A NEW SYNTHESIS FOR CYCLIC DIGUANYLIC ACID AND ITS ANALOGUES

Inaugural Dissertation

zur
Erlangung der Würde eines Doktors der Philosophie

vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

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Basel 2007

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät der Universität Basel auf Antrag der Herren

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Basel, den 16. Oktober 2007

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Dekan

The work presented here was initiated and supervised by Prof. Bernd Giese at the Chemistry Department of the University of Basel, during the time period June 2003 to August 2007.

Excerpts from this work have been published in:

N. Amiot, K. Heintz, B. Giese "New Approach for the Synthesis of c-di-GMP and its Analogues", *Synthesis*, **2006**, 24, 4230-4236

Excerpts from this work have been presented at the following Conferences:

Swiss Chemical Society – Fall Meeting, October 2006, Zürich, Switzerland

Swiss Chemical Society – Fall Meeting, October 2005, Lausanne, Switzerland

14th European Symposium on Organic Chemistry (ESOC 14), July 2005, Helsinki, Finland

24th RegioTriRhena Symposium on Organic and Bioorganic Chemistry, September 2004, Lucelle, France

À mes parents

“Patience et longueur de temps –
Font plus force ni que rage”

Jean de La Fontaine
Le Lion et le Rat (Fables, Livre II)

“Apprendre sans réfléchir est vain.
Réfléchir sans apprendre est dangereux”

Confucius

Acknowledgements

I would like to take this opportunity to extend my sincere gratitude to my thesis supervisor Prof. Dr. Bernd Giese for providing me a very interesting project and giving me the opportunity to work in his laboratory under his guidance and support. Thanks also to Prof. Dr. Wolf-Dietrich Woggon for accepting to co-referee this PhD thesis.

I am grateful to Prof. Dr. Urs Jenal, Prof. Dr. Tilman Schirmer, Prof. Dr. Serge Neunlist, and their research groups for fruitful collaborations.

I would also like to thank Dr. Nicolas Amiot whose support, advice, patience and motivation made this doctoral work not only possible, but also enjoyable.

Thank you to Dr. Wolfgang Seufert and Dr. Klaus Kulicke who recorded parts of the NMR spectra, also Dr. Heinz Nadig recorded the EI and FAB mass spectra and Werner Kirsch determined all elemental analyses. I would also like to thank the crew from the workshop for their prompt and friendly help with our everyday technical troubles and all the members of the staff who run the department and made work efficient and enjoyable.

I am indebted to those who were put under time pressure in proof-reading my dissertation - Dr. Nicolas Amiot, Dr. Jessica Bernard and Dr. Fabrizio Galbiati.

It has been a pleasure to work with the members, past and present, of the Giese and Stulz Groups. Special thanks also to Dr. Jessica Bernard and Kirsten Belser. Without their kind support, the work atmosphere they created and their comments and advice, this dissertation would not have been possible. My gratitude also goes to Stefanie Mesch for her help during her time with me as a “Wahlpraktikantin”.

Finally, it leaves me to thank my friends who have stood by me. You know who you are, and what you’ve done. Thanks a million! Last but most certainly not least - my parents and family. No words can express the unconditional support you have always given me. It makes this all worthwhile.

Also, I am grateful to the Swiss National Science Foundation and the University of Basel for their financial support.

Table of Contents

Abbreviations

ºC	Celsius degree
A	Adenine
Å	Angström
abs.	Absolute
Ac	Acetyl
ACN	Acetonitrile
All	Allyl
AMP	Adenosine monophosphate
AOC	(Allyloxy)carbonyl
ATP	Adenosine triphosphate
aq.	Aqueous
arom	aromatic
BSA	N,O-Bis(trimethylsilyl)acetamide
Bz	Benzoyl
C	Cytidine
Calcd	Calculated
CAM	Ceric Ammonium Molybdate
CAN	Ceric Ammonium Nitrate
c-di-AMP	Bis(3'-5')-cyclic diadenylic acid
c-di-GMP	Bis(3'-5')-cyclic diguanylic acid
c-di-(theo)MP	Bis(3'-5')-cyclic ditheophyllinic acid
c-di-TMP	Bis(3'-5')-cyclic dithymidic acid
COSY	Correlation Spectroscopy (NMR)
δ	Chemical shift (NMR)
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DCC	Dicyclohexylcarbodiimide
DCE	Dicloroethane
DEAD	Diethyl azodicarboxylate
DEPT	Distortionless Enhancement by Polarization Transfer (NMR)
DGC	Diguanylate cyclase
DIPEA	*N,N*-Diisopropylethylamine
DMA	Dimethylacetamide
DMAP	4-dimethylamino-pyridine
dmf	Dimethylaminoethylene
DMF	Dimethylformamide

DMSO	Dimethyl sulfoxide
DMT	Dimethoxytrityl
DNA	2'-deoxyribonucleic acid
dpa	Diphenylacetate
DUF	Domain of Unknown Function
EAL	Conserved Protein Sequence: Glutamic Acid-Alanine-Leucine
eq	Equivalent
ESI	Electron Spray Ionisation
Et	Ethyl
EtOAc	Ethylacetate
EtOH	Ethanol
Exopolysaccharide	EPS
g	Gram
G	Guanine
GGDEF	Conserved Protein Sequence: Glycine-Glycine-Aspartic Acid-Glutamic Acid-Phenylalanine
GMP	Ganosine monophosphate
GpGp	Guanylyl-(3'→5')guanosine 3'-phosphate
GTP	Guanosine triphosphate
HCTU	*O*-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HPLC	High Performance Liquid Chromatography
HMBC	Heteronuclear Multiple Bond Correlation (NMR)
HMQC	Heteronuclear Multiple Quantum Coherence (NMR)
HOBt	1-Hydroxybenzotriazole
hr	Hour
HRMS	High Resolution Mass Spectrometry
Hz	Hertz
iBu	*Iso*butyryl
iPr	*Iso*propyl
IR	Infrared
J	Coupling Constant
L	Litre
Lev	Levulinic acetate
M	Molar (mol/L)
m/z	Mass per Charge
Maldi-ToF	Matrix Assisted Laser Desorption Ionisation – Time of Flight-

	Mass Spectrometry
Me	Methyl
MeOH	Methanol
mg	Milligram
min	Minute
ml	Millilitre
mmol	Millimol
MHz	Megahertz
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
Npe	Nitrophenylethyl
NOESY	Nuclear Overhauser and Exchange Spectroscopy (NMR)
PDE	Phosphodiesterase
Ph	Phenyl
ppm	Parts per million
PTs	Pyridinium toluene-4-sulfonate
*p*TsA	*p*-Toluenesulfonic Acid
quant.	Quantitative
resp.	Respectively
R_f	Retention Factor
RNA	Ribonucleic Acid
RP	Reverse Phase
RT	Room Temperature
sat.	Saturated
sol.	Solution
t or *tert*	Tertiary
T	Thymine
TBDMS	*tert*-butyldimethylsilyl
TEAC	Triethylammonium carbonate
THF	Tetrahydrofuran
THP	Tetrahydropyranyl
TLC	Thin Layer Chromatography
TMP	Thymine Monophosphate
TMS	Tetramethylsilate
TMSOTf	Trimethylsilyl triflate
TOCSY	Total Correlation Spectroscopy (NMR)
TPSNT	1-(2,4,6-tri*iso*propylbenzenesulfonyl)-3-nitro-1*H*-1,2,4-triazole
Tom	[(Triisopropylsilyl)oxy]methyl

UV	Ultraviolet
v	Volume
µl	Microlitre

Abstract

Cyclic bis(3'-5')diguanylic acid (c-di-GMP) has been the focus of many research endeavors for biologists in the last decade. Indeed, this cyclic dinucleotide has been identified as a novel secondary messenger recently.[8-11, 49] This new discovery caused increasing interest in the regulation system which involves c-di-GMP. This insight, recently led to widespread findings about c-di-GMP in other bacteria. The cyclic bis(3'-5')-nucleotide has been shown to regulate the transition from motility to sessility in bacteria including *Caulobacter crescentus*[15], *Escherichia coli* and the pathogenic bacteria *Pseudomonas aeroginosa* and *Salmonella typhimurium*[7].

This cyclic dinucleotide also showed an influence on community behavior like biofilm formation in pathogenic bacteria including *Pseudomonas fluorescens*[16], *Yersinia pestis*[17] and *Vibrio cholerae*[18]. It is also involved in the inhibition of *Staphylococcus aureus* cell–cell interactions and biofilm formation, as well as in the reduction of the virulence of the biofilm-forming strains of the same bacterium in a mouse model of mastitis infection.[19]

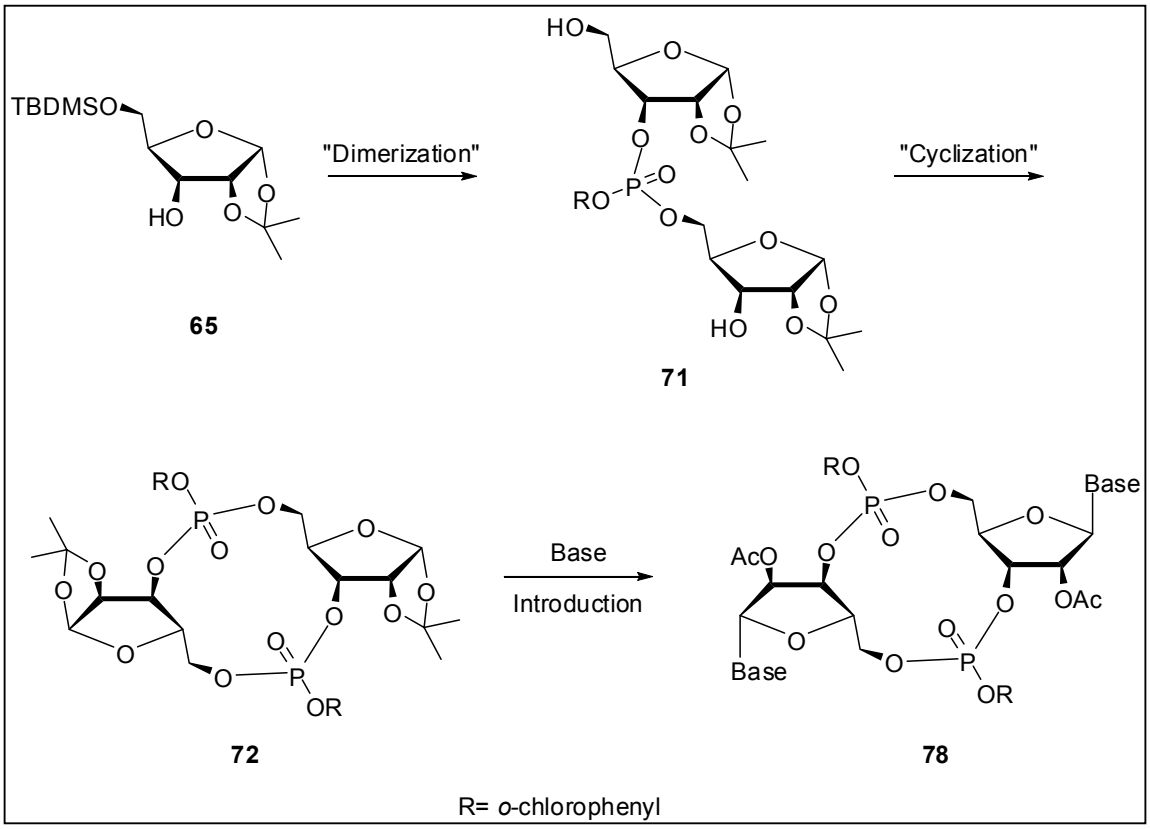
These findings suggest that cyclic diguanylic acid might be useful in preventing biofilm formation on clinically relevant surfaces such as medical devices and potentially, in the control and treatment of human and animal infection.[17] The biological activity might be even wider since reports have pointed out that this compound may have anticancer activity.[18]

Thus, c-di-GMP represents an excellent platform for drug design in medicinal chemistry and especially in the field of antibiotics where compounds with new modes of action are required. However, the mechanisms of c-di-GMP dependent signalling remain unknown, mainly because little data is available on c-di-GMP.[8,10] In order to study the biochemistry of this cyclic dinucleotide more in detail we have started this project dedicated to the synthesis of c-di-GMP and its analogues.

We intended to develop a synthetic pathway which could afford an efficient, reliable, flexible and scalable route to synthesize c-di-GMP. At the beginning of this work, the only reported synthetic route for c-di-GMP was the van Boom *et al.*[22-23] method starting from guanosine and using the phosphotriester methodology. This method was the starting point of our own synthetic investigations, even so the published synthesis needed tedious purification steps and its length rendered it only moderately suitable for eventual scale-up purposes.

In the course of this work, two more synthetic pathways were reported by Hayakawa *et al.*[29] and Jones *et al.*[30] claiming better yields, easier realization and shorter reaction sequences. We then decided to apply some of their improvements, by modifying the guanosine building block to make it less polar but still use the phosphotriester methodology towards an easier assembly of c-di-GMP. However, no previously described method afforded large quantities of c-di-GMP.

After having explored the different existing synthetic routes, it quickly became obvious that we would have to design a new method to obtain this compound in sizeable amounts to satisfy the demands for the biological investigations. We have decided to adopt a brand new approach in which we start from ribose building blocks and synthesize a sugar-phosphate backbone, and to introduce the base at a late stage (*Scheme A*). Through this route we anticipate to completely solve the difficulties, generally caused by the 2'-OH protection, by using the 1',2'-acetal protecting group.



Scheme A: New synthetic approach for the synthesis of c-di-GMP.

In order to show the flexibility of our new synthetic route, the synthesis of base-modified analogues of c-di-GMP was undertaken. The intend was to show that the synthesis is not specific for purine bases but can be applied to pyrimidine bases as well as non natural nucleobases, such as xanthine or theophylline for example. Finally, we applied the same strategy to the synthesis of internucleotide linkage modified analogues.

General Part

1. INTRODUCTION

1.1 Cyclic Nucleotides: a Background

The idea that purines could act as extracellular signaling molecules was first proposed over 80 years ago.[1] Extracellular nucleotides have since been implicated in a wide range of biological processes, including smooth muscle contraction, inflammation, platelet aggregation and pain, among many others. Cell-surface receptors for these purines and pyrimidines, termed purine receptors, have been identified and classified in two groups, P1 and P2 receptors, recognizing adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), uridine 5'-triphospahte (UTP) and uridine 5'-diphosphate (UDP).[2]

Intracellular signaling relies on the perception of such a signal at the plasma membrane by a receptor, then activating the formation of a secondary signal within the cell, which ultimately modifies the activity of an effector molecule. The lack of knowledge about the receptors and effectors in those signaling processes has often been the difficulty. Recently, a variety of novel molecular genetic approaches have been adopted, to address not only the target systems where a specific signaling molecule is active in plant cells, but also the processes in which such molecules may be involved. Hence, an increasing array of signaling molecules, their biosynthetic enzymes and effectors are emerging. In recent work, a variety of processes have been shown to be triggered by secondary signaling molecules.[3]

Nucleotides such as cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) (see *Figure 1*) have been recognized as important molecules in biological signal transduction pathways in animals and higher plants. They have also been shown to perform parallel regulatory functions in bacteria and lower organisms.[4]

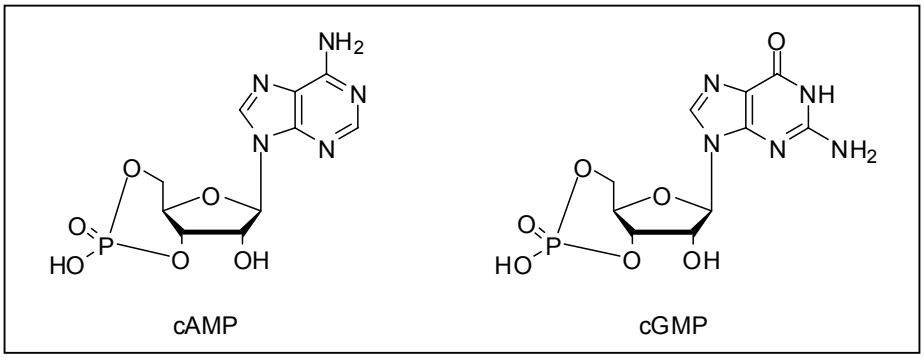


Figure 1: Cyclic ribonucleotide second messengers.

cAMP, a prototype second messenger when first discovered, has been established as a signaling molecule in both eukaryotes and prokaryotes. From its discovery, by Nobel Prize winner Earl Sutherland,[5] and the subsequent demonstration of its role in mediating the action of mammalian hormones in the liver, the idea of secondary-messengers was developed. According to this concept, mammalian hormones and neurotransmitters, acting as primary messengers, remain outside the cell. While binding to their specific receptors, a change of conformation occurs; this change transmits their signal to the interior of the cell resulting in the synthesis and release of the secondary messenger cAMP inside the cell.[6] A second cyclic nucleotide, 3',5'-cyclic guanosine monophosphate (cGMP), has been shown to have a more restricted role, in mammals at least. It has been isolated from living tissues and found to be carrying out second messenger roles.[6]

cAMP and cGMP are general cell signaling molecules of eukaryotes, such as humans, plants and fungi. In those organisms, these two molecules have been shown to be involved in a broad spectrum of cellular processes such as in the regulation of metabolic processes, cell differentiation, immunity, the transduction of olfactory and visual signals, photomorphogenesis, as well as in the regulation of complex behavioral processes of higher organisms such as learning and depression.[7]

While bacterial pathogens can interfere with the cGMP signaling of their eukaryotic host cells, prokaryotes in general do not seem to use cGMP for signaling. This suggests the existence of an alternative molecule. And indeed, recent discoveries seem to suggest that bacteria make extensive use of another cyclic guanosine compound: cyclic diguanylic acid (c-di-GMP).[8-11]

1.2 Biological Occurrence and Mode of Action of Cyclic Diguanylic Acid

Bacteria modify their cell surface in response to environmental cues. These changes can facilitate either dispersion to a new environment or adhesion to a surface, including aggregation with members of their own or other species. The particular outcome is often determined by changes in exopolysaccharides (EPS) and proteinaceous appendages. *Gluconacetobacter xylinum*, for example, produces an extracellular matrix of cellulose. In the last few decades, studies of this feature led to the identification of plant cellulose synthase genes, which then allowed the discovery of bis(3'-5')-cyclic diguanylic acid (c-di-GMP) (see *Figure 2*) as a regulatory nucleotide in the β-1,4-glucan (cellulose) biogenesis. The proteins regulating the intracellular levels of c-di-GMP for these bacteria contain two genome sequences, the so-called GGDEF and EAL domains. They are widespread domains in bacteria, but remained mostly uncharacterized signaling systems. The members of this protein family, that have been characterized, showed one common theme: regulation of bacterial cell surface adhesiveness. Since proteins containing those domains are found in diverse bacteria, it is highly likely that signaling by c-di-GMP is a conserved physiological basis for their activities.[12]

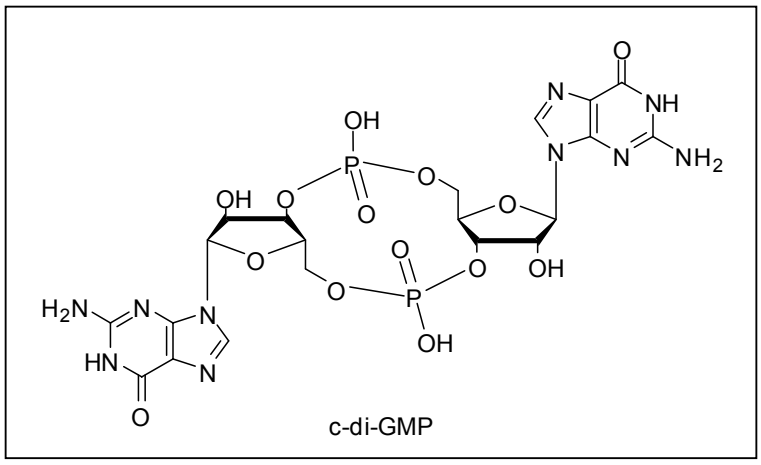


Figure 2: Cyclic diguanylic acid (c-di-GMP).

It has been shown that c-di-GMP, enzymatically formed from GTP, plays a central regulatory role in modulating the rate of cellulose-producing for bacterium *Gluconacetobacter xylinum*. The suggested model proposes that c-di-GMP functions as a reversible allosteric activator of the membrane-bound cellulose synthase. It is referred to as an allosteric effector

in the sense that it binds directly to the enzyme in a reversible manner at a regulatory site, distinct from that of the catalytic or substrate-binding sites.[13] The enzymes controlling the turnover of c-di-GMP are called diguanylate cyclase (DGC), which catalyzes its formation, and phosphodiesterase A (PDEA), which catalyzes its degradation.[14]

This insight, recently led to widespread findings about c-di-GMP in other bacteria. The cyclic bis(3'-5')-nucleotide has been shown to regulate the transition from motility to sessility in bacteria including *Caulobacter crescentus*[15], *Escherichia coli* and the pathogenic bacteria *Pseudomonas aeroginosa* and *Salmonella typhimurium*[7], and community behavior like biofilm formation in pathogenic bacteria including *Pseudomonas fluorescens*[16], *Yersinia pestis*[17] and *Vibrio cholerae*[18]. It is also involved in the inhibition of *Staphylococcus aureus* cell–cell interactions and biofilm formation, as well as in the reduction of the virulence of the biofilm-forming strains of the same bacterium in a mouse model of mastitis infection.[19]

The recent suggestion that c-di-GMP might be a novel secondary messenger[8-11] has caused increasing interest in the regulation system which involves this cyclic dinucleotide. These findings also suggest that cyclic diguanylic acid might be useful in preventing biofilm formation on clinically relevant surfaces such as medical devices and potentially, in the control and treatment of human and animal infection.[20] The biological activity might be even wider since reports have pointed out that this compound may have anticancer activity.[21]

Even though all these results have shown the implication of c-di-GMP in various biological processes, a clear understanding on the molecular level has yet to be achieved. In order to further investigate these questions, high quantities of c-di-GMP are needed.

1.3 Structure and Properties of Cyclic Diguanylic Acid

Because of the rising importance of c-di-GMP in microbiological investigations, this cyclic dinucleotide has been brought into the spotlight. This intriguing molecule has sparked interest as a result of its particular properties that might be due to the biological activity it displays. Therefore, the structure of c-di-GMP has been investigated. Bis(3'-5')-cyclic diguanylic acid is a cyclic dinucleotide where the two ribose units of guanosine monophosphates are connected *via* two intermolecular 3'-5' phosphodiester bonds. This linkage builds a 12-membered circular sugar-phosphate backbone that provides a rigid framework which holds the guanines in parallel planes, 6.8 Å apart. (*Figure 5*)

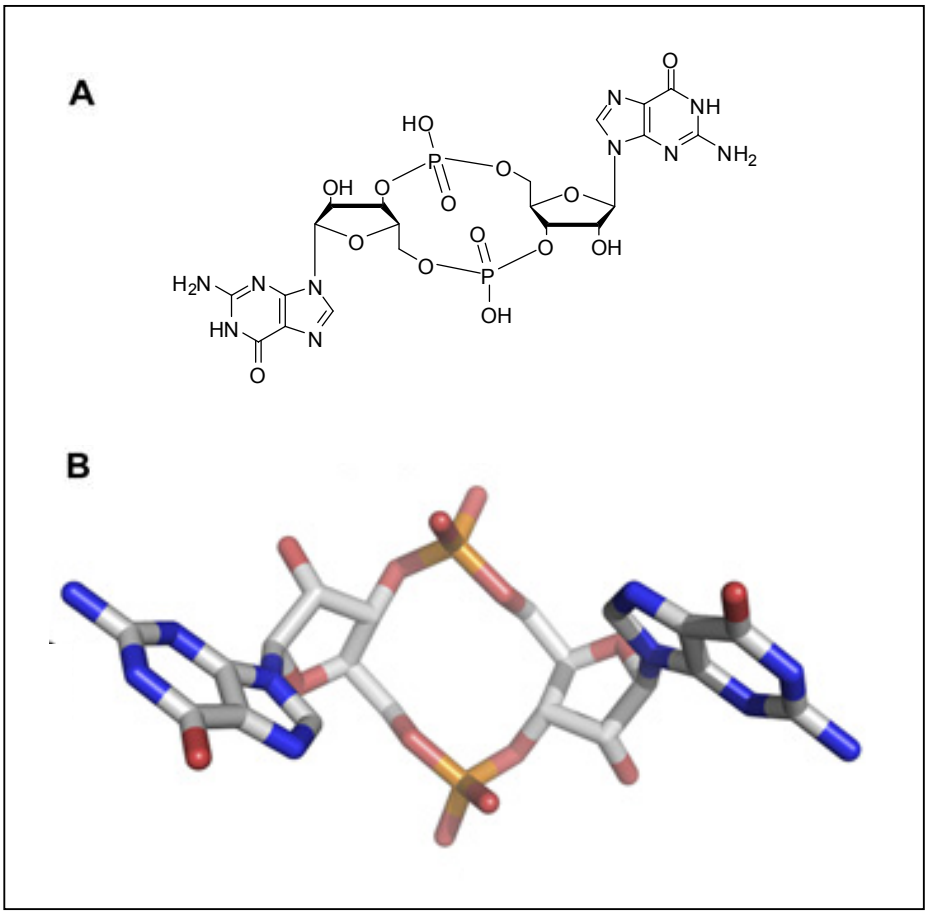


Figure 5: Structure of c-di-GMP. **A**: chemical structure of a c-di-GMP molecule. **B**: crystallographic representation of a molecule of c-di-GMP. (Nitrogens are in blue, oxygen atoms are in red and phosphorus atoms are in orange. The diagram was produced in the programme DINO.)[22]

According to published work[22-23], when crystallized in the presence of hydrated magnesium ions, two c-di-GMP units form an intercalated unit stabilized by stacks of four guanine bases, such that the imidazole ring of one guanine is positioned over the pyrimidine ring of the next. (see *Figure 6*) Each outer guanine of the four-member stack is nearly coplanar with the adjacent base in the stack. However, the two central bases are not coplanar but partially unstacked.

In addition, this dimer is also stabilized by a set of parallel hydrogen bonds between the nitrogen of the guanine and the oxygen of the phosphate of the other monomer. The hydrogen bonds also include the hydrated Mg^{2+} complex, since two of the four water molecules interact with both oxygen atoms of the phosphate groups not involved in interactions with the base and the other two water molecules form hydrogen bonds with the oxygen atoms of the two central bases. Thus hydrated magnesium ions play an integral role in the interaction between the two c-di-GMP monomers.

When considering the two independent c-di-GMP molecules, they have been shown to have very similar conformations when superposed. The 12-membered ring formed by a cyclic phosphodiester backbone, exhibits no deviations from standard torsion angles in this type of rings, which suggest that the ring closes easily. Also, a nearly perfect twofold symmetry is maintained by the backbone atoms, indicating that the conformations of the two phosphate residues in each ring are very similar.

In this structure, all the riboses adopt a 2'-exo/3'-endo conformation, as expected for RNA. Thus the intramolecular twofold symmetry is broken by the glycosyl torsion angle. The guanines are in two different orientations relative to the sugar-phosphate backbone ring, meaning the bases are not parallel but skewed at an angle, both being in an anti-conformation with respect to the riboses.[22]

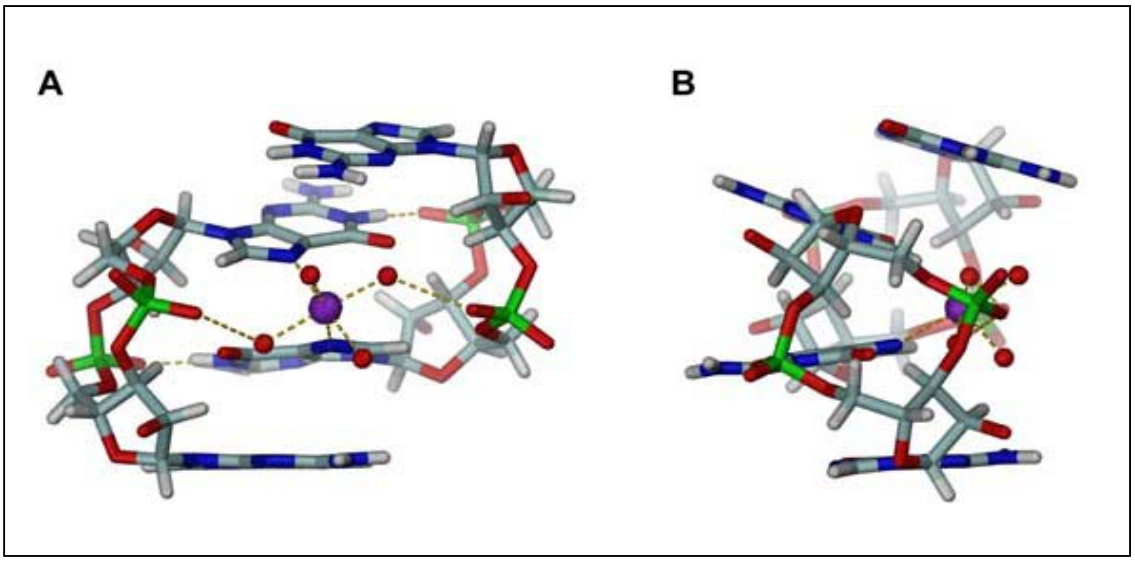


Figure 6: Crystal structure of c-di-GMP showing two intercalated c-di-GMP molecules, emphasizing base-solvent and base-backbone hydrogen bonds of c-di-GMP.[22, 23] **A**: Front view shows the alternate stacking of the guanine bases coordinated by the hydrated Mg^{2+} ion (purple). **B**: Side view shows the cyclic structure with the two phosphodiester linkages. (Water molecules are in red. Nitrogen atoms are blue, oxygen atoms are red, phosphorus atoms are green and hydrogen bonds are included as dashed lines. All diagrams are produced in the programme DINO.)

The structure of c-di-GMP might be directly related to the biological processes involving it. This is currently under investigation. It is also remarkable that the crystal structure seems to be depending on the salts used during the crystallization procedure.[22-23] This might also be one of the reasons for difficulties reportedly encountered during the purification of synthetic c-di-GMP.

1.4 Published c-di-GMP Synthetic Work

Facing growing needs in cyclic bis(3'-5') diguanylic acid, the chemical synthesis of this compound became an important option. It attracted great attention in the last two decades. Hence, it was necessary to develop an efficient chemical method for synthesizing c-di-GMP to meet those needs. Thus far, there were three different strategies developed, by three groups, with various efficiencies.

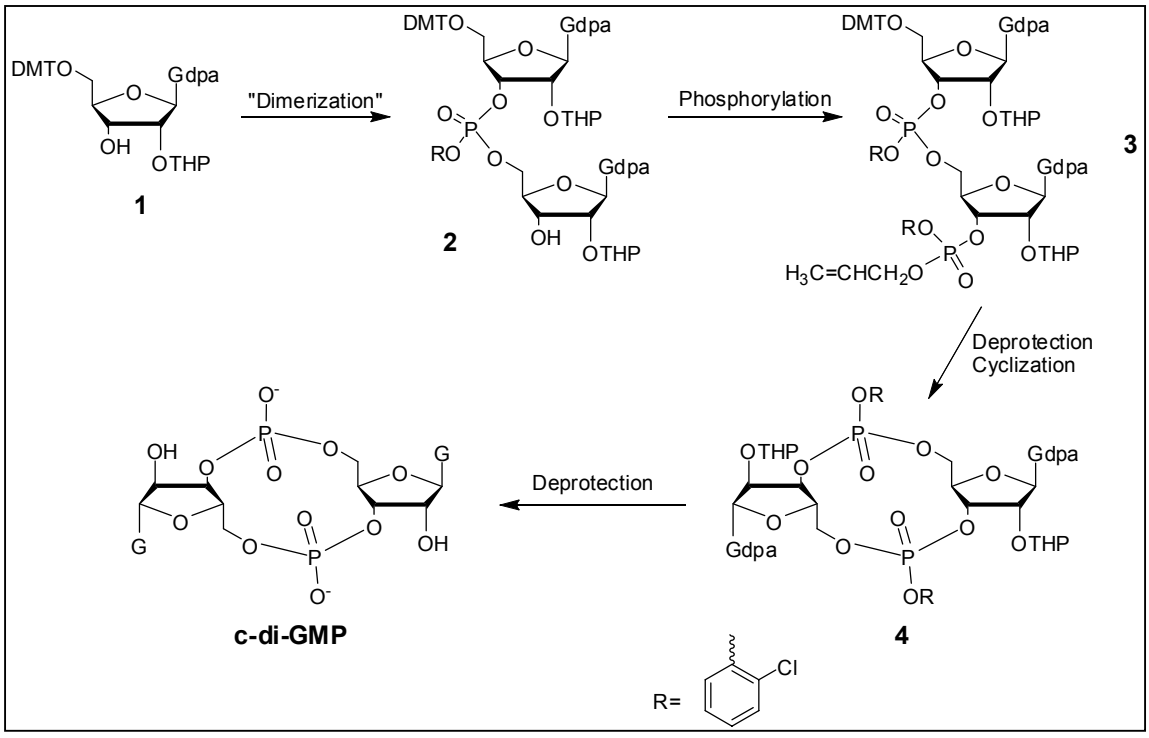
1.4.1 Phosphotriester Methodology

For oligonucleotide syntheses *via* the phosphotriester method, the synthetic intermediates are nucleotides were the internucleotidic phosphate linkages are protected by a third esterifying function. This must satisfy two main criteria: it must be selectively and readily removable from the phosphotriester functions, and secondly, it must remain intact under the conditions of acidic and basic hydrolysis which are necessary for the removal of the common protecting groups from the sugar hydroxyl functions.

The phosphotriester approach is considered the most versatile method of oligonucleotide synthesis and has a number of significant advantages over other methods that have been developed later on. First and most important, both the nucleotide building blocks and the phosphotriester intermediates are very stable and easy to handle in solution. Another positive aspect includes the possibility to use monomers, dimers as well as large nucleotide building blocks. The coupling reactions show a relative lack of sensitivity to small quantities of moisture, which are removed by the excess condensing agent. Besides, only a slight excess of nucleotide building block is needed to perform the coupling reactions. Finally, the most interesting feature of this method is its suitability for the synthesis of small DNA- and RNA-oligonucleotides in solution, therefore it is particularly interesting for the synthesis of cyclic nucleotides, a prime example being c-di-GMP.[24]

Van Boom *et al.*[25-26] were the first group to publish a chemical synthetic pathway for c-di-GMP in the late eighties. Their strategy was based on a modified hydroxybenzotriazole phosphotriester approach were two protected guanosine building blocks were coupled through a 2-chlorophenyl protected phosphotriester linkage to form the cyclic dinucleotide. (*Scheme 1*)

Through a protection-deprotection sequence, building block **1** was synthesized over six separate reaction steps in an overall yield of 65%. The 2'- and 5'-hydroxy groups of the ribose were protected with the acid-labile tetrahydropyranyl (THP) and dimethoxytrityl (DMT) groups. The 2-amine of the base was blocked with the base-labile diphenylacetyl (dpa) group.[26]



Scheme 1: van Boom synthesis pathway *via* phosphotriester methodology.

Guanosine **1** was phosphorylated *via* a bis(trifluoromethyl-hydroxybenzotriazole)-2-chlorophenyl phosphate and combined with a second unit of building block **1**, which had previously been deprotected at the 5'-OH position, to yield dimer **2**. This compound was then phosphorylated using the same method as described above; the phosphate was protected using allyl alcohol to yield intermediate **3**. After removal of the dimethoxytrityl and allyl groups, cyclization was achieved. Thus, fully protected compound **4** was obtained in 11 steps in an overall yield of 26%, starting from commercially available guanosine. The final deprotection of cyclic nucleotide **4** then afforded a small amount of c-di-GMP.[26]

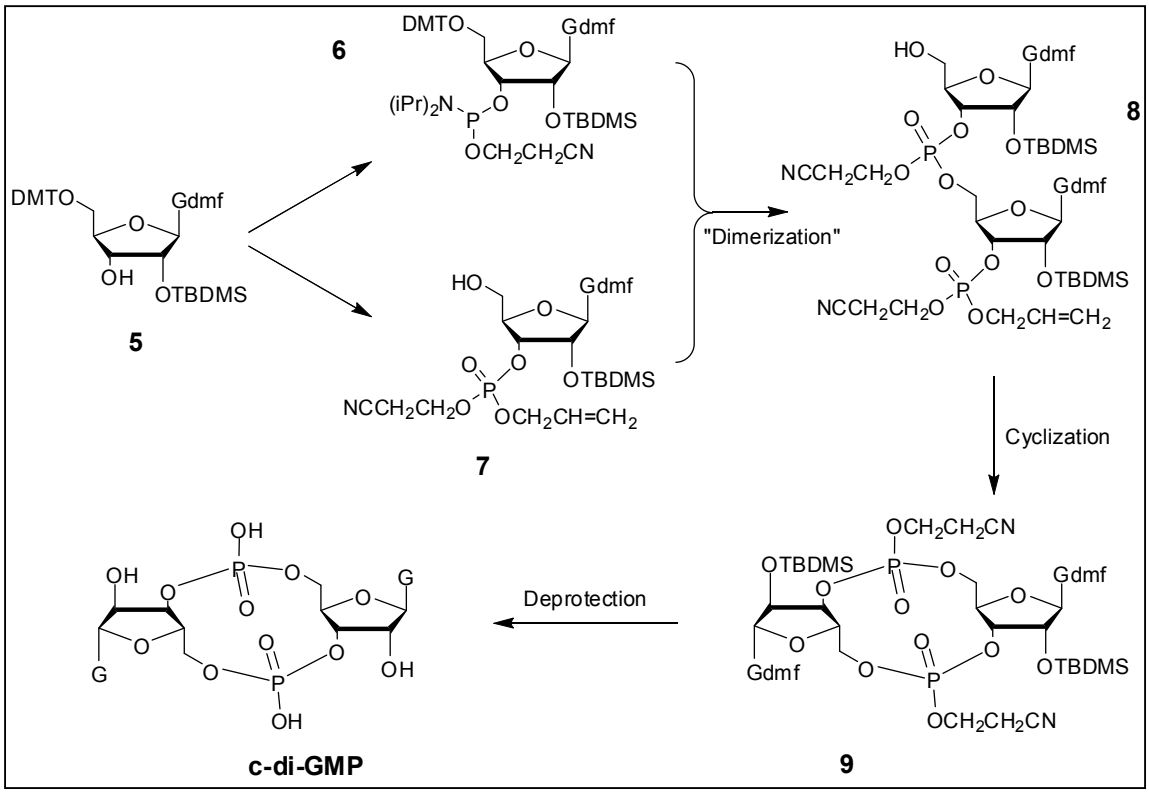
This synthetic pathway has been the first reported, but it showed some drawbacks. The synthesis of the building block was long with its accumulation of steps. The dimerization and cyclization to yield to c-di-GMP were also a rather long process with repetition steps. Furthermore, only a few of the reaction procedures were described and others were not reported in detail. Finally, only a small amount of substance was synthesized and the yield of the final deprotection step was not reported.

1.4.2 Phosphoramidite Methodology

The phosphoramidite method requires that the building blocks are nucleotides where the phosphate linkage is blocked as a trivalent moiety by two different functional groups. First, there is a di*iso*propylamine group, which is stable until the addition of the tetrazole when coupling, and serves to activate the nucleotide coupling process, and second, a cyanoethyl protecting group is used, which prevents side reactions, aids the solubility and is removed only at the final deprotection stage.[27]

The phosphoramidite approach is the most efficient as a solid-supported DNA- and RNA-oligonucleotide synthesis method.[28] Traditionally, the procedure starts with immobilizing the first nucleotide on solid-support by the 3'-hydroxy function. 3'-phosphoramidite substituted building blocks are prepared separately, and will then be used to assemble the oligonucleotides. The 5'-hydroxyl group of the immobilized nucleobase is first deprotected, then coupled with a second phosphorylated nucleotide unit, in the presence of tetrazole as an activator. Once formed the new phosphite internucleotide bond is finally oxidized to the more stable phosphotriester linkage, before the oligonucleotide can enter a new deprotection-coupling-oxidation sequence.

This method can be used in solution, even if the stability of the intermediates and building blocks is reduced. In 2004, Hayakawa *et al.*[29] published an alternative synthetic pathway to the van Boom route to prepare c-di-GMP, using modern DNA and RNA synthesis methods based on phosphoramidite coupling agents in solution (*Scheme 2*). The first step of the synthesis was the protection of the guanosine with a *tert*butyldimethylsilyl group on the 2'-OH position and a dimethyltrityl group on the 5'-OH of the sugar unit, and a dimethylaminoethylene group on the 2-*N*-amine of the base. This building block **5** was synthesized over three steps in an overall yield of 59% from guanosine.[29]



Scheme 2: Hayakawa synthesis pathway using phosphoramidite methodology.

Precursor **6** was then obtained *via* condensation of building block **5** with a cyanoethyl phosphoramidite in solution. Subsequent treatment of a part of **6** with allyl alcohol led to protected intermediate **7**. After coupling compound **7** with precursor **6** and removing the dimethoxytrityl group, dimer **8** was obtained. The allyl group of the phosphotriester moiety was removed and the resulting alcohol was converted to cyclic dinucleotide **9**. Finally, successive deprotection steps afforded c-di-GMP in an overall yield of 31% in 5 separate steps starting from building block **5**.[29]

This synthesis pathway suffers principally from the fact that the phosphoramidite methodology is not the method of choice when doing solution phase chemistry with nucleotides. Indeed, the yields are lower than in the case of solid supported couplings, and the stability of the phosphoramidite substituted nucleotides is also lower. Moreover, the synthesis of the building block, as well as the assembling of c-di-GMP, is a rather long process to obtain a small quantity of product, and the overall yield was 18% over 8 reaction steps, starting from commercially available guanosine. Another drawback is the matter of chemoselectivity when trying to protect the 2'-hydroxy group, which will be a recurring theme in the different attempt to develop a suitable synthetic route.

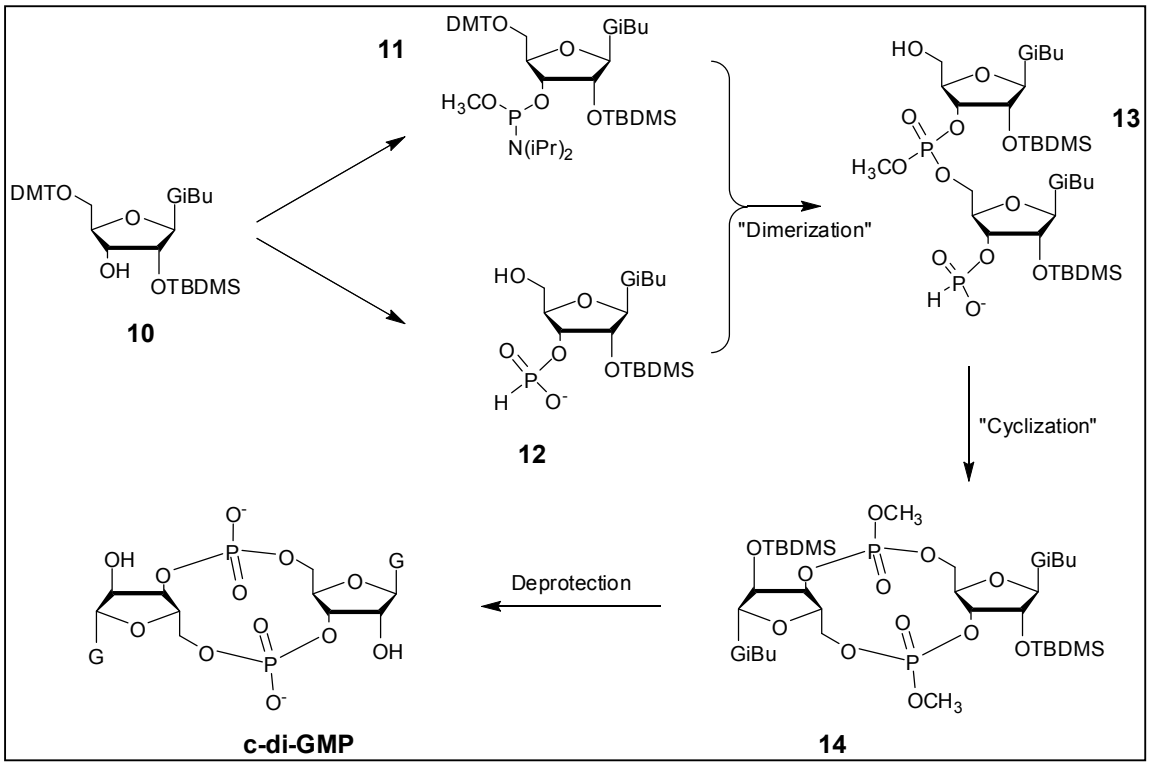
1.4.3 Hydrogen-Phosphonate Methodology

H-phosphonate monomers are useful for the preparation of internucleotide linkages that are not attainable by standard phosphoramidite chemistry. Here, the monomer used is a 5'-DMT-3'-hydrogen phosphonate-protected nucleoside that will be activated. The presence of the H-phosphonate moiety on these monomers renders phosphate protection unnecessary as it is a blocking group.

The hydrogen-phosphonate synthesis methodology for oligonucleotides, is very similar to that of the phosphoramidite approach. Slight differences result from the properties of the monomers utilized. For example, a different activating agent is used. The free oxygen on the H-phosphonate is activated and the free 5'-OH of the second monomer performs a nucleophilic attack on the activated phosphorous. In addition, the H-phosphonate diesters generated by these coupling reactions are stable to the normal reaction conditions, so oxidation at every step is unnecessary.

The H-phosphonate approach has primarily been developed as a solid-supported synthesis pathway to obtain DNA- or RNA-oligonucleotides, but it can also be applied in solution, generally for cyclic nucleotides. In 2004, Jones *et al.*[30] published a second alternative to the synthesis of c-di-GMP using the standard phosphoramidite method coupled with an H-phosphonate cyclization process. (*Scheme 3*)

The precursor used here to prepare c-di-GMP, was reported by Serebryany *et al.* in 2002.[31] The synthetic route was started with the introduction of the appropriate protecting groups on a guanosine to yield building block **10**. This consists in blocking position 2'-OH of the ribose with a *tert*butyldimethylsilyl (TBDMS) group and position 5'-OH with a dimethyltrityl group. The base was protected as an *iso*butyryl amide on the 2-*N*-amine. The building block was obtained in five steps in an overall yield of 79%.[31]



Scheme 3: Jones synthesis pathway using H-phosphonate method.

Following the preparation of precursor **10**, conversion to intermediate **11** was achieved by using the bis(di*iso*propylamino)methyl phosphoramidite and compound **12** was obtained from **10** by reaction with 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one and subsequent removal of the dimethyltrityl group. Dimer **13** was then prepared using the standard phosphoramidite methodology. After cyclization, the H-phosphonate is conveniently oxidized to a methyl triester to yield fully protected cyclic dinucleotide **14**. Finally, deprotection reactions led to c-di-GMP in an overall yield of 39% in the five steps.[30]

The main drawbacks of this method are essentially similar to the ones mentioned for the phosphoramidite approach. Indeed, this method is not a pure H-phosphonate coupling method only, but is mixed with phosphoramidite coupling steps. This means the difficulties encountered for the phosphoramidite methodology are also an issue here. One of the building blocks was substituted with a phosphoramidite, which is not ideal for “in solution” chemistry, due to the lack of stability of these building blocks. Also the matter of chemoselectivity, when trying to block the 2’-OH group is not really solved with this approach either, since 3’,5’-cyclic intermediate was prepared first. Nevertheless, c-di-GMP could be obtained in a 30% yield over 10 steps, starting from commercially available guanosine.

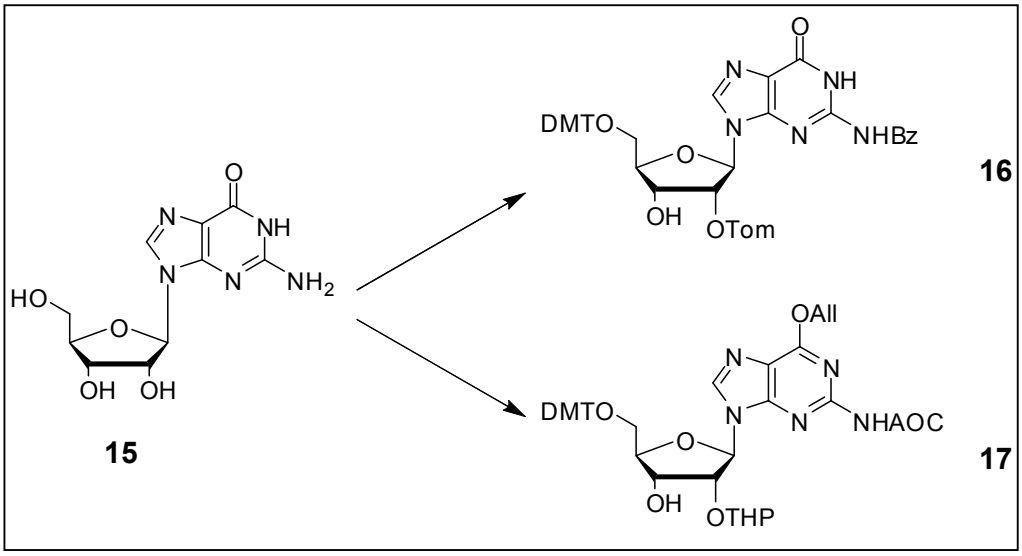
1.5 Research Project

The aim of this project was to develop a synthetic pathway which could afford an efficient, reliable, flexible and up-scalable route to synthesize c-di-GMP. No previously described method afforded large quantities of c-di-GMP. A new synthesis had to be designed to obtain this compound in sizeable amounts to satisfy the demands for the biological investigations.

One of the more predominant differences between the syntheses of DNA- and RNA-oligonucleotides is the presence of the 2'-hydroxy function. Many reports on the various preparation processes of DNA-nucleotides exist but few can be applied to RNA-nucleotides because of this functional group. The difficulty to perform a selective protection of the 2'-OH position of the ribose is one of the reasons for the small amount of reported RNA-nucleotide syntheses and one of its main drawbacks. Even if selective protecting procedures have been reported, most of them have been developed in combination with the other natural nucleotides. The existing methodologies have been reportedly more difficult to apply to the guanosine series, due to its rather unique behavior under the standard oligonucleotide chemistry.

At the beginning of this work, the only reported synthetic route for c-di-GMP was the van Boom *et al.*[25-26] method starting from guanosine and using the phosphotriester methodology (*Scheme 1*). We have decided to investigate this first route that seemed to be the best approach for short cyclic oligonucleotides. Although the phosphoramidite coupling procedure seemed more modern, it appeared less suitable for our project.

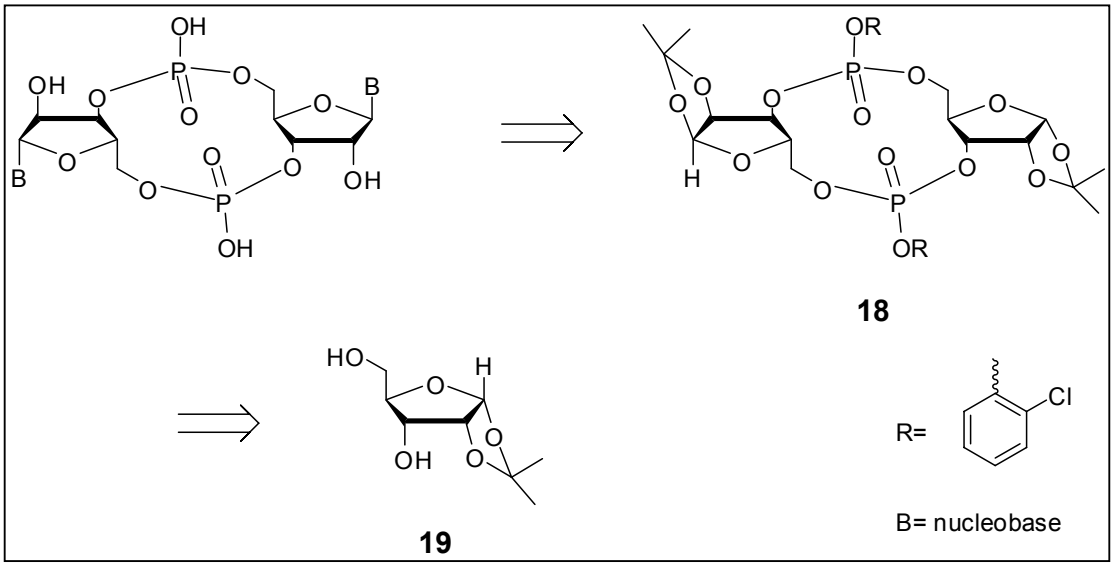
Even so, the published synthesis was cause for tedious purification steps and its length rendered it only moderately suitable for eventual scale-up purposes. At this point in the course of this work, two more synthetic pathways were reported by Hayakawa *et al.*[29] and Jones *et al.*[30] claiming better yields, easier realization and shorter reaction sequences. It was then planed to first modify the guanosine building block to make it less polar, but still use the phosphotriester methodology towards an easier assembly of c-di-GMP. (see *Scheme 4*)



Scheme 4: Examples of modifications performed on the building block.

The ultimate goal of this research project was to design a new synthetic route that could be applied to the process chemistry of c-di-GMP. The newly reported alternatives proved difficult to carry out to achieve this goal. They represented long reaction sequences to obtain small amounts or even no product. It was then decided to try a completely new approach.

Considering the difficulties inherent to the selective protection of the 2'-hydroxy group in the guanosine series, it was decided to bypass this issue by changing the starting material completely. Following the report of compound **18**[32] (see *Scheme 5*) as a by-product in the synthesis of carbohydrate phosphates, the new reactant that would be tested, would be commercially available 1,2-*O*-*iso*propylidene-α-D-xylofuranose **19**. This new approach, would lead to a late introduction of the base moiety on the ribose, which allowed for more variation potential and the facile synthesis of base-modified analogues. This modification in the strategy would also induce more flexibility in the synthetic pathway and the same approach could also be used to prepare internucleotide linkage modified analogues.



Scheme 5: Retro-synthetic overview of the carbohydrate phosphate.

2. INVESTIGATIONS TOWARDS A NEW APPROACH FOR THE SYNTHESIS OF C-DI-GMP

2.1 Synthesis of c-di-GMP Based on the van Boom Route

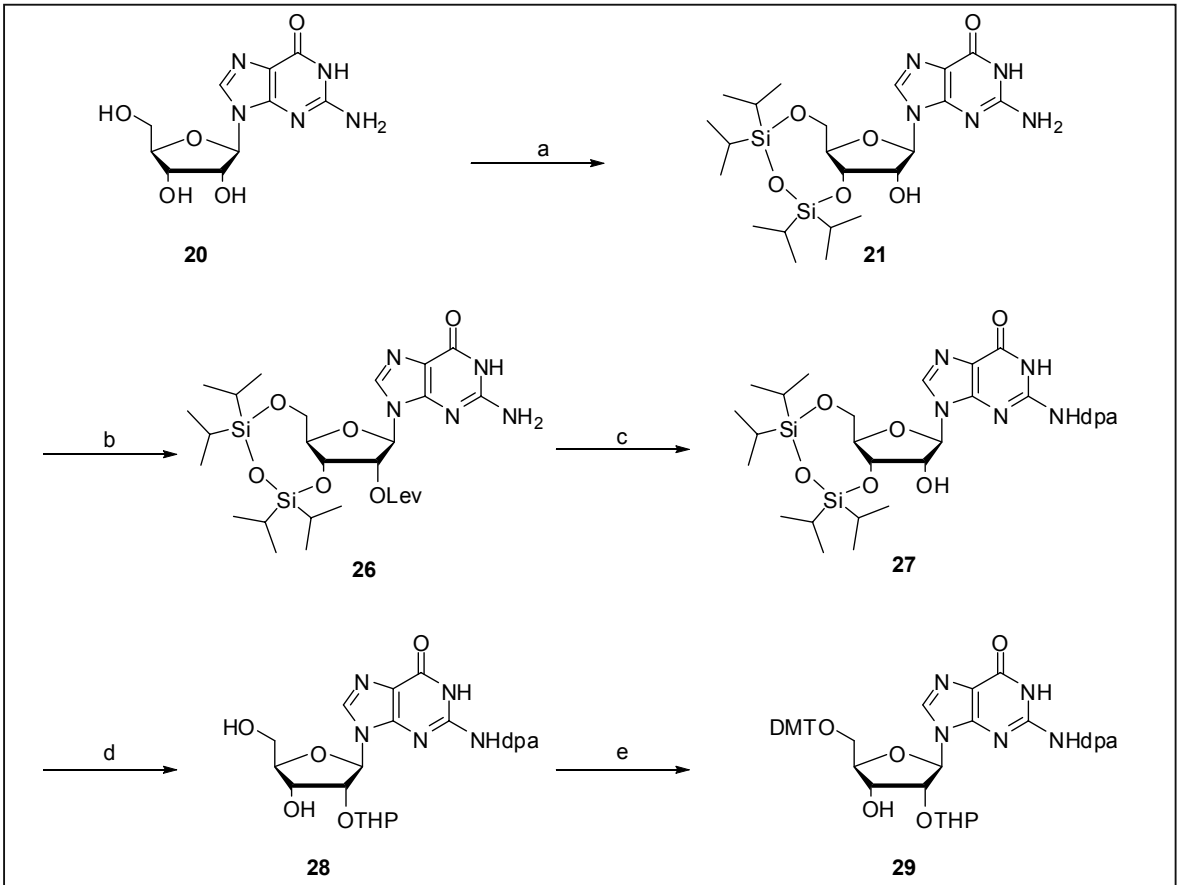
2.1.1 Synthesis of the Guanosine Building Block

At the beginning of this project, the aim was to synthesize c-di-GMP in an efficient manner. At the time, this molecule was already attracting considerable attention, due to the ongoing investigations of its biological relevance and properties, and the necessity of a synthetic method to afford this molecule became obvious. But only one chemical synthetic pathway had been reported so far. In 1990, van Boom *et al.*[26] published the total synthesis of c-di-GMP starting from guanosine, using a complex protection-deprotection strategy to afford buildings blocks which would then be assembled using the phosphotriester DNA-coupling method.

Even if this synthesis was long standing, it was the only available one and thus the start of our synthetic efforts. Indeed, we decided to tackle the synthesis of c-di-GMP by first reproducing the van Boom *et al.*[26] methodology, in order to gain more insight into the specifics the RNA-oligonucleotide synthesis and the particular behavior of guanosine.

As mentioned previously, inserting a protecting group selectively on the 2'-OH position of the ribose is a challenge due to its similar reactivity with the 3'-OH group. A large number of 2'-*O*-ribonucloeside protecting groups have been reported and can basically be divided into three categories: acid-, photo- and fluoride-labile groups.[33] The chosen group, its introduction as well as its removal, is required to be compatible with the other protecting groups used. Still, the lack of selectivity remains a primary concern. van Boom *et al.* resolved the issue of the 2'-OH protection by inserting the TiPS group which forms a cyclic link between the 5'- and 3'-alcohol functions.

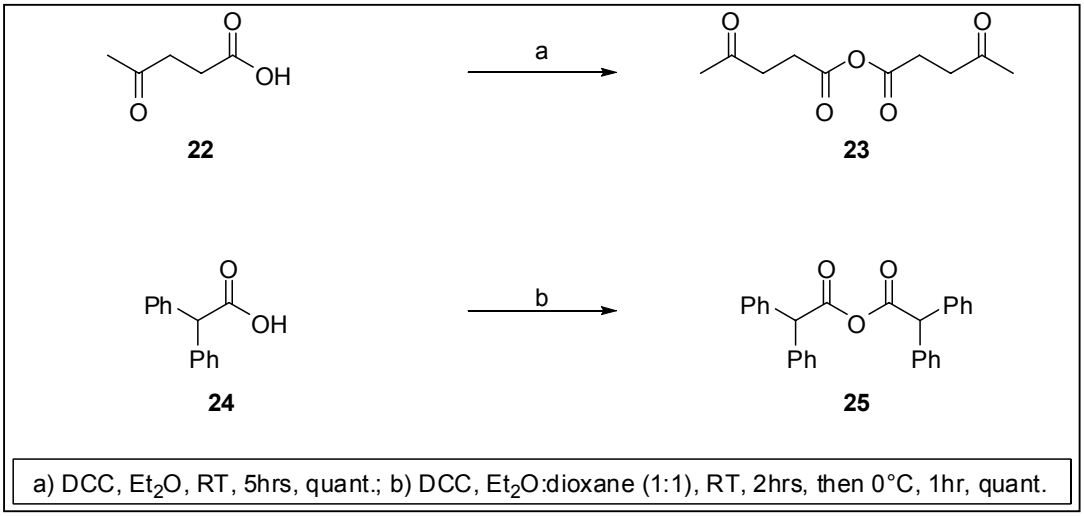
According to the procedure from van Boom *et al.*, to assemble c-di-GMP an adequately protected guanosine building block has to be prepared first. The first synthetic step consisted in the treatment of guanosine with 1,3-dichloro-1,1,3,3-tetra*iso*propyldisiloxane (TiPSCl) in DMF in the presence of imidazole to give the 3',5'-*O*-disilyl derivative **21** in 43% yield. (*Scheme 6*)[25]



a) TiPSCl, imidazole, DMF, RT, 3hrs, 43%; b) **23**, Et_3N, DMAP, DMF, RT, 15 min, 49%; c) i. **25**, pyridine, 60°C, 2hrs; ii. Hydrazine, pyridine, RT, 5 min then 2,4-pentanedione, RT, 5 min, quant.; d) i. 3,4-dihydro-2H-pyran, PPTS, DCM, RT, 4hrs; ii. HF.NEt_3, THF, RT, 16hrs, 93%; e) DMT-Cl, pyridine, RT, 16hrs, 76%.

Scheme 6: Synthesis of guanosine building block **29**.

To perform the next steps, two protecting group precursors had to be produced. Levulinic anhydride (Lev_2O) **23** was synthesized quantitatively from levulinic acid in the presence of dicyclohexyl carbodiimide (DCC) in diethyl ether. Diphenylacetic anhydride (dpa_2O) **25** was also prepared quantitatively using the same procedure in a mixture diethyl ether:dioxane (1:1). (*Scheme 7*)[25]

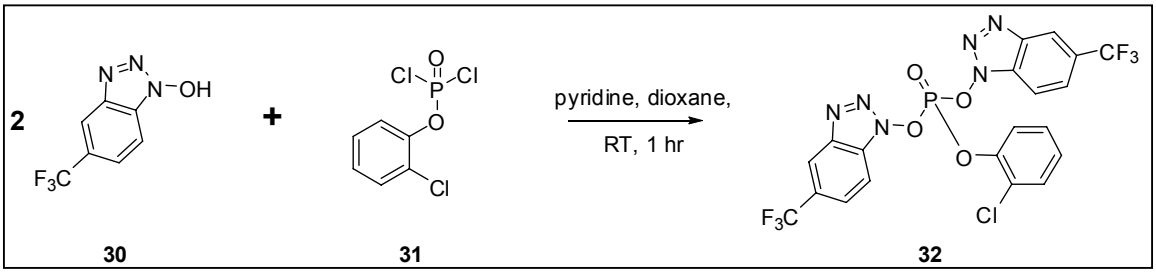


Scheme 7: Protecting group precursors Lev_2O **23** and dpa_2O **25**.

Subsequent reaction of **21** with Lev_2O **23** in DMF in the presence of 4-dimethylamino-pyridine (DMAP) and triethylamine afforded **26**, where the ribose moiety was fully protected, in 49% yield. Compound **26** was then reacted with dpa_2O **25** in pyridine, to block selectively the less reactive, free primary amine function of the guanine and directly treated with hydrazine and pentanedione in pyridine, to remove the Lev-group, thus yielding 2'-O-free derivative **27** quantitatively. Treatment with 3,4-dihydro-2*H*-pyran in dichloromethane followed directly by triethylamine trihydrofluoride in THF afforded selectively 2'-*O*-tetrahydropyranyl protected intermediate **28** in 93% yield. Finally, reaction with 4,4-dimethoxytrityl chloride in pyridine led to the desired 5'-*O*-DMT substituted guanosine building block **29** in 76% yield. Building block **29** was prepared in 15% overall yield in five reaction steps. (see *Scheme 6*)[25]

2.1.2 Synthesis of Cyclic Diguanylic Acid

To achieve the synthesis of c-di-GMP, a phosphorylating agent is needed. The one chosen by van Boom *et al.* is based on a modified benzotriazole. 2-chloro phosphoryldichloridate **31** is reacted with 1-hydroxy-6-trifluoro-methylbenzotriazole **30** in anhydrous dioxane in the presence of pyridine under inert atmosphere, to lead to activated phosphorylating agent **32** as a 0.2 M stock solution in dioxane. (*Scheme 8*)[26]

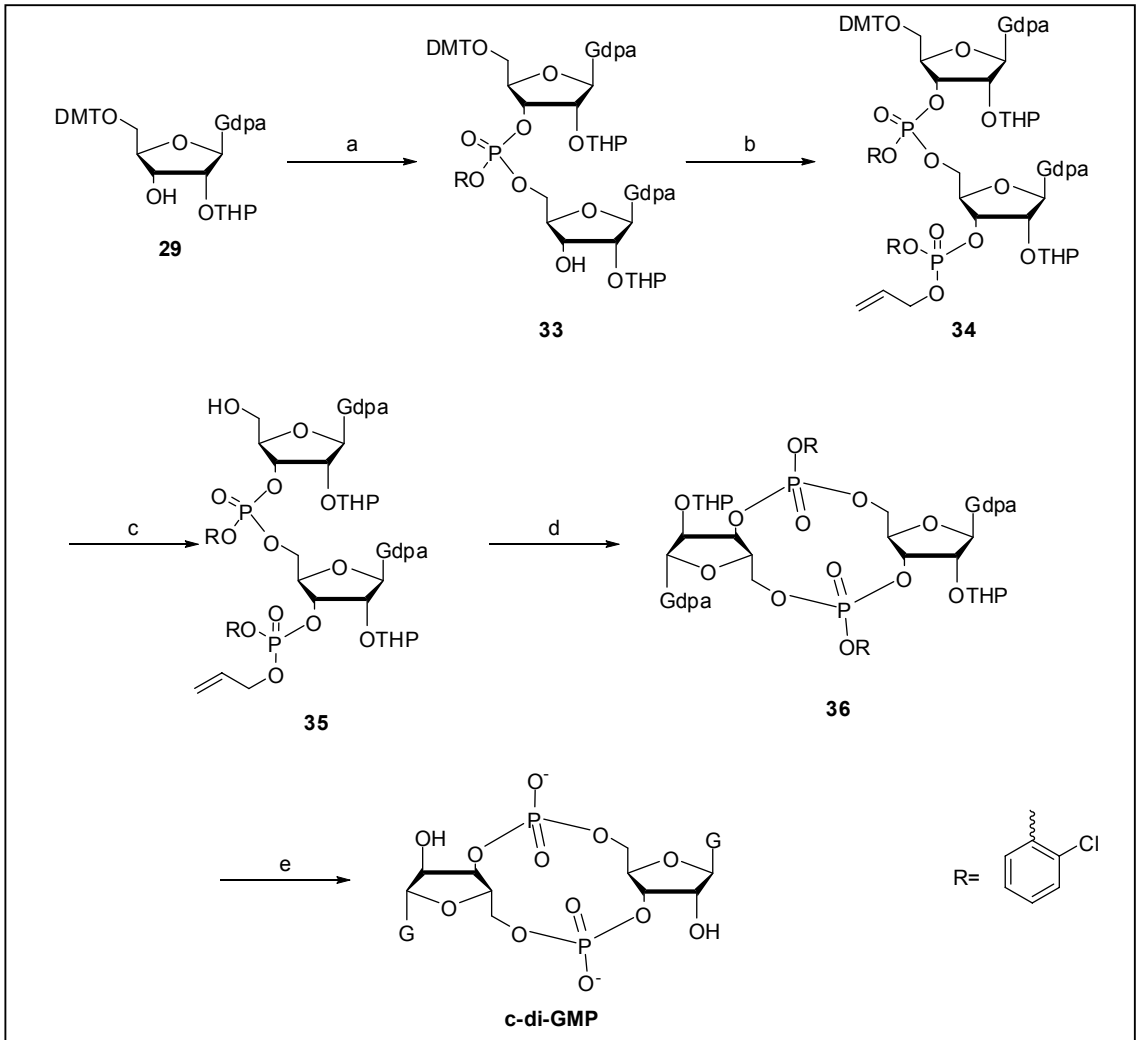


Scheme 8: Phosphorylating agent **32**.

The first step to complete the preparation of the cyclic nucleotide was assembling the two building block units **28** and **29** through reaction with phosphorylating agent **32** in dioxane to form dimer **33** in a 74% yield. In order to perform the ring closure, a second phosphate unit was necessary. Compound **33** was reacted again with phosphorylating agent **32** in pyridine to substitute the free 3'-OH, and the phosphotriester function was further protected with an allylic group *via* condensation with allylic alcohol in pyridine to give fully protected dimer **34** in 64% yield.[26]

This dinucleotide then needed to be deprotected at the 5'-*O*-DMT position. Dimer **34** was treated with *p*-toluenesulfonic acid in a mixture of dichloromethane:methanol (7:3, v/v) to afford free 5'-OH dimer **35** in 55% yield. The cyclization was performed in two steps. Treatment of compound **35** with $Pd(PPh_3)_4$ in the presence of triphenylphosphine and *n*-butylamine in THF allowed the cleavage of the allylic group on the phosphate. Afterwards, direct addition of TPSNT in pyridine activated the phosphate groups, and enabled cyclization *via* a new phosphotriester linkage to give fully protected cyclic dinucleotide **36** in 38% yield. Finally, successive deprotection with *syn*-pyridine-2-carboxaldoxime and 1,1,3,3-tetramethylguanidine in pyridine then concentrated ammonia, and treatment with hydrochloric acid led to c-di-GMP. The purification of this compound by reverse phase HPLC chromatography using triethylammonium acetate buffer and methanol (92.5:7.5, v/v) afforded

pure c-di-GMP in a 16% yield. Starting from the building block **29**, c-di-GMP was prepared in 1.5% overall yield over 5 steps (*Scheme 9*)[26]



Scheme 9: Synthesis of c-di-GMP using van Boom *et al.* methodology.

2.1.3 Discussion

The execution of the synthetic pathway as described by van Boom *et al.* showed some drawbacks. The strategy used was based on two important factors. First, the hydroxyl groups on the ribose moiety were more reactive than the amine function on the guanine moiety, which implied a need to block the alcohols before a selective protecting group for 2-NH_2 can be introduced. Second and more important, only few satisfactory protecting groups that can be inserted on the 2'-OH position have been reported. There is actually no chemoselective protecting group for the 2'-OH position, which is why the introduction of a group on 2'-OH requires having blocked positions 3'-OH and 5'-OH beforehand. One of the solutions was the use of the TiPS group, which allowed blocking the 3'- and 5'-alcohol functions with one cyclic derivative.

The use of the cyclic 3',5'-O-disilyl protecting group proved to be a challenge. The solubility of compound **25** primarily, but also, to some extend, of the other remaining intermediates where this group was present, became an issue leading to complex and lengthy purification procedures. This choice also revealed that side reactions occurred as long as the 2-NH_2 functionality was not blocked and thus drops in yields were observed.

Besides, the need to substitute selectively the 2'-hydroxy position, with a protecting group suitable for the remaining synthetic route, induced redundant steps. Indeed, this position, due to its greater reactivity compared to the 2-NH_2, was first blocked as a levulinic ester **26** and later converted again in a pyranyl ether **28**.

Another insight that we have gained was that the amide bond of the guanine was insufficiently protected. This functional group contributed to the high polarity of the building block intermediates, and thus also, to the difficulties encountered during the purification processes.

In addition, performing the phosphate ring-closure in three separate steps was a cause for low overall yields. Due to the high polarity of those compounds, the purification procedures were tedious and induced loss of material. Also, using a strategy where the 3'-OH position of dimer **33** was substituted with a phosphotriester in a primary reaction, led to the necessity to block this functionality with allylic alcohol, which had to be removed again at a later stage. Combined, those factors resulted in a 14% yield in the three steps to go from the dimer **33** to the fully protected cyclic intermediate **36**.

Furthermore, there is some evidence that the deprotection steps needed to be performed in a defined order. Indeed, as long as the phosphate linkages were blocked with the chlorophenyl groups, the molecule seemed to be sensitive to bases. Even mild conditions, such as a basic carbonate workup were enough to cleave the phosphate linkages. The free phosphate bonds on the other hand resisted basic treatment. This meant,

the chlorophenyl groups had to be the first groups to be cleaved before any basic treatment can be performed.

All the insight gained by reproducing the published van Boom *et al.*[26] synthesis, showed us that this method was a long and tedious process. Indeed, the overall yield we obtained to prepare c-di-GMP was only 0.2% over 10 steps, starting from the commercially available guanosine. This also suggested that the synthesis was ill suited for scale-up purposes. Nevertheless, through this methodology a small quantity (4 mg) of c-di-GMP could be obtained and this strategy was the base of our attempts to further develop a synthetic pathway that could bypass a few of the drawbacks mentioned above.

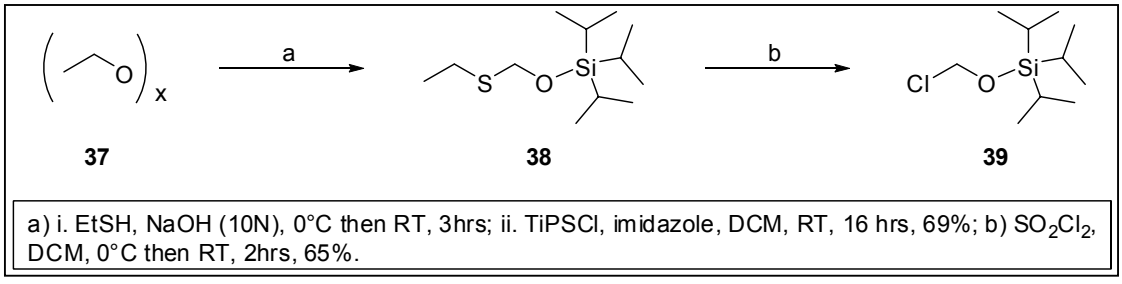
2.2 Tom Protected Building Blocks

2.2.1 Synthesis of the Building Block and Introduction of the Tom group

In order to design a new synthesis pathway using protecting groups that would withstand the reaction conditions to prepare c-di-GMP, the preparation of a new 2'-*O*-protected guanosine building block was undertaken.

In the large number of investigated protecting groups for the 2'-OH guanosine position, the *tert*butyldimethylsilyl (TBDMS) group is the one having found the widest applications, even though the nucleotide coupling yields are not the most satisfactory. A new class of protecting groups, where the steric demands are lower, has been described recently by Pitsch *et al.* The introduction of those 2'-*O*-protecting groups, derived from a formaldehyde acetal linker, such as [(tri*iso*propylsilyl)oxy]methyl chloride (TomCl), was described and the building blocks obtained, combined with the phosphoramidite chemistry coupling strategy, were shown to have a superior coupling behavior.[33] Hence, we decided to use this new protecting group to try and improve the synthesis of c-di-GMP.

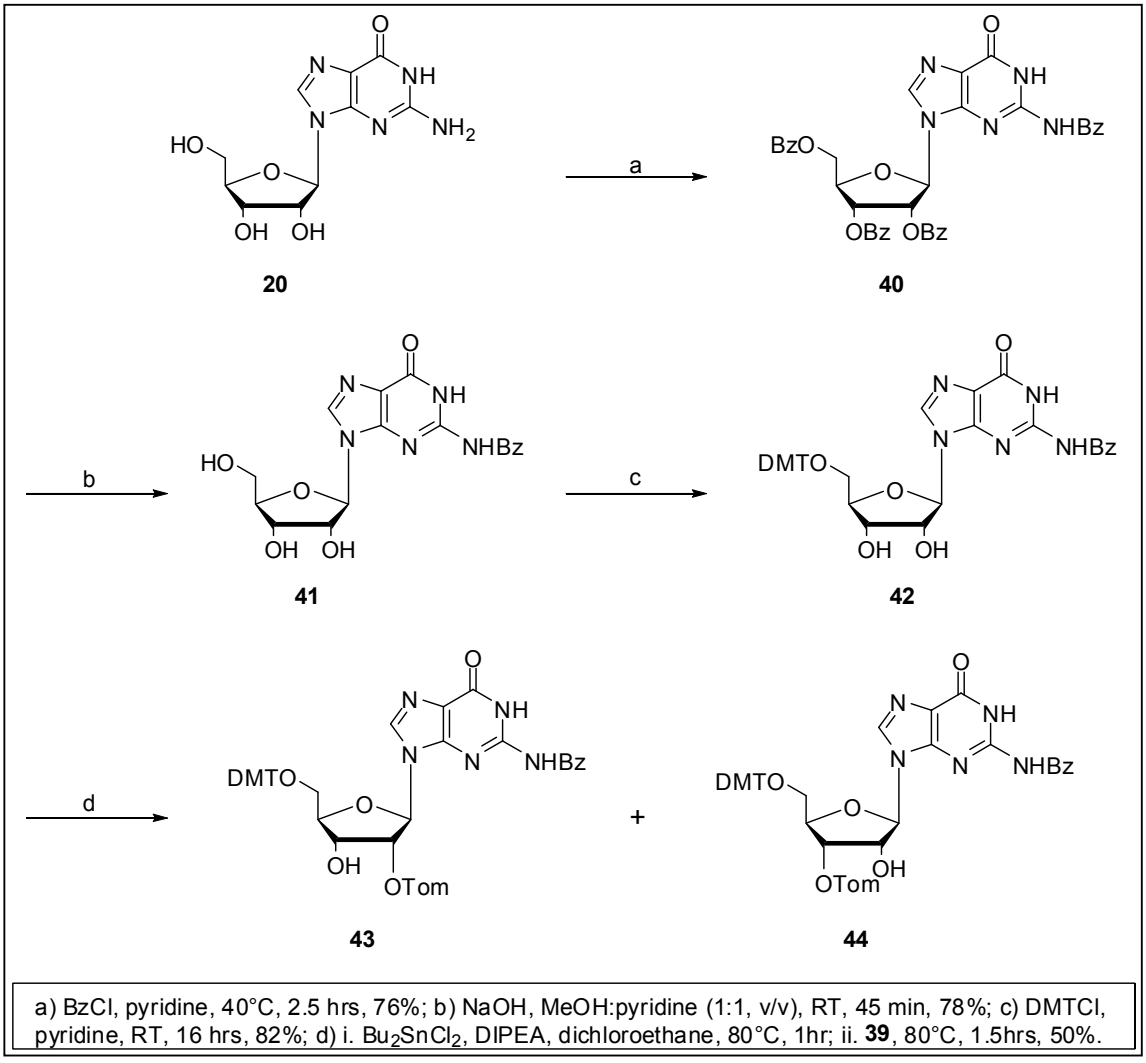
The reagent TomCl **39** was synthesized by condensation of *para*formaldehyde and (ethylthio)methanol in basic settings and silylation with tri*iso*propylsilyl chloride/imidazole in CH_2Cl_2 to give silylated derivative **38** in 69% yield. This intermediate was transformed with SO_2Cl_2 in dichloromethane into Tom-Cl **39**. By distillation *in vacuo*, compound **39** was isolated in 65% yield. (*Scheme 10*)[33]



Scheme 10: Synthesis of TomCl precursor **39**.

Guanosine **20** was reacted with benzoyl chloride in pyridine to give fully protected guanosine **40** in 76% yield. The alcohol positions were deprotected with sodium hydroxide in a mixture MeOH:pyridine (1:1, v/v) to generate 2'-, 3'- and 5'-free OH and 2-*N*-benzoyl blocked guanosine **41** in 78% yield. The 5'-OH position was then blocked with a

dimethoxytrityl group through treatment with DMTCl in pyridine in 82% yield. The final step was then the insertion of the Tom group. Compound **42** was first reacted with dibutyltindichloride and di*iso*propylethylamine in dichloroethane to generate a 2',3'-*O*-tin complex in situ which was immediately treated with TomCl **39** to give a mixture of 2'-*O*-Tom and 3'-*O*-Tom protected guanosines **43** and **44** in an overall yield of 50%. (*Scheme 11*) [34]



Scheme 11: Synthesis of the 2'-*O*-Tom protected building block **43**.

2.2.2 Discussion of the Method

The combination of the chosen protecting groups allowed for overall relatively high yields and the required deprotection processes were straightforward. Though, blocking the 2'-OH position was the least efficient step in the synthesis reported above.

Reportedly, the Tom group displays some unique properties which render it a valuable 2'-*O*-protecting group for the synthesis of oligoribonucleotides. It is stable under the reaction conditions required for the assembly and deprotection of RNA sequences. Also, the stability of the Tom group towards both strongly acidic conditions and strongly basic conditions is a consequence of the sterically very hindered tri*iso*propylsilyl moiety.

However, in our hands, the introduction of the Tom group was a cause for low yields and due to the mixture of two isomers produced, the yield was even lower. The separation of the regioisomeric Tom substituted ribonucleosides **43** and **44** proved that the favored regio-isomer was the 3'-OH Tom substituted compound **44**, which was obtained with the highest yield.

The favored isomer was not the desired one, thus the chosen protecting group (Tom), proved to be far from ideal to achieve an effective, flexible and most of all up-scalable synthetic pathway. Finally, even with this new set of protecting groups, the purification operations remained tedious, partly due to the high polarity of the intermediates, but also due to the side reactions occurring on the base moiety while reaction steps are undertaken on the ribose.

Those considerations led to the interruption of this methodology and a new strategy was devised in order to bypass those difficulties. One of the parameters that were the cause for the problems encountered in the methods described before, was the lack of adequate protection of the amide function on the guanosine base. This issue was the main focus of our next alternative for the synthesis.

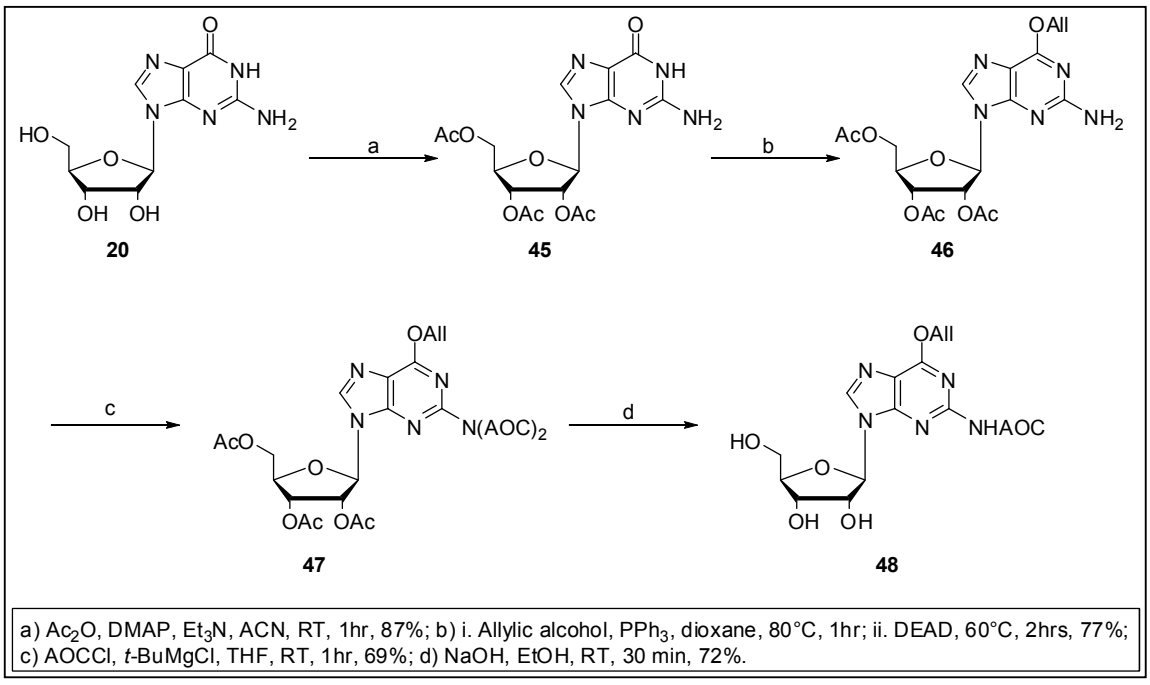
2.3 Hayakawa Based Building Blocks

2.3.1 Introduction of the Protecting Groups on the Guanine Moiety

Among the difficulties in the earlier described methods, the purification processes during the building block synthesis are major. Reasons for those complex procedures are linked to the insufficiently protected guanine moieties. Blocking the 2-NH_2 position only, leaves another free 3-NH amine and the free 4-carbonyl function, which can account for higher polarities of the intermediates, thus rendering the purification complicated.

In 1999, in an early report, Hayakawa *et al.*[35] described the synthesis of RNA building blocks (i.e **48**, **50**) where the 2-NH_2 function as well as the amide bond of the guanine moiety were protected with two separate allylic groups. By blocking both these functional groups, higher solubility can be achieved and side reactions might be avoided. In the course of our work, in 2003, the same group then published a following report, describing a new synthetic method for c-di-GMP based on these same building blocks.[37] This new pathway seemed to resolve a few of the drawbacks from the van Boom *et al.* method and achieve better yields. So we decided to apply some of the improvements showed by Hayakawa *et al.*, and develop our own new approach.

Guanosine was first acetylated in acetonitrile on all the free OH positions of the sugar to produce 2',3',5'-*O*-triacetylguanosine **45** in 87% yield. Through a Mitsunobu reaction sequence in dioxane, an allylic group was introduced on the 4-O position of the guanine moiety in a 77% yield. This intermediate **46** was then treated with allylchloroformate (AOCCl) and *tert*butylmagnesium chloride in THF to introduce the AOC protecting group to the 2-*N*-function in 69% yield. Bis-allyloxycarbonated product **47** was then treated with sodium hydroxide in ethanol, to remove the acetyl groups as well as one of the AOC groups selectively to give intermediate **48** in 72% yield. (*Scheme 12*)[35]



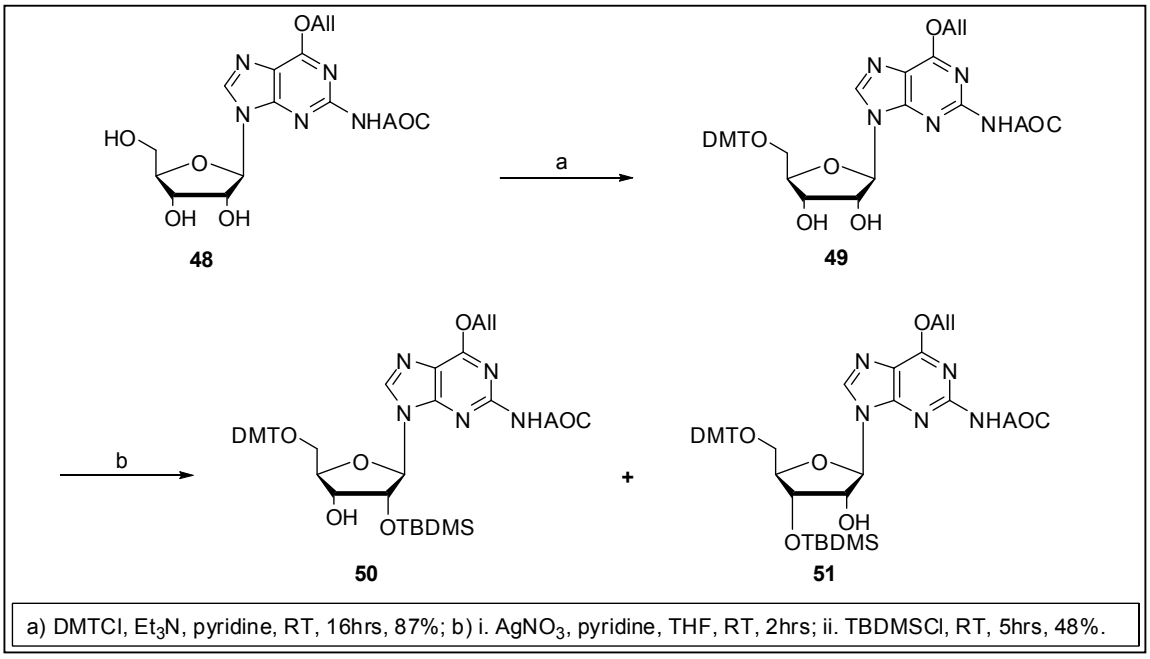
Scheme 12: Introduction of the blocking groups on the base moiety.

2.3.2 Blocking the Ribose Positions

2.3.2.1 First Alternative to the Synthesis of the Building Block

After having successfully blocked the guanine moiety, the next step was to prepare the building block for the coupling reactions, which supposed introducing satisfactory protecting groups on the ribose.

The first alternative to this end was the following: blocking the 5'-OH with a dimethoxytrityl group in pyridine to give compound **49** in an 87% yield.[36] The next step was then inserting a silyl derivative selectively on the 2'-OH position. Intermediate **49** was activated with silver nitrate and pyridine in THF then *tert*butyldimethylsilyl chloride was added to yield the two regio-isomers 2'-*O*-TBDMS-derivative **50** and 3'-*O*-TBDMS-derivative **51** in a combined 48% yield. Although, during the separation procedure the TBDMS-group was shown to migrate from the 2'-*O* to the 3'-*O*-position, which resulted in a final yield of 8% for desired isomer **50**. (*Scheme 13*)[35]



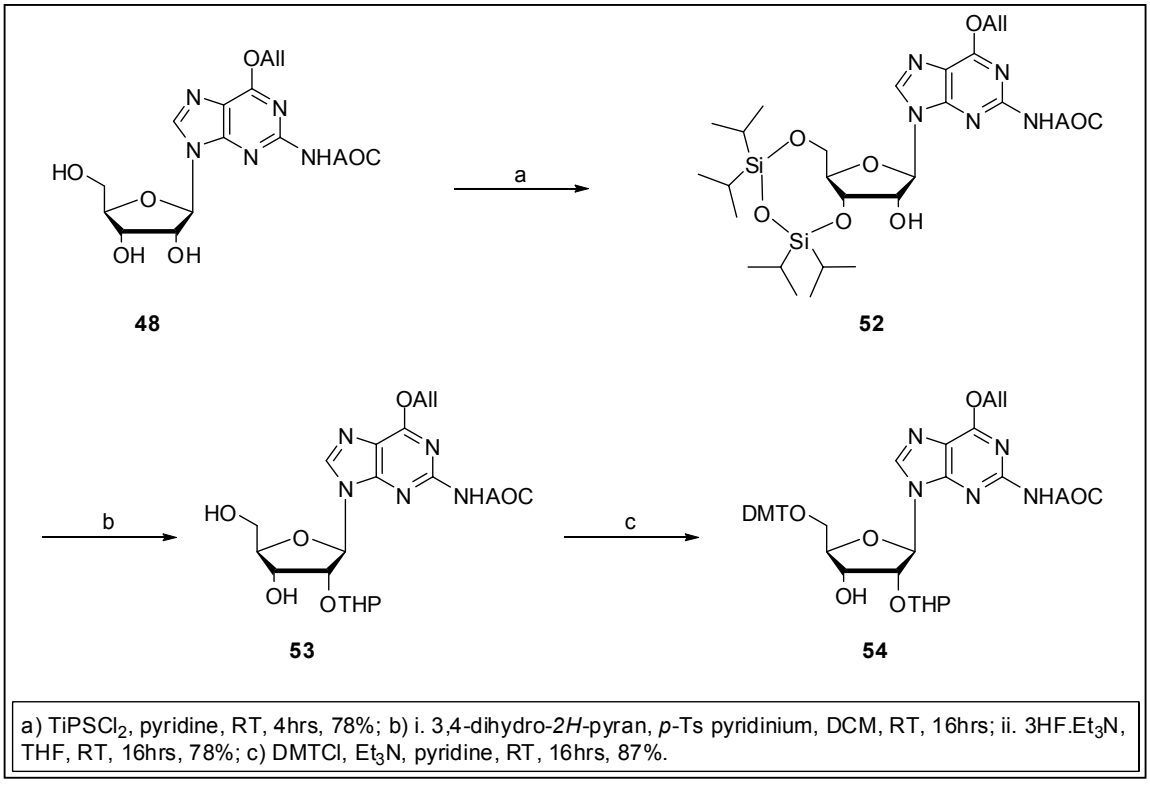
Scheme 13: Introduction of the protecting groups on the ribose moiety.

The main drawback of this method was the lack of regioselective silylation, which resulted in the formation of the mixture of 2'-O, and 3'-O silyl ethers. Although it was reported, that the use of silver nitrate or even silver perchlorate in the silylation reaction should increase the 2'-*O*-regioselectivity,[36] in the case of the guanosine derivatives, as examplified here, approximately 1:1 mixtures of the 2'- and 3'-silyl ethers were obtained. The only way to separate those regiomers was by column chromatography, but under basic conditions the silyl group can migrate. The yield could be increased by equilibration of the 3'-silyl compound to, again, form a mixture of the 2'- and 3'-isomers and separate them, but this step was time and material consuming.

2.3.2.2 Second Alternative to the Synthesis of the Building Block

Due to the setback of the TBDMS group migrating under basic conditions, a new alternative was found to complete the synthesis of the guanosine building block. The chosen protecting group for the 2'-OH function was the pyranyl group, which implied the prior use of the 3',5'-*O*-cyclic silyl group.

Starting again from intermediate **48**, the 3',5'-*O*-disilyl derivative **52** was formed in pyridine in 78% yield. In the next step, intermediate **52** was reacted with 3,4-dihydro-*2H*-pyran in dichloromethane to block the 2'-hydroxy group and directly, without further purification, treatment with triethylamine trihydrofluoride in THF removed the silyl group to yield compound **53** in 78% yield.[25] The final step was then the introduction of the dimethoxytrityl group on the 5'-hydroxy function in pyridine to give **54** in 87% yield.[36] (*Scheme 14*)



Scheme 14: Synthesis of Hayakawa-like building block **54**.

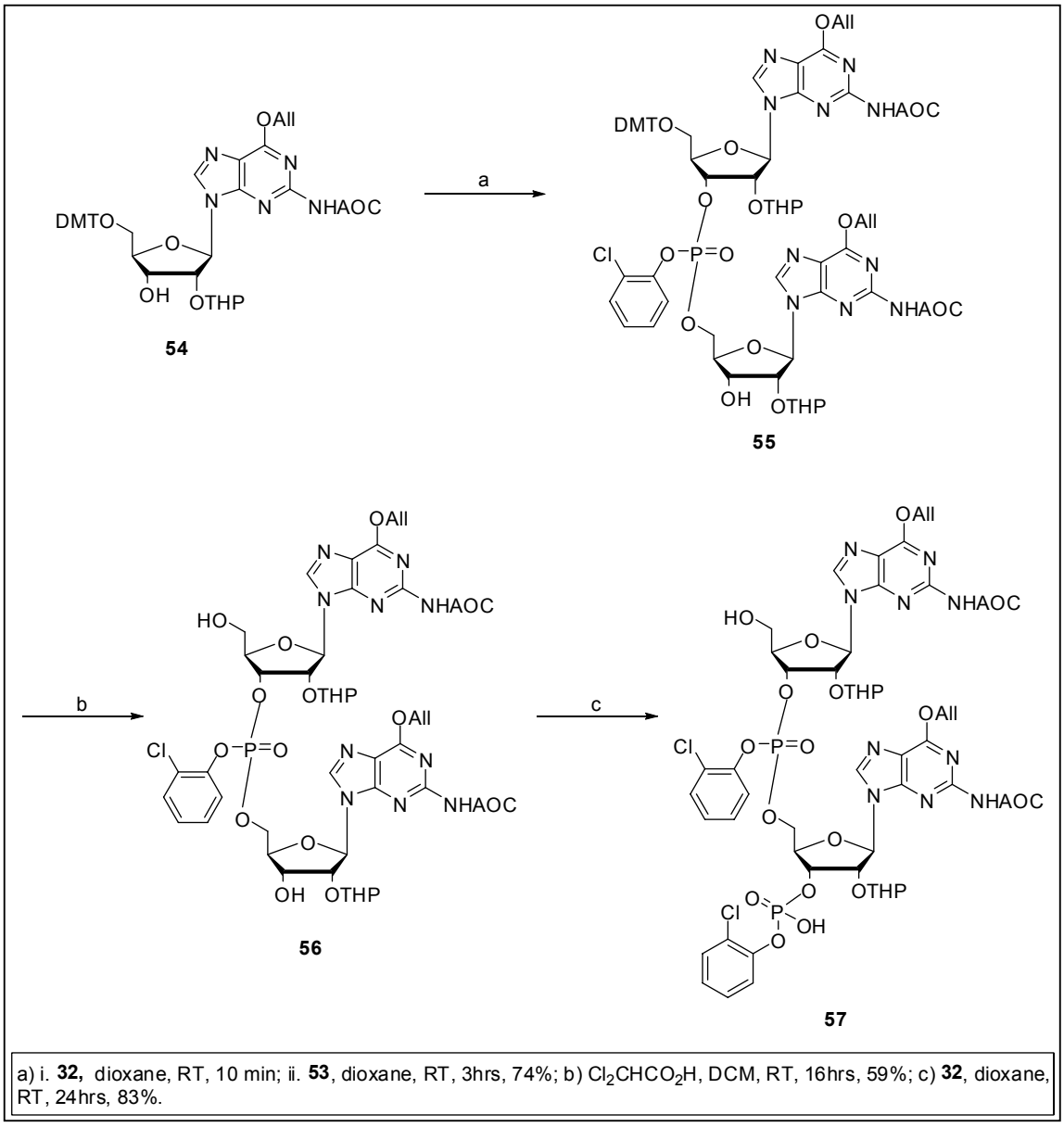
2.3.3 Synthesis of Linear Dinucleotide GpGp

After having achieved the synthesis of the two building blocks **53** and **54**, the next stage was assembling them *via* an internucleotide phosphate bond. The original Hayakawa *et al.*[37] synthesis reported the use of the phosphoramidite method. Since this kind of procedure is generally better suited for solid support preparations than for “in solution” reactions, due to the lack of long term stability of the phosphoramidite substituted intermediates, we chose instead to continue to utilize the phosphotriester methodology.

The phosphorylating agent used was the same as the one described by van Boom *et al.*[26] based on 1-hydroxy-6-trifluoro-methylbenzotriazole prepared as a 0.2M stock solution of **32** in dioxane (see chapter 2.1.2).

Precursor **54** was treated with phosphorylating agent **32** in dioxane and immediately after, nucleotide monomer **53**, in dioxane, was added. Dinucleotide **55** was obtained after easy purification processes in 74% yield. This intermediate then undergoes an acidic treatment with dichloroacetic acid in dichloromethane to remove the 5’-*O*-dimethoxytrityl group to give 3’,5’-free OH dimer **56** in 59% yield.

The last step of the synthesis was then closing the 12-membered phosphate-sugar ring. Several attempts showed that compound **56** does not undergo cyclization in the presence of phosphorylating agent **32** in dioxane, but open chain dinucleotide GpGp **57** was obtained in 87% yield. Any further attempts to cyclize the linear dimer through classic phosphate activation procedures were unsuccessful. (*Scheme 15*)[26]



a) i. **32,** dioxane, RT, 10 min; ii. **53**, dioxane, RT, 3hrs, 74%; b) Cl_2CHCO_2H, DCM, RT, 16hrs, 59%; c) **32**, dioxane, RT, 24hrs, 83%.

Scheme 15: Synthesis of the linear dimer GpGp.

2.3.4 Discussion

The advantage of preparing nucleosides with allylic protecting groups blocking the base completely was, first, the easier purification procedures as well as the reduction of redundant synthesis steps. Adding these two new protecting groups lowered the polarity of the purine base residue, thereby facilitating the purification processes and increasing the yields. The introduction of those groups was fairly straightforward and attained in high yields.

The choice of the protecting groups used on the ribose moiety was more challenging. The first alternative, where a silyl group was introduced to block selectively the 2'-hydoxy function, proved to be a poor choice, since this protecting group has been shown to migrate from 2'-OH to 3'-OH under basic conditions. The yields were low and handling this protecting group was time and material consuming, so this option was abandoned.

Nevertheless, another opportunity has been found by utilizing the 3',5'-disilyl cyclic protecting group. Even if the use of this group previously induced solubility as well as yield issues, the aforementioned allylic substituents on the base canceled those concerns nicely. This option also helped in rendering the introduction of the pyranyl group on the 2'-OH completely selective. Through this pathway, the synthesis of the two building blocks necessary, afforded high yields over six and seven steps respectively.

Assembling the precursors to form the cyclic nucleotide was performed using the phosphotriester methodology described by van Boom *et al.*[26] Unfortunately, the reported ring closure could not be reproduced. Indeed, the cyclization could not be achieved, and the phosphorylated linear dinucleotide pGpG was the only product obtained, even though a number of diverse conditions and procedures have been tried.

Several explanations can be found for this issue. The first was the sensitivity to bases of the phosphate linkage. Indeed, even mildly basic conditions, such as a work up with sodium hydrogen carbonate, were enough to open the 12-membered ring and form the linear dinucleotide. Furthermore, the introduction of the cyclizing phosphate bond was sensitive to moisture. The activated phosphorylating agent has been shown to be hydrolyzed by even small amounts of moisture present during the reaction process. And, after this hydrolysis, the phosphate formed became inactive and could not be successfully turned into an intermediate able to undergo cyclization.

In summary, the pathway to obtain c-di-GMP, described in this chapter has not led to the desired dinucleotide. Even so, it helped in resolving some of the issues, concerning solubility, chemoselectivity and purification processes, encountered during the syntheses described before. With the experience acquired during the different syntheses performed here, it became obvious that the main drawback and therefore also the biggest problem to solve, was the protection of the 2'-OH group. We were also able to apply the new insight

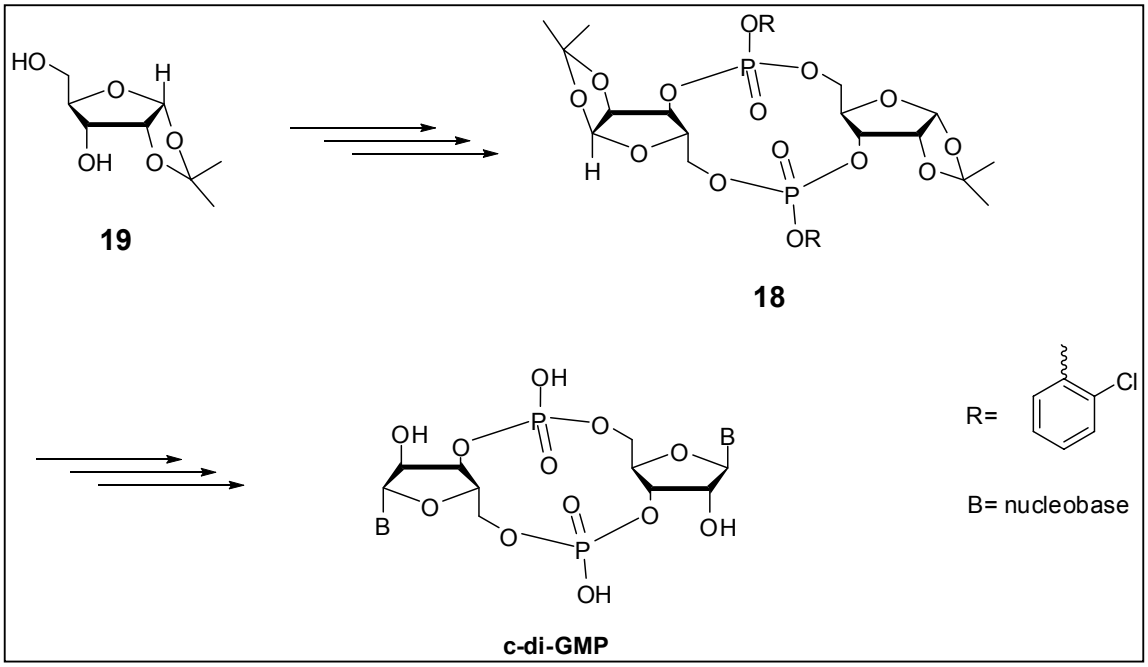
about the concerns raised by using the phosphotriester methodology, to the synthetic route investigated later.

All the methods described here so far were long and implied difficult purifications, therefore they were not suitable for scale-up synthesis processes. This coupled with the fact that the 2'-OH protection remained a problem and was expensive in material (at least half was lost), prompted us to turn our attention to a method to avoid the 2'-OH protection altogether. This new method used again the phosphotriester methodology that we estimate to be more suitable for solution phase chemistry as will be described in the next chapter. This method turned out to be suitable for scale-up and was also very flexible in terms of base modified analogues.

3. A NEW SYNTHETIC APPROACH FOR C-DI-GMP: RIBOSE BUILDING BLOCK BASED

After having explored the different existing synthetic routes, it quickly became obvious that we would have to design a new method that would be more efficient and account for all the problems we encountered previously. The main issues were finding a short and efficient synthesis for the building blocks with special attention to the 2'-OH protection of the guanine but also increasing the scale of the reaction. The choice of the phosphoramidite *versus* the phosphotriester methodology was also important. In our hands, the phosphotriester methodology seemed to be the most efficient for our project.

To solve the other problems we have decided to adopt a brand new approach in which we will synthesize a sugar-phosphate backbone and introduce the base at a late stage (as shown in *Scheme 16*). Through this route we anticipate to solve the matter of the 2'-OH protection completely by using the 1',2'-acetal protecting group. The next section will describe in detail this new and very efficient synthesis pathway.



Scheme 16: Overview of the new synthetic pathway.

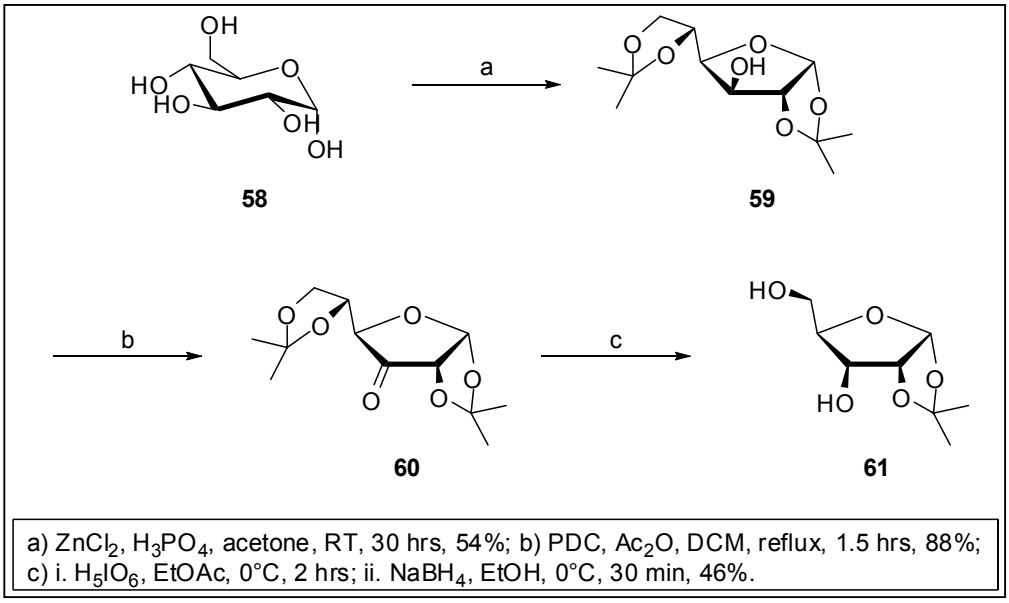
3.1 Synthesis of the Ribose Unit Starting from Glucose

The synthetic pathways described in the preceding chapter showed limitations in their efficiency and were also tedious to perform. Moreover, the described methods all started from guanosine. This meant that, to obtain analogues, the synthesis had to be started over from the very first step of the building block preparation.

Therefore, to solve all the problems encountered before, a different synthesis had to be devised. In 1983, Charollais *et al.*[32] reported the cyclic sugar backbone **18** (see *Scheme 16*) as a by-product in the synthesis of phosphate esters of ribo- and xylo-furanoses. It should be easy to convert **18** into c-di-GMP or its analogues by introducing a nucleobase, *via* modified Vorbrüggen conditions[38], at a late stage in the synthesis and starting the pathway with a furanose building block.[39]

Agrofoglio *et al.*[39] reported the synthesis of a ribo-furanose unit **61** starting from α-D-glucose. This unit will be the starting point of our new synthesis pathway. α-D-Glucose **58** was treated with acetone in the presence of zinc chloride and phosphoric acid to be converted to α-D-xylose **59** in 54% yield. Oxidation with pyridinium dichromate (PDC) and acetic anhydride in dichloromethane led to the selective dehomologation of 1',2':5',6'-di-*O*-*iso*propylidene-3'-oxo-α-D-glucofuranose **60** in 88% yield. This ketone was submitted to a one-pot sequential transformation with periodic acid in ethyl acetate and sodium borohydride in ethanol to the desired optically pure derivative **61** in 46% yield. The preparation of the first ribose building block needed **61**, showed an overall yield of 21% over 3 steps starting from α-D-glucose. (*Scheme 17*)[39]

One of the main advantages of this building block was blocking the 1'- and 2'-OH as an acetal for the whole synthesis. This allowed us to bypass all the issues inherent to the selective protection of the 2'-hydoxy group. Indeed, this was a significant drawback in the previously reported syntheses.

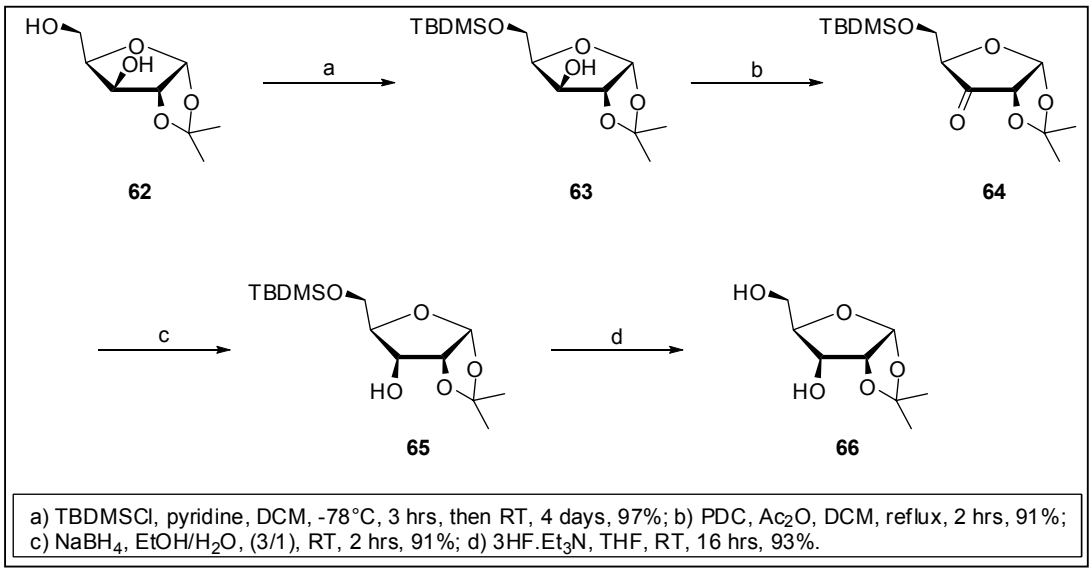


Scheme 17: Converting glucose to the ribose precursor **61**.

3.2 Synthesis of the Ribose Building Blocks

Since synthesizing the ribose building block straight from α-D-glucose did not lead to satisfactory yields, another alternative was found. This compound can be prepared more efficiently using a higher yielding pathway. Indeed, 1',2'-*O*-*iso*propylidene-α-D-xylofuranose **62** is a commercially available precursor. In order to be suitable for RNA-nucleotide synthesis, this precursor needs to be stereoselectively converted to the desired optically pure ribose sugar.

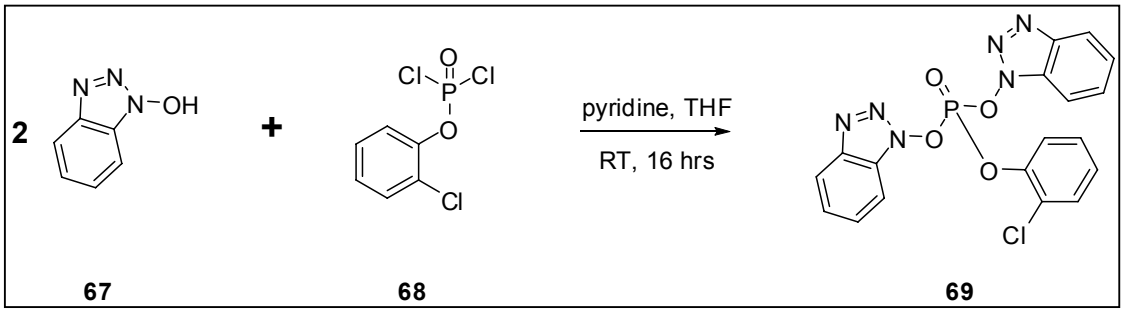
The 5'-free OH group of 1',2'-*O*-*iso*propylidene-α-D-xylofuranose **62** was first blocked with a *tert*butyldimethylsilyl (TBDMS) group via treatement with TBDMSCl in dichloromethane to afford 5-*O*-*tert*butyldimethylsilyl-1,2-*O*-*iso*propylidene-α-D-xylofuranose **63** in 97% yield.[40] Oxidation with pyridinium dichromate and acetic anhydride in dichloromethane led to the selective dehomologation at the 3'-OH position to give the 3'-oxo-xylofuranose **64** with 91% yield. The ketone was then reduced to the secondary alcohol with sodium borohydride in a mixture of ethanol:water (3:1, v/v) to yield the first building block **65** in 91% yield. Treatment with triethylamine trihydrofluoride in THF led to the removal of the 5'-TBDMS group and the second building block necessary, **66**, was obtained in 93% yield. The overall yield for this approach was 75% over 4 steps to obtain both ribose building blocks needed, **65** and **66**, starting from the commercially available xylose **62**. (*Scheme 18*)[39]



Scheme 18: Preparation of the ribose building blocks **65** and **66**.

3.3 Synthesis of the Cyclic Sugar Backbone

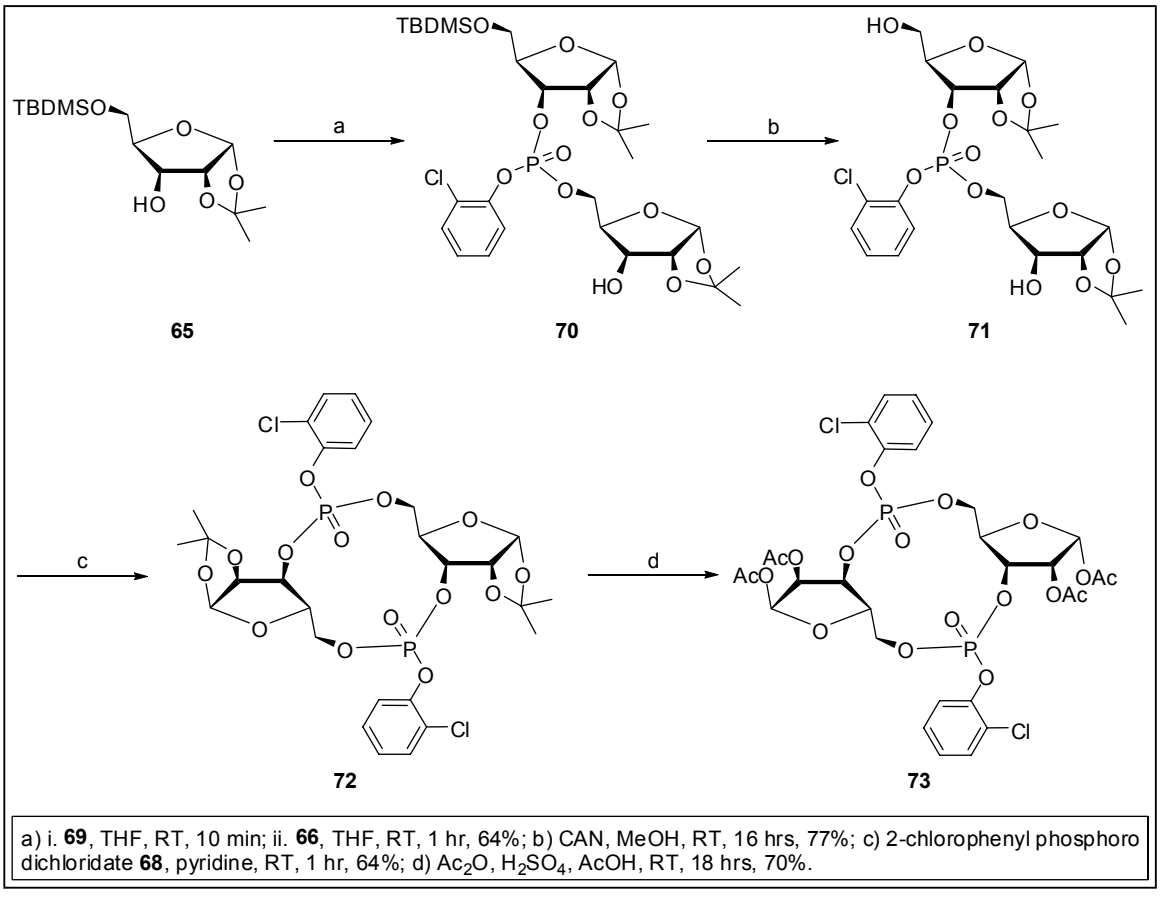
From our experience, the phosphotriester methodology was more suited in the case of an “in solution” synthesis method. Therefore, we chose to use the original van Boom phosphorylating reagent, which was based on 1-hydroxybenzotriazole (HOBt). 2-chloro phosphoryldichloridate **68** was treated with 1-hydroxybenzotriazole and pyridine in THF under inert atmosphere to yield a 1 M stock solution of phosphorylating agent **69** in THF under argon. (*Scheme 19*)[41]



Scheme 19: Synthesis of the HOBt based phosphorylating agent **69**.

The synthesis was started with 5'-*O*-*tert*butyldimethylsilyl-1',2'-*O*-*iso*propylidene-α-D-rifuranose **65**. It was treated with phosphorylating agent **69** in THF under inert atmosphere and then coupled with precursor **66** in THF to give the dinucleotide **70** in 64% yield.[26] The TBDMS protecting group was then removed by treatment with ceric ammonium nitrate (CAN) in methanol to afford 3',5'-free OH dimer **71** in 77% yield.[42] This was a rather unusual deprotection method but it was necessary here. In fact, the usual cleavage method for TBDMS involved a fluoride treatment. Classical fluoride reagent solutions, like TBAF, became basic over time, which was not compatible with the protected phosphate linkage since it was base sensitive. Another alternative had to be found and the deprotection of choice was this oxidative method involving cerium (IV).

Cyclization was then performed *via* a diluted solution of 2-chlorophenyl phosphorodichloridate **68** in pyridine to give cyclic intermediate **72** in 64% yield. Conversion of the acetal blocking groups to acetate esters was achieved through treatment with acetic anhydride in acetic acid to afford c-di-GMP precursor **73** in 70% yield. (*Scheme 20*)[39]



a) i. **69**, THF, RT, 10 min; ii. **66**, THF, RT, 1 hr, 64%; b) CAN, MeOH, RT, 16 hrs, 77%; c) 2-chlorophenyl phosphoro dichloridate **68**, pyridine, RT, 1 hr, 64%; d) Ac_2O, H_2SO_4, AcOH, RT, 18 hrs, 70%.

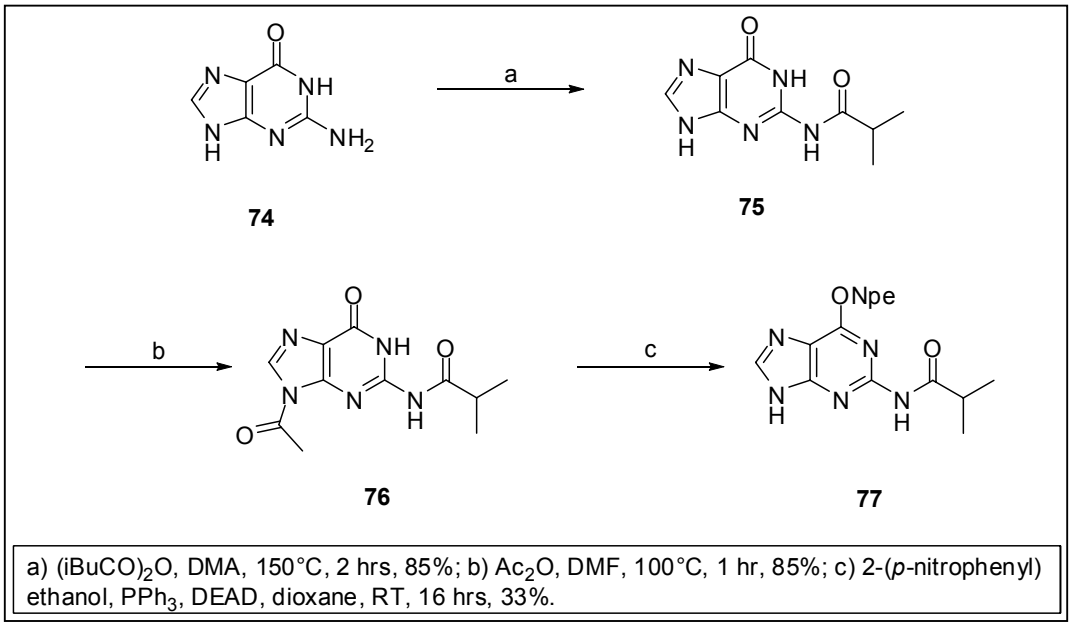
Scheme 20: Synthesis of the sugar backbone 12-membered ring **73**.

The synthesis of phosphate linked dimer **70** afforded a mixture of diastereomers due to the phosphorus atom. The R and S isomers can be distinguished in the NMR analysis but they could not be separated. The same observations were true for dimer **71**. The cyclization process added a second phosphate atom, with introduced a second stereo-centre on the molecule. The compound **72** was synthesized as a mixture of 4 diastereomers. However, two distinct fractions, which have notably different retention factors (R_f), could be obtained during the purification process and both have been identified as the desired cyclic product **72**. The NMR analysis afforded further insight into the nature of the isomers. The fraction with the highest R_f value presented two peaks in the ^{31}P NMR analysis, which lead to the belief that this fraction contained the RR and SS isomers. On the other hand, the fraction having the lowest R_f value, lead to only one peak in the ^{31}P NMR analysis. This seemed to confirm that the lowest R_f fraction consisted of the RS and SR fractions. Nevertheless, each of those fractions could be converted to the acetylated product **73** and then further used in the synthesis of c-di-GMP. To avoid the multiplication of products and facilitate the purification processes, both fractions were used separately in the following steps of the reaction pathway. The resulting final product, c-di-GMP, has been analyzed by HPLC procedures and showed the exact same retention times strating from both fractions.

3.4 Synthesis of the Guanine Unit

The greatest difficulty in the coupling of protected sugar derivatives with guanine-type bases, using the modified Vorbrüggen methodology[38], was the mixture of N7/N9 isomeric nucleotides that were produced and difficult to separate. Reportedly, coupling "directly protected" guanine derivatives has consistently produced 7/9 isomer mixtures, whereas constricting the guanine system into "4-enolate" derivatives resulted in enhancement of the 9/7 isomer ratios.[43] The problem was overcome by blocking sufficiently and appropriately the guanine.[44]

Guanine **74** was treated with *iso*butyric anhydride in dimethylacetamide (DMA) to block the 2-*N*-amine function as *iso*butyric amide **75** in 85% yield. This compound was then submitted to acetic anhydride in dimethylformamide to yield 9-*N*-acetylated intermediate **76** in 85% yield. Finally, through a Mitsunobu type reaction with 2-(*p*-nitrophenyl)ethanol, the protecting group was introduced on the free 4-oxygen and the acetyl group was removed, in a one-pot reaction, to lead to the desired appropriately blocked guanine unit **77** in 33%. (*Scheme 21*)[44]

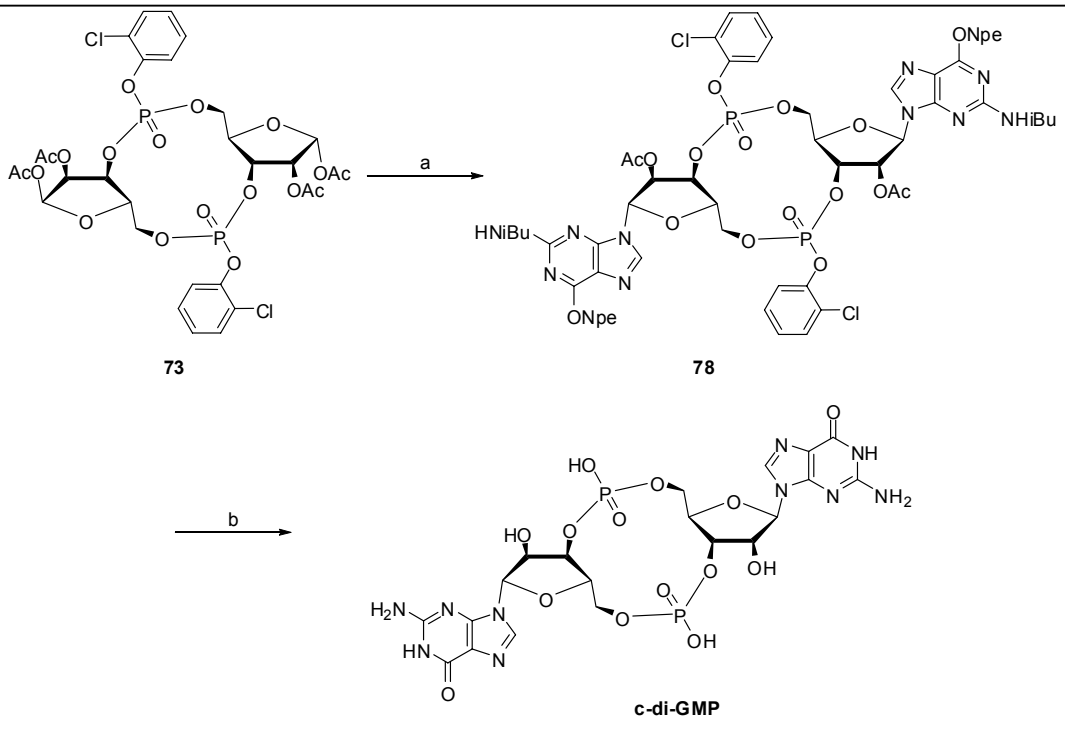


Scheme 21: Preparation of the guanine building block **77**.

3.5 Assembling c-di-GMP

The protected guanine unit **77** was persilylated with BSA in dichloroethane and condensed with the acetylated sugar-phosphate 12-membered ring **73** under modified Vorbrüggen conditions.[38] The development of these conditions helped to overcome many difficulties with the sugar-base couplings and most bases led to high regioselectivities. It was reported that the 7-isomers were formed as kinetic products of the glycosylation of persilylated guanines, whereas the more thermodynamically stable 9-isomers were obtained upon heating.[43] Using this procedure, the fully protected c-di-GMP **78** was obtained in 66% yield.

The *o*-chlorophenyl protected phosphate bond was sensitive to bases, even mild conditions could induce cleavage. Due to the nature of the other protecting groups used and the conditions necessary for their cleavage, the chlorophenyl group had to be removed first. Treatment with *syn*-pyridine-2-carbaldoxime in the presence of *N*,*N*,*N*,*N*-tetramethylguanidine in pyridine removed the chlorophenyl as well as the Npe groups. Finally, treatment with aqueous ammonium hydroxide led to the removal of the acetyl and *iso*butyryl protecting groups. A first purification step was achieved using size exclusion chromatography. This allowed the separation of the product from excess reagents and most of all the remaining nucleobase, which was responsible for internucleotide phosphate bond cleavage. An additional reverse phase HPLC purification was necessary, to separate the 7- and 9-isomers and afforded pure c-di-GMP in 84% yield. (*Scheme 22*)[45]



a) i. **77**, BSA, DCE, 80°C, 16 hrs; ii.TMSOTf, toluene, 80°C, 30 min, 66%; b) i. *syn*-pyridine-2-carbaldoxime, *N,N,N,N*-tetramethyl guanidine, pyridine, RT, 16 hrs; ii. NH_4OH, 50°C, 48 hrs, 84%.

Scheme 22: Synthesis of c-di-GMP.

3.6 Conclusions

The new synthetic pathway designed here, starting from the sugar building blocks, has successfully afforded pure c-di-GMP in high yields. Indeed, c-di-GMP was obtained in 10% overall yield in 9 steps, starting from commercially available 1',2'-*O*-*iso*propylidene-α-D-xylofuranose **62**. In comparison to the published synthesis methods, several drawbacks have been resolved and our new method affords c-di-GMP in higher amounts with high yields. For example, the overall yield we obtained to prepare c-di-GMP using the van Boom *et al.* procedure was only 0.2% over 10 steps, starting from commercially available guanosine.

One of the main concerns with the other methods was the lack of appropriate protecting groups for the 2'-OH function. This issue was completely avoided with our new strategy, since the 1'- and 2'-OH groups were blocked as an acetal during the synthesis of the sugar building blocks. This facilitated enormously the preparation of the two sugar units needed, since there were no more troubles with tedious purification processes. The synthesis of the building blocks in general, was easy to perform, and we even realized the preparation starting from 30 grams xylofuranose with high yields. The guanine precursor was also straightforward to synthesize in 3 steps with high yields, and can easily be done on a multi-gram (30 grams guanine) scale.

Assembling the sugar backbone was also easy to perform with high yields (22% over 4 steps) even when scaling-up. During the backbone assembling process, care had to be taken to avoid any basic conditions during the reactions or the work-up procedures. The chlorophenyl protected phosphate bond was particularly sensitive to bases, even weaker ones. The only problems encountered were during the deprotection of the TBDMS group prior to the cyclization. Indeed, the usual method for this step was not compatible with the protected phosphate linkage since it was base sensitive. Another efficient alternative was found using cerium (IV) ammonium nitrate.

The introduction of the base on the sugar backbone following modified Vorbrüggen conditions[38] was cause for a little more tedious work. Since we made the choice to insert the base on the sugar moiety at a late stage, the possibility of forming the 7-N-guanosine as well as the desired 9-N-guanosine existed. This trend has been known to be a cause for low yields and loss of material when trying to synthesize natural oligonucleotides. Following appropriate reports[43], heating the reaction mixture afforded preferentially the thermodynamic 9-isomer, with only small amounts of the other isomer. Even with the matter that the 9-isomer can only be obtained preferentially and not exclusively, a good yield could be achieved and c-di-GMP could be obtained in reasonable amounts.

Care had also to be taken during the final deprotection step. As mentioned before, the chlorophenyl protected phosphate was base sensitive, so it had to be the first protecting

group to be cleaved. After this, a basic treatment under rather harsh conditions with aqueous ammonia afforded c-di-GMP in fairly high yield and in 99% HPLC purity.

The new synthesis pathway described here was straightforward and suitable for scaling-up. The synthesis can be performed in a few weeks and reasonable amounts of c-di-GMP can be obtained. Indeed, using our method 500 mg product could be easily synthesized.

The only issues we encountered during this process were ones due to the purification procedures. The size exclusion chromatography purification was time consuming and poorly suitable for larger amounts of c-di-GMP. Efforts were undertaken to find more appropriate ways to purify large quantities of c-di-GMP. First tests with crystallization attempts showed promising results.

The main advantage of this new approach for the preparation of c-di-GMP was that it allowed for a fairly straightforward preparation of base modified analogues. Indeed, since the base was introduced at a late stage, it enabled us to prepare one sugar backbone and exchanging the base only required small adaptations of the method in the last steps. It could also be imagined to use the same strategy, building a cyclic sugar backbone then introducing the base, to prepare internucleotide linkage modified analogues.

4. PREPARATION OF C-DI-GMP ANALOGUES

4.1 Synthesis of Base Modified c-di-GMP Analogues

4.1.1 Base Precursors

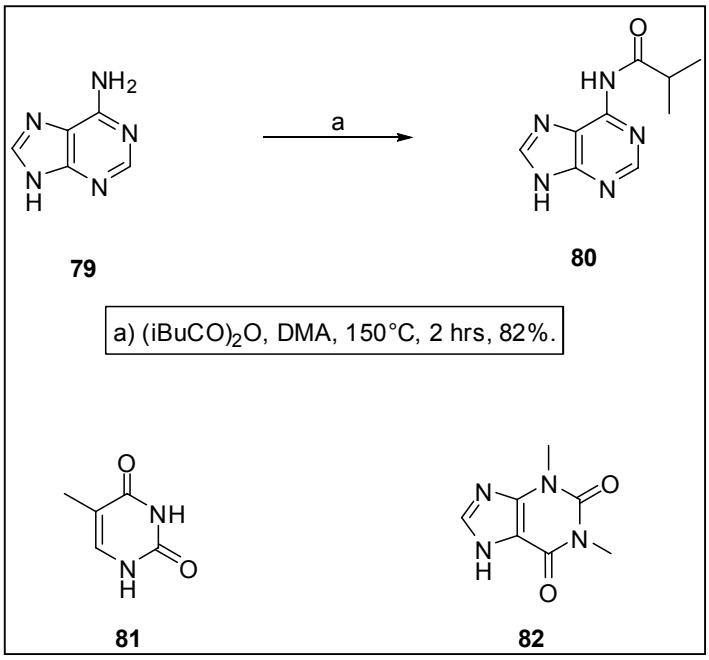
Since c-di-GMP has attracted great interest due to its various biological activities, it is important to investigate, which functions of c-di-GMP are involved in the different biological processes. The biological properties also prompted systematical investigations of the activity of c-di-GMP related compounds including derivatives with modified nucleobases, carbohydrates and internucleotide bonds.

In 2006, Hayakawa *et al.*[46] first reported the synthesis of three modified cyclic nucleotides following the same synthesis pathway they used for c-di-GMP.[29] The compounds they reported were two mixed cyclic dinucleotides, one compound where a guanosine was exchanged for an adenosine and the other where one guanosine was replaced with an inosine. They also prepared an internucleotide-bond modified cyclic diguanosine where a phosphate is replaced by a thiophosphate.

In order to show the flexibility of our own new synthetic route, the synthesis of base-modified analogues of c-di-GMP was undertaken. The intend was to show that the synthesis was not specific for purine bases but can be applied to pyrimidine bases as well as non natural nucleobases, such as xanthine or theophylline for example.

The first step to this end was preparing the bases for their introduction on the 12-membered sugar backbone. The analogues we chose to synthesize were those using adenine, thymine and theophylline as an alternative nucleobase. Thymine **81** is a pyrimidine normally found in DNA and not in RNA. It can be used directly as a nucleotide precursor and does not need any further protection steps. Theophylline **82** is an unnatural pyrimidine base that is fully protected and therefore can also be used directly as a precursor.

The only chosen nucleobase that required additional blocking steps was adenine as it has a free primary amine function on C-4 of the 6-membered ring. Adenine was treated with *iso*butyric anhydride in dimethylacetamide to afford the protected derivative **80** in 82% yield. (*Scheme 23*)[44]

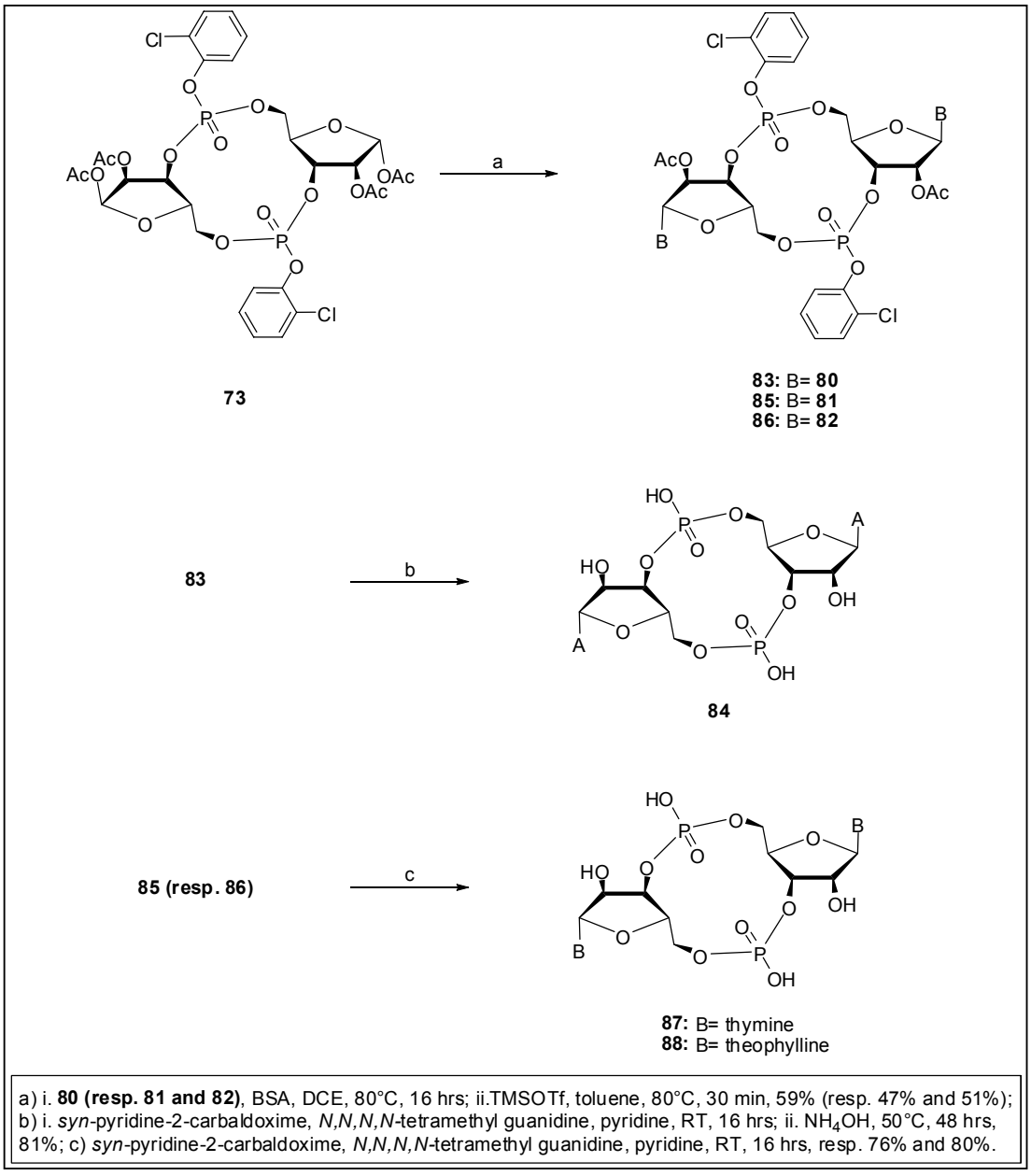


Scheme 23: Base-modified analogues precursors.

4.1.2 Synthesis of the c-di-GMP Analogues

The base introduction procedure was the same as for c-di-GMP. The protected adenine **80** was persilylated with BSA in dichloroethane and condensed with the acetylated sugar backbone **73** under modified Vorbrüggen conditions.[38] Fully protected c-di-AMP **83** was obtained in 59% yield. The phosphate protecting groups were removed using *syn*-pyridine-2-carbaldoxime in the presence of N,N,N,N-tetremaethylguanidine in pyridine. Final treatment with aqueous ammonium hydroxide led to the removal of the acetyl and *iso*butyryl protecting groups. After purification *via* size exclusion chromatography and HPLC, pure c-di-AMP **84** was obtained in 81% yield. (*Scheme 24*)[45]

The same procedure was used to prepare the analogues containing thymine **81** and theophylline **82**. Fully protected c-di-TMP **85** was obtained in 51% yield and fully protected c-di-(theo)MP **86** was obtained in 47% yield. The difference was the simplified deprotection strategy needed, due to the lack of protecting groups on the bases. The protecting groups on the ribose and on the phosphate bond were removed in one operation, using *syn*-pyridine-2-carbaldoxime in the presence of *N*,*N*,*N*,*N*-tetramethylguanidine in pyridine. After purification *via* size exclusion chromatography and HPLC, pure c-di-TMP **87** and c-di-(theo)MP **88** were obtained in 76% and 81% yield respectively. (*Scheme 24*)[45]



a) i. **80 (resp. 81 and 82)**, BSA, DCE, 80°C, 16 hrs; ii.TMSOTf, toluene, 80°C, 30 min, 59% (resp. 47% and 51%); b) i. *syn*-pyridine-2-carbaldoxime, *N,N,N,N*-tetramethyl guanidine, pyridine, RT, 16 hrs; ii. NH_4OH, 50°C, 48 hrs, 81%; c) *syn*-pyridine-2-carbaldoxime, *N,N,N,N*-tetramethyl guanidine, pyridine, RT, 16 hrs, resp. 76% and 80%.

Scheme 24: Synthesis of base modified analogues: c-di-AMP, c-di-TMP and c-di-(theo)MP.

4.1.3 Conclusions

The synthesis of base modified analogues of c-di-GMP *via* this new approach was easy since the preparation of the building blocks and the assembling of the sugar backbone was the same as for c-di-GMP and the modifications only appeared in the last two steps. This means a whole variety of molecules can be prepared starting from the same precursor, the sugar backbone.

This method was easy to apply to purine bases like guanosine or adenosine, even if those bases need to be suitably protected before being introduced on the backbone. This methodology can also be applied to pyrimidine bases, like thymine, or non natural bases like theophylline in high yields.

Another important feature was the sequence in which the deprotection step was performed. The phosphate bond was base sensitive as long as it was blocked with the chlorophenyl group. Thus, in order to preserve the cyclic dinucleotide bonds, this group had to be cleaved first, since the remaining protecting groups, on the nucleotides, were all base-labile. If this sequence was respected, the cyclic dinucleotides were obtained with high yields.

The new approach for the synthesis of c-di-GMP we described here, showed a flexibility that has not been accounted for in previously published procedures. With a little adaptation, we could even imagine preparing non symmetric compounds, where two different bases are introduced on the backbone.

Due to the apparent adaptability of this method, we decided to use the same strategy to prepare internucleotide linkage modified compounds. The modified building blocks would then be assembled to form a sugar backbone linked through carbamate, amide or non hydrolysable bonds. The introduction of the base would again be performed at a late stage to afford variation possibilities.

4.2 Towards the Synthesis of an Amide Bond Internucleotide Linked c-di-GMP Analogue

4.2.1 Synthesis of the Sugar Building Blocks

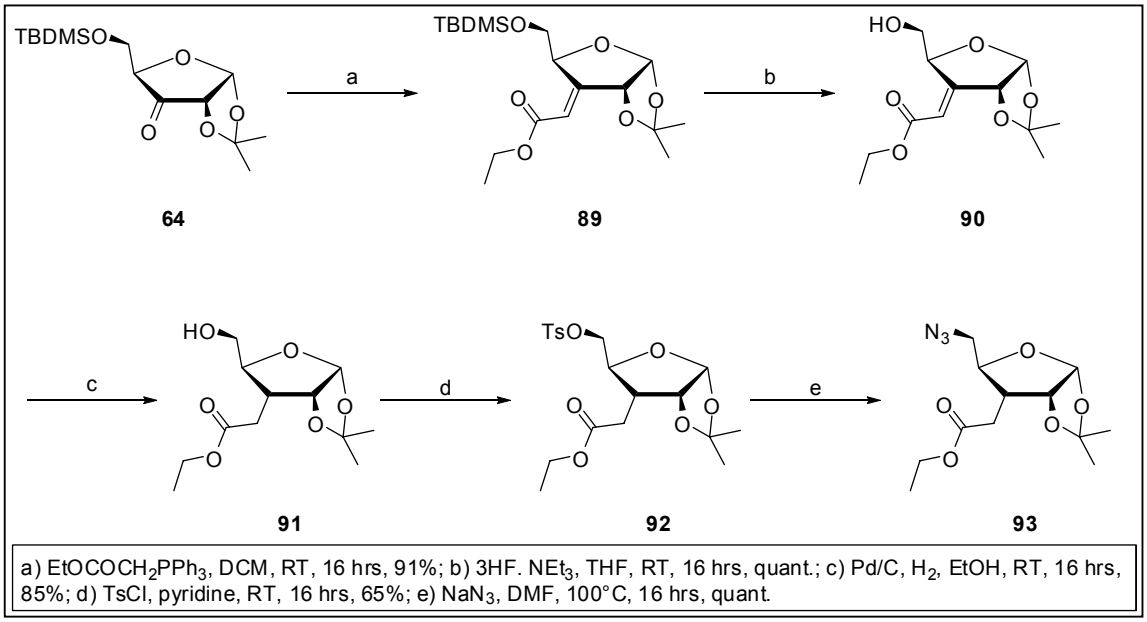
Presumably, c-di-GMP cannot pass through the cell membrane and the interactions between c-di-GMP and its target proteins during the recognition mechanisms remain unknown. Preparing analogues, whether the base or the internucleotide linkage was modified, could help to determine, which functions of c-di-GMP are involved in the various biological activities of this dinucleotide. It could also lead to the discovery of new bioactive compounds derived from c-di-GMP.

Oligoribonucleotides having amide internucleotide linkages, instead of the natural phosphodiester group, have been synthesized in the last two decades due to their potential nuclease-resistant antisense effects. The advantages of the amide bond include a greater stability under physiological conditions than the phosphodiester bond. The amide moiety is readily accessible by simple synthetic methods and also achiral, thereby avoiding diastereomeric mixtures usually obtained during the synthesis of phosphodiesters. Due to the charge reduction with respect to phosphates, neutral amide bonds should also favor the penetration of the oligonucleotides through the cellular membrane.[47]

For these reasons, exchanging the phosphodiester bond for an amide linkage seemed appropriate to gain more insight into the mode of action of c-di-GMP. After having successfully synthesized base-modified analogues, our next goal was to prepare an internucleotide amide bond dinucleotide. We decided to apply the same synthesis strategy as before, where a sugar backbone was prepared and the base was introduced at a late stage. To this end, we had to synthesize two modified building blocks starting from the commercially available 1',2'-*O*-*iso*propylidene-α-D-xylofuranose **62** shown in *Scheme 18*.

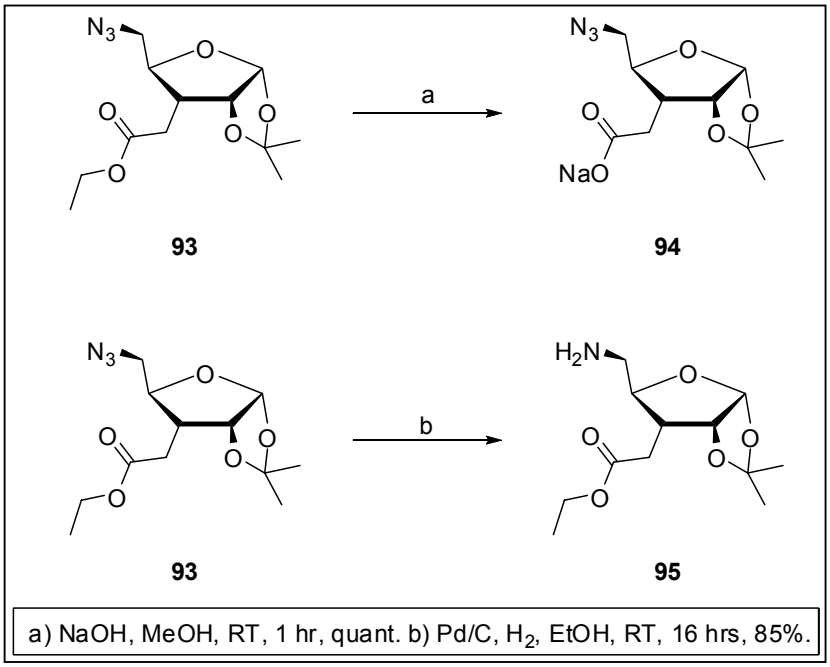
The 5'-free OH group of the xylofuranose precursor was first blocked with a *tert*butyldimethylsilyl (TBDMS) and the selective dehomologation at the 3'-OH position lead to the 3'-oxo-xylofuranose **64**, as described in chapter 3.2 (*Scheme 18*).[39] Through a Wittig reaction of the ketone with [(ethoxycarbonyl)methylene] triphenylphosphine in dichloromethane the alkene **89** was obtained in 91% yield.[48] Treatment with triethylamine trihydrofluoride in THF led to the removal of the 5'-TBDMS group and the free alcohol **90** was obtained quantitatively. Catalytic hydrogenation of the alkene function in the presence of palladium on activated charcoal in ethanol, afforded free alcohol **91** in 85% yield. The alcohol was then converted into its tosylate by treatment with tosyl chloride in pyridine, intermediate **92** was obtained in 65% yield. Finally, treatment with sodium azide in DMF yielded the building block precursor **93** quantitatively. The overall yield for this approach is 45% over the

7 steps to obtain the precursor **93** needed to prepare both building blocks, starting from the commercially available 1',2'-*O*-*iso*propylidene-α-D-xylofuranose **62**. (*Scheme 25*)[48]



Scheme 25: Preparation of the ribose precursor **93**.

In order to assemble the amide linked sugar dimer, the two different building blocks necessary were prepared from precursor **93**. Saponification of compound **93** using sodium hydroxide in methanol, gave the first building block, the 3'-(carboxymethyl) sodium salt **94**, quantitatively. Hydrogenation, in the presence of palladium on charcoal in ethanol, of azide **93** gave the second building block, the free 5'-amine **95**, in 85% yield. (*Scheme 26*)[48]

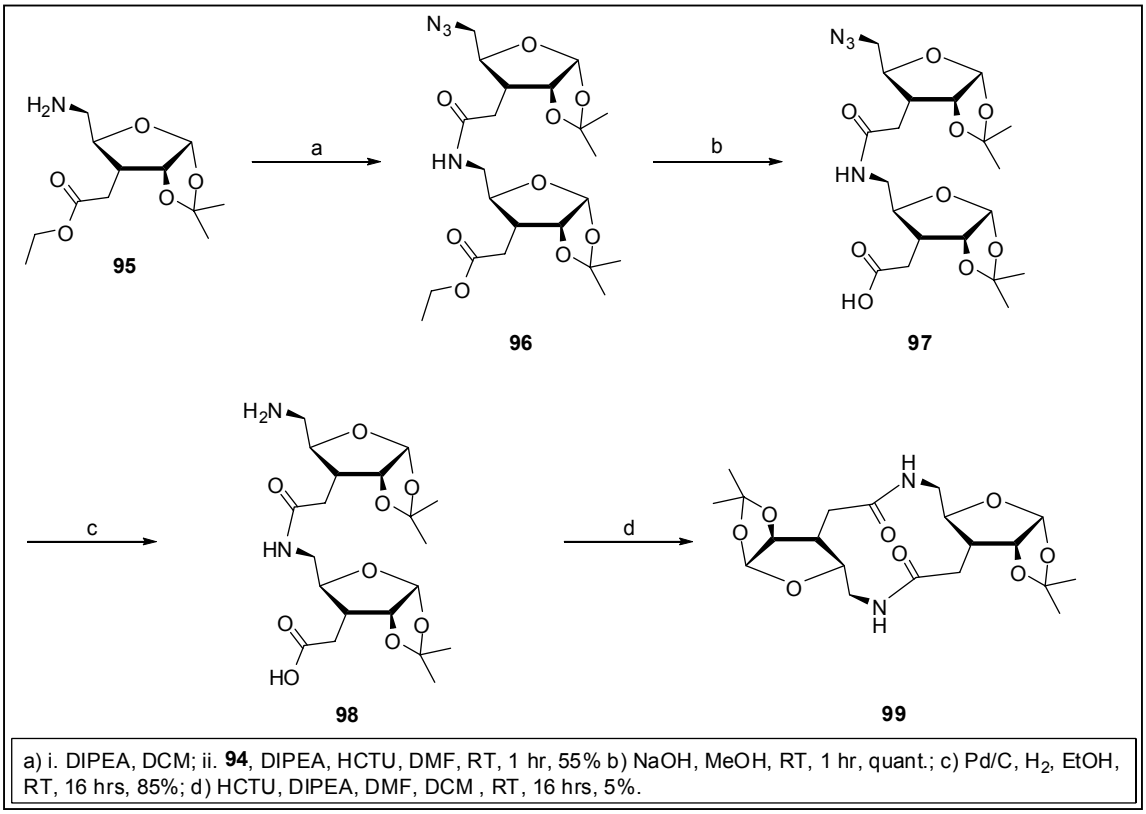


Scheme 26: Preparation of the ribose building blocks **94** and **95**.

4.2.2 Attempts to Prepare the Amide Linked Cyclic Backbone

After having prepared the necessary building blocks, the next step was the assembly of the sugar backbone. The formation of the amide bond was performed using standard peptide coupling processes. The free-acid building block **94** was activated using HCTU in DMF in the presence of Hünig's base, and added to free-amine building block **95** in dichloromethane to afford amide linked dimer **96** in 55% yield.[49] Saponification with sodium hydroxide in ethanol, followed by catalytic hydrogenation afforded dimer **98** in 85% yield.[48]

Several attempts to cyclize compound **98** were performed using HCTU as coupling reagent in the presence of Hünig's base as a diluted solution in dichloromethane.[49] Unfortunately, only trace amounts of cyclic compound **99** were obtained in yields under 5%. These findings showed that the cyclization did occur but more work was required to efficiently perform this reaction.



a) i. DIPEA, DCM; ii. **94**, DIPEA, HCTU, DMF, RT, 1 hr, 55% b) NaOH, MeOH, RT, 1 hr, quant.; c) Pd/C, H_2, EtOH, RT, 16 hrs, 85%; d) HCTU, DIPEA, DMF, DCM , RT, 16 hrs, 5%.

Scheme 27: Preparation of the cyclic backbone.

4.2.3 Discussion

The preparation of an internucleotide linkage modified analogue for c-di-GMP was thwarted by the very low yields obtained during the cyclization process when trying to build up the sugar backbone.

The macrocycle might be more difficult to form presumably due to conformational constraints in the cyclic molecule induced by the peptide bond, which would lead to the low yields. To further optimize the reaction conditions, such as using longer reaction times, other solvent systems or even changing the temperature, might improve the yields. One should also consider alternatives, such as different coupling reagents or even different reaction sequences.

Nevertheless, we managed to prepare the necessary building blocks in quite high yields as well as preparing the modified dimer efficiently. We used the same type of strategy as for the preparation of c-di-GMP where the sugar backbone was prepared first. Even if the cyclization only afforded traces of product so far, it might be interesting to continue the work in this direction.

Since the biological mechanisms, as well as the extend of the biological activities, of c-di-GMP still remain to be discovered, preparing analogues might be useful in uncovering the applications of this cyclic dinucleotide.

5. BIOLOGICAL PROPERTIES OF C-DI-GMP

5.1 The Role of c-di-GMP in *Caulobacter crescentus*

5.1.1 c-di-GMP: a Secondary Messenger in *Caulobacter crescentus*

Caulobacter crescentus is an aquatic bacterium that undergoes an obligate developmental transition that enables cells to switch between a sessile, surface-attached form (stalked cell) and a motile, flagellated form (swarmer cell). This asymmetrically dividing bacterium, produces a nonmotile and a motile cell at the end of each cycle. Upon completion of cell division, the motile swarmer cell enters a period of morphogenesis required to differentiate into a sessile stalked cell to initiate another cell division cycle. During this transition, the swarmer cell undergoes several physiological and morphological changes. As a consequence, the cell poles are constantly remodeled. One of those changes is the ejection of the polar flagellum (*Figure 3*).[16]

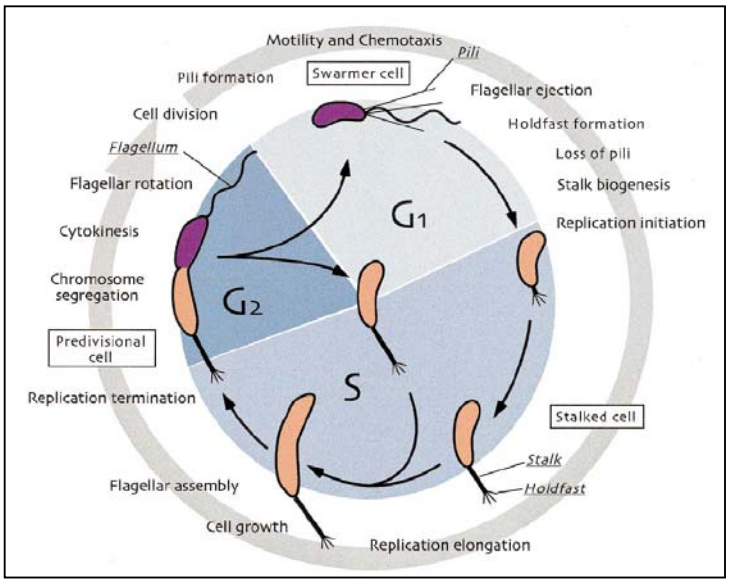


Figure 3: Schematic diagram of the *Caulobacter crescentus* cell cycle with the three different cell types: the swarmer cell, the stalked cell and the predivisional cell.[50]

PleD is an important protein involved at the flagellar-ejection stage. This unorthodox response regulator harbors two N-terminal receiver domains arranged in tandem and a C-terminal output domain with a highly conserved amino acids sequence. The putative output

domain, called “GGDEF” or “DUF1”, is widespread and highly conserved in many bacterial species. It has been demonstrated that the PleD regulator dynamically localizes to the differentiating stalked pole during the cell cycle when activated, thus postulating a local activity of PleD. This called for a mechanism that would convert the input into a readout that affects downstream targets, the production of a cyclic nucleotide could be such an output.

The function and regulation of PleD in the polar development of *C. crescentus* was investigated in collaboration with the group of Prof. Urs Jenal.[51] They demonstrated that cells lacking a functional PleD protein are hypermotile, unable to eject the flagellum and fail to synthesize a complete structure. In contrast, the presence of the constitutively active mutant protein PleD* results in elongated stalks and has a dominant negative effect on motility. In vitro experiments showed that the input comes in the form of the phosphorylation of the first receiver domain of PleD. Biochemical assays with crude extracts of *C. crescentus* containing the phosphorylated form of PleD, showed that GTP was readily converted into a novel nucleotide compound. To attribute this activity to PleD, assays with the purified protein were performed and confirmed the rapid disappearing of GTP and the formation of this new nucleotide. After analysis and comparison with the chemically synthesized di-nucleotide c-di-GMP, it was confirmed that the novel nucleotide is indeed the cyclic diguanylic acid.[51]

To further investigate the proposed enzymatic reaction, product inhibition was evaluated. When chemically synthesized c-di-GMP was added to the reaction mix in concentrations similar to GTP, strong inhibition was observed. This suggests that c-di-GMP and GTP compete for the binding site. Furthermore, monitoring the concentration of c-di-GMP by HPLC showed that neither a decrease nor a conversion of c-di-GMP into GMP or any other degradation product was observed over a prolonged period of time. Those findings confirm the absence of phosphodiesterase activity of PleD. To investigate the specificity of the activity, tests with deoxyGTP and ATP were performed. It was found that the affinity for ATP is low and both GTP and deoxyGTP bind to PleD but only GTP is converted to c-di-GMP. These experiments proved that PleD harbors an intrinsic nucleotide cyclase activity, that specifically catalyses the conversion of GTP into c-di-GMP and that this activity constitutes the output signaling action of PleD.[51]

Finally, experiments with wild-type PleD and mutated forms, where the GGDEF domain was modified, showed that the mutant proteins lack diguanylate cyclase (DGC) activity in vitro. This confirms the idea that the C-terminal GGDEF domain is responsible for the enzymatic activity. The fact that in vitro activity was observed even without phosphorylation of PleD also showed that the precise activation process remains to be elucidated but it was also clearly indicated that phosphorylation of the first receiver domain increases the activity of the dignuanylate cyclase domain.[51]

Combined with the knowledge that over 900 GGDEF domains are reported in databases, these results imply that diguanylate cyclases (DGCs) might be widespread in the bacterial kingdom and that c-di-GMP might be a common secondary messenger in prokaryotes.[8]

5.1.2 c-di-GMP as a Tool to Investigate the Structure of PleD

The importance of c-di-GMP can be understood when knowing the omnipresence of the GGDEF domain, newly identified as a diguanylate cyclase, in the bacterial genomes, where it occurs in various combinations with other sensory and/or regulatory modules. Despite the large distribution and relevance of DGC proteins, structural and functional information about this class of regulators is largely missing. Response regulators constitute a large protein family. Typically they are composed of a conserved receiver domain and a DNA-binding effector. All structurally characterized receiver domains share structural features that comprise a doubly-wound, five-stranded parallel sheet structure (see *Figure 7*).[52] PleD is an unorthodox response regulator in that it consists of three domains, two receiver domains and one output domain.

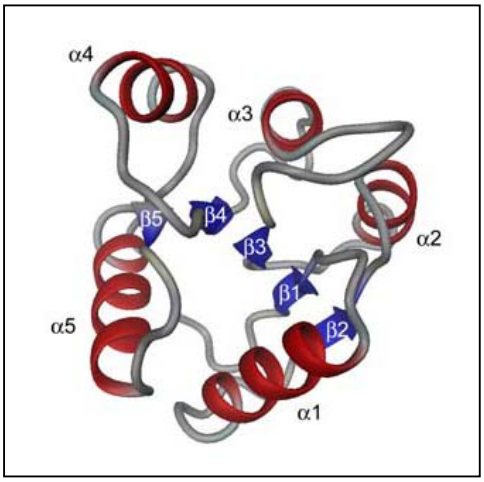


Figure 7: Typical response regulator receiver domains adopting the $(\beta/\alpha)_5$ fold. Secondary structures are labelled. All helices are marked as *α* and all sheets as *β* in this study.[52]

In a collaboration work, the group of Prof. Tilman Schirmer investigated the structure of full length PleD. Purified nonphosphorylated PleD was crystallized at room temperature in the presence of an excess synthetic c-di-GMP. After solving the crystal structure, a linear

arrangement of three structural domains (D1, D2 and DGC) that are connected by single disordered loops, was identified (*Figure 8*).[53]

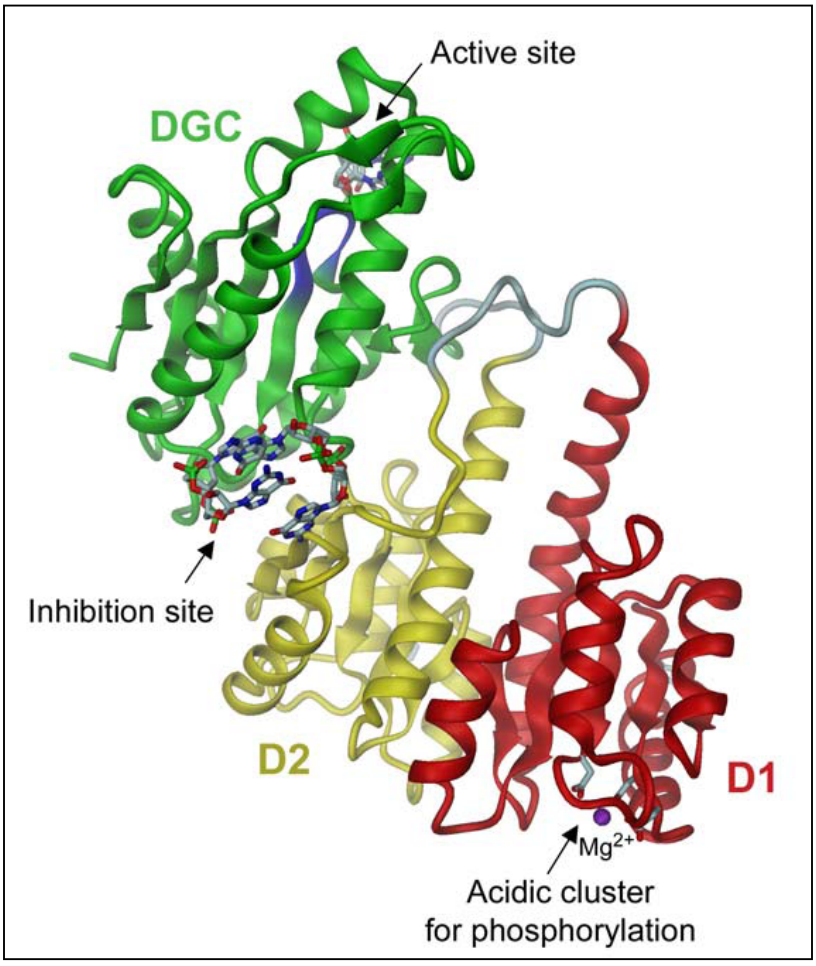


Figure 8: Crystal structure of PleD: the monomer consists of three domains, D1, D2 and DGC, connected by disordered linker peptides.[53]

The crystal structure showed that D1 and D2 have the typical response regulator fold with a $(\beta/\alpha)_5$ topology. However in both cases one α helix, the one linking to the next domain, is considerably extended beyond the globular domain. Both domains resemble each other, with the exception that D1 carries the activation domain while D2 can not be phosphorylated. The so-called “acidic-pocket” constitutes the phosphoacceptor and is shown as coordinating an Mg^{2+} ion. The DGC domain has a different structure, consisting of a five-stranded central β – sheet surrounded by α helixes. The arrangement is closely similar to that of the catalytic core of adenylate cyclase and the “palm” domain of DNA-polymerases. Obviously, not only the structure but also the function of the DGC domain are closely related to adenylate cyclase

and DNA-polymerases, as it also catalyzes the formation of a phosphodiester. The GGDEF signature motif is located on the β-hairpin (blue) and is part of the catalytic active site (A-site) where a c-di-GMP molecule is formed and can also be bound.[53]

Surprisingly, the crystal structure also showed that two c-di-GMP product molecules are bound at the D2/DGC interface, the inhibition site (I-site). (see *Figure 8*) The two molecules are intercalated in a similar structure as has been observed for crystal forms of c-di-GMP (see chapter 1.3).[22, 23] Each central guanyl moiety forming an intermolecular hydrogen-bond with a phosphate. This ligand is bound to both the D2 and DGC subunits of the protein by multiple interactions. Kinetic data revealed strong product inhibition about an order of magnitude lower than estimated for the cellular concentration. This supposes noncompetitive product inhibition i.e. independent of substrate concentration, and can thus be attributed to an allosteric effect of I-site binding.[53]

PleD catalyzes a condensation reaction between two identical substrates to yield a symmetrical product. As determined by analysis, nonactivated PleD seems to exist in solution, in the monomeric form. For the enzyme to be efficient, dimerization seems to be necessary because a reaction catalyzed by a PleD monomer would be limited by the macromolecular diffusion rate. Based on those observations, a mechanistic model was proposed (*Figure 9*). Phosphorylation of the D1 sub-unit, would induce a change of configuration at the D1/D2 interface and D1 would change its orientation with regards to D2, thereby forming a tight stem. This in turn will enhance the dimer formation mediated by interaction at the D1/D2 interfaces. Still, a large distance between the two active sites would not permit catalysis. However, when allowing flexibility of the DGC domains, a “closed” dimer conformation can be modeled, in which a complete two-fold active site is formed between DGC and DGC’. In the context of this activation-by-dimerization model, the observed allosteric effect can also be easily explained. By binding the product at the I-site, the D2/DGC interface would be stabilized, thereby preventing the active sites to come together. Recently, a secondary inhibition site on the DGC domain has also been found. This site would allow for a new form of inhibition, by crosslinking DGC and DGC’ through their respective primary and secondary inhibition sites. However in both cases, the two substrate loaded active sites are hampered from a productive encounter by the immobilization of the DGC domains through crosslinking by two product molecules.[53, 54]

The tight regulation of PleD by product inhibition probably demonstrates the importance of imposing an upper limit on the concentration of c-di-GMP. Nevertheless, it remains to be shown that, as predicted, inhibition is independent of the phosphorylation state of the enzyme. This elucidaded structure of PleD also provides insight into the molecular interactions with c-di-GMP that may recur in the recognition by other proteins.[53, 54]

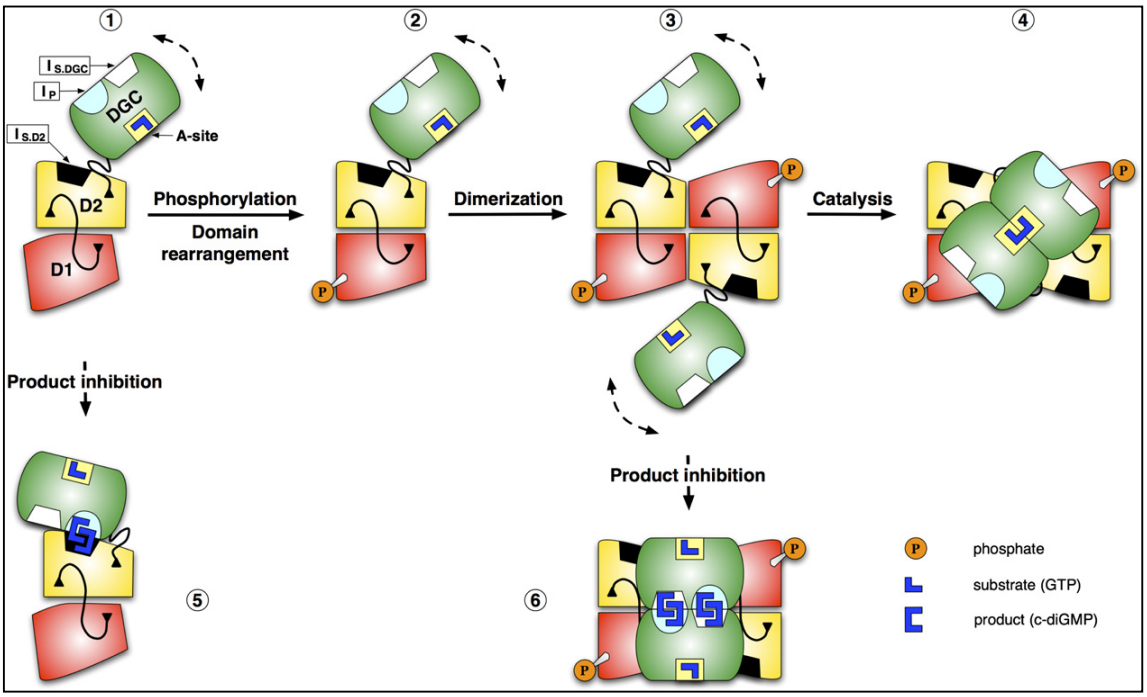


Figure 9: Mechanistic model of PleD regulation. The catalytic DGC domain (green) is connected via a flexible linker peptide (black) to the D1/D2 stem. The DGC domain is postulated to be mobile with respect to the stem, as indicated by the curved arrow (black). The bound GTP substrate is indicated in blue.[53,54]

5.2 The Role of c-di-GMP in the Biofilm Formation

5.2.1 Biofilms: Formation, Structure and Characteristics

The majority of micro-organisms favor a lifestyle where the bacteria are sessile; a surface attached state, rather than being free and isolated in their environment. After being attached to a surface, bacteria form communities called biofilms. Until recently, biofilms were known for their ability to cover and corrode water pipes or ship hulls. In the last few years, their importance in the medical field has been increasing since over 65% of bacterial infections in mankind involve biofilms. They can form on biotic or abiotic surfaces, such as catheters or implants but also on tissues such as teeth, eyes, lungs, ears and uro-genital tracts.[55]

Biofilms are organised multi-layered bacterial communities attached to a surface, and coated by an exopolysaccharide (EPS) matrix. Living in a biofilm is advantageous for the bacteria. The matrix provides a barrier against physical and chemical assaults and they are also resistant to most antibiotics, and to the immune system. The matrix creates an excellent environment for metabolic exchange, which allows multiple species to coexist in the biofilm. It also enhances the lateral transfer of genetic material, which in turn gives bacteria an advantage in dealing with environmental challenges.[56]

Recent research has focused on understanding the mechanisms and regulations of the biofilm development. Biofilm formation is not a random process. Recent studies have indicated that biofilms are a stable point in a biological cycle that includes initiation, maturation, maintenance and dissolution (see *Figure 10*).[57] In the early attachment step, movement generating appendages, such as flagella, are needed to approach the surface to be colonized. During this approach the bacteria will scan the surface and a temporary attachment will be formed. This scanning and sensing for environmental clues is called quorum sensing, if enough bacteria are present, micro-colonies are formed and their differentiation leads to the biofilm maturation. The structure is then strengthened by the creation of an exopolysaccharide matrix while still maintaining a strong plasticity. In the case of pathogenic bacteria, the virulence factors, such as toxins, are expelled at this stage. In the last step, the dissolution, bacteria detach from the structure, due to nutrient limitation or unfavorable environmental conditions, to return to a planktonic living mode. Those bacteria, free in solution, can then induce the formation of new communities in new locations but little is known about the release mechanisms.[55]

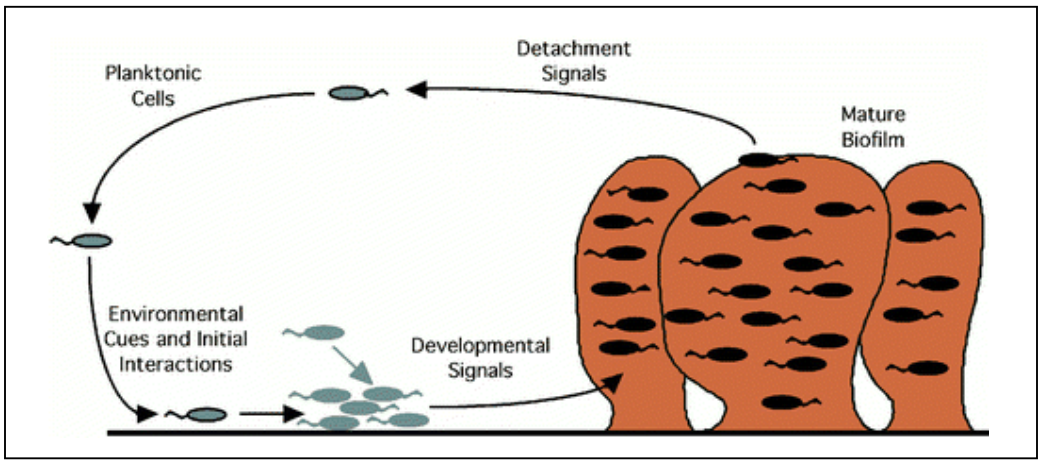


Figure 10: Biofilm formation cycle.[57]

Biofilms are complex three dimensional structures that can be composed of up to 85% of exopolysaccharides. Inside the biofilm, the micro-colonies are separated by aqueous channels serving to bring in nutrients and oxygen and remove waste in deeper areas. Indeed, it was suggested that nutrients are not evenly distributed within biofilms and thus help shape their structure. A decreasing gradient of nutrients and oxygen can be observed when reaching the deepest locations of the biofilm where bacteria are often in completely anaerobic conditions. The channel system also allows for cell-cell communication. Molecules called quorum-sensing signals help trigger and coordinate changes in the bacterial behavior. They are constantly secreted by bacteria in low levels and the receptors are triggered when there are enough bacteria to exceed a critical threshold in signal concentration. The structure and characteristics of biofilms are of prime importance, since biofilm inhabitants are up 1,000 times more resistant to antibiotics than free-floating bacteria. The explanations for this phenomenon are diverse. One of them being that: sedentary bacteria having slower metabolisms, they might be less susceptible to drugs, and the exchange of DNA is easier in the biofilm than for free-floating bacteria, which might also accelerate the transfer of antibiotic-resistance genes. For all these reasons, understanding the biofilm formation and development is important to design new strategies for the control biofilms, especially those involved in chronic diseases or infections.[55, 58]

5.2.2 Exogenous c-di-GMP Delays the Biofilm Formation of *Escherichia coli* Strains

Among the topics that gained a lot of importance in microbiology in the last few years, the involvement of c-di-GMP in the biofilm formation was central. It has been shown that an increase of intracellular c-di-GMP enhances the biofilm formation of *Pseudomonas fluorescens*[16], *Yersinia pestis*[17], *Vibrio cholerae*[59]. An over-expression of the genes involved in the exopolysaccharide synthesis was observed. This signaling molecule was also reported to be involved in the transition from motility to sessility in *Escherichia coli*, *Salmonella typhimurium*[7] and *Caulobacter crescentus*[15]. Again, c-di-GMP is involved in the biofilm formation but a different stage. The regulatory activity of this cyclic dinucleotide is apparently very complex and different biological events seem to be interconnected through this molecule. But this may not be surprising, when considering that the turnover of c-di-GMP is controlled by two types of enzymes, diguanylate cyclases and phosphodiesterases that are found in large number in bacteria.

In 2005, a more surprising effect was found with *Staphylococcus aureus*.[19] Exogenous c-di-GMP was shown to inhibit the cell-cell interactions and biofilm formation in vitro. It also exhibited antibiotic activity in vivo in a mouse model of mastitis infection.[19] This is a completely new effect. However, no obvious link has been found between this signaling molecule and the cell development of this bacterium. Although the molecular mechanisms involved in this effect are not known, the results seem promising. This prompted new interest in c-di-GMP, which could represent a new platform to develop novel antibiotic drugs with new modes of action.[20]

In collaboration with the group of Prof. S. Neunlist in Mulhouse, the study of biofilms formed by *E. coli* is ongoing and should offer more insight in the environmental parameters involved in the biofilm formation. c-di-GMP appears to play a central role; therefore it was decided to investigate the effects of the presence of this molecule in the environment of the bacteria.

Using synthetic c-di-GMP, a series of preliminary experiments were initiated to study the response of different strains of *E. coli* to an exogenous treatment. Although the response was dependant on the strain of bacteria, the trend was that c-di-GMP prevents the biofilm formation (*Figure 11*). Indeed it was observed, after a two hour incubation time, that with c-di-GMP concentrations of 100 µM or higher a visible inhibition effect on the biofilm formation could be obtained. This would suggest that c-di-GMP definitely has a positive effect in the prevention of the biofilm formation. However, it was also found that after 7 days biofilm like structures were observed. These results could suggest that the efficiency of c-di-GMP is limited in time, and is concentration dependant. Moreover, no degradation of c-di-GMP has been observed even after a three week period of incubation. This would suggest that this compound was not metabolized by the bacteria but remained in their environment.

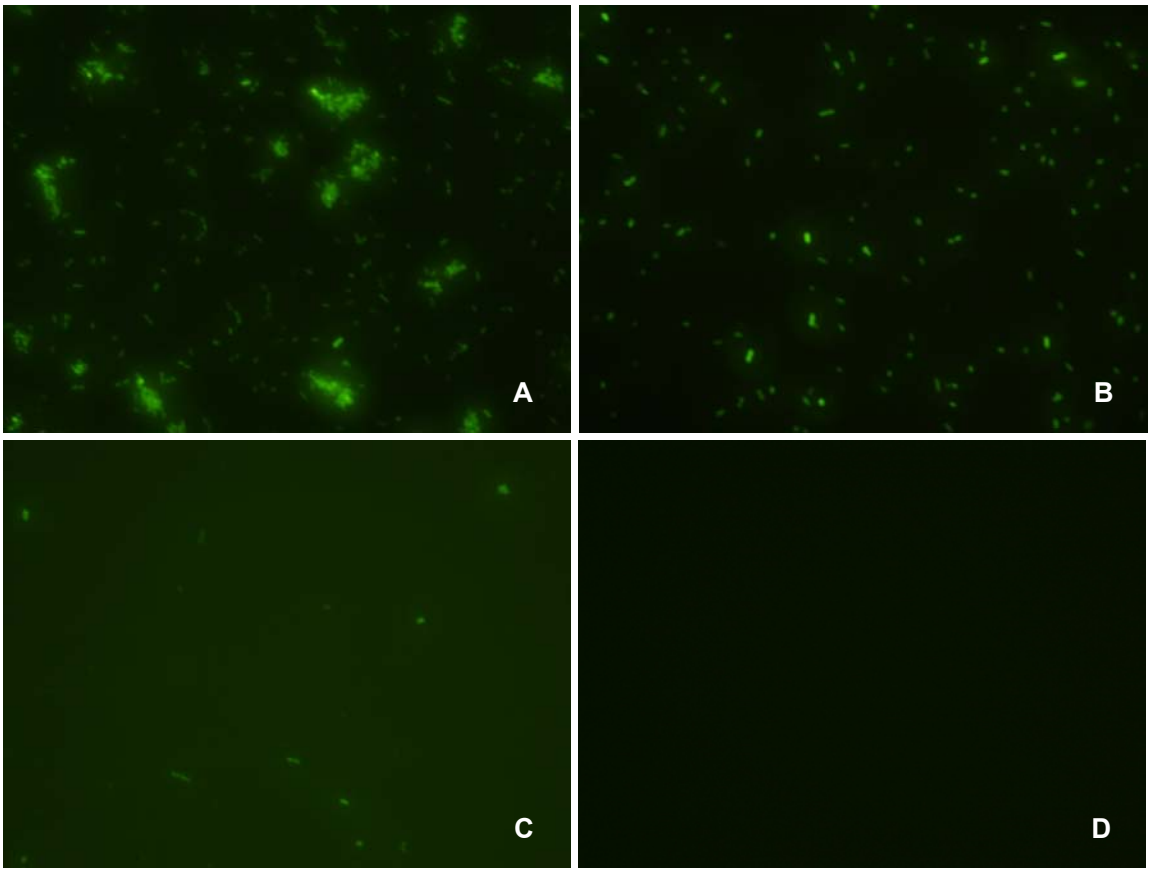


Figure 11: Effects of c-di-GMP on the biofilm formation of *E. coli*. **A**: biofilm formation after 2 hrs in the absence of c-di-GMP (blank experiment). Biofilm formation in the presence of c-di-GMP, after 2 hrs: **B**: 60 μM c-di-GMP; **C**: 100 μM c-di-GMP; **D**: 400μM c-di-GMP.

A model to comply with these results would be that c-di-GMP hindered the biofilm formation for a period of time that was longer the higher the concentration of c-di-GMP was, but did not completely inhibit the biofilm formation. It would appear that c-di-GMP might interfere with cell surface proteins involved in binding to EPS, and necessary to attach the bacteria to the EPS matrix. But with the increasing concentration of EPS in the medium, c-di-GMP might no longer be able to compete for the receptor proteins. It is believed that this was an exogenous response as the general census states that this molecule is unable to cross the cell membrane.

Based on these preliminary results, the main outcome of this kind of studies would be a better understanding of the parameters that regulate the biofilm formation. Further, once the regulation mechanisms are better known, a new class of drugs with new modes of action could be designed. A new class of antibiotics would indeed be of interest, as more and more pathogenic bacteria have become resistant to existing drugs in recent years.

6. SUMMARY AND OUTLOOK

We have successfully designed a new method, starting from sugar building blocks, for the preparation of c-di-GMP that afforded fairly high yields. This new method also proved to be applicable to the synthesis of base-modified analogues.

In comparison to the previously published synthetic methodologies, several drawbacks have been removed and our new method afforded c-di-GMP in higher amounts with higher yields. Since this new synthesis pathway was relatively straightforward, it proved to be suitable for scale-up purposes. Indeed, the synthesis can be performed in a few weeks and reasonable amounts of c-di-GMP could be obtained. We managed to perform the synthesis in such a manner that we could obtain up to 500 mg product. Considering the issues reported in previous methods, our approach seemed the most suitable for the preparation of the large quantities required to investigate the biological activities of c-di-GMP.

One of the main concerns with the other methods, and typically with the synthesis of RNA-oligonucleotides, was the lack of appropriate protecting groups for the 2'-OH function. This issue was completely avoided with our new strategy, where a sugar backbone was prepared first and the base was introduced at a late stage. Since the 1'- and 2'-OH groups were blocked as a cyclic acetal for the whole duration of the synthesis of the sugar building blocks, the preparation of the two sugar units needed was enormously facilitated. And, the synthesis of the building blocks in general, was easy to perform even on a 30 gram scale in high yields.

Assembling the sugar backbone was also easy to perform in fairly high yields even when scaling-up. During the process, care had to be taken to avoid any basic conditions since the protected phosphate linkage was base sensitive. The guanine precursor was also straightforward to synthesize in high yields and could easily be done on a multi-gram scale. The introduction of the base on the sugar backbone following modified Vorbrüggen conditions[38] also achieved high yields once the proper reaction conditions were successfully investigated.

The main advantage of this new approach for the preparation of c-di-GMP was that it allowed for a fairly straightforward preparation of base modified analogues. Indeed, we successfully prepared three analogues where the guanosine was replaced by adenosine, thymine or theophylline.

Moreover, using the synthetic c-di-GMP we prepared, our collaborators were able to perform investigations of the biological relevance of this dinucleotide. Indeed, a crystal structure of the PleD protein of *Caulobacter crescentus* was obtained, which afforded a better understanding of the regulation mechanisms of c-di-GMP in this bacterium. And also allowed for the discovery that c-di-GMP was acting as a second messenger in this same

bacterium. Preliminary investigations of the effects of exogenous synthetic c-di-GMP on the biofilm formation of *Escherichia coli* were also successful in demonstrating that the cyclic nucleotide might have an inhibiting or at least a delaying effect on the formation of biofilms.

Even though the efficiency of our new approach has been shown, it remains to be effectively applied to the preparation of internucleotide linkage modified analogues. Indeed, our attempts to prepare an amide bond linked cyclic dinucleotide have only shown little success in preparing the required cyclic backbone. Thus, more work would be necessary to further optimize the reaction conditions, such as using longer reaction times, other solvent systems or even changing the temperature. It could also be interesting to consider other alternatives, such as more potent coupling reagents or even different reaction sequences, which might afford better results and lead to the desired internucleotide bond modified c-di-GMP analogue.

We could also consider preparing other analogues such as c-di-deoxyGMP or other non-hydrolysable internucleotide linkage modified analogues. These analogues, and those already synthesized, could then be tested for their biological activity since the regulation mechanisms, the functional groups of c-di-GMP involved therein, and the numerous biological roles of c-di-GMP still remain to be uncovered.

Since preliminary results showed an inhibition effect of c-di-GMP on the biofilm formation, further investigations of this biological process and then finding ways to immobilize the cyclic dinucleotide on solid surfaces might be first steps in creating new materials that could be biofilm free.

Another way to gain more insight into the mode of action of c-di-GMP and its interaction towards proteins, might be labeling c-di-GMP (i.e with a fluorophore or a dye) and screening it against peptide libraries or receptor libraries.[60, 61]

Experimental Part

7. GENERAL PROCEDURES

7.1 Analytical Methods

NMR-Spectrometry: NMR spectra were recorded on Bruker Avance 400 (400 MHz) and Bruker Avance DRX 500 (500 MHz) NMR spectrometers, equipped with BBO broadband probeheads. The chemical shift δ is given in ppm, relative to TMS (δ = 0.00 ppm).

References were 7.26 ppm (^{1}H NMR) and 77.16 ppm (^{13}C NMR) for $CHCl_3$, 2.50 ppm (^{1}H NMR) and 39.52 ppm (^{13}C NMR) for DMSO, and 4.79 ppm (^{1}H NMR) for water.[62] 85% phosporic acid (0 ppm) was taken as an internal standard in a capillary for ^{31}P NMR (sr(CD_2Cl_2) = 94.2 Hz, sr($CDCl_3$) = 130.69 Hz, sr(C_6D_6) = 127.98 Hz) measured on the 500 MHz NMR spectrometer.

The assignment of ^{1}H- and ^{13}C-signals was made by 1D-NMR and if necessary 2D-NMR, namely COSY, HMQC, HMBC, TOCSY and NOESY-spectrometry. ^{13}C and ^{31}P, until otherwise noted, were recorded ^{1}H-decoupled. Multiplets were assigned with s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). The index br stands for broad (usually no resolution). The signal for quaternary carbons was abbreviated as: Cq.

Mass Spectrometry (MS): Mass spectra were performed by Dr. H. Nadig. Electron ionization (EI) was measured on VG70-250, fast atom bombardment (FAB) was measured on MAT 312. FAB was performed with 3-nitrobenzyl alcohol as matrix. The signals are given in mass units per charge (m/z). The fragment and intensities of the signals are given in brackets.

MALDI-ToF Mass Spectrometry: Mass Spectra were recorded on Voyager-DE PRO BioSpectrometry Workstation from Applied Biosystems. 4-Nitroaniline was used as matrix. Sample desorption and ionization was induced by a N_2-laser (337 nm, 3ns pulses). The signals are referred to the unfragmented charged molecule ions $[M-H]^-$ and $[M+H]^+$. The data are given in mass units per charge (m/z).

High Resolution Mass Spectometry (HRMS): Mass Spectra were performed by the "Service de spectrometrie de masse" at the Université Louis Pasteur, Strabourg. The data are given in mass units per charge (m/z).

Infrared Spectrometry (IR): Infrared spectra were measured on a Perkin-Elmer-1600 Series Fourier-Transform spectrometer. Solid samples were prepared as KBr wafers, liquid samples were prepared between NaCl plates. Absorption bands are given in wave numbers $v \sim [cm^{-1}]$.

Elemental Analysis (EA): Elemental analyses were carried out by Mr. W. Kirsch at the Department of Chemistry at the University of Basel, on Leco CHN-900 (C-, H-, N-detection) and Leco RO-478 (O-detection) analysers. The data are indicated in mass percent.

7.2 Purification Methods

Thin Layer Chromatography (TLC): Reactions were monitored by thin layer chromatography. Silica gel $60F_{254}$ plates from *Merck* were used. Coumpounds were visualized by UV (254 nm) and/or ceric ammonium molybdate (CAM) dip. Retention Factors (R_f) are indicated with corresponding solvent mixture in brackets.

Flash Column Chromatography (FC): For Flash Chromatography silica gel *Merck 60* (40-63 µm) was used under low pressure (~1.5 bar, membrane pump). The solvents used were of technical grade and freshly re-distilled prior to use. The ratios of solvents in the mixtures are referred to volume parts. Generally, the flash column chromatography according to Still[63] was performed.

High Performance Liquid Chromatography (HPLC): For HPLC analysis *Waters* Alliance 2690 Separations Modul and 2680 Dual Mode UV-Vis detector were used. *Merck* reverse phase RP18e Lichrospher 100, 250-4 (5µm) columns were used for analysis and *Merck* reverse phase RP18e Lichrospher 100, 250-10 (10µm) columns for separation purposes.

Size Exclusion Chromatography: Size exclusion chromatographies were performed either on Sephadex LH 20 resin or on Sephadex G15 resin purchased from *Sigma*.

7.3 Solvents and Chemicals

Solvents: Toluene, THF and dichloromethane were dried and degassed by reflux over an adequate drying agent under nitrogen.[64] Absolute solvents were purchased from *Fluka* or *Aldrich* in septum sealed bottles, kept under inert atmosphere and over molecular sieves. Deuterated solvents were purchased from *Cambridge Isotope Laboratories, Inc*.. Technical grade solvents used for extraction and purification were re-distilled prior to use. Nanopure water used for purification purposes was filtered over a *Barnstead* ultrapure water system.

Chemicals: Materials and reagents were purchased in the highest commercially available grade from *Fluka*, *Aldrich*, *Acros* and *Senn* and used without further purification.

7.4 Buffers and Solutions

TEAC buffer, 1 M, pH=7:
54 ml triethylamine, dry ice and 446 ml nanopure water

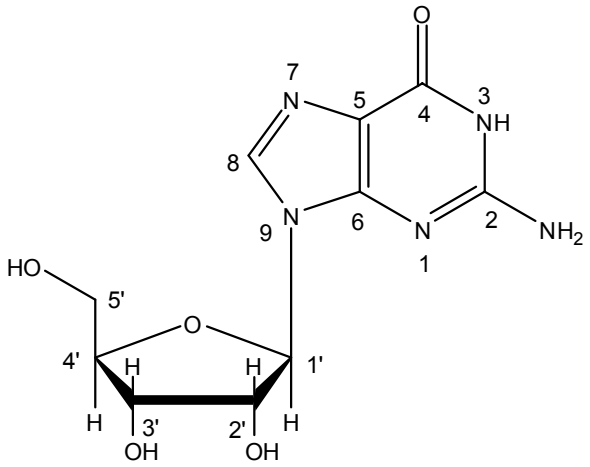
TEAA buffer, 0.1 M, pH=7:
100 ml TEAA 1M (Fluka) and 900 ml nanopure water

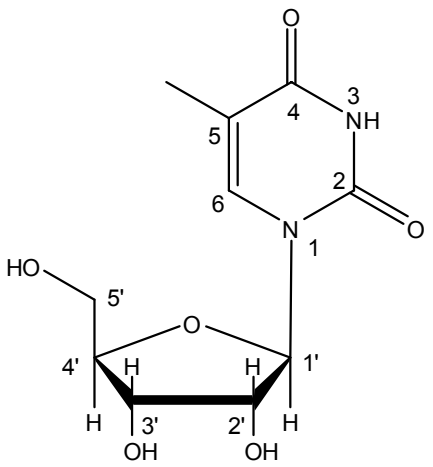
Na-phosphate buffer 0.1 M, pH=5.5:
90 g NaH_2PO_4, 32.7 g Na_2HPO_4 and 500 ml water

MALDI-ToF matrix preparation:
~12-15 mg 4-nitroaniline dissolved in 1 ml CH_2Cl_2:MeOH (9:1, v/v).

CAM-Dip:
10 g Cerium (IV)sulfate-tetrahydrate, 25 g ammonium-heptamolybdate-tetrahydrate, 100 ml 96% sulfuric acid and 900 ml distilled water

7.5 Assignment of C and H for Purine and Pyrimidine Derivatives





The protons in the ^{1}H NMR spectra are numbered the same as the related carbon or hetero atoms. Atoms within the sugar backbone of the nucleotides are marked with an additional prime. If two nucleotides were present, each was referred to separately with an additional “a” or “b” in the assignements.

8. INVESTIGATIONS TOWARDS A NEW SYNTHETIC PATHWAY FOR C-DI-GMP

8.1 Van Boom Synthesis Pathway

8.1.1 3',5'-*O*-(tetra*iso*propyldisiloxane-1,3-diyl)-guanosine (21)

20 **21**

5 g (17.66 mmol, 1 eq) guanosine **20** and 6.01 g (88.31 mmol, 5 eq) imidazole were poured in suspension in 50 ml DMF at RT under argon. 6.64 ml (21.1 mmol, 1.2 eq) 1,3-dichloro-1,1,3,3-tetra*iso*propyldisiloxane (TiPS-Cl) were added and the mixture stirred for 3 hrs. 15 ml toluene were added and the solvents evaporated under reduced pressure. The residue was taken-up in 60 ml $CHCl_3$-MeOH (3:1), washed with a saturated aqueous solution of $NaHCO_3$ (3 x 50 ml) and the combined organic solvents were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was taken up in DMF (20 ml) and the mixture poured into 1 L water. The precipitate was filtered off and re-crystallized from boiling ethanol yielding compound **21** as a white solid (3.95 g, 7.51 mmol, 43%).

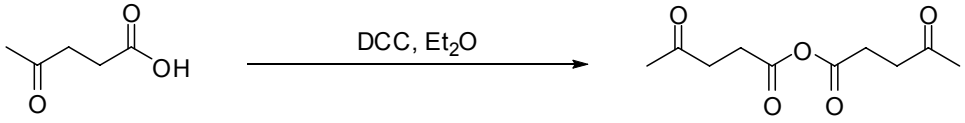
$C_{22}H_{39}N_5O_6Si_2$: 525.76 g/mol

R_f (CH_2Cl_2:MeOH, 95:5) = 0.5;

^{1}H NMR (400.0 MHz, $CDCl_3$, δ ppm): 7.73 (s, 1H, ^{8}CH), 5.65 (d, 1H, *J*= 1.76 Hz, $^{1'}$CH), 5.60 (d, 1H, *J*= 5.08 Hz, $^{2'}$OH), 4.33 (m, 1H, $^{2'}$CH), 4.23 (m, 1H, $^{3'}$CH), 4.07 (m, 1H, $^{5'}CH_2$), 3.96 (m, 1H, $^{4'}$CH), 3.90 (m, 1H, $^{5'}CH_2$), 1.03 (m, 28H, $CH_{isopropyl}$, $CH_{3\ isopropyl}$);

MS (Maldi-ToF, m/z): 526.1 ($[M]^+$).

8.1.2 Levulinic anhydride (Lev_2O) (23)

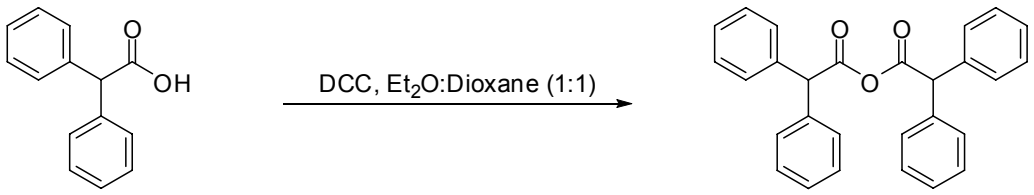


22 **23**

5.80 g (49.97 mmol, 1 eq) levulinic acid **22** and 5.15 g (24.99 mmol, 0.5 eq) *N*,*N*'-dicyclohexylcarbodiimide (DCC) were dissolved in 100 ml dry diethyl ether at RT under argon and stirred for 5 hrs. The reaction mixture was filtered over celite and the filtrate was concentrated under reduced pressure. 5.84 g of a dense oil were obtained (24.97 mmol, quant.).

$C_{10}H_{14}O_5$: 214.22 g/mol
^{1}H NMR (400.0 MHz, $CDCl_3$, δ ppm): 2.75 (m, 2H, CH_2, J_1= 1.52 Hz, J_2= 6.8 Hz), 2.68 (m, 3H, CH_2, J_1= 7.08 Hz, J_2= 1.52 Hz), 2.16 (s, 3H, CH_3);
^{13}C NMR (100.6 MHz, $CDCl_3$, δ ppm): 206.37 ($C{=}O_{ketone}$), 168.89 ($C{=}O_{anhydride}$), 37.69 (CH_2), 30.14 (CH_3), 29.42 (CH_2);
MS (Maldi-ToF, m/z): 237.4 ($[M+Na]^+$).

8.1.3 Diphenylacetic anhydride (dpa_2O) (25)



24 **25**

25.00 g (0.12 mol, 1 eq) diphenylacetic acid **24** were dissolved in a 1:1 mixture of dioxane-diethyl ether (250 ml) at RT under argon. 12.15 g (0.06 mol, 0.5 eq) DCC were added and the mixture stirred at RT for 2 hrs, then the suspension was cooled to 0°C for 1 hr. The mixture was then filtered over celite and the solvents removed under reduced pressure. The residue was re-crystallized from pentane to yield 23.90 g of **25** as a white solid (0.06 mol, quant.).

$C_{28}H_{22}O_3$: 406.49 g/mol

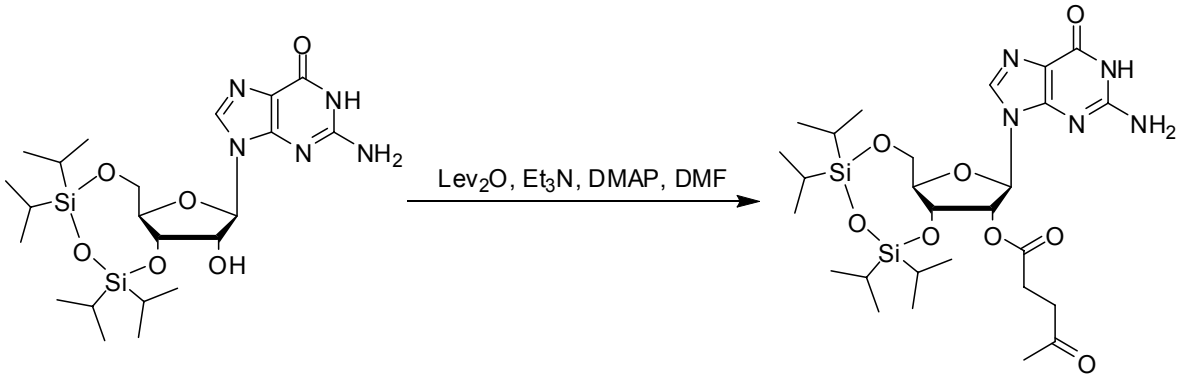
R_f (CH_2Cl_2:MeOH, 95:5) = 0.91;

^{1}H NMR (400.0 MHz, $CDCl_3$, δ ppm): 7.27 (m, 10H, CH_{arom}), 5.04 (s, 1H, CH);

^{13}C NMR (100.6 MHz, $CDCl_3$, δ ppm): 167.9 (C=O), 137.2 (C_{arom}), 129.8-128.1 (CH_{arom}), 58.2 (CH);

MS (Maldi-ToF, m/z): 429.3 ($[M+Na]^+$).

8.1.4 2'-*O*-levulinoyl-3',5'-*O*-(tetra*iso*propyldisiloxane-1,3-diyl)-guanosine (26)



21 **26**

3.00 g (5.71 mmol) 3',5'-*O*-(tetra*iso*propyldisiloxane-1,3-diyl)-guanosine **21** were dissolved in 40 ml DMF at RT under argon. 1.9 ml (13.69 mmol, 2.4 eq) triethylamine, 1.46 g (6.84 mmol, 1.2 eq) levulinic anhydride and a catalytic amount 4-dimethylamino-pyridine (DMAP) were added. The mixture was stirred for 15 min then 1 ml water as well as 10 ml toluene were added to the solution. The solvents were evaporated under reduced pressure. The residue was taken-up in 100 ml chloroform then washed with sat. aq. $NaHCO_3$ sol. (100 ml) and water (100 ml). The organic layer was dried ($MgSO_4$) and the volatiles removed under reduced pressure. Re-crystallization from ethanol yielded **26** as a white solid (471 mg, 0.75 mmol, 49%).

$C_{27}H_{45}N_5O_8Si_2$: 623.86 g/mol

R_f (CH_2Cl_2:MeOH, 90:10) = 0.55;

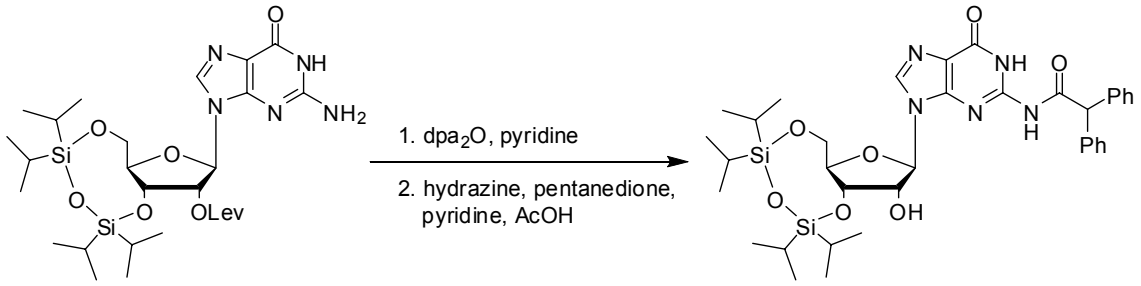
^{1}H NMR (400.0 MHz, $CDCl_3$, δ ppm): 11.99 (s, 1H, ^{3}NH), 7.81 (s, 1H, ^{8}CH), 6.40 (s, 2H, ^{2}NH), 5.70 (d, 1H, J_1= 4.04 Hz, $^{1'}$CH), 4.70 (dd, 1H, J_1= 8.45 Hz, J_2= 5.47 Hz, $^{2'}$CH), 4.13 (m, 1H,

$^{3'}$CH), 3.99 (m, 3H, $^{5'}CH_2$, $^{4'}$CH), 2.70 (m, 4H, $CH_{2\ Lev}$), 2.18 (s, 3H, $CH_{3\ Lev}$), 1.02 (m, 28H, $CH_{isopropyl}$, $CH_{3\ isopropyl}$);

^{13}C NMR (100.6 MHz, $CDCl_3$, δ ppm): 206.8 ($C{=}O_{Lev}$), 171.5 (COO_{Lev}), 137.8 (^{6}Cq), 137.7 (^{5}Cq), 128.3 (^{8}CH), 87.5 ($^{4'}$CH), 82.2 (^{3}CH), 75.8 ($^{2'}$CH), 69.5 ($^{1'}$CH), 60.9 ($^{5'}CH_2$), 38.3 (CH_2CO_{Lev}), 28.1 (CH_2COO_{Lev});

MS (Maldi-ToF, m/z): 646.5 ($[M+Na]^+$).

8.1.5 3',5'-*O*-(tetra*iso*propyldisiloxane-1,3-diyl)-2-*N*-(diphenylacetyl)-guanosine (27)



26 **27**

330 mg (0.53 mmol, 1 eq) 2'-*O*-levulinoyl-3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-guanosine **26** were dissolved in 10 ml dry pyridine at RT under argon. 640 mg (1.59 mmol, 3 eq) diphenylacetic anhydride were added and the mixture heated to 60°C for 2 hrs. The reaction was quenched with 2 ml water and the solvents were co-evaporated with 20 ml toluene. The residue was taken-up in 100 ml chloroform, washed successively with 100 ml sat. aq. $NaHCO_3$ sol. and 100 ml water. The organic phase was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was taken up in 10 ml dry pyridine. 2.65 ml (2.64 mmol, 5 eq) of a 1 M solution of hydrazine hydrate in a 3:2 mixture of pyridine:acetic acid (v/v), were added and the reaction stirred for 3 min. 1.10 ml (10.60 mmol, 20 eq) 2,4-pentanedione were added and the mixture stirred at RT for 2 min, then at 0°C for 3 min. Chloroform (35 ml) and water (15 ml) were added. The organic layer was washed with 100 ml sat. aq. $NaHCO_3$ sol., 100 ml water and 100 ml 1 M K_2HPO_4 sol. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by flash chromatography (CH_2Cl_2:MeOH, gradient from 100:0 to 95:5, v/v, +0.5% triethylamine) to yield 0.51 g of **27** as a colorless oil (0.50 mmol, 95%).

$C_{36}H_{49}N_5O_7Si_2$: 719.99 g/mol

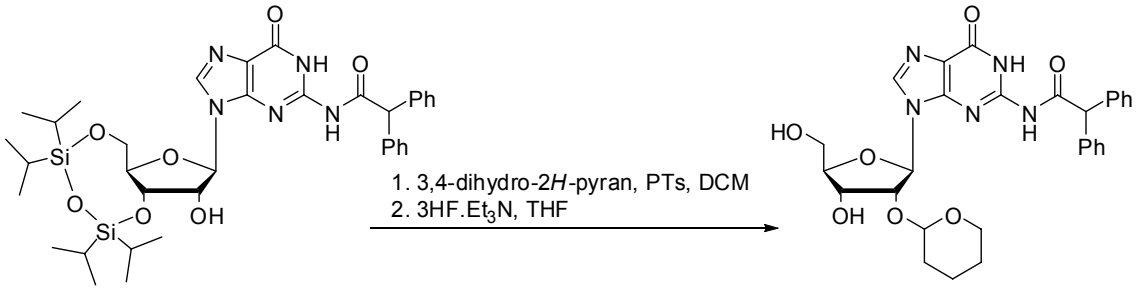
R_f (CH_2Cl_2:MeOH, 95:5) = 0.47.

^{1}H NMR (400.0 MHz, $CDCl_3$, δ ppm): 11.99 (s, 1H, ^{3}NH), 7.81 (s, 1H, ^{8}CH), 7.32 (m, 10H, CH_{arom}), 6.40 (s, 2H, ^{2}NH), 5.70 (d, 1H, J_1= 4.03 Hz, $^{1'}$CH), 5.33 (s, 1H, CH_{dpa}), 4.70 (dd, 1H, J_1= 8.44 Hz, J_2= 5.46 Hz, $^{2'}$CH), 4.13 (m, 1H, $^{3'}$CH), 3.99 (m, 3H, $^{5'}CH_2$, $^{4'}$CH), 1.02 (m, 28H, $CH_{isopropyl}$, $CH_{3\ isopropyl}$);

^{13}C NMR (100.6 MHz, $CDCl_3$, δ ppm): 179.4 ($C{=}O_{dpa}$), 147.9 (Cq_{arom}), 147.7 (Cq_{arom}), 137.8 (^{6}Cq), 137.7 (^{5}Cq), 129.4 (CH_{arom}), 129.2 (CH_{arom}), 128.9 (CH_{arom}), 128.3 (^{8}CH), 122.7 (CH_{arom}), 87.5 ($^{4'}$CH), 82.2 (^{3}CH), 75.8 ($^{2'}$CH), 69.5 ($^{1'}$CH), 60.9 ($^{5'}CH_2$), 59.2 (CH_{dpa});

MS (Maldi-ToF, m/z): 742.1 ([M+Na]$^+$).

8.1.6 2'-*O*-tetrahydropyranyl-2-*N*-diphenylacetyl guanosine (28)



27 **28**

1.38 g (1.92 mmol, 1 eq) 3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2-*N*-(diphenylacetyl)-guanosine **27** were dissolved in 15 ml dichloromethane at RT under argon. 5.22 ml (57.60 mmol, 30 eq) 3,4-dihydro-2*H*-pyran and 1.44 g (5.76 mmol, 3 eq) pyridinium toluene-4-sulfonate (PTs) were added and the mixture stirred for 4 hrs at RT. The reaction mixture was diluted with diethyl ether (80 ml) and washed with half-saturated brine (100 ml). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was dissolved in THF (20 ml), 3.12 ml (19.19 mmol, 10 eq) triethylamine trihydrofluoride were added and the mixture stirred under argon at RT overnight. The reaction was quenched with Silica gel and the solvents removed under reduced pressure. Flash chromatography (CH_2Cl_2:MeOH, gradient from 100:0 to 95:5, v/v) yielded **28** as a white solid (1.01 g, 1.78 mmol, 93%).

$C_{29}H_{31}N_5O_7$: 561.60 g/mol

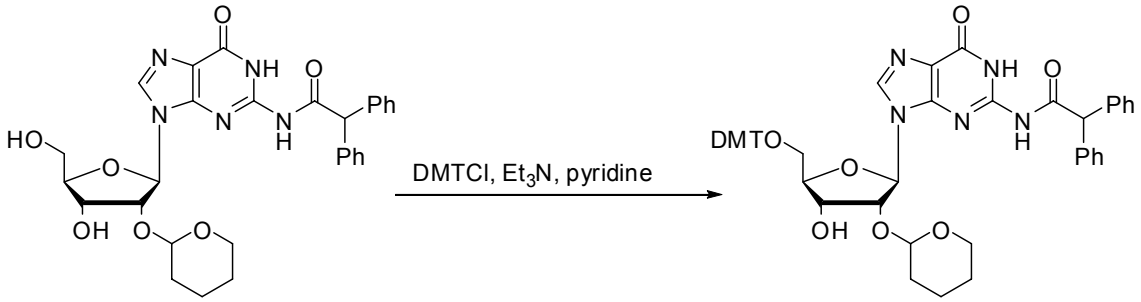
R_f (CH_2Cl_2:MeOH, 95:5) = 0.18;

^{1}H NMR (400.0 MHz, $CDCl_3$, δ ppm): 7.90 (s, 1H, ^{8}CH), 7.32 (m, 10H, CH_{arom}), 5.83 (d, 1H, J_1= 6.8 Hz, $^{1'}$CH), 5.33 (s, 1H, CH_{dpa}), 5.01 (m, 1H, $^{2'}$CH), 4.61 (m, 1H, $^{3'}$CH), 4.39 (m, 2H, $CH_{2\ THP}$), 4.22 (m, 1H, CH_{THP}), 3.85 (m, 2H, $CH_{2\ THP}$), 3.65 (m, 1H, $^{5'}CH_2$), 3.57 (m, 1H, $^{4'}$CH), 3.41 (m, 1H, $^{5'}CH_2$), 1.72 (m, 2H, $CH_{2\ THP}$), 1.46 (m, 4H, $CH_{2\ THP}$);

^{13}C NMR (100.6 MHz, $CDCl_3$, δ ppm): 179.4 ($C{=}O_{dpa}$), 174.5 (^{4}C=O), 155.6 (^{2}Cq), 147.9 (Cq_{arom}), 147.7 (Cq_{arom}), 137.8 (^{6}Cq), 137.7 (^{5}Cq), 129.4 (CH_{arom}), 129.2 (CH_{arom}), 128.9 (CH_{arom}), 128.3 (^{8}CH), 122.7 (CH_{arom}), 102.4 (CH_{THP}), 88.5 ($^{4'}$CH), 86.9 ($^{2'}$CH), 82.2 ($^{1'}$CH), 72.2 ($^{3'}$CH), 66.1 ($^{5'}CH_2$), 63.1 ($CH_{2\ THP}$), 59.2 (CH_{dpa}), 31.4 ($CH_{2\ THP}$), 31.2 ($CH_{2\ THP}$), 25.2 ($CH_{2\ THP}$), 21.4 ($CH_{2\ THP}$);

MS (Maldi-ToF, m/z): 584.5 ([M+Na]$^+$).

8.1.7 5'-*O*-dimethoxytriphenylmethyl-2'-*O*-pyranyl-2-*N*-diphenylacetyl guanosine (29)



28 **29**

300 mg (0.53 mmol, 1 eq) 2'-*O*-tetrahydropyranyl-2-*N*-diphenylacetyl guanosine **28** were co-evaporated twice with pyridine and then dissolved in 5 ml dry pyridine under argon. The solution was added with 218 mg (0.64 mmol, 1.2 eq) 4,4'-dimethoxytritylchloride (DMTCl) and stirred for 16 hrs. The reaction was quenched with 5 ml sat. aq. $NaHCO_3$ sol. then extracted with DCM. The organic fractions were dried ($MgSO_4$) and concentrated. Flash chromatography (hexane:CH_2Cl_2, 1:1, CH_2Cl_2:MeOH gradient from 100:0 to 95:5, v/v, +1% Et_3N) yielded 352 mg (0.41 mmol, 76%) of the desired product **29**.

$C_{50}H_{49}N_5O_9$: 863.98 g/mol

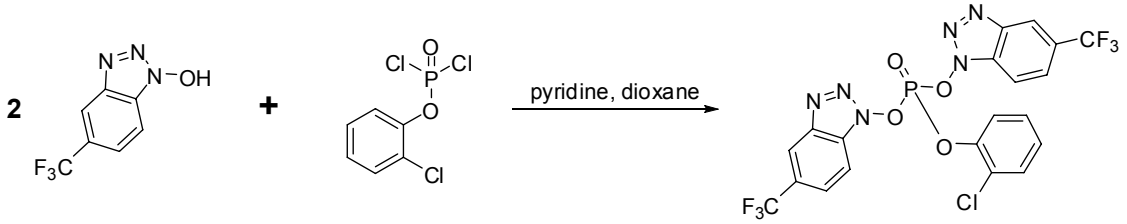
R_f (CH_2Cl_2:MeOH, 95:5) = 0.61;

^{1}H NMR (400.0 MHz, $CDCl_3$, δ ppm): 7.92 (s, 1H, ^{8}CH), 7.32 (m, 10H, CH_{arom}), 7.31 (m, 2H, CH_{arom}), 7.17 (m, 8H, CH_{arom}), 6.75 (m, 2H, CH_{arom}), 5.81 (d, 1H, J_1= 6.8 Hz, $^{1'}$CH), 5.34 (s, 1H, CH_{dpa}), 5.01 (m, 1H, $^{2'}$CH), 4.62 (m, 1H, $^{3'}$CH), 4.40 (m, 2H, $CH_{2\ THP}$), 4.21 (m, 1H, CH_{THP}), 3.87 (m, 2H, $CH_{2\ THP}$), 3.83 (s, 6H, $CH_{3\ DMT}$), 3.69 (m, 1H, $^{5'}CH_2$), 3.54 (m, 1H, $^{4'}$CH), 3.41 (m, 1H, $^{5'}CH_2$), 1.75 (m, 2H, $CH_{2\ THP}$), 1.39 (m, 4H, $CH_{2\ THP}$);

^{13}C NMR (100.6 MHz, $CDCl_3$, δ ppm): 179.5 ($C{=}O_{dpa}$), 174.5 (^{4}C=O), 158.9 (Cq_{arom}), 158.8 (CH_{arom}), 155.6 (^{2}Cq), 147.9 (Cq_{arom}), 147.7 (Cq_{arom}), 143.9 (Cq_{arom}), 137.8 (^{6}Cq), 137.7 (^{5}Cq), 136.3 (Cq_{arom}), 130.6 (CH_{arom}), 130.5 (CH_{arom}), 129.4 (CH_{arom}), 129.2 (CH_{arom}), 128.9 (CH_{arom}), 128.5 (CH_{arom}), 128.4 (^{8}CH), 127.9 (CH_{arom}), 122.7 (CH_{arom}), 113.8 (CH_{arom}), 112.9 (CH_{arom}), 102.3 (CH_{THP}), 88.5 ($^{4'}$CH), 86.8 ($^{2'}$CH), 82.3 ($^{1'}$CH), 72.3 ($^{3'}$CH), 66.0 ($^{5'}CH_2$), 63.1 ($CH_{2\ THP}$), 59.1 (CH_{dpa}), 31.6 ($CH_{2\ THP}$), 31.4 ($CH_{2\ THP}$), 25.1 ($CH_{2\ THP}$), 21.4 ($CH_{2\ THP}$);

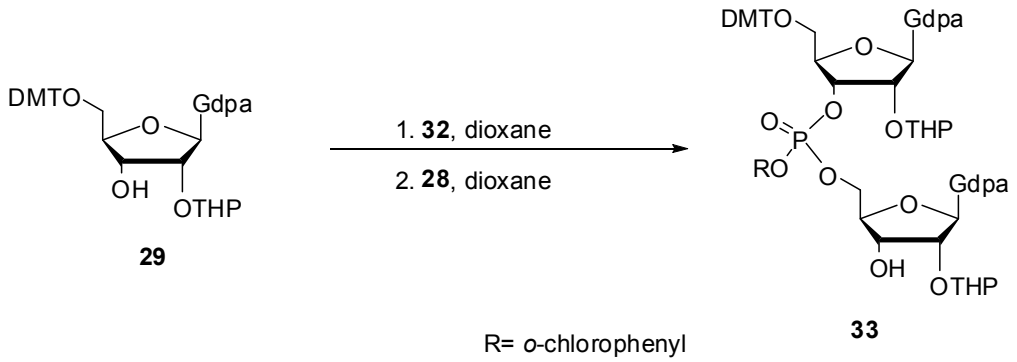
MS (Maldi-ToF, m/z): 888.1 ($[M+Na]^+$).

8.1.8 6-trifluoro-methylbenzotriazole phosphorylating agent (32)



0.50 ml (3.10 mmol, 0.5 eq) 2-chlorophosphoryldichloridate **31** were dissolved into 3 ml dry dioxane at RT and added dropwise at RT to a mixture of 1.27 g (6.25 mmol, 1 eq) 1-hydroxy-6-trifluoro-methylbenzotriazole **30** in 12.5 ml dry dioxane and 0.5 ml (6.37 mmol, 1.02 eq) dry pyridine. The obtained suspension was stirred for 1 hr then filtered under an inert atmosphere of argon. A yellow 0.2 M stock solution of phosphorylating agent **32** was obtained, and used without further purification.

8.1.9 (5'-*O*-dimethoxytriphenylmethyl-2-*N*-diphenylacetyl-2'-*O*-tetrahydropyranyl-guanosine)-(3'-5')-(2-*N*-diphenylacetyl-2'-*O*-tetrahydropyranyl-guanosine)-2-chlorophenyl phosphate (33)



344 mg (0.40 mmol, 1 eq) 5'-*O*-dimethoxytriphenylmethyl-2-*N*-diphenylacetyl-2'-*O*-tetra hydropyranyl guanosine **29** were co-evaporated with pyridine (2 x 5 ml) and then dissolved in dry dioxane (3 ml) under argon. 1.1 ml (0.44 mmol, 0.2 M, 1.1 eq) phosphorylating agent **33** were added and the mixture stirred at RT for 5 min. 268 mg (0.48 mmol, 1.2 eq) N^2-diphenylacetyl-2'-*O*-tetrahydropyranyl guanosine **28** in 0.5 ml dioxane were added and the reaction mixture stirred at RT for 1 hr. The solution was diluted with CH_2Cl_2 (20 ml) and washed with triethylammoniun acetate (TEAC) buffer (50 ml of a 1 M solution and then 50 ml of a 0.1 M solution). The organic fractions were dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography (CH_2Cl_2:MeOH, gradient from 100:0 to 95:5, v/v, +0.1% Et_3N) yielded 477 mg (0.30 mmol, 74%) of the desired product, dimer **33** as a non separable mixture of diastereomers.

$C_{85}H_{82}ClN_{10}O_{18}P$: 1598.08 g/mol

R_f (CH_2Cl_2:MeOH, 95:5) = 0.37;

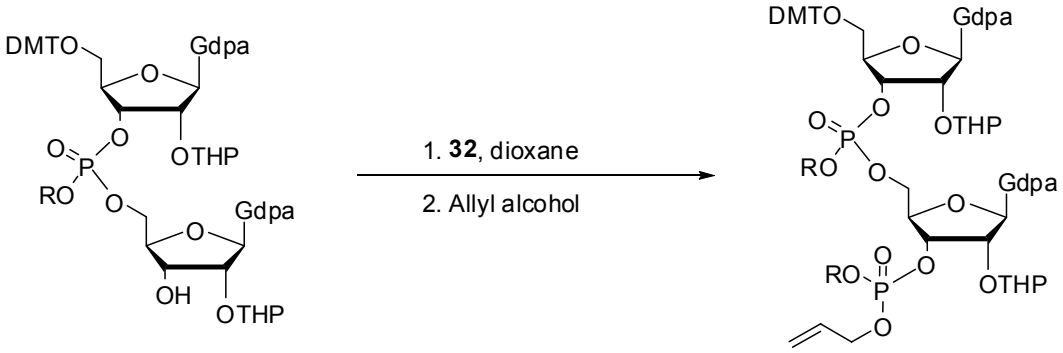
^{1}H NMR (400.0 MHz, $CDCl_3$, δ ppm): 7.94-7.92 (m, 2H, ^{8}CH), 7.57-7.15 (m, 23H, CH_{arom}), 6.85-6.75 (m, 4H, CH_{arom}), 5.84-5.81 (m, 2H, $^{1'}$CHa, $^{1'}$CHb), 5.34-5.32 (m, 2H, CH_{dpa}), 5.12 (m, 1H, $^{3'}$CHa), 5.05-4.95 (m, 2H, $^{2'}$CHa, $^{2'}$CHb), 4.63 (m, 1H, $^{3'}$CHb), 4.61-4.57 (m, 2H, $^{5'}CH_2$b), 4.44-4.38 (m, 2H, CH_{THP}), 4.27 (m, 1H, $^{4'}$CHb), 4.25-4.12 (m, 4H, $CH_{2\ THP}$), 4.13 (m, 1H, $^{4'}$CHa), 3.87-3.35 (m, 10H, $CH_{3\ DMT}$, $CH_{2\ THP}$), 3.67 (m, 1H, $^{5'}CH_2$b), 3.54 (m, 1H, $^{5'}CH_2$a), 3.41 (m, 2H, $^{5'}CH_2$a, $^{5'}CH_2$b), 2.75-2.64 (m, 4H, $CH_{2\ THP}$), 1.75-1.48 (m, 4H, $CH_{2\ THP}$);

^{13}C NMR (100.6 MHz, $CDCl_3$, δ ppm): 179.5 ($C{=}O_{dpa}$), 174.5 (^{4}C=O), 158.9 (Cq_{arom}), 158.8 (CH_{arom}), 155.6/155.5 (^{2}Cq), 148.3/143.9 (Cq_{arom}), 137.8/136.9 (^{6}Cq, ^{5}Cq), 136.3/135.9 (Cq_{arom}), 130.6-128.7 (CH_{arom}), 128.4/128.0 (^{8}CH), 127.9-113.8 (CH_{arom}), 102.3/101.9 (CH_{THP}), 88.5/88.3 ($^{4'}$CHa), 87.9/87.7 ($^{4'}$CHb), 86.8/86.6 ($^{2'}$CHa), 86.0/85.9 ($^{2'}$CHb), 82.4/82.1 ($^{1'}$CHa),

81.9/81.7 ($^{1'}$CHb), 72.3/72.1 ($^{3'}$CHa), 70.9/70.5 ($^{3'}$CHb), 66.0/65.8 ($^{5'}CH_2$b), 63.1 ($CH_{2\ THP}$), 62.3/62.0 ($^{5'}CH_2$a), 59.1/58.9 (CH_{dpa}), 55.8/55.7 (CH_3), 31.6/30.8 ($CH_{2\ THP}$), 25.3/24.8 ($CH_{2\ THP}$), 21.5/21.2 ($CH_{2\ THP}$);

MS (Maldi-ToF, m/z): 1621.2 ([M+Na]$^+$).

8.1.10 (5'-*O*-dimethoxytriphenylmethyl-2-*N*-diphenylacetyl-2'-*O*-tetrahydropyranyl-guanosine)-(3'-5')-(2-*N*-diphenylacetyl-3'-*O*-(2-chlorophenyl phosphate)-2'-*O*-tetrahydropyranyl-guanosine)-2-chlorophenyl phosphate (34)



33 **34**

R= *o*-chlorophenyl

461 mg (0.28 mmol 1 eq) of dimer **33** were co-evaporated with dioxane (2 x 5 ml) then dissolved in dry dioxane at RT under argon, added with 1.44 ml (0.31 mmol, 0.2 M, 1.1 eq) phosphorylating agent 32 and stirred for 5 min. 20 µl (0.56 mmol, 2 eq) allylic alcohol were added and the reaction mixture stirred for 1 hr at RT. The solution was diluted with DCM (20 ml) and washed with ammonium hydrogen phosphate buffer (30 ml of a 1 M solution and 30 ml of a 0.1 M solution). The organic layer was dried and the volatiles removed under reduced pressure. Flash chromatography (CH_2Cl_2:MeOH, gradient from 100:0 to 95:5, v/v, +0.1% Et_3N) led to 335 mg (0.18 mmol, 64%) of the desired product **34**, as a non separable mixture of diastereomers.

$C_{96}H_{92}Cl_2N_{20}O_{21}P_2$: 1828.63 g/mol

R_f (CH_2Cl_2:MeOH, 95:5) = 0.59;

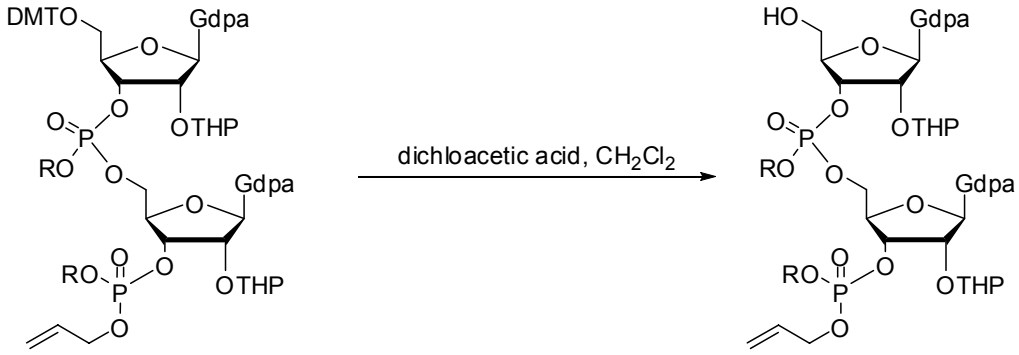
^{1}H NMR (400.0 MHz, $CDCl_3$, δ ppm): 7.96-7.93 (m, 2H, ^{8}CH), 7.58-7.18 (m, 27H, CH_{arom}), 6.88-6.77 (m, H, CH_{arom}), 5.93-5.91 (m, 1H, CH_{allyl}), 5.86-5.83 (m, 2H, $^{1'}$CHa, $^{1'}$CHb), 5.42-5.38 (m, 1H, $CH_{2\ alkene}$), 5.33-5.32 (m, 2H, CH_{dpa}), 5.27-5.25 (m, 1H, $CH_{2\ alkene}$), 5.10 (m, 1H,

$^{3'}$CHa), 5.03-4.95 (m, 2H, $^{2'}$CHa, $^{2'}$CHb), 4.63 (m, 1H, $^{3'}$CHa), 4.57-4.54 (m, 2H, $^{5'}CH_2$b), 4.52-4.49 (m, 2H, $CH_{2\ allyl}$), 4.44-4.38 (m, 2H, CH_{THP}), 4.27 (m, 1H, $^{4'}$CHb), 4.25-4.14 (m, 4H, $CH_{2\ THP}$), 4.12 (m, 1H, $^{4'}$CHa), 3.85-3.35 (m, 10H, $CH_{3\ DMT}$, $CH_{2\ THP}$), 3.67 (m, 1H, $^{5'}CH_2$b), 3.54 (m, 1H, $^{5'}CH_2$a), 3.40 (m, 2H, $^{5'}CH_2$a, $^{5'}CH_2$b), 2.75-2.64 (m, 4H, $CH_{2\ THP}$), 1.74-1.46 (m, 4H, $CH_{2\ THP}$);

^{13}C NMR (100.6 MHz, $CDCl_3$, δ ppm): 179.6 ($C{=}O_{dpa}$), 174.5 (^{4}C=O), 158.9 (Cq_{arom}), 158.8 (CH_{arom}), 155.6/155.4 (^{2}Cq), 148.2-143.9 (Cq_{arom}), 137.8-136.9 (^{6}Cq, ^{5}Cq), 136.3-135.9 (Cq_{arom}), 132.5/132.4 (CH_{allyl}), 130.5-128.7 (CH_{arom}), 128.5/128.1 (^{8}CH), 127.9-113.8 (CH_{arom}), 118.2/118.0 ($CH_{2\ alkene}$), 102.3/101.8 (CH_{THP}), 88.5/88.2 ($^{4'}$CHa), 87.9/87.7 ($^{4'}$CHb), 86.8/86.5 ($^{2'}$CHa), 86.1/85.9 ($^{2'}$CHb), 82.4/82.1 ($^{1'}$CHa), 81.8/81.7 ($^{1'}$CHb), 72.3/72.1 ($^{3'}$CHa), 70.9/70.5 ($^{3'}$CHb), 68.1/68.0 ($CH_{2\ allyl}$), 66.0/65.7 ($^{5'}CH_2$b), 63.0 ($CH_{2\ THP}$), 62.2/62.0 ($^{5'}CH_2$a), 59.1/58.9 (CH_{dpa}), 55.95/5.7 ($CH_{3\ DMT}$), 31.8/30.8 ($CH_{2\ THP}$), 25.2/24.8 ($CH_{2\ THP}$), 21.5/21.2 ($CH_{2\ THP}$);

MS (Maldi-ToF, m/z): 1850.3 ($[M+Na]^+$).

8.1.11 (2-*N*-diphenylacetyl-2'-*O*-tetrahydropyranyl-guanosine)-(3'-5')-(2-*N*-diphenylacetyl-3'-*O*-(2-chlorophenyl phosphate)-2'-*O*-tetrahydropyranyl-guanosine)-2-chlorophenyl phosphate (35)



34 **35**

R= *o*-chlorophenyl

310 mg (0.17 mmol, 1 eq) of dimer **34** were dissolved in 10 ml of a 7:3 (v/v) mixture of CH_2Cl_2:MeOH at 0°C and added with 307 mg (1.78 mmol, 10.5 eq) *p*-toluenesulfonic acid (*p*TsA) in 4 ml CH_2Cl_2:MeOH (7:3, v/v). After 10 min, the reaction mixture was poured into ammonium hydrogen phosphate buffer (30 ml, 1 M). The organic layer was dried and the volatiles removed under reduced pressure. Flash chromatography (EtOAc, 100%, gradient CH_2Cl_2:MeOH from 100:0 to 95:5, v/v) yielded 142 mg (0.09 mmol, 55%) of the desired product **35** as a non separable mixture of diastereomers.

$C_{75}H_{74}Cl_2N_{10}O_{19}P_2$: 1526.27 g/mol

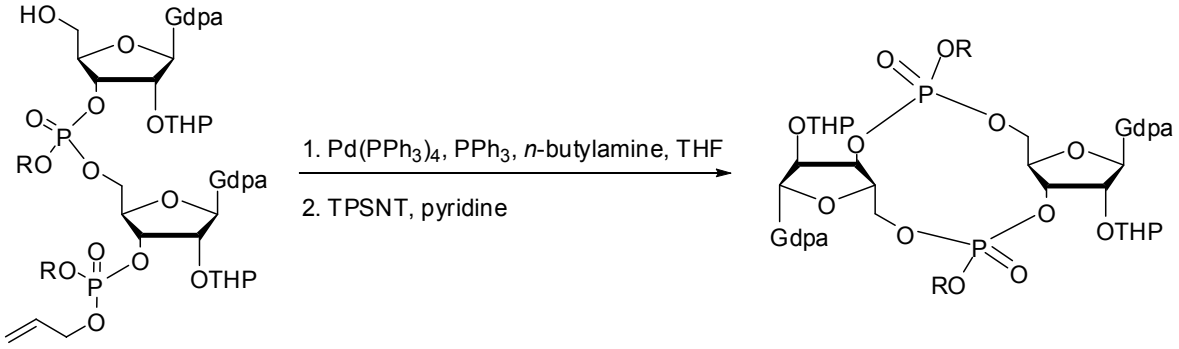
R_f (CH_2Cl_2:MeOH, 95:5) = 0.42;

^{1}H NMR (400.0 MHz, $CDCl_3$, δ ppm): 7.96-7.93 (m, 2H, ^{8}CH), 7.58-7.38 (m, 10H, CH_{arom}), 6.88-6.77 (m, 8H, CH_{arom}), 5.93-5.91 (m, 1H, CH_{allyl}), 5.86-5.83 (m, 2H, $^{1'}$CHa, $^{1'}$CHb), 5.42-5.38 (m, 1H, $CH_{2\ alkene}$), 5.33-5.32 (m, 2H, CH_{dpa}), 5.27-5.25 (m, 1H, $CH_{2\ alkene}$), 5.10 (m, 1H, $^{3'}$CHa), 5.03-4.95 (m, 2H, $^{2'}$CHa, $^{2'}$CHb), 4.63 (m, 1H, $^{3'}$CHa), 4.57-4.54 (m, 2H, $^{5'}CH_2$b), 4.52-4.49 (m, 2H, $CH_{2\ allyl}$), 4.44-4.38 (m, 2H, CH_{THP}), 4.27 (m, 1H, $^{4'}$CHb), 4.25-4.14 (m, 4H, $CH_{2\ THP}$), 4.12 (m, 1H, $^{4'}$CHa), 3.85-3.35 (m, 4H, $CH_{2\ THP}$), 3.67 (m, 1H, $^{5'}CH_2$b), 3.54 (m, 1H, $^{5'}CH_2$a), 3.40 (m, 2H, $^{5'}CH_2$a, $^{5'}CH_2$b), 2.75-2.64 (m, 4H, $CH_{2\ THP}$), 1.74-1.46 (m, 4H, $CH_{2\ THP}$);

^{13}C NMR (100.6 MHz, $CDCl_3$, δ ppm): 179.6 ($C{=}O_{dpa}$), 174.5 (^{4}C=O), 155.6/155.4 (^{2}Cq), 148.2-138.5 (Cq_{arom}), 137.8-136.9 (^{6}Cq, ^{5}Cq), 132.5/132.4 (CH_{allyl}), 130.5-128.7 (CH_{arom}), 128.5/128.1 (^{8}CH), 127.9-122.4 (CH_{arom}), 118.2/118.0 ($CH_{2\ alkene}$), 102.3/101.8 (CH_{THP}), 88.5/88.2 ($^{4'}$CHa), 87.9/87.7 ($^{4'}$CHb), 86.8/86.5 ($^{2'}$CHa), 86.1/85.9 ($^{2'}$CHb), 82.4/82.1 ($^{1'}$CHa), 81.8/81.7 ($^{1'}$CHb), 72.3/72.1 ($^{3'}$CHa), 70.9/70.5 ($^{3'}$CHb), 68.1/68.0 ($CH_{2\ allyl}$), 66.0/65.7 ($^{5'}CH_2$b), 63.0 ($CH_{2\ THP}$), 62.2/62.0 ($^{5'}CH_2$a), 59.1/58.9 (CH_{dpa}), 31.8/30.8 ($CH_{2\ THP}$), 25.2/24.8 ($CH_{2\ THP}$), 21.5/21.2 ($CH_{2\ THP}$);

MS (Maldi-ToF, m/z): 1548.3 ($[M+Na]^+$).

8.1.12 Cyclic bis(3',5')-(2-*N*-diphenylacetyl-2'-*O*-tetrahydropyranyl-guanosine)-2-chlorophenyl phosphate (36)



35 **36**

R= *o*-chlorophenyl

142 mg (0.09 mmol, 1 eq) of dimer **35** were dissolved in dry THF (2 ml) and added with 5 mg (3.5 mol%) $Pd(PPh_3)_4$, 8 mg (25 mol%) PPh_3, 0.2 ml (10% of solvent volume) *n*-butylamine and the mixture was stirred for 5 min at RT under argon. After co-evaporation with pyridine (5 x 2.5 ml), the residue was taken up in dry pyridine (20 ml) and 190 mg (0.49 mmol, 5.5 eq) 1-

(2,4,6-tri*iso*propylbenzenesulfonyl)-3-nitro-1*H*-1,2,4-triazole (TPSNT) were added. The mixture was stirred for 16 hrs at RT under argon. A few drops of water were then added to the mixture and the solvent evaporated. The residue was taken up in DCM and washed with TEAC buffer (50 ml, 1 M and 50 ml, 0.1 M). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography (CH_2Cl_2:MeOH gradient from 100:0 to 95:5, v/v) gave 52 mg (35.47 µmol, 38%) of cyclic compound **36** as a white solid.

$C_{70}H_{66}Cl_2N_{10}O_{19}P_2$: 1484.18 g/mol

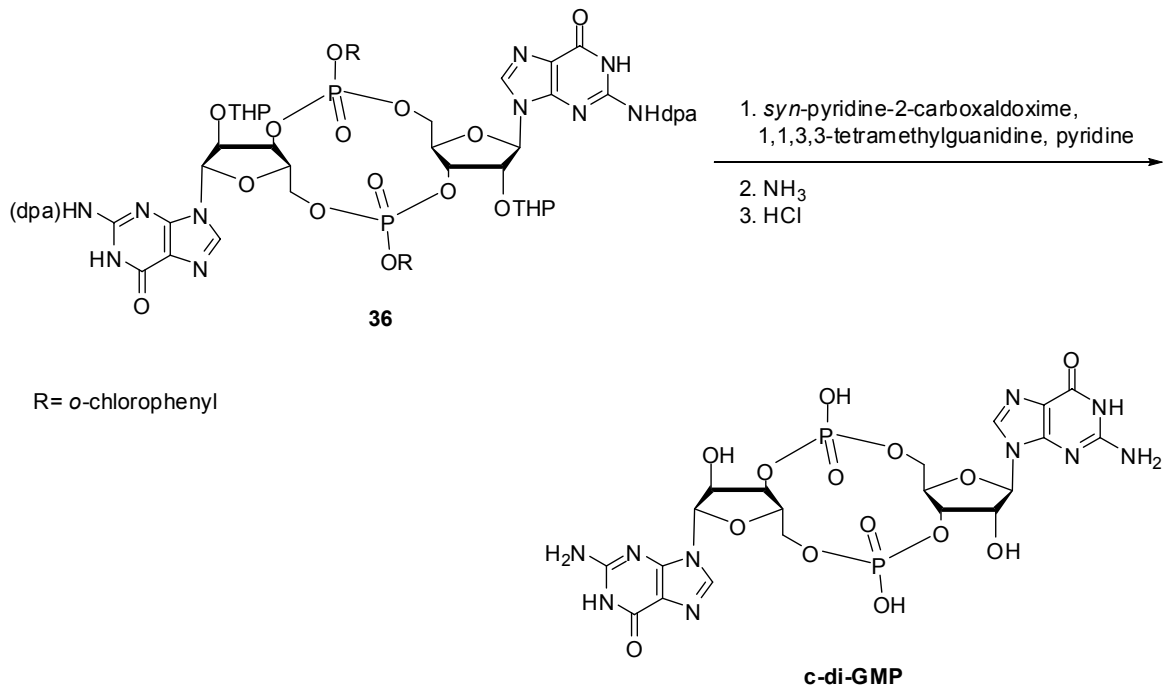
R_f (CH_2Cl_2:MeOH, 95:5) = 0.08;

^{1}H NMR (400.0 MHz, $CDCl_3$, δ ppm): 7.96-7.94 (m, 2H, ^{8}CH), 7.52-7.32 (m, 10H, CH_{arom}), 6.95-6.88 (m, 8H, CH_{arom}), 5.96-5.89 (m, 2H, $^{1'}$CHa, $^{1'}$CHb), 5.23-5.21 (m, 2H, CH_{dpa}), 5.03-4.95 (m, 2H, $^{2'}$CHa, $^{2'}$CHb), 4.65-4.62 (m, 1H, $^{3'}$CHa, $^{3'}$CHb), 4.52-4.48 (m, 2H, $^{5'}CH_2$b), 4.45-4.39 (m, 2H, CH_{THP}), 4.25-4.14 (m, 4H, $CH_{2\ THP}$), 4.12-4.05 (m, 2H, $^{4'}$CHa, $^{4'}$CHb), 3.72-3.70 (m, 1H, $^{5'}CH_2$a), 3.67-3.66 (m, 1H, $^{5'}CH_2$a), 3.64-3.35 (m, 4H, $CH_{2\ THP}$), 2.65-2.54 (m, 4H, $CH_{2\ THP}$), 1.74-1.46 (m, 4H, $CH_{2\ THP}$);

^{13}C NMR (100.6 MHz, $CDCl_3$, δ ppm): 175.6 ($C{=}O_{dpa}$), 173.5 (^{4}C=O), 154.6/154.4 (^{2}Cq), 147.6-138.5 (Cq_{arom}), 137.2/137.0 (^{8}CH), 136.8-134.9 (^{6}Cq, ^{5}Cq), 131.0-122.4 (CH_{arom}), 101.3/100.8 (CH_{THP}), 86.9/86.6 ($^{1'}$CH), 85.9/84.9 ($^{2'}$CH), 82.4/81.7 ($^{4'}$CH), 72.3/70.6 ($^{3'}$CH), 69.2/64.0 ($^{5'}CH_2$), 63.3/63.2 ($CH_{2\ THP}$), 58.7/58.1 (CH_{dpa}), 31.8/30.8 ($CH_{2\ THP}$), 25.2/24.8 ($CH_{2\ THP}$), 21.5/21.2 ($CH_{2\ THP}$);

MS (Maldi-ToF, m/z): 1489.4 ($[M+Na]^+$).

8.1.13 Cyclic diguanylic acid (c-di-GMP)



52 mg (35.47 µmol, 1 eq) of compound **36** were dissolved in dry pyridine (4 ml) at RT under argon and added with 195 mg (0.35 mmol, 10 eq) *syn*-pyridine-2-carboxaldoxime and 176 µl (1.24 mmol, 35 eq) 1,1,3,3-tetramethylguanidine and then stirred 20 hrs at RT under argon. 40 ml of an aq. ammonia solution (33%) were added and the mixture heated to 50°C for 48 hrs. The reaction mixture was concentrated to 1/10 of its volume and washed with diethyl ether (2 x 10 ml). The aqueous phase was acidified to pH 2 with 0.1 M HCl and stirred at RT for 16 hrs followed by neutralization with an aq. ammonia solution to pH 8. The reaction mixture was then concentrated to a small volume and purified by reverse phase HPLC (TEAC(0.01 M):MeOH; 92.5:7.5) to yield 4 mg (5.80 µmol, 16%) of c-di-GMP as a white foam.

$C_{20}H_{23}N_{10}O_{14}P_2$: 689.08 g/mol

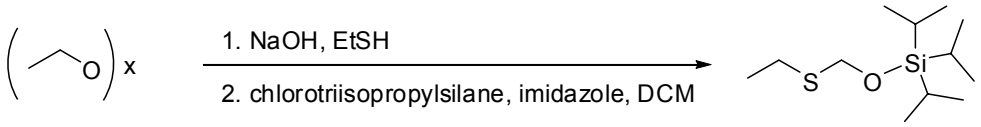
^{1}H NMR (500 MHz, D_2O, δ ppm): 7.90 (s, 2H, ^{8}CH), 5.84 (d, 2H, *J*= 1.3 Hz, $^{1'}$CH), 4.74 (dd, 2H, J_1= 8.5 Hz, J_2= 5.0 Hz, $^{3'}$CH), 4.59 (dd, 2H, *J*= 5.0 Hz,$^{2'}$CH), 4.27 (dd, 2H, *J*= 8.5 Hz, $^{4'}$CH), 4.21 (m, 2H, $^{5'}CH_2$), 3.96 (m, 2H, $^{5'}CH_2$);

^{13}C NMR (125.8 MHz, D_2O, δ ppm): 158.8, 153.8 (^{4}C=O, ^{6}Cq), 150.8 (^{2}Cq), 137.1 (^{8}CH), 116.3 (^{5}C), 89.2 ($^{1'}$CH), 79.8 ($^{4'}$CH), 73.3 ($^{2'}$CH), 70.5 ($^{3'}$CH), 62.2 ($^{5'}CH_2$);

HRMS-ESI: m/z $[M-H]^-$ calcd for $C_{20}H_{23}N_{10}O_{14}P_2$: 689.0870; Found: 689.0869.

8.2 Synthesis of Tom Protected Guanosine

8.2.1 Tri*iso*propylsilyl(ethylthio)methyl ether (38)



37 **38**

10 g (0.33 mol, 1 eq) *para*formaldehyde **37** were suspended in 24.6 ml (0.33 mol, 1 eq) ethanethiol at RT under argon and the mixture was cooled to 0°C. 1 drop of 10 M NaOH was added, the mixture allowed to slowly warm up to RT and then heated to 40°C for 3 hrs. After cooling to RT, 350 ml dichloromethane, 41.34 g (0.66 mol, 2 eq) imidazole and 67 ml (0.31 mol, 0.95 eq) chlorotri*iso*propylsilane were added and stirred overnight. The reaction mixture was diluted with 500 ml hexane and washed with 2x250 ml of a 10% aq. NaH_2PO_4 sol. The organic fractions were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was distilled in vacuo (83°C, 0.1 mbar) to give 57.10 g of **38** as a colorless liquid (0.23 mol, 69%).

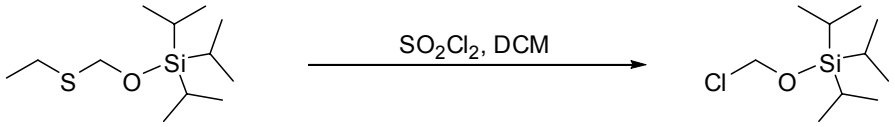
$C_{12}H_{28}OSSi$: 248.50 g/mol

^{1}H NMR (400.0 MHz, $CDCl_3$, δ ppm): 4.86 (s, 1H, CH_2), 2.69 (q, 2H, *J*= 7.32 Hz, CH_2), 1.29 (t, 3H, *J*= 7.32 Hz, $CH_{3\ ethyl}$), 1.08 (m, 26H, $CH_{isopropyl}$, $CH_{3\ isopropyl}$);

^{13}C NMR (101.0 MHz, $CDCl_3$, δ ppm): 66.4 (S-CH_2-O), 25.1 (CH_2-S), 18.2 ($CH_{3\ ethyl}$), 15.4 ($CH_{3\ isopropyl}$), 12.36 ($CH_{isopropyl}$);

MS (Maldi-ToF, m/z): 271.1 ($[M+Na]^+$).

8.2.2 [(Tri*iso*propylsilyl)oxy]methyl chloride (TomCl) (39)



38 **39**

A solution of 57 g (0.23 mol 1 eq) tri*iso*propylsilyl(ethylthio)methyl ether **38** in 200 ml dichloromethane was added with 18.7 ml (0.23 mol, 1 eq) sulfurylchloride at 0°C under argon. The yellow solution was stirred for 1 hr. The solvent was then evaporated under reduced pressure. Vacuum distillation (34°C, 0.1 mbar) yielded 31.1 g (0.14 mol, 65%) of **39** as a colorless liquid.

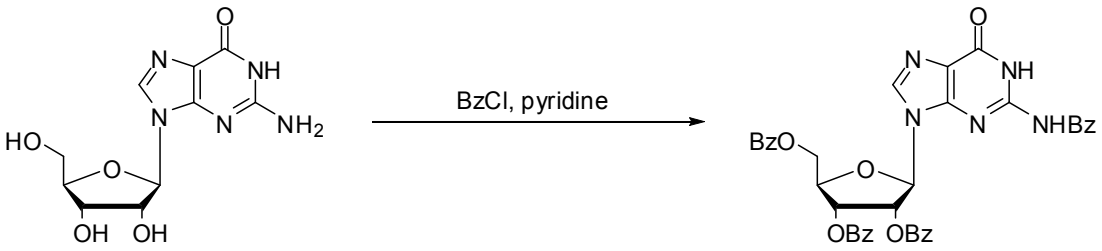
$C_{10}H_{23}ClOSi$: 222.83 g/mol

^{1}H NMR (400.0 MHz, $CDCl_3$, δ ppm): 5.65 (s, 2H, CH_2), 1.21 (m, 3H, $CH_{isopropyl}$), 1.09 (d, 18H, *J*= 7.08 Hz, $CH_{3\ isopropyl}$);

^{13}C NMR (101.0 MHz, $CDCl_3$, δ ppm): 18.1 ($CH_{3\ isopropyl}$), 14.1 ($CH_{isopropyl}$), 12.2 (CH_2);

MS (Maldi-ToF, m/z): 255.3 ([M+Na]$^+$).

8.2.3 2-*N*-2',3',5'-*O*-tetrabenzoylguanosine (40)



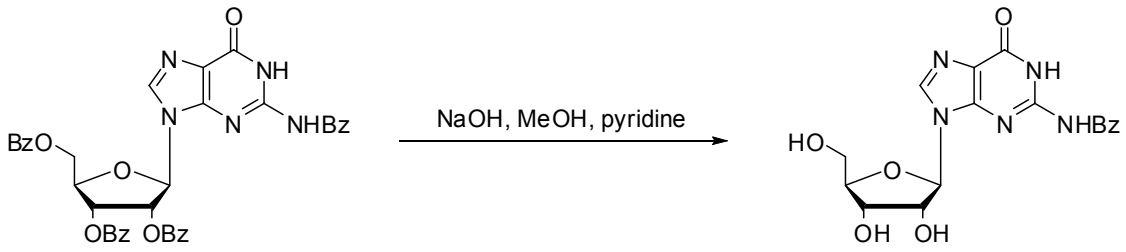
20 **40**

5 g (17.66 mmol, 1 eq) guanosine **20** were suspended in 70 ml dry pyridine at RT under argon and heated to 40°C and 15.01 g (106.84 mmol, 6.05 eq) benzoylchloride were added. An exothermic reaction occured, the temperature went up to 70°C then back to 40°C. After 2.5 hrs, 100 ml sat. aq. $NaHCO_3$ sol. as well as 35 ml chloroform were added to quench the reaction. The aqueous phase was extracted with dichloromethane (3 x 100 ml) and the

organic phase was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by flash chromatography (CH_2Cl_2:MeOH, 95:5, v/v) to yield 9.41 g of **40** as a white solid (13.42 mmol, 76%).

$C_{38}H_{29}N_5O_9$: 699.66 g/mol
R_f (CH_2Cl_2:MeOH, 95:5) = 0.55;
^{1}H NMR (400.0 MHz, $CDCl_3$, δ ppm): 11.99 (s, 1H, ^{3}NH), 9.60 (s,1H, ^{8}CH), 8.02 (m, 10H, CH_{arom}), 7.50 (m, 10H, CH_{arom}), 6.92 (dd, 1H, *J*= 7.56 Hz, *J*= 4.8 Hz, $^{3'}$CH), 6.44 (dd, 1H, J_1= 4.92 Hz, J_2= 2.1 Hz, $^{2'}$CH), 6.24 (d, 1H, *J*= 2.4 Hz, $^{1'}$CH), 4.87 (m, 2H, $^{5'}CH_2$), 4.78 (m, 1H, $^{4'}$CH);
^{13}C NMR (101.0 MHz, $CDCl_3$, δ ppm): 167.7 (C=O), 166.7 (C=O), 166.4 (C=O), 165.6 (C=O), 133-134 (CH_{arom}), 128-131 (CH_{arom}), 88.7 ($^{1'}$CH), 79.9 ($^{4'}$CH), 74.7 ($^{2'}$CH), 71.2 ($^{3'}$CH), 62.0 ($^{5'}CH_2$);
MS (Maldi-ToF, m/z): 700.1 ($[M]^+$).

8.2.4 2-*N*-benzoylguanosine (41)



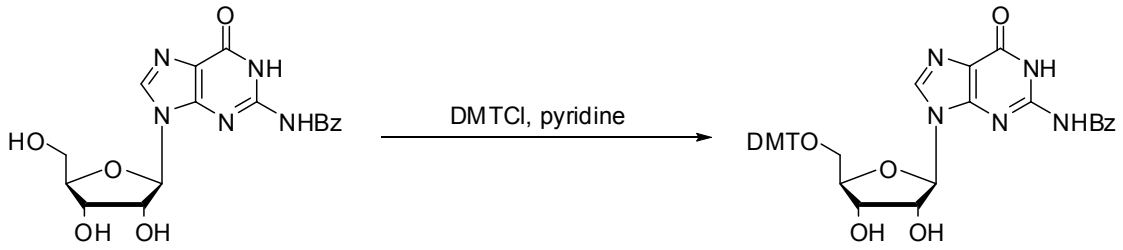
40 **41**

1 g (1.43 mmol, 1 eq) 2-*N*-2',3',5'-*O*-tetrabenzoylguanosine **40** was suspended in 20 ml methanol: pyridine (1:1) at RT. 2N NaOH (10 ml) was added to solubilize the suspension. After 35 min, the reaction was quenched with Dowex 50W till neutralization of the solution. The mixture was filtered, the resin washed with methanol and the combined solvents evaporated under reduced pressure. The residual pyridine was removed through co-evaporation with toluene. The residue was re-crystallized from boiling ethanol to yield 430 mg of **41** as a white solid (1.11 mmol, 78%).

$C_{17}H_{17}N_5O_6$: 387.35 g/mol
R_f (CH_2Cl_2:MeOH, 95:5) = 0.05;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 12.43 (s, 1H, ^{3}NH), 8.26 (s, 1H, ^{8}CH), 8.03 (m, 2H, CH_{arom}), 7.54 (m, 3H, CH_{arom}), 5.87 (d, 1H, *J*= 6.08 Hz, $^{1'}$CH), 5.48 (s, 1H, OH), 5.10 (s, 2H, OH), 4.47 (t, 1H, *J*= 5.32 Hz, $^{2'}$CH), 4.12 (dd, 1H, J_1= 4.52 Hz, J_2= 3.28 Hz, $^{3'}$CH), 3.90 (dd, 1H, J_1= 7.32 Hz, J_2= 3.8 Hz, $^{4'}$CH), 3.55 (m, 2H, $^{5'}CH_2$);
^{13}C NMR (101.0 MHz, DMSO, δ ppm): 156.1 (^{6}Cq), 149.9 (^{2}Cq), 138.7 (^{8}CH), 129-133 (CH_{arom}), 121.3 (^{5}Cq), 87.3 ($^{1'}$CH), 86.3 ($^{4'}$CH), 74.7 ($^{2'}$CH), 71.2 ($^{3'}$CH), 62.1 ($^{5'}CH_2$);
MS (Maldi-ToF, m/z): 410.2 ($[M+Na]^+$).

8.2.5 2-*N*-benzoyl-5'-*O*-dimethoxytritylguanosine (42)



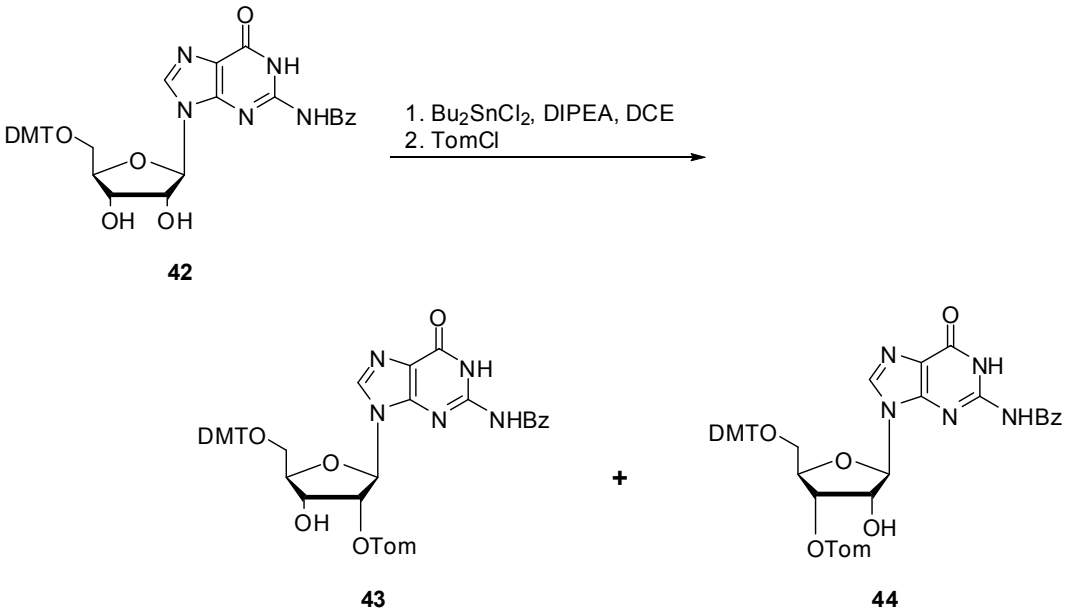
41 **42**

150 mg (0.39 mmol, 1 eq) 2-*N*-benzoylguanosine **41** were co-evaporated with pyridine (2x4 ml) then suspended in 2 ml dry pyridine at RT under argon. 145 mg (0.43 mmol, 1.1 eq) 4,4'-dimethoxytritylchloride (DMTCl) were added and the mixture was stirred overnight. The reaction was quenched with 6 ml of a 5% aq. $NaHCO_3$ sol. and extracted with dichloromethane (2 x 4 ml). The organic layer was dried and concentrated, then co-evaporated with toluene (2 x 5 ml). Flash chromatography (DCM:hexane, 1:1, CH_2Cl_2:MeOH, gradient from 100:0 to 95:5, v/v) yielded 220 mg of **42** as a white foam (0.32 mmol, 82%).

$C_{38}H_{35}N_5O_8$: 689.71 g/mol
R_f (CH_2Cl_2:MeOH, 90:10) = 0.7;
^{1}H NMR (400.0 MHz, DMSO, δ ppm): 12.43 (s, 1H, ^{2}NH), 11.84 (s, 1H, ^{3}NH), 8.14 (s, 1H, ^{8}CH), 7.97 (dd, 1H, J_1= 5.32 Hz, J_2= 1.76 Hz, CH_{arom}), 7.66 (m, 1H, CH_{arom}), 7.54 (m, 2H, CH_{arom}), 7.33 (m, 2H, CH_{arom}), 7.21 (m, 8H, CH_{arom}), 6.78 (m, 4H, CH_{arom}), 5.92 (d, 1H, *J*= 4.8 Hz, $^{2'}$OH), 5.59 (d, 1H, *J*= 5.8 Hz, $^{3'}$OH), 5.18 (d, 1H, *J*= 5.56 Hz, $^{1'}$CH), 4.55 (m, 2H, $^{2'}$CH, $^{3'}$CH), 4.19 (q, 1H, *J*= 5.04 Hz, $^{4'}$CH), 4.01 (m, 1H, $^{5'}CH_2$), 3.70 (m, 1H, $^{5'}CH_2$), 3.68 (s, 6H, CH_3);

^{13}C NMR (101.0 MHz, DMSO, δ ppm): 156.3 (^{6}Cq), 149.8 (^{2}Cq), 138.5 (^{8}CH), 129-133 (CH_{arom}), 121.1 (^{5}Cq), 87.3 ($^{1'}$CH), 86.2 ($^{4'}$CH), 74.6 ($^{2'}$CH), 71.1 ($^{3'}$CH), 62.0 ($^{5'}CH_2$);
MS (Maldi-ToF, m/z): 712.3 ([M+Na]$^{+}$).

8.2.6 2-*N*-benzoyl-5'-*O*-dimethoxytrityl-2'-*O*-[(tri*iso*propylsilyl)oxy] methylguanosine (43)



42 **43** **44**

51 mg (73.99 µmol, 1 eq) 2-*N*-benzoyl-5'-*O*-dimethoxytritylguanosine **42** were dissolved in 0.5 ml 1,2-dichloroethane (DCE) at RT under argon. 0.44 ml (0.26 mmol, 3.52 eq) di*iso*propylethylamine (DIPEA) then 25.2 mg (0.08 mmol, 1.12 eq) dibutyltindichloride were added and the mixture heated at 80°C for 1 hr. 21.7 mg (0.09 mmol, 1.32 eq) [(tri*iso*propylsilyl)oxy]methyl chloride (TomCl) were added and the mixture heated at 80°C for 1.5 hrs. The solution was diluted with 5 ml dichloromethane and extracted with 5 ml sat. aq. $NaHCO_3$ sol. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography (CH_2Cl_2, CH_2Cl_2:MeOH gradient from 100:0 to 90:10, v/v, +0.5% triethylamine) yielded 3 mg (3.73 µmol, 5%) of the 2'-O-Tom product **43** and 26 mg (32.38 µmol, 44%) of the 3'-O-Tom product **44**.

$C_{48}H_{57}N_5O_9Si$: 876.08 g/mol

2'-O-Tom isomer **43**:

R_f (CH_2Cl_2:MeOH, 95:5) = 0.63;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 12.43 (s, 1H, ^{2}NH), 11.82 (s, 1H, ^{3}NH), 8.10 (s, 1H, ^{8}CH), 7.95 (m, 2H, CH_{arom}), 7.64 (m, 1H, CH_{arom}), 7.54 (m, 2H, CH_{arom}), 7.31(m, 2H, CH_{arom}), 7.20 (m, 10H CH_{arom}), 6.78 (m, 4H, CH_{arom}), 5.60 (d, 1H, *J*= 5.7 Hz, $^{3'}$OH), 5.45 (s, 2H, $CH_{2\ Tom}$), 5.16 (d, 1H, *J*= 5.59 Hz, $^{1'}$CH), 4.64 (m, 1H, $^{2'}$CH), 4.57 (m, 1H, $^{3'}$CH), 4.19 (q, 1H, *J*= 5.1 Hz, $^{4'}$CH), 3.99 (m, 1H, $^{5'}CH_2$), 3.70 (m, 1H, $^{5'}CH_2$), 3.68 (s, 6H, CH_3), 2.14 (s, 3H, CH_{Tom}), 1.10 (s, 18H, $CH_{3\ Tom}$);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 167.5 (C=O), 157.1 (^{4}C=O), 147.8 (^{6}Cq), 147.6 (^{2}Cq), 138.5 (^{8}CH), 133.2-127.5 (CH_{arom}), 117.8 (^{5}Cq), 114.8-114.5 (CH_{arom}), 95.9 (Cq_{Tom}), 90.1 ($CH_{2\ Tom}$), 86.9 ($^{1'}$CH), 86.0 ($^{4'}$CH), 82.6 ($^{2'}$CH), 71.1 ($^{3'}$CH), 62.0 ($^{5'}CH_2$), 30.3 (CH_{Tom}), 18.1 ($CH_{3\ Tom}$);

MS (Maldi-ToF, m/z): 899.5 ($[M+Na]^+$).

3'-O-Tom isomer **44**:

R_f (CH_2Cl_2:MeOH, 95:5) = 0.59;

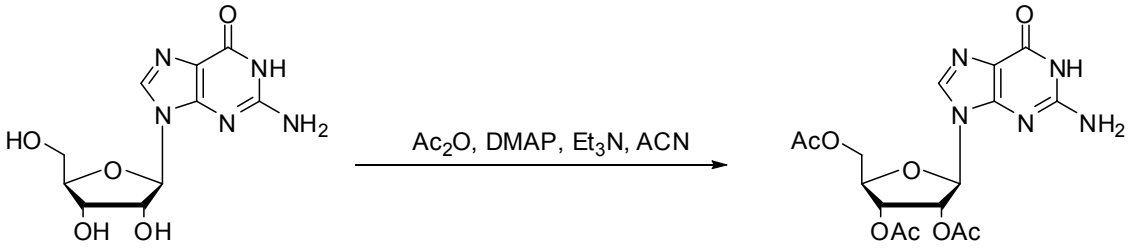
^{1}H NMR (400.0 MHz, DMSO, δ ppm): 12.43 (s, 1H, ^{2}NH), 11.82 (s, 1H, ^{3}NH), 8.10 (s, 1H, ^{8}CH), 7.95 (m, 2H, CH_{arom}), 7.64 (m, 1H, CH_{arom}), 7.54 (m, 2H, CH_{arom}), 7.31(m, 2H, CH_{arom}), 7.20 (m, 10H CH_{arom}), 6.78 (m, 4H, CH_{arom}), 5.60 (d, 1H, *J*= 5.7 Hz, $^{2'}$OH), 5.43 (s, 2H, $CH_{2\ Tom}$), 5.16 (d, 1H, *J*= 5.59 Hz, $^{1'}$CH), 4.67 (m, 1H, $^{3'}$CH), 4.54 (m, 1H, $^{2'}$CH), 4.19 (q, 1H, *J*= 5.09 Hz, $^{4'}$CH), 3.98 (m, 1H, $^{5'}CH_2$), 3.70 (m, 1H, $^{5'}CH_2$), 3.68 (s, 6H, CH_3), 2.15 (s, 3H, CH_{Tom}), 1.11 (s, 18H, $CH_{3\ Tom}$);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 167.5 (C=O), 157.3 (^{4}C=O), 147.9 (^{6}Cq), 147.7 (^{2}Cq), 138.5 (^{8}CH), 133.5-127.8 (CH_{arom}), 117.9 (^{5}Cq), 114.8-114.5 (CH_{arom}), 95.7 (Cq_{Tom}), 89.9 ($CH_{2\ Tom}$), 86.9 ($^{1'}$CH), 86.0 ($^{4'}$CH), 82.6 ($^{3'}$CH), 71.1 ($^{2'}$CH), 62.0 ($^{5'}CH_2$), 30.2 (CH_{Tom}), 18.1 ($CH_{3\ Tom}$);

MS (Maldi-ToF, m/z): 899.6 ($[M+Na]^+$).

8.3 Synthesis of the Guanosine Dinucleotide Following the Hayakawa Methodology

8.3.1 2',3',5'-*O*-triacetylguanosine (45)



20 **45**

10 g (35.32 mmol, 1 eq) guanosine **20** were suspended in 200 ml acetonitrile at RT under argon. 0.43 g (3.53 mmol, 0.1 eq) 4-dimethylamino-pyridine (DMAP), 19.5 ml (139.86 mmol, 3.96 eq) triethylamine and 13 ml (127.15 mmol, 3.6 eq) acetic anhydride were added and the mixture stirred for 1 hr. The reaction was quenched with 50 ml methanol, the solvents removed under reduced pressure and the oily residue re-crystallized from *iso*propanol. The suspension was filtered and successively washed with ethanol and diethyl ether to yield 12.51 g (30.58 mmol, 87%) of **45** as a white solid.

$C_{16}H_{19}N_5O_8$: 409.35 g/mol

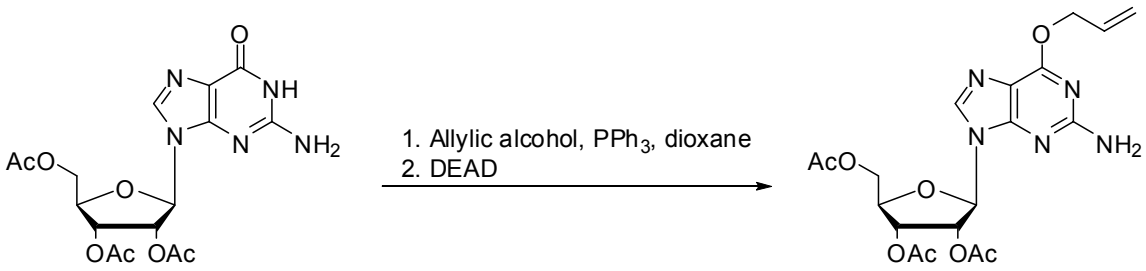
R_f (CH_2Cl_2:MeOH, 95:5) = 0.33;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 10.72 (s, 1H, ^{3}NH), 7.91 (s, 1H, ^{8}CH), 5.96 (d, 1H, *J*= 6.04 Hz, $^{1'}$CH), 5.76 (t, 1H, *J*= 6.08 Hz, $^{2'}$CH), 5.46 (dd, 1H, J_1= 6.08 Hz, J_2= 4.04 Hz, $^{3'}$CH), 4.35 (m, 1H, $^{4'}$CH), 4.26 (m, 2H, $^{5'}CH_2$), 2.09 (s, 3H, $CH_{3\ acetyl}$), 2.02 (s, 3H, $CH_{3\ acetyl}$), 2.01 (s, 3H, $CH_{3\ acetyl}$);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 85.2 ($^{1'}$CH), 80.4 ($^{4'}$CH), 72.9 ($^{2'}$CH), 71.1 ($^{3'}$CH), 63.9 ($^{5'}CH_2$), 21.4 ($CH_{3\ acetyl}$), 21.2 ($CH_{3\ acetyl}$), 21.0 ($CH_{3\ acetyl}$);

MS (Maldi-ToF, m/z): 432.2 ($[M+Na]^+$).

8.3.2 2',3',5'-*O*-triacetyl-4-*O*-allyloxyguanosine (46)



45 **46**

10.89 g (26.62 mmol, 1 eq) 2',3',5'-*O*-triacetylguanosine **45** were suspended in 150 ml dry dioxane at RT under argon, added with 11.86 g (45.26 mmol, 1.7 eq) triphenylphosphine and 16.7 ml (244.96 mmol, 9.2 eq) of freshly distilled allylic alcohol. This mixture was heated to 80°C for 1hr. After cooling, 21.9 ml (46.41 mmol, 1.8 eq) diethyl azodicarboxylate (DEAD) were added dropwise and the resulting solution heated to 60°C for 2 hrs. The solvents were evaporated under reduced pressure. The oily residue was taken-up in 80 ml dichloromethane and stored at 4°C. The crystalline triphenylphosphine oxide was filtered and the solvents evaporated under reduce pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 4:6; v/v) yielded 9.21 g of **46** as a white foam (20.49 mmol, 77%).

$C_{19}H_{23}N_5O_8$: 449.41 g/mol

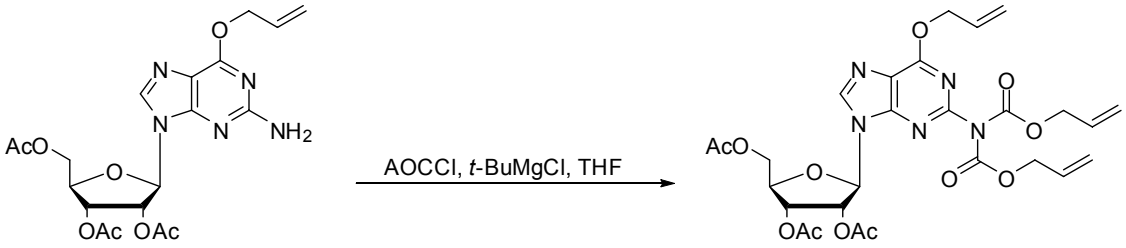
R_f (hexane:EtOAc, 3:1) = 0.3;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 8.10 (s, 1H, ^{8}CH), 6.55 (s, 2H, NH_2), 6.07 (m, 2H, CH_{alkene},$^{1'}$CH), 5.85 (t, 1H, *J*= 6.08 Hz, $^{2'}$CH), 5.52 (dd, 1H, J_1= 5.8 Hz, J_2= 4.04 Hz, $^{3'}$CH), 5.39 (dq, 1H, J_1= 17.44 Hz, J_2= 1.8 Hz, $CH_{2\ alkene}$), 5.25 (dq, 1H, J_1= 10.86 Hz, J_2= 1.12 Hz, $CH_{2\ alkene}$), 4.93 (dt, 2H, J_1= 5.56 Hz, J_2= 1.48 Hz, $CH_{2\ allyl}$), 4.40-4.23 (m, 3H, $^{5'}CH_2$, $^{4'}$CH), 2.11 (s, 3H, $CH_{3\ acetyl}$), 2.03 (s, 3H, $CH_{3\ acetyl}$), 2.01 (s, 3H, $CH_{3\ acetyl}$);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 170.9-170.1 ($C{=}O_{acetyl}$), 119.0 ($CH_{2\ alkene}$), 114.7 (CH_{alkene}), 85.3 ($^{1'}$CH), 80.4 ($^{4'}$CH), 72.7 ($^{2'}$CH), 71.2 ($^{3'}$CH), 66.9 ($^{5'}CH_2$), 63.9 ($CH_{2\ allyl}$), 21.4-21.0 ($CH_{3\ acetyl}$);

MS (Maldi-ToF, m/z): 473.6 ($[M+Na]^+$).

8.3.3 2',3',5'-*O*-triacetyl-4-*O*-allyloxy-2-*N*-di[(allyloxy)carbonyl] guanosine (47)



46 **47**

9.21 g (20.46 mmol, 1 eq) 2',3',5'-O-triacetyl-4-allyloxyguanosine **46** were dissolved in 200 ml dry THF at RT under argon, then cooled to 5°C and added with 6.5 ml (61.40 mmol, 3 eq) allylchloroformate. 35.1 ml (53.21 mmol, 2.6 eq) *t*-butylmagnesium chloride were added dropwise under stirring at 5°C. After completion of the addition, the ice bath was removed and the mixture allowed to reach RT and stirred for 1 hr. The reaction was quenched with 15 ml methanol and diluted with 150 ml ethylacetate. The solution was washed with sat. aq. NH_4Cl sol. (2 x 150 ml), sat. aq. $NaHCO_3$ sol. (2 x 150 ml) and brine (2 x 150 ml). The organic phase was dried ($MgSO_4$) and concentrated in vacuo. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 4:6; v/v) yielded 8.76 g of **47** as a white foam (14.11 mmol, 69%).

$C_{27}H_{31}N_5O_{12}$: 617.56 g/mol

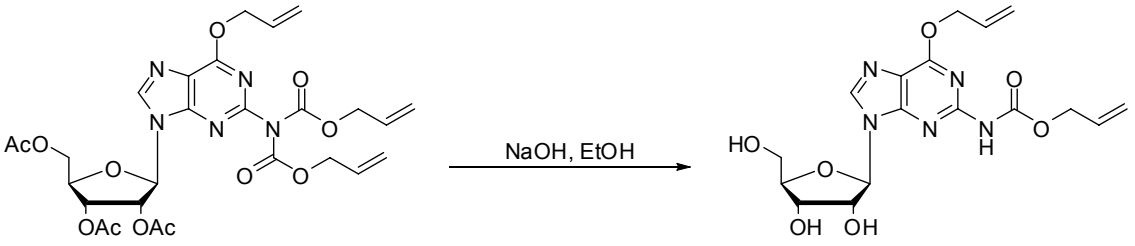
R_f (hexane:EtOAc 1:3) = 0.55;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 8.70 (s, 1H, ^{8}CH), 6.26 (d, 1H, *J*= 5.08 Hz, $^{1'}$CH), 6.08 (m, 1H, CH_{allyl}), 5.85 (m, 3H, 2xCH_{AOC}, $^{2'}$CH), 5.58 (t, 1H, *J*= 5.56 Hz, $^{3'}$CH), 5.41 (dq, 1H, J_1= 17.16 Hz, J_2= 1.52 Hz, $CH_{2\ alkene\ allyl}$), 5.29 (dq, 1H, J_1= 10.36 Hz, J_2= 1.52 Hz, $CH_{2\ alkene\ allyl}$), 5.21-5.12 (m, 4H, 2x$CH_{2\ alkene\ AOC}$), 5.03 (d, 2H, *J*= 5.8 Hz, $CH_{2\ allyl}$), 4.66 (m, 4H, CH_{2AOC}), 4.38-3.98 (m, 3H, $^{5'}CH_2$, $^{4'}$CH), 2.09 (s, 3H, $CH_{3\ acetyl}$), 2.01 (s, 3H, $CH_{3\ acetyl}$), 1.95 (s, 3H, $CH_{3\ acetyl}$);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 170.1 ($C{=}O_{acetyl}$), 160.3 (^{4}C-O), 154.1 (^{6}Cq), 152.7 (^{2}Cq), 152.3 ($C{=}O_{AOC}$), 142.8 (^{8}CH), 133.6 (CH_{allyl}), 133.5 (CH_{AOC}), 119.8 (^{5}Cq), 118.7 ($CH_{2\ alkene\ AOC}$), 118.5 ($CH_{2\ alkene\ allyl}$), 87.9 ($^{1'}$CH), 86.4 ($^{4'}$CH), 74.5 ($^{2'}$CH), 71.2 ($^{3'}$CH), 67.5 ($CH_{2\ AOC}$), 65.9 ($CH_{2\ allyl}$), 62.3 ($^{5'}CH_2$), 21.4-21.0 ($CH_{3\ acetyl}$);

MS (Maldi-ToF, m/z): 640.4 ($[M+Na]^+$).

8.3.4 4-*O*-allyloxy-2-*N*-[(allyloxy)carbonyl] guanosine (48)



47 **48**

8.76 g (14.19 mmol, 1 eq) 2',3',5'-*O*-triacetyl-4-*O*-allyloxy-2-*N*-di[(allyloxy)carbonyl] guanosine **47** were dissolved in 150 ml ethanol at RT under argon, added with 0.7 ml (7.09 mmol, 0.5 eq) 10 M NaOH and stirred for 30 mins. The reaction was quenched with Dowex 50 WX 8-200 (till pH=7), filtered and evaporated in vacuo. Flash chromatography (hexane:CH_2Cl_2 1:1; CH_2Cl_2:MeOH gradient from 1:0 to 9:1, v/v) yielded 4.13 g of **48** as a white foam (10.19 mmol, 72%).

$C_{17}H_{21}N_5O_7$: 407.38 g/mol

R_f (CH_2Cl_2:MeOH, 9:1) = 0.05;

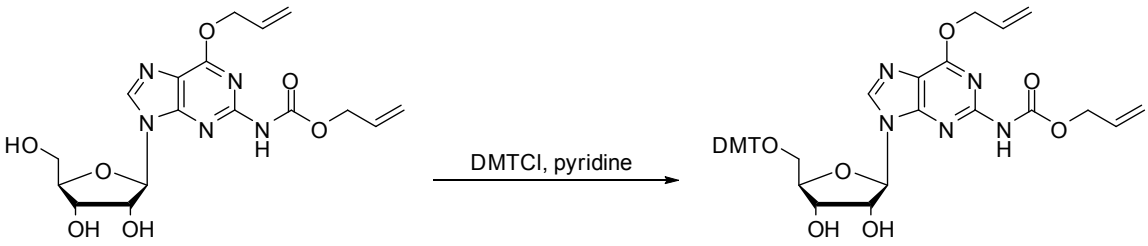
^{1}H NMR (400.0 MHz, DMSO, δ ppm): 10.38 (s, 1H, NH), 8.42 (s, 1H, ^{8}CH), 6.15 (m, 1H, CH_{allyl}), 5.95 (m, 1H, CH_{AOC}), 5.87(d, 1H, *J*= 6.08 Hz, $^{1'}$CH), 5.46 (d, 1H, *J*= 7.56 Hz, $^{2'}$OH), 5.46-5.39 (dq, 2H, J_1= 15.68 Hz, J_2= 1.52 Hz, $CH_{2\ alkene\ allyl}$), 5.29-5.19 (dq, 2H, J_1= 3.28 Hz, J_2= 1.76 Hz, $CH_{2\ alkene\ AOC}$), 5.16 (d, 1H, *J*= 4.08 Hz, $^{3'}$OH), 5.04 (d, 2H, *J*= 5.04 Hz, $CH_{2\ allyl}$), 4.92 (t, 1H, *J*= 5.52 Hz, $^{5'}$OH), 4.59 (m, 3H, $CH_{2\ AOC}$, $^{2'}$CH), 4.16 (q, 1H, *J*= 4.56 Hz, $^{3'}$CH), 3.89 (q, 1H, *J*= 4.28 Hz, $^{4'}$CH), 3.65-3.49 (m, 2H, $^{5'}CH_2$), 2.09 (s, 3H, $CH_{3\ acetyl}$), 2.01 (s, 3H, $CH_{3\ acetyl}$), 1.95 (s, 3H, $CH_{3\ acetyl}$);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 160.5 (^{4}C-O), 154.0 (^{6}Cq), 152.9 (^{2}Cq), 152.6 ($C{=}O_{AOC}$), 142.2 (^{8}CH), 133.9 (CH_{allyl}), 133.8 (CH_{AOC}), 119.8 (^{5}Cq), 118.3 ($CH_{2\ alkene\ AOC}$), 118.0 ($CH_{2\ alkene\ allyl}$), 87.9 ($^{1'}$CH), 86.5 ($^{4'}$CH), 74.3 ($^{2'}$CH), 71.3 ($^{3'}$CH), 67.8 ($CH_{2\ AOC}$), 65.6 ($CH_{2\ allyl}$), 62.3 ($^{5'}CH_2$);

MS (Maldi-ToF, m/z): 430.4 ([M+Na]$^+$).

8.3.5 First Building Block Possibility

8.3.5.1 4-*O*-allyloxy-2-*N*-[(allyloxy)carbonyl]-5'-*O*-dimethoxytriphenyl methyl-guanosine (49)



48 **49**

500 mg (1.23 mmol, 1 eq) 4-*O*-allyloxy-2-*N*-[(allyloxy)carbonyl] guanosine **48** were co-evaporated with dry pyridine (2x5 ml). dissolved in 8 ml dry pyridine at RT under argon and added with 541 mg (1.59 mmol, 1.3 eq) triethylamine, 0.25 ml (2.46 mmol, 2 eq) DMTCl and stirred for 16 hrs. The reaction was quenched with methanol (2 ml) and the solvents evaporated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 1:1, v/v, +0.5% triethylamine) yielded 760 mg of **49** as a white foam (1.06 mmol, 87%).

$C_{38}H_{39}N_5O_9$: 709.74 g/mol

R_f (hexane:EtOAc, 3:1) = 0.38;

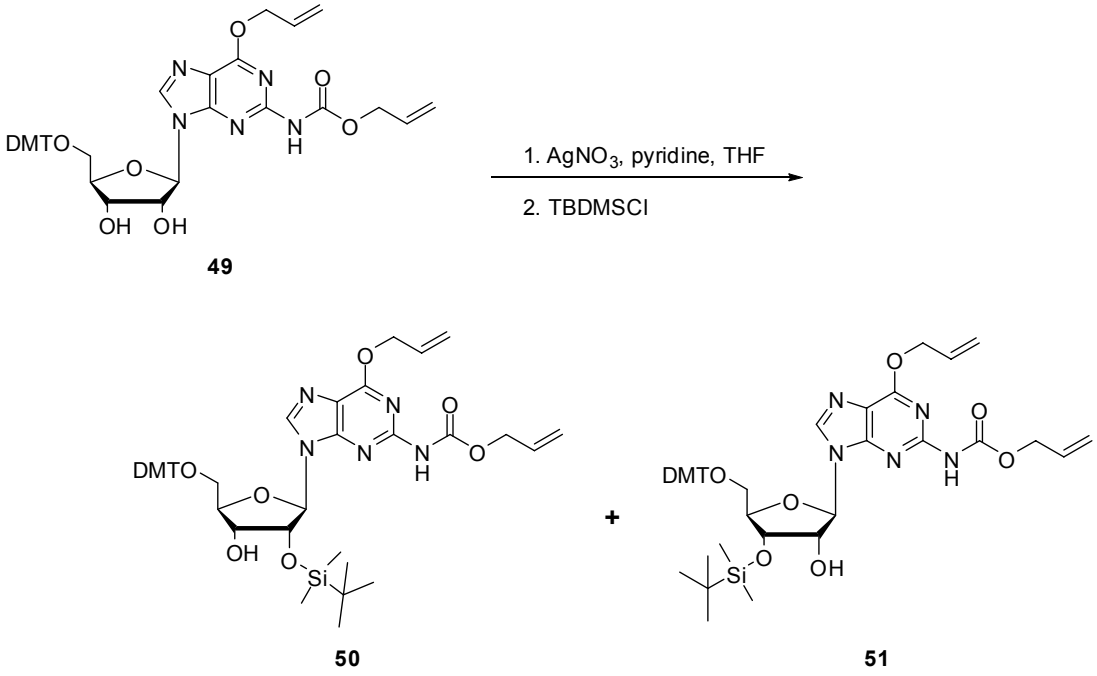
^{1}H NMR (400.0 MHz, DMSO, δ ppm): 10.37 (s, 1H, ^{2}NH), 8.35 (s,1H, ^{8}CH), 7.30 (m, 2H, CH_{arom}), 7.20 (m, 2H, CH_{arom}), 7.18 (m, 4H, CH_{arom}), 7.17 (m, 1H, CH_{arom}), 6.78 (m, 4H, CH_{arom}), 6.17 (ddt, 1H, J_1= 17.2 Hz, J_2= 10.4 Hz, J_3= 5.8 Hz, CH_{allyl}), 5.97 (ddt, 1H, J_1= 17.2 Hz, J_2= 10.6 Hz, J_3= 5.2 Hz, CH_{AOC}), 5.94 (d, 1H, J= 4.4 Hz, $^{1'}$CH), 5.61 (s, 1H, $^{2'}$OH), 5.48 (dq, 2H, J_1= 17.2 Hz, J_2= 1.6 Hz, $CH_{2\ alkene\ allyl}$), 5.39 (dq, 2H, J_1= 17.2 Hz, J_2= 1.7 Hz, $CH_{2\ alkene\ AOC}$), 5.31 (bdq, 1H, J_1= 10.4 Hz, J_2= 1.3 Hz, $CH_{2\ allyl}$), 5.23 (dq, 1H, J_1= 10.6 Hz, J_2= 1.5 Hz, $CH_{2\ allyl}$), 5.13 (s, 1H, $^{3'}$OH), 5.06 (dt, 2H, J_1= 5.8 Hz, J_2= 1.0 Hz, $CH_{2\ AOC}$), 4.72 (q, 1H, J= 5.0 Hz, $^{2'}$CH), 4.61 (dt, 1H, J_1= 5.2 Hz, J_2= 1.3 Hz, $CH_{2\ AOC}$), 4.37 (q, 1H, J= 5.2 Hz, $^{3'}$CH), 4.00 (m, 1H, $^{4'}$CH), 3.71 (s, 3H, CH_3), 3.70 (s, 3H, $CH_{3\ DMT}$), 3.20 (dd, 1H, J_1= 10.6 Hz, J_2= 6.3 Hz, $^{5'}CH_2$), 3.18 (dd, 1H, J_1= 10.6 Hz, J_2= 3.0 Hz, $^{5'}CH_2$);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 159.6 (^{4}C-O), 158.0 (Cq_{arom}), 157.9 (CH_{arom}), 152.8 (^{6}Cq), 152.0 (^{2}Cq), 151.5 ($C{=}O_{AOC}$), 144.9 (Cq_{arom}), 141.5 (^{8}CH), 135.6 (Cq_{arom}), 133.1 (CH_{allyl}), 132.9 (CH_{AOC}), 129.7 (CH_{arom}), 129.6 (CH_{arom}), 127.7 (CH_{arom}), 127.6 (CH_{arom}), 126.6 (CH_{arom}), 118.9 ($CH_{2\ AOC}$), 117.4 ($CH_{2\ allyl}$), 117.3 (^{5}Cq), 113.0 (CH_{arom}), 112.9 (CH_{arom}), 88.3

($^{1'}$CH), 85.4 (Cq), 83.7 ($^{4'}$CH), 73.1 ($^{2'}$CH), 70.6 ($^{3'}$CH), 66.9 ($CH_{2\ AOC}$), 64.7 ($CH_{2\ allyl}$), 64.2 ($^{5'}CH_2$), 55.0 ($CH_{3\ DMT}$), 54.9 ($CH_{3\ DMT}$);

MS (Maldi-ToF, m/z): 708.4 ([M]$^+$).

8.3.5.2 4-*O*-allyloxy-2-*N*-[(allyloxy)carbonyl]-5'-*O*-dimethoxytriphenyl methyl-2'-*O*-tertbutyldimethylsilyl-guanosine (50)



760 mg (1.06 mmol, 1 eq) 5'-*O*-dimethoxytrityl-4-*O*-allyloxy-2-*N*-[(allyloxy)carbonyl] guanosine **49** were dissolved in 10 ml dry THF at RT under argon and added with 0.32 ml (3.95 mmol, 3.7 eq) pyridine. 272 mg (1.60 mmol, 1.5 eq) silver nitrate were added and the suspension stirred to obtain a clear solution. 274 mg (1.82 mmol, 1.7 eq) *t*-butyldimethylsilyl chloride were added and stirred for 5 hrs. The reaction was filtered over celite and the filtrate poured into 5% aq. $NaHCO_3$ sol. (25 ml) and extracted with DCM (3 x 15 ml). The organic layer was dried ($MgSO_4$) and the solvents evaporated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 2:8, v/v,+0.5% NEt_3) yielded two isomers as white foams. 70 mg of $^{2'}$O-TBDMS substituted compound **50** (0.08 mmol, 8%) and 349 mg of $^{3'}$O-TBDMS substituted compound **51** (0.42 mmol, 40%) were obtained.

$C_{44}H_{53}N_5O_9Si$: 824.01 g/mol

2'-O-TBDMS isomer **50**:

R_f (hexane:EtOAc, 1:3) = 0.66;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 10.38 (s, 1H, ^{2}NH), 8.42 (s,1H, ^{8}CH), 7.32 (m, 2H, CH_{arom}), 7.19 (m, 8H, CH_{arom}), 6.79 (m, 4H, CH_{arom}), 6.14 (m, 1H, CH_{allyl}), 5.94 (m, 2H, CH_{AOC}, $^{1'}$CH), 5.48 (ddq, 2H, J_1= 39.12 Hz, J_2= 17.4 Hz, J_3= 1.52 Hz, $CH_{2\ alkene\ allyl}$), 5.24 (ddq, 2H, J_1= 32.08 Hz, J_2= 10.36 Hz, J_3= 1.52 Hz, $CH_{2\ alkene\ AOC}$), 5.03 (d, 1H, J= 5.8 Hz, $^{3'}$OH), 4.99 (d, 2H, J= 6.08 Hz, $CH_{2\ allyl}$), 4.88 (t, 1H, J= 5.04 Hz, $^{2'}$CH), 4.57 (d, 2H, J= 4.8 Hz, $CH_{2\ AOC}$), 4.24 (q, 1H, J= 4.8 Hz, $^{3'}$CH), 4.00 (q, 1H, J= 7.08 Hz, $^{4'}$CH), 3.38-3.18 (m, 2H, $^{5'}CH_2$), 0.73 (s, 9H, $CH_{3\ TBDMS}$), -0.05 (s, 6H, $CH_{3\ TBDMS}$);

^{13}C NMR (101.0 MHz, DMSO, δ ppm): 159.5 (^{4}C-O), 157.0 (Cq_{arom}), 156.9 (CH_{arom}), 153.5 (^{6}Cq), 151.0 (^{2}Cq), 151.5 ($C{=}O_{allyl}$), 142.9 (Cq_{arom}), 141.9 (^{8}CH), 133.9 (Cq_{arom}), 133.1 (CH_{allyl}), 133.5 (CH_{AOC}), 130.6 (CH_{arom}), 130.5 (CH_{arom}), 128.5 (CH_{arom}), 127.9 (CH_{arom}), 126.6 (CH_{arom}), 119.9 ($CH_{2\ allyl}$), 117.4 ($CH_{2\ AOC}$), 117.2 (^{5}Cq), 113.8 (CH_{arom}), 112.7 (CH_{arom}), 88.5 ($^{1'}$CH), 86.3 (Cq), 83.1 ($^{4'}$CH), 73.5 ($^{2'}$CH), 70.9 ($^{3'}$CH), 67.8 ($CH_{2\ AOC}$), 64.5 ($CH_{2\ allyl}$), 64.0 ($^{5'}CH_2$), 55.8 ($CH_{3\ DMT}$), 54.9 ($CH_{3\ DMT}$), 26.6 ($CH_{3\ TBDMS}$), 21.6 (Cq_{TBDMS}), -4.1 ($CH_{3\ TBDMS}$);

MS (Maldi-ToF, m/z): 823.9 ($[M]^+$).

3'-O-TBDMS isomer **51**:

R_f (hexane:EtOAc, 1:3) = 0.59;

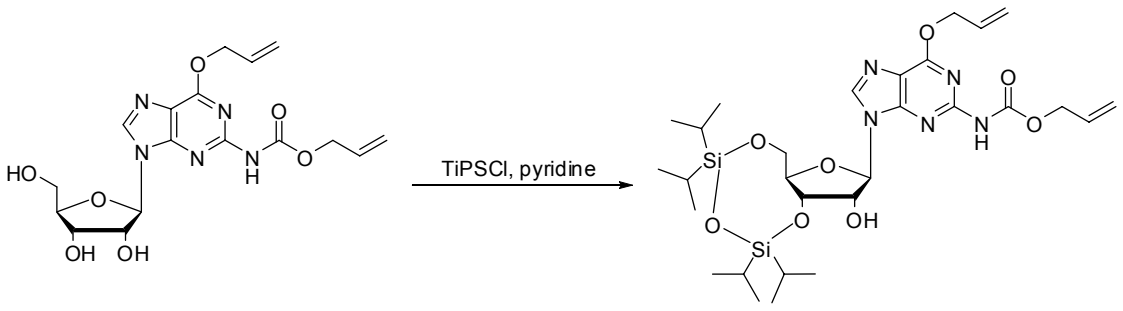
^{1}H NMR (400.0 MHz, DMSO, δ ppm): 10.37 (s, 1H, ^{2}NH), 8.42 (s,1H, ^{8}CH), 7.32 (m, 2H, CH_{arom}), 7.19 (m, 8H, CH_{arom}), 6.79 (m, 4H, CH_{arom}), 6.15 (m, 1H, CH_{allyl}), 5.94 (m, 2H, CH_{AOC}, $^{1'}$CH), 5.48 (ddq, 2H, J_1= 39.12 Hz, J_2= 17.4 Hz, J_3= 1.52 Hz, $CH_{2\ alkene\ allyl}$), 5.24 (ddq, 2H, J_1= 32.1 Hz, J_2= 10.36 Hz, J_3= 1.52 Hz, $CH_{2\ alkene\ AOC}$), 5.03 (d, 1H, J= 5.8 Hz, $^{3'}$OH), 4.99 (d, 1H, J= 6.08 Hz, $CH_{2\ allyl}$), 4.58 (t, 1H, J= 5.04 Hz, $^{2'}$CH), 4.54 (d, 1H, J= 4.8 Hz, $CH_{2\ AOC}$), 4.34 (q, 1H, J= 4.8 Hz, $^{3'}$CH), 4.00 (q, 1H, J= 7.08 Hz, $^{4'}$CH), 3.36-3.20 (m, 2H, $^{5'}CH_2$), 0.73 (s, 9H, $CH_{3\ TBDMS}$), -0.06 (s, 6H, $CH_{3\ TBDMS}$);

^{13}C NMR (101.0 MHz, DMSO, δ ppm): 159.5 (^{4}C-O), 157.1 (Cq_{arom}), 156.9 (CH_{arom}), 153.5 (^{6}Cq), 151.0 (^{2}Cq), 151.5 ($C{=}O_{allyl}$), 142.9 (Cq_{arom}), 141.9 (^{8}CH), 133.9 (Cq_{arom}), 133.1 (CH_{allyl}), 133.4 (CH_{AOC}), 130.6 (CH_{arom}), 130.5 (CH_{arom}), 128.5 (CH_{arom}), 127.9 (CH_{arom}), 126.6 (CH_{arom}), 119.9 ($CH_{2\ allyl}$), 117.4 ($CH_{2\ AOC}$), 117.2 (^{5}Cq), 113.8 (CH_{arom}), 112.7 (CH_{arom}), 88.6 ($^{1'}$CH), 86.3 (Cq), 83.5 ($^{4'}$CH), 73.9 ($^{2'}$CH), 71.5 ($^{3'}$CH), 67.8 ($CH_{2\ AOC}$), 64.5 ($CH_{2\ allyl}$), 64.0 ($^{5'}CH_2$), 55.8 ($CH_{3\ DMT}$), 54.9 ($CH_{3\ DMT}$), 26.6 ($CH_{3\ TBDMS}$), 21.6 (Cq_{TBDMS}), -4.0 ($CH_{3\ TBDMS}$);

MS (Maldi-ToF, m/z): 823.9 ($[M]^+$).

8.3.6 Second Building Block Possibility

8.3.6.1 3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-4-*O*-allyloxy-2-*N*-[(allyloxy)carbonyl] guanosine (52)



48 **52**

250 mg (0.61 mmol, 1 eq) 4-*O*-allyloxy-2-*N*-[(allyloxy)carbonyl] guanosine **48** were dissolved in 5 ml dry pyridine at RT under argon, added with 231 µl (0.73 mmol, 1.2 eq) 1,3-dichloro-1,1,3,3-tetra*iso*propyldisiloxane (TiPSCl) and stirred for 4 hrs. The reaction was quenched with 5 ml methanol and the mixture stirred for 10 mins. The solvents were evaporated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 7:3, v/v) yielded 310 mg of **52** as a white foam (0.48 mmol, 78%).

$C_{29}H_{47}N_5O_9Si$: 649.88 g/mol

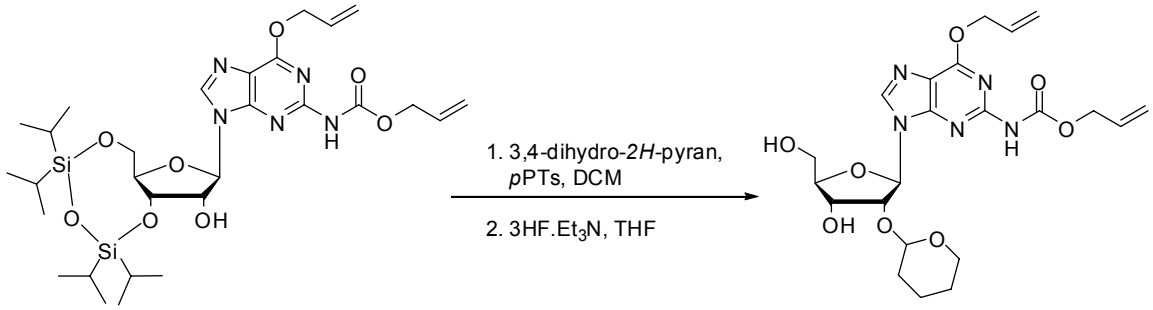
R_f (hexane:EtOAc, 1:3) = 0.37;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 10.35 (s, 1H, ^{2}NH), 8.26 (s,1H, ^{8}CH), 6.13 (m, 1H, CH_{allyl}), 5.93 (m, 1H, CH_{AOC}), 5.84 (d, 1H, *J*= 1.24 Hz, $^{1'}$CH), 5.51 (d, 1H, *J*= 4.56 Hz, $^{2'}$OH), 5.40 (ddq, 2H, J_1= 32.32 Hz, J_2= 17.16 Hz, J_3= 1.52 Hz, $CH_{2\ alkene\ allyl}$), 5.25 (ddq, 2H, J_1= 25.48 Hz, J_2= 11.6 Hz, J_3= 1.24 Hz, $CH_{2\ alkene\ AOC}$), 5.02 (d, 1H, *J*= 5.8 Hz, $CH_{2\ allyl}$), 4.58 (m, 2H, $^{2'}$CH, $^{3'}$CH), 4.55 (d, 2H, *J*= 5.8 Hz, $CH_{2\ AOC}$), 4.12-3.87 (m, 3H, $^{4'}$CH, $^{5'}CH_2$), 1.02 (m, 28H, $CH_{isopropyl}$, $CH_{3\ isopropyl}$);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 159.4 (^{4}C-O), 152.7 (^{6}Cq), 151.8 (^{2}Cq), 151.3 ($C{=}O_{AOC}$), 141.2 (^{8}CH), 133.9 (CH_{allyl}), 133.7 (CH_{AOC}), 119.8 ($CH_{2\ alkene\ AOC}$), 118.2 ($CH_{2\ alkene\ allyl}$), 116.6 (^{5}Cq), 87.5 ($^{1'}$CH), 84.1 ($^{4'}$CH), 73.1 ($^{2'}$CH), 70.2 ($^{3'}$CH), 67.8 ($CH_{2\ AOC}$), 65.4 ($CH_{2\ allyl}$), 64.1 ($^{5'}CH_2$), 18.1 (CH_3), 13.3 (CH);

MS (Maldi-ToF, m/z): 648.9 ($[M]^+$).

8.3.6.2 4-*O*-allyloxy-2-*N*-[(allyloxy)carbonyl]-2'-*O*-tetrahydropyranyl-guanosine (53)



52 **53**

658 mg (1.01 mmol, 1 eq) 3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-4-*O*-allyloxy-2-*N*-[(allyloxy)carbonyl] guanosine **52** and 764 mg (3.04 mmol, 3 eq) *p*-toluenesulfonyl pyridinium (*p*PTs) were dissolved in 20 ml dry dichloromethane under argon, added with 2.75 ml (30.46 mmol, 30 eq) 3,4-dihydro-*2H*-pyran and stirred at RT for 16 hrs. The reaction mixture was diluted with diethyl ether (50 ml) and washed with a half saturated brine sol. (2 x 50 ml). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The resulting oily residue was dissolved in 15 ml dry THF under argon, added with 1.77 ml (10.15 mmol, 10 eq) triethylamine trihydrofluoride and stirred for 16 hrs. The reaction was quenched with silica gel and the solvents were evaporated under reduced pressure. Flash chromatography (hexane,hexane:EtOAc gradient from 1:0 to 4:6, then CH_2Cl_2:MeOH gradient from 100:0 to 95:5, v/v) yielded 253 mg of **53** a white foam (0.51 mmol, 78%).

$C_{22}H_{29}N_5O_8Si$: 491.49 g/mol

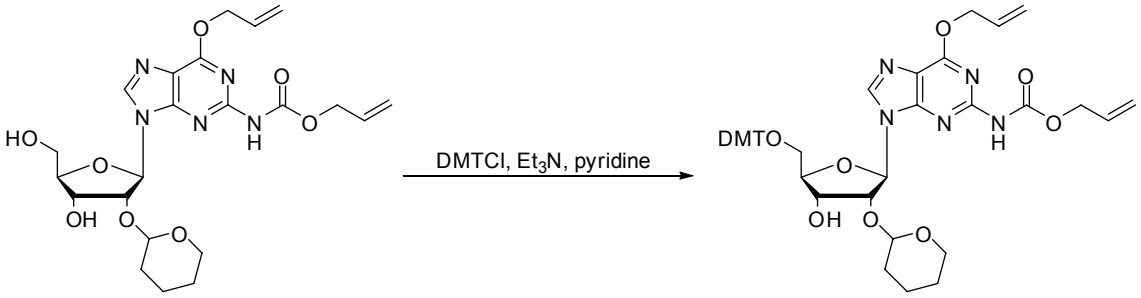
R_f (hexane:EtOAc, 6:4) = 0.08;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 10.37 (d, 1H, *J*= 8.6 Hz, ^{2}NH), 8.45 (d,1H, *J*= 13.12 Hz, ^{8}CH), 6.16 (m, 1H, CH_{allyl}), 6.01 (m, 2H, CH_{THP}, CH_{AOC}), 5.41 (ddq, 2H, J_1= 32.04 Hz, J_2= 17.4 Hz, J_3= 1.76 Hz, $CH_{2\ alkene\ allyl}$), 5.21 (m, 3H, $^{1'}$CH, $CH_{2\ alkene\ AOC}$), 5.04 (m, 3H, $CH_{2\ allyl}$, $^{2'}$CH), 4.98 (t, 1H, *J*= 5.56 Hz, $^{5'}$OH), 4.70 (m, 2H, $^{3'}$CH, $^{3'}$OH), 4.60 (m, 2H, $CH_{2\ AOC}$), 4.29 (m, 1H, $CH_{2\ THP}$), 3.94 (q, 1H, *J*= 4.04 Hz, $^{4'}$CH), 3.68-3.52 (m, 2H, $^{5'}CH_2$), 3.09 (m, 1H, $CH_{2\ THP}$), 1.74 (m, 1H, $CH_{2\ THP}$), 1.54 (m, 2H, $CH_{2\ THP}$), 1.37 (m, 2H, $CH_{2\ THP}$), 1.22 (m, 1H, $CH_{2\ THP}$);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 160.4 (^{4}C-O), 154.0 (^{6}Cq), 153.8 (^{2}Cq), 152.9 ($C{=}O_{AOC}$), 152.5 (CH_{THP}), 143.5 (^{8}CH), 133.9 (CH_{allyl}), 133.7 (CH_{AOC}), 119.8 ($CH_{2\ alkene\ AOC}$), 119.8 ($CH_{2\ alkene\ allyl}$), 117.1 (^{5}Cq), 97.9 ($^{1'}$CH), 86.6 ($^{4'}$CH), 78.1 ($^{2'}$CH), 70.7 ($^{3'}$CH), 67.8 ($CH_{2\ AOC}$), 65.5 ($CH_{2\ allyl}$), 62.5 ($^{5'}CH_2$), 30.9 (CH_2), 30.4 (CH_2), 25.7 (CH_2), 25.5 (CH_2), 19.4 (CH_2);

MS (Maldi-ToF, m/z): 490.1 ($[M]^+$).

8.3.6.3 4-*O*-allyloxy-2-*N*-[(allyloxy)carbonyl]-5'-*O*-dimethoxytriphenyl methyl-2'-*O*-tetrahydropyranyl-guanosine (54)



53 **54**

368 mg (0.73 mmol, 1 eq) 4-*O*-allyloxy-2-*N*-[(allyloxy)carbonyl]-2'-*O*-tetrahydropyranyl-guanosine **53** were co-evaporated with dry pyridine (2x5 ml), dissolved into 6 ml dry pyridine, added with 150 µl (1.50 mmol, 2 eq) triethylamine, 330 mg (0.97 mmol, 1.3 eq) DMTCl and stirred overnight at RT under argon. The reaction was quenched with methanol (5 ml) and the solvents evaporated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 2:3, v/v, +1% triethylamine) yielded 515 mg of **54** as a white solid (0.65 mmol, 87%).

$C_{43}H_{47}N_5O_{10}Si$: 793.86 g/mol

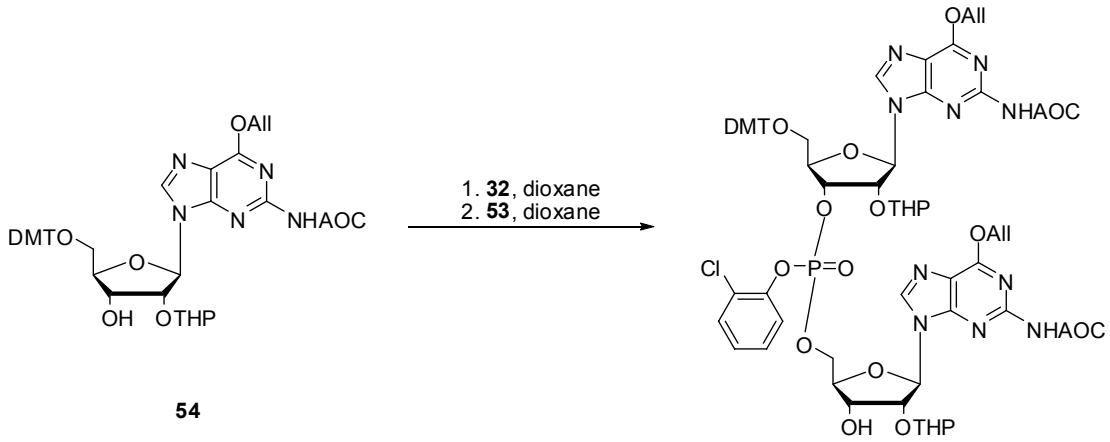
R_f (hexane:EtOAc, 2:3) = 0.33;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 10.32 (s, 1H, ^{2}NH), 8.31 (s ,1H, ^{8}CH), 7.31 (m, 2H, CH_{arom}), 7.17 (m, 8H, CH_{arom}), 6.77 (m, 4H, CH_{arom}), 6.18 (m, 2H, CH_{THP}, CH_{allyl}), 5.91 (m, 1H, CH_{AOC}), 5.38 (dd, 2H, J_1= 37.88 Hz, J_2= 17.16 Hz, $CH_{2\ alkene\ allyl}$), 5.27 (m, 3H, $^{1'}$CH, $CH_{2\ alkene\ AOC}$), 5.03 (m, 3H, $CH_{2\ allyl}$, $^{2'}$CH), 4.81 (t, 1H, J= 5.04 Hz, $^{3'}$OH), 4.73 (m, 1H, $^{3'}$CH), 4.59 (m, 2H, $CH_{2\ AOC}$), 4.48 (m, 1H, $CH_{2\ THP}$), 4.03 (m, 1H, $^{4'}$CH), 3.88 (m, 1H, $CH_{2\ THP}$), 3.37-3.15 (m, 2H, $^{5'}CH_2$), 1.63 (m, 1H, $CH_{2\ THP}$), 1.54 (m, 2H, $CH_{2\ THP}$), 1.44 (m, 2H, $CH_{2\ THP}$), 1.21 (m, 1H, $CH_{2\ THP}$);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 161.1 (^{4}C-O), 158.9 (Cq_{arom}), 158.8 (CH_{arom}), 155.0 (^{6}Cq), 154.1 (^{2}Cq), 151.9 ($C{=}O_{AOC}$), 151.8 (CH_{THP}), 143.9 (Cq_{arom}), 143.3 (^{8}CH), 136.3 (Cq_{arom}), 133.9 (CH_{allyl}), 133.7 (CH_{AOC}), 130.6 (CH_{arom}), 130.5 (CH_{arom}), 128.5 (CH_{arom}), 127.9 (CH_{arom}), 126.6 (CH_{arom}), 119.8 ($CH_{2\ alkene\ AOC}$), 118.2 ($CH_{2\ alkene\ allyl}$), 117.7 (^{5}Cq), 113.8 (CH_{arom}), 112.9 (CH_{arom}), 98.5 ($^{1'}$CH), 87.1 ($^{4'}$CH), 77.9 ($^{2'}$CH), 70.1 ($^{3'}$CH), 67.8 ($CH_{2\ AOC}$), 65.5 ($CH_{2\ allyl}$), 62.1 ($^{5'}CH_2$), 30.5 ($CH_{2\ THP}$), 30.3 ($CH_{2\ THP}$), 25.8 ($CH_{2\ THP}$), 25.2 ($CH_{2\ THP}$), 18.9 ($CH_{2\ THP}$);

MS (Maldi-ToF, m/z): 793.2 ($[M]^+$).

8.3.7 (2-*N*-(allyloxy)carbonyl-4-*O*-allyloxy-5'-*O*-dimethoxytriphenyl methyl-2'-*O*-tetrahydropyranyl-guanosine)-(3'-5')-(2-*N*-(allyloxy) carbonyl-4-*O*-allyloxy-2'-*O*-tetrahydropyranyl-guanosine)-2-chlorophenyl phosphate (55)



54 **55**

820 mg (1.03 mmol, 1 eq) 4-*O*-allyloxy-2-*N*-[(allyloxy)carbonyl]-5'-*O*-dimethoxy triphenylmethyl-2'-*O*-tetrahydropyranyl-guanosine **54** were dissolved in 9 ml dry dioxane over activated powdered 4Å MS and added with 5.6 ml (1.14 mmol, 1.1 eq) of a 0.2 M phosphorylating agent **32** sol. at RT under argon. After 10 mins, 913 mg (1.86 mmol, 1.8 eq) 4-*O*-allyloxy-2-*N*-[(allyloxy)carbonyl]-2'-*O*-tetrahydropyranyl-guanosine **53** in dry dioxane (5 ml) over activated powdered 4Å MS were added and the mixture stirred at RT under argon for 3 hrs. The reaction mixture was diluted with DCM (25 ml), filtered over celite and washed with 0.1 M triethylammonium carbonate (TEAC) (2 x 25 ml). The aqueous phase was extracted with DCM (3 x 40 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 1:1, v/v. +0.5% Et_3N) lead to 1.12 g (0.77 mmol, 74%) of **55** a colorless foam.

$C_{71}H_{78}ClN_{10}O_{20}P$: 1457.86 g/mol

R_f (hexane:EtOAc, 9:1) = 0.66;

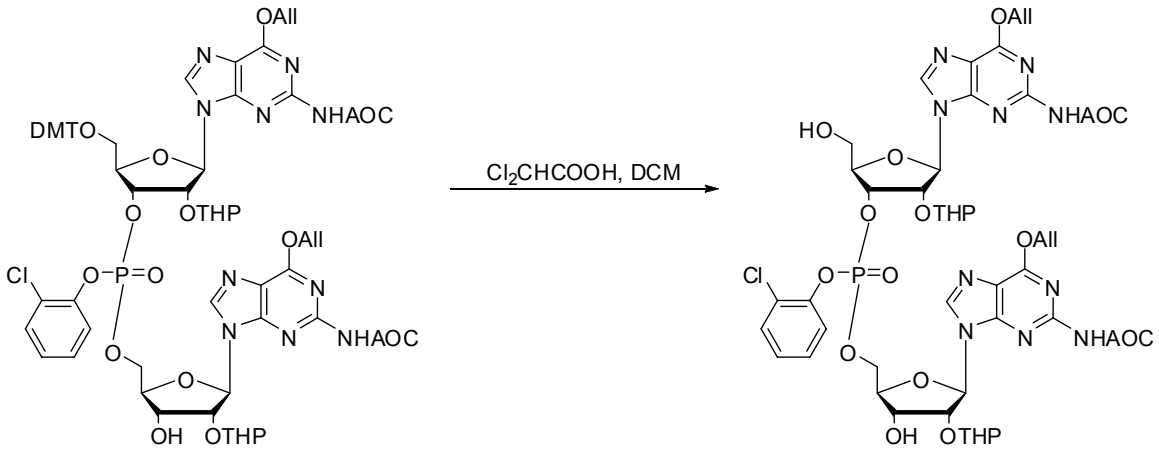
^{1}H NMR (500.0 MHz, DMSO, δ ppm): 10.42-10.37 (m, 2H, ^{2}NH), 8.47-8.38 (m, 2H, ^{8}CH), 7.55-7.17 (m, 12H, CH_{arom}), 6.77 (m, 4H, CH_{arom}), 6.20-6.09 (m, 2H, CH_{AOC}), 6.12-6.10 (m, 2H, $^{1'}$CHa, $^{1'}$CHb), 6.03-5.92 (m, 2H, CH_{allyl}), 5.50-5.43 (m, 2H, $CH_{2\ alkene\ AOC}$), 5.41-5.34 (m, 2H, $CH_{2\ alkene\ allyl}$), 5.31-5.27 (m, 2H, $CH_{2\ alkene\ AOC}$), 5.31 (m, 1H, $^{3'}$OHb), 5.24-5.21 (m, 2H, $CH_{2\ alkene\ allyl}$), 5.22 (m, 1H, $^{5'}$OH), 5.19 (m, 1H, $^{3'}$CHa) 5.15 (m, 1H, $^{2'}$CHa), 5.05-5.01 (m, 4H, CH_{2AOC}), 4.83 (m, 1H, $^{2'}$CHb), 4.71-4.67 (m, 2H, CH_{THP}), 4.62-4.60 (m, 4H, $CH_{2\ Allyl}$), 4.59 (m,

3H, $^{3'}$CHb, $^{5'}CH_2$b), 4.25 (m, 1H, $^{4'}$CHb), 4.18 (m, 1H, $^{4'}$CHa), 3.87-3.35 (m, 4H, $CH_{2\ THP}$), 3.71-3.25 (m, 4H, $CH_{2\ THP}$), 3.60 (m, 1H, $^{5'}CH_2$a), 3.52 (m, 1H, $^{5'}CH_2$a), 3.29-3.10 (m, 4H, $CH_{2\ THP}$), 2.89-2.83 (m, 4H, $CH_{2\ THP}$);

^{13}C NMR (125.8 MHz, DMSO, δ ppm): 160.1 ($C{=}O_{AOC}$), 153.7/152.9 (^{6}Cq), 152.8/152.5 (^{2}Cq), 152.1/151.9 (^{4}C-O), 146.5 (Cq_{arom}), 142.3/141.4 (^{8}CH), 136.3/133.5 (CH_{allyl}), 133.3 (CH_{AOC}), 131.0-124.8 (CH_{arom}), 119.5/119.3 ($CH_{2\ alkene\ AOC}$), 118.0/117.9 ($CH_{2\ alkene\ allyl}$), 117.7/117.5 (^{5}Cq), 113.8-112.9 (CH_{arom}), 87.7/87.0 ($^{1'}$CHa), 85.1/84.8 ($^{1'}$CHb, $^{4'}$CHa), 83.5/83.1 ($^{4'}$CHb), 77.8/77.7 ($^{2'}$CHa), 77.4/77.2 ($^{2'}$CHb), 76.2/75.9 ($^{3'}$CHa), 70.3/69.3 ($^{3'}$CHb), 69.7/69.5 ($^{5'}CH_2$b), 67.5/67.4 ($CH_{2\ AOC}$), 65.2 ($CH_{2\ allyl}$), 62.5-60.1 ($CH_{2\ THP}$), 61.3 ($^{5'}CH_2$a);

MS (Maldi-ToF, m/z): 1456.57 ($[M]^+$).

8.3.8 (2-*N*-(allyloxy)carbonyl-4-*O*-allyloxy-2'-*O*-tetrahydropyranyl-guanosine)-(3'-5')-(2-*N*-(allyloxy)carbonyl-4-*O*-allyloxy-2'-*O*-tetrahydropyranyl-guanosine)-2-chlorophenyl phosphate (56)



55 **56**

370 mg (0.25 mmol, 1 eq) dimer **55** were dissolved in 3 ml DCM, added with 60 µl (0.73 mmol, 1.2 eq) dichloroacetic acid and stirred for 16 hrs at RT under argon. The reaction mixture was oured into 20 ml sat. aq. $NaHCO_3$ sol. and extracted with dichloromethane (3 x 20 ml). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 1:9, v/v) yielded 173 mg of dimer **56** as a white foam (0.15 mmol, 59%).

$C_{50}H_{60}ClN_{10}O_{18}P$: 1155.49 g/mol

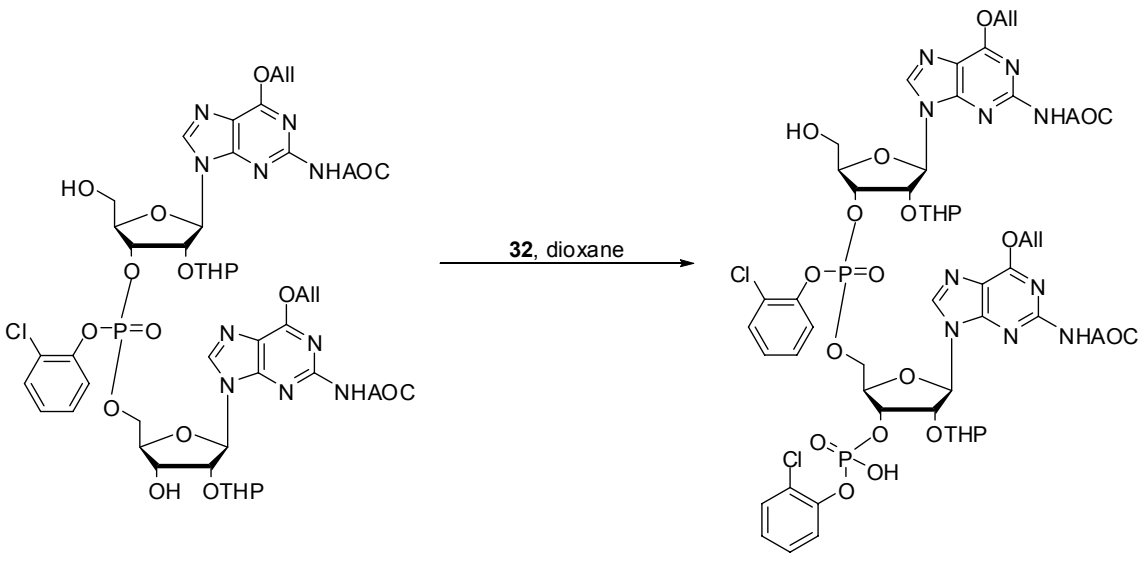
R_f (hexane:EtOAc, 1:9) = 0.1;

^{1}H NMR (500.0 MHz, DMSO, δ ppm): 10.42-10.35 (m, 2H, ^{2}NH), 8.47-8.38 (m, 2H, ^{8}CH), 7.55-7.22 (m, 4H, CH_{arom}), 6.20-6.09 (m, 2H, CH_{AOC}), 6.12-6.10 (m, 2H, $^{1'}$CHa, $^{1'}$CHb), 6.00-5.92 (m, 2H, CH_{allyl}), 5.50-5.43 (m, 2H, $CH_{2\ alkene\ AOC}$), 5.41-5.34 (m, 2H, $CH_{2\ alkene\ allyl}$), 5.31-5.27 (m, 2H, $CH_{2\ alkene\ AOC}$), 5.30 (m, 1H, $^{3'}$OHb), 5.24-5.21 (m, 2H, $CH_{2\ alkene\ allyl}$), 5.22 (m, 1H, $^{5'}$OH), 5.19 (m, 1H, $^{3'}$CHa) 5.16 (m, 1H, $^{2'}$CHa), 5.05-5.01 (m, 4H, $CH_{2\ AOC}$), 4.83 (m, 1H, $^{2'}$CHb), 4.71-4.67 (m, 2H, CH_{THP}), 4.62-4.60 (m, 4H, $CH_{2\ Allyl}$), 4.59 (m, 3H, $^{3'}$CHb, $^{5'}CH_2$b), 4.25 (m, 1H, $^{4'}$CHb), 4.17 (m, 1H, $^{4'}$CHa), 3.87-3.35 (m, 4H, $CH_{2\ THP}$), 3.71-3.25 (m, 4H, $CH_{2\ THP}$), 3.61 (m, 1H, $^{5'}CH_2$a), 3.52 (m, 1H, $^{5'}CH_2$a), 3.29-3.10 (m, 4H, $CH_{2\ THP}$), 2.89-2.84 (m, 4H, $CH_{2\ THP}$);

^{13}C NMR (125.8 MHz, DMSO, δ ppm): 160.1 ($C{=}O_{AOC}$), 153.7/152.9 (^{6}Cq), 152.8/152.5 (^{2}Cq), 152.1/151.9 (^{4}C-O), 146.3 (Cq_{arom}), 142.3/141.4 (^{8}CH), 133.5 (CH_{allyl}), 133.3 (CH_{AOC}), 131.0/124.8 (CH_{arom}), 119.5/119.3 ($CH_{2\ alkene\ AOC}$), 118.0/117.9 ($CH_{2\ alkene\ allyl}$), 117.7/117.5 (^{5}Cq), 87.7/87.0 ($^{1'}$CHa), 85.1/84.8 ($^{1'}$CHb), 85.1/84.8 ($^{4'}$CHa), 83.5/83.1 ($^{4'}$CHb), 77.8/77.7 ($^{2'}$CHa), 77.4/77.2 ($^{2'}$CHb), 76.1/75.9 ($^{3'}$CHa), 70.3/69.3 ($^{3'}$CHb), 69.7/69.5 ($^{5'}CH_2$b), 67.5/67.4 ($CH_{2\ AOC}$), 65.2 ($CH_{2\ allyl}$), 62.5-60.2 ($CH_{2\ THP}$), 61.3 ($^{5'}CH_2$a);

MS (Maldi-ToF, m/z): 1155.54 ($[M]^{+}$).

8.3.9 (2-*N*-(allyloxy)carbonyl-4-*O*-allyloxy-2'-*O*-tetrahydropyranyl-guanosine)-(3'-5')-(2-*N*-(allyloxy)carbonyl-4-*O*-allyloxy-3'-*O*-(2-chlorophenylphosphate)-2'-*O*-tetrahydropyranyl-guanosine)-2-chlorophenyl phosphate (57)



56 **57**

130 mg (0.11 mmol, 1 eq) dimer **56** were dissolved in 55 ml dry dioxane, over activated powdered 4Å MS, added with 620 µl (0.12 mmol, 1.1 eq) of a 0.2 M phosphorylating agent **32** solution and the mixture stirred at RT under argon for 24 hrs. The reaction mixture was filtered over celite. The residue was taken up in DCM (20 ml) and washed with 1 M TEAC (20 ml). The aqueous phase was then extracted with DCM (3 x 20 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 2:8, then DCM:MeOH gradient from 1:0 to 9:1, v/v) lead to 127 mg (0.09 mmol, 83%) of open-chain dinucleotide GpGp **57** as a colorless foam.

$C_{56}H_{64}Cl_2N_{10}O_{21}P_2$: 1346.02 g/mol

R_f (CH_2Cl_2:MeOH, 1:9) = 0.08;

^{1}H NMR (500.0 MHz, DMSO, δ ppm): 10.42-10.35 (m, 2H, ^{2}NH), 8.47-8.37 (m, 2H, ^{8}CH), 7.57-7.20 (m, 8H, CH_{arom}), 6.20-6.09 (m, 2H, CH_{AOC}), 6.12-6.10 (m, 2H, $^{1'}$CHa, $^{1'}$CHb), 6.00-5.92 (m, 2H, CH_{allyl}), 5.50-5.44 (m, 2H, $CH_{2\ alkene\ AOC}$), 5.39-5.34 (m, 2H, $CH_{2\ alkene\ allyl}$), 5.31-5.27 (m, 2H, $CH_{2\ alkene\ AOC}$), 5.24-5.21 (m, 2H, $CH_{2\ alkene\ allyl}$), 5.22 (m, 1H, $^{5'}$OH), 5.19 (m, 1H, $^{3'}$CHa) 5.16 (m, 1H, $^{2'}$CHa), 5.05-5.01 (m, 4H, $CH_{2\ AOC}$), 4.83 (m, 1H, $^{2'}$CHb), 4.76 (m, 1H, $^{3'}$CHb), 4.71-4.67 (m, 2H, CH_{THP}), 4.62-4.60 (m, 4H, $CH_{2\ Allyl}$), 4.59 (m, 2H, $^{5'}CH_2$b), 4.25 (m, 1H, $^{4'}$CHb), 4.17 (m, 1H, $^{4'}$CHa), 3.87-3.35 (m, 4H, $CH_{2\ THP}$), 3.71-3.27 (m, 4H, $CH_{2\ THP}$), 3.61 (m, 1H, $^{5'}CH_2$a), 3.52 (m, 1H, $^{5'}CH_2$a), 3.29-3.10 (m, 4H, $CH_{2\ THP}$), 2.88-2.83 (m, 4H, $CH_{2\ THP}$);

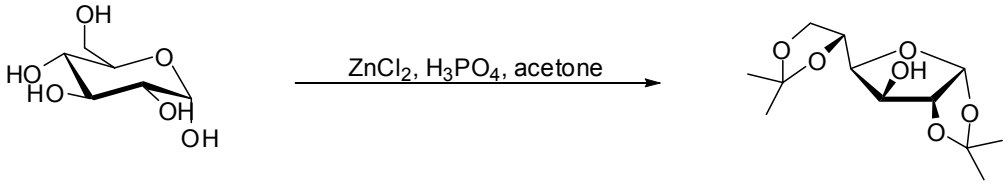
^{13}C NMR (125.8 MHz, DMSO, δ ppm): 160.1 ($C{=}O_{AOC}$), 153.7/152.9 (^{6}Cq), 152.8/152.5 (^{2}Cq), 152.1/151.9 (^{4}C-O), 146.3 (Cq_{arom}), 142.3/141.6 (^{8}CH), 133.5 (CH_{allyl}), 133.3 (CH_{AOC}), 131.0-124.8 (CH_{arom}), 119.6/119.2 ($CH_{2\ alkene\ AOC}$), 118.0/117.9 ($CH_{2\ alkene\ allyl}$), 117.7/117.5 (^{5}Cq), 87.7/87.1 ($^{1'}$CHa), 85.1/84.8 ($^{1'}$CHb), 85.1/84.8 ($^{4'}$CHa), 83.5/83.1 ($^{4'}$CHb), 77.8/77.7 ($^{2'}$CHa), 77.4/77.2 ($^{2'}$CHb), 76.1/75.9 ($^{3'}$CHa), 72.3/71.9 ($^{3'}$CHb), 69.7/69.5 ($^{5'}CH_2$b), 67.5/67.3 ($CH_{2\ AOC}$), 65.2 ($CH_{2\ allyl}$), 62.5-60.2 ($CH_{2\ THP}$), 61.3 ($^{5'}CH_2$a);

MS (Maldi-ToF, m/z): 1347.63 ($[M]^+$).

9. A NEW SYNTHESIS FOR C-DI-GMP

9.1 Building Block Synthesis from Glucose

9.1.1 1',2':5',6'-di-*O-iso*propylidene-α-D-glucofuranose (59)



58 **59**

5 g (27.8 mmol, 1 eq) α-D-glucose **58** were suspended in 50 ml dry acetone at RT under argon. 4 g (41.7 mmol, 1.5 eq) anhydrous zinc chloride were added, followed by 0.15 ml 85% aq. phosphoric acid and the mixture was stirred for 30 hrs at RT. The reaction mixture was filtered to remove the unreacted glucose. 10 M NaOH was added (pH=8). The suspension was filtered and the filtrate concentrated under reduced pressure. The residue was taken up in water (15 ml) and extracted with DCM (3 x 50 ml). The combined organic layers were dried ($MgSO_4$) and concentrated. The residue was re-crystallized from hexane to yield 3.90 g (15.01 mmol, 54%) of **59** as a white solid.

$C_{12}H_{20}O_6$: 260.28 g/mol

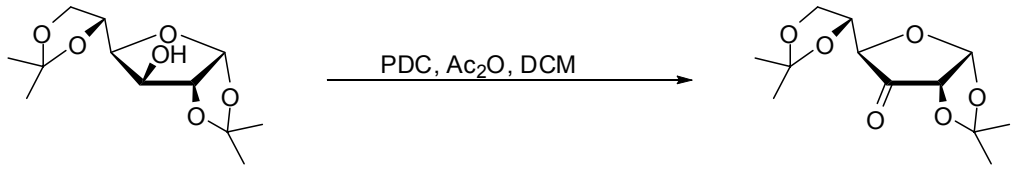
R_f (hexane:EtOAc, 1:1) = 0.55;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 5.79 (d,1H, J_1= 3.54 Hz, $^{1'}$CH), 5.35 (s, 1H, $^{3'}$OH), 4.37 (d, 1H, J_1= 3.79 Hz, $^{2'}$CH), 4.19 (dd, 1H, J_1= 12.88 Hz, J_2= 6.32 Hz, $^{3'}$CH), 3.95 (m, 2H, $^{6'}$CH), 3.92 (m, 1H, $^{5'}$CH), 3.78 (dd, 1H, J_1= 8.34 Hz, J_2= 6.07 Hz, $^{4'}$CH), 1.35 (s, 3H, CH_3), 1.29 (s, 3H, CH_3), 1.24 (s, 3H, CH_3), 1.20 (s, 3H, CH_3);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 111.5 (Cq), 108.7 (Cq), 105.4 ($^{1'}$CH), 85.8 ($^{2'}$CH), 81.7 ($^{4'}$CH), 74.0 ($^{3'}$CH), 73.1 ($^{5'}$CH), 66.9 ($^{6'}CH_2$), 27.6 (CH_3), 27.4 (CH_3), 26.9 (CH_3), 26.1 (CH_3);

MS (Maldi-ToF, m/z): 259.6 ($[M-H]^+$).

9.1.2 1',2':5',6'-di-*O*-*iso*propylidene-3'-oxo-α-D-glucofuranose (60)



59 **60**

1 g (3.85 mmol 1 eq) 1',2':5',6'-di-*O*-*iso*propylidene-α-D-glucofuranose **59** was dissolved in DCM (8 ml) at RT under argon and added portionwise with 868 mg (2.31 mmol, 0.6 eq) pyridinium dichromate (PDC) and 1.2 ml (11.55 mmol, 3 eq) acetic anhydride. The mixture was refluxed for 1.5 hrs. After cooling to RT, the reaction mixture was evaporated and EtOAc (10 ml) was added. The suspension was applied to a short silica pad and eluted with EtOAc. The solvent was evaporated under reduced pressure, to yield 0.87 g (3.37 mmol, 88%) of **60** as a colorless oil.

$C_{12}H_{18}O_6$: 258.27 g/mol

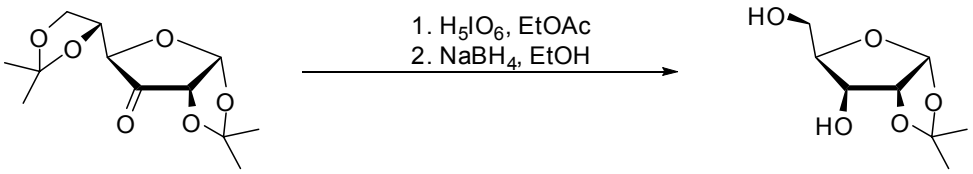
R_f (hexane:EtOAc, 1:1) = 0.5;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 5.80 (d,1H, J_1= 3.54 Hz, $^{1'}$CH), 4.41 (d, 1H, J_1= 3.79 Hz, $^{2'}$CH), 3.95 (m, 2H, $^{6'}$CH), 3.92 (m, 1H, $^{5'}$CH), 3.79 (dd, 1H, J_1= 8.34 Hz, J_2= 6.07 Hz, $^{4'}$CH), 1.35 (s, 3H, CH_3), 1.29 (s, 3H, CH_3), 1.24 (s, 3H, CH_3), 1.20 (s, 3H, CH_3);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 111.5 (Cq), 108.7 (Cq), 105.4 ($^{1'}$CH), 85.8 ($^{2'}$CH), 81.7 ($^{4'}$CH), 73.1 ($^{5'}$CH), 66.9 ($^{6'}CH_2$), 27.6 (CH_3), 27.4 (CH_3), 26.9 (CH_3), 26.1 (CH_3);

MS (Maldi-ToF, m/z): 257.6 ($[M-H]^+$).

9.1.3 1',2'-*O*-*iso*propylidene-α-D-ribofuranose (61)



60 **61**

10 g (38.72 mmol, 1 eq) 1',2':5',6'-di-*O*-*iso*propylidene-3-oxo-α-D-glucofuranose **60** were dissolved in dry EtOAc (500 ml) at 0°C under argon, added portionwise with 10.6 g (46.46 mmol, 1.2 eq) periodic acid. After 2 hrs 0°C, the reaction mixture was filtered over celite and the filtrate concentrated under reduced pressure. The residue was dissolved in EtOH at 0°C and 3.87 g (102.22 mmol, 2.64 eq) sodium borohydride were added in small portions under vigorous stirring. After 30 mins the reaction was quenched with a 10% aq. acetic acid sol. and volatiles were evaporated. The residue was dissolved in EtOAc (400 ml), the organic phase washed with H_2O (350 ml), dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography (hexane:DCM, 1:1, CH_2Cl_2:MeOH gradient from 1:0 to 8:2, v/v) yielded 3.39 g of **61** as a white foam (17.82 mmol, 46%).

$C_8H_{14}O_5$: 190.20 g/mol

R_f (hexane:EtOAc, 1:9) = 0.30;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 5.62 (d, 1H, *J*= 3.8 Hz, $^{1'}$CH), 4.97 (d, 1H, *J*= 6.6 Hz, $^{3'}$OH), 4.63 (t, 1H, *J*= 5.28 Hz, $^{5'}$OH), 4.41 (t, 1H, *J*= 2.84 Hz, $^{2'}$CH), 3.61 (m, 3H, $^{3'}$CH, $^{4'}$CH, $^{5'}CH_2$), 3.35 (m, 1H, $^{5'}CH_2$), 1.41 (s, 3H, CH_3), 1.23 (s, 3H, CH_3);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 111.99 (Cq), 104.15 ($^{1'}$CH), 81.14 ($^{2'}$CH), 79.92 ($^{4'}$CH), 71.33 ($^{3'}$CH), 61.02 ($^{5'}CH_2$), 27.46-27.26 (CH_3);

MS (FAB, m/z, %): 39 (100), 192 ($[M+H]^+$, 42), 229 ($[M+K]^+$, 58);

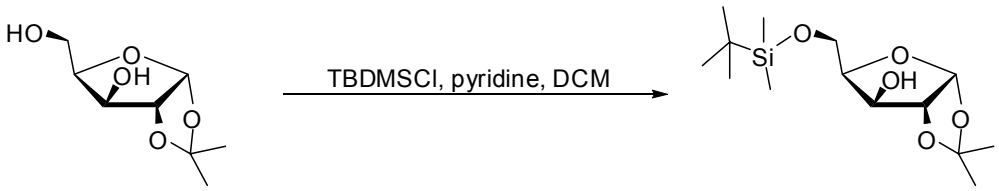
Elemental Analysis

$C_8H_{14}O_5$ (190.20): Calcd: C 50.52 H 7.42 O 42.06

Found: C 50.63 H 7.38 O 41.99

9.2 Building Block Synthesis from Xylose

9.2.1 5'-*O*-*t*-butyldimethylsilyl-1',2'-*O*-*iso*propylidene-α-D-xylofuranose (63)



62 **63**

30 g (0.16 mol, 1 eq) 1',2'-*O*-*iso*propylidene-α-D-xylofuranose **62** were dissolved in dry dichloromethane (1.2 L), added dropwise with 25.4 ml (0.31 mol, 2 eq) pyridine at RT under argon. 30.90 g (0.20 mol, 1.3 eq) *t*-butyldimethylsilyl chloride (TBDMSCl) were added portionwise after cooling to -78°C. After the addition was completed, the reaction was stirred at -78°C for 3 hrs and for 4 days at RT. Water (700 ml) was added and the aqueous phase was extracted with EtOAc (4 x 500 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 8:2, v/v) yielded 46.67g of **63** as a white foam (0.15 mol, 97%).

$C_{14}H_{28}O_5Si$: 304.46 g/mol

R_f (hexane:EtOAc, 1:1) = 0.88;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 5.78 (d, 1H, *J*= 3.8 Hz, $^{1'}$CH), 5.17 (d, 1H, *J*= 4.8 Hz, $^{3'}$OH), 4.35 (d, 1H, *J*= 3.56 Hz, $^{2'}$CH), 3.96 (m, 1H, $^{3'}$CH), 3.93 (dd, 1H, J_1= 4.8 Hz, J_2= 2.8 Hz, $^{4'}$CH), 3.79 (dd, 1H, J_1= 10.2 Hz, J_2= 5.28 Hz, $^{5'}CH_2$), 3.64 (dd, 1H, J_1= 10.88 Hz, J_2= 6.32 Hz, $^{5'}CH_2$), 1.35 (s, 3H, CH_3), 1.20 (s, 3H, CH_3), 0.84 (s, 9H, $CH_{3\ TBDMS}$), 0.03 (s, 6H, $CH_{3\ TBDMS}$);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 111.2 (Cq), 105.2 ($^{1'}$CH), 85.8 ($^{2'}$CH), 81.9 ($^{4'}$CH), 74.1 ($^{3'}$CH), 61.8 ($^{5'}CH_2$), 27.5-26.6 (CH_3), 18.9 ($Cq_{\ TBDMS}$), -4.3 ($CH_{3\ TBDMS}$);

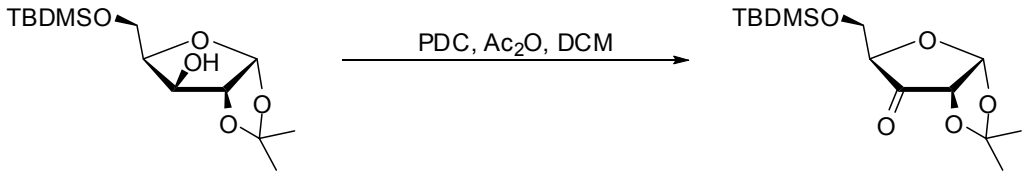
MS (FAB, m/z, %): 73 (100), 305 ($[M]^+$, 19), 343 ($[M+K]^+$, 11);

Elemental Analysis

$C_{14}H_{28}O_5Si$ (304.46): Calcd: C 55.23 H 9.27 O 26.27 Si 9.22

Found: C 55.14 H 9.36 O 26.28

9.2.2 5'-*O*-*t*-butyldimethylsilyl-1',2'-*O*-*iso*propylidene-3'-oxo-α-D-xylofuranose (64)



63 **64**

13.2 g (43.64 mmol 1 eq) 5'-*O*-*t*-butyldimethylsilyl-1',2'-*O*-*iso*propylidene-α-D-xylofuranose **63** were dissolved in dry dichloromethane (200 ml), added with 13.3 ml (0.130 mol, 3 eq) acetic anhydride and cooled to 0°C under argon. 9.79 g (26.18 mmol, 0.6 eq) PDC were added portion wise and after the addition was completed, the reaction mixture was refluxed for 2 hrs. After cooling to RT, the reaction mixture was concentrated under reduced pressure. The residue was taken up in ETOAc (200 ml), filtered over a short pad of silica gel and washed with ethylacetate. The filtrate was concentrated under reduced pressure to yield 11.88g of **64** as a colorless oil (39.27 mmol, 91%).

$C_{14}H_{26}O_5Si$: 302.45 g/mol

R_f (hexane:EtOAc, 7:3) = 0.82;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 6.06 (d, 1H, *J*= 4.56 Hz, $^{1'}$CH), 4.48 (d, 1H, *J*= 1.28 Hz, $^{2'}$CH), 4.35 (dd, 1H, J_1= 4.56 Hz, J_2= 1.28 Hz, $^{4'}$CH), 3.75 (dq, 2H, J_1= 9.12 Hz, J_2= 2.56 Hz, $^{5'}CH_2$), 1.33 (s, 6H, CH_3), 0.80 (s, 9H, $CH_{3\ TBDMS}$), 0.01 (s, 6H, $CH_{3\ TBDMS}$);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 212.0 (Cq), 113.8 (Cq), 103.8 ($^{1'}$CH), 81.5 ($^{2'}$CH), 77.2 ($^{4'}$CH), 64.2 ($^{5'}CH_2$), 28.1-26.5 (CH_3 , 18.7 ($Cq_{\ TBDMS}$), -4.6 ($CH_{3\ TBDMS}$);

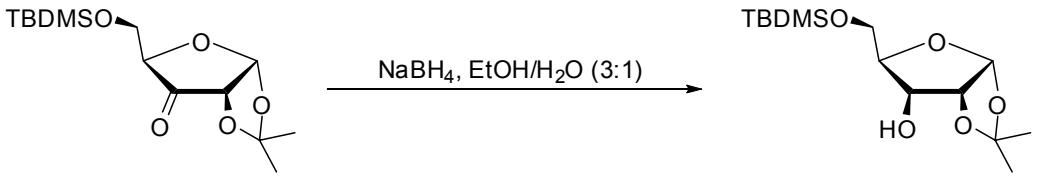
MS (FAB, m/z, %): 73 (100), 307 ($[M+H]^+$, 7);

Elemental Analysis

$C_{14}H_{26}O_5Si$ (302.45): Calcd: C 55.60 H 8.67 O 26.45 Si 9.29

Found: C 55.36 H 8.56 O 26.79

9.2.3 5'-*O*-*t*-butyldimethylsilyl-1',2'-*O*-*iso*propylidene-α-D-ribofuranose (65)



64 **65**

33.7 g (0.11 mol) 5'-*O*-*t*-butyldimethylsilyl-1',2'-*O*-*iso*propylidene-3'-oxo-α-D-xylofuranose **64** were dissolved in 500 ml ethanol:water (3:1) and cooled to 0°C. 27.4 g (0.72 mol, 6.5 eq) sodium borohydride were added portionwise to the solution. After the addition was completed, the reaction mixture was stirred at RT for 1 hr. The reaction was quenched with water (400 ml) and the mixture extracted with EtOAc (4 x 600 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 8:2, v/v) yielded 30.86 g of **65** as a colorless oil (0.10 mol, 91%).

$C_{14}H_{28}O_5Si$: 304.46 g/mol

R_f (hexane:EtOAc, 7:3) = 0.75;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 5.61 (d, 1H, *J*= 3.8 Hz, $^{1'}$CH), 5.01 (d, 1H, *J*= 6.56 Hz, $^{3'}$OH), 4.42 (t, 1H, *J*= 4.04 Hz, $^{2'}$CH), 3.80 (dd, 1H, J_1= 11.84 Hz, J_2= 1.24 Hz, $^{5'}CH_2$), 3.68 (m, 2H, $^{3'}$CH, $^{4'}$CH), 3.58 (dd, 1H, J_1= 11.64 Hz, J_2= 4.32 Hz, $^{5'}CH_2$), 1.41 (s, 3H, CH_3), 1.23 (s, 3H, CH_3), 0.84 (t, 9H, $CH_{3\ TBDMS}$), 0.01 (d, 6H, $CH_{3\ TBDMS}$);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 112.0 (Cq), 104.2 ($^{1'}$CH), 80.8 ($^{2'}$CH), 79.8 ($^{4'}$CH), 71.0 ($^{3'}$CH), 62.8 ($^{5'}CH_2$), 27.4-26.6 (CH_3), 18.9 ($Cq_{\ TBDMS}$), -4.3 ($CH_{3\ TBDMS}$);

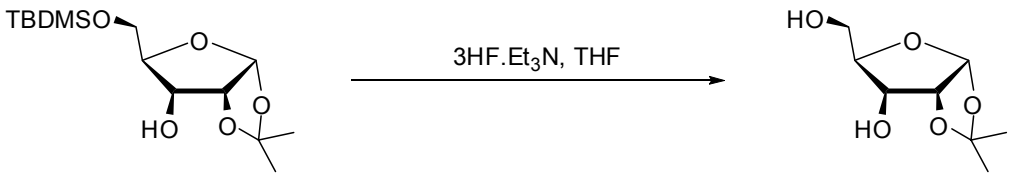
MS (FAB, m/z, %): 73 (100), 307 ($[M+H]^+$, 11), 343 ($[M+K]^+$, 17);

Elemental Analysis

$C_{14}H_{28}O_5Si$ (304.46): Calcd: C 55.23 H 9.27 O 26.27 Si 9.22

Found: C 55.23 H 9.13 O 26.42

9.2.4 1',2'-*O*-*iso*propylidene-α-D-ribofuranose (66)



65 **66**

5.68 g (18.65 mmol, 1 eq) 5'-*O*-*t*-butyldimethylsilyl-1',2'-*O*-*iso*propylidene-α-D-ribofuranose **65** were dissolved dry THF (150 ml) at RT under argon. 26.7 ml (0.18 mol, 10 eq) trihydrofluoro triethylamine were added dropwise. After the addition was completed, the solution was stirred at RT for 16 hrs. The reaction mixture was quenched with silica gel and the solvent concentrated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 1:9, v/v) yielded 2.91 g of **66** as a white foam (15.37 mmol, 93%).

$C_8H_{14}O_5$: 190.20 g/mol

R_f (hexane:EtOAc, 3:7) = 0.18;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 5.62 (d, 1H, *J*= 3.8 Hz, $^{1'}$CH), 4.97 (d, 1H *J*= 6.6 Hz, $^{3'}$OH,), 4.63 (t, 1H, *J*= 5.28 Hz, $^{5'}$OH), 4.41 (t, 1H, *J*= 2.84 Hz, $^{2'}$CH), 3.61 (m, 3H, $^{3'}$CH, $^{4'}$CH, $^{5'}CH_2$), 3.35 (m, 1H, $^{5'}CH_2$), 1.41 (s, 3H, CH_3), 1.23 (s, 3H, CH_3);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 111.9 (Cq), 104.1 ($^{1'}$CH), 81.1 ($^{2'}$CH), 79.9 ($^{4'}$CH), 71.3 ($^{3'}$CH), 61.0 ($^{5'}CH_2$), 27.4-27.2 (CH_3);

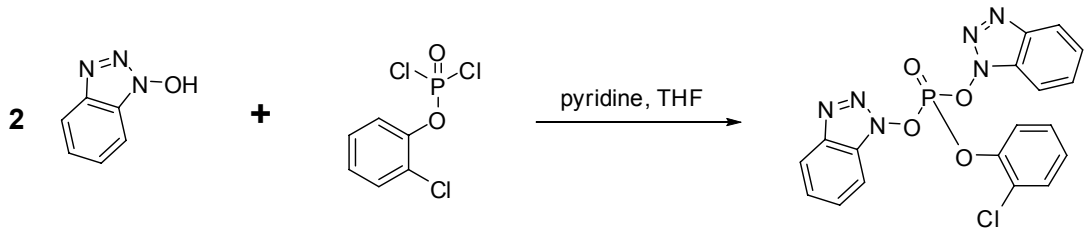
MS (FAB, m/z, %): 39 (100), 192 ($[M+H]^+$, 42), 229 ($[M+K]^+$, 58)

Elemental Analysis

$C_8H_{14}O_5$ (190.20):	Calcd: C 50.52 H 7.42 O 42.06
	Found: C 50.63 H 7.38 O 41.99

9.3 Assembling the Sugar Backbone

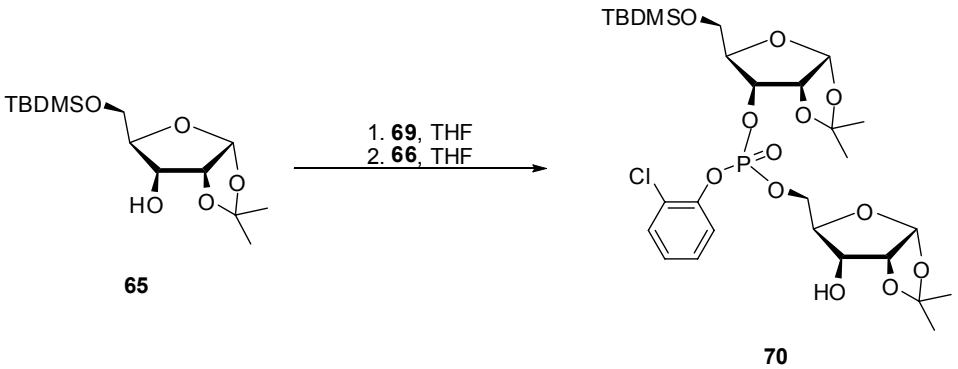
9.3.1 HOBt phosphorylating agent (69)



67 **68** **69**

16.2 g (0.12 mol, 1 eq) hydroxybenzotriazole (HOBt) **67** were suspended in 50 ml dry THF at RT under argon and added with 10 ml (60.10 mol, 0.5 eq) 2-chlorophenylphosphoro dichloridate, 10 ml (0.12 mol, 1.05 eq) pyridine and 12 ml dry THF. The reaction mixture was stirred at RT overnight then filtered under inert atmosphere to yield 50 ml of a 1 M stock solution of phosphorylating agent **69**.

9.3.2 (5'-*O*-t-butyldimethylsilyl-1',2'-bis-*O*-*iso*propylidene-D-ribo furanosyl)-(3'-5')-(1',2'-bis-*O*-*iso*propylidene-D-ribofuranosyl)-2-chlorophenyl phosphate (70)



65 **70**

2.28 g (7.49 mmol, 1 eq) 5'-*O*-*t*-butyldimethylsilyl-1',2'-*O*-*iso*propylidene-α-D-ribofuranose **65** were dissolved in 65 ml dry THF over activated powdered 4Å MS and added with 8 ml (8.98 mmol, 1.2 eq) of a 1 M HOBt phosphorylating agent **69** sol. at RT under argon. After 10 mins, 1.86 g (9.78 mmol, 1.3 eq) 1',2'-*O*-*iso*propylidene-α-D-ribofuranose **66** in dry THF (20 ml)

over activated powdered 4Å MS were added and the mixture stirred for 1 hr. The reaction mixture was filtered over celite and washed with 0.1 M TEAC (2 x 110 ml). The aqueous phase was extracted with DCM (3 x 140 ml). The combined organic layers were dried and concentrated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 1:1, v/v) lead to 3.19 g (4.78 mmol, 64%) of a colorless foam, the desired product **70** as a mixture of diastereomers.

$C_{24}H_{44}ClO_{12}PSi$: 667.17 g/mol
R_f (hexane:EtOAc, 4:6) = 0.75;

Diastereomer 1 of **70**:

^{1}H NMR (500.0 MHz, DMSO, δ ppm): 7.58 (d, 1H, *J*= 7.93 Hz, CH_{arom}), 7.44 (d, 1H, *J*= 11.73 Hz, CH_{arom}), 7.38 (m, 1H, CH_{arom}), 7.26 (m, 1H, CH_{arom}), 5.75 (d, 1H, *J*= 0.28 Hz, $^{1'}$CHa), 5.67 (d, 1H, *J*= 3.57 Hz, $^{1'}$CHb), 5.32 (d, 1H, *J*= 6.76 Hz, $^{3'}$OHb), 4.72 (t, 1H, *J*= 4.34 Hz, $^{2'}$CHa), 4.63 (m, 1H, $^{3'}$CHa), 4.47 (m, 1H, $^{2'}$CHb), 4.43 (m, 1H, $^{5'}CH_2$b), 4.16 (sx, 1H, *J*= 5.76 Hz, $^{5'}CH_2$b), 4.01 (m, 1H, $^{4'}$CHa), 3.95 (m, 1H, $^{4'}$CHb), 3.83 (m, 1H, $^{5'}CH_2$a), 3.75 (m, 1H, $^{3'}$CHb), 3.71 (m, 1H, $^{5'}CH_2$a), 1.43 (s, 6H, CH_3a, CH_3 b), 1.26 (s, 6H, CH_3 a, CH_3 b), 0.82 (s, 9H, $CH_{3\ TBDMS}$), 0.015 (s, 3H, $CH_{3\ TBDMS}$), 0.008 (s, 3H, $CH_{3\ TBDMS}$);

^{13}C NMR (125.8 MHz, DMSO, δ ppm): 145.9 (Cq_{arom}), 130.5 (CH_{arom}), 128.4 (CH_{arom}), 126.60 (CH_{arom}), 124.3 (CH_{arom}), 121.3 (Cq_{arom}), 112.2 (Cq), 111.6 (Cq), 103.5 ($^{1'}$CHa), 103.4 ($^{1'}$CHb), 78.9 ($^{2'}$CHb), 78.2 ($^{4'}$CHa), 77.5 ($^{4'}$CHb), 77.2 ($^{2'}$CHa), 74.2 ($^{3'}$CHa), 70.4 ($^{3'}$CHb), 67.6 ($^{5'}CH_2$b), 60.6 ($^{5'}CH_2$a), 26.6 (CH_3a), 26.6 (CH_3b), 26.4 (CH_3a), 26.3 (CH_3b), 25.7 ($CH_{3\ TBDMS}$), 17.9 (Cq), -5.3 ($CH_{3\ TBDMS}$), -5.4 ($CH_{3\ TBDMS}$);

Diastereomer 2 of **70**:

^{1}H NMR (500.0 MHz, DMSO, δ ppm): 7.58 (d, 1H, *J*= 7.93 Hz, CH_{arom}), 7.44 (d, 1H, *J*= 11.73 Hz, CH_{arom}), 7.38 (m, 1H, CH_{arom}), 7.26 (m, 1H, CH_{arom}), 5.75 (d, 1H, *J*= 0.28 Hz, $^{1'}$CHa), 5.62 (d, 1H, *J*= 3.56 Hz, $^{1'}$CHb), 5.32 (d, 1H, *J*= 6.79 Hz, $^{3'}$OHb), 4.76 (t, 1H, *J*= 4.25 Hz, $^{2'}$CHa), 4.59 (m, 1H, $^{3'}$CHa), 4.46 (m, 1H, $^{2'}$CHb), 4.43 (m, 1H, $^{5'}CH_2$b), 4.16 (sx, 1H, J= 5.76 Hz, $^{5'}CH_2$b), 4.01 (m, 1H, $^{4'}$CHa), 3.95 (m, 1H, $^{4'}$CHb), 3.79 (m, 1H, $^{5'}CH_2$a), 3.74 (m, 1H, $^{3'}$CHb), 3.60 (m, 1H, $^{5'}CH_2$a), 1.46 (s, 6H, CH_3a, CH_3b), 1.29 (s, 6H, CH_3a, CH_3b), 0.81 (s, 9H, $CH_{3\ TBDMS}$), -0.011 (s, 3H, $CH_{3\ TBDMS}$), -0.015 (s, 3H, $CH_{3\ TBDMS}$);

^{13}C NMR (125.8 MHz, DMSO, δ ppm): 145.9 (Cq_{arom}), 130.6 (CH_{arom}), 128.5 (CH_{arom}), 126.67 (CH_{arom}), 124.4 (CH_{arom}), 121.3 (Cq_{arom}), 112.4 (Cq), 111.6 (Cq), 103.6 ($^{1'}$CHa), 103.6 ($^{1'}$CHb), 78.9 ($^{2'}$CHb), 78.3 ($^{4'}$CHa), 77.6 ($^{4'}$CHb), 77.2 ($^{2'}$CHa), 74.3 ($^{3'}$CHa), 70.5 ($^{3'}$CHb), 68.0 ($^{5'}CH_2$b), 60.7 ($^{5'}CH_2$a), 26.6 (CH_3a, CH_3b), 26.4 (CH_3a), 26.4 (CH_3b), 25.7 ($CH_{3\ TBDMS}$), 17.9 (Cq), -5.4 ($CH_{3\ TBDMS}$), -5.4 ($CH_{3\ TBDMS}$);

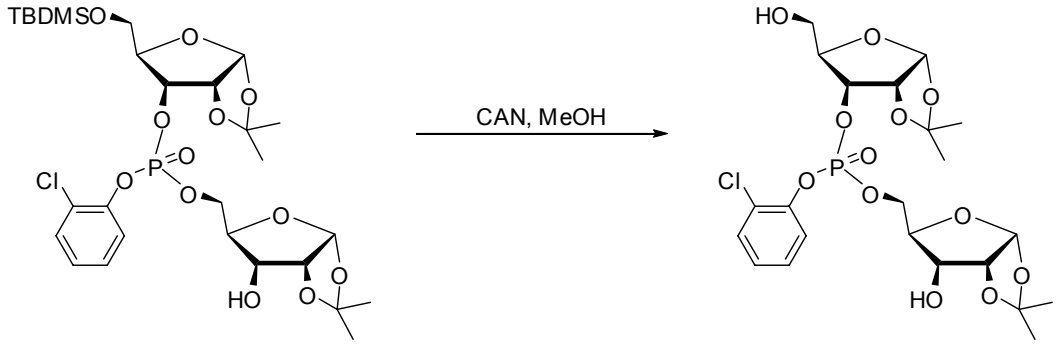
MS (FAB, m/z, %): 154 (100), 705 ($[M+K]^+$, 12);

Elemental Analysis

$C_{24}H_{44}ClO_{12}PSi$ (667.17): Calcd: C 50.41 H 6.65 O 28.78 Si 4.21

Found: C 50.45 H 6.62 O 28.77

9.3.3 (1',2'-bis-*O*-*iso*propylidene-D-ribofuranosyl)-(3'-5')-(1',2'-bis-*O*-*iso*propylidene-D-ribofuranosyl)-2chloro-phenyl phosphate (71)



70 **71**

0.74 g (1.11 mmol, 1 eq) protected dimer **70** were dissolved in 30 ml dry methanol, added with 0.67 g (1.22 mmol, 1.1 eq) cerium ammonium nitrate (CAN) and stirred for 16 hrs at RT under N_2. The reaction mixture was diluted with EtOAc (90 ml) and washed with TEAC (0.1 M, 2 x 100 ml). The aqueous phase was extracted with DCM (3 x 100 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc, gradient from 1:0 to 1:9, v/v) lead to 0.47 g (0.85 mmol, 77%) of a white foam of dimer **71** as a non-separable mixture of diastereomers.

$C_{22}H_{30}Cl_2O_{12}P$: 552.90 g/mol

R_f (CH_2Cl_2:MeOH, 95:5) = 0.23;

Diastereoisomer 1 of **71**:

^{1}H NMR (500.0 MHz, DMSO, δ ppm): 7.59 (m, 4H, H_{arom}), 5.77 (d, 1H, *J*= 3.65 Hz, $^{1'}$CHa), 5.68 (d, 1H, *J*= 3.54 Hz, $^{1'}$CHb), 5.32 (dd, 1H, J_1= 4.21 Hz, J_2= 2.58 Hz, $^{3'}$OHb), 4.89 (m, 1H, $^{5'}$OHa), 4.71 (m, 1H, $^{2'}$CHa), 4.61 (m, 1H, $^{3'}$CHa), 4.48 (bt, 1H, $^{2'}$CHb), 4.44 (m, 1H, $^{5'}CH_2$b), 4.18 (m, 1H, $^{5'}CH_2$b), 3.99 (m, 1H, $^{4'}$CHa), 3.96 (m, 1H, $^{4'}$CHb), 3.79 (m, 1H, $^{3'}$CHb), 3.69 (m, 1H, $^{5'}CH_2$a), 3.47 (m, 1H, $^{5'}CH_2$a), 1.44 (s, 6H, CH_3a, CH_3b), 1.27 (s, 6H, CH_3a, CH_3b);

^{13}C NMR (125.8 MHz, DMSO, δ ppm): 146.0 (Cq_{arom}), 130.6 (CH_{arom}), 128.6 (CH_{arom}), 126.6 (CH_{arom}), 121.4 (Cq_{arom}), 112.2 (Cq), 111.6 (Cq), 103.6 ($^{1'}$CHa), 103.4 ($^{1'}$CHb), 78.9 ($^{2'}$CHb), 78.6 ($^{4'}$CHa), 77.6 ($^{4'}$CHb), 77.3 ($^{2'}$CHa), 74.5 ($^{3'}$CHa), 70.3 ($^{3'}$CHb), 67.9 ($^{5'}CH_2$b), 59.1 ($^{5'}CH_2$a), 26.6 (CH_3a, CH_3b), 26.4 (CH_3a, CH_3b);

Diastereoisomer 2 of **71**:

^{1}H NMR (500.0 MHz, DMSO, δ ppm): 7.59 (m, 4H, H_{arom}), 5.77 (d, 1H, *J*= 3.65 Hz, $^{1'}$CHa), 5.68 (d, 1H, *J*= 3.54 Hz, $^{1'}$CHb), 5.32 (dd, 1H, J_1= 4.21 Hz, J_2= 2.58 Hz, $^{3'}$OHb), 4.89 (m, 1H, $^{5'}$OHa), 4.71 (m, 1H, $^{2'}$CHa), 4.61 (m, 1H, $^{3'}$CHa), 4.48 (bt, 1H, $^{2'}$CHb), 4.44 (m, 1H, $^{5'}CH_2$b), 4.18 (m, 1H, $^{5'}CH_2$b), 3.99 (m, 1H, $^{4'}$CHa), 3.96 (m, 1H, $^{4'}$CHb), 3.79 (m, 1H, $^{3'}$CHb), 3.69 (m, 1H, $^{5'}CH_2$a), 3.47 (m, 1H, $^{5'}CH_2$a), 1.44 (s, 6H, CH_3a, CH_3b), 1.27 (s, 6H, CH_3a, CH_3b);

^{13}C NMR (125.8 MHz, DMSO, δ ppm): 146.0 (Cq_{arom}), 130.6 (CH_{arom}), 128.6 (CH_{arom}), 126.6 (CH_{arom}), 121.4 (Cq_{arom}), 112.2 (Cq), 111.6 (Cq), 103.6 ($^{1'}$CHa), 103.4 ($^{1'}$CHb), 78.9 ($^{2'}$CHb), 78.6 ($^{4'}$CHa), 77.6 ($^{4'}$CHb), 77.3 ($^{2'}$CHa), 74.5 ($^{3'}$CHa), 70.3 ($^{3'}$CHb), 67.9 ($^{5'}CH_2$b), 59.1 ($^{5'}CH_2$a), 26.6 (CH_3a, CH_3b), 26.4 (CH_3a, CH_3b);

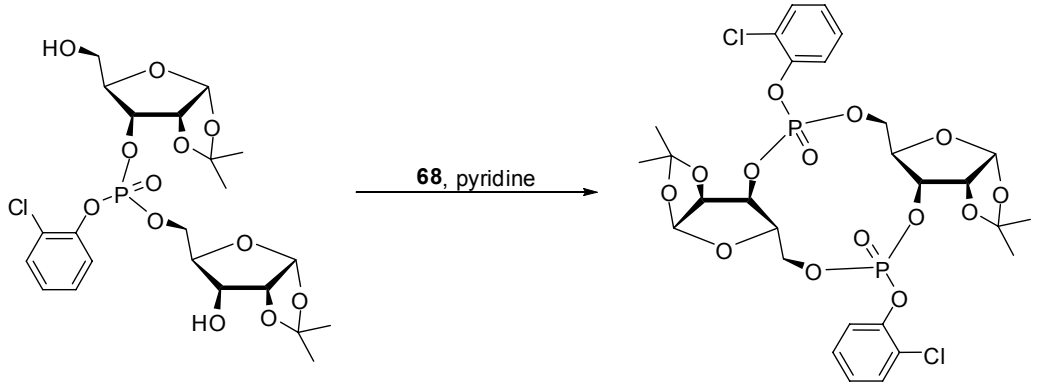
MS (FAB, m/z, %): 97 (100), 591 ($[M+K]^+$, 26);

Elemental Analysis

$C_{22}H_{30}Cl_2O_{12}P$ (552.90): Calcd: C 47.79 H 5.47 O 34.72

Found: C 47.62 H 5.67 O 34.70

9.3.4 Cyclic bis(3'-5')-(1',2'-bis-*O*-*iso*propylidene-D-ribo-furanosyl)-2-chloro phenyl phosphate (72)



71 **72**

1.80 g (3.26 mmol, 1 eq) dimer **71** were co-evaporated with pyridine (2 x 10 ml), dissolved in pyridine (900 ml), added with 0.79 ml (4.89 mmol, 1.5 eq) 2-chlorophenyl phosphoro dichloridate and the reaction mixture was stirred at RT under N_2 for 1 hr. The solvent was evaporated, the residue taken up in CH_2Cl_2 (200 ml) and washed with 0.25 M TEAC buffer. The organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. Flash silica gel chromatography (hexane, gradient from hexane 1:0 to hexane:EtOAc, 1:1, v/v) yielded two fractions containing the four possible stereoisomers of **72**. 683 mg (0.94 mmol, 29 %) of high R_f stereoisomer and 826 mg (1.13 mmol, 35 %) of low R_f stereoisomer were isolated as colorless oils.

$C_{28}H_{32}Cl_2O_{14}P_2$: 725.41 g/mol

R_f (hexane:EtOAc, 3:7) = 0.71.

High R_f stereoisomer of **72**:

^{1}H NMR (500 MHz, DMSO, δ ppm): 7.73 (m, 8H, CH_{arom}), 5.88 (d, 1H, *J*= 3.51 Hz, $^{1'}$CHa), 5.75 (d, 1H, *J*= 3.61 Hz, $^{1'}$CHb), 4.93-4.87 (m, 2H, $^{3'}$CHa, $^{2'}$CHb), 4.85-4.81 (m, 1H, $^{3'}$CHb), 4.74 (t, 1H, *J*= 4.17 Hz, $^{2'}$CHb), 4.52-4.45 (m, 2H, $^{5'}$CHa, $^{5'}$CHb), 4.34-4.24 (m, 4H, $^{5'}$CHa, $^{5'}$CHb, $^{4'}$CHa, $^{4'}$CHb), 1.52 (s, 3H, CH_3), 1.43 (s, 3H, CH_3), 1.32 (s, 3H, CH_3), 1.27 (s, 3H, CH_3);

^{13}C NMR (125.8 MHz DMSO, δ ppm): 145.7(Cq_{arom}), 145.6 (Cq_{arom}), 130.8(CH_{arom}), 130.6(CH_{arom}), 128.8(CH_{arom}), 128.5(CH_{arom}), 127.0(CH_{arom}), 126.8(CH_{arom}), 121.4, 121.0 (CH_{arom}), 112.8(Cq), 112.6 (Cq), 103.6 ($^{1'}$CHa), 103.5 ($^{1'}$CHb), 77.4 ($^{2'}$CHa), 77.3 ($^{2'}$CHb), 74.4 ($^{4'}$CHa), 74.3 ($^{4'}$CHb), 74.1($^{3'}$CHa), 74.1 ($^{3'}$CHb), 65.2/65.1 ($^{5'}CH_2$a, $^{5'}CH_2$b), 26.6 (CH_3), 26.4(CH_3), 26.3 (CH_3);

^{31}P NMR (202 MHz, DMSO, δ ppm): -8.35, -8.52;

MS (ESI) m/z = 725.5 $[M+H]^+$;

Elemental Analysis

$C_{28}H_{32}Cl_2O_{14}P_2$ (725.41): Calcd: C 46.36 H 4.45 O 30.88
Found: C 46.27 H 4.49 O 30.95

R_f (hexane:EtOAc, 3:7) = 0.49.

Low R_f stereoisomer of **72**:

^{1}H NMR (500 MHz, DMSO, δ ppm): 7.60-7.28 (m, 8H, CH_{arom}), 5.85 (d, 2H, *J*= 3.36 Hz, $^{1'}$CHa, $^{1'}$CHb), 4.82-4.78 (m, 4H, $^{2'}$CHa, $^{2'}$CHb, $^{3'}$CHa, $^{3'}$CHb), 4.46-4.45 (m, 4H, $^{5'}$CHa, $^{5'}$CHb), 4.25-4.22 (m, 2H, $^{4'}$CHa, $^{4'}$CHb), 1.42 (s, 6H, CH_3), 1.28 (s, 6H, CH_3);

^{13}C NMR (125.8 MHz, DMSO, δ ppm): 145.6 (Cq_{arom}), 130.6(CH_{arom}), 128.9(CH_{arom}), 128.4(CH_{arom}), 121.3 (CH_{arom}), 112.5(Cq), 112.8 (Cq), 103.7 ($^{1'}$CH), 77.3 ($^{2'}$CH), 74.6 ($^{4'}$CH), 73.5 ($^{3'}$CH), 65.7 ($^{5'}CH_2$), 26.5 (CH_3), 26.3 (CH_3);

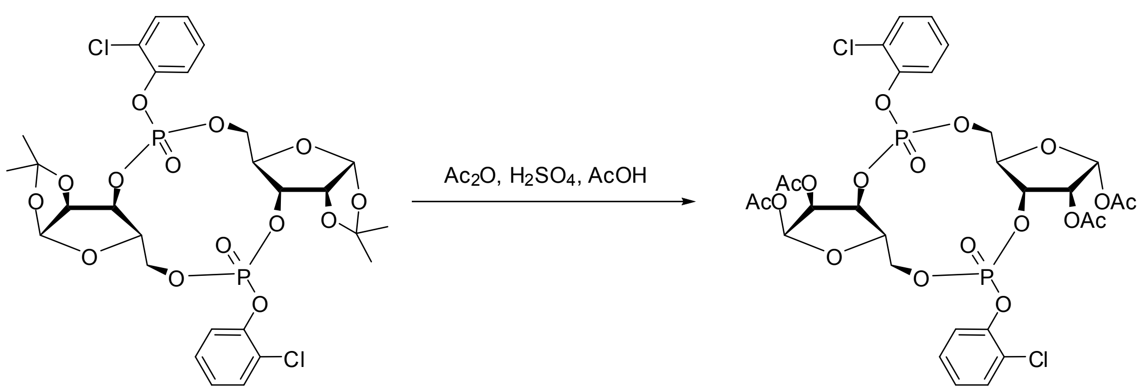
^{31}P NMR (202.5 MHz, DMSO, δ ppm): -8.08;

MS: m/z = 725.5 $[M+H]^+$;

Elemental Analysis

$C_{28}H_{32}Cl_2O_{14}P_2$ (725.41):	Calcd: C 46.36 H 4.45 O 30.88
	Found: C 46.27 H 4.49 O 30.95

9.3.5 Cyclic bis(3'-5')-(1',2'-*O*-diacetyl-D-ribo-furanosyl)-2-chlorophenyl phosphate (73)



72 **73**

670 mg (0.92 mmol, 1 eq) cyclic bis(3'-5') (1',2'-bis-*O*-isopropylidene-D-ribofuranosyl)-2-chlorophenyl phosphate **72** were dissolved in glacial acetic acid (10 ml). Acetic anhydride (0.1 ml) and sulfuric acid (0.4 ml) were added and the reaction mixture stirred for 18 hrs at RT under atmosphere of N_2. The reaction mixture was poured in ice water bath and the aqueous phase extracted with DCM (4 x 50 ml). The organic phase was washed with sat. aq. $NaHCO_3$ sol. (100 ml). The organic phase was dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was dissolved in dichloromethane, filtered through a short pad of silica gel, yileding 525 mg (0.64 mmol, 70%) of the acetylated product **73** were obtained.

$C_{30}H_{32}Cl_2O_{18}P_2$: 813.42 g/mol

R_f (hexane:EtOAc, 3:7) = 0.79;

High R_f stereoisomer of **73**:

^{1}H NMR (500 MHz, DMSO, δ ppm): 7.73 (m, 8H, CH_{arom}), 5.88 (d, 1H, *J*= 3.51 Hz, $^{1'}$CHa), 5.75 (d, 1H, *J*= 3.61 Hz, $^{1'}$CHb), 4.93-4.87 (m, 2H, $^{3'}$CHa, $^{2'}$CHb), 4.85-4.81 (m, 1H, $^{3'}$CHb), 4.74 (t, 1H, *J*= 4.17 Hz, $^{2'}$CHb), 4.52-4.45 (m, 2H, $^{5'}$CHa, $^{5'}$CHb), 4.34-4.24 (m, 4H, $^{5'}$CHa, $^{5'}$CHb, $^{4'}$CHa, $^{4'}$CHb), 2.10 (s, 3H, CH_3a), 2.06 (s, 3H, CH_3b);

^{13}C NMR (125.8 MHz DMSO, δ ppm): 145.7(Cq_{arom}), 145.6 (Cq_{arom}), 130.8(CH_{arom}), 130.6(CH_{arom}), 128.8(CH_{arom}), 128.5(CH_{arom}), 127.0(CH_{arom}), 126.8(CH_{arom}), 121.4, 121.0 (CH_{arom}), 112.8(Cq), 112.6 (Cq), 103.6 ($^{1'}$CHa), 103.5 ($^{1'}$CHb), 77.4 ($^{2'}$CHa), 77.3 ($^{2'}$CHb), 74.4 ($^{4'}$CHa), 74.3 ($^{4'}$CHb), 74.1($^{3'}$CHa), 74.1 ($^{3'}$CHb), 65.2/65.1 ($^{5'}CH_2$a, $^{5'}CH_2$b), 21.8 (CH_3), 21.4 (CH_3);

^{31}P NMR (202 MHz, DMSO, δ ppm): -8.34, -8.51;

MS (ESI) m/z = 812.4 $[M-H]^+$;

R_f (hexane:EtOAc, 3:7) = 0.70.

Low R_f stereoisomer of **73**:

^{1}H NMR (500 MHz, DMSO, δ ppm): 7.60-7.28 (m, 8H, CH_{arom}), 5.85 (d, 2H, *J*= 3.36 Hz, $^{1'}$CHa, $^{1'}$CHb), 4.82-4.78 (m, 4H, $^{2'}$CHa, $^{2'}$CHb, $^{3'}$CHa, $^{3'}$CHb), 4.46-4.45 (m, 4H, $^{5'}$CHa, $^{5'}$CHb), 4.25-4.22 (m, 2H, $^{4'}$CHa, $^{4'}$CHb), 2.10 (s, 3H, CH_3a), 2.06 (s, 3H, CH_3b);

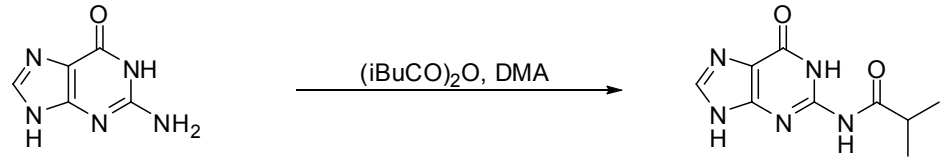
^{13}C NMR (125.8 MHz, DMSO, δ ppm): 145.6 (Cq_{arom}), 130.6(CH_{arom}), 128.9(CH_{arom}), 128.4(CH_{arom}), 121.3 (CH_{arom}), 112.5(Cq), 112.8 (Cq), 103.7 ($^{1'}$CH), 77.3 ($^{2'}$CH), 74.6 ($^{4'}$CH), 73.5 ($^{3'}$CH), 65.7 ($^{5'}CH_2$), 21.8 (CH_3), 21.4 (CH_3);

^{31}P NMR (202.5 MHz, DMSO, δ ppm): -8.09;

MS (ESI): m/z = 812.4 $[M-H]^+$;

9.4 Synthesis of the Protected Base

9.4.1 2-*N*-*iso*butyrylguanine (75)



74 **75**

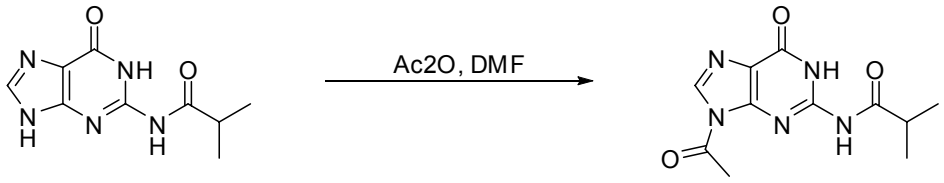
10 g (66.20 mmol, 1 eq) guanine **74** were suspended in *N*,*N*-dimethylacetamide (DMA) (140 ml) at RT under argon and added with 29.6 ml (0.18 mol, 2.7 eq) *iso*butyric anhydride. The mixture was refluxed at 150°C for 2 hrs. The clear solution was cooled to RT and the solvent evaporated to 1/10 of its volume. The precipitated crude product was filtered and re-crystallized from 1.5 L boiling ethanol:water (1:1) to give 13.31 g (56.51 mmol, 85%) of **75** as a white solid.

$C_5H_5N_5O$: 151.05 g/mol

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 8.01 (s, 1H, ^{8}CH), 2.72 (t, 1H, *J*= 6.8 Hz, CH), 1.09 (d, 6H, *J*= 6.8 Hz, CH_3);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 180.8 (C=O), 148.1 (Cq), 35.6 (CH), 19.7 (CH_3).

9.4.2 9-*N*-acetyl-2-*N*-*iso*butyrylguanine (76)



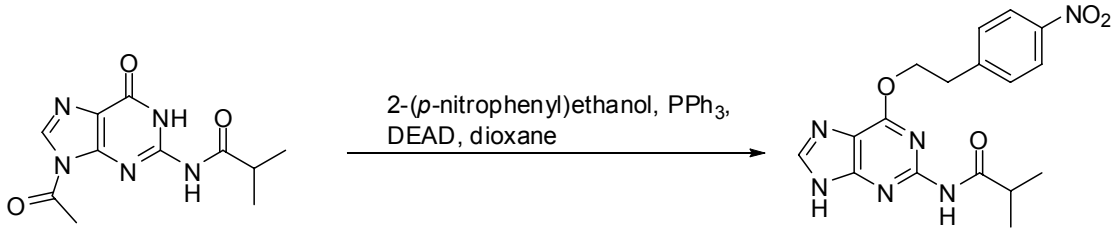
75 **76**

13.31 g (56.51 mmol, 1 eq) 2-*N*-*iso*butyrylguanine **75** were suspended in dry DMF (75 ml) at RT under argon and added with 15.3 ml (0.15 mol, 2.65 eq) acetic anhydride. The mixture was refluxed at 100°C for 1 hr. The solution was cooled to RT and the solvent evaporated under reduced pressure. The residue was re-crystallized from ethanol (30 ml) to give 13.41 g (50.9 mol, 85%) of **76** as a white solid.

$C_{11}H_{13}N_5O_3$: 263.25 g/mol

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 8.44 (s, 1H, ^{8}CH), 2.80 (s, 3H, $CH_{3\ acetyl}$), 2.79 (m, 1H, $CH_{isobutyryl}$), 1.12 (d, 6H, *J*= 6.8 Hz, $CH_{3\ isobutyryl}$);
^{13}C NMR (100.6 MHz, DMSO, δ ppm): 181.3 (C=O), 168.9 (^{6}Cq), 155.5 (^{5}Cq), 149.5 (^{2}Cq), 148.1 (^{8}Cq), 138.4 (Cq), 122.4 (Cq), 35.6 ($CH_{isobutyryl}$), 25.6 ($CH_{3\ acetyl}$), 19.7 ($CH_{3\ isobutyryl}$);

9.4.3 2-*N*-isobutyryl-4-*O*-[2-(*p*-nitrophenyl)ethyl]guanine (77)



76 **77**

16.56 g (62.3 mmol) 9-*O*-acetyl-2-*N*-*iso*butyrylguanine **76** were suspended in dioxane (625 ml), added with 24.5 g triphenylphosphine (93.4 mmol, 1.5 eq) and 15.1 g 2-(*p*-nitrophenyl)-ethanol (93.42 mmol, 1.5 eq). 24.2 ml DEAD (0.14 mol, 2.2 eq) were added dropwise and the reaction mixture stirred at RT for 16 hrs. The volatiles were removed under reduced pressure

and the residue re-crystallized in a 1:1 mixture of EtOH/H_2O (9 L). The residual triphenylphosphinoxide was washed away with DCM (100 ml) and the crude product re-crystallized again in a 1:1 mixture of EtOH/H_2O (9 L) to yield 7.8 g of **77** as a white solid (21.06 mmol, 33%).

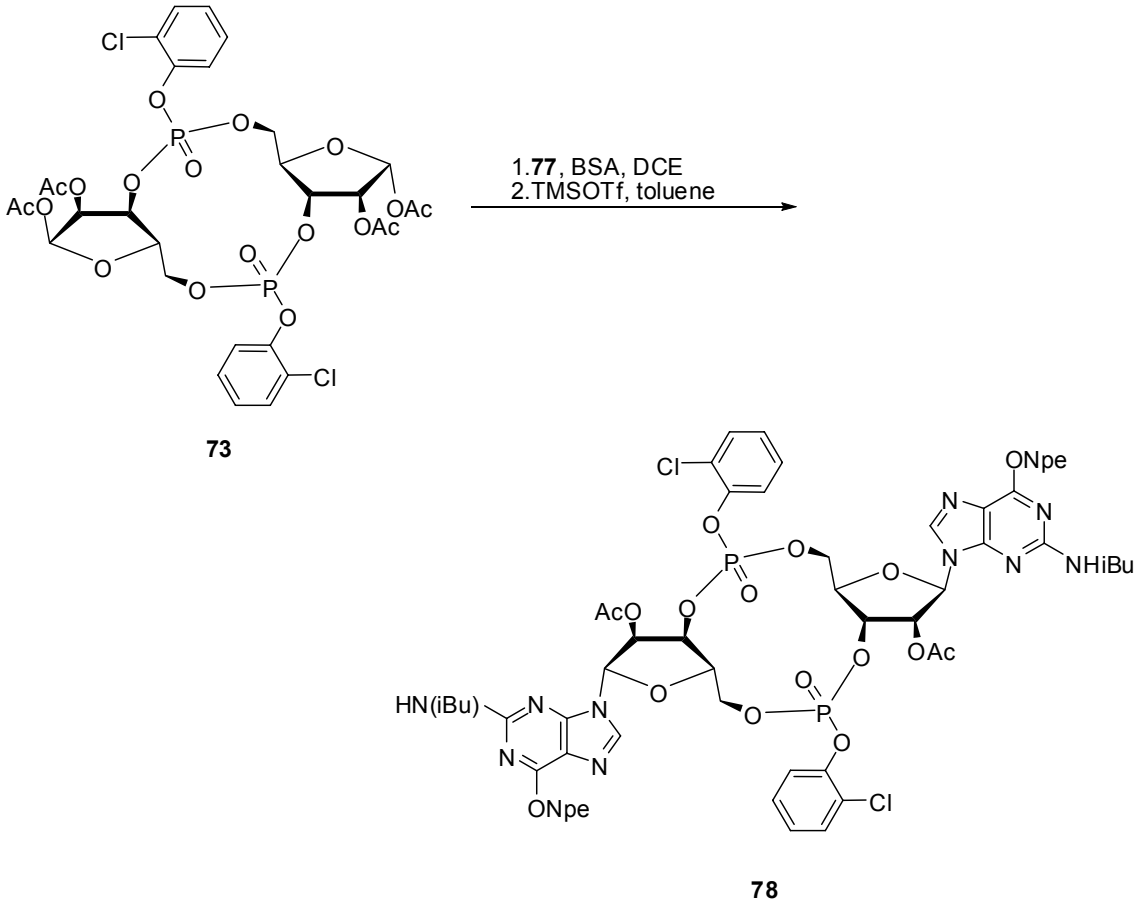
$C_{17}H_{18}N_6O_4$: 370.86 g/mol

^{1}H NMR (400 MHz, DMSO, δ ppm): 13.18 (s, 1H, ^{9}NH), 10.27 (s, 1H, ^{2}NH), 8.19 (s, 1H, ^{8}CH), 8.18 (d, 2H, *J*= 8.9 Hz, CH_{arom}), 7.66 (d, 2H, *J*= 8.7 Hz, CH_{arom}), 4.77 (t, 2H, *J*= 6.8 Hz, $CH_{2\ Npe}$), 3.32 (t, 2H, *J*= 6.8 Hz, $OCH_{2\ Npe}$), 2.85 (sept, 1H, *J*= 6.8 Hz, CH), 1.09 (s, 3H, CH_3), 1.08 (s, 3H, CH_3);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 175.8 (C=O), 152.7 (^{6}Cq), 147.5 (^{5}Cq), 147.1 (^{2}Cq), 131.2 (CH_{arom}), 124.3 (CH_{arom}), 67.1 ($OCH_{2\ Npe}$), 35.2 ($CH_{2\ Npe}$), 35.1 (CH), 20.2 (CH_3).

9.5 Base introduction and Deprotection of c-di-GMP

9.5.1 Cyclic bis(3'-5')-(2'-*O*-acetyl-2-*N*-isobutyryl-4-*O*-p-nitrophenyl ethyl-guanosine)-2-chlorophenyl phosphate (78)



73

78

820 mg (2.21 mmol, 3 eq) 2-*N*-isobutyryl-4-*O*-[2-(*p*-nitrophenyl)ethyl]guanine **77** were suspended in dichloroethane (DCE) (20 ml). 1.08 ml (4.42 mmol, 6 eq) *N*,*O*-bis(trimethylsilyl)acetamide (BSA) were added and the reaction mixture was heated to 80°C for 16 hrs in a sealed flask. The excess of BSA and DCE were removed by evaporation under reduced pressure. The residue was dissolved in toluene (20 ml). 0.70 ml trimethylsilyl triflate (TMSOTf) (3.69 mmol, 5 eq) and 600 mg cyclic sugar **73** (0.73 mmol, 1 eq), dissolved in toluene (10 ml), were added and stirred for 30 min at 80°C in a sealed flask. The reaction mixture was diluted with EtOAc (60 ml) and the solution washed with 0.25 M TEAC buffer (100 ml). The aqueous layer was extracted with EtOAc (2 x 100 ml). The combined organic phases were dried ($MgSO_4$), filtered and the solvent was evaporated under reduced pressure. Flash chromatography (CH_2Cl_2, CH_2Cl_2:MeOH gradient from 100:0 to 95:5, v/v)

followed by a final purification step, using size exclusion chromatography with Sephadex LH20 and a 1:1 mixture of nanopure water: CH_2Cl_2 as eluent, yielded 779 mg of **78** as a white oily residue (0.54 mmol, 66 %).

$C_{60}H_{60}Cl_2N_{12}O_{22}P_2$: 1434.07 g/mol

R_f (CH_2Cl_2:MeOH, 9:1) = 0.53;

^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 8.78 (s, H, ^{2}NHa), 8.70 (s, H, ^{2}NHb), 8.14 (m, 4H, CH_{arom}), 7.85 (s, 2H, ^{8}CHa), 7.76 (s, 2H, ^{8}CHb), 7.52-7.00 (m, 16H, CH_{arom}), 6.33 (m, 1H, $^{3'}$CHa), 6.19 (dd, 1H, J_1= 5.6 Hz, J_2= 4.6 Hz, $^{2'}$CHa), 6.15 (dd, 1H, J_1= 5.2 Hz, J_2= 4.0 Hz, $^{2'}$CHb), 6.02 (d, 1H, J= 4.4 Hz, $^{1'}$CHb), 5.99 (m, 1H, $^{3'}$CHb), 5.86 (d, 1H, J= 3.6 Hz, $^{1'}$CHa), 4.98 (ddd, 1H, J_1= 10.7 Hz, J_2= 7.8 Hz, J_3= 6.5 Hz, $^{5'}$CHb), 4.91-4.80 (m, 4H, O-$CH_{2\ Npe}$), 4.70 (m, 1H, $^{4'}$CHb), 4.65-4.49 (m, 3H, $^{5'}$CHa, $^{4'}$CHa, $^{5'}$CHa), 4.45 (ddd, 1H, J_1= 10.7 Hz, J_2= 5.0 Hz, J_3= 3.6 Hz, $^{5'}$CHb), 3.34-3.30 (m, 4H, $CH_{2\ Npe}$), 2.92 (bd, 2H, CH), 1.99 (s, 3H, $CH_{3\ acetyl}$), 1.94 (s, 3H, $CH_{3\ acetyl}$), 1.21-1.15 (m, 12H, $CH_{3\ isobutytryl}$);

^{13}C NMR (125.8 MHz, $CDCl_3$, δ ppm): 175.9 ($C{=}O_{isobutyryl}$), 169.4 ($C{=}O_{acetyl}$), 169.2 ($C{=}O_{acetyl}$), 160.8 (^{6}Cq), 152.4/152.3(^{2}Cq), 152.1/151.9 (^{4}C-O), 146.9-145.7 (Cq_{arom}), 141.1 (^{8}CH), 130.9-120.9 (CH_{arom}), 118.9/118.6 (^{5}Cq), 88.0/87.0 ($^{1'}$CH), 81.2/79.0 ($^{4'}$CH), 75.6/74.6 ($^{3'}$CH), 72.4/72.2 ($^{2'}$CH), 66.1/65.9 ($^{5'}CH_2$), 35.8/35.0 ($CH_{isobutyryl}$), 20.4/20.3 ($CH_{3\ isobutyryl}$), 19.4/19.3 ($CH_{3\ acetyl}$);

^{31}P NMR (202.5 MHz, $CDCl_3$, δ ppm): -5.8, -9.5;

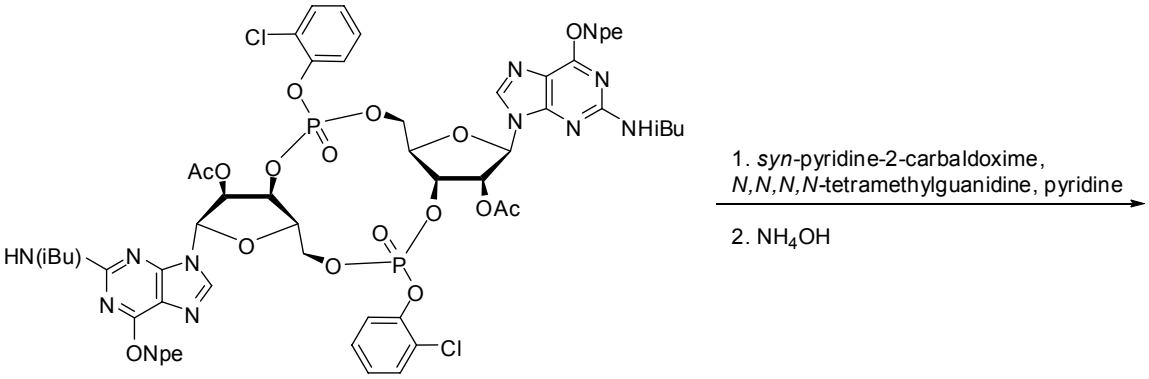
MS (Maldi-ToF, m/z): 1434.2 $[M]^+$;

Elemental Analysis

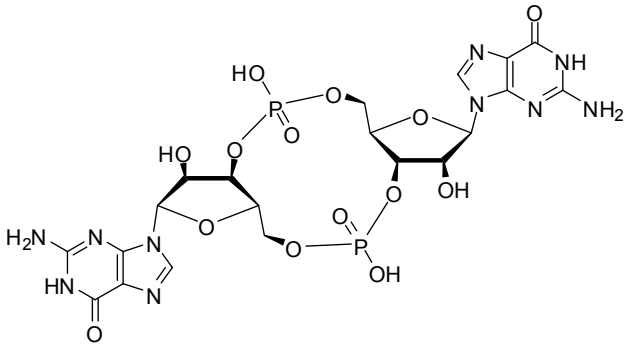
$C_{60}H_{60}Cl_2N_{12}O_{22}P_2$ (1434.07): Calcd: C 50.25 H 4.22 O 24.55.

Found: C 50.14 H 4.29 O 24.64.

9.5.2 c-di-GMP



78



c-di-GMP

430 mg (0.29 mmol, 1 eq) fully protected c-di-GMP **78** in pyridine (5 ml) were treated with 0.21 ml (1.40 mmol, 4.5 eq) 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) at 0°C. The reaction mixture was stirred at RT for 8 hrs and neutralized to pH=7 with glacial acetic acid. The solvents were evaporated under reduced pressure. The residue was dissolved in pyridine (5 ml), 1.5 g (11.99 mmol, 40 eq) *syn*-pyridine-2-carbaldoxime and 1.35 ml (10.49 mmol, 35 eq) *N*,*N*,*N*,*N*-tetramethylguanidine were added. The reaction mixture was stirred for 16 hrs at RT. 14 M ammonium hydroxide (100 ml) was added and the mixture stirred for 2 days at 50°C in a sealed flask. The solution was concentrated to 1/10 of its volume and washed with DCM (2 x 20 ml). Size exclusion chromatography with Sephadex G15 and nanopure water as eluent, followed by reverse phase HPLC chromatography (TEAC(0.01 M):MeOH; 92.5:7.5, isocratic) yielded 173 mg of pure c-di-GMP (84%, 99.99% pure).

$C_{20}H_{23}N_{10}O_{14}P_2$: 689.08 g/mol

^{1}H NMR (500 MHz, D_2O, δ ppm): 7.90 (s, 2H, ^{8}CH), 5.84 (d, 2H, *J*= 1.3 Hz, $^{1'}$CH), 4.74 (dd, 2H, J_1= 8.5 Hz, J_2= 5.0 Hz, $^{3'}$CH), 4.59 (dd, 2H, *J*= 5.0 Hz,$^{2'}$CH), 4.27 (dd, 2H, *J*= 8.5 Hz, $^{4'}$CH), 4.21 (m, 2H, $^{5'}CH_2$), 3.96 (m, 2H, $^{5'}CH_2$);

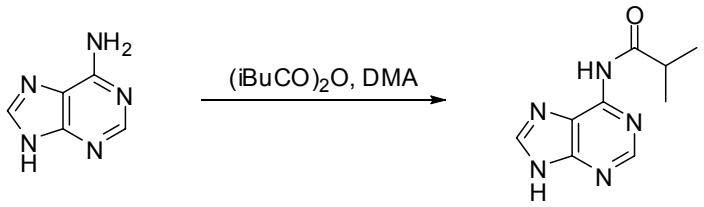
^{13}C NMR (125.8 MHz, D_2O, δ ppm): 158.8, 153.8 (^{4}C=O, ^{6}Cq), 150.8 (^{2}Cq), 137.1 (^{8}CH), 116.3 (^{5}C), 89.2 ($^{1'}$CH), 79.8 ($^{4'}$CH), 73.3 ($^{2'}$CH), 70.5 ($^{3'}$CH), 62.2 ($^{5'}CH_2$);

HRMS-ESI: m/z $[M-H]^-$ calcd for $C_{20}H_{23}N_{10}O_{14}P_2$: 689.0870; Found: 689.0869.

10. ANALOGUES

10.1 Synthesis of Base-Modified Analogues

10.1.1 2-*N*-*iso*butyryladenine (80)



79 **80**

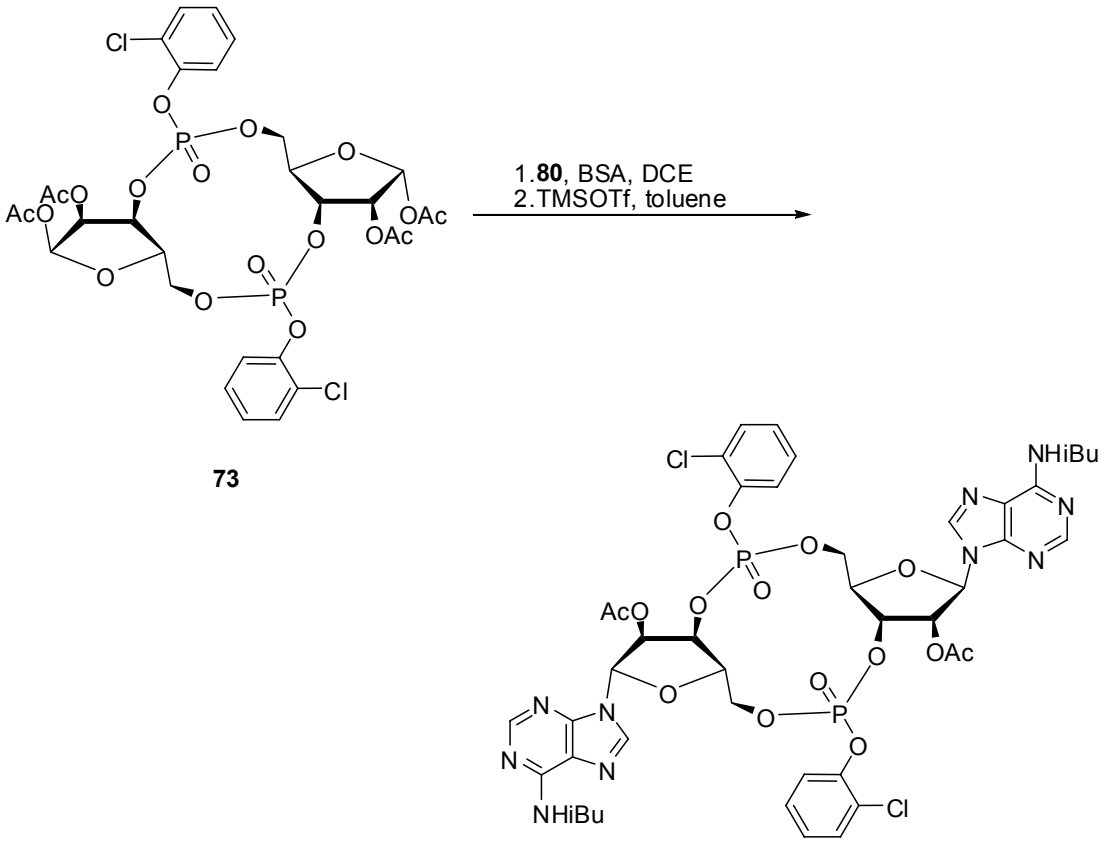
10 g (74.04 mmol, 1 eq) adenine **79** were suspended in DMA (140 ml) at RT under argon and added with 33.2 ml (0.20 mol, 2.7 eq) *iso*butyric anhydride. The mixture was refluxed at 150°C for 2 hrs. The reaction mixture was cooled to RT and the solvent concentrated to 1/10 of its volume. The precipitated crude product was filtered and re-crystallized from EtOH/H_2O (1:1) (1.5 L), to give 12.45 g (60.71 mmol, 82%) of **80** as a white solid.

$C_9H_{11}N_5O$: 205.22 g/mol

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 8.68 (s, 1H, ^{8}CH), 2.92 (t, 1H, *J*= 6.8 Hz, CH), 1.15 (d, 6H, *J*= 6.8 Hz, CH_3);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 175.5 (C=O), 150.5 (Cq), 39.2 (CH), 19.3 (CH_3).

10.1.2 Cyclic bis(3'-5')-(2'-O-acetyl-4-N-isobutyryl adenosine)-2-chloro phenyl phosphate (83)



73 **83**

28.3 mg (0.13 mmol, 2.0 eq) 2-*N-iso*butyryl-adenine **80** were suspended in DCE (3 ml), 63 µl (0.26 mmol, 4.2 eq) BSA were added and the reaction mixture was heated at 80°C for 16 hrs in a sealed flask. The excess of BSA and DCE were removed by evaporation under reduced pressure. The residue was dissolved in toluene (3 ml), 83 µl TMSOTf (0.46 mmol, 7.5 eq), 50 mg (0.06 mmol, 1 eq) cyclic sugar **73**, dissolved in toluene (1.5 ml), were added and the reaction mixture stirred for 30 min at 80°C in a sealed flask. The reaction mixture was diluted with EtOAc (10 ml) and washed with 0.25 M TEAC buffer (10 ml). The aqueous layer was extracted with EtOAc (2 x 10 ml). The combined organic phases were dried ($MgSO_4$), filtered and the solvent was evaporated under reduced pressure. Flash chromatography (CH_2Cl_2, CH_2Cl_2:MeOH gradient from 100:0 to 95:5, v/v) yielded 39.6 mg of **83** as a white residue (35.93 µmol, 59%).

$C_{44}H_{46}Cl_2N_{10}O_{16}P_2$: 1103.75 g/mol

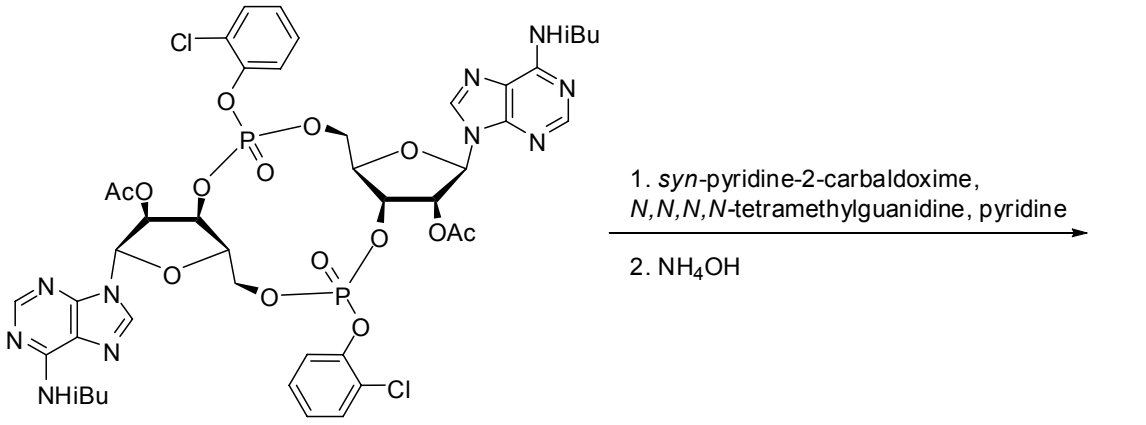
R_f (CH_2Cl_2:MeOH, 9:1) = 0.48;

^{1}H NMR (500 MHz, DMSO, δ ppm): 10.74, (s, 1H, ^{2}NHa), 10.70 (s, 1H, ^{2}NHb), 8.74 (s, 1H, ^{8}CHa), 8.68 (s, 1H, ^{2}CHa), 8.66 (s, 1H, ^{8}CHb), 8.60 (s, 1H, ^{2}CHb), 7.64-7.15 (m, 8H, CH_{arom}), 6.44 (d, 1H *J*= 6.44 Hz, $^{1'}$CHa,), 6.38 (m, 1H, $^{1'}$CHb), 6.36 (m, 1H, $^{2'}$CHa), 6.17 (d, 1H, *J*= 5.18 Hz, $^{2'}$CHb), 5.86 (m, 1H, $^{3'}$CHb), 5.74 (m, 1H, $^{3'}$CHa), 4.87 (m, 1H, $^{4'}$CHb), 4.80 (m, 1H, $^{4'}$CHa), 4.60 (m, 2H, $^{5'}CH_2$a, $^{5'}CH_2$b), 4.50 (m, 2H, $^{5'}CH_2$a, $^{5'}CH_2$b), 2.94 (q, 2H, *J*= 6.88 Hz, $CH_{isobutyryl}$), 1.95 (s, 3H, $CH_{3\ acetyl}$a),1.94 (s, 3H, $CH_{3\ acetyl}$b), 1.13 (2s, 12H, $CH_{3\ isobutyryl}$);

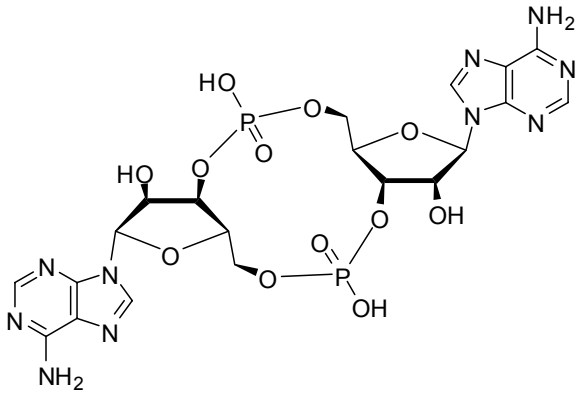
^{13}C NMR (125.8 MHz, DMSO, δ ppm): 175.3 ($C{=}O_{isobutyryl}$), 169.2/169.1 ($C{=}O_{acetyl}$), 151.9/151.8 (^{6}Cq), 151.6/151.3 (^{2}CHb), 150.2 (^{5}Cq), 145.6 (^{4}Cq), 145.5 (Cq_{arom}), 143.3/143.2 (^{8}CH), 130.8-126.8 (CH_{arom}), 124.3 (Cq_{arom}), 121.7-120.8 (CH_{arom}), 85.7 ($^{1'}$CHa), 85.1 ($^{1'}$CHb), 80.5 ($^{4'}$CH), 78.2 ($^{3'}$CH), 71.4 ($^{2'}$CHa), 70.8 ($^{2'}$CHb), 65.5 ($^{5'}CH_2$a), 65.4 ($^{5'}CH_2$b), 39.0 ($CH_{isobutyryl}$), 20.2 ($CH_{3\ acetyl}$), 19.3 ($CH_{3\ isobutyryl}$);

MS (Maldi-ToF, m/z): 1125.13 ($[M+Na]^+$).

10.1.3 Cyclic diadenylic acid (c-di-AMP) (84)



83



84

29 mg (22.68 µmol, 1 eq) fully protected c-di-AMP **83** were dissolved in pyridine (2 ml), 118 mg (0.91 mmol, 40 eq) *syn*-pyridine-2-carbaldoxime and 10 µl (0.79 mmol, 35 eq) *N*,*N*,*N*,*N*-tetramethyl guanidine were added, and the reaction mixture stirred for 16 hrs at RT. 14 M ammonium hydroxide (30 ml) was added and the mixture stirred for 2 days at 50°C in a sealed flask. The solution was concentrated to 1/10 of its volume and washed with CH_2Cl_2 (2 x 10 ml). Size exclusion chromatography with Sephadex G10 and a 1:1 mixture of MeOH-nanopure water as eluent, followed by reverse phase HPLC chromatography (TEAC(0.01 M):MeOH; 92.5:7.5, isocratic) yielded 14 mg of pure c-di-AMP **84** (21.3 µmol, 81%).

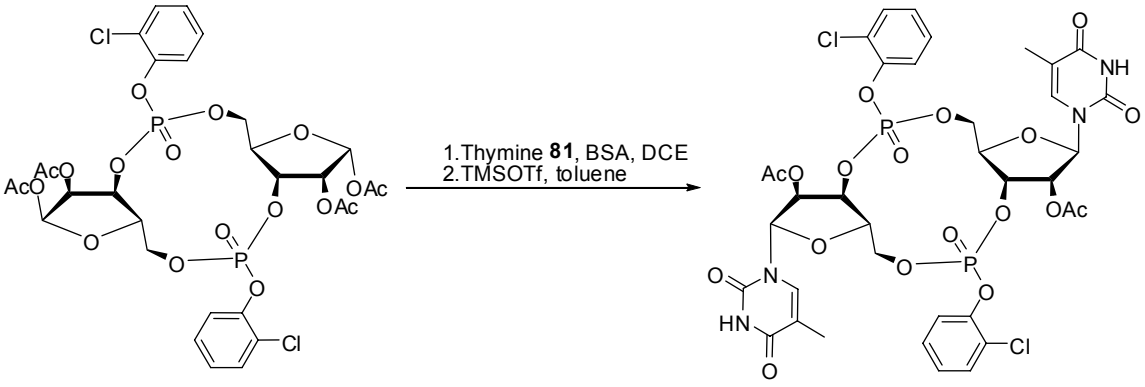
$C_{20}H_{23}N_{10}O_{12}P_2$: 657.09 g/mol

^{1}H NMR (500 MHz, DMSO, δ ppm): 8.45 (s, 2H, ^{8}CH), 8.14 (s, 2H, ^{2}CH), 7.32 (s, 4H, NH_2), 5.88 (d, 2H, *J*= 4.4 Hz, $^{1'}$CH), 4.93 (m, 2H, $^{2'}$CH), 4.68 (t, 2H, *J*= 5.3 Hz, $^{3'}$CH), 4.22 (dd, 2H, J_1= 9.7 Hz, J_2= 5.3 Hz, $^{4'}$CH), 4.00-3.98 (m, 4H, $^{5'}CH_2$);

^{13}C NMR (125.8 MHz, DMSO, δ ppm): 156.1 (^{4}C=O), 152.7 (^{6}Cq), 149.6 (^{5}Cq), 139.7 (^{8}CH), 119.2 (^{2}CH), 87.0 ($^{1'}$CH), 81.1 ($^{2'}$CH), 73.5 ($^{3'}$CH), 71.3 ($^{4'}$C), 63.9 ($^{5'}$CH$_2$);

HRMS-ESI: m/z [M-H]$^-$ calcd for $C_{20}H_{23}N_{10}O_{12}P_2$: 657.0972; Found 657.0967.

10.1.4 Cyclic bis(3'-5')-(2'-*O*-acetyl-thymidine)-2-chlorophenyl phosphate (85)



73 **85**

22.8 mg (0.18 mmol, 2.1 eq) thymine **81** were suspended in DCE (3 ml), 88 µl (0.36 mmol, 4.2 eq) BSA were added and the reaction mixture heated at 80°C for 16 hrs in a sealed flask. The excess of BSA and DCE were removed by evaporation under reduced pressure. The resulting residue was dissolved in toluene (3 ml), 117 µl (0.65 mmol, 7.5 eq) TMSOTf, 70 mg cyclic sugar **73** (0.086 mmol, 1 eq), dissolved in toluene (1.5 ml), were added and the reaction mixture stirred for 30 min at 80°C in a sealed flask. The reaction mixture was diluted with EtOAc (10 ml) and washed with 0.25 M TEAC buffer (10 ml). The aqueous layer was extracted with EtOAc (2 x 10 ml). The combined organic phases were dried over $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. Flash chromatography (CH_2Cl_2, CH_2Cl_2:MeOH gradient from 100:0 to 95:5, v/v) lead to 41.4 mg of **85** as a white oily residue (22.67 µmol, 51%).

$C_{36}H_{36}Cl_2N_4O_{18}P_2$: 945.54 g/mol

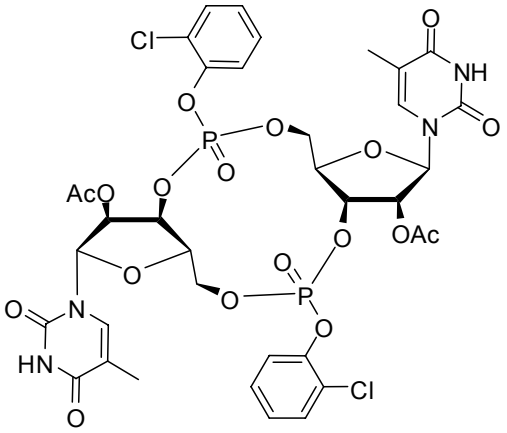
R_f (CH_2Cl_2:MeOH, 9:1) = 0.41;

^{1}H NMR (500.0 MHz, DMSO, δ ppm): 7.73 (m, 2H, ^{6}CH), 7.63 (m, 2H, CH_{arom}), 7.46 (m, 2H, CH_{arom}), 7.43 (m, 2H, CH_{arom}), 7.31 (m, 2H, CH_{arom}), 5.92 (d, 2H, *J*= 5.92 Hz, $^{1'}$CH), 5.63 (t, 2H, *J*= 5.87 Hz, $^{2'}$CH), 5.36 (m, 2H, $^{3'}$CHa), 4.70 (m, 2H, $^{5'}$CH$_2$), 4.38 (m, 4H, $^{4'}$CH, $^{5'}$CH$_2$), 1.89 (s, 6H, CH_3), 1.76 (s, 6H, $CH_{3\ acetyl}$);

^{13}C NMR (125.8 MHz, DMSO, δ ppm): 169.1 ($C{=}O_{acetyl}$), 163.6 ($^{4}C{=}O$), 150.4 ($^{2}C{=}O$), 145.6 (Cq_{arom}), 136.9 (^{6}CH), 130.8 (CH_{arom}), 128.8 (CH_{arom}), 127.2 (CH_{arom}), 124.4 (Cq_{arom}), 121.6 (CH_{arom}), 110.1 (^{5}Cq), 86.7 ($^{1'}CH$), 79.1 ($^{4'}CH$), 73.5 ($^{3'}CH$), 70.6 ($^{2'}CH$), 65.5 ($^{5'}CH_2$), 20.2 ($CH_{3\ acetyl}$), 12.0 (CH_3);

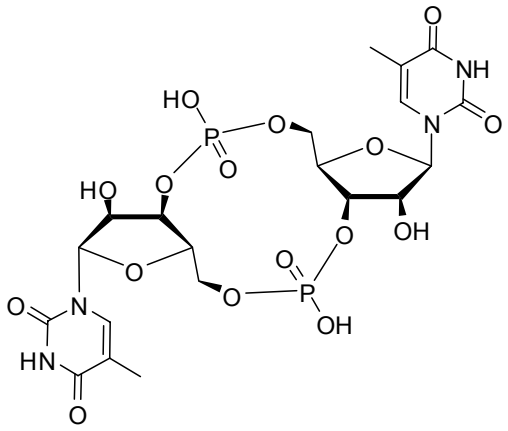
MS (Maldi-ToF, m/z): 967.76 ($[M+Na]^+$).

10.1.5 Cyclic dithymidic acid (c-di-TMP) (87)



85

1. *syn*-pyridine-2-carbaldoxime, *N,N,N,N*-tetramethylguanidine, pyridine
2. NH_4OH



87

29 mg (30.72 µmol, 1 eq) cyclic bis (3'-5') (2'-*O*-acetyl-thymidine)-2-chlorophenyl phosphate **81** were dissolved in pyridine (2 ml), 150 mg (1.22 mmol, 40 eq) *syn*-2-pyridinecarbaldoxime, 134 µl (1.07 mmol, 35 eq) *N,N,N,N*-tetramethyl guanidine were added and the reaction mixture stirred for 16 hrs at RT. The solvents were evaporated and the residue taken up in H_2O (10 ml) then washed with DCM (2 x 10 ml). The aqueous phase was then evaporated under reduced pressure. Size exclusion chromatography with Sephadex G10 and a mixture

of nanopure H_2O:MeOH (1:1) as eluent, followed by reverse phase HPLC chromatography (TEAC(0.01 M):MeOH; 92.5:7.5, isocratic) yielded 15.7 mg of pure c-di-TMP **87** (24.57 µmol, 80%, 99.99% pure).

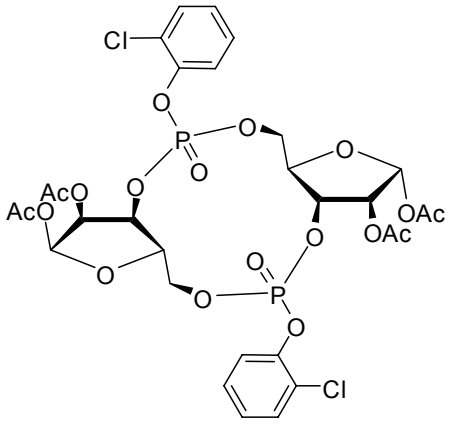
$C_{20}H_{25}N_4O_{16}P_2$: 639.07 g/mol

^{1}H NMR (500.0 MHz, DMSO, δ ppm): 11.32 (s, 2H, NH), 9.33 (s, 1H, OH), 8.38 (s, 1H, OH), 7.73 (s, 2H, CH_{alkene}), 5.75 (d, 2H, *J*= 5.08 Hz, $^{1'}$CH), 4.47 (m, 2H, $^{3'}$CH), 4.19 (t, 2H, *J*= 4.88 Hz, $^{2'}$CH), 4.07 (m, 2H, $^{4'}$CH), 3.95 (m, 2H, $^{5'}CH_2$), 3.87 (m, 2H, $^{5'}CH_2$), 1.77 (s, 6H, CH_3);

^{13}C NMR (125.8 MHz, DMSO, δ ppm): 163.6 (^{4}C=O), 150.4 (^{2}C=O), 135.6 (CH_{alkene}), 109.7 (^{5}Cq), 87.6 ($^{1'}$CH), 80.0 ($^{4'}$CH), 72.0 ($^{3'}$CH), 71.7 ($^{2'}$CH), 62.9 ($^{5'}CH_2$), 11.9 (CH_3);

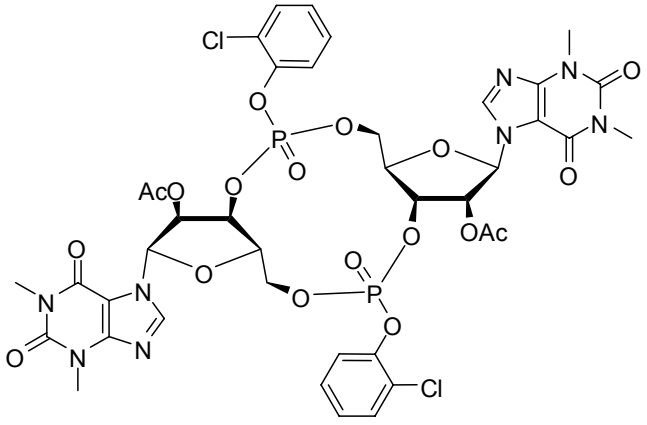
HRMS-ESI: m/z $[M-H]^-$ calcd for $C_{20}H_{25}N_4O_{16}P_2$: 639.0741; Found 639.0735.

10.1.6 Cyclic bis(3'-5')-(2'-*O*-acetyl-theophylline)-2-chloro-phenyl phosphate (86)



73

1.Theophylline **82**, BSA, DCE
2.TMSOTf, toluene



86

32.5 mg (0.18 mmol, 2.1 eq) theophylline **82** were suspended in DCE (3 ml), 88 µl (0.36 mmol, 4.2 eq) BSA were added and the reaction mixture heated at 80°C for 16 hrs in a sealed flask. The excess of BSA and DCE were removed by evaporation under reduced pressure. The residue was dissolved in toluene (3 ml), 117 µl TMSOTf (0.65 mmol, 7.5 eq), 70 mg cyclic sugar **73** (86.10 µmol, 1 eq), dissolved in toluene (1.5 ml), were added and stirred for 30 min at 80°C in a sealed flask. The reaction mixture was diluted with EtOAc (10 ml) and washed with 0.25 M TEAC buffer (2 x 10 ml). The aqueous layer was extracted with EtOAc (2 x 10 ml). The combined organic phases were dried ($MgSO_4$), filtered and the solvent was evaporated under reduced pressure. Flash chromatography (CH_2Cl_2, CH_2Cl_2:MeOH gradient from 100:0 to 95:5, v/v) lead to 42 mg of **86** a white solid (40.46 mmol, 47 %).

$C_{40}H_{40}Cl_2N_8O_{18}P_2$: 1053.64 g/mol

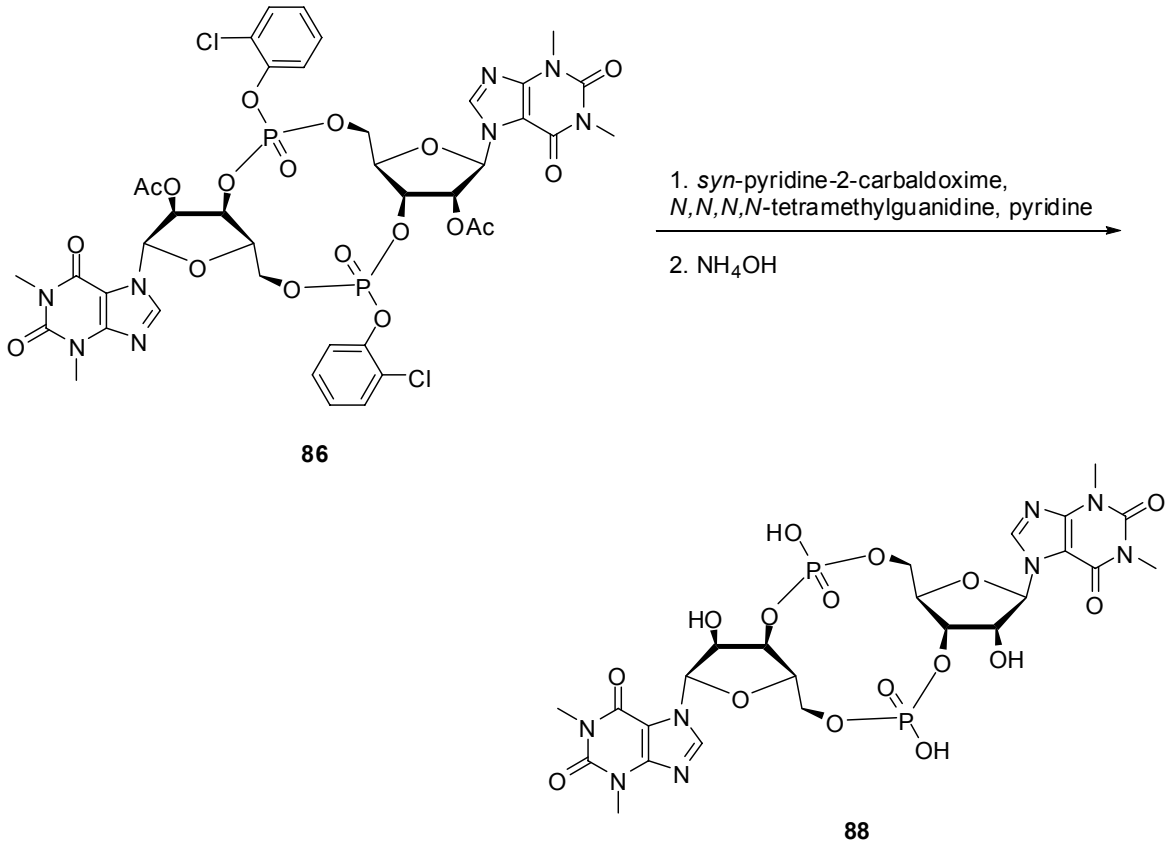
R_f (CH_2Cl_2:MeOH, 9:1) = 0.32;

^{1}H NMR (500 MHz, DMSO, δ ppm): 8.49 (s, 1H, ^{8}CHa), 8.44 (s, 1H, ^{8}CHb), 7.63 (md, 1H, *J*= 7.55 Hz, CH_{arom}), 7.56 (d, 1H, *J*= 7.89 Hz, CH_{arom}), 7.47-7.24 (m, 6H, CH_{arom}), 6.39 (d, 2H, *J*= 6.70 Hz, $^{1'}$CHa, $^{1'}$CHb), 6.08 (m, 1H, $^{2'}$CHb), 6.04 (t, 1H, *J*= 6.29 Hz, $^{2'}$CHa), 5.62 (m, 1H, $^{3'}$CHb), 5.58 (m, 1H, $^{3'}$CHa), 4.79 (m, 1H, $^{5'}CH_2$a), 4.76 (m, 1H, $^{5'}CH_2$b), 4.51 (m, 1H, $^{4'}$CHb), 4.50 (m, 1H, $^{4'}$CHa), 4.33 (m, 2H, $^{5'}CH_2$a, $^{5'}CH_2$b), 3.43 (s, 3H, $^4CH_3$a), 3.41 (s, 3H, $^4CH_3$b), 3.24 (s, 3H, $^2CH_3$a), 3.23 (s, 3H, $^2CH_3$b), 1.95 (s, 3H, $CH_{3\ acetyl}$),1.93 (s, 3H, $CH_{3\ acetyl}$);

^{13}C NMR (125.8 MHz, DMSO, δ ppm): 169.1 ($C{=}O_{acetyl}$), 168.8 ($C{=}O_{acetyl}$), 154.1/153.9 (^{3}C=O), 150.7/150.6 (^{1}C=O), 149.7/149.6 (^{5}Cq), 145.7/145.6 (Cq_{arom}), 143.6 (^{8}CHb), 142.9 (^{8}CHa), 130.8-126.7 (CH_{arom}), 124.6 (Cq_{arom}), 124.2 (Cq_{arom}), 121.7 (CH_{arom}), 120.7 (CH_{arom}), 105.6/105.5 (^{6}Cq), 86.8 ($^{1'}$CHb), 86.5 ($^{1'}$CHa), 80.5 ($^{4'}$CHa), 80.0 ($^{4'}$CHb), 75.4 ($^{3'}$CHb), 74.4 ($^{3'}$CHa), 71.7 ($^{2'}$CHa), 71.6 ($^{2'}$CHb), 65.5 ($^{5'}CH_2$a), 64.8 ($^{5'}CH_2$b), 29.7 ($^4CH_3$), 27.9 ($^2CH_3$), 20.2 ($CH_{3\ acetyl}$), 20.1 ($CH_{3\ acetyl}$);

MS (Maldi-ToF, m/z): 1075.17 ($[M+Na]^+$).

10.1.7 Cyclic bis(3'-5')-theophylline monophosphate (88)



86

88

22 mg (20.91 µmol, 1 eq) cyclic bis (3'-5') (2'-*O*-acetyl-theophyllosine)-2-chlorophenyl phosphate **86** were dissolved in pyridine (2 ml), 150 mg (1.22 mmol, 40 eq) *syn*-2-pyridine carbaldoxime, 134 µl (1.07 mmol, 35 eq,) *N*,*N*,*N*,*N*-tetramethyl guanidine were added and stirred for 16 hrs at RT. The solvents were evaporated under reduced pressure, the residue taken up in H_2O (10 ml) and washed with DCM (2 x 10 ml). The aqueous phase was evaporated under reduced pressure. Size exclusion chromatography with Sephadex G10 and a mixture of nanopure H_2O:MeOH (1:1) as eluent, followed by reverse phase HPLC chromatography (TEAC(0.01 M):MeOH; 92.5:7.5, isocratic) yielded 11.8 mg of pure c-di-(theophyllosine)MP **88** (15.79 µmol, 76%, 99.99% pure).

$C_{24}H_{29}N_8O_{16}P_2$: 747.11 g/mol

^{1}H NMR (500 MHz, DMSO, δ ppm): 8.51 (s, 1H, ^{8}CH), 7.17 (s, 1H, $^{2'}$OH),6.12 (d, 1H, *J*= 5.80 Hz, $^{1'}$CH), 4.57 (m, 2H, $^{2'}$CH, $^{3'}$CH), 4.18 (m, 1H, $^{4'}$CH), 3.91 (m, 2H, $^{5'}CH_2$), 3.43 (s, 3H, $^4CH_3$), 3.23 (s, 3H, $^2CH_3$);

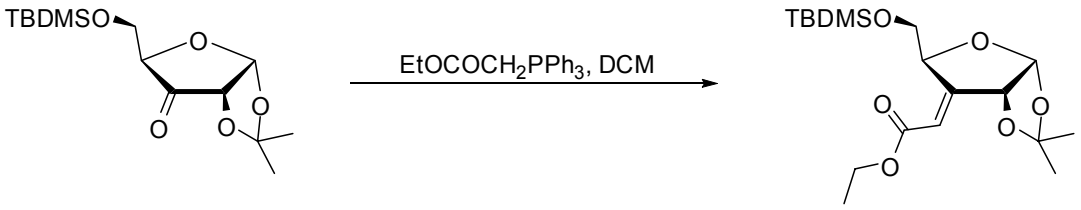
^{13}C NMR (125.8 MHz, DMSO, δ ppm): 154.1 (^{1}C=O), 150.9 (^{3}C=O), 148.9 (^{5}Cq), 141.0 (^{8}CH), 105.9 (^{6}Cq), 89.1 ($^{1'}$CH), 81.2 ($^{4'}$CH), 73.0 ($^{3'}$CH), 72.4 ($^{2'}$CH), 63.3 ($^{5'}CH_2$), 29.5 ($^{4}CH_3$), 27.7 ($^{2}CH_3$);

HRMS-ESI: m/z $[M-H]^-$ calcd for $C_{24}H_{29}N_8O_{16}P_2$: 747.1177; Found 747.1171.

10.2 Synthesis of an Internucleotide Bond Modified Analogue: an Amide Linked Dinucleotide

10.2.1 Synthesis of the Building Blocks

10.2.1.1 5'-*O*-*t*-butyldimethylsilyl-3'-deoxy-3'-[(ethoxycarbonyl) methylene]-1',2'-*O*-*iso*propylidene-α-D-*erythro*-pentofuranose (89)



64 **89**

2 g (6.61 mmol, 1 eq) 5'-*O*-*t*-butyldimethylsilyl-3'-oxo-1',2'-*O*-*iso*propylidene-α-D-*erythro*-pentofuranose **64** were dissolved in dry DCM (50 ml), added with 2.79 g (7.60 mmol, 1.15 eq) [(ethoxycarbonyl)methylene] triphenylphosphorane and stirred for 16 hrs at RT under N_2. After evaporation of the volatiles, flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 8:2; v/v) yielded 2.25 g (6.04 mmol, 91%) of **89** as a colorless oil as an *E/Z* mixture.

$C_{18}H_{32}O_6Si$: 372.53 g/mol

R_f (hexane:EtOAc, 7:3) = 0.82;

Major isomer:

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 6.05 (t, 1H, *J*= 1.76 Hz, CH_{alkene}), 5.84 (d, 1H, *J*= 4.28 Hz, $^{1'}CH$), 5.49 (m, 1H, $^{2'}CH$), 4.84 (m, 1H, $^{4'}CH$), 4.15 (m, 2H, $CH_{2\ EtO}$), 3.72 (m, 2H, $^{5'}CH_2$), 1.35 (s, 3H, CH_3), 1.29 (s, 3H, CH_3), 1.21 (t, 3H, *J*= 7.04 Hz, CH_3), 0.84 (s, 9H, $CH_{3\ TBDMS}$), 0.01 (t, 6H, *J*= 4.04 Hz, $CH_{3\ TBDMS}$);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 165.6 (C=O), 157.5 ($^{3'}CH$), 116.9 ($^{1'}CH$), 112.5 (CH_{alkene}), 105.8 (Cq), 82.5 ($^{2'}CH$), 79.2 ($^{4'}CH$), 66.1 ($^{5'}CH_2$), 61.0 ($CH_{2\ Et}$), 28.4-27.9 (CH_3), 18.6 (Cq_{TBDMS}), 14.8 ($CH_{3\ TBDMS}$), -4.7- -4.9 ($CH_{3\ TBDMS}$);

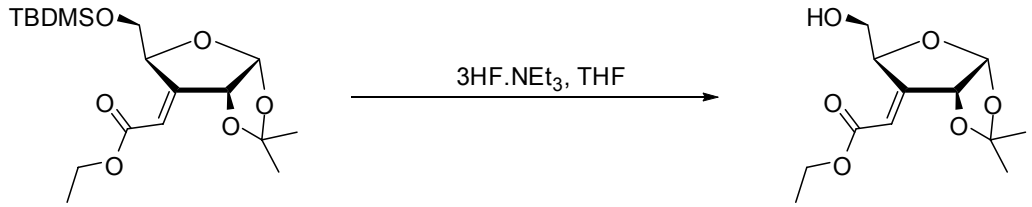
MS (Maldi-ToF, m/z): 395.4 $[M+Na]^+$.

Elemental Analysis

$C_{18}H_{32}O_6Si$ (372.53): Calcd: C 58.03 H 8.66 O 25.77 Si 7.54

Found: C 57.61 H 8.50 O 25.51

10.2.1.2 3'-deoxy-3'-[(ethoxycarbonyl)methylene]-1',2'-*O*-*iso*propylidene -α-D-*erythro*-pentofuranose (90)



89 **90**

500 mg (1.34 mmol, 1 eq) 5'-*O*-*t*-butyldimethylsilyl-3'-deoxy-3'-[(ethoxycarbonyl) methylene]-1',2'-*O*-*iso*propylidene-α-D-*erythro*-pentofuranose **89** were dissolved dry THF (15 ml) at RT under argon. 2.2 ml (13.4 mmol, 10 eq) trihydrofluoro triethylamine were added dropwise and, after the addition was completed, the solution stirred at RT for 16 hrs. The reaction mixture was quenched with silica gel and the solvent evaporated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 4:6, v/v) yielded 348 mg of **90** as a colorless oil (1.34 mmol, quant.).

$C_{12}H_{18}O_6$: 258.27 g/mol

R_f (hexane:EtOAc, 3:7) = 0.39;

Major isomer:

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 6.02 (t, 1H, *J*= 1.76 Hz, CH_{alkene}), 5.88 (d, 1H, *J*= 4.32 Hz, $^{1'}$CH), 5.53 (m, 1H, $^{2'}$CH), 4.92 (t, 1H, *J*= 5.84 Hz, $^{5'}$OH), 4.75 (m, 1H, $^{4'}$CH), 4.14 (m, 2H, $CH_{2\ EtO}$), 3.56 (m, 2H, $^{5'}CH_2$), 1.35 (s, 3H, CH_3), 1.30 (s, 3H, CH_3), 1.21 (t, 3H, *J*= 7.32 Hz, CH_3);

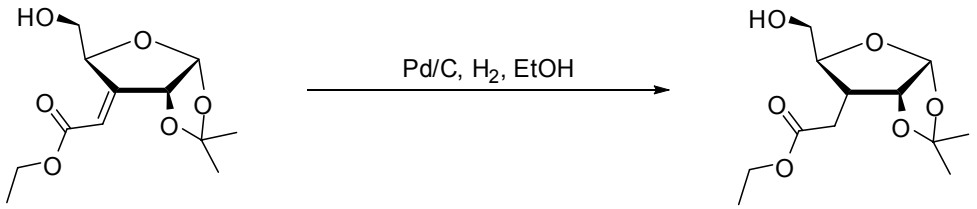
^{13}C NMR (100.6 MHz, DMSO, δ ppm): 165.2 (C=O), 157.6 ($^{3'}$CH), 116.6 ($^{1'}$CH), 112.3 (CH_{alkene}), 105.4 (Cq), 81.7 ($^{2'}$CH), 78.9 ($^{4'}$CH), 63.6 ($^{5'}CH_2$), 60.9 (CH_2), 28.1-27.9 (CH_3), 14.9 (CH_3);

MS (Maldi-ToF, m/z): 281.5 $[M+Na]^+$.

Elemental Analysis

$C_{12}H_{18}O_6$ (258.27):	Calcd: C 55.81 H 7.02 O 37.17
	Found: C 55.03 H 6.88 O 38.09

10.2.1.3 3'-deoxy-3'-[(ethoxycarbonyl)methyl]-1',2'-*O-iso*propylidene-α-D-ribofuranose (91)



90 **91**

348 mg (1.35 mmol, 1 eq) 3'-deoxy-3'-[(ethoxycarbonyl) methylene]-1',2'-*O-iso*propylidene-α-D-*erythro*-pentofuranose **90** were dissolved in ethanol (15 ml) at RT under argon, added with 35 mg (10% weight) palladium on activated charcoal and flushed with argon. The reaction mixture was shaken under H_2 pressure (3 bar) for 16 hrs. The suspension was filtered over celite, washed with ethanol and the volatiles evaporated under reduced pressure, to yield 299 mg of **91** as a colorless oil (1.15 mmol, 85%).

$C_{12}H_{20}O_6$: 260.29 g/mol

R_f (hexane:EtOAc, 7:3) = 0.03;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 5.76 (d, 1H, *J*= 3.8 Hz, $^{1'}$CH), 4.72 (m, 2H, $^{2'}$CH, $^{5'}$OH), 4.07 (m, 2H, CH_2), 3.67 (m, 1H, $^{4'}$CH), 3.56 (m, 1H, $^{5'}CH_2$), 3.44 (m, 1H, $^{5'}CH_2$), 2.46 (d, 2H, *J*= 7.32 Hz, $^{3'}CH_2$), 2.20 (m, 1H, $^{3'}$CH), 1.38 (s, 3H, CH_3), 1.23 (s, 3H, CH_3), 1.17 (t, 3H, *J*= 5.04 Hz, CH_3);

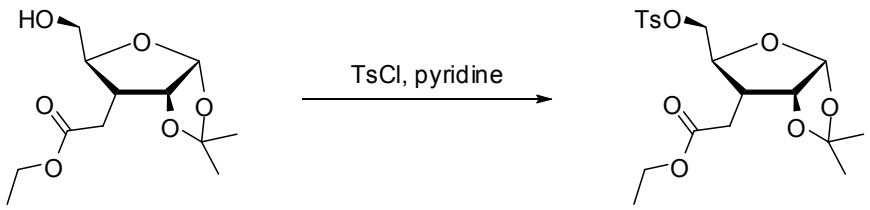
^{13}C NMR (100.6 MHz, DMSO, δ ppm): 171.5 (C=O), 110.4 (Cq), 104.3 ($^{1'}$CH), 80.9 ($^{2'}$CH), 80.6 ($^{4'}$CH), 60.5 ($^{5'}CH_2$), 59.9 (CH_2), 40.2 ($^{3'}$CH), 29.3-26.3 (CH_3), 14.0 (CH_3);

MS (Maldi-ToF, m/z): 283.4 $[M+Na]^+$.

Elemental Analysis

$C_{12}H_{20}O_6$ (260.29):	Calcd: C 55.37 H 7.74 O 36.88
	Found: C 54.14 H 7.53 O 38.33

10.2.1.4 3'-deoxy-3'-[(ethoxycarbonyl)methyl]-1',2'-*O*-*iso*propylidene-5'-*O*-*p*-toluenesulfonyl-α-D-ribofuranose (92)



91 **92**

4.02 g (15.44 mmol, 1 eq) 3'-deoxy-3'-[(ethoxycarbonyl)methyl]-1',2'-*O*-*iso*propylidene-α-D-*erythro*-pentofuranose **91** were dissolved in abs. pyridine (140 ml), added with 3.53 g (18.53 mmol, 1.2 eq) TsCl and stirred for 16 hrs at RT under N_2. The reaction mixture was diluted with water (100 ml) and extracted with EtOAc (3 x 200 ml). The combined organic phases were washed with 1 M HCl (300 ml) then dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 3:7, v/v) yielded 4.36 g of **92** as a colorless oil (10.12 mmol, 65 %).

$C_{19}H_{26}O_9S$: 430.48 g/mol

R_f (hexane:EtOAc, 7:3) = 0.54;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 7.78 (dd, 2H, J_1= 1.52 Hz, J_2= 6.6 Hz, CH_{arom}), 7.47 (d, 2H, *J*= 8.08 Hz, CH_{arom}), 5.71 (d, 1H, *J*= 3.52 Hz, $^{1'}$CH), 4.66 (t, 1H, *J*= 4.32 Hz, $^{2'}$CH), 4.25 (m, 1H, $^{5'}CH_2$), 4.05 (m, 3H, CH_2, $^{5'}CH_2$), 3.80 (m, 1H, $^{4'}$CH), 2.41 (m, 5H, $^{3'}CH_2$, CH_3), 2.15 (m, 1H, $^{3'}$CH), 1.34 (s, 3H, CH_3), 1.21 (s, 3H, CH_3), 1.17 (t, 3H, *J*= 7.08 Hz, CH_3);

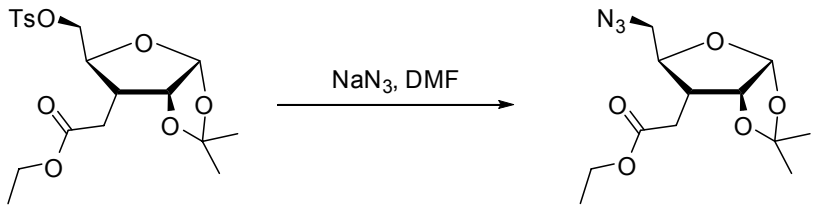
^{13}C NMR (100.6 MHz, DMSO, δ ppm): 172.0 (C=O), 145.90 (Cq), 133.0 (Cq), 131.0 (CH_{arom}), 128.5 (CH_{arom}), 111.7 (Cq), 105.2 ($^{1'}$CH), 81.1 ($^{2'}$CH), 78.4 ($^{4'}$CH), 70.22 ($^{5'}CH_2$), 60.9 ($^{3'}CH_{2\ EtO}$), 41.0 ($^{3'}$CH), 29.8 (CH_2), 27.3-26.9 (CH_3), 14.9 (CH_3);

MS (Maldi-ToF, m/z): 453.8 $[M+Na]^+$.

Elemental Analysis

$C_{19}H_{26}O_9S$ (430.48):	Calcd: C 53.01 H 6.09 O 33.45
	Found: C 54.85 H 6.30 O 38.85

10.2.1.5 5'-azido-3',5'-dideoxy-3'-[(ethoxycarbonyl)methyl]-1',2'-*O*-*iso*propylidene-α-D-ribofuranose (93)



92 **93**

4.36 g (10.12 mmol, 1 eq) 3'-deoxy-3'-[(ethoxycarbonyl)methyl]-1',2'-*O*-*iso*propylidene-5'-*O*-*p*-toluenesulfonyl-α-D-*erythro*-pentofuranose **92** were taken up in abs. DMF (250 ml), added with 3.29 g (50.60 mmol, 5 eq) NaN_3 and stirred for 16 hrs at 100°C under N_2. After cooling to RT, the reaction mixture was added with water (200 ml) and extracted with ethylacetate (4 x 250 ml). The combined organic phases were dried and the volatiles evaporated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 1:1, v/v) yielded 3.0 g o **93** as a colorless oil (10.10 mmol, quant.).

$C_{12}H_{19}N_3O_5$: 285.30 g/mol

R_f (hexane:EtOAc, 7:3) = 0.87;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 5.79 (d, 1H, *J*= 3.52 Hz, $^{1'}$CH), 4.71 (t, 1H, *J*= 4.28 Hz, $^{2'}$CH), 4.06 (m, 2H, CH_2), 3.86 (m, 1H, $^{4'}$CH), 3.60 (m, 1H, $^{5'}CH_2$), 3.30 (m, 1H, $^{5'}CH_2$), 2.47 (d, 2H, *J*= 7.08 Hz, $^{3'}CH_2$), 2.22 (m, 1H, $^{3'}$CH), 1.38 (s, 3H, CH_3), 1.23 (s, 3H, CH_3), 1.18 (t, 3H, *J*= 7.04 Hz, CH_3);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 172.1 (C=O), 111.7 (Cq), 105.3 ($^{1'}$CH), 81.4 ($^{2'}$CH), 80.1 ($^{4'}$CH), 60.9 ($^{3'}CH_2$), 51.8 ($^{5}CH_2$), 41.8 ($^{3'}$CH), 29.8 (CH_2), 27.2-26.9 (CH_3), 14.8 (CH_3);

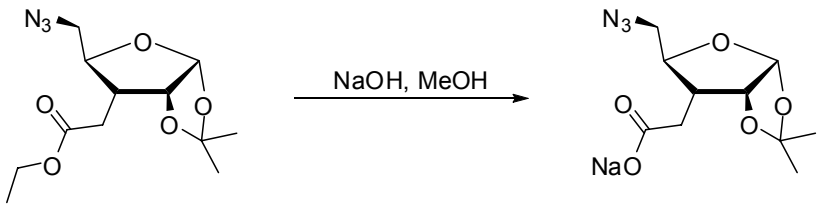
IR (ν_{max}/cm^{-1}, neat): 2102;

MS (Maldi-ToF, m/z): 308.8 $[M+Na]^+$.

Elemental Analysis

$C_{12}H_{19}N_3O_5$ (285.30):	Calcd: C 50.52 H 6.71 N 14.73 O 28.04
	Found: C 50.50 H 6.68 N 14.60 O 28.22

10.2.1.6 5'-azido-3',5'-dideoxy-3'-[(carbonyl)methyl]-1',2'-*O*-*iso*propylidene-α-D-ribofuranose sodium salt (94)



93 **94**

500 mg (1.75 mmol, 1 eq) 5'-azido-3',5'-dideoxy-3'-[(ethoxycarbonyl)methyl]-1',2'-*O*-*iso*propylidene-α-D-ribofuranose **93** were dissolved in 5 ml methanol, added with 10 M NaOH (0.3 ml) and stirred for 2 hrs at RT under argon. The reaction mixture was evaporated in vacuo to yield 491 mg of **94** as a sodium salt (1.74 mmol, quant.).

$C_{10}H_{15}N_3NaO_5$: 279.23 g/mol

R_f (hexane:EtOAc, 2:8) = 0.05;

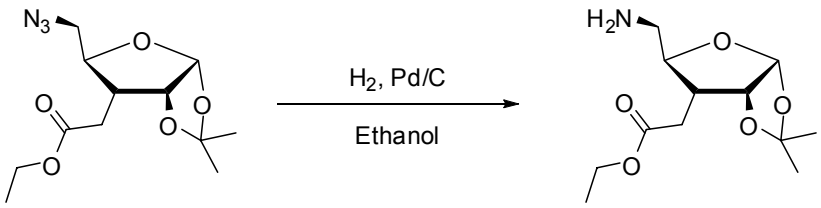
^{1}H NMR (400.0 MHz, DMSO, δ ppm): 5.72 (d, 1H, *J*= 3.52 Hz, $^{1'}$CH), 4.69 (t, 1H, *J*= 4.04 Hz, $^{2'}$CH), 3.78 (m, 1H, $^{4'}$CH), 3.57 (m, 1H, $^{5'}CH_2$), 3.25 (m, 1H, $^{5'}CH_2$), 2.15 (m, 1H, $^{3'}$CH), 2.06 (m, 1H, $^{3'}CH_2$), 1.85 (m, 1H, $^{3'}CH_2$), 1.38 (s, 3H, CH_3), 1.21 (s, 3H, CH_3);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 174.2 (C=O), 110.3 (Cq), 104.1 ($^{1'}$CH), 81.9 ($^{2'}$CH), 80.2 ($^{4'}$CH), 51.8 ($^{5}CH_2$), 43.3 ($^{3'}$CH), 33.4 ($^{3'}CH_2$), 26.7-26.3 (CH_3);

IR (ν_{max}/cm^{-1}, neat): 2101;

MS (ESI, m/z): 257.3 $[M-Na]^-$, 535.5 $[2M+Na]^-$.

10.2.1.7 5'-amino-3',5'-dideoxy-3'-[(ethoxycarbonyl)methyl]-1',2'-*O*-*iso*propylidene-α-D-ribofuranose (95)



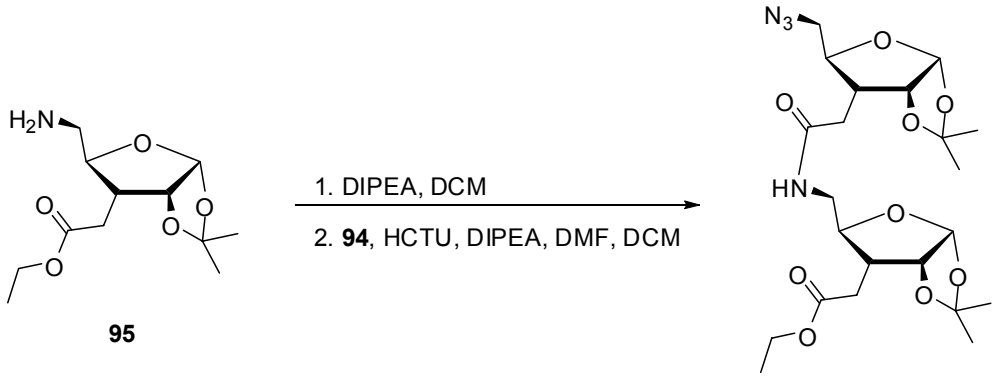
93 **95**

2 g (7.01 mmol, 1 eq) 5'-azido-3',5'-dideoxy-3'-[(ethoxycarbonyl)methyl]-1',2'-*O*-*iso*propylidene-α-D-ribofuranose **93** were dissolved in ethanol (15 ml), added with 200 mg (10% weight) palladium on activated charcoal and flushed with argon. The reaction mixture was shaken under H_2 pressure (3 bar) for 16 hrs at RT. The suspension was filtered over celite, washed with methanol and the volatiles evaporated under reduced pressure, to yield 1.6 g of compound **95** as a colorless oil (6.17 mmol, 84%).

$C_{12}H_{21}NO_5$: 259.30 g/mol
R_f (hexane:EtOAc, 2:8) = 0.18;
^{1}H NMR (400.0 MHz, DMSO, δ ppm): 5.76 (d, 1H, *J*= 3.8 Hz, $^{1'}$CH), 4.69 (t, 1H, *J*= 4.32 Hz, $^{2'}$CH), 4.06 (m, 2H, CH_2), 3.62 (m, 1H, $^{4'}$CH), 2.75 (m, 1H, $^{5'}CH_2$), 2.54 (m, 1H, $^{5'}CH_2$), 2.45 (d, 2H, *J*= 7.32 Hz, $^{3'}CH_2$), 2.17 (m, 1H, $^{3'}$CH), 1.38 (s, 3H, CH_3), 1.23 (s, 3H, CH_3), 1.18 (t, 3H, *J*= 7.04 Hz, CH_3);
^{13}C NMR (100.6 MHz, DMSO, δ ppm): 171.6 (C=O), 110.3 (Cq), 104.2 ($^{1'}$CH), 81.7 ($^{2'}$CH), 80.9 ($^{4'}$CH), 59.9 ($^{3'}CH_2$), 42.6 ($^{5}CH_2$), 40.6 ($^{3'}$CH), 29.4 (CH_2), 26.4-26.3 (CH_3), 14.0 (CH_3);
MS (Maldi-ToF, m/z): 282.1 $[M+Na]^+$.

10.2.2 Assembling the Backbone

10.2.2.1 Amide Linked Blocked Dimer (96)



2.82 g (6.83 mmol, 2 eq) HCTU were dissolved in 3 ml DMF, added with 1.17 ml (6.83 mmol, 2 eq) DIPEA, 3 ml DCM and 956 mg (3.41 mmol, 1 eq) of the 5'-azido-3',5'-dideoxy-3'-[(carbonyl)methyl]-1',2'-*O*-*iso*propylidene-α-D-ribofuranose sodium salt **94** at RT under N_2. The mixture was added with a solution of 885 mg (3.41 mmol, 1 eq) 5'-amino-3',5'-dideoxy-3'-[(ethoxycarbonyl)methyl]-1',2'-*O*-*iso*propylidene-α-D-ribofuranose **95** and 1.17 ml (6.83

mmol, 2 eq) *N*,*N*-diisopropylethylamine (DIPEA) in 5 ml DCM and stirred for 1.5 hrs at RT under N_2. The reaction mixture was washed with phosphate buffer (pH=5.5) (3 x 100 ml) and brine (100 ml). The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 2:8, v/v) yielded 958 mg of **96** as a white solid (1.86 mmol, 55%).

$C_{22}H_{34}N_4O_9$: 498,23 g/mol

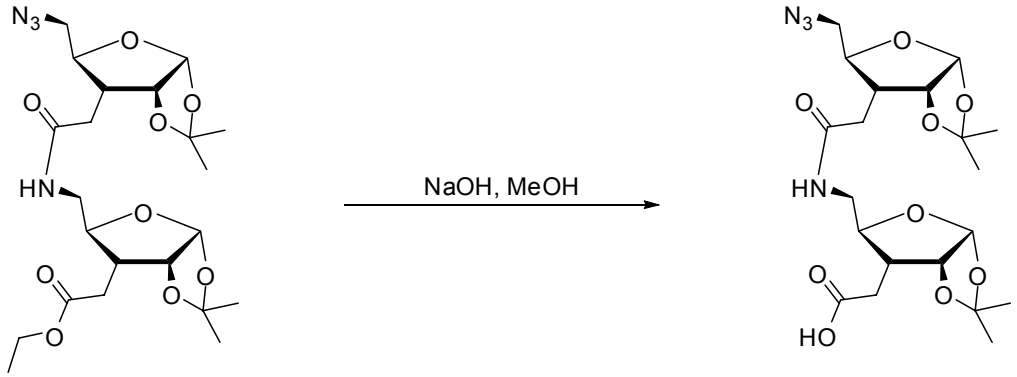
R_f (hexane:EtOAc, 2:8) = 0.71;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 8.08 (s, 1H, NH), 5.76 (s, 1H, $^{1'}$CHa, $^{1'}$CHb), 4.69 (m, 1H, $^{2'}$CHa), 4.61 (m, 1H, $^{2'}$CHb), 4.07 (m, 2H, CH_2), 3.86 (m, 1H, $^{4'}$CHb), 3.72 (m, 1H, $^{4'}$CHa), 3.57 (m, 1H, $^{5'}CH_2$b), 3.28 (m, 3H, $^{5'}CH_2$b, $^{5'}CH_2$a), 2.55 (m, 1H, $^{3'}CH_2$a), 2.43 (m, 1H, $^{3'}CH_2$a), 2.33 (m, 1H, $^{3'}CH_2$b), 2.22 (m, 2H, $^{3'}CH_2$b, $^{3'}$CHb), 2.02 (m, 1H, $^{3'}$CHa), 1.40 (s, 3H, CH_3), 1.38 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.23 (s, 3H, CH_3), 1.19 (t, 3H, *J*= 5.56 Hz, CH_3);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 171.4 (C=Ob), 170.7 (C=Oa), 110.8/110.6 (Cq), 104.2/104.1 ($^{1'}$CH), 81.1 ($^{2'}$CHb), 80.5 ($^{2'}$CHa), 79.7 ($^{4'}$CHb), 78.9 ($^{4'}$CHa), 60.0 (CH_2), 51.3 ($^{5'}CH_2$b), 41.5 ($^{3'}$CHb), 41.2 ($^{3'}$CHa), 38.2 ($^{5'}CH_2$a), 30.5 ($^{3'}CH_2$b), 29.2 ($^{3'}CH_2$a), 26.7-26.2 (CH_3), 14.0 (CH_3);

MS (ESI, m/z): 521.6 $[M+Na]^+$.

10.2.2.2 Amide Linked Free Acid Dimer (97)



96 **97**

400 mg (0.78 mmol, 1 eq) dimer **96** were dissolved in 7 ml methanol, added with 10 M NaOH (0.1 ml) and stirred for 4 hrs at RT under argon. The reaction mixture was evaporated in vacuo to yield 375 mg of **97** as a white foam (0.77 mmol, quant.).

$C_{20}H_{30}N_4O_9$: 470.20 g/mol

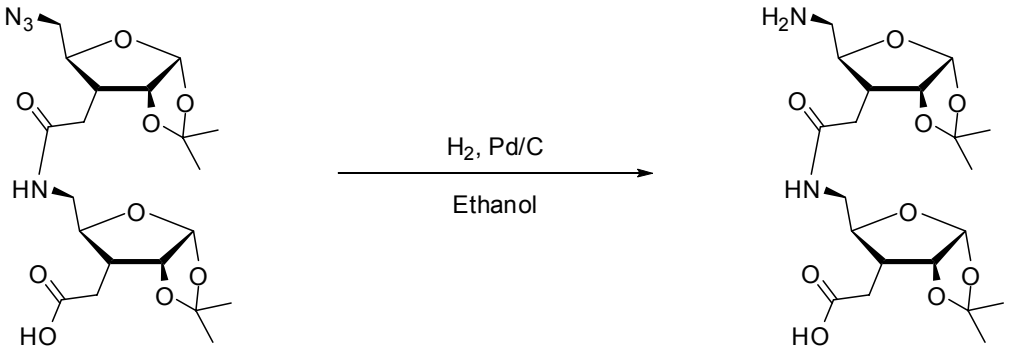
R_f (hexane:EtOAc, 2:8) = 0.1;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 5.75 (d, 1H, *J*= 3.56 Hz, $^{1'}$CHa), 5.66 (s, 1H, *J*= 3.76 Hz, $^{1'}$CHb), 4.66 (m, 2H, $^{2'}$CHa, $^{2'}$CHb), 3.87 (m, 1H, $^{4'}$CHb), 3.62 (m, 1H, $^{4'}$CHa), 3.56 (m, 1H, $^{5'}CH_2$b), 3.35 (m, 1H, $^{5'}CH_2$b), 3.25 (m, 1H, $^{5'}CH_2$a), 3.00 (m, 1H, $^{5'}CH_2$a), 2.25 (m, 2H, $^{3'}CH_2$a), 2.13 (m, 2H, $^{3'}CH_2$b), 1.99 (m, 2H, $^{3'}$CHa, $^{3'}$CHb), 1.40 (s, 3H, CH_3), 1.35 (s, 3H, CH_3), 1.24 (s, 3H, CH_3), 1.20 (s, 3H, CH_3);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 174.2 (C=Ob), 170.8 (C=Oa), 110.7/110.5 (Cq), 104.2/104.1 ($^{1'}$CH), 81.0 ($^{2'}$CHb), 80.6 ($^{2'}$CHa), 79.5 ($^{4'}$CHb), 79.0 ($^{4'}$CHa), 51.4 ($^{5'}CH_2$b), 41.4 ($^{3'}$CHb), 41.2 ($^{3'}$CHa), 38.1 ($^{5'}CH_2$a), 30.7 ($^{3'}CH_2$b), 29.2 ($^{3'}CH_2$a), 26.6-26.2 (CH_3);

MS (ESI, m/z): 493.1 $[M+Na]^+$.

10.2.2.3 Amide Linked Free Acid and Free Amine Dimer (98)



97 **98**

375 mg (0.80 mmol, 1 eq) dimer **97** were dissolved in ethanol (15 ml), added with 38 mg (10% weight) palladium on activated charcoal and flushed with argon. The reaction mixture was shaken under H_2 pressure (3 bar) for 16 hrs at RT. The suspension was filtered over celite, washed with ethanol and the volatiles evaporated under reduced pressure, to yield 300 mg of compound **98** as a white solid (0.67 mmol, 85%).

$C_{20}H_{32}N_2O_9$: 444.48 g/mol

R_f (hexane:EtOAc, 2:8) = 0.1;

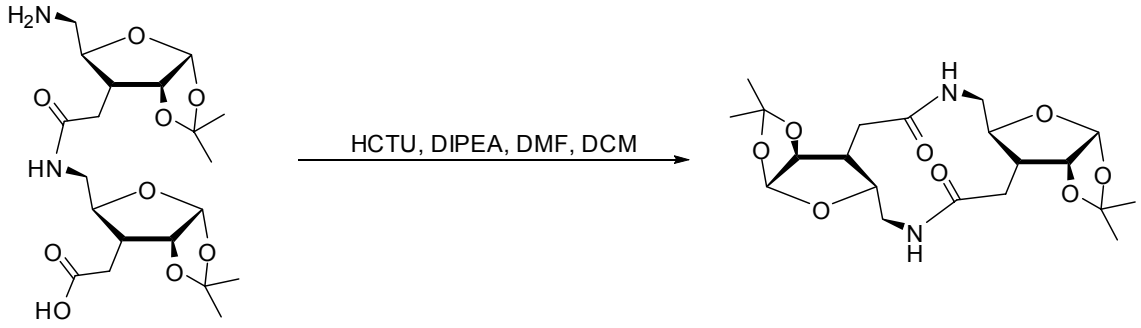
^{1}H NMR (400.0 MHz, DMSO, δ ppm): 5.66 (s, 2H, $^{1'}$CHa, $^{1'}$CHb), 4.65 (s, 2H, $^{2'}$CHa), 4.61 (s, 1H, $^{2'}$CHb), 3.66 (m, 3H, $^{4'}$CHb, $^{4'}$CHa, $^{5'}CH_2$b), 3.41 (m, 1H, $^{5'}CH_2$b), 3.29 (m, 1H, $^{5'}CH_2$a),

2.96 (m, 1H, $^{5'}CH_2$a), 2.20 (m, 2H, $^{3'}CH_2$a), 2.12 (m, 2H, $^{3'}CH_2$b), 2.00 (m, 2H, $^{3'}$CHa, $^{3'}$CHb), 1.38 (s, 3H, CH_3), 1.36 (s, 3H, CH_3), 1.22 (s, 3H, CH_3), 1.20 (s, 3H, CH_3);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 174.2 (C=Ob), 170.8 (C=Oa), 110.8/110.6 (Cq), 104.2/104.0 ($^{1'}$CH), 81.3 ($^{2'}$CHb), 80.8 ($^{2'}$CHa), 79.5 ($^{4'}$CHb), 79.1 ($^{4'}$CHa), 51.8 ($^{5'}CH_2$b), 41.2 ($^{3'}$CHb), 41.1 ($^{3'}$CHa), 38.8 ($^{5'}CH_2$a), 30.5 ($^{3'}CH_2$b), 29.3 ($^{3'}CH_2$a), 26.6-26.2 (CH_3);

MS (ESI, m/z): 467.3 $[M+Na]^+$.

10.2.2.4 Cyclic Amid Linked Sugar Backbone (99)



98 **99**

1.13 g (2.73 mmol, 9 eq) HCTU were dissolved in 2 ml DMF and added with 0.15 ml (0.91 mmol, 3 eq) DIPEA and 60 ml DCM at RT under N_2. 135 mg (0.30 mmol, 1 eq) dimer **98** were dissolved in 2.5 ml DCM under N_2 then added over 1 hr to the first mixture. The reaction was then stirred at RT under N_2 for an additional 16 hrs. The reaction was washed with phosphate buffer (pH=5.5) (3 x 70 ml) and brine (70 ml). The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. Flash chromatography (hexane, hexane:EtOAc gradient from 1:0 to 2:8 then CH_2Cl_2:MeOH gradient from 1:0 to 9:1, v/v) yielded 6.5 mg of **99** as a white solid (0.01 mmol, 5%).

$C_{20}H_{30}N_2O_8$: 426.46 g/mol

R_f (CH_2Cl_2:MeOH, 9:1) = 0.54;

^{1}H NMR (400.0 MHz, DMSO, δ ppm): 7.59 (t, 2H, *J*= 3.56 Hz, NH), 5.69 (d, 1H, *J*= 4.28 Hz, $^{1'}$CHa, $^{1'}$CHb), 4.59 (t, 2H, *J*= 4.32 Hz, $^{2'}$CHa, $^{2'}$CHb), 3.77 (m, 2H, $^{4'}$CHb, $^{4'}$CHa), 3.38 (m, 2H, $^{5'}CH_2$b), 3.14 (m, 2H, $^{5'}CH_2$a), 2.34 (m, 2H, $^{3'}CH_2$a), 2.24 (m, 2H, $^{3'}CH_2$b), 2.00 (m, 2H, $^{3'}$CHa, $^{3'}$CHb), 1.38 (s, 3H, CH_3), 1.36 (s, 3H, CH_3), 1.22 (s, 3H, CH_3), 1.20 (s, 3H, CH_3);

^{13}C NMR (100.6 MHz, DMSO, δ ppm): 174.2 (C=Ob), 173.8 (C=Oa), 110.8/110.6 (Cq), 104.2/104.0 ($^{1'}$CH), 81.3 ($^{2'}$CHb), 80.8 ($^{2'}$CHa), 79.4 ($^{4'}$CHb), 79.1 ($^{4'}$CHa), 51.8 ($^{5'}$CH$_2$b), 51.7 ($^{5'}$CH$_2$a), 41.3 ($^{3'}$CHb), 41.1 ($^{3'}$CHa), 30.5 ($^{3'}$CH$_2$b), 29.8 ($^{3'}$CH$_2$a), 26.6-26.2 (CH$_3$);
MS (ESI, m/z): 449.2 $[M+Na]^+$.

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