New methods for the synthesis of RNA, novel RNA pro-drugs and RNA microarrays

Jeremy G. Lackey

Department of Chemistry, McGill University

Montreal, QC, Canada

January, 2010

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

For Ge, Keira, Michael, and Peanut.

Copyright statement

Abstract

The demand for synthetic oligonucleotides has grown exponentially over the past decades as genome sequencing, functional genomics, polymerase chain reaction (PCR)-based detection methods, and gene silencing via RNA interference (RNAi) consume enormous numbers of DNA and RNA oligonucleotides. Although various RNA synthesis chemistries now allow oligoribonucleotides to be produced routinely, the higher complexity and cost of RNA (over DNA) has somewhat limited its availability.

A major goal of this thesis work was aimed at finding ribonucleoside synthons that potentially benefit two critical aspects of RNA manufacturing: yield and ease of post-synthesis processing. Towards these goals, we developed methods for the synthesis of RNA using 2'-*O*-Lv and 2'-*O*-acetal Lv (ALE) ribonucleoside derivatives. Deprotection of the RNA chains consisted of a three-step deprotection scheme, which eliminated the need for any harsh basic hydrolytic steps, generally composed of: (1) treatment with anhydrous NEt_3 (r.t., 1 h) to deblock the phosphate's cyanoethyl groups; (2) hydrazinolysis (r.t., 30 min – 4 h) to simultaneously deprotect the nucleobases and 2'-OH positions, and (3) fluoride treatment (r.t., 30 min) to effect cleavage from the controlled pore glass solid support. Significantly, the rather mild conditions to remove 2'-*O*-Lv or 2'-*O*-ALE protecting groups did not lead to RNA strand scission. Furthermore, in the case of 2'-*O*-ALE protection, higher step-wise monomer coupling yields (~98.7%) was possible, since the ALE protection is less bulky than conventional silyl protection, *i.e.* TBDMS. Furthermore, both 2'-*O*-Lv or 2'*O*-ALE chemistries are completely compatible with the synthesis cycles used by all automated gene synthesizers.

With adjustments in protecting group strategies for the 5'-OH, exocyclic amino nucleobase groups and the development of a light-labile solid support, two other major goals were achieved: (1) the first *in situ* synthesis of RNA on microarrays, and (2) synthesis of chemically modified RNA strands with 2'-*O*-acetal ester and 2'-*O*-acetal ester pyrrolidines in order to increase lipophilicity and

cellular permeability over native RNA. When RNA synthesis was carried out with 5'-*O*-NPPOC 2'-*O*-ALE monomers on a microarray ("chip"), deprotection typically involved (1) cleavage of the photolabile 5'-protecting group; (2) treatment with anhydrous NEt_3 (r.t., 1 h) to deblock the phosphate's cyanoethyl groups; (3) hydrazinolysis (r.t., 30 min – 4 h) to simultaneously deprotect bases and 2'-OH positions. The latter step could also be accomplished with ethylenediamine at room temperature. An RNase A assay was performed as "proof-of-principle" to demonstrate the value of a DNA-RNA microarray for studying enzyme kinetics and specificity on oligonucleotide based libraries. We showed that RNase A acts effectively on a DNA-RNA substrate with measurable kinetics analogous to those of the reference substrates.

The novel 2'-*O*-modified RNA were tested as short interfering RNA pro-drugs ("pro-siRNA") that would cross the cell membrane and be hydrolyzed (at the 2'-*O*-ester groups) by ubiquitous esterases to release the active (siRNA) molecules. Indeed, both siRNA and pro-siRNA prepared via 2'-*O*-ALE chemistry were shown to be active in an RNAi luciferase gene knockdown assay, confirming the integrity of the synthesized RNA strands and the promise of the pro-siRNA approach.

Abrégé

Durant les deux dernières décennies, la demande pour la production d'oligonucléotides synthétiques a connu une croissance exponentielle. Ceci est essentiellement dû à l'utilisation de quantités d'ADN et d'ARN, de plus en plus importantes, pour des applications telles que : le séquençage du génome, la génomique fonctionnelle, les méthodes de détection basées sur les réactions de polymérisation en chaîne (PCR) ou encore l'extinction de l'expression génétique *via* le mécanisme d'interférence de l'ARN (ARNi). Malgré l'existence de plusieurs voies de synthèse chimique des oligoribonucléotides, permettant actuellement leur production en routine, la complexité et le coût supérieur de l'ARN (comparé à l'ADN) représentent toujours une limitation importante.

Le travail effectué au cours de cette thèse avait pour objectif principal la mise au point de nouveaux synthons ribonucléosidiques, conçus afin de permettre l'amélioration de deux aspects critiques dans la fabrication de l'ARN : son rendement et sa transformation post-synthétique. Dans cette optique, nous avons développé une nouvelle voie de synthèse de l'ARN utilisant les unités ribonucléotidiques de construction comportant des groupements 2'-*O*-Lv et 2'-*O*-acétal Lv (ALE). La déprotection de la chaîne d'ARN ainsi synthétisée s'est alors effectuée en trois étapes, permettant d'éviter l'emploi de conditions basiques drastiques. Ces trois étapes ont été généralement composées de : (1) un traitement par la triéthylamine anhydre (1 h, à t.a.), permettant l'hydrolyse des groupements cyanoéthyle du squelette phosphate ; (2) une hydrazinolyse (30 min à 4 h, à t.a.), permettant la déprotection complète des nucléobases et des groupements en position 2' ; (3) un traitement aux ions fluorures (30 min à t.a.), permettant la libération de l'ARN du support solide CPG. De manière significative, l'emploi des conditions douces pour l'hydrolyse des groupements 2'-*O*-Lv et 2'-*O*-ALE, n'a pas conduit à une scission de l'ARN. De plus, dans le cas de l'utilisation du groupement 2'-*O*-ALE, des rendements des étapes de couplage supérieurs à ceux de la synthèse d'ARN standard ont pu être obtenus (~98.7%), car le groupement 2'-*O*-ALE présente un moindre encombrement stérique que le groupement

TBDMS couramment utilisé. L'utilisation des groupements 2'-*O*-Lv et 2'-*O*-ALE a également été entièrement compatible avec les cycles de synthèse standard, employés par les synthétiseurs automatiques disponibles sur le marché.

L'ajustement dans la stratégie de protection des groupements 5'-OH et des amines exocycliques, ainsi que la mise au point d'un support solide photo-labile, ont permis de réaliser deux autres objectifs majeurs : (1) le première synthèse *in situ* de l'ARN, réalisée sur une puce (microréseau) ; et (2) la synthèse de brins d'ARN chimiquement modifiés par des groupements 2'-*O*-acétal ester ou 2'-*O*-acétal ester pyrrolidine, présentant un caractère lipophile plus important, ainsi qu'une meilleure pénétration cellulaire, par rapport à l'ARN naturel. Lorsque la synthèse d'ARN a été effectuée en utilisant des unités monomères 5'-*O*-NPPOC-2'-*O*-ALE sur une puce (microréseau), les conditions de déprotection ont consisté en : (1) la déprotection du groupement photo-labile en position 5' ; (2) le traitement par la triéthylamine anhydre (1 h, à t.a.), permettant l'hydrolyse des groupements cyanoéthyle du squelette phosphate ; (2) l'hydrazinolyse (30 min à 4 h, à t.a.), permettant la déprotection complète des nucléobases et des groupements en position 2'. Cette dernière étape a pu être également effectuée en utilisant de l'éthylènediamine à température ambiante. Un test à la RNase A a ensuite été effectué, afin de réaliser la « preuve de concept » des puces à ADN-ARN, ayant pour objectif de démontrer leur valorisation potentielle pour les études de spécificité et de cinétique enzymatique. Ainsi, nous avons montré que la RNase A agit de façon efficace sur un substrat ADN-ARN, avec une cinétique comparable à celle mesurée pour les substrats de référence.

Les ARN modifiés en position 2' ont été évalués en tant que prodrogues de siARN (« pro-siARN »), qui, après passage de la membrane cellulaire, sont hydrolysés au niveau des groupements 2'-ester par des estérases cellulaires, libérant ainsi les molécules de siARN active. Ainsi, les siARN et les pro-siARN, préparés en utilisant la stratégie 2'-*O*-ALE, ont montré une activité lors de leur évaluation dans un test d'extinction du gène de la luciférase *via* l'ARNi. Ces résultats confirment l'intégrité des ARN synthétisés et montrent leur potentiel dans l'approche de pro-siARN.

Acknowledgements

I would first like to thank my mentor Professor Masad J. Damha for his guidance and patience with me. He was a major factor in the development of my creativity, ability to think critically, curiosity, professionalism and ambition. For this, I'll be forever grateful to him.

Next I'd like to thank God for looking out for me over these past years. I don't think he'll mind if I thank him second since he created Professor Damha (The Damha-nator) anyways.

I'd also like to thank Professor Franco Cerrina from The University of Madison-Wisconsin for inviting me to collaborate on the RNA Microarray project as well as Dr. Mark Somoza and Dr. Debbie Mitra for doing the synthesis and characterization of the RNA microarrays (Chapter 4). I'd also like to thank Dr. Francis Robert and Professor Jerry Pelletier from McGill Biochemistry, who carried out the luciferase assays (Chapter 2, 3, and 5).

I need to give special thanks to the Damha lab members past and present for all their help, friendship and kindess. Especially David Sabatino (The Italian Stallion) for showing me the ropes in my first year and for being a great fan of The Toronto Maple Leafs; Alexander Wahba (Wabs) for always taking the time to teach me new things and for being a great friend; Paul Peng (Penger) and Robert Donga (Dings) for great science talk and always having clean glassware; Mathew Hassler (Quagmire) for keeping the equipment in the lab running and always changing his pump oil; Pascal Gallant (Pasqually) for reminding me of the principles of chemistry and for being able to enjoy a good practical joke; Glen Deleavey (Glenster) for keeping me excited about new discoveries and for being down for whatever (as long as it was past 5 pm); Richard Johnsson (The Swede) for teaching me a lot about chemistry and for always being down for a Timmy Ho's; Adam Katolik for being a good friend and for not going postal.

I'm also very grateful to Colleen McNamee and Chantal Marotte and all of the office ladies for their help with administrative tasks. Thanks are also due for Paul Xia for his expertise in NMR and Nadim Saade for all things MS.

Finally, I'd like to thank my wife Genevieve Deschenes. She has made me a better person in every way, and I could not have written this thesis without her.

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Abbreviations

2'F-RNA	2'-deoxy-2'-fluororibonucleic acid
4-NBOM	4-nitrobenbenzyloxymethyl
A	adenosine
Å	Angstrom
Ac	acetate
Ac_2O	acetic anhydride
ACE	bis(acetoxyethoxy)methyl
AcOH	acetic acid
Ade	adenine
AGO2	Argonaute2, the endonuclease at the heart of human RISC
ALE	acetal levulinyl ester
B	base
BMT	5-benzylmercaptotetrazone
Bn	benzyl
bp	base pair
Bz	benzoyl
C	cytidine
cDNA	complementary DNA
CE	2-cyanoethyl
CEM	2-cyanoethyloxymethyl
CMPI	2-chloromethyl-pyridinium iodide
COSY	correlation spectroscopy, homonuclear (NMR)
CPG	controlled pore glass
Ctmp	1-(2-chloro)-4-methoxypiperidin-4-yl
Cy3	cyanine3
Cy5	cyanine5
Cyt	cytosine
d	doublet
DABCO	1,4 diazabicyclo-[2.2.2]octane

DBU	1,8-diazabicycloundec-7-ene
DCC	N,N'-dicyclohexylcarbodiimide
DCI	4,5-dicyanoimidazole
DCM	dichloromethane
dd	doublet of doublets
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DEPC	diethylpyrocarbonate
DIPEA	*N*,*N*-diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMEM	Dulbecco's modified Eagle medium
dmf	*N*,*N*-dimethylformamidine
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	2-tert-butyldithiomethyl
DTT	dithiothreitol (*threo*-1,4-dimercapto-2,3-butanediol)
EDA	ethylenediamine
EEDQ	2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
ESI-MS	electrospray ionization mass spectrometry
Et	ethyl
EtOAc	ethyl acetate
EtOH	ethanol
ETT	5-ethylthio-1H-tetrazole
FBS	fetal bovine serum
FMOC	9-fluorenylmethoxycarbonyl
Fpmp	1-(2-fluorophenyl)-4-methoxypiperidin-4-yl

G	guanosine
Gua	guanine
HPLC	high performance (or high pressure) liquid chromatography
J	scalar coupling contstant (in Hz)
Lv	levulinyl
MABOM	4-(*N*-dichloroacetyl-*N*-methylamino)benzyloxymethyl
MALDI	matrix-assisted laser desorption/ionization (mass spectrometry)
Me	methyl
MeCN	acetonitrile
MeOH	methanol
miRNA	microRNA
MMTr	4-monomethoxytrityl
mRNA	messenger ribonucleic acid
MsCl	mesyl chloride
Mthp	4-methoxytetrahydropyran-4-yl
NBOM	nitrobenzyloxymethyl
NMP	*N*-methylpyrrolidinone (*i.e.*, 1-methyl-2-pyrrolidinone)
NMR	nuclear magnetic resonance
NPPOC	nitrophenylpropyloxycarbonyl
nt	nucleotide
ODU	optical density units, defined as the hypothetical A_{260} of a solution of the sample of interest in 1 mL water, in a 1-cm path cuvette.
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Ph	phenyl
piRNA	PIWI interacting RNA
PivOM	pivaloyloxymethyl

PO	phosphodiester (linkage)
PS	phosphorothioate (linkage)
pyr	pyridine
r.t.	room temperature
R_f	retention factor (in TLC, the ratio of the distance traveled by the center of a spot to the distance from the baseline to the solvent front)
RISC	RNA-induced silencing complex, the effector complex of RNA interference
rN	ribonucleoside
RNA	ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal RNA
s	singlet
SELEX	systematic evolution of ligands by exponential enrichment
shRNA	short hairpin RNA
siRNA	small interfering RNA
snoRNAs	small nucleolar RNAs
SNP	single-nucleotide polymorphism
snRNPs	small nuclear ribonucleoproteins
ss	single-stranded
ssRNA	single stranded RNA
t	triplet
T	thymidine
TBAF	tetra-n-butylammonium fluoride
TBDMS	tert-butyldimethylslyl
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
TFE	2,2,2-trifluoroethanol
THF	tetrahydrofuran
Thf	tetrahydrofuran-2-yl
Thp	tetrahydropyran-2-yl
Thy	thymine
TLC	thin-layer chromatography
T_m	melting temperature, the temperature at which (for a simple two-state transtition) both the mole fraction of nucleic acid in the duplex form and the mole fraction of denatured nucleic acid are 0.5
TOM	*tris*(isopropylsilyl)oxy)methyl
TPS-Cl	triisopropylbenzenesulfonyl chloride
TREAT-HF	triethylamine trihydrofluoride
tRNA	transfer RNA
U	uridine
Ura	uracil
UTR	untranslated region
UV	ultraviolet (spectroscopy)

Chapter 1. Introduction

1.1 Biological Importance of Nucleic Acids

To put it simply, nucleic acids are the fundamental building blocks of life. In general, the flow of genetic information is typically as follows: The storage of all genetic information is carried out by deoxyribonucleic acid (DNA) which is determined by a sequence of bases adenine (A), guanine (G), cytidine (C) and thymine (T). This genetic blueprint is in turn transcribed into messenger ribonucleic acid (mRNA) which is the platform for protein expression. The mRNA can then be translated into protein molecules by the help of transfer RNA (tRNA, amino acid carriers) and ribosomes (made up of RNA and protein) which stitch the amino acids together to form proteins, the major machinery of life.

For many years RNA was regarded as a rather apathetic member of gene expression and its role was simple and well understood. However, in the past two decades it has become very apparent that RNA plays a much more complex role.

1.2 DNA and RNA Structure

1.2.1 Nucleoside and Nucleotide Structure

Nucleotides are the phosphate esters of nucleosides (**Figure 1.1**) and these are the constituents of both DNA and RNA. DNA is made up of 2'-deoxyribonucleotides and RNA is made up of ribonucleotides. The nucleosides contain a nitrogenous heterocyclic base and a ribose (pentose) sugar in the furanose configuration; more precisely, 2-deoxyribose for DNA and ribose for RNA. It is interesting to note that in aqueous solution ribose exists predominantly in the pyranose form, but the furanose form is also very common for biomolecules.[1] The major bases are either monocyclic pyrimidines (cytosine (Cyt), thymine (Thy) for DNA and Cyt, uracil (Ura) for RNA) or bicyclic purines (adenine (Ade), and guanine (Gua) for both DNA and RNA).

1.2.2 Nucleoside Conformation

The conformational space available to nucleosides is based on their sugar pucker and *syn-anti* orientation of the nucleobases. Sugar "puckering" is described by the displacement of the carbons-2' and -3' from the median plane of C1'-O4'-C4'. Deoxyribonucleosides favor the C-2'-*endo* conformation, whereas the ribonucleosides favor the C-3'-*endo* conformation (**Figure 1.2**). The *endo* face is on the same side as the C5' and the base whereas the *exo* is on the opposite face. In terms of the pseudorotation cycle[2] of the furanose ring, the sugar puckers of ribose and deoxyribonucleosides are located in the north (N) and south (S) puckers, which also reflect the shape of the C-C-C-C bonds in the C-2'-*endo* and C-3'-*endo* puckers, respectively (**Figure 1.2**).

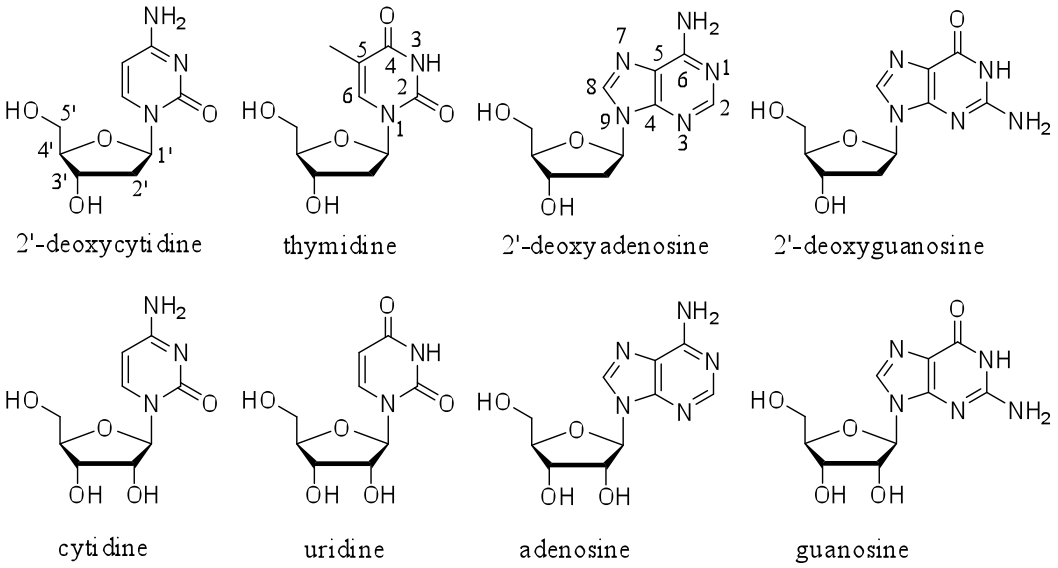


Figure 1.1. Structures of the four deoxyribonucleosides (top) and ribonucleosides (bottom). Note: The IUPAC numbering also applies to the ribonucleosides.

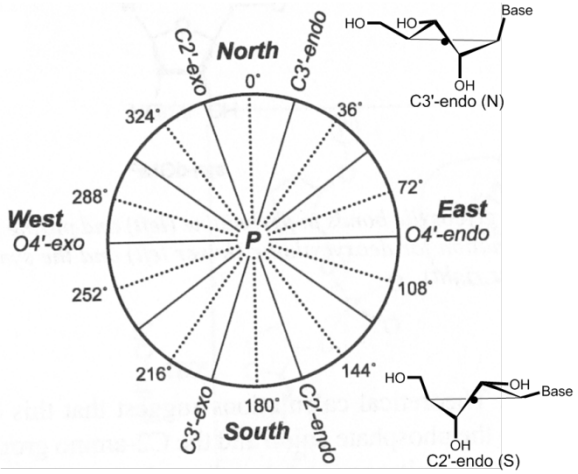


Figure 1.2. Pseudorotation wheel showing the preferred conformations of RNA (C3'-*endo* (N)) and DNA (C2'-*endo* (S)).

1.2.3 Primary Structure of DNA and RNA

The nucleotide units of both DNA and RNA oligonucleotides are joined together by 3'-5'-phosphodiester linkages. (**Figure 1.3**). At neutral pH the phosphates are negatively charge (pK_a ~ 1.6). The presence of a 2'-hydroxyl group in RNA has significant impact on both its conformation, folding, and hydrolytic stability.[3]

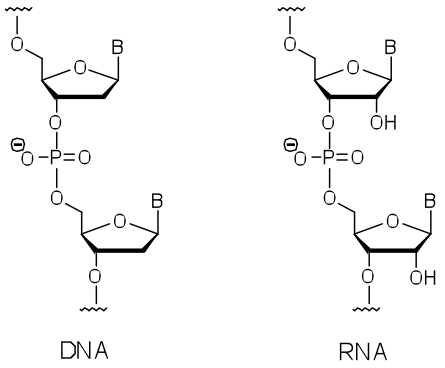


Figure 1.3. Primary structure of DNA and RNA.

1.2.4 Secondary Structure of DNA and RNA

Double stranded DNA and RNA typically adopt a right-handed helical conformation where the strands run anti-parallel to each other. DNA is most commonly found in the B-helical form[4] with its sugars puckered in the C2'-*endo* conformation. It is now widely recognized that DNA is polymorphic, adopting many right-handed helical conformations denoted by letters A to T[1]. At low humidity and high salt it favours the A-form, which is associated with the C3'-*endo* sugar pucker. RNA almost exclusively adopts an A-form helix and is unable to switch to the B-form largely due to steric interactions of 2'-OH groups.[5] Both DNA and RNA can adopt a rare left-handed helix termed the Z-form.[6,7] The A-RNA and B-DNA helical structures and conformation parameters are shown in **Figure 1.4** and **Table 1.1**.

	A-RNA	B-DNA
Helical sense	Right	Right
Residues per turn	11	10
Twist per bp	32.7	36
Displacement by bp/Å	4.4	-0.2 - -1.8
Base tilt	16-19	-6
Rise per bp/Å	2.8	3.3-3.4
Major groove width (Å)	2.7	11.7
Major groove depth (Å)	13.5	8.8
Minor groove width (Å)	11	5.7
Minor groove depth (Å)	2.8	7.5

Table 1.1. Helical parameters of A-RNA and B-DNA.[1]

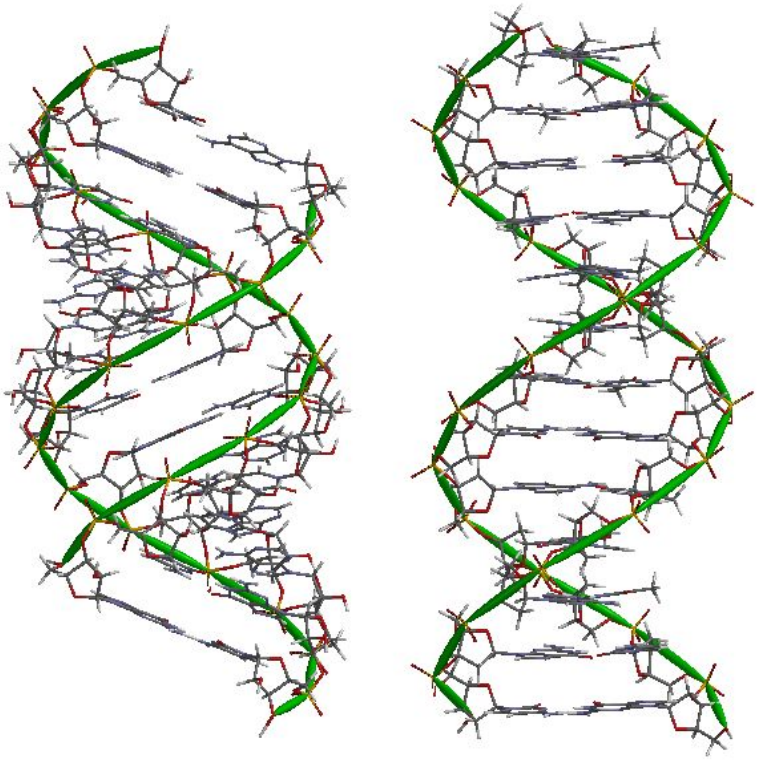


Figure 1.4. Helical conformation of A-RNA (left) and B-DNA (right). Figure generated by Spartan '08 V.2.0.

1.3 Salient Biological Functions of RNA

1.3.1 The Role of RNA in Translation

By the early 1950s it was clear that DNA was localized in the eukaryotic nucleus whereas proteins were being synthesized in the cytoplasm in the presence of three major types of RNA: mRNA, transfer RNA (tRNA), and ribosomal RNA (rRNA). [8] Ribosomes are composed of both rRNA and protein,[9] and are the cell's "work bench" upon which proteins are assembled from amino acid building blocks.[10] Francis Crick proposed the existence of 'adaptor molecules' that were able to bind to the nucleotide code of mRNA, thereby facilitating the transfer of amino acids to growing polypeptide chains.[10] He went on to argue this molecule was RNA as it is well suited as a recognition molecule through base

pairing. This hypothesis was later confirmed by Hoagland who showed a specific portion of RNA in the cytoplasm was covalently bound to amino acids – tRNA.[11] Today we know that there are many other flavours of RNA with very important and versatile biological functions. These are described in the following section.

1.3.2 Non-Coding RNAs in Human Gene Regulation

1.3.2.1 Small Nuclear and Nucleolar RNAs (snRNAs, snoRNAs)

After RNA transcription, the nascent precursor mRNA strand is processed (spliced) before it can be used as template in translation. In this process, non-coding intervening sequences or introns are removed (spliced out) and exons (coding region) are joined together to form the mature mRNA. RNA splicing takes place within a multi-component complex term the "splicesome", a collection of tightly bound small nuclear RNAs (snRNAs) and small nuclear ribonucleoproteins (snRNPs, or "snurps").[12,13] Another large group of snRNAs termed small nucleolar RNAs (snoRNAs) guide chemical modifications of rRNAs, tRNAs and snRNAs which affect gene regulation.[14]

1.3.2.2 RNA interference (RNAi) and siRNA

RNA interference (RNAi)[15] is a gene silencing pathway that has attracted much attention recently from both academia and the pharmaceutical industry. RNAi is triggered by the presence of exogenous long double-stranded RNA which is in turn cleaved by the enzyme Dicer into smaller double stranded RNA duplexes (21-23 nucleotides long) often termed small interfering RNA (siRNA). These molecules contain 5'-phosphates and two nucleotide overhangs at the 3'-ends.[16] The presence of small double stranded RNA triggers the activation of the RNA Induced Silencing Complex (RISC). At the heart of RISC is Argonaute 2 (AGO2) which is a multi-functional protein that unwinds the small double stranded RNA taking up one of the strands termed the guide strands, and cleaving the other termed the passenger strand.[17] The antisense strand (guide strand) remains bound to RISC and activates the complex which seeks out and destroys

mRNA that is complementary to the guide strand (**Figure 1.5**).[18] The cleavage of mRNA occurs between nucleotides 10 and 11 on the guide strand relative to the 5'-end.[19] RISC is then free to seek out and destroy more mRNA complementary to the guide strand. In 2006 Craig Mello and Andrew Fire received the Nobel prize in Physiology or Medicine for the discovery of RNAi.

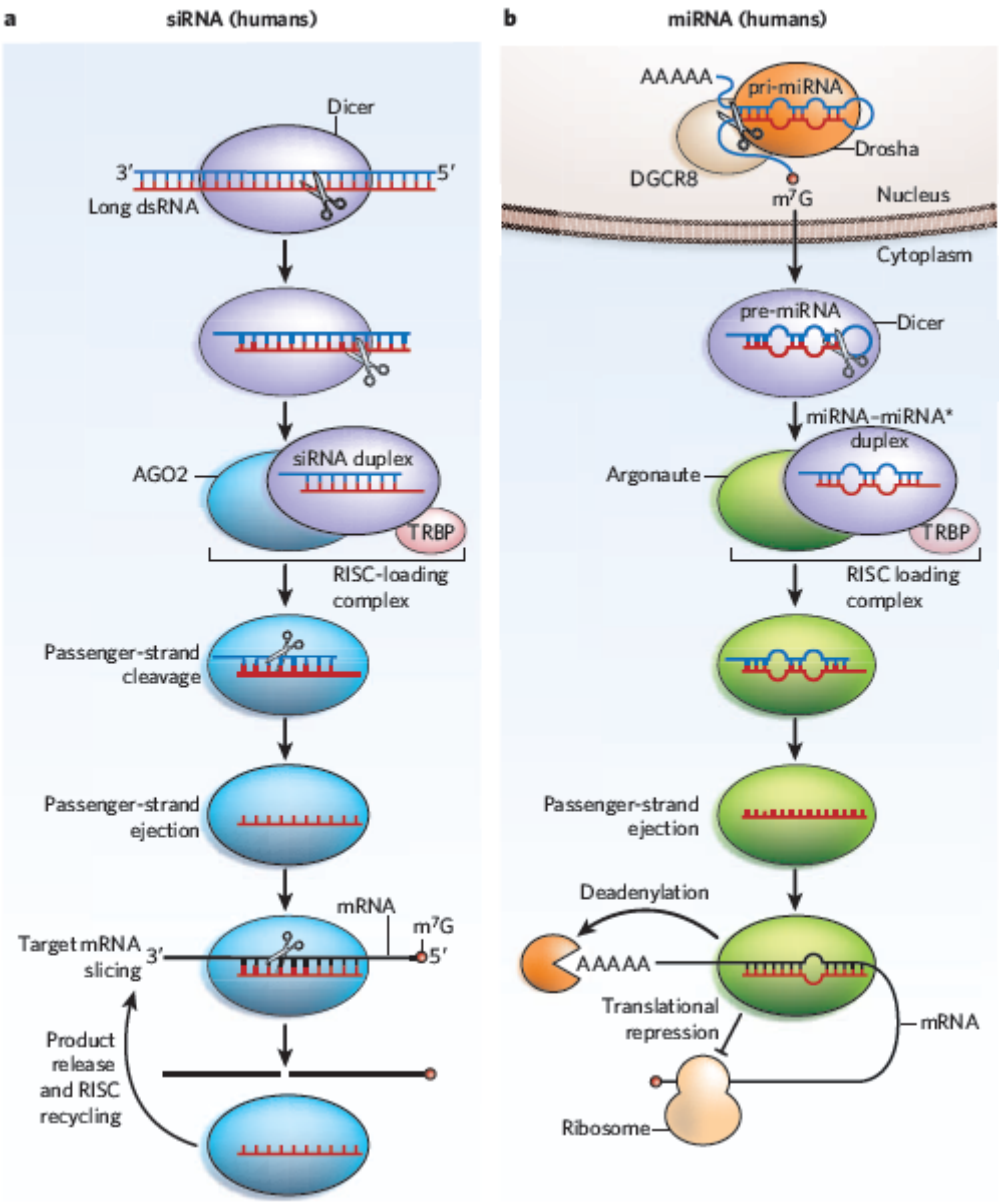


Figure 1.5. Biogenesis and mechanism of action of A) siRNA and B) miRNA in humans. Figure was adapted from ref (18).

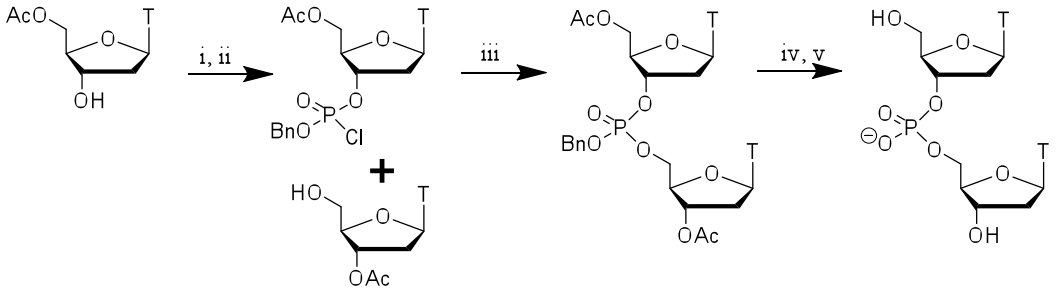
Exogenous synthetic siRNA also enters the RNAi pathway and has much promise for therapeutic applications.[20] It should also be noted that single stranded RNA can cause gene knockdown and may act through RNAi,[21] although this phenomenon is still somewhat controversial. In addition, there are other classes of 'siRNA-like' molecules that affect gene regulation such as PIWI-interacting (piRNAs) which are 24-29 nt single stranded RNAs (ssRNAs) that are germ cell specific.[22,23] There are several other classes of small regulatory RNAs[24] and more will undoubtedly be discovered.

1.3.2.3 microRNA (miRNA)

MicroRNA (miRNA) is the most well known endogenous small double stranded RNA that uses RISC. The first member of the miRNA family was discovered in 1993 by Ambros[25] and Ruvkun[26] however their role in the regulation of nearly half the human genome was not realized until more than a decade later.[27,28] The biogenesis and gene silencing activity of miRNA is extremely complex,[24] and will be briefly summarized here. Nascent pri-miRNA transcripts are first processed into ~70 nucleotide (nt) pre-miRNAs by the enzyme Drosha[29] in the nucleus, which are then transported to the cytoplasm by the protein Exportin 5.[30] Here, they are processed into duplexes 22-29 nt in length by Dicer.[16,31] They are then loaded in to RISC which acts on its mRNA target (usually in the 3'-untranslated region (3'-UTR) by translational repression or mRNA destabilization,[32-34] as opposed to siRNA which guides RISC to target cleavage only. Also, miRNA binds to its target with partial sequence complementarity, whereas siRNA has absolute sequence complementarity. Because miRNA are involved in the regulation of ~ 50% of the entire human genome, they have become the focus of an intense research effort and have great promise for therapeutic agents.

1.4. Chemical Synthesis of Oligonucleotides

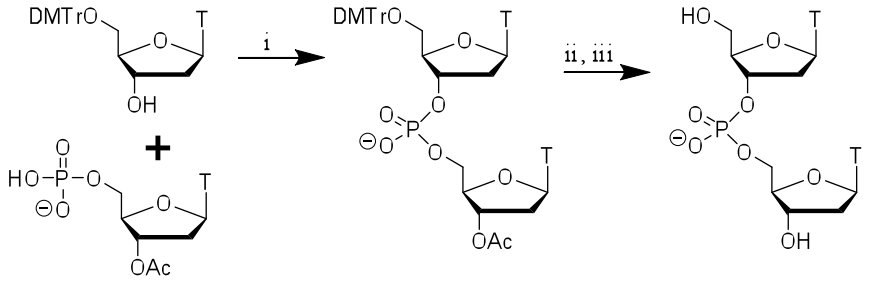
After the elucidation of the structure of DNA by Watson and Crick in 1953,[4] the field of chemical synthesis of oligonucleotides was born. The major challenge was how to stitch together nucleotides via a 5'-3'-internucleotide phosphate linkage. The first reported success was achieved in 1955 by Michelson and Todd.[35] This synthesis required the use of protected nucleoside and nucleotide monomers which would prove to be a requirement for successful oligonucleotide synthesis (**Scheme 1.1**).



Scheme 1.1. i) Product obtained from the reaction between ammonium monobenzyl phosphate and $(PhO)_2P(O)Cl$, 2,6-lutidine, benzene; ii) NCS, MeCN, benzene; ii) 2,6-lutidine, MeCN; iv) H_2SO_4, EtOH, H_2O; v) $Ba(OH)_2$, H_2O.

1.4.1 The Phosphodiester Approach

In the late 1950's Khorana and co-workers developed the phosphodiester approach, a synthetic strategy that left the internucleotide linkages completely unprotected during chain assembly. The first published synthesis is illustrated in **Scheme 1.2.**[36-38]



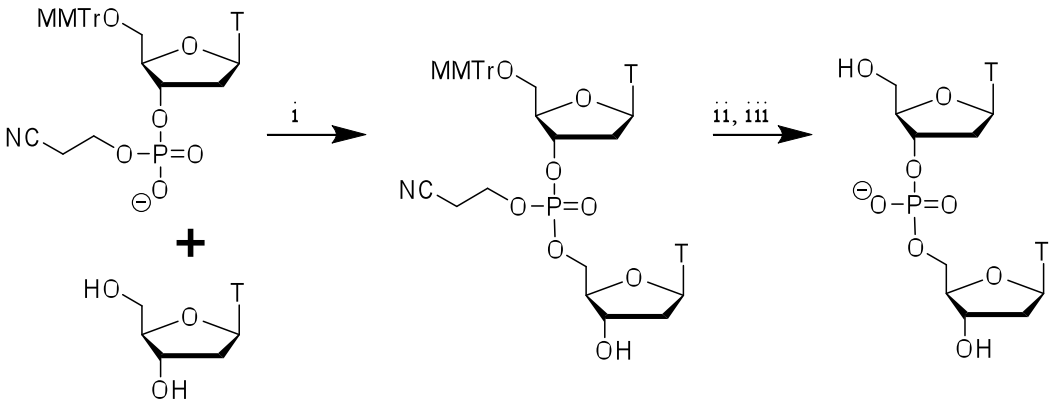
Scheme 1.2. i) DCC, pyridine; ii) AcOH, H_2O; iii) NaOH, H_2O.

This approach was also used in the first total synthesis of the yeast alanine tRNA gene[39] and the *E. coli* tyrosine suppressor gene.[40] Khorana's greatest contribution to the field of oligonucleotide chemistry was arguably the development of protecting groups for the hydroxyl and exocyclic amino groups of nucleos(t)ide building blocks.[41] He realized that for successful synthesis of long sequences, there was a need to develop orthogonal protecting groups that could be removed at specific times during synthesis without affecting coupling or cause internucleotide strand cleavage. To this end, the 5'-hydroxyl group was protected with a monomethoxy-trityl (MMTr) or dimethoxy-trityl group (DMTr) which increased acid lability 10 and 100 times, respectively, to the parent trityl (Tr) group.[42] Acyl groups were introduced as the protecting groups of choice for the exocyclic amines. Namely benzoyl for adenine,[43] isobutyryl for guanine,[44] and anisoyl for cytidine.[44] In fact, 5'-DMTr and N-acyl protecting groups are still used in modern oligonucleotide synthesis.[45] However, a myriad of other 5'-hydroxyl[46] and exocyclic amino protecting groups[47] have been investigated over the years. In Khorana's work, the 3'-hydroxyl was protected with an acyl group (e.g. Ac).[35,36] This way either the 5'-DMTr group or the 3'-acyl group could be selectively removed without hydrolyzing the internucleotide linkage. The major drawback of the phosphodiester approach was the ionic nature of the starting materials and products which led to solubility problems, side reactions, and made purification tedious and time-consuming.

1.4.2 The Phosphotriester Approach

To alleviate the problems of the phosphodiester approach, the phosphotriester approach originally introduced by Todd[48] (see Scheme 1.1) was reinvestigated by Letsinger and co-workers in the late 1960s. Here, the phosphate group is protected to prevent branching and increase solubility of the nucleotides and the growing chain (**Scheme 1.3**). As for the diester method, it requires and activator to achieve internucleotide coupling. In the original report, triisopropylbenzenesulfonyl chloride (TPS-Cl) was used. The β-cyanoethyl

group, first introduced by Tenner[49] and reinvestigated here by Letsinger,[50-52] proved to be the protecting group of choice for the phosphate moiety. In solution, this group proved to be fairly stable during nucleotide coupling, and could be removed under mild basic conditions. However, this could be problematic for solution phase synthesis if other base-labile protecting groups were used for the hydroxyl functions.



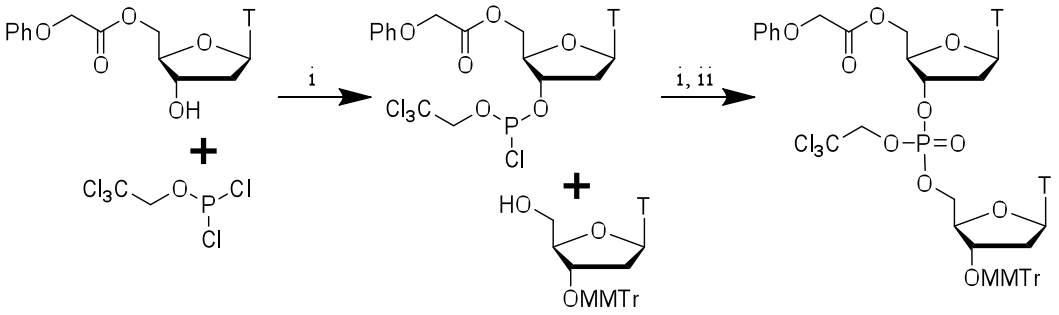
Scheme 1.3. i) TPS-Cl, pyridine; ii) 80% acetic acid iii) ammonium hydroxide.

To this end Eckstein and Rizk[53] developed the 2,2,2-trichloroethyl phosphate (TCE) group which could be removed with Zn/Cu in DMF. This group proved to be useful in the synthesis of longer oligonucleotides,[54] but it was later discovered that its complete removal was problematic.[55] Other protecting groups that were thoroughly investigated by Reese and co-workers were the phenyl[56] and substituted phenyl groups[57] (the most studied being the ortho-chlorophenyl group[58-60]). While it promoted good coupling yields, its removal required the use of hydroxide ion which not only led to phenoxide ion cleavage as desired, but also internucleotide strand cleavage.[61] The introduction of an electron withdrawing substituent on the phenyl group (i.e. ortho-chlorophenyl) decreased the phenol pK_a and led to improved selectivity of cleavage.[57] Another strategy using oximates as the cleavage nucleophile proved to be a significant improvement.[60] However, the major drawback of this approach was the low

yields of nucleoside phosphorylation[62-64] and internucleotide condensation[65-69] as well as very slow coupling times.

1.4.3 The Phosphite Triester Approach

In the mid-1970s Letsinger and co-workers[70,71] revolutionized oligonucleotide phosphorylation methodology by showing that phosphorus in the P(III) state was significantly more reactive than the classic P(V) state. In this approach a 5'-protected nucleoside was reacted with an alkyl phosphorodichloridite to generate an intermediate nucleoside-3'-phosphorochloridite within 5 min at -78 °C. The addition of a 3'-protected nucleoside resulted in the rapid formation of a dinucleoside phosphate triester which could be subsequently oxidized to a phosphate triester by an aqueous iodine solution (**Scheme 1.4**). Unfortunately, the nucleoside chlorophosphite intermediates were extremely unstable to moisture and were difficult to handle under normal conditions. However, this approach was successfully used by Ogilvie and co-workers in the first automated commercially available DNA synthesizer.[72]



Scheme 1.4. i) 2,6-lutidine, THF, -78 °C; ii) 2,6-lutidine, I_2/H_2O in THF.

1.4.4 The Phosphoramidite Approach

In the early 1980's Caruthers and co-workers[73-75] made a simple, yet revolutionary change to the phosphite triester method where a chloride leaving group was exchanged for an alkylamine. The resulting nucleoside

phosphoramidite derivatives could be made in advance, isolated and purified, and stored as a solid until needed. Just prior to internucleotide coupling, the phosphoramidite moiety is activated with a weak acid such as 1-*H*-tetrazole (**Figure 1.6**).

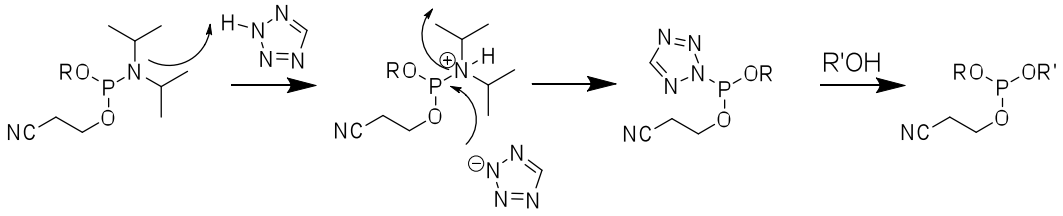
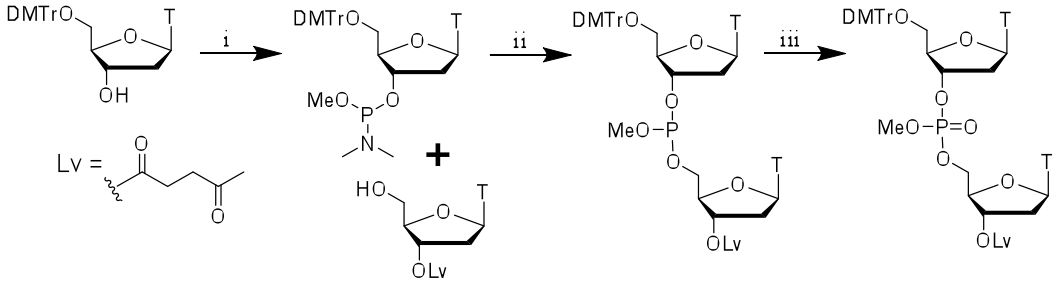


Figure 1.6. Mechanism of activation and coupling of phosphoramidite synthons as elucidated by ^{31}P NMR.[76,77]

The newly formed phosphite triester was then oxidized with I_2/H_2O/pyridine/THF (**Scheme 1.5**). Today, the 2-cyanoethyl *N,N*-diisopropylphosphoramidities[78] have been used virtually exclusively in solid phase oligonucleotide synthesis. This breakthrough also lead to a variety of new automated oligonucleotide synthesizers in the market.[79]

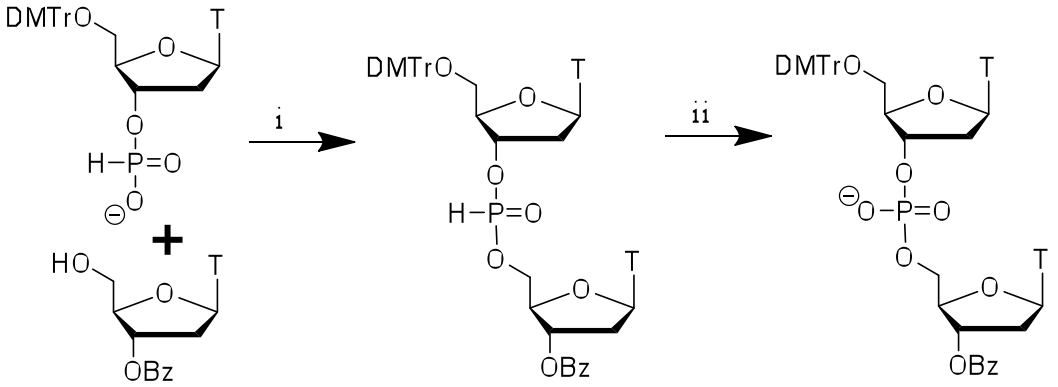


Scheme 1.5. i) MeOP(Cl)NMe$_2$, DIPEA, THF; ii) 1-*H*-tetrazole, THF; iii) I_2/H_2O/pyridine/THF.

1.4.5 The H-Phosphonate Approach

Like the phosphotriester approach, the H-phosphonate was also introduced by Todd and co-workers in the 1950's.[80] However, its usefulness was not realized until nearly 30 years later when Garegg and co-workers showed that a 5'-protected nucleoside-3'-*O*-hydrogen phosphonate reacted rapidly with a 3'-*O*-

protected nucleoside in the presence of an activating agent such as TPS-Cl, benzenesulfonyl chloride or diphenylphosphochloridate giving the correct 3'-5'-dinucleoside hydrogen phosphonate. This intermediate is then oxidized with $I_2/H_2O/NEt_3/THF$[71] to give the phosphate (**Scheme 1.6**).



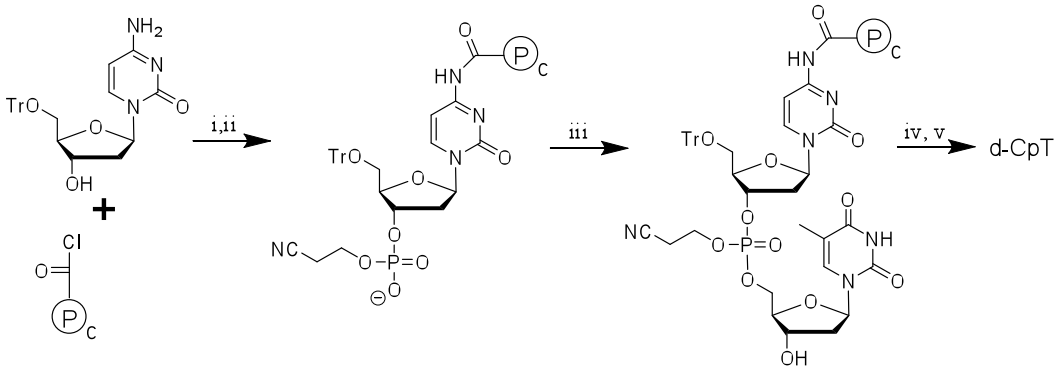
Scheme 1.6. i) $(PhO)_2P(O)Cl$, 2,6-lutidine, MeCN. ii) $I_2/H_2O/NEt_3/THF$.

The activators more commonly used today are pivaloyl chloride[81] or adamantoyl chloride[82] which allow for reliable synthesis of high molecular weight oligonucleotides on solid support.[81,83] However, this method is more amenable to solution phase synthesis with some notable examples appearing in the literature, particularly the synthesis of the first FDA-approved antisense oligonucleotide therapeutic.[84] However, this method is accepted to be less efficient than the phosphoramidite approach for syntheses carried out on solid supports.[85]

1.4.6 Solid Phase Synthesis of Oligonucleotides

In the past 25 years or so, nearly all oligonucleotide synthesis has been done on solid supports.[45] The solid-phase approach allows for iterative oligonucleotide synthesis to be carried out without isolation or purification of any intermediates.[50,86] Reactants and reagents are removed simply by washing the insoluble support with an organic solvent. It is by far the fastest, most efficient, and reliable way to synthesize small amounts of oligonucleotides required for biological research. In addition, it has also been used for multi-gram synthesis of oligonucleotides required for clinical studies.[87]

Solid phase synthesis was first introduced by Merrifield[86] for peptide synthesis, and soon after in the oligonucleotide area by Letsinger and Mahadevan in 1965.[50] Letsinger made use of the 'popcorn' solid support,[88,89] a copolymer from styrene (99.5%), and divinylbenzene (0.5%). It was insoluble in water, acidic/basic solutions, and organic solvents used for oligonucleotide synthesis, but had the unfortunate property of swelling in many solvents. Nonetheless, a 5'-DMTr-cytidine could be appended to the solid support through its exocyclic amine. The 3'-end was phosphorylated and coupled to thymidine according the phosphate triester approach. This material was then cleaved from the solid support and fully deprotected to give the free dimer (**Scheme 1.7**).



Scheme 1.7. i) pyridine; ii) pyridinium β-cyanoethyl phosphate, DCC; iii) Ms-Cl, thymidine, pyridine; iv) 0.2 M NaOH; v) 80% HOAc.

Modern methods now attach the 3'OH[90] (or less frequently the 5'OH[91]) position of the first nucleoside to glass solid supports (see below) through a linker arm consisting of succinic acid, diglycolic acid, or hydroquinone-*O*,*O*′-diacetic acid and an ethylene glycol spacers.[45]

To avoid the polymer swelling issue of the 'popcorn' copolymer solid supports, Caruthers and Matteucci,[92,93] and independently Ogilvie and co-workers,[94] introduced the use of an inorganic polymer support. Originally chromatography grade silica was used, but this was later replaced with controlled pore glass (CPG).[95] Highly cross-linked polystyrene[96] has also been used, but CPG remains the solid support of choice for routine (small and medium scale) oligonucleotide

synthesis. Controlled pore glass is typically functionalized with a long-chain alkyl amine and polystyrene with an aminomethyl group. The 5'-DMTr-nucleoside is usually appended through the 3'-end via a succinoyl group.[45] This strategy combined with phosphoramidite chemistry gave birth to the modern oligonucleotide solid phase synthesis cycle (**Figure 1.7**). The cycle begins with the removal of the 5'-DMTr group with an acid, usually 3% TCA/DCM. The free 5'-hydroxyl is then coupled with a nucleoside phosphoramidite monomer in the presence of an activator, e.g., 1-*H*-tetrazole, giving a phosphite triester intermediate. Because this reaction does not always go to completion, unreacted 5'-hydroxyl groups are capped off with acetic anhydride in the presence of *N*-methyl-imidazole to prevent truncated sequences from growing in subsequent steps which can later complicate purification of the full-length target oligomer. Also, capping at this stage alleviates the damage of side reactions that may have formed during coupling, particularly at the O-6 position of guanine.[97,98] The newly formed phosphite triester is then oxidized to a phosphate triester with I_2/H_2O/pyridine/THF. The cycle may then be repeated numerous times until the oligonucleotide of desired length is achieved. In fact, oligonucleotides over 200 units in length have been achieved using this method.[45]

1.4.7 Chemical Synthesis of RNA – How and Why?

In the synthesis of oligonucleotides, the choice of selectively removable protecting groups has been key to developing reliable synthetic strategies. RNA synthesis is much more difficult than DNA synthesis because of the difficulty in finding a compatible 2'-protecting group which could remain stable throughout the assembly and be removed selectively at the end of synthesis without chain migration or degradation.[99,100] Of course RNA polymerases may be used to synthesize RNA from a DNA template, but this method is only efficient on a very small scale (pmol or less) and would be too costly on a larger scale.

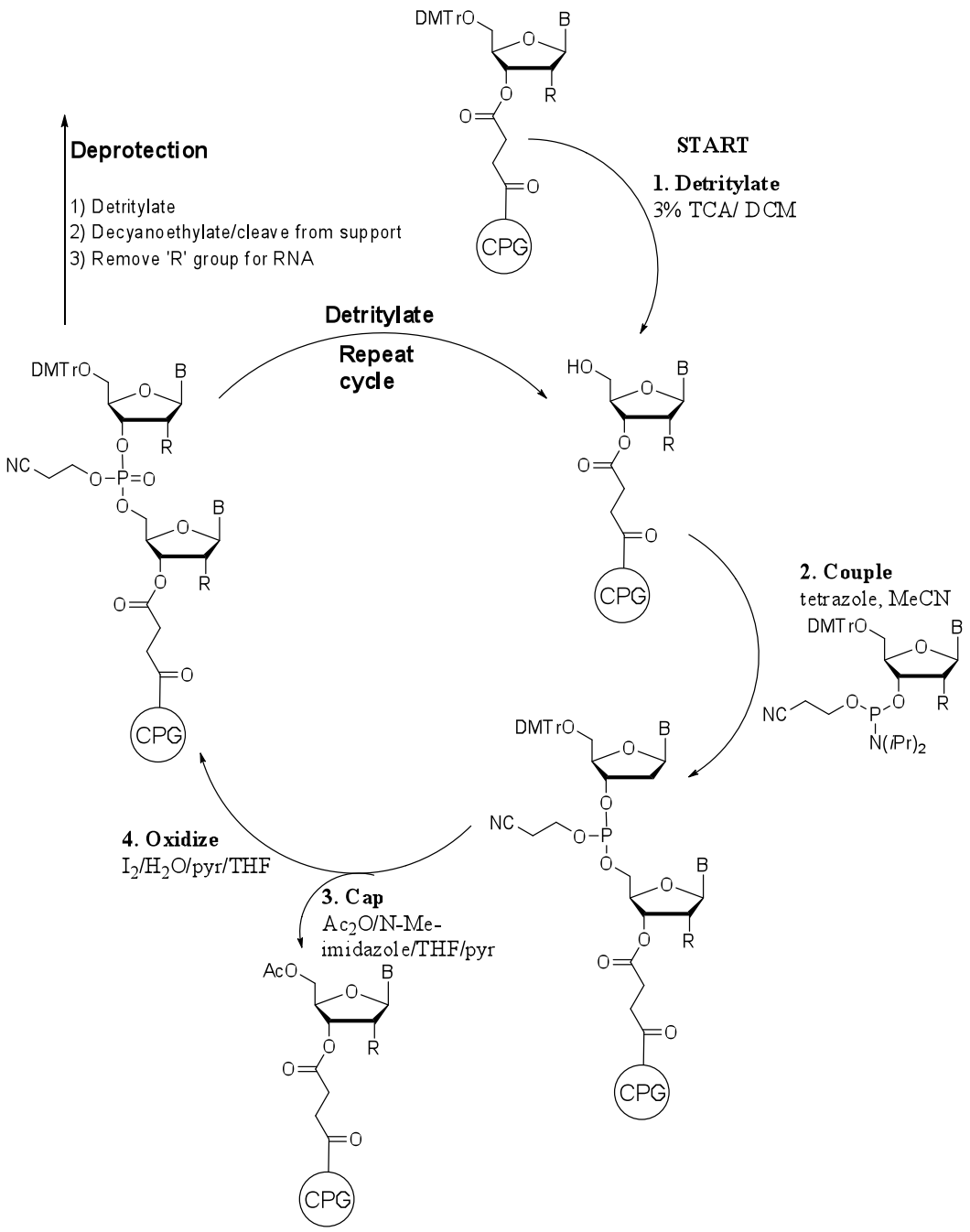


Figure 1.7. The four step modern solid phase synthesis cycle. B = T; C^{Ac}; A^{Bz}; G^{ibu}. Note : R = H for DNA, R = 2'-O-protecting group for RNA.

In the 1970s, there were no RNA protection schemes of practical utility, no truly effective coupling methods, and only the shortest of oligoribonucleotides could be produced. Luckily, there were early pioneers in oligonucleotide synthesis who, unlike most in the field, decided to tackle the much more difficult

problem of RNA synthesis. [99-103] This was a decision which required both courage and vision since, at that time, even small DNA oligonucleotides were terribly laborious to make. Furthermore, the chemical synthesis of oligonucleotides was considered to be just a curiosity without serious practical utility.

Exciting discoveries in the 1980s such as RNA splicing and RNA enzymes created a renaissance in the field of RNA synthesis to find efficient methods for its synthesis.[85] The ability to chemically synthesize oligoribonucleotides has recently acquired even greater importance with the discovery of gene silencing and control by small interfering RNAs (siRNAs) and micro RNAs (miRNAs).[15] As mentioned above, these relatively short oligoribonucleotides are proving to be important research tools that allow cell and molecular biologists to manipulate gene expression in cell culture. In addition, there is now tremendous interest in developing these molecules as therapeutic agents (siRNAs).[104]

1.4.8. RNA Stability in Acid and Base

RNA is susceptible to internucleotide cleavage under relatively mild basic[99] or acidic conditions,[100] and internucleotide migration[100] under relatively acidic conditions (**Figure 1.8**). Under basic conditions, the internucleotide linkage undergoes 2'-OH mediated cleavage giving an intermediate terminal 2'-3'-cyclic phosphate and a free 5'-hydroxyl fragment. The unstable cyclic phosphate then undergoes hydrolysis to give a mixture of 2' and 3'-monophosphates. Under acidic conditions, the internucleotide linkage undergoes reversible migration to the vicinal 2'-position.

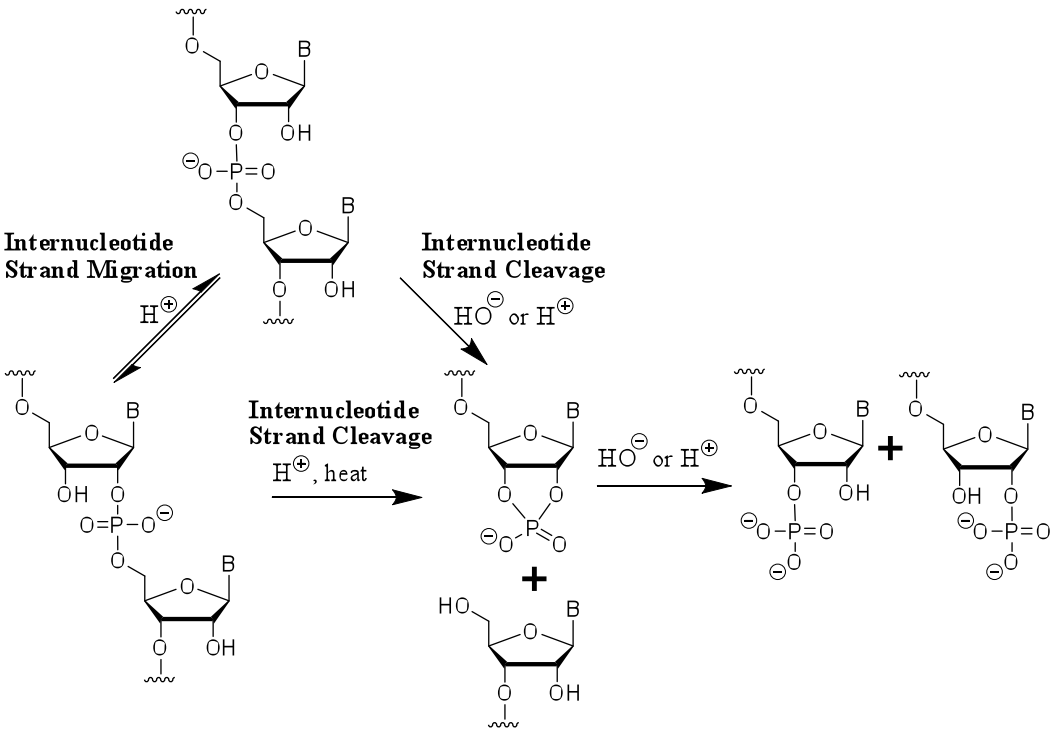


Figure 1.8. Internucleotide strand cleavage and internucleotide strand migration catalyzed by acid or base, and acid respectively.

1.4.9 Chemical Synthesis of RNA: The 2'-Protecting Group

This section describes the variety of 2'-hydroxyl protecting groups that have been developed for RNA synthesis, focusing mainly on the solid phase methods. All the monomers discussed here are protected with *N*-acyl protecting groups (i.e. A^{Bz}, C^{Ac}, and G^{ibu}) or derivatives thereof.

1.4.9.1 The 2'-*tert*-Butyldimethylsilyl (2'-TBDMS) Protecting Group

When Professor Kelvin Ogilvie began his research in the 1960's there were no RNA protection schemes of practical utility, no truly effective coupling methods, and only the shortest of oligoribonucleotides could be produced. While his research group proceeded to make significant contributions in automated DNA synthesis, his most important contribution to the field of modern oligoribonucleotide synthesis was his selection of *t*-butyldimethylsilyl protection for the 2'-OH position (**Table 1.2**, **entry 1**). [101] Unlike many of the protecting groups employed up to that time to synthesize oligoribonucleotides, the silyl

moiety proved to be a truly orthogonal protecting group that remained in place throughout the synthesis of the oligoribonucleotide and which could be removed under mild conditions at the end of the synthesis. The Ogilvie group developed procedures for synthesizing 2'-silyl protected ribonucleosides and their phosphoramidite derivatives, and then demonstrated how these reagents could be used to prepare oligoribonucleotides of defined sequence.[105] Eventually, his group produced the first solid-phase synthesis of a full-length (77 bases) transfer RNA molecule, an accomplishment which served as the precedent for today's widespread use of synthetic RNA's.[106]

Despite the advantages of TBDMS protection, great care must be taken during the synthesis of the silylated nucleosides, as the TBDMS group readily interconverts between the 2'-/3'-positions under basic conditions[107] and in protic solvents. [108] However, it is possible to obtain pure material if such conditions are avoided[105,108]

Similarly, strong bases must be avoided during the phosphorylation step so as to avoid the formation of inseparable 2'-/3'-phosphoramidite mixtures. For example, the use of 4-dimethylaminopyridine (DMAP)[105] or a mixture of 2,4,6-collidine and 1-methylimidazole is not recommended.[109] In addition, due to the bulky nature of the 2'-TBDMS group, it is difficult to obtain coupling efficiencies in excess of 97-98% during RNA synthesis. To this end, 5-ethylthio-1*H*-tetrazole (ETT) has been shown to be a superior phosphoramidite activator than 1*H*-tetrazole, and coupling efficiencies of up to 98.5% can be achieved (versus >99% for DNA synthesis).[110] Problems have also arisen during the final unblocking of the 2'-TBDMS protected RNA sequences. Standard unblocking procedures for DNA adapted for RNA synthesis, namely 55 °C in concentrated aqueous ammonia to remove the *N*-acyl base protecting groups, the 2-cyanoethyl protecting groups from the internucleotide linkage, and cleavage from the solid support,[45] leads to some TBDMS cleavage and hence fragmentation of the RNA chain.[111] It is therefore recommended to use 3:1 aqueous ammonia/EtOH at room temperature for 1-2 days, since ethanol slows down the rate of silyl cleavage under basic (ammonia) conditions.[112] A similar beneficial effect is observed when using aqueous methylamine,[113,114] a base that speeds up *N*-acyl protecting

group removal (e.g. *N*-phenoxyacetyl[115]). In the final step, the 2'-TBDMS groups are cleaved with an excess of tetra-*n*-butylammonium fluoride (1M in THF),[105] which makes it difficult to purify the desired oligomer to homogeneity.[116] This problem is somewhat alleviated by the use of neat triethylamine trihydrofluoride (TREAT-HF)[117] or a mixture of TREAT-HF in 1-methylpyrrolidone (NMP) and triethylamine (TEA).[114] If all these precautions are taken into account, the 2'-TBDMS group can be very effective at yielding high quality RNA sequences.

1.4.9.2 The 2'-(*tris*(isopropylsilyl)oxy)methyl (2'-TOM) Protecting Group

This protecting group was introduced as an improvement over the TBDMS group but has not proved to have any significant advantages.[118] Like the TBDMS, the 2'-TOM group (**Table 1.2**, **entry 2**) is orthogonal to the other protecting groups of the RNA chain. Since the triisopropylsilyl moiety is linked to the 2'-oxygen through a oxymethyl (acetal) spacer, it does not undergo 2'-/3'-isomerization making it easier to obtain diasteormerically pure 2'-TOM-3'-phosphoramidite monomers. In addition, because the 2'-TOM group is less bulky than the 2'-TBDMS group, higher coupling yields (>98.5%) are routinely reported.[119] Finally, the preferred two-step deprotection of 2'-TOM RNA sequences, namely methylamine in ethanol followed by fluoride (TBAF) results in no concomitant degradation of the RNA chain.[119] The 2'-TOM phosphoramidite monomers are commercially available and widely used, but are more expensive ($65/g) than TBDMS monomers ($30/g).

1.4.9.3 The 2'-bis(acetoxyethoxy)methyl (2'-ACE) Protecting Group

Scaringe and Caruthers developed a completely new approach to solid phase RNA synthesis by introducing the 2'-bis(acetoxyethoxy)methyl (2'-ACE) (**Table 1.2**, **entry 3**) protecting group.[103] Because ACE orthoester group is acid labile, the traditional 5'-DMTr group was replaced with a fluoride labile 5'-silyl moiety. Since the β-cyanoethyl internucleotide protecting group is unstable to fluoride treatment,[120] it needed to be replaced with the corresponding methyl

derivative which is cleavable with solution of disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate (S_2Na_2) in DMF.[103] An advantage of the ACE protecting group is that it can be introduced selectively at O2' without the formation of any 3'-ACE isomer. In addition, the 2'-ACE method has yielded the best coupling efficiencies to date (yield >99% per step) and produces oligonucleotides of the highest quality.[120] Unfortunately, because the synthesis/deprotection scheme of 2'-ACE is so different than the one used for standard DNA/RNA chemistry, it cannot be combined with a large set of modified monomers or labeling reagents used to conjugate oligonucleotides.[121] In addition, 2'-ACE chemistry requires modification of existing synthesizers (i.e. all glass equipment must be replaced due to incompatibility with the 5'-desilylation reagent). While 2'-ACE phosphoramidite monomers have recently become commercially available, their main use is in custom oligonucleotide synthesis.

1.4.9.4 Acid-Labile 2'-Protecting Groups

These types of protecting groups are not very widely used today, but they were some of the first introduced for RNA synthesis. Their main drawback is that the acidic conditions used to remove them may lead to isomerisation (3' to 2') of the internucleotide linkage.

The tetrahydropyran-2-yl (THP) group (**Table 1.2**, **entry 4**) was first examined in the early 1960s by Khorana and co-workers.[122] They applied the phosphodiester method to prepare UpU and UpA dimers. Removal of the 2'-THP groups with 80% acetic acid over 8-12 hr led to formation of 2',5'-linked dimers. The extent of isomerisation under these conditions was 1.5%. Optimization of deblocking conditions by Griffin and Reese[100] (0.01 M HCl, pH 2.0, 5 hr, r.t.) led to no more than 0.02% isomerization and less than 0.01% internucleotide strand cleavage. A major disadvantage of the THP group is that it is chiral, leading to the manipulation of diastereomeric nucleoside (2 isomers) and nucleoside 3'-phophoramidite synthons (4 isomers) during their synthesis and structural characterization.

To this end, an achiral version of the THP, namely 2'-4-methoxytetrahydropyran-4-yl (2'-Mthp) group (**Table 1.2**, **entry 5**), was studied by Reese and co-workers in the late 1960s.[123,124] This group was found to be more labile than THP under acidic conditions ($t_{1/2}$ = 6.1 hr and 19.9 hr respectively; 0.01 M HCl, pH 2, r.t.).[125] The 2'-Mthp group was successfully applied to the preparation of a 37-mer RNA fragment in solution corresponding to the yeast alanine transfer RNA.[126,127]

Another version of the THP group, the tetrahydrofuran-2-yl (Thf) group (**Table 1.2**, **entry 6**) was shown to be compatible with the 5'-DMTr provided that $ZnBr_2$ in dry DCM/isopropanol was used instead a protic acid during 5'-deblocking steps.[128] This is because the conditions normally used to deblock trityl groups during solid phase synthesis would also partially remove Thf (as well as 2'-THP, -Mthp) groups.[129-131] However, even $ZnBr_2$ can be problematic during the synthesis of long RNA sequences, as repeated exposures with this reagent leads to partial removal of both exocyclic amine protecting and 2'-OH groups (Thf). Replacing the trityl groups with the orthogonal 5'-levulinyl (Lv) group provided a solution to the premature removal of N- and 2'-protecting groups during chain assembly.[132] The 5'-Lv group can be removed by treatment with hydrazine hydrate in a pyridine/acetic acid solution, conditions in which the 2'-Thf seems to be stable.[133] An RNA 21-mer was prepared using the 5'-Lv/2'-Thf combination. Also, the 5'-9-fluorenylmethoxycarbonyl (FMOC) has been used as a substitute for the 5'-DMTr to allow RNA solid phase synthesis with 2'-Mthp protected building blocks.[134] In this case, the FMOC is removed with a solution of 0.1 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in acetonitrile leaving the 2'-Mthp group intact. However these conditions will also remove the β-cyanoethyl protecting group which may be problematic for subsequent phosphoramidite coupling steps.

Because the 5'-DMTr group is conveniently removed with acid and its carbocation is easily quantified spectrophotometrically, there was some effort to find an acetal based 2'-protecting group that would be unaffected by the conditions required for 5'-DMTr removal (e.g., dichloroacetic acid). To this end,

Reese and co-workers[102,135,136] reasoned that if the ring oxygen of the Mthp group was replaced with a tertiary aryl amine (pK_a ~ 2), then the derivatized Mthp group should be protonated and stable during 5'-DMTr deblocking with excess of trichloroacetic acid (pK_a 0.66). Furthermore, they hypothesized that this new "Mthp" derivative would remain largely unprotonated during conditions that effect its removal (i.e. pH 3) in the final deprotection step. The first such group tested was the 2'-1-(2-chloro)-4-methoxypiperidin-4-yl (2'-Ctmp) group[102] (**Table 1.2, entry 7**).[135] The Ctmp was later replaced by the structurally similar 2'-1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (2'-Fpmp) group (**Table 1.2**, **entry 8**), which afforded better yields of the required 2'-Fpmp 3'-phosphoramidite derivatives.[136] The 2'-Fpmp group was also successfully used in solid phase RNA synthesis with 5'-DMTr protection.[137] It is stable to detritylation conditions, and removed at the end of the synthesis under conditions (pH 3.25) that do not lead to either internucleotide isomerization or cleavage. Furthermore work in the Reese lab led to a robust and effective acetal, the 2'-Cpep protecting group (**Table 1.2**, **entry 9**).[138] It has a better stability to acid hydrolysis in the pH range of 0.5 - 2.5, but it is more labile than the Fpmp group in the pH range of 3.25 - 3.75. It has also been used in the large scale synthesis of siRNA.[139] A disadvantage of this chemistry, is the multi-step synthesis of the required Cpep monomers; furthermore, the bulkier Cpep group slows down somewhat monomer coupling rate (>10 min) and efficiency (~98.5%).

Very recently Beaucage and co-workers[140] have examined the use of 2'-[4-(*N*-dichloroacetyl-*N*-methylamino)benzyloxy]methyl (2'-MABOM) group **(Table 2, entry 10**). The 5'-DMTr 3'-phosphoramidite monomers used in solid phase synthesis were coupled for 3 min with an average stepwise efficiency of 99%. Deblocking of the RNA strands synthesized required a two step deprotection scheme, namely: 1) Simultaneous deblocking of the nucleobases and phosphate protecting groups and cleavage from the solid support with aqueous ammonia, 10 hours at 55 °C; and 2) cleavage of the 2'-MABOM groups with 0.1M AcOH, pH 3.8 in *N,N,N′,N′*-tetramethylethylenediamine (TEMED), 30 minutes at 90 °C. The *N*-methyliminoquinone generated is quickly quenched by water to avoid

adduct formation with the nucleobases. The formation of 2',5'-internucleotide linkages was assessed by treating a small sample of the mixed base RNA with snake venom phosphodiesterase and bovine spleen phosphodiester. This treatment selectively degrades 3'-5'-linkages, leaving 2',5'-linkages intact. According to HPLC analysis, the presence of 2',5'-linkages was minimal, being detectable only when the baseline of the HPLC trace was amplified. Several 21-mer RNAs were prepared using this very promising 2'-protection chemistry.

1.4.9.5 Fluoride-Labile 2'-Acetal Protecting Groups

In more recent years there have been several examples of fluoride labile 2'-acetal protecting groups, but not of the silyl variety (i.e. the 2'-TBDMS and 2'-TOM above). The first one to appear in the literature was from Gough and co-workers[141] in 1995, where a homo-uridylic 13-mer was synthesized using the 2'-(4-nitrobenzyloxymethyl) (2'-4-NBOM) group (**Table 1.2**, **entry 11**). In fact, this type of acetal inspired the next generation of 2'-protecting groups because, for the first time, the coupling times were close to DNA-like (~2min) without decreased coupling yields (98%).[141,142] It was rationalized that reduced steric crowding around the 2'-OH was responsible for the observed increase in coupling efficiency.[142] This protecting group was removed in a two-step fashion. First, ammonolysis to remove the β-cyanoethyl internucleotide protecting groups followed by 1 M TBAF in THF for 24 hours to deblock the 2'-4-NBOM moiety. This protecting group was also revisited by Beaucage[143] for its potential as a reductively labile group, and will be discussed later. Interestingly, when the nitro group is moved from the *para* to the *ortho*-position, the resulting 2'-2-NBOM is removed photochemically, and has been used to prepare RNA strands by solid phase synthesis (see below).[142]

The 2'-2-cyanoethyloxymethyl (2'-CEM) group (**Table 1.2**, **entry 12**) has been thoroughly investigated by Ohgi and co-workers.[144,145] In their initial report, a 55-mer mixed based RNA was synthesized by solid phase.[144] They used coupling times of only 2.5 min, and ETT as the activator. The fully protected RNA was first treated with aqueous ammonia/ethanol to simultaneously remove

the nucleobase and phosphate (β-cyanoethyl) protecting groups. It is noteworthy to point out that in contrast to cyanoethyl groups attached to a phosphate, the CEM groups at O2' are fairly stable under these conditions, with less than 5% cleavage observed. This underscore the leaving group abilities of a phosphate diester (pK_a 1.2) compared to the conjugate base of an acetal. The latter decomposes to produce formaldehyde and releasing the 2'-alkoxide *in situ* (pK_a of 2'-OH group ~ 13).[146,147] The 2'-CEM is removed with 1 M TBAF in THF, and the resulting acrylonitrile is scavenged with 10% *n*-propylamine and 1% bis(2-mercaptoethyl) ether (or 0.5% nitromethane) to avoid alkylation of the nucleobases.[144] The true power of this approach was realized when a 110-mer miRNA was synthesized and validated through a miRNA gene knockdown assay. This appears to be the longest RNA chemically synthesized in a stepwise manner, to date. [145]

The cyanoethyl (CE) group (**Table 1.2**, **entry 13**) was examined as 2'-protecting group by Sekine and co-workers.[148,149] However, the installation of the 2'-CE group required acrylonitrile and caesium carbonate which could cause alkylation of the nucleobases and thus reduced the overall yield of the monomers; thus the 2'-CE protection does not seem to provide any advantage over the 2'-CEM chemistry.

The 2'-2-(4-tolylsulfonyl)ethoxymethyl (2'-TEM) group (**Table 1.2**, **entry 14**) was introduced by Chattopadhyahya and co-workers.[150] The 2'-TEM protected phosphoramidite monomers were activated with ETT and the best yields were obtained when monomers were allowed to couple for only 2 minutes. Oligomers of various lengths were prepared (14-38mers) with stepwise coupling yields ranging from 97-99%.[150] RNA strands were deprotected under similar conditions as 2'-CEM protected oligomers. The 2'-TEM group had the advantage over 2'-CEM of being much more stable to ammonolysis conditions, but the disadvantage of generating more nucleobase adducts due to the generation of a more reactive Michael-acceptor.[150]

1.4.9.6 Reductively Cleavable 2'-Protecting Groups

The first reductively cleavable 2'-protecting group was suggested by Trentham and co-workers in 1966[151] and later re-examined by Pfleiderer and co-workers nine years later.[152] Several dimers were prepared using the 2'-benzyl (2'-Bn) group (**Table 1.2**, **entry 15**), but it was discovered the hydrogenation conditions necessary to remove the group, i.e., catalytic hydrogenolysis on Pd/C, caused reduction of uracil's 5,6-double bond. Later, Takaku and co-workers investigated substituted benzyl ethers, namely 4-methoxybenzyl[153,154] (**Table 1.2**, **entry 16**) and 3,4-dimethoxybenzyl[155] (**Table 2**, **entry 17**). However, the conditions necessary to remove these 2'-protecting groups (0.1 mM triphenylmethyl tetrafluoroborate in 4:1 acetonitrile/water) caused incomplete unblocking of those groups and even cleavage of the glycosidic bonds.[153,154] The 2'-(3,4-dimethoxybenzyl) group could be completely removed by treatment with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in wet dichloromethane and was reported to avoid glycosidic bond cleavage.[155] These milder reductive conditions appear to be suitable for deprotection of the group, but it has not been validated on a long mixed-base RNA sequence to date.

Recently, Kwiatkowski and co-workers[156] examined the 2'-2-*tert*-butyldithiomethyl (2'-DTM) group (**Table 1.2**, **entry 18**). This group had excellent coupling efficiencies (98.5 – 99.8%) with 2.5 min coupling times when activated with ETT. It was stable to the removal of β-cyanoethyl phosphate and nucleobase protecting groups under basic conditions, and removed at 55 °C in a buffer of 1,4-dithiothreitol or tris-(2-carboxyethyl)phosphine. Several RNA mixed-base RNA strands were prepared including a 45-mer. One major limitation of this approach is the instability of the phosphoramidite monomers in solution, especially the guanine monomer which decomposes after 12 hours.

The 2'-4-NBOM group (**Table 1.2**, **entry 11**) originally developed by Gough,[141] and recently re-examined by Beaucage[143] deserves a mention here. Reduction of the NBOM's nitro group with 0.1 M titanium chloride ($TiCl_4$) at pH 6 provides an aminobenzyloxymethyl group at O2', which in turn is cleaved by treatment with 0.1 M acetic acid, 40 min, at 90 °C.[143] U_{19}dT prepared this way

afforded a very clean product with no sign of internucleotide strand migration or cleavage. However, when the synthesis of a mixed-base 20-mer was attempted, the reduction step did not work as effectively, and the NBOM protecting group was abandoned in favour of the 2'-MABOM group (**Table 1.2**, **entry 10**) described previously.

1.4.8.7 Light-labile 2'-Protecting Groups

The 2'-nitrobenzyl group (**Table 1.2**, **entry 19**) was introduced by Ohtsuka and co-workers in the late 1970s.[157] It was used to synthesize various heptamers via the phosphotriester approach and was shown to be stable to both acid and base catalyzed hydrolysis. It could be removed photochemically by irradiation with ultraviolet (U.V.) light ($\lambda > 280$ nm) in water. This unblocking step was later shown to be much more effective in a slightly acidic solution of 0.1 M ammonium formate, pH 3.5.[158] However, coupling times of these phosphoramidite monomers were over 10 min and photolytic cleavage of high molecular weight RNA did not always proceed quantitatively.[158] Furthermore, extended U.V. exposure lead to nucleobase modifications,[1] such as photo-dimerization.[1,159] A major improvement in this chemistry came from Gough and co-workers[142] when they introduced the 2'-nitrobenzyloxymethyl (2'-NBOM) group (**Table 1.2**, **entry 20**). This was in fact the first 2'-protecting group to have a flexible 'methylenedioxy' spacer that projects the aromatic group away from the reactive phosphoramidite coupling centre. As a result coupling times were shorter (~2 min) while providing good coupling efficiencies (>98.5%). Photodeprotection is carried out in 0.2 M formic acid, pH 3.7 in 50% aqueous 2-methyl-2-propanol, and irradiation using a U.V. lamp for 4.5 hours. This leads to complete deprotection and minimal damage due to U.V. exposure. Oligomers up to 33 nucleotides long have been prepared by this method.[160]

1.4.9.8 Base-Labile 2'-Protecting Groups

For the most part, base-labile 2'-protecting groups have been the least examined in the literature due to the fact that RNA is sensitive to internucleotide strand cleavage under basic conditions. However, there have been a few reports of their use with varying success. Reese and co-workers[161] reported the synthesis of a series of dimers starting from N^2,5',2'-tribenzoylguanosine and N^4,5',2'-triacetylcytidine nucleosides which were condensed using the phosphotriester approach. These nucleosides could be crystallized and were apparently free of 3'-benzoyl contamination. The benzoyl groups were subsequently removed using saturated ammonia in methanol for 2 hours, with no 3' to 2'-strand migration detected. This was a major achievement since 2'-acyl groups are known to rapidly interconvert to the 3'-isomer under the mildest conditions and these isomers are usually inseparable.[162] This is the main reason why 2'-ester groups are rarely used for RNA synthesis. The 2'-benzoyl strategy was later tried using phosphoramidite chemistry by Peterson and co-workers (**Table 1.2**, **entry 21**).[163] Several chimeric DNA-RNA oligonucleotides were prepared, but the integrity of the strands was compromised due to contamination of the phosphoramidite monomers with 1-3% isomeric 2'-phosphoramidites which were inseparable by chromatography. Apparently little internucleotide strand cleavage was observed using a two-step deprotection procedure: 1) 1:2:2 thiophenol/TEA/dioxane, 1.5 hr, r.t.; 2) aqueous ammonia, 1.5 hr, r.t.

Other monomers that have been recently examined by Rozners and co-workers are nucleoside 2'-(2-chlorobenzoyl)-3'-H-phosphonate derivatives (**Table 1.2**, **entry 22**).[164,165] A major advancement was the purification of the desired monomers by column chromatography, free of 2'-H-phosphonate contamination.[164] Several short RNA strands were prepared using this chemistry. The deprotection conditions were solely concentrated aqueous ammonia during 5 hours without appreciable degradation of the target RNA sequences.

The 2'-pivaloyloxymethyl (2'-PivOM) group (**Table 1.2**, **entry 23**) has recently been evaluated by Debart and co-workers[166] and appears to be among the best base-labile 2'-protecting groups. Because the pivaloyl functionality is spaced

by an acetal linker, it cannot undergo 2'-3'-isomerization. Various 21-mer oligoribonucleotides were prepared using this chemistry with 5-benzylmercaptotetrazole (BMT) as an activator; coupling times were set at 3 minutes achieving stepwise yields in excess of 99.0%. The preferred 2-step deprotection protocol involves a treatment with 10% anhydrous piperidine in acetonitrile to remove the β-cyanoethyl groups followed by 28% aqueous ammonia for 3 hours to remove the PivOM groups. Just before evaporation, 15% (v/v) isopropylamine is added to the mixture to avoid the reaction of guanine with the formaldehyde released during deprotection (**Figure 1.9**).[166]

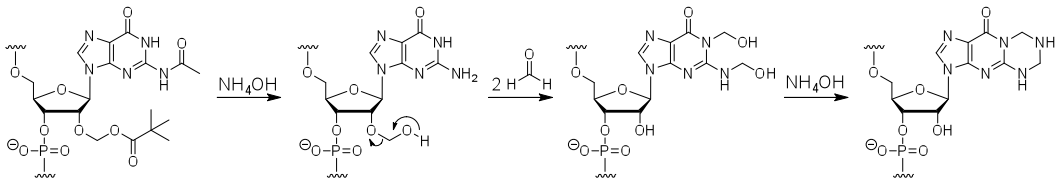
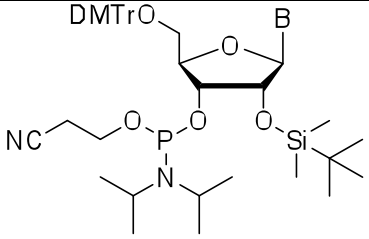
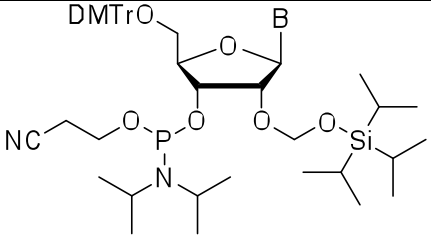
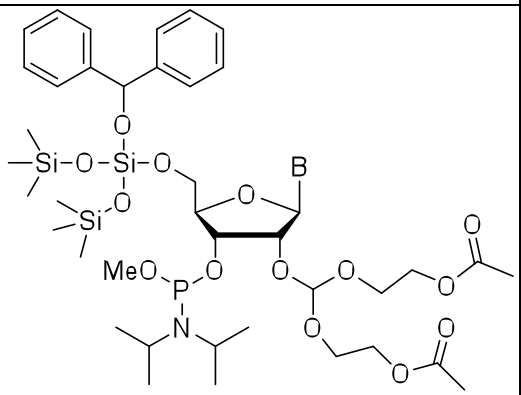
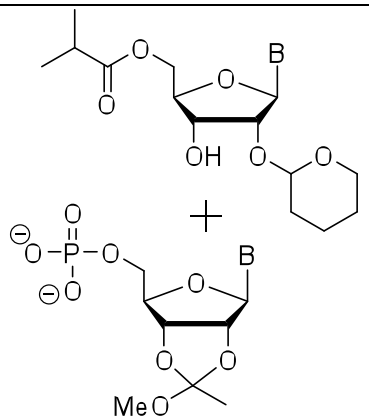
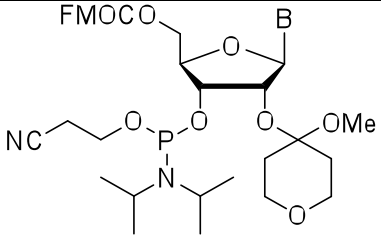
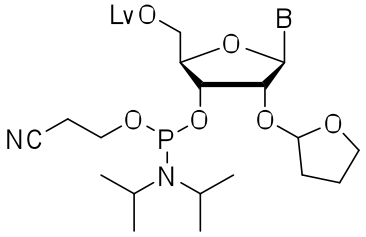
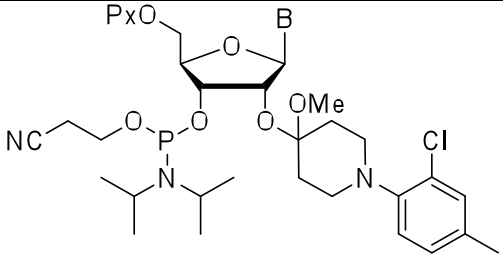
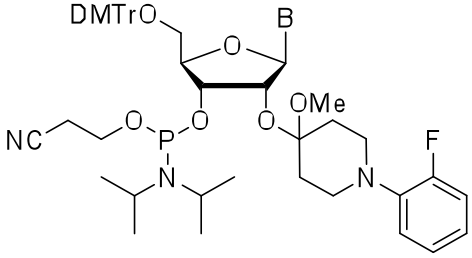


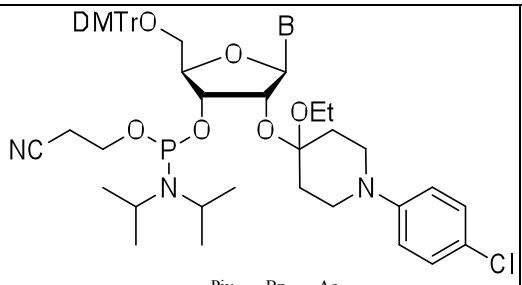
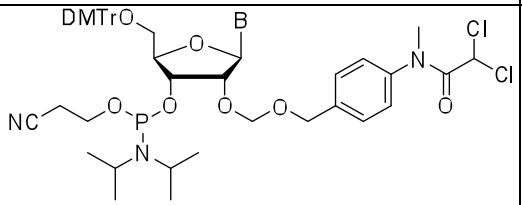
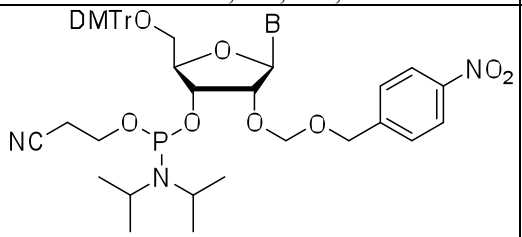
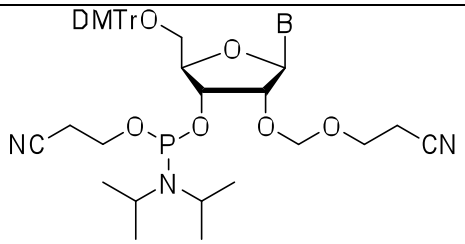
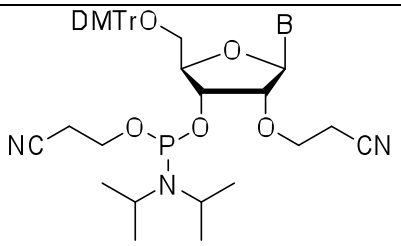
Figure 1.9. Guanine adduct formation in the presence of formaldehyde and ammonia.

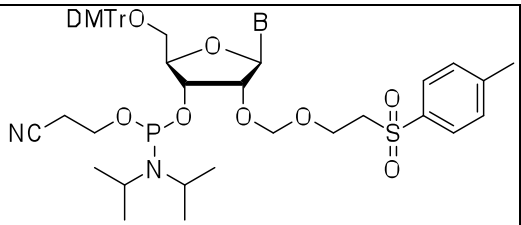
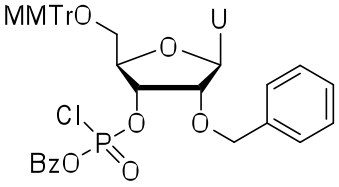
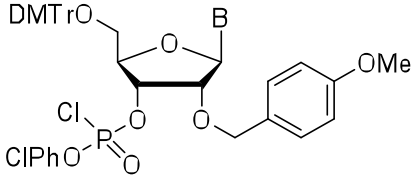
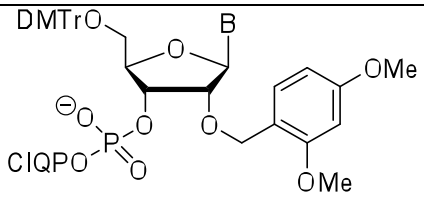
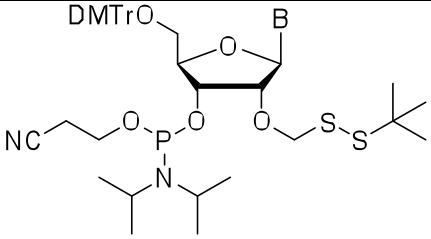
The newest protecting group to date is the 2'-(1,1-dioxo-1λ6-thiomorpholine-4-carbothioate) (2'-TC) group (**Table 1.2**, **entry 24**), developed by Caruthers and co-workers.[167] The commercial supplier indicates that phosphoramidite monomers need only be coupled for 25 seconds using ETT as an activator, resulting in ~ 98% average stepwise coupling yields. The unblocking of the RNA appears to be straightforward, requiring a simple treatment of 10% diethylamine in MeCN to remove the β-cyanoethyl groups followed by ethylenediamine treatment (1:1 EDA/toluene) for 2 hours at room temperature to simultaneously cleave the 2'-TC group, the nucleobase protecting groups, and the oligomer from the solid support. In a recent conference presentation, the researchers indicated that the free oligonucleotide remains trapped in the pores of the solid support (CPG), facilitating the removal of impurities by simple washing steps.[168] At the appropriate time, the CPG is then washed with acetonitrile, releasing the free oligonucleotide into solution thus simplifying the overall purification process. To date several 21-mers have been successfully synthesized,

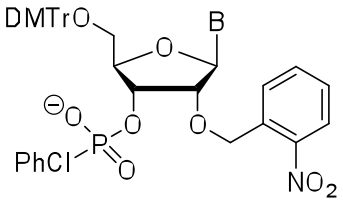
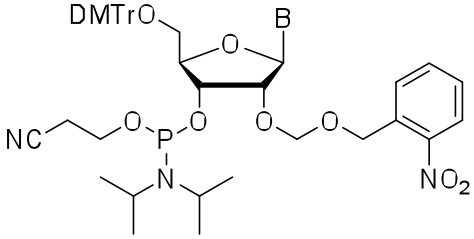
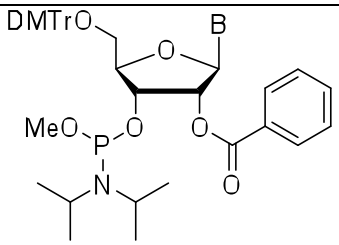
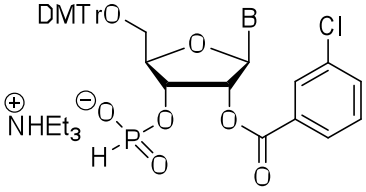
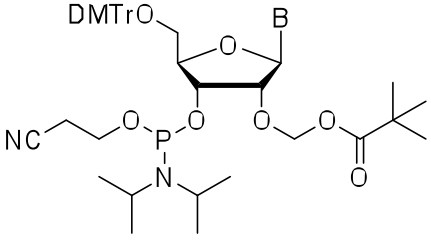
as well as several 80-mers showing the promise of this chemistry. These monomers are commercially available, but unfortunately are significantly more expensive ($150/g) than 2'-TBDMS phosphoramidite monomers ($30/g).

Entry	Nucleoside Building Block	Oligoribonucleotide Deprotection Conditions	Reference
1	 B = A^{Bz}; C^{Ac}; G^{ibu}; U	1) 3:1 NH_4OH/EtOH, r.t.,24 hr or 1) 1:1 NH_4OH/40% $MeNH_2$ in water 2) 1 M TBAF, r.t., 24 hr or 2) NEt_3:3HF, 24 hr.	ref[105,112,113,117]
2	 B = A^{Ac}; C^{Ac}; G^{Ac}; U	1) 10 M $MeNH_2$ in 1:1 H_2O/EtOH, r.t., 1 hr 2) 1 M TBAF, r.t., 14 hr	ref[119]
3	 B = A^{Ac} C^{Ac}; G^{Ac}; U	1) 1 M S_2Na_2 in 9:1 DMF/H_2O, r.t, 10 min 2) 40% $MeNH_2$ in water, 60 °C, 12 min 3) 0.1 M HOAc, pH 3.8 adjusted with TEMED, 60 °C, 30 min	ref[103,120]

4	 B = A$^{p\text{-anisoyl}}$; C$^{p\text{-anisoyl}}$; G$^{p\text{-anisoyl}}$; U	1) 1:1 NEt_4OH/MeOH, r.t. 8 hr 2) 0.01 M HCl, r.t., 6 hr	ref[100]
5	 B = A^{Bz}; C^{Bz}; G^{ibu}; U	1) NH_4OH, 55 °C, 16hr 2) 0.01 M HCl, pH 2, r.t., 4 hr.	ref[126,127,134]
6	 B = A^{Bz}; C^{Bz}; G^{ibu}; U	1) NH_4OH, 55 °C, 5hr 2) 0.01 M HCl, pH 2, r.t., 4 hr.	ref[132]
7	 B = A$^{4\text{-tBuPh}}$;C$^{4\text{-tBuPh}}$; G-*O*6$^{2\text{-ClPh}}$-*N*2^{CH_2Ph}; U-*O*4$^{2\text{-ClPh}}$	1) 0.3 M E-2-nitrobenzaldoximate in acetonitrile, 35 °C, 17 hr 2) NH_4OH, 60 °C, 17hr 3) 0.1 M HCl, pH 2, 24 hr	ref[135]
8	 B = A^{Pac}; C^{Ac}; G^{Pac}; U	1) 25% NH_4OH, 60 °C, 5hr 2) 0.1 M HCl, pH 2.5, r.t., 20 hr	ref[137]

9	 B = A^{Piv}; C^{Bz}; G^{Ac}; U	1) 3:1 NH_4OH/EtOH, r.t. 48 hr 2) 2:3 Formate buffer (0.5 M, pH 2.5)/dimethylacetamide, 40 °C, 5hr.	ref[138,139]
10	 B = A^{Bz}; C^{Bz}; G^{ibu}; U	1) NH_4OH, 55 °C, 10 hr 2) 0.1 M ACOH, pH 3.8 adjusted with TEMED, 90 °C, 30 min	ref[140]
11	 B = A^{Bz}; C^{Bz}; G^{ibu}; U	1) 3:1 NH_4OH/EtOH, r.t., 24 hr 2) 1 M TBAF, r.t., 24 hr Or 1) 3:1 NH_4OH/EtOH, r.t., 30 min 2) 0.1 M $TiCl_3$, r.t., 1hr 3) conc. NH_3 in MeOH 4) 0.1 M AcOH pH 3.8 adjusted with TEMED, 90 °C, 40 min	ref[141,143]
12	 B = A^{Ac}; C^{Ac}; G^{Pac} U	1) 1:1 NEt_3/MeCN 2) 3:1 NH_4OH/EtOH, 35 °C, 24 hr 3) 0.5 M TBAF in DMSO with 5% nitromethane , r.t., 5 hr	ref[144,145]
13	 B = A^{Pac}; C^{Ac}; G^{dmf} U	1) 10:1 NH_4OH/NH_4OAc, r.t., 90 min 2) 20:1 1 M TBAF/*n*-$PrNH_2$, r.t. 5 hr	ref[148,149]

14	 B = A^{Pac}; C^{Ac}; G^{dmf} U	1) 25% NH_3/MeOH, r.t., 20 hr 2) 1 M TBAF with 10% *n*-$PrNH_2$ and 1% bis-(2-mercaptoethyl) ether, r.t. 20 hr	ref[150]
15		1) 0.3 M NaOH, r.t., 5 min 2) 3:1:6 dioxane/water/formic acid, r.t., 4 hr 3) H_2/Pd in H_2O, r.t., 2hr	ref[152]
16	 B = A^{Bz}; C^{Bz}; G^{Bz}; U	1) 0.18 M ZnCl, aqueous pyr, r.t., 30 hr 2) NH_3/MeOH, r.t., 36 hr 3) 0.2 umol Ph_3CBF_4 in 4:1 MeCN/H_2O	ref[153,154]
17	 B = A^{Bz};; G^{ibu}; U^{MEM}	1) 0.18 M ZnCl, aqueous pyr, r.t., 24 hr 2) DDQ in 18:1 DCM/H_2O, r.t., 1 hr 3) 9:1 NH_3/pyr, 50 °C, 6 hr 4) 80% HOAc, 1hr	ref[155]
18	 B = A^{Bz}; C^{Bz}; G^{ibu}; U	1) conc. NH_4OH, 55 C, 16 hr 2) 0.5 M DTT, 55 °C, 6 hr	ref[156]

19	 B = A^{Bz}; C^{Bz}; G^{ibu}; U	1) 1 M tetramethylguanidium pyridine-2-aldoximate in 1:1 dioxane/water 2) 9:1 NH_4OH/pyr 3) 4:1 HOAc/H_2O 4) 0.1 M ammonium formate, pH 3.5, UV, 1 hr	ref[157,158]
20	 B = A^{Bz}; C^{Bz}; G^{ibu}; U	1) 3:1 NH_4OH/EtOH, 55 C, 24 hr 2) 0.2 M formic acid pH 3.7, r.t., UV, 4.5 hr	ref[160]
21	 B = A^{Bz}; C^{Bz}; G^{ibu}; U	1) 1:2:2 PhSH/NEt_3/dioxane, r.t., 1.5 hr 2) NH_4OH, 55 °C, 5 hr 3) 1:1:2 $BuNH_2$/MeOH/dioxane, 40 °C, 7 hr	ref[163]
22	 B = A^{Bz}; C^{Bz}; G^{ibu}; U	1) NH_4OH, r.t., 1.5 hr	ref[164]
23	 B = A^{Pac}; C^{Ac}; G^{ibuPac}; U	1) 10% piperidine in MeCN, 15 min 2) 28% NH_4OH, r.t., 3 hr	ref[166]

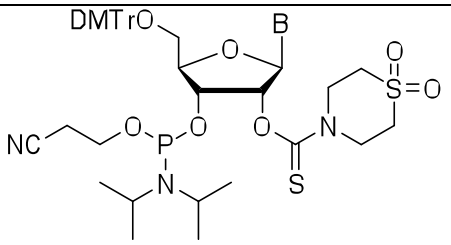
24	 B = A^{Bz}; C^{Bz}; G^{ibu}; U	1) 10% diethylamine in MeCN, 3 min 2) 1:1 EDA/toluene, 2 hr.	ref[167]

Table 1.2. Monomers used in the synthesis of RNA.

1.5 Thesis Objectives

1.5.1 The 2'-*O*-Levulinyl (Lv) Group for the Solid Phase Synthesis of RNA

The discovery of RNAi[15] and the therapeutic potential of siRNA[20,104] has spurred a renaissance in the chemical synthesis of RNA, particularly the 2'-protecting group.[85]

In **Chapter 2**, the 2'-levulinyl (Lv) protecting group strategy is examined for RNA solid phase synthesis. It was reasoned that since Rozners et al. were able to separate 2'-(2-chlorobenzoyl)-3'-H-phosphonate derivatives by column chromatography,[164,165] the same may be true for 2'-levulinyl-3'-phosphoramidite derivatives. In addition, since the levulinyl group may be removed under relatively mild deprotection conditions, 0.5 M hydrazine hydrate in 3:2 pyridine/acetic acid (v/v), internucleotide strand cleavage should be minimized. The integrity of RNA will be determined by various biophysical characterization methods as well as an RNAi luciferase gene knockdown assay. A very important feature of this approach is the possibility of removing the 2'-Lv protecting group on-column after solid phase synthesis. This would greatly simplify subsequent workup conditions relative to standard chemistry such as 2'-TBDMS, which requires removal of that group in solution and many extra tedious desalting steps to isolate the oligonucleotide.

1.5.2 The 2'-Acetal Levulinyl Ester (ALE) Group for RNA Synthesis

The inevitable problem of using esters as 2'-protecting groups in RNA synthesis is inseparable 2'-3'-isomeric mixtures.[162] Even the slightest contamination of a 3'-ester-2'-phosphoramidite will dramatically affect the integrity of a 3'-5'-RNA synthesized by solid phase.

With this in mind, **Chapter 3** will examine the use of the novel 2'-Acetal Levulinyl Ester (ALE) group for RNA synthesis. The idea here is the acetal moiety should distance the ester linkage from the 3'-OH during monomer synthesis and should therefore be too far for 2'-3'-isomerization.[166,169,170] This should greatly increase the yield of the 2'-ALE-3'-phosphoramidite monomers, and obtain isomerically pure 3'-5'-RNA. Since the 2'-ALE group encompasses a levulinyl ester, it should be removed using 0.5 M hydrazine hydrate in 3:2 pyridine/acetic acid (v/v) with concomitant release of formaldehyde. The integrity of RNA synthesized will be determined by various biophysical characterization methods as well as an RNAi luciferase gene knockdown assay. This approach should also be amenable to on-column deprotection. Basically ALE should have all the strengths of the 2'-Lv group, but none of its weaknesses.

1.5.3 The Light Directed Synthesis of RNA Microarrays

In recent years RNA microarrays ('chips') have emerged has powerful combinatorial tools as a result of increasing interest in RNAi,[171] RNA aptamers,[172] protein-RNA interactions,[173] and small molecule-RNA interactions.[174] However, unlike DNA chips,[175-178] there are only a handful of methods for fabricating RNA chips,[179-184] and their reliability and complexity is extremely limited.

In **Chapter 4**, novel 5'-(2-(2-nitrophenyl)propoxycarbonyl) (NPPOC)-2'-ALE-3'-phosphoramidite monomers are examined for their use in the fabrication of RNA chips by light directed synthesis. Various experiments will be carried out to define the optimal coupling conditions of these monomers on the chip as well as optimal deprotection conditions to remove the 2'-ALE group. The integrity of

the RNA grown and deprotected on the chip will be verified through fluorescence hybridization experiments as well as an RNase A assay.

1.5.4 The Synthesis and Biological Evaluation of Pro-siRNA

The major stumbling block for widespread use of siRNA therapeutics is delivery of the cellular delivery of these biomolecules.[185] In general siRNAs are too large (~ 13 kDa), too negatively charged (40 negative charges) and not hydrophobic enough to cross cellular membranes. Various strategies have been devised to address these problems,[185,186] but none have proven to be ideal.

In **Chapter 5**, novel N-FMOC-2'-ALE-3'-phosphoramidite monomers are examined for their use in synthesizing 2'-ALE-siRNA. The idea here is if the siRNA can be deprotected in such a way leaving the 2'-ALE group intact, this will increase the lipophilicity of the siRNA and thus increase cellular delivery. Once these 2'-ALE-siRNA have crossed the cell membrane, the 2'-ALE groups should be removed by endogenous esterases giving rise to the active siRNA molecule. This would be an example of an siRNA prodrug (or pro-siRNA). In addition, the ability to reductively aminate the ketone moiety of the ALE group will be examined. Our reasoning here is the resulting amine should be protonated at biological pH, thus reducing the overall charge of this 'zwitterionic' siRNA while maintaining lipophilicity. Various strategies will be examined for the synthesis of these molecules as well as their ability to perform RNAi in cell culture in the presence and absence of a transfecting agent.

1.6 References

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Chapter 2. The 2'-*O*-Levulinyl (Lv) Group for the Solid Phase Synthesis of RNA

2.1 Introduction

The advent of the RNAi[1] methodology and its application to therapeutics[2] has created an urgent and growing need for the synthesis of large quantities of native and chemically modified siRNA for animal and human studies.[3] For many years, RNA synthesis has been regarded as far more challenging than DNA synthesis because of the difficulty in finding a compatible 2'-protecting group that is stable throughout chain assembly and can be removed selectively at the end of synthesis without phosphodiester bond isomerization or degradation (**Chapter 1.4.7**). In fact, finding a satisfactory 2'-protecting group is a research problem that has spanned more than five decades (**Chapter 1.4.9**). Most of these 2'-protecting groups all share the same requirements for manual solution-phase 2'-deprotection. The usual steps for oligoribonucleotide deprotection are: 1) Base treatment to remove the nucleobase protecting groups, the phosphate protecting group, and cleavage from the solid support. 2) The removal of the 2'-protecing group in solution. Thus, the need for manual 2'-protecting group deprotection is time and labor intensive, particularly for large-scale synthesis, and a potential source for material losses and ribonuclease contamination. With that in mind, we have re-evaluated 2'- (and 3'-) *O*-acyl ribonucleosides, as possible synthons for RNA synthesis on solid supports.[4] The key goal was to develop monomer synthons that allowed "on-column" deprotection of the RNA chain once assembled on solid support. This approach would leave the unprotected oligomer covalently attached to the support making it possible to wash off excess deblocking reagents, salts and protecting groups. In a final step, the oligomer would be released to afford the crude RNA strand. For this, we considered two solid supports: Pon's "Q" controlled pore glass (Q-CPG), described later in this Chapter, and a light labile linker that forms the basis for the synthesis of siRNA prodrugs described in Chapter 5.

At the onset, we recognized that 2'-*O*-acyl protecting groups in RNA synthesis had been largely unsuccessful due to ease of 2'-3' migration[5] (**Figure 2.1**) and lack of specificity and compatibility with other groups. In fact, for these reasons, 2'-*O*-acyl protecting groups have only very rarely been used in oligoribonucleotide synthesis.[4,6-9]

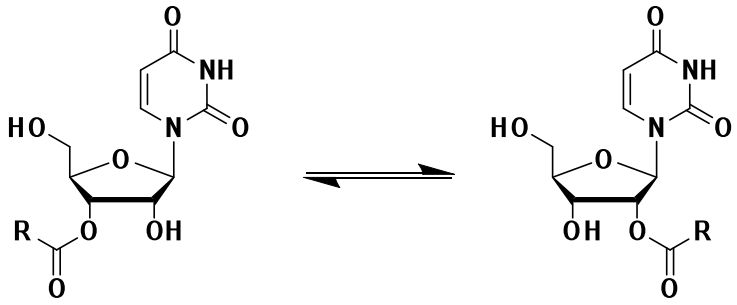


Figure 2.1. The 2'-3'-acyl group interconversion of nucleosides, uridine for example.

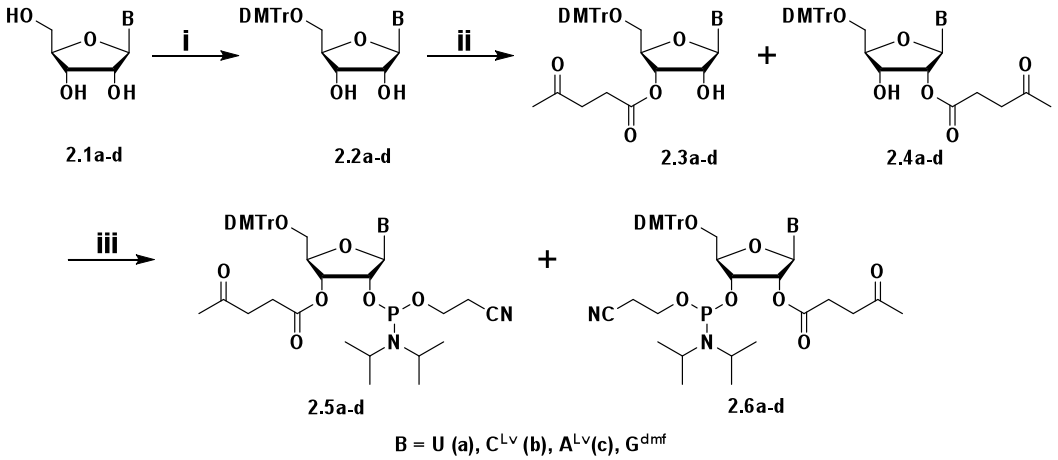
The levulinyl (Lv) group has been previously described by van Boom and co-workers[10] and Ohtsuka and co-workers[11] as an alternative to the 5'-protecting group for ribonucleosides. Ogilvie and co-workers were the first to utilize the Lv group for transient 3'-protection in conjunction with the procedures their group developed for block coupling of 3',5'-oligoribonucleotides[12] and also as an exocyclic amine protecting group of the nucleobases.[13] Here, we build upon this early work by examining the application of 3'- (and 2'-) *O*-Lv ribonucleosides as synthons for RNA synthesis. As documented in an earlier study with 3'/2'-*O*-(2-chlorobenzoyl) ribonucleosides,[8,9] we reasoned that *O*-phosphitylation of a mixture of interconverting 3'-*O*- and 2'-*O*-Lv ribonucleoside isomers would provide separable, regioisomerically stable, phosphoramidite monomers that would be suitable for RNA (and 2',5'-RNA) synthesis. In addition, the use of *N*-levulinyl nucleobase protecting groups will allow for simultaneous deblocking of base and 2'-hydroxyl groups which would simplify the workup.

2.2 3'/2'-*O*-Lv-3'-*O*-Phosphoramidite Monomer Synthesis

The synthesis of 3'/2'-*O*-Lv-3'-phosphoramidite monomers is summarized in **Scheme 2.1**. Uridine (**2.1a**), N^4-Lv-cytosine (**2.1b**) and N^6-Lv-adenine[13] (**2.1c**), and N^2-dmf-guanine[14] (**2.1d**) are first treated with DMTrCl in pyridine forming the 5'-*O*-DMTr protected nucleosides **2.2a-d** (88-90%). Because *N*-levulination of guanine proceeded sluggishly (20%),[13] the dimethylformamidine (dmf) group was used instead as it has been found that it can be efficiently introduced (>95%) and removed under the same conditions as the *N*-Lv and *O*-Lv groups.[15]

Compounds **2.2a-2.2d** were then reacted with 2-chloromethylpyridinium iodide (CMPI) and 1,4 diazabicyclo[2.2.2]octane (DABCO) to afford inseparable mixtures 2'/3'-*O*-Lv regioisomers (70-78%) favoring the 2'-*O*-Lv (**2.4a-d**) isomers (ca. 2:1 ratio for U, C, and A; 3:1 for G, after column chromatography as determined by ^{1}H NMR). The mixture of 3'-*O*-Lv (**2.3a-d)** + 2'-*O*-Lv (**2.4a-d**) was then carried on to the final phosphitylation step to afford mixtures of 3'-*O*-Lv-2'-*O*-phosphoramidites (**2.5a-d**) and 2'-*O*-Lv-3'-*O*-phosphormaidites (**2.6a-d**) in 77-85% yields. Interestingly, there was a higher percent of **2.5a-d** obtained relative to **2.6a-d**, in fact it was a near reversal in the isomeric ratio of **2.3a-d** + **2.4a-d**. It is proposed that under the phosphitylation conditions, compounds **2.3a-d** and **2.4a-d** are in a rapid equilibrium. As the 2'-OH which has a lower pK_a (*ca.* 13) and higher reactivity compared to the 3'-OH,[16] it reacts preferentially with the phosphitylating reagent to afford an excess of the 2'-amidite isomer (**2.5a-d**). As the 3'-*O*-Lv isomer (**2.3a-d**) is consumed, more of the of the 2'-*O*-Lv isomer (**2.4a-d**) converts to the 3'-*O*-Lv (**2.3a-d**) to restore the equilibrium (Le Chatelier Principle).[17] Several reaction conditions were tried to increase the 3'-amidite/2'-amidite ratio, such as temperature, solvent, and base, but without much success. While tedious to do, separation of the 2'-*O*- and 3'-*O*-phosphoramidite regioisomers was possible by flash silica gel column chromatography yielding **2.5a** (42%), **2.6a** (19%), **2.5b** (7%), **2.6b** (10%), **2.5c** (35%), **2.6c** (29%), **2.5d** (35%), and **2.6d** (22%) in isomerically pure forms. These isolated yields are generally lower than those obtained with TBDMS nucleosides, particularly for the adenosine derivatives, and reflect the very similar (TLC) chromatographic

properties of each **2.5a-d**/**2.6a-d** pair (*Rf* ~ 0.1). Nevertheless, we considered these results and those described below to be most encouraging. The identities of **2.5a-d** and **2.6a-d** were confirmed with ESI-MS in conjunction with CIGAR ^{31}P 2D NMR (^{31}P-^{1}H) experiments.

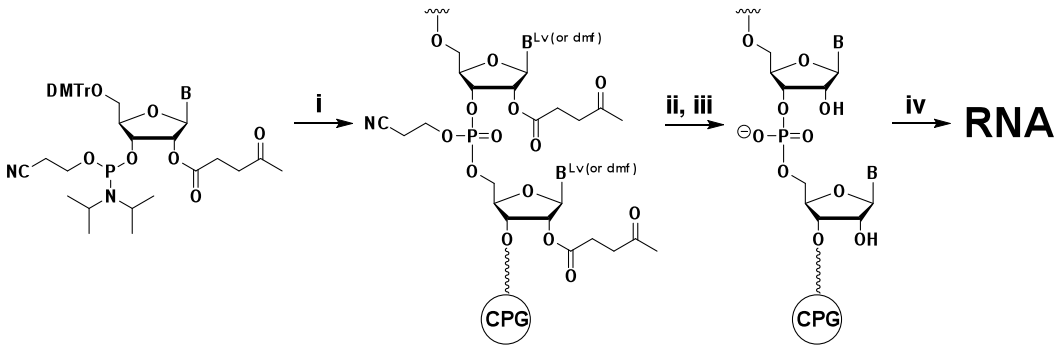


Scheme 2.1. Synthesis of 3'/2'-*O*-Lv monomers Reagents and conditions: i) DMTrCl, pyr; ii) LvOH, CMPI, DABCO, MeCN/dioxane; iii) CEtOP(Cl)NiPr$_2$, iPr$_2$NEt, CH_2Cl_2

2.3 Solid Phase Synthesis of Oligonucleotides Using 2'-*O*-Lv Chemistry

2.3.1 Synthesis of 12-mer Polyuridylic RNA

With phosphoramidite monomers **2.6a-d** in hand several oligonucleotides were synthesized to test coupling efficiencies and our novel *on-column*, or *on-line* deprotection protocol (**Scheme 2.2**).



Scheme 2.2. *On-lin*e deprotection of 2'-*O*-Lv protected RNA. i) Standard solid phase synthesis; ii) 2:3 NEt_3/CH_3CN (v/v), r.t., 60 min; iii) 0.5 M hydrazine hydrate in 3:2 pyr/HOAc (v/v), r.t., 25 min; iv) 1 M TBAF in THF, r.t., 15 min.

We first synthesized a 12-mer 5'-(U_{10})-d(TT)-3' from monomer **2.6a** and commercially available dT phosphoramidite, and compared it 5'-(U_{10})-d(TT)-3' synthesized via standard 2'-*O*-TBDMS chemistry. The oligonucleotides were synthesized on a 1 μmol scale on 500 Å CPG solid support derivatized with a 5'-*O*-DMTr-thymidine unit (45 μmol/g) appended through a hydroquinone-*O*,*O*'-diacetic acid linker (Q-linker).[18] Unlike the standard succinoyl linker, the Q-linker makes it possible to release an oligonucleotide chain with fluoride ions under conditions that do not lead to internucleotide cleavage (1 M TBAF in THF; r.t., 15 min). The phosphoramidite monomer (**2.6a** and 2'-*O*-TBDMS-rU) concentration was 0.15 M in MeCN, the activator was 0.25 M 5-ethylthiotetrazole (ETT), and coupling times of 1 min or 10 min were used. Oxidation of the phosphite triester intermediates was achieved using the standard 0.1 M iodine/pyridine/water treatment. At 1 min coupling, the average stepwise coupling yields were 95.9% and 97.0% for 2'-*O*-TBDMS-rU and **2.6a**, respectively (**2.7, 2.8, Table 2.1**). When the coupling time was increased to 10 min, these yields increased to 99.1% and 97.2%, respectively (**2.9, 2.10, Table 2.1**). Following chain assembly, the fully deprotected oligomers were obtained by *on-line* deprotection by: 1) treatment with anhydrous 2:3 NEt_3/CH_3CN (v/v) (r.t., 60 min) to deblock the β-cyanoethyl phosphate protecting groups; 2) washing the solid

support (acetonitrile, 5 min); 3) hydrazinolysis (0.5 M hydrazine hydrate in 3:2 pyridine/ acetic acid (v/v), 25 min, r.t) to remove the levulinyl group; 4) washing the solid support with acetonitrile and dichloromethane to fully remove excess hydrazine solution. At this stage, the deprotected RNA strand is attached to the CPG support through the Q-linker. A final fluoride treatment (1 M TBAF, r.t., 15 min) releases the oligonucleotide chain. The oligonucleotides synthesized by standard 2'-*O*-TBDMS chemistry[19] were deprotected as follows: 1) 3:1 NH_4OH/EtOH (v/v), 55 °C, 16 hr; 2) 1 M TBAF, r.t., 16 hr. The HPLC traces in the 15-30 min range, where the by-products or failure sequences elute, are slightly different. This could be due to poor coupling efficiency or partial deprotection of the 2'-*O*-Lv group. However, the retention time (Rt) of the full-length oligonucleotide were shown to be identical by ion-exchange HPLC (**Figure 2.2**). The TBDMS method gave far superior results at 10 min coupling times, affording ca. 20% more product than the Lv method (**Figure 2.2**). The advantage of the Lv chemistry is seen at shorter coupling times.

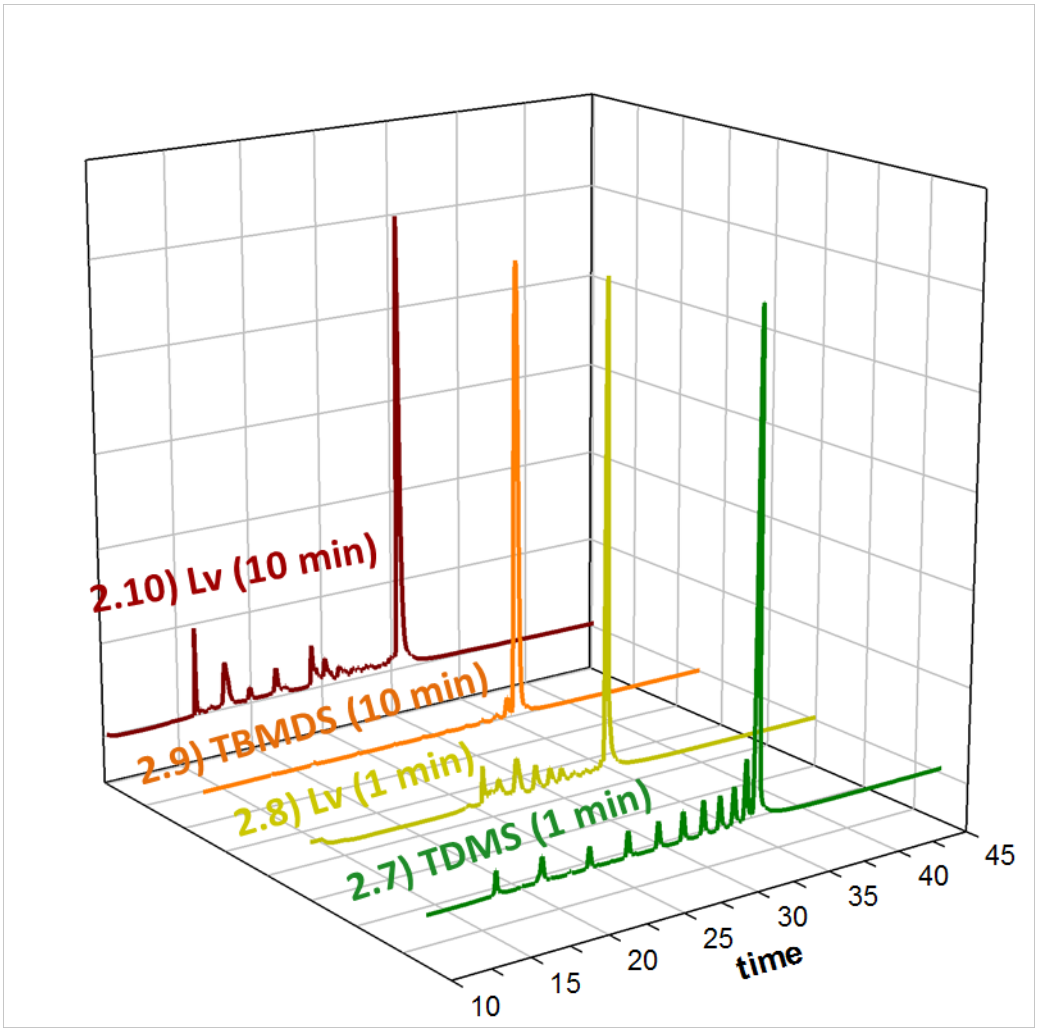


Figure 2.2. HPLC chromatogram of 12-mer 5'-(U_{10})-d(TT)-3' synthesized from 2'-*O*-Lv **(2.6a)** and 2'-*O*-TBMDS phosphoramidite monomers with 1 min and 10 min coupling times.

2.3.2 Synthesis of Mixed Base siRNA

Satisfied with the initial results obtained with the synthesis of 12-mer 5'-$U_{10}dT_2$-3' (**2.8, 2.10**), we then synthesized a mixed base 21-mer siRNA sequence using 2'-*O*-TBDMS (**2.11, Figure 2.3, Table 2.1**) and 2'-*O*-Lv chemistries (**2.12, Figure 2.3, Table 2.1**). The solid phase synthesis conditions were the same as described above (**2.3.1**), except the coupling times were extended to 14 min. (Initial tests at 1 min coupling afforded only 95.9% stepwise coupling yields;

Figure 2.3, Table 2.1). After solid phase synthesis, oligonucleotide **2.12** was deprotected as described above and purified**.** Under these conditions, both TBDMS and Lv methods afforded the same average stepwise coupling yield of 98.5% (**Figure 2.3**, **Table 2.1**).

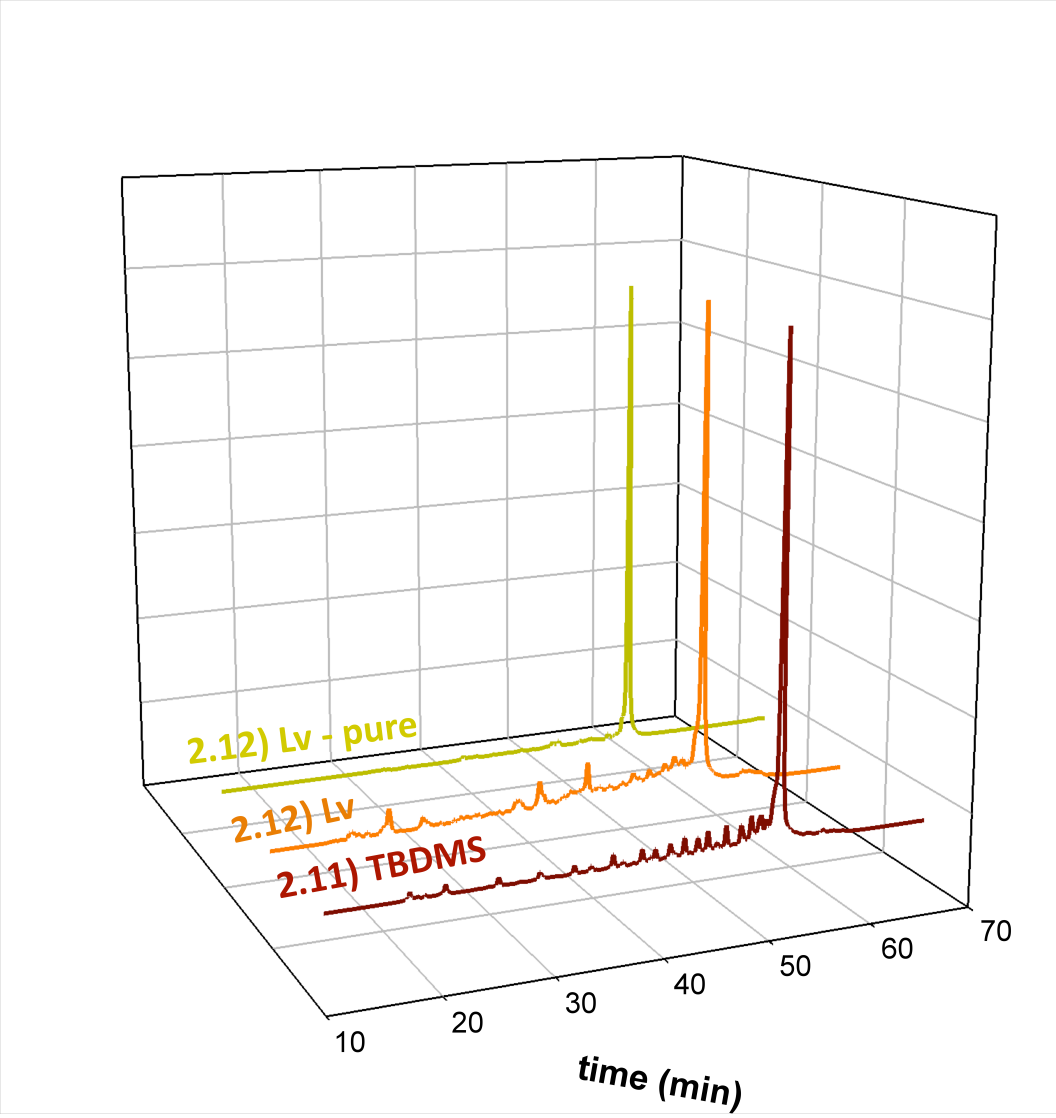


Figure 2.3. HPLC chromatograms of 5'-(GCUUGAAGUCUUUAAUUAA)-d(TT)-3' RNA synthesized from 2'-*O*-TBDMS (front, red), 2'-*O*-Lv (middle, orange) and from purified 2'-*O*-Lv (back, yellow).

These isolated full-length oligonucleotides were the same as assessed by HPLC (Rt) and MALDI-TOF mass spectrometry (calc. 6616, found: 6617 and 6614 for **2.11** and **2.12**, respectively). The T_m values of duplexes formed by the

hybridization of **2.11** and **2.12** with a complementary RNA strand (synthesized via TBDMS chemistry) were 51.7 and 49 °C, respectively (values within +/- 1 °C).

No.	5'-sequence-3'	Protecting group	CT (min)[a]	%[b]	Avg. coup. Yield (%)
2.7	(U_{10})-d(TT)	TBDMS	1	60.7	95.9
2.8	(U_{10})-d(TT)	Lv	1	69.6	97.0
2.9	(U_{10})-d(TT)	TBDMS	10	90.1	99.5
2.10	(U_{10})-d(TT)	Lv	10	71.2	97.2
2.11	(GCUUGAAGUCUUUAAUUAA)-d(TT)	TBDMS	14	72.8	98.5
2.12	(GCUUGAAGUCUUUAAUUAA)-d(TT)	Lv	14	72.5	98.5

Table 2.1. Data for oligoribonucleotides synthesized. [a]Coupling time. [b]% yield of oligomer in crude material (HPLC).

2.3.3 Integrity of the Phosphodiester Linkage Upon 2'-*O*-Lv Cleavage

To further confirm the integrity of the phosphodiester linkages during RNA synthesis and deprotection, the regioisomeric strand dTTTT-[2',5'-rU]-dTTTT and dTTTT-[3',5'-rU]-dTTTT were also prepared. These oligomers were separable by Clarity reverse-phase HPLC (Rt, 42.8 and 44.2 min, respectively) and were free from their isomeric form (detection limit <1%).

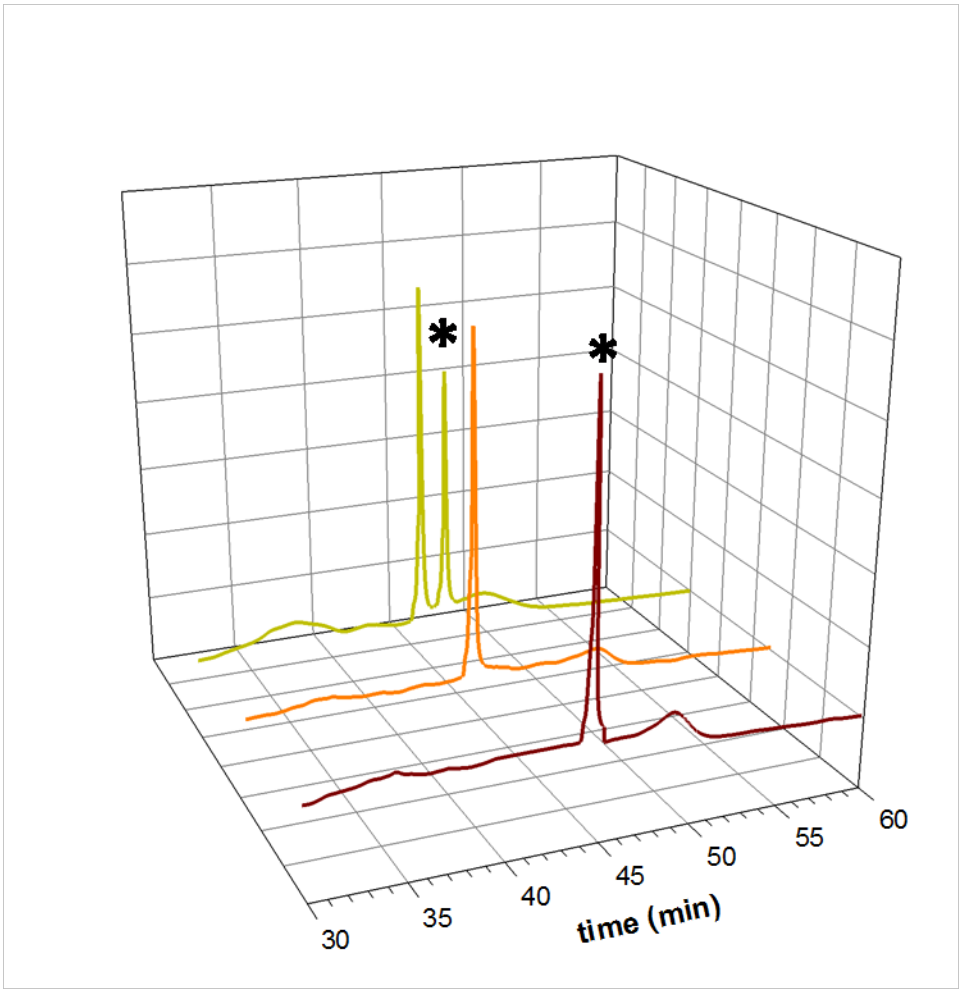


Figure 2.4: Clarity reverse phase HPLC of dTTTT-[3',5'-rU]-dTTTT (red trace; Rt = 49.2 min), prepared from 2'-*O*-Lv amidite (**2.6a**), dTTTT-[2',5'-rU]-dTTTT (orange trace; Rt = 48.8 min), prepared from 3'-*O*-Lv amidite (**2.5a**). The trace of a pre-mix sample of both oligomers is also shown (yellow trace). The elution gradient was 98 :2 to 85 :15 100 mM triethylammonium acetate/acetonitrile over 40 minutes, 60 °C.

2.3.4 RNAi Luciferase Assay

To further demonstrate the reliability of our synthetic methods, we evaluated the biological activity of siRNAs synthesized with monomers **2.6a-d** in an RNAi assay that targets luciferase mRNA.[20] The siRNA duplex [**2.12**/complement] had the same gene silencing activity [IC_{50} 0.08 nM] as the reference siRNA duplex prepared via TBDMS chemistry [**2.11**/complement] (**Figure 2.5**). This assay was performed by Dr. Francis Robert, a group member in the Pelletier lab (Biochemistry Department, McGill University).

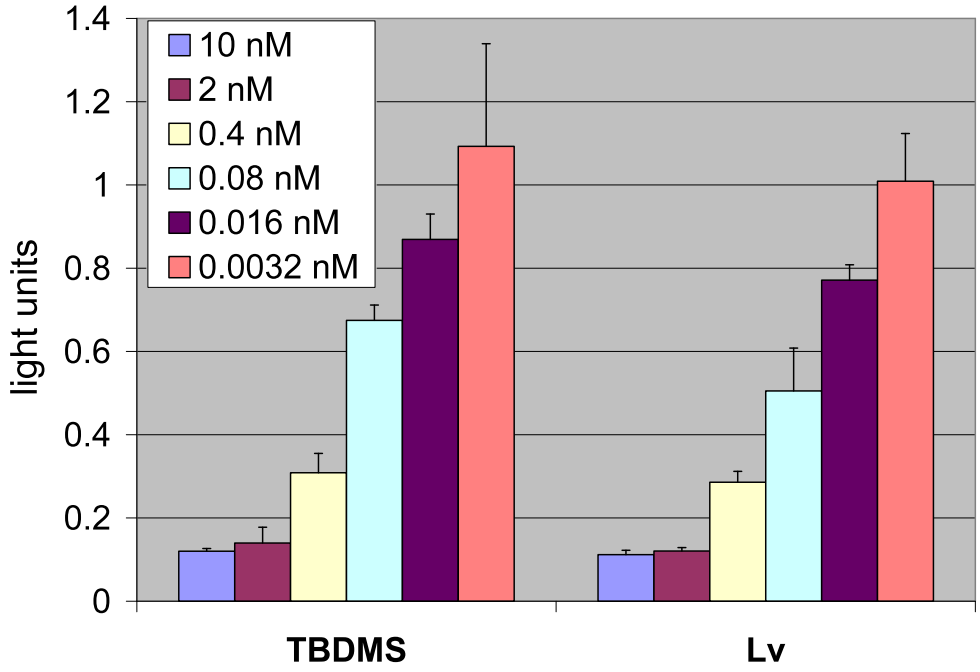


Figure 2.5. Silencing of luciferase mRNA expression by siRNA duplexes (light units are relative to a scrambled siRNA control). The antisense strands were synthesized via TBDMS and Lv chemistries (sequences **2.11** and **2.12**, respectively, Table 1), whereas the sense strand was obtained via TBDMS chemistry.

2.5 Conclusions

We have shown that the Lv group is a suitable protecting group for the 2'-hydroxy functions of ribonucleoside building blocks. Its major advantage over other 2'-protecting groups is in the *on-column* unblocking step at the end of the synthesis which greatly simplifies and speeds up post-synthetic processing. While the final deblocking still requires a fluoride step to cleave the oligomer from the Q-linker, this step can be carried out at room temperature with a minimum amount of fluoride reagent. Ideally, it would be desirable to eliminate this step altogether, and efforts to do so are described in **Chapter 5**. With regard to the introduction of the Lv group, the reagent required, 4-oxopentanoic acid, is inexpensive and readily available (prepared in >70% yield by treating starch or cellulose with acid).[21] Although this provides a clear cost advantage over some current protection schemes, the arduous separation of derivatives such as **2.5a-**

d/2.6ad are a major disadvantage. In addition, we would like to note that the orthogonal deprotection conditions of 2'-TBDMS and a transient 2'-Lv group lend themselves to the synthesis of branched RNA. This strategy was exploited by a former member of our group (Dr. David Sabatino) in the synthesis of branched and dendritic nucleic acid structures. [22]

2.6 General Methods

^{1}H NMR spectra were recorded at 500 MHz and the chemical shifts were measured from the solvent peak as an internal standard (in $CDCl_3$, CD_3CN or DMSO-d_6). ^{31}P NMR spectra were recorded at 202 MHz and the chemical shifts were measured from 85% H_3PO_4 as an external standard. CIGAR ^{31}P NMR correlation spectra were recorded at 500MHz. Mass spectra were recorded using low resolution ESI. 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. All reactions were run under argon or nitrogen atmosphere in flame-dried glassware.

The solid-phase synthesis of oligonucleotides was carried out on an Applied Biosystems DNA/RNA 3400 synthesizer using normal phosphoramidite protocol. Standard 2'-TBDMS and 3'-TBDMS-amidites were purchased from Chemgenes. Anion-exchange HPLC was performed on a Waters Alliance system with a Waters 3D UV detector and a Waters Protein Pack DEAE-5PW column (7.5 mm x 7.5 cm). Reverse-phase HPLC was performed on a semi-prep Clarity® Phenomonex column. MALDI-TOF mass spectrometry was carried out on a Kratos Kompact III instrument.

2.6.1 Synthesis of 2'/3'-*O*-Levulinyl Monomers

Example for 2-*N*-Dimethylaminomethylene-5'-*O*-(4,4'-dimethoxytrityl)-3'/2'-*O*-(levulinyl)-guanosine (2.3d/2.4d)

2-Chloro-*N*-methylpyridinium iodide (1.36 g, 5.3 mmol) was dissolved in acetonitrile (30 mL) under argon atmosphere followed by the addition of 1,4-diazabicyclo[2.2.2]octane (1.34 g, 12 mmol) in 1,4-dioxane (15 mL). Levulinic acid (32.4 mg, 2.92 mmol) dissolved in 1,4-dioxane (5 mL) was then added dropwise to the stirring reaction mixture. The resulting slurry was then stirred at 0 ºC. In a separate round bottom flask 2-*N*-dimethylaminomethylene-5'-*O*-(4,4'-dimethoxytrityl) (**2.2d**) (1.7 g, 2.7 mmol) was dissolved in 1,4-dioxane (30 mL) under argon atmosphere and stirred at 0 ºC. The former reaction slurry was then slowly cannulated into the latter and allowed to stir at 0 ºC for 30 minutes and then warmed to room temperature and stirred for an additional 30 minutes. The reaction mixture was then diluted with chloroform and washed with saturated sodium bicarbonate and brine. The organic layer was dried with Na_2SO_4 and filtered. The solvent was evaporated providing a sticky reddish gew. For G only, the oil was then dissolved in a minimum amount of chloroform and added dropwise to vigorously stirring ether (500 mL). The solid was then filtered and washed with ether, water, and ether. Chromatography was performed on a column of silica gel (50g/g of product) neutralized with 2% triethylamine and eluted with gradient up to 97:3 dichloromethane/methanol. Evaporation of solvent yielded a white foam in 75% yield (1.47 g) of two inseparable 2'/3'-*O*-Lv isomers in a 3:1 ratio.

This general procedure was also employed for the *O*-levulinylation of nucleosides **2.2a-d**. Chromatographic purification of the inseparable 3'/2'-O-Lv mixture is as follows**: 2.3a/2.4a**, gradient up to 97:3 CH_2Cl_2/MeOH (70%, 1:2 3'-Lv/2'-Lv); **2.3b/2.4b**, gradient up to 98:2 CH_2Cl_2/MeOH (78%, 1:2 3'-Lv/2'-Lv); **2.3c/2.4c**, gradient up to 98:2 CH_2Cl_2/MeOH (72%, 1:2 3'-Lv/2'-Lv);.

The following ^{1}H NMR data describes a regioisomeric mixture.

5'-*O*-(4, 4'-dimethoxytrityl)-3'/2'-*O*-(levulinyl)uridine (2.3a/2.4a)

^{1}H NMR (DMSO, 500 MHz) δ 11.41 (2H, s), 7.68 (1H, d, J = 8), 7.66 (1H, d, J = 8.5) 7.38-7.21 (18H, m), 6.90-6.87 (8H, m), 5.89 (1H, d, J = 4), 5.79 (1H, s), 5.75 (1H, d, J = 5.5), 5.53 (1H, s), 5.43 (1H, d J = 8), 5.40 (1H, d, J = 8.5), 5.25-5.23 (1H, m), 5.13-5.11 (1H, m), 4.39-4.37 (1H, m), 4.34-4.32 (1H, m), 4.1-4.07 (1H, m), 3.98-3.95 (1H, m), 3.72 (12H, m), 3.30-3.26 (2H, m), 3.22-3.20 (2H, m), 2.72-2.70 (4H, m), 2.54-2.52 (4H, m), 2.09 (6H, s); ESI MS: calcd for $C_{35}H_{36}N_2O_{10}Na$ [M + Na^+] 667.2, found 667.0.

6-*N*-levulinyl-5'-*O*-(4, 4'-dimethoxytrityl)-3'/2'-*O*-(levulinyl)adenosine (2.3b/2.4b)

^{1}H NMR (DMSO, 500 MHz) δ 10.78 (2H, s), 8.59 (1H, s), 8.57 (1H, s), 8.56 (1H,s), 8.52 (1H, s), 7.36-7.17 (18H, m), 7.22-6.77 (8H, m), 6.23 (1H, d, J = 4.5), 6.01 (1H, d, J = 6), 5.95-5.85 (2H, m), 5.63 (1H, d, J = 5.5), 5.35-5.34 (1H, m), 5.16-5.12 (1H, m), 4.72-4.67 (1H, m), 4.22-4.18 (1H, m), 4.12-4.08 (1H, m), 3.71 (6H, s), 3.70 (6H, s), 3.33-3.24 (8H, m), 2.80-2.68 (8H, m), 2.58-2.53 (1H, m), 2.13-2.11 (6H, m), 2.07-2.05 (6H, m); ESI MS: calcd for $C_{41}H_{43}N_5O_{10}$ Na [M + Na^+] 788.20, found 788.1.

4-*N*-levulinyl-5'-*O*-(4, 4'-dimethoxytrityl)-3'/2'-*O*-(levulinyl)cytidine (2.3c/2.4c)

^{1}H NMR (DMSO, 500 MHz) δ 10.95 (2H, m), 8.27-8.10 (2H, m) 7.41-7.22 (18H, m), 7.05-7.03 (2H, m), 6.91-6.89 (8H, m), 5.88 (1H, m), 5.81 (1H, m), 5.16 (1H, m), 5.09 (1H, m), 4.34 (1H, m), 4.23 (1H, m), 3.73 (12H, m), 3.32 (4H, m), 2.71-2.58 (16H, m), 2.11 (12H, m); ESI MS: calcd for $C_{39}H_{42}N_6O_9Na$ [M + Na^+] 764.20, found 764.10.

2-*N*-Dimethylaminomethylene-5'-*O*-(4,4'-dimethoxytrityl)-3'/2'-*O*-(levulinyl)guanosine (2.3d/2.4d)

^{1}H NMR (DMSO, 500 MHz) δ 11.41 (2H, s), 8.61 (2H, s), 8.46 (1H, s), 8.30 (1H, s), 7.95 (1H, s), 7.89 (1H, s), 7.32-7.13 (18, m), 6.81-6.78 (8H, m), 6.01 (1H, d, J = 5), 5.79 (1H, d, J = 4), 5.79-5.69 (1H, m), 5.56-5.41 (1H, m), 4.98 (1H, s), 4.56 (1H, s), 4.12-4.09 (1H, m), 4.12-4.09 (1H, m), 3.70-3.20 (16 H, m), 3.19-3.14 (4H, m), 3.05 (3H, s), (9H, m), 2.73-2.53 (4H, m), 2.09 (6H, s), 2.06 (6H, s); ESI MS: calcd for $C_{39}H_{42}N_6O_9Na$ [M + Na^+] 738.30, found 738.50.

Synthesis of 2'-*O*-levulinated Phosphoramidites: Example for 2-*N*-Dimethylaminomethylene-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-(levulinyl)guanosine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (2.6d)

Inseparable isomers **2.3d/2.4d** (1.17 g, 1.58 mmol) were dissolved in dry CH_2Cl_2 (15 mL) under argon at room temperature. Diisopropylethylamine (0.65 mL, 6.6 mmol) was then added to the stirring solution followed by dropwise addition of chloro(2-cyanoethoxy)(*N,N*-diisopropylamino)phosphine (0.46 mL, 6.32 mmol). After stirring for 4 h, the reaction mixture is diluted with CH_2Cl_2 and the solution is washed once with 5% $NaHCO_3$ and brine. The organic layer was dried with Na_2SO_4, filtered, and evaporated *in vacuo* yielding an off-white foam. This material was first passed through a short silica gel column (20g/g product) using 70:30 $CHCl_3$/acetone (1.16 g, 77%) to remove impurities. Isomers **2.5d** and **2.6d** were then separated by column chromatography on silica gel (200 g/g of product) neutralized with 2% triethylamine and eluted with a gradient up to 80:19.5:0.5 v/v $CHCl_3$:acetone:triethylamine to give **2.5d** (408 mg, 35%),and **2.6d** (249.8 mg, 22%), as a white foams after evaporation. This general procedure was also employed for the phosphitylation of nucleosides **2.3a/2.4a** – **2.3d/2.4d**. Crude material was first passed through a short silica gel column (20g/g product): **2.5a/2.6a**, 95:5 $CHCl_3$/acetone (1.22 g, 80%); **2.5b/6b**, 7:3 EtOAc/hexanes (1.2 g, 85%); **5c/6c** 85:15 $CHCl_3$/acetone (1.16 g, 77%). The 2'-*O*-Lv isomers are separated from the 3'-*O*-Lv isomers by silica gel column

chromatography using the following solvent systems: **2.5a/2.6a**, gradient up to 97:3 $CHCl_3$/acetone (**2.5a**, 842 mg, 42%; **2.6a** 298.2 mg, 19%,); **2.5b/2.6b**, gradient up to 4:1 EtOAc/hexanes (**2.5b** 111.5 mg, 10%,; **2.6b**, 115 mg, 7%); **2.5c/2.6c** gradient up to 90:10 $CHCl_3$/acetone (**2.5c**, 282 mg, 35%; **2.6c**, 234 mg, 29%).

5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-(levulinyl)uridine-2'-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (2.5a)

^{1}H NMR (DMSO, 500 MHz) δ 7.65–7.62 (2H, m), 7.38–7.25 (10H, m), 7.23–7.18 (8H, m), 6.91-6.87 (8H, m), 5.90-5.84 (2H, m), 5.50-5.41 (2H, m), 5.24-5.21 (1H, m), 5.20-5.17 (1H, m), 4.52-4.48 (2H, m), 4.11 (2H, m), 3.63 (12H, s), 3.58-3.42 (4H, m), 3.32-3.21 (4H, m), 2.71-2.61 (4H, m), 2.38-2.19 (4H, m), 2.06 (3H, s), 2.04 (3H, s), 1.05–0.98 (24H, m); ^{31}P NMR ($CDCl_3$, 202 MHz) δ 156.29, 156.21; ESI MS: calcd for $C_{44}H_{53}N_4O_{11}PNa$ [M + Na^+] 867.3, found 867.2.

6-*N*-levulinyl-5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-(levulinyl)adenosine-2'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (2.5b)

^{1}H NMR (CD_3CN, 500 MHz) δ 8.95 (2H, s), 8.52 (1H, s), 8.49 (1H, s), 8.28 (1H, s), 8.26 (1H, s), 7.45-7.23 (18H, m), 6.87-6.82 (8H, m), 6.18 (1H, d, J = 5), 6.16 (1H, d, J = 6), 5.59-5.57 (1H, m), 5.55-5.53 (1H, m), 5.43-5.39 (1H, m), 5.33-5.29 (1H, m), 4.35-4.28 (2H, m), 3.77 (12H, s), 3.57-3.38 (6H, m), 3.04-3.01 (4H, m), 2.86-2.75 (12H, m), 2.65-2.62 (4H, m), 2.39-2.37 (4H, m), 2.19 (3H, s), 2.17 (3H, s), 2.15 (3H, s), 2.14 (3H, s), 1.11-1.08 (24H, m); ^{31}P NMR ($CDCl_3$, 202 MHz) δ 151.942, 151.46; ESI MS: calcd for $C_{50}H_{60}N_7O_{11}PNa$ [M + Na^+] 965.4, found 965.2.

4-*N*-levulinyl-5'-*O*-(4, 4'-dimethoxytrityl)-3'-*O*-(levulinyl)cytidine 2'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (2.5c)

^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.25 (1H, d, J = 7.5), 8.20 (1H, d, J = 7), 7.45-7.27 (18H, m), 7.08 (1H, d, J = 7.5), 7.05 (1H, J = 7), 6.91-6.89 (8H, m), 6.04 (1H, d, J = 3), 6.00 (1H, d, J = 3), 5.36-5.25 (1H, m), 5.30-5.28 (1H, m), 4.68-4.66 (1H, m),

4.63-4.60 (1H, m), 4.30-4.27 (2H, m), 3.87-3.83 (2H, m), 3.79 (12H, s), 3.66-3.61 (4H, m), 3.48-3.41 (4H, m), 2.77-2.54 (16H, m), 2.15-2.12 (12H, m), 1.27-1.03 (24H, m); ^{31}P NMR ($CDCl_3$, 202 MHz) δ 152.29, 151.14; ESI MS: calcd for $C_{49}H_{60}N_5O_{12}PNa$ [M + Na^+] 964.3, found 964.2.

2-*N*-Dimethylaminomethylene-5'-*O*-(4, 4'-dimethoxytrityl)-3'-*O*-(levulinyl)guanosine 2'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (2.5d)

^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.28 (1H, s), 8.70 (1H, s), 7.68 (1H, s), 7.36-7.15 (8H, m), 6.78-6.77 (5H, m), 5.92 (1H, d, J = 4), 5.75-5.73 (1H, m), 5.23-5.20 (1H, m), 4.23-4.22 (1H, m), 3.84-3.80 (2H, m), 3.76 (6H, s), 3.54-3.48 (2H, m), 3.44 (1H, m), 3.31-3.29 (1H, m), 3.05 (3H, s), 3.03 (3H, s), 2.75-2.73 (2H, m), 2.66-2.53 (4H, m), 2.17 (3H, s), 1.12 (3H, s), 1.11 (3H, s), 0.94 (3H, s), 0.93 (3H, s); ^{31}P NMR ($CDCl_3$, 202 MHz) δ 151.89; ESI MS: calcd for $C_{48}H_{59}N_8O_{10}PNa$ [M + Na^+] 962.0, found 962.2.

5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-(levulinyl)uridine-3'-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (2.6a)

^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.17 (1H, s), 7.70 (1H, d, J = 8.5), 7.39-7.23 (9H, m), 6.85-6.83 (4H, m), 6.15 (1H, d, J = 6), 5.41-5.39 (1H, m), 5.34 (1H, d, J = 8), 4.67-4.64 (1H, m), 4.30 (1H, s), 3.80 (6H, s), 3.69-3.63 (1H, s), 3.62-3.52 (2H, m), 3.44-3.42 (2H, m), 2.78-2.75 (2H, m), 2.66-2.63 (2H, m), 2.42-2.38 (2H, m), 2.18 (3H, s), 1.17 (6H, s), 1.16 (6H, m); ^{31}P NMR ($CDCl_3$, 202 MHz) δ 151.80; ESI MS: calcd for $C_{44}H_{53}N_4O_{11}PNa$ [M + Na^+] 867.3, found 867.2.

6-*N*-levulinyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-(levulinyl)adenosine-3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (2.6b)

^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.82 (2H, s), 8.61 (2H, s), 8.18 (2H, s), 7.41-7.26 (18H, m), 6.81-6.80 (8H, m), 6.31 (1H, d, J = 5), 6.25 (1H, d, J = 6), 5.53-5.52 (2H, m), 5.18-5.15 (2H, m), 4.35-4.32 (2H, m), 3.78 (12H, m), 3.56-3.42 (6H, m), 2.92-2.90 (4H, m), 2.81-2.57 (16H, m), 2.40-2.33 (4H, m), 2.24 (6H, s), 2.19 (6H,

s), 1.25-1.10 (24H, m); ^{31}P NMR ($CDCl_3$, 202 MHz) δ 152.74, 152.47; ESI MS: calcd for $C_{50}H_{60}N_7O_{11}PNa$ [M + Na^+] 965.4, found 965.2.

4-*N*-levulinyl-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-(levulinyl)cytidine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (2.6c)

^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.40 (2H, m), 8.21 (1H, d, J = 7.5), 8.14 (1H, d, J = 7.5), 7.50-7.25 (18H, m), 7.07 (1H, d, J = 8), 6.98 (1H, d, J = 8), 6.9-6.87 (8H, m), 5.97-5.93 (2H, m), 5.57-5.49 (2H, m), 4.69-4.64 (1H, m), 4.63-4.59 (1H, m), 4.27-4.26 (1H, m), 4.22-4.20 (1H, m), 3.78 (12H, s), 3.78-3.71 (4H, m), 2.74-2.2.48 (16H, m), 2.14-2.13 (12H, m), 1.31-1.03 (24H, m); ^{31}P NMR ($CDCl_3$, 202 MHz) δ 150.38, 150.21; ESI MS calcd for $C_{49}H_{60}N_5O_{12}PNa$ [M + Na^+] 964.3, found 964.2.

2-*N*-Dimethylaminomethylene-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-(levulinyl)guanosine 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (2.6d)

^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.76 (2H, m), 8.69 (1H, s), 8.56 (1H, s), 7.74 (1H, s), 7.72 (1H, s), 7.43-7.16 (18H, m), 6.81-6.77 (8H, m), 6.06 (1H, d, J = 5.5), 5.95 (1H, d, J = 5.5), 5.87-5.58 (1H, m), 5.68-5.66 (1H, m), 5.26-5.23 (1H, m), 4.69-4.67(1H, m), 4.36 (1H, m), 4.21-4.20 (1H, s), 3.77-3.76 (12H, m), 3.59-3.42 (4H, m), 3.33-3.29 (2H, m), 3.06 (6H, s), 3.03 (3H, s), 2.99 (3H, s), 2.78-2.70 (4H, m), 2.64-2.60 (2H, m), 2.34-2.31 (2H, m), 2.17 (3H, s), 2.15 (3H, s), 1.16-1.12 (24H, m); ^{31}P NMR ($CDCl_3$, 202 MHz) δ 149.30, 147.52; ESI MS calcd for $C_{48}H_{59}N_8O_{10}PNa$ [M + Na^+] 962.00, found 962.20.

2.6.2 Synthesis of Oligoribonucleotides

RNA sequence (**2.12**) was synthesized on a 1-μmol scale using monomers **2.6a-d** (0.15 M in MeCN) as in **Table 2.2**. The solid support used was controlled pore glass (500 Å CPG) with a 5'-*O*-DMTr-thymidine unit (45 g/μmol) appended through a hydroquinone-*O*,*O'*-diacetic acid linker ('Q linker').[16] 3% Trichloroacetic acid (TCA) in dichloromethane is used to detritylate, 0.25 M 5-ethylthiotetrazole (ETT) as an activator, with 14-min coupling times. Capping was performed using CAP A (acetic anhydride/pyridine/THF, 10:10:80), and CAP B (*N*-methyl imidazole/THF, 10:90). Oxidation of the phosphite triester intermediates was achieved using the standard 0.1M iodine in pyridine/water/THF (8:16:76) treatment. The deprotection conditions are shown in **Table 2.3**. First, 2:3 triethylamine/MeCN is passed through the column for 60 min to remove cyanoethyl groups. The column is then washed with acetonitrile for 10 min. Delevulination is then performed by running 0.5 M hydrazine hydrate in 3:2 pyridine/acetic acid through the column for 25 min. The column is then washed with acetonitrile for 10 min and dried by flushing with argon. The CPG is then transferred to an eppendorf tube where the oligomer is then cleaved from the solid support using 500 μL of 1 M tetrabutylammonium fluoride (TBAF) in THF for 15 min. The CPG is spun down and the supernatant is transferred to another eppendorf tube. The CPG is washed 4x with 250 uL water and added to the eppendorf with the supernatant. This is then lyophilized to dryness. The oligomers are then precipitated using 25 μL of 3M sodium acetate and 1 mL of Butanol which is chilled on dry ice for 1 hr. The eppindorf is then spun down at 4º C for 15 min and the supernatant is removed and discarded. The remaining white pellet is then washed 2x with 100 uL of cold ethanol and evaporated to dryness. It is then dissolved in 1 mL water and quantitated before purification.

Note: we have found that a significant amount of oligoribonucleotide remains trapped in the supernatant during butanol precipitation, and hence it would be advantageous to skip this step. We recommend evaporating the TBAF solution to dryness, redissolving in 1 mL water, and subjecting the solution to desalting using

sephadex G-25. This way, the recovery of the oligonucleotide will be much higher.

Step	Operation	Reagent	Time (s)
1	Detritylation	3% TCA in CH_2Cl_2	120
2	Coupling	0.15 M amidite in MeCN 0.25 M ETT in MeCN	840
3	Capping	CAP A, CAP B	20
4	Oxidation	0.1M iodine in pyridine/water/THF	30

Table 2.2. RNA synthetic conditions

Step	Operation	Reagent	Time (min)
1	decyanoethylate	2:3 NEt_3/MeCN	60
2	delevulinate	0.5 M hydrazine hydrate in 3:2 pyridine/acetic acid	25
3	cleave from support	1 M TBAF in THF	15

Table 2.3. RNA deprotection conditions

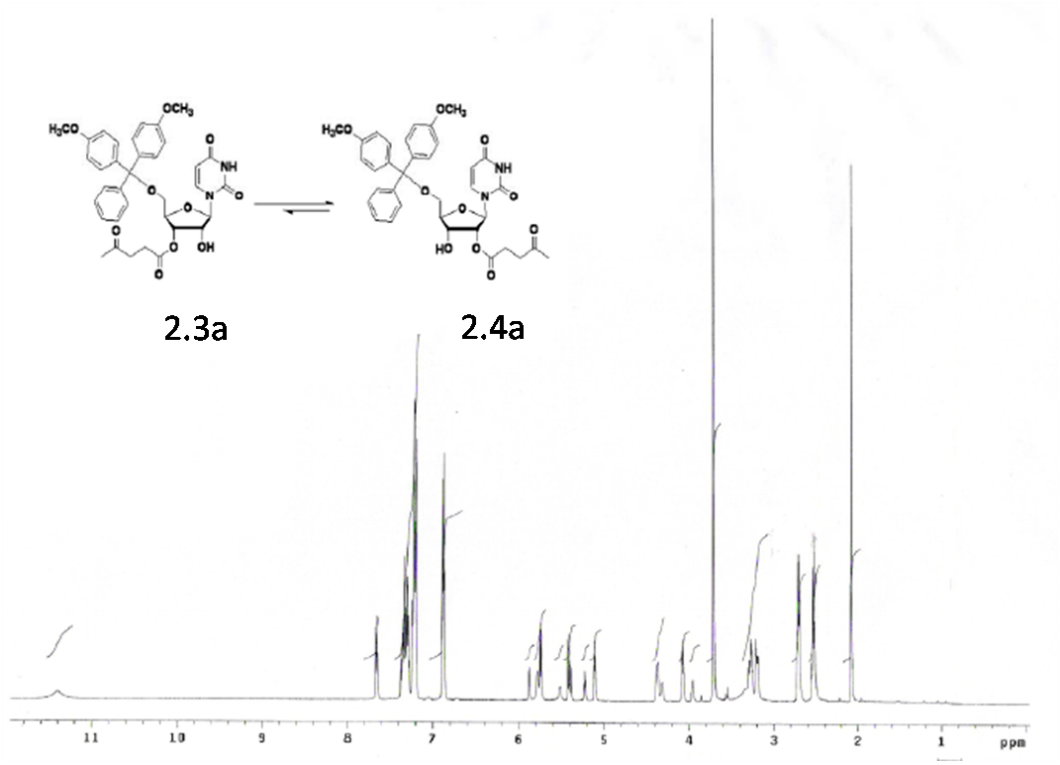
2.6.3 Purification of Oligoribonucleotides

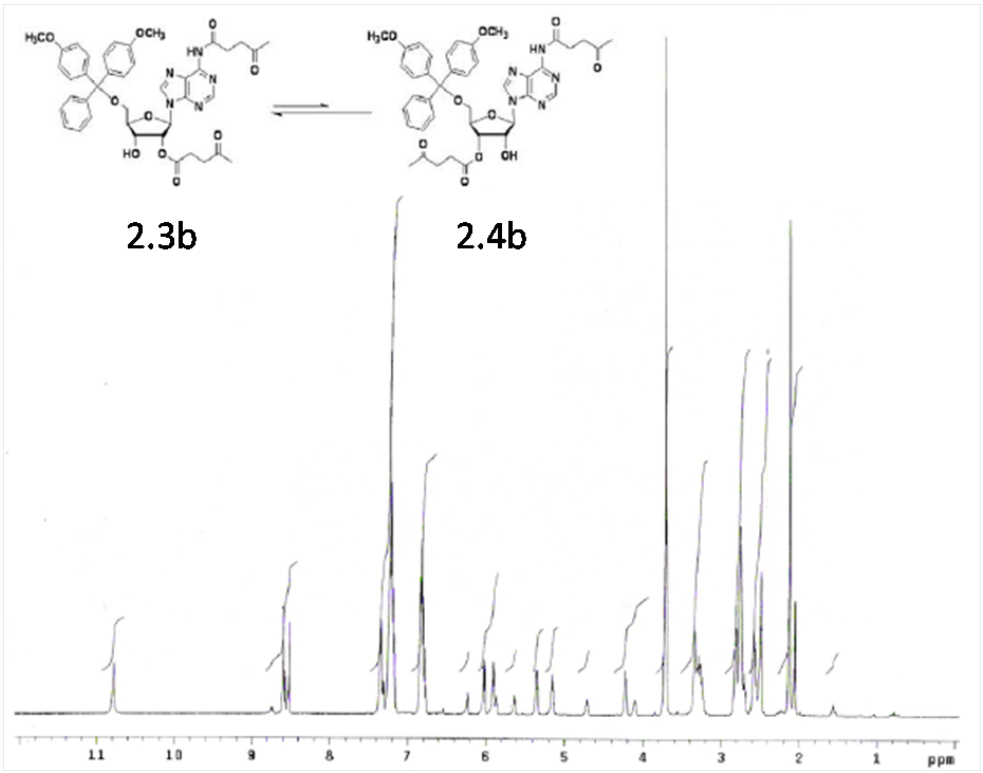
The oligoribonucleotides were either purified by ion-exchange HPLC chromatography, or PAGE. Using the former method, a gradient of 98:2 to 80:20 $H_2O/LiClO_4$ was employed with a flow rate of 1 mL/min over 60 min. The solution containing the oligonucleotide was then lyophilized to dryness and dissolved in 1 mL of water. The purified sample was then desalted using sephadex G-25 and lyophilized to dryness. Using the latter method, the oligoribonucleotides were loaded onto a 20% acrylamide gel using 98:2 10x TBE/formamide and subjected to PAGE. The band containing the purified oligoribonucleotide was then excised, and then suspended in water and shaken overnight. The solution was then filtered, and the filtrate concentrated. It was then dissolved in 1 mL of water, desalted using sephadex G-25, and lyophilized to dryness.

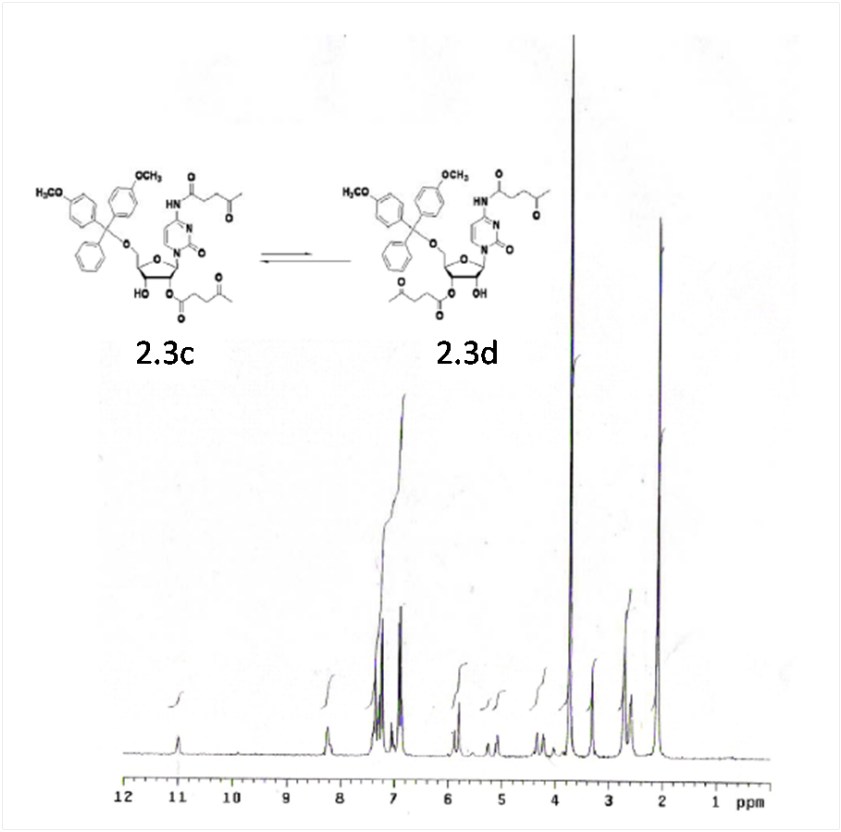
2.6.4 siRNA Assays

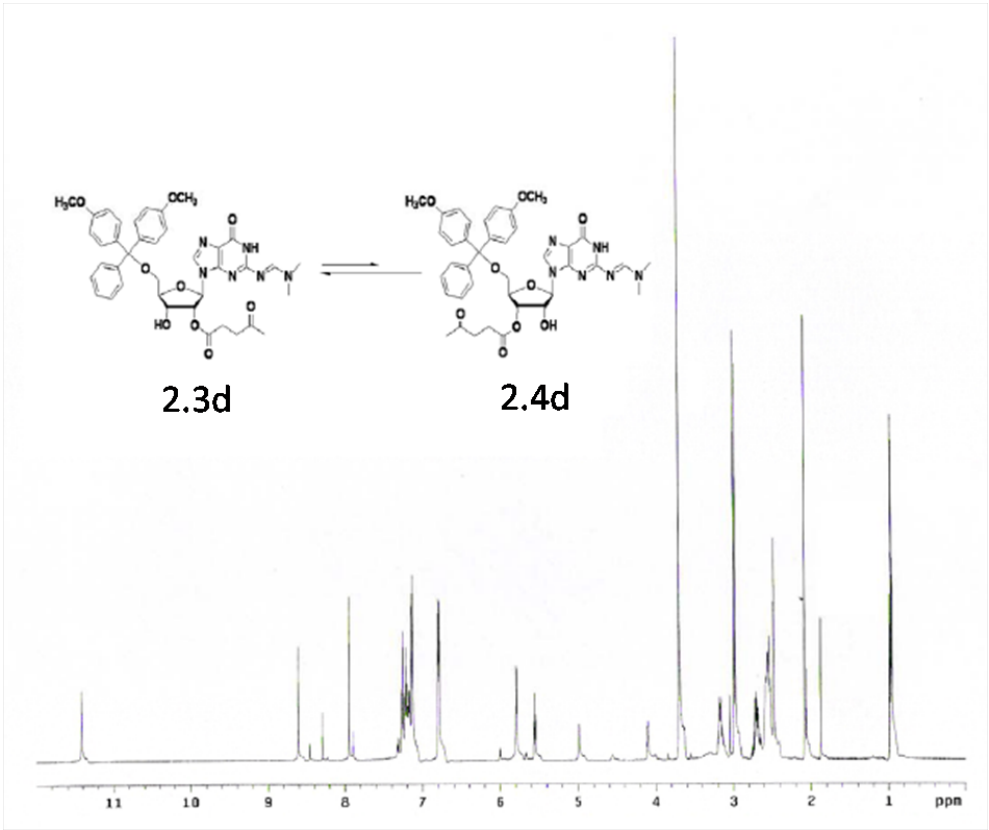
HelaX1/5 cells that stably express firefly luciferase were grown as previously described.[23] The day prior to transfection, 0.5 x 10^5 cells were plated in each well of a 24-well plate. The next day, the cells were incubated with increasing amounts of siRNAs premixed with lipofectamine-plus reagent (Invitrogen) using 1 μL of lipofectamine and 4 μL of the plus reagent per 20 pmol of siRNA (for the highest concentration tested). For the siRNA titrations, each siRNA was diluted into dilution buffer (30 mM HEPES-KOH, pH 7.4, 100 mM KOAc, 2 mM $MgOAc_2$) and the amount of lipofectamine-plus reagent used relative to the siRNAs remained constant. One day after transfection, the cells were lysed in hypotonic lysis buffer (15 mM K_3PO_4, 1 mM EDTA, 1% Triton, 2 mM NaF, 1 mg/ml BSA, 1 mM DTT, 100 mM NaCl, 4 μg/mL aprotinin, 2 μg/mL leupeptin and 2 μg/mL pepstatin) and the firefly light units were determined using a Fluostar Optima 96-well plate bioluminescence reader (BMG Labtech) using firefly substrate.[24] The luciferase counts were normalized to the protein concentration of the cell lysate as determined by the DC protein assay (BioRad). Error bars represent the standard deviation of two transfections.

2.6.5 Selected NMR






2.3c
2.3d
12 11 10 9 8 7 6 5 4 3 2 1 ppm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2.4d
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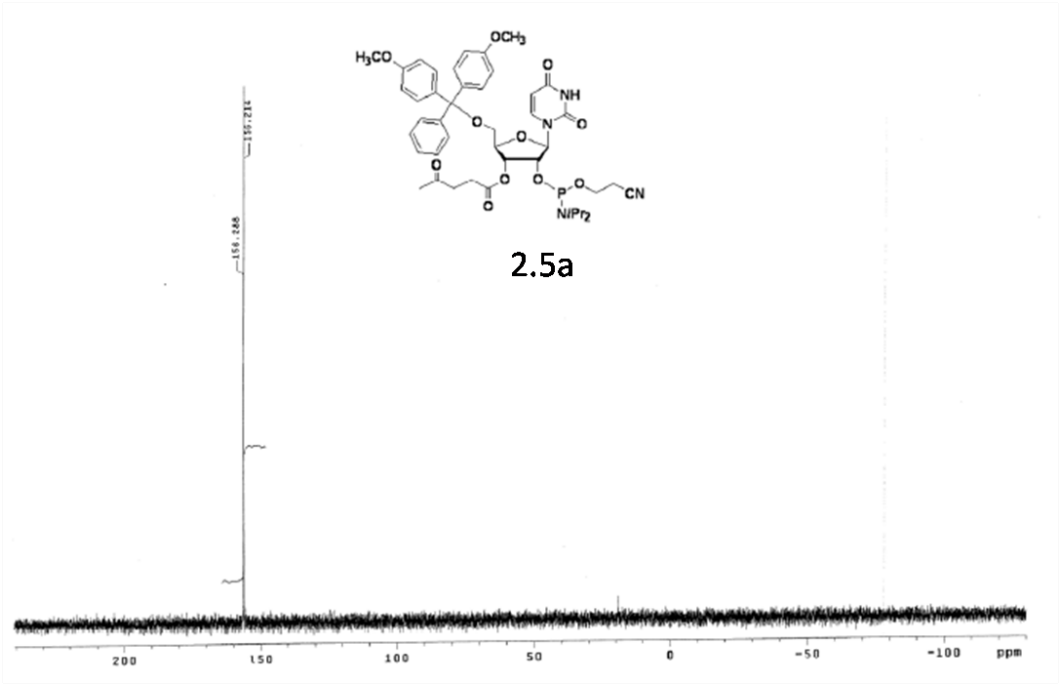
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0
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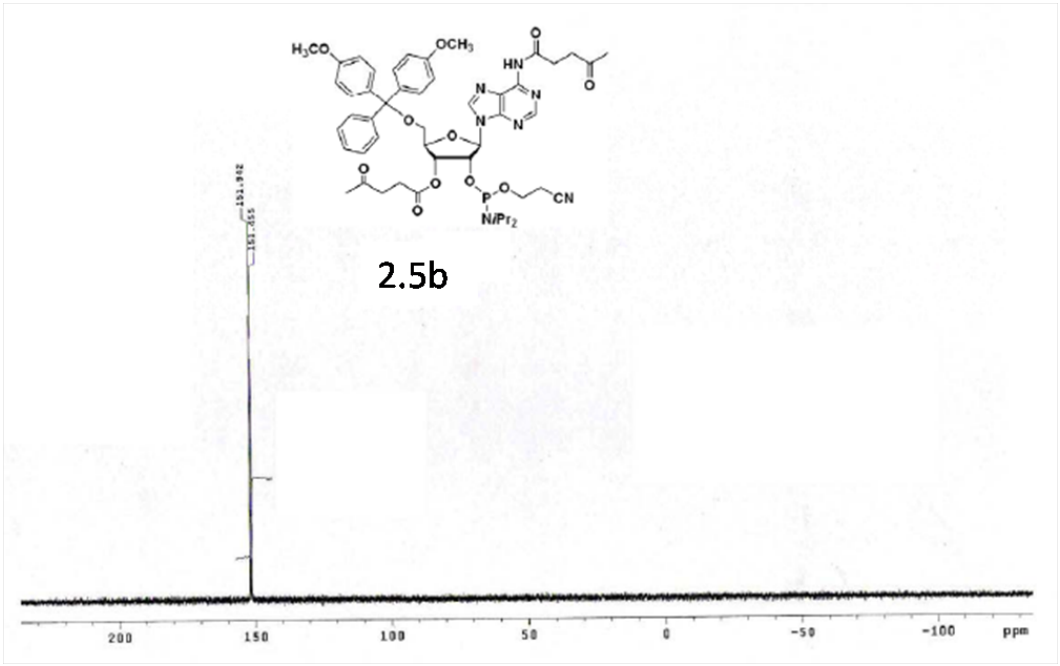
-100
ppm

2.5a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CN
N(iPr)2
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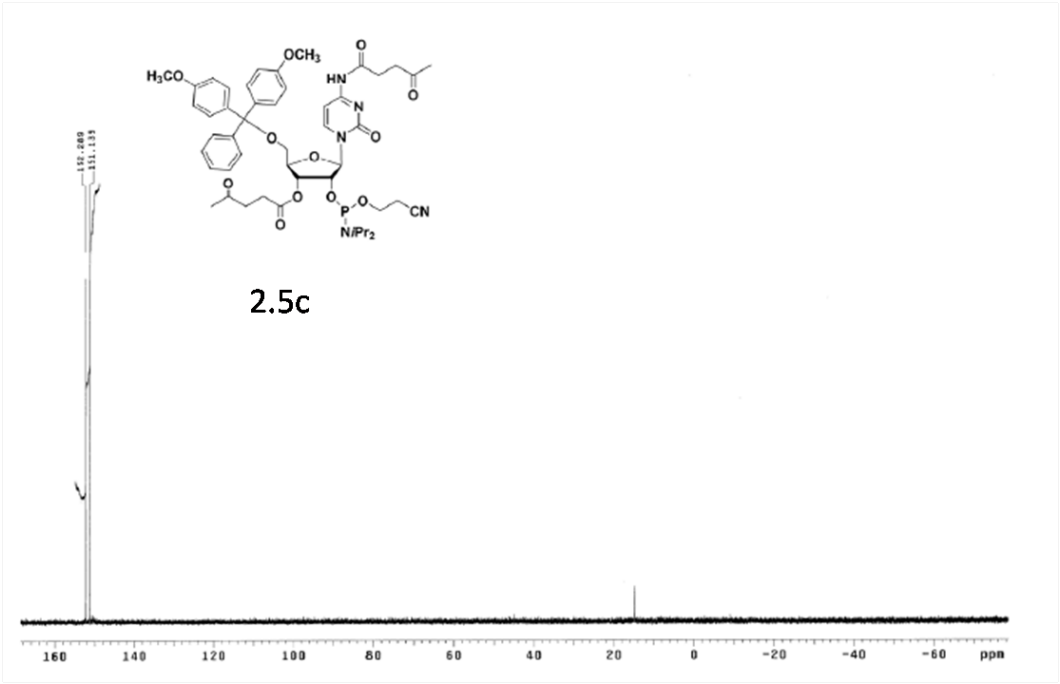
150
100
50
0
-50
-100
ppm

2.5b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H3CO
HN
O
N
O
O
O
O
O
O
O
O
P
O
CN
N/Pr2
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140
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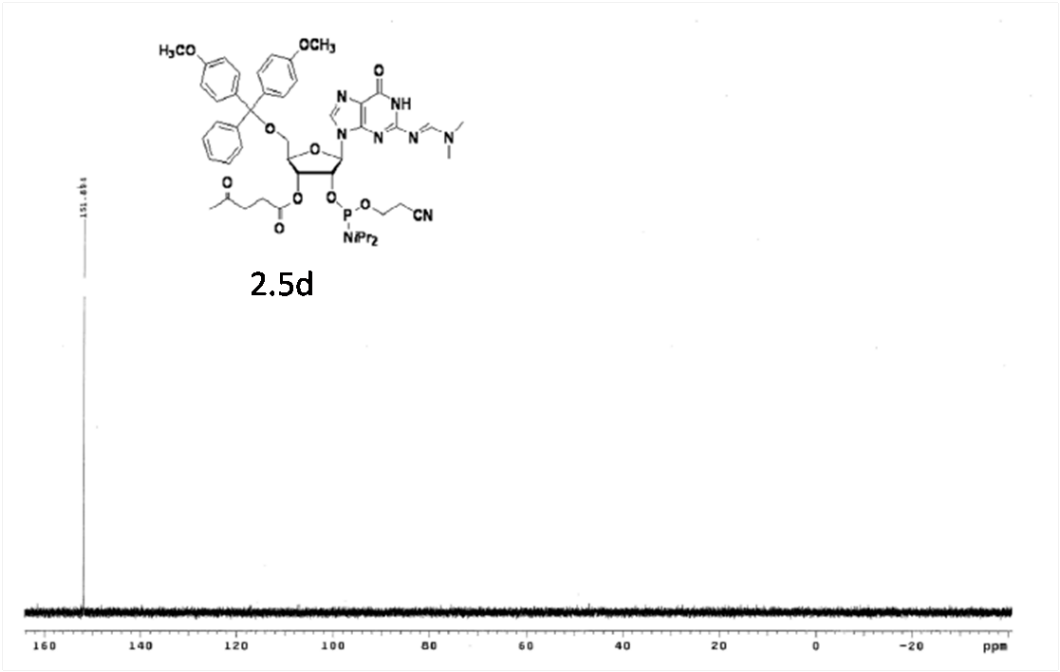
80
60
40
20
0
-20
-40
-60
ppm

2.5c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OCH3
O
N
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N
N
N
N
O
O
O
O
O
P
O
CN
N/Pr2
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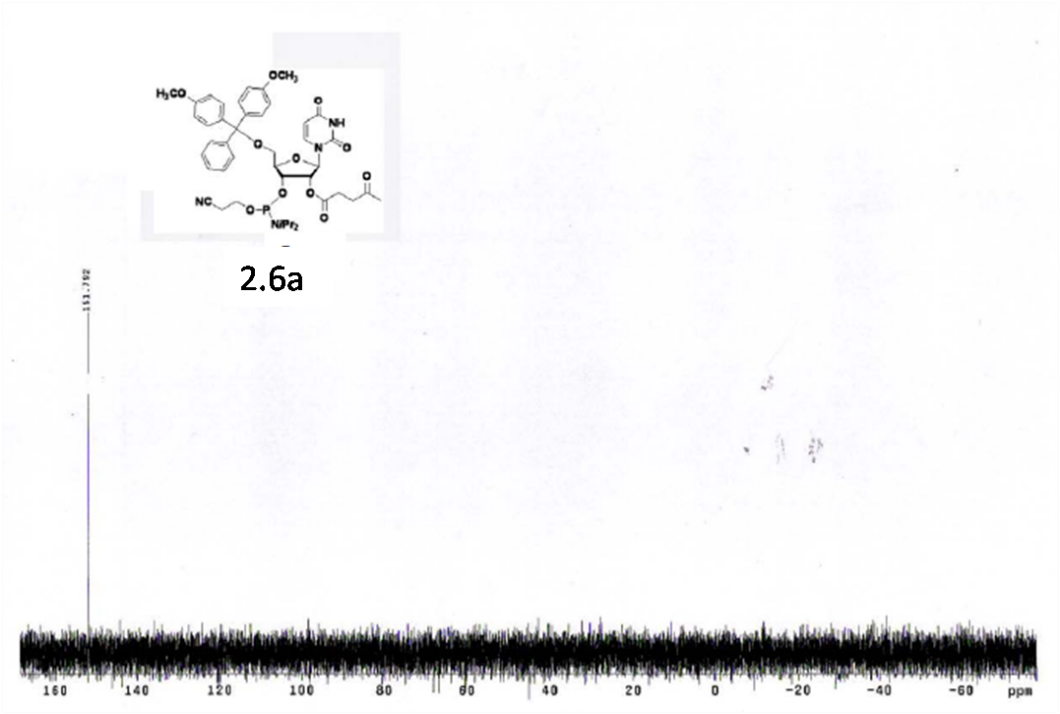
160
140
120
100
80
60
40
20
0
-20
ppm

2.5d


OCH3
H3CO
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NC
O-P
NiPr2
111.792
160
140
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100
80
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40
20
0
-20
-40
-60
ppm

2.6a

2.7 References

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Chapter 3. The 2'-Acetal Levulinyl Ester (ALE) Group for RNA Synthesis

3.1 Introduction

In the previous chapter, I described how 2'-*O*-levulinyl (Lv) ribonucleoside phosphoramidites could be used in the solid phase synthesis of RNA oligonucleotides.[1] Great care had to be exercised in the purification of the 2'-*O*-Lv-3'-phosphoramites in order to avoid contamination with the isomeric 3'-*O*-Lv-2'-phosphoramidites, the presence of which would inevitably lead to 2', 5'-internucleotide linkages in the final RNA sequence.

To overcome this limitation, this chapter describes a novel 2'-acetal levulinyl ester (ALE) 2'-hydroxyl protection strategy for the synthesis of RNA.[2,3] The ALE group may be regarded as an alternative to the levulinyl (Lv) group in that it is also removed on-column by treatment with buffered hydrazine solution; however, it has two advantages over the Lv group in that (i) it cannot migrate by virtue of its acetal function (see figure 2.1);[4] and (ii) the 5'-*O*-DMTr-2'-*O*-ALE-3'-*O*-phosphoramidite monomers can be prepared in higher yields.

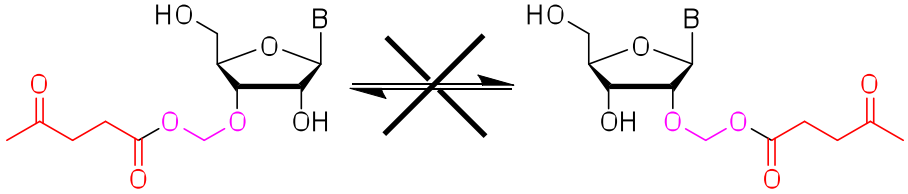


Figure 3.1. 2'/3'-Isomerization of the ALE protecting group cannot take place because the levulinyl group is linked to the ribose 2'/3'-oxygen through an oxymethyl acetal linkage.

Azhaev and co-workers were the first to introduce acetal ester modifications at the 2'-hydroxyl group of ribonucleosides.[4] To achieve this, they synthesized ribonucleosides with a 2'-*O*-CH_2-*S*-CH_3 moiety that was further elaborated to several other 2'-*O*-CH_2-X, modifications (X = OAc, F, N_3, OPh, OCH_3, etc). This approach led to the synthesis of 2'-acetal ribonucleosides as alternatives to 2'-*O*-TBDMS ribonucleosides by several research teams (**Table 1.2, Chapter 1**). [2,3,5-8]

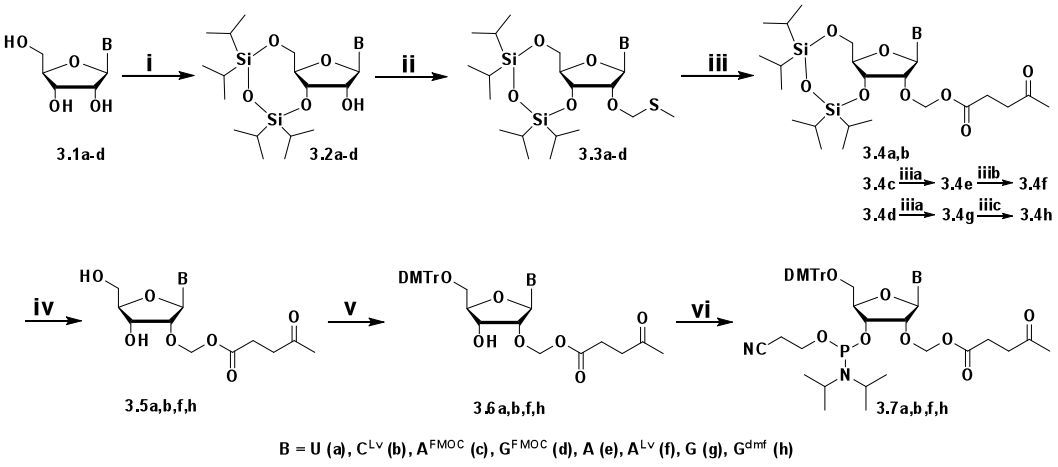
We also became aware of interesting articles originating from the laboratory of Debart and co-workers[7] at Université Montpellier 2 (Montpellier, France), describing the use of 2'-*O*-acyloxymethyl protected ribonucleosides in the synthesis of oligoribonucleotides. One of these articles reported the preparation of 2'-*O*-acyloxymethyl protected oligo-rU strands that could be converted to the "naked" oligo-rU strands upon exposure to pig liver esterase, exemplifying the first 2'-*O*-biolabile protecting group for RNA.[7] The potential applications of these derivatives and those subsequently developed by our laboratory are discussed in **Chapter 5**. Debart and co-workers later introduced another 2'-acetal ester protecting group, 2'-*O*-PivOM,[8] which intriguingly could be deprotected under aqueous ammonia conditions without any observable internucleotide cleavage (see **Table 1.2, 23, Chapter 1**). This work prompted us to combine both acetal and Lv groups into a new group for the protection of 2'-hydroxyl of ribonucleosides in the synthesis of oligoribonucleotides on solid supports. The protocol developed permits both the growth and deprotection of RNA chains that remain attached to a solid polymer support. The key synthons, 5′-*O*-DMTr 2′-acetal levulinyl ester (2′-*O*-ALE) ribonucleoside 3′-phosphoramidite derivatives coupled to Q-CPG solid support with excellent coupling efficiency (~98.7%). A three-stage mild deprotection strategy releases the RNA oligomers in very good yields. These efforts also culminated in the *in situ* synthesis of RNA on microarrays ("RNA chip") and are described in **Chapter 4.**

3.2 5'-*O*-DMTr-2'-*O*-ALE-3'-*O*-Phosphoramidite Monomer Synthesis

The synthesis of 2'-*O*-ALE-3'-phosphoramidite monomers is summarized in **Scheme 3.1**. Uridine (**3.1a**), N^4-Lv cytidine (**3.1b**), N^6-FMOC adenosine (**2.1c**), and N^2-FMOC guanosine (**3.1d**) were treated with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane in pyridine to give **3.2a-d** in near quantitative yield.[9] These materials were then reacted with DMSO, AcOH, and Ac_2O giving the 2'-*O*-thiomethyl ethers **3.3a-d** in 63-88% yield.[16,5] Compounds **3.3a-c** were treated with sulfuryl chloride for 1h, and the resulting 2'-*O*-CH_2Cl intermediates were

combined with sodium levulinate (NaOLv) and 15-crown-5 ether to afford **3.4a-c** in 78-94% yield. These conditions did not work well for the N^2-FMOC guanosine **3.3d**, and therefore it was reacted with sulfuryl chloride in the presence 4-chloro-styrene to avoid possible side reactions occurring at the guanine moiety. Without product isolation, this mixture was added to cesium carbonate and levulinic acid to provide **3.4d** in 85% yield.

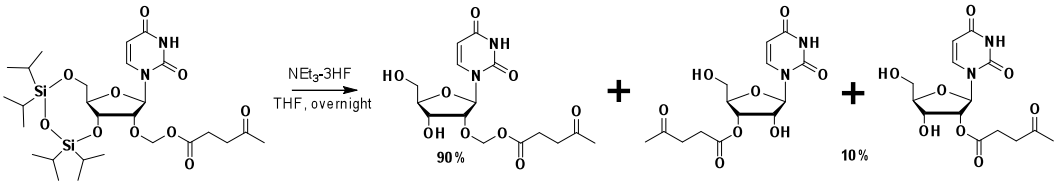
At this point, the *N*-FMOC protected purines **3.4c** and **3.4d** were converted into the desired *N*-Lv (**3.4f**) and *N*-dimethylformamidine (dmf) (**3.4h**) derivatives. This "transient" FMOC protection was necessary as *N*-Lv and *N*-dmf groups on Ade and Gua, respectively, do not survive the conditions used to install the 2′-*O*-thiomethyl ether or 2′-*O*-ALE moieties (e.g., **3.2→3.3** and **3.3→3.4**). It is presumed that neither N-Lv or N-dmf derivatives provides enough stability against depurination under the acidic conditions required in these transformations.[10] As a result, we considered *N*-FMOC protection a very attractive option for solid-phase synthesis of siRNA pro-drugs (**Chapter 5**). However, it was shown to be incompatible with the 5′-*O*-NPPOC protection required in microarray fabrication (**Chapter 4**). Thus, compounds **3.4c, 3.4d** were treated with 2:3 triethylamine/pyridine (v/v) to remove the FMOC group in quantitative yield. Next, the resulting Ade **3.4e** was reacted with EEDQ and levulinic acid to give the N^6-Lv Ade[11] **3.9f** (86%), whereas Gua **3.4g** was treated with *N,N*-dimethylformamide dimethyl acetal to give N^2-dmf Gua[12] **3.4h** also in quantitative yield. Compounds **3.4a**,**b**,**f**,**h** were then treated with NEt_3-3HF to afford **3.5a**,**b**,**f**,**h** in nearly quantitative yields. To obtain monomers suitable for standard synthesis on CPG solid supports, these nucleosides were treated with DMTrCl/pyr to afford **3.6a**,**b**,**f**,**h** (78-90%), which were then 3′-phosphitylated under standard conditions to give **3.7a**,**b**,**f**,**h** (70-90%).



Scheme 3.1. Synthesis of 2'-*O*-ALE monomers. Reagents and conditions: (i) TIPDSCl, pyr; (ii) DMSO, AcOH, Ac_2O; (iii) 1 M SO_2Cl_2, CH_2Cl_2, NaOLv, 15-C-5, CH_2Cl_2 (2 steps); for **3d**, 1 M SO_2Cl_2, CH_2Cl_2, 4-Cl-styrene, Cs_2CO_3, levulinic acid; (iiia) 2:3 TEA/pyr (v/v); (iiib) levulinic acid, EEDQ, THF; (iiic) *N,N*-dimethylformamide dimethyl acetal, THF; (iv) NEt_3-3HF, THF; (v) DMTrCl, pyr; (vi) CEtOP(Cl)NiPr_2, iPr_2NEt, CH_2Cl_2.

3.3 Rearrangement of 2'-*O*-ALE Nucleosides to 2'-*O*-Lv Nucleosides

While optimizing reaction conditions for conversion **3.4a→3.5a** (**Scheme 2.1**), it was discovered that overnight exposure of uridine **3.2a** to an excess of NEt_3-3HF (6 equivalents) led to 2'-*O*-ALE uridine, as expected, as well as a mixture of 2'/3'-*O*-Lv uridine (**Scheme 3.2**) in ~ 10% yield (estimated by TLC; structure confirmed by ^{1}H NMR and ESI-TOF). This side reaction was also observed for C, U and A nucleosides.



Scheme 3.2. Rearrangement of 2'-*O*-ALE-uridine to a mixture of 2'/3'-*O*-Lv uridine catalyzed by NEt_3-3HF.

This rearrangement can be avoided provided that the desilylation reaction is carried out within 3 hours with a small excess (1.5 equivalents) of NEt_3-3HF. To determine the extent of this rearrangement under other conditions, nucleosides **3.6a,b,f,h** were subjected to: 1) 1:1 DCM/TEA (v/v), 2) 1:1 DCM/MeOH (v/v), 3) 1:1 DCM/pyr (v/v) for 16 hours and the reaction progress monitored by TLC. All nucleosides **3.6a,b,f,h** were stable to conditions 2) and 3). However, condition 1) led to the formation of 2'/3'-*O*-Lv isomers in 20% (from **3.6a**), 10% (from **3.6b**), 90% (from **3.6f**), and 60% (from **3.6h**), as estimated by TLC. In addition, Robert Donga (a graduate student in our laboratory) noticed that considerable amounts of 2'/3'-*O*-Lv isomers would form if compound **3.6f** was not purified soon after work-up. These results suggest that the rearrangement is promoted by abstraction of the 3'-OH proton by a strong base (e.g. triethylamine; $pK_b \sim 11$), followed by intramolecular transesterification via a tetrahedral intermediate (**Figure 3.2**). This intermediate then releases formaldehyde and the 3'-*O*-Lv nucleoside, which isomerizes to produce a mixture of the 2'/3'-*O*-Lv nucleosides. Small amounts of NEt_3 (1%) such as the amounts used during silica gel column chromatography of **3.6a,b,f,h** did not lead to any detectable rearrangement. Since pyridine is weaker base ($pK_b \sim 5.21$), it is preferred over NEt_3 for use during silica gel column chromatography.

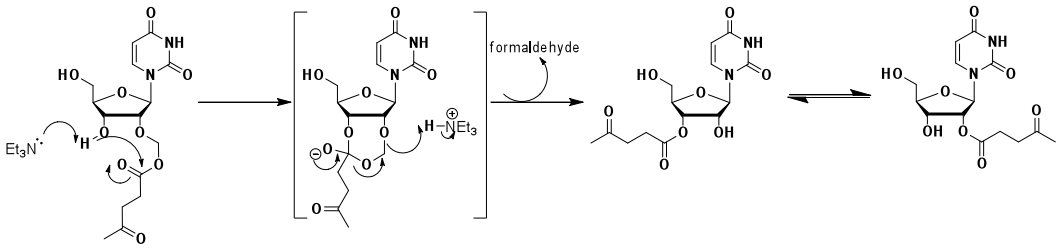


Figure 3.2. Proposed rearrangement mechanism from 2'-*O*-ALE-uridine to a mixture of 2'/3'-*O*-Lv.

When the above precautions are taken into consideration, pure 2'-*O*-ALE ribonucleosides can be obtained free from side products and in very good yield.

3.4 Oligoribonucleotide Synthesis Using 5'-*O*-DMTr-2'-*O*-ALE-3'-*O*-Phosphoramidite Monomers **3.7a**,**b**,**f**,**h**

3.4.1 Solid Phase Synthesis - Single Insert Studies

As an initial test to demonstrate the suitability of 2'-*O*-ALE monomers for RNA synthesis, we synthesized four DNA-RNA chimeric oligomers, i.e., dT_9-rN-dT_5 (rN = U, C, A and G), on a Q-CPG solid support (1 μmol scale). The Q-CPG support consisted of 5'-*O*-DMTr-dT (loading: 45 μmol/g) appended through a hydroquinone-*O*,*O'*-diacetic acid ('Q-linker'),[13] making it possible to release an oligonucleotide chain by a brief fluoride treatment.[1] The phosphoramidites **3.7a**,**b**,**f**,**h** (0.1 M in MeCN) were activated with DCI (0.25 M in MeCN) and allowed to couple to the support for 1 min. Standard capping, oxidation and detritylation steps followed the coupling step. After the completion of each synthesis, the Q-CPG was treated with 2:3 TEA/MeCN (v/v) for 1 h to effect removal of the β-cyanoethyl phosphate protecting groups. Half of the solid support was treated with 0.5 M $NH_2NH_2 \cdot H_2O$ in 3:2 v/v pyr:AcOH (v/v) for 1 h (*N*-Lv/dmf and 2'-*O*-ALE removal) followed by 1 M TBAF in THF overnight to release the oligonucleotide from the Q-CPG. The remaining solid support was treated directly with 1 M TBAF (16 h) to release the protected oligonucleotide from the support. This material was purified on a RP-HPLC column, and then treated under hydrazinolysis conditions for time-varying periods (1-24 h). Both methods of deprotection yielded the same results with no indication of base modification or internucleotide strand cleavage occurring even after extended periods of time **(Figure 3.3-3.6, Table 3.1-3.5).**

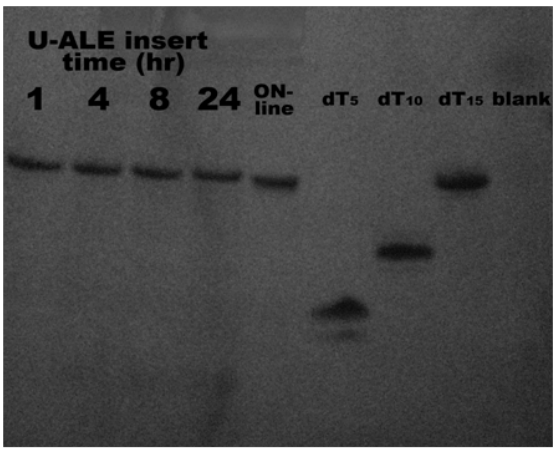


Figure 3.3. 24% denaturing gel (8.3 M urea) visualized by UV shadowing of dT_9-rU-dT_5 prepared from **3.7a**. The material was treated by the in-solution approach (1-24 hr) or the on-line approach.

Sample	Calc. MW	found MW
1 hr	4502.9	4502.6
4 hr	4502.9	4502.5
8 hr	4502.9	4502.5
24 hr	4502.9	4502.4
ON-line*	4502.9	4503.1

Table 3.1. ESI-TOF of dT_9-rU-dT_5 oligonucleotides using 2'-*O*-ALE chemistry.

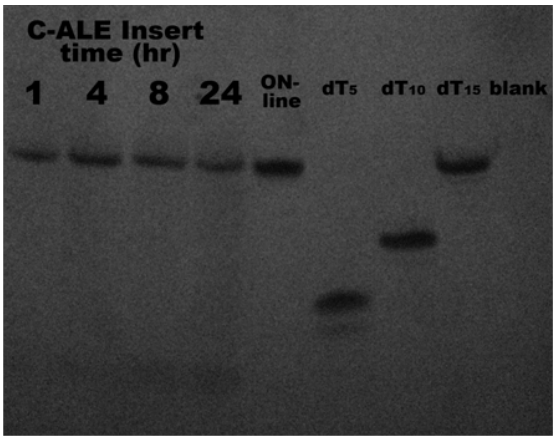


Figure 3.4. 24% denaturing gel (8.3 M urea) visualized by UV shadowing of dT_9-rC-dT_5 prepared from **3.7b**. The material was treated by the in-solution approach (1-24 hr) or the on-line approach.

Sample	Calc. MW	found MW
1 hr	4501.9	4501.5
4 hr	4501.9	4501.2
8 hr	4501.9	4501.3
24 hr	4501.9	4541.8 ($+K^+$)
ON-line*	4501.9	4501.9

Table 3.2. ESI-TOF of dT_9-rC-dT_5 oligonucleotides using 2'-*O*-ALE chemistry.

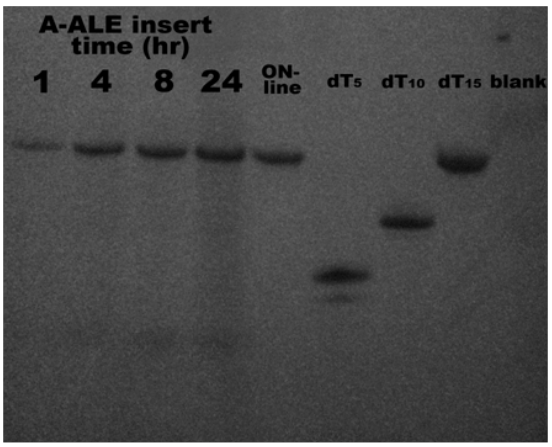


Figure 3.5. 24% denaturing gel (8.3 M urea) visualized by UV shadowing of dT_9-rA-dT_5 prepared from **3.7f**. The material was treated by the in-solution approach (1-24 hr) or the on-line approach.

Sample	Calc. MW	found MW
1 hr	4525.9	4525.5
4 hr	4525.9	4525.5
8 hr	4525.9	4525.4
24 hr	4525.9	4525.6
ON-line*	4525.9	4525.9

Table 3.3. ESI-TOF of dT_9-rA-dT_5 oligonucleotides using 2'-*O*-ALE chemistry.

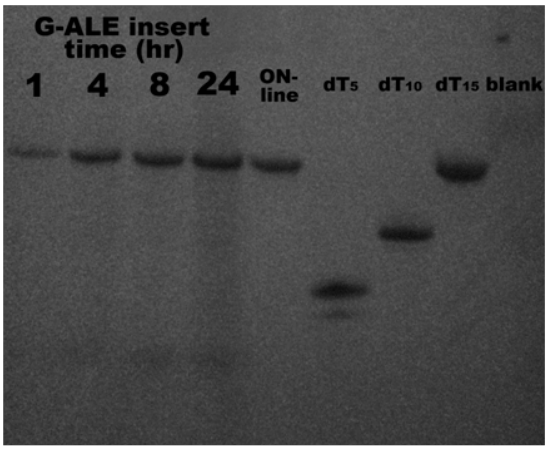


Figure 3.6. 24% denaturing gel (8.3 M urea) visualized by UV shadowing of dT_9-rG-dT_5 prepared from **3.7g**. The material was treated by the in-solution approach (1-24 hr) or the on-line approach.

Sample	Calc. MW	found MW
1 hr	4542	4541.9
4 hr	4542	4541.8
8 hr	4542	n.d.
24 hr	4542	n.d
ON-line*	4542	4541.7

Table 3.4. ESI-TOF of dT_9-rG-dT_5 oligonucleotides using 2'-*O*-ALE chemistry.

As a further check for the integrity of the oligonucleotide chains, each strand obtained by *on-column* deprotection of 2'-*O*-ALE, N-Lv/dmf groups was hybridized to its complementary (dA_5-rN-dA_9) strand. The thermal stability of the resulting duplexes, as assessed by their T_m value, was the same as the hybrids synthesized from 2'-*O*-TBDMS monomers (**Table 3.5**). Furthermore, appropriate molecular weights were observed for the various oligomers that were synthesized.

Chemistry	Sequence	T_m (°C)	Found MW	Calc. MW
TBDMS	ttt ttt ttt **A**tt ttt	29.9	4525.8	4525.9
ALE	ttt ttt ttt **A**tt ttt	30.0	4525.9	4525.9
TBDMS	ttt ttt ttt **G**tt ttt	35.2	4541.9	4542.0
ALE	ttt ttt ttt **G**tt ttt	35.3	4541.7	4542.0
TBDMS	ttt ttt ttt **C**tt ttt	34.7	4502.1	4501.9
ALE	ttt ttt ttt **C**tt ttt	35.8	4501.9	4501.9
TBDMS	ttt ttt ttt **U**tt ttt	33.0	4502.7	4502.9
ALE	ttt ttt ttt **U**tt ttt	32.6	4503.1	4502.9

Table 3.5. Sequence and properties of oligonucleotides. dT_9-**rN**-dT_5 strands were prepared from TBDMS and ALE monomers. Oligonucleotides were dissolved to give a concentration of 1μM of [dA_5-rN-dA_9]:[dT_9-rN-dT_5] hybrid in 140 mM KCl, 5 mM $MgCl_2$, 3mM Na_2HPO_4 buffer (pH 7.2)

3.4.2 Solid Phase Synthesis of Mixed Base 21-mer siRNA

Next, we compared the 2'-acetal levulinyl ester (ALE) 2'-hydroxyl protection strategy to the classical methods for RNA synthesis on CPG supports. Thus, we synthesized and compared luciferase gene knockdown of four 21-nt siRNA duplexes derived from ALE and the well established RNA methods; namely, TBDMS,[14-16] TOM[17] and ACE[18] chemistries (**Table 1.2**, entries **1**, **2**, **3** respectively). Antisense strands of these duplexes were prepared in-house from 2'-*O*-TBDMS, 2'-*O*-TOM, or 2'-*O*-ALE monomers. The sense strand was

assembled by 2'-TBDMS chemistry. Syntheses of mixed-nucleobase siRNAs via ALE monomers (0.1 M) was carried out as above, whereas those derived from TBDMS (0.15 M) and TOM (0.1 M) monomers followed literature procedures.[19] As our synthesizer was not compatible with the ACE phosphoramidite protocols, a crude sample of the same RNA sequence was obtained from Dharmacon. Detailed protocols for synthesizing RNA via ACE chemistry have been reported by Scaringe, Marshall and co-workers,[18,19] and we assume that similar procedures were followed during the preparation and isolation of our commercial sample. Normally, once the 2'-*O*-ACE oligomer is synthesized, deprotection of the methyl phosphate group is effected by S_2Na_2 (10 min); followed by treatment with aqueous 40% methylamine at 60°C for 15 min, that produces an intermediate 2'-*O*-orthoester, which is heated to 60°C for 30 min in a pH 3.8 buffer. This final step cleaves any remaining 2'-*O*-formyl groups that result from the orthoester deprotection. Coupling efficiencies of 99% and higher have been reported with coupling times of <1 min. Typically this method produces a crude product of high purity.

To assess coupling and kinetic efficiencies, monomer coupling times were set at 1 and 10 min. Deprotection of the 2'-*O*-TOM and 2'-*O*-TBDMS RNA oligomers from the CPG support was achieved with 29% *aq*. NH_3/ethanol; 3:1 (v/v); 55 °C, 30 min followed by 1 M TBAF in THF (16 h; r.t.). Deprotection of the ALE oligomer was carried out *on-column* as described above, except that the hydrazine treatment was extended to 4 h to achieve complete deprotection of the mixed sequence. This was achieved by pulsing the hydrazine solution though the column 16 × 15 min. At 1 min coupling, average stepwise coupling yield for the 2'-*O*-ALE monomers, (**3.7a**,**b**,**f**,**h**) were higher (97.7%) than those obtained with 2'-*O*-TOM (96.3%) and 2'-*O*-TBDMS (94.7%) monomers **(Figure 3.7, Table 3.6).**

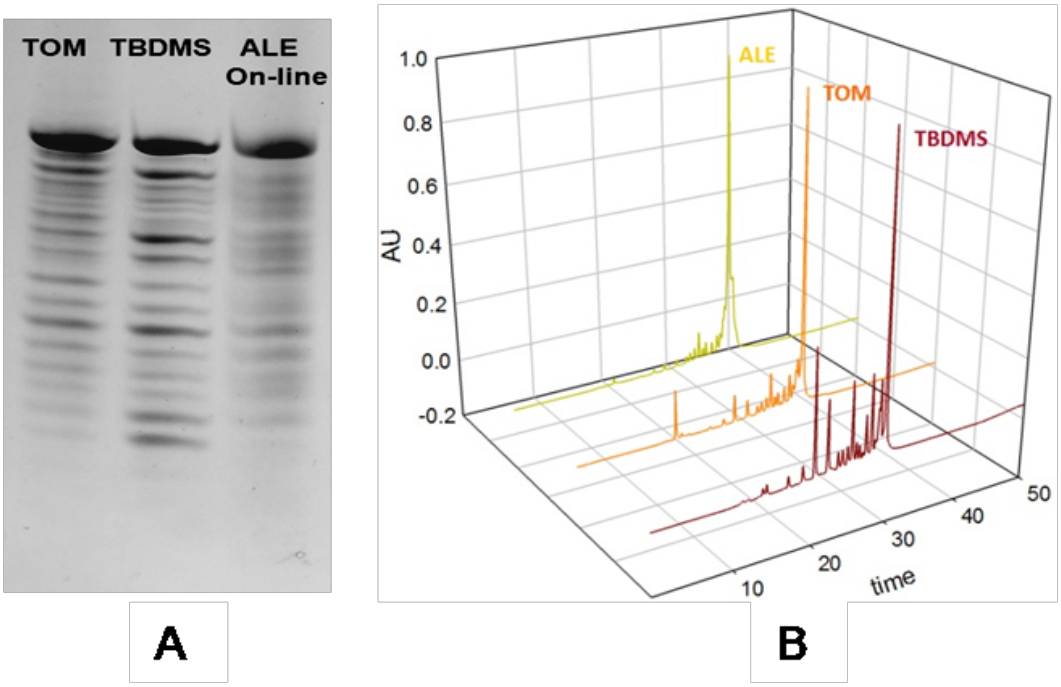


Figure 3.7. **(A)** 24% denaturing (8.3 M Urea) PAGE analysis and (**B**) Anion exchange HPLC of crude antisense siRNA strand synthesized using 2'-*O*-TBDMS (red) , 2'-*O*-TOM (orange) and 2'-*O*-ALE (yellow) using 1 min coupling times.

At longer coupling times (10 min) the values obtained were 98.7, 98.1, and 98.4%, for 2'-*O*-ALE, 2'-*O*-TOM, and 2'-*O*-TBDMS respectively. The quality of the HPLC trace of the ACE oligomer is excellent (purity 81.8%; unknown coupling time), from which an average coupling efficiency of 99% was calculated (**Figure 3.8**). As expected, extensive degradation of the RNA prepared by 2'-*O*-ALE occurred if the fully protected oligonucleotide were treated with 29% aq. NH_3/ethanol; 3:1 (v/v); r.t., 30 min. However, if a β-cyanoethylation step is carried out first with 2:3 triethylamine/acetonitrile (v/v), a subsequent aq. ammonia treatment does not cause appreciable degradation. The stability of the RNA strand under these conditions is in agreement with the results obtained by Debart and co-workers with 2'-*O*-PivOM protected RNA. It has been suggested that the unusual stability of RNA under these conditions arises from the *partial* deprotection of the acetal ester to give the partially deprotected RNA 2'-*O*-CH_2OH hemiacetals. Release of the naked RNA is believed to occur during the ammonia evaporation step which presumably also releases formaldehyde from the

2'-hydroxyl position (**figure 1.9**, **Chapter 1**).[8] It is difficult, however, to account for the enhance stability of the 2'-*O*-CH_2OH hemiacetal intermediate under these basic conditions.

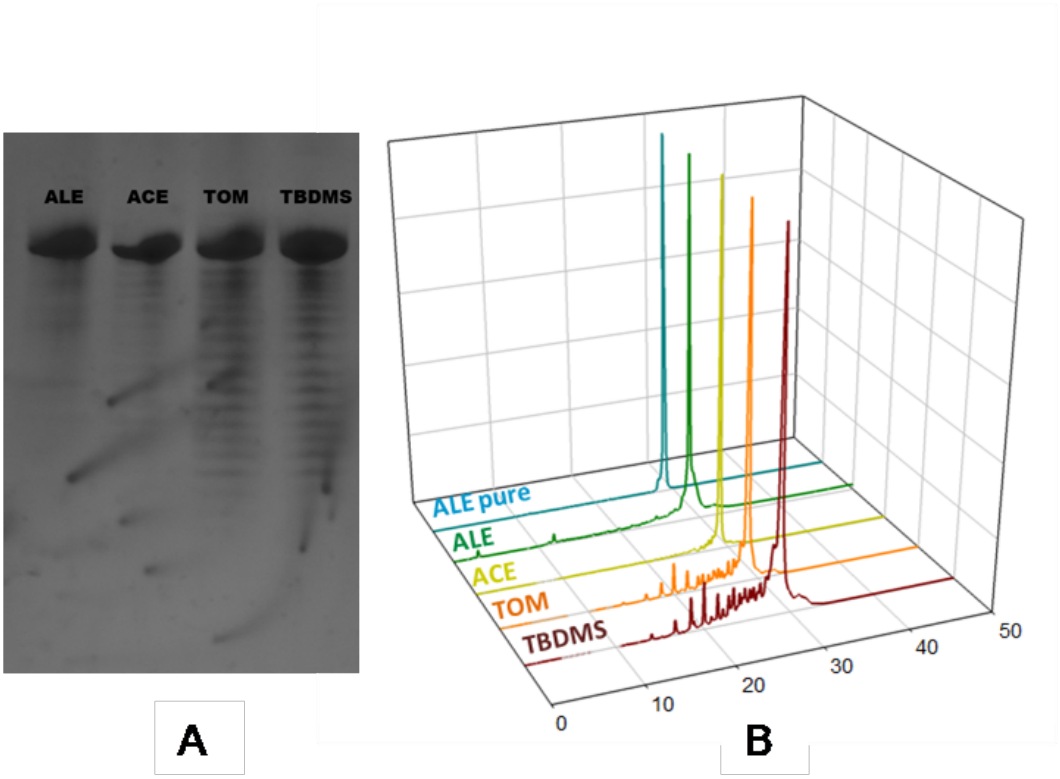


Figure 3.8. **(A)** 24% denaturing (8.3 M Urea) PAGE analysis and **(B)** Anion exchange HPLC traces of crude antisense siRNA strands synthesized from 2'-*O*-TBDMS (red), 2'-*O*-TOM (orange), 2'-*O*-ACE (yellow), and 2'-*O*-ALE (green) chemistries. Purified oligomer from 2'-*O*-ALE chemistry is shown in blue.

Careful analysis of the deprotected oligomers by ESI-TOF and thermal denaturation showed in each case that there was no base modification (**Table 3.6**).

2'-*O*-PG	Found MW[b]	T_m (°C)	10 min coupling % purity[c]	Avg. coupling yield[d]	1 min coupling % purity[c]	Avg. coupling yield[e]
TBDMS	6616.4	59.8	70.6	98.4	45.4	96.3
TOM	6616.5	60.1	67.2	98.1	32.0	94.7
ACE	6616.5	59.5	81.8[f]	99.0	n.d.	n.d.
ALE	6616.2	59.4	76.2	98.7	61.8	97.7

Table 3.6. Comparative study of 21-nt RNAs synthesized from various chemistries.[a] [a]Base sequence: r(GCUUGAAGUCUUUAAUUAA)-d(TT); [b]Calc. molecular weight: 6617 g/mol; [c]% yield calculated by HPLC (% area of major peak);[d]Calculated from 10 min. coupling time; [e]Calculated from 1 min coupling time; [f]Coupling time unknown.

3.4.3 RNAi Luciferase Assay

Next, we evaluated the activity of all RNAs synthesized in an RNAi assay that targets luciferase mRNA.[20] Each of the antisense strands prepared by the various chemistries were allowed to anneal to a common sense strand. As shown in **Figure 3.9**, the siRNA duplex prepared by 2'-*O*-ALE chemistry had the same gene silencing activity as the siRNA duplexes derived from TBDMS, TOM, and ACE protocols, further confirming the integrity of the synthesized RNA strands.

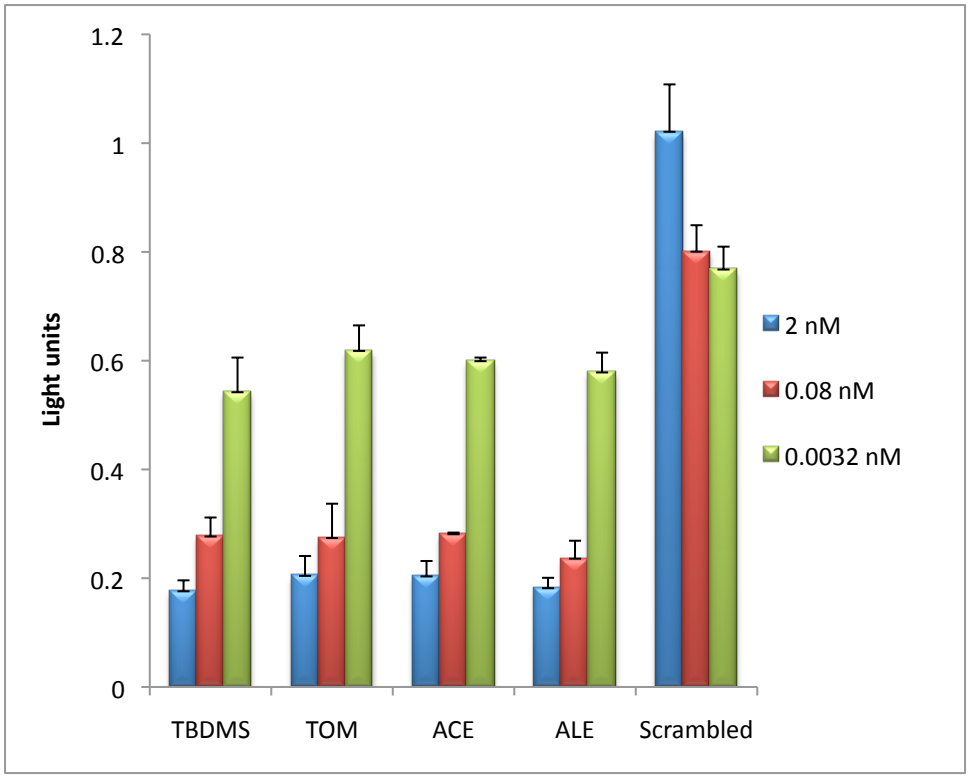


Figure 3.9. Luciferase gene knockdown by siRNA duplexes (light units are relative to Renilla control). The fully deprotected antisense strands were synthesized by TBDMS, TOM, ACE and ALE chemistries, whereas the complementary sense strand was synthesized by TBDMS chemistry.

3.5 Conclusions

In summary, the 2'-*O*-ALE and N-Lv/dmf protecting group combination provides unique ribonucleoside 3'-phosphoramidite synthons for RNA synthesis that couple with excellent rates and efficiencies. In addition, this protecting group strategy provides two distinct advantages: 1) it prevents the common 2' to 3'-isomerization that can occur with acyl protecting groups, and 2) the removal of the protecting groups can be efficiently performed *on the solid support*, which simplifies post-synthesis deprotection of RNA chains and minimizes the potential for degradation of the oligomers by RNases.

3.6 Experimental Methods

3.6.1 General Remarks

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. ^{1}H NMR spectra were referenced to residual undeuterated solvent. ^{31}P NMR spectra were measured from 85% H_3PO_4 as an external standard. Mass spectra were recorded using low resolution ESI.

The solid-phase synthesis of oligonucleotides was carried out on an Applied Biosystems DNA/RNA 3400 synthesizer. Standard 2'-TBDMS were purchased from Chemgenes Corporation (Wilmington, MA, USA) and standard 2'-TOM amidites were purchased from Glen Research (Sterling, VA, USA). Crude oligonucleotide obtained using 2'-ACE chemistry was purchased from Dharmacon (Lafayette, CO, USA). Anion-exchange HPLC was performed on a Waters Alliance system with a Waters 3D UV detector and a Waters Protein Pack DEAE-5PW column (7.5 mm x 7.5 cm). Reverse-phase HPLC was performed on a Varian C18 semi-prep column. ESI-TOF mass spectrometry was carried out on a QTOF22 (Micromass) from Waters. 24% denaturing PAGE (8.3 M urea) was carried out in a standard Hoeffer SE600.

3.6.2 Synthetic Protocols and Characterization of Nucleoside Monomers

Procedure for the preperation of N^4-levulinyl-cytidine (3.1b)

For the preparation of **3.1b**, see (a) Lackey, J.G.; Sabatino, D.; Damha, M.J. *Org lett*. **2007**, 9, 789-792; (b) Ogilvie, K. K.; Nemer, M. J.; Hakimelahi, G. H.; Proba, Z. A.;Lucas, M. *Tetrahedron Lett.* **1982**, *23*, 2615-2618.

Procedure for the preparation of N^6-(9-Fluorenylmethoxycarbonyl)-adenosine (3.1c)

For the preparation of **3.1c**, see Happ, E.; Scalfi-Happ, C.; Chladek, S. *J. Org. chem*. **1987**, 52, 5387-5391.

Procedure for the preparation of N^2-(9-Fluorenylmethoxycarbonyl)-guanosine (3.1d)

For the preparation of **3.1d**, see Heikkla, J., Chattopadhyaya. *J. Acta. Chem. Scand. Ser. B*, **1983**, B37, 263-271 and Hagen, J.; Scalfi-Happ.; Happ, E.; Chladek, S. *J. Org. Chem.* **1988**, 53, 5040-5045.

General procedure for the preparation of 3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl) ribonucleosides (3.2a-d)

(See Markiewicz, W. T. *J. Chem. Res. (S)* **1979**, 24-25. See also Markiewicz, W. T. *J. Chem. Res. (M)* **1979**, 181-197).

For example, uridine (41 mmol) was dissolved in 100 mL of pyridine. The Markiewicz reagent (43 mmol) was added dropwise under a dry nitrogen environment over 25 min. After 3hr, the reaction has gone to completion. It was quenched with 20 mL of brine, and then concentrated to an oil under reduced pressure. This residue was then redissolved in 200 mL of DCM and washed once with 50 mL of brine. The aqueous layer was then washed 3x with 50 mL of DCM. Organic extracts are then pooled and dried over $MgSO_4$, filtered and concentrated. The resulting sticky foam was then coevaporated 3x with 30 mL of

benzene and pumped on high vacuum to give a white foam in near quantitative yield. This material was used without further purification in the next synthetic step.

General procedure for the preparation of 3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-(methylthio)methyl ribonucleosides (3.3a-d)

(See Semenyuk, A.; Földesi, A.; Johansson, T.; Estmer-Nilsson, C.; Blomgren, P.; Brännvall, M.; Kirsebom, L. A.; Kwiatkowski, M. *J. Am. Chem. Soc*. **2006**, *128*, 12356-12357). For example, 3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-uridine **(3.2a)** (20 mmol) was dissolved in 30 mL of DMSO followed by the addition of 30 mL glacial acetic acid and 20 mL of acetic anhydride. This reaction mixture was stirred for 20 hr at room temperature and then heated for 4 hr at 50 ºC to drive the reaction to completion. The reaction was then cooled to room temperature and poured into a 2 L Erlenmeyer flask. This material was then stirred vigorously and a solution of K_2CO_3 (100 g in 1 L) is added. The white precipitate was filtered and dissolved in 200 mL of DCM. This material was transferred to a separatory funnel and the excess aqueous material was removed. The organic material was dried over $MgSO_4$ and the solvent was removed under reduced pressure to give a yellowish foam. This material was purified by column chromatography (0→2% MeOH in DCM) and 3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-(methylthio)methyl uridine **(3.3a)** was obtained in an 88% yield. The characterization of **3.3a** was in agreement with Semenyuk et. al.

Compound	mmol (starting)	Column conditions	Yield
3.3b	26	0→2% MeOH in DCM	82
3.3c	16	60:40 hexanes/EtOAc	65
3.3d	18	40:60 hexanes/EtOAc	63

Table 3.7: Column chromatography conditions and yields of **3.3b-3.3d**

N^4-Levulinyl-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-(methylthio)methyl cytidine (3.3b)

^{1}H NMR (500 MHz, DMSO-*d*6): δ 10.94 (s, 1H), 8.07 (d, 1H, J = 9.5), 7.18 (d, 1H, J = 9.5), 5.65 (s, 1H), 5.00-4.96 (m, 2H), 4.32 (d, 1H, J = 5.5), 4.22 (d, 1H, J = 16.5), 4.18-4.14 (m, 1H), 4.09-4.07 (1H, m), 3.93-3.90 (1H, d, J = 16.5), 2.72-2.69 (m, 2H), 2.59-2.56 (m, 2H), 2.09 (s, 3H), 2.10 (s, 3H), 1.05-0.95 (m, 28H). ^{13}CNMR (125 MHz, DMSO-*d*6): 207.7, 207.2, 173.8, 163.2, 154.8, 143.9, 95.8, 89.7, 81.8, 77.7, 73.7, 67.8, 60.0, 34.1, 31.0, 30.3, 28.6, 17.9, 17.8, 17.6, 17.5, 17.4, 13.4, 13.2, 13.1, 13.0, 12.6. ESI-TOF calc for $C_{28}H_{49}N_3O_8SSi_2$ 666.30 ($+Na^+$) found 666.32.

N^6-(9-Fluorenylmethoxycarbonyl)-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-(methylthio)methyl adenosine (3.3c)

^{1}H NMR (500 MHz, $CDCl_3$): δ 9.45 (s, 1H), 8.77 (s, 1H), 8.34 (s, 1H), 7.74-7.72 (m, 2H), 7.61-7.58 (m, 2H), 7.38-7.35 (m, 2H), 7.26-7.22 (m, 2H), 6.06 (s, 1H), 5.05, 4.98 (abq, 1H each, J = 11, 11.5), 4.72-4.69 (m, 1H), 4.67-4.59 (m, 2H), 4.38-4.31 (m, 1H), 4.21, 4.02 (abq, 1 H each, J = 13.5, 13.5), 4.17-4.14 (m, 1H), 2.17 (s, 3H), 1.11-0.94 (m, 28 H). ^{13}CNMR (125 MHz, $CDCl_3$): δ 153.1, 151.6, 150.7, 149.8, 143.9, 143.7, 141.5, 141.5, 141.2 128.0, 128.0, 127.9, 127.4, 125.3, 125.2, 122.9, 120.3, 120.2, 88.8, 82.0, 75.0, 69.1, 68.0, 59.9, 47.15, 17.7, 17.6,

17.5, 17.45, 17.4, 17.38, 17.32, 17.3, 17.2, 17.1, 13.7, 13.6, 13.2, 13.0, 12.9. ESI-TOF calc for $C_{39}H_{53}N_5O_7SSi_2$ 814.32 ($+Na^+$) found 814.28.

N^2-(9-Fluorenylmethoxycarbonyl)-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-(methylthio)methyl guanosine (3.3d)

^{1}H NMR (500 MHz, $CDCl_3$): δ 11.29 (s, 1H), 8.32 (s, 1H), 7.98 (s, 1H), 7.75-7.73 (m, 2H), 7.57-7.54 (m, 2H), 7.41-7.37 (m, 2H), 7.31-7.26 (m, 2H), 5.86 (s, 1H), 4.98, 4.94 (abq, 1 H each, J = 11.5, 11.5), 4.63-4.56 (m, 1H), 4.52-4.49 (m, 1H), 4.44 (d, 1H, J = 5), 4.25-4.20 (m, 2H), 4.13-4.10 (m, 1H), 4.00-3.97 (m, 1H), 2.14 (s, 3H), 1.10-0.92 (m, 28H). ^{13}CNMR (125 MHz, $CDCl_3$): δ 155.8, 153.7, 147.6, 146.8, 143.1, 141.5, 136.6, 128.3, 127.5, 125.0, 121.7, 120.4, 87.92, 82.0, 78.2, 74.6, 68.7, 68.5, 59.9, 46.9, 17.7, 17.5, 17.4, 17.3, 17.25, 17.2, 17.1, 13.7, 13.6, 13.2, 13.1, 12.8. ESI-TOF calc for $C_{39}H_{53}N_5O_8SSi_2$ 830.32 ($+Na^+$) found 830.32.

General procedure for the preparation of 3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-acetal levulinyl ester nucleosides (3.4a-d)

For example, the synthesis of 3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-acetal levulinyl ester uridine **(3.4a)** is described as follows. Compound **3.3a** (17 mmol) was freeze dried in dry benzene. It was then dissolved with 170 mL DCM under a dry nitrogen environment and is cooled to 0°C. 17 mL of a freshly prepared 1 M solution of sulfuryl chloride is then added dropwise over 15 minutes. The reaction was stirred for an additional 30 minutes and then warmed to room temperature. It was then stirred for an additional 30 minutes. The solvent was then removed under reduced pressure and is repressurized with dry nitrogen giving a yellow foam. This material was then redissolved with 85 mL of DCM and sodium levulinate (43 mmol) was added to the stirring solution followed by the addition of 15-crown-5 (10 mmol). The reaction mixture was stirred for 2 hr and was then diluted with 250 mL of DCM. The solution was then washed once with 150 mL of water. The aqueous layer was then washed with 3x

100 mL of DCM. The organic extracts were pooled and dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure to give yellowish foam. This crude material was then purified by column chromatography (0 → 1% MeOH in CH_2Cl_2) giving **3.4a** as a white foam in 86% yield. The same chromatography conditions apply to **3.4b**, 78% yield. For **3.4c** use 70:30 hexanes/EtOAc → 60:40 hexanes/EtOAc, 94%.

3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-acetal levuliny ester uridine (3.4a)

^{1}H NMR (400 MHz, DMSO-*d*6): δ 11.39 (s, 1H), 7.62 (d, 1H, J = 8.4), 5.56 (s, 1H), 5.52 (d, 1H, J = 8), 5.38, 5.36 (abq, 1H each, J = 6.4, 6.4), 4.41 (d, 1H, J = 4.8), 4.30-4.26 (m, 1H), 4.15 (d, 1H, J = 12.4), 3.31-3.88 (m, 2H), 2.71-2.69 (m, 2H), 2.48-2.46 (m, 2H), 2.08 (s, 3H), 1.03-0.93 (m, 28H). ^{13}C NMR (125 MHz, DMSO-*d*6): 172.6, 164.0, 150.7, 140.5, 101.8, 90.0, 88.2, 82.5, 80.6, 68.8, 60.4, 37.9, 30.2, 28.4, 18.0, 17.9, 17.8, 17.7, 17.6, 17.55, 17.5, 17.4, 13.4, 13.0, 12.9, 12.6. ESI-TOF calc for $C_{27}H_{46}N_2O_{10}Si_2$ 637.27 ($+Na^+$) found 637.26.

N^4-Levulinyl-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-acetal levuliny ester cytidine (3.4b)

^{1}H NMR (400 MHz, DMSO-*d*6): δ 10.98 (s, 1H), 8.02 (d, 1H, J = 7.6), 7.16 (d, 1H, J = 7.2), 5.64 (s, 1H), 5.45 (s, 1H), 4.31 (d, 1H, J = 4.8), 4.21-4.15 (m, 2H), 4.02 (d, 1H, J = 10), 3.90 (d, 1H, 13.2), 2.72-2.69 (m, 4H), 2.58-2.55 (m, 2H), 2.51-2.48 (m, 2H), 2.09 (s, 3H), 2.08 (s, 3H), 1.04-0.95 (m, 28H). ^{13}C NMR (125 MHz, DMSO-*d*6): 207.8, 207.4, 173.7, 172.5, 163.1, 154.8, 144.2, 95.8, 90.2, 87.9, 81.7, 80.7, 70.6, 68.0, 37.7, 31.0, 30.3, 30.2, 28.4, 18.0, 17.9, 17.8, 17.7, 17.6, 17.5, 17.4, 13.2, 13.0, 12.6. ESI-TOF calc for $C_{32}H_{53}N_3O_{11}Si_2$ 734.32 ($+Na^+$) found 734.30.

N^6-(9-Fluorenylmethoxycarbonyl)-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-acetal levuliny ester adenosine (3.4c)

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.71 (s, 1H), 8.52 (s, 1H), 8.27 (s, 1H), 7.76 (d, 2H, J = 7.6), 7.63 (d, 2H, J = 7.2), 7.42-7.38 (m, 2H), 7.32-7.26 (m, 4H), 6.04 (s, 1H), 5.58, 5.40 (abq, 1H each, J = 6.4, 6.4), 4.95-4.91 (m, 1H), 4.67 (d, 1H, J = 4.4), 4.62 (d, 1H, J = 6.8), 4.34-4.31 (t, 1H, J = 6.8), 4.19, 3.99 (abq, 1H each, J = 13.6, 13.2), 4.10 (d, 1H, J = 9.2), 2.74-2.71 (m, 2H), 2.58-2.55 (m, 2H), 2.10 (s, 3H), 1.09-1.00 (m, 28H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 206.6, 172.6, 152.9, 151.2, 150.6, 149.5, 143.7, 142.0, 141.6, 120.0, 127.4, 125.3, 122.9, 120.3, 89.1, 88.6, 81.6, 81.4, 77.5, 77.15, 77.0, 76.9, 69.5, 68.0, 60.0, 47.1, 37.9, 30.0, 28.2, 17.7, 17.5, 17.4, 17.3, 17.2, 17.1, 13.6, 13.2, 13.0, 12.9. ESI-TOF calc for $C_{43}H_{57}N_5O_{10}Si_2$ 882.36 ($+Na^+$) found 882.34.

Procedure for the preparation of N^2-(9-fluorenylmethoxycarbonyl)-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-acetal levulinyl ester guanosine (3.4d)

In flask A, compound **3.3d** (9.5 mmol) was freeze dried in dry benzene. It was then dissolved in 95 mL DCM under a dry nitrogen environment and cooled to 0°C. 9.5 mL of a freshly prepared 1 M solution of sulfuryl chloride was then added dropwise over 15 minutes. This was immediately followed by the addition of 10.45 mmol 4-Cl-styrene. This reagent was used to quench the chloromethylether by-product. The reaction was stirred for an additional 30 minutes and then warmed to room temperature. It was then stirred for an additional 30 minutes. In flask B, cesium carbonate (14.25 mmol) was suspended in 20 mL of dry DMF followed by the addition of levulinic acid (28.5 mmol). The reaction mixture was refluxed for 2 hr and then cooled to room temperature. Flask A is then canulated into flask B. The reaction mixture was then stirred for 2 hours. Upon completion of the reaction, the solution was then washed 3 x 100 mL of 5% $NaHCO_3$. The aqueous layer was washed with 3x 100

mL of DCM. The organic extracts were pooled and dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure to give reddish gew. This crude material was then purified by column chromatography 2% MeOH in DCM giving **3.4d** as a white foam in 85% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.53 (s, 1H), 9.43 (s, 1H), 8.02 (s, 1H), 7.78 (d, *J* = 7.5, 2H), 7.67 – 7.53 (m, 2H), 7.42 (t, *J* = 7.5, 2H), 7.34 (t, *J* = 7.4, 2H), 6.05 (s, 1H), 5.54, 5.43 (abq, 2H, *J* = 6.4, 6.41), 4.72 – 4.58 (m, 2H), 4.51 (dd, *J* = 4.6, 9.2, 1H), 4.40 (d, *J* = 4.6, 1H), 4.34 (t, *J* = 6.5, 1H), 4.20, 4.00 (d, 1 H each, *J* = 13.1, 13.3), 4.12 (d, *J* = 9.2, 1H), 2.63-2.59 (m, 2H), 2.50-2.45 (m, 2H), 2.06 (s, 3H), 1.20 – 0.86 (m, 28H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 209.2, 172.6, 155.9, 154.2, 147.7, 147.3, 143.2, 141.6, 128.3, 127.5, 125.0, 124.9, 121.7, 120.3, 88.37, 88.2, 81.5, 81.1, 69.1, 67.9, 59.8, 47.0, 30.3, 28.5, 17.7, 17.5, 17.4, 17.3, 17.2, 13.6, 13.2, 12.8. ESI-TOF calc for $C_{43}H_{57}N_5O_{11}Si_2$ 898.36 ($+Na^+$) found 898.46.

Procedure for the preparation of 3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-acetal levulinyl ester adenosine (3.4e)

3.4c (5.4 mmol) was dissolved in 60 mL of a dry solution of 2:3 triethylamine/pyridine. The reaction was stirred at room temperature until completion, approximately 8 hr. The reaction mixture was then evaporated to dryness and flash chromatography was performed in a gradient of 0→ 4% MeOH in DCM. The final product, **3.4e** appeared as a white foam, > 99% yield.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.28 (s, 1H), 8.08 (s, 1H), 7.26 (s, 1H), 6.01 (s, 1H), 5.65-5.660 (m, 2H), 5.41 (d, *J* = 6.5, 1H), 4.92 (dd, *J* = 4.8, 9.3, 1H), 4.66 (d, *J* = 4.8, 1H), 4.19 (d, *J* = 13.2, 1H), 4.09 (d, *J* = 9.3, 1H), 4.01 (dd, *J* = 2.5, 13.2, 1H), 2.78-2.71 (m, 2H), 2.63-2.57 (m, 2H), 2.14 (s, 3H), 1.04 (m, 28H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 206.58, 172.58, 155.51, 153.24, 153.19, 149.43, 139.72, 120.63, 88.95, 88.69, 81.52, 81.48, 69.48, 60.18, 37.94, 29.99, 28.20, 17.67, 17.57, 17.53, 17.44, 17.32, 17.27, 17.15, 13.60, 13.18, 13.01, 12.88. ESI-TOF calc for $C_{28}H_{47}N_5O_8Si_2$ 660.30 ($+Na^+$) found 660.32.

Procedure for the preparation of 3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-acetal levulinyl ester guanosine (3.4g)

3.4d (6 mmol) was dissolved in 65 mL of a dry solution of 2:3 triethylamine/pyridine. The reaction mixture was stirred at room temperature until completion, approximately 8 hr. When the reaction had finished, it was evaporated to dryness flash chromatography was performed in a gradient of 0→ 5% MeOH in DCM. The final product, **3.4g** was obtained as a white foam, > 99% yield.

^{1}H NMR (500 MHz, CDCl3) δ 12.07 (s, 1H), 7.82 (s, 1), 6.22 (s, 2H), 5.89 (s, 1H), 5.68, 5.44 (abq, 1H each, *J* = 6.4, 5.44), 4.62-4.50 (m, 1H), 4.50-4.40 (m, 1H), 4.27-4.16 (m, 1H), 4.10 (d, *J* = 9.1, 1H), 4.02 – 3.95 (m, 1H), 2.85 – 2.48 (m, 4H), 2.17 (s, 3H), 1.20 – 0.83 (m, 28H). ^{13}C NMR (125 MHz, CDCl3) δ 207.11, 172.68, 159.52, 153.83, 151.12, 89.06, 88.31, 81.54, 81.47, 68.93, 60.01, 37.82, 30.12, 28.27, 17.74, 17.69, 17.56, 17.53, 17.51, 17.42, 17.36, 17.30, 17.25, 17.11, 13.66, 13.16, 13.08, 12.76. ESI-TOF calc for $C_{28}H_{47}N_5O_9Si_2$ 676.29 ($+Na^+$) found 676.46.

Procedure for the preparation of N^6-levulinyl-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-acetal levulinyl ester adenosine (3.4f)
3.4e

(5.4 mmol) is dissolved in 60 mL of THF. This was followed by the addition of 21.4 mmol of EEDQ and then the addition of 27 mmol levulinic acid. The reaction mixture was stirred at room temperature for 1hr and then heat at 60 °C for 5 hours. When the reaction had gone to completion, it was quenched with 20 mL of 5% $NaHCO_3$ and diluted with 200 mL of ethyl acetate. The organic layer was washed 3 X 50 mL 5% $NaHCO_3$. It was then dried over $MgSO_4$, filtered and evaporated. It was then purified by column chromatography, 0→3% MeOH in DCM. The final product **3.4f** was obtained as a white foam, 86% yield in addition to a small N^6-bislevulinylated impurity that is inseparable by column chromatography at this stage.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.79 (s, 1H), 8.35 (s, 1H), 7.83 (s, 1H), 5.72 (d, *J* = 7.5, 2H), 5.06 (d, *J* = 6.4, 1H), 4.71-4.65 (m, 1H), 4.30 (d, *J* = 4.3, 1H), 4.06 (s, 1H), 3.66 (d, *J* = 12.7, 2H), 3.46 (d, *J* = 11.8, 1H), 3.14 (s, 1H), 2.93-2.83 (m, 2H), 2.63 – 2.55 (m, 3H), 2.49-243 (m, 1H), 2.42 – 2.37 (m, 1H), 2.10-2.02 (m, 2H), 1.92 (s, 3H), 1.86 (s, 3H). ^{13}C NMR (125 MHz, DMSO) δ 207.75, 207.39, 173.68, 172.53, 163.06, 154.81, 144.22, 95.77, 90.23, 87.88, 81.72, 80.64, 67.99, 60.11, 37.87, 37.65, 30.98, 30.23, 30.20, 28.40, 17.99, 17.87, 17.82, 17.74, 17.59, 17.51, 17.42, 13.28, 13.01, 12.97, 12.56. ESI-TOF calc for $C_{33}H_{53}N_5O_{10}Si_2$ 758.33 ($+Na^+$) found 758.32.

Procedure for the preparation of *N*^{2}-dimethylformamidine-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-acetal levulinyl ester guanosine (3.4h)

3.4g (1.9 mmol) was dissolved in 20 mL of THF. 7.6 mmol of dimethylformamidine dimethylacetal was added to the stirring reaction and stirred overnight at room temperature. It was then evaporated to dryness and purified by column chromatography using a gradient of 0→ 5% MeOH in $CHCl_3$. The final product, **3.4h**, was obtained as a white foam >99%.

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.01 (s, 1H), 8.60 (s, 1H), 7.87 (s, 1H), 5.99 (s, 1H), 5.62, 5.54 (abq, 1H each, J = 4.1, 4.0), 4.51 (s, 1H), 4.38 (s, 1H), 4.28-4.16 (m, 1H), 4.11 (d, 1H, *J* = 9.2), 4.04-3.93 (m, 1H), 3.20 (s, 3H), 3.10 (s, 3H), 2.75-3.60 (m, 2H), 2.57-2.43 (m, 2H), 2.12 (s, *J* = 1.9, 3H), 1.26 – 0.81 (m, 28H). ^{13}C NMR (125 MHz, CDCl3) δ 206.35, 206.31, 172.38, 158.42, 157.92, 157.12, 149.49, 135.60, 135.54, 121.07, 88.52, 87.63, 81.56, 81.44, 69.04, 59.99, 41.58, 37.86, 35.43, 35.36, 29.95, 28.18, 17.69, 17.54, 17.50, 17.36, 17.26, 17.10, 13.67, 13.17, 13.14, 12.75. ESI-TOF calc for $C_{31}H_{52}N_6O_9Si_2$ 731.33 ($+Na^+$) found 731.38.

General procedure for the preparation of 2'-*O*-acetal levulinyl ester nucleosides (3.5a,b,f,h)

The procedure is demonstrated here for the synthesis of 2'-*O*-acetal levulinyl ester uridine (**3.5a**). Compound **3.4a** (10.1 mmol) was dissolved in 30 mL dry THF and stirred under nitrogen atmosphere. NEt_3:3HF (15.1 mmol) was added dropwise and the reaction was monitored by TLC (5% MeOH in CH_2Cl_2). After 2hr, **3.5a** precipitates as a white solid. It is filtered off and washed with 100 mL of ether and dried under high vacuum in near quantitative yield.

2'-*O*-acetal levulinyl ester uridine (3.5a)

^{1}H NMR (500 MHz, DMSO-*d*6): δ 11.34 (s, 1H), 7.88 (d, 1H, J = 8), 5.84 (d, 1H, J = 5), 5.64 (d, 1H, J = 8.5), 5.30 (d, 1H, J = 6), 5.28 (d, 1H, J = 10.5), 5.21 (d, 1H, J = 7), 5.13 (t, 1H, J = 5), 4.21 (t, 1H, J = 5.5), 4.10 (t, J = 5.5), 3.83 – 3.82 (m, 1H), 3.63 – 3.30 (m, 2H), 2.67 (t, 2H, J = 6), 2.43 (t, 2H, J = 6), 2.08 (s, 3H). ^{13}CNMR (125 MHz, DMSO-*d*6): δ 207.3, 172.5, 163.8, 151.3, 141.2, 102.6, 88.5, 86.9, 85.6, 81.4, 69.3, 61.2, 37.9, 30.2, 28.4. ESI-TOF calc for $C_{15}H_{20}N_2O_9$ 395.12 ($+Na^+$) found 395.23.

***N*^{4}-levulinyl-2'-*O*-acetal levulinyl ester cytidine (3.5b)**

^{1}H NMR (400 MHz, DMSO) δ 10.96 (s, 1H), 8.38 (d, 1H, J = 7.6), 7.15 (d, 1H, J = 9.5), 5.81 (s, 1H), 5.38 (d, 1H, J = 6.4), 5.31 (d, 1H, J = 8), 5.25 (d, 1H, J = 6), 5.20 (s, 1H), 4.16 (s, 1H), 4.07 (d, 1H, J = 4.8), 3.86 (s, 1H), 3.75-3.72 (m, 1H), 3.60-3.56 (m, 1H), 2.71-2.44 (m, 8H), 2.09 (s, 3H), 2.08 (s, 3H). ^{13}C NMR (125 MHz, DMSO) δ 207.68, 207.4, 173.8, 172.5, 163.1, 155.2, 145.8, 96.0, 89.2, 88.4, 84.9, 82.1, 68.2, 60.2, 37.9, 37.7, 31.1, 30.3, 28.4. ESI-TOF calc for $C_{20}H_{27}N_3O_{10}$ 492.17 ($+Na^+$) found 492.20.

N^6-levulinyl-2'-*O*-acetal levulinyl ester adenosine (3.5f)

^{1}H NMR (500 MHz, CDCl3) δ 8.79 (s, 1H), 8.35 (s, 1H), 7.83 (s, 1H), 5.72 (d, *J* = 7.5, 2H), 5.06 (d, *J* = 6.4, 1H), 4.71 – 4.64 (m, 2H), 4.30 (d, *J* = 4.3, 1H), 4.06 (s, 1H), 3.71-3.62 (m, 1H), 3.51-3.45 (m, 1H), 3.14 (s, 1H), 2.92-2.82 (m, 1H), 2.63 – 2.56 (m, 1H), 2.53-2.43 (m, 2H), 2.42 – 2.34 (m, 2H), 2.14 – 1.99 (m, 4H), 1.92 (s, 3H), 1.86 (s, 3H). ^{13}C NMR (125 MHz, CDCl3) δ 207.7, 207.6, 207.5, 207.5, 172.2, 172.1, 152.1, 150.2, 150.1, 143.7, 123.5, 89.2, 88.22, 82.9, 72.2, 63.3, 54.0, 38.0, 37.9, 32.2, 30.2, 29.8, 29.4, 27.9. ESI-TOF calc for $C_{21}H_{27}N_5O_9$ 516.18 ($+Na^+$) found 516.27.

N^2-dimethylformamidine-2'-*O*-acetal levulinyl ester guanosine (3.5h)

^{1}H NMR (500 MHz, DMSO) δ 11.46 (s, 1H), 8.54 (s, 1H), 8.15 (s, 1H), 5.92 (d, *J* = 5.6, 1H), 5.29 (d, *J* = 6.3, 1H), 5.23 (d, *J* = 6.5, 1H), 4.77 – 4.66 (m, 1H), 4.29 (s, 1H), 3.91 (s, 1H), 3.74-3.46 (m, 2H), 3.15 (s, 3H), 3.03 (s, 3H), 2.57 (t, *J* = 6.5, 3H), 2.28 (t, 2H, *J* = 6.2), 2.04 (s, 3H). ^{13}C NMR (125 MHz, DMSO) δ 207.3, 172.4, 158.8, 158.1, 157.9, 150.43 137.5, 119.7, 88.5, 86.4, 85.7, 81.5, 69.7, 61.7, 41.4, 37.7, 35.4, 30.1, 28.2. ESI-TOF calc for $C_{21}H_{27}N_5O_9$ 489.45 ($+Na^+$) found 489.25.

General procedure for the preparation of 5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester ribonucleosides (3.6a,b,f,h)

The procedure is demonstrated here for 5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester uridine (**3.6a**). Compound **3.5a** (7.3 mmol) was dissolved in 10 mL of pyridine under a nitrogen atmosphere followed by the addition of DMTCl (8.8 mmol). The reaction was stirred at room temperature until completion, 3 hr. The reaction was then quenched with 2 mL of 5% $NaHCO_3$ and concentrated under vacuum. It was then redissolved in 50 mL DCM and washed with 25 mL 5% $NaHCO_3$. The aqueous layer was then washed 2 x 50 mL DCM. The organic extracts were then combined, dried over $MgSO_4$ and filtered. The solvent was removed by evaporation and the material was then purified by column

chromatography 0→ 3% MeOH in DCM with 0.5% triethylamine. The final product **3.6a**, appeared as a white foam, 90% yield.

Compound	mmol (starting)	Column conditions	Yield
3.6b	3.5	0→2% MeOH in DCM (0.5% TEA)	82
3.6f	8	0→2% MeOH in DCM (0.5 % TEA)	85
3.6h	5	0→3% MeOH in DCM (0.5 % TEA)	78

Table 3.8. Column chromatography conditions and yields of **3.6b, 3.6f, 3.6h**

5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester uridine (3.6a)

^{1}H NMR (500 MHz, DMSO) δ 11.39 (s, 1H), 7.69 (d, *J* = 8.1, 1H), 7.36 (d, 2H, *J* = 7.3), 7.30 (t, 2H, *J* = 7.6), 7.23 (dd, *J* = 2.4, 9.0, 5H), 6.88 (d, *J* = 8.9, 4H), 5.79 (d, *J* = 3.7, 1H), 5.43-5.33 (m, 2H), 5.31 (d, *J* = 8.1, 1H), 5.26 (d, *J* = 6.5, 1H), 4.34 – 4.26 (m, 1H), 4.26-4.19 (m, 1H), 3.94 (s, 1H), 3.72 (s, 6H), 3.29-3.16 (m, 2H), 2.68 (t, 2H, *J* = 6.6), 2.45 (t, 2H, *J* = 6.5), 2.06 (s, 3H). ^{13}C NMR (125 MHz, DMSO) δ 207.3, 172.5, 163.6, 158.8, 151.0, 145.3, 141.2, 136.0, 135.7, 130.4, 128.6, 128.5, 128.4, 127.4, 102.2, 88.6, 88.2, 86.5, 83.2, 81.2, 69.2, 67.7, 63.4, 55.7, 37.9, 30.1, 28.3, 25.8. ESI-TOF calc for $C_{36}H_{38}N_2O_{11}$ 697.25 ($+Na^+$) found 697.13

N^4-levulinyl-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester cytidine (3.6b)

^{1}H NMR (400 MHz, DMSO) δ 10.97, 8.24 (d, 1H, J = 8), 7.37 (d, 2H, J = 8.4), 7.31 (t, 2H, J = 7.6), 7.24-7.20 (m, 5H), 6.96 (d, 1H, J = 7.6), 6.88-6.86 (m, 4H),

5.79 (s, 1H), 5.43-5.41 (m, 1H), 5.36-5.33 (m, 2H), 4.30-4.26 (m, 1H), 4.18 (d, 1H, J = 5.2), 3.99 (d, 1H, J = 8), 3.72 (s, 6H), 3.32 (s, 2H), 2.70-2.68 (m, 4H), 2.58-2.56 (m, 2H), 2.41-2.39 (m, 2H), 2.09 (s, 3H), 2.08 (s, 1H). ^{13}C NMR (125 MHz, DMSO) δ 207.6, 207.4, 173.7, 172.5, 163.0, 158.8, 154.94, 145.2, 136.2, 135.8, 130.4, 130.3, 128.6, 128.4, 127.5, 113.9, 96.0, 90.0, 89.9, 88.4, 88.3, 88.2, 86.6, 82.5, 81.9, 81.8, 68.3, 62.3, 62.2, 55.8, 55.7, 55.6, 55.5, 37.9, 37.6, 31.0, 30.2, 30.1, 28.4. ESI-TOF calc for $C_{41}H_{45}N_3O_{12}$ 794.30 ($+Na^+$) found 794.21.

N^6-levulinyl-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester adenosine (3.6f)

^{1}H NMR (500 MHz, CH_2Cl_3) δ 8.97 (s, 1H), 8.57 (s, 1H), 8.20 (s, 1H), 7.41 (d, 2H J = 8), 7.31-7.30 (m, 7H), 6.80-6.79 (m, 4H), 6.18 (d, 1H, J = 4.5), 5.42, 5.34 (abq, 1 H each, J = 6, 6), 5.23 (s, 1H), 5.07 (t, 1H, 5), 4.61-5.06 (m, 1H), 4.27-4.24 (m, 1H), 3.77 (s, 6H), 3.52-3.49 (m, 1H), 3.43-3.40 (m, 1H), 3.19-3.16 (m, 2H), 2.99 (d, 1H, J = 5.5), 2.91-2.88 (m, 2H), 2.76-2.71 (m, 2H), 2.47-2.23 (m, 2H), 2.30 (s, 3H), 2.15 (s, 3H). ^{13}C NMR (75 MHz, CD3CN) δ 207.51, 207.27, 172.34, 158.89, 145.21, 136.01, 130.25, 128.23, 128.05, 113.23, 88.73, 86.38, 84.12, 63.40, 55.18, 55.09, 37.51, 37.46, 31.47, 28.96, 27.88.ESI-TOF calc for $C_{42}H_{45}N_5O_{11}$ 818.31 ($+Na^+$) found 818.29.

N^2-dimethylformamidine-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester guanosine (3.6h)

^{1}H NMR (500 MHz, CD_3CN) δ 9.83 (s, 1H), 8.55 (s, 1H), 7.77 (s, 1H), 7.42 (d, *J* = 7.7, 2H), 7.36 – 7.08 (m, 7H), 6.94-6.68 (m, 4H), 6.01 (d, *J* = 4.6, 1H), 5.43, 5.31 (abq, 1H each, *J* = 6.5, 6.6), 4.88 (t, *J* = 4.8, 1H), 4.61-4.55 (m, 1H), 4.10 (s, 1H), 3.76 (s, 6H), 3.41-3.20 (m, 2H), 3.08 (s, 3H), 3.05 (s, 3H), 2.72 – 2.51 (m, 2H), 2.45-2.29 (m, 2H), 2.06 (s, 3H). ^{13}C NMR (125 MHz, CD_3CN) δ 207.2, 172.4, 158.9, 158.6, 158.0, 157.7, 150.6, 145.2, 136.9, 136.0, 130.2, 130.1, 128.2, 128.1, 127.1, 120.5, 117.5, 113.2, 88.6, 86.4, 86.3, 83.7, 81.6, 70.1, 63.7, 55.1, 40.9, 37.4, 34.5, 29.0, 28.9, 27.9. ESI-TOF calc for $C_{40}H_{44}N_6O_{10}$ 791.31 ($+Na^+$) found 791.35.

General procedure for the preperation of 5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidites (3.7a,b,f,h)

The procedure is demonstrated here for 5'-*O*-(4, 4'-dimethoxytrityl)-2'-O-acetal levulinyl ester uridine-3'-*O*-2-(cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (**3.7a**). Compound **3.6a** (5 mmol) was dissolved in 20 mL of dry THF under a dry nitrogen environment. Diisopropylethylamine (21 mmol) was then added followed by the dropwise addition of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (6 mmol). The reaction was monitored by TLC (5% MeOH in DCM) and was complete after 2hrs. The reaction mixture was then diluted with 200 mL of DCM and washed once with 40 mL 5% $NaHCO_3$. The aqueous mixture was extracted 3x with 50 mL DCM. The pooled extracts were dried over $MgSO_4$ and filtered and evaporated under reduced pressure. This crude material was then purified on a short column neutralized with 0.5% triethylamine using a gradient of 6:4 ethyl acetate/hexanes (0.5% triethylamine) → 7:3 ethyl acetate/hexanes (0.5% triethylamine). The final product, **3.7a** was obtained as a white foam in 90% yield.

Compound	mmol (starting)	Column conditions	Yield
3.7b	6	7:3 ethyl acetate/hexanes (0.5% TEA)	84
3.7f	2.5	0 → 90% EtOAc in hexanes (0.5% TEA)	81
3.7h	1.1	0 → 100% EtOAc in hexanes (0.5% TEA)	70

Table 3.9. Column chromatography conditions and yields of **3.7b, 3.7f, 3.7h**

5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester uridine-3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (3.7a)

^{31}P NMR (80 MHz, CD_3CN): δ 151.24, 149.88. ESI-TOF calc for $C_{45}H_{55}N_4O_{12}P$ 897.36 ($+Na^+$) found 897.41.

***N*^{4}-levulinyl-5'-*O*-(4, 4'-dimethoxytrityl)- 2'-*O*-acetal levulinyl ester cytidine-3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (3.7b)**

^{31}P NMR (80 MHz, CD_3CN): δ 151.30, 149.26. ESI-TOF calc for $C_{60}H_{62}N_5O_{13}P$ 994.41 ($+Na^+$) found 994.38.

***N*^{6}-levulinyl-5'-*O*-(4, 4'-dimethoxytrityl)- 2'-*O*-acetal levulinyl ester adenosine-3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (3.7f)**

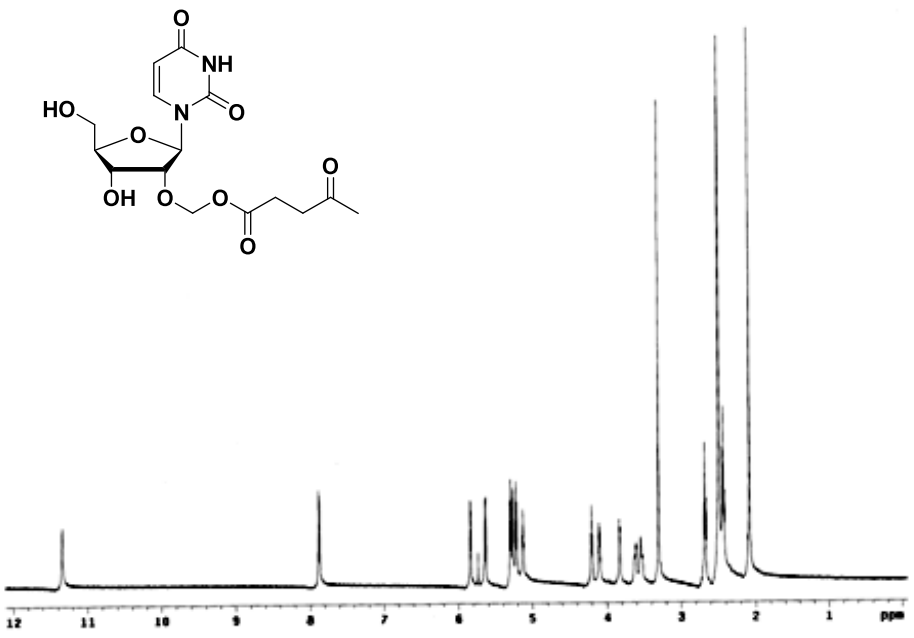
^{31}P NMR (80 MHz, CD_3CN): δ 151.05, 150.52. ESI-TOF calc for $C_{51}H_{62}N_7O_{12}P$ 1018.42 ($+Na^+$) found 1018.35.

***N*^{2}-dimethylformamidine-5'-*O*-(4, 4'-dimethoxytrityl)- 2'-*O*-acetal levulinyl ester guanosine-3'-*O*-2-(cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (3.7h)**

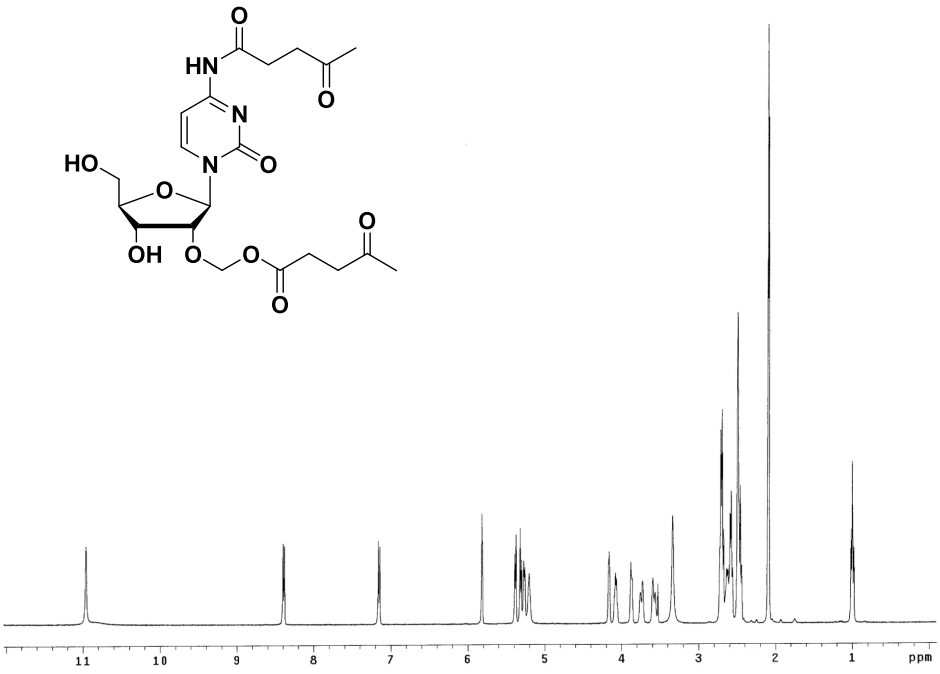
^{31}P NMR (80 MHz, CD_3CN): δ 151.5, 150.9. ESI-TOF calc for $C_{49}H_{61}N_8O_{11}P$ 1014.42 ($+Na^+$) found 1014.38.

3.6.3 Selected NMR spectra

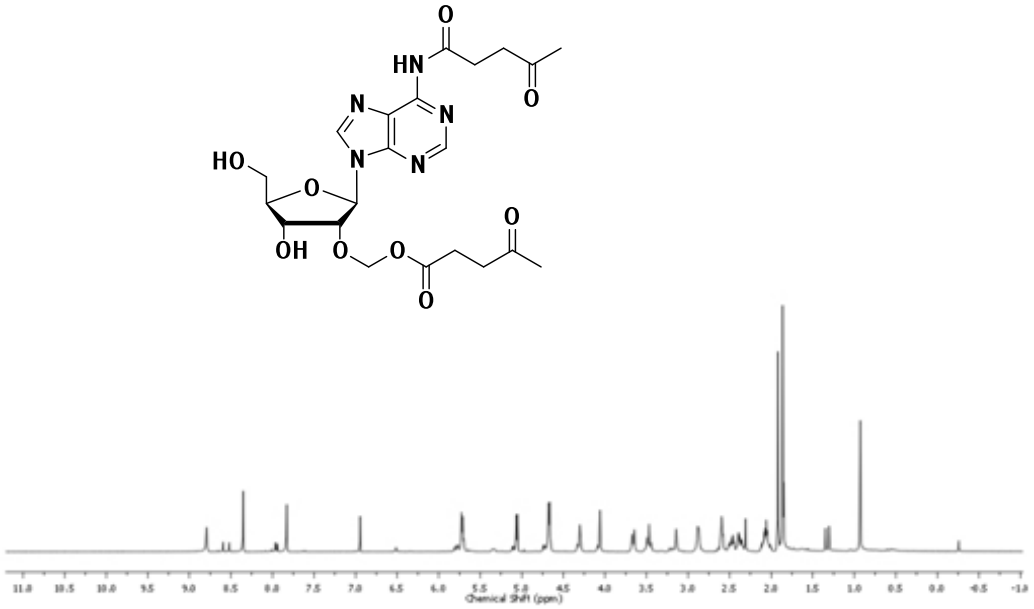
^{1}H NMR (500 MHz, DMSO-*d*6) (3.5a)



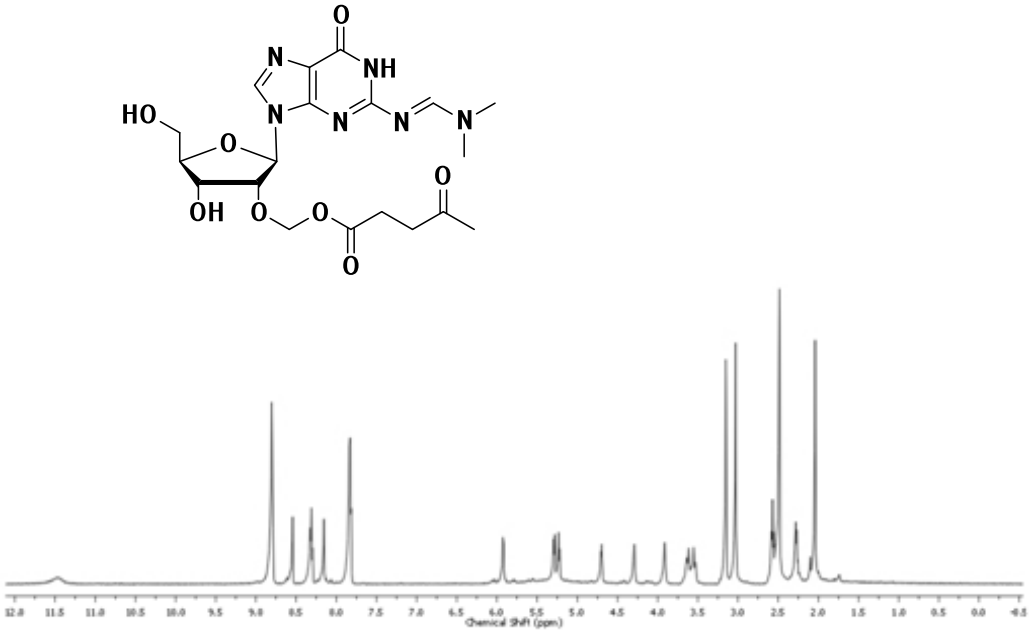
^{1}H NMR (400 MHz, DMSO-*d*6) (3.5b)



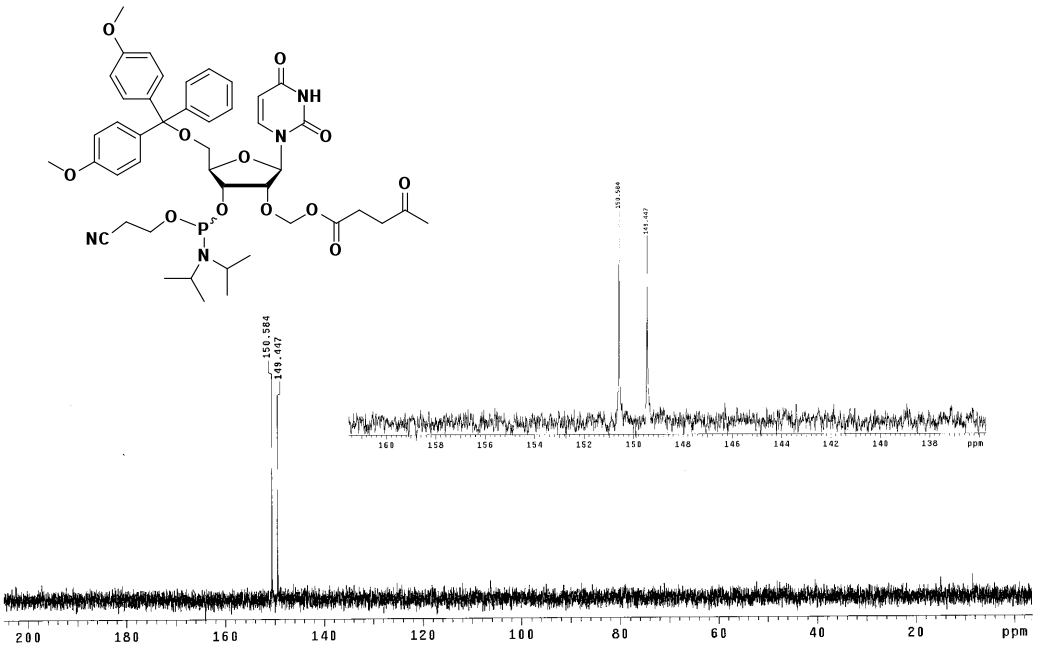
^{1}H NMR (500 MHz, $CDCl_3$) (3.5f)



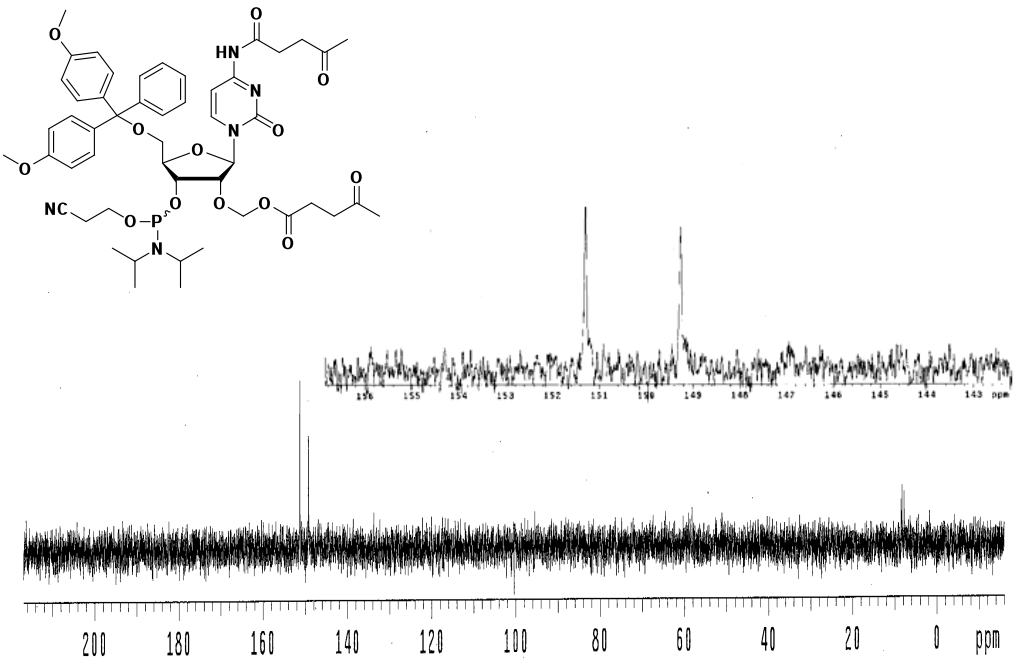
^{1}H NMR (500 MHz, DMSO-*d*6) (3.5h)



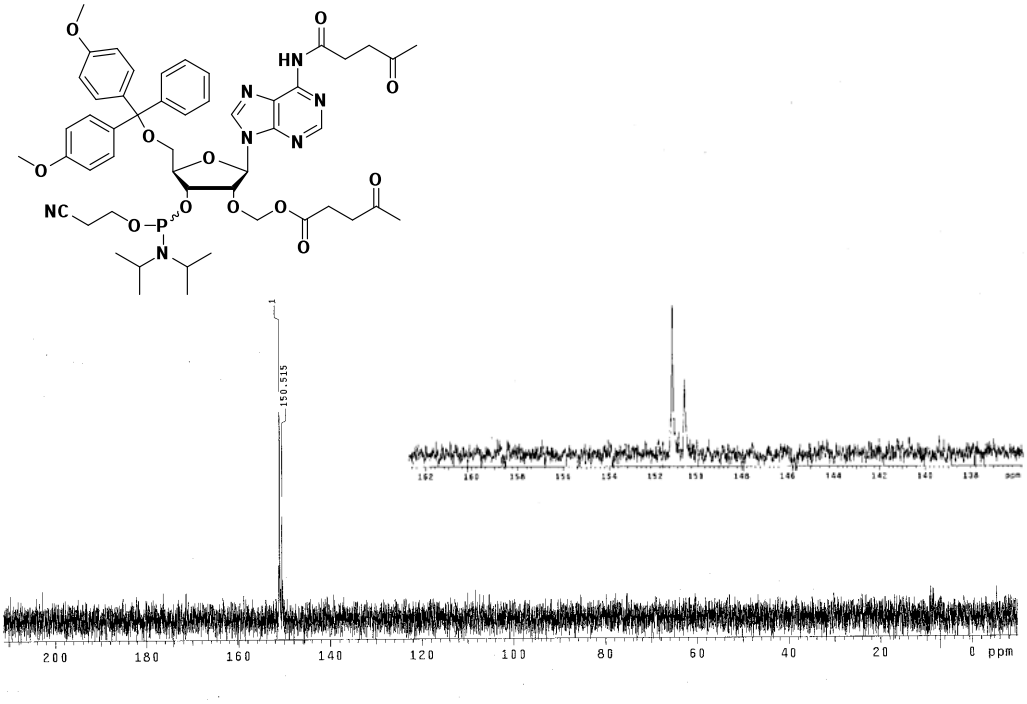
^{31}P NMR (80 MHz, CD_3CN) (3.7a)



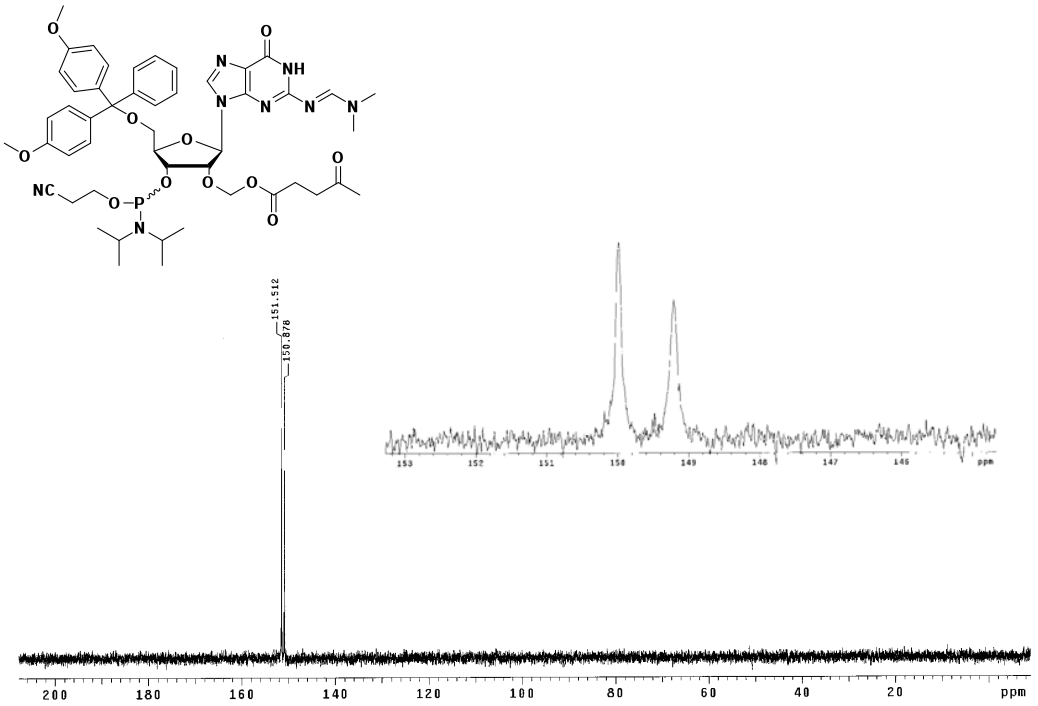
^{31}P NMR (80 MHz, CD_3CN) (3.7b)



^{31}P NMR (80 MHz, CD_3CN) (3.7f)



^{31}P NMR (80 MHz, CD_3CN) (3.7h)



3.6.4 Solid Phase Oligonucleotide Synthesis via 2′-*O*-ALE Chemistry

The solid-phase synthesis of r(GCUUGAAGUCUUUAAUUAA)-d(TT) was performed on an ABI-3400 DNA/RNA synthesizer. A 1 μmol scale was conducted in the trityl-off mode using 500 Å 5′-DMTr-dT-Q-linker long chain alkylamine controlled-pore glass (LCAA-CPG). The support was first subjected to a standard capping cycle, CAP A solution (Ac_2O/pyr/THF) and Cap B solution (10% 1-methylimidazole in THF) for 3 × 180 s to acetylate and dry the solid support. RNA synthesis was carried out using 0.1 M solutions of phosphoramidites **3.7a,b,f,h** in dry ACN with 0.25 M DCI as the activator. All other ancillary agents necessary for oligonucleotide synthesis were obtained commercially. The detritylation step used 3% trichloroacetic acid (TCA) for 80 s. Each phosphoramidite coupling step was set for 1 min, or 10 min. The capping step (using CAP A and CAP B) was set for 20 s and the oxidization step using 0.1 M iodine/pyridine/water/THF was 30 s. 2′-*O*-TBDMS phosphoramidite monomers were used at 0.15 M concentration in ACN.[21] The RNA synthesized using 2′-*O*-TOM phosphoramidite monomers were obtained commercially and treated as above except a 0.10 M phosphoramidite concentration in acetonitrile was used, as recommended by Glen Research. Crude RNA synthesized from 2′-*O*-ACE chemistry was purchased from Dharmacon. The synthetic conditions are unknown, but are assumed to be similar to reported procedures.[19]

3.6.5 On-Column Deprotection of *N*-Lv/dmf-2′-*O*-ALE RNA

After completion of the synthetic cycle, the fully protected oligomer was treated with anhydrous 2:3 TEA/MeCN (v/v) (1h; r.t.) through the column to deblock the cyanoethyl phosphate groups. The column was then washed thoroughly with ACN and dried under high vacuum. Next, the *N*-Lv/dmf and 2'-*O*-ALE groups are removed simultaneously by pulsing a solution of 0.5 M $NH_2NH_2 \cdot H_2O$ in 3:2 pyr:HOAc (v/v), 4hr (16 x 15 min), r.t. through the column. This was followed by washing the solid support with CH_2Cl_2 and MeCN, and evacuation of trace solvents on high vacuum. At this stage, the naked RNA strand bound to the Q-CPG was transferred to a 1 mL eppendorf tube. The RNA was released from the Q-CPG support using fluoride treatment (1 mL of 1 M TBAF, 16h, r.t.). The material is then centrifuged (14 000 rpm) and the supernatant was removed. The CPG was subsequently washed 4 × 250 μL with 1:1 water/ethanol (v/v). This material was evaporated to dryness and redissolved in water. It was then passed through a sephadex G-25 column to remove salts and purified further by denaturing polyacrylamide gel electrophoresis (24% acrylamide, 8.3 M urea).

3.7. References

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Chapter 4. The Light Directed Synthesis of RNA Microarrays

4.1 INTRODUCTION

DNA microarrays (or DNA 'chips') have been used in a wide range of applications such as gene expression[1,2] and genotyping,[3] and are becoming a powerful tool for biologists[4]. Like DNA microarrays, RNA microarrays have also emerged as combinatorial tools as a result of the increasing interest in RNAi,[5] RNA aptamers,[6] protein-RNA interactions,[7] and small molecule-RNA interactions.[8] The fabrication of DNA chips is generally achieved through spotting of pre-synthesized oligonucleotides,[9] or the *in situ* synthesis via ink-jet printing[10,11] or photolithographic synthesis.[12-14] Two key parameters of a microarray are the number of different probe *sites* (spots or features) per unit area, which is reflective in the information density (also called complexity), and the number of probe *molecules* (oligonucleotides) per unit area within an individual probe site (density, but often conflated in the literature with complexity). To minimize the array size, the features and the spacing between them (pitch) are as small as possible while still allowing reliable molecular recognition. (**Figure 4.1**).[15]

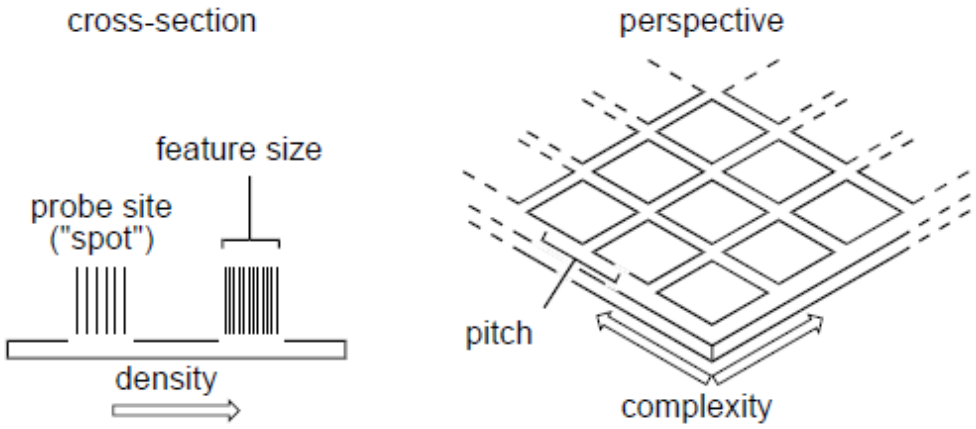


Figure 4.1. Key parameters used to define microarrays. Adapted from ref (15).

Spotted arrays that are in use today are commonly referred to as 'cDNA microarrays'. In general, custom made cDNA libraries are synthesized with a reactive group at their 5'- or 3'-end termini such as amines, thiols, aldehydes, or epoxides,[15] and then 'spotted' onto a glass slide with fine pointed pins or

needles. Addition of an appropriate buffer triggers their covalent attachment onto the chip's surface. Using this approach, cDNAs 300 – 3000 nucleotides in length can be fabricated on microarrays containing from 5 up to 30 thousand features. However, quality, reproducibility, and relatively low oligonucleotide coverage limits the application and wide-spread use of these chips.[16]

Ink-jet printing allows for *in situ* chemical synthesis of DNA via standard phosphoramidite chemistry.[17] In this approach, large area substrates containing several thousands of 100 μm "wells", formed by hydrophobic barriers are used as solid support. Tiny droplets (pL) of standard phosphoramidite monomers are then deposited onto the wells via microfabricated ink-jet pumps, similarly to a color printer, but with the A, C, T, G monomers replacing the inks. The droplet surface tension prevents undesired monomer mixing, producing only the desired nucleotide sequence. After oxidation to the phosphate triester, the wafer is then washed with acid to remove the 5'-protecting group and start the next chain extension. Using this strategy, it is possible to synthesize up to 100,000 distinct DNA strands up to 200 nt in length.

In situ photolithographic synthesis allows for the fabrication of DNA microarrays of unparalleled complexity. The synthetic strategy is analogous to the ink-jet approach, except that the 5'-protecting group is a photolabile benzyl derivative.[18] A photogenerated acid approach has also been used with a 5'-DMTr protecting group, but to a much lesser extent.[19] Light transmitted through a photolithographic mask,[12,13] or reflected by mirrors of a digital light processor (DLP),[14] is imaged onto the synthesis surface. Upon absorption of a photon, the photolabile group drops off, leaving a 5'-hydroxyl terminus which is able to react with an activated phosphoramidite. Each synthesis cycle consists of selective light deprotection followed by phosphoramidite coupling. The sequence of mask patterns and the flow order of phosphoramidites determine the layout and oligonucleotide sequences on the microarray. The physical mask approach is handicapped by the need to fabricate many individual chrome masks for each chip, which can be time consuming and expensive. The use of "virtual masks" generated by the DLP greatly

simplifies microarray synthesis by eliminating the need to make and position physical masks.[14]

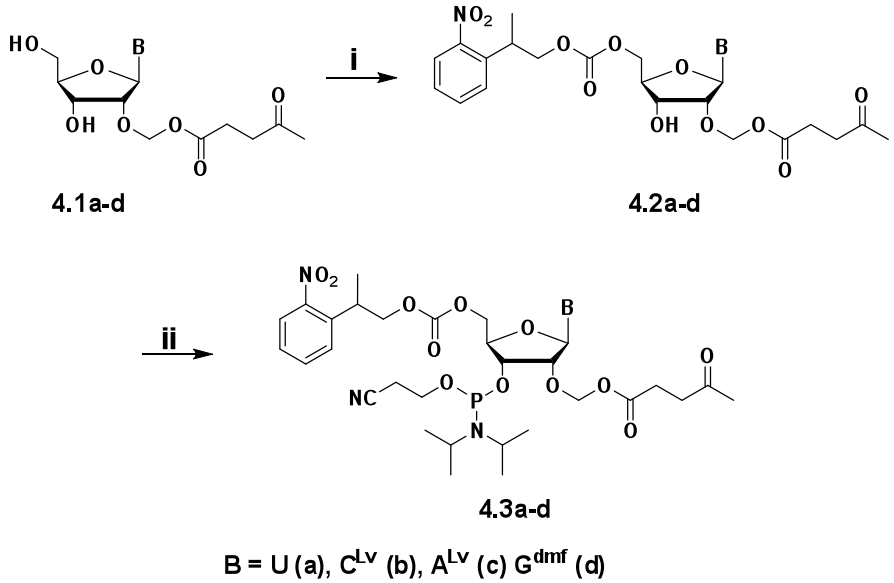
Fabrication of RNA microarrays has severely lagged behind. The few reports available in the literature describe the immobilization of several pre-synthesized RNA strands directly on the chip. This requires expensive synthesis and purification of biotin,[20] amino,[21,22] or thiol[23] terminally modified RNA which subsequently limit chip complexity. In addition, such methods leave RNA oligonucleotides vulnerable to degradation as they are extremely fragile in the deprotected form. An alternative strategy uses surface RNA-DNA ligation chemistry to create RNA microarrays from 5'-phosphate modified DNA microarrays.[24] This strategy involves expensive and elaborate procedures that are limited by reliability and complexity. Other strategies involve the direct attachment of RNA to gold surfaces via a 5'-thiophosphate group.[25] Until very recently there have been no reports of *in situ* synthesis of RNA chips. *In situ* synthesis is complicated by the need to protect the 2'-hydroxyl group of RNA during synthesis. Fluoride labile 2'-protecting groups[26,27] are unsuitable as they are incompatible with glass substrates used in oligonucleotide chip fabrication. A photolabile protecting group[28] at the 2'-position is also undesirable since it would interfere with photodeprotection of the 5'-protecting group. Also, 2'-acetal[29] and 2'-orthoester[30] protecting groups may be suitable, but in their current form, the base required to deblock standard N-protecting groups from the nucleobase (i.e. NH_3 or $MeNH_2$) would cause detachment of the RNA from its surface.[31] However, they may be compatible with this platform if the ultramild N-protecting groups were used.

This chapter will describe the *in situ* synthesis of RNA using novel 5'-2-(2-nitrophenyl)propoxycarbonyl) (NPPOC)-2'-acetal levulinyl ester (ALE)-3'-phosphoramidite monomers ("ALE RNA monomers").[32] The 2'-acetal-levulinyl(ALE) chemistry was originally designed to allow for complete deblocking of RNA while remaining bound to a solid support[32,33] and is an improvement over the 2'-levulinyl (Lv) chemistry reported in **Chapter 2**.[34] When 5'-dimethoxytrityl protection is used, the 2'-ALE monomers are amenable to RNA synthesis on conventional synthesizers using the same synthesis cycles as those used for routine DNA synthesis. Following synthesis, a two-stage deprotection strategy is employed to fully deblock the

oligoribonucleotide chain from bulk solid supports such as controlled pore glass or polystyrene.[35] First, the β-cyanoethyl phosphate protecting groups are removed with a solution of NEt_3 in acetonitrile (ACN) (2:3 v/v; 1 h, r.t.) followed by removal of the 2'-ALE (or 2'-Lv) groups under hydrazinolysis conditions (0.5 – 4hr, r.t.). This last treatment also removes the Lv group on adenine (N^6) and cytosine (N^4) and the dmf group on guanine (N^2). A washing step removes small molecule by-products leaving behind an RNA strand which is completely deprotected and still covalently bound to the solid support. A final step (1 M TBAF in THF, 16 hr, r.t.) releases the RNA strand from the solid support.[35] ALE monomers permit reduced coupling times, high coupling efficiencies (> 99%), and high percentage full-length RNA products. Herein, we examine our method for the *in-situ* synthesis of RNA chips. This required a change of 5'-protecting group strategy from DMTr to the light labile NPPOC group.

4.2 5'-*O*-NPPOC-2'-*O*-ALE-3'-Phosphoramidite Monomers Synthesis

The synthesis of the 5'-NPPOC-2'-ALE-3'-phosphoramidite monomers is summarized in **Scheme 4.1**. The synthesis up to compounds **4.1a-d** was discussed in **Chapter 3.2**.



Scheme 4.1. Synthesis of 5'-*O*-NPPOC-2'-*O*-ALE monomers. Reagents and conditions: (i) NPPOCCl, pyr; (ii) $CEtOP(Cl)NiPr_2$, iPr_2NEt, CH_2Cl_2.

Compounds **4.1a-d** were reacted with NPPOCCl/pyr to afford the 5'-*O*-NPPOC monomers **4.2a-d** in 30-65% yields. This is then followed by reaction with CEtOP(Cl)N*i*Pr$_2$/DIPEA to give the required amidite monomers **4.3a-d** (85-88%).

4.3 Synthesis of RNA on a Microarray

The following experiments were carried out with the collaboration of Dr. Debbie Mitra and Mark Somoza, both postdoctoral students in the laboratory of Dr. Franco Cerrina, of the University of Madison-Wisconsin.

4.3.1 Examination of Microarray Compatibility with 2'-*O*-ALE Deprotection Conditions.

This experiment was designed to show that the conditions necessary for RNA deprotection using 2'-*O*-ALE chemistry (discussed above) do not cause damage to the glass slide or cause oligonucleotide cleavage from the surface. First, a control 20-mer dT microarray was synthesized using conventional 5'-*O*-NPPOC-thymidine-3'-*O*-phosphoramidites.[14] It was then deprotected by exposing the chip to a solution of ethylenediamine (EDA) in ethanol (1:1, v/v, 2 hr, r.t.) and the resulting dT oligomer hybridized to a dA complement tagged with a 5'-*O*-Cyanine5 (Cy5) dye (**Figure 4.2A,B**). The dye allows for visualization and quantification of the fluorescence emission of the complementary strand, which is scanned and analyzed on an Applied Precision ArrayWorx Biochip reader. **Figure 4.2B** shows a quadrant from the DNA chip where the white boxes are the image seen as a result of successful 5'-Cy5dA$_{20}$:dT$_{20}$ hybridization as expected. Another dT$_{20}$-mer DNA chip was fabricated, except this time it was exposed to the conditions necessary to remove the 2'-*O*-ALE group as above. If the hybridization is performed immediately after the hydrazine treatment, there appears to be a 10% loss in emission intensity from salt formation on the chip (**Figure 4.2C**). This was improved by washing the chip with pyr/HOAc (1:1 v/v, 30 sec) prior to hybridization with 5'-*O*-Cy5-dA$_{20}$ (**Figure 4.2D**) and 0% loss of emission intensity was observed.

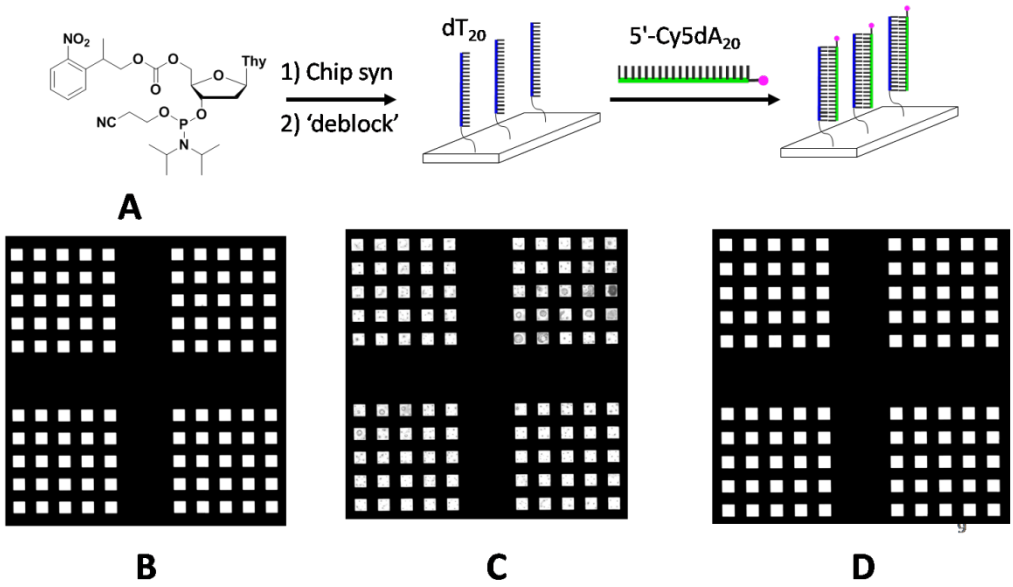


Figure 4.2. Microarray compatibility with 2'-*O*-ALE deprotection conditions. **A.** Illustration of the dT_{20} chip synthesis and subsequent hybridization with a 5'-$Cy5A_{20}$. Applied Precision ArrayWorx Biochip reader scan of the fluorescence intensity of a $Cy5dA_{20}$:dT_{20} hybridization from **B.** EDA in Ethanol (1:1, v/v, 2 hr, r.t.) deprotection conditions; **C.** 1) NEt_3 in MeCN (2:3 v/v; 1 hr, r.t.); 2) 0.5 M hydrazine hydrate in pyr/HOAc (3:2 v/v; 0.5 hr, r.t.) deprotection conditions; **D.** 1) NEt_3 in MeCN (2:3 v/v; 1 hr, r.t.); 2) 0.5 M hydrazine hydrate in pyr/HOAc (3:2 v/v; 0.5 hr, r.t.); 3) pyr/HOAc (1:1 v/v, 30 sec) deprotection conditions.

4.3.2 Coupling Time Optimization of 5'-*O*-NPPOC-2'-*O*-ALE-3'-Phosphoramidite Monomers

With monomers **4.3a-d** in hand, RNA microarray synthesis was carried on a maskless array synthesizer (MAS) with glass substrates ('chips') encased in a flow cell connected to an Expidite DNA/RNA synthesizer. To determine the coupling efficiency of the RNA monomers, sequences of one to twelve nucleotides in length were synthesized onto chips and terminally labeled with a Cyanine 5 (Cy5) phosphoramidite. dT_5 linker strands synthesized from commercially available 5'-NPPOC-dT phosphoramidites were used to distance the RNA strand from the chip surface. All the monomers (0.05 to 0.06 M in MeCN) were activated with 4,5-dicyanoimidazole (DCI; 0.25 M in MeCN) and allowed to couple to the support for 1 to 15 minutes. Standard capping (Ac_2O) was performed followed by oxidation (0.02M I_2/water/pyridine). A UV light energy dose of 6.5 J/cm^2 (up to 250 s) at 365 nm was required for a complete exposure of the photolabile 5'-*O*-NPPOC group. Fluorescence

intensities from the coupling steps were fit with a single exponential decay to determine average coupling efficiency. The optimal coupling times for **4.3a-d** are listed in **Table 4.1**.

Monomer	Concentration (mM)	Coupling time (min.)	Coupling efficiency (%)
rU, (**4.3a**)	50	10	97
rC, (**4.3b**)	50	10	95
rA, (**4.3c**)	50	10	86[a]
rG, (**4.3d**)	60	15	96

[a]unoptimized

Table 4.1. Microarray synthesis coupling parameters and efficiencies.

For example, to determine the optimal coupling time of **4.3a** the sequence 3'-d-TTTTT-$\mathbf{U_{10}}$-**Ux**-Cy5-5' was prepared where $\mathbf{U_{10}}$ is an rU-10 mer with 10 min coupling times and **Ux** is **4.3a** that is coupled at varying times, 1, 2, 5 and 10 min. The sequence is terminally labeled with Cy5 and evaluated by a fluorescence emission scan. The scan is shown on **Figure 4.3A** (left panel) while the graphical representation (average of all values) is shown in **Figure 4.3B** (right panel). Reactions were conducted at 0.05 M in MeCN with 0.25 M DCI as the activator. The emission intensity at each coupling time does not seem to vary significantly. Thus 1 minute coupling times are sufficient for effective coupling of **4.3a**.

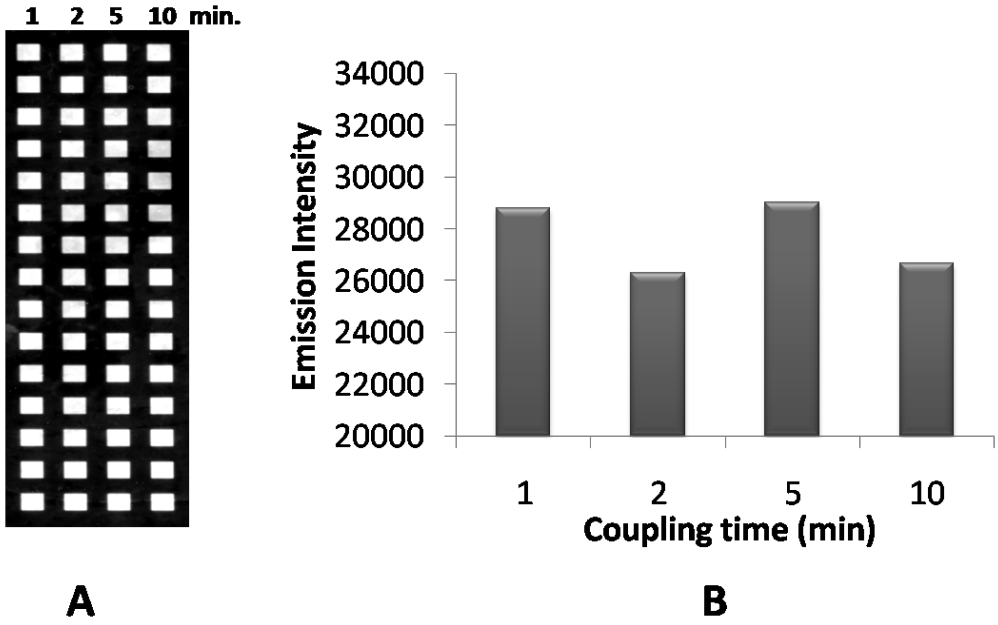


Figure 4.3. **A**. A Cy3 fluorescence emission scan of **Ux** (**4.3a**) coupling at 1, 2, 5 and 10 min (lanes 1-4); and **B**. Graphical representation of coupling time for rU (**4.3a**) of the following sequence 3'-d-TTTTT-**U10-Ux**-Cy3-5'.

4.3.3 5'-*O*-NPPOC Photodeprotection Analysis

To determine the optimal exposure of UV light (measured in Joules, J) to remove the 5'-*O*-NPPOC group from a growing RNA chain on the chip, we synthesized rU_{20} appended through a 3'-dT_5 linker on silanized glass slides with 0.5 M **4.3a** in MeCN, and 0.25 M DCI as the activator with 1 min coupling times. The array was then subjected to an exposure gradient of UV light from 0 – 15 J (or 0-250 s, 55 mW/cm^2 time of exposure) at each N + 1, 5'-*O*-NPPOC deprotection step. The chip was hybridized to a dA_{20}-Cy5 probe and analyzed by fluorescence. Each quadrant is identical where the lower left feature of the quadrants (darkest or lowest emission intensity) was 0.6 J of energy followed by an incremental increase in energy by 0.6 J from left to right up to the 25th feature to a total exposure of 15 J. An average emission intensity profile of the four quadrants was taken to determine the extent of photodeprotection. The results indicate that the optimal exposure for rU NPPOC deprotection occurs at approximately 6.5-7 J (**Figure 4.4**). This was similar to the standard deprotection for NPPOC-DNA monomers which was approximately 6 J.[14] The emission intensity values are excellent and

comparable to that of a DNA exposure gradient control (dA_{20}:dT_{20},-data not shown).

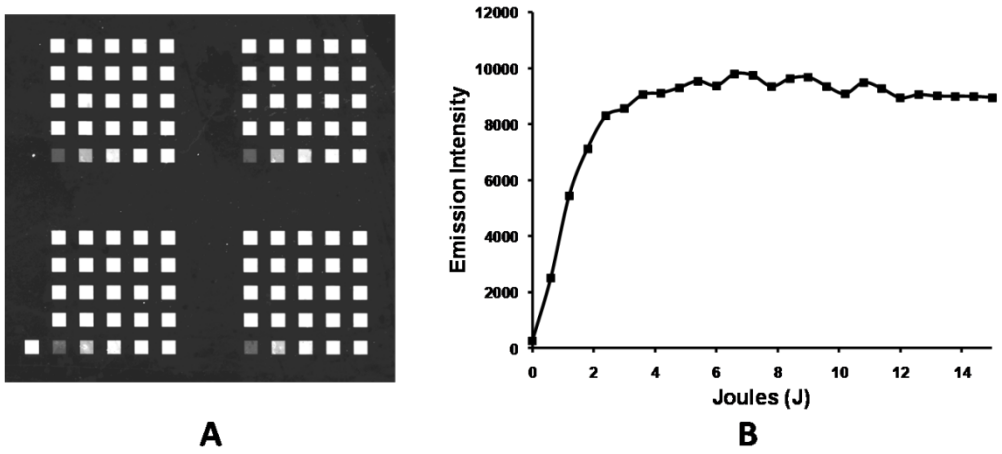


Figure 4.4. Exposure gradient of rU_{20}, 0-15 J. **A**. Cy5 emission scan of rU_{20}: dA_{20}-Cy5. **B**. Graphical representation of exposure rU_{20}:dA_{20}-Cy5.

4.3.4 Synthesis and Hybridization of RNA microarrays

Following the determination of coupling efficiencies, two microarrays (rU_{12} and rA_{12}), were synthesized and deprotected as follows: 1) Decyanoethylation was first conducted by immersing the synthesized microarrays in 2:3 NEt_3/MeCN, 80 min with agitation at room temperature. The slides were rinsed five times in anhydrous MeCN and dried under Argon. 2) The 2'-*O*-ALE protecting groups are removed by treatment with 0.5 M $NH_2NH_2{\cdot}H_2O$ (3:2 v/v pyr:AcOH), 1 h at r.t. The slides were then washed with 1:1 pyr:AcOH (pH > 5) to remove any salts formed on the glass substrate. Following deprotection, the oligonucleotides on the chip were hybridized with either Cy5-labeled dA_{20} or Cy5-labeled dT_{20} (**Figure 4.5**).

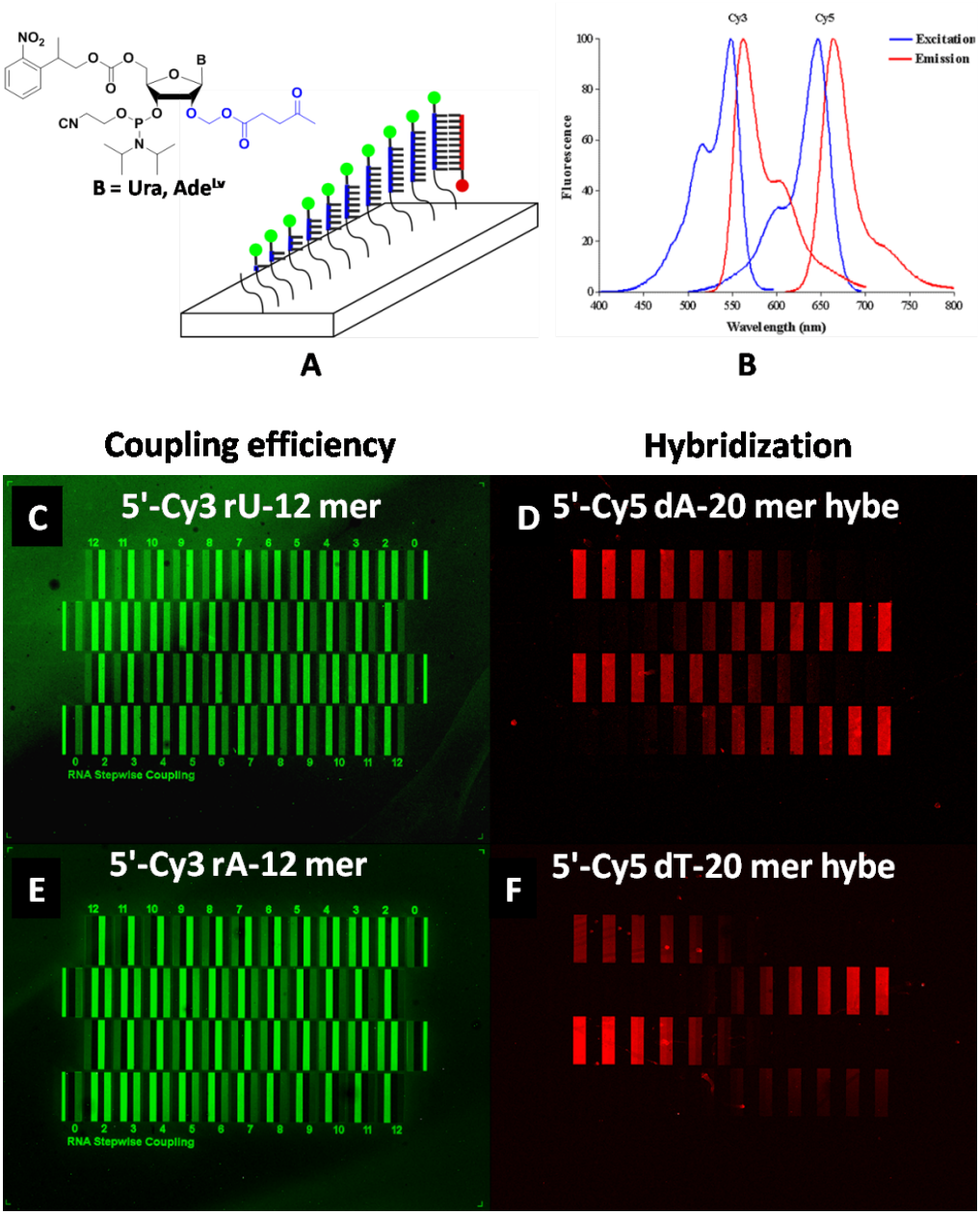


Figure 4.5. **A**. Illustration of coupling and hybridization of rU and rA microarrays. **B**. UV-vis spectra showing the emission and excitation of Cy3 and Cy5. **C**. Coupling efficiency microarray for rU with zero (blank) through twelve coupling steps and 5′-terminal Cy3 label. Each coupling step feature includes an adjacent area with the same number of couplings but no terminal label, as well as a one coupling reference. Intensity data was fit with a single exponential to obtain the average coupling efficiency for rU in **Table. 1**. **D.** The same microarray in **C**, hybridized with Cy5-labeled dA_{20}. **E.** Equivalent microarray with rA couplings. **F.** rA chip hybridized with Cy5-labeled dT_{20}.

The microarray features in the fluorescence micrographs shown in **Figure 4.5** are arranged in such a way that the length of the oligomers increases progressively (n=0 to 12), that is, the sequences on the chip above or below a

numbered label *n* are: (surface)-dT_5-r_n, where n is between 0 and 12. Zero coupling means that the area was subject to a complete coupling cycle, but without monomer, and shows that capping on the chip is ~90% efficient. The chip surface corresponding to each "*n*" labeled coupling step is subdivided into four sections: (1) single RNA coupling (dT_5-rN) is followed by (2) a very bright, single terminally-labeled RNA coupling (dT_5-rN-Cy3), followed by (3) dT_5-rN_n and (4) dT_5-rN_n-Cy3. The unlabeled regions are used for background subtraction of the fluorescence signal. The numbers and label on the chip have the terminally-labeled, single RNA coupling pattern (dT_5-rN-Cy3). Both the terminally labeled and unlabeled *n*-mers are visible upon hybridization with the probes (Cy5-labeled dA_{20} or Cy5-labeled dT_{20}), the longer (and more stable) duplexes provide, as expected, the brightest signal, which gradually decreases as the length of probes decreases.

4.3.5 RNase A Assay on Chip

RNA degradation is an important process as demonstrated by the multiple classes of RNases present in many organisms. In addition to providing a defense against viral RNA, RNases function within the cell to degrade coding or non-coding RNA once these have served their purpose. Here, an RNase A biological assay was conducted to demonstrate the value of using microarrays for studying enzyme kinetics and specificity on ribonuclease substrate libraries.

The RNase A family of endoribonucleases cleave optimally after the pyrimidine in sequences of the form purine-pyrimidine-purine-purine (**Figure 4.6a)**. Following this scheme, the enzymatic cleavage activity of RNase A has been measured for several substrates.[36] In **Table 4.2** the sequences that were chosen from Kelemen *et al.*[36] for the RNase A substrate RNA microarray, along with the reference substrate and activity thereof are listed. The primary difference between the microarray and references substrates is the fluorescence detection scheme, which in the case of the microarray is based on a loss of fluorescence from Cy3 following cleavage by RNase A, while for the reference substrates, cleavage leads to increased fluorescence from the dye 6-FAM due to separation from a quenching chromophore. In addition, the microarray sequences are tethered to the glass surface with a thymidine 15-

mer. Like the reference substrates, the microarray sequences consist of rU and DNA nucleotides, and therefore serves also as a test for the synthesis and deprotection of microarrays containing DNA/rU chimeric sequences.

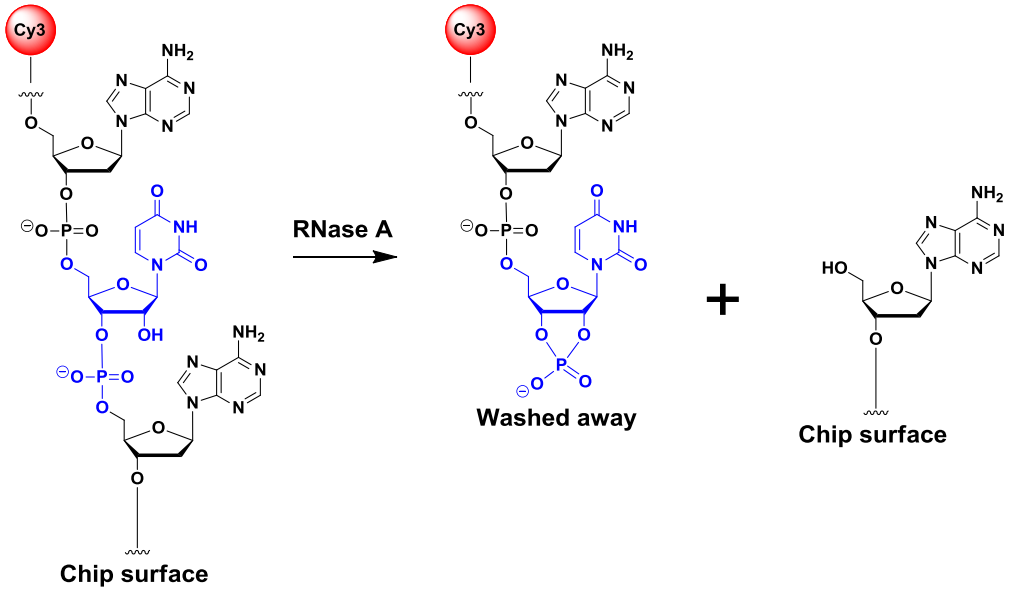
Name	Microarray* sequence 5'- 3'	Reference substrate 5' -3'*	Reference activity k_{cat}/K_m (10^7 $M^{-1}s^{-1}$)*
A	Cy3-rUdA-dT_{15}	6-FAM-rUdA-6-TAMRA	2.5 ± 0.3
B	Cy3-dArU$(dA)_2$-dT_{15}	6-FAM-dArU(dA_2) - 6-TAMRA	3.6 ± 0.4
C	Cy3-$(dA)_2$-rU$(dA)_3$-dT_{15}	6-FAM-$(dA)_2$rU(dA_3)-6-TAMRA	4.7 ± 0.6
D	Cy3-$(dA)_3$-rU$(dA)_4$-dT_{15}	6-FAM-$(dA)_3$rU(dA_4)-6-TAMRA	4.8 ± 0.5

* Reference substrates are taken from reference (36).

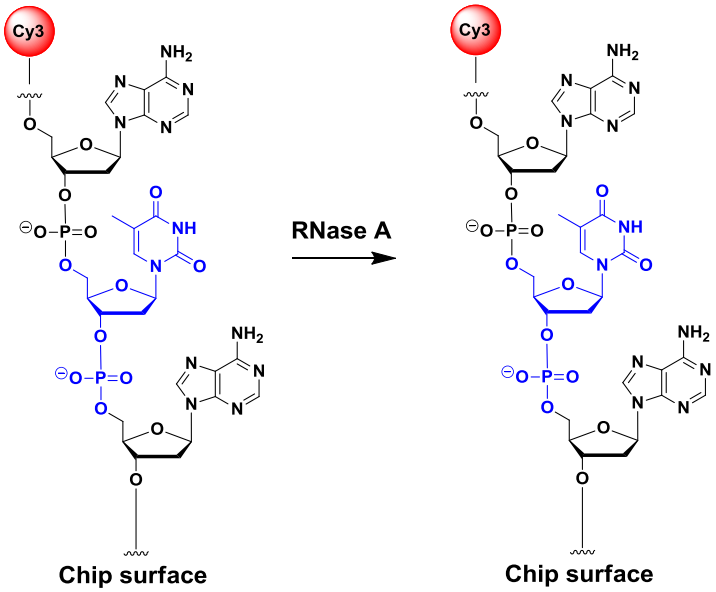
Table 4.2 : Sequences in RNase A substrate microarray

The synthesis of this microarray was done in the usual way as described above, except 5'-NPPOC-2'-ALE-3'-uridine phosphoramidite (**4.3a**) was used along with commercially available 5'-NPPOC-dT and N^6-tac-5'-NPPOC-dA 3'-phosphoramidites. The dT monomer was also used to grow a 15-mer tether from the surface as well as a substitute for uridine for four control sequences that are synthesized adjacent to the four RNase A substrates listed in **Table 4.2**. Following synthesis, RNase A substrate microarrays were deprotected in one of two ways. One method deprotected the DNA bases with 1:1 (v/v) EDA-ethanol for 4 hours, followed by deprotection of the 2'-*O*-ALE rU monomer with 2:3 (v/v) NH_3/MeCN (100 min) then 0.5 M hydrazine hydrate in 3:2 (v/v) pyridine-acetic acid (100 min). The second method was to remove all protecting groups with the ethylenediamine-ethanol solution for 4 hours. Both methods lead to microarrays with the same sensitivity to RNase A. Following deprotection, enzyme kinetics were studied on the microarray. The chip was immersed in a 50 mL Falcon tube containing a 40 mL solution

of 100 nM RNase A in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer. The tube was gently mechanically agitated for 1 min and then the microarray was quickly removed and washed in water and immediately dried with Argon. The microarray was then scanned with a GenePix 4000B microarray scanner. This procedure was repeated multiple times with various immersion times in the RNase solution. **Figure 4.6** shows the initial scan before treatment with RNase A (**C**) and terminal scan after treatment with RNase A (**D**). The loss of signal for the rU-DNA substrate (**Figure 4.6A,D**) is indicative of RNase A activity, whereas the retention of signal for the DNA substrate (**Figure 4.6 B,D**) serves as an internal control as it should not be cleaved by RNase A.


Cy3
RNase A
Washed away
Chip surface
Chip surface

A


Cy3
RNase A
Chip surface
Chip surface

B

Figure 4.6. Illustration of required purine-pyrimidine (RNA)-purine sequence necessary for RNase A activity bound to the chips surface that will undergo cleavage resulting in a loss of fluorescence (**A**) The analogous DNA control is not an RNase A substrate and will not undergo a loss of fluorescence (**B**). Scanner image of the RNase A substrate microarray before (**C**) and after (**D**) exposure to RNase A. Exposure to RNase A cleaves the end of the sequence with the fluorescent dye (see **Table 4.2**) and therefore portions of the microarray with RNase A substrate darken with increasing exposure time. Each RNase A substrate is labeled according to the scheme in **Table 4.2** and is adjacent to a control substrate with thymine replacing uracil in the nucleotide sequence.

The fluorescent data from the scans was then extrapolated using the GenePix Pro software. The plotted data for all four RNase substrates is shown in **Figure 4.7**, which shows fluorescent intensity from the substrate sequences (normalized to the fluorescent intensity from the corresponding control sequences) as a function of exposure time to the RNase A. **Figure 4.6-4.7** demonstrates that RNase A acts effectively on rU-DNA microarray substrates with kinetics comparable to those of the reference substrates. The results also indicate that mixed rU-DNA microarrays have compatible synthesis and deprotection schemes. Residual fluorescence at longer exposure time may be due to several factors, including optical alignment drift resulting in NPPOC removal and terminal labeling of regions of the array surface initially corresponding to the gaps between micromirrors. We do not believe is due to incomplete deprotection of the rU-DNA strand since there was similar residual fluorescence with more or less aggressive deprotection conditions. Nevertheless, the data demonstrate that RNase A acts effectively on the rU containing microarray substrates with measurable kinetics analogous to those of the reference substrates.

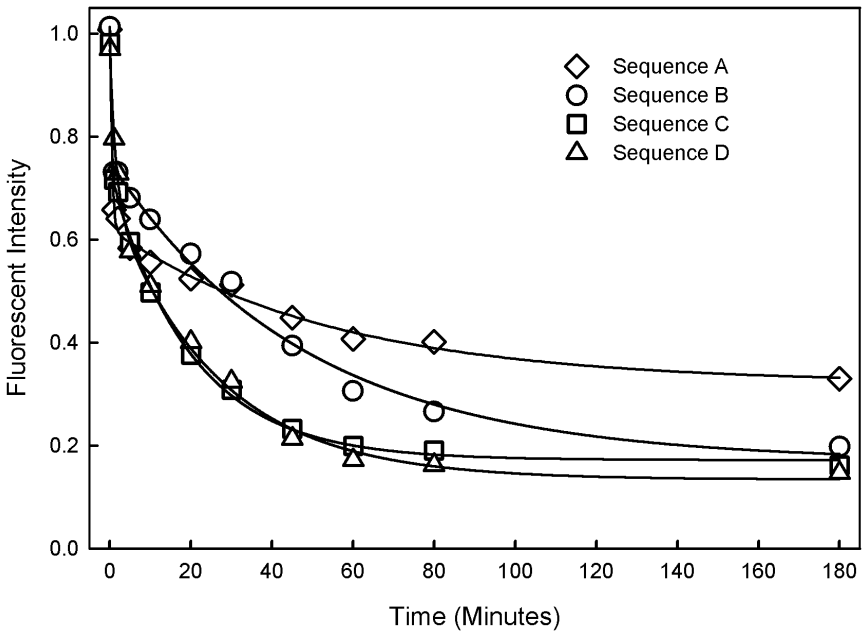


Figure 4.7. Fluorescence emission intensity from the four RNase A substrates in **Table 4.2**, normalized to the control DNA sequences, at various intervals following exposure to a dilute RNase A solution.

4.4 CONCLUSIONS

We have developed a new set of RNA monomers utilizing 2'-*O*-ALE chemistry that are suitable for the *in situ* light directed synthesis of RNA on microarrays. We have shown that phosphoramidite monomers **4.3a-d** couple with good efficiency (86 – 97%) and the 5'-*O*-NPPOC group can be removed under standard deprotection conditions. In addition, the conditions necessary to remove the 2'-*O*-ALE are not detrimental to the microarray and do not cause cleavage of the oligonucleotide from the surface. Several RNA sequences have been synthesized on chip and hybridized to their complementary sequences confirming RNA integrity and chip compatibility. In addition, an RNase A assay was conducted which demonstrates the value of these RNA microarrays for studying enzyme kinetics and specificity on ribonuclease substrate libraries.

4.5 Experimental Methods

4.5.1 General Remarks

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. ^{1}H NMR spectra were measured from undeuterated solvent. ^{31}P NMR spectra were measured from 85% H_3PO_4 as an external standard. Mass spectra were recorded using low resolution ESI.

For the microarray synthesis, the DNA synthesis reagents including Cy3-phosphoramidite, and low water acetonitrile (ACN) were obtained from Glen Research. NPPOC-DNA phosphoramidites used for control experiments and exposure solvent were purchased from Roche NimbleGen. Substrates were prepared by silanizing Superclean glass microscope slides from ArrayIt using monohydroxysilane (Gelest Inc). The slides were functionalized with a 2% *N*-(triethoxysilylpropyl)-4-hydroxybutyramide in 95% EtOH, pH 4-5 (adjusted with glacial acetic acid) for 4 h under agitation. The slides were washed twice for 20 min in 95% EtOH (pH 4-5), dried under vacuum at 120 °C for 12 h and stored in a desiccator prior to use.

4.5.2 Synthetic Protocols and Characterization of Nucleoside Monomers

General procedure for the preparation of 5'-*O*-2-(2-nitrophenyl)propoxycarbonyl-2'-*O*-acetal levulinyl ester nucleosides (4.2a-d).

For example, 5'-*O*-2-(2-nitrophenyl)propoxycarbonyl-2'-*O*-acetal levulinyl ester uridine. Compound **4.1a** (7.3 mmol) was dissolved in a 1:1 mix of THF/pyridine (20 mL) under a dry nitrogen atmosphere and the reaction mixture was cooled to 0 ºC. 2-(2-nitrophenyl)propoxylchloroformate (8.8 mmol) in 5 mL of pyridine was added dropwise to the stirred reaction and the

reaction was monitored by TLC (ethyl acetate). After 3 hrs the reaction was complete. The reaction mixture was quenched with 5 mL of water and the solvent was removed under reduced pressure. The remaining residue was dissolved in 150 mL of DCM and washed with 50 mL of 5% $NaHCO_3$. The aqueous layer was washed 3x with 50 mL DCM. The organic extracts were pooled and dried over magnesium sulphate. After filtration, the solvent was removed under reduced pressure giving a yellowish foam. This crude material was purified by flash chromatography in a gradient of 3:2 ethyl acetate/hexanes → ethyl acetate. The final diasteriomeric mixture of **4.2a** was obtained as a yellow foam in 65% yield.

Since these compounds appear as diasteriomeric mixtures, only the diagnostic peaks ^{1}H NMR peaks will be reported below, but the full ^{1}H NMR spectra are located in **4.5.6**.

Compound	mmol (starting)	Column conditions	Yield (%)
4.2b	8	0→2% MeOH in DCM	52
4.2c	2.8	80:20 EtOAc/hexanes	45
4.2d	1.5	80:20 DCM/acetone	30

Table 4.3. Column chromatography conditions and yields of **4.2b-d**

5'-*O*-2-(2-nitrophenyl)propoxycarbonyl-2'-*O*-acetal levulinyl ester uridine (12a)

Diagnostic ^{1}H NMR (500 MHz, DMSO-*d*6) of diasteriomers: δ 5.82 (d, H-1'), 5.76 (d, H-1'), 4.34-4.23 (m, 2'-*O*-CH_2-*O*- x2), 2.68-2.65 (2'-CH_2-C(O)), 2.63-2.61 (2'-CH_2-C(O)), 2.43-2.40 (m, 2'-*O*-C(O)-CH_2-), 2.36-2.33 (m, 2'-*O*-C(O)-CH_2-), 2.06 (s, 2'-C(O)-CH_3 x2). ESI-TOF calc for $C_{25}H_{29}N_3O_{13}$ 602.17 ($+Na^+$) found 602.16.

N^4-levulinyl-5'-*O*-2-(2-nitrophenyl)propoxycarbonyl-2'-*O*-acetal levulinyl ester cytidine (4.2b)

Diagnostic ^{1}H NMR (500 MHz, DMSO-*d*6): δ 5.88 (d, H-1'), 5.77 (d, H-1'), 5.19-5.01 (m, 2'-*O*-CH_2-O- x2), 2.72-2.66 (m, N^4- CH_2-C(O)- x2, 2'-CH_2-C(O) x2), 2.56-2.32 (m, N4-C(O)- CH_2- x2, 2'-*O*-C(O)-CH_2- x2), 2.10-2.05 (m, 2'-C(O)-CH_3 x2, N^4-C(O)-CH_3 x2). $C_{30}H_{36}N_4O_{14}$ 699.22 ($+Na^+$) found 699.2.

N^6-levulinyl-5'-*O*-(2-nitrophenyl)propoxycarbonyl -2'-*O*-acetal levulinyl ester adenosine (4.2c)

Diagnostic ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.24-6.19 (m, H-1' x2), 5.19-5.01 (m, 2'-*O*-CH_2-O- x2), 2.82-2.57 (m, N^6- CH_2-C(O)- x2, 2'-CH_2-C(O) x2, N6-C(O)- CH_2- x2, 2'-*O*-C(O)-CH_2- x2), 2.20-1.80 (m, 2'-C(O)-CH_3 x2, N^6-C(O)-CH_3 x2). $C_{31}H_{36}N_6O_{13}$ 723.23 ($+Na^+$) found 723.29.

N^2-dimethylformamidine-5'-*O*-(2-nitrophenyl)propoxycarbonyl-2'-*O*-acetal levulinyl ester guanosine (4.2d)

Diagnostic ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.15-6.11 (m, H-1' x2), 5.41 (d, 2'-*O*-CH-O-, J = 6.5), 5.10-5.06 (m, 2'-*O*-CH_2-O-), 5.02 (d, 2'-*O*-CH-O-, J = 6.5), 2.82-2.79 (m, 2'-CH-C(O) x2), 2.69-2.63 (m, 2'-CH-C(O) x2) 2.44-2.34 (m, 2'-*O*-C(O)- CH_2- x2), 2.18 (s, 2'-C(O)-CH_3 x2). $C_{29}H_{35}N_7O_{12}$ 696.23 ($+Na^+$) found 696.30.

General procedure for the preperation of 5'-*O*-2-(2-nitrophenyl)propoxycarbonyl-2'-*O*-acetal levulinyl ester 3'-*O*-2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidites (4.3a-d).

For example, 5'-*O*-2-(2-nitrophenyl)propoxycarbonyl-2'-*O*-acetal levulinyl ester uridine-3'-*O*-2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (**4.3a**). Compound **4.2a** (2.67 mmol) was dissolved in 10 mL of dry THF under a dry nitrogen environment. Diisopropylethylamine (10.7 mmol) was then added followed by the dropwise addition of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (3.2 mmol). The reaction was monitored by TLC (ethyl acetate) and was complete after 2hr. The reaction mixture was

then diluted with 150 mL of DCM and washed once with 25 mL 5% $NaHCO_3$. The aqueous mixture was extracted 3x with 25 mL DCM. The pooled extracts were dried over $MgSO_4$ and filtered and evaporated under reduced pressure. This crude material was then purified on a short column neutralized with 0.5% triethylamine using a gradient of 3:2 ethyl acetate/hexanes (0.5% triethylamine) → ethyl acetate (0.5% triethylamine). The final product, **4.3a** was obtained as a yellowish foam in 88% yield.

Compound	Column conditions	Yield
4.3b	4:1 ethyl acetate/hexanes (0.5% TEA)	85%
4.3c	80:20 EtOAc/hexanes→70:30 EtOAc/hexanes (0.5% TEA)	86%
4.3d	EtOAc (1% TEA)	85%

Table 4.4. Column chromatography conditions and yields of **4.3b-d**

5'-*O*-2-(2-nitrophenyl)propoxycarbonyl-2'-*O*-acetal levulinyl ester uridine-3'-*O*-2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (4.3a)

^{31}P NMR (80 MHz, CD_3CN): δ 151.30, 150.89, 150.17, 149.98. ESI-TOF calc for $C_{34}H_{46}N_5O_{14}P$ 802.28 ($+Na^+$) found 802.23.

***N*^{4}-levulinyl-5'-*O*-2-(2-nitrophenyl)propoxycarbonyl-2'-*O*-acetal levulinyl ester cytidine-3'-*O*-2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (4.3b)**

^{31}P NMR (80 MHz, CD_3CN): δ 151.15, 150.32, 150.10, 149.79. ESI-TOF calc for $C_{34}H_{46}N_5O_{14}P$ 802.28 ($+Na^+$) found 802.23. ESI-TOF calc for $C_{39}H_{53}N_6O_{15}P$ 899.33 ($+Na^+$) found 899.31.

***N*^{6}-levulinyl-5'-*O*-2-(2-nitrophenyl)propoxycarbonyl-2'-*O*-acetal levulinyl ester adenosine-3'-*O*-2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (4.c)**

^{31}P NMR (80 MHz, CD_3CN): δ 151.64, 151.41, 151.25, 151.03. ESI-TOF calc for $C_{40}H_{53}N_8O_{14}P$ 923.34 ($+Na^+$) found 923.31.

N^2-dimethylformamidine-5'-*O*-2-(2-nitrophenyl)propoxycarbonyl-2'-*O*-acetal levulinyl ester guanosine-3'-*O*-2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (4.3d)

^{31}P NMR (80 MHz, CD_3CN): δ 151.27, 151.06, 149.72, 149.59. ESI-TOF calc for $C_{40}H_{53}N_8O_{14}P$ 896.34 ($+Na^+$) found 896.37.

4.5.3 MAS (Maskless Array Synthesizer) Light Directed Synthesis

Experiments were conducted according to methods described by Cerrina and co-workers.[14] A MAS instrument and a Perspective Biosystems Expedite 8909 DNA pump system were used in the light direct synthesis approach.[14] The MAS instrument was equipped with a Texas Instrument's digital light processor (DLP) with dimensions of a 768×1024 array of 13 μm wide micromirrors. An exposure wavelength of 365 nm by a 1000 W Hg lamp was used for 5'-NPPOC deprotection during DNA and RNA synthesis (Roche NimbleGen exposure solvent). DNA microarrays were prepared according to standard protocol[37] using NPPOC-phosphoramidites (30 mM, 60 s coupling times) with photodeprotection at 6 J for 111 s at 54 mW/cm^2. Deprotection of DNA microarrays was effected using a solution of ethylenediamine in EtOH (1:1 v/v; r.t. 2h) followed by EtOH washings. Slides were dried under Argon prior to hybridization experiments. Both DNA and RNA microarrays are prepared with a 3′-dT_5 linker on the glass substrate.

4.5.4 RNA Microarrays

As demonstrated by fluorescence measurement of hybridized samples, sufficient 5'-NPPOC deprotection occurs at 6.5 J/cm^2 exposure. Standard acetic anhydride capping (CAP A and CAP B) was performed, followed by oxidation in aqueous iodine solution (0.02M). Prior to hybridization, the protecting groups were removed as follows. Decyanoethylation was effected by immersing the synthesized RNA microarray in a 2:3 (v/v) solution of anhydrous NEt_3:MeCN for 80 min with agitation at room temperature. The slide was rinsed five times in anhydrous acetonitrile and dried with argon. The 2′-*O*-ALE protecting groups were removed under buffer conditions, 0.5 M $NH_2NH_2{\cdot}H_2O$ in (3:2 v/v pyr: AcOH), and shaken for 60 min at room

temperature. The slide was washed in a 1:1 pyr: AcOH (pH >5) to remove any salts formed on the glass substrate. The slide was then flushed repeatedly with ACN and dried under argon prior to hybridization.

4.5.5 Hybridization

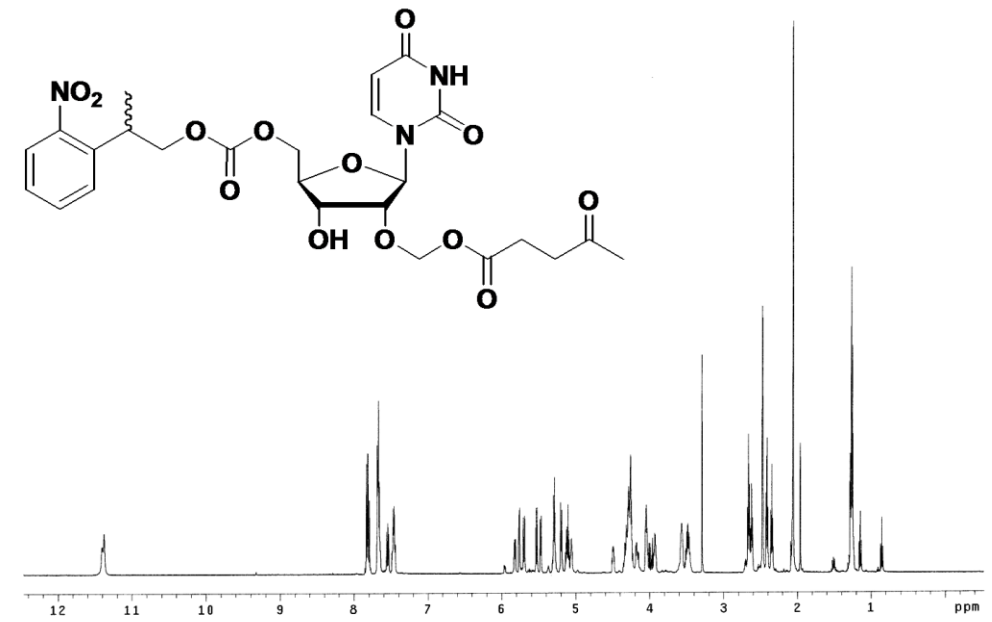
Water was treated with diethylpyrocarbonate and then autoclaved to inactivate RNase and thus prevent enzymatic degradation of the RNA microarrays. Hybridization experiments were carried out in a buffer consisting of 40 mM TRIS-HCl, 10 mM $MgCl_2$, pH 7.2. A 300 μL solution of 500 nM DNA probes (dA_{10}-5′-Cy5 and dA_{20}-5′-Cy5) were hybridized to the respective rU complements. Hybridizations were conducted for 1 h at 4 ºC for rU_{10}:dA_{10} and ambient temperature for rU_{20}:dA_{20}. The slides were washed with 300 μL of buffer (0.5 M NaCl, 0.03 M phosphate, 0.3 mM EDTA, 0.01% Tween-20) prior to fluorescence scanning. The hybed chips were scanned and analyzed on an Applied Precision ArrayWorx Biochip reader.

4.5.6 RNase A Assay

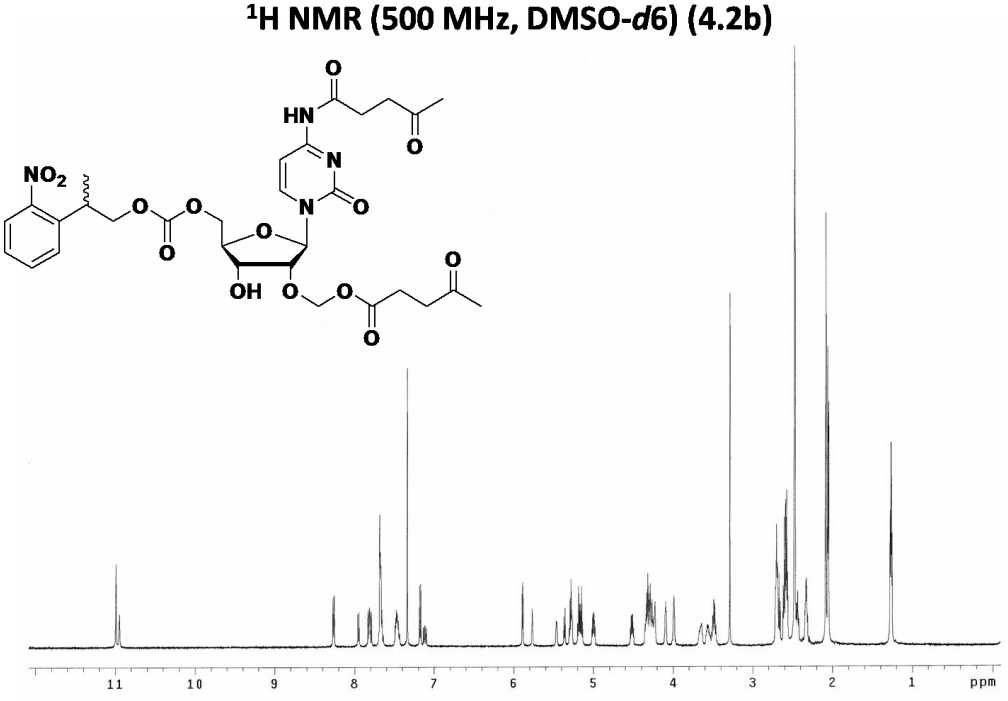
The enzyme kinetics were studied on the microarray. The chip was immersed in a 50 ml Falcon tube containing 40 ml of 100 nM RNase A in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer. The tube was gently mechanically agitated for 1 min and then the microarray was quickly removed and washed in water and immediately dried with Argon. The microarray was then scanned with a GenePix 4000B microarray scanner. This procedure was repeated multiple times with various immersion times in the RNase solution.

4.5.7 Selected NMR Spectra

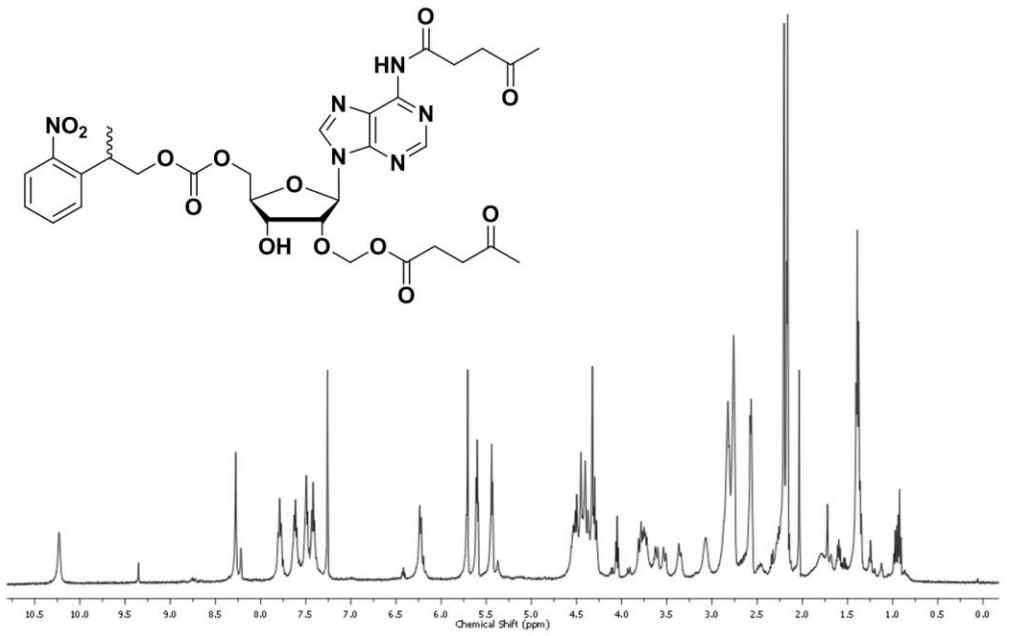
^{1}H NMR (500 MHz, DMSO-*d*6) (4.2a)



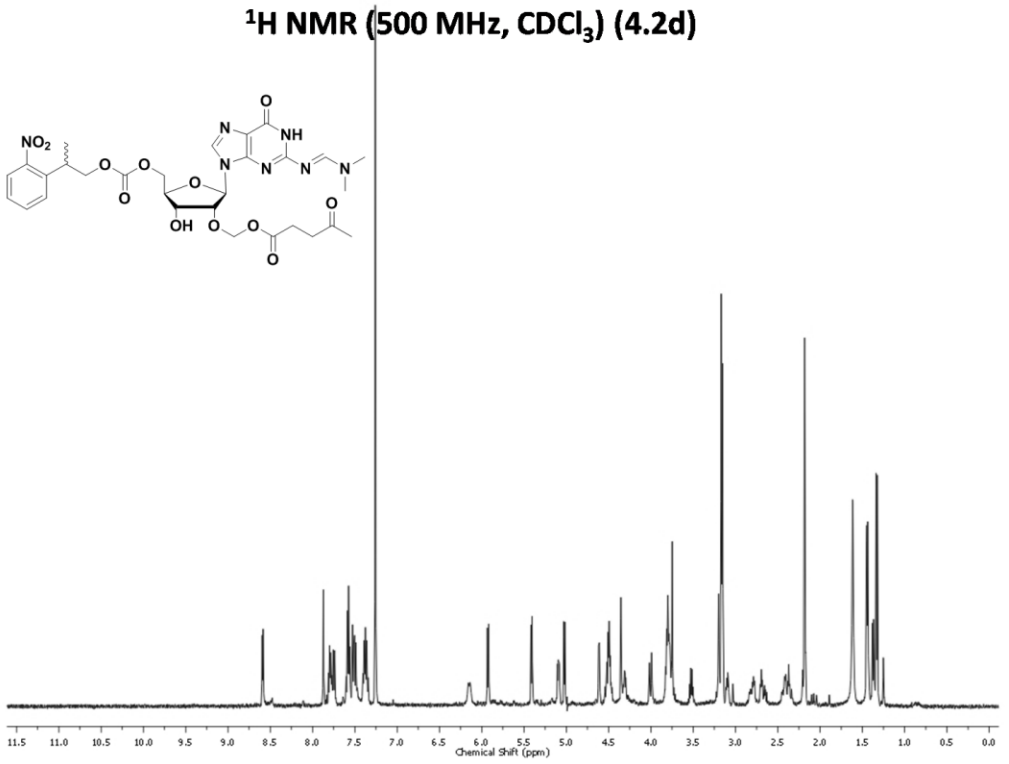
^{1}H NMR (500 MHz, DMSO-*d*6) (4.2b)



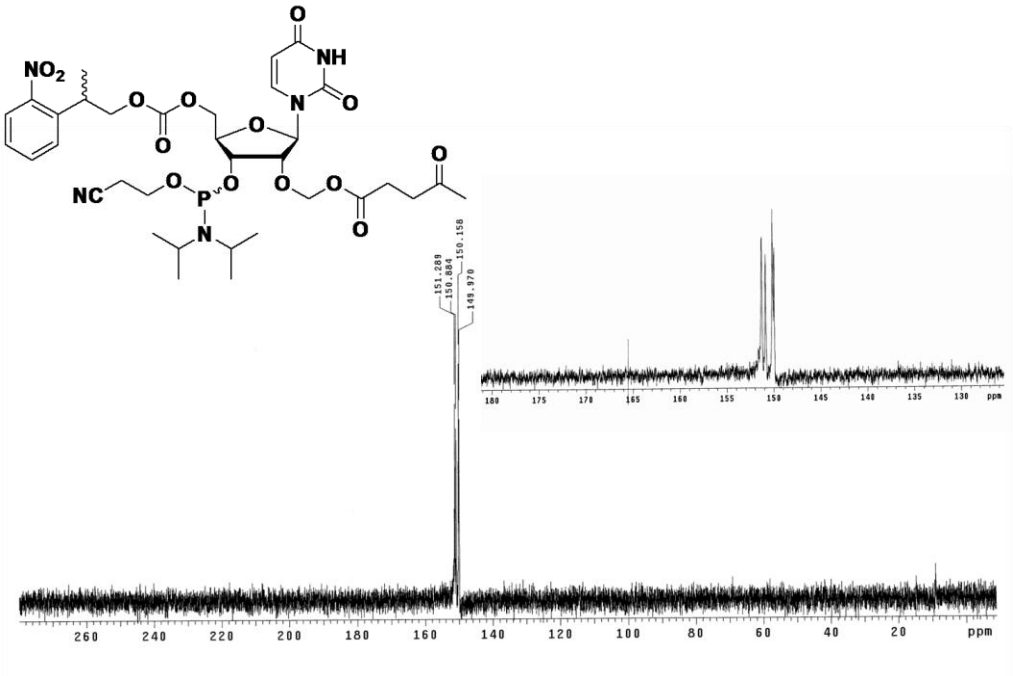
^{1}H NMR (500 MHz, $CDCl_3$) (4.2c)



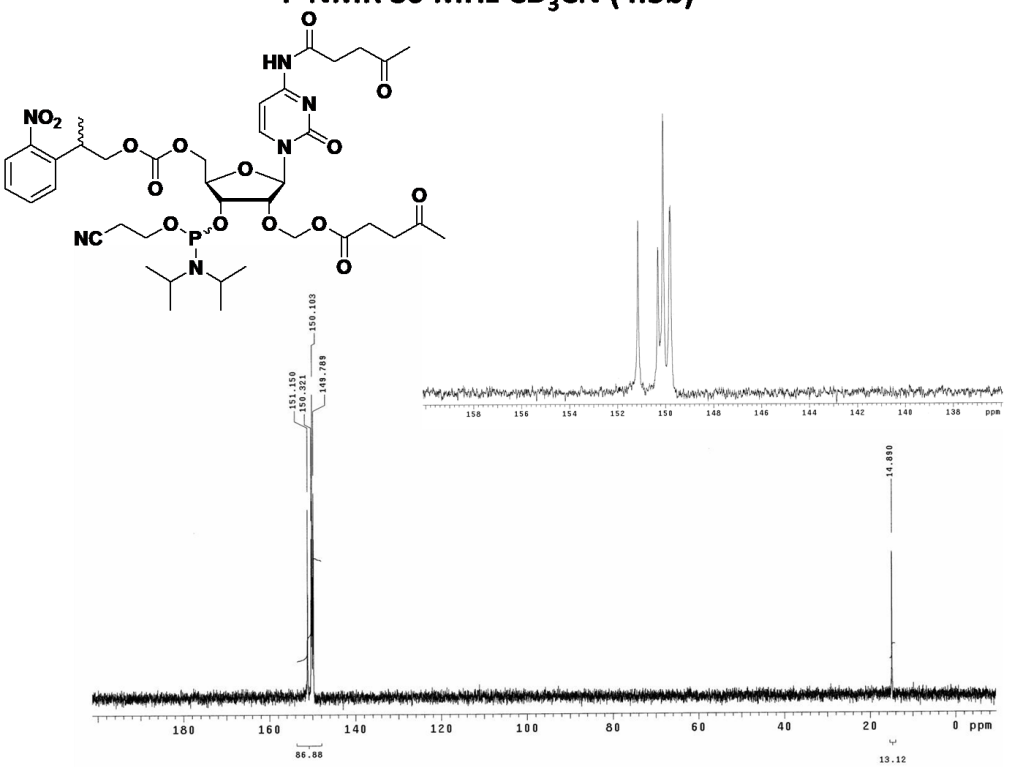
^{1}H NMR (500 MHz, $CDCl_3$) (4.2d)



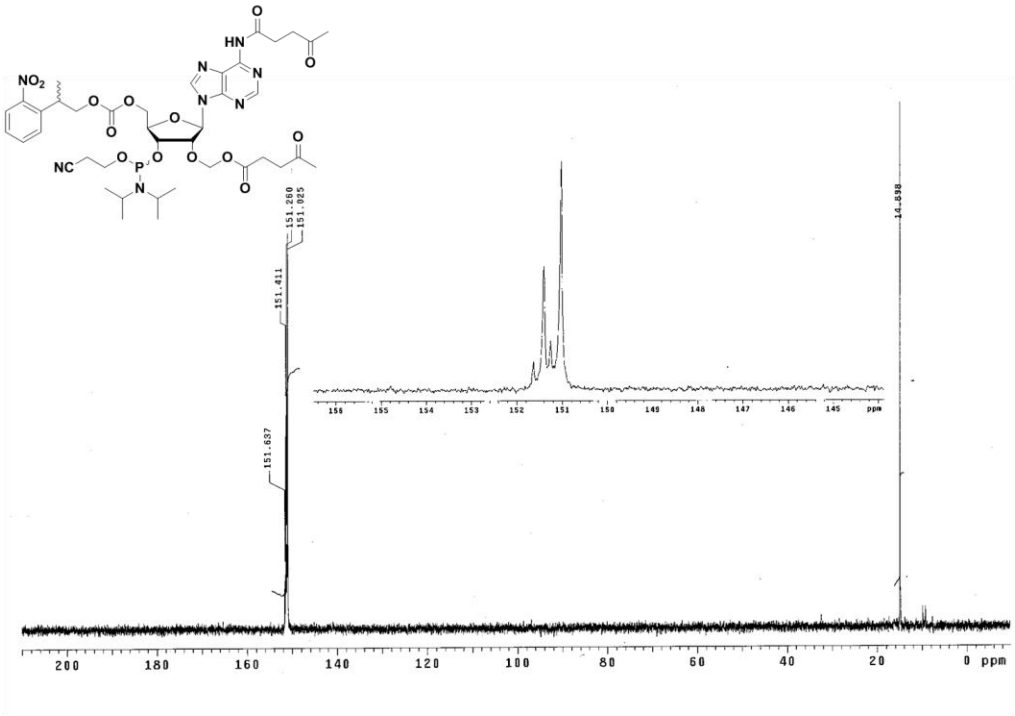
^{31}P NMR 80MHz, $CDCl_3$) (4.3a)



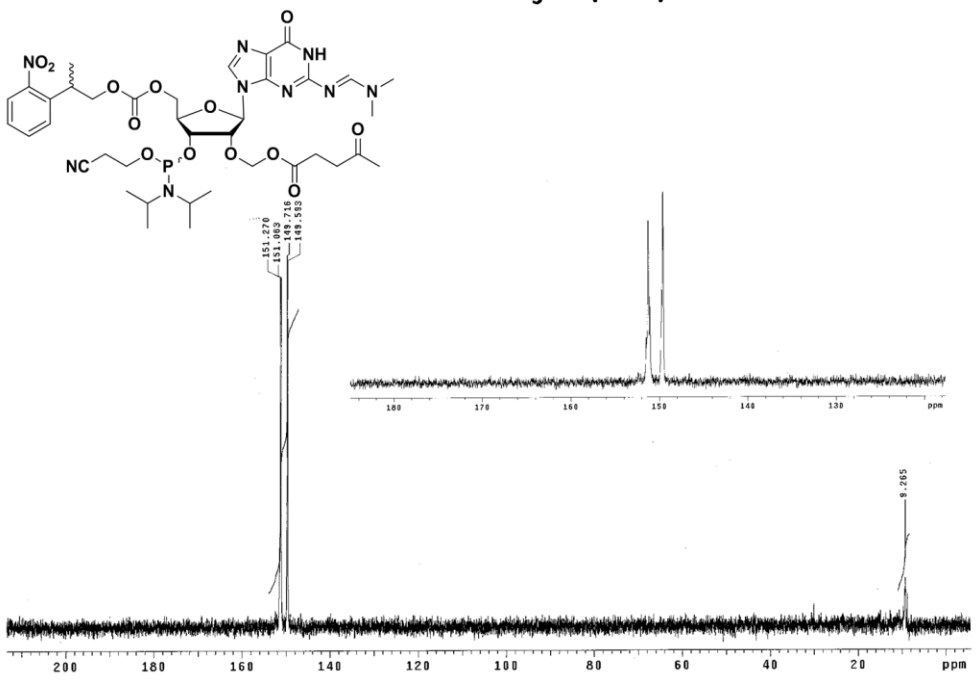
^{31}P NMR 80 MHz CD_3CN (4.3b)



^{31}P NMR 80 MHz CD_3CN (4.3c)



^{31}P NMR 80 MHz CD_3CN (4.3d)



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Chapter 5. The Synthesis and Biological Evaluation of Pro-siRNA

5.1 Introduction

Ever since the Nobel Prize-winning discovery of RNAi by Fire et al.[1] in 1998, there have been significant advances in human gene therapeutic application of small interfering RNA (siRNA).[2] However, the major obstacle of RNAi based therapeutics is the cellular delivery of siRNA.[3] In general siRNAs are too large (~ 13 kDa), too negatively charged and not hydrophobic enough for efficient intracellular uptake. They also activate the immune response and are rapidly degraded by endogenous enzymes and cleared from the body. To circumvent these problems, many groups have focused on the use of delivery vehicles such as liposomes and lipids, cationic polymer complexes or lipophilic conjugates.[4] In addition, chemically modified siRNA has been used to increase both serum and cellular stability, and to prevent the activation of the immune response.[5]

We were interested in investigating siRNA prodrugs (pro-siRNA) to enhance cellular uptake and increase chemical stability. The idea was to synthesize siRNA conjugated to bio-labile lipophilic groups that would enhance permeability across cellular membranes, but be cleaved by endogenous enzymes releasing the active siRNA once inside the cell.

Prodrugs are bioreversible derivatives of drug molecules that undergo an enzymatic and/or chemical transformation *in vivo* to release the active parent drug which can then exert the desired pharmacological effect.[6] For example, prodrugs can help to overcome the barriers related to drug formulation and delivery such as membrane permeability, aqueous solubility, chemical/enzymatic instability, toxicity, pharmacology, drug targeting, et cetera (**Figure 5.1**.). In most cases the prodrugs are simple chemical derivatives of the active drug molecule. The functional group used to modify the active drug is termed the promoiety and can be, for example, an ester, phosphate ester, amide, oxime, imine or carbamate. Basically, any group that can be removed through biocatalysis or chemically (e.g., hydrolysis) within the cell may be used. As of 2002, 5-7% of all drugs approved worldwide are prodrugs, and 15% of the drugs approved that year were prodrugs.[7]

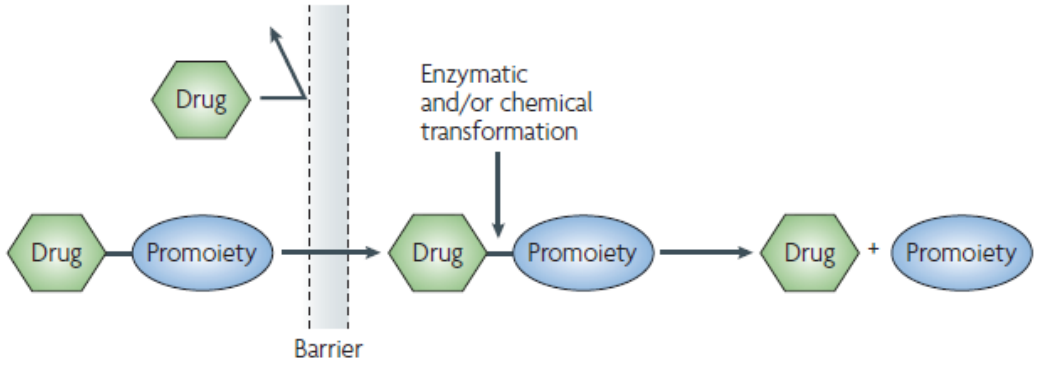


Figure 5.1. The prodrug strategy. Figure adapted from reference (7).

Though the prodrug approach has been widely successful for small molecules, this strategy has been sparingly used for oligonucleotides therapeutics, and there are no reports for its use in siRNA silencing experiments. Considering how complex oligonucleotide synthesis is in general, this is not surprising. Nevertheless, there are several positions on an oligoribonucleotide chain that may be amenable to conjugation with a promoiety.

The internucleotide phosphate linkage has received the most attention due to its negative charge. If it can be blocked, then cell uptake should be improved. The most studied modification for DNA strands is the *S*-acylthioethyl moiety (SATE, **Figure 5.2A**) and derivatives thereof.[8-10] This group masks the DNA's phosphate charge imparting more lipophilicity, nuclease resistance in serum, and better cell permeability. Other similar protecting groups are the *S*-acyloxymethyl (SAM, **Figure 5.2B**)[11] and the *O*-(4-acyloxybenzyl)-phosphate(thioate), **Figure 5.2C**.[12] The 5-nitro-2-furylmethyl and 5-nitro-2-thienylmethyl groups[13] (**Figure 5.2D**) have also shown to increase cell permeability. These groups are activated by an endogenous nitroreductase which in turn starts a cascade reaction releasing the active drug molecule.

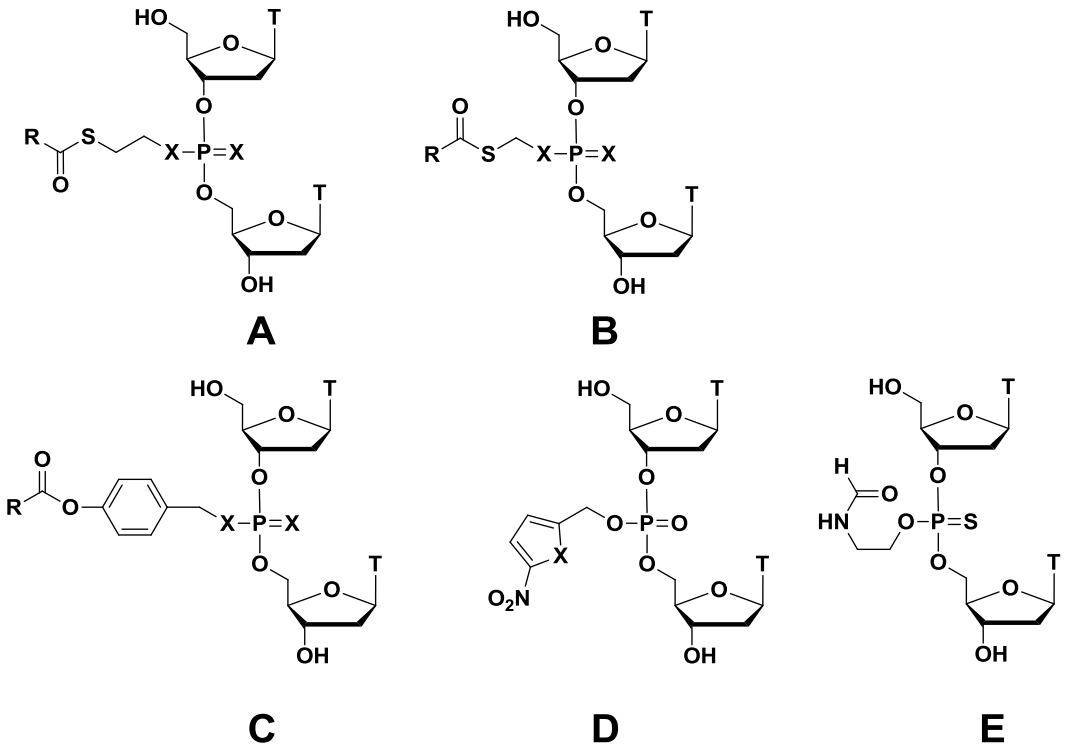


Figure 5.2. Internucleotide phosphate protecting groups for the prodrug approach. **A**. S-Acylthioethyl (SATE), activated by an esterase. **B**. *S*-Acyloxymethyl (SAM), activated by an esterase. **C**. *O*-(4-Acyloxybenzyl)-phosphate(thioate), activated by an esterase. **D**. 5-Nitro-2-furylmethyl and 5-nitro-2-thienylmethyl groups, activated by a nitroreductase. R = Me, Et, Piv, iBu, X = S or O. **E**. 2-(*N*-Formyl-*N*-methyl)aminoethyl thiophosphate protecting group, activated by temperature.

The enzymatically activated internucleotide protecting groups have been shown to work *in vitro*, but have never been tested *in vivo* as a therapeutic agent. The most successful internucleotide protecting group to date is the 2-(*N*-formyl-*N*-methyl)aminoethyl thiophosphate (**5.2E**) protecting group developed by Beaucage.[14] This group is unique as it is activated by the temperature of the cellular environment (37 °C) where it has a half-life of 73 hr. Its prodrug ability was tested as an immunomodulatory CpG ODN in mice and was shown to be effective viral protection.[14]

The 5'/3'-hydroxyl positions at the terminal ends of an oligonucleotide have been extensively used for covalent attachment of a variety of groups to aid in cellular uptake,[5] but because there are only two positions available for modification, it has received no attention for the prodrug approach. Similarly,

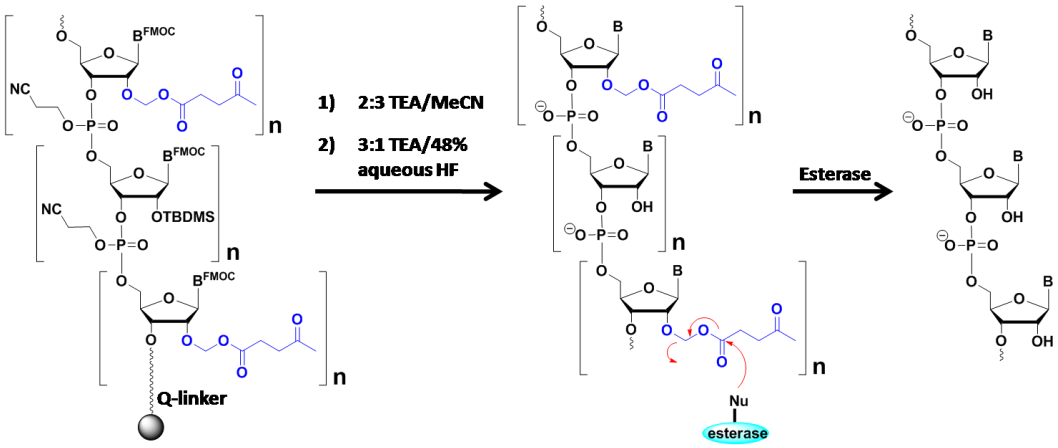
though there are many more exocyclic amines on the nucleobases, which could in principle be modified with a promoiety, these groups would interfere with duplex formation (especially with double stranded siRNA) and have received little to no attention.

The ribose 2'-hydroxyl position has traditionally been a very popular position to make "permanent" chemical modifications (e.g. alkylation) on RNA chains, particularly to enhance hybridization affinity, lipophilicity and nuclease stability.[15,16] Surprisingly, it has received little attention for the prodrug strategy. To the best of our knowledge, the 2'-acetal ester from Debart and co-workers[17,18] was the first example. These researchers have shown that a polyuridylic acid can be constructed with a 2'-acetal ester that are removed by esterases *in vitro* or in cell lysate. This approach has major potential as a novel delivery strategy, but it has yet to be tested on a mixed base sequence, or used *in vitro* or *in vivo*. Herein, a novel protecting group strategy for the synthesis of 2'-acetal levulinyl ester (2'-*O*-ALE) pro-siRNA will be presented, along with their physicochemical and biological characterization that include nuclease serum stability and preliminary *in vitro* gene silencing assays.

5.2 2'-*O*-ALE Pro-siRNA Design Considerations

The major challenge of synthesizing 2'-*O*-ALE modified RNA strands was to develop a synthetic strategy that allows for the selective deprotection of the exocyclic amines and cleavage from the solid support under conditions that releases the intact 2'-modified RNA prodrug. With this in mind, we considered FMOC protection for the purines (A, G) and pyrimidine (C) exocyclic amines as we previously showed (**Chapter 3**) that *N*-FMOC groups can be removed with 3:2 MeCN/TEA (v/v) without causing cleavage of the 2'-*O*-ALE groups. Also, these conditions had the added advantage of also removing the phosphate's cyanoethyl groups thus simplifying the overall process. As the solid support, we again considered the Q-linker[19] LCAA CPG support (**Chapter 3**) because it allows release of the bound oligonucleotide chain by a brief fluoride treatment. In fact, during the course of our studies, Debart and co-workers[20] described

conditions to successfully release several different 2'-acetal ester protected RNA oligonucleotides from Q-linker CPG, namely 3:1 TEA/ 48% aqueous HF (v/v). Since introducing too many ALE groups on an RNA may render it insoluble or cause self-aggregation, we also devised a synthetic strategy that allowed us to synthesize partially esterified RNA strands. This required the use of *N*-FMOC-2'-*O*-TBDMS synthons which turned out to be fully compatible (and orthogonal) with the *N*-FMOC-2'-*O*-ALE ribonucleoside monomers. The FMOC groups would be removed with the same 3:2 MeCN/TEA (v/v) treatment, whereas the TBDMS groups would be removed under 3:1 TEA/48% HF (v/v) conditions, keeping the 2'-*O*-ALE groups intact. The fluoride treatment would also cleave the desired pro-RNA strand from the Q-CPG solid support (**Scheme 5.1**).

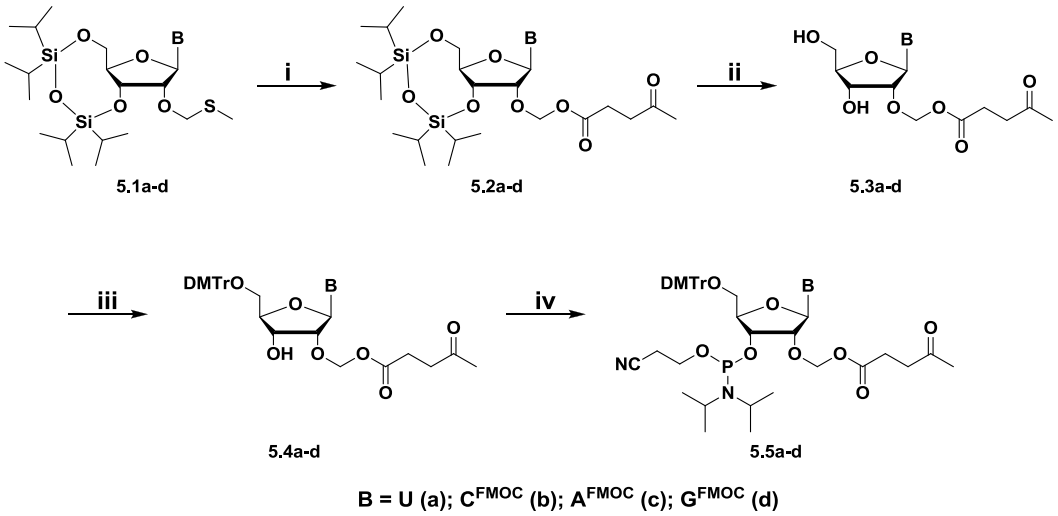


Scheme 5.1. The 2'-*O*-ALE pro-siRNA strategy using *N*-FMOC-2'-ALE and *N*-FMOC-2'-TBDMS chemistry and Pon's Q-linker CPG support.

5.3 Synthesis of *N*-FMOC-5'-*O*-DMTr-2'-*O*-ALE-3'-Phosphoramidite Monomers

The synthesis of 2'-*O*-ALE-3'-phosphoramidite monomers is summarized in **Scheme 5.2**. The syntheses of compounds **5.1a-d** was described in **Chapter 3.2**. Treatment of **5.2a-d** with sulfuryl chloride (2 hr, r.t.) gave the 2'-*O*-CH_2Cl intermediate, which were reacted *in situ* with cesium carbonate and levulinic acid to provide **5.2a-d** in 60-92% yields. Treatment of **5.2a-d** with HF-pyridine

afforded **5.3a-d** in 65-86% yields. Other reagents such as TBAF or NEt_3-3HF could not reliably be used as they caused some cleavage of the N-FMOC protecting groups. To obtain monomers suitable for standard RNA synthesis, these nucleosides were treated with DMTrCl/pyridine to afford **5.4a-d** (80-86%), which were then 3'-phosphitylated under standard conditions to give the desired phosphoramidite monomers **5.5a-d** (75-90%).

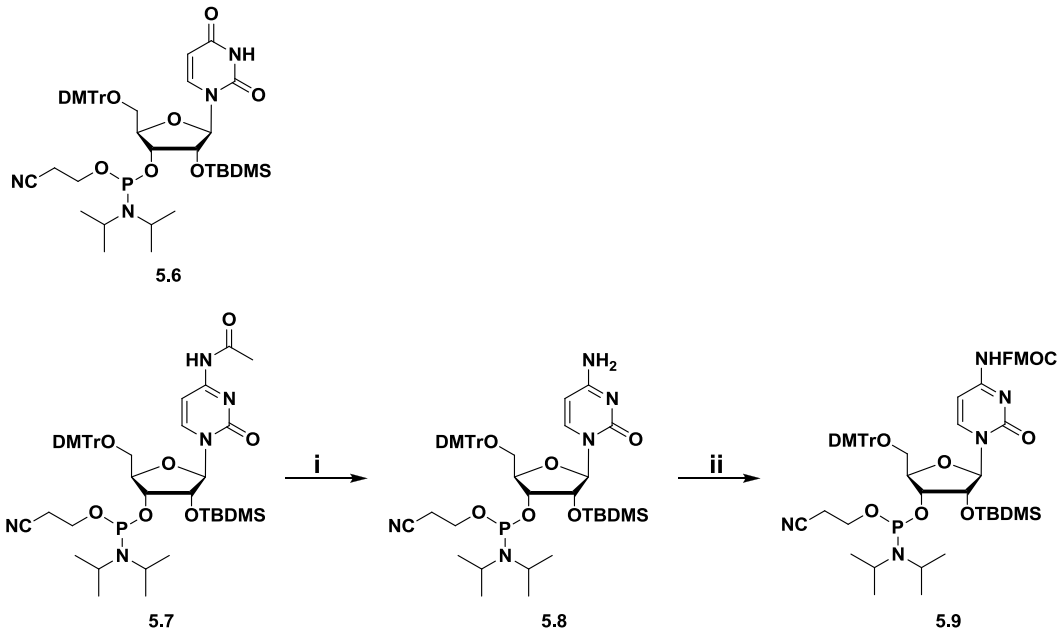


Scheme 5.2. Synthesis of N-FMOC-2'-*O*-ALE monomers. Reagents and conditions: (i) 1 M SO_2Cl_2, CH_2Cl_2, Cs_2CO_3, levulinic acid, 4-chloro-styrene was needed for **5.2d**; ii) HF-pyr, THF; (ii) DMTrCl, pyr; (iv) CEtOP(Cl)N*i*Pr_2, *i*Pr_2NEt, CH_2Cl_2.

5.4 Synthesis of *N*-FMOC-5'-*O*-DMTr-2'-*O*-TBDMS-3'-Phosphoramidite Pyrimidine Monomers

The synthesis of the pyrimidine *N*-FMOC-2'-*O*-TBDMS-3'-phosphoramidite monomers is summarized in **Scheme 5.3**. Compound **5.6** was obtained from a commercial source. Compound **5.9** was prepared in one-pot starting from commercially available **5.7** which was first treated with 1:1:1 NH_4OH/$MeNH_2$/THF (v/v/v) to generate *N*-unprotected cytosine nucleoside **5.8**.[21] This material was then treated with FMOCCl in the presence of *N*,*N*-diisopropylethylamine in THF to give **5.9** in 90% yield. Though this reaction sequence could be applied to the purine series as well, *N*-FMOC carbamation

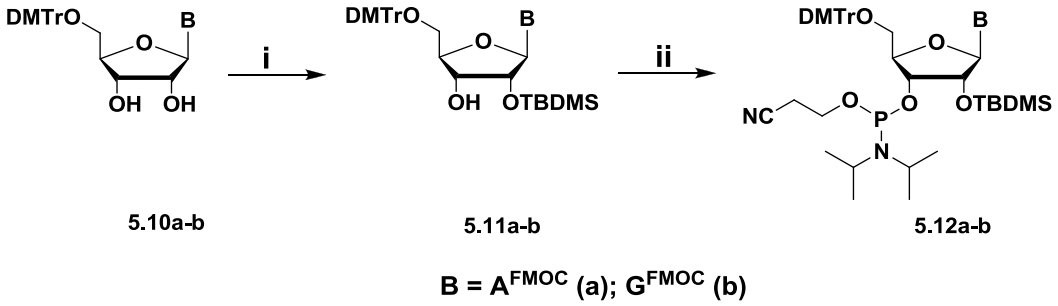
following the *N*-deprotection step could not be reliably reproduced, and a more conventional route was employed instead (**Scheme 5.4**).



Scheme 5.3. Synthesis of N^4-FMOC-2'-*O*-TBDMS cytidine monomer. Reagents and conditions: (i) 1:1:1 $NH_4OH/MeNH_2$/THF; ii) FMOCCl iPr_2NEt, THF.

5.5 Synthesis of *N*-FMOC-5'-*O*-DMTr-2'-*O*-TBDMS-3'-Phosphoramidite Purine Monomers

Starting from *N*-FMOC-5'-*O*-DMTr protected nucleosides **5.10a-b**,[22] compounds **5.11a-b** were formed by reaction with TBDMSCl and $AgNO_3$ in 9:1 THF/pyr (v/v) in 32-40% yield.[23] As expected, this step also generated some 3'-*O*-TBDMS monomer along with some bis-silylated material. Separation of these by-products from the 2'-*O*-TBDMS isomer was readily achieved via silica-gel column chromatography. It should be noted that the classical nucleoside silylating conditions, namely TBDMSCl/imidazole in DMF cannot be used as FMOC cleavage occurred under these conditions. Compounds **5.11a-b** were then subjected to standard phosphitylation conditions to yield the final product **5.12a-b** in 80-85% yield.



Scheme 5.4. Synthesis of *N*-FMOC-2'-*O*-ALE purine monomers. (i) TBDMSCl, $AgNO_3$, THF/pyr; (ii) CEtOP(Cl)N*i*Pr_2, *i*Pr_2NEt, CH_2Cl_2.

5.6 Solid Phase Synthesis: Single Insert Studies

As an initial test to demonstrate the suitability of 2'-*O*-ALE (**5.5a-d**) and 2'-*O*-TBDMS (**5.6, 5.9, 5.12 a-b**) monomers for prodrug RNA synthesis, we first prepared a series of 15-nt long DNA-RNA chimeric strands, namely dT_9-2'-*O*-ALE-rN-dT_5 (2'-*O*-ALE-rN = Ura (**5.13**), Cyt (**5.14**), and Ade (**5.15**)) from **5.5a-c** as well as the corresponding ("naked") control strands dT_9-rN-dT_5 (rN = Ura (**5.16**), Cyt (**5.17**), and Ade (**5.18**) from **5.6, 5.9, 5.12a**), on a Q-linker polystyrene solid support (1 µmol scale).[19] Oligonucleotides containing guanosine inserts were not synthesized to conserve material. We chose polystyrene as the solid support as opposed to the classical CPG because polystyrene, unlike CPG, is completely stable to the final fluoride treatment that releases the oligonucleotides into solution. The Q-linker polystyrene support was prepared in house and appended to a 5'-*O*-DMTr-dT (loading: 35 µmol/g).[19] The phosphoramidites **5.5a-c** and **5.6, 5.9, 5.12a** (0.1 M in MeCN) were activated with ETT (0.25 M in MeCN) and allowed to couple to the support for 15 min off the DNA/RNA synthesizer machine to conserve the phosphoramidite material. Standard capping (Ac_2O), oxidation (I_2/H_2O/pyr/THF) and detritylation (3% TCA in DCE) steps followed the coupling step. After the completion of each synthesis, the Q-linker polystyrene supports were treated with 2:3 NEt_3/MeCN (v/v) for 16 hr to effect removal of the β-cyanoethyl phosphate and *N*-FMOC protecting groups. These materials were then treated with 3:1 NEt_3/48% aqueous HF (v/v) and analyzed by C-18 reverse phase HPLC using a gradient of 0 → 15% MeCN in 0.1 M

triethylammonium acetate (TEAA) (pH 7) (**Figure 5.3**) and MALDI-TOF mass spectrometry (**Figure 5.4**, **Table 5.1**). The chromatographic run of the crude oligonucleotides (**5.13-15**, **Figure 5.3 A-B**) yielded two major peaks. Material corresponding to the minor peak in the profile had the same mass and co-eluted with authentic fully deprotected (naked) oligonucleotide. The major peak (labeled *, **Figure 5.3**) eluted 1-2 min later suggesting that this compound

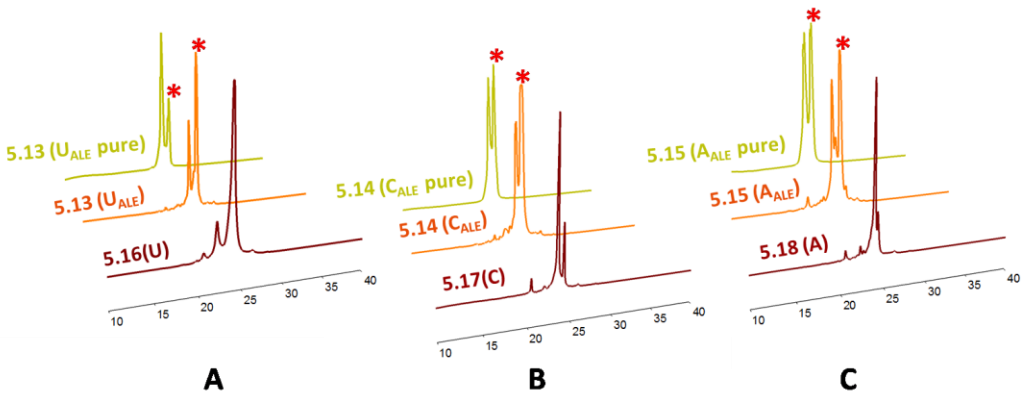


Figure 5.3. C-18 reverse phase HPLC analysis of 5'-dT$_9$-rN-dT$_5$-3' with a gradient of 0 → 15% MeCN in 0.1 M TEAA (pH 7). **A**. rN = 2'-*O*-ALE rU; **B.** rN = 2'-*O*-ALE rC; **C.** rN = 2'-*O*-ALE rA. The asterisks indicate the peaks corresponding to the 2'-*O*-ALE containing oligonucleotide.

was the desired 2'-*O*-ALE modified strand (**5.16-5.18**, **Figure 5.3**). When this compound was isolated, lyophilized, redissolved in water and re-injected into the HPLC column (**Figure 5.3 A-B**, yellow traces), the material eluted as a mixture of the 2'-*O*-ALE containing oligonucleotide (**5.13-5.15**) and the naked oligonucleotide (**5.16-5.18**) (**Figure 5.4**, **Table 5.1**), suggesting that the single ALE group was cleaving off spontaneously in solution or during HPLC purification.

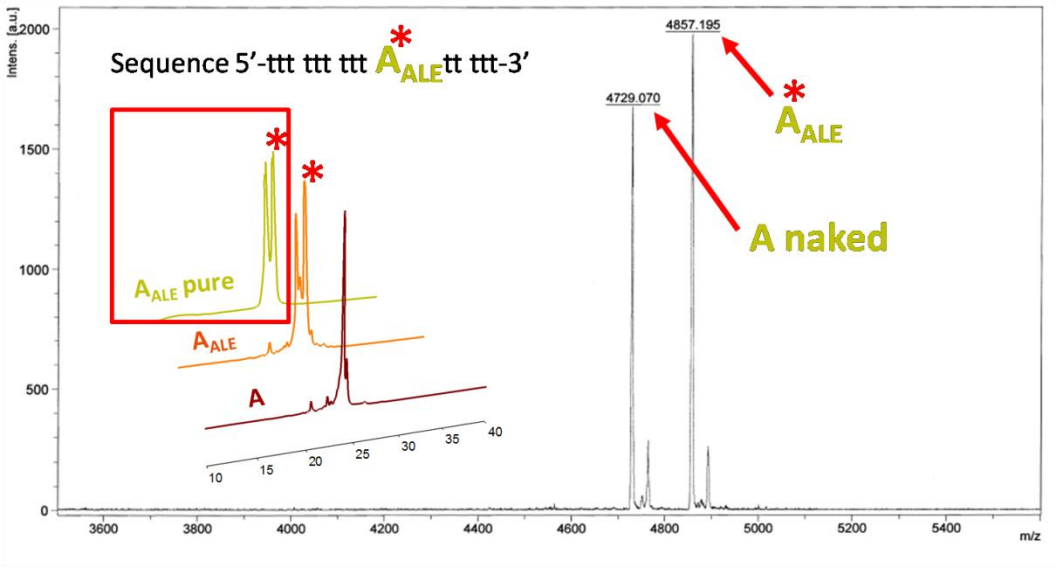


Figure 5.4. Example of a MALDI-TOF spectrum for purified 5'-dT_9-2'-*O*-ALE-rA-dT_5-3' oligonucleotide **5.15**.

No.	Oligonucleotide	Calc. MW (g/mol) + spermine	Found MW (g/mol) + spermine
5.13	5'-ttt ttt ttt **U**$^{2'\text{-ALE}}$ tt ttt-3'	4834.27	4706.19, 4834.42*
5.14	5'-ttt ttt ttt **C**$^{2'\text{-ALE}}$ tt ttt-3'	4832.34	4704.35, 4832.68*
5.15	5'-ttt ttt ttt **A**$^{2'\text{-ALE}}$ tt ttt-3'	4857.37	4729.07, 4857.20*
5.16	5'-ttt ttt ttt **U**tt ttt-3'	4706.27	4706.20
5.17	5'-ttt ttt ttt **C**tt ttt-3'	4704.28	n.d.
5.18	5'-ttt ttt ttt **A**tt ttt-3'	4729.31	4729.47

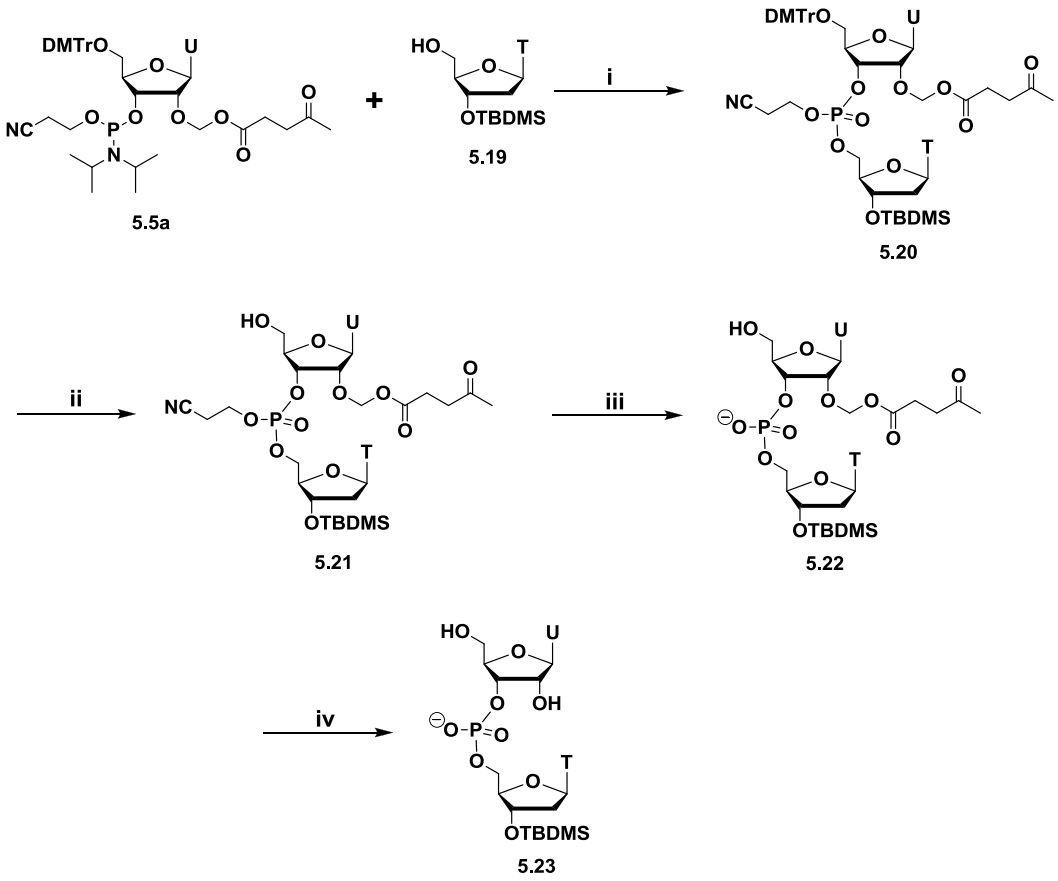
Table 5.1. MALDI-TOF analysis of dT_9-2'-*O*-ALE-rN-dT_5 (2'-*O*-ALE-rN = Ura, Cyt, and Ade) and naked dT_9-rN-dT_5 (rN = Ura, Cyt, and Ade)**.** The MALDI-TOF matrix was 6-aza-thiothymine with spermine and fucose as co-matrix.[24]

5.7 Synthesis of a 2'-*O*-ALE Containing Dimer

To investigate the decomposition of the 2'-*O*-ALE group further, we synthesized and examined a simple 2'-*O*-ALE containing dimer system. The fate

of such a compound could then be monitored via ^{31}P NMR and mass spectrometry upon exposure to the reagents and conditions used during solid phase synthesis, deprotection and purification steps of the above-mentioned 2'-*O*-ALE oligonucleotides.

The synthesis of the 2'-*O*-ALE containing dimer is summarized in **Scheme 5.5**. Compound **5.5a** was reacted with **5.19** in the presence of DCI in THF. Upon condensation, the phosphite triester intermediate was oxidized *in situ* (I_2/H_2O/pyr/THF) forming the dimer **5.20** in quantitative yield. This material was then treated with 3% TFA in CH_2Cl_2 to form **5.21** quantitatively.



Scheme 5.5. Synthesis of a 2'-*O*-ALE containing dimer. Reagents and conditions. i) 1) DCI, THF, 2) I_2/H_2O/pyr/THF; ii) 3% TFA in CH_2Cl_2; iii) 2:3 NEt_3/MeCN. iv) 0.5 M hydrazine hydrate in 3:2 pyr/HOAc (v/v).

5.8 Decyanoethylation of 2'-*O*-ALE Dimer 5.21

To test if the conditions necessary to remove the β-cyanoethyl phosphate protecting group were causing 2'-*O*-ALE cleavage, namely 2:3 NEt_3/MeCN (v/v), r.t., we monitored this reaction on dimer **5.21** by ^{31}P NMR. As expected, the diastereomeric peaks of triester **5.21** were converted into one new peak corresponding to phosphate diester **5.22** [**Figure 5.5**; ESI-TOF MS: calc. for $C_{31}H_{46}N_4O_{16}PSi$ 790.24 (-H) found 790.22]. To our surprise, the time needed for the reaction to go to completion was over 24 hr. When this reaction was conducted at 50 °C, removal of the β-cyanoethyl phosphate protecting group was complete within 1 hour with no trace of ALE cleavage.

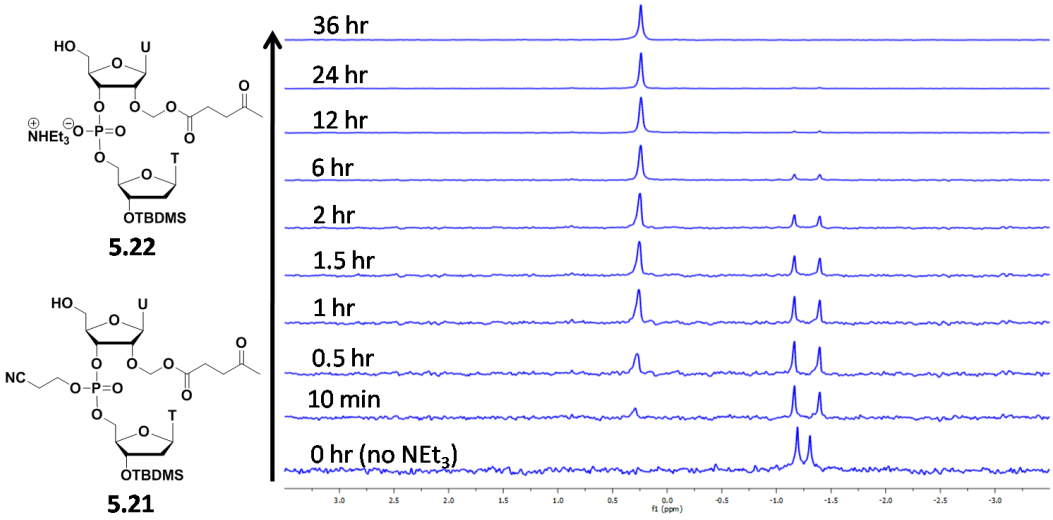


Figure 5.5. ^{31}P NMR spectra of the decyanoethylation of **5.21** to **5.22** in 2:3 NEt_3/CD_3CN (v/v), r.t. over time.

Dimer **5.22** was then treated with 0.5 M hydrazine hydrate in 3:2 pyridine/acetic acid to remove the 2'-*O*-ALE group and the reaction was again monitored by ^{31}P NMR (**Figure 5.6**). The key observation is that the peak corresponding to **5.22** converts into a single upfield peak, suggesting that 2'-3'-isomerization or internucleotide strand cleavage does not occur during 2'-*O*-ALE cleavage.

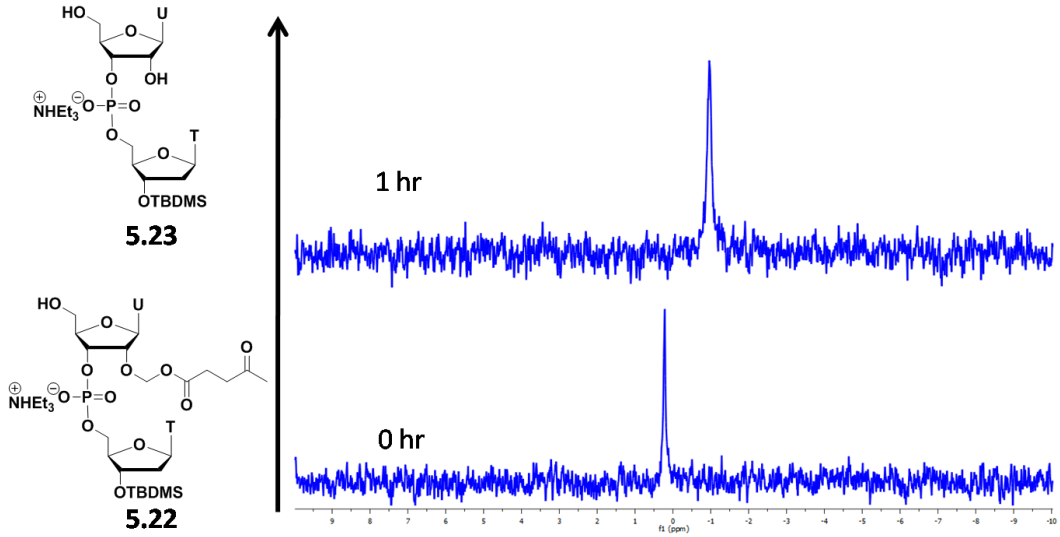


Figure 5.6. ^{31}P NMR spectra of delevulination of **5.22** to **5.23** in 0.5 M hydrazine hydrate in 3:2 pyr/HOAc (v/v), r.t. over 1 hr.

5.9 Stability of 2'-*O*-ALE in Aqueous Media

The last step in the release of a 2'-*O*-ALE oligonucleotide from the solid support is cleavage from the Q-linker using 3:1 TEA/48% aqueous HF (v/v), conditions that according to Debart should leave the 2'-*O*-acetal esters intact.[20] After this treatment, the oligonucleotide is usually manipulated in water for several hours, before purification by HPLC using aqueous 0.1 M TEAA buffer. To test if either water or the 0.1 M TEAA buffer were causing ALE cleavage, we subjected **5.22** to these conditions and monitored its stability by ^{31}P NMR over time. **Figure 5.7** shows the ^{31}P NMR spectra of **5.22** in D_2O over a 7 day period. After 1 day there is 18% ALE cleavage, and there appears to be no change in that ratio after 7 days. This material was then evaporated to dryness, redissolved in D_2O and analyzed by ^{31}P NMR. To our initial surprise, the extent of ALE cleavage had more than doubled.

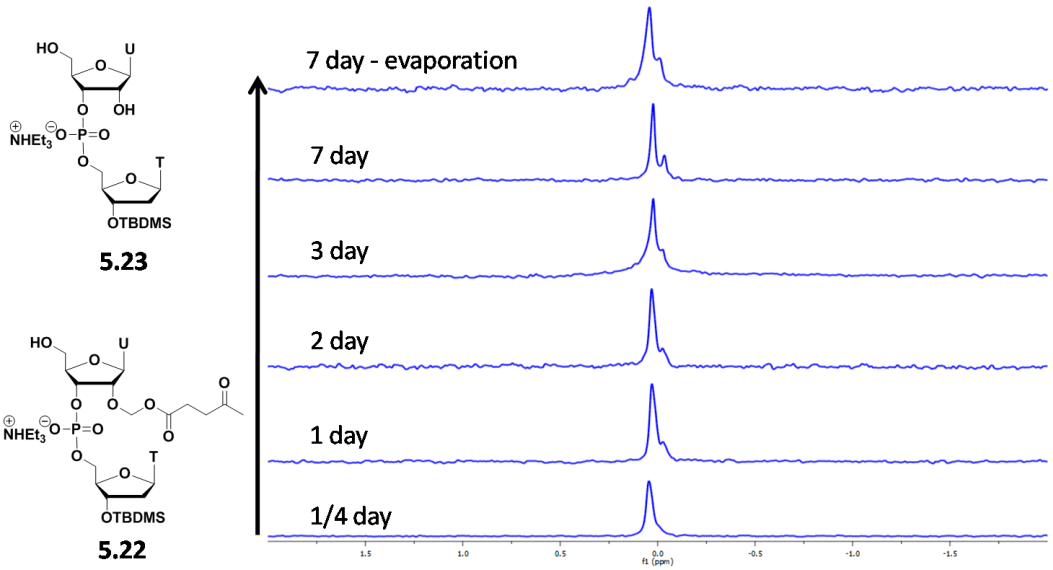


Figure 5.7. ^{31}P NMR spectra of **5.22** stability in D_2O, r.t. over time.

Figure 5.8 shows the stability of **5.22** in 0.1 M TEAA in D_2O, pD 7.2 over 7 days. After 1 day the extent of ALE cleavage is 22%, and 30% after 7 days. This material was then evaporated to dryness, redissolved in D_2O and analyzed by ^{31}P NMR resulting in 44% ALE cleavage in total.

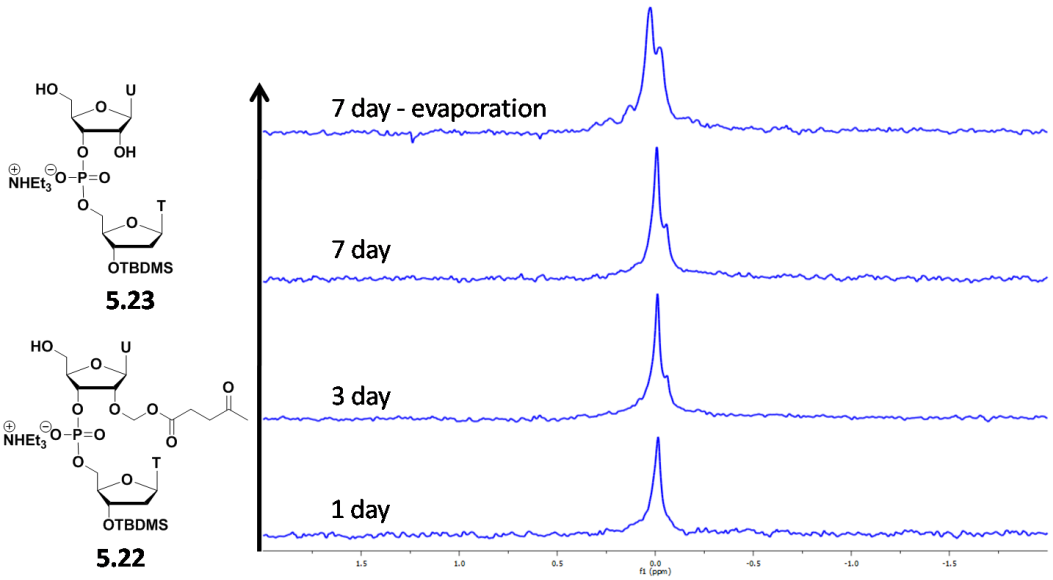


Figure 5.8. ^{31}P NMR spectra of **5.22** exposed to 0.1 M TEAA in D_2O, pD 7.2, r.t. over a 1 week period.

These results indicate that the ALE group is cleaved in D_2O, and even more rapidly under the buffer conditions used during HPLC purification(0.1M TEAA, pH 7). Since there was an increase in the rate of ALE removal upon evaporation and redissolution in D_2O, these results may also suggest that the counter ion, triethylammonium, may also play a role in the rate of ALE cleavage. With this in mind, we decided to modify our synthesis strategy such that the 2'-*O*-ALE oligonucleotide would not be exposed to water prior to HPLC analysis. This also meant we had to cleave the oligonucleotide from the support under anhydrous conditions (note the Q-linker CPG requires aqueous HF for releasing the oligonucleotide).

5.10 Synthesis of a Novel Light-Labile Linker for Oligonucleotide Synthesis

The use of light labile linkers for oligonucleotide synthesis was developed by Greenberg and co-workers[25] (**Figure 5.9**). To get >85% oligonucleotide release, the solid support is incubated in a transilluminator where the irradiation source emits maximally in the 350-365 nm range for 2-3 hours in a mixture of 9:1 MeCN/H_2O. Unfortunately, these conditions were reported to cause the formation of 3-13% thymine-thymine photodimers.

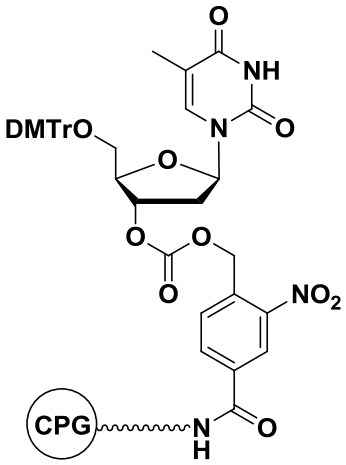
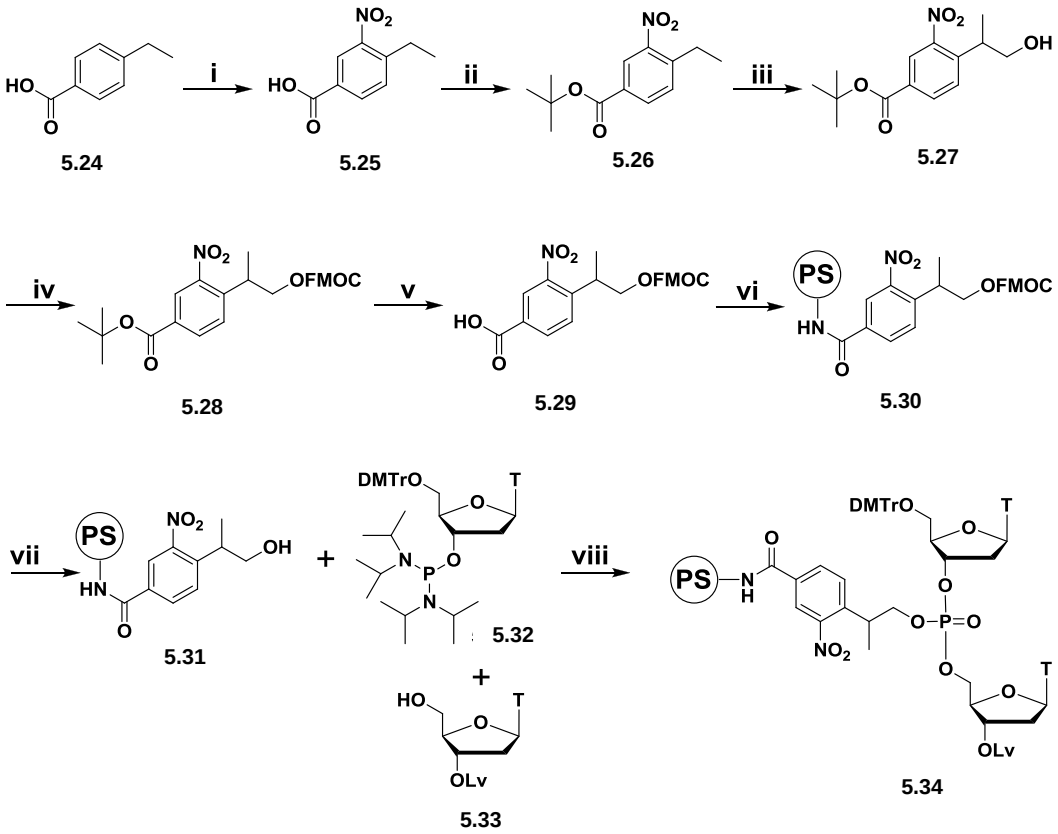


Figure 5.9. Light labile linker developed by Greenberg and co-workers.[25]

We liked the idea of photolysis for our system since, generally, photolabile linkers are cleaved under very mild conditions. However, we wanted to speed up the photolysis reaction to avoid photodimerization, and keep the conditions anhydrous to avoid ALE cleavage. With this in mind, we synthesized a novel 'NPPOC'-like photolabile linker, inspired from the microarray work described in **Chapter 4**. We already knew that the 5'-*O*-NPPOC group could be cleaved quantitatively from a nucleoside within 5 min (350-365 nm irradiation) in anhydrous acetonitrile without causing any detectable photodimerization.[26,27] In addition, the presence of small amounts of a hindered base such as *N*,*N*-diisopropylethylamine (DIPEA) speeds up the photolysis reaction, and in some cases is required for cleavage.[28]

The synthesis of our novel light labile linker is summarized in **Scheme 5.6**. Compound **5.24** is reacted with fuming nitric acid at -10 °C generating **5.25** in 95% yield.[29] This material is then protected as the *t*-butyl ester **5.26** by reaction with DCC and *t*-butanol in quantitative yield, followed by treatment with paraformaldehyde in the presence of potassium *t*-butoxide to form **5.27** in 90% yield.[30] Compound **5.27** was then reacted with FMOCCl in pyridine to generate **5.28** in 95% yield followed by treatment with 80% TFA in dichloromethane to liberate the free acid **5.29**. This compound is then ready for coupling to the polystyrene solid support. To achieve this, **5.29** is reacted with aminomethyl polystyrene (stratosphere for DNA synthesis, 1000 Å) in the presence of HATU and DMAP in pyridine followed by capping of unreacted amines with CAP A and CAP B solutions (Ac_2O/*N*-methyl-imidazole/THF) to give **5.30**. This support is then treated with 10% 4-methylpiperidine in DMF to give **5.31** with a loading of 250 μmol/g. To obtain solid support **5.34**, compounds **5.32** and **5.33** were allowed to react with 0.5 M DCI in MeCN followed by the addition of **5.31**. The phosphite triester intermediate is then oxidized with I_2/H_2O/pyr/THF and the unreacted free hydroxyl groups are capped with Ac_2O/N-methyl-imidazole/THF. Polystyrene **5.34** was obtained with a loading of 55 μmol TpT per gram of support.



Scheme 5.6. Synthesis of a light labile linker. Reagents and conditions. i) HNO_3, -10 ºC; ii) *t*-BuOH, DCC, THF; iii) paraformaldehyde, *t*-BuOK, DMF; iv) FMOCCl, pyr; v) 80% TFA in CH_2Cl_2; vi) 1) aminomethyl polystyrene (PL-AMS resin), HATU, DMAP, pyr, 2) Ac_2O/N-Me-imidazole/THF; vii) 4-methyl piperidine, DMF; viii) 1) 0.25 M DCI, 2) I_2/H_2O/pyr/THF 3) CAP A/CAP B.

5.11 Solid Phase Synthesis of a Single 2'-*O*-ALE Insert on Polystyrene Appended Through Light Labile Linker 5.34

As an initial test of this new strategy, a 1 μmol solid phase synthesis of dT_9-rN-dT_5 **5.35** and **5.36** (where rN = **5.5a** and **5.6** respectively) was performed using the same conditions as described in **Section 5.6**, except that the light labile solid support **5.34** was used. Upon completion of the synthesis cycle, the solid supports were treated with anhydrous 2:3 TEA/MeCN for 16 hr, r.t. , warmed to 50 °C for 1 hr, washed with anhydrous MeCN, and dried under vacuum. Support **5.37** was further treated with 1.5:0.75:1 NEt_3-3HF/NMP/NEt_3 for 3 hours, 50 °C, washed with MeCN and dried under vacuum yielding a naked oligonucleotide still bound to the solid support. Oligonucleotides bound to the solid support were

placed in a quartz test tube where 1% DIPEA in MeCN was added. These materials were then subjected to photolysis in a transilluminator with an irradiation source emitted maximally in the 350-365 nm range for 20 min. Both materials were then purified by C-18 reverse phase HPLC using a gradient of 0 → 15% MeCN in 0.1 M TEAA (pH 7) and immediately treated with Na^+ ion-exchange resin to change the triethylammonium counter ion to the sodium counter ion. This material was then reinjected into the C-18 reverse phase HPLC and the results are shown in **Figure 5.10**.

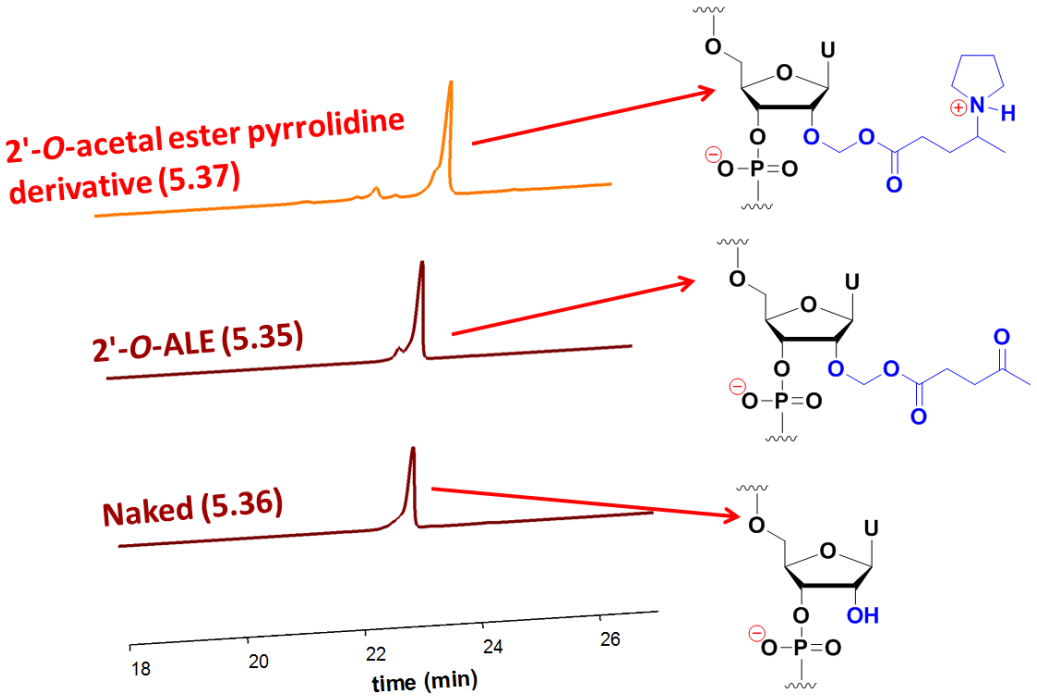


Figure 5.10. C-18 reverse phase HPLC analysis of 5'-dT_9-rN-dT_5-3' **5.36** (front), **5.35** (middle), and **5.37** (back) where rN = rU, rU-2'-*O*-ALE , and 2'-*O*-acetal ester pyrrolidine derivative respectively with a gradient of 0 → 15% MeCN in 0.1 M TEAA .

As evident the 2'-*O*-ALE oligonucleotide **5.35** was obtained in significantly greater purity than **5.13** synthesized using the Q-linker and without ion-exchange treatment (**Figure 5.3**). Under these conditions, partial (~10%) hydrolysis of ALE hydrolysis occurred, and is likely unavoidable given the number of manipulations of the oligonucleotide during purification, analysis, and eventually hybridization and transfections required for gene silencing

experiments. Since acetal ester hydrolysis was not observed by Debart and co-workers[18,20] in their system (ex. 2'-*O*-PivOM, 2'-*O*-MeOM, 2'-*O*-EtOM, 2'-*O*-butylOM, 2'-*O*-pentylOM) it is possible that the ketone functionality of the ALE moiety is playing a role here. With that in mind, we performed "on column" reductive amination of the ketone by treating the solid support bound 2'-*O*-ALE oligonucleotide **5.37** with a solution of pyrrolidine, acetic acid and sodium triacetoxyborohydride in dichloroethane. We hypothesized that 2'-*O*-acetal ester pyrrolidine moieties would impart pro-siRNAs with better hydrolytic stability and cellular uptake by virtue of its positive charge at physiological pH. The oligomer, **5.37** was deprotected and purified as described above and analyzed and characterized by HPLC (**Figure 5.10**) and ESI-TOF MS (**Figure 5.11**, calc. 4686.07, found 4686.30). The pyrrolidine containing oligo had a longer retention time than both the naked and 2'-*O*-ALE containing oligonucleotide in HPLC, but the chromatogram also appeared to contain a small amount of hydrolyzed product which was also detected in the ESI-TOF spectrum. Overall, these results were encouraging and we proceeded to synthesize a fully modified pro-siRNA of mixed base composition.

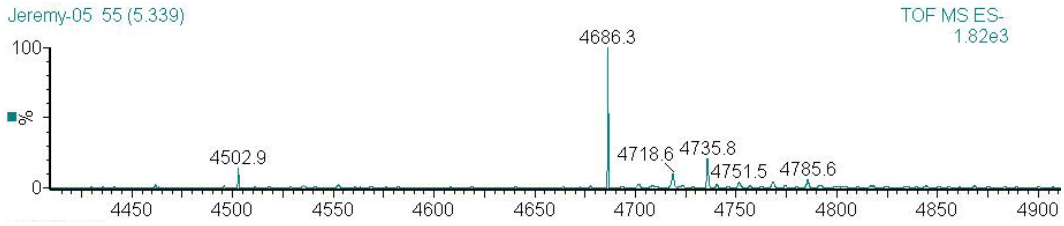


Figure 5.11. ESI-TOF of 5'-ttt ttt ttt **rU**(2'-'acetal ester pyrrolidine') tt ttt-3' **5.37**.

5.12 Synthesis of a Mixed-Base 2'-*O*-ALE siRNA and 2'-*O*-'Acetal Ester Pyrrolidine' siRNA

With phosphoramidites in hand, a 2'-*O*-ALE modified siRNA sense strand **5.38** was synthesized on our novel light labile polystyrene support **5.34** (loading of 55 µmol/g) on a 1 µmol scale. Phosphoramidite monomers **5.5a-d** (0.1 M in MeCN) were activated with DCI (0.25 M in MeCN) and allowed to couple to the support for 10 min. Standard capping (Ac_2O), oxidation (I_2/H_2O/pyr/THF) and

detritylation (3% TCA in dichloroethane) steps followed the coupling step. After the completion of the synthesis, some of the material was saved for full deprotection. The rest of the material was treated with 2:3 NEt_3/MeCN for 16 hours, r.t., then 50 °C for 1 hour. It was then washed with anhydrous MeCN and dried under vacuum. At this point some of the 2'-*O*-ALE material was exposed to the reductive amination conditions described in section **5.12** to form fully modified 2'-*O*-'acetal ester pyrrolidine' siRNA **5.39**. Both materials were then placed in a solution of 1% DIPEA in anhydrous MeCN and subjected to photolysis in a transilluminator where the irradiation source emitted maximally in the 350-365 nm range for 20 min. The materials were then analyzed by C-18 reverse phase HPLC using a gradient of 0 → 40% MeCN in 0.1 M TEAA (pH 7) (**Figure 5.12**) and compared to naked **5.40** control synthesized by standard TBDMS chemistry.

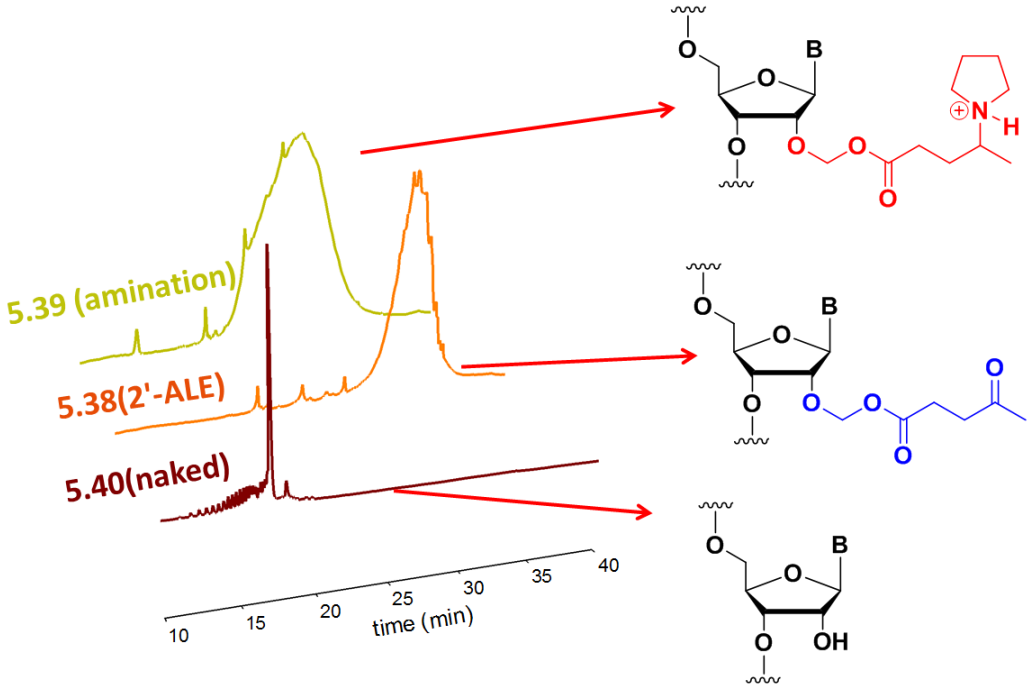


Figure 5.12. C-18 reverse phase HPLC analysis of 5'-UUAAUUAAAGACUUCAAGUCtt-3' synthesized by standard TBDMS chemistry (**5.40**, red), 2'-*O*-ALE containing siRNA (**5.38**, orange), and 2'-*O*-'aceltal ester pyrrolidine' siRNA (**5.39**, yellow) using a gradient of 0 → 40% MeCN in 0.1 M TEAA (pH 7).

As evident in the HPLC traces shown in **Figure 5.12**, both the 2'-*O*-ALE (**5.38**) and 2'-*O*-'acetal ester pyrrolidine' (**5.39**) crude oligos are more retained than the naked control (**5.40**) and appeared as a broad envelope of peaks. This is indicative of partial acetal ester hydrolysis which could occur at any one (or more) of 19 positions resulting in a mixture of inseparable compounds. The broader envelope observed for the pyrrolidine containing RNA may be due to incomplete reductive amination reaction. A mass spectrum could not be obtained for this oligonucleotide, but the ESI-TOF MS obtained for the crude 2'-*O*-ALE RNA oligo appeared as an envelope of peaks as expected. As it was not possible to separate oligos from these mixtures, we hybridized the crude oligomers to their complementary RNA strand and measured the relative thermal stability of the resulting pro-siRNA duplexes by T_m experiments. The T_m value of the control RNA duplex is 63.6 °C (**5.40**), whereas those of the 2'-*O*-ALE (**5.38**) and 2'-*O*-'acetal ester pyrrolidine' (**5.39**) containing duplexes were only 38.8 °C and 46.6 °C, respectively (Figure, **5.13** Table **5.2**).

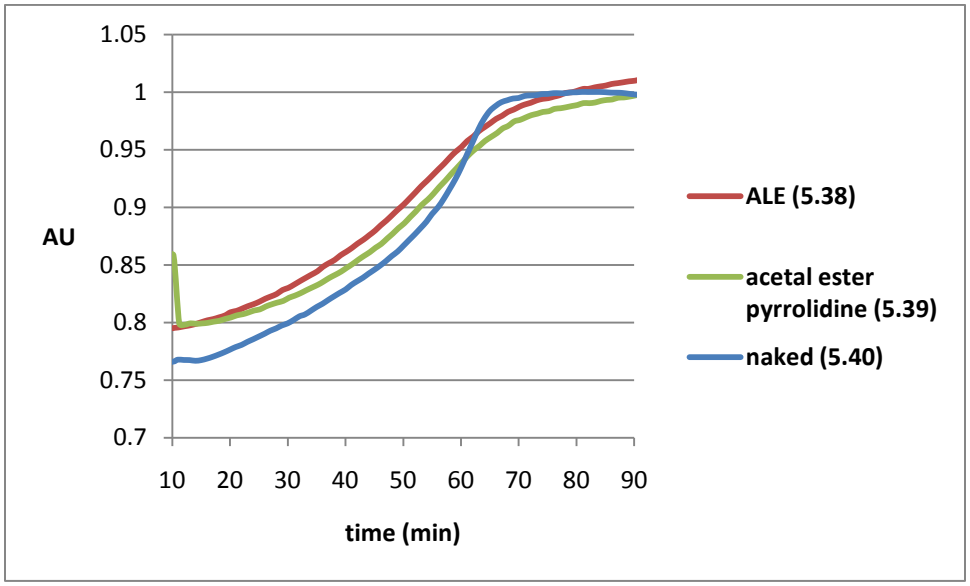


Figure 5.13. Thermal denaturation of 5'-UUAAUUAAAGACUUCAAGUCtt-3' containing 2'-*O*-ALE (**5.38**), 2'-*O*-'acetal ester pyrrolidine' (**5.39**) and 2'-OH groups (**5.40**). Complementary RNA strand was prepared from TBDMS chemistry. Oligonucleotides were dissolved to give a concentration of 1 μM hybrid in 140 mM KCl, 5 mM $MgCl_2$, and 3 mM Na_2HPO_4 buffer (pH 7.2).

No.	Oligonucleotide	T_m (°C)
5.38	2'-*O*-ALE	38.8 (56.2*)
5.39	2'-*O*-acetal ester pyrrolidine	46.6 (57.1*)
5.40	2'-OH	63.6

Table 5.2. Thermal denaturation of 5'-UUAAUUAAAGACUUCAAGUCtt-3' containing 2'-*O*-ALE (**5.38**), 2'-*O*-'acetal ester pyrrolidine' (**5.39**) and 2'-OH groups (**5.40**). Complementary RNA strand was prepared from TBDMS chemistry. Oligonucleotides were dissolved to give a concentration of 1 μM hybrid in 140 mM KCl, 5 mM $MgCl_2$, and 3 mM Na_2HPO_4 buffer (pH 7.2).
*indicates Tm for 2'-F RNA complementary strand.

Unfortunately these values indicate that a significant population of single strands would be present at the temperature in which gene knockdown cell based assays are carried out (37 °C). To counteract the destabilization caused by the 2'-*O*-acetal ester groups, we hybridized the modified strands with a 2'-deoxy-2'-fluororibonucleic acid (2'F-RNA) complementary strand. The 2'F-RNA modification is known to increase the thermal stability of siRNA duplexes (ca. +2-3 ^{o}C per rF-N modification) and be compatible with the RNAi machinery.[5] Indeed, stability of our 2'-*O*-acetal RNA/2'F-RNA duplexes were significantly higher with T_m values of 56.2 °C (2'-*O*-ALE) and 57.1 °C (2'-*O*-acetal ester pyrrolidine), respectively (**Figure 5.14, Table 5.2**). The higher T_m value of the latter relative to the 2'-*O*-ALE modified duplex is consistent with the compensatory stabilization that would be provided by positively charged (pyrrolidine) pending groups at C2'.[31]

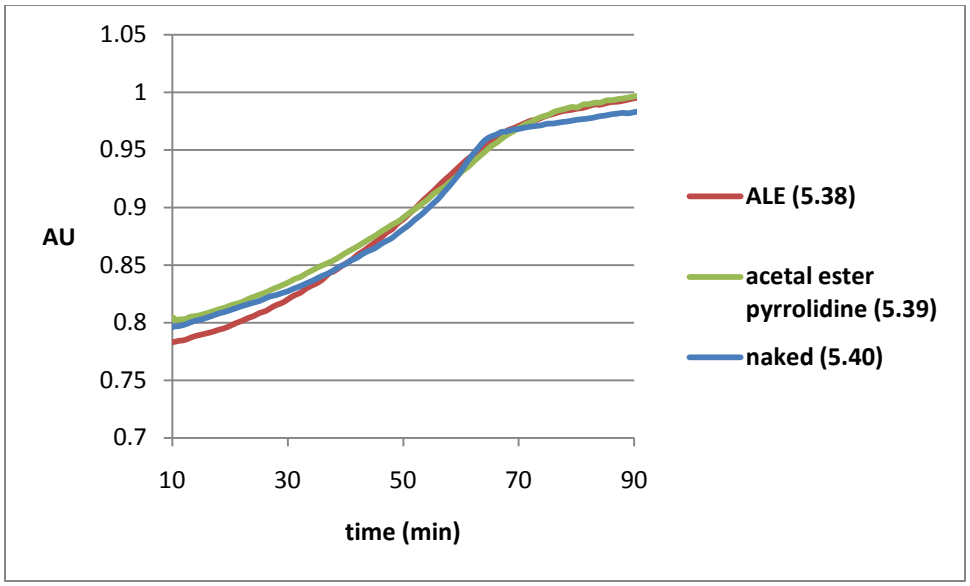


Figure 5.14. Thermal denaturation of 5'-UUAAUUAAAGACUUCAAGUCtt-3' containing 2'-*O*-ALE (**5.40**), 2'-*O*-'acetal ester pyrrolidine' (**5.41**) and 2'-OH groups (**5.42**). Complementary strand is 2'-F RNA-dTT . Oligonucleotides were dissolved to give a concentration of 1 μM hybrid in 140 mM KCl, 5 mM $MgCl_2$, and 3 mM Na_2HPO_4 buffer (pH 7.2).

5.13 RNAi Luciferase Assay

Next, we evaluated the gene silencing potency of our modified pro-siRNA (2'-*O*-acetal esterified RNA/2'F-RNA) in cell cultures. Specifically, we used a previously described HeLa X1/5 cell line which stably expresses luciferase, to assess the ability of the pro-siRNAs to regulate the intracellular levels of the luciferase mRNA target.[32-34] The corresponding RNA/2'F-RNA duplex and a scrambled siRNA was also tested for comparison. Duplexes were delivered to cells with and without lipofectamine. In the presence of delivery agent, we were hoping to see at least the same potency for the acetal esterified RNA/2'F-RNA duplexes as for the RNA/2'F-RNA control as presumably the 2'-acetyl ester groups would be cleaved following cell uptake. In the absence of lipofectamine we were expecting to see a much greater potency for 2'-acetal esterified siRNAs as their greater lipophilic character would hopefully enhance their cellular uptake. As shown in **Figure 5.15**, the ALE (**5.38**) and pyrrolidine containing (**5.39**) siRNA duplexes had similar gene silencing activity as the control (**5.40**) when

pre-treated with lipofectamine confirming their ability to enter the RNAi pathway. This data suggests that the 2'-acetal esters are hydrolyzed *in vitro* as, generally, heavily modified sense strands do not show RNAi activity.[35] The ALE and acetal ester pyrrolidine siRNA without lipofectamine treatment showed no detectable difference in RNAi activity relative to the control at 0.010 and 0.050 mmol, but a higher strand concentration and/or other cell lines were not tried.

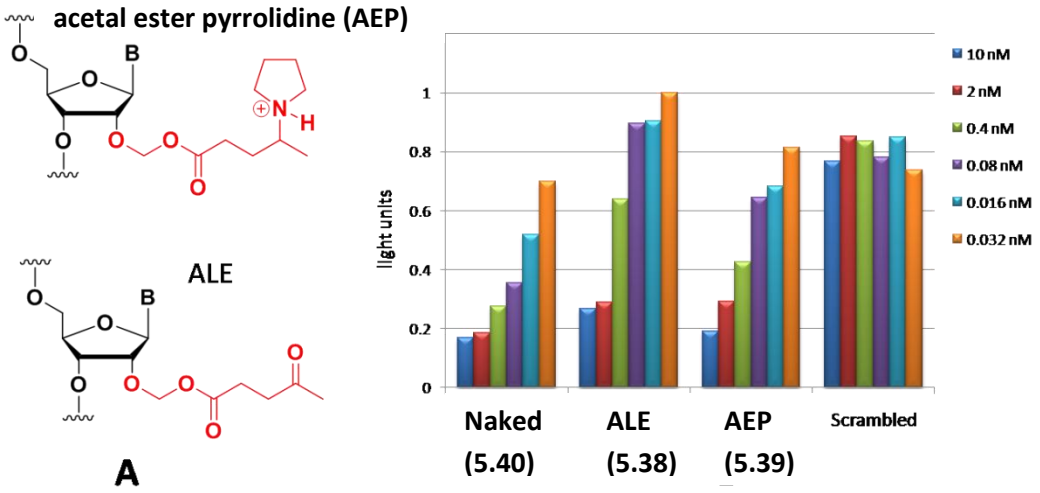


Figure 5.15. Luciferase gene knockdown by **A**. ALE **(5.38)** or acetal ester pyrrolidine **(5.39)** 2'-modified sense strand hybridized to a 2'-F antisense strand for stability. The control is a naked siRNA duplex **(5.40)**. **B**. Dose dependant response of RNAi activity with the control **(5.40)**, ALE **(5.38)** and acetal ester pyrrolidine **(5.39)** siRNA duplexes (light units are relative to Renilla control).

5.14 RNA Synthesis via *N*-FMOC- 2'-*O*-ALE Chemistry

A major advantage of using *N*-FMOC-2'-*O*-ALE (**5.5a-d**) phosphoramidite monomers for RNA synthesis on the light labile linker **5.34** is that the material can be deprotected on-column, and released using mild conditions without fluoride treatment. The material saved from the solid phase synthesis described in section **5.12** was deprotected as follows: 1) 2:3 NEt_3/MeCN for 16 hr, r.t., then 50 °C for 1 hour. The material was then split into 3 and treated with 2) A. 0.5 M hydrazine hydrate in 3:2 pyr/HOAc, 4 hour, r.t.; or B. NH_4OH, 3 hour, r.t.; or C. 1:1 EDA/toluene, 2 hour, r.t. 3) Photolysis in a transilluminator where the irradiation source emitted maximally in the 350-365

nm range in 1% DIPEA in anhydrous MeCN for 20 min. This material was then analyzed by HPLC and PAGE (**Figure 5.16**). For comparative purposes, the same siRNA mixed base sequence was prepared using 2'-*O*-TBDMS, 2'-*O*-TOM, 2'-*O*-ACE, and 2'-*O*-TC chemistry (refer to section 1.4.8, Chapter 1). The optimized synthesis and deprotection conditions were described for all of these chemistries in **Chapter 3,** except for the TC chemistry. In this case, the 2'-*O*-TC phosphoramidite monomers are dissolved in 4:1 MeCN/CH_2Cl_2 (0.1 M), and allowed to couple for 10 min with 0.25 ETT as an activator. Standard capping (Ac_2O) and oxidation (1_2/H_2O/pyr/THF), and detritylation (3% TCA) conditions were also employed. Upon completion of the synthetic cycle, the oligonucleotide was first treated with diethylamine (3 min) to remove the β-cyanoethyl phosphate protecting groups. This was followed by treatment with 1:1 EDA/toluene for 2 hours to remove the nucleobase protecting groups, the 2'-*O*-TC group, and to cleave the oligonucleotide from the solid support. The solid support was then washed with MeCN to remove all of the EDA and other impurities. Because the oligonucleotide is insoluble in MeCN, it remains trapped within the pores of the solid support. Finally, the oligonucleotide was taken up in water and then analyzed by HPLC, PAGE and mass spectrometry (**Figure 5.16, Table 5.3.**). The amount of crude material recovered was 30.1 (1/3 material) and 20.6 ODU (1/3 material) for EDA and NH_4OH deprotection, respectively. From these values, average "stepwise coupling efficiencies" of 98.8% and 98.5%, respectively, were estimated. These values were comparable to those obtained via 2'-*O*-TBDMS (70.6 ODU, 98.3%), 2'-*O*-TOM (76.8 ODU, 98.6%), 2'-*O*-ACE (99.1%), and 2'-*O*-TC (68.4 ODU, 98.1% at 10 min coupling;, 42.0 ODU, 98.0 % at 4 min coupling) chemistries. In addition, all siRNA strands had the same molecular weight and T_m when hybridized to their RNA target (**Table 5.3.**).

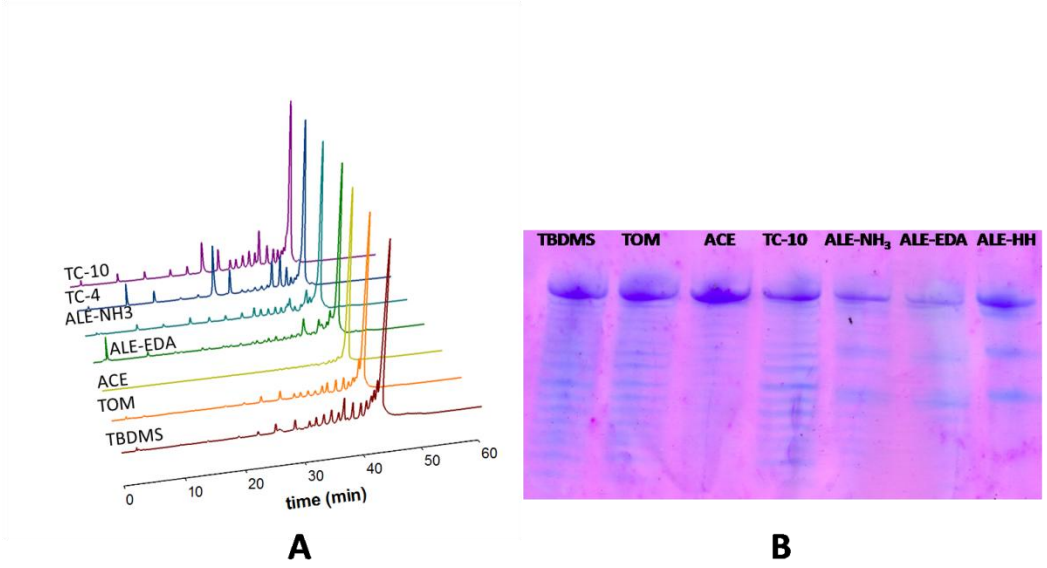


Figure 5.16. **(A)** Anion exchange HPLC traces of crude siRNA strands synthesized from 2'-*O*-TBDMS (red), 2'-*O*-TOM (orange), 2'-*O*-ACE (yellow), and 2'-*O*-ALE deprotected with EDA (green), 2'-*O*-ALE deprotected with NH3 (light blue), 2'-*O*-TC 4 min coupling times (dark blue), 2'-*O*-TC 10 min coupling times (purple) chemistries. **(B)** 24% denaturing (8.3 M Urea) PAGE analysis.

Chemistry	CT (min)	Solid support	Crude ODU	% main peak[c]	Avg. step (%)	Tm (°C)	Exp. MW
TBDMS	10	CPG	70.6	70.1	98.3	63.6	6616.4
TOM	10	CPG	76.8	73.8	98.6	62.8	6616.5
ACE	??[d]	??[d]	??[d]	83.2	99.1	63.0	6616.5
ALE-EDA*	10	PS	30.1 (1/3)	76.4	98.8	63.7	6616.5
ALE-NH_3*	10	PS	26.1 (1/3)	73	98.5	62.3	6616.7
TC-4min	4	CPG	42	65.5	98.0	63.1	n.d.
TC-10min	10	CPG	68.4	68.1	98.1	63.3	n.d.

Table 5.3. Comparative study of 21-nt RNAs synthesized from various chemistries. [a]Base sequence: r(GCUUGAAGUCUUUAAUUAA)-d(TT); [b]Calc. molecular weight: 6617 g/mol; [c]% yield calculated by HPLC (% area of major peak); [d]Coupling time unknown

In addition, the oligonucleotide synthesized by *N*-FMOC-2'-*O*-ALE (**5.5a-d**) and deprotected according to A. (hydrazinolysis conditions) had an HPLC profile that differed from all of the others (**Figure 5.17A**). This was an unexpected result because the PAGE analysis (**Figure 5.17B**) shows the hydrazine treatment to be the similar to the others (NH_4OH and EDA). This suggests that the counter ion to the RNA phosphate after hydrazinolysis may be affecting HPLC analysis using ion exchange chromatography or it may not be completely deprotected. This phenomenon was not observed in the past due the choice of solid support. Previous HPLC chromatograms using ALE chemistry and the Q-linker CPG support have given clean HPLC traces (**Chapter 3**, **Figure 3.9A, B**). The difference being cleavage from the Q-linker requires the use of fluoride whereas the RNA cleaved from solid support **5.34** uses U.V. light. So, prior to U.V. cleavage the material was treated with TBAF overnight and subjected to HPLC analysis (**Figure 5.17B**). As evident, the HPLC trace resembles that of ALE cleavage using NH_4OH or EDA from Figure **5.16A**.

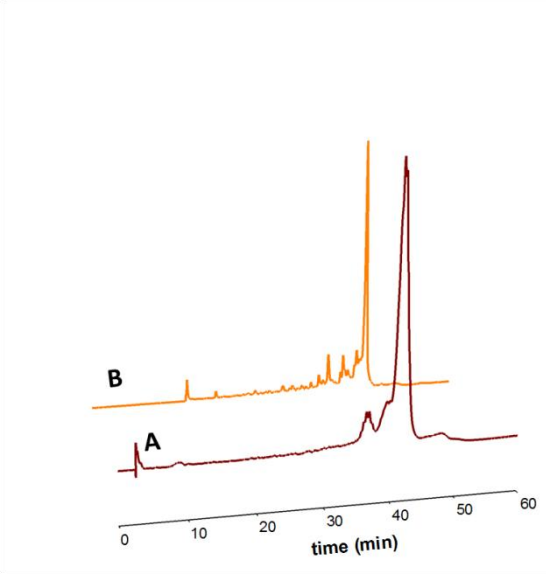


Figure 5.17. Anion exchange HPLC traces of crude siRNA strands synthesized from 2'-*O*-ALE chemistry deprotected by 1) 2:3 NEt_3/MeCN for 16 hr, r.t., then 50 °C for 1 hr 2) **A.** 0.5 M hydrazine hydrate in 3:2 pyr/HOAc, 4 hr, r.t. or **B.** i) 0.5 M hydrazine hydrate in 3:2 pyr/HOAc, 4 hr, r.t.; ii) 1 M TBAF in THF, overnight, r.t. 3) 350-365 nm U.V. in 1% DIPEA in anhydrous MeCN for 20 min.

5.15 Conclusions

In this chapter 2'-*O*-ALE RNA was examined as a siRNA prodrug. To achieve the synthesis of these siRNAs, a novel *N*-FMOC-2'-*O*-ALE protecting group strategy was developed. Single 2'-*O*-ALE RNA insert experiments along with NMR studies revealed that the 2'-*O*-ALE group spontaneously hydrolyzes in aqueous media but was stable in anhydrous organic solvent. It was possible to reductively aminate RNA strands containing 2'-*O*-ALE groups, but this did not fully alleviate the instability of 2'-*O* acetal ester moieties in aqueous media. In addition, a novel light labile linker was developed to prevent any aqueous work-up of the 2'-*O*-ALE pro-siRNA until the very last step. The 2'-*O*-ALE pro-siRNA was transfected as a crude 2'-*O*-ALE RNA:2'-F RNA hybrid with lipofectamine and was shown to be effective for RNAi gene silencing. In addition, the 2'-*O*-ALE RNA strand was subjected to reductive amination and the resulting 2'-*O*-acetal ester pyrrolidine pro-siRNA was also effective for RNAi gene silencing. Finally, on-column RNA synthesis was achieved using the light labile linker and *N*-FMOC-2'-*O*-ALE-3'-*O*-phosphoramidites. This strategy revealed that the conditions originally reported[26] to remove the 2'-*O*-ALE group, i.e., (1) 2:3 TEA/MeCN; 2) 0.5 M hydrazine hydrate in 3:2 pyr/HOAc) actually requires 1 M TBAF for complete deprotection. In fact, it appears that 1) 2:3 TEA/MeCN; 2) NH_4OH or 1) 2:3 TEA/MeCN; 2) 1:1 EDA/toluene appear to be the optimal reaction conditions for on-column deprotection of RNA via ALE chemistry.

5.16 Experimental Methods

5.16.1 General Remarks

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. ^{1}H NMR spectra were measured relative to an undeuterated solvent. ^{31}P NMR spectra were measured relative to undeuterated 85% H_3PO_4. Mass spectra were recorded using low resolution ESI.

The solid-phase synthesis of oligonucleotides was carried out on an Applied Biosystems DNA/RNA 3400 synthesizer using normal phosphoramidite protocol. Standard 2'-*O* TBDMS RNA phosphoramidites were purchased from Chemgenes and standard 2'-*O*-TOM amidites were purchased from Glen Research. The 2'-*O*-TC amidites were a gift from Link Technologies. Crude oligonucleotide obtained using 2'-*O*-ACE chemistry was purchased from Dharmacon. Anion-exchange HPLC was performed on a Waters Alliance system with a Waters 3D UV detector and a Waters Protein Pack DEAE-5PW column (7.5 mm x 7.5 cm). Reverse-phase HPLC was performed on a Varian C-18 analytical column. ESI-TOF mass spectrometry was carried out on a QTOF22 (Micromass) from Waters. MALDI-TOF was carried out a Bruker Daltonics MALDI-TOF. 24% denaturing PAGE (8.3 M urea) was carried out in a standard Hoeffer SE600 apparatus.

5.16.2 Synthetic Protocols and Characterization of Nucleoside Monomers and Other Compounds

The synthesis and characterization of compounds up until **5.2a-d** is discussed in **Chapter 3.6.2.**

General procedure for the preperation of *N*-(9-fluorenylmethoxocarbonyl)-2'-*O*-acetal levulinyl ester nucleosides (5.3b-d)

For example, N^4-(9-fluoroenylmethoxocarbonyl)-2'-*O*-acetal levulinyl ester cytidine (**5.3b**). Compound **5.2b** (0.84 mmol) was dissolved in 8 mL of THF and stirred. Pyridine-HF (168 mmol) was then added dropwise and the reaction is stirred at room temperature until completion (~ 2 hr). The mixture was then evaporated to dryness and purified by column chromatography using 4% MeOH in dichloromethane. The final compound (**5.3b**) was obtained as a white foam in 65% yield. For **5.3c**, use 3% MeOH in chloroform, white foam, 75%. For **5.3d** use 3:7 hexanes/acetone, white foam, 86%.

N^4-(9-fluorenylmethoxocarbonyl)-2'-*O*-acetal levulinyl ester cytidine (5.3b)

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.17 (d, *J* = 7.5, 1H), 7.79 (d, *J* = 7.5, 2H), 7.61 (d, *J* = 7.5, 2H), 7.43 (t, *J* = 7.5, 2H), 7.33 (t, *J* = 7.4, 2H), 7.22 (d, *J* = 7.4, 1H), 5.75 (d, *J* = 3.0, 1H), 5.59 (d, *J* = 6.3, 1H), 5.44 (d, *J* = 6.3, 1H), 4.61 (s, 1H), 4.51 (d, *J* = 7.1, 2H), 4.38 (s, 1H), 4.28 (s, 1H), 4.15 (d, *J* = 6.1, 1H), 4.05 (s, 1H), 3.90 (d, *J* = 1.9, 1H), 3.69 (s, 1H), 2.83 – 2.76 (m, 1H), 2.69 (s, 1H), 2.55 (m, 2H), 2.18 (s, *3*H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.59, 160.79, 158.32, 150.25, 149.35, 147.54, 134.21, 133.41, 131.15, 131.13, 126.37, 100.48, 95.98, 88.27, 83.90, 80.56, 74.22, 73.65, 65.58, 52.88, 23.69, 23.63, 23.52, 23.49, 23.22, 23.18, 23.14, 23.06, 19.66, 19.47, 19.27, 19.09, 18.74. ESI-TOF calc for $C_{30}H_{31}N_3O_{10}$ 616.20 ($+Na^+$); found 616.20.

N^6-(9-fluorenylmethoxocarbonyl)-2'-*O*-acetal levulinyl ester adenosine (5.3c)

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.76 (s, 1H), 8.68 (s, 1H), 8.08 (s, 1H), 7.77 (d, *J* = 7.6 Hz, 2H), 7.65 (d, *J* = 7.5 Hz, 2H), 7.45 – 7.35 (m, 2H), 7.34 – 7.28 (m, 2H), 6.02 (d, *J* = 7.6 Hz, 2H), 5.38 (d, *J* = 6.4 Hz, 1H), 5.04 – 4.98 (m, 1H), 4.96 (d, *J* = 6.4 Hz, 1H), 4.69 – 4.58 (m, 3H), 4.39 (s, 1H), 4.33 (m, 1H), 3.98 (m,1H), 3.79 (m, 1H), 3.43 (s, 1H), 2.86 – 2.75 (m,1H), 2.75 – 2.65 (m, 1H), 2.48 – 2.32 (m, 2H), 2.18 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 213.04, 178.67, 168.67, 164.89, 160.94, 158.19, 156.05, 150.79, 150.32, 149.45, 149.36, 147.54, 142.14, 141.82, 141.53, 136.34, 136.27, 134.53, 134.48, 134.23, 133.42, 133.35, 131.11, 129.92, 126.39, 119.53, 100.95, 96.13, 93.97, 93.25, 89.15, 88.27, 74.19, 73.74, 66.90, 61.41, 61.40, 52.89, 44.09, 35.96, 34.23. ESI-TOF calc for $C_{31}H_{31}N_5O_9$ 640.21 ($+Na^+$); found 640.26.

N^2-(9-fluorenylmethoxocarbonyl)-2'-*O*-acetal levulinyl ester guanosine (5.3d)

^{1}H NMR (500 MHz, $CDCl_3$) δ 11.47 (s, 1H), 9.38 (s, 1H), 8.25 (s, 1H), 7.78 – 7.71 (m, *J* = 7.2 Hz, 2H), 7.59 – 7.52 (m, *J* = 7.4, 2.9 Hz, 2H), 7.44 – 7.35 (m, *J* = 7.3 Hz, 2H), 7.33 – 7.27 (m, 3H), 5.94 (d, *J* = 4.0 Hz, 1H), 5.40 (abq, *J* = 15.6, 6.5 Hz, 2H), 4.67 (t, *J* = 4.9 Hz, 1H), 4.60 (t, *J* = 4.3 Hz, 1H), 4.59 – 4.49 (m, *J* = 6.6 Hz, 3H), 4.22 (d, *J* = 6.5 Hz, 3H), 4.06 – 3.96 (m, *J* = 10.8 Hz, 1H), 3.96 – 3.86 (m, *J* = 11.9 Hz, 1H), 2.83 – 2.72 (m, *J* = 3.5 Hz, 1H), 2.69 – 2.57 (m, *J* = 4.5 Hz, 1H), 2.57 – 2.48 (m, 1H), 2.48 – 2.39 (m, 1H), 2.14 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 213.42, 178.57, 164.80, 161.66, 159.72, 154.34, 152.84, 150.71, 149.09, 147.48, 143.99, 141.89, 141.83, 134.38, 134.27, 134.11, 133.44, 133.41, 133.21, 131.13, 131.09, 126.33, 119.42, 95.26, 93.27, 92.77, 89.86, 88.32, 76.47, 74.63, 69.57, 52.78, 43.90, 35.88, 34.27. ESI-TOF calc for $C_{31}H_{31}N_5O_{10}$ 656.21 ($+Na^+$); found 656.24.

General procedure for the preperation of *N*-(9-fluorenylmethoxocarbonyl)-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester nucleosides (5.4b-d)

For example, *N4*-(9-fluorenylmethoxocarbonyl)- 5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester cytidine (**5.4b**). Compound **5.3a** (0.52 mmol) was dissolved in 5 mL of dry pyridine and stirred under inert atmosphere. DMTrCl (0.57 mmol) was then added as a solid and the reaction mixture was stirrred until completion (~ 3 hr). The reaction was then quenched with 50 mL aqueous 5% sodium bicarbonate and extracted 3 x 50 mL of dichloromethane. The combined organic extracts were then dried with magnesium sulfate, filtered, and concentrated. This crude material was then purified by flash chromatography using 2% MeOH in dichloromethane with 1% pyridine. It was imporant to neutralize the column with pyridine and not a stronger base such as triethylamine to avoind FMOC cleavage. Compound **5.4b** was obtained as a white solid in 86% yield. For **5.4c**, use 3% MeOH in dichloromethane with 1% pyridine, white solid, 80% yield. For **5.4d**, use 4% MeOH in chloroform with 1% pyridine, white solid, 82%.

N^4-(9-fluorenylmethoxocarbonyl)-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester cytidine (5.4b)

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.50 (d, *J* = 7.4 Hz, 1H), 7.79 (d, *J* = 7.5 Hz, 2H), 7.57 (d, *J* = 7.5 Hz, 2H), 7.46 – 7.21 (m, 13H), 6.86 (d, *J* = 7.7 Hz, 4H), 5.92 (s, 1H), 5.63 (abq, *J* = 49.9, 6.2 Hz, 2H), 4.53 – 4.39 (m, 3H), 4.33 – 4.24 (m, *J* = 8.4, 6.3 Hz, 2H), 4.11 – 4.02 (m, *J* = 9.1 Hz, 1H), 3.80 (s, 3H), 3.64 – 3.58 (m, *J* = 9.9 Hz, 1H), 3.58 – 3.51 (m, *J* = 11.2, 2.3 Hz, 1H), 2.80 – 2.73 (m, *J* = 9.7, 6.3 Hz, 2H), 2.61 – 2.51 (m, *J* = 6.4 Hz, 2H), 2.18 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 213.04, 178.67, 168.67, 164.89, 160.94, 158.19, 156.05, 150.79, 150.32, 149.45, 149.36, 147.54, 142.14, 141.82, 141.53, 136.34, 136.27, 134.53, 134.48, 134.23, 133.42, 133.35, 131.11, 129.92, 126.39, 119.53, 100.95, 96.13, 93.97, 93.25, 89.15, 88.27, 74.19, 73.74, 66.90, 61.41, 61.40, 52.89, 44.09, 35.96, 34.23. ESI-TOF calc for $C_{51}H_{49}N_3O_{12}$ 918.29 ($+Na^+$); found 918.28.

N^6-(9-fluorenylmethoxocarbonyl)-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester adenosine (5.4c)

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.67 (s, 1H), 8.35 (s, 1H), 8.21 (s, 1H), 7.78 (d, *J* = 7.5 Hz, 2H), 7.66 (d, *J* = 7.4 Hz, 2H), 7.45 – 7.16 (m, 13H), 6.80 (d, *J* = 8.7 Hz, 4H), 6.20 (d, *J* = 4.7 Hz, 1H), 5.40 (abq, *J* = 46.2, 6.4 Hz, 2H), 5.09 (t, *J* = 4.9 Hz, 1H), 4.63 (d, *J* = 6.7 Hz, 4H), 4.37 – 4.31 (m, 1H), 4.31 – 4.24 (m, 1H), 3.78 (s, 3H), 3.56 – 3.49 (m, 1H), 3.47 – 3.40 (m, 1H), 2.77 – 2.71 (m, 2H), 2.49 – 2.42 (m, 2H), 2.15 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 213.02, 178.33, 164.78, 159.00, 157.23, 157.14, 156.05, 155.49, 150.70, 149.73, 149.71, 148.26, 147.55, 142.14, 141.85, 141.79, 136.30, 134.53, 134.38, 134.07, 133.39, 133.15, 131.24, 129.93, 128.79, 126.29, 119.38, 94.94, 93.59, 92.86, 90.32, 88.32, 76.79, 74.02, 69.26, 61.43, 53.13, 43.97, 35.87, 34.07. ESI-TOF calc for $C_{52}H_{49}N_5O_{11}$ 942.34 ($+Na^+$); found 942.30.

N^2-(9-fluorenylmethoxocarbonyl)-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester guanosine (5.4d)

^{1}H NMR (500 MHz, $CDCl_3$) δ 11.42 – 11.14 (m, 1H), 7.82 (s, 1H), 7.76 – 7.69 (m, *J* = 7.5 Hz, 2H), 7.51 – 7.45 (m, *J* = 6.7 Hz, 2H), 7.45 – 7.15 (m, 16H), 6.82 – 6.74 (m, *J* = 8.8, 2.3 Hz, 4H), 5.98 (d, *J* = 4.0 Hz, 1H), 5.47 (abq, *J* = 64.0, 6.3 Hz, 2H), 4.84 (t, *J* = 4.6 Hz, 1H), 4.60 – 4.54 (m, *J* = 4.7 Hz, 1H), 4.54 – 4.44 (m, 2H), 4.25 – 4.20 (m, 1H), 4.20 – 4.14 (m, 1H), 3.75 (s, 6H), 3.53 – 3.46 (m, 1H), 3.42 – 3.35 (m, *J* = 4.3 Hz, 1H), 2.76 – 2.70 (m, *J* = 6.0 Hz, 2H), 2.58 – 2.44 (m, 2H), 2.12 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 213.42, 178.57, 164.80, 161.66, 159.72, 154.34, 152.84, 150.71, 149.09, 149.03, 147.48, 143.99, 141.89, 141.83, 136.26, 136.23, 134.38, 134.27, 134.11, 133.44, 133.41, 133.21, 131.13, 131.09, 127.75, 126.33, 119.42, 95.26, 93.27, 92.77, 89.86, 88.32, 76.47, 74.63, 69.57, 61.43, 52.78, 43.90, 35.88, 34.27. ESI-TOF calc for $C_{52}H_{44}N_4O_{12}$ 958.34 ($+Na^+$); found 958.29.

General procedure for the preperation of *N*-(9-fluorenylmethoxocarbonyl)-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite nucleosides (5.5b-d)

For example, N^4-(9-fluorenylmethoxocarbonyl)- 5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester cytidine 3'-*O*-2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (**5.5b**). Compound **5.5b** (0.44 mmol) was dissolved in 5 mL of dry THF under a dry nitrogen environment. Diisopropylethylamine (1.76 mmol) was then added followed by the dropwise addition of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.53 mmol). The reaction was stirred until completion (~ 3 hr). The reaction mixture was then quenched with 5% $NaHCO_3$ and extracted 3x with 50 mL DCM. The pooled extracts were dried over $MgSO_4$, filtered, and evaporated under reduced pressure. The crude material was then purified by flash chromatography using 85:15 CH_2Cl_2/acetone with 1% pyridine, white foam, 75%. For **5.5c** use 9:1 CH_2Cl_2/acetone with 1% pyridine, white foam, 90%. For **5.5d** use 8:2 CH_2Cl_2/acetone with 1% pyridine, white foam, 82%.

N^4-(9-fluorenylmethoxocarbonyl)-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-acetal levulinyl ester cytidine-3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (5.5b)

^{31}P NMR (81 MHz, $CDCl_3$) δ 151.88, 150.47. ESI-TOF calc for $C_{60}H_{66}N_5O_{13}P$ 1118.44 ($+Na^+$) found 1118.38.

N^6-(9-fluorenylmethoxocarbonyl)-5'-*O*-(4, 4'-dimethoxytrityl)- 2'-*O*-acetal levulinyl ester adenosine-3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (5.5c)

^{31}P NMR (81 MHz, $CDCl_3$) δ 152.11, 150.10. ESI-TOF calc for $C_{61}H_{66}N_7O_{12}P$ 1047.37 ($+Na^+$); found 1047.51.

N^2-(9-fluorenylmethoxocarbonyl)-5'-*O*-(4, 4'-dimethoxytrityl)- 2'-*O*-acetal levulinyl ester guanosine-3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (5.5d)

^{31}P NMR (81 MHz, $CDCl_3$) δ 151.78, 150.59. ESI-TOF calc for $C_{61}H_{66}N_7O_{13}P$ 1158.45 ($+Na^+$), found 1158.40.

Procedure for the preparation of 5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-tert-butyldimethylsilyl cytidine-3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (5.8).

Compound **5.7** (11 mmol) was dissolved in 15 mL of THF and a mixture of 1:1 $NH_4OH/MeNH_2$ was added. The reaction was stirred at room temperature until completion (~ 3 hr). All volatiles are then removed under reduced pressure, and the material was then freeze dried in benzene to give **5.8** in quantitative yield and was used without further purification.
^{31}P NMR (81 MHz, DMSO) δ 149.63, 149.24. ESI-TOF calc for $C_{45}H_{62}N_2O_8PSi$ 882.41 ($+Na^+$) found 882.36.

N^4-(9-fluorenylmethoxocarbonyl)-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-*tert*-butyldimethylsilyl cytidine-3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (5.9)

Compound **5.**8 (11 mmol) was dissolved in 10 mL of THF followed by the addition of diisopropylethylamine (44 mmol) under inert atmosphere. Solid FMOCCl (14.4 mmol) was added and the reaction was stirred until completion (~ 5 hr). The reaction was quenched with 50 mL of 5% sodium bicarbonate and extracted 3 x 100 mL of DCM. The combined organic extracts are dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography using 3:2 hexanes/EtOAc with 2% pyridine. The final product **5.9** was obtained as a white solid in 90% yield.
^{31}P NMR (81 MHz, $CDCl_3$) δ 152.11, 150.10. ESI-TOF calc for $C_{60}H_{72}N_5O_{10}PSi$ 1104.50 ($+Na^+$) found 1104.50.

Procedure for the preparation of 5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-*tert*-butyldimethylsilyl nucleosides (5.11a-b)

For example, **5.11a**. Compound **5.10a** (2.45 mmol) was dissolved in 25 mL of dry pyridine. This was followed by the addition of TBDMSCl (2.7 mmol) and silver nitrate (2.7 mmol). The reaction was stirred for 4 hr and quenched with 100 mL of 5% sodium bicarbonate and extracted 3 x 150 mL of dichloromethane. The organic extracts were dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude material was then purified by flash chromatography with 85:15 dichloromethane/ether. The final product **5.11a** was obtained as a white foam in 40% yield. For **5.12b**, use 7:3 hexanes/EtOAc, white foam, 32% yield.

***N*^{6}-(9-fluorenylmethoxocarbonyl)-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*- *tert*-butyldimethylsilyl adenosine**

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.69 (s, 1H), 8.19 (s, 1H), 8.07 (s, 1H), 7.79 (d, *J* = 7.6 Hz, 2H), 7.68 (d, *J* = 7.4 Hz, 2H), 7.48 – 7.19 (m, 13H), 6.82 (d, *J* = 8.8 Hz, 4H), 6.09 (d, *J* = 5.3 Hz, 1H), 5.01 (t, *J* = 5.1 Hz, 1H), 4.64 (d, *J* = 6.6 Hz, 2H), 4.40 – 4.31 (m, 2H), 4.31 – 4.25 (m, *J* = 3.2 Hz, 1H), 3.78 (s, 6H), 3.59 – 3.50 (m, *J* = 10.5, 3.1 Hz, 2H), 3.46 – 3.37 (m, *J* = 10.7, 3.8 Hz, 2H), 2.70 (d, *J* = 3.9 Hz, 1H), 0.84 (s, 9H), -0.01 (s, 3H), -0.15 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.82, 159.12, 157.36, 156.99, 155.44, 150.71, 149.71, 147.68, 147.57, 141.76, 136.29, 134.33, 134.13, 134.07, 133.40, 133.21, 131.22, 126.31, 119.43, 94.61, 92.95, 90.52, 81.97, 77.78, 74.05, 69.51, 61.44, 53.13, 31.76, 24.09, 1.23, 1.04. ESI-TOF calc for $C_{52}H_{55}N_5O_8Si$ 928.38 ($+Na^+$) found 928.29.

***N*^{2}-(9-fluorenylmethoxocarbonyl)-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*- tert-butyldimethylsilyl guanosine**

^{1}H NMR (500 MHz, $CDCl_3$) δ 11.23 (s, 1H), 7.81 (s, 1H), 7.77 (d, *J* = 7.6 Hz, 2H), 7.50 (d, *J* = 7.8 Hz, 2H), 7.46 – 7.19 (m, 13H), 6.86 – 6.75 (m, *J* = 8.7, 3.3 Hz, 4H), 5.79 (d, *J* = 6.8 Hz, 1H), 5.07 – 4.99 (m, 1H), 4.51 – 4.43 (m, *J* = 8.6 Hz,

2H), 4.36 – 4.29 (m, 1H), 4.24 (s, 1H), 4.14 (t, *J* = 6.6 Hz, 1H), 3.76 (s, 6H), 3.56 – 3.49 (m, *J* = 10.5 Hz, 1H), 3.30 – 3.22 (m, *J* = 10.6, 3.0 Hz, 1H), 2.75 (d, *J* = 1.7 Hz, 1H), 0.86 (s, 8H), 0.04 (s, 3H), -0.15 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.90, 161.58, 159.30, 154.62, 152.36, 150.92, 148.96, 147.50, 147.46, 144.39, 142.01, 141.75, 136.22, 136.20, 134.29, 134.27, 134.24, 133.48, 133.33, 131.03, 130.99, 128.10, 126.38, 126.37, 119.52, 93.97, 92.72, 90.55, 81.27, 77.69, 74.91, 69.87, 61.45, 52.72, 31.75, 24.09, 1.21, 1.14. ESI-TOF calc for $C_{52}H_{55}N_5O_9Si$ 944.38 ($+Na^+$) found 944.30.

Procedure for the preparation of 5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-*tert*-butyldimethylsilyl 3'-*O*-(2-cyanoethyl *N*,*N*-diisopropyl)phosphoramidites (5.12a-b)

For example, compound **5.12a**. Compound **5.11a** (0.33 mmol) was dissolved in 4 mL of THF under inert atmosphere. Diisopropylethylamine (1 mmol) was then added to this stirring solution followed by the dropwise addition of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.4 mmol). This reaction mixture was then stirred until completion (~ 3 hr). The reaction mixture was then quenched with 50 mL of 5% sodium bicarbonate and extracted 3 x 50 mL of dichloromethane. The combined organic extracts were then dried over magnesium sulfate, filtered, and concentrated under reduced pressure. This crude material was then purified by flash chromatography using 7:3 hexanes/EtOAc with 1% pyridine. The final product **5.12a** was obtained as a white foam in 85%. For **5.12b**, use 9:1 chloroform/ether with 2% pyridine, white foam, 80%.

***N*^{6}-(9-fluorenylmethoxocarbonyl)-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*-*tert*-butyldimethylsilyl adenosine-3'-*O*-2-(cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (5.12a)**

^{31}P NMR (81 MHz, $CDCl_3$) δ 152.11, 150.10. ESI-TOF calc for $C_{61}H_{72}N_7O_9PSi$ 1128.49 ($+Na^+$) found 1128.49.

N^2-(9-fluorenylmethoxocarbonyl)-5'-*O*-(4, 4'-dimethoxytrityl)-2'-*O*- *tert*-butyldimethylsilyl guanosine-3'-*O*-2-(cyanoethyl *N*,*N*-diisopropyl)phosphoramidite (5.12b).

^{31}P NMR (81 MHz, $CDCl_3$) δ 151.38, 149.92. ESI-TOF calc for $C_{61}H_{72}N_7O_{10}PSi$ 1144.41 ($+Na^+$) found 1144.41.

Procedure for the preparation of 4-ethyl-3-nitrobenzoic acid (5.25)

This compound was prepared according to Charles, P. S.; Henderson, J. C. *ChemInform* 2004, *35*. (ref 29).

Procedure for the preparation of tert-butyl-4-ethyl-3-nitrobenzoate (5.26)

This compound was prepared according to Buhler, S.; Lagoja, I.; Giegrich, H.; Stengele, K. P.; Pfleiderer, W. *Helvetica Chimica Acta* **2004**, *87*, 620-659. (ref 30).

Procedure for the preparation of *tert*-butyl-4-(1-hydroxypropan-2-yl)-3-nitrobenzoate (5.27)

This compound was prepared according to Buhler, S.; Lagoja, I.; Giegrich, H.; Stengele, K. P.; Pfleiderer, W. *Helvetica Chimica Acta* **2004**, *87*, 620-659. (ref 30).

Procedure for the preparation of (9H-fluoren-9-yl)methyl 2-(2-nitro-4-pivaloylphenyl)propyl carbonate (5.28)

Compound **5.27** (90.4 mmol) was dissolved in 150 mL of dry pyridine under inert atmosphere. FMOCCl (95 mmol) was then added in four portions over 20 min. The reaction mixture was then stirred for 2 hr until the reaction had gone to completion. At this point, approximately 100 mL of pyridine ws removed under reduced pressure and was then diluted with 300 mL of ethyl acetate and washed 3 x with brine. The aqueous layer was then washed 3 x 100 mL ethyl acetate and the combined organic extracts were dried over magnesium sulfate,

filtered, and concentrated under reduced pressure. The crude material was then purified by flash chromatography using 95:5 hexanes/EtOAc to give **5.28** as a yellow oil in 95% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.37 (s, 1H), 8.21 – 8.12 (m, 1H), 7.76 (d, *J* = 7.5 Hz, 2H), 7.59 – 7.49 (m, 2H), 7.41 (t, *J* = 7.4 Hz, 1H), 7.35 – 7.27 (m, 1H), 4.42 – 4.27 (m, 3H), 4.22 (t, *J* = 7.3 Hz, 1H), 3.86 – 3.75 (m, *J* = 13.3, 6.8 Hz, 1H), 1.59 (s, 9H), 1.40 (d, *J* = 6.9 Hz, 3H). Note: peaks are reported for only one diastereomer.

Procedure for the preparation of 4-(1-(((9H-fluoren-9-yl)methoxy)carbonyloxy)propan-2-yl)-3-nitrobenzoic acid (5.29)

Compound **5.28** (76 mmol) was dissolved in 80% TFA in DCM (50 mL) and stirred until the reaction had gone to completion (~ 30 min). The volatile components were removed under reduced pressure and the crude material was purified by flash chromatography first with 8:2 hexanes/EtOAc to removed impurities, then with 6:4 hexanes/EtOAc to elute the product. The final compound **5.29** was obtained as a yellow solid in 93% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.53 (d, *J* = 1.7 Hz, 1H), 8.29 (dd, *J* = 8.2, 1.7 Hz, 1H), 7.76 (d, *J* = 7.5 Hz, 2H), 7.65 (d, *J* = 8.2 Hz, 1H), 7.56 (dd, *J* = 7.4, 3.5 Hz, 2H), 7.41 (t, *J* = 7.2 Hz, 2H), 7.31 (td, *J* = 7.4, 4.0 Hz, 2H), 4.38 (pd, *J* = 10.9, 6.6 Hz, 5H), 4.23 (t, *J* = 7.3 Hz, 1H), 3.86 (dd, *J* = 13.3, 6.7 Hz, 1H), 2.06 (s, 1H), 1.43 (d, *J* = 6.9 Hz, 3H). Note: peaks are reported for 1 diastereomer. ESI-TOF calc for $C_{25}H_{21}NO_7$ 470.13 ($+Na^+$) found 470.12.

Procedure for the preparation of 4-(1-(((9H-fluoren-9-yl)methoxy)carbonyloxy)propan-2-yl)-3-nitro-N-polystyrene-benzamide (5.30)

Compound **5.29** (1 g) was reacted with aminomethyl polystyrene (stratosphere for DNA synthesis, 1000 Å) in the presence of HATU (1 g) and DMAP (50 mg) in pyridine. This reaction mixture was then shaken overnight. The solid support

was then filtered and washed with 100 mL of dichloromethane, methanol, and ether. This was followed by capping of unreacted amines with CAP A (5 mL) and CAP B (5 mL) solutions (Ac_2O/N-methyl-imidazole/THF) for 20 min followed by filtration. The solid support was then washed with 100 mL of acetonitrile to give **5.30**.

Procedure for the preparation of 4-(1-hydroxyl-propan-2-yl)-3-nitro-N-polystyrene-benzamide (5.31)

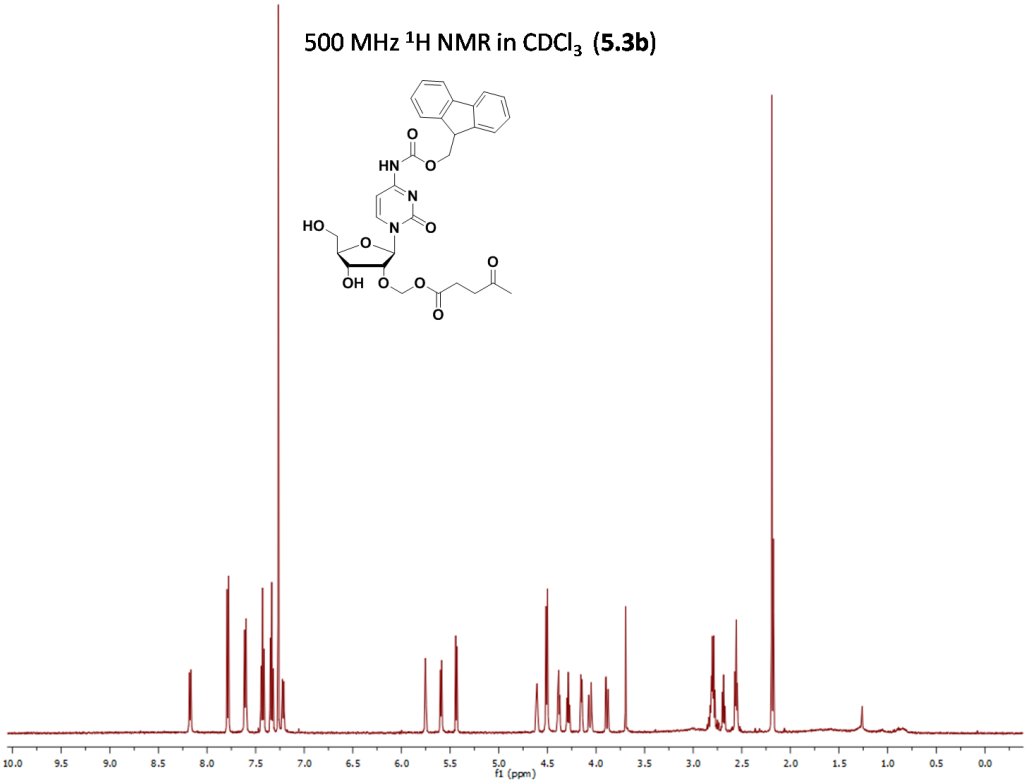
Solid support **5.30** (~ 1g) was treated with 10 mL of 10% 4-methylpiperidine in DMF for 30 min. The support was then filtered and washed with 100 mL of dichloromethane, methanol, and ether. It was then dried to give **5.31** with a loading of 250 μmol/g assayed by fulvene analysis.

Procedure for the preparation of 5'-DMTr-TpT-light labile inker (5.34)

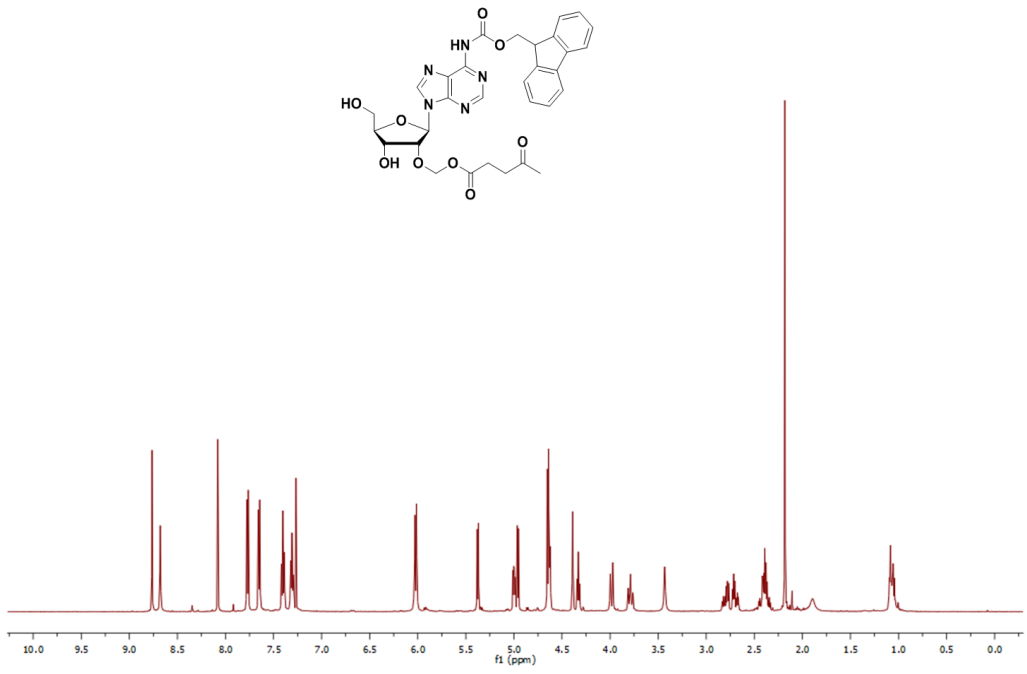
5'-DMTr-3'-phosphorodiamidite-Thy (**5.32)**, 3'-*O*-levulinyl-Thy (**5.33**), and 0.25 M DCI were mixed together in a respective molar ratio of (1:1:3) in anhydrous MeCN. The putative dinucleoside phosphorotetrazolide was generated within 10 min. Without isolation, this intermediate was mixed with polystyrene support **5.31** and was allowed to react for 20 min at room temperature. The support was then oxidized with 0.5 M I_2 in THF/pyridine/H_2O to give **5.34**. The unreacted hydroxyl groups were capped with equal volumes of Cap A and Cap B solutions. Compound 5.34 was obtained with a loading of **55** μmol TpT per gram of support assayed by trityl analysis.

5.16.3 Selected NMR Spectra

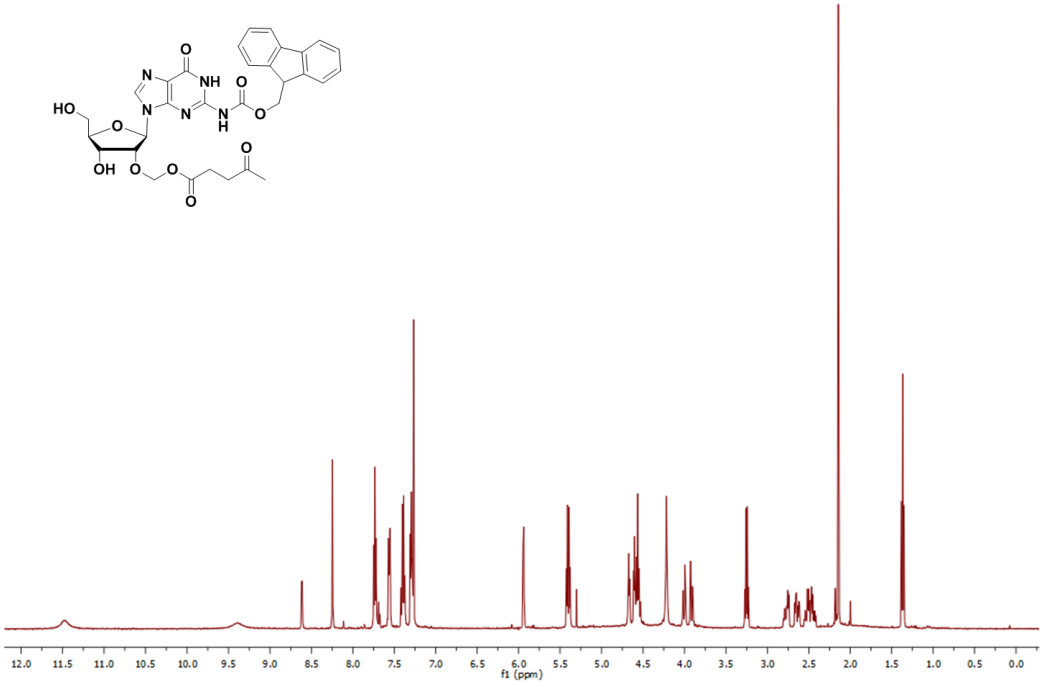
500 MHz ^{1}H NMR in $CDCl_3$ (**5.3b**)



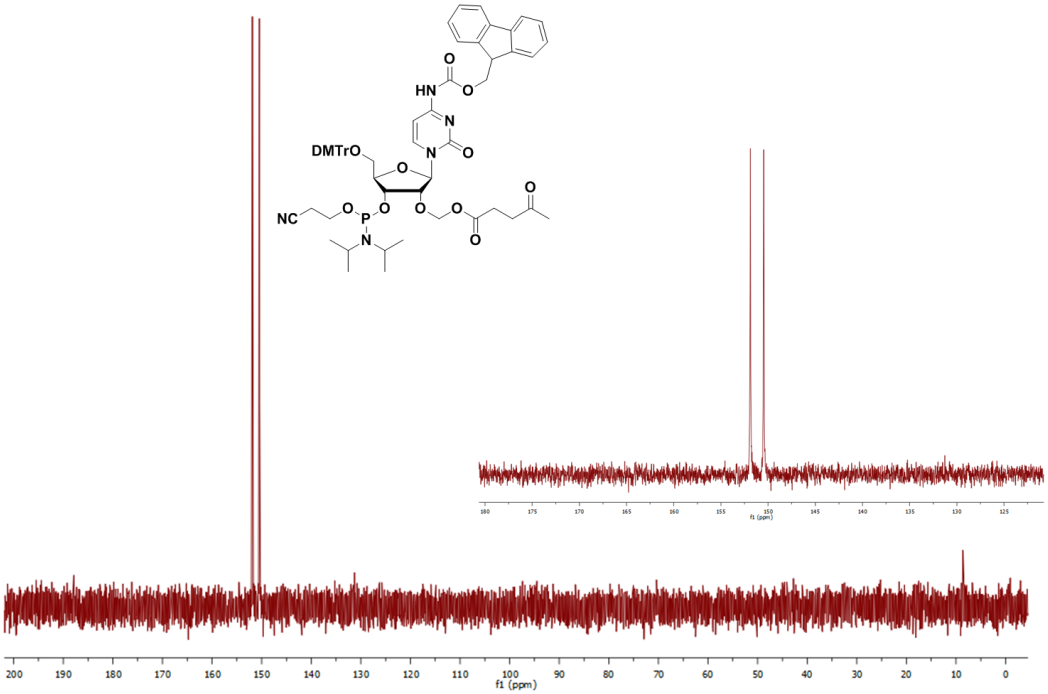
500 MHz ^{1}H NMR in $CDCl_3$ (**5.3c**)



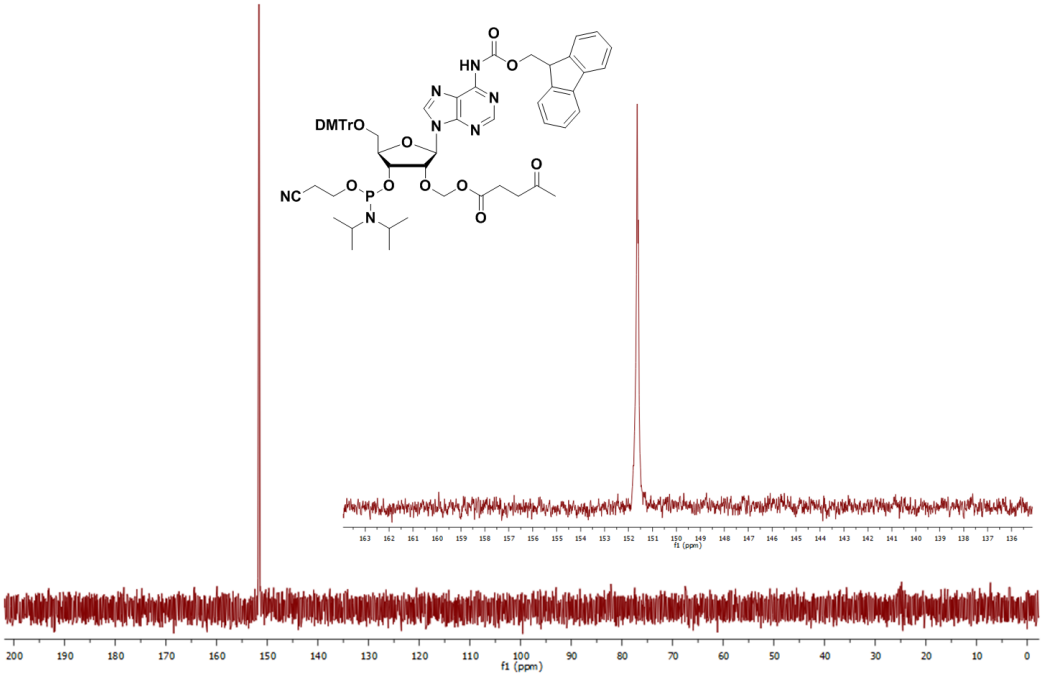
500 MHz ^{1}H NMR in $CDCl_3$ (**5.3d**)



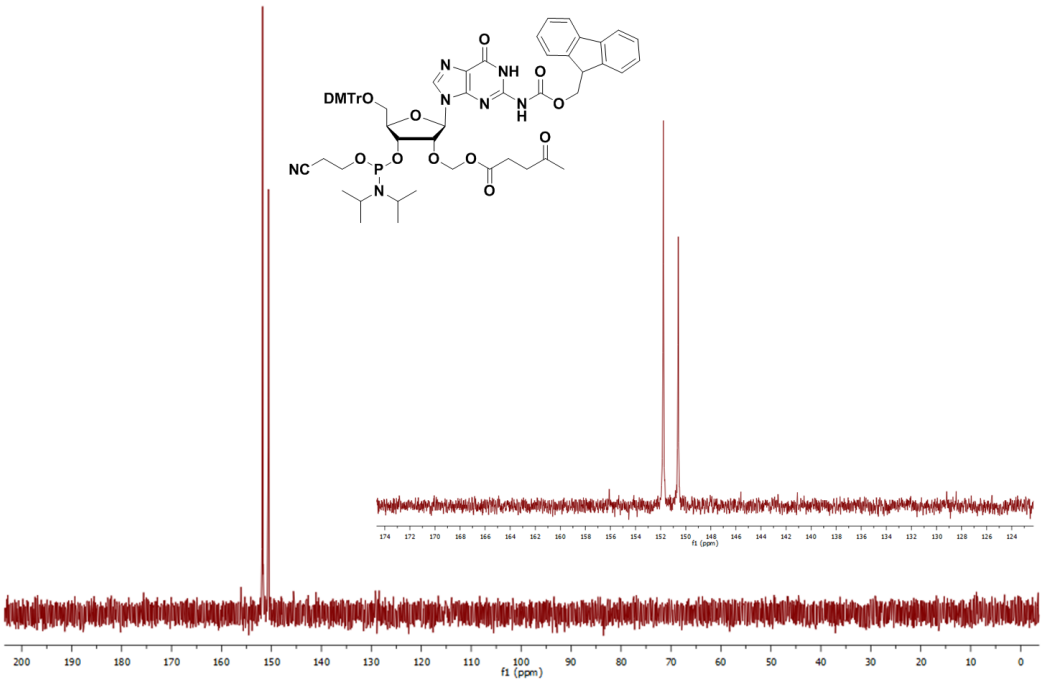
200 MHz ^{31}P NMR in $CDCl_3$ (**5.5b**)



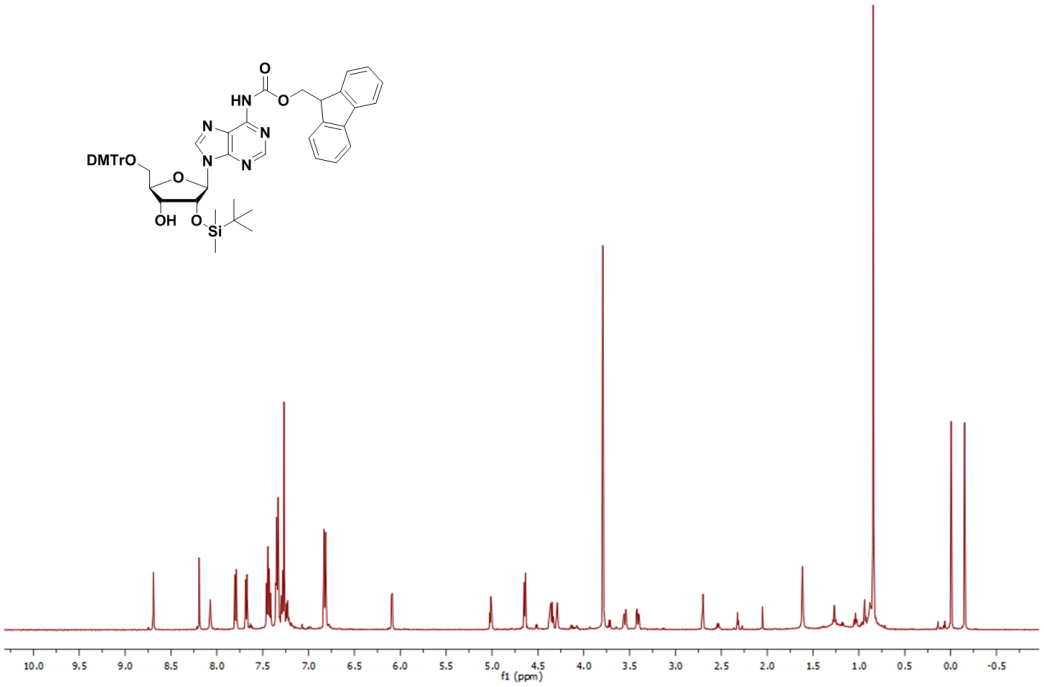
200 MHz ^{31}P NMR in $CDCl_3$ (**5.5c**)



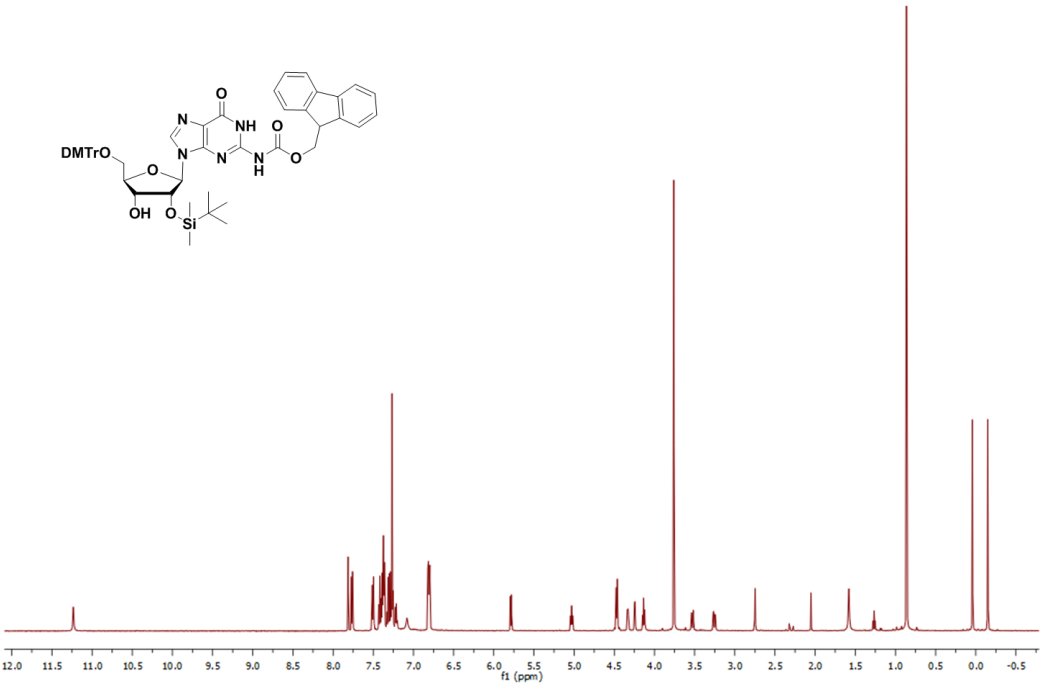
200 MHz ^{31}P NMR in $CDCl_3$ (**5.5d**)



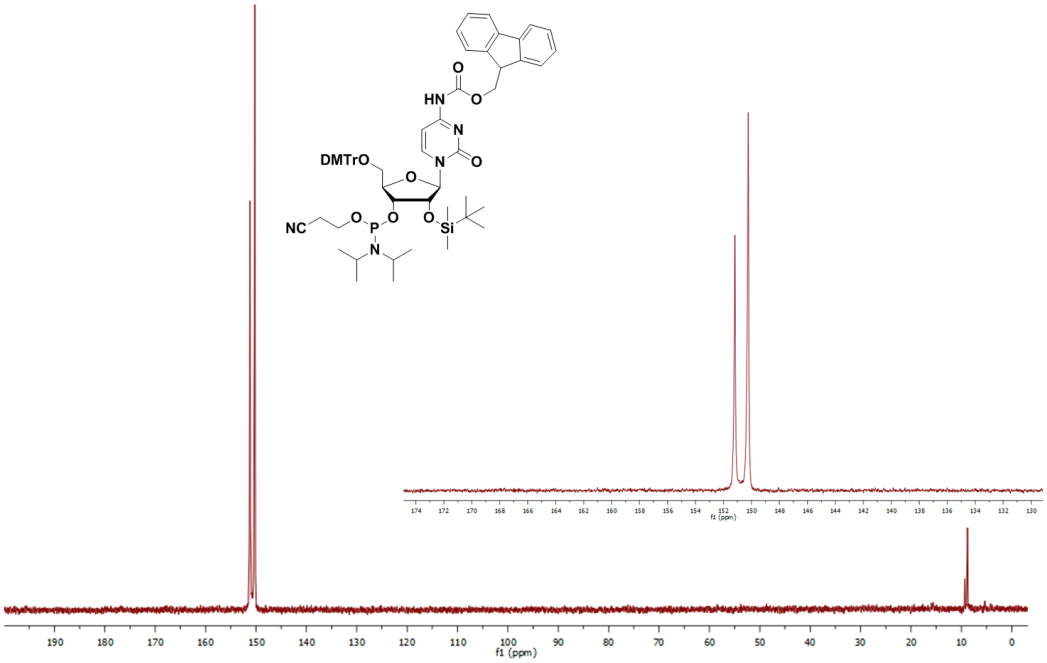
500 MHz ^{1}H NMR in $CDCl_3$ (**5.11a**)



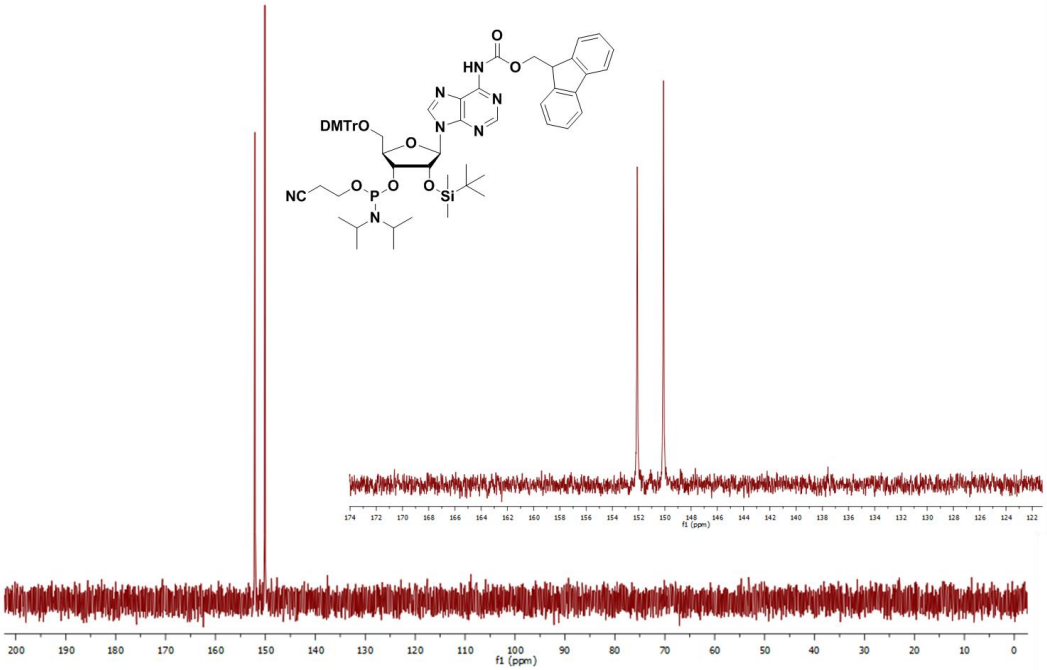
500 MHz ^{1}H NMR in $CDCl_3$ (**5.11b**)



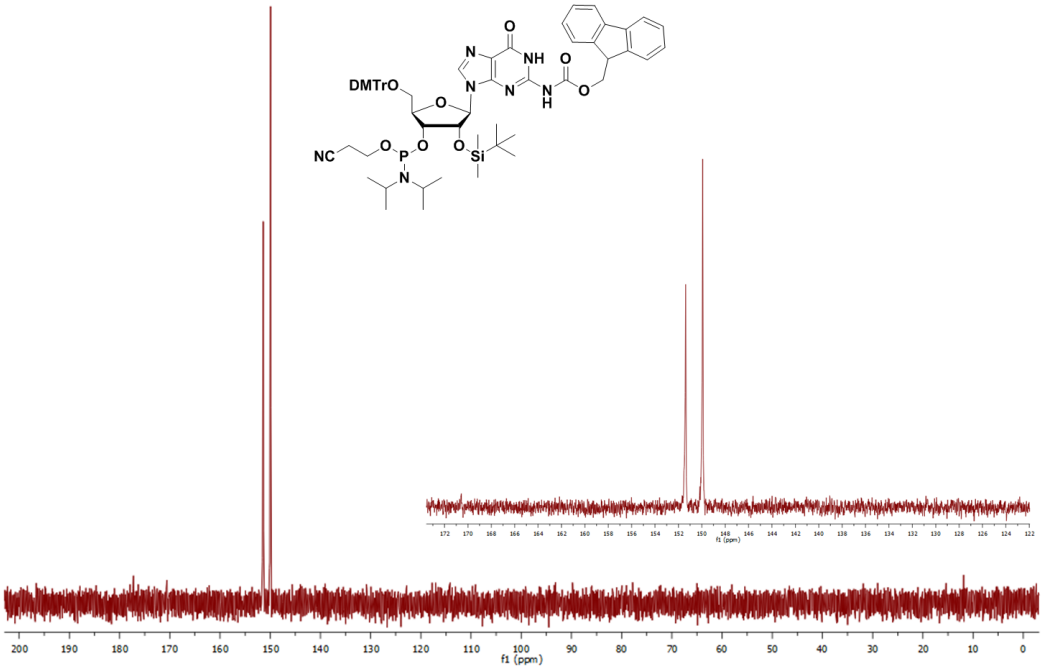
200 MHz ^{31}P NMR in $CDCl_3$ (**5.9**)



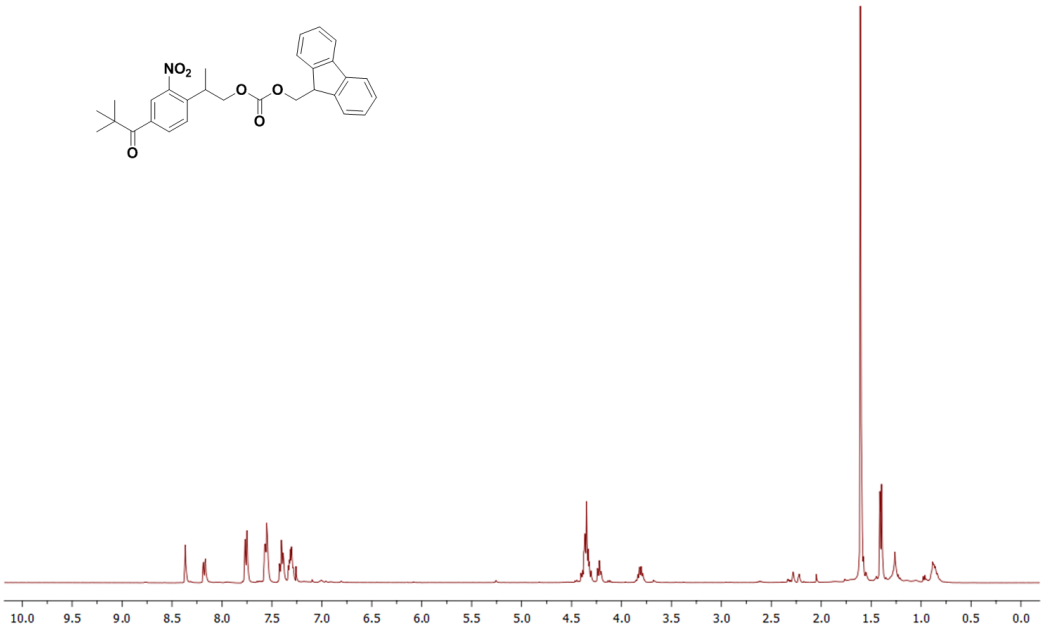
200 MHz ^{31}P NMR in $CDCl_3$ (**5.12a**)



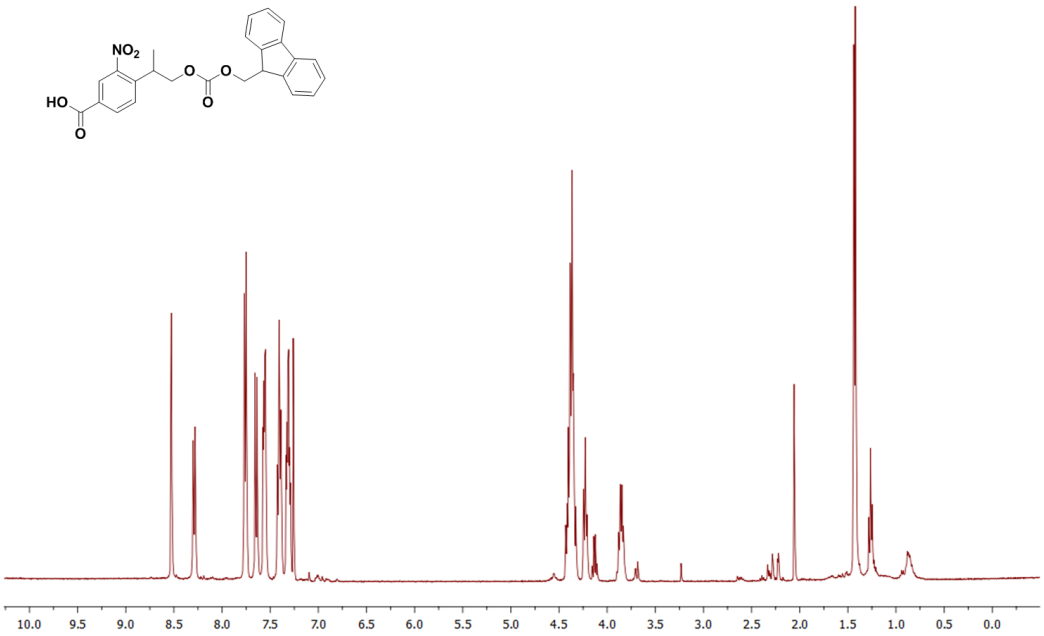
200 MHz ^{31}P NMR in $CDCl_3$ **(5.12b)**



400 MHz ^{1}H NMR in $CDCl_3$ **(5.29)**



400 MHz ^{1}H NMR in $CDCl_3$ **(5.30)**



5.16.4 Solid-phase Oligonucleotide Synthesis Using *N*-FMOC-2′-*O*-ALE Chemistry

The solid-phase synthesis of r(GCUUGAAGUCUUUAAUUAA)-d(TT) was performed on an ABI-3400 DNA/RNA synthesizer. A 1 μmol scale was conducted in the trityl-off mode using a 5'-*O*-DMTr-dT-dT-linker appended as a phosphate triester internucleotide linkage with aminomethyl polystyrene (PL-AMS Resin) with a loading of 55 μmol. The support was first subjected to a standard capping cycle, CAP A solution (Ac_2O/pyr/THF) and Cap B solution (10% 1-methylimidazole in THF) for 3 × 180 s to acylate and dry the solid support. RNA synthesis was carried out using 0.1 M solutions of phosphoramidites **5.5a-d** in dry ACN with 0.25 M ETT as the activator. All other ancillary agents necessary for oligonucleotide synthesis were obtained commercially. The detritylation step used 3% trichloroacetic acid (TCA) for 80 s. Each phosphoramidite coupling step was set for 10 min. The capping step (using CAP A and CAP B) was set for 20 s and the oxidization step using 0.1 M iodine/pyridine/water/THF was 30 s.

5.16.5 Release of 2'-*O*-ALE Oligonucleotide From Solid Support

The fully protected oligonucleotide is first treated with a solution of 2:3 TEA/MeCN on column for 16 hr by pulsing the solution through the column every hour. The solid support is then transferred to an eppindorf tube and 1 mL of 2:3 TEA/MeCN is added and the material is heated at 50 °C for 1 hr. The material is then centrifuged at 14 000 rpm and the supernatant is removed and discarded. The solid support is then washed 3 x 1 mL of MeCN and is dried. The material is then transferred to a quartz test tube and 1mL of 1% DIPEA in MeCN is added. This mixture is then placed in a transilluminator and irradiated using U.V. light in the 350-365 nm range for 20 min. The mixture was then transferred to an eppindorf tube and centrifuged at 14 000 rpm. The supernatant was removed an placed in a 2 mL eppindorf tube and the solid support was washed 4 x 250 uL of 1:1 EtOH/H_2O (v/v) and the washes were combined with the original supernatant and evaporated. The material was then ready for analysis.

5.16.6 Release of 2'-*O*-'Acetal Ester Pyrrolidine' Oligonucleotide From Solid Support

The fully protected oligonucleotide is first treated with a solution of 2:3 TEA/MeCN on column for 16 hr by pulsing the solution through the column every hour. The solid support is then transferred to an eppindorf tube and 1 mL of 2:3 TEA/MeCN is added and the material is heated at 50 °C for 1 hr. The material is then centrifuged at 14 000 rpm and the supernatant is removed and discarded. The solid support is then washed 3 x 1 mL of MeCN and is dried. Dichloroethane (250 uL) is added to the solid support followed by a mixture of 3 uL of pyrrolidine, 3.4 uL of HOAc in 50 uL of dichloroethane. This is allowed to mix at room temperature for 20 min followed by the addition of 5 mg of sodium triacetoxy borohydride. The entire mixture is allowed to shake for 8 hr followed by centrifugation. Because the solid support floats on the surface, the liquid is carefully sucked out and the solid support is washed 4 x 1 mL with MeCN followed by 4 x 1 mL water and it is dried. The acetal ester pyrrolidine material is then transferred to a quartz test tube and 1mL of 1% DIPEA in MeCN is added. This mixture is then placed in a transilluminator and irradiated using U.V. light in the 350-365 nm range for 20 min. The mixture was then transferred to an eppindorf tube and centrifuged at 14 000 rpm. The supernatant was removed an placed in a 2 mL eppindorf tube and the solid support was washed 4 x 250 uL of 1:1 EtOH/H_2O (v/v) and the washes were combined with the original supernatant and evaporated. The material was then ready for analysis.

5.16.7 siRNA Assays

HelaX1/5 cells that stably express firefly luciferase were grown as previously described [36]. The day prior to transfection, 0.5 x 10^5 cells were plated in each well of a 24-well plate. The next day, the cells were incubated with increasing amounts of siRNAs premixed with lipofectamine-plus reagent (Invitrogen) using 1 μL of lipofectamine and 4 μL of the plus reagent per 20 pmol of siRNA (for the highest concentration tested). For the siRNA titrations, each siRNA was diluted into dilution buffer (30 mM HEPES-KOH, pH 7.4, 100 mM KOAc, 2 mM $MgOAc_2$) and the amount of lipofectamine-plus reagent used relative to the siRNAs remained constant. The crude 2'-*O*-ALE (1 nmol) and 2'-*O*-'amino' (1 nmol) siRNA was hybridized to a 2'-F complementary strand (1 nmol). 24 hours after transfection, the cells were lysed in hypotonic lysis buffer (15 mM K_3PO_4, 1 mM EDTA, 1% Triton, 2 mM NaF, 1 mg/ml BSA, 1 mM DTT, 100 mM NaCl, 4 μg/mL aprotinin, 2 μg/mL leupeptin and 2 μg/mL pepstatin) and the firefly light units were determined using a Fluostar Optima 96-well plate bioluminescence reader (BMG Labtech) using firefly substrate.[37] The luciferase counts were normalized to the protein concentration of the cell lysate as determined by the DC protein assay (BioRad). Error bars represent the standard deviation of two transfections.

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Chapter 6. Contributions to Knowledge

6.1 General Conclusions and Future Work

6.1.1 The 2'-*O*-Levulinyl (Lv) Group for the Solid Phase Synthesis of RNA

The 2'-*O*-Lv group was investigated as a 2'-OH protecting group for the synthesis of RNA on solid support. *N*-Lv (dmf) protected 2'-*O*-Lv 3'-*O*-phosphoramidite monomers were efficient in the solid-phase synthesis of oligoribonucleotides. Homo-oligomers and mixed-nucleobase RNA oligomers of up to 21 nt in length were prepared in reasonable yields and with a high degree of purity. The RNA strands synthesized by this approach were examined by HPLC, mass spectrometry, thermal denaturation and RNAi gene knockdown. The major advantage of the Lv group over other 2'-protecting groups was its *on-column* unblocking at the end of the synthesis which greatly simplified post-synthetic processing. However, the final deblocking step required a fluoride source to cleave the unprotected RNA strand from a hydroquinone-*O*,*O*'-diacetate (Q-linker) controlled-pore glass solid support. In addition, 2'-*O*-Lv/3'-*O*-Lv isomerization led to low yields of the desired 2'-*O*-Lv phosphoramidite monomers. These findings paved the way to the development of the 2'-Acetal Levulinyl Ester (ALE) Group.

6.1.2 The 2'-Acetal Levulinyl Ester (ALE) Group for RNA Synthesis

The acetal levulinyl ester (ALE) group for the 2'-hydroxyl protection was developed to circumvent the drawbacks of the 2'-*O*-Lv protecting group associated with its isomerization and difficult separation of the isomeric 3'-*O*- and 2'-*O*-Lv-protected ribonucleoside phosphoramidites. Indeed we found that the 2'-*O*-ALE group does not undergo 2'/3'-isomerization and hence allows for the preparation ribonucleoside phosphoramidites in higher yields. These synthons were used in the solid-phase synthesis of various oligoribonucleotides on Q-linker CPG support with coupling efficiency over 99%. The applicability of the 2'-*O*-ALE protection to the preparation of mixed base siRNA that effected gene

silencing in cell based assays was also demonstrated. The compatibility of the ALE group with the acidic conditions employed for the iterative cleavage of the 5'-*O*-DMTr group during chain assembly, and the facile cleavage of the ALE groups from 2'-*O*-ALE-protected RNA chain under essentially neutral conditions (i.e., buffered hydrazine/pyr/AcOH) are attractive features of this approach. The applicability of the 2'-*O*-ALE protection to the preparation of RNA of mixed base composition was also demonstrated.

As observed previously with 2'-*O*-Lv monomers, deprotection of RNA chains made from ALE amidites can be effected while the oligonucleotide is immobilized on the CPG support. Final release of the RNA chain necessitates overnight treatment with 1 M TBAF at room temperature. This treatment also completed the removal of any ALE groups remaining after the hydrazine treatment. While the presence of fluoride did not pose problems in the purification of the desired oligomers, this treatment is undesirable if the RNA strand is to be released from the support with its ALE groups still attached to the ribose moieties (see below).

A remaining aspect of ALE chemistry that needs improvement is the multiple steps required to install Lv and dmf groups at the exocyclic amino functions. As these protecting groups fall off during installation of the 2'-*O*-ALE group, it was necessary to transiently protect the amines as *N*-carbamate (*N*-FMOC) derivatives. One way to circumvent this would be to switch to the conventional *N*-acyl (Bz, iBu) protecting groups, as these can be removed at the end of synthesis during the same conditions that unblock the phosphate and 2'-hydroxyl functions, e.g., A) 1) 2:3 triethylamine/acetonitrile, 60 min, r.t.; 2) ammonium hydroxide, 3 hr, r.t. or B) 1) 2:3 triethylamine/acetonitrile, 60 min; 2) 1:1 EDA/toluene, 3 hr, r.t. In addition, this protecting group strategy would allow for novel *on-column* conjugations since, in principle, the orthogonal 2'-*O*-ALE group could be selectively removed with 0.5 M hydrazine hydrate in 3:2 pyridine/acetic acid in the presence of other 2'-*O*-TBDMS RNA units without affecting the nucleobase protecting groups (*N*-Ac Cyt, N-Bz Ade, and N-iBu Gua). These research endeavors are being pursued in the Damha lab.

6.1.3 The Light Directed Synthesis of RNA Microarrays

In an effort to grow and fully deprotect RNA on any surface, specifically for the creation of 'RNA chips', we synthesized and investigated the properties novel 2'-*O*-ALE RNA monomers. The results demonstrated the first example of *in situ* RNA synthesis on microarrays, onto which nucleic acid hybridization and protein-nucleic acid interaction could readily be monitored fluorometrically. This effective strategy necessitated the introduction of a photolabile protecting group at 5'-*O*-position (NPPOC), in conjunction with our in-house ALE chemistry previously developed. Ultimately we hope that these RNA chips can become a routine part of biochemical studies that examine RNA-RNA, RNA-protein, and RNA-small molecule interactions of many different biological systems. For example, many proteins in the cell have a regulatory function and must bind to specific RNA sequences. Binding affinities of proteins to selected libraries of RNA sequences on microarrays provides valuable data on RNA-protein interaction and recognition, and therefore, on the regulatory mechanism. These studies are ongoing in collaboration with Mark Somoza from the University of Geneva, Franco Cerrina from Boston University, and Marvin Wickens from the University of Madison-Wisconsin.

6.1.4. Synthesis and Biological Evaluation of Pro-siRNA

A major consideration in nucleic acid-based drug development is the delivery of an siRNA drug candidate to the right tissue/organ, right cell type, and right cell compartment (cytoplasm). Towards the development of siRNA as drugs, one attractive alternative to the use of liposomal formulations is the covalent conjugation of an siRNA in order to enhance its cell uptake and deliver it to the desired cellular substructures where mRNA is found. These chemically modified siRNAs can in principle address other shortcomings of siRNA therapeutics,

namely, nuclease stability can be improved, and immunostimulation and off-targeting can be reduced.

In this regard, 2'-*O*-acetalester protecting groups are being developed for production of "proRNA," which remains protected until unmasked by cellular esterases. These molecules may find application as "pro-*siRNA*". In a similar fashion, we have adapted 2'-*O*-Lv and ALE chemistry to create potential siRNA prodrugs. Synthesis of these pro-siRNAs required the design and preparation of N-FMOC 5'-*O*-DMTr 2'-*O*-ALE (and 2'-*O*-TBDMS) ribonucleoside 3'-*O*-phosphoramidite synthons, as well as deprotection conditions that remove phosphate and base protecting groups without cleaving the 2'-*O*-acetal ester moieties. We succeeded at synthesizing 2'-*O*-ALE and 2'-*O*-'acetal ester pyrrolidine' siRNAs, as confirmed by ^{31}P NMR, HPLC and mass spectrometry. However, the 2'-*O*-acetal groups underwent cleavage under neutral and slightly basic aqueous solution making purification of these pro-siRNAs a major challenge. To alleviate this problem, the RNA strands were grown onto a novel solid support with a light sensitive linker moiety. Release of the 2'-*O*-modified RNA strand by photolysis in an organic solvent afforded the desired modified siRNAs. However, as soon as the pro-siRNA was taken up on water it began to gradually cleave its 2'-*O*-acetal ester groups.

In a HeLa cell assay that over expresses luciferase, we were able to inhibit gene expression with native and modified siRNAs. However, under the experimental conditions, gene silencing could not be observed in the absence of a liposomal transfecting agent. A pro-siRNA containing a modified sense strand (2'-*O*-ALE or 2'-*O*-'acetal ester pyrrolidine') had gene silencing activity, *albeit* slightly less than the unmodified siRNA. These results suggest that either 2'-*O*-ALE and 2'-*O*-'acetal ester pyrrolidine' moieties are fairly well-tolerated by the RNAi machinery or that these moieties undergo extensive (but not quantitative) cleavage during transfection. In collaboration with Dr. Cy Stein (Albert Einstein Institute) we are currently delivering our pro-siRNA under conditions that do not require a transfecting agent. Stein's group have found that if factors such as cell plating density, oligonucleotide chemistry and concentration, and experimental

duration are considered, that naked (= gymnos, from Greek, hence gymnotic) delivery of oligos can produce >95% silencing of protein and mRNA expression (Stein, C. A.; Hansen, J. B.; Lai, J.; Wu, S.; Voskresenskiy, A.; Hog, A.; Worm, J.; Hedtjarn, M.; Souleimanian, N.; Miller, P.; Soifer, H. S.; Castanotto, D.; Benimetskaya, L.; Orum, H.; Koch, T. *Nucl. Acids Res.* **2009**, online.). Gymnotic delivery and sequence-specific silencing have been demonstrated with multiple targets, including Bcl-2, survivin and the androgen receptor in numerous cell lines, including, among others, six melanoma lines, and the PC3 and LNCaP prostate cancer lines (personal communication). With optimal experimental manipulation, these researchers have demonstrated continuous silencing in 518A2 melanoma cells for >180 days, with complete reversal after 3 days in the absence of added oligo. These assays are therefore ideal to assess whether our pro-siRNA undergo conversion to unmodified siRNA, and to compare the uptake and potency of pro-siRNA vs siRNA. These experiments are in progress.

Overall, the utility of novel 2'-O-Lv and ALE monomers for the synthesis of RNA and 2'-modified RNAs, as well as RNA on microarrays has been suitably demonstrated. The future looks even more promising.

6.2. Contributions to Knowledge

As a direct result of the studies described herein, the following publications and patent filings have recently emerged, have been submitted for publication, or are currently in preparation:

6.2.1 Manuscripts

Lackey, J.G.; Johnsson, R.; Damha†, M.J. Synthesis and biological evaluation of 2'-*O*-acetal esterified siRNA prodrugs, in preparation.

Lackey, J.G.; Somoza, M.M.; Mitra, D.; Cerrina, F.; Damha†, M.J. In-situ Chemical Synthesis of RNA-DNA Chimeras on Chips and Enzymatic Recognition. *Chemistry Today*. **2009**, 27(6), 30-33.

Lackey, J.G.; Mitra, D.; Somoza, M.M.; Cerrina, F.; Damha, M.J. Acetal Levulinyl Ester (ALE) Groups for 2'-Hydroxyl Protection of Ribonucleosides in the Synthesis of Oligoribonucleotides on Glass and Microarrays. *Journal of the American Chemical Society*. **2009**, 131(24), 8496-8503.

Lackey, J.G.; Damha, M.J. The acetal levulinyl ester (ALE) group for the 2'-hydroxyl protection of ribonucleosides and the synthesis of oligoribonucleotides. *Nucleic Acids Symposium Series*. **2008**, 52, 35-36.

Lackey, J.G.; Ron, D.; Damha, M.J; Harding, H.P. Toward the discovery of new antifungal agents: The design and validation of a novel 2'P-RNA probe and HTS assay against 2'-phosphotransferase Tpt1p. *Nucleic Acids Symposium Series*. **2008**, 52, 475-476.

Harding, H.P.; Lackey, J.G.; Hsu, H.; Zhang, Y.; Deng, J.; Xu, R.; Damha, M.J.; Ron, D. An intact unfolded protein response in Trpt1 knockout mice reveals phylogenic divergence in pathways for RNA ligation. *RNA*. **2008**, 14, 225-232.

Lackey, J.G.; Sabatino, D.; Damha, M.J. Solid-phase synthesis and on-column deprotection of RNA from 2'- (and 3'-) O-Levulinated (Lv) ribonucleoside monomers. *Organic Letters*. **2007**, 9(5), 789-792.

6.2.2. Patents

Lackey, J.G.; Mitra, D.; Wickens, M.; Damha, M.J.; Cerrina, F. International patent application PCT/CA2006/001244 entitled *"RNA Monomers and Microarrays"* filed September 4, 2009 claiming priority to United States provisional patent application US 61/181,562 and United States provisional patent application US 61/094,525 of the same title filed May 7, 2009 and September 5, 2008, respectively.

6.2.3 Conference Presentations

Lackey, J.G.; Damha, M.J. *The synthesis and biological evaluation of siRNAs, RNA microarrays and siRNA prodrugs*. CSC, Hamilton, May 30 – June 3, **2009**.

Lackey, J.G.; Mitra, D.; Cerrina, F.; Damha, M.J. *The acetal levulinyl ester (ALE) group for the synthesis of siRNA and light directed synthesis of RNA microarrays*. 18th IRT and the 35th International Symposium on Nucleic Acids Chemistry, Kyoto University, Kyoto, Japan, Sept. 8-12, **2008**.

Lackey, J.G.; Damha, M.J. *2'-O-ALE (acetal levulinyl ester) and 2'-O-ester groups for 2'-hydroxyl protection in the solid-phase synthesis and delivery of siRNA.* 234th ACS National Meeting, Boston, MA, USA, Aug. 19-23, **2007**.

Lackey, J.G.; Damha, M.J. *Solid-phase RNA synthesis from 2'-O-levulynyl (Lv) protected ribonucleosides*. 17th QOMSBOC, University of Western Ontario, London, Ontario, Canada, Nov. 3-5, **2006**.

Lackey, J.G.; Mitra, D.; Cerrina, F.; Damha, M.J. *Light Directed Synthesis of RNA Microarrays.* 4th Annual Meeting of the Oligonucleotide Therapeutics Society, New York Academy of Science, Boston, MA, U.S.A., Oct. 15-18, **2008.**

Lackey, J.G.; Ron, D.; Damha, M.J.; Harding, H.P. *Toward the discovery of new antifungal agents: The design and validation of a novel 2'P-RNA probe and HTS assay against yeast Tpt1p*. 18th IRT and the 35th International Symposium on Nucleic Acids Chemistry, Kyoto University, Kyoto, Japan, Sept. 8-12, **2008**.