SYNTHESIS OF CYCLIC DIPURINIC ACIDS AS POTENTIAL INHIBITORS TARGETING DIGUANYLATE CYCLASES

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TABLE OF CONTENTS

SUMMARY

Five analogues with the c-di-GMP backbone structure but with varied bases, including c-di-GMP itself were synthesized with reasonable yield of 10-15% after 8 steps. They were designed as antimicrobial agents to inhibit biofilm formation, which is responsible for diseases such as chronic cystic fibrosis and nosocomial bacteremia. These compounds were subsequently tested for biological activities on Slr 1143, diguanylate cyclase of *Synechocystis sp*., that was overexpressed from the recombinant plasmid which contained the gene of interest and subsequently, purified by affinity chromatography. A new HPLC method was optimised and it applied in the analysis of the synthetic compounds, which was coincidentally capable of eluting out the compounds earlier than the product, hence separating the two peaks, with good resolution. Results have shown that cyclic di-inosinylic acid **9b** exhibited a higher inhibition of 60% on Slr1143 than c-di-GMP. **9b** can also be assayed on diguanylate cyclases of other bacteria species to determine its potential as an inhibitor of biofilm formation. Future work can also be done to improve the inhibitory activity of **9b** by synthesising libraries that contain compounds with pharmacophores similar to **9b.** It is our firm belief that various diseases caused by biofilm formation can be treated with an alternative therapeutic method of using strong inhibitors in the near future.

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

δ	chemical shift in ppm
ACN	acetonitrile
aq	aqueous
calcd	calculated
d	doublet
dd	doublet of doublets
dt	doublet of triplets
DCM	dichloromethane
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
DMTCl	4,4'-Dimethoxytritylchloride
equiv.	equivalent
EA	ethyl acetate
h	hour
IC_{50}	half maximal inhibitory concentration
IMP	imidazolium perchlorate
J	coupling constant
m	multiplet
MBP	maltose binding protein
Me	methyl

min	minutes
MS	mass spectroscopy
N-MeIm	*N*-methylimidazole
NMR	nuclear magnetic resonance
o/n	overnight
q	quartet
rt	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
s	singlet
temp.	temperature
t	triplet
TBDMSCl	*tert*-Butylchlorodimethylsilane
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TPSCl	2,4,6-triisopropylbenzenesulfonylchloride

CHAPTER 1: INTRODUCTION

Bacteria biofilm, which is defined as a community of bacteria that is attached to a surface and encased in a self-produced polymeric matrix[1,2], has long been a cause of concern for medical specialists and researchers as many chronic bacterial infections are a result of biofilm formation. Some of the pathogenic bacteria involved are *Vibrio cholerae*[3,4], *Yersiia pestis*, *Pseudomonas aeruginosa*[5] and *Staphylococcus aureus*[6], to list a few. For instance, *Staphylococcus aureus* is an important human and animal pathogen that is found on the skin and mucosal surfaces of humans, specifically in the anterior nares. It is the primary and most common cause of surgical infections and nosocomial bacteremia due to biofilm-based infections[6,7], which can increase hospital stay by duration of up to 2-3 days, incurring billions of added cost per year. In addition, biofilm formation can also lead to antibiotic resistance[6], which is a pressing problem that needs to be overcome. *Pseudomonas aeruginosa*, on the other hand, is responsible for systemic infections in individuals with low immunity and chronic respiratory diseases in patients with cystic fibrosis whilst *Vibrio cholerae* can cause acute intestinal infection cholera through two main virulence factors; cholera toxin (CT) is responsible for the profuse secretory diarrhoea while toxin co-regulated pilus (TCP) is required for colonization of the small intestine. Its ability to form biofilm, which can withstand environmental stresses, not only increases its chances of survival in aquatic environments between cholera epidemics, but may also result in bacterial resistance to the innate host immune system functions.

Biofilms are resistant to antimicrobial and antibiotics for a myriad of reasons. The altered living conditions of the biofilm (e.g. low pH, low pO_2, high pCO_2, low hydration level, etc) may result in low metabolic activity and hence low antimicrobial activity.[1,8] Furthermore, the antimicrobial agents may be trapped as waste or chelated by inactivating enzymes. Horizontal gene transfer within bacteria in biofilms also allows bacteria to gain resistance[1]. Quorum sensing signalling systems allow synchronisation of the target gene expression within the biofilm, allowing the bacteria to evade the effects of antimicrobial agents[9,10]. In addition, a fraction of bacteria may differentiate into persister cells which have extremely low metabolic rate and are non growing. These cells are resistant and may regenerate the biofilm once the therapy ended[11].

Current therapies to reduce the rate of biofilm infections include prophylactic use of antibiotics and microbiocides through methods such as device coatings, device immersion and surgical site irrigation[12]. Quorum quenching enzymes or inhibitors[13], as well as antimicrobials to destroy persister cells, have been developed over the years. In addition, strategies such as enhancing the activity of the antimicrobial agents via electromagnetic field[14], radiofrequency electrical current[15] and ultrasound[16] have been explored besides destroying and reducing the growth of the biofilm matrix[1].

Cyclic purine ribonucleotides such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are well-studied examples of second messengers - small molecules that play an important role in cellular signalling and function. Synthesis of these molecules is regulated by enzymes like adenyl/guanyl

cyclases, whereas the breakdown of second messengers via hydrolysis is catalysed by phosphodiesterases. This cycle of synthesis and hydrolysis allows the regulation of the intracellular levels of cyclic purine ribonucleotide molecules, which has been elucidated to be responsible for bacterial response to external stimuli such as change in temperature, light, pH, oxygen levels and nutrients . In addition, cyclic purine ribonucleotides are also responsible for the mediation of cellular processes such as vision and activities of a wide range of protein kinases, GTPases and ion channels in eukaryotes. Although cGMP is commonly involved in cellular signalling in eukaryotic cells, prokaryotes do not seem to use it as a signalling molecule. Instead they utilise an alternative, cyclic guanosine monophosphate (c-di-GMP), also known as cyclic bis(3'-5')diguanylic acid in cellular signalling.

Although c-di-GMP, was first discovered in 1990 to be an activator of a cellulose synthetase complex in *Gluconacetobacter xylinus*[17], its role as a signalling molecule only attracted widespread interest recently in view of the growing bacterial genome sequence that has been decoded. It has since been established as an ubiquitous signalling molecule in bacteria, but not in eukaryotic cells or archaea.[18] The signalling transduction function of c-di-GMP, allowing surface-cell and cell-cell interaction is further demonstrated by the spanning of diguanylate cyclase across the cell membrane. The structure of c-di-GMP is illustrated below (Figure 1).

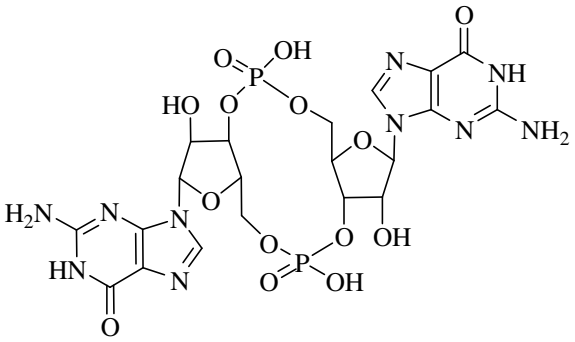

Figure 1. Structure of c-di-GMP

The synthesis of c-di-GMP involves the conversion of two guanosine triphosphate (GTP) molecules by diguanylate cyclases (DGC)s into c-di-GMP. Degradation of c-di-GMP is achieved via hydrolytic cleavage of the cyclic compound into guanosine monophosphate (GMP)[17,20,21] by phosphodiesterases (PDE)s through the GGDEF domain of DGCs and the EAL domains in PDEs respectively. There are a number of bacterial proteins that are bifunctional, meaning that they contain both the GGDEF and EAL domains. Other enzymes show greater specificity in their activity and they function either as DGCs or PDEs.[22] However, the cellular level of c-di-GMP is not regulated by one protein but a myriad of different proteins; the *Pseudomonas aeruginosa* genome encodes 17 different DGC domains, 5 PDE domains, and 16 that contain both DGC domains and PDE domains (DGC-PDE). Each performs its roles and regulate DGC and PDE levels differently such that an appropriate balance is achieved.[5]

c-Di-GMP has the ability to regulate motility and virulence gene expression of bacteria and at high intracellular concentrations of c-di-GMP, biofilm formation and exopolysaccharide movement.[19] Studies of *Pseudomonas aeruginosa* have shown that an increase in the diguanylate cyclases (DGCs) levels but not the phosphodiesterases (PDEs)

levels, accelerated bacterial production, resulting in adhesion to surfaces[19]. Increase in c-di-GMP concentration will also result in formation of multilayer pellicle which is a hyperbiofilm phenotype.[5]

It was initially proposed that, based on studies done between 2004 and 2006[17,20-22], allosteric product inhibition of DGCs is of fundamental importance for c-di-GMP signalling as this mechanism allows cellular levels of c-di-GMP in bacterial cells to be regulated. This hypothesis is further established by the elucidation of the crystal structure of the *Caulobacter crescentus* response regulator protein, PleD[17,20,22], illustrating the dimeric structure of two GGDEF domains of the enzymatically active form of DGC, with a catalytic site formed between the two subunits. Allosteric product inhibition occurs when two intercalated molecules of c-di-GMP bind to the D2/DGC interface (I-site) and immobilise the DGC domain with respect to the D1/D2 stem (Fig 2).[22] This prevents the DGC enzyme from approaching its counterpart in the dimer, thus preventing the formation of c-di-GMP. Clearly, inhibition of PleD is non-competitive i.e. independent of substrate concentration, and can thus be attributed to an allosteric effect of the I-site binding.[22]

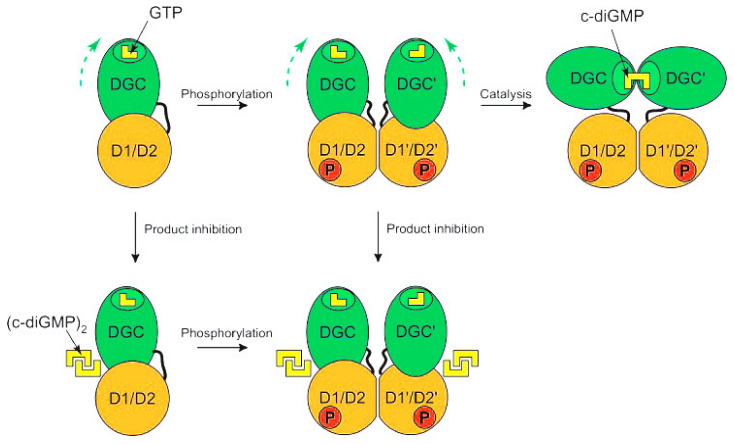

Figure 2. Mechanistic model of PleD regulation[22].

It is also interesting to note that biofilm formation and other virulence-related traits of pathogenic bacteria were not controlled by the same diguanylate cyclase across all bacteria species. For example, late stage biofilm formation by *Burkholderia cepacia* involves a protein with a DGC domain whereas *Yersinia pestis* regulates this process through HmsT protein (DGC) and HmsP protein (PDE).[23] In the *Salmonella* family of bacteria, the cellulose component of biofilm is regulated by GcpA protein[24] whereas the biofilm formation and motility is controlled by AdrA protein, which has a DGC domain and is responsible for the synthesis of c-di-GMP[25].

In addition to the properties mentioned above, c-di-GMP has been regarded to possess many biological activities. In 2005, an increase in the concentration of extracellular c-di-GMP was discovered by Karaolis and coworkers to inhibit *S. aureus* intercellular adhesive interactions, hence inhibiting biofilm formation.[6] This was also observed in methicillin resistant *S. aureus* and human and bovine intramammary mastitis isolates of *S.aureus*.[6] Furthermore, inhibition of *S. aureus'* ability to form biofilm in human epithelial cells HeLa was also observed.[6] In other words, c-di-GMP acts as a potential antimicrobial and antipathogenic agent[26] *in vivo* as it reduces bacterial colonization, hence reducing the survival of bacteria within the host. When human colon cancer cells were treated with 50μM of c-di-GMP, *in vitro* basal and growth factor-stimulated proliferation was inhibited. Studies on human neuroblastoma cells have shown that c-di-GMP is non-cytotoxic up to 100 μM which will inhibit human colon cancer cells proliferation[27]. Subsequent research by the same group unravelled the

immunostimulatory property of c-di-GMP[28] and proved that it is an effective immunomodulator and vaccine adjuvant against pneumococcal infection[29,30].

Hence, the above examples proved that c-di-GMP has numerous biological potential. With the emergence of resistant-strain bacteria, traditional antimicrobial agents and antibiotics may lose their viability. Hence, there is an urgent need to search for an alternate therapeutic approach to inhibit infections and diseases caused by bacteria. Most importantly, the main root of the problem, biofilm formation, should be addressed. Since biofilm formation can be controlled via regulation of c-di-GMP levels and c-di-GMP has shown to exhibit numerous biological potential, it is of our interest to synthesise cyclic dinucleotides with the same backbone structure but different bases and evaluate their inhibitory activities against diguanylate cyclases.

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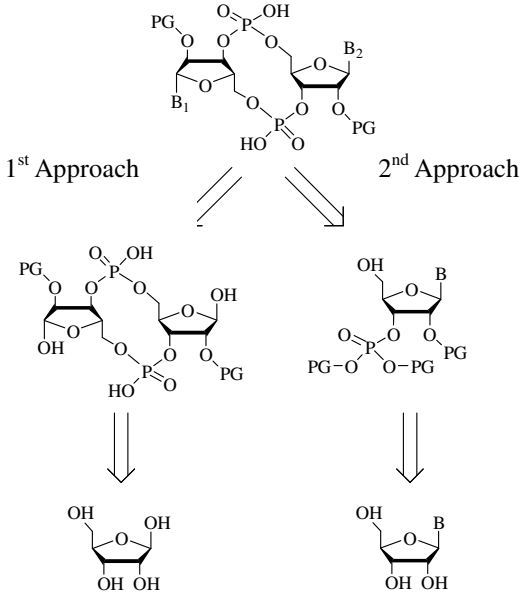
CHAPTER 2: SYNTHESIS OF CYCLIC DIGUANYLIC ACID (C-DI-GMP) AND ITS ANALOGUES

2.1 Introduction

Since the late 1950s, a few synthetic methodologies have been developed for the synthesis of oligonucleotides in general. In chronological order, they are the phosphodiester approach, phosphotriester approach, phosphite triester approach, phosphoramidite approach and the H-phosphonate approach.[1] Van Boom *et al*[2] was the first group to publish a synthetic pathway for c-di-GMP in the late 1980s. Their strategy was based on the modified hydroxybenzotriazole phosphotriester approach. In 2004, Hayakawa *et al.*[3] published the first alternative synthetic pathway. Their strategy was based on the phosphoramidite approach. In 2004 too, Jones *et al.*[4] published a second alternative to the synthesis of c-di-GMP using the phosphoramidite approach coupled with a H-phosphonate cyclization process. In 2006, Giese et *al.*[5] used the modified hydroxybenzotriazole phosphotriester approach in which the starting material was a ribose (rather than a nucleoside as in other reported methods); the base moiety was introduced onto the ribose only after cyclization of two ribose monophosphate molecules. In 2007, Yan *et al.*[6] utilized the H-phosphonate approach for their synthesis of c-di-GMP.

This project aims to synthesize c-di-GMP and four other analogues. The analogues consist of the c-di-GMP's cyclic ribose template that is attached to two of the three different bases: guanine, uridine and inosine, instead of two guanine bases.

There are two general approaches towards the synthesis of c-di-GMP as shown in Scheme 1.



Scheme 1: Retrosynthetic analysis of two major approaches for synthesizing c-di-GMP

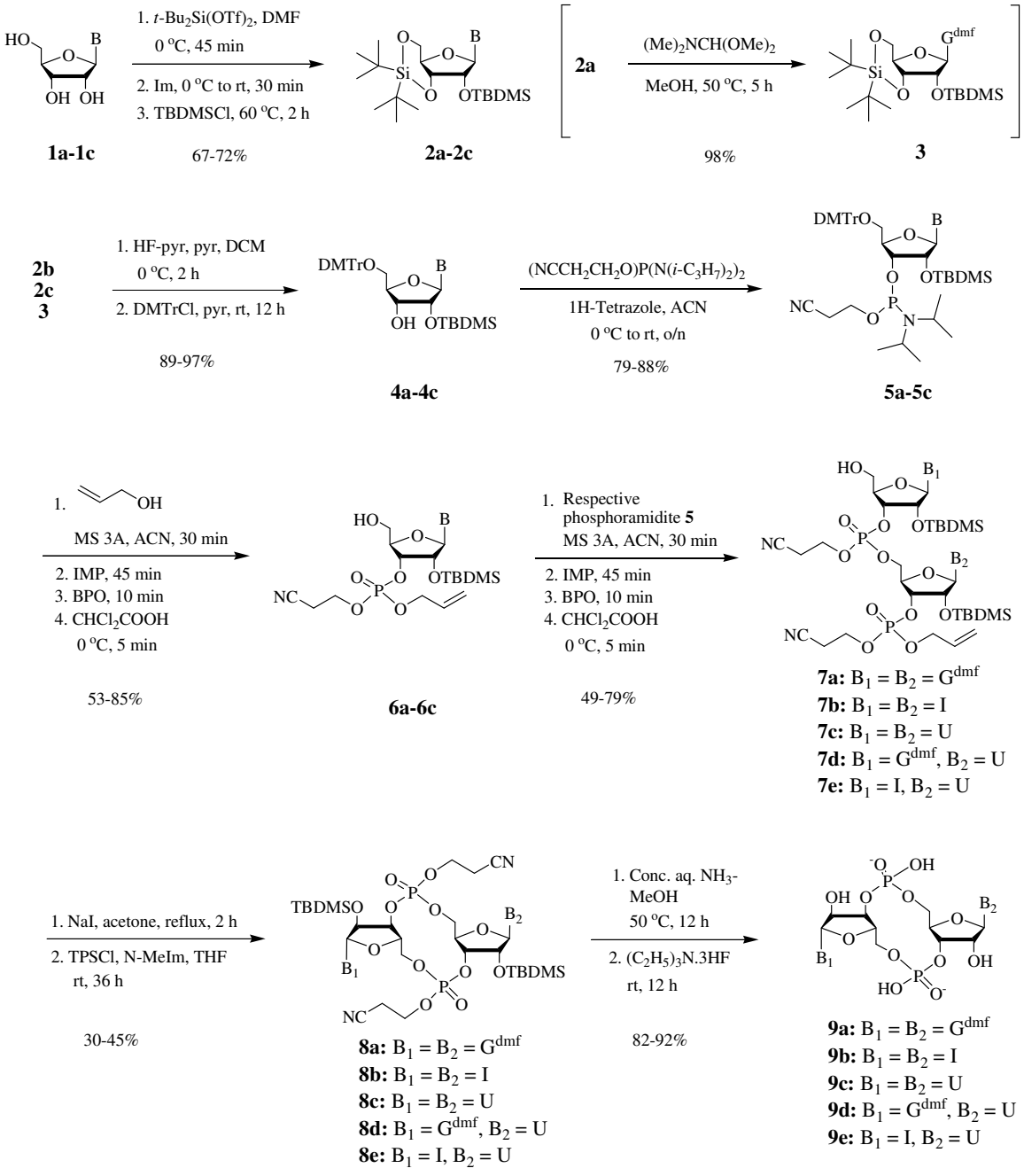
The first approach, the phosphoramidite strategy, synthesizes the cyclic backbone first, with the base being introduced subsequently in the route, as demonstrated by Giese[5]. Although this method allowed base derivatives of c-di-GMP to be made from a common synthetic intermediate, the analogues thus proposed can only have identical bases on both riboses due to the symmetrical nature of the cyclic sugar backbone.

The second approach utilized a nucleoside as the starting material, with the base present right at the beginning of synthesis. As such, besides obtaining analogues with identical nucleosides, analogues with different combinations of nucleosides could also be prepared. This allowed a more diverse library of c-di-GMP analogues to be formed,

thereby explaining our choice of utilizing the second approach. The synthetic route which was used in this project was adapted and optimised from the paper written by Hayakawa *et al.*[7]

2.2 Results and Discussion

The synthetic route is presented in Scheme 2 and the synthesized analogues are shown in Figure 1. Synthesis of 2'-*O*-TBDMS-protected nucleoside derivatives was achieved in the first step by regioselectively protecting the 3'- and 5'-hydroxyls of nucleosides, as discussed by Corey[8] and Trost[9], before protecting the 2'-OH with TBDMSCl. To prepare **2a**, guanosine **1a** was first suspended in DMF at 0 °C. Di-*tert*-butylsilanediyl ditriflate (1.2 eq,) was then added dropwise to the suspension. As the reaction progressed, the primary alcohol (5'-OH) preferentially reacted with the triflate first, then with 3'-OH, to form the protected diol that was soluble in DMF. This allowed more guanosine to dissolve, resulting in a clear solution 45 minutes later. Imidazole was then added to neutralize the triflic acid formed and to serve as a nucleophilic catalyst for the protection of 2'-OH by TBDMSCl to form a white precipitate **2a**. The 3',5'- protected diol was favourably formed instead of the 2',5'-diol because the resultant diol formed a 6-membered ring which was highly stable.



- From **1** to **6**, guanosine derivatives are denoted by '**a**'; uridine derivatives by '**b**'; inosine derivatives by '**c**'.
- The general base is denoted by 'B'; guanine base by 'G'; dimethylformamidine-protected G by G^{dmf}; uracil base by 'U', hypoxanthine base by 'I'.

Scheme 2: Synthetic Route of c-di-GMP and analogues

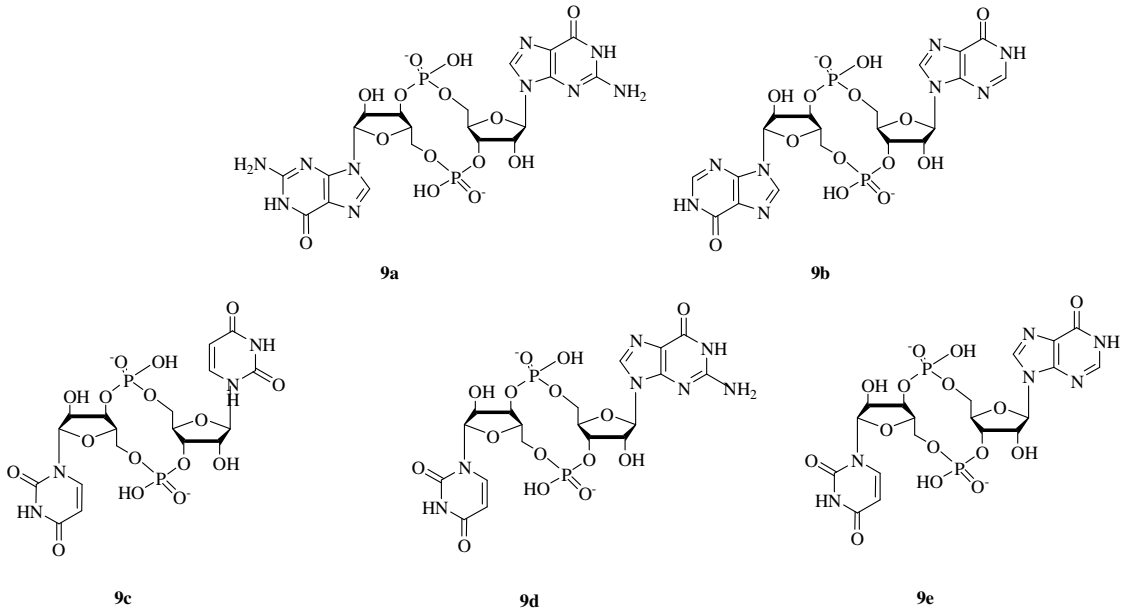


Figure 3: Analogues of **9**

For uridine, it was soluble in DMF but not inosine and interestingly, **2b** and **2c** did not precipitate out of the reaction mixture. Hence, alternative workup – extraction and flash column chromatography – was performed to purify the crude product.

The free amine on the purine ring of guanosine was subsequently protected with a dimethylformamidine protecting group. This imine protecting group was chosen as it was susceptible to strong bases and would be cleaved at the end of the route, together with the *β*-cyanoethyl protecting group[10-12].

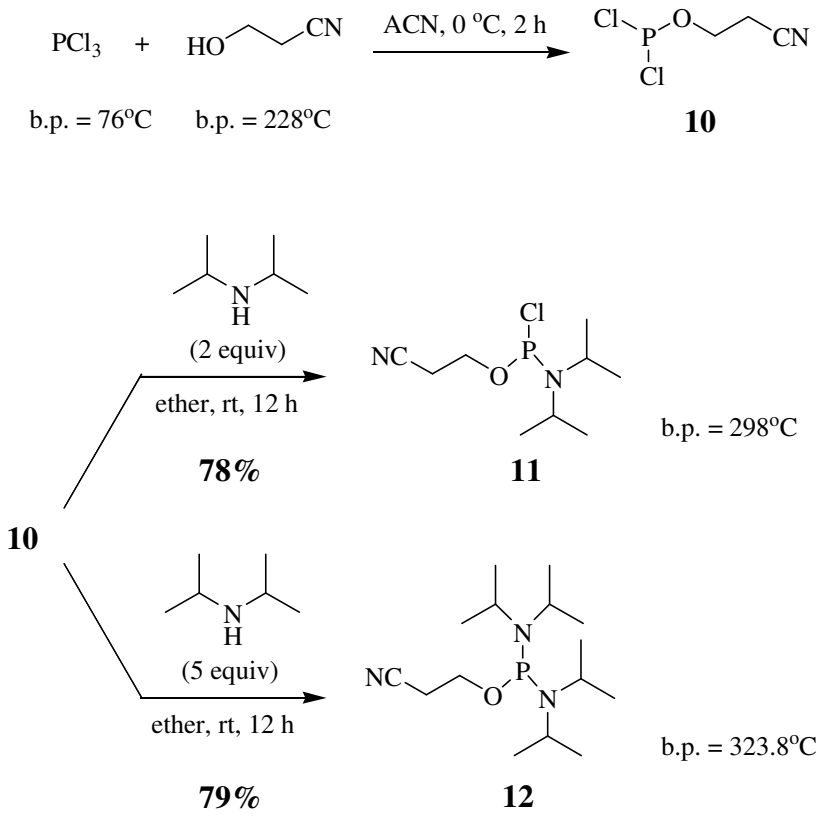
To allow 3'-OH to be phosphorylated, the silyl diol protecting group was first cleaved with hydrogen fluoride-pyridine complex in dichloromethane to free the 3'-OH and 5'-OH. The product after extraction, which was of high purity as observed from TLC,

was protected at the 5'-OH with DMTrCl under anhydrous conditions. Only the 5'-OH protected regioisomer was observed as the secondary alcohol 3'-OH was too sterically hindered to attack the tertiary dimethoxytrityl carbocation. To prevent the undesired protection of the secondary alcohol, the concentration of the reagent was diluted.

For the 2'-OH-protected inosine, it was insoluble in DCM unlike the other two bases. Workup was hence simplified as only filtration was needed and the solid was washed thoroughly with saturated $NaHCO_3$ to neutralize the acid and to remove the pyridine.

2-Cyanoethyl *N,N*-diisopropylchloro-phosphoramidite **11** was initially synthesized to be used for coupling with the free 3'OH on the protected nucleoside in the presence of collidine and N-methylimidazole to form **5a-5c**.[7] The synthesis of **11** (Scheme 3) is as follows: 3-hydroxypropionitrile was added dropwise to a mixture of PCl_3 and acetonitrile at 0 °C. Nitrogen gas was then bubbled into the reaction mixture to purge out the gaseous HCl evolved during the reaction. The mixture was then concentrated *in vacuo* to remove PCl_3 and acetonitrile. Subsequent addition of 2 equivalence of diisopropylamine to the mixture yielded **11**.[13,14] An additional equivalent of the base was necessary to form a salt with the remaining HCl present in the reaction mixture. Its counterpart 2-cyanoethyl bis-*N,N*-diisopropylphosphordiamidite[15] **12** was also synthesized as shown in Scheme 3 and replaced **11** in the synthesis of **5a-c** as both gave similar yields of approximately 78% and most importantly, **12** solved all the problems that were encountered. **11** was difficult to handle as it was highly moisture-sensitive unlike **12** which could undergo extractive

workup. Most importantly, extractive workup performed for the phosphitylation reaction utilizing excess **11** hydrolysed the monochlorophosphoramidite to monohydroxyphosphoramidite. This hydroxyl compound was difficult to remove by column chromatography during the purification of **5a-c** as it tailed seriously and would eventually elute together with the product.



Scheme 3: Synthesis of **11** and **12**

12 was synthesized by adding 5 instead of 2 equivalence of diisopropylamine to 2-cyanoethyl dichlorophosphoramidite to ensure the substitution of all chloro groups with diisopropylamine. With **12**, coupling was achieved in the presence of 1H-tetrazole in anhydrous acetonitrile. Tetrazole extracted the proton from the free 3'OH, increasing the rate of the nucleophilic attack to form **4a-c**. Tetrazole also converted diisopropylamine to

diisopropylammonium tetrazolide salt, which precipitated out of the reaction mixture, thus driving the reaction forward.

Allyl alcohol was added to a mixture of **5** and molecular sieves in acetonitrile. The displacement of diisopropylamine by allyl alcohol was promoted by the acid azole, imidazolium perchlorate (IMP). The postulated mechanism[16,17] is as follows: IMP first acted as an acid by protonating the phosphoramidite at nitrogen to form an activated species. The free imidazole then attacked the phosphorous centre, displacing diisopropylamine to form a phosphorazolidite. Being a good leaving group, the azole was then easily displaced by allyl alcohol. Subsequent oxidation of the diribonucleoside phosphite to diribonucleoside phosphate was achieved with butanone peroxide. Dichloroacetic acid was added to cleave the DMT protecting group to afford compounds **6a-e** in high yields; this resulted in a orange-red solution, which indicated the presence of the DMT-OH.

Different nucleosides can be coupled together in the presence of the promoter IMP and molecular sieves as moisture scavenger[16]. The mechanism of this reaction is the same as the previous step. The free 5'-OH group on **6a** displaces the diisopropylamine group on **5a** to form a coupled product via the phosphorazolidite intermediate. Subsequent oxidation and detritylation gave compound **7a**. The other analogues were synthesized in the same manner using the respective phosphoramidite and 5'-*O*-free nucleoside phosphate.

With sodium iodide in refluxing acetone, the iodide attacked the partially electron deficient allylic alcohol oxygen on **7** to form the alkoxide, with allyl iodide as side product. This intermediate was highly hygroscopic. Attempts to isolate it by filtration was only partially successful as most of the precipitate dissolved during the course of filtration. Hence, an alternative isolation procedure was attempted. Cold anhydrous acetone was added to the concentrated reaction mixture to precipitate more white solid, which was quickly filtered, washed with cold anhydrous acetone and dissolved in methanol. Removal of methanol *in vacuo* afforded the dried intermediate, which was further dried overnight *in vacuo* at 45 °C before suspending it in anhydrous THF under high-dilution conditions at room temperature, with *N*-methylimidazole (*N*-MeIm) as the nucleophilic catalyst and 2,4,6-triisopropylbenzenesulfonylchloride (TPSCl) as the condensing agent.

The yield of this cyclization step was generally very low. In all theattempts, the cyclization reaction did not go to completion even after 60 hrs. The long hours of reaction also resulted in the formation of many side products (as shown by TLC analysis). To circumvent these problems, we proceeded to modify the procedure by adding moisture scavenger, molecular sieves (3Å) to the reaction mixture and using 5 – 7 equivalence of TPSCl and *N*-MeIm. These modifications enabled the reaction to go to completion with minimal side products in 36 hours. This made the isolation of the cyclized product easier.

Finally, the fully protected cyclic dinucleotide was treated with a 1:1 mixture of concentrated aqueous ammonia and methanol to remove the dimethylformamidine and *β*-

cyanoethyl protecting groups. Subsequent treatment with triethylamine trihydrofluoride removed the silyl group. The resulting crude product was purified by reverse-phase HPLC. The purified c-di-GMP and its analogues were obtained in 10 – 15% average overall yields.

2.3 Experimental Section

3.1 Materials

All solvents and reagents were purchased from commercial sources and used without further purification. Powdery molecular sieves (MS) 3Å were used without further treatment. Imidazolium perchlorate[16] and 2-cyanoethyl bis-*N,N*-diisopropylphosphoramidite[15] were prepared according to reported methods.

3.2 General

Moisture-sensitive reactions were carried out under nitrogen atmosphere with commercially obtained anhydrous solvents. Reactions were monitored by thin layer chromatography (TLC) using precoated plates (Merck silica gel 60, F254) and visualized with UV light or by charring with ninhydrin or phosphomolybdic acid. Flash column chromatography was performed with silica (Merck, 230 – 400 mesh). ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR spectra were recorded at 298 K on either a Bruker ACF300, Bruker AMX500 or Bruker DPX-300 NMR spectrometer calibrated using residual undeuterated

solvent as an internal reference. The following abbreviations were used to explain the multiplicities: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). The number of protons (*n*) for a given resonance was indicated as *n*H. ESI mass spectra were acquired using Finnigan LCQ or Finnigan TSQ7000 spectrometer. HPLC analysis was carried out using a Phenomenex Luna 3μ-C18 column [4.6 (diameter) × 50 (height) mm]. Semi-preparative HPLC was achieved using a COSMOSIL 5C18-AR-300 column [10 (diameter) × 250 (height) mm].

3.3 Preparation of Compounds 2 – 9

2.3.1 General Procedure for Preparation of 2

To a stirred suspension of the respective nucleoside **1** (15 mmol) in DMF (30 mL) at 0 °C, di-*tert*-butylsilanediyl ditriflate (6.6 mL, 18 mmol) was added dropwise over 15 min. After stirring at the same temperature for 30 min, imidazole (75 mmol) was added. This resulting mixture was stirred at 0 °C for 5 min and then at room temperature for 45 min. When the reaction has ended, *tert*-butyldimethylchlorosilane (TBDMSCl) (22.5 mmol) was added and the resulting mixture stirred at 60 °C for 2 hrs. The occurring precipitate was collected by filtration, washed with cold methanol and dried *in vacuo* to give pure **2a**. For **2b** and **2c**, the reaction mixture remained clear at the end of the reaction. After DMF was partially removed *in vacuo*, the resulting material was partitioned between water (30 mL) ether (60 mL). The layers were separated and the aqueous layer back-extracted with ether (3 × 20 mL). The combined organic extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting

residue was purified by flash column chromatography. Elution using a gradient from 1:30 ethyl acetate: dichloromethane to 1:20 ethyl acetate: dichloromethane afforded pure **2b**. Elution using a gradient from 1:50 methanol: dichloromethane to 1:40 methanol: dichloromethane afforded pure **2c**.

2'-*O*-(*tert*-Butyldimethylsilyl)-3',5'-*O*-(di-*tert*-butylsilanediyl)guanosine (2a)

^{1}H NMR (300 MHz, DMSO–d_6) δ 0.07 (s, 3H), 0.09 (s, 3H), 0.86 (s, 9H), 1.01 (s, 9H), 1.06 (s, 9H), 3.93 – 4.01 (m, 2H), 4.26 – 4.35 (m, 2H), 4.57 (d, J = 5.1 Hz, 1H), 5.72 (s, 1H), 6.35 (s, br, 2H), 7.91 (s, 1H), 10.65 (s, 1H); $C_{24}H_{44}O_5N_5Si_2^+$ (M + H^+) calcd *m/z* 538.2876, found *m/z* 538.2895. Yield = 72%.

2'-*O*-(*tert*-Butyldimethylsilyl)-3',5'-*O*-(di-*tert*-butylsilanediyl)uridine (2b)

^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ 0.17 (s, 3H), 0.21 (s, 3H), 0.96 (s, 9H), 1.06 (s, 18H), 4.10 – 4.16 (m, 3H), 4.46 (d, J = 4.2 Hz, 1H), 4.52 (d, J = 4.2 Hz, 1H), 5.62 (d, J = 8.1 Hz, 1H), 5.77 (s, 1H), 7.60 (d, J = 8.1 Hz, 1H), 10.27 (s, br, 1H); $C_{23}H_{42}O_6N_2NaSi_2^+$ (M + Na^+) calcd *m/z* 521.2474, found *m/z* 521.2490. Yield = 67%.

2'-*O*-(*tert*-Butyldimethylsilyl)-3',5'-*O*-(di-*tert*-butylsilanediyl)inosine (2c)

^{1}H NMR (300 MHz, $CDCl_3$) δ 0.06 (s, 3H), 0.08 (s, 3H), 0.85 (s, 9H), 0.98 (s, 9H), 1.06 (s, 9H), 4.00 – 4.08 (m, 2H), 4.35 – 4.36 (m, 1H), 4.50 – 4.55 (m, 1H), 4.58 – 4.59 (m, 1H), 5.93 (s, 1H), 8.04 (s, 1H), 8.28 (s, 1H), 12.43 (s, br, 1H); $C_{24}H_{42}O_5N_4NaSi_2^+$ (M + Na^+) calcd *m/z* 545.2586, found *m/z* 545.2612. Yield = 80%.

2.3.2 Preparation of N^2-(Dimethylamino- methylene)-2'-*O*-(*tert*-butyldimethyl- silyl)-3',5'-*O*-(di-*tert*-butylsilanediyl) guanosine (3)

N,*N*-Dimethylformamide dimethyl acetal (52 mmol) was added to a suspension of **2a** (13 mmol) in methanol (78 mL). The reaction mixture was stirred at 50 °C for 5 hrs. After the solvent was removed *in vacuo*, hexane was added to precipitate out a white solid under cooling. The solid was collected by filtration, washed with cold hexane and dried *in vacuo* to afford pure **3**.

^{1}H NMR (300 MHz, DMSO-d_6) δ 0.08 (s, 3H), 0.11 (s, 3H), 0.87 (s, 9H), 1.01 (s, 9H), 1.05 (s, 9H), 3.04 (s, 3H), 3.12 (s, 3H), 3.93 – 4.08 (m, 2H), 4.31 – 4.40 (m, 2H), 4.59 (d, J = 4.9 Hz, 1H), 5.88 (s, 1H), 7.99 (s, 1H), 8.48 (s, 1H), 11.40 (s, 1H); $C_{27}H_{49}O_5N_6Si_2^+$ (M + H^+) calcd *m/z* 593.3298, found *m/z* 593.3311. Yield = 96%.

2.3.3 General Procedure for Preparation of 4

To a solution of **3**, **2b** and **2c** (10.7 mmol) respectively in dichloromethane (42 mL) at 0 °C, a chilled solution of hydrogen fluoride-pyridine complex (42.7 mmol) in pyridine (5.1 mL) was added dropwise over 15 min. The reaction mixture was stirred at 0 °C for 2 hrs. The reaction mixture was then washed with saturated sodium bicarbonate solution (2 × 30 mL) and extracted with dichloromethane (3 × 30 mL). The combined organic extracts were washed with brine (40 mL), dried over anhydrous Na_2SO_4, concentrated and dried *in vacuo* to give the detritylated product of **2b** and **3** as white solids respectively.

For detritylated **2c**, it precipitated out of the reaction mixture. Saturated sodium bicarbonate solution was added to the stirred reaction mixture till the pH was alkaline. The white solid of detritylated **2c** was collected by filtration and washed with water followed by cold dichloromethane. The resulting dried solid of detritylated **2b, 2c** and **3** were then respectively dissolved in anhydrous pyridine (100 mL). Dimethoxytrityl chloride (21.4 mmol) was added and the reaction mixture stirred at room temperature for 12 hrs. The reaction was quenched by addition of methanol (3 mL). Concentration of the reaction mixture gave a viscous liquid. This material was partitioned between saturated sodium bicarbonate solution (30 mL) and dichloromethane (30 mL). The layers were separated and the aqueous layer back-extracted with dichloromethane (3 × 20 mL). The combined organic extracts were washed with brine (30 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by flash column chromatography. Elution using a gradient from 1:1 ethyl acetate-hexane to 1:20 methanol-dichloromethane afforded pure **4a**, **4b** and **4c** respectively.

N^2-(Dimethylaminomethylene)-2'-*O*-(*tert*-butyldimethylsilyl)-5'-*O*-(*p*,*p'*dimethoxytrityl) guanosine (4a)

^{1}H NMR (300 MHz, CD_3OD) δ -0.06 (s, 3H), 0.03 (s, 3H), 0.82 (s, 9H), 2.95 (s, 3H), 3.02 (s, 3H), 3.32 – 3.46 (m, 2H), 3.70 (s, 6H), 4.17 – 4.18 (m, 1H), 4.36 – 4.39 (m, 1H), 4.71 (t, *J* = 5.0 Hz, 1H), 6.00 (d, *J* = 5.0 Hz, 1H), 6.79 (d, *J* = 8.9 Hz, 4H), 7.16 – 7.44 (m, 9H), 8.02 (s, 1H), 8.49 (s, 1H); $C_{40}H_{51}O_7N_6Si^+$ ($M + H^+$) calcd *m/z* 755.3583, found *m/z* 755.3601. Yield = 89%.

2'-*O*-(*tert*-butyldimethylsilyl)-5'-*O*-(*p*,*p'*-dimethoxytrityl)uridine (4b)

^{1}H NMR (300 MHz, (CD_3OD) δ 0.14 (s, 6H), 0.92 (s, 9H), 3.46 – 3.47 (m, 2H), 4.04 (s, 6H), 4.11 (m, 6H), 4.32 – 4.33 (m, 2H), 5.25 (d, *J* = 8.1 Hz, 2H), 5.89 (s, 1H), 6.84 (d, *J* = 8.7 Hz, 4H), 7.20 – 7.42 (m, 9H), 8.00 (d, *J* = 8.1 Hz, 1H); $C_{36}H_{44}O_8N_2NaSi^+$ ($M + Na^+$) calcd *m/z* 683.2759, found *m/z* 683.2766. Yield = 90%.

2'-*O*-(*tert*-butyldimethylsilyl)-5'-*O*-(*p*,*p'*-dimethoxytrityl)inosine (4c)

^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ 0.54 (s, 3H), 0.64 (s, 3H), 1.44 (s, 9H), 3.39 – 3.42 (m, 2H), 4.02 – 4.04 (m, 2H), 4.37 (s, 6H), 4.82 – 4.85 (m, 1H), 5.00 – 5.03 (m, 1H), 5.52 – 5.55 (m, 1H), 6.64 – 6.66 (m, 1H), 7.38 – 7.48 (m, 4H), 7.79 – 8.11 (m, 9H), 8.63 (s, 1H), 8.73 (s, 1H); $C_{37}H_{44}O_7N_4NaSi^+$ ($M + Na^+$) calcd *m/z* 707.2871, found *m/z* 707.2884. Yield = 89%.

2.3.4 General Procedure for Preparation of 5

To a solution or suspension of **4a**, **4b** and **4c** (5.5 mmol) in anhydrous acetonitrile (33 mL) respectively, 1H-tetrazole (5.5 mmol) was added. $NCCH_2CH_2OP(N(i\text{-}C_3H_7)_2)_2$ (11 mmol) was then added dropwise at room temperature and allowed to stir for 12 hrs. The occurring white solid was removed by filtration and washed with ethyl acetate. The filtrate was concentrated, diluted with ethyl acetate (30 mL) and washed with saturated sodium bicarbonate (20 mL). The aqueous layer was back-extracted with ethyl acetate (3 × 20mL). Combined organic extracts were then washed with brine (30 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by

flash column chromatography. Elution using 1:2 ethyl acetate-hexane afforded pure **5b**. Elution using a gradient of 3:2 ethyl acetate-hexane to 2:1 ethyl acetate-dichloromethane afforded pure **5a** and **5c** respectively.

N^2-(Dimethylaminomethylene)-2'-*O*-(*tert*-butyldimethylsilyl)-5'-*O*-(*p*,*p'*-dimethoxytrityl) guanosine 3'-[(2-Cyano-ethyl) *N*,*N*-Diisopropylaminophospho-ramidite] (5a)

^{1}H NMR (300 MHz, CD_3OD) δ -0.13, -0.09 (2s, 3H), 0.03, 0.04 (2s, 3H), 0.80, 0.83 (2s, 9H), 1.03 – 1.23 (m, 12H), 2.40 – 2.44 (m, 1H), 2.72 – 2.78 (m, 1H), 2.94, 2.96, 3.08 (3s, 6H), 3.47 – 3.68 (m, 4H), 3.77, 3.78 (2s, 6H), 3.84 – 4.01 (m, 1H), 4.29 – 4.40 (m, 1H), 4.46 – 4.51 (m, 1H), 4.88 – 4.97 (m, 1H), 5.99 – 6.05 (m, 1H), 6.81 – 6.87 (m, 4H), 7.21 – 7.48 (m, 9H), 8.02, 8.04 (2s, 1H), 8.48, 8.51 (2s, 1H); ^{31}P NMR (121.5 MHz, CD_3OD) δ 150.21, 151.72; $C_{49}H_{68}O_8N_8PSi^+$ ($M + H^+$) calcd *m/z* 955.4662, found *m/z* 955.4658. Yield = 79%.

2'-*O*-(*tert*-butyldimethylsilyl)-5'-*O* (*p*,*p'*-dimethoxytrityl)uridine 3'-[(2-Cyanoethyl) *N*,*N*-Diisopropylamino-phosphoramidite] (5b)

^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ 0.27, 0.29 (2s, 6H), 1.03, 1.04 (2s, 9H), 1.16 – 1.31 (m, 12H), 2.69 (t, *J* = 6.0 Hz, 1H), 2.84 – 2.88 (m, 1H), 3.57 – 3.82 (m, 5H), 3.87 (s, 6H), 3.94 – 4.02 (m, 1H), 4.38 – 4.46 (m, 1H), 4.50 – 4.57 (m, 1H), 4.62 – 4.68 (m, 1H), 5.41 (dd, *J* = 6.6, 8.1 Hz, 1H), 6.04 – 6.11 (m, 1H), 6.99 – 7.03 (m, 4H), 7.35 – 7.62 (m, 9H), 8.02 (dd, *J* = 8.2, 16.8 Hz, 1H) 10.27 (s, br, 1H); ^{31}P NMR (121.5 MHz, $(CD_3)_2CO$) δ

150.80, 151.22, 151.43; $C_{45}H_{61}O_9N_4NaPSi^+$ (M + Na^+) calcd *m/z* 883.3838, found *m/z* 883.3850. Yield = 84%.

2'-*O*-(*tert*-butyldimethylsilyl)-5'-*O* (*p*,*p'*-dimethoxytrityl)inosine 3'-[(2-Cyanoethyl) *N*,*N*-Diisopropylamino-phosphoramidite] (5c)

^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ -0.10 (s, 3H), 0.05, 0.06 (2s, 3H), 0.83 (s, 9H), 1.14 – 1.34 (m, 12H), 2.55 – 2.63 (m, 2H), 2.79 – 2.83 (m, 1H), 3.43 – 3.48 (m, 1H), 3.55 – 3.62 (m, 2H), 3.66 – 3.72 (m, 2H), 3.79 (s, 6H), 4.01 – 4.08 (m, 1H), 4.50 (m, 2H), 5.10 (m, 1H), 6.07 – 6.09 (m, 1H), 6.88 – 6.92 (m, 4H), 7.24 – 7.57 (m, 9H), 8.10 – 8.11 (m, 1H), 8.16 – 8.19 (m, 1H); ^{31}P NMR (121.5 MHz, $(CD_3)_2CO$) δ 149.35, 150.90; $C_{46}H_{61}O_8N_6NaPSi^+$ (M + Na^+) calcd *m/z* 907.3950, found *m/z* 907.3962. Yield = 87%.

2.3.5 General Procedure for Preparation of 6

To a solution of **5a**, **5b** and **5c** (2.5 mmol) in anhydrous acetonitrile (9 mL) respectively, powdery MS 3Å (115 mg) and allyl alcohol (3 mmol) were added. The resulting mixture was stirred at room temperature for 30 min. Imidazolium perchlorate (5 mmol) was then added and stirring continued for an additional 45 min. To the resulting mixture, a 31% solution of 2-butanone peroxide/dimethyl phthalate in toluene (1.1 mL) was added. This reaction mixture was stirred for 10 min, after which the MS 3Å was removed by filtration through a Celite 545 pad. The filtrate was diluted with ethyl acetate (20 mL) and then washed with saturated sodium bicarbonate solution (20 mL). The aqueous layer was back-extracted with ethyl acetate (2 × 20 mL). Combined organic extracts were washed with brine (20 mL), dried over anhydrous Na_2SO_4 and concentrated

in vacuo to obtain a viscous liquid. This liquid was then dissolved in dichloromethane (19 mL) and cooled to 0 °C. Dichloroacetic acid (50 mmol) was then added dropwise to the reaction mixture. This was stirred for 5 min after which the reaction was quenched by addition of saturated sodium bicarbonate solution to the stirred reaction mixture till the pH was alkaline. The aqueous layer was extracted with dichloromethane (3 × 20 mL). Combined organic extracts were washed with brine (30 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by flash column chromatography. Elution using a gradient of 1:30 methanol-dichloromethane to 1:10 methanol-dichloromethane afforded pure **6a**, **6b** and **6c** respectively.

N^2-(Dimethylaminomethylene)-2'-*O*-(*tert*-butyldimethylsilyl)guanosine 3'-(Allyl 2-Cyanoethyl Phosphate) (6a)

^{1}H NMR (300 MHz, CD_3OD) δ -0.18, -0.17 (2s, 3H), -0.01, 0.00 (2s, 3H), 0.78, 0.79 (2s, 9H), 2.92 – 2.94 (m, 2H), 3.11 (s, 3H), 3.20 (s, 3H), 3.85 – 3.88 (m, 2H), 4.31 – 4.34 (m, 2H), 4.42 – 4.44 (m, 1H), 4.66 – 4.72 (m, 2H), 4.98 – 5.02 (m, 2H), 5.31 – 5.48 (m, 1H), 5.43 – 5.50 (m, 1H), 5.98 (d, J = 6.42 Hz, 1H), 6.05 – 6.11 (m, 1H), 8.14 (s, 1H), 8.59 (s, 1H); ^{31}P NMR (121.5 MHz, CD_3OD), δ -1.02, -0.99; $C_{25}H_{41}O_8N_7PSi^+$ ($M + H^+$) calcd *m/z* 626.2518, found *m/z* 626.2507. Yield = 85%.

2'-*O*-(*tert*-butyldimethylsilyl)uridine 3'-(Allyl 2-Cyanoethyl Phosphate) (6b)

^{1}H NMR (300 MHz, CD_3OD) δ 0.09, 0.10, 0.13, 0.14 (4s, 6H), 0.91 (s, 9H), 2.93 (t, J = 5.9 Hz, 2H), 3.82 – 3.85 (m, 2H), 4.28 – 4.37 (m, 3H), 4.54 – 4.58 (m, 1H), 4.65 – 4.71 (m, 2H), 5.31 – 5.35 (m, 1H), 5.42 – 5.49 (m, 1H), 5.77 (d, J = 8.1 Hz, 1H), 5.99 – 6.09

(m, 2H), 8.05 (d, J = 8.1 Hz, 1H); ^{31}P NMR (121.5 MHz, CD_3OD) δ -1.83; $C_{21}H_{34}O_9N_3NaPSi^+$ (M + Na^+) calcd *m/z* 554.1694, found *m/z* 554.1706. Yield = 78%.

2'-*O*-(*tert*-butyldimethylsilyl)inosine 3'-(Allyl 2-Cyanoethyl Phosphate) (6c)

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.22, 0.25 (2s, 6H), 0.93, 0.96 (2s, 9H), 2.78 – 2.87 (m, 1H), 2.89 – 2.94 (m, 1H), 3.84 – 3.91 (m, 1H), 4.14 – 4.19 (m, 1H), 4.33 – 4.34 (m, 2H), 4.42 – 4.46 (m, 2H), 4.65 – 4.67 (m, 2H), 5.28 – 5.44 (m, 4H), 5.94 – 6.10 (m, 2H), 8.51 (s, 1H), 9.87 – 9.96 (m, 1H), 13.59 (s, br, 1H); ^{31}P NMR (202.5 MHz, $CDCl_3$) δ -1.23, -1.08; $C_{22}H_{34}O_8N_5NaPSi^+$ (M + Na^+) calcd *m/z* 578.1807, found *m/z* 578.1820. Yield = 80%.

2.3.6 General Procedure for Preparation of 7

A mixture of **5a** and **6a**, **5a** and **6b**, and **5c** and **6c** respectively (0.9 mmol of each compound), were dissolved in anhydrous acetonitrile (7 mL). MS 3Å (80 mg) was then added and the reaction mixture stirred at room temperature for 30 min. Imidazolium perchlorate (1.8 mmol) was then added and stirring continued for an additional 45 min. To the resulting mixture, a 31% solution of 2-butanone peroxide/ dimethyl phthalate in toluene (1.2 mL) was added. This reaction mixture was stirred for 10 min, after which the MS 3Å was removed by filtration through a Celite 545 pad. The filtrate was concentrated to obtain a viscous liquid. This liquid was then dissolved in dichloromethane (11 mL) and cooled to 0 °C. Dichloroacetic acid (18 mmol) was then added dropwise to the reaction mixture. This was stirred for 5 min after which the reaction was quenched by addition of

saturated sodium bicarbonate solution to the stirred reaction mixture till the pH was alkaline. The aqueous layer was back-extracted with dichloromethane (3 × 20 mL). Combined organic extracts were washed with brine (30mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by flash column chromatography. Elution using a gradient of 1:30 methanol-dichloromethane to 1:5 methanol-dichloromethane afforded pure **7a**, **7b** and **7c** respectively.

Guanylyl (3'-5')guanosine 3'-Phosphate (7a)

^{1}H NMR (300 MHz, $CDCl_3$) δ -0.23 – 0.09 (m, 12H), 0.75 – 0.85 (m, 18H), 2.80 – 2.82 (m, 4H), 3.09, 3.12, 3.19, 3.23 (4s, 9H), 3.72 – 3.82 (m, 2H), 4.31 – 4.63 (m, 10H), 4.89 – 5.04 (m, 4H), 5.29 – 5.45 (m, 2H), 5.78 – 6.02 (m, 3H), 7.80 – 7.91 (m, 2H), 8.40 (s, 1H), 8.61 (s, 1H); ^{31}P NMR (121.5 MHz, $CDCl_3$), δ -1.68, -1.41, -1.13, -0.88; $C_{44}H_{68}O_{14}N_{14}NaP_2Si_2^+$ (M + Na^+) calcd *m/z* 1157.3945, found *m/z* 1157.3958. Yield = 57%.

Inosinylyl(3'-5')inosine 3'-Phosphate (7b)

^{1}H NMR (300 MHz, MeOD) δ -0.21 – 0.09 (m, 12H), 0.74 – 0.97 (m, 18H), 2.89 – 2.95 (m, 4H), 3.77 – 3.78 (m, 2H), 4.33 – 4.37 (m, 4H), 4.96 – 4.99 (m, 2H), 5.17 (m, 1H), 5.33 – 5.36 (m, 1H), 5.44 – 5.50 (m, 1H), 6.04 – 6.09 (m, 3H), 7.61 – 7.67 (m, 2H), 7.71 – 7.75 (m, 2H), 8.15 (d, *J* = 5.8 Hz, 1H) 8.31 (s, 1H), 8.42 (s, 1H); ^{31}P NMR (202.5 MHz, $CDCl_3$) δ -1.64, -1.51, -1.13, -1.03, -0.95; $C_{41}H_{62}O_{15}N_{10}NaP_2Si_2^+$ (M + Na^+) calcd *m/z* 1075.3302, found *m/z* 1075.3303. Yield = 60%.

Uridylyl(3'-5')uridine 3'-Phosphate (7c)

^{1}H NMR (300 MHz, $(CD_3)CO$) δ 0.12 - 0.16 (m, 12H), 0.91 (s, 18H), 2.95 - 3.01 (m, 6H), 3.88 (s, 2H), 4.27 - 4.68 (m, 12H), 4.94 - 4.96 (m, 2H), 5.33 (d, *J* = 10.4 Hz, 1H), 5.44 (d, *J* = 15.8 Hz, 1H), 5.66 - 5.72 (m, 2H), 5.91 - 5.98 (m, 3H), 7.74 (d, *J* = 8.2 Hz, 1H), 8.00 (d, *J* = 5.4 Hz, 1H); ^{31}P NMR (121.5 MHz, $(CD_3)CO$) δ -0.99- -1.20 (m); $C_{39}H_{62}N_6NaO_{17}P_2Si_2^+$ ($M + Na^+$) calcd *m/z* 1027.3078 found m/z 1027.3092 Yield = 79%

Guanylyl(3'-5')uridine 3'-Phosphate (7d)

^{1}H NMR (500 MHz, $CDCl_3$) δ -0.25 – 0.12 (m, 12 H), 0.78 – 0.89 (m, 18H), 2.82 (m, 4H), 3.11 (s, 3H), 3.19 (s, 3H), 3.72 – 3.91 (m, 4H), 4.29 – 4.63 (m, 10H), 4.93 – 5.07 (m, 3H), 5.32 – 5.43 (m, 2H), 5.68 – 5.78 (m, 2H), 5.95 (m, 2H), 7.47 (m, 1H), 7.86 (s, 1H), 8.43 (s, 1H), 9.15 (m, 1H); ^{31}P NMR (202.5 MHz, $CDCl_3$) δ -1.96, -1.80, -1.31, -0.99, -0.88; $C_{43}H_{69}O_{16}N_{10}P_2Si_2^+$ ($M + H^+$) calcd *m/z* 1099.3901, found *m/z* 1099.3899. Yield = 49%

Inosylyl(3'-5')uridine 3'-Phosphate (7e)

^{1}H NMR (300 MHz, MeOD) δ -0.18 - 0.16 (m, 12H), 0.84 (dd, *J* = 2.4, 43.5 Hz, 18H), 1.99 (s, 1H), 2.90 - 2.98(m, 4H), 3.86 (m, 2H), 4.31 - 4.71 (m, 11H), 5.03 (d, *J* = 4.5 Hz, 2H), 5.33 (d, *J* = 10.4 Hz, 1H), 5.45 (d, *J* = 17.1 Hz, 1H), 5.78 - 6.00 (m, 2H), 6.01 - 6.09 (m, 2H), 7.68 - 7.73 (m, 1H), 8.12 (s,1H), 8.40 (s, 1H); ^{31}P NMR (202.5 MHz, MeOD) δ 8.0414, 8.6787; $C_{40}H_{62}O_{16}N_8NaP_2Si_2^+$ ($M + H^+$) calc *m/z* 1051.3190 found *m/z* 1051.3220. Yield = 53%

2.3.7 General Procedure for Preparation of 8

To a solution of **7a**, **7b** and **7c** (0.24 mmol) in anhydrous acetone (7.2 mL) respectively, sodium iodide (2.4 mmol) was added. The resulting mixture was stirred under reflux for 2 – 3 hrs. The reaction mixture was then concentrated. Addition of chilled anhydrous acetone precipitated a white solid which was filtered, washed with chilled anhydrous acetone and subsequently dissolved in methanol. Methanol was then removed *in vacuo* to obtain a colourless crystalline solid. This solid was then suspended in anhydrous THF (41 mL), to which MS 3Å (50 mg), *N*-methylimidazole (12 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (12 mmol) were added. The resulting mixture was stirred at room temperature for 36 – 48 hrs. Water (18 mL) was then added and stirring continued for an additional 1 hr. The reaction mixture was concentrated *in vacuo*. The residual material was dissolved in ethyl acetate (20 mL) and washed with brine (20 mL). Combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by flash column chromatography. Elution using a gradient of 1:30 methanol:dichloromethane to 1:10 methanol:dichloromethane afforded pure **8a**, **8b** and **8c** respectively.

Fully Protected Cyclic Bis(3'-5')diguanylic Acid (8a)

^{1}H NMR (300 MHz, MeOD) δ -0.17 – 0.06 (m, 12H), 0.79 – 0.83 (m, 18H), 2.91 – 2.95 (m, 4H), 3.13 (s, 6H), 3.21 (s, 6H), 4.08 – 4.13 (m, 2H), 4.29 – 4.38 (m, 4H), 4.66 – 4.73 (m, 4H), 5.31 (m, 1H), 5.34 (m, 1H), 5.42 (m, 1H), 5.47 (m, 1H), 5.96 (d, J = 6.6 Hz, 2H),

8.62 (s, 2H), 8.90 (s, 2H); ^{31}P NMR (121.5 MHz, MeOD) δ -1.72, -1.69; $C_{44}H_{68}O_{14}NaP_2Si_2^+$ (M + Na^+) calcd *m/z* 1157.3945, found *m/z* 1157.3958. Yield = 40%.

Fully Protected Cyclic Bis(3'-5')diinosinic Acid (8b)

^{1}H NMR (500 MHz, MeOD) δ -0.14 (s, 6H), 0.11 (s, 6H), 0.78 (s, 18H), 2.98 – 3.00 (m, 4H), 4.24 – 4.28 (m, 2H), 4.58 (dd, *J* = 4.4, 10.7 Hz, 4H), 4.74 – 4.78 (m, 4H), 5.33 (dd, *J* = 5.1, 8.2 Hz, 2H), 5.41 – 5.45 (m, 2H), 6.04 (d, *J* = 7.6 Hz, 2H), 8.12 (s, 2H), 8.26 (s, 2H); ^{31}P NMR (202.5 MHz, MeOD) δ 0.88; $C_{38}H_{56}O_{14}N_{10}NaP_2Si_2^+$ (M + Na^+) calcd *m/z* 1017.2884, found *m/z* 1017.2842. Yield = 45%.

Fully Protected Cyclic Bis(3'-5')diuridylic Acid (8c)

^{1}H NMR (300 MHz, MeOD) δ 0.13 (s, 6H), 0.19 (s, 6H), 0.91 (s, 9H), 0.94 (s, 9H), 2.95 (t, *J* = 5.9 Hz, 4H), 4.24 - 4.68 (m, 10H), 5.70 - 5.76 (d, 2H), 5.82 (d, *J* = 6.1 Hz, 2H), 7.68 (d, *J* = 8.2 Hz, 2H); ^{31}P NMR (121.5 MHz, MeOD) δ -0.6641; $C_{36}H_{56}N_6O_{16}NaP_2Si_2^+$ (M + Na^+) calcd *m/z* 969.2659 found *m/z* 969.2688. Yield = 30%

Fully Protected Cyclic (3'-5')guanylic/uridylic Acid (8d)

^{1}H NMR (300 MHz, MeOD) δ -0.10, 0.10, 0.20 (3s, 12H), 0.79, 0.90 (2s, 18H), 2.91 – 3.00 (m, 4H), 3.20 (s, 6H), 4.10 – 4.68 (m, 12H), 5.13 – 5.19 (m, 1H), 5.32 – 5.38 (m, 1H), 5.76 (d, 1H, *J* = 8.1 Hz), 5.86 – 5.94 (m, 2H), 7.66 (d, *J* = 8.1 Hz, 1H), 7.95 (s, 1H), 8.69 (s, 1H); ^{31}P NMR (121.5 MHz, MeOD) δ 0.34, 0.68; $C_{40}H_{62}N_{10}O_{15}NaP_2Si_2^+$ (M + Na^+) calc *m/z* 1063.3 found *m/z* 1063.3. Yield = 39%.

Fully Protected Cyclic (3'-5')inosylyl/uridylic Acid (8e)

^{1}H NMR (300 MHz, MeOD) δ -0.14 - 0.19 (m, 12H), 0.78 - 1.68 (m, 18H), 2.95 - 3.00 (m, 4H), 4.21 - 4.76 (m, 11H), 4.90 - 5.37 (m, 3H), 5.74 (d, *J* = 8.3 Hz, 1H), 5.85 (d, *J* = 7.3 Hz, 1H), 6.03 (d, 1H, *J* = 7.0 Hz), 7.66 (d, *J* = 8.0 Hz, 1H), 8.11 (s, 1H), 8.25 (s, 1H); ^{31}P NMR (202.5 MHz, $(CD_3)_2CO$) δ 0.88 - 3.71 (m); $C_{37}H_{56}O_{15}N_8NaP_2Si_2^+$ calc *m/z* 993.2771 found *m/z* 993.2804. Yield = 30%

2.3.8 General Procedure for Preparation of 9

To a solution of **8a**, **8b** and **8c** (0.05mmol) in methanol (8 mL) respectively, concentrated aqueous ammonium hydroxide (8 mL) was added. The resulting mixture was stirred at 50 °C for 12 hrs, after which it was then concentrated and dried *in vacuo* to obtain a residual material. This was then dissolved in $(C_2H_5)_3N.3HF$ (1.0 mL) and the mixture stirred at room temperature for 12 hrs. To the reaction mixture was added a 1M ammonium acetate buffer solution (10 mL). The reaction mixture was stirred vigorously at 40 °C to precipitate a pale yellow solid. After the removal of the precipitate, the aqueous solution was subjected to semi-preparative HPLC using a COSMOSIL 5C18-AR-300 column [20 (diameter) × 250 (height) mm]. Elution was carried out under the following conditions to obtain **9a**, **9b** and **9c** respectively: [A = water with 1% TFA, B = 20:80 mixture of water and acetonitrile with 1% TFA] gradient: 0 – 3 min: 100% A, 3 – 35 min: (linear gradient) 100% A to 85% A / 15% B, 35 – 45 min: 100% B, 45 – 55 min: 100% A; detection at 254 nm; flow rate 3 mL/min. Relevant fractions were collected, concentrated and subsequently washed with acetonitrile. Centrifugation afforded a white

solid which was dissolved in 1M aqueous ammonium acetate. Average overall yields of 10 – 15% were observed.

Cyclic Bis(3'-5')diguanylic Acid (9a)

^{1}H NMR (500 MHz, D_2O), δ 4.01 – 4.04 (m, 2H), 4.32 – 4.40 (m, 4H), 4.83 (s, 2H), 5.04 (m, 2H), 5.81 (s, 2H), 7.95 (s, 2H); ^{31}P NMR (202.5 MHz, D_2O) δ -1.05; $C_{20}H_{23}O_{14}N_{10}P_2^-$ ($M - H^-$) calcd *m/z* 689.0865, found *m/z* 689.0849. Yield = 84%.

Cyclic Bis(3'-5')diinosylic Acid (9b)

^{1}H NMR (500 MHz, D_2O) δ 3.92 – 3.98 (m, 2H), 4.12 (m, 2H), 4.51 – 4.60 (m, 4H), 6.09 (s, 1H), 6.21 (s, 1H), 8.10 (s, 1H), 8.30 (s, 1H), 8.63 (s, 2H); ^{31}P NMR (202.5 MHz, D_2O) δ -1.04; $C_{20}H_{23}O_{14}N_8P_2^+$ ($M + H^+$) calcd *m/z* 661.0804, found *m/z* 661.0828. Yield = 85%.

Cyclic Bis(3'-5')diuridylic Acid (9c)

^{1}H NMR (300MHz, MeOD) δ 3.94 -3.97 (m, 2H), 4.28 - 4.40 (m, 10H), 4.48 - 4.56 (m, 4H), 5.5 - 5.70 (m, 4H), 7.90 (d, *J* = 7.7 Hz, 2H); ^{31}P NMR (121.5 MHz, D_2O) δ -1.08; $C_{18}H_{20}O_{16}N_4P_2^-$ ($M - H^-$) calcd *m/z* 611.0433, found *m/z* 611.0452. Yield = 90%

Cyclic (3'-5')guanylic/uridylic Acid (9d)

^{1}H NMR (500 MHz, D_2O) δ 4.08 (m, 2H), 4.41 – 4.52 (m, 4H), 5.34 (m, 2H), 5.60 (m, 1H), 5.93 (m, 1H), 7.49 (s, 1H), 7.92 (m, 2H), 8.45 (m, 1H), 8.70 (s, 1H); ^{31}P NMR (202.5 MHz, D_2O) δ -0.89; $C_{19}H_{23}N_7O_{15}NaP_2^+$ ($M + Na^+$) calcd 673.0547 found *m/z* 673.0568. Yield = 82%.

Cyclic (3'-5') inosylic/uridylic Acid (9e)

^{1}H NMR (500 MHz, D_2O) δ 4.05 (m, 2H), 4.51-4.36 (m, 7H), 5.42 (s, 1H), 6.16 (s, 1H), 7.89 (s, 1H), 8.16 (s, 1H); ^{31}P NMR (202.5 MHz, D_2O) δ -0.97(s); $C_{19}H_{21}O_{15}N_6P_2$ (M^-) calc *m/z* found *m/z* 635.0535. Yield = 92%

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CHAPTER 3: BIOLOGICAL SCREENING OF COMPOUNDS

3.1 Introduction

To achieve the aim of screening the synthesized compounds for biological activity, an enzyme that contains only the GGDEF domain (diguanylate cyclase activity) but not the EAL domain (phosphodiesterase activity) has to be obtained, so as to assay the amount of c-di-GMP formed. Gomelsky and coworkers have isolated PCR-amplified bacterial DNA fragments coding for GGDEF domain-containing proteins-and cloned them into vector pMAL-c2x (New England Biolabs) in strain *E. coli* DH5α[1]. Hence, his recombinant bacterial cells were requested for and upon receipt, the cells were streaked on LB agar plates and kept at 4^{o}C for subsequent overexpression of the protein.

Out of the five proteins isolated from the delivered recombinant bacteria, only Slr 1143 from oxygenic phototroph *Synechocystis sp. (Cyanobacteria)* and DRB0044 from the radiation- and desiccation-resistant soil bacterium *D. radiodurans (Deinococcus/Thermus)* were highly overexpressed. However, optimal purification of DRB0044 could not be achieved due to a lack of time and resources, thus our efforts were channeled into the purification of Slr1143. Not much is known about Slr1143, except that it is a diguanylate cyclase consisting of 344 amino acids[2].

The vector pMAL-c2x was designed such that after the insertion of the protein of interest, it would fuse to the maltose binding protein (MBP) to form a fusion protein. This

facilitated the purification of the desired protein via affinity chromatography whereby the maltose binding protein was captured by the amylose resin (*New England Biolabs*), thus allowing the unwanted proteins to be washed off. Washing of the resin with buffer solution containing maltose allowed the isolation of pure protein. To obtain the protein of interest, the fusion protein was cleaved from the MBP with the specific protease Factor Xa. Subsequently, the maltose was removed from the protein mixture, before it was passed through the amylose resin once again to remove the MBP. However, in our experiment, the cleavage of the MBP was not performed, as Gomelsky had illustrated that the protein is viable without the removal of MBP[1]. The *mal*E gene on the vector pMAL-c2x was also deleted from the signal sequence, so the fusion protein produced would remain in the cytoplasm and not be exported out into the media.

The compounds synthesized were screened to determine the biological activities they may possess against Slr1143. After the enzymatic assay in which the enzyme was incubated with both its substrate and inhibitor, the mixture of GTP, c-di-GMP and inhibitor was separated by reverse phase HPLC and the product peak area was determined by software, LCsolution Ver 1.2. To determine the inhibitory activity of the compounds, the product of the enzyme, c-di-GMP, was assayed relative to the control. For preliminary screening, the enzyme was screened with 100µM of inhibitor and 100µM of GTP, against the control, which did not contain any inhibitor.

3.2 Material and Methods

All methods of preparation of media and solutions can be obtained from New England Biolabs pMAL™ Protein Fusion and Purification System Instruction Manual. Plasmid coding for Slr1143 was kindly provided for by Gomelsky[1]. Enzymatic assay buffer was prepared according to reported method[1]. Protein concentration was determined to be 2.069mg/ml by Bradford assay. HPLC analysis was carried out using a Phenomenex Luna 3μ-C18 column [4.6 (diameter) × 50 (height) mm].

3.2.1 Large scale overexpression of protein

1L rich LB broth with 2g glucose and ampicillin (100 μg/ml) was inoculated with 10ml of overnight culture of *E.coli* cells and incubated at 37°C to an OD of 0.6-0.8 at 600nm. 3ml of 0.1M Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added next to the subculture to a final concentration of 0.3mM and further incubated for 2 hours to induce the expression of protein. After which, the cells were centrifuged at 4°C for 20 min at 4000*g* and the supernatant was discarded. 25ml of column buffer was added to resuspend the cells before Halt Protease Inhibitor Cocktail (*Pierce*) was added and kept at -20°C overnight. Lysozyme was added at 1mg/ml of buffer and incubated for 30 min. To further disrupt the cells to facilitate the release of protein into the supernatant, the cells were sonicated for 10min, 10sec on, 15sec off, on ice. The crude was then centrifuged at 9000*g* for 30 min. The supernatant was subsequently incubated with 1ml of amylose beads (*New England Biolabs*), which were washed beforehand according to the instructions, for an hour at 4°C.

3.2.2 Purification of protein

The amylose beads were poured into affinity column and the flow through was collected. As the amylose beads would only bind to the maltose binding protein (MBP) within the fusion protein, the unwanted protein were washed out with 10 column volumes of column buffer before elution of the fusion protein with 10mM of maltose in column buffer was performed. Small fractions of 0.6ml of eluant were collected. SDS-PAGE was carried out on all the fractions to determine which fractions contained the fusion protein. Glycerol was added at a concentration of 20% before a Bradford assay was performed to determine the concentration of the protein. Subsequently, the protein was aliquoted into smaller fractions of 20ul.

3.2.3 Analysis of Slr1143 diguanylate cyclase with different GTP concentration.

Enzyme (2µl, 1µM) (kept on ice) in enzymatic assay buffer was incubated at 30^{o}C for 5min. Following which, GTP (kept on ice) was added to desired concentration and incubated at 30 ^{o}C. 50µl of the reaction mixture was pipetted out at 0.5, 1, 1.5 and 2 min and quenched by heating it at 95^{o}C. Each sample was filtered with 0.2µM HPLC filter, before 10µl was analysed by HPLC reversed phase. The experiment was then repeated for other GTP concentrations.

3.2.4 Analysis of compounds on Slr1143 diguanylate cyclase.

5µl of a 1mM compound in enzymatic assay buffer was added to a mixture of enzyme (1µM, 2µl) (kept on ice) in enzymatic buffer (38µl) to a final concentration of 100µM in 50µl and the mixture was incubated at 37^{o}C for 5min. Following that, 5µl of 1mM GTP (kept on ice) was added to a final concentration of 100µM and the reaction was quenched at 2 min, by heating it at 95^{o}C. Each sample was filtered with 0.2µM HPLC filter, before 10µl was analysed by HPLC reversed phase. The experiment was then repeated for the other compounds.

3.2.5 HPLC method

Buffer A: 20mM Triethylammonium bicarbonate buffer. Buffer B: Methanol.
0-2 min: A: 100%, 2-10 min: B: 12% 10-12min B: 18% 12-20min B: 30%

3.3 Results and Discussion

3.3.1 Overexpression and purification of enzyme

A small scale experiment was initially performed according to the protocol by *New England Biolabs* for pMAL-c2 vectors to determine the expression level of the maltose-binding fusion protein and the feasibility of the method. Uninduced cells, pellet of lysed crude cell extract were resuspended in 1ml of column buffer and its supernatant were kept for SDS PAGE to determine the expression levels. This was similarly done for the large scale preparation to determine the amount of protein released into the supernatant and the amount remaining in the pellet.

The cells were initially lysed with B-PER Bacterial Protein Extraction Reagent II (*Pierce*) but the lysis was shown to be ineffective as seen from the absence of the protein in the supernatant as shown in Figure 4. Effective lysis will release the fusion protein into the supernatant and a band at 80kDa would be observed after running SDS-PAGE.

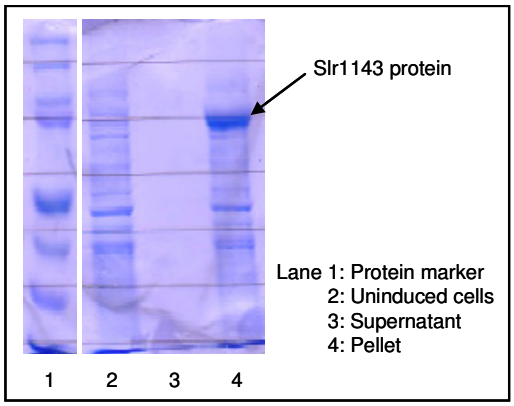

Figure 4: SDS-PAGE showing absence of protein in supernatant.

An alternative attempt at cell lysis was performed using sonication on ice, for two pulses of 10 seconds on and 10 seconds off. However, it was only effective for the small scale but not on the large scale experiment. Even sonication for 30 minutes did not result in a significant amount of protein being released into the supernatant as compared to the pellet. This could be due to the fact that the sonicator was attached to a microprobe and too much buffer was added initially (50 mL), resulting in the crude cell extract being too dilute. Coupled with the fact that the disruptive forces of the ultrasound waves became weaker as they travelled further away from the microprobe, the cells could not be lysed effectively. Feliu *et al*[3] have shown that cell concentration has no effect on the rate at which the protein is released whereas the volume of the extract has an inverse relationship on the rate. Hence, in subsequent attempts to lyse the cells, only 25 mL of

buffer was added. In addition, a major consideration when ultrasonicating is sample heating. Cavitation caused by the ultrasonicator can raise the temperature quickly, and the solution may get very hot in the vicinity of the tip of the microprobe. The heat thus generated could lead to protein denaturation and aggregation. For this reason, one can do many short pulses rather than one long continuous pulse. An additional precaution in preventing the cell suspension from getting too hot is to make sure that equipment and samples are kept cold at all times.

A third attempt at cell lysis was the freeze/thaw method which was repeated six times, before under going sonication cycle of 10 seconds on, 15 seconds off, for 10 min. This proved to be a very effective but time consuming method. Hence, to enhance cell lysis before subjecting the cells to ultrasound waves, lysozyme (1 mg/ml) was added and incubated for 30 minutes at 37 °C, the temperature at which the activity of lysozyme is optimal. Thereafter, the crude cell extract was subjected to centrifugation. The supernatant was retained and subjected to affinity chromatography to obtain the pure Slr1143 protein as shown in Figure 4.

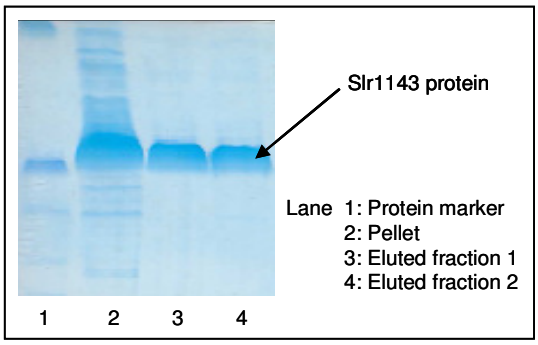

Figure 4: SDS-PAGE showing pure protein in eluted fractions 1 and 2

3.3.2 HPLC elution buffer solution

Phosphate buffers are common buffers used for the separation of GTP and c-di-GMP peaks with HPLC[1,4-7]. Initially, phosphate buffer was used but the baseline was very noisy and there was another problem of column clogging due to precipitation of phosphate salts at low room temperatures or when the column was not properly washed with non-buffered solvents. Hence, re-preparation of the buffer solution was necessary. As adjustment of buffer pH was necessary, the temperature at which the adjustment of pH was done would need to be the same as that during analysis. Therefore, alternatives were considered.

One such method would be to use [^{32}P] GTP and separate the reaction mixture on polyethyleneimine-cellulose plates. The concentration of the compound was determined by comparing the intensity of the radioactive compounds as seen on a phosphorimaging screen, against a standard[8]. However, radioactive materials are hazardous and should best be avoided. The standard buffer system of ACN/H_2O and 0.1% TFA in ACN/0.1% TFA in H_2O were used but GTP peaks were not observed at 254nm. However, the usage of triethylammonium carbonate[9] was a good alternative as it is liquid at room temperature and would thus solve the problem of potential column clogging. As triethylammonium carbonate was not easily available, triethylammonium bicarbonate was experimented as a possible alternative and to our surprise, a smoothened baseline was observed (Figure 5). The method was subsequently optimized to avoid the hassle of adjusting pH, to obtain a good resolution of the peaks and to cut down on the running time from 50 min to 30 min.

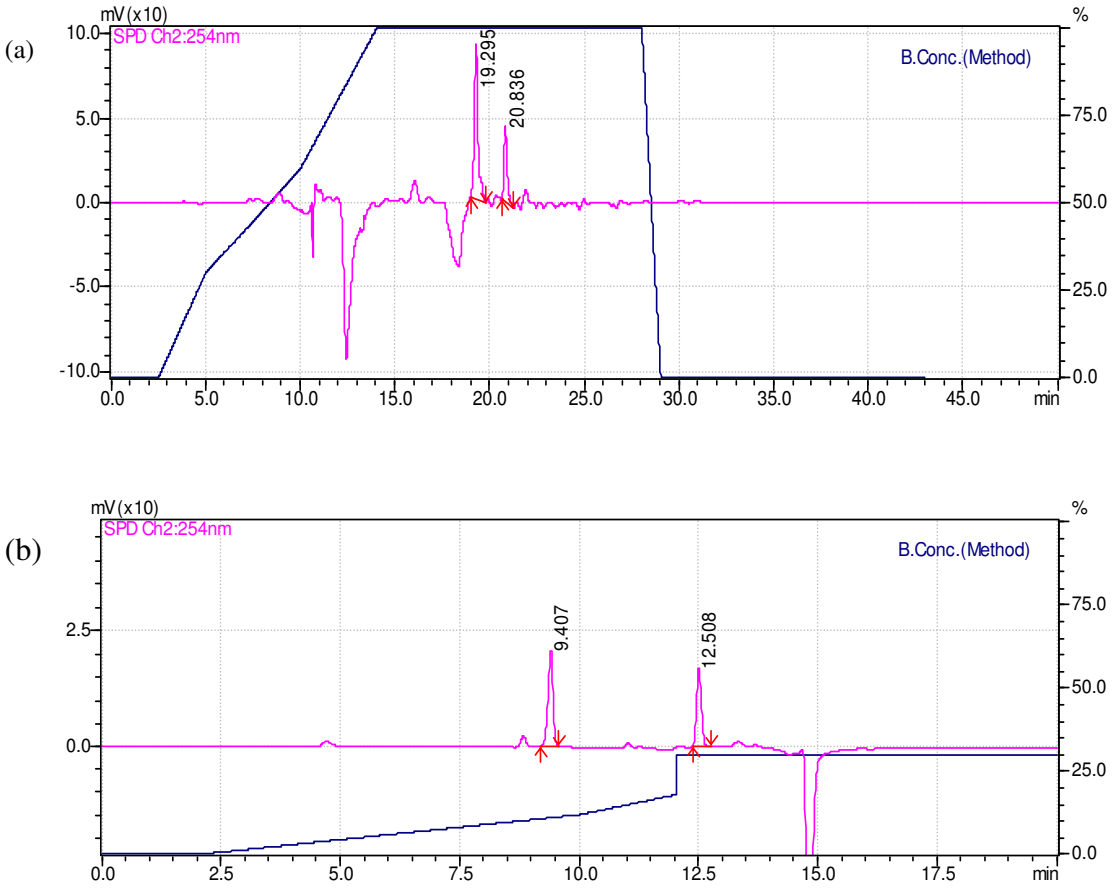

Figure 5: (a) Chromatogram obtained when phosphate buffer was used. (b) Chromatogram obtained after optimization.

3.3.3 Analysis of effect of compounds on the activity of Slr1143 diguanylate cyclase.

Prior to the actual assay, a Michaelis-Menten graph was necessary to determine if this diguanylate cyclase also possessed intrinsic allosteric inhibitor properties like other DGCs. If Slr1143 exhibited allosteric inhibition, the optimum concentration substrate could be elucidated from the curve. To obtain the graph, different concentrations of substrate were introduced to a mixture of 1µM of pre-incubated enzyme and assay buffer for 2 mins. Aliquots of the reaction mixture were taken out every 30s and quenched by

heating at 95°C for 3 mins. The experiment was repeated twice and a graph of product peak area was plotted against time to obtain the rate of reaction. Subsequently, the average rate of reaction was plotted against the concentration of substrate.

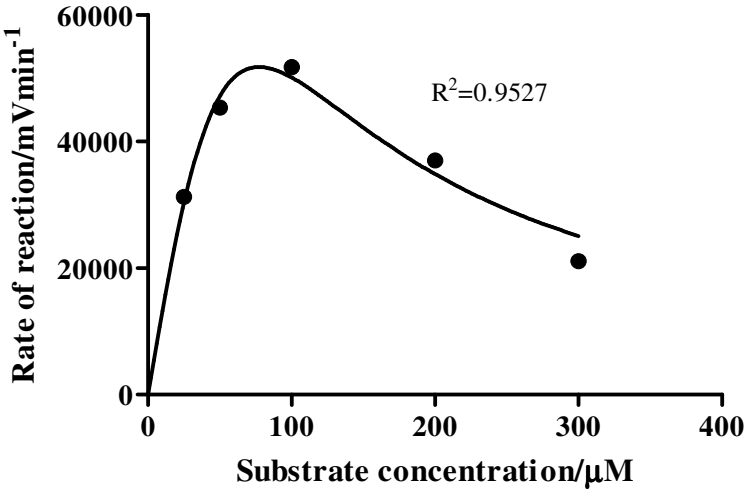

Figure 6: Activity of Slr1143 at 30°C in the presence of different substrate concentration.

From the results obtained, an initial increase in the concentration resulted in an increase in rate of reaction. However, as the concentration increased, the rate of the reaction would reach a maximum at around 100µM before undergoing a decline. This confirmed that Slr1143, like other diguanylate cyclases, exhibited allosteric product inhibition[10]. At low concentrations of substrate, the amount of product formed initially was too low to inhibit the enzyme allosterically. As the concentration of substrate increased, the frequency of collision between enzyme and substrate increased, hence rate of reaction increased. However, as concentration increased further, the rate of reaction would reach a maximum and subsequently, the amount of product synthesized would be high enough to inhibit the activity of Slr1143 drastically, resulting in the curve (Figure 6). From the graph, the optimum substrate concentration at which the enzyme activity

exhibited the maximum rate, without the product interfering with the rate of reaction was determined to be at 100µM. Now that the optimum concentration has been determined, assay of the analogues was carried out in duplicates with 100µM of compound and 100µM of substrate.

From Figure 7 shown below, it could be concluded that **9b** was a much better inhibitor than c-di-GMP **9a**, with an inhibition of 60% at 100µM, with an IC50 value of 68.9µM (Figure 8). The only difference between **9b** and **9a** was the presence of an amino group on each of the two bases. Interestingly, two compounds, **9c** and **9d**, which have uridine bases in common, were found to activate the enzyme.

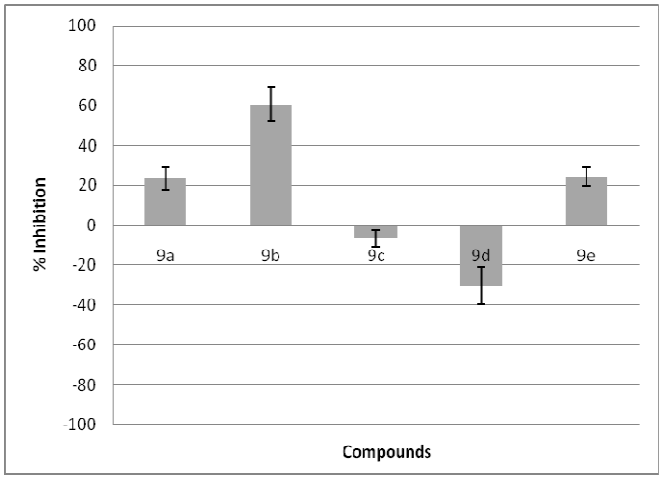

Figure 7: Inhibitory activities of compounds **9a-e**

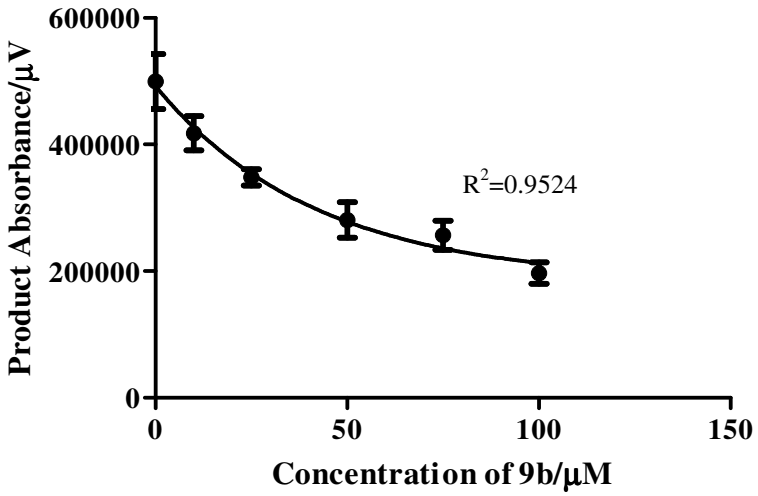

Figure 8: Graph of absorbance vs concentration of **9b**

However, DGC activity of the GGDEF domains of Slr1143 protein was reported to be lower than that of full length proteins[1]. Though the diguanylate cyclase has the intrinsic capability of forming dimers and trimers, similar to its parent, it is strongly affected by neighbouring proteins or protein domains that interact with it[1]. Other factors that might affect its activity would be the composition of the enzymatic buffer, which was not optimized. Hence, although the results obtained would definitely differ from an experiment using a full length protein, they were still able to give us a good idea of the inhibitory activities of the compounds, thus justifying future *in vivo* studies to determine if the compounds inhibit biofilm formation.

3.4 References

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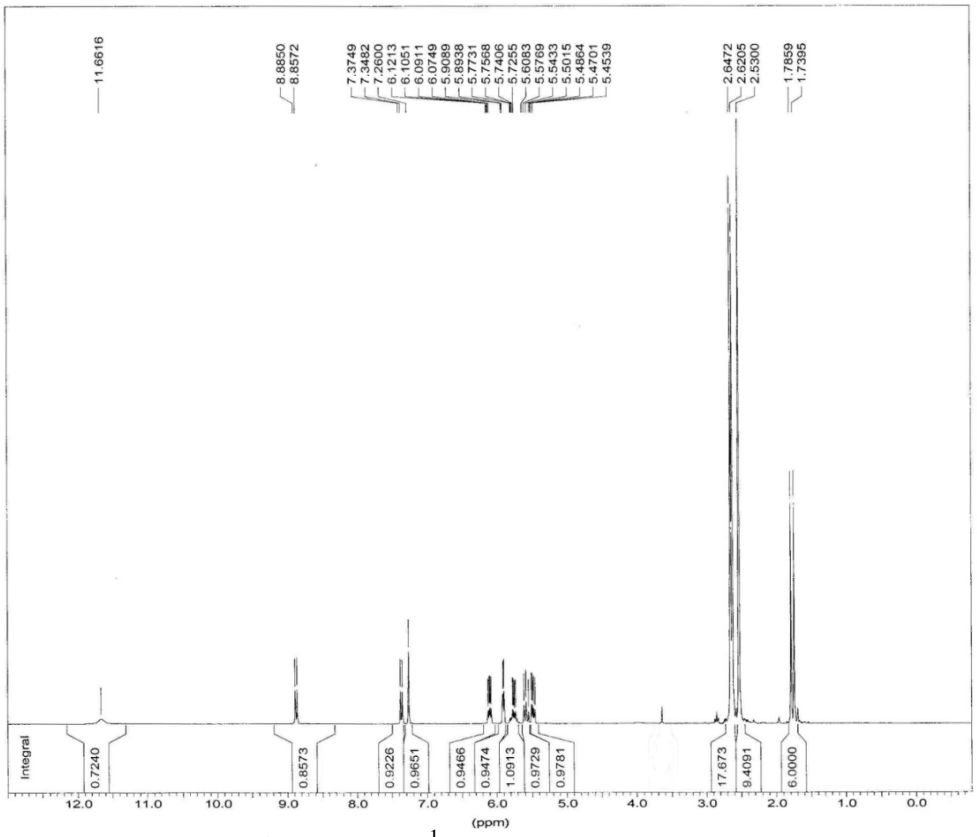
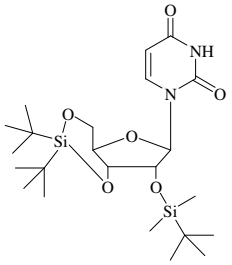
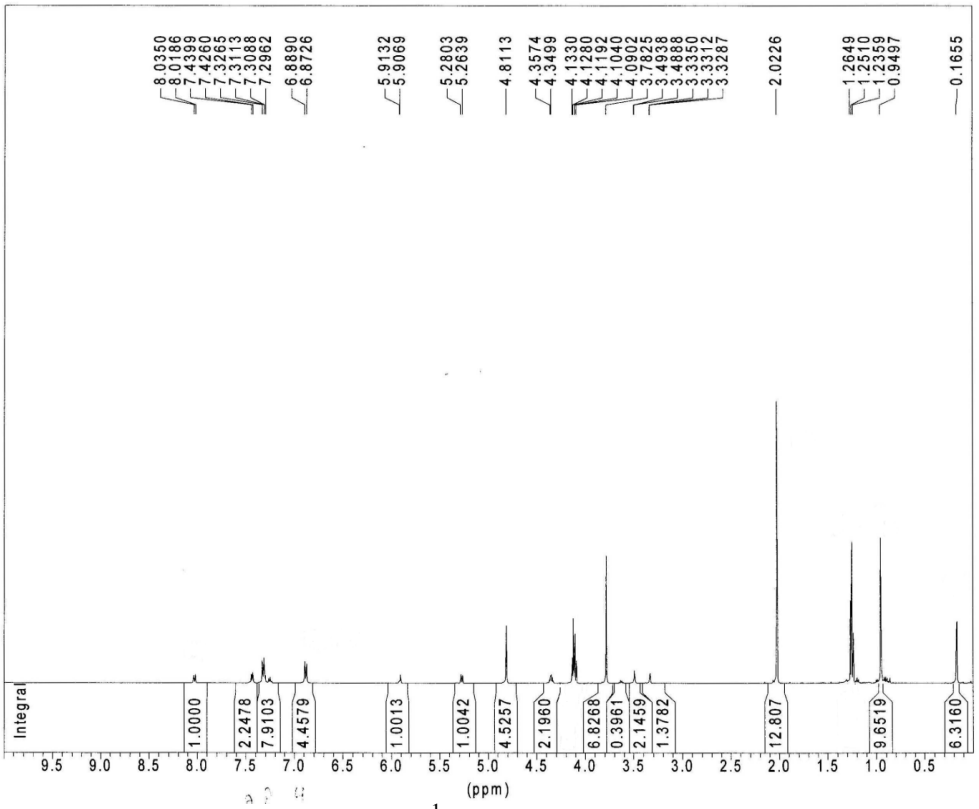
APPENDIX A: SPECTRAL ANALYSES



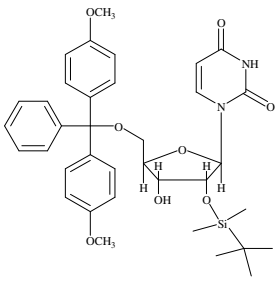
Spectrum 1: ^{1}H NMR spectrum of compound **2b**



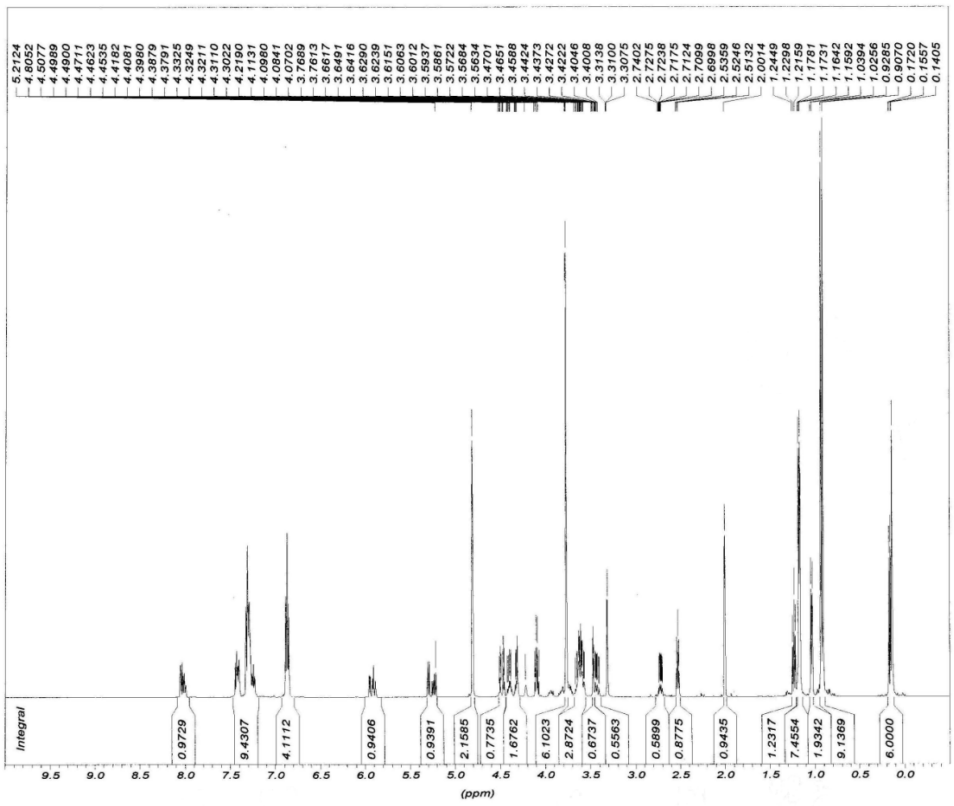
2b

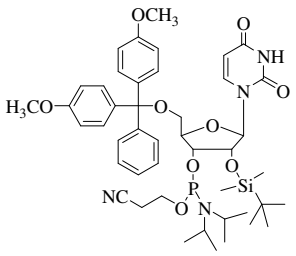


Spectrum 2: ^{1}H NMR spectrum of compound **4b**



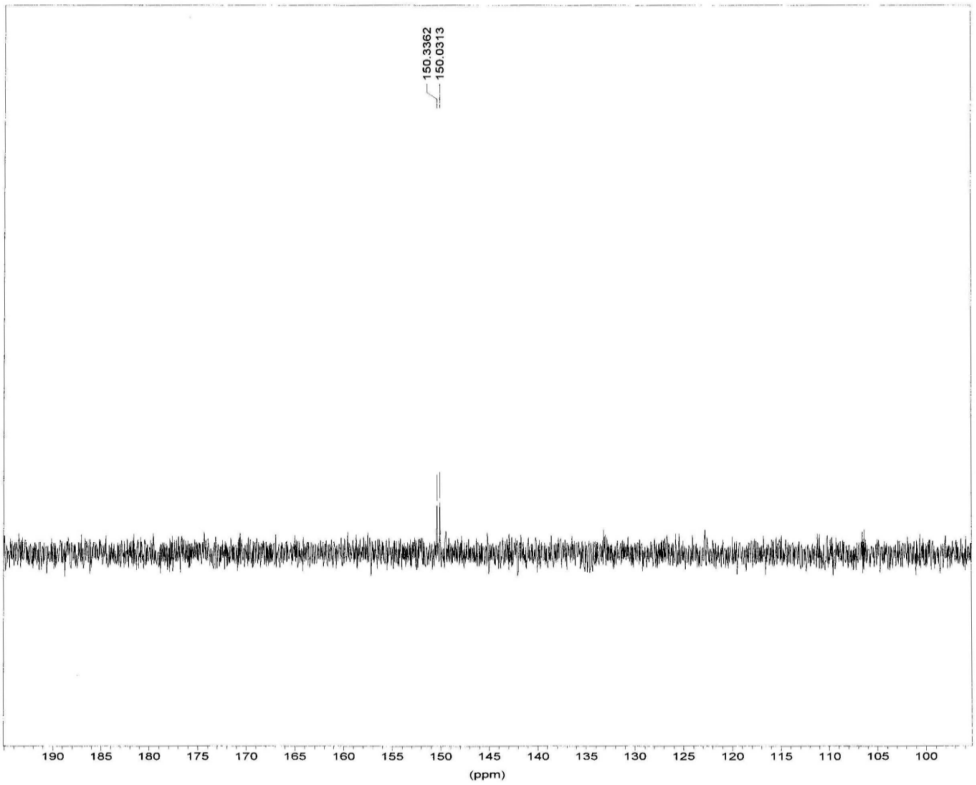
4b



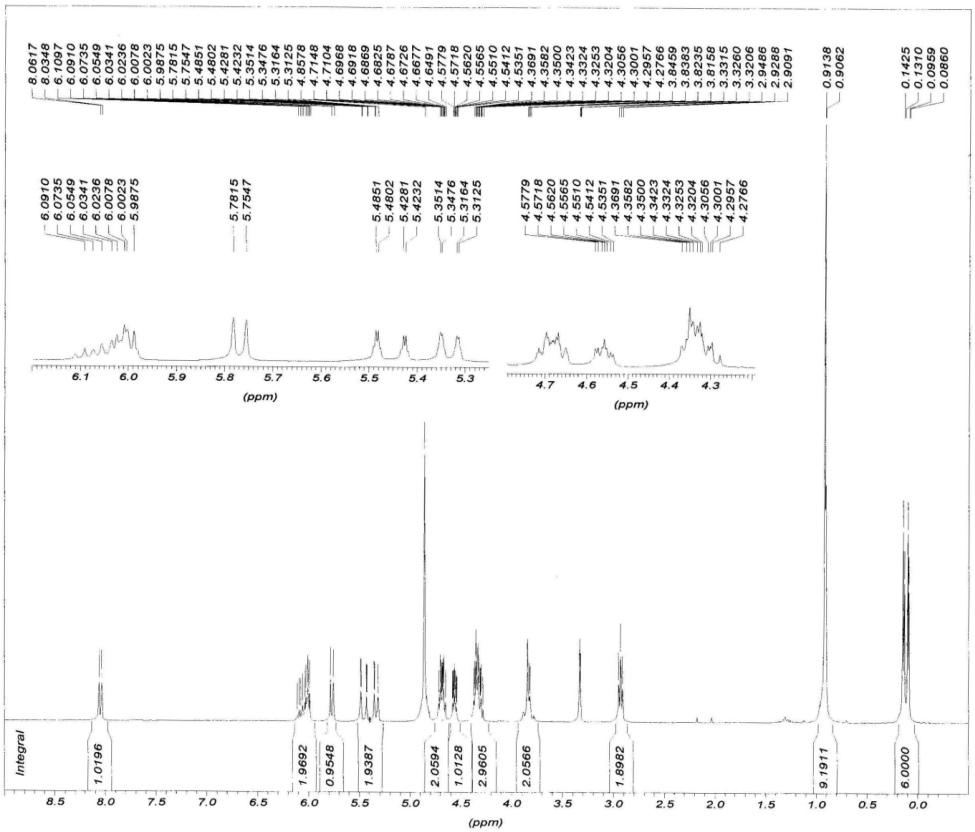


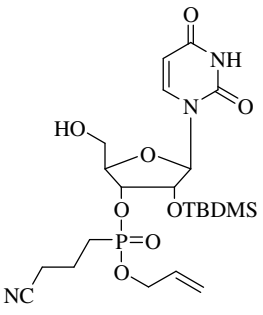
5b

Spectrum 3: ^{1}H NMR spectrum of compound **5b**



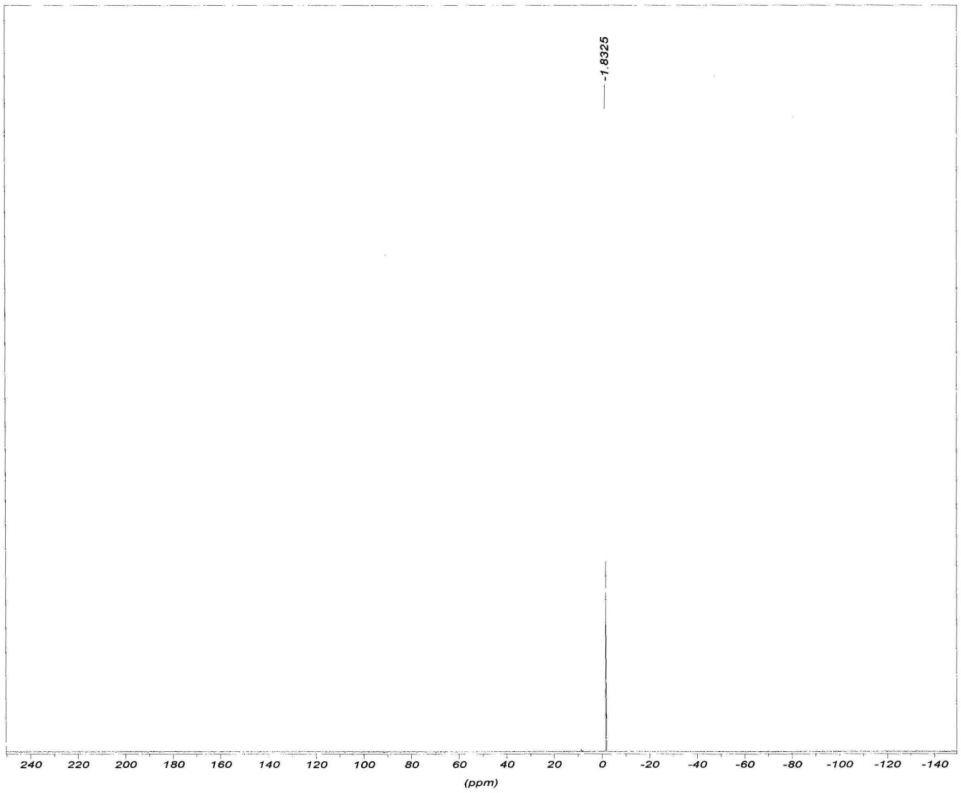
Spectrum 4: ^{31}P NMR spectrum of compound **5b**



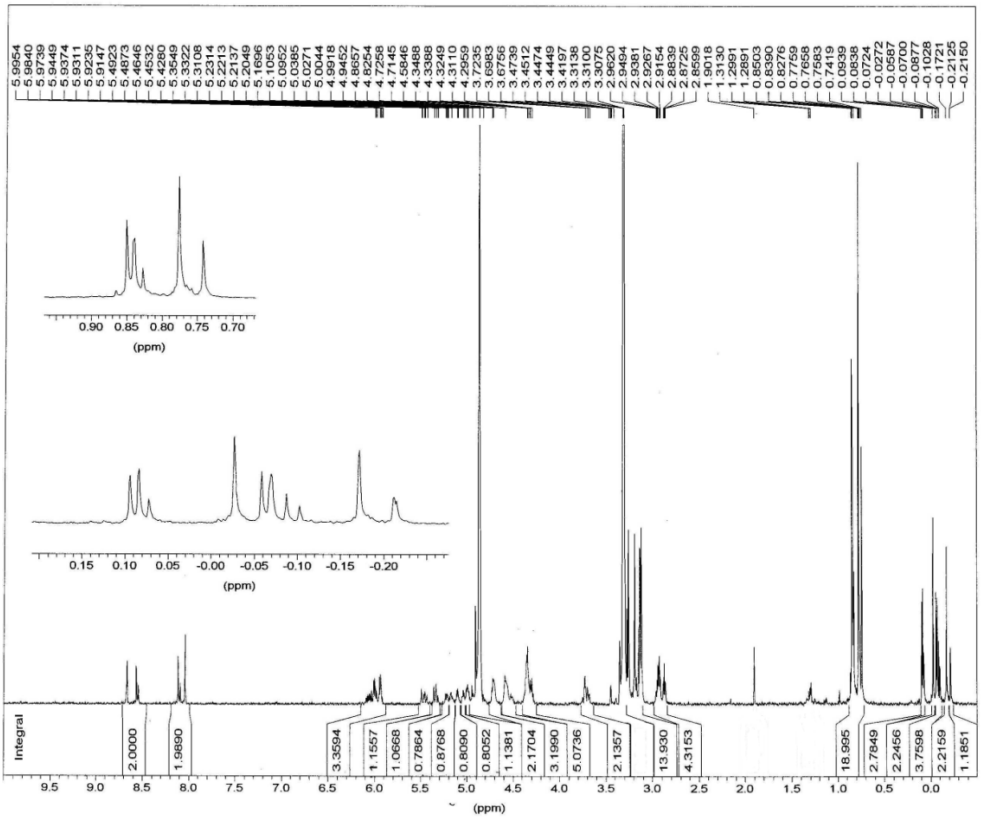


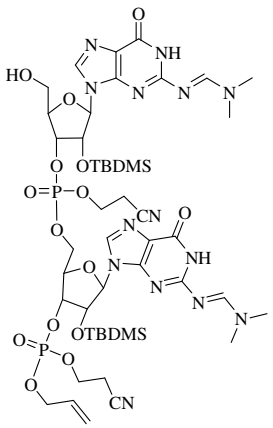
6b

Spectrum 5: ^{1}H NMR spectrum of compound **6b**



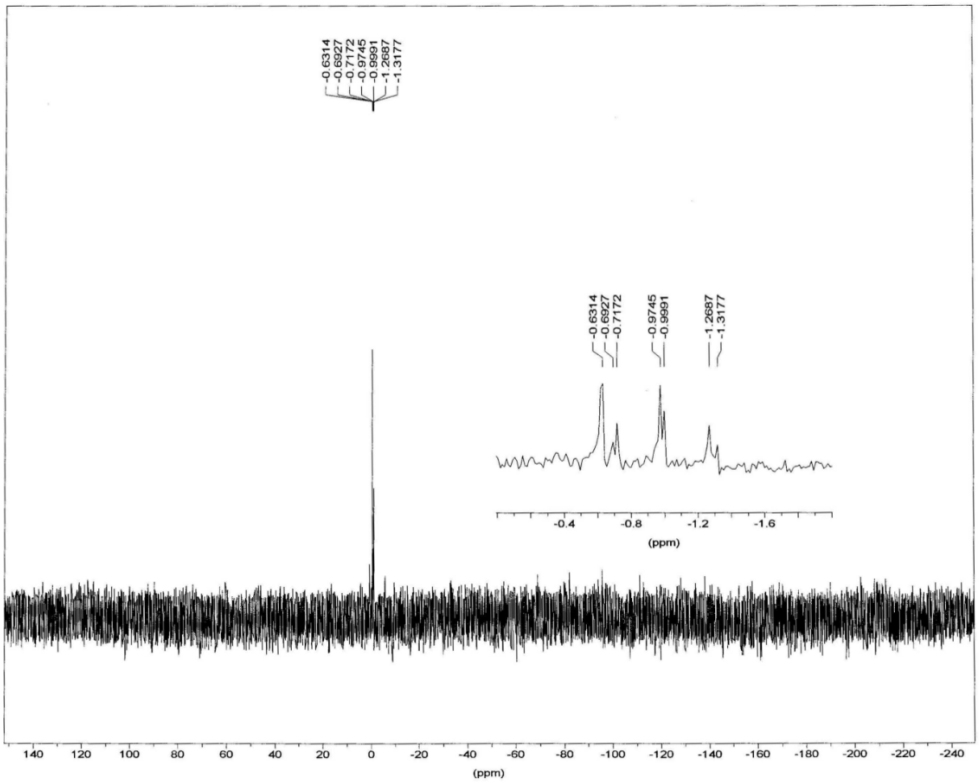
Spectrum 6: ^{31}P NMR spectrum of compound **6b**





7a

Spectrum 7: ^{1}H NMR spectrum of compound **7a**



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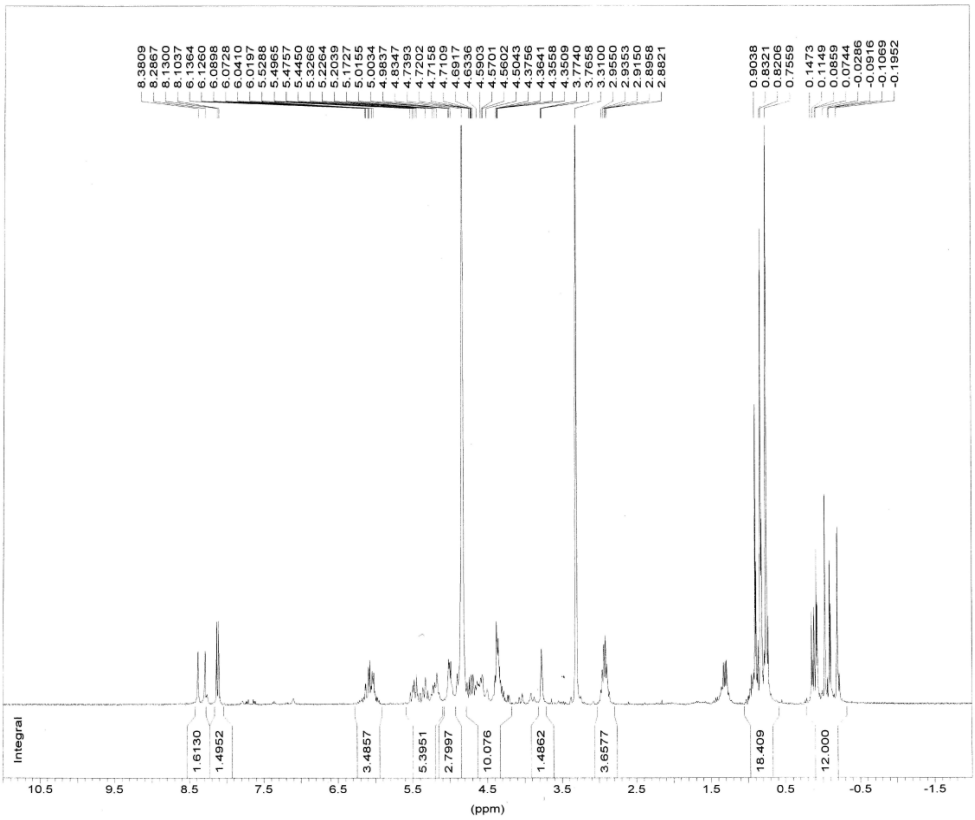
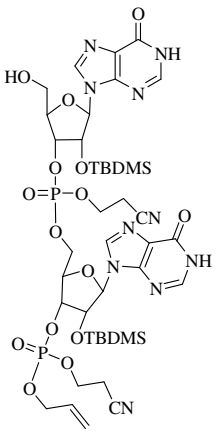
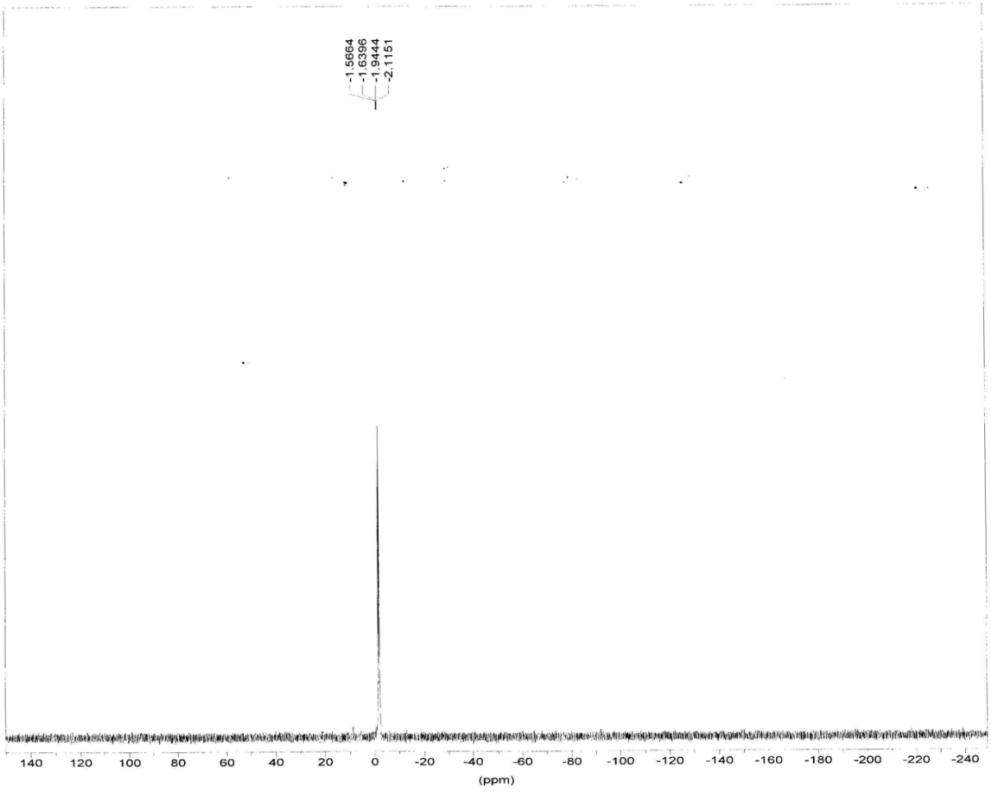


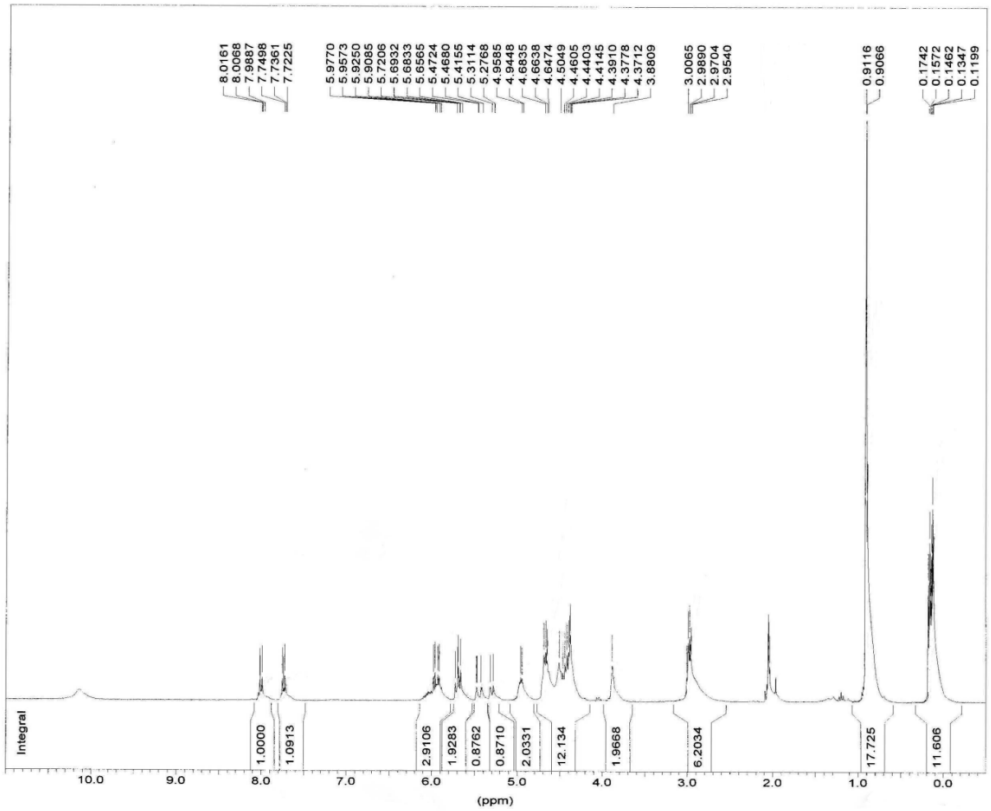


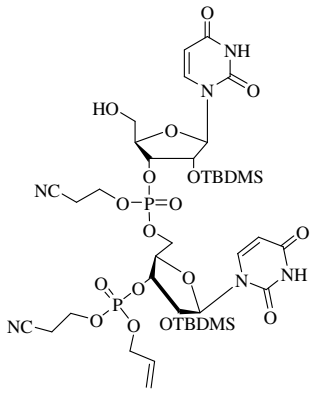
7b

Spectrum 9: ^{1}H NMR spectrum of compound **7b**



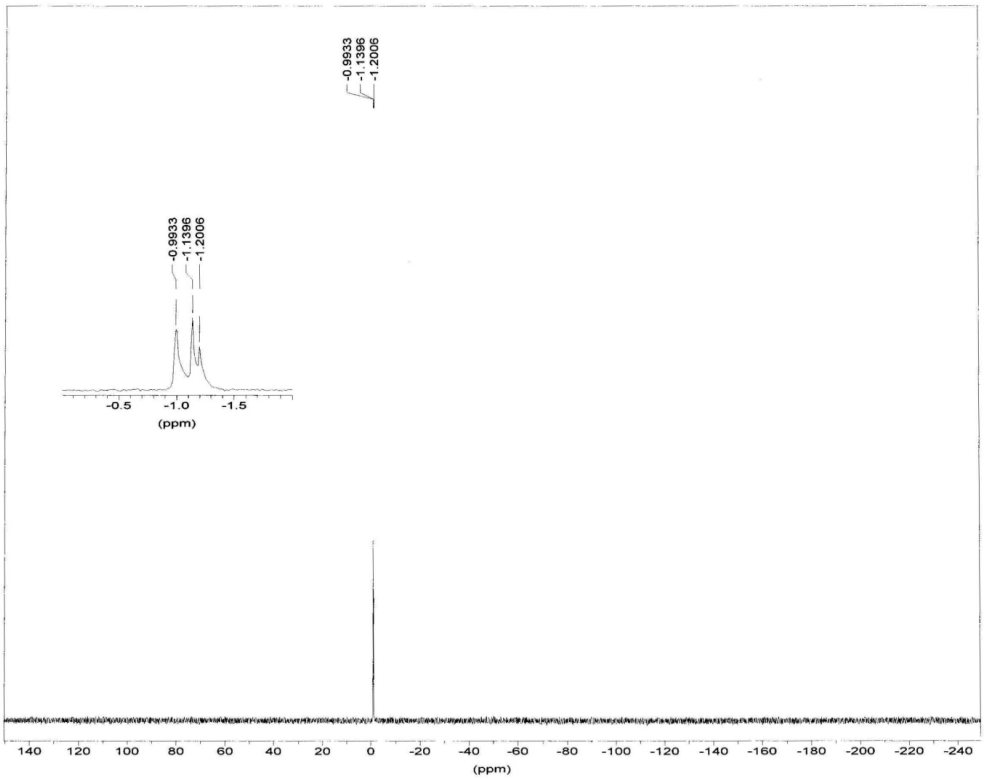
Spectrum 10: ^{32}P NMR spectrum of compound **7b**



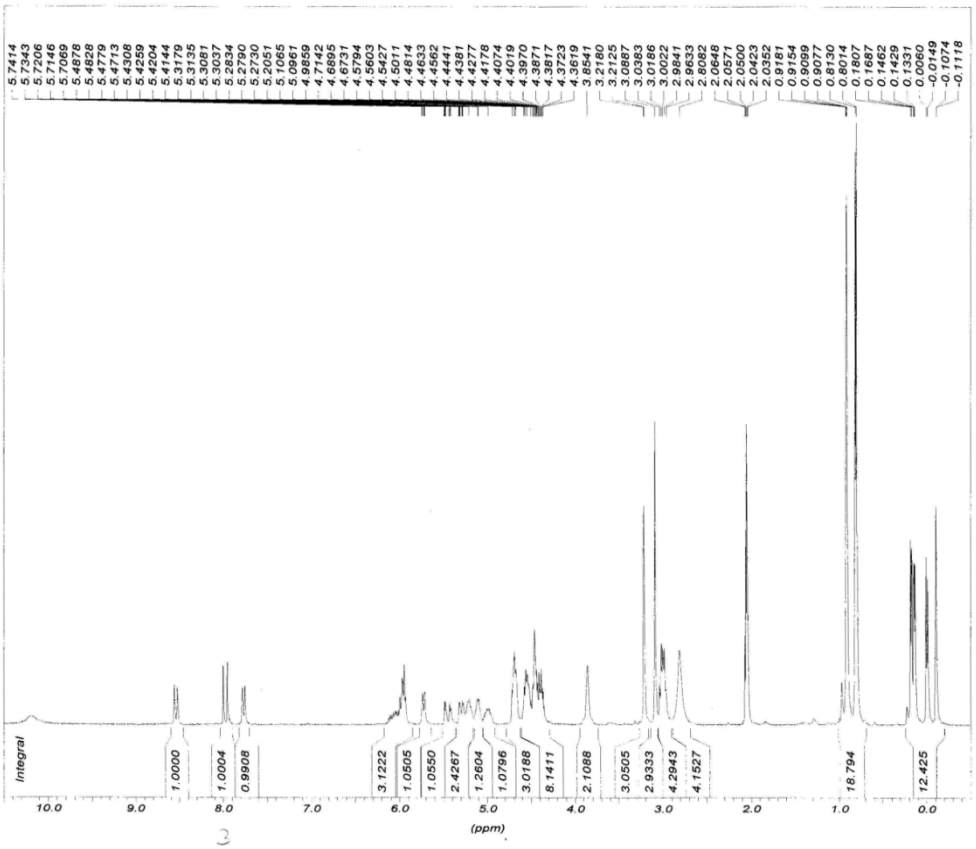


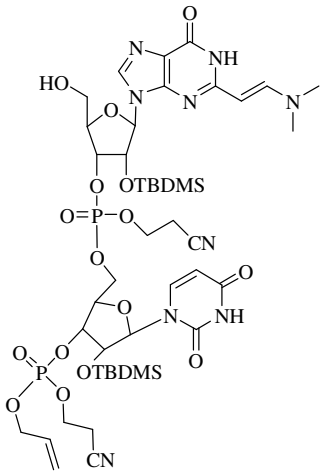
7c

Spectrum 11: ^{1}H NMR spectrum of compound **7c**



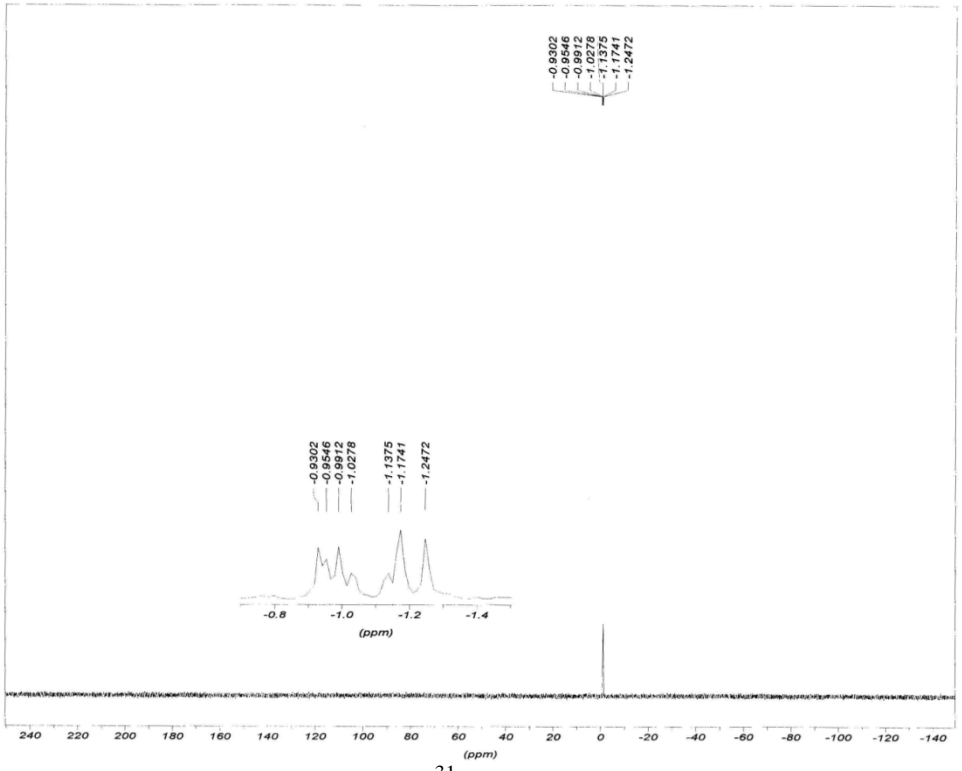
Spectrum 12: ^{31}P NMR spectrum of compound **7c**



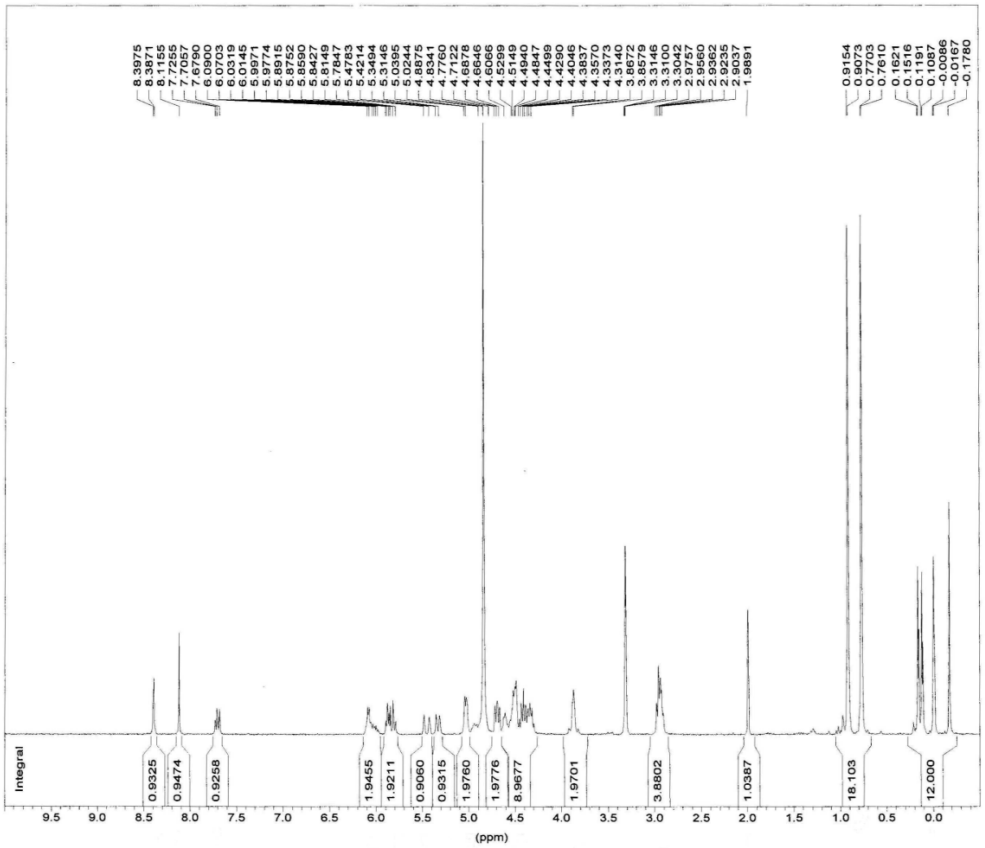


7d

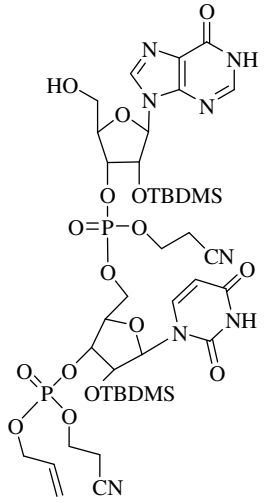
Spectrum 13: ^{1}H NMR spectrum of compound **7d**



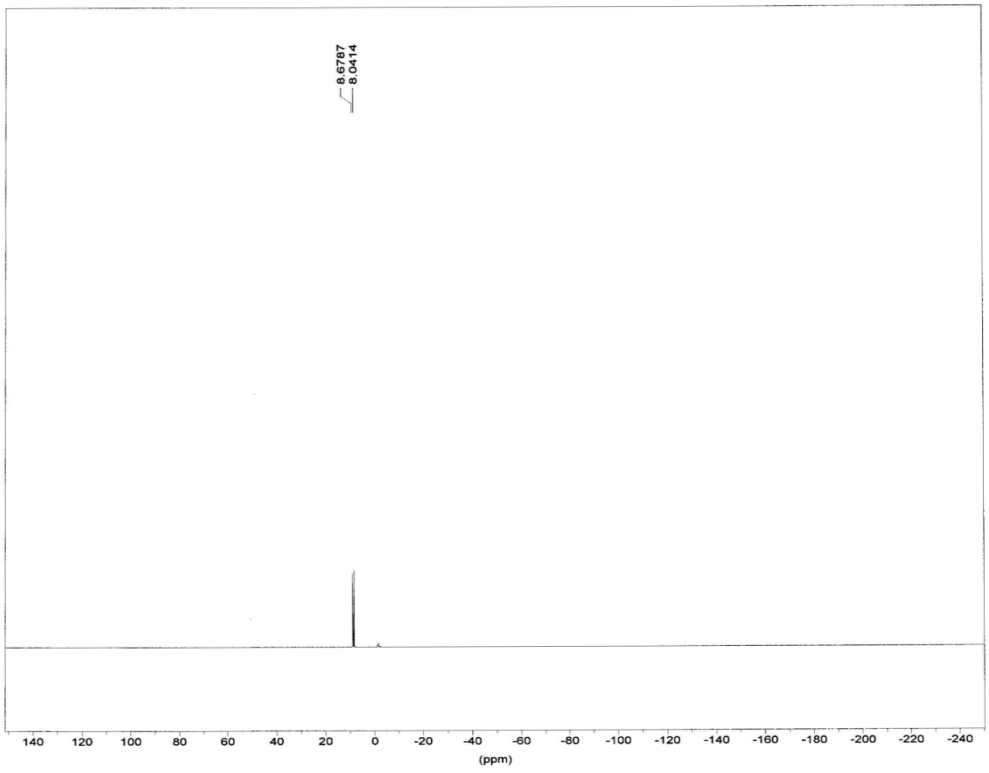
Spectrum 14: ^{31}P NMR spectrum of compound **7d**



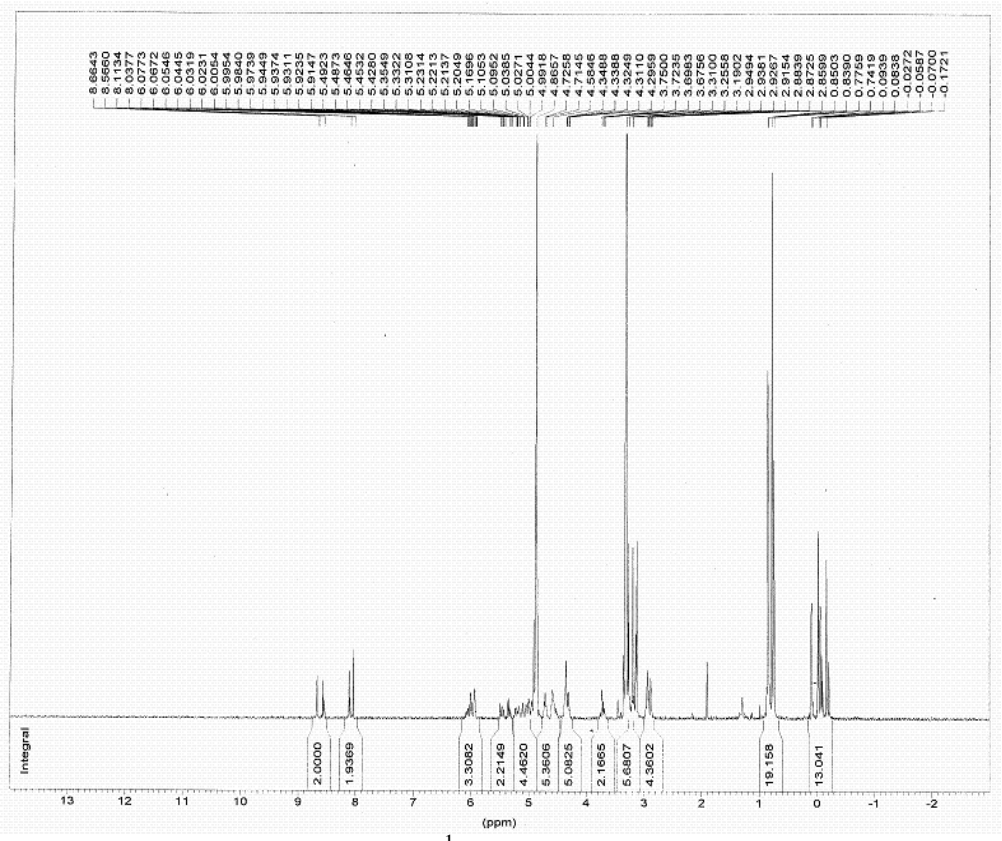
Spectrum 15: ^{1}H NMR spectrum of compound **7e**

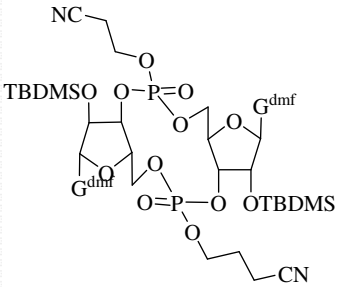


7e



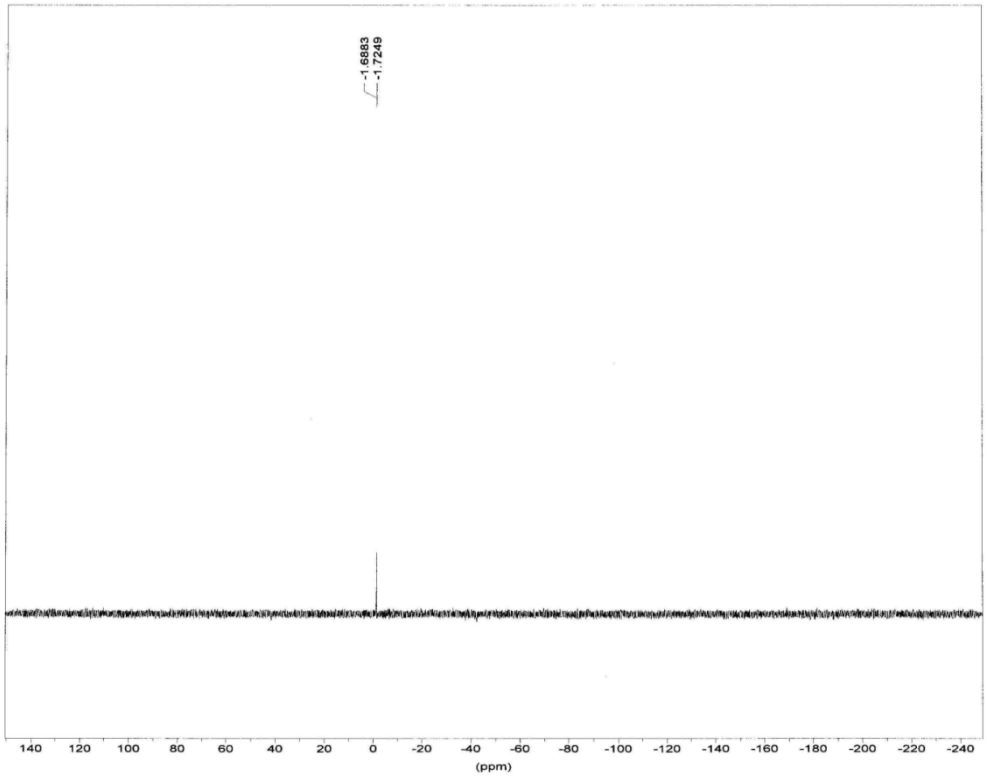
Spectrum 16: ^{31}P NMR spectrum of compound **7e**



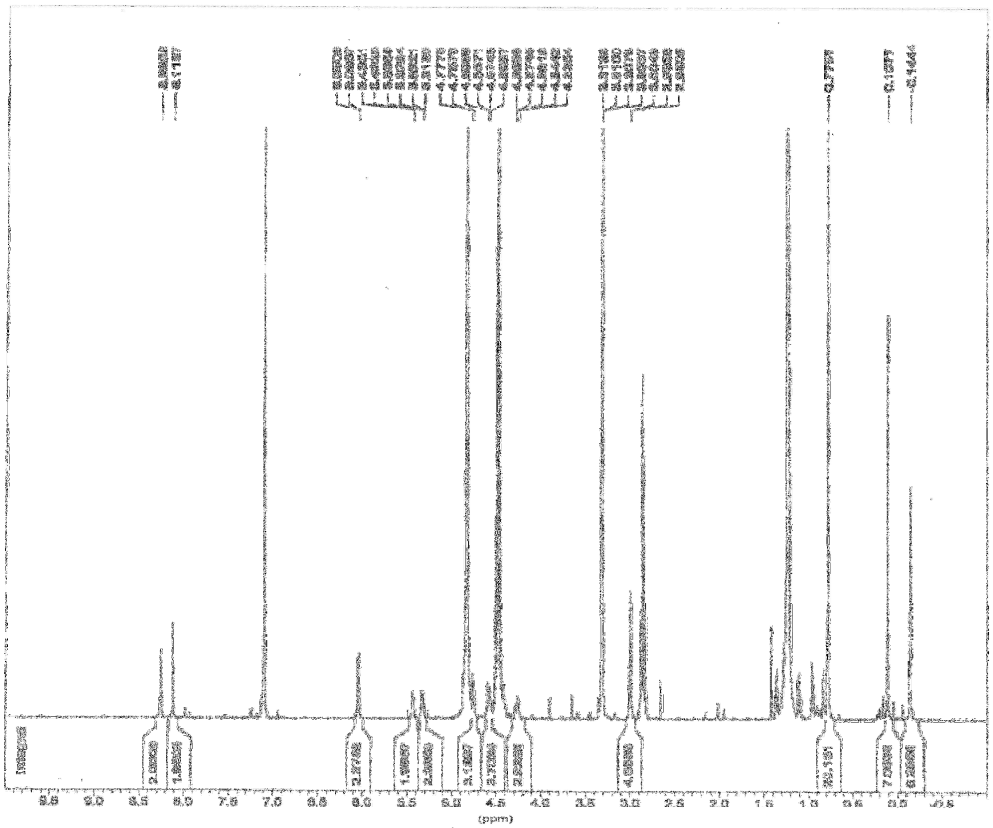


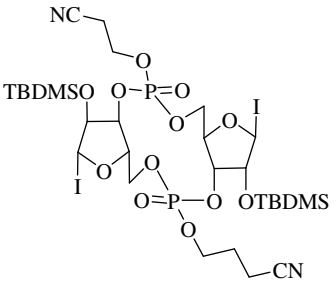
8a

Spectrum 17: ^{1}H NMR spectrum of compound **8a**



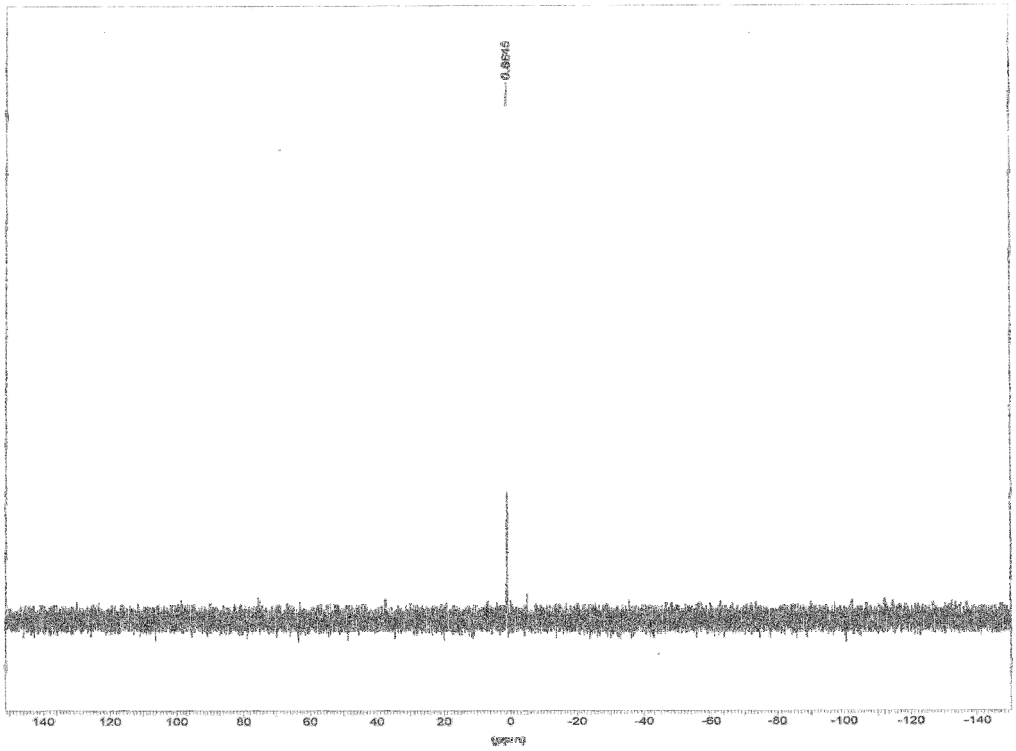
Spectrum 18: ^{31}P NMR spectrum of compound **8a**



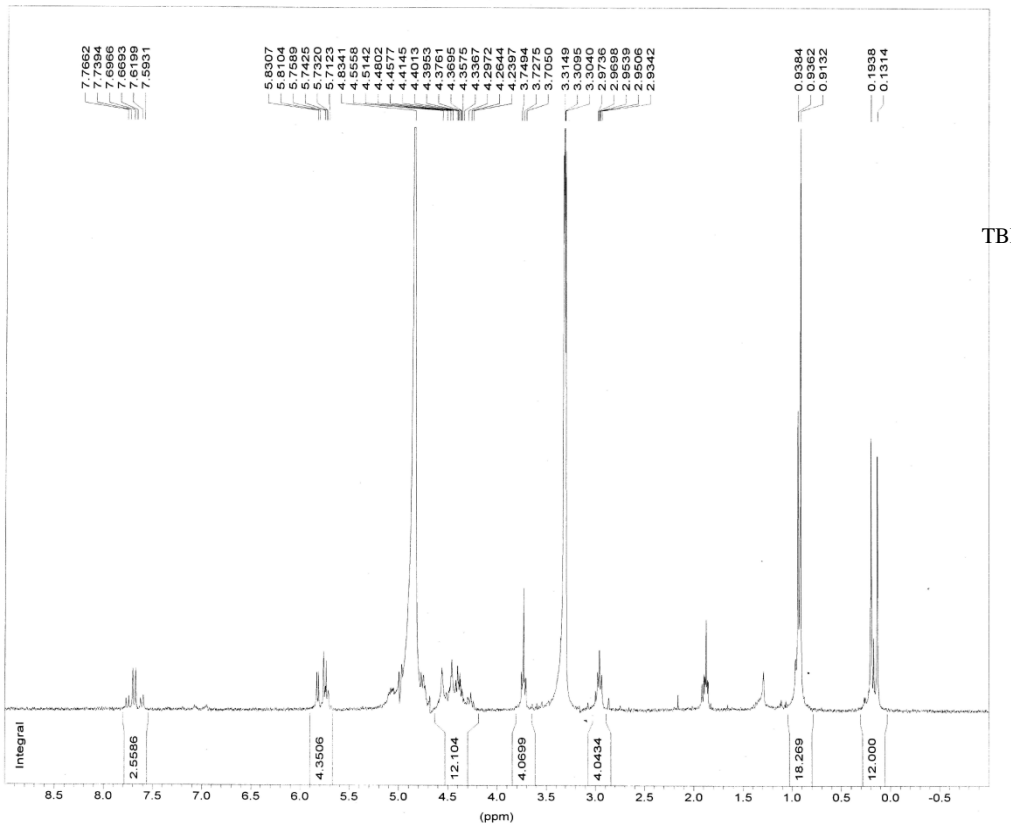


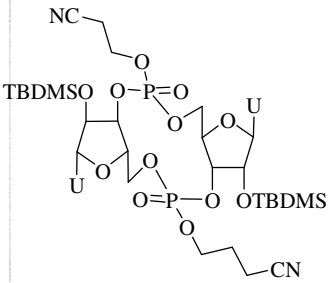
8b

Spectrum 19: ^{1}H NMR spectrum of compound **8b**



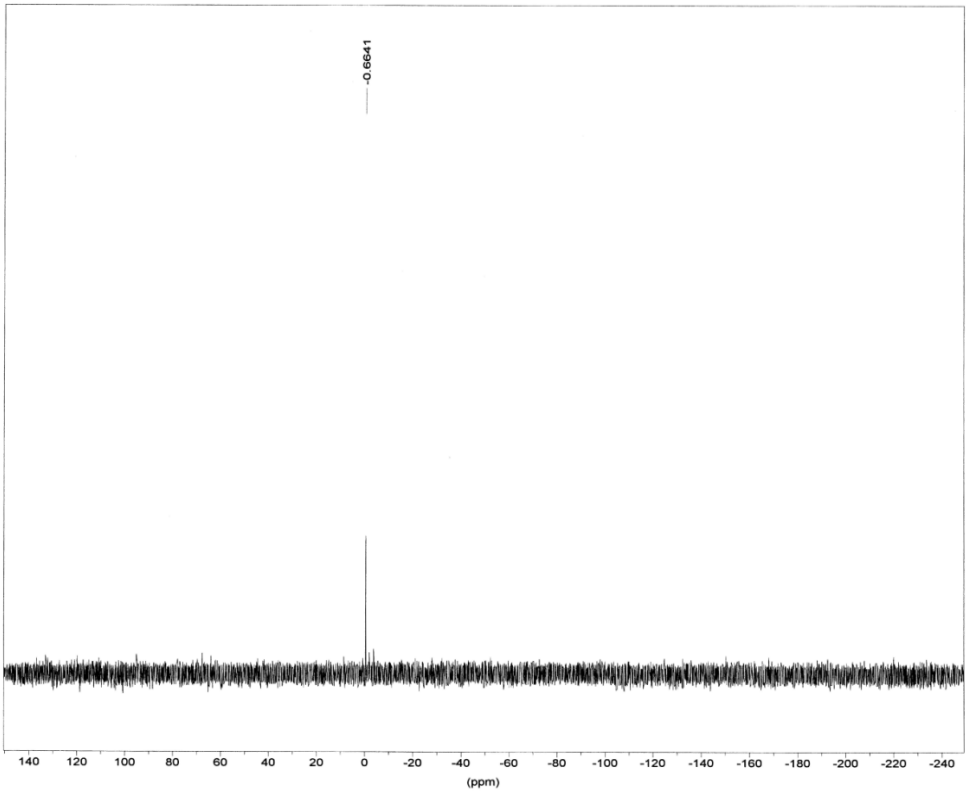
Spectrum 20: ^{31}P NMR specrum of compound **8b**



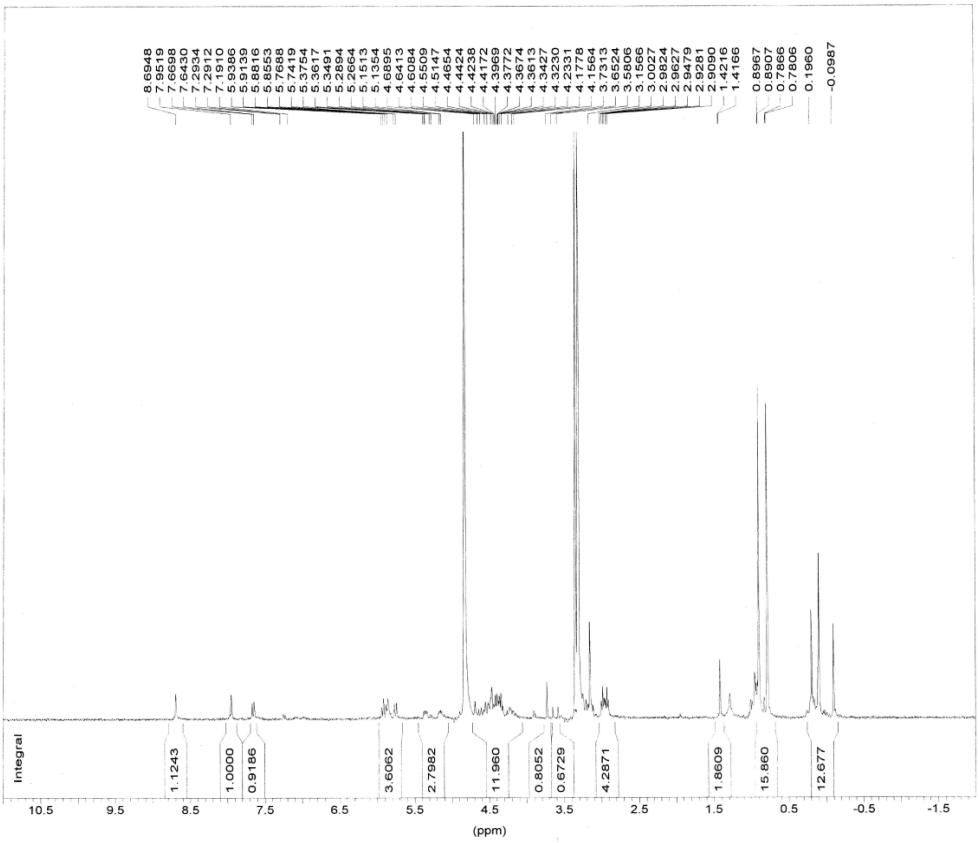


8c

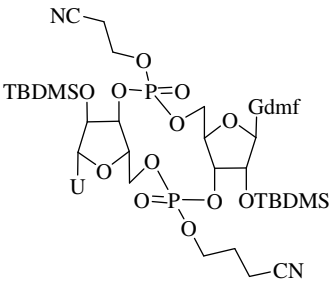
Spectrum 21: ^{1}H NMR spectrum of compound **8c**



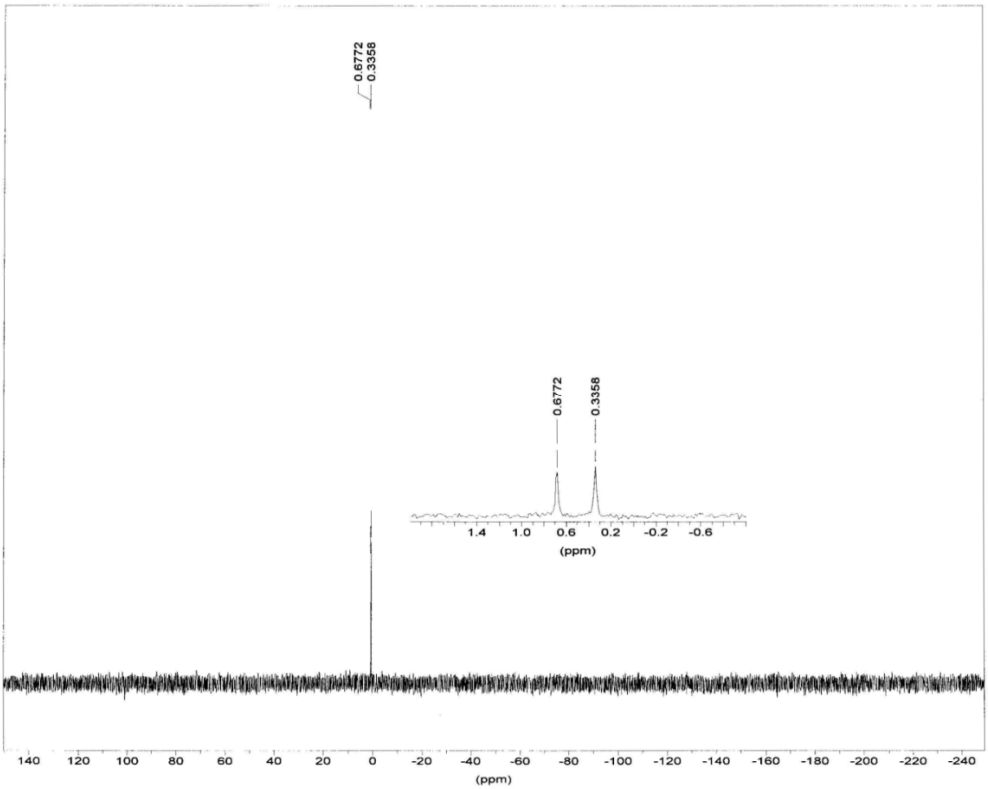
Spectrum 22: ^{31}P NMR spectrum of compound **8c**



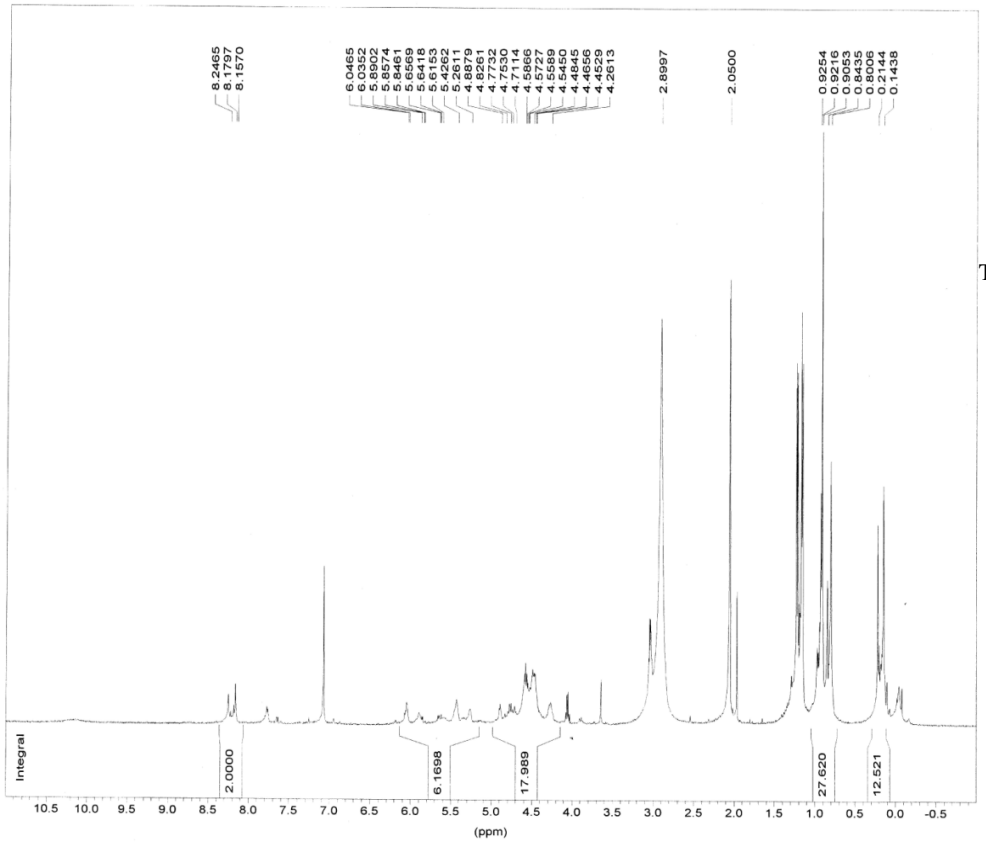
Spectrum 23: ^{1}H NMR spectrum of compound **8d**



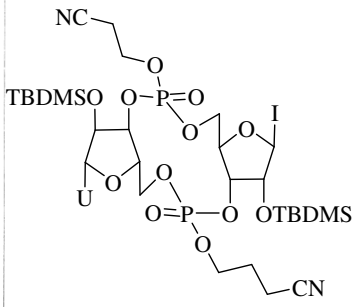
8d



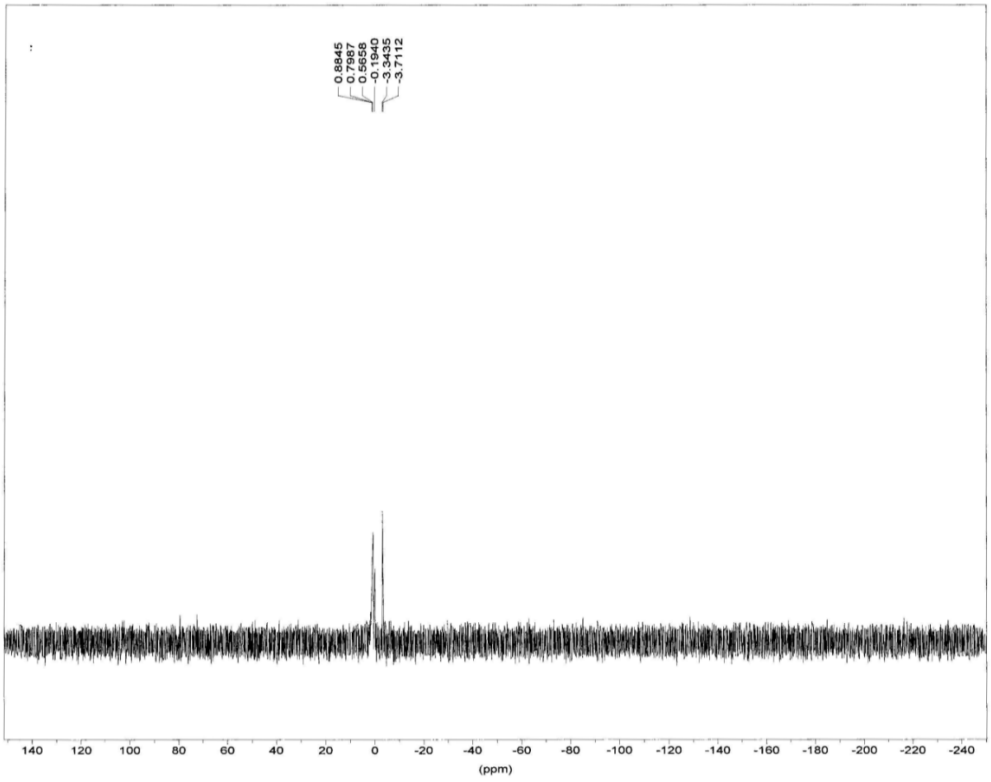
Spectrum 24: ^{31}P NMR spectrum of compound **8d**



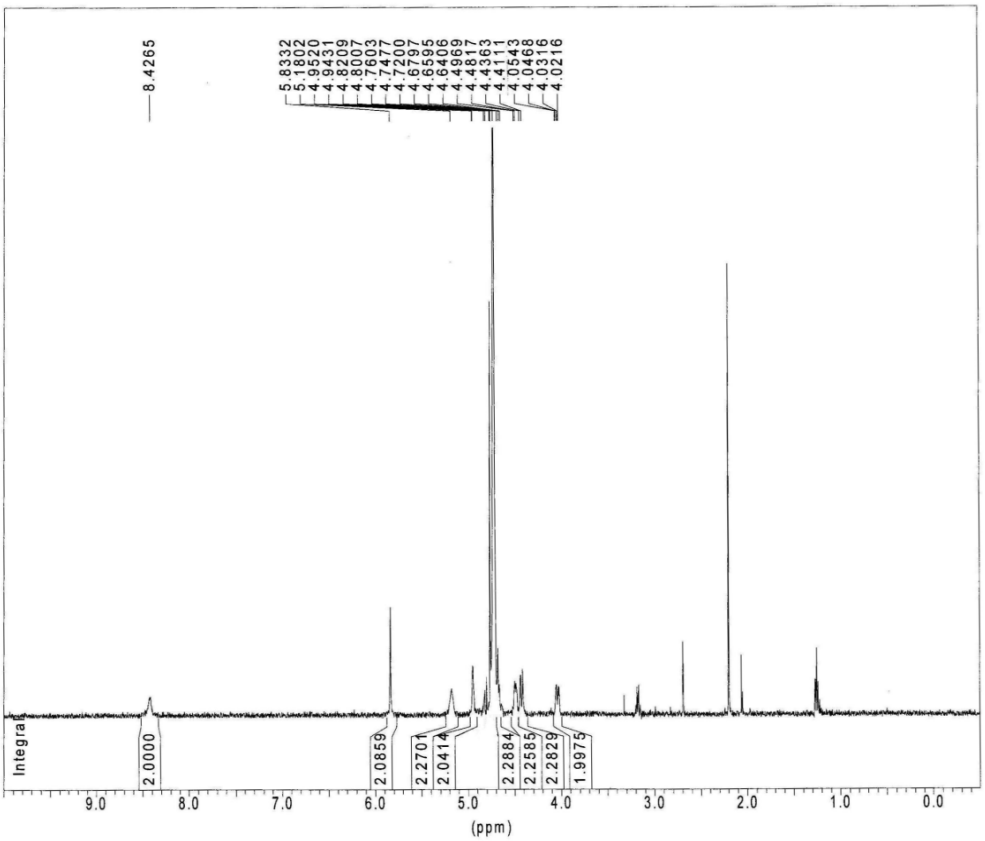
Spectrum 25: ^{1}H NMR spectrum of compound **8e**



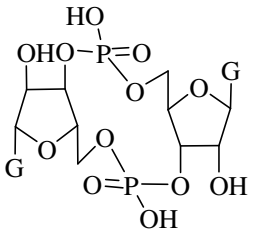
8e



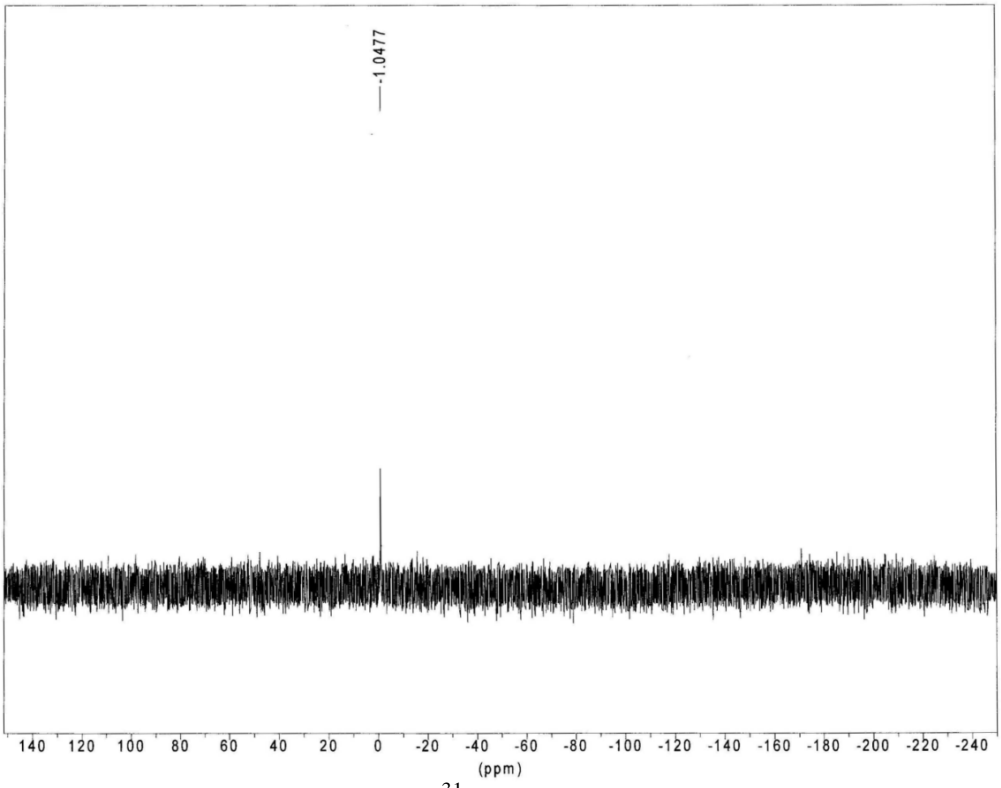
Spectrum 26: ^{31}P NMR spectrum of compound **8e**



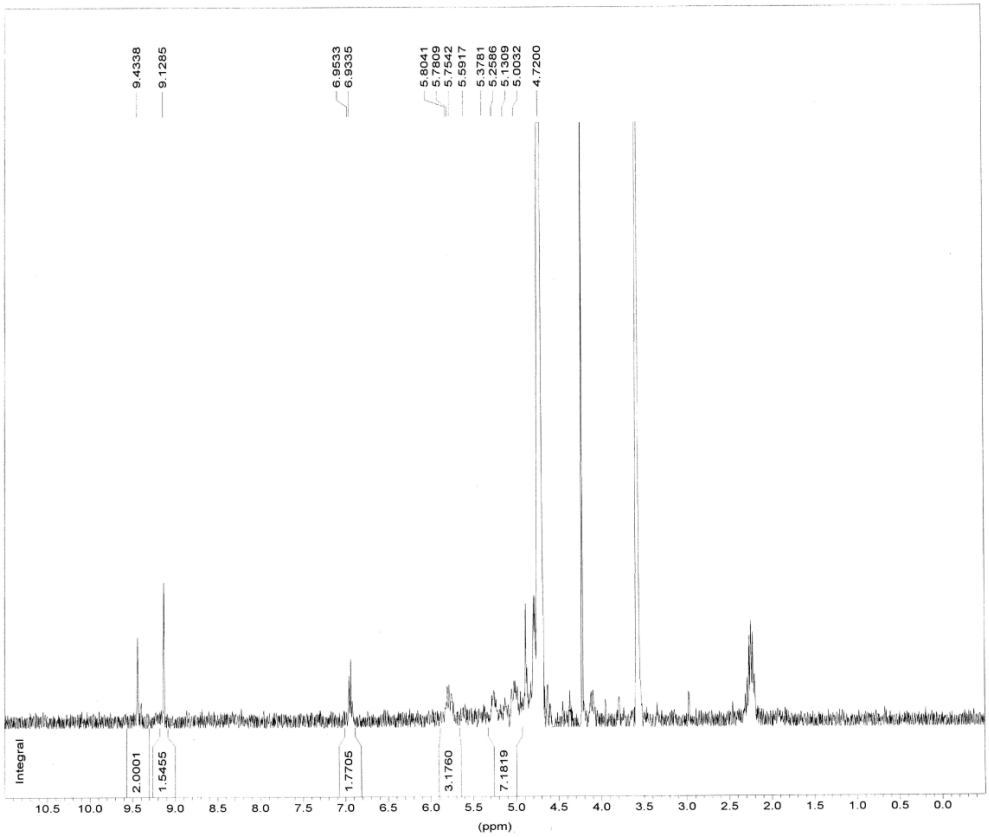
Spectrum 27: ^{1}H NMR spectrum of compound **9a**

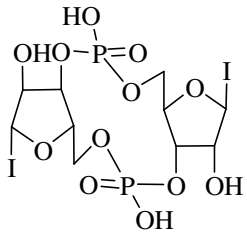


9a



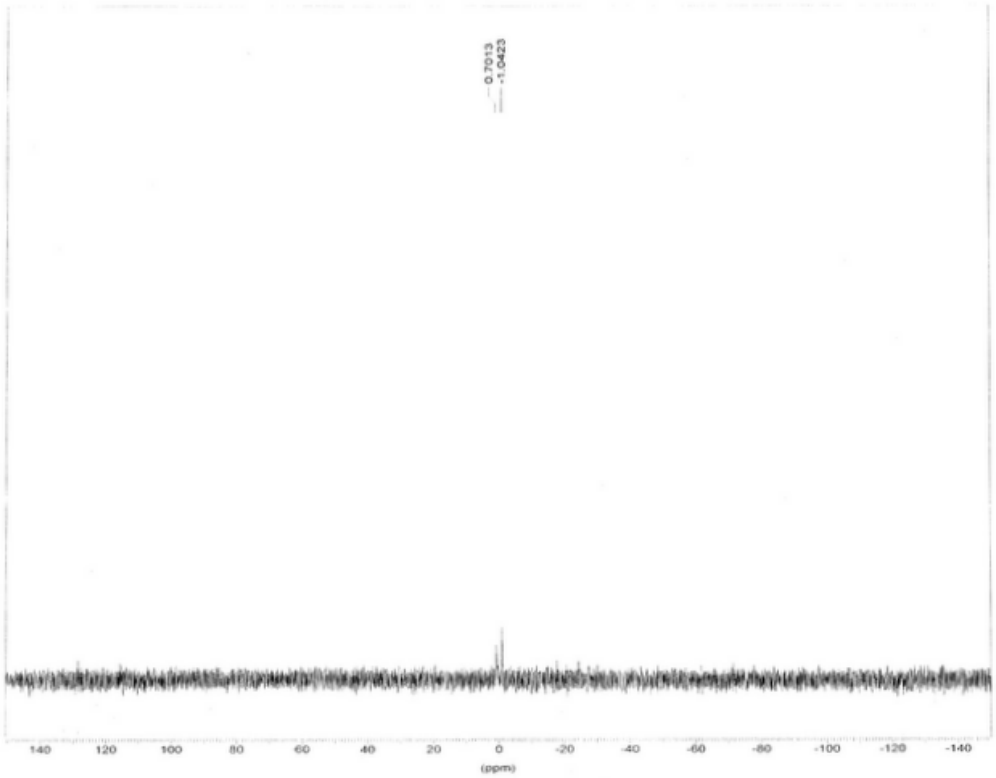
Spectrum 28: ^{31}P NMR spectrum of compound **9a**



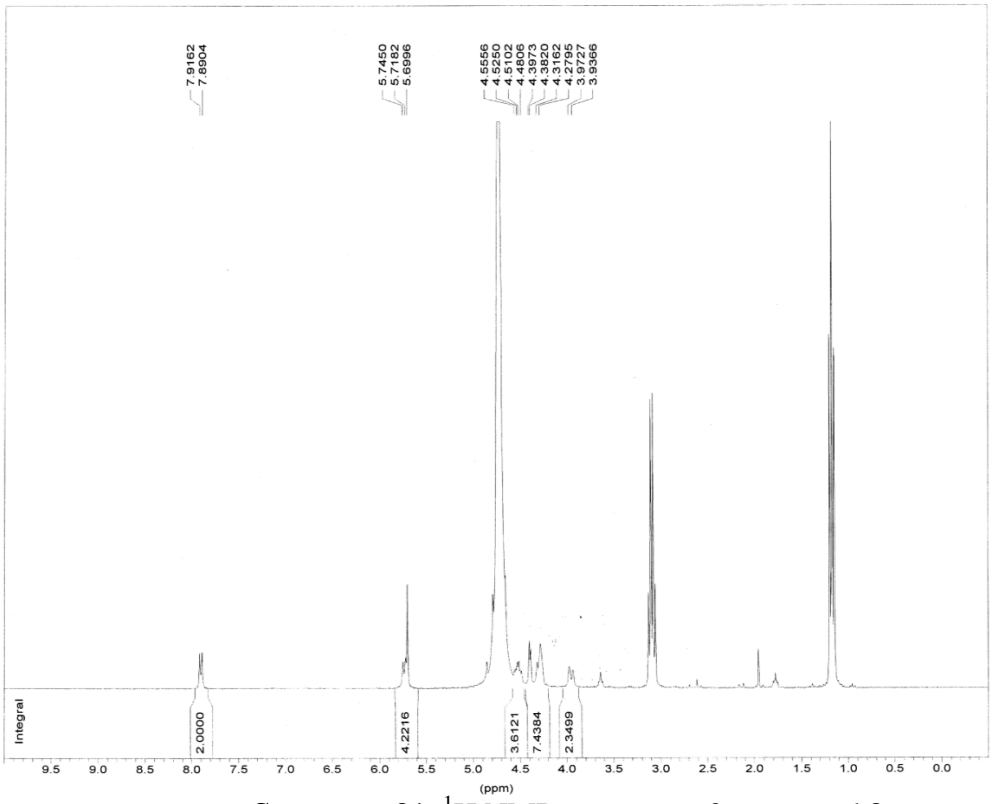


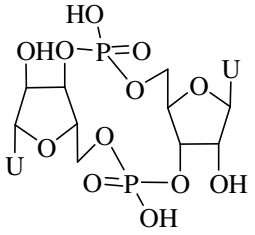
9b

Spectrum 29: ^{1}H NMR spectrum of compound **9b**



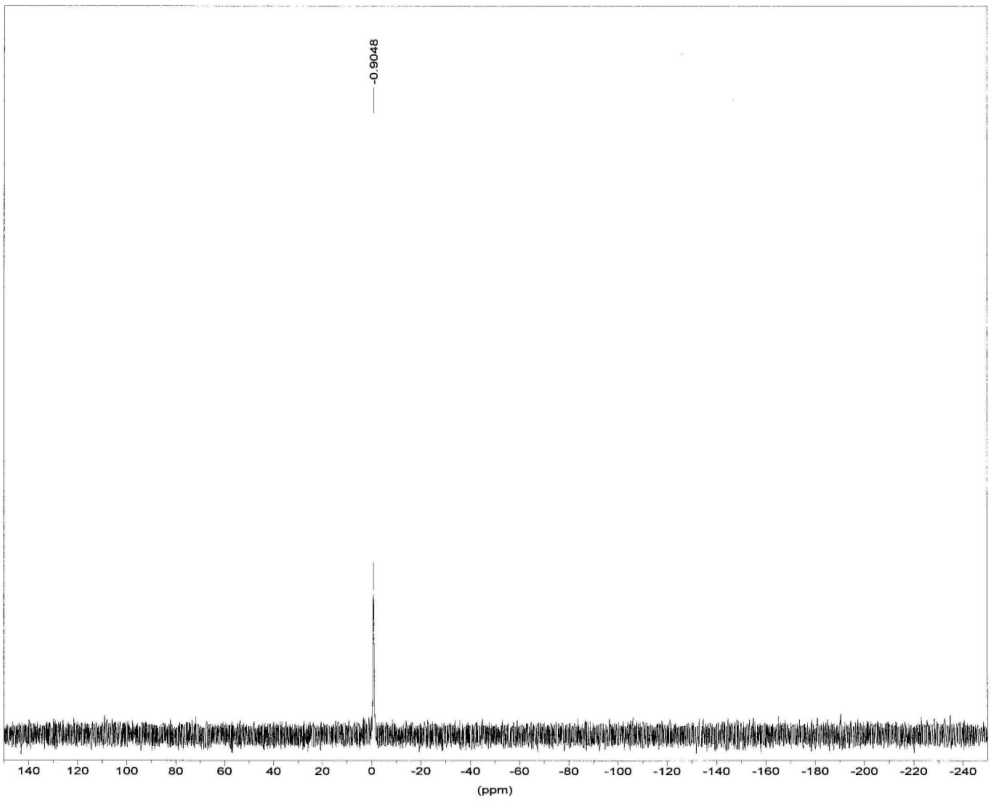
Spectrum 30: ^{32P} NMR spectrum of compound **9b**



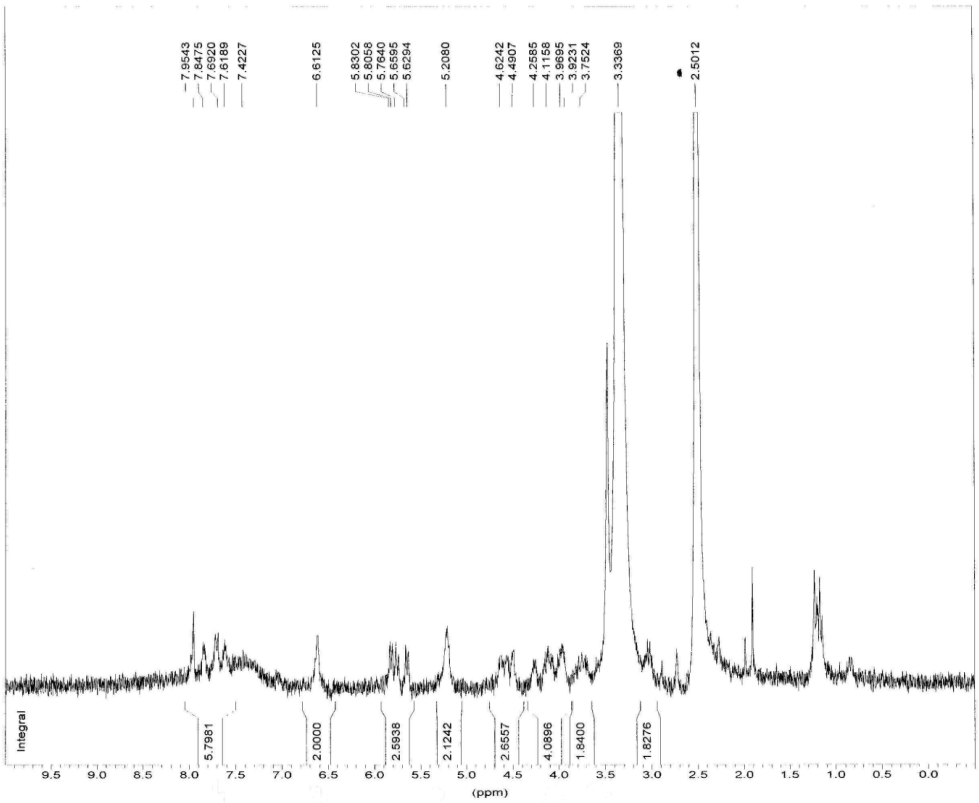


9c

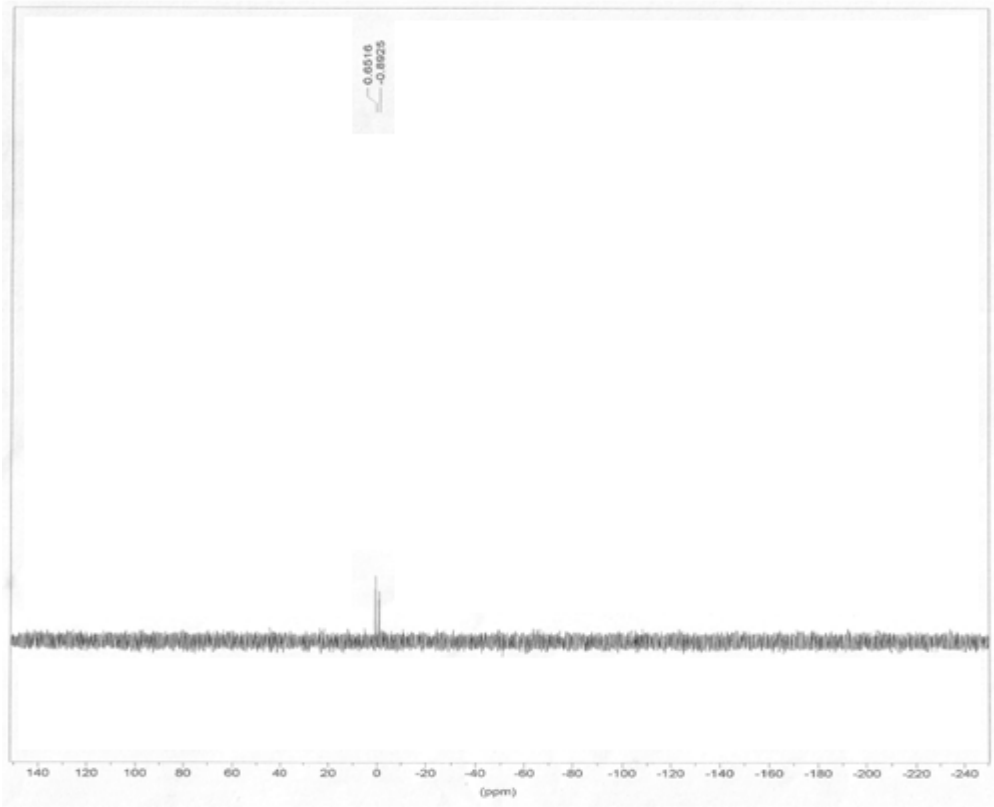
Spectrum 31: ^{1}H NMR spectrum of compound **9c**



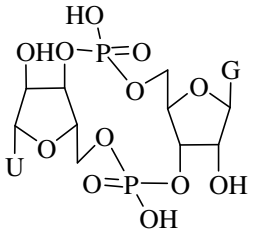
Spectrum 32: ^{32P} NMR spectrum of compound **9c**



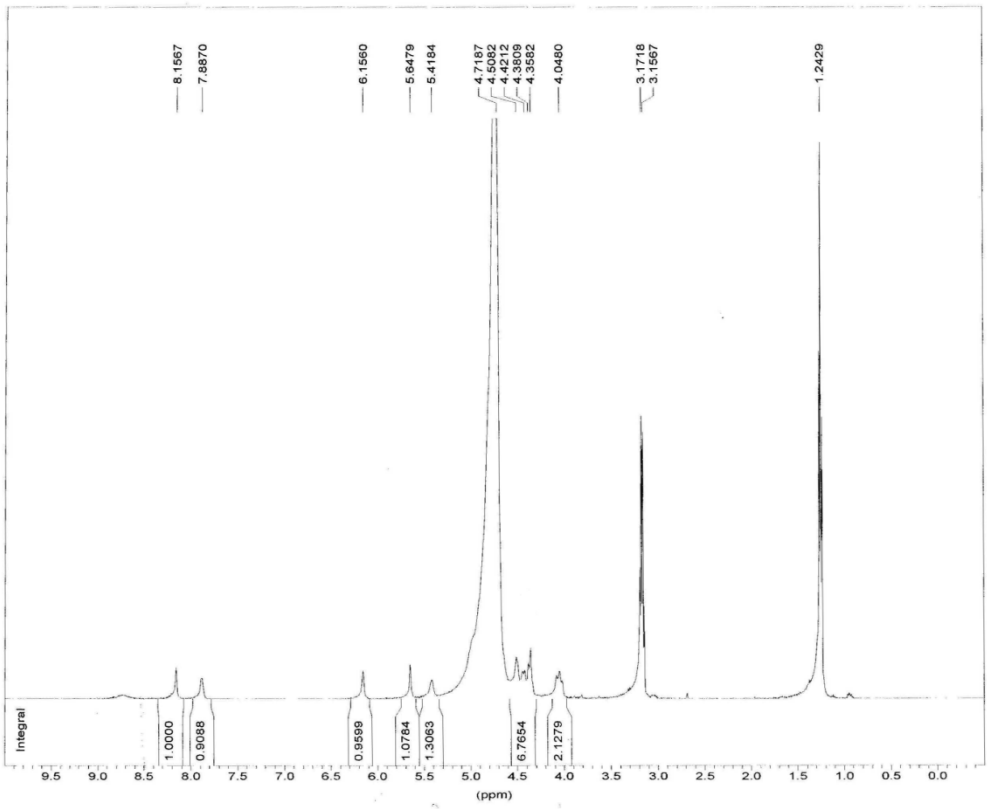
Spectrum 33: ^{1}H NMR spectrum of compound **9d**



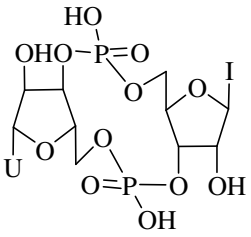
Spectrum 34: ^{32}P NMR spectrum of compound **9d**



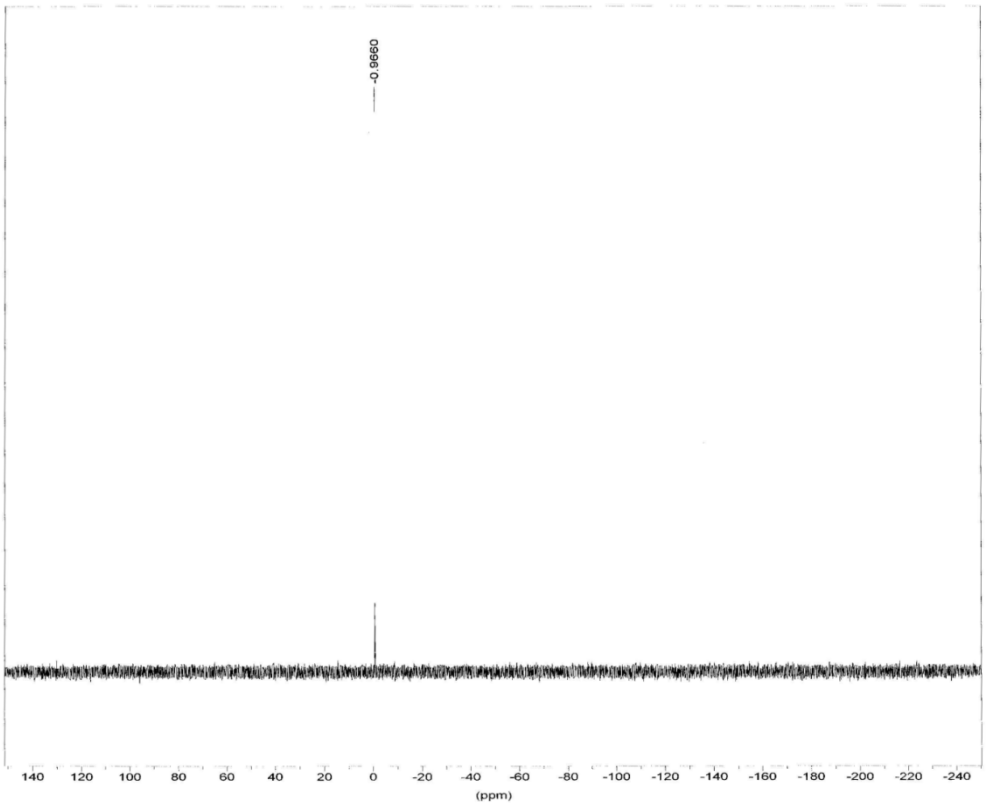
9d



Spectrum 35: ^{1}H NMR spectrum of compound **9e**



9e



Spectrum 36: ^{32}P NMR spectrum of compound **9e**