)

Compound **3.40** (0.31 g, 0.90 mmol) was dissolved in 5 mL of acetonitrile and mixed with 1,2-dimethylimidazole (0.13 g, 1.3 mmol). The reaction was warmed to 50 °C and stirred overnight. The reaction mixture was then cooled to room temperature and the solvent was then removed under reduced pressure. The resultant oil was rinsed several times with diethyl ether and then the compound was again subjected to reduced pressure. This gave **3.41** (0.38 g, 95% yield) as a colourless oil. ^{1}H NMR (500MHz, DMSO-d_6) δ = 7.63 (d, *J* = 2.2 Hz, 1 H, CH=CH), 7.60 (d, *J* = 2.2 Hz, 1 H, CH=CH), 4.09 (t, *J* = 7.2 Hz, 2 H, N**CH$_2$**), 4.04 (q, *J* = 7.2 Hz, 2 H, COOC**H$_2$**), 3.73 (s, 3 H, NC**H$_3$**), 3.31 (q, *J* = 6.9 Hz, 4 H, Ethyl acetal C**H$_2$**), 2.56 (s, 3 H, C**H$_3$**CN$_2$), 2.20 (t, *J* = 8.1 Hz, 2 H, CH$_2$C**H$_2$**COOEt), 1.76 (t, *J* = 8.1 Hz, 2 H, C**H$_2$**CH$_2$COOEt), 1.71 - 1.63 (m, 2 H, NCH$_2$C**H$_2$**CH$_2$CH$_2$), 1.49 (t, *J* = 7.6 Hz, 2 H, NCH$_2$CH$_2$CH$_2$C**H$_2$**), 1.24 - 1.19 (m, 2 H, NCH$_2$CH$_2$C**H$_2$**CH$_2$), 1.16 (t, *J* = 7.2 Hz, 3 H, COOCH$_2$C**H$_3$**), 1.05 (t, *J* = 6.9 Hz, 6 H, Ethyl acetal C**H$_3$**), ^{13}C NMR (126MHz ,DMSO-d_6) d = 173.6, 122.8, 121.3, 102.5, 60.7, 55.6, 48.6, 35.3, 33.2, 29.9, 29.3, 28.8, 20.8, 15.2, 14.1, 9.7$C_{19}H_{35}N_2O_4^{1+}$ low resolution ESI-MS calculated: 355.26, found: 355.28.

8-(2,3-dimethyl-1*H*-imidazol-1-yl-bromo)-4-oxooctanoic acid (**3.42**)

Compound **3.41** (0.44 g, 1.0 mmol) was mixed with 1 M aqueous sodium hydroxide (5 mL) and stirred overnight. The solution was then acidified to pH 1 by addition of concentrated aqueous HCl. The aqueous solution was then rinsed with diethyl ether, followed by the removal of water under reduced pressure. The resulting solid was dissolved in a minimum of cold acetone and dichloromethane any undissolved material was removed by filtration. The solvent was then removed under reduced pressure to give **3.42** (0.34 g, > 100% yield) as an off-white solid, likely mixed with a small amount of sodium chloride. ^{1}H NMR (400MHz, DMSO-d_6) δ = 7.63 (d, *J* = 2.1 Hz, 1 H, CH=CH), 7.61 (d, *J* = 2.1 Hz, 1 H, CH=CH), 4.09 (t, *J* = 7.3 Hz, 2 H, N**CH$_2$**), 3.73 (s, 3 H, NC**H$_3$**), 2.61 (t, *J* = 6.3 Hz, 2 H, C**H$_2$**CH$_2$COOH), 2.56 (s, 3 H, C**H$_3$**CN$_2$), 2.49 (t, *J* = 7.3 Hz, 2 H, NCH$_2$CH$_2$CH$_2$C**H$_2$**), 2.37 (t, *J* = 6.3 Hz, 2 H, CH$_2$C**H$_2$**COOH), 1.65 (m, 2 H, NCH$_2$C**H$_2$**CH$_2$CH$_2$), 1.43 (m, 2 H, NCH$_2$CH$_2$C**H$_2$**CH$_2$), ^{13}C NMR (126MHz ,Acetonitrile-d_3) d = 208.9, 173.4, 122.3, 120.9, 48.0, 41.0, 36.7, 34.8, 28.6, 27.7, 19.9, 9.2, $C_{13}H_{21}N_2O_3^{1+}$ high resolution ESI-MS required: 253.15467, found: 253.15459.

General procedure for the derivatization of **3.42** to DNA and RNA nucleosides

Compound **3.42** (0.4 mmol) was dissolved in 15% v/v DMF/acetonitrile and mixed with DCC (0.6 mmol), DMAP (0.2 mmol), and the desired nucleoside (0.5 mmol). The reaction mixture was stirred overnight and was then precipitated from MTBE. The resultant solid was removed by filtration and recovered from the

filter by dissolving it in acetonitrile. The solvent was removed under reduced pressure and the resulting solid was taken up in dichloromethane and washed with dilute aqueous sodium tetrafluoroborate to achieve ion metathesis. The organic phase was dried over magnesium sulphate and the solvent was removed under reduced pressure. The resultant solid was then dissolved in acetonitrile and the solution was precipitated from MTBE, followed by filtration and recovery in acetonitrile. The solvent was then removed under reduced pressure to yield the desired tagged nucleoside in 80-90% yield, containing the DCC failure product (**3.45**) in quantities up to 10% w/w.

3.44 ^{1}H NMR (500MHz, DMSO-d_6) δ = 11.37 (s, 1 H, H3), 7.59 (d, *J* = 2.1 Hz, 1 H, tag CH=CH), 7.57 (d, *J* = 2.1 Hz, 1 H, tag CH=CH), 7.49 (s, 1 H, H6), 7.39 – 7.36 (m, 2 H, DMT), 7.34 - 7.30 (m, 2 H, DMT), 7.29 (t, *J* = 7.1 Hz, 2 H, DMT), 7.25 - 7.19 (m, 5 H), 6.90 – 6.85 (m, 4 H, DMT), 6.17 (dd, *J* = 5.9 Hz, 1 H, H1'), 5.25 (d, *J* = 6.3 Hz, 1 H, H3'), 4.08 (t, *J* = 7.1 Hz, 2 H, tag NCH_2), 4.01 (dd, *J* = 3.5 Hz, 1 H, H4'), 3.72 (s, 6 H, trityl OCH_3), 3.71 (s, 3 H, tag NCH_3), 3.33 – 3.27 (m, water and H5'), 3.22 - 3.17 (dd, *J* = 7.2, 3.3 Hz, 1 H, H5''), 2.69 (t, *J* = 6.1 Hz, 2 H, tag C**H$_2$**CH_2COO), 2.60 (s, 3 H, tag CH_3CN_2), 2.55 – 2.44 (m, DMSO and C5-CH_3 and H2' and tag CH_2C**H$_2$**COO and tag $NCH_2CH_2CH_2C$**H$_2$**), 2.53 (dd, *J* = 8.1, 5.4 Hz , 1 H, H2''), 1.72 - 1.61 (m, 2 H, tag NCH_2C**H$_2$**CH_2CH_2 and **3.45** impurity), 1.48 - 1.42 (m, 2 H, tag, NCH_2CH_2C**H$_2$**CH_2 and **3.45** impurity), 1.19 – 1.28 (m, **3.45** impurity), $C_{44}H_{51}N_4O_9^{1+}$ high resolution ESI-MS required: 779.36506, found: 779.36526.

3.48 ^{1}H NMR (500MHz, DMSO-d_6) δ = 11.47 (s, 1 H, H3), 8.22 - 8.16 (m, 1 H, DMT), 7.72 - 7.70 (br. s, 1 H, H6), 7.69 - 7.68 (m, 1 H, tag CH=CH), 7.63 - 7.60 (m, 1 H, tag CH=CH), 7.60 - 7.58 (m, 2 H, DMT), 7.58 - 7.56 (m, 2 H, DMT), 7.36 - 7.27 (m, 2 H, DMT), 7.26 - 7.18 (m, 2 H, DMT), 6.91 - 6.84 (m, 4 H, DMT), 5.87 (d, *J* = 6.8 Hz, 1 H, H1'), 5.49 (dd, *J* = 2.0, 5.9 Hz, 1 H, H5), 5.16 (t, *J* = 2.3 Hz, 1 H, H3'), 4.64 (t, *J* = 6.2 Hz, 1 H, H2'), 4.06 (t, *J* = 7.4 Hz, 2 H, NCH_2), 3.73 (s, 6 H, DMT OCH_3), 3.70 (s, 3 H, tag NCH_3), 3.29-3.15 (m, water and H5'&5''), 2.70 (t, *J* = 6.7 Hz, 2 H, tag C**H$_2$**CH_2COO), 2.63 - 2.58 (m, 3 H, tag CH_3CN_2), 2.55 (m, DMSO and tag CH_2C**H$_2$**COO and tag $NCH_2CH_2CH_2C$**H$_2$**), 2.54 - 2.45 (m, 5 H), 1.73 - 1.61 (m, tag NCH_2C**H$_2$**CH_2CH_2 and **3.45** impurity), 1.49 - 1.35 (m, tag, NCH_2CH_2C**H$_2$**CH_2 and **3.45** impurity), 1.28 - 1.13 (m, **3.45** impurity), 0.99 - 0.90 (m, 9 H), $C_{52}H_{69}N_4O_{10}Si^{1+}$ high resolution ESI-MS required: 937.47775, found: 937.47680.

5.4 Chapter 4

^{DMT}dTpT$_{TBDPS}$ (phosphate deprotected) dimer (**4.4**)

3'-*O*-TBDPS protected thymidine nucleoside (0.24 g, 0.5 mmol) was mixed with DCI (0.73 g, 6.1 mmol) and DMT-thymidine phosphoramidite (0.57 g, 0.8 mmol) and dissolved in acetonitrile (4 mL). The reaction was stirred for 2 hours and then oxidation was performed by adding collidine (0.2 mL) and then adding a solution of iodine (0.1 M in 2:1 v/v THF/water) dropwise until the iodine colour

remained. The reaction mixture was then diluted with dichloromethane and washed with 5% w/v aqueous sodium bisulphite to quench the excess iodine followed by water and brine. The organic layer was dried over sodium sulphate and the solvent was removed under reduced pressure to give crude **4.3**. The crude product was then dissolved in 1:9 v/v triethylamine/acetonitrile and stirred for 2 hours. The solvent was then removed under reduced pressure and the product was purified by flash column chromatography using a dichloromethane/methanol gradient elution system to give **4.4** (0.33 g, 62% yield for 2 steps) as a white foam. ^{1}H NMR (500MHz, Acetonitrile-*d*$_3$) δ = 7.75 - 7.72 (m, Ar), 7.67 - 7.62 (m, 2 H, H6), 7.47 - 7.35 (m, Ar), 7.29 (m, DMT), 6.90 - 6.82 (m, DMT), 6.39 (t, *J* = 5.7 Hz, 1 H, H1'A), 6.22 (dd, *J* = 2.6, 5.3 Hz, 1 H, H1'B), 4.67(m, 1 H, H3'B), 4.38 (d, *J* = 4.6 Hz, 1 H, H3'A), 3.81 - 3.78 (m, 2 H, H4'A&B), 3.77 (s, 6 H, DMT OCH_3), 3.75 - 3.42 (m, H5'&5"A&B), 2.91 – 2.54 (m, C5-CH_3), 2.28 - 1.94 (m, H2'&2"A&B), 1.07 (s, 9 H, *t*-BuSi), A=3'-nucleotide, B=5'-nucleotide, $C_{57}H_{62}N_4O_{14}PSi^{1-}$ low resolution ESI-MS calculated: 1085.38, found: 1085.30, ^{31}P NMR (Acetonitrile-*d3*) -1.058.

General procedure for the preparation of trinucleotide trimers **4.5a-c**

Compound **4.4** (0.1 mmol) was mixed with 2,4,6-triisopropylbenzenesulphonyl chloride (0.4 mmol) and the appropriate nucleoside (0.5 mmol). The mixed solids were dissolved in acetonitrile (5 mL) along with 1-methylimidazole (0.06 mL) as a catalyst. The reaction mixture was allowed to stir overnight and then the solvent was removed under reduced pressure. The

products were isolated by flash column chromatography using a dichloromethane/methanol gradient elution system to give the trinucleotide phosphotriesters **4.5a-c**, in 80-85% yields.

4.5a $C_{88}H_{93}N_6O_{20}PSiNa^{1+}$ low resolution ESI-MS calculated: 1635.58, found: 1635.36, ^{31}P NMR (Acetonitrile-d_3) -1.335.

4.5b $C_{101}H_{99}N_{10}O_{21}PSiNa^{1+}$ low resolution ESI-MS calculated: 1869.64, found: 1869.26, ^{31}P NMR (Acetonitrile-d_3) -1.577, -1.788.

4.5c $C_{95}H_{96}FN_9O_{19}PSiNa^{1+}$ low resolution ESI-MS calculated: 1744.63, found: 1744.16, ^{31}P NMR (Acetonitrile-d_3) -3.44, -3.47.

^{HO}rG$_{diAIL}$ (**4.8**)

The ribonucleoside *N*-*i*Bu -5'-*O*-DMT-guanosine (2.0 g, 3.1 mmol) was mixed with TBTU (4.88 g, 15.2 mmol) and dissolved in acetonitrile (15 mL). To this mixture was added **3.06** (2.4 g, 9.2 mmol) as a solution with triethylamine (2.2 mL) in acetonitrile (5 mL). The reaction was allowed to stir for 3 days then approximately half of the solvent was removed under reduced pressure. The solution was then precipitated for MTBE, filtered and recovered from the filter in acetone. The solvent was removed under reduced pressure and the resulting solid was taken up in dichloromethane. The organic layer was washed with water and then dried over magnesium sulphate. The solvent was removed under reduced pressure and the solid was again dissolved in acetonitrile and precipitated from MTBE to give the tagged nucleoside **4.7**, DMT-rG-diAIL (3.02 g,

86% yield), as a pale-brown foam. The tagged nucleoside was then dissolved in 30 mL of acetonitrile and TFA (2.0 mL) was added. The reaction was stirred for 10 minutes and then several millilitres of methanol were added to quench the trityl cation. The reaction mixture was then precipitated from MTBE, filtered and the solid was recovered from the filter in acetone. The entire detritylation procedure was repeated two more times and then a final precipitation was performed without any acid addition to give **4.8** (2.10 g, 95% yield) as an off-white foam. **4.7** $C_{51}H_{59}BF_4N_9O_{10}^{1+}$ low resolution ESI-MS calculated: 1044.44, found: 1044.38. **4.8** ^{1}H NMR (400MHz, DMSO-*d*$_6$) δ = 12.10 (s, 1 H, H1), 11.65 (s, 1 H, NH*i*Bu), 9.09 (m, 2 H, tag CHN_2), 8.29 (s, 1 H, H8), 7.81 - 7.60 (m, 4 H, tag imidazolium), 6.03 (d, *J* = 7.4 Hz, 1 H, H1'), 5.81 - 5.75 (m, 1 H, H2'), 5.47 - 5.43 (m, 1 H, H3'), 4.39 - 4.33 (m, 1 H, H4'), 4.23 - 4.15 (m, 2 H, H5'&5''), 4.12 – 4.08 (m, 4 H, tag NC**H$_2$**CH_2CH_2-COO), 3.70 - 3.64 (m, 1 H, 5'-OH), 2.81 - 2.69 (m, 1 H, N*i*Bu-CH), 2.51 - 2.42 (m, 4 H, NCH_2CH_2C**H$_2$**-COO), 2.11 - 2.00 (m, 4 H, NCH_2C**H$_2$**CH_2-COO), 1.17 - 1.05 (m, 6 H, N*i*Bu-CH_3), $C_{30}H_{41}BF_4N_9O_8^{1+}$ low resolution ESI-MS calculated: 742.31, found: 742.27.

^{DMT}rApG$_{diAIL}$ cyanoethyl protected phosphate (**4.9**)

Compound **4.8** (0.75 g, 0.90 mmol) was mixed with *N*-Bz-5'-*O*-DMT-3'-*O*-TBS adenosine 2-*O*-phosphoramidite (1.14 g, 1.2 mmol) and DCI (0.22 g, 1.9 mmol). The mixed solids were dissolved in acetonitrile (15 mL) and stirred for 45 minutes. The excess phosphoramidite was then quenched by the addition of *t*-butanol (0.1 mL) and after 15 more minutes of stirring, the reaction mixture was

precipitated twice from MTBE, as previously described. The material was then re-dissolved in acetonitrile (15 mL) and Cap A and Cap B solutions (1 mL each) were added. The reaction was stirred for 10 min and then *t*-butyl hydroperoxide (0.79 mL) was added. After a further 10 minutes of stirring, the reaction mixture was again precipitated twice from MTBE to give **4.9** (1.37 g, 75% yield when impurity is considered, by NMR) as an off-white foam, with 5'-*O*-Ac-*N*-*i*Bu-2',3'-diAIL guanosine as a contaminant. $C_{77}H_{92}BF_4N_{15}O_{17}PSi^{1+}$ low resolution ESI-MS calculated: 1644.63, found: 1644.46, ^{31}P NMR (Acetonitrile-d_3) -0.407, -0.850.

^{DMT}rApG$_{diAIL}$ deprotected phosphate, 3'-*O*-TBS Ade (**4.10a**)

Compound **4.9** (0.68g, 0.3 mmol when corrected for impurity) was dissolved in 1:9 v/v triethylamine/acetonitrile and the reaction was stirred for 1 hour. The solvent was then removed under reduced pressure to give **4.10a** (0.67 g, 98% yield when corrected for impurity) as an off-white foam. $C_{74}H_{88}N_{14}O_{17}PSi^{1+}$ low resolution ESI-MS calculated: 1503.60, found: 1503.53, ^{31}P NMR (Acetonitrile-d_3) -1.13.

^{DMT}rApG$_{diAIL}$ deprotected phosphate, 3'-OH Ade (**4.10b**)

Compound **4.10a** (0.67g , 0.33 mmol adjusted for impurity) was dissolved in THF (3 mL) and mixed with TREAT HF (0.21 g, 1.3 mmol). The reaction was stirred for 2 hours and then the solvent was removed under reduced pressure. The oil obtained was taken up in acetone and precipitated twice from MTBE to

give **4.10b** (0.61 g, 99% yield after adjusting for impurity) as an off-white foam. $C_{68}H_{74}N_{14}O_{17}P^{1+}$ low resolution ESI-MS calculated: 1389.51, found: 1389.55, ^{31}P NMR (Acetonitrile-d_3) -2.042.

^{HO}rApG$_{diAlL}$ cyanoethyl protected phosphate, 3'-*O*-TBS Ade (**4.11**)

Compound **4.9** (0.68 g, 0.33 mmol after adjusting for impurity) was dissolved in acetonitrile (5 mL) and then TFA (0.15 mL) was added. The reaction was stirred for 10 minutes and then a small amount of methanol was added to quench the trityl cation, as appreciated by the disappearance of colour. The mixture was then precipitated from MTBE and the process was repeated 3 more times. A final precipitation was then performed, without the addition of acid, to give **4.11** (0.54 g, 95% yield after adjusting for the presence of impurity) as a pale yellow foam. $C_{56}H_{74}BF_4N_{15}O_{15}PSi^{1+}$ low resolution ESI-MS calculated: 1342.50, found: 1342.42, ^{31}P NMR (Acetonitrile-d_3) -2.18, -2.25.

^{Ac}rApG$_{diAlL}$ cyanoethyl protected phosphate, 3'-*O*-TBS Ade (**4.12**)

Compound **4.11** (0.54 g, 0.31 mmol after adjusting for impurity) was dissolved in acetonitrile (5 mL) and then Cap A and Cap B solutions (0.6 mL each) were added. The reaction was stirred for 30 minutes and then precipitated from MTBE to give **4.12** (0.54 g, 100% yield after adjusting for impurity) as a pale yellow foam. $C_{58}H_{76}BF_4N_{15}O_{16}PSi^{1+}$ low resolution ESI-MS calculated: 1384.51, found: 1384.31, ^{31}P NMR (Acetonitrile-d_3) -2.14, -2.36.

^{Ac}rApG$_{diAlL}$ deprotected phosphate, 3'-*O*-TBS Ade (**4.13a**)

Compound **4.12** (0.54 g, 0.31 mmol when corrected for impurity) was dissolved in 1:9 v/v triethylamine/acetonitrile and the reaction was stirred for 1 hour. The solvent was then removed under reduced pressure to give **4.13a** (0.52 g, 98% yield when corrected for impurity) as a pale yellow foam. $C_{55}H_{72}N_{14}O_{16}PSi^{1+}$ low resolution ESI-MS calculated: 1243.48, found: 1243.32, ^{31}P NMR (Acetonitrile-d_3) -1.35.

^{Ac}rApG$_{diAlL}$ deprotected phosphate, 3'-OH Ade (**4.13b**)

Compound **4.13a** (0.52 g, 0.30 mmol adjusted for impurity) was dissolved in THF (3 mL) and mixed with TREAT HF (0.19 g, 1.2 mmol). The reaction was stirred for 2 hours and then the solvent was removed under reduced pressure. The oil obtained was taken up in acetone and precipitated twice from MTBE to give **4.13b** (0.47 g, 97% yield after adjusting for impurity) as a pale yellow foam. $C_{49}H_{58}N_{14}O_{16}P^{1+}$ low resolution ESI-MS calculated: 1129.39, found: 1129.28, ^{31}P NMR (Acetonitrile-d_3) -0.99.

^{Ac}rCpApG$_{diAlL}$ deprotected ApG phosphate, 2'/3' branched- Ade (**4.14**)

Compound **4.13b** (0.47 g, 0.29 mmol after adjusting for impurity) was mixed with *N*,2',3'-tribenzoyl cytidine 5'-*O*-phosphoramidite (0.88 g, 1.2 mmol) and DCI (0.44 g, 3.7 mmol). The mixture was dissolved in 15% v/v DMF/acetonitrile (3.5 mL) and stirred overnight. The excess phosphoramidite

was then quenched by the addition of *t*-butanol (0.1 mL) and after 15 minutes of stirring, the reaction mixture was precipitated twice from MTBE, as previously described. The material was then re-dissolved in 15% v/v DMF/acetonitrile (3.5 mL) and Cap A and Cap B solutions (0.3 mL each) were added. The reaction was stirred for 10 min and then *t*-butyl hydroperoxide (0.3 mL) was added. After a further 10 minutes of stirring, the reaction mixture was again precipitated twice from MTBE to give **4.14** (0.64 g, 96% yield after adjusting for impurity) as a pale yellow foam. $C_{82}H_{85}N_{18}O_{26}P_2^{1+}$ low resolution ESI-MS calculated: 1799.54, found: 1799.07, ^{31}P NMR (Acetonitrile-d_3) -2.052, -2.080, -2.370, -2.799.

^{Ac}rCpApA$_{2'F}$-G$_{diAlL}$ trinucleotide phosphotriester AAG, 2'/3' branched- Ade (**4.15c**)

Compound **4.14** (0.14 g, 0.07 mmol) was mixed with 2,4,6-triisopropylbenzenesulphonyl chloride (0.11 g, 0.3 mmol) and the *N*-Bz-5'-DMT 2'-deoxy-2'-ribofluoroadenosine (0.5 mmol). The mixed solids were dissolved in acetonitrile (4 mL) along with 1-methylimidazole (0.05 mL) as a catalyst. The reaction mixture was allowed to stir overnight and then it was precipitated twice from MTBE to give **4.15c** as an off-white foam. $C_{120}H_{118}FN_{23}O_{31}P_2^{2+}$ high resolution ESI-MS required: 1228.89062 found 1228.88843, ^{31}P NMR (Acetonitrile-d_3) -2.03, -2.08, -2.38, -2.49, -2.64, -2.72, -2.83, -2.95.

Chapter 6 Contributions to Knowledge

6.1 Summary of contributions to knowledge

6.1.1 ILSS approach to oligonucleotide synthesis

A new solution-phase method for oligonucleotide synthesis based on a soluble ionic tag as a support has been developed that attempts to address the eventual need for a scalable procedure of DNA and RNA oligomer generation, once therapeutic agents are approved for use. The initial methodology demonstrated that the technique was suitable for all of the standard 2'-deoxyphosphoramidite building blocks and that couplings could afford high quality products in excellent yields very rapidly. It was determined that, for the synthesis of oligodeoxyribonucleotides, alkyl amide protecting groups for the nucleobases were superior to aryl protecting groups, in that they enhanced the solubility of the by-products in the anti-solvent, thus facilitating the purification of the growing oligomer. In addition, it was demonstrated that the presence of the charge allowed for facile monitoring of the growing oligomer to be performed by mass spectrometry, even when the molecular weight of the oligomer exceeded the upper limit of detection for the instrument since multiply charged species could be observed.

The effects of reactions involving the generation of positive charge, such as the cleavage of the trityl ether by addition of acid, were noted to be more difficult

for the tagged system, owing to the presence of the positive charge in the tag. Alternative systems such as lewis acids or stronger Brønsted acids were found to be effective but were difficult to remove. The ideal cleavage conditions were proposed to involve either a solid-supported acid source or an acid that was extremely soluble in the anti-solvent. TFA, though often requiring multiple treatments to afford complete deprotection, was preferred since it was volatile and could be removed under reduced pressure if need be.

It was found that it was necessary to quench the excess phosphoramidite added during the coupling reaction in order to facilitate purification of the growing oligomer and that the nature of the quenching alcohol was critical, since the newly formed phosphite triester linkage was susceptible to transesterification by the quenching agent. If quenching was not performed prior to oxidation, the excess amidite could be converted to an H-phosphonate or a phosphomonoester, which was insoluble in the anti-solvent, thus difficult to remove from the desired material. The tertiary alcohol, *t*-butanol was found to be a suitable quenching agent, reacting with the excess amidite, allowing its removal, and did not cause transesterification of the desired phosphite triester, which was stable enough to be isolated with no cleavage observed.

The oxidation was also optimized for this solution-phase approach from that commonly used in the conventional solid-phase synthesis. The change to organic peroxide from an aqueous iodine based system allowed the removal of a post-oxidation extraction, such that purification at all steps could be performed through precipitation.

The method has been shown to be effective for oligoribonucleotides as well and the TBDMS group commonly used was found to enhance the purification of the growing oligomer by increasing the solubilites of the excess amidites relative to their 2'-deoxy analogues. The approach was demonstrated to be much faster than that of the traditional solution-phase approach, which relies on silica-gel based chromatography to purify the products, allowing for the synthesis of a target pentamer in a fraction of the time.

Exchange of the counter-ion to the tag moiety was observed but this was determined to be of little consequence as the solubility profiles of the various ion-pairs were similar to that of the initial system.

The ILSS approach has been demonstrated to be functional up to the decamer stage by linear synthesis when the concentration of the oligonucleotide was kept above 0.06M and the phosphoramidite was kept above 0.12M. This was found to be difficult in neat acetonitrile so DMF was determined to be a suitable co-solvent at 15% v/v concentration.

Although the ultimate achievable length by stepwise synthesis is yet unknown the risk of failure prior to achieving the therapeutically relevant henicosamer (21-mer) grows with each step making this approach less attractive and an alternative approach, that of block condensation was proposed for study. Approaches to synthesizing these polymeric building blocks were explored in the subsequent chapter.

6.1.2 Approaches to synthesizing oligomeric block phosphoramidites

A novel set of ionic tags was proposed that would allow an ILSS base synthesis of oligomeric block phosphoramidites. Initially, this was attempted with a simple tag, AIL, with the intention of selectively cleaving the ester attaching it to the oligomer. A single methodology was discovered to achieve this aim but it generated the desired material in poor yield. It was found that alkyl esters were too similar to the phosphate esters employed to be cleaved selectively with high enough yields of the desired oligomeric species.

A new generation of ionic tags were proposed that would be orthogonally cleavable from the other protecting groups used in oligonucleotide synthesis, exploiting the *γ*-keto ester moiety's reactivity with hydrazine to achieve release. To this end, an approach was explored to synthesize a molecule of this form directly from the commonly used levulinyl protecting group. Though an ionic tag was synthesised by this approach, it was found that it could not derivatized to a nucleoside due to its ability to internally cyclize. This molecule did however demonstrate that ionic tags of this type would undergo ester cleavage when treated with hydrazine hydrate.

An alternate synthetic route to a product of the desired form was explored, using Grignard reagents to perform necessary carbon-carbon bond formation. It was found that over-addition was a serious drawback of this approach until Weinreb amides were used as the electrophiles. A molecule was synthesised using this route but it was found it could not be separated from an interfering impurity after the final deprotection.

Another path to the desired type of molecule employed the Stetter reaction to effectuate carbon-carbon bond formation. An aryl precursor to an ionic tag was synthesised but it was found that aryl based *γ*-keto esters were slow to cleave upon treatment with hydrazine and were resistant to formation of the desired ionic moiety. An alternative alkyl based *γ*-keto ester of the desired form was successfully synthesised using the Stetter reaction. It was found that the ionic tag was unstable as the tetrafluoroborate salt but could be stored long term as the bromide salt. The bromide salt was successfully derivatized to both DNA and RNA nucleosides, with anion metathesis being performed *in situ* after derivatization had occurred, and then it was demonstrated that the ionic tag could be easily cleaved using hydrazine hydrate.

The rate of cleavage of the ionic tag was compared to that of a levulinated nucleoside and it was found that the rate determining steps for the two species were found to be different. The source of this difference appears to stem from the presence of the positive charge in the tag, which slows the formation of the intermediate hydrazone. This is similar to the effect the tag has on the ease of 5'-detritylation discovered earlier.

The cleavage was demonstrated to be effective for both DNA and RNA tagged nucleosides. When the 2'-O-TIPS protecting group is used for RNA it was found that little or no isomerization occurred with the 3'-position upon tag cleavage. This indicates that this would be a successful approach to synthesising oligomeric block phosphoramidites. Alternate routes were finally proposed to improve the synthesis of the ionic tag.

6.1.3 Synthesis of a novel oligonucleotide structure based on RNA-X

A series of studies were proposed to examine RNA-X, a molecule discovered by Manley and co-workers having an unusually stable motif of a free 2'-hydroxyl vicinal to a phosphotriester linkage. The first study constructed the core of the molecule *in silico* and used it to perform a conformational search in an effort to elucidate the source of the unusual stability observed in the parent molecule. The lowest energy conformer found suggested that the unusual stability might be due to conformational rigidity within the molecule, arising from favourable steric and electronic factors that hold the vicinal 2'-hydroxyl away from the phosphotriester moiety, making the first step in the decomposition of the molecule, namely attack of the hydroxyl on the phosphotriester linkage, difficult.

A synthetic route to two other analogues of the core of RNA-X, was developed. First, model trinucleotide phosphotriesters were used to determine the feasibility of the proposed synthetic route to form the desired phosphotriester with all the necessary nucleosides and then they were used to show that the phosphotriester was stable to the transformations, such as detritylation and desilylation, required for the synthesis of the full analogues. The full synthetic route, which employed both phosphoramidite and phosphotriester strategies was then attempted. It was found that formation of the branched trinucleotide was hindered by the steric bulk of the 5'-trityl protecting group. When this trityl was exchanged for the much smaller acetyl protecting group, the formation of the branched trinucleotide was possible. Finally, one of the fully protected analogues was successfully generated using the developed procedure. The final studies

were then outlined to examine the rate of cleavage and the overall conformation of the core of RNA-X. It is worthwhile noting that the synthetic route used was greatly simplified by the use of the ILSS approach.

6.2 Papers, patents, and conference proceedings

6.2.1 Published papers

Donga, R. A.; Chan, T. H.; Damha, M. J. (2007) Ion-tagged synthesis of an oligoribonucleotide pentamer - The continuing versatility of TBDMS chemistry. *Canadian Journal of Chemistry*, 85(4), 274-82.

Donga, R. A.; Hassler, M.; Chan, T. H.; Damha, M. J. (2007) Oligonucleotide Synthesis using Ionic Liquids as Soluble Supports. *Nucleosides, Nucleotides, and Nucleic Acids*, 26, 1287–93.

Donga, R. A.; Khaliq-Uz-Zaman, S. M.; Chan, T. H.; Damha, M. J. (2006) A Novel Approach to Oligonucleotide Synthesis Using an Imidazolium Ion Tag as a Soluble Support. *Journal of Organic Chemistry*, 71(20): 7907-10.

6.2.2 Manuscripts in preparation

Donga, R. A.; Damha, M. J. “Synthesis of a novel *γ*-keto ester based ionic tag for DNA and RNA synthesis”

Donga, R. A.; Hassler, M.; Reddy, N. M. K.; Chan, T. H.; Damha, M. J. “Preparation of oligomeric block phosphoramidites of DNA and RNA using an ILSS strategy’

Donga, R. A.; Damha, M. J. “Synthesis and stability properties of RNA-X core analogues”

6.2.3 Patents

Chan, T. H.; Damha, M. J.; Miao, W.; **Donga, R. A.**; He, X. (2006), PCT Int. Appl., WO 2006096963, ‘Ionic liquid-supported peptide, oligosaccharide, and oligonucleotide synthesis.’

6.2.4 Conference proceedings

Hassler, M.; Reddy, N.M.K.; **Donga R. A.**, Chan, T. H.; Damha M. J. (2010), New Developments in the Synthesis of Oligoribonucleotides: “Ionic Tag” Soluble Supports and RNA Dimer Bloc Phosphoramidites, 19th International Roundtable on Nucleosides, Nucleotides and Nucleic Acids, Lyon, France - August 29 – September 3.

Damha, M. J.; **Donga, R. A**.; Lackey, J.; Hassler, M.; Gallant, P.; Khaliq-Uz-Zaman, S. M.; Chan, T. H. (2006), A Novel Approach to Oligonucleotide Synthesis Using Ionic Liquids, 17th International Roundtable on Nucleosides, Nucleotides and Nucleic Acids, Bern, Switzerland, September 3-7.

Donga R. A., Damha M. J.; Investigating the Structure and Stability of Branched Ribonucleic Acid X (RNA X), 39th IUPAC Congress and 86th Conference of The Canadian Society for Chemistry, August 10-15 2003, Ottawa, Ontario.

Donga R. A., Damha M. J.; Determining the Structure and Stability of Branched Ribonucleic Acid X (RNA X), 6th Annual Chemistry and Biochemistry Graduate Research Conference of Concordia University, November 14-16 2003, Montreal,

Quebec.

Donga R. A., Damha M. J.; Probing the Structure and Stability of Nucleoside Phosphotriesters: Toward an Understanding of RNA-X, 87th Conference of The Canadian Society for Chemistry, May 29-June 1 2004, London, Ontario.

References

1. Hershey, A. D.; Chase, M. *Journal of General Physiology* **1952,** *36*, 39-56.
2. Avery, O. T.; MacLeod, C. M.; McCarty, M. *Journal of Experimental Medicine* **1944,** *79*, 137-58.
3. Watson, J. D.; Crick, F. H. C. *Nature* **1953,** *171*, 737-8.
4. Voet, D.; Voet, J. G. *Biochemistry: Second Edition*; John Wiley & Sons: New York, 1995.
5. Reese, C. B. *Organic & Biomolecular Chemistry* **2005,** *3*, 3851-68.
6. Brown, D. M.; Todd, A. R. *Journal of the Chemical Society* **1952**, 52-8.
7. Carter, C. E. *Journal of the American Chemical Society* **1951,** *73*, 1537-9.
8. Brown, D. M.; Magrath, D. I.; Todd, A. R. *Journal of the Chemical Society* **1954**, 1442-7.
9. Brown, D. M.; Todd, A. R. *Journal of the Chemical Society* **1953**, 2040-9.
10. Brown, D. M.; Magrath, D. I.; Todd, A. R. *Journal of the Chemical Society* **1955**, 4396-401.
11. Michelson, A. M.; Todd, A. R. *Journal of the Chemical Society* **1955**, 2632-8.
12. Khorana, H. G.; Tener, G. M.; Moffatt, J. G.; Pol, E. H. *Chemistry & Industry (London)* **1956**, 1523.
13. Khorana, H. G.; Razzell, W. E.; Gilham, P. T.; Tener, G. M.; Pol, E. H. *Journal of the American Chemical Society* **1957,** *79*, 1002-3.
14. Khorana, H. G. *Pure and Applied Chemistry* **1968,** *17*, 349-81.
15. Khorana, H. G.; Agarwal, K. L.; Buechi, H.; Caruthers, M. H.; Gupta, N. K.; Kleppe, K.; Kumar, A.; Ohtsuka, E.; RajBhandary, U. L.; van de Sande, J. H.; Sgaramella, V.; Terao, T.; Weber, H.; Yamada, T. *Journal of Molecular Biology* **1972,** *72*, 209-17.
16. Khorana, H. G.; Agarwal, K. L.; Besmer, P.; Buechi, H.; Caruthers, M. H.; Cashion, P. J.; Fridkin, M.; Jay, E.; Kleppe, K.; Kleppe, R.; Kumar, A.; Loewen, P. C.; Miller, R. C.; Minamoto, K.; Panet, A.; RajBhandary, U. L.;

Ramamoorthy, B.; Sekiya, T.; Takeya, T.; van de Sande, J. H. *Journal of Biological Chemistry* **1976,** *251*, 565-70.

17. Agarwal, K. L.; Yamazaki, A.; Cashion, P. J.; Khorana, H. G. *Angewandte Chemie, International Edition in English* **1972,** *11*, 451-9.
18. Schaller, H.; Weimann, G.; Lerch, B.; Khorana, H. G. *Journal of the American Chemical Society* **1963,** *85*, 3821-7.
19. Brown, D. M. *Methods in molecular biology (Clifton, N.J.)* **1993,** *20*, 1-17.
20. Jacob, T. M.; Khorana, H. G. *Journal of the American Chemical Society* **1964,** *86*, 1630-5.
21. Lohrmann, R.; Khorana, H. G. *Journal of the American Chemical Society* **1966,** *88*, 829-33.
22. Reese, C. B.; Saffhill, R. *Chemical Communications (London)* **1968**, 767-8.
23. Reese, C. B. *Colloques Internationaux du Centre National de la Recherche Scientifique* **1970,** *No. 182*, 319-28.
24. Letsinger, R. L.; Mahadevan, V. *Journal of the American Chemical Society* **1965,** *87*, 3526-7.
25. Letsinger, R. L.; Ogilvie, K. K. *Journal of the American Chemical Society* **1967,** *89*, 4801-3.
26. Eckstein, F.; Rizk, I. *Angewandte Chemie, International Edition in English* **1967,** *6*, 695-6.
27. Eckstein, F.; Rizk, I. *Angewandte Chemie, International Edition in English* **1967,** *6*, 949.
28. Daub, G. W.; Van Tamelen, E. E. *Journal of the American Chemical Society* **1977,** *99*, 3526-8.
29. Adamiak, R. W.; Arentzen, R.; Reese, C. B. *Tetrahedron Letters* **1977**, 1431-4.
30. Ogilvie, K. K.; Beaucage, S. L.; Entwistle, D. W. *Tetrahedron Letters* **1976**, 1255-6.
31. Itakura, K.; Katagiri, N.; Bahl, C. P.; Wightman, R. H.; Narang, S. A. *Journal of the American Chemical Society* **1975,** *97*, 7327-32.

32. Reese, C. B.; Titmas, R. C.; Yau, L. *Tetrahedron Letters* **1978**, 2727-30.
33. Green, A. L.; Saville, B. *Journal of the Chemical Society* **1956**, 3887-92.
34. Reese, C. B. *Tetrahedron* **2002,** *58*, 8893-920.
35. Reese, C. B.; Zard, L. *Nucleic Acids Research* **1981,** *9*, 4611-26.
36. Eckstein, F.; Rizk, I. *Chemische Berichte* **1969,** *102*, 2362-77.
37. Catlin, J. C.; Cramer, F. *Journal of Organic Chemistry* **1973,** *38*, 245-50.
38. Katagiri, N.; Itakura, K.; Narang, S. A. *Journal of the American Chemical Society* **1975,** *97*, 7332-7.
39. Berlin, Y. A.; Chakhmakhcheva, O. G.; Efimov, V. A.; Kolosov, M. N.; Korobko, V. G. *Tetrahedron Letters* **1973**, 1353-4.
40. Katagiri, N.; Itakura, K.; Narang, S. A. *Journal of the Chemical Society, Chemical Communications* **1974**, 325-6.
41. Stawinski, J.; Hozumi, T.; Narang, S. A. *Canadian Journal of Chemistry* **1976,** *54*, 670-2.
42. Letsinger, R. L.; Lunsford, W. B. *Journal of the American Chemical Society* **1976,** *98*, 3655-61.
43. Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Letters* **1981,** *22*, 1859-62.
44. Adams, S. P.; Kavka, K. S.; Wykes, E. J.; Holder, S. B.; Galluppi, G. R. *Journal of the American Chemical Society* **1983,** *105*, 661-3.
45. McBride, L. J.; Caruthers, M. H. *Tetrahedron Letters* **1983,** *24*, 245-8.
46. Hall, R. H.; Todd, A.; Webb, R. F. *Journal of the Chemical Society* **1957**, 3291-6.
47. Dreef, C. E.; Dreef-Tromp, C. M.; Van der Marel, G. A.; Van Boom, J. H. *Synlett* **1990**, 481-3.
48. Merrifield, R. B. *Journal of the American Chemical Society* **1963,** *85*, 2149-54.
49. Letsinger, R. L.; Kornet, M. J. *Journal of the American Chemical Society* **1963,** *85*, 3045-6.
50. Letsinger, R. L.; Mahadevan, V. *Journal of the American Chemical Society* **1966,** *88*, 5319-24.

51. Van der Marel, G.; Van Boeckel, C. A. A.; Wille, G.; Van Boom, J. H. *Tetrahedron Letters* **1981,** *22*, 3887-90.
52. Caruthers, M. H.; Barone, A. D.; Beaucage, S. L.; Dodds, D. R.; Fisher, E. F.; McBride, L. J.; Matteucci, M.; Stabinsky, Z.; Tang, J. Y. *Methods in Enzymology* **1987,** *154*, 287-313.
53. Alvarado-Urbina, G.; Sathe, G. M.; Liu, W. C.; Gillen, M. F.; Duck, P. D.; Bender, R.; Ogilvie, K. K. *Science* **1981,** *214*, 270-4.
54. Gough, G. R.; Brunden, M. J.; Gilham, P. T. *Tetrahedron Letters* **1981,** *22*, 4177-80.
55. McCollum, C.; Andrus, A. *Tetrahedron Letters* **1991,** *32*, 4069-72.
56. Scaringe, S. A.; Wincott, F. E.; Caruthers, M. H. *Journal of the American Chemical Society* **1998,** *120*, 11820-1.
57. Pon, R. T.; Damha, M. J.; Ogilvie, K. K. *Nucleic Acids Research* **1985,** *13*, 6447-65.
58. Pon, R. T.; Usman, N.; Damha, M. J.; Ogilvie, K. K. *Nucleic Acids Research* **1986,** *14*, 6453-70.
59. Smith, M.; Rammler, D. H.; Goldberg, I. H.; Khorana, H. G. *Journal of the American Chemical Society* **1962,** *84*, 430-40.
60. Smrt, J. *Collection of Czechoslovak Chemical Communications* **1973,** *38*, 3642-7.
61. Griffin, B. E.; Reese, C. B. *Tetrahedron Letters* **1964**, 2925-31.
62. Reese, C. B. In *Current protocols in Nucleic Acid Chemistry;* Beaucage, S. L.; Bergstrom, D. E.; Glick, G. D.; Jones, R. A. Eds.; Wiley: New York, 2000; pp. 2.2.1-2.2.24.
63. Ohtsuka, E.; Tanaka, S.; Ikehara, M. *Journal of the American Chemical Society* **1978,** *100*, 8210-13.
64. Ogilvie, K. K.; Sadana, K. L.; Thompson, E. A.; Quilliam, M. A.; Westmore, J. B. *Tetrahedron Letters* **1974**, 2861-3.
65. Zamecnik, P. C.; Stephenson, M. L. *Proceedings of the National Academy of Sciences of the United States of America* **1978,** *75*, 280-4.

66. Agrawal, S.; Mayrand, S. H.; Zamecnik, P. C.; Pederson, T. *Proceedings of the National Academy of Sciences of the United States of America* **1990,** *87*, 1401-5.
67. Furdon, P. J.; Dominski, Z.; Kole, R. *Nucleic Acids Research* **1989,** *17*, 9193-204.
68. Reese, C. B.; Yan, H. *Journal of the Chemical Society, Perkin Transactions 1: Organic and Bio-Organic Chemistry* **2002**, 2619-33.
69. Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. *Nature* **1998,** *391*, 806-11.
70. Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. *Nature* **2001,** *411*, 494-8.
71. Filipowicz, W.; Jaskiewicz, L.; Kolb, F. A.; Pillai, R. S. *Current Opinion in Structural Biology* **2005,** *15*, 331-41.
72. Valencia-Sanchez, M. A.; Liu, J.; Hannon, G. J.; Parker, R. *Genes & Development* **2006,** *20*, 515-24.
73. Sontheimer, E. J. *Nature Reviews Molecular Cell Biology* **2005,** *6*, 127-38.
74. Blank, M.; Blind, M. *Current Opinion in Chemical Biology* **2005,** *9*, 336-42.
75. Lee Jennifer, F.; Stovall Gwendolyn, M.; Ellington Andrew, D. *Current opinion in chemical biology* **2006,** *10*, 282-9.
76. Que-Gewirth, N. S.; Sullenger, B. A. *Gene therapy* **2007,** *14*, 283-91.
77. Tuerk, C.; Gold, L. *Science* **1990,** *249*, 505-10.
78. Eckstein, F. *Expert opinion on biological therapy* **2007,** *7*, 1021-34.
79. Ogilvie, K. K.; Usman, N.; Nicoghosian, K.; Cedergren, R. J. *Proceedings of the National Academy of Sciences of the United States of America* **1988,** *85*, 5764-8.
80. Damha, M. J.; Ogilvie, K. K. *Methods in Molecular Biology* **1993,** *20*, 81-114.
81. Bellon, L.; Wincott, F. *Solid-Phase Synthesis* **2000**, 475-528.
82. Letsinger, R. L.; Hamilton, S. B. *Journal of the American Chemical Society* **1959,** *81*, 3009-12.
83. Bayer, E.; Mutter, M. *Nature* **1972,** *237*, 512-3.

84. Bonora, G. M.; Scremin, C. L.; Colonna, F. P.; Garbesi, A. *Nucleic Acids Research* **1990,** *18*, 3155-9.
85. Douglas, S. P.; Whitfield, D. M.; Krepinsky, J. J. *Journal of the American Chemical Society* **1991,** *113*, 5095-7.
86. Pon, R. T.; Yu, S.; Guo, Z.; Deshmukh, R.; Sanghvi, Y. S. *Journal of the Chemical Society, Perkin Transactions 1* **2001**, 2638-2643.
87. Sanghvi, Y. S. **2006**, personal communication.
88. Gravert, D. J.; Janda, K. D. *Chemical Reviews (Washington, D. C.)* **1997,** *97*, 489-509.
89. Toy, P. H.; Janda, K. D. *Accounts of chemical research* **2000,** *33*, 546-54.
90. Horvath, I. T.; Rabai, J. *Science* **1994,** *266*, 72-5.
91. Studer, A.; Hadida, S.; Ferritto, R.; Kim, S.-Y.; Jeger, P.; Wipf, P.; Curran, D. P. *Science* **1997,** *275*, 823-6.
92. Horvath, I. T. *Accounts of Chemical Research* **1998,** *31*, 641-50.
93. Betzemeier, B.; Knochel, P. *Angewandte Chemie, International Edition in English* **1997,** *36*, 2623-4.
94. Wende, M.; Meier, R.; Gladysz, J. A. *Journal of the American Chemical Society* **2001,** *123*, 11490-1.
95. Holbrey, J. D.; Seddon, K. R. *Journal of the Chemical Society, Dalton Transactions: Inorganic Chemistry* **1999**, 2133-40.
96. Wilkes, J. S. *Green Chemistry* **2002,** *4*, 73-80.
97. Castner, E. W., Jr.; Wishart, J. F. *Journal of Chemical Physics* **2010,** *132*, 120901/1-9.
98. Sheldon, R. *Chemical Communications (Cambridge)* **2001**, 2399-407.
99. Sheldon, R. A.; Lau, R. M.; Sorgedrager, M. J.; van Rantwijk, F.; Seddon, K. R. *Green Chemistry* **2002,** *4*, 147-51.
100. Wasserscheid, P.; Keim, W. *Angewandte Chemie, International Edition in English* **2000,** *39*, 3772-89.
101. Fuller, J.; Carlin, R. T.; Osteryoung, R. A. *Journal of the Electrochemical Society* **1997,** *144*, 3881-6.

102. Fuller, J.; Breda, A. C.; Carlin, R. T. *Journal of Electroanalytical Chemistry* **1998,** *459*, 29-34.
103. Huddleston, J. G.; Rogers, R. D. *Chemical Communications (Cambridge)* **1998**, 1765-6.
104. Boesmann, A.; Datsevich, L.; Jess, A.; Lauter, A.; Schmitz, C.; Wasserscheid, P. *Chemical Communications (Cambridge)* **2001**, 2494-5.
105. Ye, C.; Liu, W.; Chen, Y.; Yu, L. *Chemical Communications (Cambridge)* **2001**, 2244-5.
106. Welton, T. *Chemical Reviews (Washington, D. C.)* **1999,** *99*, 2071-83.
107. Wasserscheid, P.; Welton, T.; Editors *Ionic Liquids in Synthesis*, 2003.
108. Chan, T. H. L., L.-H.; Yang, Y.; Lu, W. *Clean Solvents: Alternative Media for Chemical Reactions and Processing. (Proceedings of a Meeting held November 2000 in San Francisco, California.) [In: ACS Symp. Ser., 2002; 819]*, 2002.
109. Law, M. C.; Wong, K.-Y.; Chan, T. H. *Journal of Organic Chemistry* **2005,** *70*, 10434-10439.
110. Miao, W.; Chan, T. H. *Organic Letters* **2003,** *5*, 5003-5005.
111. Fraga-Dubreuil, J.; Bazureau, J. P. *Tetrahedron* **2003,** *59*, 6121-6130.
112. Miao, W.; Chan, T.-H. *Journal of Organic Chemistry* **2005,** *70*, 3251-3255.
113. He, X. C., Tak Hang. *Synthesis* **2006,** *10*, 1645-1651.
114. Huang, J.-Y.; Lei, M.; Wang, Y.-G. *Tetrahedron Letters* **2006,** *47*, 3047-50.
115. Alvarado-Urbina, G.; Sathe, G. M.; Liu, W. C.; Gillen, M. F.; Duck, P. D.; Bender, R.; Ogilvie, K. K. *Science (Washington, DC, United States)* **1981,** *214*, 270-4.
116. Damha, M. J.; Ogilvie, K. K. *Methods in molecular biology (Clifton, N.J.)* **1993,** *20*, 81-114.
117. Usman, N.; Ogilvie, K. K.; Jiang, M. Y.; Cedergren, R. J. *Journal of the American Chemical Society* **1987,** *109*, 7845-54.
118. Krotz, A. H.; McElroy, B.; Scozzari, A. N.; Cole, D. L.; Ravikumar, V. T. *Organic Process Research & Development* **2003,** *7*, 47-52.

119. Hadden, C. E.; Martin, G. E.; Krishnamurthy, V. V. *Magnetic Resonance in Chemistry* **2000,** *38*, 143-147.
120. Streiwieser, A.; Heathcock, C. H.; Kosower, E. M. *Introduction to Organic Chemistry*, 4th ed.; Macmillan Publishing Company: NY, USA, 1992.
121. Reese, C. B.; Serafinowska, H. T.; Zappia, G. *Tetrahedron Letters* **1986,** *27*, 2291-4.
122. Kierzek, R.; Ito, H.; Bhatt, R.; Itakura, K. *Tetrahedron Letters* **1981,** *22*, 3761-4.
123. Hayakawa, Y.; Uchiyama, M.; Noyori, R. *Tetrahedron Letters* **1986,** *27*, 4191-4.
124. Damha, M. J. Doctoral Thesis, McGill University, 1988.
125. Donga, R. A.; Khaliq-Uz-Zaman Syed, M.; Chan, T.-H.; Damha, M. J. *Journal of Organic Chemistry* **2006,** *71*, 7907-10.
126. Roger, M.; Hotchkiss, R. D. *Proceedings of the National Academy of Sciences of the United States of America* **1961,** *47*, 653-69.
127. Bellon, L.; Workman, C.: USA, 2000.
128. Dowler, T.; Bergeron, D.; Tedeschi, A.-L.; Paquet, L.; Ferrari, N.; Damha, M. J. *Nucleic Acids Research* **2006,** *34*, 1669-75.
129. Ohkubo, A.; Kasuya, R.; Sakamoto, K.; Miyata, K.; Taguchi, H.; Nagasawa, H.; Tsukahara, T.; Watanobe, T.; Maki, Y.; Seio, K.; Sekine, M. *Nucleic Acids Research* **2008,** *36*, 1952-64.
130. Weimann, G.; Khorana, H. G. *Journal of the American Chemical Society* **1962,** *84*, 419-30.
131. Weimann, G.; Schaller, H.; Khorana, H. G. *Journal of the American Chemical Society* **1963,** *85*, 3835-41.
132. Schaller, H.; Khorana, H. G. *Journal of the American Chemical Society* **1963,** *85*, 3841-51.
133. Ohtsuka, E.; Moon, M. W.; Khorana, H. G. *Journal of the American Chemical Society* **1965,** *87*, 2956-70.
134. Koessel, H.; Moon, M. W.; Khorana, H. G. *Journal of the American Chemical Society* **1967,** *89*, 2148-54.

135. Koessel, H.; Buechi, H.; Khorana, H. G. *Journal of the American Chemical Society* **1967,** *89*, 2185-94.
136. Ohtsuka, E.; Khorana, H. G. *Journal of the American Chemical Society* **1967,** *89*, 2195-202.
137. Schott, H.; Koessel, H. *Journal of the American Chemical Society* **1973,** *95*, 3778-85.
138. Berlin, Y. A.; Efimov, V. A.; Kolosov, M. N.; Korobko, V. G.; Shingarova, L. N. *Bioorganicheskaya Khimiya* **1975,** *1*, 1738-45.
139. Agarwal, K. L.; Buchi, H.; Caruthers, M. H.; Gupta, N.; Khorana, H. G.; Kleppe, K.; Kumar, A.; Ohtsuka, E.; Rajbhandary, U. L.; Van de Sande, J. H.; Sgaramella, V.; Weber, H.; Yamada, T. *Nature* **1970,** *227*, 27-34.
140. Weber, H.; Khorana, H. G. *Journal of Molecular Biology* **1972,** *72*, 219-49.
141. Buechi, H.; Khorana, H. G. *Journal of Molecular Biology* **1972,** *72*, 251-88.
142. Kumar, A.; Ohtsuka, E.; Khorana, H. G. *Journal of Molecular Biology* **1972,** *72*, 289-307.
143. Ohtsuka, E.; Kumar, A.; Khorana, H. G. *Journal of Molecular Biology* **1972,** *72*, 309-27.
144. Kumar, A.; Khorana, H. G. *Journal of Molecular Biology* **1972,** *72*, 329-49.
145. Agarwal, K. L.; Kumar, A.; Khorana, H. G. *Journal of Molecular Biology* **1972,** *72*, 351-73.
146. Caruthers, M. H.; Van de Sande, J. H.; Khorana, H. G. *Journal of Molecular Biology* **1972,** *72*, 375-405.
147. Caruthers, M. H.; Khorana, H. G. *Journal of Molecular Biology* **1972,** *72*, 407-26.
148. Sgaramella, V.; Khorana, H. G. *Journal of Molecular Biology* **1972,** *72*, 427-44.
149. Sgaramella, V.; Kleppe, K.; Terao, T.; Gupta, N. K.; Khorana, H. G. *Journal of Molecular Biology* **1972,** *72*, 445-56.
150. Van de Sande, J. H.; Caruthers, M. H.; Sgaramella, V.; Yamada, T.; Khorana, H. G. *Journal of Molecular Biology* **1972,** *72*, 457-74.

151. Caruthers, M. H.; Kleppe, K.; Van de Sande, J. H.; Sgaramella, V.; Agarwal, K. L.; Buchi, H.; Gupta, N. K.; Kumar, A.; Otsuka, E.; RajBhandary, U. L.; Terao, T.; Weber, H.; Yamada, T.; Khorana, H. G. *Journal of molecular biology* **1972,** *72*, 475-92.
152. Van de Sande, J. H.; Caruthers, M. H.; Kumar, A.; Khorana, H. G. *Journal of Biological Chemistry* **1976,** *251*, 571-86.
153. Minamoto, K.; Caruthers, M. H.; Ramamoorthy, B.; Van de Sande, J. H.; Sidorova, N.; Khorana, H. G. *Journal of Biological Chemistry* **1976,** *251*, 587-98.
154. Agarwal, K. L.; Caruthers, M. H.; Fridkin, M.; Kumar, A.; Van de Sande, J. H.; Khorana, H. G. *Journal of Biological Chemistry* **1976,** *251*, 599-608.
155. Jay, E.; Cashion, P. J.; Fridkin, M.; Ramamoorthy, B.; Agarwal, K. L.; Caruthers, M. H.; Khorana, H. G. *Journal of Biological Chemistry* **1976,** *251*, 609-23.
156. Agarwal, K. L.; Caruthers, M. H.; Buchi, H.; van de Sande, J. H.; Khorana, H. G. *Journal of Biological Chemistry* **1976,** *251*, 624-33.
157. Sekiya, T.; Besmer, P.; Takeya, T.; Khorana, H. G. *Journal of Biological Chemistry* **1976,** *251*, 634-41.
158. Loewen, P. C.; Miller, R. C.; Panet, A.; Sekiya, T.; Khorana, H. G. *Journal of Biological Chemistry* **1976,** *251*, 642-50.
159. Panet, A.; Kleppe, R.; Kleppe, K.; Khorana, H. G. *Journal of Biological Chemistry* **1976,** *251*, 651-7.
160. Caruthers, M. H.; Kleppe, R.; Kleppe, K.; Khorana, H. G. *Journal of Biological Chemistry* **1976,** *251*, 658-66.
161. Kleppe, R.; Sekiya, T.; Loewen, P. C.; Kleppe, K.; Agarwal, K. L.; Buchi, H.; Besmer, P.; Caruthers, M. H.; Cashion, P. J.; Fridkin, M.; Jay, E.; Kumar, A.; Miller, R. C.; Minamoto, K.; Panet, A.; RajBhandary, U. L.; Ramamoorthy, B.; Sidorova, N.; Takeya, T.; van de Sande, J. H.; Khorana, H. G. *Journal of biological chemistry* **1976,** *251*, 667-75.
162. Arentzen, R.; Reese, C. B. *Journal of the Chemical Society, Perkin Transactions 1: Organic and Bio-Organic Chemistry* **1977**, 445-60.

163. Ohtsuka, E.; Ubasawa, M.; Ikehara, M. *Journal of the American Chemical Society* **1971,** *93*, 2296-301.
164. Werstiuk, E. S.; Neilson, T. *Canadian Journal of Chemistry* **1972,** *50*, 1283-91.
165. Ohtsuka, E.; Sugiyama, T.; Ikehara, M. *Chemical & Pharmaceutical Bulletin* **1975,** *23*, 2257-62.
166. Werstiuk, E. S.; Neilson, T. *Canadian Journal of Chemistry* **1976,** *54*, 2689-96.
167. Van Boom, J. H.; Burgers, P. M. J.; Van der Marel, G.; Verdegaal, C. H. M.; Wille, M. G. *Nucleic Acids Research* **1977,** *4*, 1047-63.
168. Adamiak, R. W.; Biala, E.; Grzeskowiak, K.; Kierzek, R.; Kraszewski, A.; Markiewicz, W. T.; Okupniak, J.; Stawinski, J.; Wiewiorowski, M. *Nucleic Acids Research* **1978,** *5*, 1889-905.
169. Ogilvie, K. K.; Nemer, M. J. *Canadian Journal of Chemistry* **1980,** *58*, 1389-97.
170. Ohtsuka, E.; Fujiyama, K.; Ikehara, M. *Nucleic Acids Research* **1981,** *9*, 3503-22.
171. Ohtsuka, E.; Yamane, A.; Ikehara, M. *Nucleic Acids Research* **1983,** *11*, 1325-35.
172. Ohtsuka, E.; Yamane, A.; Doi, T.; Ikehara, M. *Tetrahedron* **1984,** *40*, 47-57.
173. Gait, M. J.; Singh, M.; Sheppard, R. C.; Edge, M. D.; Greene, A. R.; Heathcliffe, G. R.; Atkinson, T. C.; Newton, C. R.; Markham, A. F. *Nucleic Acids Research* **1980,** *8*, 1081-96.
174. Duckworth, M. L.; Gait, M. J.; Goelet, P.; Hong, G. F.; Singh, M.; Titmas, R. C. *Nucleic Acids Research* **1981,** *9*, 1691-706.
175. Efimov, V. A.; Reverdatto, S. V.; Chakhmakhcheva, O. G. *Nucleic Acids Research* **1982,** *10*, 6675-94.
176. Iwai, S.; Koizumi, M.; Ikehara, M.; Ohtsuka, E. *Tetrahedron* **1987,** *43*, 59-67.

177. Virnekas, B.; Ge, L.; Plueckthun, A.; Schneider, K. C.; Wellnhofer, G.; Moroney, S. E. *Nucleic Acids Research* **1994,** *22*, 5600-7.
178. Lyttle, M. H.; Napolitano, W.; Calio, B. L.; Kauvar, L. M. *BioTechniques* **1995,** *19*, 274-8, 280-1.
179. Ono, A.; Matsuda, A.; Zhao, J.; Santi, D. V. *Nucleic Acids Research* **1995,** *23*, 4677-82.
180. Zehl, A.; Starke, A.; Cech, D.; Hartsch, T.; Merkl, R.; Fritz, H.-J. *Chemical Communications (Cambridge)* **1996**, 2677-8.
181. Kayushin, A. L.; Korosteleva, M. D.; Miroshnikov, A. I.; Kosch, W.; Zubov, D.; Piel, N. *Nucleic Acids Research* **1996,** *24*, 3748-55.
182. Eleuteri, A.; Capaldi, D. C.; Cole, D. L.; Ravikumar, V. T. *Nucleosides & Nucleotides* **1999,** *18*, 475-83.
183. Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992,** *48*, 2223-311.
184. Kayushin, A.; Korosteleva, M.; Miroshnikov, A.; Kosch, W.; Zubov, D.; Piel, N.; Weichel, W.; Krenz, U. *Nucleosides & Nucleotides* **1999,** *18*, 1531-3.
185. Kayushin, A.; Korosteleva, M.; Miroshnikov, A. *Nucleosides, Nucleotides & Nucleic Acids* **2000,** *19*, 1967-76.
186. Yagodkin, A.; Azhayev, A.; Roivainen, J.; Antopolsky, M.; Kayushin, A.; Korosteleva, M.; Miroshnikov, A.; Randolph, J.; Mackie, H. *Nucleosides, Nucleotides & Nucleic Acids* **2007,** *26*, 473-97.
187. Kuijpers, W. H.; Huskens, J.; Koole, L. H.; van Boeckel, C. A. *Nucleic Acids Research* **1990,** *18*, 5197-205.
188. Iyer, R. P.; Yu, D.; Jiang, Z.; Agrawal, S. *Tetrahedron* **1996,** *52*, 14419-36.
189. Alul, R. H.; Singman, C. N.; Zhang, G. R.; Letsinger, R. L. *Nucleic Acids Research* **1991,** *19*, 1527-32.
190. Fei, Z.; Zhao, D.; Geldbach, T. J.; Scopelliti, R.; Dyson, P. J. *Chemistry--A European Journal* **2004,** *10*, 4886-93.
191. Ueki, M.; Aoki, H.; Katoh, T. *Tetrahedron Letters* **1993,** *34*, 2783-6.
192. MacDonald, S. F. *Canadian Journal of Chemistry* **1974,** *52*, 3257-8.
193. Ronald, R. C.; Wheeler, C. J. *Journal of Organic Chemistry* **1983,** *48*, 138-9.

194. Sato, F.; Inoue, M.; Oguro, K.; Sato, M. *Tetrahedron Letters* **1979**, 4303-6.
195. Eberle, M. K.; Kahle, G. G. *Tetrahedron Letters* **1980,** *21*, 2303-4.
196. Nahm, S.; Weinreb, S. M. *Tetrahedron Letters* **1981,** *22*, 3815-18.
197. Uchiyama, K.; Hayashi, Y.; Narasaka, K. *Tetrahedron* **1999,** *55*, 8915-30.
198. Petit, S.; Azzouz, R.; Fruit, C.; Bischoff, L.; Marsais, F. *Tetrahedron Letters* **2008,** *49*, 3663-5.
199. Stetter, H. *Angewandte Chemie, International Edition in English* **1976,** *15*, 639-47.
200. Letsinger, R. L.; Caruthers, M. H.; Miller, P. S.; Ogilvie, K. K. *Journal of the American Chemical Society* **1967,** *89*, 7146-7.
201. Letsinger, R. L.; Miller, P. S. *Journal of the American Chemical Society* **1969,** *91*, 3356-9.
202. Letsinger, R. L.; Ogilvie, K. K.; Miller, P. S. *Journal of the American Chemical Society* **1969,** *91*, 3360-5.
203. Van Boom, J. H.; Burgers, P. M. J. *Tetrahedron Letters* **1976,** *17*, 4875-8.
204. Dugave, C.; Demange, L. *Chemical Reviews (Washington, D. C.)* **2003,** *103*, 2475-532.
205. Hassler, M.; Damha, M. J. **2010**, unpublished results.
206. Leisvuori, A.; Poijarvi-Virta, P.; Virta, P.; Lonnberg, H. *Tetrahedron Letters* **2008,** *49*, 4119-21.
207. Wada, T.; Ohkubo, A.; Mochizuki, A.; Sekine, M. *Tetrahedron Letters* **2001,** *42*, 1069-72.
208. Crick, F. H. *Symposia of the Society for Experimental Biology* **1958,** *12*, 138-63.
209. Crick, F. H. C. *Nature* **1970,** *227*, 561-3.
210. Berget, S. M.; Moore, C.; Sharp, P. A. *Proceedings of the National Academy of Sciences of the United States of America* **1977,** *74*, 3171-5.
211. Cech, T. R.; Zaug, A. J.; Grabowski, P. J. *Cell* **1981,** *27*, 487-96.
212. Cech, T. R.; Tanner, N. K.; Tinoco, I., Jr.; Weir, B. R.; Zuker, M.; Perlman, P. S. *Proceedings of the National Academy of Sciences of the United States of America* **1983,** *80*, 3903-7.

213. Zaug, A. J.; Grabowski, P. J.; Cech, T. R. *Nature* **1983,** *301*, 578-83.
214. van der Veen, R.; Arnberg, A. C.; van der Horst, G.; Bonen, L.; Tabak, H. F.; Grivell, L. A. *Cell* **1986,** *44*, 225-34.
215. Valadkhan, S.; Manley, J. L. *Nature* **2001,** *413*, 701-7.
216. Valadkhan, S.; Manley, J. L. *RNA* **2003,** *9*, 892-904.
217. Williams, N. H.; Takasaki, B.; Wall, M.; Chin, J. *Accounts of Chemical Research* **1999,** *32*, 485-93.
218. Kosonen, M.; Hakala, K.; Lonnberg, H. *Journal of the Chemical Society, Perkin Transactions 2: Physical Organic Chemistry* **1998**, 663-70.
219. Lonnberg, T.; Mikkola, S. *Journal of Organic Chemistry* **2004,** *69*, 802-10.
220. Lonnberg, T.; Korhonen, J. *Journal of the American Chemical Society* **2005,** *127*, 7752-8.
221. Lonnberg, T.; Kiiski, J.; Mikkola, S. *Organic & Biomolecular Chemistry* **2005,** *3*, 1089-96.
222. Westheimer, F. H. *Accounts of Chemical Research* **1968,** *1*, 70-8.
223. Jarvinen, P.; Oivanen, M.; Lonnberg, H. *Journal of Organic Chemistry* **1991,** *56*, 5396-401.
224. Damha, M. J.; Ogilvie, K. K. *Biochemistry* **1988,** *27*, 6403-16.
225. Chaulk, S. G.; MacMillan, A. M. *Nucleic Acids Research* **1998,** *26*, 3173-8.
226. Braich, R. S.; Damha, M. J. *Bioconjugate Chemistry* **1997,** *8*, 370-7.
227. Ivanova, E. M.; Khalimskaya, L. M.; Romanenko, V. P.; Zarytova, V. F. *Tetrahedron Letters* **1982,** *23*, 5447-50.
228. Kierzek, R.; Kopp, D. W.; Edmonds, M.; Caruthers, M. H. *Nucleic Acids Research* **1986,** *14*, 4751-64.
229. Damha, M. J.; Ogilvie, K. K. *Journal of Organic Chemistry* **1988,** *53*, 3710-22.
230. Prashad, M.; Prasad, K.; Repic, O. *Nucleosides & Nucleotides* **1994,** *13*, 945-52.
231. Lackey, J. G.; Sabatino, D.; Damha, M. J. *Organic Letters* **2007,** *9*, 789-92.

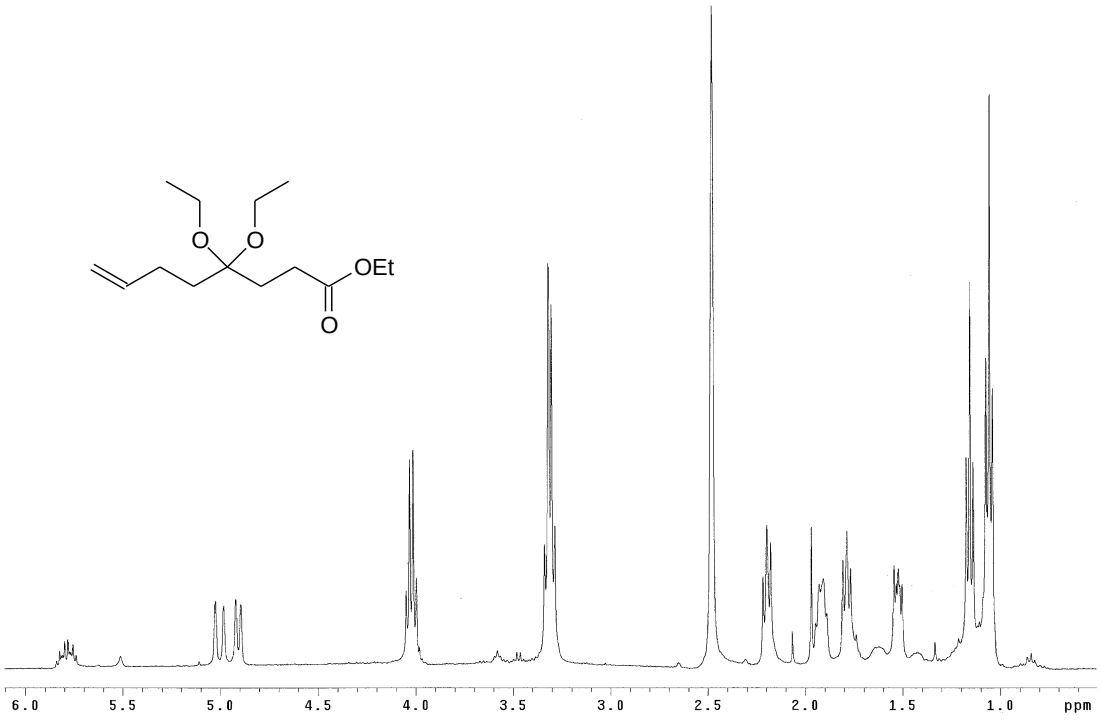
232. Lackey, J. G.; Mitra, D.; Somoza, M. M.; Cerrina, F.; Damha, M. J. *Journal of the American Chemical Society* **2009,** *131*, 8496-502.
233. Westheimer, F. H.; Huang, S.; Covitz, F. *Journal of the American Chemical Society* **1988,** *110*, 181-5.
234. Karplus, M. *Journal of the American Chemical Society* **1963,** *85*, 2870-1.
235. van Wijk, J.; Haasnoot, C. A. G.; de Leeuw, F. A. A. M.; Huckriede, B. D.; Hoekzema, A. W.; Altona, C.: *PSEUROT 6.3*; Leiden Institute of Chemistry, Leiden University; Leiden, The Netherlands, 1999.

Appendix

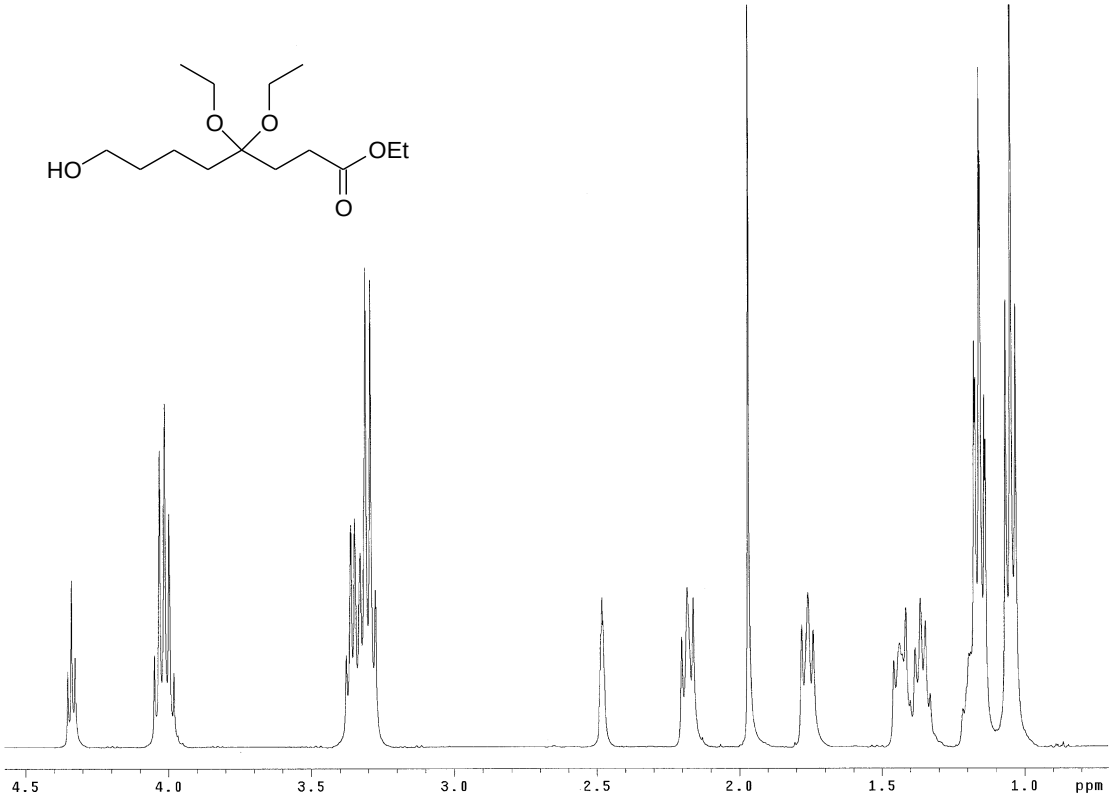
Representative ^{1}H NMR of synthesis of orthogonally cleavable tag

(Scheme 3-25, Scheme 3-26)

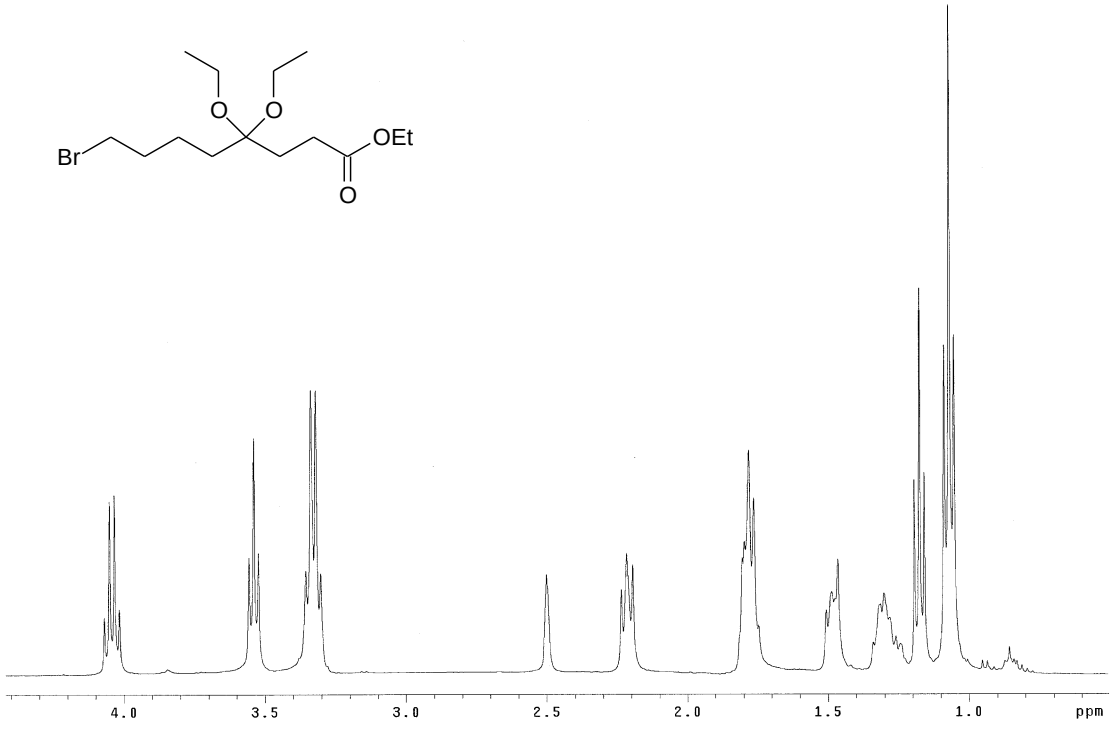
3.38



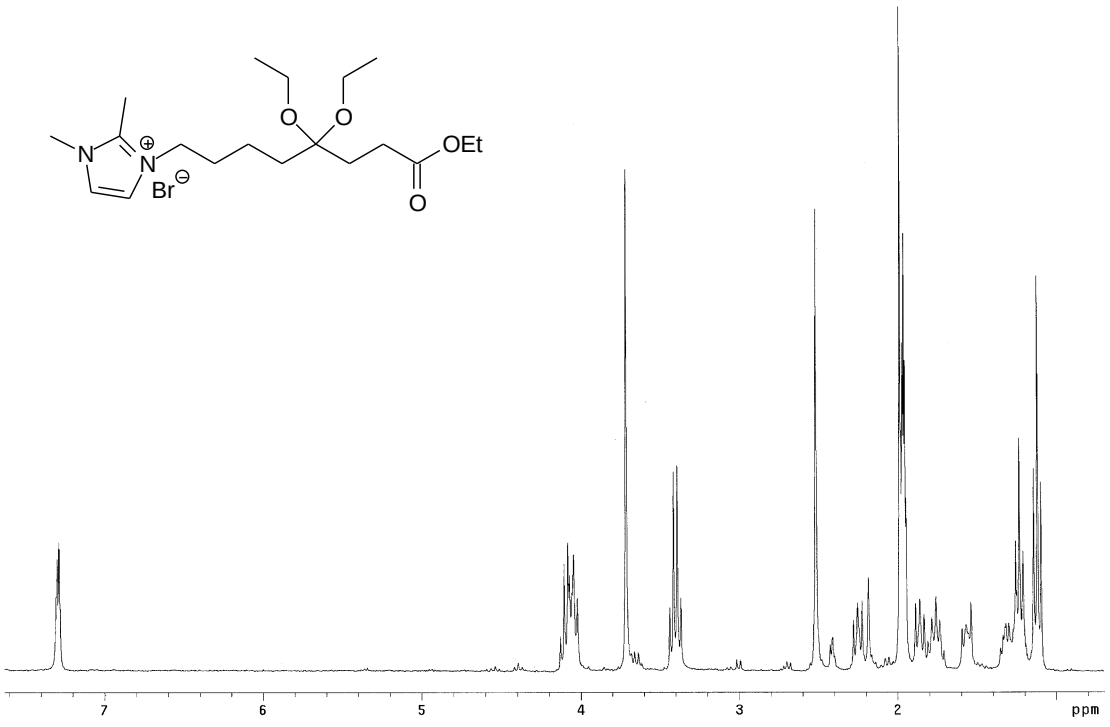
3.39



3.40



3.41



3.42