INAUGURAL–DISSERTATION

Zur Erlangung der Doktorwürde

der

Naturwissenschaftlich–Mathematischen Gesamtfakultät

der

Ruprecht-Karls-Universität

Heidelberg

Vorgelegt von

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Tag der mündlichen Prüfung: 09.07.2009

Studies on the Chemical Biology of Natural and Chemical Ribonucleotide Modifications

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Die vorliegende Arbeit wurde in der Arbeitsgruppe von PD. Dr. Mark Helm in der Zeit von Nov. 2004 bis August 2008 am Institut für Pharmazie und Molekulare Biotechnologie (IPMB), Abteilung Chemie der Fakultät für Biowissenschaften der Ruprecht-Karls-Universität Heidelberg angerfertigt.

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Salifu Seidu-Larry

Acknowledgement

I first give thanks to the Almighty God whose grace has been part of this long journey away from the bigger family and relations.

I would like to thank PD Dr. Mark Helm who gave me the opportunity to undertake my PhD thesis in his group and for being a dedicated and patient supervisor whom I could consult for any questions. I really appreciated the interesting discussions in his office during the later-months of the PhD. I am also very thankful to PD Dr. Mark Helm for his patient and also the interesting project that I was in charge of and especially the freedom of creating my own ideas during the work. I should say that I did not only learn science but human relations as well.

I want to say profoundly from the bottom of my heart a very big thank you to the following persons: Prof. Dr. A Jäschke, first for being a co-supervisor, and creating a scientifically conducive atmosphere for work and lastly for the knowledge impacted during my stay in Heidelberg. I also say a big thank you to Prof. Dr. Jürgen Reichling and Prof. Dr. Gert Fricker who both opted to be part of the final defence examining board. To Frau Viola Funk and and Frau Karin Weiss whose help in all spheres (work and social) are unquantifiable I say (Nye yi wala do on) thank you.

Thanks also go to Herrn Heiko Rudy, Tobias Timmerman and Frau Sandra Suhm for the technical and friendly support during my work here. I would very much express my feeling of warmth to Markus Petermeier and Ayan Samanta who do manage out of the precious time of a Phd student to spend some time with my family. Also of whose special visit to my two little girls I consider invaluable is Florentine Wahl. The ‘Seidu-Larrys’ do appreciate it and we thank you immensely. Many thanks to all the former lab members who helped me acclimatize to Heidelberg: Julius M., Felix V-H., Roberto F., etc to mention a few. Thanks also go to Felix V.-H., Niklas F. and Barbara S. W. for the night life in Heidelberg before I got married. The Wednesday morning soccer section which got me hospitalized also send memories of the nice time we shared among Pierre Fournier, Richard Wombacher, Markus L Petermeier and the members of the AK Metzler-Nolte. Many thanks to Mihaela who got me oriented into the use of most of the gadgets in the lab not forgetting Steffi Pfander too. Those of us who share a common reading desk or area: Martin Hengesbach who helped for the correction of the diffult ‘stress-induced grammar’, Armine Hayrapetyan who coached me on UV melting curves and a lot more, thank you soo soo much. Markus Hirsch welcome back to AK Helm. Thanks go also to the

following: Alexander Nierth, Anna Wiesmayr, Juliane Schoch, Marco Singer, Marie-Luiz Winz, Michaela Kotaskova, Olwen Domingo, Steffi Draeger, Stefanie Gehrig, Steffi Kraut and Valeska Dernedde for creating a friendly working environment. To Mr. 'Sandy'Ameta Sandeep, I am more than grateful for you keeping vigil with me in order to get this thesis into a printable manuscript. Enver Cagri, Lars Ludicke, Stefanie Kellner, Dominic Riedel, Anne Neef and Chandrabali Bhattacharya who contributed in every small way during the time we spent together in the Lab, to you I say (Ayekoo) thank you in abundance. To those who by virtue of me being stress-hung I forgot that we spend some valuable time during this work, thank you too.

To the very 'old padies' Kristof Lindner and Karifala Dumbuya your friendship have I always cherished. To my Ghanaian links in Heidelberg, Maame Manful Theresa, Ernest Osei and David Ankrah thanks for the nice times spent together here in Heidelberg. My final and my hearthy gratitude go to my wife Frau Belinda S.-Larry for the patience, love and warmth we shared. I do not forget the two 'lovely girls' Moindan and Todenn who tried helping Papa type during the night. Before I finally say Tschuss, I say a big thank you to my family back home in Ghana especially my mum whose constant encouragement makes life worth a journey.

Summary **Seidu-Larry, Salifu; M. Sc. Chem.**

Title: Studies on the chemical biology of natural and chemical ribonucleotide modifications

1. Examiner: Prof. Dr. Andres Jäschke
2. Examiner: PD. Dr. Mark Helm

This work is centered on the synthesis and chemical recognition of modified RNA. Phosphoramidites of m^5U, Ψ, m^1G, m^2G and $m^2{}_2G$ were synthesized and were incorporated into RNA oligomers by standard *SPS*. The synthesized oligomers were used for two purposes: (i) the total construction of tRNA *via* enzyme catalyzed ligation, and (ii) investigations of modifications in siRNAs.

The construction of human mitochondrial $tRNA^{Ile}$ (i) with and without modification was achieved in a one-pot 3 fragments ligation using T4-DNA-ligase with a DNA-splint. The yield for the ligation was 20-30 % and the products were further investigated for the stability of the tertiary structure *via* UV-melting curve analysis. The unmodified tRNA was found to be significantly less stable than the fully modified tRNA.

In the second case (ii) RNAi efficiency and the immunostimulation in cells were investigated with 22 nucleotide long double-stranded siRNAs that were synthesized containing the modifications m^1G, m^2G, $m^2{}_2G$, rT and Ψ in varying stochiometry. The investigations showed differential effects in knock-down as well as immunostimulation.

In a related perspective, compounds for the chemical recognition of modifications in RNA were synthesized. Since the recognition of pseudouridine by 4-bromomethyl-coumarin derivates is of particular interest, 7-azido-4-bromomethyl-coumarin was obtained with an overall yield of 44 % in a 5-step synthesis. Another derivative, the biotinylated 4-bromomethyl-coumarin spaced with jeffamine-148 was obtained in a 6-step synthesis with an overall yield of 17 %, to allow the use of strepavidin for affinity separation.

In the course of further investigations of coumarine conjugates, mild and bio-compatible conditions for the syntheses of rhodols and rhodamines were discovered. The conversion of fluorescein into the 3'-mesylated or 3'-6'-dimesylated derivate and the subsequent displacement of the mesylate with a nitrogenous nucleophile gave rise to rhodols or rhodamines, respectively. As these reaction conditions are compatible with biomolecular labeling, the tagging of polypeptides was investigated. Tri-and pentapeptides were synthesized, and dye-labeled via 3'/6'-xanthene substitution, and the product identity confirmed by MALDI-TOF-MS. Further syntheses via 3'/6'-xanthene substitution lead to moderate yields of 3',6'-bis(dimethylamino)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-3-one, 3',6'-di(piperidin-1-yl)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-3-one and the 3',6'-bis(2-(2-(2-aminoethoxy)ethoxy)ethyl amino)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-3-one.

Zusammenfassung **Seidu-Larry, Salifu; M. Sc. Chem.**

Title: Untersuchungen zur Chemischen Biologie von natürlichen und chemischen Ribonukleotidmodifikationen

1. Gutachter: Prof. Dr. Andres Jäschke
2. Gutachter: PD. Dr. Mark Helm

Im Mittelpunkt dieser Arbeit stehen die Synthese und chemische Erkennung von modifizierter RNA. Die Phosphoramidite von m^5U, Ψ, m^1G, m^2G und $m^2{}_2G$ wurden synthetisiert und mittels Standard-Festphasensynthese in RNA-Oligonukleotide inkorporiert. Die synthetisierten RNA Oligomere wurden für zwei Zwecke verwendet: (i) die Totalsynthese von tRNA mittels enzymkatalysierter Ligation und (ii) Untersuchungen zu Modifikationen in siRNAs.
Die Synthese von humaner mitochondrialer $tRNA^{Ile}$ (i) mit und ohne Modifikationen wurde in einer Eintopfligation von drei Fragmenten durch T4-DNA-Ligase mit einem DNA-Splint erzielt. Die Ausbeuten lagen zwischen 20-30 %. Die Produkte wurden durch UV-Schmelzkurvenanalyse auf Stabilität der Tertiärstrukturen untersucht. Hierbei zeigte sich, dass modifizierte tRNA signifikant stabiler als die unveränderte tRNA war.
Im zweiten Fall, (ii) wurden RNAi Effizienz und zelluläre Immunstimulation von, doppelsträngigen siRNAs (22 Nukleotide) untersucht, welche mit den Modifikationen m^5U, Ψ, m^1G, m^2G und $m^2{}_2G$ in unterschiedlicher Stöchiometrie synthetisiert worden waren. Die Untersuchungen zeigten differenzierte Effekte in Knock-Down und Immunostimulation.
Vor diesem Hintergrund wurden Verbindungen zur chemischen Erkennung von Modifikationen in RNA synthetisiert. Weil der Erkennung von Pseudouridin durch Derivate von 4-Brommethylcumarin eine besondere Bedeutung zukommt, wurde 7-Azid-4-brommethyl-coumarin mit einer Gesamtausbeute von 44 % in einer fünfstufigen Synthese erhalten. Ein weiteres Derivat wurde in einer sechsstufigen Synthese mit einer Gesamtausbeute von 17 % erhalten. Dieses biotinylierte 4-Brommethylcumarin, welches ein Jeffamine-148 als Abstandshalter enthält, ist für den Gebrauch in der Affinitätsreinigung mittels Streptavidin einsatzbereit.
Während weiterer Untersuchungen zu Konjugaten von Cumarinen wurden schonende und bio-kompatible Bedingungen für die Synthesen von Rhodolen und Rhodaminen entdeckt. Die Umwandlung von Fluorescein in 3'-mesylierte beziehungsweise 3'-6'-dimesylierte Derivate, gefolgt von einer nukleophilen Substitution der Mesylate durch Stickstoffnukleophile führte zu Rhodolen beziehungsweise Rhodaminen. Da sich diese Bedingungen als kompatibel mit der Markierung von Biomolekülen erwiesen, wurden Konjugationsreaktionen mit Peptiden untersucht. Tri- und die Pentapeptide wurden synthetisiert, per 3'/6'-Xanthen-Substitution markiert, und die Identität der Produkte wurde durch MALDI-TOF-MS bestätigt. Weitere Untersuchungen zur 3'/6'-Xanthen-Substitution führten zu Synthesen von 3',6'-Bis(dimethylamino)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-3-on, 3',6'-Di(piperidin-1-yl)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-3-on und 3',6'-Bis(2-(2-(2-aminoethoxy)ethoxy)ethyl amino)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-3-on in moderaten Ausbeuten.

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List of Abbreviations

δ	Chemical shift
ε	Molar extinction coefficient
λ	Wavelength
A	Adenosine; Peak area
A260	Absorbance at 260 nm
Ac	Acetyl
AcOH	Acetic acid
AN	Acetonitrile
AIBN	2,2′-azobisisobutyronitrile
ATP	Adenosine triphosphate
Av	Average
bd	Broad doublet
$BenzN_2^+$	*p*-*N*-dimethylaminobenzendiazonium cation
bpa	*N*,*N*′-Bis(2-picolyl)amine
bpy	2,2′-Bipyridine
bs	Broad singlet
BTT	5-Benzylthio-(1*H*)-tetrazole
c	Concentration
C	Cytidine
CEP-Cl	2-cyanoethyl diisopropylphosphoramidochloridite
Ci	Curie; 1Ci = 37 MBq
CID	Collision-induced dissociation
cod	1,5-cyclooctadiene
CPG	Controlled pore glass
CMCT	1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate
d	Doublet
dA	2′-Deoxy-adenosine

DAD	Diode-array detector
dC	2′-Deoxycytidine
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DCE	1,2-dichloroethane
DEA	Diethylamine
DEAD	Diethyl azodicarboxylate
dG	2′-Deoxy-guanosine
DIAD	Diisopropyl azodicarboxylate
DIEA	Diisopropylethylamine
DIPA	Diisopropylamine
DMAP	*N,N*-dimethylpyridin-4-amine
DMF	*N,N*′-Dimethylformamide
DMSO	Dimethyl sulfoxide
DMT	4,4′-Dimethoxytrytil
DMT-Cl	4, 4′-Dimethoxytrityl chloride
DNA	Deoxyribonucleic acid
dppz	Dipyridophenazine
EA	Ethylacetate
EDC	*N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride
EDTA	Ethylenediamine tetraacetate
ee	Enantiomeric excess
EI	Electron impact
equiv	Equivalent
ESI	Electrospray ionization
EtOH	Ethanol
FAB	Fast atomic bombardment
fCC	flash Column Chromatography
FLD	Flourescence detector
FT-ICR	Fourier-transform ion cyclotron resonance
g	Gram

G	Guanosine
h	Hour(s)
Hepes	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
Hex	Hexane
HPLC	High Pressure Liquid Chromatography
I	Spin quantum number
I	Light intensity
J	Coupling constant
K	Reaction rate constant
l	Length of the light path
L	Liter; Ligand
LG	Leaving group
m	Meter; Multiplet
M	Mol/L; Molar; Transition metal
MALDI-TOF	Matrix assisted laser desorption ionization time-of-flight
MS 4Å	4Å molecular sieves (activated overnight at 180 °C and 0.01 mbar)
Me	Methyl
Me2-dppz	7,8-Dimethyldipyridophenazine
min	Minute(s)
MS	Mass spectrometry
Ms	Methane sulfonyl
MVS	methyl vinyl sulfone
n	number of nucleotides in a chain
NHS	*N*-hydroxysuccinimide, *N*-hydroxysuccinimidyl
nm	Nanometer
NMR	Nuclear magnetic resonance
Nu	Nucleophile
ODN	Oligodeoxynucleotide
Pa	Pascal
PAGE	Polyacrylamide gel electrophoresis

PCR	Polymerase chain reaction
PG	Protecting group
Ph	Phenyl
PhCN	Benzonitrile
phen	10-Phenanthroline
phi	9,10-Phenanthrenequinone diimine
PHOX	2-(2-Diphenylphosphino-phenyl)-4,5-dihydrooxazole
PNK	Polynucleotide kinase
ppm	Parts per million
PPTS	pyridinium p-toluene-sulfonate
Pr	Propyl
PTFE	Polytetrafluoroethane
Pyr	Pyridine
RBF	Round Bottom Flask
RedAl	Sodium bis(2-methoxyethoxy)aluminium hydride
RNA	Ribonucleic acid
rpm	Rotations per minute
rt	Room temperature (ca. 20 °C)
RT	Reverse transcription
s	Singlet
SDS	Sodiumdodecyl sulfate
sec	Second
S_N	Nucleophilic substitution
Solv	Solvent
SPOS	Solid phase oligomeric synthesis
SPPS	Solid phase peptide synthesis
SPS	Solid phase synthesis
t	Triplet
T	Thymidine; Temperature
TAC	(*t*-Butyl)phenoxyacetyl

tap	1,4,5,8-Tetraazaphenantrene
TBDMS-Cl	tert-butyldimethylsilyl chloride
TBE	Tris-borate-EDTA buffer
TCA	Trichloroacetic acid
TEA	Triethylamine
TEAA	Triethylammonium acetate
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
Tm	Melting temperature
TMS-Cl	Trimethylsilyl chloride(Me_3Si-Cl)
TOM-Cl	triisopropylsilyloxymethyl chloride
TPPDS	Bis(4-sulfonatophenyl)phenylphosphine
tpyr	2,2′:6′,2″-Terpyridine
tR	Retention time
Tris	Trishydroxymethylaminomethane; 2-amino-2-hydroxymethyl-1,3-propanediol
U	Uridine; Unit
UV	Ultraviolet

1 Introduction

1.1 Modification of RNA by Enzymes.

1.1.1 RNA

Ribonucleic acid (RNA) is a type of chain polymeric molecule that consists of nucleotides as monomeric units. They were discovered by Friedrich Miescher in 1868 in the nucleus and were called 'nuclein' [1]. Each nucleotide consists of a purine or pyrimidine base, a ribose sugar and a phosphate. RNA is very similar to DNA, but differs in a few important structural details: in the cell RNA is usually single-stranded whilst DNA is double stranded. On the chemical level RNA nucleotides contain the ribose whilst the DNA contains deoxyribose, also in RNA the base uracil is replaced with thymine in DNA as shown in Figure 1. The bases in nucleic acids do pair by H-bond formation resulting in structural rigidity through folding.

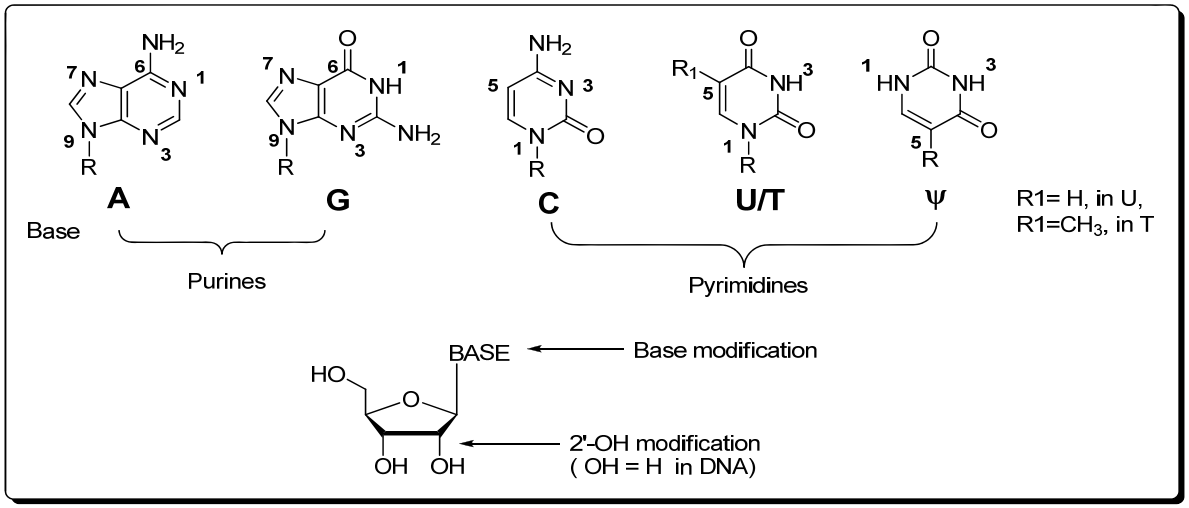


Figure 1 Schematic representation of nucleic acids

The H-bonds in nucleic acids do follow Watson-Crick rule where the bases may form H-bonds between cytosine and guanine, and that between adenine and uracil/thymine. There is also the case of the wobble H-bonding between guanine and uracil which mainly occurs in RNA as

shown in Figure 2 [2]. It is known that for equivalent complementary sequences, the RNA duplex is more stable than the DNA duplex under most laboratory and physiological conditions. Traditionally, this has been explained by a variety of factors, including differential stacking, sugar puckering, and solvation [3-5].

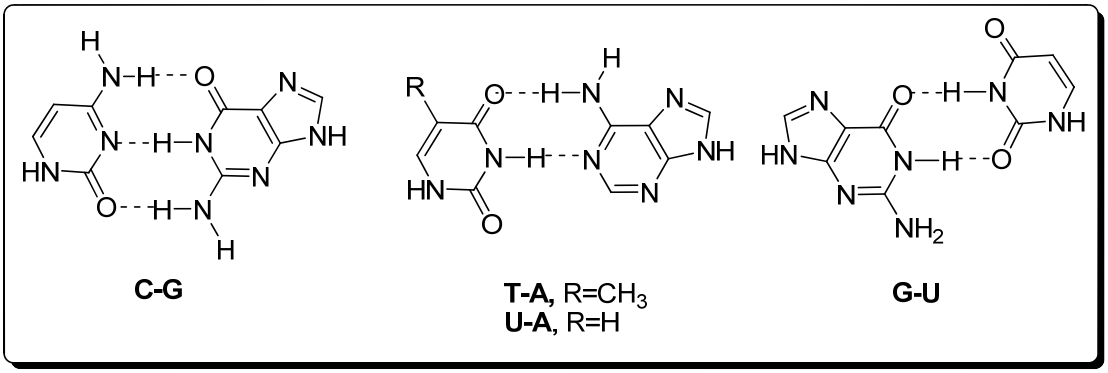


Figure 2 Base pairing in RNA and DNA

Recently, it was shown in non-base-paired mononucleotides that the difference in pKa values of rA and rU is less than that for dA and dT, from which it was inferred that RNA hydrogen bonds can be stronger than those of DNA [6]. In the cell the synthesis of RNA is performed on a DNA template *via* a process called transcription by enzymes called RNA polymerases. The transcribed RNA is further processed by other enzymes where several other activities are involved such as 5′-capping, poly(A) tailing and removal of introns by spliceosomes. There is messenger RNA which carries the code responsible for protein synthesis. Those RNAs that however do not code for proteins are called non-coding RNAs. Prominent examples of the non-coding RNAs are the transfer RNA (tRNA) and the ribosomal RNA (rRNA), both of which are involved in the process of translation. There are other non-coding RNAs that are implicated in the regulation of gene expression, RNA processing and other roles. Certain RNAs are able to catalyze chemical reactions such as the scissoring and joining of other RNA molecules [7], peptide bond formation in ribosomes [8] and that of the Diels-Alder reaction [9, 10], these are known as ribozymes. In general the role of RNA could be translational, regulatory, genomic, processing and in some cases transcriptional.

1.1.2 Natural Modification of RNA by Enzymes

Natural modification of RNAs was first reported in the works of Cohn *et al*. [11]. In 1951, a modified nucleoside differing from the four canonical nucleosides was isolated. This identified modification was later characterized as pseudouridine, a 5-ribosyl isomer of uridine [12]. This modification was found to be widely distributed in most types of RNAs (1-2 % of total RNA), with the noticeable exception of mRNA (although its absence remains to be definitively proven [13]. Other modified nucleosides such as m^1G, m^2G, $m^2{}_2G$ and m^5U are present in much smaller quantities, but they represent a large diversity of original structures and or functions and are mainly found in tRNAs with 66 % of the known modifications in RNA [13, 14]. There are about 32 known uridine modifications including Ψ. The known modified uridines include the following variations: Ψ m, $m^1Ψ$, $m^3Ψ$, and $m^1acp^3Ψ$. These modifications are found in rRNA, mRNA and mainly tRNA. There are about 13 different types of modifications in cytidine, about 23 in adenosine and over 30 of varying degrees of modifications in guanosine. A recent list of natural modifications in nucleic acids can be found in book chapter [15]. The structural and functional effects of these modifications are investigated using chemical and biological procedures. The chemical synthesis of RNA oligomers including base modifications *via* phosphoramidite chemistry, the specific reagents for recognition of such modifications, and the incorporation of fluorescence tagging moieties such as coumarin, rhodamine, cy3 etc which would allow the use of FRET for their investigation e. g. [16].

These are the four main areas that will be treated thoroughly, however, from the diverse nature of this topic; there is a general introduction to each of the four specific areas mentioned.

1.2 Phosphoramidite Synthesis including Modified Bases

1.2.1 Chemical Synthesis of Oligonucleotides

This technology, first developed for the preparation of DNA oligonucleotides and then adapted, taking into consideration the 2′-OH, to the synthesis of RNA oligonucleotides, is similar to other solid-phase methods, such as the solid-phase peptide synthesis (*SPPS*). A controlled pore-glass (CPG) solid support, with a long chain alkyl spacer functionalized with an amine is bonded with the first nucleoside *via* the terminal 3′-OH. The solid-phase synthesis is a stepwise coupling of

single building blocks, which are suitably orthogonally protected and functionalized. The product of the synthesis is then cleaved from the solid support, deprotected and then purified. As shown in Figure 3, such a single reaction cycle is schematically illustrated; the number (n) of repetition of this cycle leads to the desired oligonucleotide (n+1).

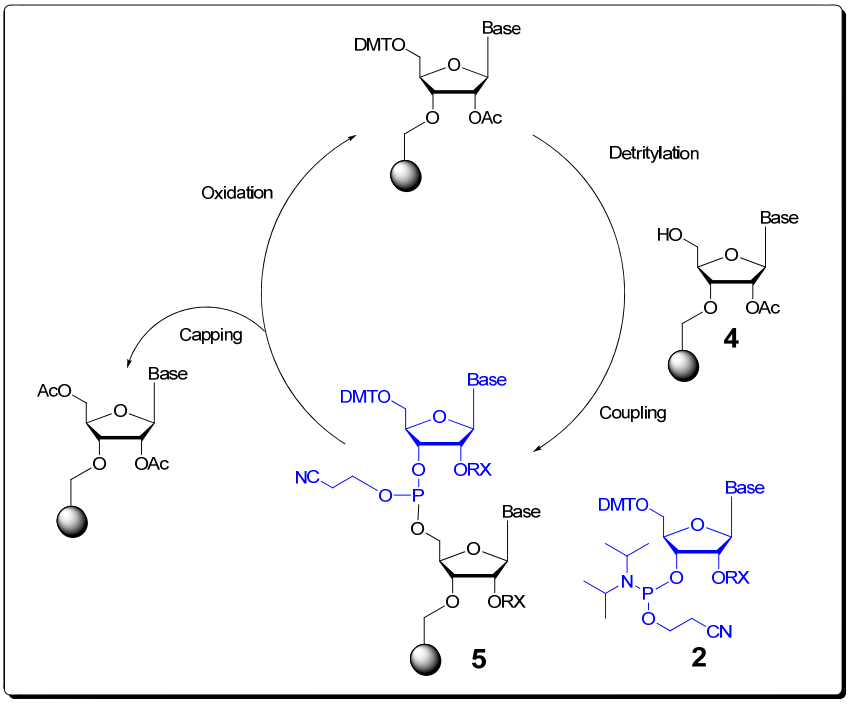


Figure 3 Schematic representation of SPOS.

The phosphoramidite building blocks are 5′-*O*-protected with a trityl moiety; usually the 4,4′-dimethoxytrityl (5′-*O*-DMT) protecting group, which is removed under acidic conditions Figure 3. This cleavage reaction, which constitutes the first step of the cycle, liberates the 5′-OH functionality. After washing to get rid of the cleaved trityl cation, a mixture of an appropriate phosphoramidite and 1*H*-benzylthio tetrazole (BTT) is added, which *in situ* react with each other under the formation of the activated form of the nucleotide; phosphorotetrazolide **3** shown in Figure 4. This reactive intermediate is then allowed to react with the previously liberated 5′-OH group of the growing chain.

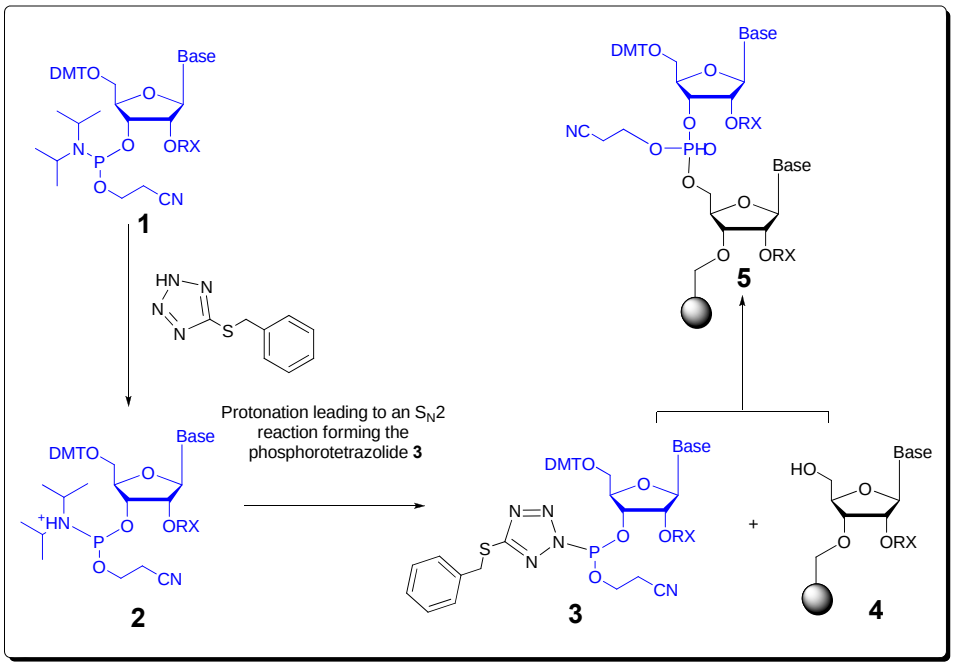


Figure 4 Activation and coupling during the *SPS*.

Although quite efficient, with usually 90-99 % yield per a cycle, this coupling step leaves a few unreacted 5′-OH groups, which later could lead to the formation of (shorter fragments) sequences that contaminate the full-length product. In order to prevent an accumulation of these undesired sequences, the unreacted nucleosides are blocked in a process called 'capping' by acetylation with acetic anhydride in the presence of *N*-methylimidazole and 2,6-lutidine. The phosphate triester obtained after coupling is prevented from degradation by oxidation with a mixture of iodine, lutidine and water. The considerations required to achieve a high-speed synthesis of RNA oligomer were described by Marshall *et al.* [17].

1.2.2 General Syntheses of Modified Phosphoramidites

The general considerations in phosphoramidite synthesis will be discussed bearing in mind the following three considerations:

1. Protection of *exo*-cyclic amines and/or other reactive functionalities on the nucleobase. In this respect base modification to achieve a desired goal is done under the protection of the 2′, 3′ and 5′-OHs after which they are cleaved.

2. The orthogonal protection of the 5′-OH and the 2′-OH groups taking into consideration the compatibility with the *SPS*.
3. Finally, the phosphitylation at the 3′-OH position allowing chain elongation.

For the complete list of available modifications synthesized or commercially available see the publication book chapter [15]. The detailed protocol for the synthesis of a particularly modified phosphoramidite will be discussed taking into consideration the type of modification, orthogonality of protection groups and introduction of the phosphitylation moiety as shown in Figure 5.

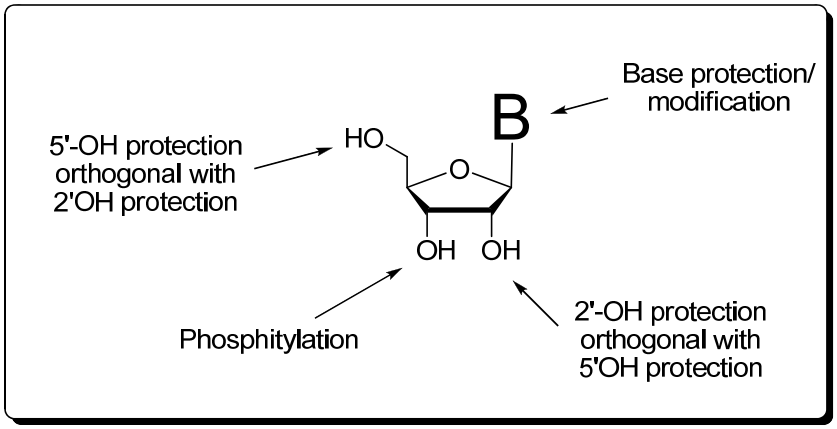


Figure 5 General scheme of phosphoramidite synthesis from nucleoside.

The differences in procedure for the synthesis of phosphoramidite depend on it being either

(i) a non base-protected phosphoramidite or
(ii) a base protected or modified phosphoramidite.

To illustrate examples of published syntheses of the two cases given will be discussed below i.e. synthesis of (i) m^5U and pseudouridine and (ii) m^1G, m^2G and $m^2{}_2G$ respectively.

1.2.3 Synthesis of m^5U Modified Phosphoramidite

In 1995 Soell *et al.* reported that 5-methyluridine (ribothymidine, rT), one of the most conserved modified nucleosides found in tRNAs is located at position 54 in the TψC loop [18]. This modification has been implicated to be introduced by methylation of uridine with the (m^5U54) methyltransferase. The mechanism of modification has simply been an extrapolated one [19] since

the sequence of the enzyme is similar to the enzyme thymidylate synthase, which generates (d)TMP from (d)UMP. A mechanism that involves a cysteine residue attacking the C6, generating an enolate and a nucleophilic C5, which subsequently allows reaction with an electrophilic methyl group has been implicated.

Alluringly, the nature of the methylating reagent depends on the organism. It is often SAM but can also be 5,10-methylenetetrahydrofolate which was reported by Delk *et al.*[20]. Furthermore, although the identification elements involved in the process depend on the organism, same dependence is observed for the nature of identity elements involved in the editing process. Whereas in yeast some specific nucleobases have been identified, in E.*coli* no such sequence specificity has been observed and the interaction depends on the structure of the T arm as was reported by Becker *et al.* [21].

The m^5U nucleoside is readily available commercially and as such the synthesis of the phosphoramidite involves standard procedures. The reported synthesis of the m^5U phosphoramidite involves the protection of the 2′-OH with TOM. The formation of the 2′-*O*-TOM protected 5-methyluridine **8** from the parent nucleoside is done in two steps, by first introducing the trityl-group with $(MeO)_2$Tr-Cl in pyr according to Gasparutto *et al.* [22], and Pitsch *et al.* [23], followed by alkylation of the product **7** with TOM-Cl under standard conditions as shown in Figure 6. Finally the 3′-OH unprotected product was converted to the final phosphoramidite **9** by *CEP*-Cl [23]. For the synthesis of similar and other natural phosphoramidites one could refer to the book chapter [15].

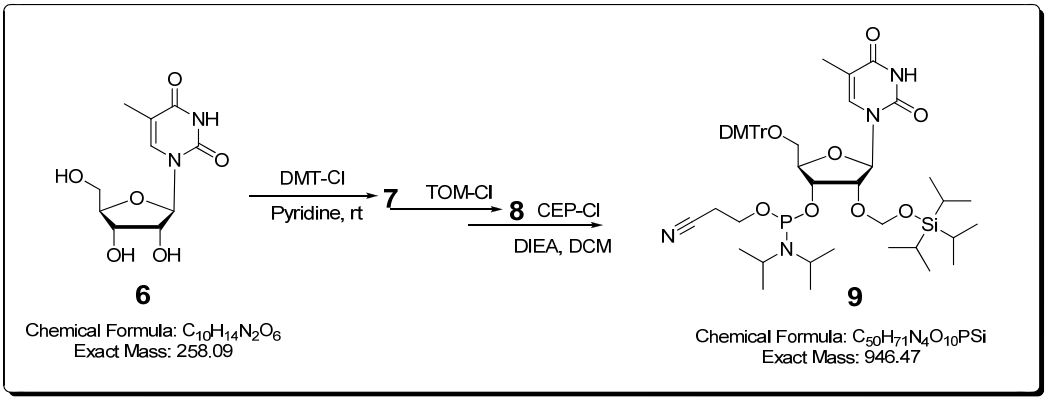


Figure 6 Synthesis of the m^5U phosphoramidite

1.2.4 Synthesis of Pseudouridine Phosphoramidite

Pseudouridine; a chemically simple modification [24], present in all variety of RNAs (rRNA, snRNA, snoRNA, tRNA) is mainly obtained by isolation from natural sources resulting in a prohibitive price. Pseudouridine is highly conserved at position 55 in most of the tRNAs in the TψC loop and is also found at other positions where it appears to influence the three-dimensional structure, cell viability and or aminoacylation [25, 26]. This 5-ribosyl isomer of uridine is unique by having a C-C, rather than the usual N-C glycosyl bond that links base and sugar [27]. This feature confers different hydrogen bonding capacities to it and influences the conformational preferences. Whereas the free nucleotide shows no real preferential conformation [28],[29], NMR and X-ray experiments have demonstrated that it exhibits an extraordinary conformational rigidity in RNA sequences due to the coordination of a water molecule between N1-H and the 5′-phosphate group [25, 30]. This feature improves base stacking interactions and contributes significantly to the structural stability of RNAs [28, 29, 31, 32]. Among the modified nucleosides of tRNA, pseudouridine possesses the unique feature to be also present in the middle of the anticodon (position 35) where it is supposed to reinforce the base pairing strength [33]. One can describe its presence to be vaguely universal and as such its role extensive.

Consequently, some attention was given to its chemical preparation in milligram scale, but the stereoselective formation of C-nucleosides is still and remains a challenging task. The first of these syntheses was reported in 1961 by the work of Shapiro *et al.* [34]. The synthetic strategy makes use of an ether-protected 2,4-dimethoxy-bromopyrimidine which was bromo-substituted into a lithiated analogue and used as a nucleophile in an S_N2 type reaction with tri-benzoyl-protected D-ribofuranosyl chloride. The β-anomer of the pseudouridine was synthesized in a 5-step reaction with an overall yield of 2 %.

The work of Brown *et al.* in 1981 uses the benzyl protected ribofuranose instead of the benzoyl analogue. Even though the yield of the final steps of the reactions was not given, the 6-step reaction was described as successful [35].

A process, involving the coupling of an iodinated pyrimidine with a suitably protected ribonolactone was presented by Grohar *et al.* [36, 37]. In their approach, a protected 5′-*O*-TBDMS-2,3-*O*-isopropylidene-D-ribolactone was used instead of the protected ribose used in the first two cases. The pyrimidine surrogate used in this work was 2,4-dimethoxy-1-iodopyrimidine, whose methyl-ether deprotection could be challenging. There was however a moderate improvement in

the ratio of the α to β in the mixture after coupling. An inproportionate decrease in the yield of the final purified product was observed and attributed probably to the difficulty in the cleavage of the methoxy groups. The most current synthetic reported work on the synthesis of pseudouridine was by Hanessian *et al* in 2003 [38]. The synthetic strategy was not different from that of Grohar *et al.* except for the use of the very easily deprotectible *tert*-butyl group. The reducing agent used in the step before the cyclization reaction leading to the formation of the ribofuranose was also investigated. The overall reported yield showed 10 % increase in the deprotection step. This indicates an advantage when the methyl-ether was substituted for with the easily cleavable *tert*butyoxy group.

The conversion of pseudouridine to its phosphoramidite as reported by Chui *et al.* involves the formation of the cyclic bridging of the 5′-OH and the 3′-OH by the use of TIPDSCl. The protection of the 2′-OH with TMS and the successive deprotection of the 3′,5′-*O*-TIPDS afforded the 2′-*O*-TMS-pseudouridine which was converted to the 5′-*O*-BzH-2′-*O*-ACE pseudouridine and finally to the phosphoramidite by action of *MEP*-Cl [37, 39]. Gasparutto *et al.* reported the synthesis of the pseudouridine phosphoramidite. The synthetic strategy made use of protected 5′-*O*-DMT-2′-*O*-TBDMS-ribonucleoside which was finally phosphitylated using 2-Cyanoethyl-*N*-ethyl-*N*-methyl-chlorophosphoramidite [22, 40].

1.2.5 Synthesis of m^1G, m^2G and $m^2{}_2G$ Modified Phosphoramidites

The m^2G and $m^2{}_2G$ nucleosides have been already incorporated into RNA sequences as 2′-*O*-TOM/*O*-C(6) nitrophenylethyl protected phosphoramidite building blocks. They have been prepared by nucleophilic aromatic substitution reaction of a protected 2-fluoroinosine derivative with the appropriate amine, $MeNH_2$ for m^2G and Me_2NH for $m^2{}_2G$, respectively. The conversion to the 5′-*O*-DMT and the protection of the 2′-OH with the fluoride labile silyl moiety and the final conversion to the phosphoramidite using CEP-Cl could be achieved as was reported by Hoebartner *et al* [41]. There was also the reported work of Beier *et al.* where they employed an azomethane, to a protected 2′-*O*-mthp guanosine ($G^{npe,\ npeoc}$), as the alkylating reagent. The 5′-OH protection and the phosphitylation follow similar procedure as reported by Beier *et al.* [42].

In contrary, a route proposed by Sekine and Satoh was adopted giving mixed products. The introduction of the methyl group into the protected guanosine derivative in a one-pot synthesis

was chosen. First, the treatment of nucleoside in pyr with Me_3SiCl (silylation of *O*-C(6) and *O*-C(3′)), and then subsequently with 1,3-benzodithiozolium tetrafluoroborate [43, 44]. The resulting N^2-benzodithiol-2-yl derivative was filtered on silica gel and treated with $(Me_3Si)_3SiH$ (Ballestri et al. 1991) and AIBN in refluxing benzene. Finally, cleaving the Me_3Si-group off with NH_3 in MeOH resulted in the formation of the modified nucleoside with an overall yield of 38 %. The protected N^2,N^2-dimethylguanosine nucleoside could be prepared from the crude N^2-monomethylated intermediate by repeating the sequential treatment above. After cleavage of the Me_3Si-group with HF-pyridine as was reported on a similar system by Iwase *et al.*, the fully modified nucleoside could be obtained [45].

1.3 Reagents for Specific Recognition of RNA Modifications

1.3.1 Specific Recognition of Modified RNA

The specific reactions of certain electrophiles with nucleophiles e.g the exo-cyclic amines in nucleic acids have been known for some time and have been subjected to research for decades with several applications.

Reagent(s)	Nucleophile	Comment(s)
Dimethyl sulfate (DMS) chloroethylamine	G(N-7), A(N-3), C(N-3)	Order of reactivity G(N-7)> A(N-3)> C(N-3)
Ethyl nitrosourea (ENU), methyl nitrosourea (MNU)	phosphates	Other nucleophiles are also affected
β-ethoxy-α-ketobutyraldehyde (Ketoxal)	G (N1 and N2) in ssRNA	N-1 and N-2 involved in ring
Diethyl pyrocarbonate (DEPC)	A(N-7)	N-7 of adenine, N-7 and C-8 opening of ring upon aniline treatment
1-cyclohexyl-3-(2-morpholino-4-ethyl)carbodiimide methoptosylate (CMCT)	Ψ(N-3), G(N-1), D(N-3)	Reacts with Ψ, G and U but Ψ-adduct is resistant to alkaline treatment
$BenzN_2^+$ and derivates/*hυ*	phosphates	Statistical modification
Tritiated water	HC-8Py	Complete modification
1-Fluoro-2,4-dinitrobenzene	2′-OH ss A	Complete modification

Table 1 Probing reagents and reacting nucleophile.

These applications include site-specific modification of RNAs leading for structural probing. Table 1 shows some of these reagents used in these modification recognition reactions. These reagents are specific to recognition of modified bases, and as such some are being used in combination with mass spectrometry which has become an increasingly powerful tool in the identification of modified nucleotides. The combination of site specific modification and mass spectrometry has taken an important place in the detection and mapping of post-transcriptionally modified nucleosides [46].

The mass spectrometry of pseudouridine is not straightforward because: (1) pseudouridine has the same elemental composition and hence is isobaric with uridine, which is present in excess in RNA from natural sources, (2) pseudouridine does not readily fragment at the glycosidic bond of the base and (3) pseudouridine is poorly retained on reversed phase columns [47] and tends to elute in the solvent front making it difficult to spot. Several methods have been proposed for the specific sequence placement of pseudouridine. A method based on the reaction of pseudouridine with CMCT was described by Bakin and Ofengand [48], and later applied with matrix assisted laser desorption ionization (MALDI) mass spectrometry as the detection method by Patteson *et al.* [49]. More recently, Mengel-Jorgensen *et al.* reported a different approach based on the Michael type addition of acrylonitrile in weakly alkaline conditions followed by MALDI mass spectrometry [50]. It is conceivable that, because the addition reaction proceeds through a deprotonated nucleobase, the acidity of the base protons determines largely the reactivity of pseudouridine and its unmodified counterpart, uridine. Both these methods allow specific sequence placement of pseudouridine in the RNA strand, but so far no data is provided about any increase in detection sensitivity of pseudouridine in nucleoside mixtures. The most recent report was a derivatization reagent for pseudouridine, methyl vinyl sulfone (MVS). The derivatization reaction is also a Michael type addition and the reaction product has a higher electrospray ionization efficiency compared to non-derivatized pseudouridine, allowing lower detection limits. The moderate specificity (more than 4:1 of Ψ to U) makes this reagent useful for sensitive and specific detection of pseudouridine in presence of high amounts of uridine [51].

1.3.2 Coumarins and Derivatives

The specific recognition of 4-thiouridine in pure *E. coli* tRNA ^{fMet} using 4-bromomethyl-7-methoxycoumarin (BMB) as the reacting counterpart was reported first by Yang *et al.* about 35 years ago [52]. In a follow-up publication, Yang *et al.* reported that in the presence of pseudouridine and thiouridine containing mixture of nucleotides, the reaction of BMB was evident with 2-thio-5-(*N*-methylaminomethy1)uridine as well as with pseudouridine and thus this could be extended to a total tRNA containing pseudouridine [53]. The attractive part of this choice of reagent is that the covalent attachment of pseudouridine is multi-purposeful. The multifunctional role of coumarin both for specific labeling of pseudouridine and also for spectrofluorometric detection might be extended by further tailor-functionalizing the coumarin without changing the reactivity of the bromide. In this direction one could envisage an azido-functionalized coumarin that could be employed in a 'click' chemistry with a counterpart functionalized with a terminal alkyne [54, 55]. A biotin-functionalized coumarin that could be used as a separation tool taking advantage of the high affinity between biotin and strepavidin (avidin) is illustrated in Figure 7 [56]. For the synthesis of these multi-functionalized or derivatized coumarins, one might look to the established and published protocols.

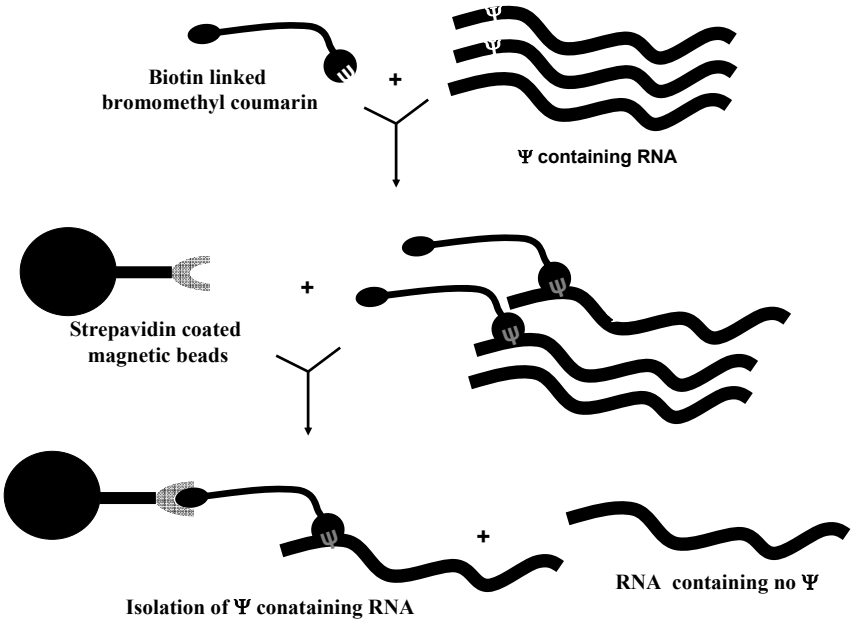


Figure 7 Using strepavidin to capture biotin linked RNA

Coumarins and their derivatives form a class of compounds, occupying an important place in the realm of natural products and synthetic organic chemistry. Their application runs a broad spectrum; ranging from additives in food, perfumes, cosmetics, pharmaceuticals, and in the preparation of insecticides [57], optical brightness [58, 59], and dispersed fluorescent and tuner laser dyes. It shows a variety of bioactivities including its application in the inhibition of platelet aggregation, as an anticancer agent with inhibitory properties against the 5α-reductases, anthelmintic and hypnotic properties. These properties have made coumarins interesting targets for organic chemists. The last decade witnessed a series of publications on the development of a synthetic methodology for this important heterocyclic scaffold. A typical example of the synthesis of 7- functionalized coumarin is shown in Figure 8. Thus, it is evidently required to be circumspective, when one needs to accommodate important functionalities which are broad in scope, and therefore the need for development of new and flexible protocols are required. The synthesis of coumarin has been performed following one of the following routes; Pechmann [60], Perkin [61], Knoevenagel [62-65], Reformatsky [66] and Wittig reactions [67, 68] among others including flash vacuum pyrolysis [69]. Among these, the Pechmann reaction is the most widely used method as it involves the use of simple starting materials in the presence of acidic condensing agents. The use of Lewis acids in the Pechmann condensation reaction has been reported in several publications including that of Bose et al. [70], Smitha et al. [71], Kimura et al. [72] and that of Varughese et al. [73] The use of Lewis acids such as $AlCl_3$, $FeCl_3$, $ZnCl_2$, $POCl_3$, P_2O_3, $ZrCl_4$ and that of Bismuth (III) derivatives has been reported in the catalysis of the Pechmann condensation of phenols with β-ketoesters. There is also the use of Brønsted acids as catalyst in the condensation reaction leading to the formation of coumarin as shown in Figure 8.

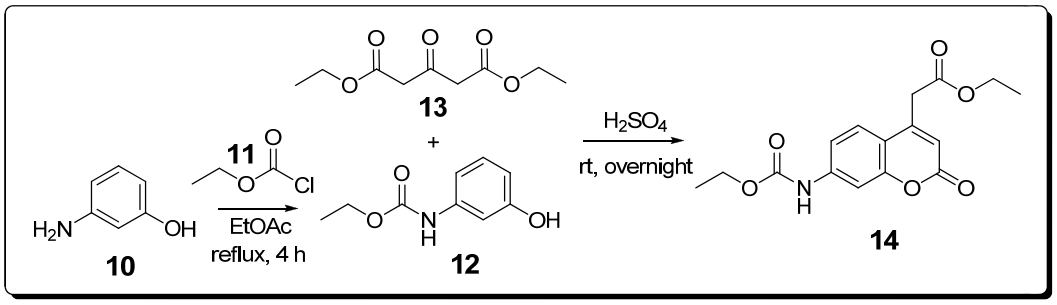


Figure 8 Synthesis of coumarin.

1.4 Synthesis of Xanthene and Related Derivatives

1.4.1 General Introduction to Xanthenes

Xanthene (9*H*-xanthene, 10*H*-9-oxaanthracene) is a yellow heterocyclic compound with the chemical formula $C_{13}H_{10}O$. It is soluble in ether. Its melting point is 101-102 °C and its boiling point is 310-312 °C. Xanthene is used as a fungicide and it is also a useful intermediate in organic synthesis. Xanthene is relatively easy to synthesize; heating of phenyl salicylate **23** in the presence of a lewis acid affords the xanthone which is then reduced to the xanthene **24** under standard reduction condition using $LAlH_4$ as illustrated in Figure 9 [74].

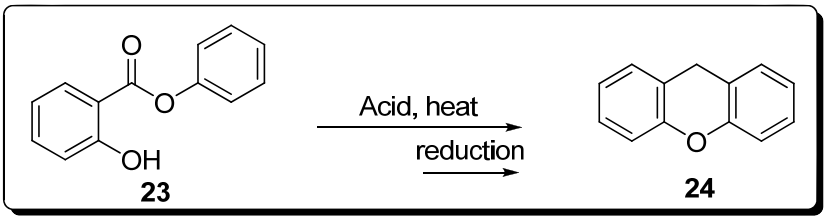


Figure 9 Synthesis of the core xanthene moiety

Xanthene and derivatives among others are the basis of a class of dyes which includes fluorescein, eosins, rhodols and rhodamines. Xanthene dyes tend to be fluorescent, yellow to pink to bluish red, brilliant dyes. Many xanthene dyes can be prepared by condensation of derivates of phthalic anhydride **16** with derivates of resorcinol **15** or 3-aminophenol **12**. A typical example of the synthesis of xanthene is shown in Figure 10. Mixture of phthalic anhydride **16** and 2 equivalent of resorcinol **15** is heated usually in a solvent free Lewis acid catalyzed reaction. The resulting mixture is purified by re-crystallization from di-ethylether; however flash chromatography is frequently required for purification of xanthene dye and derivatives.

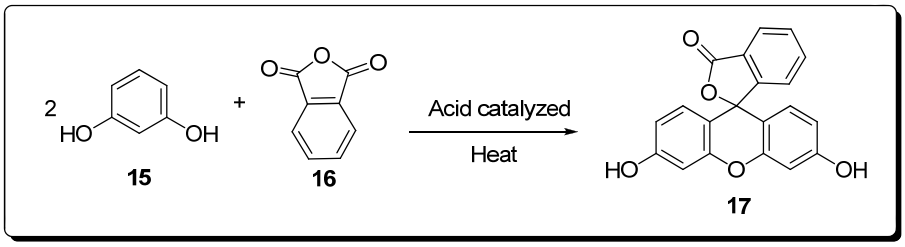


Figure 10 Synthesis of fluorescein

1.4.2 Synthesis of Rhodols

Rhodols are highly fluorescent dyes that form a structural hybrid of fluorescein **17** and rhodamine **18**, as shown in Figure 11. They are useful for oligonucleotide, antibody and protein labeling studies and for the preparation of peptidase and esterase substrates. Rhodols retain the pH-sensitive properties conferred by a phenolic hydroxyl group, whereas the amine substituent reduces the pK_a values. Rhodols exhibit high extinction coefficient (usually >70,000 $cm^{-1}M^{-1}$) and high quantum yield (~0.80–0.99) in aqueous solution, with absorption maxima at approximately 490 nm and emission maxima between 520 and 545 nm. As with fluorescein-based dyes, the fluorescence excitation profiles of rhodol-based dyes are pH dependent, which can be useful for ratiometric pH determinations intracellularly. The dyes, however, show less spectral sensitivity to pH in the physiological range than does fluorescein. Rhodols have higher solubility in non-polar solvents and have improved photostability.

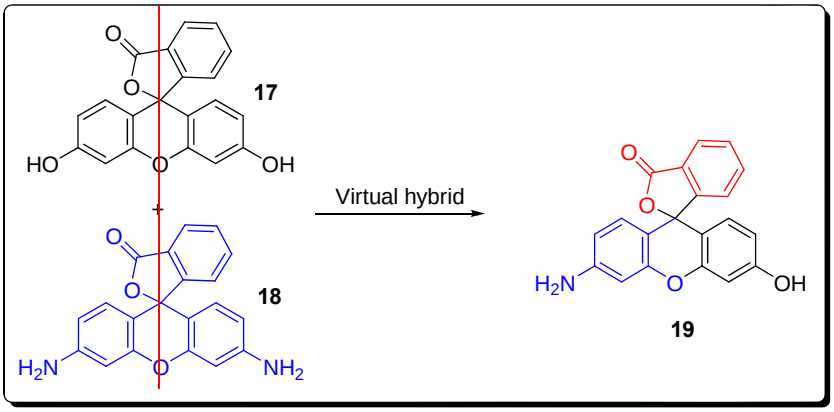


Figure 11 Rhodol, a hybridized product of fluorescein and rhodamine

Rhodols are synthesized chemically by a stepwise condensation reaction. This usually involves a sequential condensation of phthalic anhydride **16** with resorcinol **15** followed by addition of 3-aminophenol **12**, as shown in Figure 12. The reaction is catalyzed by a Lewis acid and the product is purified by re-crystallization or chromatography [75]. Even though rhodols could be described as hybridized products of fluorescein and rhodamines, there is no reported data that show the conversion of fluorescein to rhodol or vice versa under mild conditions.

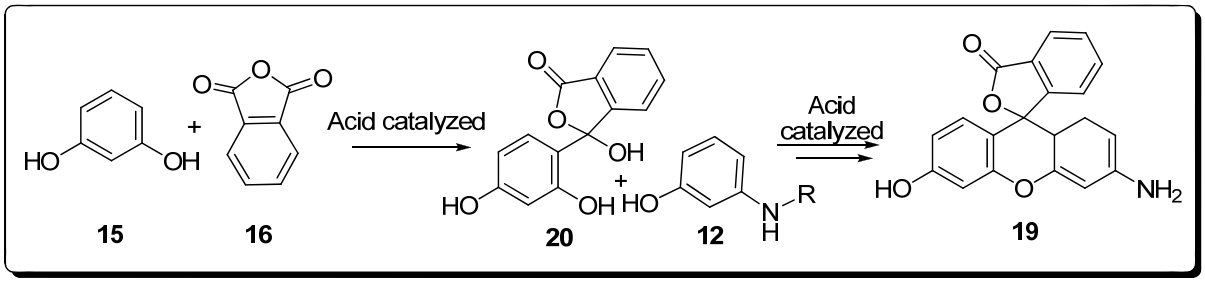


Figure 12 Synthesis of rhodol *via* a stepwise condensation

1.4.3 Synthesis of Rhodamines

Rhodamines are a family of related chemical compounds, fluorone dyes. Just like fluorescein and rhodols they contain the xanthene core moiety with two aminyl moieties at the 3′ and 6′-position of the xanthene. Examples include among others the well known Rhodamine 6G and Rhodamine B. They are used for labeling and also as dye laser gain media. They are also often used as a tracer dye within water to determine the rate and direction of flow and transport. Rhodamine dyes, just like fluorescein, fluoresce and can thus be easily detected with fluorometers. Rhodamine dyes are used extensively in biotechnology applications such as fluorescence microscopy, flow cytometry, fluorescence correlation spectroscopy and ELISA. Rhodamine dyes are generally toxic, and are soluble in water, methanol and ethanol. They are usually synthesized in a condensation reaction of phthalic anhydride **16** and the particular 3-alkyl aminophenol **21**, as shown in Figure 13 [76]. This reaction however is conducted at high temperature > 150 °C which is not compatible with most biochemical investigations.

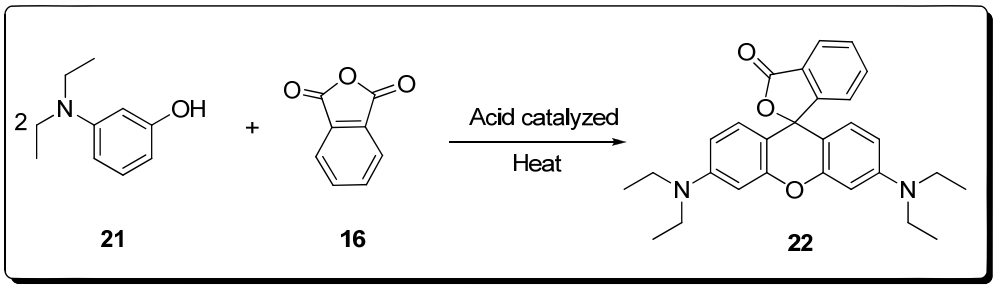


Figure 13 The synthesis of rhodamine by Obata *et al*. [76].

Kim *et al.* reported the use of a new rhodamine-derived boronic acid as derivatization agent for the detection of a variety of sugars and related biomolecules. This dye is formed from reaction between rhodamine and formylphenylboronic acid after which the product is reduced with $NaBH_4$. The detection of sugars by forming reversible fluorescent complexes has been implicated in mixtures containing the dye and sugars [77]. Chang *et al.* reported the synthesis and use of xanthene core peroxyfluor-1 (PF1), a new type of optical probe for intracellular imaging of hydrogen peroxide in living biological samples. The PF1 Utilizes a boronate deprotection mechanism to provide unprecedented selectivity and optical dynamic range for detecting H_2O_2 in aqueous solution. Also in imaging changes in $[H_2O_2]$ in living mammalian cells [78]. Dickinson *et al.* reported the synthesis, via derivatization of 3′,6′-diiodofluorescein, of a boronic acid functionalized xanthene core-moiety for an *in vitro* hydrolysis assay to monitor the H_2O_2 production in the mitochondria of living cells, in a case of rendering a non-fluorescent boronic acid fluorescent [79]. Miller *et al.* reported the synthesis and application of Peroxy Green (PG) and Peroxy Crimson (PC), two new fluorescent probes that show high selectivity for H_2O_2 and are capable of visualizing endogenous H_2O_2 produced in living cells by growth factor stimulation, including the first direct imaging of peroxide produced for brain cell signaling [80]. Li *et al.* reported the use of 'Singapore Green' a peptide coupled rhodol-like molecule that allows the indirect measurement of peptidase activity as an increase of the fluorescence intensity in a microarray-based protease substrate profiling and living-cell experiments as illustrated in Figure 14 [130].

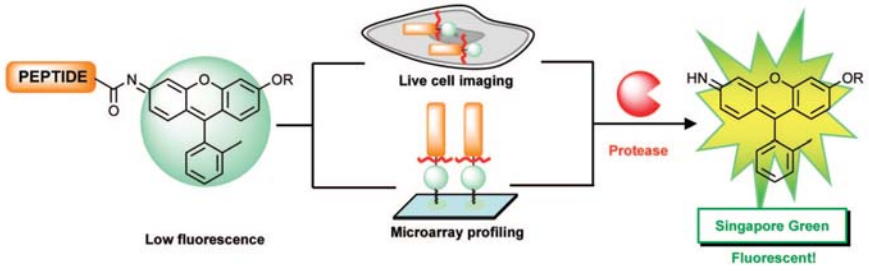


Figure 14 'Singapore Green' for cell-profiling by Li e*t al.*

The conversion of 3′, 6′-dichloro- or difluorofluorescein to rhodamine has been reported as patent data. The reaction involves heating of the respective amine with the 3′,6′-dihalofluorescein at elevated temperature in high boiling solvent such as sulfolane [81]. Similarly, a patented report of the reaction between fluorescein and thionyl chloride followed by the refluxing of the crude mixture in high boiling solvents with an amine results in the formation of the desired rhodamine. The use of high boiling solvents coupled with the high temperature of reaction in the synthesis of rhodamines do not allow the use of similar procedure where biomolecular investigation of proteins, nucleic acids and RNA in particular are of concern. There is therefore the need for a mild and compatible approach that allows the incorporation of rhodamines into RNA, peptides etc.

2 Aims and Scope

The objective of this thesis is the use of chemical biology as a tool to investigate modifications in RNA including the synthesis of particular nucleotide modifications and its conversion into phosphoramidites for later incorporation into RNA oligonucleotides. The synthesis of the following phosphoramidite and their incorporation into RNA were pursued; m^5U, Ψ, m^1G, m^2G and $m^2{}_2G$. The synthesized modified phosphoramidites were then to be fused into RNA oligonucleotides *via* the SPOS which will then be utilized for the construction of full length tRNA and for siRNA studies.

One particular project was to investigate the recognition of these modifications and in particular the chemical recognition of pseudouridine by various multifunctional derivatives of 4-bromomethyl-coumarin.

In investigating different approaches to modify the detection sensitivity of the presumably pseudouridine selective bromomethyl-coumarin reaction, there was a serendipitous discovery of the possibility to directly transform fluorescein into rhodols and rhodamines. This found method occurs under mild reaction condition which could allow for biomolecular labeling which are usually difficult to be performed under harsh conditions. In addition the novelty of the reaction prompted us to further investigate it into more detail.

3 Results and Discussions

3.1 2′-Hydroxy Protection in Phosphoramidite Synthesis

The protection of the 2′-hydroxy functionality could either be done by either of the fluoride labile silyl protecting groups widely used in RNA phosphoramidite synthesis: TBDMS-Cl or TOM-Cl. The latter was chosen due to the 2′-3′ non migration character of silyl moiety in basic condition as shown in Figure 15. TOM-Cl was synthesized according to the published protocol by Pitsch *et al.* [23].

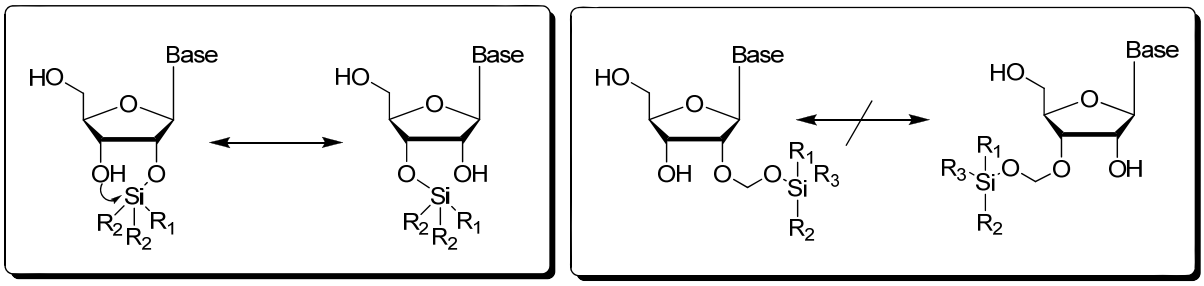


Figure 15 1,2-Silyl Migration in basic medium.

The 2′-OH protection with TOM-Cl as was introduced by Pitsch *et al.* went successful with yields in the range 30-45 %. However, the formation of a foamy suspension which makes the work-up very difficult needs to be mentioned. The suspension is conceived to be as a result of the coordination of water molecules to di*tert*butyltin dichloride which then gets distributed between the phases. DIEA catalyzed S_N2 reaction between TOM-Cl and the 2′-OH group proceeded *via* the formation of the tin mediated complex. The sterically less hindered side which is usually the 2′-OH section allows selectivity of the 2′-OH over the 3′-OH. In our case the selectivity enhancement was not pronounced, yielding a mixture of 2′-*O*- and the 3′-*O*- isomers which were separated by fCC. The extraction of the organic layer with DCM resulted into formation of an inseparable scum which made the whole work-up difficult to handle. This problem was circumvented by the filtration of the scum and re-suspending the mixture in the separating funnel for separation.

3.2 Synthesis of m^{5}U Phosphoramidite

This Section describes the synthesis of 5-methyluridine (m^{5}U) phosphoramidite starting with commercially available 5-methyluridine. These synthesized phosphoramidite was finally used in the SPOS of which detailed procedure is described in chapter 3.6.

The synthesis of the m^{5}U phosphoramidite was achieved in a 3-step synthetic strategy starting from the commercially available 5-methyluridine which is schematically represented in Figure 16. The 5′-OH was protected with the acid labile DMT-Cl in pyridine at rt for 5 h reaction time after which the dried crude was purified by fCC giving a yield of 85 %.

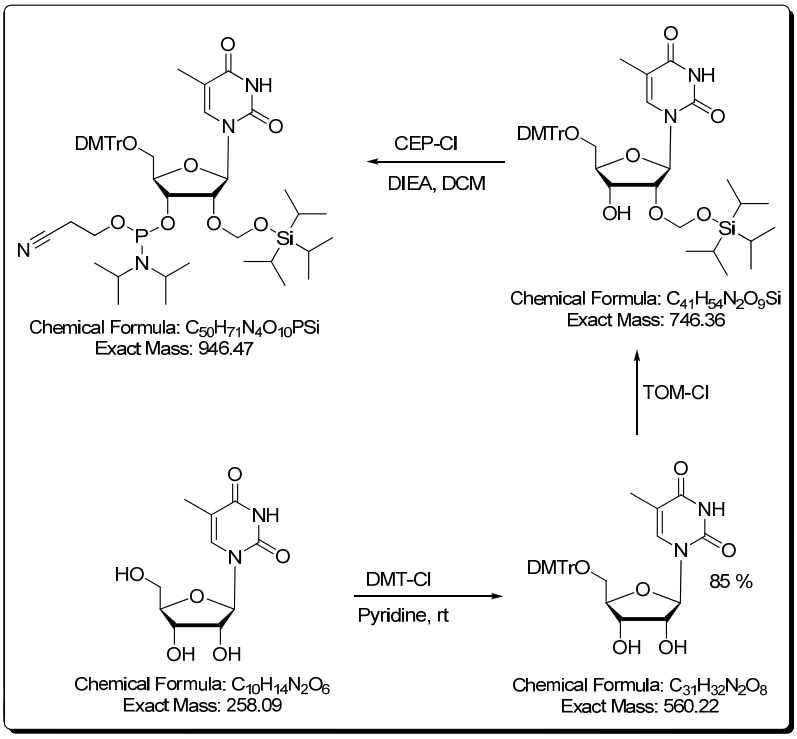


Figure 16 Synthesis of m^{5}U phosphoramidite.

The 5′-*O*-DMT protected m^{5}U was subjected to the fluoride labile protection of the 2′-hydroxy group in pyridine basified with imidazole at rt. The reaction mixture was quenched after overnight reaction when no starting material was detected by TLC. The reaction products were purified by fCC resulting in the desired 2′-*O*-TOM isomer, and the undesired 3′ isomer in equal ratio. There was also very little amount of the 2′,3′-di-*O*-TOM which was not quantified. The attachment of the phosphoryl group to the 3′-*O*H was finally achieved in a 2 h reaction in DCM

using DIEA as base for the reaction at rt. The product of this final step was 86 % after fCC purification on silica gel with a gradient eluent system of Hex:EtOAc; (20:1, 5:1). This phosphoramidite was used in the SPS of the RNA Oligonucleotides which is described in chapter 3.6.

3.3 Synthesis of Pseudouridine

This section describes the synthesis of Pseudouridine starting with the commercially available 5-bromouracil. Subsequently the obtained Pseudouridine is converted into the required phosphoramidite which was finally used in the SPOS described in detail in chapter 3.6.

3.3.1 Known Synthetic Approaches to the Synthesis of Pseudouridine

At the onset of this work, there were four reported total syntheses of pseudouridine. The first of these syntheses was reported in 1961 by the work of Shapiro and Chambers [34]. The synthetic strategy makes use of an ether-protected 2,4-dimethoxy bromopyrimidine which was bromo-substituted into a lithiated analogue and used as a nucleophile in an S_N2 type reaction with tri-benzoyl protected D-ribofuranosyl chloride, as represented schematically in Figure 17.

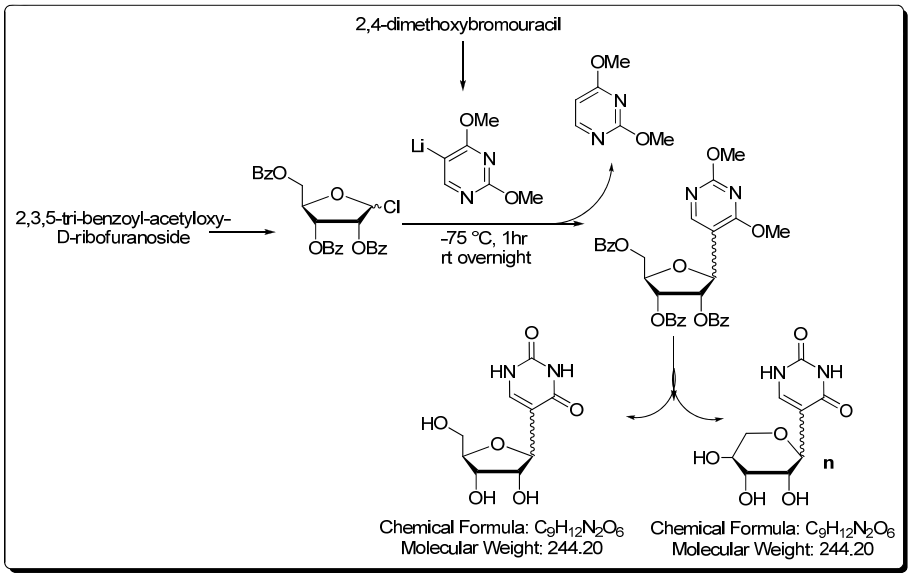


Figure 17 Synthesis of Pseudouridine by *Chambers et al.* 1961.

The pseudouridine surrogate product which was finally cleaved off its protection groups afforded the undesired 6-membered heterocyclic hexose **n** together with the desired α and β anomers in a total overall synthesis of 5 steps with a yield of 3.6 % for all the various isomers Figure 17. The side product may have been formed as a result of protonation of the ribofuranose bridge oxygen leading to ring expansion. The difficulty in this strategy was in the coupling and the deprotection steps. The coupling makes use of the very short-lived intermediate; the lithiated pyrimidine, which often gets reduced by protonation within a short time of formation. It is therefore an important decision exactly when should the two coupling parts be put together in order to achieve maximum yield, forming less of the undesired 2,4-dimethoxyuracil. The second problem in this synthesis involves the difficulty in the cleavage of the methoxy group. There was incomplete cleavage after refluxing in 20 % (v/v) of dichloroacetic acid for 4 h. A lower pH condition could effect fast deprotection but this could as well speed up the isomerisation of the product. The final pure product was obtained after two times re-crystallization of the obtained crude from the deprotection using 95 % refluxing ethanol. The β-anomer of the pseudouridine was synthesized in a 5-step reaction with an overall yield of 2 %.

The second total synthesis of pseudouridine was reported by Brown and Ogden in 1981. Their synthetic strategy uses the benzyl protected ribofuranose instead of the *benzoyl* moiety as was reported separately in the works of Buchanan *et al.* and Moffat *et al*. in C-C nucleoside synthesis. Also the very acid labile moiety *tert*butoxy group was use for protection instead of the very difficult to cleave methyl ether analogue. The reaction proceeded in THF at -78 °C giving rise to the two D-*allo* and D-*altro* isomers shown in Figure 18.

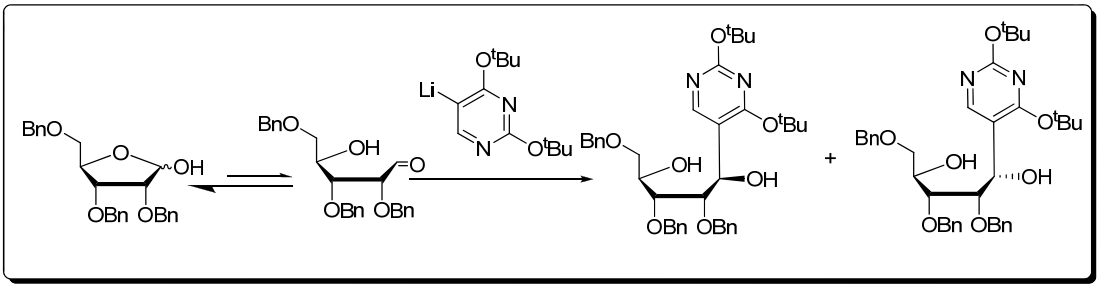


Figure 18 The chemical synthesis of Pseudouridine by Brown & Odgen (1981).

The two epimeric mixtures were cyclisized under the condition of ethanolic hydrochloric acid giving rise to the respective α and β forms of the nucleoside in the same ratio as the starting material suggestion stereospecificity in reaction as shown schematically in Figure 19. The non-isomerization and stereospecificity of the reaction makes this condition superior compared to the S_N2 mechanism shown in the method executed by Shapiro *et al*. [34]. The benzyl groups were later cleaved from the surrogate pseudouridine by boron trichloride in DCM at -78 °C without any isomerisation as shown in Figure 19, after several failed attempts using catalytic hydrogenation procedure resulting in mainly 5-ribityluracil.

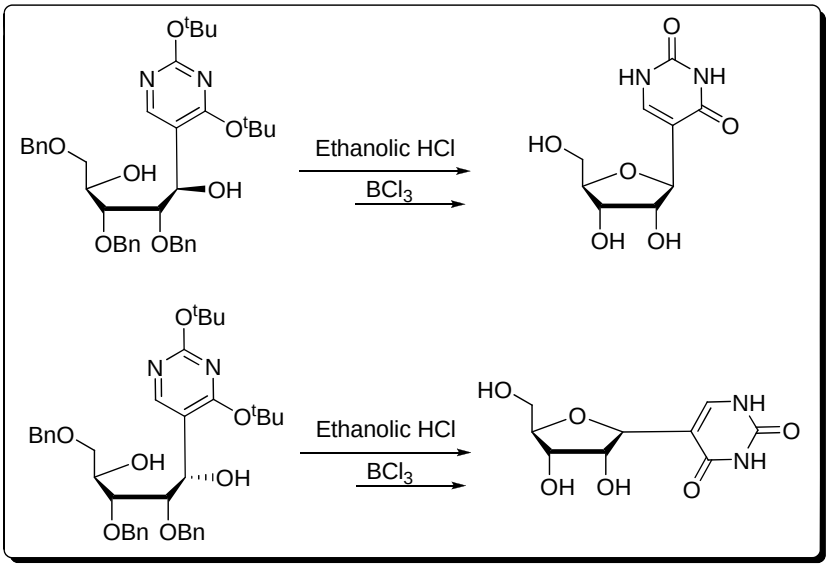


Figure 19 Cyclization and deprotection leading to α and β pseudouridine

The application of the above discussed synthetic strategy using the methyl-protected ribose analogue lead to an increase in the D-*altro* anomer. The incomplete deprotection; a problem with the cleavage of the methyl-group, after the acidic cyclization condition, using the boron trichloride was observed. The use of the harsher boron tribromide led to the isomerisation of the product formed after a prolonged deprotection time of 2 days. The reaction of 2,3-*O*-isopropylidene-D-ribose with lithiated di*tert*-butoxyuracil was also investigated. There was however no formation of any of the polyol derivatives. The unusual product which was the lithiated reagent with acetone as could be confirmed by high resolution mass spectrometry and ^{1}H NMR as is being depicted in Figure 20. This was further confirmed when acetone was solely used as the electrophilic carbonyl component in the reaction instead of the 5′-*O*-trityl-2,3-*O*-

isopropylidene-D-ribose. In summary the use of the benzyl ribose was successful in forming the desired β-pseudouridine in very good yield even though the percentage is not given.

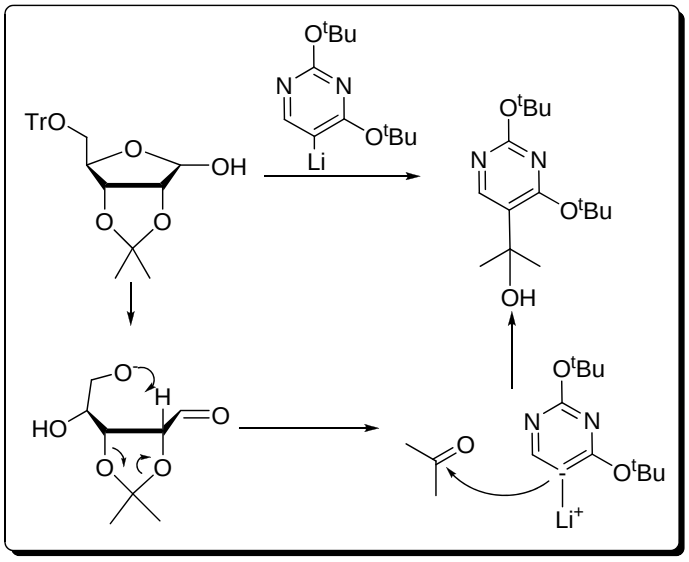


Figure 20 Using 2,3-*O*-isopropylidene-D-ribose as the electrophile.

The third synthetic work of pseudouridine was published by Grohar *et al.* in 1999 [36]. This synthetic work used a protected 5′-*O*-TBDMS-2, 3-*O*-isopropylidene-D-ribolactone instead of the protected ribose used in the first two cases. The pyrimidine surrogate used in this work was rather the difficult to cleave methyl ether; 2,4-dimethoxy-1-iodopyrimidine. Surprisingly, there was an improvement in the ratio of the α to β anomers in the mixture after coupling which was 1:8 as compared to 2:5 in the previous synthesis by Chambers *et al.* shown in Figure 21.

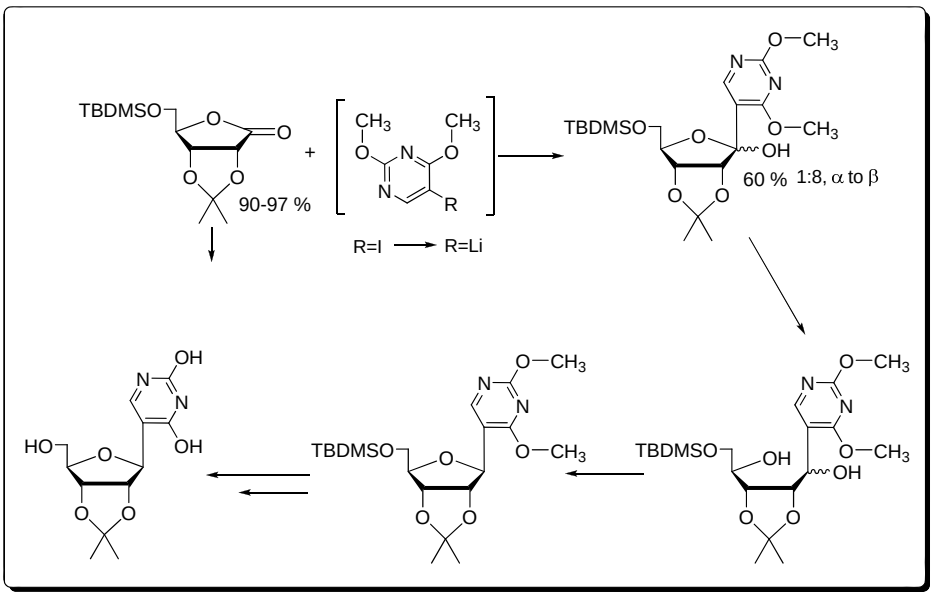


Figure 21 Synthesis of pseudouridine by Chow *et al*.

There was however the isomerisation of the product during the reduction process leading to a 1:1 mixture of both the α and β forms. The successful deprotection of the methyl ether using NaI in a refluxing acetic acid without any isomerisation within 25 min reaction time was an important step in the synthetic strategy. The final deprotection of the TBDMS was achieved using methanolic HCl with yield greater than 95 %. The final β-pseudouridine was obtained in 10-20 % after 7 steps.

The most current reported work on the synthesis of pseudouridine was published by Hanessian *et al.* in 2003 [38]. The synthetic strategy was very similar to that reported by Chow *et al.* except in the fact that the very acid labile non-cyclic acetal protection of the 5′-OH functionality was achieved as a one-port protection during the formation of the stable isopropylidene moiety. The 2 and 4 positions of the pyrimidine were also protected as *tert*butyl ether in order to achieve easy and complete deprotection as and when it was needed. The reported α to β ratio in the synthesis was same (1:8) as reported in the work of Chow *et al.* confirming the proposed stereochemical environment of the reaction and the reproducibility of the synthetic condition. The reduction of the diasteromeric lactols was achieved with the aid of L-selectride in the presence of $ZnCl_2$ giving the final acyclic-D-*altro*-hexitol as a single isomer in 85 % yield. The absence of $ZnCl_2$ results in the formation of the single D-*allo*-hexitol isomer suggesting the *Si*-face attack as illustrated by the scheme in Figure 22.

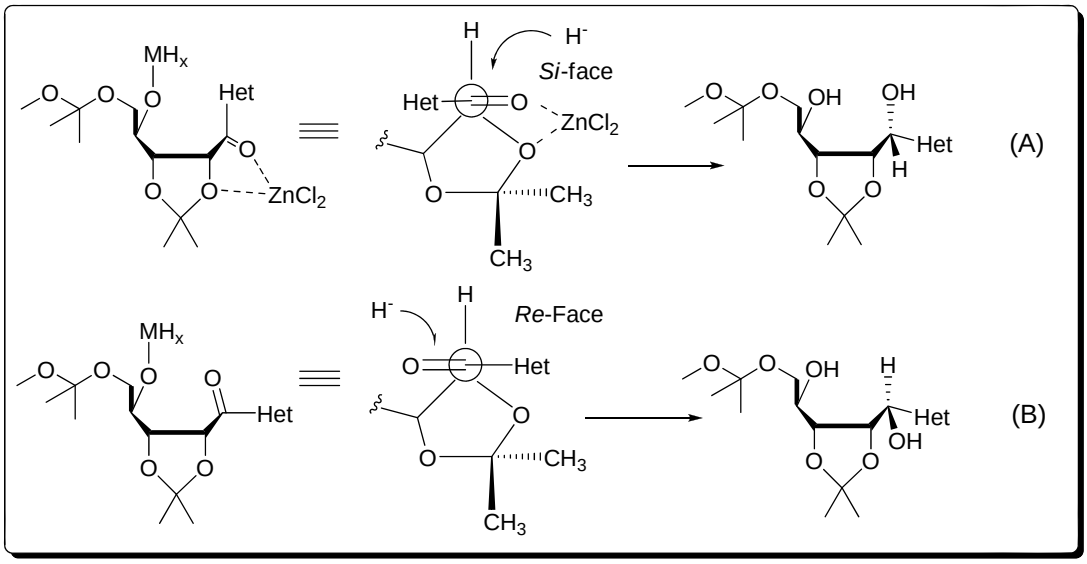


Figure 22 Possible pathways of stereoselective reduction.

The synthesis of the *allo* form of this hexitol could follow the pathway where there is no $ZnCl_2$-complex formation due to the absence of $ZnCl_2$. The resulting *Re*-face attack leads to the product as illustrated schematically in Figure 22 (B). The cycloetherification reaction was achieved under the Mitsunobu reaction conditions using DIAD and triphenylphosphine in THF with yield greater than 70 %. The final deprotection using 70 % acetic acid gave no isomerized product but rather more than 93 % of β-pseudouridine. It is however very important to note that the non-isomerizable reduction condition, using the L-selectride, reported in this work failed in our case. There was therefore the need to re-strategize in order to circumvent the problems.

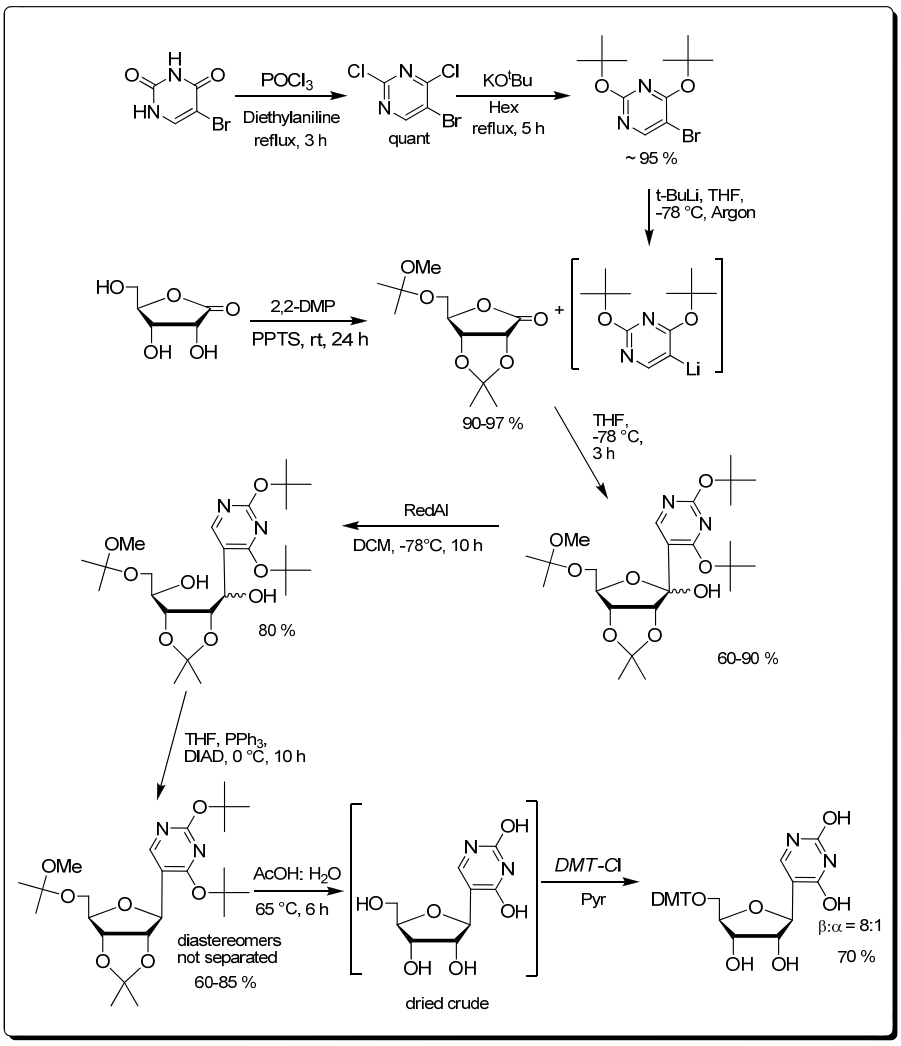


Figure 23 Total synthesis of pseudouridine in the present work.

3.3.2 Own Synthetic Approach to the Synthesis of Pseudouridine

Considering the synthetic strategies used in the syntheses of β-pseudouridine described in the four cases above, we endeavored to incorporate the advantages as well as minimized the disadvantages in order to synthesize β-pseudouridine in as few steps as possible. By considering the most current work by Hanessian *et al*. in 2003, we envisaged a similarly plausible route due to the improved yield in the number of steps as shown in Figure 23. The synthesis of pseudouridine started from the commercially available material 5-bromouracil. The conversion of this substance to the 2,4-dichloro-5-bromouracil was achieved in excellent yield, using diethylaniline and phosphorous oxychloride under reflux, and the subsequent conversion of the

2,4-dichloro-5-bromouracil to the ditert-butoxy analogue was also achieved without much difficulty using potassium di*tert*-butoxide in hexane under reflux for 5 h. The schematic representation for these reactions is shown in Figure 24. Phosphorus oxychloride has been used as a phosphorylating agent in the nucleotide chemistry [82]. In the contrary, we employed it as a chlorinating agent. Based on the reported work of Zhang *et al.*, Bobosik *et al.* and Szulc *et al.* the use of phosphorus oxychloride for reductive chlorination results in yields of not less than 70 % [83-85].

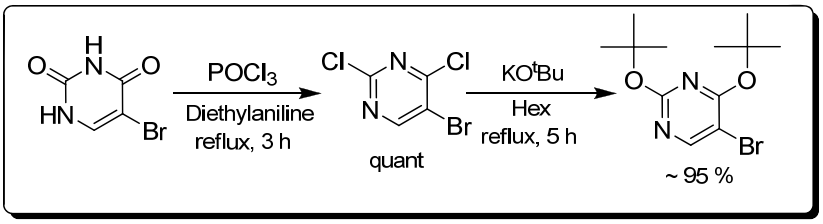


Figure 24 Synthesis of 2,4-ditert-butoxy-5-bromouracil.

The conversion of the 2,4-di-tertbutoxy-5-bromouracil reagent into the lithiated derivative resulting into a charge inverted center usually termed 'Umpolung' was very quick. The subsequent reaction of this intermediate with the protected lactone gave variable yields depending on the time at which the lactone is added to the organo-lithiated intermediate.

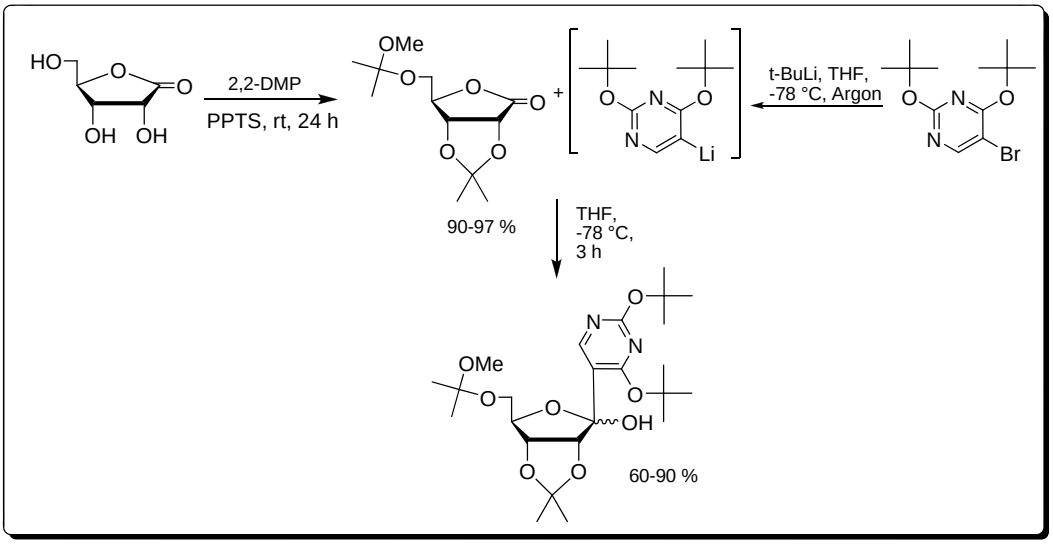


Figure 25 Coupling of 2,4-di*tert*-butoxy-5-bromouracil to the protected lactone

The lithiated intermediate which is bright yellow in color can survive within 30 min of the reaction time. The amount then decreases very quickly forming the proton substituted analogue. The optimal condition for the coupling is within 20 min when the brightest yellow is first observed. The isolated yield of the coupling reaction is between 60-90 %, shown in Figure 25. As was reported by the work of Gazivoda *et al.*, the organolithiated-mediated coupling of uracils usually gives lower yields due to the fast protonation reaction in the absence of a nucleophile [86-88]. The reduction of the pseudouridine surrogate, formed by the coupling reaction, needs to be done under conditions compatible with the stability of the acetal-protected hydroxy group. The ether functionality is known to cleave off under acidic conditions.For this reason pHs lower than 7 need to be avoided. There are quite a number of reducing agents that could be used for this purpose including Et_3SiH-$BF_3{\cdot}OEt_2$ [89, 90], $LiAlH_4$ [91], H-DIBAL [92], DIBAL [93], ReDAL [94, 95], and L-Selectride [96].

However the stability of the final reduced product will be compromised if available options are not carefully considered.

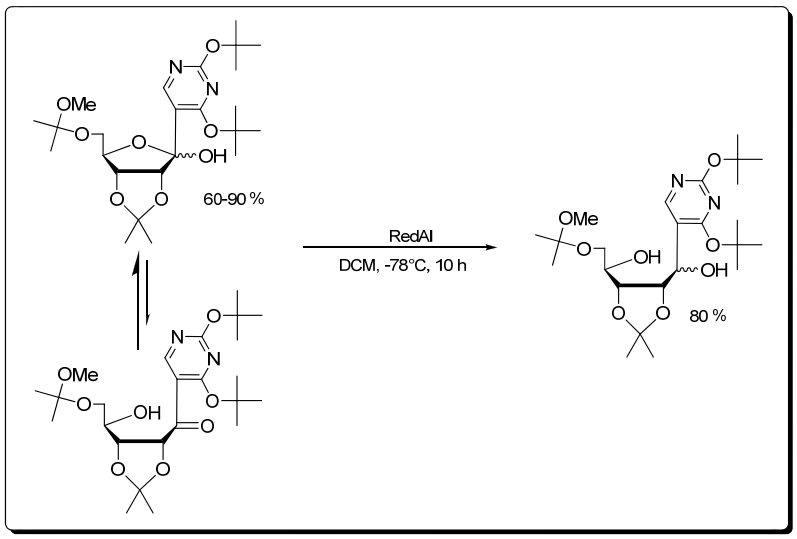


Figure 26 Reduction of pseudouridine surrogate with RedAL.

The use of RedAL was reported separately by Satoh *et al.* [94] and Eland *et al.* [95]. Other reported alternatives include H-DiBaL, L-selectride and $LiAlH_4$. Red-Al (sodium bis(2-methoxyethoxy)aluminum hydride) is primarily use as a reducing agent or a source of hydride very similar to $LiAlH_4$.

The reduction of the equilibrium deprived form of the coupling product was achieved after several failed attempts. The use of H-DIBAL [92] in THF failed and so also was the use of L-Selectride. Strangely the use of Et_3SiH-$BF_3{\cdot}OEt_2$ [89] gave several spots from the TLC and so the reaction mixture was discarded. The solvent dependency of this reaction needs to be mentioned; in THF the reaction worked very slowly and was usually finished after 3 days without allowing the reaction to warm up above 0 °C. There were unidentified side-products though. The reaction was successful using DCM as solvent after an over-night reaction at 0 °C as shown schematically in Figure 26. Subsequently, the cyclization of this product under the Mitsunobu conditions using Ph_3P and DIAD [97] resulted in the formation of the diastereomeric mixtures which were not separated during fCC purification and were used in the next step of the reaction as shown in Figure 27. The cleavage of the acetal and ethereal protection groups was achieved in one step using refluxing acetic acid for 6.5 h. The crude product which precipitated was not further purified. It was used in the next reaction after extensive drying under *vacuo*.

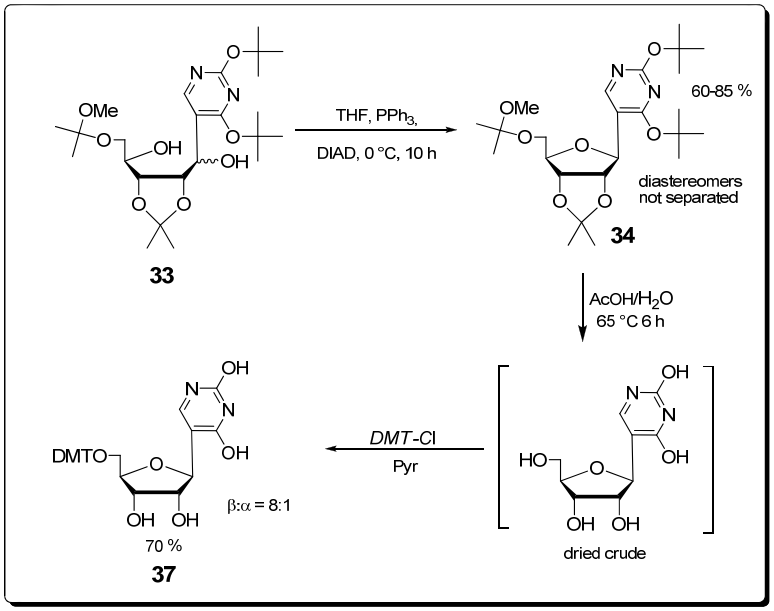


Figure 27 Cyclization leading to pseudouridine surrogate.

The α and β forms of the pseudouridine were produced after cleavage of the protection groups. They have very similar R_f-value and were therefore difficult to separate after several failed attempts with various solvent combinations. We therefore proceeded with the mixture to form the 5′-*O*-protected DMT-product which eventually allowed us to separate α and β pseudouridine

(ratio 1:8 respectively) by fCC as shown in Figure 27. The *β*-5′-*O*-DMT-pseudouridine was synthesized in an 8–steps reaction with an overall yield of 18 %. The 8:1 ratio of the *β* to *α* anomers reported separately by Grohar *et al*. [36] and Hanessian *et al.* [38] was confirmed.

3.4 Synthesis of Pseudouridine Phosphoramidite

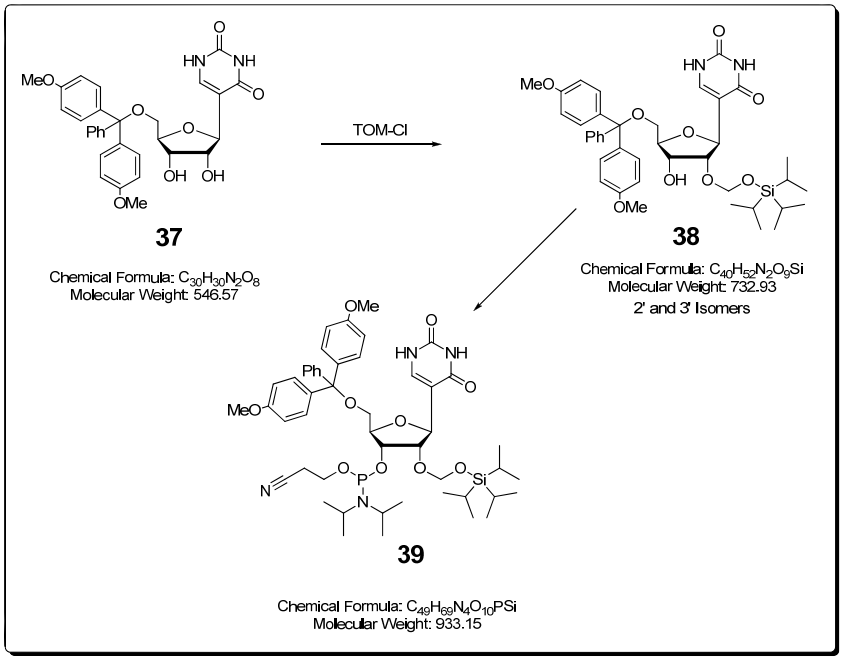


Figure 28 Synthesis of pseudouridine phosphoramidite.

The synthesis of the pseudouridine phosphoramidite started with the conversion of the diastereomeric mixture of α and β pseudouridine to the *α*– and *β*-5′-*O*-DMT-pseudouridine allowing us to separate the diastereomeric mixtures by fCC. The β-5′-*O*-DMT-Pseudouridine was used in the synthesis of the desired phosphoramidite whilst the undesired 3′-product was cleaved off its 3′-protection by the action of TBAF and was further re-used for synthesis.

The 2′-OH protection of the *β*-5′-*O*-DMT-pseudouridine was achieved using TOM-Cl in DCE shown in Figure 28. The reaction proceeded forming both the 2′-*O*- and 3′-*O*-TOM products in equal amounts, together with some of the 2′,3′-di-*O*-TOM, which were separated and the 2′-*O*-TOM-5′-*O*-DMT-pseudouridine dried under vacuum overnight giving a yield of 48 %. The final reaction that involves the attachment of the phosphitylation moiety to the 3′-OH functionality was achieved in a very successful 95 % yield reaction. The addition of the 2-day vacuum-dried 2′-*O*-

TOM-5′-*O*-DMT-pseudouridine in dry DCM at 0 °C pre-equipped with DIEA and the subsequent addition of the *CEP*-Cl as a solution in THF resulted in the diastereomeric products which were purified by fCC and subsequently used in the SPOS which is described in-depth in chapter 3.6.

3.5 Synthesis of m^1G, m^2G and $m^2{}_2G$ Phosphoramidites

This Section describes the synthesis of N^1-methylguanosine (m^1G), N^2-methylguanosine (m^2G) and N^2,N^2-dimethyguanosine ($m^2{}_2G$) phosphoramidites starting with commercially available guanosine. These synthesized phosphoramidites were finally used in the SPOS of which the detailed procedure is described in Chapter 3.6.

3.5.1 Synthesis of m^1G Phosphoramidite

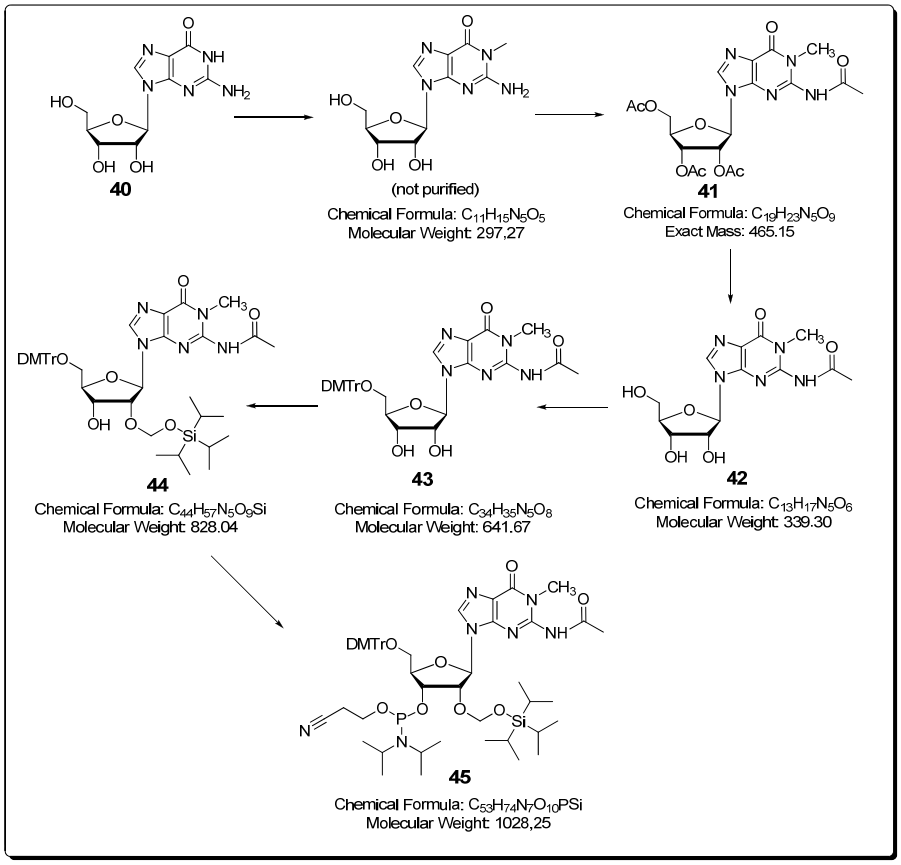


Figure 29 Synthesis of the m^1G phosphoramidite.

The synthesis of the phosphoramidite m^1G was achieved from commercially available guanosine **40** according to an already established method described by Hörbartner *et al*. with a few changes leading to improved yields as shown in Figure 29.

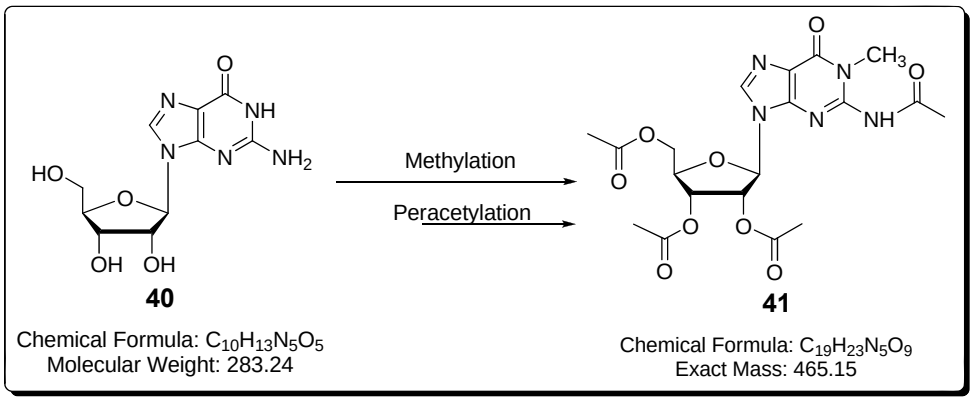


Figure 30 Synthesis of 2′, 3′, 5′-*O*-triacetyl-N^2-acetyl-N^1-methylguanosine.

The methylation of the N^1-postion of the purine base was achieved using NaH in anhydrous DMSO at rt to which the methylating reagent, MeI, was added. This product was not purified and was used in the next step after 5 h reaction time when the mixture was evaporated under *vacuo* at 80 °C. The peracetylation of the N^1-methylguanosine was achieved using refluxing mixture containing DMSO, Ac_2O and pyr in the ratio 1:1:1 (40 equivalents) in 14 h reaction time. The variation of the ratio of the DMSO, pyr and the acetic anhydride could lead mainly to the tri-esterified product. The resulting product **41** was very sticky and very difficult to purify. The dissolution of the crude in MeOH, addition of coarse silica and evaporation in *vacuo* finally ensured the successful packing onto an already prepared column which was eluted with a mixture of DCM and MeOH to give the product with yield for both steps being greater than 60 %. The scheme of these reactions is shown in the Figure 30.

The ester functionalities of the peracetylated product were cleaved off selectively in the presence of the amide functionality by careful addition of a 2 M solution of NaOH to a vigorously stirring mixture of the peracetylated product in a mixture of THF/MeOH/ H_2O in ratio 5:4:2. The saponification reaction was usually finished within 15 min and the reaction mixture was brought to pH 6.5-7 and kept at 4 °C overnight to precipitate. The precipitate was filtered and washed with a cold mixture of THF/MeOH/ H_2O in ratio 5:4:2 and dried under *vacuo*. The product **42**, a fluffy yellow solid, was obtained in a yield of 72 %.

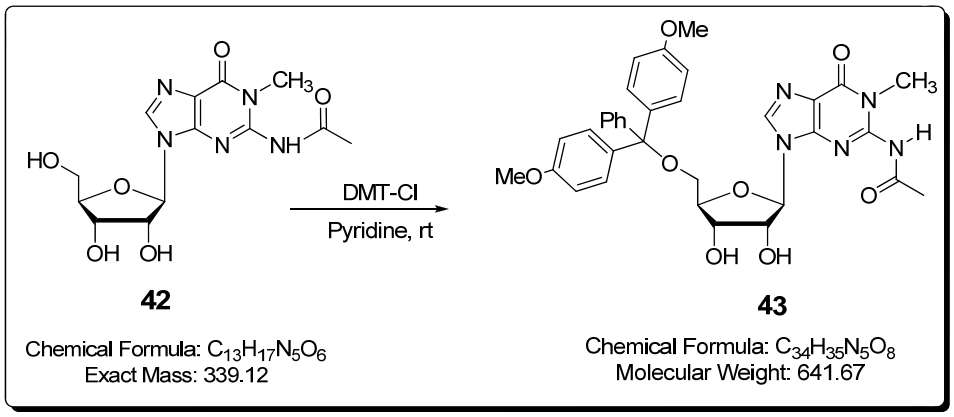


Figure 31 Attaching the trityl moiety to the 5′-*O*-position of m^1G.

The synthesis of the 5′-*O*-DMT-1-methylguanosine was achieved using the already established procedure used by Micura *et al.* [41]. The N^2-acetyl-1-methylguanosine **42** was dissolved in pyridine and the pyridine evaporated under reduced pressure, this was repeated three times to make the reaction mixture H_2O-free. To the finally dried mixture, molecular-sieve dried-pyridine was added and stirred at rt for 30 min. To this stirring mixture *DMT*-Cl as a solution in pyridine was then added slowly within 1 h. The reaction mixture was stirred and monitored by the aid of TLC. The reaction was finished within 8 h and was quenched with MeOH. The solvent was removed under *vacuo* and the crude product purified by fCC to yield the 5′-*O*-DMT–N^2-acetyl-N^1-methylguanosine in a yield of 85 % as shown in Figure 31. The product was allowed to dry under vacuum for 3 days and was used in the next reaction.

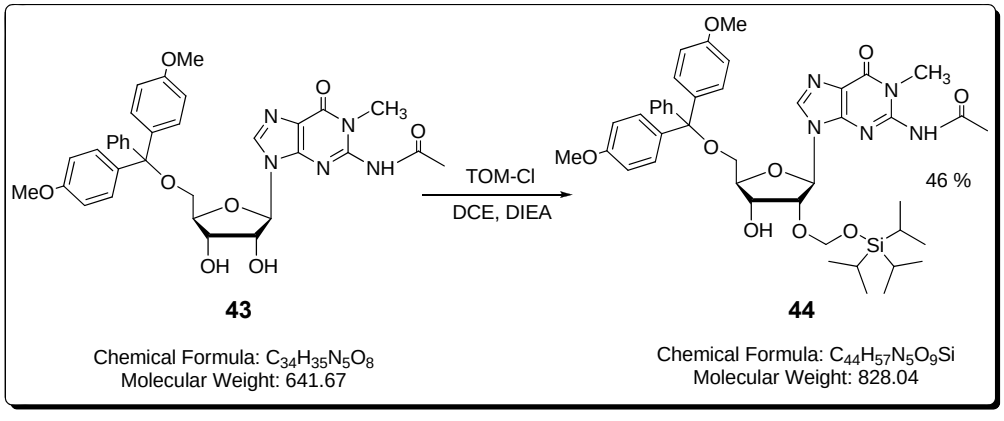


Figure 32 Attaching the TOM-group to the 2′-O position of m^1G.

The 2′-*O*H protection of the *β*-5′-*O*-DMT-N^2-acetyl-1-methylguanosine was achieved using TOM-Cl in dichloroethane in a similar reaction condition as described in the case of pseudouridine above. The reaction proceeded forming both the 2′-*O* and 3′-*O*-TOM protected products in non-equal amounts, but in this case no detectable amount of the 2′,3′-di-TOM product. The crude mixture was separated and the 2′-*O*-TOM-5′-*O*-DMT-N^2-acetyl-1-methylguanosine dried under vacuum overnight, giving a fluffy white solid with a yield of 46 % illustrated in Figure 32. The 3′-diastereomer was also formed with a yield of 36 %. This minor preference of the 2′-*O*- over the 3′-*O*-diastereomer was however not reproducible.

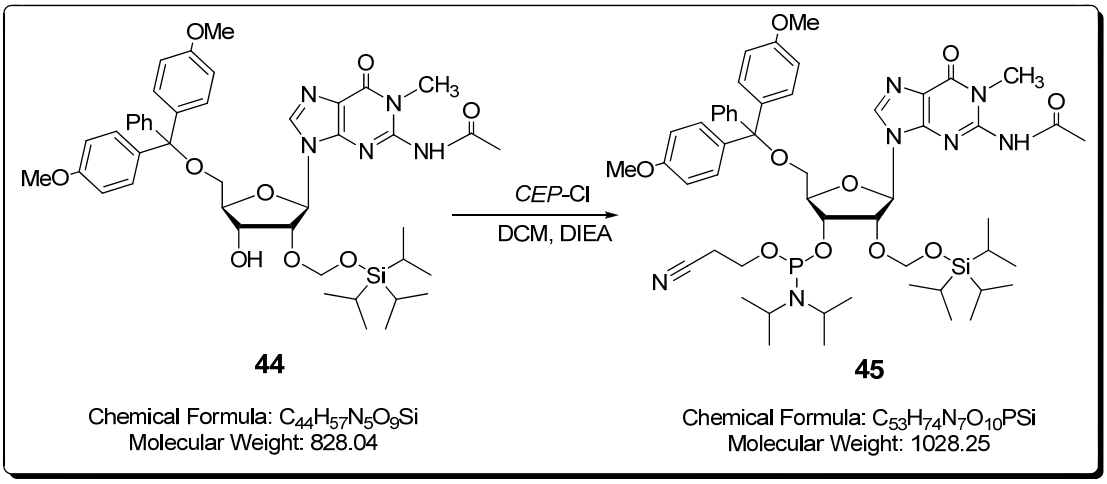


Figure 33 Ultimate step in the formation of the phosphoramidite m^1G.

The ultimate step which is the conversion of the 2′-*O*-TOM-5′-*O*-DMT-N^2-acetyl-1-methylguanosine into the phosphoramidite reagent that could be used in the *SP-RNA-S* was executed based on similar conditions used in the synthesis of the pseudouridine phosphoramidite using DCM and *CEP*-Cl at 0 °C to rt. The yield of this final step after fCC purification and subsequent drying under *vacuo*, was 85 %. The reaction scheme is illustrated in Figure 33. Care must be taken in order not to cleave off the DMT protection of the 5′-OH. An addition of 1-3 % Et_3N in the elution solvent mixture was therefore used in the fCC.

3.5.2 Synthesis of Precursor for m^2G and $m^2{}_2G$ Phosphoramidite

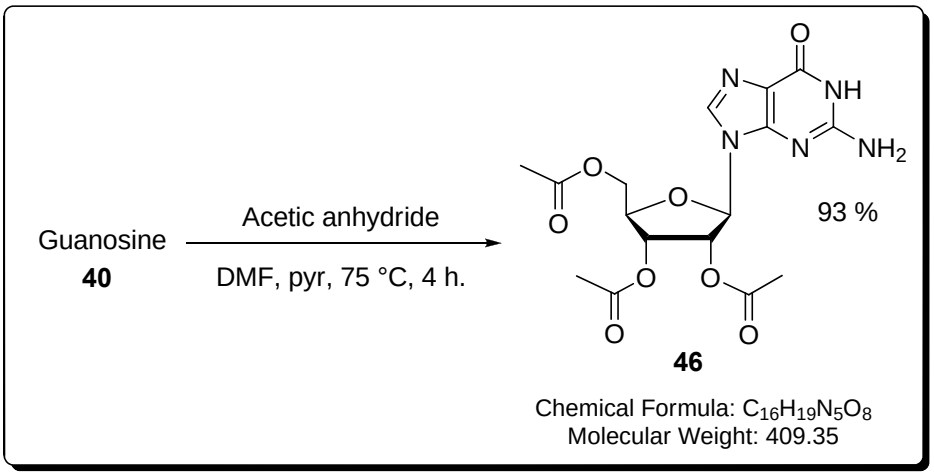


Figure 34 Synthesis of 2′,3′,5′-*O*-triacetylguanosine.

The 2′,3′,5′-tri-*O*-acetyl-O^6-npe-guanosine is a common precursor for both m^2G and $m^2{}_2G$ synthesis. The synthesis of this precursor follows a procedure reported by Robins *et al*. in which the esterification reaction occurs selectively in the presence of the free *exo*-cyclic amino functionality of the purine [98]. A reaction mixture containing acetic anhydride, pyridine and DMF in the ratio 6:3:8 was heated together with guanosine at 75 °C for 4 h giving rise to only the tri-ester with no detectable amount of the N^2-acetyl side product, as shown in Figure 34. The purification involves hot filtering and allowing the warm solution to cool to rt. The precipitate formed is filtered and re-crystallized in hot isopropanol. The yield for this reaction was in the range of 90-95 %.

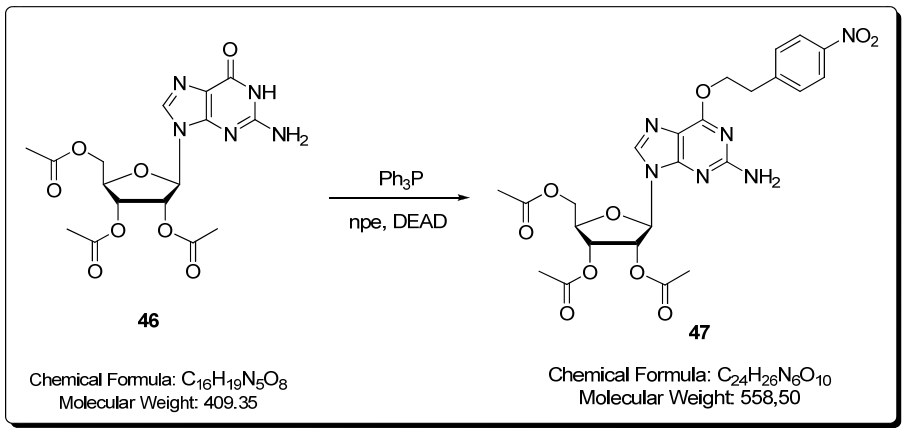


Figure 35 Protection of O^6-carbonyl using *npe* under Mitsunobu reaction condition.

3.5.3 Synthesis of m^2G Phosphoramidite

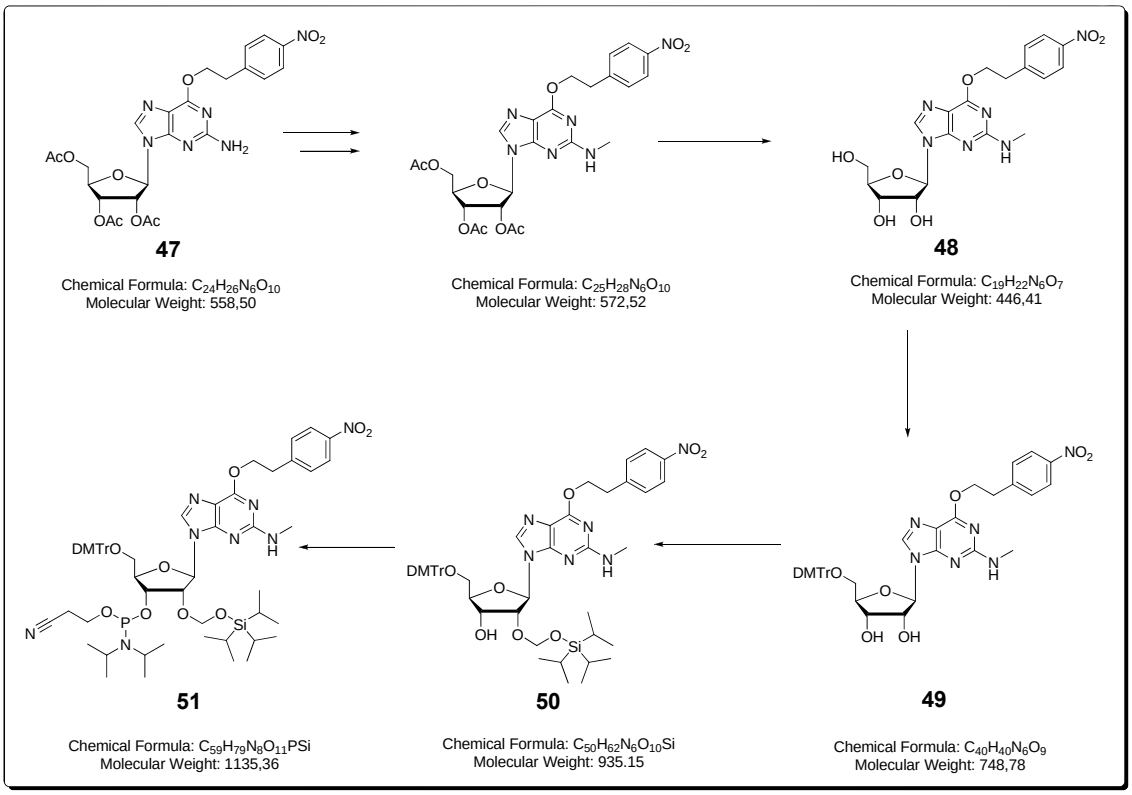


Figure 36 Synthesis of the m^2G phosphoramidite.

The general scheme for the synthesis of the m^2G phosphoramidite is illustrated in Figure 36. Using the 2′,3′,5′-tri-*O*-acetylguanosine, the protection of the O^6-hydroxy functionality was achieved using Mitsunobu reaction conditions as shown in Figure 35. The two reaction parts are 2-(4-nitrophenyl)ethanol and the 2′,3′,5′-tri-*O*-acetylguanosine in the presence of PPh_3 and DEAD as promoters for the etherification reaction. The reaction was carried out in dioxane at 80 °C and allowed to cool down to 60 °C within 1 h, after which the clear solution was reduced under *vacuo* when TLC shows no starting nitrophenyl alcohol left. The product of this etherification reaction was purified by fCC using Et_2O and DCM mixture to yield of 80 % as a semi-solid.

The methylation of the N^2-*exo*-cyclic amine functionality was achieved through the indirect convertible nucleotide strategy. The addition of HBF_4 first and subsequent addition of $NaNO_2$ resulted in the exchange reaction between the formed *nitroso* and the fluoride functionality. The fluoride solution formed was neutralized with NaOH. Afterwards it was extracted into DCM and

dried further with anhydrous Na_2SO_4 after which it was evaporated under *vacuo*. The crude product was not further purified but used in the next step. The crude material was dissolved in 8 M CH_3NH_2 in ethanol and stirred overnight after which it was evaporated to dryness under reduced pressure. During the fluoride methylamine exchange reaction, the acetyl group of the ester was cleaved off in a one-pot reaction giving the product **48** with a yield of 51 %.

The 5′-*O*-DMT protection was achieved using the general procedure applied in the synthesis of the m^1G phosphoramidite. There was in this case the addition of the base DMAP due to the very slow rate of reaction.

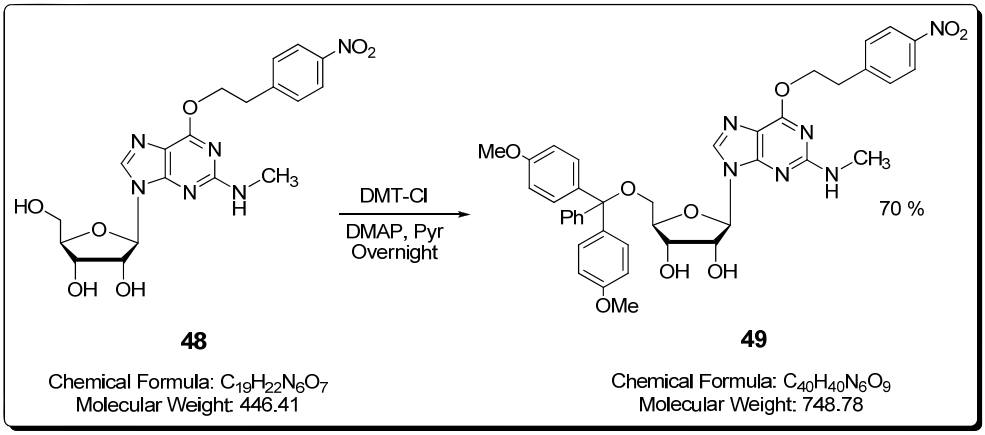


Figure 37 Selective protection of 5′-OH of the m^2G with DMT-Cl.

Typically, 5′-*O*-DMT protection reaction was completed within 8 h. However, we had to consider an overnight reaction due to the difficulty in the dissolution of the modified G and the resulting decrease in the reaction rate. The yield for this reaction was 70 %, a fluffy pale yellow product **49**. Typical yield for the 5′-*O*-DMT protection was 85 % or greater.

The 2′-*O*H protection was achieved using the fluoride labile non-migrating TOM-Cl. This reaction just like the 5′-*O*-DMT protection gave rather low yields compared to other 2′-OH protections. Longer reaction time was not resulting into an appreciable increase in the amount of product formed.

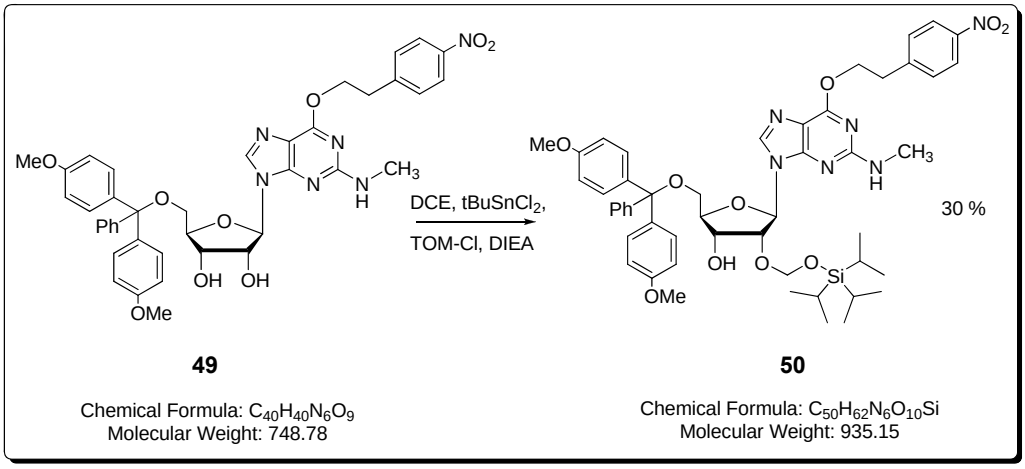


Figure 38 Protection of the 2′-*O*- with *TOM*.

The reason for this low yield will be attributed to the low dissolution of the starting material in DCE or DCM. The regioselectivity of the 2′-*O*-alkylated over 3′-*O*-alkylated product was not different. The product **50**, a foamy white solid was obtained after fCC with 3 % Et_3N, giving a yield of 30 %. The scheme of the reaction is as shown in Figure 38.

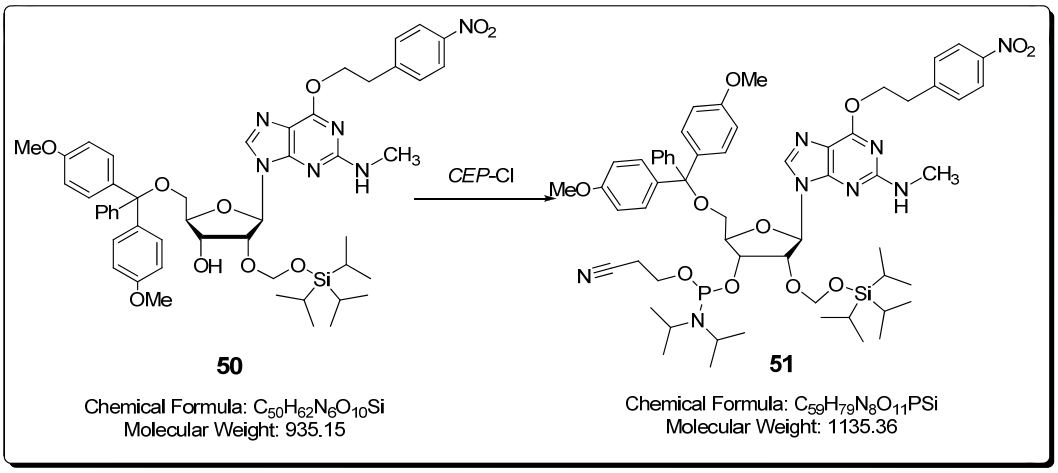


Figure 39 The phosphitylation reaction of m^2G.

The conversion of the 5′-*O*-DMT-2′-*O*-TOM-m^2G **50** to the m^2G-phosphoramidite **51** was achieved using DCM to which DIEA was pre-dissolved at rt. The addition of the starting material during cooling after which the mixture was brought to rt and then the successive addition of *CEP*-Cl as a solution in THF within 15 min resulted in the formation of the m^2G-

phosphoramidite **51** shown in Figure 39. This phosphoramidite was purified by fCC giving a yield of 65 % and used in the *SPOS* which is described in detail in chapter 3.6.

3.5.4 Synthesis of $m^2{}_2G$ Phosphoramidite

Using the 2′,3′,5′-tri-*O*-acetylguanosine, the protection of the O^6-hydroxy functionality after tautomerization from the keto form was achieved using the Mitsunobu reaction condition. The reaction condition was similar to the one used in the synthesis of the m^2G. The resulting yield was 82 % as a semi-solid. The di-methylation of the N^2-*exo*-cyclic amine functionality was achieved through the indirect convertible nucleotide strategy.

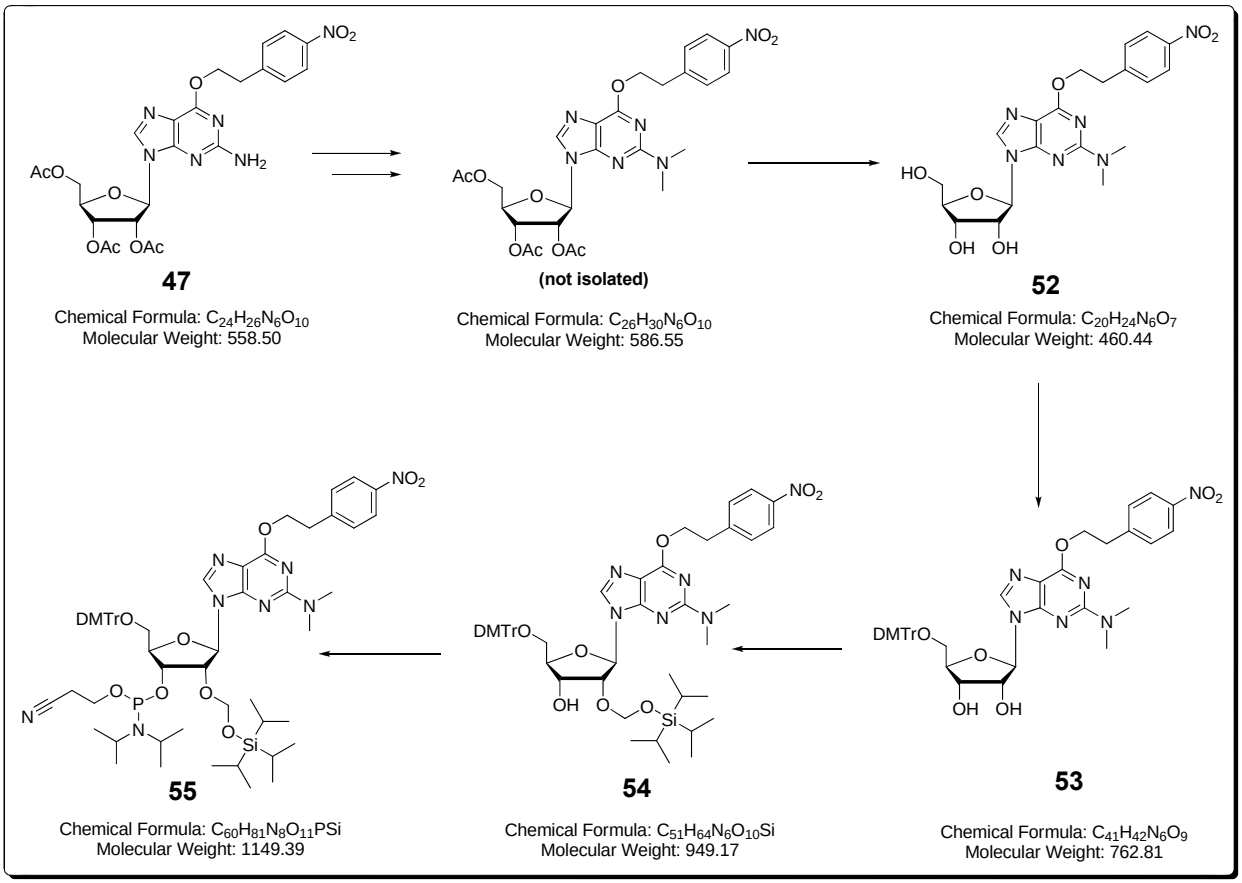


Figure 40 **Synthesis of $m^2{}_2G$ phosphoramidite.**

The successive addition of HBF_4 and $NaNO_2$ resulted in the exchange reaction between the nitroso and the fluoride functionality. The mixture was neutralized with NaOH after which it was extracted into DCM and dried furthermore with Na_2SO_4, evaporated under *vacuo*. The crude

product was used in the next step without further purification as shown in Figure 40. The crude material was dissolved in ~12M $(CH_3)_2NH$ in ethanol and stirred overnight after which water was added to speed up the saponification of the product and then evaporated to dryness under reduced pressure. During the fluoride-dimethylamine exchange reaction, the acetyl groups of the ester were cleaved off in the one-pot reaction giving the product with yield of 51 % shown in Figure 40.

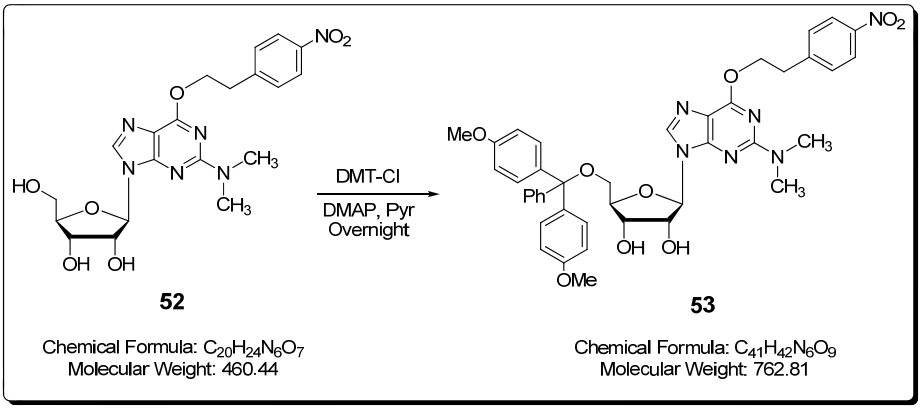


Figure 41 Protection of the 5′-OH for the synthesis of the $m^2{}_2G$ phosphoramidite.

The 5′-*O*-DMT protection was achieved using the general procedure applied in the synthesis of the m^2G phosphoramidite. As described earlier, an overnight reaction was considered due to the difficulty in the dissolution of the modified G and the resulting decrease in the reaction rate which is similar to the case of the m^2G. The yield for this reaction, a fluffy pale yellow product **53,** was 68 % as shown in Figure 41.

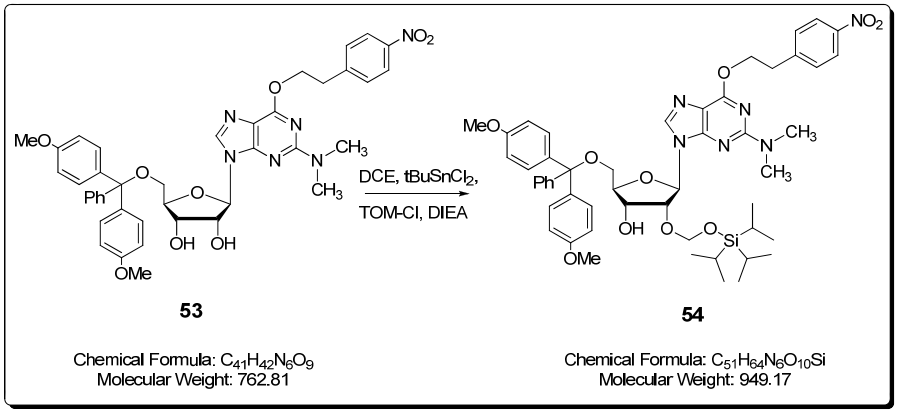


Figure 42 Fluoride labile protection of the 2′-OH for the synthesis of $m^2{}_2G$ phosphoramidite.

The 2′-hydroxy protection was achieved using the fluoride labile non-migrating *TOM*-Cl. This reaction just like the case in the m^2G gave rather low yields compared to other 2′-protections. Similarly, longer reaction time was not resulting into an appreciable increase in the amount of product formed. The reason for this low yield may be attributed to the low dissolution of the starting material in DCE or DCM. The regioselectivity of the 2′-*O*-alkylated over 3′-*O*-alkylated product was 5:3. The product, a foamy off-white solid was obtained after fCC with 3 % Et_3N in an Hexane-EtOAc mixture with a yield of 32 %. The scheme of the reaction is as shown in Figure 42.

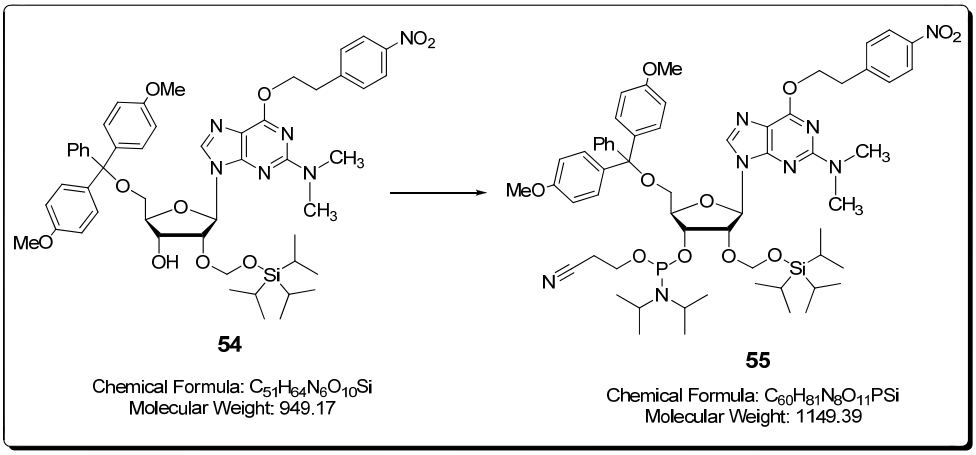


Figure 43 The ultimate step in the synthesis of $m^2{}_2G$ phosphoramidite.

The ultimate step, which was the conversion of the 5′-*O*-DMT-2′-*O*-TOM-$m^2{}_2G$ to its phosphoramidite, was achieved using DIEA activation of the 3′-OH functionality in DCM prior to the addition of the *CEP*-Cl as a solution in THF at rt. The finally formed diastereomeric crude phosphoramidite was purified by fCC giving a yield of 69 %, illustrated in Figure 43, which was used in the *SPOS* described in detail in chapter 3.6.

3.6 RNA Oligonucleotide Syntheses

This Section describes the Solid-Phase RNA Oligomeric Synthesis (SPOS) of the various RNA fragments that were used for both tRNA fragments for the ligation construction kit and those for the siRNA investigations. The synthesis employs the use of both modified synthesized and commercially available phosphoramidites.

3.6.1 Construction Kit to Investigate Effects of Modification on tRNA

The Solid phase RNA synthesis was performed using both the purchased and the self-synthesized modified phosphoramidites. The incorporation of the modified m^5U and Ψ phosphoramidites was equally efficient as that of the unmodified counterparts A, C, G and U used in the *SPS*. There was however low coupling efficiency from the modified guanosine phosphoramidite. This reduction of coupling efficiency might be due to the difficulty in the dissolution of the modified guanosine phosphoramidite in the solvent (AN) used for the synthesis and hence the low reactivity. The synthesis failed for the cases where more than one modified guanosine was to be incorporated. This problem was finally solved by making a dilute solution of the modified phosphoramidite (0.1 M instead of the 0.25 M in AN) and repeating each step in the cycle of the protocol for the modified guanosine phosphoramidites.

The RNA oligonucleotides were synthesized for two main research purposes. In the first instance, our idea of a construction kit shown in Figure 44, that allows the total chemical synthesis of full length tRNA fragments which are themselves products of finally ligated shorter fragments was envisaged [99].

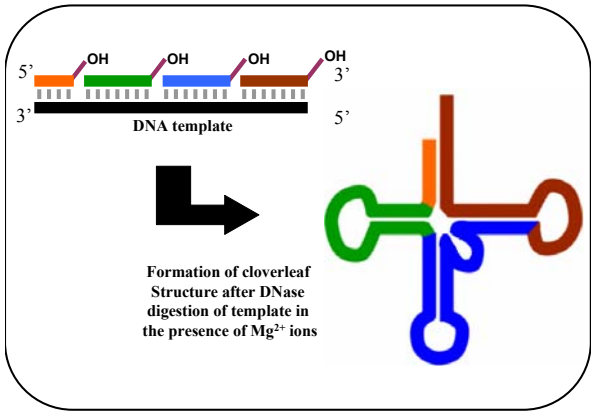


Figure 44 Concept of the ligation kit.

In this respect, the synthesis was strategized to achieve RNA fragments as long as possible but as well without compromising on the practicability of the process. Typically, oligomeric units of up to 40 nucleotides length could be synthesized but for a very efficient synthesis, nucleotide lengths of 20 to 30 are optimal. The construction kit was designed to synthesize tRNAs using fragments of oligonucleotides with varying degree of bases modifications. The construction of the $tRNA^{Ile}$ with total modification and without any modification was achieved in a 3-fragment T4 DNA ligase catalyzed synthesis with yields between 20-30 %.

3.6.2 Ligation Experiment

The ligation reaction was conducted overnight at 16 °C and the crude mixture was purified by GEL electrophoresis after digestion of the DNA splint using a DNase. The digestion was monitored by analyzing the result of 1 µL on using PAGE. The result of the PAGE is displayed in the experimental section. The following scheme was used for synthesis of $tRNA^{Ile}$ Table 2.

	oligo1	**oligo2**	**oligo3**	**Splint**	**L-premix**	**ligase**	**H_2O**	**T. vol**	
MH	272	274	276	329	4x				
Conc	100	100	100	100					Ile1
µL	20	20	20	20	62.5	40	67.5	250	
MH	273	275	276	329					
Conc	100	100	100	100					Ile4
µL	20	20	20	20	62.5	40	67.5	250	

Table 2 The ligation scheme for the synthesis of $tRNA^{Ile}$.

3.6.3 Analysis of $tRNA^{Ile}$ by Thermal Melting Curves

The stability of a tertiary structure of the RNA was measured by thermal denaturation experiments on a UV-visible spectrophotometer, by recording the absorbance at 260 nm as a function of temperature. Heating a RNA sample results in a change in absorbance properties, which reflects a conformational change of the molecule in solution, and allows the determination

of RNA secondary structure stability. Denaturation leads to a hyperchromism of 15-20%. Cooling the sample leads to a renaturation of the structure.

A thermal denaturation experiment of RNA yields the melting temperature value T_m, which corresponds to the temperature at which half of the sample is base-paired and half is unwinded. T_m deteremination implies the measurement of the absorbance properties of the folded and unfolded forms as a function of temperature.

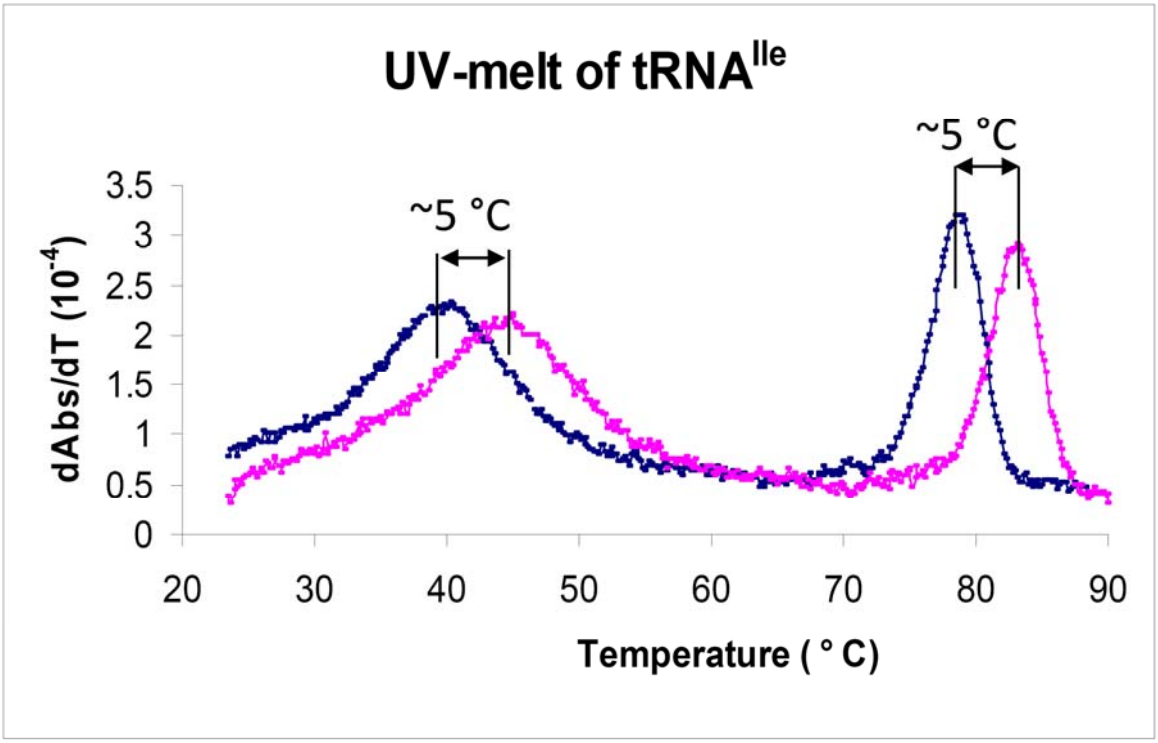


Figure 45 The first derivative of the UV melts of tRNA ^{Ile} with (purple) and without (blue) modifications.

The results of the melting curve analysis show that the unmodified tRNA (T_m ~ 77.1 °C) were (~ 4.5 °C) less stable as compared to the fully modified tRNA (T_m ~ 81.6 °C) as shown in Figure 45. This was however not surprising since the modification will result into more stable tertiary structure hence the stability.

3.6.4 Effects of Base Modifications on siRNA Studies

Table 3 Synthesized siRNAs for knock-down and immunostimulation studies.

Synthesized Oligomers	Code
r(GAACUψCAGGGUCAGCUUGCCG)	MH278
r(GAACUψCAGGGψCAGCUUGCCG)	MH279
r(GAACUψCAGGGψCAGCUψGCCG)	MH280
r(GAACUUCAGGGUCAGCUTGCCG)	MH281
r(GAACUUCAGGGTCAGCUTGCCG)	MH282
r(GAACUTCAGGGTCAGCUTGCCG)	MH283
r(GAACUUCAGGGUCAGCUUm^{1}GCCG)	MH284
r(GAACUUCAGGm^{1}GUCAGCUUm^{1}GCCG)	MH285
r(m^{1}GAACUUCAGGm^{1}GUCAGCUUm^{1}GCCG)	MH286
r(GAACUUCAGGGUCAGCUUm^{2}GCCG)	MH287
r(GAACUUCAGGm^{2}GUCAGCUUm^{2}GCCG)	MH288
r(m^{2}GAACUUCAGGm^{2}GUCAGCUUm^{2}GCCG)	MH290
GCAAGCUGACCCUGAAGUUCAU	MH294
GCAAGCψGACCCUGAAGUUCAU	MH295
GCAAGCUGACCCUGAAGUψCAU	MH296
GCAAGCUGACCCψGAAGUUCAU	MH297
GCAAGCUGACCCUGAAGψψCAU	MH298
GCAAGCψGACCCψGAAGUψCAU	MH299

In the second case we endeavored to investigate the effect of modified bases in siRNA. The synthesis of the following RNAs with the modifications shown were achieved, (see Table 3), using the modified phosphoramidites that were synthesized and purified as described in Chapter 3.

The synthesized RNA oligomeric units were used for the investigation of the knock-down effects and also tested for immunostimulation efficiency. This was done in a collaborative work with

Dalpke *et al.* (Florian Eberle). The results of the knock-down and the immunostimulation efficiency are shown in the following.

In a series where the antisense strand of the GFP siRNA has been made to contain 1, 2 or 3 pseudouridines or no pseudouridine, the influence of these pseudouridine moieties on immunostimulation was negligible. From the bar graph shown in **Figure 46**, the extent of immunostimulation was highest with the strand containing no modification. However there was no significant decrease in immune stimulation in the number of introduced pseudouridines in the strand.

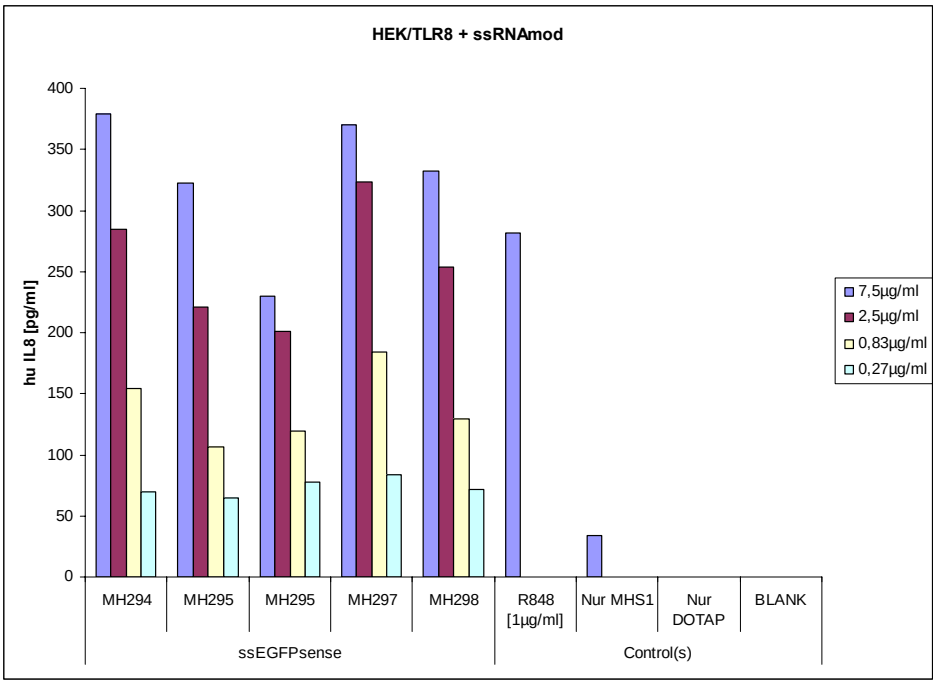


Figure 46 Immunostimulation of IL8 in the presence pseudouridine**:** Effect of varying concentration of siRNA on sensitivity of IL8 induction as a measure of varying the position and the number of Ψ in siRNA. Increasing concentration results in increase in immune response.

R-848 an immune response modifier, which is a known immunostimulator was used as a positive control for the experiment. Also for the negative control DOTAP which has no immunostimulation effect was used. There was also the blank which contains only the cell line shown in **Figure 46**.

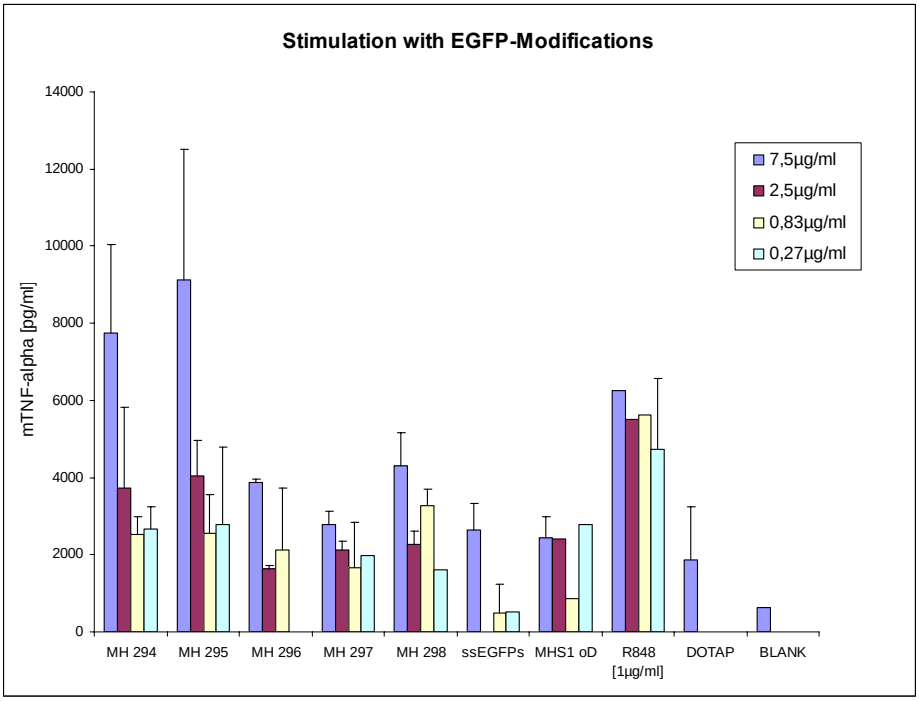


Figure 47 TNF-alpha stimulation using single strand siRNA with modifications: Effect of varying concentration of siRNA on sensitivity of mTNF-α induction as a measure of fixed position and the number of Ψ in siRNA.

In another series where the antisense strand with a sequence complementary of the GFP containing either no pseudouridine or 1, 2 or 3 pseudouridines moieties were tested for the expression of mTNF-alpha induction on a cell line as shown in the bar graph in **Figure 47**. There was an inexplicably high immunostimulation with the MH294 and MH295 containing no modification and 1 modification at position 7 respectively. There was however no such expression with similar siRNA containing 1 modification at position 19 (MH296) and position 13 (MH297). The results of the test of the expression of interleukins by the induction of the cells with the siRNAs containing various modifications of pseudouridine did not give very clear results.

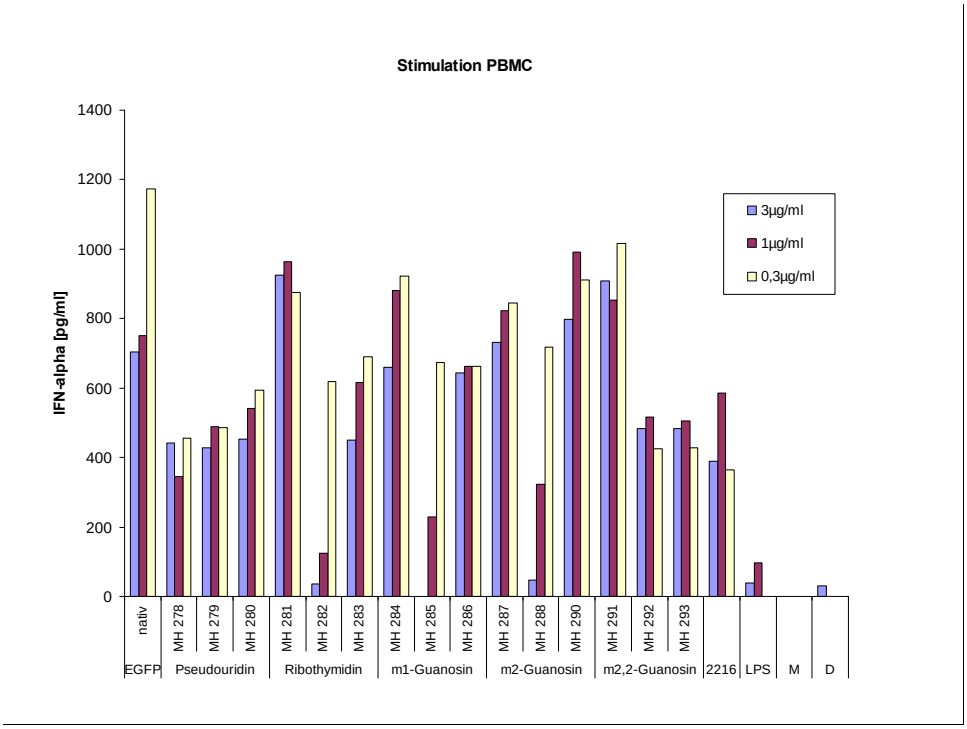


Figure 48 IFN-alpha expressions in the presence of modified siRNA: Sensitivity and effect of modification types on the stimulation of INF-alpha using siRNA containing 1, 2 or 3 Ψ, rT, m^2G, $m^2{}_2G$ and m^1G. The results show a proportionate responds with respect to incressing concentration of modified siRNA.

We therefore decided to consider various modifications using one, two or three modifications of pseudouridine, ribothymidine (T), m^1G, m^2G and $m^2{}_2G$. The synthesized siRNAs were tested for their immunostimulation in concentrations of 0.3, 1.0 and 3 µg/mL. The amount of interferon alpha expressed was measured in each case. The result of this investigation is shown in Figure 48. There was a proportionate increase in the amount of expressed IFN-alpha in the case of the m^1G and the ribothymidine introduced modifications. That could however not be stated for the others. Based on this finding, the m^1G and the ribothymidine modified siRNAs were re-investigated.

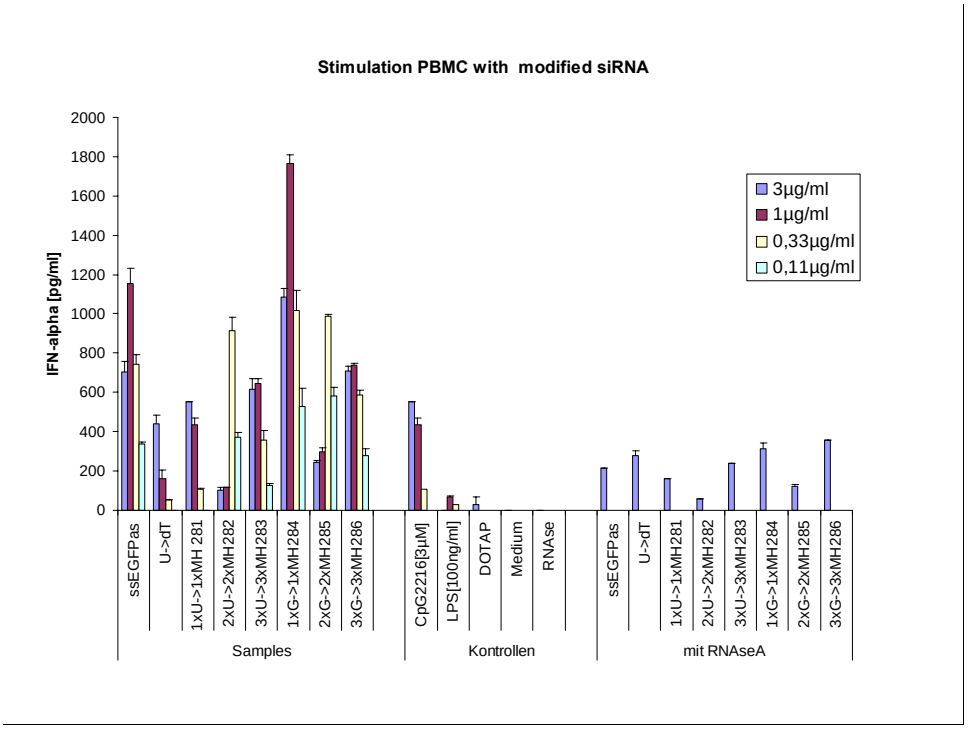


Figure 49 IFN-alpha expression in the presence of modified siRNA containing rT and m^1G: Effects of varying concentration of modified siRNA as anti sense strand on IFN-alpha stimulation showing inexplicable high induction in the presence of m^1G.

In a series of experiments where the antisense strand has been made to contain one, two or three ribothymidine and m^1G, the immunostimulation was measured as the amount of secreted interferon-alpha. There was a higher expression of interferon by the siRNA containing the m^1G as compared to the others shown in Figure 49.

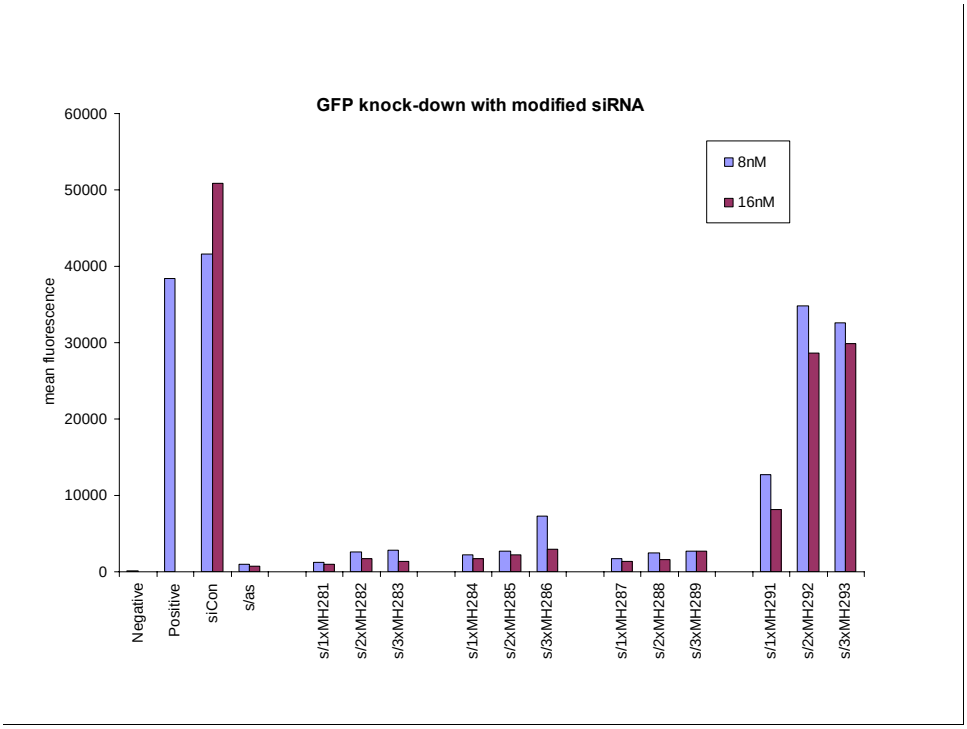


Figure 50 Knockdown of GFP protein in the presence of modified siRNA: GFP knockdowns vs. type and number of modification using siRNA containing 1, 2 or 3 rT, m^2G, m^1G and $m^2{}_2G$. Results show efficient knock down with rT, m^2G and m^1G but very poor knock down with $m^2{}_2G$.

The knock-downs of GFP by the siRNA with modifications introduced were measured by the amount of fluorescence emitted by the cells. The m^1G, m^2G and the rT modified siRNAs showed very high knock-down efficiency and the results were proportionate with the amounts used in the investigation as shown in Figure 50. The $m^2{}_2G$ containing siRNA as expected showed a poor knock-down of the GFP and hence a high mean fluorescence.

3.7 Syntheses of Bromocoumarins and Derivatives

This Section describes the synthesis of the various bromomethylcoumarins and their derivatives to be used for the qualitative and quantitative investigations of pseudouridine containing oligonucleotides.

3.7.1 Synthesis of 7-azido-4-bromomethylcoumarin

In our bid to synthesize the 4-bromomethyl-coumarin compound, we performed the following trial reactions in order to ascertain the feasibility and ease of reaction taking into consideration the susceptibility to hydrolysis of the benzylic-like bromide functionality. The reactions are usually performed under solventless condition. However reported work of the synthesis of 7-acetamide-4-bromomethylcoumarin was employed using 75 % H_2SO_4 with the dual role of catalyst and solvent. This alternative allows the uniform mixing of reagents and therefore was considered superior even though an additional step was required for the removal of the sulfuric acid.

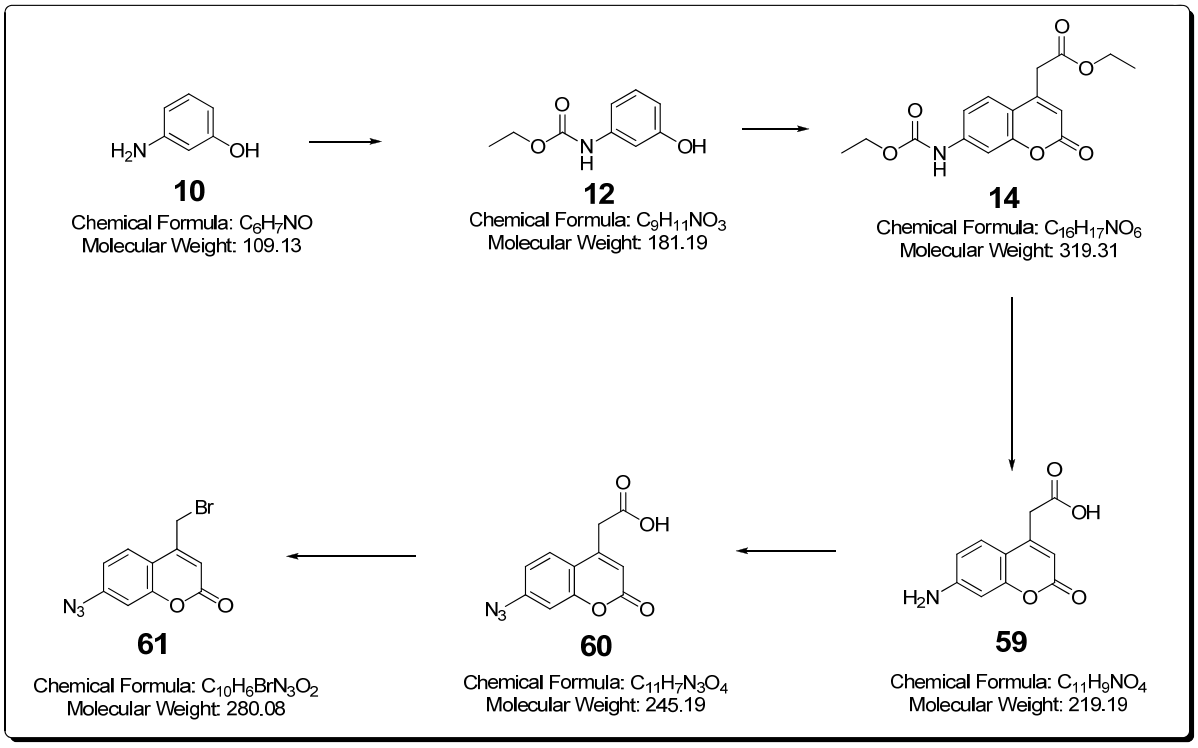


Figure 51 Scheme of 7-azido-4-bromomethylcoumarin.

The protection of the amino functionality of the 3-aminophenol was achieved following the already established protocol published by Maly *et al* [100]. The mixture was filtered whilst hot and the hot filtrate cooled to rt and then evaporated to dryness. The isopropanol recrystallized product was obtained in a yield greater than 95 % shown in Figure 51. This did not differ from the reported yield of the published work. The product of this reaction was analyzed after thorough drying under reduced pressure and used in the next step. A critical step in this synthesis was the formation of the coumarin moiety by the Pechmann condensation reaction. This critical reaction, was conducted using the ethyl 3-hydroxyphenylcarbamate formed and the heat sensitive cyclization partner diethyl 3-oxopentane-1,5-dioate in an acid catalyzed reaction. The reaction was typically finished after 10 h and was therefore left overnight after which it was checked for the formation of the product by TCL. The product formed was dissolved in water and extracted into EtOAc, the H_2O dried with anhydrous Na_2SO_4 evaporated to dryness and used in the next step without further purification. The yield of this important step shown in Figure 51 was 90 %.

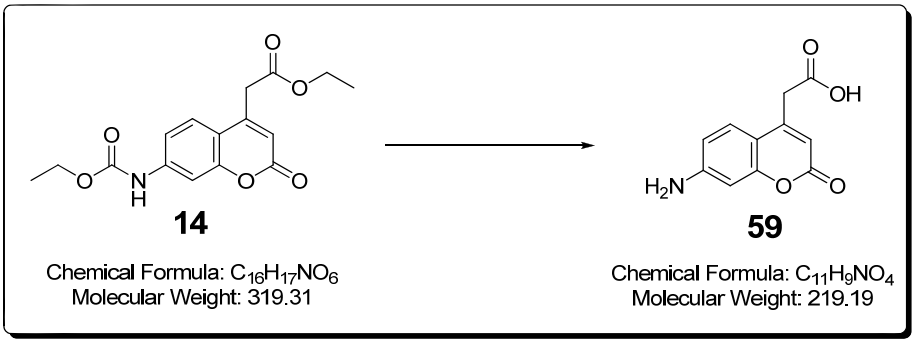


Figure 52 Hydrolysis and cabarmoyl deprotection.

The deprotection of the amino-functionality was achieved in a one pot reaction that also removes the ethyl ester protection on the *exo*-cyclic carboxylic acid. A 16 h reflux of the carbamate-ethyl protected product in a 4 M solution of NaOH resulted in the formation of the product; 2-(7-amino-2-oxo-2*H*-chromen-4-yl)acetic acid shown in Figure 52. The formation of the azido-functionality was executable at this stage. Consecutively, the addition of 2-(7-amino-2-oxo-2*H*-chromen-4-yl) acetic acid, conc. H_2SO_4 and ice slurry, and the temperature of the mixture was maintained at 0 °C for 15 min. Furthermore, $NaNO_2$ was added portion-wise as a solution in ice-water whilst stirring vigorously and subsequently NaN_3 was added in one portion (as a solution in

ice-water). The reaction mixture was stirred overnight after which it was extracted with EtOAc. The organic layer was washed with brine and then dried with anhydrous sodium sulfate, filtered and concentrated under *vacuo*.

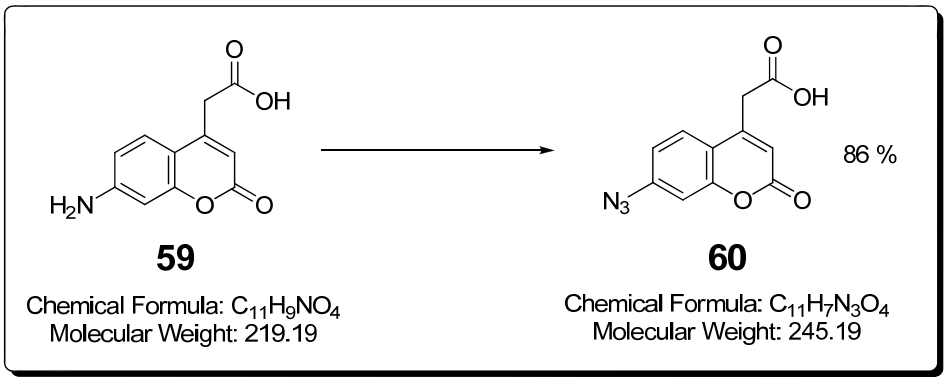


Figure 53 Formation of 7-azido-4-methylcarbamoylcoumarin.

The product was pure enough (from NMR) and was therefore used in the next step without further purification, as shown in Figure 53. Part of this crude mixture was purified by fCC with a yield of 86 %.

The ultimate step involves the decarboxylic bromination reaction under acidic condition. It was reported by Secrist *et al*. that under refluxing acetic acid the methylcarbamoyl-coumarin was converted into its bromo product as shown in Figure 54 [101]. The reported yield of the reaction using equal equivalents of bromine and 7-methoxycoumarin-4-methylcarboxylic acid was 51 %.

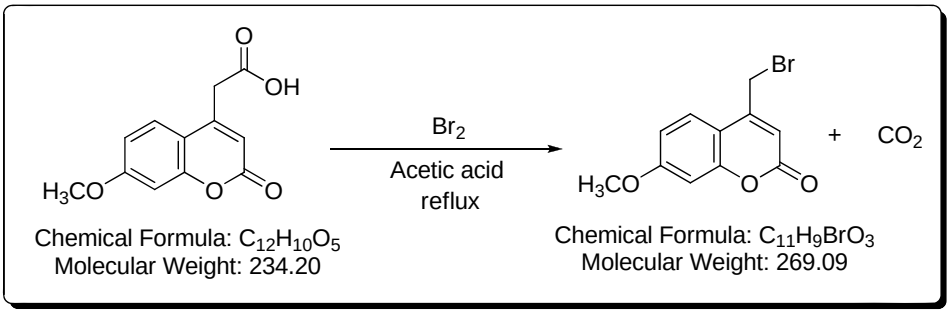


Figure 54 Decarboxylative bromination reaction by Secrist *et al*. (1971).

We endeavored to achieve the decarboxylative bromination reaction following the reported protocol by Secrist *et al.* with very minor modification in order to increase the yield. A refluxing

solution containing 2-(7-azido-2-oxo-2H-chromen-4-yl)acetic acid in acetic acid was stirred for 10 min. Br_2 was added as a solution in acetic acid and the reaction mixture was refluxed for 1 h after which TLC reveals large amount of starting material. Additionally, Br_2 (1 equiv) was added and the refluxing was continued for 1 h after which TCL still showed starting material. Br_2 was then added again and the mixture refluxed overnight. The mixture was concentrated under reduced pressure and with the aid of a short fCC, the product was eluted with ethyl acetate. The total amount of bromine used was 3 equivalents and the yield after purification was 93 %. The reaction scheme is shown in Figure 55. The product **61** was analyzed for the integrity of the azido-functionality by IR-spectroscopy

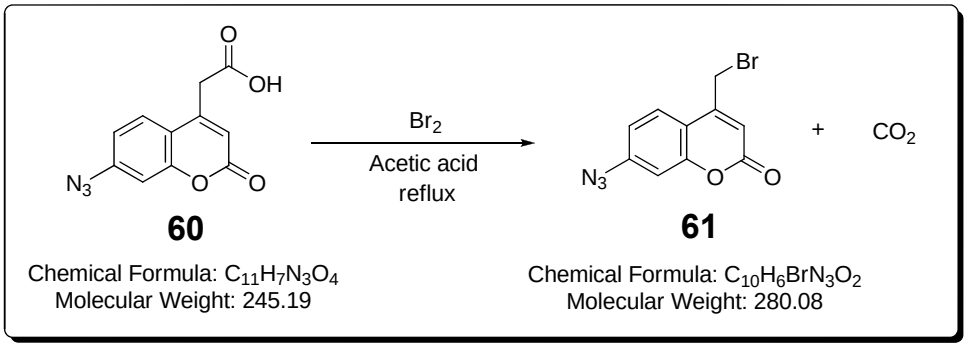


Figure 55 Decarboxylative bromination of 7-azidocoumarin-4-methylcarboxylic acid.

The product, a yellowish-brown solid, is light sensitive and decomposes upon exposure to light. It is stored under argon in a light-opaque flask at -20 °C. The 7-azido-4-bromomethylcoumarin **61** is currently used in the studies of specific recognition of pseudouridine in the Helm group {unpublished results}.

3.7.2 Synthesis of 7-(biotinyl-jeffamine-succinyl-4-bromomethylcoumarin

The retrosynthetic scheme of the synthesis of biotinylated bromomethyl coumarin spaced with jeffamine succinate is shown in Figure 56. The reported work of the Hong *et al.* [102] where the coupling of the 7-hydroxy-4-bromomethycoumarin and the activated carboxylic acid functionality as reported was carefully studied for its applicability to our case. The easily hydrolizable bromide functionality need to be preserved during the reaction and purification of the crude product. In the reported work of Hong *et al*., the polydispersed and high molecular

weight of the carboxylic acid functionalized moiety allows the product of the reaction to be separated by radial chromatography.

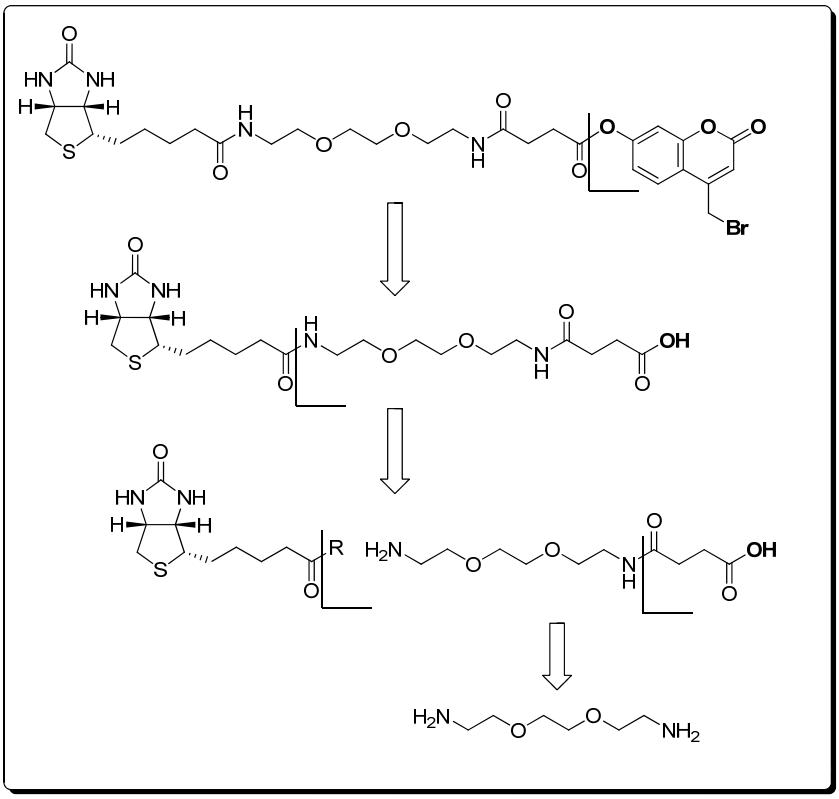


Figure 56 Retrosynthetic scheme of 7-(biotin-jeffaminyl-succinoyl)-4-bromomethyl coumarin.

3.7.3 Synthesis of Biotin–NHS Ester

The conversion of biotin to the biotin-NHS ester was performed according to published result from the work of Charvet *et al* [103]. The synthetic strategy involves the use of DCC to activate the carboxylic acid functionality of biotin after which the NHS is added to form the semi-stable biotin-NHS ester intermediate **63**; shown in Figure 57. The reaction durations are typically very long, 2-3 days at rt. There is often an increase in yield of the side product when the temperature is increased due to re-arrangement of the very stable DCC activated product. The filtration of the DCU formed and the subsequent dissolution of the mixture in water and extracting with DCM/MeOH (1:1) mixture, drying the organic phase with anhydrous Na_2SO_4 and evaporation under reduced pressure gave the crude.

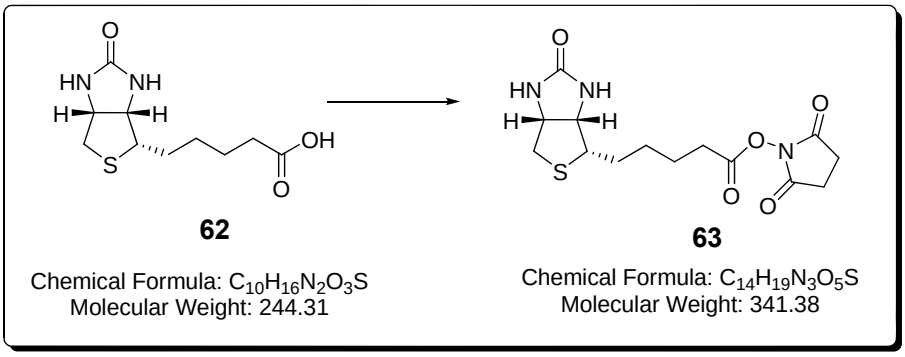


Figure 57 Synthesis of biotin–NHS ester.

Re-crystallization of the crude from hot isopropanol and allowing the mixture to cool to rt results in the prism-like product with a yield of 92 %.

3.7.4 Synthesis of *3-(2-(2-(2-aminoethoxy)ethoxy)ethylcarbamoyl)propanoic acid*

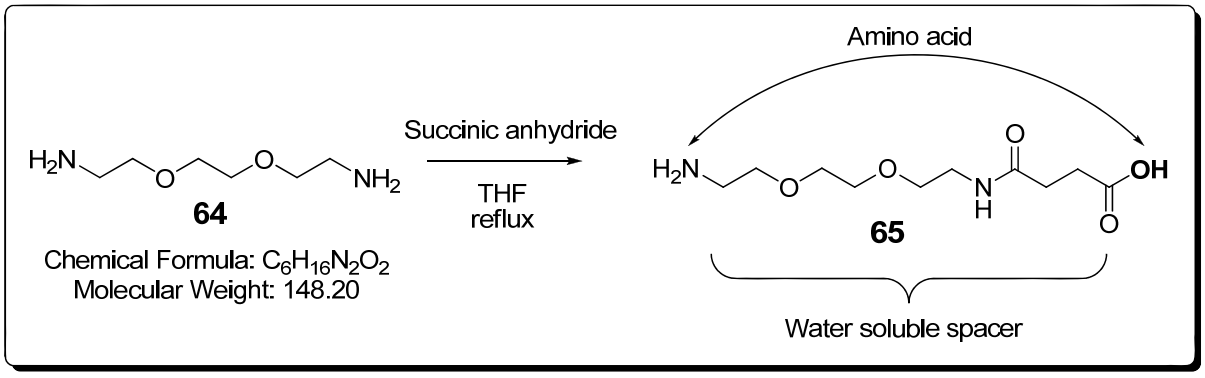


Figure 58 Synthesis of jeffaminylsuccinylate.

In order to maintain the water solubility of the final molecule, the desired linker should be as short as possible and hydrophilic in nature. We started with oxalic acid, attempting to protect one of the two carboxylic acid functionalies with *tert*-butanol, as had been described for malonic acid by Gao *et al.* [104]. This reaction failed, probably due to the instability of such oxalic acid derivatives in general. It was proceeded with analogous protection of malonic acid and subsequent formation of a succinimide at the free acid functionality. However, this was accompanied by side product formations which led to low yields. Succinic acid was then chosen as dicarboxylic acid linker, as the amide bond formation with jeffamine 148 could easily be achieved by simply adding succinic anhydride to the latter diamine without an additional

protection step. The mono-protection of the diamine functionalized water soluble compound, Jeffamine-148 **64**, was achieved without much difficulty. The drop-wise addition of 1 equivalent of succinic anhydride, as a solution in THF, to a refluxing mixture of containing 4 equivalent of 2,2′-(ethane-1,2-diylbis (oxy)) diethanamine in THF. The refluxing was stopped and the mixture was allowed to cool to rt after which the formed precipitates were filtered and washed several times with EtOAc and allowed to dry in air. This crude product **65** was pure enough and was used in the next stage of the reaction without further purification. The yield of this reaction was estimated as quantitative as no succinic anhydride was observed; the scheme of the reaction is as shown in Figure 58. The product, an amino-acid like compound, could act as a water soluble linker for most syntheses that require a hetero-bifunctional group for orthogonal coupling.

3.7.5 Synthesis of Biotinylated-jeffaminylsuccinylate

The attachment of the hydrophilic spacer-linker to biotin was achieved using the NHS-activated strategy. The biotin-NHS ester was dissolved in DCM to which the amino-like acid was added as a solution in MeOH and the reaction mixture stirred and monitored by the aid of TLC. The reaction was finished within 1 h after which it was evaporated to dryness and purified by fCC. The yield of this penultimate step was greater than 80 %. The scheme of this reaction is shown in Figure 59.

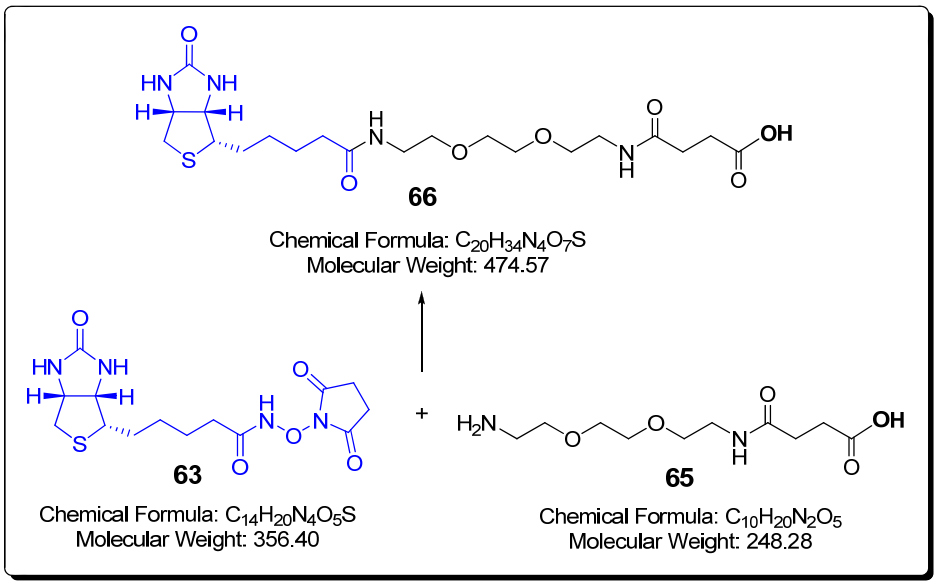


Figure 59 Synthesis of biotin-jeffaminylsuccinylate.

The ultimate step of the reaction involves the attachment of the coumarin moiety bearing the easily hydrolysable bromine functionality to the water soluble spacer-linked biotin. The 7-hydroxy-4-bromomethyl coumarin **56** was synthesized from the 7-acetoxy-4-bromomethyl coumarin **57** by selectively saponifying the acetyl moiety with sodium perborate in MeOH without hydrolyzing the bromide. This final step in our synthetic strategy was successful after several failed attempts. Ester bond formation between **66** and **56** was attempted with dicyclohexylcarbodiimide and DMAP according to Neises *et al.* [105]and Besheer *et al*. [106] as well as by using a large excess (10 equiv) of DCC without further additives [102]. Both reactions failed; instead of ester formation only hydrolysis of the bromide could be observed as confirmed by TLC. The Steglich reaction condition which is usually employed in the synthesis of amides usually works for the synthesis of esters too. The use of the combination of EDC-HCl and DMAP as activator in dioxane for this coupling also failed. Most of the conditions that allow the readily formation of an ester functionality e.g. the use of inorganic base or acid catalyzed aqueous media reaction was not a matter of choice since this will as equally lead to the hydrolysis of the bromide functionality. The use of pentafluorophenol in combination with DCC was also investigated but there was basically no product formation after an overnight reaction time. In our bid to adopting an alternative strategy that would lead to the formation of same compound or with little modification in structure, we decided to try the combination of DCC and HOBt, which is also used in the activation of sterically hindered carboxylic acid functionalities. This trial investigation finally gave the product **67** in a yield of around 30 % as shown in Figure 60.

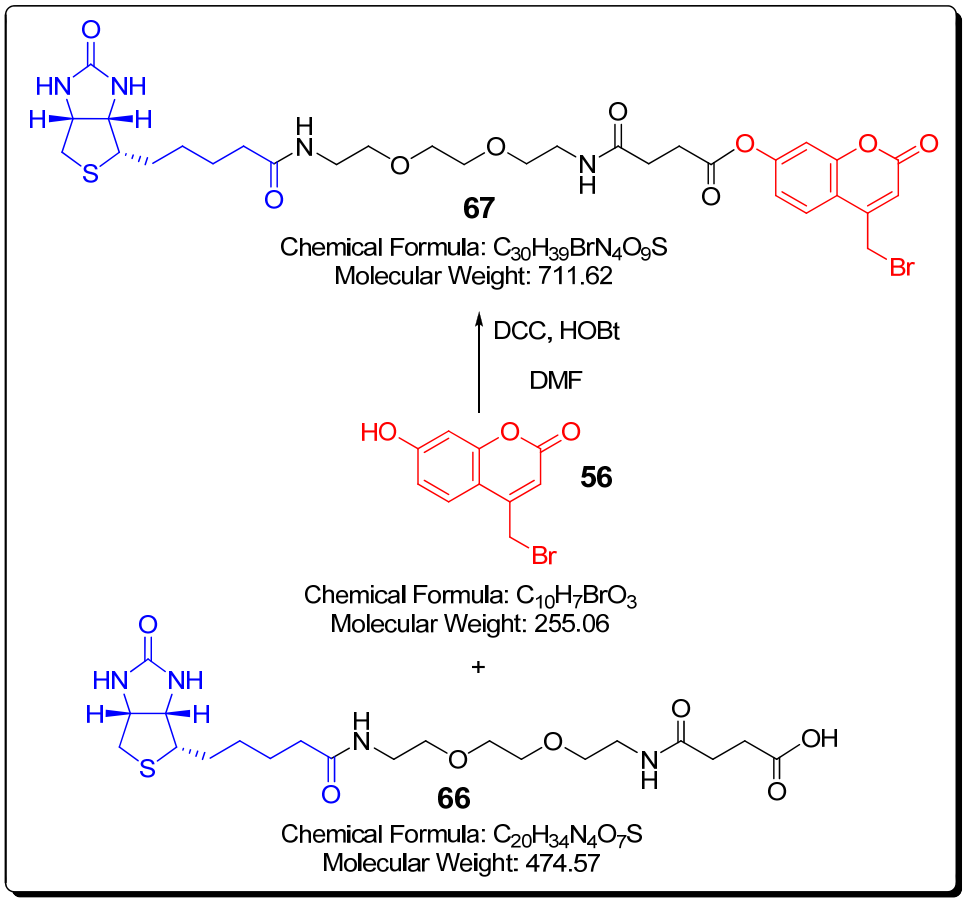


Figure 60 Scheme of the ultimate reaction leading to the final product.

This result was not very different from the work of Delmotte *et al.* where they reported 45 % yield, using the DCC/HOBt combination as compared to the case of TBTU/HOBt combination with a yield of 90 %, on a very similar system [107]. There was therefore the need for testing the use of TBTU/HOBt combination for the activation of the carboxylic acid functionality. The test experiment showed the formation of product within 6 h. We would however not hesitate to say that the possibility of the hydrolysis of the benzylic-like bromide of the coumarin as was observed with the DCC/DMAP combination occurred for longer reaction times.

The concept of the applicability of the finally synthesized multi-functionalized compound **66** is illustrated in Figure 61. Functionalization of 4-bromomethyl coumarin with a biotin handle (fishable end in blue), *via* a water soluble linker, could allow easy detection and isolation respectively.

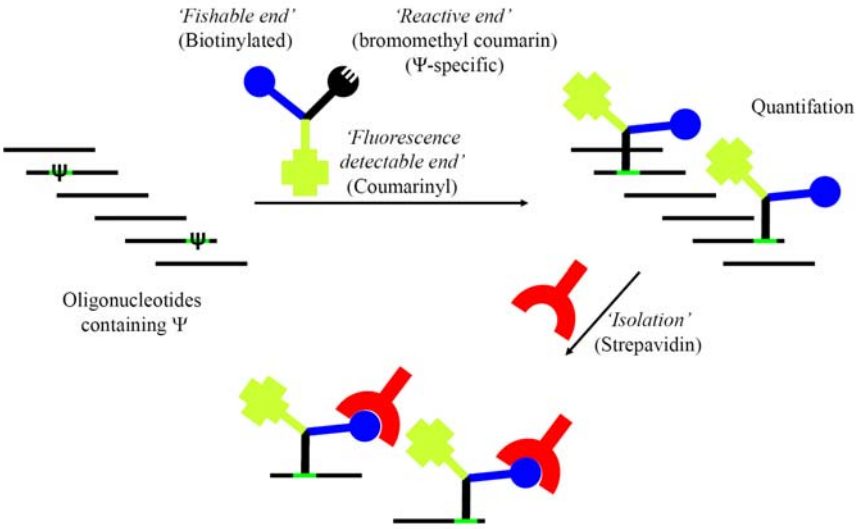


Figure 61 Concept of using biotinylated coumarin for 'fishing out' pseudouridine.

The biotin will allow isolation *via* immobilization of the coupled product onto a strepavidin resin whilst the fluorescence nature of the coumarin moiety will allow for easy detection.

3.8 Syntheses of Xanthenes and its Multi-Functionalization for Bio-conjugation

This Section describes the syntheses of xanthenes and its multi-functionalization for bio-conjugation. The serendipitous discovery of the synthetic route for the syntheses of multifunctional xanthenes and related dyes including the classes: rhodols and rhodamines.

3.8.1 Synthetic Route to the Rhodols

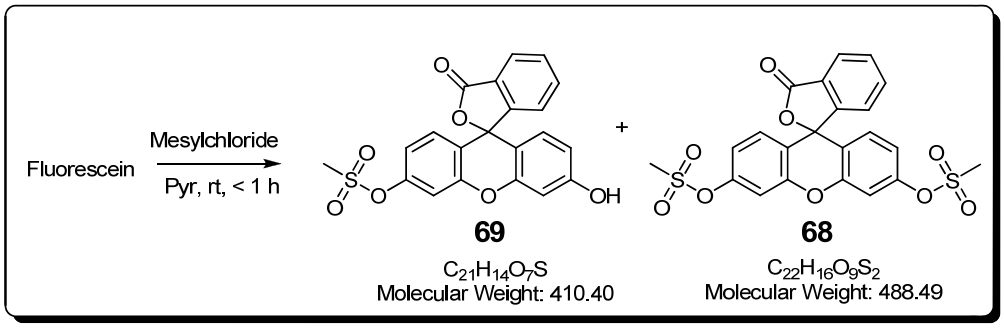


Figure 62 Synthesis of mono- and di-mesylfluorescein.

The discovery of the synthetic route to this compound was serendipitous. During the process in the investigation of the successive protection and attachment of fluorescein to bromo-coumarin in order to enhance sensitivity and hence the quantum yield this discovery was made. The synthesis of the 3,6-di-mesylfluorescein was made under general conditions using pyr and MsCl yielding both the disubstituted and the undesired mono-substituted products as shown in Figure 62.

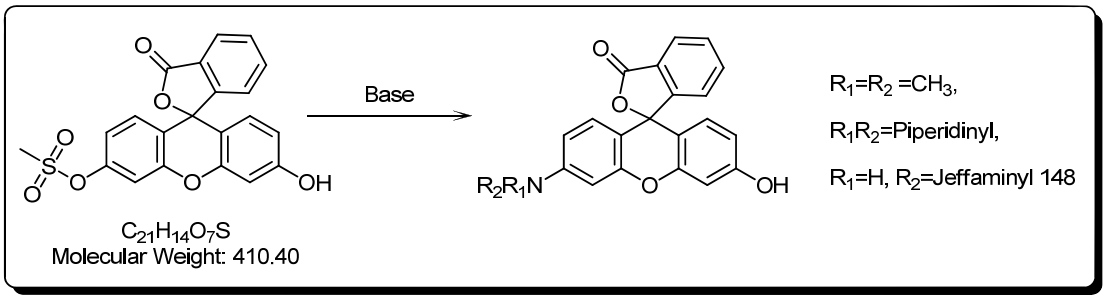


Figure 63 Synthesis of rhodols.

In the investigations for the deprotection conditions of these mesylated products using different bases, as shown in Figure 63, the formation of either the hydrolyzed and or the amine substituted products were observed. The results of the reaction of the bases with the 3-mesylfluorescein at rt is summarized in Table 4.

Base	Product	Hydrolysis(%)
Piperidine	Mono-substituted product	~10
Methylamine	Mono-substituted product	~15
dimethylamine	Mono-substituted product	~10+

Table 4 Results of reaction of 3-mesylfluoran with bases.

There was also a similar trend of results when the dimesyl-protected fluorescein was investigated for hydrolysis. Based on these preliminary findings we decided to investigate the synthetic plausibility that might lead to the formation of the asymmetric rhodols with variations in the type of the amine functionality used in the synthesis.

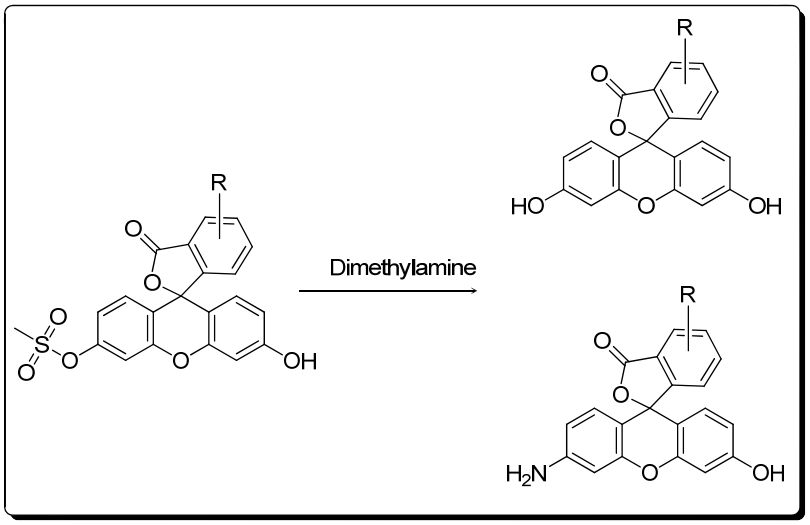


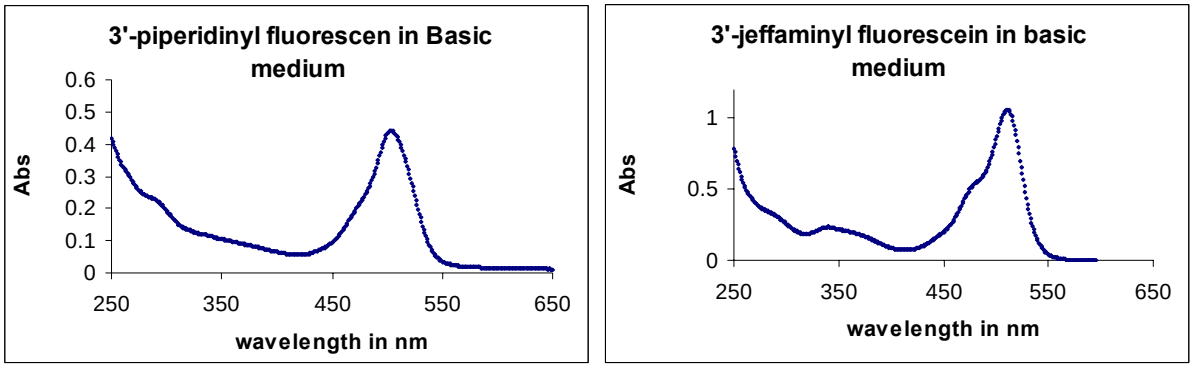
Figure 64 Synthesis of rhodol.

The mono-mesylation of fluorescein was carried out under the condition of pyridine and 0.9 equivalent of the mesylating reagent: MsCl. The yield for this reaction is usually between 80-90 % with the remainder being recovered starting material. The reaction mixture was then evaporated in *vacuo* to effect the removal of unreacted MsCl after which the mixture was re-suspended in pyridine and cooled to 0 °C. Subsequently the addition of the nucleophyllic bases result in the production of the following rhodols as shown in Table 5.

R	Name
jeffamine	3'-jeffaminyl-fluorescein
methylamine	3'-methylaminyl-fluorescein
dimethylamine	3'-dimethylaminyl-fluorescein

Table 5 Reaction of bases with 3-monomesyl-fluorescein

The synthesized Rhodols; 3-dimethylaminyl-fluorescein, 3-methylaminyl-fluorescein and 3-jeffaminyl-fluorescein were then quantified and characterized by HPLC, MS and NMR. Typical UV spectra are as shown below.



Graph showing the typical absorption spectra from Rhodols: 3'-piperidinyl and 3'-jeffaminyl fluorescein respectively.

3.8.2 Synthetic Route to the Rhodamines

Rhodamines are a family of related chemical compounds, fluorone dyes. Just like fluorescein and rhodols they contain the Xanthene core moiety with two aminyl moieties at the 3 and 6-postion of the Xanthene. Examples include among others: Rhodamine 6G and Rhodamine B. They are used as dyes and also as dye laser gain media. They are also often used as a tracer dye within water to determine the rate and direction of flow and transport. Rhodamine dyes, like fluorescein, fluoresce and can thus be detected easily and inexpensively with fluorometers. Rhodamine dyes are used extensively in biotechnology applications such as fluorescence microscopy, flow cytometry, fluorescence correlation spectroscopy and ELISA. Rhodamine dyes are generally toxic, and are soluble in water, methanol and ethanol.

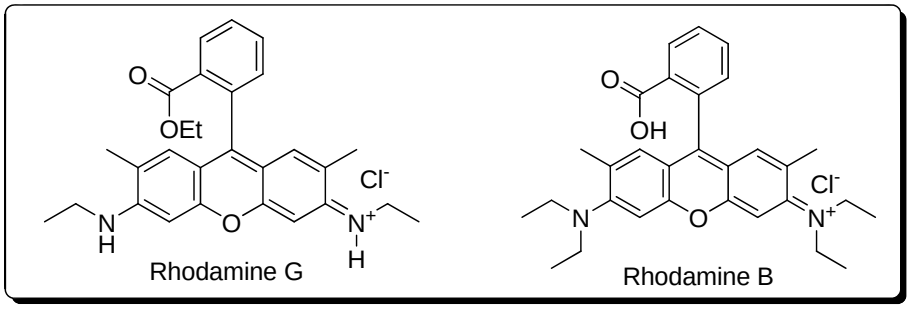


Figure 65 Typical examples of rhodamine.

The rhodamine dyes are relatively expensive and there is basically not very much literature freely accessible on their synthesis. Most of the published data and of the synthesis of this class of compounds are patented [81]. The rhodamine dyes are usually sold as salts of HCl (as shown in the Figure 65) possibly due to the fact that their free base might be less stable.

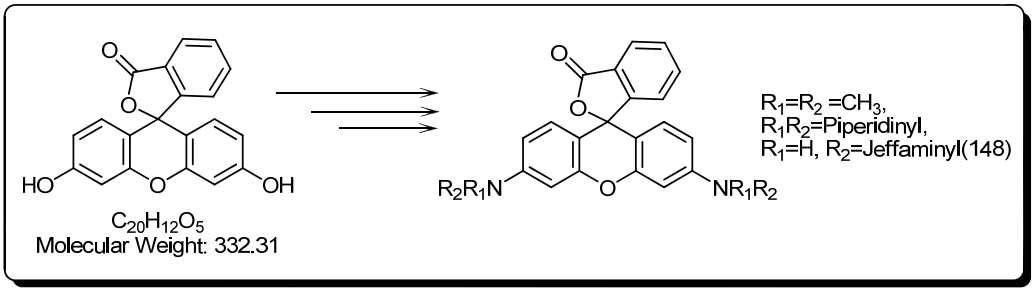


Figure 66 Synthetic pathways to rhodamines.

The di-mesylation of fluorescein was successfully carried out under the condition of molecular-sieved dried pyridine and 4 equivalents of the mesylating reagent MsCl at 0 °C. The yield for this reaction is usually above 90 %. After the removal of unreacted MsCl by evaporation of mixture in *vacuo*, the formed di-mesylfluorescein was re-suspended in pyr and cooled to 0 °C. Subsequently, the addition in excess (4-6 equivalents) of the nucleophyllic bases piperidine, dimethylamine and Jeffamine-148 resulted in the formation of the following rhodamines as shown in Figure 67. The conversion rates for these reactions are usually in the range of 40-60 %.

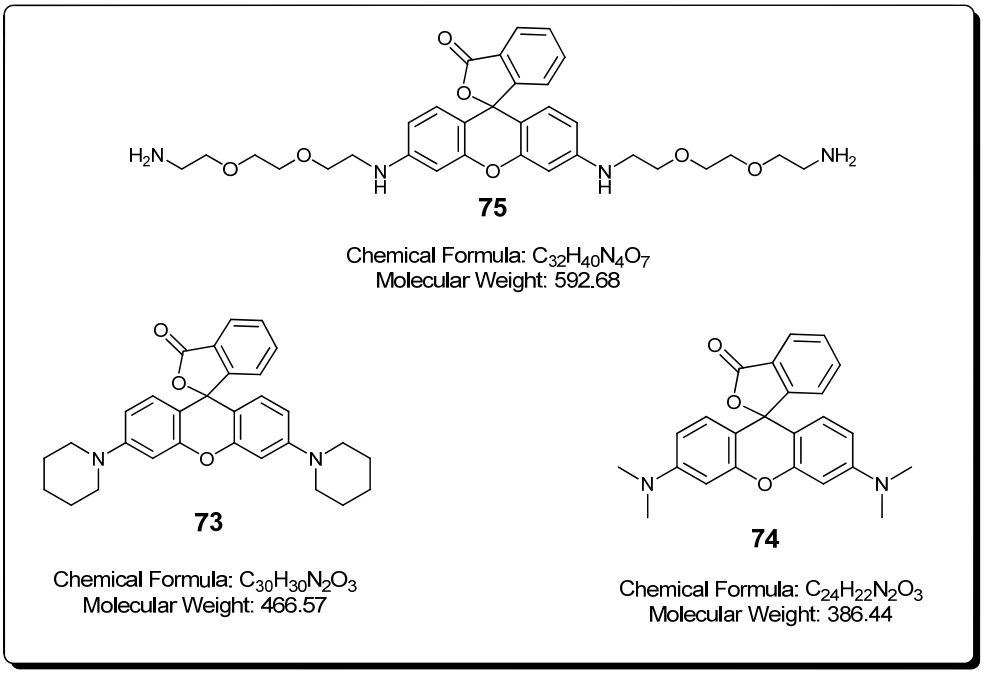
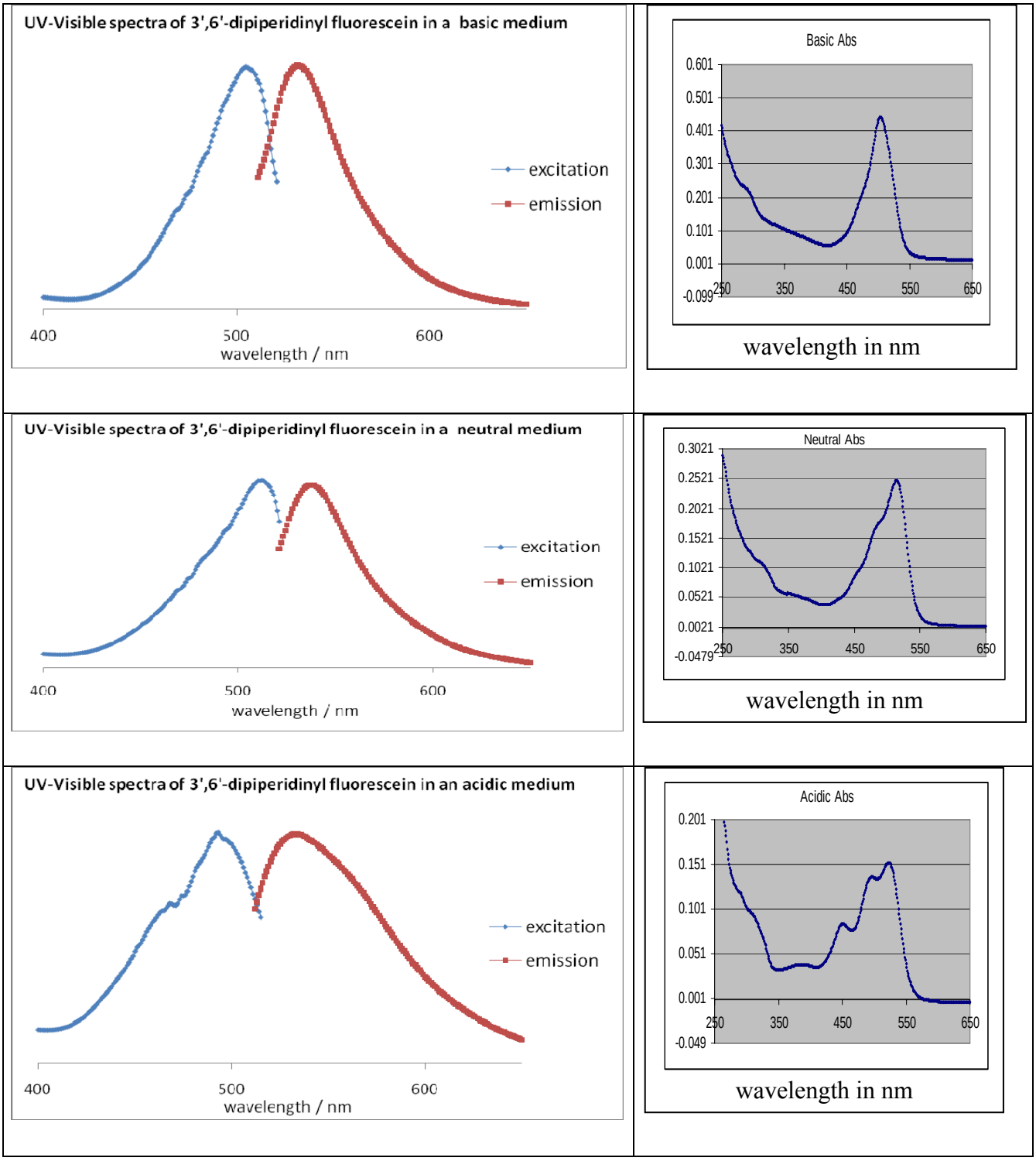


Figure 67 Some of the synthesized rhodamines.

The yields of these reactions are between 35-45 % as were characterized and quantified by NMR and HPLC respectively. The absorption, emission and excitation spectra are shown in the Table 6.

Table 6 Characterisation of dyes by Abs, Excitation and Emission spectra.

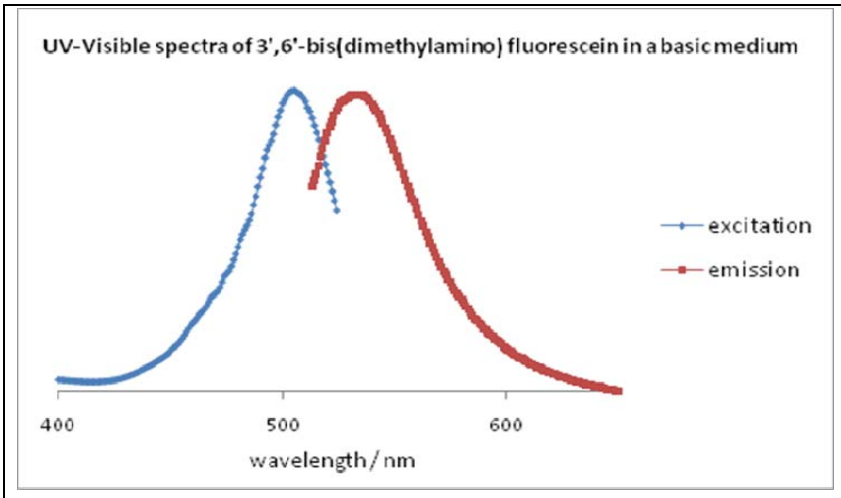
3,6-dipiperidinyl fluorescein	Abs. max wavelength(s)/ nm	Excitation/ nm	Emission/ nm
Acidic condition	519, 492, 448	456, 504	546
Neutral condition	512	516	545
Basic condition	500	510	537

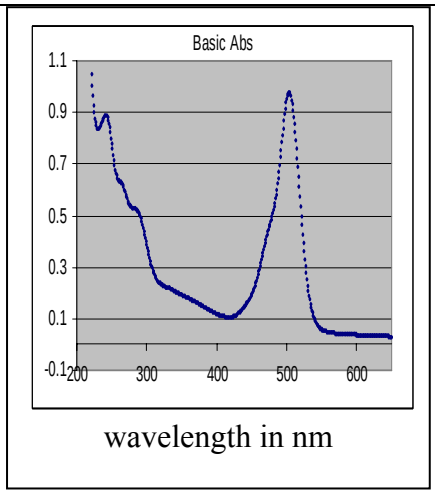


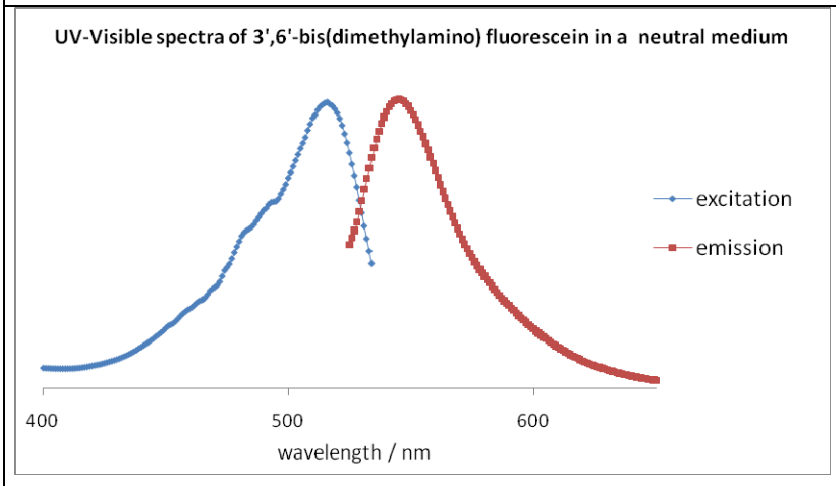
UV-Visible spectra of 3',6'-dipiperidinyl fluorescein showing absorption on right hand side and excitation and emission spectra on the left hand side in basic, neutral and acidic media respectively.

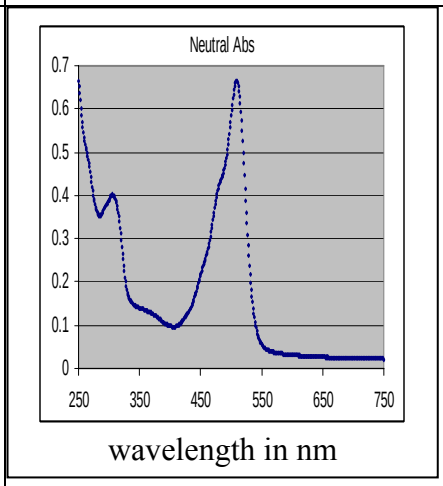
3,6-bis(dimethylamino) fluorescein	Abs. max wavelength(s)/ nm	Excitation / nm	Emission / nm
Acidic condition	508	318	508
Neutral condition	507	512	537
Basic condition	502	507	533

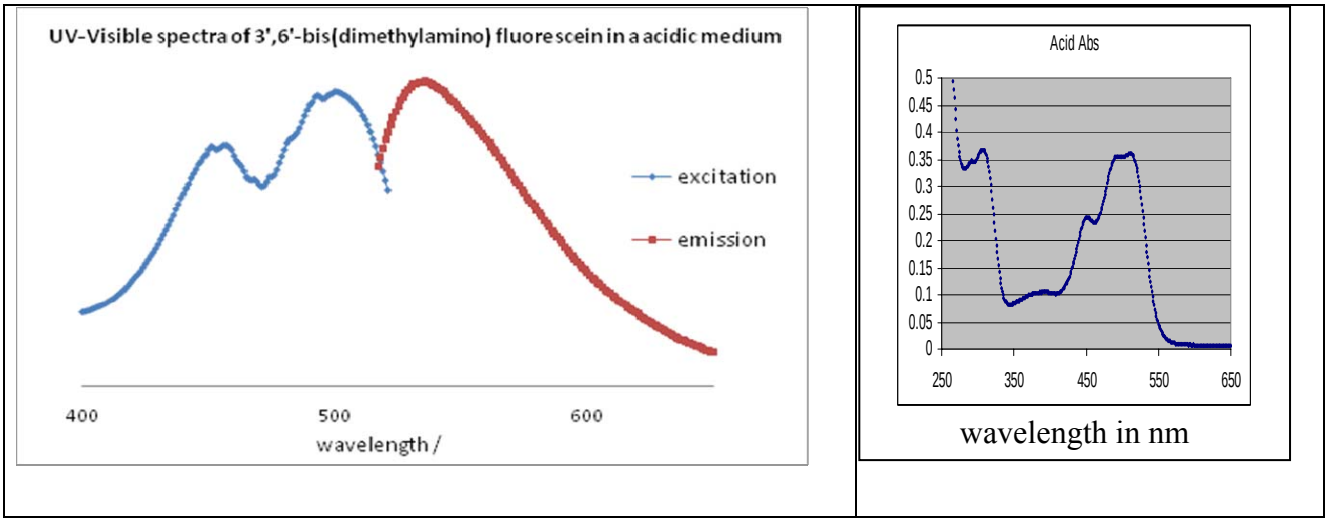
Table showing the absorption excitation and emission maxima for 3',6'-bis(dimethylamino) fluorescein







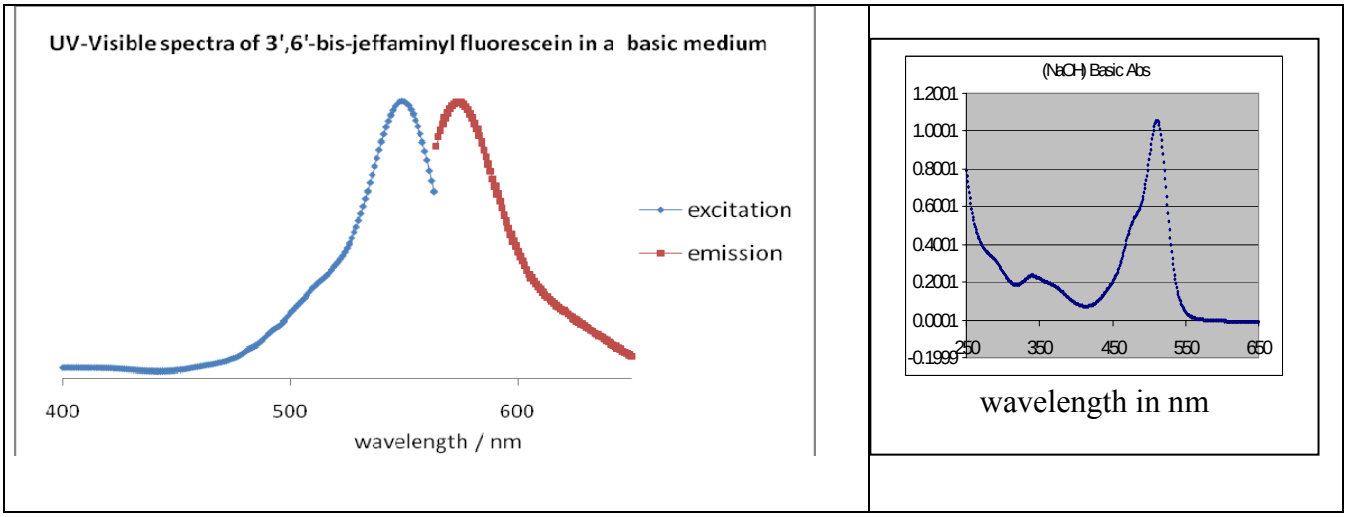


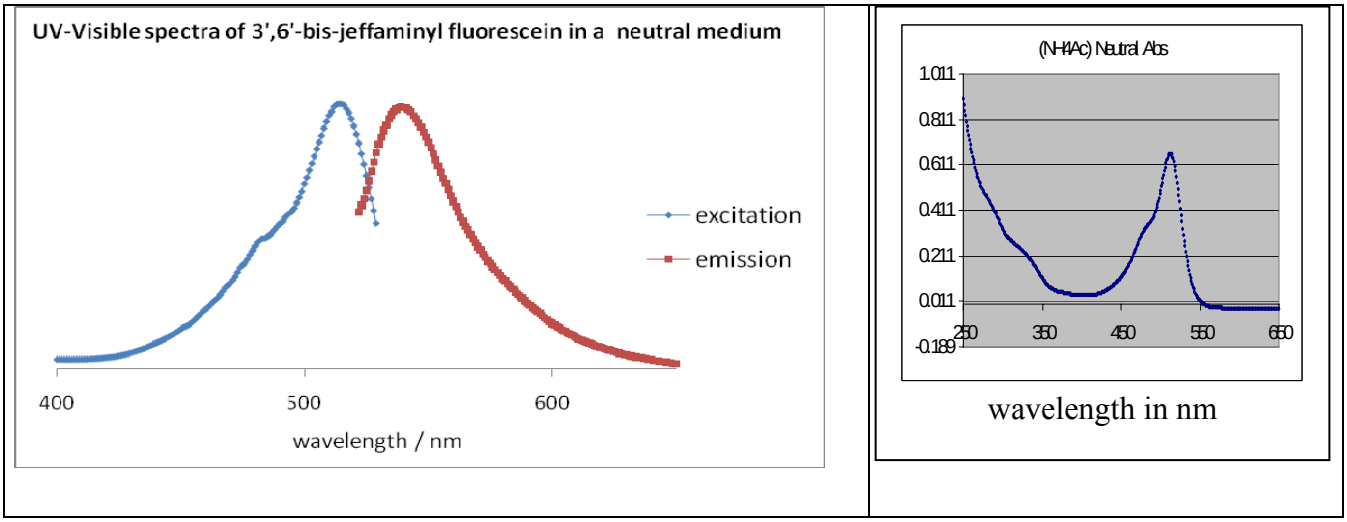


UV-Visible spectra of 3',6'-bis(dimethylamino) fluorescein showing absorbtion on righthand side and excitation and emission spectra in left hand side in the basic, neutral and acidic media respectively.

3',6'-bisjeffaminyl fluorescein	Abs. max wavelength(s)/ nm	Excitation / nm	Emission / nm
Acidic condition	514, 492, 455	-	525
Neutral condition	508	515	538
Basic condition	500	550	573

Table showing the absorption excitation and emission maxima for 3',6'-bisjeffaminyl fluorescein





UV-Visible spectra of 3',6'-bisjeffaminyl fluorescein showing absorbtion on righthand side and excitation and emission spectra in left hand side in the basic and neutral media respectively.

3'-jeffaminyl fluorescein	Abs. max wavelength(s)/ nm	Excitation / nm	Emission / nm
Acidic condition	-	440	512, 548
Neutral condition	-	500	525

Table showing the absorption excitation and emission maxima for 3'-jeffaminyl fluorescein

3'-Piperidinyl fluorescein	Abs. max wavelength(s)/ nm	Excitation / nm	Emission / nm
Acidic condition	492,	461, 502	506
Neutral condition	512	506	515
Basic condition	490	502	525

Table showing the absorption excitation and emission maxima for 3'-Piperidinylfluorescein

3'-Glycinyl-6'-jeffaminyl fluorescein	Abs. max wavelength(s)/ nm	Excitation / nm	Emission / nm
Acidic condition	514, 492, 446	456, 504	516
Neutral condition	508	512	515
Basic condition	494	508	517

Table showing the absorption excitation and emission maxima for 3'-Glycinyl-6'-jeffaminyl fluorescein.

3'-Piperidinyl-6'-Sulfonyl fluorescein	Abs. max wavelength(s)/ nm	Excitation / nm	Emission / nm
Acidic condition	508	504	506
Neutral condition	502	496	501
Basic condition	500	510	517

Table showing the absorption excitation and emission maxima for 3'-Piperidinyl-6'-Sulfonyl fluorescein

3.8.3 Peptide Labeling using Fluorescein Surrogates

The monitoring of most biological reactions requires the use of a 'moiety' that allows sensitive detection and measurable changes in both concentration and in certain cases its physical environment like pH and or temperature. Frequently used moieties include radio-labeling, fluorophore labeling or the use of an affinity tag like biotin. Labeling peptides with fluorophore reporters has always been fraught with limitations: poor dye selection, dye isomer contamination, inefficient dye incorporation and poor sensitivity. Labeling and detection allows the preparation of custom peptide conjugates utilizing conventional fluorescent dyes, quenchers. The exact specifications and purity are usually a challenge in some cases whilst, the expertise to conjugate a peptide to other molecules including labeled antibodies remains not that trivial. In addition to the common fluorophores like fluorescein and rhodamine the increasing list includes also; Atto, Alexa Fluor, BODIPY, Cyanine, Oregon Green and Texas Red dyes, QSY quenchers, Biotin, desthiobiotin (DSB) and Qdots nanocrystals.

Based on the success of the syntheses of the rhodols and the rhodamines, we endeavored to seek for a simple and pragmatic use to which the mono- and the dimesylfluorescein could be put to. The usual methods by which peptides are labeled with a fluorophore apply one of the following chemistries: *amide, isocyanate, thiol, epoxide* or *phosphine etc*. There is therefore the need for one to include a tailored-reactive group which is compatible with the choice one makes with the chemistry. The free amino functionality of the amino acid units within the peptide could be employed in our case as an anchor to which the fluorophore could be attached directly in a one-pot synthesis. In principle there is also the possibility to pre-attach the fluorophore to a resin before attaching the peptide via the *mesyl*-elimination as shown in Figure 68B or alternatively attaching the fluorophore as the ultimate step in the synthesis as shown in Figure 68A.

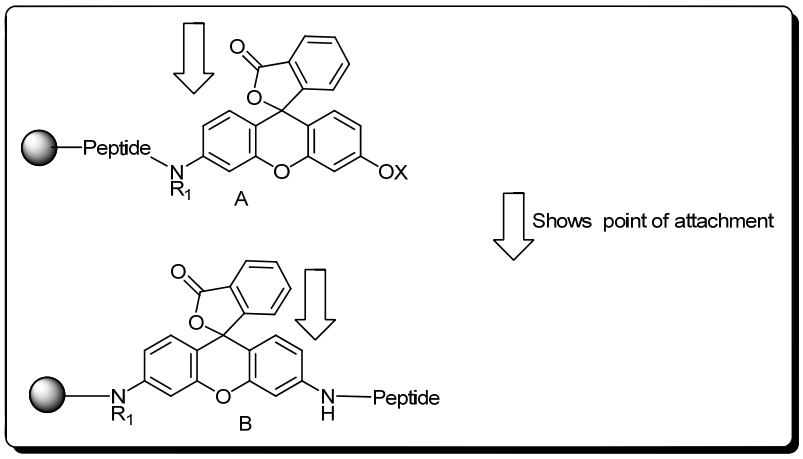


Figure 68 Labeling of peptides *via* rhodamine formation.

We investigated both labeling either before or after the synthesis of the peptide. The penta- and tri-peptides shown in Figure 69 were synthesized according to standard solid phase peptide synthesis on a Rink AM resin [108]. After cleavage of the final fmoc from the amino functionality the resin was dried after which dimesylfluorescein was added as a solid in excess (> 4 equiv.) to which pyridine was then aspirated and shaken overnight at rt. The resulting purplish-orange beads were washed several times with DMSO and then swirled with DCM after which it was dried under vacuum overnight awaiting cleavage. The addition of 95 % TFA in DCM to the dried resin, swirling for 15 minutes and filtering resulted in the formation of the product after 3 repetitions. Care must be taken since an irreversible coupling of the resin and the product has been reported in such similar cleavage condition [109], [110]. The collected filtrate was cooled and dried overnight by freeze drying. Purity and also the level of incorporation of the fluorophore was assessed using HPLC and MALDI–TOF-MS

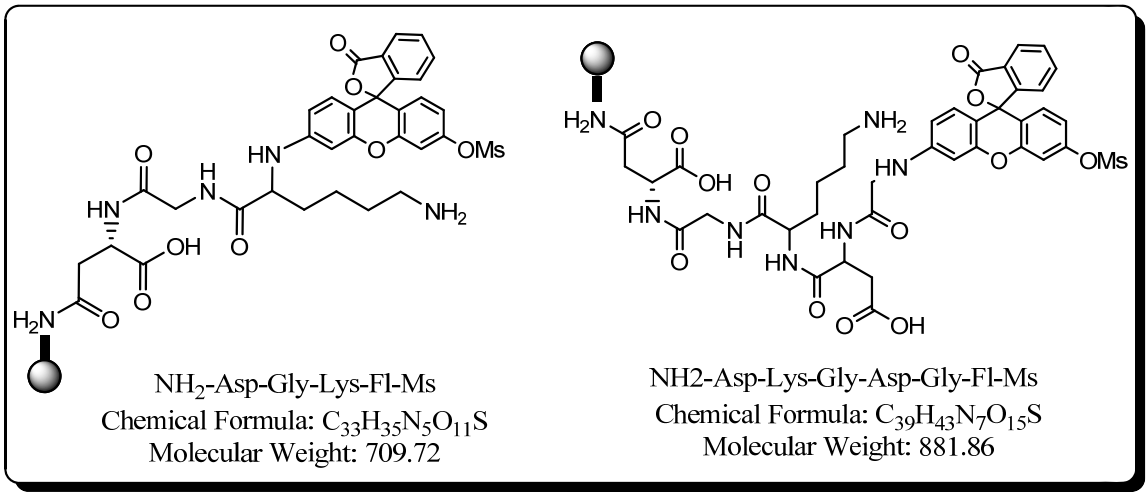


Figure 69 Synthesis of Rhodamine labeled peptide.

The results of an analytical HPLC using isocratic mixture of water/acetonitrile (4:1)containing 3 % TFA at 45 °C with a flow rate of 1 mL/min resulted in the separation of all the various components which was detected with the aid of both the DAD and FLD. The purified product from the HPLC was then analysed by MALDI-TOF-MS. The measured mass was 882.9 amu which was not different from the expected mass as shown in Figure 71.

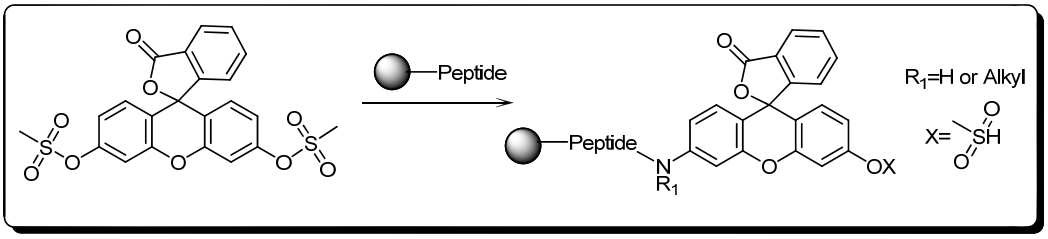


Figure 70 Post labeling of peptide on a resin.

The structure of the tri- and pentapeptide is shown in Figure 69. Polypeptides with sterically free amino functionality can be synthesized on a resin and be allowed to react with a dimesylfluorescein. The fluorescence product could be use as a means of monitoring the diffusion of the peptide in cells. Fluorophore labeling of the polypeptide as we have done with tri and pentapeptide could be extended to other amine containing biomolecules.

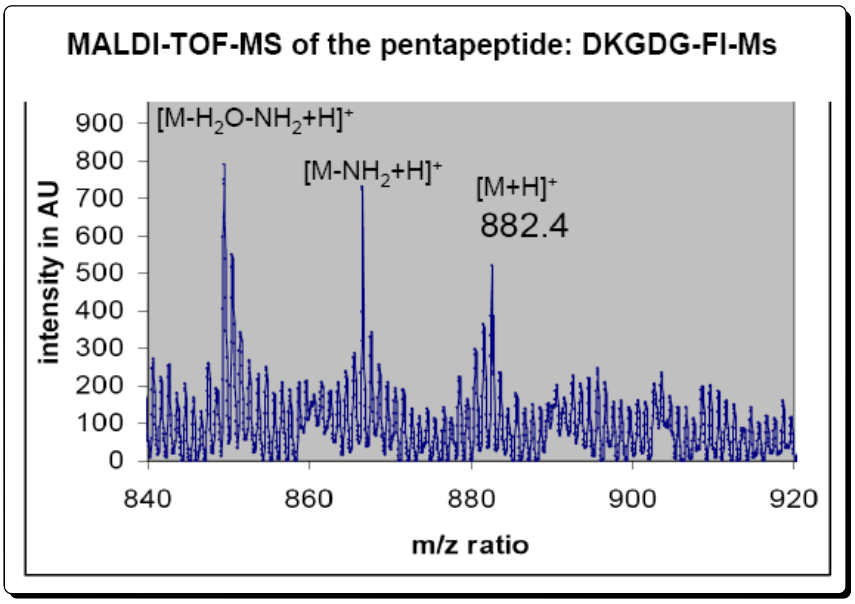


Figure 71 MALDI-TOF-MS of pentapeptide coupled with diMsFl.

The pre-labeling of the amino functionalized resin (AM Resin) with dimesylfluorescein was achieved using overnight reaction time following the procedure discussed in the post synthetic method Figure 68B. The product of the reaction which is mesylated at one end, after extensive drying and weighing, was used in the next step as it was still coupled to the resin without further purification. To this dried mixture was added a tripeptide which has both the amino and carboxylic acid functionality free to react. An overnight reaction time resulted after several washing steps with DMF and DCM and then drying under reduced pressure a mass gain corresponding to 40 % coupling.

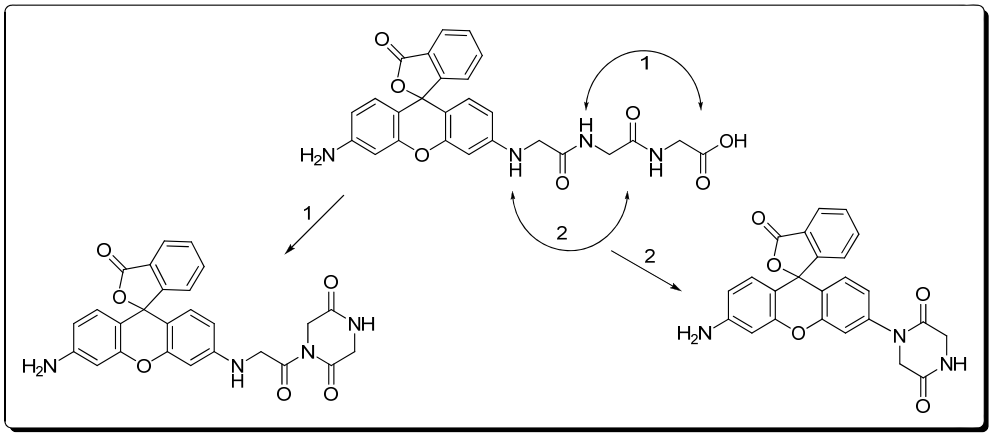


Figure 72 Possible decomposition route of coupled product.

In the first instance, the cleavage of the product from the resin and analysis of the crude product by MALDI-TOF-MS shows higher masses which do not correspond to the expected calculated masses. Upon purification by HPLC and analysis, there were masses corresponding to the intra-molecular cyclization, as well as some decomposed product, as one could envisage through the scheme in Figure 72.

4 Conclusions & Outlook

4.1 Synthesis, incorporation and effects of nucleotide modifications

In order to investigate the effects of modifications in RNA, the syntheses of the modified RNA phosphoramidites m^5U, Ψ, m^1G m^2G and $m^2{}_2G$ were successfully executed. They were then incorporated into RNA oligomers using standard *SPS*. The synthesized oligomers were used in the total construction of tRNA *via* enzyme catalyzed ligation. The construction of $tRNA^{Ile}$ with and without modification was achieved in a one-pot 3-fragment ligation using a T4 DNA ligase with a DNA-splint [99]. The products were further investigated for the stability of the tertiary structure *via* UV melting curve analysis. The results of the melting curve analysis show that the unmodified tRNA was more stable compared to the fully modified tRNA.

In the case of siRNA, synthesis and incorporation of modified nucleotides as phosphoramidites was achieved and the knock down of gene and the immunostimulation in cells were investigated. In a series of experiments, the antisense strand of the siRNA has been made to contain 1, 2 or 3 rT, m^1G, m^2G and $m^2{}_2G$ modifications. The induction of interferon by the siRNA containing the m^1G was a higher as compared to the others. The knock down of GFP by the siRNA with the various modifications was measured by the amount of fluorescence emitted by the cells. The m^1G, m^2G and the rT modified siRNAs showed very high knock down efficiency whereas those with $m^2{}_2G$ were less efficient.

Future work should include the chemical synthesis of the phosphoramidites of s^2U, mnm^5U, cmo^5U, mnm^5s^2U, m^5C, m^6A and t^6A just to mention a few. This may throw light on the mystery surrounding these modifications by their selective incorporation into full-length tRNA. The inclusion of hypermodified nucleotides for siRNA studies and its effect on knock-down and immunostimulation may open the door to gene silencing therapy.

4.2 Specific recognition of modification in RNA

The recognition of the modifications in RNA and in particular the chemical recognition of pseudouridine by 4-bromomethyl-coumarin was also investigated. The specific reaction between pseudouridine and 4-bromomethyl coumarin at optimal conditions in the presence of the four

main canonical nucleosides has been optimized in the Helm group {unpublished results}. The reaction mixture of bromomethyl coumarin, pseudouridine and the other nucleosides at optimal temperature and pH results in a coupled product with the pseudouridine nucleoside. The scheme of the extension of the concept to full-length tRNA containing pseudouridine with bromomethyl coumarin is shown in Figure 73.

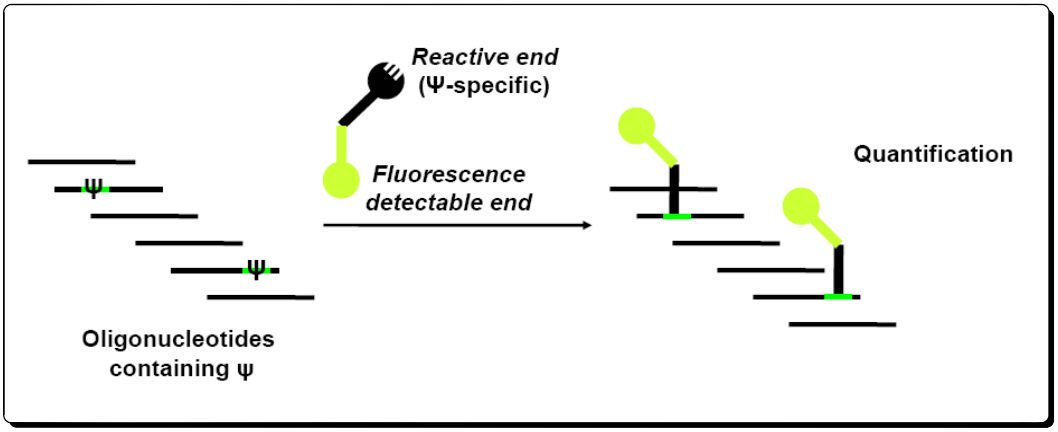


Figure 73 Concept of pseudouridine detection by bromomethyl coumarin

The synthesis of 7-azido-4-bromomethyl-coumarin **61** was executed, which can be used to investigate the click chemistry of the product of the bromomethyl coumarin and pseudouridine containing modified nucleotide. In a 5-step synthesis, **61** was obtained with a yield of 44 %. The results of preliminary investigation of the reaction of **61** with pseudouridine containing nucleosides shows the formation of the product which has been confirmed from the fragmentation pattern of the LC-MS/MS {unpublished results}.

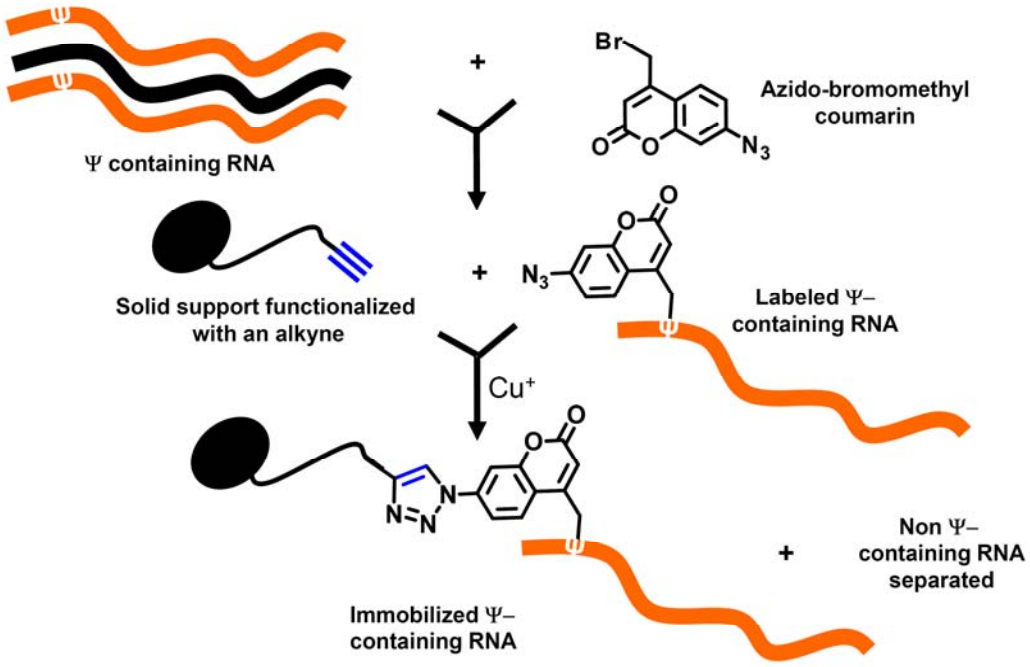


Figure 74 Perspective of immobilzing of Ψ containinig RNA by 'click' chemistry.

The reaction of an aryl-azido moiety with a terminal alkynyl group in the presence of Cu^{+} ions has been known and studied for some time now [111-117]. The use of a solid support which is functionalized with an alkyne can be made to react with an azido functionality of the product of the reaction between pseudouridine and an azido-4-bromomethyl coumarin. Further studies may be conducted to illucidate if this method illustrated in Figure 74 can be used as a means of immobilizing pseudouridine containing RNAs onto a solid support.

A 6-step synthesis of the biotinylated 4-bromomethyl-coumarin spaced with jeffamine-148 was also carried out. The difficulty in this work was in the final attachment of the coumarin moiety to the biotin *via* the succinyl-jeffamine moiety with a yield of 29 %. The overall yield of the 6 step synthesis was 17 %. This product **67**, which is water soluble, may allow both the detection as well as the separation of product of the tagging of pseudouridine with bromomethyl coumarin taking advantage of the affinity between strepavidin and biotin [118]. Future work should be focused on the improvement of the yield in the final step of the synthesis. In this direction, the use of cocktail mixtures of the peptide coupling reagents could be adopted for the ester bond formation reaction. The other possibility would be to use a more reactive nucleophile e.g. 7-amino-4-bromomethylcoumarin whose amino group could be made to react with the carboxylic

acid functionality of the biotin directly or via a spacer to form an amide. These would ultimately lead to the identification of modifications in RNA by sequencing

4.3 Rhodols, Rhodamines and their Derivatives

A mild and bio-compatible condition for the syntheses of rhodols and rhodamines was discovered. This invention, a serendipitous one, occurred in our bid to extend the conjugation of the double bonds connected to the coumarin moiety in order to increase the extinction coefficient of the tagging molecule which will eventually lead to an increase in sensitivity. The conversion of fluorescein into the mesylated product at the 3-position only **(69)** or both 3 and 6 –position(s) **(68)** as shown in Figure 75, and the subsequent displacement of the mesylate with a nucleophile gave rise to rhodols or rhodamines respectively.

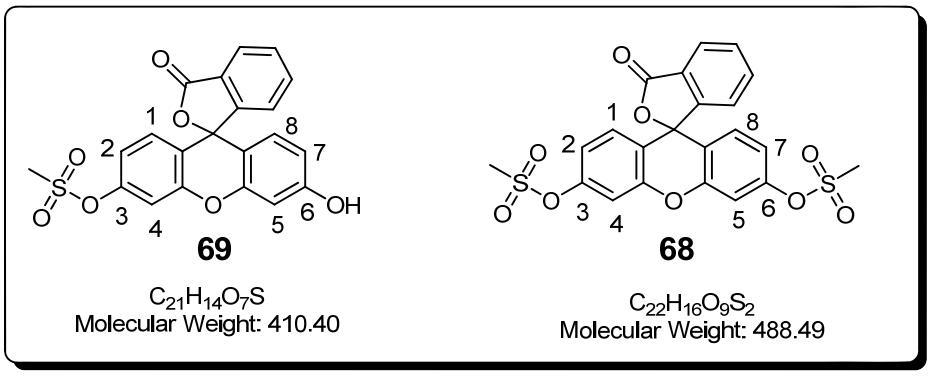


Figure 75 3-Mono- and 3,6-di-mesyl-fluorescein

The mild reaction conditions compared with traditional methods of synthesis [119] and even with the recent one via microwave assisted reactions [120] could allow for biomolecular labeling. The synthesis of rhodols with variation in the type of amine functionality could give rise to several derivatives with particular attention of it being either primary, secondary (e.g. amine functionality is jeffamine-148 or mono-alkylamine derivative) or tertiary (e.g. amine functionality is piperidine or di-alkylamine derivative) as shown Figure 76. With these variations the pH dependency of the rhodols fluorescence can be fine-tuned.

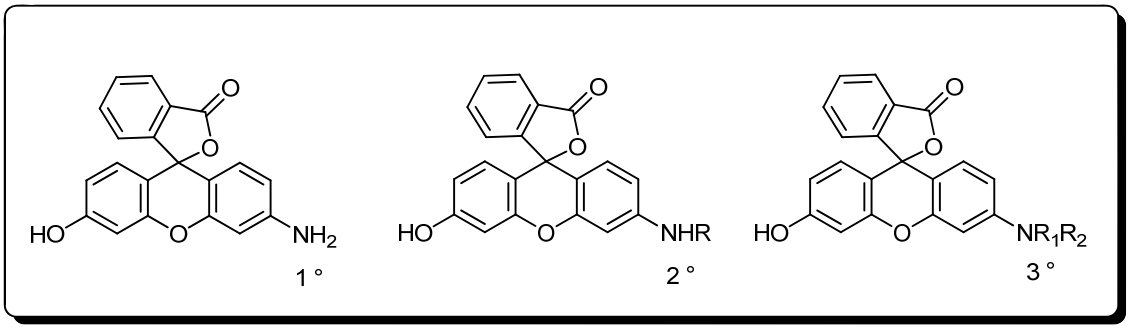


Figure 76 Variation of amine functionality leading to pH dependent fluorescence

The synthesis of 3,6-bis-dimethylaminofluorescein, 3,6-dipiperidinylfluorescein and 3,6-dijeffaminylfluorescein were all achieved at rt within a couple of hours. On the contrary, these rhodamine derivatives are not accessible at rt using 3-aminophenol functionalized at the 3-position, reacting with phthalic anhydride. The mild conditions of this discovery prompted us to further investigate the tagging of polypeptides.The tagged tri and pentapeptide whose mass were confirmed by MALDI-TOF-MS, suggest that the condition could be adopted for several applications. The tagging of molecules containing an accessible amino functionality could be investigated. The use of other nucleophiles such as the *thiol* is currently being investigated. The use of carbon nucleophiles such as the alkyl metal halides found in the Grignard reaction [121-123], the Blaise reaction [124], the Reformatsky reaction [125, 126], and the Barbier reaction [127, 128] could be investigated in the presence of the mesylfluorescein shown in Figure 77.

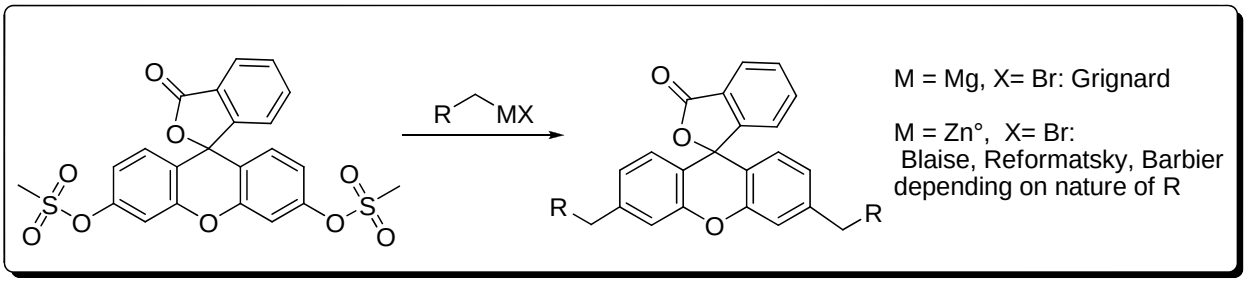


Figure 77 Alkylmetal halides as nucleophile reacting with mesylfluorescein.

Also very interesting could be the investigation of the reaction between organolithium reagents of terminal alkynes with the dimesylfluorescein. This could lead to the extension of the conjugation of the double bonds of the xanthene as well as providing an alkynyl moiety for further extension of the side chain as shown in Figure 78.

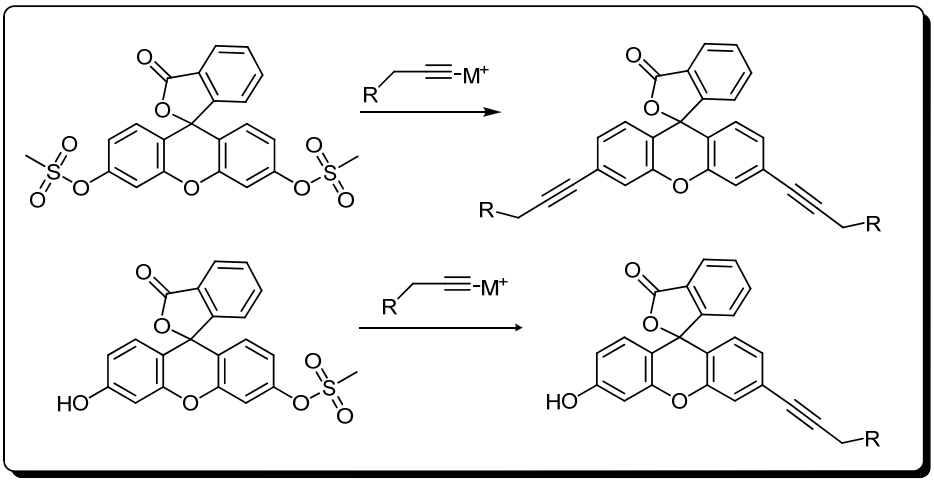


Figure 78 Perspective of conjugation extention on the xanthene moiety.

The extension of conjugation of the xanthene may lead to novel compounds with characteristic absorption and excitation spectra. The alkyne moiety which is formed could be used as a reacting partner in an alkyne-azide huisgen cycloaddition reaction. There may be the need to use Cu^{+} and also slightly higher temperature for the non-terminal nature of the alkyne.

5 Experimental Section

Reagents and solvents of highest purity were purchased from various commercial suppliers and used without further purification. TOM-Cl and the 2'-O-TOM-protected ribonucleoside and the corresponding phosphoramidites were prepared according to Pitsch *et al.* [129]. The reactions conducted at -20 °C were attained by mixing MeOH and dry ice (solid CO_2) and the one at -78 °C was obtained using liquefied nitrogen.

Workup implies distribution of the reaction mixture between DCM and satd. aq. $NaHCO_3$ solution, drying of the organic layer by $MgSO_4$ or Na_2SO_4, and evaporation of solvent under reduced pressure using a rotary evaporator.

Thin layer chromatography (TLC): Precoated silica gel plates from Merck. Staining was achieved by (i) dipping into a soln. of Blue Shift, constituents of which are anisaldehyde (10 mL), H_2SO_4 (10 mL), and AcOH (2 mL) in EtOH (180 mL) and subsequently warming with a heat-gun until color/stain appears (ii) other methods of staining.

Column chromatography (CC): Silica gel 60 (230–400 mesh) from Fluka.

NMR-spectroscopy: Varian 300 MHz (^{1}H: 300 MHz, ^{13}C: 75 MHz, ^{31}P: 121.5 MHz) and Bruker 500 MHz (^{1}H: 500 MHz, ^{13}C: 125 MHz, ^{31}P: 202.5 MHz). Chemical shift δ in ppm, relative to external standards (^{1}H- and ^{13}C:, ^{31}P: 85 % aq. H_3PO_4) and Me_4Si as internal standard; coupling constants J in Hz; multiplicities (^{13}C) according to DEPT-spectra.

ESI-MS (positive mode): SSQ 710 (Finnegan), measurements in MeCN/H2O/AcOH 50:50:1.

EI-MS: The GC analysis was performed with a WCOT Fused silica column (30m) coupled to a Varian Saturn 2200 mass spectrometer (Varian) for the GC-MS analysis (CP3800).

MALDI-MS (positive mode): Axima CFR Plus (Kratos/Shimadzu), matrix: 2,4,6-trihydroxyacetophenone, $(NH_4)_2$-citrate.

ESI-MS (negative mode): Q-Tof-Ultima (Micromass/Waters) coupled to Cap-LC (Waters), injection: 2 µL aq. sample (c (RNA) = 2.5 µM, c (EDTA) = 1 mM); chromatography on Xterra 186

RP-C18 column (Waters, 5 µm, 0.32 x 50 mm; flow 8 µL/min, eluent A: 25 mM aq. $Me_2NBu.H_2CO_3$ (pH 8.4); eluent B: MeCN, elution at 60 °C, sheath-flow 25 µL/min (MeCN)); gradient A → A/B 1:1 (15 min.); deconvolution by MaxEnt1-software.

Standard desalting

The purified sequences were desalted by treating with 1M aq. $Et_3N.AcOH$ (pH 7, 5 mL) and applied to Sephadex column-cartridges (GE): after elution of the salts with 0.1 M aq.

Et_3N.AcOH (pH 7, 10 mL), followed by H_2O (20 mL), the Et_3NH^+-salt of the sequences were eluted with MeCN/H_2O 1:1 (5 mL) and the solvent evaporated to dryness.
Other measurements include the following on the Varian spectrophotometer and the Cary 100 UV

5.1 Instruments and Special Materials

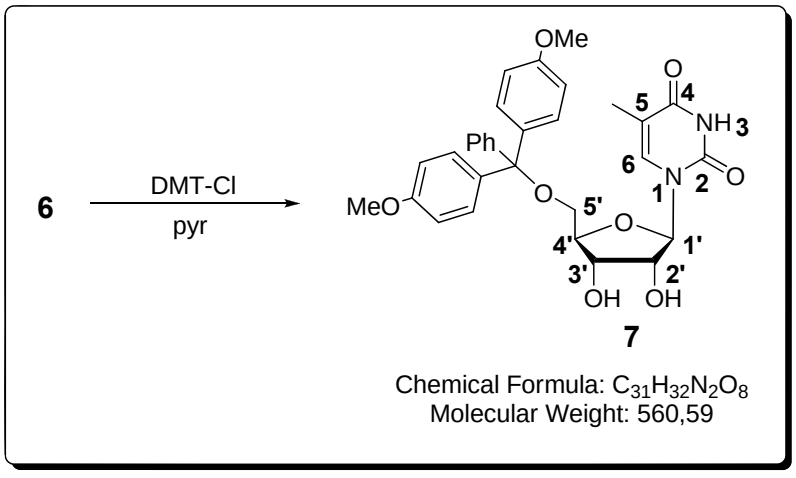
Analytical balance	AX 204 and B3001-S Mettler Toledo
Centrifuges	Eppendorf 5804 R and Mikro 120 Hettich
Electrophoresis chamber	GIBCO BRL Sequencing System LIFE
Eppendorf and PCR tubes, silanized	Biozym
Exposure cassettes	For 35 × 43 cm Kodak imaging screens
Freeze dryer	BenchTop K Series, VirTis Ismatec
Gel Documentation equipment	AlphaImagerTM 2200 Alpha Innotech
Greiner tubes	CellStar
HPLC	Agilent 1100 Series
HPLC Columns:	
- Luna C18, 5 µm, 4.6 250 mm and 15.0 × 250 mm	Phenomenex®
- Chiralcel OJ-H, 4.6 × 250 mm	Daicel
Mass Spectrometer:	
- MALDI-TOF	Bruker BIFLEX III
- FAB and EI	JEOL JMS-700
- ESI	Finnigan MAT TSQ 700
- ESI FT-ICR	Bruker APEX IV
Minicentrifuges	Kiesker
NAP columns, Sephadex G-25	GE Healthcare (Amersham Biosciences)
NMR Spectrometer	Mercury Plus 300, Varian VNMR S 500, Bruker AC-300, DRX-300
pH-Meter	MP 220 Mettler Toledo
Phosphorimager	Typhoon 9400 Amersham Biosciences
Pipettes	Abimed P2, P20, P200, P1000
Scintillation counter	Beckman LS 6500
Silica gel 40 µm	J.T. Baker
Silica gel plates Polygram® Sil G/UV254	

40 × 80 mm	Macherey-Nagel
Speed vac	Univapo 100 ECH
Spin filters	Nanosep® MF Centrifugal devices, 0.2 μm PALL, Life Sciences
DNA/RNA Syntheziser	Applied Biosystems ExpediteTM 8909
Syringe filters	PTFE, 13 mm, 0.2 μm, Carl Roth
Thermomixer	Eppendorf, Thermomixer 5436
Ultrapure Water Purification System	Milli-Q, Millipore
UV Cuvettes	Quarzglas SUPRASIL, HELLMA
UV-Lamp 254 nm	Benda NU-8 KL
UV-Transilluminator	254 nm, 300 × 200 mm Carl Roth
UV/VIS Spectrophotometer	
- Ultrospac 2100 pro	Amersham Pharmacia Biotech
- NanoDrop ND-1000	Peqlab Biotechnologie
- Cary 100 Bio	Varian

5.2 Chemical Synthesis of m^5U phosphoramidite

This section describes the synthesis of the m^5U phosphoramidite starting from the commercially available 5-methylribothymidine.

5.2.1 Synthesis of *5'-O-DMT-5-methyluridine* (7)

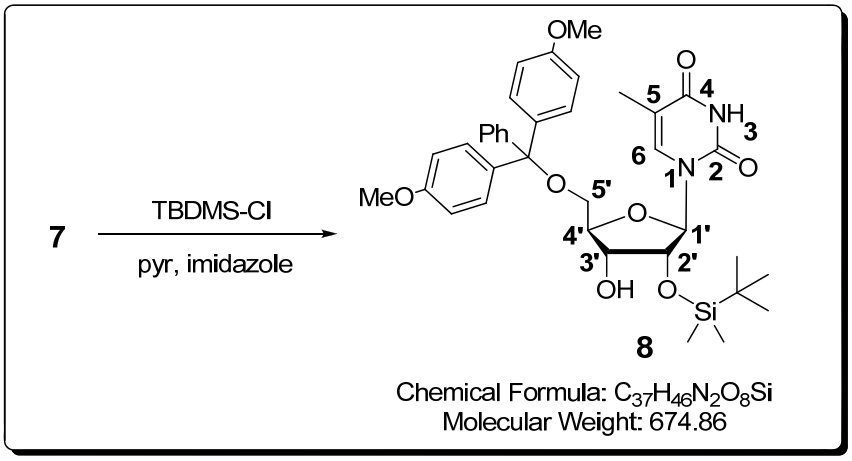


To a stirred solution of ***5-methyluridine*** (**6**) (1.0 g, 4.1 mmol, 1.0 equiv) in 30 mL of anhydrous pyr was added DMT-Cl (1.53 g, 2.4 mmol, 1.2 equiv) in pyr (3 mL) portion-wise

over 2h at rt. The reaction mixture was stirred at rt and monitored by TLC. The reaction was complete after 5 h after which it was quenched with water. The mixture was then evaporated to dryness under reduced pressure. The resulting solid was redissolved in 5 mL of DCM and purified by fCC on silica gel with a gradient eluent system of DCM:MeOH, 60:1 to 40:1 to yield **7** (0.72 g, 85 %).

TLC (silica gel, DCM:MeOH, 25:1): R_f = 0.38. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ= 1.86 (s, 3H, H5), 3.25 (m, 1H, H5'), 3.62 (m, 2H, H5'), 3.66 (s, 6H, 2x CH_3O-trityl), 4.11 (m, 1H, H3'), 4.18 (m, 1H, H2'), 4.38 (t, 1H, H4'), 5.90 (s, 1H, H1'), 6.70 (d, 4H, trityl), 7.13 (d, 4H, trityl), 7.16 (d, 2H, trityl), 7.24 (d, 2H, trityl), 7.52 (s, 1H, H6) ppm. FAB-MS: 561.60 $[M+H]^+$, 303.1 (32, $[(MeO)_2Tr]^+$).

5.2.2 Synthesis of *5'-O-DMT-2'-O-TBDMS-5-methyluridine* (8)



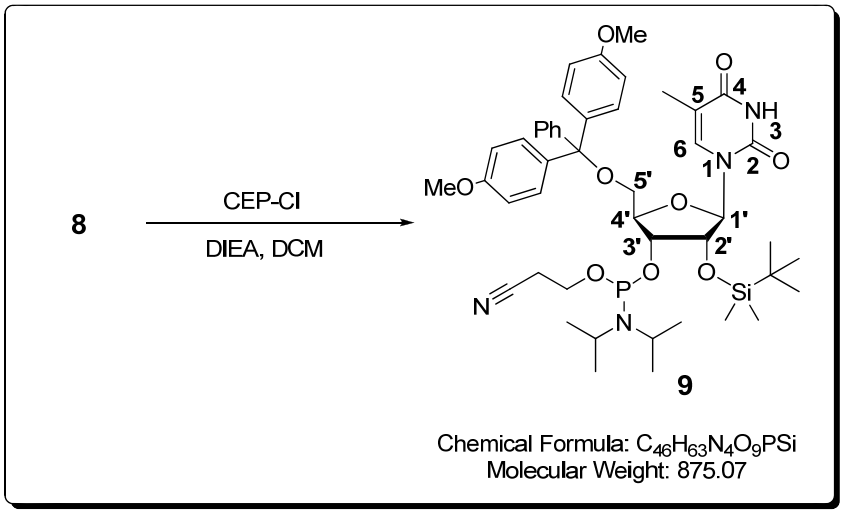
To a stirred solution of ***5'-O-DMT-5-methyluridine*** **(7)** (60 mg, 0.12 mmol, 1.0 equiv) in 10 mL of anhydrous pyr was added imidazole (30 mg, 0.48 mmol, 4.0 equiv) in 5 mL pyr at rt. The mixture was stirred for 15 min after which TBDMS-Cl (18 mg, 0.12 mmol, 1.1 equiv) was added portion-wise and stirring was continued overnight at rt to ensure completion of reaction (TLC) followed by quenching with MeOH. The reaction mixture was then evaporated to dryness under reduced pressure to give a yellow gum. The resulting solid was redissolved in 5 mL of DCM and purified by fCC on silica gel with a gradient eluent system of DCM:MeOH, 60:1 to 40:1 to yield **8**.(0.38 mg, 50 %) and the ***3'-O-*** isomer (0.32 mg, 48 %).

TLC (silica gel, DCM:MeOH, 25:1): R_f = 0.35. ^{1}H NMR (300 MHz, $CDCl_3$, 25°C): δ= 0.00 (s, 3H, CH_3,TBDMS), 0.01 (s, 3H, CH_3,TBDMS), 0.79 (s, 9H, TBDMS), 1.86 (s, 3H, 5-CH_3),

2.62 (m, 1H, H1), 3.25 (m, 2H, H5), 3.66 (s, 6H, 2x CH_3O-trityl), 4.11 (m, 1H, H3'), 4.18 (m, 1H, H2'), 4.38 (t, 1H, H4'), 5.90 (s, 1H, H1'), 6.70 (d, 4H, trityl), 7.13 (d, 4H, trityl), 7.16 (d, 2H, trityl), 7.24 (d, 2H, trityl), 7.52 (s, 1H, H6) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25°C): δ=-1.8 (2C, 2 x $SiCH_3$-) 12.9 (C-C5) 26.6 (C, $3CH_3$-*tert*Bu) 58.82 (C, 4C-CH_3O-trityl), 64.48 (C, CH_2O-C5), 71.99 (C, C2), 73.94 (C, C3), 76.90 (C, C1), 81.45 (C, C4), 85.96 (C, C-$(MeOPh)_2PhC$), 110.23, 113.17 (C, 4C-MeOPh), 126.76 (C, C-phenyl), 127.72 (C, 2C-phenyl), 127.86 (C, 2C-phenyl), 128.41, 130.32 (C, 4C-MeOPh), 136.44, 136.48, 139.27, 145.72, 151.27, 158.85, 163.59. MALDI-TOF-MS: 675.83 $[M+H]^+$, 303.14 (100, $[(MeO)_2Tr]^+$).

With TOM-Cl, Light yellow foam. TLC (Hex/AcOEt 1:1): R_f 0.47. 1H-NMR (400 MHz, $CDCl_3$): 1.04 – 1.16 (*m*, iPr_3Si); 1.38 (*s*, Me-C(5)); 3.13 (br. *s*, OH_C(3')); 3.40 (*dd*, *J*=1.5, 11.4, H-C(5')); 3.53 (*dd*, *J*=1.9, 11.5, H-C(5')); 3.81 (*s*, 2 MeO); 4.19 (br. *s*, H-C(4')); 4.38 (*t*, *J*=5.1, H-C(2')); 4.48 (br. *s*, H-C(3')); 5.03 (*d*, *J*=4.5, 1 H, OCH_2O); 5.24 (*d*, *J*=4.5, 1 H, OCH2O); 6.14 (*d*, *J*=8.3, H-C(1')); 6.84 – 6.86 (*m*, 4 arom. H); 7.26 – 7.43 (*m*, 9 arom. H); 7.66 (br. *s*, H-C(6)); 8.47 (br. *s*, H-N(3)). ^{13}C-NMR (75 MHz, $CDCl_3$): 12.1 (*d*, Me_2CH); 12.3 (*q*, *Me*-C(5)); 18.2 (*q*, Me_2CH); 55.7 (*q*, MeO); 63.5 (*t*, C(5')); 70.8 (*d*, C(2')); 82.9 (*d*, C(3')); 84.3 (*d*, C(4')); 86.2 (*s*, arom. C); 87.4 (*d*, C(1')); 91.2 (*t*, OCH2O); 111.7 (*s*, C(5)); 113.6 (*d*, arom. C); 127.6, 128.2, 128.5, 130.6 (4*d*, arom. C); 135.6, 135.8 (2*s*, arom. C); 144.7 (*s*, C(6)); 150.7 (*s*, C(2)); 159.2 (*s*, arom. C); 163.9 (*s*, C(4)). ESI-MS: 373.29 (100, $[M+H]^{2+}$).

5.2.3 Synthesis of *5'-O-DMT-2'-O-TBDMS-3'-O-CEP-5-methyluridine* (9)

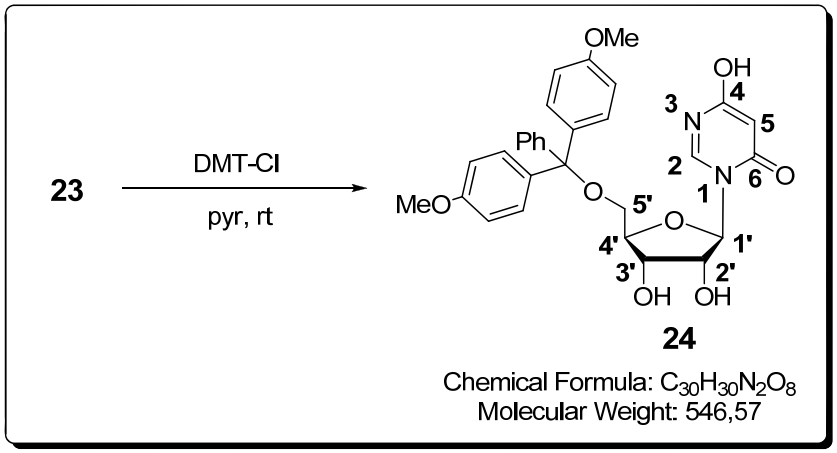


In a 2-neck rb flask equipped with a stirrer at rt was added ***5'-ODMT-2'-OTBDMS-5-methyluridine*** **(8)** (189 mg, 0.21 mmol) and DCM (5 mL). The reaction mixture was treated consecutively with DIEA (160 mg, 2.2 mmol, 1.01 equiv) and CEP-Cl (79 mg, 0.33 mmol, 1.5 equiv). The reaction was stirred at rt for 2 h (TLC). The mixture was then evaporated to dryness under reduced pressure to give a yellow foam. The resulting solid was redissolved in Hex: EtOAc (2 mL, 10:1) and purified by fCC on silica gel with a gradient eluent system of Hex: EtOAc, 60:1 to 40:1. The yield of the reaction was 86 %.

TLC (silica gel, Hex/EtOAc, 25:1): R_f = 0.44. ^{1}H NMR (300 MHz, $CDCl_3$/DMSO-*d6*, 25°C): δ= 0.01 (s, 3H, CH_3,TBDMS), 0.02 (s, 3H, CH_3,TBDMS), 0.78 (s, 9H, TBDMS), 1.09 (d, 12H-4xCH_3-*N*-isopropyl), 1.86 (s, 3H, 5-CH_3), 2.61 (m, 2H-NCCH_2-*CEP*), 2.63 (m, 1H, H1), 2.98 (m, 2H- *N*-isopropyl), 3.25 (m, 2H, H5), 3.66 (s, 6H, 2x CH_3O-trityl), 3.92 (m, 2H-CH_2O*CEP*), 4.11 (m, 1H, 3'), 4.18 (m, 1H, H2'), 4.38 (t, 1H, H4'), 5.91 (s, 1H, H1'), 6.70 (d, 4H, trityl), 7.13 (d, 4H, trityl), 7.16 (d, 2H, trityl), 7.23 (d, 2H, trityl), 7.53 (d, 1H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25°C): δ=-1.2 (2C, 2 x SiCH_3-) 12.9 (C-C5) 26.6 (C, 3CH_3-*tert*Bu), 21.2, 24.8, 24.9 52.3, 56.74, 58.82 (C, 4C-CH_3O-trityl), 64.48 (C, CH_2O-C5), 71.99 (C, C2), 73.94 (C, C3), 76.90 (C, C1), 81.45 (C, C4), 85.96 (C, C-$(MeOPh)_2$PhC), 110.23, 113.17 (C, 4C-MeOPh), 119.12, 126.76 (C, C-phenyl), 127.72 (C, 2C-phenyl), 127.86 (C, 2C-phenyl), 128.41, 130.32 (C, 4C-MeOPh), 136.44, 136.48, 139.27, 145.72, 151.27, 158.85, 163.59. MALDI-TOF-MS: 876.83 $[M+H]^+$, 303.14 (100, $[(MeO)_2Tr]^+$).

5.3 Chemical Synthesis of Uridine Phosphoramidite

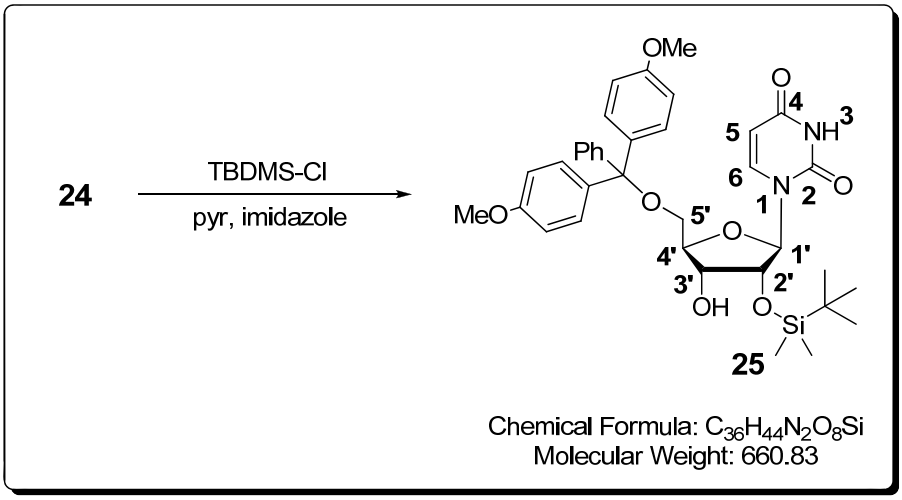
5.3.1 Synthesis of *5'-O-DMT-Uridine* (24)



To a stirred solution of ***uridine* (23)** (1.0 g, 4.1 mmol, 1.0 equiv) in 30 mL of anhydrous pyr was added DMT-Cl (1.53 g, 2.4 mmol, 1.2 equiv) in pyr (3 mL) portion-wise over 2h at rt. The reaction mixture was stirred at rt and monitored by TLC. The reaction was complete after 5 h after which it was quenched with water. The mixture was then evaporated to dryness under reduced pressure. The resulting solid was redissolved in 5 mL of DCM and purified by fCC on silica gel with a gradient eluent system of DCM:MeOH, 60:1 to 40:1 to yield **24** (0.74 g, 86 %).

TLC (silica gel, DCM:MeOH, 25:1): R_f = 0.25. ^{1}H NMR (300 MHz, $CDCl_3$, 25°C): δ= 3.40 (dd, 1H, *J*=3.4, 10.9 Hz), 3.50 (dd, 1H, *J*=2.9, 10.9 Hz), 3.79 (s, 2 OCH_3), 4.28 (m, 1H-C4'), 4.34 - 4.38 (m, 2H, H3' & H2'), 5.56 (d, 1H, *J* = 8.2 Hz, H5), 5.80 (d, 1H, *J* = 3.6 Hz, H1'), 6.83 (m, 4H, trityl-H), 7.23-7.36 (m, 9H, trityl-H) ppm ^{13}C NMR (125MHz, $CDCl_3$, 25°C): δ= 55.16 (2 CH_3O), 62.52 (C5), 71.25 (C3), 76.49 (C2), 84.92 (C4), 87.01, 92.06 (C1), 101.37 (C5'), 113.20, 127.06, 127.89, 127.95, 127.97 (trityl C), 135.06, 135.15, 137.30 (C6'), 144.15, 152.12, 158.66, 162.53 ppm. MALDI-TOF-MS: 569.60 $[M+Na]^+$.

5.3.2 Synthesis of *5'-O-DMT-2'-O-TBDMS-Uridine* (25)

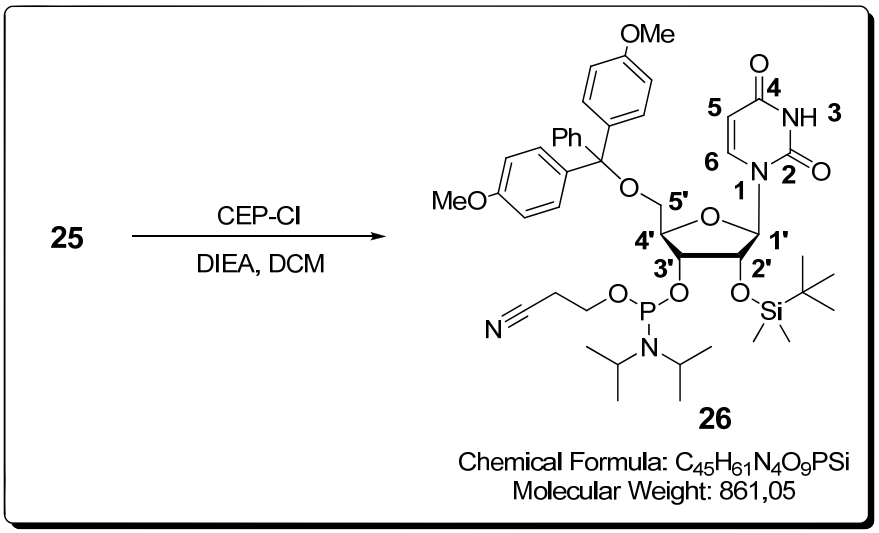


To a stirred solution of ***5'-O-DMT-uridine* 24** (60 mg, 0.12 mmol, 1.0 equiv) in 10 mL of anhydrous pyr was added imidazole (30 mg, 0.43 mmol, 4.0 equiv) in pyr 5 mL at rt. The mixture was stirred at rt for 15 min after which TBDMS-Cl (18 mg, 0.12 mmol, 1.1 equiv) was added portion-wise and stirring was continued. The reaction was monitored by TLC. The reaction was complete usually overnight, and then quenched with water. The mixture was then evaporated to dryness under reduced pressure to give a yellow gum. The resulting solid

was redissolved in 5 mL of DCM and purified by fCC on silica gel with a gradient eluent system of DCM:MeOH, 60:1 to 40:1 to yield **25** (0.42 mg, 48%).

TLC (silica gel, DCM:MeOH, 40:1): R_f = 0.31. ^{1}H NMR (300 MHz, $CDCl_3$, 25°C): δ = 0.28 (s, 6H, 2xCH_3Si), 1.02 (s, 9H, CH_3-tertBu), 3.40 (dd, 1H, *J*=3.4, 10.9 Hz, H5'), 3.50 (dd, 1H, *J*=2.9, 10.9 Hz, H5'), 3.79 (s, 2 OCH_3), 4.28 (m, 1H, H4'), 4.33 – 4.38 (m, 2H, H3' & H2'), 5.56 (d, 1H, *J* = 8.2 Hz, H-C5), 5.80 (d, 1H, *J* = 3.6 Hz, H1'), 6.83 (m, 4H, trityl-H), 7.23-7.36 (m, 9H, trityl-H) ppm ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ= 27.12 (CH_3-*tert*Bu), 30.23 (*tert*Bu), 55.21 (2 CH_3O), 62.52 (C5), 71.25 (C3), 76.49 (C2), 84.92 (C4), 87.01, 92.06 (C1), 101.37 (C5'), 113.20, 127.06, 127.89, 127.95, 127.97 (trityl C), 135.06, 135.15, 137.30 (C6'), 144.15, 152.12, 158.66, 162.53 ppm. MALDI-TOF-MS: 661.81 $[M+H]^+$.

5.3.3 Synthesis of *5'-ODMT-2'-OTBDMS-3'-OCEP-Uridine* (26)



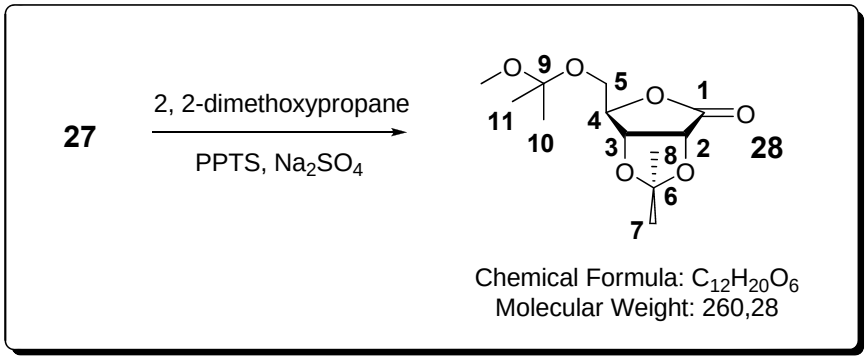
In a 2-neck rb flask equipped with a stirrer at rt was added ***5'-O-DMT-2'-O-TBDMS-Uridine*** **(25)** (189 mg, 0.21 mmol, 1 equiv) and DCM (5 mL). The reaction mixture was treated consecutively with DIEA (160 mg, 2.2 mmol) and CEP-Cl (79 mg, 0.33 mmol, 1.5 equiv). The reaction was monitored by TLC and was usually complete after 2 h. The mixture was then evaporated to dryness under reduced pressure resulting into a yellow foamy solid. The resulting solid was redissolved in Hex: EtOAc (2 mL, 10:1) and purified by fCC on silica gel with a gradient eluent system of Hex: EtOAc, 60:1 to 30:1 to yield **26** (0.42 g, 55 %).

TLC (silica gel, Hex:EtOAc, 20:1): R_f = 0.37. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 0.28 (s, 6H, 2xCH_3Si), 1.02 (s, 9H, CH_3-tertBu), 1.09 (d, 12H-4xCH_3-N-isopropyl), 2.62 (m, 2H-

$NCCH_2$-CEP), 2.98 (m, 2H- N-isopropyl), 3.40 (dd, 1H, J=3.4, 10.9 Hz), 3.50 (dd, 1H, J=2.9, 10.9 Hz), 3.78 (s, 6H-3xCH_3O-), 3.92 (m, 2H-CH_2O*CEP*), 4.28 (m, 1H-C4'), 4.32 -4.38 (m, 2H, H3' & H-C2')), 5.56 (d, 1H, J = 8.2 Hz, H5), 5.80 (d, 1H, J = 3.6 Hz, H1'), 6.83 (m, 4H-trityl), 7.22 (m, 1H-phenyl), 7.30 (m, 2H-phenyl), 7.36 (m, 4H-trityl), 7.52 (m, 2H-pheny), 7.54 (s, 1H-C6'). ^{13}C NMR (75 MHz, $CDCl_3$, 25°C): δ = 27.12 (CH_3-*tert*Bu), 30.23 (tertBu), 58.82 (C, 4C-CH_3O-trityl), 64.06 (C, CH_2O-C5), 71.25 (C, C2), 73.94 (C, C3), 76.69 (C, C1), 81.45 (C, C4), 85.96 (C, C-$(MeOPh)_2$PhC), 110.23, 113.17 (C, 4 C-MeOPh), 126.81 (C, C-phenyl), 127.91 (C, 2C-phenyl), 128.36 (C, 2C-phenyl), 130.29 (C, 4 C-MeOPh), 136.29, 136.40, 138.37, 144.15, 152.12, 158.66, 163.54 ppm. FAB-MS: 862.10 $[M+H]^+$, 303.1 (34, $[(MeO)_2Tr]^+$).

5.4 Chemical Synthesis of Pseudouridine

5.4.1 Synthesis of *6-((2-methoxypropan-2-yloxy)methyl)-2,2-dimethyldihydrofuro[3,4-d][1,3]dioxol-4(3aH)one* (28)

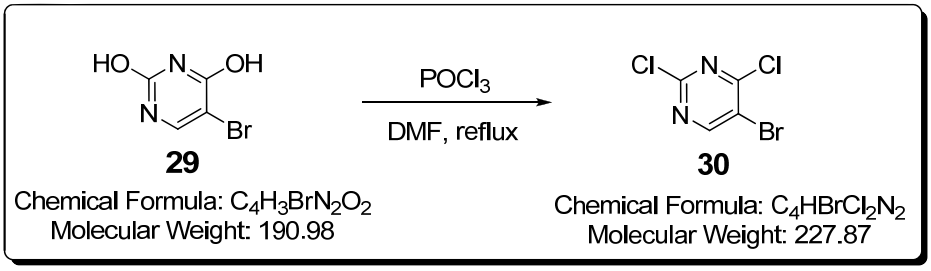


To a stirred solution of ***ribonic acid*** **(27)** (1.50 g, 7.5 mmol) in 2, 2-dimethoxypropane (15 mL), at rt, was added anhydrous Na_2SO_4 (3.0 g), and PPTS (0.2 g), and the resulting mixture was refluxed for 8 h (TLC). The reaction mixture was concentrated under reduced pressure to dryness and purified by fCC on silica gel with a gradient eluent system of EtOAc:Hex, 20:1 to 10:1. The silica gel was pre-treated in MeOH overnight. The afforded protected lactone **28** was a colorless liquid, with a yield of 95 %.

TLC (silica gel, EtOAc:Hex=1:5): R_f=0.42; ^{1}H NMR (300 MHz, $CDCl_3$, 25°C) δ= 1.29 (s, 3H-CH_3), 1.31 (s, 3H-CH_3), 1.38 (s, 3H-CH_3), 1.47 (s, 3H-CH_3), 3.14 (s, 3H-CH_3), 3.52 (d, 1H, J=9.0 Hz), 3.75 (d, 1H, J=8.44 Hz), 4.7 (m, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 24.1

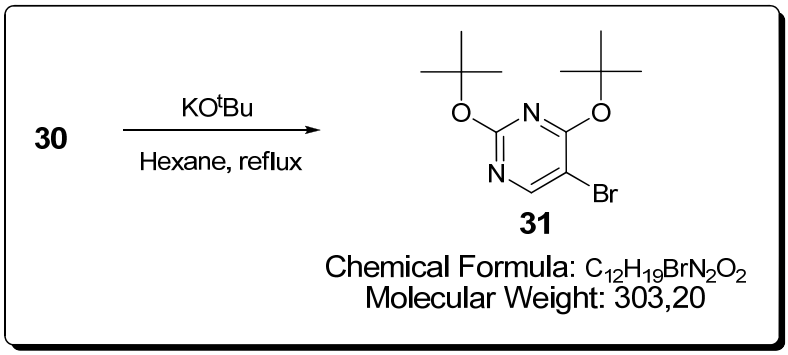
2(C8), 25.0 2(C7), 26.0(C10 & C11), 28.0(C-Me), 62.2, 78.5, 80.4, 83.9, 103.7, 114.8, 172.2, 173.4; FAB-MS: 261.15 $[M+H]^+$.

5.4.2 Synthesis of *5-bromo-2,4-dichloropyrimidine* (30)



In a rb flask equipped with ***5-bromouracil* (29)** (2.3 g, 12.1 mmol) was added DMF (25 mL) and phosphorous oxychloride(4.08 g, 26.6 mmol, 2.2 equiv). The mixture was then refluxed for 3 h (TLC). The DMF was then evaporated and the resulting solid purified by fCC on silica gel with a gradient eluent system of Hex: EtOAc 100:1, 100:2. The pure product **30** was recrystallized, after removal of solvent in *vacuo*, in Hex. The yield for this reaction was 98 %. TLC (silica gel, $CHCl_3$:MeOH=2:1), R_f = 0.42; ^{1}H NMR (DMSO-d_6): δ = 8.67 (s, 1H), FAB-MS: 225.59, 227.06 and 229.34 $[M+H]^+$.

5.4.3 Synthesis of *5-bromo-2, 4-di-tert-butoxypyrimidine* (31)

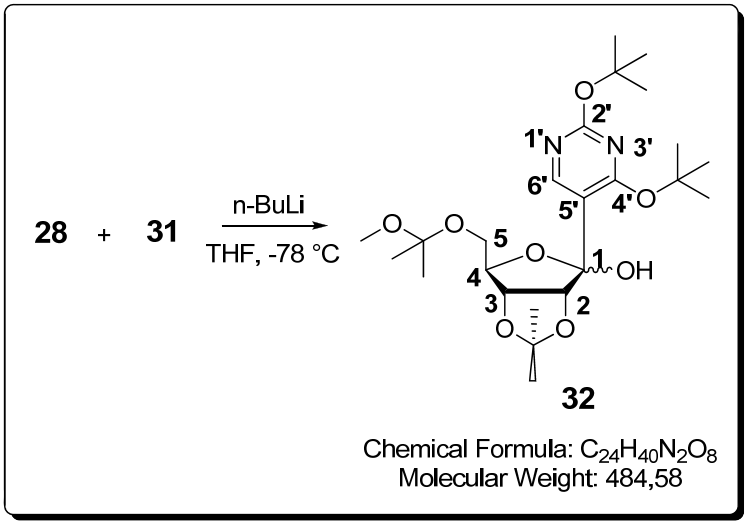


In a rb flask was added potassium tert-butyloxide (KO^{t}Bu) (0.99 g, 8.85 mmol), Hex (50 mL) and refluxed for 1 h under argon after which 2,4-dichloro-5-bromopyrimidine **(30)** (1.0 g, 4.42 mmol) was slowly added during 10 min. The mixture was reflux for further 2 h. The solution was allowed to cool afterwards and the solvent evaporated under reduced pressure.

The resulting mixture was dissolved in water and quickly extracted with diethyl ether (5x50 mL). The organic extract was then washed with brine (sat. NaCl solution), and subsequently dried with Mg_2SO_4. The ether was then evaporated and the resulting solid purified by fCC on silica gel with a gradient eluent system of Hex: EtOAc: 100:1, 100:2. Pure product **31** crystallized after removal of solvent. The yield was 87 %.

TLC (silica gel, Hex:EtOAc=10:1), R_f=0.40; ^{1}H NMR (DMSO-*d6*): δ= 1.48 (s, 9H), 1.52 (s, 9H), 8.22 (s, 1H), ^{13}C NMR (75 MHz, CD_3OD): δ = 28.48 (C-CH_3(*tert*Bu)), 28.52 (C-CH_3(*tert*Bu)), 80.94 (C- (*tert*Bu)), 83.42 (C- (*tert*Bu), 99.74 (C- CBr), 159.15 (C- CH), 163.14 (C- CO*tert*Bu), 165.87 (C- CO *tert*Bu) ppm. MALDI-MS: 304.2.$[M+H]^+$.

5.4.4 Synthesis of *4-(2,4-di-tert-butoxypyrimidin-5-yl)-6-((2-methoxypropan-2-yloxy)methyl)-2,2 dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-ol* (32)

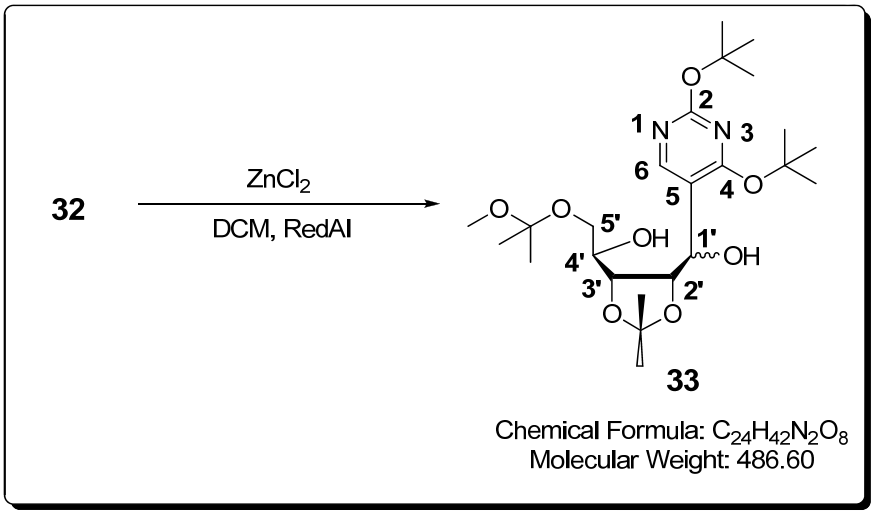


In an rb flask containing 2,4-di-*tert*butoxy-5-bromopyrimidine **31** (0.200 g, 0.66 mmol) and n-butyl-lithium (0.73 mmol, 1.1 equiv.) in dry oxygen free THF (10 mL) at -78 °C, stirred for 25 min, was added during 20 min the pre-cooled protected lactone **28** (700 mg, 2.7 mmol, 1 equiv.) in 40 mL of THF at -78 °C. After 4 h at -78 °C the solution was allowed o warm to rt and left overnight. The THF was removed under reduced pressure after TLC shows no starting material. The crude product was purified by fCC on silica gel with a gradient eluent system of DCM: MeOH, 19:1, 10:1.

TLC (silica gel, $CHCl_3$:MeOH=10:1), R_f = 0.35. ^{1}H NMR ($CDCl_3$): δ= 1.26 (s, 3H CH_3-cyclic aceta), 1.28 (s, 3H-CH_3-cyclic acetal), 1.40 (s, 6H-2xCH_3-open acetal), 1.61 (s, 9H-

3xCH$_3$*tert*Bu), 1.65 (s, 9H-3xCH$_3$*tert*Bu), 3.24 (s, 3H-CH$_3$O), 3.64 (d, *J* = 6.3 Hz, 2H-C5), 4.42 (m, 1H), 4.68 (s, 1H-OH), 4.87 (m, 1H-H2), 4.91 (m, 1H-H4), 8.46 (s, 1H-H6'). ^{13}C NMR (125MHz, CDCl$_3$, 25°C): δ=24.60 (C-CH$_3$-cyclic acetal), 24.66 (C-CH$_3$-cyclic acetal), 25.54 (C-CH$_3$-open acetal), 28.22 (C-CH$_3$-open acetal), 28.65 (C-3xCH$_3$*tert*Bu), 28.75 (C-3xCH$_3$*tert*Bu), 48.94 (C-CH$_3$O), 62.88 (C-C5), 66.46 (C-C1), 69.13 (C-C4), 77.68 (C-C2), 79.84 (C-C3), 80.34 (C- *tert*Bu), 82.19 (C- *tert*Bu), 100.64 (C-C5'), 108.85 (C-cyclic acetal), 115.36 (C-open acetal), 157.90 (C-C6'), 163.70 (C-C2'), 168.04 (C-C4').484.6 [M+H]$^+$.

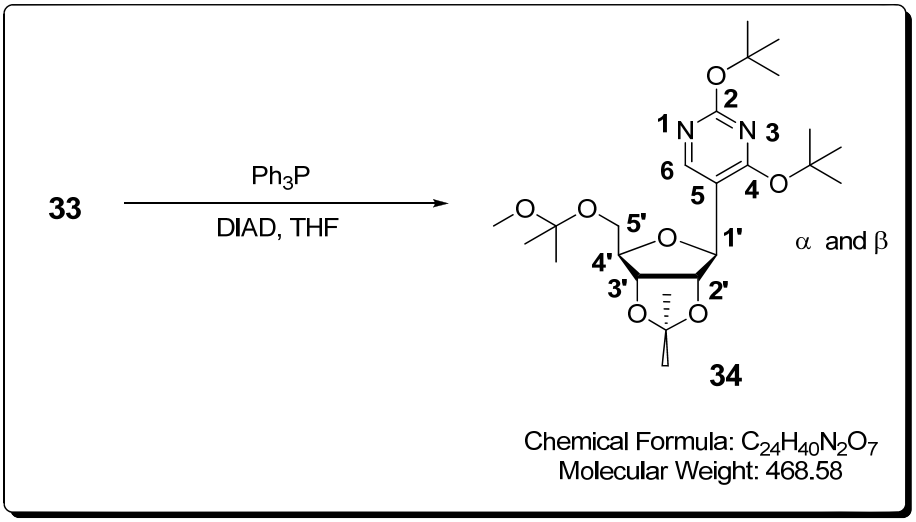
5.4.5 Synthesis of *1-(5-((2,4-di-tert-butoxypyrimidin-5-yl)(hydroxy)methyl)-2,2-dimethyl-1,3-dioxolan-4-yl)-2-(2-methoxypropan-2-yloxy)ethanol* (33)



To **32** (340 mg , 0.70 mmol) in dry DCM (30 mL) at -78 °C was added ZnCl$_2$ (0.95 mL, 0.95 mmol, 1 M in Et$_2$O) and the mixture was then stirred for 30 min. RedAl (2.38 mL, 2.38 mmol, 1 M in THF) was then added dropwise and portion-wise over a period of 30 min at -78 °C. The mixture was then slowly brought to rt and stirred overnight. It was then quenched with EtOH (0.40 mL), H$_2$O (0.09 mL), NaOH 6 M (0.35 mL) and H$_2$O$_2$ (30 % v/v, 0.35 mL). It was then stirred for 30 min and diluted with EtOAc (40 mL) and H$_2$O (40 mL). Standard workup gave colorless oil which was purified by fCC on silica gel with a gradient eluent system of Hex: EtOAc (1:1, 2:1) giving the product as a white foamy solid **33** with yield of.
TLC (silica gel, CHCl$_3$:MeOH=10:1), R_f = 0.33. ^{1}H NMR (CDCl$_3$): δ= 1.26 (s, 3H CH$_3$-cyclic acetal), 1.28 (s, 3H-CH$_3$-cyclic acetal), 1.40 (s, 6H-2xCH$_3$-open acetal), 1.61 (s, 9H-3xCH$_3$*tert*Bu), 1.65 (s, 9H-3xCH$_3$*tert*Bu), 3.24 (s, 3H-CH$_3$O), 3.64 (d, *J* = 6.3 Hz, 2H-H5'), 411 (m, 1H), 4.42 (m, 1H), 4.67 (s, 1H-OH), 4.87 (m, 1H-H2'), 4.91 (m, 1H-H4'), 8.46 (s, 1H-

H6). ^{13}C NMR (125MHz, $CDCl_3$, 25°C): δ=24.60 (C-CH_3-cyclic acetal), 24.66 (C-CH_3-cyclic acetal), 25.54 (C-CH_3-open acetal), 28.22 (C-CH_3-open acetal), 28.65 (C-3xCH_3*tert*Bu), 28.75 (C-3xCH_3*tert*Bu), 48.94 (C-CH_3O), 62.88 (C-C5), 66.48 (C-C1), 69.03 (C-C4), 77.67 (C-C2), 79.81 (C-C3), 80.34 (C- *tert*Bu), 82.19 (C- *tert*Bu), 100.64 (C-C5'), 108.85 (C-cyclic acetal), 115.36 (C-open acetal), 157.90 (C-C6'), 163.70 (C-C2'), 168.04 (C-C4'). MALDI-TOF-Ms: 487.61 $[M+H]^+$

5.4.6 Synthesis of *2,4-di-tert-butoxy-5-(6-((2-methoxypropan-2-yloxy)methyl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)pyrimidine* (34)

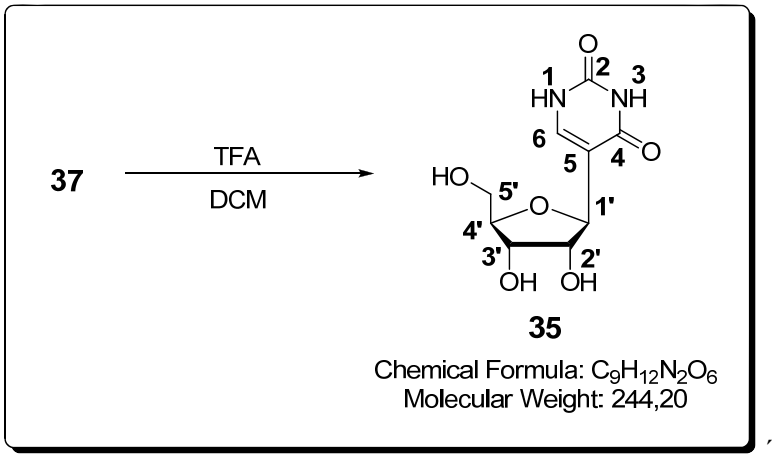


To a solution of **(33)** (3 g, 7.5 mmol) in THF (0.3 L) was added Ph_3P (4 g, 15.1 mmol) at 0 °C under argon atmosphere. DIAD (12 mL, 59.4 mmol) was added, and the mixture was stirred overnight and concentrated. The residue was purified by fCC on silica gel with a gradient eluent system of Hex:EtOAc: (10:1, 2:1) to afford the bis-acetal as a white foamy solid **34**. (2.5 g, 26.4 mmol, 86%).

TLC (silica gel, $CHCl_3$:MeOH=10:1), R_f = 0.38 ^{1}H NMR (300 MHz, $CDCl_3$, 25°C): δ = 1.28 (s, 6H-2xCH_3-open acetal), 1.32 (s, 3H-CH_3-cyclic acetal),- 1.40 (s, 3H-CH_3-cyclic acetal), 1.52 (s, 9H-3xCH_3*tert*Bu), 1.62 (s, 9H-3xCH_3*tert*Bu), 3.14 (s, 3H-CH_3O), 3.49-3.52 (m, 2H-H5'), 4.04 (m, 1H), 4.54 - 4.60 (m, 1H-H3'), 4.86 (m, 1H-H2'), 5.14 (m, 1H-H4'), 8.24 (s, 1H-H6) ppm. ^{13}C NMR (125MHz, $CDCl_3$, 25°C): δ = 24.60 & 24.62 (C-CH_3-cyclic acetal), 24.66 & 24.72 (C-CH_3-cyclic acetal), 25.54 & 25.62 (C-CH_3-open acetal), 28.22 & 28.24 (C-CH_3-open acetal), 28.65 & 28.72 (C-3xCH_3*tert*Bu), 28.75 & 28.83 (C-3xCH_3*tert*Bu), 48.94 (C-CH_3O), 61.52 - 62.88 (C-C5), 67.48 (C-C1), 71.03 (C-C4), 77.67 & 77.72 (C-C2), 79.71 &

79.82 (C-C3), 80.32 & 80.36 (C- *tert*Bu), 82.19 & 82.23 (C- *tert*Bu), 100.64 (C-C5'), 111.65 & 112.49 (C-cyclic acetal), 113.97 & 114.40 (C-open acetal), 157.04 & 157.70 (C-C6'), 163.70 & 163.91 (C-C2'), 166.51 & 167.47 (C-C4') ppm. FAB-MS: 470.01 $[M+H]^+$. The reported chemical shifts are for the mixed α and β anomers and not respective values for the α and β forms.

5.4.7 Synthesis of *5-(3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl) pyrimidine-2,4(1H,3H)-dione* (35)

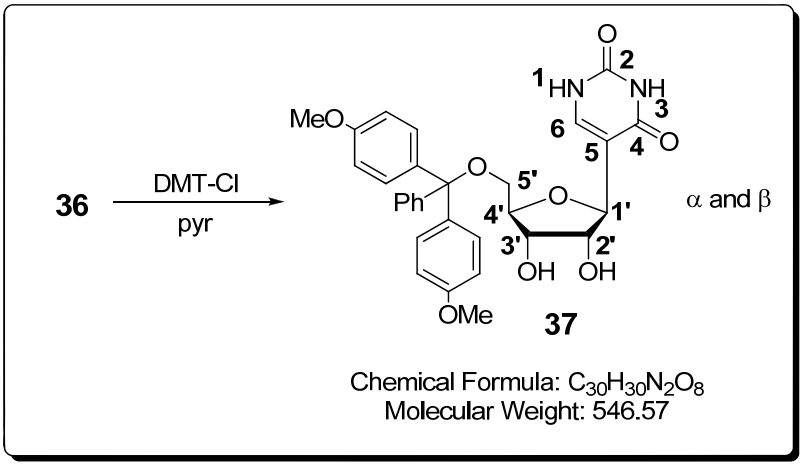


To ***β-5'-ODMT-Pseudouridine*** **(37)** (300 mg , 0.70 mmol) in Dry DCM (30 mL) at rt was added a 20 % solution of trifluoroacetic acid in DCM. The mixture was then slowly stirred and monitored by TLC every 5 min. The reaction was usually finished after 15 min after which it was quenched by the neutralization of the acid using sodium bicarbonate. Standard workup gave a solid which was purified by fCC on silica gel with a gradient eluent system of Hex: EtOAc (1:1, 1:2) to afford *β*-pseudouridine **35** as a white powder in70 % yield.

TLC (silica gel, Hexane: EtOAc (1:2)), R_f = 0.18 as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ = 3.68 (dd, 1H, *J*=12.0, 4.4 Hz), 3.82 (dd, 1H, *J*=12.4, 3.2 Hz), 3.97 (m, 1H), 4.10 (t, 1H, J= 5.6 Hz), 4.23 (t, 1H, *J* = 5.4 Hz), 4.67 (d, 1H, *J* = 5.6 Hz), 7.61(s, 1H) ppm. ^{13}C NMR (125MHz, $CDCl_3$, 25°C): δ=61.51, 70.80, 73.46, 79.81, 83.16, 110.06, 139.34, 153.16, 164.74 ppm. MALDI-TOF- MS: 245.19 $[M+H]^+$.

5.5 Chemical Synthesis of Pseudouridine Phosphoramidite

5.5.1 Synthesis of *5'-O-DMT-Pseudouridine* (37)



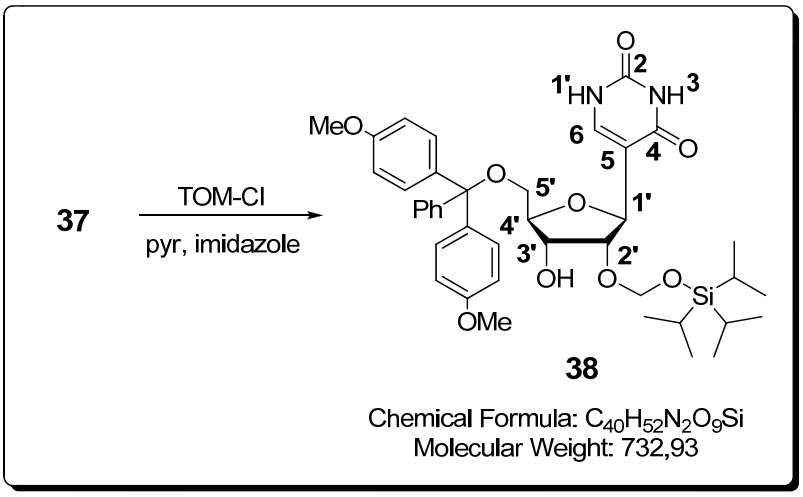
To a stirring mixture of α and β-***Pseudouridine* 36** (1.0 g, 4.1 mmol, 1.0 equiv) dissolved in 30 mL of anhydrous pyr was added DMT-Cl (1.53 g, 2.4 mmol, 1.2 equiv) in pyr (3 mL) portion-wise over 2 h at rt. The reaction mixture was stirred at rt and monitored by TLC. The reaction was complete after 5 h after which it was quenched with water. The mixture was then evaporated to dryness under reduced pressure. The resulting solid was dissolved in 5 mL of DCM and purified by fCC on silica gel with a gradient eluent system of DCM:MeOH, (60:1 to 30:1) resulting in **37** (0.72 g, 85 %).

Alpha-product. TLC (silica gel, DCM:MeOH, 40:1), R_f = 0.24. ^{1}H NMR (300 MHz, Acetone-d_6, 25 °C): δ = 3.21 (m, 1H), 3.30 (m, 1H), 3.78 (s, 6H-3xCH_3O), 4.09 (m, 1H), 4.14 (m, 1H), 5.21(m, 1H), 5.62 (s, 1H), 6.89 (m, 4H-trityl), 7.22 (m, 1H-phenyl), 7.30 (m, 2H-phenyl), 7.36 (m, 4H-trityl), 7.52 (m, 2H-pheny), 7.54 (s,1H-C6') ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25°C): δ= 58.82 (C, 4C-CH_3O-trityl), 64.48 (C, CH_2O-C5), 71.99 (C, C2), 73.94 (C, C3), 76.90 (C, C1), 81.45 (C, C4), 85.96 (C, C-$(MeOPh)_2$PhC), 110.23, 113.17 (C, 4C-MeOPh), 126.76 (C, C-phenyl), 127.72 (C, 2C-phenyl), 127.86 (C, 2C-phenyl), 128.41, 130.32 (C, 4C-MeOPh), 136.44, 136.48, 139.27, 145.72, 151.27, 158.85, 163.59 ppm. MALDI-TOF-MS: 547.61 $[M+H]^+$, 303.14 (100, $[(MeO)_2Tr]^+$).

Beta-product. TLC (silica gel, DCM:MeOH, 40:1): R_f = 0.22. ^{1}H NMR (300 MHz, Acetone-d_6, 25°C): δ = 3.21 (m, 1H), 3.30 (m, 1H), 3.78 (s, 6H-3xCH_3O), 4.09 (m, 1H), 4.14 (m, 1H), 4.71(m, 1H), 5.62 (s, 1H), 6.89 (m, 4H-trityl), 7.22 (m, 1H-phenyl), 7.30 (m, 2H-phenyl),

7.36 (m, 4H-trityl), 7.52 (m, 2H-pheny), 7.54 (s,1H-C6') ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25°C): δ= 58.82 (C, 4C-CH_3O-trityl), 64.06 (C, CH_2O-C5), 72.00 (C, C2), 73.94 (C, C3), 76.90 (C, C1), 81.45 (C, C4), 85.96 (C, C-$(MeOPh)_2$PhC), 110.23, 113.17 (C, 4C-MeOPh), 126.81(C, C-phenyl), 127.91(C, 2C-phenyl), 128.36 (C, 2C-phenyl), 130.29 (C, 4C-MeOPh), 136.29, 136.40, 138.37, 145.55, 151.27, 158.89, 163.59 ppm. MALDI-TOF-MS: 547.60 $[M+H]^+$, 303.14 (100, $[(MeO)_2Tr]^+$).

5.5.2 Synthesis of *5'-O-DMT-2'-O-TOM- Pseudouridine* (38)

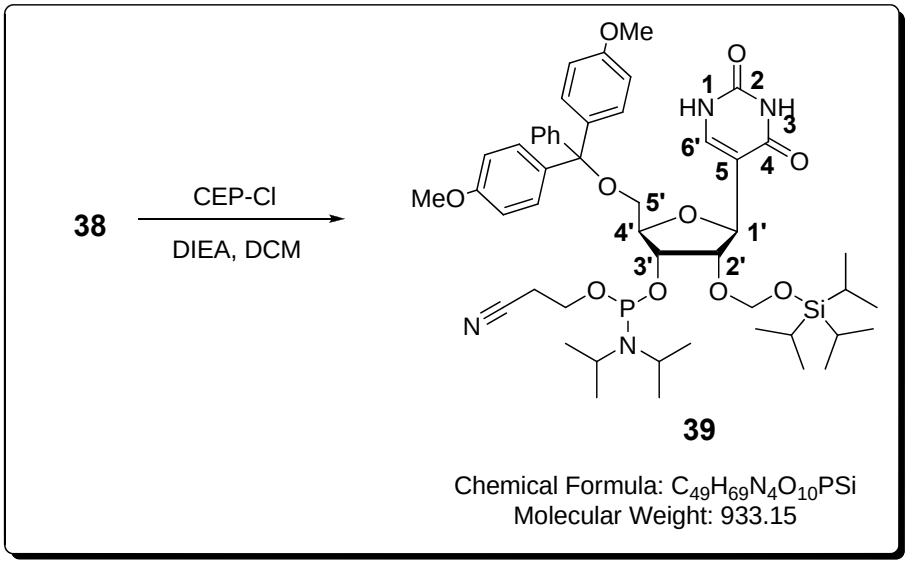


To a stirred solution of ***5'-O-DMT- Pseudouridine*** **(37)** (60 mg, 0.12 mmol, 1.0 equiv) in anhydrous pyr (10 mL) was added imidazole (30 mg, 0.43 mmol, 4.0 equiv) in pyr (5 mL) at rt. The mixture was stirred at rt for 10 min after which TOM-Cl (18 mg, 0.12 mmol, 1.1 equiv) was added portion-wise and stirring was continued. The reaction was complete usually overnight (TLC), and then quenched with water. The mixture was then evaporated to dryness under reduced pressure to give a yellow gum. The resulting solid was dissolved in 2 mL of DCM and purified by fCC on silica gel with a gradient eluent system of DCM:MeOH, (80:1 to 40:1) to afford **38** (0.42 mg, 55 %)

TLC (silica gel, DCM:MeOH, 40:1): R_f = 0.25. ^{1}H NMR (300 MHz, Acetone-d_6, 25 °C): δ= 1.02 (s, 18H-6xCH_3-isopropyl), 1.81 (m, 3H-isopropyl), 3.21 (m, 1H), 3.30 (m, 1H), 3.78 (s, 6H-3xCH_3O), 4.09 (m, 1H), 4.14 (m, 1H), 4.71 (m, 1H), 5.62 (s, 1H), 5.86 (s, 2H-OCH_2O-TOM), 6.89 (m, 4H-trityl), 7.22 (m, 1H-phenyl), 7.30 (m, 2H-phenyl), 7.36 (m, 4H-trityl), 7.52 (m, 2H-pheny), 7.54 (s,1H-C6'). ^{13}C NMR (75 MHz, $CDCl_3$, 25°C): δ= 18.2 (C, 4C-4xCH_3-isopropyl), 23.12 (C, 3C-isopropyl), 58.82 (C, 4C-CH_3O-trityl), 64.06 (C, CH_2O-C5),

72.00 (C, C2), 73.94 (C, C3), 76.90 (C, C1), 81.45 (C, C4), 85.96 (C, C-$(MeOPh)_2$PhC), 110.23, 113.17 (C, 4C-MeOPh), 126.81 (C, C-phenyl), 127.91 (C, 2C-phenyl), 128.36 (C, 2C-phenyl), 130.29 (C, 4C-MeOPh), 136.29, 136.40, 138.37, 145.55, 151.27, 158.89, 163.59. MALDI-TOF-MS: 733.92 $[M+H]^+$, 303.14 (100, $[(MeO)_2Tr]^+$).

5.5.3 Synthesis of *5'-O-DMT-2'-O-TOM-3'-O-CEP-pseudouridine* (39)



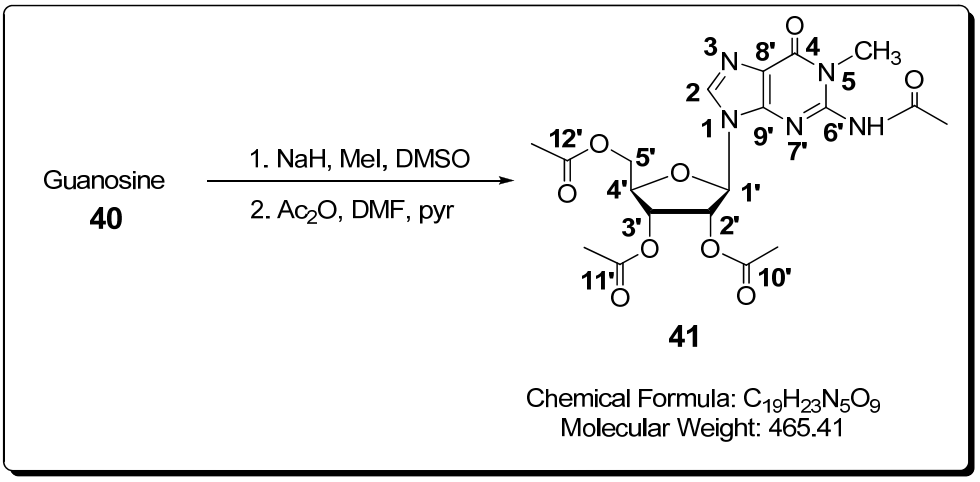
In a 2-neck rb flask equipped with a stirrer at rt was added ***5'-O-DMT-2'-O-TOM-pseudouridine* (38)** (189 mg, 0.21 mmol, 1.0 equiv) in 5 mL dry DCM was added DIEA (160 mg, 2.2 mmol) and CEP-Cl (79 mg, 0.33 mmol, 1.5 equiv) at rt. The reaction was stirred at rt for 2 h (TLC). The mixture was then evaporated to dryness under reduced pressure to giving yellow foam. The resulting solid was dissolved in Hex: EtOAc (2 mL, 10:1) and purified by fCC on silica gel with a gradient eluent system of (Hex: EtOAc, 60:1 to 40:1) resulting in the product **39** (0.42 g) as a diastereomeric mixture in 75 %.

TLC (silica gel, Hex:EtOAc, 25:1): R_f = 0.32. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ= 1.02 (s, 18H-6xCH_3-isopropyl), 1.09 (d, 12H-4xCH_3-*N*-isopropyl), 1.81 (m, 3H-isopropyl), 2.62 (m, 2H-NCCH_2-*CEP*), 2.98 (m, 2H- *N*-isopropyl), 3.21 (m, 1H), 3.30 (m, 1H), 3.78 (s, 6H-3xCH_3O), 3.92 (m, 2H-CH_2O*CEP*), 4.09 (m, 1H), 4.14 (m, 1H), 4.71(m, 1H), 5.62 (s, 1H), 5.86 (s, 2H-OCH_2O-TOM), 6.89 (m, 4H-trityl), 7.22 (m, 1H-phenyl), 7.30 (m, 2H-phenyl), 7.36 (m, 4H-trityl), 7.52 (m, 2H-pheny), 7.54 (s,1H-C6') ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25°C): δ= 18.2 (C, 4C-4xCH_3-isopropyl), 23.12 (C, 3C-isopropyl), 58.82 (C, 4C-CH_3O-trityl), 64.06 (C, CH_2O-C5), 72.00 (C, C2), 73.94 (C, C3), 76.90 (C, C1), 81.45 (C, C4), 85.96 (C, C-

$(MeOPh)_2PhC$), 110.23, 113.17 (C, 4C-MeOPh), 126.81(C, C-phenyl), 127.91(C, 2C-phenyl), 128.36 (C, 2C-phenyl), 130.29 (C, 4C-MeOPh), 136.29, 136.40, 138.37, 145.55, 151.27, 158.89, 163.59 ppm. MALDI-TOF-MS: 956.12 $[M+Na]^+$, 303.14 (100, $[(MeO)_2Tr]^+$).

5.6 Chemical Synthesis of m^1G Phosphoramidite

5.6.1 Synthesis of *2',3',5',N^2-tetraacetyl-N^1-methylguanosine* (41)

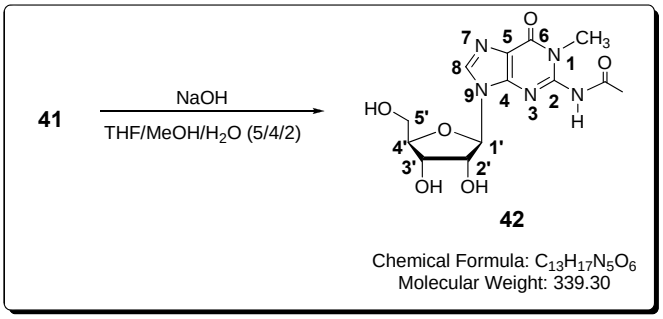


NaH (254mg, 10.6mmol, 1.0 equiv) was added to a stirred solution of guanosine **(40)** (3 g, 10.6mmol, 1.0 equiv) in 20 mL of anhydrous DMSO at rt. The reaction mixture was stirred until evolution of hydrogen gas ceased followed by the addition of MeI (1.5 g, 10.6mmol, 1.0 equiv) in 1 mL of DMSO was added dropwise and stirred for 5 h at rt. The mixture was evaporated to dryness under reduced pressure at 80°C and re-dissolved in a mixture of pyr/DMF/acetic anhydride (50 mL each) and heated to 140 °C for 10 h. Thin Layer Chromatography (TLC) showed no starting material after which reaction mixture was evaporated under reduced pressure. The resulting solid was dissolved in 200 mL of MeOH and 70 g of silica gel was added. The MeOH was evaporated, the silica gel coated by the crude product was dried under vaccum and purified by fCC on silica gel with a gradient eluent system of (silica gel, DCM:MeOH, 40:1 to 25:1) resulting in **41** (4.2 g, 85%).

TLC (silica gel, DCM:MeOH = 40:1): R_f = 0.25; ^{1}H NMR (500MHz, $CDCl_3$, 25 °C): δ = 2.07, 2.08, 2.13, 2.33 (4s, $COCH_3$), 3.60 (s, 1-CH_3), 4.40–4.46 (m, H1–C(5'), H–C(4')), 4.53 (dd, J=5.2, 11.0 Hz, H2– C(5')), 5.67 (t, H–C(3')), 5.91 (t, H–C(2')), 5.99 (d, J=5.2Hz, H–C(1')), 7.80 (s, H–C(8)), 8.76 (br s, NH) ppm. ^{13}C NMR (125MHz, CDCl3, 25°C): δ =20.37, 20.54,

20.79, 23.78 ($COCH_3$), 31.90 (1-CH_3), 63.19 (C(5')), 70.72 (C(3')), 72.95 (C(2')), 80.23 (C(4')), 87.00 (C(1')), 122.90, 138.80 (C(8)), 145.73, 146.90, 157.29, 169.34, 169.62, 170.10, 171.04 ppm. MALDI-TOF-MS: 466.45$[M+H]^+$ and 488.5 $[M+Na]^+$.

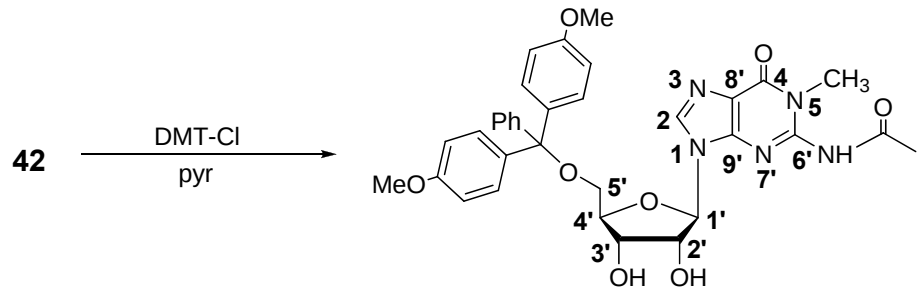
5.6.2 Synthesis of *N^2-acetyl-N^1-methylguanosine* (42)



To a vigorously stirred solution of **41** (2.0 g, 4.3 mmol) in 24 mL of a mixture of THF/MeOH/H_2O (5/4/2) aqueous NaOH (2 mL, 10 M) was added at rt. The reaction was monitored by TLC ($CHCl_3$: MeOH, 1:1) and was completed typically after 15 min. The mixture was quenched with ~2 mL of acetic acid to give a final pH of 6.5. The product **42** (1.18 g, 81 %) precipitated as a pale yellow solid after overnight storage at 4 °C and was collected by filtration and washed twice with 10 mL of cold THF/MeOH/H_2O (-20 °C) and dried under vacuum for 3 days. Yield of the reaction was 72 %.

TLC (silica gel, $CHCl_3$:MeOH = 2:1): R_f = 0.4; ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ = 2.13 (s, $COCH_3$), 3.41 (s, 1-CH_3), 3.63 (dd, J=3.9, 11.9Hz, H1–C(5')), 3.53 (dd, J=4.0, 11.9 Hz, H2–C(5')), 3.91 (q, H–C(4')), 4.12 (t, H–C(3')), 4.45 (t, H–C(2')), 4.99 (br s, HO–C(5')), 5.15, 5.44 (2br s, HO–C(2'), HO–C(3')), 5.79 (d, J=5.8Hz, H–C(1')), 8.31 (s, H–C(8)) ppm; ^{13}C NMR (75MHz, d^6-DMSO, 25°C): δ=23.8 ($COCH_3$), 31.9 (1-CH_3), 62.1 (C(5')), 71.2 (C(3')), 74.9 (C(2')), 86.5 (C(4')), 87.8 (C(1')), 139.8 (C(8)), 122.0, 147.3, 148.0, 157.7, 171.0 ppm; ESI MS: 340.1 $[M+H]^+$.

5.6.3 Synthesis of *5'-O-DMT-N^2-acetyl-N^1-methylguanosine* (43)



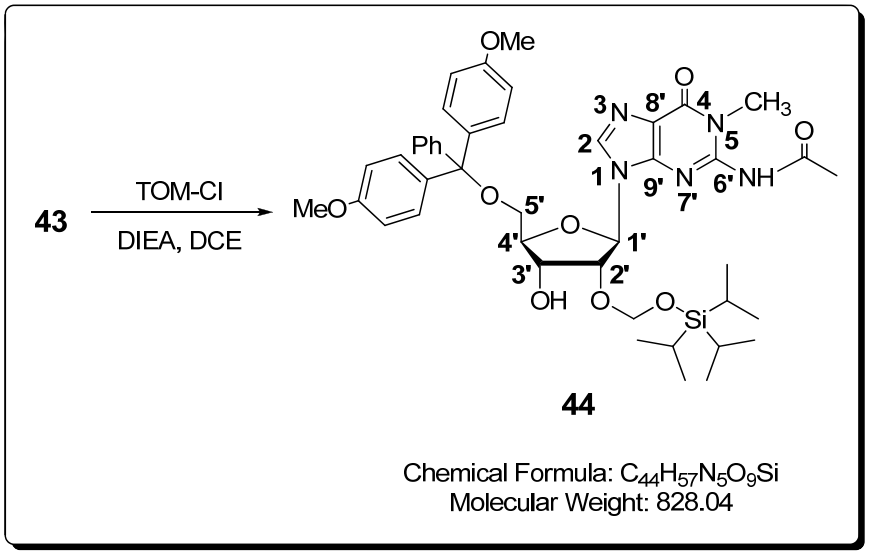
43

Chemical Formula: $C_{34}H_{35}N_5O_8$
Molecular Weight: 641.67

To a stirred solution of ***N^2-acetyl-N^1-methylguanosine*** **(42)** (0.5 g, 1.5 mmol, 1.0 equiv) in 6 mL of anhydrous pyr and 3 mL of anhydrous DMF at rt was added DMT-Cl (585 mg, 1.73 mmol, 1.2 equiv) in 10 mL pyr. The reaction mixture was stirred and monitored by TLC. The reaction was complete after 8 h. The mixture was then evaporated to dryness under reduced pressure at 40 °C. The resulting solid was dissolved in 20 mL of DCM/MeOH (10:1) and purified by fCC on silica gel with a gradient eluent system of (DCM:MeOH, 40:1 to 25:1) resulting in **43** with yield (4.2 g, 85 %).

TLC (silica gel, DCM:MeOH=10:1): R_f = 0.4; ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ = 1.84 ($COCH_3$), 3.22 - 3.42 (m, 2H–C5'), 3.45 (1-CH_3), 3.71 (s, -OCH_3), 3.72 (s, -OCH_3), 4.29 (t, H–C4'), 4.42 (t, H–C3'), 4.88 (t, H–C2'), 5.86 (d, J=6.1Hz, H–C1'), 6.25 (br q, NH), 6.72–6.80 (m, 4H, trityl-H), 7.10–7.42 (m, 9H, trityl-H), 7.85 (s, H–C(8)) ppm; ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ=23.62 ($COCH_3$), 31.30 (1-CH_3), 55.20 (2 CH_3O), 63.68 (C5'), 71.91 (C(3')), 74.88 (C2'), 85.27 (C4'), 86.40, 89.58 (C1'), 113.19 (trityl-C), 121.2, 126.96, 127.90, 128.03, 129.99, 130.05 (trityl-C), 135.55, 135.63, 138.93 (C(8)), 144.66, 146.05, 146.43, 157.35, 158.54, 170.25 ppm. FAB-MS: m/z=642.2 (100, $[M+H]^+$), 303.1 (39, $[(MeO)_2Tr]^+$).

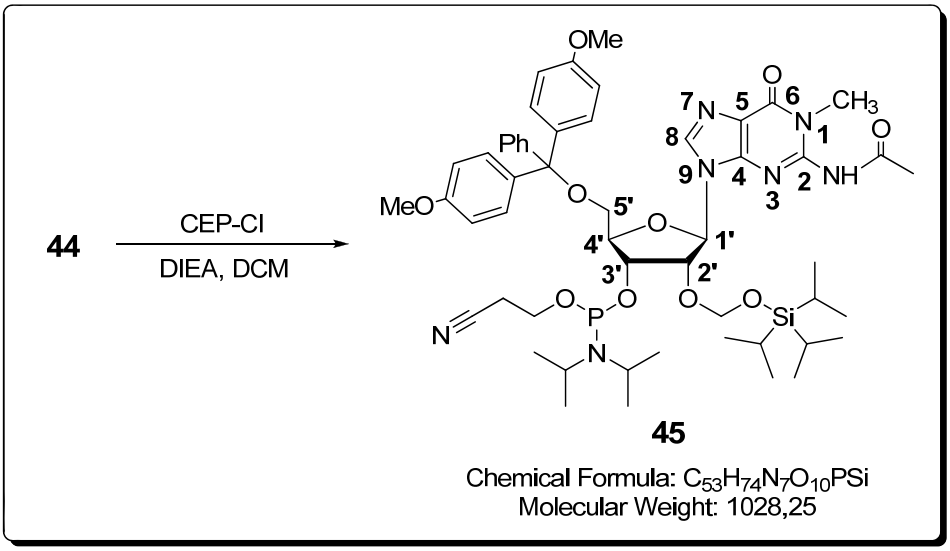
5.6.4 Synthesis of *5'-ODMT-2'-OTOM-N^2-acetyl-N^1-methylguanosine* (44)



In a rb flask equipped with a stirrer under argon was added ***5'-ODMT-N^2-acetyl-N^1-methylguanosine*** **(43)** (400 mg, 0.56 mmol, 1.0 equiv), DCE (4 mL), DIEA (285 mg, 2.25 mmol, 4.0 equiv), and di-*tert*-butyltindichloride (200 mg, 0.65 mmol, 1.2 equiv). The mixture was heated to 70 °C and stirred for 15 min and then allowed to cool to rt again, and treated with TOM-Cl (145 mg, 0.6 mmol, 1.2 equiv). The mixture was further stirred for additional 1 h followed by the addition of MeOH (0.3 mL) after which TLC showed no starting material. The mixture was evaporated to dryness after which it was subjected to fCC (silica gel, DCM:MeOH, 60:1 to 40:1) to afford the fluffy white solid **44** (204 mg, 46 %) and the ***5'-O-DMT-3'-OTOM-N^2-acetyl-N^1-methylguanosine*** (154 mg, 36 %).

TLC (silica gel, DCM:MeOH = 25:1): R_f = 0.37; ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ=0.98–1.07 (m, iPr_3Si), 1.42 (s, $COCH_3$), 3.00 (d, J=1.5Hz, HO–C3'), 3.17 (dd, J=3.2, 10.5 Hz, H1–C5'), 3.52 (s, 1-CH_3), 3.55 (dd, J=1.8, 10.5Hz, H2–C5'), 3.78, 3.79 (2s, 2 OCH_3), 4.25 (m, H–C4'), 4.52 (m, H–C3'), 4.94 (q, H–C2'), 4.90, 5.11 (2d, J=4.7Hz, OCH_2O), 5.93 (d, J=7.3Hz, H–C1'), 6.80 (m, 4H, trityl-H), 6.98 (br s, NH), 7.17–7.54 (m, 9H, trityl-H), 7.83 (s, H–C8) ppm; ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ = 11.80 ($(CH_3)_2CH$), 17.75 ($(CH_3)_2CH$), 22.89 ($COCH_3$), 31.79 (1-CH_3), 55.28 (2 CH_3O), 63.78 (C5'), 70.90 (C3'), 81.96 (C2'), 84.37 (C4'), 86.37 (C1'), 86.50, 91.07 (OCH_2O), 113.26, 113.32 (trityl-C), 123.05, 127.16, 128.01, 128.08, 129.97, 130.12 (trityl-C), 135.68, 135.89, 139.58 (C(8)), 145.12, 146.24, 157.30, 158.72, 169.68 ppm; FAB-MS: m/z=829.2 (83, $[M+H]^+$), 303.1 (100, $[(MeO)_2Tr]^+$).

5.6.5 Synthesis of *5'-O-DMT-2'-O-TOM-3'-O-CEP-N^2-acetyl-N^1-methylguanosine* (45)

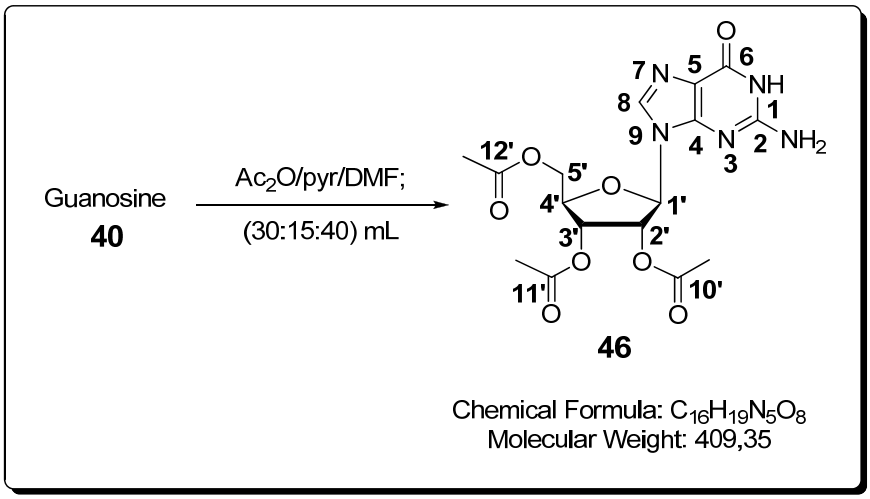


In a 2-neck rb flask equipped with a stirrer at rt was added **44** (189 mg, 0.21 mmol, 1 equiv) and DCM (5 mL). The reaction mixture was treated consecutively with DIEA (160 mg, 2.2 mmol) and CEP-Cl (79 mg, 0.33 mmol, 1.5 equiv). The reaction mixture was monitored by TLC, stirred for 3 h and quenched with MeOH (0.3 mL) , evaporated under vacuum to dryness. Workup and purified by fCC on silica gel with a gradient eluent system of DCM:MeOH (80:1, 40:1), containing 2 % Et_3N afforded **45** (192 mg, 85 % as diastereoisomeric mixture).

TLC (silica gel, DCM:MeOH=25:1): R_f = 0.5; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 0.86 – 1.19 (m, 30H, iPr_3Si (18H), $((CH_3)_2CH)_2N$ (12H)), 1.44, 1.66 (br s, 6H, $COCH_3$), 2.27, 2.69 (2m, 4H, CH_2CN), 3.21 (m, 2H, H1– C(5')), 3.50–3.55 (m, 4H, $((CH_3)_2CH)_2N$), 3.51, 3.52 (2s, 6H, 1-CH_3), 3.53, 3.59 (m, 2H, $POCH_2$), 3.51, 3.62 (m, 2H, H2–C(5')), 3.77, 3.78 (2s, 12H, OCH_3), 3.90, 3.97 (2m, 2H, $POCH_2$-), 4.27, 4.34 (2q, 2H, H–C(4')), 4.52, 4.57 (2m, 2H, H–C(3')), 4.89–4.96 (4d, 4H, J=5.2Hz, OCH_2O), 5.00, 5.06 (t, q, 2H, H–C(2')), 5.89, 5.99 (2d, 2H, J=7.1Hz, H–C(1')), 6.76–6.82, 7.20–7.31, 7.35–7.53 (m, 26H, trityl-H), 7.82, 7.84 (2s, 2H, H–C(8)) ppm; 31P NMR (200MHz, $CDCl_3$, 25°C): δ= 149.98, 150.50 ppm; FAB-MS: m/z=1028.5 (44, $[M+H]^+$), 821.5 (100), 303.1 (35, $[(MeO)_2Tr]^+$).

5.7 Synthesis of Precursor for m^2G and $m^2{}_2G$ Phosphoramidite

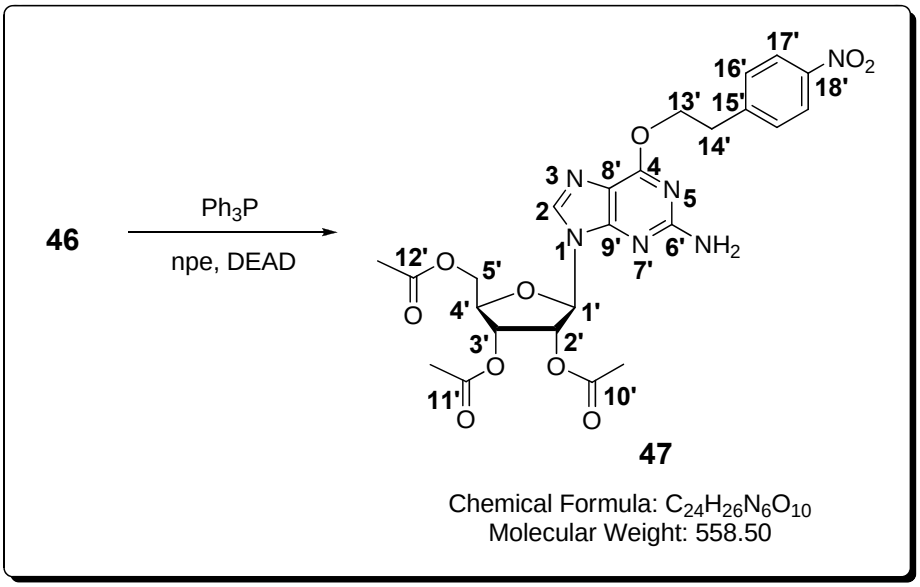
5.7.1 Synthesis of *2',3',5'-tri-O-acetylguanosine* (46)



A rb flask equipped with a stirring bar containing a mixture of guanosine **(40)** (14.2 g, 50 mmol, 1 equiv) acetic anhydride (30 mL), pyr (15 mL), and DMF (40 mL) was heated whilst stirring at 75 °C for 4 h. The resulting clear solution was filtered hot, cooled to rt and evaporated *in vacuo* to afford a heavy crystalline suspension. A portion of hot isopropanol (50 mL) was added and the mixture cooled to 4 °C and the suspension filtered and rewashed with isopropanol and dried under vacuum overnightto afford crystalline sample **46**, with a yield of (19.03 g, 46.6 mmol) 93 %.

TLC (silica gel, $CHCl_3$:MeOH=20:1): R_f = 0.24; ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ=2.03 (s, 3H-COCH3), 2.05 (s, 3H-COCH3), 2.11 (s, 3H-$COCH_3$), 4.27 (3H, m, H-C4' & 2H-C5'), 5.49 (1H, t, H-C3'), 5.79 (1H, t, H-C2'), 5.99 (1H, d, H-C1'), 6.53 (2H, s, H-NH_2), 7.93 (1H, s, H-C2'), 10.72 (1H, s,H-C5') ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ= 20.63 ($\underline{C}H_3$-CO), 20.82 ($\underline{C}H_3$-CO), 20.97 ($\underline{C}H_3$-CO), 63.51 (C5), 70.75 (C4), 72.49 (C3), 79.99 (C2), 84.85 (C1), 117.27 (C8'), 151.54 (C2'), 154.31 (C6' & 9'), 157.06 (C4'), 169.69 (C10'), 169.86 (C11'), 170.51(C12') ppm MS-FAB 410.13 $[M+H]^+$.

5.7.2 Synthesis of *2',3',5'-tri-O-acetyl-O^6-nitrophenylethylguanosine* (47)

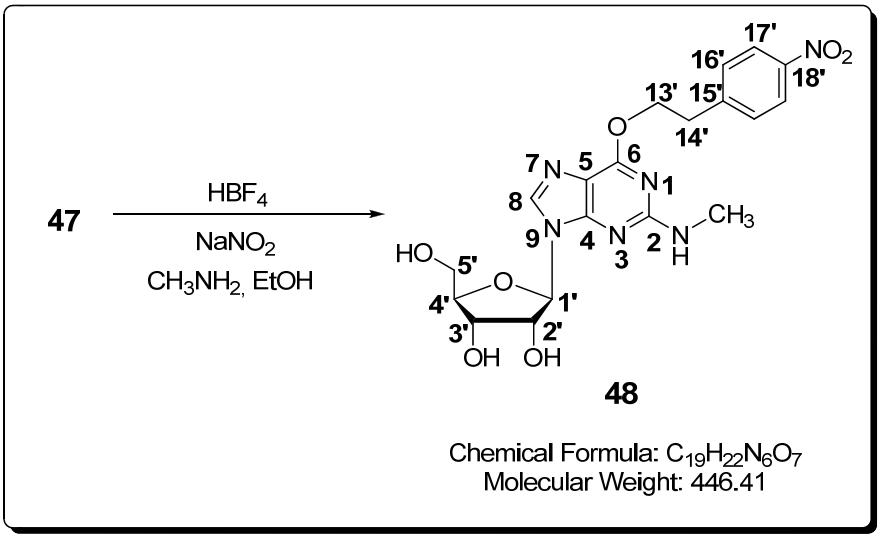


A suspension of ***2',3',5'-O-triacetylguanosine*** **(46)** (340 mg, 24.6 mmol, 1.0 equiv) in dry dioxane (150 mL) and triphenylphosphine (PPh_3) (440 mg, 37.5 mmol, 1.5 equiv) and 2-(4-nitrophenyl)ethanol (54.8 mg, 56 mmol, 2.0 equiv) was stirred at 80 °C for 45 min. DEAD (45 mg, 37.5 mmol, 1.5 equiv) was added during which a clear solution was formed. The mixture was stirred at 60 °C for 1 h after which it was cooled to rt and evaporated under *vacuo*. The residual oil was purified by fCC on silica gel with a gradient eluent system of Et_2O/DCM, 9:1 (200 mL), Et_2O/DCM, 4:1 (200 mL), Et_2O/DCM; 1:1 (200 mL), DCM (300 mL), DCM/MeOH; 49:1 (300 mL), and finally DCM/MeOH; 19:1. The product **47** was obtained as a semi-solid with a yield of 58 %.

TLC (silica gel, $CHCl_3$:MeOH=20:1): R_f = 0.28. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 2.08 (s, 3H-$COCH_3$), 2.10 (s, 3H-$COCH_3$), 2.12 (s, 3H-$COCH_3$), 3.26 (2H, t, H-C14'), 4.27 (3H, m, H-C4 & 2H-C5), 4.73 (2H, t, H-C13'), 5.75 (1H, t, H-C3), 5. 93 (1H, t, H-C2), 5.99 (1H, d, H-C1), 7.45 (2H, s, H-NH_2), 7.48 (2H, m, H-C16'), 7.66 (2H, m, H-C17'), 8.14 (1H, s, H-C2') ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25°C): δ = 20.63 ($\underline{C}H_3$-CO), 20.82 ($\underline{C}H_3$-CO), 20.97 ($\underline{C}H_3$-CO), 63.51 (C5), 70.75 (C4), 72.49 (C3), 79.99 (C2), 84.85 (C1), 117.27 (C8'), 151.54 (C2'), 154.31 (C6' & C9'), 157.06 (C4'), 169.69 (C10'), 169.86 (C11'), 170.51(C12') ppm, MS-FAB 559.63 $[M+H]^+$.

5.8 Chemical Synthesis of m^2G Phosphoramidite

5.8.1 Synthesis of *N^2-methyl-O^6-nitrophenylethylguanosine* (48)

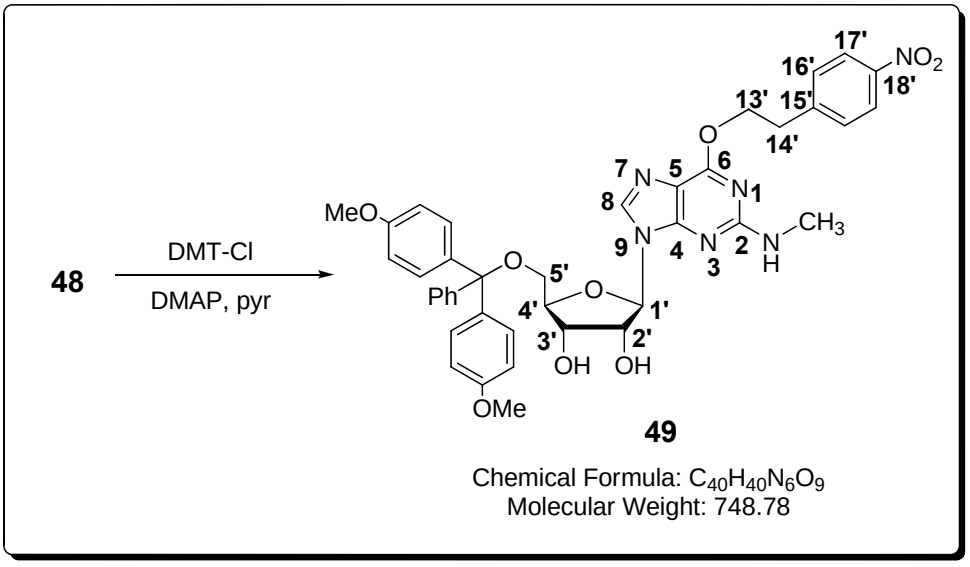


A suspension of ***2',3',5'-tri-O-acetyl-O^6-nitrophenylethylguanosine*** **47** (5.3 g, 9.4 mmol) in 40 mL of acetone was cooled to -20 °C and treated with 100 mL of 50 % HBF_4 (1.0 mol in water). Under vigorous stirring $NaNO_2$ (1.84 g, 23.5 mmol) in 22 mL of water were added dropwise. The reaction mixture was allowed to warm to room temperature, stirred for 4 h, and neutralized with 50 % NaOH. The reaction mixture was extracted two times with DCM, the organic layers were dried with Na_2SO_4 and evaporated to dryness. The crude material was dissolved in 8 M CH_3NH_2 in ethanol and stirred overnight. The reaction mixture was evaporated to dryness. Column chromatography (silica gel, DCM/MeOH; 15:1) afforded **48** (2.46 g, 5.51 mmol, 51 %).

TLC (silica gel, $CHCl_3$:MeOH=20:1): R_f = 0.24; ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ=2.82 (d, J=4.8Hz, 3H-N^2-CH3), 3.26 (t, J=6.8Hz, 2H-CH_2–C_6H_4–NO_2), 3.55 - 3.63 (2m, 2H–C5'), 3.90 (q, 1H–C4'), 4.14 (q, 1H–C3'), 4.57 (q, 1H–C2'), 4.71 (t, J=6.8Hz, 2H-O^6–CH_2), 4.84 (t, HO–C5'), 4.99 (d, J=4.9Hz, HO–C3'), 5.23 (d, J=6.1Hz, H-HO–C2'), 5.79 (d, J=5.9Hz, H–C1'), 6.75 (br q, NH), 7.61 (d, J=8.5Hz, 4-nitrophenyl H–C(2)/H–C(6)), 8.02 (s, 1H–C8), 8.16 (d, J=8.5Hz, 4-nitrophenyl H–C(3)/H–C(5)) ppm; ^{13}C NMR (75 MHz, d^6-DMSO, 25 °C): δ=28.65 (N^2–CH_3), 34.82 (CH_2–C_6H_4–NO_2), 62.05 (C(5')), 65.79 (O^6–CH_2), 70.96 (C(3')), 73.56 (C(2')), 85.74 (C(4')), 87.49 (C(1')), 123.78 (4-nitrophenyl C(3)/C(5)),

114.33, 130.63 (4-nitrophenyl C(2)/C(6)), 138.73, 138.79 (C(8)), 146.74, 147.08, 154.67, 159.76, 160.34 ppm, FAB-MS, 447.52 $[M+H]^+$.

5.8.2 Synthesis of *5'-O-DMT-N^2-methyl-O^6-nitrophenylethylguanosine* (49)

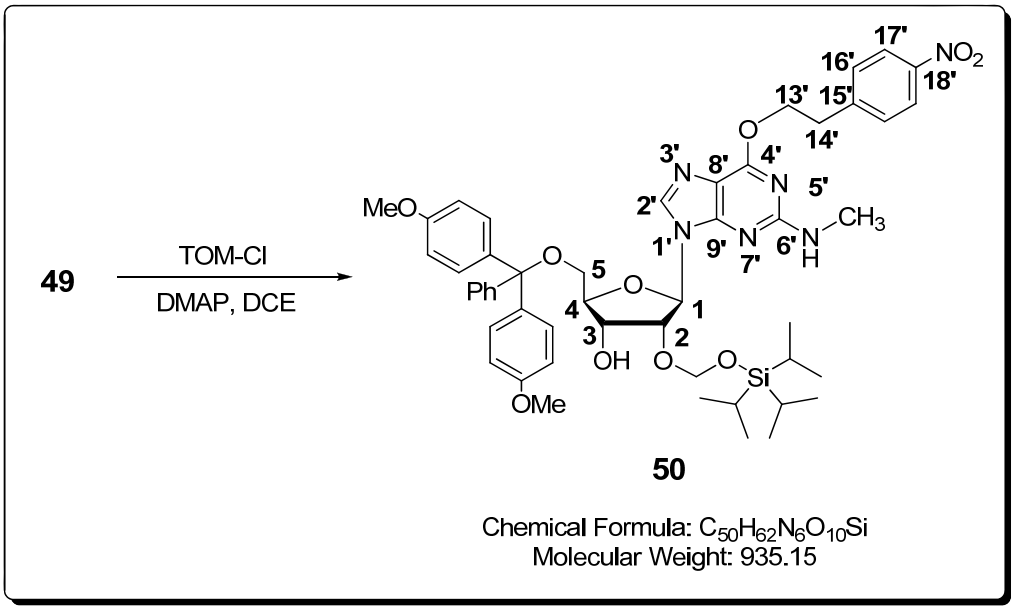


In a rb flask equipped with compound **(48)** (2.1 g, 4.72 mmol) and a magnetic stirring bar was coevaporated three times with anhydrous pyr and finally dissolved in 20 mL of pyr. Then, DMT-Cl (1.76 g, 4.85 mmol) was added portion-wise over a period of 4 h. DMAP (0.21 g, 1.67 mmol) was added and stirring was continued overnight. The reaction mixture was quenched with MeOH, evaporated to dryness, workup, and purified by fCC on silica gel with a gradient eluent system of DCM:MeOH; (50:1, 20:1) to yield **49** (2.45 g, 3.20 mmol) as a pale yellow foam (70 %).

TLC (silica gel, DCM:MeOH=10:1): R_f = 0.4; ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ = 2.73 (d, J=4.8Hz, N^2-CH_3), 3.22 (d, J=4.7Hz, 2H–C(5')), 3.25 (t, J=6.7Hz, 2H-CH_2–C_6H_4–NO_2), 3.71(s, 3H-OCH_3), 3.72 (s, 3H-OCH_3), 4.01 (q, 1H–C4'), 4.32 (q, 1H–C3'), 4.64 (q, 1H–C2'), 4.71 (t, J=6.7Hz, 2H-O^6–CH_2), 4.97 (d, J=5.8Hz, 1H-HO–C3'), 5.28 (d, J=5.0Hz, 1H-HO–C2'), 5.82 (d, J=4.6Hz, H–C1'), 6.65 (br q, NH), 6.81 (m, 4H, trityl-H), 7.17–7.27, 7.33–7.37 (m, 9H, trityl-H), 7.60 (d, J=8.7Hz, 4- nitrophenyl H–C(2)/H–C(6)), 7.90 (s, H–C(8)), 8.15 (d, J=8.7Hz, 4-nitrophenyl H–C(3)/H–C(5)) ppm; ^{13}C NMR (125 MHz, DMSO-d_6, 25 °C): δ = 28.54 (N^2-CH_3), 34.78 (CH_2–C_6H_4–NO_2), 55.45 (2 CH_3O), 64.46 (C(5')), 65.81 (O^6–CH_2), 70.98 (C(3')), 73.26 (C(2')), 83.46 (C(4')), 85.98, 88.25 (C(1')), 113.56 (trityl-C), 114.49, 123.73 (4-nitrophenyl C(3)/C(5)), 126.98, 128.05, 128.17 (trityl-C), 130.03, 130.08,

130.58 (trityl-C, 4-nitrophenyl C(2)/C(6)), 136.08, 138.71 (C(8)), 145.22, 146.83, 147.06, 158.52, 158.55, 159.80, 160.40 ppm, FAB-MS, 748.81 $[M+H]^+$, 303.01 $[(MeO)_2Tr]^+$).

5.8.3 Synthesis of *5'-O-DMT-2'-O-TOM-N^2-methyl-O^6-nitrophenylethylguanosine* (50)

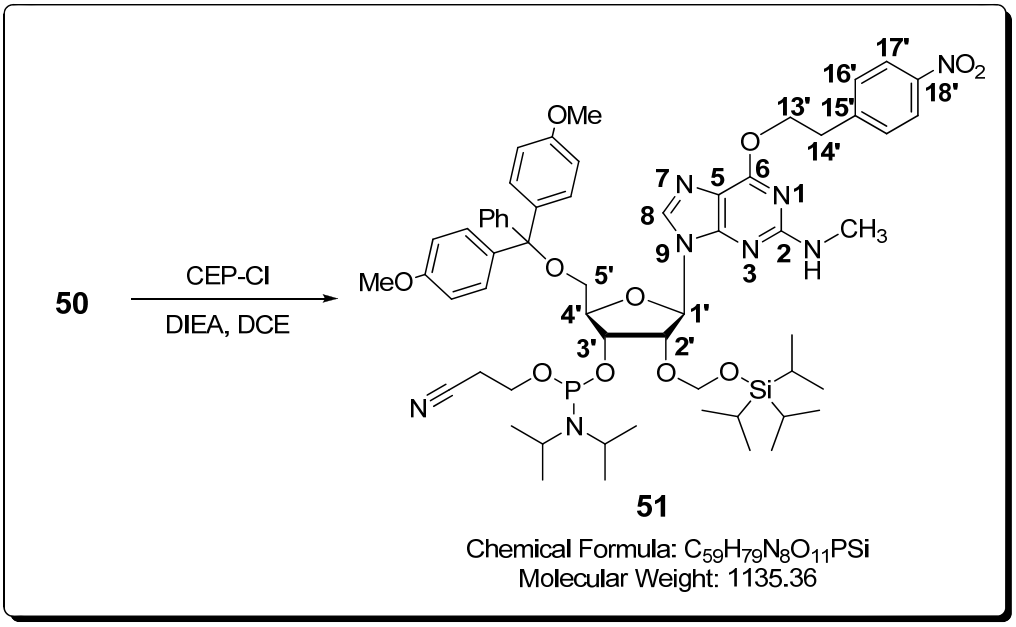


A stirred solution of of **(49)** (1.63 g, 1.76 mmol) and DIEA (2.26 g, 17.42 mmol) in 40 mL of anhydrous DCE, di-*tert*-butyltindichloride (1.60 g, 2.61mmol) was added. The mixture was heated to 70 °C for 15 min, allowed to cool to rt again and treated with TOM-Cl (0.53 g, 2.40 mmol). Stirring was continued for further 3 h, followed by addition of 0.5 mL of MeOH, evaporation to dryness and workup. Purified by fCC on silica gel with a gradient eluent system of Hex:EtOAc=3:1 to 1:1 afforded of **50** (0.70 g, 0.6 mmol) as white solid foamwith yield of 55 %.

TLC (silica gel, DCM:MeOH=10:1): R_f = 0.4; ^{1}H NMR (350 MHz, DMSO-d_6, 25 °C): δ= 2.73 (d, J=4.8Hz, 3H-N^2-CH_3), 3.22 (d, J=4.7Hz, 2H–C(5')), 3.25 (t, J=6.7Hz, 2H-CH_2–C_6H_4–NO_2), 3.71 s, 2H-OCH_3), 3.72 (s, 3H-OCH_3), 4.01 (q, 1H–C(4')), 4.32 (q, 1H–C(3')), 4.64 (q, 1H–C(2')), 4.71 (t, J=6.7Hz, 2H-O^6–CH_2), 4.97 (d, J=5.8Hz, 1H-HO–C(3')), 5.28 (d, J=5.0Hz, HO–C(2')), 5.82 (d, J=4.6Hz, 1H–C(1')), 6.65 (br q, NH), 6.81 (m, 4H, trityl-H), 7.17–7.27, 7.33–7.37 (m, 9H, trityl-H), 7.60 (d, J=8.7Hz, 4- nitrophenyl H–C(2)/H–C(6)), 7.90 (s, H–C(8)), 8.15 (d, J=8.7Hz, 4-nitrophenyl H–C(3)/H–C(5)) ppm; ^{13}C NMR (125MHz, DMSO-d_6, 25 °C): δ = 28.54 (N^2-CH_3), 34.78 (CH_2–C_6H_4–NO_2), 55.45 (2 CH_3O), 64.46 (C(5')), 65.81

(O^6–CH_2), 70.98 (C(3')), 73.26 (C(2')), 83.46 (C(4')), 85.98, 88.25 (C(1')), 113.56 (trityl-C), 114.49, 123.73 (4-nitrophenyl C(3)=C(5)), 126.98, 128.05, 128.17 (trityl-C), 130.03, 130.08, 130.58 (trityl-C, 4-nitrophenyl C(2)=C(6)), 136.08, 138.71 (C(8)), 145.22, 146.83, 147.06, 158.52, 158.55, 159.80, 160.40 ppm. FAB-MS, 936.27 $[M+H]^+$ 303.01 $[(MeO)_2Tr]^+$.

5.8.4 Synthesis of *5'-O-DMT-2'-O-TOM-3'-O-CEP-N^2-methyl-O^6-nitrophenylethylguanosine* (51)



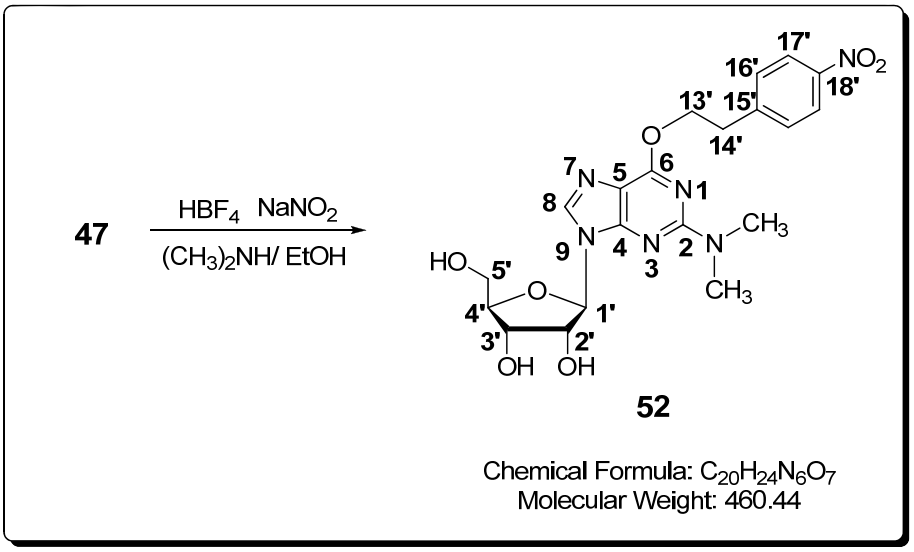
A stirred solution of of **(50)** (1.63 g, 1.76 mmol) in 40 mL of anhydrous DCE was added DIEA (2.26 g, 17.42 mmol). The mixture was stirred for 15 min at 0 °C, allowed to warm to rt again and was added CEP-Cl (0.53 g, 2.40 mmol). Stirring was continued for 3 h, followed by addition of 0.5 mL of MeOH, evaporation to dryness and workup. Purified by fCC on silica gel with a gradient eluent system of (silica gel, Hex:EtOAc=3:1 to 1:1) to afford **51** (0.70 g, 0.6 mmol) as white solid foam with a yield of 34 %.

TLC (silica gel, Hex:EtOAc=1:1): R_f = 0.4; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 0.98–1.10 (m, iPr_3Si), 2.86 (d, J=5.1Hz, 3H-N^2-CH_3), 2.98 (d, J=4.1Hz, 1H-HO–C(3')), 3.28 (t, J=6.9Hz, 2H-CH_2–C_6H_4–NO_2), 3.36 (dd, J=4.5, 10.5 Hz, 1H–C(5')), 3.46 (dd, J=3.9, 10.5Hz, 2H–C(5')), 3.78 (s, 3H-OCH_3), 3.79 (s, 3H-OCH_3), 4.22 (q, 1H–C(4')), 4.58 (q, 1H–C(3')), 4.67 (br s, NH), 4.73 (t, J=6.9Hz, O^6–CH_2), 4.95 (t, 1H–C(2')), 4.98, 5.13 (2d, J=4.8Hz, 2H-OCH_2O), 6.03 (d, J=5.4Hz, 1H–C(1')), 6.79 (m, 4H, trityl-H), 7.19–7.32, 7.42– 7.43 (m, 9H, trityl-H), 7.47 (d, J=8.7Hz, 4-nitrophenyl H–C(2)/H–C(6)), 7.71 (s, H–C(8)), 8.16 (d,

J=8.7Hz, 4-nitrophenyl H–C(3)/H–C(5)) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 11.85 ($(CH_3)_2CH$), 17.74 ($(CH_3)_2CH$), 28.59 (N^2-CH_3), 35.24 (CH_2–C_6H_4–NO_2), 55.16 (2 CH_3O), 63.55 (C(5')), 65.83 (O^6–CH_2), 70.99 (C(3')), 81.22 (C(2')), 83.74 (C(4')), 86.42, 86.70 (C(1')), 90.79 (OCH_2O), 113.13 (trityl-C), 115.33, 123.68 (4-nitrophenyl C(3)/C(5)), 126.81, 127.79, 128.20 (trityl- C), 129.88, 130.08 (trityl-C, 4-nitrophenyl C(2)/C(6)), 135.77, 135.83, 138.02 (C(8)), 144.64, 146.11, 146.84, 154.10, 158.52, 159.64, 160.51 ppm; MALDI-MS: m/z=1158.41 (5, $[M+Na]^+$), 303.1 (100, $[(MeO)_2Tr]^+$)

5.9 Chemical Synthesis of $m^2{}_2G$ Phosphoramidite

5.9.1 Synthesis of *(2R,3R,4S,5R)-2-(2-(dimethylamino)-6-(4-nitrophenethoxy)-9H-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol* (52)

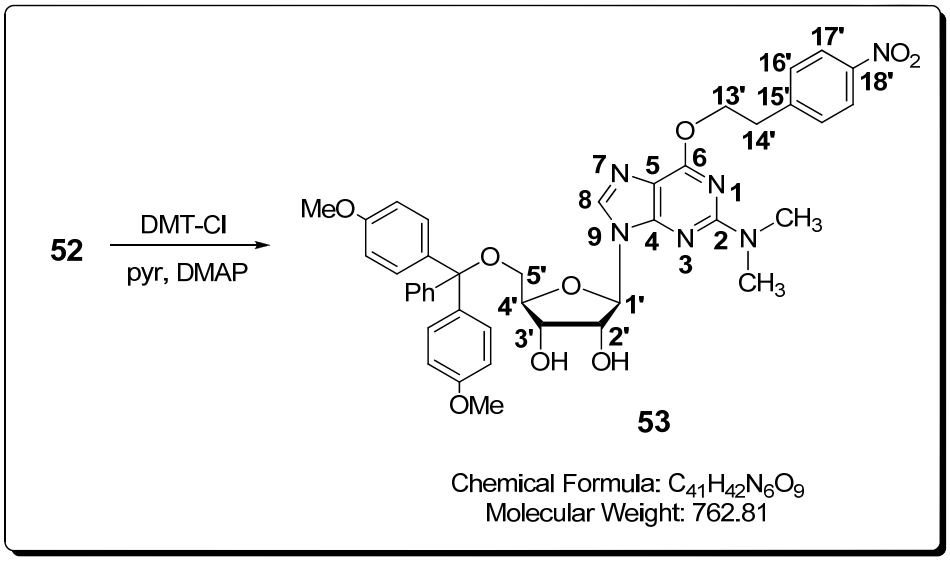


In rb flask containing a suspension of ***2',3',5'-tri-O-acetylguanosine*** **(47)** (3.0 g, 5.3 mmol) in 20 mL of acetone was cooled to -20 °C and treated with 50 mL of 50 % HBF_4 (0.55 mol). Under vigorous stirring 0.92 g of $NaNO_2$ (13.4 mmol) in 13 mL of water was added dropwise. The reaction mixture was allowed to warm to rt, stirred for 3 h, and neutralized with 50 % NaOH. The reaction mixture was extracted twice with DCM, the organic layers dried with Na_2SO_4 and evaporated to dryness. The crude material was dissolved in a stirred solution of 33 % $(CH_3)_2NH$ in 20 mL of ethanol. After 1 h, a solution of 40 % $(CH_3)_2NH$ in 20 mL of water was added and stirring was continued for another 3 h. The reaction mixture was evaporated to dryness and co-evaporated three times with MeOH/toluene/DCM and purified

by fCC on silica gel with a gradient eluent system of (DCM: MeOH=40:1, 15:1) to afford **52** **(**1.24 g, 2.7 mmol) with a yield of 50 %.

TLC (silica gel, $CHCl_3$:MeOH=10:1): R_f=0.5; ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ = 3.12 (s, N2-$(CH_3)_2$), 3.27 (t, J=6.7Hz, CH_2–C_6H_4–NO_2), 3.50, 3.61 (2m, H2–C(5')), 3.87 (q, H– C(4')), 4.14 (q, H–C(3')), 4.59 (q, H–C(2')), 4.73 (t, J=6.7Hz, O^6–CH_2), 4.90 (t, J=5.6Hz, HO– C(5')), 5.15 (d, J=4.9Hz, HO–C(3')), 5.35 (d, J=6.2Hz, HO–C(2'), 5.80 (d, J=5.9Hz, H–C(1')), 7.60 (d, J=8.7Hz, 4-nitrophenyl H–C(2)=H–C(6)), 8.08 (s, H–C(8)), 8.16 (d, J=8.7Hz, 4-nitrophenyl H–C(3)=H–C(5)) ppm; ^{13}C NMR (125 MHz, DMSO-d_6, 25 °C): δ=34.77 (CH_2–C_6H_4–NO_2), 37.53 (N^2-$(CH_3)_2$), 61.99 (C(5')), 65.81 (O^6–CH_2), 70.89 (C(3')), 73.41 (C(2')), 85.58 (C(4')), 87.40 (C(1')), 113.69, 123.85 (4-nitrophenyl C(3)/C(5)), 130.64 (4-nitrophenyl C(2)/C(6)), 139.29, 139.31 (C(8)), 146.68, 147.12, 154.71, 158.87, 159.86 ppm. FAB MS:461.12 $[M+H]^+$.

5.9.2 Synthesis of *(2R,3S,4R,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(2-(dimethylamino)-6-(4-nitrophenethoxy)-9H-purin-9-yl)tetrahydrofuran-3,4-diol* (53)

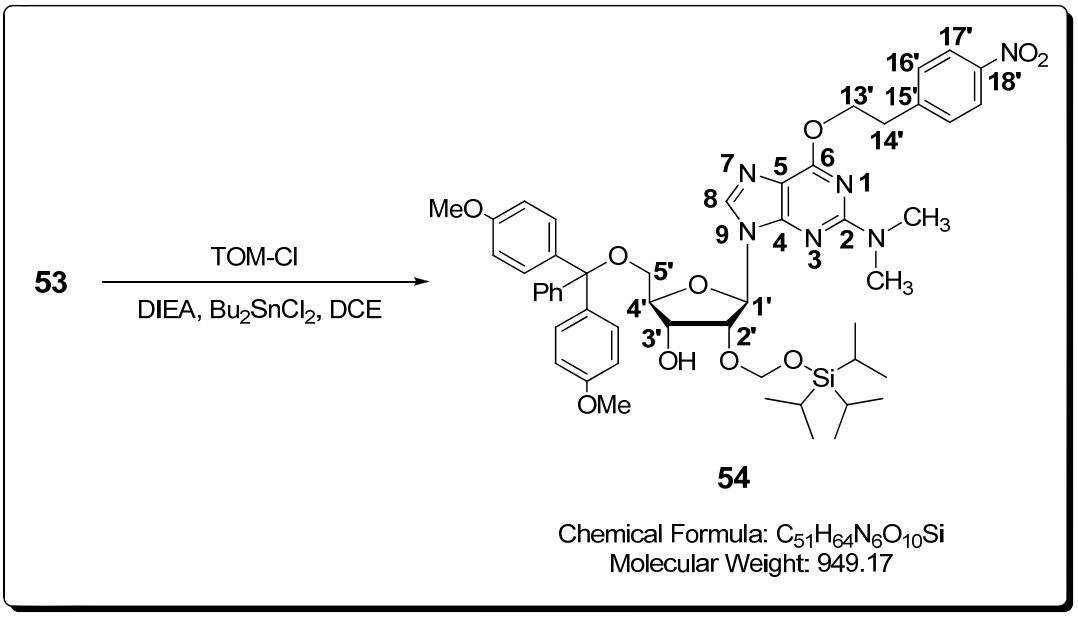


In rb flask containing compound **52** (0.5 g, 1.09 mmol) was co-evaporated three times with anhydrous pyr was dissolved in 4 mL of the same solvent. Then, 0.41 g of DMT-Cl (1.21 mmol) was added in three portions over a period of 1 h. DMAP (10 mg, 0.08 mmol) was added and stirring was continued overnight. Quenching by the addition of MeOH, evaporation

to dryness, workup, and purified by fCC on silica gel with a gradient eluent system of DCM: MeOH; (50:1, 35:1) yielded 690 mg of the product **53** as pale yellow foam (70 %).

TLC (silica gel, DCM:MeOH=20:1): R_f = 0.37; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ=3.04 (s, HO–C(3')), 3.18 (s, N^2-$(CH_3)_2$), 3.24 (dd, J=3.5, 10.4 Hz, 1H–C(5')), 3.31 (t, J=6.8Hz, CH_2–C_6H_4–NO_2), 3.41 (dd, J=3.8, 10.4 Hz, 2H–C(5')), 3.76 (s, 3H-OCH_3), 3.77 (s, 3H-OCH_3), 4.39 (d, 1H–C(3')), 4.44 (t, 1H–C(4')), 4.68 (t, 1H–C(2')), 4.78 (t, J=6.8Hz, 2H-O^6–CH_2), 5.83 (d, J=6.6Hz, 1H–C(1')), 6.73–6.79 (m, 4H, trityl-H), 7.16–7.22, 7.26–7.30 (m, 9H, trityl-H), 7.49 (d, J=8.9Hz, 4-nitrophenyl H–C(2)/H–C(6)), 7.84 (s, H–C(8)), 8.16 (d, J=8.9Hz, 4-nitrophenyl H–C(3)/H–C(5)) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 35.16 (CH_2–C_6H_4–NO_2), 37.78 (N^2-$(CH_3)_2$), 55.10 (2 CH_3O), 63.73 (C(5')), 65.89 (O^6–CH_2), 72.84 (C(3')), 76.21 (C(2')), 86.34 (C(4'), 86.38, 90.41 (C(1')), 113.06 (trityl-C), 114.10, 123.70 (4-nitrophenyl C(2)=C(6)), 126.77, 127.76, 127.97 (trityl-C), 129.82, 129.83, 129.89, 129.93 (trityl-C and 4-nitrophenyl C(3)/C(5)), 135.38, 135.55, 136.83 (C(8)), 144.27, 145.90, 146.81, 153.22, 158.45, 160.10 ppm; MALDI- MS: m/z=785.55 ($[M+Na]^+$), 303.1 (100, $[(MeO)_2Tr]^+$).

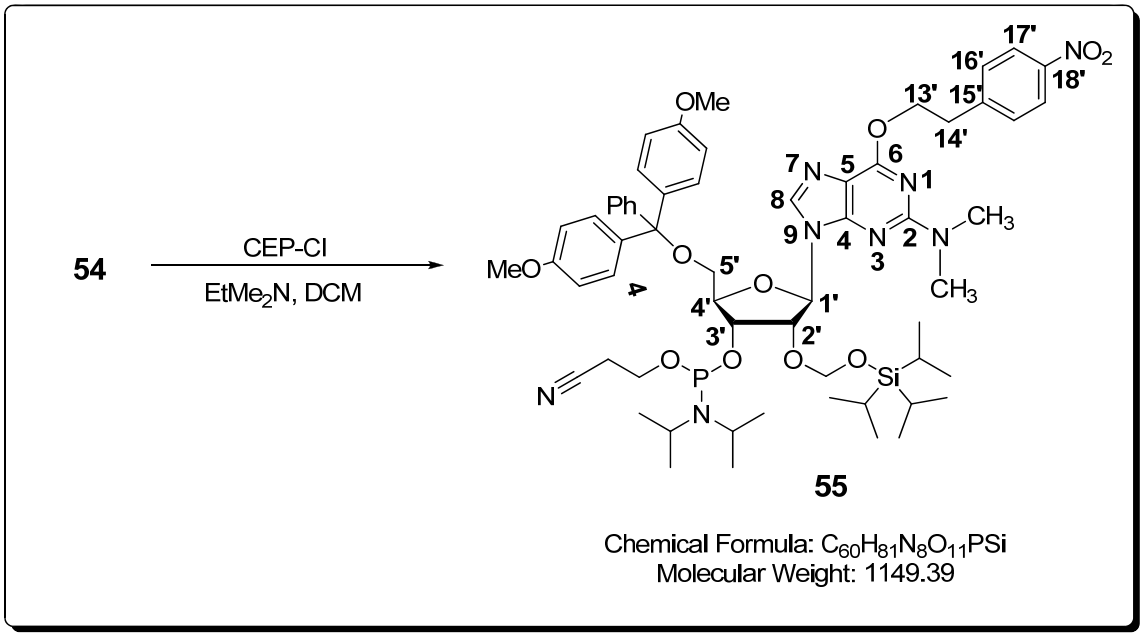
5.9.3 Synthesis of *(2R,3R,4R,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy) methyl)-5-(2-(dimethylamino)-6-(4-nitrophenethoxy)-9H-purin-9-yl)-4-((triisopropylsilyloxy) methoxy)tetrahydrofuran-3-ol* (54)



In rb flask containing a stirring solution of **53** (500 mg, 0.70 mmol) and DIEA (366 mg, 2.84 mmol) in 6 mL of anhydrous DCE, di-*tert*-butyltindichloride (258 mg, 0.85 mmol) was added. The mixture was heated to 70 °C for 15 min, allowed to cool to rt again and treated with [(triisopropylsilyl)oxy]methylchloride (173 mg, 0.8mmol). Stirring was continued for 4 h, quenched by addition of 0.5 mL of MeOH, evaporation to dryness and workup and purified by fCC on silica gel with a gradient eluent system of Hex:EtOAc = 3:1 to 1:1 to afford **54 (**222 mg, 0.23 mmol) as a white solid foam with a yield of 33 %. The regioselectivity of ***2'-O-*** alkylated over ***3'-O-*** alkylated product was approximately 5:3 as judged from the isolated products from the fCC.

TLC (silica gel, Hex: EtOAc=1:1): R_f = 0.4; ^{1}H NMR (300MHz, $CDCl_3$, 25 °C): δ = 1.02–1.12 (m, iPr_3Si), 2.99 (d, J=4.7, HO–C(3')), 3.10 (s, 3H-N^2-$(CH_3)_2$), 3.30 (t, J=6.9Hz, 2H-CH_2–C_6H_4–NO_2), 3.46 (d, H2–C(5')), 3.76 (s, 3H-OCH_3), 3.78 (s, 3H-OCH_3), 4.20 (q, 1H–C(4')), 4.57 (q, 1H–C(3')), 4.76 (t, J=6.9Hz, 2H-O^6–CH_2), 4.92 (t, 1H–C(2')), 4.99, 5.14 (2d, J=4.8Hz, 2H-OCH_2O), 6.05 (d, J=4.9Hz, 1H–C(1')), 6.78 (m, 4H, trityl-H), 7.19–7.33, 7.38–7.42 (m, 9H, trityl-H), 7.48 (d, J=8.4Hz, 4-nitrophenyl C(2)/C(6)), 7.71 (s, H–C(8)), 8.17 (d, J=8.4Hz, 4-nitrophenyl H–C(3)/H–C(5)) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 11.78 ($(CH_3)_2CH$), 17.66 ($(CH_3)_2CH$), 35.23 (CH_2–C_6H_4–NO_2), 37.33 (N^2-$(CH_3)_2$), 55.08 (2 CH_3O), 63.59 (C(5')), 65.57 (O^6–CH_2), 70.80 (C(3')), 81.27 (C(2')), 83.49 (C(4')), 86.34, 86.75 (C(1')), 90.72 (OCH_2O), 113.06 (trityl-C), 114.16, 123.65 (4-nitrophenyl C(3)/C(5)), 126.71, 127.71, 128.11 (trityl-C), 129.81, 129.99 (trityl-C, 4-nitrophenyl C(2)/C(6)), 135.70, 135.79, 137.74 (C(8)), 144.56, 146.11, 146.79, 154.13, 158.43, 159.04, 159.86 ppm; MALDI-MS: m/z=950.2 (45, $[M+H]^+$), 303.1 (100, $[(MeO)_2Tr]^+$).

5.9.4 Synthesis of *(2R,3R,4R,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy) methyl)-5-(2-(dimethylamino)-6-(4-nitrophenethoxy)-9H-purin-9-yl)-4 ((triisopropylsilyloxy)methoxy) tetrahydrofuran-3-yl 2-cyanoethyl diisopropylphosphoramidite* (55)

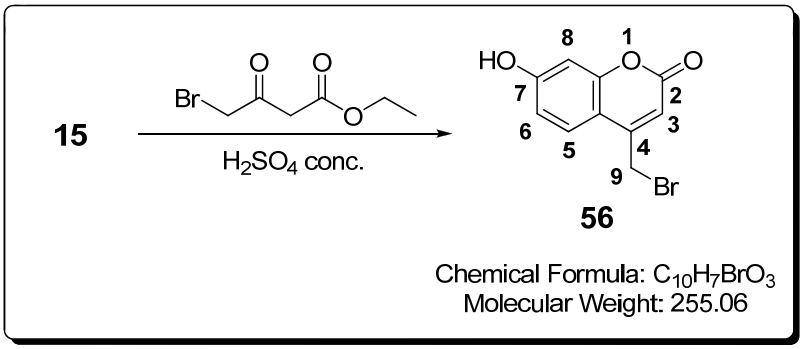


In rb flask containing a stirring solution of **54** (444 mg, 0.47 mmol) in 4 mL of anhydrous DCM was treated consecutively with ethyldimethylamine (343 mg, 4.7 mmol) and CEP-Cl (116 mg, 0.49 mmol). The mixture was stirred for 3 h, quenched with 0.15 mL of MeOH, and evaporated to dryness. Workup and purified by fCC on silica gel with a gradient eluent system of Hex:EtOAc= 5:1, 3:1 (+2 % Et_3N)) afforded the product **55 (**469 mg, 0.41 mmol) as a white solid foam with yield of 70 %. (1:1 mixture of diastereoisomers).

TLC (silica gel, Hex:EtOAc=1:1): R_f = 0.63; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 0.86–1.28 (m, 42H, iPr_3Si, 24H, $((CH_3)_2CH)_2N$), 2.32, 2.64 (2m, 4H, CH_2CN), 3.06, 3.07 (2s, 12H, $N^{2-}(CH_3)_2$), 3.29 (t, 4H, J=6.4Hz, CH_2–C_6H_4–NO_2), 3.35–3.47 (m, 4H, 2H–C(5')), 3.48–3.65 (m, 4H, $((CH_3)_2CH)_2N$, 2H, $POCH_2$), 3.77, 3.78 (2s, 12H, OCH_3), 3.84–3.96 (2m, 2H, $POCH_2$), 4.30, 4.33 (2q, 2H, H–C(4')), 4.62 (m, 2H, H–C(3')), 4.75 (t, J=6.4Hz, O^6–CH_2), 4.88–4.97 (q, 4H, J=6Hz, OCH_2O), 5.06 (m, 2H, H–C(2')), 6.05, 6.09 (2d, 2H, J=5.9Hz, H–C(1')), 6.75–6.79, 7.20–7.40 (m, 26H, trityl-H), 7.48 (2d, J=8Hz, 4-nitrophenyl H–C(2)/H–C(6)), 7.70 (s, 2H, H–C(8)), 8.16 (d, J=8Hz, 4-nitrophenyl H–C(3)/H–C(5)) ppm; ^{31}P NMR (f00MHz, $CDCl_3$, 25°C): δ=150.22, 150.59 ppm; MALDI-MS: m/z= 1171.53 (5, $[M+Na]^+$), 303.12 (100, $[(MeO)_2Tr]^+$).

5.10 Chemical Syntheses of Coumarins and Related Derivatives

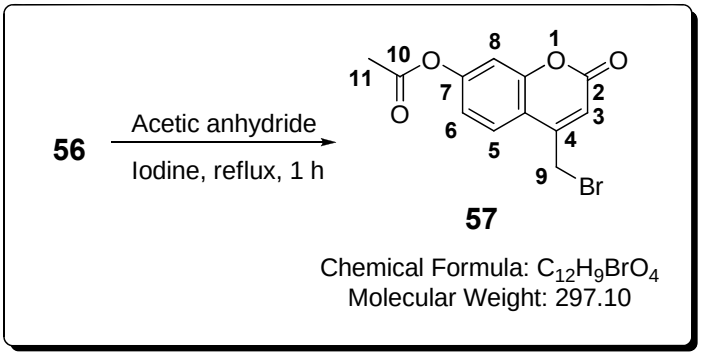
5.10.1 Synthesis of *4-(bromomethyl)-7-hydroxy-2H-chromen-2-one* (56)



In a rb flask equipped with a stirrer was added 1,3-dihydroxybenzene **15** (2 g, 18.2 mmol, 1 equiv) and ethyl 4-bromo-3-oxobutanoate (3.8 g, 18.2 mmol, 1.0 equiv) and the mixture was homogenized by carefully mixing after which the mixture was cooled to 0 °C and then sulfuric acid 70 % (1 mL) was added in drops. The reaction mixture was stirred until the disappearance of the purple coloration. Cooled water and ice (0 °C) (20 mL) was added and the resulting granular solid was carefully triturated into fine particles and filtered afterwards, followed by washing with cold water. The solid was dried under *vacuo* to give a final off white product **56** (3.7 g, 14.50 mmol) 79 %.

TLC (silica gel, Hex:EtOAc=10:1): R_f=0.24, ^{1}H NMR (300 MHz, DMSO-*d6*, 25 °C): δ=, 4.61 (s, 2H, -CH_2-Br, H9),6.45 (s, 1H, Ar H3), 7.42 (m, 1H, Ar H5), 7.56 (s, 1H, Ar H8), 7.75 (m, 1H, Ar H6), 8.05 (s br, 1H, Ar-OH),^{13}C NMR (75 MHz, DMSO-d_6, 25 °C): δ= 28.56, 105.56 (C(4)), 112.66, 114.44 (C(6)), 115.50, 127.27 (C(5)), 143.87 (C(7)), 153.99 (C(3)), 160.61 (C(2)) ppm. FAB-MS: 253.2, 255.2$[M+H]^+$

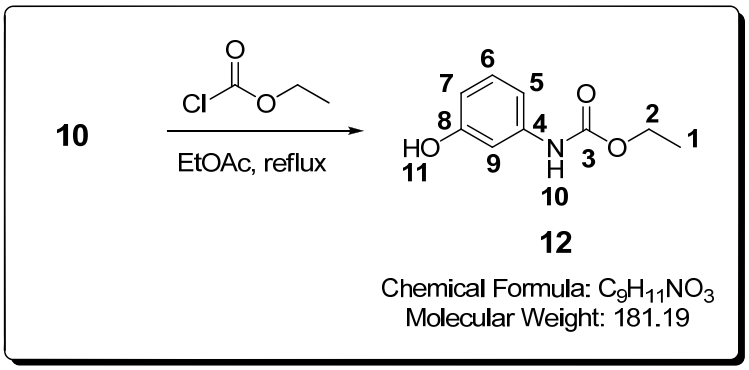
5.10.2 Synthesis of *4-(bromomethyl)-2-oxo-2H-chromen-7-yl acetate* (57)



In an rb flask containing ***4-(bromomethyl)-7-hydroxy-2H-chromen-2-one*** **56** (3 g, 11.7 mmol) was added acetic anhydride (5 mL). The reaction mixture was heated to 40 °C and iodine (20 mg; as catalyst) was added and heating was continued for further 1 h. The reaction was usually finished after 1 h. EtOAc (5 mL) was added and cooled to 4 °C overnight for precipitation. The precipitate was filtered and washed several times with cold EtOAc (50 mL) and dried under vacuum. The yield of the product **57** (2.99 g, 10.06 mmol) was 86 %.

TLC (silica gel, Hex:EtOAc=10:1): R_f = 0.34, ^{1}H NMR (300 MHz, DMSO-d$_6$, 25 °C): δ= 2.32 t, 3H-H11), 4.62 (s, 2H, -CH$_2$-Br, H9), 6.45 (s, 1H, Ar H3), 7.42 (m, 1H, Ar H5), 7.58 (s, 1H, Ar H8), 7.65 (m, 1H, Ar H6), 8.05 (br, 1H, Ar-OH),^{13}C NMR (75 MHz, DMSO-d$_6$, 25 °C): δ= 22.32 (C(11)), 28.54, 105.54 (C(4)), 112.67, 114.44 (C(6)), 115.50, 127.27 (C(5)), 144.87 (C(7)), 154.98 (C(3)), 160.61 (C(2)), 169.61 (C(10)) ppm. FAB-MS: 295.1, 297.1[M+H]$^+$

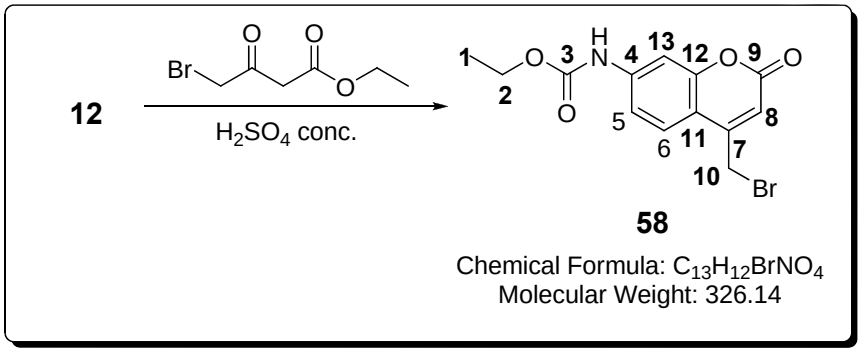
5.10.3 Synthesis of *ethyl 3-hydroxyphenylcarbamate* (12)



This reaction was performed based on the reported protocol from Maly *et al.* [100]In an rb flask containing ***3-aminophenol*** **(10)** (15 g, 137.4 mmol, 1.0 equiv) was added EtOAc (50 mL) and the mixture was refluxed for 30 min. To the refluxing mixture was added ethyl chloroformate (9 mL, 94.1 mmol, 0.7 equiv) in drops over 30 min. The reaction mixture was further refluxed for 1 h. The mixture was filtered whilst hot and the hot filtrate cooled to rt and then evaporated to dryness. To the off-white residue was added hot isopropanol and the dissolved mixture kept at 4 °C overnight after which the precipitate **12** was filtered and dried under vacuum with total yield of 95.6 % (23.4 g, 129 mmol).

TLC (silica gel, DCM:MeOH=40:1): R_f=0.47, ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ= 1.20 (t, J = 9.3 Hz, 3H (CH_3)), 4.07 (q, J = 9.3 Hz, 2H (CH_2-)), 6.38 (m, 1H, ar H6), 6.85 (m, 1H, ar H7), 6.92-7.08 (m, 2H, ar-H5 and ar-H9), 9.43 (s, 1H, NH, H10), ^{13}C NMR (300 MHz, $CDCl_3$, 25°C): δ = 15.79 (C(1)), 60.66 (C(2)), 106.61 (C(9)), 109.52 (C(7)), 110.54 (C(5)), 130.82 (C(6)), 140.95 (C(4)), 154.09 (C(3)), 158.35 (C(8)) ppm. FAB-MS: 182.09 $[M+H]^+$

5.10.4 Synthesis of *ethyl 4-(bromomethyl)-2-oxo-2H-chromen-7-ylcarbamate* (58)

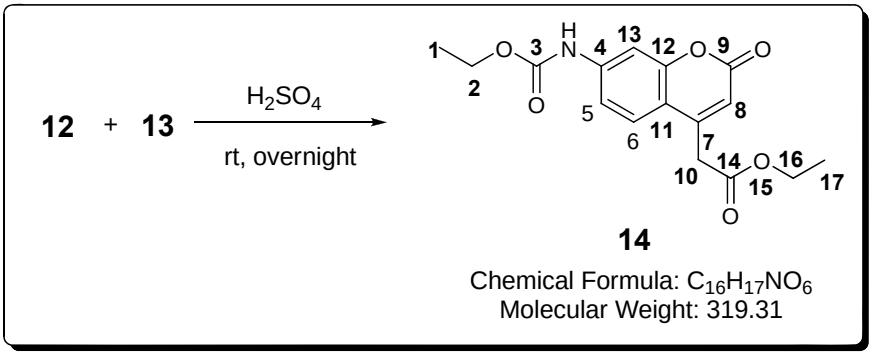


Ethyl 3-hydroxyphenylcarbamate **(12)** (3.3 g,18.2 mmol, 1 equiv), ethyl 4-bromo-3-oxobutanoate (3.8 g, 18.2 mmol, 1.0 equiv) in a rb flask was homogenized by carefully mixing after which the mixture was cooled to 0 °C and then pre-cooled sulfuric acid 70 % (5 mL) at 0 °C was added portion-wise. The reaction mixture was stirred at 0 °C for 4 h and was allowed to slowly warm up to rt overnight. The reaction mixture was diluted with water (10 mL) and extracted with EtOAc (4 times). The product **58** (5.2 g, 16.02 mmol) was obtained after drying under vacuum overnight with 88 % yield.

TLC (silica gel, DCM:MeOH=40:1): R_f= 0.47, ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ= 1.25 (t, 3H, -CH_3 H1), 4.15 (q, 2H, -CH_2- H2), 4.81 (s, 2H, -CH_2-Br, H10),6.54 (s, 1H, Ar H8), 7.42 (m, 1H, Ar H6), 7.56 (s, 1H, Ar H13), 7.75 (m, 1H, Ar H5), 10.17 ((s, 1H, NH); ^{13}C

NMR (300 MHz, DMSO-d_6, 25 °C): δ=15.78 (C(1)), 28.56, 61.45 (C(2)), 105.56 (C(7)), 112.66, 114.44 (C(5)), 115.50, 127.27 (C(6)), 143.87 (C(4)), 151.81 (C(3)), 153.99, 155.11, 161.62 (C(8)) ppm. FAB-MS: 326.1, 328.1 $[M+H]^+$

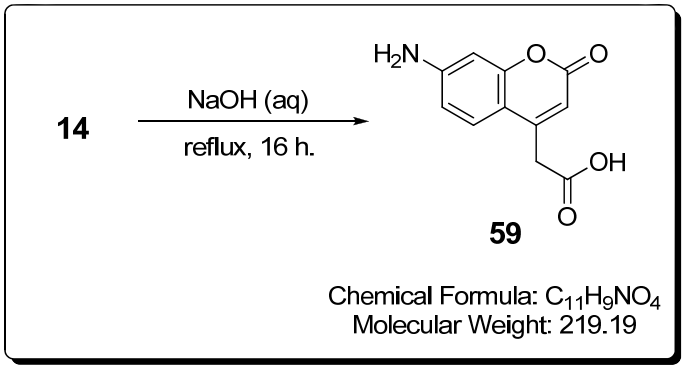
5.10.5 Synthesis of *ethyl 2-(7-(ethoxycarbonylamino)-2-oxo-2H-chromen-4-yl)acetate* (14)



In a rb flask equipped with a stirrer was placed ***ethyl 3-hydroxyphenylcarbamate*** **(12)** (1.1 g, 6.1 mmol, 1.0 equiv) and diethyl-1,3-acetone dicarboxylate (1.23 g, 6.1 mmol, 1.0 equiv) and the mixture was homogenized. Concentrated sulfuric acid (H_2SO_4) (3 drops) was added and the stirring was continued overnight. TLC shows no starting material. NMR reveals product in > 90 % yield. This was used in the next reaction without further purification.

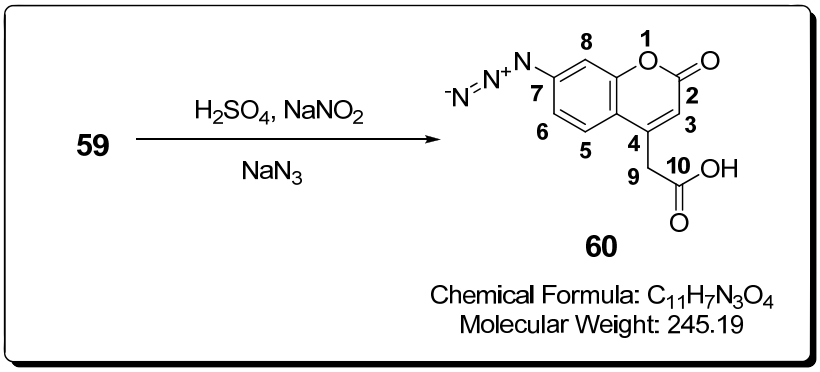
TLC (silica gel, DCM:MeOH=25:1): R_f= 0.38 ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ= 1.25 (t, 3H, -CH_3-H1), 1.27 (t, 3H, -CH_3-H17),3.21 (s, 2H, -CH_2-, H10), 4.14-4.17 (m, 4H-H2 & H16), 6.54 (s, 1H, Ar H8), 7.42 (m, 1H, Ar H6), 7.56 (s, 1H, Ar H13), 7.75 (m, 1H, Ar H5), 10.17 ((s, 1H, NH); ^{13}C NMR (300 MHz, DMSO-d_6, 25 °C): δ =15.62, 15.78 (C(1)), 35.56, 61.45 (C(2)), 61.65 (C(16)), 105.54 (C(7)), 112.65, 114.44 (C(5)), 115.50, 127.27 (C(6)), 143.87 (C(4)), 151.81 (C(3)), 153.99(C(8)), 160.61 (C(9)), 170.61 (C(14)) ppm. FAB-MS: 319.3, 320.3 $[M+H]^+$

5.10.6 Synthesis of *2-(7-amino-2-oxo-2H-chromen-4-yl)acetic acid* (59)



In a rb flask equipped with a stirrer was placed ***ethyl 2-(7-(ethoxycarbonylamino)-2-oxo-2H-chromen-4-yl)acetate*** **(14)** (2.6 g, 8.1 mmol, 1.0 equiv) and aqueous sodium hydroxide (25 mL, 4 M) and the mixture was refluxed overnight; approximately 16 h. After cooling to rt, the pH was adjusted to 2.0 by dropwise addition of concentrated H_2SO_4. The resultant yellow fluffy precipitate was collected by filtration and dried under vacuum. The filtrate was cooled to 4 °C overnight and the resulting precipitate was refiltered. The total yield after purification was 72 % (1.28 g, 5.83 mmol). This was used in the next reaction without further purification. TLC (silica gel, Hex:EtOAc=10:1): R_f = 0.12, ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ= 3.20 (s, 2H, -CH_2-COOH, H9), 6.50 (s, 1H, Ar H3), 7.52 (m, 1H, Ar H5), 7.25 (s, 1H, Ar H8), 7.53 (m, 1H, Ar H6), ^{13}C NMR (75 MHz, DMSO-d_6, 25 °C): δ= 28.56, 105.56 (C(4)), 101.66(C(8)), 114.44 (C(6)), 115.50, 128.27 (C(5)), 154.87 (C(7)), 153.99 (C(3)), 155.21, 160.61 (C(2)), 172.01 (C(10)) ppm. FAB-MS: 242.2 $[M+Na]^+$

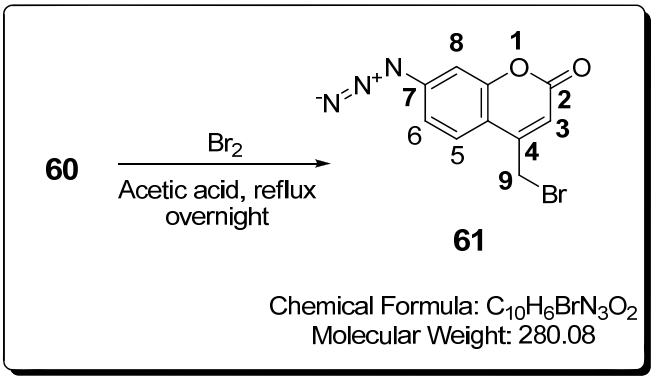
5.10.7 Synthesis of *2-(7-azido-2-oxo-2H-chromen-4-yl)acetic acid* (60)



In a rb flask equipped with a stirrer was placed ***2-(7-amino-2-oxo-2H-chromen-4-yl)acetic acid* 59** (650 mg, 2.97 mmol, 1.0 equiv), concentrated H_2SO_4 (20 mL), ice slurry (80 g), and the temperature was maintained at 0 °C. Sodium nitrite ($NaNO_2$) (308 mg, 4.45 mmol, 1.5 equiv) was added portion-wise as a solution in iced-water (20 mL). Stirring was continued for 10 min after which sodium azide (1.2 g, 17.8 mmol, 6.0 equiv) was added in one-portion as a solution in icold water (20 mL). The reaction mixture was stirred overnight after which it was extracted with EtOAc (5x100 mL). The organic layer was washed with brine and then dried with anhydrous sodium sulfate. After filtration, the organic phase was concentrated under vacuum. The product **60** was pure enough and so was used in the next step without further purification. The part of the crude was purified by fCC and was used for analysis (yield was estimated as 86 % by proportion (625 mg, 2.55 mmol)).

TLC (silica gel, Hex:EtOAc=10:1): R_f = 0.18, ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ= 3.18 (s, 2H, -CH_2-CO_2H, H9), 6.51 (s, 1H, Ar H3), 7.42 (m, 1H, Ar H5), 7.55 (s, 1H, Ar H8), 7.73 (m, 1H, Ar H6), ^{13}C NMR (75 MHz, DMSO-d_6, 25 °C): δ= 28.56, 105.56 (C(4)), 112.66, 114.44 (C(6)), 115.50, 127.27 (C(5)), 143.87 (C(7)), 151.23, 154.00 (C(3)), 160.60 (C(2)), 172.01 (C(10)) ppm. FAB-MS: 245.2 $[M+H]^+$

5.10.8 Synthesis of *7-azido-4-(bromomethyl)-2H-chromen-2-one* (61)

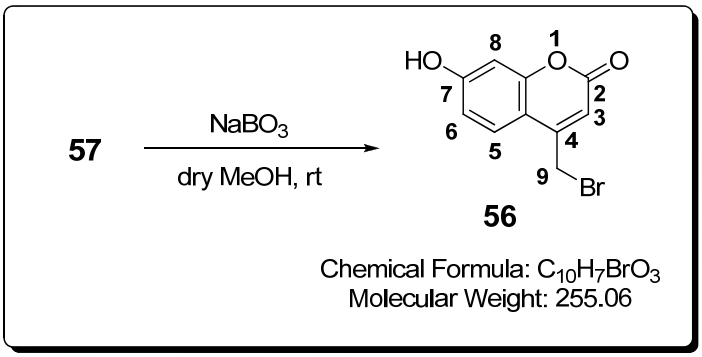


In a rb flask equipped with a stirrer was placed ***2-(7-azido-2-oxo-2H-chromen-4-yl)acetic acid* (60)** (350 mg, 1.43 mmol, 1.0 equiv) and AcOH (50 mL) and stirred for 10 min. Bromine (230 mg, 1.42 mmol, 1.0 equiv) was added as a solution in acetic acid (10 mL) and the reaction mixture was refluxed for 1 h, TLC reveals large amount of starting material. Additionally, Br_2 (230 mg, 1.42 mmol, 1.0 equiv) was added and the refluxing was continued for further 1 h after which TCL shows still starting material. Br_2 (230 mg, 1.42 mmol, 1.0

equiv) was then added again and the mixture refluxed overnight. The mixture was concentrated under reduced pressure and with the aid of a short fCC, the product was eluted with EtOAc. The total yield of the product formed **61** after purification was (330 mg, 1.18 mmol) 83 %.

TLC (silica gel, Hex:EtOAc=10:1): R_f = 0.28, ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ= 4.81 (s, 2H, -CH_2-Br, H9),6.55 (s, 1H, Ar H3), 7.42 (m, 1H, Ar H5), 7.56 (s, 1H, Ar H8), 7.75 (m, 1H, Ar H6), ^{13}C NMR (75 MHz, DMSO-d_6, 25 °C): δ= 28.56, 105.56 (C(4)), 112.66, 114.44 (C(6)), 115.50, 127.27 (C(5)), 143.87 (C(7)), 153.99 (C(3)), 160.61 (C(2)) ppm. FAB-MS: 278.1, 280.1 $[M+H]^+$

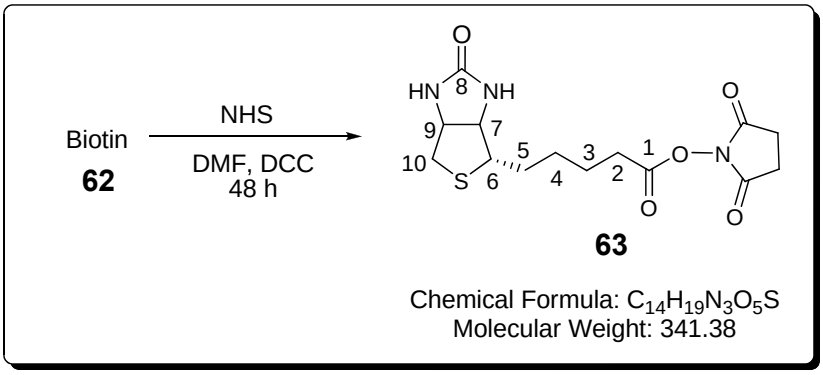
5.10.9 Synthesis of *4-(bromomethyl)-7-hydroxy-2H-chromen-2-one* (56)



In an rb flask equipped with a stirrer, were added 7-acetyloxy-4-bromomethyl-coumarin **(57)** (1 g, 3.37 mmol, 1.0 equiv), and sodium perborate (403 mg, 4.04 mmol, 1.2 equiv). MeOH (17 mL) was added and stirred at rt. The reaction was monitored by TLC and was usually finished after 2.5 h. Filtration to remove sodium perborate, evaporation of solvent and then drying under vacuum to yield a yellow crystalline product **56** which was used for further reaction without any purification.

TLC (silica gel, DCM:MeOH=25:1): R_f=0.37 FAB MS (3-NBA): $[M+H]^+$ calculated 253.96, found 254.0; $[M+H]^+$ calculated 254.97, found 255.0.

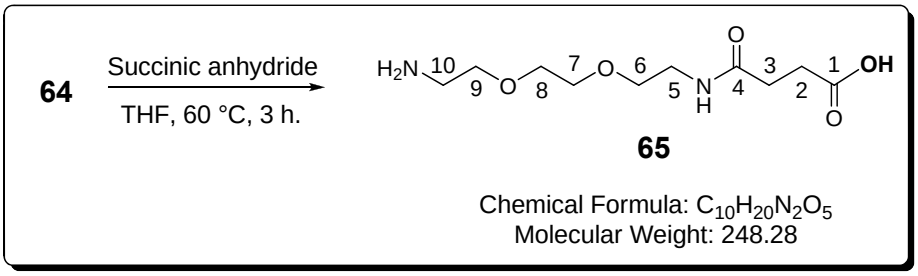
5.10.10 Synthesis of *N-(2,5-dioxopyrrolidin-1-yloxy)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide* (28)



In a rb flask equipped with a stirrer was placed ***biotin(27)*** (5 g, 20.5 mmol, 3.0 equiv) and anhydrous DMF (20 mL) and ***N-hydroxysuccinimide*** (3.1 g, 26.6 mmol, 1.3 equiv) The mixture was stiired for 15 min after which ***dicyclohexylcarbodiimid***e (DCC) (5.5 g, 26.6 mmol, 1.3 equiv) was added as a solution in DMF (20 mL). The mixture was stirred for additional 48 h after which TLC shows starting biotin. The mixture was stirred for further 14 h after which it was filtered to remove the DCU. Water (120 mL) was added and the organic layer extracted with DCM /MeOH (9:1) (3 x 250 mL). The organic phase was dried with Na_2SO_4 and evaporated to dryness under vacuum. The crude precipitate was recrystallized using isopropanol 50 mL) to afford the final product in 92 % yield.

TLC (silica gel, DCM:MeOH=40:1): R_f = 0.36 ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ = 6.42, 6.36 (2 s, 2H, 2x NHCO), 4.31 (dd, 1H, H^7), 4.29 (m, 1H, H^9), 3.11 (m, 1H, H^6), 2.85 (dd, 1H, H^{10}), 2.81 (s, 4H, 2x CH_2 in NHS), 2.67 (t, 2H, 2x H^2), 2.58 (d, 1H, $H^{9'}$), 1.35-1.70 (m, 6H, 2x H^3, 2x H^4, 2x H^5); ^{13}C-NMR (DMSO-d_6): δ = 170.2 (2x (CO)NH in NHS), 168.8 (C^1), 162.6 (C^8), 60.9 (C^9), 59.1 (C^7), 55.1 (C^6), 39.8 (C^{10}), 29.9 (C^2), 27.7, 27.5, 25.4, 24.2 (C^3, C^4, C^5, 2x CH_2 in NHS). FAB MS (3-NBA): $[M+H]^+$ calculated 342.1, found 342.0.

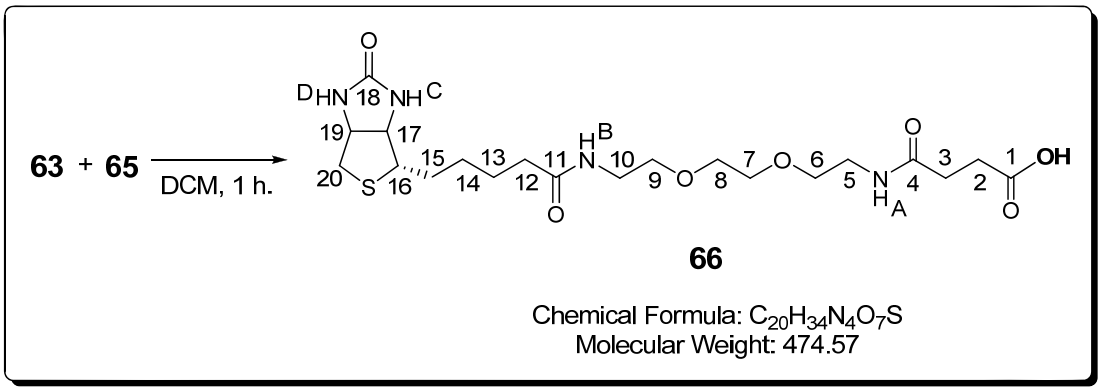
5.10.11 *Synthesis of 4-(2-(2-(2-aminoethoxy)ethoxy)ethylamino)-4-oxobutanoic acid* (26)



In a rb flask equipped with a stirrer was placed ***2,2'-(ethane-1,2-diylbis(oxy))diethanamine* (64)** (27 g, 180 mmol, 3.0 equiv) and anhydrous THF (50 mL) and the mixture heated up to 60 °C. Succinic anhydride (6.0 g, 60 mmol, 1.0 equiv) was added dropwise as a solution in THF (65 mL) using a dropping funnel over a period of 1 h. The mixture was then allowed to cool to rt whilst stirring and stirring was continued for additional 2 h after which it was cooled to 4 °C and maintained at this temperature overnight. The precipitate was filtered and dried under reduced pressure giving a sticky semi-solid. The total yield was quantitative (15 g, 60 mmol). NMR reveals product in acceptable purity. This was used in the next reaction without further purification.

TLC (silica gel, DCM:MeOH=40:1): R_f = 0.27 ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ = 8.21 (t, 1H, N<u>H</u>CO), 3.60-3.49 (m, 6H, 2x H^6, 2x H^7, 2x H^8), 3.40 (t, 2H, 2x H^9), 3.18 (dd, 2H, 2x H^5), 2.87 (t, 2H, 2x H^{10}), 2.25-2.23 (m, 4H, 2x H^2, 2x H^3); ^{13}C-NMR (d_6-DMSO): δ = 176.1 (C^1), 172.6 (C^4), 69.7, 69.5, 69.2 (C^6, C^7, C^8), 67.8 (C^9), 38.8, 38.5 (C^5, C^{10}), 32.5, 32.1 (C^2, C^3). FAB MS (3-NBA): $[M+H]^+$ calculated 249.1, found 249.2.

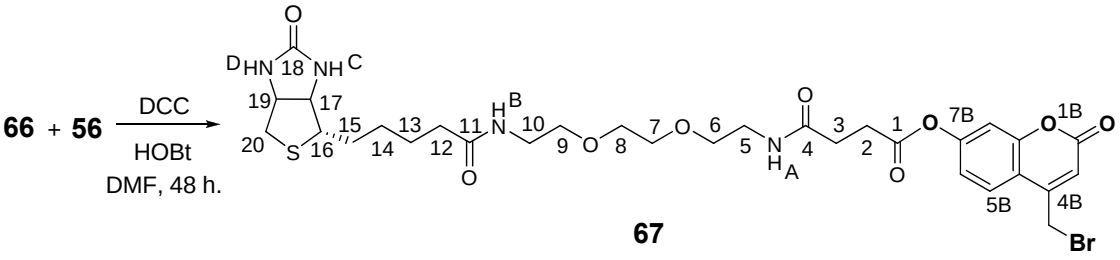
5.10.12 Synthesis of *N-(2-(2-(2-(4,4-dihydroxybutanamido)ethoxy)ethoxy) ethyl -5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide* (66)



In a rb flask equipped with a stirrer was placed ***4-(2-(2-(2-aminoethoxy)ethoxy)ethylamino)-4-oxobutanoic acid*** **(65)** (2 g, 8.1 mmol, 1.1 equiv) as a solution in methanol (20 mL) and ***N-(2,5-dioxopyrrolidin-1-yloxy)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide*** **63** (2.5 g, 7.32 mmol, 1.0 equiv) in DCM (80 mL) as a suspension. The mixture was stirred until a clear solution was obtained. The reaction was usually finished after 1 h as shown by TLC. Evaporation of the solvent under reduced pressure and the subsequent purification by fCC; (silica gel, (DCM, MeOH; 8:1 to 2:1)) gave the product **65**(2.9 g, 6.1 mmol) with a yield of 65 %.

TLC (silica gel, DCM:MeOH=40:1): R_f=0.26 ^{1}H NMR (300 MHz, DMSO-*d6*, 25°C): δ = 7.90, 7.83 (2 t, 2H, H^A, H^B), 6.42, 6.36 (2 s, 2H, H^C, H^D), 4.30 (dd, 1H, H^{17}), 4.12 (m, 1H, H^{19}), 3.50 (s, 4H, 2x H^7, 2x H^8), 3.39 (t, 4H, 2x H^6, 2x H^9), 3.18 (dd, 4H, 2x H^5, 2x H^{10}), 3.09 (m, 1H, H^{16}), 2.81 (dd, 1H, H^{20}), 2.57 (d, 1H, $H^{20'}$), 2.35 (m, 4H, 2x H^2, 2x H^3), 2.06 (t, 2H, 2x H^{12}), 1.70-1.20 (m, 6H, 2x H^{13}, 2x H^{14}, 2x H^{15}); ^{13}C-NMR δ = (d_6-DMSO): 173.8 (C^1), 172.1 (C^4), 171.0 (C^{11}), 162.7 (C^{18}), 69.5, 69.0 (C^6, C^7, C^8, C^9), 61.0 (C^{19}), 59.1 (C^{17}), 55.3 (C^{16}), 38.5, 38.4 (C^5, C^{10}, C^{20}), 35.0 (C^{12}), 29.9, 29.1, 28.1, 28.0, 25.2 (C^2, C^3, C^{13}, C^{14}, C^{15}). MALDI-TOF MS (DHB): $[M+H]^+$ calculated 475.2, found 475.4; $[M+Na]^+$ calculated 497.2, found 497.4.

5.10.13 Synthesis of *the compound* (67)

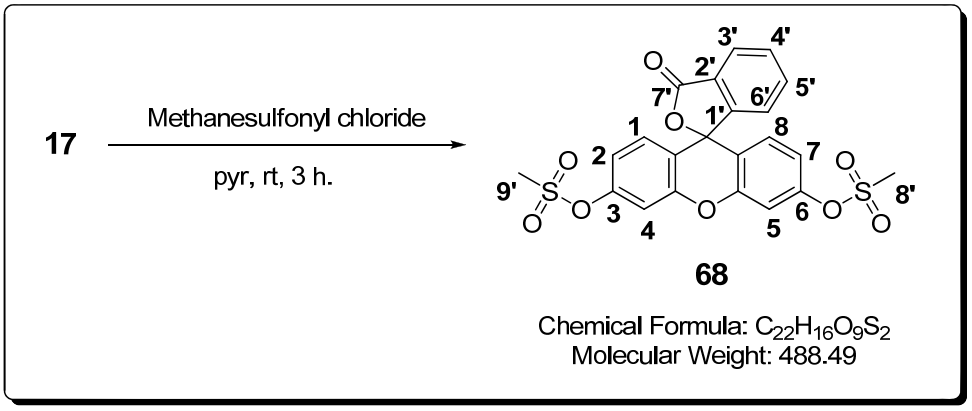


In a rb flask equipped with a stirrer was placed **(66)** (960 mg, 2.02 mmol, 1.2 equiv) as a solution in DMF (15 mL), DCC (694 mg, 3.36 mmol, 2.0 equiv) and (HOBt) 454 mg, 3.36 mmol, 2.0 equiv) and the solution stirred for 15 min under argon. ***4-(bromomethyl)-7-hydroxy-2H-chromen-2-one* 56** (640 mg, 1.68 mmol, 1.0 equiv) was added as a solution in DMF (5 mL) and rinsed with additional DMF (5 mL). The mixture was stirred until a clear colorless solution was obtained. The reaction was usually finished after 48 h as shown by TLC. Evaporation of the solvent, and the subsequent purification by fCC; (silica gel, DCM/MeOH 5:1, 1:1).The yield for this reaction was 29 %.

TLC (silica gel, DCM:MeOH=40:1): R_f = 0.21 ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ = 7.90 (m, 1H-H^{5B}), 7.83 (2 t, 2H, H^A, H^B),7.48 (m, 2H-H^{6B} & H^{8B}), 6.52 (s, 1H,-H^{3B}), 6.42, 6.36 (2 s, 2H, H^C, H^D), 4.62 (s, 2H,-CH_2Br), 4.30 (dd, 1H, H^{17}), 4.12 (m, 1H, H^{19}), 3.50 (s, 4H, 2x H^7, 2x H^8), 3.39 (t, 4H, 2x H^6, 2x H^9), 3.18 (dd, 4H, 2x H^5, 2x H^{10}), 3.09 (m, 1H, H^{16}), 2.81 (dd, 1H, H^{20}), 2.57 (d, 1H, $H^{20'}$), 2.35 (m, 4H, 2x H^2, 2x H^3), 2.06 (t, 2H, 2x H^{12}), 1.70-1.20 (m, 6H, 2x H^{13}, 2x H^{14}, 2x H^{15}); ^{13}C-NMR δ = (d_6-DMSO): 173.8 (C^1), 172.1 (C^4), 171.0 (C^{11}), 163.6 (C^{2B}), 162.7 (C^{18}), 154.7, 117.8, 117.2 (C^{3B}), 69.5, 69.0 (C^6, C^7, C^8, C^9), 61.0 (C^{19}), 59.1 (C^{17}), 55.3 (C^{16}), 42.16 (C-CH_2Br), 38.5, 38.4 (C^5, C^{10}, C^{20}), 35.0 (C^{12}), 29.9, 29.1, 28.1, 28.0, 25.2 (C^2, C^3, C^{13}, C^{14}, C^{15}). ES-MS: $[M+H]^+$ calculated 712.0, found 712.4; $[M+H]^+$

5.11 Chemical Syntheses of Xanthene and Related Derivatives

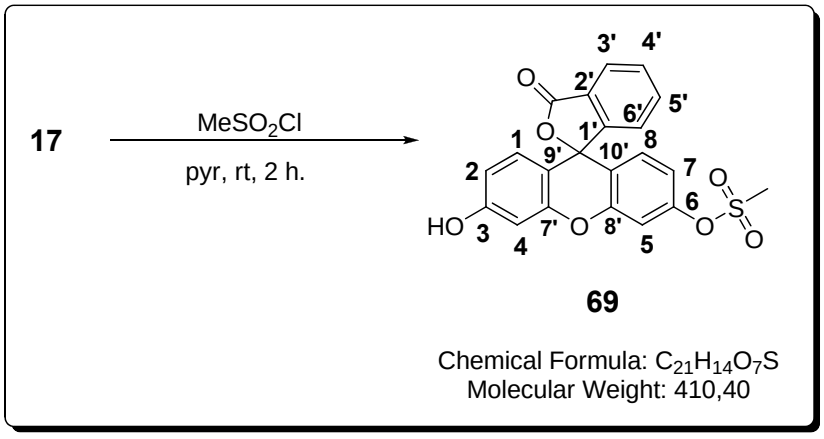
5.11.1 Synthesis of *3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dimethanesulfonate* (68)



In a 2-neck-rb flask equipped with a stirrer and fluorescein **(17)** (2 g, 6.0 mmol, 1.0 equiv) dried overnight under reduced pressure was added pyr (40 mL) at rt. The mixture was then stirred for 30 min until the fluorescein was completely dissolved. Methanesulfonyl chloride (4.14 g, 2.80 mL, 6.0 equiv) was then slowly added as a solution in pyr during 1 h whilst the mixture was being stirred with cooling using ice. The reaction was monitored by TLC and was usually finished after 2 h. Purification of the product **68** involves removal of pyr under reduced pressure and then recrystallizing using DCM containing 2 %MeOH. The yield of the reaction was 80 %.

TLC (silica gel, DCM:MeOH=80:1): R_f = 0.35; ^{1}H NMR (300 MHz, CD_3OD-d_4, 25 °C): δ = 3.47 (s, 6H, 2xCH_3, Ms-C8' & C9'), 6.99 (d, J=9.6 Hz, 2H, C(1) & C(8)), 7.15 (dd, J=2.7, 9.6 Hz, 2H, C(2) & C(7)), 7.40 (dt, J=8.0, 1.3 Hz, 1H–C(6')), 7.50 (d, J=2.7 Hz, 2H, C(4) & C(5)), 7.62 (td, J=8.0, 1.3 Hz, 1H, C(4')), 7.82 (td, J=8.0, 1.3 Hz, 1H1, C(5')), 8.07 (dt, J=8.0, 1.3 Hz, 1H, C(3')) ppm, ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 38.32 (Ms- C8' & C9'), 81.08 (1-CH_0 bridge), 111.64 C(4')) and (C(5')), 118.38 (C(3')), 119.61 (C(2')), 124.84 (C(4')), 125.86 (C(1')), 126.40 (C(4')), 130.60 (C(1')), 131.42 (C(8)), 136.81 (C(8)), 142.36 (C(8)), 146.15 (C(8)), 150.92 (C(8)), 151.48 (C(1')), 168.98 (C(7')) ppm. MALDI-TOF-MS: 489.23 $[M+H]^+$.

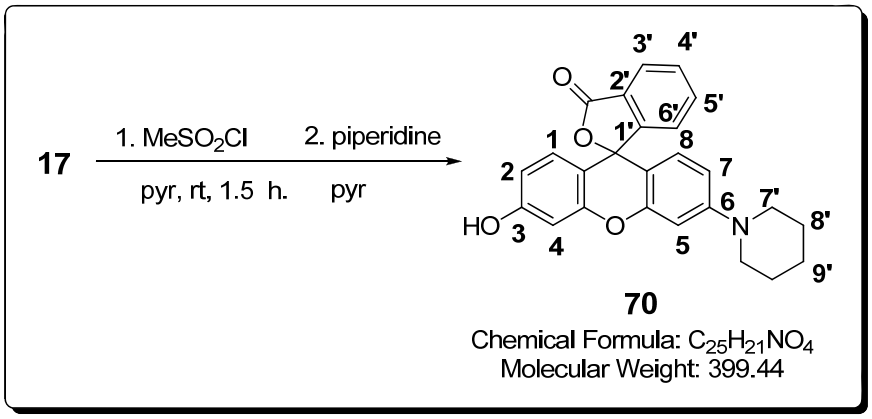
5.11.2 Synthesis of *3'-hydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6'-yl methanesulfonate* (69)



In a 2-neck-rb flask (250 mL) equipped with a stirrer and fluorescein **17** (1 g, 3.0 mmol, 1.0 equiv) dried overnight under reduced pressure was added pyr (20 mL) at rt. The mixture was then stirred for 10 min until the fluorescein was completely dissolved and cooled to 0 °C. Methanesulfonyl chloride (676 mg, 0.46 mL, 0.98 equiv) was then slowly added as a solution in pyr during 1 h whilst the mixture is being stirred maintaining 0 °C. The reaction mixture was stirred for further 30 min after which it was allowed to warm to rt. The reaction was monitored by TLC and was usually finished after 1 h. The removal of pyr was achieved under reduced pressure and the crude recrystallized using DCM containing 2 % MeOH to obtain the product **69** in 80 % yield.

TLC (silica gel, DCM:MeOH=80:1): R_f = 0.25; ^{1}H NMR (300 MHz, CD_3OD-d_4, 25 °C): δ = 3.17 (s, 3H, CH_3, Ms), 6.58 (dd, J=2.7, 9.6 Hz, 1H–C(2), 6.78 (m, 1H-C(4)), 6.74 (d, J=2.7 Hz, 1H–C(5)), 6.80 (d, J=9.6 Hz, 1H–C(8)), 6.92(dd, J=2.7, 9.6 Hz, 1H–C(7)), 7.14 (m, 1H-C(6')), 7.20 (d, J=9.6 Hz, 1H–C(1)),7.32(m, 1H-C(1')), 7.61 (m, 1H-C(4')), 7.67 (m, 1H-C(5')), 8.00 (m, 1H-C(3')), 8.60 (s, br, 1H-OH) ppm, ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 38.32 (SO_2CH_3), 81.08 (1-CH_0 bridge), 111.64 C(4)) and (C(5)), 118.38 (C(9')), 119.61 (C(10')), 124.84 (C(2)), 125.86 (C(7)), 126.40 (C(6')), 130.60 (C(4')), 131.42 (C(3')), 136.81(C(2')), 142.36(C(1)), 146.15(C(8)), 150.92 (C(5)), 151.48 (C(6)), 152.71 (C(1')), 156.32 (C(7')), 156.36 (C(8')),168.98 (C(3)), 172.45 (CO')) ppm. MALDI-TOF-MS: 411.23 $[M+H]^{+\cdot}$

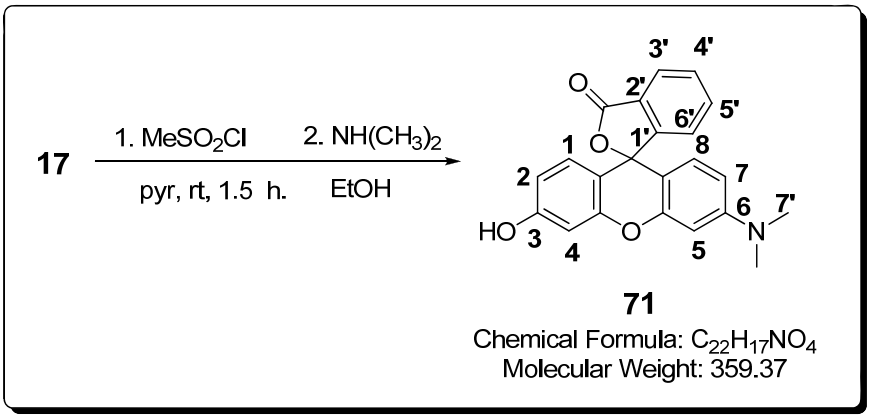
5.11.3 Synthesis of *3'-hydroxy-6'-(piperidin-1-yl)-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one* (70)



In a 2-neck-rb flask (100 mL) equipped with a stirrer and fluorescein **(17)** (0.5 g, 1.50 mmol) dried overnight under reduced pressure was added pyr (10 mL) at rt. The mixture was then stirred for 10 min until the fluorescein was completely dissolved and cooled to 0 °C. Methanesulfonyl chloride (338 mg, 0.23 mL, 0.98 equiv) was then added as a solution in pyr during 1 h whilst the mixture was being stirred maintaining 0 °C. The reaction mixture was stirred for further 30 min after which it was allowed to warm to rt. The reaction was monitored by TLC and was usually finished after 1 h. To the above solution was added piperidine in excess (10 equiv) in one portion during cooling with ice and the stirring was continued for further 1h. The reaction mixture was evaporated after TLC showed no intermediate mesylated. The crude product was purified by fCC on silica gel with a gradient eluent system of DCM)/MeOH, 9:1, 5:1, 1:1. The produc **70** was obtained with a yield of 60 %.

TLC (silica gel, DCM:MeOH =10:1): R_f = 0.20; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 1.23 (m, 4H-H8'), 1.55 (m, 2H-H9'), 3.27 (m, 4H-H7'), 5.30 (br, 1H-OH), 6.42-6.56 (m, 4H-H2, H4, H5, & H7), 6.62 (m, 2H-H1 & H8), 7.24 (m, H-H6'), 7.65-7.72 (m 2H-H4' & H5'), 7.98 (m, 1H-H3') ppm. MALDI-TOF-MS: 400.59 $[M+H]^+$.

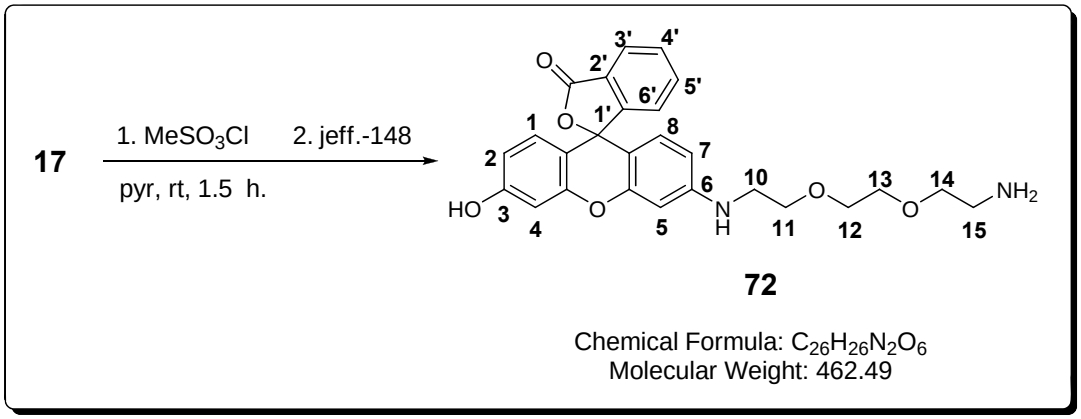
5.11.4 Synthesis of *3'-(dimethylamino)-6'-hydroxy-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one* (71)



In a 2-neck-rb flask (100 mL) equipped with a stirrer and fluorescein **(17)** (0.025 g, 0.075.0 mmol, 1 equiv) dried overnight under reduced pressure was added pyr (10 mL) at rt. The mixture was then stirred for 10 min until the fluorescein was completely dissolved and cooled to 0 °C. Methanesulfonyl chloride (17 mg, 0.012 mL, 0.98 equiv) was then added as a solution in pyr during 1 h whilst the mixture is being stirred maintaining 0 °C. The reaction mixture was stirred for further 30 min after which it was allowed to warm to rt. The reaction was monitored by TLC and was usually finished after 1 h. To above solution was added dimethylamine (as a solution in ethanol) in excess (10 equiv) in one portion during cooling with ice and allowed to warm to rt. The stirring was continued for 2days. The reaction mixture was evaporated after TLC showed no intermediate mesylated product.The product **71** of this reaction purified by recrystalisation using DCM/MeOH with a yield of 50 %.

TLC (silica gel, DCM: MeOH =10:1): R_f= 0.18; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 3.42 (s, 6H, -N(CH_3)$_2$), 5.30 (br, 1H-OH), 6.42-6.56 (m, 4H-H2, H4, H5, & H7), 6.62 (m, 2H-H1 & H8), 7.24 (m, H-H6'), 7.65-7.72 (m 2H-H4' & H5'), 7.98 (m, 1H-H3') ppm. MALDI-TOF-MS: 360.4 $[M+H]^+$.

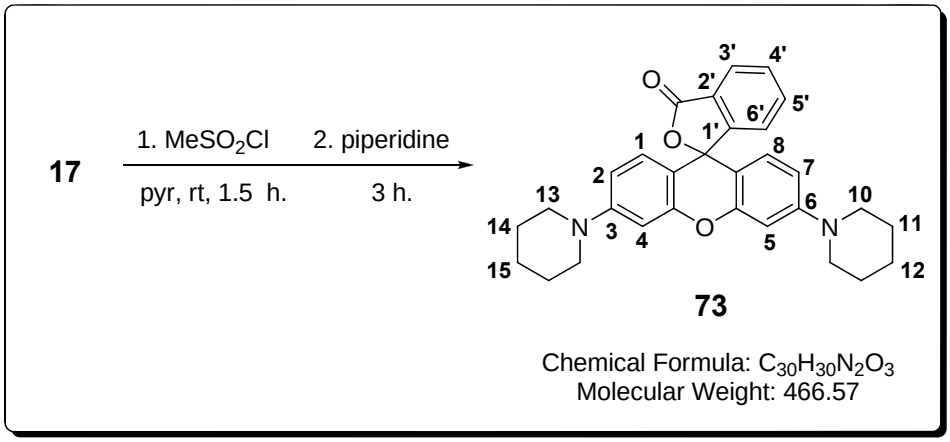
5.11.5 Synthesis of *3'-(2-(2-(2-aminoethoxy)ethoxy)ethylamino)-6'-hydroxy-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one* (72)



To a stirring mixture of fluorescein **(17)** (0.025 g, 0.075.0 mmol) and pyr was added methanesulfonyl chloride (17 mg, 0.011 mL, 0.98 equiv) and the reaction mixture stirred at rt for 1.5 h. The unreacted methanesulfonyl chloride was evaporated after which Jeffamine-148 (11.1 mg, 1.5 equiv) was then added in one portion during cooling with ice and the stirring continued for further 1h. The reaction mixture was evaporated after TLC showed no intermediate mesylated product. Flash column chromatography purification was achieved with (DCM/MeOH) (9:1, 5:1, 1:1, MeOH). The yield of the product **72** (after over week-end drying) was 50 %.

TLC (silica gel, DCM: MeOH =2:1): R_f = 0.21; ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ = 2.67 (m, 4H), 3.37 (t, 4H), 3.57 (m, 4H), 5.75 (s, 1H-NH_2), 6.40 (dd, J = 4.7 and 7.8 Hz, 2H-H5 & H7,) 6.46 (d, J = 4.7 Hz, 1H), 6.58 (d, J = 7.8 Hz, 2H-H1 & H7), 7.20 (m, 1H-H6'), 7.61-7.64 (m, 2H-H4' & H5'), 7.97 (m, 1H-H3') ppm. ^{13}C NMR (75 MHz, DMSO-d_6, 25 °C): δ =42.43 (C(15), 47.64 C(10)), 72.52 (C(11) 72.58 C(12)), 73.32 C(13)), 74.08 (C(14)), 81.09 (1-CH_0 bridge), 113.65 (C(7) 119.48, 120.62 (C(2)), 124.86 (C(6')), 125.80 (C(3')), 126.41 (C(4')), 130.60 (C(2')), 131.44 (C(8)), 136.82 (C(1)), 142.33 (C(5')), 146.16 (C(6)), 150.94, 151.49 (C(1')), 152.71 (C3)), 171.98 (C(CO.)) ppm. MALDI-TOF-MS: $[M+H]^+$. 463.0, $[M+Na]^+$ 485.0

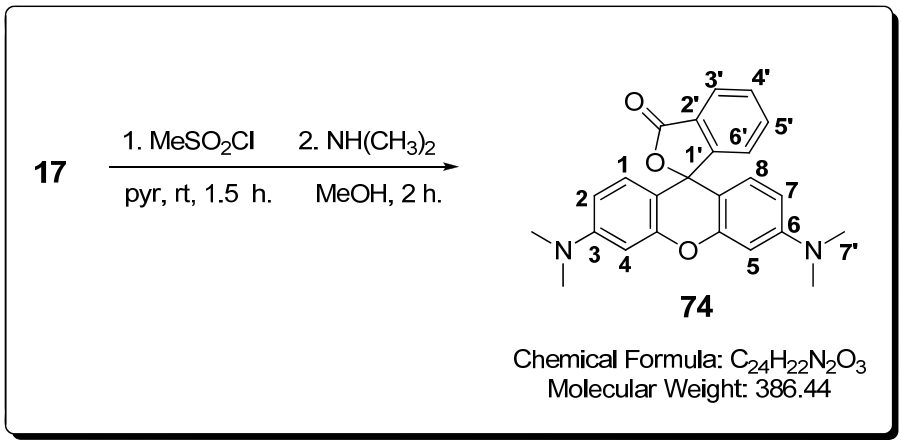
5.11.6 Synthesis of *3',6'-di(piperidin-1-yl)-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one* (73)



In a 2-neck-rb flask (100 mL) equipped with a stirrer and fluorescein **(17)** (0.5 g, 1.5.0 mmol, 1.0 equiv) dried overnight under reduced pressure was added pyr (10 mL) at rt. The mixture was then stirred for 10 min until the fluorescein was completely dissolved and cooled to 0 °C. methanesulfonyl chloride (1.4 g, 0.94 mL, 4.0 equiv) was then added as a solution in pyr during 1 h whilst the mixture is being stirred maintaining the reation at 0 °C. The reaction mixture was stirred for further 30 min after which it was allowed to warm to rt. The reaction was monitored by TLC and was usually finished after 2 h. To the above solution was added piperidine in excess (10.0 equiv) in one portion during cooling with ice and the stirring continued for further 1h. The reaction mixture was evaporated after TLC shows no intermediate mesylated product. Purification by fCC on silica gel with a gradient eluent system of DCM:MeOH (9:1, 5:1, 1:1). The product **73** was obtained with a yield of 60 %.

TLC (silica gel, DCM: MeOH =10:1): R_f=0.24; ^{1}H NMR (300MHz, CD_3OD-d4, 25 °C): δ=1.28 (m, 2x4H-H11 & H14), 1.42 (m, 2x2H-H12 & H15), 3.37 (m, 2x4-2H-CH_2-H10 & H13), 6.72 (d, J=9.6 Hz, 2xH– C(1) & C(8)), 6.55 (dd, J=2.7, 9.6 Hz, 2xH–C(2) & C(7)), 7.40 (dt, J=8.0, 1.3 Hz, 1H–C(6')), 6.58 (d, J=2.7 Hz, 2xH– C(4) & C(5)), 7.61 (td, J=8.0, 1.3 Hz, 1H–C(4')), 7.72 (td, J=8.0, 1.3 Hz, H1–C(5')), 8.02 (dt, J=7.8, 1.3 Hz, H1–C(3')) ppm, ^{13}C NMR (75MHz, $CDCl_3$, 25°C): δ =25.04 (C(12) &C(15)), 26.24 (C(11) &C(14)), 58.32 (C(10) &C(13)), 82.08 (1-CH_0 bridge), 112.64 (C(4')), 113.64 (C(5')), 118.48 (C(3')), 119.62 (C(2')), 124.84 (C(4')), 125.86 (C(1')), 126.40 (C(4')), 130.60 (C(1')), 131.42 (C(8)), 136.81 (C(8)), 142.36 (C(8)), 146.15 (C(8)), 150.92 (C(8)), 151.48 (C(1')), 152.71 (C(1')), 168.98 (C(7')) ppm. MALDI-TOF-MS: calc. 466.56, found 467.5 $[M+H]^+$.

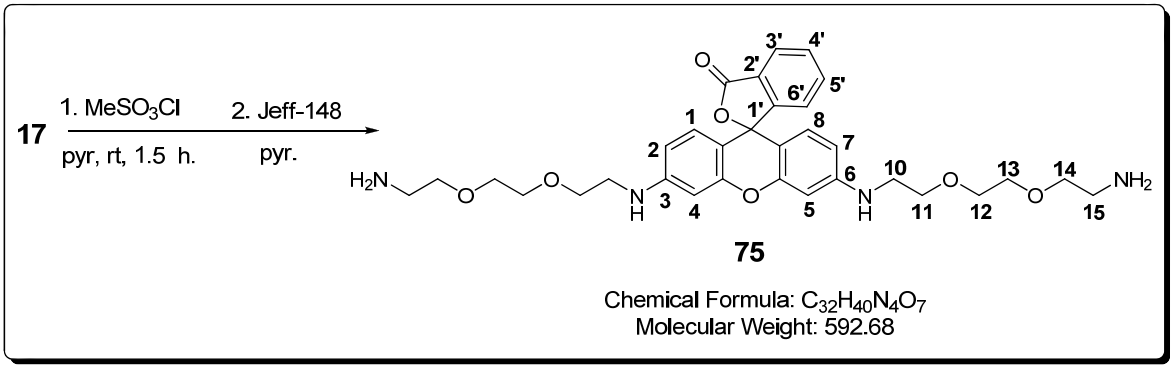
5.11.7 Synthesis of *3',6'-bis(dimethylamino)-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one* (74)



To a stirring mixture of fluorescein **(17)** and was added methanesulfonyl chloride (4.0 equiv) and the reaction mixture was stirred at rt for 1.5 h. The excess methane sulfonyl chloride was then removed by evaporation under reduced pressure The reaction mixture was then cooled to 0 °C during which excess dimethylamine in methanol was then added in one portion and stirring was continued for further 2 h. The reaction mixture was evaporated after TLC showed no intermediate mesylated product. The product **74** was purified by fCC on silica gel with a gradient eluent system of DCM:MeOH (9:1, 5:1, 1:2) with a yield of 40 %.

TLC (silica gel, DCM: MeOH =10:1): R_f = 0.22; ^{1}H NMR (300 MHz, CD_3OD-d_4, 25 °C): δ = 3.32 (s, 12H, 4xCH_3, -NMe_2), 6.99 (d, J=9.6 Hz, 2xH– C(1) & C(8)), 7.14 (dd, J=2.7, 9.6 Hz, 2xH–C(2) & C(7)), 7.41 (dt, J=8.0, 1.3 Hz, H–C(6')), 7.50 (d, J=2.7 Hz, 2xH– C(4) & C(5)), 7.76-7.82 (m, 2xH–C(4')), 7.72 (td, J=8.0, 1.3 Hz, H–C(5')), 8.05-8.09 (m, H–C(3')) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 25.04 (C(12) &C(15)), 26.24 (C(11) &C(14)), 58.32 (C(10) &C(13)), 82.08 (1-CH_0 bridge), 112.64 (C(4')), 113.64 (C(5')), 118.48 (C(3')), 119.62 (C(2')), 124.84 (C(4')), 125.86 (C(1')), 126.40 (C(4')), 130.60 (C(1')), 131.42 (C(8)), 136.81 (C(8)), 142.36 (C(8)), 146.15 (C(8)), 150.92 (C(8)), 151.48 (C(1')), 152.71 (C(1')), 168.98 (C(7')) ppm. MALDI-TOF-MS: 387.5 $[M+H]^+$.

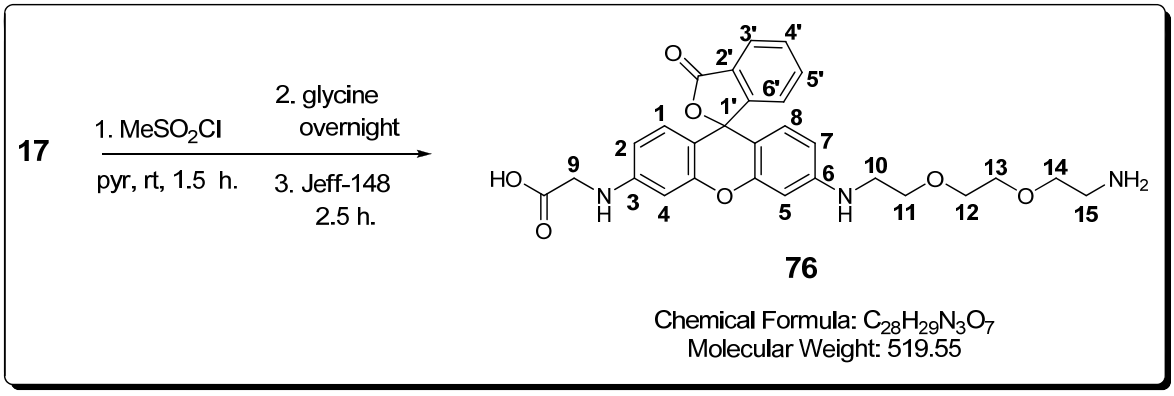
5.11.8 Synthesis of *3',6'-bis(2-(2-(2-aminoethoxy)ethoxy)ethylamino)-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one* (75)



To a stirring mixture of fluorescein (**17)** and pyr was added methanesulfonyl chloride (4.0 equiv) and the reaction mixture stirred at rt for 1.5 h. The unreacted methanesulfonyl chloride was evaporated after which jeffamine-148 (6.0 equiv) was added in one portion during cooling with ice and the stirring continued for further 1 h. TLC showed no complete conversion and so reaction was stirred overnight during which a slight green solution was formed. The reaction mixture was evaporated after TLC showed no intermediate mesylated product. Prep. TLC purification by fCC on silica gel with a gradient eluent system of DCM:MeOH (DCM, 9:1, 5:1, 1:1, MeOH). Product formation confirmed by MS and NMR but very unstable.

TLC (silica gel, DCM: MeOH =2:1): R_f= 0.14; ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ= 2.65 (t, 4x2H-H10 & H15), 3.35 (t, 4x2H-H11 & H14), 3.46 (s, 4x2H-H12 & H13), 6.28 (m, 2H), 6.32 (m, 2H), 6.58-6.60 (m, 2H), 7.13 (m, 1H), 7.54-7.58 (m, 2H), 7.94-7.98 (m, 2H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ =25.04 (C(12) &C(15)), 26.24 (C(11) &C(14)), 58.32 (C(10) &C(13)), 82.08 (1-CH_0 bridge), 112.64 (C(4')), 113.64 (C(5')), 118.48 (C(3')), 119.62 (C(2')), 124.84 (C(4')), 125.86 ppm. MALDI-TOF-MS: calc. 593.7, found 593.6 $[M+H]^+$.

5.11.9 Synthesis of *2-(3'-(2-(2-(2-aminoethoxy)ethoxy)ethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6'-ylamino)acetic acid* (76)

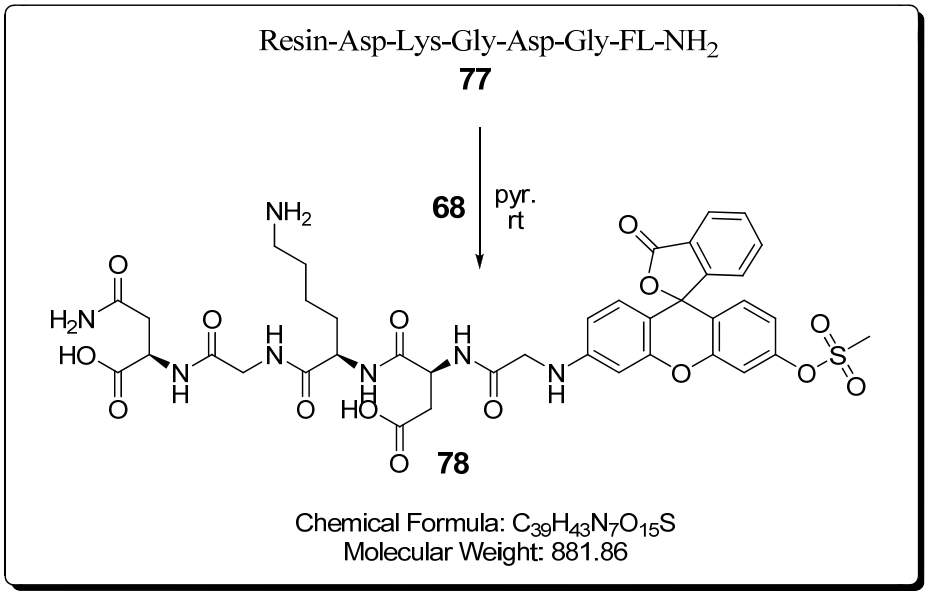


To a stirring solution of dried fluorescein **(17)** in pyr was added methanesulfonyl chloride (3.0 equiv) and the reaction mixture was stirred until no colour could be detected by the unaided eye (TLC confirmed this). The excess methanesulfonyl chloride was evaporated together with the pyr. Glycine 1.0 equivalent was added and the reaction mixture was diluted with ethanol to ensure complete dissolution of the glycine. The reaction mixture was stirred overnight. A slight purple coloration results after which 2.5 equivalent jeffamine was added and reaction stirred for further for 2 h. TLC showed complete consumption of starting material. Work up involves evaporation of pyr and ethanol and purified by fCC on silica gel with a gradient eluent system of

DCM/MeOH (100 % DCM, 100% MeOH). Product formation confirmed by mass Spectrometry, (but mainly di-jeffamine by comparing tlc).

TLC (silica gel, DCM: MeOH =1:1): R_f = 0.18; ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ = 2.72 (m, 4H-H10 & H15), 3.40 (m, 4H-H11 & H14), 3.46 (s, 4H-H12 & H13), 5.44 (br, 2H-NH_2), 6.32-6.39 (m, 2H-H4 & H5), 6.42 (m, 2H-H2 & H8), 6.62 (m, 2H-H1 & H8) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ =25.04 (C(12) &C(15)), 26.24 (C(11) &C(14)), 58.32 (C(10) &C(13)), 82.08 (1-CH_0 bridge), 112.64 (C(4')), 113.64 (C(5')), 118.48 (C(3')), 119.62 (C(2')), 124.84 (C(4')), 125.86 ppm. MALDI-TOF-MS: calc. 520.6, found 520.5 $[M+H]^+$.

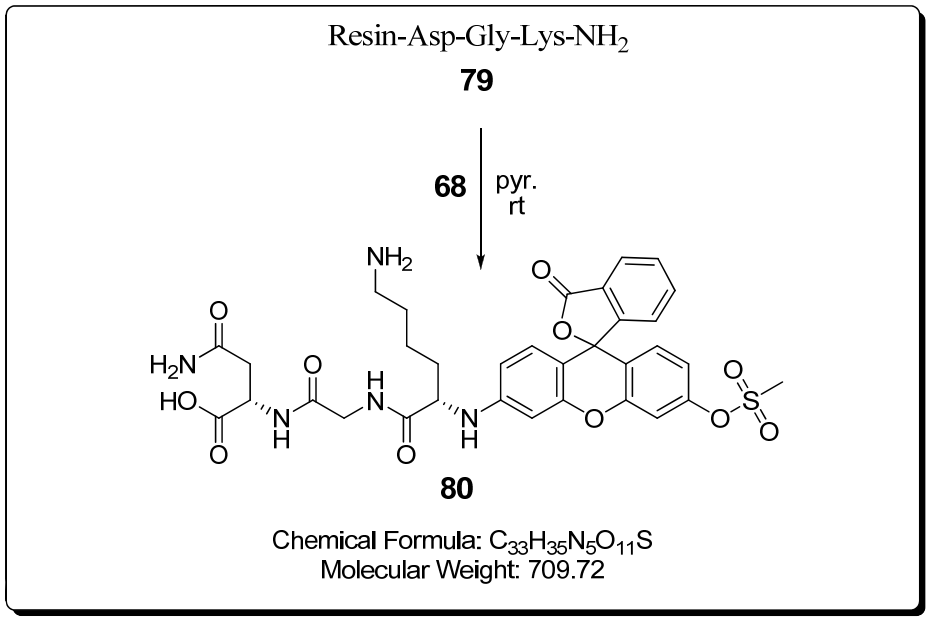
5.11.10 Synthesis of *(4S,7R,13R)-15-amino-7-(4-aminobutyl)-4-(carboxymethyl)-1-(3'-(methylsulfonyloxy)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6'-ylamino)-2,5,8,11,15-pentaoxo-3,6,9,12-tetraazapentadecane-13-carboxylic acid* (78)



The penta-peptide **77** was synthesized according to standard SPPS (novabiochem 2008/9) on a Rink AM resin. After cleavage of the final fmoc from the amino functionality, **68** was added as a solid to which pyr was then aspirated and shaken overnight. The purplish-orange beads were then washed several times after which they were swirled with DCM and then dried under vacuum overnight. To the dried resin was added 95% TFA in DCM and filtered 3 times within 1 h. The combined filtrate was cooled and dried overnight by freeze drying. MS confirms product **78**, estimated yield was 20 %.

TLC (silica gel, DCM: MeOH =1:1): R_f=0.11; ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): MALDI-TOF-MS: calc. 881.9, found 882.8 $[M+H]^+$.

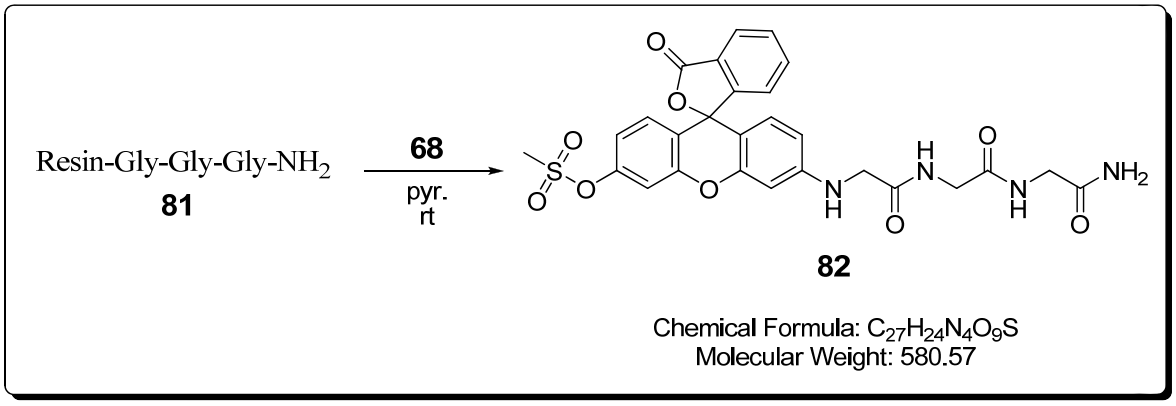
5.11.11 Synthesis of *(2S)-4-amino-2-(2-((2S)-6-amino-2-(3'-(methylsulfonyloxy)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6'-ylamino)hexanamido)acetamido)-4-oxobutanoic acid* (80)



The tripeptide **79** was synthesized according to standard SPPS (novabiochem 2008/9) on a Rink AM resin. After cleavage of the final fmoc, **(68)** was added as a solid to which pyr was then aspirated and shaken overnight. The purplish-orange beads was then washed several times after which was swirled with DCM and then dried under vacuum overnight. To the dried resin was added 95% TFA in DCM and filtered 3 times within 1 h. The combined filtrate was cooled and dried overnight by freeze drying. MS and NMR confirms product **80**.

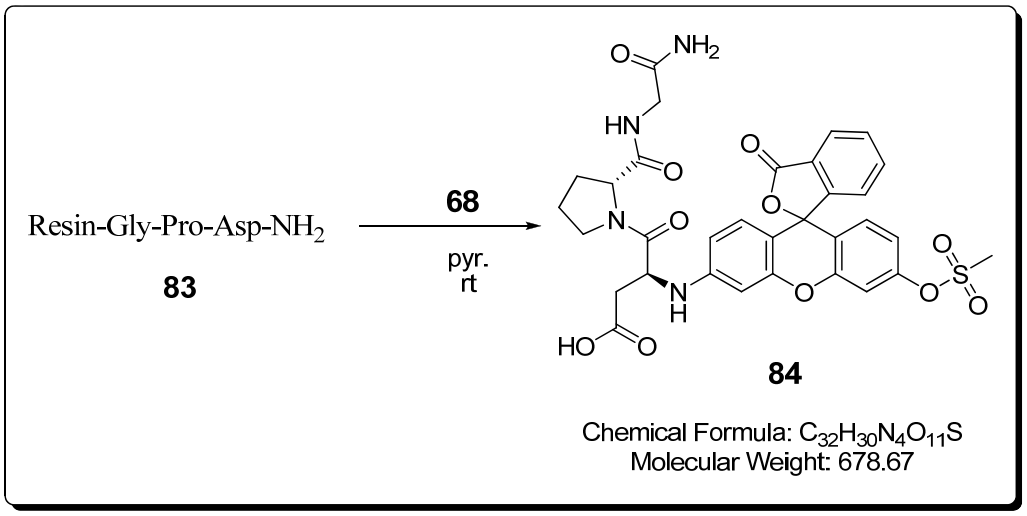
TLC (silica gel, DCM:MeOH=40:1): R_f = 0.47 ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ = 1.42 (s, 18H), 4.20 (s, 4H), 4.37 (q, J = 9.3 Hz, 1H), 4.50 (dt, J = 9.8 and 5.6 Hz, 1H), 4.72-4.76 (m, 1H), 5.05 (t, J = 4.7 H. MALDI-TOF-MS: calc. 709.7, found 710.8 $[M+H]^+$.

5.11.12 Synthesis of *3'-(2-(2-(2-amino-2-oxoethylamino)-2-oxoethylamino)-2-oxoethylamino)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6'-yl methanesulfonate* (81)



The tripeptide **81** was synthesized according to standard SPPS (novabiochem 2008/9) on a Rink AM resin. After cleavage of the final fmoc, **68** was added as a solid to which pyr was then aspirated and shaken overnight. The purplish beads were then washed several times after which they were swirled with DCM and then dried under vacuum overnight. To the dried resin was added 95% TFA in DCM and filtered 4 times within 1 h. Thecombined filtrate was cooled and dried overnight by freeze drying. MS confirms product **82**. MALDI-TOF-MS: 581.6 $[M+H]^+$.

5.11.13 Synthesis of *(3S)-4-((R)-2-(2-amino-2-oxoethylcarbamoyl)pyrrolidin-1-yl)-3-(3'-(methylsulfonyloxy)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-6'-ylamino)-4-oxobutanoic acid* (84)



The tri-peptide **(83)** was synthesized as described previously. After cleavage of the final fmoc from the amino functionality, **68** was added as a solid to which pyr was then aspirated and shaken overnight. The purplish beads were then washed several times after which it was swirled with DCM, filtered and then dried under vacuum overnight. To the dried resin was added 95% TFA in DCM and filtered 4 times within 1 h. The combined filtrate was cooled and dried overnight by freeze drying. The crude was then purified by HPLC. The product **84** is confirmed by MS and NMR

TLC (silica gel, DCM:MeOH=2:1): R_f = 0.14 ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C): δ= 2.22 (m, 4H-H5, H5', H6 & H6'), 3.02 (m, 2H-H12 & H12'), 3.14 (m, 2H-H7 & H7'), .3.89 (m, 2H-H10 & H4), 4.12 (m, 2H-H1 & H1'), 6.58 (m, H1–C(2)-Ar), 6.78 (m, 1H-C(4)-Ar), 6.74 (m, H1–C(5)-Ar), 6.80 (m, H1–C(8)-Ar), 6.92(m, H1–C(7)-Ar), 7.14 (m, 1H-C(6')-Ar), 7.20 (d, H1– C(1)-Ar),7.32(m, 1H-C(1')-Ar), 7.61 (m, 1H-C(4')-Ar), 7.67 (m, 1H-C(5')-Ar), 8.00 (m, 1H-C(3')-Ar) ppm. MALDI-TOF-MS: 701.6 $[M+Na]^+$

5.12 Synthesis of RNA Oligomers

Step	Function	Mode	Amount	Time	Description
Deblocking	144/*Index Fract. Coll.	NA	1	0	'Event out ON'
	0/*Default	WAIT	0	15	'Wait'
	38/*Diverted Wsh A	PULSE	15	0	'Flush system with Wsh A'
	141/*Trityl Mon. On/Off	NA	1	1	'START data collection'
	16/*Dblk	PULSE	20	0	'Dblk to column'
	0/*Default	WAIT	0	20	'Default'
	16/*Dblk	PULSE	40	40	'Deblock'
	38/*Diverted Wsh A	PULSE	60	0	'Flush system with Wsh A'
	141/*Trityl Mon. On/Off	NA	0	1	'STOP data collection'
	38/*Diverted Wsh A	PULSE	20	0	'Flush system with Wsh A'
	144/*Index Fract. Coll	NA	2	0	'Event out OFF'
Coupling	1/*Wsh	PULSE	1	0	'Flush system with Wsh A'
	2/*Act	PULSE	0	15	'Flush system with Act'
	18/*A+Act	PULSE	15	0	'Monomer + Act to Column'
	18/*A+Act	PULSE	1	1	'Couple monomer'
	2/*Act	PULSE	20	0	'Couple monomer'
	18/*A+Act	PULSE	0	20	'Couple monomer'
	2/*Act	PULSE	40	40	'Deblock'
	0/*Default	WAIT	60	0	'Flush system with Wsh A'
	1/*Wsh	PULSE	0	1	'STOP data collection'
	1/*Wsh	PULSE	20	0	'Flush system with Wsh A'
Capping	12/*Act	PULSE	20	20	'Event out ON'
	13/*Caps	PULSE	0	0	'Wait'
	12/*Wsh	PULSE	40	40	'Flush system with Wsh A'
	12/*Wsh	PULSE	60	60	'START data collection'
Oxidizing	15/*Act	PULSE	20	20	'Event out ON'
	0/*Default	WAIT	0	0	'Wait'
	12/*Wsh A	PULSE	40	40	'Flush system with Wsh A'
Capping	13/* Caps	PULSE			'Event out ON'
	12/* Wsh A	PULSE			'Wait'

[a] Deblocking reagent = dblk, acetonitrile = Wsh, and WshA, argon = Gas B, activator = act, capping reagents = Caps, oxidizer = Ox, [b] ! PULSE = 16 μL.

5.12.1 Synthesized RNA Oligomers

Table 7 The RNA oligomeric units were synthesized using the 1 μmol synthetic protocol

Sequence no.	Sequencse (5'-3')	Av. coupling yield %	Mass Meas. (MALDI)
MH278	GAACUΨCAGGGUCAGCUUGCCG	42	7034.3
MH279	GAACUΨCAGGGΨCAGCUUGCCG	38	7034.4
MH**280**	GAACUΨCAGGGΨCAGCUΨGCCG	37	7034.4
MH281	GAACUUCAGGGUCAGCUTGCCG	40	7048.5
MH282	GAACUUCAGGGTCAGCUTGCCG	36	7071.2
MH283	GAACUTCAGGGTCAGCUTGCCG	38	7072.4
MH284	GAACUUCAGGGUCAGCUUm^{1}GCCG	32	7047.7
MH285	GAACUUCAGGm^{1}GUCAGCUUm^{1}GCCG	30	7061.3
MH**286**	m^{1}GAACUUCAGGm^{1}GUCAGCUUm^{1}GCCG	26	7075.4
MH**287**	GAACUUCAGGGUCAGCUUm^{2}GCCG	29	7047.2
MH**288**	GAACUUCAGGm^{2}GUCAGCUUm^{2}GCCG	29	7061.3
MH**290**	m^{2}GAACUUCAGGm^{2}GUCAGCUUm^{2}GCCG	28	7075.2
MH**291**	GAACUUCAGGGUCAGCUUm$^{2}_{2}$GCCG	24	7061.2
MH**292**	GAACUUCAGGm$^{2}_{2}$GUCAGCUUm$^{2}_{2}$GCCG	20	7089.3
MH**293**	m$^{2}_{2}$GAACUUCAGGm$^{2}_{2}$GUCAGCUUm$^{2}_{2}$GCCG	19	7117.1
MH**294**	GCAAGCUGACCCUGAAGUUCAU	35	7002.3
MH295	GCAAGCΨGACCCUGAAGUUCAU	32	7002.4
MH296	GCAAGCUGACCCUGAAGUΨCAU	34	7003.3
MH297	GCAAGCUGACCCΨGAAGUUCAU	34	7004.0
MH298	GCAAGCUGACCCUGAAGΨΨCAU	24	7002.3
MH299	GCAAGCΨGACCCΨGAAGUΨCAU	22	7002.4
MH300	GAACUUCAGGGUCAGCUUGCCG	37	7034.3
MH343	GAACUUCAGGGUCAGCUUm^{1}GCCG	32	7049.1
MH344	GAACUUCAGGGUCAm^{1}GCUUm^{1}GCCG	30	7063.2
MH259	GAAAAAGUCAUGGAGGUCAUGGGGUUG	26	8817.5
MH260	GAAAAAGUCAUGGAGGUCAUGGGGΨUG	22	8831.2
MH261	GCUUGAAACCAGCUUUGGG	36	6078.7
MH262	GGGUUCGAUUCCUUCAUUUUUUGCCA	38	8157.9

MH263	GGGTUCGAUUCCUUCAUUUUUUGCCA	34	8171.4
MH264	CAGAGGUUCAAUUCCUCUUCUUAACACCA	30	9110.7
MH265	CAGAGGUUCAATUCCUCUUCUUAACACCA	23	9125.2
MH266	AAACUUAAAACUUUACUGU	41	5976.7
MH267	AAACUUAAAACΨUUACUGU	24	5976.8
MH268	CCCGGUAAUCGCAUA	34	4736.0
MH269	CCCGGUAAUCGCAΨA	35	4737.1
MH270	GUUAAGAUGGCAGAG	33	4880.06
MH271	GUUAAGAUm^{1}GGCAGAG	29	4895.7
MH272	AGAAAUAUGUCUGAUAAA	32	5773.8
MH273	AGAAAUAUm^{1}GUCUGAUAAA	32	8000.3
MH274	AGAGUUACUUUGAUAGAGUAAA	35	7076.4
MH275	AGAm$^{2}_{2}$GΨΨACUUUGAUAGAGUAAA	17	8015.2
MH276	UAAUAGGAGCUUAAACCCCCUUAUUUCU ACCA	23	10075.2
MH277	GGUACCCAAAAU	33	3803.4

Synthesized Oligomers	Code	Mass Calc.	Mass Meas. (MALDI)
r(GAACUψCAGGGUCAGCUUGCCG)	**MH278**	7034.29	7034.3
r(GAACUψCAGGGψCAGCUUGCCG)	**MH279**	7034.29	7034.4
r(GAACUψCAGGGψCAGCUψGCCG)	**MH280**	7034.29	7034.4
r(GAACUUCAGGGUCAGCUTGCCG)	**MH281**	7048.29	7048.5
r(GAACUUCAGGGTCAGCUTGCCG)	**MH282**	7062.29	7071.2
r(GAACUTCAGGGTCAGCUTGCCG)	**MH283**	7076.29	7077.4
r(GAACUUCAGGGUCAGCUUm^{1}GCCG)	**MH284**	7048.29	7048.4
r(GAACUUCAGGm^{1}GUCAGCUUm^{1}GCCG)	**MH285**	7062.29	7063.3
r(m^{1}GAACUUCAGGm^{1}GUCAGCUUm^{1}GCCG)	**MH286**	7076.29	7080.1
r(GAACUUCAGGGUCAGCUUm^{2}GCCG)	**MH287**	7048.29	7049.0
r(GAACUUCAGGm^{2}GUCAGCUUm^{2}GCCG)	**MH288**	7062.29	7063.3
r(m^{2}GAACUUCAGGm^{2}GUCAGCUUm^{2}GCCG)	**MH290**	7076.29	7078.2

5.12.2 Preparation of RNA Sequences

Typical synthesis of oligomers by SPOS using the following as an example: RNA-sequence r(GAACUψCAGGGUCAGCUUGCCG) **MH278**,

The sequence was assembled from 34 mg of solid support (loading 30 μmol/g) on an **Expedite 8909 synthesizer** using the standard conditions for the assembly of 2′-O-TOM-protected ribonucleoside phosphoramidites, employing a combination of 2′-O-TOM and TBDMS protected phosphoramidite building block.. After the final coupling, the solid support was washed with $(iPr)_2NH$/MeCN 1:9 for 20 min (flow-rate 2.5 mL/min). Cleavage from the solid support and deprotection was carried out with 12 M NH_3 in MeOH (1 mL) for 14 h at 20 °C. The supernatant was removed by centrifugation and evaporated to dryness; the residue was treated with a THF soln. (1 mL) of $Bu_4NF{\cdot}3H_2O$ (1 M) for 14 h at 20 °C, diluted with aq. Tris.HCl buffer (1 mL, 1 M, pH 7.4) and evaporated to a volume of 1 mL. After desalting on a NAP cartridge, the main peak of the crude product was isolated by semi-preparative HPLC and characterize by MALDI-TOF-MS analysis:

These oligomers shown in Table 7 were syntheiszed by modification of the synthetic protocol in order to take into consideration the insoluble nature of the modified G. The modified G-phosphoramidite was dissolved to make 0.2 M solution (usually 0.4 M for the other nucleosides). The dilution of the G was taken into consideration and hence the coupling times and the injection volume were all increased by a factore of two.

5.12.3 Phosphorylation Reactions

Table 8 The phosphorylation of the 5' of the ligation fragments.

Conc(μM)	MH	Oligo (μL)	5X KL	ATP 100mM	DTT 0.1M	H_2O	PNK	Total Volume
100	272	26	20	2	5	39.6	7.6	100
100	273	28.6	20	2	5	37.0	7.6	100
100	274	22.2	20	2	5	43.4	7.6	100
100	275	27	20	2	5	38.6	8	100
100	276	65.6	20	2	5	0.0	7.6	100

The Fragment components for the ligation experiment were phosphorylated at the 5`end before subjecting to the ligation condition. A typical phosphorylation reavtion scheme for a 100µM scale synthesis is as shown in Table 8. The final mixture was incubated at 37°C for 1h at 300 rpm after which it was cooled to rt. The phosphorylated products were used without further purification.

5.12.4 Ligation Experiments

RNA-sequence r(GCUUGAAACCAGCUUUGGG) **MH261, and that of MH 262 and 263**). The 21mer MH262, 13mer MH2635 and 24mer template MH261 were mixed to a final concentration of 40 µM for the substrates and 40 µM for the template (1.0 eq). After the addition the master mix and water (~54 µl), the mixture was heated at 75° for 4 min and subsequently cooled down to r.t within 10min. After the addition of ligation buffer (40mM TrisHCl, 2mM MgCl2, 10mM DTT, 0.5mM ATP) and 8 units of ribonuclease inhibitor (Fermentas), the reaction was initiated by adding T4 DNA ligase and kept at 16 °C overnight stirring at 300 rpm.

Table 9 The ligation scheme for the synthesis of $tRNA^{Ile}$.
A typical ligation scheme for an 8 µM scale synthesis is shown below.

	oligo1	**oligo2**	**oligo3**	**Splint**	**L-premix**	**ligase**	**H_2O**	**T.vol**	
MH	272	274	276	329	4x				
Conc	100	100	100	100					Ile1
µL	20	20	20	20	62.5	40	67.5	250	
MH	273	275	276						
Conc	100	100	100	100					Ile4
µL	20	20	20	20	62.5	40	67.5	250	

The scheme above shows the amount of substance used for the ligation experiment. The amount of oligomers (shown above), the DNA splint, the ligation premix and H_20 were pipetted into an eppendorf tube. The reaction tube together with its content was then heated to

75 °C for 5 min after which it was allowed to cool to rt within 10 min. The ligase was then added and the mixture kept at 16 °C and shaken at 300 rpm overnight.
NB: The ligation premix has the following constituent.80 µL of 5xKL, 10µL of H_2O, 8µL of 0.1M ATP and 2µL of 1M DTT.)

For the ligation kinetic, 5µL aliquots was taken after which 12µL of the DNAse I was added to the remaining portion and stirred at 37°C for an hour. A 12 % analytical PAGE gel reveals formation of the full length ligated product and also the digestion of the DNA splint. The product of the ligation was purified finally on a 12% PAGE after which the bands were excised and eluted overnight in an ammonium acetate solution.

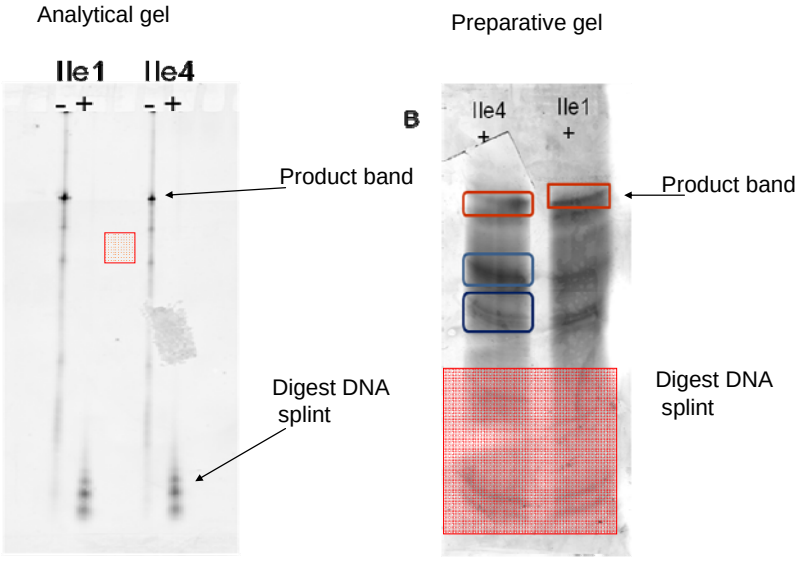


Figure 79 PAGE chromatogram after DNase digestion.

5.12.5 General Concentration of RNA by Alcohol Precipitation

RNA can be purified for RNA sequencing or for ligation or restriction digestion by precipitation in an alcohol/water mixture in the presence of a high concentration of inorganic salt. RNA is recovered from the aqueous solution by addition of salt to final concentrations of 0.8 M LiCl, 0.3-0.5 M NaCl, NaOAc, or 2.5 M NH_4OAc and an appropriate volume of alcohol (30 %-50 % final percentage isopropanol; 60 %-80 % final percentage ethanol), storage for a brief period of time at –20 °C or –70 °C, followed by centrifugation. Subsequent

desalting of the RNA pellet involves rinsing in 70 % alcohol, recentrifugation and re-suspension in appropriate buffer or water.

About 1/2 as much isopropanol as ethanol is required to precipitate RNA, although higher concentrations may be helpful when the RNA is at low concentration. Since isopropanol is less volatile than ethanol, and since many salts are less soluble in isopropanol than in ethanol, a second 70% alcohol rinse of the pellet is recommended to more efficiently desalt the RNA pellet. NaCl is not as soluble as NH_4OAc, NaOAc or LiCl in ethanol/water or isopropanol/ water; it can be replaced by the latter salts when feasible. Precipitations are commonly performed at –70 °C, -20 °C, 0 °C, or room temperature for periods of 5 min to overnight. For low RNA concentrations: higher final concentrations of alcohol, longer precipitations (1 h to overnight), lower temperatures (-20 °C to –70 °C) and longer centrifugation times (up to 30 min) may be required for efficient recovery. At the lower temperatures, the viscosity of the alcohol is greatly increased and centrifugation for longer times may be required to effectively pellet the precipitated RNA. The efficiency of precipitation for small concentrations or amounts of RNA may be increased by incubation at –70 °C, but these reactions should be brought to 0 °C before centrifugation.

When SDS is present, one must take care not to precipitate it along with the RNA. By adding NaCl to a final concentration of only 0.2 M or using LiCl (0.5 M) before adding the alcohol, the SDS will remain in the supernatant. Finally, one should take care to remove all NH_4^+ ions if subsequent enzymatic steps are to be utilized, as it inhibits many enzymes (especially T4 polynucleotide kinase).

5.12.6 Isopropanol Precipitation

(1) Add to the RNA solution 1/2 vol. of 7.5 M NH_4OAc and 2-2.5 vol. isopropanol (based on volume of RNA + salt solution) (1 vol. isopropanol is sufficient to precipitate RNA).

(2) Incubate at room temperature for 10 minutes.

(3) Spin for 10 minutes at highest speed and remove the supernatant solution. Wash with 70 % ethanol. The volume of ethanol should be sufficient to at least cover the pellet and wet the sides of the tube when vortexed (there is no volume too large). Vortex the sample for 1

minute; the pellet should come loose from the tube and be broken up in the EtOH. Centrifuge the sample 10 minutes highest speed, to recollect the pellet.

(4) Gently pour off ethanol being careful not to dislodge the pellet. Re-spin for 1 min and remove remaining ethanol using 100 µL pipette.

(5) Air dry for 10 min. Resuspend in volume of water or TE buffer to achieve desired final concentration of RNA.

General Notes:

(1) Advantage of isopropanol over ethanol - less volume, fewer salts co-precipitated, done at room temperature, and may be more effective in separating primers (which is not precipitated) from PCR products than ethanol.

(2) For improved recovery of RNA from dilute solutions (<10 ng/ml), overnight incubation and extended (30 min) centrifugation is recommended.

(3) In general, the length of time of centrifugation is more important for precipitation DNA than chilling the solution in -20 or -70 °C freezer.

(4) Addition of ammonium acetate to 2.5 M (without ethanol) has been shown to be effective in precipitating proteins while leaving the RNA in solution.

(5) NaOAc precipitation can precipitate large amount of protein together with theRNA.

5.12.7 Mass Spectrometry Analysis of Oligonucleotides

Conditions for MALDI-TOF MS analysis. Oligonucleotides were dissolved in water to a final concentration of ~10 µM and desalted by using C18-ZipTips (Millipore Corporation, Bedford, MA, USA). The C18 resin was wetted using 50% aqueous acetonitrile solution (4 x 10 µL) and then equilibrated by washing with 0.1 M TEAA (4 x 10 µL). For binding of the oligonucleotide to the resin, the sample was aspirated and dispensed (approx. 5-8 times). The salts were removed by washing with 0.1 M TEAA. The desalted DNA was eluted by aspirating and dispensing about 3 times 10 µL 60% acteonitrile/water in another vial. The samples for analysis were prepared using the dried droplet method with the

following matrix solutions: 1) 6-aza-2-thiothymine/diammonium hydrogen citrate in 1:2 v/v water/acetonitrile (detection in negative mode); 2) 3-hydroxy-picolinic acid/diammonium hydrogen citrate in 1.2:1 v/v water/acetonitrile (detection in positive mode) The results of the Maldi is summarized in Table 7.

6 Publications and Poster

Patent Application

Seidu-Larry, **S**., & Helm**, M.**, (**2008**) " Reaktive Fluoreszenzfarbstoffe und Leuko-Fluoreszenzfarbstoffe auf Xanthenbasis und Verfahren zur Herstellung derselben "

Book Chapters

Seidu-Larry, **S**., Porcher, S., Micura, R., & Helm, M. (**2008**): List of available phosphoramidites of modified nucleotides for chemical DNA/RNA synthesis.In Grosjean, H. (Ed.), DNA and RNA Modification Enzymes: Comparative Structure, Mechanism, Functions, Cellular Interactions and Evolution. *Landes Bioscience, Austin.* ***in press.***

Hayrapetyan, A., **Seidu-Larry**, **S**., & Helm, M**.* (2008)**: Function of modified nucleosides in RNA stabilization. In Grosjean, H. (Ed.), DNA and RNA Modification Enzymes: Comparative Structure, Mechanism, Functions, Cellular Interactions and Evolution. *Landes Bioscience, Austin.* ***in press.***

Poster:

Seidu-Larry, **S**.[#] Voigts-Hoffmann, F., & Helm, M. (**2006**): A Construction Kit for the Total Synthesis of tRNA including post-transcriptional Modifications. GBM meeting in Kassel, Germany.

7 References

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