Advancements in the synthesis of oligoribonucleotides on solid support and in solution: Ionic soluble supports, blockmer ribonucleotide amidites, and orthogonal linkers

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Copyright statement

Contributions

I would like to thank and acknowledge that Dr. Mallik Reddy contributed to the synthesis of the adenosine dimers presented in Chapter 3 and assisted in the solution phase synthesis of oligoribonucleotides using dimer phosphoramidites.

Prudence, (Yi Qiao Wu) a summer student in our laboratory, assisted and significantly contributed to the NMR delevulination study of the monomers and dimers presented in Chapter 3.

This work is dedicated to my family.
Even though I have been gone for a long time, and this endeavor has certainly not brought us any closer together, your constant encouragement and support from afar in letting me find my way means more to me than you know.

Abstract

This thesis is focused on the investigation of synthetic approaches for the preparation of oligonucleotides in solution. We describe our efforts to produce these valuable molecules in a more cost effective manner relative to the current industrial standards employing solid supported synthesis. We demonstrate that an ionic tag supported synthesis technique can be successfully applied to oligonucleotide synthesis using a phosphonium-based ionic tag, using simple precipitation and phase separation methods without the need for chromatography to obtain reasonably high product purity at each step.

We also explore the optimization of the technique as it is moved to the gram scale and investigate the use of dimer and trimer blocks in the assembly of RNA. This strategy significantly reduced the total number of steps required in the synthesis of a target RNA sequence, provided more material, and simplified separation of the product from shorter failure sequences. The procedure was illustrated by the synthesis of rUpU, rApA, and rUpUpU phosphoramidite blocks and their use in the synthesis of 2-19 nt oligoribonucleotides both in solution and on solid phase. The building blocks utilized a 2′-*O*-triisopropylsilyl (TIPS) protecting group for the 3′-termini instead of the standard 2′-*O*-tert-butyldimethylsilyl (TBDMS) protection, in order to eliminate silyl migration during synthesis of dimer and trimer phosphoramidites.

The synthesis and use of two novel, orthogonally cleavable linkers for solid and soluble supported synthesis are also described. The linkers are derived from the well known levulinyl and 3′-nitrophenylpropyloxycarbonyl (NPPOC) protecting groups, and expand utility to our methods by providing ready access to dimer and trimer phosphoramidite blocks. As the orthogonal linkers are removed under mild conditions, they will likely find use in the post-synthesis modification of oligonucleotides, the synthesis of oligonucleotides with base sensitive modifications, and siRNA or antisense pro-drugs.

Résumé

Cette thèse se penche principalement sur l'étude d'approches synthétiques pour la préparation d'oligonucléotides en solution. Nous décrivons nos efforts pour produire ces molécules précieuses par une méthode plus économiquement rentable comparativement aux normes industrielles courantes qui utilisent la synthèse en phase solide. Nous démontrons qu'une synthèse employant une technique de tag ionique supporté peut être appliquée avec succès à la préparation d'oligonucléotides employant un tag ionique basée sur le phosphonium. L'utilisation de méthodes simples de purification, telles que la précipitation et la séparation de phases, nous a permis d'obtenir des composés raisonnablement purs à chaque étape sans avoir recours à la chromatographie.

Nous avons également exploré l'optimisation de la technique pendant l'extrapolation à grande échelle et investigué l'utilisation de dimère et trimère dans l'assemblage de l'ARN. Cette stratégie réduit de façon significative le nombre d'étapes totales requises pour la synthèse d'une séquence d'ARN cible, produit plus de matériel et simplifie la séparation du produit d'autres séquences tronquées plus courtes. Cette procédure a été illustrée par la synthèse de blocs phosphoramidites rUpU, rApA et rUpUpU ainsi que par leur utilisation dans la synthèse d'oligoribonucléotides de 2 à 19 nt tant en solution qu'en phase solide. Les synthons disposaient d'un groupement protecteur 2′-*O*-triisopropylsilyle (TIPS) sur l'extrémité 3′ de l'ADN au lieu du 2′-*O*-tert-butyldiméthylsilyle (TBDMS) habituel afin d'éliminer la migration de groupe silyle durant la synthèse de dimère et trimère de phosphoramidites.

La synthèse et l'utilisation de deux nouveaux liens clivables orthogonalement pour la synthèse en phase solide et en solution est aussi décrite. Les liens ont été dérivés des groupes bien connus lévulinyl et 3′-nitrophénylpropyloxycarbonyle (NPPOC), et élargissent notre méthode en donnant l'accès facile aux dimères et trimères de blocs phosphoramidites. Comme les liens orthogonaux sont enlevés sous des conditions douces, ils vont fort probablement trouver une utilisation dans la modification post-synthétique

d’oligonucléotides, la synthèse d’oligonucléotides possédant une modification basosensible et le petit ARN interférant ou de promédicament antisens.

Acknowledgements

Dr. Damha, I remember very clearly my first week in Montreal, sitting in your office, discussing my options for choice of supervisor, while you were still the graduate student coordinator. While I was struggling with my decisions, you asked me if I had considered you as an option for my supervisor, and I remember looking right at you and saying "You work here?" While not the most informed response, I am extremely grateful of the relationship we have developed as a member of your group and your constant attention to the ever persistent challenge of making me a little less misinformed. I can confidently say (to myself) that I am a capable, insightful, and diligent scientist because of the mentoring that you have provided to me over these years. I know that I must have tested your patience at times (or many); I will be forever grateful for the time that you spend ensuring we perform the right experiments, reference the right work, and present our work in the best way possible…even though you were often met with a lot of resistance. Even though you are the only PhD supervisor I have ever had, certainly making it easier to say, you are the best! You are an inspiration for the type of supervisor I would hope to one day become. Thank you for giving me this wonderful, fun, and challenging learning experience.

Dr. (Bill) Chan, I feel very fortunate to have been able to get to know you through our collaborative work. The time and consideration you showed me, while guiding me through our work together is something that I will try to emulate while working with others as I move forward with my career. Also, I have really enjoyed the fact that you insist on us calling you Bill, which has had the effect of driving Dr. Damha crazy. I think I see his eye twitch every time I address you in our meetings.

Damha lab members, past and present, you have certainly made this great experience what it is. Alex, Rob, Jon, Debbie, Paul, Dave, Sierra and Jer, thank you so much for welcoming me into the group and always being there to help me with any problems and showing me how to get things done properly. I have done my best to pass forward the warm welcome, and continue the sense of "we are all in this together" environment you harbored when I first arrived. Jer, thanks for

showing me the ropes in the lab and getting me off to a productive start, and giving me advice whenever needed. Thanks to Jon, Alex, Rob and Dave, for confirming that said advice was in fact correct, or not. Jon, thanks for the shirt, you are a well dressed man. More current members, thanks for the diverse work environment our lab has become. I have learnt so much about the world by working with all of you, who have come from all over it. Julia, PAULA!, Nuria, other Nuria and Saul: All of our Spanish friends, I hope you enjoyed our Canadian experience as much as I enjoyed the Spanish experience you gave me during my travels. Karolina, Richard, Mallik, briefly that Russian guy.. Macgyver?, The Iran trio – Maryam, Maryam and Fereshteh, Dilip, and Hala and everyone else in the department I have met from seemingly every corner of the planet: while I am not a well traveled man, thank you for introducing me to so many different cultures, thoughts, and ideas. You have all made the world much less of a foreboding place for this small town prairie kid, and I'm now anxious to go out and explore it. Thank you to the whole group: Hala, Adam, Maryam, Maryam, Jovanka, Glen, Danielle, Saul, Ken who have put up with me in the time leading up to, and while writing my dissertation. Danielle, thanks for grammar proofing my document, and Ken for your very insightful comments. To Glen and Adam in particular, it has been a great experience learning to keep our lab operational; looking back it has been great fixing, poking, prodding, and fine tuning all the equipment – these unintentional skills we have developed will no doubt be valuable in the future.

To all my friends in the department, you have been an essential part of my learning experience with constant support and advice during every single "road block" I have encountered during the course of my studies. For risk of forgetting anyone, I'll refrain from a list, but you know who you are; I think we have all done a great job mutually helping each other to keep our sanity during the rough times, and celebrated all the successes. You are a wonderful group of people, and I'm sure that we are not all just friends of circumstance, and will continue to keep up with each other over the years to come.

There are, however few people I must mention in particular, who have had a particular influence on my time here. Ryan and Val, thank you for taking me

under your wing and making the start of my very memorable journey into Montreal and McGill such a great experience. Katerina, your friendship, constant help and advice throughout my time here has made this degree possible, the extent of which I'm sure you already know, and will continue to remind me of. While we do bicker constantly, know that it's all in fun, and that your friendship is and has always been very important to me. Rodrigo, man, thanks for all the advice and time you gave me while I bounced every possible idea off of you. Countless times I walked away from our conversations with a new approach or a fresh perspective.

Thank you to everyone and all of whom we have forgotten; experiencing and exploring the city with you will always remain "those great times" that I'm sure I will often look back and reflect on. While we have all moved on across the globe I'm sure there will be many more great experiences yet to come, I look forward to seeing you all there.

-*Matthew Hassler*

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Abbreviations

A adenosine

Å Angstrom

A_{260}	UV absorbance measured at 260 nm
Ac	acetate
Ac_2O	acetic anhydride
ACE	bis(acetoxyethoxy)methyl
ACN	acetonitrile
AcOH	acetic acid
Ade	adenine
AGO2	Argonaute 2, the endonuclease at the heart of human RISC
ALE	acetal levulinyl ester
AON	antisense oligonucleotide
B	base
BMT	5-benzylmercaptotetrazole
Bn	benzyl
bp	base pair
Bz	benzoyl
C	cytidine
ca.	circa (approximately)
calc.	calculated
CE	2-cyanoethyl
CEM	2-cyanoethyloxymethyl
CMPI	2-chloromethyl-pyridinium iodide
CMV	Cytomegalovirus retinitis
CNE	cyanoethyl
COSY	correlation spectroscopy, homonuclear (NMR)
Cpep	1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl
CPG	controlled pore glass
Ctmp	1-(2-chloro)-4-methoxypiperidin-4-yl
DBU	1,8-diazabicycloundec-7-ene
DCC	N,N′-dicyclohexylcarbodiimide
DCI	4,5-dicyanoimidazole
DCM	dichloromethane
dd	doublet of doublets
DIPEA	*N,N*-diisopropylethylamine

DMAP	4-(dimethylamino)pyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DMTr	4,4′-dimethoxytrityl
dN	2′-deoxyribonucleoside
DNA	2′-deoxyribonucleic acid
ds	double-stranded
dt	doublet of triplets
dT	2′-deoxythymidine
DTM	dithiomethyl
ESI-MS	electrospray ionization mass spectrometry
Et	ethyl
et al.	and others
EtOAc	ethyl acetate
EtOH	ethanol
Fmoc	9-fluorenylmethoxycarbonyl
G	gram
G	guanosine
Gua	guanine
H	hours
HPLC	high performance (or high pressure) liquid chromatography
HRMS	high resolution mass spectrometry
I.E.	ion exchange
J	scalar coupling contstant (in Hz)
Lv	levulinyl
m/z	mass to charge ratio
MALDI	matrix-assisted laser desorption/ionization (mass spectrometry)
NBOM	nitrobenzyloxymethyl
Me	methyl
MeCN	acetonitrile
MeOH	methanol
miRNA	microRNA
MMTr	4-monomethoxytrityl

mRNA	messenger ribonucleic acid
MTBE	methyl *tert*-butyl ether
n-bu	1-butyl
NH_4OH	ammonium hydroxide
NMP	*N*-methylpyrrolidinone (*i.e.*, 1-methyl-2- pyrrolidinone)
NMR	nuclear magnetic resonance
NPPOC	nitrophenylpropyloxycarbonyl
nt	nucleotide
OD	optical density units, defined as the hypothetical A260 of a solution of the sample of interest in 1 mL water, in a 1-cm path cuvette.
p	phosphate
PEG	polyethylene glycol
PivOM	pivaloyloxymethyl
PO	phosphodiester (linkage)
pyr	pyridine
R.P.	reverse phase
r.t.	room temperature
R_f	retention factor (in TLC, the ratio of the distance
RISC	RNA-induced silencing complex, the effector complex of RNA interference
r	ribo
rN	ribonucleoside
RNA	ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal RNA
s	singlet
shRNA	short hairpin RNA
siRNA	small interfering RNA
ss	single-stranded
ssRNA	single stranded RNA
t	triplet
T	thymidine
TBAF	tetra-n-butylammonium fluoride

TBDMS	*tert*-butyldimethylsilyl
TBTU	O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate
TC	1,1-dioxo-1,6-thiomorpholine-4-carbothioate
TCA	trichloroacetic acid
TCE	2,2,2-trichloroethyl
TEA	triethylamine
TEAA	triethylammonium acetate
TEM	2-(4-tolylsulfonyl)ethoxymethyl
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
THF	tetrahydrofuran
Thy	thymine
TMS	trimethyl silyl
TIPS	triisopropylsilyl chloride
TLC	thin-layer chromatography
TOM	*tris*(isopropylsilyl)oxy)methyl
TPS	triphenylsilyl
TPS-Cl	triisopropylbenzenesulfonyl chloride
TREAT-HF	triethylamine trihydrofluoride
tRNA	transfer RNA
U	uridine
Ura	uracil
UV	ultraviolet (spectroscopy)

Chapter 1 Introduction

"If I have seen further it is by standing on the shoulders of giants"

-Sir Isaac Newton

1.1 Nucleic Acids

As was common with most findings in the biological world, nucleic acids were initially discovered in 1871, without any knowledge of their function, purpose or utility.[1] This oversight was perhaps understandable, as only a few years prior in 1867 the world had just unraveled the very important relationship between germs and disease,[2,3] as well as other important discoveries such as the electric light bulb,[4] and the telephone.[5] So it was not until after almost a hundred years of other important innovations that nucleic acids were to be studied in earnest. This resulted in the fundamental discovery by Oswald Avery in 1944, who established the relationship between nucleic acids, DNA, and the carrier of genetic information.[6] This was followed by numerous findings about the nucleic acid function in cellular activity. Of course the most notable amongst them was made by Rosalind Franklin[7,8] and Maurice Wilkins[9] in collaboration with the famed James Watson and Francis Crick, namely the discovery of the structure of DNA, the double helix.[10] The seminal discovery of the structure of RNA followed just three years later in 1956 by Alexander Rich and David Davies.[11] These discoveries helped form the initial hypotheses for cellular replication, which is now the accepted theory we know today.

The fields of genomics and later molecular biology were thus formed, investigating these important discoveries, unifying and broadening our knowledge of how genetic information is stored, replicated and later used to produce functional proteins. Our understanding of the operation of life was forever changed by arguably some of the most important discoveries in our quest to understand the function of life.

1.2 The Structure of Nucleotides and Nucleic Acids

Long before the structure of DNA and RNA was discovered, much work was done investigating the basic building blocks of these nucleic acids, the

individual nucleotides of which they are composed. The basic structural differences between DNA and RNA are primarily in the sugar component of the nucleotide shown in Figure 1.1.

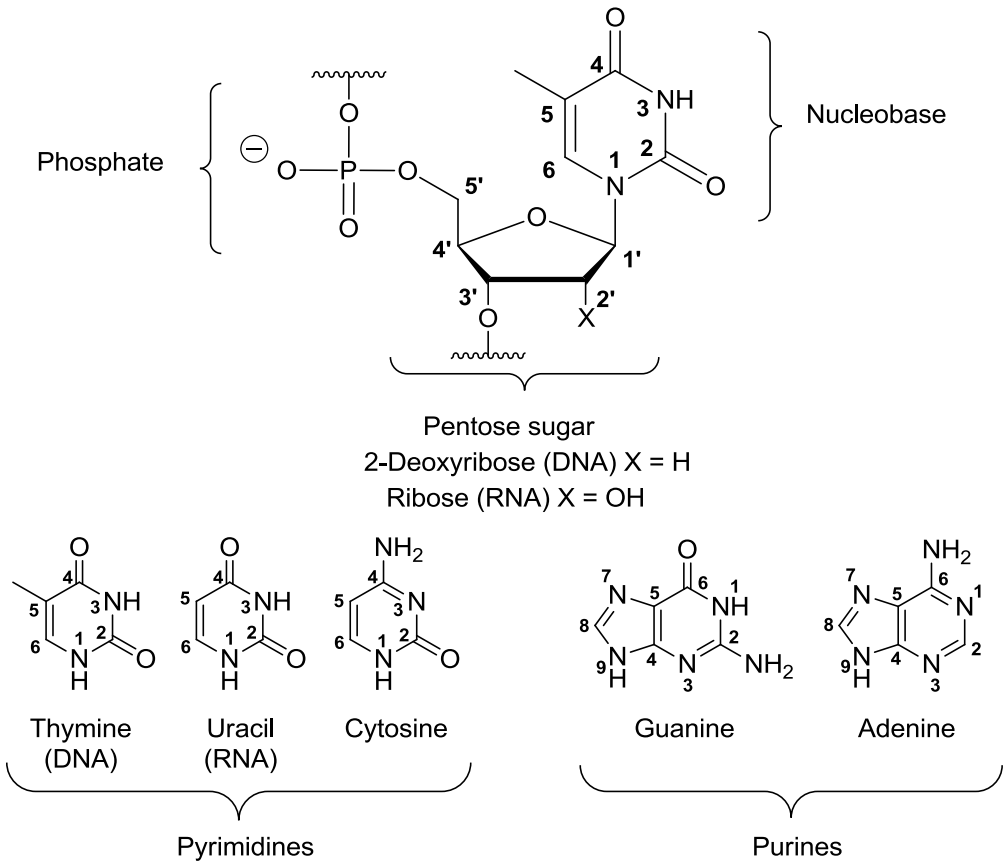


Figure 1.1. Basic structure of the nucleotide components of both DNA and RNA

Each nucleotide unit consists of three major components: a pentose sugar (ribose), a nucleobase, and a phosphate group. RNA, or ribonucleic acids, possesses a hydroxyl functionality at the 2′-position of the sugar ring. The ribose sugar component in DNA lacks the hydroxyl group at the 2′-position, hence its name **deoxy**ribonucleic acid. The nucleobases in DNA and RNA are primarily the same and consist of either purines or pyrimidines. The only difference is that thymine found in DNA is substituted with uracil in RNA. The nucleobases are attached to the sugar through a β-glycosidic linkage from the C1′ to either the N1 of the pyrimidines, or the N9 of the purines. The phosphate moiety is present at both the 5′ and the 3′ hydroxyl groups of the ribose in DNA and RNA and serves

to connect the nucleoside units. A unit that consists of a sugar ring and a nucleobase is referred to as a nucleoside unit. When a phosphate group is present at the 5', 3', or 2'-position, the unit is a nucleotide. Each oligonucleotide strand of either DNA or RNA (primary structure) is connected through a 3′-5′-phosphodiester linkage that has a negative charge (pK_a~1.6) at physiological pH, and in the presence of a sequence of complementary nucleobase pairs will form a duplex, known as a double helix.

The double helix has been studied extensively since its discovery in 1953.[10,12,13] Much has been learned about its structure and the differences that exist between a DNA double helix and an RNA double helix (secondary structure). As described below, the difference in the helical structure between DNA and RNA lie in the propensity of the individual sugars to favor one conformation (sugar pucker) over another.

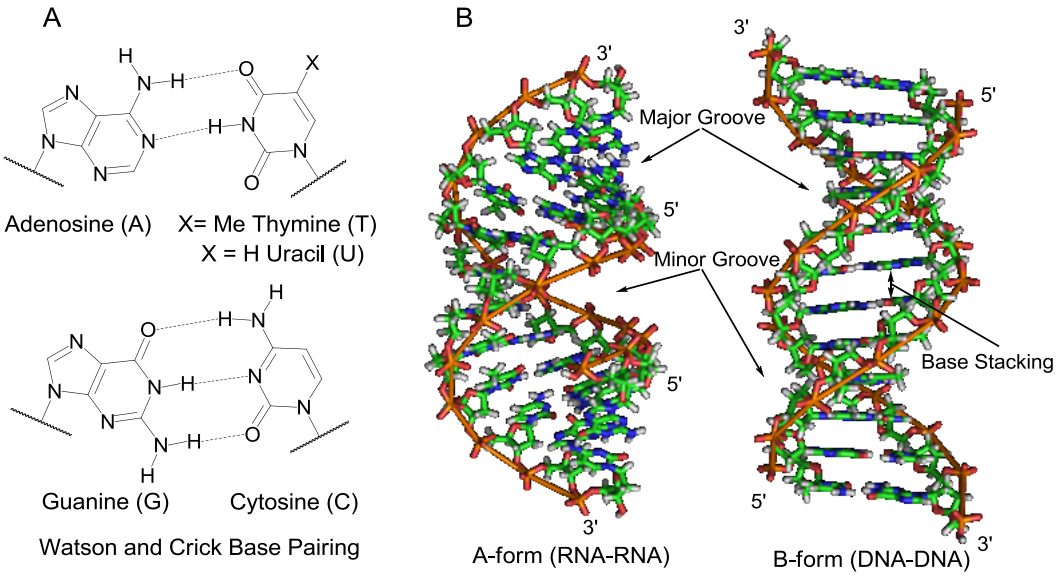


Figure 1.2. A) Watson and Crick GC and AT base pairs B) A and B form of helices. Image drawn by Richard Wheeler and used with permission.

An important discovery was that DNA and RNA form duplexes that bind in an anti-parallel orientation, where two strands hybridize in a "head to tail" orientation. This allows for the nucleobase to form strong "Watson and Crick" hydrogen bonding between their nucleobase pairs (A-T (or U) and G-C).[13,14] The hydrogen bonding between A-T base pairs are typically weaker in comparison to

the G-C pair. This is explained by two points of hydrogen bonding in the A-T pair compared to three in the G-C pair. Another important contribution to the helix structure is the favorable hydrophobic base stacking interactions between adjacent base pairs located within the core of the helix (Figure 1.2 B). This interaction has been found to contribute significantly to the stability of the helical structure.[15-17]

Two of the most prominent helical structures, the "A-form" and the "B-form" are shown in Figure 1.2. They differ primarily as a result of the sugar conformation adopted by the individual nucleotide units that comprise the duplexes (Figure 1.3).[18-20] RNA nucleotides prefer the C3′-endo conformation, or the "northern pucker", which helps to pre-organize the more compact "A-form" double stranded helix that RNA is typically found in.[21] The "northern pucker" results in an intra-nucleotide phosphate distance of 5.9Å which is responsible for the more compact A-form helix. DNA nucleotides prefer the C2′-endo conformation, or the "southern pucker" which has the effect of further extending the intra-nucleotide phosphate distance by 1.1 Å, which results in the more widely recognized image of the double stranded helix, the B-form.[7,8,22]

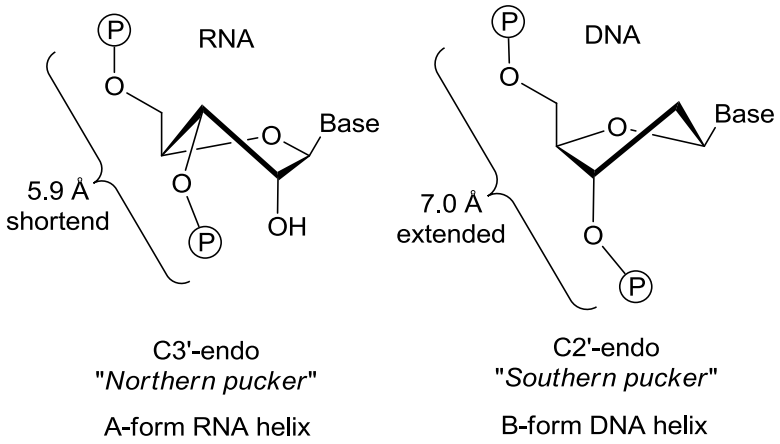


Figure 1.3. Favored sugar pucker conformation of RNA and DNA**.**

1.3 The Role of Nucleic acids

The primary role of nucleic acids is best described in the so called "*Central Dogma*" of molecular biology. This details how DNA is the carrier of all genetic

information which goes through two major processes. It postulates that DNA is the carrier of all genetic information, which is transferred through two major processes: 1) Replication of all information by DNA-polymerase in order to pass information from one generation to the next (of course with the occasional nucleotide misincorporation, allowing for Darwinian evolution to occur). 2) Transcription of the genetic information into single stranded RNA (pre-mRNA), which is further processed into mRNA, and finally translated by cellular ribosomes into functional proteins which carry out the processes that necessitate life.[23]

If only it were that simple. Over the last 20 years it has been determined that DNA, and particularly RNA, play much larger role than just carriers of genetic information. It has been discovered that both DNA and RNA also play a central role in the regulation of gene expression.[24]

1.3.1 Antisense oligonucleotides (AON)

In 1978 Zamecnik and Stephenson demonstrated for the first time that a short (13 nt) single stranded DNA was able to inhibit viral replication by hybridizing to a viral RNA.[25] The initial observation opened the door for a whole new approach to treat disease because traditionally, therapeutics focused on inhibiting a particular protein or enzyme (not a nucleic acid) that contributed to the disease to be treated. With the discovery of the antisense mechanism, the translation of the particular protein or enzyme of interest could be retarded or altered by cleaving pre-mRNA or altering its splicing pathway.[26]

Much later after the initial discovery of antisense oligonucleotides (AON), the mechanism(s) was determined by many collaborative efforts, and found to operate primarily with the assistance of an endogenous cellular protein, RNase H (see Figure 1.4). This enzyme is particularly important since it potentiates the biological activity of AONs by degrading the mRNA portion of the AON-mRNA duplex and releasing the AON.[27-29]

Many problems related to the antisense approach, such as large scale production of AONs, stabilization of the AON against degradative nucleases, or

efficient transport and delivery, are gradually being overcome through major advances in chemistry.[30-33]

In 1998 the Food and Drug Administration approved the first AON drug for the treatment of cytomegalovirus retinitis, a virus that can cause blindness in AIDS patients.[34] Unfortunately, this drug was used only to a limited extent because the incidence of CMV retinitis declined as AIDS patients lived longer, healthier lives thanks to effective antiretroviral drugs. Furthermore, ganciclovir, a nucleoside analogue, is quite effective against CMV[35] and cheaper to produced compare to the AON compound. Early this year, another AON-based therapeutic was introduced by ISIS Pharmaceuticals, the same company that brought Vitravene to the market. "Kynamro", known generically as mipomersen, inhibits action of the apolipoprotein B, a gene that is involved in the formation of particles that carry cholesterol in the blood.[36] Because of some toxicity exhibited by Kynamro, this drug will be primarily used to treat a rare inherited disorder that causes extremely high cholesterol levels and heart attacks.

1.3.2 Small interfering RNA (siRNA)

The discovery of RNA interference (RNAi) in 1998 by Fire and Mello[37] was awarded the Nobel Prize in Physiology and Medicine in 2006, only 8 years after it was first described. Their studies unraveled a completely new mechanism for the regulation of gene expression outlined in Figure 1.4

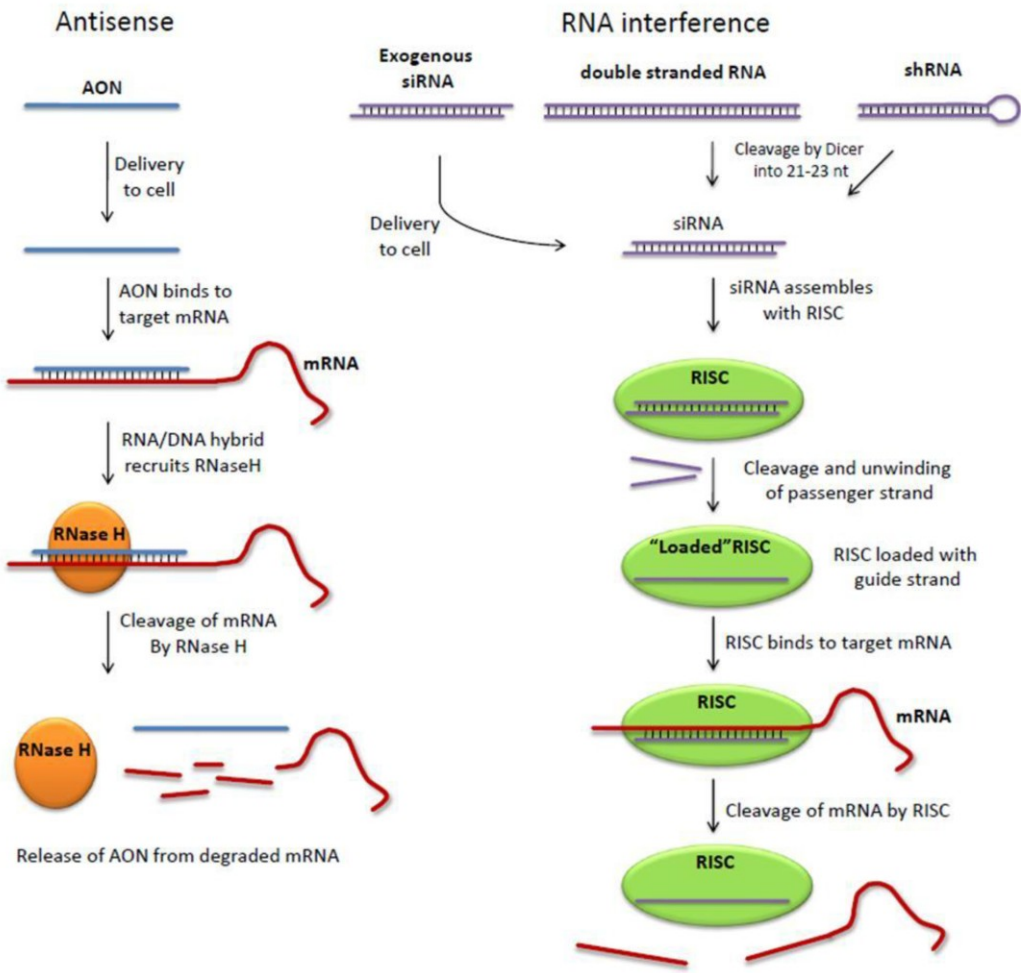


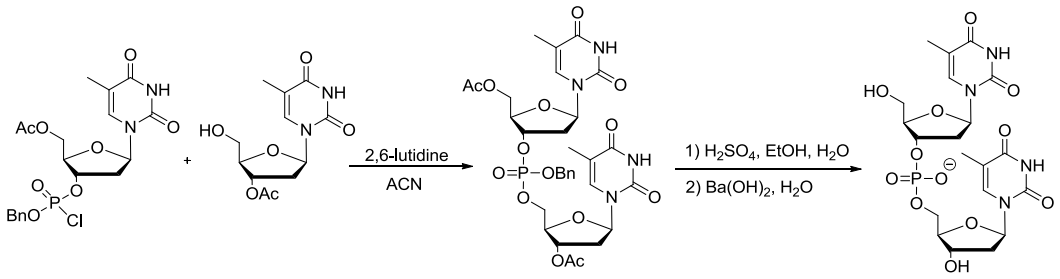
Figure 1.4. Diagram of antisense and RNA interference. This figure was created by Dr. Alexander Wahba, of our group, and used with permission.

Small interfering RNAs (siRNAs) are the key regulatory molecules, and can originate from three difference sources/pathways. Synthetic siRNAs can be introduced exogenously, or produced endogenously by "Dicer" from either long double stranded RNA or short hairpin RNA (shRNA). The resulting small RNA duplexes (19-21 bp) are taken up by the RNA Induced Silencing Complex (RISC). The RISC complex unravels the RNA duplex retaining one strand, termed the guide strand (or the antisense strand),[38] while the other, termed the passenger strand (sense strand), is cleaved by Argonaut 2 (AGO2; or "slicer") and released.[39] The active "loaded RISC complex" then seeks out mRNA sequences complementary to the loaded guide strand and cuts the mRNA between the 10th and 11th nucleotides relative to the 5′-end of the guide strand.[40]

While no siRNA therapeutics have received regulatory approval, many potential candidates are in clinical trials, and the nucleic acid and medical communities remain hopeful that siRNA-based therapeutics like AON drugs will soon be a reality.[33]

1.4 Synthesis of Nucleic Acids

The first reported synthesis of a DNA dimer nucleotide was accomplished by Todd *et al* in 1955.[41] They successfully coupled a thymidine 3′-benzylchlorophosphate to the 5′-hydroxyl of a 3′-acetylated thymidine, creating a thymidine homodimer (Scheme 1.1). This approach is known as the phosphotriester method and will be described in greater detail in the following sections.



Scheme 1.1. First reported chemical synthesis of a thymidine homodimer.[41]

1.4.1 The phosphodiester method

Shortly after Todd's dTpT synthesis, Khorana and co-workers developed alternative synthesis and protecting group strategies for the synthesis of oligodeoxynucleotides. Many of the new protecting groups they introduced remain in use today (Figure 1.5).[42,43]

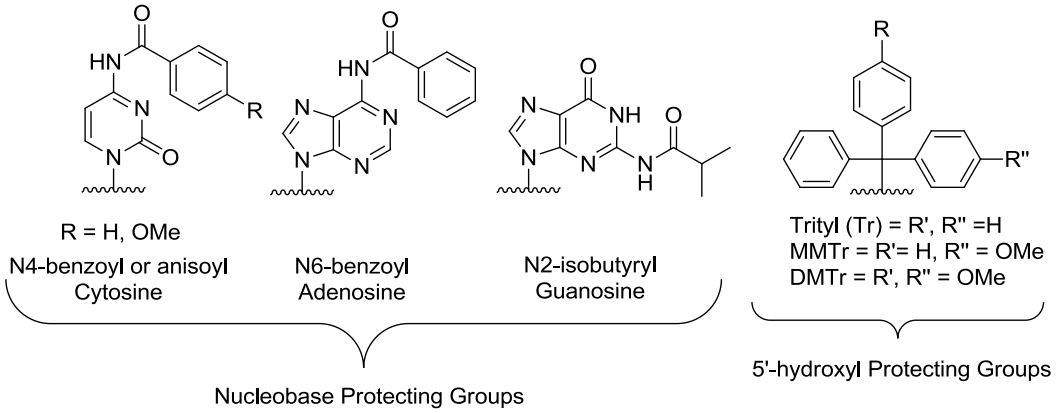
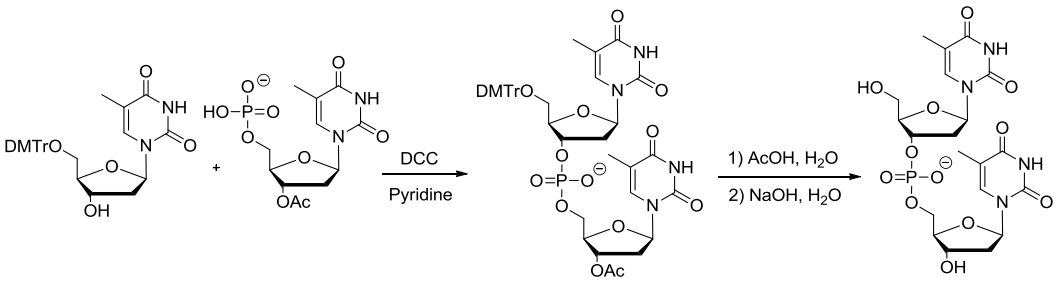


Figure 1.5. Standard protecting groups used for nucleoside/nucleotide protection introduced by Khorana *et al.*

These include the exocyclic amine protecting groups, such as benzoyl for adenine,[44] isobutyryl for guanine,[45] and anisoyl (and later acetyl)[46] for cytosine,[45] which in combination with the 5′-trityl groups (MMTr and DMTr) led to synthesis of oligomers via negatively charged phosphodiester intermediates. [40,41]



Scheme 1.2. Phosphodiester approach to the synthesis of oligonucleotides introduced by the Khorana research group.[47]

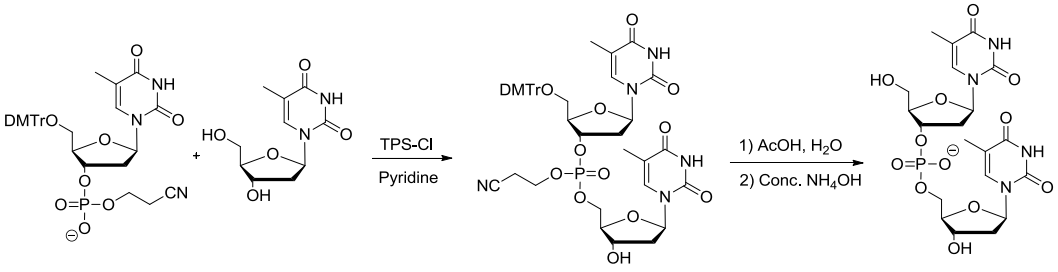
The milder coupling of a tritylated nucleoside to the 5′-phosphate group of a nucleotide was accomplished using N,N′-dicyclohexylcarbodiimide (DCC) as coupling reagent. This was followed by acid deprotection to yield the deprotected phosphate dimer dTpT (Scheme 1.2).[47-49] Short oligonucleotide fragments were made in solution, purified, and then connected by specific annealing and enzymatic ligation to generate longer duplexes. Their initial approach involved successive condensations in solution between "blocks" of protected di-, tri-, and tetra-nucleotides (bearing a 5′-phosphate monoester) and the 3′-hydroxyl end of the growing fully-protected oligonucleotide chain. At each step, the products were

separated by anion exchange chromatography and then verified for purity by paper chromatography upon removal of all remaining protecting groups. This work culminated in the total synthesis of the structural gene for the alanine transfer ribonucleic acid found in yeast.[50] The duplex was 77 bp in length and it took over three years to be made. When the results were published, they occupied the entire issue of the Journal of Molecular Biology (283 pages!).[50]

Khorana's pioneering work culminated with the 1968 Nobel Prize in Physiology or Medicine, which he shared with Robert W. Holley and Marshall W. Nirenberg "for their interpretation of the genetic code and its function in protein synthesis." Impressive progress in this area continues to be made (sections 1.4.6 and 1.5). In 2002, the first *de novo* synthesis of Poliovirus' 7.5k bp genome was constructed from synthetic oligonucleotides. The molecule exhibited its own life cycle.[51]

1.4.2 The phosphotriester method

The unprotected phosphates generated in the phosphodiester method were difficult to work with as they were mostly insoluble in conventional solvents. Building on the coupling procedure performed by the Todd group[41] and the protecting group strategies developed by Tener[52] and Khorana,[42,44,53] the Letsinger group reintroduced a phosphate protecting group, the 2-cyanoethyl moiety, which remains in modern use. Thus, this methodology was termed the phosphotriester method as inter-nucleoside phosphotriester linkages are formed (Scheme 1.3).[54-56]



Scheme 1.3. Phosphotriester method produced by the Letsinger group.[54,55]

The cyanoethyl protected phosphate of the 5′-tritylated thymidine was activated by 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl), and selectively

coupled to the 5′-hydroxyl of unprotected thymidine. This resulted in a more soluble dimer phosphotriester that formed faster and in higher yield relative to dimers prepared by the phosphodiester method. The internucleotide cyanoethyl protecting group was easily removed by treatment with a mild base, yielding after 5′-*O*-detritylation, the fully deprotected thymidine homodimer.

1.4.3 Phosphate protecting groups

A suitable phosphate protecting group must withstand the multiple 5′-*O*-deprotections during oligonucleotide chain growth and be cleaved under conditions that does not lead to internucleotide cleavage (and/or 3′,5′- to 2′,5′-isomerization in the case of RNA). Alexander Todd first used the benzyl phosphate protecting group,[41] but it was found to be too labile under basic conditions.

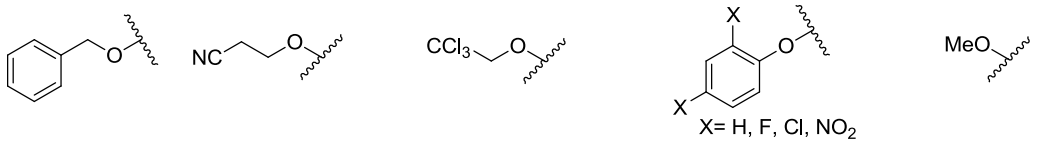


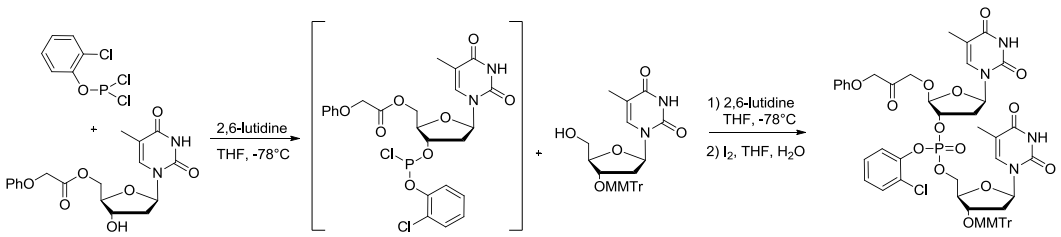
Figure 1.6. Phosphate protecting groups.

Later, Tener developed the cyanoethyl phosphate protecting group which was cleaved under basic conditions.[52] In fact, it was found to be too labile for solution phase synthesis, and it was not widely adopted until the phosphoramidite coupling method was developed in the early 1980's (Section 1.4.6). The trichloroethyl protecting group used for the synthesis of nucleotides by Eckstein[57] and Ogilvie.[58,59] Reese and Saffhill intensively studied the use of aryl and substituted aryl protecting groups with great success.[60-63] The *o*-chlorophenyl derivative, in particular, was found to be significantly stable during solution phase synthesis via the phosphotriester method, and later using the phosphite triester method. It additionally facilitated the mild cleavage post oligonucleotide synthesis.[64] The methyl phosphate protecting group was introduced much later by

Daub.[65] It was found to be stable to solution phase synthesis via the phosphotriester,[65] and the phosphite triester[66] and phosphoramidite[67] methods described below.

1.4.4 Phosphite triester method

In the mid 1970′s the Letsinger group continued to make invaluable contributions to the development of oligonucleotide synthesis. In two seminal papers, they took advantage of the higher reactivity of trivalent phosphorus (^{III}P), as compared to pentavalent phosphorus (^{V}P), and the facile oxidation of ^{III}P to ^{V}P with aqueous elemental iodine (Scheme 1.4).[68,69] The method was based on the in situ generation nucleosides 3'-phosphorochloridite which could then be rapidly coupled, at -78°C, with an appropriately protected nucleoside.



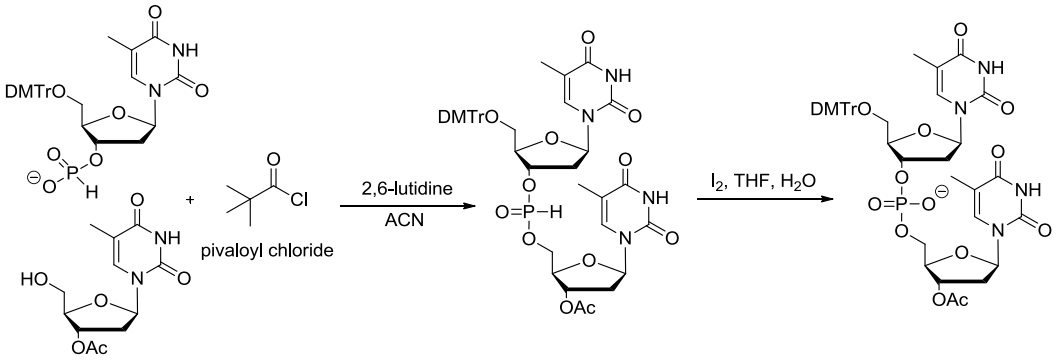
Scheme 1.4. Letsinger phosphite triester dTpT synthesis[68,69]

In 1981, Ogilvie and co-workers reported the use of this approach in the automated synthesis of gene fragments on silica based solid supports. Each monomer unit was added in a 30 min cycle, permitting a 14 nt DNA sequence to be constructed in 6.5 hours.[70] This approach was subsequently adapted with the use of nucleoside phosphoramidites described below. The technical ease and operation of such DNA synthesizers ("gene machines") led to their very successful commercialization.

1.4.5 The H-phosphonate method

The H-Phosphonate method was first developed by Michelson and Todd in 1957.[71] It was reintroduced nearly 30 years later by Garegg *et al.*[72] making use of pivaloyl chloride instead of N-chlorosuccinimide as activator, and the more modern protecting groups (Scheme 1.5). [73] An important feature of this approach

is that oxidation of all H-phosphonate linkages is carried in a single step after assembly of the oligonucleotide chain has been completed. This makes it an attractive choice for the large scale synthesis of oligonucleotides, as removal of one whole step during the synthetic cycle, results in 19-23 less steps, significantly reducing the overall time of production.

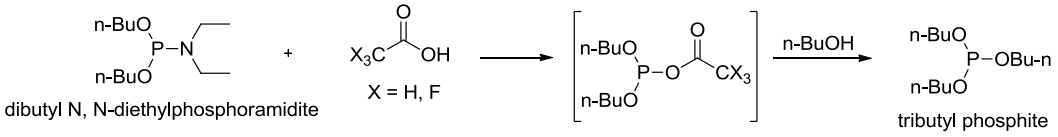


Scheme 1.5. The H-phophonate approach described the Garegg group.

An elegant application of this method was carried out by Reese and Yan in the solution phase synthesis of Vitravene, an antisense drug developed by ISIS for the treatment of CMV retinitis.[34] This method has also been adapted to the solid-phase supported synthesis of oligoribonucleotides.

1.4.6 The phosphoramidite method

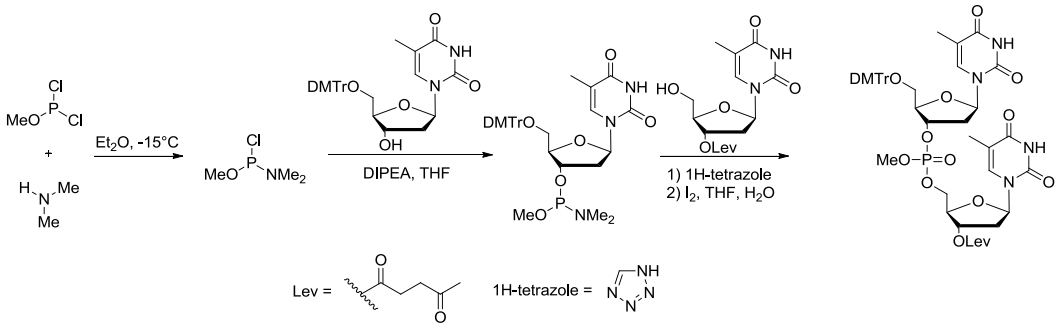
The first examples of (^{III}P) phosphoramidite derivatives were described by Evdakov *et al.*[74,75] in the 1970's. They demonstrated that dibutyl N,N-diethylphosphoramidite in the presence of an organic acid and butanol very rapidly converted to the tributylphosphite triester (Scheme 1.6).



Scheme 1.6. First example of organic acid activation of a phosphoramidite.

It was not till 1981, however, that Beaucage and Caruthers elegantly exploited this chemistry in DNA synthesis. They reported the use of nucleoside N,N-dialkylphosphoramidites which when activated by weak acids such as

tetrazole (pK_a 4.9) would couple nearly quantitatively within minutes to an appropriately protected nucleoside.[76] This approach provided an improvement to the phosphite triester approach by Letsinger. While the nucleoside phosphoramidites coupled very quickly, they were isolable as stable solids which, unlike nucleoside phosphochloridites, could be purified by column chromatography, and able to be stored for long periods. Later Caruthers[77-80] and Ogilvie[81,82] applied this very efficient approach to the automated solid supported method, forever changing how oligo-DNA and RNA are produced.



Scheme 1.7. The phosphoramidite procedure as described by Beaucage and Caruthers.

1.5 RNA Synthesis

To date, there have been many attempts to design protecting groups and methods that embody the conditions required for the construction of high quality oligoribonucleotides.[63,83] In fact, for many years, RNA synthesis has been regarded as far more complicated than DNA synthesis because of the difficulty in finding a compatible 2′-protecting group that (a) affords high step-wise coupling yields, (b) is stable throughout chain assembly, and (c) can be removed selectively at the end of synthesis without phosphodiester bond isomerization or degradation.

The presence of the 2′ hydroxyl group in RNA is responsible for its susceptibility to internucleotide cleavage under either mild basic[84] or mild acidic conditions.[85] Thus, the 2′-hydroxyl protecting group must have cleavage conditions mild enough to suppress the degradation of the deprotected oligonucleotide.

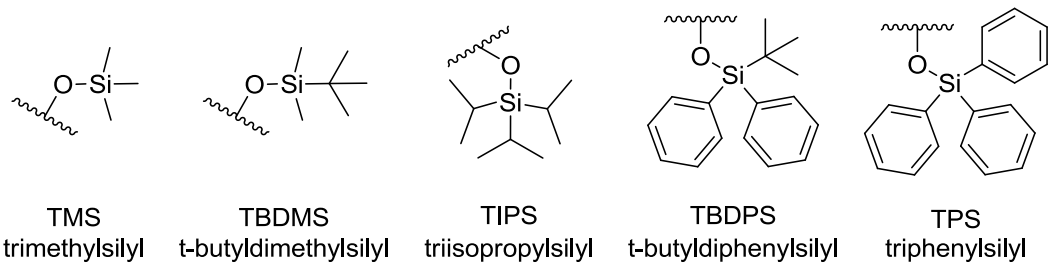


Figure 1.7. Common silyl protecting groups.

Silyl ether-based protecting groups have been amply described for the protection of nucleosides.[86,87] These include the trimethylsilyl (TMS), triisopropylsilyl (TIPS), methyldiisopropylsilyl (MDIPS), and *t*-butyldiphenylsilyl (TBDPS) as well as cyclic alkylsilyl and other silyl groups (Figure 1.7).[67,88] Among these, TIPS protection has been described primarily for 5′-monomethoxytrityl-N-isobutyrylguanosine derivatives, as the 2′,3′-*O*-TIPS isomers are more readily separated from each other by silica gel chromatography.[67,87] The smaller steric bulk of the TBDMS group relative to TIPS, TBDPS and other bulkier silyl ethers favors TBDMS as a 2′-protecting group for RNA synthesis. In fact, the 2′-*O*-TBDMS protecting group has been used the most compared to other protecting groups for RNA synthesis. When coupled with the phosphoramidite condensation procedure, 2′-*O*-TBDMS monomers have allowed for a highly efficient synthesis of oligoribonucleotides.[81,82] It is removed at the end of RNA chain assembly in the presence of fluoride ions.

A potential drawback of silyl ethers for the protection of the 2′-hydroxyl group lies in their widely recognized ability to undergo 2′- to 3′-*O*-isomerization under the influence of protic solvents, nucleophilic catalysts, or basic conditions. For example, the TBDMS group migrates from the O2′ to O3′ position (and vice versa) in the presence of either methanol, imidazole, pyridine/water, or aqueous ammonia, thereby generating a mixture of nucleoside O2′ and O3′ silyl regioisomers.[67,89,90]

Silyl isomerization is characteristic of other *O*-silyl ether protecting groups. *O*-TIPS derivatives of uridine and 7-deazaguanosine also undergo isomerization

in methanol, albeit more slowly than their *O*-TBDMS counterparts.[88,91] 5′-Monomethoxytrityl-N2-isobutyryl-2′-TIPS guanosine undergoes isomerization under ethanolic aq. ammonia conditions to give a mixture of 2′/3′-TIPS regioisomers which can be separated by chromatography. This provides a method to convert (recycle) the unwanted 3′-isomer into the more useful 2′-isomer.[67]

2′-*O*-Silyl groups do not normally migrate to O3′ in dry aprotic solvents. When these conditions are strictly adhered to, it is possible to prepare 2′-silylated ribonucleoside-3′-*O*-phosphoramidite derivatives in regioisomerically pure form.[92,93] This is clearly an important requirement during RNA synthesis as the presence of even traces of the 3′-silyl (2′-phosphoramidite) regioisomer will impact on the quality and biological activity of the desired RNA sequence.

Many other protecting groups for the 2′-hydroxyl position have been used in the synthesis of RNA.[63,83] RNA synthesis using monomers containing the 2′-triisopropylsilyloxymethyl (TOM) group, the 2′-acetal-levulinyl group, and the 2′-*O*-bis(2-acetoxyethoxy)methyl (ACE) group have been reported to yield higher coupling efficiency, because these protecting groups exhibit lower steric hindrance than the 2′-TBDMS group.[94] Like the TBDMS group, the TOM protecting group is removed using fluoride ions.

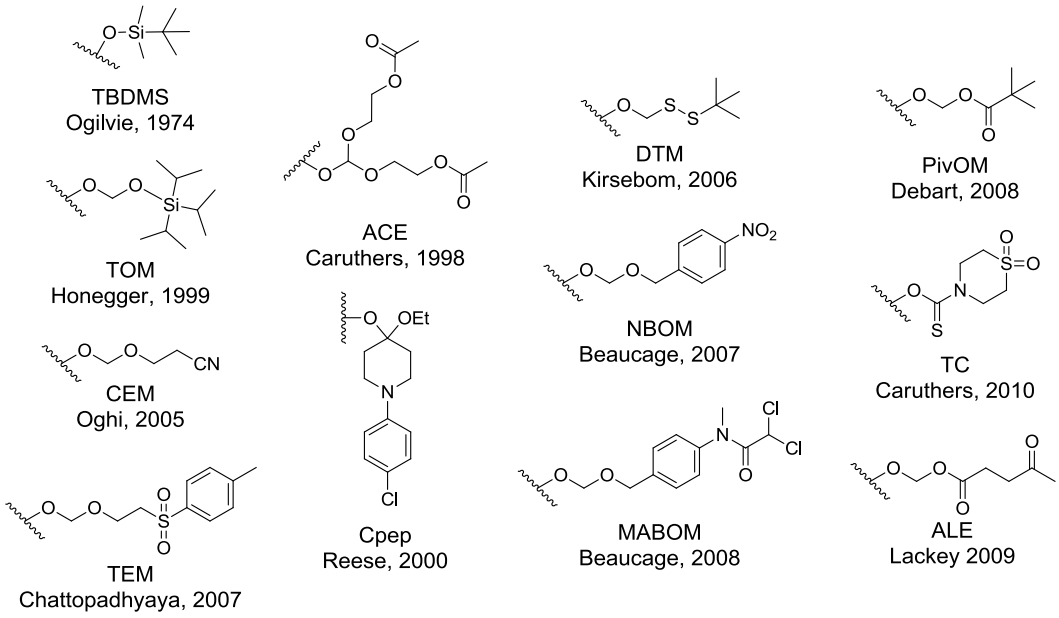


Figure 1.8. Selected 2′-protecting group used in the synthesis of oligoribonucleotides (RNA).

As is for DNA synthesis, the synthesis of oligoribonucleotides is an elaborate multistep process, which comprises assembly of the oligonucleotide chain typically from monomeric phosphoramidite building blocks (e.g., 5′-*O*-dimethoxytrityl-N-protected-2′-*O*-*tert*-butyldimethylsilyl-nucleoside-3′-*O*-phosphoramidites), deprotection of the base labile nucleobase protecting groups (e.g., benzoyl, isobutyryl, acetyl, phenoxyacetyl, etc.), cleavage from the support (e.g., glass beads or polystyrene), and finally, removal of the 2′-hydroxyl protecting groups. This approach allowed Ogilvie and co-workers to carry out the total solid phase chemical synthesis of a 77-nucleotide long RNA sequence having methionine acceptance activity.[82]

1.6 Solid phase synthesis of nucleic acids

As described above, current methods for DNA and RNA synthesis rely on stepwise addition of monomeric phosphoramidite units.[70,95] Chain elongation from 3′- to 5′-end is preferable and can be achieved by coupling a nucleoside unit having a 3′-phosphorus (III) group (in its activated form) to a free 5′-hydroxyl group of another nucleoside unit. As solid support, 500 to 1000 Å long chain

alkyl amine (LCAA), controlled pore glass (CPG) support, or polystyrene support PS200, are used in combination with a linker that covalently attaches the first nucleotide unit to the solid support. Traditionally, the free 3′-hydroxyl of the 3′-nucleoside is linked to the solid support by an ester bond through a succinyl linker.

A typical synthesis cycle is shown in Figure 1.9. Chain elongation begins by cleavage of the 5′-O-dimethoxytrityl group with an organic acid, liberating a nucleophilic 5′-hydroxyl group. This terminal nucleophile is then coupled to a protected nucleoside 3′-*O*-phosphoramidite monomer in the presence of an activator. In the case of RNA synthesis, suitable protection of the 2′-hydroxyl group is required (see Section 1.5). Any unreacted 5′-hydroxyl groups are acetylated in a process referred to as 'capping'. The most commonly used group used for this purpose is an acetyl ester. Thus, 'capping' with acetic anhydride esterifies any unreacted 5′-hydroxyl groups and halts the accumulation of by-products (or "failure" sequences). 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 sequence is obtained.

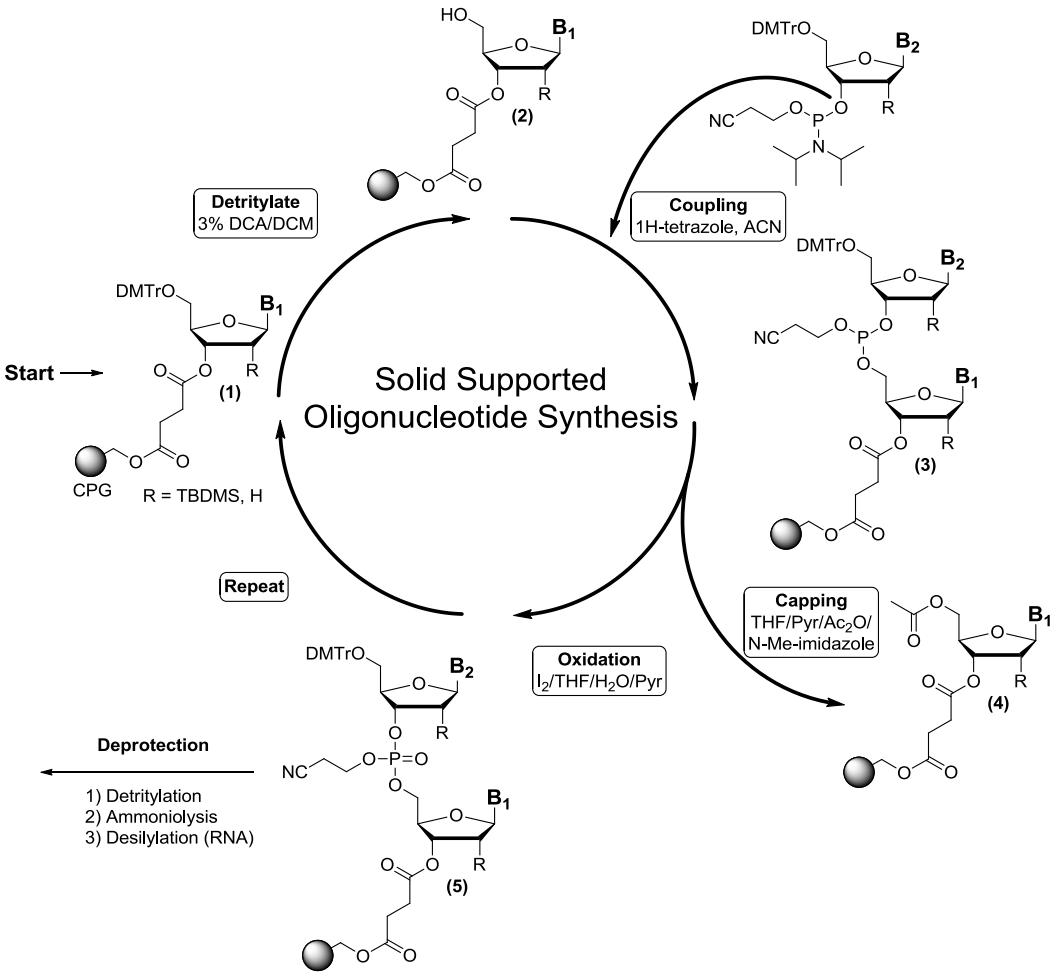


Figure 1.9. Solid supported oligonucleotide synthesis cycle.

Cleavage of the oligomer from the solid support and removal of the protecting groups from the sugars, phosphates and nucleobases provides the desired target oligomer, which is then separated from shorter failure sequences by ion exchange high pressure liquid chromatography (HPLC), ion-pair reverse phase HPLC, or polyacrylamide gel electrophoresis (PAGE). The full length oligomer is then characterized by mass spectrometry. Meanwhile, a large number of DNA oligomers can be synthesized in parallel on DNA microarrays or "gene chips",[96] although these methods are currently limited to the picomolar scale.[97]

Irrespective of the chemistry of coupling used, a problem associated with current methods for DNA synthesis is the high frequency of sequence errors. The longer the target oligonucleotide sequence, the more defects there are as a result of repeated exposure of the immobilized DNA sequence to deblocking reagents.

For example, repeated acidic treatments required to remove the 5′-dimethoxytrityl protecting group during assembly of the DNA chain lead to loss of the nucleobase (e.g., depurination); as a result, this process is only practical for making relatively short sequences of nucleotides. The current practical limit of the solid-phase phosphoramidite method is about 200 nt for a DNA oligonucleotide of sufficient quality for a biological application. For RNA synthesis on a solid support, the limit appears to be just over 100 nt, but for a different reason: stepwise coupling efficiencies of RNA 3′-phosphoramidite monomers are less (96-99%) than DNA phosphoramidite couplings (98-100%), in part due to steric effects created by the 2′-protecting group of the RNA building blocks.

1.7 The development of solid supported linkers for the synthesis of oligonucleotides

The immobilization of organic compounds on solid supports was first described by Letsinger and co-workers in 1959.[98] A few years later, the Merrifield and Letsinger groups reported, respectively, the solid supported synthesis of a polypeptide and oligonucleotide.[99,54] This approach offered the advantage of simply filtering off excess reagents and reactants for the first time. The popularity of this approach grew rapidly for the synthesis of peptides as the successful synthetic techniques gleaned from solution phase chemistry transferred well to the solid supported method. However, the solid supported method for the synthesis of nucleic acids lagged behind, since the techniques used to create nucleic acids were not well developed or suited for solid supported synthesis at that time. The phosphodiester and triester approaches simply did not provide pure enough or high enough yields for this approach. Once the phosphite triester and phosphoramidite approach were developed, the yields of the solution and solid phase reactions increased significantly over phosphodiester and phosphotriester approaches. The longest synthesis resulting from this development was a 21mer in 17% yield in 1980[100,101] which was considered an exceptional result at the time.

Another factor limiting the successful application of nucleic acids was the availability of an appropriate solid support. The polystyrene supports initially

used were found to have better results in less polar solvents that were not suitable for solvation of the reactants and reagents used in nucleotide synthesis at that time. Many types of solid supports were investigated with various nucleotide condensation methods: polydimethylacrylamide,[101] polyacryloylmorpholide,[102-105] and even cellulose[106-108] were attempted with limited success. The most successful solid support at the time was found to be derivatized silica gel, which was applied, first with the phosphite triester method,[109,110] and later with the phosphoramidite method, used in combination with the automated solid phase synthesizer.[70] Soon after the adoption of SiO_2 based supports, controlled pore glass (CPG) was discovered as a superior solid support, which was fortunate, as the preparation of functionalized silica gel was long and tedious.[111] The CPG offered superior and consistent flow of reagents and was easily functionalized without inconsistent and problematic swelling, as was the case with other polymer supports.[111] CPG and highly crosslinked polystyrene beads quickly became the solid supports of choice by the late 1980's and early 1990's, owing to their use in the synthesis of long, high yielding oligonucleotides in combination with the phosphoramidite method.[81] Thus, it took approximately 20 years of intensive research for an appropriate coupling method and solid support to be discovered to permit the successful solid phase synthesis of oligonucleotides.

The covalent attachment of the oligonucleotides was not extensively investigated during the development of solid supports. However, it was known that the length, rigidity, and hydrophobicity of the linker arm significantly affected coupling efficiency.[112,113] Eventually, the long-chain alkyl amine (LCAA)–succinate linker was found to be one of the most effective for DNA and RNA synthesis on the majority of the supports investigated, including CPG.[81] Presently, the majority of solid supports conjugate the 5′- or 3′-hydroxyl of the terminal nucleoside *via* a succinate ester as shown in Figure 1.10.

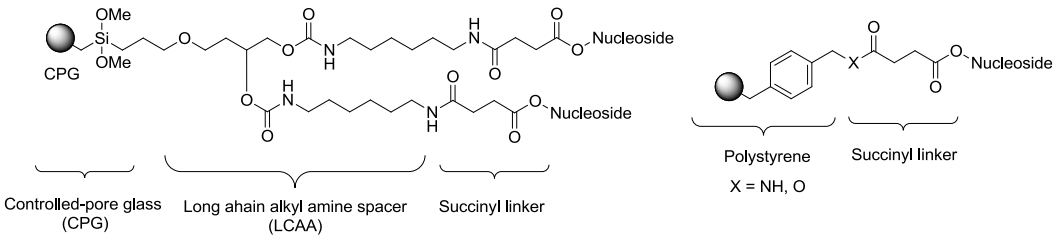


Figure 1.10. Standard CPG and polystyrene solid supports used in oligonucleotide synthesis.

The basic conditions that are used to cleave the nucleotide protecting groups post-oligonucleotide synthesis are often strong enough to simultaneously remove the oligo from the solid support. For some applications, however, the oligo may need to be retained on solid support (e.g., DNA microarrays), or cleaved at a different point of the synthesis or deprotection process. A few of these linkers are shown in Figure 1.11 categorized by the conditions necessary for oligonucleotide release. The majority of the linkers, particularly those containing ester functionalities, are of course cleavable by the standard ammonolysis, but may also have a secondary mode of oligonucleotide release.

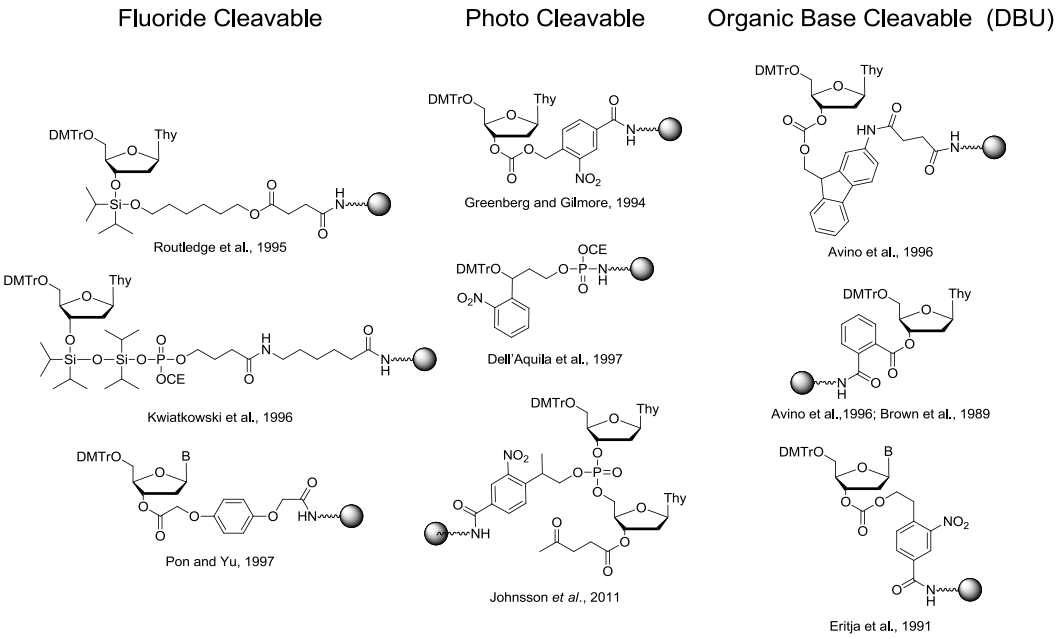


Figure 1.11. Selected linkers cleavable by fluoride, photolysis or organic base.

1.8 Thesis objectives

With oligonucleotide based therapeutics on the horizon there is a great need to develop approaches that produce these molecules on a large scale in a cost efficient manner. Over the last 30 years the solid supported method has become the method of choice for DNA and RNA synthesis due to the simplicity of work up and isolation of the target molecules. While significant improvements have been made in the large scale solid phase synthesis and purification of oligonucleotides,[30,31,114-116] solution phase synthesis offers the ability to significantly increase the scale of the reactions without using costly and specialized equipment.

In this thesis we will investigate alternative methods to produce oligoribonucleotides in solution using the phosphoramidite approach, which has not been extensively studied as a solution phase method. Our goal is to use the phosphoramidite method in conjunction with soluble ionic supports to develop a method that rivals the well-established solid phase method both in terms of yield and purity of the target oligonucleotides. Thus, Chapter 2 describes our attempts to use trialkylphosphonium ionic tags for gram scale synthesis of short RNA oligomers in solution. Satisfyingly, the method produced 2-8 nt sequences in comparable yield and purity to products generated using standard automated synthesis techniques. It also provided a convenient route to characterize (spectroscopic and MS techniques) oligonucleotides during chain assembly as well as straightforward, precipitation based purifications of the intermediates during chain elongation. We also describe the difficulties encountered and limitation of this approach.

In Chapter 3 and 4 we investigate the preparation and use of dimer and trimer building "blocks" for RNA synthesis. The goal of this work is to develop efficient procedures for the synthesis of isomerically pure RNA both in solution and on solid support, by eliminating the problem of 2′- to 3′-*O*-silyl migration during synthesis of the starting materials. The blockmer approach has the advantage of improving speed, purification and cost for producing RNA oligomers.

Chapters 5 and 6 will describe the development of two orthogonally cleavable linkers intended to facilitate the preparation of oligonucleotide block phosphoramidites in solution. The linkers developed were a photocleavable 2-(2-nitrophenyl) propoxycarbonyl (NPPOC) derivative and a γ-keto ester (Lev) linker susceptible to removal by treatment with hydrazine. Various routes to these linkers will be explored and several other routes will be proposed. It will be shown that both linkers can be removed easily from DNA and RNA nucleosides that are attached to an ionic tag or solid support, in a way similar to that which is used for the NPPOC and Lev protecting groups. It is shown that the NPPOC linker allows for the complete synthesis, rapid deprotection and photo-induced release from the support. Similarly, the levulinyl linker makes it possible to a) fully deprotect RNA while it is still attached to the solid-support, releasing the oligonucleotide by a mild hydrazinolysis step; or b) cleave a protected RNA strand for further post-synthesis modification. We foresee that these linkers will find use in RNA synthesis, post-synthesis 3'-conjugation of oligonucleotides assembled on solid-phase or ion tag, and synthesis of oligonucleotides containing base sensitive moieties along the sugar-phosphate backbone (e.g., oligonucleotide pro-drugs).

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Chapter 2. Solution Phase Synthesis of Oligoribonucleotides

2.1 Introduction

The discovery that RNA interference[1] (RNAi) can be harnessed for targeted gene silencing using short synthetic double stranded RNA has resulted in an explosion of research focused on the development of RNA-based drugs for treating a spectrum of disease from cancer to chronic viral infections.[2] These activities have created a demand for synthetic siRNA comprising of 19-23 nucleotides in substantial quantities,[3] a need that is difficult to be met by current approaches in a cost effective manner. Thus, we can foresee that there will be a high manufacturing demand once these products undergo late-stage clinical trials and particularly after they reach the market. While the synthesis of DNA has become routine and cost-efficient, there are a lot of challenges associated with large-scale RNA production.[3-5]

Currently almost all synthetic oligonucleotides are produced via solid-supported synthesis. Over 40 years of dedicated research in this field have made it possible to make this approach a fast, reliable, high yielding, and fully automated method.[6] From its initial conception significant improvements continue to be made with respect to solid supports, solvents, protecting groups, phosphitylating reagents, activators, coupling times, and a multitude of deprotection conditions designed to yield more product.[7] It is likely that besides mechanical advances, there is very little to improve upon chemically, and any further improvement will produce only marginal gains. The limitations of the solid phase approach are fundamental to the nature of the method itself, where the scale is limited primarily by the nature of the solid support. The maximum surface functionalization density is between 250-350 μmol/g for polystyrene solid support and far less (30-100 μmol/g) for controlled pore glass (CPG)[5]. Therefore, the physical amount of solid support becomes prohibitively large and very specialized equipment becomes necessary. In order to produce 1 kg of pure material the synthesis should be split into three parts to reduce the risk of a

possible failure.[8] For example, the maximum scale attempted ranges from 50 to 100 mmol, which yields over 300 g of a typical RNA strand (21-mer; ~6000 g/mol) in approximately 50-60% yield. Another disadvantage of solid phase synthesis is the large quantity of solvent used, reaching at times 20-30,000 L of acetonitrile for every 5 mmol of 21-mer produced. This translates to approximately 400,000 L of solvent when 300 g of a typical siRNA strand is produced.[8] One last limitation of the method that should be mentioned is the high cost of the solid support itself, which may account for up to 40% of the overall synthesis cost.[4,9,10]

An alternative method is homogeneous solution-phase synthesis on soluble supports. This process works on the principle that a molecule of interest is covalently attached to a moiety that serves to selectively "pull" it out from solution leaving behind all other reagents and reactants. This method combines the advantages of standard homogeneous solution chemistry with the ease of purification that accompanies solid support synthesis. It has been applied to the synthesis of small molecules,[11] oligosaccharides,[12] oligopeptides,[12] and only in a few cases to oligonucleotides.[13-18] Amongst some of the benefits of using soluble supports are the significantly reduced amounts of organic solvents and support needed, as well as the ability to use reaction vessels that are ubiquitous to the standard manufacturing of fine chemicals. As a comparison, 1 g of CPG solid support is expected to yield 0.65 g of a 21-nt oligomer, whereas the same mass of soluble support would produce 15-20 g of the same oligomer.

The success of soluble supports is dependent on the simplicity, cost effectiveness, and their ability to selectively purify the molecule of interest without the use of chromatography. A variety of soluble supports have been developed with some interesting but limiting properties (Figure 2.1).[16]

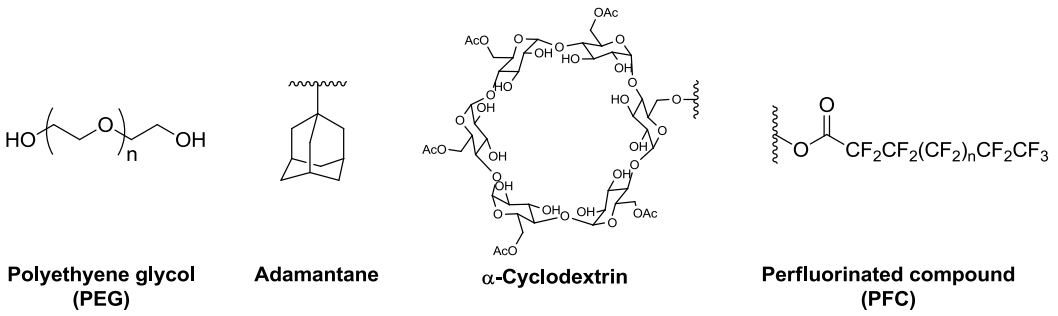


Figure 2.1. Structures of commonly used soluble supports.

Despite its limitations, the most commonly used soluble support is polyethylene glycol (PEG).[15,19-21] PEG has a high molecular weight and only 2 sites of functionalization which severely limits the loading capacity of the polymer. Additionally, temperature cycling is required to encourage precipitation of the polymer which is a significant and costly drawback of this material.[11]

Adamantane[22] and cyclodextrins[23] have been used in place of PEG to overcome the loading limitation, but temperature cycling, thermal instability, and the use of chromatography limit their effectiveness.[24,25] A particularly versatile method is to functionalize the molecule of interest with a long perfluorinated carbon chain.[26] Thus, its isolation is achieved by passing the reaction mixture over a fluorinated solid support which retains the molecule of interest and is later recovered by extraction with perfluorinated solvents.[27] The advantages of this "fluorous phase" synthesis approach are great for complicated reaction mixtures but a major drawback is the high costs of the perfluorinated solid support and solvents making it currently prohibitively expensive on an industrial scale.

Recently, Chan and co-workers introduced low molecular weight ionic tags as supports for solution phase synthesis.[11,12,28,29] A catalyst, or the reagent, or the substrate, can be covalently bound to an organic soluble ionic compound (the ionic tag). The homogeneous reaction involving the ion-tagged species is usually carried out in a relatively polar solvent such as acetonitrile. After the reaction, the ion-tagged species are easily separated from the other reagents by precipitation with a less polar solvent such as ethers or hexanes. In the case of ion-tagged substrate, the ion tag is cleaved after the reaction (or sequence of reactions) to

give the desired product, very often without the need for further purification. Compared to insoluble solid support, "ion-supported synthesis" offers the advantages of homogeneous reaction conditions, easier monitoring of reaction processes and easier scalability to large-scale synthesis. The Chan group has demonstrated examples for the use of ion-supported species as catalysts, reagents and substrates.[11,30]

Advantages of ionic soluble supports over other soluble supports are their high thermal and chemical stability, high loading capacity, and no measurable vapor pressure; this makes them superior to the "fluorous phase" in not contributing to the "volatile organic contaminant" burden in the environment. Most importantly, they have easily tunable solubility by virtue of their greater structural variations (Figure 2.2). This allows for specific separation of the molecule coupled to the ionic tag over all other reagents in ethereal or other non polar solvents without the use of column chromatography. Thus, no special apparatus, exotic solvents or temperature cycling are necessary when ionic tags are used. Many of these ionic organic salts are also known to be non-toxic and relatively cheap to produce or purchase (Cytec industries). These properties should make ionic soluble supports of particular interest to the manufacturing industry.

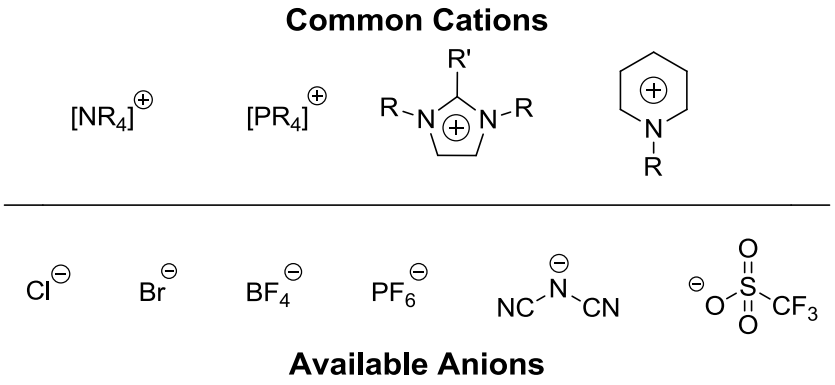
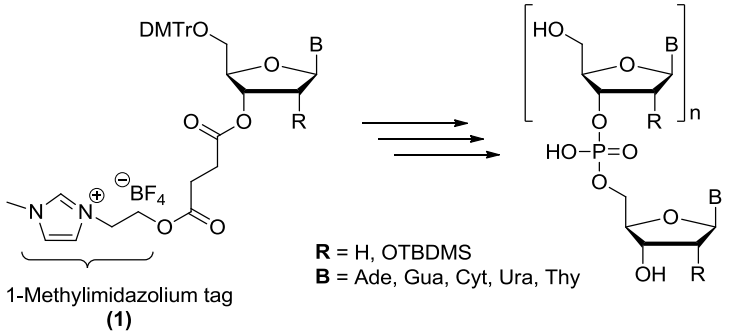


Figure 2.2. Common core cations and anions used as ionic liquids and ionic tags

Recently our research group has been interested in applying the ionic tags as soluble support for the synthesis of oligonucleotides. Initial studies were conducted by Dr. Robert Donga of our laboratory in collaboration with Prof. Tak-

Hang Chan, where the 1-methylimidazolium ionic tag **(1)** was tested as a soluble support for the synthesis of short DNA (4-nt) and RNA (5-nt) oligomers (Scheme 2.1).[16,17] The imidazolium tag was found to be stable to all conditions of the oligonucleotide synthesis cycle, such as detritylation (3% TFA in DCM), phosphoramidite coupling (DCI activator in acetonitrile), and iodine/water oxidation. Detritylation with TFA was problematic, however, requiring several acid treatments to effect removal of the 5′-*O*-DMTr protecting group. When a stronger acid such as benzene sulfonic acid was used, its complete removal was not possible, and the remaining acid interfered with subsequent steps.[17]

The method demonstrated important advantages, especially for the synthesis of chemically modified oligomers which contain un-natural or expensive monomeric units. Specifically, and in contrast to the solid phase synthesis, a great excess of nucleoside phosphoramidite monomer during coupling reactions was not required. While it was clear that the imidazolium-supported synthesis was compatible with many of the methods developed for efficient oligonucleotide synthesis, these studies were limited to tetra (DNA) and pentanucleotide (RNA) sequences. Yields of each of the chemical steps in the tetra and pentamer synthesis were comparable to those of normal solution phase reactions. The added polarity of the imidazolium tag allowed for the selective precipitation of the growing oligomer from diethyl ether or methyl *tert*-butyl ether at every reaction step in the cycle over all other reactants and reagents used. This afforded the pure tagged oligomers without resorting to any other purification methods.



Scheme 2.1. Imidazolium tagged monomer phosphoramidite coupling.

However, the imidazolium tag has some limitations. For example, the tagged nucleoside was not soluble in acetonitrile when chloride or bromide was used as the imidazolium counter ion. Exchange to a softer anion such as tetrafluoroborate or hexafluorophosphate was necessary for the monomer to be dissolved in acetonitrile, which is costly and time consuming. Purity of the starting tagged nucleoside is of high importance; it must be separated from the coupling reagent, excess imidazole, and other by-products before the start of the oligonucleotide synthetic cycle. This requires column chromatography, which in the case of the very polar imidazolium tagged mononucleoside was not possible. Nevertheless, these important and thorough studies served as an excellent starting point to explore other ionic tags for the synthesis of target RNA sequences.

In this chapter we report on the design and preparation of new ionic organic soluble supports and their application in the solution phase synthesis of oligoribonucleotides. Among the soluble supports we tested, the tributylphosphonium bromide showed the best results. We attached a nucleoside to this phosphonium tag through a succinyl linker, and used the resulting material as a starting building block for the synthesis of a decaribonucleotide. This sequence, corresponding to a 10-nt segment of the "sense" strand of a siRNA duplex, targets the firefly luciferase protein (Figure 2.3). Some of the issues that impeded the growth of the full length 21-nt oligonucleotide will be addressed later in this chapter.

Figure 2.3. Decaribonucleotide target for ion-supported synthesis.

The protocol we developed followed the general steps of the solid phase cycle, introducing small modifications to make this method suitable for solution phase synthesis. The new method afforded gram quantities of the ribonucleotides with purity and yields attainable by solid phase synthesis. Furthermore, we discuss the characterization as well as the challenges of elongation we

encountered during the synthesis of this oligoribonucleotide. The synthesis was carried out in collaboration with Dr. N.M. Reddy of our laboratory.

2.2 Results and Discussion

2.2.1 Synthesis and general properties of the phosphonium ionic tag

Driven by our interest in using ionic tags to replace solid supports in the synthesis of oligonucleotides, we investigated several ionic tags and assessed their suitability for ion-supported oligonucleotide synthesis (Figure 2.4). Initial motivations were based on finding an ionic liquid that could be converted into an ionic tag capable of circumventing some of the limitations of the imidazolium ionic tag, previously developed in our lab.[17]

The first concern was to address the solubility of the ionic tag in acetonitrile while maintaining its ability to be precipitated in ethereal solvents. The imidazolium chloride tag **(1)** was much too polar, making it poorly soluble in acetonitrile. In order to increase the solubility in acetonitrile, a tag with more lipophilic properties would be necessary. After some investigation we discovered that tributylammonium and tributylphosphonium ionic tags were best suited for their stability and low cost. In Figure 2.4 we show the different ionic tags prepared for our studies.

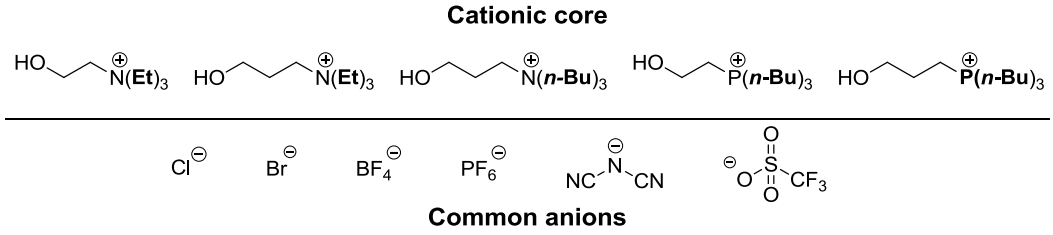


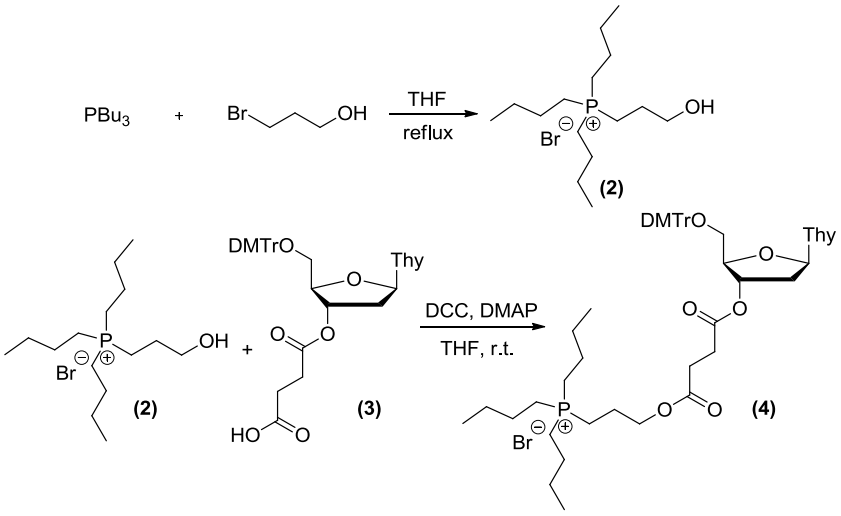
Figure 2.4. Ammonium and phosphonium ionic tags.

The ammonium tags were our initial choice due to low cost and availability. However, we discovered that, compared to the phosphonium tags, they undergo β-elimination more readily under alkaline conditions. Additionally, the higher polarizability of phosphorus allows for greater solubility of the phosphonium tags in a wider range of solvents.[19,27] Furthermore, phosphonium tags have higher thermal stability relative to ammonium derivatives (decomposition of

phosphonium tags occur above 250 °C, or ca. 100 °C higher relative to analogous ammonium derivatives).[20,21] As a result of these initial considerations attention was focused on the phosphonium species.

As shown below, the tributylphosphonium tag had all of the desirable characteristics necessary for solution phase oligonucleotide synthesis. It also had a few distinct advantages over the imidazolium tag previously used. The phosphonium salts alone and their nucleoside conjugates were easily purified by traditional column chromatography due to their increased lipophilicity. Additionally, the anionic counterion did not have any significant impact on the solubility in acetonitrile (chloride vs. triflourosulphate). This is a significant advantage over the imidazolium tag **(1)** as anion exchange is costly, wasteful, and time consuming. Lastly, the phosphonium salts were not soluble in ethereal solvents, allowing for selective precipitations in these solvents.

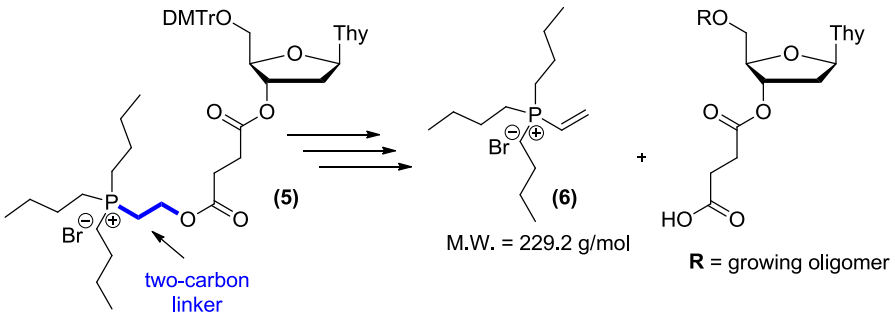
The synthesis of phosphonium species is generally achieved by a nucleophilic substitution (S_N2) of an alkylhalide by a tertiary phosphine.[21,31,32] We chose tributylphosphine and reacted it with an excess 3-bromopropanol in THF under reflux, to quantitatively form tributyl(3-hydroxypropyl)phosphonium bromide **(2)**, (Scheme 2.2). The hydroxyl group that was introduced at this step, provides for a chemical handle from which the oligonucleotide synthesis could start.



Scheme 2.2. Synthesis of tributyl(3-hydroxypropyl)phosphonium bromide **(2)** and subsequent coupling to succinylated thymidine.

The phosphonium tag **(2)** was condensed to a succinylated thymidine derivative **(3)** using standard coupling conditions (DCC and catalytic DMAP). The resulting ionically tagged thymidine monomer **(4)** was significantly more polar than the starting nucleoside and excess DCC and DCU by-product, making it possible to isolate **(4)** in high purity by passing the reaction mixture through a short silica gel column. Although the goal of ion-supported synthesis is to use precipitation as the sole purification method during the oligonucleotide synthesis, as demonstrated below, silica gel purification of the ion-tagged monomer was necessary to ensure absolute purity of this key starting material. By contrast, imidazolium tag **(1)** did not allow for silica purification after nucleoside coupling, requiring multiple precipitations and aqueous extractions at this stage to achieve a high degree of purity.[16]

Initially, ion-tagged monomer **(5)** (Scheme 2.3), which contains a two-carbon (ethylene) linker instead of the 3-carbon linker present in **(4)**, was prepared. However, **(5)** was found to undergo elimination during assembly of the oligonucleotide chain, as ascertained by the detection of compound **(6)** (MS 229.2 m/z) in oligomer samples isolated after each coupling step. Switching to the longer propyl linker appeared to eliminate this side reaction completely.

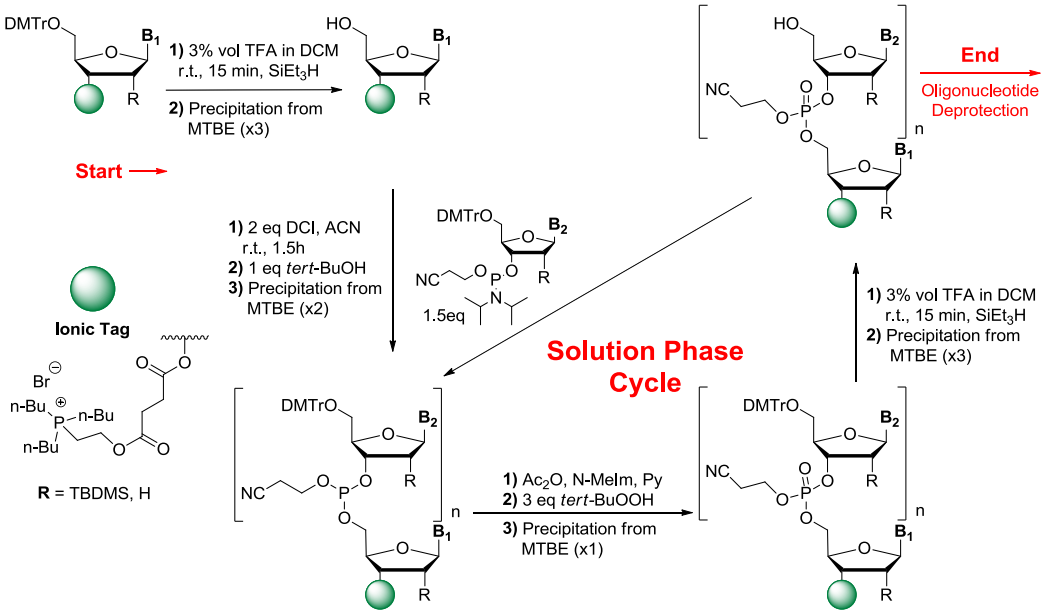


Scheme 2.3. Elimination reaction at the phosphonium linker during oligonucleotide synthesis leading to the formation of side product **(6)**.

2.2.2 Solution phase synthesis of oligoribonucleotides via the phosphonium tag

To test if the phosphonium tag could be used in solution as an analogue to a solid support we next attempted the synthesis of an oligoribonucleotide. The strategy follows the same steps of the solid-phase synthesis cycle, i.e.,

detritylation, phosphoramidite coupling, capping, and oxidation of the newly formed phosphite triester internucleotide linkage to the more stable phosphate triester. The target sequence is grown from the 3′ to the 5′ end by the stepwise addition of phosphoramidite monomers. The cycle of the stepwise addition of nucleotide units to the growing oligomer (with modified reaction conditions) is shown in Scheme 2.4. The various improvements and refinements are discussed in the sections below.



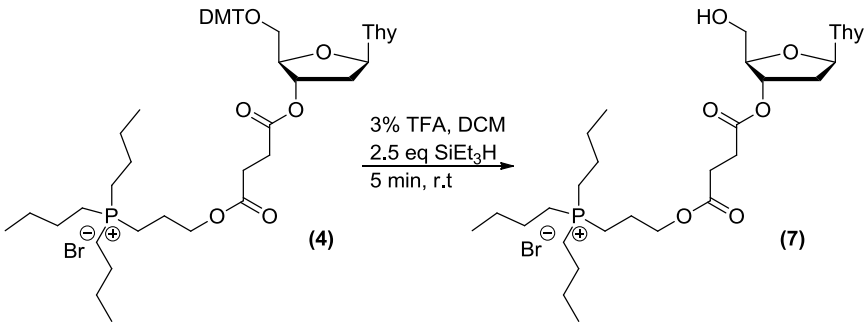
Scheme 2.4. Phosphonium tagged solution phase oligonucleotide synthesis cycle.

To determine whether the phosphonium tag was compatible with synthetic protocols developed for oligonucleotide synthesis, the 3′-terminal dTT fragment was assembled first.

2.2.2.1 Detritylation of compound (4)

The first step of the oligonucleotide cycle is the detritylation of the starting nucleoside to yield a free 5′-hydroxyl group for subsequent phosphoramidite coupling (Scheme 2.5). The starting monomer was the tributylphosphonium tagged 5′-*O*-DMTr thymidine **(4)**. Standard trityl deprotection conditions were used, whereby **(4)** was treated with 3% TFA in dichloromethane for 5 minutes. The presence of the phosphonium tag allowed for purification of the reaction

mixture by direct precipitation into methyl *tert*-butyl ether (MTBE) to afford phosphonium tagged 5′-OH thymidine **(7)**. However, the product obtained after the precipitation contained approximately 5-10 % of tritylated starting nucleoside **(4)**. This was eliminated only after 4 repetitions of the TFA treatment. The difficulty in removing the DMTr group can be explained by the reversibility of the detritylation reaction. The acid mediated removal of DMTr is an equilibrium reaction, where the forward reaction is strongly favoured by the excess acid used. The cleavage of the trityl group under acidic conditions leads to the formation of a trityl cation, which can react with the 5′-OH of the deprotected nucleoside (competing S_N1 reaction) to form the starting DMTr protected nucleoside. This is very problematic, as at every step of the oligonucleotide synthetic process, the 5′-*O*-protecting group must be completely removed, or otherwise undesired short-mers are created, making the purification step of the full length product very difficult and low yielding.



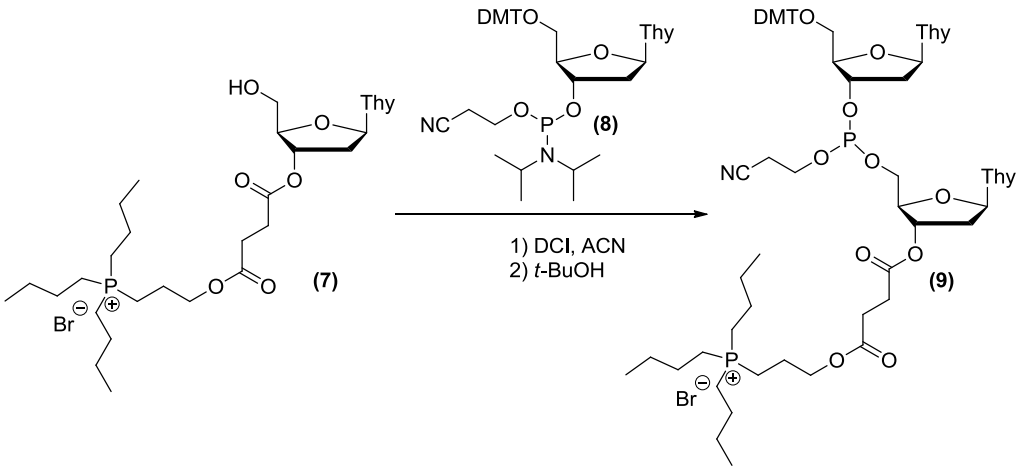
Scheme 2.5. Detritylation of the starting phosphonium tagged monomer **(4)**.

The trityl re-association problem is not generally encountered in solid-phase synthesis because the acid solution flows through the column containing the solid support-coupled nucleoside continuously removing the trityl cation as it is generated. In an attempt to remedy the re-tritylation of the nucleoside, dry methanol was added to the reaction mixture to quench the trityl cation formed. Even under these conditions, detritylation of **(4)** still required 3 acidic treatments and methanol quenching in order to maximize the yield of **(7)**. As a result of these complications alternative methods were investigated.

In the past, 1-H-pyrrole has been used successfully as a cationic scavenger.[33] Limitation to this method is the unavoidable polymerization of pyrrole that occurs under acidic conditions, which could cause major problems particularly on a large (e.g. industrial) scale. Alternatively, the mild reducing agent triethylsilane (a hydride donor) has been shown to quench the trityl cation[22] or speed up the acid catalysed hydrolysis of *tert*-butyl ester derivatives.[34] We implemented the use of triethylsilane in our deprotection procedure with success (Scheme 2.5). Here, two and half equivalents of triethylsilane were sufficient to rapidly quench the trityl cation, requiring only two brief (15 min) treatments with 3% TFA solution in the presence of triethylsilane to completely eliminate the re-tritylation reaction. Purification of the product after each TFA/Et_3SiH treatment was achieved by precipitation from MTBE. The resultant white precipitate was filtered over Celite and recovering the precipitate from the filter by dissolving in THF. Interestingly, the repeated TFA/Et_3SiH treatments were only necessary at the dimer and trimer stage; once the length of the growing oligonucleotide is above three units, the detritylation was complete after only one TFA treatment. This may be ascribed to the proximity of the ion tag which makes protonation of the 5′-*O* rate limiting step.[16,17]

2.2.2.2 Phosphoramidite coupling step

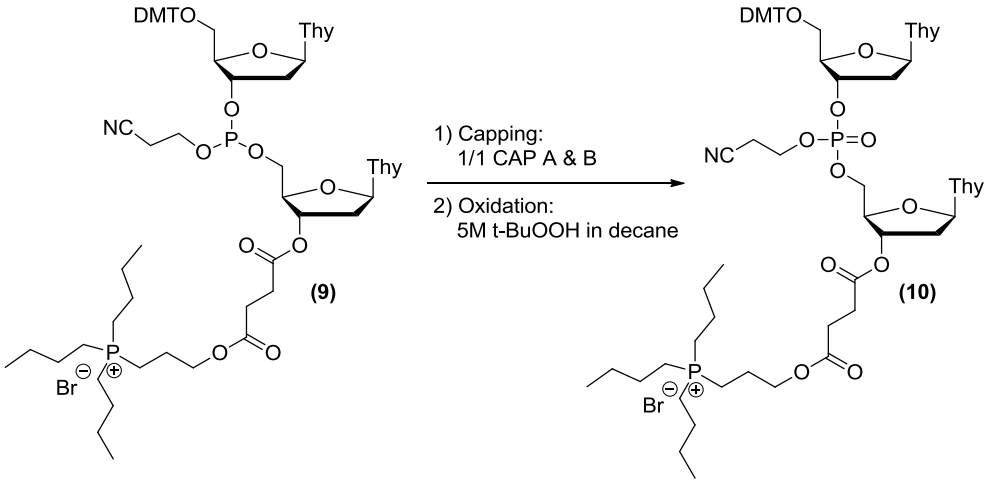
The next step in the oligonucleotide synthesis is the coupling of the free 5′-hydroxyl group to a nucleoside 3′-*O*-phosphoramidite unit. To accomplish this, phosphonium tagged thymidine **(7)** was reacted with 5′-*O*-DMTr-thymidine 3′-*O*-phosphoramidite **(8)** (1.5-3 equivalents) using 4,5-dicyanoimidazole (2-3.5 equivalents) as an activator under standard coupling conditions (Scheme 2.6).



Scheme 2.6. Coupling of thymine phosphoramidite **(8)** to the 5′-hydroxyl group of the phosphonium tagged thymidine **(7)**.

The progress of the reaction was readily monitored by electrospray ionization mass spectrometry (ESI-MS) since the presence of permanent positive charge on the phosphonium tag gives rise to a very strong MS signal. After stirring for 3 hours, dry *tert*-butanol (10 equivalents) was added to quench the excess phosphoramidite and enhance the solubility of the resulting 5′-*O*-DMTr-thymidine 3′-*O*-(*t*-butyl-2-cyanoethyl)phosphite triester by-product in the precipitation medium (MTBE). As before, the intermediate dTT dimer **(9)** was isolated and purified by adding the reaction mixture dropwise to stirred MTBE. The resultant precipitate was filtered over Celite® and recovered from the filter by dissolving in THF yielding **(9)** in 93 % yield.

2.2.2.3 Capping and oxidation



Scheme 2.7. Capping and oxidation of dimer phosphite triester **(9)**, where CAP A is 40% Ac_2O in 1/1 pyridine/THF and CAP B is 16% N-methylimidazole in THF.

In standard solid-phase synthesis capping of the unreacted 5′-hydroxyl group is necessary in order to avoid the formation of shorter ("failure") sequences during subsequent coupling steps. Given the high yield of the coupling reactions, as assessed by oxidizing a small sample of dTT dimer and analyzing by HPLC after deprotection (see below), capping may not be necessary for the synthesis of short oligomers (e.g., 2-6 nt). To be safe, however, we chose to cap after each chain elongation step with a equal amount of acetic anhydride:pyridine:THF, 1:2:2 ratio ("Cap A") and N-methylimidazole (16% v/v in THF; "Cap B") (Scheme 2.7). Following stirring for 10 min, the oligonucleotide was again isolated via precipitation from MTBE.

Capping is followed by oxidation of the phosphite triester linkage to the more robust phosphate triester linkage. Traditionally, the reagent of choice for this step is an iodine-water mixture in THF/pyridine solution. It is a fast, reliable, and very cheap method for oxidation. However, this method is incompatible with our purification strategy due to the immiscibility of water and MTBE. Instead, the oxidation was performed by reacting **(9)** with *tert*-butyl hydroperoxide (5 M solution in decane), and precipitating the resulting phosphate triester **(10)** in MTBE. When incomplete oxidation was evident, as assessed by ^{31}P-NMR, the material was again treated with oxidizing agent; this is crucial since phosphite

triester linkages are not stable to the acidic conditions of subsequent detritylation steps.

Dimer phosphate triester **(10)** was further subjected to the four steps described in the previous sections (detritylation, phosphoramidite coupling, capping, and oxidation). At each step the purification of the growing oligomer was achieved by simple precipitation from MTBE (Figure 2.5). The synthetic cycle was repeated until the 10-nt oligonucleotide sequence was created, after which deprotection of the 5′-*O*-DMTr group was performed.

Figure 2.5. Precipitation of phosphonium-tagged oligonucleotide from MTBE and subsequent filtration over Celite®.

A noteworthy issue that was encountered during the synthesis was that of solubility of longer oligomers. As the growing oligonucleotide gained mass, and the number of moles stayed the same, it became increasingly difficult to dissolve the molecule in required amount of ACN to maintain the proper molar concentration. At the 10mer point (5′-rUUAAUUAAdtt-3′) it was required to dissolve 3.1g of the oligomer, 1.0g of the rU amidite and 0.2g of DCI in only 9.6 mL of ACN. This proved to be impractical, as significant manipulation, sonication and agitation was necessary to solvate all the reactive species. Often the DCI was not completely dissolved and visibly suspended in the viscous reaction mixture. This was an unexpected problem. To overcome this issue a 15% dimethylformamide (DMF)/ACN mixture was used as the solvent for the coupling reaction.

Table 2.5 summarizes the stepwise synthesis of the growing oligonucleotide using the general procedure described above. Amidites used, coupling reagents,

conditions, and yields are presented. A great advantage of the solution-phase method is that it can be readily carried out in the 1-2 mmol scale, providing gram quantities of the intermediates. Furthermore, as pointed out previously, great excess of phosphoramidite monomer was not required during coupling reactions compared to solid phase synthesis. This is an important advantage especially for the synthesis of chemically modified siRNA and antisense oligonucleotides which generally contain un-natural or expensive monomeric units.

Starting Comp.	Mass (g)	St. mat. (mmol)	mmol of amidite	Amidite (g)	ACN (mL)	DCI (mmol)	DCI (g)	Rex. Time (h)	% yield
t-OH	1.78	1.74	4.35	3.24 (dt)	29.0	5.22	0.62	3	65.0
tt	2.52	1.55	3.89	3.84 (rA)	25.9	4.66	0.55	3	93.4
ttA	3.61	1.62	4.06	4.01 (rA)	27.1	4.87	0.57	3	102.7
ttAA	2.54	0.94	2.36	2.03 (rU)	15.7	2.83	0.33	3	97.3
ttAAU	2.30	0.73	1.81	1.56 (rU)	12.1	2.18	0.26	5	92.7
ttAAUU	2.49	0.66	1.65	1.63 (rA)	11.0	1.98	0.23	12	90.2
ttAAUUA	2.60	0.59	1.49	1.46 (rA)	9.91	1.78	0.21	12 (x2)*	90.0
ttAAUUAA	2.69	0.56	1.39	1.19 (rU)	9.25	1.67	0.20	12 (x2)*	91.0
ttAAUUAAU	1.52	0.29	0.71	0.62(rU)	4.76	0.86	0.10	12 (x2)*	91.0

Table 2.1. Stepwise synthesis reagents and yields of oligonucleotide synthesis *the coupling reaction was repeated to ensure the complete coupling. % yield (far right column) is calculated based on recovered material (by weight). Yield of product was determined from the HPLC analysis of the crude oligomers after deprotection (see Figure 2.6A).

To follow chain growth during synthesis, a small sample was removed after each detritylation step, fully deprotected (General deprotection conditions **A**) and analyzed by ion exchange chromatography (Figure 2.6A). To assess overall synthesis efficiency and purity of the crude oligonucleotides by HPLC, we also synthesized each oligonucleotide intermediate (4-mer to the 10-mer) using standard solid-phase synthesis (Figure 2.6B).

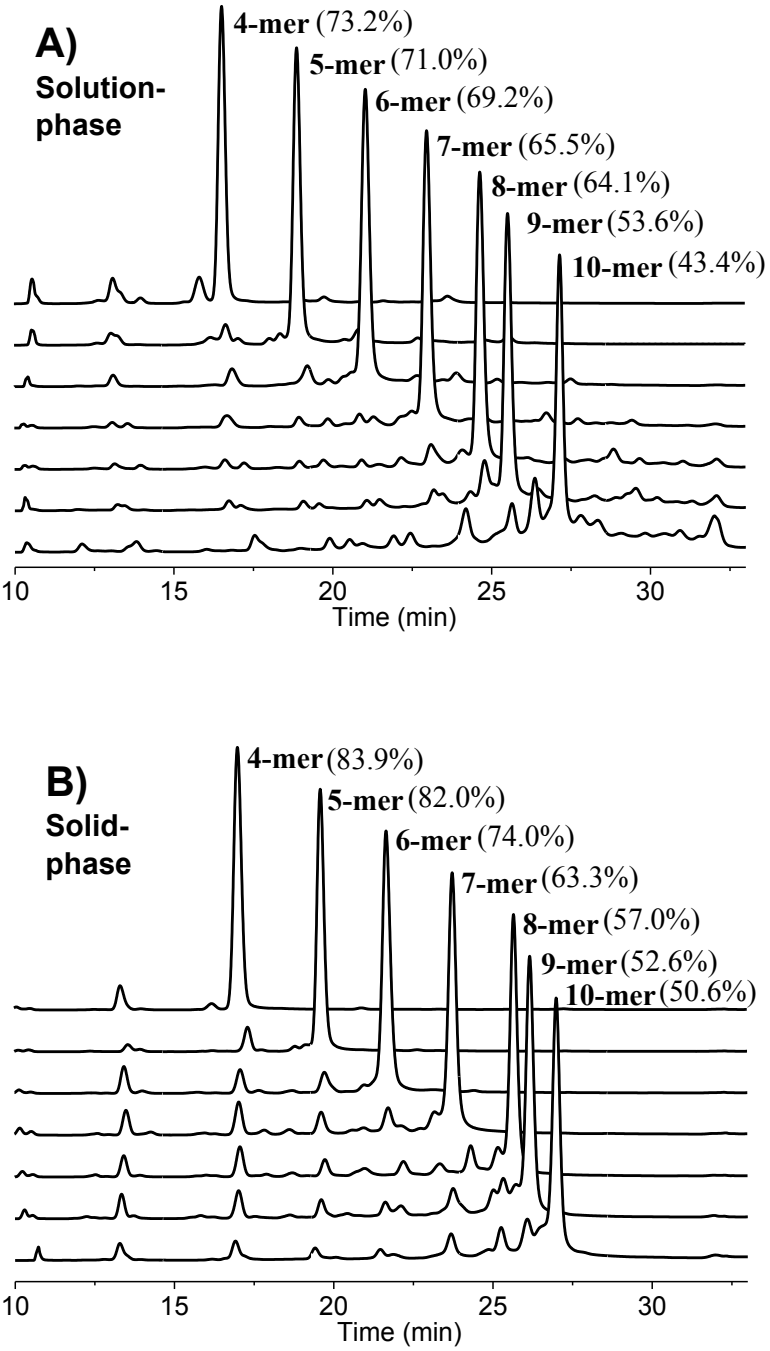


Figure 2.6. Ion exchange HPLC chromatograms of oligoribonucleotides. The percent purity of the full length product, calculated by integration of the whole chromatogram, is shown in brackets. A) Oligomers synthesized by the solution-phase method B) Oligomers synthesized by solid-phase method. All oligomers were deprotected using general deprotection procedure (A).

The percent purity of each target oligomer was calculated from their respective HPLC peak areas. A comparison between the two sets of chromatograms shows that the solution-phase procedure, produces samples that are comparable to the standard solid-support method; for example, for the tetramer, the yields are 73.2 % and 83.9 %, respectively; for the 10-mer, yields were 43.4 % and 50.6%, respectively. The failure sequences (eluting before the main peak) are slightly more abundant for syntheses performed via the solution-phase method. Peaks eluting after the target sequence (referred to as "longmers") were also more pronounced for the solution-phase method and became progressively more abundant as the length of the oligonucleotide increased.

There was some variability from batch to batch preparation; Figure 2.7 shows the HPLC traces of different 6-mer batches prepared by the solution and solid phase methods. In this case, the results are nearly indistinguishable, providing respectively 85% and 88% of the desired product.

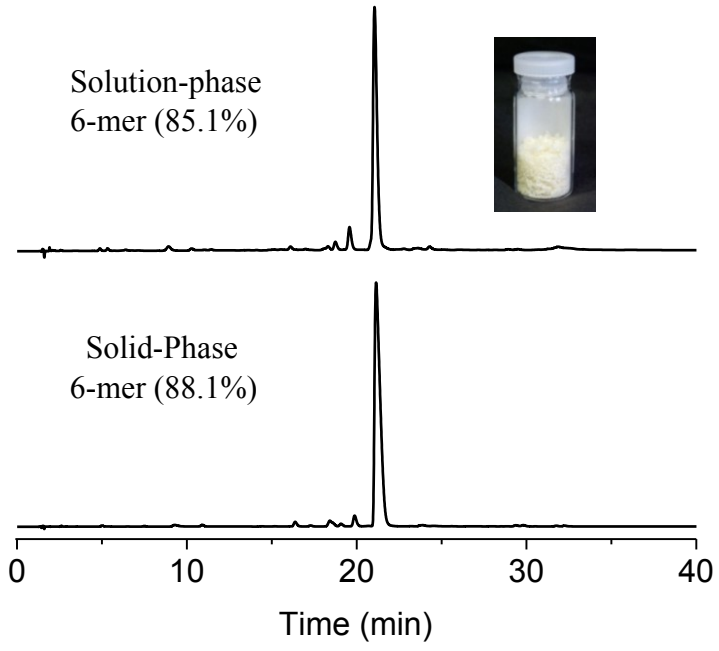


Figure 2.7. Ion exchange chromatograms of hexamer (5′-UUAAtt-3′) prepared by the solid phase (top trace) and solution phase methods (bottom trace). Percent yield of full length product is given in brackets. The pentamers were deprotected using general deprotection procedure (B).

It was clear from the above results, that while synthesis of a 10-nt oligomer works reasonably well, that linear (step-wise) synthesis of longer oligomers would not be able to provide the target oligomer in acceptable yields (50-70%).

2.2.3 Monitoring and characterization of oligoribonucleotides during chain growth

The ability to quickly monitor and characterize reaction intermediates and products by traditional means such as TLC and ^{1}H and/or ^{13}C NMR becomes difficult or impossible as the oligonucleotide becomes larger and more complex. We were able to monitor reactions by TLC up to the tetramer stage. The identification of the integrity of the growing oligonucleotide is of the utmost importance, as each step must go to completion in order to achieve reasonable overall yield of the desired full length product. This leaves only a few characterization methods available for our use. The phosphorus ionic tag allows for two non-destructive techniques to be used: mass spectrometry and ^{31}P-NMR. This is a significant advantage over the solid phase method which does not allow identification of the growing oligomer at each step during chain growth. Traditionally the RNA sequence is analyzed by full deprotection after its synthesis is completed.[35]

2.2.3.1 Mass spectrometry

This is the method of choice for the characterization of ion-tagged oligonucleotides. The permanent positive charge on the molecule provided by the phosphonium tag significantly increases the sensitivity of detection in MS because no ionization of the molecule is necessary before reaching the detector. This high sensitivity allows us to follow the disappearance and emergence of reactants and products with a much higher degree of accuracy than any other method. Thus, we were able to readily monitor the progress of the detritylation, coupling, and oxidation reactions during chain assembly (Table 2.2). The MS instruments available to us had a maximum range of 2000 m/z. This is somewhat limiting as the growing oligomer surpasses this limit at the 4 nt stage (with all

protecting groups on). Nevertheless, from the tetramer onward, multiple charging occurred pulling the analytes back into the observable range, albeit with lower sensitivity. Unfortunately, with the conditions and instruments used, we were unable to detect ions beyond the protected hexamer stage. Despite our own limitations, we believe that MS is the best choice for monitoring oligonucleotide chain growth.

Length (nt)	Sequence (5′ to 3′)	Phosphite triester (m/z)		Phosphate triester (m/z)		Detritylated (m/z)	
		exact mass calc.	mass found	exact mass calc.	mass found	exact mass calc.	mass found
1	t	N/A	N/A	N/A	N/A	585.33	585.4
2	tt	1228.54	1228.5	1244.53	1244.5	942.50	942.51
3	Att	1828.73	1828.8	1844.72	1844.8	1542.59	1542.7
4	AAtt	2428.92	1215.0; 1225.9	2444.92	1223.0; 1233.9	2142.79	1071.8,
5	UAAtt	2902.06	n.d.	2918.06	n.d.	2615.92	1319.5
6	UUAAtt	3375.2	n.d.	3391.19	1696.2	3089.06	1545.1

Table 2.2. MS data of fully protected oligonucleotides.

To confirm the identity of all of oligonucleotides, the samples were analyzed by MS after complete deprotection and desalting. Gratifyingly, the observed masses agreed with the expected (calculated) values (Table 2.3).

Length (nt)	Sequence (5′ to 3′)	Mol. Weight calc. (g/mol)	Mol. Weight Found (g/mol)
4	AAtt	1204.8	1203.2
5	UAAtt	1511.0	1509.2
6	UUAAtt	1817.2	1815.2
7	AUUAAtt	2146.4	2144.3
8	AAUUAAtt	2475.6	2473.2
9	UAAUUAAtt	2781.8	2779.3
10	UUAAUUAAtt	3087.9	3085.4

Table 2.3. HPLC-HRMS data of fully deprotected oligoribonucleotides.

2.2.3.2 ^{31}P-NMR characterization

Alternatively, the terminal phosphonium moiety can be used as a convenient "internal standard" to monitor chain extensions by ^{31}P-NMR. By integrating the signals at ca. 35 ppm. (phosphonium, P′) and ca. 1 ppm. (phosphate triester, P), the chain length is provided by the following equation: 1+ P/P′ (Figure 2.8 and 2.9).

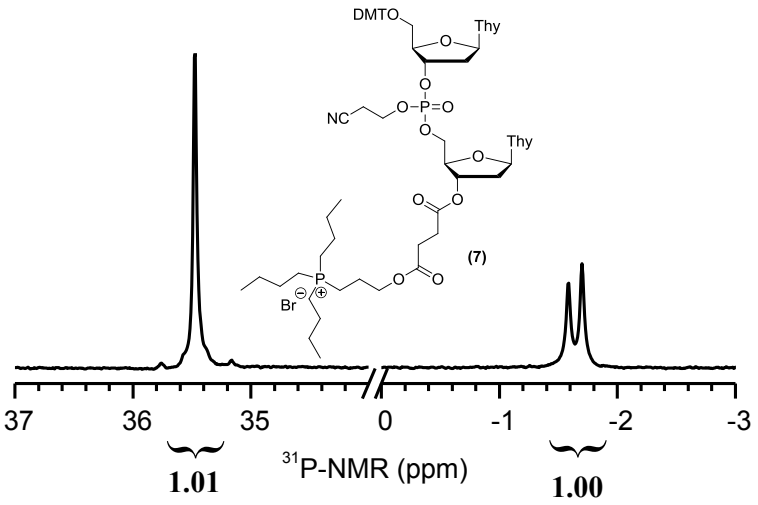


Figure 2.8. ^{31}P-NMR of thymidine dimer **(11)**. The phosphorus signals at -1.6 ppm and 35.4 ppm correspond to the phosphonium and phosphate moieties, respectively. Peak areas (integration) are in agreement with dimer **(7)**; 1 + P/P′ = 2.

All ^{31}P-NMR spectra were collected after each oxidation step (Figure 2.9). To obtain reliable integrations, the NMR spectra were acquired in a way that saturation is avoided by adding a pulse delay between scans allowing for the phosphorus to completely "relax" and signals to be adequately recorded. In general, integration errors caused by saturation effects will depend on the relative relaxation rates of the phosphorus nuclei in the oligomers. Interestingly, as the length of the oligonucleotide grew the length of the delay had to be increased as well. For example, to obtain accurate integration the time delay between pulses was increased to 2 seconds for dimer **(7)** and 3 seconds for the pentamer (the default is 1 second). Additionally, as the length of the oligomer increased the ability to obtain proper ^{31}P-NMR signals became more difficult resulting in broad peaks that did not integrate reliably. Changing the solvent from deuterated acetone to a faster relaxing solvent such as deuterated chloroform did not significantly help the shimming and tuning process.

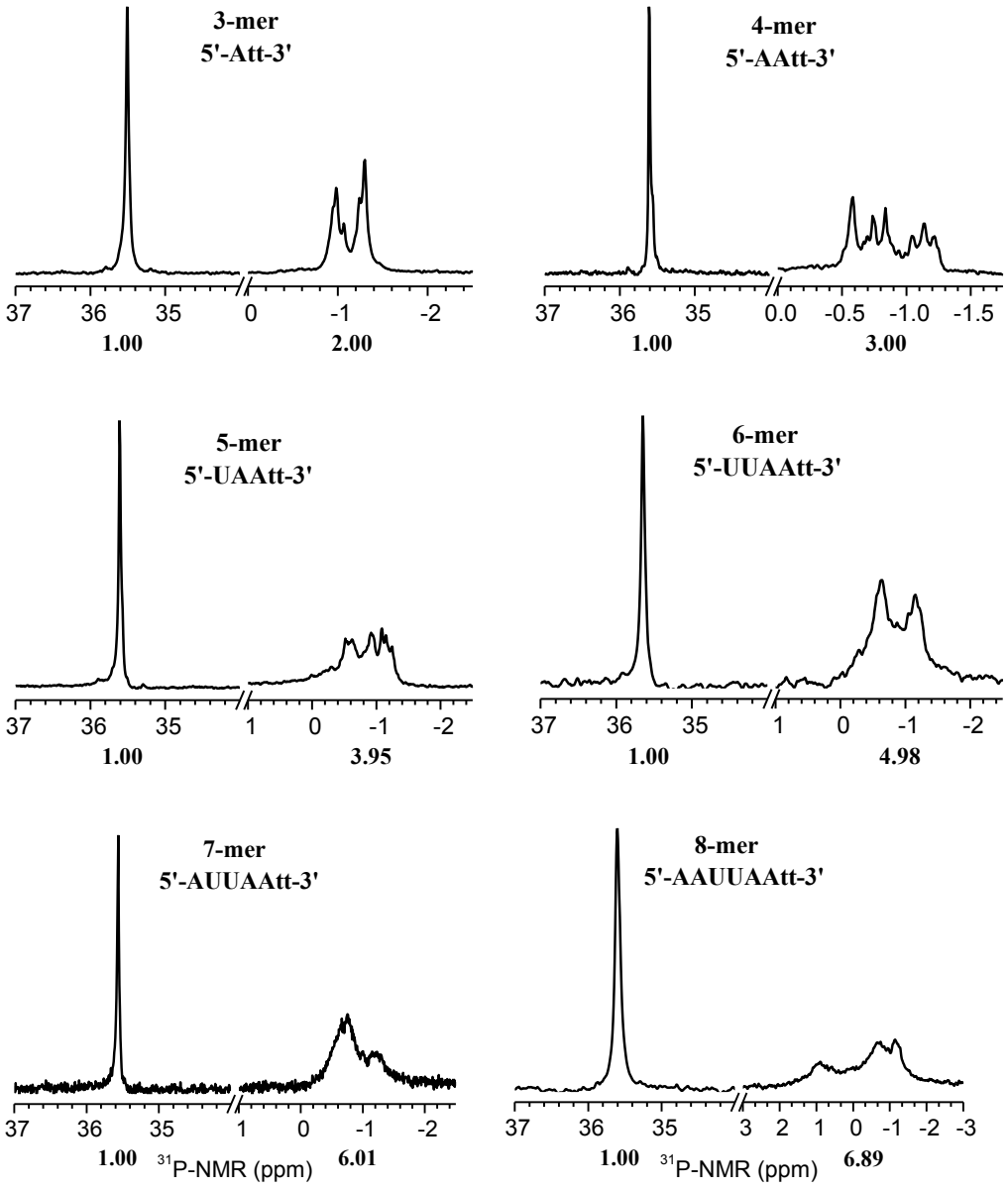


Figure 2.9. ^{31}P-NMR spectra of protected oligonucleotides (2 through 8 nt) providing the expected integration of phosphonium (P′, ca. 35.6 ppm) and phosphate (P; ca. +1 to -2 ppm) signals. The experimentally determined value of 1 + P/P′ correlates well with the expected chain length.

2.2.4 Troubleshooting the solution phase synthesis method

During our initial attempt at the solution phase synthesis, 5% TFA in DCM was used to deblock the 5′-hydroxyl of the growing oligomer. The higher than necessary acidic concentration in combination with lack of a trityl scavenger resulted in oligonucleotide quality below acceptable levels (Figure 2.10).

After the third nucleotide unit was added we noticed that only one treatment of TFA was necessary, in stark contrast to 2-3 treatments for the previous two units. The ease of detritylation continued using only one acid treatment until the 6th and 7th unit. After this length, detritylations required 2-3 treatment of TFA, as was the case for the 2nd and 3rd units. Unfortunately, lack of due diligence in combination with the reduced ability to monitor the reaction process by MS (section 2.2.3.1) resulted in an unacceptably low purity of the reaction mixture and the synthesis was halted at the 12-mer step (Figure 2.10).

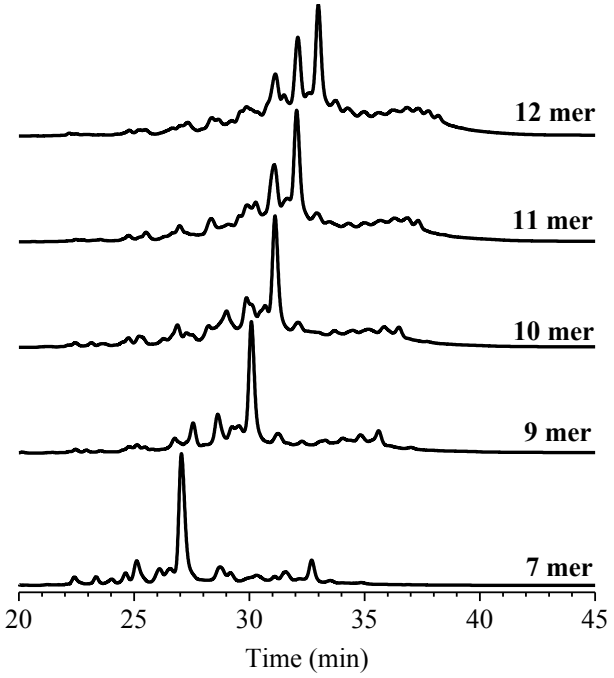


Figure 2.10. Ion exchange chromatograms of the 7mer (5′-AUUAAtt-3′) to the 12mer (5′-CUUUAAUUAAtt-3′) from the first attempt at the solution phase synthesis.

Using longer and multiple treatments of 5% TFA on the oligomer shown in Figure 2.10 resulted in significant increases in the amount of "longmers" seen later in the chromatogram past the full length product. The length of time in the acid media is defiantly problematic. Two potential issues were identified and addressed:

1) Residual TFA remaining after the detritylation step. If the TFA is not completely removed there is risk of multiple successive couplings during addition

of a given phosphoramidite coupling (i.e., coupling, detritylation, and further chain extensions). As a precautionary measure, we introduced an extra MTBE precipitation step after detritylation to ensure that any residual TFA (free or trapped in the solid precipitate) is removed.

2) Partial desilylation during our detritylation. TBDMS protecting groups can be removed under acidic conditions (e.g. 1% aq. HCl); therefore the presence of TFA and moisture may have the same effect (Figure 2.11).

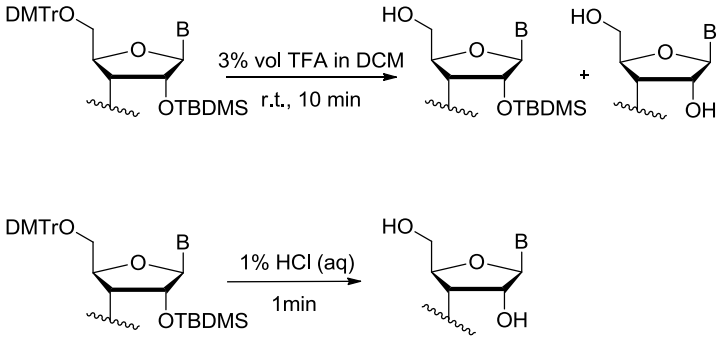


Figure 2.11. The removal of TBDMS group under acidic conditions.

The unmasking of the 2′-hydroxyl at any position within the oligonucleotide would provide additional reaction sites during phosphoramidite couplings leading to "longmers" (Figure 2.12).

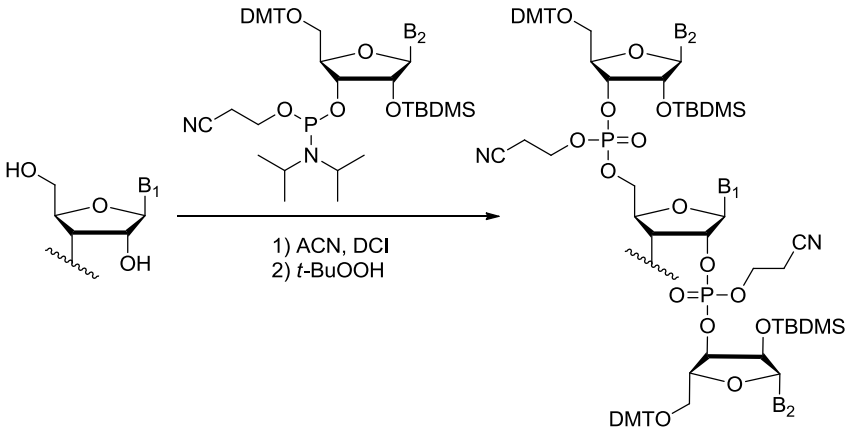


Figure 2.12. Phosphoramidite coupling to the 2′-hydroxyl: creation of longmers.

To minimize this possible side reaction we increased the amount of triethylsilane while decreasing reaction time and concentration of TFA from 5 to

3%. To prevent moisture from entering the reaction mixture, the TFA solution should be stored over 3Å molecular sieves.

2.3 Conclusions

A viable method for the synthesis of short (2-10 nt) oligoribonucleotide sequences with acceptable purity was demonstrated. The goal to use conventional compounds and reagents in combination with our ionically tagged monomer to produce short oligonucleotides in solution was achieved. The phosphonium ionic tag was found to be effective for precipitating the growing oligonucleotide, while being stable to all conditions used in standard oligonucleotide synthesis cycles. Key limitations and points of improvement to the solution phase method have been identified and can be implemented in future experiments.

2.4 Future Work

It has been determined that extended acid treatments during detritylation reactions (at early and late stages of syntheses) are a significant limitation to the solution based approach. An obvious solution would be to use an alternative protecting group that has increased acid sensitivity or that is cleaved under non-acidic conditions. Possible groups to consider in this regard are the pixyl group[36] the benzhydryloxy*bis*-(trimethylsilyloxy)silane,[37] or the levulinyl group.[38] However, the goal of the current research is to produce a viable approach to making oligonucleotides in solution using standard conditions and common reagents. Because our method has shown promise in producing short oligonucleotides (e.g., 2-7 nt), one may invision the assembly of 21-nt RNAs via "block" couplings, such as 7x 3-nt, or 3x 7-nt, rather than the more conventional stepwise synthesis approach (21x 1-nt). This possibility and other advancements will be discussed in the following chapters.

2.5 Experimental

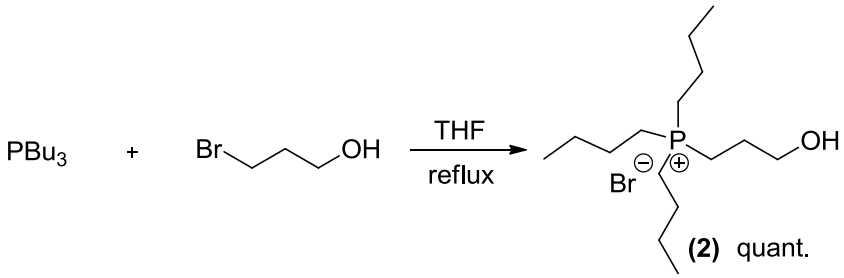
Materials

Thin layer chromatography was performed on EM Science Kieselgel 60 F-254(1mm) plates. Silicycle 40-63 μm (230-400 mesh) silica gel was used for flash chromatography. Pyridine, acetonitrile, and dichloromethane were distilled fromCaH_2 after refluxing for several hours. THF was distilled from benzophenone and sodium after refluxing for several hours. All other anhydrous solvents were purchased from Sigma-Aldrich. Chemicals and reagents were purchased from either Sigma-Aldrich (Oakville, Ontario, Canada). Phosphoramidites were purchased from ChemGenes Inc.

Instrumentation

The solid phase syntheses of oligonuc1eotides were conducted on an ABI synthesizer. UV spectra for oligonuc1eotide quantitation (absorbance measurements) were measured at 260 nm on a Varian Cary I or 300 UV-VIS dual beam spectrophotometer. Anion-exchange HPLC were performed on an Agilent 1200 series machine. Ion exchange column used was a Waters Protein Pack DEAE-5PW column (7.5 mm x 7.5 cm). Purified samples were desalted by size exclusion chromatography on G-25 Sephadex purchased from GE-Healthcare. ^{1}H NMR and ^{13}C-NMR were recorded on a Varian 300, 400 or 500 MHz spectrophotometer with chemical shift values reported in ppm. ^{1}H-NMR and ^{13}C-NMR spectra were referenced to residual undeuterated solvent. ^{31}P-NMR were collected at 80 MHz with a Varian 200MHz spectrophotometer and were measured from 85% H_3PO_4 as an external standard.

Synthesis procedures:



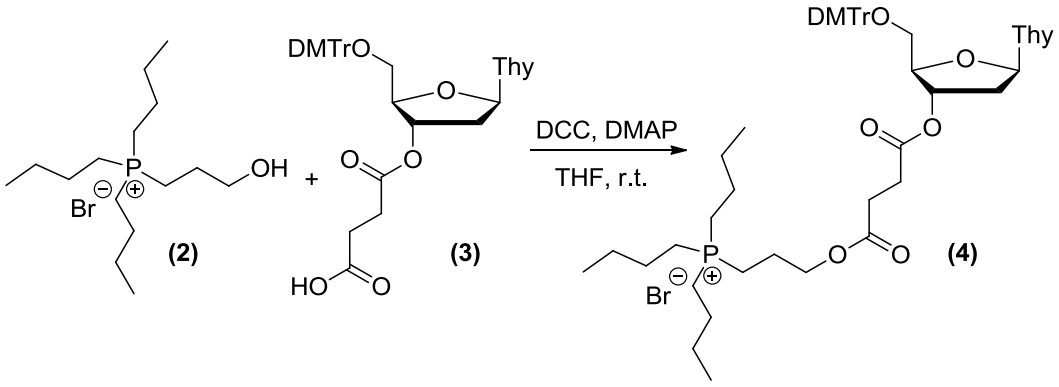
3-hydroxypropyl(tributyl)phosphonium bromide (2)

Tributylphosphine (5 mL, 4.10g, 0.02 moles) was mixed with 2 eq of 3-bromopropanol (3.63 mL, 5.58 g, 0.04 moles) dissolved in 20 ml of dry tetrahydrofuran (THF). The reaction mixture was refluxed under argon overnight for 16 h. The reaction was monitored by TLC (9:1 dichloromethane-methanol) using polymolybdenum acid stain (PMA) to visualize the spots. After the reaction was complete, the solvent was removed under reduced pressure and the product was re-dissolved in acetonitrile (ACN) and extracted with hexanes (4x100ml). The ACN layer was collected and condensed to yield a colorless viscous liquid in quantitative yield

^{1}H NMR (200 MHz, CD_3CN) δ ppm 0.85 - 1.07 (m, 9 H) 1.32 - 1.66 (m, 13 H) 1.67 - 1.90 (m, 2 H) 2.07 - 2.45 (m, 9 H) 3.58 (t, *J*=5.32 Hz, 2 H)

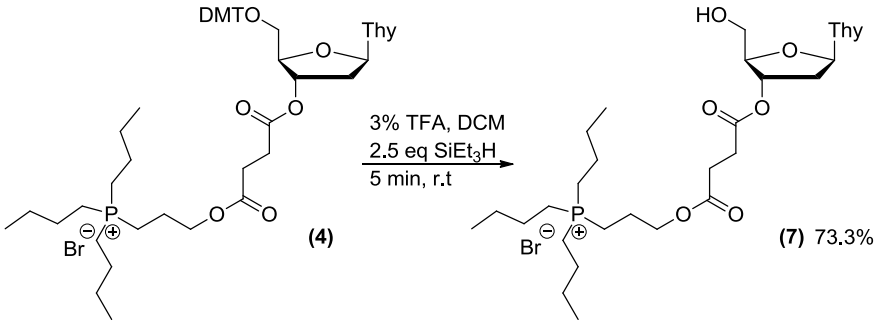
^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 13.21, 18.23, 18.86, 19.22, 23.28, 23.54, 23.74, 26.48, 28.66, 31.98, 37.70, 48.53, 54.25.

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 35.0 (s, 1 P)



5′-*O*-(4,4′`-dimethoxytrityl)-3′-(1-succinyl-4-[oxyethyltributylphosphonium bromide])-thymidine (4)

Compound **(2)** (12 g, 18.6 mmol) was added to a stirring solution of **(3)** (12 g, 18.6 mmol) and freshly distilled dicyclohexylcarbodiimide (DCC) (2 eq) in 100ml of dry THF. The reaction mixture was stirred for 3 days at room temperature. TLC analysis in 9:1 dichloromethane-methanol was performed for the disappearance of compound (**95**). The crude reaction mixture was filtered to remove the precipitated dicyclohexylurea (DCU). This crude mixture was dissolved in 1:1 acetone, acetonitrile and precipitated in MTBE followed by silica gel purification (0-10% MeOH in DCM) to afford **(4)** in 83% yield (14.9 g).
^{1}H NMR (400 MHz, CD_3CN) δ ppm 0.87 - 1.03 (m, 8 H) 1.37 - 1.61 (m, 16 H) 1.75 - 1.90 (m, 3 H) 2.05 - 2.26 (m, 20 H) 2.64 (s, 5 H) 3.36 (dd, *J*=15.26, 3.13 Hz, 2 H) 3.71 - 3.84 (m, 6 H) 4.02 - 4.24 (m, 3 H) 5.39 (d, *J*=6.26 Hz, 1 H) 6.28 (dd, *J*=8.80, 5.67 Hz, 1 H) 6.79 - 6.96 (m, 4 H) 7.20 - 7.55 (m, 10 H) ^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 35.0 (s, 1 P)



5′-hydroxyl-3′-(1-succinyl-4-[oxypropyl tributylphosphonium bromide])-thymidine (7)

Detritylation of compound **(4)** (2.78 g, 2.88 mmol) was performed by the addition of 5 eq of trifluoroacetic acid as a 3% v/v solution in DCM and 2.5 eq triethylsilane and stirring for 5-10 min. Two trifluoroacetic acid treatments were required to remove the complete trityl group. The reaction mixture was precipitated from MTBE ether after each trifluoroacetic acid treatment and finally twice to make sure that no traces of acid., the compound **(7)** (1.4 g, 73.3%) was condensed into a 250 mL round bottom flask and dried on high vacuum for a

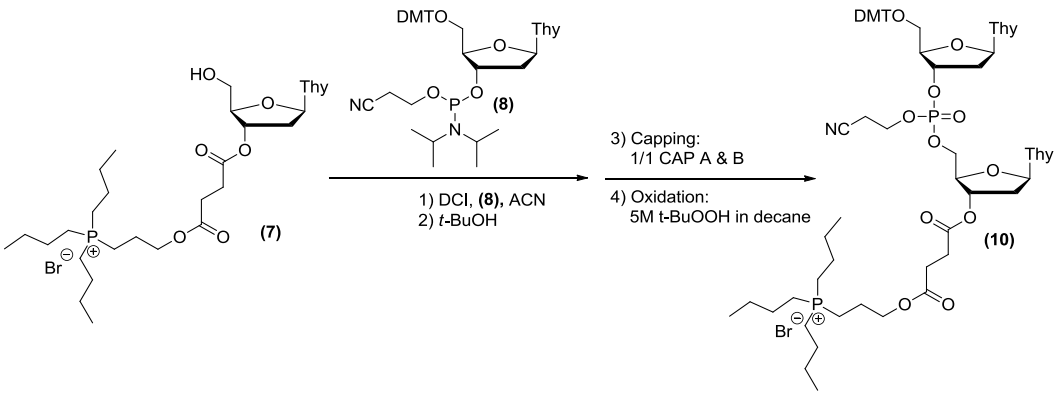
minimum of one hour to remove residual solvent, TFA and water, as any of these will adversely affect the next coupling step. The detritylated compound was highly hygroscopic so it should not exposed to air.

^{1}H NMR (400 MHz, CD_3CN) δ ppm 0.85 - 1.07 (m, 9 H) 1.32 - 1.66 (m, 13 H) 1.67 - 1.90 (m, 2 H) 2.07 - 2.45 (m, 9 H) 2.65 (s, 3 H) 3.63 - 3.82 (m, 1 H) 4.04 (d, *J*=1.96 Hz, 1 H) 4.16 (t, *J*=6.06 Hz, 1 H) 5.29 (d, *J*=5.48 Hz, 1 H) 6.21 (dd, *J*=8.41, 6.06 Hz, 1 H) 7.63 (s, 1 H) 8.94 (br. s., 1 H)

^{13}C NMR (400 MHz, CD_3CN) δ 173.5, 173.1, 165.1, 152, 137.4, 118.8, 86.3, 85.8, 76.9, 65, 64.8, 62.8, 38.3, 30.3, 30.1, 27.5, 24.9, 24.7, 24.2, 22, 19.5, 19, 16.8, 16.3, 14, 13.

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 35.0 (s, 1 P)

ESI-MS for $C_{29}H_{50}N_2O_8P^+$: Calc. 585.33, Found 585.33



[5′-hydroxyl-thymidine]-[3′-p(CyEt)-5′]-[3′-(1-succinyl-4-(oxypropyl tributylphosphonium bromide))-thymidine] (7)

Amidite **(8)** (2.35g, 3.15mmol) and 4, 5-dicyanoimidazole (DCI) (0.49g, 4.2mmol) was co-evaporated with benzene and dried under vacuum for 2 hours prior to starting the reaction. Compound **(7)** (1.4g, 2.1 mmol) was co-evaporated with benzene and dried under vacuum for 2 hours. 15% v/v dimethylformamide (DMF) in acetonitrile (ACN) was added to make a 0.06 M solution of oligonucleotide (0.12 M in amidite). 26 mL of the solvent was added to the amidite and DCI with syringe. The above solution was transferred to the oligomer with a cannula. The above reaction mixture was stirred for 3 hours.

After three hours, *tert*-butanol was melted (m.p. 25°C), 5 eq were added and allowed to stir for 10 min to quench the excess amidite, making it more soluble in ether. This solution was then added to a small dropper funnel, rinsing the flask with ACS grade acetone to final volume of about 40ml. This solution was precipitated by dropwise addition to 500-600 mL of methyl *tert*-butyl ether (MTBE) over about 15min. The solution was then filtered over Celite® to collect the precipitated dimer DMTr-dTT Succ-IL **(9)**. The MTBE was then condensed under reduced pressure and the remaining solution was once again precipitated from ether to recover oligomer that was dissolved in the MTBE. After the sample was collected and condensed to dryness, the sample was re-suspended in about 30ml of acetone and THF and precipitated once more to ensure complete removal of excess amidite before oxidation.

The phosphite triester **(9)** oligomer was dissolved in same amount of 15% v/v DMF in acetonitrile and 0.5 eq each of Cap A and Cap B solution (16% N-methylimidazole in THF, and acetic anhydride:pyridine:THF, 1:2:2 ratio) was added. The reaction was allowed to stir for 10min. Make sure to only allow 10min as longer reaction times discolor the reaction mixture to a dark yellow/brown color. Next, 5 eq of 6M *tert*-butyl hydroperoxide was added in decane to oxidize. Then it was allowed to stir for a further 15min. This solution was then again precipitated as described above, recovering compound **(10)** from the filter in a 250 mL round bottom flask. This solid compound was then placed under a high vacuum for 15-20 min to make sure there was no residual *tert*-butyl hydroperoxide or pyridine remaining. Compound 10 was isolated in 93.4% yield. ^{1}H NMR (400 MHz, CD_3CN) δ ppm 0.95 (t, *J*=6.85 Hz, 9 H) 1.37 (d, *J*=6.26 Hz, 3 H) 1.42 - 1.62 (m, 13 H) 1.70 - 1.91 (m, 5 H) 1.91 - 2.02 (m, 5 H) 2.06 - 2.27 (m, 7 H) 2.31 (br. s., 2 H) 2.50 (br. s., 1 H) 2.57 (d, *J*=5.48 Hz, 1 H) 2.65 (br. s., 3 H) 2.70 - 2.85 (m, 2 H) 3.36 (d, *J*=11.74 Hz, 3 H) 3.78 (d, *J*=1.96 Hz, 6 H) 4.11 - 4.34 (m, 7 H) 5.12 - 5.33 (m, 2 H) 6.20 (t, *J*=7.04 Hz, 2 H) 6.25 - 6.36 (m, 1 H) 6.89 (dd, *J*=8.80, 2.15 Hz, 4 H) 7.12 - 7.36 (m, 7 H) 7.36 - 7.54 (m, 5 H) 9.65 (br. s., 2 H)

^{13}C NMR (400 MHz, CD_3CN) δ 208, 173.4, 173.2, 165.3, 160, 151.9, 145.9, 136.7, 131.3, 129.2, 128.3, 114.4, 111.9, 87.9, 86.1, 85.5, 83.3, 75.2, 68.8, 64.9, 64.4, 64.1, 56.2, 48.2, 39.4, 37.5, 31.2, 30, 29.8, 24.8, 24.7, 24, 21.8, 20.5, 19.3, 18.8, 16.6, 16.1, 13.9, 12.9, 12.5.

^{31}P NMR (200 MHz, CD_3CN) δ 35.457, -1.599, -1.720.

ESI-HRMS for $C_{63}H_{84}N_5O_{17}P_2^+$: Calc. 1244.52, found 1244.5

General procedure for the synthesis of oligoribonucleotides in solution

Beginning with the detritylated, tagged oligonucleotide, which was dried under vacuum for at least 2 hours, the mass is determined and a small portion is removed for deprotection and analysis. The mass is re-determined and the next coupling is prepared. The next amidite to be coupled is removed from the freezer and allowed to warm to room temperature in a dessicator before the appropriate weight is measured out (2.5 eq). The coupling reagent, 2,3-dicyanoimidazole (DCI) has been dried under vacuum and stored in a dessicator and is weighed as is.

Both the DCI and phosphoramidite are added as solids to the dried oligo and are kept under nitrogen or argon. 15% v/v dimethylforamide (DMF) in ACN is added to make a 0.06M solution of oligonucleotide. This solution must be mixed for up to 10-15min to completely solvate the solid oligomer. This solution is then stirred for 4 hours with periodic manual agitation to ensure complete mixing with droplets adhering to the sides of the flask.

After four hours, *tert*-butanol is melted (m.p. 25^{o}C), and 25 eq are added and allowed to stir for 10 min to quench the excess amidite, making the excess amidite into a *t*-butyl phosphite triester which is more soluble in the ether layer. This solution was then added to a small dropper funnel, rinsing the flask with ACS grade acetone to final volume of about 40ml. The solution was precipitated by dropwise addition to 500-600ml of methyl *tert*-butyl ether (MTBE) over 15min. The solution was then filtered over Celite® to collect the precipitated phosphite triester. The precipitate is quite fine and packs hard over the Celite® causing the flow through the filter to be greatly reduced. Once the sample was

almost completely filtered, you must add more MTBE to rinse the solid. It is extremely important to not let the filter run dry, as the hard packed solid will crack and fold up (Figure 2.13) which will then not allow you to rinse away impurities and the precipitation process will have to be repeated.

Figure 2.13. Oligonucleotide filtered over Celite® that has been allowed to dry forming cracks in the solid cake that allow rinsing solvent to pass right through.

It was imperative to remove all the excess amidite. If any amidite was retained it will grow just as the oligomer, forming significant N-1 mers. This poses two significant problems 1) The full length target oligomer will be harder to purify, and 2) The excess amidite will consume phosphoramidite in subsequent coupling steps taking away equivalence from the growing oligomer.

The solid precipitate is dissolved off the filter into a 500 mL or 1 L round bottom flask using both ACS acetone and THF. As the oligonucleotide grows it becomes less and less soluble. Previously, only acetone and acetonitrile were used to dissolve the sample off the filter, but as the oligomer grows acetonitrile proved to not have the solvating power necessary. It was found that THF is the best choice because it is quite inert, unlike using methanol or DMF which is more difficult to remove.

After the sample was collected and condensed to dryness, the sample was re-suspended in about 30ml of acetone and THF and precipitated once more to ensure complete removal of excess amidite before oxidation.

Once the purified phosphite triester was dry the oligomer was dissolved in 15% v/v DMF in acetonitrile to a concentration of approximately 0.08M, 16 eq each of Cap A and Cap B solution (16% N-methylimidazole in THF, and acetic anhydride:pyridine:THF, 1:2:2 ratio) was added. The reaction was allowed to stir for 10min. Make sure to only allow 10min as longer reaction times discolor the reaction mixture to a dark yellow/brown color. Next, 36 eq of 6M *tert*-butyl hydroperoxide in decane was added to oxidize. The mixture was allowed to stir for a further 15min. This solution was then once again precipitated as described above, recovering the sample from the filter in a 250ml round bottom flask. The solid was then placed under a high vacuum for 15-20min to make sure there is no residual pyridine remaining.

To induce detritylation, 20 eq of 3% v/v triflouroacetic acid in acetonitrile is added, followed by 2.5 eq of triethylsilane (a quencher of trityl cation) and the solution is stirred for 15 to 20 min until the orange trityl color disappears to a light orange huge. The solution was again precipitated, rinsing the reaction vessel with dry ACS acetone.

Once the material is detritylated, the solubility of the molecule is significantly decreased. Dissolving it off the Celite® filter then becomes much more difficult. More solvent and time is necessary to ensure complete removal. Also, at this point the molecule seems to be much more hygroscopic, thus exposure to atmosphere should be minimized or avoided completely.

To ensure that the reaction was detritylated completely, spot a TLC plate with the solution just before it is completely condensed on the rotovap, as a more concentrated solution is necessary to see trace amounts of trityl color. Burn this TLC after an acid treatment. If you see any trityl color you must repeat the TFA treatment cycle. Alternatively, a small portion can be removed and placed in acidic DCM and run on a low resolution mass spectrometer and look for the appearance of the trityl cation.

After the necessary number of detritylations are completed, the material was condensed into a 250ml round bottom flask and dried on high vacuum for a minimum of one hour to remove residual solvent, TFA and water, as any of these

would adversely affect the next coupling step. The solution phase synthesis cycle is a time consuming process and extreme care must be taken at each step of the reaction, as any failure would require the whole synthesis to be repeated. In Table 2.4 is an approximate time allocation for each coupling step.

Step	Time (h)
Coupling	3-24
ppt. of phosphite triester	2 (x2)
Capping/oxidation	2
detritylation with ppt**	2 (x2)
Drying on high vacuum	3
Total hours per coupling	16-38

Table 2.4. Approximate time of each step in the synthesis cycle **assumes x2 detritylations and one precipitation without acid treatment.

In Table 2.5 we have summarised the stepwise synthesis of the growing oligonucleotide using the general procedure described above. Amidites used, coupling reagents, conditions, and yields are presented.

Starting Comp.	Mass (g)	St. mat. (mmol)	mmol of amidite	Amidite (g)	ACN (mL)	DCI (mmol)	DCI (g)	Rex. Time (h)	% yield
t-OH	1.781	1.74	4.35	3.24 (dt)	29.0	5.22	0.62	3	65.0
tt	2.523	1.55	3.89	3.84 (rA)	25.9	4.66	0.55	3	93.4
ttA	3.61	1.62	4.06	4.01 (rA)	27.1	4.87	0.57	3	102.7*
ttAA	2.546	0.94	2.36	2.03 (rU)	15.7	2.83	0.33	3	97.3
ttAAU	2.301	0.73	1.81	1.56 (rU)	12.1	2.18	0.26	5	92.7
ttAAUU	2.493	0.66	1.65	1.63 (rA)	11.0	1.98	0.23	12	90.2
ttAAUUA	2.6	0.59	1.49	1.46 (rA)	9.91	1.78	0.21	12 (x2)*	90.0
ttAAUUAA	2.69	0.56	1.39	1.19 (rU)	9.25	1.67	0.20	12 (x2)*	91.0
ttAAUUAAU	1.52	0.29	0.71	0.62(rU)	4.76	0.86	0.10	12 (x2)*	91.0

Table 2.5. Stepwise synthesis reagents and yields of oligonucleotide synthesis *the coupling reaction was repeated to ensure the complete coupling. *the yields were calculated by mass, it can be believed that ion exchange of the phosphonium has occurred to produce the higher than 100% yield.

Synthesis of Standard oligonucleotides, solid-phase synthesis

Solid-phase oligonucleotide synthesis was performed on an Applied Biosystems ABI 3400 synthesizer using previously published methods[35] using standard TBDMS protected phosphoramidites (ChemGenes). Below a table of reagents and coupling times is presented.

Step	*Operation*	*Reagent*	*time (s)*
1	Detritylation	3% TCA in CH_2Cl	120
2	Coupling	0.15 M or 0.1M in MeCN 0.25 M DCI in MeCN	600
3	Capping	CAP A, CAP B	20
4	Oxidation	3M *tert*-butylhydroperoxide	20

Table 2.6. ABI synthesizer reagents and conditions for the synthesis of standard oligonucleotides.

Oligonucleotide deprotection conditions.

General procedure (A)

Each sample was treated with 1mL of 3:1 aqueous NH_4OH:EtOH at 55°C for 16h for nucleobase and phosphate deprotection. The samples were vented for 2 hours, after which they were either decanted in the case of solid supported reagents, or chilled directly chilled on dry ice for 15 min, then lyophilized to dryness in a speed-vac concentrator. The resulting solid was then treated with 200 µL of triethylamine trihydrofluoride (TREAT-HF) and placed on a shaker for 48 h to remove all silyl protecting groups. To quench the reaction mixture 50 µL of 3M sodium acetate (NaAc) pH 5.5 was added to the solution, vortexed thoroughly. The oligonucleotide was precipitated by the addition of 1 mL of cold (-20°C) n-butanol and placed on dry ice for 30min to encourage complete precipitation of all oligonucleotide in solution. This was particularly important for short oligonucleotides (4-6mer) as significant amount of product would remain in solution. The sample was then vortexed and decanted. The residual n-butanol was evaporated in the speed-vac lyophilizer. The samples were then desalted using G-25 Sephadex® size exclusion columns (GE healthcare), samples were pooled and condensed to dryness.

General procedure (B)

The conditions to deprotect oligonucleotides here are identical to that of conditions A above, except during the of 3:1 aqueous NH_4OH:EtOH treatment is not heated, but rather left at room temperature for 48h. Under these conditions it is know that there is significant reduction in the amount of cleavage product.

Ion exchange HPLC conditions

HPLCs were performed on an Agilent 1200 series machine. Ion-exchange HPLC were performed with a Waters PROTEIN-PAK DEAE 5PW (7.5 x 150 mm) HPLC column using a 0-23.5% gradient of 1M $LiClO_4$ in Mili-Q water as solvent B over 45 minutes. Mili-Q water was used as solvent A. (Table 2.7).

	Time (min)	%A	%B	Flow (mL/min)
1	0.00	100	0.00	1.00
2	3.00	100	0.00	1.00
3	45.00	76.5	23.5	1.00
4	48.00	50	50	1.00
5	54.00	50	50	1.00
6	56.00	100	0.00	1.20
7	66.00	100	0.00	1.20

Table 2.7. Ion exchange HPLC gradient A= Milli-Q water; B=1M $LiClO_4$ in Milli-Q water.

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Chapter 3. Blockmer Amidites for Oligoribonucleotide Synthesis

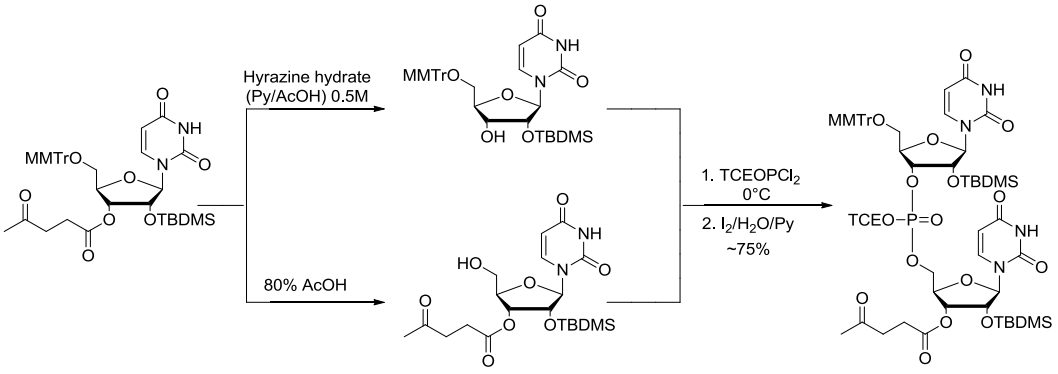
3.1 Introduction

Current methods for solid-supported RNA synthesis rely on stepwise additions of monomeric phosphoramidite units. The solid support method facilitates the removal of excess reagents following each coupling step. However, careful chromatographic purification of the final product is required after the oligomer is detached from the solid support and fully deprotected, due to contamination with failure sequences arising from incomplete coupling. Failure sequences one nucleotide shorter than the desired oligonucleotide, so-called "(N-1)-mers", present a particularly difficult purification challenge due to their similar retention times on HPLC, and electrophoretic mobility on polyacrylamide gels. An alternative approach that circumvents this problem is the assembly of oligonucleotide chains through "block" condensation reactions. This method was pioneered by Khorana, where protected "blocks" of di-, tri- , and tetranucleotides (bearing a 5′ -phosphate monoester) were utilized to prepare a fully-protected oligodeoxyribonucleotide chain in homogeneous solution.[1,2] The yields of pure products at various steps were low, in the range of 33-54 %.[1] At each step, the products were separated by anion exchange chromatography and verified for purity by paper chromatography after removal of the protecting groups. The yields decreased as the chain lengths increased, even when a large excess of "blocks" was used. Nevertheless, this approach demonstrates two advantages of block over monomer coupling: block couplings. They reduce the number of synthetic steps speeding up the oligonucleotide assembly. They also generate crude oligonucleotide samples that are more easily purified since failure couplings are a "N-2(or 3)" mer rather than "N-1" mer in the monomer based approach. Later, the Reese group demonstrated a more efficient RNA blockmer stradegy using the phosphotriester approach,[3] improving on the work of Khorana. The application of the phosphoramidite block condensation method was later

demonstrated by Ravikumar *et al.* in the successful solid phase synthesis of a 20-mer oligodeoxyribonucleotide using dimer and trimer synthons.[4]

Despite the many advantages of the block condensation method, it has not been applied to solid phase RNA synthesis, likely due to the difficulty of obtaining block-mer ribonucleotide phosphoramidites in an isomerically pure form, and the perceived inefficiency of their couplings on solid-phase supports.

The generation of oligoribonucleotide block-mers is made difficult by the presence of the 2′-hydroxyl group, which requires protection. The Ogilvie group had described the synthesis of 5′-*O*-MMTr-2′-*O*-TBDMS-3′-*O*-levulinyl ribonucleoside monomers, and their use in the assembly of a hexadecauridylic acid via the phosphodichloridite procedure in 51% yield.[5] It is important to note that the levulinyl (Lev) ester group was introduced as a transient and orthogonal protection for the 3′ position of ribonucleotides. Further, the authors show that the 5′-*O*-MMTr-2′-*O*-TBDMS-3′-*O*-levulinyl ribonucleotide monomers could be quantitatively delevulinated under mild conditions by treatment with hydrazine hydrate buffered in pyridine:acetic acid (3:2) without isomerization of the 2′ TBDMS group. The ribonucleotide monomers were initially used to synthesize dimer blocks via the phosphodichloridite procedure shown in Scheme 3.1. This protocol was used to convert the dimers into tetramers, which were then condensed to form octamers and finally a hexadecamer.[5]



Scheme 3.1. Synthesis of [5′-MMTr-2′TBDMS-Ura]-[3′-p(OTCE)-5′]-[rUra-2′TBDMS-3′-Lev] dimer)[5]. TCE is the tricholoroethyl phosphate protecting group.

This and other reports[6,7] utilize earlier coupling methods, which generally provide yields far too low to be considered effective synthetic strategies,

especially for large scale RNA synthesis. Since then, the phosphoramidite approach has proven to be the coupling method of choice for solid phase synthesis of oligonucleotides,[8] offering higher yields and faster reaction times. Although the block-wise solid-phase synthesis of DNA using phosphoramidite block-mers has been well-demonstrated,[9,10] to date there is only one report describing a hypothetical synthesis of RNA phosphoramidite block-mers for use in solid-phase synthesis in a patent application, but to our knowledge never successfully produced.[11] Given the efficiency of phosphoramidite chemistry, it is highly desirable to have access to block (dimer and trimer) phosphoramidites for RNA synthesis. Such an approach should benefit from the advantages of a block-mer coupling strategy, without suffering from low coupling yields.

Herein, we describe our efforts in preparing isomerically pure dimer and trimer phosphoramidite synthons for the block synthesis of oligoribonucleotides. For this purpose, we explore the use of 2′-*O*-triisopropylsilyl (TIPS) protecting group. The 2′-*O*-TIPS protected dimer and trimer synthons provide several distinct advantages over previously reported 2′-*O*-TBDMS-3′-phosphoramidite dimer synthons.[11] We show for the first time through extensive NMR studies that in nucleotides the 2′-*O*-TBDMS protecting group undergoes 2′ to 3′ migration under standard delevulination conditions. We further demonstrate that the use of the TIPS protecting group completely eliminates the 2′ to 3′ silyl migration that occurs with 2′-*O*-TBDMS protecting groups employed in previously reported synthons.[5,11]

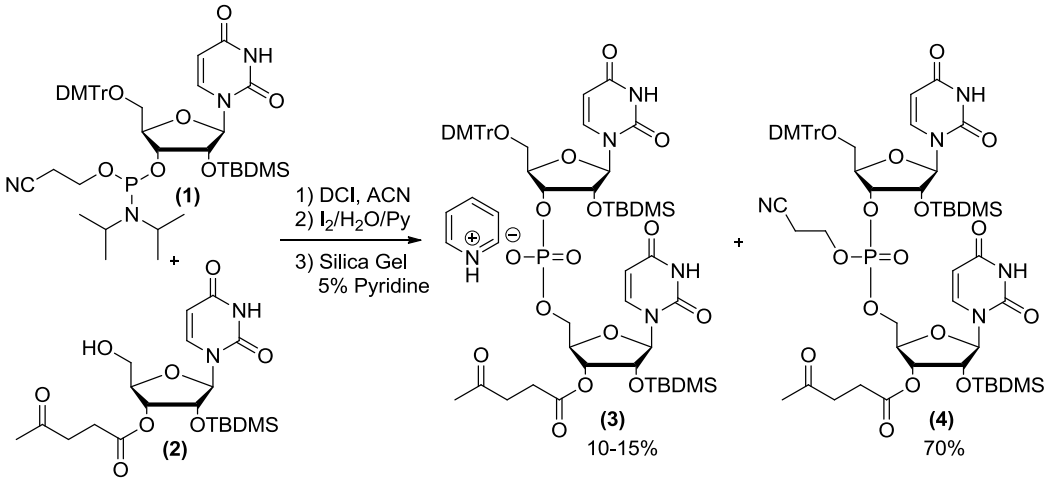
The synthetic procedures described herein provide facile access to isomerically pure dimer and trimer synthons in high yields. We show that this method is readily amenable to the synthesis of both purine and pyrimidine dimer and trimer block-mers. These synthons will allow for a quick and high yielding chain extension for the solution or solid phase synthesis of RNA oligomers.

3.2 Results and Discussion

3.2.1 Choice of phosphate protecting group

Our method builds upon the contributions of the Ogilvie group,[5] who used the levulinyl group as a transient protection for the 3′-position and the trichloroethyl[12] group as phosphate protecting group. The 3′-levulinyl protection was retained, but the phosphate protecting group was replaced with the industry standard cyanoethyl group, a more electron withdrawing group allowing quantitative cleavage under much more mild conditions.

We first attempted the synthesis of a rUU dimer. This was accomplished by coupling the commercially available 5′-*O*-DMTr-2′-*O*-TBDMS-uridine-3′-*O*-cyanoethyl phosphoramidite **(1)** with 3′-*O*-Lev-2′-*O*-TBDMS-uridine **(2)** (Scheme 3.1). While the desired dimer product **(4)** was obtained, we observed up to 15% decyanoethylation during column chromatography caused by the basic elutent system (pyridine) which was necessary in order to avoid removal of the 5′-DMTr protecting group. These chromatography conditions are also required to prevent the premature activation of the phosphoramidite moiety. The 10 to 15% loss of material due to decyanoethylation is not considered an unacceptable reduction in yield when small quantities of material are needed. However, our process is intended for the production of large scale quantities of material, requiring robust and high yielding reactions.

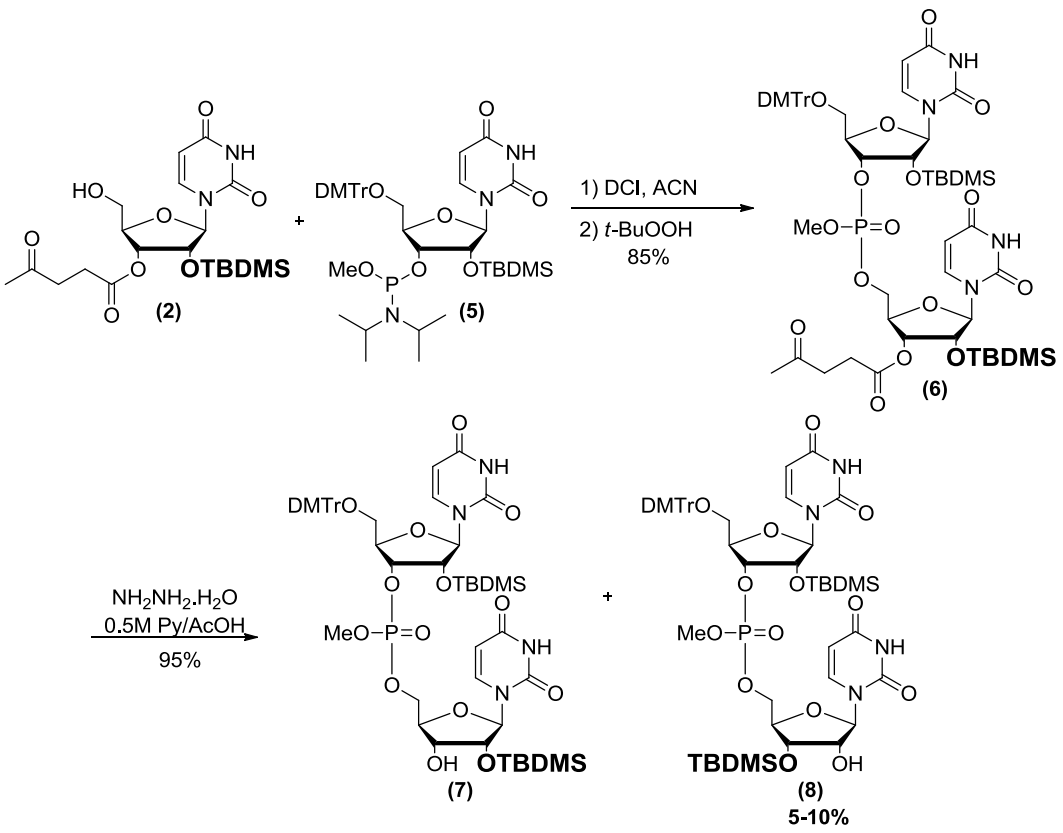


Scheme 3.2. Base-mediated decyanoethylation of dimer synthons.

This led us to pursue alternative phosphate protecting groups compatible with our synthetic methods. Over the years, many phosphate protecting groups compatible with oligonucleotide synthesis have been developed (see Chapter 1 Section 1.4.3). Of these, the methyl phosphate protecting group was chosen due to its stability towards the basic conditions necessary during silica gel purification, and ease of cleavage from a full length oligonucleotide under very mild conditions.[13-16]

3.2.2 2′ to 3′ migration of TBDMS protecting groups upon delevulination in monomer and dimer nucleotides

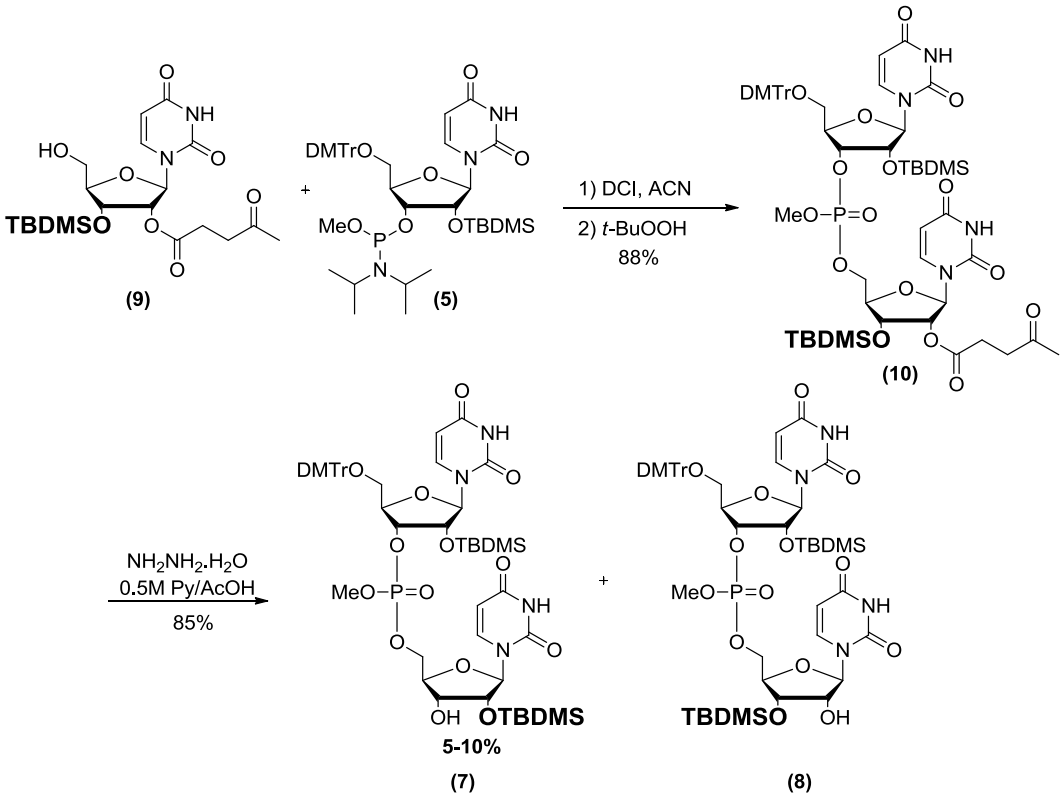
With the alternative methyl phosphate protecting group used in place of the cyanoethyl group, we proceeded with the synthesis of the dimer rUpU. The synthetic approach is outlined in Scheme 3.3.[5,17] We took the commercially available 5′-*O*-DMTr-2′-*O*-TBDMS-uridine-3′-*O*-cyanoethyl phosphoramidite **(1)** and coupled it to 3′-*O*-levulinyl-2′-*O*-TBDMS-uridine using DCI as a phosphoramidite activator at room temperature, followed by an *in situ* oxidation of the phosphite triester to the corresponding phosphate triester with *tert*-butyl hydroperoxide. The use of iodine/water for the oxidation should be avoided when methyl phosphate protection is used as premature demethylation via an Arbuzov-like reaction can occur under these conditions.[18] The fully protected dimer **(6)** was isolated by column chromatography with no observable loss of the phosphate protecting group. Next, the 3′-*O*-Lev group was removed by treatment with a 0.5 M solution of hydrazine hydrate in a solution of pyridine/acetic acid (3:2). After isolation and purification of the delevulinated material, a significant amount (5-10%) of a contaminant was observed in the ^{31}P-NMR. We suspected that the 2′-*O*-TBDMS protecting group migrates to the 3′-position during the delevulination process, a known issue with silyl protecting groups on vicinal diols.[19]



Scheme 3.3. Synthesis of [5′-DMTr-2′-TBDMS-U]-[3′-p(OMe)-5′]-[U-2′-TBDMS] dimer **(7)**. Compound **(7)** was contaminated with 5-10% of regioisomer **(8)**, which likely forms during the delevulination step.

To confirm that the observed impurity was in fact the 3′-*O*-TBDMS dimer **(8)**, we treated the sample with mixture of aqueous base (aqueous pyridine) for 2h to encourage silyl migration. As expected, the minor peaks in the ^{31}P-NMR increased significantly, which indirectly gives evidence for the occurrence of TBDMS migration during levulinyl deprotection.

To further investigate this theory, we prepared predominantly the 3′-*O*-TBDMS dimer **(8)** using an identical procedure to that of the 2′-*O*-TBDMS dimer scheme, but starting with 3′-OTBDMS-2′-*O*-Lev uridine **(9)** instead (Scheme 3.1.[5,17] We further used **(8)** as a spectroscopic standard in NMR product studies, comparing it to the correct product 2′-*O*-TBDMS dimer **(7)**.



Scheme 3.4. Synthesis of [5′-DMTr-2′-TBDMS-U]-[3′-p(OMe)-5′]-[U-3′-TBDMS] dimer **(8)**. Compound **(8)** was contaminated with 5-10% of the 3′ to 2′ TBDMS migration product **(7)** upon delevulination.

The 2′- to 3′-*O*-TBDMS isomerization was confirmed by ^{1}H and ^{31}P-NMR studies, where the chemical shifts of isomers **(6)** and **(7)** were compared to isomers **(10)** and **(8)**, respectively. We first tested the purity of the levulinated dimers **(6)** and **(10)** to show that the previously observed impurities in the NMR of the final dimers were not a result of isomerically impure starting materials. The two peaks for the phosphotriester diastereomers of the 2′-*O*-TBDMS and 3′-*O*-TBDMS dimers can be clearly distinguished in the ^{31}P-NMR spectrum (Figure 3.1).

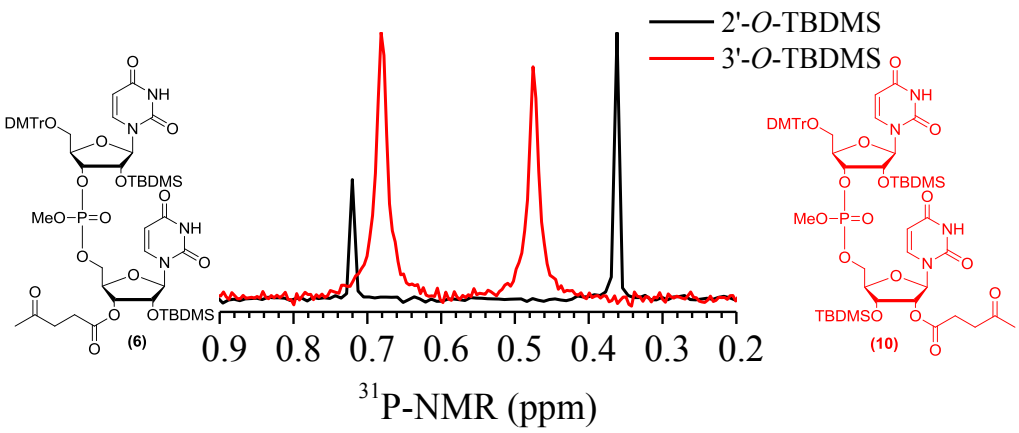


Figure 3.1. Overlay of ^{31}P-NMR of 2′-*O*-TBDMS (black) and 3′-*O*-TBDMS (red) dimers.

Dimers **(6)** and **(10)** were next delevulinated by treatment with 10 equivalents of hydrazine hydrate buffered in a 3/2 ratio of pyridine/acetic acid. After five minutes, the excess hydrazine was quenched by the addition of 2,4-pentadione. Dimers **(7)** and **(8)** were isolated by silica gel flash chromatography. The ^{31}P-NMR chemical shifts of each dimer confirmed the observed 2′- to 3′-*O*-TBDMS isomerization. Panel A and B in Figure 3.2 show the ^{31}P-NMR of 2′- and 3′-*O*-TBDMS dimers, respectively. Their overlay in panel C clearly demonstrates that that the two small peaks in the ^{31}P-NMR of 2′-*O*-TBDMS dimer **(7)** correspond to the peaks in the ^{31}P-NMR of 3′-*O*-TBDMS dimer **(8)** (Figure 3.2 C). The isomerization is further exemplified by the ^{31}P-NMR of a mixture of the two isomers combined in a 1:1 ratio (Figure 3.2 D).

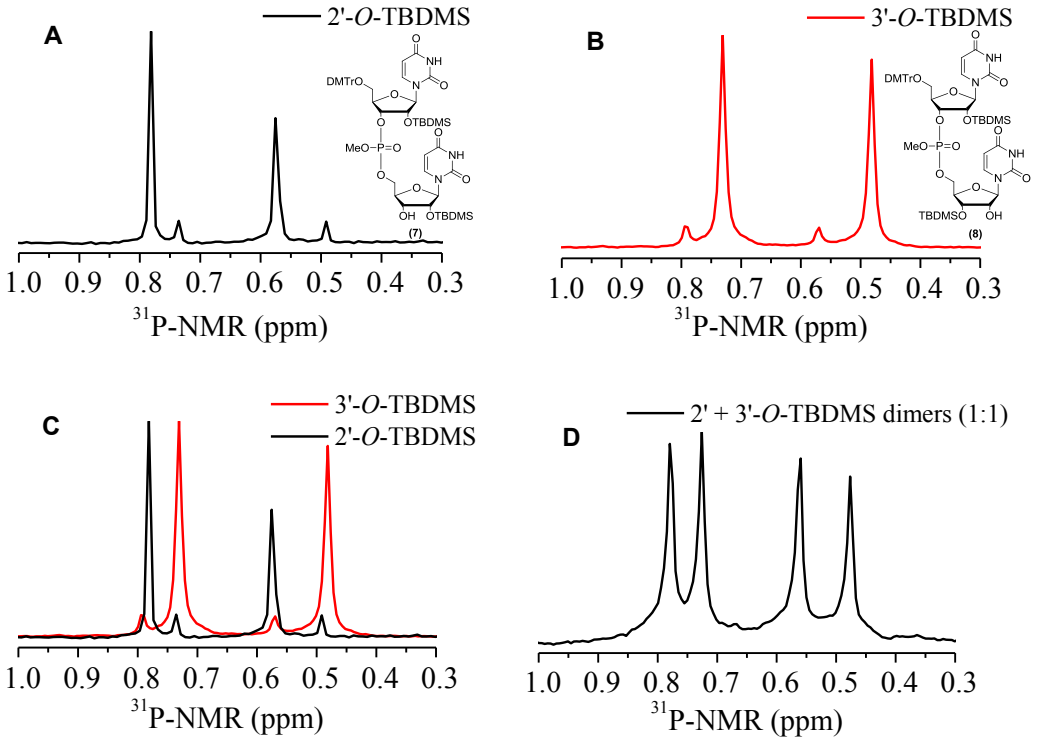


Figure 3.2. ^{31}P-NMR of: A) 2′-*O*-TBDMS dimer **(7)**; B) 3′-*O*-TBDMS dimer **(8)**; C) overlay of A and B; D) mixture of the two isomers combined in a 1:1 ratio. Solvent: d_3-ACN.

After we confirmed by ^{31}P-NMR that the observed 5-10 % impurity in the 2′-*O*-TBDMS dimer is the 3′-regioisomer, we attempted to purify the dimer. However, after many attempts, we found that it was not possible to separate the two regioisomers by silica gel flash chromatography or reverse-phase HPLC. We also established that the regioisomers could not be resolved by TLC. TLC is the only method of characterisation used by Ogilvie and co-workers in 1980,[5] which may explain why previous reports did not detect TBDMS isomerization under the delevulination conditions used.

3.2.3 NMR study of the rates of delevulination and isomerization of the TBDMS protecting group in a fully protected nucleoside and dimer

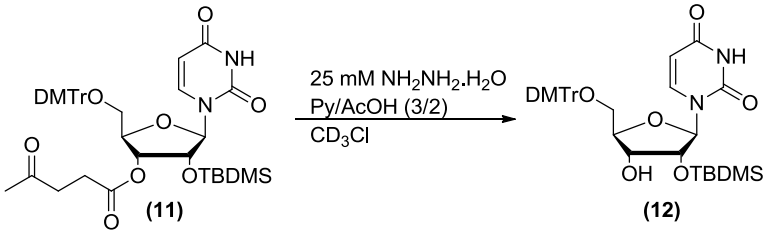
We proceeded with an NMR study of the relative rates of delevulination and TBDMS isomerization. We monitored the two reactions under hydrazine/ pyridine/ acetic acid conditions using both ^{1}H-NMR and ^{31}P-NMR. The study

was conducted on the fully protected mononucleoside **(11)** and the dimer nucleotide **(6)**. In order to be able to observe the reactions in the NMR time scale, the concentration of hydrazine used was reduced to 25 mM compared to the 0.5 M, originally used by Ogilvie and co-workers.[5]

No 2′ to 3′ TBDMS isomerization is expected to occur in the fully protected nucleoside **(11)** under standard delevulination conditions, as they have not been observed by our or other groups in the past.[5,20] Thus, the nucleoside was used as a standard to test the delevulination conditions used for the NMR study. Since the monomer does not have phosphorus-containing groups, the study was performed using ^{1}H-NMR. We further applied the same delevulination conditions to monitor the TBDMS isomerization in the dimer using ^{31}P-NMR.

3.2.3.1 TBDMS isomerization study for 5′-*O*-DMTr-3′-OH-2′-*O*-TBDMS-Uridine (12)

First the 5′-*O*-DMTr-3′-*O*-Lev-2′-*O*-TBDMS uridine **(11)** was treated with 5 equivalents of hydrazine hydrate (0.25 M) in a 1/1 mixture of deuterated chloroform and 3/2 pyridine/acetic acid buffer to yield the deprotected nucleoside **(12)** (Scheme 3.5). The reaction was followed by ^{1}H-NMR (Figure 3.3). The 3′-*O*-Lev group was fully deprotected after 20 min, as indicated by the disappearance of the peaks at 4 and 4.3 ppm. The reaction was monitored for 11 hours. The reaction mixture was compared to the 5′-*O*-DMTr-3′-*O*-TBDMS-2′-OH uridine isomer in order to detect any isomerization. Over the 11 hours of the experiment, no isomerization of the 2′-*O*-TBDMS monomer was observed, as indicated by the lack of peaks at 3.8 and 4.9 ppm (Figure 3.3). The concentration of hydrazine was doubled to 0.5M (10 equivalents), and still no detectable isomerization occurred.



Scheme 3.5. Delevulination conditions for ^{1}H-NMR study of TBDMS isomerization in mononucleoside **(12)**.

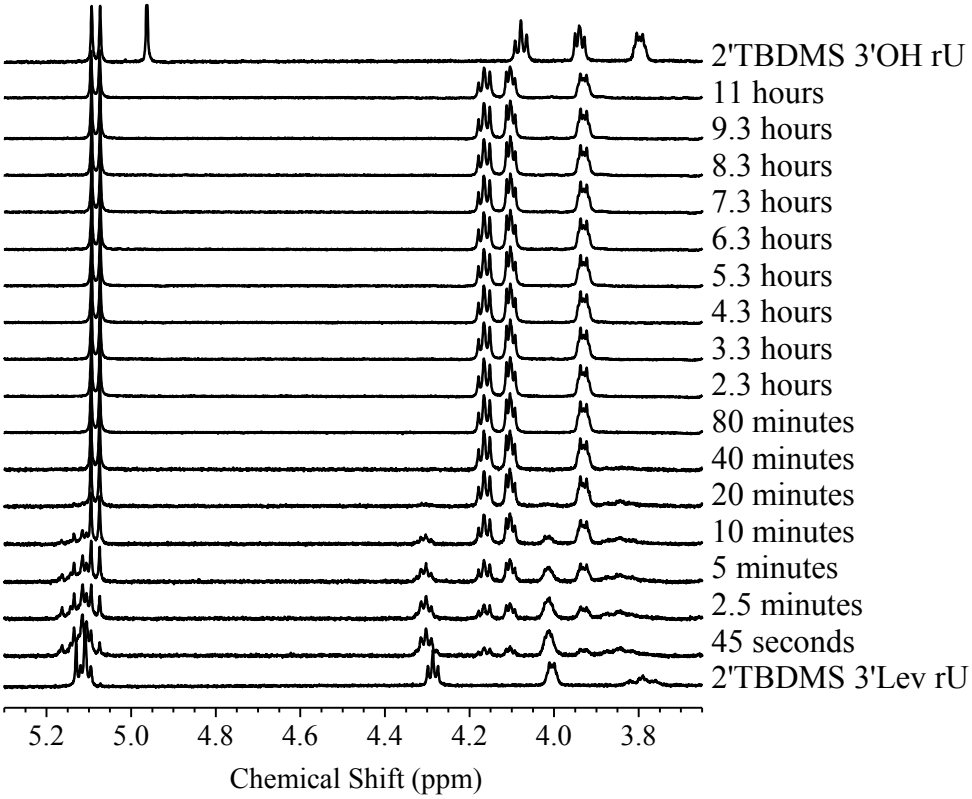


Figure 3.3. ^{1}H-NMR study of the delevulination of 5′-*O*-DMTr-2′-*O*-TBDMS-3′-*O*-Lev uridine **(11)**. 5′-*O*-DMTr-3′-*O*-TBDMS uridine was used as a standard to monitor possible 2′ to 3′ TBDMS isomerization. No TBDMS isomerization was observed under the deprotection conditions used.

3.2.3.2 TBDMS isomerization study for [5′-*O*-DMTr-2′-*O*-TBDMS-U]-[3′-p(OMe)-5′]-[U-2′-*O*-TBDMS] dimer (7) under delevulination conditions

Next, we deprotected [5′-*O*-DMTr-2′-*O*-TBDMS-U]-[3′-p(OMe)-5′]-[U-3′-*O*-Lev-2′-*O*-TBDMS] dimer **(6)** under the delevulination conditions used for the

3′-*O*-Lev deprotection of monomer nucleoside **(11)**. We then followed the 2′ to 3′ TBDMS isomerization in [5′-*O*-DMTr-2′-*O*-TBDMS-U]-[3′-p(OMe)-5′]-[U-2′-*O*-TBDMS] dimer nucleotide **(7)** that occurs during the deprotection. We previously showed that the two regioisomers could easily be identified by ^{31}P-NMR (Figure 3.2). Thus, we used ^{31}P-NMR to monitor the delevulination and subsequent isomerization (Figure 3.4). This additionally allows minimizing the amount of interfering peaks that are normally observed in ^{1}H-NMR.

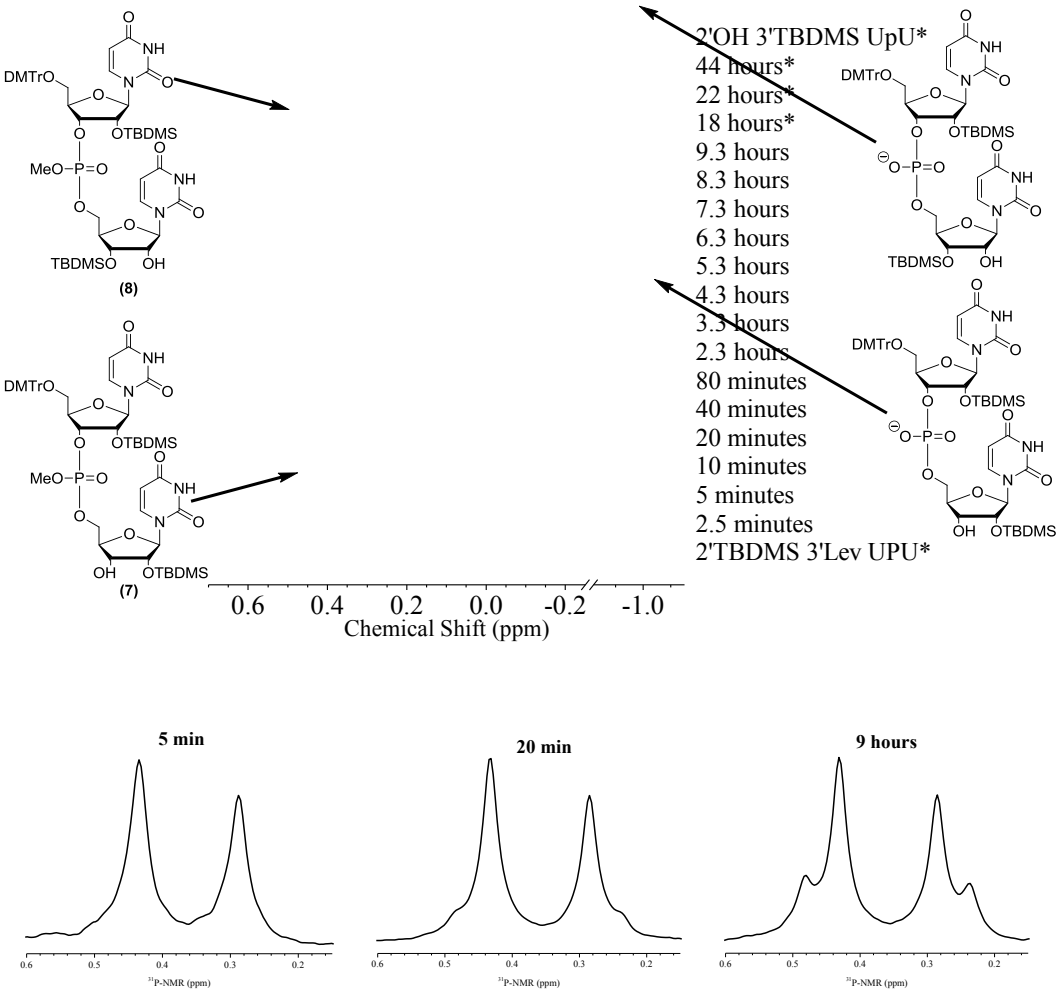


Figure 3.4. A) ^{31}P-NMR study of 2′ to 3′ TBDMS isomerization in [5′-*O*-DMTr-2′-*O*-TBDMS-U]-[3′-p(OMe)-5′]-[U-3′-OH-2′-*O*-TBDMS] dimer **(7)** under delevulination conditions (0.25 M hydrazine, 5 eq in 1/1 mixture of deuterated chloroform and 3/2 pyridine/acetic acid buffer). Full deprotection of the 3′-*O*-Lev in [5′-*O*-DMTr-2′-*O*-TBDMS-U]-[3′-p(OMe)-5′]-[U-3′-*O*-Lev-2′-*O*-TBDMS] dimer nucleotide **(6)** was

observed after 2.5 min. Noticeable TBDMS isomerization was detected after 20 min. The spectrum of [5′-*O*-DMTr-2′-*O*-TBDMS-U]-[3′-p(OMe)-5′]-[U-3′-*O*-TBDMS] dimer nucleotide **(8)** is shown for comparison. B) Zoomed ^{31}P-NMR of the reaction mixture after 5 min, 20 min, and 9 hours (shown is the region between 0.15 and 0.6 ppm). Solvent: Py:AcOH:$CDCl_3$.

Removal of the 3′-levulinyl group in the 2′-*O*-TBDMS dimer nucleotide **(6)** was monitored for 22 hours. Under the conditions used, complete cleavage of the levulinyl group from dimer **(6)** was observed after 2.5 min (Figure 3.4 A). This is in stark contrast with the delevulination of monomer **(11)** which is completed after 20 min (Figure 3.3).

Dramatic difference was also observed in the isomerization rates of nucleoside **(12)** when compared to dimer **(7)**. Upon closer inspection of the NMR data in Figure 3.4, we noticed the appearance of a shoulder at 0.23 and another at 0.48 ppm after 20 min. The shoulders increased substantially overtime. After comparison with the ^{31}P-NMR of the 3′-*O*-TBDMS regioisomer, the shoulders were identified to be the resonances of the isomeric 3′-*O*-TBDMS dimer (Figure 3.4).

The data also showed that the methyl group is partially cleaved by the hydrazine/pyridine/acetic acid reagent, converting the chiral phosphotriester linkage (two diastereoisomers, Rp and Sp) into an achiral phosphodiester linkage (one isomer). The emergence of the demethylated product became apparent after 80 min (Figure 3.3).

This poses an interesting question: why is the isomerization rate of the monomer so different from that of dimer? Likely, the 5′- phosphate triester affects the 3′-hydroxyl reactivity, but how? Potential explanations include inductive effect, solvation effect, and/or conformational and stereoelectronic changes that allow for increased reactivity as well as steric bulk. The relevant information extracted from these experiments is that the delevulination conditions cause TBDMS isomerization much more readily on dimers than on monomers.

3.2.4 Various delevulination conditions for 2′-*O*-TBDMS dimer (6); Attempts in avoiding isomerization

As indicated above, ^{31}P-NMR proved to be a very useful technique to determine the extent of 2′ to 3′ TBDMS isomerization in rUpU dimer **(6)**. This allowed us to quickly evaluate various delevulination conditions in an attempt to develop conditions that do not lead to isomerization.

We tested several different conditions, where we varied the concentration and equivalents of hydrazine, the pyridine/acetic acid ratio, and work-up conditions. The conditions used are summarized in Table 3.1. Unfortunately, we observed that while some conditions slightly reduced the isomerization, each attempt resulted in approximately 3-10%, and it was never completely eliminated.

Equivalents of hydrazine	**1**	**1.5**	**2**	**2.5**	**10**	**10**
Concentration of hydrazine (M)	**0.1**	**0.5**	**0.2**	**0.2**	**0.1**	**0.5**
Solvent Conditions						
Py:AcOH 3:2	**X**	**X**	**X**	**X***	**X**	**X**
Py:AcOH 3:3				**X**	**X**	
THF				**X**	**X**	
Work-up Conditions						
Direct concentration of reaction mixture	**X**	**X**	**X**	**X***	**X**	**X**
NH_4Cl extraction	**X**	**X**	**X**	**X**	**X**	**X**
Crude mixture placed directly on silica gel column				**X**		**X**
$CuSO_4$ extraction to remove pyridine				**X***	**X**	**X**

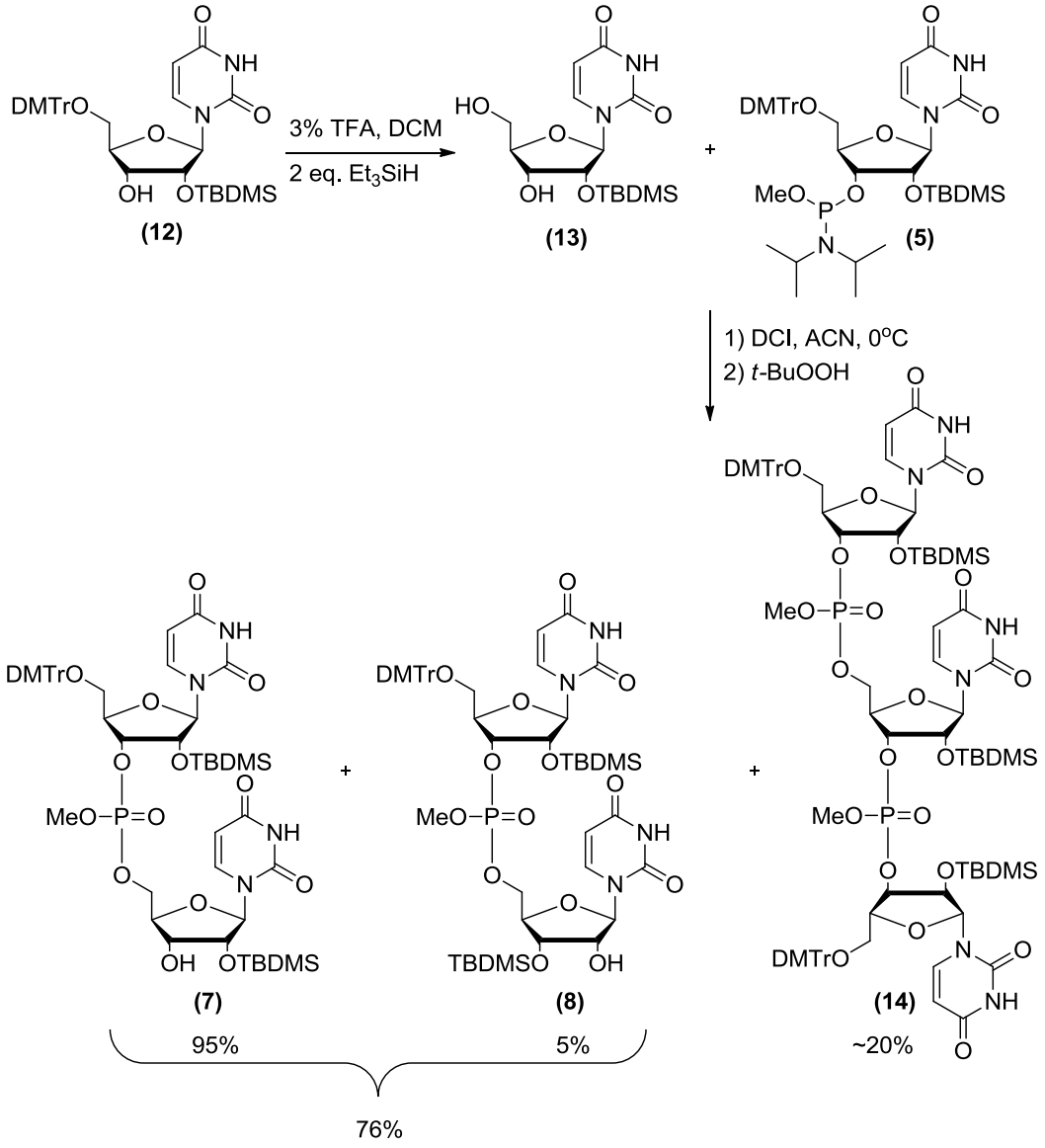
Table 3.1. Conditions tested for removal of 3′-*O*-Lev group from dimer nucleotide **(6)**. The concentration of dimer **(6)** was 0.1 M in all reactions. In all cases the excess hydrazine was quenched with 2,4-pentanedione. *Reaction and work-up were done at 0°C. "X" indicates the conditions that were tried; in all cases isomerization was detected to approximately the same extent.

3.2.5 Alternative approaches for the synthesis of dimer nucleotides

The various conditions of delevulination of the 2′-*O*-TBDMS dimer nucleotide described Table 3.1 led to the formation of the inseparable 3′-*O*-TBDMS isomer. Thus, we decided to develop alternative approaches, described

below, to synthesize dimer nucleotides. Two of these circumvent the use of 3′-protection completely and rely on the greater steric bulk around the secondary hydroxyl to reduce its reactivity, allowing selective coupling with a phosphoramidite at the less unhindered 5′-hydroxyl.

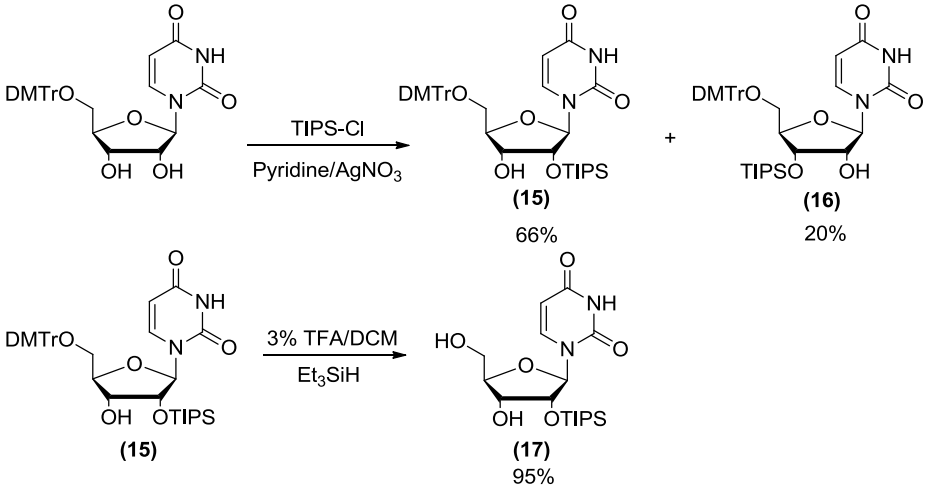
3.2.5.1 Regioselective coupling of 2′-*O*-TBDMS uridine (no 3′-protecting group) to uridine phosphoramidite



Scheme 3.6. Synthesis of [5′-*O*-DMTr-2′-*O*-TBDMS-U]-[3′-p(OMe)-5′]-[U-2′-*O*-TBDMS] dimer **(7)** using 5′-OH-3′-OH-2′-*O*-TBDMS-uridine mononucleoside **(13)**. Attempt at selective phosphoramidite coupling to the 5′-OH over the 3′-OH group in mononucleoside **(13)**.

An alternative synthetic route tested circumvented the use of the 3′-*O*-Lev protecting group altogether, described in Scheme 3.6. This in turn would avoid the isomerization that occurs during the hydrazinolysis conditions. The main step is the coupling of a fully protected phosphoramidite preferentially to the primary 5′-hydroxyl group over the more sterically hindered 3′-hydroxyl group of 2′-*O*-TBDMS uridine (Scheme 3.6). First, 2′-*O*-TBDMS uridine **(13)** was obtained by treating 5′-*O*-DMTr-2′-*O*-TBDMS-uridine with 3% TFA in DCM. Next, 5′-*O*-DMTr-2′-*O*-TBDMS-uridine-3′-methylphosphoramidite **(5)** was pre-activated with DCI, and added dropwise to a stirred solution of nucleoside **(13)** in ACN at °0 C. The resulting mixture was allowed to warm up to room temperature and then oxidized with *tert*-butyl hydroperoxide *in situ* to provide **(7)**. Upon isolation of the products, the isomerised dimer **(8)** was detected (5%) along with trimer **(14)** (~20%). The desired dimer **(7)** was obtained in 71 % yield after separation by column chromatography. Notably, no strictly 3′-3′ linked dimer was detected.

3.2.5.2 Regioselective coupling of 2′-*O*-TIPS uridine (no 3′-protecting group) to uridine phosphoramidite



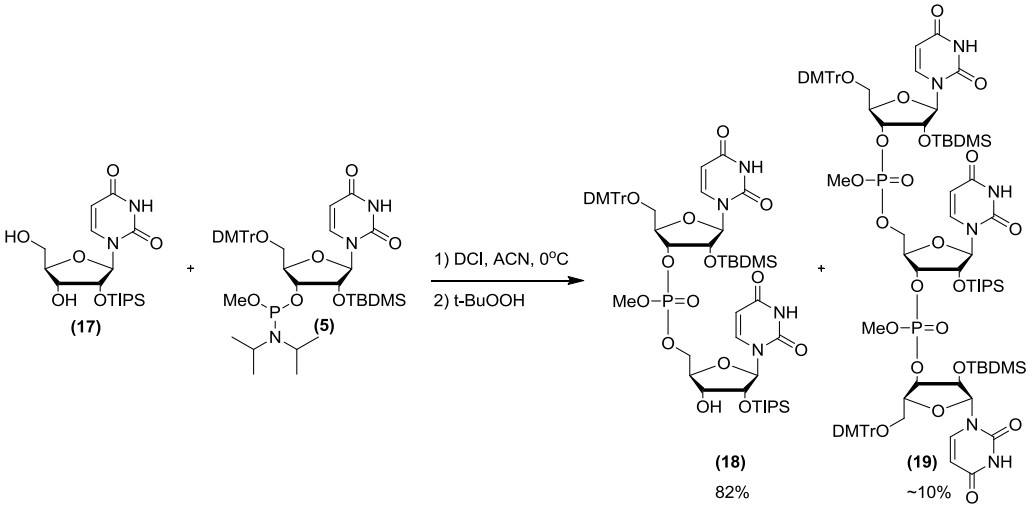
Scheme 3.7. Synthesis of 2′-*O*-TIPS uridine **(17)**.

We envisioned that the formation of the trimer by-product **(14)** could be significantly reduced by increasing the steric bulk around the 3′-hydroxyl group in the nucleoside monomer. This, in turn, should also reduce or eliminate the silyl

migration observed. Hence we went on to prepare 2′-*O*-TIPS uridine **(17)** as outlined in Scheme 3.7. The bulky triisopropylsilyl (TIPS) group was considered because it had successfully been used for protecting the 2′-hydroxyl group of guanosine monomers.[17,21,22]

Silylation of 5′-*O*-DMTr uridine with TIPS-Cl in the presence of pyridine/$AgNO_3$ afforded the two regioisomers 5′-*O*-DMTr-2′-*O*-TIPS uridine **(15)** and 5′-*O*-DMTr-3′-*O*-TIPS uridine **(16)** in 66% and 20% yield, respectively. The regioisomers were easily separated by silica gel column chromatography. The position of the TIPS group in isomers **(15)** and **(16)** was confirmed by heteronuclear chemical shift correlation (HETCOR), where we monitored the coupling of ^{29}Si (-4.84 ppm) to $^{1}H2'$ (4.4 ppm). The 5′-*O*-DMTr group was removed from **(15)** using 3% trifluoroacetic acid (TFA) at room temperature for 5 min followed by addition of triethylsilane and excess methanol, yielding the desired 2′-*O*-TIPS mononucleoside **(17)** in 95%.

We next proceeded with the coupling of 5′-*O*-DMTr-2′-*O*-TBDMS-uridine-3′-methylphosphoramidite **(5)** with 2′-*O*-TIPS uridine **(17)** (Scheme 3.8). The reaction was carried out using the same conditions described in Section 3.2.5.1. TIPS protection afforded increased yield of dimer **(18)** (82 %), less of the undesired trimer byproduct **(19)** (~10 %), with no indication of isomerization.

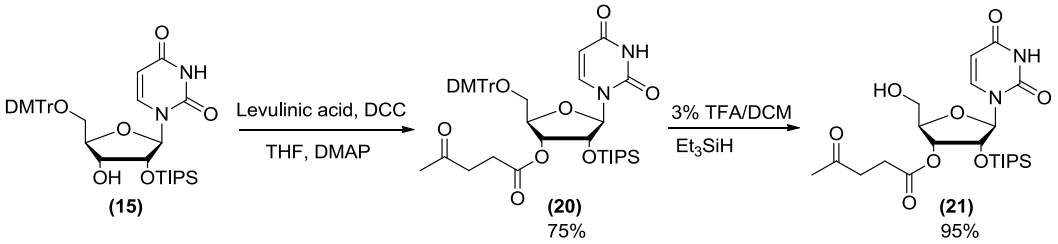


Scheme 3.8. Synthesis of [5′-*O*-DMTr-2′-*O*-TBDMS-U]-[3′-p(OMe)-5′]-[U-2′-*O*-TIPS] dimer **(18)** using 5′-OH-3′-OH-2′-*O*-TIPS-uridine mononucleoside **(17)**. Selective phosphoramidite coupling to the 5′-OH over the 3′-OH group in mononucleoside **(17)**.

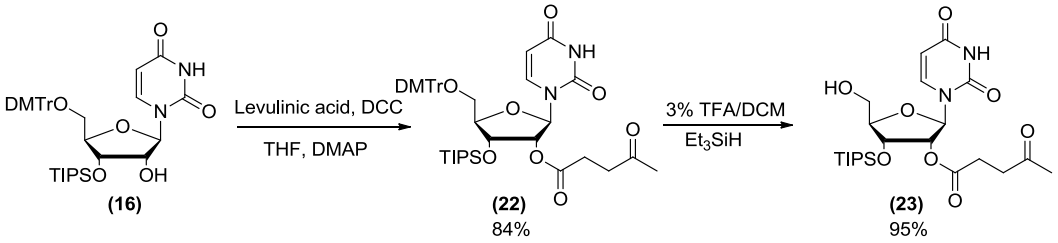
3.2.5.3 Use of 2′-*O*-TIPS and 3′-Lev protecting groups in the mononucleoside used for dimer nucleotide synthesis

As shown above, TIPS protection provides a great improvement over TBDMS protection with respect to isomerization and coupling regioselectivity. Also, unlike TBDMS, the TIPS group is uniquely resistant to silyl isomerization during the delevulination conditions. While the TIPS regioselective approach eliminated isomerization, it led to 10% of trimer by-product **(19)**, reducing the yield of the desired dimer. In addition, special care had to be exercised when combining the monomers. Therefore, instead of optimizing the reaction further, we resorted again to 3′-Lev protection, using 2′-*O*-TIPS in the place of 2′-*O*-TBDMS protection (Scheme 3.3).

The required monomer, 2′-*O*-TIPS-3′-*O*-Lev uridine **(21)**, was obtained by treating **(15)** with levulinic acid and dicyclohexylcarbodiimide (DCC), followed by treatment with TFA as shown in Scheme 3.9. No isomerization of the 2′-*O*-TIPS group occurred during these reactions. This was verified by comparison with an authentic sample of the isomeric compound **(23)** (prepared as outlined in Scheme 3.10).



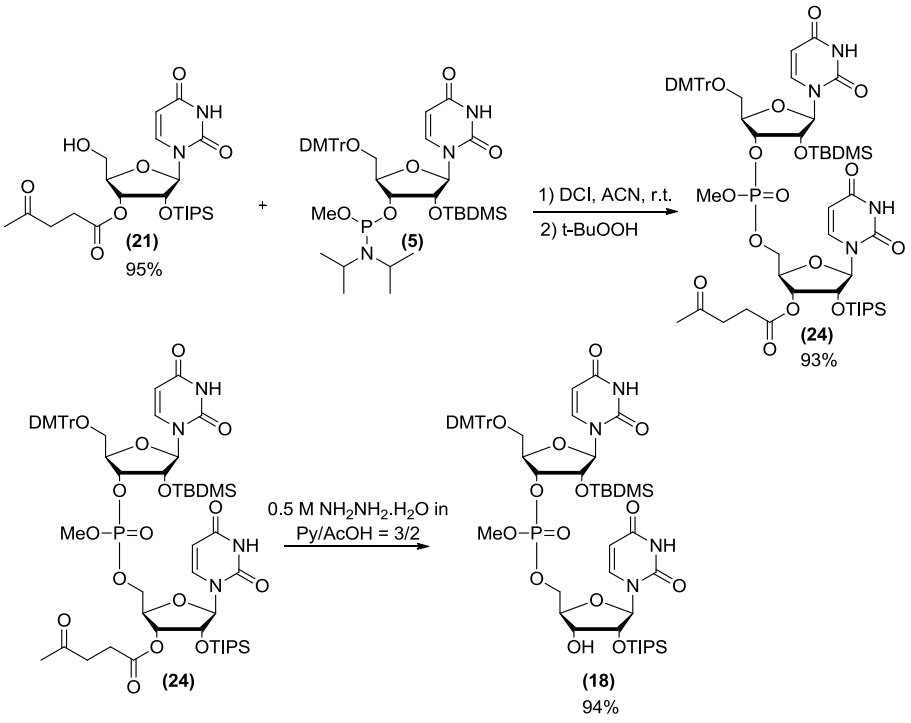
Scheme 3.9. Synthesis of 2′-*O*-TIPS-3′-*O*-Lev uridine **(21)**.



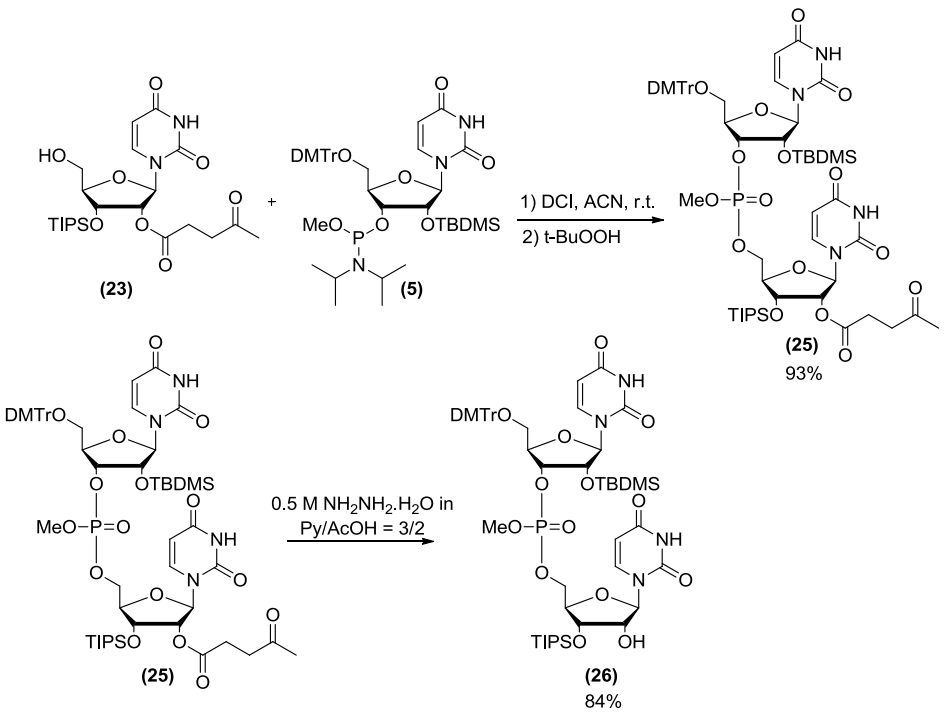
Scheme 3.10. Synthesis of 2′-*O*-Lev-3′-*O*-TIPS uridine **(23)**

Next, both the 2′ and 3′-*O*-TIPS dimer **(18)** and **(26)** were synthesized according to Scheme 3.11 and Scheme 3.12, respectively. Coupling of

phosphoramidite **(5)** with 2′-*O*-TIPS-3′-*O*-Lev uridine **(22)** (Scheme 3.11) or 2′-*O*-Lev-3′-*O*-TIPS uridine **(23)** (Scheme 3.12) in the presence of DCI, followed by *in situ* oxidation with 6M *tert*-butyl hydroperoxide afforded dimers **(24)** and **(25)**, both in 93 % yield. Further treatment with a 0.5 M (10 eq) solution of hydrazine hydrate in pyridine/acetic acid (3:2 v/v) for 10 min afforded dimers **(18)** and **(26)** quantitatively. Under these conditions no isomerization of the TIPS protecting group was detected. The issue of isomerization is thoroughly investigated in the following section.



Scheme 3.11. Synthesis of dimer **(18)** starting from 2′-*O*-TIPS-3′-*O*-Lev uridine **(21)**.



Scheme 3.12. Synthesis of control dimer **(26)** starting from 2′-*O*-Lev-3′-*O*-TIPS uridine **(23)**.

3.2.5.4 TIPS: isomerization study

The use of the TIPS protecting group circumvented its migration to the vicinal hydroxyl position during delevulination. This is true when the hydrazine treatment is carried out within a short period of time; however, isomerization and eventually demethylation occurs if the dimers are treated with the hydrazine reagent for extended periods of time (e.g., hours) as established by ^{1}H-NMR and ^{31}P-NMR (see Section 3.2.3).

3.2.5.5 ^{1}H-NMR study - isomerization of (15)

5′-*O*-DMTr-3′-*O*-Lev-2′-*O*-TIPS uridine **(20)** was treated with 0.25 M hydrazine hydrate (3/2 pyridine/acetic acid buffer) in deuterated solvent and the formation of **(15)** was monitored over time by ^{1}H-NMR. Complete delevulination of the nucleoside **(20)** was observed after 40 min, as indicated by the disappearance of the peaks at 4.4 and 5.1 ppm (Figure 3.5). The corresponding TBDMS monomer (11) is deprotected within 20 min under the same conditions,

probably reflecting the smaller steric bulk of TBDMS vs TIPS. No isomerization of **(15)** occurred during the delevulination step since none of the resulting resonances corresponded to those seen for isomer **(16)**.

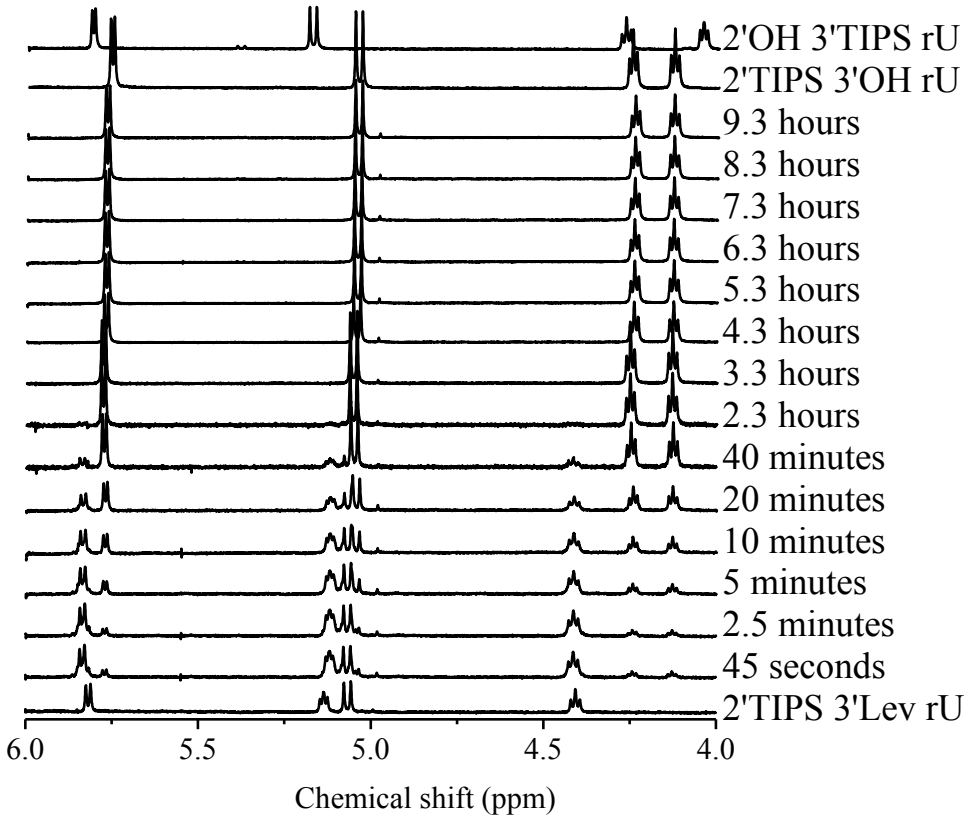


Figure 3.5. ^{1}H-NMR study of 2′ to 3′ TIPS migration in 5′-*O*-DMTr-2′-*O*-TIPS-U mononucleoside **(15)** under delevulination conditions (0.25 mM hydrazine hydrate in 1/1 mixture of deuterated chloroform and 3/2 pyridine/acetic acid buffer). Delevulination of 5′-*O*-DMTr-3′-*O*-Lev-2′-*O*-TIPS-U mononucleoside **(20)** was complete after 40 min. No isomerization was observed for up to 9.3 hours.

3.2.5.6 ^{31}P-NMR study – isomerization of dimer nucleotide (18)

Cleavage of the levulinyl group from dimer **(24)** was carried out under the previously described conditions for nucleoside **(20)** (Figure 3.6). Complete cleavage of the levulinyl group occurred within 20 min, as indicated by the disappearance of the peaks at 0 and 0.5 ppm and the appearance of two new peaks (0.25 and 0.49 ppm) corresponding to the delevulinated product **(18)**. As for

TBDMS protection, delevulination occurred more rapidly in the dimer form than in the monomer form.

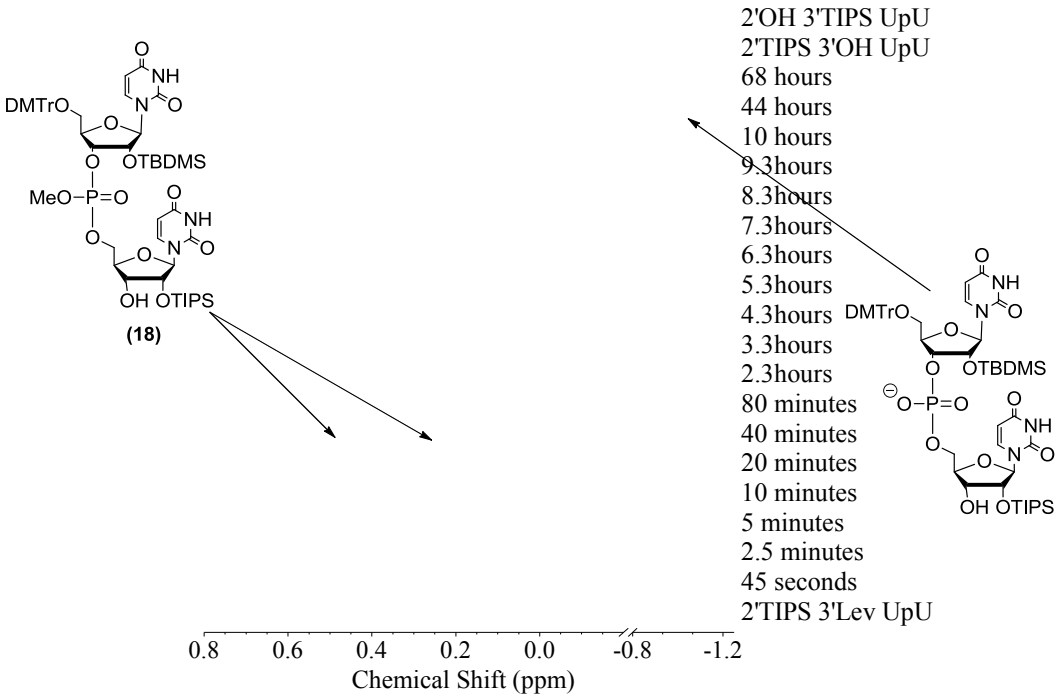


Figure 3.6. ^{31}P-NMR analyses of 2′ to 3′ TIPS isomerization in [5′-*O*-DMTr-2′-*O*-TBDMS-U]-[3′-p(OMe)-5′]-[U-2′-*O*-TIPS] dimer **(18)** under delevulination conditions (0.25 M final hydrazine hydrate concentration in 1/1 mixture of deuterated chloroform and 3/2 pyridine/acetic acid buffer). The deprotection reaction of [5′-*O*-DMTr-2′-*O*-TBDMS-U]-[3′-p(OMe)-5′]-[U-3′-*O*-Lev-2′-*O*-TIPS] dimer **(24)** was complete after 10 min. No isomerization was detected for up to 68 hours.

While detectable isomerization of the TBDMS group was observed within 20 min, the TIPS dimer **(18)** was resistant to silyl isomerization for at least 44 h under the same conditions. A tiny amount (ca. 1%) isomerization was detected after 68 hours (^{31}P NMR detection limit: 0.4 mol %). As for TBDMS protection, the emergence of demethylated products in the TIPS dimer became apparent after 2.3 h by the appearance of the single resonance at -1.00 ppm; demethylation of the TBDMS dimer occurred after only 40 min. The presence of a single peak for

the TIPS demethylated dimer also confirmed that no isomerization occurred during the delevulination step.

3.2.6 Effect of reaction work-up conditions on the 2′ to 3′ silyl isomerization in TBDMS and TIPS protected dimers

To carry out delevulination reactions within a reasonable timeframe, the above mentioned NMR studies were conducted using lower concentrations of reagents than those used in the actual synthetic reactions (i.e., 0.25M instead of 0.5M hydrazine). Additionally, in the NMR experiments no work-up of the reaction was performed. Thus, we were interested in determining the extent of silyl isomerization under "real" reaction conditions, followed by work-up, for both TBDMS and TIPS protected dimer **(6)** and **(24)**.

Four identical hydrazine reactions were set up, two with compound **(6)** and two with (**24)**. The reaction conditions were: 0.08 M dimer, 0.25 M hydrazine hydrate in 1/1 acetonitrile with pyridine/ acetic acid buffer (3/2). The reaction times were 10 minutes or 42 hours for each dimer. The work-up was the same for all reactions, where the excess hydrazine was quenched at 0 °C with 10 equivalents of 2,4-pentanedione,[5] followed by 5% aqueous ammonium chloride wash. After the work-up, all four reaction mixtures were subjected to ^{31}P-NMR. The results are summarized in Figure 3.7

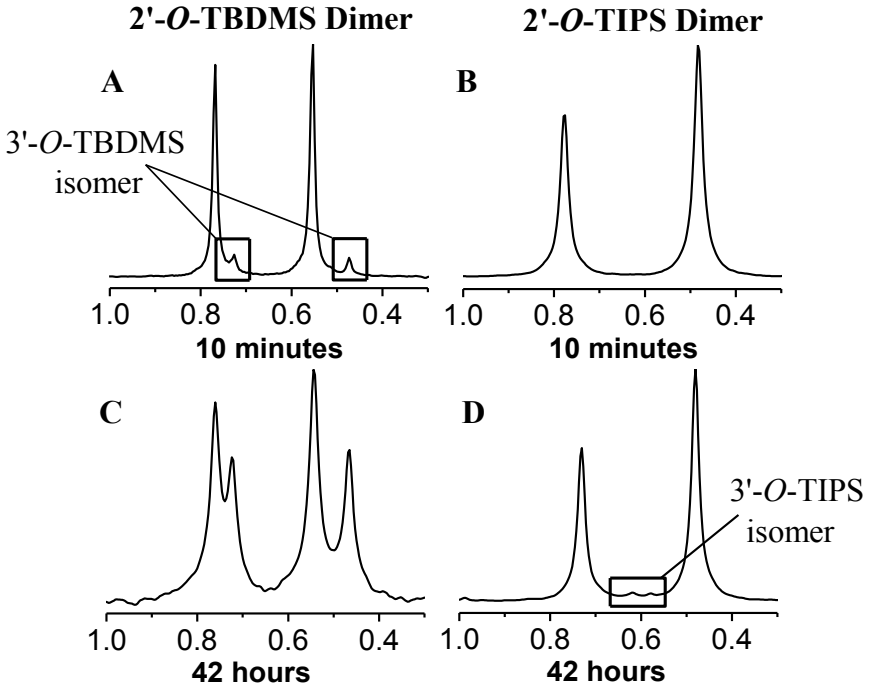


Figure 3.7. ^{31}P-NMR spectra of dimer nucleotides **(6)** and **(24)** subjected to delevulination (0.08 M dimer, 0.25 M hydrazine hydrate buffer) A) 2′-*O*-TBDMS dimer after 10 min delevulination (isomerization detected); B) 2′-*O*-TIPS dimer after 10 min delevulination (no isomerization); C) 2′-*O*-TBDMS dimer after 42 h delevulination (increased isomerization); D) 2′-*O*-TIPS dimer after 42 h delevulination (very little isomerization detected). For better peak separation and visualization, the NMR for 2′-*O*-TBDMS dimer **(6)** was acquired in deuterated acetonitrile, whereas the spectra for 2′-*O*-TIPS dimer **(24)** was acquired in deuterated chloroform.

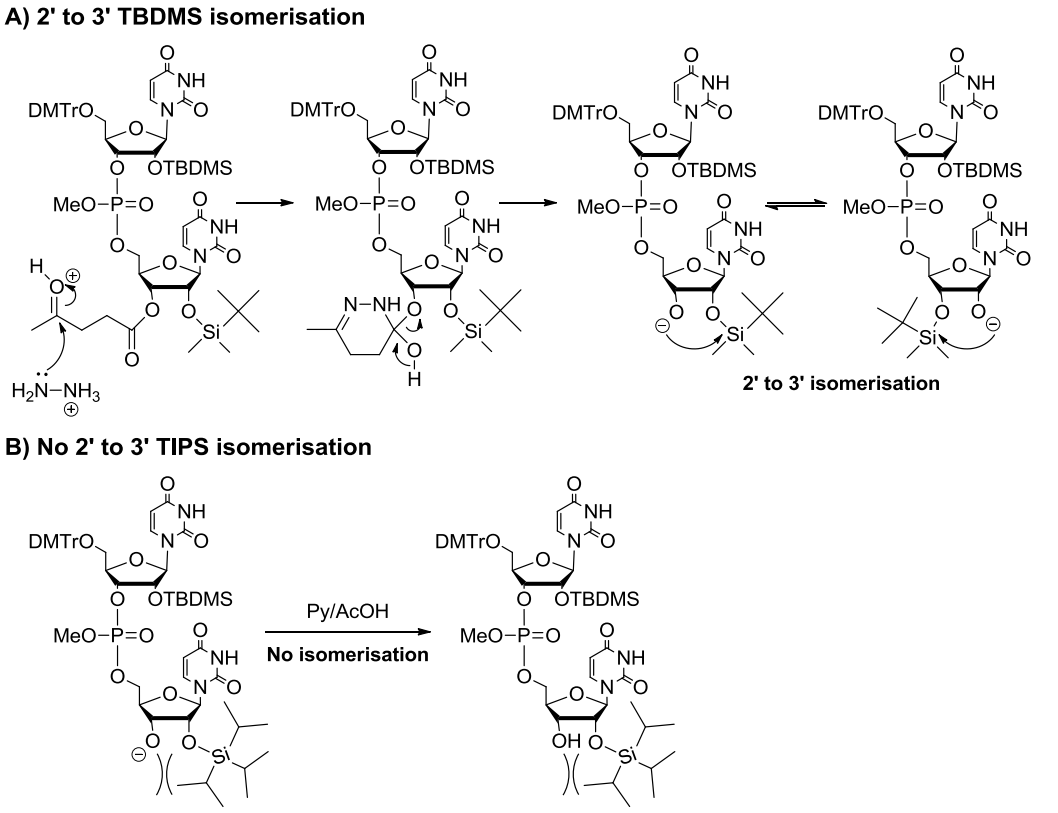
The ^{31}P-NMR spectra shown in Figure 3.7 indicate that the TBDMS protected dimer **(6)** undergoes significant silyl isomerization, whereas the TIPS protected dimer **(24)** is not affected by either the reaction time or work-up conditions. There is a detectable amount of isomerization occurring for the 2′-*O*-TBDMS dimer after 10 min (Figure 3.7 A), but no measurable amount was observed for the 2′-*O*-TIPS protected dimer (Figure 3.7 B). After 42 hours of reaction, up to 40% of the delevulination product had isomerized for the 2′-*O*-TBDMS dimer (Figure 3.7 C). For the 2′-*O*-TIPS dimer even isomerization was barely detectable even after 42 hours of reaction (Figure 3.7 D). Additional information gained from this experiment is that isomerization is not affected by the work-up conditions used but rather it depends on the time the deprotected

dimer is exposed to the delevulination reagents. Comparison between the two protecting groups illustrates the higher resistance to isomerization of the TIPS group. This further confirms that TIPS protected dimers in combination with the 3′-*O*-levulinyl protecting group are significantly more promising for obtaining isomerically pure compounds.

In summary, these experiments confirmed that the use of TIPS protection is significantly more likely to produce isomerically pure dimers within the time required for delevulination. Since removal of the levulinyl group of TIPS dimer is complete within 10 min, there is little chance of either isomerization or demethylation during the conversion of dimer **(24)** into **(18)** and neither was observed.

3.2.7 Proposed explanation for contrasting migration behavior of TBDMS vs TIPS groups

In the ^{31}P-NMR studies we conducted with 2′-*O*-TBDMS and 2′-*O*-TIPS protected dimer nucleotides **(7)** and **(18)** under delevulination conditions, we observed a much higher resistance to 2′ to 3′ silyl migration when the more sterically hindered TIPS group was employed. We looked into the mechanism of 3′-*O*-Lev deprotection in order to elucidate the reasons for this difference in reactivity (Scheme 3.13). We suggest that the 2′ to 3′ isomerization occurs following an intramolecular attack of the 3′-alkoxide to the 2′-*O*-silyl group. However, the delevulination reaction is done in pyridine/ acetic acid buffer which is likely to protonate the 3′-alkoxide, forming a hydroxyl group with diminished nucleophilicity. We can conclude that the rates of these two competing processes would determine the extent of silyl isomerization. We propose that due to the great steric bulk and lipophilicity of the TIPS group, the protonation of the 3′-alkoxide out-competes its nucleophilic attack on the Si in the 2′-*O*-TIPS group, leading to a much slower isomerization. The 2′-*O*-TBDMS group is less sterically hindered, thus allowing for a faster nucleophilic attack from the adjacent 3′-alkoxide and favoring isomerization.



Scheme 3.13. Proposed mechanism of 2′ to 3′ silyl migration in dimer nucleotides. A) Isomerization upon delevulination of dimer **(6)**; B) Dimer is resistant to silyl migration due to increased bulk of TIPS versus TBDMS.

Additionally, we observed throughout the ^{1}H and ^{31}P-NMR delevulination studies that the dimer nucleotides are more susceptible to 2′ to 3′ silyl isomerization than the corresponding mononucleotide units. This difference in reactivity could be explained with the possible difference in the ribose conformation in the monomer vs. the dimer. The ribose in a fully deprotected mononucleoside adopts more of a north conformation, where the 2′-OH is in an pseudoaxial position and the 3′-OH is in an pseudoequatorial position (See Figure 3.8 A for clarity). We would thus expect that the 5′-*O*-DMTr-3′-OH-2′-*O*-silyl nucleoside would have a conformation close to the standard northern conformation as well. This would keep the vicinal 2′ and 3′ hydroxyls farther apart and out of plane in respect to each other, impeding the nucleophilic attack of the 3′-OH to the 2′-*O*-SiR_3 (See Figure 3.8 B). In contrast, we suggest that the

presence of the 5′-phosphate as well as a tritylated nucleoside, in the dimer nucleotide, would force the sugar ring into a more eastern orientation (Figure 3.8A). This would place the 2′-OH and the 3′-OH groups in a more eclipsed orientation, closer to each other, which would facilitate the nucleophilic attack of the 3′-OH to the 2′-*O*-SiR_3 and thus the silyl migration. In order to support this theory, further studies are required including calculations of the most energetically favored conformations of the monomers and dimers via NMR and PSEUROT.[23]

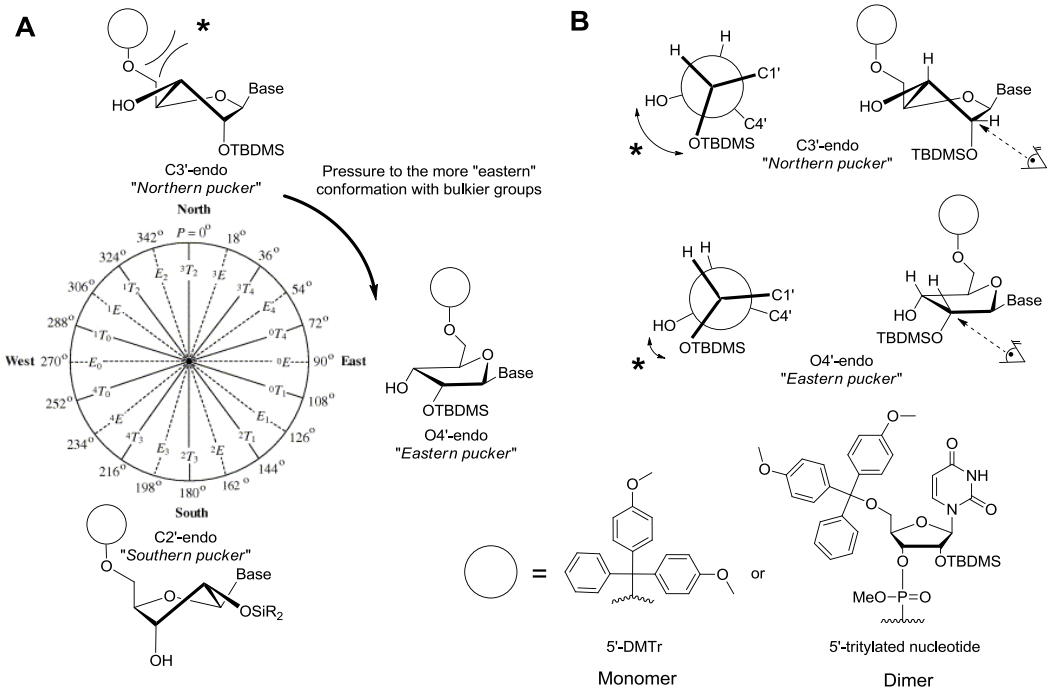
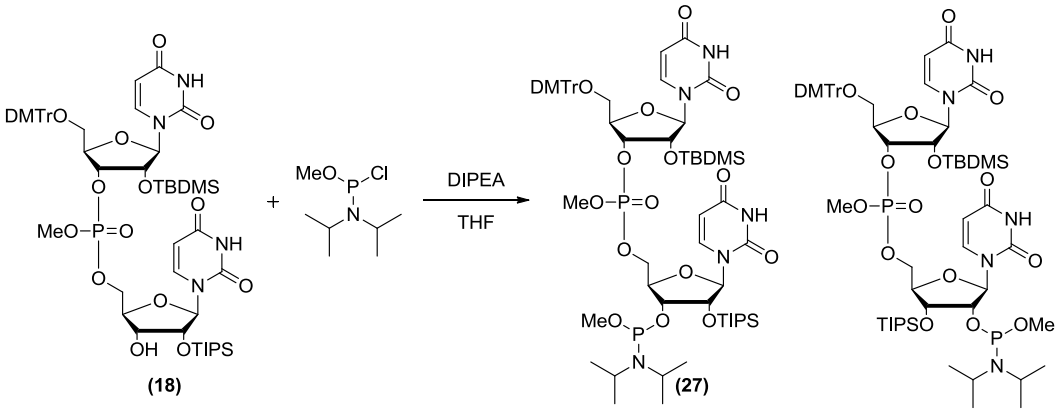


Figure 3.8. A) Pseudorotational furanose wheel illustrating the possible conformation shift of ribose with a bulky 5′-group from "north" to a more "eastern" conformation. B) Newman projection through the C2′ to the C3′ of the North and East furanose conformations. This shows the dihedral angle reduction between the 2′ silylated hydroxyl and the 3′ hydroxyl in the eastern conformation, a possible explanation for the observed increased silyl migration rates of TBDMS versus TIPS in monomer and dimers.

3.2.8 Phosphitylation of dimer (18)

2′-*O*-TIPS dimer **(18)** was first subjected to standard phosphitylation conditions,[24] namely N,N-diisopropylaminomethoxyphosphonamidic chloride in the presence of the non-nucleophilic base DIPEA (Scheme 3.14). While this method produced the desired phosphitylated dimer **(27)**, the reaction required over

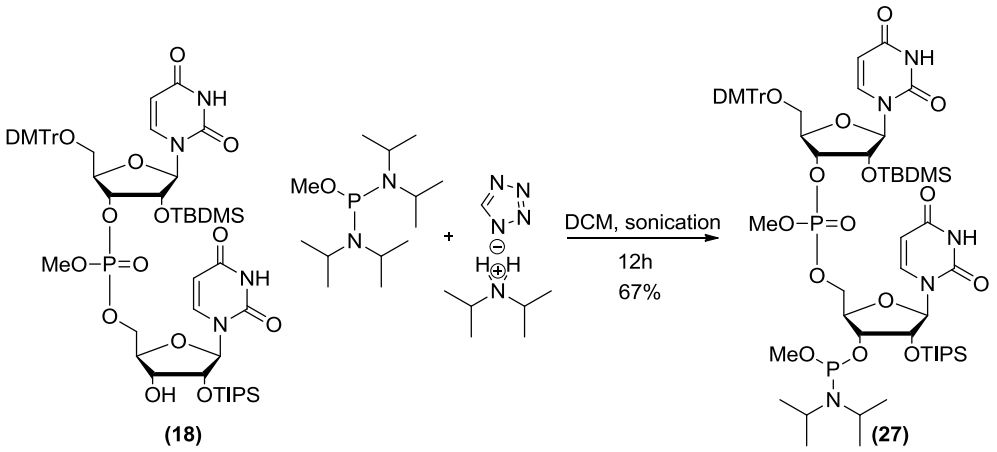
12 hours to go to completion. This length of time in basic media led to small amount (~5% by ^{31}P-NMR) of 2′ to 3′ TIPS isomerization, producing an inseparable mixture. To avoid isomerization, the reaction had to be quenched and worked-up within 4 hours, however, this resulted in considerable amounts of un-reacted starting dimer, lowering the overall yield.



Scheme 3.14. Phosphitylation of dimer **(18).**

An alternative phosphitylation method involves the use of bis-(N,N-diisopropylamino)-methoxyphosphine in the presence of a less basic activator, namely N,N-diisopropylammonium tetrazolide. Commonly referred to as the "acidic" method of phosphitylation,[25] it requires the conjugate acid of DIPEA paired with a weak base (tetrazolide). Caruthers and co-workers showed that N,N-diisopropylammonium tetrazolide activates only one of the two N,N-diisopropylamine moieties, allowing for high fidelity mono-substitution of the phosphine reagent (Scheme 3.15).[25]

To test the viability of the acidic phosphitylation method, the tetrazolide salt was combined with dimer **(18)** in DCM and the mixture allowed to stir for 12h at room temperature with no detectable silyl migration.



Scheme 3.15. Phosphitylation of [5′-*O*-DMTr-2′-*O*-TBDMS-U]-[3′-p(OMe)-5′]-[U-2′-*O*-TIPS] dimer **(18)** via the acidic phosphitylation method.

Sonication was necessary to speed up the reaction by breaking up the insoluble tetrazolide salt. After work up and purification, the desired dimer **(27)** was isolated in 67 % yield. Since compound **(27)** is a mixture of four diastereomers, its NMR spectrum displays multiple 8 peaks in the ^{31}P-NMR spectrum; 4 peaks in the phosphate region (0-1 ppm) and 4 peaks in the phosphoramidite region (151-154 pm) (Figure 3.9). The isomeric purity was confirmed by synthesis and isolation of the opposite regioisomer (3′-*O*-TIPS phosphitylated dimer) and comparison of the corresponding ^{31}P-NMR spectra (not shown). The data confirmed that, within the detection limit of the NMR experiment, no silyl migration had occurred.

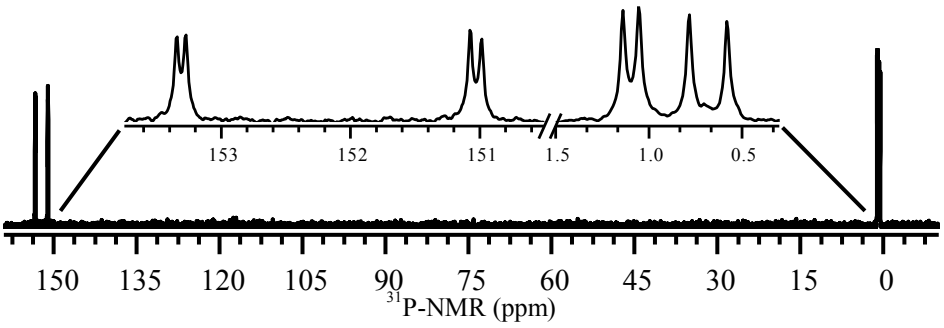
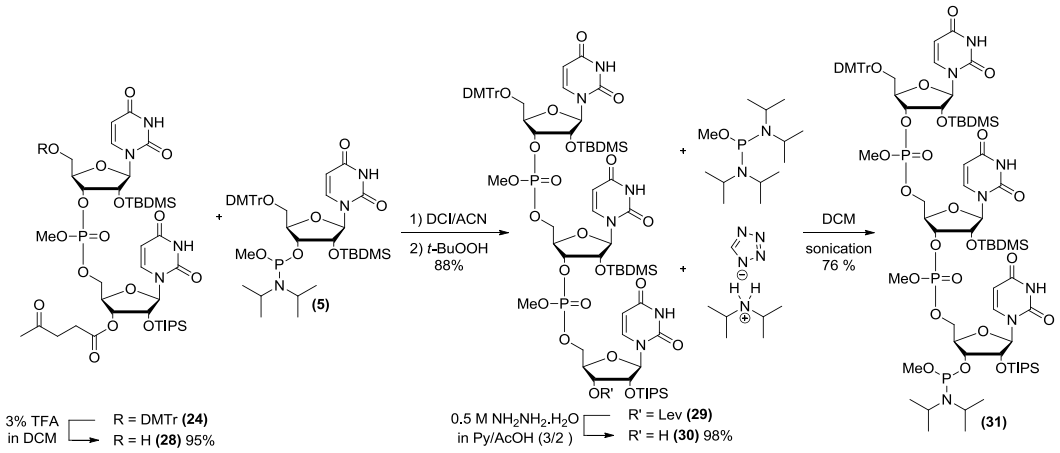


Figure 3.9. ^{31}P-NMR of rUpU dimer phosphoramidite **(27)**.

3.2.9 Synthesis of trimer ribonucleotide phosphoramidites.

Once we have established a viable way to synthesize RNA dimer phosphoramidite blocks, we proceeded with the synthesis of RNA trimer phosphoramidites. The synthetic procedure is outlined in Scheme 3.16.



Scheme 3.16. Synthesis of trimer phosphoramidite, rUpUpUp **(31)**.

The fully protected dimer nucleotide **(24)** was subjected to 5′-detritylation by treatment with 3% TFA in DCM to afford **(28)** in 95% yield. Dimer **(28)** was then coupled to phosphoramidite **(5)** using standard coupling conditions (DCI activation), followed by *in situ* oxidation with t-BuOOH to produce fully protected trimer ribonucleotide **(29)** in 88% yield. Subsequent 3′-delevulination, followed by 3′-phosphitylation using the acidic phosphitylation method afforded the desired trimer phosphoramidite rUpUpU **(31)** in 80% yield (over two steps) without any detectable 2′ to 3′ silyl isomerization (Scheme 3.16).

The ^{31}P-NMR spectra in $CDCl_3$ of the delevulinated trimer **(30)**, as well as the phosphitylated trimer **(31)**, confirmed the isomeric purity if the isolated compounds (Figure 3.10 and Figure 3.11).

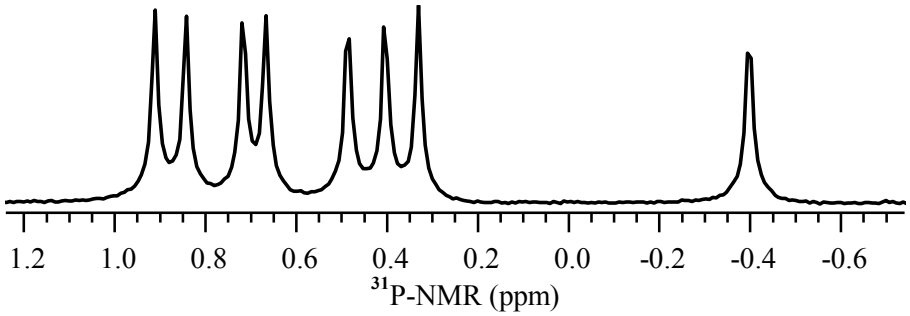


Figure 3.10. ^{31}P-NMR of delevulinated trimer rUpUpU **(30)**. The eight peaks observed is due to the diastereomeric nature of this compound (total of 2^2 =4 isomers, each exhibiting 2 signals (total = 8 resonances).

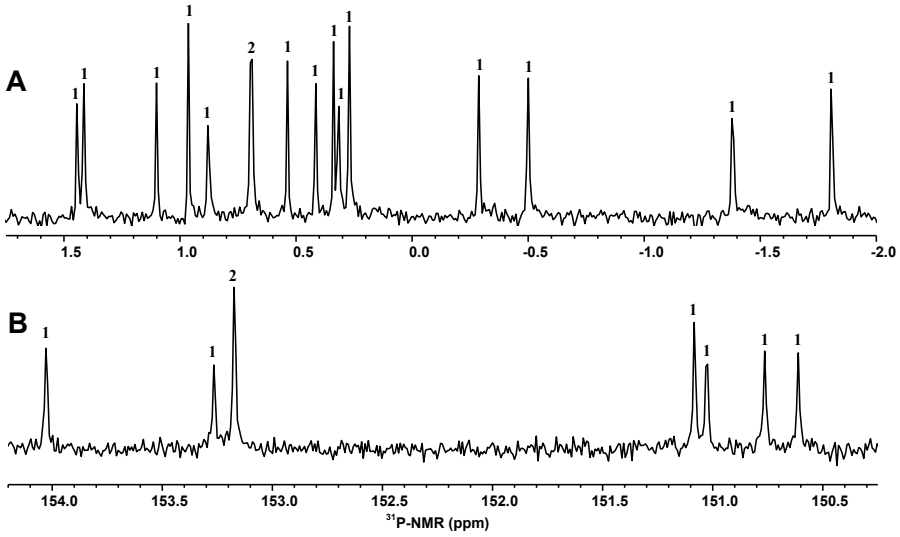
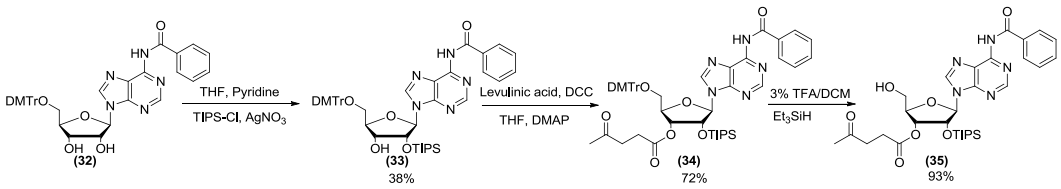


Figure 3.11. ^{31}P-NMR of trimer phosphoramidite **(31)**. This compound consist of a mixture of 8 diastereomers. The chiral phosphorus moieties provide 2^3 = 8 isomers, each exhibiting 3 signals (total = 24 resonances). A) Up-field region showing the expected 16 phosphate resonances B) Downfield region showing the expected 8 phosphoramidite resonances. Peak integrations are shown above the peaks.

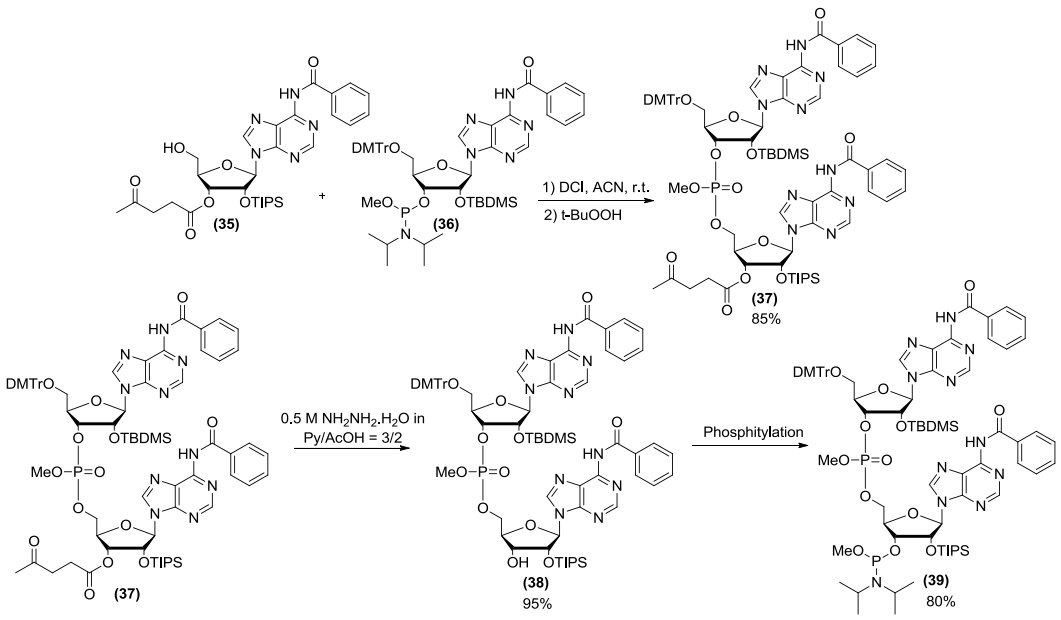
3.2.10 Synthesis of purine dimer phosphoramidite (rApAp)

To ensure that the dimer phosphoramidite synthetic method described above applies to both pyrimidines and purines, we also synthesized the adenosine dimer phosphoramidite (Scheme 3.17 and 3.18).



Scheme 3.17. Synthesis of the 2′-*O*-TIPS protected adenosine nucleoside monomers.

The synthesis proceeded smoothly, starting from N-benzoly-5′-*O*-DMTr adenosine. Treatment with TIPS chloride in the presence of pyridine and silver nitrate afforded a mixture of 2′ and 3′-*O*-TIPS regioisomers that was easily separated by column chromatography. Levulination of the free 3′ hydroxyl was achieved by DCC mediated coupling of levulinic acid/DMAP and **(33)**. Here we noted that if DCC and levulinic acid are added in large excess, partial levulination of the protected exocyclic amine occurs. Lastly, the 5′-*O*-DMTr protecting group was removed by treatment with 3% TFA in DCM in the presence of triethylsilane to give the required compound **(35)** in very good yield.



Scheme 3.18. Synthesis of 2′-*O*-TIPS protected adenosine phosphoramidite dimer **(39)**.

The rApA dimer was synthesized, as with the uridine derivative, by coupling **(35)** with amidite **(36)** in the presence of DCI. Delevulination and phosphitylation gave dimer phosphoramidite in 80% yield.

3.2.11 Dimer and trimers as probes for examining the interaction of RNA with the 5′ binding MID domain of human argonaute protein (hAGO2).

As described in the Introduction (Chapter 1), small interfering RNAs (siRNAs) can trigger potent gene silencing through the RNA interference (RNAi) pathway. The RISC complex is key to this targeted mRNA degradation, and the human Argonaute2 (hAgo2) endonuclease component of RISC is responsible for the actual mRNA cleavage event. During RNAi, hAgo2 becomes loaded with the siRNA guide strand, making several key nucleic acid-enzyme interactions. Native and chemically modified siRNAs are now widely used, and understanding how these compounds interact with hAgo2 has become particularly important.

Previous research into the binding conformations and affinities of oligonucleotides in the 5′ binding MID domain of hAgo2 were focused on 5′ mononucleotides and purified hAgo2 MID domain.[26] To further these studies and expand this model system into a more biologically relevant context, we produced dinucleotide and trinucleotide molecules suitable for NMR titration and crystallography, which served as better mimics of true siRNA strands in this model system than the previously studied nucleoside 5′-monophosphates.

In collaboration with Dr. Bhushan Nagar (Biochemistry Dept., McGill University) our laboratory examined the binding affinities of both chemically modified dimers (Glen Deleavey, Ph.D. candidate of our laboratory) and native dimers and trimers (Matthew Hassler, Figure 3.12) for the 5′ binding site in the MID domain of Ago2. This collaborative study employed NMR titration experiments and crystallographic techniques to determine binding affinities. Preliminary results suggest that dimers have particularly tight binding affinity for the hAgo2 MID domain, and siRNAs featuring native and 2′F modifications at their 5′ ends are well tolerated. 5′-*O*-phosphorylated dimers bind 2.5 times more tightly than 5′-*O*-phosphorylated nucleosides, with preference for dimers bearing 5′-uracil and 5′-adenine nucleobases. Structural insights gained from these studies suggest that important interactions exist between the 5′-nucleotide nucleobase and a rigid loop in the MID domain,[26] which the Nagar-Damha labs are currently

trying to optimize. A wide variety of nucleobase modifications for siRNA applications exist,[27] some of which will certainly prove useful in these ongoing research efforts. Since the interaction between Ago2 and siRNA is required for proper gene silencing activity,[28] optimizing this interaction will allow for the design of more potent siRNAs. The details of these studies fall outside the scope of this thesis. Preliminary results of our combined efforts can be found in our recent publication.[29]

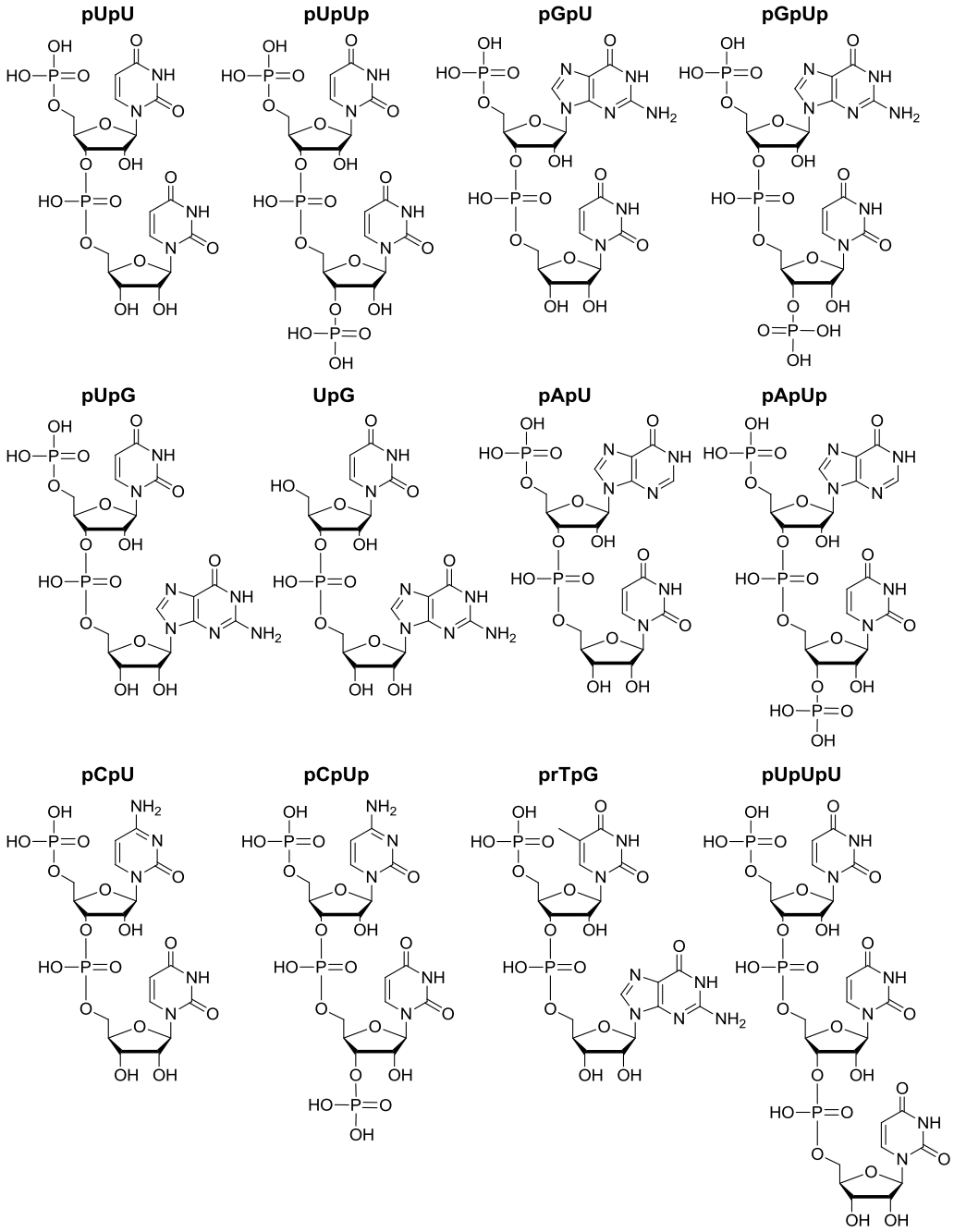


Figure 3.12. Library of dimer and trimers prepared for studying the 5′ binding MID domain of human Argonaute2**.**

3.3 Conclusions

In this chapter we described the development of a synthetic method for the preparation of isomerically pure dimer and trimer phosphoramidites and ribonucleotides. We utilized the 2′-*O*-TIPS protecting group, which proved to be

superior to the commonly used 2′-*O*-TBDMS protection due to its resistance towards 2′ to 3′ isomerization under standard delevulination conditions. The isomeric purity of the newly prepared dimers and trimers was confirmed by ^{31}P-NMR.

Furthermore, we conducted extensive NMR studies on the relative rates of delevulination, 2′ to 3′ silyl migration, and phosphate demethylation in 2′-*O*-TBDMS and 2′-*O*-TIPS protected ribonucleosides and RNA dimers. 2′-*O*-TBDMS and 2′-*O*-TIPS protected nucleosides undergo 3′-delevulination within 40 min without any detectable silyl isomerization. In contrast, the 2′-*O*-TBDMS and 2′-*O*-TIPS dimers exhibited a dramatic difference in both the rate of delevulination and the rate of silyl isomerization. While the 2′-*O*-TBDMS protected dimers underwent fast delevulination (2.5 min) and fast isomerization (20 min), the 2′-*O*-TIPS protected dimers were much more stable (20 min delevulination) with no isomerization observed for up to 42 h. In all cases, however, phosphate demethylation of the internucleotide linkage was detected after 2.3 hours under the experimental conditions used.

Finally, we investigated direct applications of this new synthetic methodology by preparing a library of RNA dimers and trimers containing purine and pyrimidine nucleotides. The synthetic procedures described herein provided facile access to isomerically pure dimer and trimer synthons in high yields.

Interactions between the 5′ nucleotide binding domain of hAgo2, MID, and native RNA dimers and trimers were investigated. These studies shed some light on the use of native (and chemically modified nucleotide analogues) in siRNA, and contributed to the rapidly advancing field of oligonucleotide-based gene silencing therapeutics.

Chapter 3 describes the use of dimer and trimer blocks for the synthesis of oligoribonucleotides in solution and on solid supports. These compounds allow longer chain extensions at each coupling stage of RNA synthesis, significantly reducing the total number of steps required in the synthesis of target RNA oligomers. Furthermore, they produce crude RNA oligomers that are generally more readily separated from failure sequences.

3.4 Experimental

Materials

Thin layer chromatography was performed on EM Science Kieselgel 60 F-254(1mm) plates. Silicycle 40-63 μm (230-400 mesh) silica gel was used for flash chromatography. Pyridine, acetonitrile, and dichloromethane were distilled from CaH_2 after refluxing for several hours. THF was distilled from benzophenone and sodium after refluxing for several hours. All other anhydrous solvents were purchased from Sigma-Aldrich. Chemicals and reagents were purchased from Sigma-Aldrich. Cyanoethyl phosphoramidites and methoxyphosphoramidic chloride phosphitylation reagents were purchased from ChemGenes Corp.

Instrumentation

^{1}H NMR and ^{13}C-NMR were recorded on a Varian 300, 400 or 500 MHz spectrophotometer with chemical shift values reported in ppm. ^{1}H-NMR and ^{13}C-NMR spectra were referenced to residual solvent. ^{31}P-NMR were collected at 80 MHz with a Varian 200MHz spectrophotometer and were measured from 85% H_3PO_4 as an external standard. TOF mass spectrometry was carried out on a Kratos Kompact III instrument.

NMR delevulination study conditions

Before each experiment, the 200 MHz NMR instrument used was shimmed and tuned with a sample containing deuterated chloroform, pyridine/acetic acid and nucleoside to be delevulinated in the exact concentration and volume used in the reaction. Once this is complete the tuning sample is removed. The reaction mixture is then combined in the NMR tube in the following order. First the nucleoside is dissolved by the deuterated chloroform and put in to the NMR tube, then the required amount of hydrazine hydrate in pyridine acetic acid, pre made just before the reaction is added to the tube. The tube is inverted x2, placed in the NMR and the computer begins to acquire at the scheduled time point automatically. A summary of the conditions used and the results of each NMR-delevulination reaction are summarized in Table 3.2.

	TBDMS			TIPS	
	Monomer		Dimer	Monomer	Dimer
Compound	**(11)**	**(11)**	**(6)**	**(20)**	**(24)**
Amount (mg, mmol)	50 (0.066)	50 (0.066)	53 (0.044)	57 (0.071)	50 (0.040)
Vol. CDCl3 (mL)	0.5	0.5	0.44	0.5	0.4
Vol. Hydrazine hydrate (mL)	0.66 (@ 1M)	0.66 (@ 0.5M)	0.44	0.71	0.4
Final Conc. of hydrazine (M)	0.5	0.25	0.25	0.25	0.045
Hydrazine hydrate (eq.)	10	5	5	5	5
Results					
Complete delevulinylation	5 min	10 min	<2.5 min	10 min	5min
Detection of isomerization	2.3 hours	None	20 min	None-	Non
Demethylation	N/A	N/A	20 min	N/A	2.3 h

Table 3.2. Conditions and results of NMR isomerization studies from Figures 3.4 to 3.6.

Limits of detection of ^{31}P-NMR.

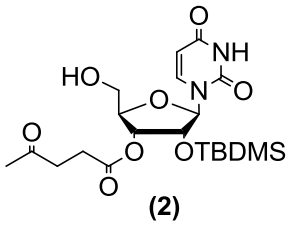
To determine the approximate limit of detection region isomers by ^{31}P-NMR, a solution of delevulinated 2′-*O*-TIPS dimer **(18)** (73mg, in 0.75ml of CD_3CN) was prepared and spectra taken with a 45 degree pulse, 1.6 second acquisition time, and 200 transients. Compound (**26)**, the 3′-*O*-TIPS regioisomer of compound **(3)**, was added to the NMR tube, gradually increasing its concentration until the compound was detected (Table 3.3)

	Mass of **(26)** in solution (mg)	Mol. % of **(26)**	Mass of **(18)** (mg)	**Detection of (26) (-/+)**
1	0	N/A	73	-
2	0.018	0.025	73	-
3	0.036	0.05	73	-
4	0.073	0.1	73	-
5	0.146	0.2	73	-
6	0.292	0.4	73	+
7	0.584	0.8	73	++
8	0.730	1	73	+++

Table 3.3. Limit of detection determination of regioisomers by ^{31}P-NMR.

From the data in Table 2 it shows that the limit of detection by NMR is between 0.2 and 0.4 mol %.

Synthesis procedures:

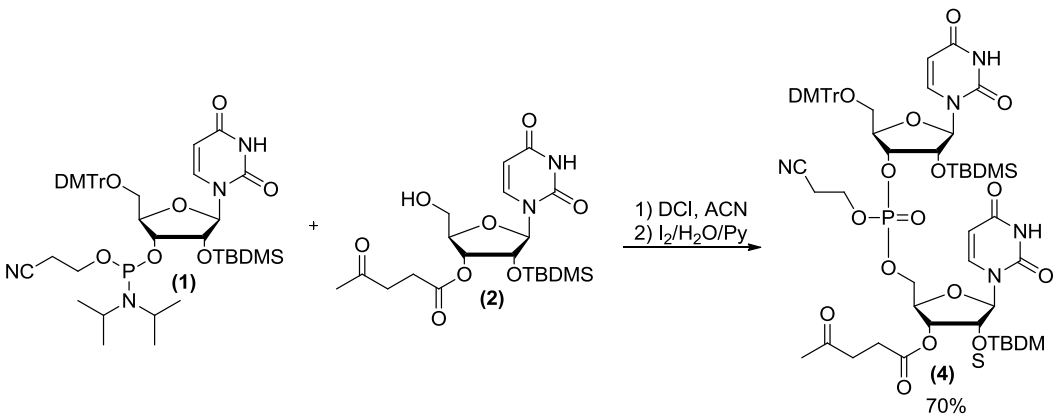


(2)

2′-TBDMS-3′-levulinyl-uridine (2)

5′-DMTr-2′-TBDMS-3′-levulinyl-uridine **(11)** (10g, 13.2 mmol) was dissolved in 100 mL of 3% trifloroacetic acid (TFA) in DCM (vol/vol) and allowed to stir for 10 min followed by 10 mL of MeOH. The solution was immediately condensed to an oil and the process was repeated with another 100 mL of DCM/TFA. The sample was then re-suspended in DCM and loaded on a column. The DCM was washed off with hexanes and the tritanol was eluted with hexanes:ethyl acetate (50:50) the product was eluted with 75% ethyl acetate. Yield: 5.68 g (94%).

^{1}H NMR (500 MHz, CD_3CN) δ ppm 0.10 (s, 8 H) 0.13 (s, 6 H) 0.80 - 0.97 (m, 9 H) 2.12 (s, 2 H) 2.48 - 2.64 (m, 2 H) 2.67 - 2.82 (m, 2 H) 3.38 (br. s., 1 H) 3.65 - 3.86 (m, 2 H) 3.98 - 4.04 (m, 1 H) 4.38 - 4.49 (m, 1 H) 5.25 (t, *J*=5.32 Hz, 1 H) 5.48 (s, 1 H) 5.66 (d, *J*=8.44 Hz, 1 H) 5.95 (d, *J*=5.50 Hz, 1 H) 7.82 (d, *J*=8.07 Hz, 1 H) Calc. Mass: 456.19 Mass found: 479.29 ($M+Na^+$).

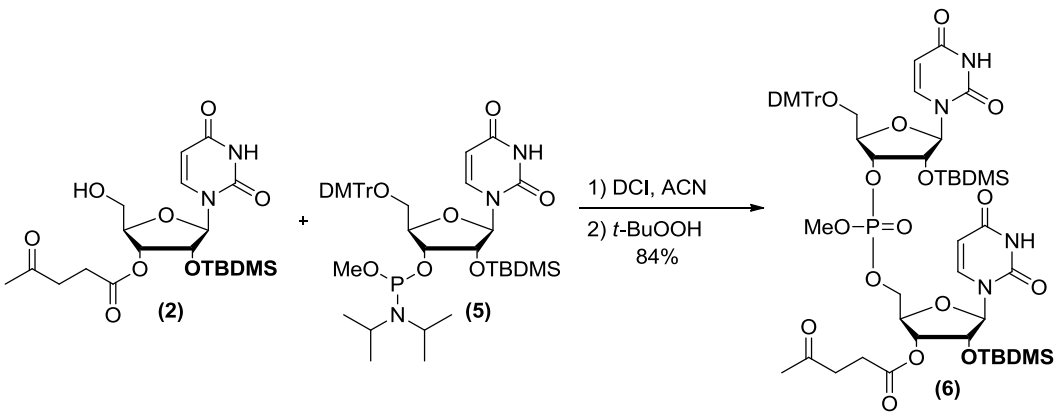


[5′-DMTr-2′TBDMS-rU]-[3′-p(CNEt)-5′]-[rU-2′TBDMS-3′-Lev] dimer (4)

2′-TBDMS-3′-levulinyl-uridine **(2)** (2.85g, 6.25mmol) was dissolved in 60mL of THF with 2eq of DCI (1.48g, 12). To this stirring solution 1.2eq (6.46g 7.50mmol) 5′-DMTr-2′TBDMS-uridine 3′-cyanoethylphosphoramidite **(1)** was added and allowed to stir at room temperature for 2h. The reaction was monitored by TLC (5%MeOH in DCM) and after 2 hours a small amount of compound **(1)** remained and another 0.2 eq (0.11 g, 0.124 mmol)of amidite were added and allowed to stir for one hour until the starting material had been consumed. At this point 10 eq (10 mL of a 6 M solution in decane) of *tert*-butyl hydroperoxide was added and allowed to stir for 20 min, until the phosphite triester was converted to the phosphate, as monitored by TLC (5% MeOH in DCM). The solution was condensed to approximately 10 mL and then dissolved in ethyl acetate and extracted with 5% sodium sulfite (x2) and brine (x1). The organic layer was dried and condensed and put on a short column (0→5%MeOH in DCM) yielding 70 % pure material. The column was not treated with triethylamine to avoid loss of the cyanoethyl phosphate protecting group.

^{1}H NMR (400 MHz, CD_3CN) δ ppm -0.04 - 0.22 (m, 5 H) 0.77 - 0.97 (m, 7 H) 1.96 (dt, *J*=4.94, 2.47 Hz, 1 H) 2.07 - 2.18 (m, 2 H) 2.66 - 2.90 (m, 2 H) 3.44 (d, *J*=2.74 Hz, 2 H) 3.79 (d, *J*=1.37 Hz, 4 H) 4.15 - 4.56 (m, 6 H) 4.88 (dd, *J*=7.95, 3.84 Hz, 1 H) 5.03 - 5.15 (m, 1 H) 5.36 (dd, *J*=8.23, 5.21 Hz, 1 H) 5.43 - 5.50 (m, 3 H) 5.64 (d, *J*=8.23 Hz, 1 H) 5.72 - 5.93 (m, 2 H) 6.91 (dd, *J*=8.91, 2.88 Hz, 3 H)

7.20 - 7.56 (m, 9 H) 7.69 (dd, *J*=8.23, 1.92 Hz, 1 H) 9.29 (s, 2 H); ^{31}P NMR (81 MHz, Acetone) δ ppm 0.58 (s, 1 P) 0.96 (s, 1 P).

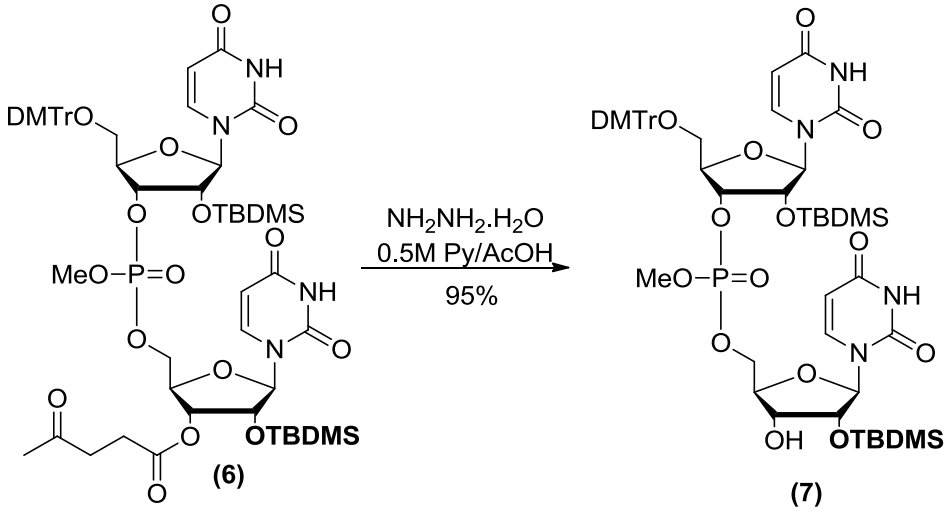


[5′-DMTr-2′TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′TBDMS-3′-Lev] (6)

Compound **(2)** (1.55 g**,** 3.39 mmol) was dissolved in 26 mL of THF (0.15M) with 2eq of DCI. To this stirring solution (3.21 g, 3.90 mmol) of phosphoramidite **(5)** was added all at once and allowed to stir at room temperature for 2h. The reaction was monitored by TLC (5%MeOH in DCM) and after 2 hours the reaction was complete. At this point 10eq of *tert*-butyl hydroperoxide was added and allowed to stir for 20min, until all the phosphite triester was converted to the phosphate, as monitored by TLC (5% MeOH in DCM) observing a more polar spot appear. The solution was condensed to approximately 10 ml and then dissolved in ethyl acetate and extracted with 5% sodium sulfite (x2) and brine (x1). The organic layer was dried and condensed and put on a short column (0→5%MeOH in DCM). Yield: 3.12 g (84%).

^{1}H NMR (500 MHz, CD_3CN) δ ppm 0.05 - 0.17 (m, 6 H) 0.88 - 0.97 (m, 11 H) 1.97 (dt, *J*=4.95, 2.54 Hz, 1 H) 2.03 (s, 1 H) 2.12 (d, *J*=2.45 Hz, 2 H) 2.53 - 2.64 (m, 2 H) 2.72 - 2.79 (m, 1 H) 3.39 - 3.48 (m, 2 H) 3.51 - 3.55 (m, 1 H) 3.56 - 3.61 (m, 1 H) 3.63 - 3.66 (m, 1 H) 3.70 (d, *J*=11.25 Hz, 1 H) 3.77 (s, 1 H) 3.78 - 3.82 (m, 4 H) 4.03 - 4.13 (m, 1 H) 4.13 - 4.26 (m, 2 H) 4.29 - 4.36 (m, 1 H) 4.41 (dt, *J*=13.88, 5.53 Hz, 1 H) 4.47 (t, *J*=4.89 Hz, 1 H) 4.78 - 4.84 (m, 1 H) 4.85 - 4.91 (m, 1 H) 5.24 (t, *J*=4.89 Hz, 1 H) 5.37 (dd, *J*=8.19, 5.26 Hz, 1 H) 5.59 (dd, *J*=12.23, 8.07 Hz, 1 H) 5.77 (d, *J*=4.40 Hz, 1 H) 5.81 (d, *J*=4.65 Hz, 1 H) 5.87 (dd, *J*=13.94, 5.38 Hz, 2 H) 6.88 - 6.94 (m, 2 H) 7.26 - 7.42 (m, 11 H) 7.42 - 7.49

(m, 1 H) 7.69 (dd, *J*=10.64, 8.19 Hz, 1 H) 9.20 - 9.36 (m, 2 H); ^{31}P NMR (81 MHz, CD_3CN) δ ppm 0.47 (s, 1 P) 0.67 (s, 1 P); HRMS (ESI) for $C_{57}H_{77}N_4O_{18}PSi_2$ calc: 1192.4509, found: 1215.32 ($M + Na^+$).

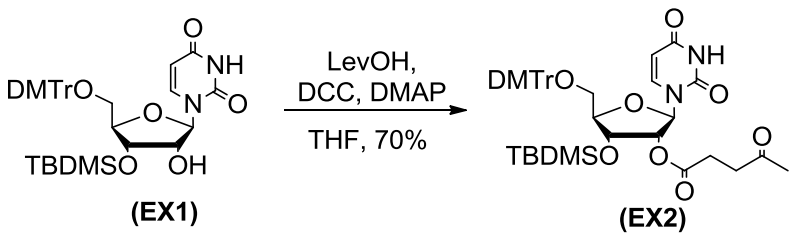


[5′-DMTr-2′TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′TBDMS] (7)

Dimer nucleotide **(6)** (1.26g, 1.06mmol) was dissolved in minimal amounts of ACN, just enough to get it into solution. It was then treated with ten equivalents (21ml, 10.6mmol) of a 0.5M hydrazine hydrate solution in 3:2 pyridine:acetic that was prepared immediately before the reaction. The reaction mixture was allowed to stir for 10min before it was chilled to 0°C in an ice bath and ten equivalents (0.728ml, 10.6mmol) of 2,4-pentanedione which was allowed to stir for an additional 5min before the reaction was diluted with MTBE and extracted x3 with ammonium chloride (5%w/v) and once with cupric sulfate, and once with brine. The sample was then dried over MgSO4 and condensed to dryness. The sample was put on a short column and run with MeOH 0→5% in chloroform. Yield: 1.20g (95%) (impure, contaminated with compound **(8)**).

^{1}H NMR (400 MHz, CD_3CN) of major component δ ppm -0.11 - 0.07 (m, 7 H) 0.07 - 0.25 (m, 7 H) 0.80 - 0.94 (m, 20 H) 1.84 - 2.05 (m, 3 H) 2.07 - 2.31 (m, 6 H) 2.48 - 2.70 (m, 3 H) 2.75 (m, 3 H) 3.33 - 3.54 (m, 3 H) 3.66 - 3.85 (m, 10 H) 4.15 - 4.41 (m, 6 H) 4.47 (t, *J*=4.89 Hz, 2 H) 4.73 - 4.99 (m, 2 H) 5.00 - 5.24 (m, 2 H) 5.37 (dd, *J*=8.22, 1.57 Hz, 2 H) 5.42 - 5.54 (m, 1 H) 5.64 (d, *J*=7.83 Hz, 1 H) 5.72 - 5.91 (m, 3 H) 6.80 - 6.98 (m, 5 H) 7.24 - 7.40 (m, 8 H) 7.40 - 7.57 (m, 4 H) 7.68 (dd, *J*=8.22, 5.09 Hz, 2 H) 9.30 (br. s., 3 H); ^{31}P NMR (81 MHz, CD_3CN) δ

ppm 0.49 (s, 1 P) 0.58 (s, 1 P) 0.74 (s, 1 P) 0.78 (s, 1 P)); Mass calc: 1094.41, found: 1117.42 ($M + Na^+$).



(EX1) (EX2)

5′-DMTr-2′-levulinyl-3′-TBDMS-uridine (EX2)

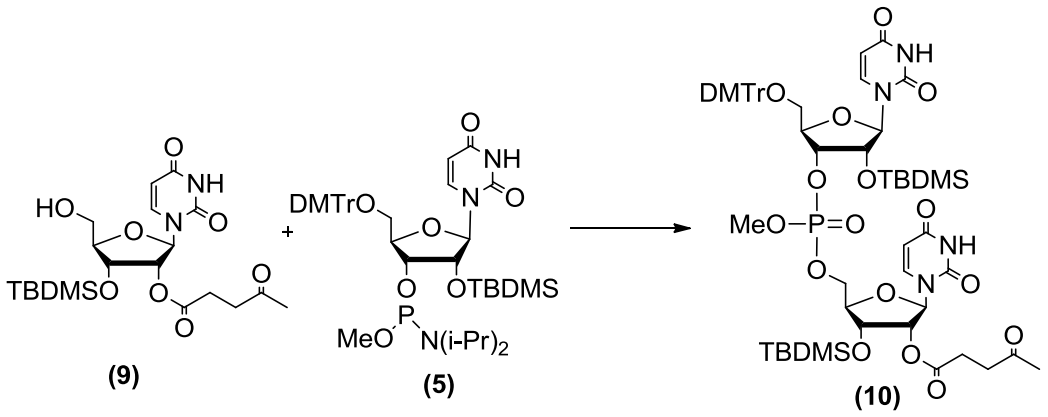
Levulinic acid (4.44 g, 6.72 mmol), DCC (2.64 g, 12.8 mmol) and DMAP (0.1g, 0.82 mmol) were dissolved in 45mL of THF and stirred for 15 min. Compound **(EX1)** (4.44 g, 7.30 mmol) was then added directly to the above solution. After 5h the reaction was completely brown/red and complete by TLC (50:50 ethyl acetate:hexanes). The reaction mixture was filtered over Celite®. The organic layer was washed with 5% $NaHCO_3$, and saturated NaCl. The compound was dried with $MgSO_4$ and purified by column chromatography 0 →30% ethylacetate:hexanes. Yield: 3.11 g (70%). Mass calc. 758.32 Mass found: 781.32 ($M+Na^+$).

(EX2) (9)

2′-Levulinyl-3′-TBDMS uridine (9)

5′-DMTr-2′-levulinyl-3′-TBDMS-uridine **(EX2)** (2.40g, 3.16 mmol) was dissolved in 25ml of 3% trifloroacetic acid (TFA) in DCM (vol/vol) and allowed to stir for 5min followed by 10ml of MeOH. The solution was immediately condensed to a oil and the process was repeated with another 15ml of DCM/TFA. The sample was then re- suspended in DCM and loaded on a column. The DCM was washed off with Hexanes and the tritanol was eluted with Hexanes:ethyl acetate 50:50, the product was eluted with 80% ethylacetate. Yield: 1.36g (94%).

^{1}H NMR (500 MHz, CD_3CN) δ ppm 0.04 (d, *J*=3.67 Hz, 15 H) 0.74 - 0.91 (m, 23 H) 1.97 (dt, *J*=4.86, 2.52 Hz, 9 H) 2.15 (s, 10 H) 2.54 - 2.68 (m, 6 H) 2.72 - 2.82 (m, 6 H) 3.64 - 3.79 (m, 7 H) 4.07 - 4.19 (m, 3 H) 4.45 (dd, *J*=6.05, 5.32 Hz, 4 H) 5.19 (dd, *J*=5.14, 3.30 Hz, 4 H) 5.70 (dd, *J*=8.07, 2.20 Hz, 3 H) 5.86 (d, *J*=6.24 Hz, 3 H) 7.84 (d, *J*=8.44 Hz, 3 H) 9.13 (br. s., 4 H). Mass calc. 456.19 Mass found: 479.29 ($M+Na^+$)



[5′-DMTr-2′TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′TBDMS-3′-Lev] (10)

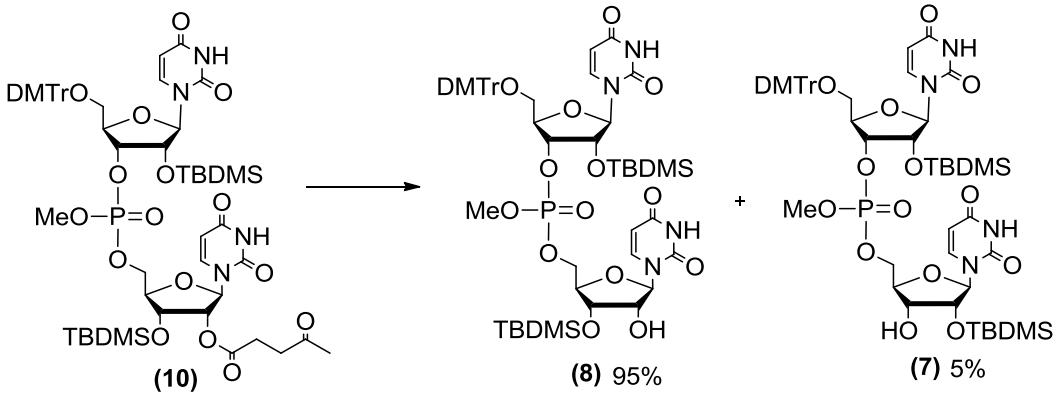
Compound **(9)** (1.55 g**,** 3.39 mmol) was dissolved in 26 mL of THF (0.15M) with 2eq of DCI. To this stirring solution (3.21 g, 3.90 mmol) of phosphoramidite **(5)** was added all at once and allowed to stir at room temperature for 2h. The reaction was monitored by TLC (5%MeOH in DCM) and after 2 hours the reaction was complete. At this point 10eq of *tert*-butyl hydroperoxide was added and allowed to stir for 20min, until all the phosphite triester was converted to the phosphate, as monitored by TLC (5% MeOH in DCM) observing a more polar spot appear. The solution was condensed to approximately 10ml and then dissolved in ethyl acetate and extracted with 5% sodium sulfite (x2) and brine (x1). The organic layer was dried and condensed and put on a short column (0→5%MeOH in DCM). Yield: 3.12 g (84%).

^{1}H NMR (500 MHz, CD_3CN) δ ppm 0.05 - 0.17 (m, 6 H) 0.88 - 0.97 (m, 11 H) 1.97 (dt, *J*=4.95, 2.54 Hz, 1 H) 2.03 (s, 1 H) 2.12 (d, *J*=2.45 Hz, 2 H) 2.53 - 2.64 (m, 2 H) 2.72 - 2.79 (m, 1 H) 3.39 - 3.48 (m, 2 H) 3.51 - 3.55 (m, 1 H) 3.56 - 3.61

(m, 1 H) 3.63 - 3.66 (m, 1 H) 3.70 (d, *J*=11.25 Hz, 1 H) 3.77 (s, 1 H) 3.78 - 3.82 (m, 4 H) 4.03 - 4.13 (m, 1 H) 4.13 - 4.26 (m, 2 H) 4.29 - 4.36 (m, 1 H) 4.41 (dt, *J*=13.88, 5.53 Hz, 1 H) 4.47 (t, *J*=4.89 Hz, 1 H) 4.78 - 4.84 (m, 1 H) 4.85 - 4.91 (m, 1 H) 5.24 (t, *J*=4.89 Hz, 1 H) 5.37 (dd, *J*=8.19, 5.26 Hz, 1 H) 5.59 (dd, *J*=12.23, 8.07 Hz, 1 H) 5.77 (d, *J*=4.40 Hz, 1 H) 5.81 (d, *J*=4.65 Hz, 1 H) 5.87 (dd, *J*=13.94, 5.38 Hz, 2 H) 6.88 - 6.94 (m, 2 H) 7.26 - 7.42 (m, 11 H) 7.42 - 7.49 (m, 1 H) 7.69 (dd, *J*=10.64, 8.19 Hz, 1 H) 9.20 - 9.36 (m, 2 H);

^{31}P NMR (81 MHz, CD_3CN) δ ppm 0.47 (s, 1 P) 0.67 (s, 1 P);

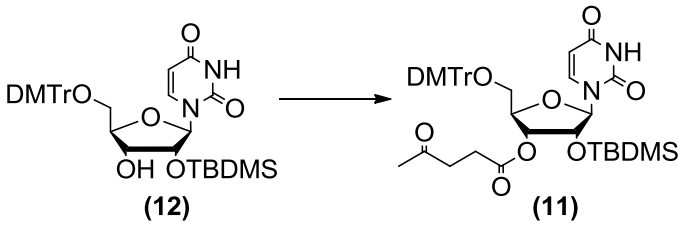
MS (ESI) for $C_{57}H_{77}N_4O_{18}PSi_2$ calc: 1192.4509, found: 1215.32 ($M + Na^+$).



[5′-DMTr-2′-TBDMS-rU]-[3′-p(OMe)-5′]-[rU-3′-TBDMS] (8)

Compound (10) (0.210g, 0.176 mmol) was dissolved in minimal amounts of ACN, just enough to get it into solution. It was then treated with ten equivalents (3.52 mL) of a 0.5 M hydrazine hydrate solution in 3:2 pyridine:acetic that was prepared immediately before the reaction. The reaction mixture was allowed to stir for 10 min before it was chilled to 0oC in an ice bath and ten equivalents (0.18 mL, 1.75 mmol) of 2,4-pentanedione which was allowed to stir for an additional 5 min before the reaction was diluted with MTBE and extracted x3 with ammonium chloride (5%w/v) and once with cupric sulfate, and once with brine. The sample was then dried over MgSO4 and condensed to dryness. The sample was put on a short column and run with MeOH 0 → 4 % in chloroform. Yield: 0.162g (85%).

^{1}H NMR (500 MHz, CD_3CN) δ ppm -1.93 - 0.51 (m, 13 H) 0.88 - 0.96 (m, 11 H) 1.89 (s, 3 H) 1.97 (dt, J=5.07, 2.48 Hz, 1 H) 2.03 (s, 1 H) 2.21 (s, 3 H) 3.41 - 3.48 (m, 1 H) 3.50 - 3.67 (m, 2 H) 3.78 (m, J=1.47 Hz, 4 H) 3.98 - 4.29 (m, 4 H) 4.34 (dd, J=5.62, 3.18 Hz, 1 H) 4.48 (t, J=5.01 Hz, 1 H) 4.67 - 5.15 (m, 2 H) 5.39 (dd, J=8.19, 1.83 Hz, 2 H) 5.62 (t, J=8.19 Hz, 1 H) 5.76 (dd, J=12.84, 4.28 Hz, 1 H) 5.85 (br. s., 1 H) 5.89 (t, J=5.87 Hz, 1 H) 6.23 (br. s., 2 H) 6.91 (dd, J=8.93, 3.30 Hz, 4 H) 7.13 - 7.41 (m, 5 H) 7.41 - 7.59 (m, 2 H) 7.71 (t, J=8.07 Hz, 4 H) 9.78 (br. s., 3 H); 31P NMR (81 MHz, CD_3CN) δ ppm 0.48 (s, 1 P) 0.57 (s, 1 P) 0.73 (s, 1 P) 0.79 (s, 1 P



5′-DMTr-2′-TBDMS-3′-levulinyl-uridine (11)

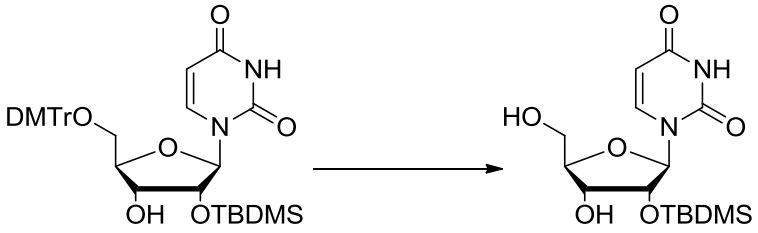
Levulinic acid (1.70g, 14.6 mmol), DCC (3.03 g, 14.5 mmol) and DMAP (0.23 g, 1.85mmol) were dissolved in 50mL of THF and stirred for 15 min. 5′-DMTr-2′-TBDMS-uridine **(12)** (4.83 g, 7.30 mmol) was then added directly to the above solution. After 5h the reaction was completely brown/red and complete by TLC (50:50 ethyl acetate:hexanes). The reaction mixture was filtered over Celite®. The organic layer was washed with 5% $NaHCO_3$, and saturated NaCl. The compound was dried with $MgSO_4$ and purified by column chromatography 0 →30% Ethylacetate:hexanes. Yield: 4.88g (87%)

^{1}H NMR (500 MHz, CD_3CN) δ ppm 0.09 (d, *J*=3.00 Hz, 6 H) 0.85 - 0.94 (m, 9 H) 2.50 - 2.67 (m, 2 H) 2.68 - 2.83 (m, 2 H) 3.40 (d, *J*=3.00 Hz, 1 H) 3.53 - 3.66 (m, 1 H) 3.80 (s, 6 H) 3.92 - 4.05 (m, 1 H) 4.19 (d, *J*=3.86 Hz, 1 H) 4.50 (s, 1 H) 5.30 (d, *J*=0.86 Hz, 1 H) 5.40 (d, *J*=8.14 Hz, 1 H) 5.87 (d, *J*=5.57 Hz, 1 H) 6.87 - 7.01 (m, 2 H) 7.25 - 7.40 (m, 4 H) 7.41 - 7.49 (m, 1 H) 7.70 (d, *J*=8.14 Hz, 1 H) ESI MS, Calc. 758.32 found: 781.32 ($M+Na^+$)

5′-DMTr-2′-TBDMS-uridine (12)

5′-*O*-DMTr-uridine (27.88 g, 51.0 mmol) was dissolved in dry THF (340 mL). Dry pyridine (16.2 mL, 0.20 mol) and $AgNO_3$ (9.53 g, 56.1 mmol) were added and stirring was continued for 15 min. Once almost all $AgNO_3$ dissolved, *tert*-butyldimethylsilyl chloride (TBDMS-Cl) (8.46 g, 56.1 mmol) was added all at once and the resulting cloudy milky solution was stirred at room temperature for 12h. The reaction mixture was filtered into 5% $NaHCO_3$ solution to avoid deprotection of DMTr. This mixture was condensed on the rotovap to remove approx half the solvent (200ml). This mixture was then diluted with ethyl acetate and extracted with sat. $NaHCO_3$ (x3). The organic layer was dried with $MgSO_4$ and condensed to dryness. This mixture was purified by column chromatography in *tert*-butyl-methyl ether: Hexanes 0→50%. Yield of 5′-DMTr-2′-TBDMS-uridine **(12)** 21.92g (65%)

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm -0.14 (s, 5 H) -0.04 (s, 6 H) 0.74 (s, 15 H) 1.07 (t, *J*=7.04 Hz, 2 H) 3.26 (d, *J*=4.30 Hz, 9 H) 3.70 (s, 10 H) 4.12 (d, *J*=4.70 Hz, 3 H) 4.26 (d, *J*=5.48 Hz, 2 H) 4.88 (t, *J*=5.09 Hz, 2 H) 5.19 (d, *J*=6.26 Hz, 2 H) 5.74 (s, 2 H) 6.06 (d, *J*=5.09 Hz, 1 H) 6.83 (dd, *J*=9.00, 2.74 Hz, 7 H) 7.03 - 7.29 (m, 12 H) 7.37 (d, *J*=7.43 Hz, 4 H) 7.45 - 7.57 (m, 4 H) 7.62 (d, *J*=7.43 Hz, 2 H) 8.02 (d, *J*=7.04 Hz, 4 H) 8.58 (s, 2 H) 8.65 (s, 2 H) 11.20 (br. s., 3 H) mass calc. 546.20 mass found: 569.32 ($M+Na^+$).



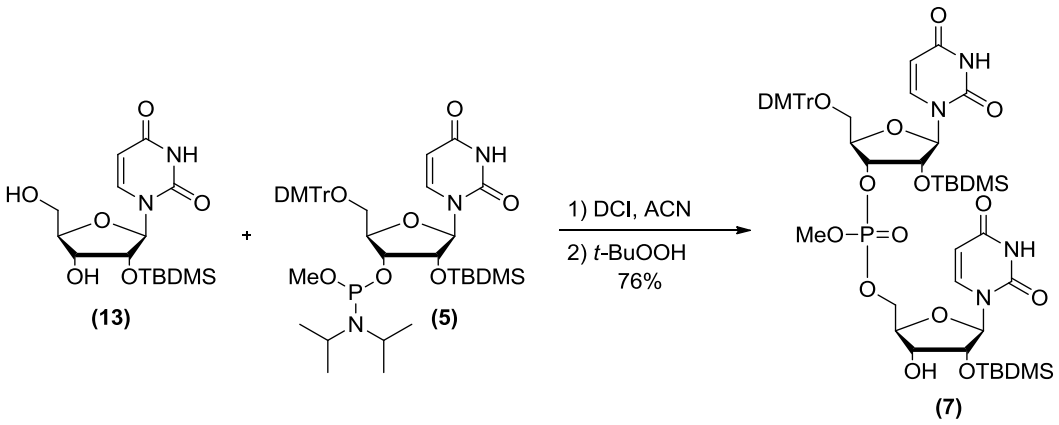
(12) **(13)**

2′-TBDMS-uridine (13)

A solution of 3% triflouroacetic acid (TFA) in DCM (15mL) was added directly to compound 5′-DMTr-2′-TBDMS-uridine **(12)** (1.26 g, 1.91 mmol)**.** The solution was quenched with 5 mL of MeOH after 5 min and condensed to dryness on a rotovap. By TLC there was a very small amount of starting material so the

process was repeated again. The compound was purified by column chromatography. 0 →75% Ethyl acetate: hexanes. Yield: 0.65 g (94%).

^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 0.02 (s, 10 H) 0.00 (s, 10 H) 0.82 (s, 30 H) 3.55 (d, *J*=11.98 Hz, 4 H) 3.63 (d, *J*=11.25 Hz, 4 H) 3.86 (br. s., 4 H) 3.92 (d, *J*=4.16 Hz, 4 H) 4.01 (d, *J*=7.34 Hz, 3 H) 4.12 (br. s., 4 H) 4.96 (d, *J*=5.14 Hz, 4 H) 5.12 (br. s., 5 H) 5.63 (d, *J*=8.07 Hz, 4 H) 5.76 (d, *J*=4.40 Hz, 4 H) 7.92 (d, *J*=8.07 Hz, 1 H) 11.30 (br. s., 1 H) Mass calc. 358.16 Mass found: 381.20 ($M+Na^+$).



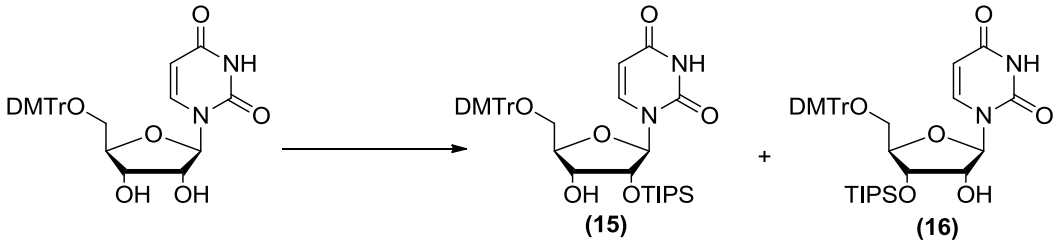
Selective coupling reaction, according to Scheme 3.6

[5′-DMTr-2′-TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′-TBDMS] (8)

A concentrated solution of phosphoramidite **(5)** (4.40g, 5.35mmol, 1.01 eq), DCI (1.25g, 10.6mmol, 2 eq) in acetonitrile (ACN, 26.5mL) were added to a flamed dried flask and allowed to stir until all reagents were dissolved. In a separate vessel the 2′-TBDMS nucleoside **(13)** (1.9g, 5.30mmol, 1.0eq) was dissolved in 20 mL of ACN. The solution of activated amidite was then cannulated slowly (drop-wise) into the flask with the nucleoside. This mixture was allowed to stir for 20 min before a TLC was run (10% MeOH in DCM) showing complete consumption of the amidite. *tert*-butyl hydroperoxide (6M in decane, 5.3 mL, 5 eq) was then added to the solution and allowed to stir for another 20 min. The sample was then diluted with MTBE and extracted x3 with a 5% aqueous solution of sodium sulphite and once with brine. The mixture was then dried over magnesium sulphate and evaporated to dryness. The sample was then purified by silica gel chromatography using 0-4% MeOH in DCM as eluting solvent to give

the pure product in reasonable yield (Note this was a difficult column as the starting nucleoside had similar polarity to the oxidized dimer): 3.7g (68%) (impure).

^{1}H NMR (400 MHz, CD_3CN) δ ppm -0.11 - 0.07 (m, 7 H) 0.07 - 0.25 (m, 7 H) 0.80 - 0.94 (m, 20 H) 1.84 - 2.05 (m, 3 H) 2.07 - 2.31 (m, 6 H) 2.48 - 2.70 (m, 3 H) 2.75 (q, *J*=6.26 Hz, 3 H) 3.33 - 3.54 (m, 3 H) 3.66 - 3.85 (m, 10 H) 4.15 - 4.41 (m, 6 H) 4.47 (t, *J*=4.89 Hz, 2 H) 4.73 - 4.99 (m, 2 H) 5.00 - 5.24 (m, 2 H) 5.37 (dd, *J*=8.22, 1.57 Hz, 2 H) 5.42 - 5.54 (m, 1 H) 5.64 (d, *J*=7.83 Hz, 1 H) 5.72 - 5.91 (m, 3 H) 6.80 - 6.98 (m, 5 H) 7.24 - 7.40 (m, 8 H) 7.40 - 7.57 (m, 4 H) 7.68 (dd, *J*=8.22, 5.09 Hz, 2 H) 9.30 (br. s., 3 H); ^{31}P NMR (81 MHz, CD_3CN) δ ppm 0.56 (s, 1 P) 0.77 (s, 1 P).



5′-DMTr-2′-TIPS-uridine (15)

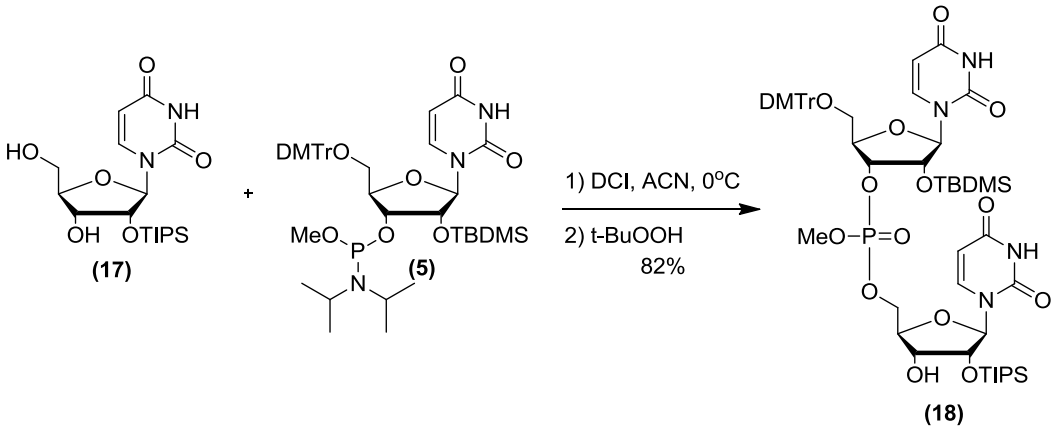
5′-DMTr-uridine (8.55 g, 15.6 mmol) was dissolved in dry THF 156 mL. Dry pyridine (5.10 mL, 62.5 mmol) and $AgNO_3$ (2.92 g, 17.2 mmol) were added and stirring was continued for 5 min, antil almost all $AgNO_3$ dissolved. TIPS-Cl (3.6 mL, 17.2 mmol) was added all at once and the resulting cloudy milky solution was stirred at room temperature. After 12h TLC was analysed and there was still a lot of starting material in the reaction mixture. So, one half more equivalent of $AgNO_3$ and TIPS-Cl were added and the mixture was sonicated for another 6 h. The reaction mixture was filtered into 5% $NaHCO_3$ solution to avoid deprotection of DMTr. The reaction mixture was extracted with DCM, evaporated and purified through column chromatography. The unreacted starting material was recovered. Yield 2′ TIPS nucleoside **(15)**: 7.25g (66%); 3′ TIPS nucleoside **(16)** 2.19g (20%).

^{1}H NMR (300 MHz, CD_3CN) δ ppm 1.05 - 1.22 (m, 21 H) 3.17 (d, *J*=5.22 Hz, 1 H) 3.36 (dd, *J*=10.89, 2.97 Hz, 2 H) 3.79 (s, 6 H) 4.09 (d, *J*=3.96 Hz, 1 H) 4.30 (d, *J*=4.68 Hz, 1 H) 4.51 (t, *J*=4.95 Hz, 1 H) 5.34 (d, *J*=8.10 Hz, 1 H) 5.91 (d, *J*=4.86 Hz, 1 H) 6.90 (dd, *J*=9.00, 0.90 Hz, 2 H) 7.26 - 7.37 (m, 7 H) 7.41 - 7.46 (m, 2 H) 7.75 (d, *J*=8.28 Hz, 1 H). Mass calc. 702.33, found 725.36.

2′-TIPS-uridine (17)

A solution of 3% triflouroacetic acid (TFA) in DCM (30mL) was added directly to compound **(15)** (3.54 g, 5.04 mmol)**.** The solution was quenched with 10 mL of MeOH after 5 min and condensed to dryness on a rotovap. By TLC there was a very small amount of starting material so the process was repeated again. The compound was purified by column chromatography. 0 →75% Ethyl acetate: hexanes. Yield: 1.96 g (97%).

Mass calc. 702.33, found 725.38. ($M+Na^+$)



[5′-DMTr-2′-TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′-TIPS] (18)

Selective coupling procedure according to Scheme 3.8

A concentrated solution of phosphoramidite **(5)** (4.40g, 5.35mmol, 1.01 eq), DCI (1.25g, 10.6mmol, 2 eq) in acetonitrile (ACN, 26.5mL) were added to a flamed dried flask and allowed to stir until all reagents were dissolved. In a

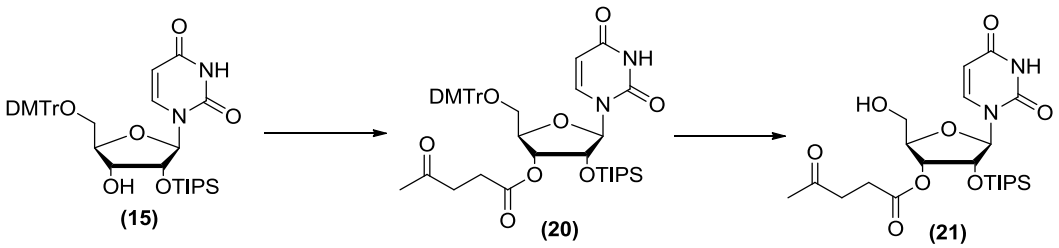
separate vessel the 2′-TIPS nucleoside **(17)** (1.9g, 5.30mmol, 1.0eq) was dissolved in 20 mL of ACN (note: for rA nucleoside a small amount of THF was added due to solubility issues). The solution of activated amidite was then cannulated slowly (drop-wise) into the flask with the nucleoside. This mixture was allowed to stir for 20 min before a TLC was run (10% MeOH in DCM) showing complete consumption of the amidite. *tert*-butyl hydroperoxide (6M in decane, 5.3 mL, 5 eq) was then added to the solution and allowed to stir for another 20 min. The sample was then diluted with MTBE and extracted x3 with a 5% aqueous solution of sodium sulphite and once with brine. The mixture was then dried over magnesium sulphate and evaporated to dryness. The sample was then purified by silica gel chromatography using 0-4% MeOH in DCM as eluting solvent to give the pure product in 82% yield (3.7 g).

^{1}H NMR (300 MHz, CD_3CN) δ ppm 0.09 - 0.15 (m, 6 H) 0.87 - 1.20 (m, 40 H) 1.94 - 2.00 (m, 1 H) 2.10 - 2.20 (m, 2 H) 3.44 (br. s., 3 H) 3.67 - 3.79 (m, 12 H) 4.04 - 4.23 (m, 5 H) 4.28 - 4.35 (m, 2 H) 4.38 - 4.48 (m, 2 H) 4.81 - 4.90 (m, 1 H) 5.38 (d, *J*=8.21 Hz, 1 H) 5.64 (d, *J*=8.20 Hz, 1 H) 5.81 - 5.91 (m, 2 H) 6.90 (d, *J*=7.91 Hz, 5 H) 7.24 - 7.37 (m, 9 H) 7.41 - 7.52 (m, 4 H) 7.71 (dd, *J*=8.20, 2.64 Hz, 1 H) 9.62 (br. s., 1 H)

^{13}C NMR (126 MHz, $CDCl_3$) δ ppm -5.08, -5.00, -4.90, -4.87, 12.02, 12.05, 17.66, 17.69, 17.76, 17.79, 25.56, 25.59, 54.59, 54.92, 54.97, 55.21, 55.25, 61.72, 67.06, 67.29, 70.17, 70.26, 74.72, 74.84, 75.68, 75.99, 81.50, 82.14, 87.53, 87.57, 88.18, 88.33, 90.51, 90.57, 102.45, 102.92, 102.96, 113.33, 127.35, 127.76, 128.05, 128.21, 128.23, 129.12, 130.17, 130.23, 130.25, 130.36, 134.58, 134.66, 134.79, 139.83, 140.37, 140.45, 143.95, 144.06, 150.24, 150.28, 150.50, 150.58, 158.77, 158.78, 158.82, 163.29, 163.34, 163.39.

^{31}P NMR (81 MHz, CD_3CN) δ ppm 0.58 (s, 1 P) 0.83 (s, 1 P).

Mass calc. 1137.36 Mass found. 1159.53 ($M+Na^+$).



5′-DMTr-2′-TIPS-3′-levulinyl-uridine (20)

Levulinic acid (1.05g, 9.05 mmol), DCC (1.601 g, 7.76 mmol) and DMAP (0.23 g, 1.85 mmol) were dissolved in 26 mL of THF and stirred for 10 min. Compound 5′-DMTr-2′-TIPS-uridinen **(15)** (2.73 g, 3.88 mmol) was then added directly to the above solution. After 5h the reaction was completely brown/red and complete by TLC (50:50 ethyl acetate:hexanes). The reaction mixture was filtered over Celite®. The organic layer was washed with 5% $NaHCO_3$, and saturated NaCl. The compound was dried with $MgSO_4$ and purified by column chromatography 0 →30% ethyl acetate:hexanes. Yield: 1.79 g (75%).

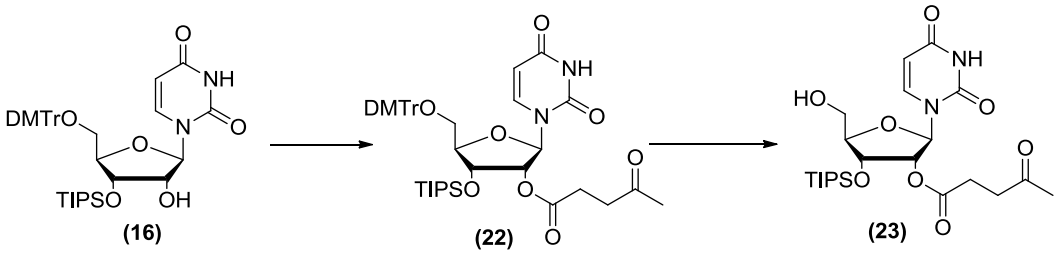
^{1}H NMR (300 MHz, CD_3CN) δ ppm 1.02 - 1.36 (m, 21 H) 2.13 (s, 3 H) 2.54 - 2.62 (m, 2 H) 2.71 - 2.78 (m, 2 H) 3.37 - 3.40 (m, 2 H) 3.78 (s, 6 H) 4.15 (d, *J*=2.77 Hz, 1 H) 4.63 - 4.67 (m, 1 H) 5.31 (dd, *J*=5.14, 2.96 Hz, 1 H) 5.40 - 5.46 (m, 1 H) 5.42 (s, 1 H) 5.99 (d, *J*=6.32 Hz, 1 H) 6.87 - 6.93 (m, 4 H) 7.27 - 7.37 (m, 7 H) 7.41 - 7.45 (m, 2 H) 7.75 (d, *J*=8.30 Hz, 1 H).

2′-TIPS-3′-levulinyl-uridine (21)

A solution of 3% triflouroacetic acid (TFA) in DCM (20mL) was added directly to compound **(20).** The solution was quenched with 5mL of MeOH after 5 min and condensed to dryness on a rotovap. By TLC there was a very small amount of starting material so the process was repeated again. The compound was purified by column chromatography. 0 →75% ethyl acetate: hexanes. Yield: 0.92 g (95%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.96 - 1.11 (m, 20 H) 2.20 (s, 3 H) 2.52 - 2.88 (m, 5 H) 3.82 (d, *J*=2.05 Hz, 1 H) 3.88 - 3.95 (m, 1 H) 4.16 - 4.21 (m, 1 H) 4.82

(t, *J*=5.27 Hz, 1 H) 5.17 (dd, *J*=4.98, 3.52 Hz, 1 H) 5.68 (d, *J*=5.57 Hz, 1 H) 5.75 (dd, *J*=7.91, 2.34 Hz, 1 H) 7.71 (d, *J*=8.21 Hz, 1 H) 8.43 (s, 1 H).



5′-DMTr-2′-levulinyl-3′-TIPS-uridine (22)

Levulinic acid (0.49 g, 4.24 mmol), DCC (0.834 g, 4.04 mmol) and DMAP (0.09 g, 0.73 mmol) were dissolved in 13 mL of THF and stirred for 10 min. Compound **(16)** (1.42 g, 2.02 mmol) was then added directly to the above solution. After 5h the reaction was completely brown/red and complete by TLC (50:50 ethyl acetate:hexanes). The reaction mixture was filtered over Celite®. The organic layer was washed with 5% $NaHCO_3$, and saturated NaCl. The compound was purified by column chromatography 0 →30% ethylacetate:hexanes. Yield: 1.36g (84%).

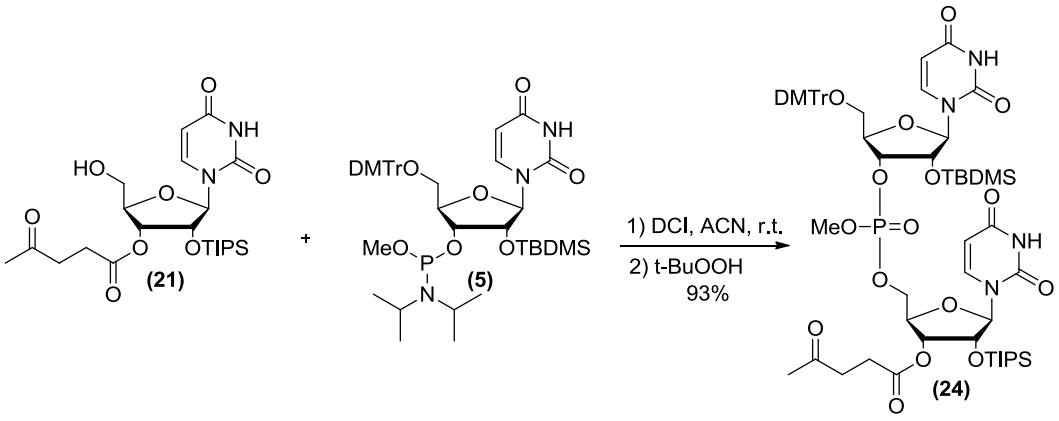
^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.72 - 1.13 (m, 21 H) 2.14 - 2.19 (m, 3 H) 2.60 - 2.69 (m, 2 H) 2.69 - 2.84 (m, 2 H) 3.38 (dd, *J*=10.84, 2.93 Hz, 1 H) 3.47 - 3.56 (m, 1 H) 3.79 (s, 6 H) 4.08 - 4.16 (m, 1 H) 4.58 (dd, *J*=4.98, 2.93 Hz, 1 H) 5.38 - 5.47 (m, 2 H) 6.16 (d, *J*=6.15 Hz, 1 H) 6.83 (d, *J*=8.50 Hz, 4 H) 7.21 - 7.53 (m, 10 H) 7.74 (d, *J*=8.21 Hz, 1 H) 8.53 - 8.64 (m, 1 H). Mass calc. 800.37, found 823.24

2′-Levulinyl-3′-TIPS-uridine (23)

A solution of 3% triflouroacetic acid (TFA) in DCM (20mL) was added directly to compound **(22)** (1.10 g, 1.37 mmol)**.** The solution was quenched with 5ml of MeOH after 5 min and condensed to dryness on a rotovap. By TLC there was a very small amount of starting material so the process was repeated again. The compound was purified by column chromatography. 0 →75% Ethyl acetate: hexanes. Yield: 0.65g (95%).

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.87 - 1.22 (m, 23 H) 2.13 (s, 3 H) 2.46 - 2.62 (m, 2 H) 2.66 - 2.85 (m, 2 H) 3.37 (br. s., 1 H) 3.70 - 3.95 (m, 2 H) 4.09 (d, *J*=2.20 Hz, 1 H) 4.64 (dd, *J*=4.89, 3.18 Hz, 1 H) 5.41 (t, *J*=5.62 Hz, 1 H) 5.69 (dd, *J*=7.95, 1.34 Hz, 1 H) 5.82 (d, *J*=6.11 Hz, 1 H) 7.59 (d, *J*=8.07 Hz, 1 H) 9.59 (s, 1 H)

^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 12.16, 17.84, 27.77, 29.70, 37.78, 61.83, 71.18, 74.62, 86.99, 89.90, 102.56, 142.32, 150.53, 163.80, 172.35, 206.66.



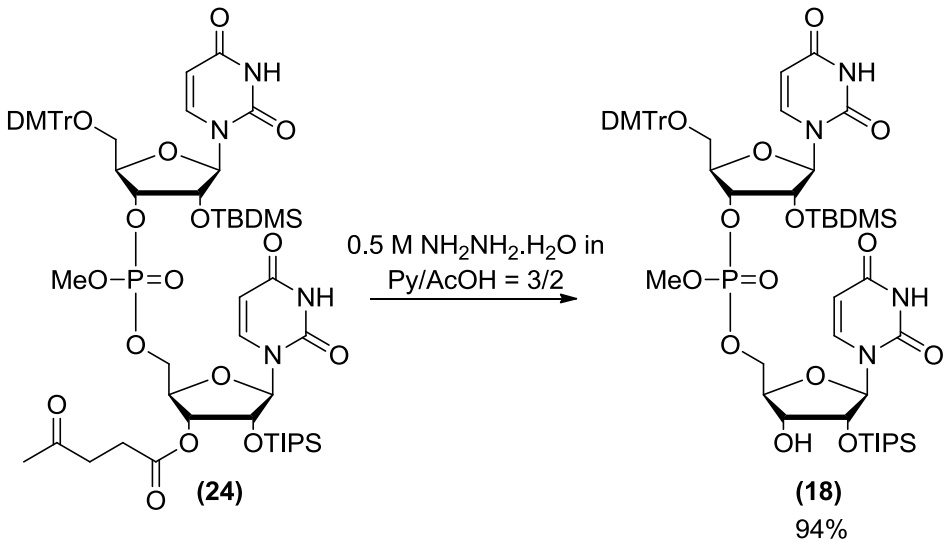
[5′-DMTr-2′TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′-TIPS-3′-Lev] (24)

DCI (1.08 g, 9.18 mmol, 2 eq) was added to a 30 mL ACN solution of the phosphoramidite **(5)** (5.66 g, 6.89 mmol, 1.5 eq) and the nucleoside **(21)** (2.289 g, 4.59 mmol, 1.0 eq). The mixture was allowed to stir for 20-30 min until the starting nucleoside was completely consumed. One equivalent of tert-butanol (0.5 mL) was added to quench any remaining active amidite (this helps with the next purification step as the quenched amidite becomes less polar and easier to separate from the desired dimer). Five equivalents of tert-butyl hydroperoxide (6 M; 3.06 mL) were added and the resulting mixture allowed to stir for an additional 15 min until all of the phosphite triester was consumed (TLC analysis with 10% MeOH in DCM). The sample was then diluted with MTBE and extracted x3 with a 5% solution of sodium bisulphite and once with brine. The mixture was then dried over magnesium sulphate and condensed to dryness. The sample was then purified by column chromatography (0-75% gradient of ethyl acetate in hexane). Yield: 5.29 g (93%).

^{1}H NMR (500 MHz, CD_3CN) δ ppm -0.07 - 0.13 (m, 6 H) 0.75 - 0.86 (m, 8 H) 0.87 - 1.04 (m, 19 H) 1.08 (s, 4 H) 1.99 - 2.10 (m, 3 H) 2.28 (s, 23 H) 2.44 - 2.59 (m, 3 H) 2.69 (dd, *J*=10.76, 4.16 Hz, 3 H) 3.08 (s, 2 H) 3.26 - 3.45 (m, 3 H) 3.62 (d, *J*=11.25 Hz, 2 H) 3.66 - 3.77 (m, 7 H) 4.06 - 4.30 (m, 5 H) 4.34 - 4.56 (m, 3 H) 4.73 (dt, *J*=7.76, 3.82 Hz, 1 H) 4.81 (dt, *J*=7.58, 4.04 Hz, 1 H) 5.04 (ddd, *J*=8.86, 5.20, 3.30 Hz, 1 H) 5.30 (d, *J*=7.58 Hz, 1 H) 5.58 (d, *J*=8.07 Hz, 1 H) 5.77 (d, *J*=5.14 Hz, 1 H) 5.79 - 5.89 (m, 2 H) 6.73 - 6.87 (m, 4 H) 7.07 (d, *J*=8.80 Hz, 1 H) 7.15 - 7.30 (m, 6 H) 7.31 - 7.39 (m, 2 H) 7.45 (dd, *J*=12.72, 8.07 Hz, 1 H) 7.56 - 7.65 (m, 1 H) 7.78 (dd, *J*=8.19, 3.30 Hz, 1 H) 9.40 (br. s., 2 H)

^{13}C NMR (126 MHz, CD_3CN) δ ppm -5.87, -5.74, -5.72, -5.64, -5.56, -5.51, 11.92, 11.94, 11.95, 17.10, 17.12, 17.20, 17.22, 17.71, 17.73, 25.02, 25.04, 25.06, 26.25, 27.77, 28.93, 37.36, 37.38, 48.52, 54.73, 54.88, 54.94, 54.99, 62.21, 71.71, 71.75, 73.11, 73.13, 80.67, 87.02, 87.08, 87.46, 88.43, 88.51, 102.12, 102.25, 102.66, 102.74, 112.88, 113.24, 113.27, 126.78, 127.14, 127.64, 127.65, 128.03, 129.03, 130.11, 130.15, 130.17, 135.13, 135.20, 135.24, 135.30, 139.87, 139.94, 139.98, 140.11, 144.55, 144.58, 150.50, 150.57, 150.60, 150.62, 158.53, 158.83, 158.84, 162.77, 162.79, 162.88, 162.94, 172.23, 206.73.

31P NMR (81 MHz, CD_3CN) δ ppm 0.39 (s, 1 P) 0.81 (s, 1 P).



Procedure according to Scheme 3.11

[5′-DMTr-2′-TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′-TIPS] (18)

To a solution of **(11a)** 9.53g (7.71 mmol) in ACN (10 mL) was added 77 mL (5 eq) of 0.5 M hydrazine hydrate in pyridine/acetic acid (3:2) and the resulting mixture allowed to stir for 20 min. The reaction was worked up by cooling the reaction mixture to 0°C in an ice bath and adding 5eq of 2,4-pentanedione (3.96 mL, 38.6 mmol) to quench excess hydrazine. At this point, the mixture was removed from the ice bath and allowed to stir for an additional 10 min at room temperature. The mixture was diluted with MTBE or ethyl acetate and extracted x4 with 5% ammonium chloride to keep the aqueous medium slightly acidic, then once with brine to remove any residual ammonium chloride. Then the mixture was extracted twice with 3% aqueous $CuSO_4$ solution (to mop up excess pyridine) then once with 1% EDTA and 10% NaCl to remove any residual copper from the organic layer. The organic layer was then dried with magnesium sulphate and condensed to dryness. This mixture was then purified by column in ethyl acetate/hexanes (0-75% gradient). Yield: 8.25 g (94%).

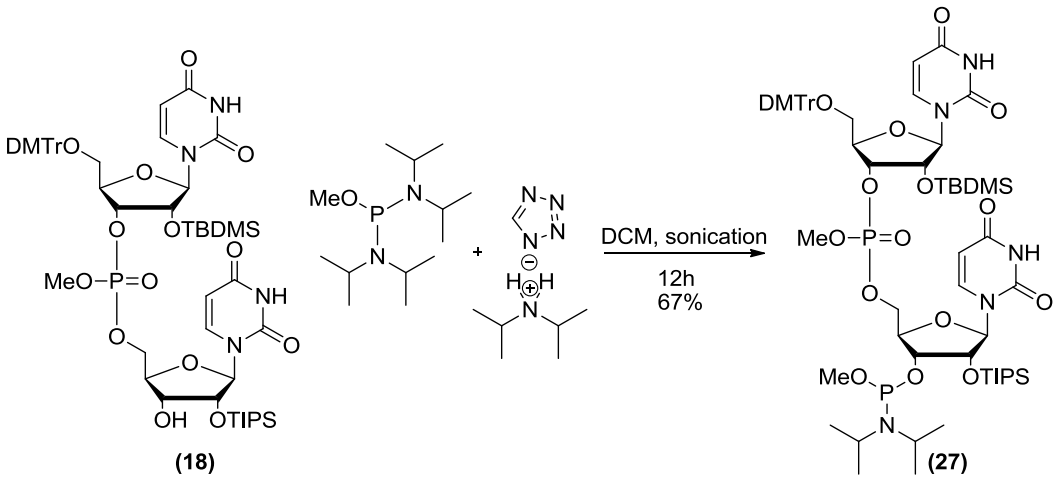
^{1}H NMR (300 MHz, CD_3CN) δ ppm 0.09 - 0.15 (m, 6 H) 0.87 - 1.20 (m, 40 H) 1.94 - 2.00 (m, 1 H) 2.10 - 2.20 (m, 2 H) 3.44 (br. s., 3 H) 3.67 - 3.79 (m, 12 H) 4.04 - 4.23 (m, 5 H) 4.28 - 4.35 (m, 2 H) 4.38 - 4.48 (m, 2 H) 4.81 - 4.90 (m, 1 H) 5.38 (d, *J*=8.21 Hz, 1 H) 5.64 (d, *J*=8.20 Hz, 1 H) 5.81 - 5.91 (m, 2 H) 6.90 (d,

J=7.91 Hz, 5 H) 7.24 - 7.37 (m, 9 H) 7.41 - 7.52 (m, 4 H) 7.71 (dd, *J*=8.20, 2.64 Hz, 1 H) 9.62 (br. s., 1 H)

^{13}C NMR (126 MHz, $CDCl_3$) δ ppm -5.08, -5.00, -4.90, -4.87, 12.02, 12.05, 17.66, 17.69, 17.76, 17.79, 25.56, 25.59, 54.59, 54.92, 54.97, 55.21, 55.25, 61.72, 67.06, 67.29, 70.17, 70.26, 74.72, 74.84, 75.68, 75.99, 81.50, 82.14, 87.53, 87.57, 88.18, 88.33, 90.51, 90.57, 102.45, 102.92, 102.96, 113.33, 127.35, 127.76, 128.05, 128.21, 128.23, 129.12, 130.17, 130.23, 130.25, 130.36, 134.58, 134.66, 134.79, 139.83, 140.37, 140.45, 143.95, 144.06, 150.24, 150.28, 150.50, 150.58, 158.77, 158.78, 158.82, 163.29, 163.34, 163.39,

^{31}P NMR (81 MHz, CD_3CN) δ ppm 0.58 (s, 1 P) 0.83 (s, 1 P).

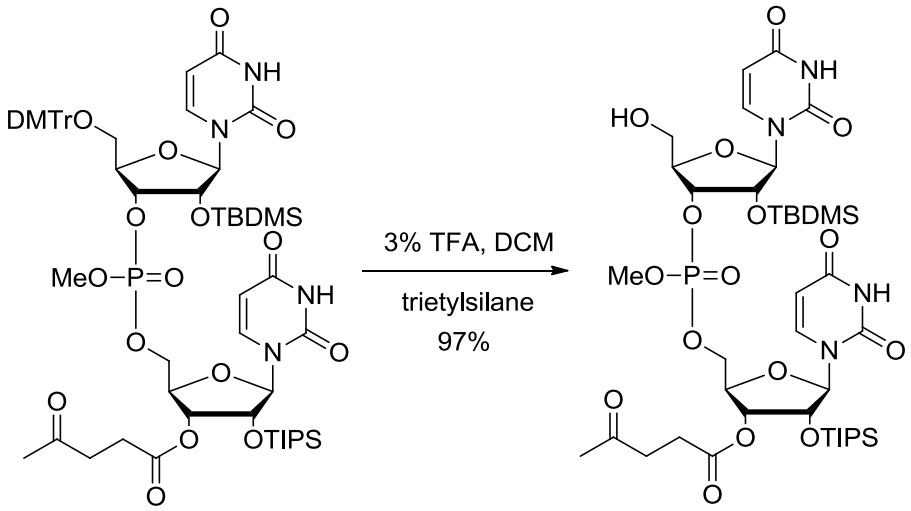
Mass calc. 1137.36 Mass found. 1159.53 ($M+Na^+$).



[5′-DMTr-2′-TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′-TIPS]-3′-methylphosphoramidite (27)

N,N-Diisopropyl ammonium tetrazolide (14.1 mmol, 2.41 g) and bis (N,N-diisopropylamino)methylphosphine (14.1 mmol, 3.69 g) were added to 26.8mL of ACN. After stirring for a few minutes, the mixture was sonicated for 0.5h until the majority of the tetrazolide salt had dissolved. The dimer **(18)** (4.01 mmol, 4.566 g) was then added directly to this solution and sonicated for an additional 8h; finally the mixture was allowed to stir overnight at room temperature. Sonication was preformed to help dissolve the activator and considerably speed up the reaction. The mixture was then diluted with either MTBE or ethyl acetate

(the ethyl acetate must be prewashed with saturated sodium bicarbonate to remove residual acetic acid) and extracted with a 10% NaCl solution x2 then once with brine. The organic layer was then dried with magnesium sulphate and condensed to dryness. The mixture was then taken up in minimal amounts of DCM and precipitated in petroleum ether to remove amines (-78^{0}C). The ppt was filtered over Celite® and collected with DCM. The solution was evaporated and the resulting solid purified by column chromatography. The column was first loaded with 3% triethylamine in hexanes. The sample was then dissolved with 17% hexanes, 3% triethylamine, 80% DCM and loaded on the column. The compound was eluted with 0-10% Ethyl acetate/DCM/3%triethylamine. Yield: 3.5 g (67%). ^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 0.59 (s, 1 P) 0.77 (s, 1 P) 1.09 (s, 1 P) 1.14 (s, 1 P) 151.25 (s, 1 P) 151.38 (s, 1 P) 151.99 (s, 1 P) 152.05 (s, 1 P).

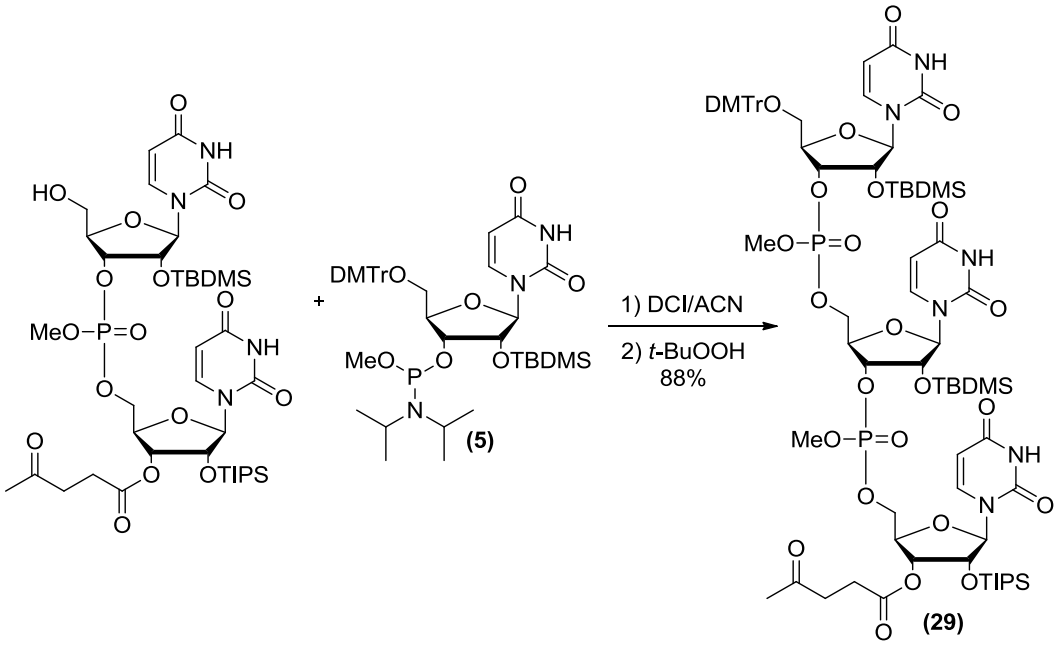


[5′-OH-2′-TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′-TIPS-3′-Lev] (28)

Compound **(24)** (5.29g, 4.28 mmol) was dissolved in 75ml of DCM and 3% trifluoroacetic acid (TFA) (vol/vol) and allowed to stir for 5min then 20ml of MeOH was added. The solution was immediately condensed to a oil and the process was repeated with another 25ml of DCM/TFA. The sample was then re-suspended in DCM and loaded on a column. The DCM was washed off with Hexanes and the tritanol was eluted with Hexanes :ethyl acetate 50:50, the product was eluted with 80% ethyl acetate. Yield: 3.88g (97%).

^{1}H NMR (400 MHz, CD_3CN) δ ppm -0.08 - 0.13 (m, 8 H) 0.75 - 0.95 (m, 13 H) 0.96 - 1.10 (m, 27 H) 1.20 (s, 1 H) 1.78 - 1.91 (m, 1 H) 1.91 - 2.04 (m, 2 H) 2.07 - 2.32 (m, 5 H) 2.52 - 2.71 (m, 3 H) 2.71 - 2.97 (m, 4 H) 3.70 - 3.87 (m, 7 H) 4.22 - 4.40 (m, 6 H) 4.41 - 4.63 (m, 4 H) 4.70 - 4.87 (m, 2 H) 5.15 (ddd, *J*=14.18, 5.38, 3.13 Hz, 2 H) 5.23 - 6.21 (m, 4 H) 5.67 - 5.95 (m, 6 H) 7.59 (dd, *J*=8.22, 5.09 Hz, 2 H) 7.85 (dd, *J*=8.22, 2.74 Hz, 3 H) 9.50 (s, 1 H) 9.46 (s, 2 H).

^{13}C NMR (126 MHz, CD_3CN) δ ppm -5.87, -5.81, -5.63, -5.58, 11.93, 11.95, 17.11, 17.12, 17.20, 17.21, 17.71, 17.72, 25.04, 25.06, 27.79, 27.80, 28.93, 28.94, 37.38, 54.77, 54.81, 54.94, 54.98, 60.48, 60.69, 66.86, 66.90, 67.25, 71.69, 71.87, 73.14, 74.10, 74.12, 74.15, 76.13, 76.17, 76.90, 76.94, 80.40, 80.46, 80.56, 80.62, 83.32, 83.34, 83.61, 83.64, 87.86, 88.31, 88.42, 88.58, 102.14, 102.33, 102.70, 102.84, 140.05, 140.25, 140.48, 140.55, 150.72, 150.75, 150.77, 150.82, 163.04, 163.06, 163.22, 163.26, 172.27, 172.32, 206.90, 206.93,

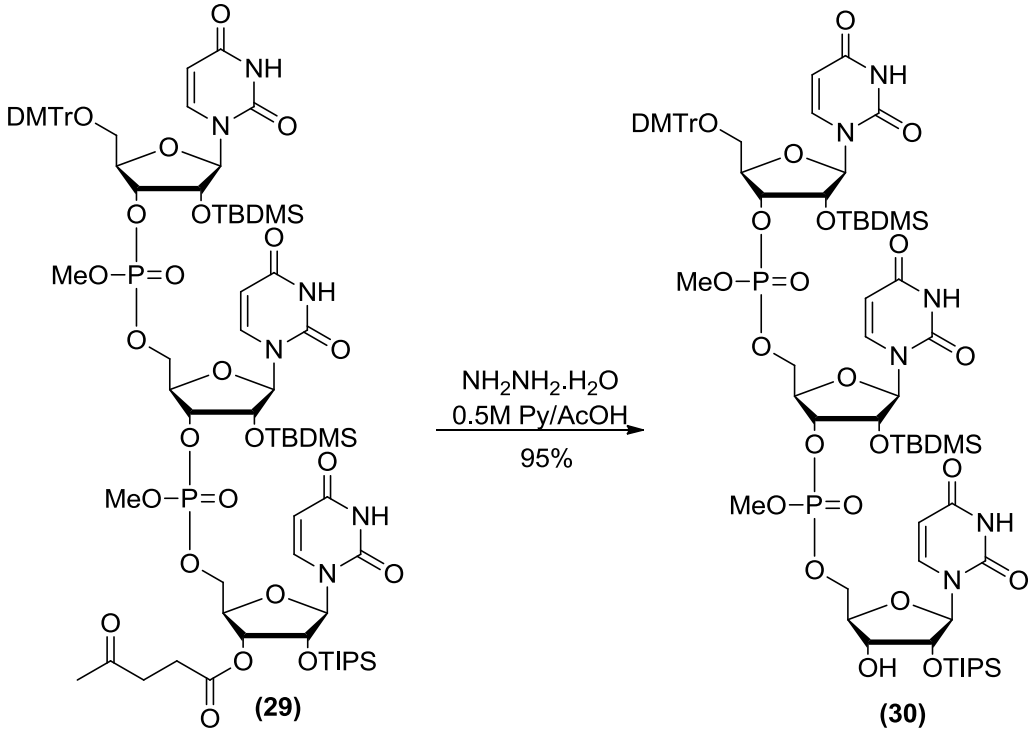


[5′-DMTr-2′-TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′-TBDMS]-[3′-p(OMe)-5′]-[rU-2′-TIPS-3′-Lev] (29)

Compound **(28)** (3.88g, 4.16mmol) was dissolved in 15ml of THF with 2eq of DCI (0.982g, 8.32mmol). Immediately phosphoramidite **(2a)** (4.10g 4.99mmol) was added to the solution and allowed to stir at room temperature for 2h. The reaction was monitored by TLC (5%MeOH in DCM) and after 2 hours

the reaction was usually complete. At this point 10eq (2.5ml of a 6M solution in decane) of *tert*-butyl hydroperoxide was added to solution and allowed to stir for 20min until all the phosphite triester was converted to the phosphate, as monitored by TLC (5% MeOH in DCM) observing a more polar spot appear. The solution was condensed to approximately 10ml and then dissolved in ethyl acetate and extracted with 5% sodium sulfite (x2) and brine (x1). The organic layer was dried and condensed and put on a short column (0→5%MeOH in DCM) yielding 6.10g of (**13**) (88%).

^{1}H NMR (500 MHz, CD_3CN) δ ppm -0.06 - 0.14 (m, 12 H) 0.68 - 0.87 (m, 13 H) 0.87 - 1.07 (m, 15 H) 1.88 (dt, *J*=4.89, 2.45 Hz, 5 H) 2.06 (s, 0 H) 2.46 - 2.58 (m, 2 H) 2.63 - 2.84 (m, 3 H) 3.28 - 3.50 (m, 3 H) 3.60 - 3.75 (m, 9 H) 4.16 - 4.22 (m, 3 H) 4.23 - 4.35 (m, 3 H) 4.44 - 4.56 (m, 2 H) 4.57 - 4.76 (m, 2 H) 4.76 - 4.93 (m, 1 H) 5.03 - 5.13 (m, 1 H) 5.21 - 5.35 (m, 2 H) 5.39 (s, 1 H) 5.47 - 5.60 (m, 2 H) 5.60 - 5.70 (m, 2 H) 5.72 - 5.76 (m, 1 H) 5.77 - 5.86 (m, 2 H) 6.83 (dd, *J*=8.80, 2.45 Hz, 4 H) 7.16 - 7.31 (m, 6 H) 7.31 - 7.53 (m, 4 H) 7.54 - 7.67 (m, 1 H) 9.25 - 9.44 (m, 4 H)



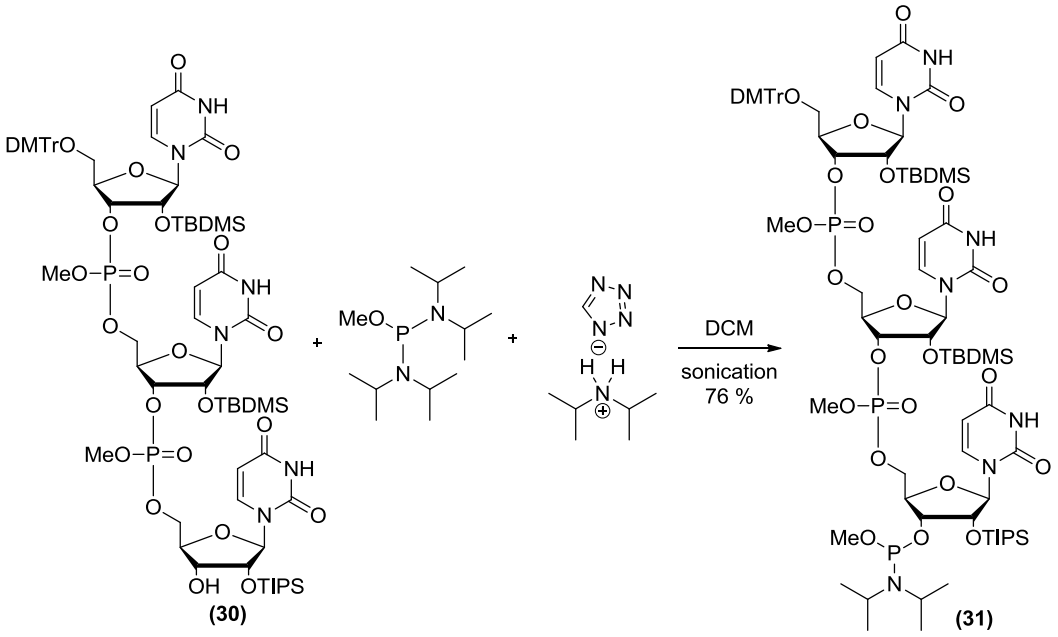
[5′-DMTr-2′-TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′-TBDMS]-[3′-p(OMe)-5′]-[rU-(2′-TIPS] (30)

The levulinyl protected trimer (**29)** (6.01g 3.64mmol) was dissolved in minimal amounts of ACN, just enough to get it into solution. It was then treated with 5 equivalents (36.4ml, 18.2mmol) of a 0.5M hydrazine hydrate solution in 3:2 pyridine:acetic acid that was prepared immediately before the reaction. The reaction mixture was allowed to stir for 10min before it was chilled to 0°C in an ice bath and 5 equivalents (1.87ml, 18.2mmol) of 2, 4-pentanedione was added which was allowed to stir for an additional 5min before the reaction was diluted with MTBE and extracted x3 with ammonium chloride (5%w/v) and once with cupric sulfate, then once with a 1% solution of EDTA in 10% NaCl (w/vol) and lastly once with brine. The sample was then dried over MgSO4 and condensed to dryness. The sample was re-suspended in chloroform, loaded on a column and eluted with ethyl acetate:hexanes 0→80%. Yield: 5.58g (95%).

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.00 - 0.20 (m, 12 H) 0.75 - 0.95 (m, 16 H) 1.04 (m, *J*=5.10 Hz, 16 H) 3.40 - 3.63 (m, 3 H) 3.67 (d, *J*=11.49 Hz, 2 H) 3.71 - 3.86 (m, 8 H) 4.07 - 4.42 (m, 9 H) 4.44 - 4.60 (m, 3 H) 4.65 - 4.85 (m, 2 H) 4.85 - 5.05 (m, 2 H) 5.24 (dt, *J*=16.75, 8.25 Hz, 1 H) 5.35 (d, *J*=8.07 Hz, 1 H) 5.47 - 5.66 (m, 2 H) 5.66 - 5.84 (m, 3 H) 5.85 - 6.08 (m, 2 H) 6.73 - 6.90 (m, 4 H) 7.16 (d, *J*=8.56 Hz, 1 H) 7.20 - 7.37 (m, 8 H) 7.38 - 7.44 (m, 1 H) 7.50 (br. s., 0 H) 7.56 (d, *J*=8.07 Hz, 1 H) 7.77 (d, *J*=8.07 Hz, 1 H) 7.82 (d, *J*=5.87 Hz, 1 H) 9.38 - 9.79 (m, 2 H) 10.00 (br. s., 1 H) 10.17 (br. s., 1 H)

^{13}C NMR (126 MHz, $CDCl_3$) δ ppm -5.14, -5.08, -5.06, -5.05, -5.03, -5.00, -4.99, -4.96, -4.91, -4.90, -4.88, 12.00, 12.04, 17.66, 17.70, 17.73, 17.79, 18.02, 18.06, 18.13, 25.54, 25.55, 25.57, 25.59, 54.91, 55.10, 55.23, 55.27, 62.10, 67.16, 70.09, 70.51, 73.67, 73.93, 74.45, 74.57, 81.86, 82.22, 87.57, 87.62, 87.96, 102.55, 102.74, 102.90, 113.12, 113.35, 127.36, 127.75, 127.81, 128.09, 128.16, 128.20, 129.11, 130.10, 130.14, 130.17, 130.28, 130.34, 134.60, 134.69, 134.78, 139.46, 139.79, 143.97, 144.01, 144.15, 150.00, 150.14, 150.16, 150.24, 150.29, 150.39, 150.58, 151.11, 158.57, 158.72, 158.78, 158.82, 162.98, 163.04, 163.14, 163.20, 163.22, 163.30, 163.86,

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm -0.52 (s, 1 P) 0.27 (s, 1 P) 0.40 (s, 1 P) 0.44 (s, 1 P) 0.64 (s, 1 P) 0.71 (s, 1 P) 0.83 (s, 1 P) 0.94 (s, 1 P).



[5′-DMTr-2′-TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′-TBDMS]-[3′-p(OMe)-5′]-[rU-2′-TIPS]-3′-methylphosphoramidite (31)

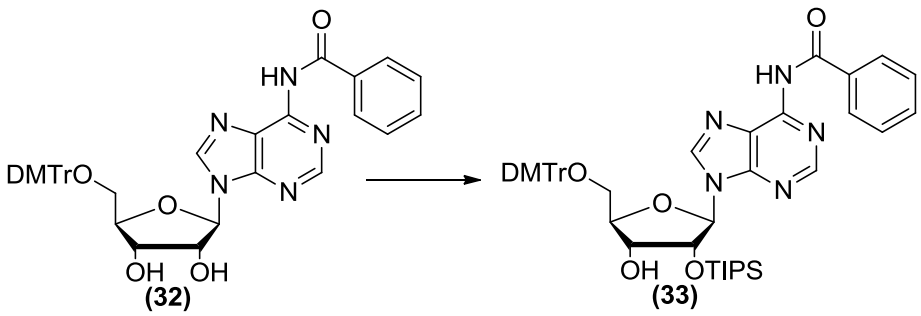
N,N-Diisopropylammonium tetrazolide 3.6eq (2.52g, 14.7 mmol) and bis (N,N-diisopropylamino)methylphosphine 3.5eq (3.76 g, 4.11ml, 14.3 mmol) were added to 27.3mL of DCM. After stirring for a few minutes, the mixture was sonicated for 0.5 h until the majority of the tetrazolide salt had dissolved. The trimer [5′-DMTr-2′-TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′-TBDMS]-[3′-p(OMe)-5′]-[rU-(2′-TIPS] **(30)** (6.433g, 4.09 mmol) was then added directly to this solution and sonicated for an additional 8 h; finally the mixture was allowed to stir overnight at room temperature. The mixture was then diluted with either MTBE or ethyl acetate (the ethyl acetate must be prewashed with saturated sodium bicarbonate to remove residual acetic acid) and extracted with a 10% NaCl solution x2 then once with brine. The organic layer was then dried with magnesium sulphate and condensed to dryness. The mixture was then taken up in minimal amounts of DCM and precipitated in petroleum ether to remove amines (-78^{0}C). The precipitate was filtered over Celite® and collected with DCM. The

solution was evaporated and the resulting solid purified by column chromatography. The column was first loaded with 3% triethylamine in hexanes. The sample was then dissolved with 17% hexanes, 3% triethylamine, 80% DCM and loaded on the column. The compound was eluted with 0-10% ethyl acetate/DCM/3%triethylamine. Yield: 5.433 g (76%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm -0.13 - 0.04 (m, 8 H) 0.67 - 0.83 (m, 14 H) 0.85 - 0.97 (m, 15 H) 1.05 (m, *J*=5.00 Hz, 9 H) 1.09 - 1.27 (m, 3 H) 1.72 (m, *J*=6.40, 6.40 Hz, 2 H) 2.98 (s, 1 H) 3.13 - 3.42 (m, 6 H) 3.43 - 3.72 (m, 12 H) 4.05 (m, *J*=16.70 Hz, 2 H) 4.15 - 4.29 (m, 4 H) 4.30 - 4.48 (m, 2 H) 4.49 - 4.66 (m, 1 H) 4.70 - 4.93 (m, 1 H) 5.05 - 5.42 (m, 1 H) 5.48 - 5.74 (m, 2 H) 5.80 - 6.09 (m, 1 H) 6.66 - 6.80 (m, 3 H) 7.07 - 7.26 (m, 9 H) 7.39 (d, *J*=19.93 Hz, 1 H) 7.47 - 7.62 (m, 1 H) 7.64 - 7.76 (m, 1 H)

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm -5.16, -5.07, -5.01, -4.94, -4.91, -4.51, 7.68, 7.87, 12.19, 12.24, 12.26, 12.33, 12.45, 17.78, 17.82, 17.86, 17.95, 18.01, 18.05, 18.14, 18.18, 24.49, 24.59, 24.70, 24.78, 25.54, 25.58, 25.65, 25.71, 42.71, 42.89, 43.11, 43.28, 47.01, 49.62, 55.01, 55.15, 55.21, 55.26, 55.67, 67.91, 72.07, 72.37, 72.56, 74.57, 74.59, 74.61, 74.64, 81.74, 87.51, 87.53, 87.61, 87.64, 102.55, 103.07, 113.35, 127.37, 128.07, 128.21, 130.21, 130.28, 134.54, 134.64, 134.75, 134.80, 134.92, 139.75, 143.96, 144.37, 149.91, 150.21, 150.33, 150.43, 150.50, 150.62, 151.02, 151.57, 158.62, 158.78, 158.82, 163.13, 163.20, 163.32, 163.49, 163.55, 164.09.

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm -1.19 (s, 1 P) -0.71 (s, 1 P) -0.09 (s, 1 P) 0.23 (s, 1 P) 0.32 (s, 2 P) 0.64 (s, 1 P) 0.74 (s, 2 P) 0.79 (s, 1 P) 0.86 (s, 1 P) 0.93 (s, 1 P) 1.08 (s, 1 P) 1.15 (s, 2 P) 1.23 (s, 1 P) 150.66 (s, 1 P) 150.80 (s, 1 P) 151.06 (s, 2 P) 153.00 (s, 1 P) 153.06 (s, 2 P) 153.57 (s, 1 P).

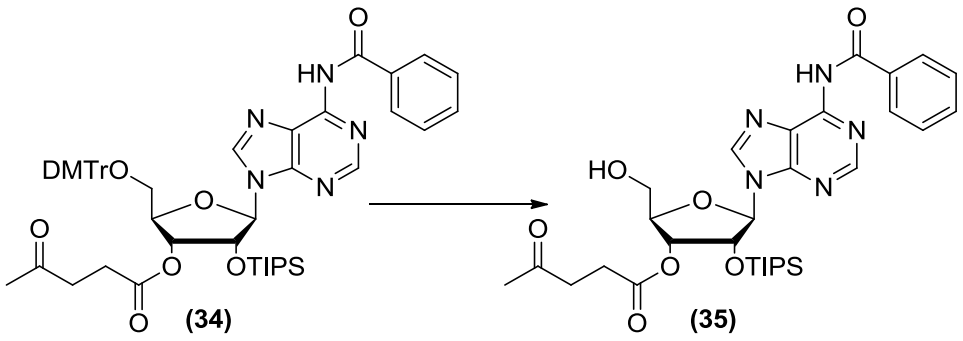


5′-DMTr-2′-TIPS-N-benzoyl-adenosine (33)

5′-DMTr-N-benzoyl-adenosine **(32)** (10 g, 14.8 mmol) was dissolved in dry THF 150 mL. Dry pyridine and $AgNO_3$ were added and stirring was continued for 5 min, until almost all $AgNO_3$ dissolved. TIPS-Cl (3.5 mL, 16.62 mmol) was added all at once and the resulting cloudy milky solution was stirred at room temperature. After 12h, TLC analysis revealed there was still a lot of starting material in the reaction mixture. So, one more equivalent of $AgNO_3$ and TIPS-Cl was added and the mixture was stirred for another 12 h. The reaction mixture was filtered into 5% $NaHCO_3$ solution to avoid deprotection of DMTr. The reaction mixture was extracted with DCM, evaporated and purified through column chromatography. The unreacted starting material was recovered and reused for the next time. Yield 2′ TIPS nucleoside 4.7 g, 38%, 3′ TIPS nucleoside 1.5 g, 12%.

^{1}H NMR (400 MHz, Acetone) δ ppm 9.40 (s, 1H), 8.68 (s, 1H), 8.41 (s, 1H), 8.12 (d, *J* = 7.2, 2H), 7.82 – 7.27(m, 12H), 7.03 – 6.86 (m, 4H), 6.19 (d, *J* = 5.1, 1H), 5.33 (t, *J* = 4.7, 1H), 4.55 (d, *J* = 4.2, 2H), 4.41 – 4.28 (m, 2H), 3.87 (s, 6H), 3.61 – 3.18 (m, 0H), 2.28 (br s, 1H), 1.25 – 0.91 (m, 21H).

^{13}C NMR (300 MHz, Acetone) δ ppm 171.17, 158.73, 151.86, 150.18, 145.16, 143.18, 135.87, 134.29, 132.61, 130.16, 128.69, 128.27, 128, 126.99, 124.95, 117.49, 113.19, 89.53, 86.38, 83.54, 75.4, 70.8, 63, 59.3, 55, 20.4, 17.4, 17.2, 13.8, 12, 0.59.



5′-DMTr-2′-TIPS-3′-levulinyl-N-benzoyl-adenosine (34)

Levulinic acid (0.21 g, 1.84 mmol), DCC (0.371 g 1.84 mmol) and DMAP (0.034 g, 0.30mmol) were dissolved in 3mL of THF and stirred at 0 °C for 10 min. Compound 5′-DMTr-2′-TIPS-N-benzoyl-adenosine **(33)** (0.5 g, 0.613 mmol) was added drop wise to the above solution slowly at 0 °C. The reaction mixture was slowly warmed to room temperature and stirred an additional 3h. After the 3 h, the reaction mixture was slowly changing its colour to dark brown. TLC was monitored and the reaction was completed. The reaction mixture was filtered over Celite®. The organic layer was washed with saturated $NaHCO_3$, and saturated NaCl. The compound was purified through column chromatography, 72% yield

^{1}H NMR (400 MHz, Acetone) δ ppm 9.99 (br s, 1H), 8.66 (s, 1H), 8.6 (s, 1H), 8.54 (d, *J* = 6.8, 2H), 8.13 (d, *J*=7.3, 2H), 7.85-7.18 (m, 10H), 6.86 (d, *J* = 10.1, 4H), 6.20 (d, *J* = 5.7, 1H), 5.73 – 5.53 (m, 1H), 4.37-4.3 (m, 1H), 3.78 (s, 6H), 3.62-3.3.36 (m, 2H), 3.11 – 2.52 (m, 4H), 2.16 (s, 3H), 1.29 (br s, 1H), 1.06 – 0.72 (m, 21H).

^{13}C NMR (300 MHz, $CDCl_3$) δ ppm 206, 174.6, 171.9, 170.8, 164.89, 158.5, 153.2, 152.5, 152, 150.7, 149.6, 144.5, 141.9, 135.4, 135.3, 134.1, 133.5, 132.6, 129.99, 129.3, 129.1, 128.63, 128.4, 128, 127.9, 126.8, 123.6, 113.1, 88, 86.7, 82.5, 73.75, 63.1, 60.1, 55, 37.6, 31.5, 29.7, 27.8, 20.8, 17.6, 17.4, 14.1, 11.9.

HRMS (ESI) for $C_{52}H_{61}N_5O_9Si$ calc: 927.42, found: 928.02

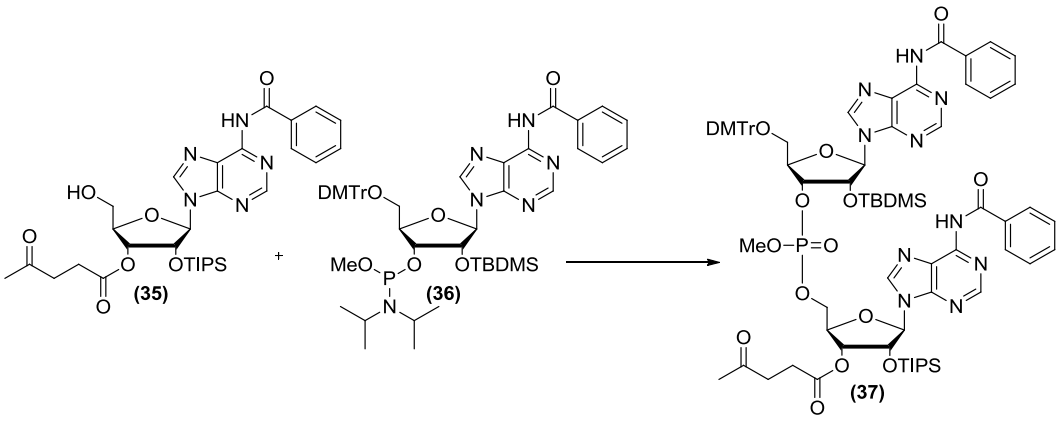
2′-TIPS-3′-levulinyl-N-benzoyl-adenosine (35)

5′-DMTr-2′-TIPS-3′-levulinyl-N-benzoyl-adenosine **(34)** (2.8 g, 3.02 mmol) was solvated in 12 mL of DCM and 5 eq of 3% trifluoroacetic acid (TFA) (vol/vol) and allowed to stir for 5min, then a few drops of MeOH was added. The solvent was evaporated and the process was repeated one more time. The sample was then re- suspended in DCM and loaded on a column. The DCM was washed off with Hexanes and the tritanol was eluted with Hexanes :ethyl acetate 50:50, the product was eluted with 80% ethyl acetate. Yield 1.75 g, 93%.

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.0 (br s, 1H), 8.81 (s, 1H), 8.17 – 7.93 (m, 3H), 7.7-7.47 (m, 3H), 5.91 (d, *J* = 7.1, 1H), 5.48-5.32 (m, 2H), 4.29 (s, 1H), 3.90 (dd, *J* = 40.4, 1.9, 2H), 3.01 – 2.50 (m, 5H), 2.22 (s, 3H), 0.82 (d, *J* = 1.2, 21H).

^{13}C NMR (300 MHz, $CDCl_3$) δ ppm 206.2, 172, 165, 151.9, 150.5, 150.4, 143.3, 133.3, 132.6, 128.6, 127.9, 124.1, 90.7, 85.7, 74.6, 73, 62.4, 37.57, 29.66, 27.7, 17.3, 11.7.

HRMS (ESI) for $C_{31}H_{43}N_5O_7Si$ calc: 625.29, found: 648.25 (M+23)



[5′-DMTr-2′TBDMS-N-Bz-rA]-[3′-p(OMe)-5′]-[rA-2′TIPS-3′-Lev] (37)

DCI (0.223 g, 1.88mmol) was dissolved in 8 mL THF/CH_3CN (1:1) and added to adenosine phosphoramidite **(36)** (1.63 g, 1.717 mmol). The above mixture was slowly added to the compound **(35)** (1.07 g, 1.717 mmol) in 8 mL THF/CH_3CN (1:1) at 0˚C. The reaction mixture was slowly warmed to room temperature. After stirring for 30 min, 10 eq of *tert*-butyl hydroperoxide (6M in

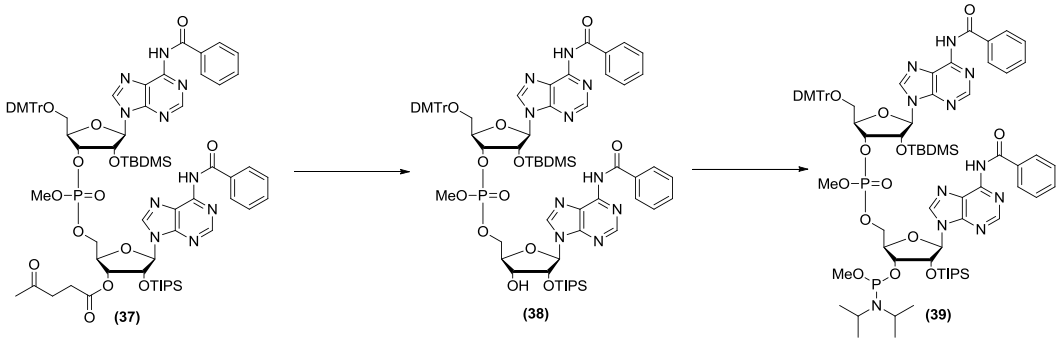
decane, 1.92 mL) was added to the reaction mixture and stirred for an additional 10 min. The reaction mixture was taken into MTBE and washed quickly with 5% sodium sulfite 2 times. The organic layer was dried over anhydrous $MgSO_4$ and subjected to column chromatography on silica gel using eluent EtOAc & DCM (0-50% EtOAc) to get the desired rAA dimer (2.15 g, 85%).

^{1}H NMR (400 MHz, Acetone) δ ppm 9.97 (m, 2H), 8.70 (s, 2H), 8.69(s, 2H), 8.52-8.41 (m, 2H), 8.12 (d, *J* = 6.6, 2H), 7.72-7.13 (m, 15H), 6.94-6.77 (m, 4H), 5.64 – 5.34 (m, 2H), 5.22 – 5.04 (m, 2H), 4.62 – 4.36 (m, 4H), 3.76 (s, 9H), 3.64-3.37 (m, 2H), 2.16 (s, *J* = 4.9, 3H), 1.08 – 0.79 (m, 3H), 0.97 (s, 9H), 0.74 (d, *J* = 8.3, 18H), -0.01 (d, *J* = 7.9, 3H), -0.19 (d, *J* = 7.7, 3H).

^{13}C NMR (300 MHz, Acetone) δ ppm 172.0, 170.1, 165.3, 158.7, 152, 150.7, 145, 143.5, 142.6, 135.6, 134, 132.3, 130.1, 128.6, 127.9, 126.8, 125.4, 125.1, 113.1, 88.7, 88.3, 86.5, 82.8, 82.7, 81.4, 81.3, 77.6, 77.5, 77.2, 77.1, 73.7, 73.5, 72.8, 72.5, 67.1, 66.9, 63, 62.8, 60, 54.8, 54.4, 37.4, 25.2, 20.2, 17.7, 17.4, 17.2, 13.8, 12, -5.3, -5.8.

^{31}P NMR (200 MHz, Acetone) δ ppm 0.97 (s, 1P), 0.78 (s, 1P).

HRMS (ESI) for $C_{76}H_{93}N_{10}O_{16}PSi_2$ calc: 1488.60, found: 1511.55 (M+23)



[5′-DMTr-2′TBDMS-N-Bz-rA]-[3′-p(OMe)-5′]-[rA-2′TIPS] (38)

Compound **(37)** (0.72 g, 0.484 mmol) was dissolved in minimum amounts of acetonitrile (1mL/mmol). 0.5 M hydrazine (0.244 mL) in pyridine/acetic acid (3:2) was added dropwise to the above reaction mixture at room temperature and stirred for 10-15 min. The reaction mixture was cooled to 0 ˚C and 2,4-pentanedione added at once (0.5 mL, 4.48mmol). The reaction mixture was warmed to room temperature and stirred for an additional 15 min. Reaction

mixture was diluted with MTBE and washed 2 times with aq. NH_4Cl and saturated NaCl. The organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated. The compound was subjected to column chromatography on silica gel using eluent DCM & EtOAc (0-50% EtAc) to get the product (0.64 g, 95%).

^{1}H NMR (400 MHz, Acetone) δ 10.01 (s, 2H), 8.67 (d, *J* = 6.1, 2H), 8.54-8.46 (m, *J* = 2.7, 2H), 8.12 (d, *J* = 5.7, 4H), 7.71 – 7.08 (m, 15H), 6.87 (d, *J* = 8.2, 4H), 6.24 (dd, *J* = 8.5, 4.3, 1H), 6.13 (dd, *J* = 13.9, 6.5, 1H), 5.49 – 5.01 (m, 4H), 4.71 – 4.15 (m, 4H), 3.76 (s, 9H), 3.64 – 3.33 (m, 3H), 3.12 (s, 1H), 1.32-0.8 (m, 3H), 1.13 (s, 9H), 0.74 (d, *J* = 3.2, 18H), -0.00 (d, *J* = 2.1, 3H), -0.20 (d, *J* = 13.0, 3H).

^{31}P NMR (200 MHz, Acetone) δ 1.15 (s, 1P), 0.89 (s, 1P).

^{13}C NMR (300MHz, Acetone) δ 164.9, 158.77, 152, 150.5, 145.0, 143.3, 143.2, 142.6, 142.4, 135.65, 134, 133.9, 132.35, 130, 128.49, 128.3, 128, 127.7, 126.7, 125.46, 125.2, 125.1, 113, 89.42, 89.3, 88.2, 88.1, 86.4, 82.78, 82.71, 77.42, 77.38, 76.93, 76.88, 75.5, 75.2, 72.8, 72.78, 71.8, 70.9, 70.8, 67.25, 67.19, 66.97, 66.91, 62.88, 54.64, 48.45, 26.3, 25, 17.3, 17.2, 12, -5.5, -5.9.

HRMS (ESI) for $C_{71}H_{87}N_{10}O_{14}PSi_2$ calc: 1390.57, found: 1413.37 (M+23)

[5′-DMTr-2′TBDMS-N-Bz-rA]-[3′-p(OMe)-5′]-[rA-2′TIPS]-3′-methylphosphoramidite (39)

N,N-Diisopropylammonium tetrazolide (0.67 g, 3.59 mmol) was added to the bis(N,N-diisopropylamino)methylphosphine (1.05 mL, 3.59 mmol) in 1ml of CH_2Cl_2. Theadenosine dimer nucleotide **(38)** (0.5 g, 0.359 mmol) was added to the above reaction mixture at room temperature and stirring was continued for 12h. The reaction mixture was diluted with DCM and washed 2 times with water and saturated NaCl. The organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated. The compound was dissolved in minimum DCM and precipitated in petroleum ether at -78 C. The precipitated compound was subjected to column chromatography on silica gel using 1:1 DCM & EtOAc to get the rAA dimer amidite (0.45 g, 80%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm -0.05 - 0.33 (m, 6 H) 1.00 (br. s., 8 H) 1.16 - 1.35 (m, 16 H) 1.39 - 1.69 (m, 16 H) 3.32 (q, *J*=6.94 Hz, 2 H) 3.51 - 3.83 (m, 4 H)

3.83 - 4.09 (m, 10 H) 4.56 - 4.90 (m, 4 H) 5.13 - 5.41 (m, 1 H) 5.54 (s, 2 H) 6.31 - 6.57 (m, 2 H) 7.09 (d, *J*=8.21 Hz, 3 H) 7.44 - 7.58 (m, 2 H) 7.58 - 7.88 (m, 11 H) 8.09 - 8.40 (m, 4 H) 8.41 - 8.73 (m, 2 H) 8.89 (d, *J*=10.84 Hz, 1 H) 8.96 - 9.34 (m, 2 H) 9.86 (br. s., 1 H)

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm -5.41, -5.01, 8.60, 12.17, 12.34, 17.36, 17.68, 17.77, 17.88, 18.85, 22.59, 22.82, 24.67, 25.34, 25.38, 29.25, 29.59, 42.66, 42.83, 43.23, 45.71, 46.18, 46.82, 50.57, 53.49, 55.13, 60.65, 62.57, 73.49, 76.87, 77.30, 77.72, 82.87, 86.78, 113.15, 123.36, 126.95, 127.86, 128.07, 128.64, 129.98, 132.62, 133.60, 135.31, 144.25, 149.68, 151.59, 152.42, 158.51, 164.82,

^{31}P NMR (200 MHz, Acetone) δ 153.41 (s, 1P), 153.34 (s, 1P), 150.96 (s, 1P), 150.65 (s, 1P), 1.20 (s, 1P), 1.05 (s, 1P), 0.92 (s, 1P), 0.86 (s, 1P).

MS (ESI) for $C_{78}H_{103}N_{11}O_{15}P_2Si_2$ calc: 1551.66, found: 1574.69 (M+23)

3.5 References

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Chapter 4 Synthesis of Oligoribonucleotides Using Dimer and Trimer Ribo-Phosphoramidites

4.1 Introduction

The principal driver of research into the synthesis of DNA and RNA in recent years has been the prospect of using oligonucleotides as therapeutic agents. The "antisense" oligonucleotides have made the most progress, by the successful approval of Vitravene (1998) and most recently, Kynamro (2013), an injectable drug for treating patients who are genetically predisposed to have high levels of LDL cholesterol. There are other oligonucleotide-based disciplines including aptamers,[1-3] siRNA,[4,5] immunomodulatory,[6] ribozymes,[7-9] and micro/non-coding RNA[10,11] research. As well, oligonucleotides have become routine research tools, in molecular biology, chemistry and even nanotechnology.[12-14] This high demand has become the reason for the urgent need for efficient and cost effective methods for the large scale synthesis of oligonucleotides.

As discussed previously, current methods for DNA and RNA synthesis rely on stepwise addition of monomeric nucleotide units on solid supports.[15,16] However, it is unlikely that the solid-phase method will be able to meet the demands for kilogram to ton scale synthesis of oligonucleotides. This goal could be achieved by solution-phase growth of the desired DNA and RNA sequences.

Different chemistries for assembly of oligonucleotide chains have been developed, however, the phosphoramidite and H-phosphonate chemistries remain the most popular approaches.[17-20] Regardless of the method used (solid vs solution-phase), a problem associated with current DNA and RNA synthesis is the high frequency of sequence errors associated with the chemistry employed. This is principally as a result of repeated exposure of the growing oligonucleotide sequence to coupling and deblocking reagents. For example, the repeated acidic treatments required for the removal of the 5′-*O*-DMTr protecting group during chain assembly leads to loss of nucleobases (e.g., depurination). Therefore, this process is only practical for making relatively short oligonucleotide sequences. The current practical limit of the solid-phase phosphoramidite method is about

200 nucleotides for a DNA oligonucleotide of sufficient quality for biological applications. For RNA synthesis on a solid support, the limit appears to be about 100 nucleotides.[21] The latter is a result of the lower stepwise coupling efficiencies of RNA (96-99%) compared to DNA monomers (98-100%). This is primarily due to steric effects created by the protecting groups used to mask the 2′-hydroxyl group of the RNA building blocks.

As described in Chapter 3, an alternative strategy to assemble oligonucleotide chains more rapidly is through "block" condensation reactions in solution. This approach is based on the coupling of dimer, trimer, or tetramer block units, instead of monomers, thus reducing the number of couplings necessary to prepare the desired sequence. This strategy was pioneered by Khorana and co-workers[22,23] in the total synthesis of an alanine transfer ribonucleic acid (tRNA) gene sequence.[24] To re-iterate, short oligonucleotide fragments were made in solution, purified, and then connected by specific annealing and enzymatic ligation steps to generate longer duplexes and finally the alanine tRNA gene sequence. Their initial approach involved successive condensations of "blocks" of protected di-, tri-, and tetra-nucleotides (bearing a 5′-phosphate monoester) and the 3′-hydroxyl of the growing oligonucleotide chain. At each step, the products were separated by anion exchange chromatography and then verified for purity by paper chromatography after removal of the protecting groups. The yields decreased as the chain lengths increased even when a large excess of "blocks" was used. Nevertheless, this approach demonstrated two key advantages of block over monomer coupling: block couplings reduce the number of synthetic steps during the elongation and yield more appreciable differences between the starting and the final oligonucleotide chain.

Ikehara and co-workers coupled RNA trimers and tetramers via the phosphotriester method to achieve 30% yield of final sequence.[25] Werstiuk and Nielson reported the coupling of an RNA tetramer and an RNA pentamer affording the desired nona-nucleotide RNA sequence in 50% yield after 16 days.[26] Van Boom and co-workers condensed an RNA tetramer and an RNA decamer in

58% yield in a 3.5 days reaction.[27] Ogilvie and co-workers described the synthesis of 5′-*O*-monomethoxytrityl-2′-*O*-*tert*-butyldimethylsilyl-3′-*O*-levulinyl ribonucleoside monomers and their use in the assembly of a hexadecauridylic acid via the phosphodichloridite procedure.[28] Reese and Yan demonstrated, in their synthesis of Vitravene, that block condensation of trimer units in solution using the less common H-phosphonate method was an achievable goal.[29]

Currently, the method of choice for synthesizing DNA and RNA on solid supports is the phosphoramidite method using monomeric phosphoramidite units. In 1986, Miroshnikov *et al.* were the first to report the preparation of DNA trinucleotide phosphoramidites which are compatible with automated synthesizers; since then, there have been many more examples.[30-38] DNA trinucleotide phosphoramidites are generally used to prepare DNA libraries site specific or random mutagenesis in proteins through the replacement of entire codon in a synthetic gene without the accidental introduction of non-amino acid code or a stop codon.[37] These DNA oligonucleotide libraries have successfully been transcribed to RNA and translated into functional proteins. The trinucleotide DNA "codons" for each amino acid are now available commercially as chlorophenylphosphotriester 3′-CNE phosphoramidites (Glen Research).

There are very few reports on the successful synthesis of RNA phosphoramidite dimer or trimer blocks in solution. Given the efficiency of the phosphoramidite chemistry, it is highly desirable to have access to block phosphoramidites for RNA synthesis, as they would permit longer chain extensions at each step during chain assembly, significantly shortening the time required for synthesis. Additionally, the block coupling strategy should produces crude RNA oligomers that are more readily separated from shorter failure sequences seen in monomer based synthesis.

In Chapter 3 we described a novel synthetic procedure that provides facile access to isomerically pure dimer and trimer ribonucleotides synthons in high yields. Here, we will explore the utility of these building blocks for the synthesis of RNA sequences (19-mer) using standard automated solid-phase synthetic techniques.

In Chapter 2 we discussed the development of a soluble ionic support (tributyl phosphonium bromide) that successfully substitutes the solid support in a solution-phase synthesis of medium size oligoribonucleotides via monomer couplings. Here, we also investigate the use of the tributyl phosphonium tagged nucleotides together with the dimer ribonucleotides for the block synthesis of RNA in solution. As shown below, the oligoribonucleotide sequences prepared using the dimer method gave high purity and yields, surpassing those achieved by the monomer coupling method.

4.2 Results and Discussion

4.2.1 Solid phase synthesis of oligoribonucleotides using blockmer ribo phosphoramidites under un-optimized conditions

One of the issues associated with the sequential addition of monomeric units using the solid-support method to grow oligonucleotides is the purification of the final sequence from the shorter sequences arising from incomplete coupling during the synthesis, e.g. the N-1,-2, etc. "failure" sequences. In principle, the use of dimer and trimer building blocks prevents the formation of the "N-1" by product (and the "N-2" by-product if the trimer block is used). In order to illustrate this, we synthesised a homopolymeric sequence [5′-$(rU)_{18}$dT-3′], via monomer, dimer and trimer coupling using synthons (**1-3**) under sub-optimal coupling conditions, i.e., using lower concentrations of the building blocks (**1-3**) and shorter coupling times. After chain assembly and deprotection, the resulting failure sequences were resolved by ion exchange HPLC.

The first unit in the sequence, 5′-*O*-DMTr-dT, was linked to long chain alkylamine controlled pore glass (LCAA-CPG) via a succinyl ester linkage. The chain elongation was carried out by the stepwise addition of: A) 18 monomer phosphoramidite units, B) 9 dimer phosphoramidite units, or C) 6 trimer phosphoramidite units. The couplings for the different units were done under identical conditions (0.1 M in dry acetonitrile) and coupling times (10 min). DCI was used as coupling activator. The standard oxidation solution

(iodine/pyridine/THF/H_2O) was replaced with 3 M *tert*-butyl hydroperoxide to avoid premature demethylation of the protected phosphate groups.[39,40]

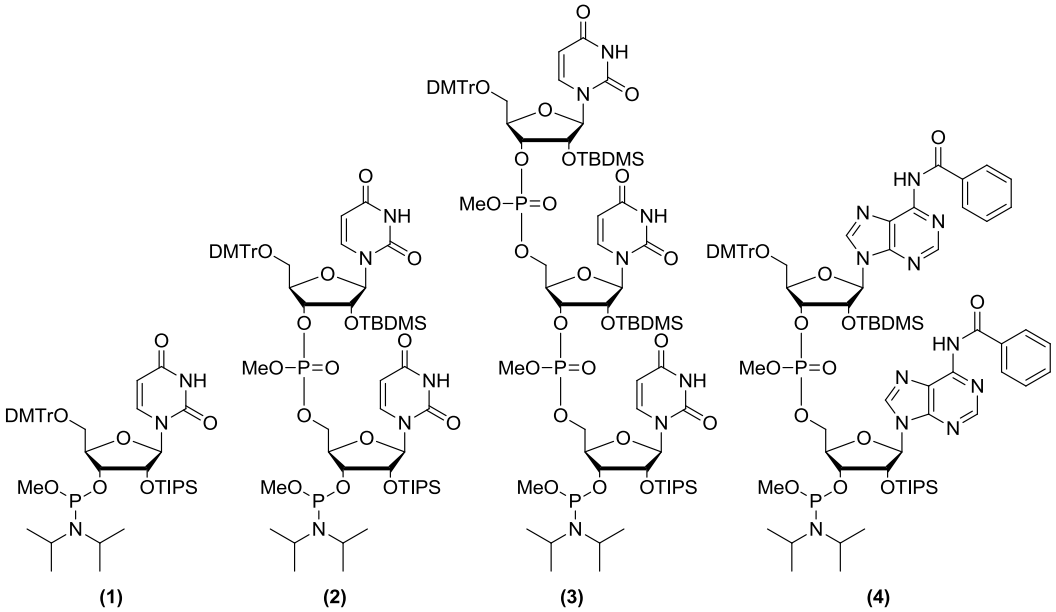

Figure 4.1. Mono-, di-, and trimer 2′-*O*-TIPS protected methylphosphoramidites used for the synthesis of oligoribonucleotide sequences.

Following chain assembly, the final oligomers were fully deprotected by: 1) treatment with 5-*tert*-butyl-2-methylthiophenol (r.t., 2 h) to deblock the methyl protecting groups on the phosphates; 2) ammonolysis with 3/1 NH_4OH/EtOH (r.t., 48 h) to release the oligomers from the solid support; 3) fluoride treatment (1 ml 1M TBAF in THF, r.t., 48 h) to deprotect the 2′-*O*-TBDMS and 2′-*O*-TIPS protecting groups. The three full-length oligomers (19-nt) were identical as shown by HPLC (Figure 4.2) and MALDI-TOF (calc 5753.3, found 5752.9 m/z).

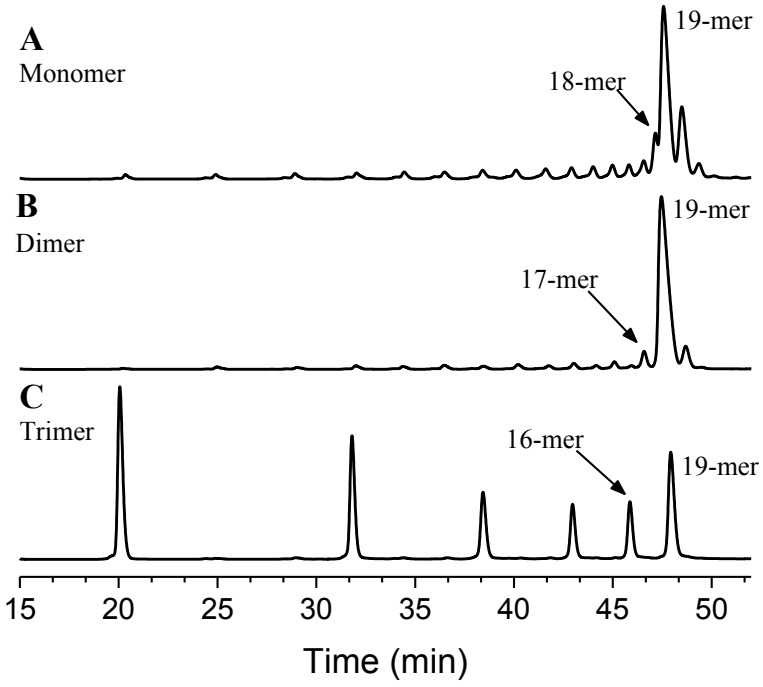

Figure 4.2. Ion exchange HPLC chromatograms of 5′-rU_{18}-dT-3′ made from A) monomer phosphoramidite (1) (x 18 couplings) B) dimer phosphoramidite (2) (x 9 couplings) and C) trimer phosphoramidite (3) (x 6 couplings), under, purposefully, sub-optimal coupling conditions (0.1M, 10 min coupling times).

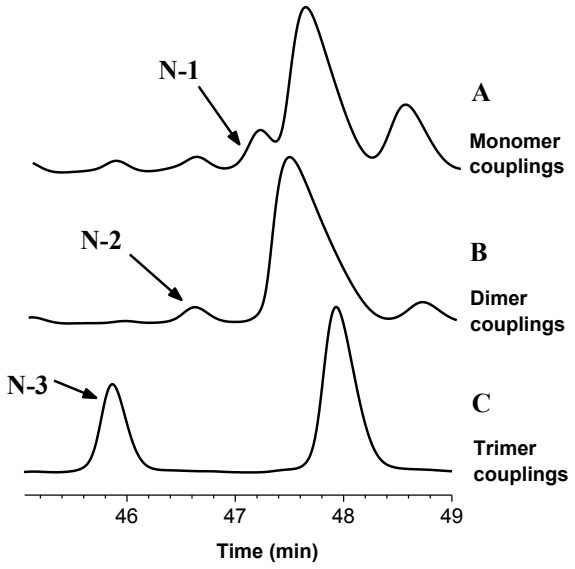

Figure 4.3. Zoomed in region of Figure 4.2 showing the visible N-1, N-2 and N-3 failure sequences formed, by incomplete coupling of monomer (1), dimer (2), and trimer (3), respectively.

As seen in Figure 2, the use of the dimer and trimer amidites led to significantly better separation of the desired oligomer from their respective failure sequences. A zoom of the chromatograms (Figure 4.3) shows the lack of peaks corresponding to (N-1) sequences when the dimer and trimer blocks are used. While these oligonucleotide sequences were synthesized under sub-optimal conditions and the coupling efficiencies obtained are not representative (see below), they demonstrate a distinct feature of our dimer and trimer phosphoramidites, i.e., the better separation of the desired oligomer from failure sequences.

4.2.2 Optimized solid phase RNA synthesis from dimer and trimer phosphoramidites

We proceeded with the synthesis of the same oligoribonucleotide sequence on solid-support, seeking for better coupling conditions. The oligonucleotides were synthesized by applying the synthetic procedure described above but with higher phosphoramidite concentration (0.15 M instead of 0.10 M) and longer coupling times (20 min instead of 10 min) to ensure more efficient coupling of the units. Also, this time we used triethylamine trihydrofluoride (TREAT-HF) instead of TBAF in the final silyl deprotection step since this provided cleaner chromatograms and simplified the work up by butanol precipitations. Coupling efficiencies and the overall yield obtained for the 19-mer sequences are tabulated in Table 4.1. These were calculated by integration of the peaks in each HPLC chromatogram given in Figure 4.4.

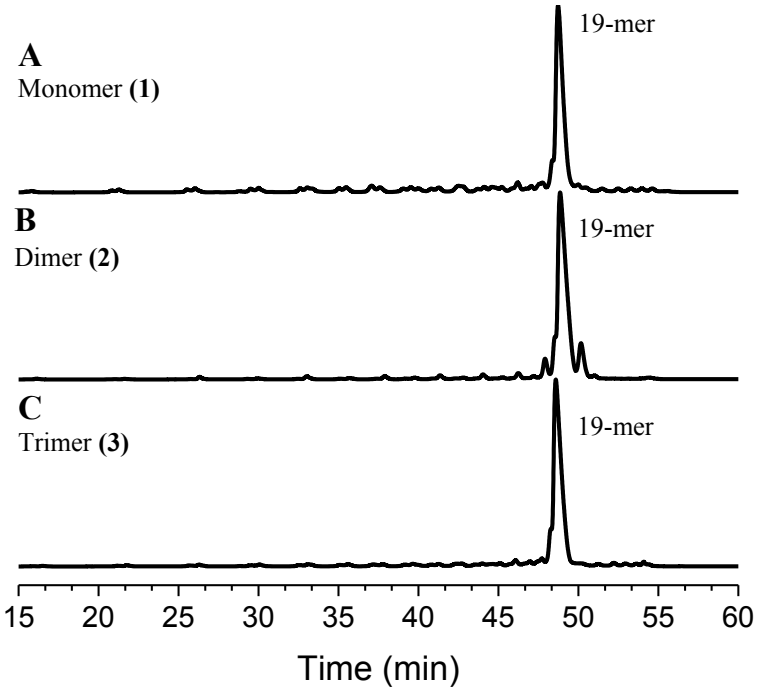

Figure 4.4. Ion exchange HPLC chromatograms of 5′-rU_{18}-dT-3′ made from: A) monomer phosphoramidite **(1)** (x 18 couplings), B) dimer phosphoramidite **(2)** (x 9 couplings), and C) trimer phosphoramidite **(3)** (x 6 couplings) under optimized coupling conditions (0.15 M phosphoramidite and 20 min coupling times).

The coupling efficiencies and yields of the oligomers were calculated for the crude mixtures by integration of the peaks in each HPLC chromatogram in Figure 4.4. The coupling efficiencies were estimated at 98.7%, 98.3%, and 97.2% for monomer, dimer and trimer phosphoramidite couplings, respectively (Table 4.1). The overall yield of 5′-rU_{18}dT-3′ prepared from dimer couplings and trimer couplings were 85.9% and 84.7 %, respectively, which are superior to the 80.1% overall yield obtained by coupling rU monomers. Thus, while the coupling efficiencies in all cases are comparable, reducing the number of coupling steps increases the overall yield of product. The increase of yield of the full length product seems trivial, but on a large industrial scale, this could represent a significant benefit.

Phosphor-phosphoramidite	Concentration (M)	Number of couplings	Time (min)	Coupling efficiency (%)[a]	Overall yield (%)[a]
rU **(1)**	0.10	18	10	98.5	76.5
rU **(1)**	0.15	18	20	98.7	80.1
rUU **(2)**	0.10	9	10	97.2	77.8
rUU **(2)**	0.15	9	20	98.3	85.9
rUUU **(3)**	0.10	6	10	86.5	41.8
rUUU **(3)**	0.15	6	20	97.2	84.7
$rA^{Bz}A^{Bz}$ **(4)**	0.15	4	20	99.0	88.8

Table 4.1. Conditions and yields for the synthesis of 5′-rU_{18}-dT-3′ via monomer, dimer and trimer phosphoramidite coupling. The yields obtained under optimized and unoptimized conditions are provided. [a] Average stepwise coupling efficiency and overall yield of oligomer are calculated from the HPLC traces of each crude mixture via peak integration. [b] Sequence 5′-rAAUUAAUUAAUUAAUUdtt-3′ made from adenosine phosphoramidite dimer **(4)** and uridine phosphoramidite dimer **(2)**. Solid support: dT-succinyl-LLAA-CPG (42 umol/g) prepared by standard protocols.[41]

Interestingly, the purity of the mixed nucleobase sequence 5′-rAAUUAAUUAAUUAAUUdtt-3′ created from the phosphoramidites dimers **(2)** and **(4)** under similar conditions was exceptional (88.8%; Figure 4.5).

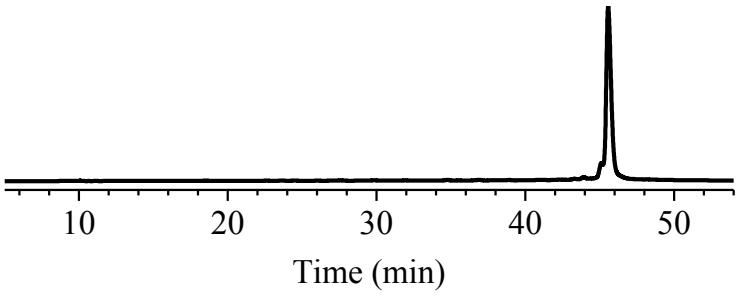

Figure 4.5. Crude 5′-rAAUUAAUUAAUUAAUUdtt3′ made from the addition of first one thymidine phosphoramidite, followed by sequential coupling of rUpUp **(2)** and rApAp **(4)** dimer phosphoramidites under optimized coupling conditions (0.15 M and 20 min coupling times).

4.2.3 Detailed analysis of the polyuridine synthesized using dimer and trimer phosphoramidites

Currently, we have not yet completely optimized the final deprotection conditions of products obtained via block couplings. We have noted in some instances the presence of *very small* amounts of (N-1) and (N-2) sequences even when using dimer and trimer phosphoramidites (Figure 4.6). Given the inherent

nature of blockmer couplings, the formation of these shorter sequences could not be due to incomplete couplings. A more likely explanation of this occurrence is the premature silyl cleavage during ammonia treatment of the oligomer under standard conditions (3/1 NH_4OH/EtOH, r.t., 48 h) at the final deprotection step (cleavage from the solid support), as first reported by Ogilvie and co-workers for a poly-rU sequence.[42,43] These would produce shorter oligomers containing 2′/3′-phosphate moieties (due to 2′-OH mediated cleavage of the internucleotide linkages) which would elute at different retention times relative to normal failure sequences (e.g., rN_nN vs rN_nNp; Figure 4.6). Conditions that greatly minimize this problem have been developed (use of anhydrous ethanolic NH_3 or NH_3 gas),[42] however their use is not routine, but will be investigated in the future. On the other hand, when the oligomer is obtained through standard monomeric addition the formation of the (N-1) products seen in the crude HPLC could be due to both incomplete coupling and cleavage of the chain during the final deprotection. The two events cannot be decoupled, which does not allow for effective optimization of the coupling and deprotection conditions. A distinct advantage of using dimer and trimer over monomer phosphoramidites is the ability to distinguish between the two processes and to quantify the amount of chain cleavage using a particular deprotection method. Being able to distinguish between incomplete coupling and cleavage products would allow for more detailed analysis of deprotection conditions. This would be of great value to manufacturers of oligonucleotides, where decreasing the amount of cleavage would directly increase the amount of full length product.

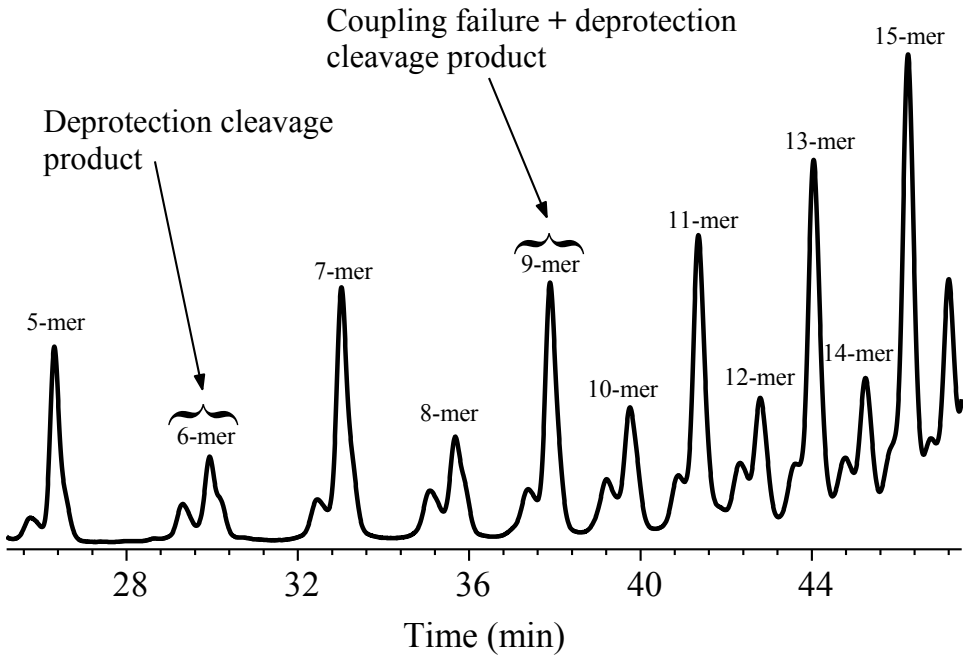

Figure 4.6. Zoomed view of the ion exchange HPLC chromatogram shown in Figure 4.2 for 5′-rU_{18}-dT-3′ synthesized from rUpUp dimer phosphoramidite **(2)** using dT derivatized CPG under un-optimized coupling conditions. The difference in peak height of the incomplete couplings and deprotection cleavage products is clearly resolved. The shoulder peaks correspond to sequences (e.g. 18-mer, 17-mer, 16-mer, etc.) arising from 2′-OH mediated cleavage of the internucleotide linkage caused by premature removal of TBDMS groups during ammonia treatment.

To illustrate this further, Figure 4.7 shows a zoomed view of the chromatograms obtained from blockmer (dimer and trimer) and monomer syntheses. Again 5′-rU_{18}-dT-3′ was prepared under the same coupling (0.1M monomer/blockmer; 10 min coupling time) and deprotection conditions. The methyl phosphate protecting groups were removed by treatment with 2-methyl-5-*tert*-butylthiophenol/NEt_3 in ACN (1/1/3; v/v/v) at ambient temperature for 2 h. Cleavage from the solid support was accomplished with 3/1 NH_4OH/EtOH for 48 hours at room temperature and the silyl protecting groups were removed by treatment with TREAT-HF for 56 h at room temperature. The increased time of silyl deprotection was to ensure complete removal of the more stable 2′-*O*-TIPS protecting group.

The peaks corresponding to failure couplings/cleave products are more pronounced in the HPLC traces for the 19-mer produced by the monomer addition method compared to the cleaner traces for the dimer and trimer method (Figure 4.7). This is consistent with the results presented above. As well, the appearance of additional peaks before and after the full length product (44-47 and 50-55 min) was clearly observed for the oligomer prepared via monomer couplings. Also, the trimer synthons afforded fewer amount of "long-mer" sequences compared to the dimer synthons (50-55 min). Although it is not clear how all of the byproducts are formed it may be related, at least in part, to the extra acidic treatments required for the 5′-*O*-DMTr protecting group removal when shorter blocks are used (i.e. monomers vs. dimers and trimers). We postulate that the use of dimer and trimer phosphoramidites reduces the cumulative negative effects associated with multiple acid treatments required during chain growth. In this regard, the benefits of block coupling (over monomer coupling) are even more striking when the solution-phase synthesis method is applied (Section 4.2.4).

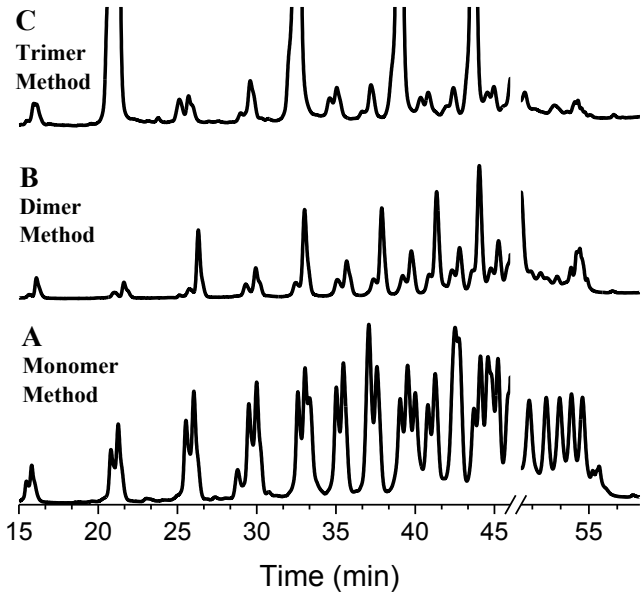

Figure 4.7. Blow up views of the ion exchange HPLC chromatograms from Figure 4.2 of polypyrimidine 5′-rU_{18}-dT-3′ oligonucleotide sequences synthesized from monomer (A), dimer (B), and trimer (C) phosphoramidites using un-optimized coupling conditions. In all three chromatograms the main product (19-mer) peak is not shown as it is off-scale, impeding the comparison.

4.2.4 Solution phase synthesis of oligoribonucleotides using dimer phosphoramidite blocks.

The utility of the dimer phosphoramidite synthons described in the previous sections were next applied to the solution phase (ion supported) synthesis of a decaribonucleotide corresponding to a portion of the 21-nt siRNA strand shown in Figure 4.8. The ion-tagged synthesis of this 10-mer, 5′-rUUAAUUAA-dTT-3′, via stepwise monomer coupling was described in Chapter 2, and hence provided an opportunity to directly compare synthesis efficiencies of monomer versus dimer blocks. The 10-mer was assembled by coupling rApA and rUpU 3′-phosphoramidites successively to the previously synthesized ion tagged dTpT (Figure 4.8). These syntheses were executed by Dr. Reddy, of our laboratory, adapting the procedures described in Chapter 2. A brief summary of the findings are provided below.

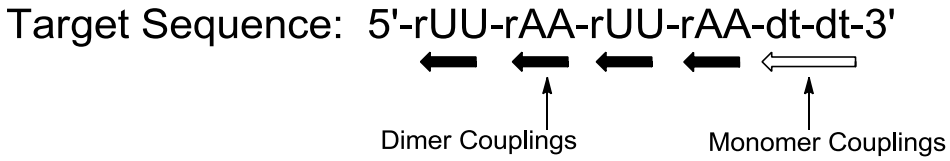

Figure 4.8. Target siRNA strand for solution-phase synthesis, grown from the 3′ to the 5′ end.

Synthesis started by coupling rApA phosphoramidite **(4)** to ion tagged dTT as shown in Figure 4.8. In a test reaction, 0.1 mmol of ion-tagged dTpT was combined with 1.5 equivalents of rApA phosphoramidite **(4)** in the presence of 4,5-dicyanoimidazole (DCI) (2.0 eq.) using ACN as the solvent. Unlike monomer couplings, this produced a difficult to dissolve (and very viscous) mixture; hence coupling was allowed to proceed overnight at room temperature. After sequential precipitation, oxidation (t-BuOOH), and another precipitation, 220 mg of crude tetramer rA_2dT_2 were isolated (97.3% yield). To check for coupling efficiency, the crude material was deprotected by a sequence of treatment consisting of 1) removal of the phosphate protecting groups (2-methyl-5-tert-butylthiophenol:NEt_3:ACN, 1:1:3; v/v/v, 2h, r.t.), 2) removal of the benzoyl groups (ethanolic aqueous ammonia, 16 h, 55°C), 3) removal of the silyl protecting groups (triethylamine trihydrofluoride/ N-methylpyrrolidone/

triethylamine, 1.5:0.75:1 by volume, 2.5 h, 65 ˚C). HPLC analysis revealed that coupling of rApA amidite to ion-tagged dTpT had proceeded with only 60% efficiency to produce a mixture of unprotected tetramer rA_2dT_2 and unprotected dTpT (data not shown). The identity of the dimer and tetramer were confirmed by MS and comparison to standard samples prepared via solid-phase synthesis. To achieve the same coupling efficiency and reaction rates as monomer units, reactions had to be sonicated and stirred for an additional 24 hours (total of up to 48 h).

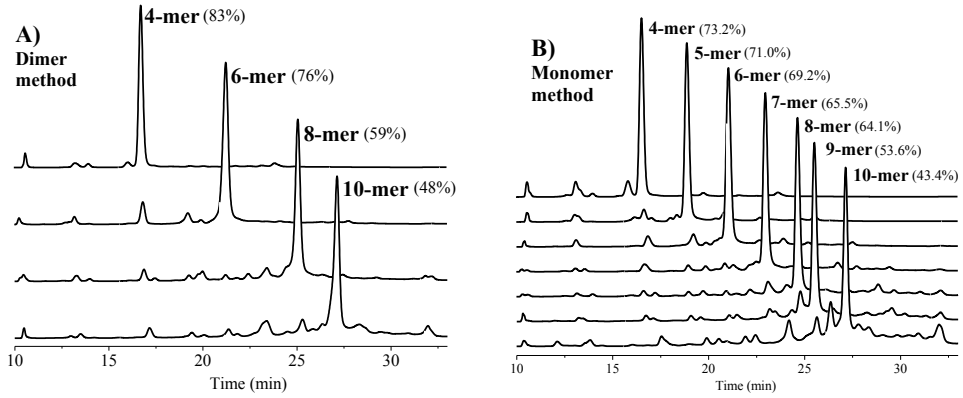

Figure 4.9. Step wise ion exchange HPLC of the solution phase synthesis of the 10mer oligoribonucleotides of the sequence 5′- rUUAAUUAAdTT-3′ A) Solution-phase synthesis using dimer phosphoramidtes **(2)** and **(4)** B) Solution-phase synthesis using monomer cyanoethyl TBDMS phosphoramidites.

The formation of the 8-mer and 10-mer presented a particular challenge. For these, multiple coupling cycles were necessary, as one coupling with 2 equivalents of dimer was not enough to achieve good results. The reaction time for each coupling iteration increased significantly as well, reaching 48 h at this stage. To shorten coupling time while increasing yield, sonication, mild heating (38°C), and agitation was necessary. In all cases coupling reaction progress was monitored by removing a very small sample from the reaction mixture, oxidizing the newly formed internucleotide phosphite triester linkages, and analyzing the crude material after deprotection. If there was significant starting material left unreacted, the coupling procedure was repeated. This was possible because the

capping step normally used in solid-phase synthesis was removed, allowing us to repeat the coupling reaction till an acceptable amount (>70%) of product formed.

When all of these precautions were implemented, the dimer coupling strategy produced generally cleaner product compared to the standard monomer strategy (Figure 4.9). Reduction of the number of the detritylation steps is likely the primary reason for this improvement. While these results are preliminary, the data is indeed encouraging. An area that needs attention, if this strategy were to find general use, is the synthesis cost of the dimer (and trimer) phosphoramidite blocks themselves. As described earlier (Chapter 3), their synthesis requires silica gel column chromatography after each deblocking and coupling step. This issue is addressed in the following two chapters by introduction of orthogonally protected ion tags.

4.3 Conclusion

The utility of the regioisomerically pure dimer and trimer phosphoramidites for solid-phase and solution-phase synthesis of RNA has been demonstrated. Under optimal conditions, dimer and trimer units couple with excellent efficiency, increasing the overall yield of the target sequence by decreasing the number of coupling steps required for chain assembly. Oligomers produced using the 'blockmer' approach are devoid of (N-1) failure products, which greatly facilitates and simplifies purification of products.

Extension of this strategy to solution-phase synthesis was more challenging; however, conditions were found that provided acceptable yields and purity of products. We believe that this strategy will find use in the large scale synthesis of RNA based therapeutics.

4.4 Experimental

Materials

Thin layer chromatography was performed on EM Science Kieselgel 60 F-254(1mm) plates. Silicycle 40-63 μm (230-400 mesh) silica gel was used for flash chromatography. Acetonitrile and dichloromethane were distilled fromCaH_2 after

refluxing for several hours. THF was distilled from benzophenone and sodium after refluxing for several hours. All other anhydrous solvents were purchased from Sigma-Aldrich. Chemicals and reagents were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Cyanoethyl phosphoramidites were purchased from ChemGenes Inc.

Instrumentation

The solid phase syntheses of oligonuc1eotides were conducted on an ABI 3400 synthesizer. UV spectra for oligonuc1eotide quantitation (absorbance measurements) were measured at 260 nm on a Varian Cary I or 300 UV-VIS dual beam spectrophotometer. Anion-exchange HPLC were performed on an Agilent 1200 series machine. Ion exchange column used was a Waters Protein Pack DEAE-5PW column (7.5 mm x 7.5 cm). Purified samples were desalted by size exclusion chromatography on G-25 Sephadex purchased from GE-Healthcare. ^{1}H NMR and ^{13}C-NMR were recorded on a Varian 300, 400 or 500 MHz spectrophotometer with chemical shift values reported in ppm. ^{1}H-NMR and ^{13}C-NMR spectra were referenced to residual solvent. ^{31}P-NMR were collected at 80 MHz with a Varian 200MHz spectrophotometer and were measured from 85% H_3PO_4 as an external standard

Solid-phase Oligonucleotide Synthesis:

Slight modifications to the standard phosphoramidite solid-phase synthesis conditions were used for the synthesis of all oligonucleotides. Each synthesis was performed on a 1 μmole scale using controlled pore glass (500 Å CPG) with a 5′-*O*-DMTr-thymidine derivatized monomer attached by a succinyl linker, prepared according to literature procedure.[41] All DNA and RNA phosphoramidites were prepared as 0.10M and 0.15 M solutions in acetonitrile (ACN) respectively, unless otherwise stated. 4,5-Dicyanoimidazole (0.25 M in ACN) was used to activate phosphoramidites for coupling. Detritylations were accomplished with 3% trichloroacetic acid in CH_2Cl_2 (110 s). Capping of failure sequences was achieved with a 16% N-methylimidazole in THF(CAP A) and Acetic

anhydride:Pyridine:THF, (1:2:2) (CAP B) for (30 s). Oxidation was done using a 3M solution of *tert*-butyl hydroperoxide in toluene (40 s). See Table 4.2 for reaction condition summary.

Step	*Operation*	*Reagent*	*time (s)*
1	Detritylation	3% TCA in CH_2Cl	120
2	Coupling	0.15 M or 0.1M in MeCN 0.25 M DCI in MeCN	1200 or 600
3	Capping	CAP A, CAP B	20
4	Oxidation [44]	3M *tert*-butyl hydroperoxide	40

Table 4.2. Reaction conditions for oligonucleotide synthesis on an automated ABI 3400 synthesizer.

General procedure for the synthesis of oligoribonucleotides in solution

Beginning with the detritylated, tagged oligonucleotides, which was dried under vacuum for at least 2 hours, the mass is determined and a small portion is removed for deprotection and analysis. The mass is re-determined and the next coupling is prepared. The next amidite to be coupled is removed from the freezer and allowed to warm to room temperature in a dessicator before the appropriate weight is measured out (Table 4.3). The activator, 2,3- dicyanoimidazole (DCI) has been dried under vacuum and stored in a dessicator and is weighed as is.

Both the DCI and phosphoramidite are added as solids to the dried oligomer and are kept under nitrogen or argon. 15% v/v dimethylformamide (DMF) in ACN is added to make a 0.06M solution of oligonucleotide. This solution must be mixed for up to 10-15min to completely solvate the solid oligomer. This solution is then stirred for 4 hours with periodic manual agitation to ensure complete mixing with droplets adhering to the sides of the flask. In the case of more viscous solutions, as well as with longer sequences (6-10mer) the solution is placed into the sonicator to encourage dissolution, and increase the rate of reaction.

After the required time of coupling, *tert*-butanol is melted (m.p. 25^{o}C), and 25 equivalence are added and allowed to stir for 10 min to quench the excess amidite, making the excess amidite into a *t*-butyl phosphite triester which is more

soluble in the ether layer. This solution was then added to a small dropper funnel, rinsing the flask with ACS grade acetone to a final volume of about 40ml. The solution was precipitated by dropwise addition to 500-600ml of methyl *tert*-butyl ether (MTBE) over 15min. The solution was then filtered over Celite® to collect the precipitated phosphite triester. The precipitate is quite fine and packs hard over the Celite® causing the flow through the filter to be greatly reduced. Once the sample was almost completely filtered, you must add more MTBE to rinse the solid. It is extremely important to not let the filter run dry, as the hard packed solid will crack and fold up which will then not allow you to rinse away impurities and the precipitation process will have to be repeated.

The solid precipitate is dissolved off the filter into a 500 mL or 1 L round bottom flask using both ACS acetone and THF. As the oligonucleotide grows it becomes less and less soluble. Previously, only acetone and acetonitrile were used to dissolve the sample off the filter, but as the oligomer grows acetonitrile proved to not have the solvating power necessary. It was found that THF is the best choice because it is quite inert, unlike using methanol or DMF which is more difficult to remove.

After the sample was collected and condensed to dryness, the sample was re-suspended in about 30ml of acetone and THF and precipitated once more to ensure complete removal of excess amidite before oxidation.

Once the purified phosphite triester was dry the oligomer was dissolved in 15% v/v DMF in acetonitrile to a concentration of approximately 0.08M, 16 equivalence each of Cap A and Cap B solution (16% N-methylimidazole in THF, and Acetic anhydride:pyridine:THF, 1:2:2 ratio) was added. The reaction was allowed to stir for 10min. Make sure to only allow 10min, as longer reaction times discolor the reaction mixture to a dark yellow/brown color. Next, 36 equivalence of 6M *tert*-butyl hydroperoxide in decane was added to oxidize the triester. The mixture was allowed to stir for a further 15min. This solution was then once again precipitated as described above, recovering the sample from the filter in a 250ml round bottom flask. The solid was then placed under a high vacuum for 15-20min to make sure there is no residual pyridine remaining.

To induce detritylation, 20 equivalence of 3% v/v trifluoroacetic acid in acetonitrile is added, followed by 2.5 equivalence of triethylsilane (a quencher of trityl cation) and the solution is stirred for 15 to 20 min until the orange trityl color disappears to a light orange huge. The solution was again precipitated, rinsing the reaction vessel with dry ACS acetone.

Once the material is detritylated, the solubility of the molecule is significantly decreased. Dissolving it off the Celite® filter then becomes much more difficult. More solvent and time is necessary to ensure complete removal. Also, at this point the molecule seems to be much more hygroscopic, thus exposure to atmosphere should be minimized or avoided completely.

To ensure that the reaction was detritylated completely, spot a TLC plate with the solution just before it is completely condensed on the rotovap, as a more concentrated solution is necessary to see trace amounts of trityl color. Burn this TLC after an acid treatment. If you see any trityl color you must repeat the TFA treatment cycle. Alternatively, a small portion can be removed and placed in acidic DCM and run on a low resolution mass spectrometer and look for the appearance of the trityl cation.

After the necessary number of detritylations are completed, the material was condensed into a 250ml round bottom flask and dried on high vacuum for a minimum of one hour to remove residual solvent, TFA and water, as any of these would adversely affect the next coupling step.

A summary of the reagents and conditions used to form the 10-mer (Figure 4.9) in solution using dimer phosphoramidites is shown in Table 4.3.

Starting Comp.	Mass (g)	St. mat. (mmol)	mmol of amidite	Amidite (g)	ACN (mL)	DCI (mmol)	DCI (mg)	Rex. Time (h)*	% yield
tt	1.43	0.88	1.76	2.74	14.6	2.64	310	8h	93.4
ttAA	0.42	0.16	0.31	0.39	2.59	0.47	55	12	97.3
ttAAUU	0.58	0.15	0.31	0.48	2.56	0.46	54	12 (x2)	90.2
ttAAUUAA	0.24	0.049	0.10	0.12	0.82	0.15	18	12 (x2)	91.0

Table 4.3. Summary of reaction conditions for the solution phase synthesis of oligoribonucleotides in solution from the 2-mer (5′-dTT-3′) to the 10-mer (5′-rUUAAUUAAdTT-3′).

Deprotection of Oligonucleotides:

The oligonucleotides were demethylated by treatment with a solution of 2-methyl-5-tert-butylthiophenol:triethylamine: acetonitrile (1:1:3; v/v/v) at ambient

temperature for 2 h. Cleavage from the solid support was accomplished with either 3:1 NH_4OH:EtOH for 48 at room temperature.

Desilylation was achieved by two different methods for two different set of oligonucleotides in this chapter:

1) **Un-optimised conditions**

   Oligonucleotides synthesized under un-optimised conditions, (0.1M, 600s) presented in Figure 4.2 and Figure 4.3 were desilylated by 1M TBAF in THF for 36 h at room temperature. Work up of the TBAF desilylation is achieved by addition of 50ul of a 3M sodium acetate solution, concentrate slightly to remove excess THF and the sample is passed through a bead of autoclaved Na^+ ion exchange resin to remove the tributyl ammonium ion. The sample is then lyophilised and desalted on a Nap-25 Sephadex® columns from GE Healthcare

2) **Optimised conditions**

   Oligonucleotides synthesized under optimised conditions, (0.15M, 1200s) presented in Figure 4.4 and Figure 4.5 were desilylated with neat triethylamine trihydrofluoride (TREAT-HF) for 60 hours at room temperature. The crude oligonucleotides were precipitated from the TREAT-HF by quenching the solution with 30µL of a 3M sodium acetate solution (pH 5.5). The quenched solution was washed away by the addition of dry, cold (-20°C) n-butanol. The sample was centrifuged to a pellet and the n-butanol was decanted. This procedure was repeated once more and the sample was either immediately analyzed by HPLC or it was desalted with Nap-25 Sephadex® columns from GE Healthcare.

Ion exchange HPLC conditions

HPLCs were performed on an Agilent 1200 series machine. Ion-exchange HPLC were performed with a Waters PROTEIN-PAK DEAE 5PW (7.5 x 150 mm) HPLC column.

Oligoribonucleotides synthesized on solid support using monomer, dimer, and trimer phosphoramidites presented in Table 4.1 were analyzed using a 0-16.5%

gradient of 0.5M $LiClO_4$ in Milli-Q water as solvent B over 60 minutes. Milli-Q water was used as solvent A. (Table 4.4)

	Time (min)	%A	%B	Flow (mL/min)
1	0.00	100	0.00	1.00
2	2.00	100	0.00	1.00
3	50.00	83.5	16.5	1.00
4	55.00	60	40	1.00
5	63.00	60	40	1.00
6	65.00	100	0.00	1.20
7	75.00	100	0.00	1.20

Table 4.4. Ion exchange HPLC gradient for oligoribonucleotides made on solid support A= Milli-Q water; B=1M $LiClO_4$ in Milli-Q water.

Oligoribonucleotide synthesized in solution phase using dimer phosphoramidites presented in Figure 4.9 were analyzed using a 0-15% gradient of 0.5M $LiClO_4$ in Milli-Q water as solvent B over 60 minutes. Milli-Q water was used as solvent A. (Table 4.5)

	Time (min)	%A	%B	Flow (mL/min)
1	0.00	100	0.00	1.00
2	3.00	100	0.00	1.00
3	28.00	85	15	1.00
4	31.00	50	50	1.00
5	36.00	50	50	1.00
6	39.00	100	0.00	1.20
7	51.00	100	0.00	1.20

Table 4.5. Ion exchange HPLC gradient for solution phase synthesized oligos. A= Milli-Q water; B=1M $LiClO_4$ in Milli-Q water.

Analysis of Oligoribonucleotides:

The oligoribonucleotides were purified by ion-exchange HPLC chromatography. The solution containing the purified oligonucleotide was then lyophilized to dryness and dissolved in 1 mL of water. The purified sample was then desalted using Sephadex® G-25 and lyophilized to dryness, and analysed by high resolution HPLC MS.

Entry	Amidite used	Sequence (5′ to 3′)	Mass calc.	Mass found
Oligoribonucleotides synthesized on solid support				
1	rU (1)	(rUx18)-dT	5753.3	5752.9
3	rUU (2)	(rUx18)-dT	5753.3	5753.1
5	rUUU (3)	(rUx18)-dT	5753.3	5752.9
7	rUU and rAA	AAUUAAUUAAUUAAUUtt	5629.4	5629.3
Oligoribonucleotides synthesized in solution				
8		AAtt	1204.8	1203.2
9		UUAAtt	1817.2	1815.2
10		AAUUAAtt	2475.6	2473.2
11		UUAAUUAAtt	3087.9	3085.4

Table 4.6. High resolution LC MS data for oligoribonucleotides.

The sequence of all oligonucleotides were also confirmed by the synthesis of standards using standard protocols and co-injected in ion exchange HPLC.

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Chapter 5 RNA Blockmers with Orthogonally Cleavable Ionic Tags

5.1 Introduction

Chemical modifications to oligoribonucleotides have been used to improve the pharmacological properties of RNA strands (e.g. siRNA) *in vitro* and *in vivo*. Masking the negative charge of the phosphate backbone has been a popular strategy in terms of increasing bioavailability and cellular uptake, and significantly enhancing resistance towards degradation by nucleases in serum and in tissue homogenates.[1,2-4] Chemically modified siRNA are being developed not only as gene silencing therapeutics but also as research tools to study the biological processes in which they are involved. Sugar modifications, particularly at the 2′ position of the furanose can result in enhanced hybridization affinity, lipophilicity, and nuclease stability.[3,5] The 5′- and 3′-hydroxyl positions at the termini of oligoribonucleotides have been used for covalent attachment of a variety of groups such as cholesterol[2] and lauric acid[6,7] in order to increase cellular uptake. The so-called "prodrug" strategy involves the introduction of labile groups ("pro-moieties") in the RNA structure such as esters,[8] carbonates,[8] oximes,[8] imines[8] or even thermally cleavable functionalities[9,10,11] in order to enhance both bioavailability and cellular uptake of such RNAs (e.g., siRNAs). Ideally, once the RNA permeates the cell membrane, such groups would be cleaved by the cell's machinery to release the active (unmodified) siRNA molecule. Syntheses of such pro-siRNAs are challenging, however, because current solid-phase methods require the use of a base to deprotect and release the RNA product from the solid support. For example, the oligonucleotides are typically connected to the solid support via a 3′-succinate ester (Figure 5.1). This ester remains untouched during the iterative stepwise synthesis of both DNA and RNA, and is cleaved at the end of the synthesis along the other protecting groups (i.e., phosphate base protecting groups) by an ammonolysis step (*aq*. NH_3/alcohol; heat). Such conditions would also cleave the "pro-moieties" prematurely. The

replacement of the 3′-succinate linker by an orthogonally cleavable linker would offer a partial solution to this problem.

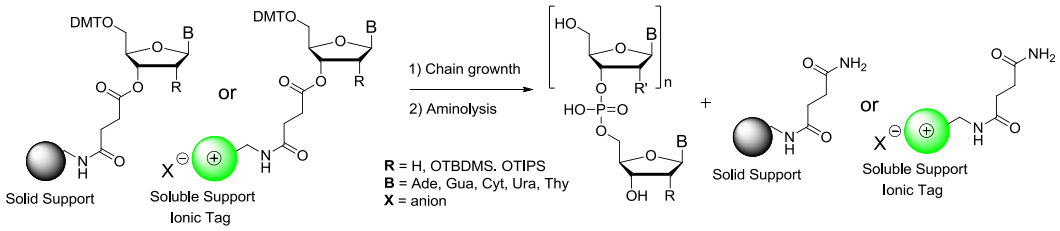


Figure 5.1. Cleavage of oligonucleotides from soluble or solid support by ammonolysis.

A variety of different linkers have now been developed for attaching nucleosides to solid supports. A particularly versatile linker is the Unylinker™ which does not require derivatization of the nucleoside to the support prior to automated oligonucleotide synthesis. Other alternatives include the Q-linker developed by Pon *et al.*[12] and silicon-based linkers developed by Ohkubo *et al.*[13] and Boehm *et al.*[14], which are cleaved by a fluoride source. These linkers, however, suffer from the same limitation as the traditional succinyl ester linker in that they do not allow for selective modification of the oligonucleotide post cleavage from the solid or soluble support. The option of using linkers which are cleaved orthogonally (under conditions that leave other protecting groups intact) would be of particular interest to those attempting post-synthetic 3′-hydroxyl modifications of oligoribonucleotides, or those working with particularly sensitive moieties.

There are a few protecting groups that are known to be orthogonally cleavable in the presence of all traditional RNA and DNA protecting groups. These include the photocleavable o-nitrophenyl derivatives and the hydrazine cleavable levulinyl group.

The use of photocleavable protecting groups in nucleoside and nucleotide chemistry is very well established for both the 2′ and 5′ hydroxyl protection.[15-17] The most common variety used is the *o*-nitrophenyl group (and derivatives), due to their ability to be released by UV light. As long as UVA radiation is used for a short time period no modifications (e.g. formation of photodimers) to the DNA or RNA is observed.[18,19] These protecting groups have been adopted for the

production of DNA[20-22] and RNA[23,24] oligonucleotide microarrays, two very powerful diagnostic tool in molecular biology[25] and genomics.[26,27] More recently, Pfleiderer and co-workers have developed the 2-(2-nitrophenyl) propoxycarbonyl (NPPOC) protecting group, which is up to 8 times more labile than other o-nitrophenyl derivatives.[28] The increased reactivity of the NPPOC group, makes it an attractive choice for use in the protection of nucleosides and nucleotides.[29] Hence the development of orthogonal linkers based on the NPPOC group becomes highly attractive.

The other orthogonal protecting group mentioned above is the levulinyl ester. The levulinyl group possesses the same stability as traditional esters, but can be cleaved under relatively mild conditions by hydrazinolysis. We have shown in Chapter 3 that the 3′-levulinyl group in the place of a more traditional esters moiety (Ac, Bz) can be removed selectively in conjunction with a 2′-TIPS protecting group without migration of the silyl group, allowing for subsequent chain elongations.[30,31] This method is compatible with all protecting groups used in DNA and RNA synthesis, as well as many protecting groups used in classical synthetic organic chemistry. Exceptions are any ketone functional group, as it will readily react with hydrazine to form hydrazone derivatives; in fact, this occurrence is fundamental in the cleavage mechanism of the levulinyl protecting group.

Unlike the bi-functional succinic acid linker, the levulinyl group requires further modification if it were to serve as a "linker" in solution (ion tag) or solid phase syntheses. In 2008, the Lonnberg group introduced the use of levulinyl-like linker, namely 2-oxoheptanedioic (4-ketopimelic acid), for the solid-phase synthesis DNA oligonucleotides containing base sensitive heterocycles (Figure 5.2).[32] Two years later, while our work was in progress, Overkleeft group introduced an alternative γ-keto ester linker to link peptides to a biotin tag.[33]

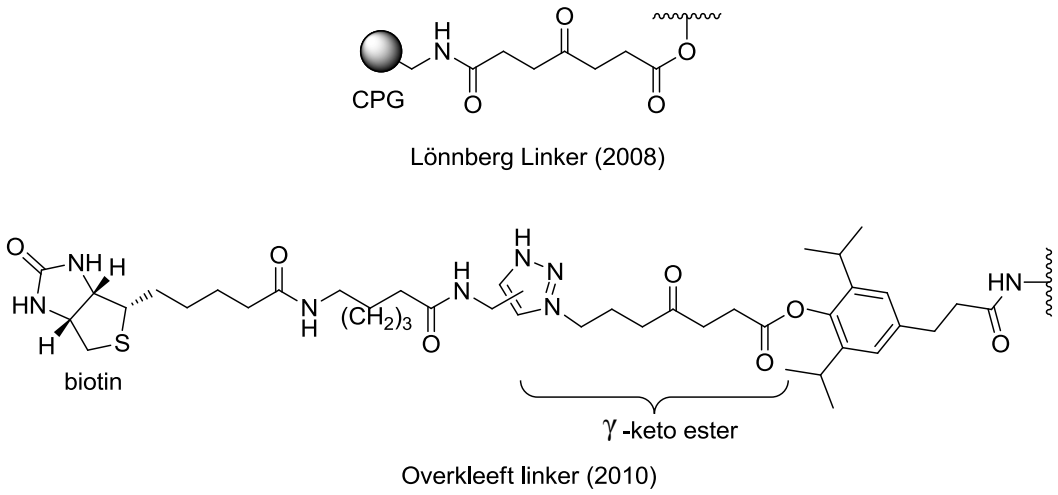


Figure 5.2. Previously reported γ-keto ester linkers by the Lonnberg[32] and Overkleeft[33] groups.

We sought to combine the solution phase oligoribonucleotide synthesis using the soluble ionic-tag approach we developed (and discussed in Chapter 2) with linkers that can be used in place of standard ester protecting groups for orthogonal cleavage from the support. This would provide both a mild and selective release of the 3′-hydroxyl group and the opportunity to carry out post-synthetic 3′-*O*-modifications.

The use of ionic soluble supports allows for selective precipitation of the growing oligoribonucleotide chain from all other reagents used in the oligoribonucleotide synthesis cycle, significantly simplifying intermediate purification steps (Chapter 2). This selective precipitation of products over reagents can be used in a variety of reactions,[34-38] and has been shown to be effective as an alternative to chromatography. This method of product isolation is therefore beneficial with respect to both cost and time when purifying compounds, as it can circumvent the use of traditional chromatographic methods used to purify organic compounds. This can be advantageous, particularly in the context of a large scale industrial process where chromatography can be prohibitively expensive.[38]

Here we report on the synthesis of two novel semi-orthogonal ionically-tagged linkers: i) a levulinyl like linker, cleavable by hydrazinolysis; ii) an NPPOC derivative, which is cleaved by photolysis (Figure 5.3). The linkers are

modified to allow for attaching the tributyl phosphonium ionic tag that we developed on one side and the 3′-*O*-hydroxyl moiety of a ribonucleoside on the other.

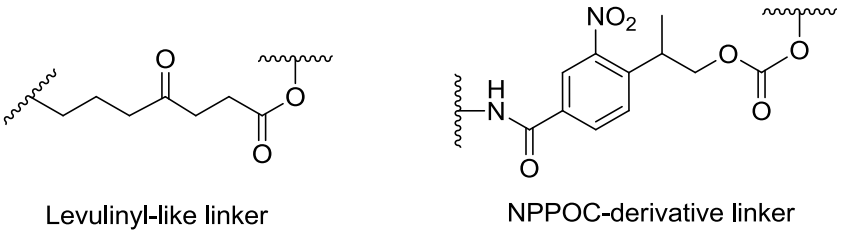


Figure 5.3. Levulinyl and NPPOC derivative linkers.

We show the synthesis of a monomer and dimer ribonucleotides coupled to the ionic tag through either the levulinyl or NPPOC linker. We further test for the ability of selective post-dimerization cleavage of the linker without simultaneous deprotection of any other protecting groups, nor migration of the vicinal 2′-*O*-silyl protecting group. Isomerically pure ribonucleotide blocks were obtained that had a free 3'-hydroxyl position and otherwise untouched protecting groups at all other positions. Finally, we convert the protected dimers obtained through this method into block dimer 3′-phosphoramidites.

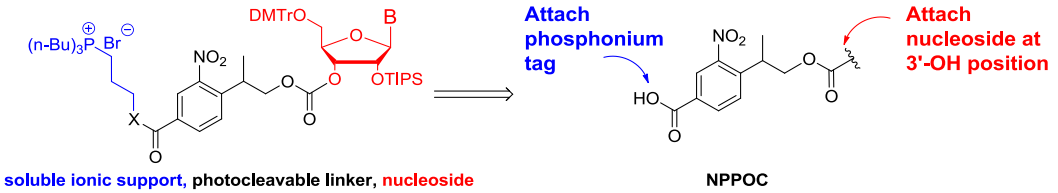
5.2 Results and discussion

5.2.1 Synthesis of the NPPOC photocleavable ionically tagged linker

Chapter 2 described the ion-tagged solution phase synthesis of oligoribonucleotides up to 10 units in length. The tributylphosphonium tag proved to be a suitable solution soluble substitute for solid supports. It was attached to the first nucleotide through a non-orthogonal succinyl ester linker. We wanted to apply the same strategy and methodology to prepare oligonucleotides connected through an orthogonally cleavable linker.

The first approach we investigated involved the use of a light labile group as a linker. The general concept of this strategy is outlined in Scheme 5.1. We sought to utilize the well studied light labile NPPOC derivative as a linker between the ribonucleoside and the ionic tag. The NPPOC-like linker has a carboxylic acid on one end that would serve as a place to couple the phosphonium

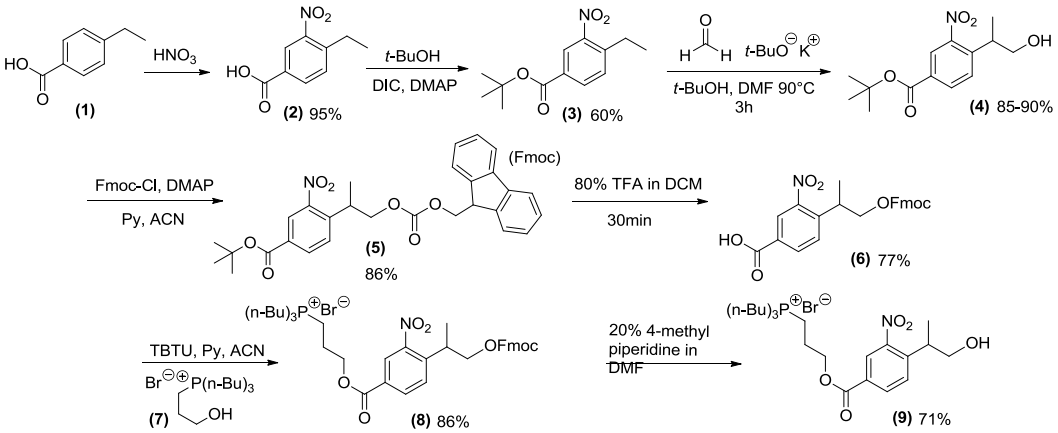
tag and a hydroxyl group on the other that could be attached to the nucleoside through a carbonate group. This construct would allow for the cleavage of the linker carrying the phosphonium tag upon UV irradiation, releasing the free 3'-OH ribonucleoside or oligoribonucleotide.



Scheme 5.1. Design of a nucleoside connected to a soluble ionic support (ionic tag) through the light labile linker NPPOC.

Photocleavable protecting groups, and the NPPOC group in particular[39,40], have been previously used with great success as 5′-*O*-protecting groups in deoxyribonucleotides,[39-41] and ribonucleotide phosphoramidites[24] and subsequent fabrication of DNA[42,43] and RNA microarrays.[24] The NPPOC derivatives demonstrate high efficacy as a light cleavable linker,[44] as well as that can be removed in the presence of all standard nucleotide protecting groups without causing photo-mediated dimerization of the nucleobases.

There are several reports for the synthesis of NPPOC derivatives that possess all the desired characteristics of a linker.[39-41] We chose to adapt the protocols developed Pfleiderer and co-workers.[41] The synthetic steps are presented in Scheme 5.2.

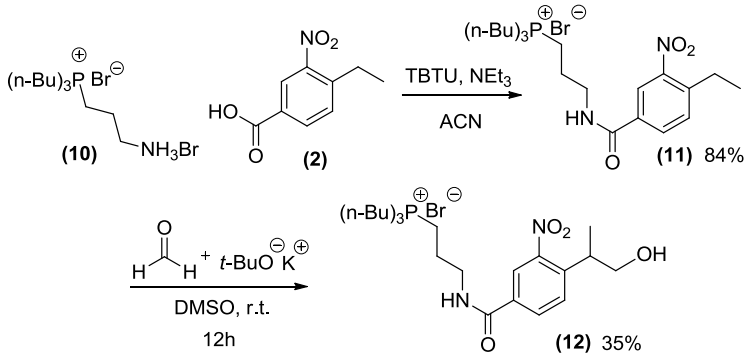


Scheme 5.2. Synthesis of ionically tagged NPPOC derivative **(9)**.

In the first step a nitro group was introduced in an *o*-position to the ethyl group (intrinsic to the photoreactivity of the NPPOC group) in the commercially available 4-ethylbenzoic acid **(1)** by treatment with fuming nitric acid at -10°C to yield 3-nitro-4-ethylbenzoic acid **(2)** in 95%. The carboxylic acid was then protected as a *tert*-butyl ester **(3)** under standard DCC coupling conditions with a moderate yield of 60%. The formation of the 2-substituted propan-1-ol derivative **(4)** was achieved by treating compound **(3)** with *para*-formaldehyde and a catalytic amount of potassium *tert*-butoxide in an aprotic dipolar solvent, such as DMF or DMSO, at 90°C. The reaction yielded the desired product **(4)** in 85-90% yield. The newly formed primary hydroxyl group was then protected with Fmoc-Cl, an acid stable protecting group, to form compound **(5)**. This allowed for cleavage of the *t*-butyl ester with 80% TFA in DCM without deprotection of the primary hydroxyl group. The reaction was attempted without Fmoc protection (i.e., on compound **4**), however, dehydration of the newly formed hydroxyl was found to occur, forming a propene derivative. After the installation of the Fmoc group, it is imperative that the compounds in the subsequent steps are not exposed to sunlight or tungsten light for long periods of time, as they undergo photolytic cleavage as per the design of the molecules. The newly formed free acid **(6)** was then coupled with the tributylphosphonium ionic tag using TBTU as a coupling reagent in ACN affording the tagged species **(8)** in a moderate yield (65%). The reaction mixture was wrapped in aluminum foil for the extent of the reaction. Next, the Fmoc group was removed under standard conditions by treatment with 20% 4-methylpiperidine in DMF for 2h, yielding compound **(9)** in good yield. This compound should be kept in the dark at all times. Some degradation was observed to occur over time, albeit much more slowly than when the Fmoc group was present.

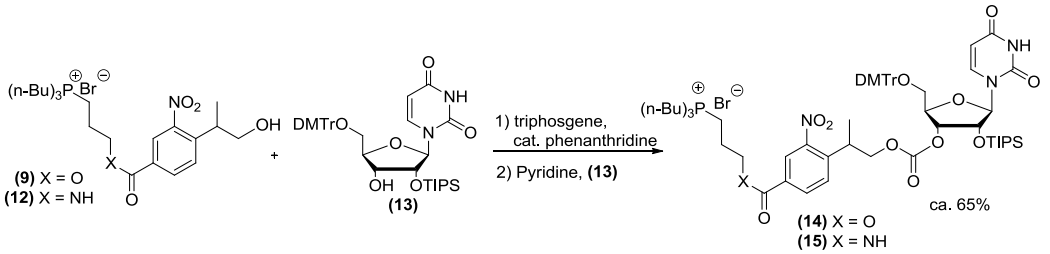
While the synthesis described above (Scheme 5.2) provided the desired tag-linker (21% overall yield), which we later found to be effective for our desired purposes, it is somewhat long and required the use of an expensive transient protecting group (Fmoc). In an attempt to shorten the synthesis, we were able to avoid protection, deprotection, followed by re-protection of the carboxylic acid

moiety, while moderately increasing overall yields. This was accomplished by directly conjugating a modified phosphonium ionic tag **(10)** containing a primary amine instead of a hydroxyl group, creating an amide bond in compound **(11)** (Scheme 5.3). The amide linkage of **(11)** was formed by treatment of compound **(2)** with TBTU as a coupling reagent, excess triethylamine and phosphonium tag **(10)**. The tagged nitrobenzyl derivative **(11)** was isolated in 84% yield. The formation of the 2-substituted propan-1-ol derivative **(12)** was achieved as described above (see Scheme 5.2; 29% yield over two steps).



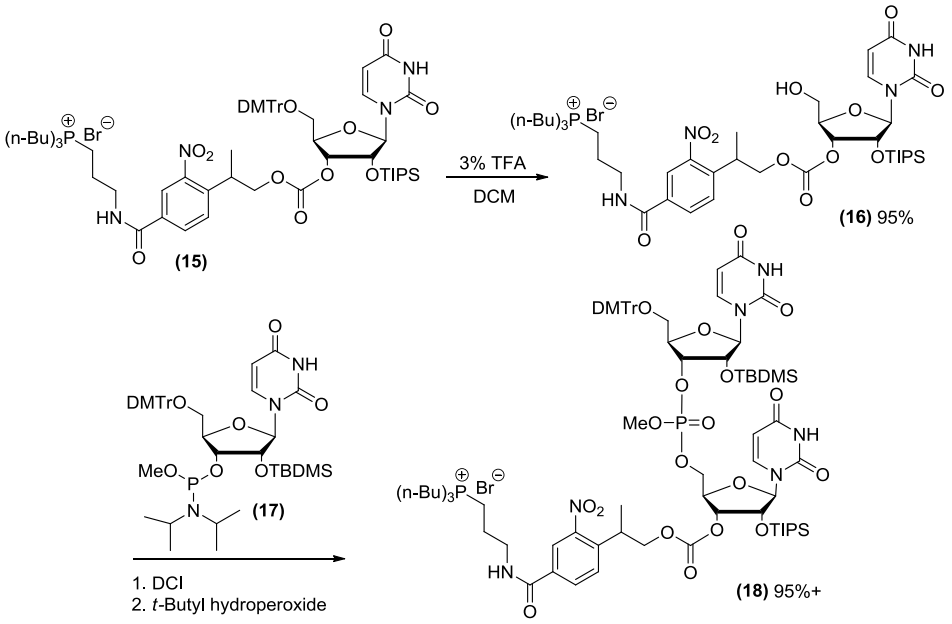
Scheme 5.3. Shortened NPPOC tag-linker synthesis.

We next attempted the coupling of the NPPOC linker to a nucleoside through a carbonate. This was achieved by first phosgenation of the hydroxyl group in the NPPOC linker followed by reaction with the 3′-OH group in the nucleoside. Triphosgene was used to generate phosgene *in situ* to form the chlorocarbonate of **(9)** or **(12)** (Scheme 5.4). Phosgenation was carried out by the "safe phosgene" procedure developed by H. Eckert.[45] Thus, a solution of nucleoside **(13)** in acetonitrile was added directly to the mixture of DIPEA and chlorocarbonate intermediate, and allowed to stir for 6 h at room temperature. Because of the presence of the positively charged ionic tag, compounds **(14)** or **(15)** could be purified by precipitation from MTBE to remove excess (unreacted) nucleoside and DIPEA.



Scheme 5.4. Conjugation of 2′-*O*-TIPS protected nucleoside **(13)** to the photolabile tag linker.

After successfully conjugating the ionically tagged photocleavable linker to a nucleoside through a carbonate linkage, we applied the newly formed monomer to synthesize oligoribonucleotides as described in Chapters 2 and 4.



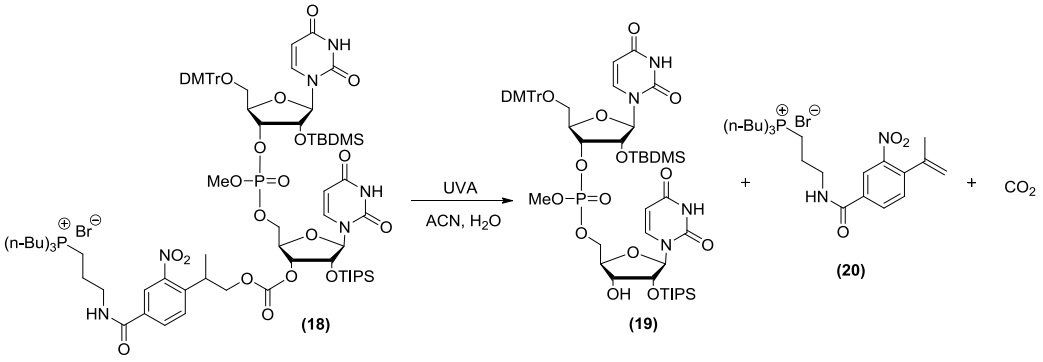
Scheme 5.5. Dimerization of the ionically tagged NPPOC linked nucleoside **(15)**.

Detritylation of the 3′-NPPOC-tagged-uridine **(15)** was achieved using the same procedure developed for the solution phase synthesis of oligoribonucleotides and described in Chapter 2. Compound **(15)** was treated with 3% TFA in DCM and triethylsilane, and the mixture was allowed to stir for 5 min. The crude product was then precipitated in MTBE to remove the trityl and excess triethylsilane, affording deprotected monomer **(16)** in 95% yield. The synthesis of dimer rUpU **(18)** was carried out by coupling uridine phosphoramidite **(17)** to compound **(16)** in the presence of 4,5-dicyanoimidazole (DCI) in ACN. The resulting solution was allowed to stir at room temperature for 3 h. Ten

equivalents of *tert*-butanol were added to quench excess phosphoramidite, followed by ten equivalents of 6 M *tert*-butyl hydroperoxide in decane in order to oxidize the internucleotide phosphite triester to the more stable phosphate triester. The reaction mixture was again purified by precipitation from MTBE to remove all excess reagents. Tagged rUpU dimer **(18)** was isolated in 95% yield.

It was absolutely imperative to keep any NPPOC derivative away from all sunlight, to avoid premature deprotection. Even then, we found that the ambient light in the laboratory still caused deprotection of the NPPOC derivative during manipulations, such as transferring, weighing, and rotovap concentrating of the sample. Samples that were stringently wrapped in aluminum foil did not undergo premature deprotection, disproving our concern of spontaneous degradation of the linker.

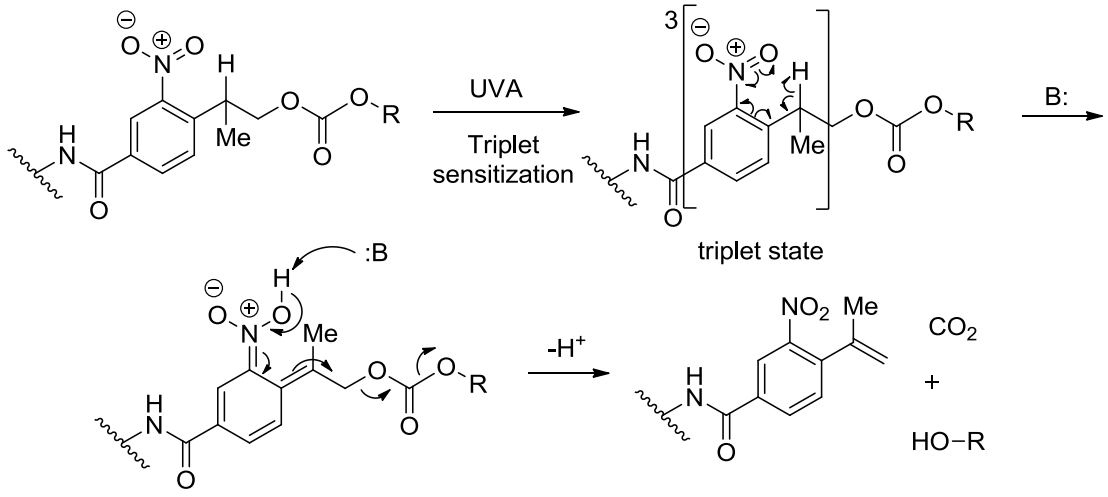
To ensure that the light-mediated cleavage of the NPPOC linker did not cause migration of the 2′-*O*-silyl protecting group, dimer rUpU **(19)** was used, rather than monomer **(15),** as we have previously shown that 2′-*O*-silyl groups of nucleotides dimers are far more likely to undergo 2′ to 3′-migration than nucleoside monomers (Chapter 2). Dimer **(18)** was deprotected at the 3′ hydroxyl by photolysis using UVA light (316-400 nm) as outlined in Scheme 5.6.



Scheme 5.6. Photolysis and removal of the NPPOC tagged linker **(18)**.

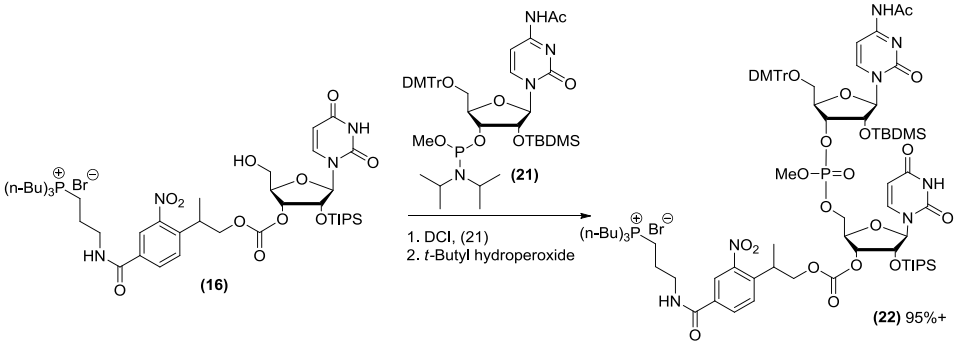
Dimer **(18)** was dissolved in wet ACN (3200 ppm of H_2O), in a Pyrex® round bottomed flask. The flask was placed inside a photoreactor equipped with UVA (316-400 nm) bulbs and reacted for 35 min with stirring at a concentration of 0.01 M. The mixture was concentrated to 1-2 mL of ACN and the cleaved tag was removed by precipitation with methyl *t*-butyl ether (MTBE). The desired

dimer **(19)** was found in the MTBE solution. Concentration *in vacuo* of the MTBE gave the isolated product **(19)** in 92% yield. There was no detectable isomerization as confirmed by ^{31}P-NMR or ^{1}H-NMR, compared to the standards previously synthesized (see Chapter 3). The proposed mechanism for the photo induced cleavage of the NPPOC linker is shown in Scheme 5.7.

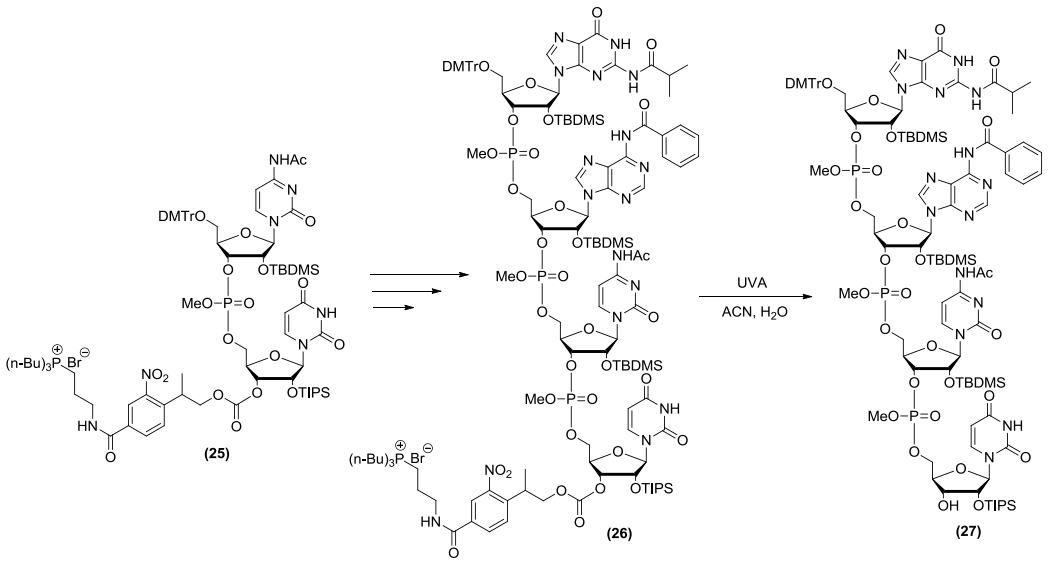


Scheme 5.7. Proposed photo induced cleavage mechanism by Pfleiderer and co-workers.[40]

The synthesis of dimer rCpU **(22)** was carried out similarly by coupling of the 3′-tagged uridine **(16)** with rC phosphoramidite monomer **(21)** (Scheme 5.8). In an attempt to prepare tetramer **(27)** the above process was repeated two more times using the appropriate rA and rG phosphoramidites as shown in Scheme 5.9.



Scheme 5.8. Synthesis for the rCpU NPPOC tagged dimer **(22)**.



Scheme 5.9. Proposed scheme to produce the tetra-nucleotide **(27)** containing all four nucleobases.

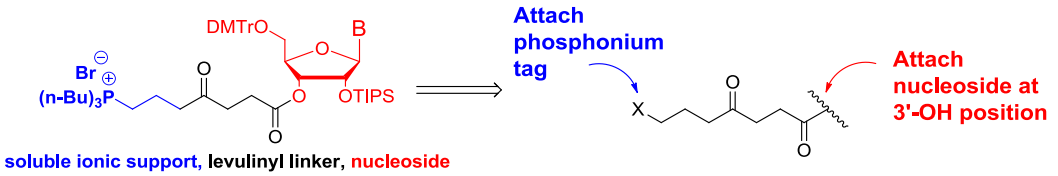
Unfortunately, the synthesis of the target tetramer was unsuccessful. After every precipitation step of the synthesis, significant amounts of the untagged oligoribonucleotide were found in the ether layer, as well as in the precipitated solid. We determined that the NPPOC derivative, while perfectly chemically stable to all conditions of the oligoribonucleotide synthesis cycle, was far too labile to ambient light during the unavoidable manipulations outside of the covered reaction flasks. We have further determined that this linker is in fact cleavable under very mild conditions and does not promote silyl migration upon deprotection as demonstrated in Scheme 5.6.

Since we did not achieve our goal in producing a robust ionically tagged, orthogonally cleavable linker using the NPPOC derivative to be used in solution phase synthesis, we next focused our attention on the levulinyl-like linker.

5.2.2 Levulinyl-like linker synthesis

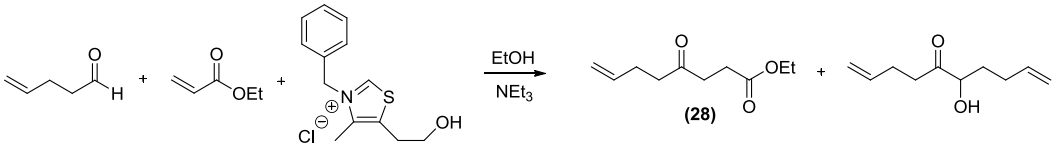
We sought to utilize the γ-keto ester moiety of the levulinyl protecting group which is cleavable by hydrazinolysis and attach to it our ionic soluble support. The general concept of this strategy is outlined in Scheme 5.10. The carboxylic acid functionality will connect the nucleoside, in the same way a levulinyl protecting group does, while the other end of the linker would be

extended and modified to serve as a place to couple the phosphonium ionic tag. This construct would allow for the cleavage of the linker carrying the phosphonium ionic tag by treatment with hydrazine hydrate, releasing the free 3'-OH ribonucleoside or oligoribonucleotide.



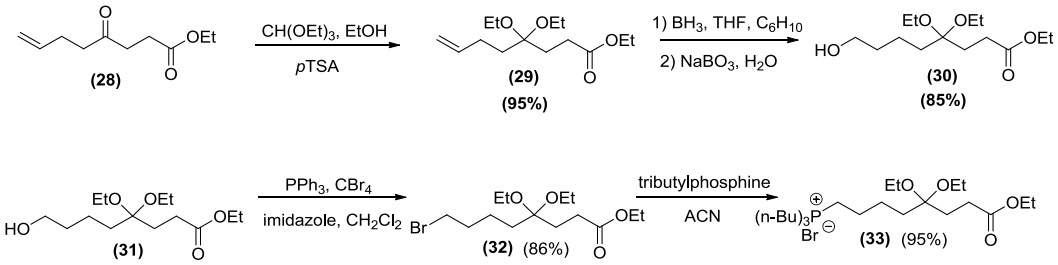
Scheme 5.10. Design of a nucleoside connected to a soluble ionic support (ionic tag) through the a γ-keto ester or levulinyl moiety.

Several approaches have been applied to obtain the desired γ-keto-acid linker**.** The first such attempt was completed by a previous group member, Dr. Robert Donga, who employed a Stetter reaction[46] as the first step to create the γ-keto-ester moiety, shown in Scheme 5.11.[47] This transforms aldehydes into nucleophiles using catalytic amounts of a thiazolium salt, in the presence of a mild base.



Scheme 5.11. Stetter approach to the γ-keto ester.

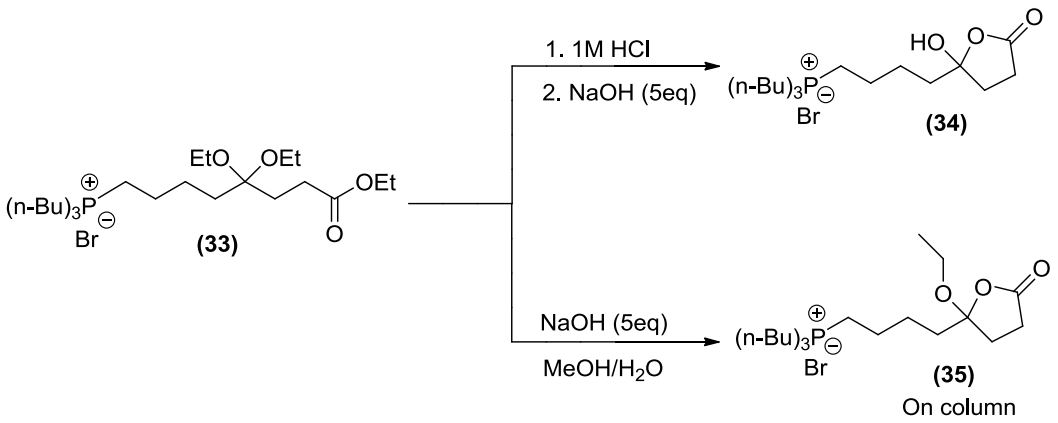
Using the alkene **(28)** previously synthesized,[47] we attempted a synthesis of the tagged levulinyl linker with our phosphonium ionic tag, in place of the imidazolium tag (Scheme 5.12).



Scheme 5.12. Synthesis of phosphonium ionic tagged γ-keto ester derived from the Stetter reaction.

In the first step the ketone **(28)** was protected by forming the diethyl ketal by acid-catalyzed dehydration using a Dean Stark apparatus; in 95% yield. The alkene was then subjected to a regio-selective hydroboration-oxidation by treatment with borane, cyclohexene, and sodium borate as the oxidant. This produced great selectivity in producing the primary hydroxyl in compound **(30)** in 85% yield. The primary hydroxyl was then converted to the alkyl bromide by treatment with triphenylphosphine and carbon tetrabromide. The phosphonium ionic species was then formed by treatment of compound **(32)** with excess tributylphosphine at 65°C for 6 hours, producing the phosphonium tagged compound **(33)** in excellent yield. The resulting ionic compound was isolated by precipitation in methyl *tert*-butyl ether (MTBE).

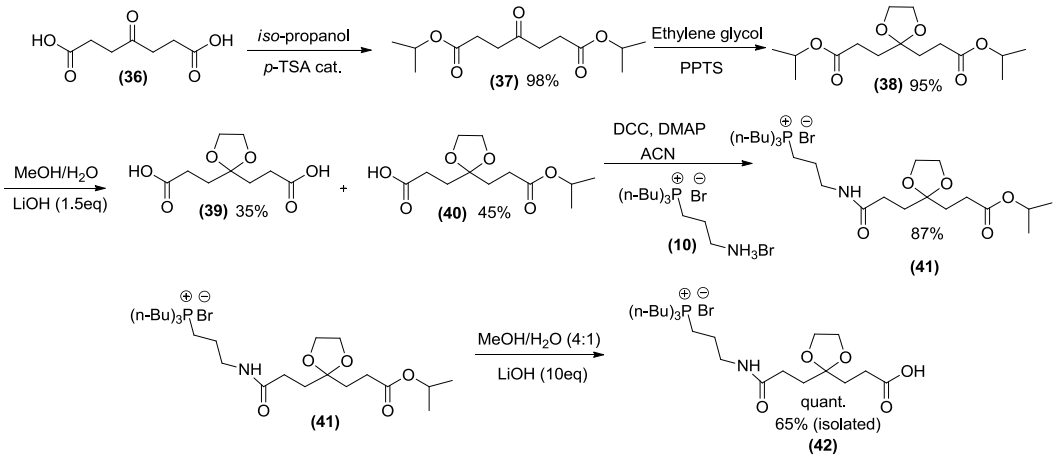
Unfortunately, the simultaneous ketal and ester hydrolysis procedure outlined by Dr. Donga[47] led to an undesired cyclic hemi-lactone **(34)**, in Scheme 5.13. If only the ester was removed and purified, compound **(35)** was isolated. In either case, attempts to conjugate the isolated products **(34)** and **(35)** to the 3′-hydroxyl of a nucleoside, using either TBTU or DCC proved to be unsuccessful, yielding only 5-10% of the desired nucleoside conjugate.



Scheme 5.13. Intramolecular cyclization of diethyl ketal **(33)** during deprotection.

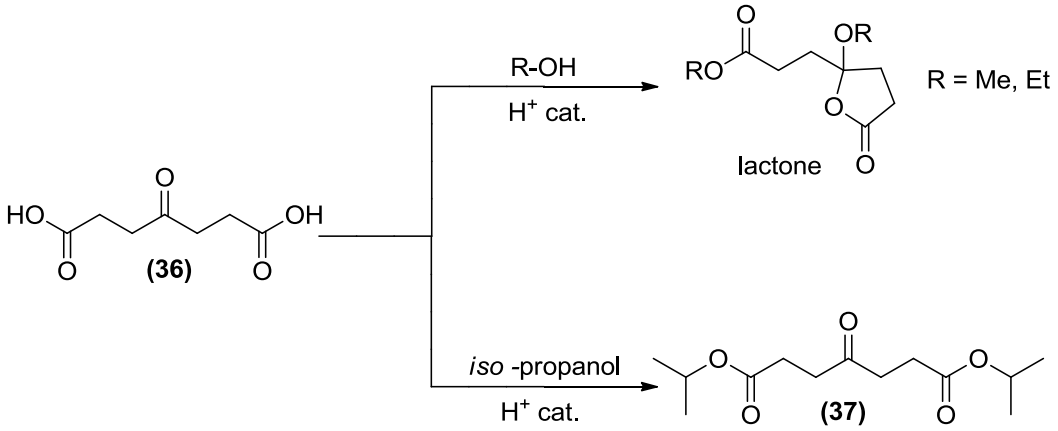
We determined that the diethyl ketal derivative used in the route described above is too labile to use with a γ-ester functionality and allows intermolecular cyclization, significantly inhibiting the coupling to a nucleoside. This is an intrinsic problem with γ-keto acid moieties as they have a propensity to cyclize

into the stable 5 member ring.[48-50] This cyclic form may be more favored with the presence of the ionic tag, as other γ-keto acids (such as the levulinyl group) couple without any issues. To overcome this obstacle we attempted to use a 1,3-dioxolane derivative as a more stable alternative, which was prepared from 4-ketopimelic acid, as shown in Scheme 5.14.



Scheme 5.14. 4-ketopimelic acid route to the ionically tagged γ-keto acid.

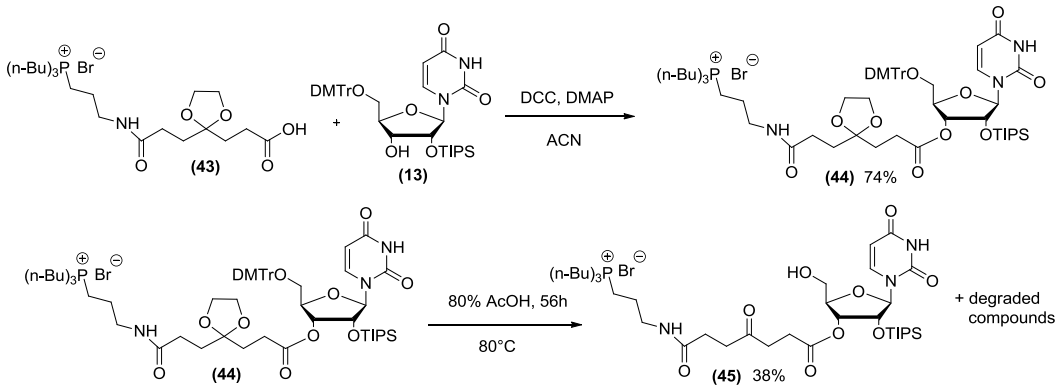
The synthesis started with treatment of 4-ketopimelic acid **(36)** with *iso*-propanol and catalytic *para*-toluenesulfonic acid using a Dean-Stark trap to esterify both the terminal carboxylic acid moieties, affording the diester product **(37)** in 98% yield. The use of methanol and ethanol in lieu of *iso*-propanol in this reaction resulted in lactone formation as the major product instead of the desired diester **(37)** (Scheme 5.15). This reaffirmed our hypothesis that the diethyl ketal is an inappropriate protecting group for these types of molecules



Scheme 5.15. Acid catalyzed esterification of ketopemillic acid **(36)**.

The diisopropyl ester **(37)** in Scheme 5.14 was isolated and subsequently reacted with ethylene glycol and catalytic pyridinium *para*-toluenesulfonate by refluxing in a Dean-Stark trap to form the cyclic ketal **(38)** in 96% yield over two steps. Attempts to use ethylene glycol directly to form both the cyclic ketal and glycol ester directly from 4-ketopimelic acid resulted in the undesired lactone derivative, necessitating that esterification and acetal formation be carried out in two separate steps.

Cyclic ketal diester **(38)** was taken up in methanol and 0.4 equivalents of aqueous lithium hydroxide were added to preferentially hydrolyze one of the two ester moieties, producing a mixture of compounds **(39)** and **(40),** which were separated in yields of 35% and 45%, respectively. Coupling the phosphonium ionic tag **(10)** to the cyclic ketal monoester **(41)** was accomplished using DCC and catalytic DMAP, affording the phosphonium tagged compound **(42)** in 84% yield. After isolation, the isopropyl ester was hydrolyzed by LiOH in MeOH-water mixture to afford compound **(43)** in nearly quantitative yield. After column chromatography, the yield was reduced to 65%, likely due to the phosphonium tag's affinity for silica.



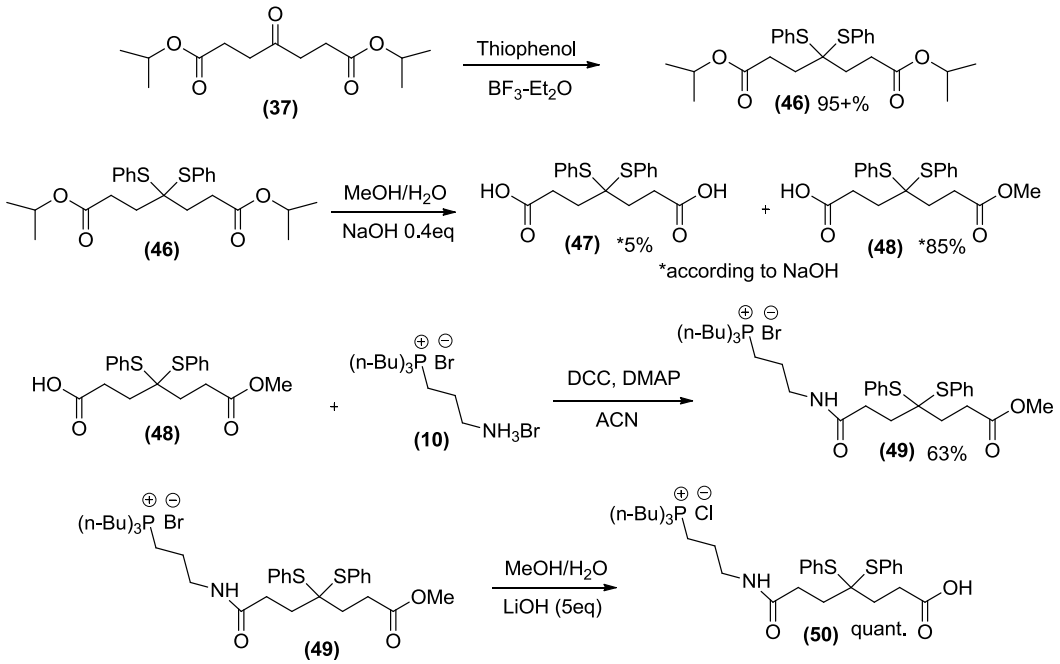
Scheme 5.16. Synthesis of ion tagged nucleoside **(45)**.

Before conjugation of the ionically tagged species **(43)** to the nucleoside **(13)** (Scheme 5.16) the sample was combined with NaBr and taken up in methanol to exchange any acetate ions that had exchanged previously during silica gel/AcOH (1%) purification. If this step were skipped, a significant amount of 3′-acetylation occurred in the subsequent esterification step. After ion

exchange, the ionic tag **(43)** was then conjugated to the 3′-hydroxyl moiety of nucleoside **(13)** using standard coupling conditions (DCC/cat. DMAP, 4 hours), affording compound **(44)** in good yield (74%).

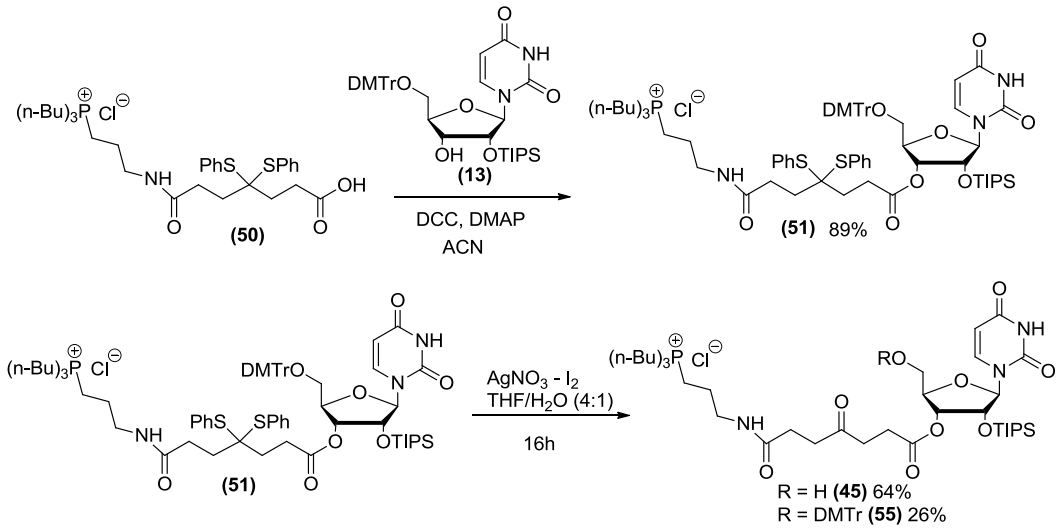
Simultaneous removal of the cyclic ketal and DMTr protecting groups was attempted by treatment with either 80% AcOH in water or 5.0 eq. of pyridinium *p*-toluenesulfonate in 50:50 acetone:water (60-80°C, 4 days). The reaction mixture was neutralized and purified *via* column chromatography to yield only 38% of compound **(45)** and other material lacking 3′-TIPS protection. Evidently, the hot acidic solution was causing desilylation as well.

To overcome this limitation, the ketone was protected as the thioketal as it is more stable to acidic conditions (Scheme 5.17). Thus, a mixture of thiophenol and compound **(37)** were treated with boron trifluoride etherate to afford compound **(46)** in 92% yield. Next, selective hydrolysis of the diester was achieved by treatment with MeOH/water (4:1) and with 0.4 eq. of NaOH at 0°C. Once the NaOH was consumed, the reaction mixture was condensed and extracted with ethyl acetate and 1M HCl which afforded the desired product, **(48)**, in 85% yield (in reference to the NaOH added). The phosphonium ionic tag **(10)** was then conjugated to compound **(48)** under standard coupling conditions (DCC/ cat. DMAP in pyridine:ACN) to afford compound **(49)** in 87% yield. Lastly, the ester of compound (**49**) was hydrolyzed with 5.0 eq. of NaOH in methanol/water to afford compound **(50)** in near quantitative yield.



Scheme 5.17. Thioketal protected ionically tagged γ-keto acid synthesis **(50)**.

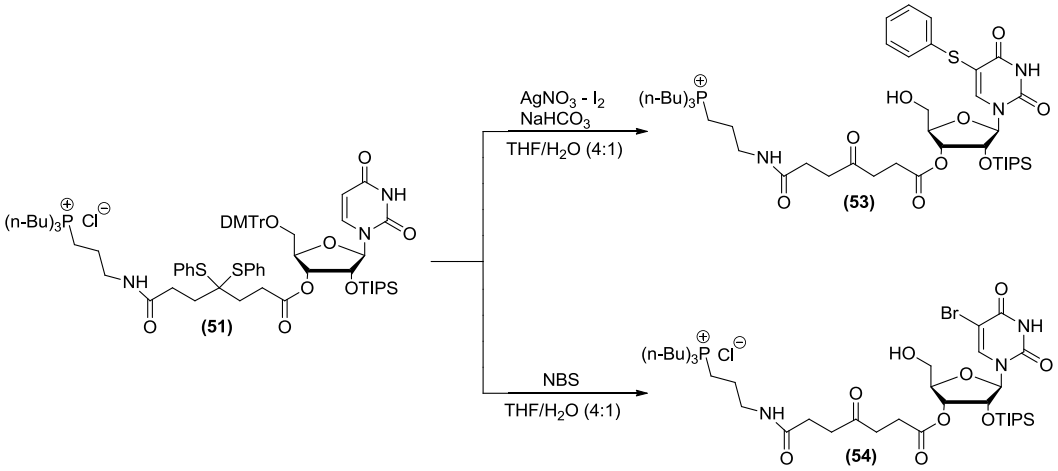
With compound **(50)** in hand, the nucleoside conjugation was achieved (Scheme 5.18), again with the use of DCC and DMAP to afford the nucleoside conjugate **(51)** in good yield, 89%.



Scheme 5.18. Thioketal protected ionically tagged γ-keto acid nuceloside conjugation.

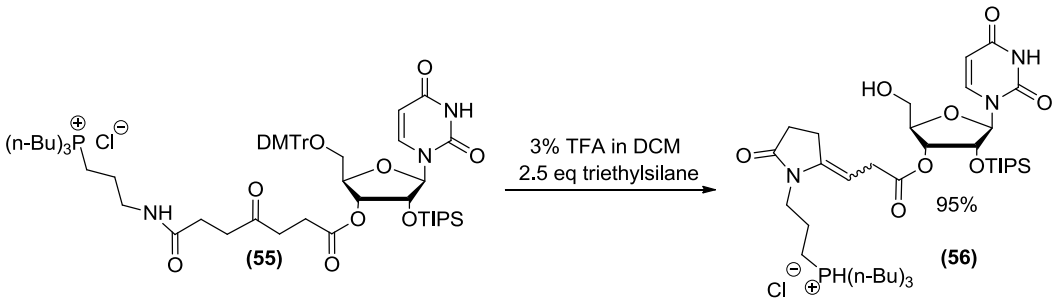
After conjugation, the thioketal of **(51)** was removed by treatment with silver nitrate and molecular iodine in 80% THF/water, resulting in a mixture of tritylated and detritylated versions of compound **(45)**. The absence of any base

produced a mixture of tritylated and detritylated material, which were easily separable by column chromatography. When the same reaction was attempted in the presence of sodium bicarbonate as a base, a very different product was observed. In this case, the C-5 thiophenyl derivative **(53)** formed as the major product (Scheme 5.19). This compound likely forms *via* electrophilic addition of iodine to C-5, followed by substitution with thiophenol. The use of collidine in place of sodium bicarbonate in the silver nitrate/iodine method reduced but did not totally eliminate the formation of the C-5 thiophenyl derivative **(53).** These conditions afforded compound **(45)** in 64% yield (Scheme 5.18). In another deprotection strategy, NBS was used as a source of bromonium ion in order to cleave the thioketal; instead, however, bromination of the C-5 position occurred to produce **(54)** as the major product (Scheme 5.19).



Scheme 5.19. Side product formation during thioketal deprotection strategies

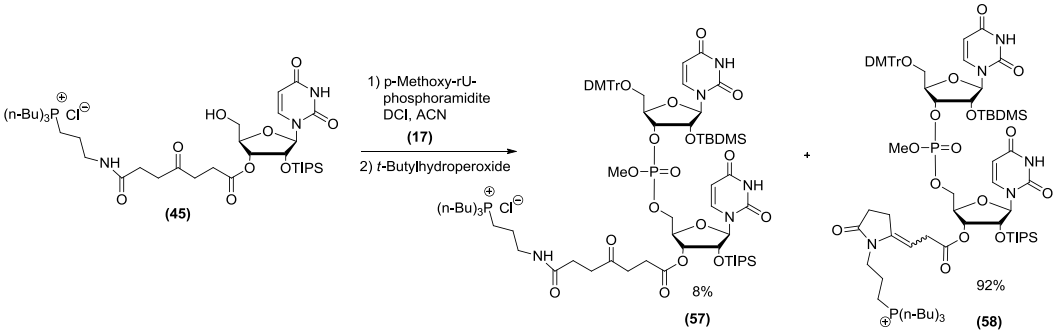
The tritylated linker nucleoside conjugate **(55)** was treated with 3% TFA in DCM for 10 min. Toluene was added, followed by concentration *in vacuo*. The sample was then taken up in acetone and precipitated from methyl *tert*-butyl ether (MTBE), producing an interesting but undesired enamine product, compound **(56)**, in 95% yield (Scheme 5.20). To minimize formation of this condensation product, the experiment was repeated without the toluene concentration step, and precipitating the reaction mixture directly into MTBE. However, similar results were obtained, with the major product, compound **(56)**, obtained in 60% yield, and the remainder being the desired 5′-hydroxyl product **(45)**.



Scheme 5.20. Formation of the undesired cyclic enamine nucleoside conjugate **(56)**.

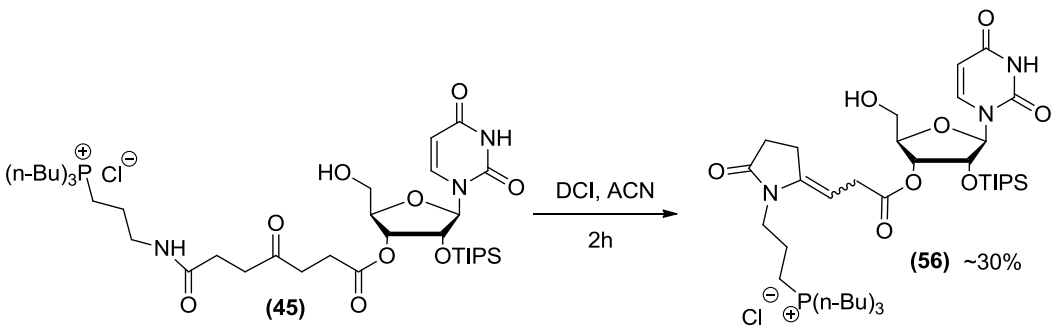
After ruling out that toluene dehydration was not the cause of the cyclization reported in Scheme 5.20, we assumed that perhaps the acidic conditions activated the ketone moiety, causing intra-molecular cyclization of the amide.

Without pursuing a solution to the intramolecular cyclization observed above, we attempted a phosphoramidite coupling (Scheme 5.21) with the small amounts of compound **(45)** we had collected from the previous experiments. Unfortunately, intramolecular cyclization also occurred during the phosphitylation reaction (Scheme 5.21).



Scheme 5.21. Phosphoramidite coupling of the deprotected tagged nucleoside conjugate **(45)**

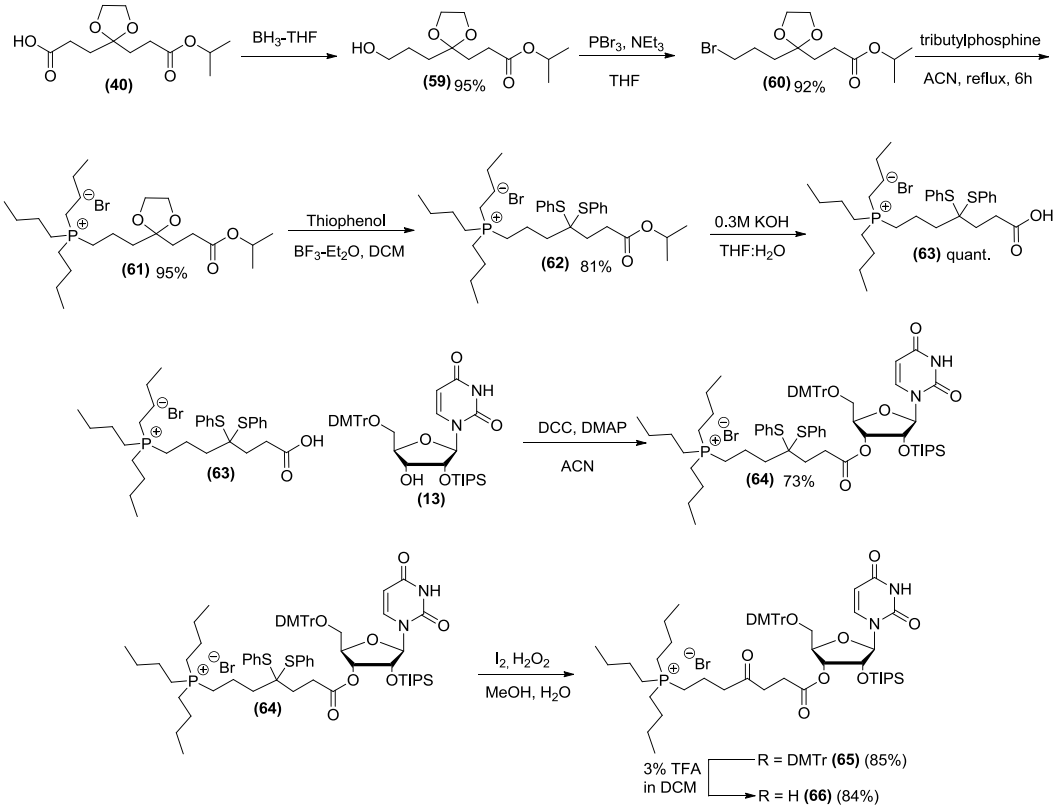
Next, compound **(45)** (derived from the hydrolysis of the thioketal in the absence of base; Scheme 5.18) was coupled with rU-methylphosphoramidite **(17)**, followed by oxidation, to produce mainly the undesired cyclized linker dimer nucleotide **(58);** formation of this by-product was promoted by DCI alone (determined by monitoring the reaction by mass spectrometry). A few examples of this type of reaction, catalyzed only by sodium acetate[51] or acetic acid,[52] have been reported in the literature.



Scheme 5.22. DCI treatment of deprotected nucleoside conjugate **(45)** to produce the undesired cyclic enamine product **(56)**.

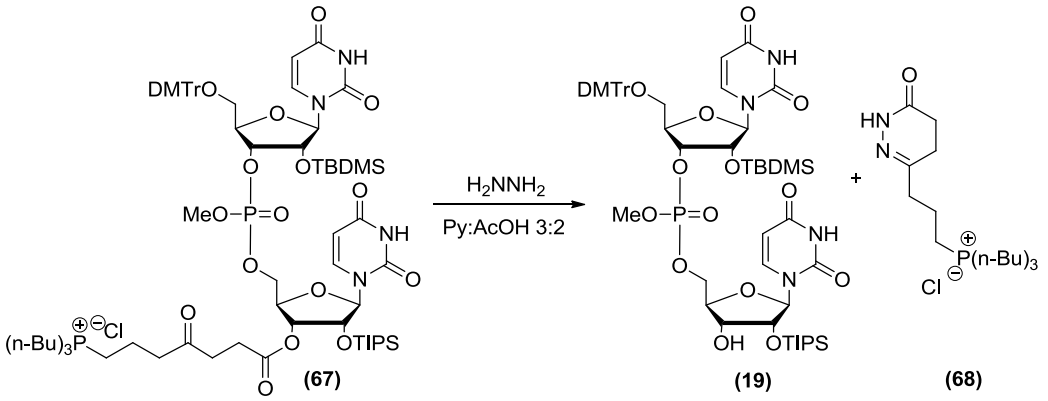
Consistent with the structure of cyclic enamine **(56),** this compound was inert to 0.5 M hydrazine hydrate buffered in pyridine acetic acid (3:2).

With the knowledge that acyclic and cyclic acetals are a poor choice for a ketone protecting group on our levulinyl-like linker, and that γ-keto amides are also incompatible with standard phosphoramidite couplings conditions, an alternative synthetic route was pursued (Scheme 5.23).



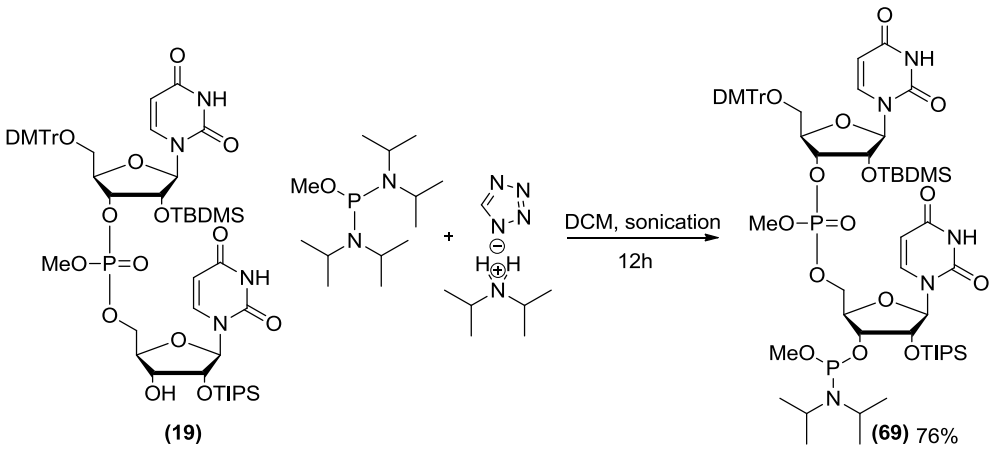
Scheme 5.23. Successful synthesis of ionically tagged levulinyl-like nucleoside conjugate

The previously synthesized compound **(40)** (Scheme 5.23) was dissolved in dry THF, cooled to 0°C, and reduced with borane-THF. After quenching with methanol, the reaction mixture was condensed to dryness. Purification of the crude product by a column chromatography yielded over 95% of **(59)**. Treatment of **(59)** with phosphorus tribromide (added dropwise at 0°C over 10 min, followed by stirring for 1 hour and work out) gave the alkyl bromide **(60)** in 92% yield. Compound **(60)** was then dissolved in minimal amounts of acetonitrile, and tributylphosphine was added slowly over 10 min. After refluxing for an additional 6 hours, the mixture was concentrated and precipitated into vigorously stirring hexanes. The hexanes layer was passed through a Celite® filter and discarded; the remaining viscous oil, found both on the filter and in the flask, was pure phosphonium tagged compound **(61)**, isolated in quantitative yield. Next, the ketal of compound **(61)** was replaced with the more stable and more easily removed thioketal, by treatment with thiophenol, borane trifluoride etherate in the presence of molecular sieves for 6 hours at room temperature. Purification by column chromatography afforded **(62)** in 81% yield. Ester hydrolysis of **(62)** gave **(63)**, which was coupled TIPS protected nucleoside **(13)** under standard (DCC/DMAP) to give, after precipitation, the tagged nucleoside **(64)** (73%). Finally, the thioketal was removed under very mild conditions by treatment with 20 mol% of molecular iodine in a solution of methanol, water and hydrogen peroxide, providing, after aqueous work-up and isolation by precipitation, the deprotected monomer **(65)** in 85% yield. The new conditions to remove the thioketal is an improved, milder and cheaper alternative[53] to the silver nitrate used previously. Furthermore, these conditions does not remove the 5′-DMTr protecting group or promote modification of the uracil base.



Scheme 5.24. Selective removal of the ionically tagged levulinyl-like linker providing isomerically pure rUpU dimer **(19)**.

Finally, dimer **(67)** was prepared by detritylation of **(65)** followed by coupling of the 3′-tagged nucleoside **(66)** with nucleoside phosphoramidite **(17)**. Treatment of dimer **(67)** with hydrazine hydrate (0.5 M in pyridine:acetic acid 3:2) afforded isomerically pure dimer **(19)** in almost quantitative yield. Phosphitylation on dimer **(19)** under the acidic phosphitylation conditions (Chapter 2), afforded **(69)** in 76% yield after a chromatography step (Scheme 5.25).



Scheme 5.25. Phosphitylation of [5′-O-DMTr-2′-O-TBDMS-U]-[3′-p(OMe)-5′]-[U-2′-O-TIPS] dimer (19) via the acidic phosphitylation method

With the successful phosphitylation of dimer **(19)** (0.65 mmol scale; 740 mg), we have demonstrated that the ionically tagged levulinyl linker provides a viable route to produce block RNA block phosphoramidites.

5.3 Conclusions

We have described the synthesis of two unique, ionically tagged orthogonally cleavable linkers. The levulinyl derivative was shown to provide the desired regio-isomerically pure dimer **(19)**. Although the NPPOC derivative was far too sensitive for manual manipulation in the laboratory, resulting in premature deprotection due to ambient light, this linker may find use in solid phase synthesis, where manipulations are automated and the support is protected from light inside a synthesis column. Chapter 6 highlights this approach.

5.4 Experimental

Materials

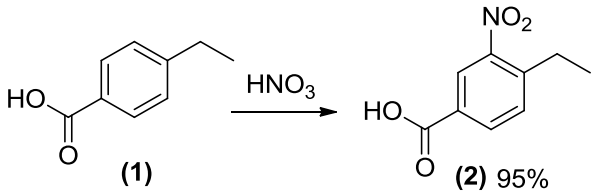
Thin layer chromatography was performed on EM Science Kieselgel 60 F-254(1mm) plates. Silicycle 40-63 μm (230-400 mesh) silica gel was used for flash chromatography. Pyridine, acetonitrile, and dichloromethane were distilled from CaH_2 after refluxing for several hours. THF was distilled from benzophenone and sodium after refluxing for several hours. All other anhydrous solvents were purchased from Sigma-Aldrich. Chemicals and reagents were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Cyanoethyl phosphoramidites and methylphosphoramidic chloride were purchased from ChemGenes Inc.

Instrumentation

The solid phase syntheses of oligonuc1eotides were conducted on an ABI synthesizer. UV spectra for oligonuc1eotide quantitation (absorbance measurements) were measured at 260 nm on a Varian Cary I or 300 UV-VIS dual beam spectrophotometer. ^{1}H NMR and ^{13}C-NMR were recorded on a Varian 300, 400 or 500 MHz spectrophotometer with chemical shift values reported in ppm. ^{1}H-NMR and ^{13}C-NMR spectra were referenced to residual undeuterated solvent. ^{31}P-NMR were collected at 80 MHz with a Varian 200MHz spectrophotometer

and were measured from 85% H_3PO_4 as an external standard. Photolysis was performed in a Luzchem LZC photoreactor using their LZC-UVA lamps, which have a range of 316-400 nm.

Synthesis procedures



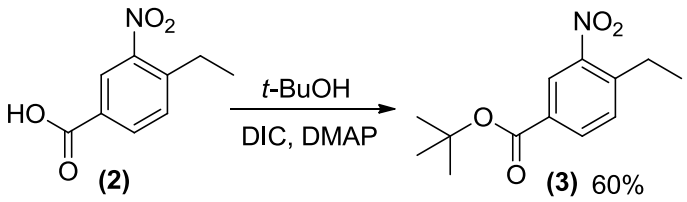
(1) **(2)** 95%

3-Nitro-4-ethyl-benzoic acid (2)

Fuming nitric acid (90%) (150 ml) was cooled with stirring to -10°C and 4-ethyl benzoic acid **(1)** (30 g, 0.2 moles; Sigma-Aldrich) was added slowly over 30min directly into the sitting solution (1.33 mmol/mL of **(1)** to fuming nitric acid). The mixture was then allowed to stir for 30min after addition was complete. The mixture was then poured over approximately 600 g of crushed ice to quench the reaction. The product formed a white precipitate which can be filtered over a sintered glass funnel. The excess ice melted by washing the product with water. The sample was then re-crystallized from ethyl acetate/hexanes. Two rounds of crystallization were preformed. Yield: 37.2 g (95%).

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 1.33 (t, *J*=7.58 Hz, 3 H) 2.99 (q, *J*=7.58 Hz, 2 H) 7.52 (d, *J*=8.07 Hz, 1 H) 8.23 (dd, *J*=8.07, 1.71 Hz, 1 H) 8.58 (d, *J*=1.71 Hz, 1 H) ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 14.62, 26.38, 126.37, 128.29, 131.67, 133.87, 144.85, 149.36, 170.40

$C_9H_9NO_4Na^{1+}$ low resolution ESI-MS calculated: 195.05, found: 218.2.

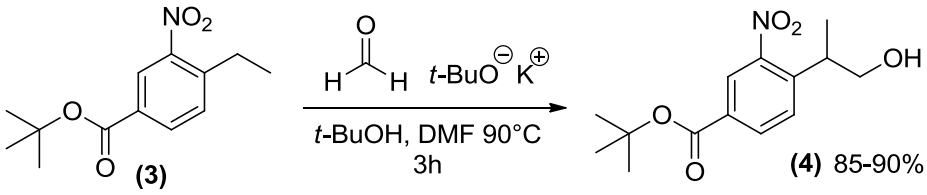


***tert*-Butyl 3-nitro-4-ethyl-benzoate (3)**

Compound **(2)** (19.65 g 0.10 moles) was dissolved in 500ml of THF (0.2 M) followed by 1.15 eq (10.14 g, 0.15 moles) of diisopropylcarbodimide. This mixture was allowed to stir for 5min followed by 1.5 eq of *tert*-butanol (17.9 mL) and catalytic amounts of 4-(dimethylamino)-pyridine. The mixture was allowed to stir for 60 h before the reaction was complete. The reaction was diluted with diethyl ether and filtered to remove the diisopropylurea (DIU) and condensed to dryness. The mixture was dissolved in ethyl acetate and extracted with 5% $NaHCO_3$. The product was separated from **(2)** by column chromatography (solvent system: hexanes:DCM 100:0 →0:100). Yield of **(3)**: 15.1g (60%)

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.26 (t, *J*=7.47 Hz, 3 H) 1.57 (s, 11 H) 2.91 (q, *J*=7.33 Hz, 2 H) 7.40 (d, *J*=7.91 Hz, 1 H) 8.08 (dd, *J*=7.91, 1.47 Hz, 1 H) 8.38 (d, *J*=1.47 Hz, 1 H) ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 14.70, 26.18, 28.05, 76.64, 77.06, 77.49, 82.13, 125.42, 131.12, 131.18, 133.23, 143.00, 149.12, 163.56.

$C_{13}H_{17}NO_4Na^{1+}$ low resolution ESI-MS calculated: 251.11, found: 274.32.



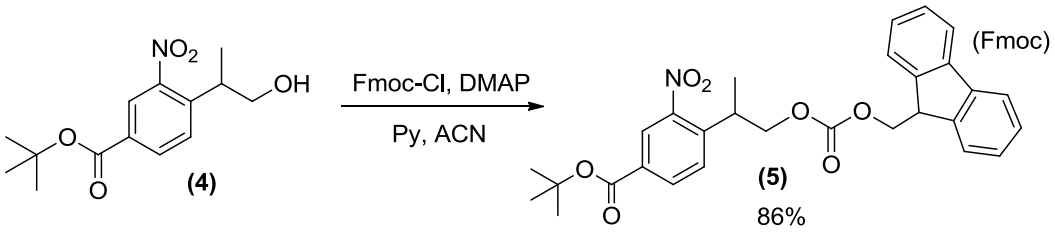
***tert*-Butyl 4-(1-hydroxypropan-2-yl)-3-nitrobenzoate (4)**

Compound **(3)** (10.5 g, 41.7 mmol) was dissolved in 19 mL of DMF (2.2M) to which 1.5 eq (1.88 g) of paraformaldehyde was added followed by a solution of potassium *tert*-butoxide (0.12 eq, 0.56 g) in *tert*-butanol (5.7 mL). This mixture

was allowed to stir at room temperature for 10 min before being brought up 90^0C for 3h. The mixture was then acidified to neutrality by the addition of a 1M HCl monitored by a pH meter. This mixture was then diluted with sat. NaCl and ethyl acetate (x2). Compound **(4)** was purified by column chromatography in DCM:ethyl acetate 100:0 →90:10. Yield: 9.4 g (85%).

^{1}H NMR (400 MHz, CD_3CN) δ ppm 1.41 (d, *J*=7.03 Hz, 3 H) 3.80 (sxt, *J*=6.88 Hz, 1 H) 4.49 - 4.67 (m, 2 H) 7.40 (d, *J*=7.91 Hz, 1 H) 8.08 (dd, *J*=7.91, 1.47 Hz, 1 H) 8.38 (d, *J*=1.47 Hz, 1 H) ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 17.35, 27.96, 36.57, 67.01, 82.27, 124.77, 128.50, 131.04, 131.06, 132.81, 142.62, 150.41, 163.58.

$C_{14}H_{19}NO_5Na^{1+}$ low resolution ESI-MS calculated: 281.12, found: 304.02.



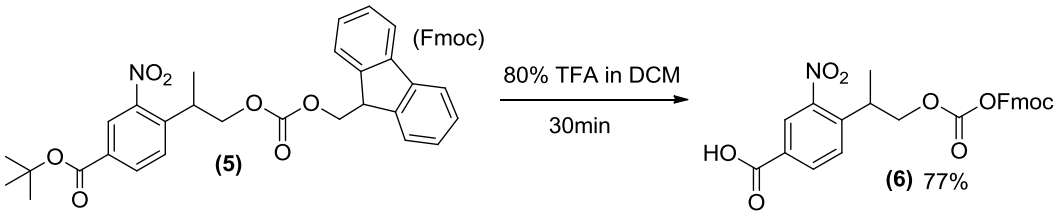
***tert*-Butyl 4-(1-(F-moc)propan-2-yl)-3-nitrobenzoate (5)**

Compound **(4)** (5.38 g, 20.2 mmol) was co-evaporated with pyridine (x3) and dissolved in 97 mL of ACN (0.2M) and 2eq of pyridine (3.13 mL, 38.7 mmol). Fmoc-Cl (5.0g, 19.33 mmol) was added directly to solution and was allowed to stir for 16h in the dark, covered with aluminum foil. The reaction went to completion by TLC. The solution was extracted (x3) with 5% ammonium chloride and once with brine and purified by column chromatography Hex/EtAc 100:0 → 75:25. Isolated yield: 8.19 g (86%).

^{1}H NMR (500 MHz, CD_3CN) δ ppm 1.30 (d, *J*=7.09 Hz, 3 H) 1.60 (s, 9 H) 3.65 (sxt, *J*=6.94 Hz, 1 H) 4.19 (t, *J*=6.24 Hz, 1 H) 4.23 - 4.34 (m, 2 H) 4.39 - 4.53 (m, 2 H) 7.27 - 7.34 (m, 2 H) 7.41 (t, *J*=7.46 Hz, 2 H) 7.53 (d, *J*=7.34 Hz, 2 H) 7.62 (d, *J*=8.31 Hz, 1 H) 7.80 (d, *J*=7.58 Hz, 2 H) 8.13 (dd, *J*=8.19, 1.59 Hz, 1 H) 8.28 (d, *J*=1.71 Hz, 1 H) ^{13}C NMR (126 MHz, CD_3CN) δ ppm -0.10, 0.06, 0.23, 0.40, 0.56, 0.73, 0.89, 16.83, 27.30, 27.32, 33.47, 46.64, 68.89, 70.90, 82.13, 117.34,

120.06, 124.56, 124.87, 124.90, 127.17, 127.18, 127.81, 127.83, 128.87, 131.72, 132.75, 141.03, 141.17, 143.50, 143.55, 150.42, 154.54, 163.26.

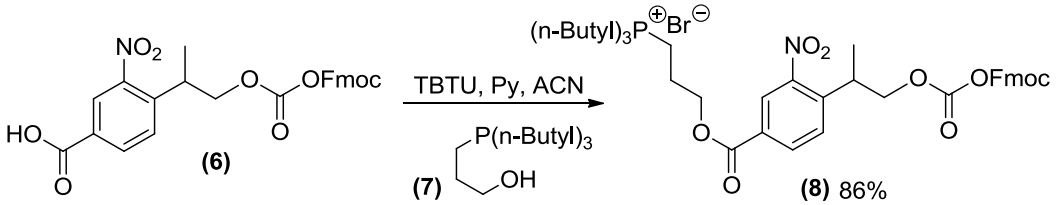
$C_{29}H_{29}NO_7Na^{1+}$ low resolution ESI-MS calculated: 503.19, found: 526.41.



4-(1-(Fmoc)propan-2-yl)-3-nitrobenzoic acid (6)

Compound **(5)** (8.9 g, 17.7 mmol) was directly dissolved in a solution of 80% TFA in DCM (50 mL) and allowed to stir for 30min, until all starting material had been consumed. The sample was then evaporated to dryness on the rotovop and purified by column chromatography, Hex/EtAc 100:0 → 60:40. Isolated yield: 6.09 g (77%).

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 1.43 (d, *J*=6.85 Hz, 3 H) 3.86 (sxt, *J*=6.80 Hz, 1 H) 4.32 - 4.46 (m, 4 H) 7.28 - 7.36 (m, 2 H) 7.41 (t, *J*=7.58 Hz, 2 H) 7.57 (dd, *J*=6.97, 4.52 Hz, 2 H) 7.65 (d, *J*=8.07 Hz, 1 H) 7.76 (d, *J*=7.34 Hz, 2 H) 8.29 (dd, *J*=8.07, 1.71 Hz, 1 H) 8.53 (d, *J*=1.71 Hz, 1 H) 11.69 (br. s., 1 H) ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 17.58, 33.71, 46.62, 70.08, 71.14, 76.80, 77.05, 77.31, 120.06, 125.10, 125.11, 126.16, 127.16, 127.17, 127.91, 127.93, 128.87, 129.00, 133.71, 141.26, 141.27, 142.82, 143.14, 143.20, 150.39, 155.00, 169.79. $C_{25}H_{20}NO_7^{1-}$ low resolution ESI-MS calculated: 447.13, found: 446.0.



Phosphonium tag 4-(1-(Fmoc)propan-2-yl)-3-nitrobenzoate (8)

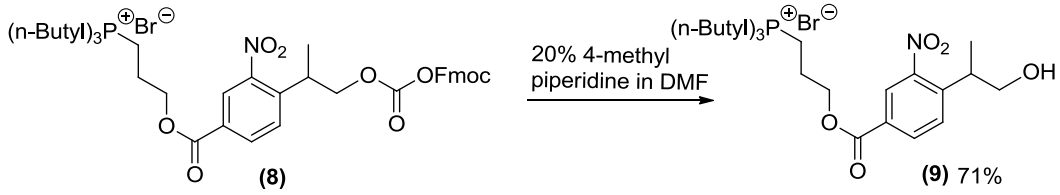
Compound **(6)** (3.726 g, 8.33 mmol) was dissolved in half the solvent (ACN:Py, 28 mL:1.25 mL) to which was added a solution of the phosphonium tag **(7)** (3.86 g, 9.16 mmol) in the other half of the solvent, followed directly by TBTU (4.0 g, 12.5 mmol). The solution was allowed to stir overnight and by

morning the reaction was complete (12 h), and was concentrated to half solvent volume then extracted with ethyl acetate and 5% $NaHCO_3$ (x3) and once with brine. The organic layer was dried with $MgSO_4$ and condensed to dryness. The compound was then precipitated in 500 mL of MTBE to remove the excess pyridine and TBTU byproduct. The precipitated white goo was filtered and collected over Celite® then purified by column chromatography, DCM:MeOH 100:0 → 92:8. Isolated yield of **(79)**: 5.52 g (86%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.81 - 0.99 (m, 8 H) 1.35 (d, *J*=7.03 Hz, 3 H) 1.38 - 1.61 (m, 11 H) 1.99 - 2.38 (m, 8 H) 2.66 (br. s., 2 H) 3.54 - 3.83 (m, 3 H) 4.10 - 4.41 (m, 5 H) 7.21 - 7.44 (m, 4 H) 7.57 (dd, *J*=10.11, 8.06 Hz, 3 H) 7.72 (d, *J*=7.33 Hz, 2 H) 8.55 (d, *J*=1.76 Hz, 1 H) 8.70 (dd, *J*=8.20, 1.76 Hz, 1 H)

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 13.35, 17.68, 18.59, 19.22, 23.55, 23.62, 23.81, 24.01, 33.40, 46.62, 69.91, 71.36, 76.67, 77.09, 77.52, 119.96, 124.31, 125.21, 127.18, 127.83, 128.61, 131.93, 133.41, 139.59, 141.19, 143.27, 143.33, 150.18, 154.87, 165.19, ^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 35.07 (s, 1 P)

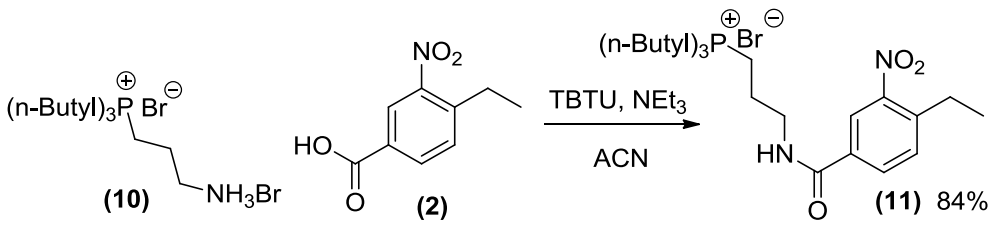
$C_{40}H_{53}NO_7P^{1+}$ low resolution ESI-MS calculated: 690.30, found: 690.35.



Phosphonium tag 4-(1-hydroxypropan-2-yl)-3-nitrobenzoate (9)

To compound **(8)** (6.408 g, 9.29 mmol) was added a 20% solution of 4-methylpiperidine in DMF (20ml). After 2 h the reaction was complete by TLC and the solution was evaporated to dryness, then taken up in DCM and precipitated in 500ml of MTBE to yield 3.09 g of **(9)** (71%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.91 (t, *J*=7.03 Hz, 11 H) 1.26 (d, *J*=7.03 Hz, 3 H) 1.38 - 1.59 (m, 13 H) 1.98 (d, *J*=7.03 Hz, 2 H) 2.11 - 2.28 (m, 7 H) 2.36 - 2.51 (m, 2 H) 3.40 - 3.59 (m, 3 H) 3.64 - 3.82 (m, 2 H) 7.51 (d, *J*=8.21 Hz, 1 H) 8.22 (d, *J*=8.21 Hz, 1 H) 8.26 (s, 1 H) $C_{25}H_{43}NO_5P^{1+}$ low resolution ESI-MS calculated: 468.28, found: 468.28.



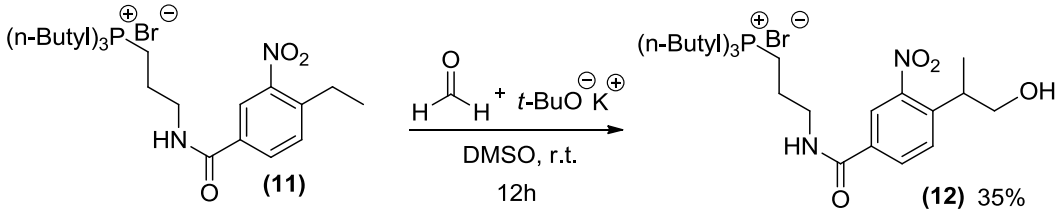
Tributyl(3-(4-ethyl-3-nitrobenzamido)propyl)phosphonium bromide (11)

To a solution of 3-nitro-4-ethyl-benzoic acid **(2)** (3.15 g, 16.1 mmol) and diisopropylethylamine (4 eq, 11.25 ml) in ACN (30 mL) was added compound **(10)** (1.3 eq, 20.9 mmol) and TBTU (1.3 eq, 6.74 g, 20.9 mmol). This mixture was allowed to stir for 12 h. The dark brown solution was concentrated to a viscous oil, and taken up in DCM and precipitated in 500 ml of MTBE. The solid was collected and re-purified by silica gel column chromatography (DCM:MeOH, 100:0 → 85:15) eluting as very dark yellow oil. Isolated yield: 7.07 g (84%).

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.90 (t, *J*=6.97 Hz, 9 H) 1.24 (t, *J*=7.46 Hz, 3 H) 1.35 - 1.56 (m, 12 H) 1.96 (d, *J*=8.07 Hz, 2 H) 2.05 - 2.19 (m, 7 H) 2.25 - 2.38 (m, 2 H) 2.78 (s, 2 H) 2.87 (q, *J*=7.34 Hz, 2 H) 3.60 (q, *J*=5.79 Hz, 2 H) 7.40 (d, *J*=8.07 Hz, 1 H) 7.78 (t, *J*=5.50 Hz, 1 H) 8.07 (d, *J*=8.07 Hz, 1 H) 8.37 (s, 1 H)

^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 13.21, 14.61, 18.17, 18.56, 23.33, 23.37, 23.76, 23.88, 25.97, 38.57, 76.77, 77.02, 77.28, 123.82, 131.09, 131.47, 132.89, 141.77, 149.21, 165.48.

$C_{24}H_{42}N_2O_3P^{1+}$ low resolution ESI-MS calculated: 437.29, found: 437.30.



Tributyl(3-(4-(1-hydroxypropan-2-yl)-3-nitrobenzamido)propyl) phosphonium bromide (12)

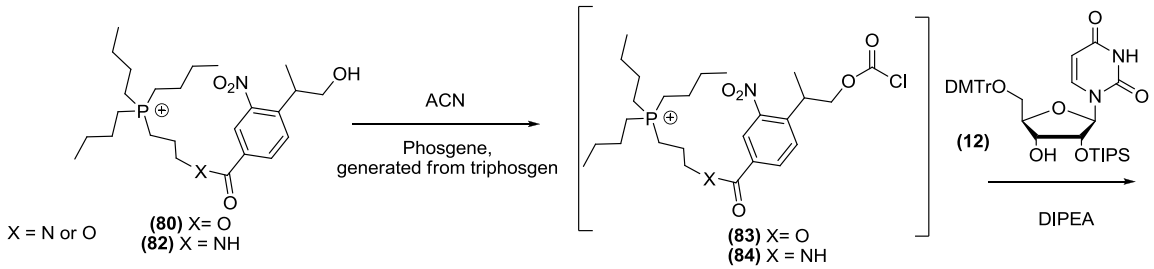
To a solution of compound **(11)** (3.56 g, 6.89 mmol) dry DMSO (13.8 mL), was added *para*-formaldehyde (2.1eq, 0.43 g, 14.4 mmol). This mixture was

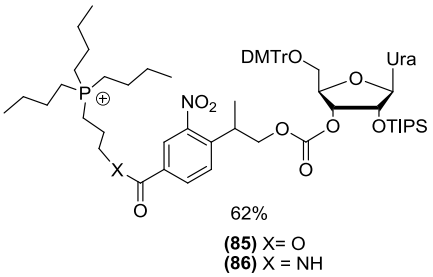
sonicated for 20 min till all of *para*-formaldehyde dissolved. The resulting mixture was treated with 1.5 eq of potassium *tert*-butoxide (1.16 g, 10.3 mmol). The reaction turned a dark purple immediately. The reaction was allowed to stir for 12 h at room temperature. The reaction was monitored by MS, showing the disappearance of the starting material. The reaction was treated with 1M HCl in MeOH to bring it to neutrality at which point the reaction was precipitated in diethyl ether, and then in DCM. This last precipitation step separated the product from unreacted para-formaldehyde. The product was purified by reverse phase chromatography, using 100 mM TEAA buffer in water (pH 7): ACN 80:20 → 20:80. Isolated yield: 1.3 g (35%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.84 - 0.98 (m, 9 H) 1.26 (d, *J*=7.03 Hz, 3 H) 1.38 - 1.59 (m, 11 H) 1.98 (d, *J*=5.86 Hz, 2 H) 2.09 - 2.27 (m, 6 H) 2.34 - 2.50 (m, 2 H) 3.39 - 3.49 (m, 1 H) 3.53 (d, *J*=5.47 Hz, 2 H) 3.64 - 3.81 (m, 2 H) 7.51 (d, *J*=8.21 Hz, 1 H) 8.22 (d, *J*=8.21 Hz, 1 H) 8.26 (s, 1 H) 8.45 (br. s., 1 H) ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 13.38, 17.65, 18.43, 19.05, 23.61, 23.68, 23.78, 23.99, 36.68, 50.06, 66.17, 76.69, 77.11, 77.54, 124.73, 128.38, 129.42, 133.08, 144.51, 150.43, 164.33

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 35.07 (s, 1 P)

$C_{25}H_{44}N_2O_4P^{1+}$ low resolution ESI-MS calculated: 467.30, found: 467.31.





5′-DMTr-2′-TIPS-3′-(tributyl(3-(4-(1-hydroxypropan-2-yl)-3-nitro benzamido)propyl) phosphonium bromide)-uridine (15)

This "safe phosgene procedure was adopted from Eckert, H. & Auerweck, J. Solvent-Free and Safe Process for the Quantitative Production of Phosgene from Triphosgene by Deactivated Imino-Based Catalysts. *Org. Process Res. Dev.* 14, 1501-1505 (**2010**). Please read for further safety instruction before attempting this or any other reaction with phosgene.

Phosgene is an extremely toxic gas, do not attempt any reactions using this reagent without proper safety, consultation, supervision and preparation.

A two necked flask containing the triphosgene and phenanthridine was connected to a distillation head and condenser. The receiving end of the condenser was attached to an ammonia trap and cooled by a dry ice/acetone bath to condense the phosgene that was produced. The bottom of the ammonia trap was connected to a three necked round bottom flask with a stir bar which was also cooled by a dry ice/acetone bath. All open necks of round bottoms were sealed by fresh septa wrapped with Teflon tape. One neck of the three necked flask was punctured with a 20G needle attached to a tygon tube and two bubblers in series; the first was empty and the second contained mineral oil. Tygon tubing was used to connect the second bubbler to a 9-inch 20G needle that was fully inserted into a saturated solution of sodium hydroxide in methanol. A second needle and tube was inserted into the septa of the methanolic sodium hydroxide, which acted as a vent up into the fume hood. Triphosgene (1.87 g, 6.33 mmol) and cat. phenanthridine were heated to 90°C, at which point the triphosgene melted and dissolved the phenanthridine catalyst, promoting the evolution of phosgene gas. After 30 min, all triphosgene was consumed and phosgene had begun condensing in the receiving flask. At this point a balloon of argon was punctured through the septa on the two necked flask, pushing any phosgene gas to the condenser and quenching solution. Once phosgene had stopped condensing, an acetonitrile solution of **(12)** (1.06 g, 1.9 mmol) was added dropwise to the stirring phosgene, then removed from the dry ice/acetone bath after 10 min. The reaction was stirred

for 2 h at room temperature. Next, argon gas was passed over the whole apparatus and also bubbled through the reaction mixture into the methanolic sodium hydroxide to remove and quench the excess phosgene. NOTE: a very low flow from a balloon was used at first to ensure the phosgene was quenched. Once all phosgene was removed, DIPEA (4 mL) was added to the mixture to quench the HCl produced in the reaction with phosgene and compound **(12)**.

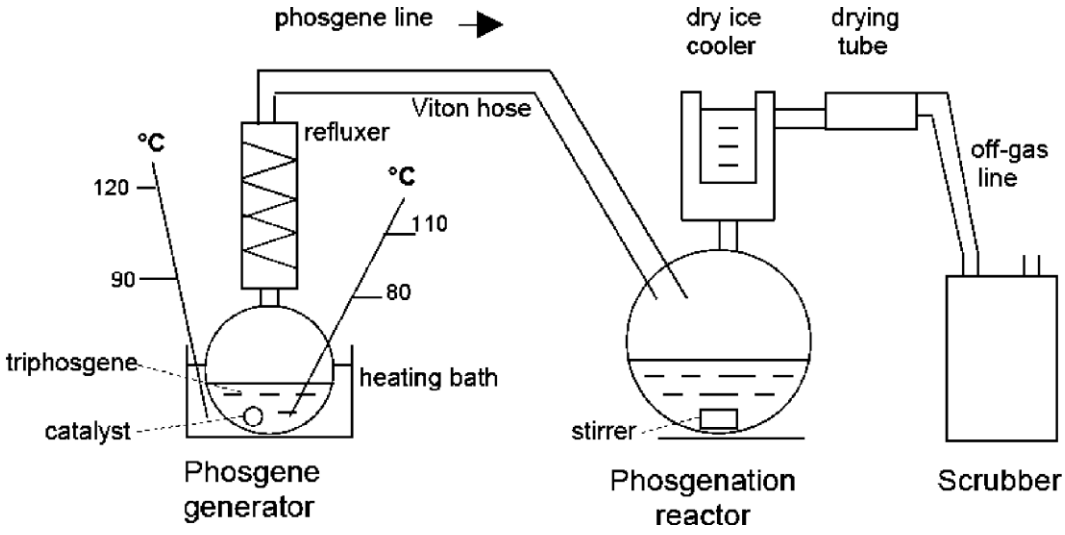


Figure 5.1. Schematic for the generation of safe phosgene using cat. Phenanthridine.

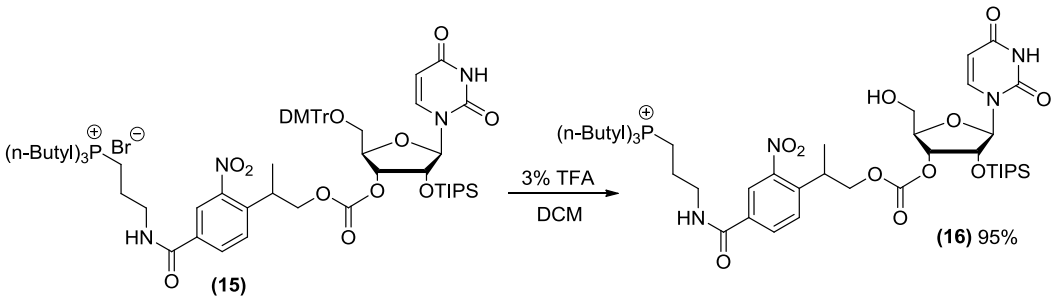
A solution of nucleoside **(13)** (1 eq, 1.35 g, 1.9 mmol) in ACN (3 mL) was added directly to the above mixture, and the resulting solution allowed to stir for 6 h at room temperature. The solution was diluted with ethyl acetate and extracted with sat. $NaHCO_3$ (x3) and once with brine. The mixture was precipitated in 300 mL of MTBE to remove excess nucleoside and DIPEA. The resulting precipitate was then purified by column chromatography (DCM:MeOH 100:0 → 90:10) to afford 1.32 g of **(15)** (62% yield).

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.85 (t, *J*=7.09 Hz, 8 H) 0.96 (t, *J*=6.85 Hz, 12 H) 1.01 (br. s., 4 H) 1.31 (dd, *J*=6.85, 2.45 Hz, 4 H) 1.34 - 1.54 (m, 10 H) 1.95 - 2.08 (m, 2 H) 2.08 - 2.27 (m, 11 H) 2.59 (s, 1 H) 2.62 - 2.83 (m, 2 H) 3.31 - 3.52 (m, 2 H) 3.54 - 3.70 (m, 3 H) 3.74 (s, 5 H) 4.11 - 4.33 (m, 3 H) 4.62 (d, *J*=2.45 Hz, 2 H) 5.15 - 5.29 (m, 2 H) 5.98 (dd, *J*=5.26, 3.06 Hz, 1 H) 6.70 - 6.85 (m, 3 H) 7.14 - 7.36 (m, 7 H) 7.53 (t, *J*=7.70 Hz, 1 H) 7.85 (dd, *J*=8.07, 2.20 Hz, 1 H) 8.54

(dd, *J*=14.43, 1.71 Hz, 1 H) 8.72 (br. s., 1 H) 9.26 (d, *J*=9.29 Hz, 1 H) 9.73 (br. s., 2 H)

^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 12.11, 13.31, 16.72, 17.11, 17.61, 17.72, 18.56, 18.94, 21.08, 23.52, 23.83, 23.95, 29.24, 30.91, 33.40, 39.94, 55.21, 62.60, 71.70, 74.42, 76.06, 76.86, 77.11, 77.37, 81.21, 87.50, 88.21, 102.47, 113.28, 124.29, 127.21, 128.01, 128.57, 130.02, 130.10, 132.08, 133.70, 134.72, 134.93, 139.06, 140.18, 144.03, 149.87, 150.04, 150.33, 154.27, 158.69, 163.09, 165.10, 207.05.

$C_{65}H_{92}N_4O_{13}PSi^{1+}$ low resolution ESI-MS calculated: 1195.61, found: 1195.60.



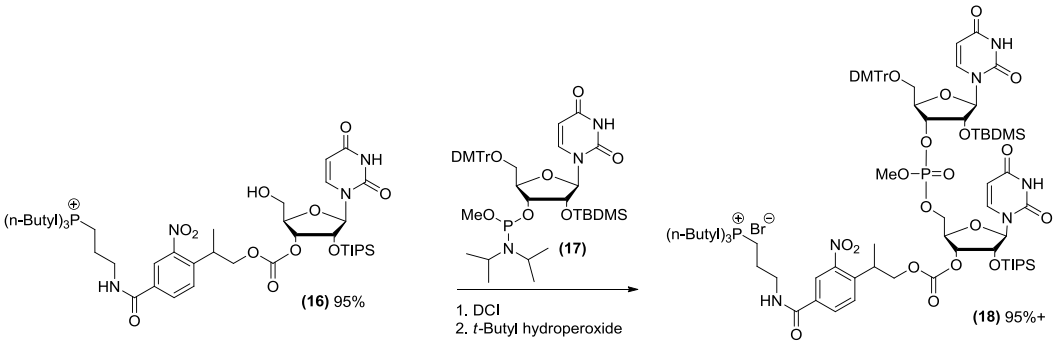
5′-OH-2′-TIPS-3′-(tributyl(3-(4-(1-hydroxypropan-2-yl)-3-nitrobenzamido) propyl)phosphonium bromide)-uridine (16)

3′-Tagged-uridine **(15)** (0.49 g, 0.38 mmol) was dissolved in 3% TFA in DCM and allowed to stir for 5 min before adding methanol to quench the trityl cation. The reaction was then concentrated to an oil taken up in minimal amounts of DCM and precipitated in MTBE to remove dimethoxytritanol. The compound was filtered over Celite®, collected in DCM and purified by column chromatography (DCM:MeOH, 100:0 →90:10%). Isolated yield of (**16):** 0.37 g (95%).

^{1}H NMR (500 MHz, CD_3CN) δ ppm 0.87 - 1.12 (m, 15 H) 1.38 (dd, *J*=6.97, 3.55 Hz, 1 H) 1.42 - 1.61 (m, 6 H) 1.96 (dt, *J*=4.89, 2.45 Hz, 1 H) 1.98 - 2.07 (m, 1 H) 2.07 - 2.17 (m, 3 H) 2.22 - 2.33 (m, 1 H) 3.49 (br. s., 1 H) 3.66 - 3.79 (m, 1 H) 4.13 (dd, *J*=13.45, 1.96 Hz, 1 H) 4.30 - 4.46 (m, 2 H) 4.57 - 4.66 (m, 1 H) 5.03 (ddd, *J*=10.82, 5.07, 1.71 Hz, 1 H) 5.70 (d, *J*=8.07 Hz, 1 H) 5.85 (dd, *J*=6.97, 4.28 Hz, 1 H) 7.73 - 7.83 (m, 1 H) 8.27 (d, *J*=8.31 Hz, 1 H) 8.39 (t, *J*=1.71 Hz, 1 H)

9.17 (br. s., 1 H) ^{13}C NMR (126 MHz, CD_3CN) δ ppm -0.16, 0.17, 0.34, 0.36, 0.50, 0.52, 0.54, 0.67, 0.83, 11.91, 11.93, 12.57, 15.13, 15.52, 16.93, 16.99, 17.00, 17.01, 17.11, 17.69, 18.07, 20.64, 20.67, 22.84, 22.88, 23.45, 23.57, 33.49, 33.64, 61.34, 61.37, 64.62, 64.76, 71.11, 71.31, 73.49, 76.99, 77.10, 82.99, 87.73, 87.76, 102.64, 102.66, 117.32, 124.83, 124.86, 129.10, 129.32, 129.84, 129.85, 133.06, 133.08, 140.43, 140.46, 141.68, 141.82, 150.41, 150.53, 150.81, 150.83, 154.10, 154.15, 162.65, 164.03, 164.04.

$C_{44}H_{74}N_4O_{11}PSi^{1+}$ low resolution ESI-MS calculated: 893.48, found: 893.50.



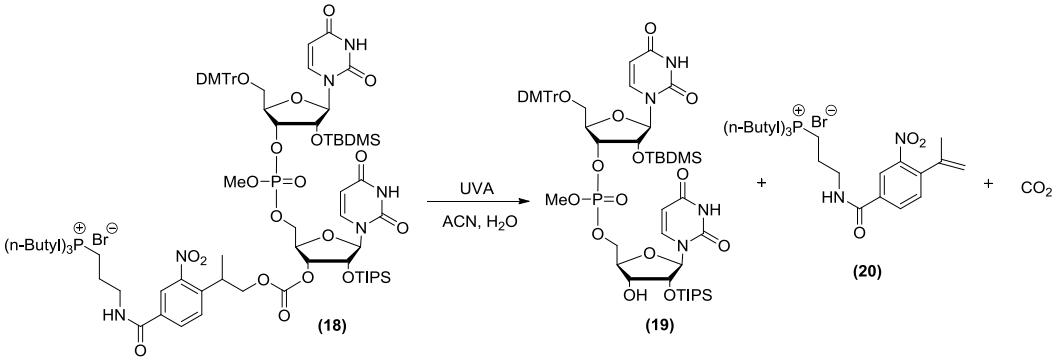
[5′-DMTr-2′-TBDMS-rU]-[3′-p(OMe)-5′]-[2′-TIPS-3′- (tributyl (3-(4-(1-hydroxypropan-2-yl)-3-nitrobenzamido) propyl)phosphonium bromide)] uridine (18)

Compound **(16)** (316 mg, 0.29 mmol) was treated with 10 mL of 3% TFA in DCM followed by 0.5 mL of triethylsilane. This mixture was allowed to stir for 10 min followed by 5 mL of MeOH to further quench the trityl cation. This mixture was then diluted with 20 mL of toluene and concentrated to near dryness. The mixture was then taken up in acetone and precipitated in MTBE, filtered and dissolved in DCM then condensed to dryness. The above process was repeated once more to ensure there was no tritylated material. The mixture was then co-evaporated with toluene to dry. To this mixture, 1.5eq of DCI was added (52 mg, 0.44 mmol) and 1.5eq of phosphoramidite **(17)** (362 mg, 0.44 mmol) was added and finally dissolved with 3 mL of ACN. This mixture was allowed to stir for 4 h until all starting material was consumed as monitored by MS. The mixture was then treated with 1 mL of *tert*-butanol to react with any excess phosphoramidite. This mixture was directly precipitated in MTBE:hexanes (75:25) to remove all

excess reagents. The precipitate was dissolved in DCM and treated with 1 mL of *tert*-butylhydroperoxide in decane and allowed to stir for 20 min until all starting material was oxidized, as monitored by MS. This mixture was then precipitated in MTBE producing compound pure **(18)** in 91% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.07 (br. s., 6 H) 0.81 - 1.03 (m, 33 H) 1.31 - 1.57 (m, 16 H) 1.98 (s, 1 H) 2.04 - 2.24 (m, 8 H) 2.37 (d, *J*=13.29 Hz, 1 H) 3.42 - 3.48 (m, 1 H) 3.55 - 3.67 (m, 3 H) 3.68 - 3.78 (m, 8 H) 4.17 - 4.24 (m, 1 H) 4.24 - 4.34 (m, 2 H) 4.47 (br. s., 2 H) 4.88 (br. s., 1 H) 4.93 - 5.06 (m, 1 H) 5.13 - 5.23 (m, 1 H) 5.69 (dd, *J*=8.21, 3.13 Hz, 1 H) 5.84 (s, 1 H) 5.90 - 6.02 (m, 1 H) 6.77 - 6.84 (m, 3 H) 7.18 - 7.34 (m, 8 H) 7.57 (d, *J*=6.64 Hz, 2 H) 8.16 - 8.19 (m, 1 H) 8.35 - 8.40 (m, 1 H) 9.29 (br. s., 1 H)

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm -0.91 (d, *J*=1.24 Hz, 1 P) -0.34 (d, *J*=4.32 Hz, 1 P) 34.08 (s, 1 P) 34.20 (s, 1 P)



[5′-DMTr-2′-TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′-TIPS] (19)

Compound **(18)** was dissolved in wet ACN (3200ppm) to a final concentration of 0.01M in a 50 mL Pyrex® round bottom flask treated with UVA for 40 min. The mixture was concentrated to dryness, re-suspended in minimal acetone and precipitated into 200 mL of MTBE. The MTBE was filtered over Celite®, leaving the cleaved ionic tag as a viscous oil on the filter. The MTBE was concentrated to dryness, leaving the cleaved dimer (19) and residual ionic tag (20). A short silica column (0 → 75% ethyl acetate/hexanes) was performed to remove the excess tag, producing pure compound (47) in 86% yield after purification by column chromatography

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.09 - 0.15 (m, 6 H) 0.87 - 1.20 (m, 40 H) 1.94 - 2.00 (m, 1 H) 2.10 - 2.20 (m, 2 H) 3.44 (br. s., 3 H) 3.67 - 3.79 (m, 12 H) 4.04 - 4.23 (m, 5 H) 4.28 - 4.35 (m, 2 H) 4.38 - 4.48 (m, 2 H) 4.81 - 4.90 (m, 1 H) 5.38 (d, *J*=8.21 Hz, 1 H) 5.64 (d, *J*=8.20 Hz, 1 H) 5.81 - 5.91 (m, 2 H) 6.90 (d, *J*=7.91 Hz, 5 H) 7.24 - 7.37 (m, 9 H) 7.41 - 7.52 (m, 4 H) 7.71 (dd, *J*=8.20, 2.64 Hz, 1 H) 9.62 (br. s., 1 H)

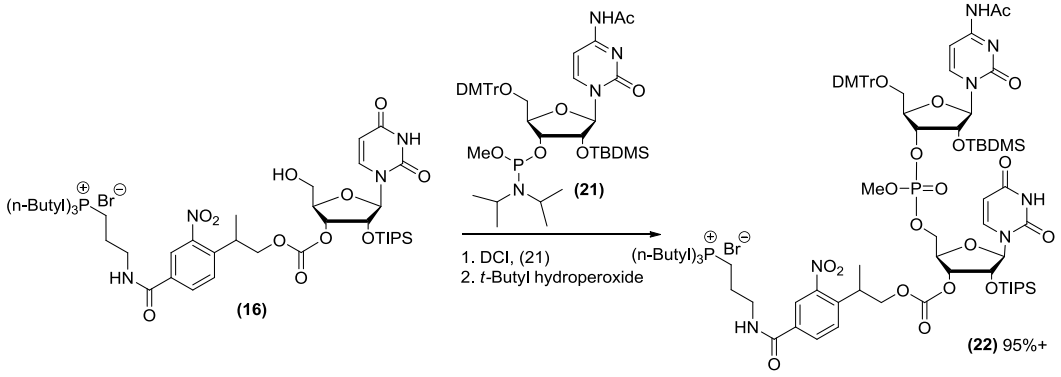
^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 0.58 (s, 1 P) 0.83 (s, 1 P).

tributyl(3-(3-nitro-4-(prop-1-en-2-yl)benzamido)propyl)phosphonium bromide (20)

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.96 (t, *J*=6.84 Hz, 22 H) 1.41 - 1.60 (m, 24 H) 2.01 - 2.25 (m, 11 H) 2.39 - 2.54 (m, 9 H) 2.69 - 2.87 (m, 3 H) 3.66 (br. s., 1 H) 3.77 (br. s., 2 H) 4.51 (t, *J*=6.06 Hz, 2 H) 4.97 (s, 2 H) 5.23 (s, 2 H) 7.47 (dd, *J*=12.11, 7.82 Hz, 2 H) 8.31 (dd, *J*=8.01, 1.76 Hz, 1 H) 8.44 (d, *J*=1.56 Hz, 1 H)

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 33.63 - 33.92 (m, 1 P)

$C_{25}H_{42}N_2O_3P^+$ low resolution ESI-MS calculated: 449.29, found: 449.30.

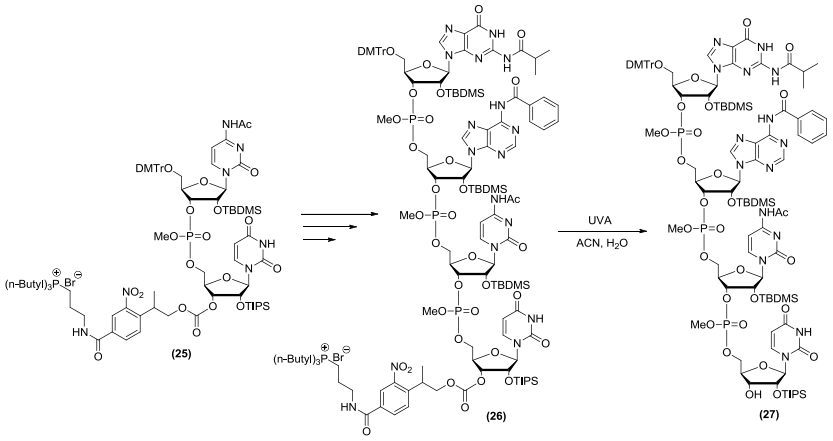


[5′-DMTr-2′-TBDMS-rC^{NHAc}]-[3′-p(OMe)-5′]-[2′-TIPS-3′- (tributyl (3-(4-(1-hydroxypropan-2-yl)-3-nitrobenzamido) propyl) phosphonium bromide)]uridine (22)

Compound **(16)** (0.37 g, 0.38mmol) was dried by two co-evaporations of dry toluene:DCM on a rotovap before placed under high vacuum. K3 was dissolved in 15 ml of ACN containing 2 eq of DCI (0.982 g, 8.32 mmol). Immediately after, phosphoramidite **(17)** (0.51 g 0.57 mmol) was added, and the

resulting solution was allowed to stir at room temperature for 3 h. MS analysis indicated that the reaction was complete (no starting material K3 present). Ten eq of *tert*-butanol was added to quench excess phosphoramidite, followed by 10 eq of *tert*-butyl hydroperoxide (1 mL of a 6 M solution in decane), and the resulting mixture allowed to stir for 20 min until all the phosphite triester was converted to the phosphate, as monitored by MS. The reaction was then concentrated to an oil, taken up in minimal amounts of DCM, and precipitated in MTBE to remove all excess reagents. The precipitation process was repeated if the presence of any quenched phosphoramidite was detected by TLC. Tagged dimer **(18)** was isolated in 95% yield (0.63 g).

$C_{63}H_{122}N_7O_{21}P_2Si_2^{1+}$ low resolution ESI-MS calculated: 1670.77, found: 1670.73.



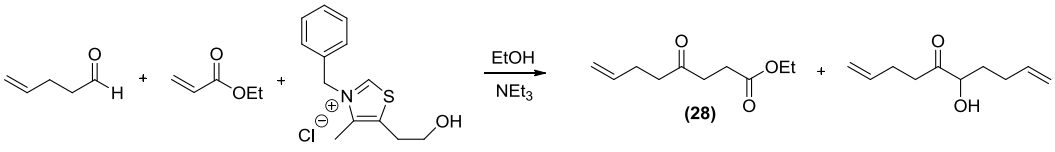
The above process described for compound **(22)** was repeated as described above, using the appropriate phosphoramidites till tetramer (**26)** was obtained. Unfortunately, the yield loss per step was unacceptable due to premature NPPOC removal, never the less; the process was continued and characterized by MS at each point during the synthesis. While we were able to actually detect the presence of the full length material by MS if we looked specifically for it, the intensity of the cleaved linker was more than 500x more intense.

Step	Compound 5′→3′	Mass cal. (m/z)	Mass found (m/z)
1	DMTr-rCU-tag (a)	1654.77	1654.71
2	DMTr-rCU-tag (b) (90)	1670.77	1670.73
3	HO-rCU-tag	1368.63	1368.59
4	DMTr-rACU-tag (a)	2215.95	1119.46 $(M^+ + Na^+)/2$
5	DMTr-rACU-tag (b)	2231.95	1127.44 $(M^+ + Na^+)/2$

6	HO-rACU-tag	1929.82	1929.80
7	DMTr-rGACU-tag (a)	2645.06	1334.11 ($M^+ + Na^+$)/2
8	DMTr-rGACU-tag (b)(91)	2661.05	1341.94 ($M^+ + Na^+$)/2

Table 1. Step wise characterization of the phosphonium tagged NPPOC linker couplings up to the tetramer 5′-(rGrArCrU)-3′. Tag = light labile phosphonium tag (a) = phosphite triester; (b) = phosphate triester.

EXPERIMENTAL FOR LEV LIKE LINKER



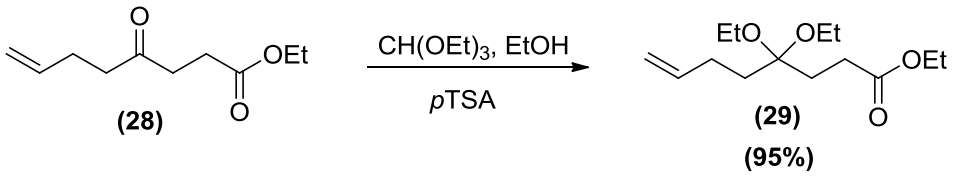
Ethyl 4-oxooct-7-enoate (28)

4-Pentenal (3.8 g, 45 mmol) was mixed with 3-benzyl-5-(2-hydroxyethyl)-4-methylthiazolium chloride (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 **(28)** (3.96 g, 25% yield) as a pale yellow oil.

^{1}H NMR (300MHz, CD_3CN) δ ppm 5.99 - 5.73 (m, 1H, with acyloin impurity), 5.15 - 5.05 (m, 2 H), 5.04 - 4.93 (acyloin impurity), 4.15 - 4.06 (q, *J* = 7.0 Hz, 2 H), 2.76 - 2.67 (m, 2 H, with acyloin impurity), 2.67 - 2.60 (acyloin impurity), 2.55 - 2.48 (m, 2H, with acyloin impurity), 2.35 - 2.26 (m, 2 H), 2.20 - 2.17

(acyloin impurity), 2.16 - 2.12 (m, 2 H), 1.99 - 1.94 (acyloin impurity), 1.21 (t, *J* = 7.0 Hz, 3 H).

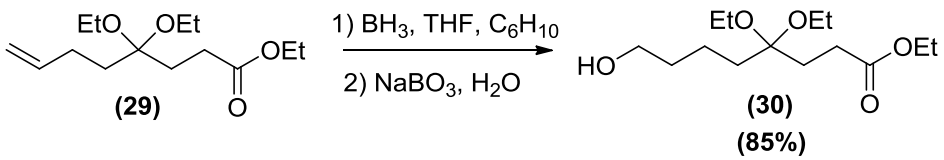
$C_{10}H_{16}O_3Na^{1+}$ low resolution ESI-MS calculated: 207.10, found: 207.31.



Ethyl 4,4-diethoxyoct-7-enoate (29)

Compound **(28)** (2.0 g,) 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 **(29)** (1.36 g, 92% yield) as a colorless oil. ^{1}H NMR (500MHz, DMSO-d_6) δ ppm 5.85 - 5.73 (m, 1 H), 5.05 - 4.88 (m, 2 H), 4.03 (q, *J* = 7.2 Hz, 2 H), 3.32 (q, *J* = 7.0 Hz, 4 H), 2.20 (t, *J* = 7.8 Hz, 2 H), 1.97 - 1.88 (m, 2 H), 1.79 (t, *J* = 7.8 Hz, 2 H), 1.53 (t, *J* = 8.4 Hz, 2 H), 1.16 (t, *J* = 7.2 Hz, 3 H), 1.06 (t, *J* = 7.0 Hz, 6 H).

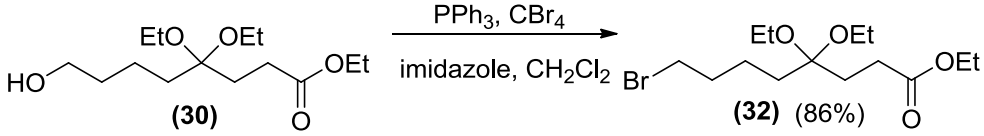
^{13}C NMR (75MHz, CD_3CN) δ ppm 173.0, 138.5, 113.8, 102.0, 60.1, 55.0, 32.3, 28.7, 28.3, 27.8, 14.6, 13.6, $C_{14}H_{26}O_4Na^{1+}$ low resolution ESI-MS calculated: 281.17, found: 281.13.



Ethyl 4,4-diethoxy-8-hydroxyoctanoate (30)

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 **(29)** (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 combined 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 **(30)** (0.45 g, 81% yield) as a colorless oil. Starting material **(29)** was also recovered. ^{1}H NMR (300MHz, DMSO-d_6) δ ppm 4.33 (t, J = 5.2 Hz, 1 H), 4.02 (q, J = 7.1 Hz, 2 H), 3.36 (m, 2 H), 3.31 (q, J = 7.3 Hz, 4 H), 2.18 (t, J = 7.7 Hz, 2 H), 1.76 (t, J = 7.7 Hz, 2 H), 1.46 - 1.41 (m, 2 H), 1.40 - 1.31 (m, 2 H), 1.25 - 1.19 (m, 2 H), 1.16 (t, J = 7.1 Hz, 3 H), 1.05 (t, J = 7.3 Hz, 6 H), ^{13}C NMR (75MHz, DMSO-d_6) δ ppm 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.



(30) (32) (86%)

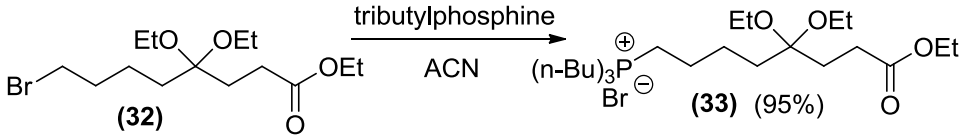
Ethyl 8-bromo-4,4-diethoxyoctanoate (32)

Compound **(30)** (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 **(32)** (0.49 g, 81% yield) as a colorless oil. ^{1}H NMR (400MHz, DMSO-d_6) δ ppm 4.04 (q, J = 7.1

Hz, 2 H), 3.54 (t, *J* = 6.5 Hz, 2 H), 3.32 (q, *J* = 6.9 Hz, 4 H), 2.22 (t, *J* = 7.8 Hz, 2 H), 1.83 - 1.74 (m, 4 H), 1.47 (t, *J* = 7.2 Hz, 2 H), 1.35 - 1.25 (m, 2 H), 1.18 (t, *J* = 7.1 Hz, 3 H), 1.07 (t, *J* = 6.9 Hz),

^{13}C NMR (126MHz ,CD_3CN) δ ppm 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.

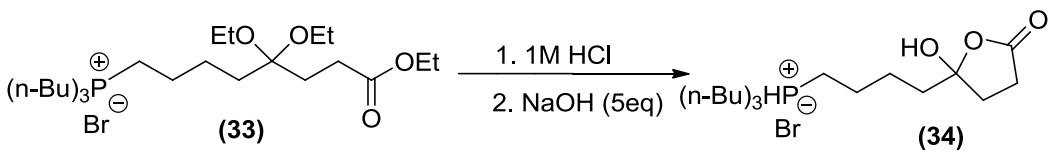


tributyl(5,5,8-triethoxy-8-oxooctyl)phosphonium bromide (33)

Alkyl-bromide **(32)** (3.8g, 11.2 mmol) was dissolved in 22.4 mL of dry ACN to which was added to 1.5eq of tributylphosphine (3.4 g, 16.8 mmol) and was stirred at 50°C for 8h. The reaction mixture was then concentrated to dryness on rotovop then precipitated in hexanes to afford pure phosphonium tagged species (33) in 93% yield, 5.64 g.

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.76 - 0.91 (m, 16 H) 0.95 - 1.07 (m, 10 H) 1.08 - 1.19 (m, 6 H) 1.27 - 1.58 (m, 33 H) 1.71 - 1.89 (m, 3 H) 2.07 - 2.21 (m, 4 H) 2.22 - 2.49 (m, 15 H) 3.18 - 3.39 (m, 8 H) 3.88 - 4.08 (m, 2 H)

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 49.63 (s, 1 P)

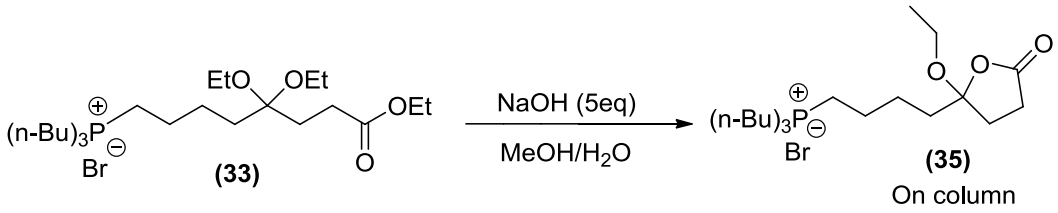


tributyl(4-(2-hydroxy-5-oxotetrahydrofuran-2-yl)butyl)phosphonium bromide (34)

Compound **(33)** (0.2 g, 0.37 mmol) was dissolved in minimal methanol approx 3 mL to which was added 3 mL of 5M HCl and was allowed to stir for 20 min, at which point the reaction was observed to be complete by TLC. The reaction was then neutralized by the addition of 3 mL of 5M NaOH, then approximately 5eq (75 mg, 1.9 mmol) of extra NaOH was added and allowed to

stir for an additional 30min. The reaction was monitored by MS, showing complete consumption of starting material. The reaction was then neutralized with 1M HCl, taken up in ethyl acetate and shaken acidified brine to remove all excess salts, and twice with brine to remove any excess HCl. The ethyl acetate was dried with magnesium sulfate and concentrated to dryness. Yielding compound **(34)** in 88% yield, 176 mg.

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.78 - 0.97 (m, 9 H) 1.45 (br. s., 17 H) 1.87 (br. s., 1 H) 1.97 - 2.07 (m, 1 H) 2.13 (m, *J*=9.38, 6.74 Hz, 1 H) 2.28 (br. s., 8 H) 2.38 - 2.72 (m, 2 H) 3.40 - 3.57 (m, 1 H)

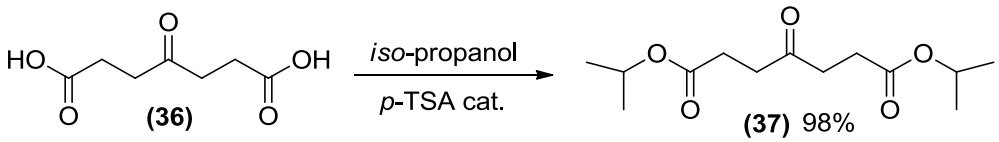


tributyl(4-(2-ethoxy-5-oxotetrahydrofuran-2-yl)butyl)phosphonium bromide (35)

Compound **(33)** (0.25 g, 0.46 mmol) was dissolved in minimal amount of methanol, 1.5 mL, and treated with approximately 5eq (80 mg, 2.1 mmol) of NaOH and allowed to stir for 1h at room temperature. The reaction mixture was neutralized to pH 8 then concentrated to dryness. The mixture was then passed through a silica column with 1% acetic acid and 10% MeOH- DCM to remove all salts and generate the acid form of the product. Yielding 74% of compound **(35)**

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.78 - 0.97 (m, 9 H) 1.07 (t, *J*=7.00 Hz, 4 H) 1.45 (br. s., 17 H) 1.87 (br. s., 1 H) 1.97 - 2.07 (m, 1 H) 2.13 (m, *J*=9.38, 6.74 Hz, 1 H) 2.28 (br. s., 8 H) 2.38 - 2.72 (m, 2 H) 3.23 - 3.39 (m, 2 H) 3.40 - 3.57 (m, 1 H)

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 13.41, 15.28, 18.38, 19.02, 23.55, 23.61, 23.69, 23.77, 23.97, 28.70, 31.54, 32.59, 35.21, 42.36, 55.17, 57.98, 102.33, 110.85, 176.42, 177.15.

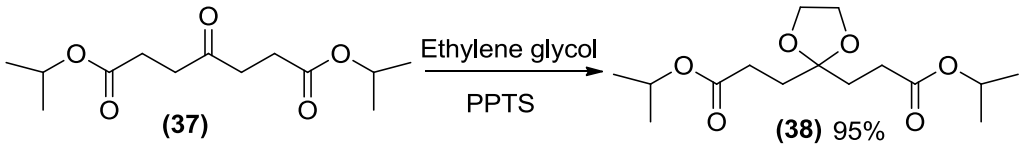


Diisopropyl 4-oxoheptanedioate (37)

4-ketopemilic acid **(36)** (10 g, 57.4 mmol; purchased from Acrose) was suspended in 50 mL of isopropanol and 50 mL of benzene. Catalytic amount of *p*-toluene solfonic acid was added to the mixture and brought to reflux using a Dean Stark trap to remove the water produced. Once the volume had decreased to approximately 50 mL in the flask, another 50 mL of 50:50 Benzene:*iso*-propanol was added and further reduced to approximately 30 ml. The mixture was then taken up in ethyl acetate and extracted with $NaHCO_3$ (x3) and once with brine. The organic layer was dried with $MgSO_4$ and condensed to dryness yielding pure **(19):** 14.2 g (95%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.06 (d, *J*=6.45 Hz, 12 H) 2.39 (t, *J*=7.00 Hz, 1 H) 2.60 (t, *J*=6.70 Hz, 4 H) 4.72 - 4.89 (m, 2 H) ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 21.57, 28.13 (s, 3 C) 28.13 (s, 3 C) 36.92 (s, 3 C) 67.66, 171.90 (s, 2 C) 206.82.

$C_{13}H_{22}O_5Na^{1+}$ low resolution ESI-MS calculated: 258.14, found: 281.21.



Diisopropyl 3,3'-(1,3-dioxolane-2,2-diyl)dipropanoate (38)

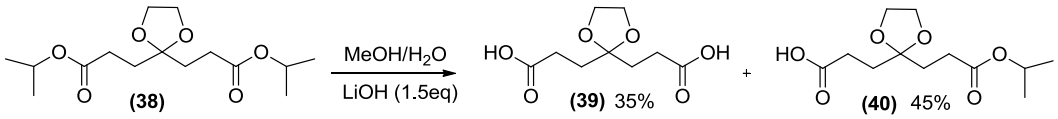
Compound **(37)** (0.55 g, 2.1 mmol) was dissolved with 5eq of ethylene glycol (0.58 mL, 10.5 mmol), 90 mL of dry toluene and catalytic amount of pyridinium *para*-toluene sulfonate. This mixture was refluxed at 140°C replacing

the toluene 3 times and finally allowing the reaction to reflux overnight. The mixture was then distilled to approximately 30 mL, removed from heat and diluted with DCM and extracted with sat. $NaHCO_3$ (x2) then water (x3) to remove any excess ethylene glycol. The product was purified by column chromatography (DCM:MeOH, 100:0 → 95:5). Isolated yield: 0.41 g (65%).

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 1.05 (d, *J*=6.36 Hz, 12 H) 1.78 (t, *J*=7.58 Hz, 15 H) 2.16 (t, *J*=7.58 Hz, 15 H) 3.76 (s, 15 H) 4.82 (dt, *J*=12.53, 6.33 Hz, 8 H)

^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 21.59, 29.02, 32.07, 64.94, 67.23, 67.26, 109.84, 172.56.

$C_{15}H_{26}O_6Na^{1+}$ low resolution ESI-MS calculated: 302.17, found: 325.0.



3-(2-(3-hydroxy-3-oxopropyl)-1,3-dioxolan-2-yl)propanoic acid (21) and 3-(2-(3-isopropoxy-3-oxopropyl)-1,3-dioxolan-2-yl)propanoic acid (22).

Compound **(38)** (2.1 g, 6.9 mmol) was dissolved in 20 mL of MeOH to which was added 1.5 eq of LiOH (0.51 g, 21.5 mmol) in 5 mL of water. This mixture was allowed to stir for 2h until all starting material was consumed. The solution was brought to neutrality by the addition of 1 M HCl. This mixture was purified by column chromatography (DCM:MeOH with 1% AcOH, 100:0 → 90:10). Isolated yield of **(39):** 0.53 g (35%). Yield of **(40):** 0.81 g (45%).

(39)

^{1}H NMR (300 MHz, METHANOL-d_4) δ ppm 1.94 (t, *J*=8.20 Hz, 4 H) 2.33 (t, *J*=7.30 Hz, 15 H) 3.94 (s, 26 H)

^{13}C NMR (75 MHz, METHANOL-d_4) δ ppm 28.19, 31.82, 64.77, 109.81, 175.90,

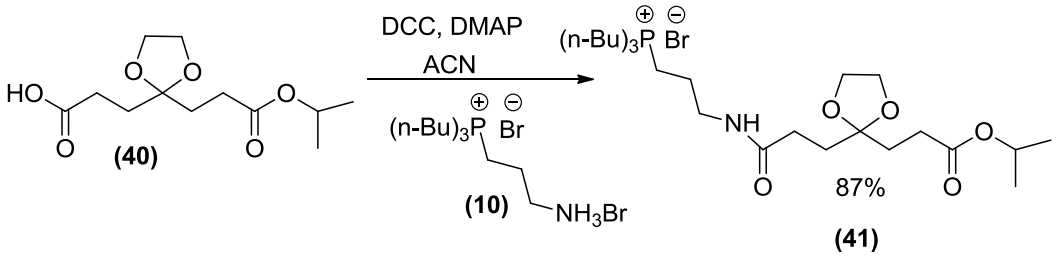
$C_9H_{14}O_6Li^{1-}$ low resolution ESI-MS calculated: 218.07, found: 224.12.

(40)

^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm 1.22 (d, *J*=6.36 Hz, 6 H) 1.87 - 2.03 (m, 4 H) 2.23 - 2.34 (m, 4 H) 3.55 (m, *J*=5.14 Hz, 3 H) 3.67 (m, *J*=5.14 Hz, 3 H) 4.89 - 5.00 (m, 2 H)

^{13}C NMR (126 MHz, METHANOL-d_4) δ ppm 7.75, 20.72, 28.80, 31.81, 60.87, 62.94, 67.60, 72.13, 109.92, 173.40, 176.73.

$C_{12}H_{20}O_6^{1-}$ low resolution ESI-MS calculated: 260.12, found: 259.03.

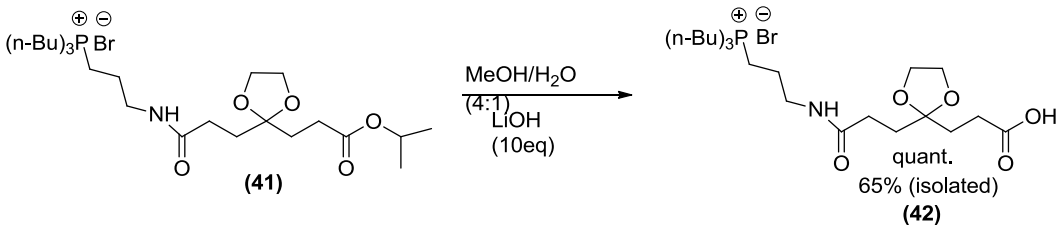


Tributyl(3-(3-(2-(3-isopropoxy-3-oxopropyl)-1,3-dioxolan-2-yl)propanamido) propyl)phosphonium bromide (41)

Compound **(40)** (0.3 g, 1.1 mmol) was dissolved in 1.5 mL of ACN followed by TBTU (0.39 g, 1.2 mmol), 2.5 eq of triethylamine (0.38 ml) and phosphonium ionic tag **(10)** (0.45 g, 1,2 mmol). This mixture was allowed to stir for 4h until the starting material **(40)** was completely consumed. The reaction mixture was diluted with ethyl acetate and extracted with 5% $NaHCO_3$ x2 and once with brine. The organic layer was dried and concentrated and purified by column chromatography. DCM:MeOH 100:0 → 95:5. Isolated yield: 0.54 g (84%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.91 (t, *J*=6.74 Hz, 9 H) 1.06 (d, *J*=6.45 Hz, 6 H) 1.25 (s, 6 H) 1.38 - 1.59 (m, 11 H) 1.80 – 1.95 (m, 7 H) 2.15 - 2.30 (m, 9 H) 2.18 (t, *J*=7.03 Hz, 8 H) 3.28 - 3.44 (m, 2 H) 3.84 (br. s., 5 H) 3.94 (s, 6 H) 4.72 - 4.89 (m, 1 H).

$C_{27}H_{53}NO_6P^{1+}$ low resolution ESI-MS calculated: 502.36, found: 502.36.

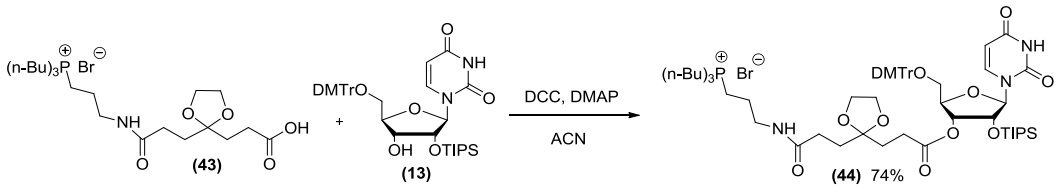


Tributyl(3-(3-(2-(3-hydroxy-3-oxopropyl)-1,3-dioxolan-2-yl)propanamido) propyl)phosphonium bromide (42)

Compound **(41)** (0.25 g, 0.4 mmol) was dissolved in 2.5 mL of MeOH to which was added 10 eq of LiOH (0.1 g, 4 mmol) in mL of water. This mixture was allowed to stir for 3h until all starting material was consumed. The solution was brought to neutrality by the addition of 1M HCl in MeOH. This mixture was purified by column chromatography (DCM:MeOH with 1% AcOH, 100:0 → 90:10). Isolated yield of **(42):** 0.20 g (95%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.91 (t, *J*=6.74 Hz, 9 H) 1.25 (s, 6 H) 1.38 - 1.59 (m, 11 H) 1.96 - 2.05 (m, 7 H) 2.15 - 2.30 (m, 9 H) 2.40 (t, *J*=7.03 Hz, 8 H) 3.28 - 3.44 (m, 2 H) 3.84 (br. s., 5 H) 3.94 (s, 6 H).

$C_{24}H_{47}NO_5P^{1+}$ low resolution ESI-MS calculated: 460.31, found: 460.30.



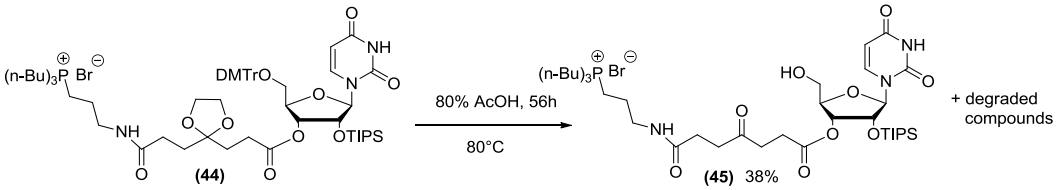
5′-DMTr-2′-TIPS-3′-[tributyl(3-(3-(2-(2-carboxyethyl)-1,3-dioxolan-2-yl)propanamido)propyl)phosphonium bromide] (44)

To a solution of compound **(43)** (0.2 g, 0.37 mmol) in ACN (1 mL) was added TBTU (0.19 g, 0.6 mmol), triethylamine (0.5 mL) and compound **(13)** (0.42 g, 0.6 mmol). The resulting mixture was allowed to stir for 12 h until the starting material **(43)** was completely consumed. The reaction mixture was diluted with ethyl acetate and extracted with 5% $NaHCO_3$ x2 and once with brine. The organic layer was dried and concentrated, taken up in minimal amounts of DCM at precipitated in 100 ml of MTBE, filtered over Celite®. Isolated yield of **(44):** 0.20g (45%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.91 (t, *J*=6.74 Hz, 9 H)1.02 - 1.59 (m, 38 H) 1.96 - 2.05 (m, 7 H) 2.15 - 2.30 (m, 9 H) 2.40 (t, *J*=7.03 Hz, 8 H) 3.37 - 3.40 (m, 4 H) 3.78 – 3.94 (br. m, 17 H) 4.15 (d, *J*=2.77 Hz, 1 H) 4.63 - 4.67 (m, 1 H) 5.31 (dd, *J*=5.14, 2.96 Hz, 1 H) 5.40 - 5.46 (m, 1 H) 5.42 (s, 1 H) 5.99 (d, *J*=6.32 Hz, 1

H) 6.87 - 6.93 (m, 4 H) 7.27 - 7.37 (m, 7 H) 7.41 - 7.45 (m, 2 H) 7.75 (d, *J*=8.30 Hz, 1 H).

$C_{63}H_{95}N_3O_{12}PSi^{1+}$ low resolution ESI-MS calculated: 1144.64, found: 1144.7.



2′-TIPS-3′-[(tributyl(3-(7-oxy-4,7-dioxoheptanamido)propyl)phosphonium chloride] uridine (45)

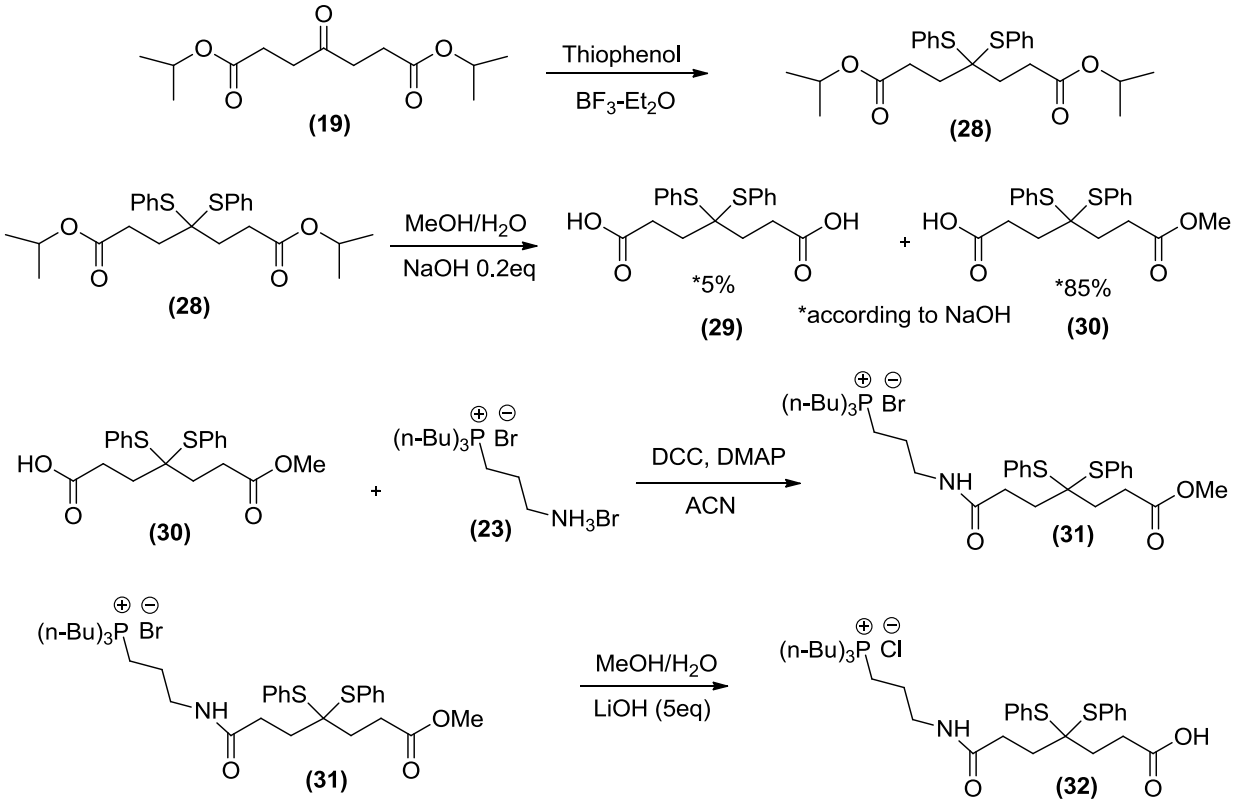
Compound **(44)** (0.228 g, 0.199 mmol) was dissolved with 15 ml of 80% acetic acid in water and first allowed to stir at room temperature for 6h showing almost no loss of the ketal, but complete loss of the trityl group. The reaction was then heated to 50°C for 12h which then showed about a 25% loss of ketal. Then the reaction was placed at 80°C for 40h which then showed almost complete consumption of starting material. The mixture was diluted with DCM and extracted with 3 portions of saturated $NaHCO_3$. The organic layers were dried with $MgSO_4$ and concentrated to dryness. The crude mixture was then dissolved in minimal amounts of DCM and precipitated in MTBE to remove trityl and some cleaved TIPS. The compound was then filtered over Celite® and collected with DCM. The resulting mixture then had to be purified by column chromatography (0-15% MeOH in DCM) affording compound 27 in moderate yield of 62%, 0.108g

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.90 - 1.13 (m, 37 H) 1.39 - 1.52 (m, 17 H) 1.78 - 1.87 (m, 2 H) 1.90 - 1.95 (m, 1 H) 2.00 - 2.03 (m, 1 H) 2.06 - 2.25 (m, 11 H) 2.27 - 2.34 (m, 5 H) 2.44 - 2.60 (m, 4 H) 2.73 - 2.80 (m, 2 H) 3.37 (d, *J*=5.47 Hz, 1 H) 3.68 - 3.92 (m, 3 H) 4.16 - 4.22 (m, 1 H) 4.64 - 4.72 (m, 1 H) 5.01 - 5.13 (m, 1 H) 5.62 - 5.72 (m, 1 H) 5.78 (dd, *J*=7.62, 4.49 Hz, 1 H) 8.07 (d, *J*=7.82 Hz, 1 H) 8.23 (d, *J*=8.21 Hz, 1 H) 8.70 - 9.11 (m, 1 H)

^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 12.05, 12.16, 13.37, 17.66, 17.67, 17.75, 17.77, 17.79, 17.85, 18.25, 18.73, 21.25, 23.44, 23.50, 23.55, 23.80, 23.94, 125.81, 128.58, 139.39, 143.40, 183.06, 207.78.

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 33.29 (s, 1 P) 33.36 (s, 1 P) 33.42 (s, 1 P) 33.51 (s, 1 P) 33.72 (s, 1 P) 33.78 (s, 1 P).

$C_40H_{73}N_3O_9PSi^{+1}$ low resolution ESI-MS calculated:798.48 , found: 798.51



diisopropyl 4,4-bis(phenylthio)heptanedioate (46)

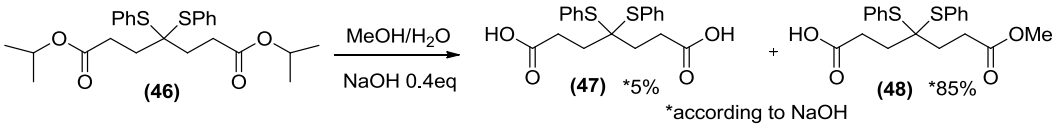
Compound **(37)** (4.69 g, 18.1 mmol) was dissolved in 36.3 mL of DCM (0.5 M) followed by 4.83 mL, 2.6eq of thiophenol (5.203 g, 47.2 mmol). This mixture was cooled down to 0°C in an ice bath then treated, drop-wise, with stirring 5.75 mL of boron trifluoride etherate ($BF_3{\cdot}O(Et)_2$) (6.44 g, 45.4 mmol). This mixture was stirred and allowed to slowly warm up to room temperature over night. After 10 hours, the mixture was cooled to 0°C and concentrated $NaHCO_3$ was added carefully to the solution quenching the Boron trifluoride followed by DCM to dilute. The mixture was extracted ×3 with saturated sodium bicarbonate, dried

with $MgSO_4$ concentrated to dryness. The compound was purified from excess thiopenol by column chromatography (0 → 20% Hexanes: ethyl acetate) yielding quantitative conversion.

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.21 (d, *J*=6.64 Hz, 12 H) 1.78 - 2.03 (m, 4 H) 2.58 - 2.84 (m, 4 H) 4.96 (dt, *J*=12.60, 6.40 Hz, 2 H) 7.30 - 7.45 (m, 6 H) 7.62 (d, *J*=6.64 Hz, 4 H).

^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 21.82, 30.20, 32.87, 67.80, 128.80, 129.38, 130.55, 136.83, 172.42.

$C_{25}H_{32}O_4S_2$ low resolution ESI-MS calculated: 460.17, found: 483.23.



4,4-bis(phenylthio)heptanedioic acid (47) and 7-isopropoxy-7-oxo-4,4-bis(phenylthio)heptanoic acid (48)

Compound **(46)** (6.43 g, 14.0 mmol) was dissolved in methanol:water 4:1 200ml and cooled to 0°C in an ice bath. 0.4 eq of NaOH (0.22g, 5.6 mmol) was dissolved in 10 mL of H_20 and added drop-wise to the above solution. This mixture was allowed to react for 12h then was concentrated to remove a significant amount of methanol, then 1M HCL was added followed by ethyl acetate and extracted ×2 with 1M HCL. The organic layer was dried with $MgSO_4$, concentrated to dryness yielding a very complex mixture of compounds. After purifying each spot by column chromatography (0 → 30% Hexanes: ethyl acetate with 1% AcOH) it was determined that not all the methanol was removed and neither was all the HCL, as no brine wash was done at the end and significant transesterification of the isopropyl ester to the methyl ester was observed. The monoester product **(48)** during this scale-up was completely converted to the methyl ester product in 85% yield.

(47)

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.45 - 2.01 (m, 4 H) 2.50 - 2.64 (m, 4 H) 7.35 - 7.49 (m, 6 H) 7.52 - 7.65 (m, 4 H) 12.19 (s, 2 H).

^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 29.62, 32.78, 38.81, 68.19, 129.54, 130.05, 130.44, 136.77, 174.01, 178.31.

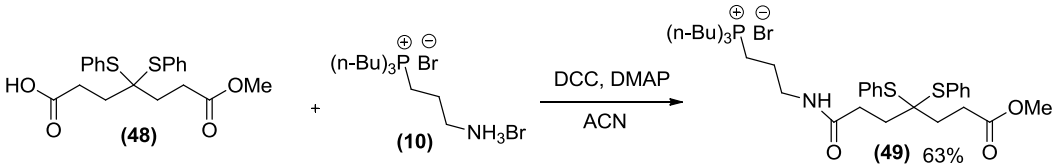
$C_{19}H_{20}O_4S_2$ low resolution ESI-MS calculated: 376.08, found: 399.21

(48)

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.82 - 2.04 (m, 4 H) 2.64 - 2.84 (m, 4 H) 3.65 (s, 3 H) 7.28 - 7.45 (m, 6 H) 7.54 - 7.67 (m, 4 H).

^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 29.61, 29.70, 32.65, 32.93, 51.86, 66.90, 128.96, 129.54, 130.30, 136.75, 173.46, 179.34, 183.06.

$C_{20}H_{22}O_4S_2$ low resolution ESI-MS calculated: 390.1, found: 413.12



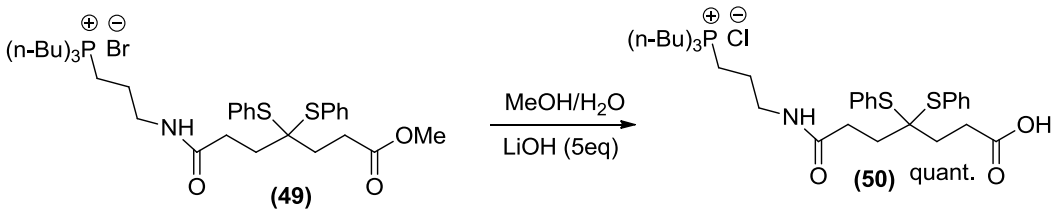
Tributyl(3-(7-methoxy-7-oxo-4,4-bis(phenylthio)heptanamido)propyl) phosphonium bromide (49)

Compound **(48)** (0.5 g, 1.28 mmol) 1.5eq of DCC (0.396 g, 1.92 mmol) were dissolved in 5 mL of ACN. Phosphonium tag **(10)** 1.15eq (0.62 g, 1.47 mmol) was dissolved in 2 mL of dry pyridine and added directly to the above solution followed by catalytic amount of DMAP. The reaction was allowed to stir for 6h until the reaction was complete by MS. The reaction was taken up in ethyl acetate and extracted ×2 with ammonium chloride and once with brine. The organic layer was dried with magnesium sulfate and concentrated to dryness. The mixture was purified by column chromatography to remove excess tag (0 → 15% MeOH:DCM) and in the process a significant amount of material was lost on the column, yielding 63%, 0.54g

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.85 - 1.05 (m, 9 H) 1.36 - 1.63 (m, 13 H) 1.80 - 2.04 (m, 7 H) 2.17 - 2.35 (m, 6 H) 2.58 - 2.81 (m, 6 H) 3.30 - 3.46 (m, 2 H) 3.60 (s, 3 H) 7.28 - 7.40 (m, 6 H) 7.59 - 7.76 (m, 4 H) 8.51 (t, *J*=5.27 Hz, 1 H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 13.44, 13.46, 16.92, 17.57, 18.59, 19.22, 21.27, 21.33, 23.63, 23.69, 23.88, 24.08, 29.66, 30.95, 31.33, 32.82, 33.40, 39.16, 39.36, 51.62, 67.92, 128.75, 129.14, 130.82, 136.91, 173.24, 173.57.

$C_{35}H_{55}NO_3PS_2^+$low resolution ESI-MS calculated: 632.92, found: 632.91

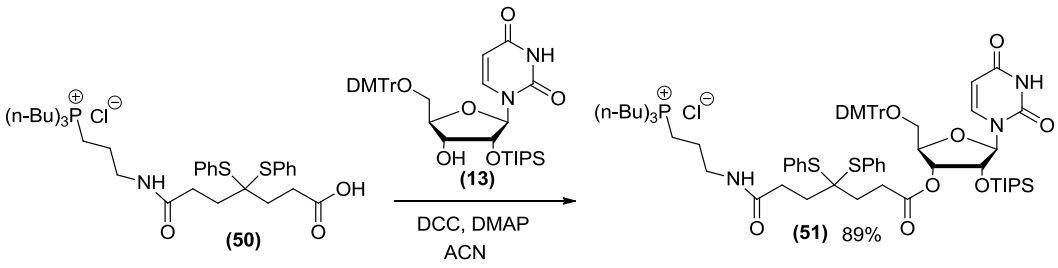


tributyl(3-(6-carboxy-4,4-bis(phenylthio)hexanamido)propyl)phosphonium chloride (50)

Compound **(49)** (0.5 g, 0.75 mmol) was dissolved in 8 mL of MeOH to which was added 5 eq of NaOH (0.15 g, 3.7 mmol) in 2 mL of water. This mixture was allowed to stir for 3h until all starting material was consumed. The solution was brought to neutrality by the addition of 1M HCl. This mixture was purified by HCl extraction followed by brine ×3, dried and concentrated. Quantitative recovery.

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.98 (t, *J*=6.59 Hz, 9 H) 1.52 (br. s., 13 H) 1.88 (d, *J*=9.67 Hz, 3 H) 2.01 (d, *J*=4.10 Hz, 4 H) 2.08 - 2.25 (m, 7 H) 2.49 - 2.64 (m, 4 H) 2.68 (t, *J*=6.59 Hz, 2 H) 3.39 (d, *J*=4.40 Hz, 2 H) 7.12 - 7.21 (m, 3 H) 7.30 - 7.41 (m, 6 H) 7.65 - 7.79 (m, 4 H) 8.00 (br. s., 1 H).

$C_{34}H_{53}NO_3PS_2^+$ low resolution ESI-MS calculated: 618.32, found: 618.32



5′-DMTr-2′-TIPS-3′-[(tributyl(3-(7-oxo-4,4-bis(phenylthio)heptanamido) propyl)phosphonium bromide] uridine (51)

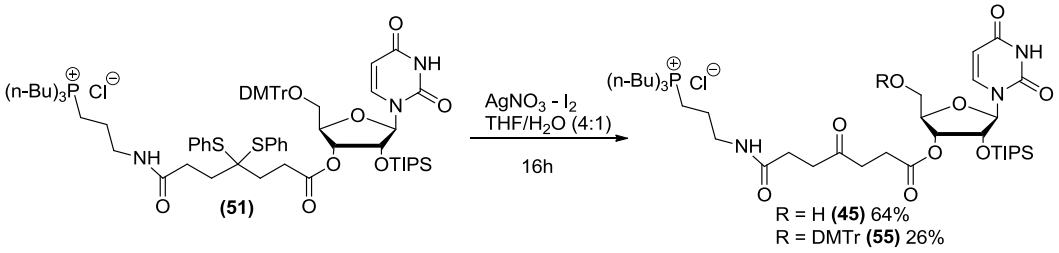
Compound **(50)** (0.56 g, 0.85 mmol) and 2eq of DCC (0.35 g, 1.7 mmol) were dissolved in 8 mL of ACN. To the above solution 2eq of nucleoside **(13)**

1.23 g, 1.7 mmol) was added followed by sub stoichiometric amounts of DMAP. The reaction was allowed to stir for 6h until all starting material was consumed, monitored by MS. The sample was diluted with ethyl acetate and extracted with ammonium chloride ×2 and once with brine. The organic layer was dried with magnesium sulphate and concentrated to dryness. The mixture was then precipitated in MTBE-Hexanes 50:50 to remove any unreacted DCC then purified by column chromatography (0 → 10% MeOH-DCM)affording a mixture of compound (34) and (33) as an inseparable mixture in 89% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.81 - 1.15 (m, 31 H) 1.35 - 1.61 (m, 13 H) 1.75 - 2.06 (m, 7 H) 2.12 - 2.35 (m, 6 H) 2.50 - 2.94 (m, 6 H) 3.26 - 3.52 (m, 4 H) 3.77 (s, 6 H) 4.19 (d, *J*=2.34 Hz, 1 H) 4.66 (t, *J*=5.47 Hz, 1 H) 5.24 (d, *J*=8.21 Hz, 2 H) 5.35 (dd, *J*=4.88, 2.54 Hz, 1 H) 6.09 (d, *J*=5.86 Hz, 1 H) 6.03 - 6.16 (m, 1 H) 6.81 (dd, *J*=8.60, 1.56 Hz, 4 H) 7.17 - 7.36 (m, 17 H) 7.27 - 7.28 (m, 1 H) 7.59 - 7.68 (m, 4 H) 7.84 (d, *J*=8.21 Hz, 1 H) 8.45 (t, *J*=5.67 Hz, 1 H) 9.36 (br. s., 1 H).

^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 12.03, 13.44, 17.60, 17.77, 18.56, 19.03, 23.52, 23.57, 23.83, 23.97, 55.20, 67.53, 76.95, 77.26, 77.48, 77.59, 87.45, 113.25, 113.28, 127.21, 128.00, 128.83, 129.25, 129.95, 130.08, 130.44, 130.58, 134.71, 134.93, 136.74, 136.82, 144.05, 150.77, 158.64, 158.67, 163.38, 172.16, 172.94.

$C_{73}H_{101}N_3O_{10}PS_2Si^+$ low resolution ESI-MS calculated: 1302.64, found: 1302.45 (DCC failure product: $C_{47}H_{75}N_3O_3PS_2$+ resolution ESI-MS calculated: 824.50, found: 824.32)



5′-OH-2′-TIPS-3′-[(tributyl(3-(7-oxy-4,7-dioxoheptanamido)propyl) phosphonium chloride] uridine (45)

AND

5′-DMTr-2′-TIPS-3′-[(tributyl(3-(7-oxy-4,7-dioxoheptanamido)propyl) phosphonium chloride] uridine (55)

Compound **(51)** (0.51 g, 0.38 mmol) was dissolved in 3.8 ml of THF:water 8:2 mixture to which 1.5eq of silver nitrate was added (96 mg, 0.57 mmol). A clowdy white precipitate was formed. Molecular iodine was then added (48 mg, 0.38 mmol). This mixture was allowed to stir for 16h until all starting material was consumed. The sample was then filtered from precipitated silver iodine over Celite® and washed with ACN. The sample was then condensed, re-suspended in DCM and precipitated in MTBE yielding a mixture of compounds **(45)** and **(55)** which were easily separable by silica gel chromatography. They were isolated in 64% and 26% yield respectively.

Compound **(45)**

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.90 - 1.13 (m, 37 H) 1.39 - 1.52 (m, 17 H) 1.78 - 1.87 (m, 2 H) 1.90 - 1.95 (m, 1 H) 2.00 - 2.03 (m, 1 H) 2.06 - 2.25 (m, 11 H) 2.27 - 2.34 (m, 5 H) 2.44 - 2.60 (m, 4 H) 2.73 - 2.80 (m, 2 H) 3.37 (d, *J*=5.47 Hz, 1 H) 3.68 - 3.92 (m, 3 H) 4.16 - 4.22 (m, 1 H) 4.64 - 4.72 (m, 1 H) 5.01 - 5.13 (m, 1 H) 5.62 - 5.72 (m, 1 H) 5.78 (dd, *J*=7.62, 4.49 Hz, 1 H) 8.07 (d, *J*=7.82 Hz, 1 H) 8.23 (d, *J*=8.21 Hz, 1 H) 8.70 - 9.11 (m, 1 H).

^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 12.05, 12.16, 13.37, 17.66, 17.67, 17.75, 17.77, 17.79, 17.85, 18.25, 18.73, 21.25, 23.44, 23.50, 23.55, 23.80, 23.94, 125.81, 128.58, 139.39, 143.40, 183.06, 207.78.

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 33.29 (s, 1 P) 33.36 (s, 1 P) 33.42 (s, 1 P) 33.51 (s, 1 P) 33.72 (s, 1 P) 33.78 (s, 1 P).

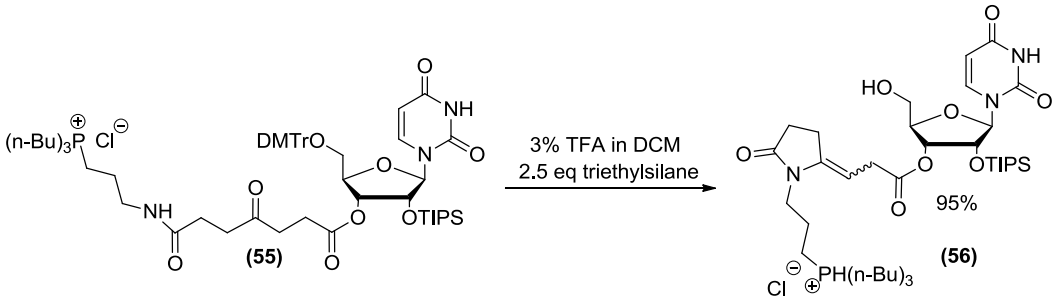
$C_{40}H_{73}N_3O_9PSi^{+1}$ low resolution ESI-MS calculated:798.48 , found: 798.51

Compound **(55)**

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.85 (t, *J*=7.09 Hz, 8 H) 0.96 (t, *J*=6.85 Hz, 12 H) 1.01 (br. s., 4 H) 1.31 (dd, *J*=6.85, 2.45 Hz, 4 H) 1.34 - 1.54 (m, 10 H) 1.95 - 2.08 (m, 2 H) 2.08 - 2.27 (m, 11 H) 2.59 (s, 1 H) 2.62 - 2.83 (m, 2 H) 3.31 - 3.52 (m, 2 H) 3.54 - 3.70 (m, 3 H) 3.74 (s, 5 H) 4.11 - 4.33 (m, 3 H) 4.62 (d, *J*=2.45 Hz, 2 H) 5.15 - 5.29 (m, 2 H) 5.98 (dd, *J*=5.26, 3.06 Hz, 1 H) 6.70 - 6.85 (m, 3 H) 7.14 - 7.36 (m, 7 H) 7.53 (t, *J*=7.70 Hz, 1 H) 7.85 (dd, *J*=8.07, 2.20 Hz, 1 H) 8.54

(dd, *J*=14.43, 1.71 Hz, 1 H) 8.72 (br. s., 1 H) 9.26 (d, *J*=9.29 Hz, 1 H) 9.73 (br. s., 2 H).

$C_{61}H_{91}N_3O_{11}PSi^+$ low resolution ESI-MS calculated: 1100.61, found 1100.58



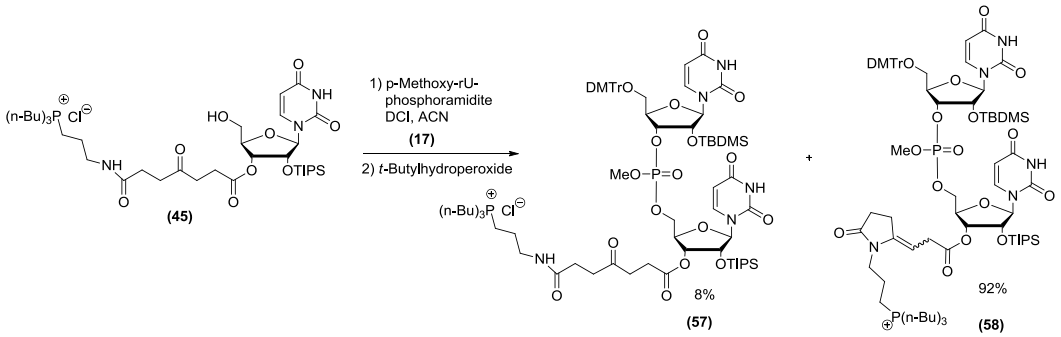
5′-OH-2′-TIPS-3′-[(E/Z)-tributyl(3-(2-carboxyethylidene)-5-oxopyrrolidin-1-yl)propyl)phosphonium chloride] uridine

Compound **(55)** (0.12 g, 0.105 mmol) was dissolved/reacted directly with 10 mL of a 3% TFA solution in DCM followed by 2.5 eq of triethylsilane to quench the trityl cation. This reaction was allowed to stir for 10 min before 4 mL of methanol was added to further quench the trityl cation. This was followed by 20 mL of toluene then the mixture was concentrated to dryness, taken up in DCM and precipitated in MTBE yielding compound **(56)** in 95% yield (62 mg, 0.1 mmol)

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.78 - 1.14 (m, 43 H) 1.47 (br. s., 18 H) 1.66 - 1.94 (m, 3 H) 1.97 - 2.86 (m, 20 H) 2.99 - 3.23 (m, 1 H) 3.34 (br. s., 1 H) 3.52 - 4.01 (m, 3 H) 4.16 (br. s., 1 H) 4.60 - 4.80 (m, 1 H) 4.93 (br. s., 1 H) 5.04 - 5.24 (m, 1 H) 5.72 (br. s., 2 H) 7.70 (d, *J*=7.91 Hz, 1 H) 7.96 - 8.28 (m, 2 H) 9.20 (br. s., 1 H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.01, 12.03, 12.42, 13.24, 16.69, 17.58, 17.68, 17.70, 18.06, 18.14, 18.69, 18.78, 21.32, 23.27, 23.33, 23.71, 23.91, 26.89, 28.18, 28.44, 29.49, 32.14, 36.84, 37.34, 61.24, 73.45, 73.66, 74.04, 83.52, 88.78, 89.46, 92.69, 102.60, 114.66, 118.55, 125.79, 127.98, 128.61, 133.49, 133.90, 141.68, 141.79, 150.96, 163.71, 163.83, 170.93, 171.95, 172.99, 176.18.

$C_{40}H_{71}N_3O_8PSi$+ low resolution ESI-MS calculated: 780.47, found: 780.58

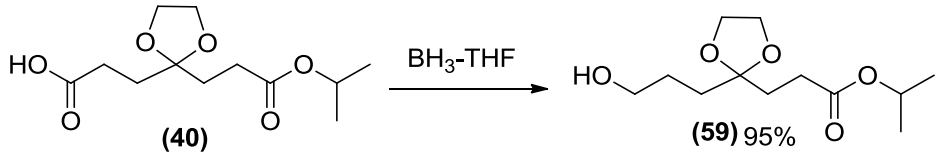


Dimer nucleoside enamine product formed during phosphoramidite coupling, compound (57) and (58) mixture

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.07 - 0.16 (m, 8 H) 0.83 - 1.05 (m, 62 H) 1.09 - 1.28 (m, 9 H) 1.33 (d, *J*=6.15 Hz, 18 H) 1.47 (br. s., 20 H) 1.91 (br. s., 4 H) 2.14 (br. s., 11 H) 2.21 - 2.38 (m, 6 H) 2.52 (br. s., 2 H) 2.64 (br. s., 2 H) 3.14 (br. s., 1 H) 3.21 (s, 1 H) 3.27 - 3.41 (m, 4 H) 3.48 (d, *J*=7.91 Hz, 3 H) 3.66 (s, 3 H) 3.62 (s, 3 H) 3.78 (d, *J*=5.57 Hz, 12 H) 3.89 (s, 2 H) 4.17 - 4.35 (m, 4 H) 4.50 (br. s., 3 H) 4.89 (br. s., 1 H) 5.13 - 5.24 (m, 2 H) 5.92 - 6.03 (m, 2 H) 6.77 - 6.87 (m, 6 H) 7.13 - 7.36 (m, 26 H) 7.51 (d, *J*=7.91 Hz, 1 H) 7.69 - 7.74 (m, 2 H) 7.80 - 7.88 (m, 2 H).

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm -0.91 (s, 1 P) -0.36 (s, 1 P) 33.91 (s, 1 P) 33.96 (s, 1 P).

$C_{77}H_{116}N_5O_{18}P_2Si_2$+low resolution ESI-MS calculated: 1516.73, found: 1516.62



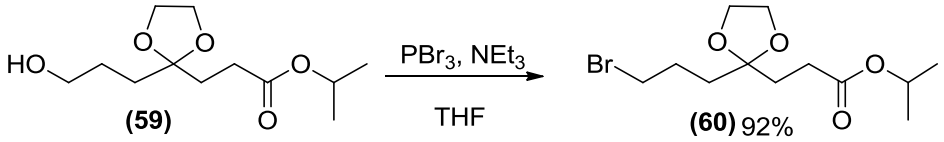
Isopropyl 3-(2-(3-hydroxypropyl)-1,3-dioxolan-2-yl)propanoate (59).

Compound **(40)** (0.334 g, 1.35 mmol) is dissolved in 13.5 mL making a 0.1M solution. This mixture is cooled to 0°C and borane, 1M in THF (1.75 mL. 1.75 mmol) is added drop wise. Once the vigorous release of hydrogen subsides, the ice bath is removed and the reaction is allowed to stir for 2 hours until the reaction is complete as shown by TLC. The mixture is quenched by first cooling the solution back down to 0°C and adding methanol slowly until the release of hydrogen subsides. The crude reaction mixture is then concentrated to dryness,

taken up in methanol once more and concentrated again to ensure removal trimethyl borate. The mixture is purified by column chromatography 100% hexanes to 50:50 hexanes:ethyl acetate yielding pure primary hydroxyl 95%, 0.315 g.

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 1.11 (d, J=6.11 Hz, 6 H) 1.46 - 1.68 (m, 6 H) 1.82 - 1.94 (m, 3 H) 2.17 - 2.32 (m, 3 H) 3.43 - 3.61 (m, 2 H) 3.84 (s, 4 H) 4.88 (dt, J=12.53, 6.33 Hz, 1 H).

^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 21.67, 26.85, 29.14, 29.69, 31.90, 33.68, 62.46, 64.91, 67.52, 110.69, 173.07.

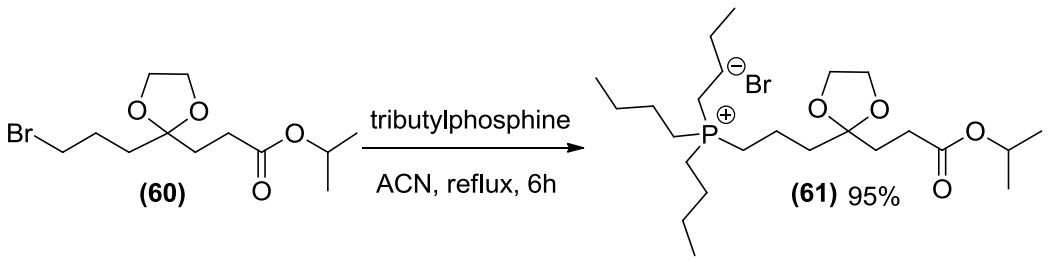


Isopropyl 3-(2-(3-bromopropyl)-1, 3-dioxolan-2-yl)propanoate (60)

Compound **(59)** (0.32 g, 1.58 mmol) was dissolved in 10 mL of DCM and 2 mL of triethylamine and cooled to 0° in an ice bath. To this solution PBr3 (0.225 mL, 2.37 mmol) was added drop wise over 10min and allowed to stir for 1 h at zero degrees until the reaction was complete by TLC. The reaction was quenched with saturated sodium bicarbonate diluted with ethyl acetate and extracted with brine twice. The organic layer was dried, concentrated and purified by a short silica plug with a 70:30 Hexanes:ethyl acetate yielding 0.45 g, 1.455 mmol of compound **(60)**

^{1}H NMR (500MHz, $CDCl_3$) δ = 5.01 - 4.87 (m, 1 H), 3.97 - 3.82 (m, 4 H), 3.36 (t, J = 6.7 Hz, 2 H), 2.33 - 2.20 (m, 2 H), 1.96 - 1.82 (m, 4 H), 1.73 - 1.64 (m, 2 H), 1.16 (d, J = 6.4 Hz, 6 H).

^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 21.75, 27.20, 29.12, 32.11, 33.85, 35.69, 64.95 - 65.08, 67.47, 110.26, 172.81.

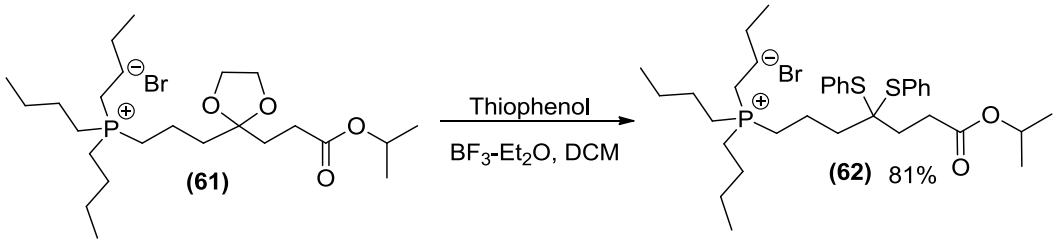


Tributyl(3-(2-(3-isopropoxy-3-oxopropyl)-1,3-dioxolan-2-yl)propyl) phosphonium bromide (61)

Compound **(60)** (0.33 g, 1.06 mmol) was dissolved with 4 mL of ACN. To this mixture tributylphosphine (0.53 ml, 2.12 mmol) was added directly and heated to 80°C. This was allowed to stir for 6 h. The mixture was concentrated and diluted with acetone then triturated into 250 mL of hexanes. The precipitate was filtered and the hexanes discarded. This process was repeated once more and the resulting goo was pure ionically tagged compound **(61)** in near quantitative yield, 0.54 g, 1.05 mmol.

^{1}H NMR ($CDCl_3$, 200MHz): δ = 4.91 (quin, J=6.3 Hz, 1 H), 3.97 - 3.82 (s, 4 H), 2.56 - 2.72 (m, 2 H), 2.23 - 2.55 (m, 10 H), 1.66 - 1.99 (m, 6 H), 1.35 - 1.61 (m, 16 H), 1.08 - 1.26 (m, 7 H), 0.80 - 1.02 ppm (m, 12 H).

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 32.96 (s, 82 P).



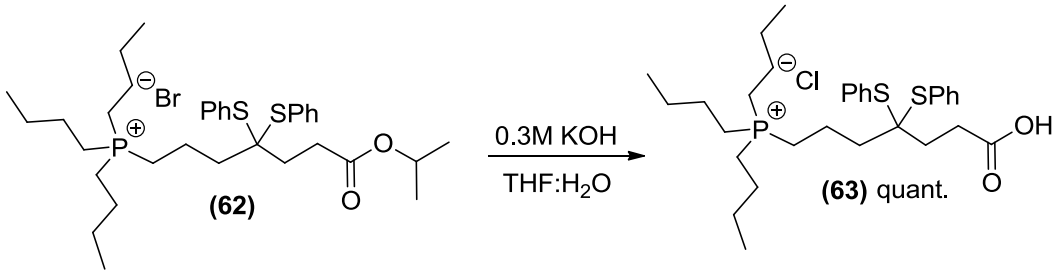
Tributyl(7-isopropoxy-7-oxo-4,4-bis(phenylthio)heptyl)phosphonium bromide (62)

Compound **(61)** (0.156 g, 0.30 mmol) was dissolved in 8 mL of dry DCM with 5eq of thiophenol (0.193 mL, 1.52 mmol) followed by 5eq of boron trifluoride etherate (0.157 mL, 1.52 mmol) and approximately 1 gram of crushed 3 Å molecular sieves. This mixture was stirred for 12h at which point the reaction was filtered over Celite® to remove the sieves. In the collection flask was 40 mL of saturated sodium bicarbonate. The biphasic mixture was transferred to a

separatory funnel and extract x3 with bicarbonate and once with brine. The organic layer was dried and concentrated; the resulting oil was purified by precipitation in hexanes/MTBE 75:25. Resulting in pure compound **62)** in. 81% recovered yield, 0.162 g, 0.24 mmol.

^{1}H NMR (200 MHz, $CDCl_3$) δ ppm 0.80 - 1.02 (m, 12 H) 1.08 - 1.26 (m, 7 H) 1.35 - 1.61 (m, 16 H) 1.66 - 1.99 (m, 6 H) 2.23 - 2.55 (m, 10 H) 2.56 - 2.72 (m, 2 H) 4.91 (quin, J=6.25 Hz, 1 H) 7.30 - 7.43 (m, 5 H) 7.52 - 7.62 (m, 4 H).

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 33.29 (s, 1 P).



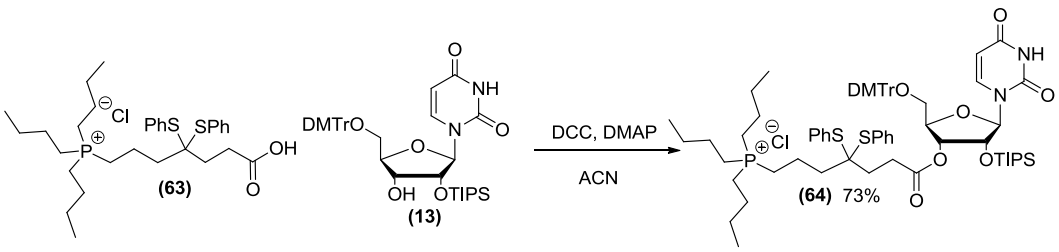
Tributyl(6-carboxy-4,4-bis(phenylthio)hexyl)phosphonium bromide (63)

Compound **(62)** (0.75 g, 1.19 mmol) is first dissolved in 3 mL of THF and then is treated with 30 mL of 0.2 M solution of KOH in water THF 50:50. This mixture is allowed to stir vigorously for 1 hour, until the hydrolysis is complete by TLC. The mixture is acidified by the addition of 20 mL of 1M HCl and excess NaCl is added as well. The organic layer is diluted with ethyl acetate and extracted x3 with brine, dried and concentrated to dryness. This affords pure tagged acid,**(63)** in quantitative yield.

^{1}H NMR 400 MHz, $CDCl_3$) δ ppm 0.82 - 1.02 (m, 9 H) 1.28 - 1.51 (m, 12 H) 1.58 (br. s., 2 H) 1.75 (br. s., 2 H) 1.94 (t, J=7.23 Hz, 2 H) 2.10 - 2.32 (m, 8 H) 2.75 (t, J=7.42 Hz, 2 H) 7.27 - 7.37 (m, 6 H) 7.65 (d, J=7.82 Hz, 4 H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 13.41, 18.19, 18.82, 21.44, 23.44, 23.50, 23.74, 23.93, 30.10, 32.76, 36.32, 68.37, 128.76, 129.14, 131.08, 136.38, 174.83.

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 32.89 (s, 1 P).



5′-DMTr-2′-TIPS-3′-[tributyl(7-oxy-4,4-bis(phenylthio)hexyl) phosphonium bromide] uridine (64)

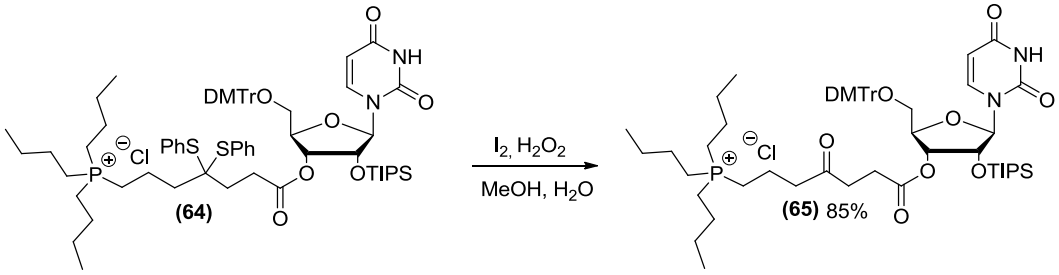
Compound **(63)** (0.294 g, 0.49 mmol) and 1.5 equivalents of DCC (0.14 g, 0.76 mmol) were dissolved in 9.5 mL of ACN. To the above solution 1.5 eq of nucleoside **(13)** (0.54 g, 0.76 mmol) was added followed by sub stoichiometric amounts of DMAP. The reaction was allowed to stir for 6h until all starting material was consumed, monitored by MS. The sample was diluted with ethyl acetate and extracted with ammonium chloride ×2 and once with brine. The organic layer was dried with magnesium sulphate and concentrated to dryness. The mixture was then precipitated in MTBE-Hexanes 50:50 to remove any unreacted DCC then purified by column chromatography (0 to 10% MeOH-DCM) affording compound **(64)** in 82% yield (0.48g, 0.40 mmol)

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.94 - 1.18 (m, 39 H) 1.32 - 1.60 (m, 21 H) 1.64 - 1.72 (m, 3 H) 1.74 - 1.84 (m, 3 H) 1.90 - 2.17 (m, 15 H) 2.76 - 2.84 (m, 2 H) 3.42 - 3.51 (m, 3 H) 3.79 (d, J=0.78 Hz, 7 H) 4.10 - 4.15 (m, 2 H) 4.67 - 4.71 (m, 1 H) 5.26 - 5.30 (m, 1 H) 5.40 (dd, J=5.08, 2.74 Hz, 1 H) 6.10 (d, J=6.25 Hz, 1 H) 6.81 - 6.85 (m, 5 H) 7.21 - 7.38 (m, 23 H) 7.60 - 7.68 (m, 5 H) 7.88 (d, J=8.21 Hz, 1 H) 8.61 (s, 1 H).

^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.13, 13.38, 17.68, 17.85, 18.01, 18.64, 23.45, 23.52, 23.76, 23.96, 25.02, 25.65, 33.86, 55.27, 67.41, 76.69, 77.11, 77.53, 87.57, 113.33, 113.36, 128.05, 129.00, 129.43, 130.02, 130.15, 130.71, 130.74, 134.75, 134.93, 136.52, 144.09, 150.66, 158.74, 158.78, 163.29, 172.20.

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 32.73 (s, 1 P).

Low resolution MS, m/z calc: 1231.6 found: 1231.5



5′-DMTr-2′-TIPS-3′-[tributyl(6-oxy-4-oxohexyl)phosphonium bromide] uridine (65)

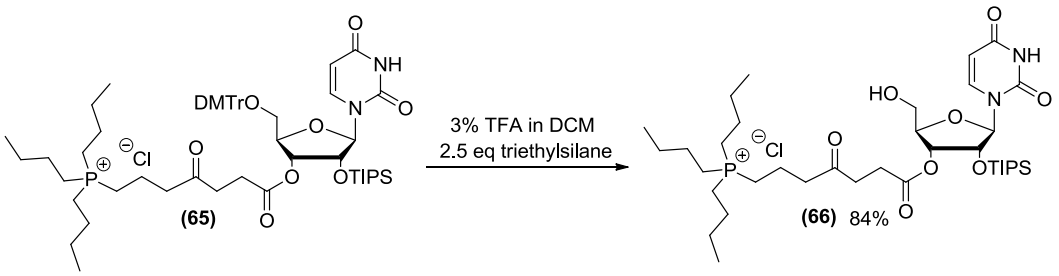
Compound **(64)** (1.44 g, 1.1 mmol) was dissolved in 10 ml of MeOH:water 8:2 mixture to which 20 mol% of molecular iodine (0.06 g, 0.22 mmol) was added. This was shortly followed by 4 equivalence of 30% hydrogen peroxide (0.5 mL, 4.4 mmol). This mixture was allowed to stir for 2 h until all starting material was consumed. This mixture was first diluted with ethyl acetate then quenched by extracting with 10% solution of sodium thiosulfite, then brine x3. Although the compound could be purified by precipitation alone, a column was run to ensure the highest purity. This provided the deprotected monomer **(65)** in good yield of 85%. This reaction does yield higher results if no column chromatography is used. 1.03 g.

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.91 - 1.10 (m, 11 H) 1.44 - 1.57 (m, 5 H) 1.63 (dd, J=15.90, 8.07 Hz, 1 H) 1.78 (dd, J=14.06, 6.97 Hz, 1 H) 2.29 - 2.40 (m, 3 H) 2.41 - 2.53 (m, 1 H) 2.56 - 2.85 (m, 3 H) 3.76 - 3.92 (m, 1 H) 3.98 (s, 1 H) 4.11 - 4.19 (m, 1 H) 4.70 - 4.78 (m, 1 H) 5.18 (dd, J=4.89, 3.42 Hz, 1 H) 5.75 (d, J=8.07 Hz, 1 H) 5.81 (d, J=5.62 Hz, 1 H) 8.00 (d, J=8.31 Hz, 1 H) 8.67 (br. s., 1 H).

^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 11.93, 11.96, 11.98, 13.37, 17.50, 17.52, 17.54, 17.57, 17.58, 17.60, 17.62, 17.65, 17.66, 17.67, 17.69, 17.71, 17.72, 17.78, 17.80, 18.59, 18.77, 18.97, 19.15, 20.80, 20.84, 23.54, 23.57, 23.77, 23.88, 24.35, 24.47, 28.00, 30.88, 36.93, 41.11, 61.25, 73.72, 73.96, 83.72, 88.91, 102.72, 141.92, 150.83, 163.77, 171.98, 208.11.

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 33.08 (s, 1 P).

Low resolution MS, m/z calc: 1029.6 found: 1029.5



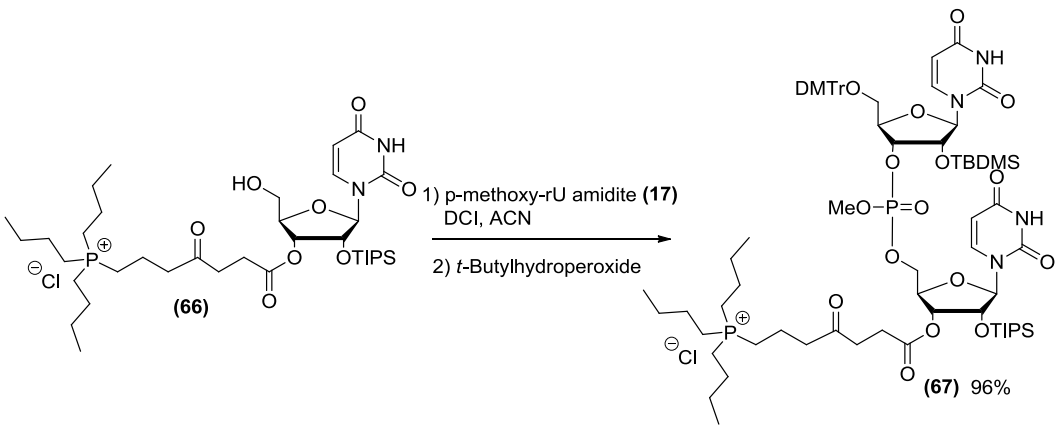
5′-OH-2′-TIPS-3′-[tributyl(6-oxy-4-oxohexyl)phosphonium chloride] uridine (66)

3′-tagged-uridine **(65)** (0.65 g, 0.61 mmol) was dissolved in 3% TFA in DCM and allowed to stir for 5 min before adding triethylsilane to quench the trityl cation. The reaction was then concentrated to an oil taken up in minimal amounts of DCM and precipitated in MTBE to remove dimethoxytritanol. The compound was filtered over Celite®, collected in DCM and purified by column chromatography (DCM:MeOH, 100:0 →90:10%). Isolated yield of (**16):** 0.39 g (84%).

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.91 - 1.10 (m, 11 H) 1.44 - 1.57 (m, 5 H) 1.63 (dd, *J*=15.90, 8.07 Hz, 1 H) 1.78 (dd, *J*=14.06, 6.97 Hz, 1 H) 2.29 - 2.40 (m, 3 H) 2.41 - 2.53 (m, 1 H) 2.56 - 2.85 (m, 3 H) 3.76 - 3.92 (m, 1 H) 3.98 (s, 1 H) 4.11 - 4.19 (m, 1 H) 4.70 - 4.78 (m, 1 H) 5.18 (dd, *J*=4.89, 3.42 Hz, 1 H) 5.75 (d, *J*=8.07 Hz, 1 H) 5.81 (d, *J*=5.62 Hz, 1 H) 8.00 (d, *J*=8.31 Hz, 1 H) 8.67 (br. s., 1 H).

^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 11.93, 11.96, 11.98, 13.37, 17.50, 17.52, 17.54, 17.57, 17.58, 17.60, 17.62, 17.65, 17.66, 17.67, 17.69, 17.71, 17.72, 17.78, 17.80, 18.59, 18.77, 18.97, 19.15, 20.80, 20.84, 23.54, 23.57, 23.77, 23.88, 24.35, 24.47, 28.00, 30.88, 36.93, 41.11, 61.25, 73.72, 73.96, 83.72, 88.91, 102.72, 141.92, 150.83, 163.77, 171.98, 208.11.

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 33.1 (s, 1 P).



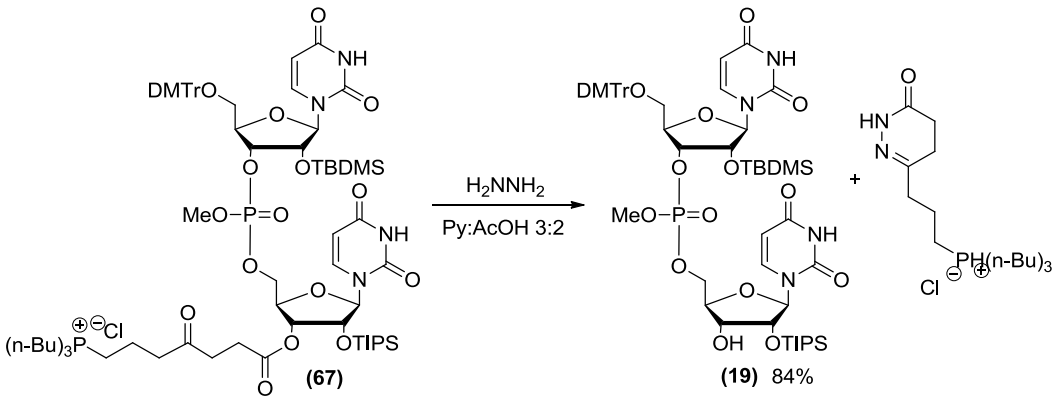
[5′-DMTr-2′TBDMS-uridine]-[3′-p(OMe)-5′]-[2′-TIPS-3′-(tributyl(6-oxy-4-oxohexyl)phosphonium chloride)-uridine (67)

Compound **(66)** (0.332 g, 0.42 mmol) was dissolved in 4.1 mL of dry ACN. To this mixture was added 1.5eq of phosphoramidite **(39)** (0.52 g, 0.63 mmol) and 1.6eq of DCI (82 mg, 0.69 mmol). This mixture was stirred for 6h at room temperature at which point an MS showed complete consumption of starting material. 0.5 mL of *tert*-butanol was then added to the mixture to react with excess phosphoramidite and make it more soluble in the MTBE mixture. The mixture was then precipitated into MTBE:hexanes 50:50 to remove the excess amidite and butanol. The solid precipitate was dissolved in DCM and treated with 0.5 mL of *tert*-buty hydroperoxide in decane (5M) to oxidize the internucleotide phosphate. The mixture was then precipitated into MTBE to remove the excess peroxide, decane, DCI, and residual amidite that might remain. This afforded pure **(67)** 0.63 g, 96% yield.

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm -0.05 - 0.20 (m, 7 H) 0.86 (s, 15 H) 0.89 - 1.02 (m, 33 H) 1.10 - 1.29 (m, 8 H) 1.35 (d, *J*=6.15 Hz, 16 H) 1.48 (br. s., 16 H) 1.74 (br. s., 2 H) 2.19 (br. s., 10 H) 2.60 (d, *J*=14.07 Hz, 4 H) 2.66 - 2.77 (m, 1 H) 3.20 (s, 1 H) 3.27 - 3.41 (m, 3 H) 3.46 (br. s., 2 H) 3.52 - 3.71 (m, 2 H) 3.71 - 3.80 (m, 7 H) 3.89 (s, 1 H) 4.05 (br. s., 1 H) 4.13 (br. s., 1 H) 4.43 - 4.56 (m, 2 H) 5.06 - 5.22 (m, 2 H) 5.60 - 5.77 (m, 1 H) 5.82 - 6.05 (m, 2 H) 6.76 - 6.87 (m, 5 H) 7.17 - 7.38 (m, 16 H) 7.41 - 7.58 (m, 2 H) 7.72 - 7.98 (m, 2 H).

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm -1.07 (s, 1 P) -0.30 (s, 1 P) 33.31 (s, 1 P) 33.35 (s, 1 P).

$C_{74}H_{113}N_4O_{18}P_2Si_2^+$ low resolution ESI-MS calculated: 1463.70 , found: 1463.8



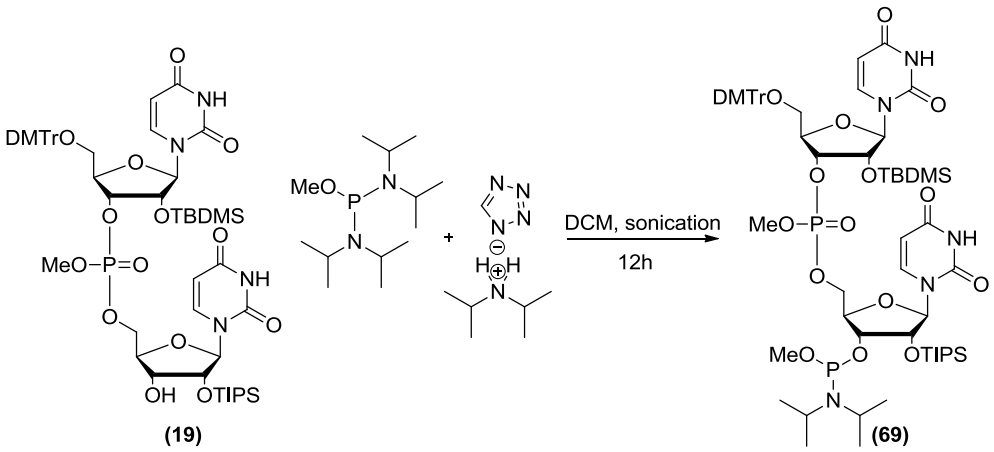
Compound **(67)** (0.114 g, 0.08 mmol) was dissolved in minimal amounts of ACN, approximately 1 ml, then treated with 10 eq of a 0.5 M hydrazine hydrate solution in pyridine:acetic acid (3:2) (40 mg, 0.8 mmol) and allowed to stir for 15 min. The reaction progress was monitored by MS to confirm consumption of starting material. At this point 10eq of 2,4-pentanedione was added to the solution to quench any excess hydrazine (80 mg, 0.8 mmol. The reaction was then diluted with ethyl acetate and extracted with first a saturated ammonium chloride solution then ×2 with a 5% solution ammonium chloride solution, then once with brine. The organic layer was dried and concentrated. The mixture was then taken up in minimal amounts of DCM and precipitated in a 75/25 mixture of MTBE/hexanes mixture. The oily precipitate was filtered off and the ether layer was concentrated to dryness. The crude concentrate was then purified by column chromatography (0 → 75% ethyl acetate:hexanes) yielding isomerically pure **(19)** in 84% yield, 76 mg.

^{1}H NMR (300 MHz, CD_3CN) δ ppm 0.09 - 0.15 (m, 6 H) 0.87 - 1.20 (m, 40 H) 1.94 - 2.00 (m, 1 H) 2.10 - 2.20 (m, 2 H) 3.44 (br. s., 3 H) 3.67 - 3.79 (m, 12 H) 4.04 - 4.23 (m, 5 H) 4.28 - 4.35 (m, 2 H) 4.38 - 4.48 (m, 2 H) 4.81 - 4.90 (m, 1 H) 5.38 (d, *J*=8.21 Hz, 1 H) 5.64 (d, *J*=8.20 Hz, 1 H) 5.81 - 5.91 (m, 2 H) 6.90 (d, *J*=7.91 Hz, 5 H) 7.24 - 7.37 (m, 9 H) 7.41 - 7.52 (m, 4 H) 7.71 (dd, *J*=8.20, 2.64 Hz, 1 H) 9.62 (br. s., 1 H).

^{13}C NMR (126 MHz, $CDCl_3$) δ ppm -5.08, -5.00, -4.90, -4.87, 12.02, 12.05, 17.66, 17.69, 17.76, 17.79, 25.56, 25.59, 54.59, 54.92, 54.97, 55.21, 55.25, 61.72, 67.06, 67.29, 70.17, 70.26, 74.72, 74.84, 75.68, 75.99, 81.50, 82.14, 87.53, 87.57, 88.18, 88.33, 90.51, 90.57, 102.45, 102.92, 102.96, 113.33, 127.35, 127.76, 128.05, 128.21, 128.23, 129.12, 130.17, 130.23, 130.25, 130.36, 134.58, 134.66, 134.79, 139.83, 140.37, 140.45, 143.95, 144.06, 150.24, 150.28, 150.50, 150.58, 158.77, 158.78, 158.82, 163.29, 163.34, 163.39.

^{31}P NMR (81 MHz, CD_3CN) δ ppm 0.58 (s, 1 P) 0.83 (s, 1 P).

Mass calc. 1137.36 Mass found. 1159.53 ($M+Na^+$).



5′-DMTr-2′-TBDMS-rU]-[3′-p(OMe)-5′]-[rU-2′-TIPS]-3′-methyl phosphoramidite (69)

N,N-Diisopropylammonium tetrazolide (1.3. mmol, 0.23 g) and bis (N,N-diisopropylamino)methylphosphine (1.3 mmol, 0.34 g) were added to 4.5 mL of ACN. After stirring for a few minutes, the mixture was sonicated for 0.5h until the majority of the tetrazolide salt had dissolved. The dimer **(19)** (0.65 mmol, 0.74 g) was then added directly to this solution and sonicated for an additional 12h. Sonication was performed to help dissolve the activator and considerably speed up the reaction. The mixture was then diluted with either MTBE or ethyl acetate (the ethyl acetate must be prewashed with saturated sodium bicarbonate to remove residual acetic acid) and extracted with a 10% NaCl solution x2 then once with brine. The organic layer was then dried with magnesium sulphate and condensed to dryness. The mixture was then taken up in minimal amounts of DCM and precipitated in petroleum ether to remove amines (-78^{0}C). The

precipitate was filtered over Celite® and collected with DCM. The solution was evaporated and the resulting solid purified by column chromatography. The column was first loaded with 3% triethylamine in hexanes. The sample was then dissolved with 17% hexanes, 3% triethylamine, 80% DCM and loaded on the column. The compound was eluted with 0-10% Ethyl acetate/DCM/3%triethylamine. Yield: 0.64 g (76%).

^{31}P NMR (81 MHz, $CDCl_3$) δ ppm 0.59 (s, 1 P) 0.77 (s, 1 P) 1.09 (s, 1 P) 1.14 (s, 1 P) 151.25 (s, 1 P) 151.38 (s, 1 P) 151.99 (s, 1 P) 152.05 (s, 1 P).

5.5 REFERENCES

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Chapter 6 Development of Orthogonal Linkers for Solid Phase Synthesis and On-Support Deprotection of Oligonucleotides

6.1 Introduction

In chapter 5 we described the design and synthesis of two ionically-tagged linkers (levulinyl and NPPOC based) that are cleaved under relatively mild conditions. To further demonstrate the utility of these linkers we set out to conjugate them to solid supports suitable for oligonucleotide synthesis, rather than the soluble ionic supports previously. Ideally, release of the oligomers will be mild enough to allow, in principle, for a) the synthesis of DNA and RNA strands containing acid or base sensitive groups (e.g., siRNA or antisense pro-drugs), or b) the ability to grow and fully deprotect the oligonucleotide while still attached to the support ("on-support" deprotection), greatly simplifying and speeding up post-synthesis processing.

To put our work in context to prior published work, we provide a brief overview below. Greenberg *et al* have extensively studied nitrobenzyl based linkers for solid phase oligonucleotide synthesis (compound **(1);** Figure 6.1). These solid supports require UVA light (1–2 hours @ 315-400 nm in ACN/water) for releasing the oligomer without causing any detectable photo-induced thymine dimerization.[1] Drawbacks of this support are the prolonged irradiation times required for cleavage, and the presence of a carbonate moiety which precludes on-support deprotection under basic conditions.

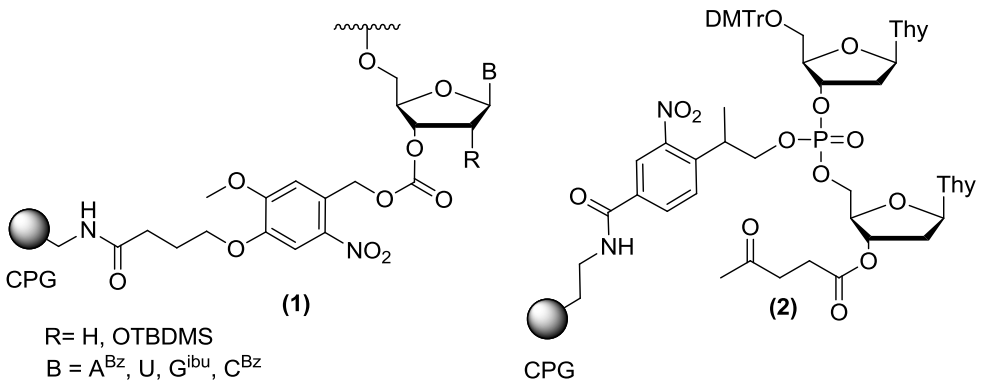


Figure 6.1. Solid support with photocleavable linkers used in the synthesis of DNA and RNA. **(1)** *o*-nitrophenyl derivative conjugated though a carbonate ester by Greenberg *et al.* **(2)** NPPOC derivative containing photocleavable linker conjugated through the first phosphate linkage developed by our group.[2]

The Imbach research group carried out the synthesis of DNA phosphotriesters on a photolabile solid support. The 2-(acylthio)ethyl (SATE) group was used to mask the phosphates,[3-5] thus allowing for the selective cleavage of the allyoxycarbonyl (AOC) nucleobase protecting groups and release from the solid support. Further development of this method involved the use of photocleavable protecting groups for the nucleobases,[6,7] permitting simultaneous deprotection and release of the oligomer by a single photolysis step (wavelength > 300 nm). RNA would not be amenable to this method, since RNA phosphotriesters (unmasked 2′OHs) undergo spontaneous cleavage and/or isomerization of the internucleotide linkage.[8]

Pfleiderer *et al* introduced in 1998 the photolabile NPPOC group (*o*-nitrophenyl derivative) as a 5′-nucleotide protecting group.[9] It was found to be much more sensitive to photolytic cleavage (up to 8 fold), addressing one of the major limitations of photocleavable groups (photolysis time). The authors further developed a series of photolabile groups which were utilized in the synthesis of "caged" nucleosides.[10-13] Based on this pioneering work, our research group recently reported on the conversion of the NPPOC protecting group into an orthogonal photocleavable linker (compound **(2)**; Figure 6.1),[2] and applied this linker to the solid-phase synthesis of chimeric dT-rU oligomers containing 2′-*O*-acetalesterified uridines.[2] The linker is cleaved more rapidly than the original linker developed by Greenberg (20 min vs 1-2 hours), but suffers from the same

limitation in that it is also cleavable under the basic conditions required to unmask the more commonly used phosphate and nucleobase protecting groups. As well, the silica based (CPG) solid support used was unsuitable for on support desilylation using fluoride.

In this Chapter, we describe our efforts in extending this work to the synthesis and on support deprotection of RNA of mixed base composition. To make the process compatible with TBDMS RNA chemistry, we decided to switch to polystyrene (NittoPhase®) solid support to which we would install variations of our improved photo-cleavable NPPOC linker, and the levulinyl like linker developed in Chapter 5. The chemical stability of the support bound NPPOC linker was assessed by the full deprotection of a mixed base RNA sequence using ammonia treatment and subsequent microwave assisted "on-support" desilylation. We also demonstrate the synthesis and on-support deprotection of a poly rU sequence immobilized via a levulinyl-like linker. With further improvements to the methods described below, we envision that this linker will be directly applicable to the synthesis of modified RNA containing base sensitive or bio-labile groups at specific positions of the sugar-phosphate backbone.

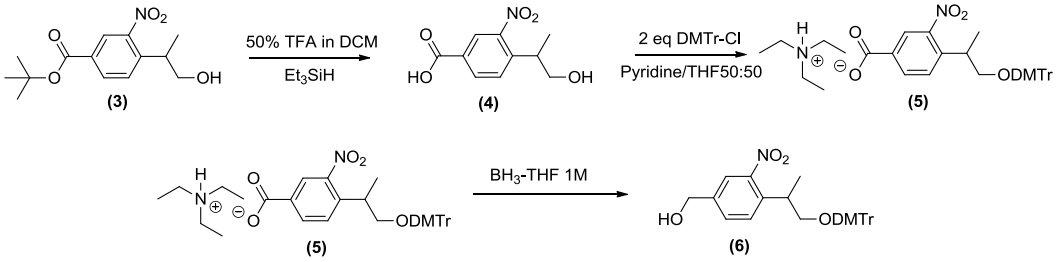
6.2 Results and discussion

6.2.1 Photocleavable NPPOC derivative synthesis and solid support conjugation

An important requirement for on support deprotection of RNA on solid supports is resistance of the orthogonal linker to the basic conditions (such as aqueous or ethanolic ammonia) used to unmask the nucleobases. We thus first focused on optimizing the chemical structure of the NPPOC linker **(2)** where we aimed to avoid the use of amide groups as connecting functionalities due to their sensitivity to ammonolysis. We settled to use a hydroxyl group that was attached to the solid support through a methyl protected phosphate (Schemes 6.1 – 6.3). We then assessed the use of common solid supports. As mentioned previously, the generally used CPG (controlled pore glass) was deemed unsuitable due to its instability to fluoride treatment during the desilylation step. Further, most

commercially available polystyrene-type supports could not be "well-wetted" by acetonitrile (ACN) which caused floating of the supports at the surface of the solution. We found that the commercially available NittoPhase® solid support is properly wetted in ACN and undergoes minimal swelling (4.0 mL/g). We thus decided to apply this polystyrene-type support to our new synthetic procedure.

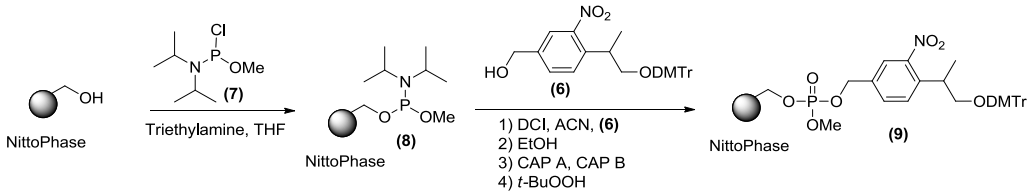
The preparation of the redesigned photocleavable NPPOC linker followed the synthetic procedure outlined in Scheme 6.1. The synthesis of the starting material t*ert*-butyl 4-(1-hydroxypropane-2-yl)-3-nitrobenzoate (**3**) was described in Chapter 5. The *tert*-butyl ester of compound **(3)** was cleaved by treatment with a 50% TFA in DCM in the presence of triethylsilane.[14] The product was obtained in 86% yield. It is important to note that the free acid should not be subjected to dehydrating conditions such as toluene co-evaporation as the acidic conditions promote polymerization of this molecule. Next the primary hydroxyl group was protected by treatment with DMTr-Cl (2 eq.) in pyridine-THF (1/1). The carboxylic acid in compound **(5)** was selectively reduced to a primary alcohol with 1.0 M BH_3-THF without any reduction of the nitro group.[15] Compound **(6)** was thus obtained in 92 % yield. The NPPOC derivatives (**5**) and (**6**) were very sensitive to ambient light. All reactions presented in Scheme 6.1 were carried out by covering the reaction flask with aluminum foil.



Scheme 6.1. Synthesis of the photocleavable linker **(6).**

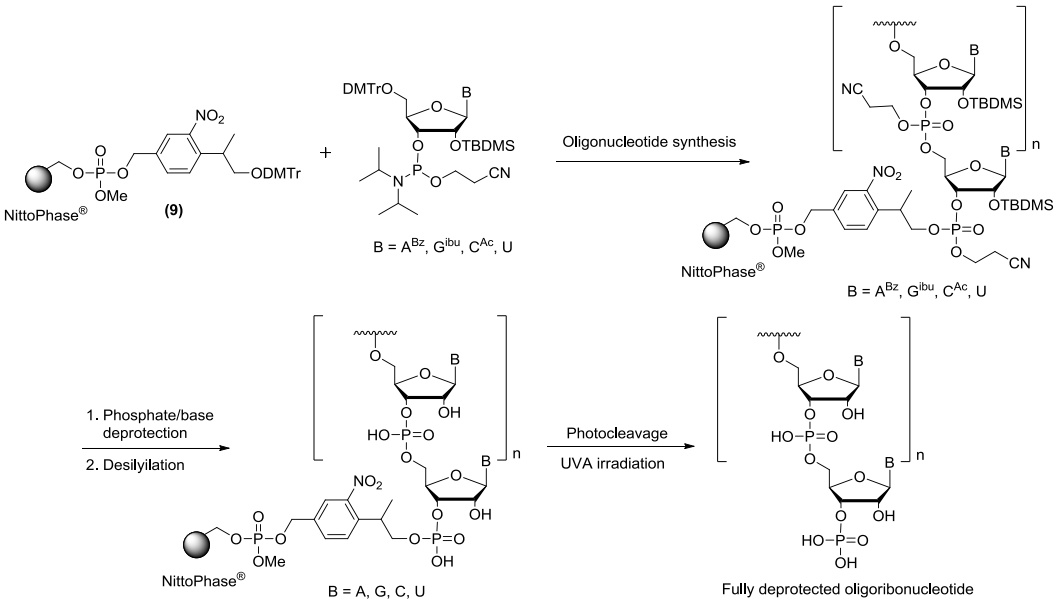
Immobilization of the NPPOC derivative (**6**) onto the solid support (NittoPhase®) was achieved utilizing phosphoramidite chemistry (Scheme 6.2). Because the internucleotide phosphates are ubiquitous to both DNA and RNA we envisioned that there would be no incompatibility in conjugating the NPPOC linker to the solid support through a phosphate group. In order to minimize

exposure to light of the tritylated NPPOC, compound **(6)**, we first phosphitylated the solid support (NittoPhase®) with **(7)** as shown in Scheme 6.2. In order to ensure dry conditions for the subsequent coupling the solid support, (**8**) was washed with dry ACN (3 times) over an argon atmosphere. To this rinsed solid support, premixed dry ACN solution of DCI and compound (**6**) were added directly to the solid support, and very gently agitated for one hour. Anhydrous ethanol was then added to the reaction mixture to quench any remaining phosphoramidite on the solid support. The mixture was agitated for an additional 10 min. Excess reagents were then filtered through the reaction vessel. Unreacted hydroxyl groups on supports were capped with acetyl group by Ac_2O/*N*-methylimidazole, and phosphite linkages were oxidized with *tert*-butyl hydroperoxide. This afforded the photolabile linker in good yields. The loading amount of the NPPOC linker on solid supports was quantitated by means of DMTr titration assay described by Damha *et al.*[16] It was found to be 143 µmol/gram of solid support.



Scheme 6.2. Immobilization of NPPOC linker (**6**) on solid supports (NittoPhase®).

6.2.2 Solid supported oligonucleotide synthesis and deprotection using the photocleavable NPPOC derivative (9)



Scheme 6.3. General scheme for the synthesis and deprotection of oligoribonucleotides using the NPPOC linked NittoPhase® solid support.

Using the photocleavable linker **(9)** standard solid phase synthesis procedure is possible; Scheme 6.3 outlines the general procedure for the synthesis and deprotection of oligoribonucleotides using this linker. First the tritylated linker on solid support **(9)** is deprotected, similarly to any nucleotide derivatized solid support. Then using any phosphoramidites standard solid supported oligonucleotide chain elongation is possible, affording the full length fully protected oligonucleotide. Next, full deprotection of the phosphates, nucleobases and 2′-*O*-silyl protecting groups of the aforementioned oligonucleotide is performed while retaining the oligonucleotide covalently bound to solid support. Finally, using UVA irradiation the fully deprotected 3′-phosphate oligonucleotide is removed from solid support.

The polystyrene-type solid support (NittoPhase®) in combination with the NPPOC-like linker **(9)** provides a unique way to deprotect DNA and RNA (Scheme 6.3). The linker, we will show, is completely inert to all deprotection conditions used in both DNA and RNA synthesis. This allows for complete deprotection while retaining the oligonucleotide covalently bound to the support.

This is advantageous in the same way that that regular solid supported synthesis is, *i.e.*, all excess reagents and by products are simply rinsed from support leaving the fully deprotected product. Traditionally the initial step of deprotection, ammonolysis, removes the oligonucleotide from the solid support.

This support-linker combination overcomes a major drawback encountered in oligonucleotide deprotection: the separation of the product from excess reagents, and protecting group by-products. It is particularly advantageous in the case of oligoribonucleotide deprotection, where removing excess fluoride reagent and salts are problematic and time consuming. An additional feature is that the counter ion of the oligo backbone phosphates can easily be exchanged to any desired cation post deprotection.

6.2.2.1 Evaluation of the chemical stability of the NPPOC linker to ammonolysis

Synthesis of oligothymidylic acid was carried out on a 1 μmol scale using 6.8 mg of NPPOC-solid support **(9)** and thymidine cyanoethylphosphoramidites under standard coupling conditions. The solid support (Figure 6.2) was treated under standard RNA/DNA deprotection conditions such as NH_4OH-EtOH (3:1, v/v) at room temperature for 48 h to evaluate the stability of the NPPOC linker under the basic condition. The reaction was covered with aluminum foil. After this treatment the solid support was subjected to photoinduced cleavage (discussed in detail in the next section).

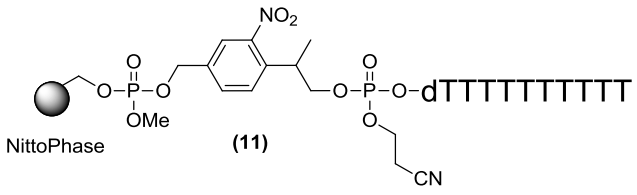


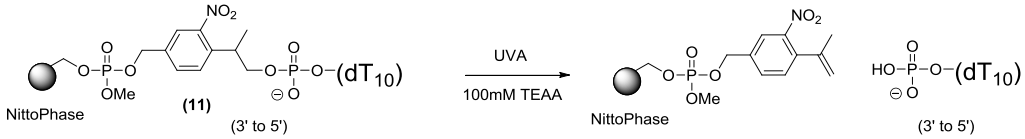
Figure 6.2. NPPOC-solid-phase synthesis of poly-dT (dT_{10}).

The ammonium hydroxide layer that was collected and lyophilized to dryness did not contain any UV active material, as determined by HPLC (λ_{max}= 260 nm), indicating that no product was cleaved from the solid support during ammonolysis.

To further validate the chemical stability of the NPPOC linker, a small sample of solid support **(11)** was treated with 40% methylamine in water at 60°C for 30 min; this is an alternative deprotection reagent for "ultra fast" deprotection of DNA and RNA sequences. The methylamine was decanted, lyophilized, and was also shown to contain no UV active material by HPLC (λ_{max}= 260 nm), confirming no oligonucleotide was released from the solid support. Thus, our NPPOC linker, unlike those previously described, is truly orthogonal to phosphate and base deprotection conditions.

6.2.2.2 Photoinduced cleavage of poly-dT from solid support (11)

The previously developed NPPOC linker by our group[2] (compound **(2)**; Figure 6.1) is cleaved from CPG solid support by photolysis in the presence of anhydrous organic base (e.g., 1% DIPEA or triethylamine in ACN),[2] which had to be removed prior to HPLC analysis of the crude oligonucleotide material.



Scheme 6.4. Photoinduced cleavage of poly-dT in 100 mM triethylammonium acetate buffer (pH=7).

To facilitate a "no work-up" procedure after the photocleavage step we used 100 mM triethylammonium acetate (TEAA) buffer (pH=8.0) which is commonly used for HPLC analysis of DNA/RNA oligonucleotides (Scheme 6.4). This buffer system showed increased efficiency in the release of the oligonucleotides compared to the basic anhydrous ACN, providing twice as much deprotected material possibly due to the increased solubility of DNA in the aqueous buffer over the ACN.

To determine the optimal irradiation time to cleave the oligonucleotide from the solid support, a time-dependent study was conducted. A 1 μmol (6.8 mg) deprotected dT_{10}-oligomer attached to the photocleavable NPPOC linked solid support (**10**) was suspended in 1 mL of 100 mM TEAA buffer in a Pyrex® glass vial (pretreated with Sigmacote®). The solution was irradiated while stirring in a photoreactor equipped with 13 UVA bulbs (315-400 nm). To measure the rate of

release, 1 µL aliquots were taken at regular intervals and the amount of released oligonucleotide was calculated over time (Figure 6.3).

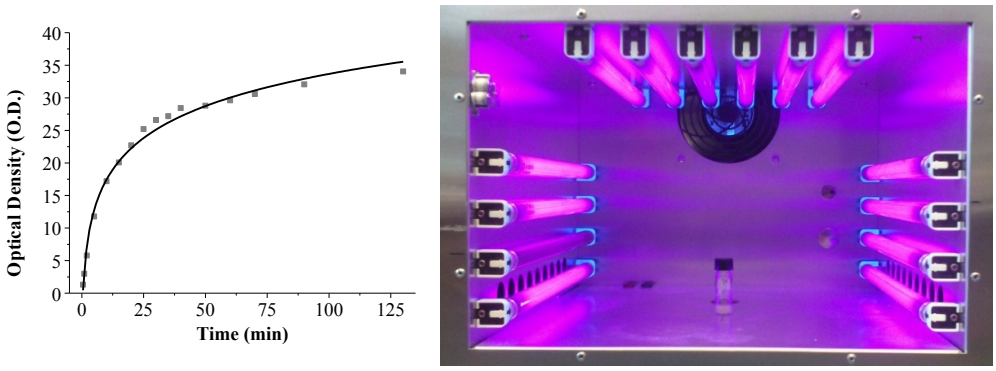


Figure 6.3. Time dependent release of dT_9T_p (3′-5′) under UVA irradiation**.**

The final amount of $(dT_p)_{10}$ collected after the photo cleavage was 0.45 µmol (34.5 OD) after UVA irradiation for 130 min, which is 42% of the 1 µmol (81.6 OD) of solid support used for the synthesis of dT_{10}. The lower yield of cleaved oligomer is likely due to two factors: 1) the high loading of the linker on solid support; this would have the effect of absorbing most of the photons at the support's surface essentially forming a "wall" that prevents deep penetration of light; and 2) the opacity of the NittoPhase® polystyrene solid support scatters the light preventing penetration deep into the beads. Unlike CPG and other polystyrene solid supports, the NittoPhase® is not translucent when placed in solvents. This slightly limits the effectiveness of this solid support. Dr. Johnsson, a former member of our group, demonstrated significantly faster deprotection times (30 ODs in 10 min) with lower loading of their photocleavable linker **(2)** on CPG solid support.[2] We determined that 60 min were necessary in the photo reactor to release the majority of the oligonucleotide that is available to be cleaved using our NittoPhase® solid support.

A standard $(dT_p)_{10}$ -oligonucleotide was synthesized using common 3′-phosphate on CPG (ChemGenes Corp.) and standard cyanoethyl protected thymidine phosphoramidites. This oligonucleotide was used as a standard to evaluate the purity of the photocleaved $(dT_p)_{10}$-oligomer. The sample was deprotected under standard conditions and analyzed by reverse-phase HPLC. The

photocleaved $(dT_p)_{10}$ showed a much cleaner trace than that of $(dT_p)_{10}$ synthesized by the conventional procedure (Figure 6.4).

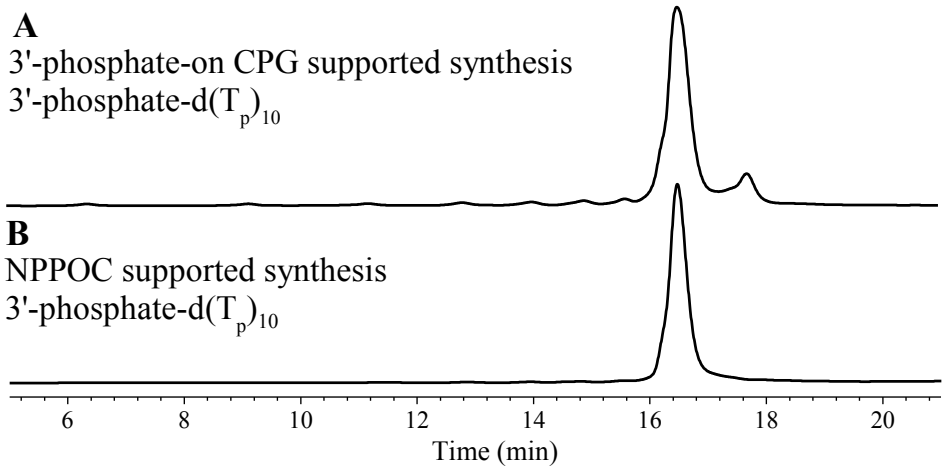


Figure 6.4. Reverse-phase HPLC analysis of crude 3′-phosphate $(dTp)_{10}$ oligonucleotide synthesized on A) 3′-phosphate ON CPG and B) on the photocleavable NPPOC linked solid support.

The new photocleavable NPPOC linked solid support **(6)** has a clear advantage over conventional solid phase synthesis of oligonucleotides. The workup procedure during deprotection of the material is exceptionally faster because the excess reagents used for deprotection and cleaved protecting groups are simply filtered off. This additionally affords much cleaner crude deprotected oligonucleotides as shown in the HPLC traces in Figure 6.4.

6.2.2.3 Synthesis of $(rU_p)_{10}$ for determination of the desilylation conditions on NPPOC solid support.

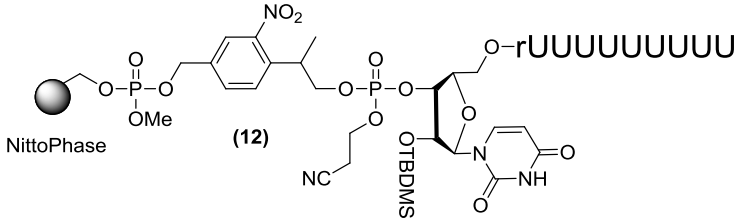


Figure 6.5. NPPOC linked poly-rU 10-mer, $(rU_p)_{10}$

To determine the optimal "on support desilylation" conditions, $(rU_p)_{10}$ **(12)** was synthesized using standard 2′-*O*-TBDMS cyanoethyl phosphoramidites on the NPPOC solid support (Figure 6.5). For comparison, $(rU_p)_{10}$ was also synthesized using the standard 3′-phosphate on CPG and deprotected and cleaved from CPG resin using standard conditions: NH_4OH for 48h; TREAT-HF (TEA-3HF) for a further 48 h at room temperature (Figure 6.6). The NPPOC support was treated with either TREAT-HF (for 48 h at room temperature) or 1.0 M TBAF-THF (for 24 h at room temperature) after the treatment with NH_4OH for 48 h. A final irradiation step released the oligonucleotide, which was analyzed by HPLC.

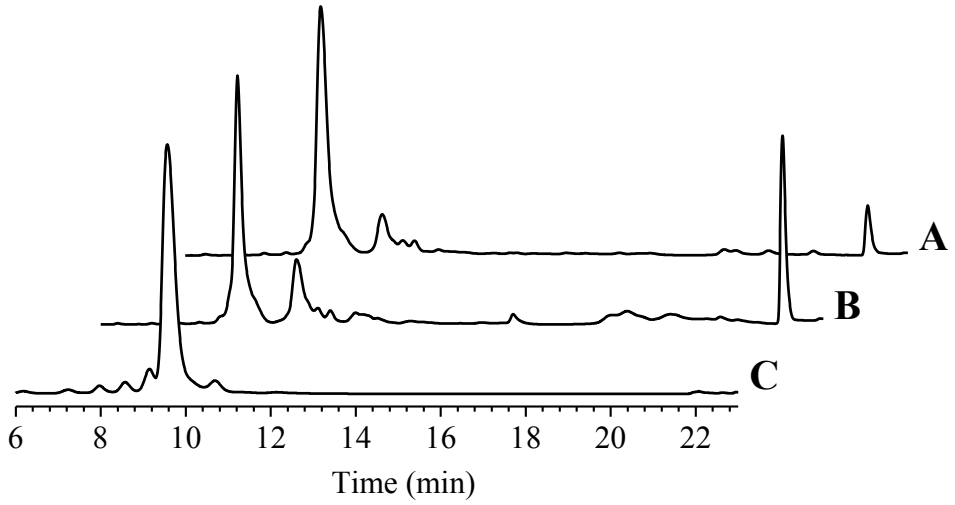


Figure 6.6. Reverse Phase HPLC analysis of $(rU_p)_{10}$ **(12)** synthesized on A)-B) NPPOC-polystyrene linked, and C) phosphate on CPG; desilylation conditions A) TREAT-HF at room temperature for 48h; B) TBAF at room temperature for 24h; C) TREAT-HF 48h room temperature.

The HPLC traces of the TREAT-HF and TBAF treated samples contained small amounts of "late" eluting peaks (Figure 6.6A and B respectively), indicative of partially silylated material resulting from incomplete desilylation. Reduced reaction kinetics of polymer bound materials, and/or poor "wetting" of the solid support (in TREAT-HF) may be responsible for these results.

Under the influence of my wise and ever persuasive supervisor, Prof. Masad Damha, I was told, and I quote, "*Matt, I challenge you to deprotect RNA as fast as*

preparing a cup of coffee.[..]..instant coffee that is! Ah Ha!" With those 'inspiring' words I was influenced to think of an alternative desilylation method, which led me to explore microwave irradiation. Like "instant coffee", I was determined to make "Instant RNA" on a new "Hassel-Free" photocleavable linker.

Hence, a series of $r(U_p)_{10}$ samples were treated with TREAT-HF under microwave irradiation for 5, 10 or 15 min at 80°C (2 Watts). As a result, all samples treated with microwave irradiation showed complete desilylation and apparent disappearance of the more retained peaks from the full length product at 10 min (Figure 6.7 B-D).

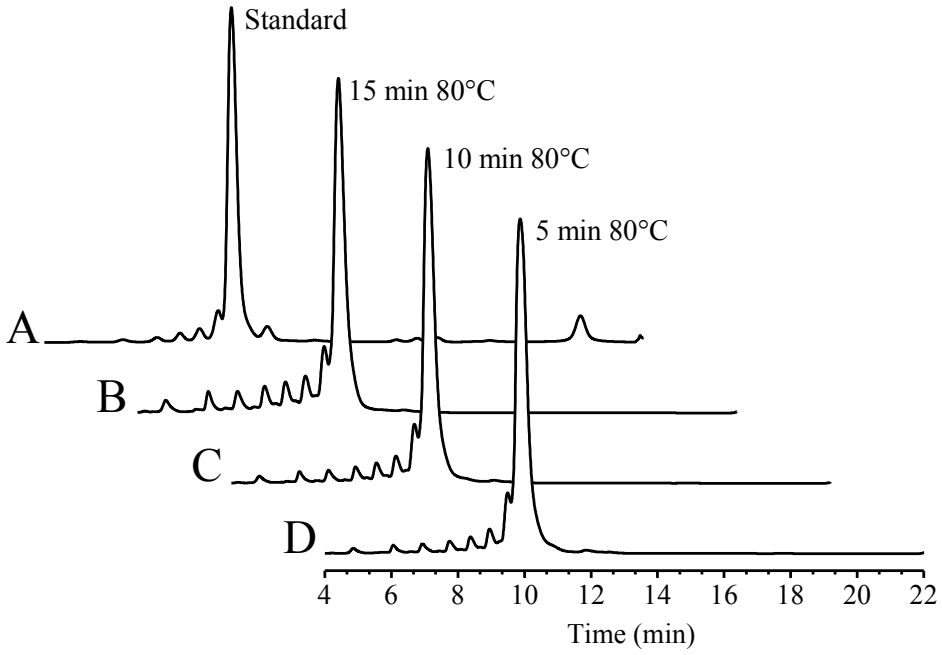


Figure 6.7. Reverse phase HPLC analysis of microwave assisted desilylation conditions of $r(U_p)_{10}$ on solid support: A) standard 3′-phosphate on CPG, TREAT-HF 48h r.t. B)-D): NPPOC-polystyrene: B) TREAT-HF, µW, @2W, 15min, 80°C; C) TREAT-HF, µW, @2W 10min, 80°C; D) TREAT-HF, µW, @2W, 5min, 80°C.

In contrast to conventional method (Figure 6.7 A), peaks eluting after $(rU_p)_{10}$ were not observed in all cases (Figure 6.9 B-C) indicating that the poly-uridine was completely desilylated on our NPPOC support. However, it is clear

that prolonged microwave treatment at 80°C causes increased amounts of chain cleavage (compare Figure 6.6 and Figure 6.7).

To the best of our knowledge, this is the first demonstration of microwave assisted deprotection of RNA 2′-silyl protecting groups. Furthermore, this method has the important advantage in that all of excess fluoride reagent and cleaved protecting groups can be washed off from the solid support, leaving behind the fully deprotected RNA bound on the solid support.

To confirm that the microwave assisted desilylation conditions are compatible with, and orthogonal to, the NPPOC linker, the TREAT-HF of the sample treated for 15 min at 80°C (Figure 6.7 B) was collected and the analyzed by RP-HPLC which is shown in Figure 6.8.

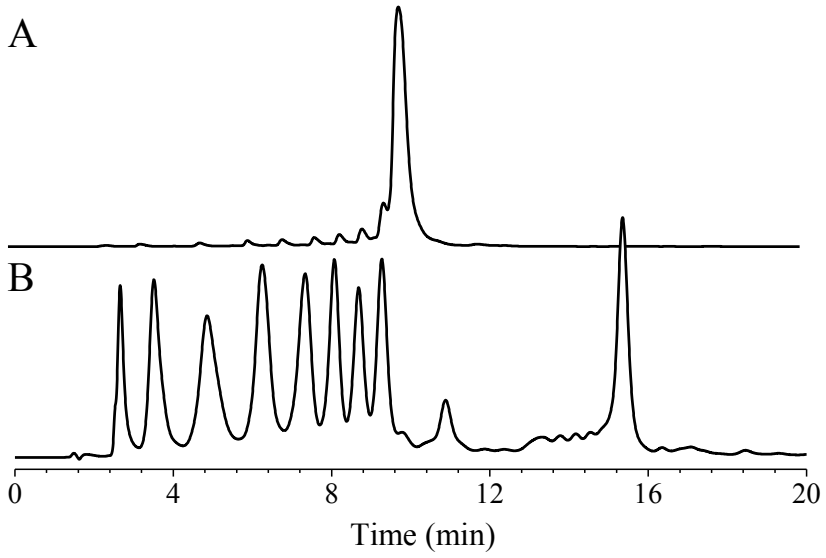


Figure 6.8. Reverse phase HPLC analysis of A) NPPOC-linked $(rU_p)_{10}$ obtained by our new procedure the components (15 min, 80°C) B) TREAT-HF layer decanted from the desilylation step.

The peaks shown in Figure 6.8 B eluting between 3 and 10 min represent cleavage products produced during the microwave-assisted desilylation. We also conclude that the linker is stable to the deprotection conditions as only short units exist and almost no full length material is observed. The total quantity of the products collected from the TREAT-HF solution was 6 ODs compared to the 42

ODs of the full length product isolated by HPLC (Figure 6.8 A), indicating that NPPOC linker is reasonably stable to the desilylation conditions.

With the synthesis of RNA the advantages of the photocleavable linker becomes even clearer in comparison to DNA. Even disregarding the increased speed associated with desilylation under microwave irradiation, the speed and simplified work-up procedure is an exceptional advantage. The conventional butanol/sodium acetate precipitation requires 2 hours of lyophilizing to remove residual butanol. Furthermore, the sample is generally re-suspended in water and passed through a size exclusion Sephadex® column to remove excess salt, collected in various amount of water, then once again lyophilized to dryness. Through these conventional procedures, an extra day can be easily added for obtaining the final product while making multi use of rather expensive autoclaved materials/reagents.

With the combination of photo-cleavable linker and on-support desilylation, the excess TREAT-HF is simply rinsed away before placing the solid support/buffer or solvent mixture in the photo reactor for 1-2 hours. The following section shows that total deprotection time can be further reduced to 40 min (yet to be optimized), which rivals the "ultrafast" deprotection conditions described by Farooqui and co-workers[17] which requires at least 1-2 days to isolate desalted RNA. While it is not quite "instant coffee", it is a step closer towards this goal!

6.2.2.4 Determination of the optimal deprotection conditions for mixed sequence RNA oligonucleotides

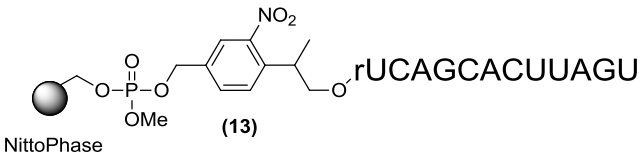


Figure 6.9. Mixed base RNA sequence used to evaluate on support deprotection conditions.

Next, the dodecanucleotide sequence, 5′-rUGAUUCACGACU-3′ **(13)** (Figure 6.9) was synthesized using RNA phosphoramidites with standard base

protection (G^{ibu}, A^{Bz}, and C^{Ac}) (Figure 6.11). In addition to determining that our new procedure can be applicable to the mixed sequence RNA, we were also interested in validating a nucleobase and internucleotide phosphate deprotection procedure on solid support. As a faster deprotection condition of internucleotide and nucleobases, we adopted the 40% aqueous $MeNH_2$ treatment outlined in current protocols for the deprotection of oligonucleotides,[18] and the "ultrafast" deprotection protocol involving 40% methylamine in water at 65°C for 15min and 15min TREAT-HF at 65°C (total time: 30 min).[17] We also tried further optimizations of our microwave-assisted desilylating condition. The results are shown in Figure 6.10 and 6.11.

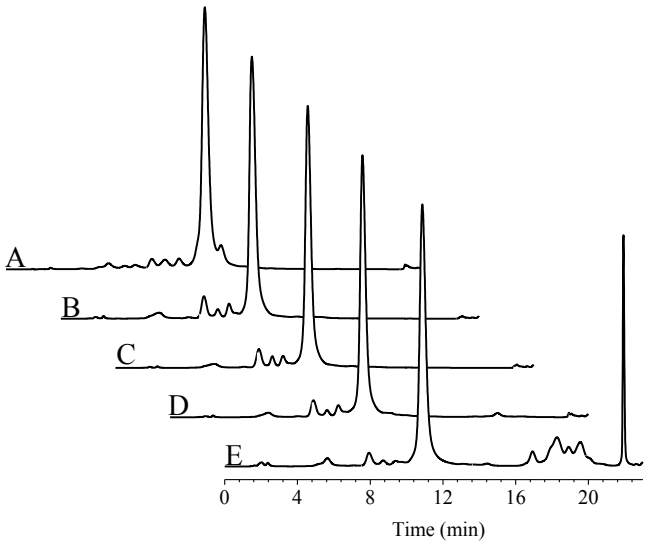


Figure 6.10. Reverse phase HPLC analysis of mixed base sequence **(13)** under various deprotection conditions. A) Standard synthesis on 3′ phosphate CPG deprotected by NH_4OH/EtOH (3:1, v/v), 48 h r.t., TREAT-HF 48h r.t. B)-D): NPPOC polystyrene synthesis of **(13)**; deprotected by B) NH_4OH/EtOH (3:1) 48h r.t. TREAT-HF μW 5 min at 80°C; C) 40% methylamine for 30 min at 60°C then TREAT-HF μW 5min at 80°C; D) 40% methylamine 30min 60°C TREAT-HF μW 10min at 60°C; E) 40% methylamine 30 min 60°C, TREAT-HF for 10 min at 65°C heat only (no μW). All sequences were characterized by HPLC-MS m/z cal. 3836.3 found 3836.8.

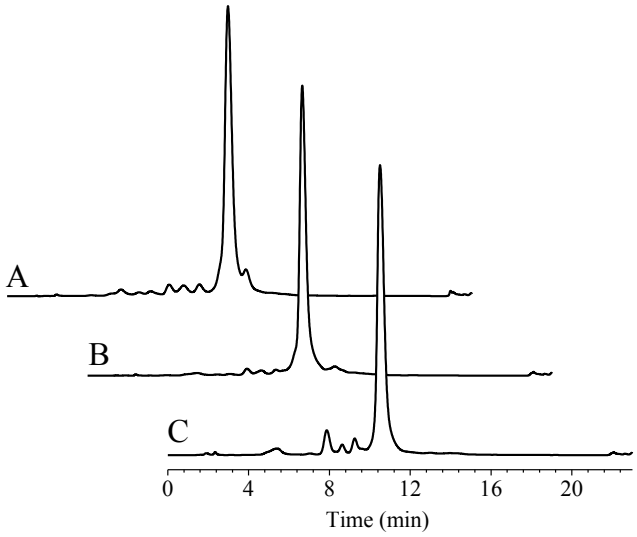


Figure 6.11 Reverse phase HPLC of the mixed base sequence **(13)** prepared on A) 3′-phosphate on CPG using standard deprotection conditions; B) 3′-phosphate on CPG deprotected by the "ultrafast" method, 40% methylamine in water at 65°C for 15min, and 15min TREAT-HF at 65°C; C) NPPOC supports and deprotected by 40% methylamine (30 min) and irradiated with TREAT-HF at 60°C for 10 min with μW.

Aqueous $MeNH_2$ deprotection gave excellent results, requiring only 30 min treatment at 60°C compared to 2 days, r.t. for aqueous ammonia deprotection (compare Figure 6.10, chromatogram C versus B). Further optimization of desilylation conditions were conducted with mixed sequence RNA **(13)** as shown in Figure 6.10.D and we found that a brief (10 min; 60°C) fluoride treatment under microwave irradiation was sufficient to effect complete desilylation. The benefits of microwave irradiation are appreciated by comparing the HPLC traces of an oligomer treated with TREAT-HF at 65°C for 10 min without microwave irradiation (Figure 6.10 E), to that of a sample treated under the same conditions with microwave irradiation (Figure 6.10 D). In addition to the deprotected product, significant amounts of silylated materials were observed in the sample not exposed to microwave irradiation (Figure 6.10 E). We recognize that complete desilylation may also occur under only thermal conditions (without the aid of microwave-irradiation), however, we have not yet investigated these conditions.

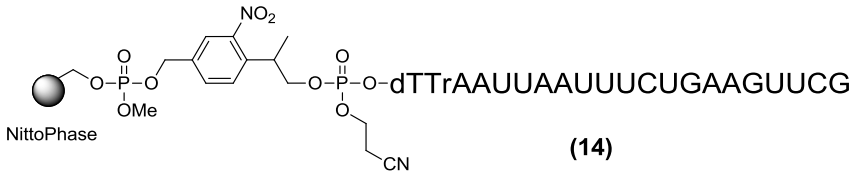


Figure 6.12. Sense strand of the luciferase siRNA duplex synthesized on the photocleavable NPPOC linker.

It is worth noting that our deprotection conditions (40 % aq. $MeNH_2$; 60 ^oC, 30 min; TREAT-HF; 10 min microwave; 60°C) rival the "ultrafast" deprotection conditions commonly employed in some laboratories[17] (Figure 6.11). While both methods are suitable, an advantage of our method is the ability to carry out the on-support unblocking of all protecting groups at the end of the synthesis which greatly simplifies and speed up post-synthesis processing. This will be particularly useful for large scale (> 1 mmol) synthesis.

To validate our method on longer RNA sequences, a 21-mer oligoribonucleotide corresponding to the sense strand of Luciferase siRNA[19,20] **(14)** was next synthesized on a 1 μmol scale (Figure 6.12). The sequence was compared to a standard synthesized on 3′-phosphate CPG using standard phosphoramidites and deprotection conditions. The NPPOC linked RNA **(14)** was deprotected with our deprotection conditions, namely, 40% $MeNH_2/H_2O$ for 30 min at 60°C, and TREAT-HF for 10 min at 60°C under MW irradiation; Cleavage: UVA irradiation in 100 mM TEAA buffer for 1 h. The results are shown in Figure 6.13. While both methods yielded excellent results our method required significantly shorter times to produce the pure isolated material (1-2 hours versus 1-2 days). The percent yields of products obtained from CPG and NPPOC polystyrene were 74.5% and 84.6%, respectively. The identity of the final product was confirmed by HPLC-MS (m/z cal. 6697.0; Found 6697.2)

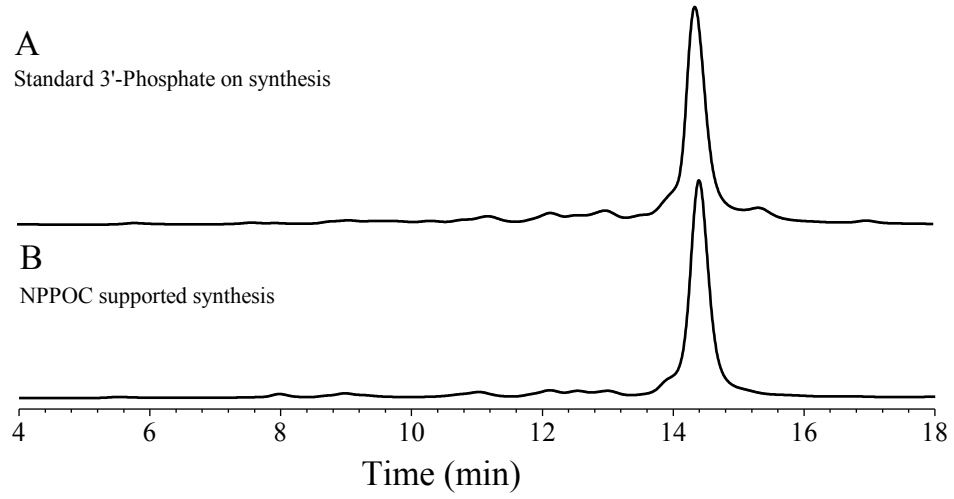
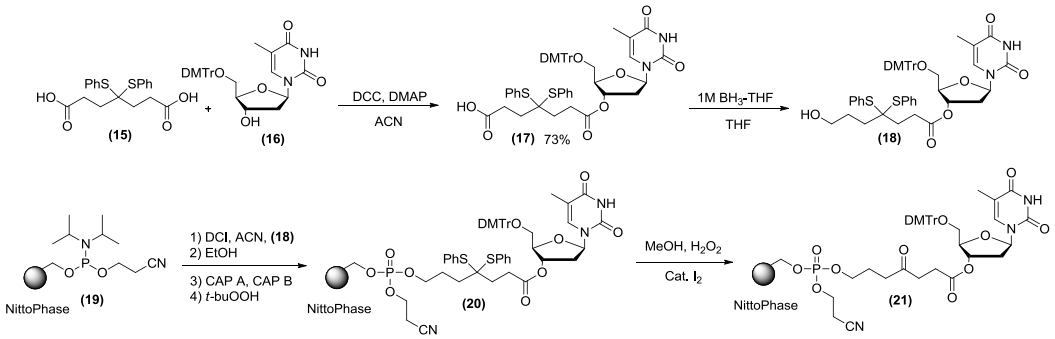


Figure 6.13. Reverse phase HPLC analysis of the 21-mer Luciferase sense strand synthesized on A) 3′-phosphate ON CPG deprotected by 48 h NH_4OH/EtOH (3:1) at room temperature. and 48 h TREAT-HF at room temperature. B) NPPOC supported synthesis of **(14)** deprotected by 40% methylamine 30min, 60°C; TREAT-HF, 10min, 60°C, microwave irradiation.

6.2.3 Synthesis of thymidine derivatized levulinyl linker and conjugation to solid support.



Scheme 6.5. Synthesis of thymidine derivatized levulinyl linker on solid support **(21)**.

4,4-Bis(phenylthio)heptanedioic acid (**15**) was condensed with 5′-*O*-DMTr-thymidine using DCC and cat. DMAP in ACN for 30 min. Next, the free acid of compound **(17)** was reduced to the primary hydroxyl group by slow addition of 1.0 M BH_3-THF at 0 °C. After work up and purification, compound **(18)** was isolated in 90% yield. To immobilize the thymidine derivative **(18)** to the solid support (NittoPhase®), the terminal hydroxyl group of resin was first

phosphitylated using 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite/TEA in THF. The solid support was rinsed with ACN over a filter, affording the phosphitylated solid support **(19)**. Using a peptide synthesizer, this solid support was mixed with compound **(18)**, DCI and ACN, and the resulting mixture gently agitated for one hour. Anhydrous ethanol was added to the reaction mixture to quench any remaining activated phosphoramidite on the solid support and agitated for an additional 10 min. The excess reagents were then filtered through the reaction vessel and remaining hydroxyl group on the solid support was capped by acetic anhydride/N-methylimidazole, and finally oxidized with *tert*-butyl hydroperoxide. The thioketal of compound **(20)** was then converted to carbonyl group by treatment with methanol, hydrogen peroxide and catalytic iodine. This mixture was allowed to react for 40 min before the excess reagents were washed off the solid support with ACN affording the thymidine-loaded solid support **(21)**. The loading amount of thymidine on solid support was quantitated as previously described[16] and found to be 172 μmol/gram.

6.2.3.1 Solid supported synthesis and deprotection of dT_{10} (22)

The synthesis and deprotection of dT_{10} **(22)** was carried out to demonstrate the stability of the levulinyl linker to phosphate deprotection conditions (triethylamine/pyridine and 4-methylpiperidine) as well as to assess cleavage conditions.

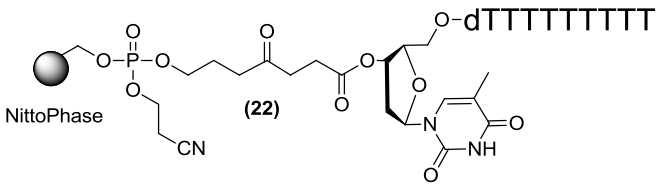


Figure 6.14. Poly-dT dT_{10} synthesized on the levnilyl linker solid support.

Synthesis of dT_{10} **(22)** was carried out using 5.8 mg (1 μmol) of solid support **(21)** and standard thymidine phosphoramidite (Figure 6.14). After solid supported synthesis, two separate supports containing **(22)** were treated with a 50:50 mixture of triethylamine/pyridine and the other with a 20% solution of 4-methylpiperidine in DMF. These mixtures were treated for 12 hours to ensure

complete decyanoethylation. The basic solution was filtered to remove excess reagents from the solid support, collected and lyophilized to dryness. The collected filtrates were analyzed by UV (λ_{max}= 260 nm) for the presence of prematurely cleaved oligomers. As none was detected, this demonstrates that the Lev-like linker is stable to triethylamine/pyridine/ 4-methylpiperidine treatment.

To cleave the deprotected DNA, the polystyrene beads were treated with 0.5 M solution of hydrazine hydrate in pyridine-AcOH (3:2, v/v) for 30 min. The excess hydrazine was then quenched by the addition of 2,4-pentanedione. The hydrazine layer was decanted from the solid support to collect the cleaved oligonucleotides. Finally, the solid support was then washed with autoclaved mili-Q water to collect residual cleaved product, and the hydrazine and water layers were combined and concentrated to dryness on a lyophilizer.

The collected samples were then analyzed by reverse phase HPLC and compared to a synthetic dT_{10} standard synthesized on Unylinker™ CPG support using standard procedures (Figure 6.15)

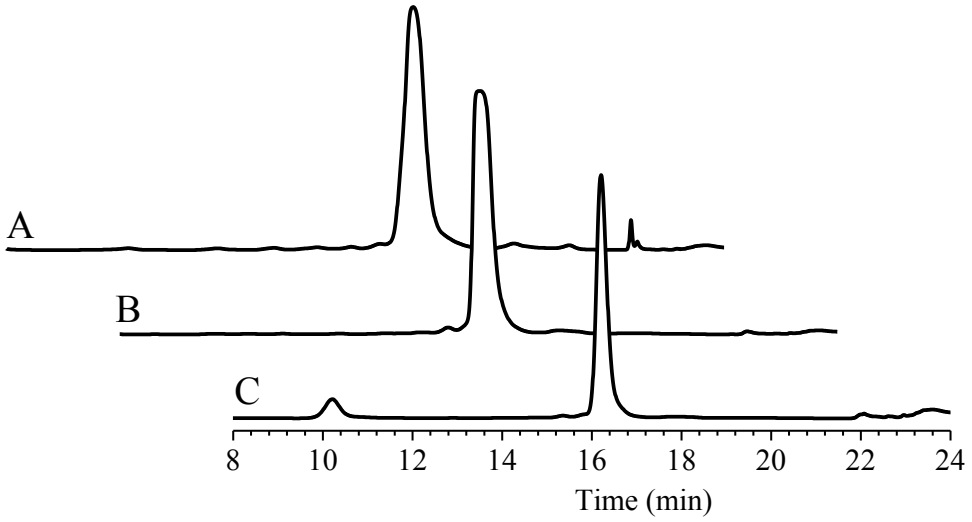


Figure 6.15. RP-HPLC profiles of dT_{10} synthesized on (A) Unylinker™ solid support, (B) levulinyl solid support (**21**) and deprotected by TEA-pyridine (50:50, v/v); (C) levulinyl solid support (**21**) and deprotected by 20% 4-methylpiperidine in DMF.

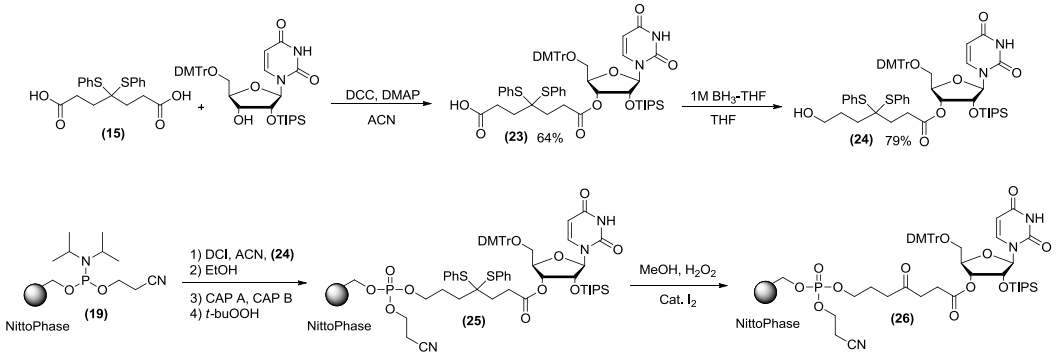
Both TEA treatment (Figure 6.15 B) and 20% 4-methylpiperidine treatment in DMF (Figure 6.15 C), for 12 hours, show complete phosphate deprotection

producing cleaner HPLC traces relative to the synthetic standard (Figure 6.15 A). (Note: treatment for 6-8 hours resulted in incomplete deprotection of the internucleotide phosphates). Each sample represented in Figure 6.15 was characterized by HPLC-MS (m/z cal. 2980.0; Found 2980.3). Yields were 85.2% (CPG) and 79% (Lev-polystyrene), respectively (as assessed by integration of HPLC traces).

The above results show that the levulinyl linker **(21)** is suitable for the solid supported synthesis, phosphate deprotection, and subsequent release of a poly-dT sequence. The linker is stable to 20% 4 –methylpiperidine (12 hours), conditions commonly used for remove the Fmoc protecting group. Hence, the use of this linker, in conjuction with Fmoc protected RNA phosphoramidites (soon to be launched by ChemGenes Inc), will permit the production of RNA of mixed base composition. This work is in progress in our laboratory.

6.2.3.2 Preparation of uridine-Lev derivatized polystyrene support

The synthesis of the uridine conjugated levulinyl linker **(26)** followed the same procedures outlined in Section 6.2.3 for the thymidine derivative **(21)** (Scheme 6.6).



Scheme 6.6. Synthesis of uridine derivatized levulinyl linker on solid support **(26)**.

Thus, 4,4-Bis(phenylthio)heptanedioic acid **(15)** was condensed with 5′-*O*-DMTr-2′-*O*-TIPS-uridine using DCC and catalytic DMAP in ACN. The carboxyl group of compound **(23)** was reduced to the primary hydroxyl group by treatment with 1.0 M BH_3-THF solution. The uridine derivative **(24)** was coupled with the phosphitylated solid support **(19)**, followed by thioketal deprotection as

previously described, producing the 5′-*O*-DMTr-2′-*O*-TIPS-uridine-loaded solid support **(26)**. The amount (loading) of uridine on the resin was found to be 160 μmol/gram.

6.2.3.3 Solid supported synthesis and deprotection of rU_{10} (27)

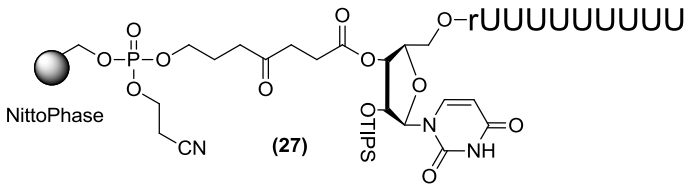


Figure 6.16. Synthesis of rU_{10} synthesized on the Lev-polystyrene solid support.

Using standard coupling conditions, rU_{10} **(27)** was synthesized on a 1μmol scale (6.2 mg of support) using the uridine derivatized solid support **(26)**. Post synthesis, rU_{10} **(27)** was treated with TEA-pyridine (1:1, v/v) for 12 h at room temperature to cleave the cyanoethyl phosphate protecting groups. The solid support was filtered, washed with ACN, and dried by a flow of Argon. The partially deprotected rU_{10} sequence on the solid support was desilylated by treatment with TREAT-HF and 10 min of microwave irradiation at 60°C.

The TREAT-HF layer was then decanted and set aside to be analyzed for uridine content. The solid support was then washed with ACN to remove any excess TREAT-HF and cleaved silyl groups. The fully deprotected rU_{10} sequence was released solid support by treatment with hydrazine hydrate (r.t., 30 min) as described previously.

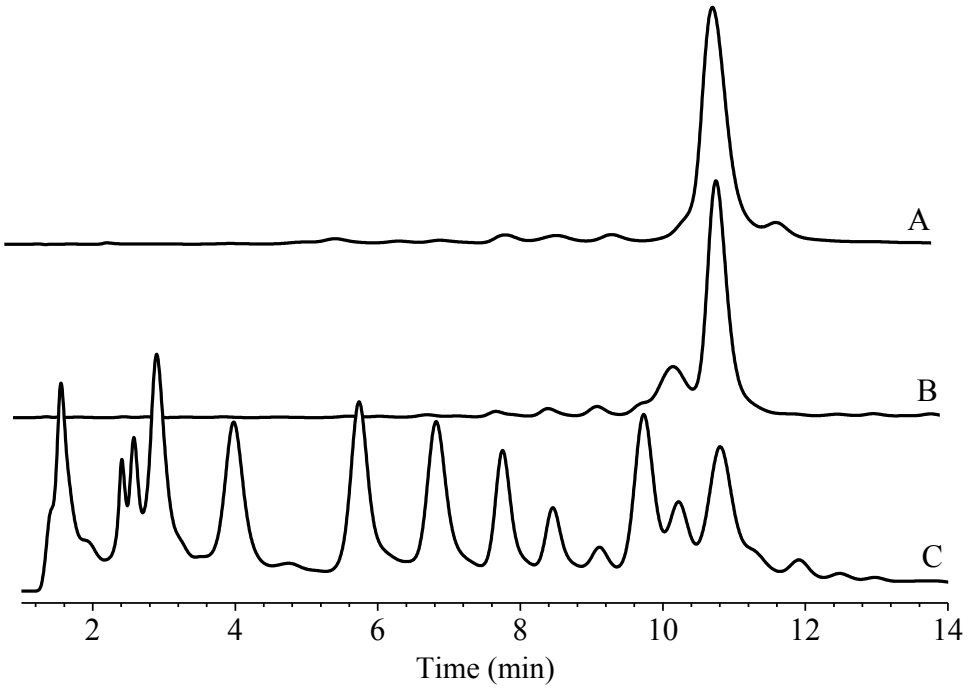


Figure 6.17. Reverse Phase HPLC analysis of rU_{10}. A) Standard synthesized on Unylinker™ support deprotected under standard conditions; B) **(27)** deprotected by triethylamine/pyridine (50/50), TREAT-HF 10min μW; C) TREAT-HF layer collected during deprotection of rU_{10} (crude shown in B above).

The HPLC analysis results of the deprotection of rU_{10} prepared via Unylinker and Lev linker solid supports are shown in Figure 6.17 A and B, respectively. It is evident that the purity and yield of product obtained from both methods are comparable: 77 ODs (0.79 μmol) versus 89 ODs (0.92 μmol), respectively.

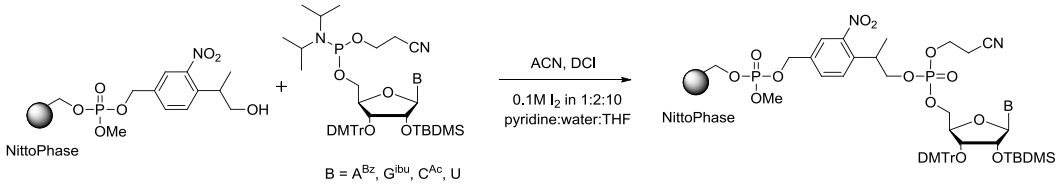
To confirm that the levulinyl linker was stable to the microwave assisted desilylation conditions, the TREAT-HF layer collected from the deprotection of **(27)** was subjected to standard RNA work up procedure (see experimental) to determine if any full-length product was cleaved during the desilylation. The HPLC trace indicated that no significant amount of the full length product was detected in the TREAT-HF layer (Figure 6.17 C). The total amount of UV absorbing material collected from the TREAT-HF layer was 8 ODs, which is significantly less than the total collected from the hydrazine cleavage treatment (89 ODs; m/z cal. 2999.7; Found 2999.0).

6.1 Conclusions

We have presented the successful synthesis of DNA and RNA oligomers on two novel polystyrene solid supports. The photocleavable NPPOC support makes it possible to synthesize, fully deprotect and isolate the oligonucleotide sequences under very mild conditions. The levulinyl support was stable to cyanoethyl phosphate and TREAT-HF microwave assisted 2′-desilylation. This protocol sets the stage for synthesis of RNA of mixed base composition using N-Fmoc protected RNA monomers. The methods described are directly applicable to the synthesis of modified RNA containing base sensitive or bio-labile groups at specific positions of the sugar-phosphate backbone. These experiments are also ongoing in our laboratory.

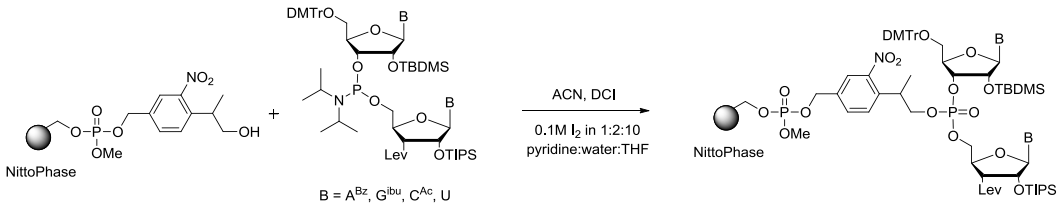
6.2 Future work

The NPPOC solid supports described above generated oligonucleotides containing a 3′ phosphate. Our support can be readily adapted to produce the more common 5′-phosphate derivatives (Scheme 6.7) or oligonucleotides lacking a terminal phosphate all together (Scheme 6.8). In the first approach, reverse phosphoramidite are used to synthesize the target strand the 5′ to 3′ direction, releasing the 5′-phosphorylated oligonucleotide in the final photolysis step.



Scheme 6.7. Method to produce 5′-phosphate oligonucleotides using the NPPOC support.

The second approach adopts the strategy developed by our group, whereby a dimer (dNpN) unit is attached to the support via its internucleotide linkage.[2] Upon synthesis and photolysis, the dimer segment becomes the "dNpN overhang" commonly present in siRNA sequences.



Scheme 6.8. Method to produce oligonucleotides with no 5′ or 3′ phosphate using the NPPOC support.

6.3 Experimental:

Materials

Thin layer chromatography was performed on EM Science Kieselgel 60 F-254 (1mm) plates. Silicycle 40-63 μm (230-400 mesh) silica gel was used for flash chromatography. Pyridine was distilled from CaH_2 after refluxing for several hours. Acetonitrile, dichloromethane, THF, and toluene were dried using and Mbraun Inc. SPS 800 solvent dryer. All other anhydrous solvents were purchased from Sigma-Aldrich. Chemicals and reagents were purchased from either Sigma-Aldrich(Oakville, Ontario, Canada). Standard cyanoethyl phosphoramidites and methoxyphosphoramidic chloride phosphitylation reagents were purchased from ChemGenes Inc. Underivatized NittoPhase® was purchased from Kinovate Life Sciences Inc., 501 Via del Monte, Oceanside, CA.

Instrumentation

The solid phase syntheses of oligonuc1eotides were conducted on an ABI 3400 synthesizer. UV spectra for oligonuc1eotide quantitation (absorbance measurements) were measured at 260 nm on a Varian Cary I or 300 UV-VIS dual beam spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian 300, 400 or 500 MHz spectrophotometer with chemical shift values reported in ppm. ^{1}H-NMR and ^{13}C-NMR spectra were referenced to residual solvent. ^{31}P-NMR were recorded at 80 MHz with a Varian 200MHz spectrophotometer and were measured from 85% H_3PO_4 as an external standard. Photolysis was performed in a Luzchem LZC photoreactor using their LZC-UVA lamps, which have a range of 316-400 nm.

Standard Solid-phase Oligonucleotide Synthesis: ABI 3400 synthesizer

Standard phosphoramidite solid-phase synthesis conditions were used for the synthesis of all oligonucleotides. Each synthesis was done at approximately 1 μmole scale using either controlled pore glass (500 Å CPG) with a 5′-*O*-DMTr-thymidine derivatized monomer attached by a succinyl linker[16] or Unylinker® solid support. All phosphoramidites were prepared as 0.15 M solutions in acetonitrile (ACN) for RNA and 0.1 M for DNA. 4,5-Dicyanoimidazole (0.25 M in ACN) was used to activate phosphoramidites for coupling. Detritylations were accomplished with 3% trichloroacetic acid in CH_2Cl_2 (110 s). Capping of failure sequences was achieved with a 16% N-methylimidazole in THF (CAP A) and acetic anhydride:pyridine:THF, (1:2:2, v/v/v) (CAP B) for (30 s). Oxidation was done using 0.1 M I_2 in pyridine-water-THF (1:2:10, v/v/v) unless otherwise stated.

Step	*Operation*	*Reagent*	*time (s)*
1	Detritylation	3% TCA in CH_2Cl	120
2	Coupling	0.15 M or 0.1M in MeCN 0.25 M DCI in MeCN	300 (DNA) 600 (RNA)
3	Capping	CAP A, CAP B	20
4	Oxidation [21]	3M *tert*-BuOOH in toluene	40

Table 6.1. Reaction times used on the ABI 3400 solid phase synthesizer.

Deprotection of Oligonucleotides General procedures:

Standard deprotection of DNA

The oligonucleotide is removed from the solid phase column and placed in a polypropylene screw cap vial. 1 mL of concentrated ammonium hydroxide is added directly and allowed to shake gently for 48h at room temperature. To work up, the vial is allowed to vent with cap removed for 1-2 h the sample is then decanted from the solid support into an eppendorf tube. The solid support is then rinsed with 500 μL of autoclaved mili-Q water, decanted and combined with the first decant. This sample is then cooled to -78°C and placed in a lyophilizer. The

sample is then resuspended in 1 mL of autoclaved water, quantitated by UV then analyzed by reverse phase HPLC.

Standard deprotection procedure for RNA

After the chain elongation of oligonucleotides, the solid support was transferred to a polypropylene screw cap vial. To the resin, 1 mL of a 3:1 mixture of concentrated ammonium hydroxide/anhydrous ethanol was added directly and allowed to shake gently for 48 h at room temperature. To work up, the vial was allowed to vent for 1-2 h by removing of screw cap, and then the sample was decanted from the solid support into an eppendorf tube. To collect cleaved oligonucleotides effectively, the solid support was then rinsed with 500 µL of a 50:50 mixture of autoclaved mili-Q water and ethanol decanted and combined with the solution collected in the first decantation. This sample was then cooled to -78°C and placed in a lyophilizer to dryness.

Desilylation

The dried down sample was then resuspended with 150 µL of TREAT-HF and allowed to gently shaked for a further 48h. Once the desilylation was completed, 50 µL of 3 M sodium acetate buffer (pH 5.5) was added to the TREAT-HF mixture and vortexed. To this mixture 1 mL of dry *n*-butanol cooled to -20°C was added to precipitate the oligonucleotide. This mixture was vortexed then placed on dry ice (-78°C) for 1 hour. After centrifugation for 5min (32,000 rpm), the butanol layer was decanted off and discarded. The *n*-butanol precipitation was then repeated to ensure complete removal of all TREAT-HF. The residual n-butanol was then removed in the lyophilizer (2-3h). Once the sample was dried, it was resuspended in 1 mL of autoclaved milli-Q water. The sample was desalted by passing it though a Sephadex® size exclusion NAP-G25 (GE Healthcare) to remove excess salt from the sample. The collected samples containing oligonucleotides was combined and concentrated to dryness, then resuspended in 1 mL of autoclaved milli-Q water to accurately assess the crude mixture.

"On support deprotection" procedure of DNA using the photocleavable linker (9)

The solid support was treated with either ammonium hydroxide for 48 h at room temperature or 40% methylamine in water for 30 min at 60°C. The excess reagents were simply removed by filtration.

To cleave the oligonucleotides from solid supports, the polystyrene beads were placed in a 1 Dram vial that has been pre-treated with Sigmacote® followed by a small stir bar and 1 mL of 100 mM triethylammonium acetate (TEAA) pH 7.5, the starting buffer used with our reverse phase HPLC column, for ease of subsequent analysis. This vial was then placed in a photoreactor equipped with UVA bulbs (315-400 nm), and the sample was exposed to the UVA with stirring for 1 hour. A small portion of the solution phase was aliquoted to quantitate the amount of sample obtained, and then subjected to reverse phase HPLC analysis.

"On-support deprotection" procedure of RNA using the photocleavable linker (9)

Post synthesis the solid support was treated with either a 3:1 mixture of ammonium hydroxide/ethanol for 48 h at room temperature or 40% methylamine in water for 30 min at 60°C. The excess reagents were simply removed by filtration and the solid support was rinsed with ACN.

Desilylation was achieved by suspending the solid support in 200 µL of TREAT-HF in a Biotage® Initiator reaction vial with the supplied stir bar, and subjected to microwave irradiation up to 60°C and held for 10 min. The excess reagents were then simply removed by filtration and the solid support was rinsed with ACN.

To cleave the oligonucleotides from solid supports, the polystyrene beads were placed in a 1 Dram vial that has been pre-treated with Sigmacote® followed by a small stir bar and 1 mL of 100 mM triethylammonium acetate (TEAA) pH 7.5, the starting buffer used with our reverse phase HPLC column, for ease of subsequent analysis. This vial was then placed in a photoreactor equipped with UVA bulbs (315-400 nm), and the sample was exposed to the UVA with stirring

for 1 hour. A small portion of the solution phase was aliquoted to quantitate the amount of sample obtained, and then subjected to reverse phase HPLC analysis.

"On-support deprotection" of DNA or RNA using the levulinyl linker (21) or (26)

Post synthesis the solid support was treated with a 50:50 mixture of triethylamine/pyridine for 12h at room temperature. The excess reagents were simply removed by filtration and the solid support was rinsed with ACN.

Desilylation in the case of RNA was achieved by suspending the solid support in 200 µL of TREAT-HF in a Biotage® Initiator reaction vial with the supplied stir bar, and subjected to microwave irradiation up to 60°C and held for 10 min. The excess reagents were then simply removed by filtration and the solid support was rinsed with ACN.

To remove the oligo from the solid support the polystyrene was treated with 1 mL of a 0.5 M solution of hydrazine hydrate in a mixture of pyridine/acetic acid (3:2) for 30 min at room temperature with gentle shaking. The excess hydrazine was quenched by the addition of 75 µL of 2,4-pentanedione and shaken for a further 5 min. The sample was then centrifuged and the solvent is decanted into a separate eppendorf tube. The solid support was then rinsed with 500 µL of autoclaved milli-Q water and combined with the previously decanted sample. This solution was cooled to -78°C on dry ice and then lyophilized to dryness. The mixture was then resuspended in 1 mL of autoclaved milli-Q water, quantitated and subjected to reverse phase HPLC analysis.

Reverse phase HPLC conditions

HPLCs were performed on an Agilent 1200 series machine. Reverse phase HPLCs were performed with a Hamilton PRP-1 5 µm 100Å 2.1 x150 mm HPLC column. Oligoribonucleotide synthesized were analyzed using a 0-15% gradient over 20 min. Solvent A: 100 mM TEAA pH 7.0. Solvent B: 100% ACN.

Time (min)	%A	%B	Flow (mL/min)

1	0.00	100	0.00	1.00
2	2.00	100	0.00	1.00
3	20.00	86	14	1.00
4	22.00	0	100	1.00
5	29.00	0	100	1.00
6	30.00	100	0.00	1.20
7	39.00	100	0.00	1.20

Table 6.2. Reverse phase HPLC gradient for oligoribonucleotides made on solid support A= 100mM TEAA pH 7.0 with 5% ACN; B=ACN.

Analysis of Oligoribonucleotides:

The oligoribonucleotides were purified by reverse phase HPLC chromatography. The solution containing the purified oligonucleotide was then lyophilized to dryness and dissolved in 1 mL of water and analysed by high resolution HPLC MS.

Entry	Sequence (5′ to 3′)	Mass calc. (m/z)	Mass found (m/z)
Oligonucleotides synthesized by the NPPOC linker			
1	dTx10	3060.0	n.d.
3	rUx10	3079.7	3079.8
5	UGAUUCACGACU	3836.3	3836.8
7	GCUUGAAGUCUUUAAUUAAdTdT	6697.0	6697.2
Oligonucleotides synthesized by the levulinyl linker			
	dTx10	2980.0	2980.3
9	rUx10	2999.7	2999.0

Table 6.3. High resolution LC MS data for oligonucleotides.

The sequence of all oligonucleotides were also confirmed by the synthesis of standards using standard protocols and co-injected in ion exchange HPLC.

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Chapter 7 Contributions to Knowledge

7.1 Summary of contributions

7.1.1 Development of a solution phase method to produce oligonucleotides, using soluble ionic supports

We developed a viable method for the synthesis of oligoribonucleotide sequences in solution. We achieved our goal to use conventional reactants and reagents in combination with a novel tetraalkylphosphonium soluble support to produce short (2-10 nt) oligonucleotides in solution. The phosphonium ionic tag was found to be effective for precipitating the growing oligonucleotide, while being stable to all conditions used in standard oligonucleotide synthesis cycles. We attached a nucleoside to this phosphonium tag through a succinyl linker, and used the resulting material as the key starting building block for synthesis. Furthermore, the phosphonium ionic tag introduced a unique method to monitor the reaction process via ^{31}P-NMR and mass spectrometry. For example, the permanent ionic nature of the molecule significantly enhances the sensitivity of the MS method (ESI-TOF), allowing for reaction progression to be monitored very accurately. Overall, the new solution phase method affords gram quantities of the ribonucleotides with purity and yields attainable by solid phase synthesis.

7.1.2 First successful synthesis of dimer and trimer ribonucleotide phosphoramidite and their use in solution phase and solid phase synthesis of oligoribonucleotides

We developed a reliable synthetic method for the preparation of isomerically pure dimer and trimer ribonucleotide phosphoramidites. We re-introduced the TIPS protecting group for 2′-hydroxyl protection, which proved to be superior when placed at the terminal unit relative to TBDMS protection. TIPS resists 2′ to 3′ migration under the conditions required to remove a vicinal (3′) Lev group, and ensuing 3′-phosphitylation. The isomeric purity of the newly prepared dimers were confirmed by ^{1}H-NMR and ^{31}P-NMR analysis.

Furthermore, we conducted extensive NMR studies on the relative rates of delevulination, 2′ to 3′ silyl migration, and phosphate demethylation in 2′-*O*-TBDMS and 2′-*O*-TIPS protected ribonucleosides and RNA dimers. Through these experiments, we established optimal conditions for TIPS removal, and confirmed the resistance of this protecting group towards migration.

The dimer and trimer phosphoramidites allow for longer chain extensions at each coupling stage of RNA synthesis, significantly reducing the total number of steps required in the synthesis and yield of target RNA oligomers. Furthermore, when precautions are taken to deprotect the oligonucleotide chain, the products produced via the 'blockmer' approach are devoid of (N-1) failure sequences, which should greatly facilitate and simplify the purification process.

7.1.3 Synthesis and application of two novel orthogonally cleavable linkers for the chromatography free synthesis of block oligoribonucleotide phosphoramidites

To combine the solution phase oligoribonucleotide synthesis strategy (discussed in Chapter 2) with the production of block RNA amidites, an alternative to the succinyl linker used to conjugate the ionic tag to the growing nucleotide was investigated. To this end we synthesized a photocleavable 2-(2-nitrophenyl) propoxycarbonyl (NPPOC) derivative and a levulinyl like, γ-keto ester linker, cleavable by hydrazinolysis. These linkers serve as a handle to attach the ionic soluble supports, as well as the nucleotide unit providing for a chromatography free synthesis of oligoribonucleotides. Once the desired sequence is obtained, the orthogonal linker is selectively removed from the 3′-hydroxyl terminus mildly removing the ionic tag. The free 3′-hydroxyl terminus is converted into a functional phosphoramidite. The orthogonality of these linkers was successfully demonstrated.

7.1.4 Synthesis, and on solid support deprotection of oligoribonucleotide using orthogonally cleavable linkers. Development of "Instant RNA"

We have presented the successful solid phase synthesis of DNA and RNA using two novel polystyrene solid supports. We show that the NPPOC linker is completely inert to all deprotection conditions used in standard RNA deprotection strategies. This allows for the complete synthesis and on support full deprotection of all protecting groups, including a novel TREAT-HF microwave assisted 2′-silyl removal, followed by photoinduced release from the support. This is accomplished with exceptional speed and purity, yielding our so-called "instant RNA." The modified NPPOC linker allows for the deprotected RNA to remain on the solid support, facilitating simple isolation from deprotection reagents by simply filtration. The levulinyl linker also introduces a method to deprotect RNA while retaining a covalent ester linkage to solid support. The levulinyl like linker was found to be stable to cyanoethyl phosphate deprotection conditions as well as on support TREAT-HF microwave assisted 2′-silyl removal, followed by mild hydrazinolysis to release the fully deprotected RNA.

7.2 Provisional patent filings

Hassler, M., Damha, M., Orthogonal ionic tags for the use in liquid synthesis, PCT/CA2012/000784 (will appear in the WIPO on August 2013) Filing number: 05001770-479USPR

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7.4 Conference presentations

94th Canadian Chemistry Conference and Exhibition in Montreal, **2011**, Quebec, Canada *Advances in The Synthesis of Oligoribonucleotides*, Matthew Hassler, N.M.K. Reddy, T.-H. Chan, M.J. Damha (Oral presentation)

McGill Organic Chemistry Lecture Series, McGill University, Montreal, QC, Canada, **2010,** *New Developments in RNA Oligonucleotide Synthesis, Novel linkers for solid supported* synthesis, Matthew Hassler, M.J. Damha (Oral Presentation)

XIX International Round Table on Nucleosides, Nucleotides and Nucleic Acids, August 29th - September 3rd, **2010**, Lyon, France, *Solution Phase Synthesis of oligoribonucleotides: "Ionic Tag" Soluble Supports and RNA Dimer Block Phosphoramidites*, Matthew Hassler, N.M.K. Reddy, R. A. Donga, T.-H. Chan and M. J. Damha (Poster presentation)

240th ACS National Meeting: Chemistry for preventing and combating disease, August 22 – 26, **2010**, Boston, Massachusetts, USA, *New developments in the synthesis of oligoribonucleotides: Use of dimer/trimer blocks in combination with an "ionic tag" soluble support*, Matthew Hassler, N.M.K. Reddy, T.-H. Chan, M.J. Damha (Oral presentation)

93rd Canadian Chemistry Conference and Exhibition in Toronto, **2010**, Ontario, Canada *New Methods in RNA Oligonucleotide Synthesis*, Matthew Hassler, N.M.K. Reddy, T.-H. Chan, M.J. Damha (Oral presentation)

92nd Canadian Chemistry Conference and Exhibition, Hamilton, ON, Canada, May **2009** *Towards Large Scale Synthesis of Oligoribonucleotides Using Ionic Soluble Supports*, Matthew Hassler, Tak-Hang Chan, Masad.J. Damha. (Oral presentation)