Synthesis of 3′, 5′-cyclic diguanylic acid (c-di-GMP) analogues

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Abstract

In the past few years, interest in signaling networks involving 3′, 5′ -cyclic diguanylic acid (c-di-GMP) has increased dramatically. Evidence started to emerge that connects c-di-GMP to the regulation of a range of biological processes in bacteria, such as bacterial biofilm formation, virulence, extracellular polysaccharide synthesis, however, much remains to be explored in the signaling pathways that involve this secondary messenger. This molecule has also been shown to be a very powerful immunostimulating agent and potent mucosal vaccine adjuvant.

Research in our lab has been directed towards the understanding of the structure – activity relationship of c-di-GMP, particularly the relevance of base residues in the bioactivity of corresponding c-di-GMP analogues. Towards this goal, the cytidine analogue of c-di-GMP, i.e. c-di-CMP, was successfully synthesized via the modified H-phosphonate approach. In this synthesis, the 2'-hydroxyl group were protected by the 1-(4-chlorophenyl)-4-ethoxy-1,2,5,6-tetrahydropyridine (Cpep) group. The activity of c-di-CMP in regulating bacterial biofilm formation and its immunostimulating properties are currently under evaluation. Towards the investigation of cellular and tissue distributions of c-di-GMP, and potentially identify c-di-GMP binding proteins, attempts were made towards the synthesis of guanosine bearing 2'-*O*-(6-azidohexyl) modification using 2,6-diaminopurine ribonucleoside as starting material. In this work, the synthesis of N^2-isobutyryl-3',5'-(1,1-dichloro-1,1,3,3-tetraisopropyldisiloxan-3',5'-diyl)-2'-*O*-(6-hydroxyhexyl)guanosine was accomplished. Future work will focus on the synthesis of 2'-*O*-modified c-di-GMP using this building block.

Acknowledgements

I would like to express my special appreciation and thanks to my supervisor Professor Tony Yan, you have been a tremendous mentor for me. I would like to thank you for encouraging my research and for allowing me to grow as a research scientist. Your advice on both research as well as on my career have been priceless.

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List of Chemical Abbreviations

Å	angstrom
ACE	bis-(2-acetoxyethoxy)methyl
ach	acetylcholine
ALE	acetal levulinyl ester
AMP	adenosine monophosphate
BAL	bronchoalveolar lavage
BPO	2-butanone peroxide
C	cytosine
°C	degree in Celsius
CFU	colony-forming unit
CDN	cyclic dinucleotide
cGAS	cyclic GMP–AMP synthase
Cpep	1-(4-chlorophenyl)-4-ethoxy-1,2,5,6-tetrahydropyridine
CT	cholera toxin
Ctmp	1-(2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl
d	doublet

DAI	Disease Activity Index
DCM	dichloromethane
DGC	diguanylate cyclase
DMF	dimethylformamide
DMOCP	5,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphinane
DMTr	dimethoxytrityl
DNA-PK	DNA-dependent protein kinase
ds	double stranded
EAL	Glu-Ala-Leu
EGF	epidermal growth factor
ESI-MS	electrospray ionization mass spectrometry
Fpmp	1-(2-fluorophenyl)-4-methoxy piperidin-4-yl
G	guanosine
g	gram
GGDEF	Gly-Gly-Asp-Glu-Phe
GMP	guanosine monophosphate
GTP	guanosine triphosphate
h	hour

HSV-1	herpes simplex virus type 1
HPLC	high performance liquid chromatography
IFN-β	interferon-β
IFI16	gamma-interferon-inducible protein Ifi-16
IKK	inhibitor of nuclear factor kappa-B kinase subunit beta
IL-6	interleukin 6
IL-8	interleukin 8
IMP	imidazolium perchlorate
IRF-3	interferon regulatory factor 3
KC	keratinocyte-derived chemokine
M	Molar
m	multiplet
MCP-1	monocyte chemotactic protein-1
mg	milligram
MHz	mega Hertz
min	minute
MIP-1β	macrophage inflammatory protein
mL	milliliter

mmol	millimoles
mol	moles
M_3R	M_3 muscarinic
Mthp	4-methoxytetrahydropyran-4-yl
2-NBO	2-nitrobenzaldoxime
2-NBOM	(2-nitrobenzyloxy)methyl
NBS	*N*-bromosuccinimide
NF-κB	nuclear factor κB
nmol	nanomole
NMR	nuclear magnetic resonance
o-	ortho
P.	*Pseudomonas*
P	Probability distribution
PAS	Periodic Aryl Single minded
PBS	phosphate buffered saline
PxCl	9-chloro-9-(*p*-tolyl)xanthene
Ph	phenyl
PsaA	pneumococcal surface adhesion A

py	pyridine
RANTES	regulated upon Activation, Normal T cell Expressed and Secreted
r.t.	room temperature
R_f	retention factor
SEM	standard error of the mean
SIL	benzyhydroxy-bis(trimethylsilyloxy)silyl chloride
SRB	sulforhodamine B
t	triplet
TBDMS	*tert*-Butyldimethylsilyl
STAT6	signal transducer and activator of transcription 6
STING	stimulator of interferon genes
TBAF	tetra-*n*-butylammonium fluoride
TBK-1	TANK-binding kinase 1
tDGC	thermophilic diguanylate cyclase domain
TEM	[2-(toluene-4-sulfonyl) ethoxy]methyl
TEMED	tetramethlethylenediamine
Th2	T helper2

Thp	tetrahydropyran-2-yl
THF	tetrahydrofuran
TIPDS	1,1-dichloro-1,1,3,3-tetraisopropyldisiloxane
TNF-α	tumor necrosis factor-alpha
TOM	[(triisopropylsilyl)oxy]methyl
TPSNT	2,4,6-triisopropylbenzenesulfonyl-3-nitro-1,2,4-triazole
TPSCl	triisopropylbenzenesulfonyl
UDP-glucose	uridine diphosphate glucose
UTRs	untranslated regions
UV-Vis	ultraviolet-visible

Chapter 1

1.1 Introduction to c-di-GMP

Research in cyclic bis-(3′,5′)diguanylic acid (c-di-GMP) began in the early 1980s in the laboratory of Moshe Benziman at the Department of Biological Chemistry of the Hebrew University of Jerusalem, Israel. The discovery of this new nucleotide in 1987 was a result of many years of research on the enzymatic mechanism and regulation of cellulose biosynthesis in the gram-negative bacterium *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) by Benziman and co-workers.[1-3] In the subsequent years and until his death in 2003, Benziman and his coworkers published several papers describing various aspects of c-di-GMP biochemistry[4, 5] and finally identifying the enzymes responsible for c-di-GMP synthesis and hydrolysis.[6] Some details of the history of the c-di-GMP discovery can be found in the 1991 review written by Benziman and his graduate students Peter Ross and Raphael Mayer.[3]

C-di-GMP is an important molecule with a role in intracellular signaling in many different kinds of bacteria. Bacteria use the signal transduction mechanisms to respond to various environmental changes, such as temperature, pH, and concentration of oxygen. In this role, c-di-GMP has been recognized as an important bacterial second messenger that relays signals from receptors on the cell surface to target molecules inside the cell, in the cytoplasm or nucleus.

There are some other second messengers that were previously identified such as, cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and guanosine tetra- and pentaphosphate((p)ppGpp) (Figure 1-1), all these second messengers participate in biological signal transduction pathways.

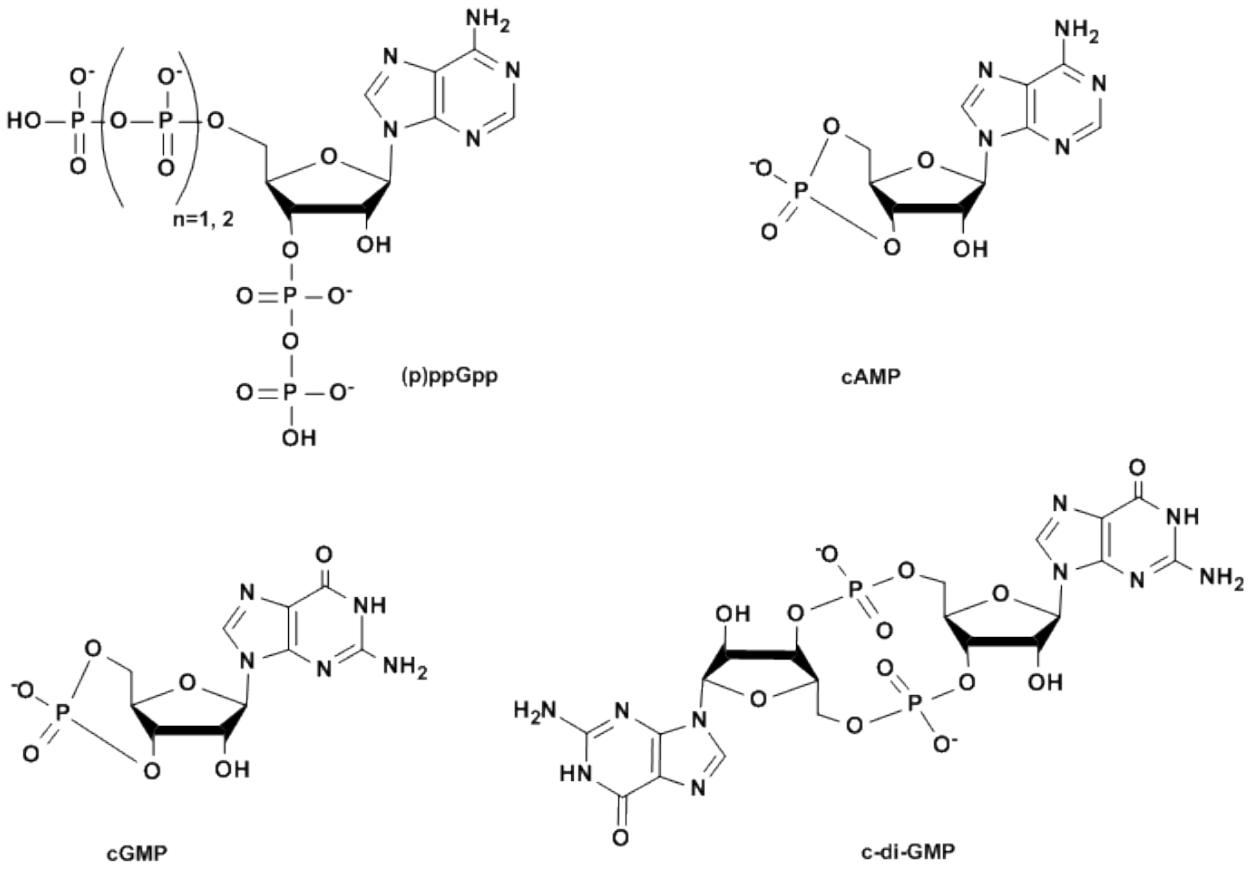


Figure 1-1 Structures of known nucleotide-based second messengers.

In the early 2000s, research emerged that connected c-di-GMP signaling to proteins that contain the GGDEF (Gly-Gly-Asp-Glu-Phe) domain, the EAL (Glu-Ala-Leu) domain, or both. The EAL domain contains approximately 240 amino acids that fold into a globular structure.The domain name originates from one of the most conserved amino acid signature motif. Like GGDEF, EAL is usually found as part of multidomain signaling proteins in combination with periplasmic membrane-embedded or cytoplasmic ligand binding sensory domains. Approximately half of all EAL domains are associated with GGDEF domains.[7] It is also important to note that although the Glu-Ala-Leu sequence motif appears to play a role in the c-di-GMP specific phosphodiesterase activity of the EAL domain, the presence of this motif in a given protein does not mean that the protein would have phosphodiesterase activities.[8] The identification of the GGDEF domain as a component of diguanylate cyclase (DGC) was a turning point for c-di-GMP research in at least two aspects. [6] First it provided

the first evidence of an enzymatic function for this widespread protein domain and paved the way to the experimental demonstration that the GGDEF domain alone was responsible for the DGC activity.[9, 10] Second, the link between this widespread domain and c-di-GMP turnover provided evidence for the participation of c-di-GMP in a variety of signaling processes.[11,12]

C-di-GMP can be synthesized by DGC [11] from two molecules of guanosine triphosphate (GTP) through a linear intermediate pppGpG. DGC enzymes are found to contain the conserved GGDEF domain. On the other hand, c-di-GMP is degraded by phosphodiesterases (PDE) to guanosine monophosphate (GMP).

C-di-GMP is an allosteric regulator of downstream proteins. Upon binding to c-di-GMP, the proteins undergoes conformational changes, and provide positive or negative regulation on some biological functions. These signal transduction pathways enable bacteria to respond to environmental cues and survive in challenging environments. It has been found that most GGDEF and EAL (or HY-GYP) domains are associated with cytoplasmic signaling domains, such as PAS (oxygen sensing). The PAS domain is assumed to have a binding pocket for heme[13] or flavin,[14] which can act as an oxygen or redox potential sensing domain. In biosynthesis of cellulose in *G. xylinus*, for example, oxygen is the first messenger that binds to the PAS domain. Depending on the level of oxygen in the environment, c-di-GMP provides a mechanism to regulate cellulose synthesis through the modulation of the activity of cellulose synthase in *G. xylinus*. In this bacterium, the PDE activity that is executed through EAL domain depends on the binding status of PAS domain towards oxygen, which serves as the first messenger. When the environmental concentration of oxygen is high, the PDE activity decreases due to the binding of PAS domain to Oxygen. Consequently, cellulose synthase is activated to produce cellulose (Figure 1-2 (b)) as a result of accumulation of cellular c-di-GMP. When the oxygen level is low in the environment, however,

phosphodiesterase activity is restored, leading to decrease in the concentration of c-di-GMP and thus inactivation of cellulose synthase (Figure 1-2(a)).[13]

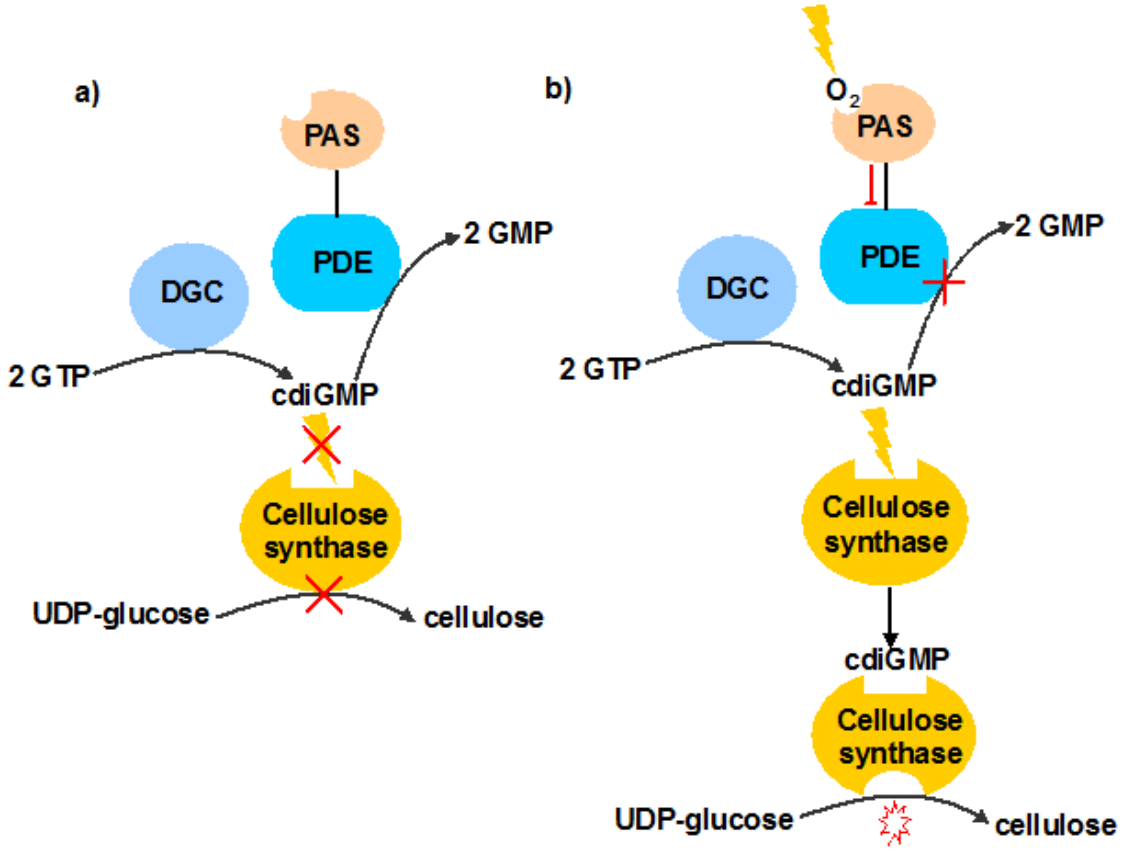


Figure1-2 C-di-GMP regulation of cellulose synthesis in *G. xylinus*.[15] (Reprinted from the *Chemical Society Review)*

1.2 Structural investigation of c-di-GMP

The structure of c-di-GMP was determined by nuclear magnetic resonance (NMR)[16] and X-ray crystallography.[17-20] In all these structures, the 12-member macrocycle formed by the ribose and phosphate moieties is found to maintain virtually the same conformation.[21] This homodimeric form of c-di-GMP has a dramatically different molecular outline from the

monomer which means that the monomeric versus dimeric forms are likely to interact with different receptors (Figure 1-3). In the dimeric form, association of c-di-GMP with Mg^{2+} or Co^{2+} is observed in some structures, where these cations are found on the two fold symmetry axis coordinated by the N-7 atoms of the two central bases, but their presence does not significantly change the structure of the dimer.[18-20]

(A) (B)

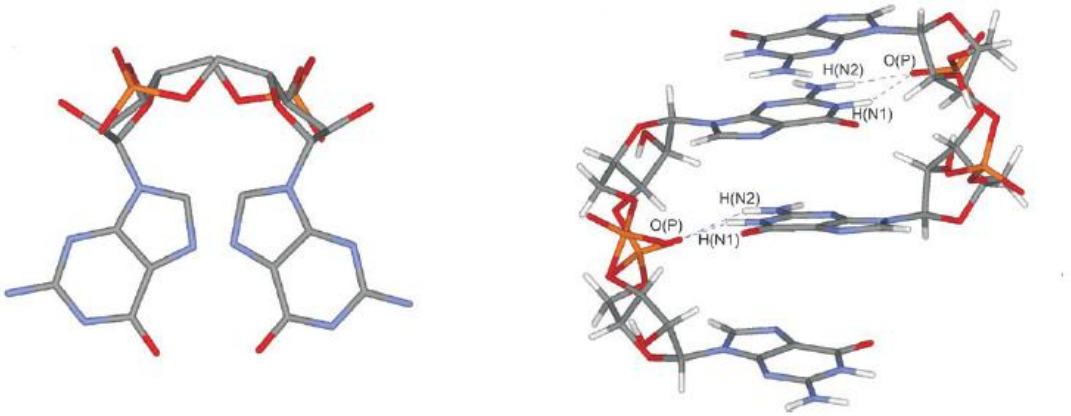


Figure 1-3 (A) Top view of c-di-GMP in the monomer form; (B) c-di-GMP in the dimer form, with hydrogen bonds shown as dash lines.[15] (Reprinted from the *Chemical Society Review*).

It was also shown that in solution, equilibrium of dimeric, tetrameric and octomeric c-di-GMP can be affected by both c-di-GMP concentration and presence of metal ions. In this respect, in lower concentrations of c-di-GMP and the presence of small metal ions such as Li^{+}, the equilibrium prefers the formation of bimolecular structures. However, in higher concentrations and presence of larger metal ions, such as K^{+}, the equilibrium prefers the formation of more complex assembly (Figure 1-4).[22]

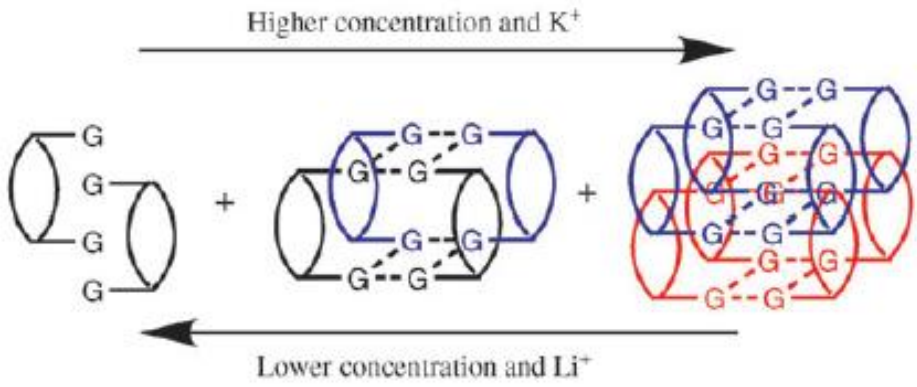


Figure 1-4 Cartoon of c-di-GMP intermolecular assembly.[22] (Reprinted from the *American Chemical Society*).

The biological significance of this equilibrium is not clear, however, it was hypothesized that dimeric c-di-GMP can provide a feed-back inhibition for the DGC domain in *Gaulobacter crescentus.* In this bacterium the PleD protein consists of a receiver domain (D1), adapter domain (D2) and also a DGC domain. When D1 is phosphorylated, PleD is activated at the differentiating pole, which catalyzes the conversion of two molecules of GTP to c-di-GMP (Figure1-5). It has been proposed that the c-di-GMP dimer can bind to the D2/DGC domain (inhibition site or I-site),[23] leading to inactivation of the DGC enzyme. This feedback regulation of PleD by product inhibition shows the importance of an upper limit on the concentration of c-di-GMP.

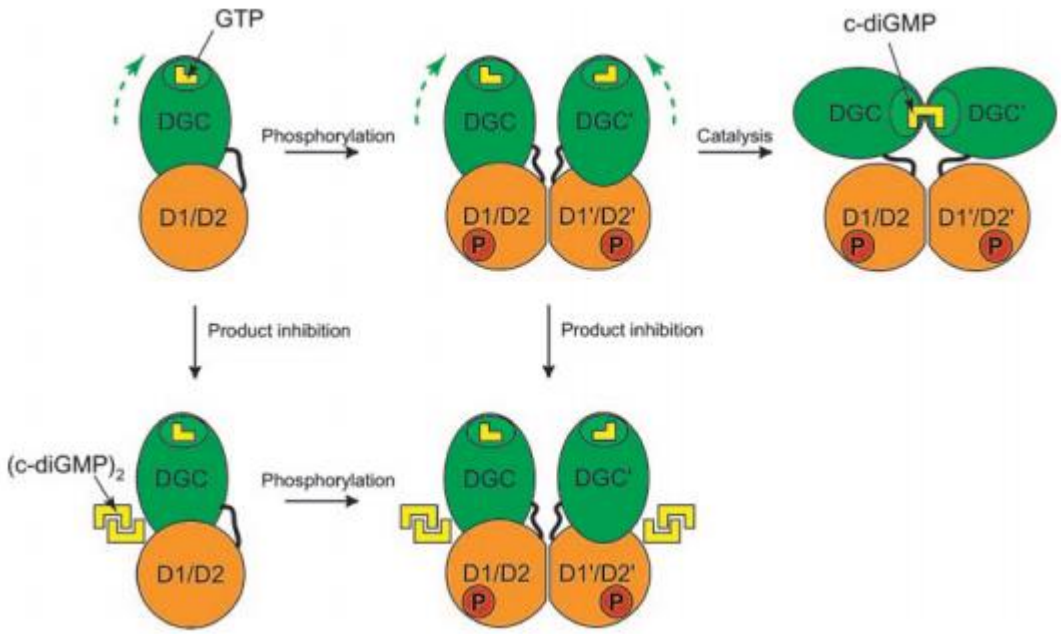


Figure 1-5 PleD regulation of c-di-GMP synthesis *in vivo*.[23]

(Reprinted from the *Proceeding of National Academy of Science. USA)*

1.3 Immunostimulatory properties of c-di-GMP

C-di-GMP recently gained significant attention among immunologists due to its extraordinary immunostimulatory and immunomodulatory properties. In the initial experiments, c-di-GMP was shown to induce the activation and maturation of human immature dendritic cells (DCs), the professional antigen-presenting cells, *in vitro*.[24] The activation of DCs was proved by the increased levels of key chemokines and cytokines upon treatment of DC cultures with c-di-GMP.

Further, intranasal treatment of mice with c-di-GMP increases the level of cytokines and chemokines in Bronchoalveolar lavage (BAL) that are essential in the recruitment of neutrophils such as IL-8, KC, RANTES, IL-6, MIP-2, MCP-1 (Top panel, Figure 1-10). On the other hand, the level of these cytokines and chemokines normalized seven days after the

treatment (Bottom panel, Figure 1-6). These data strongly suggested the immunostimulatory properties of c-di-GMP at a mucosal site.[25]

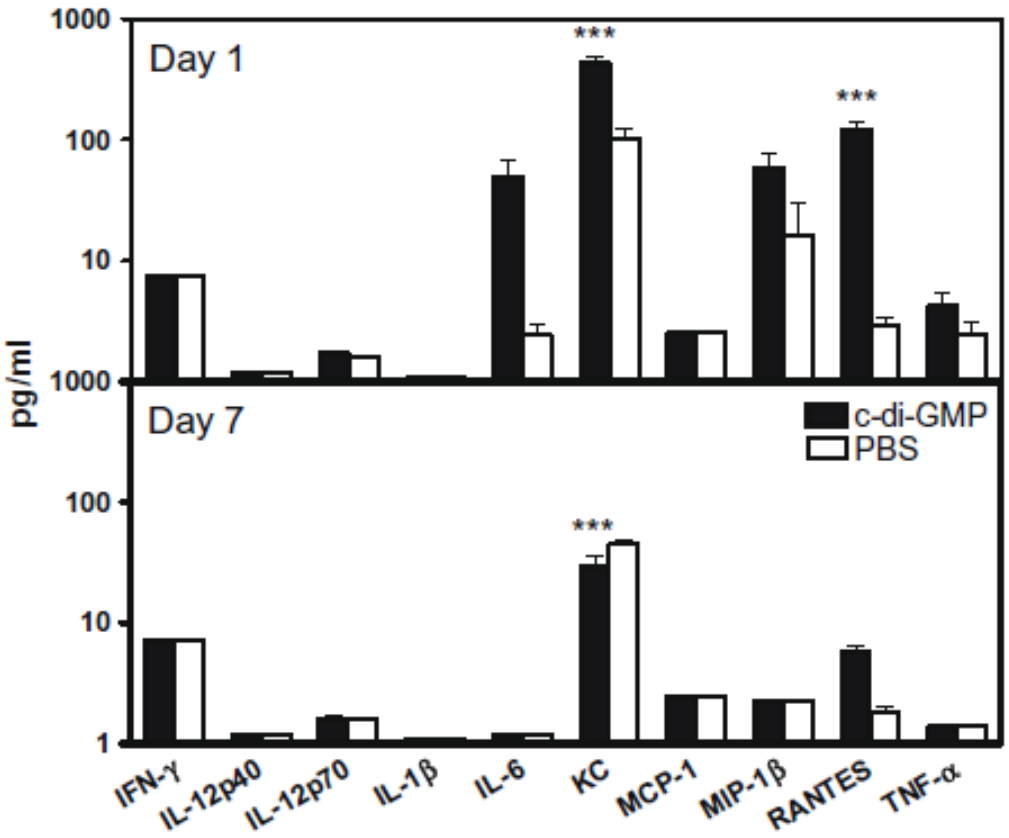


Figure 1-6 Proinflammatory cytokine/chemokine levels in bronchoalveolar lavage (BAL) fluids from c-di-GMP-(solid bars) and phosphate buffer saline PBS (open bars)-treated mice.[25] Groups of BALB/c mice were treated with 10 µg c-di-GMP or PBS at day 0. Mice were sacrificed 1 and 7 days later, their lungs were lavaged and the lavage fluid was assayed for cytokines and chemokines.[25] (Reprinted from the *Biochemical and Biophysical Research Communications)*

To investigate whether c-di-GMP had any prophylactic effect, mouse mammary glands were treated 12 h and 6 h before infection with *S. aureus* produce significant prophylactic effect of

a 1.5 and 3.8 log reduction of the bacterial burden in tissues treated with 50 and 200 nmol of c-di-GMP, respectively in comparison with untreated control. These data strongly suggest that c-di-GMP stimulates the innate immune response (Figure 1-7).[27]

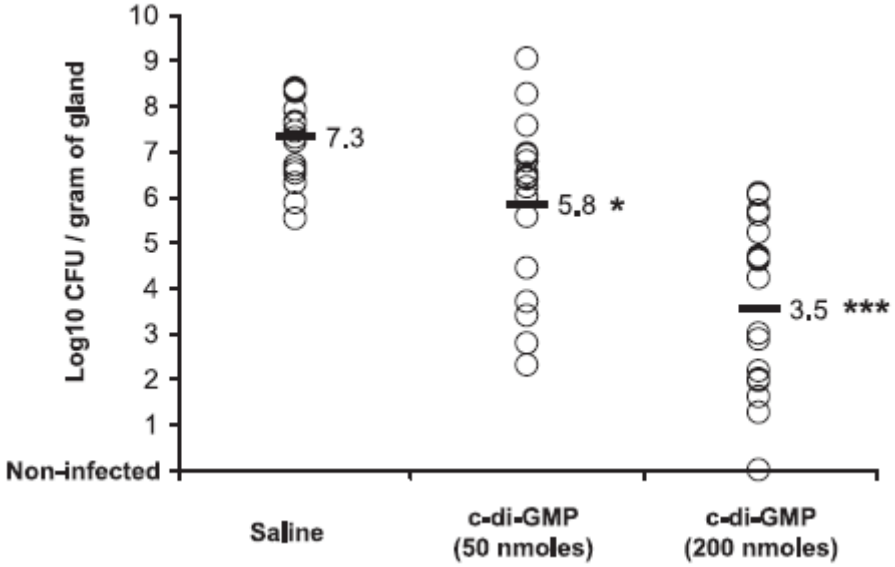


Figure 1-7 Pretreatment protective effect of c-di-GMP.[27] C-di-GMP was administered 12 and 6 h before infection with *S. aureus*. Lactating mice were infected by intramammary inoculation and the infection was allowed for 10 h before mammary glands were harvested for bacterial colony-forming unit CFU determination. Each circle on the graph corresponds to the number of CFU per gram of gland for an individual gland. Mean values are indicated and show that prophylaxis with c-di-GMP significantly reduced the level of *S. aureus* colonization in a dose-dependent manner. *, $p < 0.05$; ***, $p < 0.001$.[27] (Reprinted from the *Immunology*).

Acinetobacter baumannii is a major cause of both community-associated and nosocomial infections worldwide. *A. baumannii* has rapidly developed resistance to multiple antibiotics; as a result, it has become difficult to treat infections caused by this pathogen. Treatments of mice with diverse doses of c-di-GMP reduced the bacterial burdens in both the lungs and

spleens. Treatments with all three dosages (12.5, 25, 50 μg) reduced the bacterial burdens in the spleen almost equally efficiently.

It was shown in a recent study that mice treated intranasally with 50 μg of c-di-GMP 18 h prior to *A. baumannii* infection provided the best protection, leading to reduction in bacterial burden in both lungs and spleens as compared with mice treated with cGMP (Figure 1-8).[28]

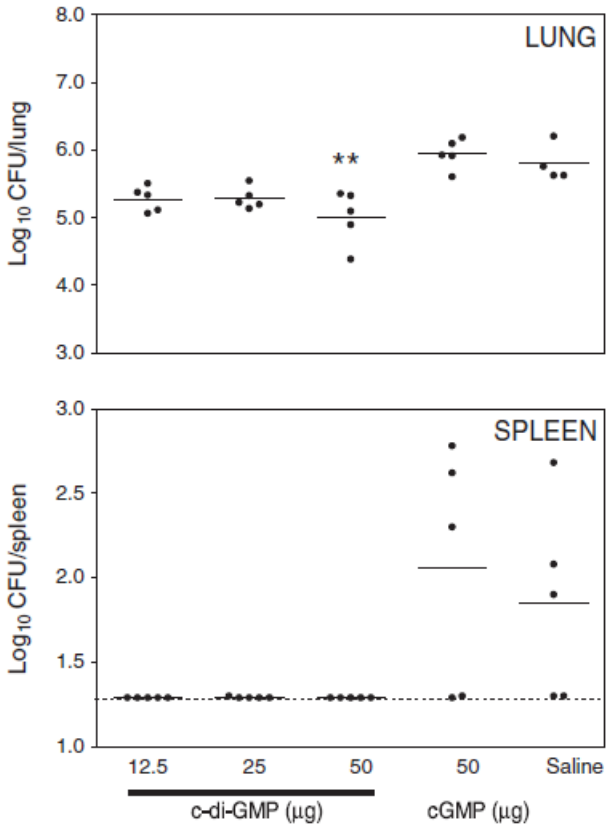


Figure 1-8 Treatment of different dosage of c-di-GMP reduce bacterial burden in lung and spleen.[28] (Reprinted from the *International Immunopharmacology*).

1.4 C-di-GMP as a mucosal vaccine adjuvant

Pathogenic microorganisms are an extremely diverse group of living entities found in different environments, including the human mucosal surfaces. Indeed, the mucosal system is the frontline of defense against bacteria and virus invasion. Significant attempt has been

invested on the development of mucosal vaccination method to improve immune responses at both systemic and mucosal levels. The immune system is composed of two primary compartments: the mucosal and systemic immune systems. Vaccination at mucosal site is through needle-free administration and is more patient-compliant; however, mucosally administered antigens are generally tolerated as the host strikes to maintain mucosal homeostasis by responding to mucosal antigens with tolerance. As such it is challenging to induce mucosal immune response effectively. Currently, only four mucosal vaccines have been approved for human use, all based on killed or live attenuated pathogens (Table 1-1).[29] Due to the safety concerns with the use of killed or live attenuated pathogens, there is an urgent need for the development of effective mucosal vaccine adjuvants that allow for the use of antigens that are not based on killed or live attenuated pathogens.[30]

Table 1-1 Known mucosal vaccines for human use.

Disease	Trademarks	Type of vaccine
Polio	Orimune (Lederle Labs/Wyeth)	Live attenuated
Rotavirus	RotaRix (GSK)	Live attenuated
Influenza	FluMist (MedImmune)	Live attenuated
Typhoid	Vivotif Berna, Swiss Serum, International Vaccine Institute	Live attenuated

The mucosal immune system has three main functions: (i) to protect the mucous membranes against colonization and invasion by potentially dangerous microbes, (ii) to evade recognition of undegraded antigens such as foreign proteins derived from absorbed food and commensal microorganisms, and (iii) to prevent the development of potentially harmful immune responses to these antigens if they do reach inside the body.[31]

The systemic immune system functions in a normally sterile milieu and often actively responds to invading foreign materials. The most routine vaccines are designed for delivery

via the systemic system, which leads to systemic immunity, but induce a lesser degree of immune response at the mucosal system. Increasing numbers of studies have shown that the respiratory mucosa provides a valuable target site for immunization against respiratory pathogens.[31]

Mucosal vaccination via the respiratory tract such as intranasal delivery shows advantages over other routes including the subcutaneous immunization. The intranasal delivery approach is easier, flexible, and more importantly, mucosal delivery demonstrate an ability to activate both mucosal and systemic immune responses.[32]

C-di-GMP has recently been recognized as a very useful immunomodulating agent in several *in vitro* and *in vivo* animal model studies. These studies showed the potent immunomodulatory (immunostimulatory and adjuvant) effect of c-di-GMP on cellular components of both the innate and adaptive or necessary arms of host immunity.[33-37]

Further, Ebensen and co-workers showed that c-di-GMP has a potential application as a mucosal vaccine adjuvant.[38] It was also shown by Yan and co-workers that vaccination of mice with pneumococcal surface adhesion A (PsaA) antigen adjuvated by c-di-GMP led to reduction of bacterial burdens in the lung when mice were subsequently challenged with *Streptococcal pneumonia.* More importantly, the level of reduction of bacterial burden in the vaccinated mice was comparable to those vaccined with PsaA + cholera toxin (CT) (Figure 1-9).[39] Note that CT has been regarded as the "golden standard" mucosal research vaccine adjuvant, however, its clinical use is restricted due to its toxicity.

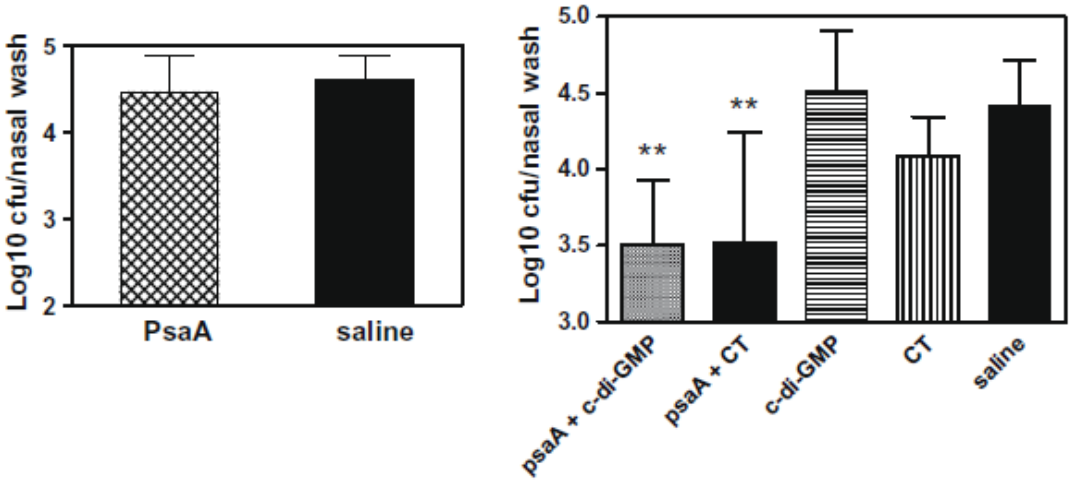


Figure 1-9 Reduction of bacterial colonization in mice immunized with PsaA-c-di-GMP.[39]

(Reprinted from the *Biochemical and Biophysical Research Communications*)

1.5 C-di-GMP and STING protein

In successful immune responses, detection of foreign materials is the first step. STING (stimulator of interferon genes) is an important signaling molecule for DNA and c-di-GMP-mediated type I interferon (IFN) production via TANK-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3) pathways.[40-43] The activation of innate immunity with second messengers suggests that sensing cyclic dinucleotides (CDNs) may be essential to host defense against bacterial infection.[44, 45] STING is the principle mediator for the second cytosolic pathway that triggers type 1 interferon, in response to sensing cytosolic dsDNA from infectious.[40] STING was discovered by Glen Barber and colleagues using cDNA expression cloning methods as a MyD88-independent host cell defense factor expressed in macrophages, dendritic cells (DCs) was found to induce expression of IFN-β and NF-κB dependent pro-inflammatory cytokines in response to sensing cytoplasmic DNA (Figure 1-10).[40]

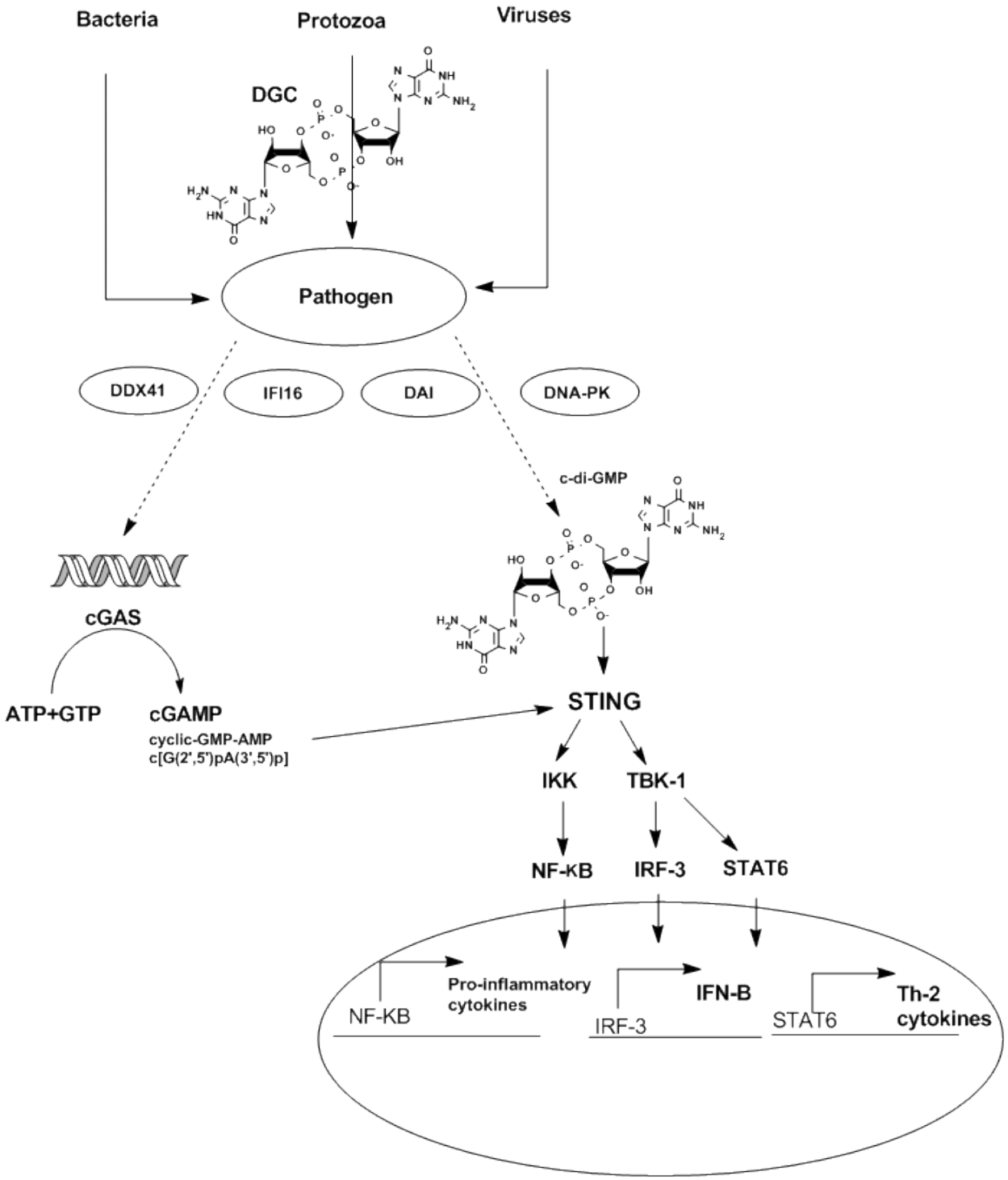


Figure 1-10 Activation of interferon genes by the STING in response to the cytosolic nucleic acids. [40] (Reprinted from the *Therapeutic Advances in Vaccine).*

STING is a central sensor of cytosolic nucleic acids. STING is a MyD88-independent host cell defense factor that senses cytosolic nucleic acids, and in response activates TBK-1/IRF-3 signaling cascades, inducing the expression of pro-inflammatory cytokines and IFN-β.[46] The activating ligands for STING are CDNs, second messengers that are produced by bacteria or by cellular cGAS in response to binding cytosolic dsDNA. CDNs produced by bacteria and cGAS are structurally distinct. Less well studied, activation of STING by CDNs has also been shown to induce the expression of STAT6 dependent Th2 cytokines.

It was recently shown that c-di-GMP can bind to STING.[47] STING contains of a cytosolic C-terminal domain (CTD) and an N-terminal region. STING CTD has been shown to specifically recognize c-di-GMP using the dimerization interface and imposes a symmetrical conformation to the bound c-di-GMP (Figure 1-11). Thus, binding of c-di-GMP does not cause significant conformational changes in STING CTD. Detailed analysis of the c-di-GMP-bound STING structure revealed some asymmetrical interactions of STING with the purine of c-di-GMP. However, the mechanism for the elicitation of IFN production through binding of c-di-GMP remains unknown.

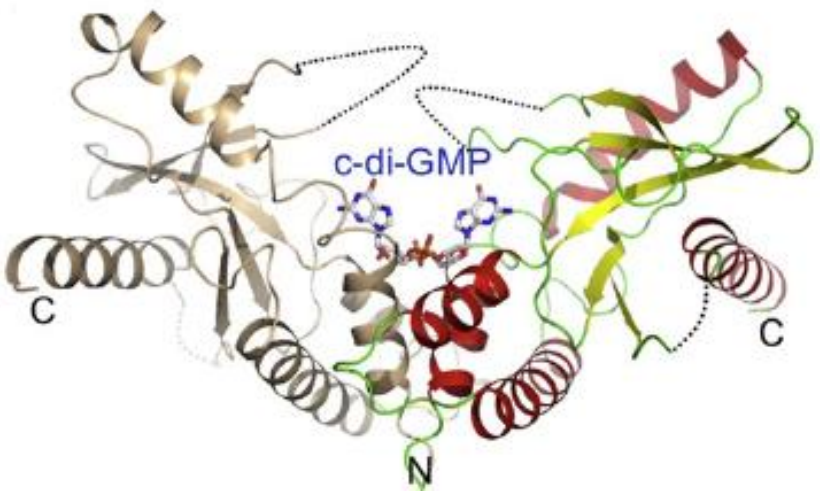


Figure 1-11 C-di-GMP binds to STING dimer at the interface.[48]

(Reproduced with permission from the *Immunity)*

To evaluate the importance of STING in host defense against select virus infection *in vivo*, $Sting^{-/-}$ (STING deficient) or control mice were infected intravenously with type 1 Herpes simplex virus (HSV-1) and then survival in both groups was monitored. All the Sting-knockout mice died in 7 days of HSV-1 infection (Figure 1-12a), however, 80% of similarly infected wild-type control mice survived. It was determined that HSV-1 was detected in the brain of majority of the infected $Sting^{-/-}$ mice, but not in the control groups at 5 days after infection (Figure 1-12b). Analysis of serum from the infected $Sting^{-/-}$ mice showed a defect in the production of type I IFN 6 h after infection (Figure 1-12c, d).[48] Furthermore, $Sting^{-/-}$ mice were found to be more sensitive to HSV-1 after intravaginal infection of HSV-1. This data indicates that STING is necessary *in vivo* for the effective production of type I IFN and is crucial for efficient protection against HSV-1 infection.[48]

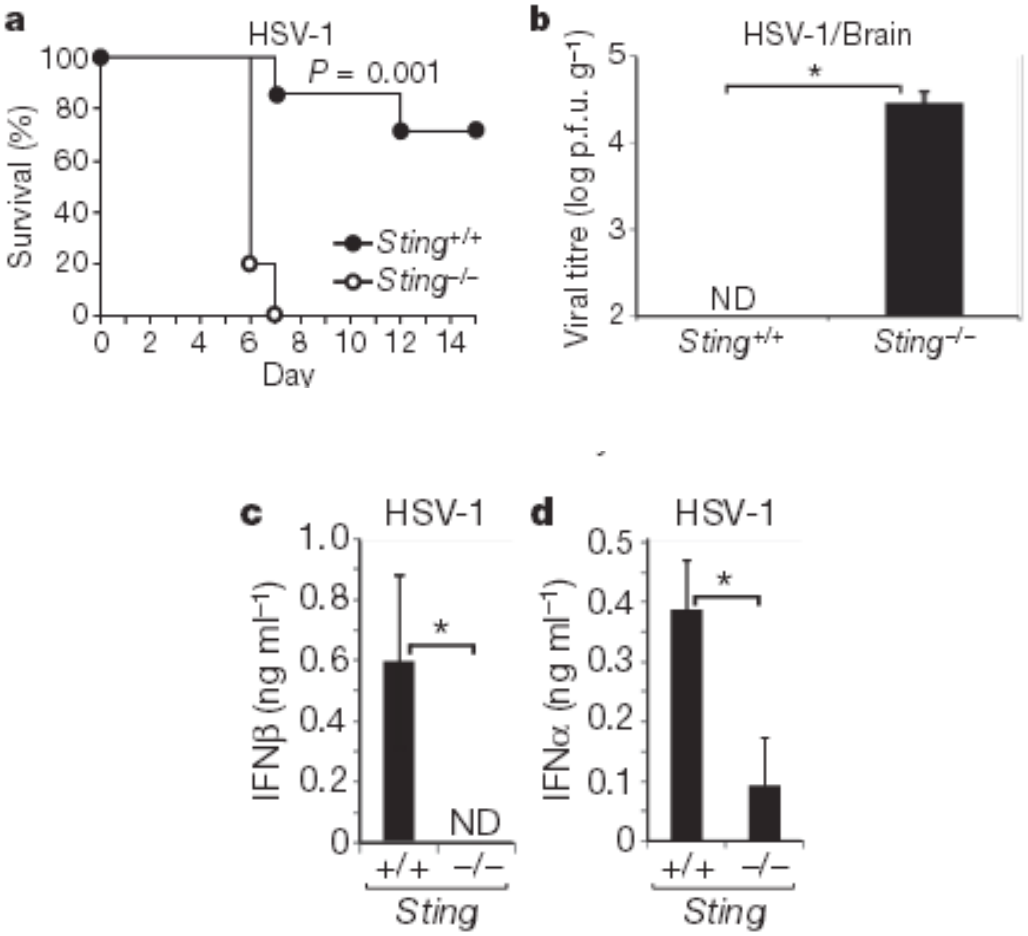


Figure 1-12 STING is required for effective *in vivo* host defense.[48] a) Sting deficient animals ($Sting^{-/-}$) or littermate controls ($Sting^{+/+}$) (n = 7; approximately 8 weeks-of-age) were infected with HSV-1 ($1x10^7$ i.v.(intravenous)) and survival was monitored. b) $Sting^{-/-}$ or control mice were infected with HSV-1 as in panel a and brains were retrieved after 5 days for HSV-1 plaque assays. (p.f.u., plaque-forming units). c, d) Serum from animals (n=3) infected with HSV-1 ($1x10^7$ i.v.) was analysed for IFNβ (c) or IFNα (d) production after 6 h. (Reprinted from the *Immunity*).

1.6 Preparation of c-di-GMP

C-di-GMP has been synthesized by both enzymatic and chemical approaches. Each method has its advantages and disadvantages.

1.6.1 Enzymatic synthesis of c-di-GMP

As a natural product, c-di-GMP could be simply extracted from a natural source.[49] It is possible to extract c-di-GMP from bacterial culture; however, low concentration of cellular c-di-GMP makes this method impractical. Enzymatic synthesis of c-di-GMP suffers from low yield due to protein instability and strong product inhibition; however, it is possible to minimize these issues through the use of thermophilic diguanylate cyclase domain (tDGC) protein.[50]

With the enzymatic approach c-di-GMP can be synthesized from GTP on multiple milligram scale. The product is typically purified by reversed phase HPLC.[51-54] One major limitation with the enzymatic approach is that it is not easily amenable for the preparation of modified c-di-GMP.

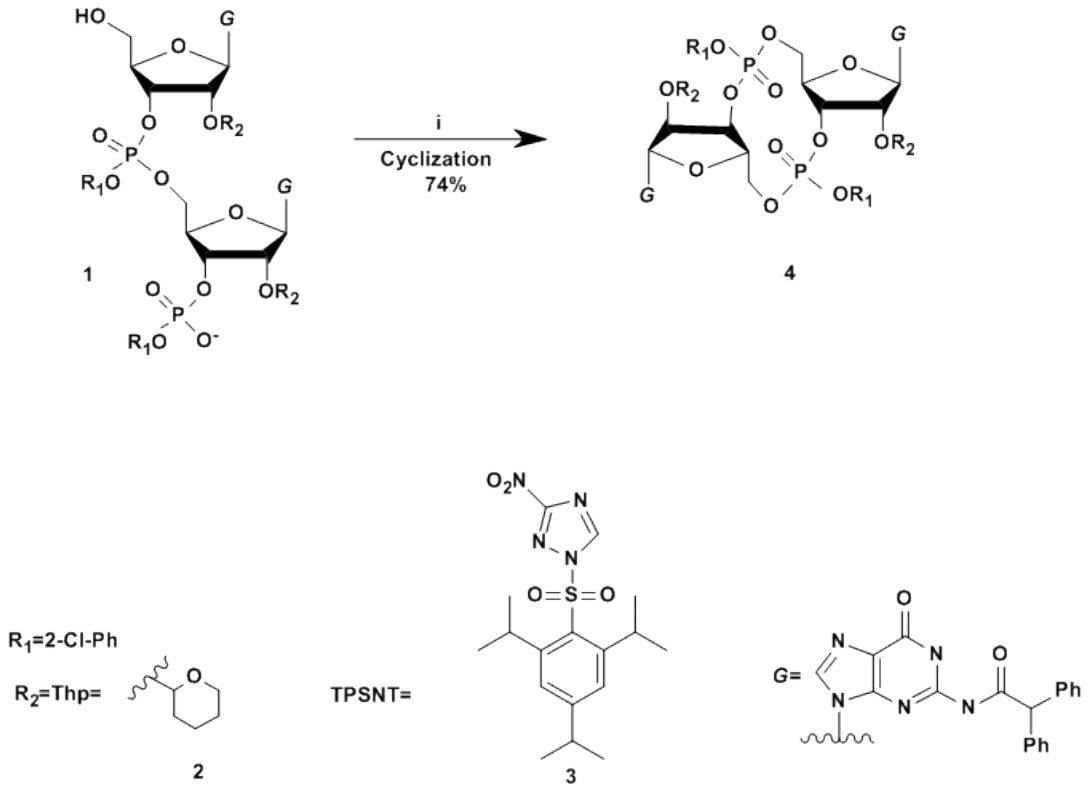
1.6.2 Chemical synthesis of c-di-GMP and analogues

Compared with enzymatic synthesis, chemical synthesis allows for the preparation of not only c-di-GMP, but also analogues, potentially on much larger scales. In this approach, protection of 2'-OH of ribonucleoside and choice of phosphorylation method are two important considerations. So far, c-di-GMP syntheses have been carried out mostly in solution using the phosphotriester, phosphoramidite, H-phosphonate, and the modified H-phosphonate approaches.

1.6.2.1 Phosphotriester approach

At the beginning of the 1980s, the most general approach to oligonucleotide synthesis both in solution and on solid supports was the phosphotriester chemistry. The first chemical synthesis of c-di-GMP via the phosphotriester approach was reported by van Boom *et al*.[55] where 2'-hydroxyls of guanosine in c-di-GMP **1** were protected with tetrahydropyran-2-yl (Thp as in **2**). The coupling and cyclization reactions were effected by 2,4,6-triisopropylbenzenesulfonyl-3-nitro-1,2,4-triazole (TPSNT) **3** to give the cyclized compound **4** (Scheme 1-1).

In this chemistry, once chain assembly was completed, the internucleotide linkages were deprotected in a two-step fashion by treatment first with oximate and then aqueous ammonium hydroxide (Scheme 1-1).



Scheme 1-1 Cyclization using the phosphotriester chemistry.[55]

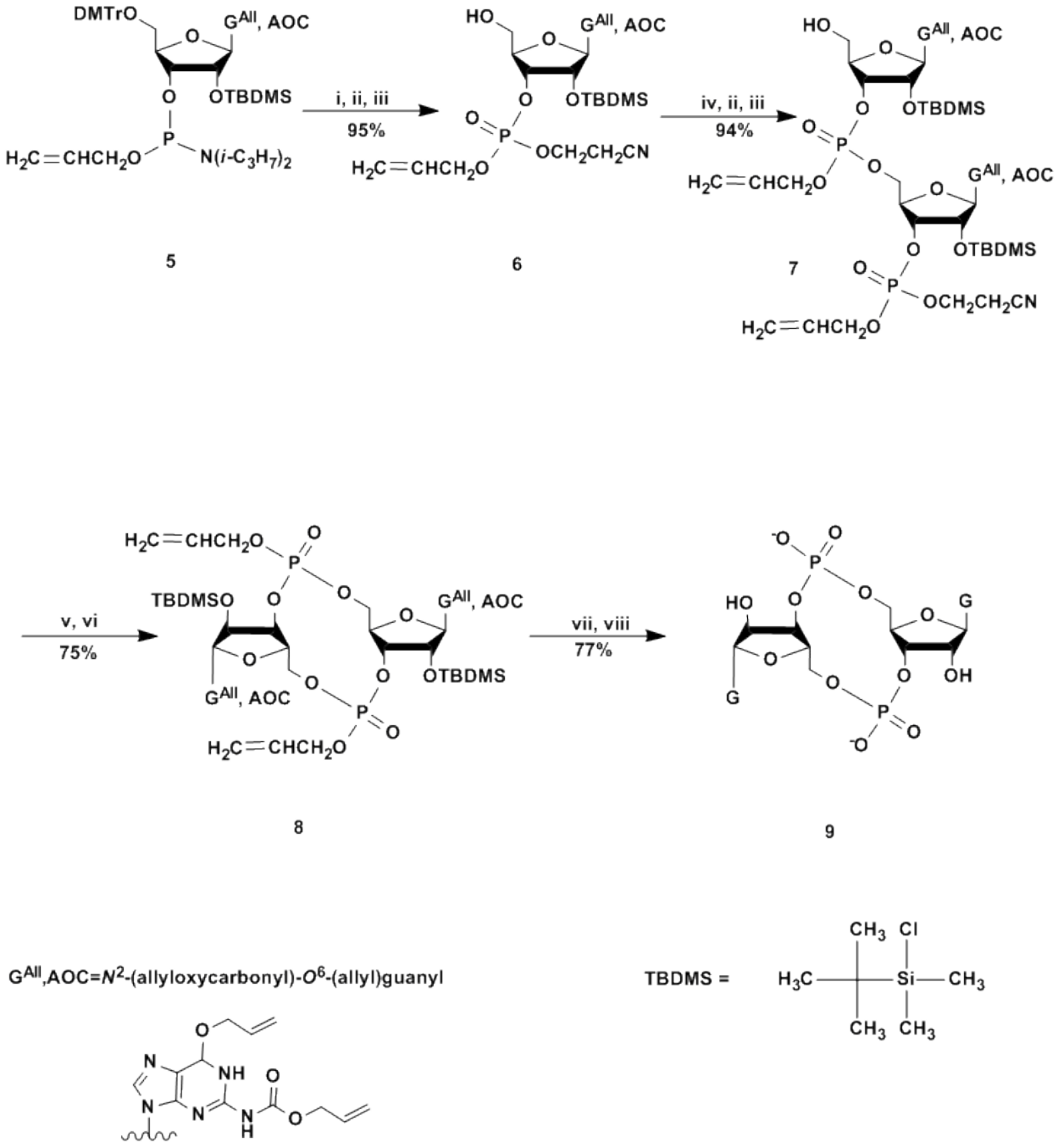
Reagents and conditions: i) pyridine, TPSNT

The phosphotriester chemistry worked well for the phosphorylation, however, coupling reactions are not as fast as some other chemistry such as phosphoramidite and H-phosphonate. Further, removal of 2'-*O*-Thp protecting groups requires prolonged treatment with acid, which is detrimental to RNA.

1.6.2.2 Modified phosphotriester approach

A modified phosphotriester approach was later used by Hayakawa *et al*. towards the chemical synthesis of c-di-GMP.[56] In this synthesis building block **6** was prepared through the imidazolium perchlorate (IMP)-promoted reaction of the nucleoside phosphoramidite **5** with 2-cyanoethanol in acetonitrile, followed by oxidation with 2-butanone peroxide (BPO) and detritylation with dichloroacetic acid in dichloromethane. Equal molar equivalents of the building blocks **5** and **6** were subsequently condensed in the presence of imidazolium perchlorate. The resulting product was oxidized with 2-butanone peroxide followed by detritylation to give linear dimer **7**.

Compound **7** was treated with aqueous ammonium hydroxide to remove the cyanoethyl group, and the resulting product was subjected to cyclization effected by a mixture of triisopropylbenzenesulfonyl chloride (TPSCl) and *N*-methylimidazole**.** The fully protected compound **8** was deprotected first by treatment with a catalytic amount of $Pd_2[(C_6H_5CH{=}CH)_2CO]_3{\cdot}CHCl_3$ in the presence of triphenylphosphine and butylammonium formate followed by exposure to $(C_2H_5)_3N{\cdot}3HF$ to give the diammonium salt of **9** (Scheme 1-2).



Scheme 1-2 Modified phosphotriester approach.

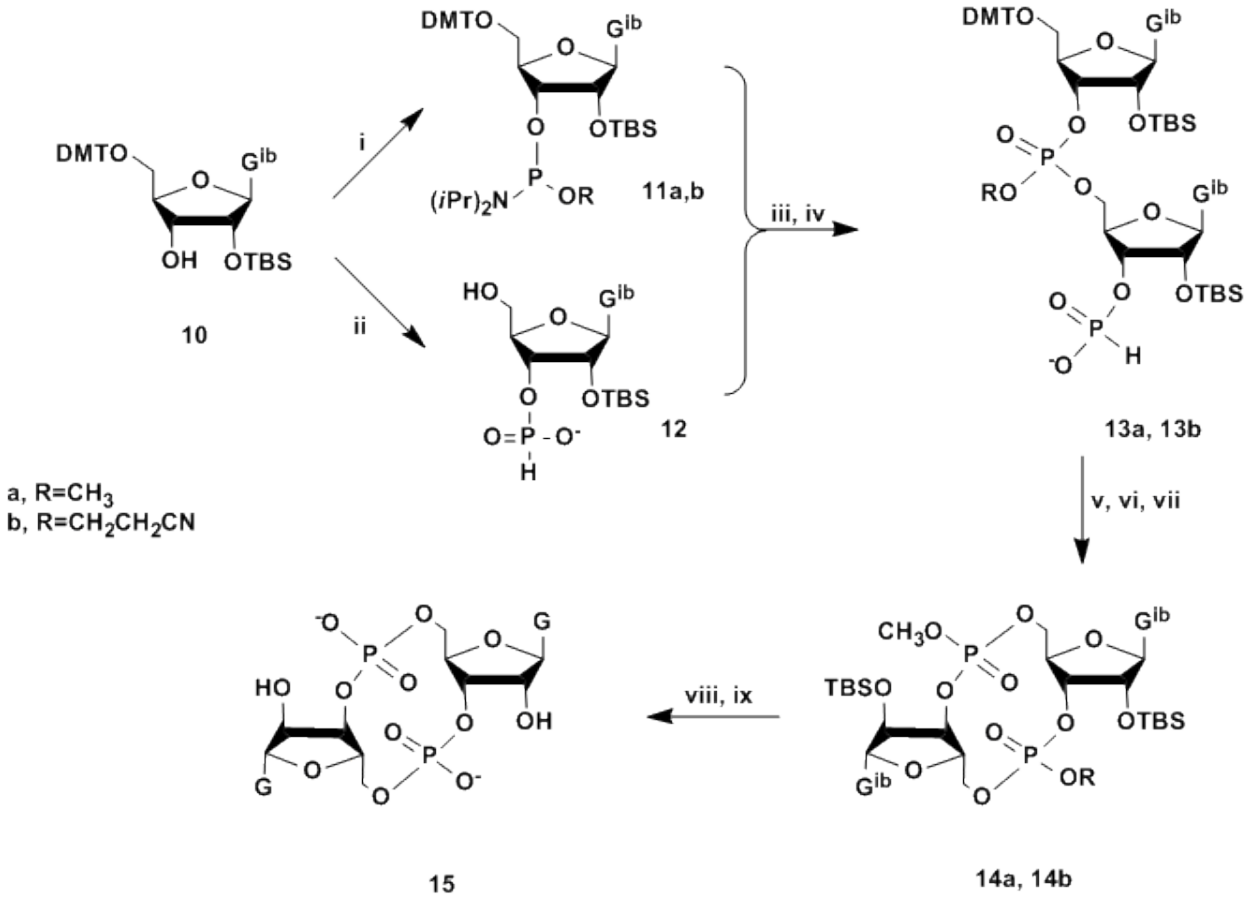
Reagents and conditions: i) $HOCH_2CH_2CN$, IMP (imidazolium perchlorate), CH_3CN, 30 min; (ii) 6.7% 2-butanone peroxide (BPO)/toluene solution, 5 min; (iii) $Cl_2CHCOOH$, CH_2Cl_2, 0ºC, 5 min; (iv) phosphoramidite **5**, IMP, CH_3CN, 30 min; (v) conc. aq. NH_3–CH_3OH (1:10 v/v), 60 min; (vi) triisopropylbenzenesulfonyl chloride (TPSCl), *N*-methylimidazole, THF, 12 h; (vii) $Pd_2(dba)_3 \cdot CHCl_3$, $P(C_6H_5)_3$, n-$C_4H_9NH_3^+HCOO^-$, THF, 10 min; (viii) $(C_2H_5)_3N \cdot 3HF$, 12 h.

The unique strategy used in this synthesis is the use of allyloxycarbonyl and allyl groups for the protection of guanine bases and two internucleotide phosphates, respectively. These allylic protecting groups can be removed all at once by the organopalladium-catalyzed reaction under neutral conditions. One problem with the TBDMS as a protecting group is that it can undergo base-catalyzed migration.[57]

1.6.2.3 H-Phosphonate approach

Using H-phosphonate approach for synthesizing c-di-GMP was proposed by Jones *et al*.[58] In this synthesis 5′, 2′-protected compound **10** was transformed into two different compounds **11a,b** and **12**. Standard *O*-cyanoethyl-protected **11b** or methyl protected phosphoramidite monomer **11a** was first coupled with 3′-H-phosphonate **12** to give a dimer H-phosphonate **13**. It is noted that the use of 5'-hydroxyl-3'-H-phosphonate **12** circumvented additional protection/deprotection steps.

Cyclization of linear dimer **13** was then effected by adamantoylcarbonyl chloride to give the corresponding cyclic H-phosphonate diester, which was oxidized *in situ* to afford the fully protected cyclic c-di-GMP **14** as a mixture of diastereomers (Scheme 1-3). The deprotection was effected by aqueous ammonia and tetraethylammonium fluoride to make fully deprotected c-di-GMP **15**. Purification of final product **15** was carried out by reverse phase HPLC (79% from **14a**).



ib: Isobutyryl

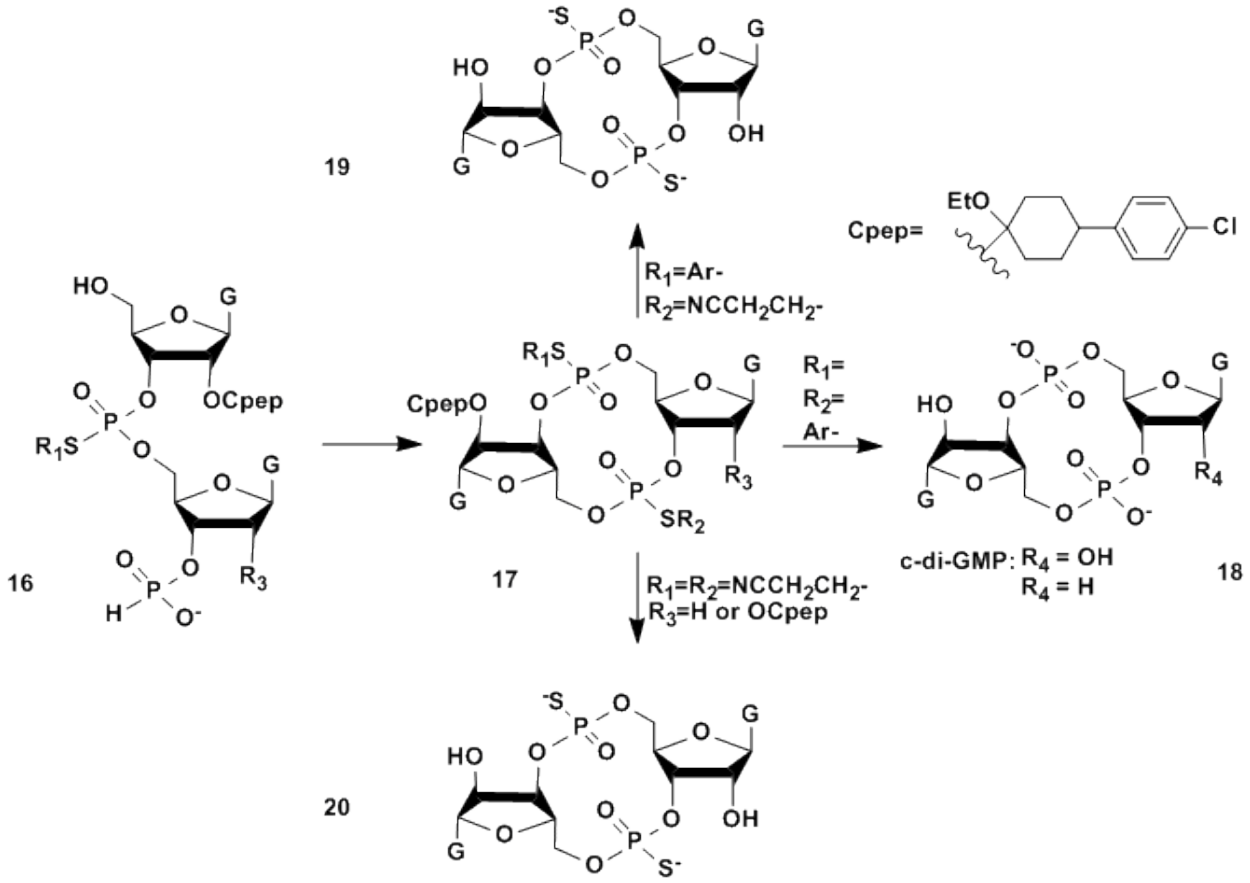
Scheme 1-3 H-Phosphonate approach for the synthesis of c-di-GMP.

Reagents and conditions: i) Bis(diisopropylamino)methyl or bis(diisopropylamino)cyanoethyl phosphoramidite and pyridinium trifluoroacetate; (ii) 2-chloro-4*H*-1,3,2-benzodiox- aphosphorin-4-one; (iii) pyridinium trifluoroacetate; (iv) *tert*-butylhydroperoxide; (v) sulfonic acid resin; (vi) adamantoylcarbonyl chloride; (vii) methanol/NBS; (viii) pyridine/aq NH_3 (1:1); (ix) TEA/HF.

1.6.2.4 Modified H-phosphonate approach

The modified H-phosphonate approach was first used by Yan and co-worker towards the synthesis of c-di-GMP in 2007.[59] In this approach 2′-OH was protected by the 1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl (Cpep) and H-phosphonate diesters were oxidized *in situ* with a sulfur transfer reagent to give the corresponding phosphorothioate triester

intermediate **16** (Scheme 1-4). The cyclization was effected by diphenyl chlorophosphate at -40ºC to give the protected cyclic compound **17**. After deprotection the final compound **18** was obtained without the need for HPLC purification. By using a different sulfur transfer reagent, it is also possible to synthesize phosphorothiaote analogus of c-di-GMP where the non-bridging oxygen is replaced by sulfur (compound **19** and **20**).[60]



Scheme 1-4 The modified H-phosphonate chemistry in c-di-GMP synthesis.

The modified H-phosphonat approach has its own advantages and disadvantages. The advantage of this approach is that the protecting groups are stable and remained intact until the end of the pathway. The Cpep protecting group precursor can be made easily with a high yield from inexpensive starting material. Like most other chemistry for the synthesis of c-di-GMP, the modified H-phosphonate approach also involves multistep synthesis, which is rather time consuming.

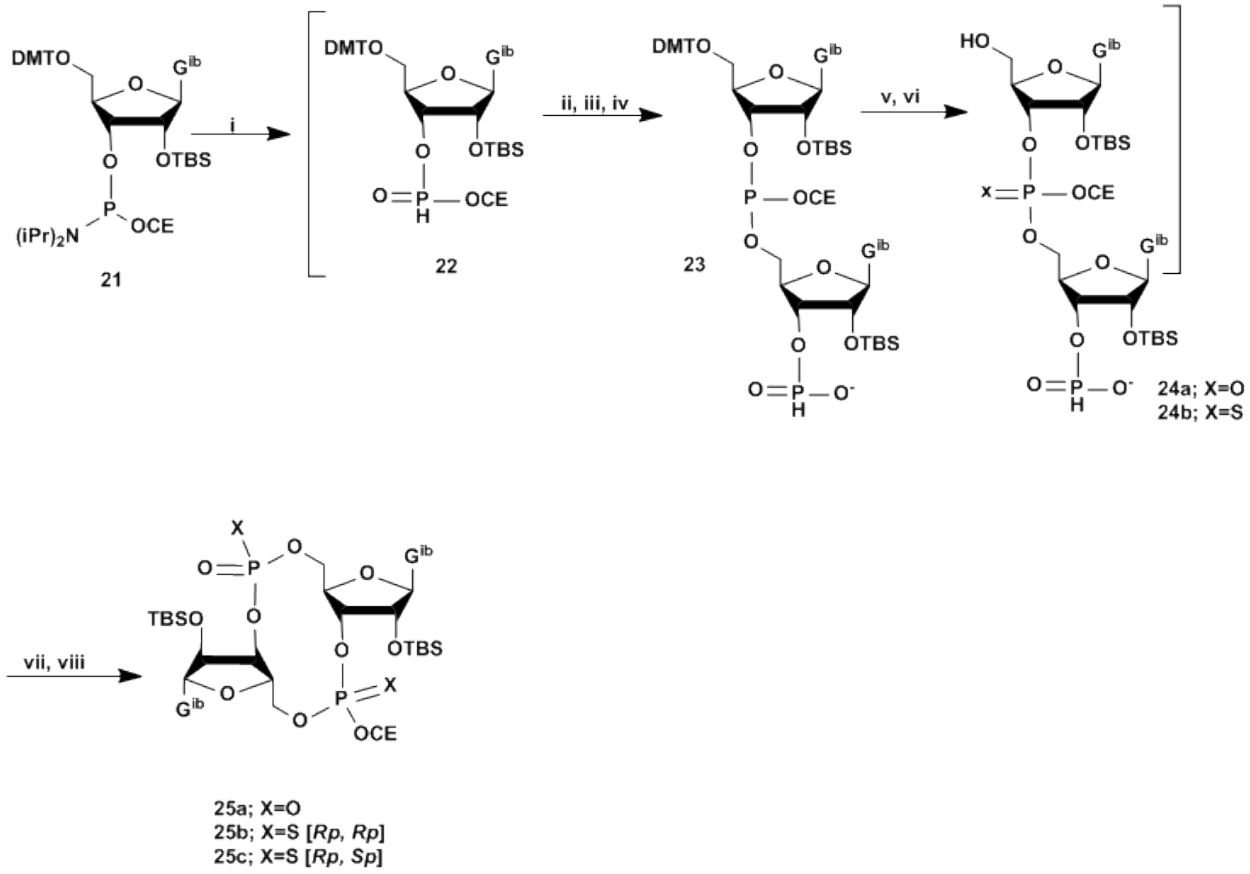
As this chemistry was used for synthesizing the modified c-di-GMP and its analogue c-di-CMP in this thesis, more detail on this chemistry will be provided in later chapters.

1.6.2.5 Large scale synthesis of c-di-GMP

In another route to synthesis c-di-GMP and analogues, the commercially available *N*-isobutyryl-2′-*O*-TBS-protected guanosine phosphoramidite **21** was used as starting material. In total, the protected cyclic derivatives **26a-c** were obtained in eight steps by extraction without isolation of intermediates.

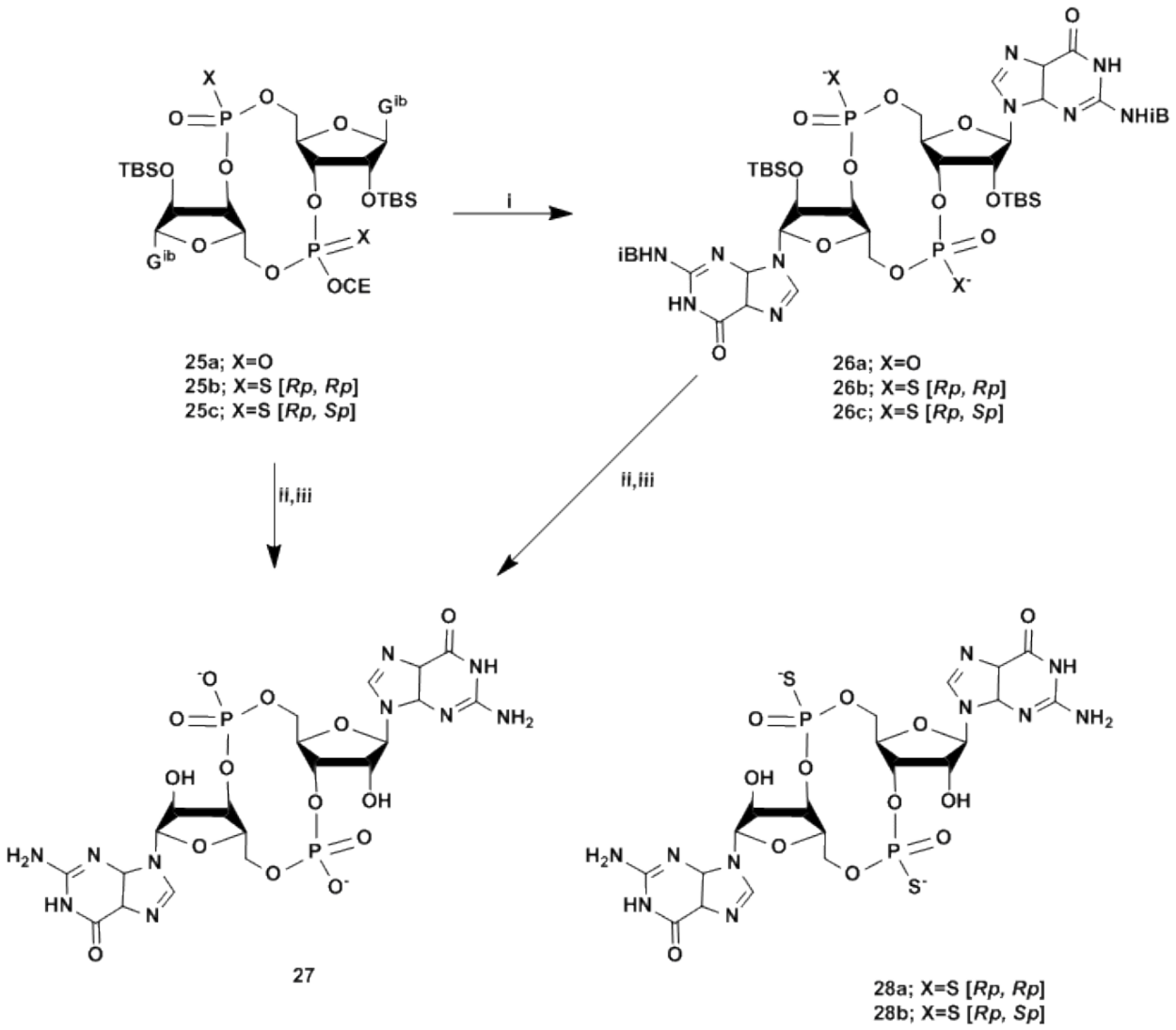
As shown in scheme 1-5, the first step in this synthesis is hydrolysis of the guanosine phosphoramidite **21** by treatment with pyridinium trifluoroacetate in acetonitrile containing two equivalents of water. The hydrolysis takes less than one min to give the corresponding H-phosphonate diester **22** which was further treated with *tert*-butylamine to remove the cyanoethyl group followed by detrityaltion with DCA, the detritylated compound was treated with the other portion of compound **21** to give a dimer **23**. Oxidation step was followed by using *tert*-butylhydroperoxide, or sulfurization using 3-((dimethylaminomethylidene)-amino)-3*H*-1,2,4-dithiazole-5-thione (DDTT). The solution is concentrated to convert the solvent to methylene chloride for the second detritylation, using DCA/water, to give the linear dimer **24a** or **24b**. The

Cyclization is effected by 3-3.5 equivalents of 5,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphinane (DMOCP) to the pyridine solution of **24a** or **24b** for 5-10 min. Pivaloyl chloride or adamantoyl chloride is used for cyclization and works well, but it was found that DMOCP gives slightly cleaner results compared with pivaloyl chloride.



Scheme 1-5 *Reagents and conditions:* i) pyridinium trifluoroacetate in acetonitrile /2 equiv of water, 1 min; ii) *tert*-butylamine, 10 min; iii) 3% dichloroacetic acid (DCA) 10 equiv water in methylene chloride, 10 min; iv) 21, 1.3 equiv 21, pyridine, acetonitrile; v) *tert*-butylhydroperoxide/3-((dimethylaminomethylidene) amino)-3*H*-1,2,4-dithiazole- 5-thione (DDTT), 30 min; vi) DCA/water; vii) 3-3.5 equiv of 5,5-dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphinane (DMOCP), 5-10 min; viii) iodine/water or 3*H*-1,2-benzodithiol-3-one, 5 min.

Final deprotection was a two-step, one-flask process was also developed where the final products (**27** and **28**) are isolated by crystallization, without the need for ion-exchange, or reverse-phase HPLC. The only chromatographic purification in this approach was the separation of the protected phosphorothioate diastereomers **25b** and **25c**, on a silica gel column.



Scheme 1-6 *Reagents and conditions:* i) *tert*-butylamine/acetonitrile; ii) methylamine in ethanol, rt, 1-2 h; iii) TEA.HF, 50°C, 60 min.

This route to c-di-GMP synthesis is based on the most common commercially available guanosine phosphoramidite. The phosphoramidites of many other nucleosides, and analogues, are also available, thus this route should be generally appropriate for preparation of a wide variety of other cyclic dinucleotides, including unsymmetrical molecules, as well as their phosphorothioate analogues.

The method reported here is significantly faster and more convenient than other approaches reported to date, allowing facile preparation of gram scale amounts of c-di-GMP.[61]

1.7 Choices of protecting group for 2′-OH and 5′-OH in RNA synthesis

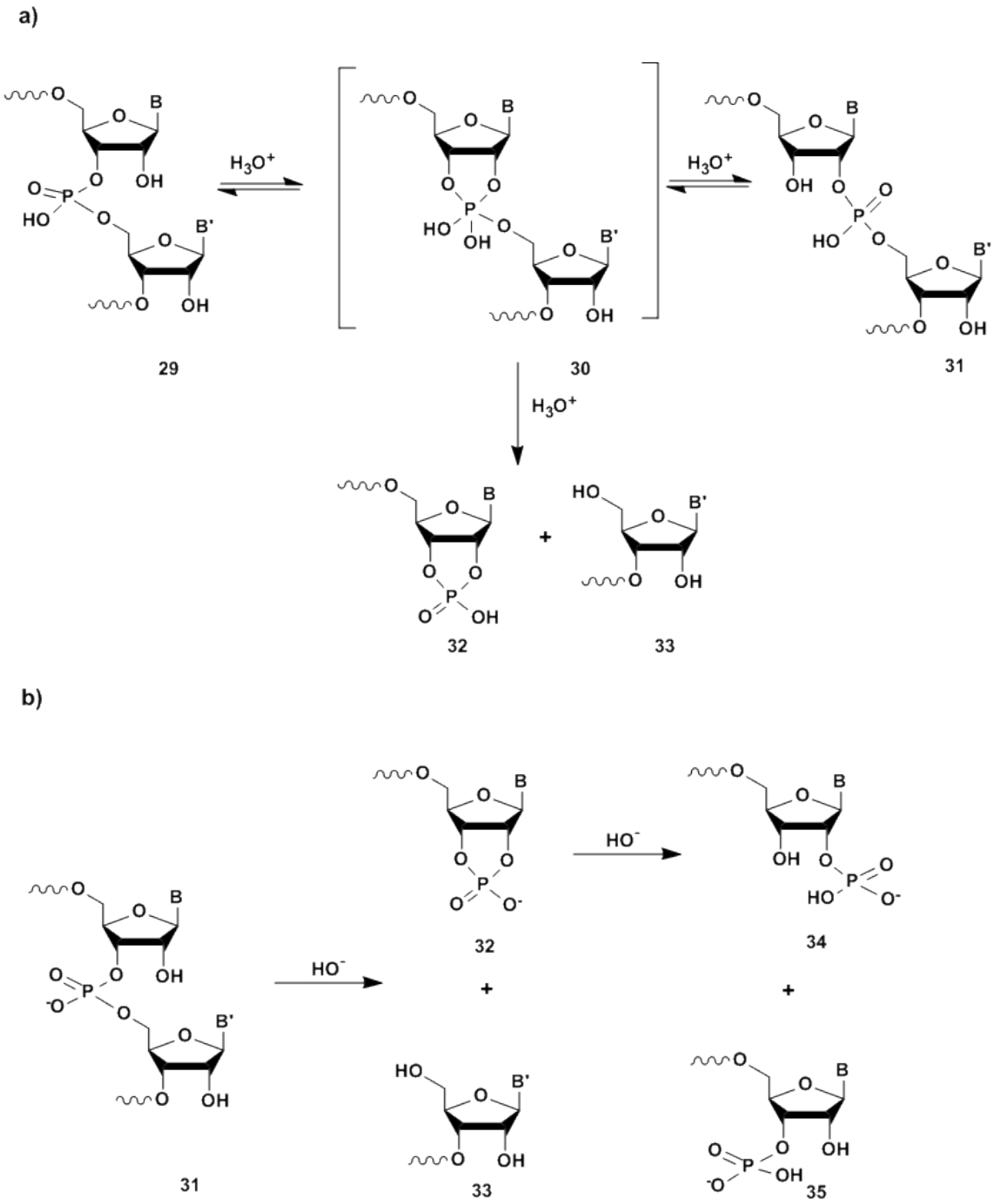
A key consideration in RNA synthesis is that 2′- and 5′-hydroxyls must be suitably masked by protecting groups that are removable under orthogonal conditions.

1.7.1 Choices of protecting group for 2′-OH

RNAs are sensitive to both acidic and basic conditions due to the presence of 2′-OH (Scheme 1-7). In both acidic and basic conditions RNA **29** cleavage occurs through an intermediate **30** to give the 2′,3′-cyclic phosphate **32** and fragment **33**. Under acidic conditions, in addition to the cleavage, migration of internucleotide phosphate linkages between 2′- and 3′-postions **31** also occurs.[62] Under basic conditions the newly formed cyclic phosphate **32** further undergoes hydrolysis to generate both 2′- and 3′-phosphates **34** and **35**, respectively.

Thus a suitable protecting group for the 2′-OH in oligoribonucleotide synthesis must meet the following criteria: 1) it must be stable under the acidic conditions used for repeated removal of dimethoxytrityl (DMTr) groups, which is the most common protecting group for 5′-OH used in both DNA and RNA synthesis. 2) It must be stable to the alkaline conditions that are used to cleave the assembled chain from the solid support and to remove the base and phosphate protecting groups. 3) It should be easily removable at the end of the synthesis under mild conditions that do not lead to modification of the nucleoside bases and cleavages or migration of the internucleotide linkages. 4) The 2′-OH protecting group should not be too bulky to affect coupling reactions. 5) The reagents employed for the removal of the 2′-OH protecting group should not make the purification of fully deprotected RNA too tedious or time-consuming.[63]

In this arena, a number of protecting groups for the 2′-OH in RNA synthesis have been reported. Depending on their deprotection conditions, these protecting groups can be classified into three broad categories: acid labile, base labile, and those that are removable under neutral conditions.



Scheme 1-7 Degradation of RNA in a) acidic and b) basic conditions.

1.7.1.1 Acid labile 2′-*O*-protecting groups

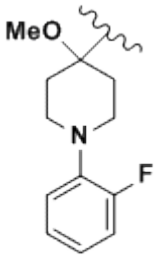
About twenty-five years ago, Reese and co-workers developed the tetrahydropyranyl (Thp)[64] and 4-methoxytetrahydropyran-4-yl (Mthp)[65] protecting groups for the 2′-hydroxyl of

ribonucleosides. However, it was later found that neither Thp nor Mthp groups are compatible with 5′-*O*-DMTr in oligoribonucleotide synthesis, as they are not stable under the acidic conditions used for detritylation.[66] Attempts to address this issue led to the development of 1-aryl-4-alkoxypiperidin-4-yl protecting groups that are more suitable in RNA synthesis, such as 1-(2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl (Ctmp)[67] and 1-(2-fluorophenyl)-4-methoxy piperidin-4-yl (Fpmp).[68] More recently, protected acetals, such as (2-nitrobenzyloxy)methyl (2-NBOM), (4-nitrobenzyloxy)methyl (4-NBOM), bis-(2-acetoxyethoxy)methyl (ACE) and acetal levulinyl ester (ALE), have been shown to be very useful protecting groups for 2′-OH in RNA synthesis.

1.7.1.2 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp)

Studies on the Ctmp protecting group led to the development of 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) **36**. Fpmp has been used successfully in the preparation of the ribonucleoside building blocks for the solid phase synthesis of RNA sequences.[69]

Studies suggest that Fpmp is compatible with the acidic conditions required for detritylation and is readily removable at pH 3.0. Only 1.1% of migration was observed during Fpmp deprotection under these conditions.



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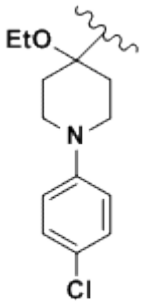
1.7.1.3 1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl (Cpep)

Compared with Ctmp and Fpmp, the rate of hydrolysis of the Cpep group **37** is virtually independent of pH between 0.5-2.5 (figure 1-13).[70] Further the Cpep protecting group can be more readily removed under acidic conditions without leading to cleavages or migration.

In this respect Cpep is 1.3 times more stable at pH 0.5 and 2.2 in comparison with Fpmp and Ctmp, while it is more acid-labile at pH 3.75, making it more suitable for the protection of 2′-OH in oligoribonucleotide synthesis. In addition, the synthesis of Cpep protecting group precursor involves an inexpensive material, *i.e. p*-chloroaniline, therefore, this group has the potential to be used in large scale synthesis of oligoribonucleotides.



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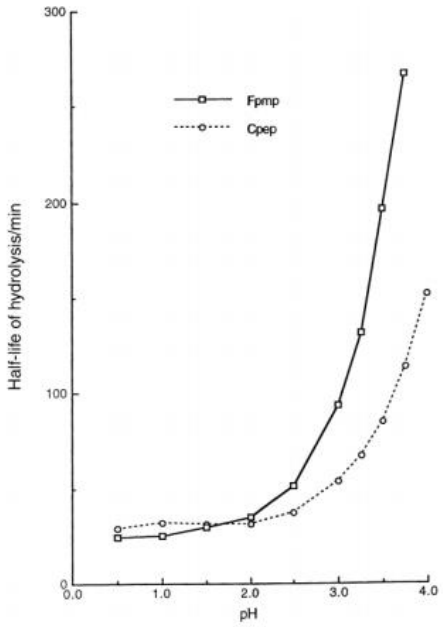


Figure 1-13 Dependence of the half-lives of hydrolysis at 30°C of the 2′-*O*-Fpmp and 2′-*O*-Cpep-uridines.[70]

1.7.1.4 2-Nitrobenzyloxymethyl (2-NBOM) and 4-nitrobenzyloxymethyl (4-NBOM)

These nitrobenzyl derivatives are unique 2′-protecting groups as their removal is achieved by photolysis. Unlike Ctmp, Fpmp and Cpep, the introduction of 2-NBOM and 4-NBOM (Figure 1-14) into ribonucleosides is not regioselective, leading to low yields in these reactions (20 to 40%).[71-74] Nevertheless coupling reactions of 2-NBOM and 4-NBOM protected ribonucleoside phosphoramidites typically give greater than 98% yields in two to three minutes. This coupling efficiency is attributed to the reduced steric bulk of these protecting groups. Complete removal of the 2-NBOM group may be achieved under photolytic conditions at pH 4.

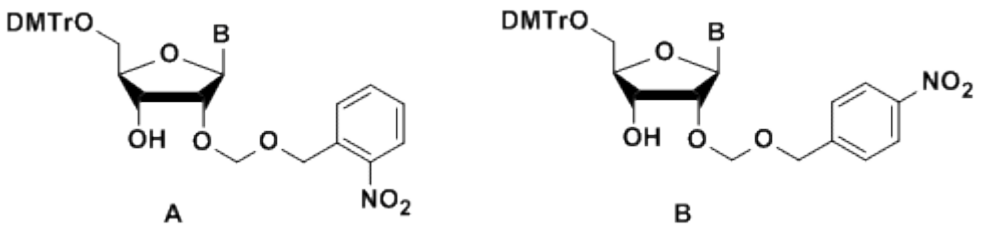
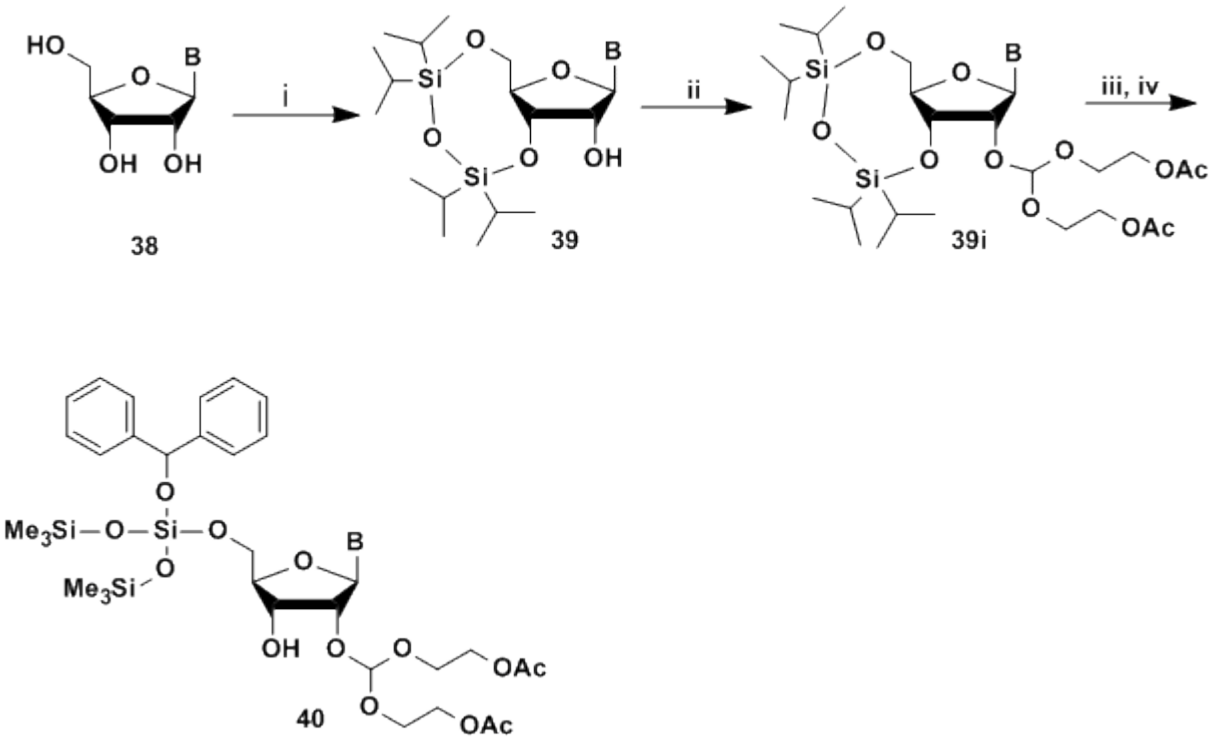


Figure 1-14 2′-(2-nitrobenzyloxy)methyl (A) and (4-nitrobenzyloxy)methyl (B) protected ribonucleoside.

1.7.2 Base labile 2′-*O*-protecting groups

1.7.2.1 Bis-(2-acetoxyethoxy)methyl (ACE)

The 2′-ACE protecting group was developed by Scaringe and co-workers.[75, 76] To introduce ACE at 2′-position, TIPDS protect 3′ and 5′ hydroxyl of compound **38**. It was followed by addition of ACE to protect 2′-hydroxyl (compound **39).** TIPDS easily would be deprotected . It was continued with protecting specifically 5′-hydroxyl with benzyhydroxy-bis(trimethylsilyloxy) silyl chloride (SIL) protecting group to reach compound **40**. (Scheme 1-8).



Scheme 1-8 5′-*O*-SIL-2′ -*O*-ACE Protected building block. *Reagents and conditions*: i) TIPDS chloride; ii) Bis-(2-acetoxyethoxy)methyl (ACE); iii) tetraethylammonium fluoride in acetonitrile (1.0 *M*, pH 8.0); iv) benzylhydroxy-bis(trimethylsilyloxy)silyl chloride, pyridine.

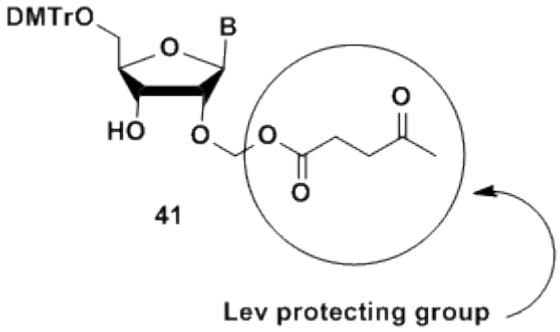
The advantage of the ACE protecting group is that it does not migrate between 2′- and 3′-positions. The coupling yield of ACE protected nucleoside phosphoramidites is as high as 99% with the coupling time of around 90 sec. Deprotection of ACE protected oligoribonucleotides is a two-step process. First, the deacetylation under basic conditions, using 40% aqueous $MeNH_2$, gives the 2′-*O*-bis(2-hydroxyethoxy)methylorthoester, which is more labile under acidic condition compared with ACE. This is followed by the treatment with tetramethylethylethylenediamine (TEMED)-acetate buffer (0.1 *M*, pH 3.8) at 60°C for 30 min to give the fully deprotected oligoribonucleotides. One disadvantage with the use of

the 2′-ACE protected building blocks is their incompatibility with conventional solid phase synthesizers due to the use of a silyl protecting group for the 5′-hydroxyls.[77]

1.7.2.2 Acetal levulinyl ester (ALE)

Levulinyl (Lev) was original proposed by Damha *et al*. as a protecting group for 2′-OH in RNA synthesis. It was not surprising; however, that migration of the Lev group between 2′- and 3′-OH became a serious concern. Damha and co-workers later reported the acetal levulinyl ester (ALE) for the protection of 2′-OH in RNA synthesis. Unlike Lev, the introduction of ALE is regiospecific with good yields. The ALE group, once installed, does not migrate either.

This protecting group was shown to give 98% stepwise coupling yield in RNA synthesis.[78] An additional utility of this group is that it allows for the preparation of siRNA pro-drugs through conjugation of amino acids with the carbonyl on the ALE group.[79]

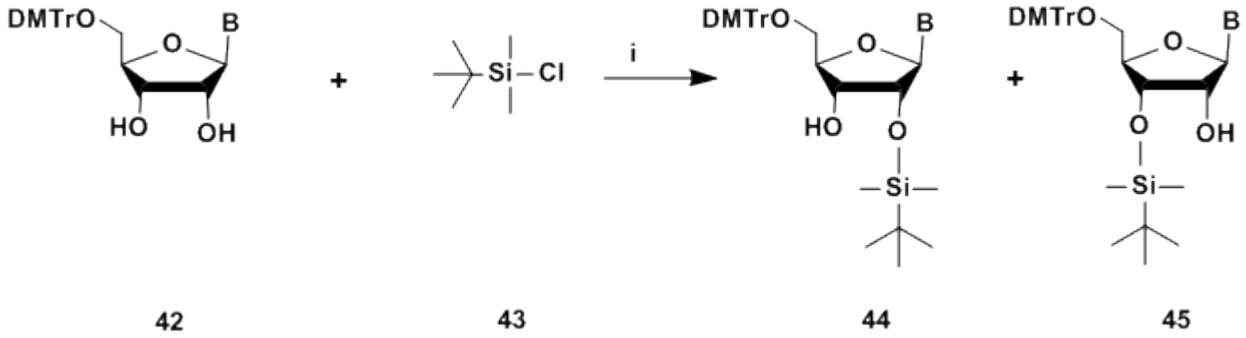


1.7.4 2′-*O*-protecting groups removable under neutral conditions

1.7.4.1 *tert*-Butyldimethylsilyl (TBDMS)

The *tert*-butyldimethylsilyl (TBDMS) protecting group is the most widely used for protecting 2′-OH of ribonucleoside and 2′-*O*-TBDMS protected ribonucleoside phosphoramidites **44** are commercially available. The TBDMS protecting group can easily be removed by the treatment with tetra-*n*-butylammonium fluoride (TBAF).[80] However, TBDMS-based ribonucleoside building blocks suffer from several disadvantages: 1) incorporation of the

TBDMS into ribonucleosides lacks regioselectivity between 2′- and 3′-hydroxyl functions. Thus, a mixture of regioisomers is obtained (Scheme 1-9). After the desired 2′-TBDMS ribonucleosides are obtained, there is a tendency for the TBDMS group to migrate between 2′- and 3′-positions. 3) The coupling time associated with 2′-TBDMS protected phosphoramidites in solid phase synthesis is relatively long, typically around 10 min, which could lead to a partial loss of the 5′-*O*-DMTr protecting group. This relatively low reactivity can be attributed to the relatively large steric hindrance of the TBDMS as a protecting group.

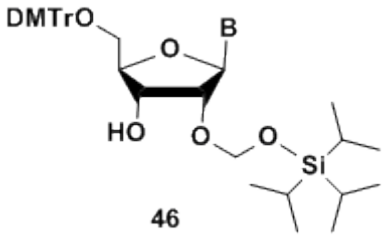


Scheme 1-9 Lack of regioselectivity in the TBDMS protection of DMTr protected riboside.

Reagents and conditions: i) DCM, triethylamine.

1.7.3.2 [(Triisopropylsilyl)oxy]methyl (TOM)

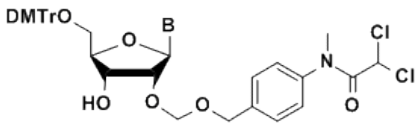
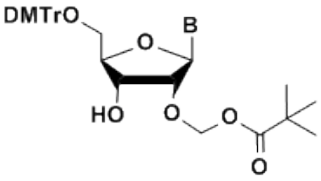
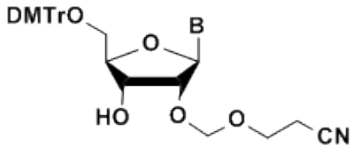
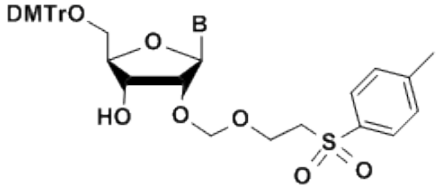
[(Triisopropylsilyl)oxy]methyl (TOM) group (as shown in **46**) is a protected protecting group. Deprotection of TOM is triggered by the treatment with fluoride. Unlike the TBDMS, the TOM group does not migrate once it is installed at the 2′-*O*-position; however, the introduction of TOM into ribonucleosides is not regiospecific, as is the case for TBDMS.[81] The coupling reaction of 2′-*O*-TOM protected phosphoramidite building blocks is very efficient, leading to stepwise coupling yields of 99% in three minutes. As such, the TOM group has been widely used in solid phase synthesis of oligoribonucleotides.



46

Some other protecting groups for the 2′-OH developed for oligonucleotide synthesis are shown in Table 1-2. Despite the availability of these protecting groups, there still remains a need for the development of RNA chemistry that meets all the requirements (Refer to 1.7.1) and that can be readily carried out on large scales to meet the increasing needs for therapeutic RNAs.

Table 1-2 Miscellaneous 2′-protecting groups for ribonucleosides.

Name	Structure	Deprotection conditions
4-(N-dichloroacetyl-N-methylamino)benzyloxymethy (4-MABOM)[82, 83]		NH_4OH, TEMED-acetate buffer (0.1 *M*, pH 3.8), 90ºC, 30 min
(pivaloyloxy)methyl (PivOM)[84, 85]		10% piperidine, ammonium hydroxide, r.t., 3 h.
(2-cyanoethoxy)methyl (CEM)[86, 87]		TBAF in DMSO (0.5 *M*) with 5% nitromethane, r.t., 5 h.
[2-(toluene-4-sulfonyl) ethoxy]methyl (TEM)[88, 89]		TBAF in THF (1.0 *M*), 10% piperidine, r.t., 20 h.

1.7.4 Choices of protecting group for 5′-OH in RNA synthesis

Protecting groups for 5′-OH must be compatible with others, particularly the 2′-*O*-protecting group in RNA synthesis. With the exception of the ACE chemistry, where a silyl protecting group is used for 5′-OH, most RNA chemistry uses an acid labile protecting group to mask 5′-OH.[90] The most popular protecting groups for 5′-OH are 9-(*p*-tolyl)xanthene (Px or pixyl) and dimethoxytrityl (DMTr) groups (Figure 1-16).

There are some advantages in using the pixyl group. The pixyl group is about 3 times more readily removable under acidic conditions compared with the DMTr group,[90] therefore

its use shortens the exposure of oligonucleotides to acids. When 2′-OH is protected by an acetal group, such as Cpep, this feature is particularly useful as shortened acid exposure minimizes the potential loss of the Cpep group. In this regard, the pixyl group is completely deprotected with trifluoroacetic acid in the presence of pyrrole in 10 seconds. Under this condition, Cpep is completely intact.

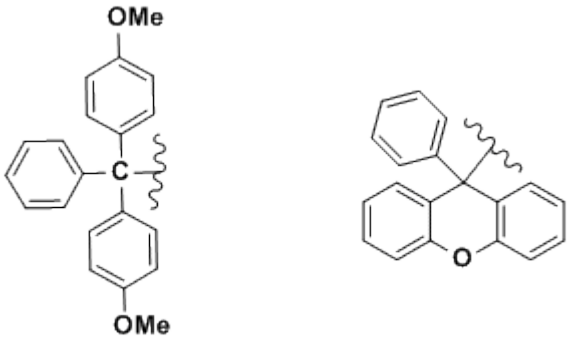
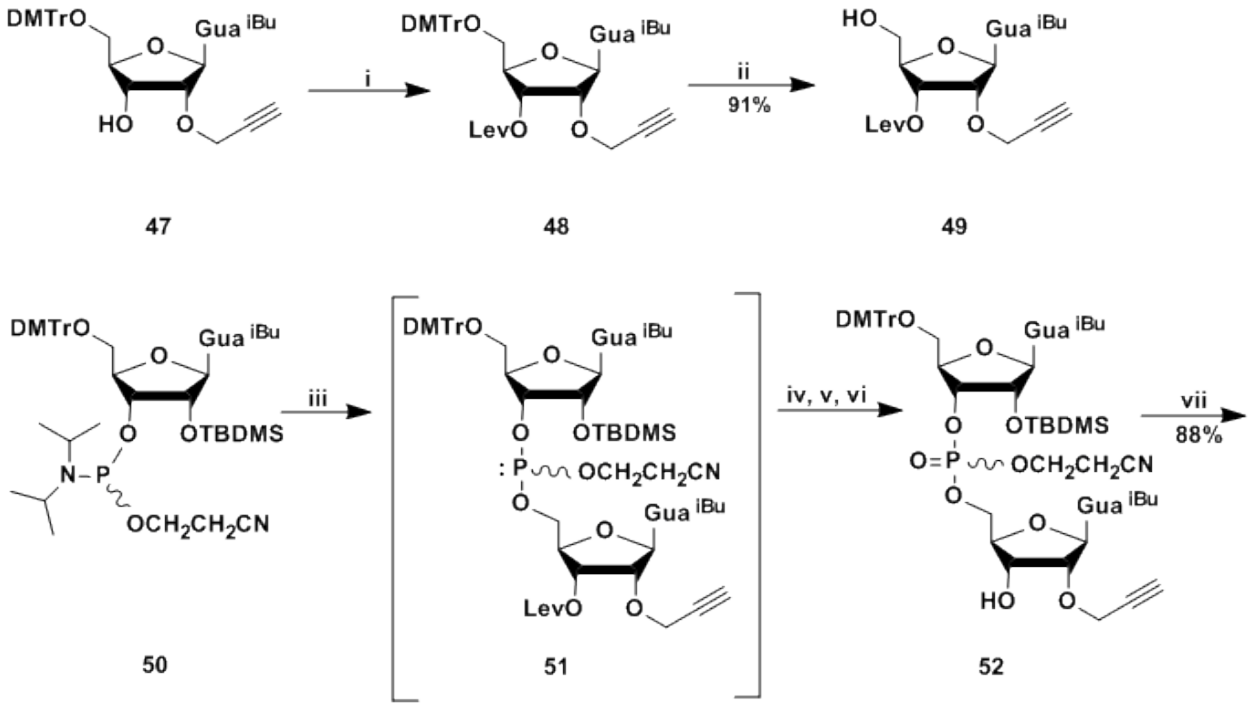


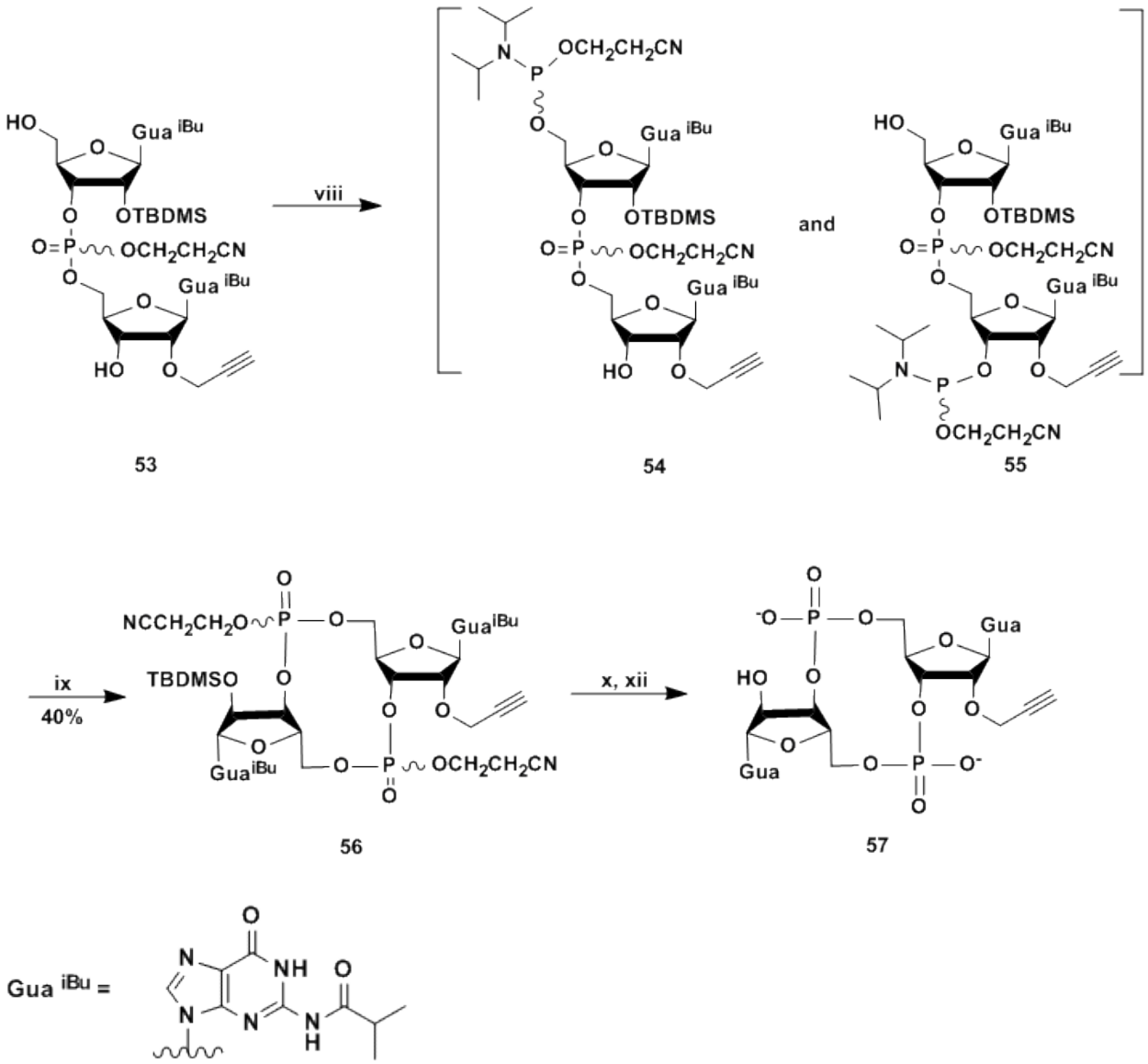
Figure 1-15 DMTr and Pixyl groups.

1.8 Labelling c-di-GMP at 2′ position

Despite the importance of c-di-GMP in regulating bacterial processes, and its potential applications in modulating immune systems in mammalian, there is a general lack of knowledge in the cellular and tissue distributions of c-di-GMP, and proteins that bind to c-di-GMP. In this respect, it would be beneficial to introduce a functional group to c-di-GMP that will allow for the tracking of c-di-GMP and identification of c-di-GMP binding proteins. In 2010 Serge L. Beaucage and co-workers demonstrated a strategy for the synthesis of the propargylated c-di-GMP **57.** The synthesis was based on two commercially available starting materials **47** and **50** as building blocks. The 2′-propargylated monomer **49** was readily accessible in two steps from 2'-*O*-propargyl-5'-*O*-DMTr-*N*2-isobutyrylguanosine. This monomer was then coupled with phosphoramidite **50** to generate the corresponding linear dimer **52**. After removal of the DMTr group, phosphitylation led to formation of a mixture of two phosphoramidites **54** and **55**, which afforded the fully protected cyclic dimer **56** upon

cyclization. Deprotection finally yielded c-di-GMP bearing a propargyl group at the 2'-position (Scheme 1-10).





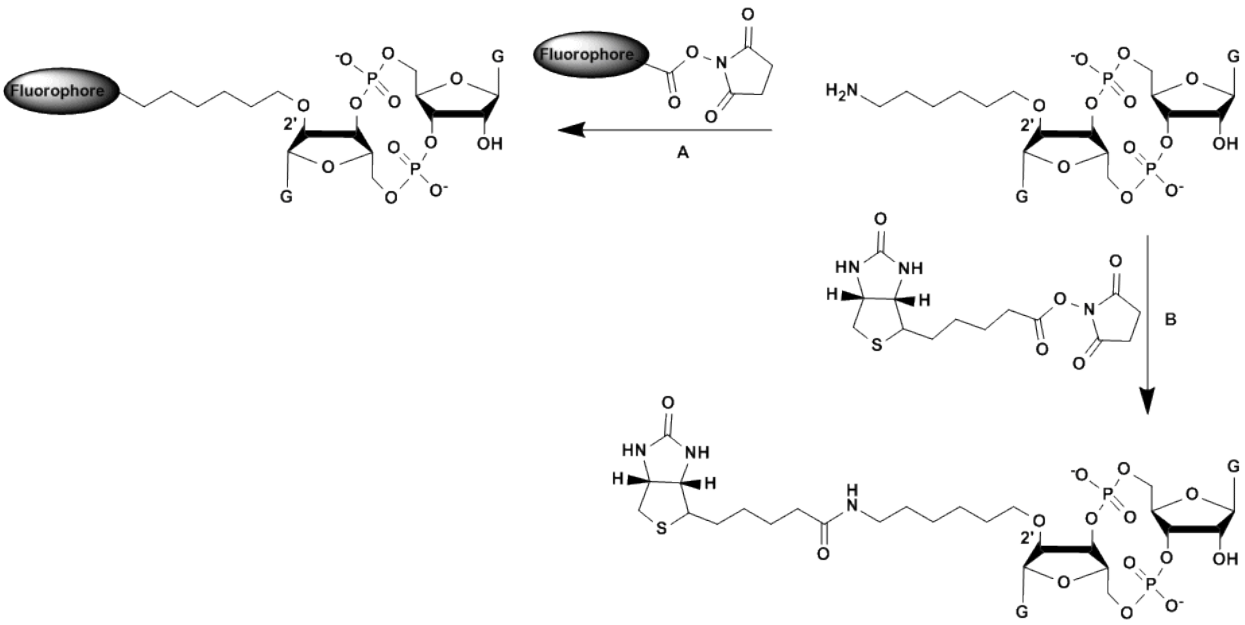
Gua, guanin-9 yl; iBu, isobutyryl; DMTr, 4,4′-dimethoxytrityl; TBDMS, *tert*-butyldimethylsilyl; Lev, levulinyl.

Scheme 1-10 *Reagents and conditions:* i) levulinic anhydride, pyridine, 25°C, 16 h; ii) 80% AcOH, 3h; (iii) 1*H*-tetrazole, MeCN, 49, 2 h, 25°C; (iv) tert-BuOOH/decane, 30 min; (v, vi) hydrazine hydrate/AcOH/C_5H_5N, 15 min, then 2,4-pentanedione, 5 min; (vii) 80% AcOH, 3 h, 25°C. (viii) $(i\text{-}Pr_2N)_2POCH_2CH_2CN$, 1*H*-tetrazole (1.0 equiv at the rate of 0.25 equiv/15 min), MeCN, 1 h; (ix) 1*H*-tetrazole (2 equiv), MeCN, 16 h; (x) conc. aq. NH_3, 30 h, 25°C; (xi) $Et_3N{\bullet}3HF$, 20 h, 25°C.

Objective

This thesis addressed two objectives. First, the cytidine analogue of c-di-GMP, *i.e*. c-di-CMP, was synthesized. As described previously in the introduction, the structural requirements for cyclic dinucleotides as immunostimulating agents and vaccine adjuvants remain unclear. Availability of c-di-GMP analogues, such as c-di-CMP, will allow for the exploration of structure-activity relationship in this vein. The cytidine analogue was chosen as the synthetic target because the immunostimulatory activity and adjuvanticity of c-di-CMP have never been evaluated.

Second, synthesis of c-di-GMP analogues that are modified at one of the 2′-OH functionalities of guanosine was attempted. The ultimate goal of this study relates to the labeling of c-di-GMP by a fluorophore or biotin (Scheme 1-11) that will allow for tracking cellular and tissue distribution of c-di-GMP, and elucidation of proteins that bind to c-di-GMP. Towards this goal, synthesis of guanosine bearing a reactive group, such as azido and amino, at 2′-OH through an alkyl linker was explored.



Scheme 1-11 Label c-di-GMP with a fluorophore (A) or biotin (B) through at 2′-position through an alkyl linker.

Chapter 2

Results and discussion

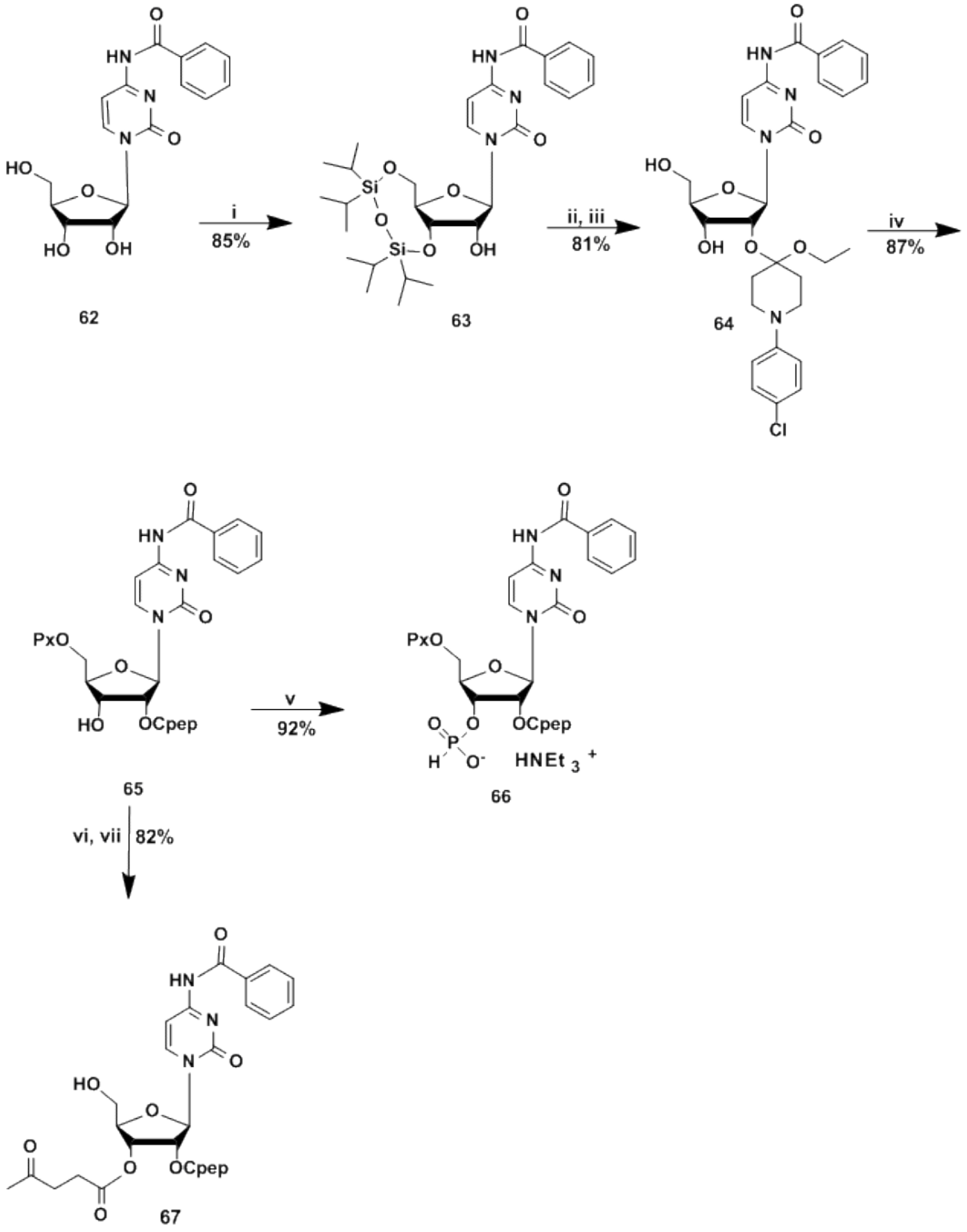
2.1 Synthesis of c-di-CMP

In the modified H-phosphonate approach, both oligonucleotide phosphates and phosphorothioates can be readily isolated in high yields due to their stability. Fully protected c-di-CMP can also be purified to very high purity. The protecting groups are chosen in a fashion where they can be removed without leading to cleavage of phosphodiester linkages or modification to the nucleobase residues. In this thesis, 1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl (Cpep) was used to mask 2′-hydroxyls due to the advantages outlined previously in Chapter 1.

2.1.1 Chemical synthesis of c-di-CMP

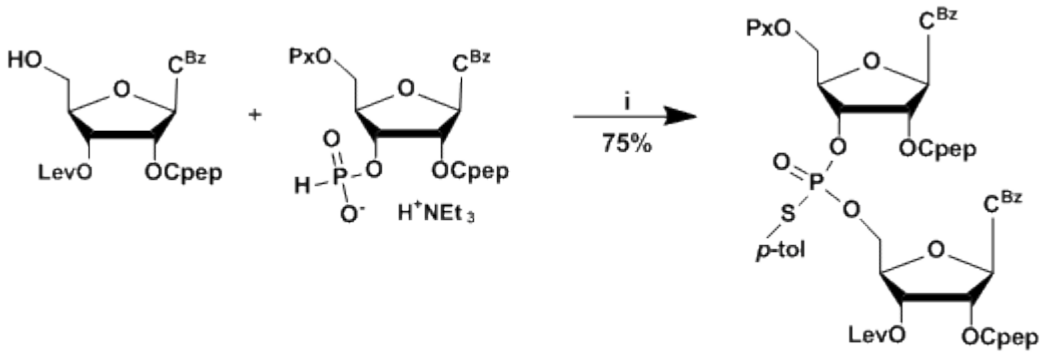
The synthesis of c-di-CMP was initiated by the TIPDS protection of the commercially available *N*4-benzoyl-cytidine **62**. This reaction was accomplished by treating *N*4-benzyol-cytidine **62** with TIPDS chloride in pyridine at 0ºC to give compound **63** in 85% yield. Subsequently the 2′-OH of **63** was protected with the 1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl **(**Cpep) group by the treatment with enol ether in the presence of trifluoroacetic acid in dry dichloromethane. Without purification the 2′-*O*-Cpep-3′,5′-TIPDS protected *N*4-benzyol-cytidine was subjected to TIPDS deprotection by the treatment with tetraethylammonium fluoride in acetonitrile (1.0 *M*, pH 8.0) to give compound **64** in 81% overall yield. The 5′-OH of the compound **64** was subsequently protected with pixyl by the treatment with pixyl chloride in pyridine to give compound **65** in 87% yield (Scheme 2-1). The monomer **65** was then further transformed into two building blocks. First, treatment of the monomer **65** with *p*-tolyl H-phosphonate and pivaloyl chloride in pyridine gives the corresponding 3′-H-phosphonate **66** in 92% yield after column chromatography. On the other hand, the monomer **65** was treated with levulinic anhydride in pyridine in the presence of a catalytic amount of

dimethylaminopyridine to give the corresponding 3′-*O*-Lev protected monomer. Removal of 5′-pixyl with trifluoroacetic acid in the presence of pyrrole led to the building block **67** in 82% yield.



Scheme 2-1 Chemical synthesis of c-di-CMP. *Reagents and conditions:* i) $(i\text{-Pr})_2Si(Cl)OSi(i\text{-Pr})_2(Cl)$, C_5H_5N; ii) Cpep enol ether, CH_2Cl_2, CF_3CO_2H, 5h; iii) tetraethylammonium fluoride (1.0 *M*, pH 8.0), iv) PxCl, C_5H_5N, 40 min; v) H-phosphonate, pivaloyl chloride, C_5H_5N, 0ºC, 1 h; vi) $(Lev)_2O$, CH_2Cl_2, dimethylaminopyridine, Et_3N, 30 min; vii) pyrrole, CH_2Cl_2, CF_3CO_2H, 30 sec.

The H-phosphonate **66** and the monomer with free 5′-OH **67** were then subjected to treatment with pivaloyl chloride to give the corresponding H-phosphonate diester, which was subsequently transformed into *S*-(*p*-tolyl)phosphorothioate triester **68** by treatment *in situ* with 2-phenylsulfanyl-1*H*-isoindole-1,3(1*H*)-dione. The phosphorothioate triester **68** is stable and was isolated in a good yield (75%) by column chromatography.



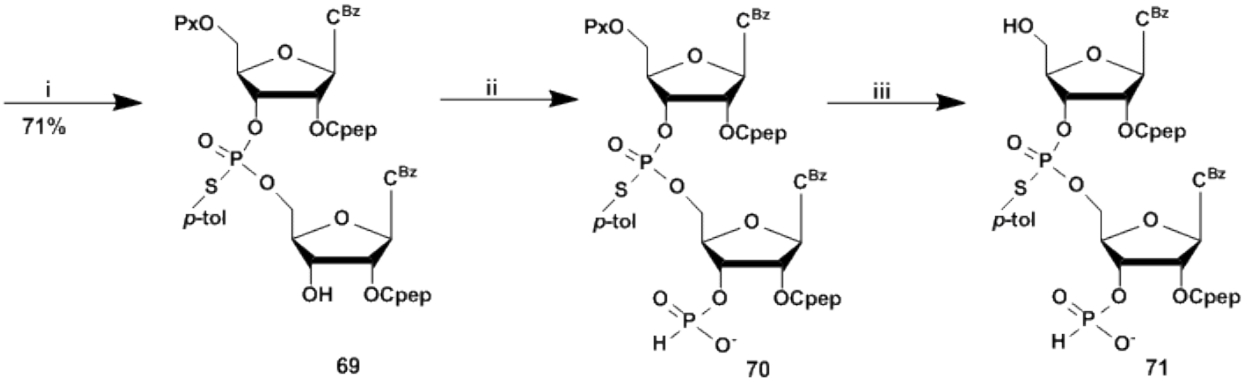
67 66 68

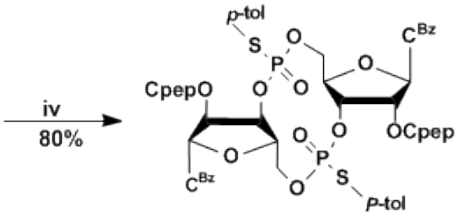
Scheme 2-2 Chemical synthesis of c-di-CMP. *Reagents and conditions:* i) $(CH_3)_3CCOCl$, C_5H_5N, sulfur transfer reagent (Scheme 2-5), 30 min.

Removal of 3′-levulinyl from the fully protected linear dimer **68** was effected by the treatment with hydrazine hydrate. The resulting product from levulinyl deprotection, Px-*Cp(s)C*-OH* **69**, was then transformed into the corresponding H-phosphonate Px-*Cp(s)C*p(H) **70** by treatment with *p*-tolyl H-phosphonate in the presence of pivaloyl chloride. Px-*Cp(s)C*p(H) **70** was subsequently treated with trifluoroacetic acid in the presence of pyrrole to remove the 5′-pixyl protecting group (compound **71**). Under this condition, pixyl group is completely removed in as short as 30 seconds.

*Abbreviation is followed for protected oligonucleotides in which nucleoside residues and internucleotide linkages are italicized if they are protected in some defined way. In the present context, *C″* represents cytidine protected on *N*-4 with a benzyl group; and *p(s ′)* represents an *S*-phenyl-protected phosphorothioate.

The purified dimer H-phosphonate HO-*Cp(s)C*p(H) **71** was subjected to cyclization effected by diphenylchlorophosphate at -40ºC. It is noted that this reaction must be carried out under high dilution conditions to avoid formation of linear oligomer products. The high dilution was effected by the slow addition of a solution of HO-*Cp(s)C*p(H) **71** in dry pyridine to a solution of diphenylchlorophosphate in dry dichloromethane. The purified fully protected cyclic dinucleotide **72** showed one signal in the ^{31}P NMR spectrum (Figure 2-1).





Scheme 2-3 Chemical synthesis of c-di-CMP. *Reagents and conditions:* i) C_5H_5N, $NH_2NH_2•H_2O$, $C_2H_4O_2$, H_2O; ii) H-phosphonate, C_5H_5N, $(CH_3)_3CCOCl$, 0ºC, 1 h; iii) CH_2Cl_2, pyrrole, CF_3CO_2H, 30 sec; iv) $(PhO)_2P(O)Cl$, CH_2Cl_2, C_5H_5N, sulfur transfer reagent, -40ºC.

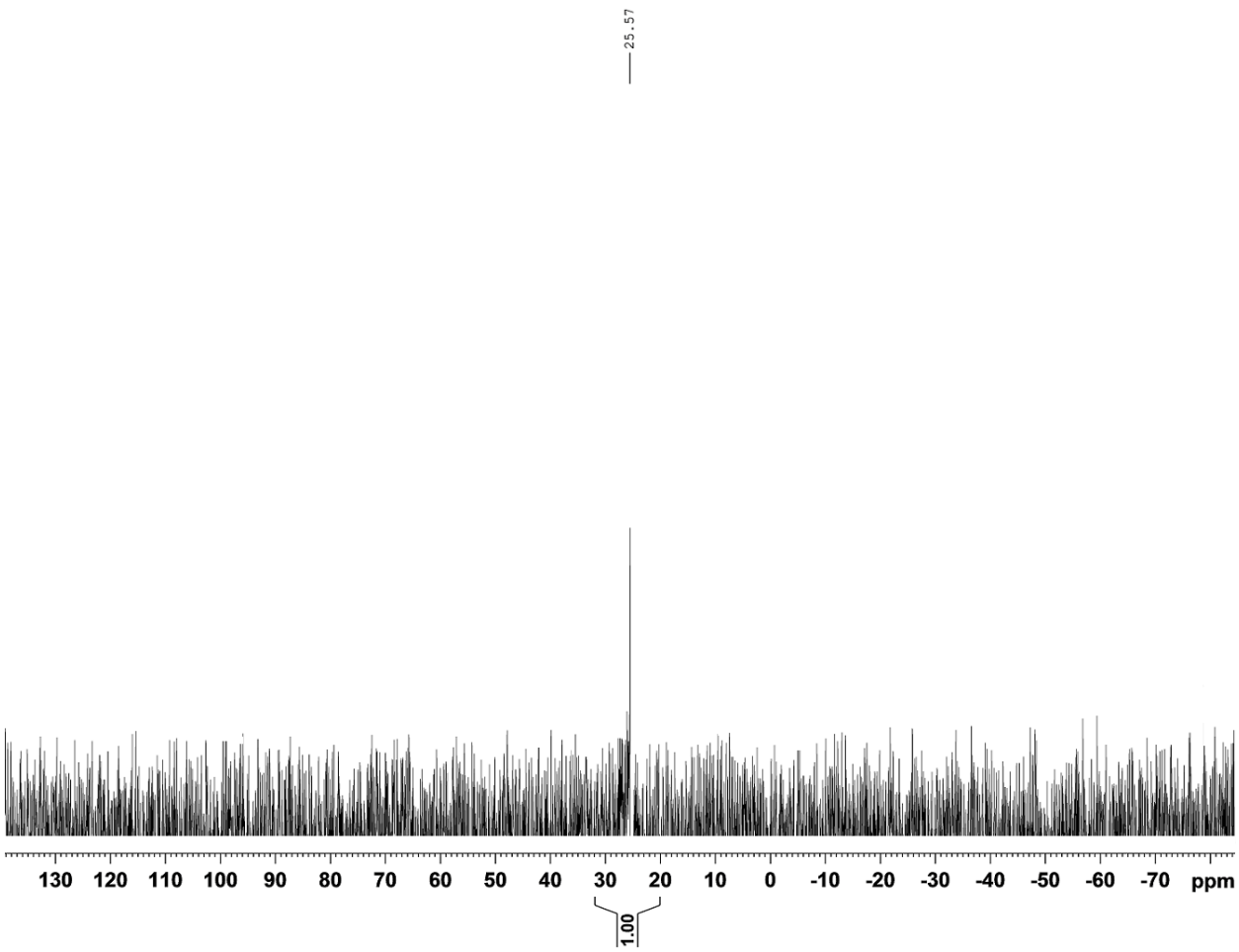
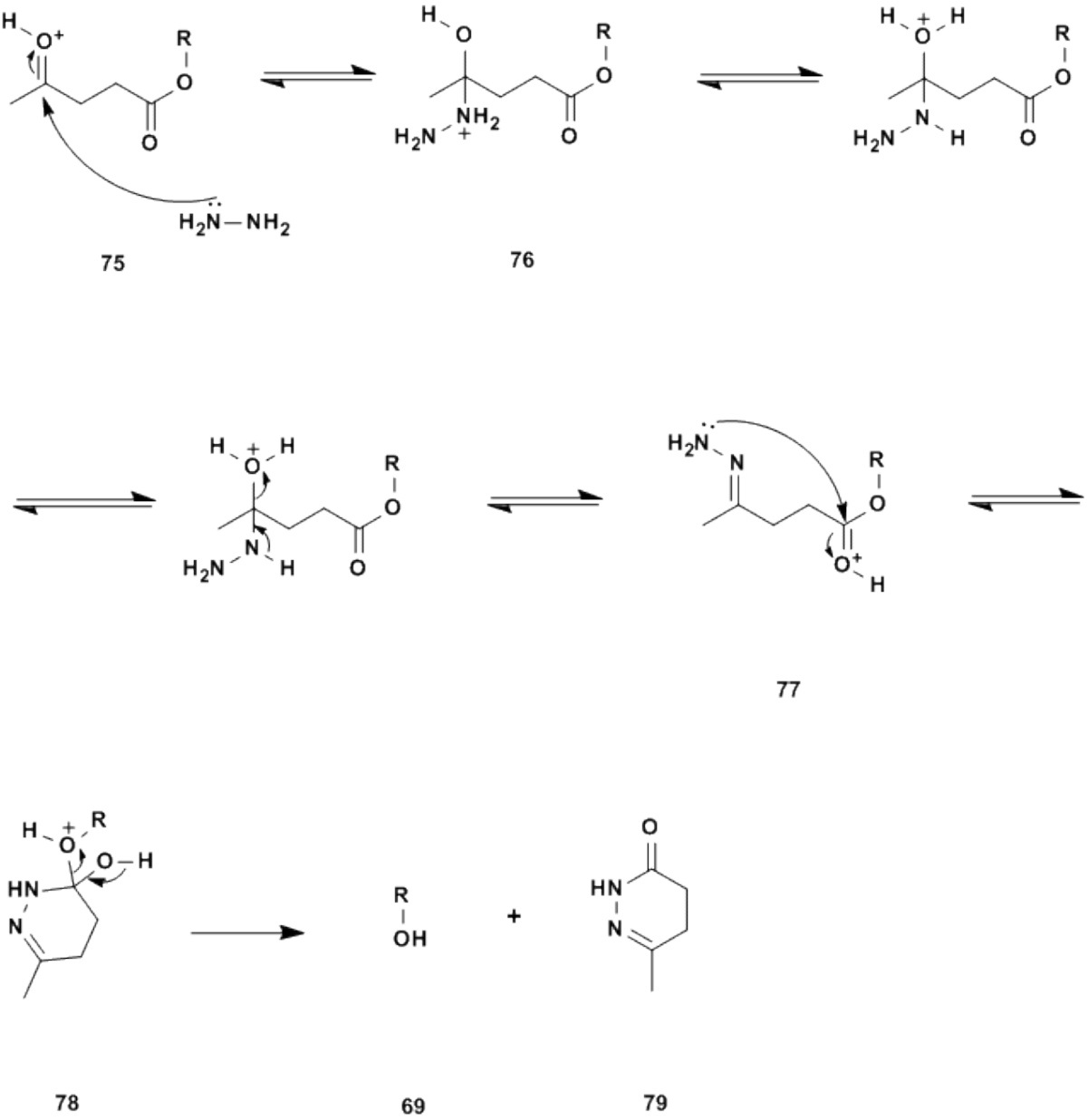


Figure 2-1 ^{31}P-NMR of fully protected c-di-CMP **69**.

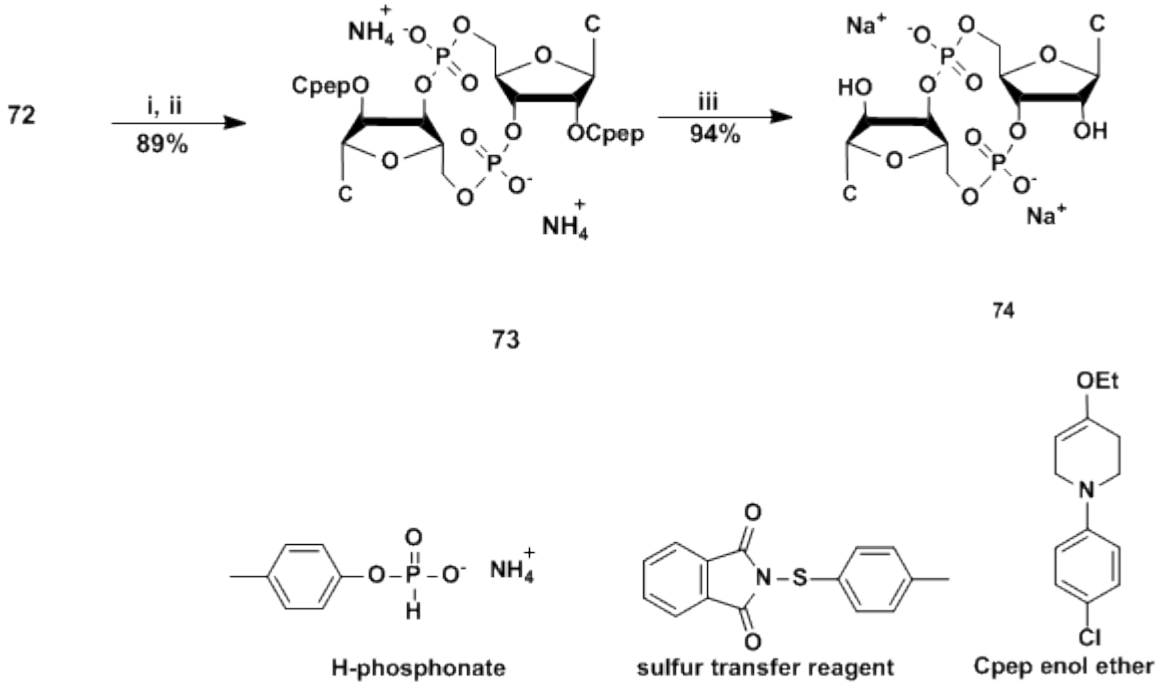
It is noted that during the removal of levulinyl group, hydrazine hydrate must be added as a pre-mixed solution with acetic acid-pyridine-water, as hydrazine can modify nucleobases if not neutralized by acetic acid. The mechanism for the deprotection of levulinyl ester is shown in scheme 2-2. Acid will activate the ketone of the lev group in compound **75**, which leads to the nucleophilic addition of hydrazine (compound **76**) and subsequent condensation to give the compound **77**. In the next step, the second nitrogen of hydrazine in compound **77** undergoes nucleophilic attach at the carbonyl group of the ester, which leads to deprotection of the lev group from the 2′-OH (compound **78)**. This reaction leads to the formation of a cyclic hydrozoneamide **79** as a by-product (shown in Scheme 2-4). The excess of hydrazine should be treated with pentane-2,5-dione prior to aqueous workup.



Scheme 2-4 Deprotection mechanism of Lev protecting group.

Deprotection of the fully protected cyclic dinucleotide **72** involved a three-step process. First, compound **72** was treated with 2-nitrobenzaldoxime in the presence of *N*,*N*,*N′*,*N′*-tetramethylguanidine. This is followed by incubation in concentrated aqueous ammonium hydroxide at 55ºC for 12 hours. The partially protected c-di-CMP, *i.e*. all protecting groups except 2′-*O*-Cpep **73** were removed, was then subjected to incubation in a mixture of

dimethylacetamide (DMA) and triethylammonium formate buffer (0.5 *M*, pH 2.52). Note that this mixture showed an apparent pH of 4.0 with a pH meter. After the reaction mixture was heated at 40ºC for 5 hours, the products were cooled, and the pH was adjusted to 7.0 with triethylamine. The products were then extracted with chloroform to remove DMA. The fully deprotected c-di-CMP was then precipitated by addition of *n*-butanol. This precipitation procedure also removes the organic salts that were generated in the deprotection process. The product was then eluted through an Amberlite (IR120, Na^+ form) cation-exchange column to give the sodium salt of c-di-CMP. The product was characterized by ^1H and ^{31}P NMR, reverse phase HPLC, and mass spectrometry. As shown in ^{31}P NMR (figure 2-2) and HPLC profile (Figure 2-3), the c-di-CMP was quite pure. The above mentioned synthetic strategy allowed us to obtain the fully deproteced c-di-CMP **74** in pure form simply by precipitation after deprotection, without using HPLC purification, which makes the process efficient for a large scale synthesis.



Scheme 2-5 Chemical synthesis of c-di-CMP. *Reagents and conditions:* i) 2-Nitrobenzaldoxime, *N*,*N*,*N*′,*N*′-Tetramethylguanidine, 16 h; ii**)** *aq*. NH_3 55ºC, 15 h; iii) acetonitrile, NEt_3-HCOOH buffer (0.5 *M*, pH 2.52), 40ºC, 5 h.

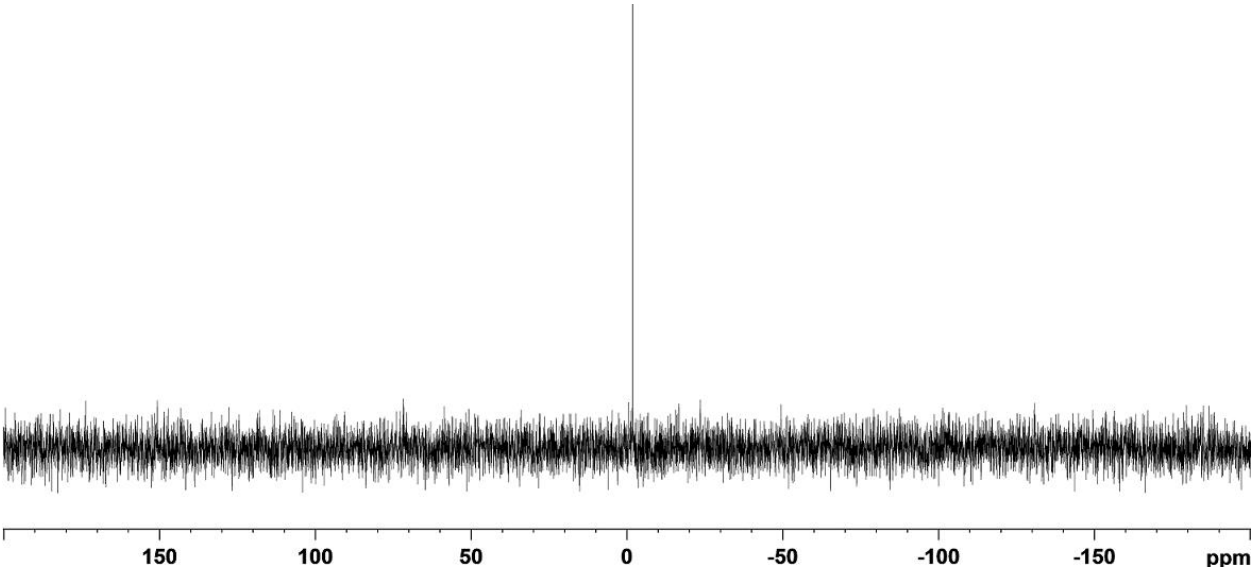


Figure 2-2 ^{31}P-NMR of fully deproteted c-di-CMP **74** (^{1}H decoupling).

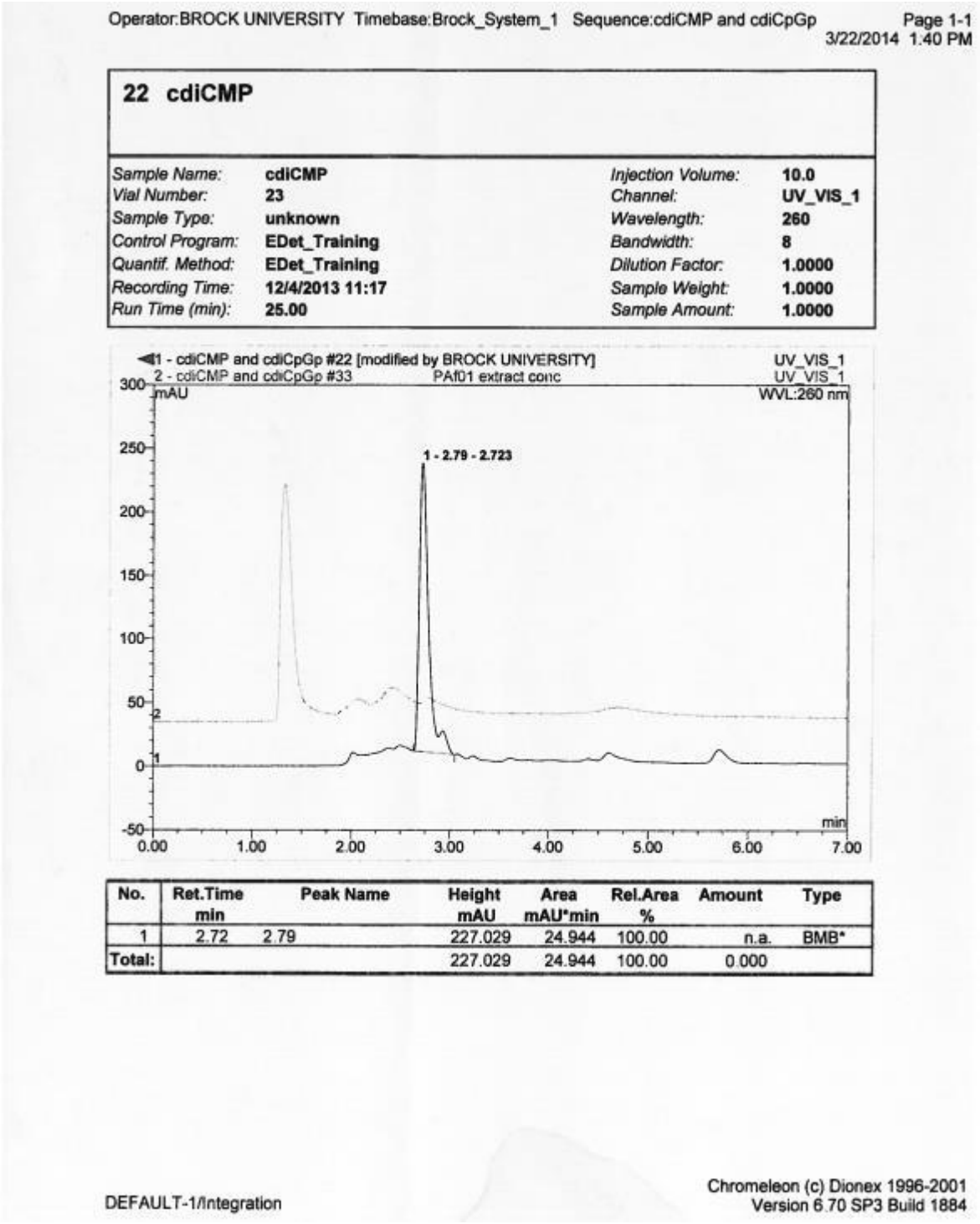
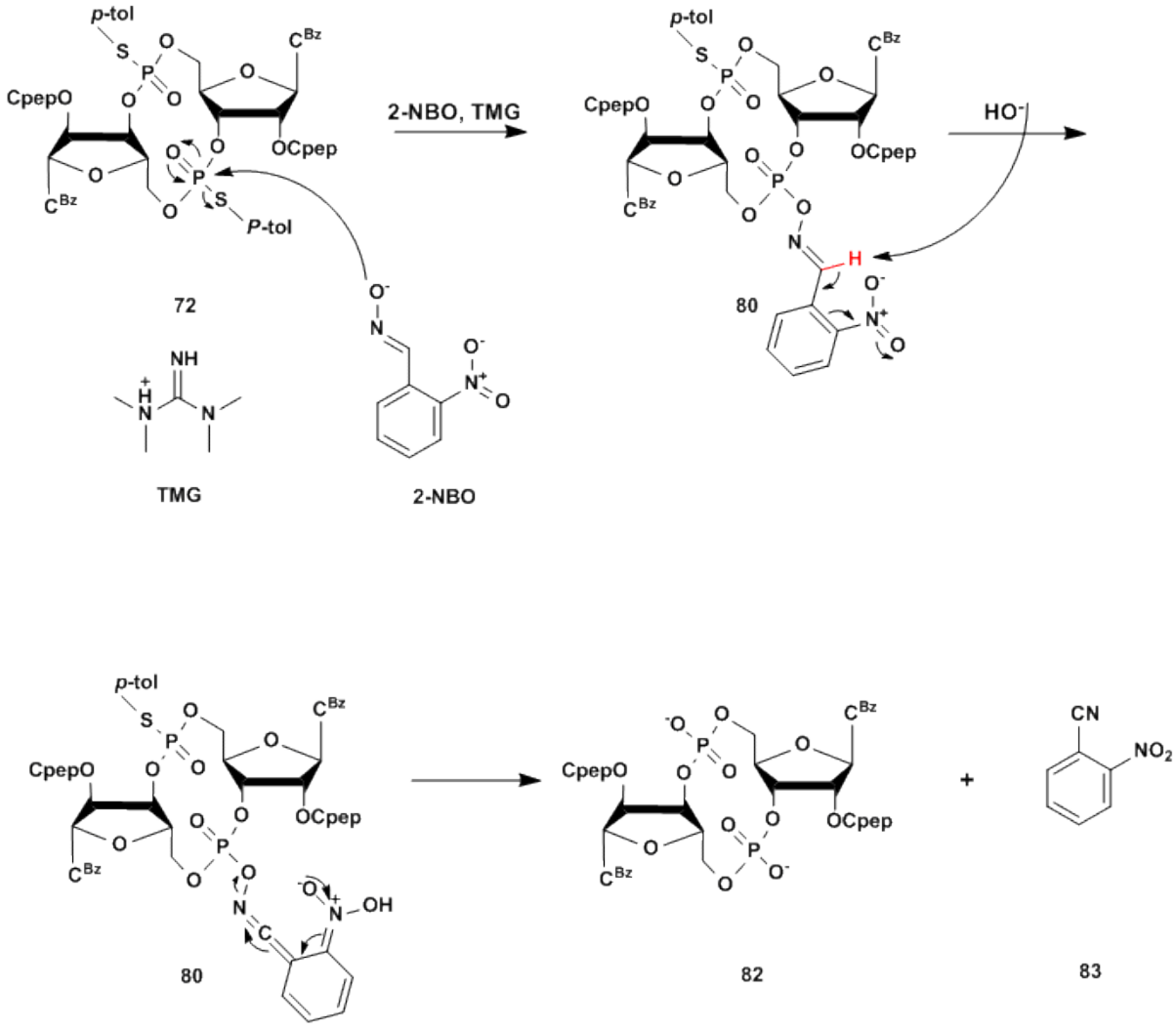


Figure 2-3 HPLC profile for c-di-CMP **74**.

The HPLC analysis was carried out on a C18 reverse phase Clarity 3u Oligo rp 3 micron column (50 x 4.60mm). The column was eluted at 0.80 mL/min with the following program: linear gradient of triethylammonium acetate buffer (10 *mM* pH 7.0) – acetonitrile (100:00 v/v to 85:15 v/v) over 10 minutes and then isocratic elution.



Scheme 2-6 Mechanism of action of 2-NBO.

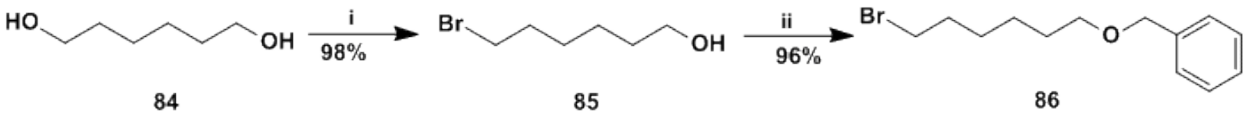
The role of 2-NBO in this deprotection sequence is shown in Scheme 2-6. The oximate first displaces the *S*-(*p*-tolyl) group on the phosphorothioate to give the corresponding oximate ester **80** (Scheme 2-6). The latter subsequently undergoes *β*-elimination upon incubation in aqueous ammonium hydroxide to give corresponding phosphate diester, releasing 2-nitrobenzonitrile as by-product (compound **83**). The use of 2-NBO to deprotect phosphorothioate triester is essential as direct treatment of phosphorothioate triesters with ammonium hydroxide leads to undesired chain cleavages on the phosphate backbone.[92]

2.2 Synthesis of c-di-GMP modified at 2′-*O*-position with a C6 linker

As we discussed in objective Scheme 1-11 the purpose of synthesizing c-di-GMP modified at 2′-*O*-position is to label it with fluorophore or biotin. The biotinated c-di-GMP entails a triazole that is very close to c-di-GMP, which could interfere with the binding of c-di-GMP with its receptor. As such, it would be advantageous to use a longer linker that is less likely to affect the binding. In this thesis, a C6-alkyl chain with terminal alkyne is used for the conjugation purpose.

2.2.1 The chemical synthesis of guanosine modified at 2'-*O*-position with a C6 linker.

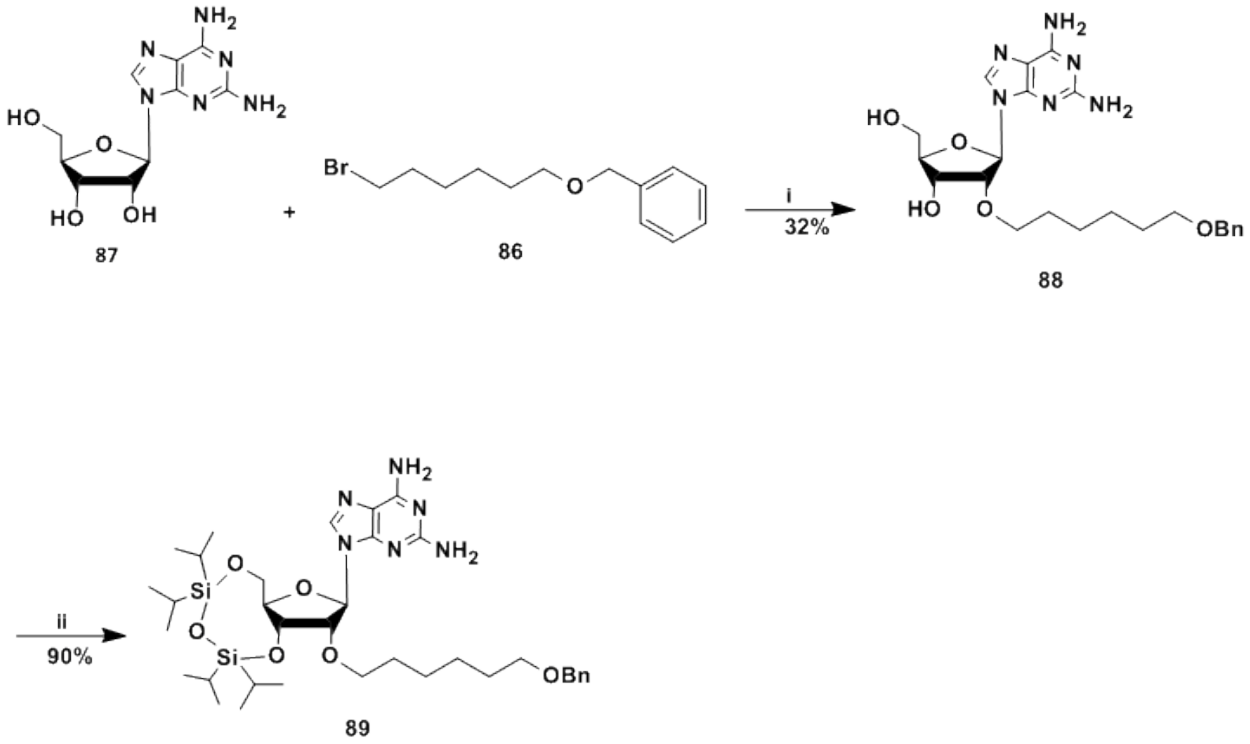
In this thesis, a C6 alkyl linker was used to attach either biotin or fluorophores to 2′-OH. The synthesis started with the preparation of 6-bromohexanol **85** by treating 1,6-hexandiol **84** with hydrobromic acid (48%) in toluene as the commercially available 6-bromohexanol is rather expensive. The hydroxyl in 6-bromohexanol was subsequently protected with a benzyl group by treatment with benzyl bromide in the presence of sodium hydride (Scheme 2-7). It is noted that the compound **86** must be distilled under reduced pressure to remove all residual moisture; or the subsequent alkylation reaction will not proceed.



Scheme 2-7 the synthesis of 6-bromohexyl benzyl ether **86**. *Reagents and conditions:* i) HBr, toluene, reflux 2 h; ii) NaH, THF, 6-bromo-hexanol, benzyl bromide, 24 h.

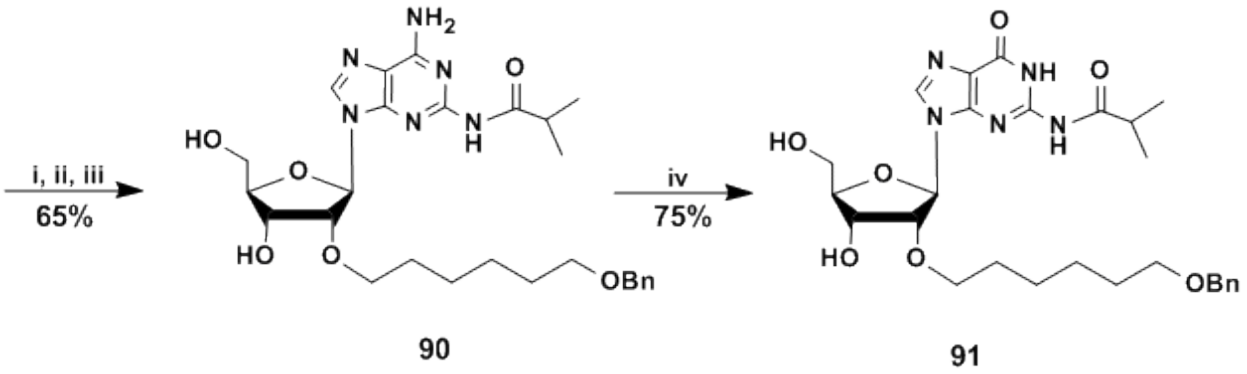
In the next step, diaminopurine riboside **87** was alkylated with 6-bromohexyl benzyl ether **86** Scheme 2-8. It is worth noting that all of the 2′-, 3′-, and 5′-hydroxyls was protected in the alkylation. As a consequence, the alkylation led to the formation of a mixture of products. Considering the fact that the 2′-OH is slightly more acidic than 3′- and 5′-hydroxyls in

ribonucleosides, it is perhaps not surprising that the 2′-*O*-alkylated product **88** was isolated in *ca*. 35% yield.[80] In earlier attempts, guanosine, instead of diaminopurine riboside was used as the starting material for this alkylation reaction, which inevitably led to alkylation at the guanine residue. Attempts were also made to protect 3′- and 5′-hydroxyls with protecting groups such as TIPDS. However, TIPDS tends to be cleaved during the alkylation reaction (It is not shown). The 2′-*O*-alkylated diaminopurine riboside **88** was subsequently protected with TIPDS at the 3′- and 5′-positions **89** in good yield (Scheme 2-8).



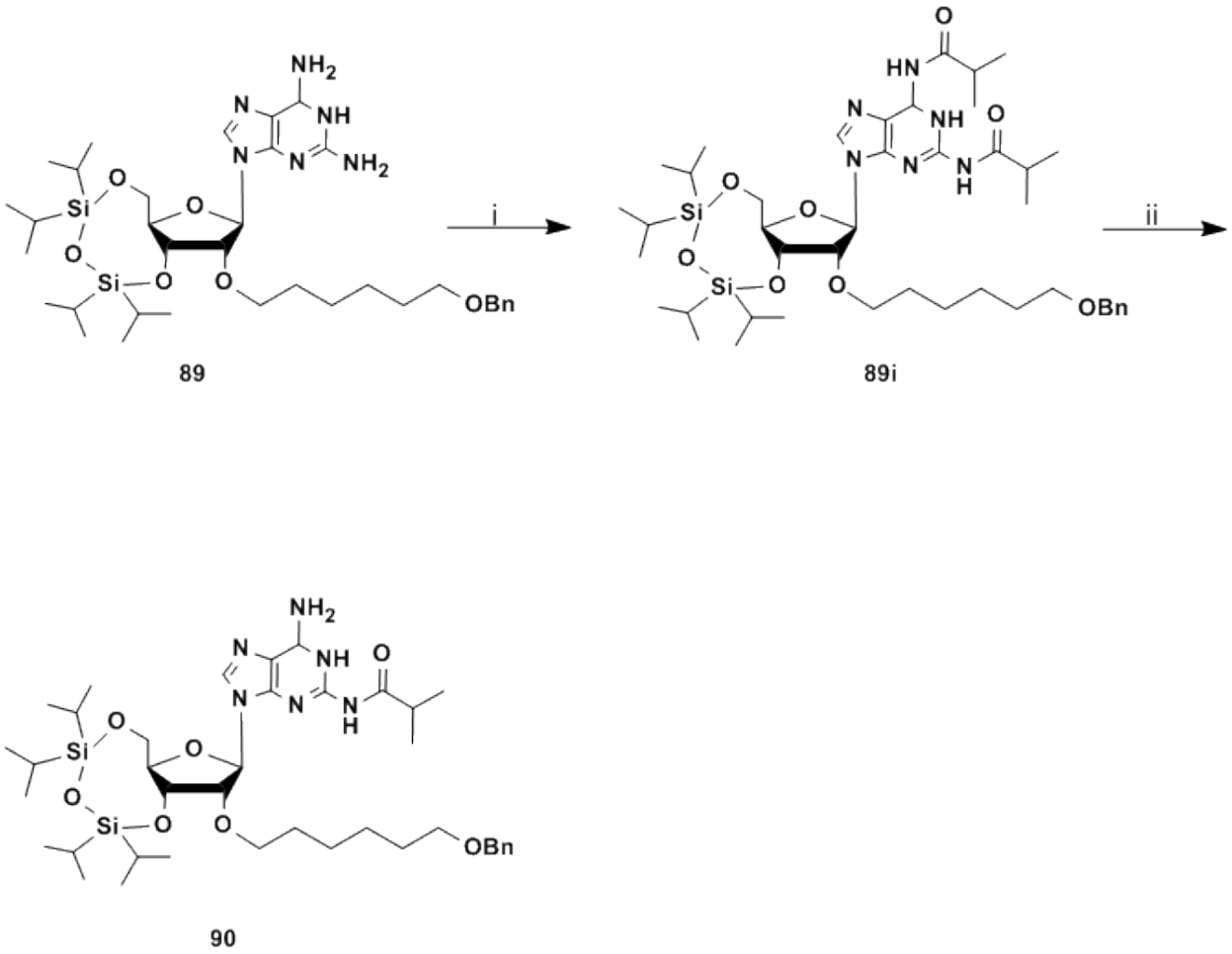
Scheme 2-8 Chemical synthesis of guanosine modified at 2'-*O*-position with a C6 linker. *Reagents and conditions:* i) NaH, $(CH_3)_2NCH$, 48 h; ii) $(i\text{-Pr})_2Si(Cl)OSi(i\text{-Pr})_2(Cl)$, C_5H_5N.

Then it was followed by acetylation of 2′-*O*-alkyl-3′,5′-*O*-TIPDS diaminopurine riboside **89** to get the desired *N*2-isobutyryl diaminopurine riboside **90**, which was obtained in 65% yield. It was continued by converting **90** to guanosine (Scheme 2-9).



Scheme 2-9 Chemical synthesis of guanosine modified at 2'-*O*-position with a C6 linker. *Reagents and conditions:* i) tetraethylammonium fluoride (1.0 *M*, pH=8.0), CH_3CN; ii) $(CH_3)_2CHCOCl$, C_5H_5N; iii) *aq*. NH_3 55ºC, 20 h; iv) $CH_3CN(CH_3)_2$, CH_3COOH, H_2O, $NaNO_2$, 7 days.

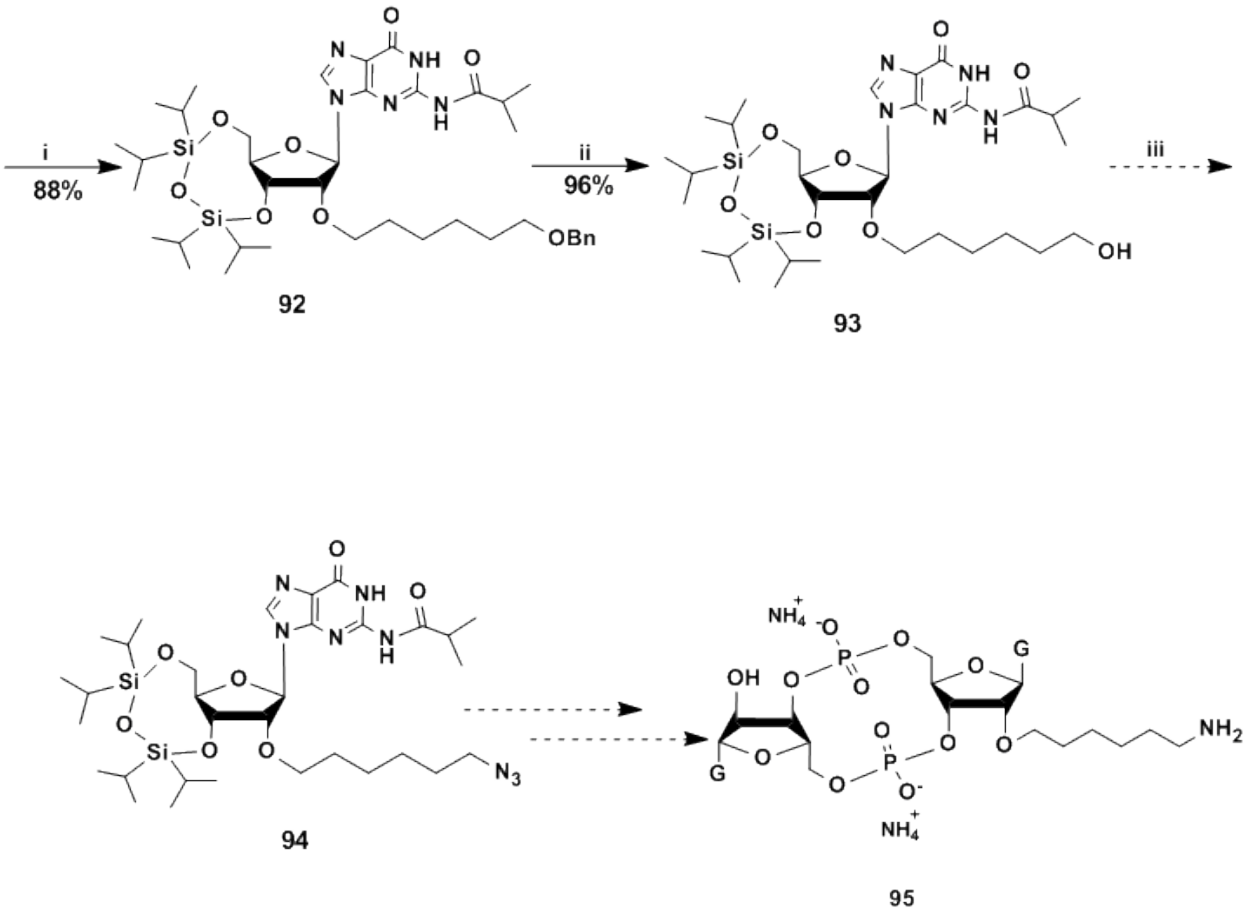
To get the desired acetylated compound **90** three steps combined to getter, as can be seen in Scheme 2-9. A literature procedure to first bisacylate 2,6-diamine followed by selective deacylation of the *N*6-acyl group was used. This was accomplished by first treating with isobutyryl chloride to acylate both 2- and 6-amine (intermediate **89i**), and then using aqueous ammonia (Scheme 2-9).



Scheme 2-10 Chemical synthesis of guanosine modified at 2'-*O*-position with a C6 linker. *Reagents and conditions:* i) C_5H_5N, $(CH_3)_2CHCOCl$; ii) *aq*. NH_3 55ºC, 20 h.

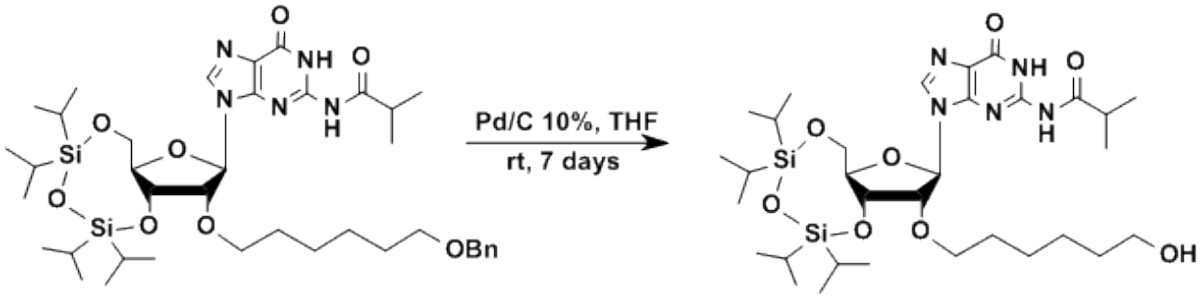
In purpose of converting **90** to guanosine, in earlier attempts to accomplish this goal, an enzymatic approach was used. Thus, either 2′-*O*-alkylated-3′,5′-*O*-TIPDS protected diaminopurine riboside **90** or 2′-*O*-alkylated diaminopurine riboside **89** was treated with adenosine deaminase in aqueous DMSO in the presence of tris(hydroxymethyl)aminomethane hydrochloride. The reactions were extremely slow (>7 days) and the conversion was very low, most probably due to the poor solubility of the substrate. An alternative deamination approach was found to be more effective. Thus 2′-*O*-alkylated-3′,5′-*O*-TIPDS protected diaminopurine riboside **90** was first treated with sodium nitrite in dimethylacetamide to generate a diazonium species, followed by treatment with aqueous acetic acid at 4°C. The reaction was also rather slow (7 days) and addition of

multiple portions of sodium nitrite was required to make compound **92**, however, deamination was complete and the procedure was found to be reproducible (Scheme 2-11). During this reaction, TIPDS protecting group was found to be partially hydrolyzed. In order to simplify purification, a treatment with TEAF was used to remove TIPDS group completely before the deamination reaction (Scheme 2-9). The purified product was then treated with TIPDS chloride to mask 3′- and 5′-OH **92**.



Scheme 2-11 Chemical synthesis of guanosine modified at 2'-*O*-position with a C6 linker. *Reagents and conditions:* i) (*i*-Pr)$_2$Si(Cl)OSi(*i*-Pr)$_2$(Cl), C_5H_5N; ii) Pd/C (10% wt), H_2; iii) a) 4-Toluenesulfonyl chloride, CH_2Cl_2 ; b) DMF, LiCl, NaN_3.

In order to install a handle for conjugation at the 2′-*O*-position, the benzyl group needs to be removed (Scheme 2-11). To our surprise, catalytic hydrogenation reaction failed under many different conditions (Table 2-1).



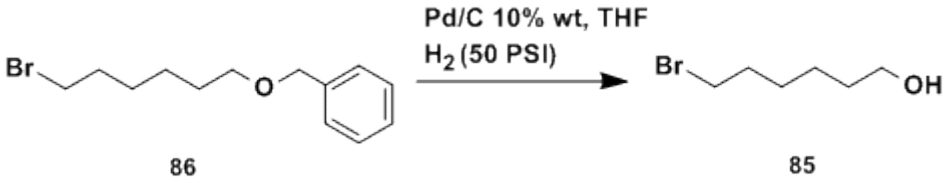
Scheme 2-12 Hydrogenation reaction condition.

Table 2-1 Different conditions attempted for the debenzylation of alkyl side chain.

Solvent	Catalyst	Hydrogen source	Other reagents	Results
Ethanol	Pd/C 10% (1.1 mol%)	H_2 (g)	50 psi	No Reaction
Methanol	Pd/C 10% (1.1 mol%)	H_2 (g)	50 psi	No Reaction
Ethyl acetate	Pd/C 10% (1.1 mol%)	H_2 (g)	50 psi	No Reaction
Methanol	Pd/C 10%, $Pd(OH)_2$ (1.1 mol%)	H_2 (g)	50 psi	No Reaction
Methanol	Pd/C 10% (1.1 mol%)	H_2 (g)	50 psi, 2,6-lutidine (0.1 eq), 45°C	No Reaction
Methanol	Pd/C 10% (1.1 mol%)	H_2 (g)	100 psi	No Reaction
Methanol	Pd/C 10% (1.1 mol%)	Triethylsilane (10.0 eq)		No Reaction
Methanol	Pd/C 10% (1.1 mol%)	H_2 (g)	50psi, Trichloroacetic acid	No Reaction
Dichloromethane			borontribromide (0.1 *M*), -78°C, 2h	No Reaction

As can be seen in table 2-1, hydrogenation reaction in different solvents such as methanol, ethanol and ethyl acetate, at 50 psi in the presence of catalytic amount of Pd/C did not lead to the removal of the *O*-benzyl group after 4 days. Other conditions, such as hydrogenation in the presence of triethylsilane or lutidine, also failed to give the desired product.

Lewis acid promoted debenzylation, on the other hand, led to the formation of a complex mixture. It was found that a excess amount of palladium catalyst was required for successful debenzylation **91**, however, only after a lengthy reaction time (7 days) (Scheme 2-12). The reason for the requirement of stoichiometric amount palladium in this reaction is unclear at this time. Attempts were made to determine if the difficulty in debenzylation is due to the presence of the alkyl chain attached to 2′-OH of ribose or not. Thus hydrogenation reactions of 6-bromohexylbenzyl ether **86** in THF were carried out under hydrogen atmosphere (50 psi) and the presence of catalytic amount of Pd/C (Scheme 2-13).



Scheme 2-13 Hydrogenation reaction condition for bromohexyl-6-benzyl ether.

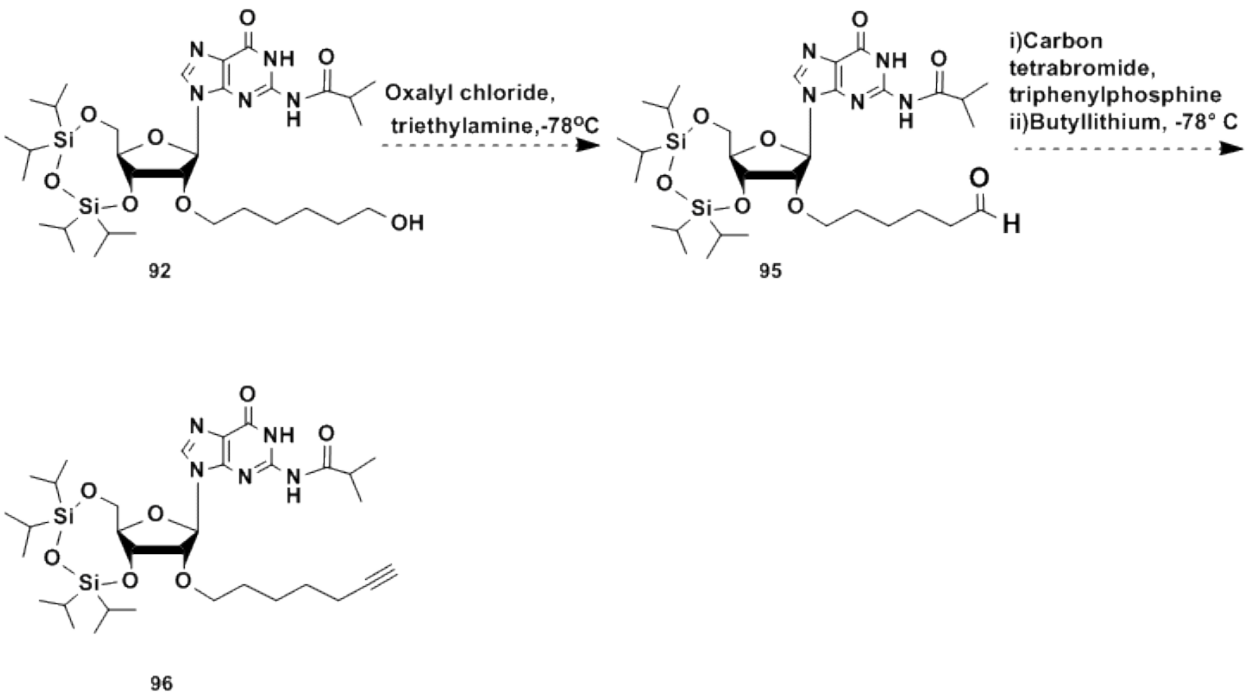
Interestingly, under these conditions, the starting material was completely consumed in 24 h to give the debenzylated product **85**.

In the subsequent steps, the hydroxyl group on the 2′-linker of compound **92** was transformed into mesylate or tosylate to facilitate the nucleophilic substitution with azide. The reaction between **92** and mesyl chloride in dichloromethane in the presence of triethylamine was completed in 10 min. Upon evaporation, the products were subsequently

treated with sodium azide in dry DMF. Surprisingly, this reaction failed to give the corresponding azido product. Addition of molar equivalence of lithium chloride or catalytic amount of 18-crown-6 to the reaction mixture led to the formation of a new product, in addition to what appear to be the hydrolyzed product (*i.e*. displacement of mesylate by OH); however, the identity of the new product could not be confirmed by mass spectrometry and NMR. Further, treatment of this product under catalytic hydrogenation conditions failed to give an amine. Note that if the desired azido product were successfully produced, then catalytic hydrogenation would have given the corresponding amine. At the time of this writing, it remains uncertain what are the causes of the failed mesylate-azido displacement reaction.

An attempt to transform **92** into the corresponding azido compound using a Mitsunobu reaction also failed. In this respect, **92** was treated with diethyl azodicarboxylate, triphenylphosphine, and diphenyl phosphoryl azide, but no reaction was observed after a day. Similarly, a literature procedure to transform alcohol into azide by treatment with *N*-(*p*-toluenesulfonyl) imidazole, tetra-*n*-butylammonium iodide, triethylamine, and sodium azide was also unsuccessful.

Due to the difficulty encountered in the above transformation, attempts were also made to convert compound **92** to the corresponding terminal alkyne **96** through a Swern oxidation – Corey Fuch sequence (Scheme 2-14).

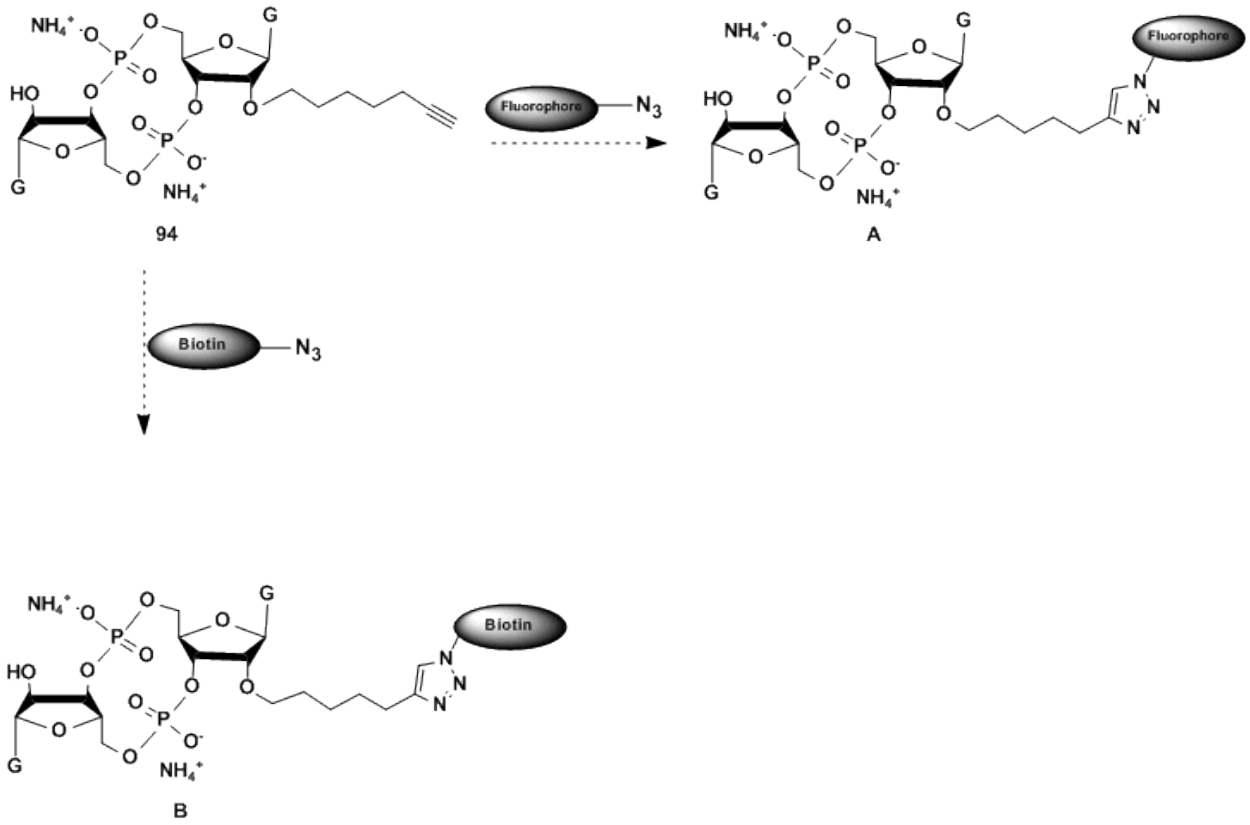


Scheme 2-14 Conversion of hydroxyl group on the 2′-linker to alkyne through a Swern oxidation – Corey Fuch sequence.

Due to reasons unknown at the time, Swern oxidation did not give the corresponding aldehyde **95**, as the ^{1}H NMR spectrum of the oxidation product did not reveal a characteristic aldehyde proton.

2.3 Conclusion and future work on labeling of c-di-GMP at 2′-position

Due to the challenges that were encountered in accessing the building block with azido substitution (compound **94**), future work will consider alkylation of diaminopurine riboside with a reagent that bears a terminal alkyne. This modification will allow for conjugation of c-di-GMP bearing the alkynyl functionality with azido biotin (B) or azido fluorophore (A) (Scheme 2-15).



Scheme 2-15 Conjugation of c-di-GMP bearing the alkynyl functionality with azido biotin (B) or azido fluorophore (A).[61]

Chapter 3

Experimental

^{1}H NMR spectra were measured at 600 MHz with a Bruker AV600 spectrometer, AV400 and AV300. ^{13}C at 75 MHz or 151 MHz, ^{31}P at 121 MHz. *J* values are given in Hz. Chemical shifts are given in ppm.

3.1 EI, FAB mass spectrometric measurements

Prior to December/2013 all samples were run on a Kratos/MSI Concept 1S instrument at 6kV accelerating potential. Resolution was 1000 for nominal mass spectra and 10,000-15,000 for high resolution runs. FAB bombardment used an Ion Tech atom gun employing Xe gas. Data were processed using Mach3 software. After May 1, 2014 all spectra were run on a Thermo DFS high resolution spectrometer at 5 kV accelerating potential. Resolution was 1000 for nominal mass spectra and 10,000-15,000 for high resolution runs. FAB bombardment used a Cs ion gun. Data were processed using Xcalibur software.

3.1.1 Electrospray mass spectrometric measurements

Samples in solution were run by infusion (syringe pump) or liquid chromatography using an Agilent 1100 LC system. All detection (ESI) was carried out on a Bruker HCT Ultra ion trap system. All qualitative and quantitative data were processed using Bruker Data Analysis software. All ESI data were low resolution due to the poor ionization of the nucleosides.

3.1.2 MALDI mass spectrometric measurements

MALDI mass spectra were generated on a Bruker Autoflex II/TOF/TOF instrument using stainless steel sample plates. Samples were run neat or in a suitable matrix determined by sample requirements. Data were processed using Bruker FLEX Analysis software.

3.2 Chromatography

Dessican 230–400 mesh silica gel 60 was used for flash column chromatography. Thin layer chromatography was performed on Silicycle Silica Plate F-254 TLC plates using the following system:

System A: methanol- dichloromethane (15:85 v/v)

System B: methanol- dichloromethane (10:90 v/v)

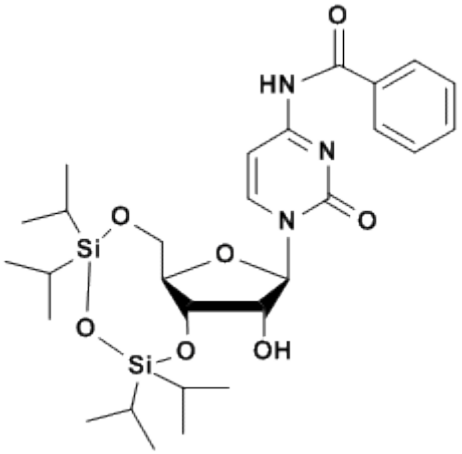
System C: methanol- dichloromethane (5:95 v/v)

3.2.1 Solvents and Chemicals

Toluene was dried by heating under reflux over sodium in the presence of benzophenone for 4 h and then distilled. Dimethylformamide was heated at 60ºC for 2 h, pyridine, was dried by heating under reflux over calcium hydride for 4 h and dichloromethane was dried by P_2O_5 and then distilled . All the distilled solvents were stored over activated 4 Å molecular sieves. All other reagents were purchased from Sigma-Aldrich or TCI America and were used without further purification unless stated otherwise.

3.3 Preparation of compounds

4-*N*-Benzoyl-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxyl) cytidine 63



N-benzoylcytidine **62** (2.20 g, 6.33 mmol) was dried at 80°C *in vacuo* for 5 h and then it was dissolved in anhydrous pyridine (15 ml), followed by addition of 1,1-dichloro-1,1,3,3-tetraisopropyldisiloxane **96** (2.2 ml, 6.97 mmol) while the temperature was kept at 0°C (ice-water bath). After 5 h the products were concentrated under reduced pressure, and the residue was partitioned between dichloromethane (60 ml) and saturated aqueous sodium hydrogen carbonate (50 ml). The layers were separated and the aqueous layer was back-extracted with dichloromethane (3x10 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane-methanol (98:2 v/v) were combined and concentrated under reduced pressure to give the compound **63** as a colorless froth (3.20 g, 85.7%). ^{1}H NMR data are in agreement with literature values.[92]

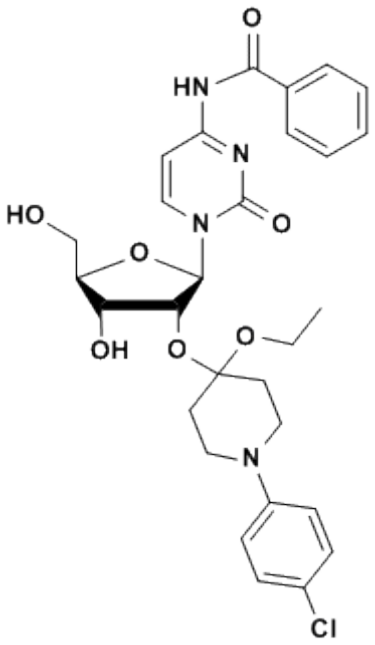
ESI-MS $[M-H]^-$ found 589.4, $C_{28}H_{43}N_3O_7Si_2$ required 589.26

δ_H [$(CD_3)_2SO$]: 11.32 (1 H, s), 8.19 (1 H, d, J = 7.5 Hz), 8.02-7.99 (1 H, m), 7.65-7.60 (1 H, m), 7.53-7.48 (1 H, m), 7.38-7.36 (1 H, m), 5.81 (1 H, d, J = 3.9 Hz), 5.64 (1 H, s), 4.23 (1 H,

d, *J* =13.5 Hz), 4.23 (1H, d, *J* = 13.5 Hz), 4.13 (3 H, s), 3.94 (1 H, d, *J* = 13.2 Hz), 0.95-1.06 (m, 28).

R_f : 0.48 (system C).

2′-*O*-[1-(4-Chlorophenyl)-4-ethoxypiperidin-4-yl]-5′-4-*N*-benzoylcytidine 64 ($C_{29}H_{32}ClN_4O_7$, 584.203)



4-*N*-Benzoyl-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxyl) cytidine **63** (4.10 g, 6.95 mmol) and 1-(4-chlorophenyl)-4-ethoxy-1,2,5,6-tetrahydropyridine **37** (4.94 g, 20.8 mmol) were evaporated with dry toluene (2x10.0 ml) and then dissolved in dry dichloromethane (20 ml), followed by addition of dry trifluoroacetic acid (1.00 ml, 13.5 mmol). After 5 h, triethylamine (1.64 ml, 11.8 mmol) was added. The products were partitioned between dichloromethane (30 ml) and saturated sodium hydrogen carbonate solution (30 ml). The organic layer was separated, dried with $MgSO_4$ and concentrated under reduced pressure. The residue was dissolved in acetonitrile (20 ml) followed by addition of a solution of tetraethyl ammonium fluoride in acetonitrile (20 ml, 1.0 *M*, pH 8.0). The products were concentrated under reduced

pressure after 30 min. The residue was purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane-methanol (97:3 v/v) were pooled and concentrated under reduced pressure to give the compound **64** as a colorless froth (3.30 g, 81%).

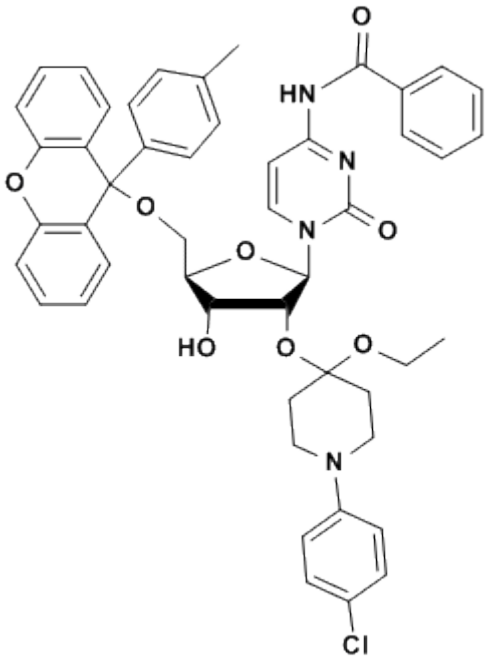
ESI-MS $[M-H]^-$ found 584.1, $C_{29}H_{32}ClN_4O_7$ required: 584.20

$\delta_H[(CD_3)_2SO]$: 11.31 (1 H, s), 8.48 (1 H, d, *J* = 7.2), 8.00-8.02 (2 H, m), 7.66-7.61 (1 H, m), 7.54-7.49 (2 H, m), 7.39 (1 H, d, *J* = 9.0), 7.18 (2 H, d, J = 9.0), 6.92 (2 H, d, *J* = 9.0), 6.13 (1 H, d, *J* = 6.0), 5.26 (1 H, t, *J* = 12.0), 5.15 (1 H, d, *J* = 6.1), 4.45-4.41 (1 H, dd, J = 5.1 and 6.6), 4.02-4.06 (1 H, m), 3.97-3.96 (1 H, m), 3.65-3.61 (2 H, m), 3.43-3.46 (2 H, m), 3.19-3.14 (1 H, m), 3.04-3.01 (1 H, m), 2.90-2.89 (1 H, m), 1.79-1.84 (4 H, m), 0.91 (3 H, t, *J* = 13.8).

$\delta_C[(CD_3)_2SO]$: 15.5, 32.4, 33.6, 45.8, 46.1, 55.4, 61.8, 71.2, 74.1, 86.9, 99.7, 117.6, 122.3, 128.9, 128.9, 129.0, 133.2, 149.7

R_f : 0.62 (system C).

4-*N*-Benzoyl-2′-*O*-[(4-chlorophenyl)-4-ethoxypiperidin-4-yl]-5′-*O*-[9-(*p*-tolyl)xanthen-9-yl] cytidine 65



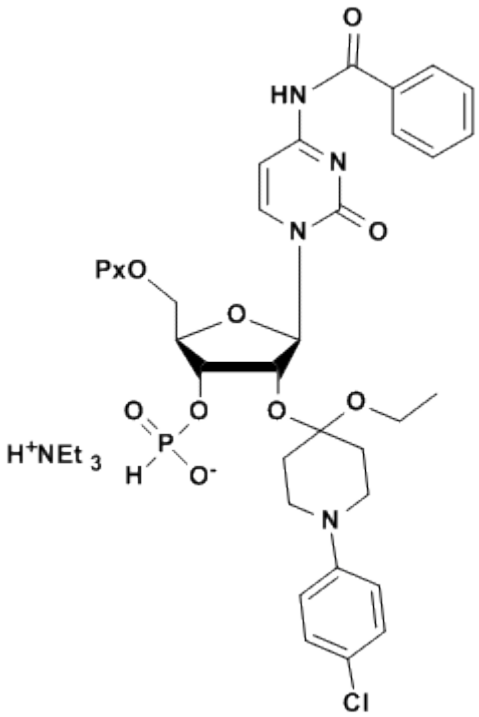
2′-*O*-[1-(4-Chlorophenyl)-4-ethoxypiperidin-4-yl]-5′-4-*N*-benzoylcytidine **64** (2.50 g, 4.28 mmol) was evaporated with dry pyridine (2x5 ml). The residue was dissolved in anhydrous pyridine (25 ml), followed by addition of 9-chloro-9-(*p*-tolyl)xanthene **98** (1.57 g, 5.12 mmol). After 40 minutes a mixture of methanol - *N*-methylmorpholine (3 ml, 1:1 v/v) was added. The products were partitioned between dichloromethane (60 ml) and saturated sodium hydrogen carbonate solution (40 ml). The organic layer was separated and the aqueous layer was back extracted with dichloromethane (2x10 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The product was purified by short column chromatography on silica gel. The appropriate fractions which were eluted with dichloromethane-methanol (99:1 v/v) were combined and concentrated under reduced pressure to give the compound **65** as a yellow foam (3.20 g, 87%).

ESI-MS $[M-H]^-$ found 855.1, $C_{49}H_{47}ClN_4O_8$ required 855.39

$\delta_H[(CD_3)_2SO]$: 11.35 (1 H, s), 8.30-8.20 (1 H, d), 8.02-7.99 (2 H, m), 7.66-7.61 (1 H, m), 7.54-7.15 (21 H, m), 6.94 (2 H, d, *J* = 9.3), 6.12 (1 H, d, J = 6.0), 5.26 (1 H, d, *J* = 5.4), 4.55 (1 H, t, *J* = 10.5), 4.06-4.05 (1 H, m), 3.57 (1 H, t, *J* = 3.0), 3.40-3.42 (1 H, m), 3.25-3.22 (3 H, m), 3.10-2.93 (3 H, m), 1.91-1.82 (4 H, m), 1.00 (3 H, t, *J* = 9.2).

R_f : 0.58 (system C)

4-*N*-Benzoyl-2′-*O*-[(4-chlorophenyl)-4-ethoxypiperidin-4-yl]-5′-*O*-[9-(*p*-tolyl)xanthen-9-yl] cytidine 3′-H phosphonate, triethylammonium salt 66



p-Tolyl H-phosphonate (1.19 g, 4.86 mmol) was co-evaporated with triethylamine (1.46 ml, 11.4 mmol) and methanol (8 ml). 4-*N*-Benzoyl-2′-*O*-[(4-chlorophenyl)-4-ethoxypiperidin-4-yl]-5′-*O*-[9-(*p*-tolyl)xanthen-9-yl] cytidine **65** (1.60 g, 1.87 mmol) was added and the mixture was evaporated with dry pyridine (2x5 ml) followed by addition of dry pyridine (40 ml). While temperature was kept at 0°C (ice-water bath) distilled pivaloyl chloride (0.80 ml, 6.54

mmol) was added dropwise in 5 min. The reaction mixture was stirred for 1 hour at 0°C (ice-water bath) then the reaction was quenched by addition of water (8 ml). After 30 min the products were concentrated under reduced pressure then the residue was dissolved in dichloromethane (80 ml) and extracted with saturated aqueous sodium hydrogen carbonate (40 ml). The layers were separated and the aqueous layer was back extracted with dichloromethane (4x20 ml). The combined organic layers were extracted with triethylammonium phosphate buffer (40 ml, 0.5 *M*, pH 7.0). The aqueous layer was back extracted with dichloromethane (20 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by short column chromatography on silica gel. The appropriate fractions which were eluted with dichloromethane-methanol (96:4 v/v) were combined and concentrated under reduced pressure to give the compound **66** as a withe froth (1.60 g, 92%).

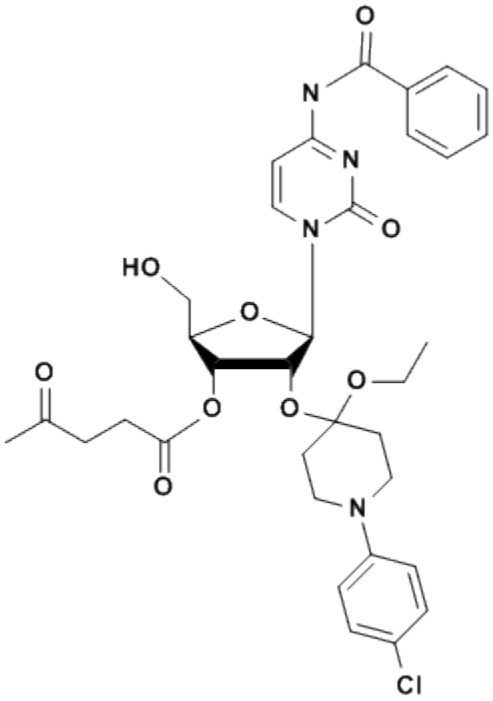
ESI-MS $[M-H]^-$ found 1019.4, $C_{48}H_{45}ClN_4O_{10}P$ required 1019.40

$\delta_H[(CD_3)_2SO]$: 11.28 (1 H, s), 8.24 (1 H, d, *J* = 7.8), 8.02-7.99 (2 H, m), 7.66-7.61 (1 H, m), 7.54-7.49 (2 H, m), 7.47-7.38 (2 H, m), 7.37-7.22 (15 H, m), 6.94 (2 H, d, J = 9.0), 6.12 (1 H, d, J = 6.0), 4.62-4.54 (2 H, m), 4.28-4.27 (1 H, m), 4.02 (1 H, dd, J = 5.4 and 10.5), 2.96 (1 H, d, J = 7.8), 3.10-2.80 (14 H, m), 1.93-1.76 (4 H, m), 1.66-1.78 (1 H, m), 1.10-1.10 (23 H, m), 1.01 (3 H, t, J = 15.0).

$\delta_P[(CD_3)_2SO]$: 0.09 (d, *J* = 56.6).

R_f : 0.55 (system B)

4-*N*-Benzoyl-2′-*O*-[(4-chlorophenyl)-4-ethoxypiperidin-4-yl]-3′-*O*-levulinylcytidine 67



4-*N*-Benzoyl-2′-*O*-[(4-chlorophenyl)-4-ethoxypiperidin-4-yl]-5′-*O*-[9-(*p*-tolyl)xanthen-9-yl] cytidine **65** (2.30 g, 2.69 mmol) was evaporated with dry pyridine (2x5 ml), and then dissolved in dry pyridine (30 ml). Levunilic anhydride (1.15 g, 5.37 mmol) was added followed by addition of *N*,*N*-dimethylaminopyridine (0.026 g, 3.22 mmol) and triethylamine (0.75 ml, 7.5 mmol). After 30 minutes the products were partitioned between dichloromethane (60 ml) and saturated sodium hydrogen carbonate solution (40 ml). The organic layer was separated and the aqueous layer was back extracted with dichloromethane (2x10 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. After the residue was co-evaporated with toluene (2x10 ml), it was dissolved in dichloromethane followed by addition of pyrrole (1.8 ml, 26.8 mmol) and trifluoroacetic acid (1.24 ml, 10.7 mmol). After 30 second the reaction was quenched by addition of *N*-methylmorpholine (2.30 ml, 22.8 mmol). The products were partitioned between dichloromethane (60 ml) and saturated sodium hydrogen carbonate solution (40 ml). The

organic layer was separated and the aqueous layer was back extracted with dichloromethane (2x10 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by short column chromatography. The appropriate fractions, which were eluted with dichloromethane-methanol (98:2 v/v) were combined and concentrated under reduced pressure to give the compound **67** as a colorless froth (1.50 g, 82%).

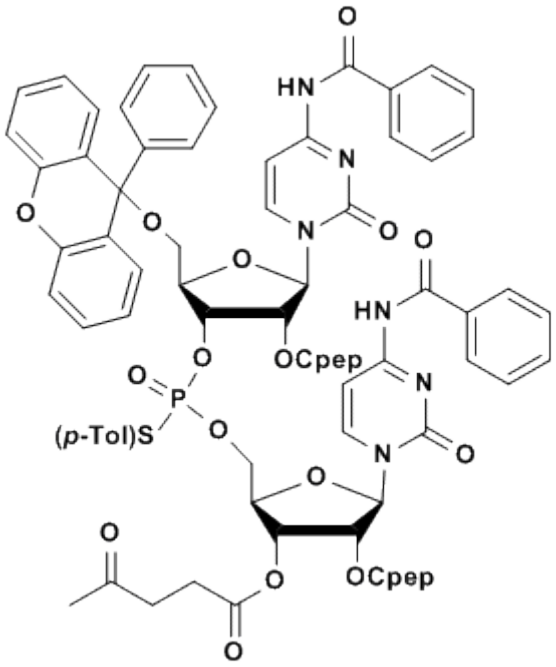
ESI-MS $[M-H]^-$ found 682.2, $C_{34}H_{39}ClN_4O_9$ required 682.24

$\delta_H[(CD_3)_2SO]$: 11.35 (1 H, s), 8.42 (1 H, d, *J* = 7.2), 7.96 (2 H, d, *J* = 7.5), 7.64 (1 H, t, *J* = 14.7), 7.52 (2 H, t, *J* = 14.7), 7.40 (1 H, s), 7.15 (2 H, d, *J* = 8.7), 6.87 (2 H, d, *J* = 9.0), 6.18 (1 H, d, *J* = 7.8), 5.50 (1 H, s), 5.16 (1 H, d, *J* = 4.8), 4.60-4.65 (1 H, m), 4.08 (1 H, s), 3.67 (2 H, s), 3.13-3.18 (3 H, m), 2.99-3.03 (1 H, m), 2.85-2.88 (1 H, m), 2.71-2.69 (3 H, m), 2.58-2.54 (2 H, m),

1.79-1.81 (4 H, m), 1.55-1.62 (1 H, m), 0.88 (5 H, t, *J* = 13.8).

R_f : 0.54 (system C).

Px-*C′′p(s′)C′′*-Lev* 68



4-*N*-Benzoyl-2′-*O*-[(4-chlorophenyl)-4-ethoxypiperidin-4-yl]-5′-*O*-[9-(*p*-tolyl)xanthen-9-yl] cytidine 3′-H phosphonate, triethylammonium salt **66** (1.50 g, 1.49 mmol) and 4-*N*-benzoyl-2′-*O*-[(4-chlorophenyl)-4-ethoxypiperidin-4-yl]-3′-*O*-levulinylcytidine **67** (0.70 g, 1.02 mmol) were co-evaporated with dry pyridine (2x5 ml) and then dissolved in dry pyridine (20 ml), while the temperature kept at 0°C (ice bath) pivaloyl chloride (0.20 ml, 1.73 mmol) was added. After 5 min 2-phenylsulfanyl-1*H*-isoindole-1,3(1*H*)-dione (0.70 g, 1.83 mmol) was added. The reaction mixture was stirred for 30 min at room temperature and the reaction was quenched by addition of water (0.2 ml). The products were partitioned between dichloromethane (60 ml) and saturated sodium hydrogen carbonate solution (40 ml). The organic layer was separated and the aqueous layer was back extracted with dichloromethane (2x10 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The product was purified by short column chromatography on silica gel. The appropriate fractions which were eluted with dichloromethane-methanol (98:2 v/v) were

combined and concentrated under reduced pressure to give the compound **68** as a colorless froth (1.30 g, 75%).

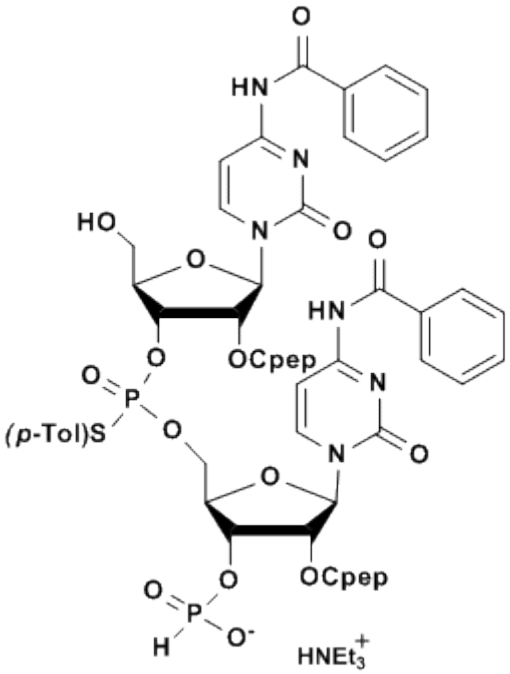
ESI-MS $[M-H]^-$ found 1690.1, $C_{89}H_{89}Cl_2N_8O_{18}$ required 1690.50

$\delta_H[(CD_3)_2SO]$: 11.35 (1 H, s), 7.99 (2 H, d, *J* = 6.8), 7.61-7.64 (1 H, m), 7.51 (3 H, t, *J* = 15.9), 7.45-7.33 (3 H, m), 7.32- 7.25 (3 H, m), 7.09-7.17 (6 H, m), 6.85-6.95 (2 H, m), 6.04-6.13 (1 H, m), 4.97-4.74 (1 H, m), 4.40 (1 H, s), 4.14-4.19 (1 H, m), 3.16 (3 H, d, *J* = 5.1), 3.04-2.89 (1 H, m), 2.88-2.77 (1 H, m), 2.65-2.71 (1 H, m), 2.24 (1 H, s), 2.08 (2 H, t, *J* = 12.0), 1.77-1.86 (3 H, m,), 1.22 (1 H, s), 1.02-0.82 (3 H, m).

R_f : 0.52 (system C).

$\delta_P[CD_3)_2SO]$: 22.83.

HO-*C′′p(s′)*C′′p(H) Chemical Formula 71



Px-*C′′p(s′)C′′*-Lev **68** (0.70 g, 0.41 mmol) was dissolved in pyridine (5.0 ml), followed by addition of a mixture of hydrazine monohydrate (0.12 ml, 4.5 mmol), acetic acid (2.6 ml), water (0.35 ml) and pyridine (5.0 ml). After 15 min the reaction was quenched by addition of pentan-2,4-dione (0.8 ml). The products were concentrated under reduced pressure after 10 min. The residue was dissolved in dichloromethane (15.0 ml) and extracted with saturated aqueous sodium hydrogen carbonate (8.0 ml). The layers were separated and the aqueous layer was back extracted with dichloromethane (4x5 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. To the residue were added *p*-tolyl H-phosphonate (0.30 g, 1.23 mmol), triethylamine (0.3 ml, 2.9 mmol) and methanol (1.0 ml) and the mixture were evaporated under reduced pressure. The residue was evaporated with dry pyridine (2x5 ml) and then dissolved in dry pyridine (5.0 ml) followed by addition of pivaloyl chloride (0.15 ml, 1.23 mmol) over a period of 5 min while temperature was kept at 0°C (ice bath). The reaction mixture was stirred for 30 min at the same temperature, and the reaction was quenched by addition of water (0.5 ml). The products were concentrated under

reduced pressure. The residue was dissolved in dichloromethane (20.0 ml) and extracted with saturated aqueous sodium hydrogen carbonate (10.0 ml). The layers were separated and the aqueous layer was back extracted with dichloromethane (2x5 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was then co-evaporated with dry toluene (3x5 ml) and then dissolved in dry dichloromethane (10.0 ml) followed by addition of distilled pyrrole (0.27 ml, 4.13 mmol) and distilled trifluoroacetic acid (0.42 ml, 3.71 mmol). After 30 sec the reaction was quenched by addition of *N*-methylmorpholine (0.43 ml, 3.71 mmol). The products were extracted with saturated aqueous sodium hydrogen carbonate (10.0 ml). The layers were separated and the aqueous layer was back extracted with dichloromethane (2x5 ml). The combined organic layer was extracted with triethylammonium phosphate buffer (10.0 ml, 0.5 *M*, pH 7.0) and aqueous layer was back extracted with dichloromethane (2x5 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane-methanol (96:4 v/v), were concentrated with a rotary evaporator to give the compound **71** as a white glass (0.55 g, 89%).

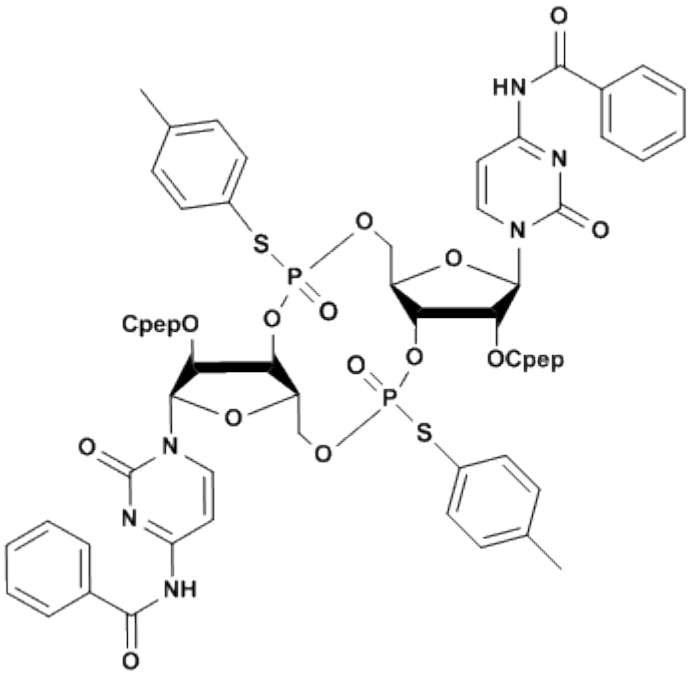
ESI-MS $[M-H]^-$ found 1501.5, $C_{65}H_{71}Cl_2N_8O_{15}$ required 1501.48

$\delta_H[(CD_3)_2SO]$: 11.35 (1 H, s), 8.01 (2 H, d, *J* = 8.4), 7.62 (1 H, d, *J* = 6.9), 7.52 (3 H, d, *J* = 7.8), 7.23 (2 H, d, J = 9.3), 7.20-7.07 (3 H, m), 6.88-6.92 (2 H), 6.23- 6.03 (1 H, m), 4.64-4.68 (1 H, m), 3.06-3.08 (12 H, m), 2.98-2.86 (1 H, m), 2.85-2.76 (1 H, m), 1.96- 1.68 (3H, m), 2.25 (2 H, s), 1.17 (16 H, t, *J* = 6.0), 0.88-0.89 (4 H, m, *J* = 3.2).

R_f : 0.87 (system A)

$\delta_P[(CD_3)_2SO]$: 2.78, and 23.15.

Preparation of fully protected c-di-CMP 72



HO-*C''p(s'')C''*pH **71** (100 mg, 0.07 mmol) was co-evaporated with dry toluene (2x3 ml) and then dissolved in dry dichloromethane (5.0 ml). This solution was then added dropwise over a period of 20 min at -40° C (dry ice-acetone bath) to a solution of diphenyl chlorophosphate (0.25 ml, 1.22 mmol) in dry pyridine (5.0 ml). The reaction mixture was stirred for 20 min followed by addition of 1-phenylsulfanyl-pyrrolidine 2,5-dione (0.22 g, 1.34 mmol). The reaction mixture was then allowed to warm up to room temperature over 30 min. After 5 min water (3.0 ml) was added, the products were extracted with saturated aqueous sodium hydrogen carbonate (10.0 ml). The layers were separated and the aqueous layer was back extracted with dichloromethane (2x5 ml). The combined organic layers were dried ($MgSO_4$) and concentrated with a rotary evaporator. The residue was purified by column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane- methanol (97:3 v/v), were concentrated with a rotary evaporator to give the compound **72** as a white glass (65 mg, 80%).

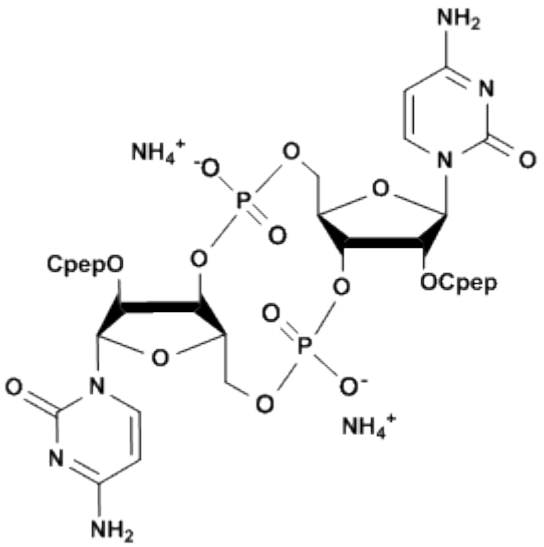
ESI-MS $[M-H]^-$ found 1504.1, $C_{72}H_{76}Cl_2N_8O_{16}P_2S_2$ required 1504.36

$\delta_H[(CD_3)_2SO]$: 11.41 (1 H, s), 8.42 (1 H, d, J = 9.0), 8.03 (3 H, d, J = 7.8), 7.84 (1 H, d, J = 7.8), 7.69-7.60 (2 H, m), 7.58-7.49 (6 H, m), 7.46 (2 H, d, J = 9.0), 7.40-7.30 (3 H, m), 7.28-7.20 (6 H, m), 7.19-7.12 (4 H, m), 6.95-6.83 (5 H, mm), 6.05-5.92 (1 H, m), 5.91-5.80 (1 H, m), 5.11-4.96 (3 H, m), 4.73-4.57 (2 H, m), 4.19-4.05 (1 H, m), 3.98 (2 H, t, J = 14.7), 3.80-3.67 (2 H, m), 3.58-3.49 (2 H, m), 3.25 (1 H, s), 3.20-3.04 (3 H, m), 3.05-2.90 (3 H, m), 2.89-2.71 (3 H, m), 2.37-2.79 (6 H, m), 2.26-2.18 (3 H, m), 2.00 (2 H, s), 1.89-1.77 (7 H, m), 1.62-1.44 (5 H, m), 1.39-1.29 (4 H, m), 1.28-1.11 (12 H, m), 1.05-0.90 (9 H, m), 0.90-0.78 (5 H, m).

R_f : 0.56 (system C).

$\delta_P[CD_3)_2SO]$: 25.57.

Preparation of partially protected c-di-CMP 73



Fully-protected c-di-CMP **72** (57 mg, 0.37 mmol) was evaporated with dry toluene (2x2 ml) and the residue was dissolved in dry acetonitrile (2 ml). 2-Nitrobenzaldoxime (0.88 mg, 0.53 mmol) followed by *N*,*N*,*N′*,*N′*- tetramethylguanidine (61 μl, 0.49 mmol) were added. The reactants were stirred at room temperature for 16 hours and were then concentrated under

reduced pressure. To the residue was added concentrated aqueous ammonia (33%, *d* 0.88, 2.0 ml) and the mixture was heated at 55°C for 15 hours. After the products had been cooled, they were concentrated to dryness followed by co-evaporation with ethanol (2x2 ml) under reduced pressure. The residue was dissolved in methanol (1 ml) and precipitated with diethyl ether (30 ml). The precipitate was collected by centrifugation. This precipitation-centrifugation process was repeated for one more time and the solid residue was dried *in vacuo* to give 2′-*O*-Cpep c-di-CMP as colorless solid (32 mg, 89%).

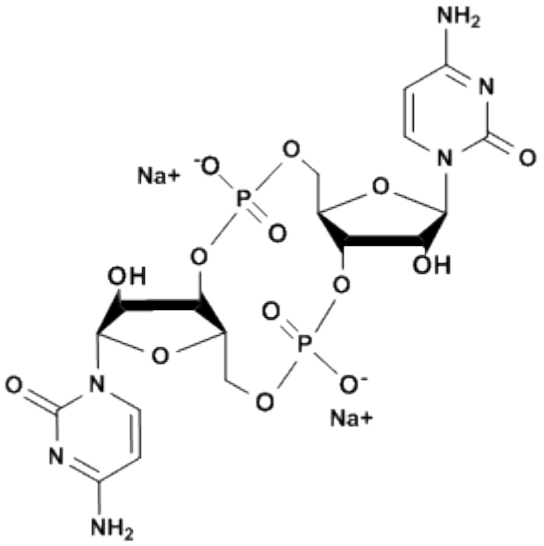
ESI-MS $[M-H]^-$ found 1083.0, $C_{44}H_{54}Cl_2N_8O_{16}P_2^-$ required 1082.25

$\delta_H[(CD_3)_2SO]$: 7.91 (1 H, d, J = 9.0), 7.53-7.40 (3 H, m), 7.27 (3 H, d, J = 9.0), 7.04 (2 H, d, J = 9.0), 5.97 (2 H, d, J = 9.0), 4.56 (1 H, s), 4.37-4.30 (1 H, m), 4.25 (1 H, d, J = 15.1), 3.97 (1 H, d, J = 13.5), 3.67 (1 H, t, J = 15.9), 3.57-3.44 (6 H, m), 3.39-3.30 (1 H, m), 3.06-2.97 (2 H, m), 2.96-2.91 (12 H, m), 2.36 (1 H, s), 2.09-2.01 (4 H, m), 1.93 (1 H, s), 1.13-1.03 (1o H, m).

R_f : 0.64 (system C).

$\delta_P[(CD_3)_2SO]$: 0.03.

Preparation of fully deprotected c-di-CMP 74



2′-*O*-Cpep protected c-di-CMP **73** (30 mg, 0.24 mmol) was dissolved in *N*,*N*-dimethylacetamide (1.8 ml) followed by addition of triethylammonium formate buffer (0.5 *M*, pH 2.52, 1.2 ml, prepared with sterile water). The reactants were then sealed and heated at 40°C. After 5 hours, the products were cooled, and the pH was adjusted to 7.0 with triethylamine. The products were then extracted with chloroform (4x2.0 ml). The organic layers were discarded. The aqueous layer was evaporated under reduced pressure at room temperature to a volume of 0.2 ml. To the residue was added *n*-butanol (2.0 ml). The mixture was vortexed vigorously and then chilled at -78°C (dry ice–acetone bath) for 10 minutes. Then it was centrifuged for 10 min. The supernatant was discarded and the pellet was re-dissolved in sterile water (0.2 ml). The above precipitation–centrifugation process was repeated two more times. The final pellet was then dried *in vacuo*.

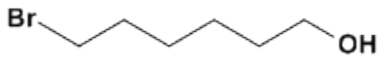
This material was dissolved in sterile water (1.0 ml) and passed through an Amberlite (IR120, Na^+ form) cation-exchange column (0.5×2.0 cm). The fractions which contained oligonucleotide were pooled and freeze-dried to give the fully unblocked c-di-CMP as a colorless froth (18.0 mg, 94%).

ESI-MS $[M-H]^-$ found 608.0, $C_{18}H_{22}N_6O_{14}P_{22}^-$ required 608.06

$\delta_H[(CD_3)_2SO]$: 8.05 (1 H, d, *J* = 7.8), 5.99 (1 H, d, *J* = 7.5), 5.71 (1 H, s), 3.95 (1 H, d, *J* = 14.4), 4.50-4.40 (2 H, m), 4.39-4.27 (4 H, m), 3.95 (1 H, d, *J* = 12.9).

$\delta_P[(CD_3)_2SO]$: 1.17.

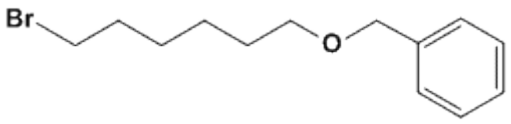
6-Bromo-1-hexanol 85



A literature procedure was followed for the preparation of this compound.ref 1,6-Hexandiol **83** (20.0 g, 0.169 mol) and hydrobromic acid (48% in water, 60.0 ml) were added to toluene (200.0 ml) and the mixture was heated under reflux with vigorous stirring for 2 h. after the reaction mixture was cooled to 0ºC (ice-water bath), the organic layer was separated and washed successively with saturated aqueous sodium hydrogen carbonate (2x50 ml) and water (70.0 ml), and then concentrated under reduced pressure to give the compound **85** as a colorless liquid (30.10 g, 98%). ^{1}H NMR data are in agreement with literature values.[98]

$\delta_H[(CD_3)_2SO]$: 4.02 (1 H, s), 3.52 (2 H, t, *J* = 18.0), 3.37 (2 H, t, *J* = 12.1), 2.51 (1 H, s), 1.85-1.75 (2 H, m), 1.49-1.23 (5 H, m).

1-Benzyloxy-6-bromohexane 86

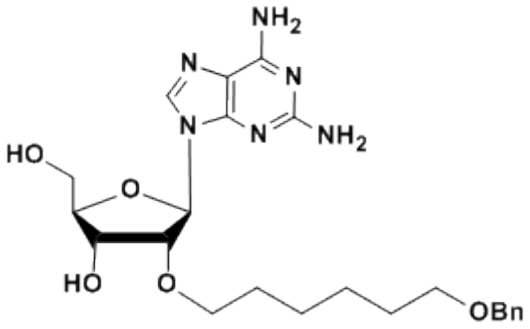


Sodium hydride (883 mg, 22.1 mmol) was suspended in dry tetrahydrofuran (20.0 ml) followed by dropwise addition of a solution of 6-bromo-1-hexanol **85** (4.01 g, 22.1 mmol) in

tetrahydrofuran (5.0 ml) while temperature was kept at 0°C (ice-water bath). After 30 min solution of benzyl bromide (3.78 g, 22.1 mmol) in tetrahydrofuran (5.0 ml) was added at the same temperature. After 24 h the products were concentrated under reduced pressure. The residue was dissolved in ethylacetate (200 ml) and extracted with water (40 ml) and brine (40 ml). The organic layer was dried ($MgSO_4$) and concentrated by a rotary evaporator. Distillation performed to get the desired product at 120°C (5.72g, 96%)(1.5×10^{-3} torr).^{1}H NMR data are in agreement with literature values.[93]

$\delta_H[CD_3)_2SO]$: 7.38-7.23 (5 H, m), 4.44 (2 H, s), 3.50 (2 H, t, *J* = 12.2), 3.41 (2 H, t, *J* = 12.3), 1.84-1.73 (2 H, m), 1.59-1.48 (2 H, m), 1.45-1.27 (4 H, m).

2′-*O*-(6-Benzoxylhexyl)-2,6-diaminopurine riboside 88



2,6- Diaminopurine riboside **87** (10.0 g, 35.4 mmol)) was dried at 80°C *in vacuo* for 5 h and then suspended in *N*,*N*-dimethylformamide (100.0 ml) followed by addition of sodium hydride (1.60 g, 39.0 mmol). The mixture was stirred for 1 h and then 1-benzyloxy-6-bromohexane **85** (7.03 ml, 35.4 mmol) was added dropwise over a period of 5 min while temperature was kept at 0°C (ice-water bath). After 48 h, the reaction was quenched by addition of water (5.0 ml). The products were concentrated under reduced pressure and the residue was purified by column chromatography on silica gel. The appropriate fractions,

which were eluted with dichloromethane-methanol (98.5:1.5 v/v), were concentrated by rotary evaporator to give the compound **88** as a white glass (5.45 g, 32%).

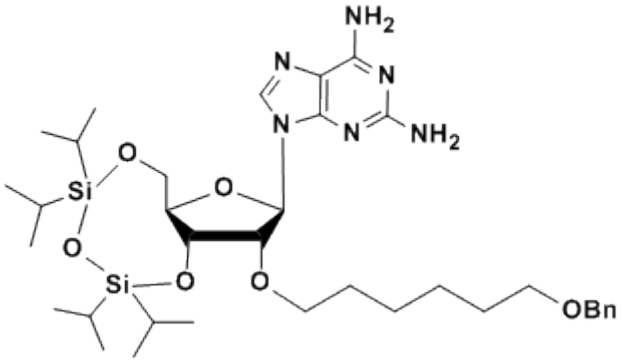
ESI-MS $[M-H]^-$ found 472.7, $C_{23}H_{32}N_6O_5$ required 472.24

$\delta_H[CD_3)_2SO]$: 7.95 (1 H, s), 7.25-7.35 (5 H, m), 6.78 (2 H, s), 5.79 (1 H, d, *J* = 6.9), 5.73 (2 H, s), 5.48-5.44 (1 H, m), 5.08 (1 H, d, *J* = 5.7), 4.42-4.35 (3 H, m), 4.26-4.20 (1 H, m), 3.93-3.92 (1 H, m), 3.64-3.59 (1 H, m), 3.56-3.49 (2 H, m), 1.45-1.38 (4 H, m), 1.33-1.21 (4 H, m).

$\delta_C[CD_3)_2SO]$: 25.6, 29.9, 29.5, 62.2, 69.7, 70.0, 72.2, 81.1, 85.8, 86.8, 113.9, 127.7, 127.8, 128.6, 136.8, 139.2, 151.8, 156.5, 160.3.

R_f : 0.57 (system B).

2′-*O*-(6-Benzyloxyhexyl)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxyl)-2,6-diaminopurine-D riboside 89



2′-*O*-(6-Benzyloxyhexyl)-2,6-diaminopurine riboside **88** (3.00 g, 4.19 mmol) was co-evaporated with dry pyridine (2x10 ml) then dissolved in anhydrous pyridine (30.0 ml), followed by addition of 1,1-dichloro-1,1,3,3-tetraisopropyldisiloxane (1.07 ml, 5.02 mmol) while the temperature was kept at 0°C (ice-water bath). After 5 h the products were concentrated under reduced pressure and then partitioned between dichloromethane (60 ml)

and saturated aqueous sodium hydrogen carbonate (50 ml). The layers were separated and the aqueous layer was back-extracted with dichloromethane (3x10 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane-methanol (98:2 v/v) were combined and concentrated under reduced pressure to give the compound **89** as a colorless froth (4.50 g, 90%).

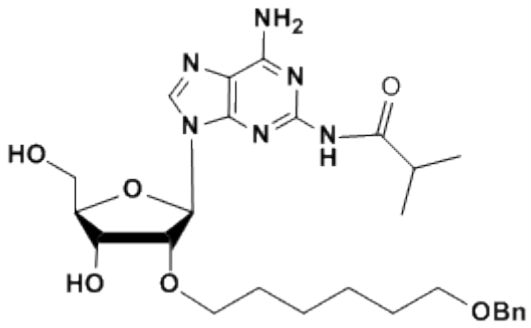
ESI-MS $[M-H]^-$ found 714.5, $C_{35}H_{58}N_6O_6Si_2$ required 714.39.

$\delta_H[(CD_3)_2SO]$: 7.76 (1 H, s), 7.37-7.21 (5 H, m), 6.79 (2 H, s), 5.75 (3 H, d, *J* = 7.8),4.52-4.53 (1 H, m), 4.42 (2 H, s), 4.25-4.30 (1 H, m),4.20-4.01 (1 H, m), 4.02-4.14 (2 H, m), 3.73-3.82 (1 H, m), 3.70-3.60 (1 H, m), 3.39-3.37 (1 H, m), 1.53-1.51 (4 H, m), 1.32-1.23 (4 H, m), 1.04-1.00 (29 H, m).

$\delta_C[(CD_3)_2SO]$: 12.7, 12.8, 13.2, 17.2, 17.3, 17.4, 17.6, 17.7, 17.8, 25.9, 26.0, 29.6, 29.8, 60.8, 70.0, 70.3, 70.9, 72.2, 81.2, 81.3, 87.0, 127.8, 128.6, 139.2,154.0, 156.4, 176.2,.

R_f : 0.38 (system B).

2′-*O*-(6-Benzyloxyhexyl)-2(*N*-isobutyryl)-6-riboside 90



2′-*O*-(6-Benzyloxyhexyl)-3′,5′–*O*-(1,1,3,3-tetraisopropyldisiloxyl)-2,6-diaminopurine-D ribosede **89** (3.00 g, 4.19 mmol) was dissolved in dry pyridine (30.0 ml) followed by addition of solution of isobutyric chloride (1.0 ml, 4.61 mmol) in dry dichloromethane (3.0

ml) dropwise at room temperature. The reaction mixture was stirred for 1 h at the same temperature and then (check the TLC) it was quenched by addition of ethanol (5.0 ml). The products were concentrated under reduced pressure. The residue was dissolved in dichloromethane (30.0 ml) and extracted with saturated aqueous sodium hydrogen carbonate (15.0 ml). The layers were separated and the aqueous layer was back extracted with dichloromethane (2x10 ml). The combined organic layers were dried by ($MgSO_4$) and concentrated with a rotary evaporator.

To the residue was added methanol (5.0 ml) and concentrated aqueous ammonia (33%, *d* 0.88, 10.0 ml) and the mixture was heated at 55ºC for 20 h. After the products had been cooled, they were concentrated to dryness. The residue was dissolved in acetonitrile (10.0 ml) followed by addition of a tetraethylammonium fluoride solution in acetonitrile (10.0 ml, 1.0 *M*, pH 8.0). The reaction mixture was stirred for 30 min and concentrated to dryness under reduced pressure. The residue was purified by column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane-methanol (96:4 v/v), were concentrated under reduced pressure to give the compound **90** as a white glass (0.50 g, 65% for three steps).

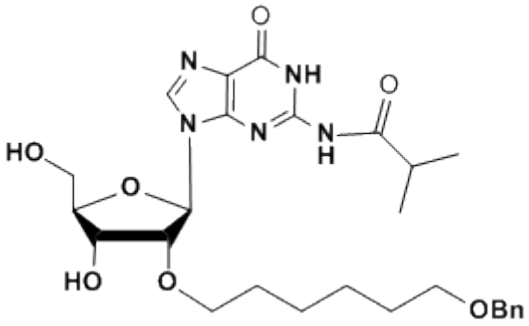
ESI-MS $[M-H]^-$ found 54.2, $C_{27}H_{38}N_6O_6$ required 542.28

$\delta_H[CD_3)_2SO]$: 9.83 (1 H, s), 8.27 (1 H, s), 7.36-7.31 (2 H, m), 7.30-7.20 (5 H, m), 5.92 (1 H, d, *J* = 6.0), 5.13 (1 H, d, *J* = 4.8), 5.05 (1 H, t, *J* = 11.4), 4.46 (1 H, t, *J* = 11.4), 4.40 (2 H, s), 4.28-4.25 (1 H, m), 3.93-3.91 (1 H, m), 3.66-3.61 (1 H, m), 3.58-3.51 (2 H, m), 3.38-3.36 (1 H, m), 2.84 (1 H, s), 1.45-138 (4 H, m), 1.23-1.11 (5 H, m), 1.06-1.03 (6 H, m), 0.93-0.78 (1 H, m).

$\delta_C[CD_3)_2SO]$: 19.8, 25.6, 25.9, 29.5, 29.5, 31.0, 31.1, 31.2, 31.3, 34.5, 55.4, 61.8, 69.3, 69.9, 70.0, 72.2, 79.6, 81.1, 85.4, 86.5, 116.7, 127.7, 127.8, 128.6, 139.1, 139.2, 150.7, 153.3, 156.6, 175.4, 207.0.

R_f : 0.69 (system B).

2′-*O*-(6-Benzyloxyhexyl)-N_2-isobutyryl-guanosine 91



2′-*O*-(6-Benzoxyhexyl)-N_2-isobutiryl-6-aminopurine riboside **90** (1.00 g, 1.84 mmol) was dissolved in *N*,*N*-dimethylacetamide (15.0 ml) followed by addition of glacial acetic acid (28.0 ml) and water (14.0 ml). A solution of sodium nitrite (1.39 g, 20.3 mmol) in water (3.0 ml) was then added dropwise while the temperature was kept at 4°C. After 24 h another portion of sodium nitrite (1.39 g, 20.28 mmol) in water (3.0 ml) was added. The reaction mixture was stirred at 4°C for 5 days with addition of the same portion of sodium nitrite in water on a daily basis (check TLC). The solvents were evaporated *in vacuo* to dryness. The residue was dissolved in dichloromethane (30.0 ml) and extracted with saturated aqueous sodium hydrogen carbonate (15.0 ml). The layers were separated and the aqueous layer was back extracted with dichloromethane (2x10 ml). The combined organic layers were dried ($MgSO_4$) and then concentrated with a rotary evaporator. The appropriate fractions, which were eluted with dichloromethane-methanol (96:4 v/v), were concentrated under reduced pressure to give the compound **91** as a white glass (0.75 g, 75%).

ESI-MS $[M-H]^-$ found 544.1, $C_{27}H_{37}N_5O_7$ required 544.27

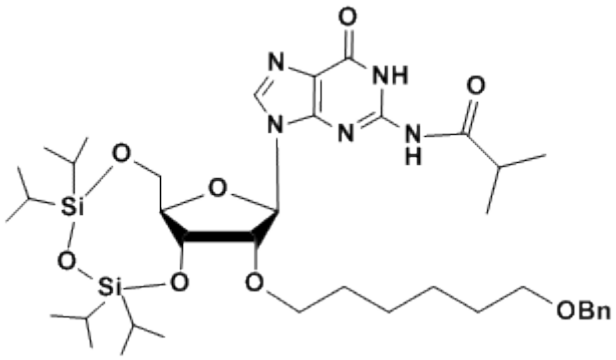
$\delta_H[(CD_3)_2SO]$: 12.10 (1 H, s), 11.70 (1 H, s), 8.30 (1 H, s), 7.35-7.24 (6 H, m), 5.89 (1 H, d, *J* = 6.8), 5.18 (1 H, d, *J* = 4.4), 5.09 (1 H, s), 4.47-4.24 (1 H, m), 4.39 (2 H, s), 4.34-4.31 (1 H, m), 4.28-4.24 (1 H, m), 3.95-3.93 (1 H, m), 3.65-3.58 (2 H, m), 3.58-3.52 (2 H, m), 3.34-

3.30 (3 H, m), 2.78-2.72 (1 H, m), 1.44-1.37 (4 H, m), 1.20-1.14 (4 H, m), 1.11 (4 H, t, *J* = 13.6).

R_f : 0.66 (system B).

$\delta_C[CD_3)_2SO]$: 19.3, 25.6, 25.9, 29.4, 29.6, 31.1, 35.2, 61.7, 69.3, 69.9, 70.1, 72.2, 81.8, 84.9, 86.8, 120.5, 127.7, 127.8, 128.6, 137.9, 139.1, 148.7, 149.5.

2′-*O*-(6-Benzyloxyhexyl)-3′,5′–*O*-(1,1,3,3-tetraisopropyldisiloxyl)-N_2-isobutyryl-guanosine 92



2′-*O*-(6-Benzyloxyhexyl)-N_2-isobutyryl-guanosine **91** (1.50 g, 2.76 mmol) was co-evaporated with dry pyridine (2x10 ml) and then it was dissolved in dry pyridine (20.0 ml), followed by addition of 1,1-dichloro-1,1,3,3-tetraisopropyldisiloxane (0.95 ml, 3.03 mmol) while the temperature was kept at 0°C (ice-water bath). After 5 h the products were concentrated under reduced pressure and then partitioned between dichloromethane (60 ml) and saturated aqueous sodium hydrogen carbonate (50 ml). The layers were separated and the aqueous layer was back-extracted with dichloromethane (3x10 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was fractionated by

short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane-methanol (98:2 v/v) were combined and concentrated under reduced pressure to give the compound **92** as a colorless froth (2.60 g, 88.0%).

ESI-MS $[M-H]^-$ found 771.2, $C_{38}H_{61}N_5O_8Si_2$ required 771.40

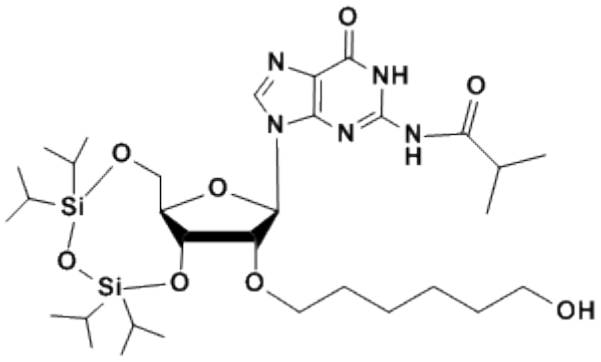
$\delta_H[CD_3)_2SO]$: 12.12 (1 H, s), 11.62 (1 H, s), 8.03 (1 H, s), 7.45-7.20 (5 H, m), 5.82 (1 H, s), 4.52-4.38 (3 H, m), 4.24-4.23 (1 H, m), 4.15-3.91 (3 H, m), 3.79-3.76 (1 H, m), 3.67-3.62 (1 H,

m), 2.89-2.70 (1 H, m), 1.60-1.49 (4 H, m), 1.32-1.30 (5 H, m), 1.05-0.95 (30 H, m).

$\delta_C[CD_3)_2SO]$: 12.5, 12.7, 12.8, 13.2, 13.3, 17.2, 17.3, 17.6, 17.6, 17.6, 17.8, 19.3, 25.8, 25.9, 29.6, 29.8, 35.2, 60.7, 70.0, 70.2, 71.0, 72.2, 81.3, 81.8, 87.0, 120.7, 127.7, 128.6, 136.7, 139.1, 148.4, 148.7, 155.2, 180.6.

R_f : 0.35 (system B).

2′-*O*-(6-Hydroxyhexyl)-3′,5′–*O*-(1,1,3,3-tetraisopropyldisiloxyl)-N_2-isobutyryl-guanosine 93



2′-*O*-(6-Benzyloxyhexyl)-3′,5′–*O*-(1,1,3,3-tetraisopropyldisiloxyl)-N_2-isobutiryl-guanosine **92** (0.30 g, 0.389 mmol) was dissolved in regular tetrahydrofuran (20.0 mL) and then it was followed by addition of excess amount of Pd/C (10%wt) (0.58 g, 0.389 mmol). The reaction

was shaken in a hydrogen atmosphere (50 psi) for 7 days. The reaction mixture was filtered through a bed of Celite. The filtrate was concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane-methanol (98:2 v/v) were combined and concentrated under reduced pressure to give the compound **93** as a colorless froth (0.24 g, 88%).

ESI-MS $[M-H]^-$ found 695.0, $C_{32}H_{57}N_5O_8Si_2$ required 695.37

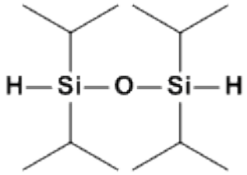
$\delta_H[CD_3)_2SO]$: 12.28 (1 H, s), 11.62 (1 H, s), 8.02 (1 H, s), 5.83 (1 H, s), 4.49 (1 H, d, *J* = 4.8), 4.48 (1 H, s), 4.25 (1 H, d, *J* = 4.8), 4.10 (1 H, d, *J* = 13.2), 4.02 (1 H, d, *J* = 8.4), 3.95 (1 H, d, *J* = 12), 3.79-3.76 (1 H, m), 3.66-3.63 (1 H, m), 2.79-2.76 (1 H, m), 1.54-1.50 (3 H, m), 1.40-1.23

(9 H, m), 1.12 (8 H, d, J = 6.6), 1.09-0.9 (24 H, m).

$\delta_C[CD_3)_2SO]$: 13.6, 14.2, 17.3, 17.5, 17.6, 19.2, 21.4, 22.5, 22.8, 23.7, 25.8, 26.0, 29.5, 30.0, 30.8, 35.1, 61.1, 67.7, 70.2, 71.2, 72.7, 81.4, 81.7, 87.1, 120.8, 125.3, 127.6, 128.4, 128.5, 128.8, 129.0, 129.6, 131.8, 132.2, 136.6, 139.5, 148.4, 148.7, 151.9, 155.2, 167.3, 174.8, 180.6.

R_f : 0.30 (system B).

1,1,3,3-Tetraisopropyldisiloxane 97



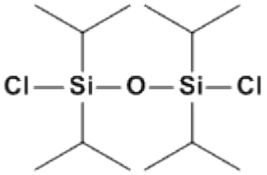
To a suspension of magnesium turnings (34.6 g, 1.43 mol), iodine (0.1 g) in dry diethylether (500 ml) was added dropwise 2-bromopropane (130 ml, 1.38 mol) at such a rate that a gentle reflux was maintained. After the addition was complete, the reactants were cooled (ice-salt bath). A solution of trichlorosilane (50 ml, 0.493 mol) in dry diethylether (400 ml) was added dropwise over a period of 2 h, while the temperature was kept below 15°C. The reaction mixture was heated under reflux for 6 h and then cooled (ice-salt bath). To this cooled mixture was added hydrochloric acid (10% w/v, 660 ml) dropwise while the temperature did not exceed 15°C.

After the addition was complete, stirring was continued for 30 min. The layers were separated and the aqueous layer was back-extracted with diethyl ether (4x100 ml). The combined organic layers were further washed with water (2x100 ml), dried ($MgSO_4$) and concentrated under reduced pressure. Distillation of the residue under reduced pressure afforded the compound **97** as a colorless liquid (53.30 g, 87%, 118-120°C/28 mmHg). ^{1}H NMR data are in agreement with literature values.[94]

$\delta_H[CD_3)_2SO]$: 4.43 (2H, s), 1.10-1.06 (28H, m).

$\delta_C[CD_3)_2SO]$: 17.15, 13.26.

1,1-Dichloro-1,1,3,3-tetraisopropyldisiloxane 98

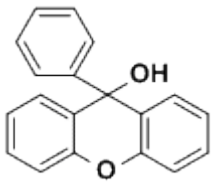


To a solution of 1,1,3,3-tetraisopropyldisiloxane (80.0 g, 0.324 mol) in dichloromethane (500 ml) cooled at 0ºC (ice-salt bath) was added dropwise a saturated solution of chlorine in dichloromethane (600 ml) until a yellow color persisted. The products were then evaporated under reduced pressure and the residue was distilled under reduced pressure to give compound **98** as a colorless liquid (88 g, 86%, 92-96°C/0.7 mmHg). ^{1}H NMR data are in agreement with literature values.[94]

$\delta_H[(CD_3)_2SO]$: 1.53-1.10 (28H, m).

$\delta_C[(CD_3)_2SO]$: 16.54, 15.44.

9-Phenylxanthen-9-ol 99

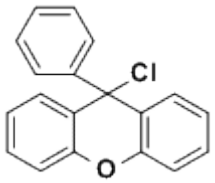


A literature procedure was followed for the preparation of this compound.[95] Magnesium turnings (2.43 g, 0.10 mol) were suspended in dry diethyl ether (30.0 ml) followed by addition of a small crystal of iodine (15 mg). Then a solution of bromobenzene (15.70 g, 10.46 ml, 0.10 mol) in dry diethyl ether (50.0 ml) was added dropwise over a period of 1 h. the reactants were then heated gently under reflux until the magnesium turnings disappeared. Then xanthen-9-one (9.81 g, 50 mmol) was added, followed by addition of dry diethyl ether

(50.0 ml). After the reactants had been heated under reflux for 2.5 h. they were allowed cool to room temperature and the precipitate was collected by filtration, followed by washing with diethyl ether (2x20 ml). The filter cake was suspended in a mixture of water (200 ml) and ice (200 g). Concentrated hydrochloric acid (37% w/v, 40 ml) was added under vigorous stirring. After 30 min, the products were filtered and washed with water until the washings were neutral. The solid product was air-dried to give a pale yellow solid (12.5 g, 86%) which was recrystallized from ethanol to give 9-phenyl-xanthen-9-ol as colorless crystals (8.20 g). ^{1}H NMR data are in agreement with literature values.[95]

$\delta_H[CD_3)_2SO]$: 6.73 (br s, 1H),7.07 (t, 2H, *J* = 15), 7.11 (t, 1H, *J* = 13.2), 7.19–7.30 (m, 8H),7.35 (d, 2H, *J* = 7.8).

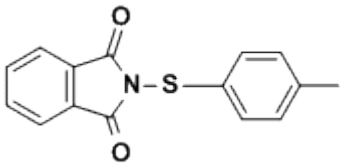
9-Chloro-9-phenylxanthene 100



A literature procedure was followed for the preparation of this compound.[96] 9-Phenyl-xanthen-9-ol **97** (4.47 g, 15.50 mmol) was evaporated with dry toluene (2x10 ml), and the residue was re-dissolved in anhydrous toluene (20.0 ml), followed by addition of acetyl chloride (13.0 ml, 0.183 mmol). After the reactants had been stirred for 16 h at room temperature under an atmosphere of argon, the products were evaporated under reduced pressure. The residue was co-evaporated with dry toluene (3x15 ml). The product was further dried *in vacuo* (oil pump) at 40ºC for 3 h to give the compound **100** as a pale yellow solid in

virtually quantitative yield (4.80 g). The product was used without further purification due to its sensitivity to moisture.

2-Methylphenylsulfanyl-1*H*-isoindole-1,3(1*H*)-dione 101



4-Methylbenzenethiol (15.19 g, 0.105 mol) and phthalimide (14.70 g, 0.10 mol) were dissolved in hot pyridine (40.0 ml) and acetonitrile (50.0 ml), and the stirred solution was cooled to room temperature. A solution of bromine (19.20 g, 6.19 ml, 0.12 mol) in acetonitrile (50.0 ml) was then added dropwise over 30 min. After a further period of 2h, methanol (200 ml) was added dropwise over 30 min. the products were cooled (ice-water bath) for 30 min, and then filtered to give compound **101** as pale yellow crystals (25.20 g, 87%). Recrystallization of this material from methanol gave pale yellow crystals. ^{1}H NMR data are in agreement with literature values.[97]

$\delta_H[CD_3)_2SO]$: 7.40 (4H, m), 7.93 (2H, m), 7.98 (2H, m).

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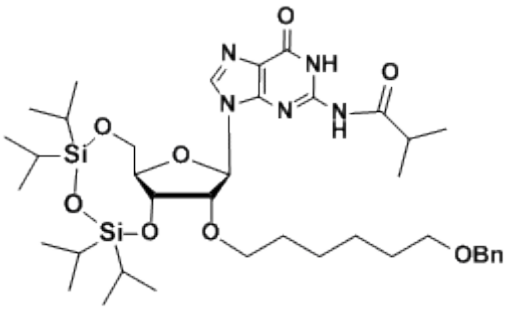
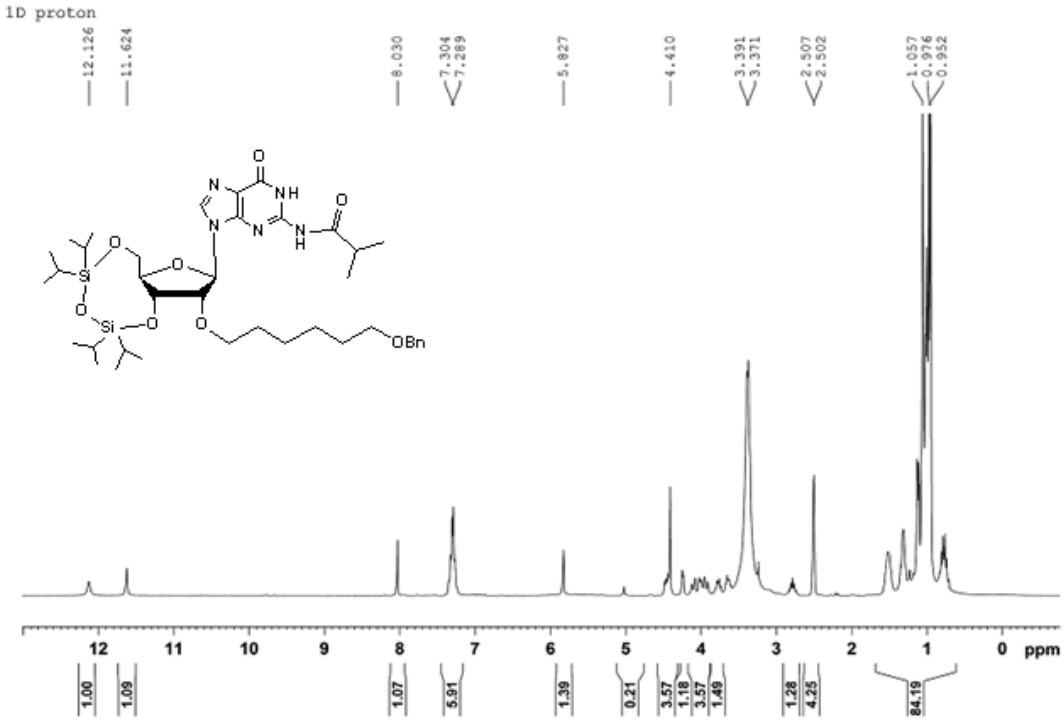
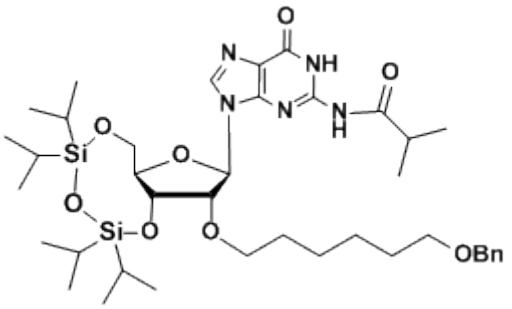
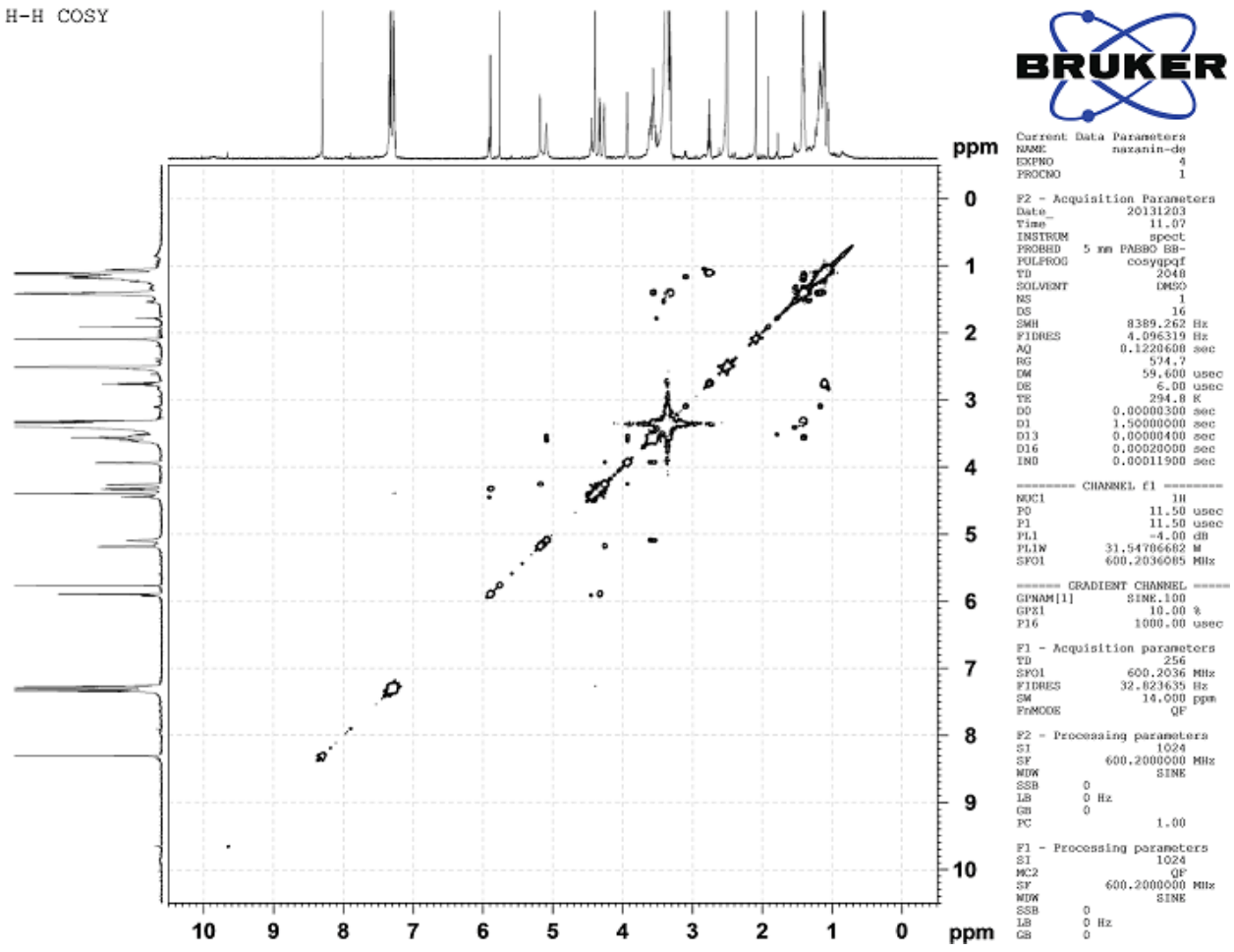
Appendix – Selected NMR Spectra



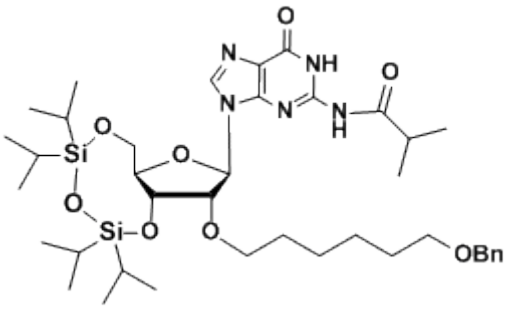


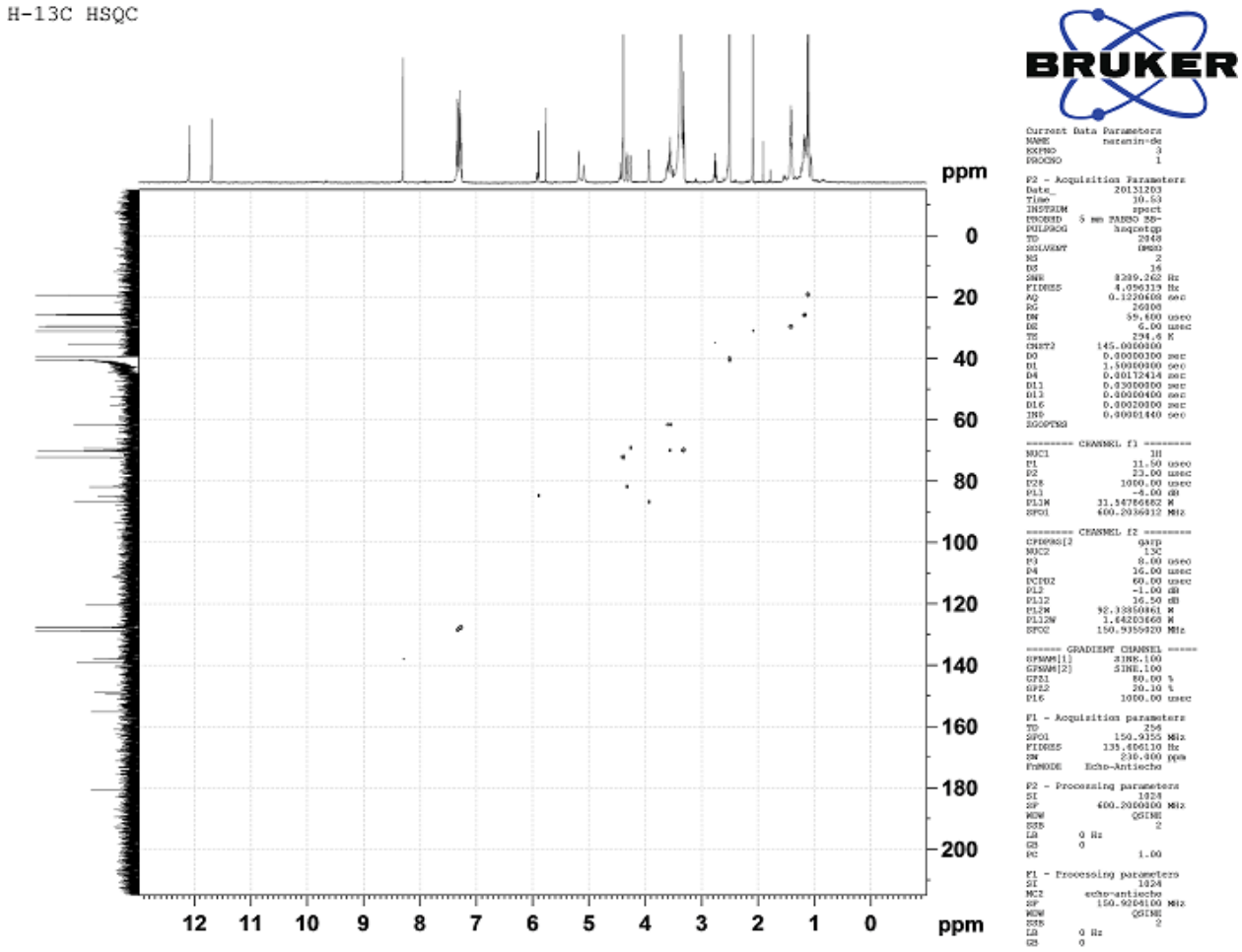
^{1}H-NMR of compound **92**.



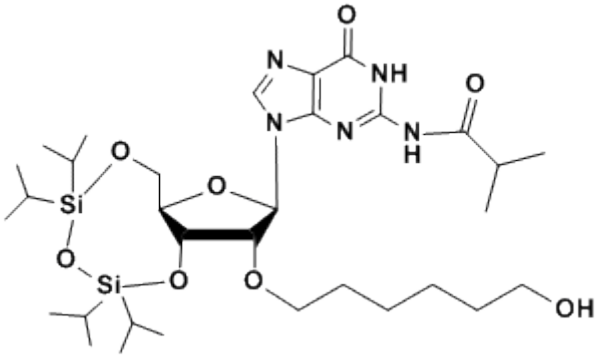


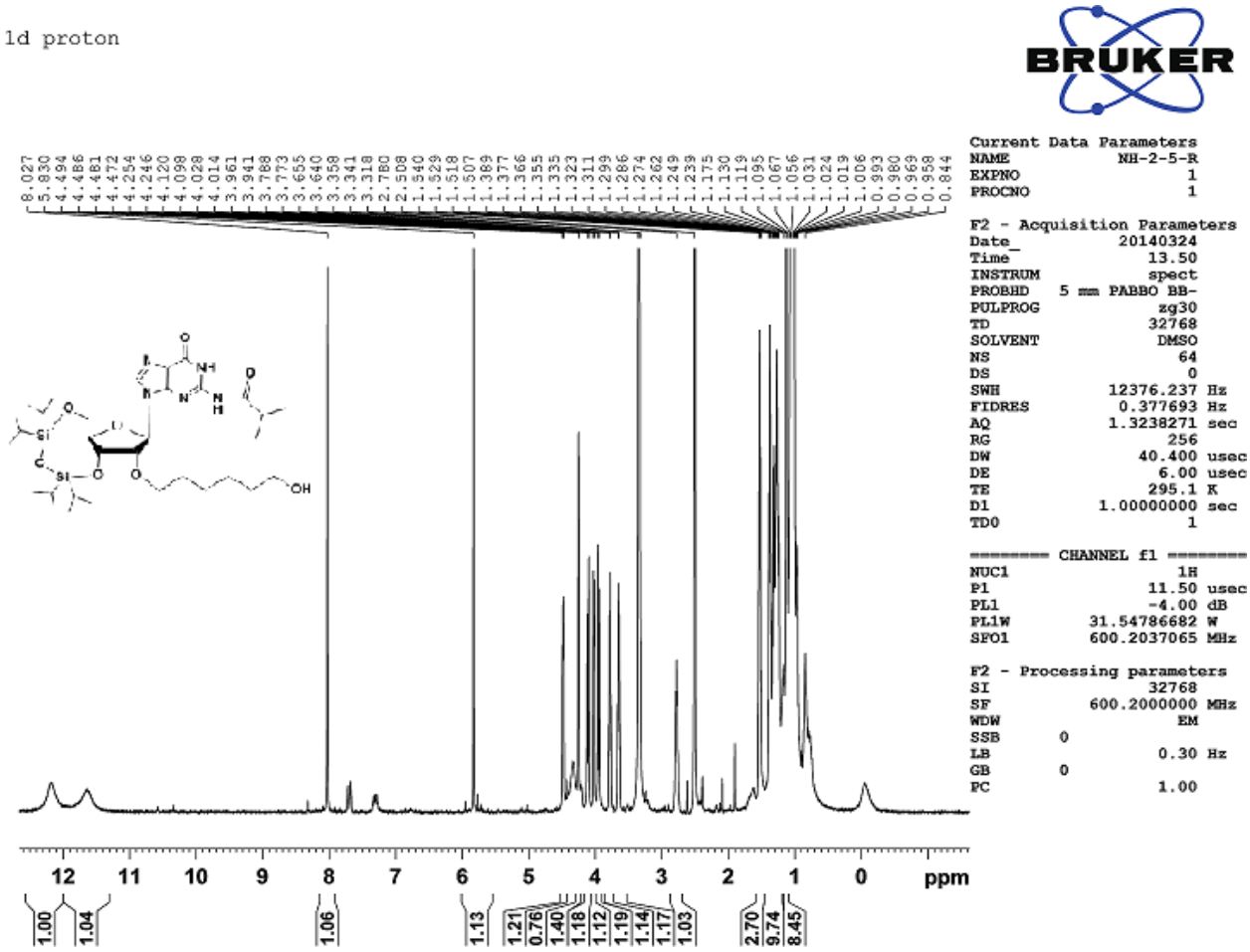
COSY-NMR of compound **92**.





HSQC-NMR of compound **92**.





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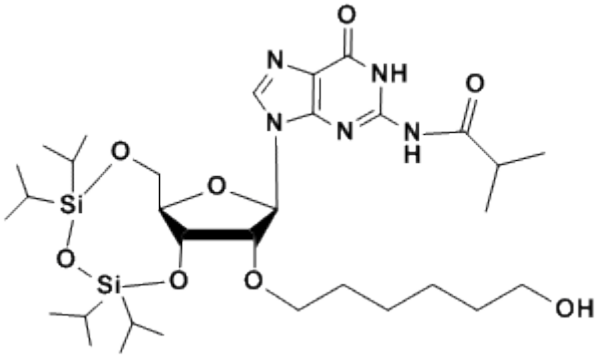

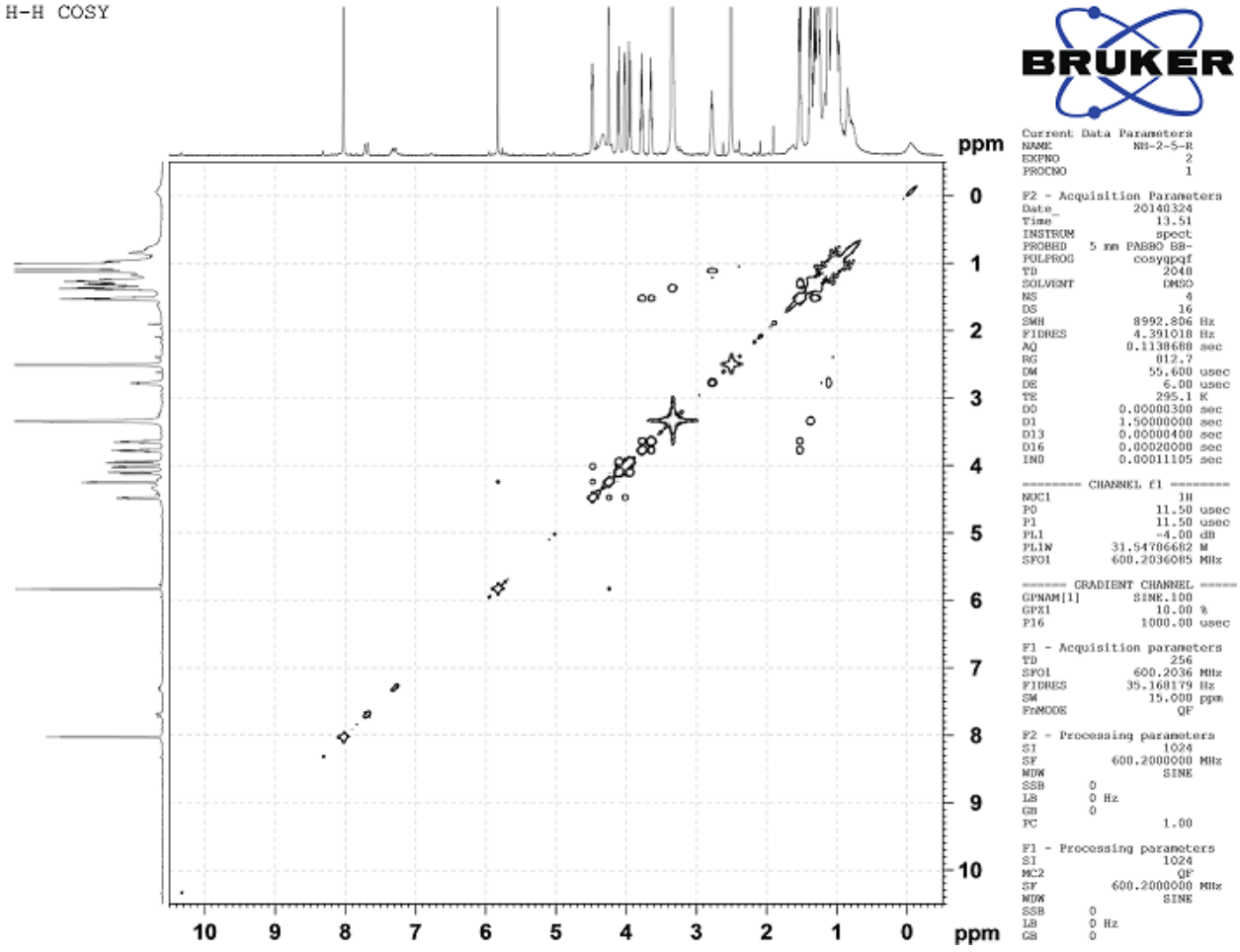

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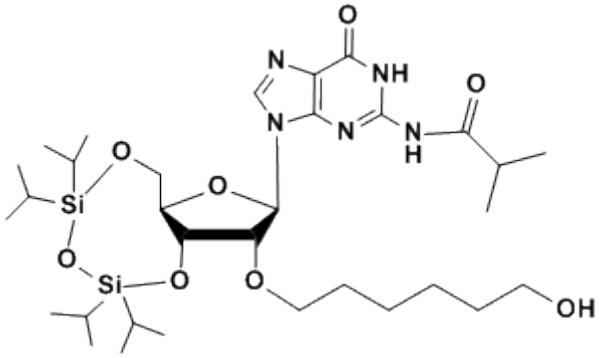
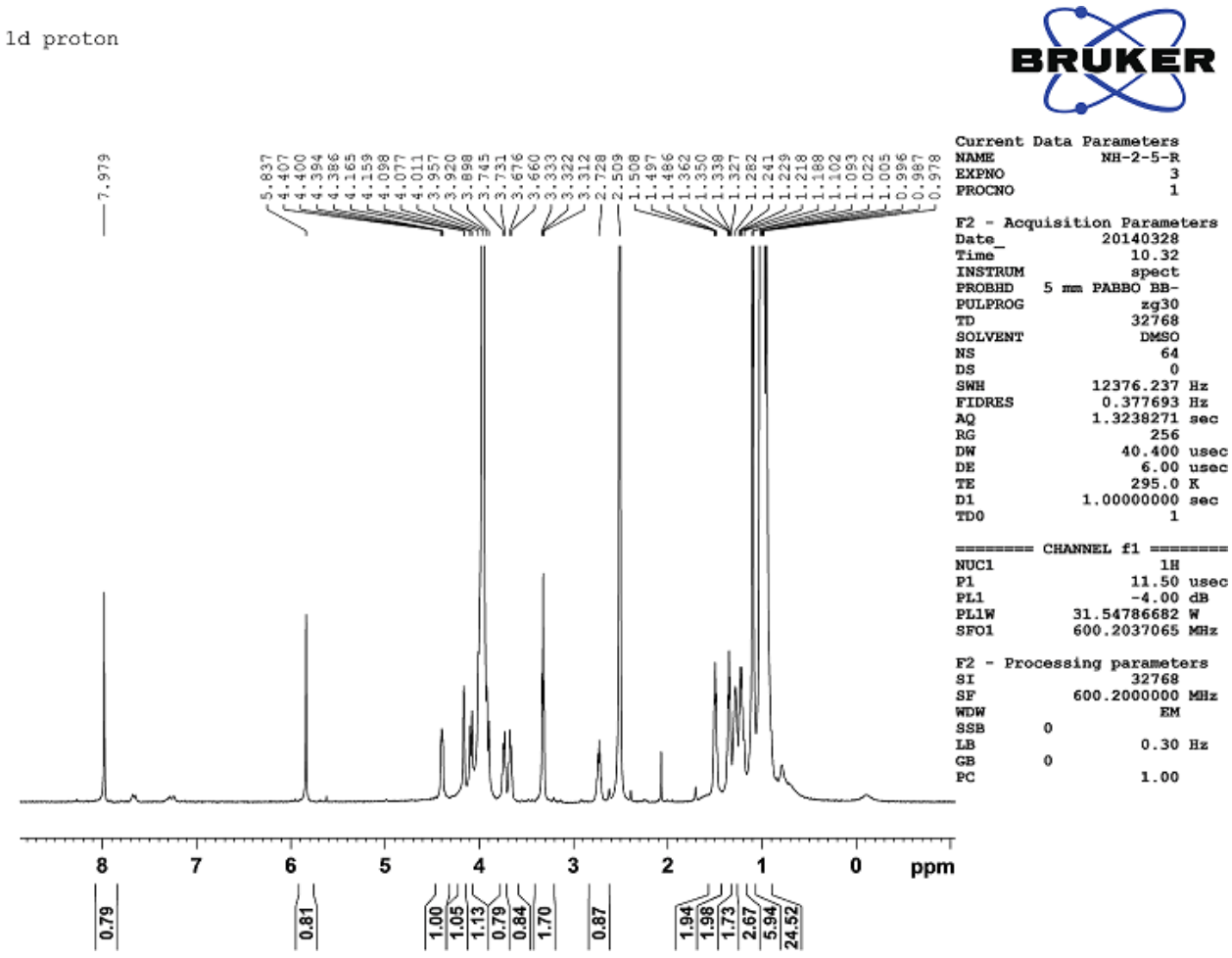
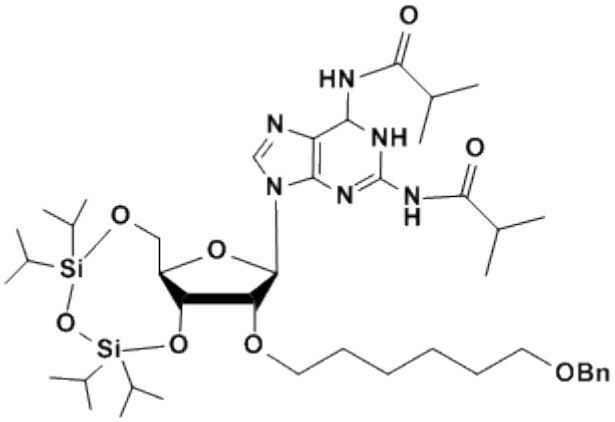
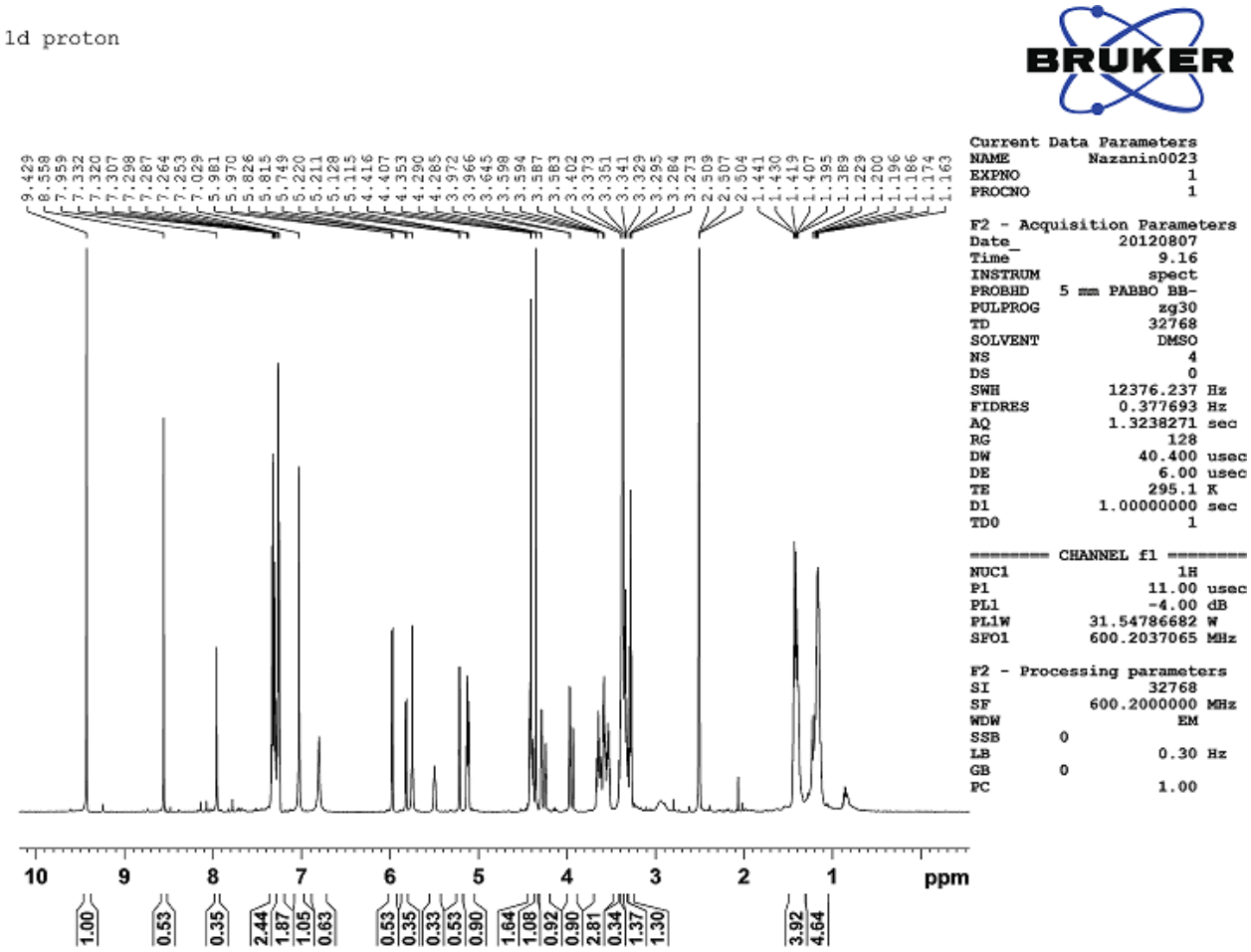


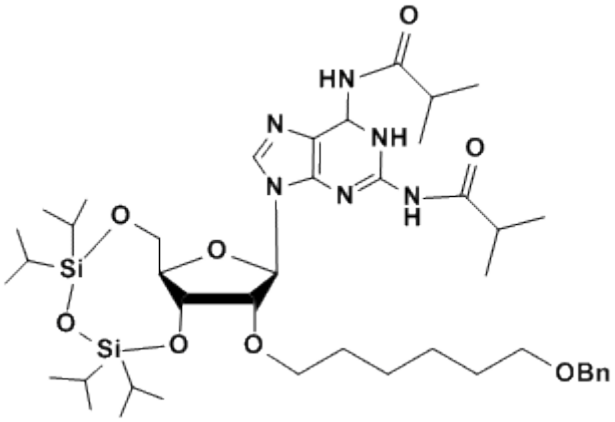


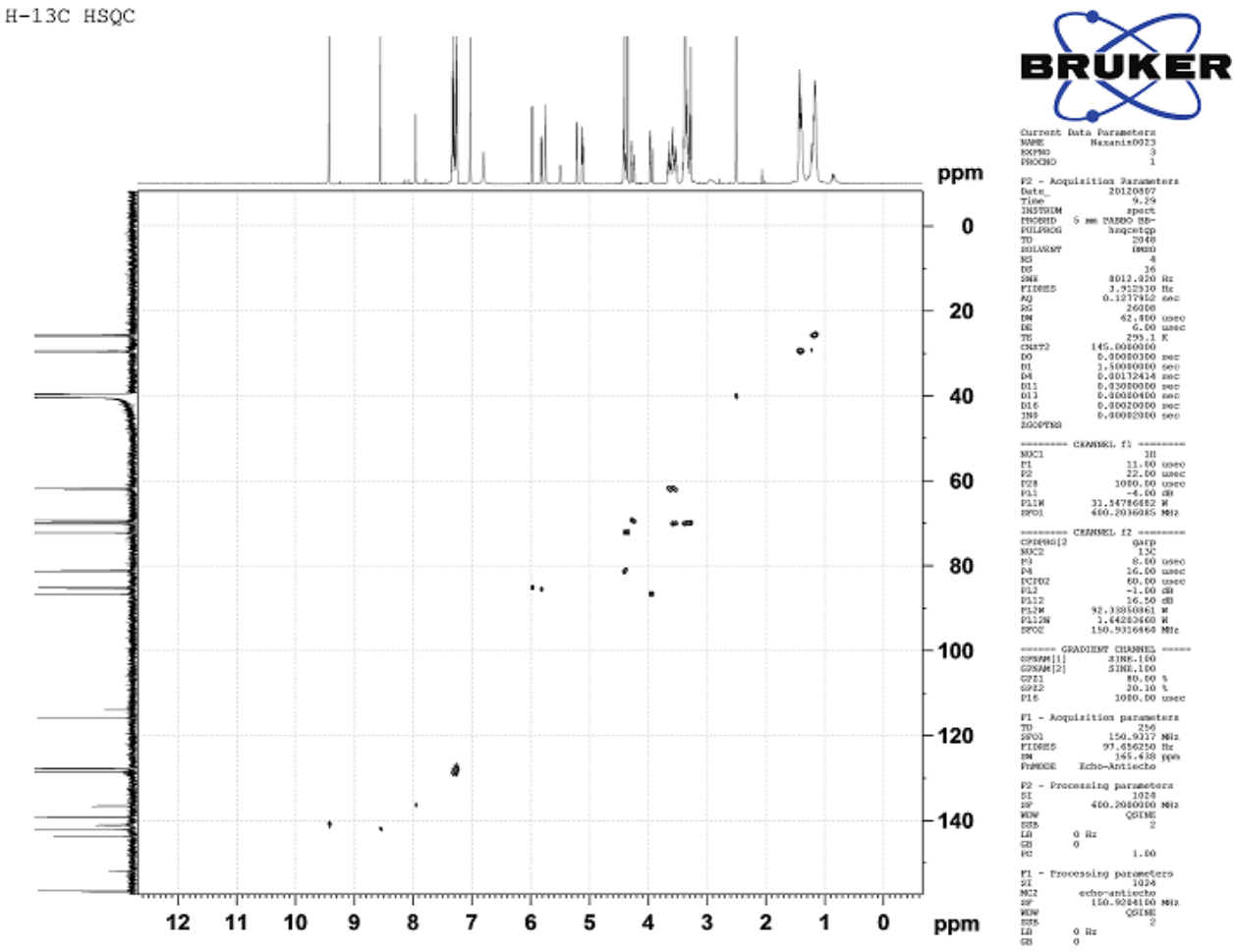
D_2O-exchange of compound **93**.



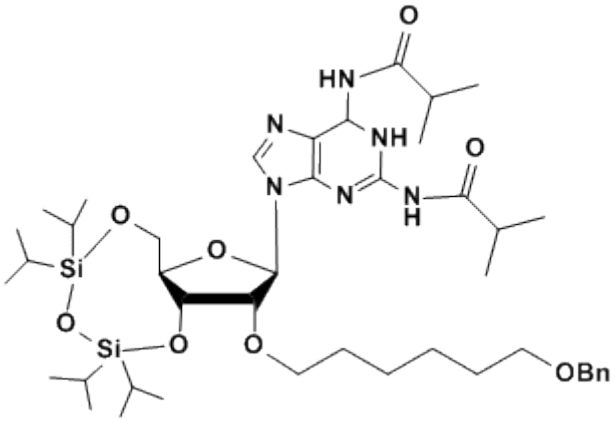


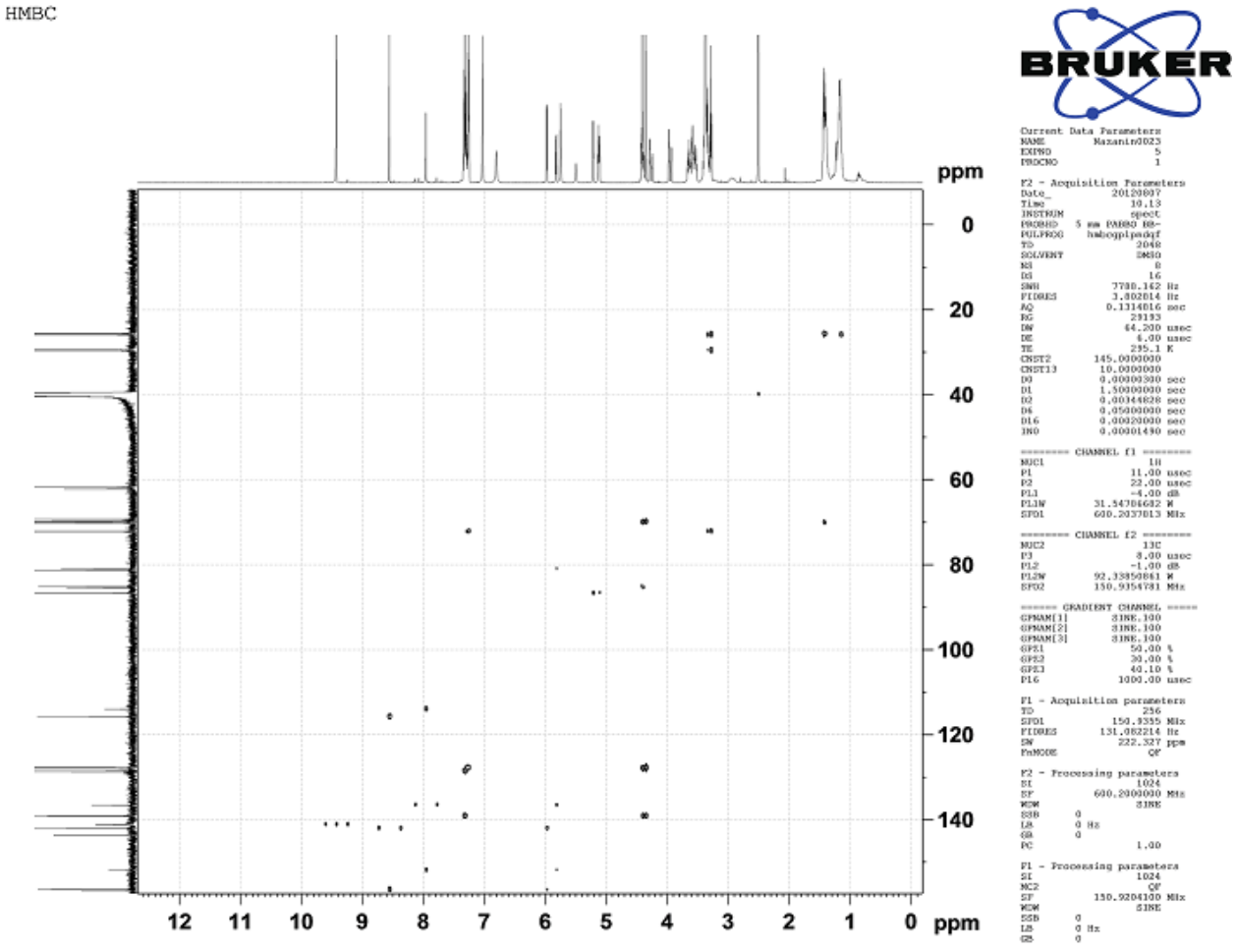
[1]H-NMR of compound **89i** (without TIPDS).



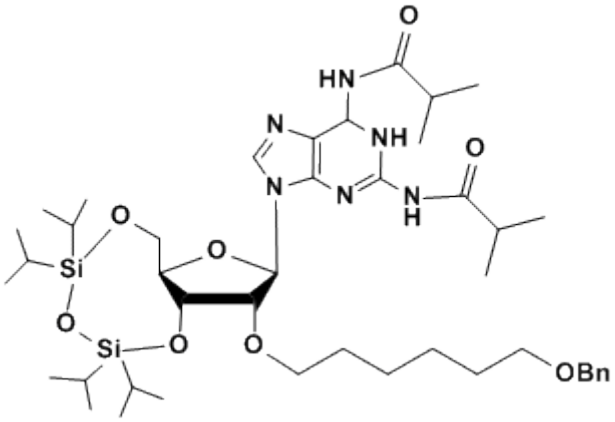


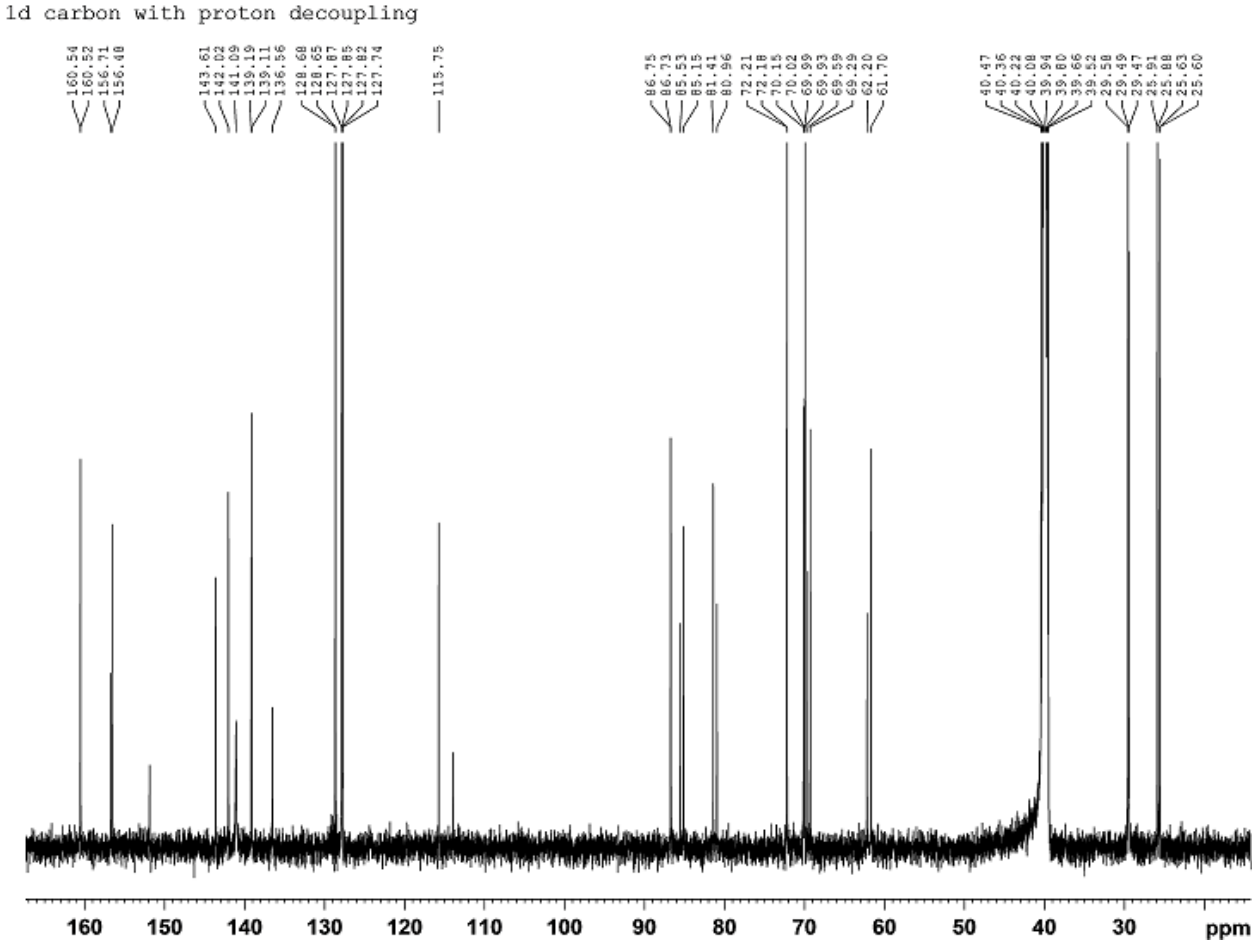
HSQC-NMR of compound **89i** (without TIPDS).



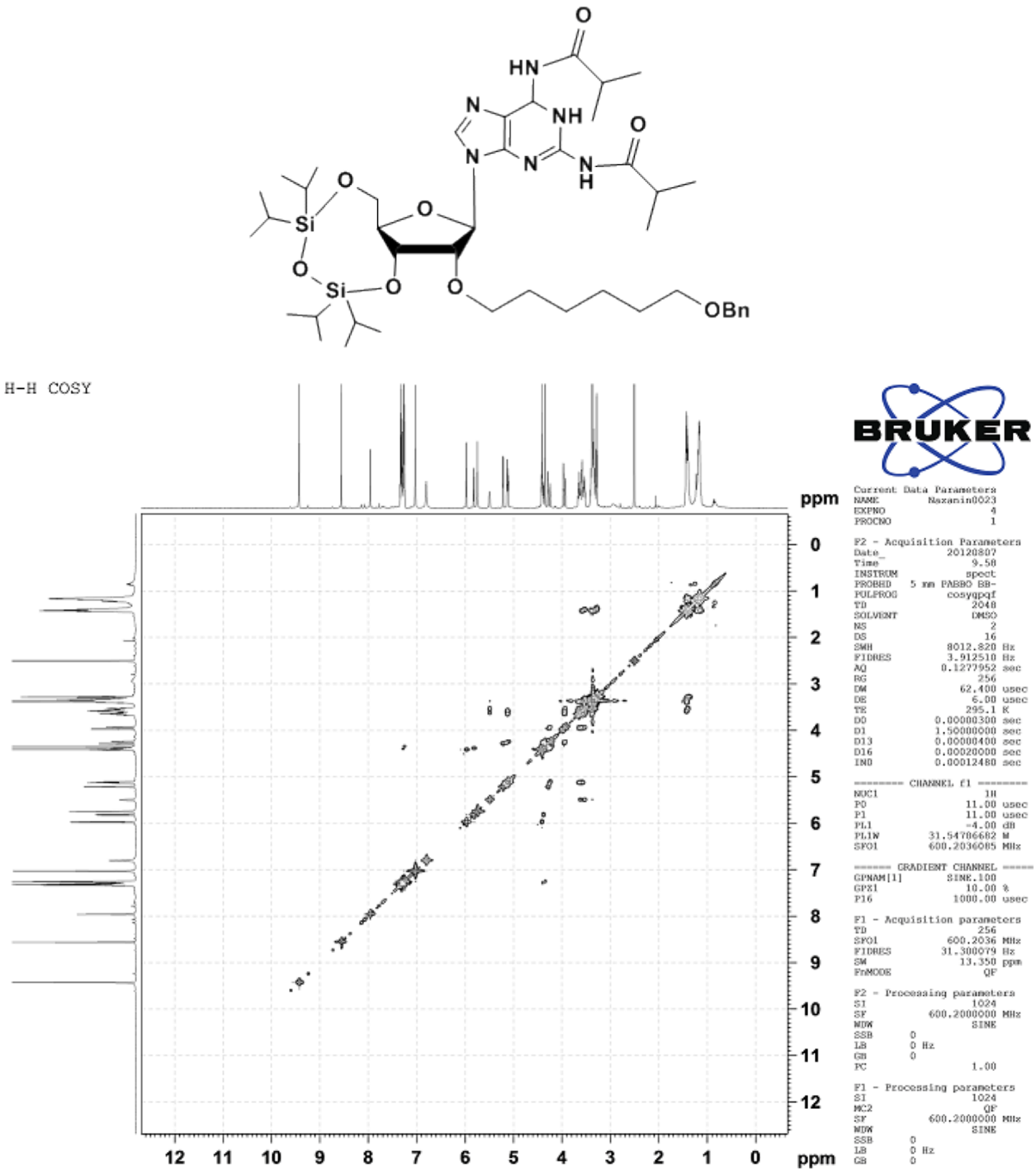


HMBC-NMR of compound **89i** (without TIPDS)

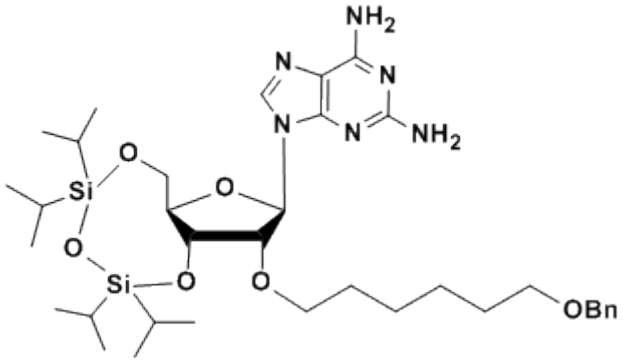


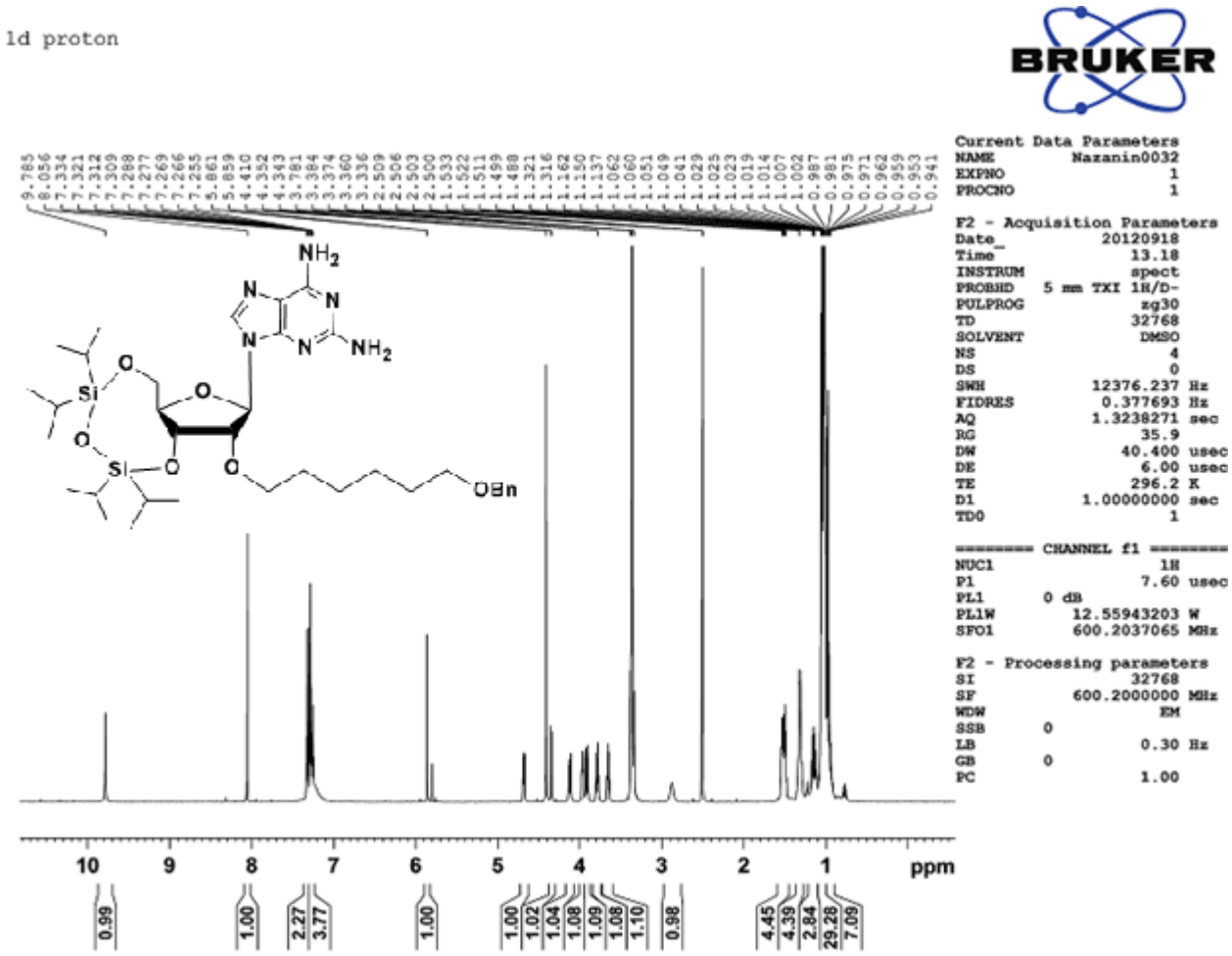


^{13}C-NMR of compound **89i** (without TIPDS).

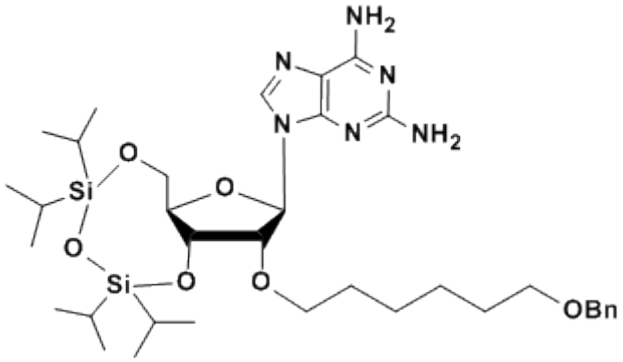


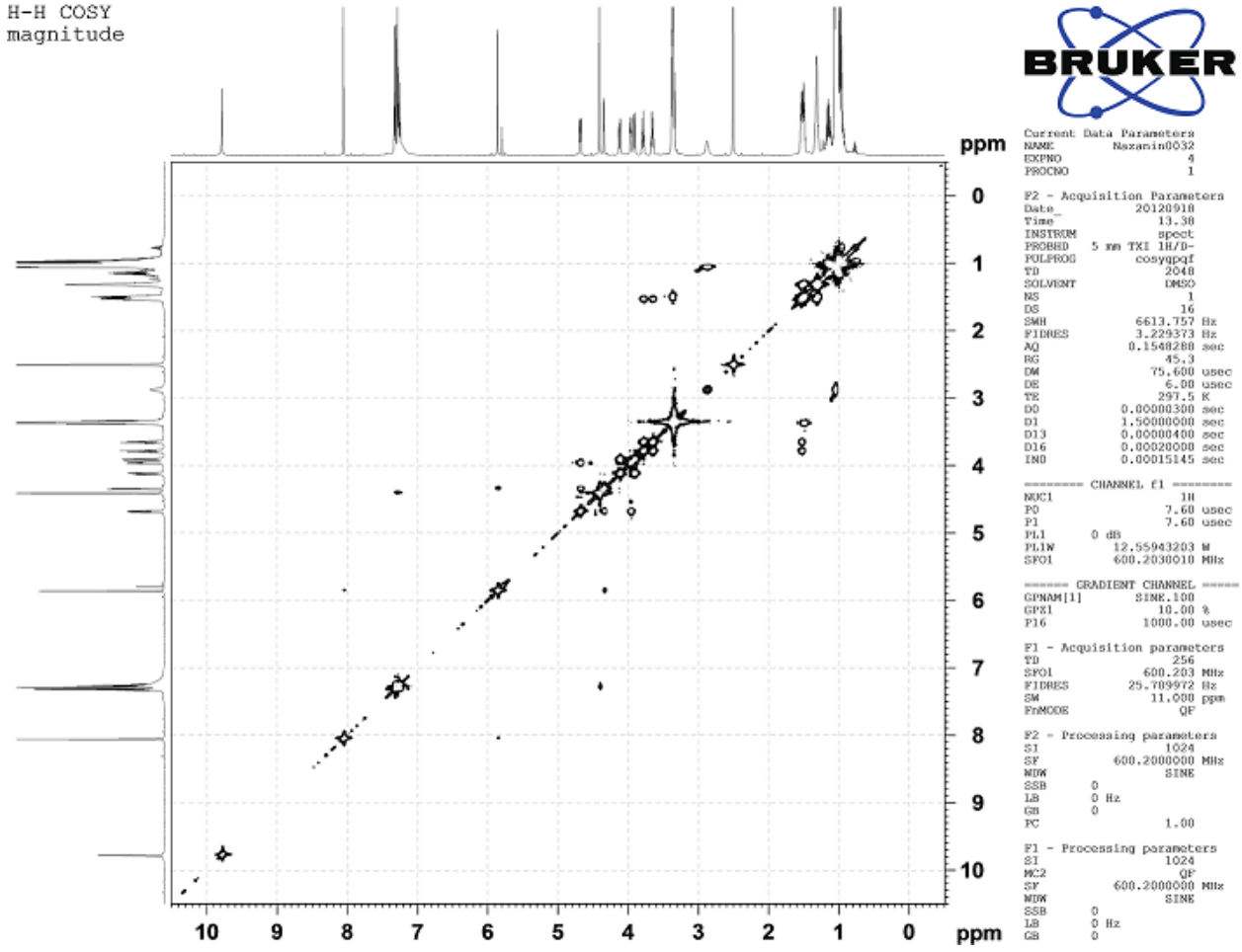
COSY-NMR of compound **89i** (without TIPDS).



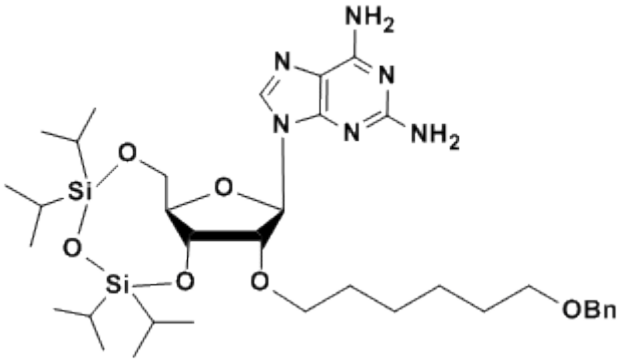


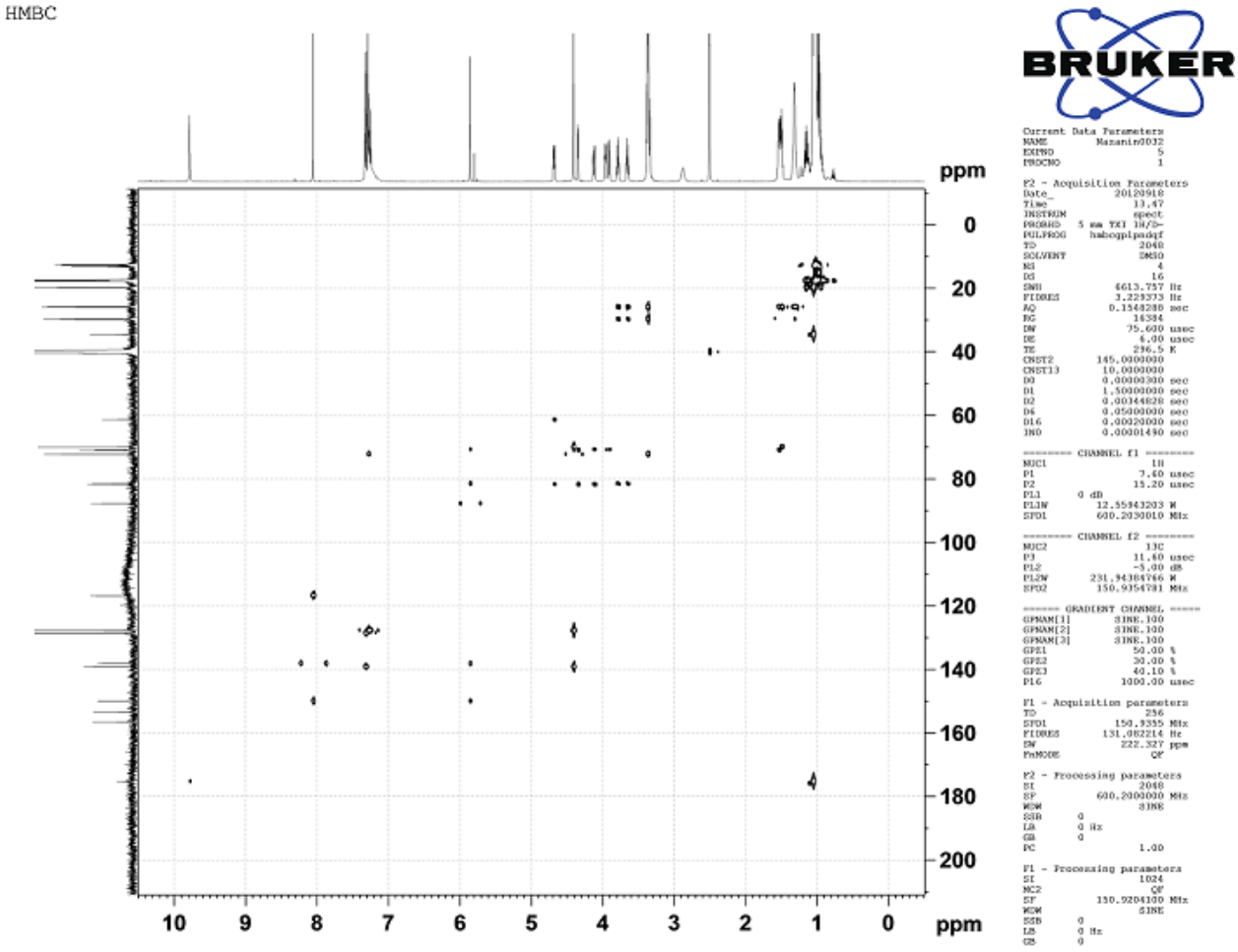
^{1}H-NMR of compound **89.**





COSY-NMR of compound **89**.





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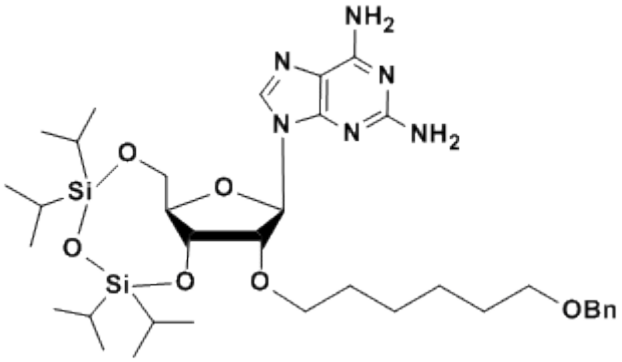
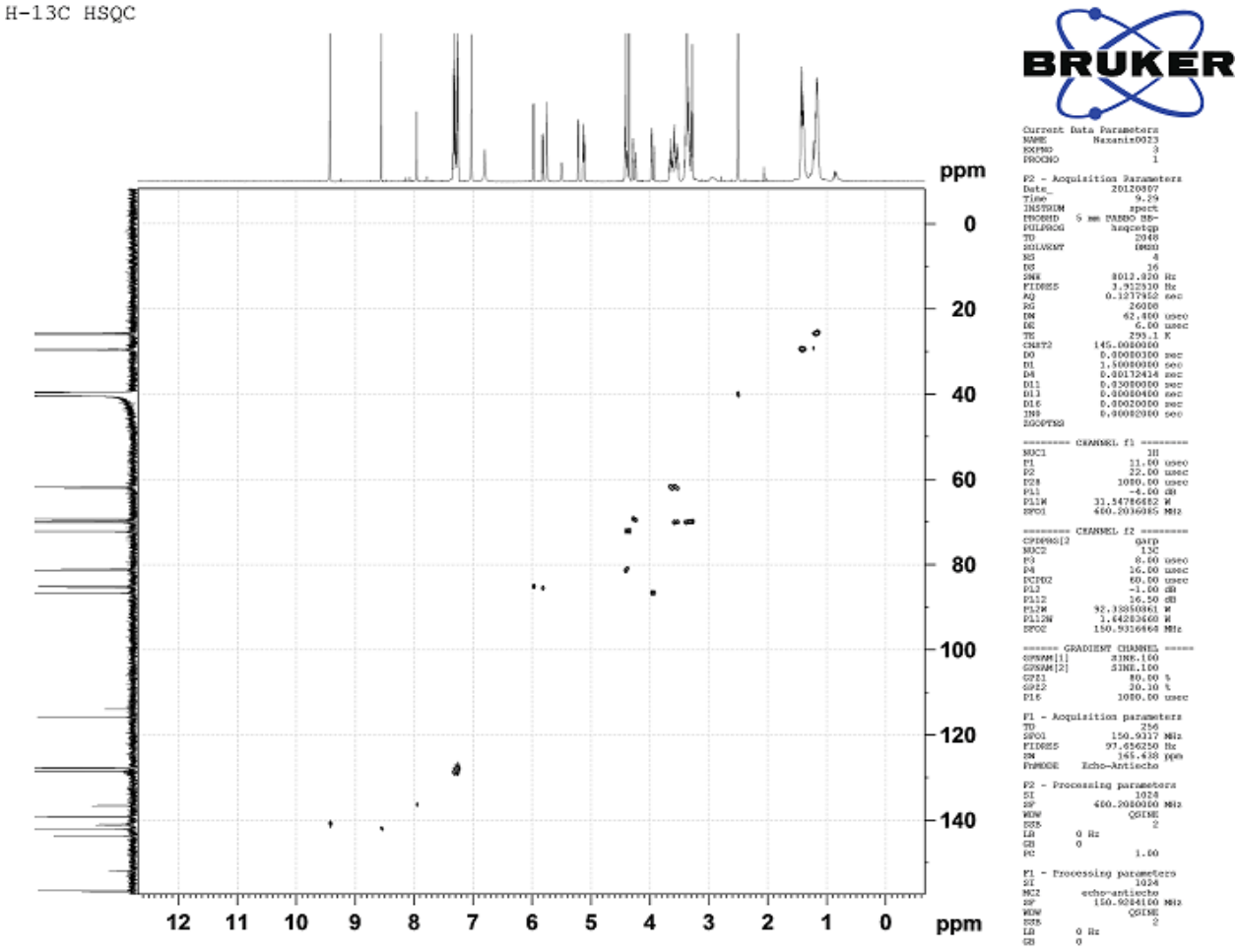
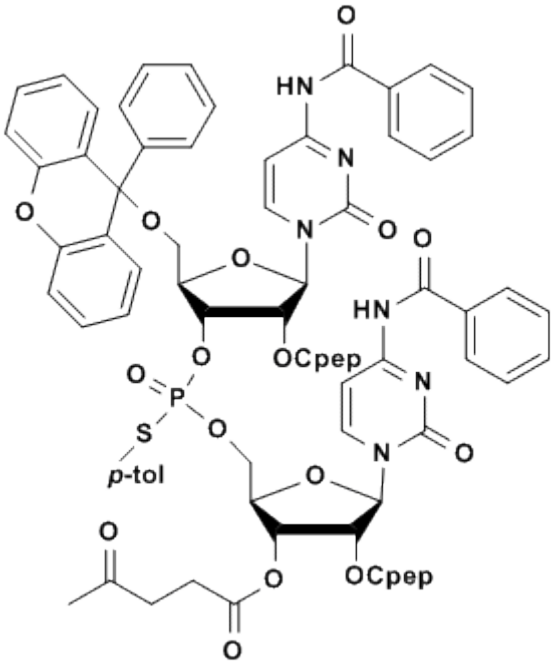
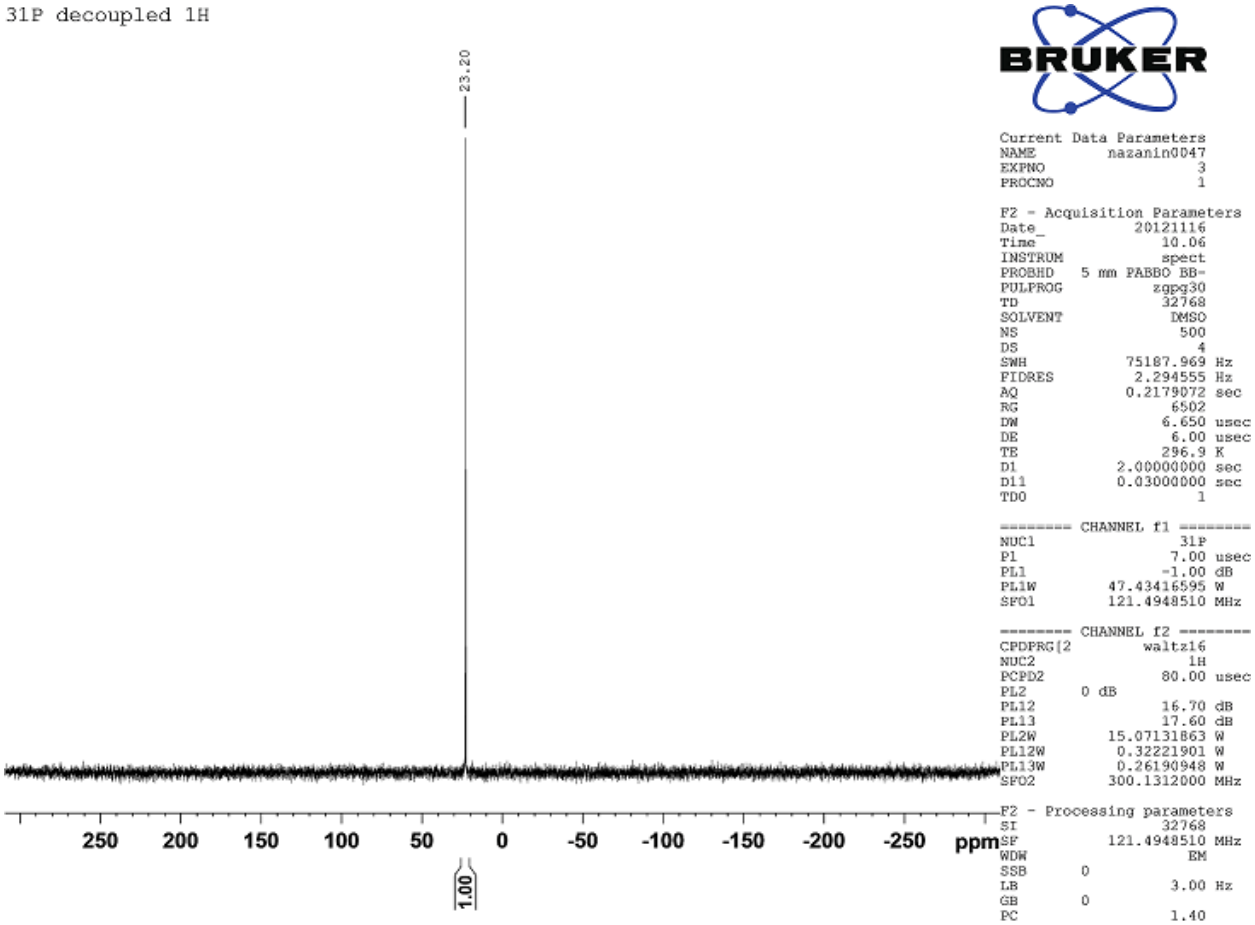


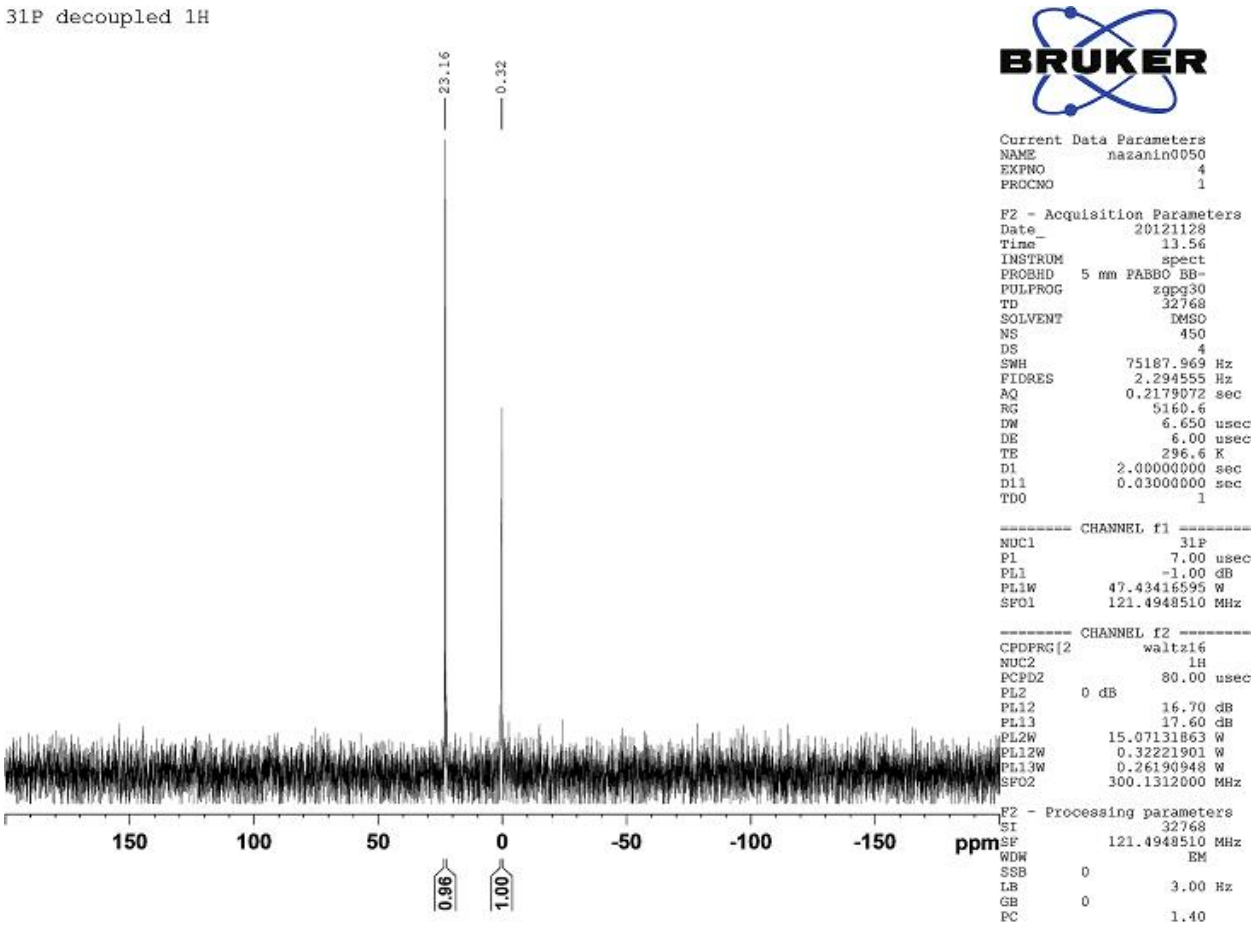


HSQC-NMR of compound **89**.





^{31}P-NMR of compound **68** (^{1}H decoupling).



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