SYNTHESIS AND BIOCHEMICAL EVALUATION OF NUCLEOSIDE TRIPHOSPHATES AND NUCLEIC ACID ANALOGUES

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I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

List of publications arising from the thesis

Journal

1. Hybrid DNA i-motif: Aminoethylprolyl-PNA (pC5) enhance the stability of DNA (dC5) i-motif structure. **Gade, C. R** and Sharma N.K. * *Bioorg. Med. Chem Lett*. **2017,** *27* 5424–5428.
2. Dideoxy Nucleoside Triphosphate (ddNTP) Analogues: Synthesis and Polymerase Substrate Activities of Pyrrolidinyl Nucleoside Triphosphates (prNTPs). **Gade, C. R,** Dixit, M.; Sharma, N.K* *Bioorg. Med. Chem.* **2016***, 24,* 4016-4022
3. Methylene Salicylicacidyl Hexamer (MSH) Has DNAse Activity. Tiwari A, **Gade C.R**, Dixit M, Sharma N.K* *Appl Biochem Biotechnol.* **2015** , *176*, 1791-800
4. Synthesis and spectroscopic studies of berberine immobilized modified cellulose material. **Gade C.R**, Sharma. N.K* *RSC Adv.* **2014** , *4*, 39337-39342

Chapters in books and lectures notes: NA

Conferences

1.Synthesis and biochemical evaluation of pyrrolidine Nucleoside Triphosphate (*pr*NTP) Analogues as new terminators for DNA sequencing **Chandrasekhar Reddy Gade**, Manjusha Dixit, and Nagendra K. Sharma in 11th Natinal Organic Symposium Trust Conference for Research Scholars at National Institute of Science Education and Research Bhubaneswar .December 14th-17th 2015. (Oral presentation)

2. "Synthesis and biochemical evaluation of pyrrolidine Nucleoside Triphosphate (*pr*NTP) Analogues as new terminators for DNA sequencing" **Chandrasekhar Reddy Gade**,

Manjusha Dixit, and Nagendra K. Sharma. International Symposium on Bioorganic Chemistry (ISBOC-10) 11th to 15th January 2015 Indian Institutes of Science Education and Research – Pune (Poster presentation)

3. "Synthesis of Prolyl Nucleoside Triphosphate (*pr*NTP) Analogues" **Chandrasekhar Reddy Gade** and Nagendra K. Sharma in Indo-German Conference on Bio-inspired Chemistry September 10-12, 2014 at Indian Institute of Science ,Bangalore (Poster presentation)

4. "Synthesis and Charecterization of Immobilized Berberine on Cellulose" **Chandrasekhar Reddy Gade,** Saikat Nandi and Nagendra K. Sharma Indo-French Symposium on Functional Metal-Organics: Applications in Materials and catalysis, held on (24-26) th February 2014, NISER Bhubaneswar, India (**Poster presentation**)

Others (Manuscripts)

1. Synthesis and polymerase activity studies of alanyl-Thymidine triphosphate. **Gade. C.R** and Sharma N.K* (Manuscript under preparation)
2. Design, synthesis, photophysical studies and polymerase activity studies of troplone conjugated 2'deoxy uridine Triphosphates. **Gade. C.R** and Sharma N.K* (Manuscript under preparation)

To

My family

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SYNOPSIS

The thesis has been organized into six chapters.

Chapter 1: Nucleosidetriphosphates (NTPs) General Introduction

Nucleic acids (DNA/RNA) are genetic material and essential to life. Nucleic acids are synthesized by DNA/RNA polymerase from respective Nucleoside triphosphates (NTP).Apart from being the substrates of polymerase NTP are the source of energy, involved in signal transduction and they are part of many enzymes and cofactors.[1] Structurally NTP contains three components a sugar ring, a nucleobase and a triphosphate group (Figure 1). If the sugar is ribose then they are called rNTP or NTP, if the sugar unit is 2'deoxy ribose then they are called dNTP and if the sugar is 2,3 dideoxy ribose then they are called ddNTP. NTP and dNTP are involved in many biological functions besides they are the substrates of respective polymerases.[2] ddNTP are the synthetic analogues which lack 2' and 3' hydroxyl group which terminates the DNA polymerization catalyzed by DNA polymerase. Sanger developed ddNTP analogues and used in DNA sequencing.[3] Sanger used radio labelled ddNTPs or primers for DNA sequencing. To advance the sequencing technologies and to improve detection methods fluorescent labelled ddNTP have been developed for DNA sequencing where nucleobase was modified at C-5 position of pyrimidine and C-7 position of 7-deazapurine to be used as fluorescently labelled ddNTP analogues for DNA sequencing. 3'OH protected dNTP analogues have been synthesized and studied as ddNTP analogues. A chemo labile/photolabile bulky steric group attached at nucleobase C-5/C-7 position can terminate the polymerase reaction and can act as 3'OH unblocked ddNTP analogues. After removing the steric group by irradiation of light or by treatment with chemical reagent again can act as dNTP analogues to

resume the DNA synthesis. These are called reversible sequence terminators.[4] We have designed ddNTP analogues derived from proline and hydroxyl proline.

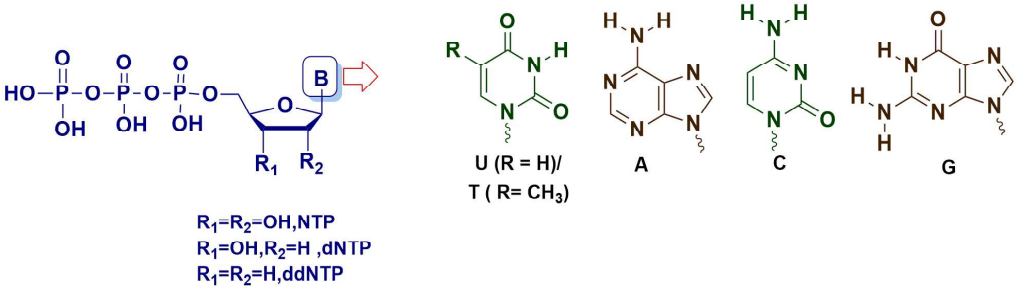


Figure 1. Structure of NTP/dNTP/ddNTP

Fluorescent nucleosides/nucleoside triphosphates are in demand in the fields of chemical biology and medicinal chemistry to monitor and visualize the biochemical processes. A variety of nucleotide analogues with well-defined photophysical properties have been developed to advance the understanding of nucleic acids.[5] We have designed, synthesized and studied the photophysical and biochemical properties of tropolone conjugated 2'-deoxyuridine triphosphate analogues.

Nucleic acid sequences containing cytosine -rich units form a tetraplex structures called cytosine i-motif. Cytosine rich nucleic acids forms a parallel hemi protonated duplexes under acidic conditions .Two such duplexes intercalate in an antiparallel manner to form an intercalated tetraplex structure called i-motif (Figure-2).[6] The i-motif structure has many biological functions as specific gene regulation.[7] Many DNA analogues have been synthesized to study the effect of modification on structure and stability of i-motif. Aminoethylglycine (*aeg*) peptide nucleic acids (PNA) are the analogues of DNA containing neutral amide back bone form stable hybrid i-motif with DNA and RNA by minimizing the negative charge repulsion in the i-motif structure.[8,9] Aminoethyl prolyl (*aep*) backbone containing PNAs form positive charge in acidic medium due to the tertiary amine of backbone.[10] Aep PNA can form more stable

hybrid i-motif with DNA and RNA. We rationally designed *aep*-C_5 pentamer to form a stable hybrid i-motif with DNA and RNA.Guanine rich nucleic acid sequences form G-quadrplexes. Four guanine units form a square planar structure by Hoogsteen hydrogen bonding called guanine tetrad. This tetrad structures stack on each other form a stable supra molecular structure called G-quadraplex (Figure-2) G-quadraplex play important role in the telomere maintenance.

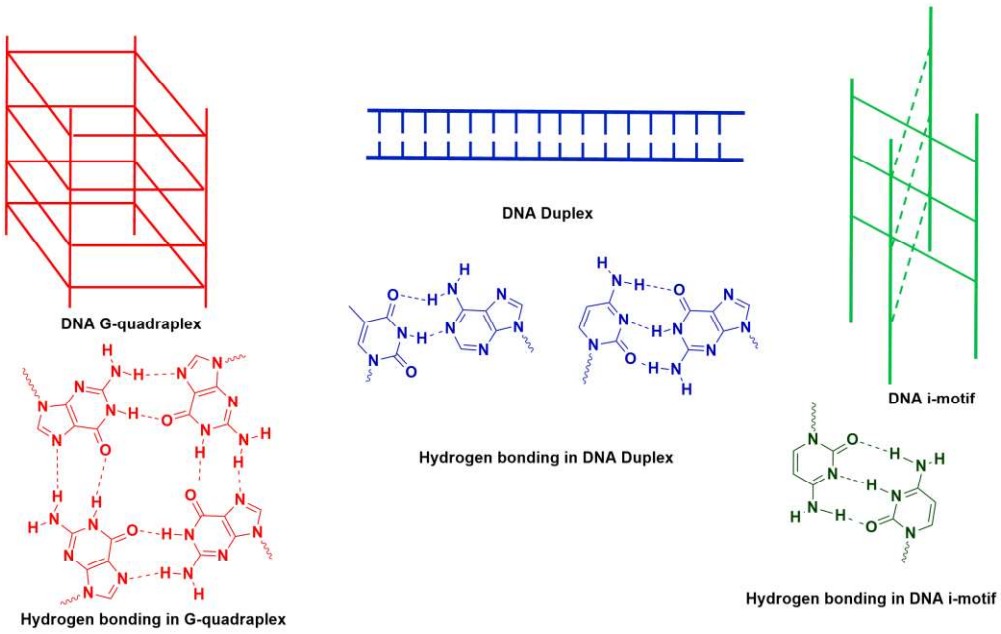


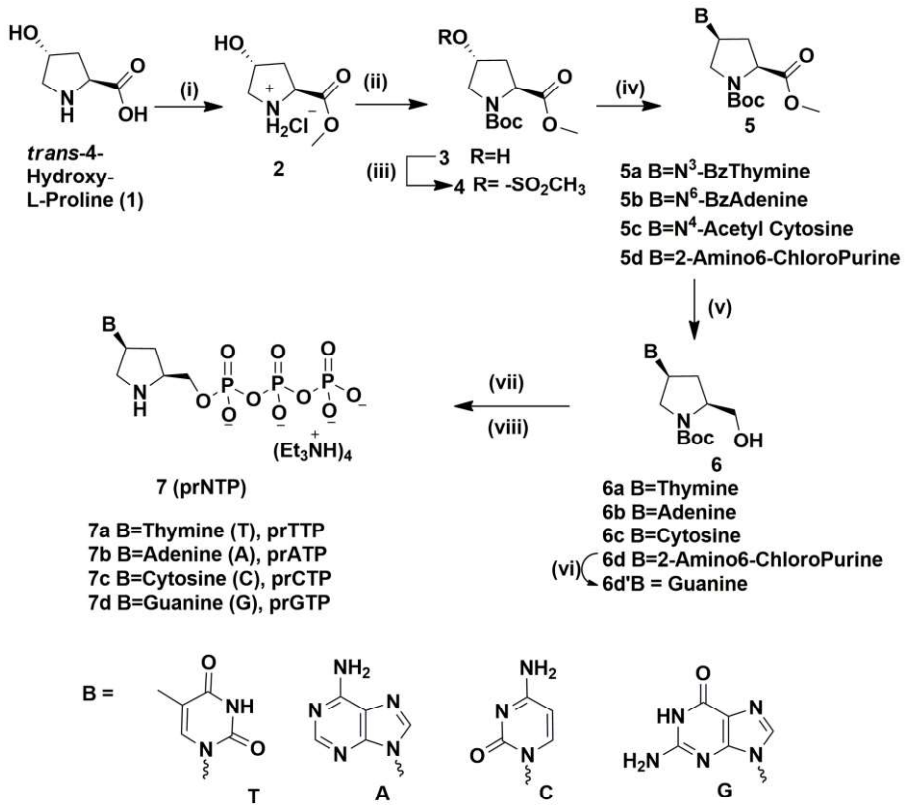
Figure 2. Structure and Hydrogen bonding of DNA duplex, G-quadraplex and i-motif and transcription regulation. G-quadraplex structures are stabilized by cation and some synthetic small molecules called ligands. There are many examples known in literature .Berberine is a natural product which selectively binds with G-quadraplex strongly. We have immobilized Berberine on cellulose surface. This material could be applied to study the G-quadraplex structure formation on solid support.

Chapter -2: Synthesis and Biochemical Evaluation of Pyrrolidine Nucleoside triphosphates (PrNTPs)

Dideoxynucleoside triphosphates (dd NTPs) are the DNA sequencing terminators for

DNA sequencing. Sanger developed the ddNTP analogues and applied in DNA sequencing. Later a variety of ddNTP analogues have been developed to improve the DNA sequencing technologies. We have designed ddNTP analogues derived from proline and hydroxyl proline as sequencing terminators. We have synthesized all the four pyrrolidine nucleoside triphosphates (Pr NTPs) starting form trans-4-hydroxy proline. (Scheme-1) The acid and amine groups were protected as ester and boc derivatives. Protected hydroxyl proline ester (**3**) was treated with N^3-benzoyl thymine under Mitsunobu conditions to obtained compound **5a**. The ester and benzamide groups of compound **5a** were reduced to alcohol and amine groups. The alcohol **6a** was employed in the triphosphate synthesis following Eckstein-Ludwig method[6]. Boc deprotection performed under acidic conditions followed by HPLC purification yielded pure Pr-TTP (**7a**). Compound **3** was treated with mesylchloride to obtain mesyl derivative **4** which was treated with N^6-benzoyl adeine, N^4-Acetyl cytosine and 2-amino-6 chloro purine to obtain compounds **5b, 5c** and **5d** which were reduced to alcohols **6b,6c** and **6d.** Alcohol **6d** was converted to **6d'** by treating with lithium hydroxide.Alcohols **6b, 6c** and **6d'** were converted into their triphophsophates(**7b, 7c and 7d**). All the triphosphates were tested with Therminator DNA polymerase and LC-MS analysis. We also tested the incorporation of PrNTPs along with control ddNTP analogues by gel electrophoresis.[11] (Figure 2). The PrNTPs are novel triphosphates with amine functionality which can be used to synthesize labelled triphosphate and bio conjugates.

Scheme 1: Synthesis of Pr-NTP



Reagents and conditions: (i) $SOCl_2$, MeOH, 0^OC-reflux, 98%; (ii) H_2O:CH_3CN (1:1), TEA, $(Boc)_2O$, (iii) Mesylchloride, Pyridine,quantitative yeild; (iv) N^3Bz-Thymine, $(Ph)_3P$, DIAD, THF, 50% (for 5a)/N^6Bz-Adenine, DMF, K_2CO_3,18-Crown-6, 75^OC,12h, 65% (for 5b)/ N^4Ac-Cytosine, DMF, K_2CO_3,18-Crown-6 ,75^OC,12h, 60% (for 5c)/2-Amino-6-chloropurine, DMF,K_2CO_3,18-Crown-6, 75^OC,12h, 50% (for 5d); (v) $LiBH_4$,THF, 0^oC-rt,87% (6a)/75% (6b), 80% (6c)/ 70% (6d); (vi) 1.0N LiOH,Dioxane,H_2O, 50%.(vii) (a) Salicylchlorophosphidite,Dioxane,Pyridine; (b) Tributylammonium pyrophosphate (TBAPP), DMF, Tributyl amine (c) I_2/Py/H_2O; (d) aq. NH_4OH. (viii) (a) 1.0N HCl;(b) aq. NH_4OH.

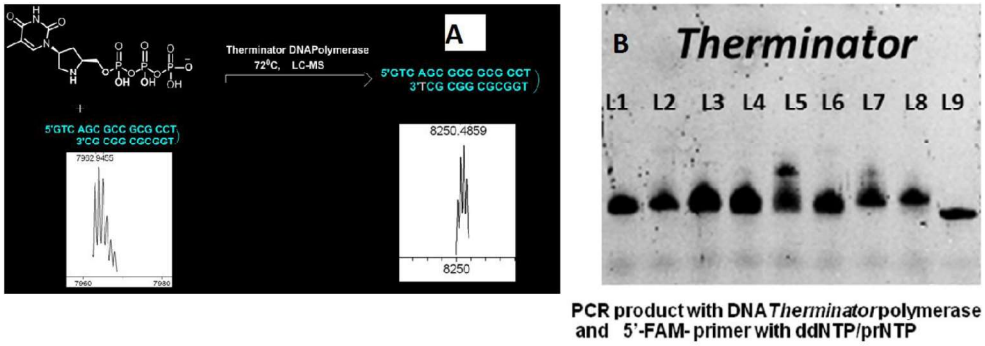


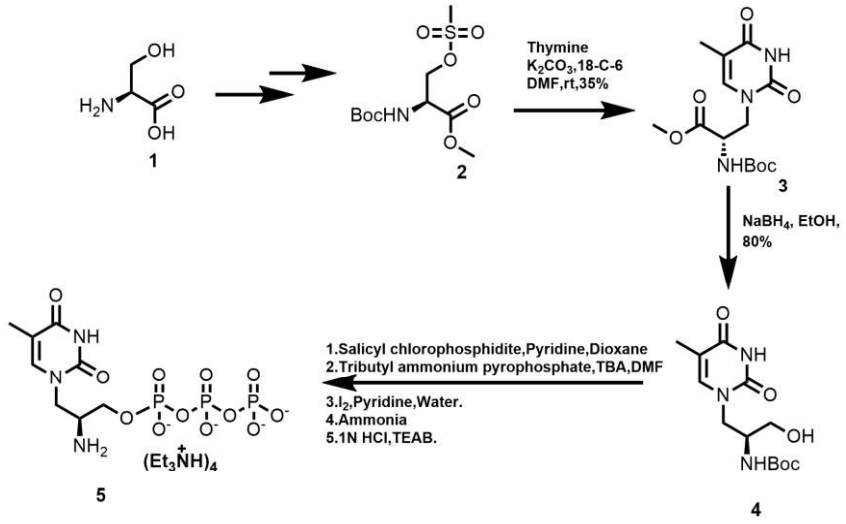
Figure 2. LC-MS analysis of incorporation of Pr-TTP (A) Primer extension reaction product withDNA Therminator polymerase 5'-FAM-P1 (P2) primer with ddNTP/prNTP:(L1)ddTTP ; (L2) prTTP; (L3) dATP; (L4) prATP; (L5) ddGTP; (L6) prGTP; (L7)ddCTP; (L8) prCTP; and (L9) Primer 5'-FAM-P1.

Chpter 3: Synthesis and Biochemical Evaluation of ProlamideThymidine/Uridine and Alanyl Thymidine Triphosphate Analogues

This chapter is divide into two parts

Part A: Part A of the chapter describes the synthesis and biochemical evaluation of Alanyl-thymidine triphosphate (Ala-TTP). Alanyl peptide nucleicacids (Ala-PNA) are the promising nucleic acid analogues which inspired us to design the corresponding Ala-TTP analogue. The synthesis of Ala-TTP started from serine (**1**).

Scheme 2. Synthesis of Alanyl Thymidine triphosphate (Ala-TTP)



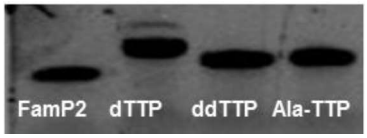


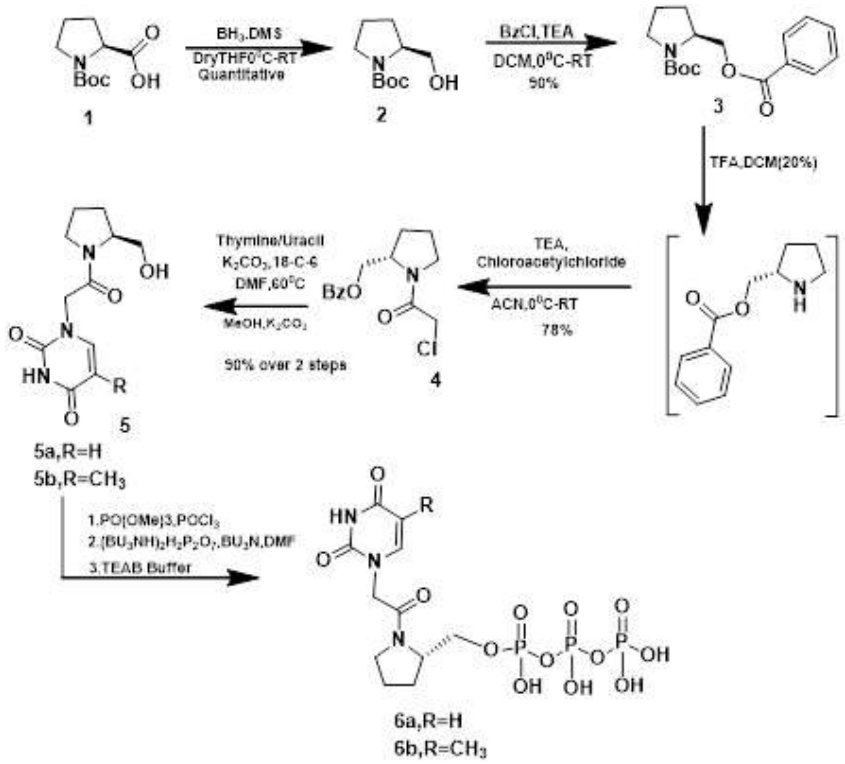
Figure 3. PAGE analysis of Ala-TTPs, control dTTP and ddTTP.

(scheme-2). The acid and amine groups were protected as ester and boc. The protected serine was treated with mesylchloride in pyridine to obtain mesylate (**2**) which was treated with thymine in DMF with K_2CO_3 and catalytic amount of 18-C-6 to obtain thymidine derivative (**3**). The ester group was reduced to alcohol (**4**) by treating with

sodium borohydride in ethanol. The alcohol was phosphorylated to its triphosphate. The purified Ala-TTP (**5**) was treated with DNA polymerase which was incorporated by Theriminator DNA polymerase. (Figure 3) Incorporation of Ala-TTP into DNA will yield 3'amino functionalized DNA. Such amino functionalized DNA can coupled with dye/ fluorophore via amide bond.

Part B: Part B of the chapter describes the synthesis and biochemical evaluation of prolamide thymidine/uridine triphosphates (scheme-4). Prolamide analogues have been synthesized and incorporated in PNA/DNA and studied their binding properties. We are interested to synthesize and study the substrate activity of prolamide NTP. The

Scheme 3: Synthesis of prolamide TTP/UTP



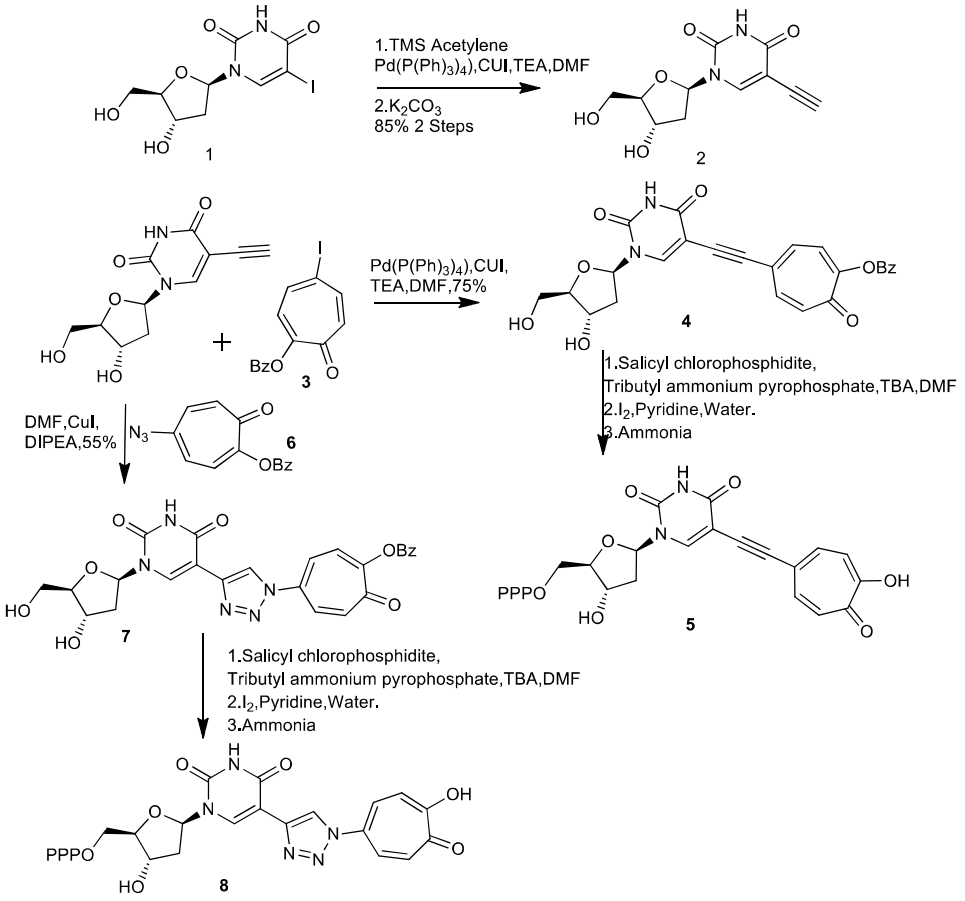
synthesis of prolamide TTP/UTP started from *N*-Boc proline. *N*-Boc proline (**1**) was reduced to Boc prolinol (**2**) by borane DMS. Boc prolinol was protected as benzoate (**3**) by treating with benzoylchloride in Dichloromethane and triethylamine. The boc group was deprotected and was treated with chloroacetyl chloride and triethylamine in acetonitrile to obtain chloro derivative (**4**) .The chloro derivative was treated with thymine/uracil in DMF using K_2CO_3 and catalytic amount of 18-crown-6 to obtain thymidine/uridine derivative which were treated with methanol and K_2CO_3 to yield thymidine/uridine alcohol (**5a&5b**). The alcohols phosphorylated to obtain corresponding triphosphate analogues (**6a&6b**). The prolamide TTP/UTP tested with several DNA polymerases to study their substrate activities. We could not find a suitable DNA polymerase to incorporate prolamide TTP/UTP into DNA by primer extension reactions.The solid state structure of alcohols **5a** and **5b** show supra molecular helical assembly by hydrogen bonding interactions.

Chapter 4: Synthesis and Biochemical Evaluation of Tropolone Conjugated 2'-deoxyuridine Triphosphates

This chapter describes the synthesis and biochemical evaluations of torpolone conjugated 2'-deoxy uridine triphosphates as fluorescent labeling DNA bulding blocks. The synthesis started from 5-iododeoxyuridine (**1**) which was coupled with trimethylsilylacetylene under Sonoghashira conditions using palladium tetrakis triphenyl phosphine ,copper iodide and triethylamine in DMF at room temperature to yield trimethylsilyl (TMS) ehtynyldeoxyuridine.TMS group was deprotected by treatment with potassium carbonate in methanol to yield 5-ethynyl uridine (**2**). 2-

benzoyloxy 5-iodo/5-azido tropolone (**3/6**) were synthesized from tropolone by following literature procedure.[12] 2-benzoyloxy5-iodotropone was coupled to 5-ethynyl 2'-deoxyuridine under Sonoghashira conditions to obtain 5-tropolonyl ethynyl 2'-deoxyuridine (**4**) which was phosphorylated to its triphosphate(**5**) . 5-Azido tropolone (**6**) was treated with 5-ethynyl 2'- deoxy uridine in DMF using copper iodide to yield 5-triazolyl tropolonyl 2'-deoxyuridine (**7**) which was phosphorylated to its triphosphate (**8**) (Scheme-4).

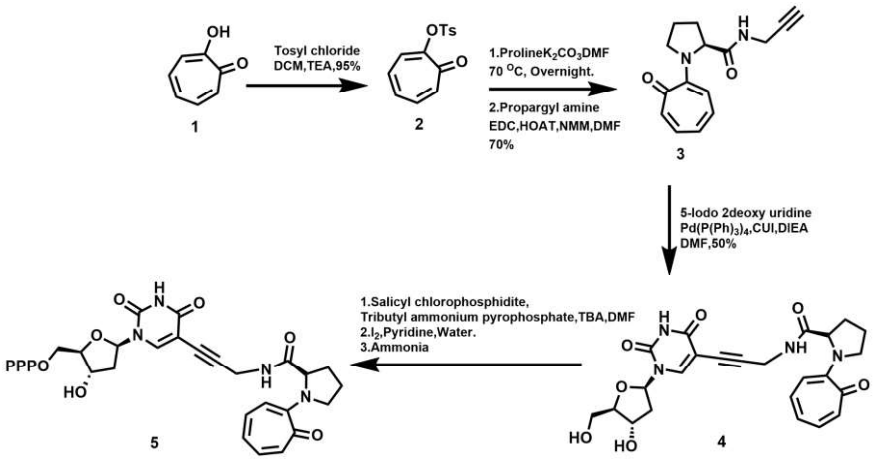
Scheme-4 Synthesis of tropolone conjugated 2'-deoxyuridine triphosphate analogues



The synthesis of another analogue, Tropolonamide conjugated 2'-deoxyuridine triphosphate is depicted in Scheme-5 synthesized.The synthesis started from tropolone (**1**) which was treated with tosylchloride and triethylamine in dichloromethane to

obtain tosyltropolone (**2**) .This tosyl tropolone (**2**) was treated with proline in DMF using K_2CO_3 to obtain tropolonamide followed by coupling with propargylamine using EDC.HCl, HOAt and NMM in DMF to obtained propargylamide proline tropolone (**3**). 5-Iodo 2'-deoxy uridine was coupled with propargylamide proline tropolone to obtain tropolonamide conjugated deoxy uridine (**4**) which was converted into its triphosphate (**5**).

Scheme-5 Synthesis of tropolonamide conjugated 2'-deoxyuridine triphosphate analogues

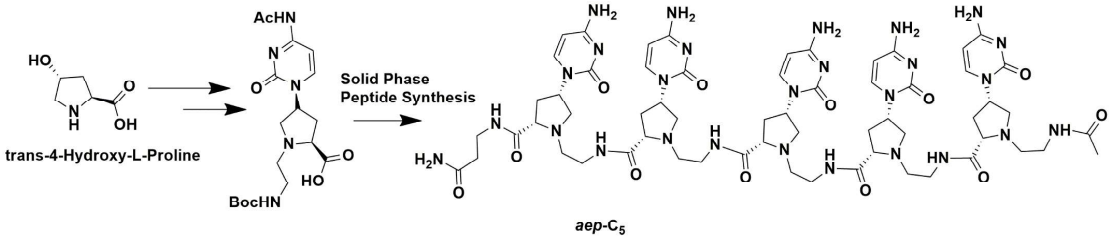


Chapter 5: Synthesis and Biophysical Studies of *aep* PNA –C_5 to Stabilize the Hybrid DNA and RNA i-motif

Cytosine i-motif plays important role in transcriptional regulation .The development of i-motif binding/stabilizing agents are in demand to study the biological processes. This chapter describes the synthesis and biophysical studies of *aep*-C_5 in the stabilization of DNA and RNA hybrid i-motif. The *aep* monomer [13] was employed in the solid phase peptide synthesis to obtaine *aep* cytosine pentamer (*aep*-C_5). (Sheme-6) *Aep*-C_5 was purified by HPLC and characterized by mass spectrometry. The formation and stability of RNA/PNA and DNA/PNA hybrid i-motif was studied by

UV, CD, NMR and mass spectrometry. The UV melting studies revealed that aep PNA forms stable hybrid i-motif with DNA/RNA in comparison to DNA/RNA i-motif. These hybrid structures are new tools for bio nanotechnology.

Scheme-6 Synthesis of aminoethyl prolyl cytosine pentamer (*aep*-C_5)



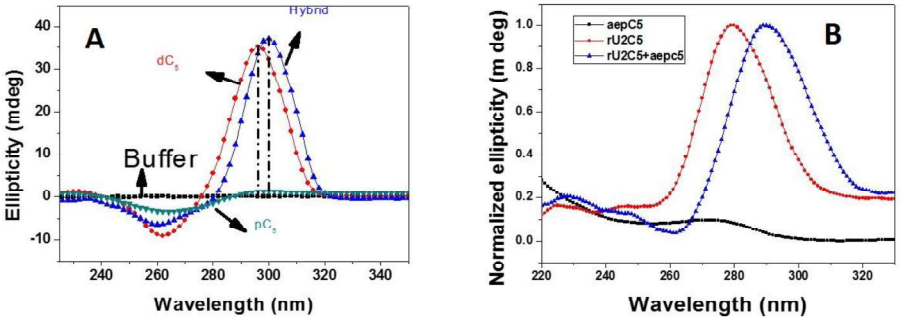
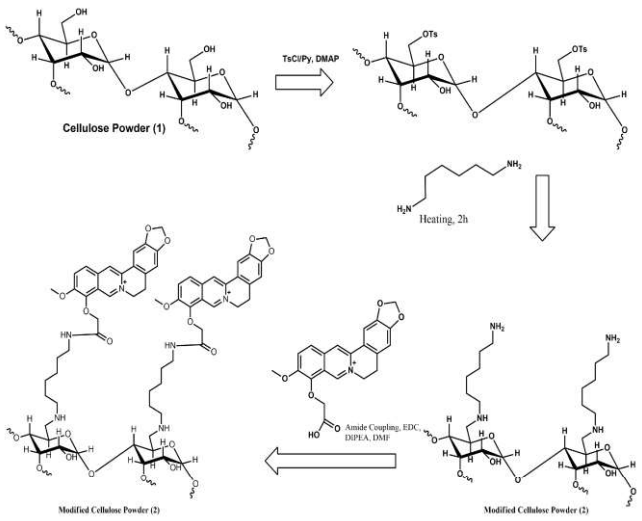


Figure: 4 CD of *aep*-C_5, dC_5 and DNA-PNA, Hybrid i-motif (A) CD of *aep*-C_5, rU_2C_5 and RNA-PNA Hybrid i-moti (B)

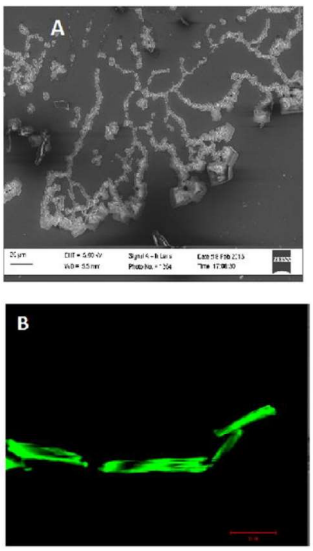
Chapter 6: Synthesis and Spectroscopic Studies of Berberine Immobilized Modified Cellulose Material

Cellulose is the most abundant biopolymer on the earth containing a long chain of anhydroglucose units, which are linked together by a β-1,4-glycosidic bond. Cellulose and related polymers are present in the plant cell wall and woody tissues.[14] Cellulose contains multiple hydroxyl groups on its surface which can be functionalised. Berberine is a natural product and G-quadraplex binding agent with fluorescent

properties. We have functionalised the berberine with acetic acid tag which was used to couple with modified cellulose. Cellulose was functionalised with tosyl group which was treated with 1,6-hexamethyl diamine to obtain amine tethered cellulose which was coupled with berberine acetic acid to obtain berberine immobilized cellulose material.(Scheme-7) The modified cellulose material was charecterized by powder X-ray, IR spectroscopy, FE-SEM (Figure 5A), Confocal microscopy (Figure 5B) and fluorescence microscopy[15]. The modified cellulose material also studied absorption and emission properties. The modified cellulose material could be used to identify G-Quadrplex structure.



Scheme-7 Immobilization of berberine on cellulose surface



Figre 5. FE-SEM Image of berberine cellulose (A) Confocal microscope image of berberine cellulose (B)

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List of Figures

List of Schemes

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List of abbreviations

A	Adenine
AEP	Aminoethyl proplyl
AEP-C_5	Amino ethyl proplyl cytosine pentamer
Ala	Alanyl
Ala-TTP	Alanyl thymine triphosphate
Å	Angstrom
ATP	Adenosine triphosphate
Ac	Acetyl
Bz	Benzyl
Boc	T*ert*-butyloxycarbonyl
Br	Broad
C	Cytosine
CTP	Cytidine triphosphate
°C	Degrees Celsius
Calcd	Calculated
CCDC	Cambridge Crystallographic Data Centre
CD	Circular Dichroism
D	doublet
d-ATP	Deoxyadenosine triphosphate
d-CTP	Deoxycytidine triphosphate
d-GTP	Deoxyguanosine triphosphate
d-TTP	Deoxythymidine triphosphate
dC_5	Deoxycytidine pentamer

d d	doublet of doublet
DNA	Deoxyribose nucleic acid
DMF	*N*,*N*-dimethylformamide
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DIPEA	Diisopropylethylamine
EDC.HCl	N-(3-Dimethylaminopropyl)-ethylcarbodiimidehydrochloride
ESI-MS	Electrospray Ionization mass spectrometry
EtOAc	Ethylacetate
FAD	Flavin adenine dinucleotide
FE-SEM	Field emission scanning electron microscopy
G	Guanine
GTP	Guanosine triphosphate
G	gram(s)
H Cl	Hydrochloric acid
HRMS	High Resolution Mass Spectroscopy
HPLC	High pressure liquid chromatography
IR	Infrared (spectroscopy)
J	Coupling constant
Λ	wavelength
LC-MS	Liquid Chromatography –Mass Spectrometry
M	multiplet or milli
m/z	mass to charge ratio
μ	micro

Me	methyl
mg	milli gram
MHz	megahertz
Mol	mole(s)
Ml	milli litre
Ml	micro litre
μM	micro molar
mM	milli molar
NAD	Nicotinamide adenine dinucleotide
NMR	Nuclear Magnetic Resonance
Nm	nanometers
NTP	Nucleosidetriphosphate
pH	hydrogen ion concentration in aqueous solution
PL	Photoluminiscence
PNA	Peptide nucleic acid
Ppm	parts per million
Pr	Pyrrolidine
Pr-ATP	Pyrrolidine ahymidinetriphosphate
Pr-CTP	Pyrrolidine cytidinetriphosphate
Pr-GTP	Pyrrolidine guanosinetriphosphate
Pr-TTP	Pyrrolidine thymidinetriphosphate
Py	Pyridine
Q	quartet
rt	room temperature

s	singlet
T	Thymine
TTP	Thymidine triphosphate
T	triplet
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	thin layer chromatography
TMS	trimethylsilyl
UPLC	Ultra performance liquid chromatography
UV	Ultra-violet

Chapter 1

Nucleosidetriphosphates (NTPs) General Introduction

Chapter 1

Nucleoside Triphosphates (NTPs) General Introduction

Contents

1.1 Introduction

Nucleotides are the biologically ubiquitous molecules which involve in almost all biochemical processes of living organism.[1] Nucleoside triphosphates (NTP) are the substrate of DNA and RNA polymerase and involved in the synthesis of DNA and RNA by respective polymerases. Apart from being the substrate of polymerase and source of energy, NTP are involved in many biological processes such as, they are involved in signal transduction and they are the part of many coenzymes. Adenosine diphosphate (ADP) is present in nicotinamide adenine dinucleotide (NADH), Nicotinamide adenine dinucleotide phosphate (NADPH) and Flavin adenine dinucleotide (FADH) .[2]

1.1.1 Structure of nucleotides, nucleosides and bases

Nucleotides structure contains 3 major components a phosphate group, a sugar ring and a nucleobase. Chemically nucleotides are the phosphate ester of nucleosides. Nucelosides contains a five membered sugar ring attached covalently to a nitrogenous base. The sugar ring is five membered ribose present in ribonucleotides and 2'-deoxy ribose present in 2'-deoxy ribonucleotides (Figure 1.1).

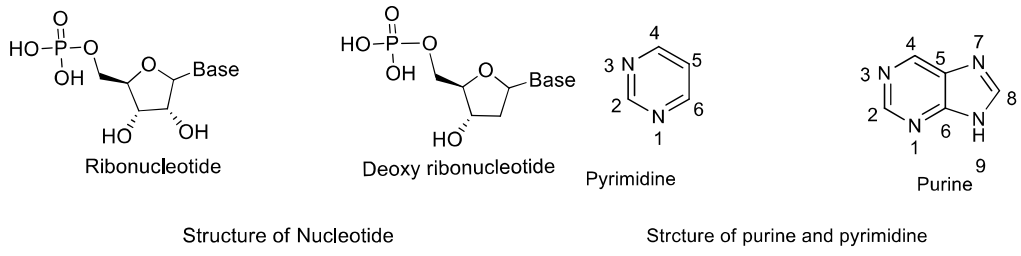


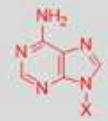
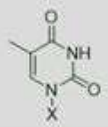
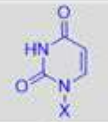
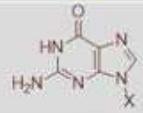
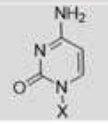
Figure 1. 1. Structure of nucleotides, purine and pyrimidine.

In nucleic acid structure the 5'-O phosphate ester is connected to the 3'-OH of another nucleotide making a phosphodiester bond. The nitrogenous bases are hetero cyclic,

aromatic and planar molecules .The nitrogenous bases are two types one is purine and another is pyrimidine. Pyrimidine is a six membered hetrocyle with nitrogen at 1 and 3 positions. Purine is a bicyclic hetrocycle in which a pyrimidine ring is fused to an imidazole ring. (Figure 1.1).The C1 of sugar attached to the pyrimidine base covalently at N1 position by glycosidic linkage. Similarly purine N9 is attached to C1 of sugar by glycosidic linkage.

The structure of nucleobase, nucleosides and nucleoside triphosphates are described in table 1.1

Table 1. 1. Structure of nucleobases nucelosides and nucleotides

Base Structure	Base X=H	Nucleoside	Nucleotide
	Adenine	X=Ribose,Adenosine X=Deoxyribose, 2,deoxy adenosine	X=Ribose 5'Triphosphate, ATP X=2' deoxyRibose 5'Triphosphate, dATP
	Thymine	X=Ribose,Adenosine X=Deoxyribose, 2,deoxyadenosine	X=Ribose 5'Triphosphate, TP X=2' deoxyribose 5'Triphosphate, dATP
	Uracil	X=Ribose, Adenosine X=Deoxyribose, 2,deoxyadenosine	X=Ribose 5'Triphosphate, ATP X=2' deoxyribose 5'Triphosphate, dATP
	Guanine	X=Ribose,Guanosine X=Deoxyribose, 2,deoxyguanosine	X=Ribose 5'Triphosphate, ATP X=2' deoxyRibose 5'Triphosphate, dATP
	Cytosine	X=Ribose,Guanosine X=Deoxyribose, 2,deoxyguanosine	X=Ribose 5'Triphosphate, ATP X=2' deoxyribose 5'Triphosphate, dATP

1.1.2 Chemical structure of nucleic acids

Chemically nucleic acids are the polymers of nucleotides. The 3'-OH of one nucleotide forms a phosphodiester bond with 5' phosphate of another nucleotide and

the 5’ triphosphate of the same nucleotide forms phosphodiester bond with 3’-OH of another nucleoride .This way repeatedly forming phosphodiester bond at 3’ and 5’ ends make a polymer. Nucleic acids maintain directionality .The 5’end and 3’end of nucleic acids contains free hydroxyl group (5’OH and 3’OH) (Figure1. 2).

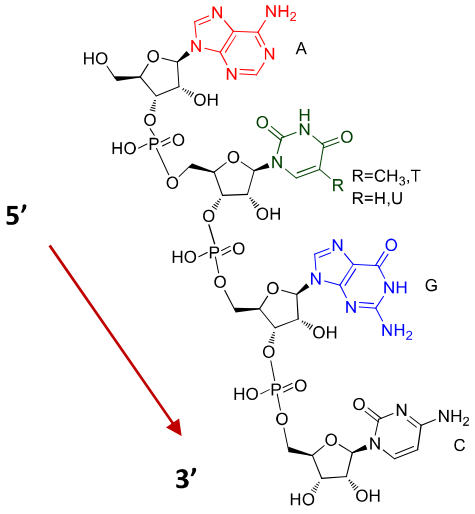


Figure 1. 2. Chemical Structure of nucleic acids.

1.1.3 Structure of DNA/RNA polymerase

The biosynthesis of nucleic acids are accomplished by polymerase .Nucleoside triphosphates are the building block of nucleic acids. Polymerase catalyses the synthesis of nucleic acids by addition of nucleotides in complimentary to the template. The structure of DNA/RNA polymerase and the mechanism of polymerization is described below. The major function of NTP being the substrate of the polymerase. They are the building block of nucleic acids. NTPs addition to the primer catalysed by polymerase in 3’ direction in complementary to the template. The reaction mechanism of this process understood by the crystal structure of polymerase with duplex DNA and NTP. [3-6] The structure of a polymerase can compare with right hand palm it contains three domains a thumb domains, fingers domain and palm domain .Palm

domain is the place where phosphoryl transfer takes place, finger domain plays important role in the interactions of the incoming nucleotide and template where as thumb domain plays important role in the positioning duplex, process and translocation of duplex.The crystal structure of DNA polymerase is depicted in

Figure 1. 3.

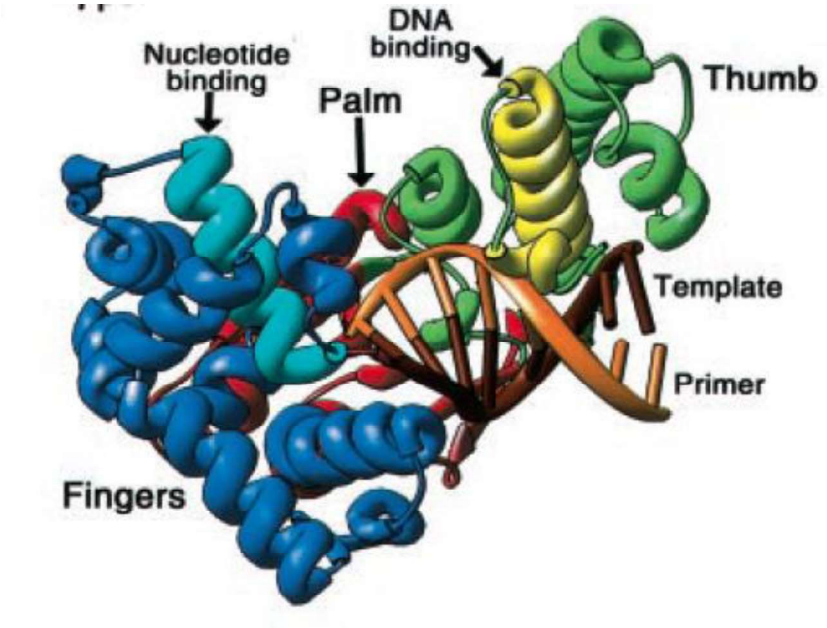
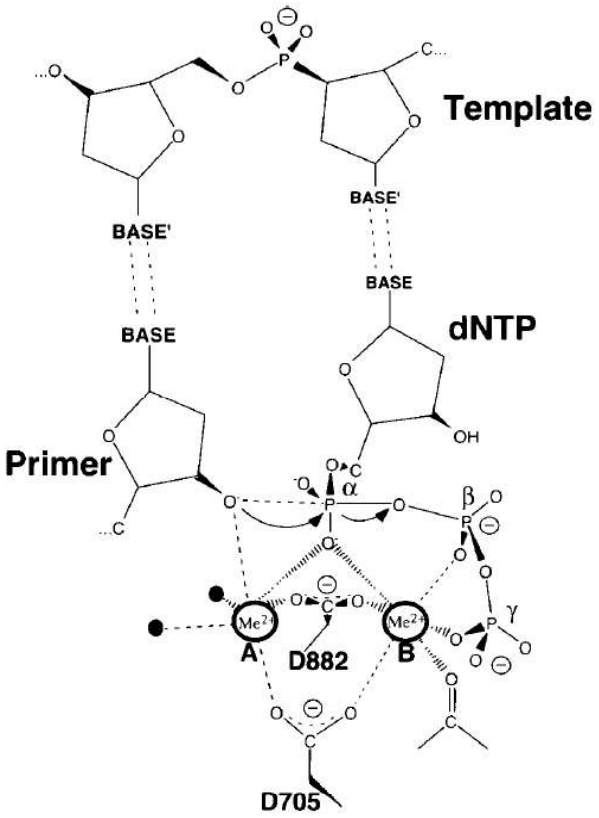


Figure 1. 3. Structure of DNA polymerase

1.1.4 Reaction mechanism of DNA polymerase catalysed phosphodiester bond formation

The mechanism is explained by two metal ions model which is widely accepted. Two Magnesium metal ions are bound by three carboxylates of asparates. Metal ion **A** interacts with 3'OH group of primer and lowers its pK_a value facilitates to attack on incoming NTP .Metal ion **A** also interacts with alpha phosphates of incoming nucleotide stabilizes its negative charge. Metal ion **B** interacts with beta and gamma phosphates of incoming NTP and stabilize the negative charge and leaving

pyrophosphate group .3'-OH of primer attacks on alpha phosphate and forms new phosphodiester bond. The detailed mechanism described in figure 1 .4



Me^{2+} in the figure is Mg^{2+}

Figure 1. 4. Reaction mechanism of polymerase catalysed Phosphodiester bond formation.

1.2 Synthesis of nucleoside triphosphates (NTPs)

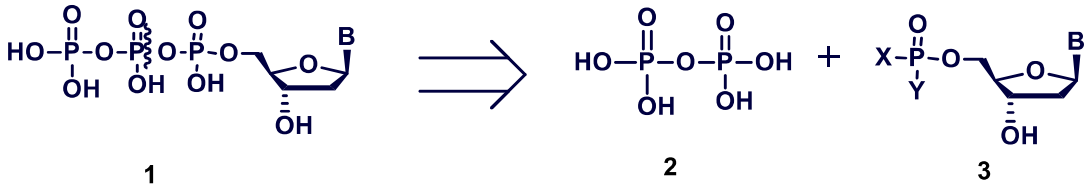
NTPs are naturally occurring biomolecules with several biological and therapeutic functions attracted chemists to synthesize NTP and their analogues for various applications. To evaluate the biological applications and therapeutic action of NTP and their analogues, the synthesis of NTP and their analogues are in demand. NTP are naturally occurring but the synthesis, storage, isolation and characterization of NTP

and their analogues is difficult due to several factors.[7] To address the difficulties in NTP synthesis and to access NTP and their analogues in adequate amounts chemists developed many synthetic methadologies. In the last two decades significant progress has been made for the synthesis of NTP and their analogues.Till to date many synthetic methodologies reported and reviewed [2] some of popular and important synthetic methods of NTP discussed here.

1.2.1 Synthesis via nucleophilic attack of pyrophosphate on an activated nucleoside monophosphate

This strategy is one of the most widely used in the synthesis of NTP (**1**). In this strategy pyrophosphate (**2**) is added to the activated monophosphate (**3**). The disconnection approach shown in scheme 1.1.

Scheme 1. 1. Synthesis of triphosphate by activation of monophosphates disconnection approach

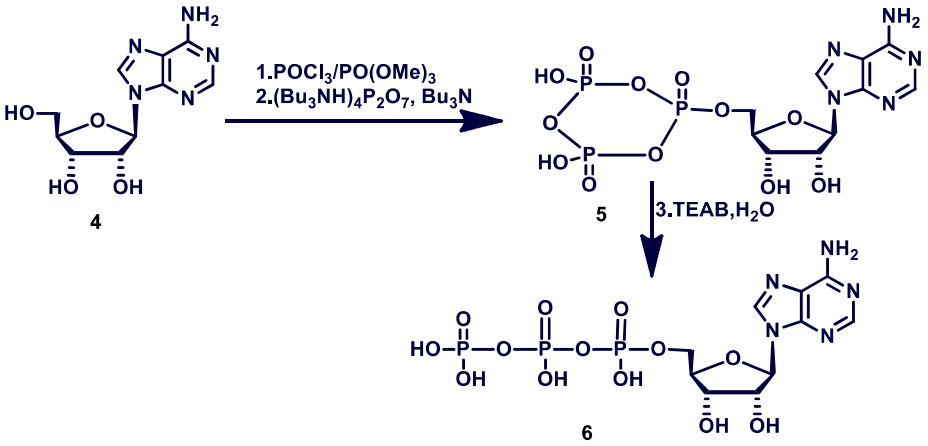


X and Y in the monophosphate structure are the activating groups which makes phosphate as good leaving group. The pyrophosphate salt is nucleophilic in nature attacks on the leaving group and forms triphosphates. In this strategy many activating groups utilised to make monophosphate a good leaving group. A few important methods described here.

Synthesis of triphosphates via dichlorophosphates: Yoshikawa and co-workers have developed a methodology to synthesize selectively 5'-monophosphates by using

phosphorylchloride and trialkyl phosphates as solvent at low temperature followed by hydrolysis.[8,9] Ludwig and Eckstein extended this method to synthesize triphosphates by one pot three steps.[10] In this method nucleoside (**4**) is treated with phosphory chloride in trialkyl phosphate. The resulting dichloro phosphate is treated with pyrophosphate to form cyclic metatriphosphate (**5**). This cyclic triphosphate is hydrolysed under basic conditions to triphosphate (**6**) by triethyl ammonium bicarbonate (TEAB) or aqueous ammonia. The synthesis is illustrated in scheme 1.2.

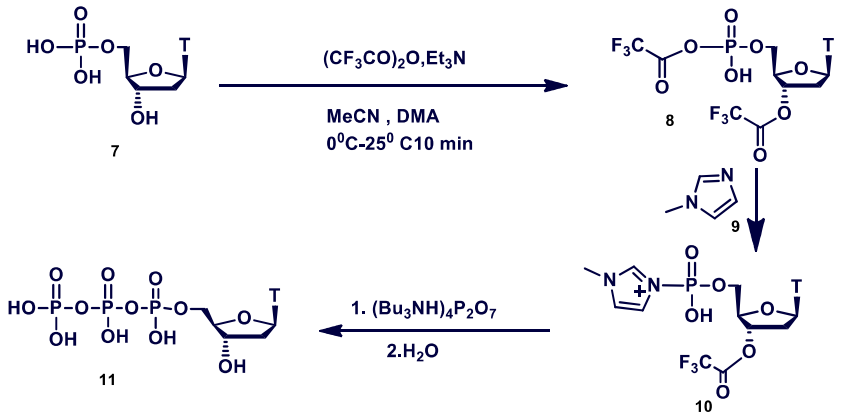
Scheme 1. 2. Synthesis of triphosphate via dichloro phosphate



Activation of monophosphate with trifluoroacetic anhydride

In this method nucleoside monophosphate (**7**) is treated with trifloroacetic anhydride to form activeated phosphate anhydride (**8**) .This anhydride is treated with N-methyl imidazole (**9**) to form activated monophosphate(**10**) .The activated monophosphateistreated with tributyl ammonium pyrophosphate to afford nucleoside triphosphates (**11**) in excellent yields.[11](Scheme1.3)

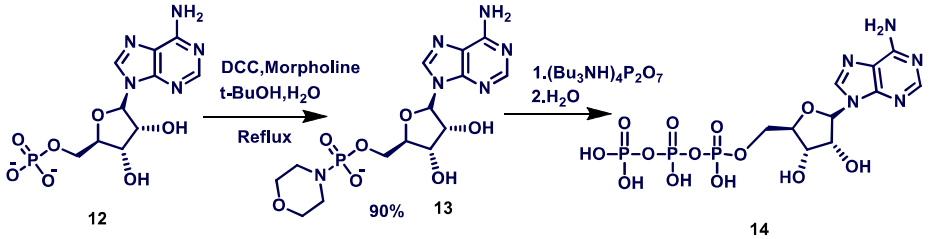
Scheme 1. 3. Synthesis of triphosphate by activation of monophosphate with trifluro aceticanhydride and imidazole



Activation of monophosphate with carbodimides via phosphoramidates

Phosphoramidates are active intermediates used in the synthesis of NTP. Phosphoramidates are prepared from monophopshates (**12**) by treatment with DCC and morpholine to give phosphoramidates (**13**). Phosphoramidate is treated nucleophilic tributylammonium pyrophosphate to yield triphosphates (**14**) in excellent yields **(s**cheme 1.4). [12-14]

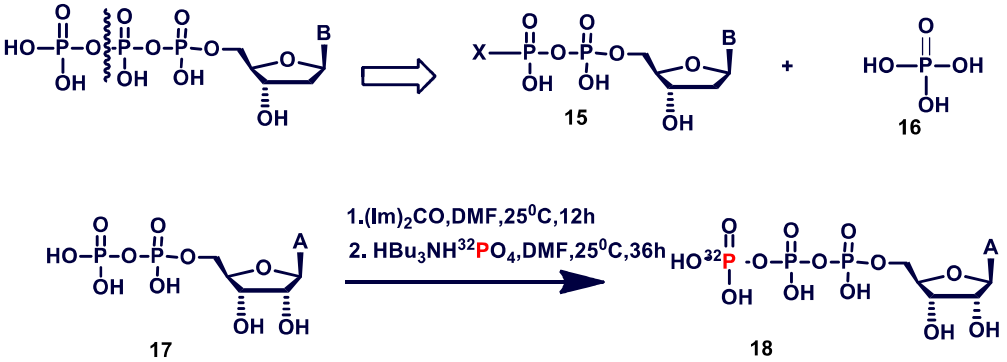
Scheme 1. 4. Synthesis of triphosphate by activation of monophosphate with morpholine and DCC



1.2.2 Synthesis via nucleophilic attack of phosphate on activated pyrophosphate

This method is used to synthesize gamma labelled triphosphate. Activation of pyrophosphate on nucleoside involves obstacles like selective activation of beta phosphate in presence of other functionalities on nucleoside. This method is used to synthesize γ ^{32}P labelled triphosphates .The activation of beta phosphate (**17**) is achived by using 1,1 carbonyl diimidazole (CDI). This activated diphosphate is treated with nucleophilic monophosphate to yield γ ^{32} P labelled triphosphate.[15](**18**) The disconnection approach involves activation of nucleoside diphosphate (**15**) and addition of monophosphate (**16**) (scheme 1.5).

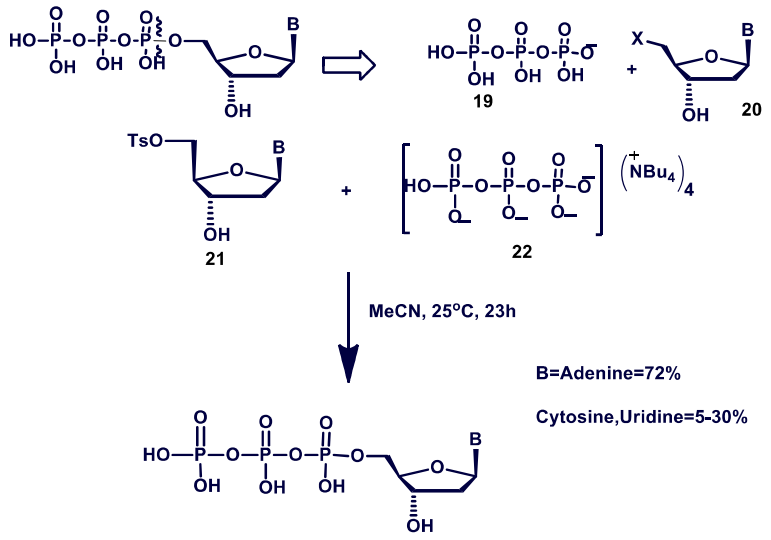
Scheme 1. 5. Synthesis of triphosphate by activation of diphosphate



1.2.3. Synthesis involving direct displacement of 5'-O leaving group by triphosphate nucleophiles

Nucleoside triphosphates (NTPs) are synthesized by treatment of 5'-O leaving group (**20**) with triphosphate salt (**19**) . 5'-O tosyl nucleosides(**21**) are treated with tetra butylammonium salt of triphosphate (**22)** in acetonitrile at room temperature to yield NTPs in good yield to low yield depending on nucleobase .This method gives good yield for adenine and low yield for other nucleosides.[16] The disconnection approach and schematic presentation of the method shown scheme1.6.

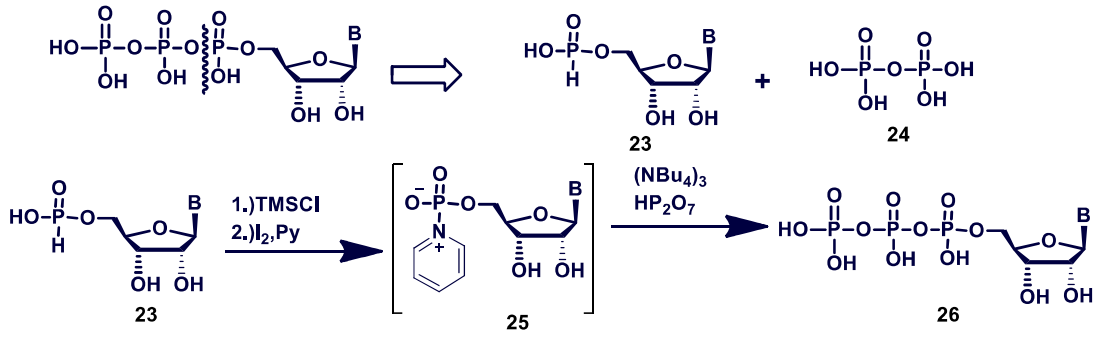
Scheme 1. 6. Synthesis of triphosphate by activation of diphosphate



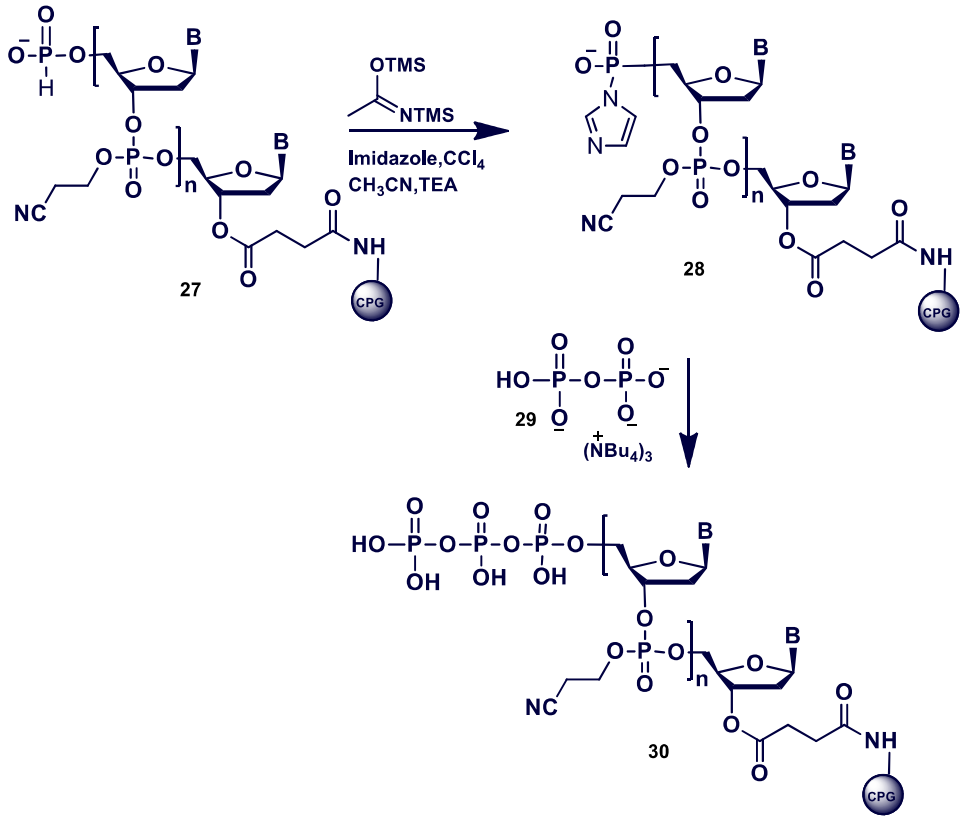
1.2.4. Synthesis involving activation of 5' H-Phosphonates

NTPs are synthesized from Nucleoside 5' H-Phosphonates (**23**) by activation followed by treatment with pyrophosphate (**24**). Nucleoside 5' H-Phosphonates are reacted with trimethyl silyl chloride, iodine and pyridine to form activated phosphonate (**25**).The activated 5' H-Phosphonates (**26**) are reacted with tetrabutyl ammonium pyrophosphate forms 5' triphosphates in good yield (scheme1.7) .[17,18] This strategy has extended to synthesize 5' triphosphates of DNA, RNA and their analogues on solid support.[19] In this strategy 5' H-Phosphonates of oligonucleotides (**27**) are activated by imidazole and the activated imidazolinium phosphanates (**28**) are treated with nucleophilic tetrabutytl ammonium pyrophosphate (**29**) to yield 5'triphosphates of oligonucleotides (**30**) and their analogues (scheme1.8.)

Scheme 1. 7. Synthesis of triphosphate by activation of 5' H-Phosphonates



Scheme 1. 8. Synthesis of oligonucleotides triphosphate by activation of 5' H-Phosphonates on solid phase.

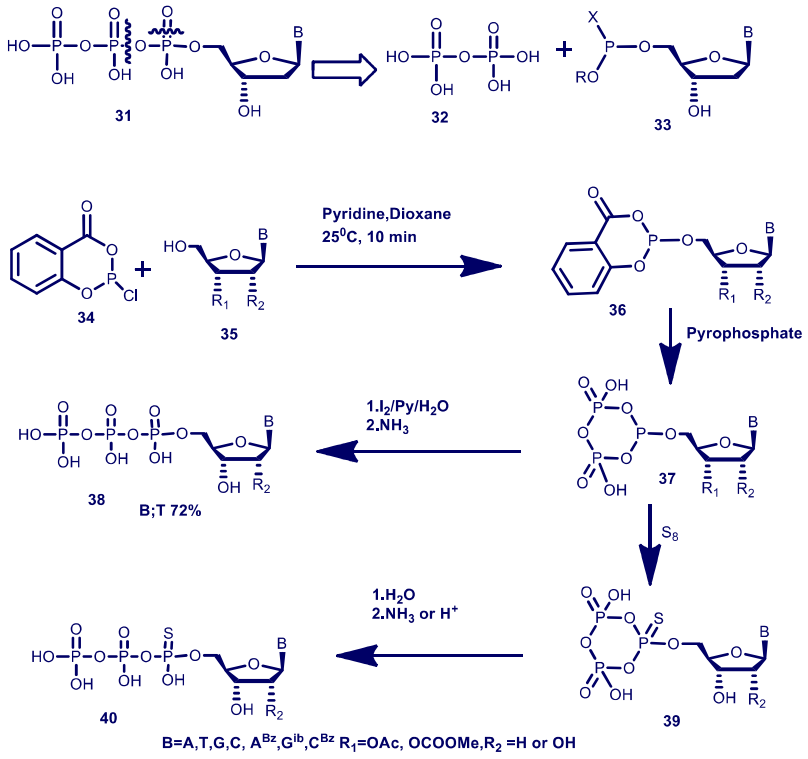


1.2.5. Synthesis involving activated phosphites derived from nucleosides

Nucleoside phosphites (**33**) are reactive intermediates which are being used in the nucleoside triphosphate (**31**) synthesis. There are some synthetic methods developed

which involved the phosphites. In late 1980 s Ludwig and Eckstein developed a synthetic method using salicyl chlorophosphidite (2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one)(**34**) .This method is popularized due to its rapidity and efficiency .In this method protected nucleosides (**35**) are treated with salicyl chlorophosphidite in pyridine and dioxane mixture to yield a reactive nucleoside phosphite. This phosphite intermediate (**36**) is treated with pyrophosphate (**32**) and tributylamine to obtain cyclic triphosphate (**37**). The cyclic triphosphite is oxidized with iodine in pyridine/water solution followed hydrolysis under basic conditions

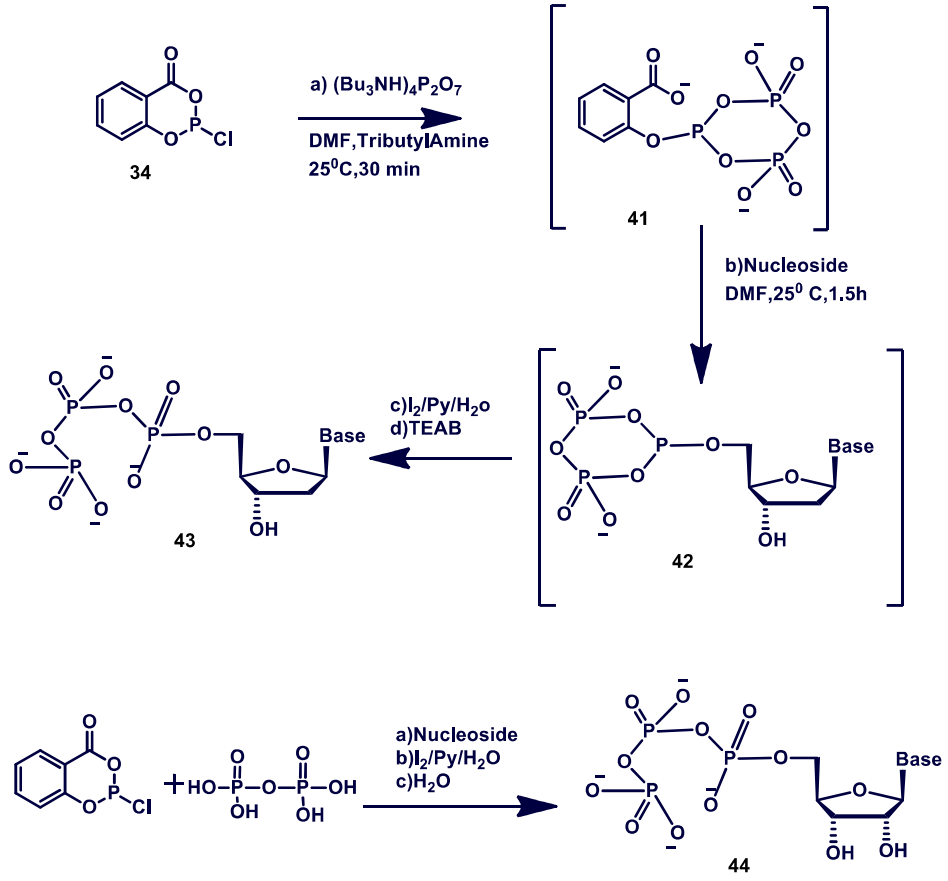
Scheme 1. 9. Synthesis of triphosphate involving activated phosphites derived from nucleosides



to produce triphosphates.[20] The cyclic triphosphites (**37**) are treated with sulphur to produce alpha thio cyclictriphosphates (**39**) which on hydrolysis gives alpha thio triphosphates (**40**) .[21] The disconnection approach depicted in the above scheme 1. 9.

In recent years Zhen Haung and co-workers have developed a protection free NTP synthesis involving phosphite intermediates.[22,23] In this method a reactive cyclic triphosphite (**41**) is produced by treating salicylchlorophosphidite, pyrophosphate and tributylamine. An unprotected nucleoside is treated with the active phosphite to produce a nucleoside triphosphate. This cyclic triphosphate is oxidized and hydrolyzed to produce triphosphate (**43**).

Scheme 1. 10. Synthesis of nucleoside triphosphate involving activated phosphites



This method also used to synthesize, rNTPs(**44**),[24] alpha thio triphosphates[25] and modified NTPs in convenient way. The disconnection approach and synthetic scheme 1. 10.

Many methodologies have been developed to synthesize NTP and their analogues but still there is no universal method for the synthesis of NTP which can give a better yield for all NTP. In view of developing new modified NTP analogues each and every analogue need to optimise the synthetic conditions and methods. One method can work well for one analogue may not give same results for the other analogues.

1.3 NTP Analogues

Chemists have modified the NTP structure at three possible sites (Figure 1. 5) to develop the structurally modified NTPs for biochemical and therapeutic applications.[26]

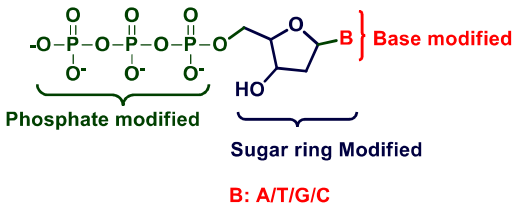


Figure 1. 5. Nucleoside triphosphate modifications

1.3.1 Sugar modified NTPs

Sugar ring modified NTP analogues have been developed for various therapeutic and biochemical application .Dideoxynucleoside triphosphates (Figure1. 6) are the sequence terminators developed by Sanger and co-workers used in the DNA sequencing technologies. [27]

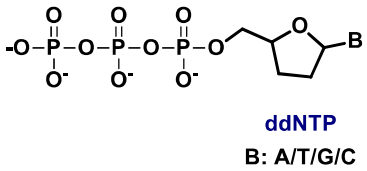


Figure 1. 6. Structure of dideoxy nucleoside triphosphates

Sugar modified 3'OH protected/functionalized dNTP analogues (**45**) (Figure1. 7) have been synthesized and tested as sequencing terminators for application in DNA sequencing technologies.[28,29] Sugar modified analogues such as 3'OH functionalized with methyl, acetyl, paranitrobenzoyl, orthoamino benzoyl, orthonitrobenzyl, allyl and tetrahydropyranyl derivatives are synthesized and evaluated as sequencing terminators. Peptide nucleic acid derivatives are also synthesized and evaluated as sequencing terminators.[30,31]

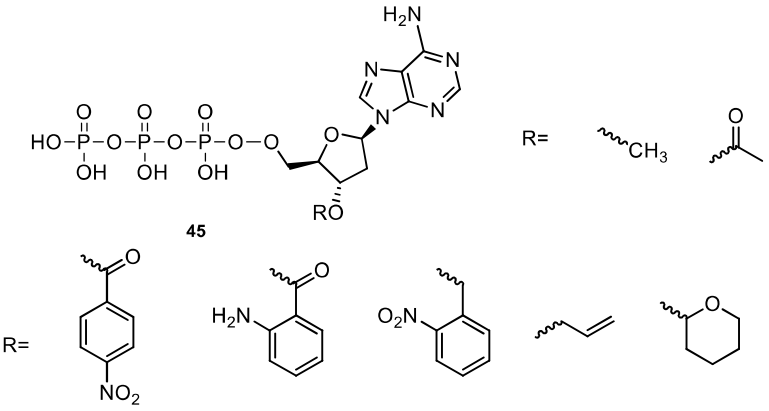


Figure 1. 7. Structure of sugar modified nucleoside triphosphates

In the last decade many sugar modified analogues such as carbocyclic nucleosides/nucleotides have been synthesized for therapeutic application as antiviral agents.[32] Recently sugar modified analogues are also developed as therapeutic drugs for various viral diseases.[33,34] Sugar modified nucleoside triphosphate analogues are functionalized with lipophilic carrier (**46-50**) (Figure1. 8) at terminal phosphorous atom synthesized and evaluated as membrane permeable antiviral prodrugs. [35]

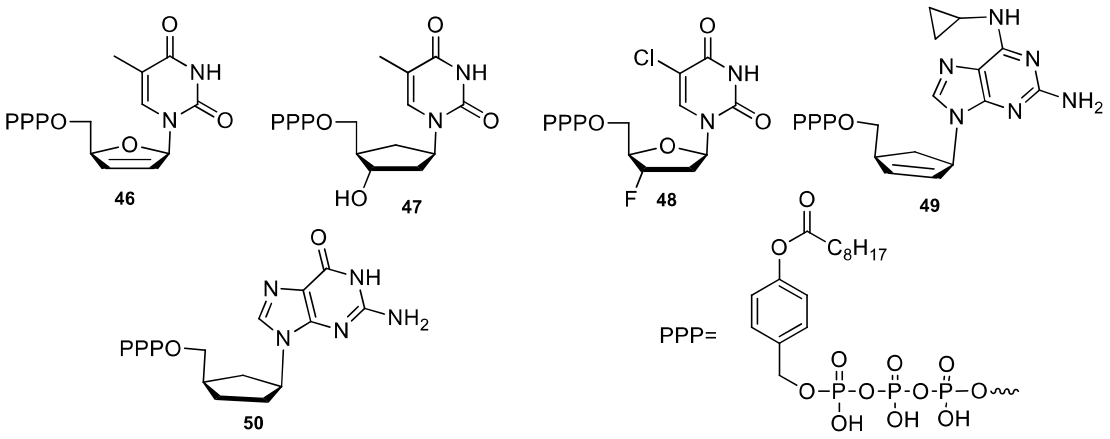


Figure 1. 8. Structure of membrane permeable sugar modified nucleoside triphosphates drugs

1.3.2 Phosphate modified NTP analogues

Several phosphate modified NTP analogues have beensynthesized and evaluated as therapeutic agents. Recently a review was published on phosphate modified nucleotides for monitoring the enzyme activity. Here the detailed methods to synthesize the labelled triphosphates and their application in evaluating the polymerase activity are reviewed.[36] γ- Phosphate substituted analogues were prepared and used to monitoring enzymatic reaction as alternative to radio labelled phosphate.The γ-thio[37](**51**) and flouoro[38] (**52**)(Figure1. 19)ATP analogues were also synthesized and used to monitor the kinase action.

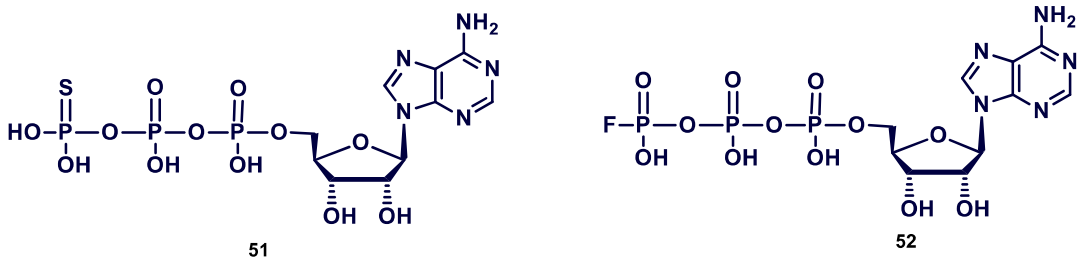


Figure 1. 9. Structure of γ-thio/fluoro modified nucleoside triphosphates

Apart from being a good substrate, the modified analogue also must be stable under reaction condition to improve the chemical stability of substrate over a wide range of pH .To address this many terminally modified polyphosphates have been prepared and studied their stability under reaction conditions.The analogues from tetra to hexa phosphates(**53-57**) (Figure1. 10) are the good substrate of polymerases and the stability of terminal nitrogen analogues are less to the hydrolysis below pH 6 while the carbon and oxygen analogues are stable from pH 2-12. [39-43]

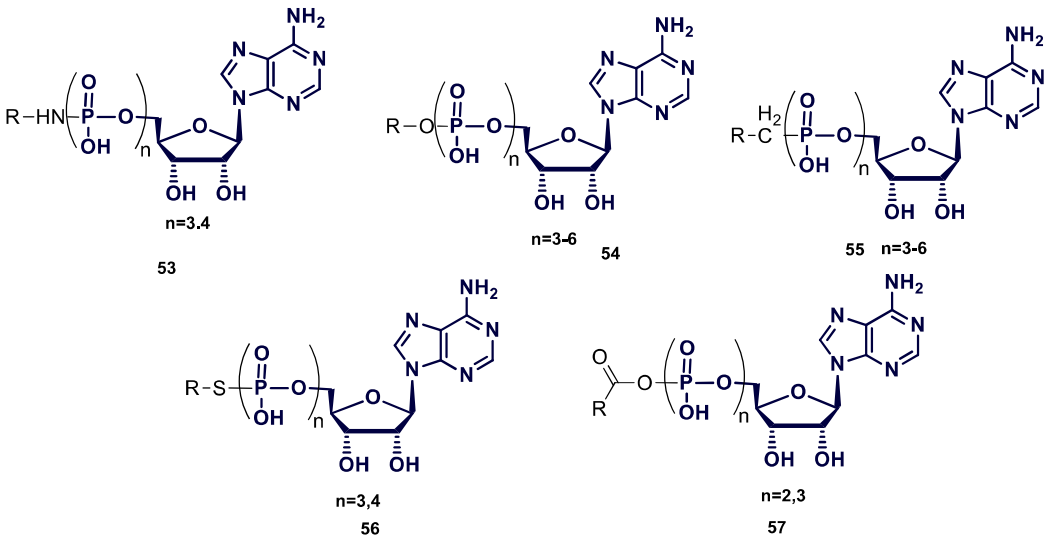


Figure 1. 10. Structure of terminally modified nucleoside polyphosphates

Biotin is a versatile reporter to detect the peptide modifications by kinases. The ATP analogues with bition at γ- phosphate (**58** & **59**) have been developed to monitor the transfer of biotin to target protein (Figure 1. 11.).[44]

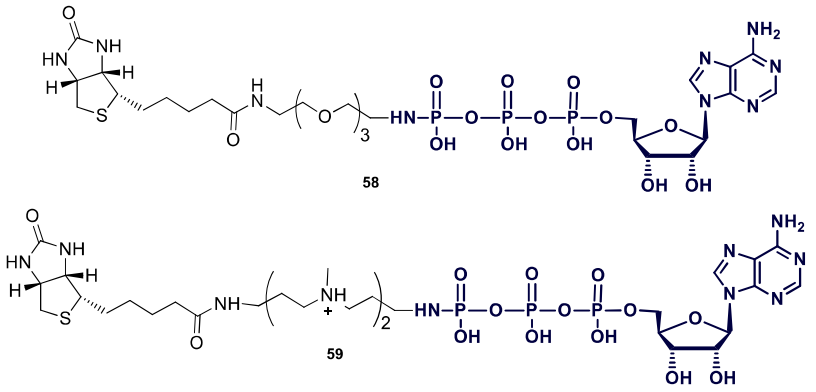


Figure 1. 11. Structure of Structure of γ-biotinylated nucleosidetriphosphates

Several ATP analogues with γ- phosphate labelled with a fluorophore (**60-62**) are developed to monitor the kinase activity by FRET technique. The labelled ATP on reaction with kinases transfers its fluorophore to the kinases.The kinase on incubation with second fluorophore gives the FRET effect (Figure 1.12).[45-47]

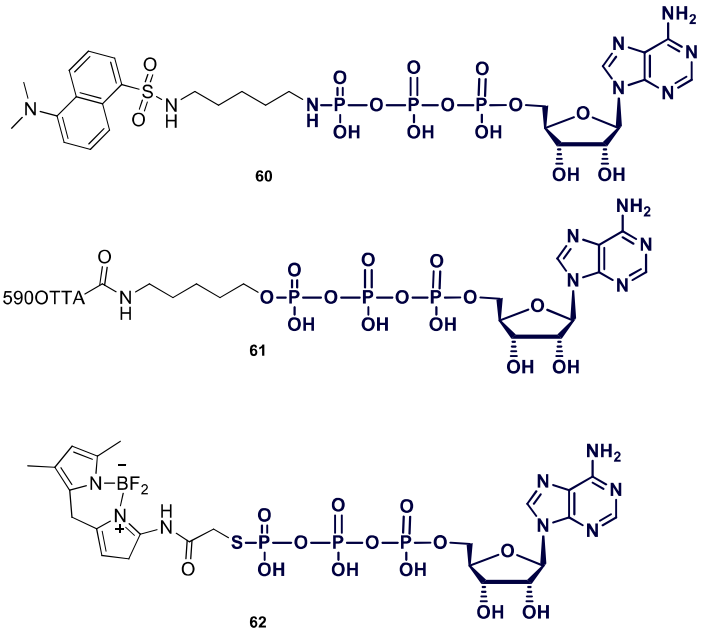


Figure 1. 12. Structure of Structure of γ-florophore attached nucleosidetriphosphates

All these substrates are terminally labelled with specific functional group these substrates on reaction with kinases will transfer the terminal group to the substrate

which will be labelled. Figure 1.13 depicts the schematic representation of transfer of terminal group to the substrate during enzyme reaction. [48]

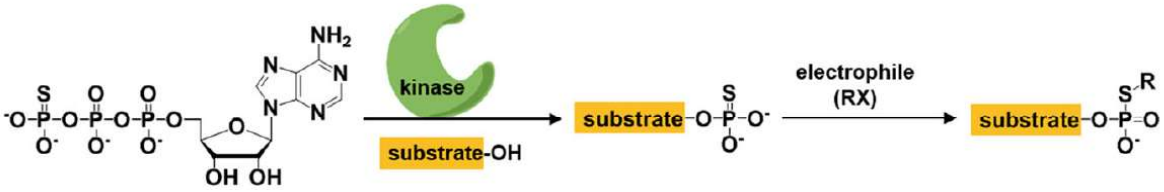


Figure 1. 13. Schematic representation of transfer of γ terminal group of nucleoside triphosphates to the substrate

γ- Phosphate substituted 2'-dNTP analogues had been synthesized and evaluated as substrates of human immunovirus (HIV) and avian myeloblastosis virus (AMV) reverse transcriptases. The γ- Phosphate group functionalized as ester, amide and phosphanate (**63** & **64**) and these analogues are tested as substrates for the reverse transcriptases and mammalian polymerases. These are the substrates of only reverse transcriptases and the mammalian polymerase. The substituent group on the γ-phosphate alters the activity of cellular DNA polymerases but not the reverse transcriptases (Figure 1.14).[49]

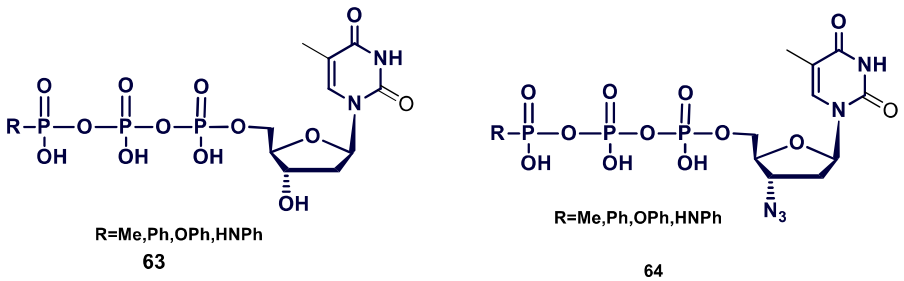
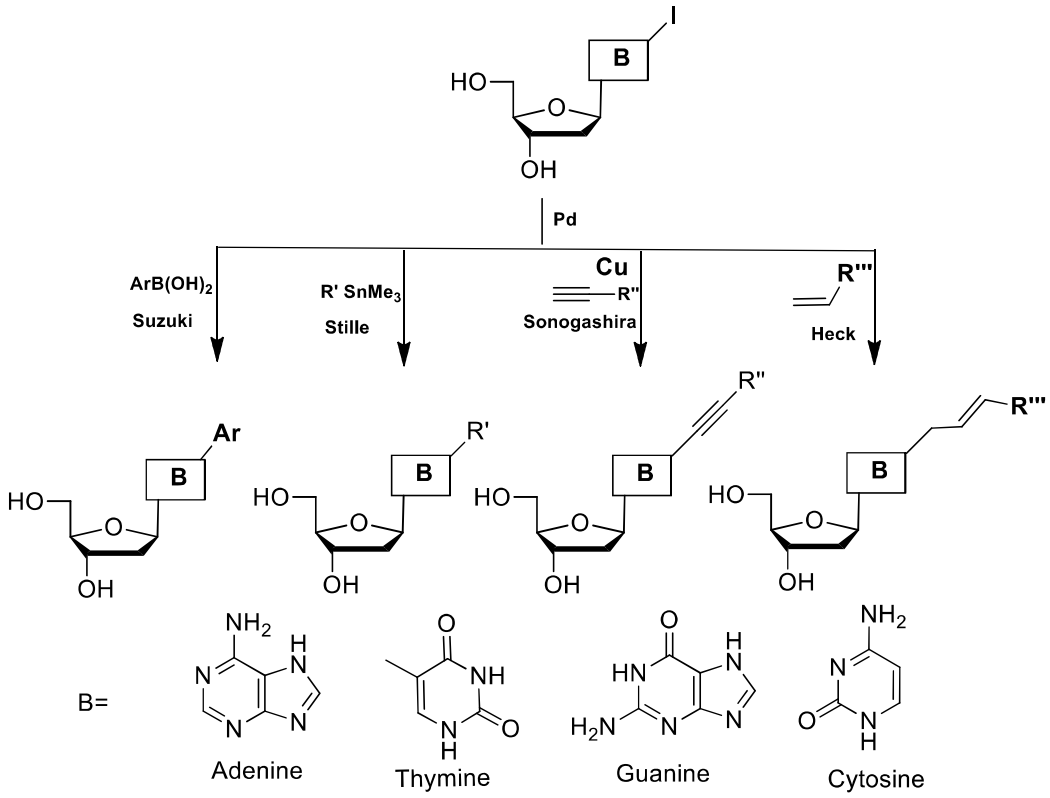


Figure 1. 14. Structure of γ modified nucleosidetriphosphate drugs

1.3.3 Nucleobase modified NTP analogues

Nucleobase modified NTPs are the substrates for polymerase to synthesize the modified nucleic acids. Oligonucleotides containing functional nucleobases are being used to synthesize the modified DNA for applications in chemical biology, nanaotechnology and material science.[50] The modified nucleic acids can be synthesized by incorporating the modified phosphoramidtes into oligo nucleotides by solid phase synthesis. But this method has the disadvantage of limitations to synthesize only upto 100-200 nucleotide and compitability of functional groups in the modified phosphoramidates to the various reagents and oxidizing agents during the synthesis. To overcome these issues researchers developed methods to incorporate

Scheme 1. 11. Synthesis of base modified nucleoside analogues

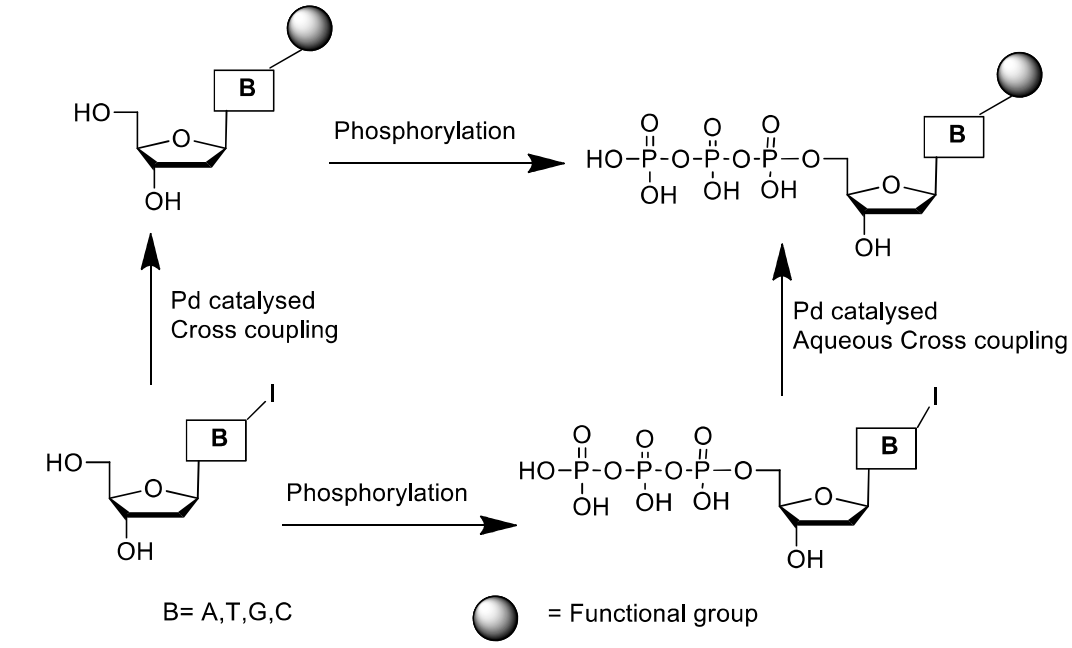


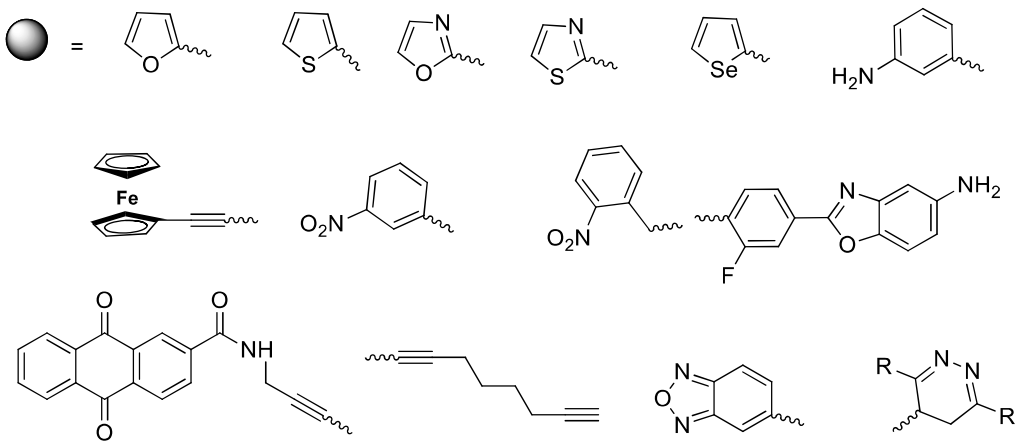
modified NTPs into oilgonucleotides by polymerase reaction with aid of designed primer and template. Common method for modifying nucleobase structure is to attach a functional group/chromophore/fluorophore/antibody at 5-position of pyrimidine/7-position of 7-deaza purines.[51](Scheme 1.11) The commonly used methods are coupling of 5-halo pyrimidines/7-deaza purines under transition metal catalysed reactions.[52] The use of organostannanes is widely used method for the functionalization of uridine derivatives. Organo boranes,[53] organostannanes,[54] alkynes[55] and alkenes[56] are also coupled with 5-halo pyrimidines/7-deaza purines by palladium catalysed reactions. [57]

The general method for attaching functional groups to NTP is to synthesize the modified nucleoside analogues and then phosphorylation of the modified nucleosides to achieve the functionalized NTP. This strategy involves the protection of all the reactive functional groups before phosphorylation and deprotection after phosphorylation involves tedious purification steps and lowering the total yield of modified NTPs. To overcome these problems methods have been developed to synthesize the modified NTP after phosphorylation of 5-halopyrimidine/ 7-halo7-deaza purines. Flouorophore conjugated dUTP/ddUTP analogues were synthesized by Sonoghashira coupling of 5-halo dUTP/ddUTP analogues. In recent years 7-halo 7-deaza purines /5-halo pyrimidines triphosphates utilised to synthesize alkene, alkyne conjugated base modified triphosphates analogues by aqueous Heck/Sonoghashira coupling reactions. [52](Scheme 1.12) The base modified nucleotides used for the synthesis of modified nucleic acids which have potential application as florescent tags in sensing the environmental changes,[58] barcoded nucelotides for diagnostic applications,[59] redox labelling for electrochemical detection,[60,61] spin labelling[62]

application bioconjugation of antibodies, proteins, fluorophores and various other applications.[50]

Scheme 1. 12. Synthesis of base modified nucleoside triphosphate by cross coupling reaction of nucleoside triphosphates





1.4 Summary and outlook

NTP are the biomolecules involved in various biological functions. Synthesis of NTP is challenging, chemists have developed many synthetic methodologies to access NTP

in adequate amounts and good yields. Still there is no universal and convenient method to synthesize NTP. Due to the biological importance and their application in therapeutics and analytical methods many NTP analogues designed, synthesized and studied for various therapeutic and analytical applications. We have planned to develop some sugar modified dideoxy NTP analogues derived from proline and hydroxyl proline as alternate substrates for the DNA sequencing technology. We have also planned to develop some base modified NTP analogues for detection of environmental changes and reversible labelling .We designed some base modified NTP analogues derived from tropolone. Tropolone is a seven member ring containing aromatic compound with hydroxyl and carbonyl groups on adjuscent cabons. Tropolone occurred in many natural products and drugs. We designed tropolone conjugated 2'dUTPs, synthesized and studied their photo physical and biochemical properties.

1.5 Thesis organization

The thesis has been organized into six chapters.

Chapter 1 Nucleosidetriphosphate (NTP) General Introduction

This chapter describes the general introduction of NTP. The structure of nucleosides, nucleotides, nucleic acids and the role of nucleotides in biological systems are discussed. Further the structure and mechanism of DNA polymerase are also discussed. Synthetic methods of NTP and NTP analogues are also discussed.

Chapter 2: Synthesis and Biochemical Evaluation of Pyrrolidine Nucleoside Tri phosphates (Pr NTPs)

This chapter describes the introduction to ddNTP analogue synthesis, Sanger sequencing methods, recent trends and development of sequencing terminator are discussed. The rational design, synthesis and bio-chemical evaluation of pyrrolidine nucleoside triphosphates are discussed.

Chapter 3 this chapter is divided into two parts

Chapter 3A: Synthesis and Biochemical Evaluation of Alanylthymidine triphosphate: This chapter describes the introduction of peptide nucleic acids, (PNA) modified peptide nucleic acid as alanyl peptide nucleic acids (ala-PNA), synthesis and bio-chemical evaluation of alanyl-thymidine triphosphate (*ala*-TTP). The self-assembly of alanyl-thymidine ester is studied by X-ray structure.

Chapter 3B: Synthesis and Biochemical Evaluation of Prolamide Thymidine /Uridine Triphosphates

Chapter 3B describes the introduction to prolinol acetyl nucleosides, synthesis and bio-chemical evaluation of prolinol acetyl thymine/uracil triphosphates. Prolinol acetyl thymine/uracil triphosphate are not the substrates of DNA polymerase. Nucleoside analogues prolinol acetyl thymine/uracil are characterized by single crystal X-ray diffraction studies .Both thymine and uracil analogues show supra molecular helical self-assembly by hydrogen bonding interaction.

Chapter 4: Synthesis, Photophysical and Biochemical Studies of Tropolone Modified 2'-deoxyuridine Triphosphates

Chapter 4 describes the general introduction to fluorescent nucleoside/nucletides, synthesis and bio-chemical evaluation of tropolone derived 2'-deoxy uridine triphosphates. Herein we describe the synthesis and biochemical evaluation of three uridine triphosphate analogues. 5-(ethynyl tropolonyl) 2'-deoxy uridine triphosphate is a substrate of DNA polymerase. The absorption and emission spectra of the compound show that this compound is highly pH responsive and can be used to synthesize a pH response oligonucleotides by DNA polymerase. 5-(triazolyl tropolonyl) 2'-deoxy uridine triphosphate is also substrate of DNA polymerase. 5-*N*-troponyl prolamide propargylamine 2'-deoxy uridine triphosphate is also substrate of DNA polymerase. The compound contains acid cleavable peptide which can be used for reversible labelling.

Chapter 5: Synthesis and Biophysical Studies of Aminoethyl prolyl Cytosine Pentamer (*aep*-C_5) in the Stabilization of Hybrid i-motif with DNA and RNA

This chapter describes the introduction, structure and analogues of i-motif. The chapter also describes synthesis and characterization of aminoethyl prolyl cytosine pentamer (aep-C_5). The *aep*-C_5 used to form hybrid i-motif with analogous DNA and RNA sequences. The resultant hybrid i-motifs are very stable compared to native DNA and RNA analogues owing to the backbone ion-ion interactions. The formation and stability of hybrid i-motif analogues are studied by UV/CD/NMR/FE-SEM/ESI-MS methods.

Chapter 6 Synthesis and Spectroscopic Studies of Berberinemmobilized Modified Cellulose Material

This chapter describes the introduction to cellulose grafted materials and their application. This chapter also describes the synthesis and characterization of berberine immobilized cellulose material. The berberine cellulose material charecterized by powder x-ray diffraction studies and FT-IR studies. The photo-physical properties of modified cellulose material are studied by UV-vissible, florescence and photoluminescence spectra .The morphology of the compound studied by scanning electron microscopy, fluorescence microscopy and confocal microscopy.

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Chapter 2

Synthesis and Biochemical Evaluation of Pyrrolidine nucleoside Triphosphates (Pr-NTPs)

Chapter 2

Synthesis and Biochemical Evaluation of Pyrrolidinenucleoside Triphosphates (Pr-NTPs)

Contents

2.1 Introduction

Deoxy nucleoside triphosphate (dNTP) is a substrate of DNA polymerase and involved as monomer in the biosynthesis of DNA polymer in the presence of complementary DNA template. Four types of dNTPs such as dATP, dTTP, dCTP, dGTP are responsible for DNA synthesis by enzymatic incorporation into DNA primer. The general mechanism of dNTP incorporation is described in chapter 1 which shows the formation of phosphodiester bond between the 3'-OH of primer DNA and the α-phosphate of the incoming dNTP in presence of DNA polymerase and DNA template.[1-3] After one dNTP incorporation, another dNTP incorporate at the 3'-OH of previous one via similar phosphodiester bond formation. Another modified NTP analogues as 2',3'-dideoxynucleoside triphosphate (ddNTP), also called as Sanger's nucleotides, are responsible for termination of DNA synthesis after single incorporation into DNA primer. Thus ddNTP are known as sequence terminator of DNA synthesis which is foundation for development of DNA sequencing technology. In first-time, Sanger and co-workers, have successfully demonstrated the sequencing of bacteriophase Φ X 174 DNA.[4] The detection of ddNTP incorporation are most challenging to chemists. Initial days, radioisotope labelled [5] ddNTPs have been used for the detection of terminated DNA sequence by measuring the disintegration of radioisotope of radiolabel ddNTP, mostly ^{32}P of ddNTP, which are hazardous to environment. Nowadays, dye/fluorescent labelled ddNTP, comparatively environment friendly, are being used in the detection of ddNTP incorporated terminated DNA primer.[6]

2.1.1 Sanger DNA sequencing methods

The sequencing of unknown long DNA become easy and routine work for molecular biologist by using DNA sequencing machine. The basic principle of DNA sequencing machine is Sanger DNA sequencing method which pictorially represented Figure 2.1 In this methods, template DNA (unknown DNA) and DNA primer (DNA oligonucleotide complimentary to template DNA), and labelled ddNTP are mixed in

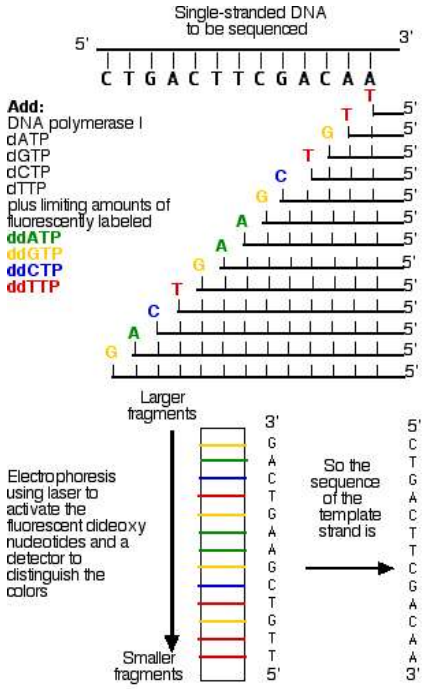


Figure 2. 1. Sanger sequencing method illustration

the presence of DNA polymerase and employed for thermal denaturation followed by annealing for incorporation of ddNTP. Since all four ddNTPs are labelled with four different fluorescent tag, so each ddNTP produces emission at specific wavelength. Thus four ddNTP incorporated primers could easily be separated separated by capillary gel electrophoresis and visualized distinctly. A reaction is performed with

DNA polymerase, mixer of dNTPs and ddNTPs. The primer DNA is terminated by ddNTPs which are fluorescently labelled. The terminated sequences of different lengths are detected by the emission of fluorescent dyes which gives the sequence of template.[7] The schematic presentation of Sanger sequencing method is illustrated in Figure 1. Human genome project[8] give lot of information of human genome. The completion human genome project encouraged researchers to develop new sequencing terminators in economical way. In this point of view reseacrhers have been developing many ddNTP analogues for DNA sequencing.[9]

2.1.2 ddNTP analogues

After development of ddNTP analogues by Sanger many ddNTP analogues have been synthesized and tested as ddNTP analogues for DNA sequencing.[10] 3'-OH modified dNTP analogues have been synthesized and tested with many DNA polymerases as terminators for DNA sequencing. Metzker and coworkers have synthesized 3'-OH methyl, acetyl, allyl, tetrahydropyranyl, para-nitrobenzoyl, 2-amino benzoyl and photo labile orthonitrobenzyl dNTPs[11] have been and tested with several DNA polymerases as alternative terminators in Sanger sequencing methods (Figure 2 .2).

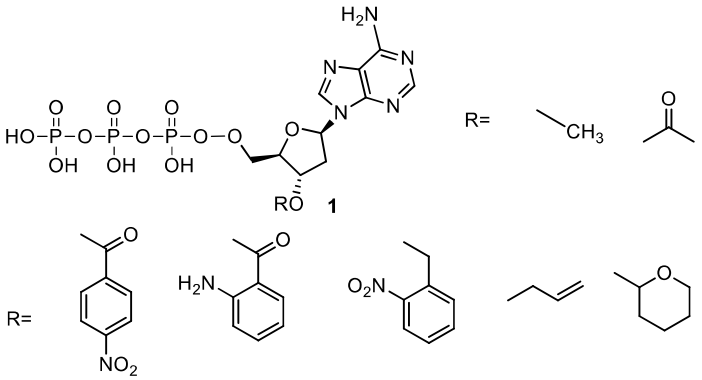


Figure 2. 2. 3'-OH modified dNTP analogues

The 3'-OH group of dNTP protected by a labile group terminates the synthesis of DNA and resume after removal of the group by treatment with a chemical reagent/UV-light. The termination is detected by a fluorescently labelled 3'-OH modified dNTP analogues. This technology is called sequencing by synthesis (SBS) and widely used in the second generation sequencing technologies. These substrates are called reversible sequencing terminators. [12]

Several research groups developed many 3'-OH modified dNTP analogues for DNA sequencing using SBS approach.

Jinguye Ju and co-workers have developed a series of 3'-OH labelled reversible sequencing terminators[13-15] for DNA sequencing by SBS approach. For example 3'O allyl group blocks the synthesis of DNA after single incorporation and the termination is detected by the fluorescently labelled dye linked via cleavable allyl bridge.[16](**2** Figure 2. 3) After detecting the fluorescent signal from the terminated dNTP the allyl group is deprotected by palladium catalysed reaction to generate free 3'OH group as well removal of the fluorescently labelled dye linked via cleavable allyl bridge. Next the newly generated 3'-OH starts the DNA synthesis and so on. The four dNTP analogues (A, T, G and C) labelled with four different dyes to detect the termination of each nucleobase. [17,18]All these steps are automated and performed on solid support constructed using molecular engineering approaches. By following same principle but different cleavable group azido methyl group(**3** Figure 2.3) by phosphine derivative another set of fluorescently labelled 3'-OH reversible dNTP analogues for DNA sequencing by SBS approach have been developed. [17]

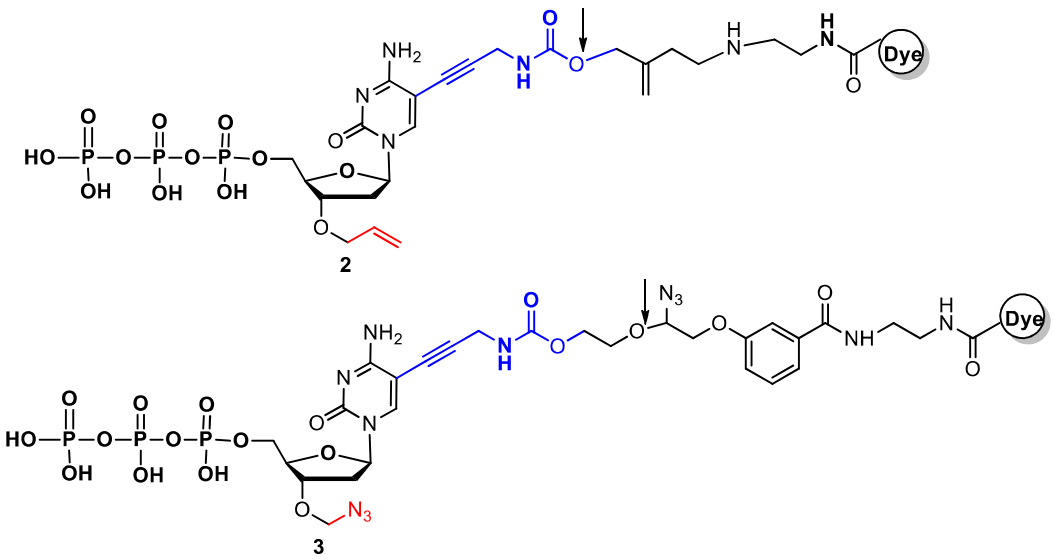


Figure 2. 3. 3'-O allyl, azido methyl 2'-deoxynucleoside triphosphates

Benner and co-workers have developed a chemo lablie 3'O-NH_2 labelled dNTP analogues with a cleavable fluorescently labelled dye as reversible dNTP analogues. The 3'O-NH_2 group is cleaved by sodium nitrite buffer. [19] They have modified the DNA polymerase by reconstructed evolutionary adaptive paths (REAP) technologies to construct a polymerase suitable for the designed dNTP analogue.[20] (Figure 2.4)

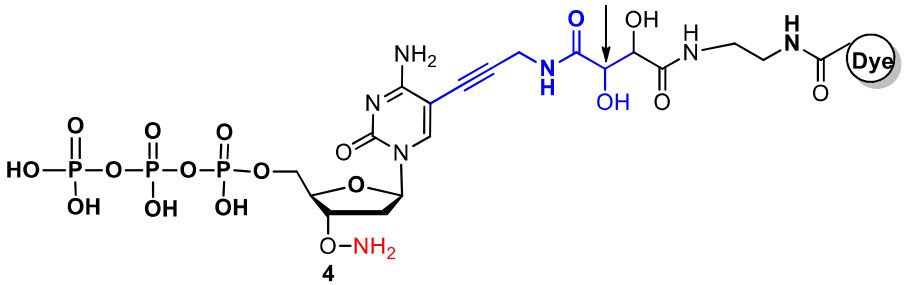


Figure 2. 4 3'-O NH_2 2'-deoxynucleoside triphosphates

An appropriate chemo labile or photo labile group attached at abasic site of nucleobase can terminate the DNA synthesis temporarily and after removal of the group the DNA synthesis can resume. These are called 3'-OH unblocked reversible terminators and next generation sequencing terminators. Metzker et al developed C-5 pyrimidine/C-7-Deaza purine hydroxyl methyl nitrobenzyl derivatives as 3'-OH unblocked reversible sequence terminators.[21-23] (**5** Figure 2.5) The nitrobenzyl group attached to the hydroxyl methyl group can deprotect by irradiating with UV- light and after removal of the nitrobenzyl group DNA synthesis can be resumed. The nitro benzyl attached modified 3'-OH unblocked reversible terminator structure shown in Figure2. 5. These are also called lighting terminators.[24]

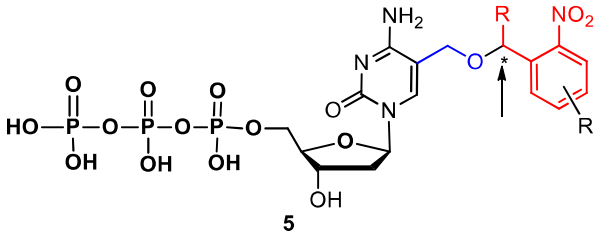


Figure 2. 5 3'-OH unblocked photo cleavable 2'-deoxynucleoside triphosphates

Recently Shen and co-workers have reported a chemo labile 3'-OH unblocked dNTP as reversible ddNTP analogues.[25] An acid labile dimethyl ketal linked dye labelled at C-5 position of 2'deoxy uridine tiphosphate (2'd-UTP) analogue (**6** Figure 2.6) as

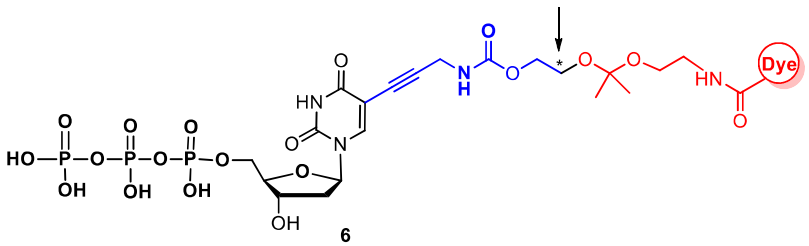


Figure 2. 6 3'-OH unblocked acid cleavable 2'-deoxynucleoside triphosphates

reversible terminator. The analogue terminates the DNA synthesis after single incorporation and can resume the synthesis after removal of the linker by treatment with acidic buffer. [26]

In the repertoire of the ddNTP analogues peptide nucleic acid derived acyclic triphosphate analogues have been developed and used as alternate DNA sequencing terminators. Burgess group developed analogues derived from PNA as acyclic nucleoside triphosphates and allylic/acyclic (**8 &9** Figure 2. 7) nucleoside triphosphate analogues derived from PNA.[27,28] These two analogues derived from aminoethyl glycinyl (*aeg*) PNA (**7** Figure 7) which are acyclic and achiral. Proline derived chiral PNA analogues are conformationally constrained and chiral which bind with complementary DNA/RNA strongly.[29]

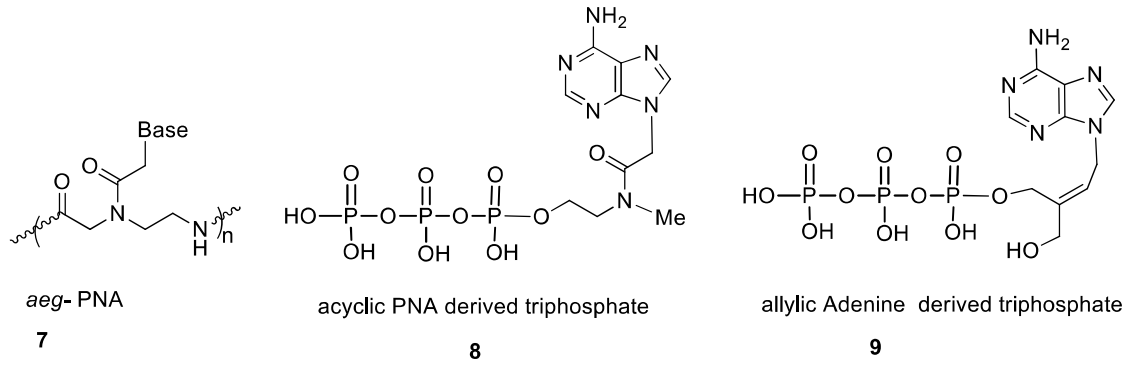


Figure 2. 7 PNA and PNA derived acyclic nucleoside triphosphates as terminators

2.1.3 Hypothesis and rational of present work

We hypothesised to develop ddNTP analogues derived from conformationally constrained proline derived nucleosides which may work as alternate ddNTP anlogues. We designed several ddNTP analogues derived from proline nucleosides. We planned to synthesize pyrrolidine nucleoside triphosphates and to study as ddNTP

analogues. Pyrrolidine derived nucleic acid analogues have been synthesized and studied their binding properties with complementary DNA/RNA analogues.[30-32] We hypothesized that we can synthesize the pyrrolidine nucleoside triphosphates (Pr-NTP) and study as ddNTP analogues. The Pr-NTP contain the free secondary amine group which could be used to label with dye via linker. First we have planned to synthesize and study the Pr-NTP as substrate of DNA polymerase then we can use Pr-NTP analogues to make labelled Pr-NTP analogues (**10-12** Figure 2.8).We have also designed a 3'-OH unblocked dNTP analogues as reversible ddNTP analogues.Recently reported that troponamide moiety containing amides are cleavable under acidic conditions (**13** Figure 2.8) .We hypothesized the tropolonamide can terminate the DNA synthesis and resume after removal of tropolonamide moiety.Detailed synthesis and biochemical evaluations described in chapter 4.

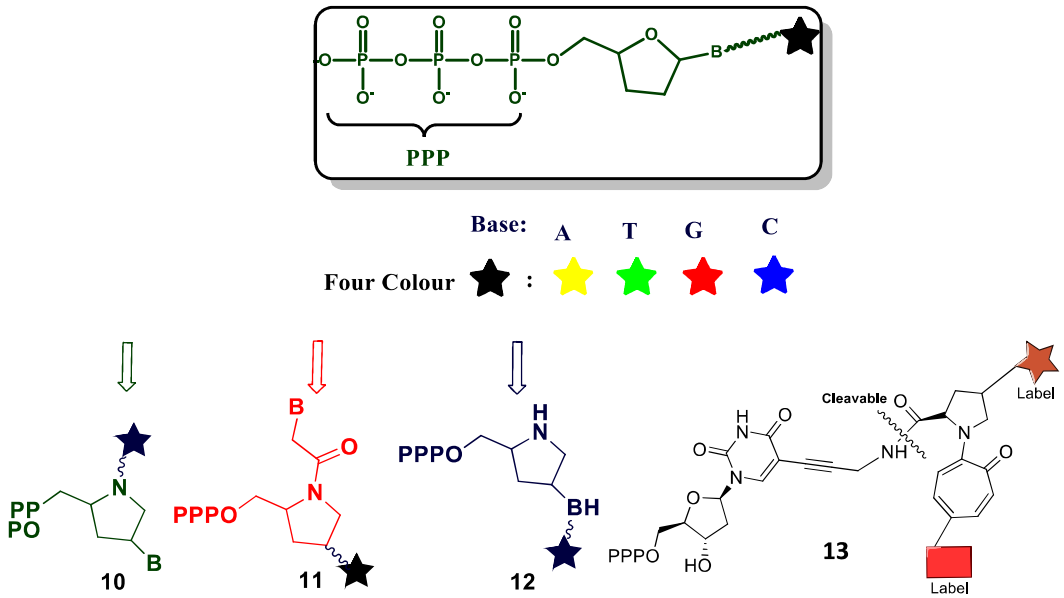


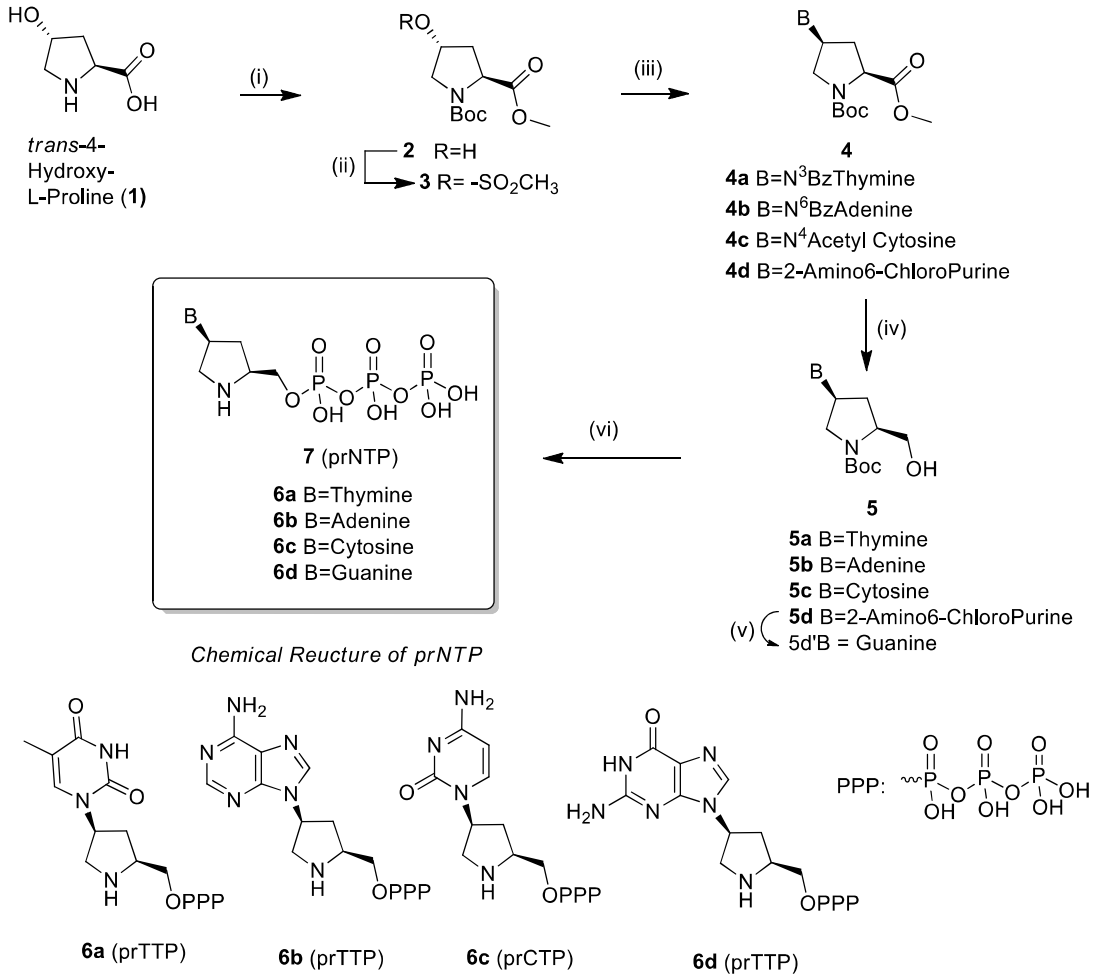
Figure 2. 8. Proline derived nucleoside triphosphates as ddNTP analogues (**10-12**) and Tropolone derived dUTP analogues as reversible ddNTP analogues (**13**)

2.2 Results and discussion

2.2.1Synthesis of pyrrolidinenucleoside triphosphates (Pr-NTPs)

We started the syntheses of rationally designed pyrrolidinenucleoside triphosphates (*pr*NTPs) as prTTP, prATP, prGTP and prCTP from commercially available

Scheme 2. 1. Synthesis of Pyrrolidine nucleoside triphosphates



Reagents and conditions: (i) (a) $SOCl_2$, MeOH, 0°C-reflux, 98%; (b) H_2O:CH_3CN (1:1), TEA, $(Boc)_2O$, 95%; (ii) Mesylchloride, pyridine,quantitative yeild; (iii) N^3 -Bz Thymine, $(Ph)_3P$, DIAD, THF, 50% (for **4a**)/N^6-Bz Adenine, DMF, K_2CO_3,18-Crown-6, 75°C,12h,65% (for **4b**)/ N^4 -Ac-Cytosine,DMF,K_2CO_3,18-Crown-6 ,75°C,12h, 60% (for **4c**)/2-Amino-6-chloropurine, DMF,K_2CO_3,18-Crown-6, 75°C,12h, 50% (for **4d**); (iv) $LiBH_4$,THF, 0°C-rt, 87% (**5a**) /75% (**5b**), 80% (**5c**)/ 70% (**5d**); (v) 1.0N LiOH, Dioxane, H_2O, 50%. (vi) (a) Salicylchlorophosphidite, dioxane, pyridine; (b) $((Bu)_3NH)_2H_2P_2O_7^{2-}$) (TBAPP), DMF, TBA;(c) I_2/Py/H_2O; (d) aq. NH_4OH. (e) 1.0N HCl.

aminoacid 4-(*R*)-hydroxy-2-(*S*)-Proline (**1**) by following the synthetic route of Scheme 1. First the functional groups (-NH-and –COOH) of aminoacid (**1**) were protected and converted into pyrrolidine derivatives (**2**) by following two steps: (i) esterification of carboxylate, and (ii) Boc-protection of amine (-NH-) groups.[33] The compound **2** was treated with N^3-Benzoyl- Thymine (N^3- Bz-T), under Mitsunobu conditions using triphenyl phosphine, diisopropyl diazadicarboxylate (DIAD) in tetrahydrofuran to obtain the nucleoside **4a**.[34] Nucleoside **4a** was reduced with lithium borohydride to obtain pyrrolidine thymidine alcohol **5a**.Pyrrolidine thymidine alcohol phosphorylated by following –Ludwig-Eckstein method[35] to synthesize the pyrrolidine thymidine triphosphate. The phosphorylation steps were monitored by mass spectrometry and identified the intermediates (Figure 2.9). Alcohol **5a** was treated with salicyl chlorophosphidite followed by addition of tributylammonium pyrophosphate which gave the cyclic phosphite derivative .The phosphite derivative on oxidation with iodine in pyridine/water gave oxidized cyclic triphosphate which on hydrolysis with aqueous ammonia yielded the triphosphate. The boc group of the triphosphate deprotected under the acidic conditions using 1N HCl and purified by HPLC to obtain the final product Pyrrolidine Thymidine Triphosphate (Pr-TTP) .The triphosphate was characterized by HRMS, ^{31}P and ^{1}HNMR before using the compound in biochemical evaluation. HPLC chromatogram and ^{31}P NMR spectrum of Pr-TTP are depicted in Figure 2.10 & 2.11.

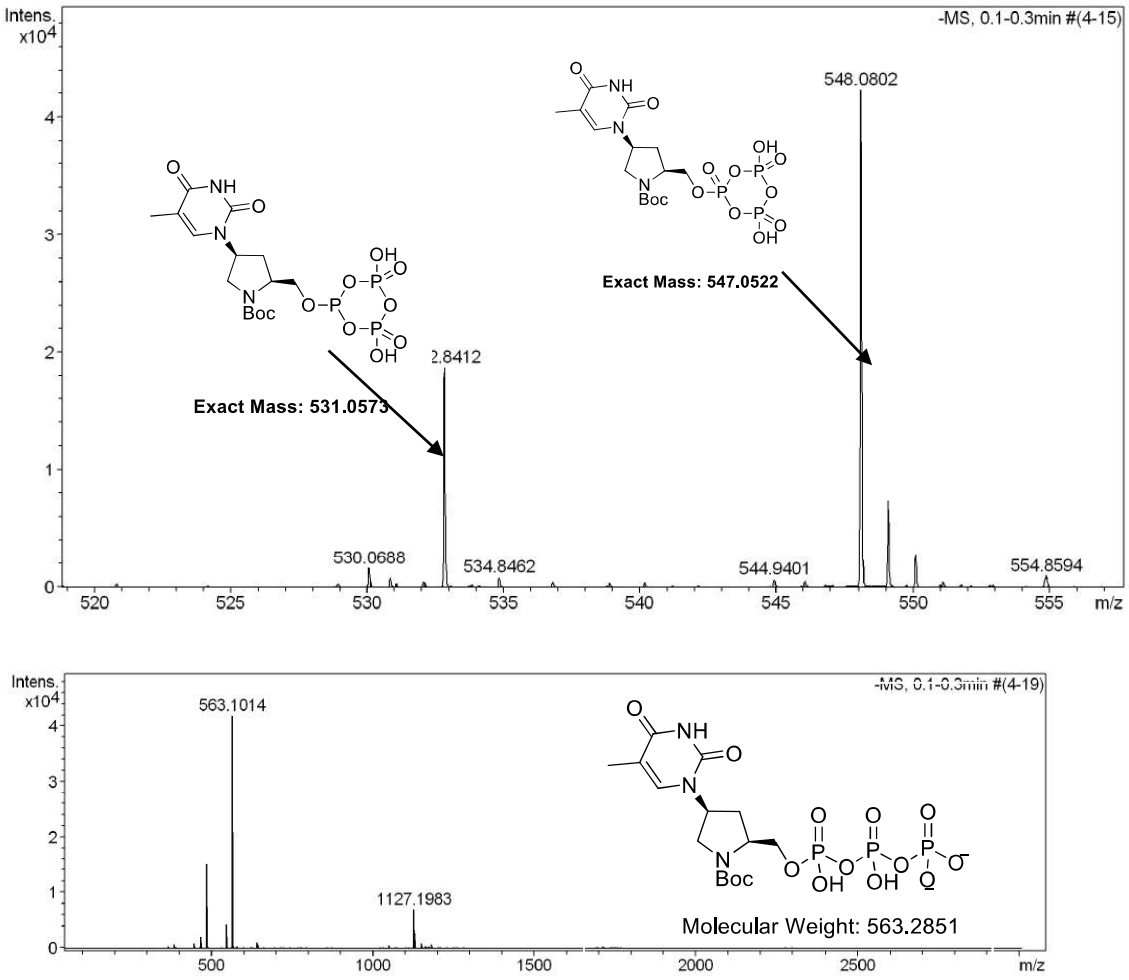


Figure 2. 9. Mass spectra of reaction intermediates of phosphorylation.

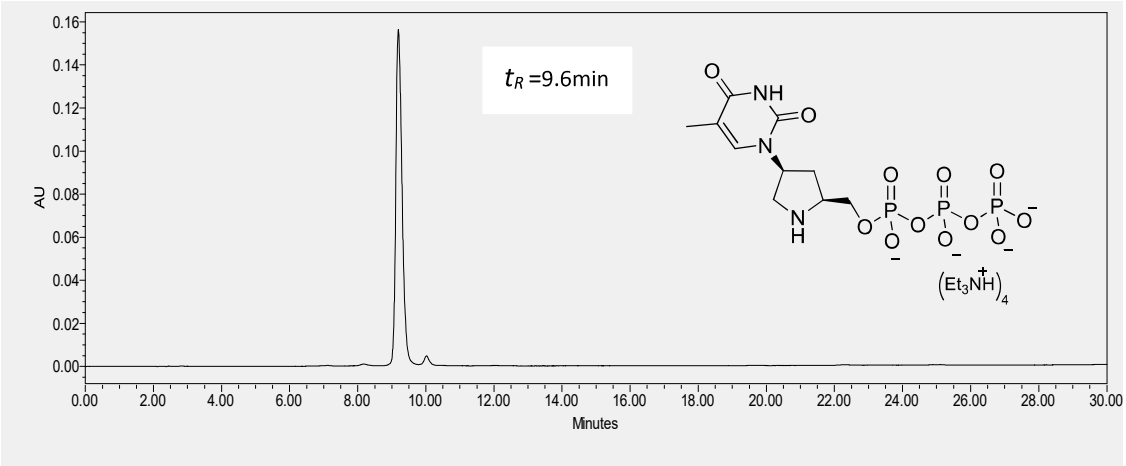


Figure 2. 10. Analytical chromatogram of Pr-TTP

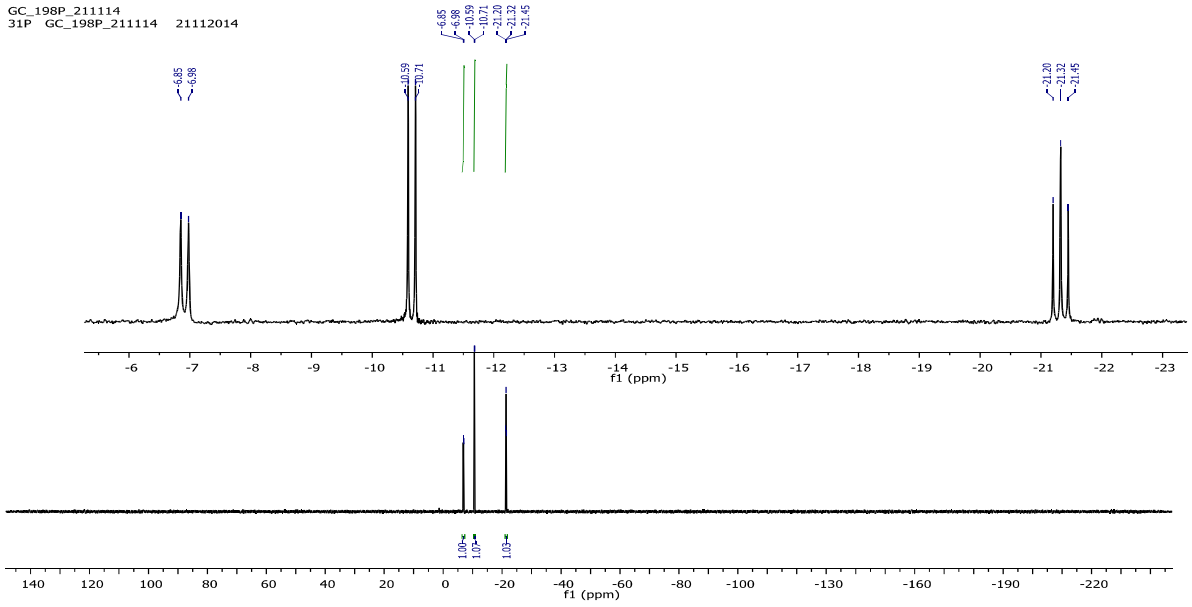


Figure 2. 11. ^{31}P NMR of Pr-TTP

After successful synthesis and characterization Pr-TTP was tested with many DNA polymerases. Finally we were able to detect the primer extension of Pr-TTP by Therminator DNA polymerase. After standardizing the synthetic methods and primer extension reaction condition by Pr-TTP we planned to synthesize the other three Pr-NTPs. Compound **2** was treated with mesylchloride to obtain the mesyl derivative (**3**). The reactive mesylderivative (**3**) was treated with N^4-Ac cytosine, N^6-Bz adenine and 2-amino 6-chloro purine, potassium carbonate, 18-crown-6, in dimethyl formamide to obtain the corresponding nucleoside derivatives. The nucleoside derivatives were reduced to nucleoside alcohols. Nucleoside derivative **5d** was treated with lithium hydroxide to convert the chloro group to oxy group. The phosphorylation and N-Boc deprotection steps of other nucleoside alcohols performed like thymidine alcohol and finally purified by HPLC to obtain the pyrrolidine cytisine triphosphate (Pr-CTP), pyrrolidine adenine triphosphate (Pr-ATP) and pyrrolidine guanine triphosphate (Pr-GTP).

2.2.2 Biochemical evaluation of Pr-NTP

To evaluate the biochemical activity of Pr-NTPs we tested the Pr-NTPs with different DNA polymerases. First we screened the activity of Pr-TTP with different DNA primer extensions of Pr-NTPs we used self-priming DNA templates designed for each NTP. (Table 2.1) We used another labelled primer (FAM P1) and template (T1) to study the primer extension reactions by gel-electrophoresis. For control studies we purchased ddNTPs from commercial sources.

Table 2.1. Sequence of primer and template

Entry	DNA Oligo nucleotides
1	Self-priming template (hairpin DNA Oligonucleotide) (**ON1**)5'-GTCA*GCGCCGCGCC-T 3'-CGCGGCGCGG-T
2	Self-priming template (**ON2**) 5'-GACT*GCGCCGCGCC-T 3'-CGCGGCGCGG-T
3	Self-priming template (**ON3**) 5'-ATCG*GCGCCGCGCC-T 3'-CGCGGCGCGG-T
4	Self-priming template (**ON4**) 5'-GATC*GCGCCGCGCC-T 3'-CGCGGCGCGG-T
5	FAM labelled self-priming template (**FAM-ON1**) FAM-5'-GTCA*GCGCCGCGCC-T 3'-CGCGGCGCGG-T
6	Primer (**P1**): 5'TGTAAAACGACGGCCAGT-3' Template (**T1**):3'ACATTTTGCTGCCGGTCAA*GTCGAGGCAT 5'
7	**FAM-P1**: FAM-5'TGTAAAACGACGGCCAGT-3' Template (**T1**):3'ACATTTTGCTGCCGGTCAA*GTCGAGGCAT 5'
8	**FAM-P1**: FAM-5'TGTAAAACGACGGCCAGT-3' Template (**T2**): 3'ACATTTTGCTGC CGG TCAT*GTCGAGGCAT
9	**FAM-P1**: FAM-5'TGTAAAACGACGGCCAGT-3' Template (**T3**): 3'ACATTTTGCTGC CGG TCAC*GTCGAGGCAT-5'
10	**FAM-P1**: FAM-5'TGTAAAACGACGGCCAGT-3' Template (**T4**): 3'ACATTTTGCTGC CGG TCAG*GTCGAGGCAT-5'

* Incorporation of complimentary dNTP/ddNTP/pr-NTP into primer

2.2.4 Mass spectrometry (LC-MS) studies of primer extension reactions

We standardized LC-MS methods[36,37] to confirm the primer extension reactions. First we standardized the methods with Pr-TTP and control ddTTP. The molecular weight of ON1 measured before and after the primer extension reactions. We found the increased molecular weight of ON1 as m/z 7961.1 $(M-5H)^-$ with Pr-TTP as m/z 8250.4 $(M-3H)^-$ like control ddTTP as m/z 8249.3$(M-5H)^-$.

The LC-MS analysis of extension of Pr-TTP with **ON 1** is depicted in Figure 2.12

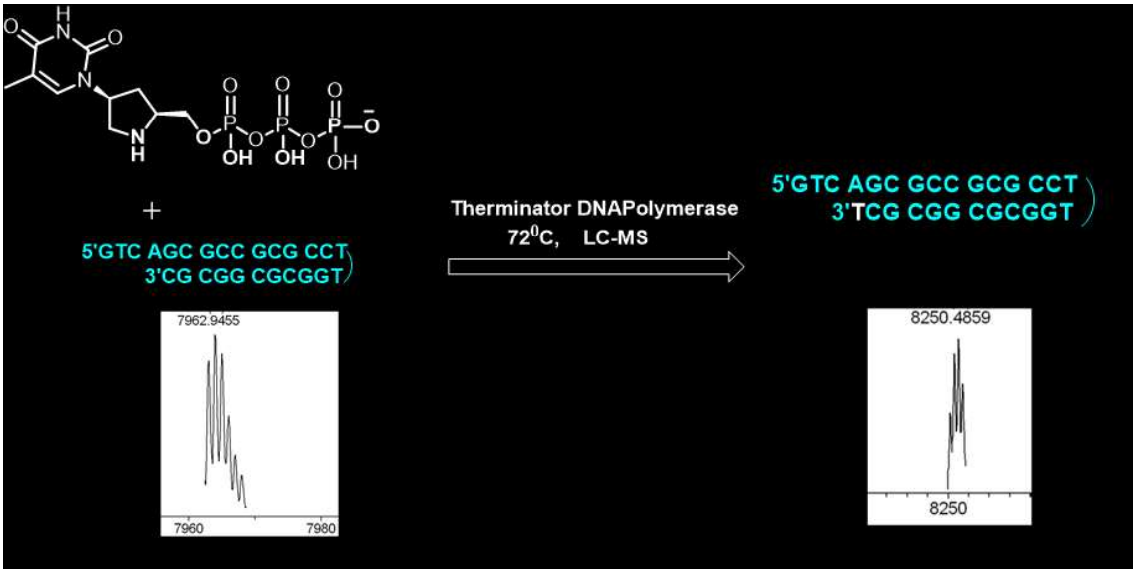


Figure 2. 12. LC-MS of primer extension reaction product of Pr-TTP with Therminator

After successful incorporation of Pr-TTP into oligonucleotide by Therminator DNA polymerase we have performed the similar experiments with other Pr-NTPs with designed self-priming DNA templates, Therminator DNA polymerase. We observed the extended mass of Pr-ATP with **ON 2** as m/z 8257.3$(m-5H)^-$,Pr-CTP with **ON 3** as

m/z 8250.2**(**M+NH4-5H)$^-$ and Pr-GTP with **ON 4** as m/z 8275.3 (m-5H)$^-$. All these results were summarized in the table 2.2. The LC-MS results strongly support that the Pr-NTPs are the substrate of Therminator DNA polymerase .To validate the termination property of Pr-NTPs we have performed an experiment with Pr-TTP, ON1 and other three dNTPs (dATP, dCTP and dGTP) in presence of Therminator DNA polymerase followed by LC-MS analysis. We observed only terminated product of ON1 with Pr-TTP. This experiment revealed that the Pr-TTP is working as terminator and cannot extend the further in presence of other dNTPs.

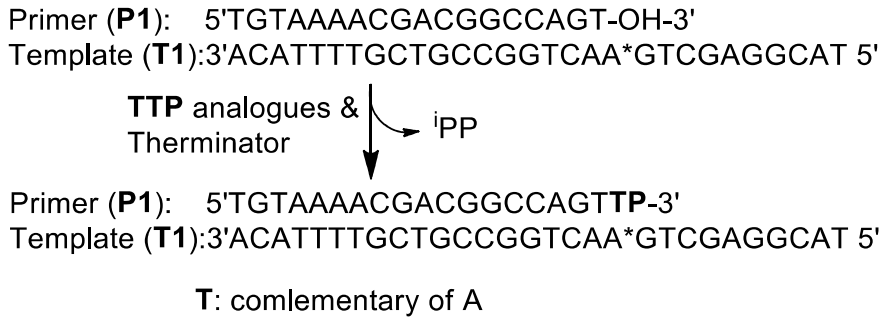
Table 2.2. ESI-MS analysis of primer extension of Pr-NTPs with Therminator

Entry	DNA Primer Observed mass [M-nH]$^-$	Nucleoside Triphosphate analogues	Mass of PCR Products (Observed from LC-MS)
1	**ON1**		(7961.604)[M-5H]$^-$)
2	**ON1**	*prTTP*	8250.485**(**[M-3H]$^-$
3	**ON1**	*ddTTP (control)*	8249.331 **(**[M-5H]$^-$
4	**ON2**	*prATP*	8257.313 **(**[M-5H]$^-$
5	**ON3**	*prCTP*	8250.241**(**[M+NH4-5H]$^-$
6	**ON4**	*prGTP*	8275.313 **(**[M-5H]$^-$

Further to ensure the incorporation of prNTPs into a typical DNA primer (**P1**) in presence of template (**T1**) and *Therminator* polymerase, we performed the similar reaction and mass studies of following mixture: DNA **P1**, DNA **T1**, *Therminator*

polymerase, and prTTP (Table 3). For control, the similar reaction was repeated with dTTP/ddTTP instead of prTTP, and studied by mass. The false incorporations of most reactive substrate dTTP into DNA **P1** were observed with *Therminator* but it was

Table 2. 3. Mass results of primer extension reaction products from DNA primer (**P1**) and NTP (dTTP/ddTTP/pr-TTP) and *Therminator* reaction



Entry	DNA Primer Observed mass $[M-nH]^-$	dTTP /ddTTP/ pr-TTP	Mass of PCR Products	No of Incorporations in **P1**
1	**P1** (5528.984 $[M-5H]^-$) &**T1**	*dTTP*	6450.137 **(**$[M+5H]^-$ &5833.1476 **(**$[M-3H]^-$,	**P1-(dTP)$_3$Three**extensions* /**P1-(dTP), one** extension**
2	**P1**& **T1**	*ddTTP*	5817.126 **(**$[M-3H]^-$	**P1-ddTP,** one extension
3	**P1**& **T1**	*pr-TTP*	5816.052 **(**$[M-3H]^-$	**P1-*Pr*TP**one extension

2.3.entry 2-3.

PCR Reaction time 30.0 minutes* like other NTP (ddTTP&dTTP) minutes and 5.0 minutes*

controlled to the single correct incorporation by reducing the reaction times of the same PCR (Table 3, Entry 1). In presence of same polymerase, single incorporation

of ddTTP/prTTP into DNA primer **P1** were also detected in their mass spectra (Table2.3)

2.2.5 Gel electrophoresis studies

Furthermore the visualization of an incorporation of prNTPs into labelled-DNA primer with *Therminator* polymerase was attempted by gel electrophoretic methods. For comparison, the similar gel-electrophoresis studies were performed with control dNTP/ddNTP and the same labelled DNA primer and polymerase. Herein, we used the FAM-labelled self-priming template (**5'-FAM-ON1**) and primer (**5-FAM-P1**) was treated with prNTP/dNTP/ddNTP derivatives in presence of *Taq*/*Therminator* DNA

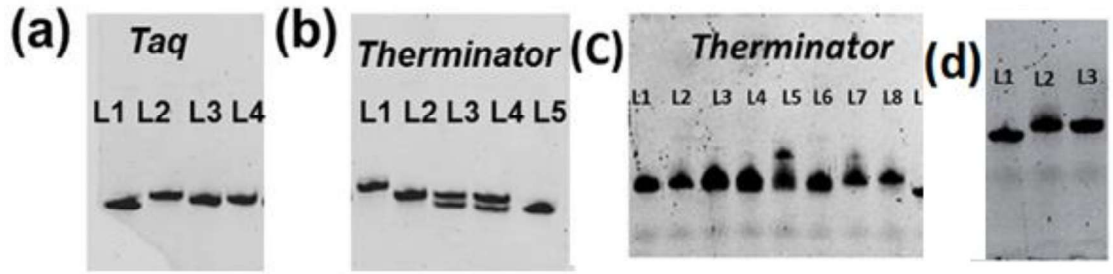


Figure 2. 13. 20% Denaturating Urea PAGE ran in 1x TBE buffer: (a) Primer extension reaction product with DNA *Taq* polymerase: (L1) **5'-FAM-ON1** primer; (L2) **5'-FAM-ON1** with dTTP; (L3) **5'-FAM-ON1** with ddTTP; (L4) **5'-FAM-ON1** with *pr*TTP. (b) Primer extension reaction product with DNA *Therminator po*lymerase: (L1) **5'-FAM-ON-1** with dTTP; (L2) **5'-FAM-ON1** with ddTTP; (L3) **5'-FAM-ON1** with *pr*TTP after PCR at 72 ºC; (L4) **5'-FAM-ON1** with *pr*TTP after PCR at 55 ºC; (L5) **5'-FAM-ON1** primer. (c) Primer extension reaction product with DNA *Therminator* polymerase 5'-FAM-P1 **(P2)** primer with ddNTP/prNTP:(L1) ddTTP ; (L2) prTTP; (L3) dATP; (L4) prATP; (L5) ddGTP; (L6) prGTP; (L7) ddCTP; (L8) prCTP; and (L9) Primer 5'-FAM-P1 **(P2)** (d) Primer extension reaction prodcucts with DNA *Therminator* polymerase L1: FAM-P1 , L2: Primer extension reaction product with prTTP, L3 :Primer extension reaction product of FAMP1 with ddTTP.

polymerase. The primer extended products were then analysed by the denaturating urea PAGE. The image of those gel-bands are illustrated in Figure 2. 13. In control,

the gel-band of primer extension product of dTTP: **FAM-ON1:** *Taq* reaction is marginally shifted from that of **FAM-ON1** (blank sample), while the gel-bands of primer extended products of ddTTP/prTTP: **FAM-ON1:***Taq* reaction matched with band of **FAM-ON1**(Figure 2.13a).The shifting of gel-band of labelled DNA only with dTTP:*Taq* support the incoporation of dTTP into DNA **FaM-ON1**. With *Therminator* polymerase, the gel-bands of Primer extended products of dTTP/ddTTP/prTTP and **5'-FAM-ON1** reaction were significantly shifted from blank but the extent of shifting are in following order: dTTP>ddTTP~prTTP>Blank (Figure 2.13 b). However, two close bands are appeared with prTTP even in two different conditions. The difference in the gel-shift band support the incorporation of dTTP/ddTTP/prTTP into DNA (**FAM-ON1**) but the amount of incorporation with dTTP was more than that of ddTTP/prTTP.

Further the incorporation of prNTPs (prATP/prCTP/prGTP) into typical labelled-primer (**FAM-P1**) with Therminator polymerase was also examined by the same gel-electrophoretic methods. First we performed the primer extension reactions with FAM-P1/T1 in presence of *Therminator* DNA polymerase and analysed by denaturing urea PAGE and the results are depicted in Figure 2.13.d. Lane 1 is the primer, lane 2 is the extended product with Pr-TTP and Lane 3 is extended product with dd-TTP. This experiment concluded that Pr-TTP gave single incorporation like control dd-TTP. We performed similar experiment with all four Pr-NTPs and control dd-NTPs using FAM-P1. Four Pr-NTPs (prTTP/prATP/prCTP/prGTP) along with control dd-NTPs (ddTTP/ddATP/ddCTP/ddGTP) were treated with respective template (**T1/T2/T3/T4**) in presence of *Therminator* polymerase and analysed by denaturing urea PAGE. The gel-band images prNTP and ddNTP are depicted in Figure 2.13 c. The gel-band of

DNA primer **FAM-P1** is significantly shifted with both prNTPs and ddNTPs. The gel-bands of extended products with prNTPs (prTTP/prATP/prCTP/prGTP) are matched with gel bands of respective four ddNTPs (ddTTP, ddATP, ddCTP and ddGTP). However, ddNTPs are known as sequencing terminators that allows single incorporation of ddTTP/ddATP/ddCTP/ddGTP into **FAM-P1**in presence of respective template (**T1-T4**).Thus prTTP, prATP, prCTP and prGTP are also singly incorporated into DNA primer **FAM-P1** in presence of respective template **T1, T2, T3 and T4** with *Therminator* polymerase. Hence gel-electrophoretic studies results, with Therminator polymerase, are consistently matching with LC-MS studies that support, prNTPs are novel DNA sequencing terminators as like ddNTPs.

2.3 Conclusion

In conclusion, we have successfully demonstrated the synthesis of the novel modified amino functionalized *pr*-NTP and their involvement in termination of DNA polymerization after single incorporation into primer with Therminator polymerase. We have also optimized the LC-MS method to confirm the incorporation of *pr*-TTP/dNTP/ddNTP into DNA primer directly from crude PCR product. Due to the amine functionality, prNTP analogues could be potential analogues for synthesis of amino functionalized NTP analogues possessing natural or modified nucleobases to develop labelled terminators.

2.4 Experimental section

Material and methods: All chemicals, solvents and reagents were purchased from commercial suppliers and used as received. DMF was distilled over CaH_2 and stored over 4Å molecular sieves, THF and Dioxnae were distille dover Na/Benzophenone and stored over sodium wire, pyridine was distilled over anhydrous potassium

hydroxide and stored over CaH_2 and tributyl amine was distiiled over potassium hydroxide and stored over 4Å molecular sieves. DNA oligos and FAM labelled primers were purchased from IDT. All enzymes and buffers for primer extension reactions were purchased from New England Bio labs.

Compounds **2-4** were synthesized by following literature report.[33]

*(2S, 4S)-4-N^3Benzoyl Thymidyl Boc prolyl methyl ester (**5a**):* NBoc L-hydroxyl proline ester (0.5 g, 2.04mmol) was dissolved in anhydrous tetrahydrofuran (10 ml) and stirred under nitrogen atmosphere for 10 minutes, N^3-Bz thymine (0.47 g, 2.04mmol) and triphenyl phosphine (0.64 g, 2.44mmol) were added and the resulting suspension stirred at $0^{O}C$ for 10 minutes under nitrogen atmosphere. Diisopropylazo dicarboxylate (0.48 ml, 2.44 mmol) was added drop wise at $0^{o}C$ and the reaction was warmed to stirr at room temperature overnight. The solvents were evaporated and the crude reaction mixture purified by column chromatography using methanol and dichloromethane on silica gel to obtain 466 mg of title compound as white foam in 50% yield. ^{1}H-NMR ($CDCl_3$, 400MHz) δ (ppm) (9H,s)1.44, (3H,s) 1.96, (1H,m,br) 2.14, (1H,m,br)2.76, (1H,m)3.55-.367, (3H,s)3.78, (1H,t *J*=8Hz)3.95, (1H,m,br)4.33-4.39, (1H,m.br) 5.21, (1H,s)7.37, (2H,m)7.46-7.50, (1H,t,*J*=8Hz)7.64, (1H,d, *J*=8Hz. ^{13}C-NMR ($CDCl_3$, 100MHz); δ (ppm) 12.76, 28.31, 35.69, 49.38, 52.63, 57.98, 70.13, 81.39, 111.72, 129.27, 130.56, 131.59, 132.50, 135.20, 136.15, 150.06, 162.54, 168.93, 173.20. HRMS (ESI-TOF): calcd. For [$C_{23}H_{27}N_3O_7Na$, M+Na] m/z 480.1741 found m/z 480.1743.

*(2S, 4S)-N-Boc-4-thyminyl prolinol (**6a**):* Anhydrous THF(10 ml) was added to $LiBH_4$ (0.038g, 1.7mmol) and cooled to $0^{O}C$ and stirred under nitrogen atmosphere for 15 minutes followed by addition of compound **4a** (0.2 g ,0.43 mmol dissolved in

tetrahydrofuran) at 0^{O} C under nitrogen atmosphere, the reaction was warmed to room temperature, and then stirred over night at room temperature . After completion the reaction was quenched with ammonium chloride solution and the reaction mixture concentrated to dryness and the residue dissolved in water, extracted with ethylacetate. Ethylacetate layer washed with water and brine solution, dried with anhydrous sodium sulphate and concentrated followed by purification on silica gel using dichloromethane and methanol afforded 123 mg of alcohol 5a as white solid in 87% yield. TLC 5% MeOH/DCM R_f =0.6 ^{1}H-NMR ($CDCl_3$, 400MHz): δ (ppm) (s, 9H) 1.47, (s,3H)1.94, (m, 1H)1.98-2.0, (m, 1H)2.39-2.46, (m, 1H)3.23-3.28, (m, 1H)3.65-3.69, (m, 1H)3.89-3.92, (m, 2H)3.99-4.01, (s,br1H) 4.30, (m, 1H)5.02-5.11, (s, 1H)7.13, (s,1H)8.73.^{13}C-NMR ($CDCl_3$, 100MHz); δ (ppm) 12.94, 28.22, 32.03, 31.67, 49.75, 51.40, 58.32, 66.63, 81.21, 112.03, 135.49, 151.06, 163.74. HRMS, (ESI-TOF): calcd for [$C_{15}H_{23}N_3O_5Na$ (M+Na)] m/z 348.1530 found m/z 348.1535.

*(2S, 4S)-Pyrrolidine Thymidine Triphosphate (pr-TTP) (**7a**):* Triphosp- horylation was performed by following Eckstein-Ludwig's procedure.[35]

*(2S, 4S) N-Boc-4-thyminyl prolinol (**6a**):* (0.03g, 0.10 mmol) dried by co-evaporation using anhydrous pyridine, followed by drying under high vacuum over P_2O_5 in a vacuum desiccator for one hour at ambient temperature. Desiccator opened under nitrogen atmosphere and round bottom flask closed with a septum and a nitrogen balloon attached. Alcohol 4 dissolved in 0.5 ml anhydrous pyridine and 1.0 ml anhydrous dioxane. Salicylchlorophosphidite (0.02g, 0.11mmol) dissolved in anhydrous dioxane (1.0 ml) was added and stirred at room temperature. After 10 minutes a well vertexed mixture of tri- butylammonium pyrophosphate (0.08g, 0.16mmol) and tri-butylamine (0.17mL, 0.74 mmol) in anhydrous dimethylformamide

(DMF) (1mL) added. After 10 minutes, Iodine solution (1% in pyridine/water 98/2) was added till the brown color remained. After 10 minutes concentrated ammonia (5 ml) was added and reaction was monitored for complete hydrolysis of cyclic triphosphate. Hydrolysis step was monitored by mass spectrometry. After three hours complete hydrolysis of cyclic triphosphate was observed, then solvents were lyophilized, the residue was again dissolved in 10 ml distilled water and then washed with ethyl acetate (15mL X 3). The aqueous layer was loaded to anion-exchange column (DEAE Sephadex A-25) column, and then eluted with triethyl ammonium bicarbonate (TEAB) buffer (50.mM-1000mM). Compound was identified by UV specroscopy and mass spectrometry. Again compound was dissolved in 5.0 mL Milli-Q-water, cooled to 0ºC before adding 0.5 ml (11.0N HCl) and warmed to room temperature to remove Boc group. After one hour complete deprotection was observed, and then quenched with dilute ammonia at 0ºC. This solution was further lyophilized and purified by HPLC to obtain the pure compound **7a** in form of triethylammonium salt.^{1}H-NMR (D_2O, 400 MHz): δ (ppm) 1.89 (s, 3H), 2.23-2.31(m, 1H), 2.62-2.70(m, 1H), 3.66-3.73(m, 2H), 4.04-4.06 (m,1H), 4.21-4.28(m,1H), 4.41-4.43(m,1H), 5.04-5.08(m,1H), 7.56(s,1H). ^{31}P-NMR (162 MHz, H_3PO_4); as external standard at δ 0.0), δ (ppm) 6.95 (d, J = 19.44Hz, 1P, P_γ), 10.65 (d, $J_{\beta\gamma}$=21.0Hz, 1P, P_α), 21.32 (t, 1P, P_β) $J_{\alpha\beta}$=19.5Hz, $J_{\beta\gamma}$=21.06Hz), HRMS (ESI-TOF) calcd for [$C_{10}H_{17}N_3O_{12}P_3$ (M-H)] m/z464.0020, found m/z464.0037.prATP, prGTP and prCTP (all 100µmol scale) were also synthesized by following the above procedure.

General procedure for nucleosidation: Mesyl compound (1.0 g ,3.07mmol) ,one of the protected nucleobases (N^6 -Bz adenine or N^4-Ac cytosine or 2-Amino 6-chloropurine (1.0 eq)),anhydrous potassium carbonate (0.63 g, 4.6 mmol) and a

catalytic amount of 18-crown-6 (0.16g, 0.61 mmol,0.2 eq, 0.5 eq in case of 2-amino 6-chloro purine) were stirred at 75^{O}C for 12 hours in 20 ml of anhydrous DMF under nitrogen atmosphere .After completion of reaction indicated by TLC, DMF removed in rotavapour under reduced pressure and purified on silica gel using dichloromethane and methanol.

Adenine methyl ester ***(5b)****:* 0.93 g of white solid obtained in 65% yield. TLC EtOAc *Rf* = 0.4[1] HNMR 400 MHz$CDCl_3$: (9H,s)1.46, (1H,m,br)2.55, (1H,m)2.97-2.99, (3H,s)3.71, (1H,m)3.94-4.07, (1H,m)4.18-4.22, (1H,m)4.45-4.52, (1H,m) 5.26, (2H,t *J*=8Hz)7.53, (1H,m)7.60-7.63, (1H,d, *J*=8Hz)8.03, (1H,s)8.21, (1H,s)8.79, (1H,s,br)9.05. ^{13}CNMR 100MHz $CDCl_3$: 28.23, 35.09, 36.16, 50.12, 50.92, 52.12, 52.45, 57.61, 81.26, 123.18, 128.01, 128.79, 132.76, 133.65, 141.18, 149.76, 152.00, 152.53, 153.31, 164.97, 172.34. HRMS (ESI-TOF): calcd for [$C_{23}H_{26}N_6O_5$ (M+H)] m/z 467.2037, found m/z 467.2044.

Cytosine methylester ***(5c):*** 0.7 g of cytosine ester obtained as white solid in 60% yield. TLC 40% $CHCl_3$/EtOAc R_f =0.33 ^{1}HNMR (400 MHz, $CDCl_3$): (9H,s) 1.38ma,1.42 mi, (3H,s) 2.18, (1H,m)2.34-2.37, (2H,m),2.56-2.61, (3H,s)3.65 mi,3.67ma, (1H,m)4.35-4.38 ma,4.57-4.60 mi, (1H,m) 5.35-5.41, (1H,m)7.74-7.76, (1H,m)8.29-8.32, (1H,s,br) 8.89 ma,8.95 mi. ^{13}C NMR (100MHz, $CDCl_3$): 24.71, 28.29, 28.42, 35.45, 36.18, 51.77, 52.25, 52.39, 57.54, 57.82, 74.10, 75.33, 80.36, 80.42, 104.2, 114.10, 153.87, 154.38, 159.40, 160.30, 163.51, 170.08, 172.60. HRMS: calcd for [$C_{17}H_{24}N_4O_6$ (M+H)] m/z 381.1769 found m/z 381.1743.

(2S, 4S) 2-Amino 6-chloro purine ester ***(5d):*** 0.61 g of obtained as white solid in 50% yield. TLC 35% EtOAc/DCM *Rf* = 0.27 ^{1}HNMR (400 MHz, $CDCl_3$): (9H,s)1.43, (1H,m,br)2.48, (1H,m,br)2.86, (3H,s) 3.72, (1H,m)3.77-3.91, (1H,m)4.09-4.15,

(1H,m)4.40-4.47, (1H,m)4.96-7.97, (1H,m,br)5.23-5.28, (1H,s)7.88. ^{13}C NMR (100MHz, $CDCl_3$): 28.31, 34.87, 35.81, 49.94, 50.49, 51.98, 52.52, 57.41, 57.70, 81.28, 125.50, 140.29, 151.65, 153.36, 153.81, 159.10, 159.16, 172.38. HRMS, (ESI-TOF): calcd for [$C_{16}H_{21}N_6O_4Cl$ (M+H)] m/z397.1386, found m/z 397.1341.

*Adenine alcohol (**6b**):* 0.5 g of N^6-Bz Adenine methyl ester was reduced to Adenine alcohol by following procedure as described for the synthesis of compound 5a. 0.284 g of 5b obtained as white powder in 75% yield. LC 10%MeOH/EtOAc *Rf* =0.27 ^{1}HNMR (400MHz, $CDCl_3$) (9H,s)1.43, (1H,m)2.30-2.41, (1H,m,br)2.65, (2H,m)3.63-3.72, (1H,m,br)3.89, (3H,m)4.1-4.18, (1H,t J=8Hz)5.0, (2H,s,br)6.55, (1H,s)7.93, (1H,s)8.26. ^{13}C NMR (100MHz, $CDCl_3$): 28.47, 33.56, 51.34, 52.22, 58.91, 66.30, 81.02, 114.34, 119.77, 124.05, 138.76, 139.34, 149.85, 152.88, 155.92, HRMS, ESI-TOF calcd for [$C_{15}H_{22}N_6O_3$ (M+H)] m/z335.1826, found m/z 335.1873.

*Cytosine alcohol (**6c**):* 0.5 g of N^4 -Ac Cytosine methyl ester was reduced to cytosine alcohol by following procedure as described for the synthesis of compound **5a**. 0.32 g of **5c** obtained as white powder in 80% yield. TLC EtOAC *Rf* = 0.42 ^{1}HNMR (400 MHz, $CDCl_3$): (9H,s)1.43, (2H,m)1.95-2.04, (1H,s)2.35-2.36, (1H,s)3.53-3.56, (2H,s)3.63-3.72, (1H,s)3.89-3.94, (1H,s)4.06-4.12, (1H,s,br)5.36, (2H,s,br)5.49, (1H,d J=4Hz)6.08-6.09, (1H,d J=4Hz)7.92-7.93.^{13}C NMR (100MHz, $CDCl_3$): 28.5, 34.37, 53.33, 59.54, 60.50, 67.50, 74.46, 80.70, 100.09, 156.82, 157.04, 164.17, 165.15, 17.31, 175.20. HRMS, (ESI-TOF) calcd for [$C_{14}H_{24}N_4O_4$ (M+H)] m/z 311.1714 found m/z 311.1726.

***(**2S,4S)2-Amino 6-chloro purine alcohol (**6d**):* 0.5g of 2-Amino 6-chloro purine ester was reduced to 2-amino 6-chloro purine alcohol by following procedure as described for the synthesis of compound **5a.** 0.32 g of compound **5d** obtained in 70% yield as a

white foam. TLC 20%EtOAc/DCM R_f =0.32 ^{1}HNMR (400MHz, $CDCl_3$ (9H,s)1.45, (1H,m)2.02-2.07, (1H,m)2.30-2.37, (1H,m)2.60-2.65, (1H,m)3.62-3.66, (1H,m)3.71-3.75, (1H,m)3.85-3.87, (2H,m)4.08-4.10, (1H,m)4.82-4.90, (2H,s,br)5.39, (1H,s)7.87 ^{13}C NMR 100MHz$CDCl_3$:28.47, 31.99, 33.25, 50.77, 52.22, 59.04, 62.71, 66.07, 81.33, 99.04, 114.15, 125.53, 139.37, 140.67, 151.72, 153.68, 159.06, 174.45. HRMS: calcd for [$C_{15}H_{21}N_6O_3Cl$ (M+H)] m/z 369.1436 found m/z 369.1406.

*(2S, 4S) 2Amino -6oxo- purine alcohol (**6d'**):* 0.2 g of *(2S, 4S)* 2-Amino 6-chloro purinyl prolyl alcohol (**5d**) was dissolved in 4 ml of 1,4 dioxane and 4 ml of 1N LiOH solution was added and stirred at room temperature overnight solvents were removed under reduced pressure and the reaction mixture dried in roto followed by purification on silicagel obtained 0.09 g of title compound as white powder in 50% yield. ^{1}H NMR (400MHz ,CD_3OD): (9H,s)1.49, (1H,m)2.01-2.13, (1H,m) 2.55-2.61, (1H,m)3.19-3.24, (1H,m)3.57 , (2H,m)3.72-3.81, (1H,m)4.00 ,(1H,m)4.16, (1H,s)7.89. ^{13}CNMR (100MHz, CD_3OD):28.69, 30.73, 33.06, 34.88, 53.40, 59.14, 63.90, 81.69, 114.68, 128.54, 128.79, 129.03, 140.14, 155.22.HRMS, ESI-TOF calcd for [$C_{15}H_{22}N_6O_4$ (M+H)] m/z 351.1775 found m/z 351.1771.

*Adenine triphosphate(**7b**):*^{1}HNMR (400 MHz ,D_2O): (1H,m)1.79, (1H,m)2.39-2.44, (2H,m)2.91-3.11, (1H,m)3.82-3.96, (1H,m)4.11-4.21, (1H,m) 4.31-4.62, (1H,m)5.46-5.51, (1H,s)8.29, (1H,s)8.32. ^{31} PNMR (162 MHz, D_2O): (-21.11, t,1P, P_β), (-10.52,-10.64,d ,1P, P_γ), (-5.32,-5.50 , d ,1P,P_α) ($J_{\alpha\beta}$=29.16Hz , $J_{\beta\gamma}$=19.44Hz) HRMS,ESI-TOF calcd for [$C_{10}H_{16}N_6O_{10}P_3$(M-H)] m/z473.0135, found m/z 473.0144.

*Cytosine triphosphate (**7c**):* ^{1}HNMR(400MHz, D_2O) : (1H,m)2.15-2.18, (1H,m)2.66-2.74, (1H,m)3.57-3.62, (1H,m)3.73-3.76, (3H,m)4.10-4.30, (1H,s,br) 5.69, (1H,d J=8Hz)6.36-6.38, (1H,d J=4 Hz)7.90-7.91. ^{31} PNMR(162 MHz, D_2O) : (-21.87, t 1P,

P_β),(-11.01-11.03 1P,P_α) (-7.59-7.72,1P,P_γ), HRMS: calcd for [$C_9H_{16}N_4O_{11}P_3$(M-H)] m/z 449.0023 found m/z 449.0095.

*Guanine triphosphate (**7d**):* ^{1}HNMR (400 MHz, D_2O): (1H,m)2.20-2.28, (1H,m)2.86-2.94, (1H,m)3.76-3.90, (1H,m)4.07-4.08, (2H,m)4.38-4.42, (1H,m)5.34-5.38, (1H,s)7.78 ^{31} PNMR (162 MHz, D_2O): (-21.17,t, 1P ,P_β), (-10.63,-10.76, d, 1P, P_α) (-0.32,-0.44P,P_γ) ($J_{\alpha\beta}$ =21.06 Hz, $J_{\beta\gamma}$=19.44 Hz) HRMS, (ESI-TOF) calcd for [$C_{10}H_{15}N_6O_{11}P_3$ (M-2H)] m/z488.0006 found m/z 488.0079.

Primer extension reactions and LC-MS Procedures

Primer extension experiments for LC-MS analysis: For all enzymatic reactions with TTP analougues self-priming DNA oligomer (ON-1). 5'-GTC AGC GCC GCG CCT TGG CGC GGC GC-3' used

Ventexo- DNA polymerase: Self-priming DNA template (5µM), dTTPorprTTP (100µM), 10X thermopol buffer (2.5µl) and Ventexo DNA polymerase (1 unit) in 25µl reaction mixture incubated at 60^{O}C for 60 minutes followed by LC-MS analysis.

Klenowexo-: Self-priming DNA template (5µM), dTTP or prTTP (100 µM), 10X NEB2 buffer (2.5µl) and Klenowexo DNA polymerase (25 units) in 25µl reaction mixture incubated at 37^O C for 60 minutes followed by LC-MS analysis.

Taq DNA Polymerase: Self-priming DNA template (5µM), dTTP or pr- TTP (100 µM), 10X standard Taq buffer (2.5µl) and Taq DNA polymerase (10 units) in 25µl reaction mixture incubated at 72^{O}C for 60 minutes followed by LC-MS analysis.

Bst DNA Polymerase: Self-priming DNA template (5µM), dTTP or pr- TTP (100 µM), 10X Thermopol buffer (2.5µl) and Bst DNA polymerase (16 units) in 25µl reaction mixture incubated at 65^{O}C for 60 minutes followed by LC-MS analysis. Boric acid

and EDTA pH 8.0) buffer and run in 1X TBE at 90V for 3 hours.Images recorded using Bio Rad Gel doc system.

Therminator DNA Polymerase: Self-priming DNA oligomer (ON-1) or FAMON1(5μM), dTTP or pr-TTP (100μM), 10X thermopol buffer (2.5μl) and Therminator DNA polymerase (2units) in 25μl reaction mixture incubated at 72^{O}C for 60 minutes followed by LC-MS analysis or denaturating PAGE analysis.

For primer extension reaction of other prNTPs following self-priming DNA templates were used: prTTP: **ON1**; prATP: **ON2**; prCTP: **ON3**; prGTP:**ON 4**.

Gel experiments were performed using 5'-FAM labelled primer (FAM-P1) in combination with suitable template for each NTP (prNTP/ddNTP).

A reaction mixture containing 5'-FAM labelled Primer P1 **FAM P1**(1μ mol) and suitable template (1μ mol) T1 for prTTP and ddTTP, T2 for pr-ATP and ddATP , T3 for pr-GTP and ddGTP , T4 for pr-CTP and ddCTP , 10xThermo pol buffer (1.0 μl), NTPs (prNTPorddNTP) (100 μ mol) and 1 unit of Therminator polymerase in total volume of 10 μl were incubated at 55^{O}C for 30 minutes and subsequently reactions were quenched with 10 μl of 2x loading dye (contains 90% formamide, 0.5% EDTA, 0.1% xylene cyanol and 0.1% bromphenol blue) by heating at 85^{O} C for 5 minutes followed by cooling at 4^{O} C. Primer extension reactions were analysed by 20% denaturating urea PAGE. Gels prepared and ran in 1X TBE (Tris Boricacid EDTA, pH 8.0) buffer.

LC-MS experiments: LC-MS experiments performed using BrukermicroTOF-Q II coupled with a Waters Acquity UPLC auto sampler. Primer extension reaction products diluted with 25μl Milli-Q water and 10.0 μl from this mixture injected by auto sampler.

LC-MS experiments performed using 5mM ammoniumacetate (bufferA pH 7.0) and methanol (buffer B) with the following programme at a flow rate 0.2ml/minute linear gradient.0-10 min80% A gradient,10-12 min 30% A gradient , at 12- 13 min30%A isocratic,13-14min 100% A gradient, at 14-15 min 100% A isocratic. A C-18 RP UPLC column (2.1 x 5.0 mm, 1.7µm, Acquity Waters) used for LC-MS experiments. Column temperature was maintained 30^{o} C during the experiments.

2.5 References

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2.6Appendix

1. $^{1}H,^{13}C$ & ^{31}P NMR Spectra and HRMS of Compounds **2-7**

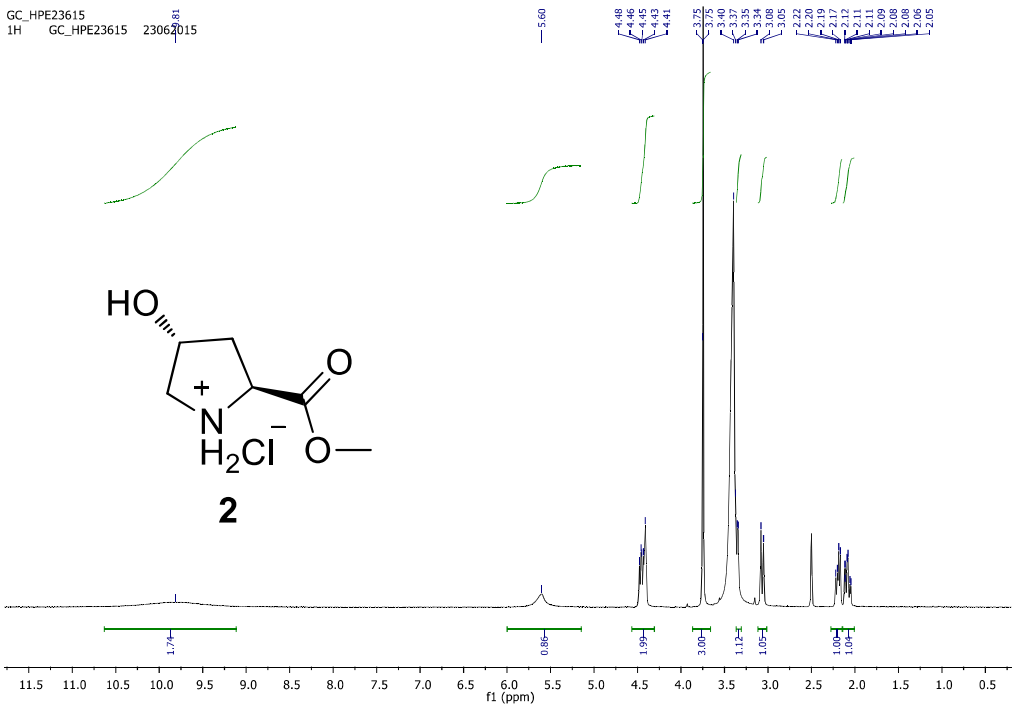


Figure A1 ^{1}HNMR Compound **2**

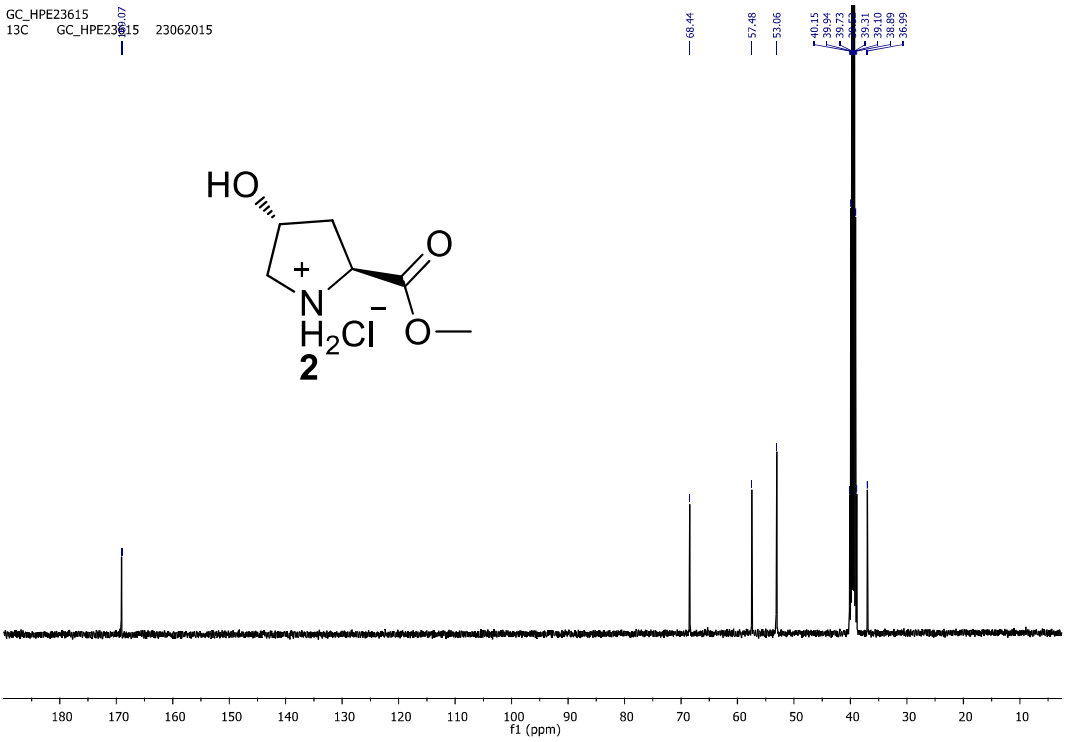


Figure A 2 ^{13}C NMR of Compound **2**

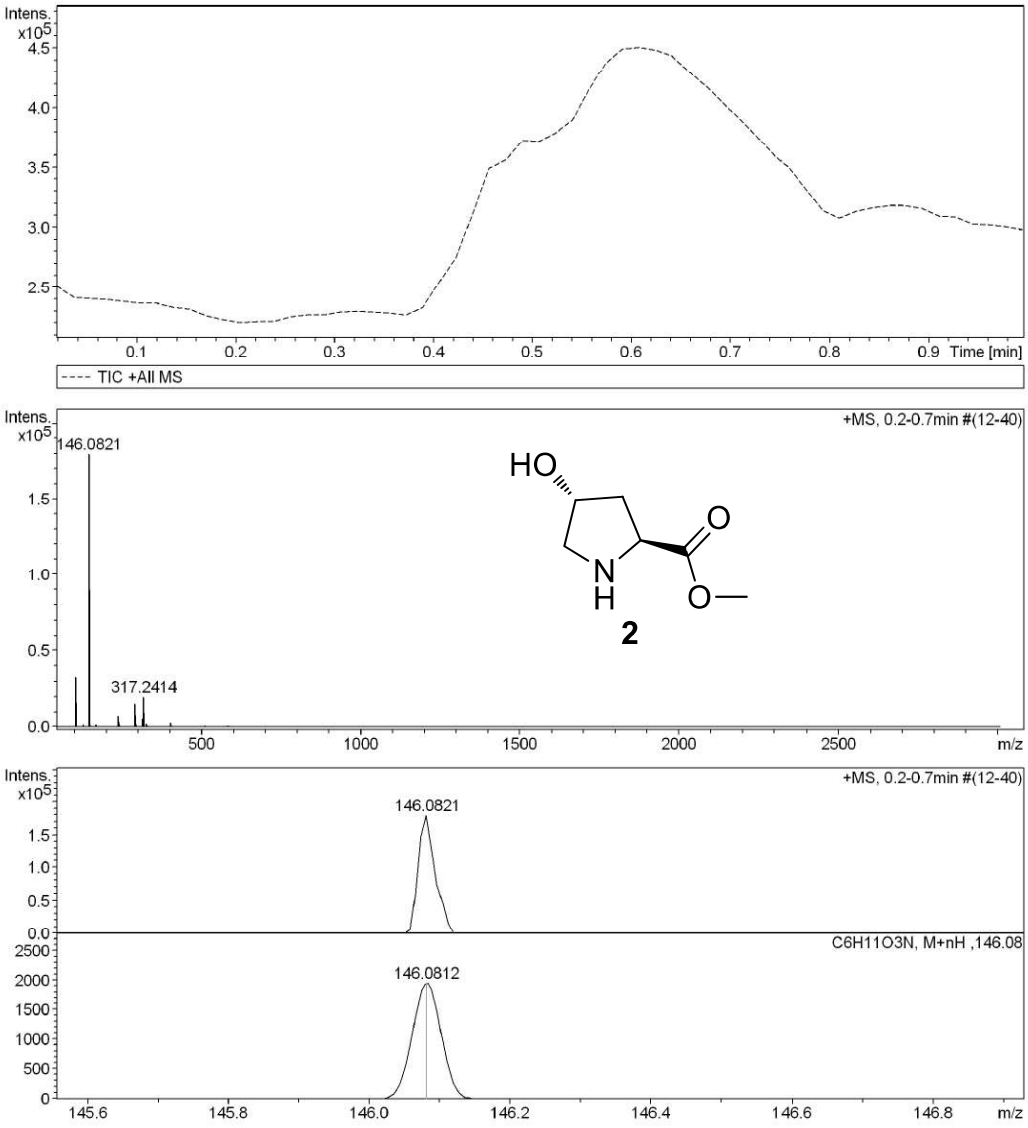


Figure A3 HRMS Compound **2**

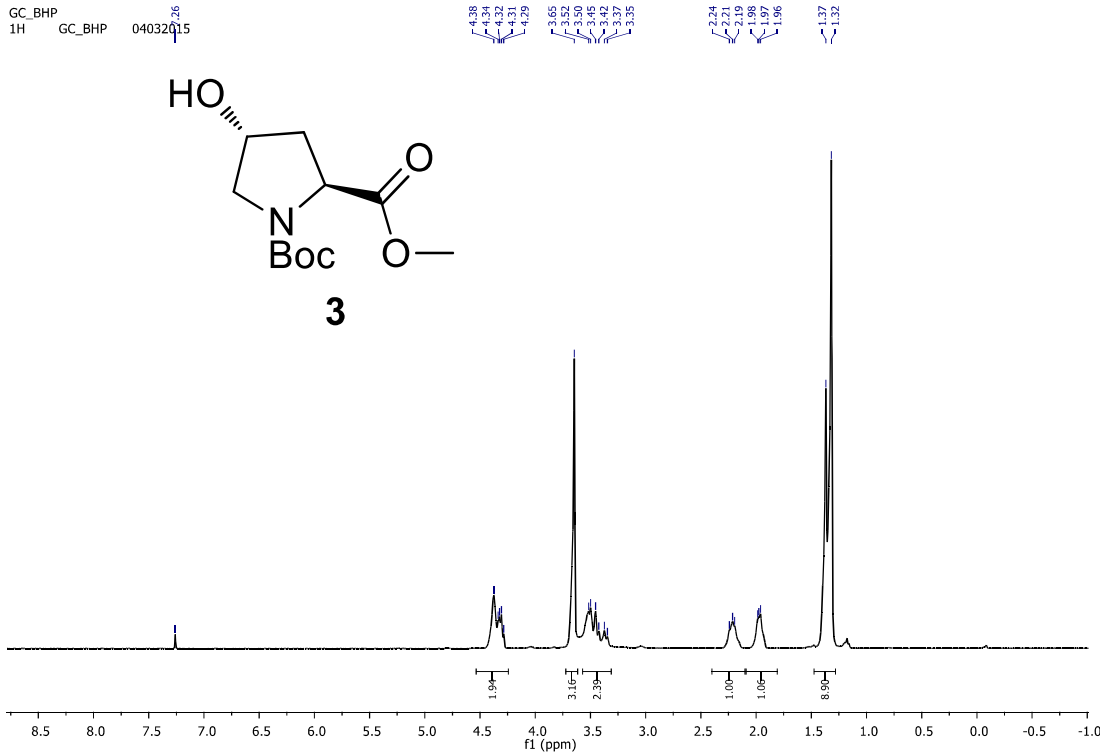


Figure A 4 ^{1}HNMR of Compound **3**

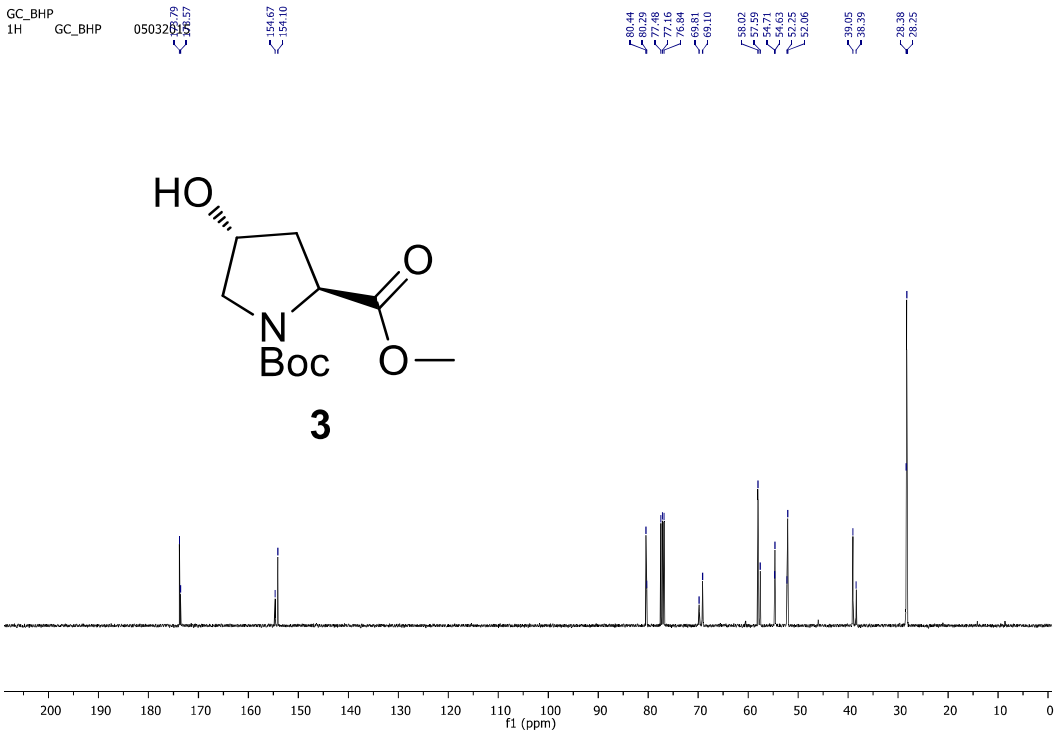


Figure A5 ^{13}CNMR of Compound **3**

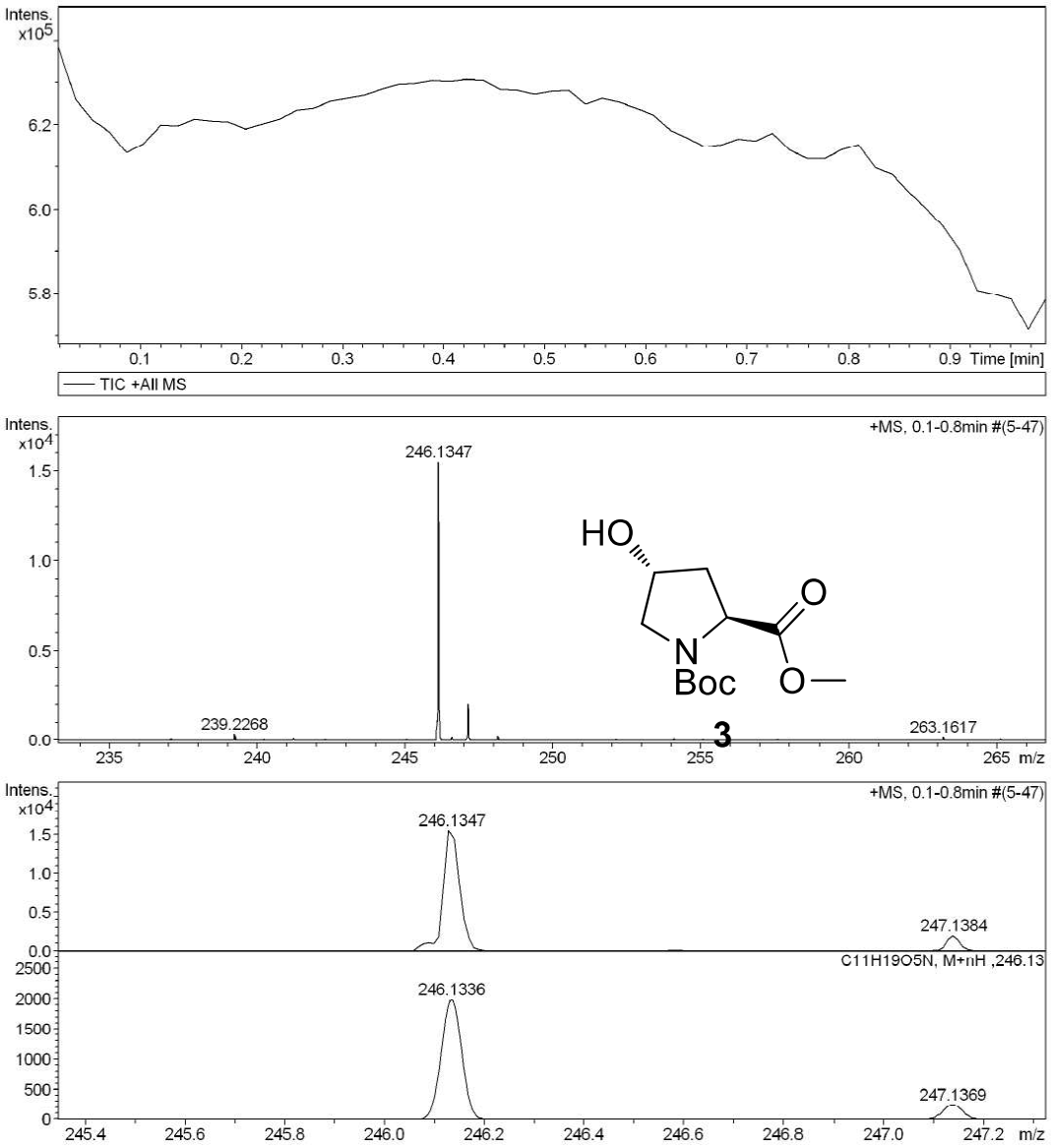


Figure A6 HRMS of Compound **3**

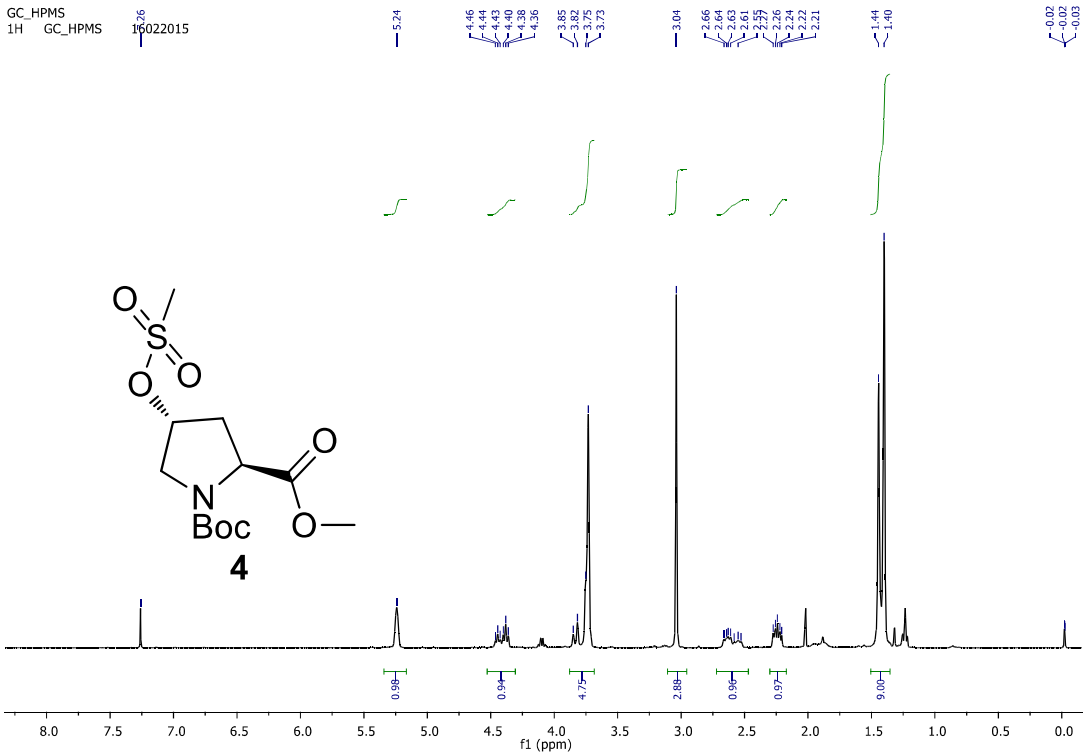


Figure A7 ^{1}HNMR of Compound **4**

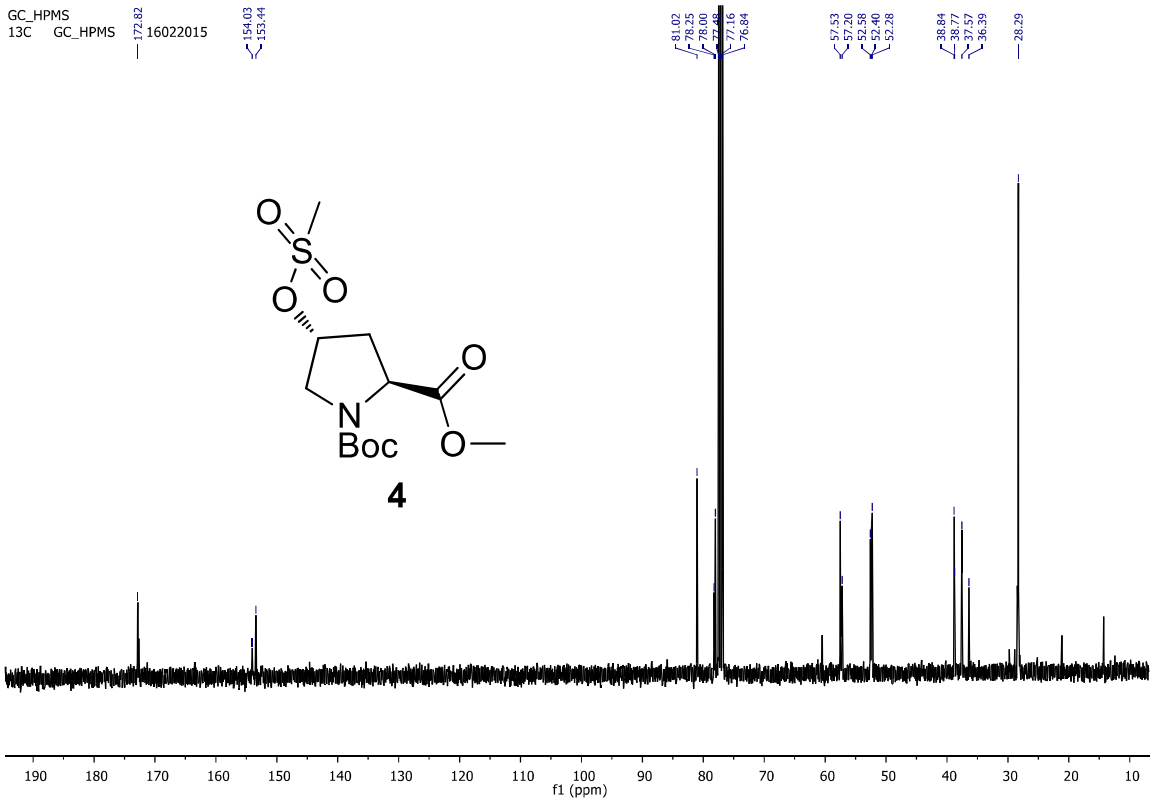


Figure A8 ^{13}CNMR of Compound **4**

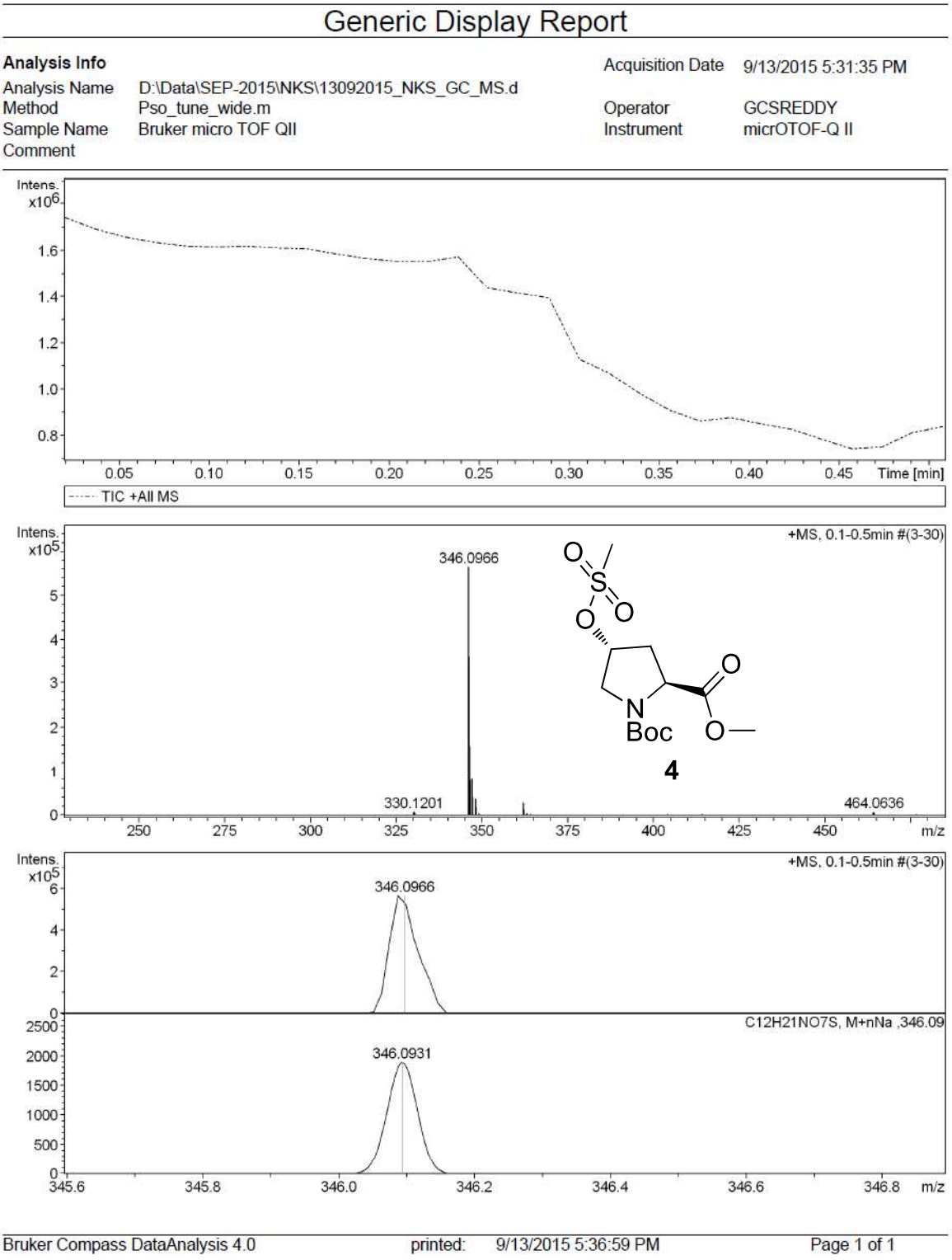


Figure A9 HRMS of Compound **4**

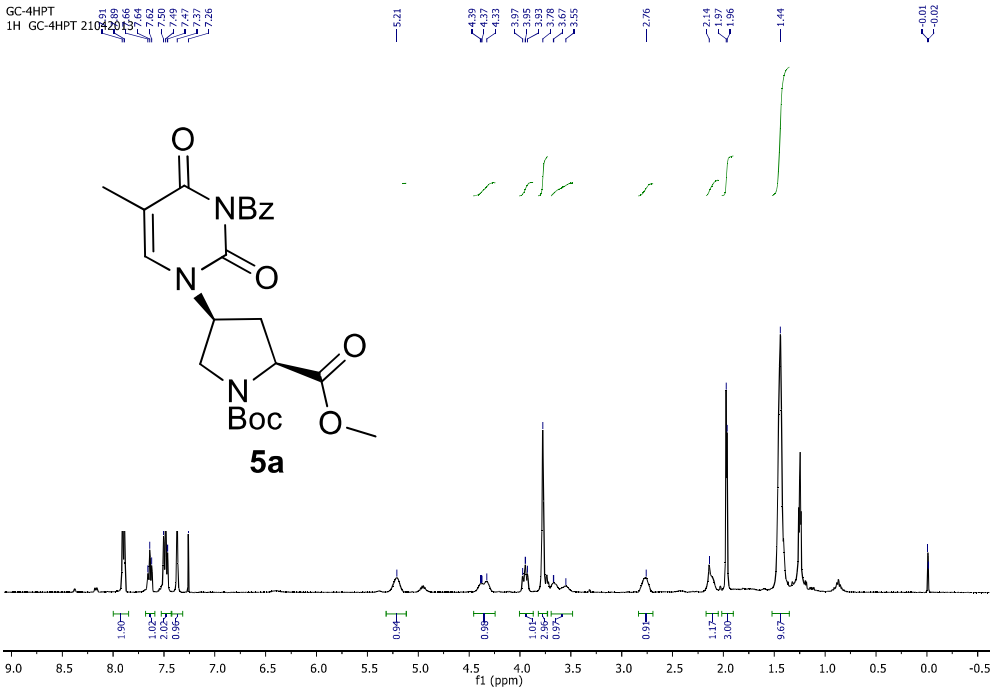


Figure A10 ^{1}H-NMR of compound **5a**

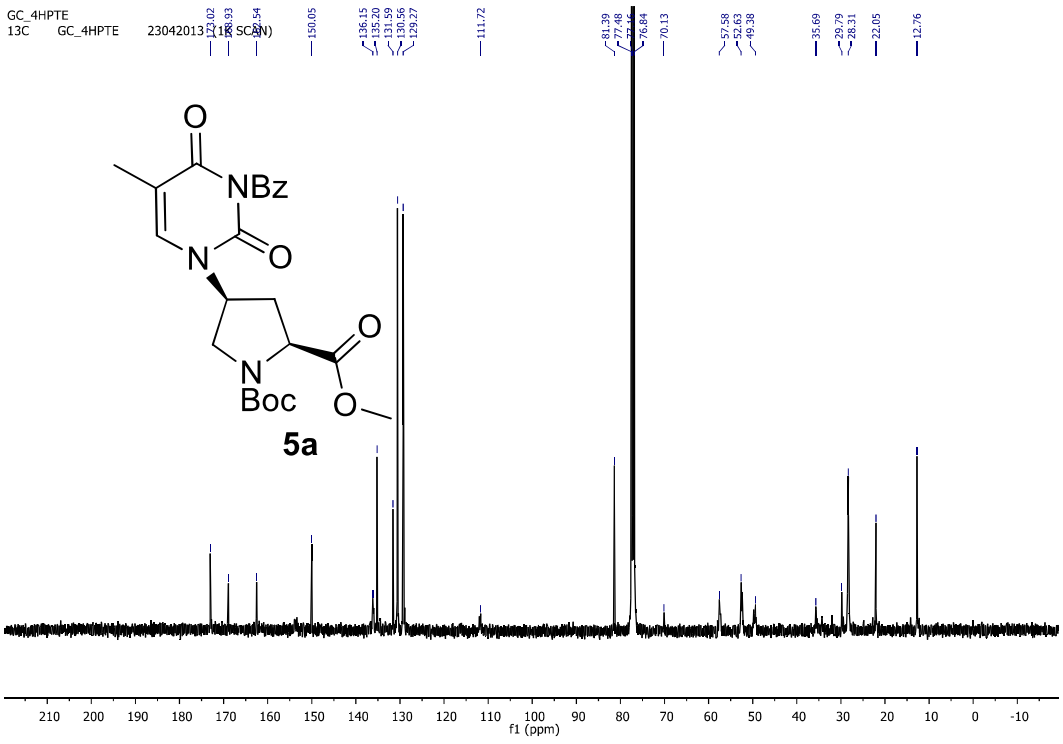


Figure A11 ^{13}C-NMR of compound **5a**

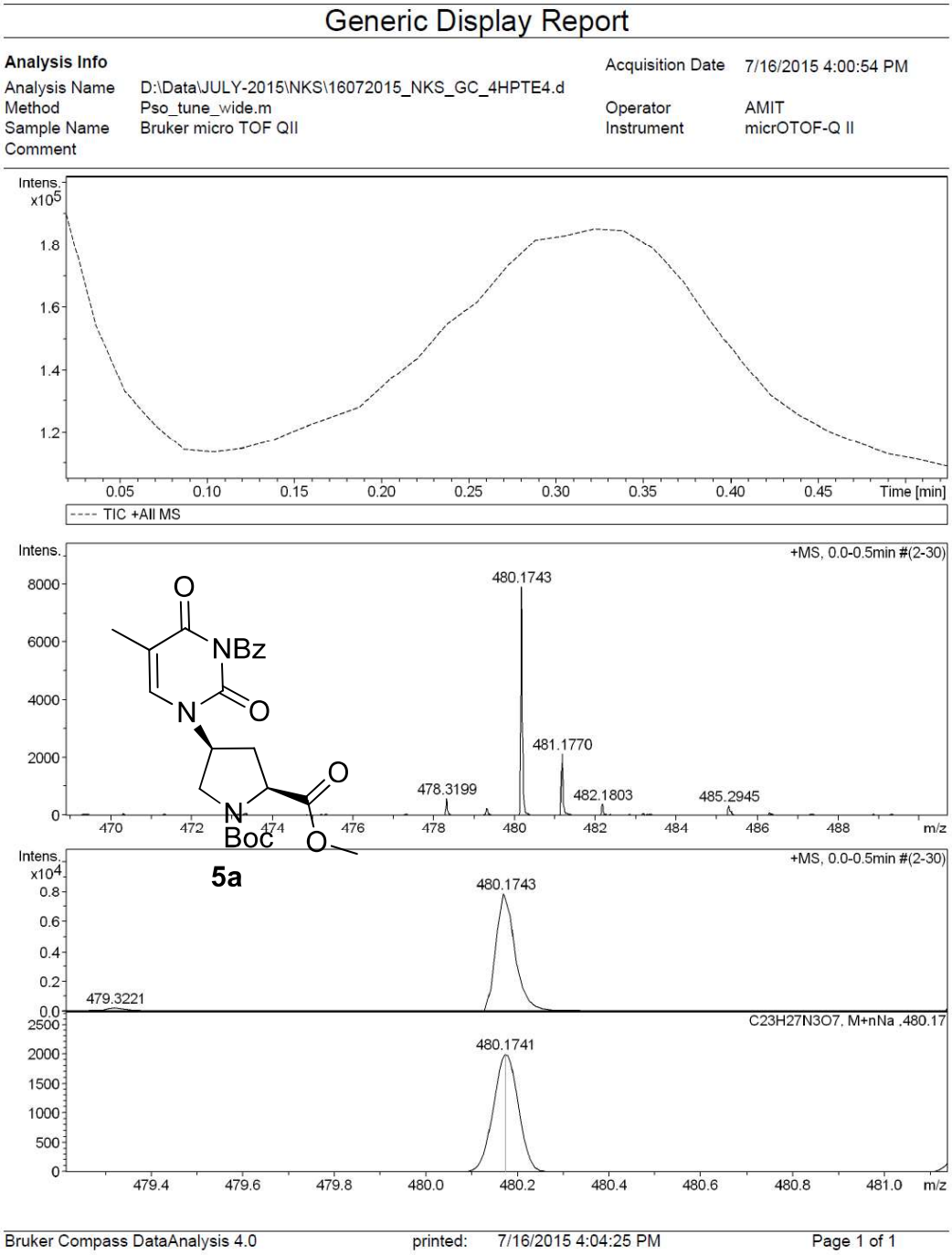


Figure A12 HRMS of compound **5a**

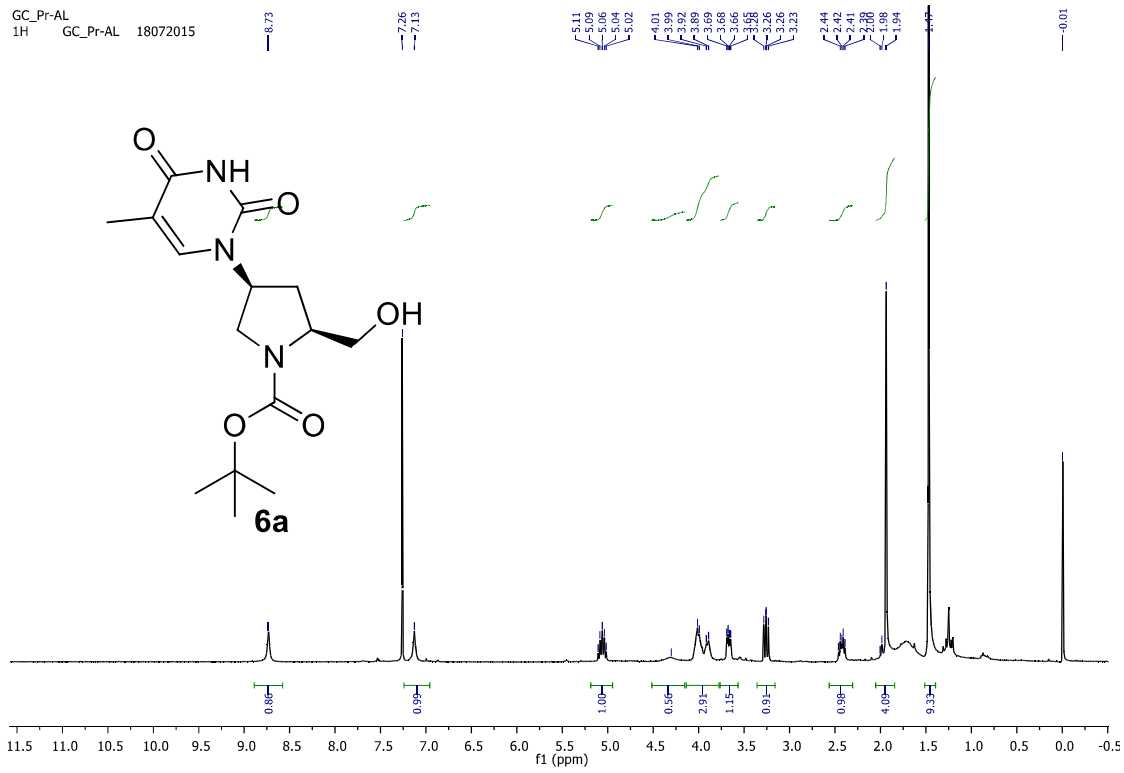


Figure A13 ^{1}H-NMR of compound **6a**

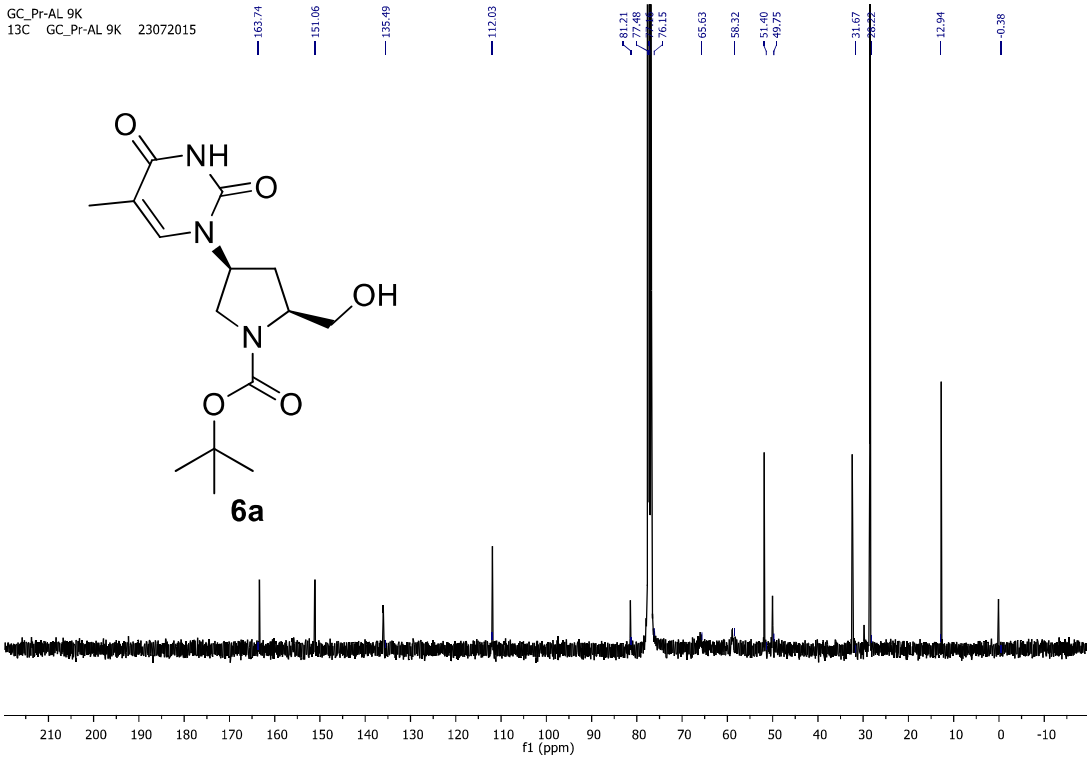


Figure AS14 ^{13}CNMR of Compound **6a**

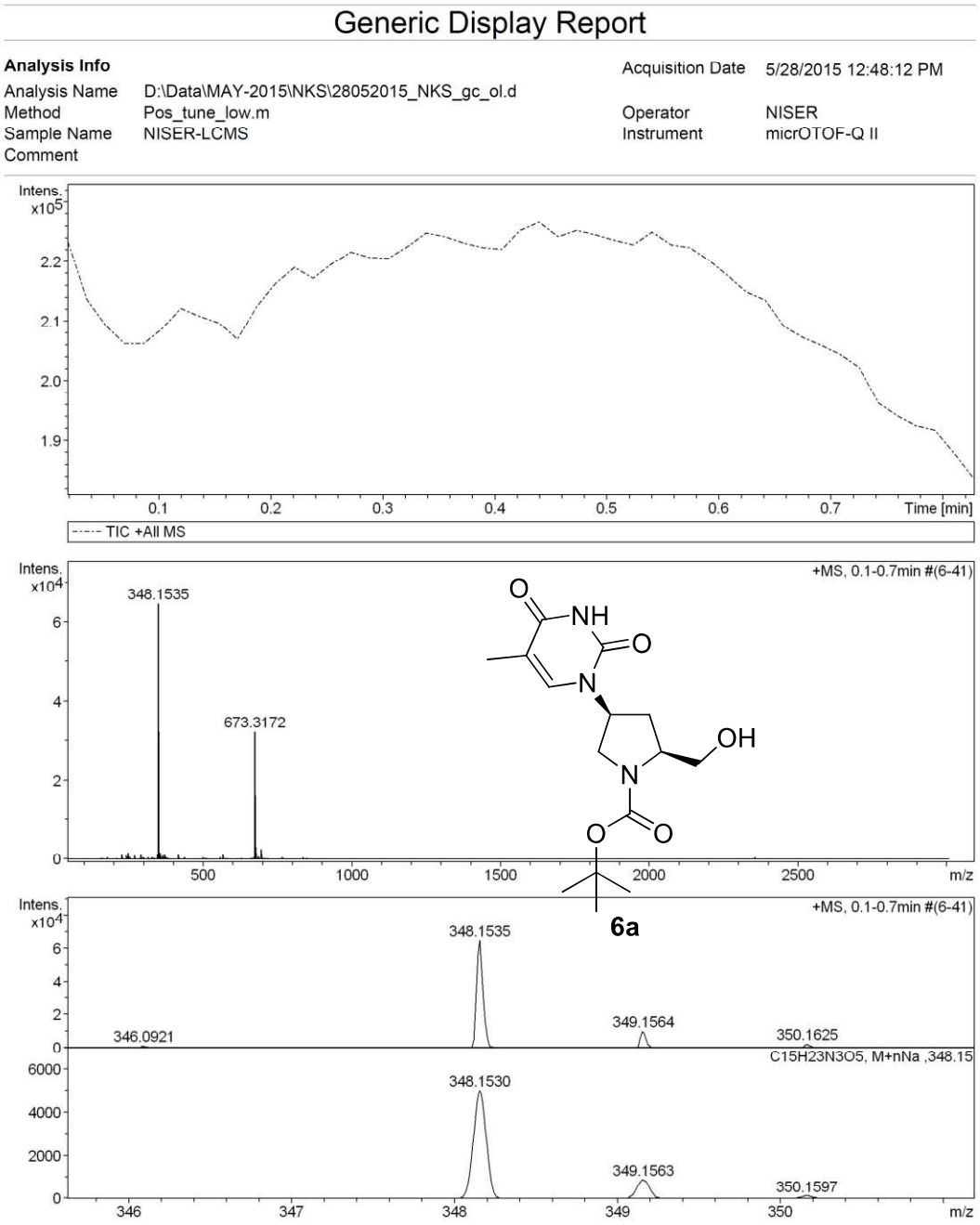


Figure A15 HRMS of compound **6a**

2. Monitoring Phosphorylation steps by Mass spectrometry

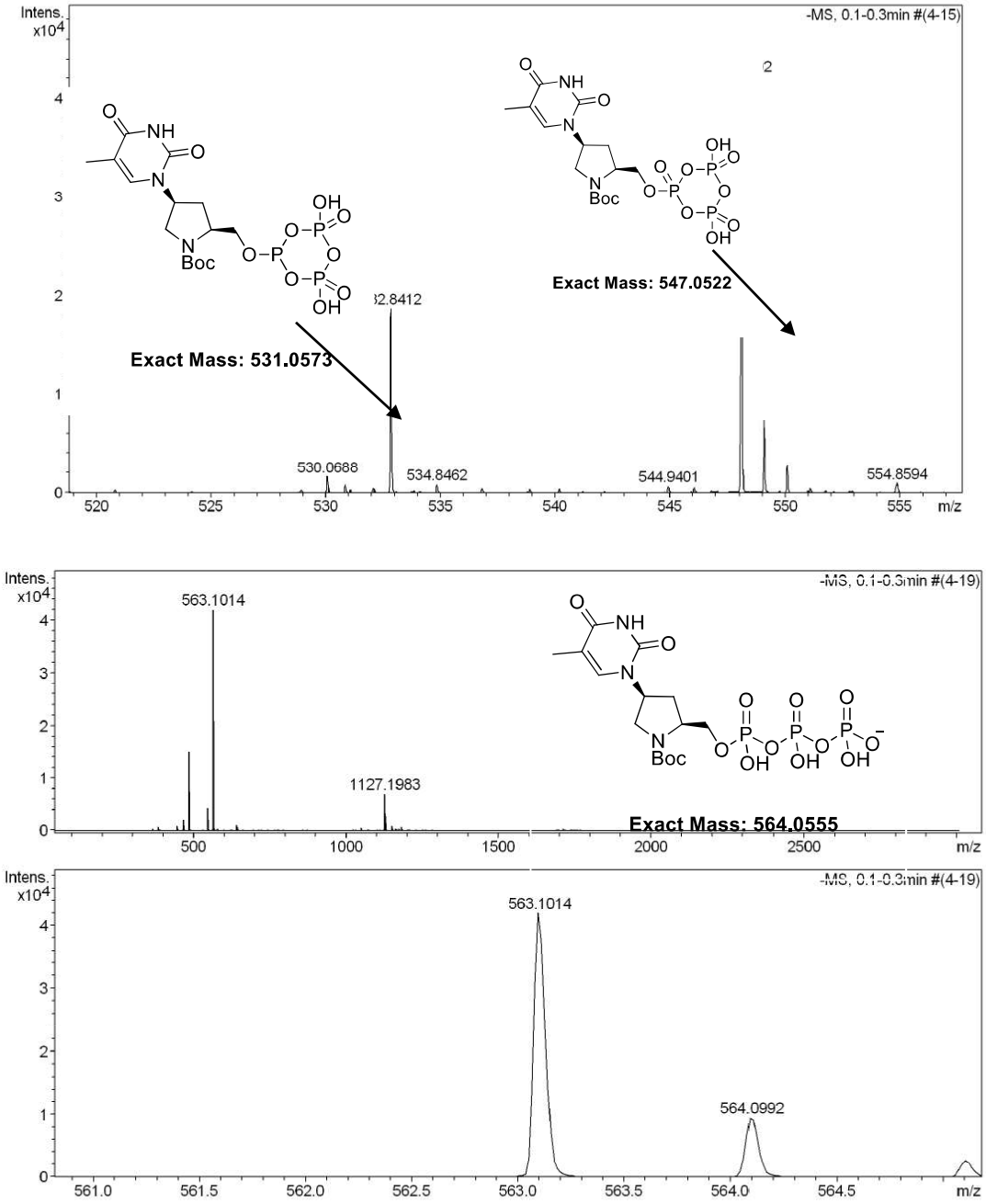


Figure A16 MS of phopshorylation intermediates

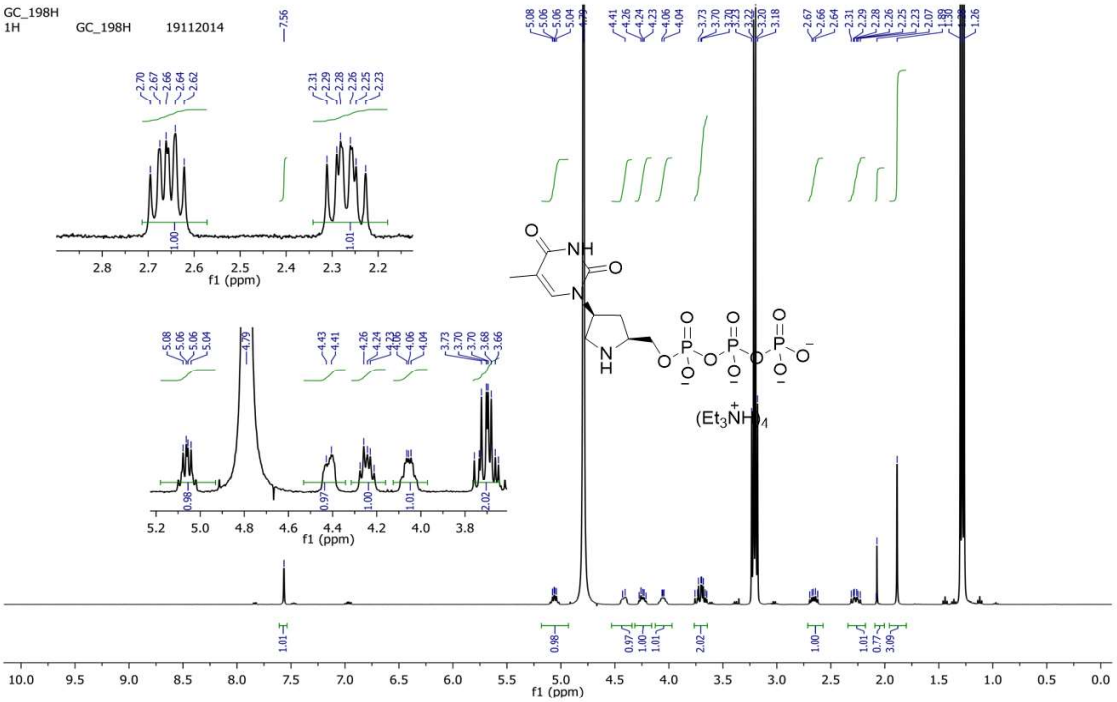


Figure A17 ^{1}H-NMR of compound **7a**

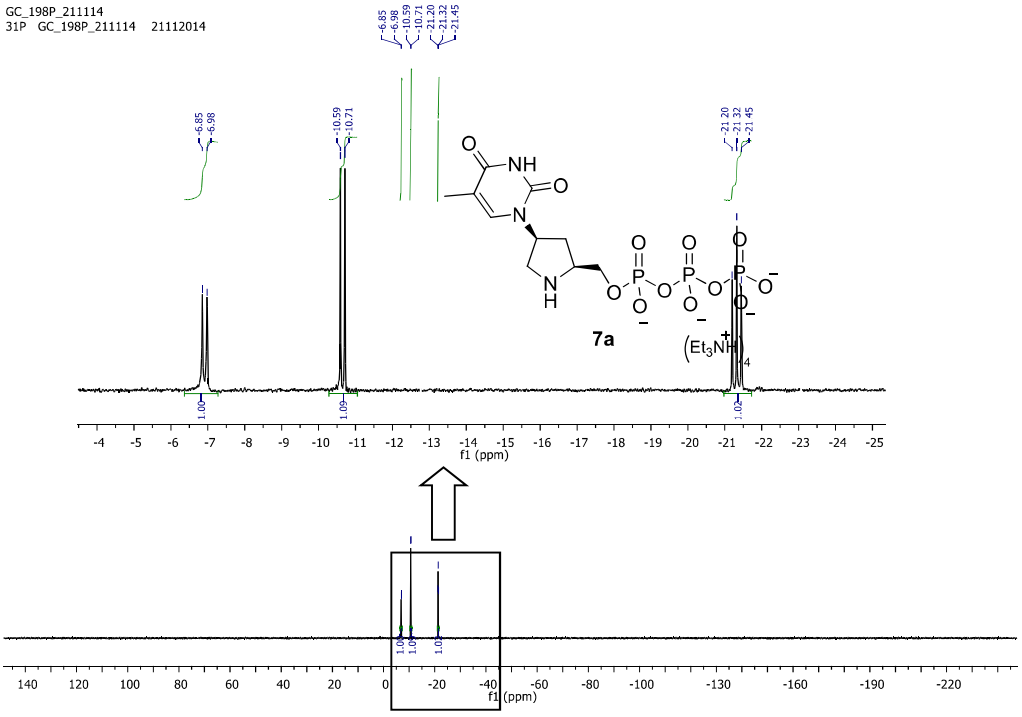


Figure A18 ^{31}P-NMR of compound **7a**

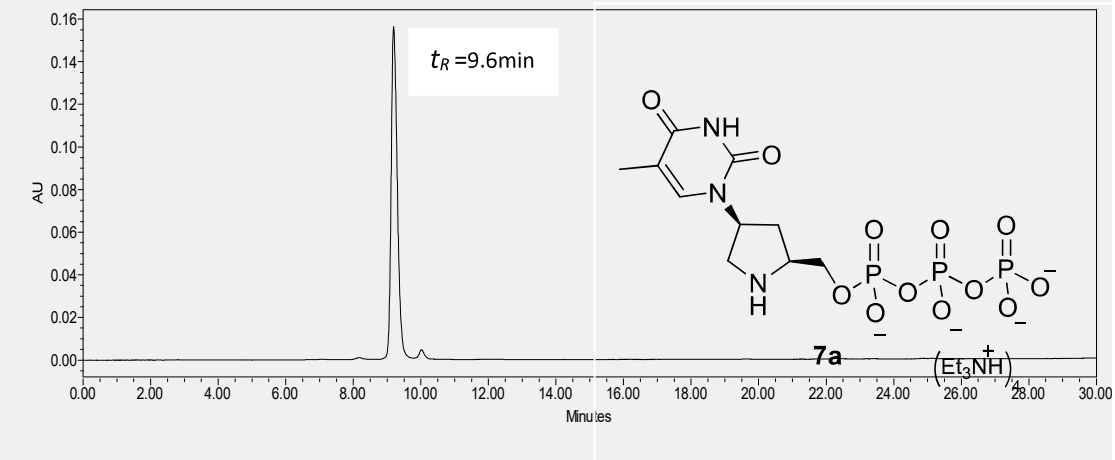


Figure A19 Analytical HPLC chromatogram of compound **7a**

HPLC performed on Waters semi preparative HPLC. 15mM TEAB (Buffer A) and Acetonitrile (Solvent B) were used as solvents .pH of TEAB buffer adjusted to 7.0 by adding aceticacid .A C-18 RP Waters sun fire column (5µm, 4.6 x150 mm) used to purify *pr*-TTP at a flow rate of 1.0 ml/min monitored at 269 nm by following the below programme.

S.NO	Time(Min)	%A	%B
1	0	100	0
2	27	90	10
3	28	100	0
4	30	100	0

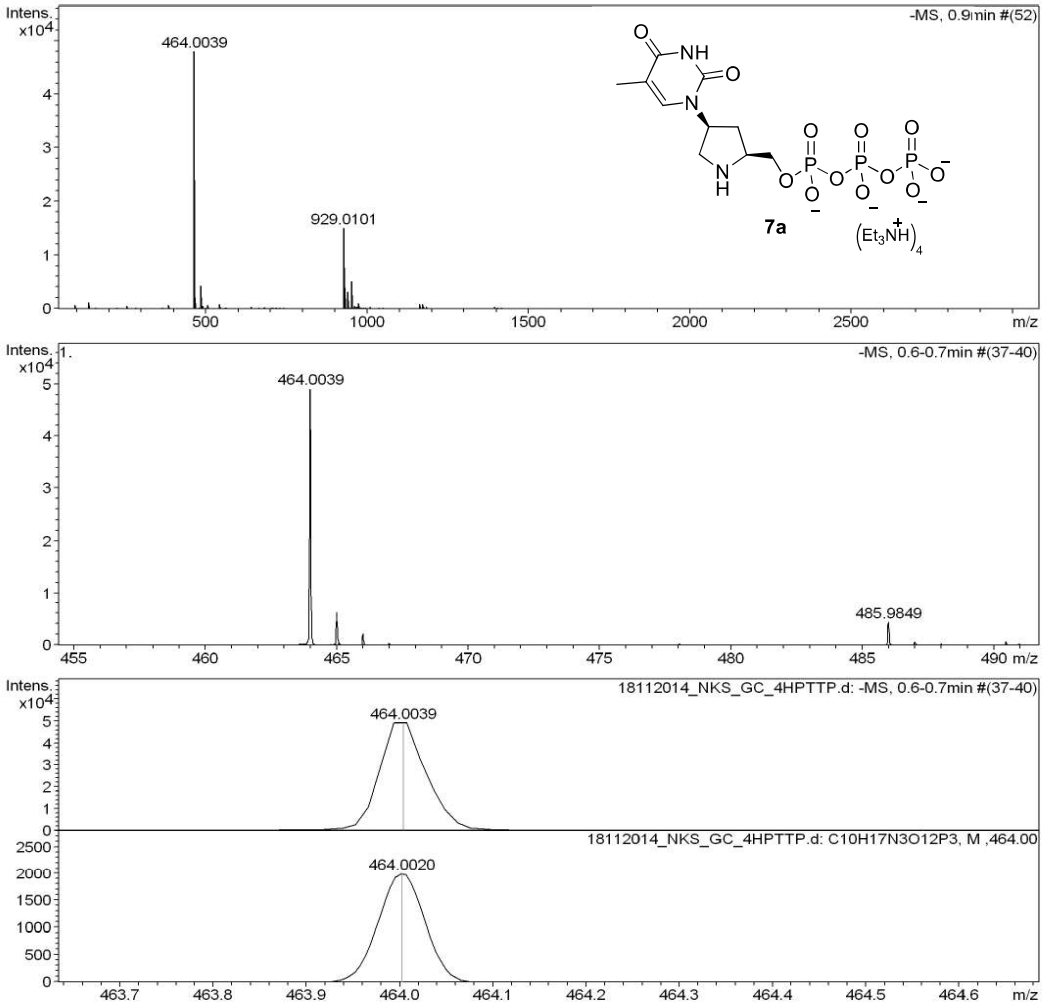


Figure A 20 HRMS spectrum of compound **7a**

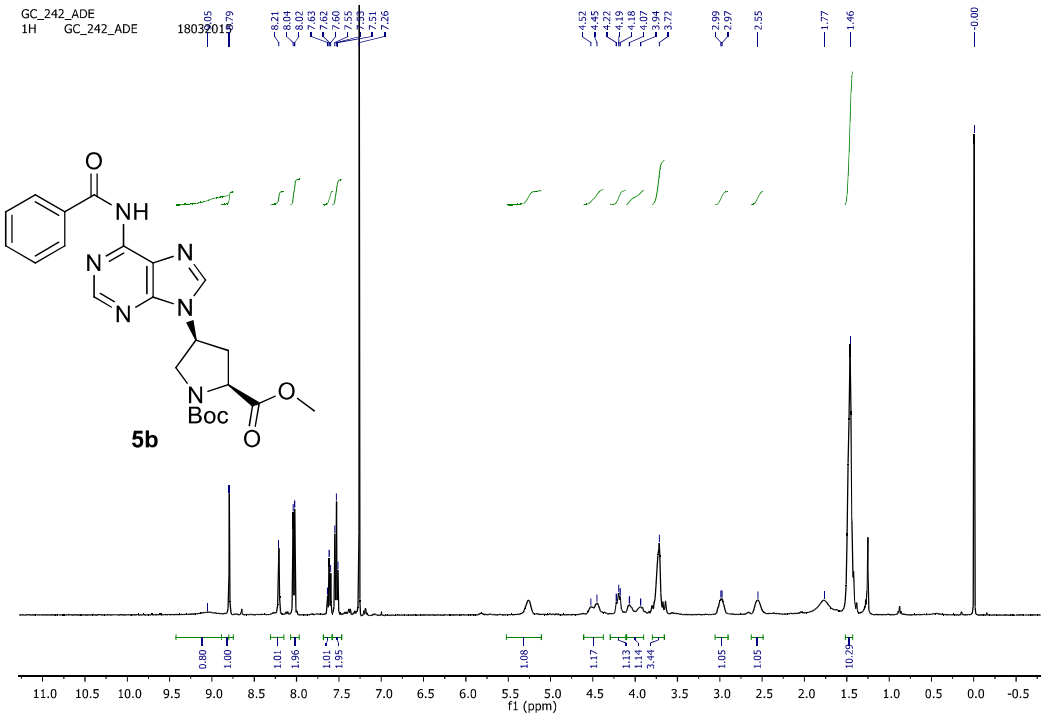


Figure A21 [1]HNMR of compound **5b**

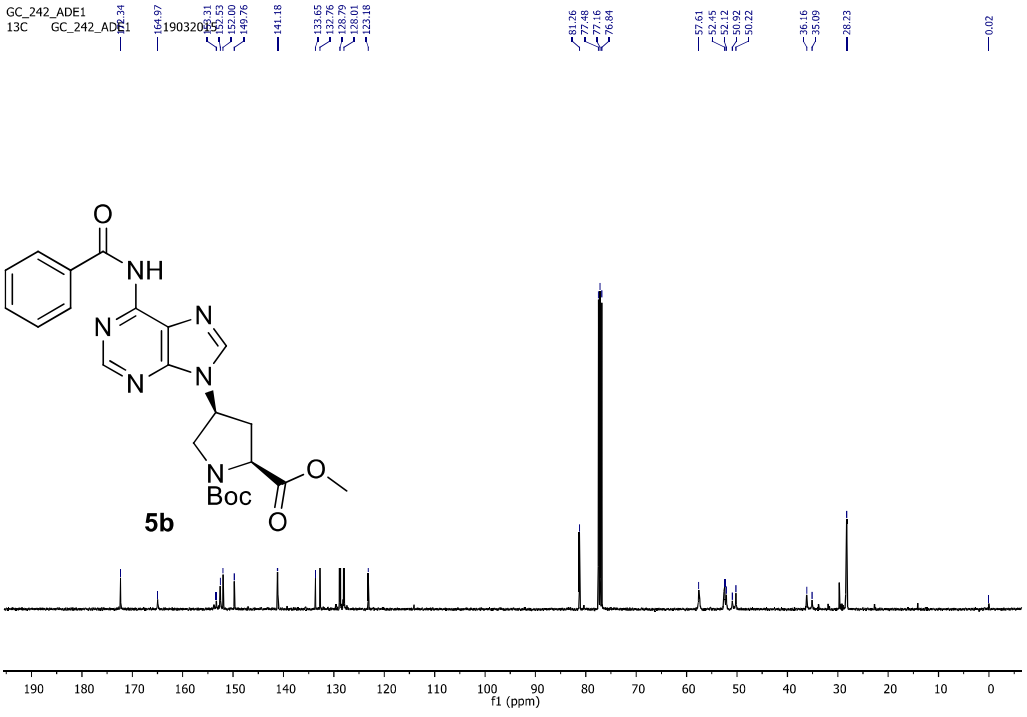


Figure A22 [13]CNMR of compound **5b**

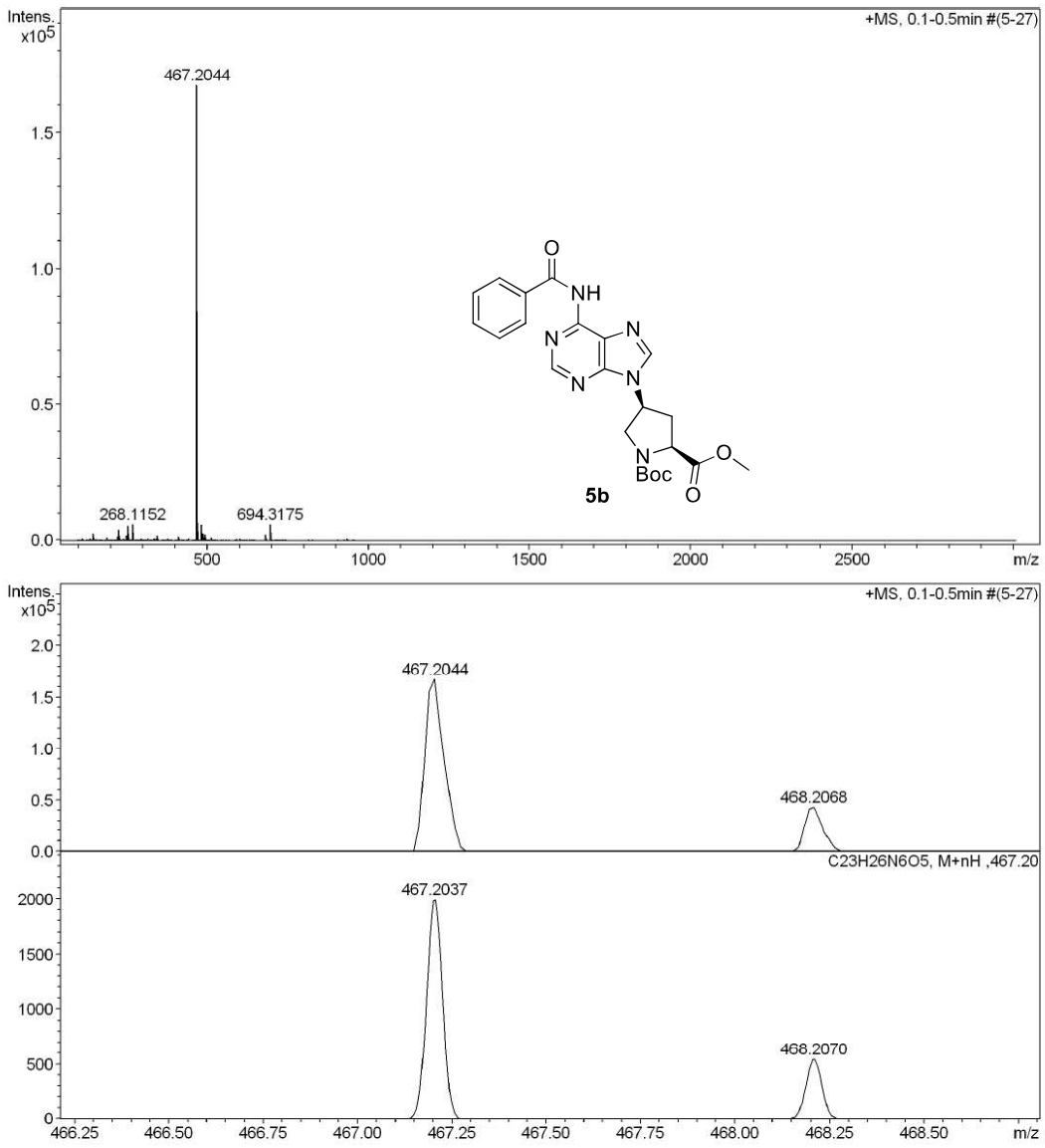


Figure A23 HRMS of compound **5b**

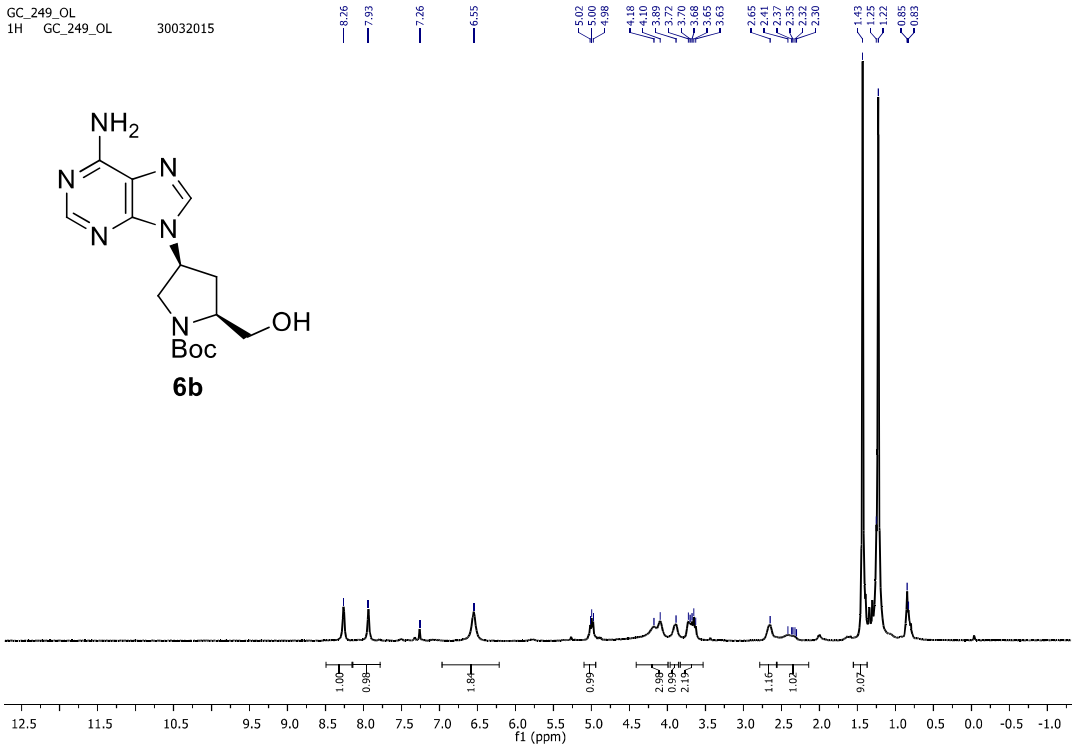


Figure A24 ^{1}HNMR of compound **6b**

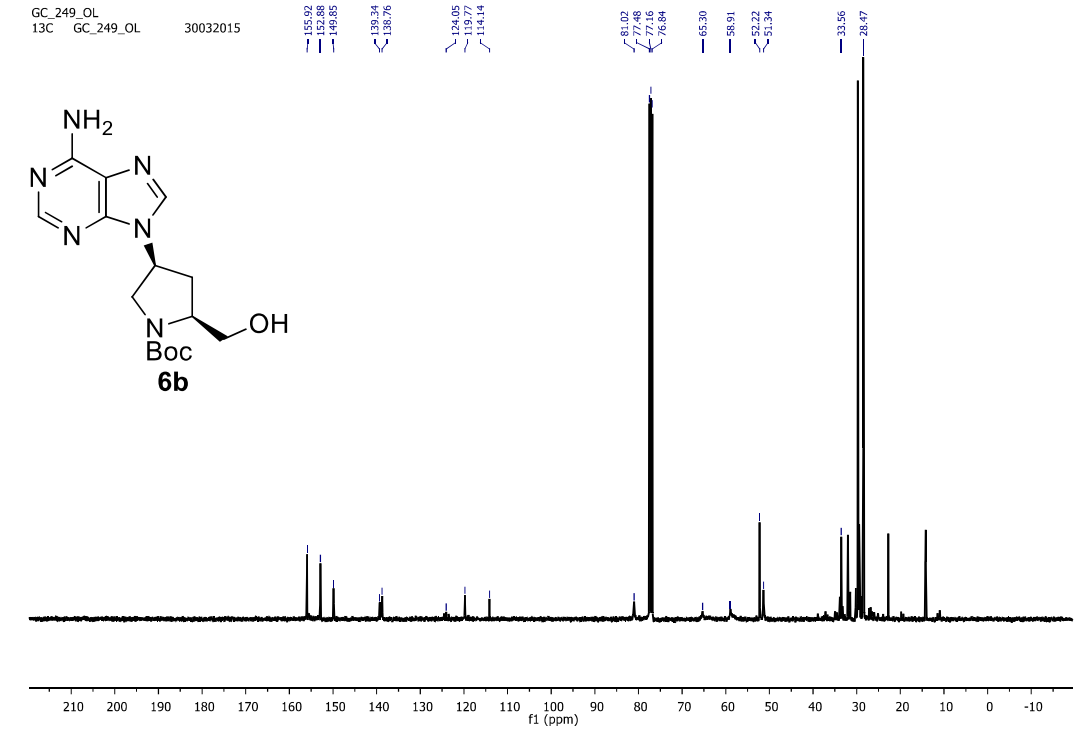


Figure A25 ^{13}CNMR of compound **6b**

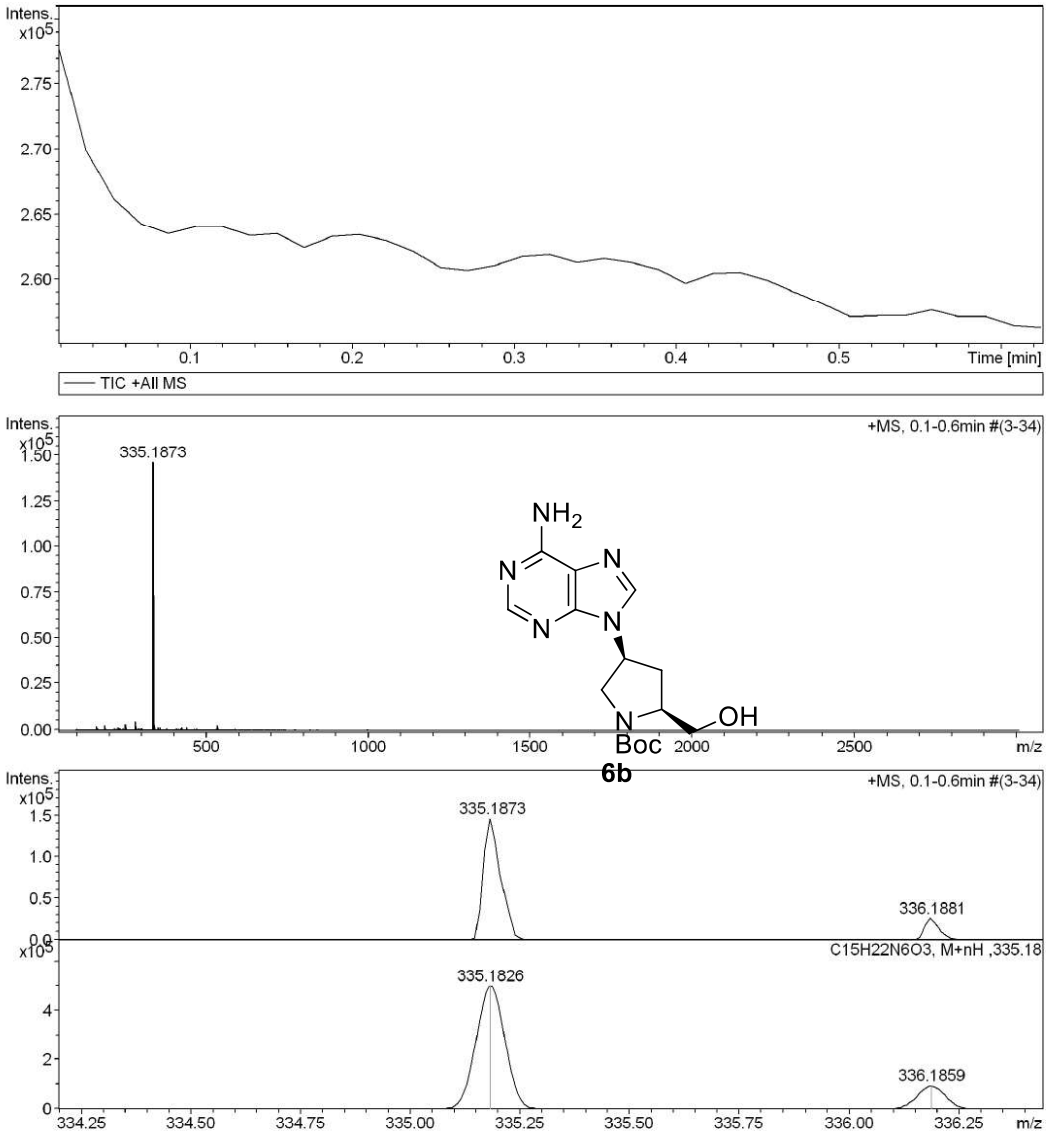


Figure A26 HRMS of compound **6b**

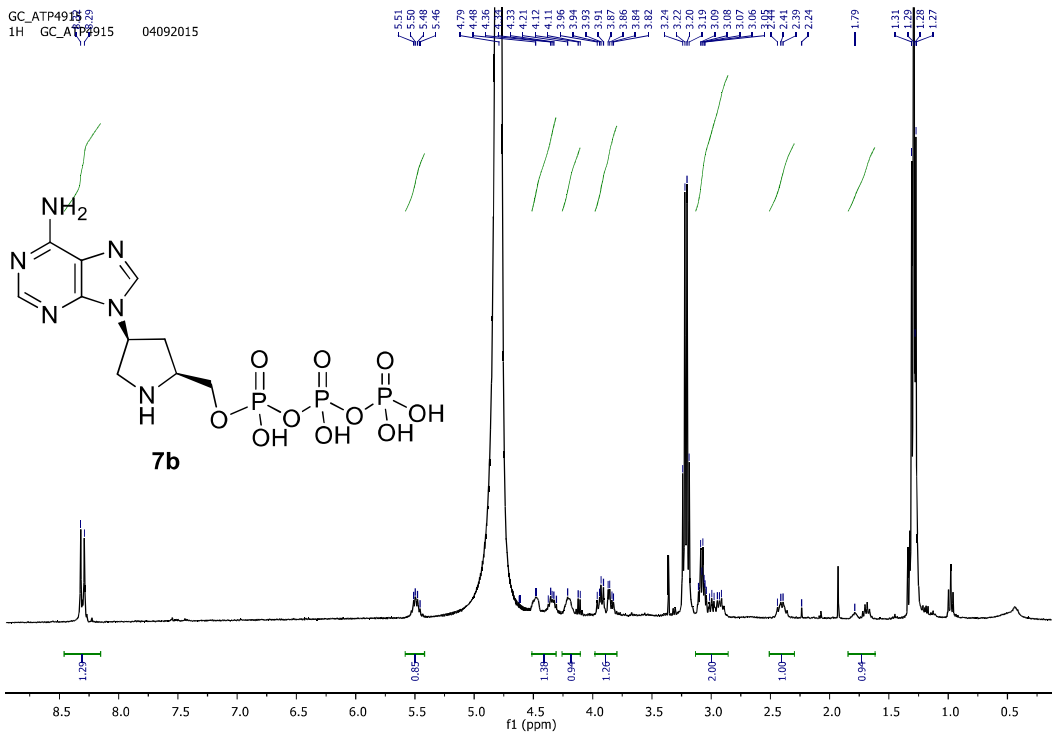


Figure A27 ^{1}HNMR of compound **7b**

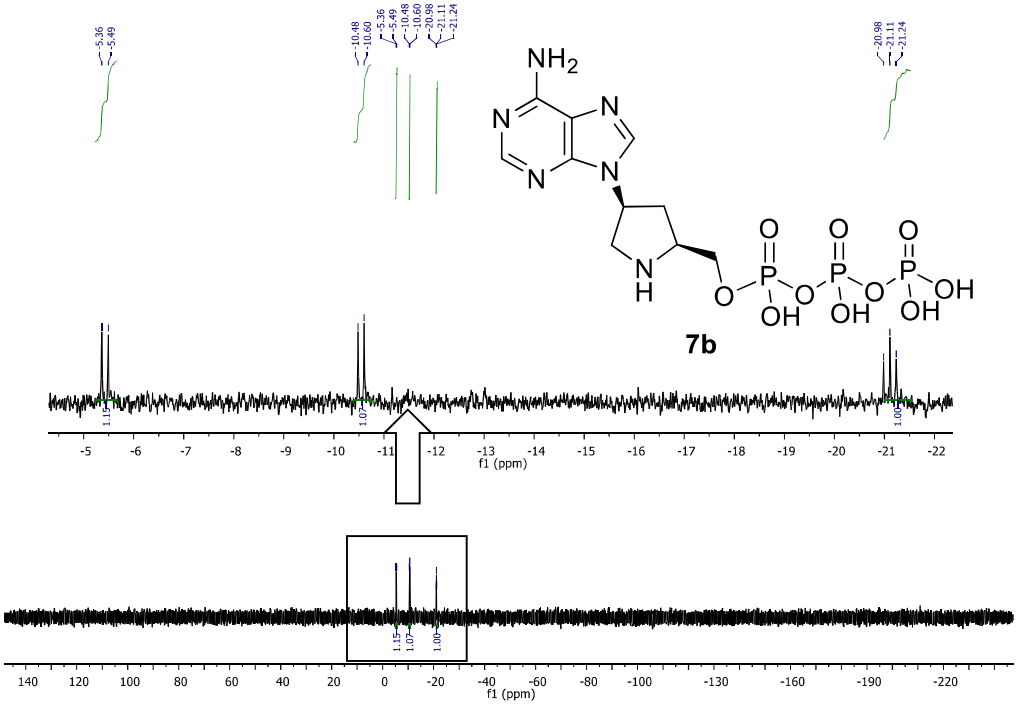


Figure A28 ^{31}PNMR of compound **7b**

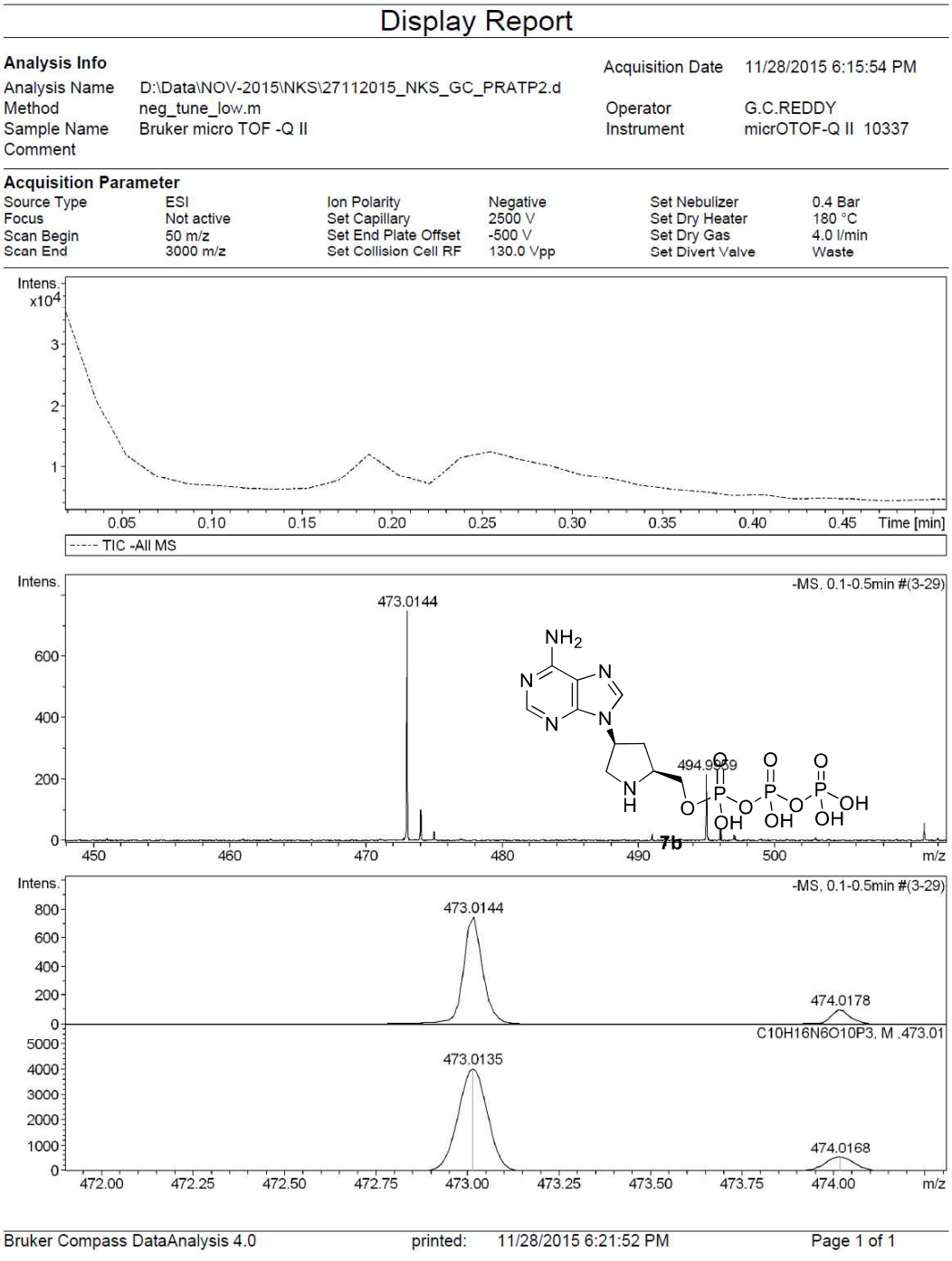


Figure A29 HRMS of compound **7b**

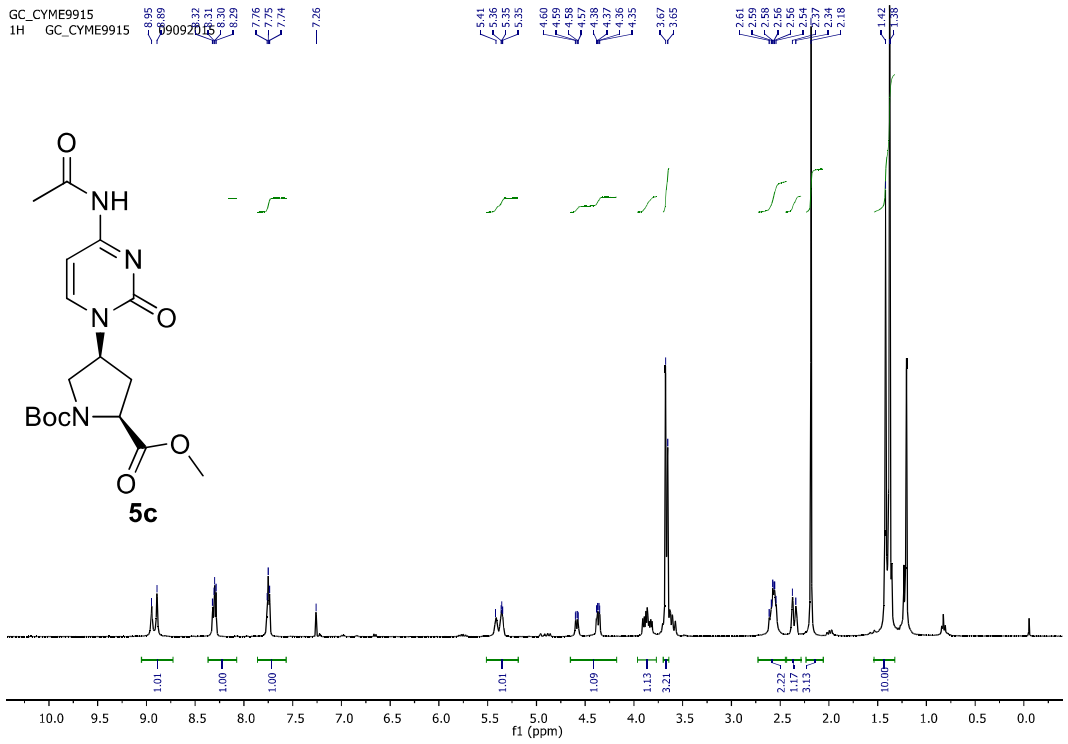


Figure A30 ^{1}HNMR of compound **5c**

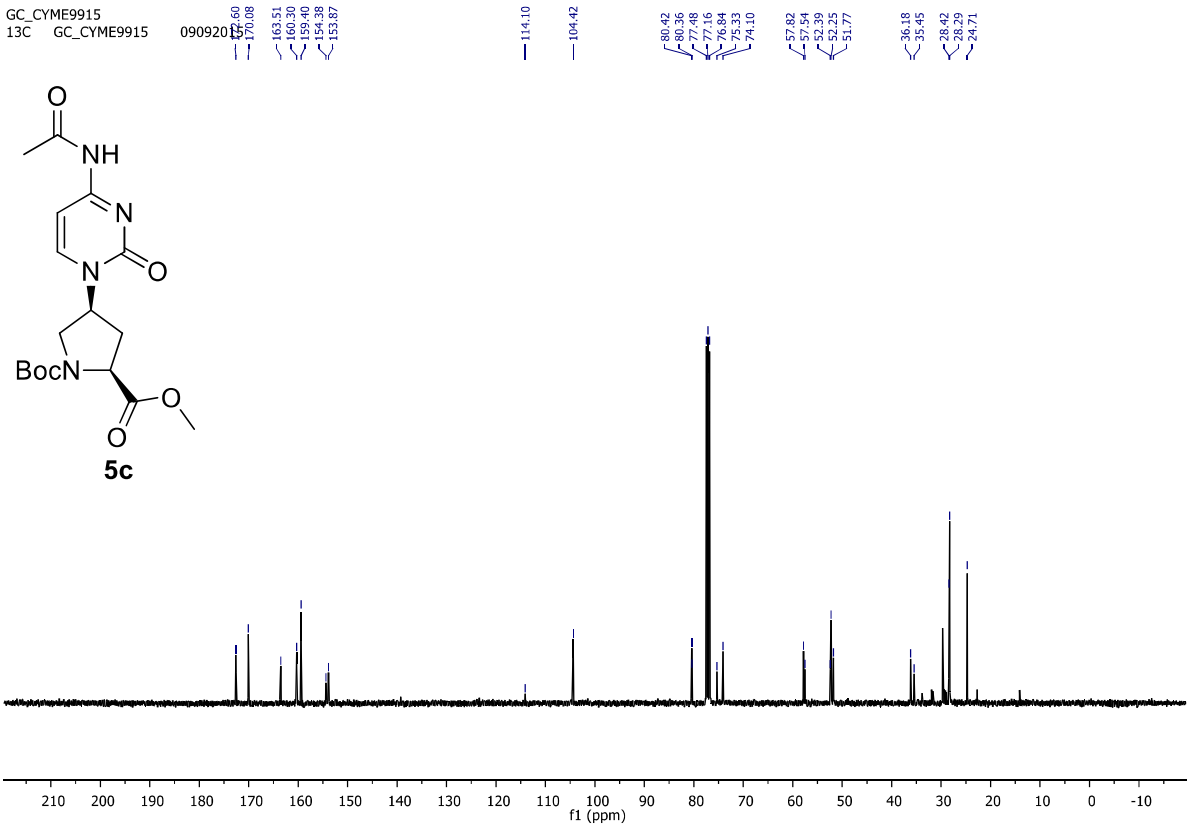


Figure A31 ^{13}CNMR of compound **5c**

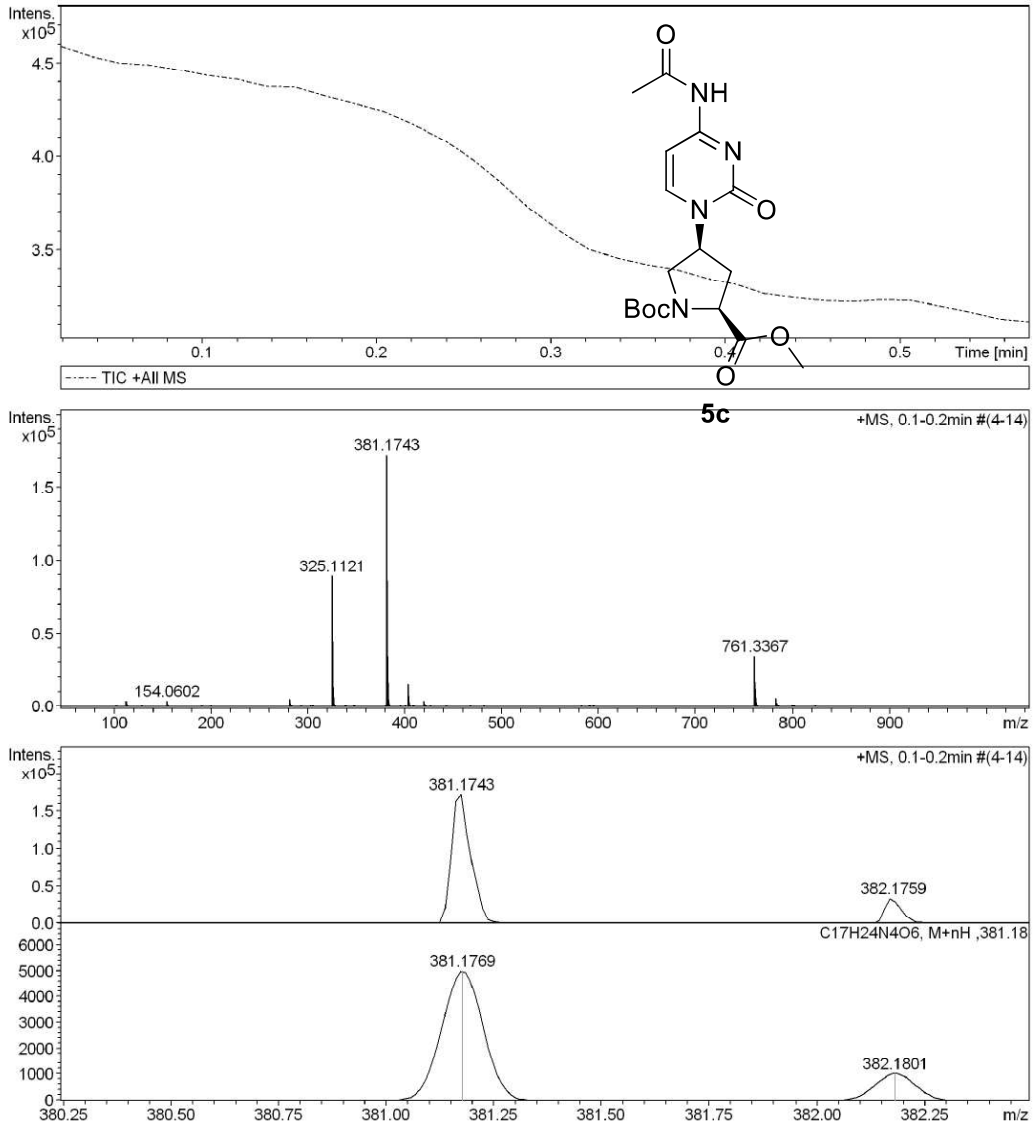


Figure A32 HRMS of compound **5c**

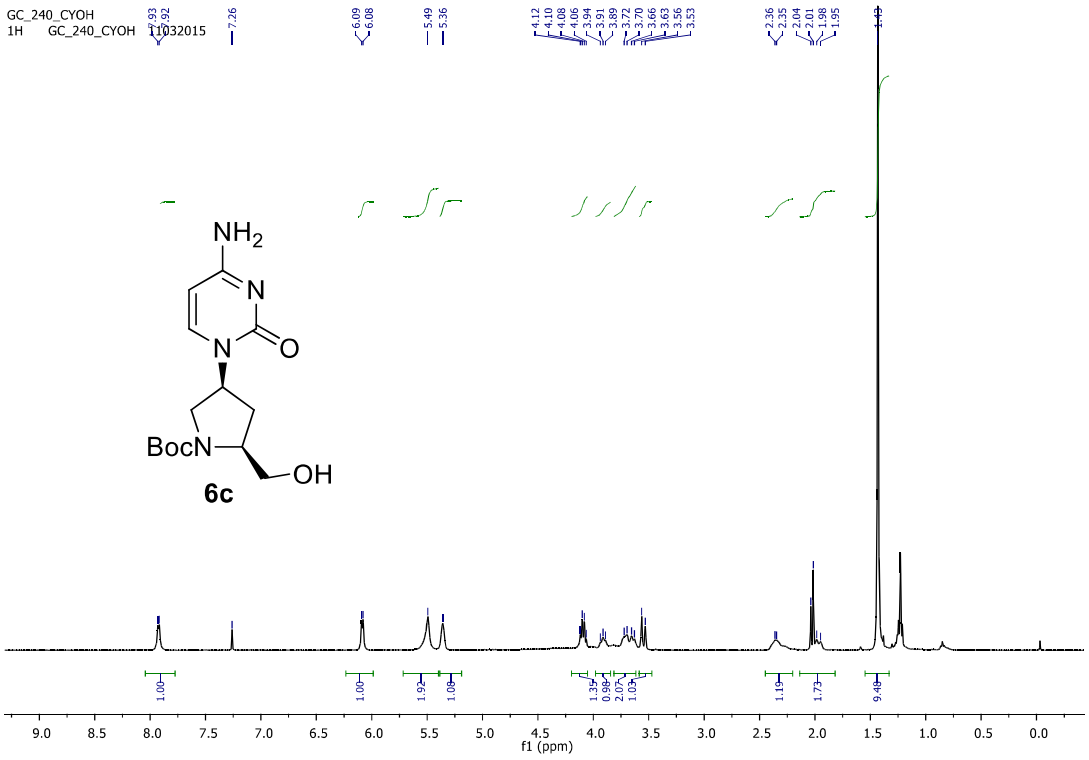


Figure A33 [1]HNMR of compound **6c**

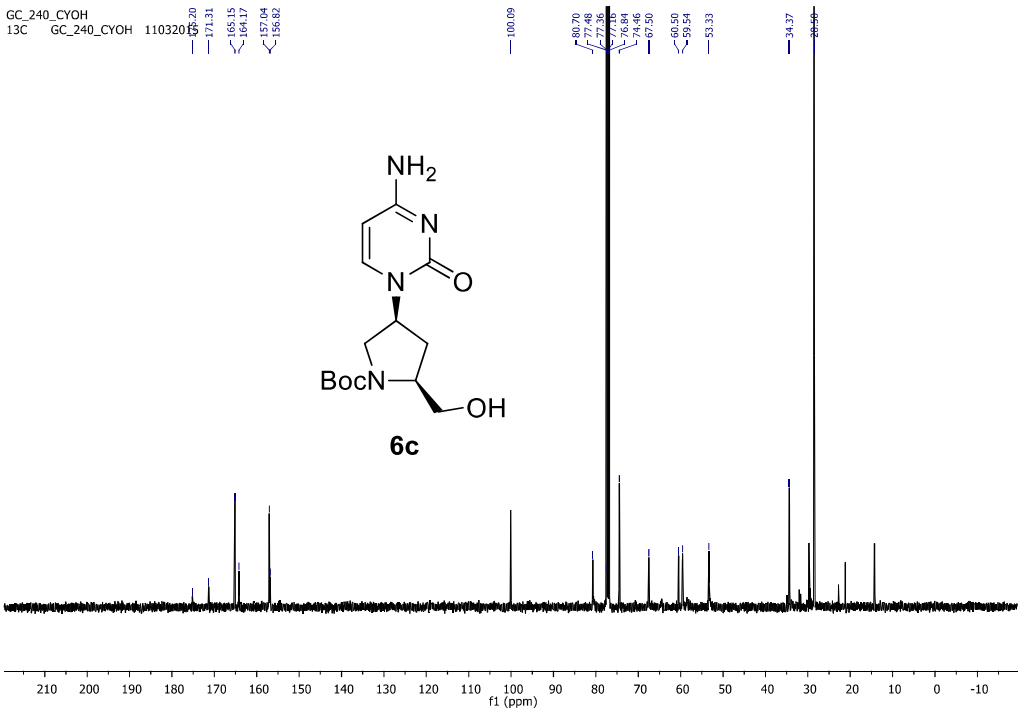


Figure A34 [13]CNMR of compound **6c**

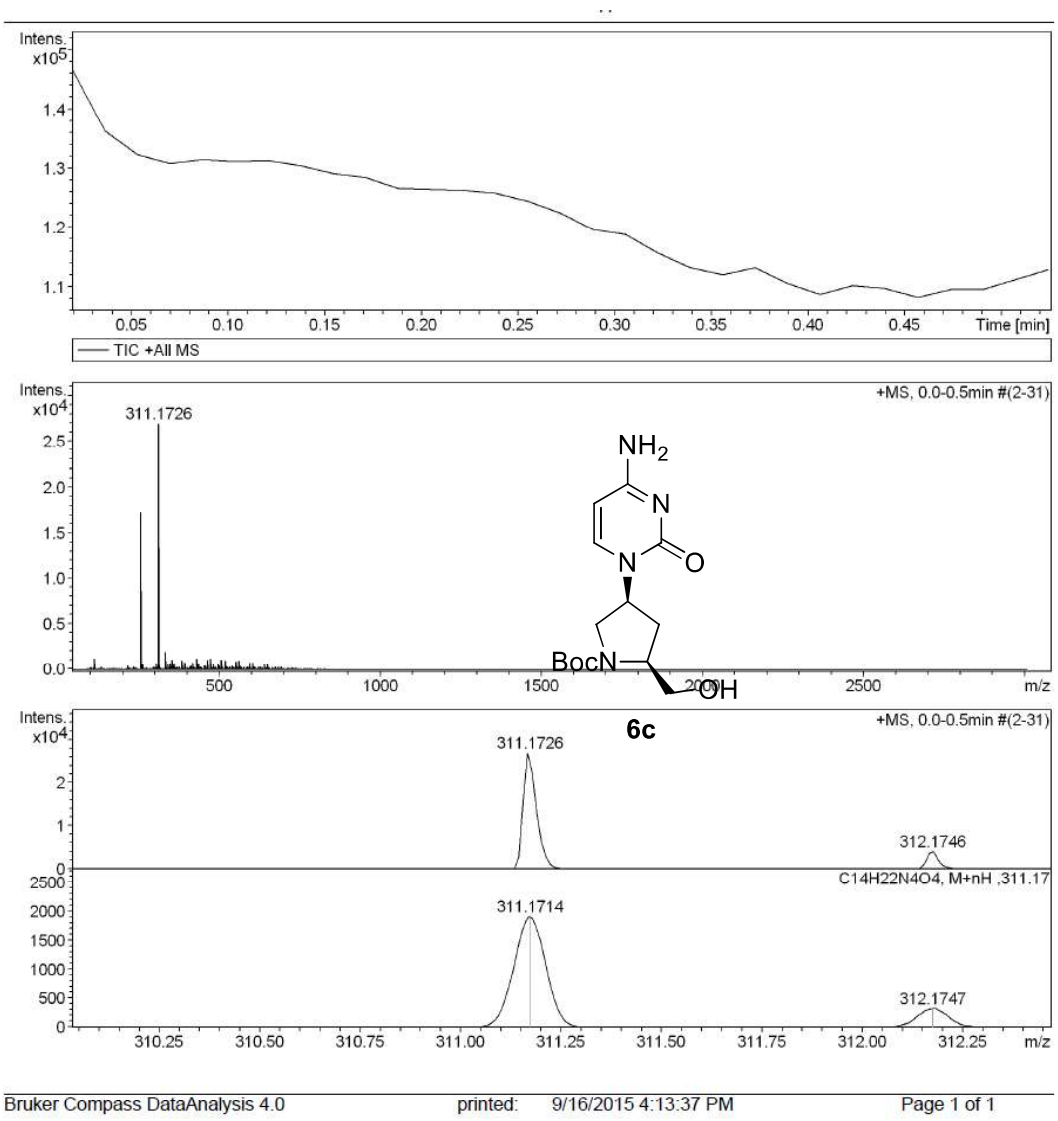


Figure A35 HRMS of compound **6c**

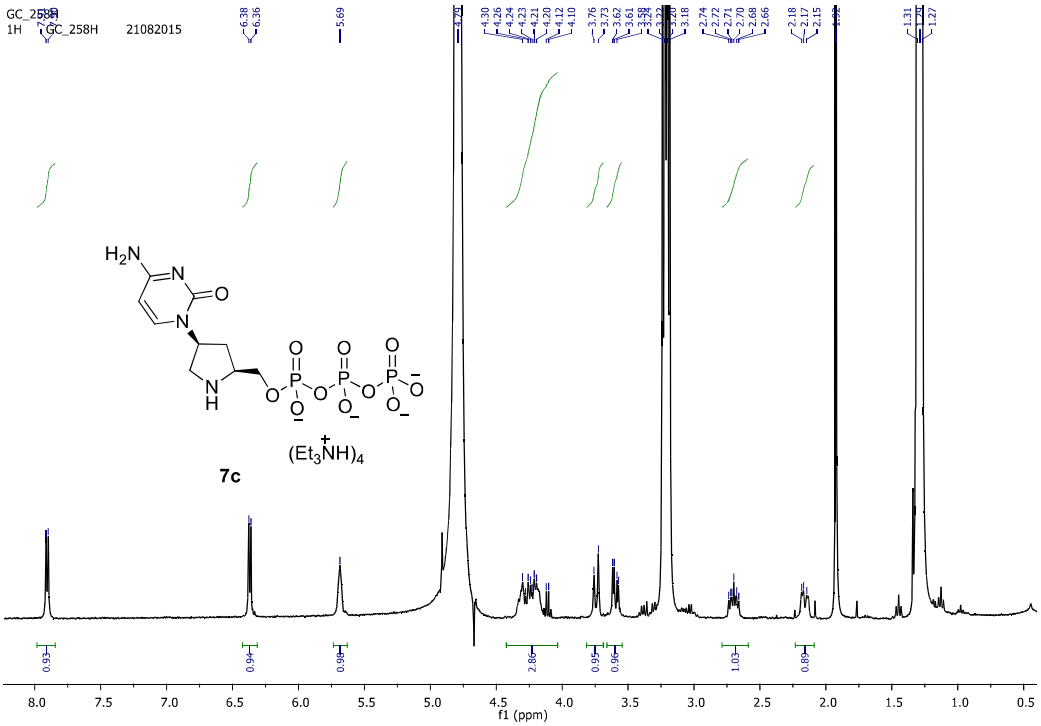


Figure A36 ^{1}HNMR of compound **7c**

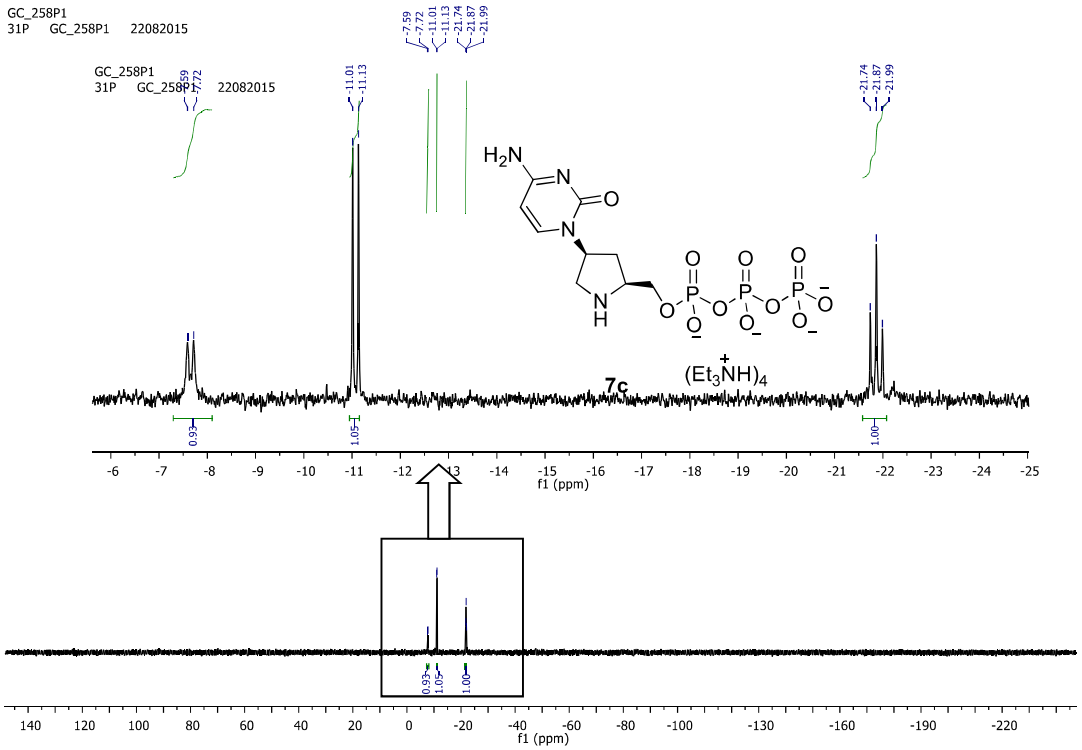


Figure A37 ^{31}PNMR of compound **7c**

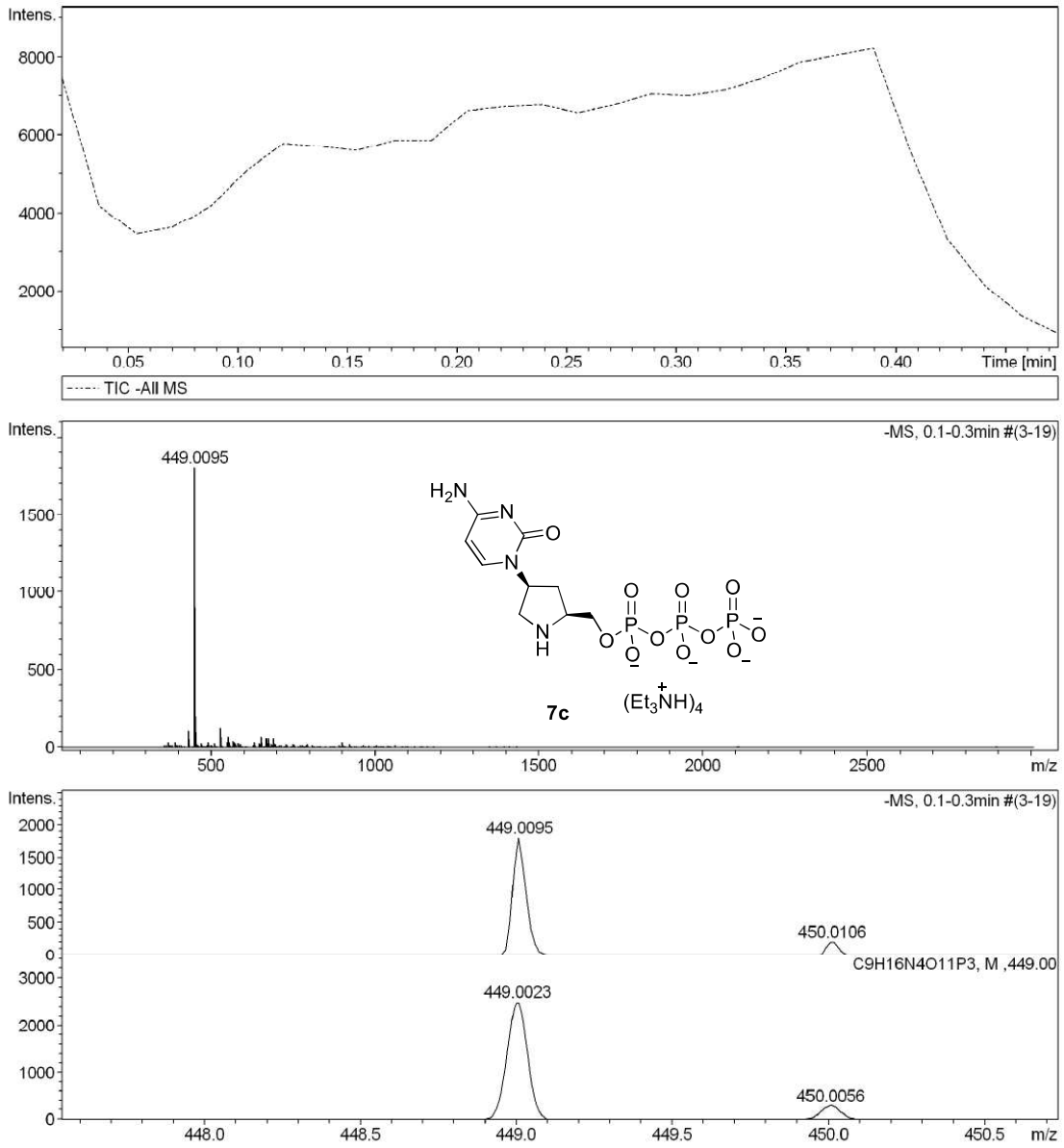


Figure A38 HRMS of compound **7c**

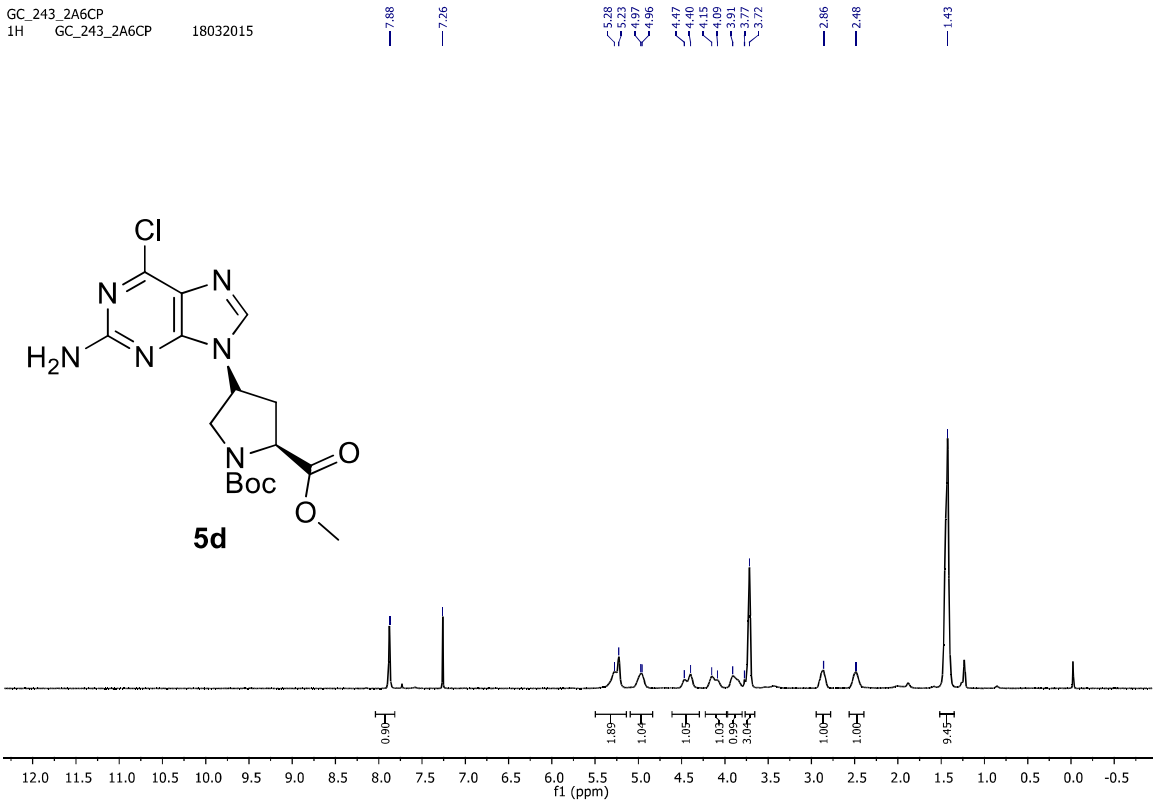


Figure A39 [1]HNMR of compound **5d**

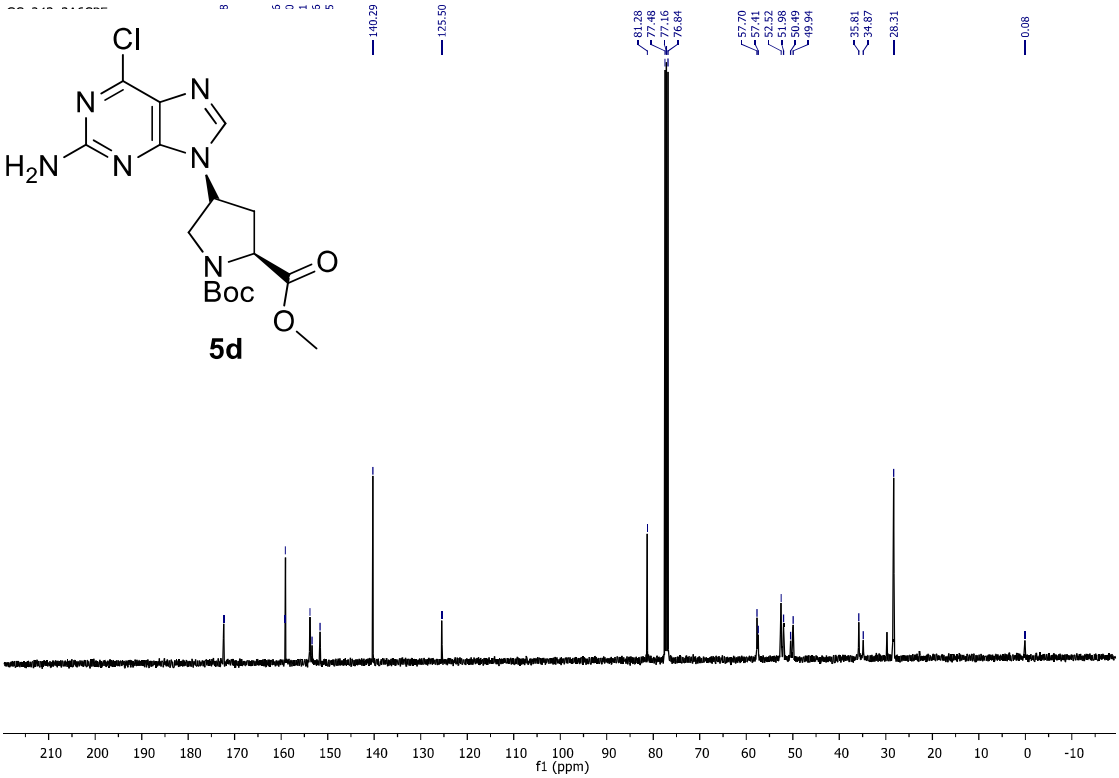


Figure A40 ^{13}CNMR of compound **5d**

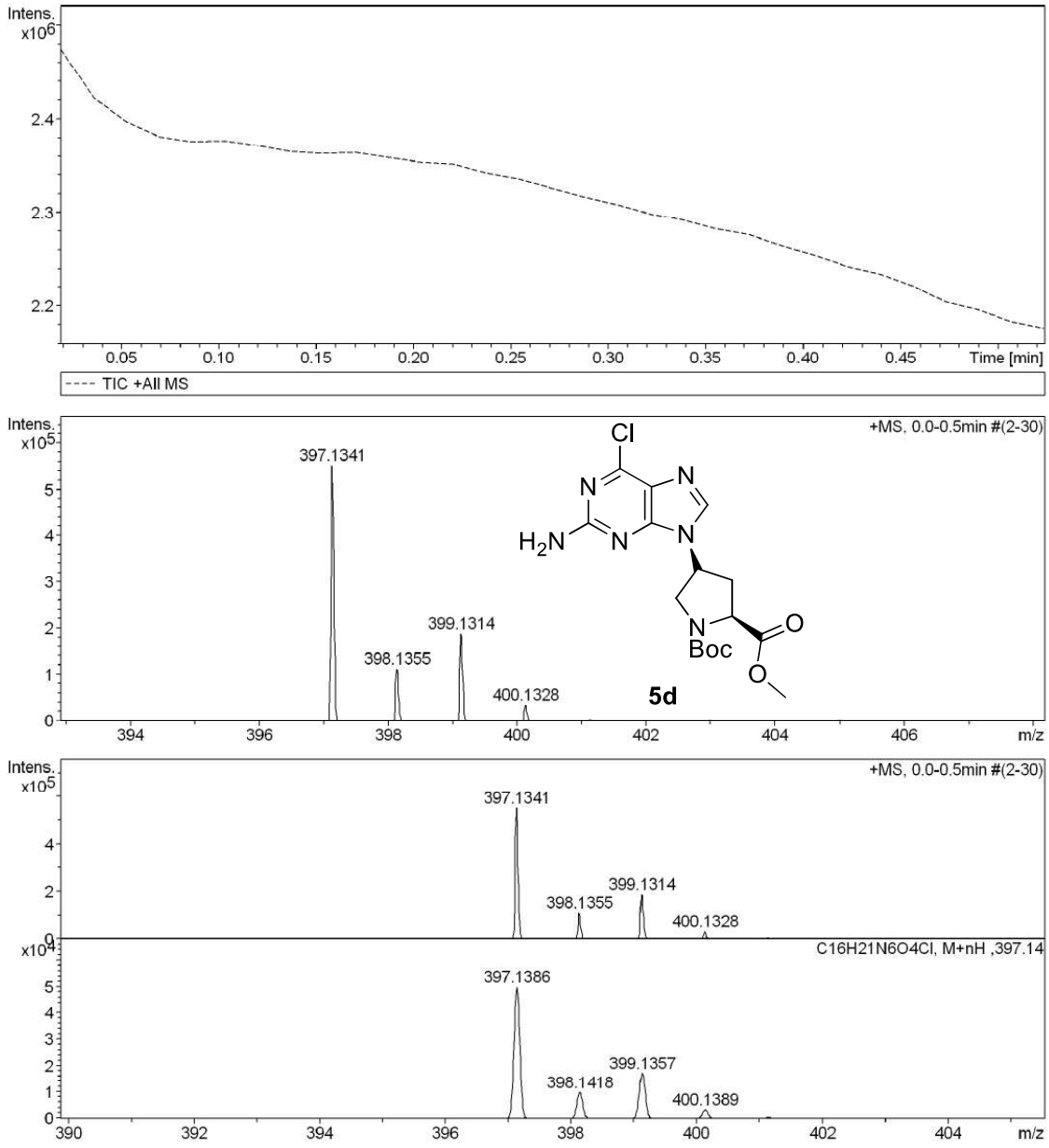


Figure A41 HRMS of compound **5d**

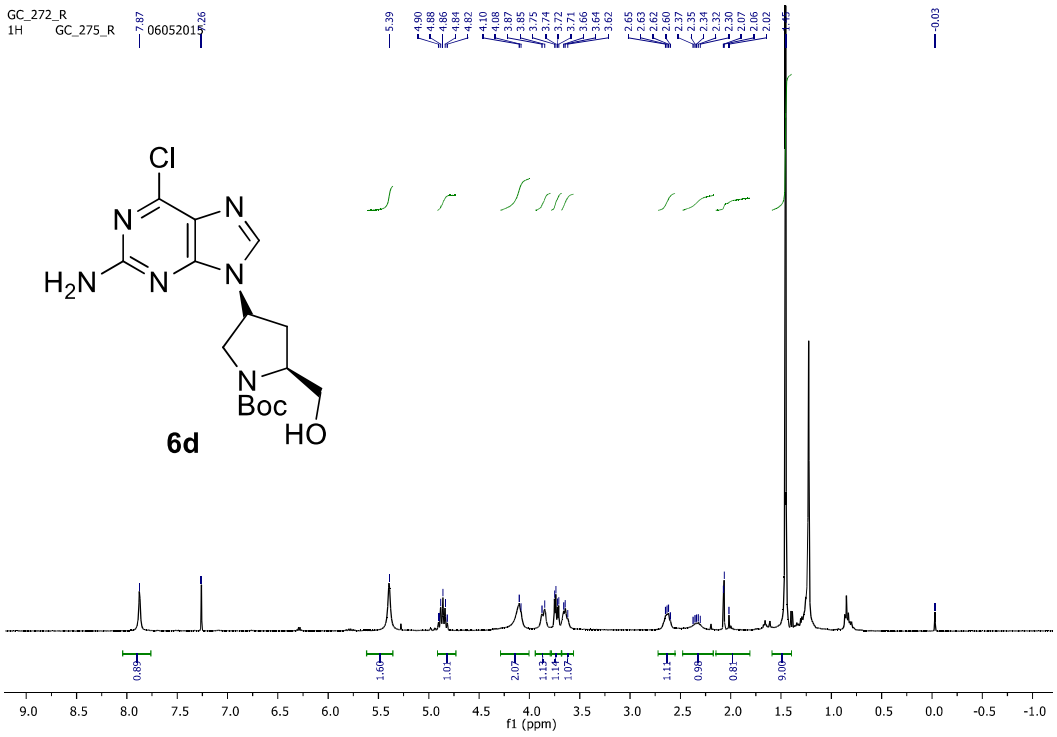


Figure A 42 [1]HNMR of compound **6d**

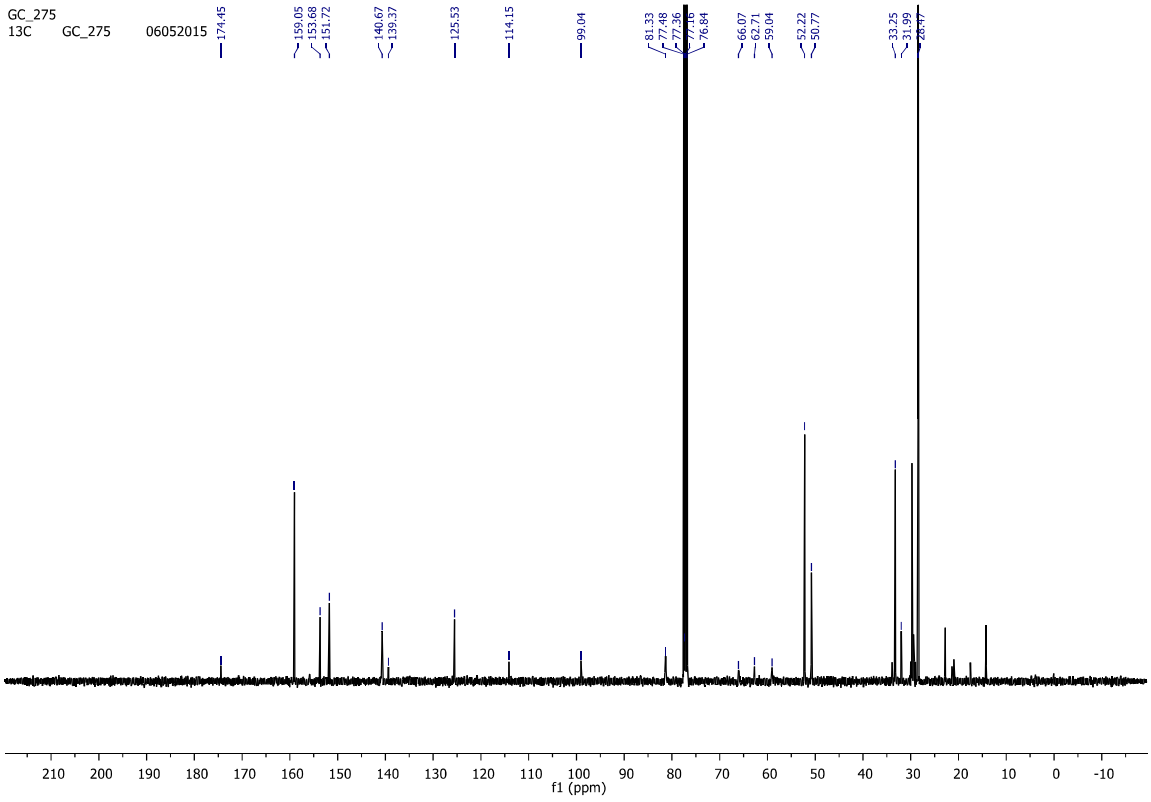


Figure A43 [1]HNMR of compound **6d**

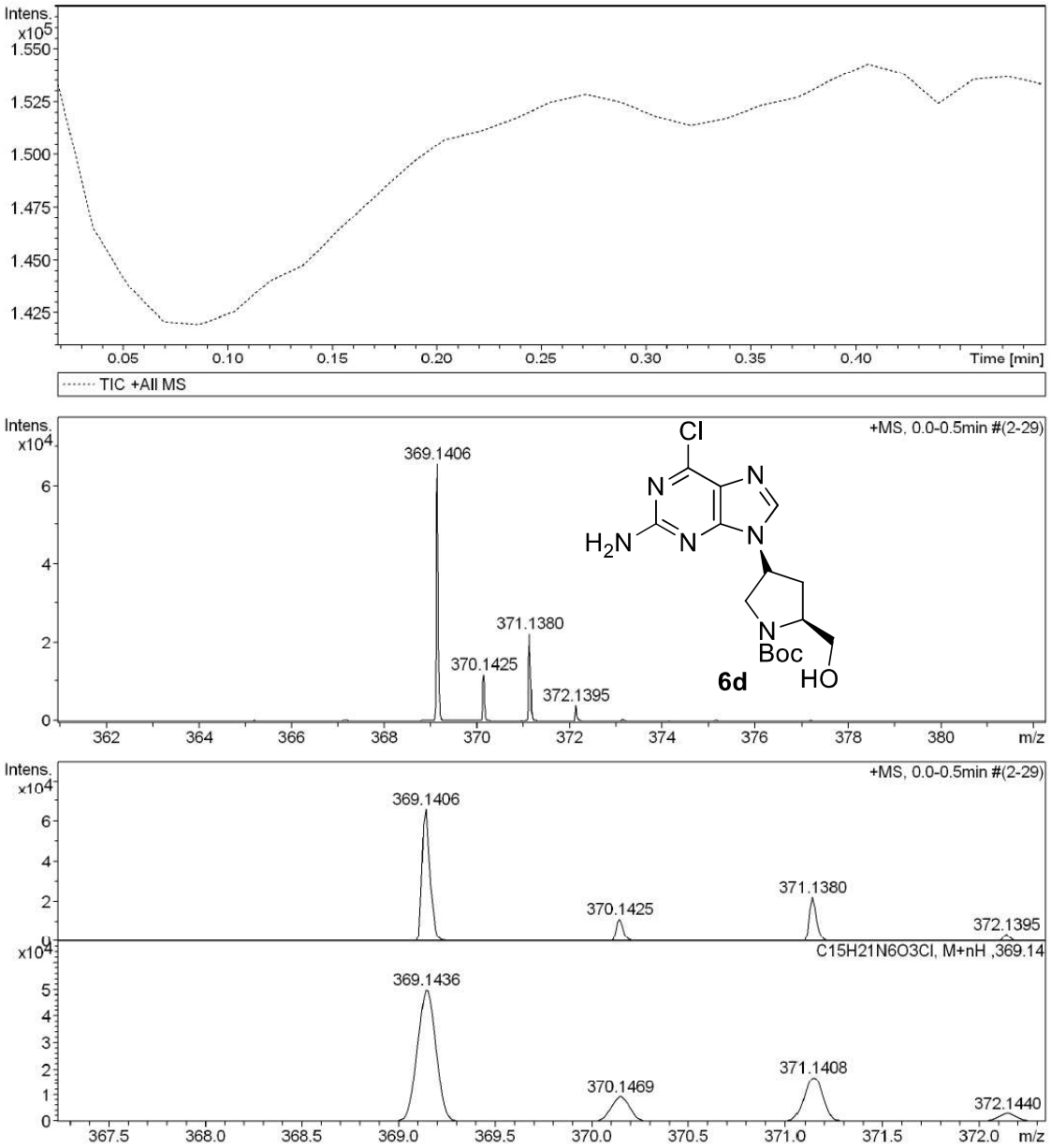


Figure A44 HRMS of compound **6d**

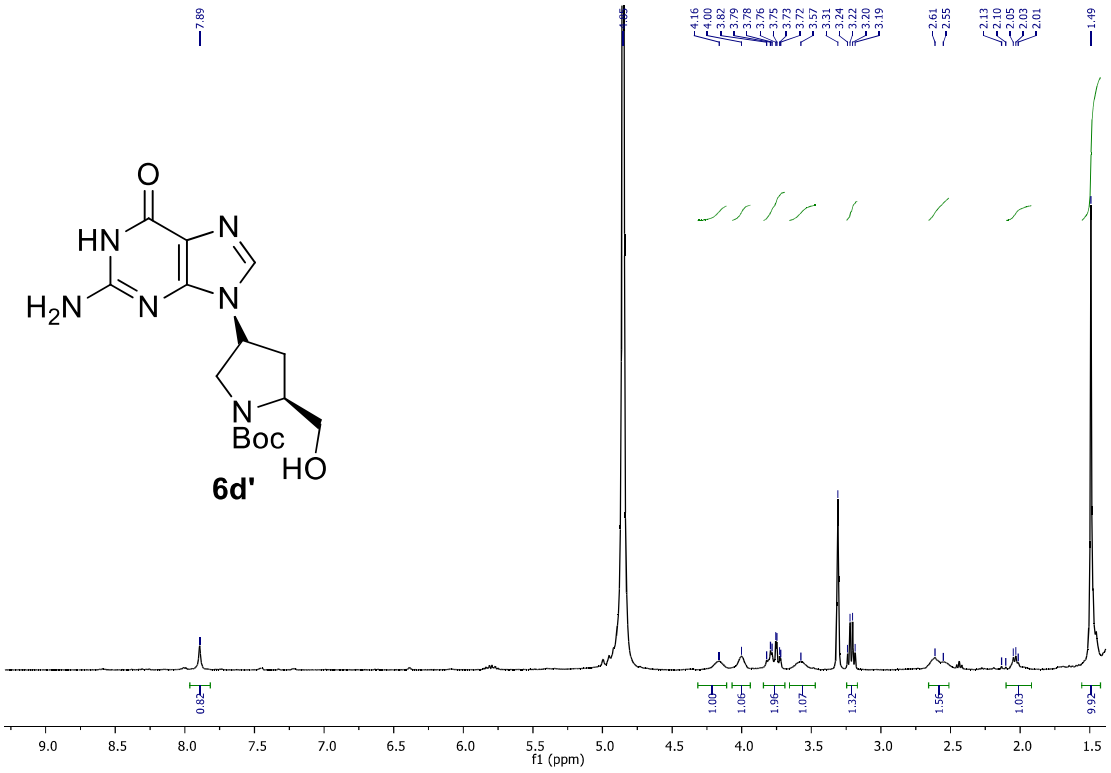


Figure A45 [1]HNMR of compound **6d'**

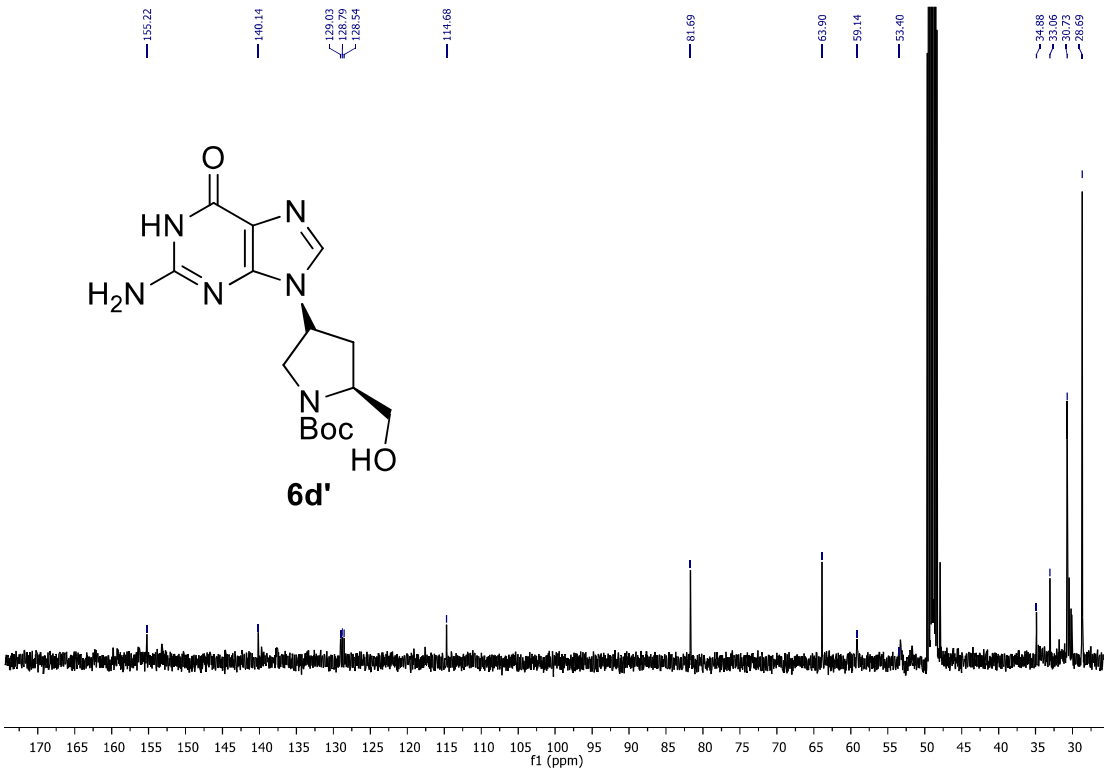


Figure A46 [13]CNMR of compound **6d'**

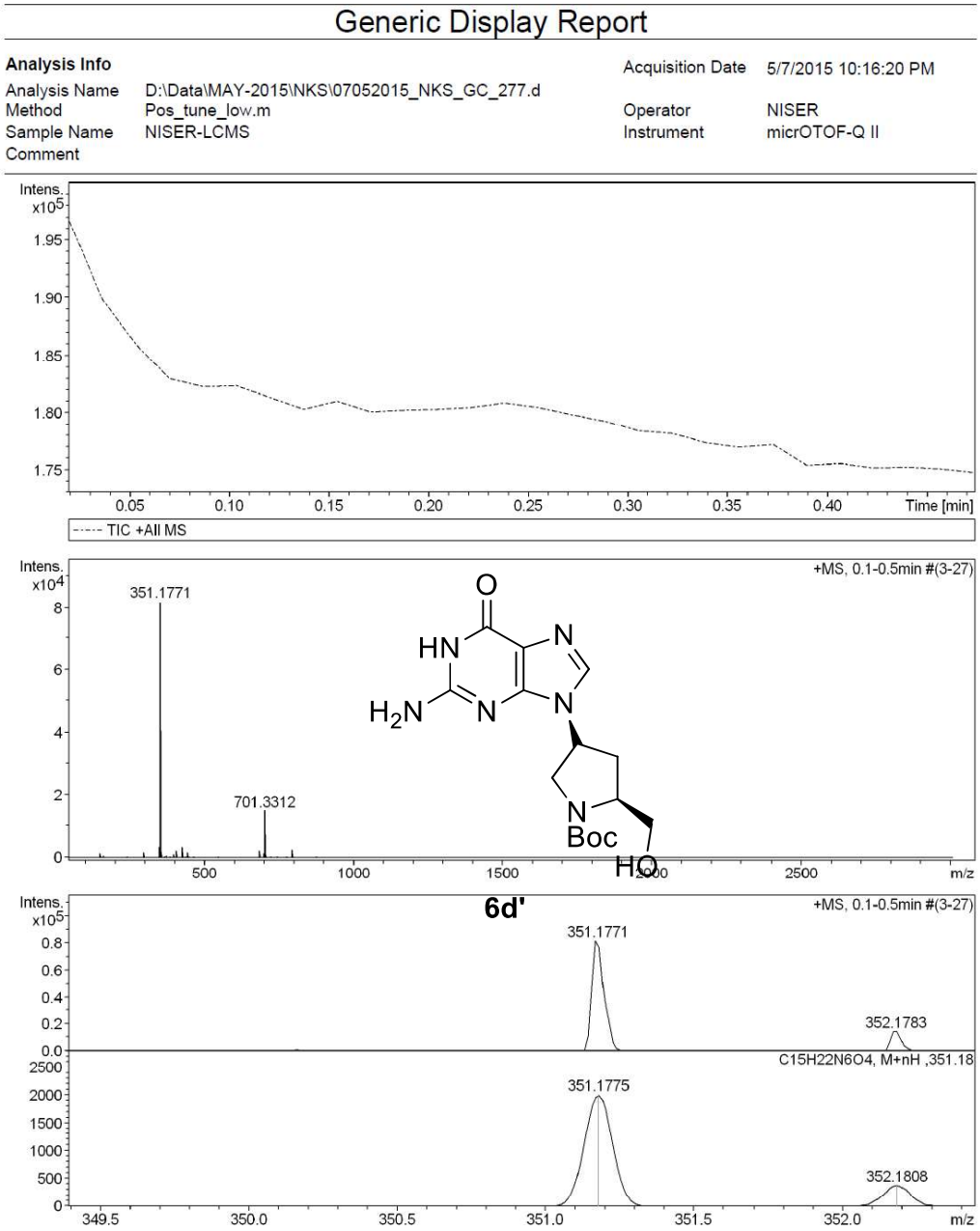


Figure A47 HRMS of compound **6d'**

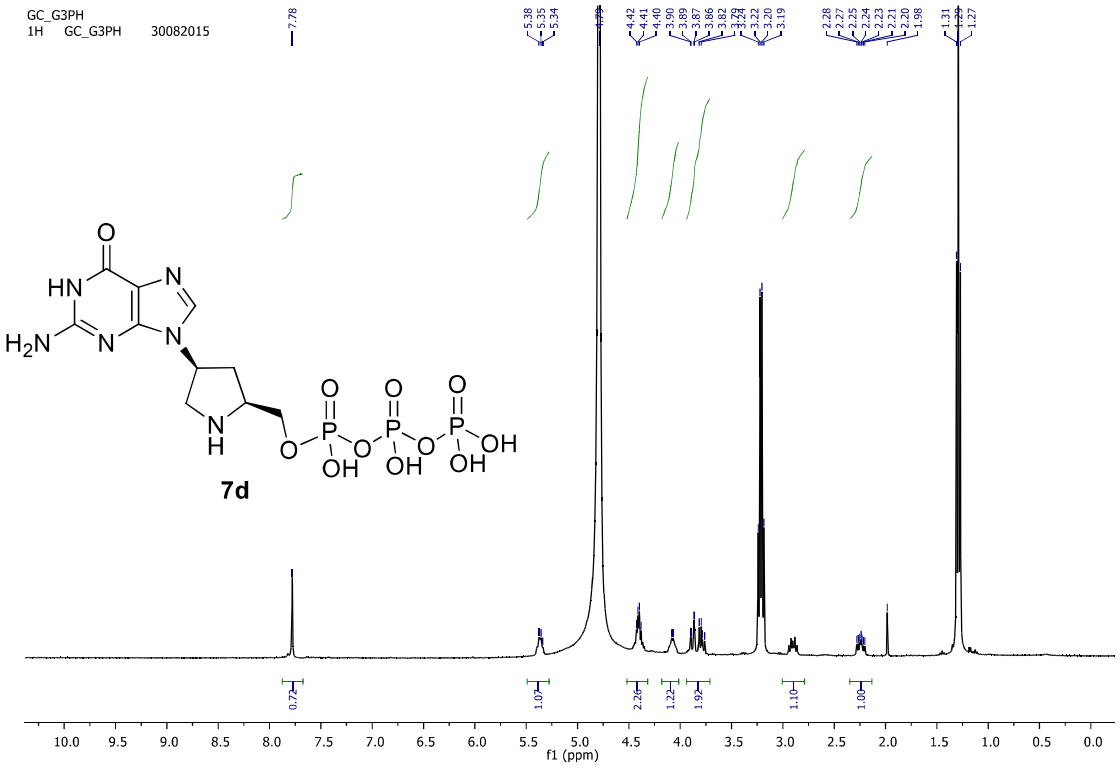


Figure A48 ^{1}HNMR of compound **7d**

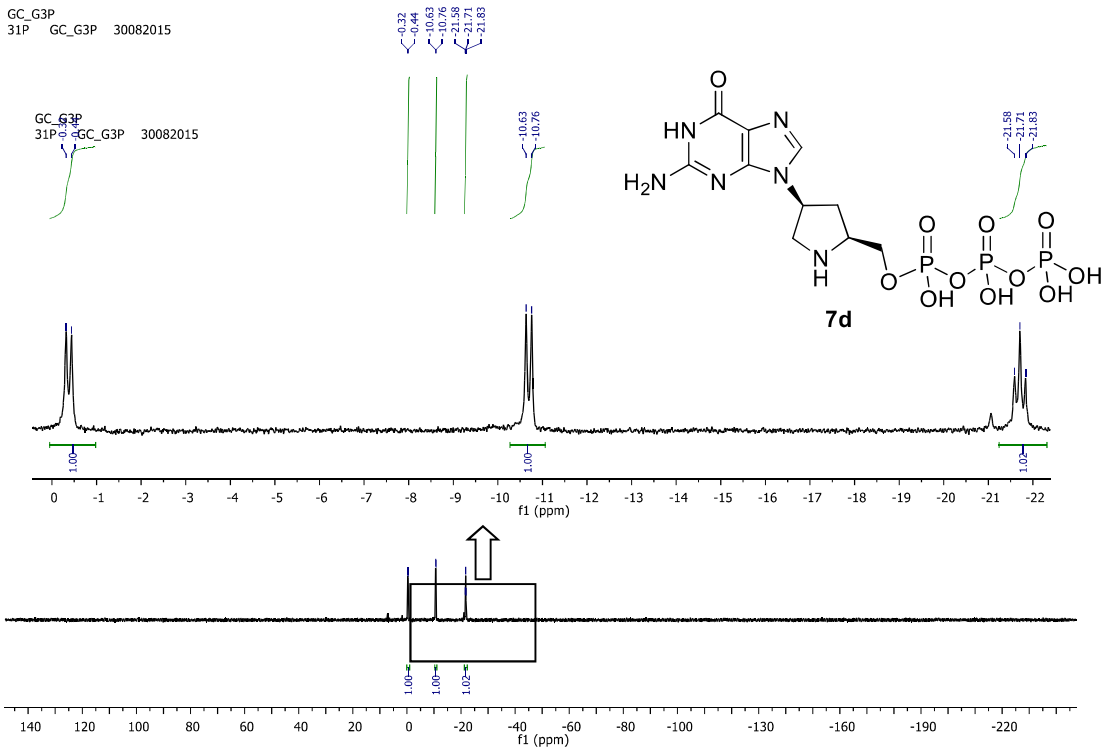


Figure A 49 P^{31}NMR of compound **7d**

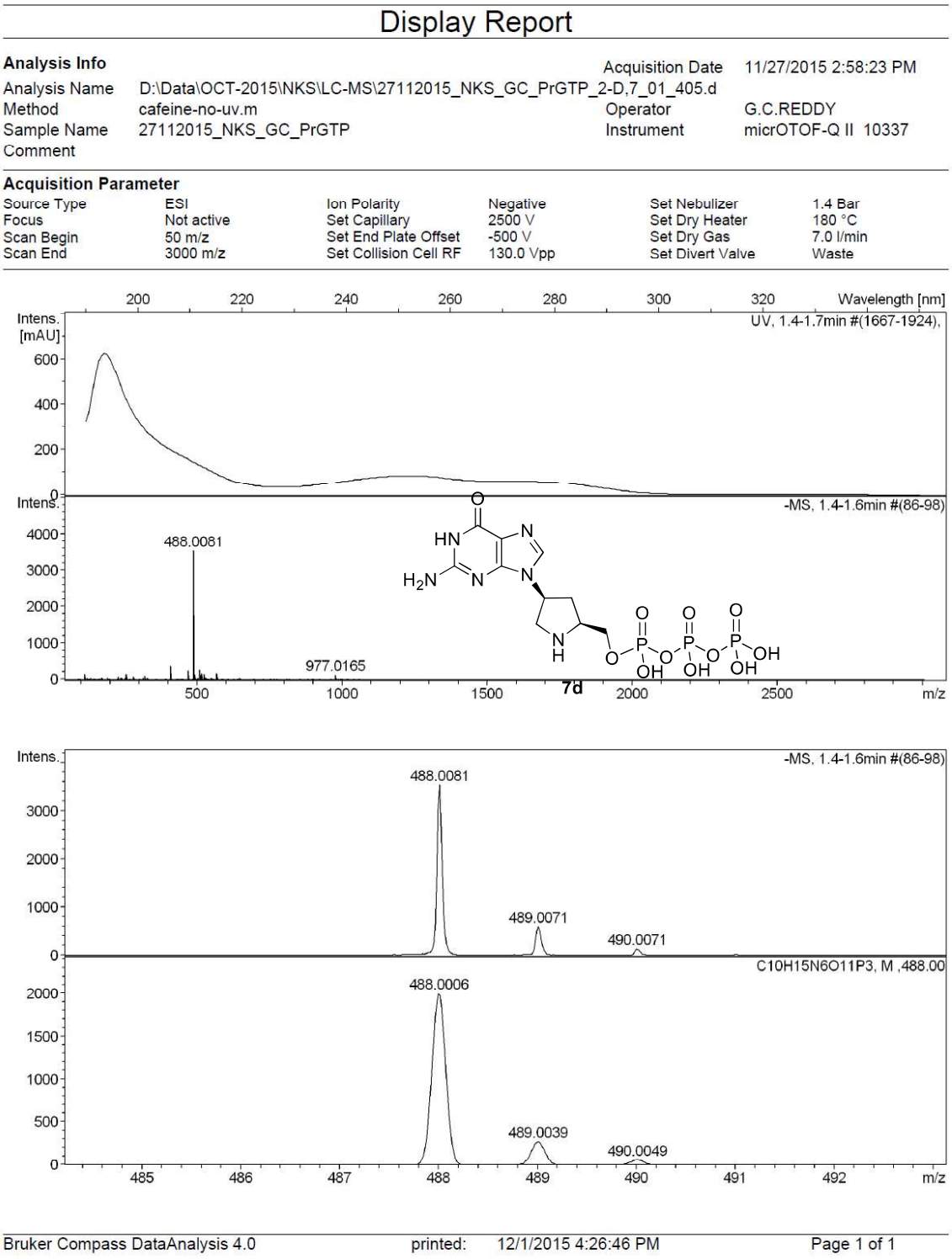


Figure A50 HRMS of compound **7d**

3. LC-MS Spectra of Primer extension reaction of pr-NTPs

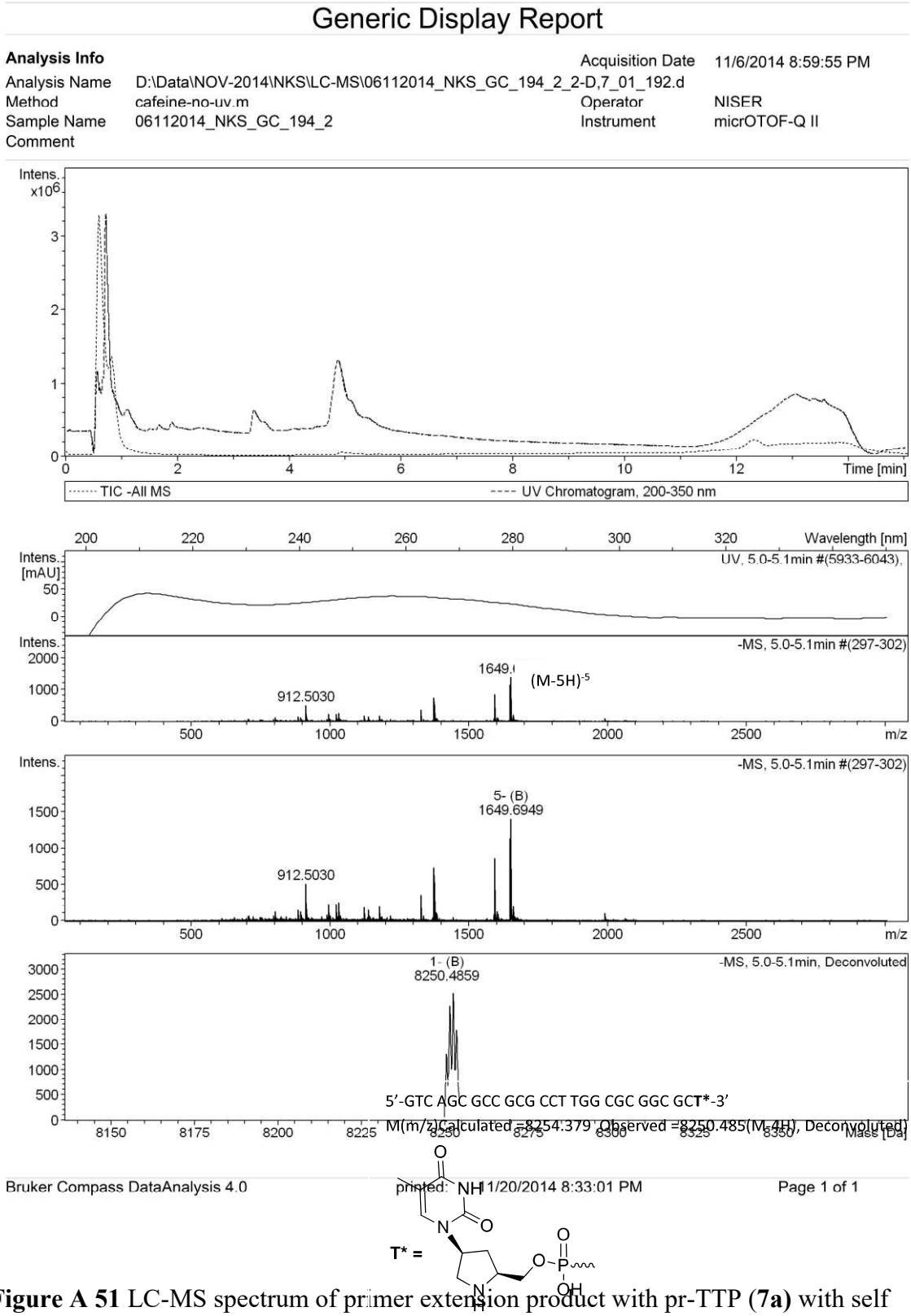


Figure A 51 LC-MS spectrum of primer extension product with pr-TTP (**7a)** with self -priming template (**ON1**)

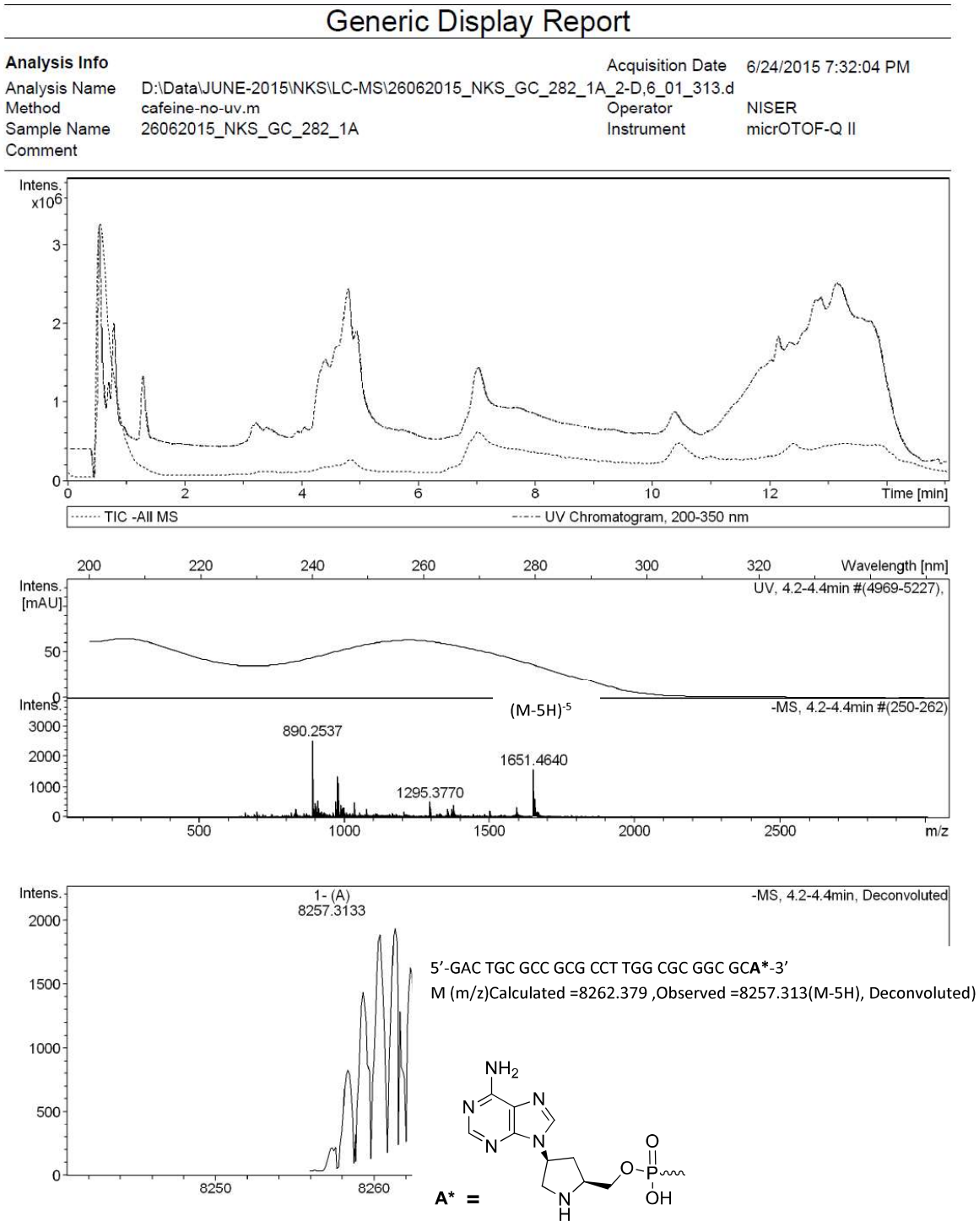


Figure A 52 LC-MS of primer extension reaction of pr-ATP **(7b)** with self-priming template (**ON2**)

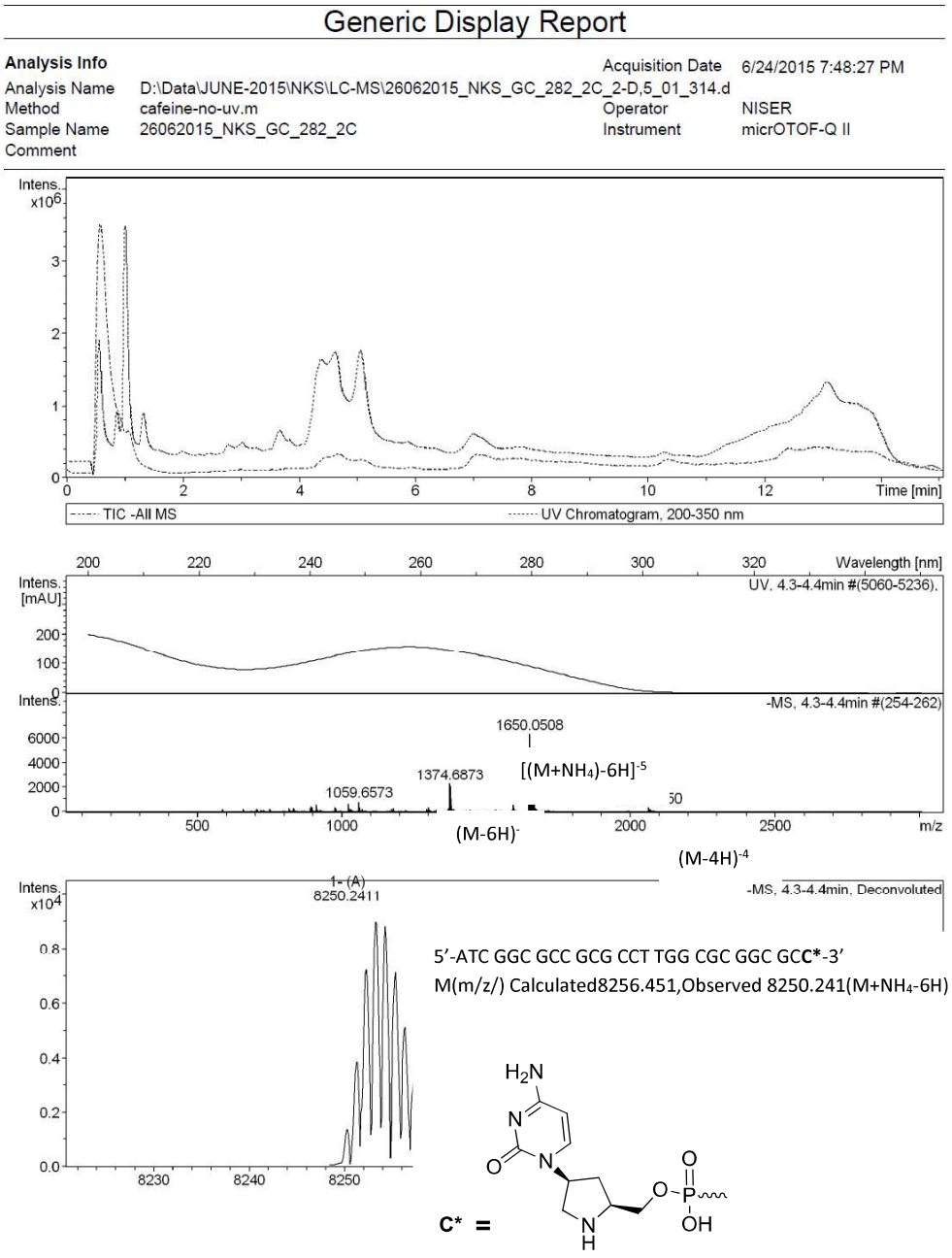


Figure A53 LC-MS of primer extension reaction of pr-CTP **(7c)** with self-priming template (**ON3**)

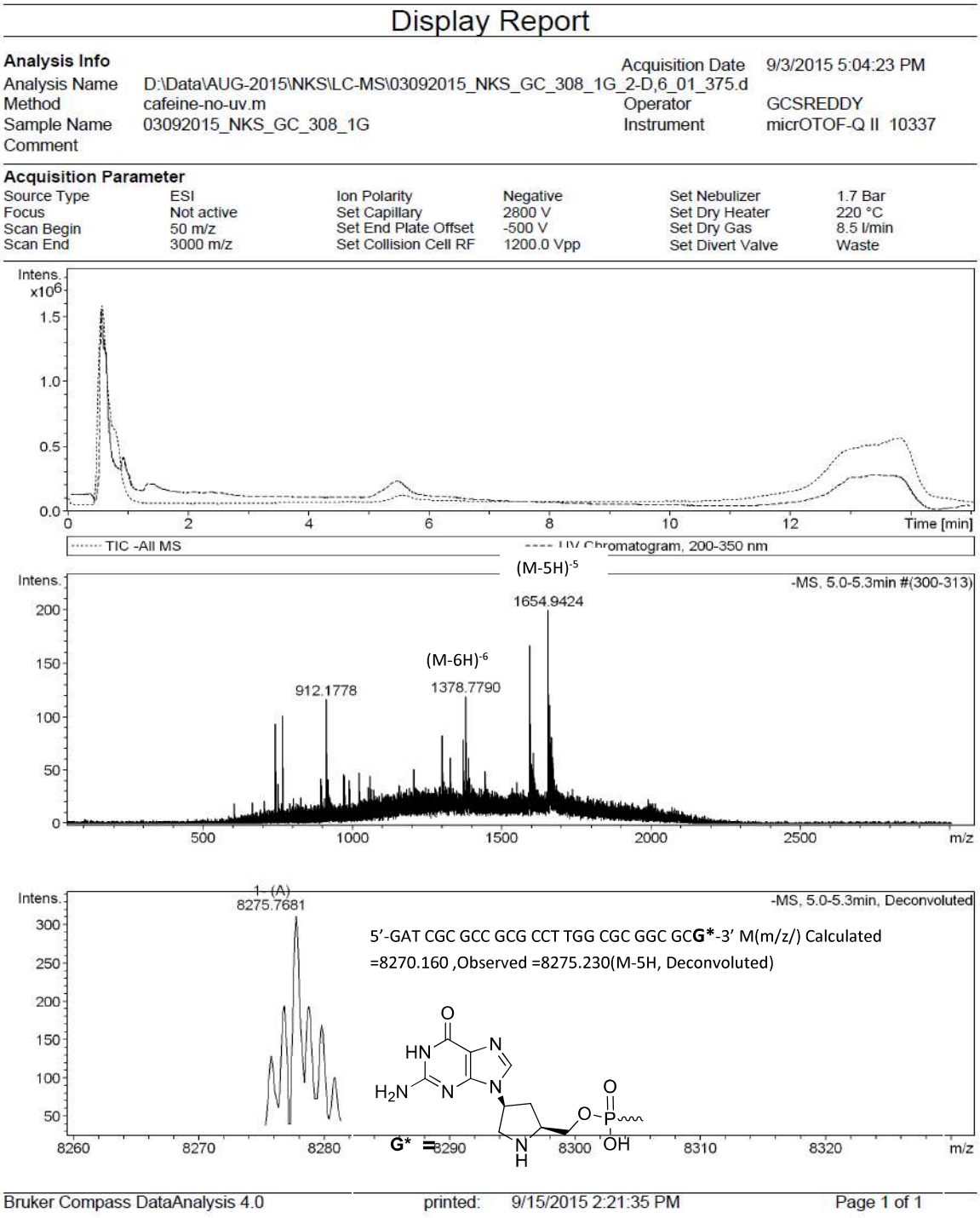


Figure A 54 LC-MS of primer extension reaction of pr-GTP **(7d)** with self -priming template (**ON4**)

4. LC-MS spectra of self priming DNA oligomers and Primer extension experiments

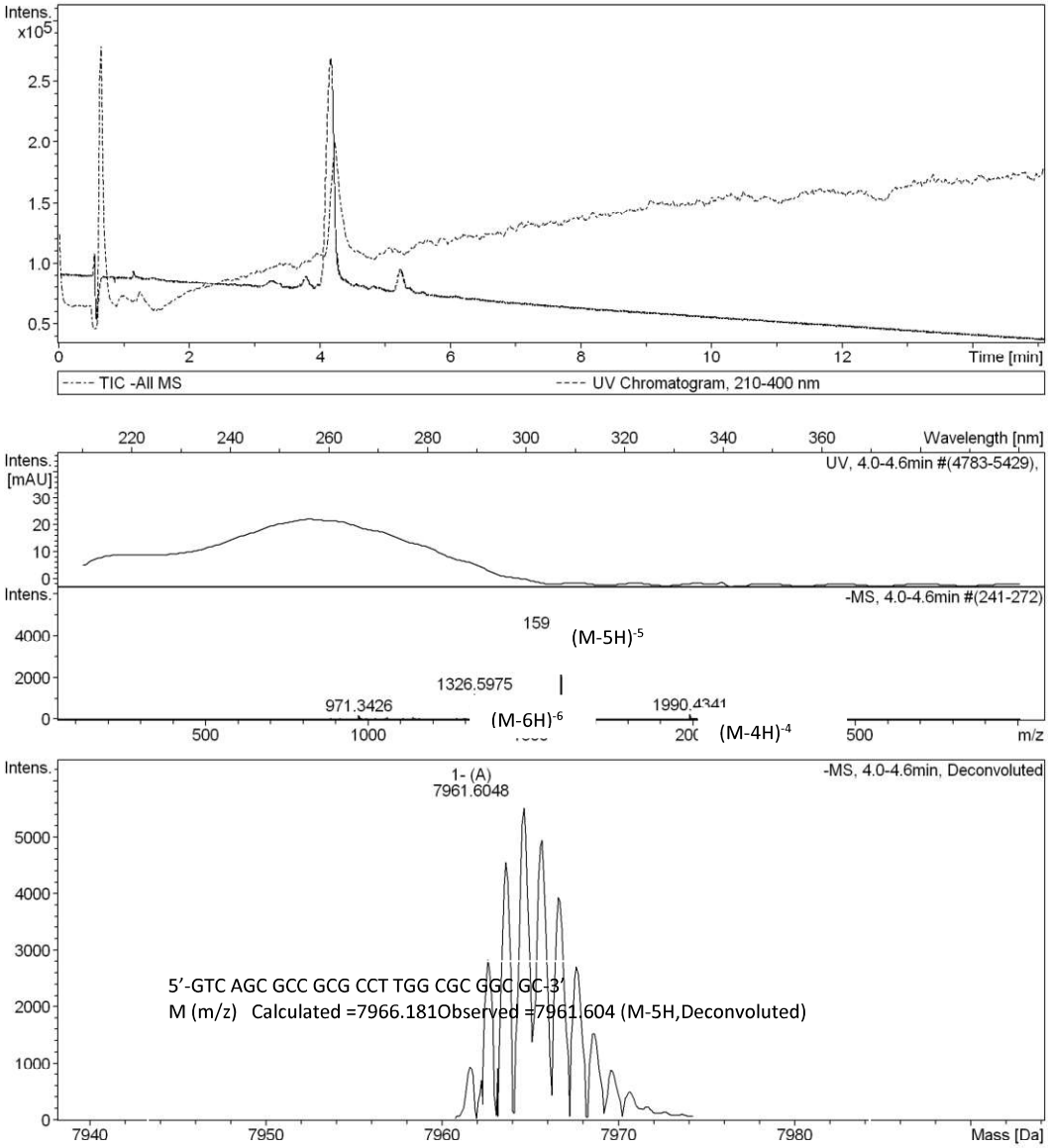


Figure A55 LC-MS spectrum of self-priming DNA oligomer (**ON-1**)

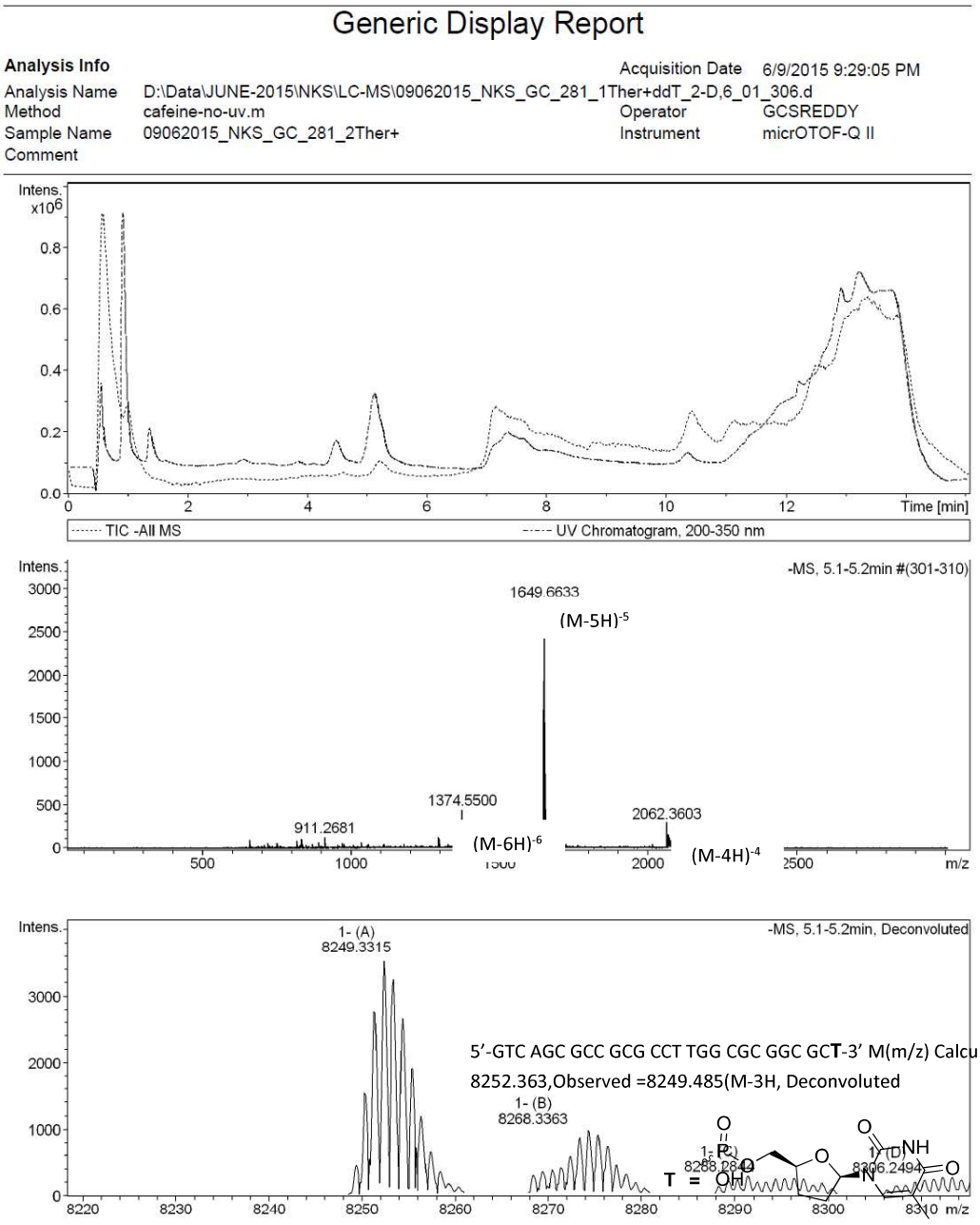


Figure A56 LC-MS spectrum of primer extension product with dd-TTP using *Therminator* DNA Polymerase

5. Comparative study of pr-TTP, d-TTP & dd-TTP towards polymerase activity

A comparative study has been done for incorporation of,*pr*-TTP and ddTTPs into a primer using template with Therminator DNA polymerase.

Reaction conditions: Template (**T1**)(5 μm), primer (**P1**) (5 μm),*pr*-TTP or ddTTP or, thrmopolbuffer10X(2.5 μl) and Therminator DNA polymerase (2 units), in a total volume of 25 μl reaction mixture incubated at 55^0C 30 min subsequently PCR products analysed by LC-MS.

Template (**T1**): 5'TAC GGA GCT GA ACT GGC CGT CGT TTT ACA 3'

Underlined sequence is complementary to primer.

Primer(**P1**): 5'TGT AAA ACG ACG GCC AGT 3'

Molecular weight (m/z) of template (**T1**): Calculated ;8908.846, observed;8903.562 (M-5H, Deconvoluted)

Molecular weight (m/z) of primer(**P1**): Calculated;5532.676,observed;5528.984(M-3H, Deconvoluted)

Molecular weight (m/z) of primer terminated with ddTTP calculated: 5820.727

observed 5817.126 (M-3H, Deconvoluted)

Molecular weight (m/z) of primer terminated with *pr-TTP* calculated: 5819.743 observed 5816.052 (M-3H, Deconvoluted)

.

6. LC-MS spectra of primer (**P1**), template (**T1**) and PCR products

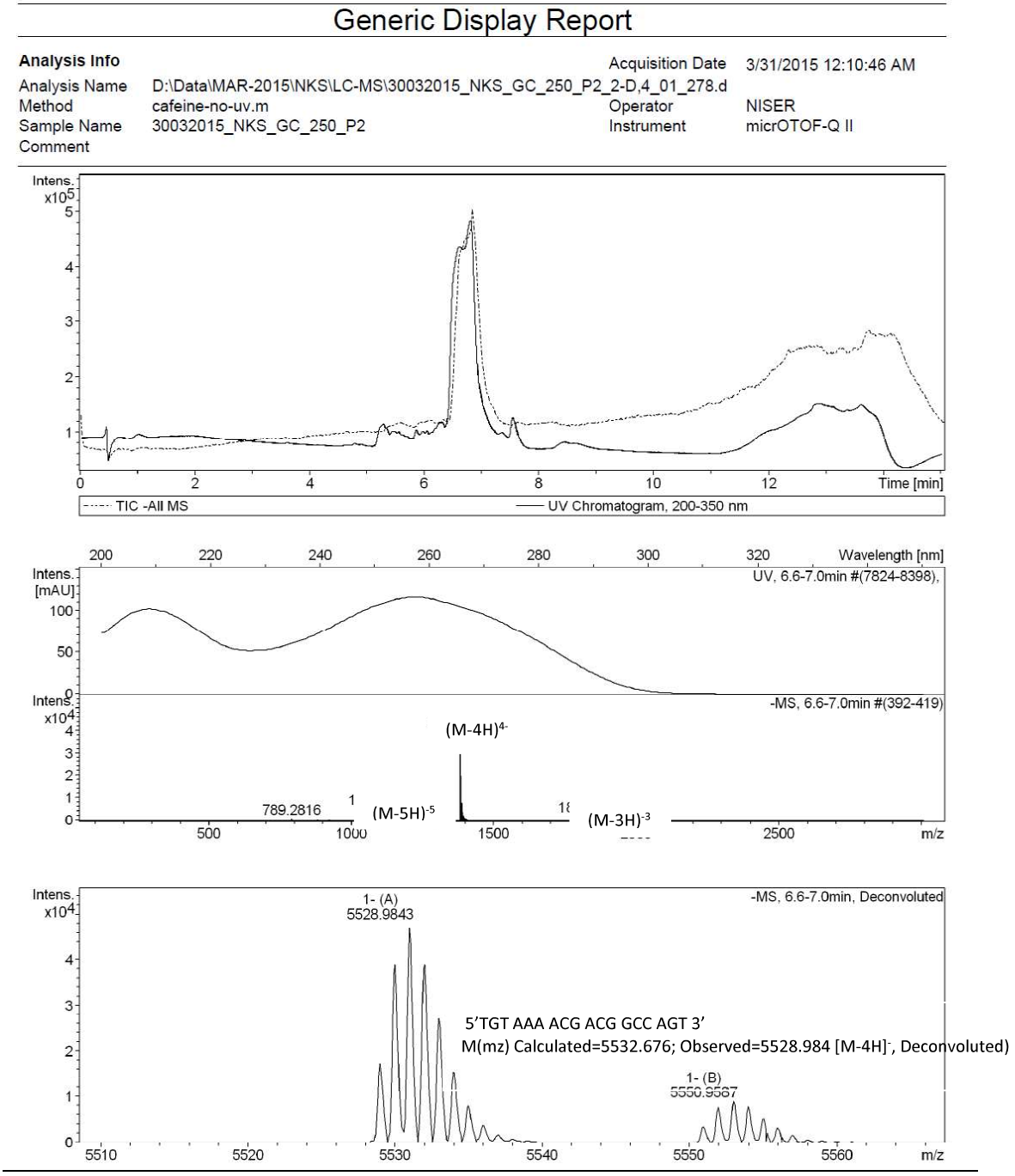


Figure A57 LC-MS spectrum of primer **(P1)**

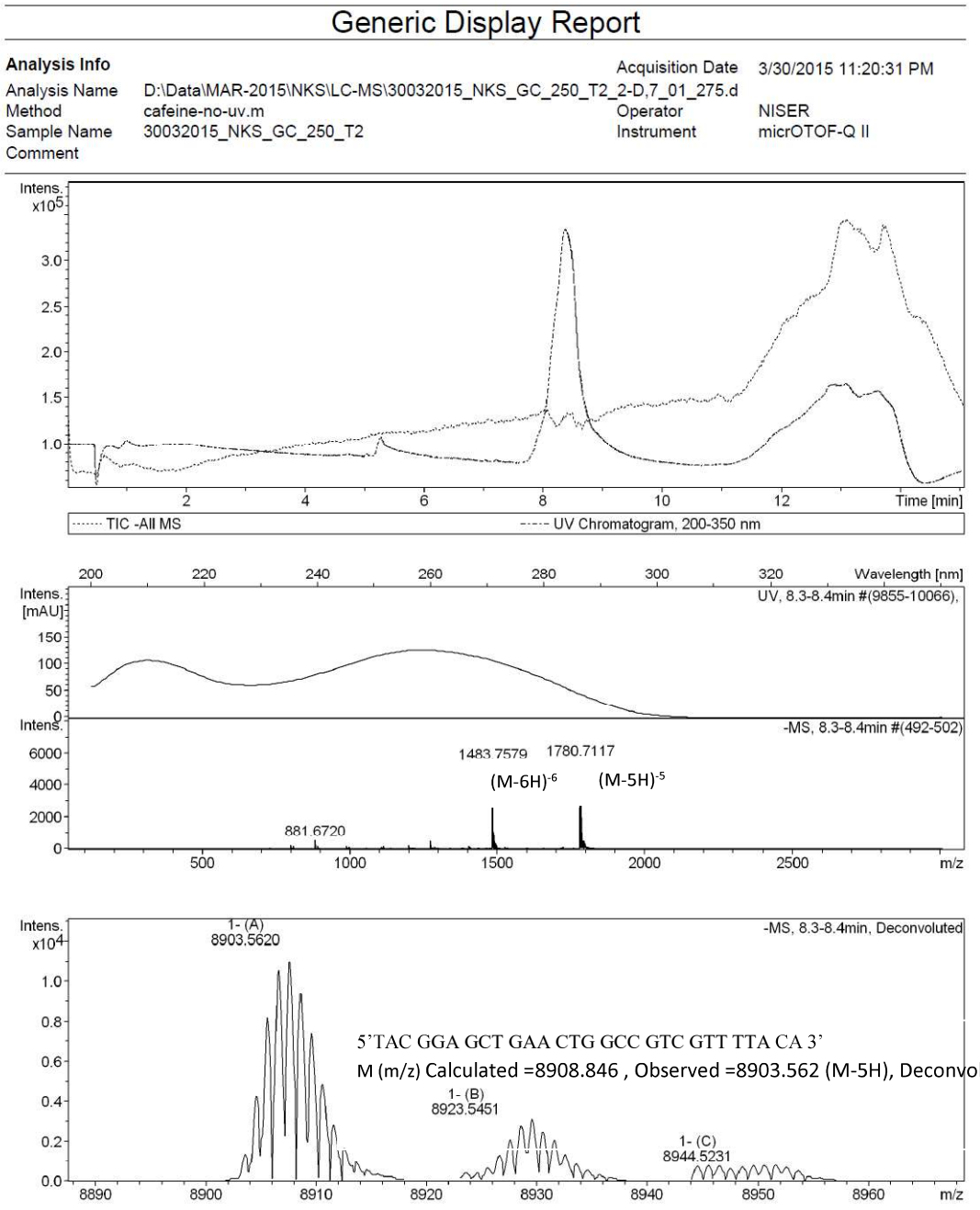


Figure A58 LC-MS spectrum of template **(T1)**

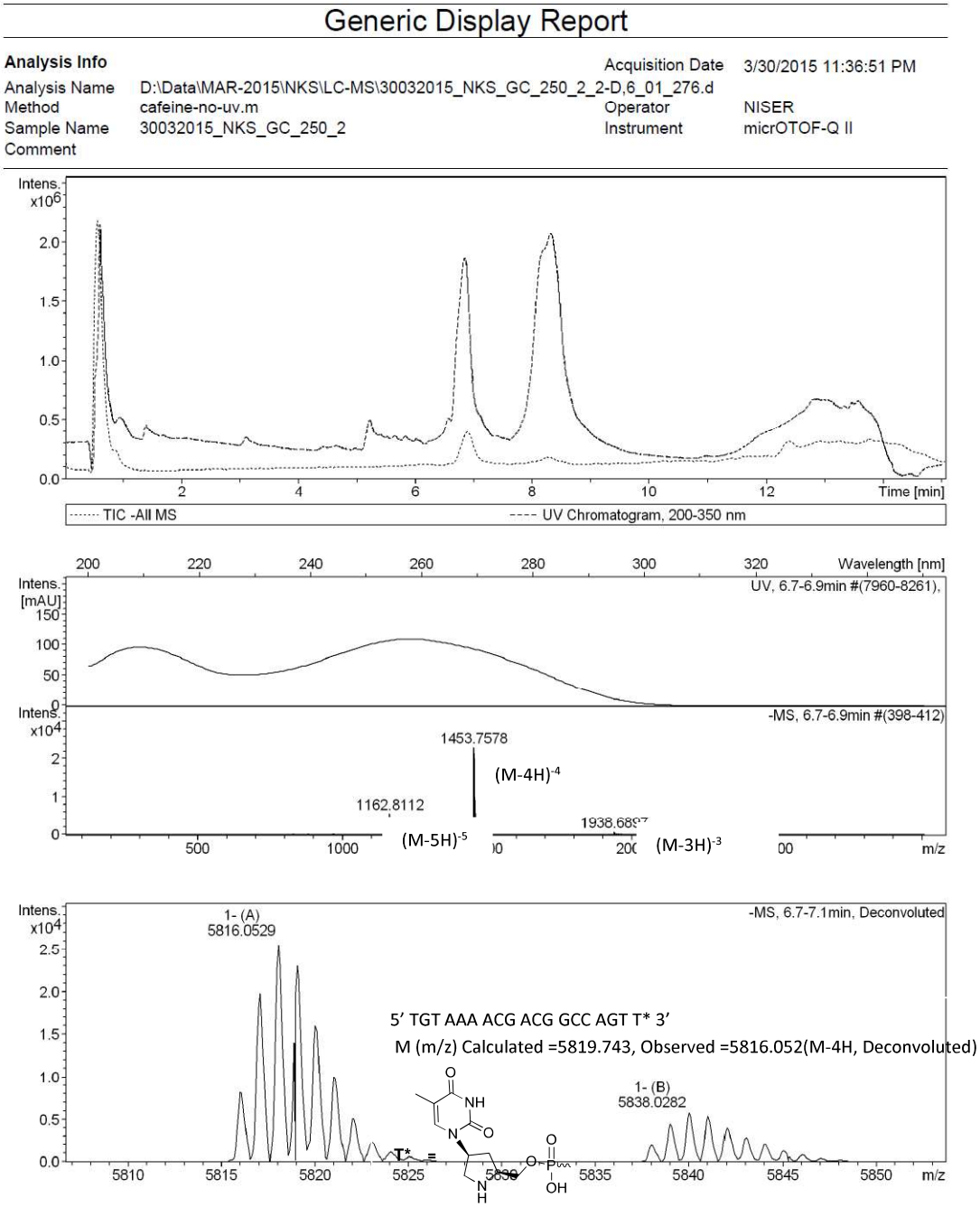


Figure A59 LC-MS spectrum of terminated primer with *pr*-TTP **(7a)** and primer (**P1**) at 30.0 minutes

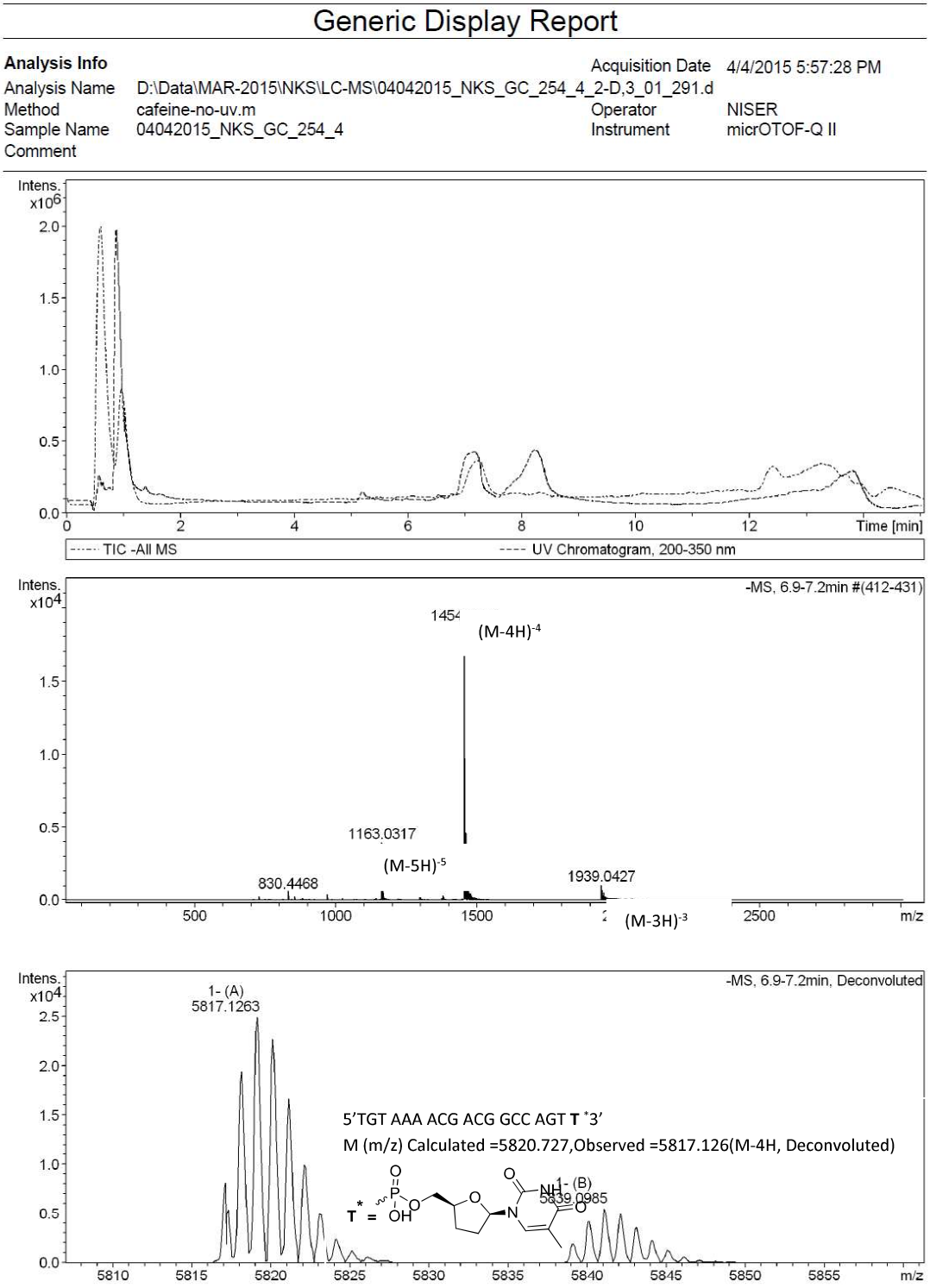


Figure A60 LC-MS spectrum of terminated primer with ddTTP and primer **(P1)** at 30.0

7. LC-MS of Primer extension product of prTTP /ddTTPwith P1/T1 in presence of other prNTPs/ddNTPs

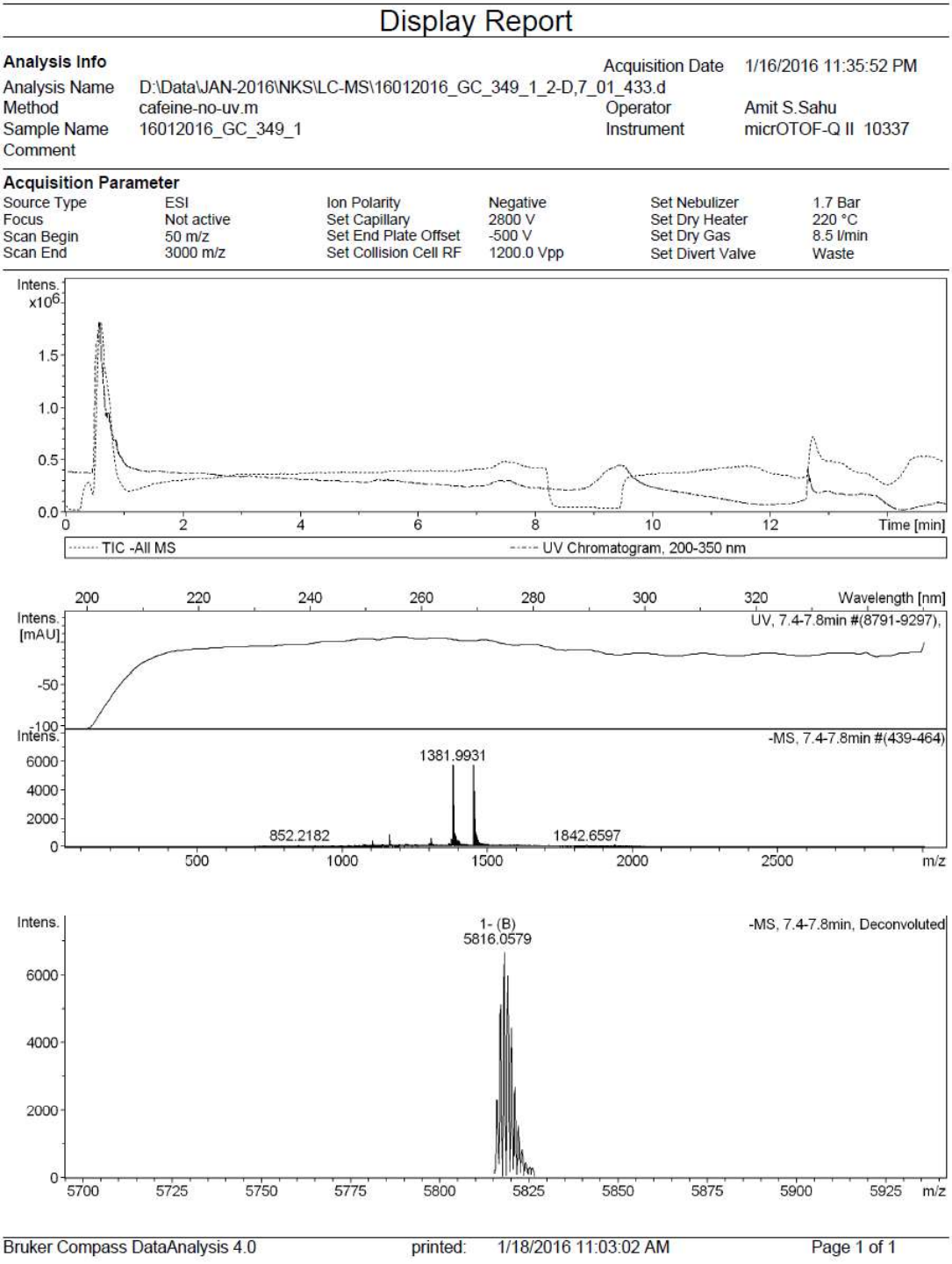


Figure A61. LC-MS of Primer extension product of prTTP with P1/T1 in presence of other prNTPs

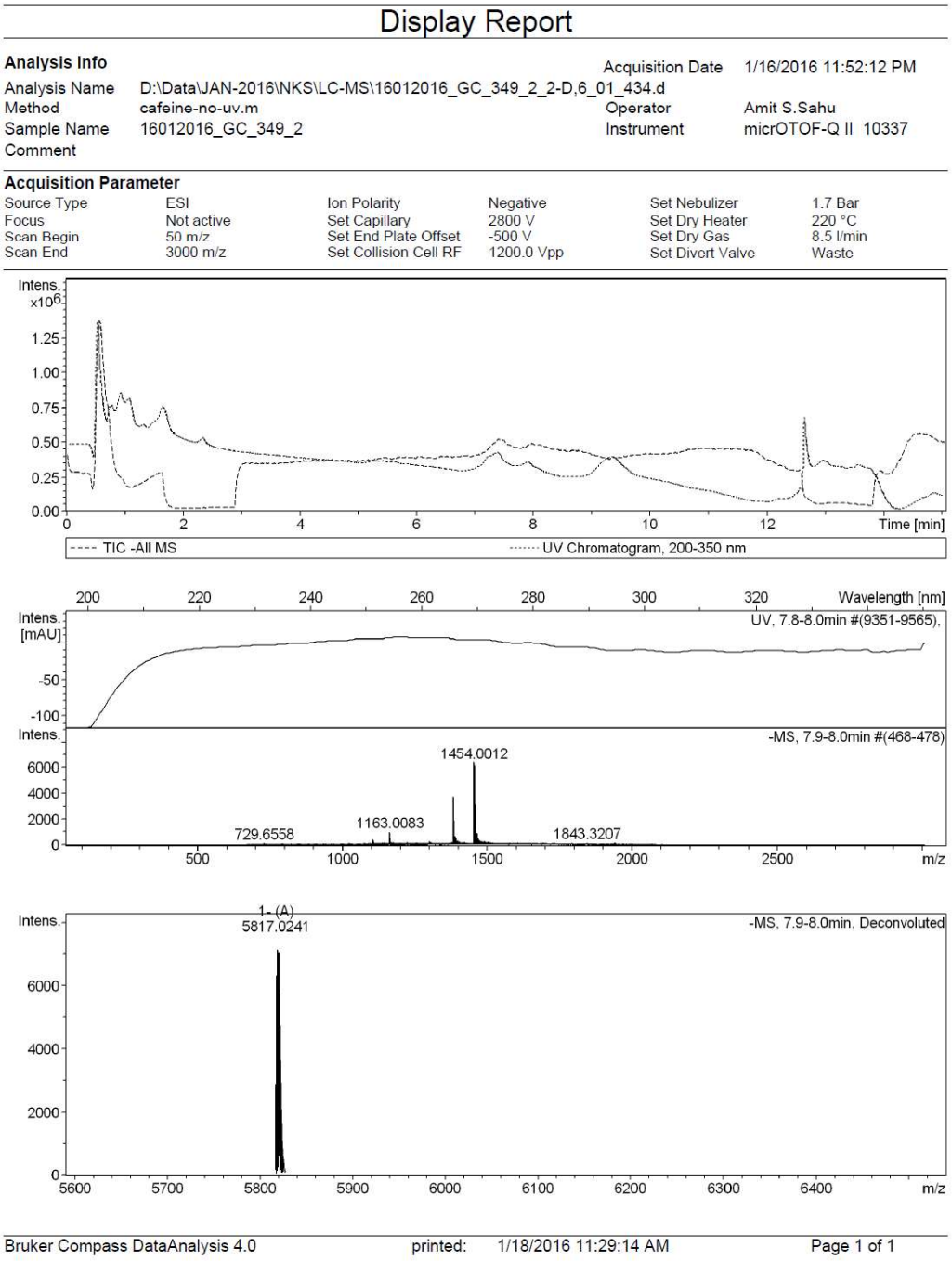
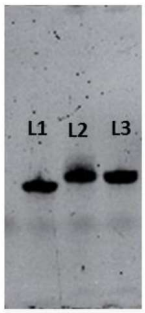


Figure A62 LC-MS of Primer extension product of ddTTP with P1/T1 in presence of other ddNTPs

8. Gel picture of FAMP1/T1 with prTTP and ddTTP



L1: 5'FAM labelled Primer P1

, **L2:** Primer extension reaction product of FAMP1 with prTTP,

L3 : Primer extension reaction product of FAMP1 with ddTTP

Reaction conditions: FAM P1 and T1 1µ mol, prTTP or ddTTP 100 1µ mol, 10 x Thermopol buffer 1.0 µl, Therminator DNA polymerase 1 unit in a reaction mixture of 10.0 µl incubated at 55^0 C for 30 minutes followed by quenching by adding 10.0 µl 2x loading dye and heated to 90^0C for 5 minutes and then analysed by 20 % denaturating urea PAGE.

Chapter 3A

Synthesis and Biochemical Evaluation of Alanyl Thymine Triphosphate (Ala-TTP)

Chapter 3A

Synthesis and Biochemical Evaluation of Alanyl Thymidine Triphosphate (Ala-TTP)

Contents

3A. 1 Introduction

Deoxyribose nucleic acid (DNA) is the genetic material and exists as double helical structure. [1] DNA has the phosphate back bone which is negatively charged. Due to the biological significance of DNA the structural analogues of DNA have been developed. Peptide nucleic acid (PNA) has emerged as potential DNA analogues which backbone is peptide oligomer from rationally designed aminoethylglycinate (*aeg*) amino acid (Figure 3A.1).[2] However the backbone of this peptide is acyclic and achiral, unlike deoxyribose sugar ring of DNA. In course of structurally modified PNA, Didersion and co-workers have reported alanyl-PNA containing alanine amino acid derivative as backbone, which is emerged a potential chiral PNA analogue.[3,4] Importantly, alanyl-PNA has less distance between nucleobase for increasing the π-π interactions and stabilizing PNA-PNA duplex/tetraplex structures.[5,6] However duplex structure from alanyl-PNA are unabled to form perfect duplex structure, unlike DNA duplex, due to short distance between nucleobases and poor flexibility in the backbone. Further consecutive repeated cytosine rich sequence as TC_8 are reportedly formed stable i-motif tetraplex type of structure.[7] Alanyl nucleobases are also incorporated into protein.[9] Further a modified PNA as alanyl Peptides nucleic acid (PNA) have been explored as the formation of stable i-motif tetraplex[12] structureand other biological applications

Moreover similar analogue as glycerol nucleic acid (GNA) are synthesized and studied in the formation /stabilizing of DNA duplex type of structure. GNA oligomers/homo oligomer are synthesized from its phosphoramidites using DNA synthesizer. [8,9] GNA units are also biosynthetically introduced into DNA primer from

its triphosphate analogue as glycerol triphosphate in presence of template DNA and DNA polymerase enzyme and however it is found to be poor substrate of various DNA polymerases.[10,11] Further alanyl nucelobase are incorporated into Peptides nucleic acid binds strongly to RNA and DNA.[12] Alanylnucelo bases incorporated into protein are synthesized and used as probe for DNA binding protein.[13]

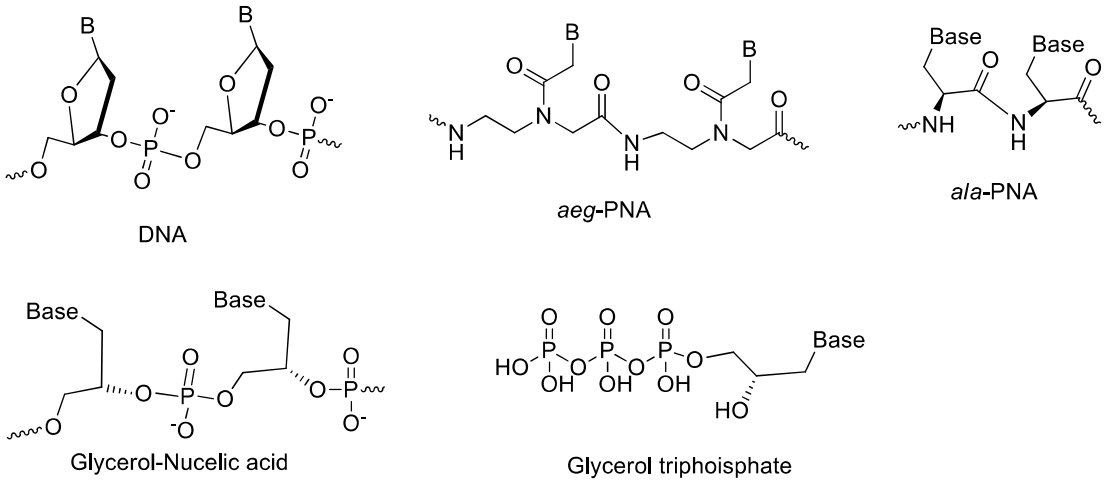
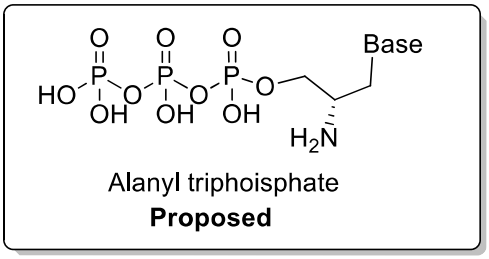


Figure 3A. 1 Structure of *aeg*-PNA, Ala-PNA, glycerol nucleic acid, and glycerol triphosphate.

3A 1.1 Hypothesis and objectives

Since *alanyl*-PNA is neutral acyclic chiral PNA analogue which encourage us to design its triphosphate analogue and incorporate into DNA primer enzymatically. This chapter describe the synthesis of rationally designed *alanyl*-nucleoside triphosphate analogue and its biochemical evaluation as enzymatic incorporation into DNA primer with DNA ploymerases.



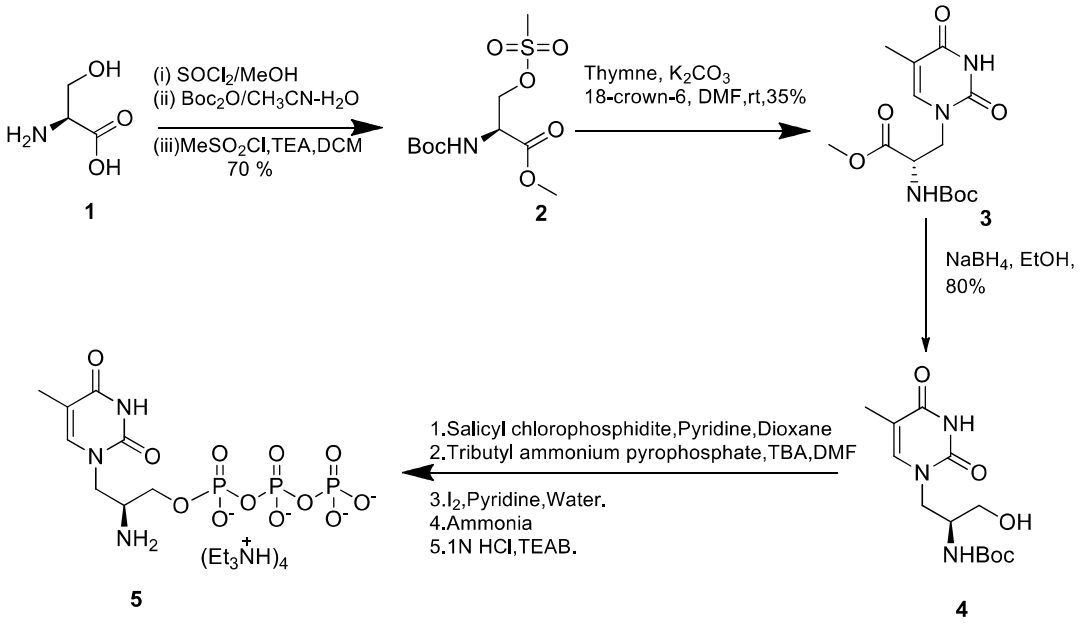
3A.2 Results and discussion

3A.2.1 Synthesis of alanyl thymidine triphosphate

The synthetic scheme of alanyl triphosphate analogue is described in Scheme 3A.1. Herein the synthesis of alanyl thymidine triphosphate was started from the naturally ocuuring amino acid L-serine (**1**). The carboxylic acid and amine groups were protected as methyl ester and *N*-Boc respectively. The hydroxyl group of protected *N*-Boc-Aminoacid-ester was modified as active mesylate derivative (**2**) with mesyl chloride. This mesyl derivative (**2**) was treated with nucleobase thymine under basic conditions (K_2CO_3/DMF) and isolated as desired *N*-Boc-thyminyl-alanyl methyl ester (**3**) after purification by column chromatography. In next, the methyl ester group of nucleoside (**3**) was reduced into alcohol as compound **4** with versatile reducing agent $NaBH_4$. This nucleoside alcohol (**4**) was phosphorylated with phosphorylating reagents by treting with salicylchlorophosphidite, tributylammonium pyrophosphate followed by oxidation with iodine pyridine/water solution then hydrolysed the cyclic triphosphate to triphosphate. Finallythen *N*-Boc group was deprotected with aq. HCl (1.0N). As resultant the desired compound as *N*-Boc-alanyl-thymidine triphosphate (ala-TTP, **5**) was isolated in moderate yield and purified by Sephadex-Gel column and

HPLC methods. All new intermediates of this scheme are well characterized by NMR ($^{1}H/^{13}C/^{31}P$) and ESI-Mass techniques.

Scheme 3A. 1 synthesis of Alanyl thymidine triphosphate



The nucleobases are known in hydrogen bonding and self-assembled with unique pattern. Thus we desired to obtain any self-assembly structure in alanyl-thymidine nucleoside for structural organizations. Thus we attempted to get single crystal from alanyl-thymidine nucleoside ester (**3**)/alcohol (**4**)/ triphosphate (**5**). However we got crystal of only alanyl nucleoside ester (**3**) in solid state after many attempts. The single crystal of nucleoside (**3**) was analysed by single X-ray diffractometer and its data are deposited to Cambridge crystallographic data centre (CCDC) with 1568315. number.

3A.2.2 Biochemical evaluation of alanyl thymidine triphosphate

The biochemical evaluation as examination of genuine substrate of DNA is studied with primer extension reaction method. In comparison, the known substrates of DNA, dTTP & ddTTP, are employed as control studies with following DNA polymerases: *Taq, Vent exo, Deep vent-exo, Bst and Therminator*. The DNA primers (**P1/FAM-P1)** and DNA template (**T1/T2**) are used for incorporation of *ala*-TTP or/& control dTTP/ddTTP under primer extension reactions. The sequence of DNA primer and templates are following: primer **(P1)** 5'-TGTAAAACGACGGCCAGT-3'; labelled primer **(FAM-P1)** Fam-5'TGTAAAACGACGGCCAGT-3'; Template **(T1)** 3'ACATTTTGCTGCCGGTCAA*GTCGAGGCAT-5'; template **(T2**) 3'ACATTTTGCTGCCGGTCAAAAAAAAAAAA-5. The underline sequence are complementary to primers.

For gel electrophoretic studies, the FAM labeled primer (5'**FAM-P1)** as **FAM-**5'TGTAAAACGACGGCCAGT-3' was treated with alanyl-TTP and enzyme (DNA polymerase) in presence of DNA template (T1) at 55^0C. The labelled DNA and its incorporated products, after primer extension reactions were visualized by denaturating PAGE. Their gel images are depicted in Figure 2. In Figure 2 A, the gel-band of L1 belongs to labelled DNA primer. While the gel-bands of L2/L3/L4 belong the extended labelled primer with respective dTTP,/ddTTP/*ala*-TTP with *therminator* DNA polymerase (Figure 2A). The remarkable gel-bands shift of FAM-DNA primer are visualized with *ala*-TTP as like control (dTTP & ddTTP). However with dTTP the shift is comparatively more than ddTTP and *ala*-TTP. In literature, the multiple incorporations of dNTP into primer are reported with high fidelity DNA polymerase.[14]

Since ddTTP is well known as terminator of DNA synthesis after single incorporation into DNA primer. In case of also the gel-shift band of extended labelled primer is closely matched with that of ddTTP. These gel-shift band analysis strongly support the only single incorporation of alanyl-TTP into DNA primer with highly efficient DNA *Therminator* polymerase. With *vent-exo* & *Taq* DNA polymerases, the similar gel-shifts of DNA primer (**FAM-P1**) are noticed with *ala*-TTP and control ddTTP (Figure 3A, 2.A L4 & L5).

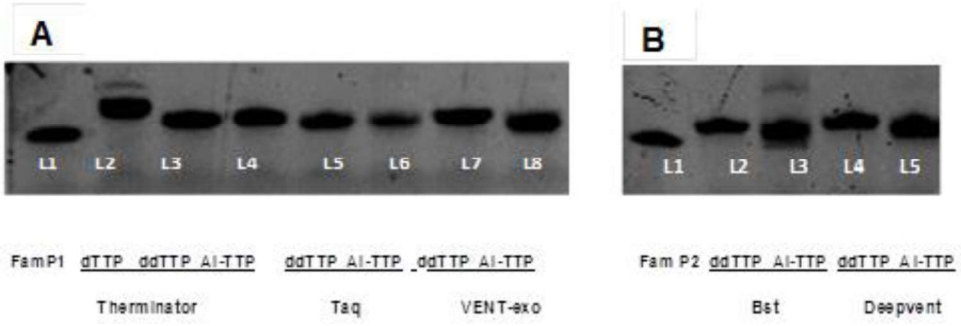
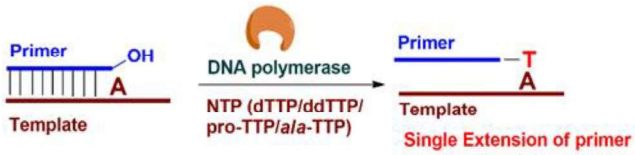


Figure 3A. 2 PAGE analysis of primer extension reactions of Ala-TTP with DNA polymerases (A) L1 FAM P1, L2, L3 and L4 extended primer with dTTP, ddTTP and Ala-TTP byTherminator DNA polymerase, L5 and L6 extended primer with ddTTP and Ala-TTP by TaqDNA polymerase, L7 and L8 extended primer with ddTTP and Ala-TTP by Vent exo-DNA polymerase (B) L1 FAM P1, L2 and L3 extended primer with ddTTP and Ala-TTP by Bst DNA polymerase, L4 and L5 extended primer with ddTTP and Ala-TTP by Deep vent DNA polymerase

With Bst & Deepvent DNA polymerses, the gel-shifts of DNA primer (**FAM-P1**)'s gel-band are consistently similar. (Figure 3A.2B; Lane 2-5). These gel studies strongly support the single incorporation of *ala*-TTP into DNA primer as like control

ddTTP with other DNA polymerases too. The incorporation of dTTP/ddTTP/alanyl-TTP into labeled primer with DNA polymerase are also summarized in Table 3A.1. As like control dTTP and ddTTP, alanyl-TTP is also get easily incorporated into DNA with all given DNA polymerase. Hence *ala*-TTP is another terminator of enzymatic DNA synthesis after single incorporation.

Table 3A. 1 DNA polymerase ativity of *alanyl*-TPP & control (dNTP &ddNTP) reaction results



Sr. No	DNA Polymerase	dTTP	ddTTP	ala-TTP
1	Bst	✓	✓	✓
2	Deepvent	✓	✓	✓
3	Taq	✓	✓	✓
4	Vent-Exo	✓	✓	✓
5	Therminator	✓	✓	✓

Further to investigate for multiple incorporations of *ala*-TTP into primer, we designed a template (**T2**) which could incorporate multiple thymidine triphosphate. Herein, we performed primer extension reactions with designed primer/template and *ala*-TTP and control dTTP by gel-electrophoretic methods. Their gel-shifts are depicted in Figure 3. In case of dTTP, the synthesis of multiple dTTP incorporated DNA, almost full

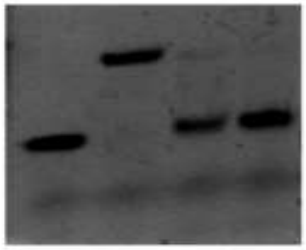


Figure 3A. 3 PAGE analysis of primer extension reactions of *ala*-TTP/dTTP/ddTTP with DNA polymerase L1 FAM P1, L2, L3 and L4 extended primer with dTTP, ddTTP and Ala-TTP byTherminator DNA polymerase

length DNA sequence were noticed (Figure 3, L2). However, with *ala*-TTP, gel-shift band (Figure 3A. 3, L3) is almost equal to that of ddTTP (Figure 3A. 3, L4). Hence *ala*-TTP also could not extend the synthesis of DNA after single incorporation like ddTTP. In literature, glycerol NTP is known for single incorporation into DNA.

3A.2.3 Liquid Chromatography –Mass Spectrometry (LC-MS) Studies

Finally the incorporation of *ala*-TTP into DNA primer was analyzed by LC-ESI-Mass analyses. We recorded the mass spectra of DNA primer (**P1**) and Template (**T1**) before and after treatment with *ala*-TTP/ddTTP in presence of one of the enzyme, *Therminator* DNA polymerase. The mass spectra of primer/extended primer with ala-TTP and control ddTTP are provided in Appendix of this chapter. The mass spectrum of DNA primer **P1** exhibited a prominent peak at 1381.99 (m/z) which belong to molecular mass ion (M_p) of primer as $[M_p\text{-}4H]^{4-}$, while the mass of template(**T1**) as $[M_T\text{-}6H]^{6-}$ is appeared at 1483.57 In presence of therminator polymerase and ala-TTP,

the mass of DNA primer (P1) is increased and appeared at 1447.28 (m/z) (Figure 3A.4) which belong to the

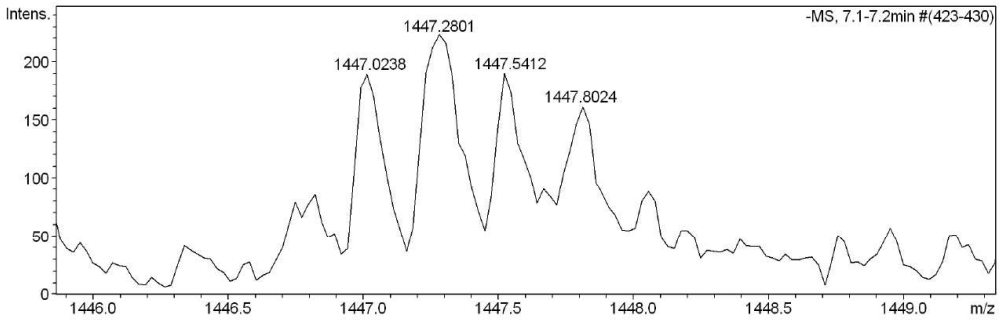


Figure 3A. 4 Mass spectra of extended mass of primer with ala-TTP

molecular mass ion of single *ala*-TTP extended DNA primer (M_p^*) as $[M_p^*\text{-}4H]^{4-}$. However, with control sample, ddTTP, the mass of DNA primer P1 is also increased and appeared at 1454.16 (m/z) as $[M_p^*\text{-}4H]^{4-}$ under similar primer extension reaction conditions which belongs to the mass of single ddTTP extended DNA primer. Hence these mass results confirm the single enzymatic incorporation of ala TTP into DNA primer in presence of DNA Template and DNA polymerase.

3A.2.4 Self-assembly study of alanyl-thymidine ester (3)

Importantly we got single crystal of *alanly* nucleoside ester (**3**), precursor of *ala*-TTP (**5**), and studied by X-ray diffractometer which confirmed the chemical structure of nucleoside ester (**3**) in solid state. The X-ray data is deposited to Cambridge crystallographic data center (CCDC) with reference CCDC 1568315. The ORTEP diagram of compound **3** are depicted in Figure 3A.5.

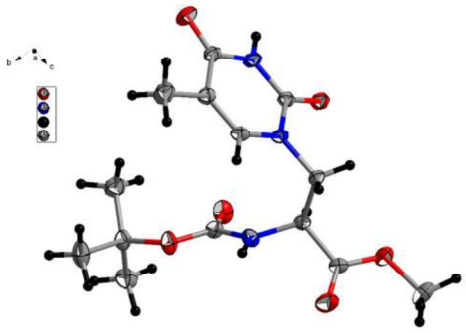


Figure 3A. 5 ORTEP diagram and Phase data of ala-thymidine ester (3): Space-group P 1 21/c 1 (14) – monoclinic; Cell a=5.2456(3) Å b=26.0994(15) Å c=12.1838(7) Å β=99.467(4)°; V=1645.33(16) Å3

After the confirmation of ala-thymidine ester (3) crystal structure, we attempted to find its (compound 3) supramolecular assembly in solid state. Using crystal visualizing software Diamond 3.2, the packing arrangement of thymidine ester **3** was obtained (Figure 3A.6, top left). The viewing from one axis 'b' indicate two thymidine ester (3) are assembled by an unusual hydrogen bonding pattern between their thymidine residues in plane's axis 'a' and 'c' (Figure 3A .6, top right). Herein a unique type of nucleobase pairs, Thymine: Thymine (T:T) are noticed by two hydrogen bonding between two thymine residues as following: N^3-H of one thymine (T) with O=C^2 of other thymine (T) and vice versa. In literature too, such type of hydrogen bonding are called as reverse Watson-Crick hydrogen bonding which are noticed in the supra molecular assemblies of Ferrocene linked Thymine/Uracil conjugated compounds.[15,16] However, Watson-Crick and Hoogsteen types of hydrogen bonding are found in nucleobase supramolecular self-assembly in many modified nucleosides.[17-21] In addition further the side view of ala-Thymidine ester supramolecular assembly are depicted in Figure 3A.6 (bottom left) which indicate the

formation of *anti-parallel self-duplex* type of structure. Each strand of this duplex is formed by hydrogen bonded backbone as –C=O-------HN of Boc- group of ester **3**. Interesting unique π-π interactions are noticed between thymine residues of one stand with that of other strand and further stabilized that self-duplex structure of *alanyl* nucleoside in solid state. The distance of hydrogen bonds and π-π interactions are extracted from the supramolecular structure of alanyl ester (**3**) (Figure3A. 6, bottom right). The hydrogen bond distances between two thymine are 2.0Å; hydrogen bond distances between two Boc groups are 2.4Å; the distance of π-π interactions in thymine are 3.7Å; the distance between two strands of that duplex are 10Å, and distance between two units of each strand are 5.2Å (Figure3A.6, bottom right). 6, bottom right).

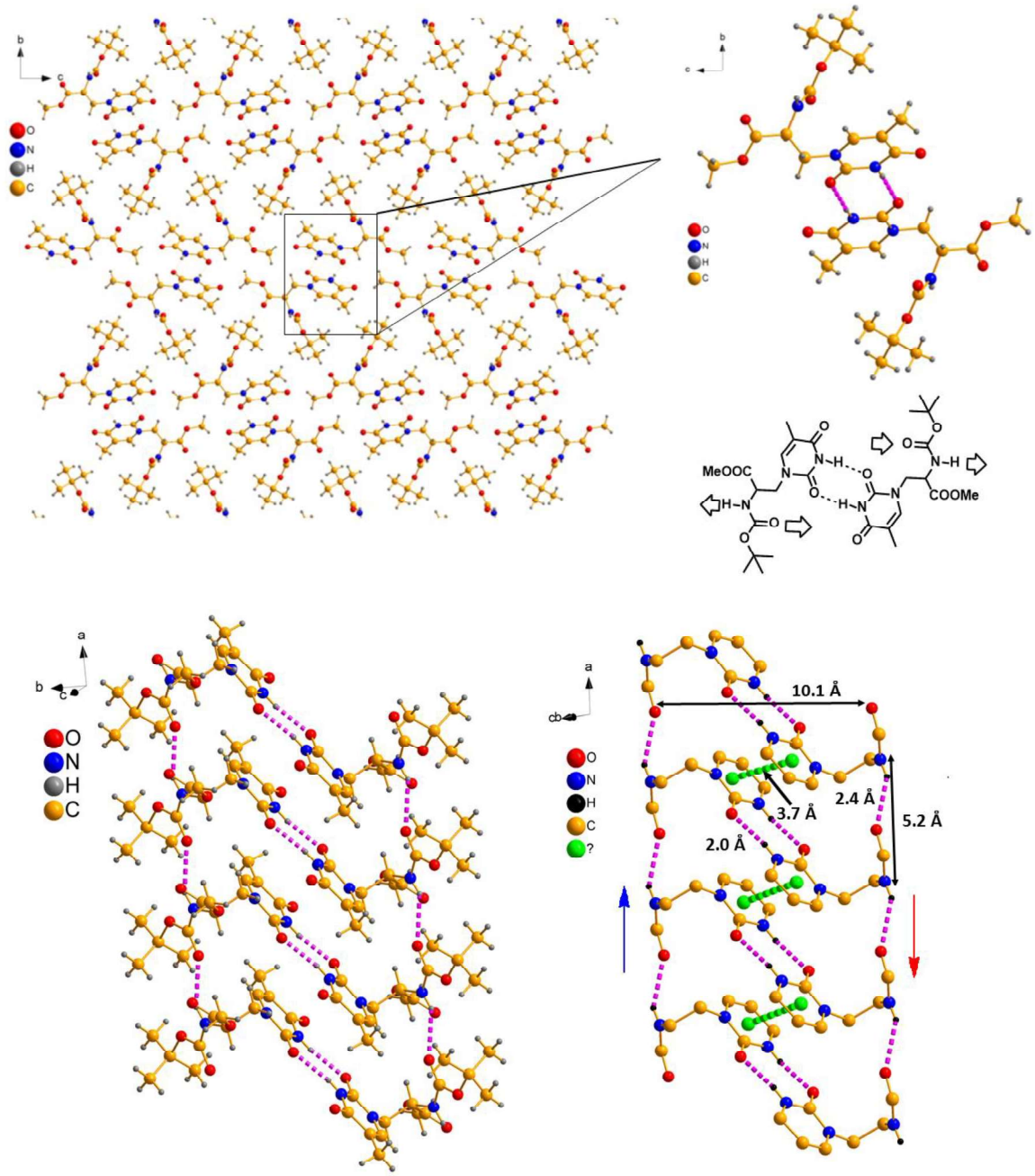


Figure 3A .6 Structural studies of single crystal of Boc-N-alanylthymidine methylate (3) by X-analyse: (a) Packing diagram (top left); (b) diagram of selected region (top right); (c) Hydrogen bonding pattern (bottom left) and (d) Detail of distance for hydrogen bonding and π-π interaction (Bottom right)

3A. 3 Conclusion

We have successfully synthesized alanyl thymidine triphosphate (*ala*-TTP), and performed its biochemical evaluation with various DNA polymerases in presence of DNA primer and DNA templates. The alanyl thymidine triphosphate is a substrate of several DNA polymerases. The ala-TTP involves in primer extension reaction and and incorporated one could extend the primer only one extension like glycerol NTP due to its flexible backbone and rotational degrees of freedom. The *ala*-thymidine triphosphate can be used to synthesize the 3'-amino functionalized DNA. We have also studied the self-assembly of crystal structure of *ala*-thymidine ester. The compound shows self-assembly and duplex type of structure aided by hydrogen bonding and pi-pi interactions.

3A.4 Experimental section

All chemicals and reagents purchased from commercial sources and used as received. Oilgo nucleotide purchased from IDT. All enzymes purchase from NEB .DMF and DCM distilled over CaH_2 and stored on molecular sieves. THF and dioxane dried over Sodium benzophenone and stored over sodium. Pyridine distilled over potassium hydroxide and stored on CaH_2.Triethyl ammonium bicarbonate (TEAB) prepared by passing CO_2 gas into 2M aqueous triethylamine solution and stored at 4ºC. HPLC performed on Waters semipreparative HPLC using sunfire column(C18 ,4.6X150 mm) using 20mM TEAB and Acetonitile (0-10 % 30 min). LC-MS experiments performed on Bruker microTOF-Q II coupled with a Waters Acquity UPLC auto sampler .Primerextension reaction products(25 µl) were diluted with 25 µl Milli-Q water and 10.0 µl from this mixture injected by auto sampler by following programme

at a flow rate 0.2ml/minute linear gradient.0-10 min80% A gradient, 10-12 min 30% A gradient, at 12- 13 min30% A isocratic, 13-14min 100% A gradient, at 14-15 min 100% A isocratic. A C-18 RP UPLC column (2.1 x 5.0 mm, 1.7µm, Acquity Waters) used for LC-MS experiments. Column temperature maintained at $30^{O}C$ during the experiments (buffer A: 5mM ammonium acetate (pH 7.0) and buffer B: methanol)

Primer extension reactions for LC-MS analysis were carried out with 5 µM of primer and template, 100 µM ala-TTP or ddTTP in a total reaction volume of 25 µl with 2 units of Vent exo- DNA polymerase

All primer extension reactions performed with 1µM FAM labelled primer, 1µM template 100 µM NTP in 10.0 µl with reaction buffer provided by the supplier. All reactions performed at $55^{o}C$ for 60 minutes. 2 units of each therminator, Vent exo- and Deep Vent exo- DNA polymerases were used in 10.0 µl reactions .Bst and Taq DNA polymerases were used 8.0 units and 10.0 units in 10.0 µl reactions.

Gel experiments performed with 20% PAGE (29: 1 Acrylamide ,bis acrylamide) and 7M urea .Gels prepared and ran in 1x TBE and visualised under trans UV using Bio-Rad gel doc system.

Experimental procedures form the synthesis of compounds 2-5

(S)-methyl 2-((tert-butoxycarbonyl)amino)-3-((methylsulfonyl)oxy)propanoate **(2):** Boc serine ester (2.0 g, 9.13 mmol) dissolved in anhydrous pyridine (10 ml) and stirred at 0ºC under nitrogen atmosphere. Mesylchloride (1.06 ml, 13.7 mmol) was added dropwise with a syringe and the reaction stirred at 0ºC for one hour. The reaction mixture concentrated in roto and extracted with water and DCM. The DCM layer washed with sodium bicarbonate solution followed by brine solution, dried with anhydrous sodium sulphate and concentrated followed by purification using ethyl acetate and hexanes on silica gel gave 1.89 g of oily compound in 70% yield. ^{1}H NMR (400MHz ,$CDCl_3$) δ ppm 1.36 (9H, s), 2.95 (3H, s),3.71(3H, s),4.39-4.42(1H, m), 4.47-4.53(2H, m),5.53-5.55(1H, m) ^{13}C NMR (100MHz ,$CDCl_3$) δ ppm 28.11,37.17, 52.89,53.01,68.99,80.43,155.07,169.15. HRMS (ESI-TOF) Calculated ($C_{10}H_{17}O_7N$ S +Na) 320.0774, Measured 320.0775

(S)-methyl2-((tert-butoxycarbonyl) amino)-3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)propanoate **(3)**: Thymine (0.2g, 1.58 mmol) dissolved in anhydrous DMF (5 ml), K_2CO_3 (0.328 g, 2.38 mmol) and 18-Crown-6 (0.083 g, 0.31mmol) added and stirred under nitrogen atmosphere for 10 minutes. Mesyl derivative (compound **2**) (0.471 g, 1.58mmol) was added to the reaction and stirred at room temperature overnight under nitrogen atmosphere. The reaction mixture concentrated followed by purification on silica gel using methanol and dichloromethane as solvents gave 0.181 g white solid in 35% yield. ^{1}H NMR (400MHz, DMSO-D_6 +CD_3OD) δ ppm 1.27 (9H, s), 1.72 (3H, s), 3.54-3.60(1H, m), 3.63(3H, s),

4.18-4.23 (1H, m), 4.44-4.46 (1H, m), 7.18(1H, m). ^{13}C NMR (100 MHz, DMSO-D_6 + CD_3OD) δ ppm 11.23, 27.49, 49.19, 51.78, 51.88, 79.40, 109.05, 142.20, 151.43, 156.01, 165.26, 17.42 HRMS (ESI-TOF) Calculated ($C_{14}H_{21}O_6N_3$ +Na) 328.1503, Measured 328.1509.

(S)-tert-butyl (1-hydroxy-3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)propan-2-yl)carbamate **(4)**: Thymine ester (0.15 g, 0.45 mmol) dissolved in anhydrous ethanol, stirred under nitrogen atmosphere for 10 minutes followed by addition of sodium borohydide (0.05 g, 1.37 mmol) and stirred overnight under nitrogen atmosphere at room temperature .The reaction mixture concentrated and extracted with ammonium chloride and ethylacetate. Ethylacetate layer washed with brine solution, dried with anhydrous sodium sulphate and concentrated .The crude compound purified on silica gel column using methanol and dichloromethane to obtain 0.11 g of white solid in 80% yield. ^{1}H NMR (400MHz ,DMSO-D_6) δ ppm 1.29 (9H, s),1.72 (3H, s),3.23-3.29(1H, m),3.34-3.41(2H, m),3.74-3.80(1H, m),3.95-4.00(1H, m), 4.80-4.83(1H, m),6.54-6.57(1H, m),7.31(1H, s),11.12(1H, s) ^{13}C NMR (100 MHz ,DMSO-D_6) δ ppm 12.51, 28.52, 49.98, 51.18, 61.90, 78.25, 107.90, 142.58, 151.43, 155.72, 164.81. HRMS (ESI-TOF) Calculated ($C_{13}H_{21}O_5N_3$) 300.1554, Measured 300.1556.

Triethylammonium (S)-2-amino-3-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)propyl triphosphate **(5)**: Triphosphorylation was performed by following reported literature. [1] Compound **4** (0.04 g, 0.13 m mol) taken in a 25 ml round bottom flask dissoloved in 3 ml of anhydrous pyridine and dried in roto followed by drying in a vacuum desiccator at ambient temperature for one hour. Descicator opened under argon atmosphere and closed with septum. All the further steps performed under argon

pressure. Salicylchlorophosphidite (0.03g, 0.160mmol) dissolved in dry dioxane (1.0 ml) was added and stirred at room temperature for 10 minutes. Immediately a cock tail containing tri- butylammonium pyrophosphate (0.14 g, 0.26 mmol) and tri-butylamine (0.26 mL, 1.07 mmol) in anhydrous dimethylformamide (DMF) (1ml) injected into the flask and stirred for 10 minutes followed by the addition of Iodine in pyridine/water solution (1% in pyridine/water 98/2). After 10 minutes 10 ml of 100 mM TEAB buffer (pH 8.0) was added and stirred for one hour followed by lyphilzation .The reaction mixture dissolved in 10 ml water and extracted with ethyl acetate. Aqueous layer lyophilized followed by anion exchange column (DEAE Sephadex A-25) chromatography eluted with TEAB buffer (0.1M-1.0M) compound containing fractions were pooled and concentrated followed by boc deprotection with 1N HCl .The reaction was quenched with aqueous ammonia and concentrated followed by HPLC purification and lyophilzation of the HPLC purified samples gave Ala-TTP as a white powder dissolved in nuclease free water and used for primer extension reactions. ^{1}H NMR (400 MHz, D2O) δ 7.57 (s, 1H), 4.41 (dd, J = 6.7, 3.1 Hz, 1H), 4.26 (dd, J = 14.7, 7.3 Hz, 1H), 4.20 – 4.04 (m, 2H), 3.94 (s, 1H), 1.94 (s, 3H). ^{31}PNMR (162 MHz, D_2O): (-22.13, t,1P, P_β), (-11.16,-11.04,d ,1P, P_γ), (-10.4,-10.16 , d ,1P,P_α) ($J_{\alpha\beta}$=19.44Hz , $J_{\beta\gamma}$=19.44Hz) HRMS, ^{1}HNMR ESI-TOF calcd for [$C_8H_{15}NO_{12}P_3$(M-H)] m/z437.9863, found m/z 437.9969.

3A. 5 Refeerences

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3A.6 Appendix

NMR and HRMS spectra of compounds 2-5

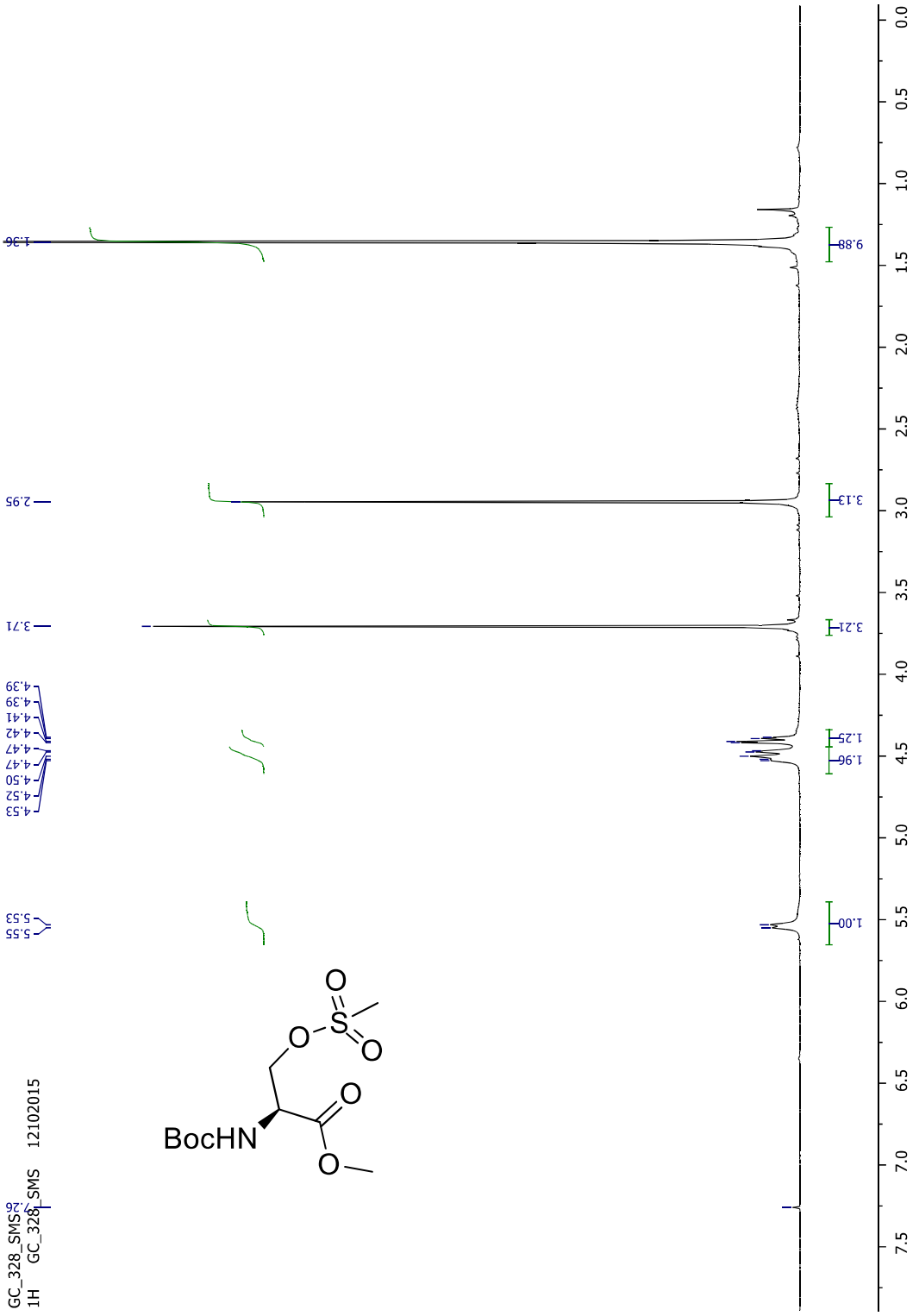


Figure A1 ^{1}H NMR of compound 2

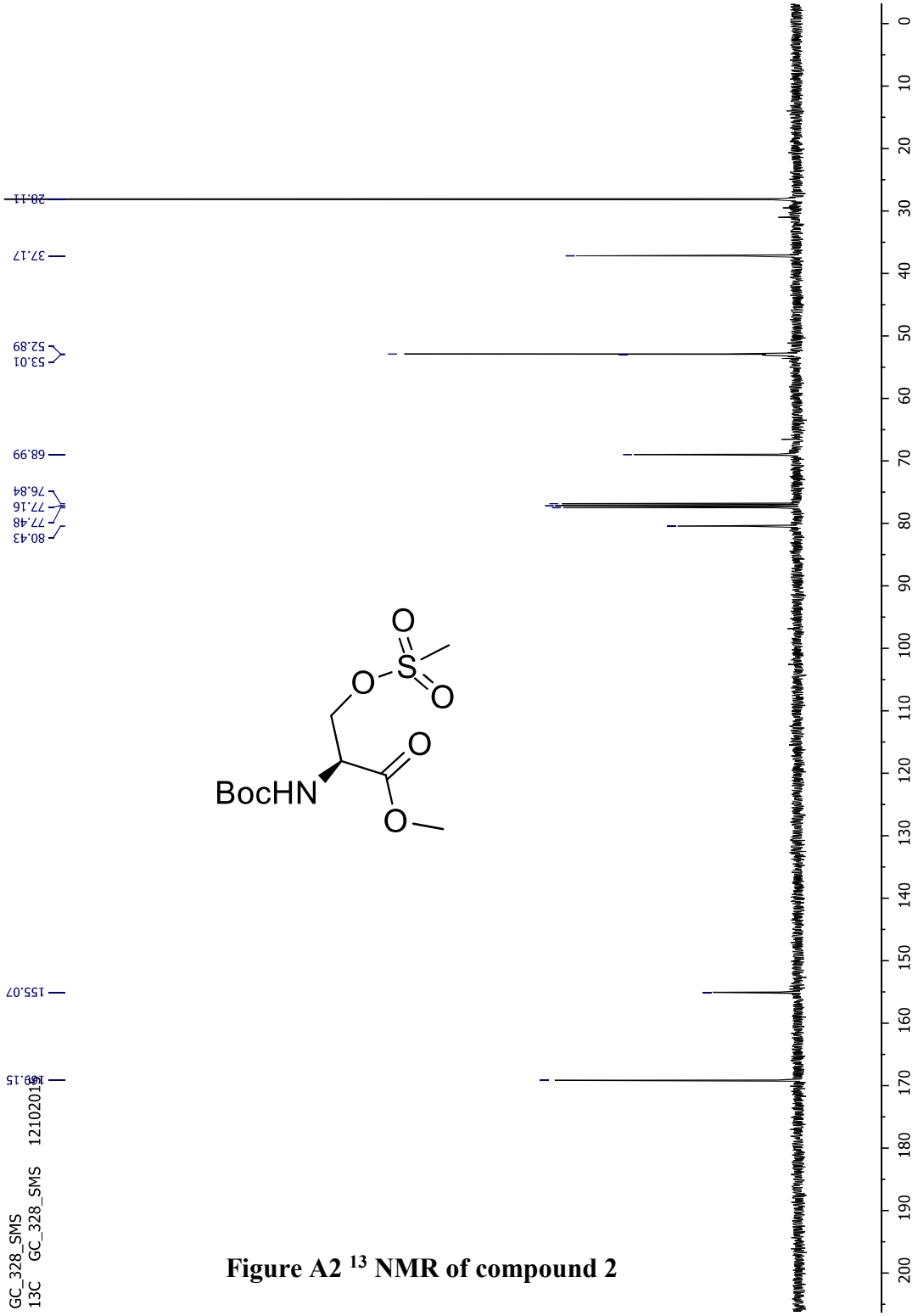


Figure A2 [13] NMR of compound 2

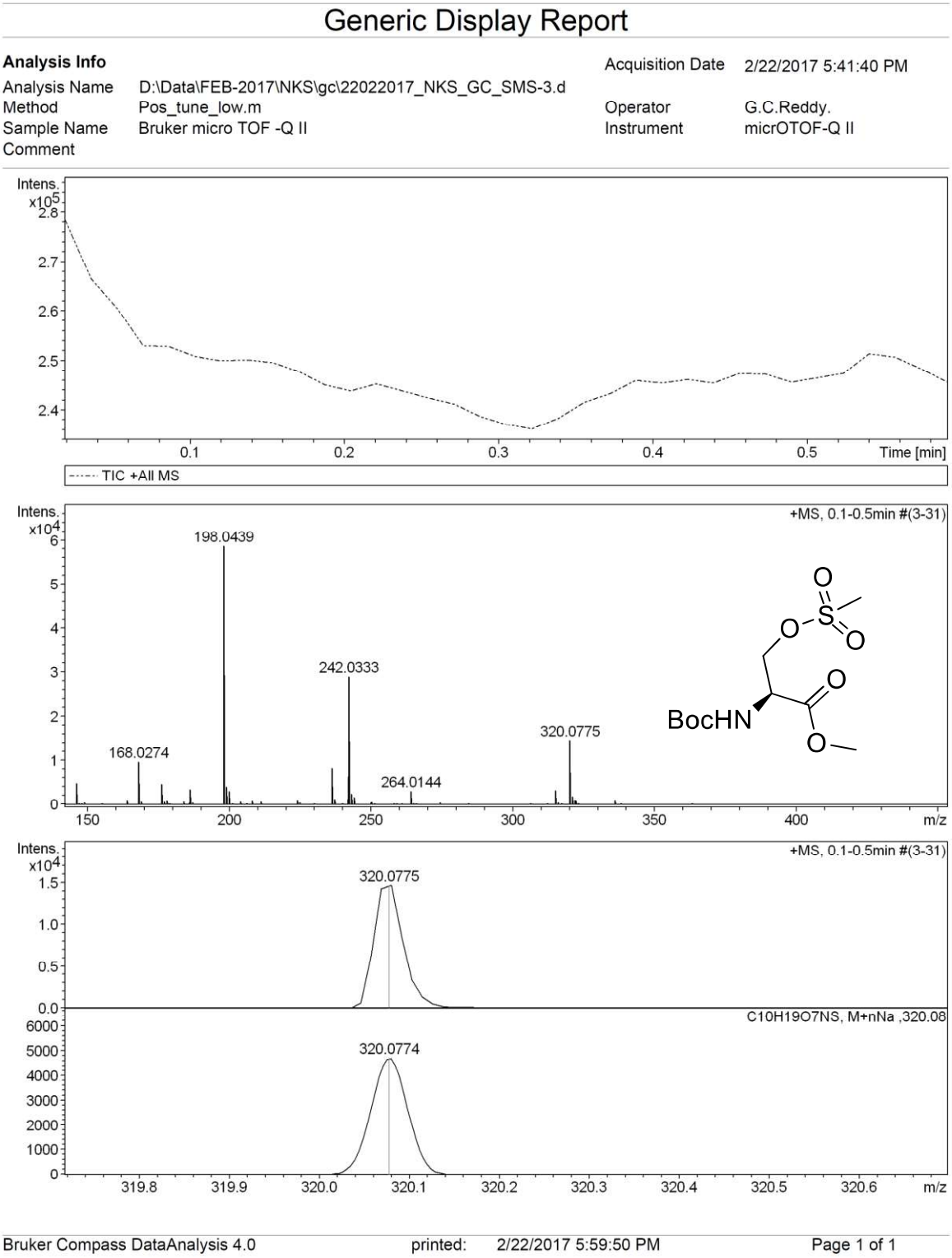


Figure A3 HRMS of compound 2

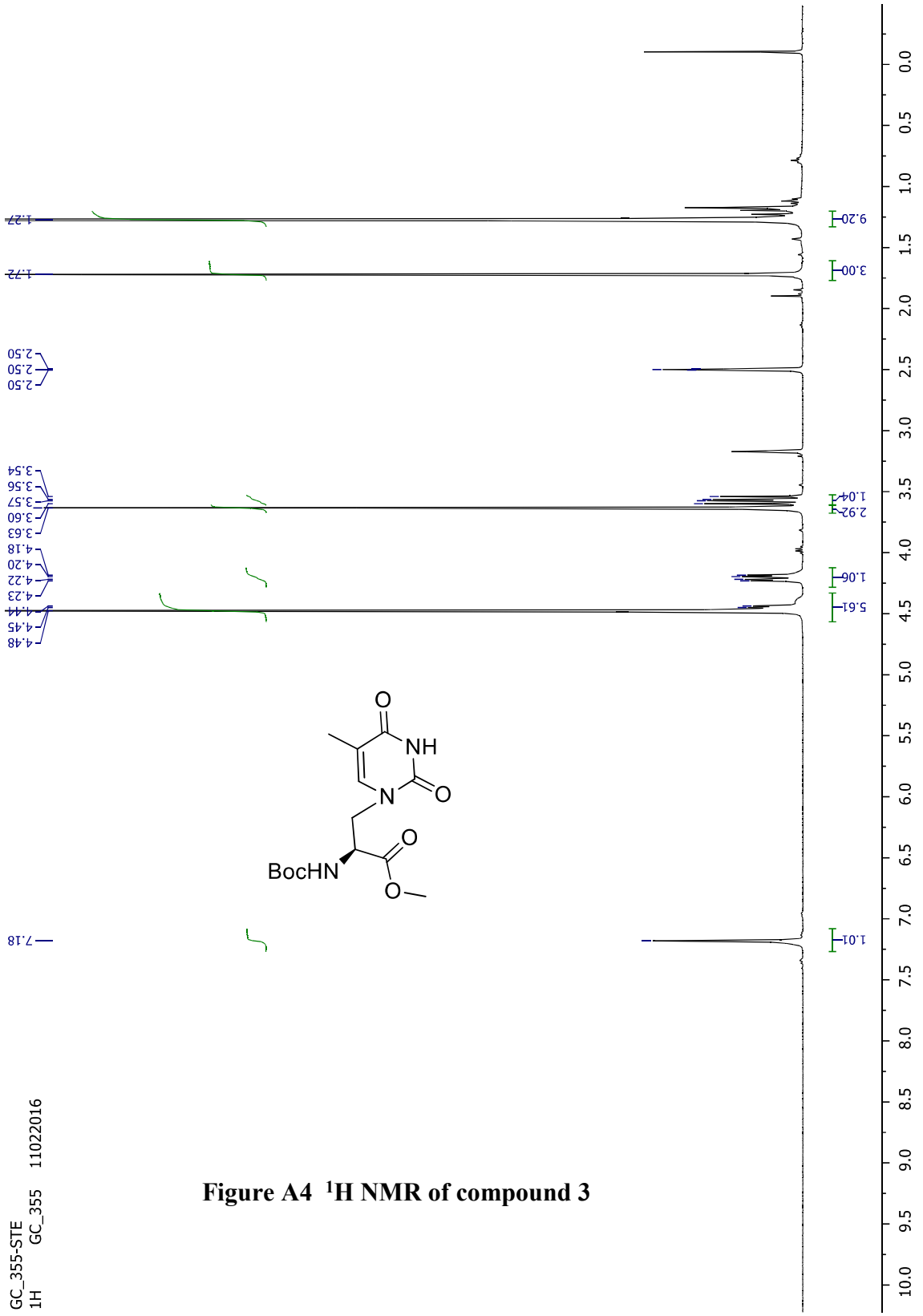


Figure A4 ^{1}H NMR of compound 3

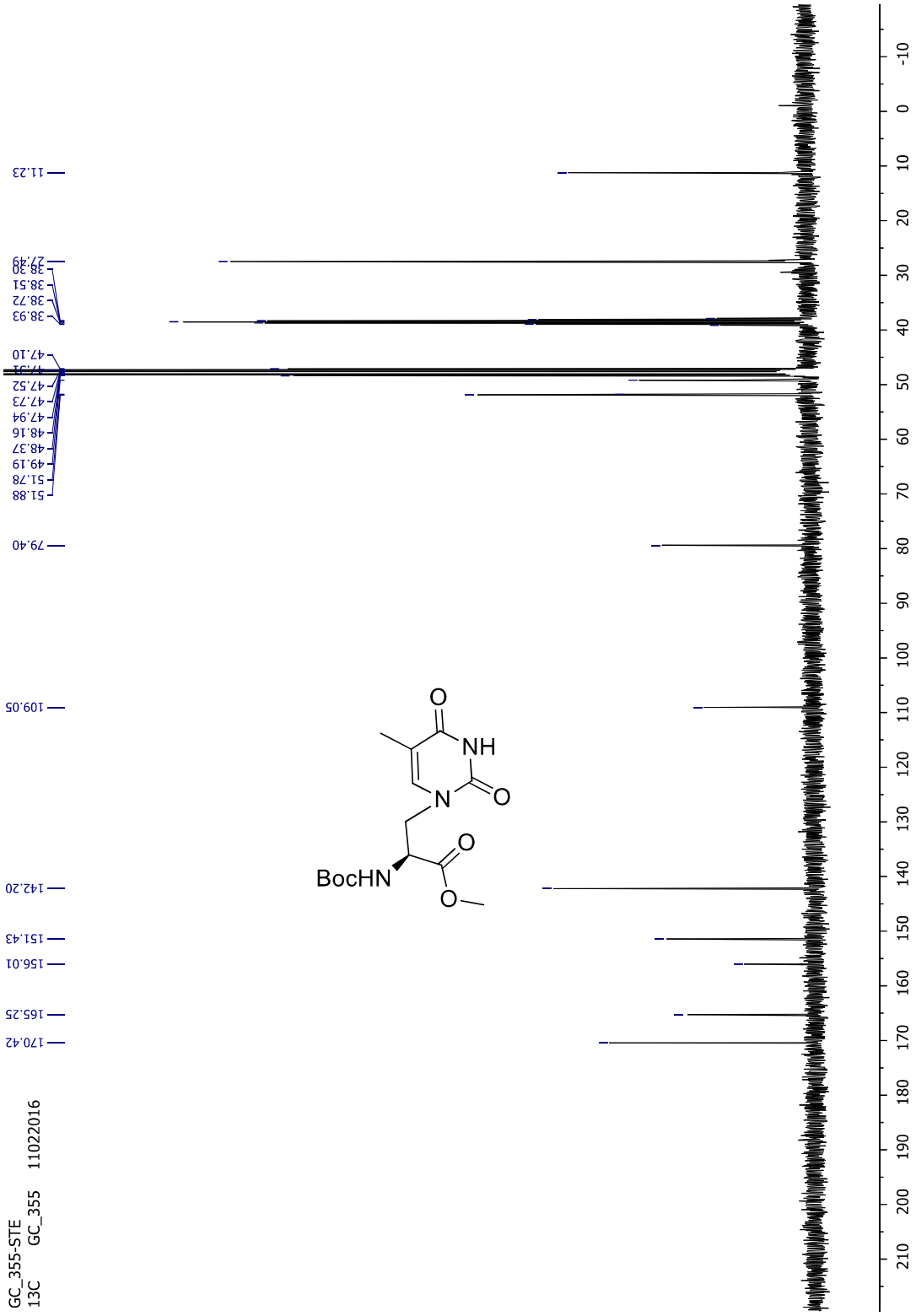


Figure A5 ^{13}C NMR of compound 3

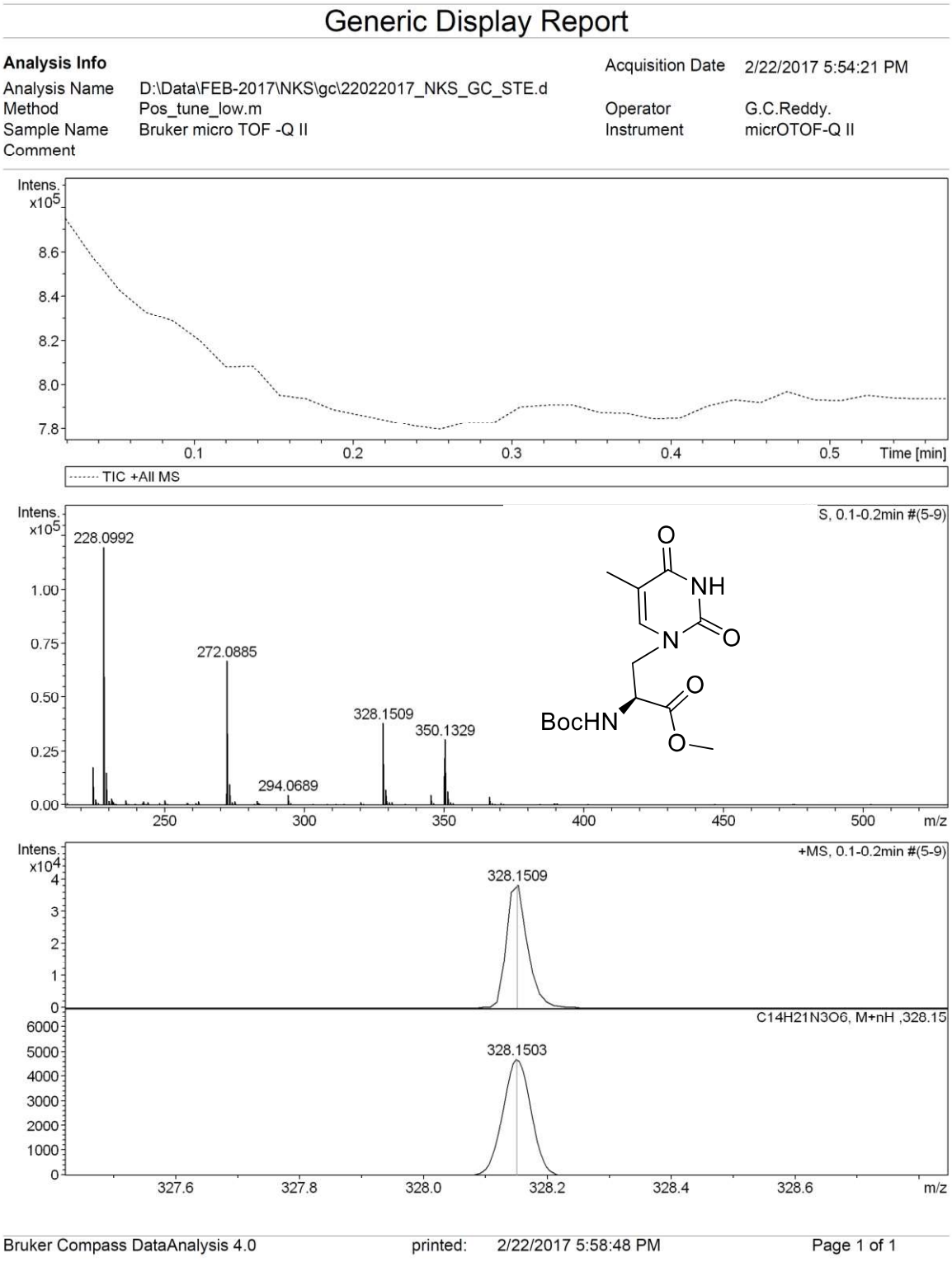


Figure A6 HRMS of compound 3

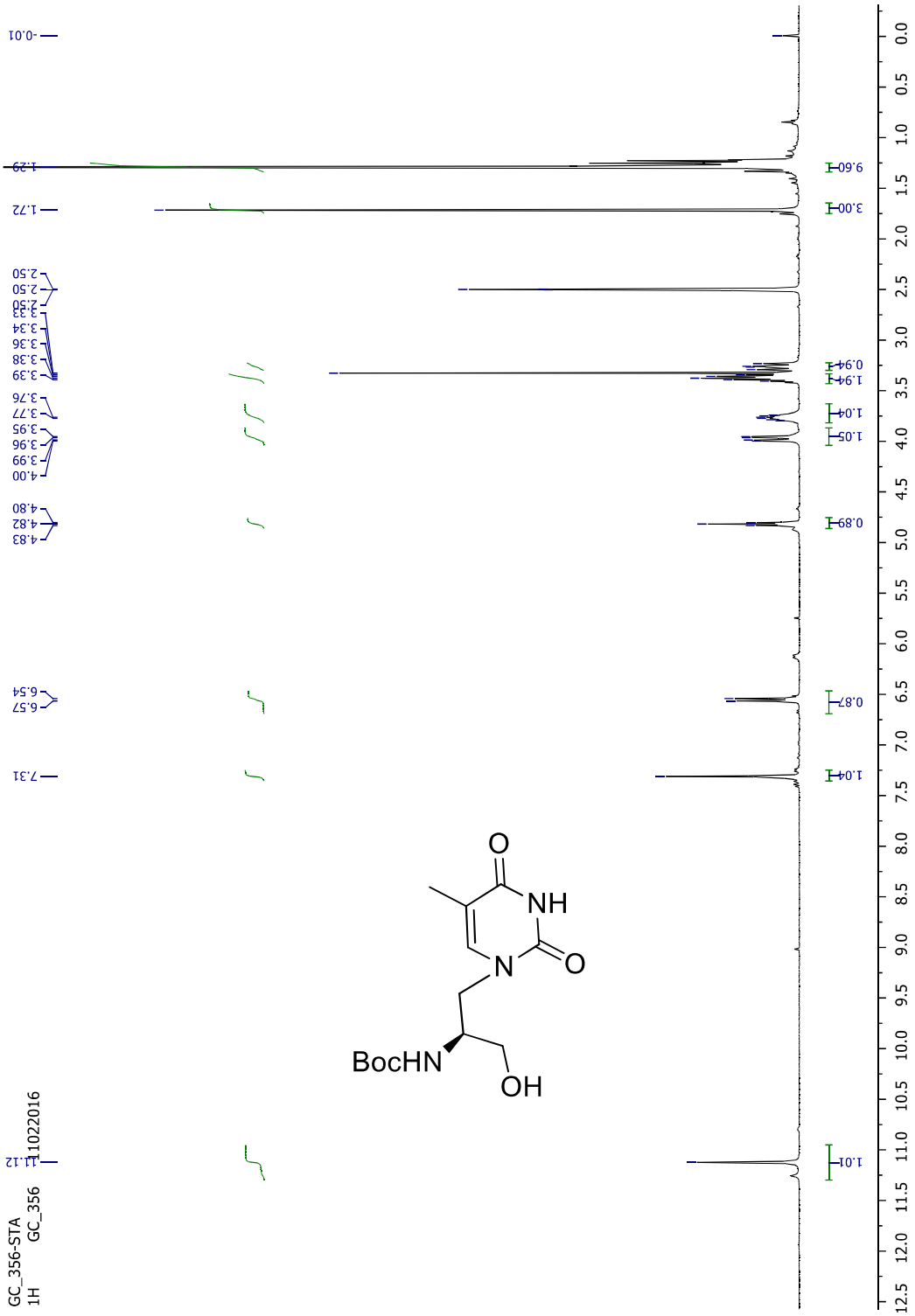


Figure A7 ^{1}H NMR of compound 4

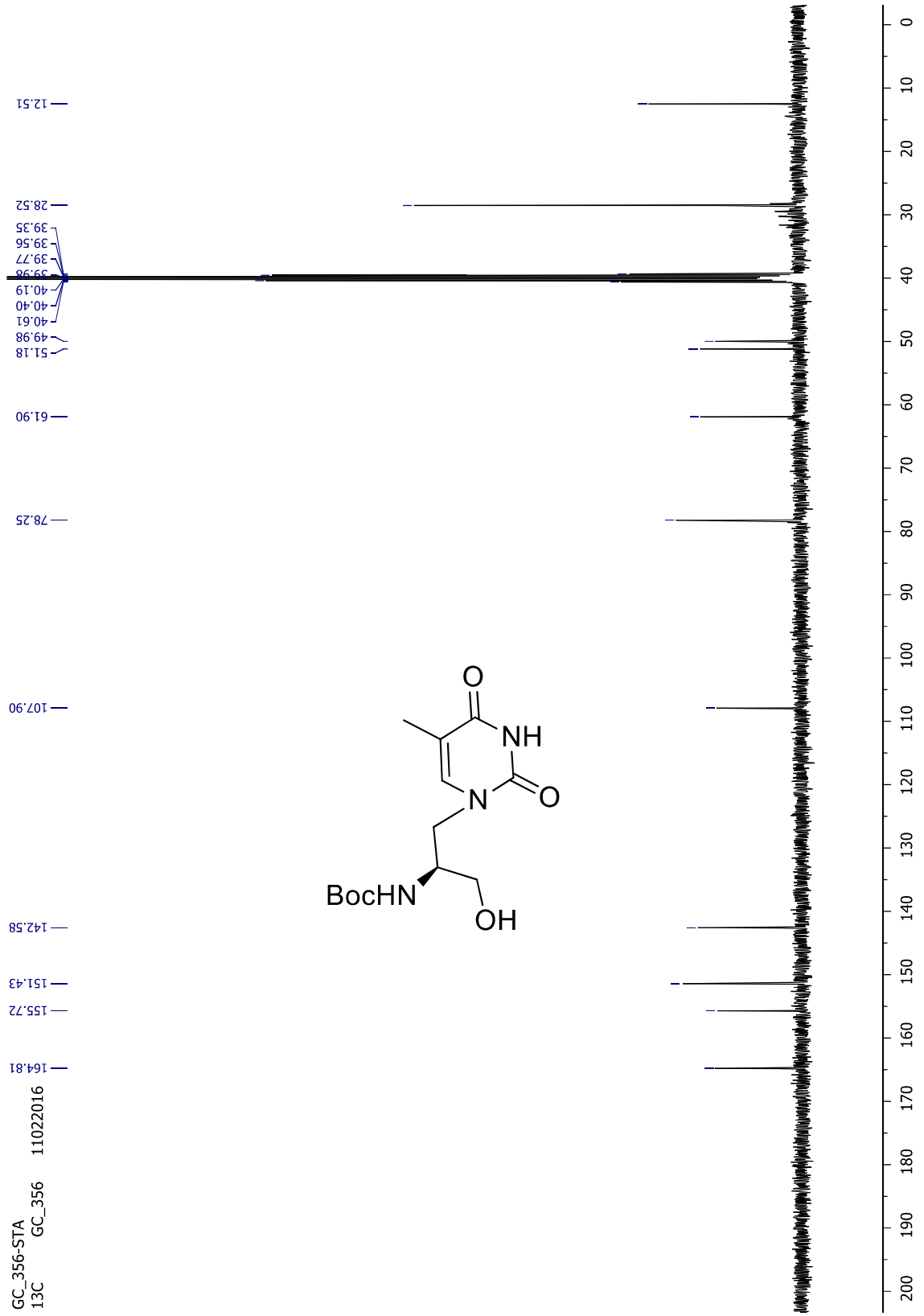


Figure A8 ^{13}C NMR of compound 4

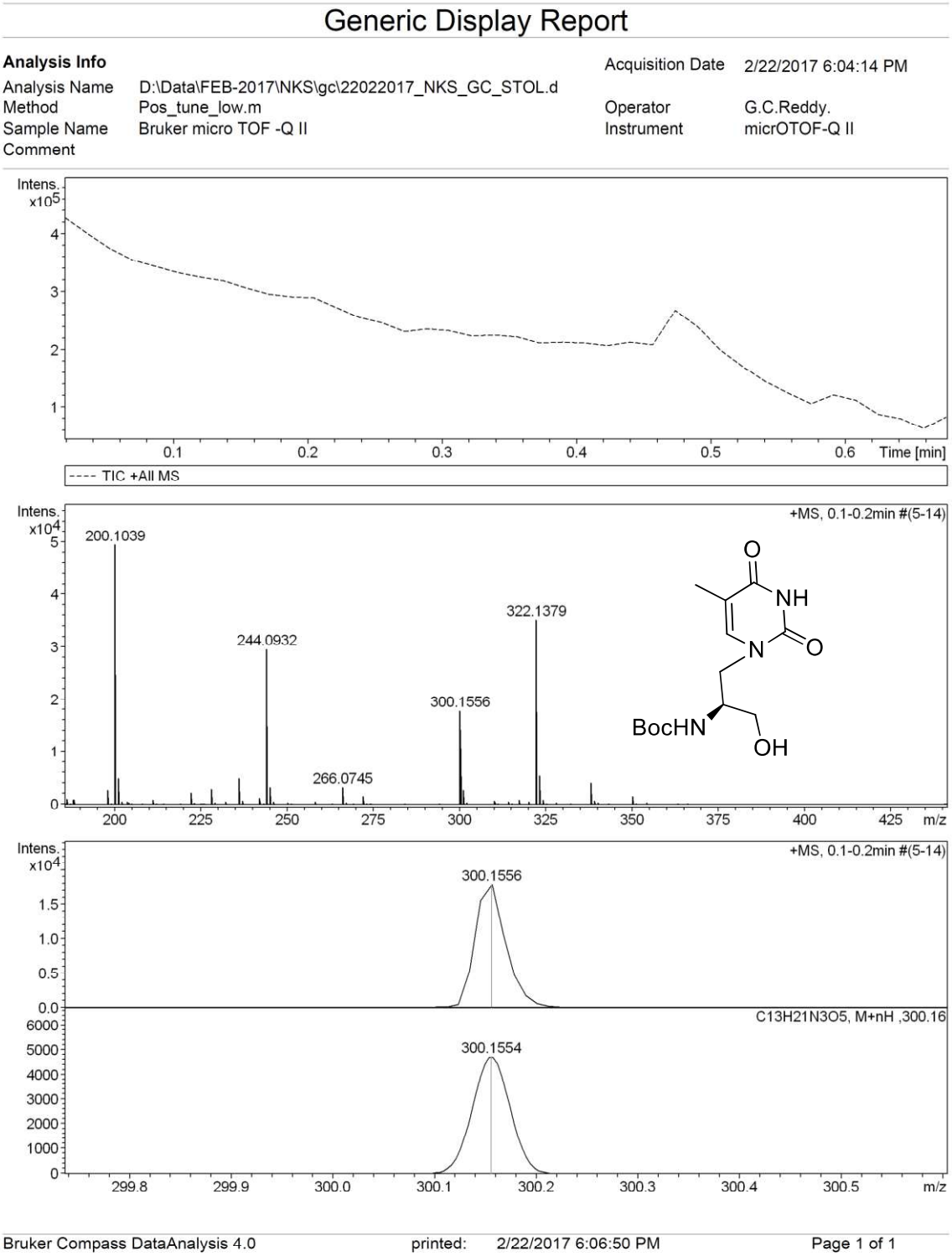


Figure A9 HRMS of compound 4

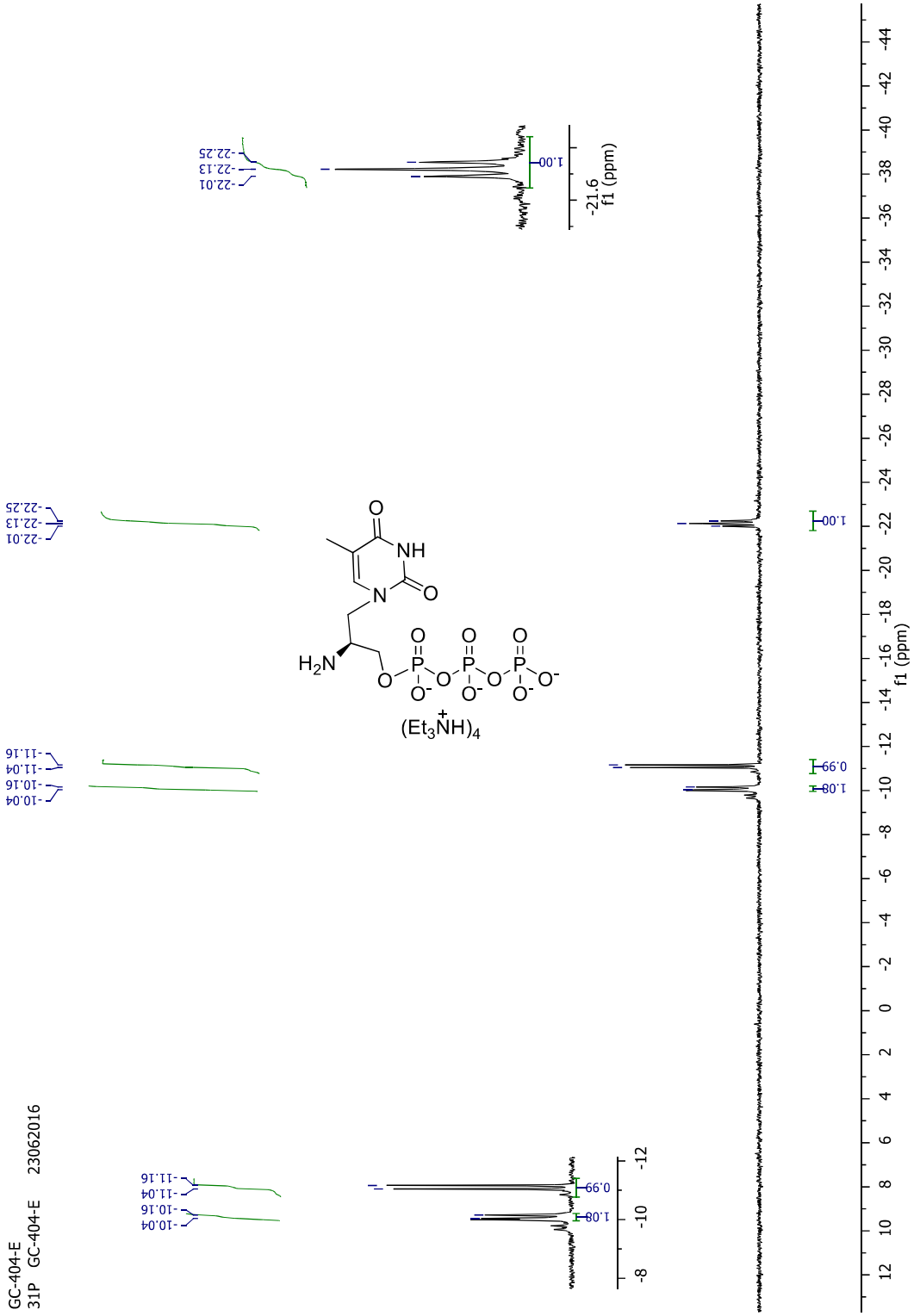


Figure A10 ^{31}P NMR of compound 5

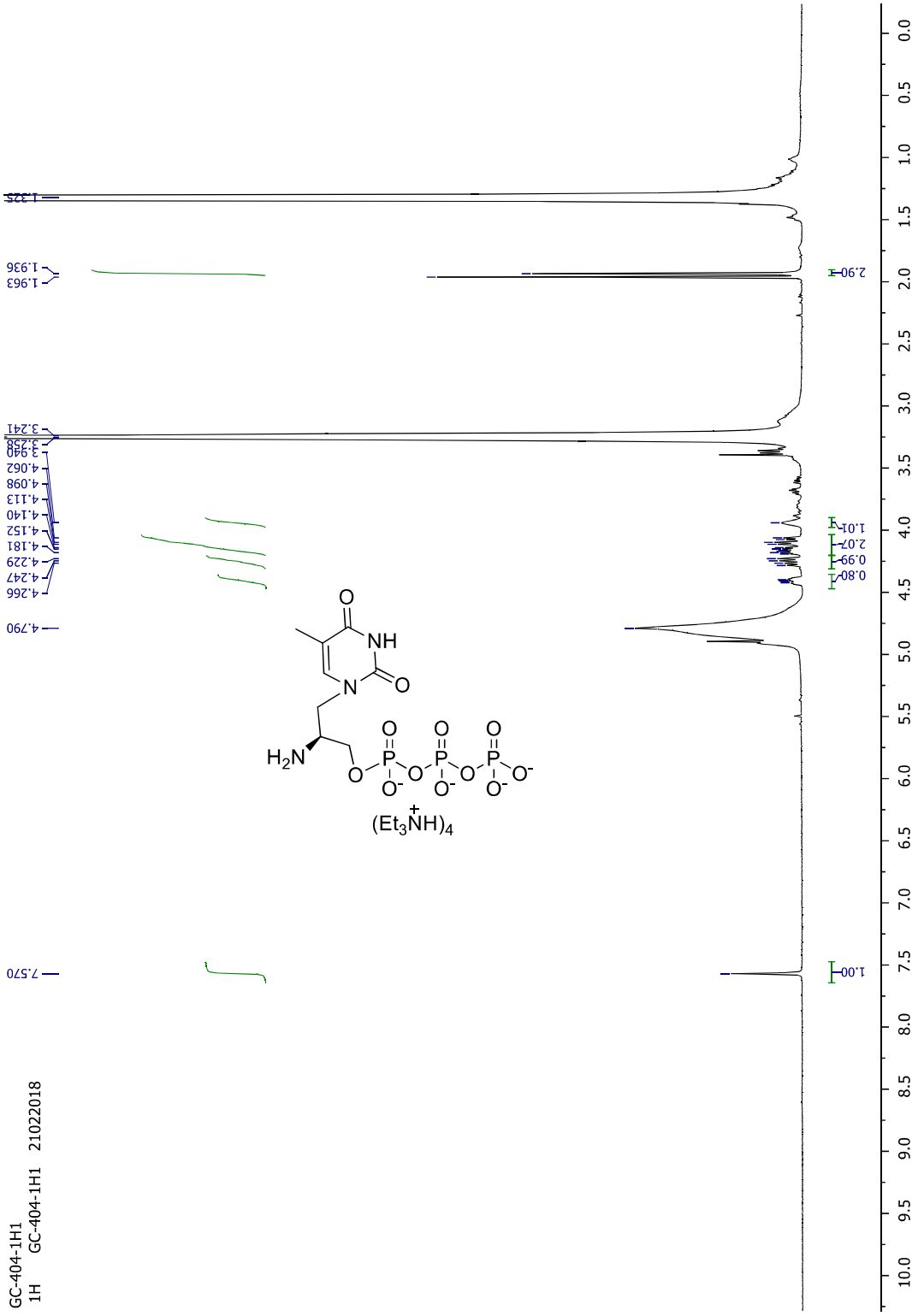


Figure A11 ^{1}H NMR of compound 5

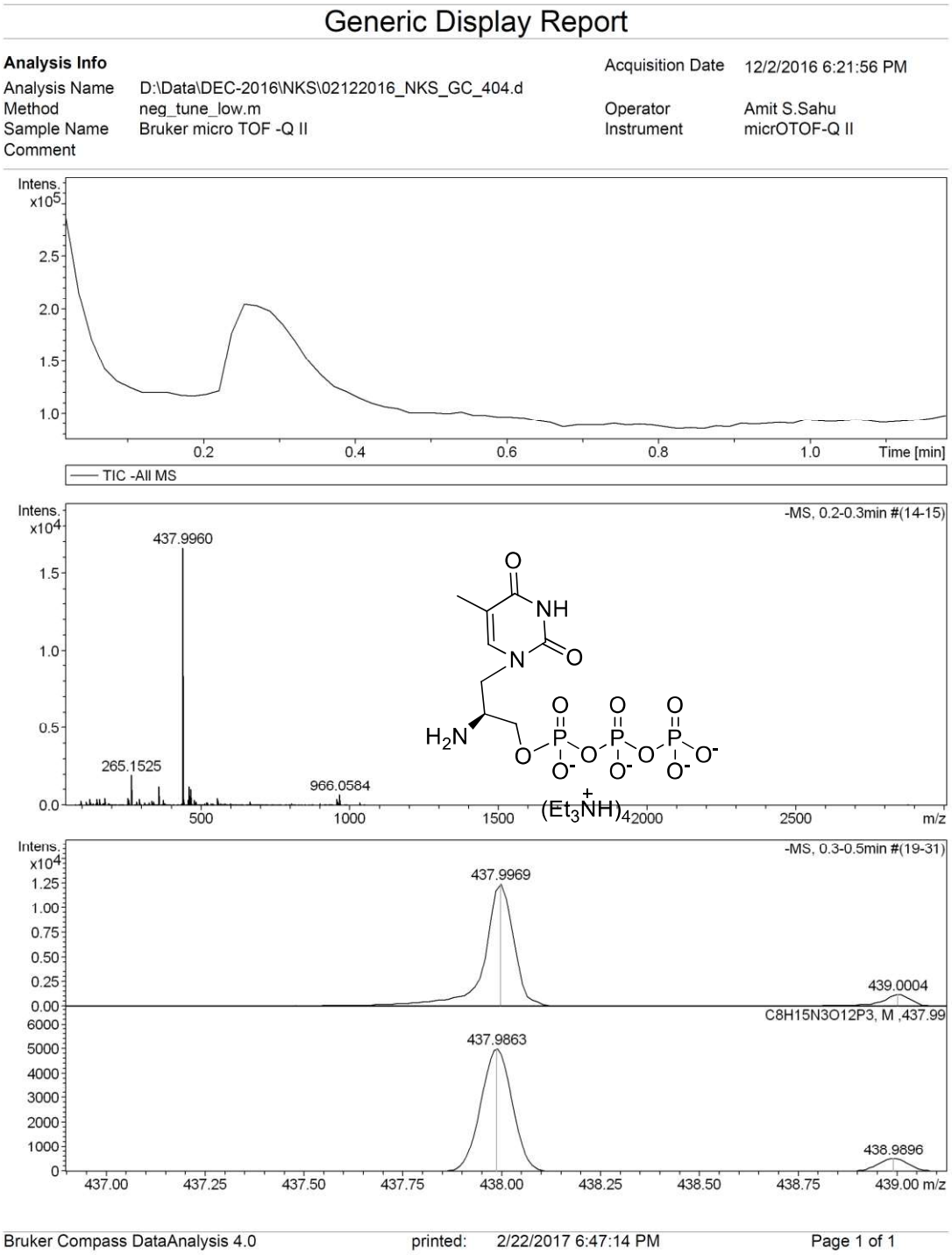
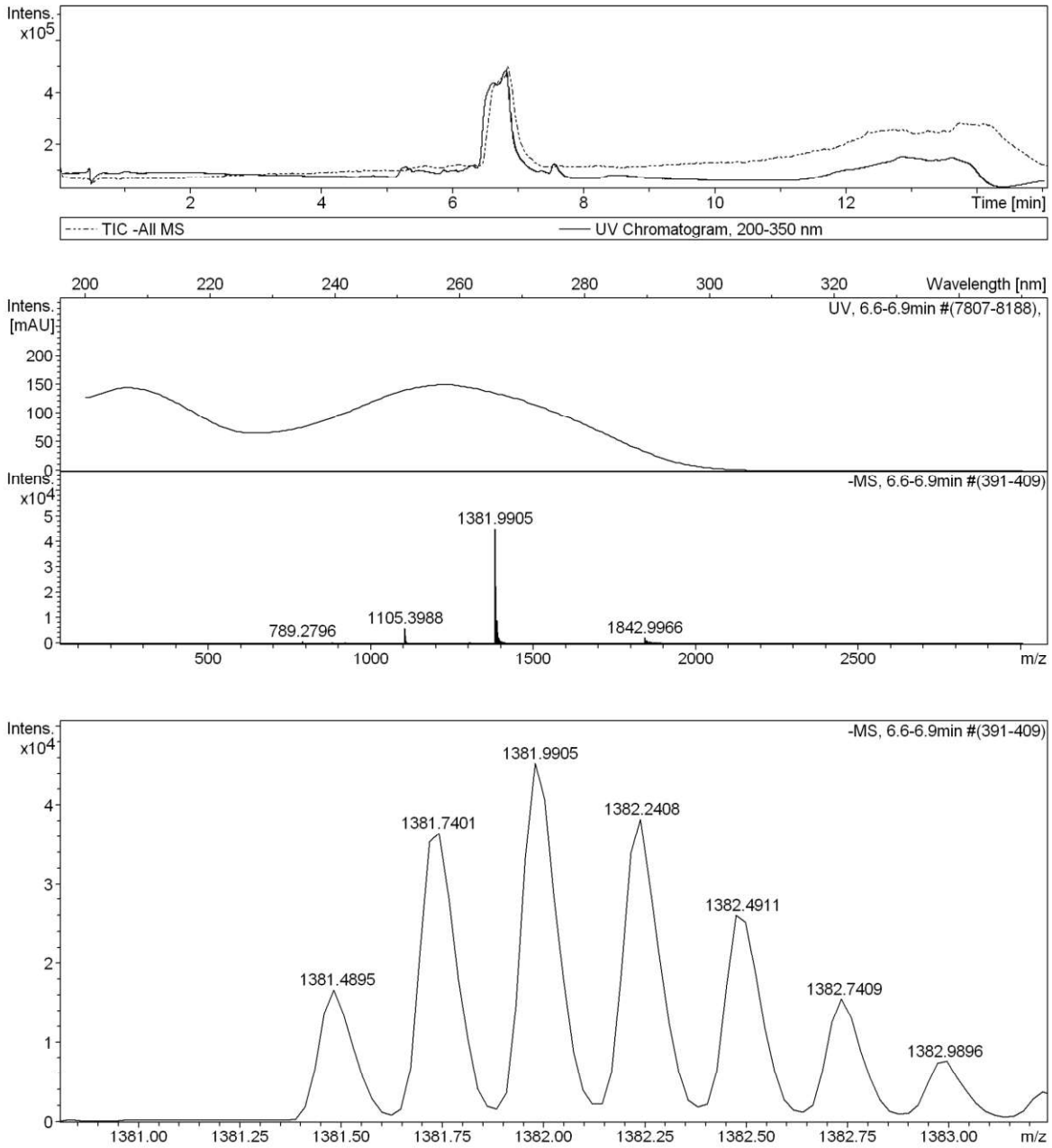


Figure A12 HRMS of compound 5

LC-MS of primer,template and extended primer with ala-TTP and ddTTP



TGTAAAACGACGGCCAGT Mass =5532.676

Calculated , $(M-4H)^{4-}$= 1382.16, observed 1381.99

Figure A13 LC-MS of primer

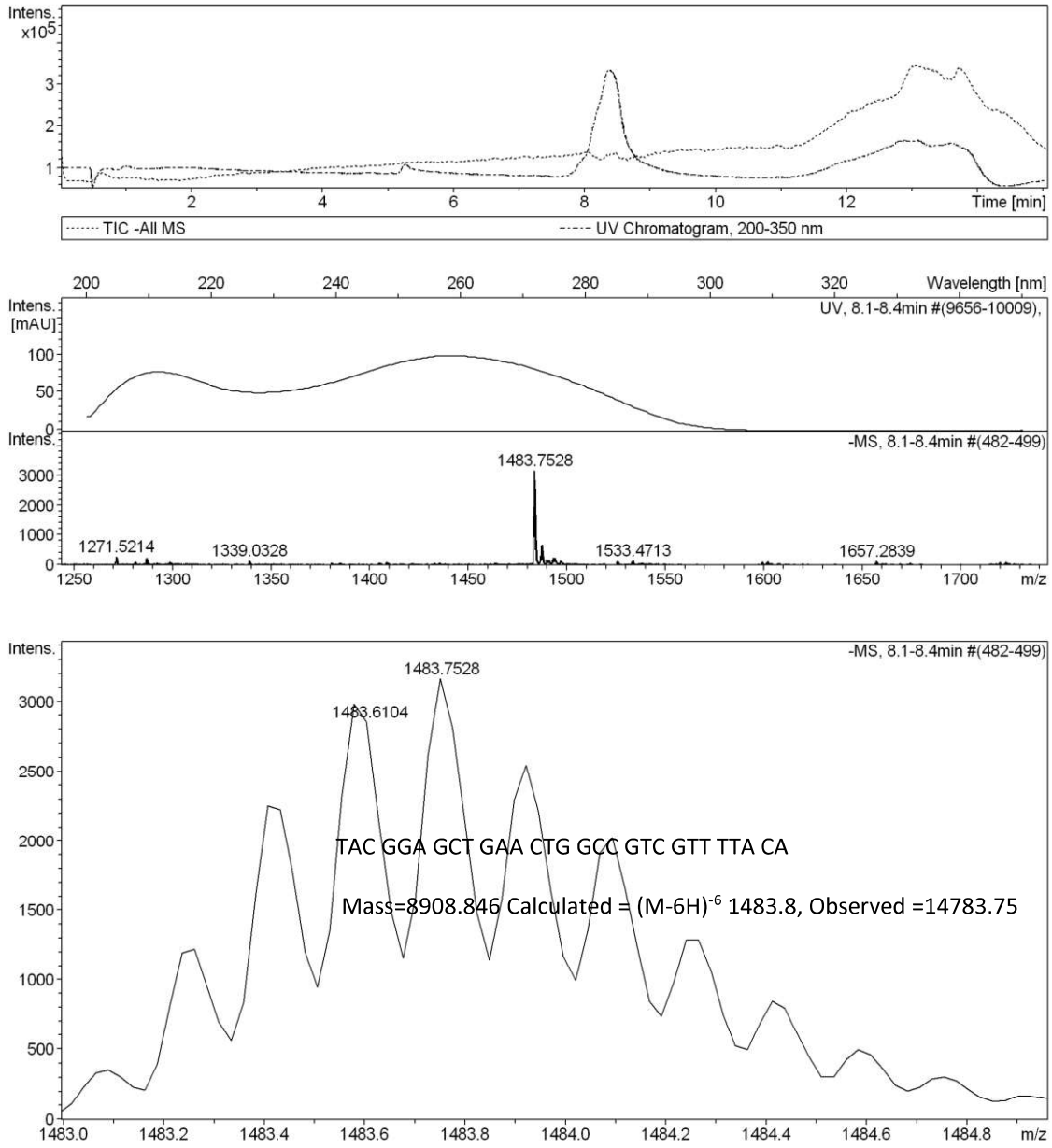
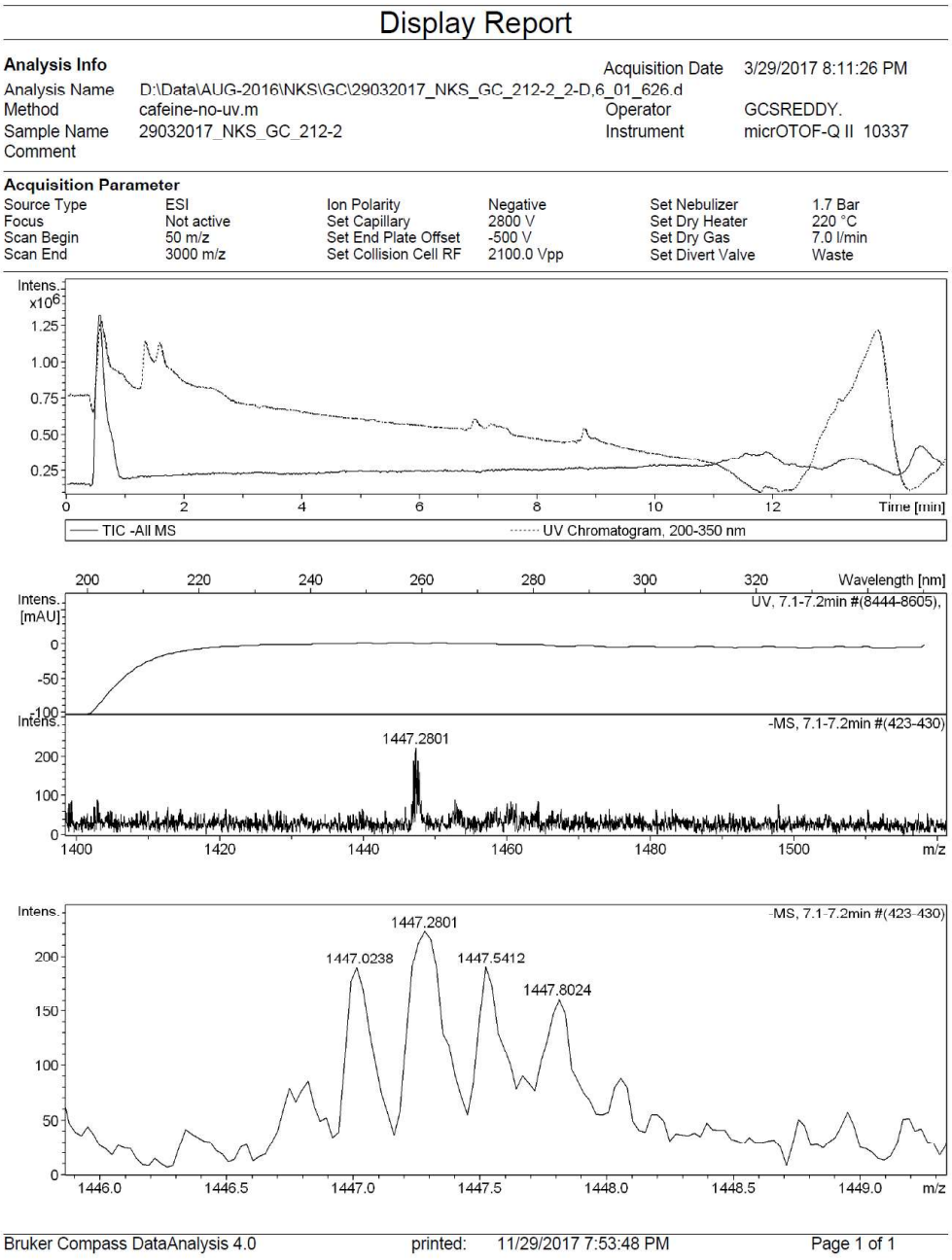


Figure A14 LC-MS of template



TGTAAAACGACGGCCAGT**T** Mass =5,789

Calculated , $(M-4H)^{4-}$ = 1447.25, observed =1447.28

T=

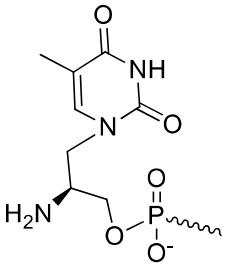
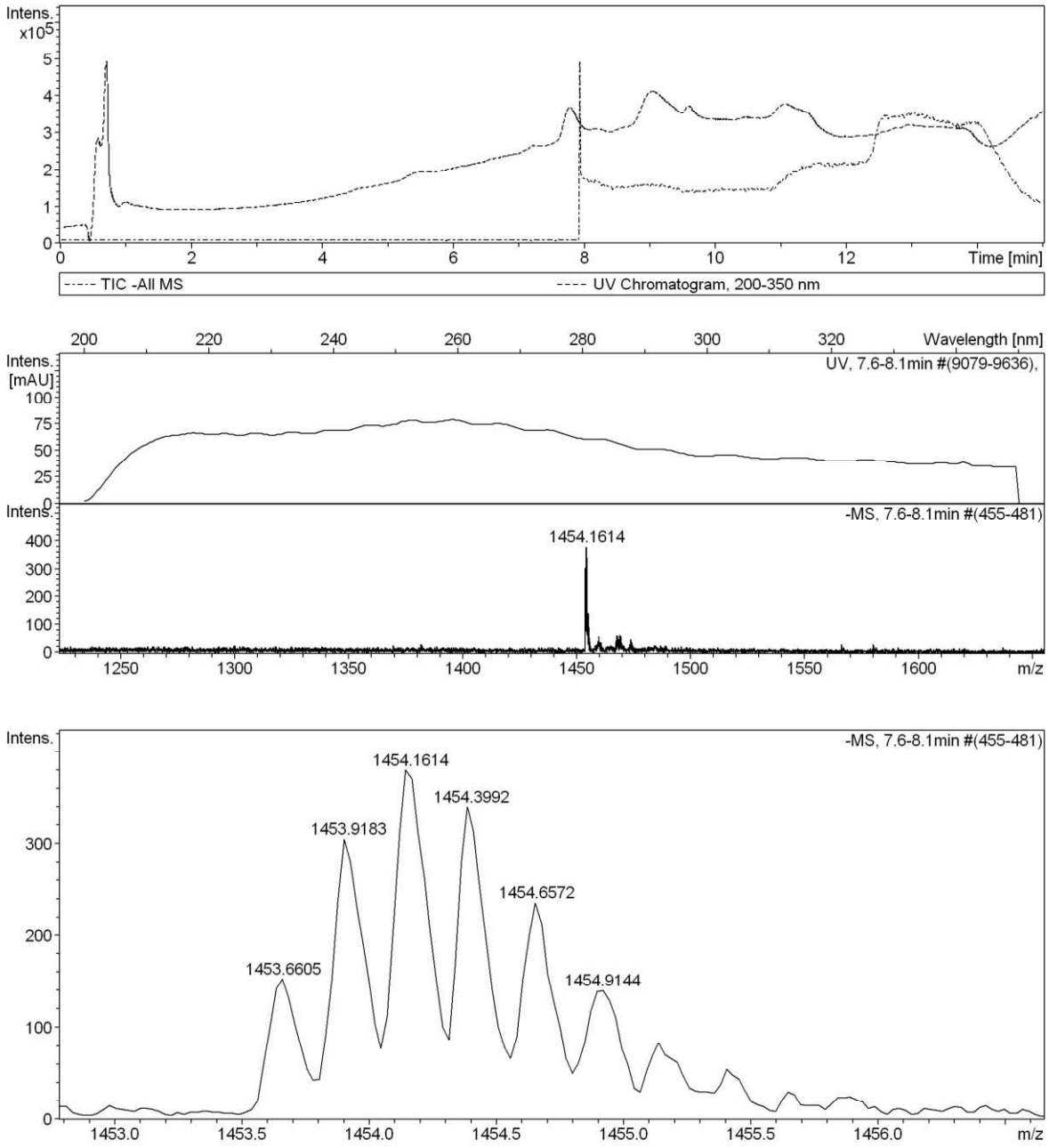


Figure A15 LC-MS of extended primer with Ala-TTP and Vent exo-



TGTAAAACGACGGCCAGT**T** Mass =5838.57

Calculated , $(M-2H)^{4-}$ = 1454.0925, observed =1454.1

T=

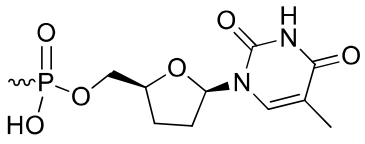


Figure A16 LC-MS of extended primer with dd-TTP and Vent exo-

Chapter 3B

Synthesis and Biochemical Evaluation of Prolamide Thymidine/Uridine Triphosphate Analogues

Chapter 3B

Synthesis and Biochemical Evaluation of Prolamide Thymidine/Uridine Triphosphate Analogues

Contents

3B.1 Introduction

Dideoxy nucleoside triphopshates (ddNTP) are the sequence terminators developed by Sanger.[1] After Sanger's report researchers have been developed alternate ddNTP analogues. Amino ethyl glycinyl (*aeg*) Peptide nucleic acids (PNA) are nucleic acid analogues where phosphate back bone is replaced by neutral amide backbone. *Aeg* derived triphosphate analogues have been synthesized and studied as ddNTP analogues.[2,3] *Aeg* PNA are the acyclic and flexible molecules. Proline derived conformationally constrained PNA analogues have been synthesized and their binding properties with complementary DNA/RNA have been studied (Figure 3B.1).[4-6] We have synthesized and studied the biochemical properties of 4-hydroxy proline derived pyrrolidine nucleoside triphosphates (Pr-NTPs) as ddNTP analogues.[7] PNA analogues with prolinol acetyl nucleobase have been synthesized and incorporated in PNA at terminal and interior positions which stabilized the duplex with complementary DNA.[8,9] [10,11]

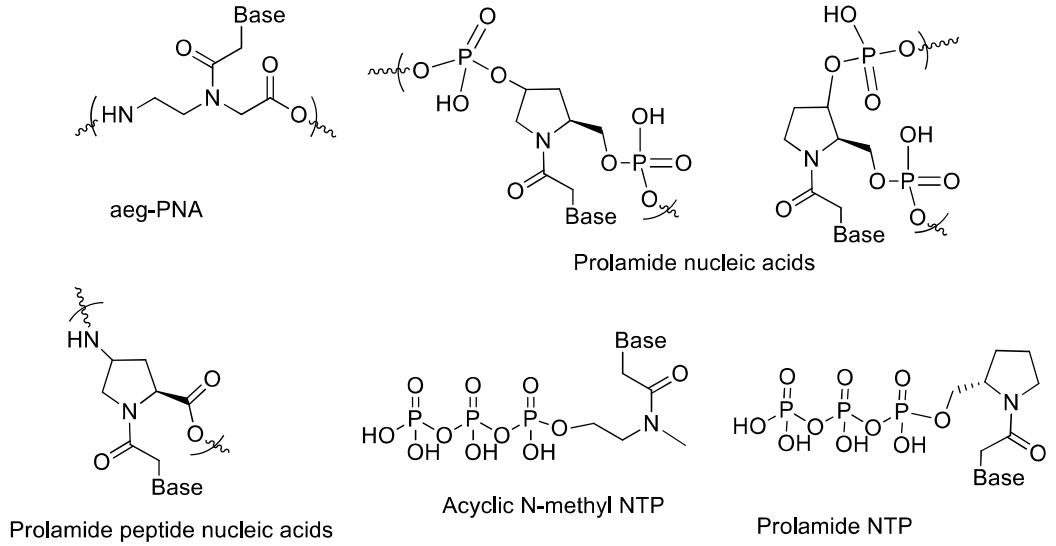


Figure 3B. 1. Structure of aeg-PNA, Prolamide nucleic acids, Prolamide PNA, Acyclic N-methyl NTP and Prolamide NTP

The 4-hydroxy /3-hydroxy prolinol acetyl nucleobase modified phosphoramidates have been synthesized and studied the stability of the hybrid duplex with complimentary DNA/RNA.[12-14]

3B.1.1 Hypothesis and rational of present work

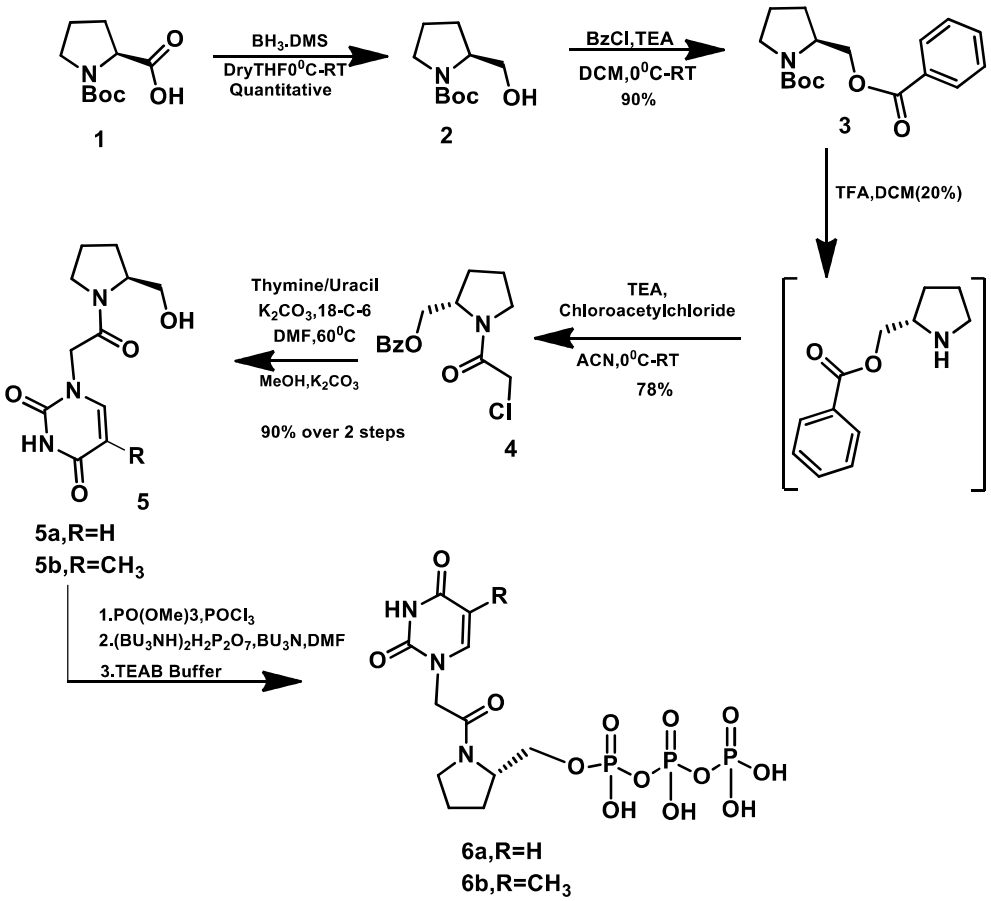
As prolinol acetyl nucleobase nucleic acid analogues forms stable duplexes with complementary DNA/RNA we hypothesized to synthesize nucleoside triphosphates derived from prolinol acetyl thymine/ uracil and study the biochemical activities of with DNA polymerase as alternate ddNTP analogues.

3B.2. Results and discussion

3B.2. 1 Synthesis of prolamide thymidine/uridine triphosphates

We started the synthesis of prolinol acetyl thymine/ uracil form N-Boc proline (**1**). *N*-Boc proline was treated with bornae dimethyl sulphide to obtain N-Boc prolinol (**2**). The alcohol was protected as benzoate (**3**).The *N*-Boc proline benzoate was treated with 20% TFA/DCM to remove the boc group and insitu it is treated with chloroacetyl chloride to afford chloro derivative (**4**). The chloro derivative treated with thymine/uracil in dimethylformamide using potassium carbonate to obtain the benzoyl protected nucleoside analogue which was treated with potassium carbonate in methanol to obtain the nucleoside alcohols **(5a&5b)**.Nucleosides **5a** & **5b** were characterized by single crystal X-ray diffraction. The nucleoside alcohols (**5a & 5b**) phosphorylated by Yoshikawa's method. The alcohol treated with phosphoryl chloride in trimethylphosphate followed by treatment with tributylammonium pyrophosphate to obtain thymine/uracil triphosphate analogues.The synthetic steps are depicted in scheme 3B.1

Scheme 3B. 1: Synthesis of prolamide thymidine/uridine triphosphate



3B.2. 2 Biochemical evaluation of prolamide thymidine/uridine triphosphate analogues

The synthesized analogues were tested with many DNA polymerases *therminator, taq, vent, deepvent, bst,* and *klenow* DNA polymerases to study the primer extension reaction. Both the thymine and uracil analogues are the substrates for none of these enzymes.

3B.2. 3 Self-assembly prolinol acetyl thymine/uracil

The single crystal structure analysis of nucleosides show the helical self-assembly by hydrogen bonding interactions. Helix is the spiral symmetrical arrangement of molecules by supramolecular interactions. Helix is the supramolecular structure with coiled topology present in biological systems. [15] The helical supramolecular assemblies are formed by hydrogen bonding interaction originated in intra or inter molecular fashion as seen in alpha- helix of proteins [16] and double helix of DNA. [17]The helices assemble to supramolecular structure by interaction with other molecules to form coil-coiled structure, DNA-protein, super helices of DNA structures to form functional supramolecular system which are vital for life and involved in many biological function such as ion-transport, molecular recognisation, catalysis and genetic information storage.[18] Hydrogen bonding is the key element in the formation and maintenance of the supramolecular structures in biomolecules. [19] Chemists used many strategies to make artificial helical systems to mimic the nature. Foldamers, small molecules, oligomers, double stranded helical polymers have been synthesized to mimic the helix topology.[20-22] Nucleic acids attain different conformations under given physiological condition to adopt a secondary structure which play crucial role in biological systems.[23] Many nucleic acid based supramolecuar structures have been developed. Small molecule based supramolecular self-assembled systems have been developed and studied their structural features by X-ray crystallography and other techniques [24]. The supramolecular self-assembly of molecules at nucleobase level have been studied by designing small molecules based on homo or hetero dimerization

/self-assembly of nucleobase derivatives. [25] [26]Adenine nucleobase derivatives forms supra molecular structures by hydrogen bonding[27,28] or metal mediated [29] self-assembled structures. Guanosine derivatives self-assemble to supramolecular structure by Hoogsteen hydrogen bonding. [30] [31]Hemi protonated cytosine and its derivatives self-assemble to form supra molecular structures by hydrogen bonding.[32] Ferrocne linked Thymine/Uracil conjugates forms supra molecular assemblies by Watson-Crick/reverse Watson-Crick hydrogen bonding.[33,34] Thyminyl/uracilyl N-acetyl prolinol derivatives have been syntheisized and incorporated in DNA/PNA analogues and their binding properties have been studied.We are interested to study the supramoleculcuar self-assembly formed by Thyminyl/uracilyl N-acetyl prolinol derivatives. Herein we describe the synthesis, self-assembly study of Thyminyl/uracilyl N-acetyl prolinol derivatives by X-ray crystallography, ESI-MS studies and SEM image techniques.

Helical self-assembly of prolinol acetyl uracil: The crystal structure of prolinol acetyl uracil is depicted in (Figure 3B .2). The supramolecular helical self-assembly in prolinol acetyl uracil originated from the hydrogen bonding interactions between the alcochol OH and carbonyl group. The helix formation from the hydrogen bonding interactions is explained here by taking 3 residues of prolinol acetyl uracil. The single crystal X-ray structure of prolinol acetyl uracil shows the arrangement of alcohol and carbonyl groups oriented in such a way that the carbonyl group can accept the hydrogen from another molecule in upward direction and the alcohol group can donate the proton to carbonyl group of another molecule in downward direction. This continuous arrangement of the residues by hydrogen bonding interactions build a helix around a pseudo axis. The puckered conformation of proline ring facilitated

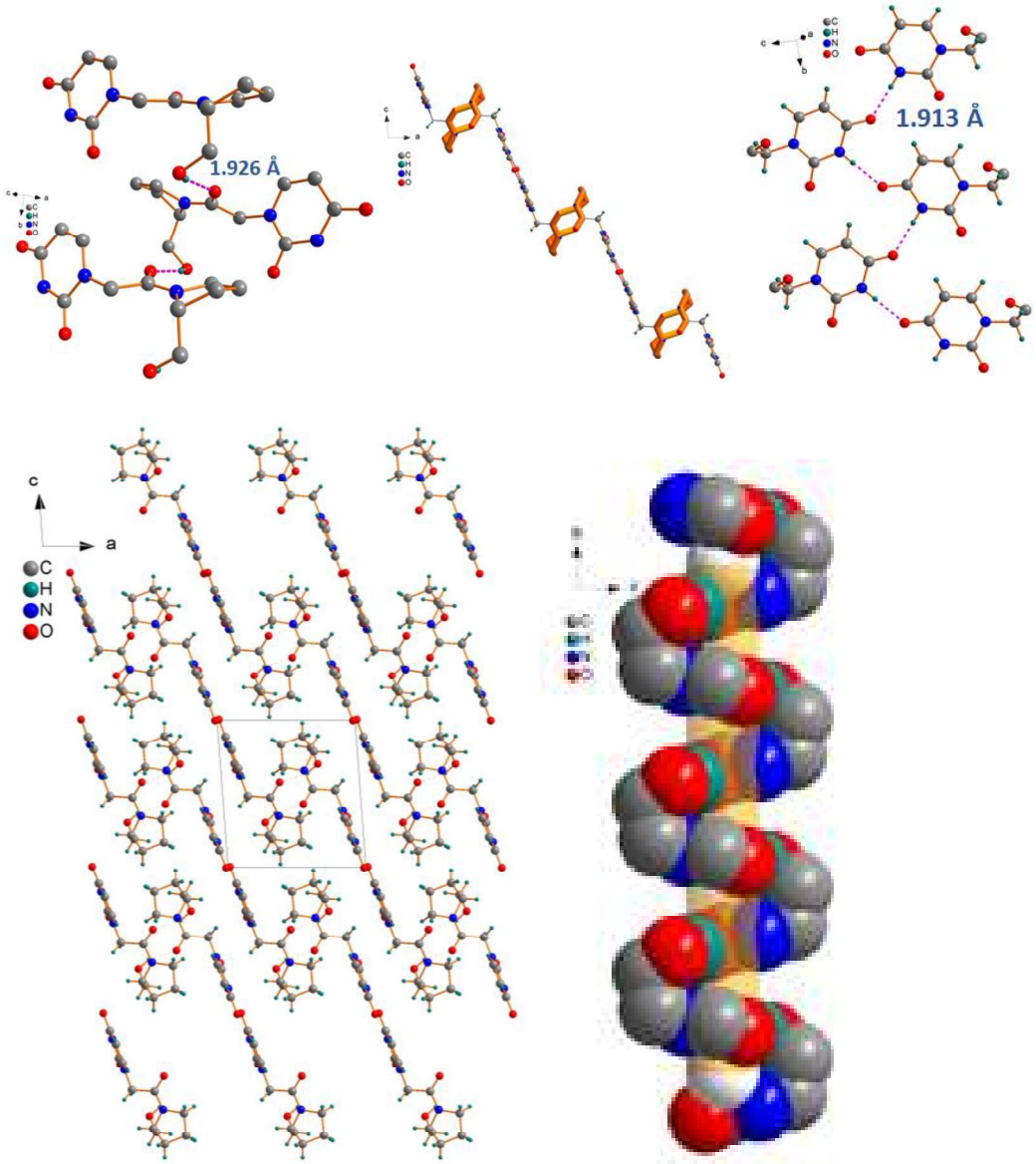


Figure 3B. 2 Crystal structure and self-assembly of prolinol acetyl uracil

such an arrangement of donor-acceptor to make stable helix. The uracil units flanked around the helix provides further supra molecular interaction with the uracil units of adjacent helixes. An uracil moiety donates its N3 H to O 4 of uracil in downward direction and accepts hydrogen form N3 H to its O4 in upward direction. The hydrogen bonding distance between the alcohol OH and carbonyl group is 1.926 Å

and the distance between N3 H and O4 is 1.913 Å both are strong hydrogen bonding interactions.

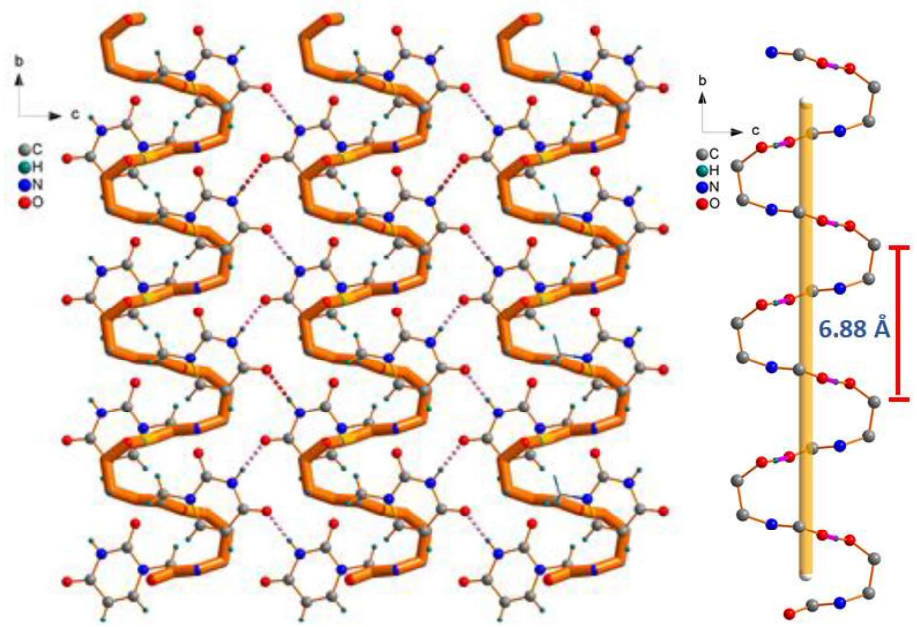


Figure 3B. 3 Crystal structure and self-assembly of prolinol acetyl uracil

Helical self-assembly of prolinol acetyl thymine: Prolinol acetyl thymine also forms helical supramolecular self-assembled structure.The crystal structure of prolinol acetyl thymine illustrated in Figures 3B.4 and 3B.4 In contrast to the prolinol acetyl uracil in thymine analogue with same backbone the carbonyl group of acetyl unit is not involved in the hydrogen bonding but here thymine take part in the helix formation and the alcohol acts as both donor by donating hydrogen to the carbonyl of thymine O 4 and acceptor by taking hydrogen from the N3 of thymine. Here the thymine residues are arranged in such a way that O4 accepts hydrogen from upward direction of alcohol and N3 accepts hydrogen from the alcohol from down ward direction and the

repeating units makes helix. Hydrogen bond distance of N3-H and alcohol oxygen is 1.902 Å and OH and O4 of thymine is 1.723 Å both are strong hydrogen bonds.

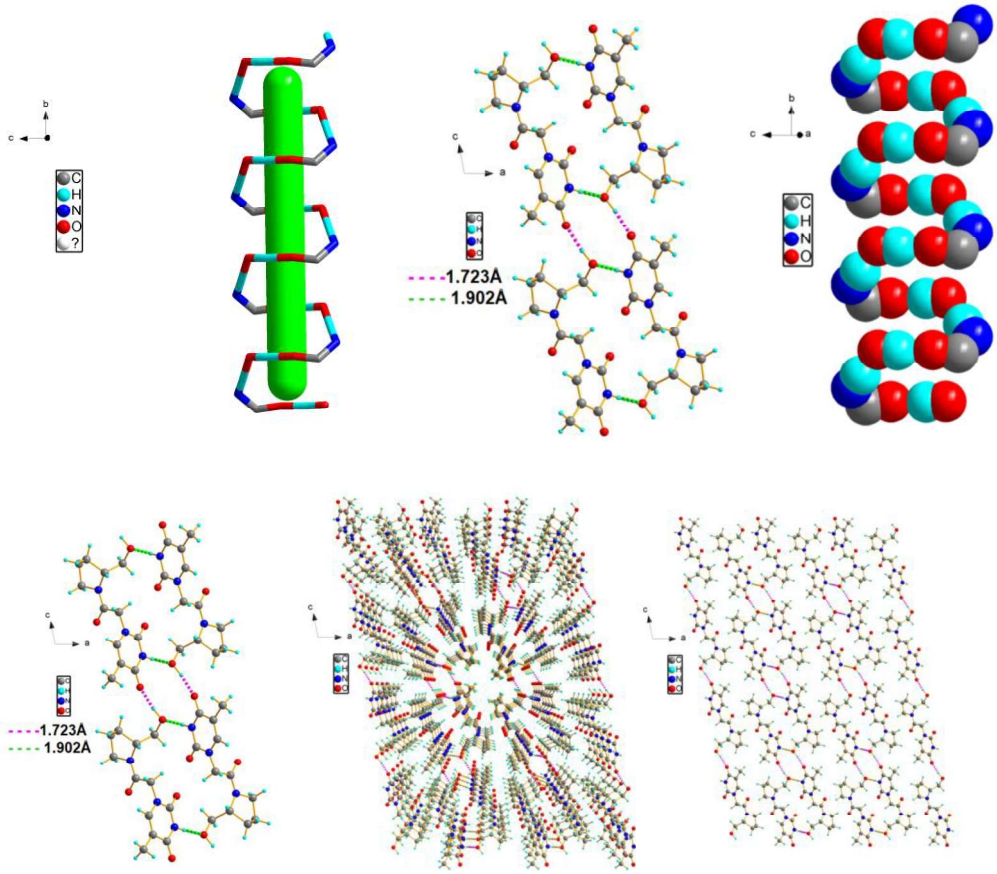


Figure 3B. 4 Crystal structure and self-assembly of prolinol acetyl Thymine

3B.2.4 Self-assembly study by scanning electron microscopy (SEM)

We recorded SEM images of prolinol acetyl thymine/uracil to study the morphology of self-assembled structuresThe SEM image of prolinol acetyl thymine show rod like structures (Figure 3B.5). The rods are in nanometer size. At micrometer size also we observed the rod like structures and some aggregation. The SEM image of prolinol acetyl uracil (Figure 3B.5) show high aggregation in comparison to the thymine analogue. The crystal structure of prolinol acetyl uracil (Figure 3B. 2) show the helical self-assembly formed by the hydrogen bonding interactions of alcohol OH and

carbonyl oxygen .The flanking uracil units around helix connects other helixes by hydrogen bonding interaction with adjacent uracil units forming a high aggregation. The crystal structure of prolinol acetyl thymine show that thymine residue is involved in the helix formation along with alcohol OH of proline ring where as in uracil case the carbonyl oxygen of acetyl group and alcohol OH of proline ring are involved in helix formation and uracil units take part in the connection of helixes by hydrogen bonding interactions. Due to extension of aggregation of helixes by joining uracil units a high aggregation seen in uracil analogue (Figure 3B.6). In thymine analogue thymine residue involves in helix formation and not available to extend the attachment of helixes as like in uracil. Due to this a symmetrical rod like structure are observed in thymine case.

Overall the SEM studies support the self-assembly of prolinol acetyl thymine/uracil and also provide an evidence to understand the nature of aggregation as seen in crystal structures.

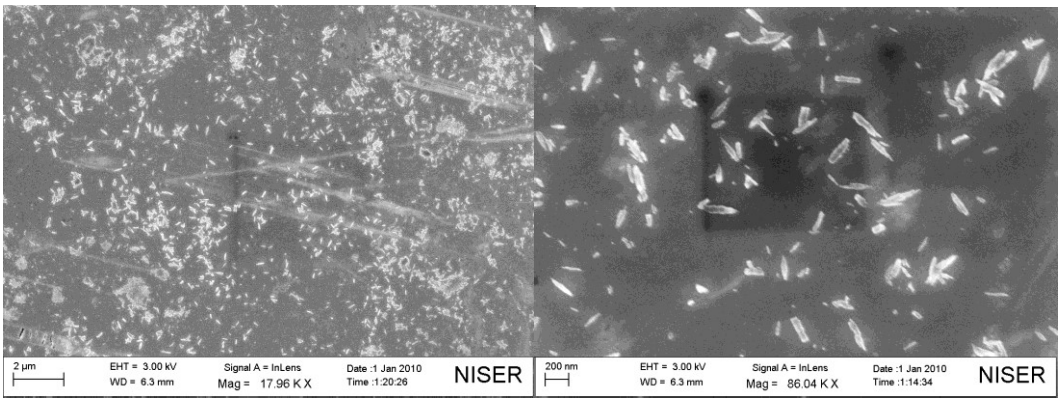


Figure 3B. 5 SEM image of prolinol acetyl thymine

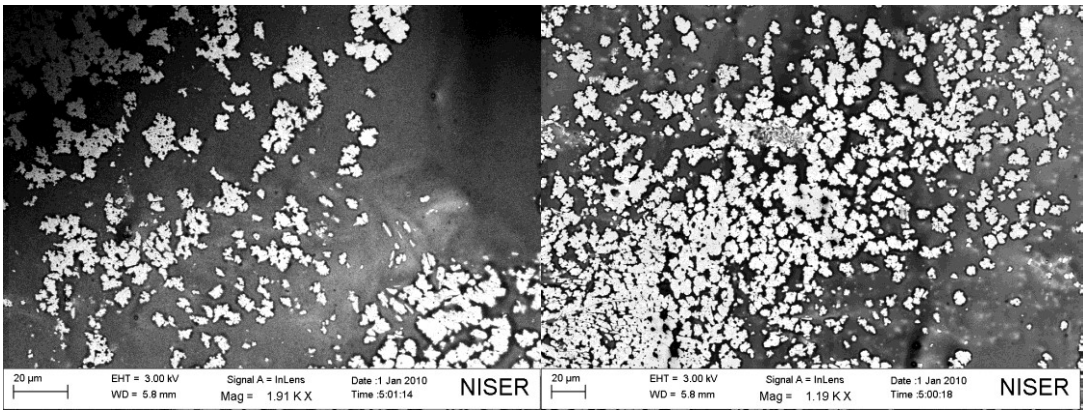


Figure 3B. 6 SEM image of prolinol acetyl uracil

3B.2.5 ESI-MS studies

The self-assembly of nucleobase/nucleosides derivative have been studied by ESI-MS. For example Guanine nucleosides forms G-quartets which formed by the self-assembly four quinine units in presence of metal ion. 5-substituted thymine and uracil derivatives also forms pentameric structures in presence of metal-ions have been studied by ESI-MS studies. As our helical structures are formed by the self-assembly we are interested to study the self-assembly by ESI-MS. We recorded the ESI-MS of thymine and uracil derivatives (Figure 3B.7& 3B8). In both cases the monomer is predominate and the dimer and trimers were also observed with less intensity. A tetramer also observed with less intensity. The higher order aggregates are not stable in ESI conditions. However ESI-MS results support the self-assembly of these analogues though they not stable enough to determine the high order self-assembled structures.

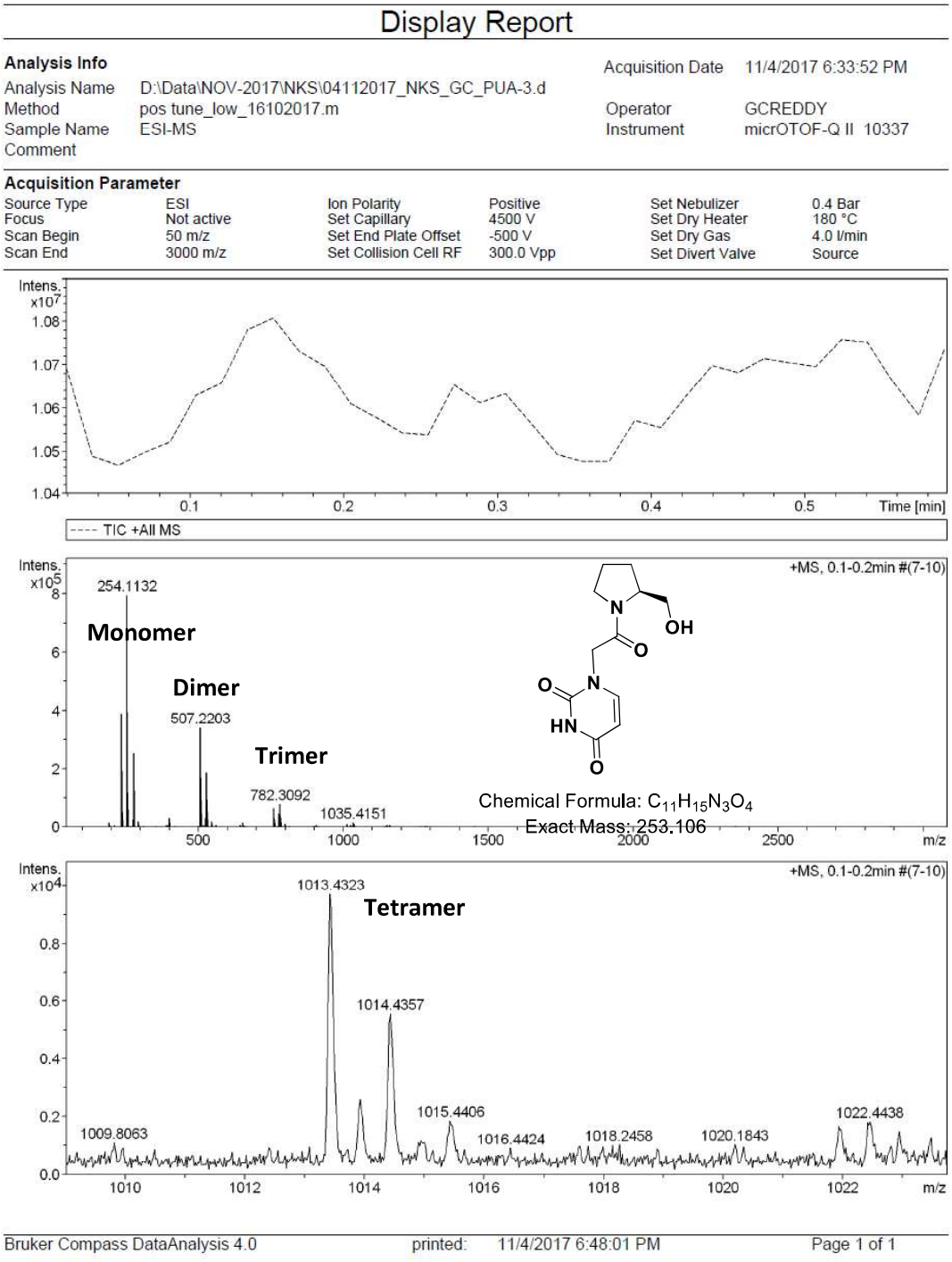


Figure 3B.7. ESI-MS of prolinol acetyl uracil

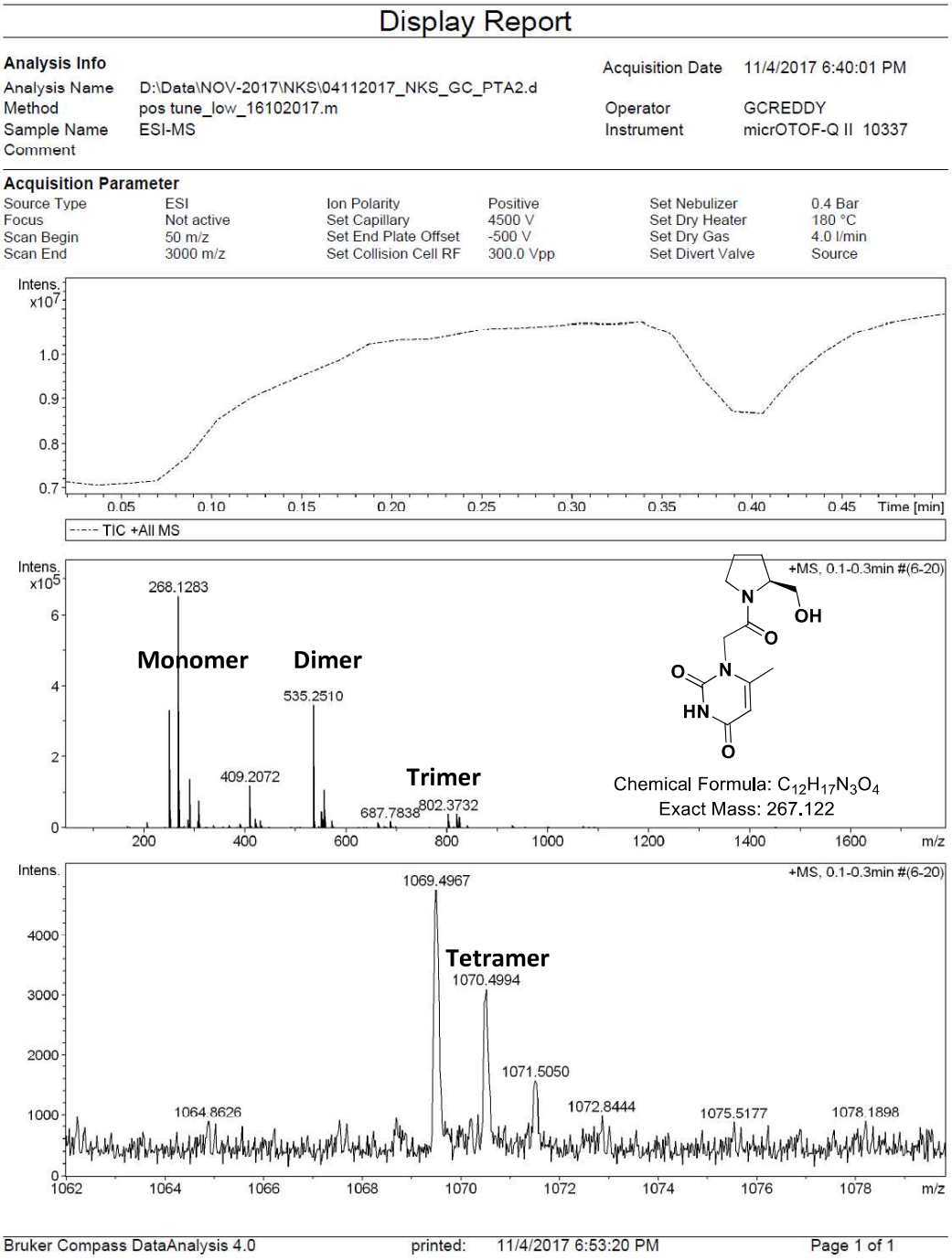


Figure 3B. 8. ESI-MS of prolinol acetyl uracil

3B.3 Conclusion

In conclusion we synthesized prolinol acetyl thymine/uracil triphosphates and studied their biological activity with DNA polymerase. The triphosphate analogues are not substrates of DNA polymerase. We have studied the self –assembly of prolinol acetyl thymine/uracil by single crystal X-ray Diffraction. The two analogues form supramolecular helical self-assembly by hydrogen bond interactions as shown crystal structure. Importantly the puckered nature of prolinol acetyl moiety plays crucial role in the orientation of helix structure. The systems can help to understand and design the helical self-assembled structures.

3B.4 Experimental section

(S)-tert-butyl 2-(hydroxymethyl)pyrrolidine-1-carboxylate **(2)** 4.0g (18.6mmol) boc proline was dissolved in 30 ml dry THF and stirred at 0ºC for 10 minutes under nitrogen atmosphere,borane dimethyl sulphide (5M in diethyl ether) 3.71ml (18.6mmol) was added drop wise with syringe at 0ºC .After 10 minutes the reaction mixture warmed to room temperature and stirred for 6 hours. The reaction mixture evaporated to dryness and coevaporated with methanol (2x30 ml) and extracted with water and ethylacetate .Ethyl acetate layer washed with brine solution and dried with anhydrous sodium sulphate and concentrated at reduced pressure. White solid 3.73 g obtained in quantitative yield used directly in next step without any purification. TLC DCM R_f =0.43 ^{1}H NMR (400MHz in $CDCl_3$) δ1.45(s,9H),1.84-1.85(m, 3H) ,1.95-2.04(m, 2H) ,3.26-3.32(m, 1H),3.41-3.49(m,1 H),3.54-3.62(m, 2H),3.85-3.94(m, 1H) 4.74(s, 1H) ^{13}CNMR(100M HZ in $CDCl_3$) δ24.18, 28.57, 28.22, 47.66, 60.30, 67.78, 80.33, 157.26 HRMS (ESI-TOF) Calcd for $(C_{10}H_{19}NO_3 + Na)^+$ 224.1257 found 224.1231

(S)-tert-butyl 2-((benzoyloxy)methyl)pyrrolidine-1-carboxylate **(3)**

To a solution of Boc prolinol 3.6 g (18.15mmol) in dry DCM (30ml), triethylamine 5.0ml(36.3mmol) was added at 0^0C followed by addition of benzoyl chloride 3.1 ml (27.2 mmol) drop wise and the reaction was stirred at room temperature overnight. 50 ml water was added to the reaction mixture and stirred for 10 min and organic layer was separated. Aqueous layer extracted with DCM (2x50 ml) .Combined organic layer washed with brine solution and dried with anhydrous sodium sulphate ,concentrated at reduced pressure and purified by silica gel column chromatography (ehtylacetate and hexane) to afford 4.9 g of white solid in 90% yield. TLC DCM R_f =0.32 ^{1}H NMR (400MHz $CDCl_3$) δ 1.45(S,9H),1.85-2.0(m,4H), 3.29-3.53(br, 2H) 4.10-4.42(m,3H),7.43(t,2H 8hz),7.55(t,1H 8hz),8.03(d ,2H 8hz). ^{13}CNMR(100 MHz $CDCl_3$) δ23.30,23.98, 28.05,28.59,29.00, 46.62,48.5, 55.79, 65.20,65.39, 79.55,79.92,128.50, 129.74, 130.18, 133.16, 154.61, 166.51. HRMS Calcd for $(C_{17}H_{23}NO_4+Na)^+$: 328.1519 found 328.1542.

S-(1-(2-Chloroacetyl)pyrrolidine-2-yl)methyl benzoate **(4):** 530 mg (1.73mmol) of compound **3** was treated with 10 ml of TFA.DCM (20%) at room temperature. After 3hours the reaction mixture was evaporated to dryness and diluted with 5ml dry acetonitrile, triethylamine 0.48 ml(3.47mmol) was added at 0^o C under N_2 atmosphere followed by chloroacetyl chloride 0.20 ml (2.6mmol) at 0^o C and stirred overnight at room temperature .The reaction mixture concentrated and extracted with ethylacetate and water .Ethyl acetate layer washed with brine solution, dried with anhydrous sodium sulphate and concentrated followed by purification on silica gel column chromatography to obtain colurless oily compound 380 mg in 78% yield. TLC DCM

R_f =0.64 ^{1}H NMR (400MHz $CDCl_3$) δ 1.96-2.13(m,4.3H), 3.57-3.64(m,2H), 4.04(s,1.42H), 4.12-4.17(m,0.45H),4.29-4.74(m, 3H), 7.44(t,2H, *J*= 8 HZ),7.56 (t,1H *J*= 8 HZ) 8.01(d,2H *J*=8 HZ) ^{13}CNMR (100MHz , $CDCl_3$) δ 21.16, 24.40, 27.39, 28.89, 41.62, 42.36, 46.33, 47.32, 56.25, 56.96, 63.93, 65.13, 128.48, 128.66, 129.33, 129.61, 129.96, 133.13, 133.57, 165.49, 165.76, 166.32, 166.36. HRMS (ESI-TOF) Cald for $(C_{14}H_{15}O_3NCl+H)^+$: 282.0891 found 282.0889.

*(S)-1-(2-(2-(hydroxymethyl)pyrrolidin-1-yl)- 2-oxoethyl) -5-methylpyrimidine 2,4(1H,3H)-dione***(5a)**

0.126 g (1.00mmol) of thymine dried under high vacuum at room temperature for 2 hours and dissolved in 4 ml anhydrous DMF followed by addition of 0.2g (1.5mmol) anhydrous potassium carbonate and 18-crown-6, 52mg (0.2mmol) and stirred at 60^0c for 20 minutes under N_2 atmosphere followed by addition of anhydrous DMF solution (2.8ml) of compound **5** 0.28g (1.0mmol) by a syringe and stirred over night at 60^0c .The reaction mixture was concentrated to dryness and extracted with water and ethyl acetate. Ethylacetate layer dried with anhydrous sodium sulphate and concentared to get white pluffy compound which was dissolved in methanol, stirred at room temperature for 10 minutes followed by addition of 0.276 g (2.0 mmol) of potassium carbonate and stirring was continued for 24 hours at room temperature, the reaction mixture was concentrated and purified by silica gel column chromatography using DCM/MeOH to obtain a white solid 0.262 g in 95% yield. TLC 10 % MeOH/DCM R_f =0.56 ^{1}H NMR (400 MHz CD_3OD) 1.88(s, 3H), 1.93-2.11(m,4H),3.41-3.66(m,4.0H),4.08-4.18(m, 1H),4.50-4.71(m, 2H),7.32(s,1H) ^{13}C NMR ($CDOD_3$100MHz) δ12.33, 22.54, 25.09, 28.01, 29.14, 60.42, 61.21, 62.92,

64.48, 110.79, 110.96, 143.74, 143.99,153.18, 166.97,167.84,168.34 HRMS (ESI-TOF) Calcd for ($C_{17}H_{12}N_3O_4Na$) 268.1292 found 268.1290

(S)-1-(2-(2-(hydroxymethyl)pyrrolidin-1-yl)-2-oxoethyl)pyrimidine-2,4(1H,3H)-dione **(5b)**: 0.112 g(1.0 mm) of uracil used to syntheisize the compound 5b in a similar procedure to obtain 0.24 g **5b** in 95% yield as white soild. TLC 10 % MeOH/DCM R_f =0.43 ^{1}H NMR (400 MHz CD_3OD)1.69-2.27,(m,4H)3.28(m, 2H),3.61-3.85(m, 3H),4.30(s, 1H) 4.56 (s,1H),5.64-5.70 (m, 1H) 7.49-7.60 (m, 1H) (CD_3OD, 100MHz) 22.67, 24.38, 25.21, 28.13, 29.72, 60.55, 61.31, 63.12, 64.57, 102.21, 102.36, 148.13, 148.38, 153.27, 167.05, 167.91, 168.35. HRMS Calcd for $C_{11}H_{15}N_3O_4Na$ 276.0955 Found 276.0970.

((S)-1-(2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetyl)pyrrolidin-2-yl)methyl trihydrogen triphosphate **(6a)**

Compound **5a** 0.03 g (0.112mmol) dried in high vacuum at room temperature for 2 hours, dissolved in 3ml trimethyl phosphate and stirred under nitrogen atmosphere at -20^{0}c for 10 minutes. 0.03 ml (0.33 mmol) of phosphorous oxy chloride was added and stirred at -5^{0}c for 2 hours followed by injection of a cocktail containing 154 mg (0.28mmol) tributylammonium pyrophosphate and 0.21ml (0.89mmol) of tributylamine in 2 ml of anhydrous DMF and stirred at room temperature for 30 minutes. 5 ml of 100ml of 100mM TEAB buffer added and stirred at room temperature for one hour the hydrolysis step was monitored by mass spectrometry after one hour another 5ml TEAB buffer added and stirred for 30 minutes. The reaction mixture lyophilized and purified by DEAE sephadex column using ammonium bicarbonate as eluent (0.1M -1.0 M) .The compound further purified by HPLC using 10 mM TEAB buffer (A) and acetonitrile (B). ^{1}HNMR (400 MHz D_2O)

δ 2.01.2.11(m,1H), 3.36-3.40(m,1H), 3.53-3.67(m,2H) 3.97-4.13(m,1H),4.29-4.44(m,3H), 4.91-5.0(m,1H), 5.87-5.89((m,1H), 7.60-7.71(m,1H) ^{31}P NMR (162MHz,D_2O): δ -22.05,-22.79 (br ,1P,), -9.91(br, 2P) HRMS: Calcd for $C_{12}H_{19}N_3O_{13}P_3$ 506.0125 Found 506.0178

((S)-1-(2-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetyl)pyrrolidin-2-yl)methyl trihydrogen triphosphate **6b**

Compound 5b 0.03 g (0.11 mmol) phosphorylated as described above to obtaine prolamiode uridine triphosphate (**6b**) ^{1}H NMR (400 MHz D_2O) 2.01-2.11(m ,4H), 3.50-3.71(m,2H), 3.94-4.17 (m,2H), 4.28(br,1H), 4.44 (br,1H), 5.83-5.89(m,1H), 7.60-7.70 (m,1H), ^{31}P NMR(162MHz,D_2O): δ -22.05,-22.44 (br ,1P,), -10.34(br, 2P) HRMS: Calcd for $C_{11}H_{17}N_3O_{13}P_3$ 499.9969 Found 499.9918

3B.5 Refferences

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3B.6 Appendix

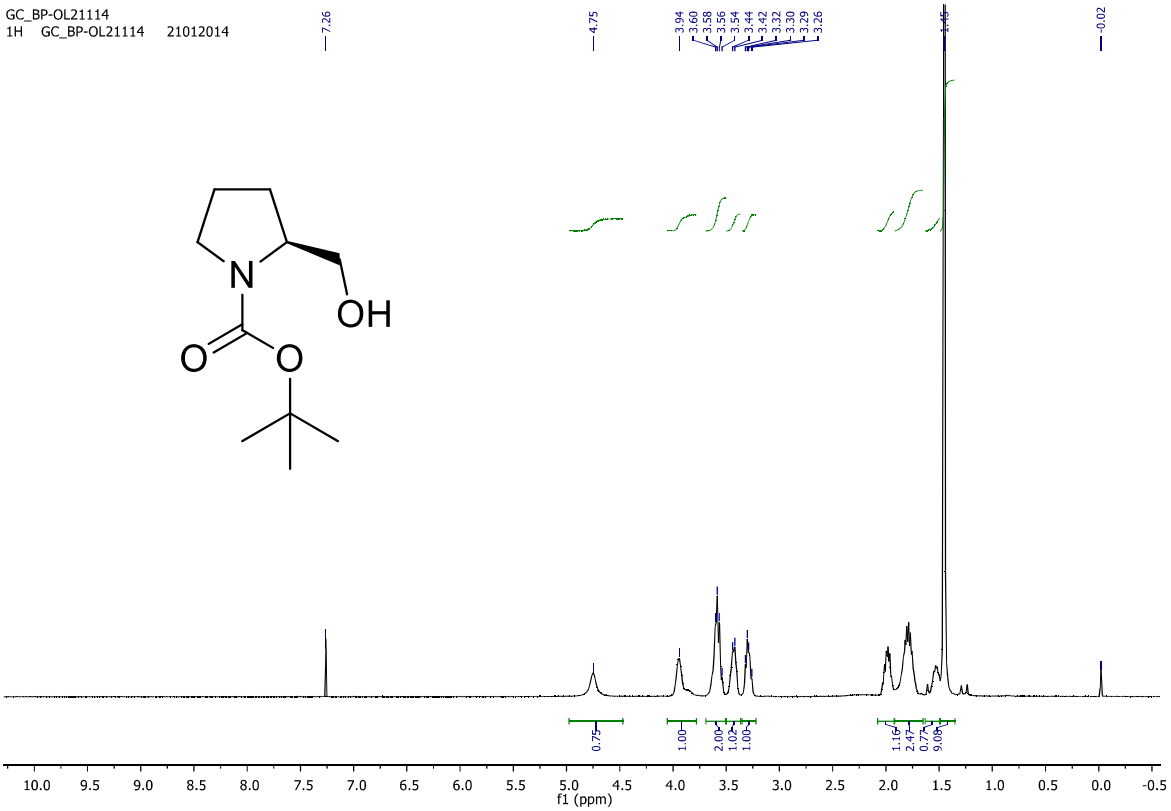


Figure A 1 ^{1}HNMR of compound 2

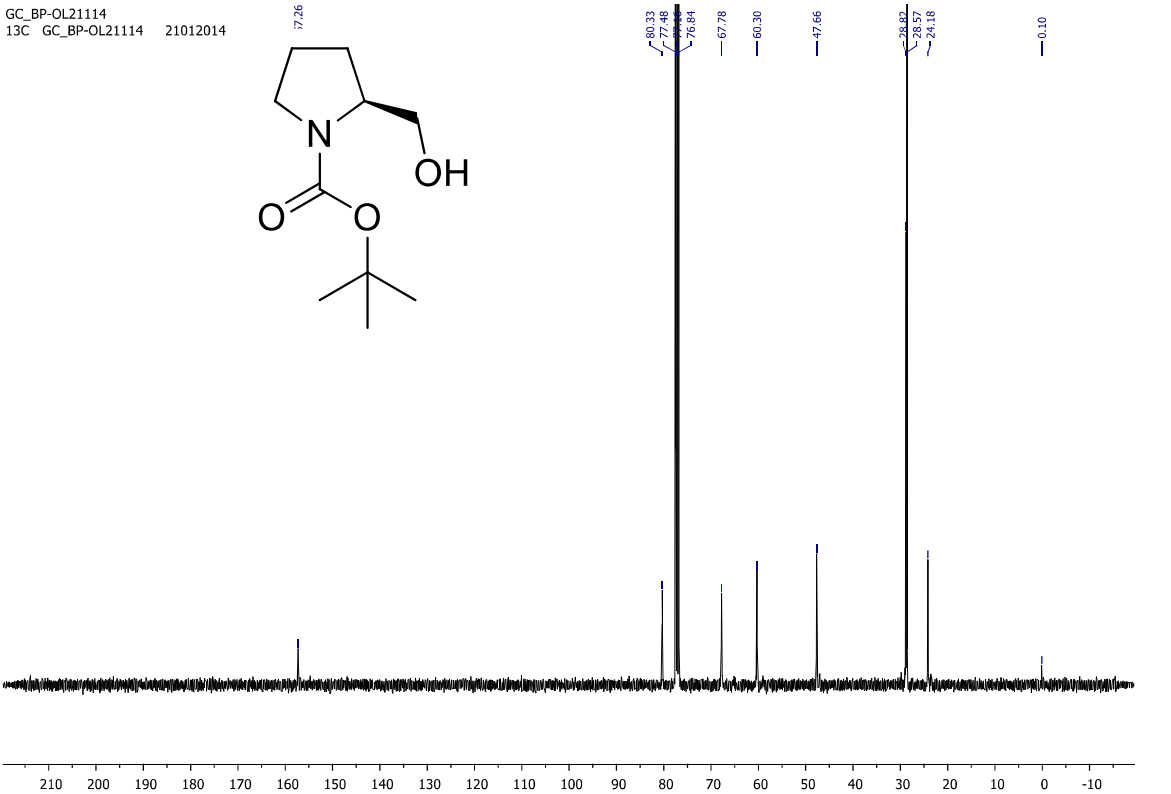
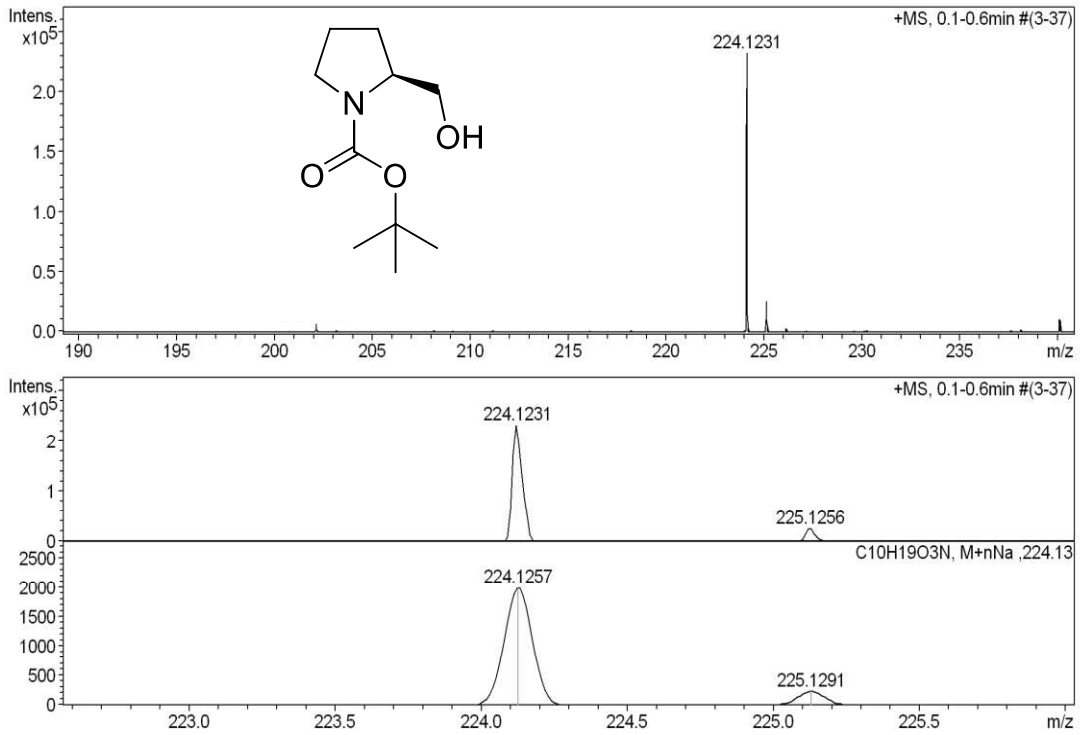


Figure A 2 ^{13}C NMR of compound 2



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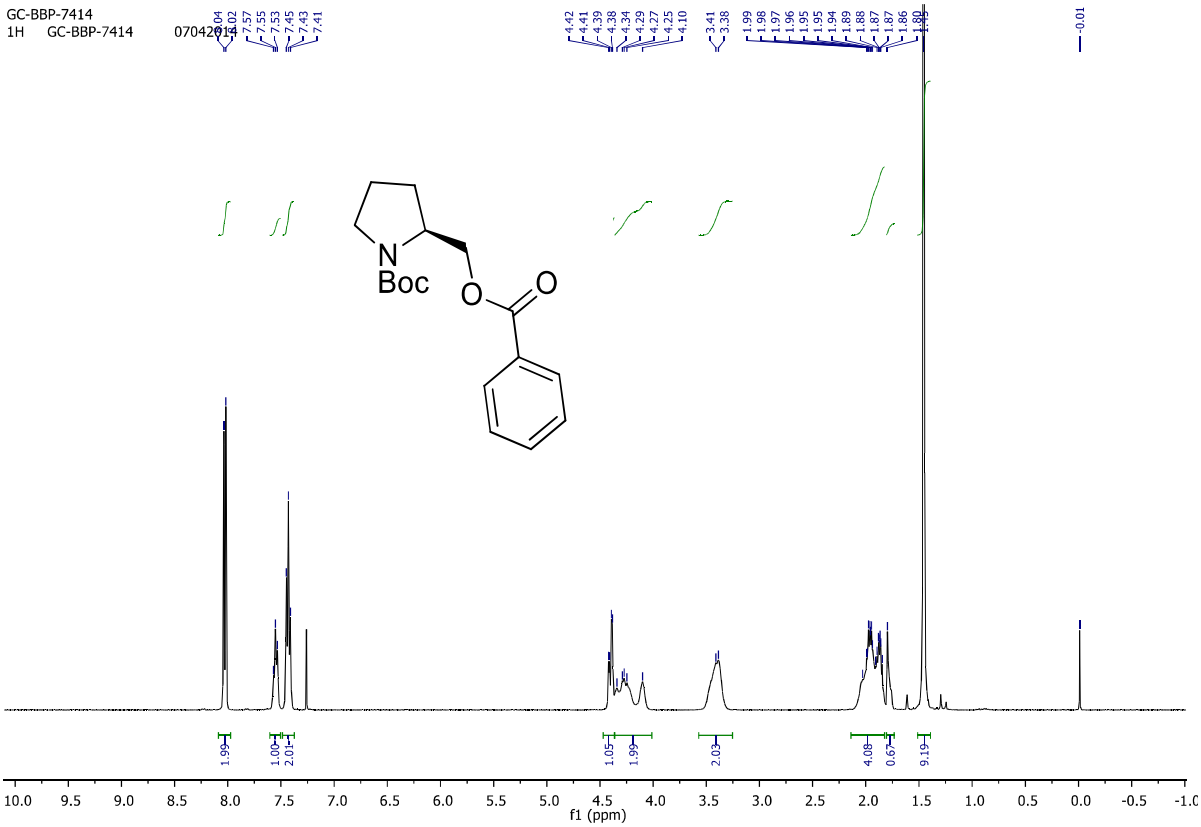
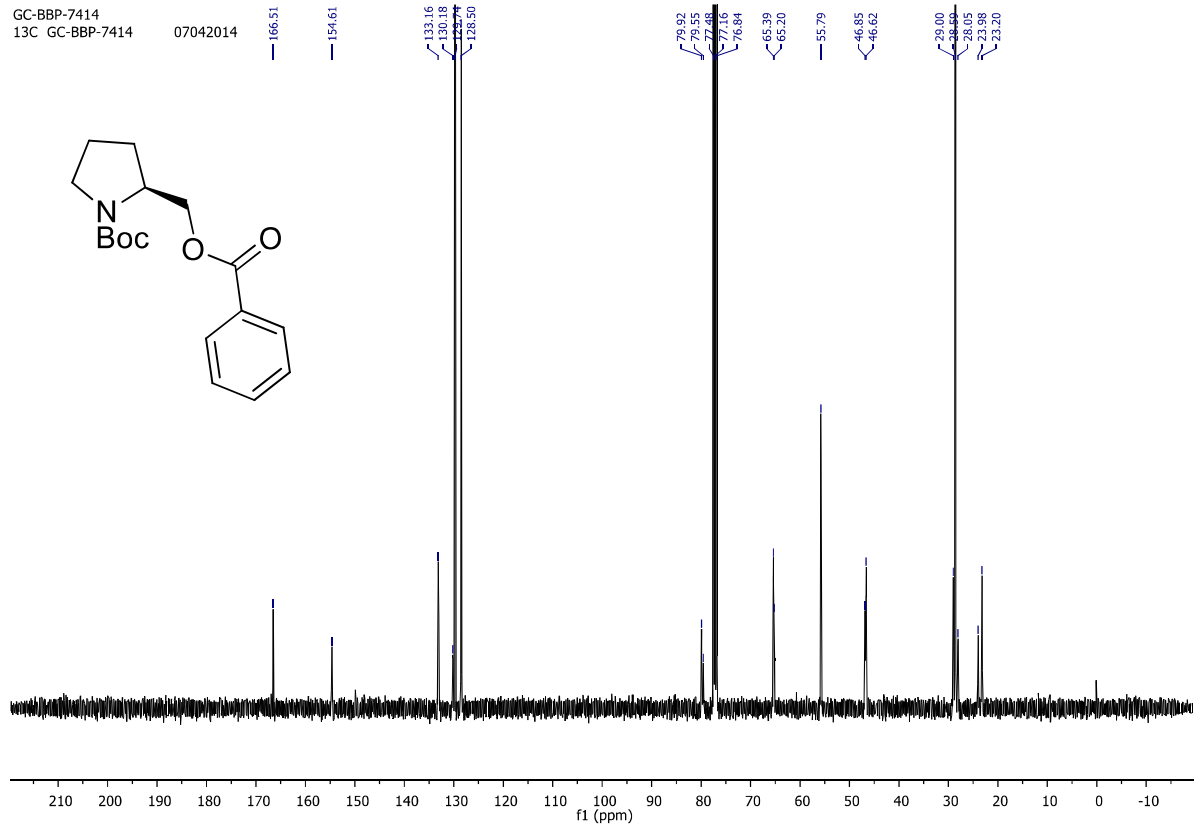


Figure A 4 [1]HNMR of compound 3



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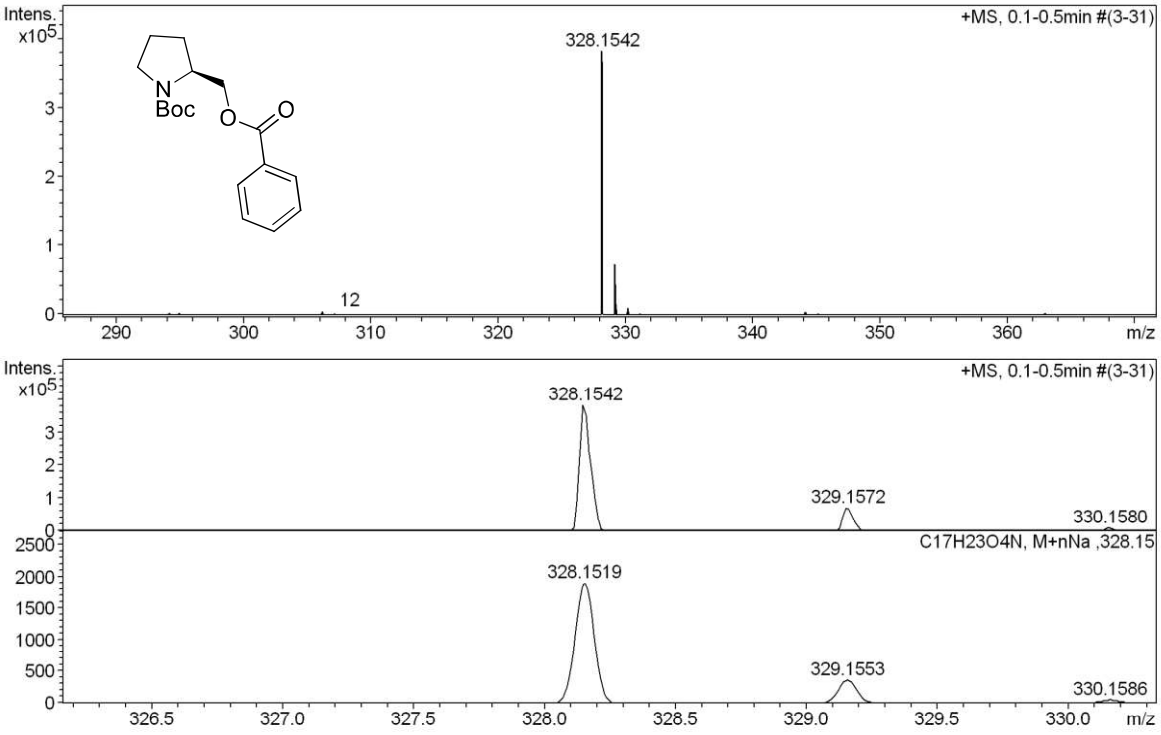

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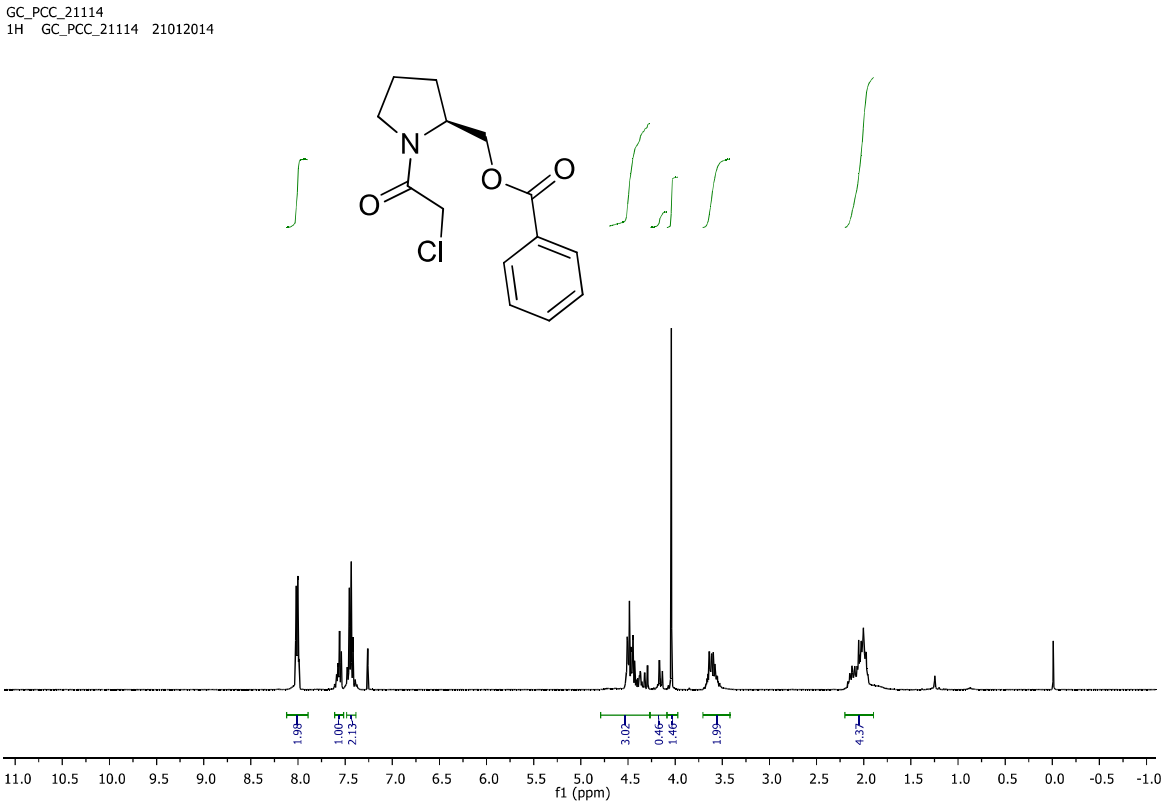
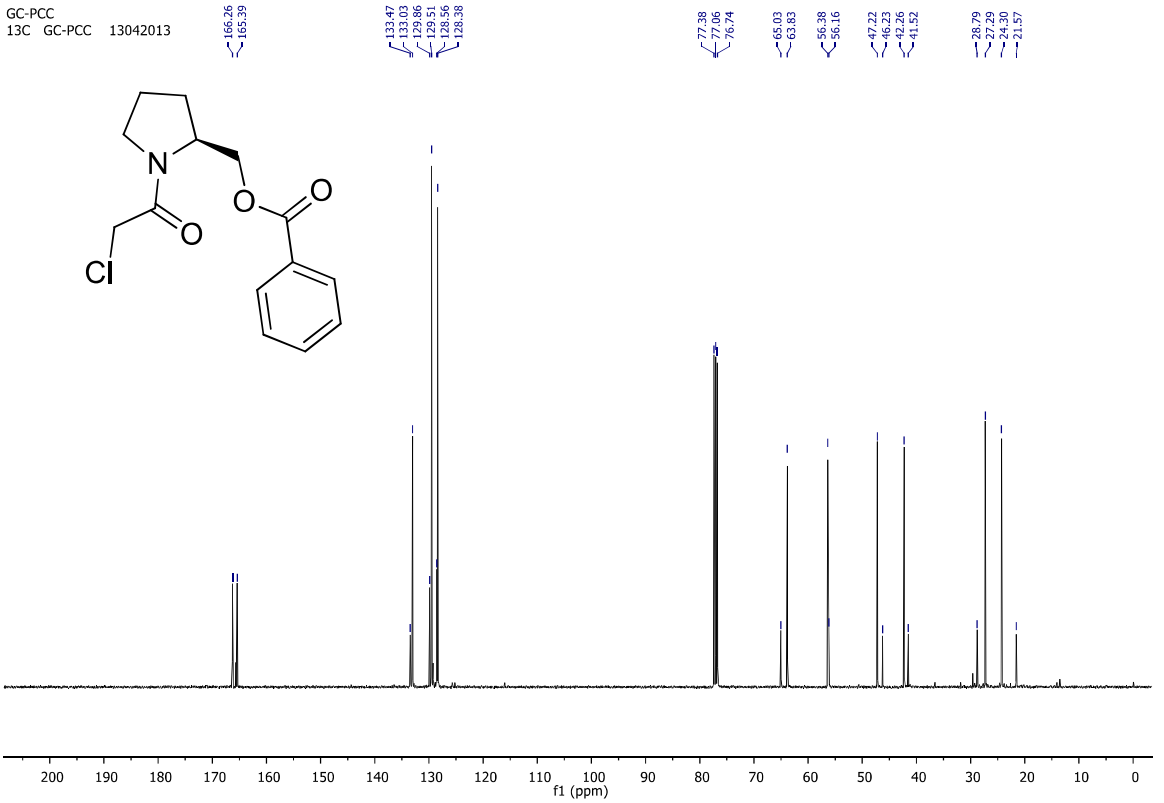


Figure A 7 ^{1}H NMR of compound 4



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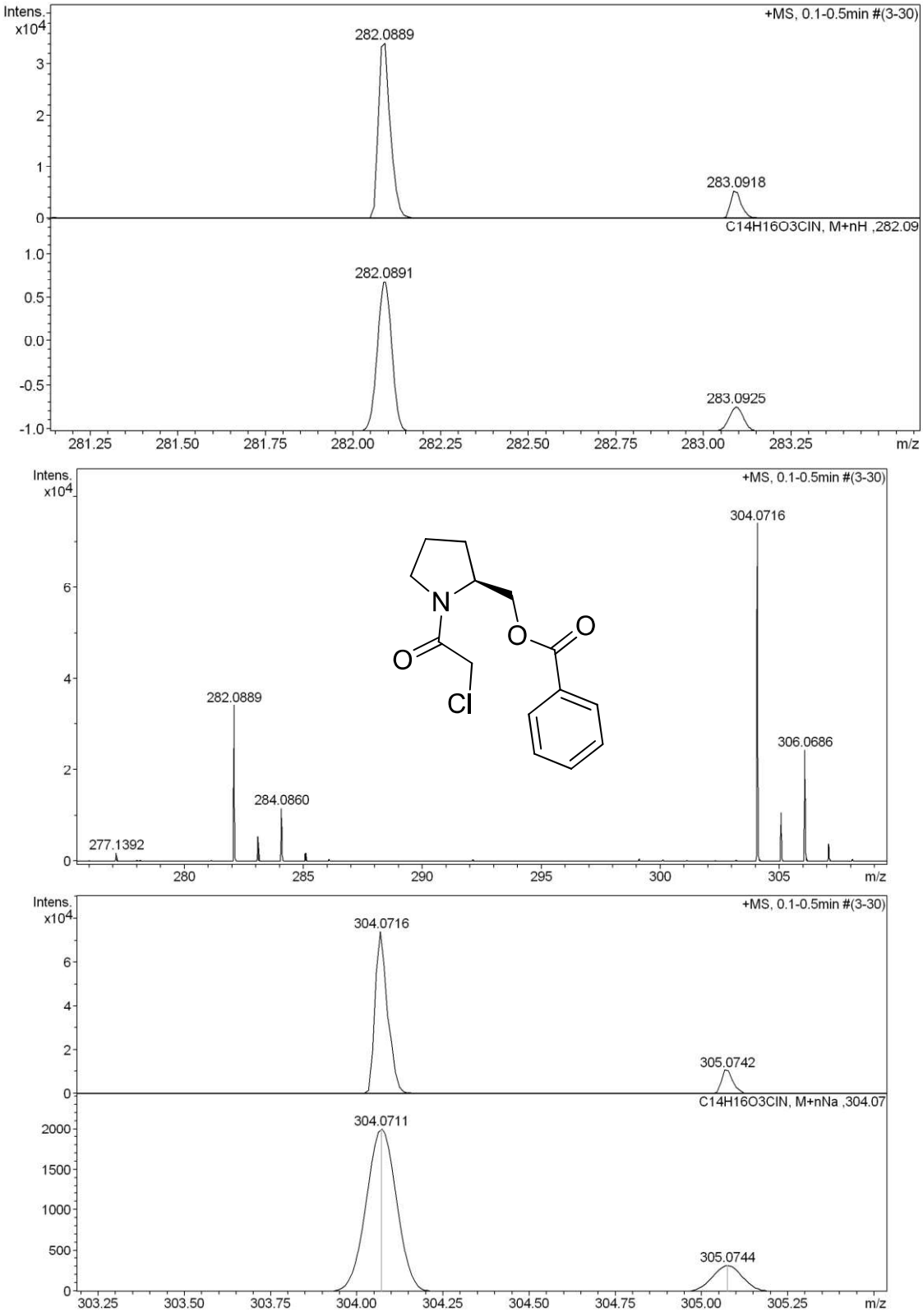

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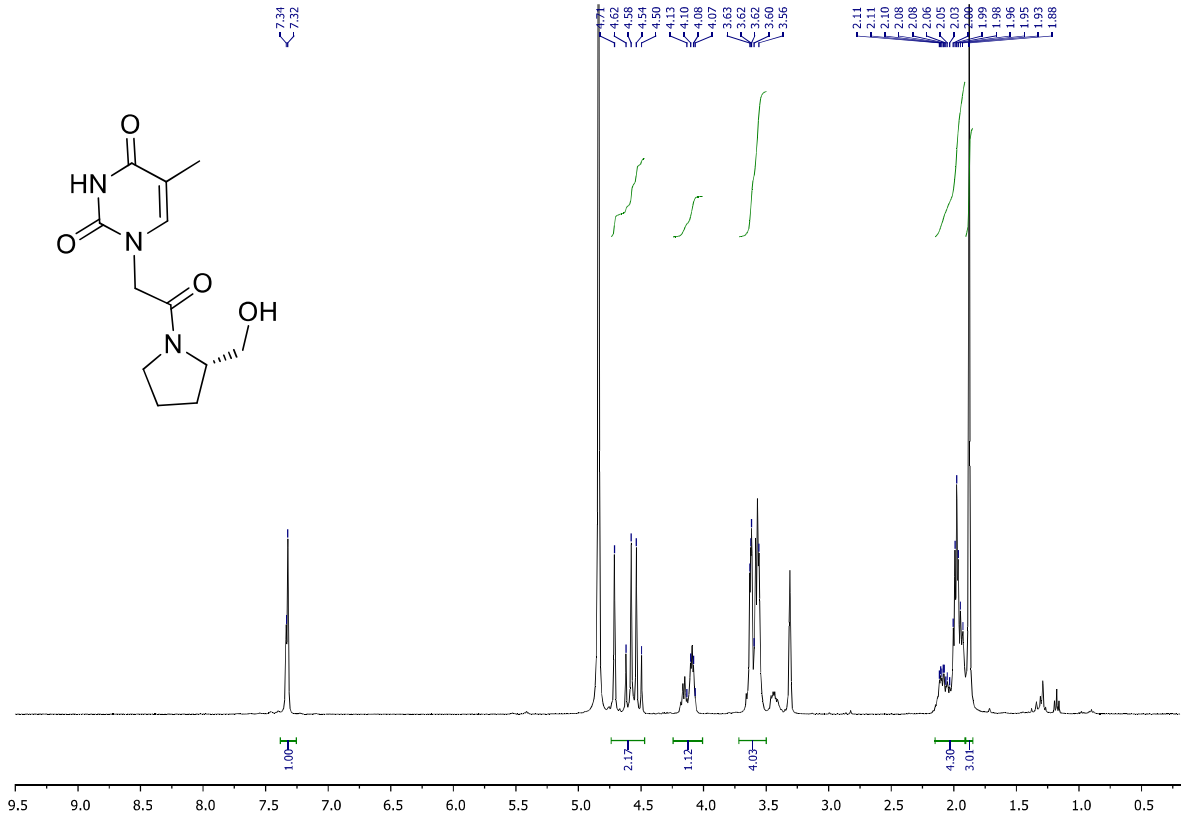


Figure A 9 ^{1}H NMR of compound 5a

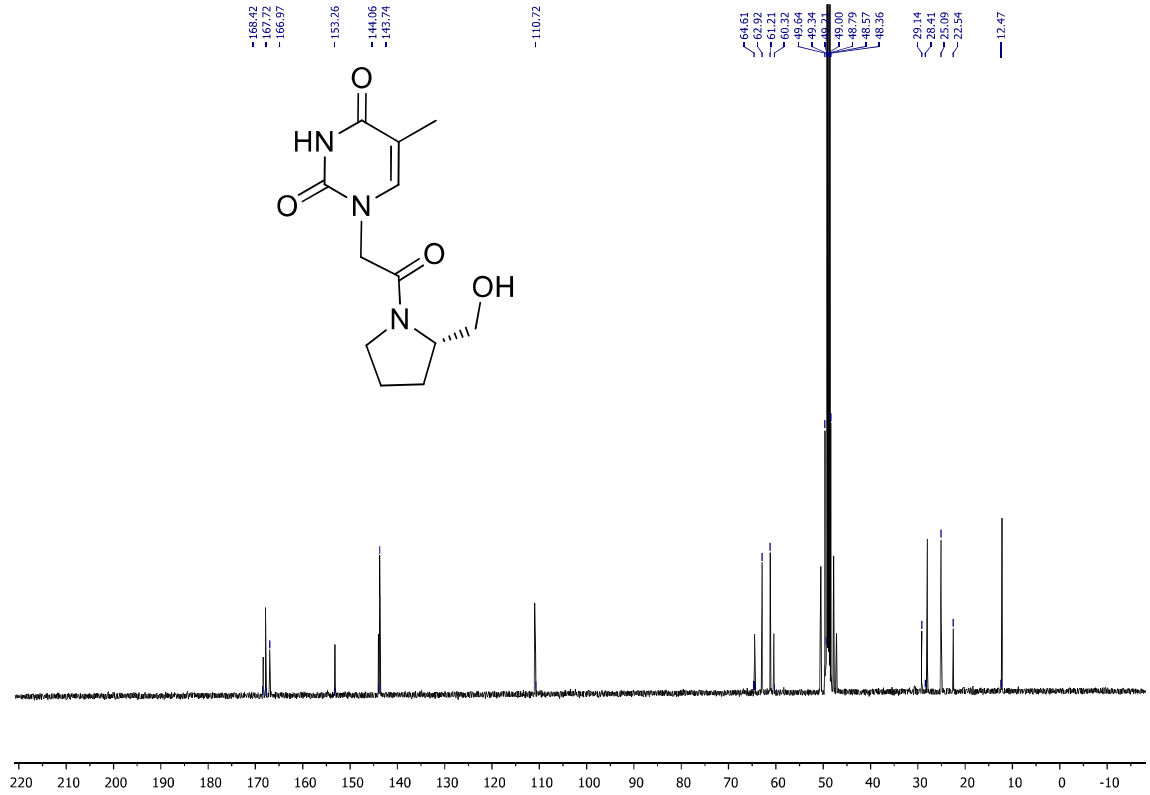
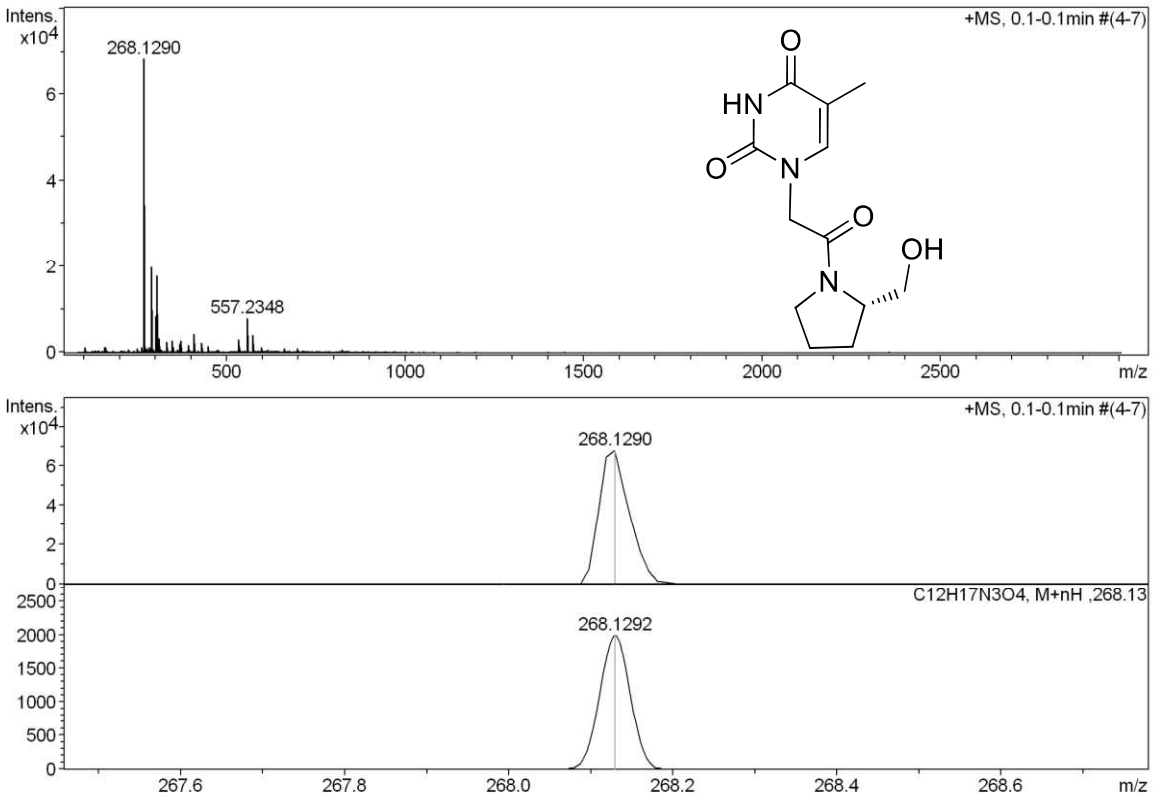


Figure A 10 ^{13}C NMR of compound 5a



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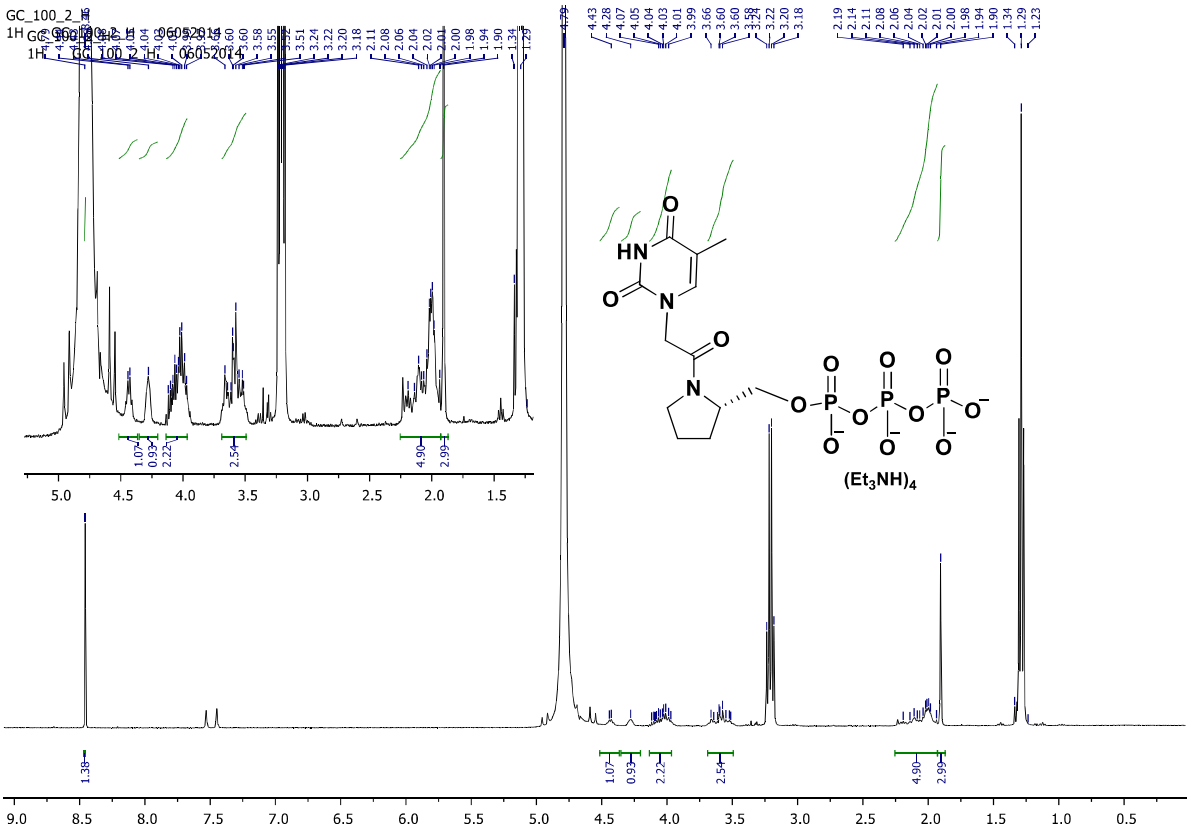


Figure A 12 ^{1}H NMR of compound 5a

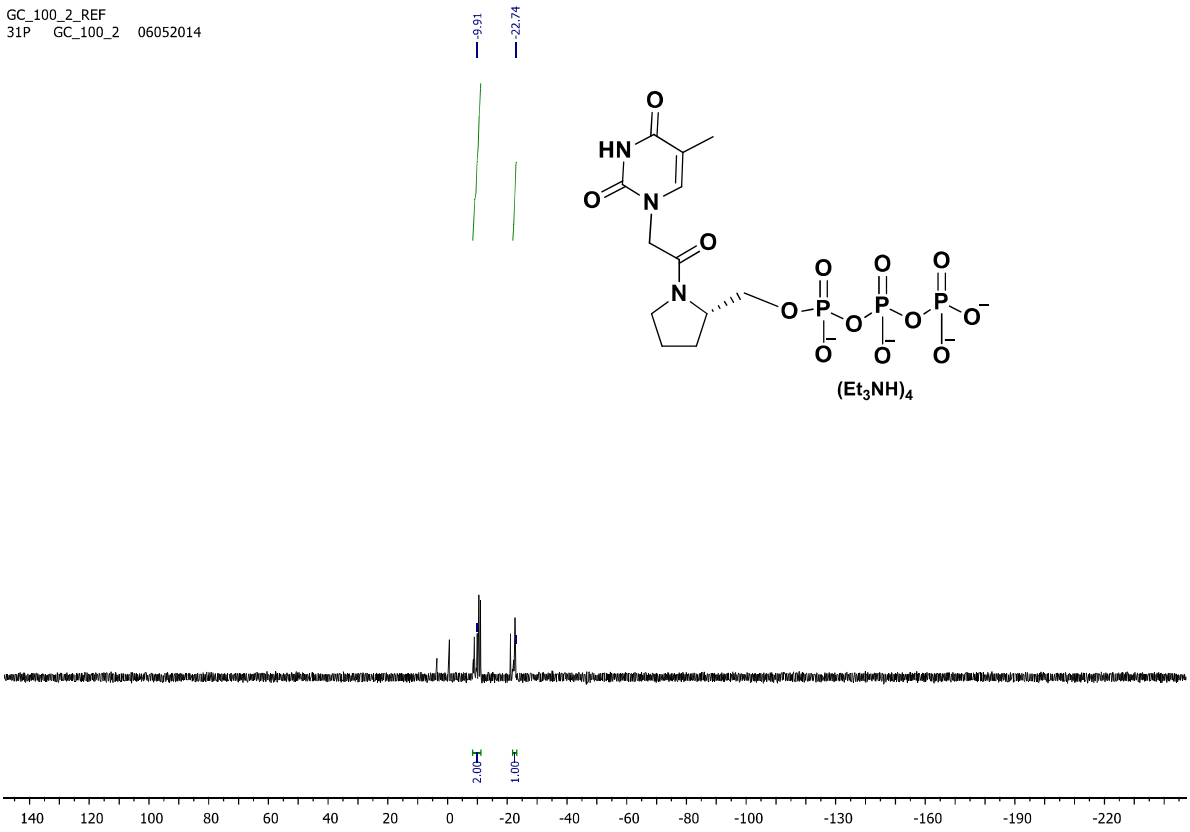


Figure A 13 ^{31}P NMR of compound 5a

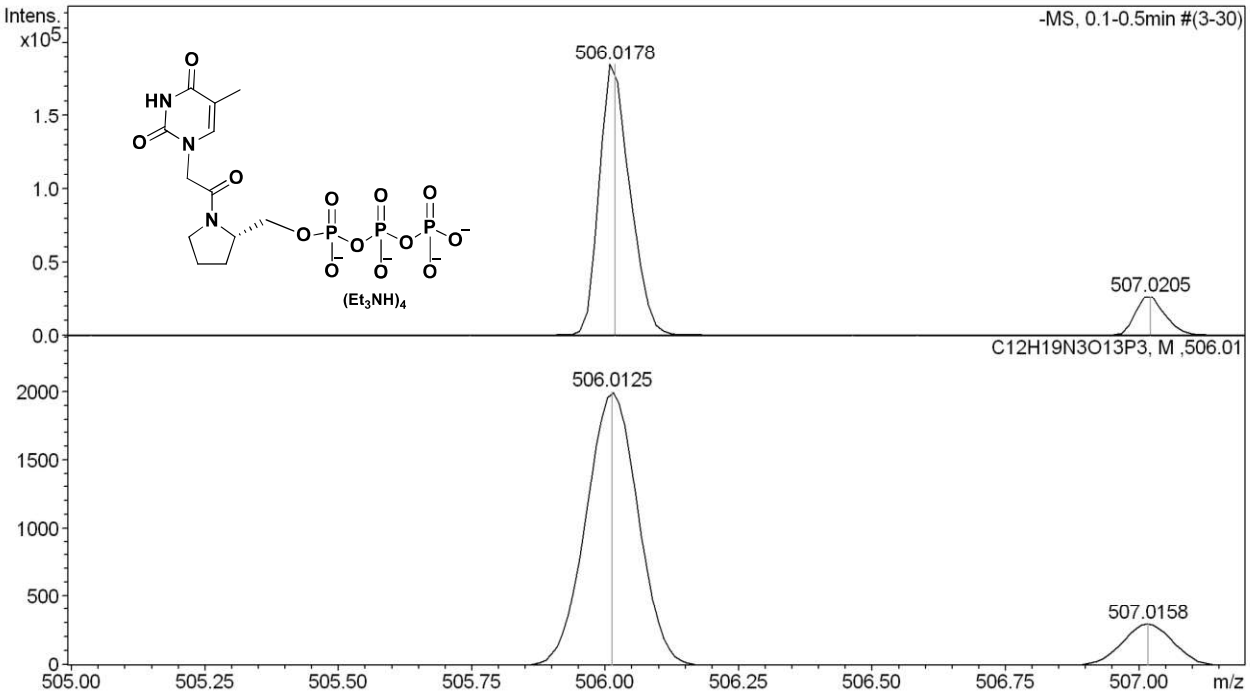


Figure A 14 HRMS of compound 5a

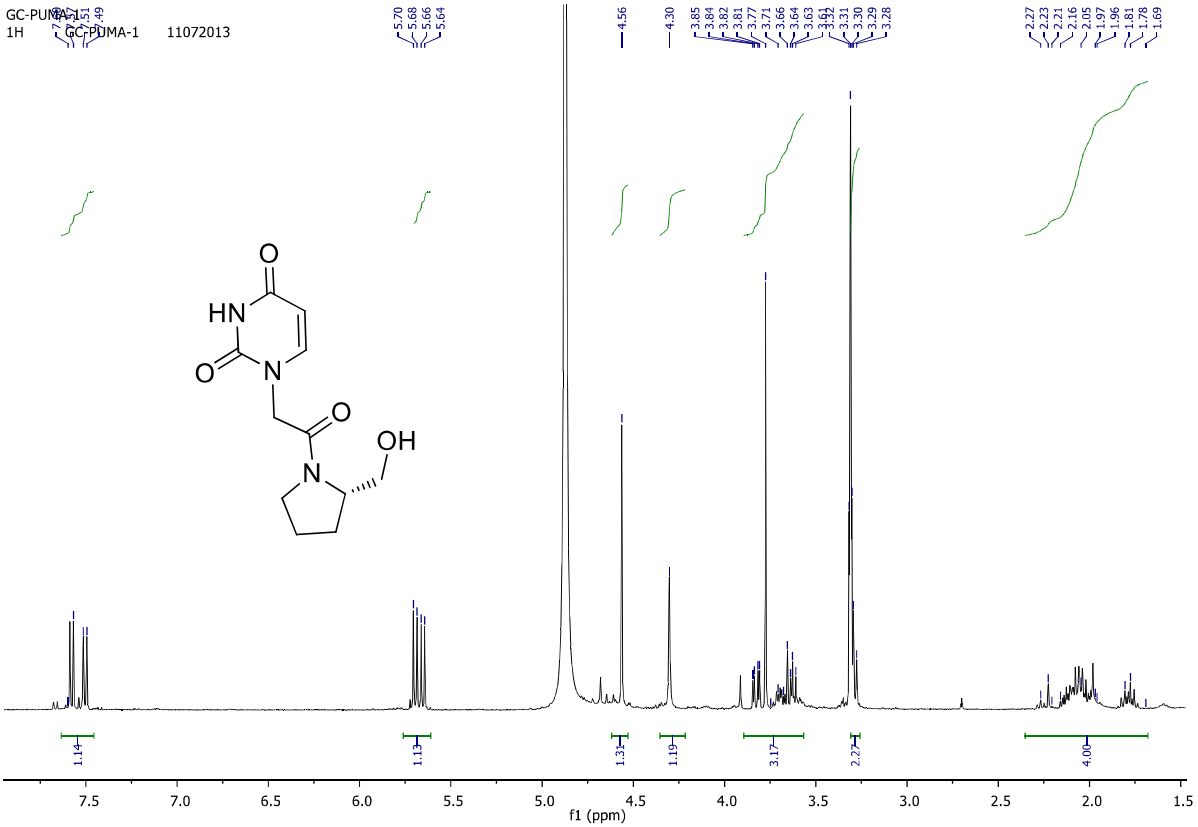


Figure A 15 ^{1}H NMR of compound 5b

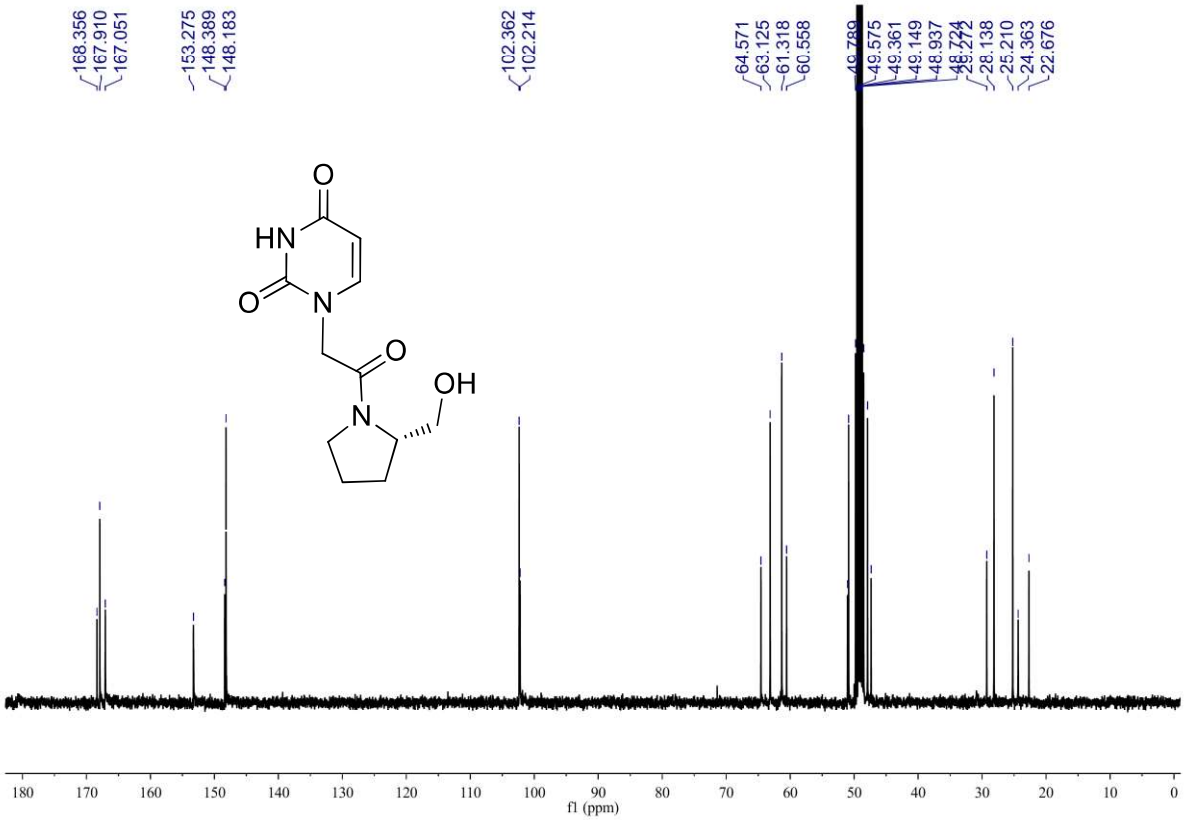


Figure A 16 ^{13}C NMR of compound 5b

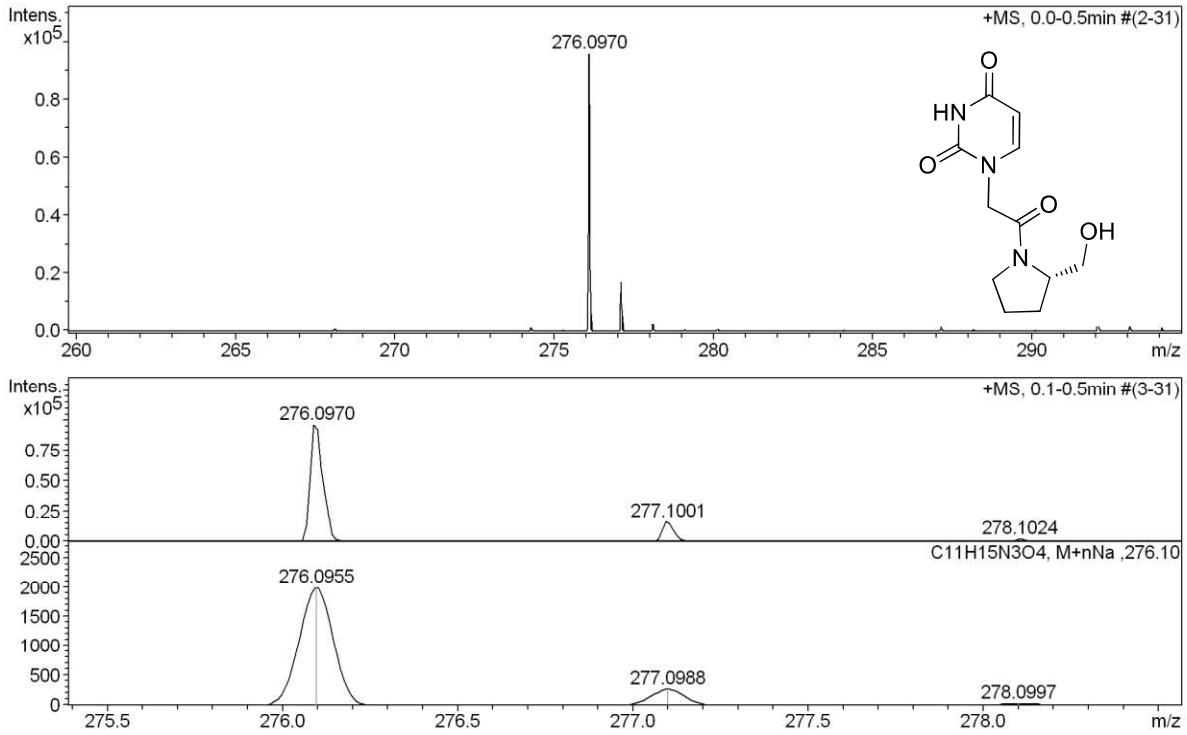


Figure A 17 HRMS of compound 5b

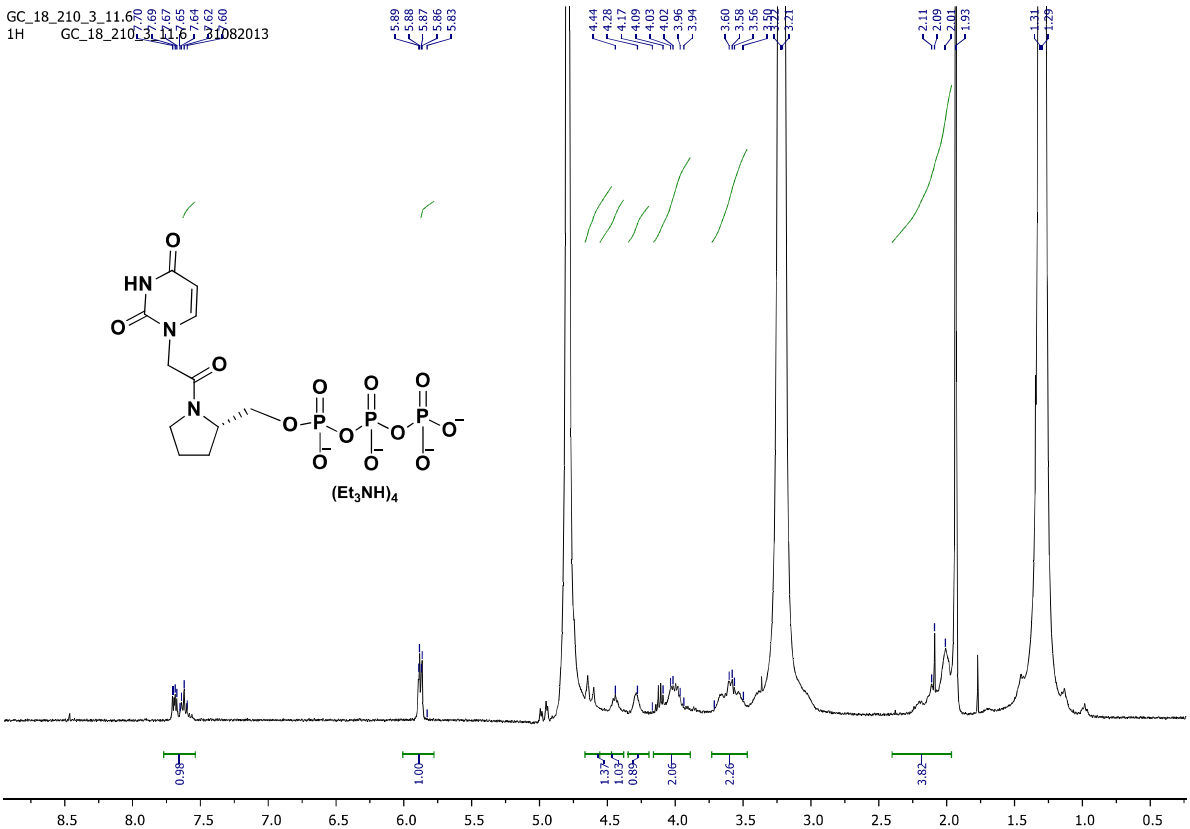


Figure A 18 ^{1}HNMR of compound 6b

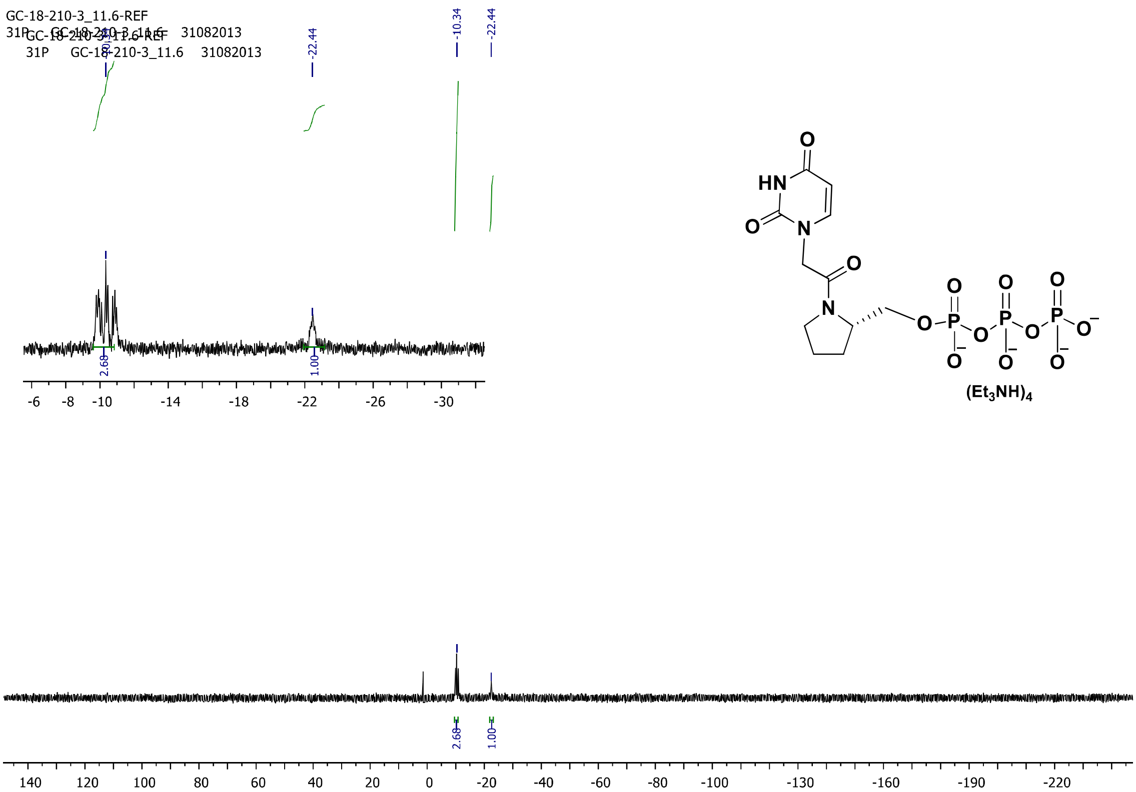


Fig S19 ^{31}P NMR of compound 6b

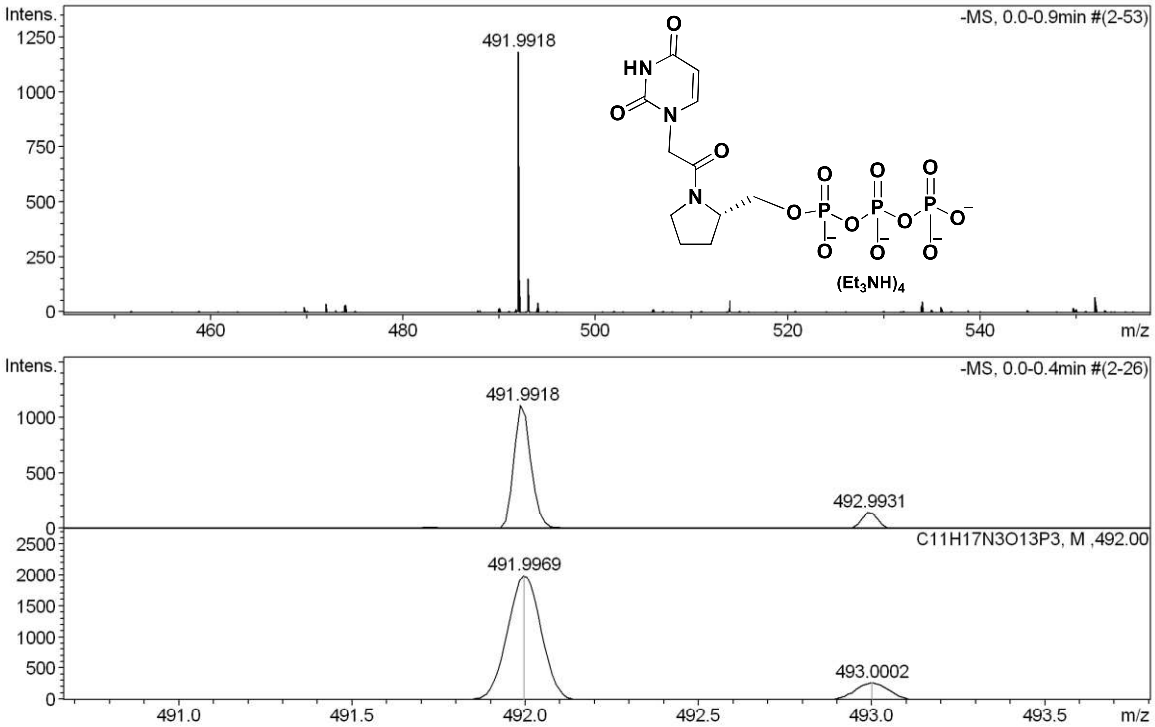


Figure A 20 HRMS of compound 6b

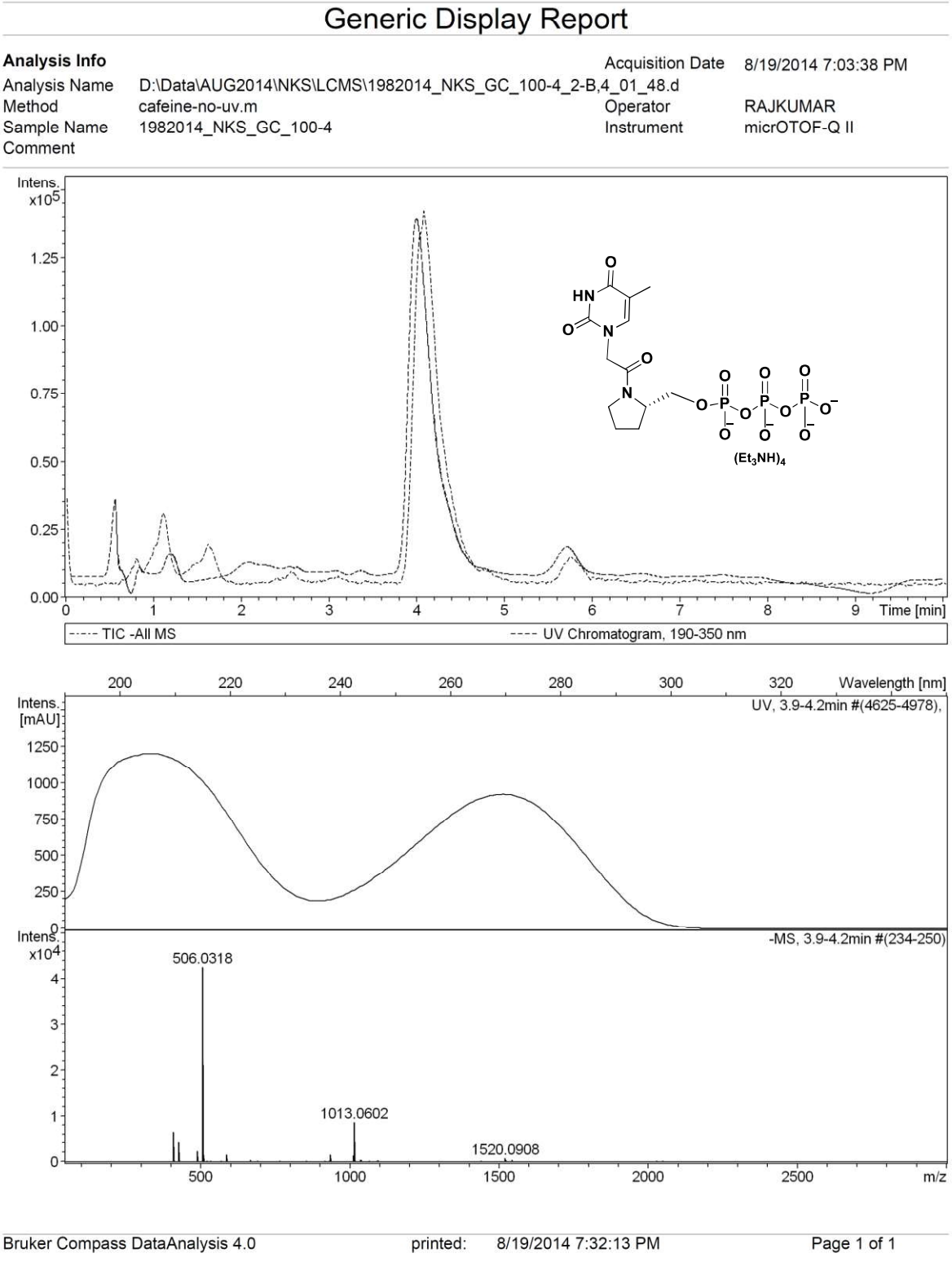


Figure A21 LC-MS of compound 6

Chapter 4

Synthesis, Photophysical and Biochemical Studies of Tropolone Modified 2'-deoxyuridine Triphosphates

Chapter 4

Synthesis, Photophysical and Biochemical Studies of Tropolone Modified 2'-deoxyuridine Triphosphates

Contents

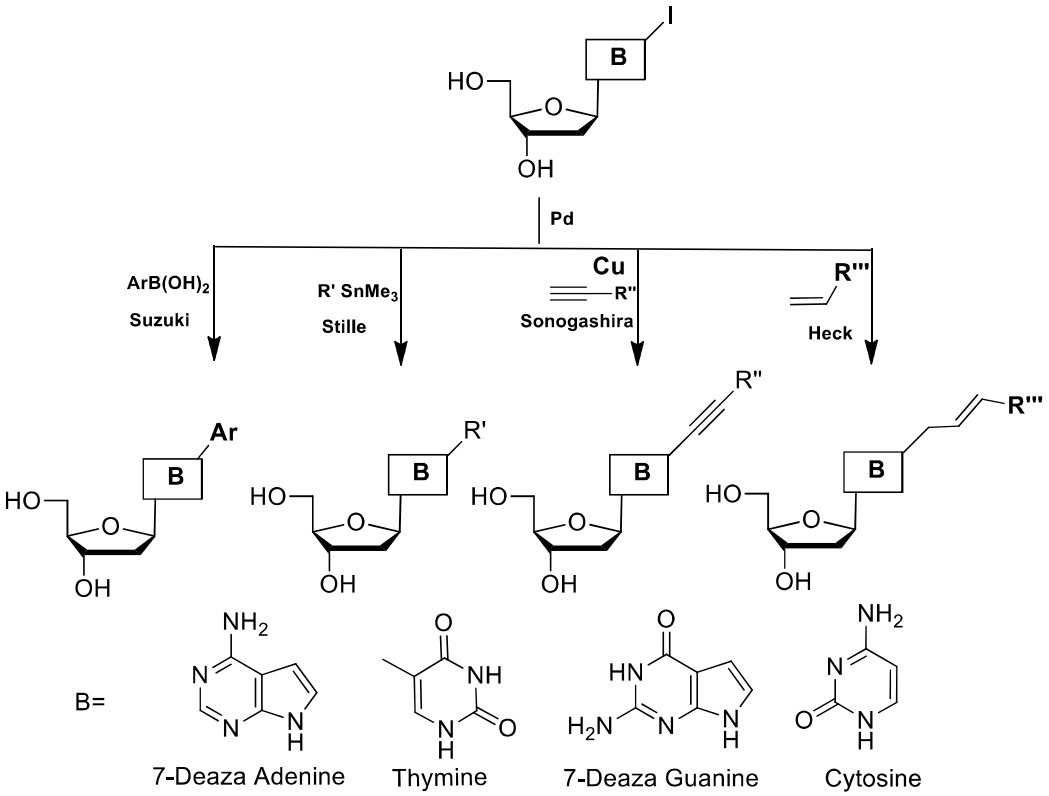
4.1 Introduction

DNA is genetic material of living systems and its biosynthesis comprises units of four dNTPs. The synthesis of labelled DNA are sought for tracking role of DNA in biological systems. Radio isotopes and dye fluorescent labelling are most commonly used methods. The radio labelled materials are biohazardous and have limited practical applications. Dyes and fluorescent labelling [1] are most encouraging however they have many other challenges such as solubilty and permeability into cells. To understand the biological process and to advance the chemical biology of nucleic acids, nucleosides and nucleotides with well-defined photo physical properties are have been developed.[2-4] Natural unmodified nucelosides are not fluorescent. Nucleoside/nucleotide analogues functionalized at C-5 position of pyrimidine/C-7position of 7 deaza purines with desired functional group with required properties have been synthesized and their photo-physical and biochemical properties have been studied.[5]

4.1.1 Synthesis of modified nucleoside/nucleotide analogues

Functionalization of nucleosides /nucleotide analogues at C-5 substituted pyrimidine and C-7 substituted 7-deaza purine analogues are commonly used for the synthesis of labelled nucleoside/nucleotide analogues. C-5 halo pyrimidines /7deaza C-7 halo purines are the commonly used precursors. Various functional groups such as aryl, heteroaryl, chromophore or fluorophore are attached by transition metal catalysed cross coupling reactions. Organo bornaes, organo stannanes, alkenes and alkynes are coupled to nucleoside halides by palladium catalysed cross coupling reactions (Scheme 4.1). [6-11]

Scheme 4. 1 Synthesis of base modified nucleoside analogues by cross coupling reactions



Pyrene and antroquinone based extended nucleoside/nucleotide analogues are synthesized and studied as fluorescent probes (Figure 4.1) .The extended and non-conjugated fluorophore are attached uridine analogues such as pyrene **1** is used for studying single base mismatch in duplexes.[12,13] A similar pyrene analogue **2** is environmental sensitive used to study change in the polarity of medium and also used to sense base mismatch.[14] Nucleoside **3** anthroquinone based nucleoside **4** are together used as molecular beacon where **3** is emitter and **4** is quencher .In close proximity 98% quenching of **3** is observed , on hybridization complementary sequence 49 times enhancement of c is observed and 23 fold enhancement is observed.[15,16]

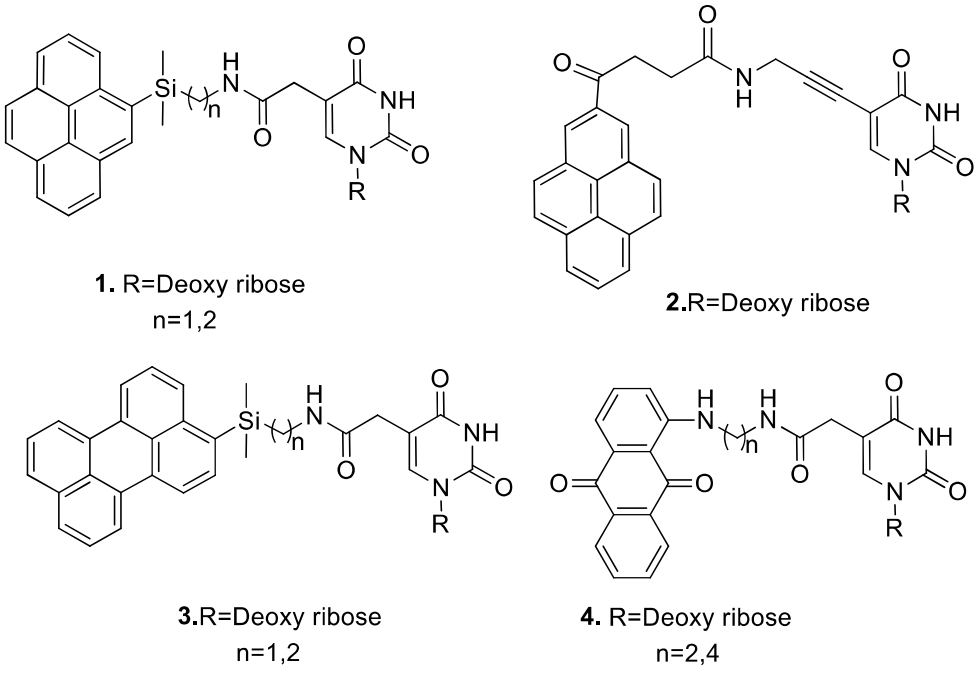


Figure 4. 1 Structure of pyrene, sillyl pyrene and anthraquinone attached 2'-deoxythymidine nucleoside

Conjugated fluorophores extending the π electronic system of uridine conjugated to the aromatic system of fluorophore have been synthesized and their photophysical properties studied. The deoxyuridine conjugated with cyanine 5 (Cy 5) and cyanine 3 (Cy 3) florophores (Figure 4. 2) synthesized and incorporated into DNA sequences to study the florescence properties of the labelled DNA oligo nucleotides. The fluorescence emission of locked cyanine analogue **7** is superior to unlocked analogues **5** and **6** due to prevention of photo induced cis-trans isomerization. Further and fluorescence quenching by dye-dye and dye-DNA interactions are prevented in locked analogue.[17,18]

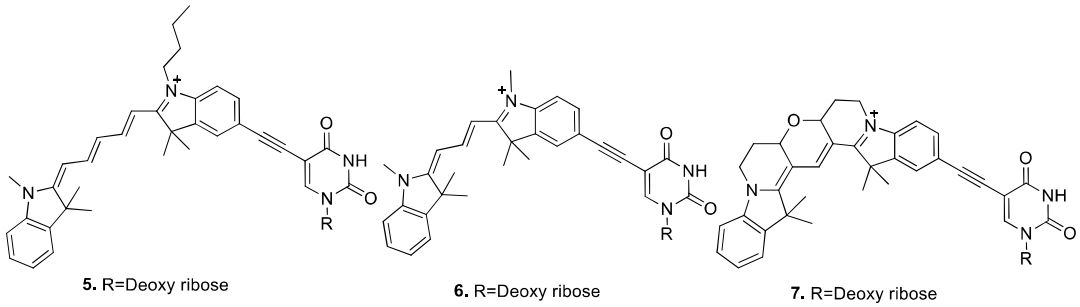


Figure 4. 2 Structure of Cy 5 and Cy 3 attached 2'-deoxythymidine nucleoside

2'-deoxyuridine analogues with, 9-fluorenone **8** di-benzofuran/dibenzothiophene **9** and 1-napthylenyl **10** moieties conjugated by ethynylbridge (Figure4.3) are incorporated into oligonucleotides and their fluorescence properties have been studied. Quantum yield of analogue **8** is low but shows the sensitivity change in polarity of the medium. Upon incorporation into DNA **8** displayed good emission sensitivity to detect nucleotide polymorphism with pyrimidines.[19] Compounds **9a** and **9b** alsoshown similar properties where fluorenone moiety has been replaced by dibenzofuran and dibenzo thiophene analogues.These compounds have been used to identifyflanking base and base mismatches.[20] Deoxyuridine conjugated 1-napthalenylethynyl or cyano group **10 a** and **10 b** provide interesting photophysical properties. Compound **10a** incorporated DNA sequences in consecutive or interstand neighbouring position show fluorescence quenching. The emission of DNA duplex sequence red shifted 85 nm but quantum yield is decresed when compound **10 a** and **10 b** are incorporated in adjacent position possibly due to exciplex formation by two different naphthalene units located in DNA major groove.[21]

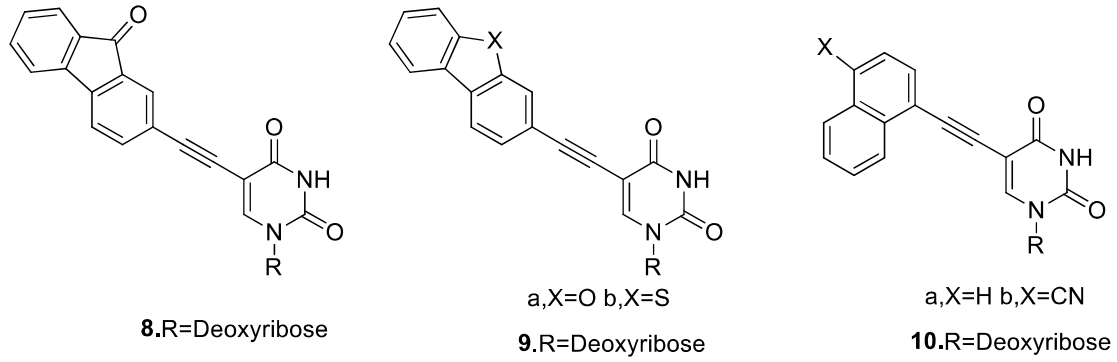


Figure 4. 3 Structure of Cy 5 and Cy 3 attached 2'-deoxythymidine nucleoside

Further a library of 2'deoxy uridine analogues with chromophores containing different substituents attached to the π system of nucleobase by ethynyl bridge **11a-c** or vinyl bridge **12 a-e** have been synthesized and incorporated into oligonucleotides as fluorescent probes[6] (Figure 4.4).The nucleosides **12** a-e displayed best properties of emission red shift upto 478 nm with a quantum yield of 12%.The nucleosides incorporated in DNA for detection of cyclin D1 mRNA in total cell RNA extract from cancerous human cells by change in emission intensity.[22]

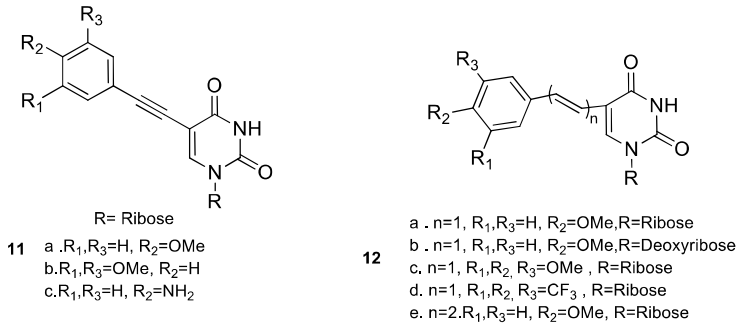


Figure 4. 4 Structure of ethynyl/vinyl bridged chromophore labelled nucleosides.

Click chemistry has been used to attach the fluorophores to the nucleosides (**13**) and nucleic acids. Azide- alkyne 3+2 cyclo addition catalysed copper (I) reagent is the most commonly used methods for click chemistry[23,24] (**13** Figure4.5). The method has the limitations to extend to use in vivo due to the cytotoxicity of

copper species. A copper free method has been developed to synthesize the conjugates by click chemistry using a strained aklyne which promte copper free alkyne-azide cycloaddition .This strategy has been used to synthesize cyanine conjugates deoxy uridine (**14& 16** Figure 4.5).[25,26] Same strategy also used to synthesize quinolinium –styryl-coumarin azide dye (**15** Figure 4.5)with far red emission (720 nm).[27]

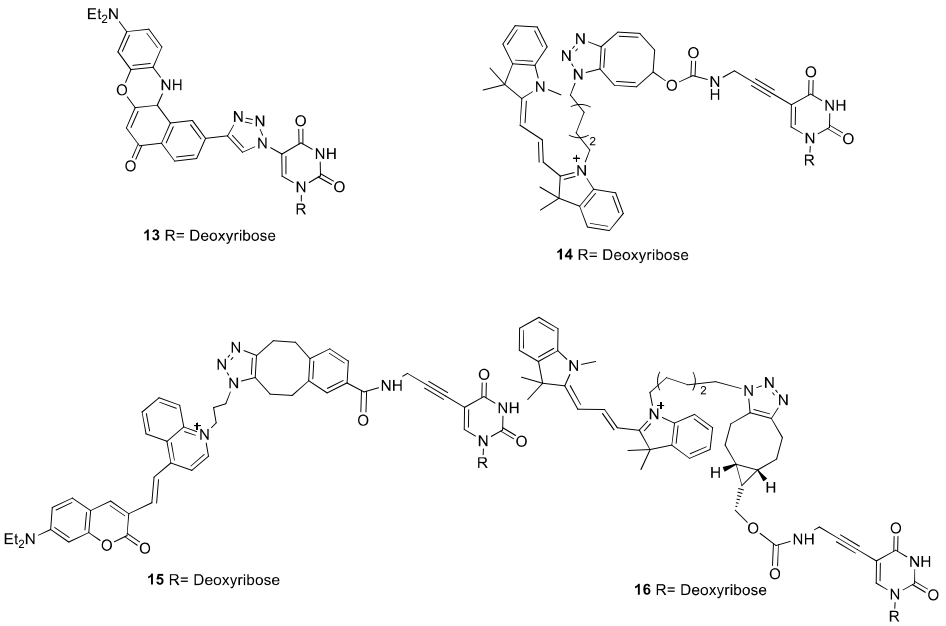


Figure 4. 5 Structure of non-conjugated extended fluorophores containing nucleoside analogues by synthesized by click chemistry

Five membered hetrocycle conjugated uridine/deoxy uridine analogues such as 5-furanyl/thiophenyl/selenophenyl/oxazolyl/thiazolyl conjugated nucleotides synthesized and incorporated into DNA/RNA by polymerase or by incorporation of modified phosphoramidates on solid phase synthesis. These 5-memeberd hetro cycle containing nucleoside/nucleic acid analogues (**17-22**)[28-33] used in various analytical studies such as sensing in environmental polarity changes and

identification of abasic mismatch site. However despite of the photophysical properties and analytical applications the quantum yield of these analogues were low. To enhance the quantum yield and to tune the absorption and emission properties to red shift iso-morphic 6-aza uridine nucleosides conjugated with 5-memebered hetero cycle at 5-position (**23**) have been synthesized and their photo physical properties were studied.[34] (Figure 4.6)

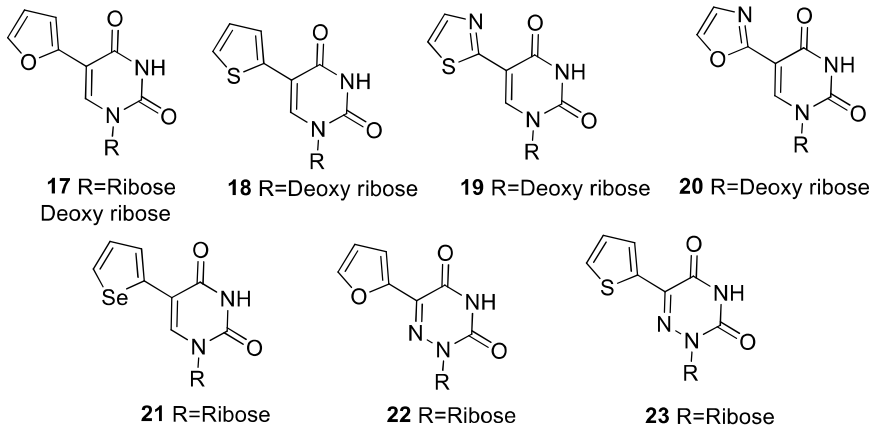
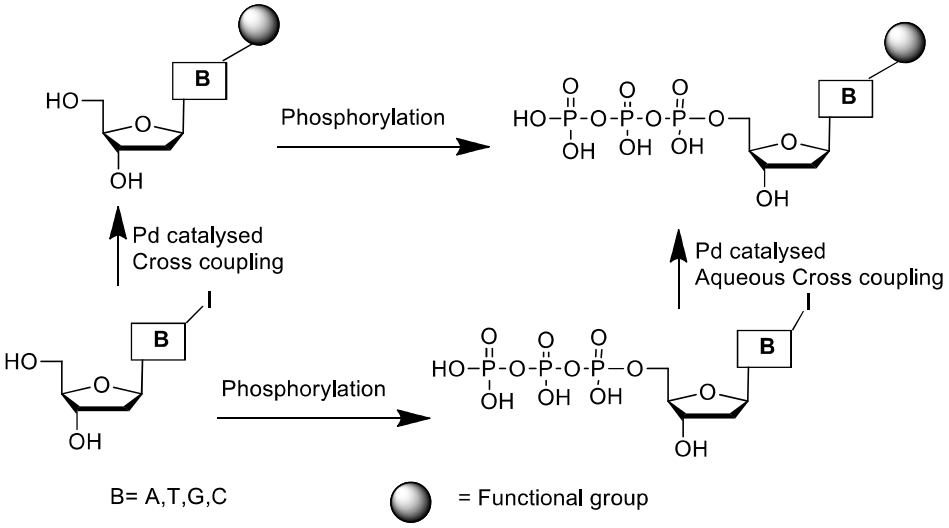


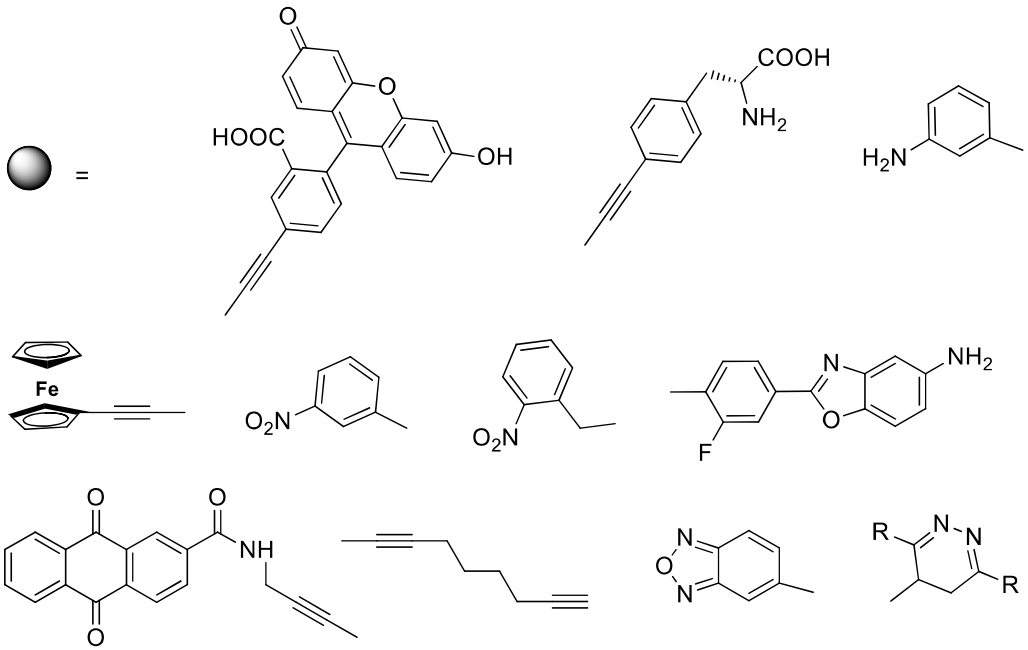
Figure 4. 6 Structure of 5-membered heterocycle conjugated uracil/6-aza uracil analogues

Nucleobase modified dNTPs are the substrates for the synthesis of modified nucleic acids .Nucleobase modified dNTPs can be synthesized by direct triphosphorylation of modified nucleosides or another way of making nucleobase modified dNTPs is to attach the functional group to the halogenated dNTPs by aqueous cross coupling reactions.[10] Recently chemists used this strategy to access the modified dNTPs in good yield. (Figure 7) Many nucleobase modified dNTPs have been developed by aqueous Suzuki coupling, Heck coupling and Sonoghashira coupling reactions. These modified nucleoside triphosphates incorporated into DNA by polymerase to synthesize flouroescent/redox/ spin

labeled DNA. (Figure 8) These modified dNTPs have been used in the synthesis of functionalized DNA primer extension reactions and polymerase chain reaction for various applications (Scheme 4.2).[35,36]

Scheme 4. 2 Synthesis of base modified nucleoside triphosphate analogues





4.1.2 Rational of the present work

Tropolone is a seven membered non-benzenoid aromatic compound. Tropolone is present in many natural products such as Colchicine, Puberulonic acid, Stipitatic acid and Thujaplicicn. Tropolone derived drugs are being used as antibacterial and antifungal agents. Chemical structure of tropolone (2 hydroxy-2,4,6 cycloheptatrien-1-one) conatins seven membered ring with carbonyl group adjacent to hydroxyl group. The carbonyl group of tropolone is polarized by getting partial negative charge on oxygen and partial positive charge on carbon. Due to the positive charge on the system (4n+2) π electrons are delocalized and stabilized the system by making it aromatic. The polarized oxygen of carbonyl group forms hydrogen bond with hydrogen of adjacent hydroxyl group making the system further stabilized. The hydrogen is sharing by two oxygen atoms makes the system to exist in two mobile tautomeric forms (Figure 4.7). Tropolone is a vinylog of acetic acid with superior acidity than aromatic phenols having a dissociation constant pKa = 6.8 more than phenols (pKa=10) but less than acetic acid (pKa=4.8).[37]

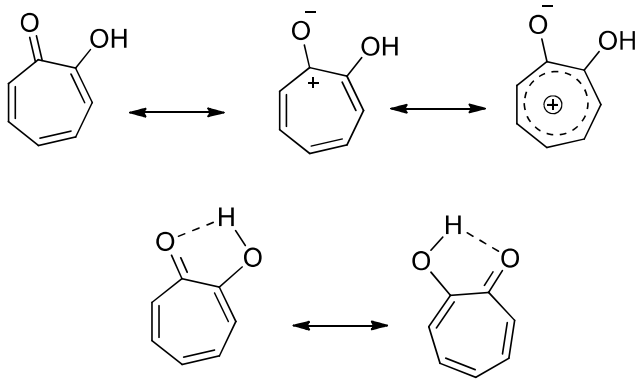


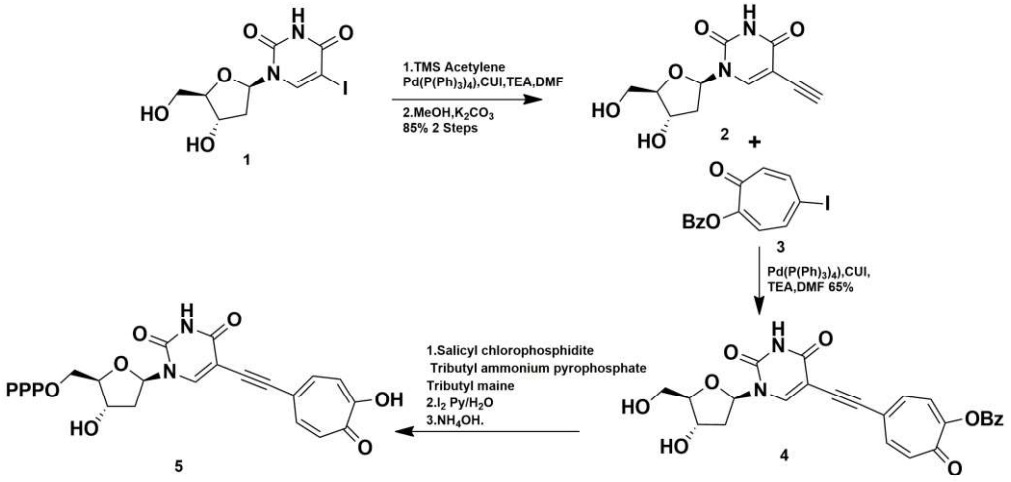
Figure 4.7 Tropolone its resonance and tautomeric structures

The photophysical properties of tropolone in condensed media have been studied.[38] Importantly the photophysical properties at different temperatures and different polarities suggest that tropolone is environmental sensitive fluorophore. Tropolone derived conjugated polymers are synthesized and their optical properties have been studied. [39] Recently boron –aminotroponimine complexes are synthesized and studied as fluorophores.[40] We hypothesized to conjugate tropolone moiety to nucleobase which could be a fluorescent analogue. The resulting fluorescent triphosphate analogue can be incorporate into DNA by polymerase to synthesize fluorescently labelled DNA. We hypothesize to conjugate tropolone moiety to 2'-deoxy uridine by ethynylbridge or triazolylbridge which may act as fluorescent building blocks. These nucleoside analogues can phosphorylate to triphosphate which could incorporate in the DNA by polymerase. We have also designed an acid labile tropolonamide conjugated 2'-deoxyuridine triphosphate for the reversible labelling of DNA.

4.2 Results and discussion

4.2.1 Synthesis of 5-(ethynyl tropolonyl) 2'-deoxyuridine triphosphate

Scheme 4. 3 Synthesis of 5-(ethynyltropolonyl) 2'-deoxyuridine triphosphate



The synthesis of 5-(ethynyl tropolonyl) 2'-deoxy uridine triphosphate started from commercially available 2'-deoxy 5-iodo uridine triphosphate (**1**). Trimethylsilyl acetylene on Sonoghashira coupling with 2'-deoxy 5-iodo uridine followed by deprotection of trimethylsilyl group obtained 5-ethynyl 2'-deoxy uridine (**2**).2 benzoyloxy 5-Iodo tropone (**3**) was synthesized starting from tropolone by following literature report which involves the nitration, reduction of nitro to amine, diazatization of amine followed by treatment of tropolone diazonium salt with potassium iodide.[41] The hydroxyl group of 5-Iodo tropolone was protected with benzoyl group. 5-ethynyl 2'-deoxy uridine was treated with benzoyl protected 5-iodo tropolone under Sonoghashira conditions to obtain 5-(ethynyl benzoyl tropolonyl) 2'-deoxy uridine (**4**) which was phosphorylated followed by removal of benzoyl group to obtain the desired 5-(ethynyl tropolonyl) 2'-deoxy uridine triphosphate (**5**). The synthetic steps are illustrated in scheme 4.3

4.2.2 Photo-physical properties of 5-(ethynyl tropolonyl) 2'-deoxyuridine triphosphate

4.2.3 Absorption spectra

The tropolone moiety attached to the uridine has carbonyl and hydroxyl group on adjacent carbons. The protonation and deprotonation of the system may have some effect on the absorption and emission properties .We wanted to investigate the changes of absorption and emission properties upon changing the pH of the medium. We prepared the samples in 1x PBS buffer of pH ranging from 4-9(Figure 4.8).

The absorption spectra of pH 4 showed absorption maxima at 382 nm, at pH 5 absorption maxima shifted to 384 nm, suddenly it shifted to 399 nm at pH 6, further shifted to 400 nm at pH 7 and remains almost same at 401 nm from pH 7 to 9.An increase in intensity was observed with increasing the pH value from 4-9.

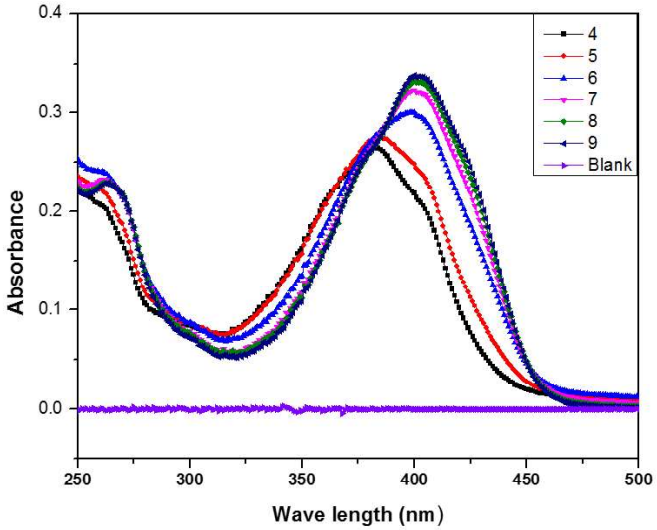


Figure 4. 8 pH dependent absorption spectra of 5-(ethynyl tropolonyl) 2'-deoxy uridine triphosphate in PBS buffer of different pH

4.2.4 Emission spectra

We recorded the pH dependent fluorescence spectra of 5-(ethynyl tropolonyl) 2'-deoxy uridine triphosphate in 1x PBS buffer from pH 4-9. The emission intensity at 518 nm is maximum at pH 4 and constantly decreased with an increase in pH from 4 to 9. pH dependence absorption and emission of compound 5 described in figure 4.10.

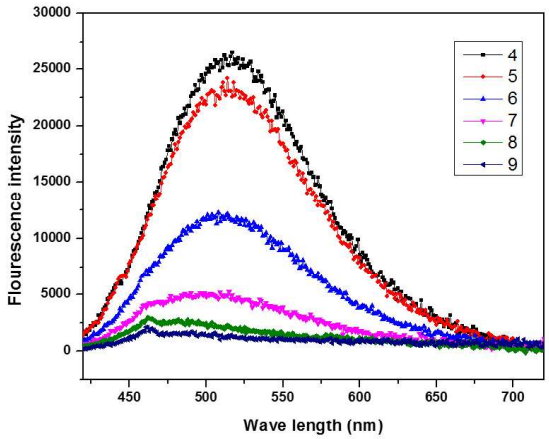


Figure 4. 9 pH dependent emission spectra of 5-(ethynyl tropolonyl) 2'-deoxy uridine triphosphate in PBS buffer of different pH.

With increasing the pH of the medium the absorption spectra shifted to red shift while with increasing the pH the emission intensity of the compound 5 decresed.

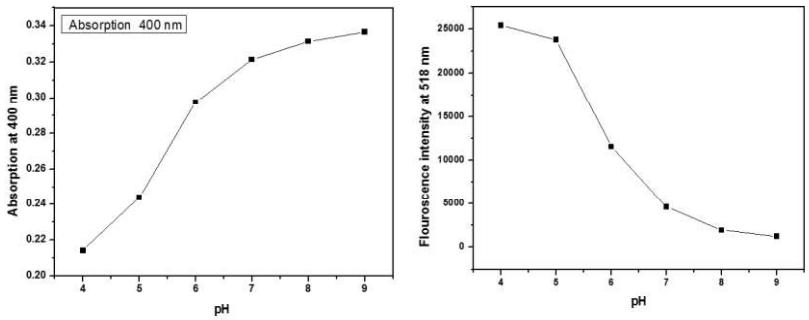


Figure 4. 10 pH dependent absorption and emission of compound **5**

4.2.5 Biochemical evaluation of 5-(ethynyl tropolonyl) 2'-deoxyuridine triphosphate

We performed the primer extension reactions of 5-(ethynyl tropolonyl) 2'-deoxy uridine triphosphate **(5)** with different DNA polymerases using FAM labelled primer The following DNA sequence are used biochemical evaluations

P1: 5'-TGTAAAACGACGGCCAGT-3'

T1:3'-ACATTTTGCTGCCGGTCA**A***GTCGAGGCAT 5'

FAM P1: FAM-5'-TGTAAAACGACGGCCAGT-3'

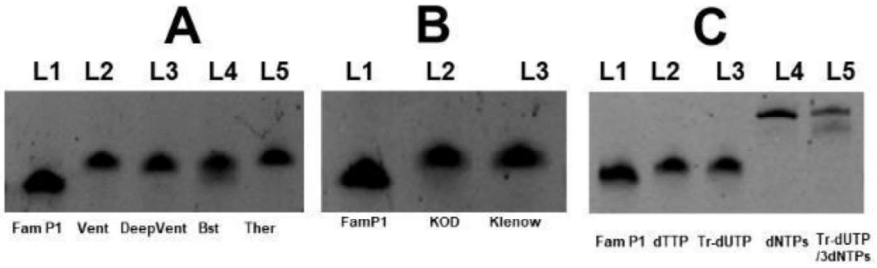


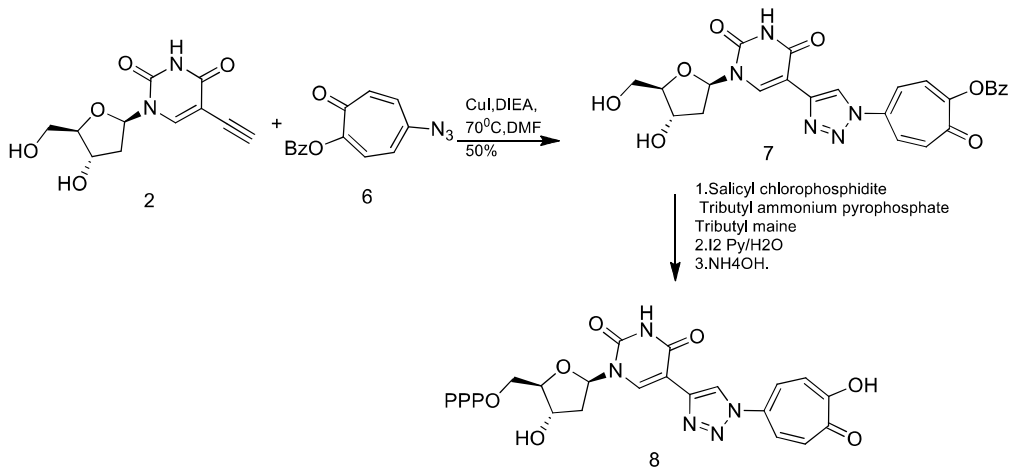
Figure 4. 11 PAGE Analysis of primer extension reactions of Tr-dUTP with different DNA polymerases. (A) FAM P1(L1), Vent exo_(L2), Deep Vent exo_ (L3), Bst (L4), Therminator (L5),(B) FAM P1 (L1), KOD-Dash(L2), Klenow (L3) , (C) FAM P1(L1), dTTP (L2), tr-dUTP(L3), dNTPs (L4) and tr-dUTP and other three dNTPs (L5) with DNA polymerase Therminator.

(**FAM P1).** We performed the primer extension reaction with following commercially available enzymes: *Vent, DeepVent, Bst, Therminator, KOD, and Klenov*. For control experiment we used known substrates dNTP (dTTP/dATP/dGTP/dCTP) in primer extension reaction. The primer extension reactions were analysed by gel electrophoresis and the results are depicted in Figure **4.11** The gel-shifts of FAM-primer (**P1**) were noticed after primer extension reaction with **5** in presence of DNA polymerases vent/DeepVent/Bst/Therminator/KOD/ Klenow (Figure 4.11 A & 2B). For control studies, the similar results were noticed with natural substrate dTTP with primer extension experiments (Figure 4.11 C). For the investigation of full length extension of primer, a mixture of **5** and other 3 dNTPs (dATP/dGTP/dCTP) were employed for primer extension reaction and compared with control experiment using all four dNTPs (dATP/dTTP/dGTP/dCTP) in presence of therminator DNA polymerse The gel-shift image of those experiments are depicted in Figure 4.11 C which supports the full length extension of primer as like control. These results strongly support the enzymatic incorporation of tr-dUTP into DNA. Hence tr-dUTP is substrate of above mentioned DNA ploymerases as like dTTP.

4.2.6 Synthesis and bio-chemical evaluation of 5-(triazolyl tropolonyl) 2'-deoxy uridine triphosphate

The synthesis of 5-(triazolyl tropolonyl) 2'-deoxy uridine triphosphate is depicted in scheme4.4. Ethynylation of 5-iodo 2'-deoxy uridine was performed by treating trimethyl sillyl acetylene with 5-iodo 2'-deoxy uridine under Sonoghashira conditions followed by deprotection of trimethylsilyl group with potassium carbonate. 5-Azido tropolone **(6)** was synthesized form tropolone by following similar procedure. The hydroxyl group was protected as benzoate. Benzoyl protected 5-azido tropolone (**6**)

Scheme 4. 4 Synthesis of 5-(triazolyltropolonyl) 2'-deoxy uridine triphosphate



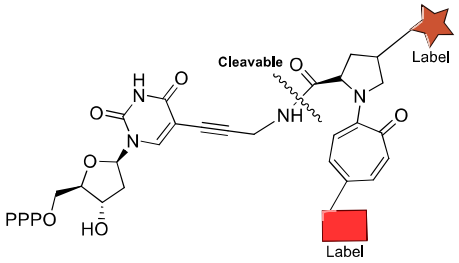
was treated with 5-ethynyl 2-deoxy uridine using copper (I) iodide to obtain cyclized product. The cyclized product was phosphorylated and benzoyl group was deprotected to obtain the desired 5-(triazolyl tropolonyl) 2'-deoxy uridine triphosphate (**8**). The triphosphate was tested with therminator DNA polymerase by primer extension reaction using primer P1 and template T1 .The triphosphate was incorporated into DNA by Therminator DNA polymerase. (Appendix Figure A22)

4.2.7 Rational design of 5-(tropoloneprolamide propargylamide) 2'-deoxy uridine triphosphate

Recently tropolone bearing aminoethyl glcycinyl moiety has shown remarkable properties under mild acidic conditions.[42] Tropolonamide containing peptides are cleavable under mild acidic conditions .We have planned to synthesize tropolonamide peptide containing 2'-deoxy uridine triphosphate which can be incorporate into DNA by polymerase. The triphosphate analogue can be labelled at amino acid residue or tropolone moiety. After incorporating into DNA the label can be removed under mild acidic conditions. In the process of development of reversible cleavable label like a fluorophore/peptide containing dNTP which can

be a substrate of a polymerase we started to synthesize a simple cleavable peptide containing dNTP as substrate of DNA polymerase (Scheme4.5).

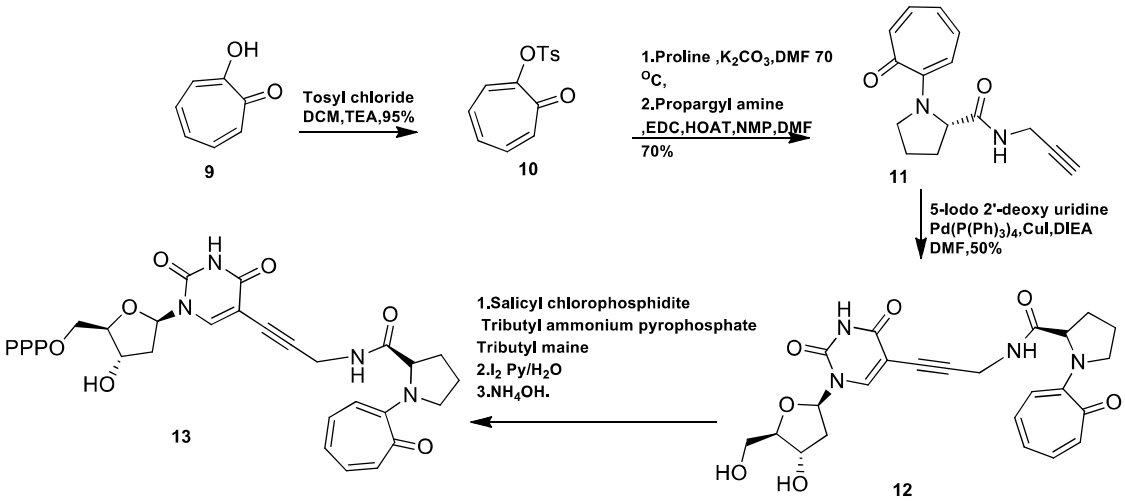
Scheme 4.5 Design of an acid cleavable troponamide containing 2'-deoxy uridine triphosphate



4.2.8 Synthesis and polymerase incorporation of 5-(propargyl amido N-tropone prolamide) 2'-deoxyuridine triphosphate into DNA

We have deigned a cleavable peptide containing an alkyne which can be couple to nucleoside by Sonoghashira cross coupling reactions. The synthesis of cleavable

Scheme 4.6 Synthesis of 5-(propargyl amido N-tropone prolamide) 2'-deoxy uridine triphosphate



peptide conating nucleotide analogue depicted in scheme 4.6. Tosyl topolone (**10**) synthesized from tropolne (**9**) by treating tropolone with tosyl chloride .Tosyl

tropolone treatedwith proline in DMF using potassium carbonate and without further purification directly treated with propargylamine EDC.HCl, HOAT and NMP in DMF to obtain propargylamide containing tropolone prolamide (**11**)which was coupled to 5-iodo 2'-deoxy uridine to obtain cleavable peptide containing 2'-deoxy uridine to obtaine nucleoside **12** which was phosphorylated to obtain its triphosphate (**13**). The triphosphate analogue was incorporated into DNA with Thermiantor DNA polymerase, primer P1 and template T1 by primer extension reaction which was confirmed by LC-MS experiments. (Appendix Figure A33)

4.3 Conclusion

In conclusion we have successfully synthesized and studied the photophysical and biochemical properties of tropolone derived 2'-deoxy uridine triphosphate analogues. All these analogues are the substrates of DNA polymerase compound **5** is pH responsive emissive compound. The emission of compound **5** depends on the pH of the medium so this substrate can be used to synthesize pH sensitive oligonucleotide by polymerase reaction. Compound **13** is a cleavable peptide containing nucleoside triphosphate analogue. This compound can be used to synthesize oligonucleotides with reversible cleavable label like a fluorophore/peptide.

4.4 Experimental section

General methods and materials: All solvents and chemicals purchased from commercial sources and used directly without further purification otherwise mentioned. DMF and DCM distilled over CaH_2 and stored on 4 Å molecular sieves .Pyridine distilled over anhydrous KOH and stored over CaH_2. NMR spectra recorded on Bruker 700 MHz, ^{31} P NMR recorded on Jeol 400 MHz. ESI-MS experiments performed on Bruker Micro-Tof Q-II .Oligo nucleotides purchased from IDT. All the

enzymes purchased from New England Biolabs. UV-Vissible and emission spectra recorded in 1X PBS buffer of different pH.

5-ethynyl-1-((2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2yl)pyrimidine-2,4(1H,3H)-dione (**2**): 5-Iodo 2'-deoxy Uridine (1.0 g, 2.82 mmol) dissolved in dry DMF (10 ml), trimethyl silyl acetylene (1.6 ml, 11.29 mmol) Tetrakis (triphenylphosphine) palladium (0.326 g, 0.028 mmol), copper (I) iodide (0.107 g, 0.564 mmol) and triethylamine (0.787 ml, 5.64 mmol) were added and stirred under argon atmosphere over night at room temperature. The solvents were removed under reduced pressure and the reaction mixture passed through silica column (using methanol and chloroform) to obtain a beige solid. The solid dissolved in dry methanol (10 ml) potassium carbonate was added and stirred overnight under argon atmosphere. The reaction mixture concentrated and purified by column chromatography on silica gel using methanol and chloroform as solvents to obtain 0.533g of 5-Ethynyl 2'-deoxy uridine as white solid in 75% yield. TLC 10% MeOH/DCM R_f =0.37 ^{1}H NMR (700 MHz, DMSO) δ ppm 2.19 – 2.08 (m, 2H), 3.59 – 3.53 (m, 1H), 3.67 – 3.59 (m, 1H), 3.79 (dd, *J* = 6.5, 3.3 Hz, 1H), 4.10 (s, 1H), 3.90 (s, 1H), 4.23 (dt, *J* = 8.6, 4.4 Hz, 1H), 5.13 (t, *J* = 4.9 Hz, 1H), 5.24 (d, *J* = 4.3 Hz, 1H), 6.10 (t, *J* = 6.6 Hz, 1H), 8.29 (s, 1H), 11.62 (s, 1H), .(^{13}C NMR , 175 MHz, DMSO –D_6) δ ppm 40.33, 60.85, 70.18,76.38, 83.58, 84.95,87.79, 97.68, 144.67,149.53, 161.81. HRMS ESI-Tof Calcd for (C_{11} $H_{12}N_2O_5$+Na) 27.0638, Found 275.0646.

4-iodo-7-oxocyclohepta-1,3,5-trien-1-yl benzoate (**3**): 5-Iodo tropolone synthesized starting from tropolone by following reported procedure.[41] 5-Iodo tropolone (1.0 g 4.0 mmol) dissolved in dry dichloromethane (10 ml) triethylamine (1.12 ml 8.09 mmol)

was added and stirred at room temperature under nitrogen atmosphere for 10 minutes. Benzoyl chloride (0.702 ml, 6.0 mmol) was added and stirred the reaction at room temperature for 1 hour. Dichloromethane (50 ml) added to the reaction and extracted with water. The organic layer washed with sodium bicarbonate and brine solutions followed by drying on anhydrous sodium sulphate and concentrated to obtain 1.35 g of 5-Iodo 2 benzoyloxy tropone as white solid in 95% yield.TLC 20% EtOAc/Hexane R_f =0.37 ^{1}H NMR (700 MHz, $CDCl_3$) δ ppm 6.86 (d, J = 11.3 Hz, 2H), 7.18 (d, J = 11.4 Hz, 2H), 7.49 (ddd, J = 22.1, 11.2, 5.1 Hz, 4H), 7.64 – 7.58 (m, 2H), 7.68 (d, J = 11.3 Hz, 2H), 8.09 (d, J = 8.0 Hz, 2H), 8.16 (dd, J = 7.5, 5.2 Hz, 3H), (^{13}C NMR , 175 MHz, $CDCl_3$ δ) ppm 103.64, 128.60, 128.63, 128.73, 129.46, 130.3, 130.65, 133.82, 134.07, 139.03, 141.1, 145.99, 163.8, 171.27. HRMS ESI-Tof Calcd for ($C_{14}H_9O_3I$+Na) 374.9489, found 374.9487

4-((1-((2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)ethynyl)-7-oxocyclohepta-1,3,5-trien-1-yl benzoate **(4):** 5-Ethynyl 2'-deoxy uridine (0.2 g, 0.793 mmol) , 5-Iodo 2benzoyloxy tropone (0.335g, 0.952 mmol) , Tetrakis (triphenylphosphine) palladium (0.0916 g, 0.0793 mmol), copper (I) iodide (0.03 g, 0.158 mmol) and triethylamine (0.221 ml, 1.58 mmol) were dissolved in dry DMF (5 ml) and stirred overnight under argon atmosphere. The solvents were evapourated under reduced pressure and the reaction mixture purified on silicagel chromatography by chloroform and methanol to obtain 0.245 g of yellow solid in 65 % yield.TLC 10% MeOH/$CHCl_3$ R_f =0.43 ^{1}H NMR (700 MHz, DMSO) δ ppm 2.24 – 2.10 (m, 2H), 3.61 (d, J = 11.8 Hz, 1H), 3.68 (d, J = 11.8 Hz, 1H), 3.83 (q, J = 3.4 Hz, 1H), 4.27 (s, 1H), 5.21 (s, 1H), 5.28 (s, 1H), 6.12 (t, J = 6.4 Hz, 1H), 7.42 (d, J = 9.9 Hz, 4H), 7.61 (t, J = 7.8 Hz, 2H), 7.77 (t, J = 7.5 Hz,

1H), 8.08 (d, *J* = 7.1 Hz, 2H), 8.53 (s, 1H), 11.84 (s, 1H), (^{13}C NMR , 175 MHz, $CDCl_3$) δ ppm 40.33, 60.7, 69.72,85.17, 87.67, 87.86, 93.55, 97.16, 128.29, 128.61, 129.08, 129.29, 129.98, 132.89, 134.34, 145.46, 149.37, 161.22,163.31, HRMS ESI-Tof Calcd for ($C_{25}H_{20}N_2O_8$+Na) 499.1112 found 499.1111.

((2R,3S,5R)-3-hydroxy-5-(5-((4-hydroxy-5-oxocyclohepta-1,3,6-trien-1-yl)ethynyl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate **(5)** : The phosphorylation reaction performed by following reported procedure.[43] Compound **4** (0.05 g, 0.105 mmol) was dissolved in anhydrous pyridine and evapourated under reduced pressure and dried under high vacuum over P_2O_5 for one hour. Salicyl chlorophosphidite (0.042 g, 0.210 mmol) and Tributyl ammonium triphosphate (0.115 g, 0.210 mmol) were dried under high vacuum over P_2O_5 for one hour in two separate round bottom flasks. Salicyl chlorophosphidite was dissolved in dry DMF (0.5 ml) and stirred under argon atmosphere. Tributylammonium pyrophosphate was dissolved in dry DMF (0.5 ml) and tributyl amine (0.5 ml) was added to this mixture and this solution was transferred to salicylchlorophosphidite soltion and stirred the reaction mixture under argon atmosphere for 30 minutes. This reaction mixture was added to the solution of nucleoside (**4**) and stirred for 2 hours at room temperature. 3% iodine solution (in 9:1, pyridine: water) was added to the reaction dropwise until the brown colour remained. Ammonia solution (10 ml, 28% aqueous) was added to the reaction mixture and stirred overnight. The reaction mixture lyophilized purified by DEAE sephadex column .The fractions containing compound were identified by UV and mass spectrometry and lyophilized followed by HPLC purification to get pure compound as yellow solid. ^{31}P NMR δ ppm (162 MHz, D_2O) -21.46 (1P, br,m) ,-10-75 (1P, br ,m),-0.25 (1P, br ,m). (^{1}HNMR , 700 MHz,

D_2O) δ ppm ^{1}H NMR (700 MHz, D_2O) δ ppm 2.53 – 2.33 (m, 1H), 2.68 (d, *J* = 20.6 Hz, 1H), 3.12 – 2.96 (m, 2H), 3.31 (dd, *J* = 17.3, 8.1 Hz, 1H), 3.90 (dd, *J* = 18.2, 9.6 Hz, 1H), 4.45 – 4.17 (m, 2H), 6.46 – 6.24 (m, 2H), 7.45 – 7.10 (m, 2H), 7.83 – 7.59 (m, 1H), 8.08 – 7.86 (m, 1H), 8.36 – 8.20 (m, 1H), 8.91 (s, 1H). HRMS ESI-Tof Calcd for ($C_{18}H_{17}N_2O_{16}P_3$ M-2H) 609.9785, Found 609.9544.

4-azido-7-oxocyclohepta-1,3,5-trien-1-yl benzoate **(6):** 5-amino tropolone synthesized starting from tropolone by following reported literature procedure. 5-amino tropolone (0.5 g, 3.64 mmol), was dissolved in 6.0 ml conc HCl and 10 ml distilled water added to the reaction mixture and the resulting solution stirred at -20^{0}C. Sodium nitrate (0.372 g, 4.3 mmol) dissolved in 9.0 ml distilled water was added slowly in 0.5 h and stirred 20 min. Sodium azide (0.284 g, 4.3 mmol) dissolved in 9.0 ml distilled water was added slowly over 0.5 h. The reaction mixture was stirred at 55^{0}C for one hour. The reaction mixture was cooled to room temperature and and extracted with dichloromethane .The aqueous layer was neutralized with sodium hydroxide solution and extracted with dichloromethane .The combined organic layers were washed with brine solution and dried with anhydrous sodium sulfate and concentrated and purified by silica gel column chromatography to obtain 0.39 g of title compound as brown colour compound in 40% yield. TLC DCM R_f =0.5 ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 6.81 (s, 2H), 7.28 (t, *J* = 7.2 Hz, 2H), 7.48 (d, *J* = 7.6 Hz, 2H), 7.63 (t, *J* = 8.7 Hz, 1H), 8.13 (d, *J* = 30.1, 7.1 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 128.58, 128.69, 128.84, 130.28, 130.62, 133.75, 133.96, 145.61, 164.12. HRMS ESI-Tof Calcd for ($C_{14}H_9N3O3$+Na) 290.0536, Found 290.0531.

4-(4-(1-((2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-1H-1,2,3-triazol-1-yl)-7-oxocyclohepta-1,3,5-trien-1-yl benzoate **(7):** 5-ethynyl 2-deoxy uridine (0.2 g, 0.793 mmol), 5-azido 2-benzoyloxy tropolone (0.211 g, 0.793 mmol), coper iodide (0.301 g, 1.58mmol), didiosopropylethylamine (0.275 ml, 1.58 mmol) disoolved in anhydrous dimethylformamide (5 ml) and stirred at 70^0C for 24 h under nitrogen atmosphere. The reaction mixture concentrated and purified by silicgel column chromatography to yield 0.205 g of yellow colour solid in 50% yield. 10% MeOH/DCM R_f =0.32 ^{1}H NMR (400 MHz, DMSO) δ ppm 2.22 (s, 2H), 3.63 (s, 2H), 3.88 (s, 1H), 4.30 (s, 1H), 5.09 (s, 1H), 5.32 (s, 1H), 6.25 (s, 1H), 7.70 (dd, *J* = 54.6, 6.7 Hz, 5H), 8.07 (t, *J* = 22.9 Hz, 4H), 8.73 (s, 1H), 8.99 (s, 1H), 11.80 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 61.33, 70.60, 84.98, 87.73, 104.29, 120.77, 128.20, 129.05, 129.98, 134.34, 137.19, 139.67, 140.56, 149.62, 161.04, 163.27. HRMS ESI-Tof Calcd for for ($C_{25}H_{21}N_5O_8$+Na) 542.1282, Found 542.12.60.

((2R,3S,5R)-3-hydroxy-5-(5-(1-(4-hydroxy-5-oxocyclohepta-1,3,6-trien-1-yl)-1H-1,2,3-triazol-4-yl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate **(8):** Phosphorylation and benzoyl deprotection of 7 was performed as like 5 to obtain title compound as yellow solid.HRMS ESI-TOF Calcd For Calcd For (M-H)C_{24} $H_{27}N_4O_{16}P_3$ 720.0629 Found 720.0632.

7-oxocyclohepta-1,3,5-trien-1-yl 4-methylbenzenesulfonate **(10):** Tropolone (5.0 g, 40.9 mmol) was dissolved in dichloromethane (50 ml) and stirred at room temperature for 10 min under nitrogen atmosphere .Tosyl chloride (9.37 g, 49.1 mmol) was added to the reaction mixture and stirred 10 min. Triethylamine (6.85 ml, 49.1 mmol) was added dropwise and the reaction stirred overnight at room temperature under nitrogen

atmosphere. The reaction mixture was diluted with 50 ml dichloromethane and extracted with saturated sodium bicarbonate solution. The organic layer was washed with brine solution and dried on anhydrous sodium sulphate and concentrated. The resulting solid was recrystallized with hexane and dichloromethane to obtain 10.2 g of pale brown solid in 90 % yield.TLC 30%EtOAc/DCM *Rf* = 0.7 ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 2.45 (s, 3H), 6.98 (t, *J* = 10.1 Hz, 1H), 7.06 – 7.16 (m, 2H), 7.18 – 7.24 (m, 1H), 7.34 (d, *J* = 8.2 Hz, 2H), 7.43 (d, *J* = 9.3 Hz, 1H), 7.91 (d, *J* = 8.3 Hz, 2H), ^{13}C NMR (101 MHz, $CDCl_3$) δ 21.82, 76.84, 77.16, 77.48, 128.63, 129.70, 130.05, 130.90, 133.44, 134.78, 136.49, 141.24, 145.61, 155.18, 179.48. HRMS, ESI-Tof Calcd for ($C_{14}H_{12}SO_4$) 277.0529, Found 277.0525

(S)-1-(7-oxocyclohepta-1,3,5-trien-1-yl)-N-(prop-2-yn-1-yl)pyrrolidine-2-carboxamide **(11):** Tosyl torpolone (.5 g, 1.80 mmol), L-proline(0.207 g, 1.80 mmol), dissolved in anhydrous dimethyl formamide (5 ml), potassium carbonate (0.498 g, 3.6 mmol) added and the reaction mixture stirred overnight at70^{0}C under nitrogen atmosphere. The reaction mixture filtered and concentrated to obtain a syrupy residue. EDC.HCl (0.519 g, 2.7 mmol), HOAT (0.368 g, 2.7 mmol), NMM (0.396 ml, 3.6 mmol) and propargyl amine (0.115 ml, 1.8 mmol), added to the residue and dissolved in anhydrous dimethyl formamide and stirred overnight at room temperature under nitrogen atmosphere. The reaction mixture concentatred and purified by silicagel column chromatography using methanol and dichloromethane to obtain yellow colour syrupy compound in 70% yield.TLC 50% EtOAc/DCM *Rf* = 0.47 ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm ^{1}H 1.85-2.28 (m, 6H), 3.31 – 3.45 (m, 1H), 3.76 – 3.88 (m, 1H), 4.00 (m, br, 2H), 4.92 (dd, *J* = 7.6, 4.8 Hz, 1H), 6.46 (d, *J* = 10.5 Hz, 1H), 6.60 (t, *J* = 9.2 Hz, 2H),6.92 (d, *J* = 11.8 Hz, 1H), 7.08 (dt, *J* = 23.4, 9.4 Hz,

2H).^{13}CNMR(101MHz, $CDCl_3$) δ 23.61, 29.24, 31.45, 52.02, 64.07, 71.52, 76.84, 77.16, 77.48, 79.70, 114.2, 123.77, 132.82, 134.6, 136.17, 155.96,1 72.96, 180.84. HRMS, ESI-TOF Calcd For (C_{15} $H_{16}N_2O_2$) 279.1104 found 279.1124.

(S)-N-(3-(1-((2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)prop-2-yn-1-yl)-1-(7-oxocyclohepta-1,3,5-trien-1-yl)pyrrolidine-2-carboxamide **(12):** N-Tropone proline propargylamide (**11**) (0.2 g, 0.781 mmol), 5-iodo 2'-deoxy uridine (0.275 g, 0.781 mmol), Tetrakis(triphenylphosphine) palladium (0) (0.09g, 0.078 mmol), coper iodide (0.029 g, 0.156 mmol) and trimethylamine(0.217 ml, 1.56 mmol) were dissolved in dry dimethyl formamide and stirred under nitrogen atmosphere overnight at room temperature. The reaction mixture concentrated and purified by silicagel column chromatography to obtain 0.226 g of yellow solid in 60% yield. TLC 5% MeOH/DCM *Rf* = 0.33 ^{1}H NMR (400 MHz, MeOD) δ ppm 1.91-2.11 (m, 3H), 2.13-2.37 (m, 3H), 3.05 (q, *J* = 7.1 Hz, 1H), 3.22 (q, *J* = 7.3 Hz, 1H), 3.47-3.60 (m, 1H), 3.75 (dt, *J* = 23.4, 11.5 Hz, 3H), 3.94 (s, 1H), 4.13 (s, 2H), 4.40 (s, 1H), 4.61 (s, 1H), 5.08 (d, *J* = 7.4 Hz, 1H), 6.23 (d, *J* = 6.2 Hz, 1H), 6.68 (dd, *J* = 20.3, 10.2 Hz, 2H), 6.85 (d, *J* = 11.7 Hz, 1H), 7.19 (dd, *J* = 20.4, 10.2 Hz, 2H), 8.33 (d, *J* = 7.4 Hz, 1H). ^{13}C NMR (101 MHz, MeOD) δ ppm 10.16, 12.47, 24.83, 31.33, 33.91, 42.62, 44.43, 54.06, 63.52, 66.59, 72.98, 76.01, 87.92, 87.98, 90.09, 91.06, 100.89, 117.0, 125.34, 133.43, 137.54, 138.8, 146.33, 152.06, 158.26,165.39,176.10,182.51.

((2R,3S,5R)-5-(2,4-dioxo-5-(3-((S)-1-(7-oxocyclohepta-1,3,5-trien-1-yl)pyrrolidine-2-carboxamido)prop-1-yn-1-yl)-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate **(13)**:The triphosphorylation of compound (**12**) was performed as like compound 5 to obtain

compound 13 as yellow solid. ^{1}H NMR (700 MHz, D_2O) δ 1.86- 2.14 (m, 4H), 2.40 (dd, *J* = 13.8, 7.0 Hz, 4H), 3.02 – 3.13 (m, 3H), 3.39 – 3.92 (m, 4H), 4.00 – 4.40 (m, 4H), 6.29 (dd, *J* = 12.5, 5.9 Hz, 1H), 6.72- 7.04 (m, 3H), 7.35 (dt, *J* = 22.9, 11.8 Hz, 2H), 8.14 (dd, *J* = 24.6, 17.6 Hz, 1H).HRMS ESI-TOF,Calcd, for ($C_{24}H_{27}N_4O_{16}P_3$)720.0629, Found720.0632.

4.5 Refferences

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4.6 Appendix

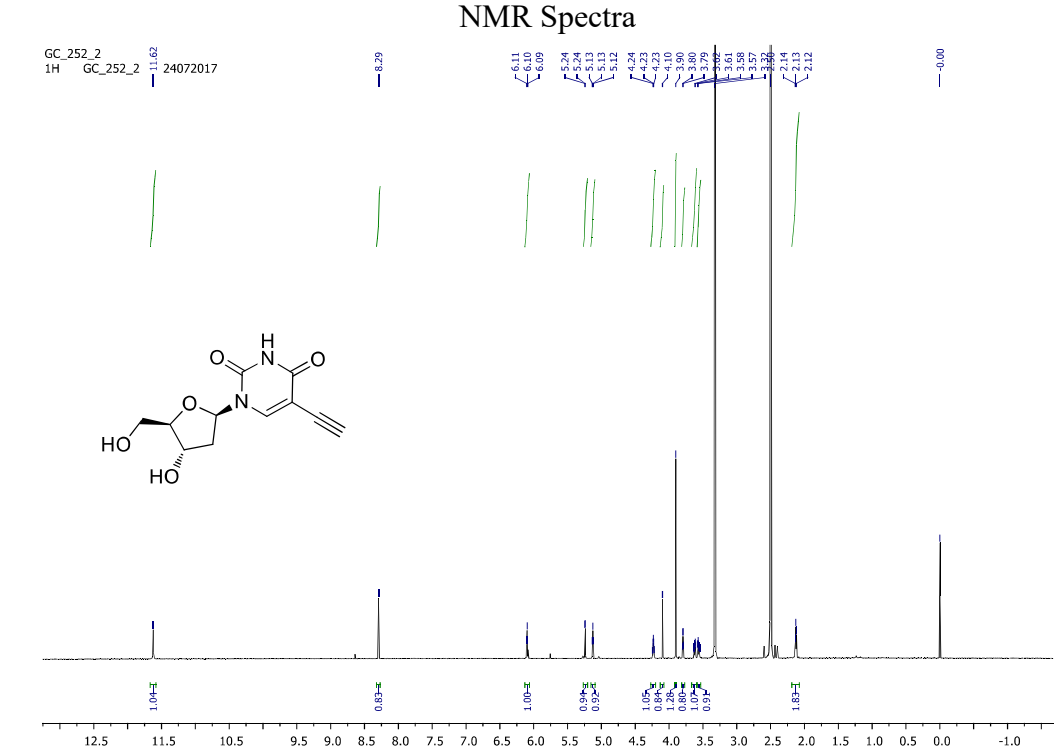

Figure A1 ^{1}H NMR of Compound **2**

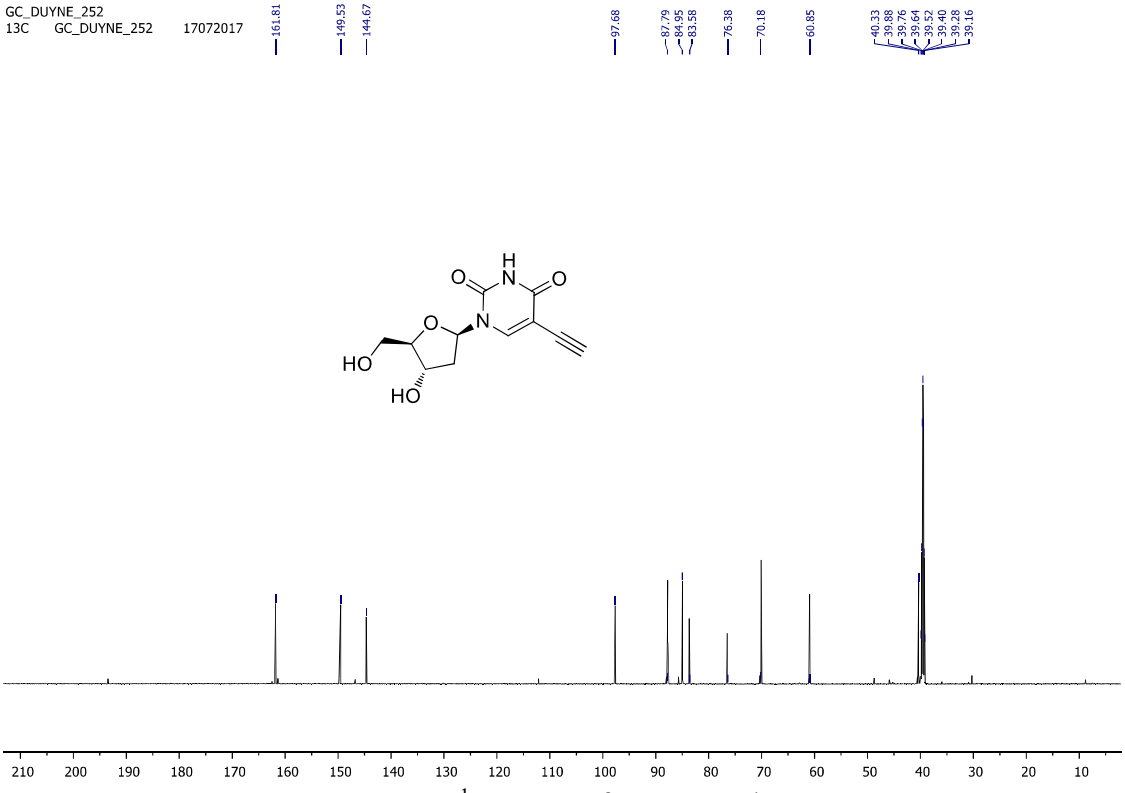

Figure A2 ^{1}H NMR of Compound **2**

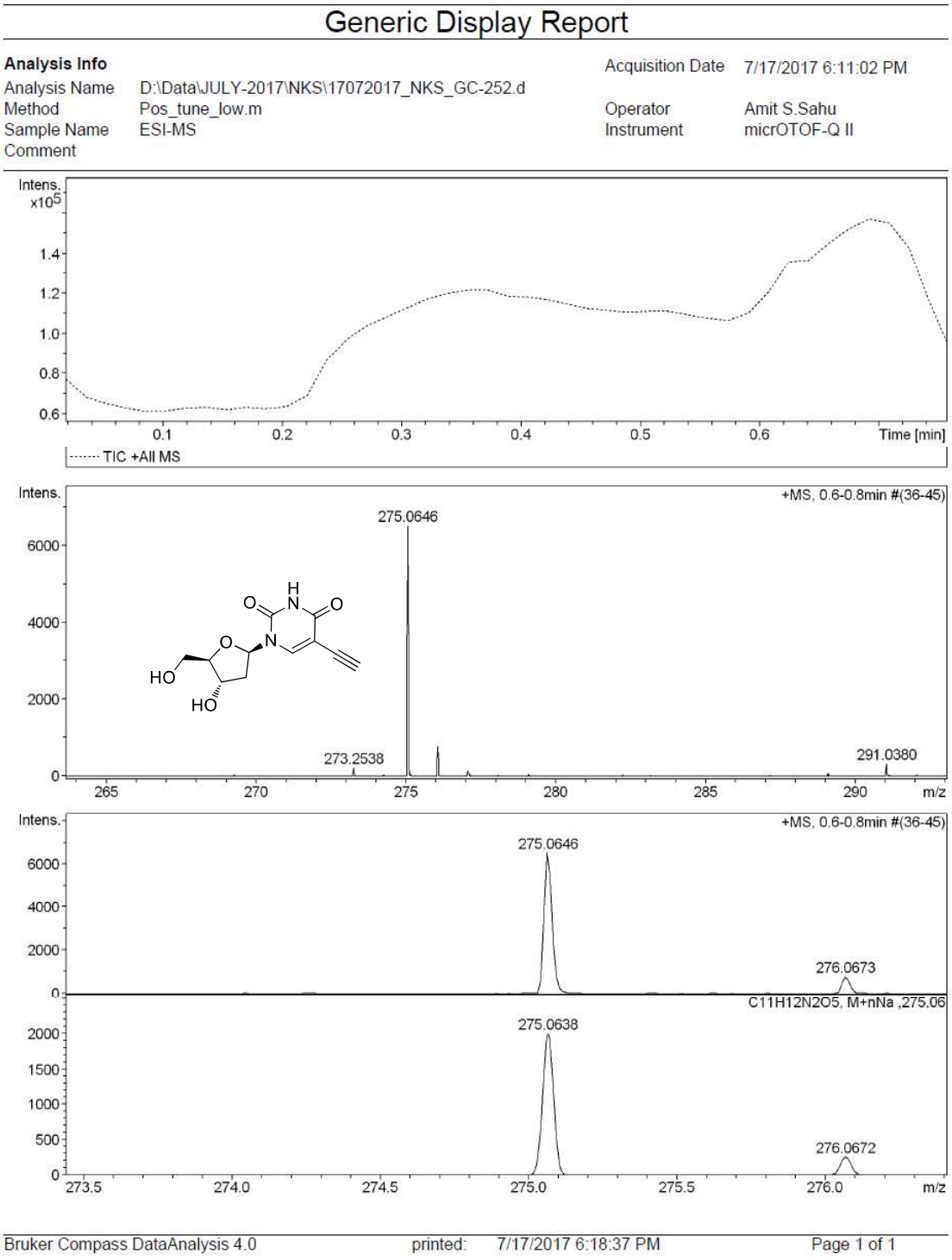

Figure A3 HRMS of Compound **2**

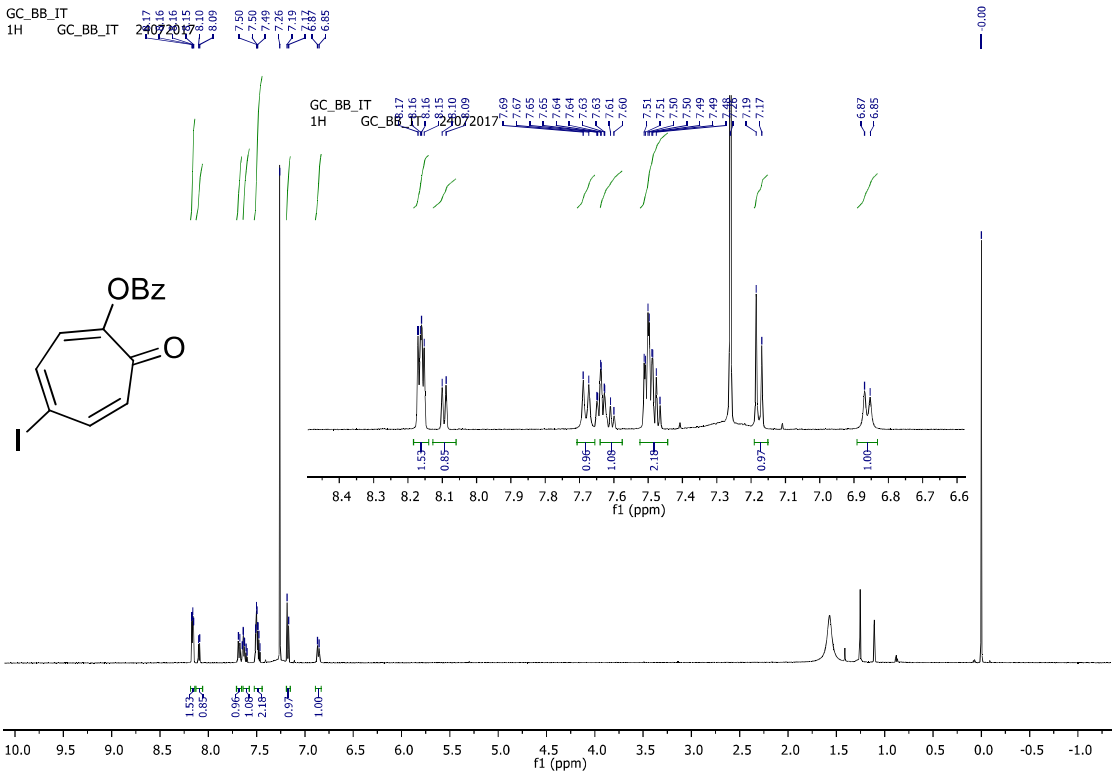

Figure A4 ^{1}H NMR of Compound **3**

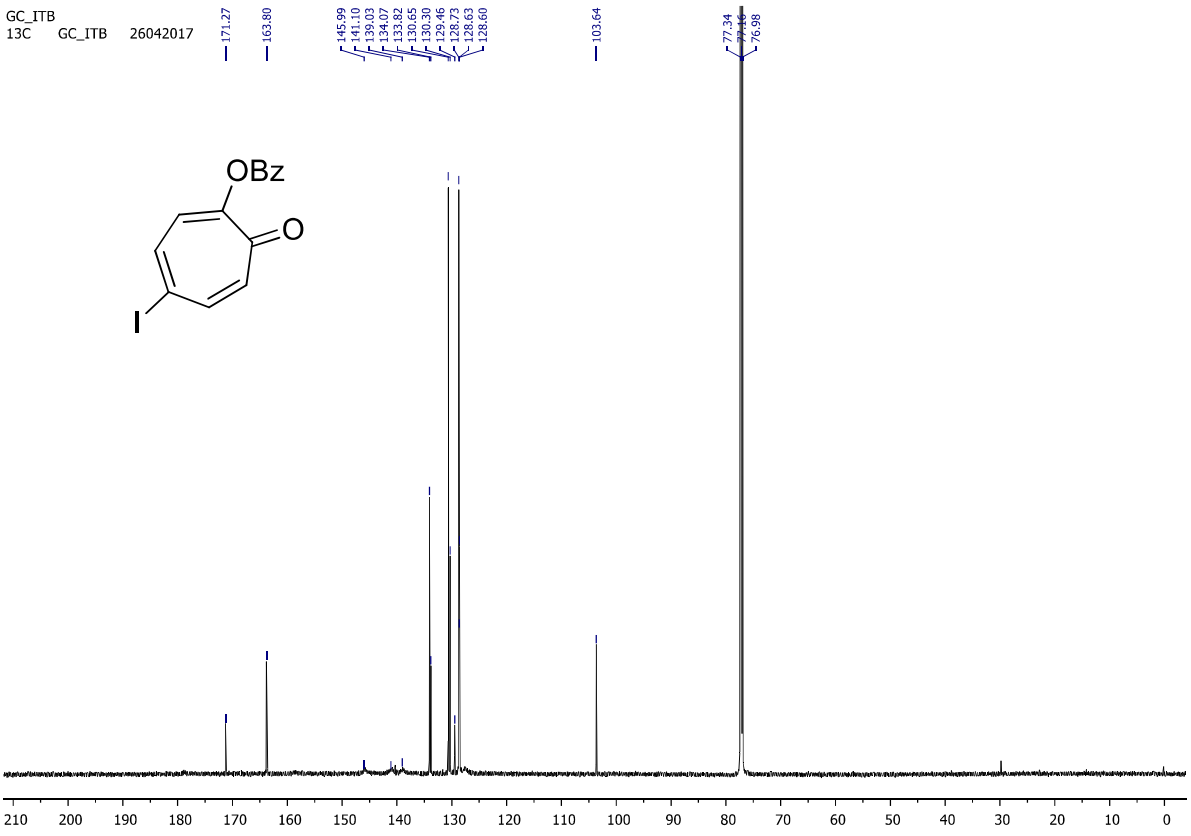

Figure A5 ^{13}C NMR of Compound **3**

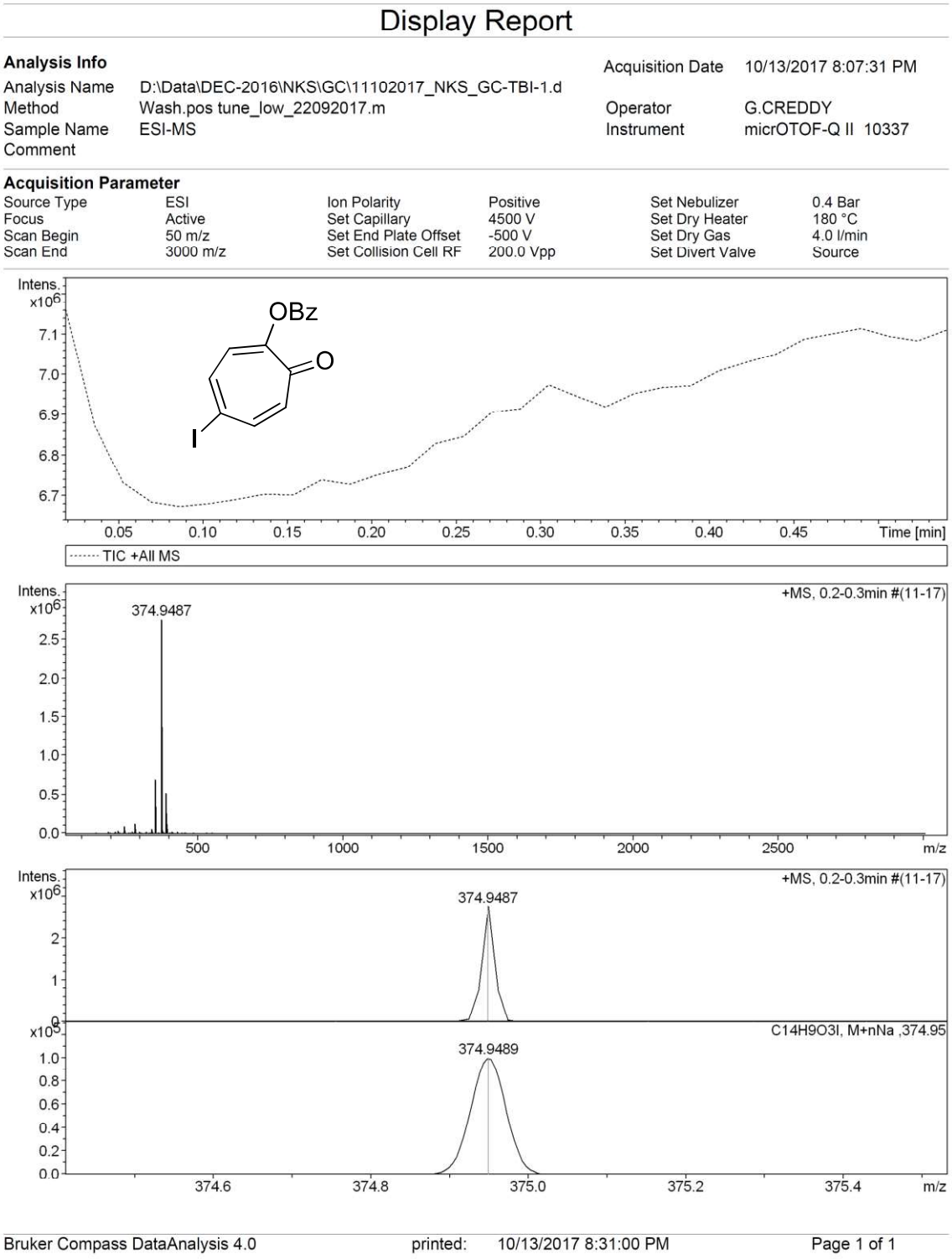

Figure A6 HRMS of Compound **3**

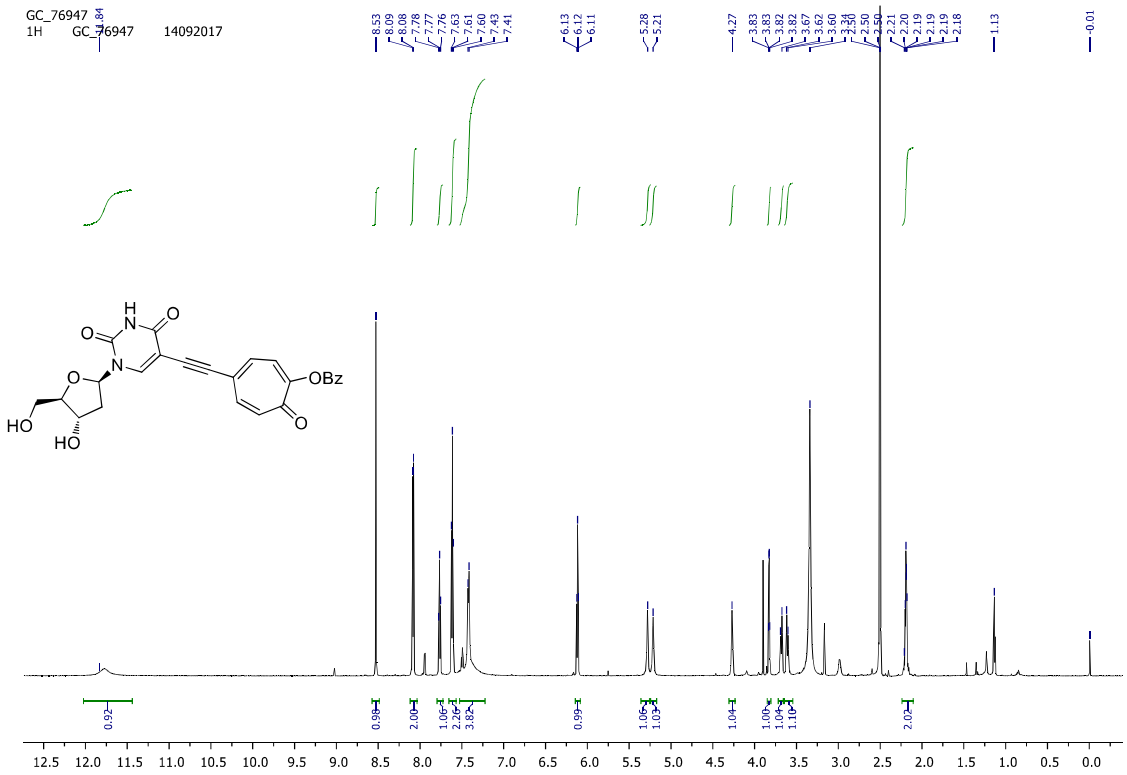

Figure A7 ^{1}H NMR of Compound **4**

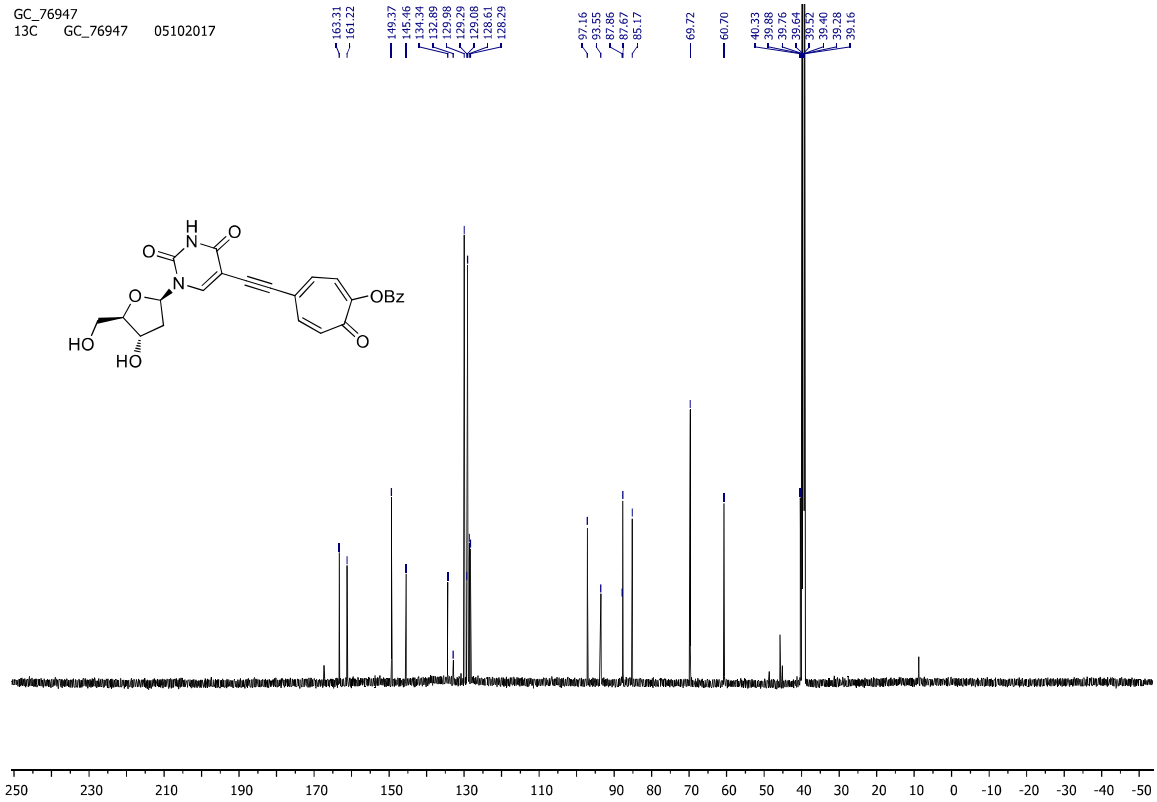

Figure A8 ^{13}CNMR of Compound **4**

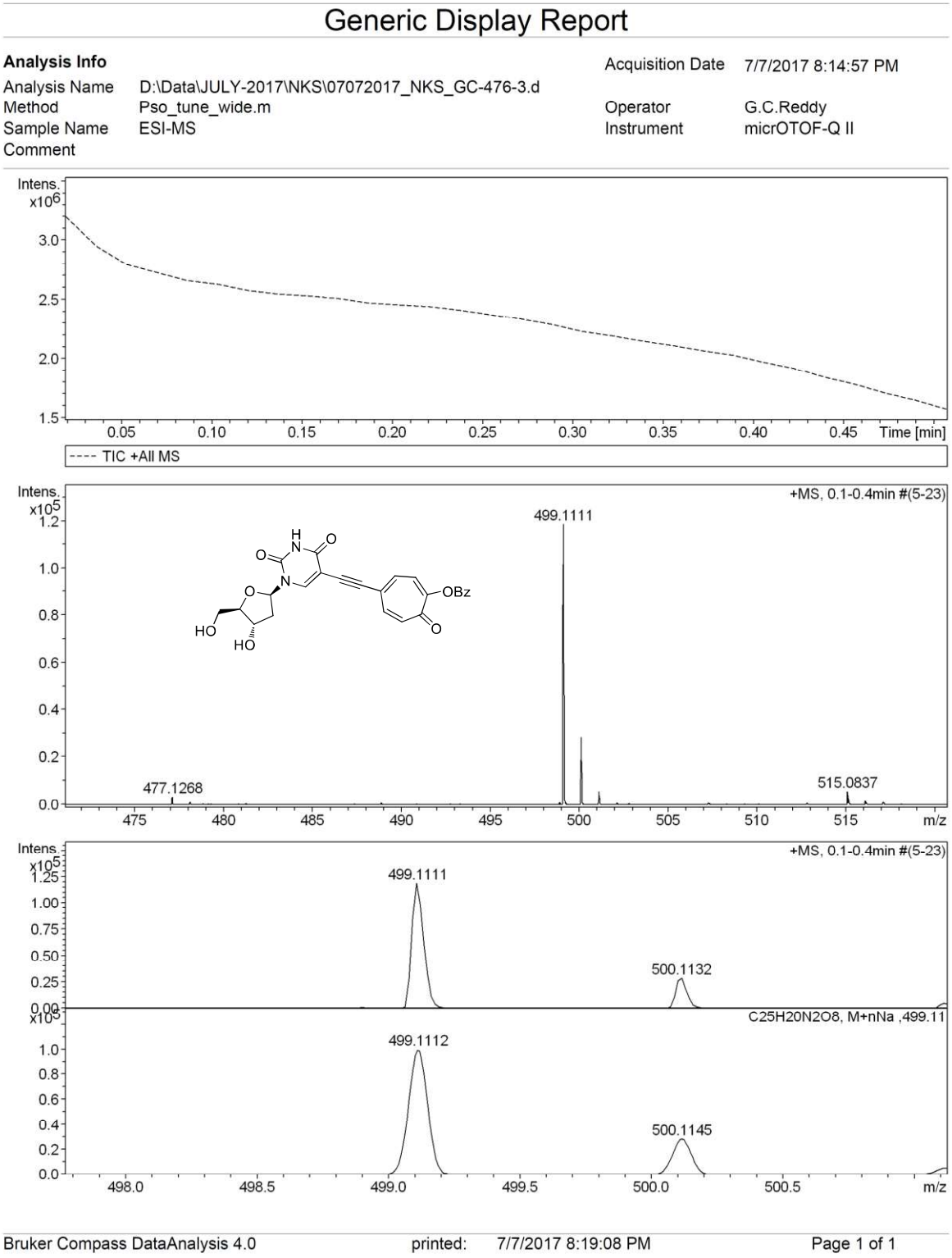

Figure A9 HRMS of Compound **4**

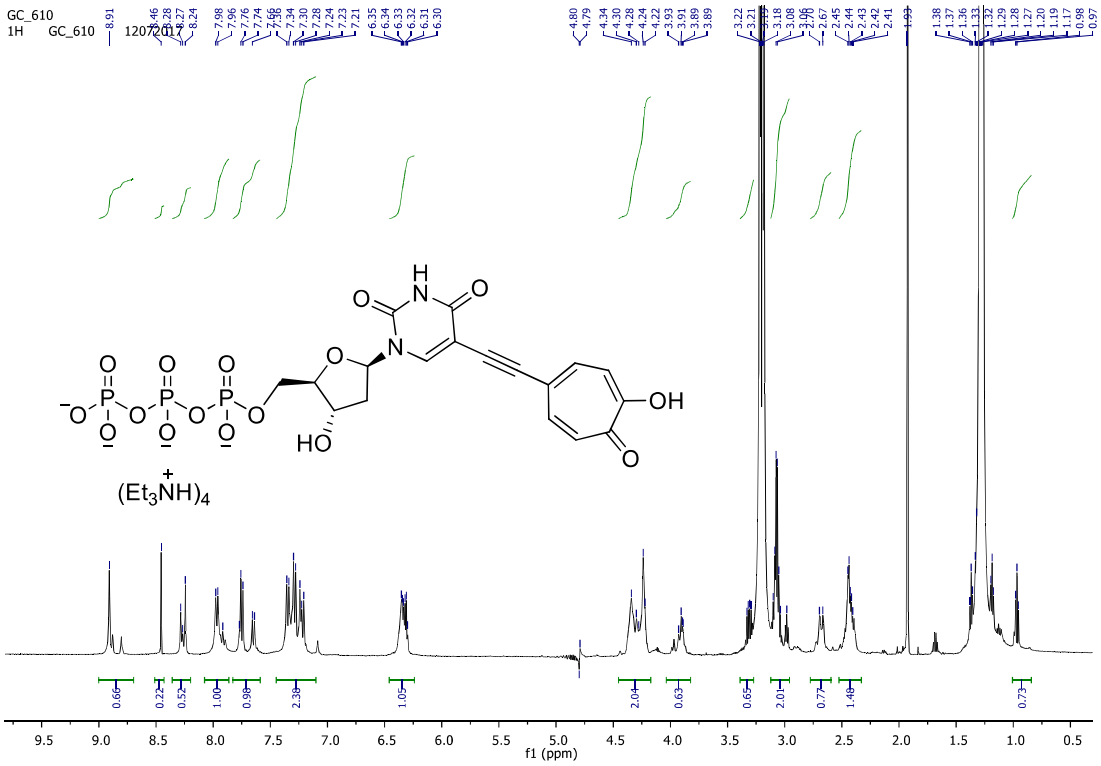

Figure A10 ^{1}H NMR of Compound **5**

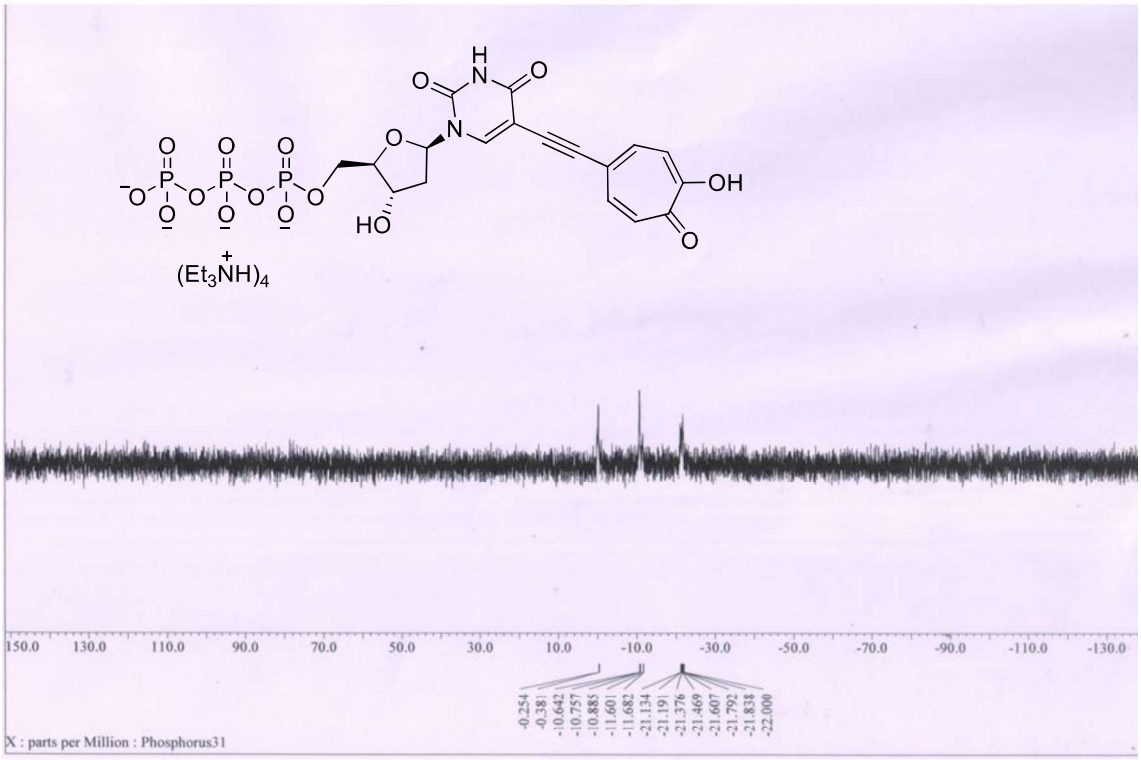

Figure A11 ^{31}P NMR of Compound **5**

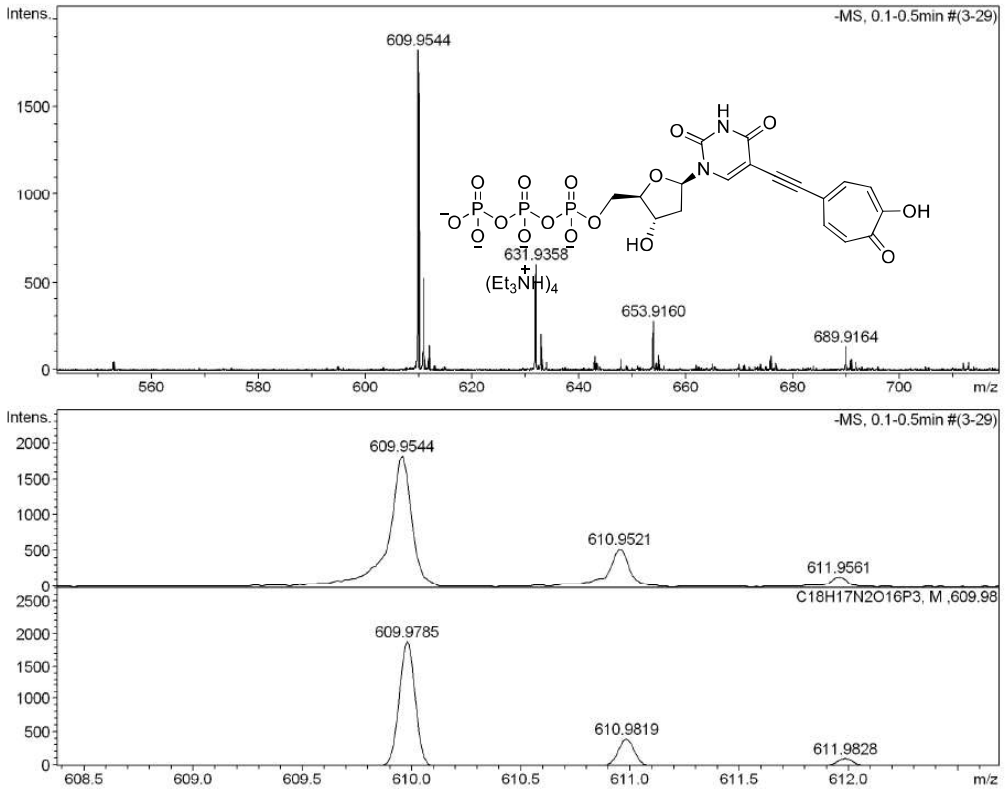

Figure A12 ^{31}P HRMS of Compound **5**

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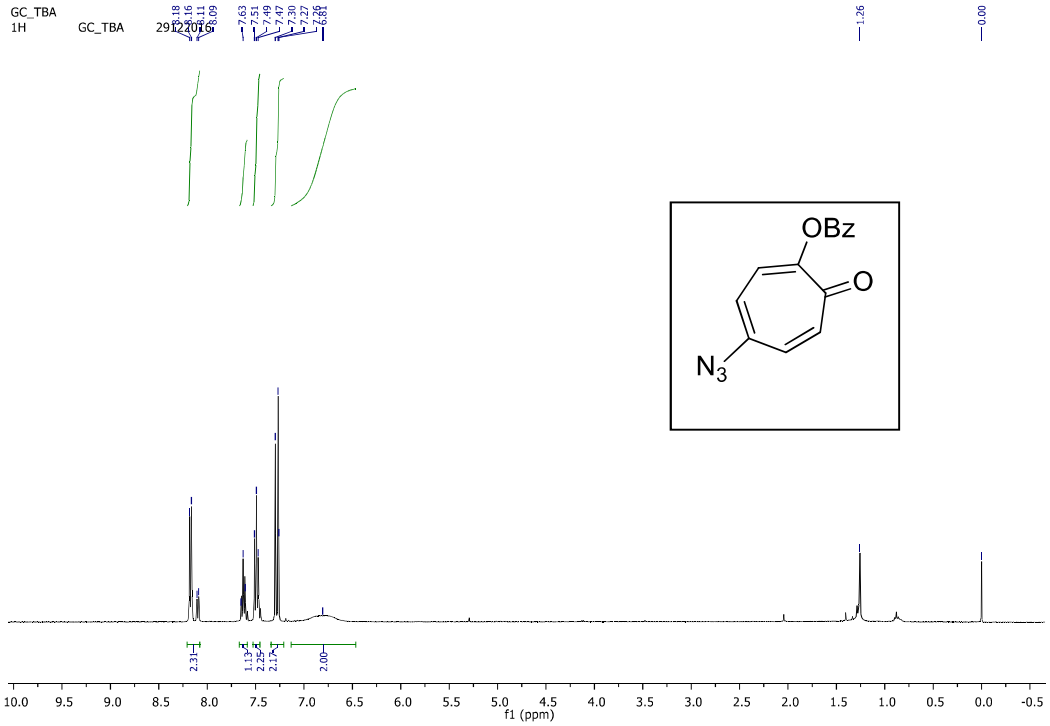


Figure A16 ^{1}H NMR of Compound **6**

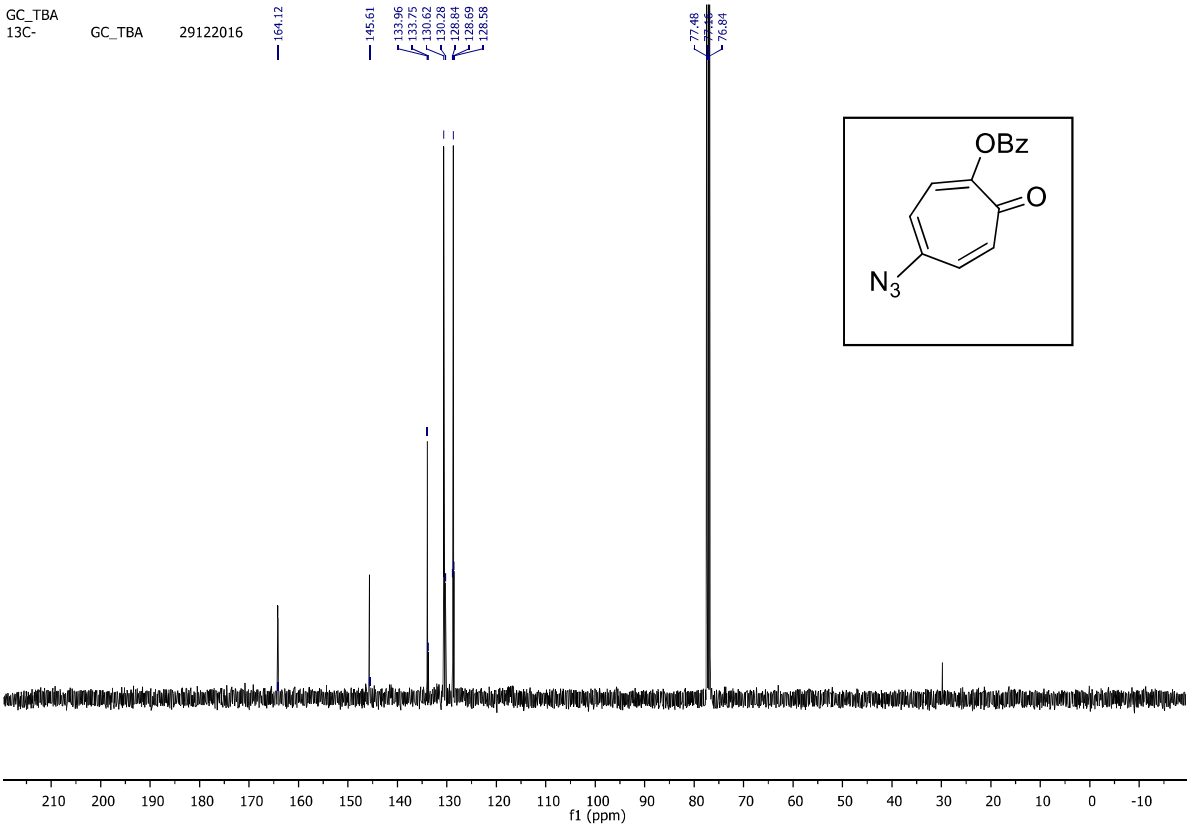


Figure A17 ^{13}C NMR of Compound **6**

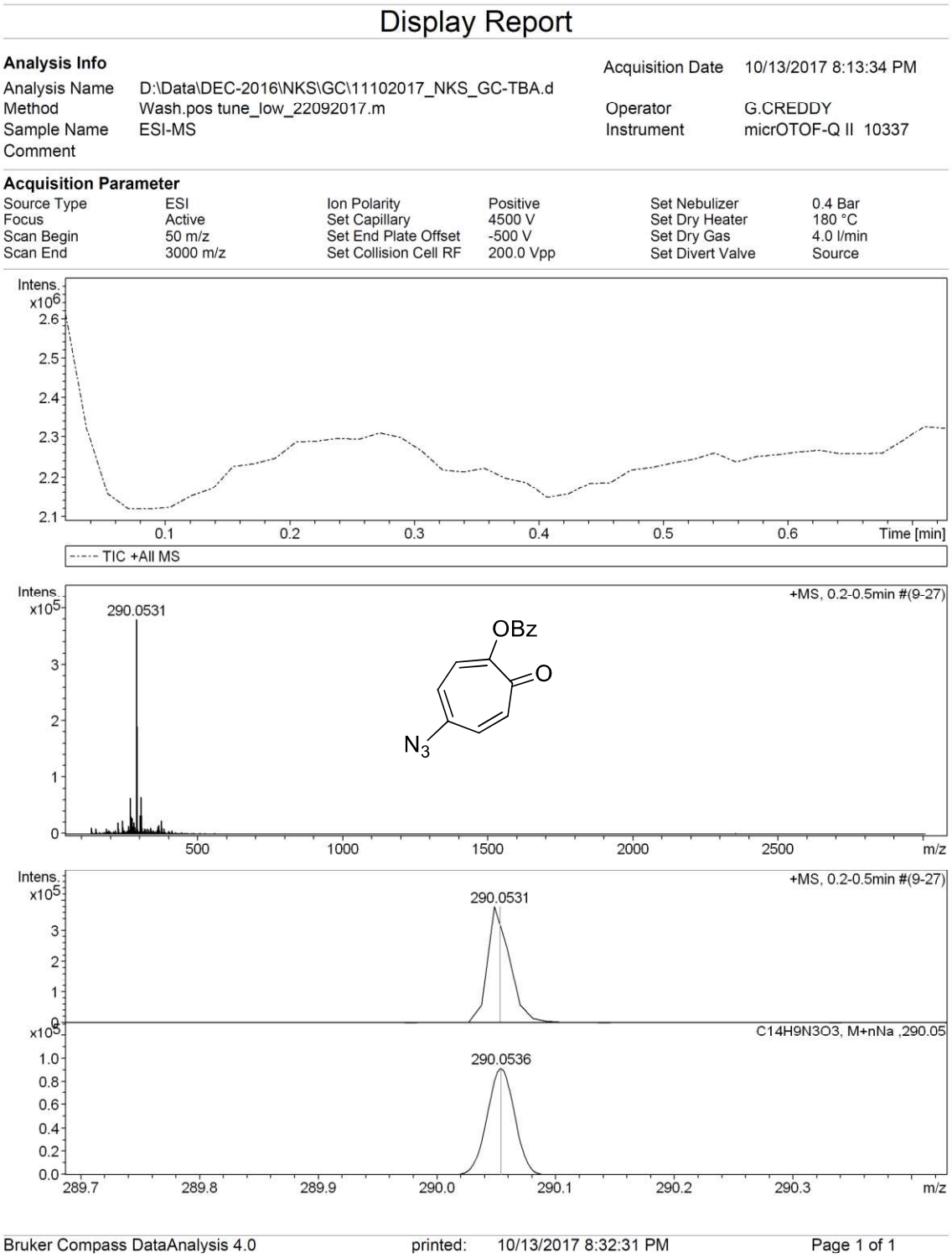


Figure A18 HRMS of Compound **6**

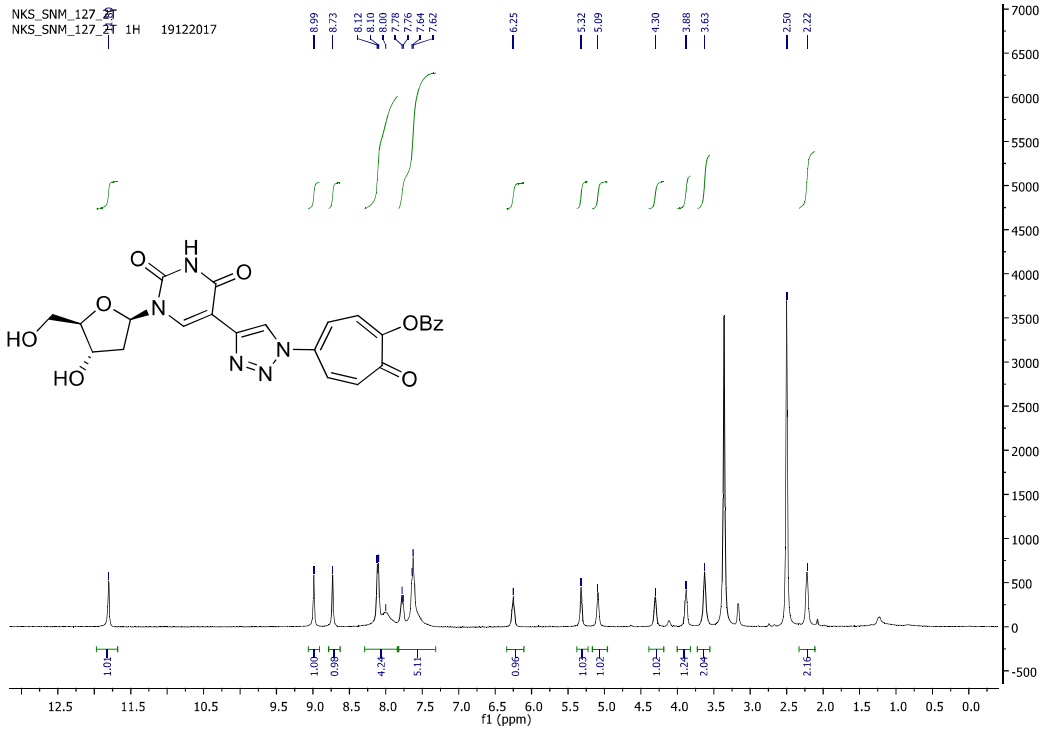


Figure A18 ^{1}H NMR of Compound **7**

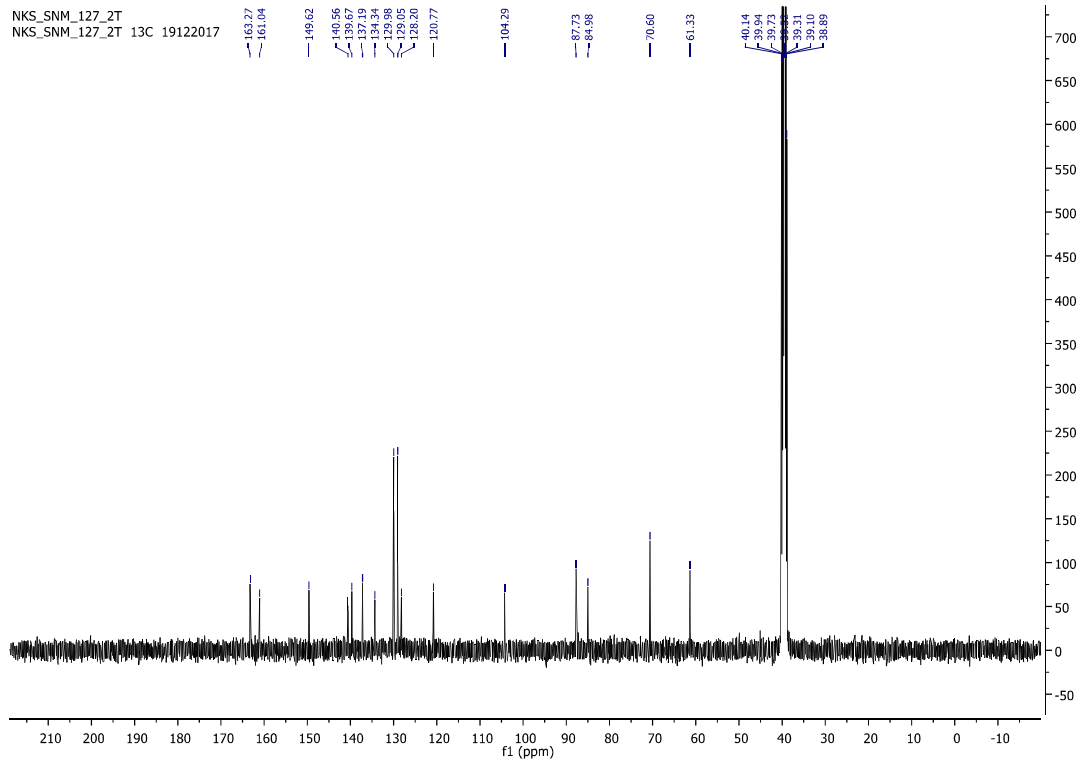


Figure A19 ^{13}C NMR of Compound **7**

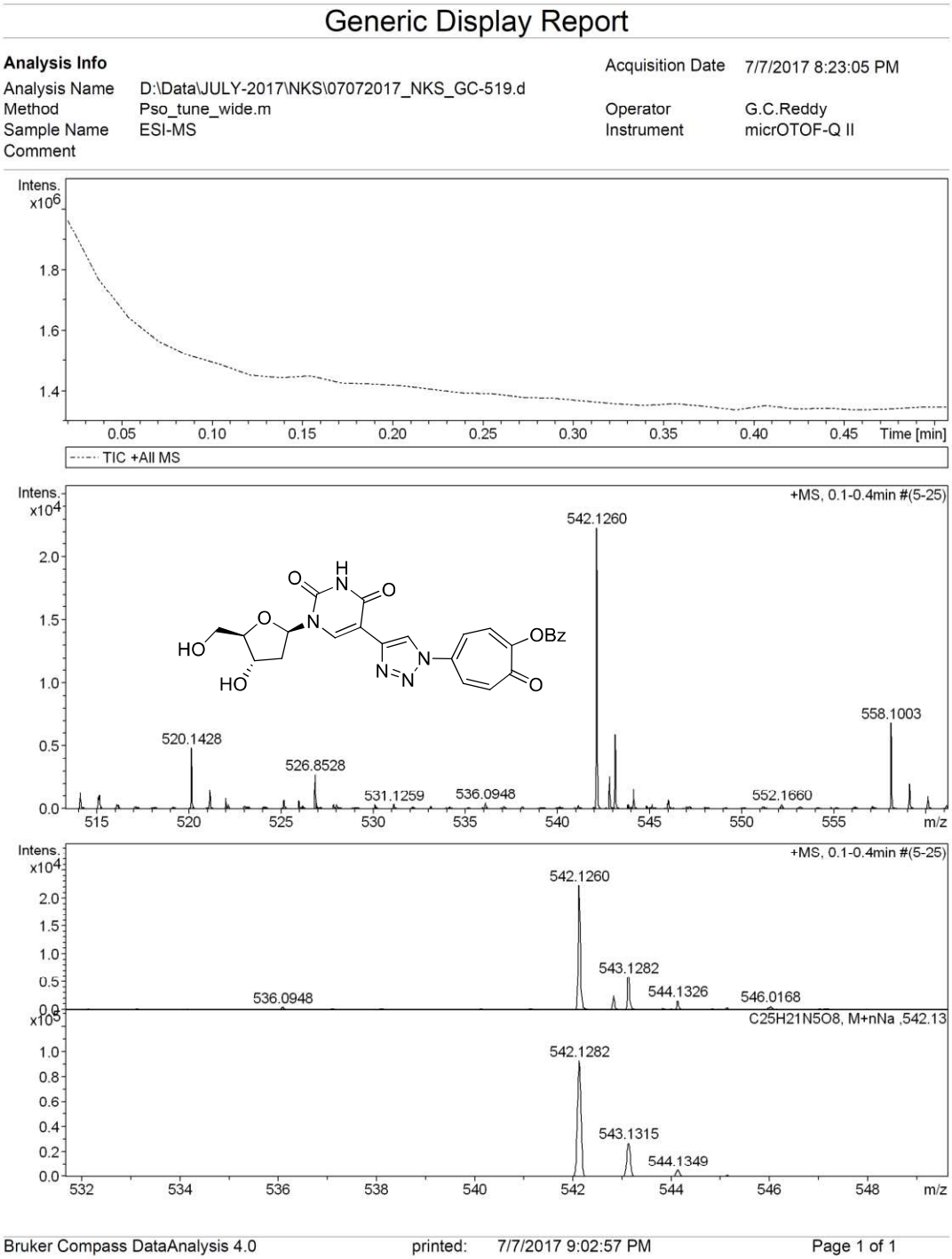


Figure A20 HRMS of Compound **7**

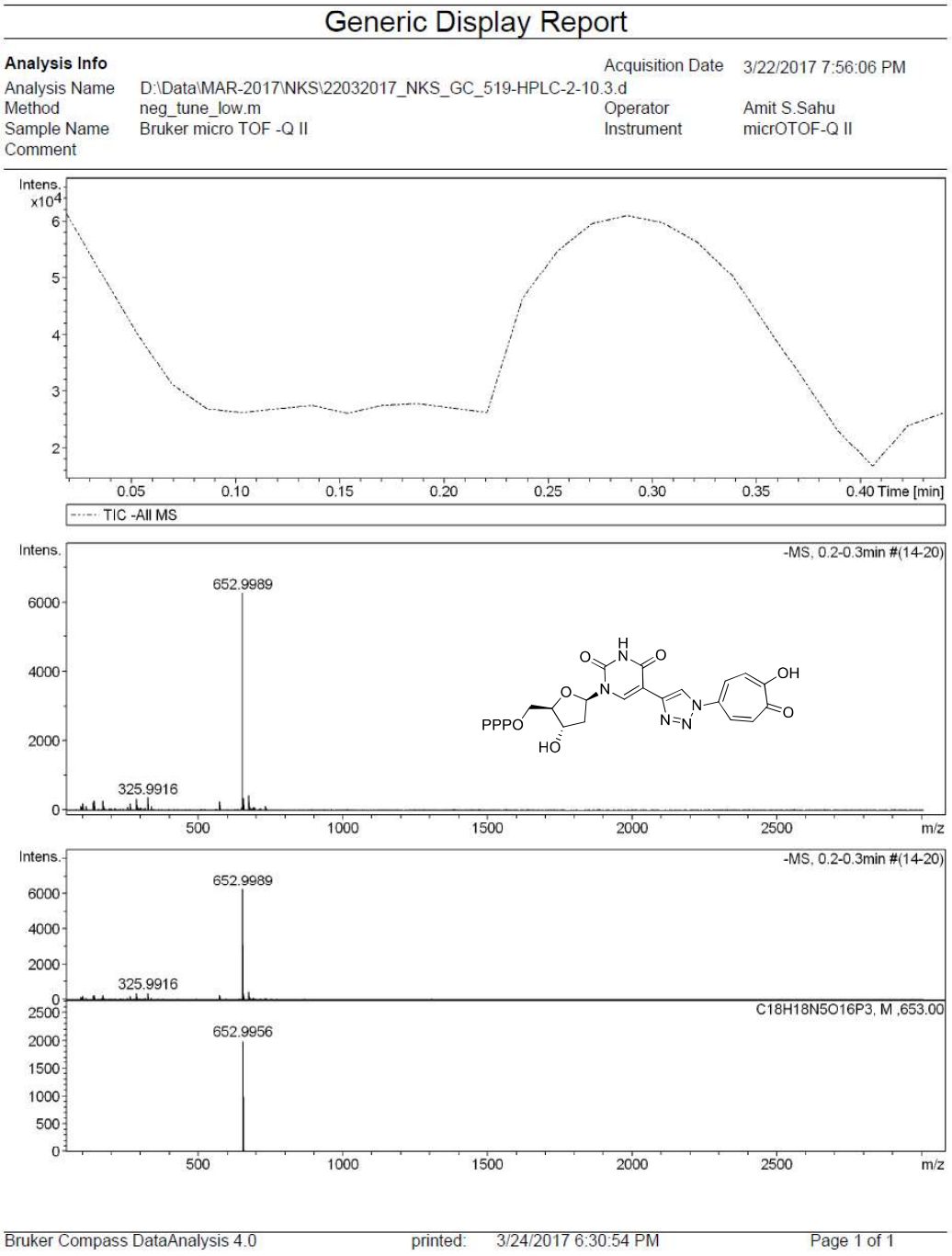


Figure A21 HRMS of Compound **8**

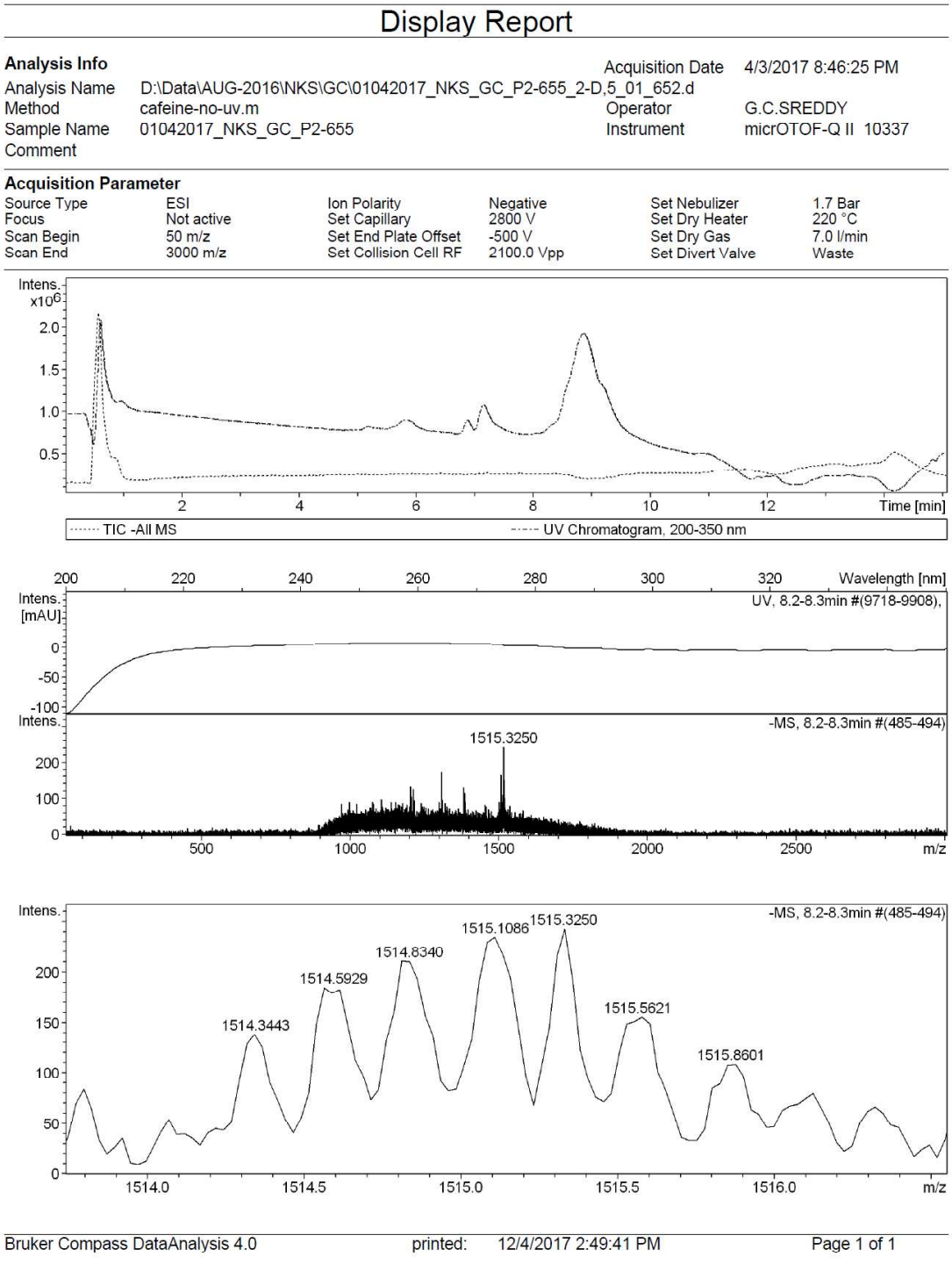


Figure A22 LC-MS of primer extension reaction of DNA with compound **8**

Calculated mass for extended primer=6009+54 (3 NH_4) = 6063 /4 =1515. 75, observed = 1515.32

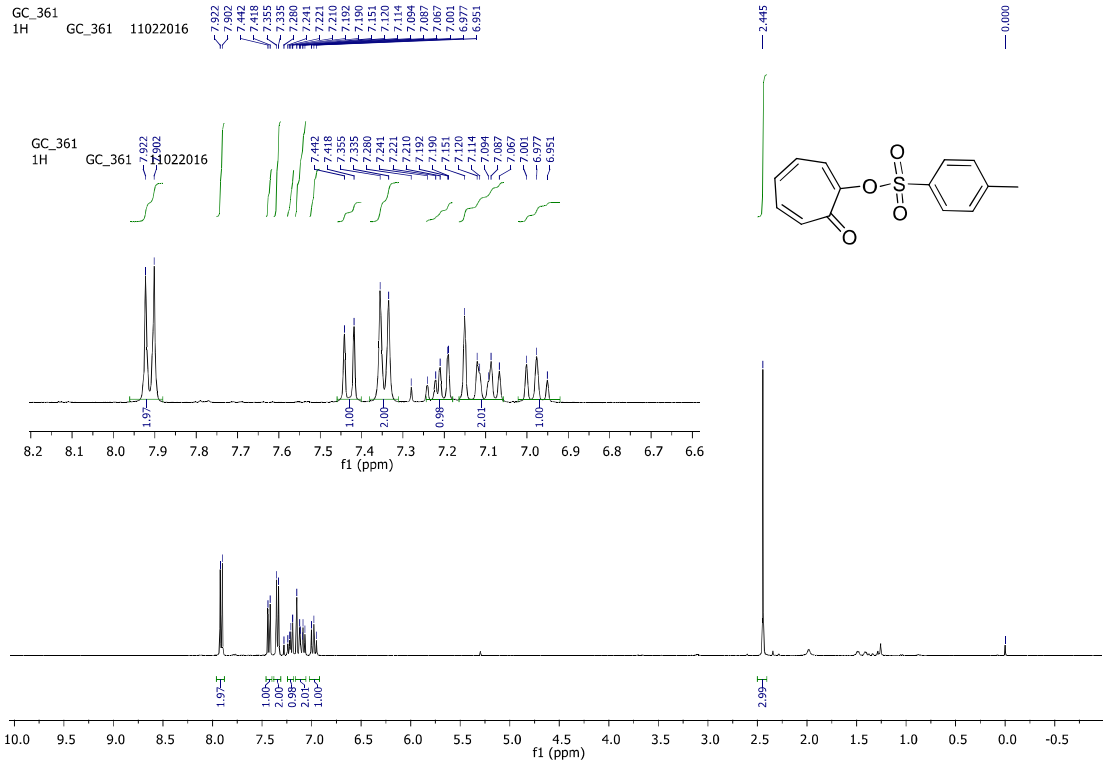


Figure A23 ^{1}H NMR of Compound **10**

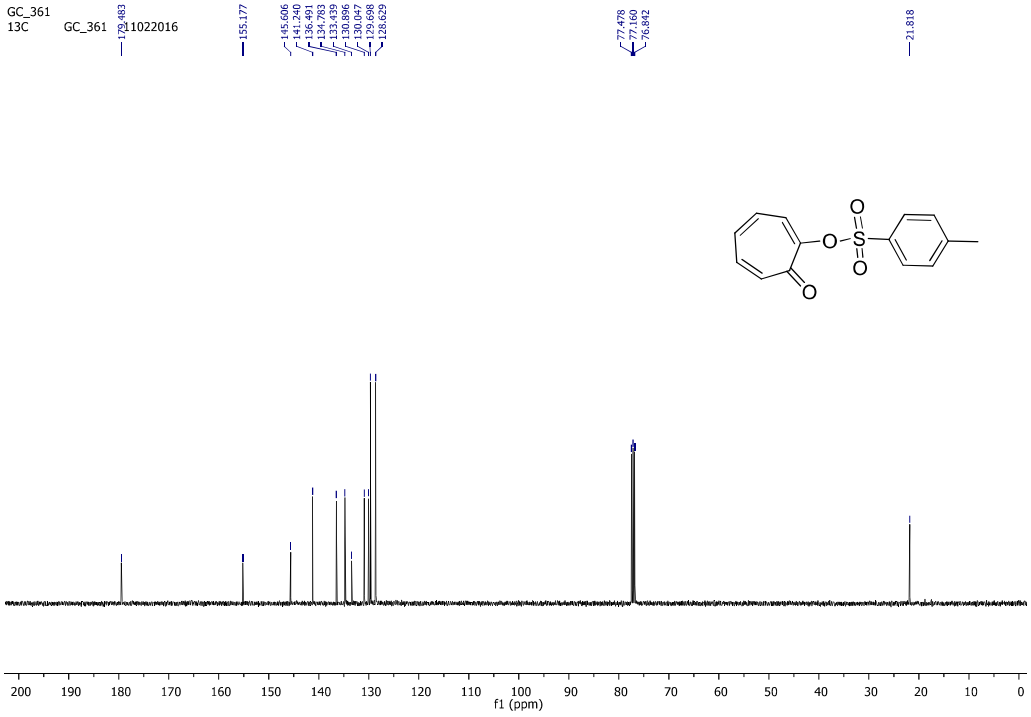


Figure A24 ^{13}C NMR of Compound **10**

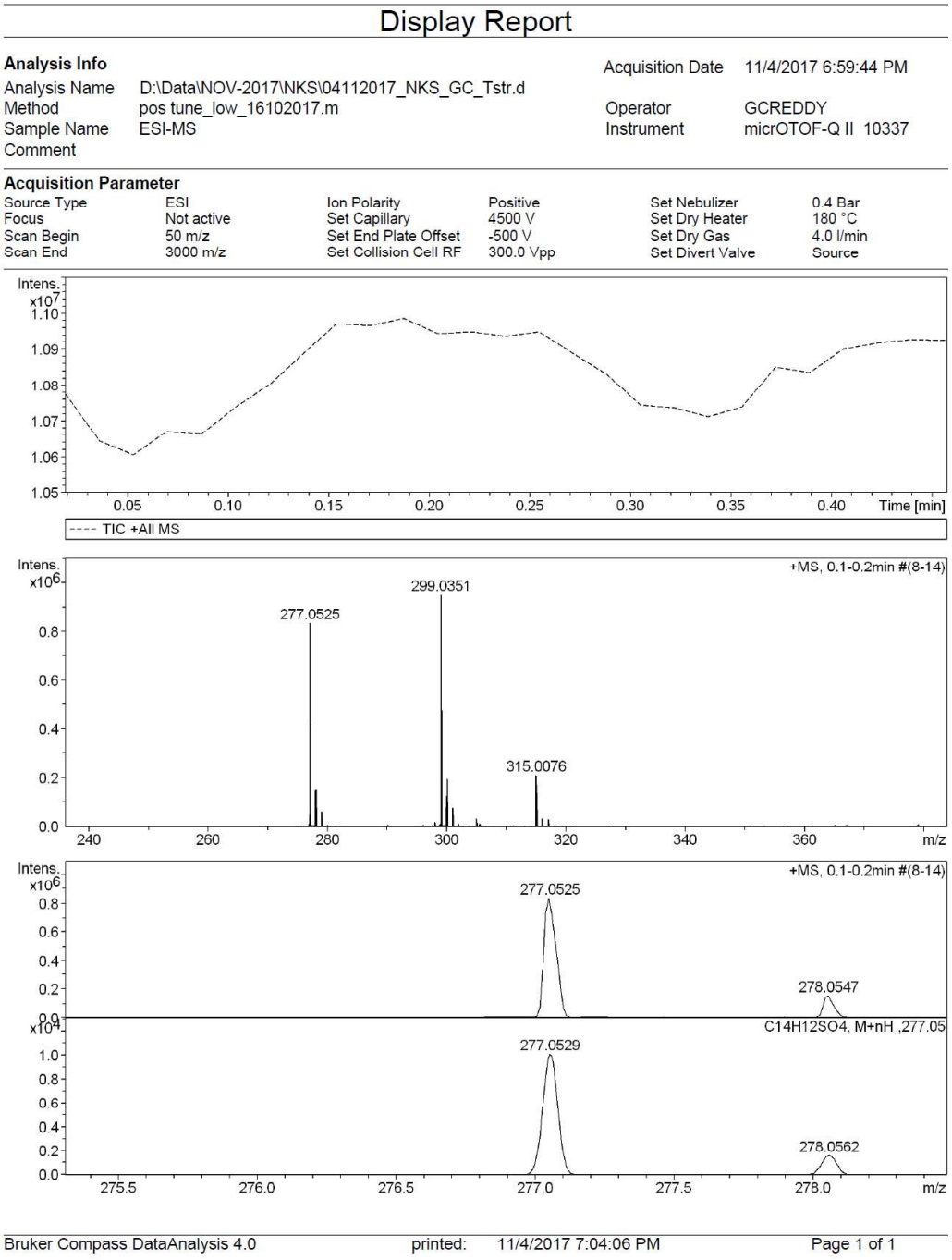


Figure A25 HRMS of Compound **10**

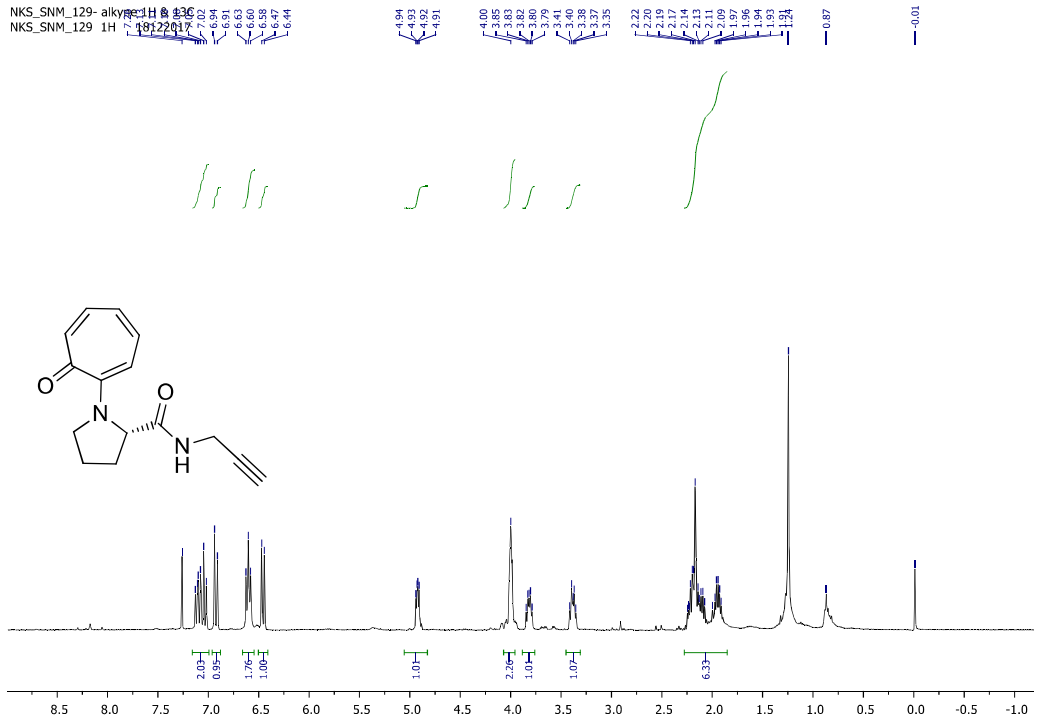


Figure A26 ^{1}H NMR of Compound **11**

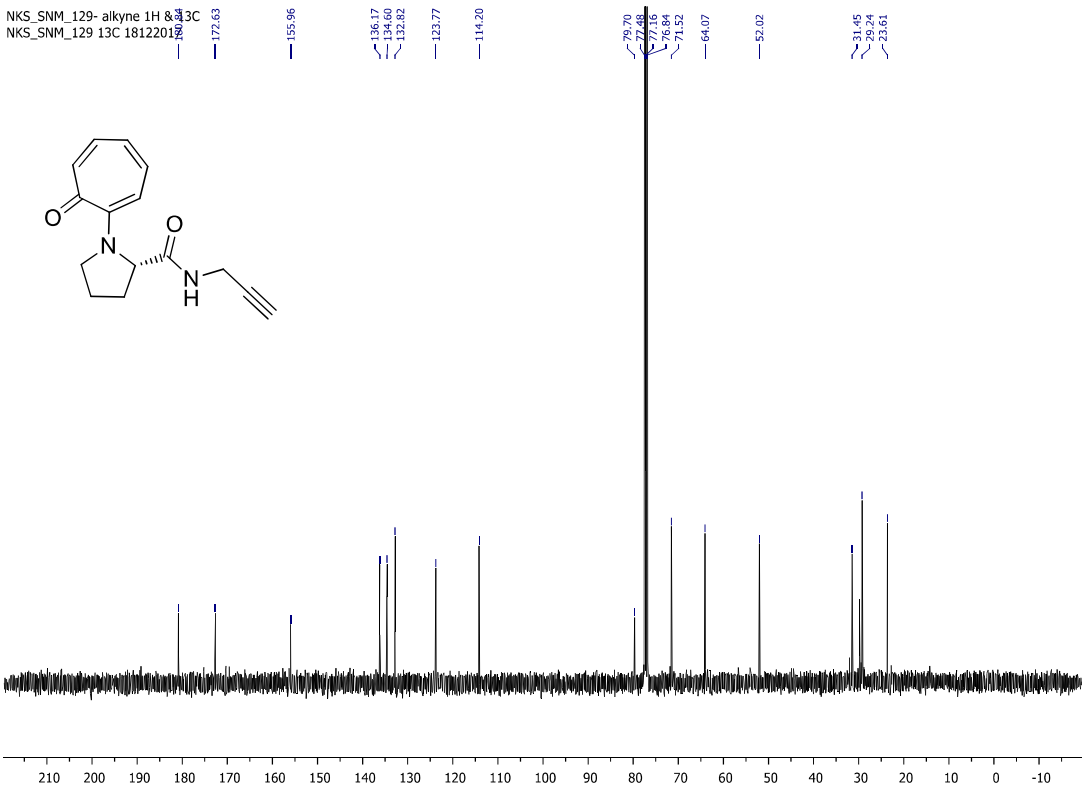


Figure A27 ^{13}C NMR of Compound **11**

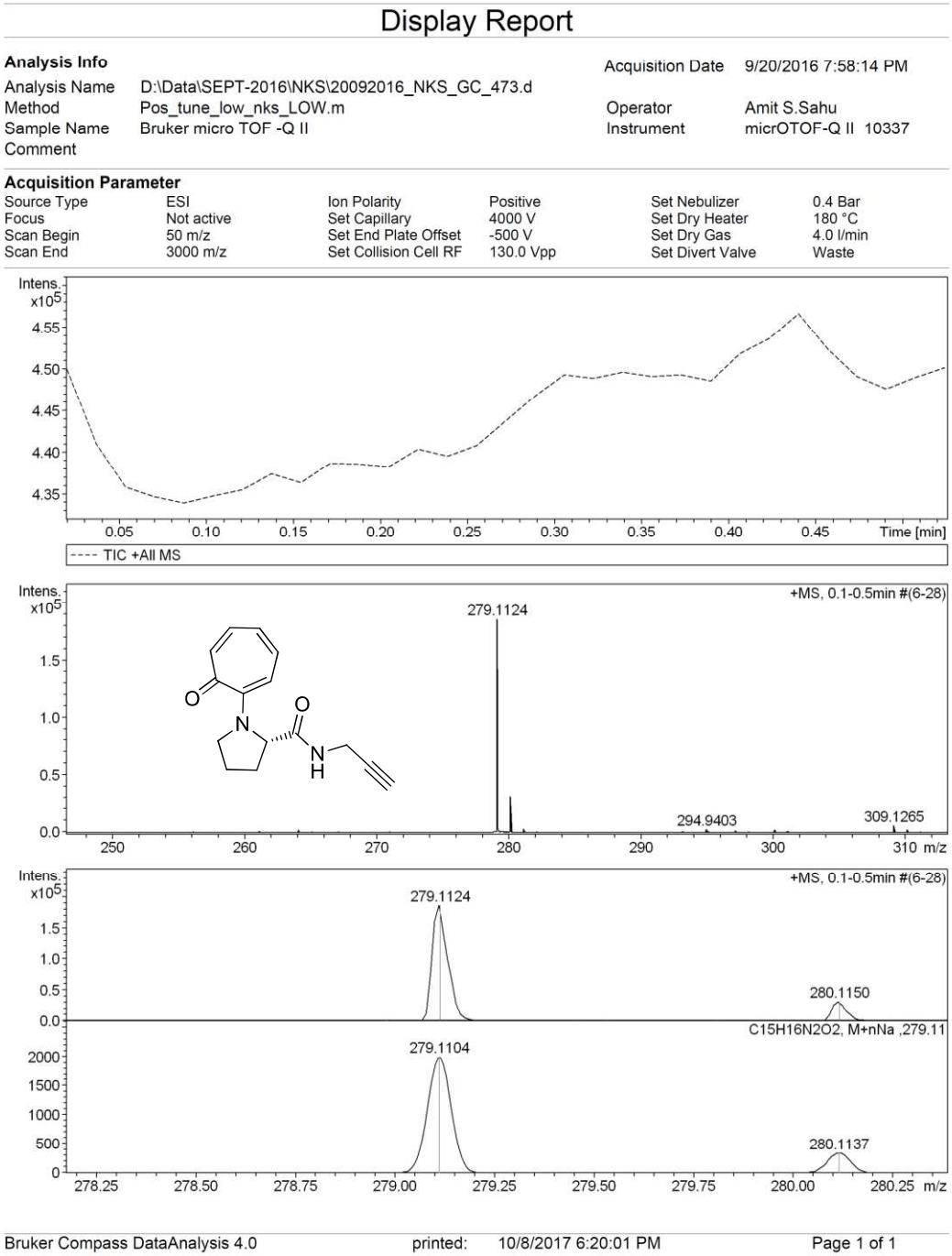


Figure A28 HRMS of Compound **11**

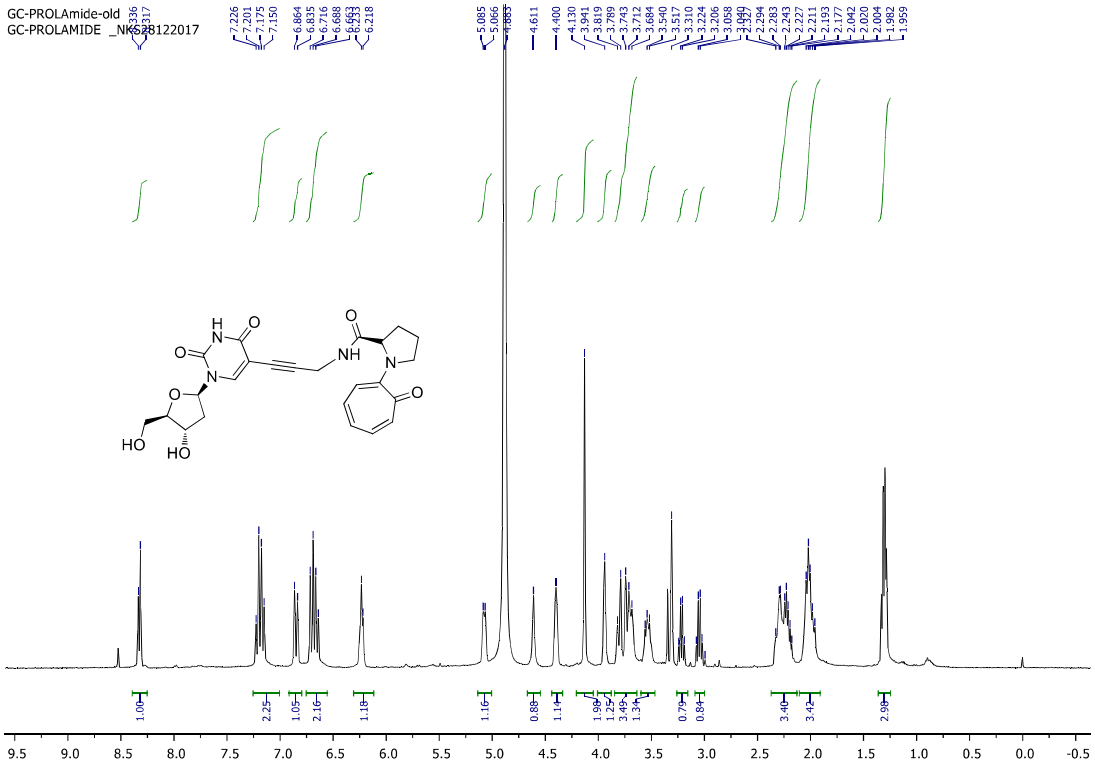


Figure A29 ^{1}H-NMR of Compound **12**

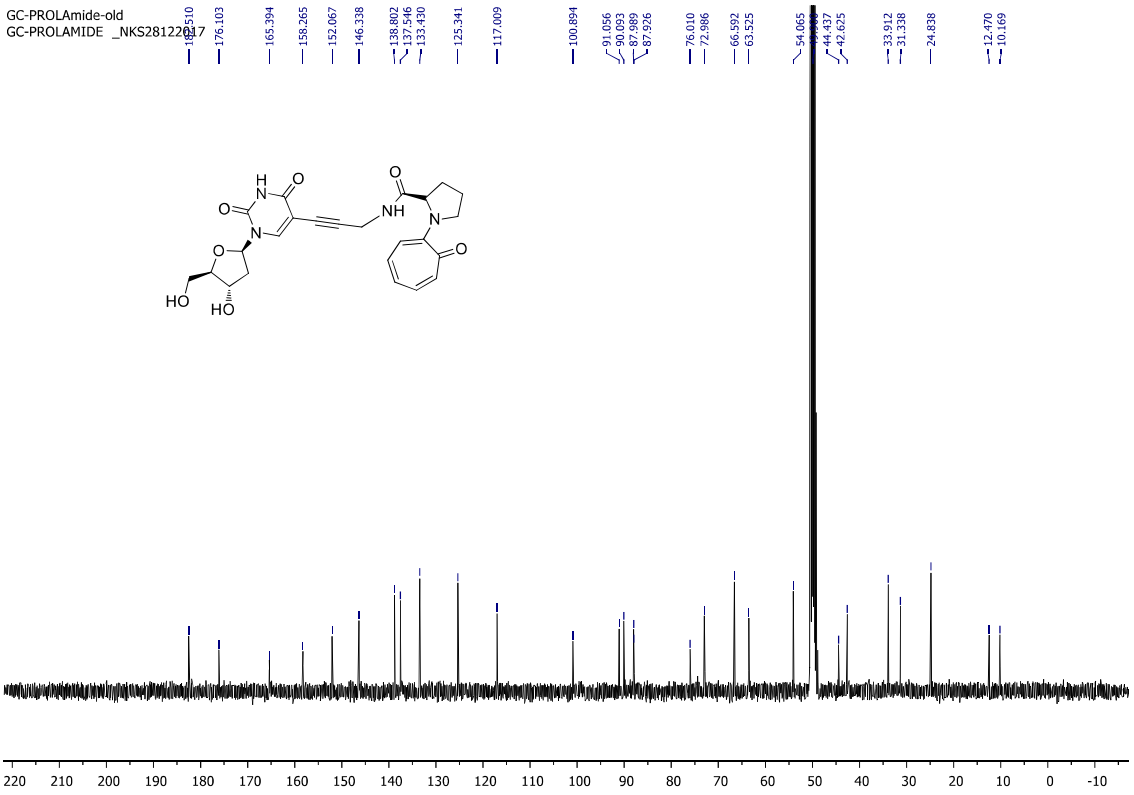


Figure A30 ^{13}C-NMR of Compound **12**

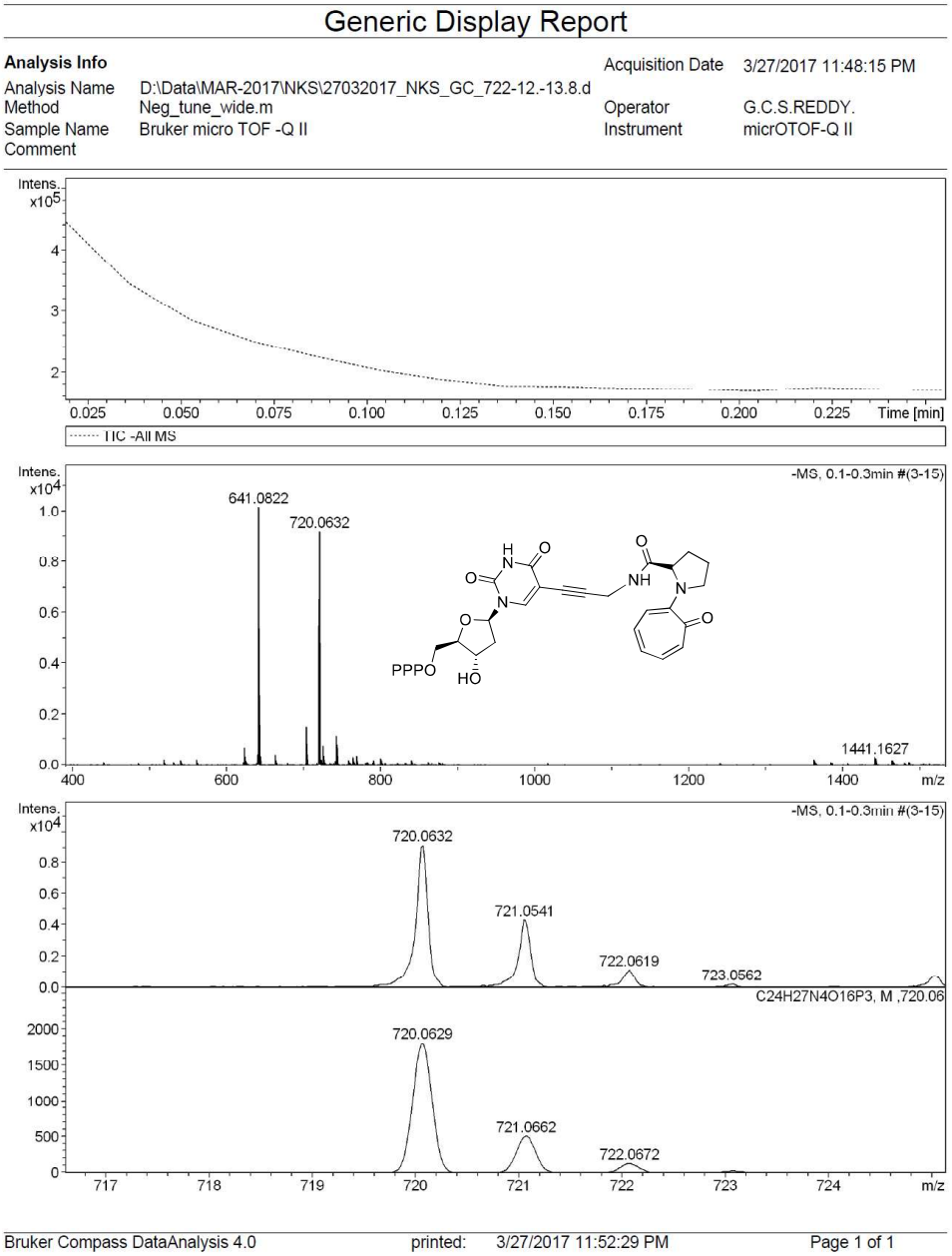


Figure A31 ESI-HRMS of Compound **13**

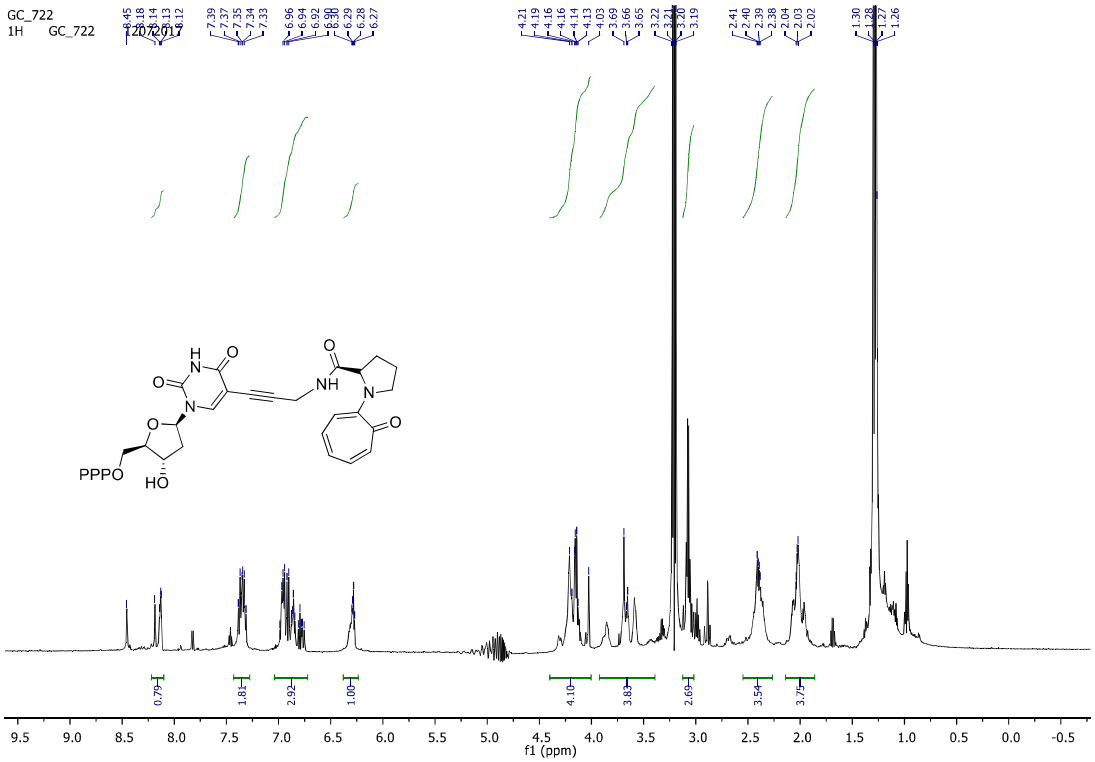
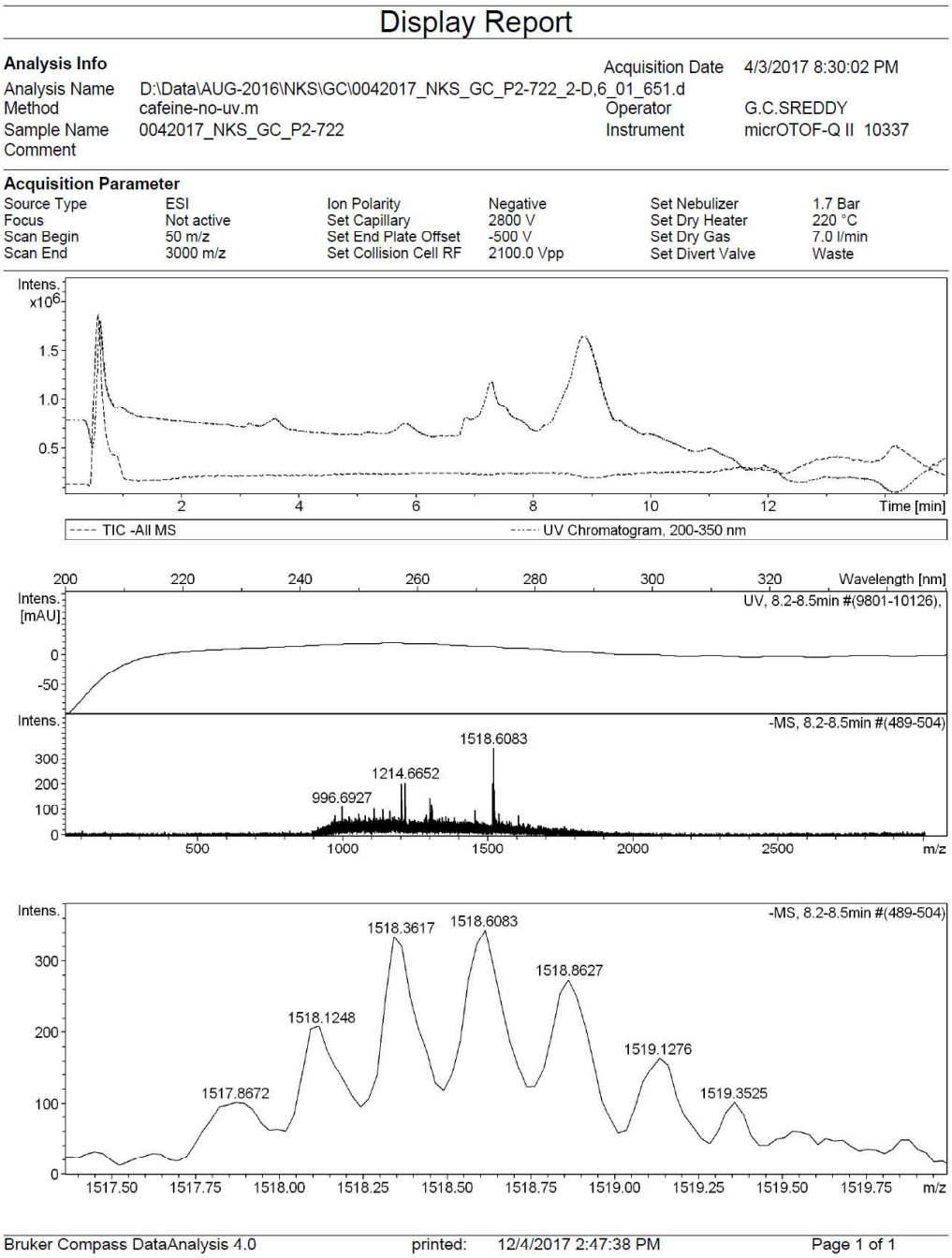


Figure A32 ^{1}H-NMR of Compound **13**



Calculated mass for extended primer=6076=6076/4=1519 observed=1518.6

Figure A33 LC-MS of primer extension reaction of DNA with compound **13.**

Chapter 5

Synthesis and Biophysical Studies of Aminoethyl prolyl Cytosine Pentamer (*aep-C_5*) in the Stabilization of Hybrid i-motif with DNA and RNA

Chapter 5

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Contents

5.1 Introduction

Deoxyribose nucleic acid (DNA) is genetic material of living organisms which carries the genetic information for the next generation. Recent studies has revealed that biological DNA has encompassed with large amount of non-coding DNA sequences. The non-coding DNA involved in many biological functions such as telomere maintenance, regulation of transcription and translation. The non-coding sequences have repeated sequences of DNA which can fold into non-B DNA structures such as tetraplex, triplex, poly A-duplex, Z-DNA, hairpin, cruciform *etc.*[1] The non-B-DNA forming sequences can induce genetic instability and cause human disease.[2] Of all the non-B-DNA conformations, G-quadraplex and Cytosine i-motif have attracted considerable attention dueto their role in telomere maintainance and regulation of gene expression.. Since Guanine rich nucleic acid sequences are known to form G-quadrplexes structure while C-rich sequence for i-motif structure. In G-quadruplex structure, four guanine units form a square planar tetrad structure by Hoogsteen hydrogen bonding and nucleobase stacking (Figure5.1). Importantly the function of G-quadraplex in biological system are known as in the telomere maintenance and transcription regulation.[3-5]

Another important DNA i-motif structure are formed by C-rich sequence under acidic conditions. The cytosine rich DNA sequences form a parallel hemi protonated duplexes under acidic conditions. Two such duplexes intercalate in an antiparallel manner to form an intercalated tetraplex structure called i-motif. [6-11] These structures occur in centromere, telomere and oncogene promoter regions. The i-motif structure is also involved in the regulation of transcription. [12-17] The i-motif structure also used

as potential materials in nanotechnology research such as the controlling of macromolecular structure assembly with pH response.[18,19]

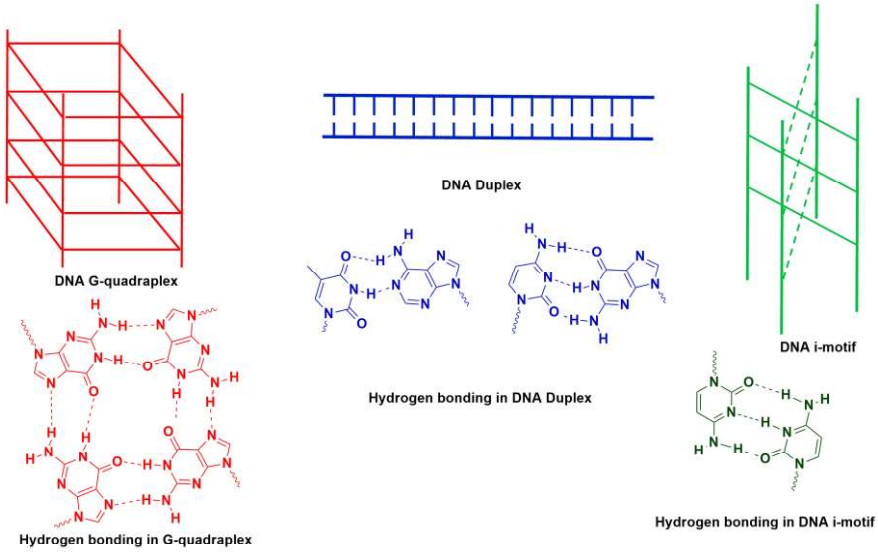


Figure 5.1 DNA duplex, G-quadraplex and i-motif structure and hydrogen bonding

5.1.1 Structure of i-motif

The fundamental unit for i-motif formation is Cytosine.Cytosine$^+$ (C.C$^+$) base pair which formed by hydrogen boding between two cytosine units one neutral and one protonated at N3. The C.C$^+$ dimer is more stable than Watson-Crick G.C pair. [20]The proton bound to C.C+ pair is not equally distributed between to cytosine units but located at the largest distance to the hydrogen of next C.C$^+$ pair. The i-motif is closely packed structure with strong interaction between cytosine base pairs.

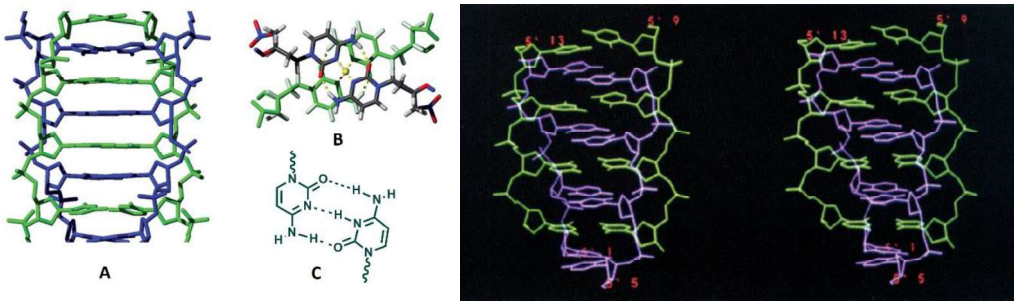


Figure 5. 2 Structure and hydrogen bonding in i-motif

The distance between two base pairs is 3.1 Å with a helical twist of 12-16^0 (Figure 5.2). [6] The stacking interaction between adjacent base pairs is due to the stacking of exocyclic carbonyl and amino groups which are oriented in opposite dipole but not due to the stacking of aromatic nucleobases.[21,22]

The i-motif structure is stable across a narrow range of pH (3.5-5.5) but the maximum stability attained at a pH equal to the *pKa* of Cytosine N3.[23,24] But *invivo* in cellular environment due to the negative super helicity and molecular crowding the i-motif structure is stable under physiological pH. [25] Chemists developed small binding molecules called ligands and metal ions to stabilize the i-motif structure at neutral pH.[26,27]

5.1.2 i-motif analogues

To modulate the stability of i-motif structure various analogues have been synthesized and studied the effect of modification on the stability. The effect of modification on nucleoside was studied in modified oligo nucleotides. Oligo nucleotides with 5-propynyl-deoxyuracil [28] forms stable i-motif in contrast modification on cytosine at 5-position such as 5-methyl cytosine or 5-halo cytosine which destabilized the DNA i-motif.[17] (Figure 5.3)

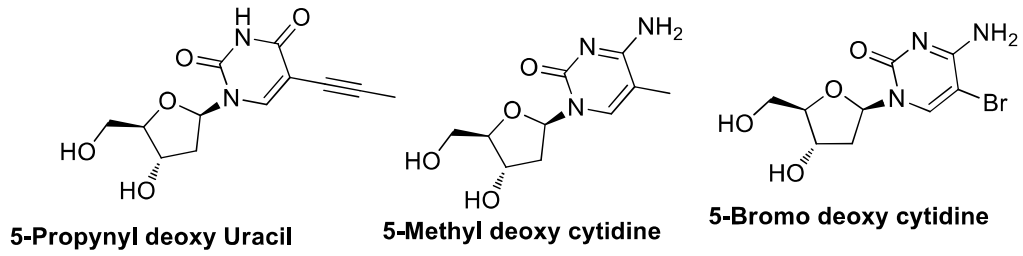


Figure 5. 3 Modified nucleobases structure used in i-motif formation

The effect of modification at the phosphate backbone on the stability of i-motif also studied in detail. The phosphoramidate (Figure 5.4a) oligonucleotides prevent the formation of i-motif at acidic pH but promote the formation of triple helices.[25] The modification of phosphate as methyl phosphate (Figure 5.4d) do not allow the formation of i-motif.[29] The thioanalogues of phosphate back bone phosprothioate [30,31] (Figure 5.4b & c) and phosphorothiolate (Figure 5.4e) allow the DNA sequences to fold into i-motif .The stability of i-motif formed by phosphorothiate was slightly lower than unmodified DNA while that of phosphorothiolate was higher due to the stabilization of C3'-*endo* sugar pucker confirmation. [32]

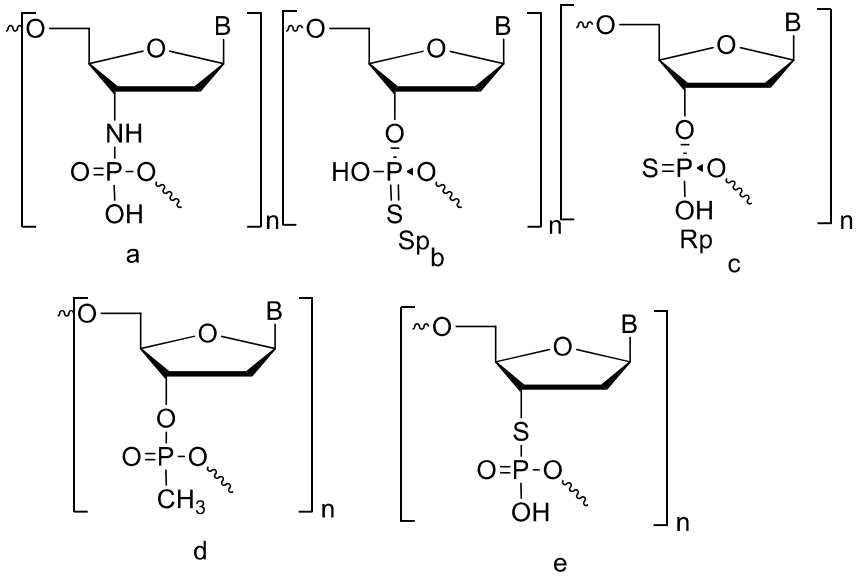


Figure 5. 4 Back bone modification of i-motif forming DNA sequence

The i-motif analogues with modification on sugar ring have been synthesized and studied their stability. Locked nucleic acids (LNA) (Figure 5.5a) are conformationally constrained analogues with 2'-O-4'-C-methylene bridge which locks the C3'-endo sugar conformation. The stability of i-motif has tuned by changing the substituent in the TC_5 sequence at different positions.[33] The effect of LNA on the stability of i-motif

in *C-myc* sequence also studied by changing the LNA at different positions.[34] Unlocked nucleic acids (UNA) (Figure 5.5b) are acyclic RNA derivatives. The effect of UNA monomers incorporated at different positions in 22-nucleotide human telomeric sequence and studied the effect on the stability of i-motif.[35,36] Significant effects are observed depending on the monomer position. Cytosine rich RNA sequences cannot form stable i-motif but forms a stable triplex formation.[37] Another analogue 2'fluoro arabinose (Figure 5.5c) derivative enhance the stability of i-motif at neutral pH due to favorable FC-H2'….O4' interactions.[38,39] Peptide nucleic acids (PNAs) (Figure 5.5d) are the neucleic acid analogues with amide back bone which are neutral and strongly bind to the DNA.[40] Alanyl PNA are the modified PNA analogues with alanyl back bone.[41] The Alanyl cytosine octamer could not form i-motif at pH 4.5 on contrary H-(Gly-AlaC)$_4$-Lys-NH$_2$ sequence formed the i-motif.[42] Amino ethyl glycinyl (*aeg*) peptide nucleic acids with TC$_8$ sequence forms i-motif with enhanced stability over the analogous dTC$_8$ sequence at acidic pH. [43] *Aeg* TC$_5$ sequence also studied in the formation of i-motif at narrow range of pH.[44] Further *aeg*TC$_5$ sequence has been studied in the formation of hybrid i-motif with analogous DNA and RNA with enhanced stability in comparison with DNA[45] and RNA[46,47] sequences. The enhanced stability of the resulting hybrid species are due to nullifying the negative charge repulsions of the DNA/RNA back bone. The PNA analogues have the net neutral/slightly positive charge reduce the repulsive interactions among the DNA strands and thus enhance the stability of the structure.

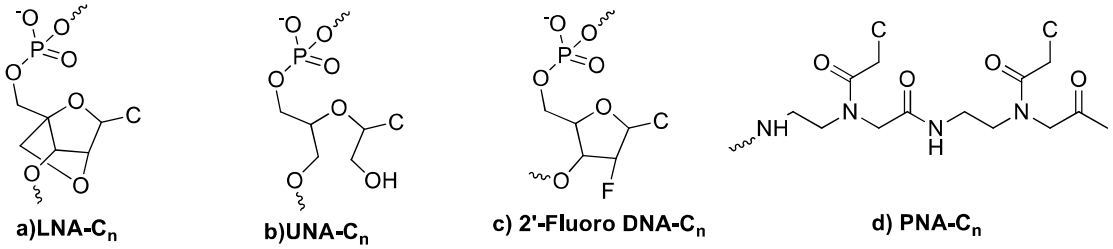


Figure 5 .5 Sugar ring modification of i-motif forming DNA sequence

5.1.3 Hypothesis and rational of present work

We hypothesized if a net positive charge can introduce in the hybrid i-motif structure which not only can reduce the negative charge repulsions but also can induce the positive-negtaive charge attractions which can stabilize the structure significantly. Amino ethyl prolyl (*Aep*) peptide nucleic acids[48-50] are conformationally constrained and can form positive charge in acidic medium. Guanine rich *aep* PNA are explored in the formation of stable G-quadraplexes.[51] We rationally designed *aep*-C_5 sequence and planned to study the hybrid i-motif formation with DNA and RNA which may stabilize the resulting structure. (Figure 5.6)

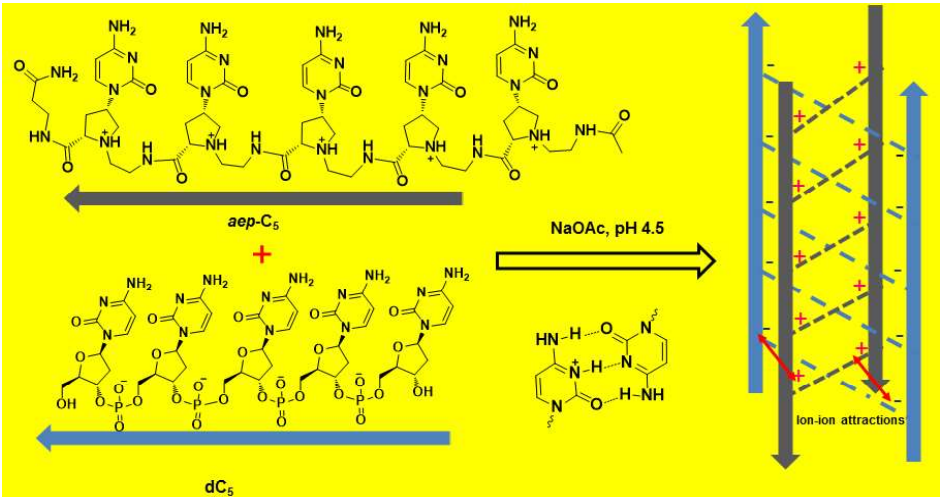
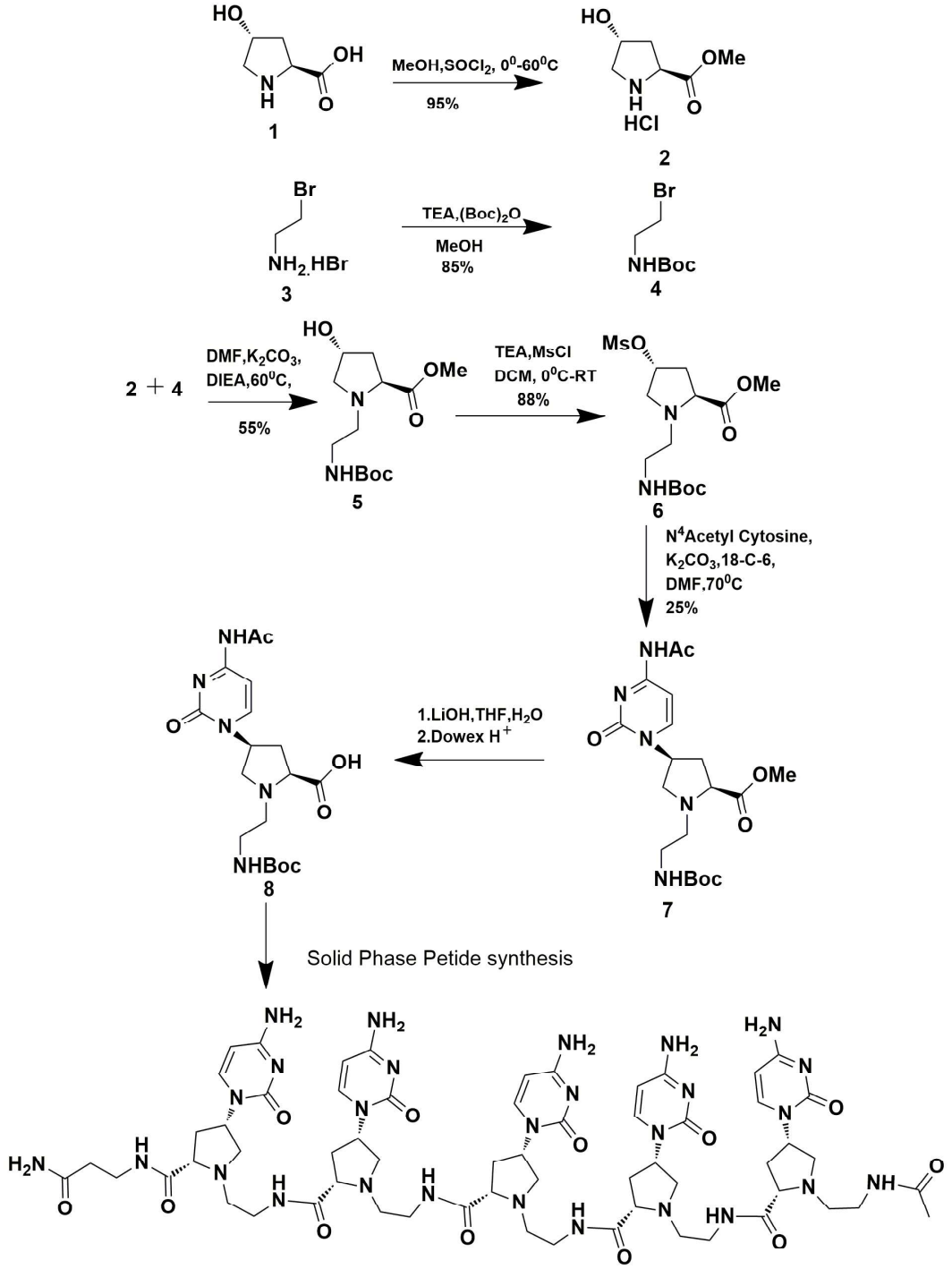


Figure 5. 6 Hybridf i-motif formation form DNA and *aep*-PNA DNA sequence

5.2. Results and discussions

5.2.1 Synthesis and of *aep*-PNA C_5

Scheme 5. 1 Synthesis of *Aep*-C_5



Aminoethyl prolyl cytosine monomer was synthesized by following reported procedure as described in scheme 5.1 2-Bromo boc ethylamine was treated with 4-hydroxy proline ester hydrochloride in anhydrous DMF ,DIEA and K_2CO_3 to obtain the amino ethyl 4-hydroxy proline (*aep*) in 55% yield which was converted to its mesyl derivative. *Aep* mesylate was treated with N^4-Acetyl cytosine, K_2CO_3 and 18-crown-6 in anhydrous DMF to obtain *aep* cytosine monomer. This *aep* cytosine monomer employed in the solid phase peptide synthesis to obtain the *aep*-cytosine pentamer (*aep*-C_5). After cleavage from the resin, PNA *aep*-C_5 was isolated by gel filtration (Sephadex G-15), and then purified by HPLC. The purified *aep*-C_5 was characterized by ESI-Mass studies (see in ESI). For comparative studies, DNA cytosine pentamer (dC_5) was purchased and directly used as control sample.

5.2.2 CD studies

45 µM dC_5, *aep*-C_5 and hybrid i-motif (22.5 µ m each strand) were dissolved in sodium acetate buffer (100 mM pH 4.5) and annealed. Annealing was performed by heating the samples to 90^{O}C for 10 minutes then left overnight for slow cooling and

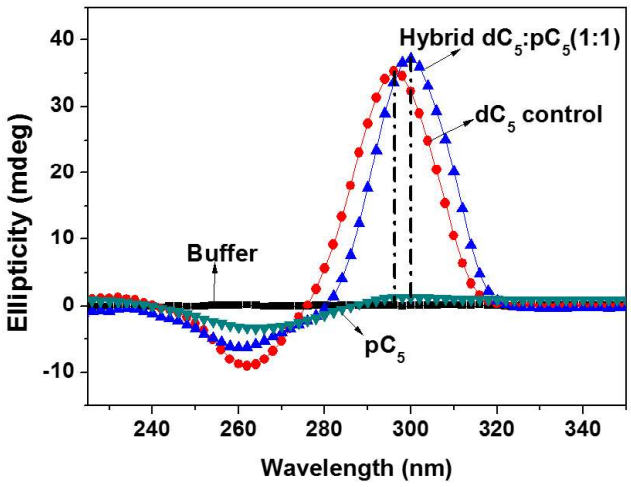


Figure 5. 7 CD-Spectra of *aep*-C_5, dC_5 and Hybrid

stored at 4 ^{O}C for 5 hours before the experiments. We recorded the CD spectra of the annealed PNA *aep*-C_5, DNA dC_5, and hybrid *aep*-C_5:dC_5 (1:1) samples. The CD spectra of DNA i-motif structure gives maxima (~296 nm) and minima (~261 nm) respectively. Similar CD spectrum is observed with control DNA dC_5 (Figure 7). CD spectrum of PNA *aep*-C_5 not matched with control DNA dC_5. The CD spectrum of hybrid dC_5:*aep*-C_5, however, exhibit characteristic CD signature with maxima (~300 nm) and minima (~261 nm) like control dC_5. Importantly the marginal red shift (~4.0 nm) is also observed in the CD maxima of i-motif in hybrid sample. These CD spectra support the formation of hybrid i-motif. i-motif formation by *aep*-C*5* was not observed under these conditions.The repulsive interactions between positive charge of prolyl amine and hemi-protonated cytosine residues may possibly destabilize i-motif. However the CD spectra of *aep*-C_5 at that pH condition has only minima at ~263 nm possibly due to the self-duplex (Figure 7).

As the i-motif formation is pH sensitive we prepared the samples (*aep*-C_5, dC_5 and hybrid) in different pH sodium acetate buffer ranging from 4 to 9. In finding of the appropriate pH conditions for the formation of hybrid i-motif, we recorded the pH dependent CD spectra of.

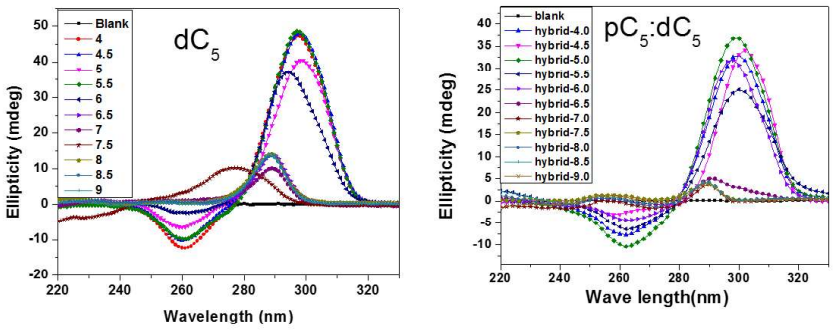


Figure 5. 8 pH dependent CD-Spectra of dC_5 and hybrid i-motf

hybrid dC_5: *aep*-C_5 (1:1), aep-C5 and control DNA dC_5. The pH dependent CD spectra of hybrid dC_5: *aep*-C_5 are depicted in Figure.8 .The pH dependent CD spectra of hybrid (*aep*-C_5:dC_5) is remarkably affecting with pH conditions as the intensity of characteristic i-motif maxima and minima are depleting with the increasing pH. These results indicate the formation of hybrid *aep*-C_5:dC_5 (1:1) i-motif are observed only at low pH range (4.5-6.0) like control dC_5

The pH dependent CD spectra of *aep*-C_5 depicted in figure 5.9. At pH 4.0 a negative peak observed the intensity of peak decreased at pH 4.5, at pH 5.0 the intensity of 264 nm peak was further decreased and a positive peak at 291 nm was observed, at pH 5.5 the negative peak at 264 nm switched to positive peak at 269 nm, at pH 6.0 the intensity of positive peak decreased and shifted to 282 nm, again at pH 6.5 the peak position shifted to 262 nm with increased intensity and again fall its intensity at pH 7.0 again the intensity of peak raised with increase in pH from pH 7.5 to 9.0 . This structure is highly pH responsive and can used as sensor for monitoring pH changes.

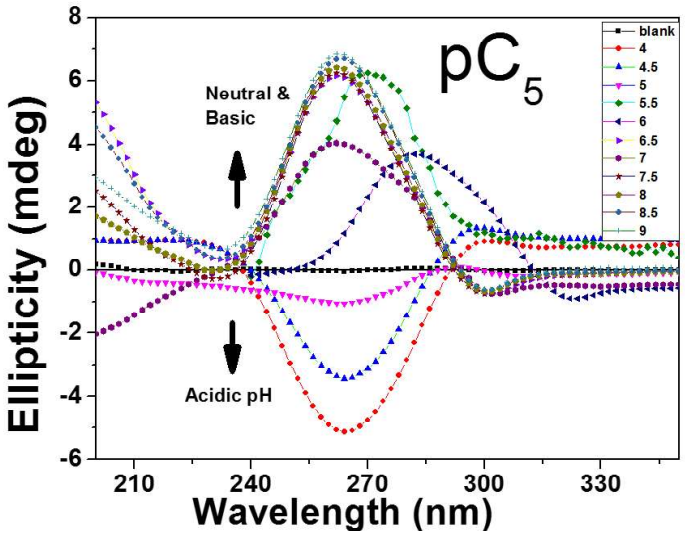


Figure 5. 9 pH dependent CD-Spectra of *aep*-C_5

Further we recorded the temperature dependent CD spectra of hybrid *aep*-C_5:dC_5 (1:1) and control dC_5 starting from 10ºC to 90ºC at an interval of 10ºC .The characteristic CD signature (of hybrid (*aep*-C_5:dC_5) are severely affected with temperature as like control dC_5 .These melting results indicate that the T_m (melting temperature) of hybrid i-motif is remarkably higher than that of control dC_5. The CD-maxima verses temperature gives inverse sigmoidal curve. For DNA the CD-maxima at 295 plotted at its temperature for hybrid the CD-maxima at 299 nm plotted against its temperature. Both control and hybrid gave inverse sigmoidal curve shown in inset. The temperature dependent CD spectra and the melting profiles of hybrid and DNA i-motif depicted below. (Figure 5.10)

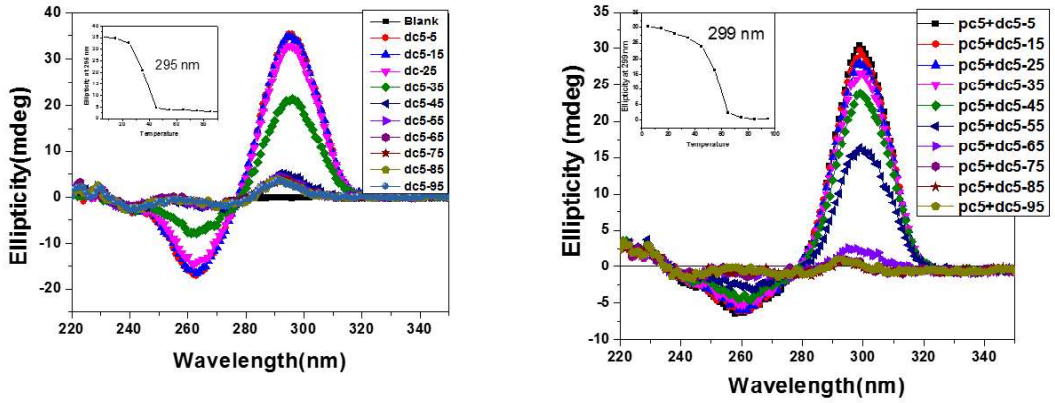


Figure 5.10 Temperature dependent CD-Spectra of dC_5 and hybrid

5.2.3 Thermal UV-studies.

The stability of DNA structures (duplex, triplex and *tetraplex*) are measured in term of their T_m (Thermal melting) values, which are extracted from respective sigmoidal melting profiles. [52] The temperature dependent UV studies are one of the inexpensive and accurate methods to determine T_m of DNA structures. The positive sigmoidal

melting profile at 260 nm or the negative sigmoidal melting profile at wavelength 300 nm are characteristic for DNA tetraplexes (G-quadruplex and i-motif) and the first derivative curve of those sigmoidal curves give accurate T_m of DNA structure. We, here, recorded the temperature dependent UV absorbance of the annealed *aep*-C_5/dC_5/hybrid *aep*-C_5:dC_5 (1:1) sample at λ_{295nm} under acidic condition (pH 4.5), and plotted their respective thermal melting profiles (Figure 5.11). The hybrid (*aep*-C_5:dC_5) and control dC_5 samples exhibit the negative sigmoidal curve (at λ_{295nm}) which are characteristic for the denaturation of i-motif structure, while alone *aep*-C_5 exhibit positive sigmoidal curve (at λ_{295nm}), non-characteristic for i-motif structure at that wavelength.

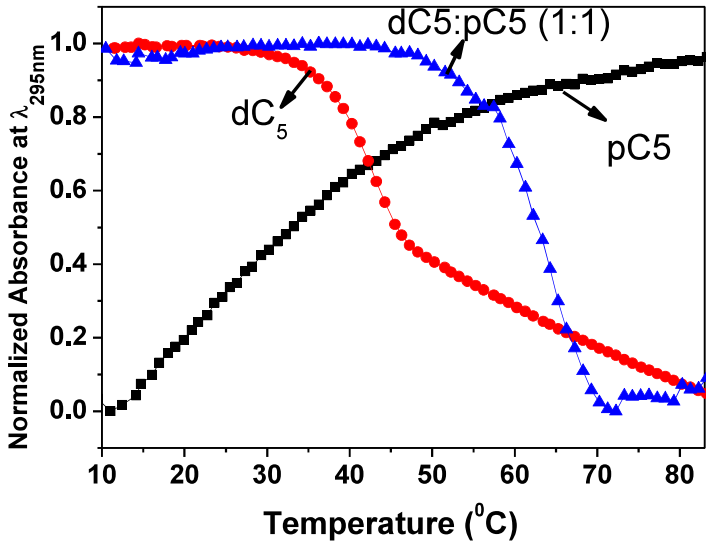


Figure 5.11 Temperature dependent UV-Spectra of annealed dC_5 , *aep*-C_5 and hybrid

T_m values of hybrid (dC_5:*aep*-C_5) and dC_5 are extracted from their respective melting profiles (Table 5.1. Entry1). The T_m value of hybrid (dC_5: *aep*-C_5) i-motif is remarkably higher than control dC_5 with temperature difference (ΔT_m) ~15.0^{0}C under that pH (4.5) conditions. However, the positive sigmoidal curves at λ_{300nm} for *aep*-C_5

is presumably due to the self-duplex formation consisting hemi-protonated cytosine complex (C^+H:C) under that pH conditions.

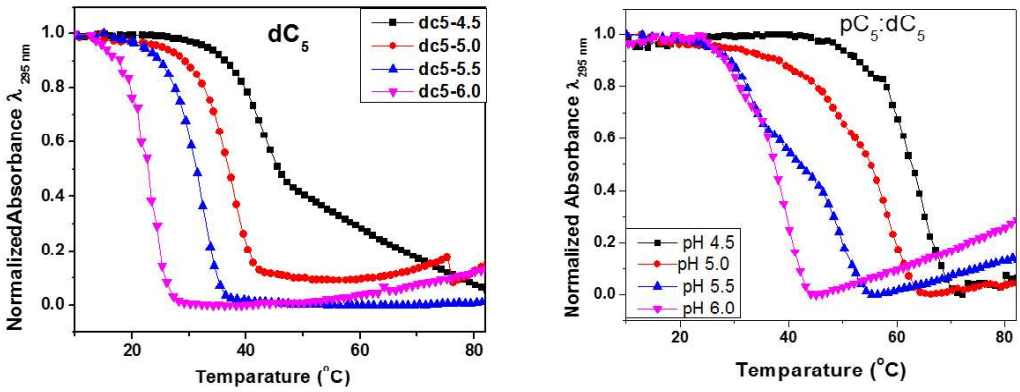


Figure 5. 12 Temperature dependent UV-Melting curves of dC_5 and hybrid

Further we investigated the range of acidic pH for the formation of i-motif structure in hybrid *aep*-C_5:dC_5/control dC_5 samples, and then determined their T_m at other acidic pH conditions (4.5-6.0). We performed thermal UV-melting experiment at λ_{295nm} with *aep*-C_5, dC_5 and hybrid *aep*-C_5:dC_5 (1:1) under different acidic pH range (4.5-6.0). UV-melting profiles of hybrid (*aep*-C_5:dC_5) at acidic pH (4.5-6.0) are depicted in the Figure 5.12, while melting profiles of *aep*-C_5 under similar pH conditions are provided in figure 5.13. Except *aep*-C_5, the melting profiles of dC_5 and hybrid *aep*-C_5:dC_5 exhibit negative sigmoidal curve at $\lambda_{295nm,}$ characteristic of i-motif under acidic pH range (4.5-6.0). A consistency as negative sigmoidal curve is observed in melting profiles of hybrid aep-C5:dC_5 as like control dC_5 under acidic pH range 4.5.0-6.0. However, we could not observed sigmoidal curve at λ_{295nm} with dC_5/ *aep*-C_5/hybrid dC_5: *aep*-C_5 at neutral pH. We extracted their T_m values from respective melting profile and summarized in Table 5.1. The comparative stability of hybrid i-motif over control are measured as ΔT_m and given in Table 5.1. Herein, the T_m value of

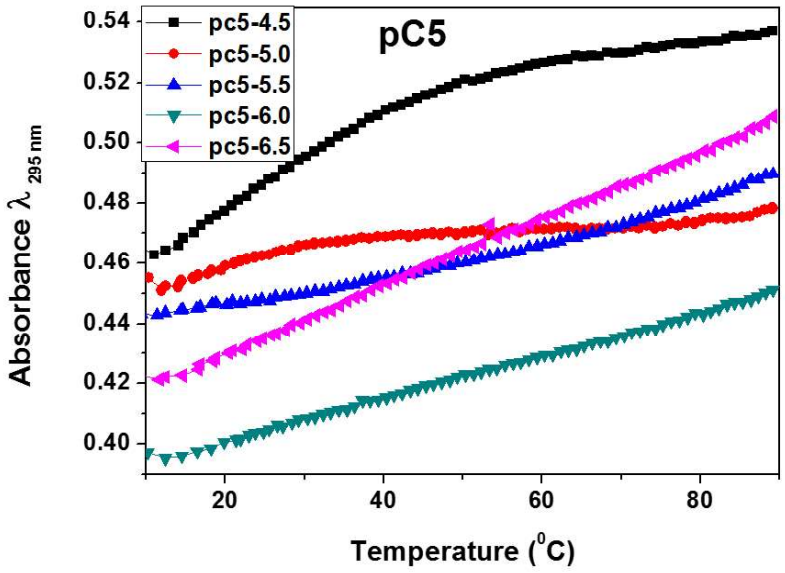


Figure 5. 13 Temperature dependent UV-Melting curves of *Aep*-C_5

Table 5. 1 pH dependent UV-Tm of dC5/Hybrid dC5:aep-C5 (1:1)

Entry	pH	T_m (ºC) of dC5	T_m (ºC) of dC5:aep-C5(1:1)	ΔT (ºC)*
1	4.5	46.73 (35.82) §	62.00 (55.4) §	15.27 (19.58)
2	5.0	36.13	54.06	17.93
3	5.5	31.26	40.11	8.85
4	6.0	22.66	35.82	13.16

* ΔT_m is difference in T_m of dC_5 and dC_5:*aep*-C_5 (1:1).
§T_m determined by CD melting.

hybrid dC_5: *aep*-C_5 i-motif structures are remarkably higher than control dC_5 i-motif under acidic pH conditions. The observed ΔT_m is following ~15.0^{0}C at pH 4.5 (Entry 1), ~18.0^{0}C at pH 5.0 (Entry 2), ~9.0ºC at pH 5.5 (Entry 3) and ~13.0ºC at pH 6.0 (Entry 4). The maximum ΔT_m of hybrid *aep*-C_5:dC_5 i-motif structure is under pH 5.0 conditions. These thermal UV-melting studies strongly support the formation of

hybrid *aep*-C_5:dC_5 (1:1) i-motif structure with remarkable stability over control dC_5 even near neutral (pH 6.0) conditions.

5.2.4 ^{1}H 1D NMR studies.

Further we studied i-motif formation by dC_5/hybrid *aep*-C_5:dc_5 by ^{1}H-NMR of hydrogen bonded *imino* N-H of protonated cytosine residues. DNA i–motif structures have the characteristic ^{1}H-NMR signal (δ 15.0-16.00) of *imino* N-H of protonated cytosine residue. We recorded the ^{1}H-NMR spectrum of annealed *aep*-C_5/dC_5/hybrid *aep*-C_5:dC_5 (1:1) at low temperature (~4.0/25.0^{o}C). The imino regions of ^{1}H-NMR spectrum of the annealed dC_5/hybrid dC_5: *aep*-C_5 hybrid are depicted in Figure **5.14**. The ^{1}H-NMR spectrum of the hybrid dC_5: *aep*-C_5 (1:1) exhibit ^{1}H-NMR signal at δ 15.0-16.00 like control dC_5 at both temperature 4.0^{o}C and 25^{o}C.

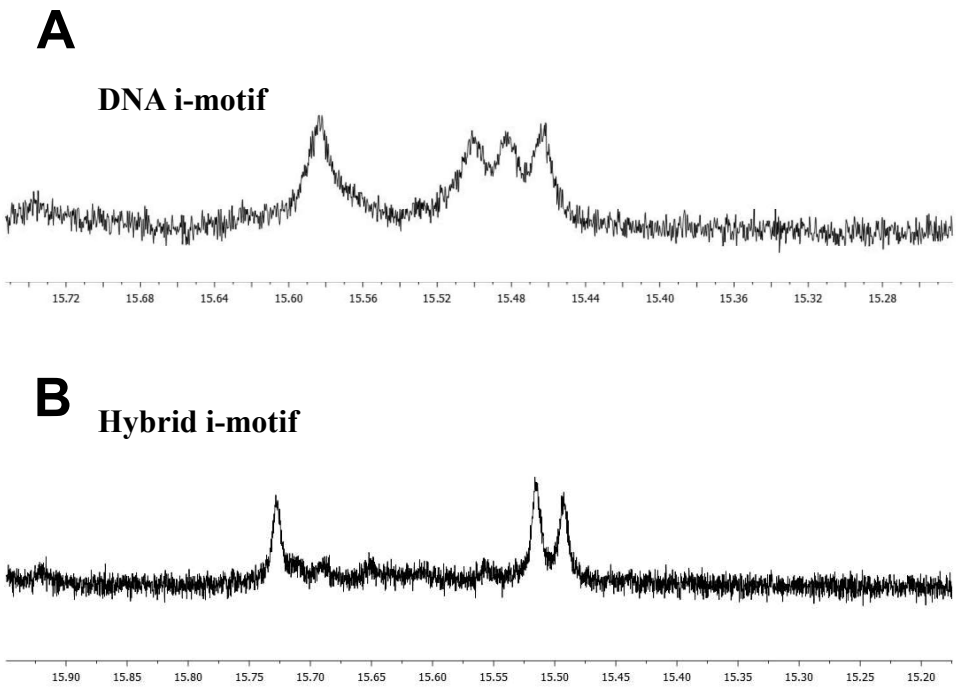
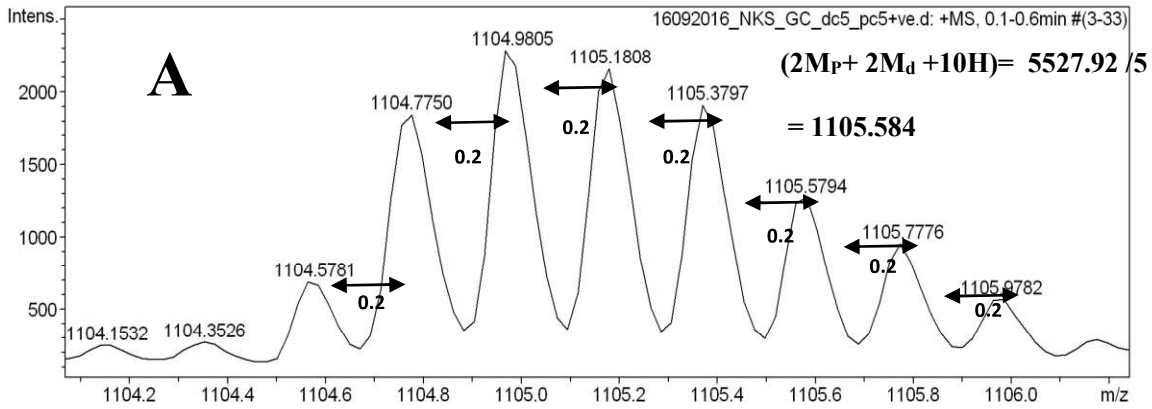


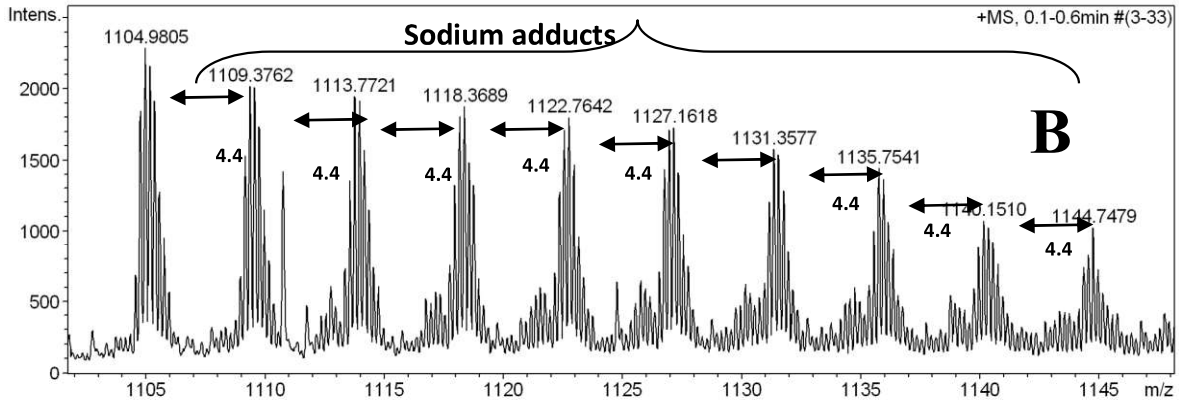
Figure 5. 14 ^{1}H-NMR of hybrogen bonded *imino* N-H of protonated cytosine at pH 4.5 at 25^{0}C (700MHz): A. DNA dC_5; and B. hybrid dC_5: *aep*-C_5 (1:1)

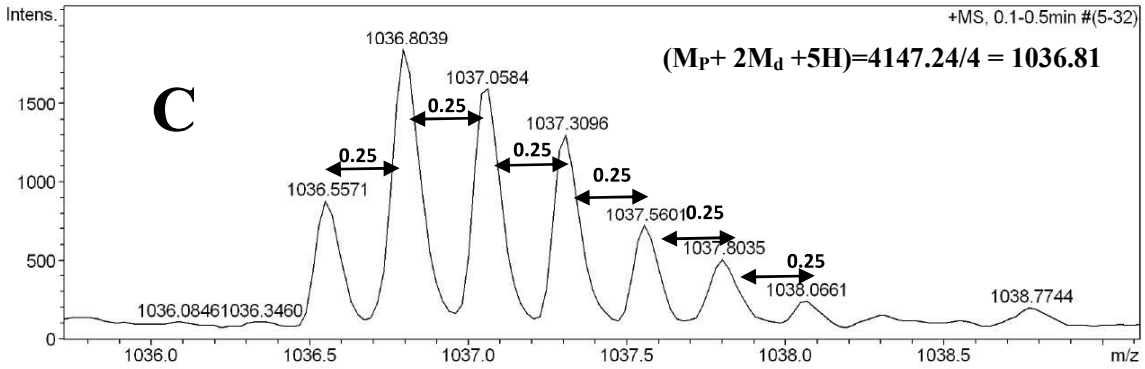
However no characteristic ^{1}H-NMR signal (δ 15.0-16.00) noticed in the ^{1}H-NMR spectra of annealed-aep-C5. These NMR results strongly support the hybrid i-motif formation by *aep*-C_5:dC_5 (1:1) like control dC_5 at low pH conditions.

5.2.5 ESI-MS analysis

For quantitative support of the hybrid (*aep*-C_5:dC_5) i-motif formation, we recorded ESI-Mass spectra of *aep*-/dC_5/hybrid *aep*- C_5:dC_5 before/after annealing under acidic (pH 4.5) conditions. The mass spectra of selected regions are given in figure 5.15. Mass analysis shows that no complex structure is observed in mass spectrum of un-annealed dC_5/*aep*-C_5 sample. The mass spectrum of annealed hybrid-*aep*- C_5:dC_5 (1:1) shows mass peaks of doubly charged dimer as m/z 1384.75(Figure 5.15 E) with isotopic pattern separated by m/z 0.50$[2M_d+5H]^{2+}$,a triply charged trimer as m/z 1382.39 (Figure 5.15 D) with isotopic pattern separated by m/z 0.33$[2M_d+ M_p+ 5H]^{3+}$,a tetra charged trimer as m/z 1036.8030 (Figure 5.15 C) with isotopic pattern separated by m/z 0.25 ,anda quintuple charged tetramer as m/z 1104.9805(Figure 5.15 A) isotopic pattern separated by m/z 0.2 $[2M_d+M_p+10H]^{4+}$, which belong to the self-dC_5-dimer, hybrid trimer as 2dC_5:*aep*- C_5 (2:1)and hybrid tetrameras 2dC_5:2*aep*-C_5 (1:1) respectively. The appearance of mass peak of hybrid trimer is probably occurred due to the separation of one *aep*-C_5 strand from hybrid *tetraplex*.We also observed quintiple charged sodium adducts of hybrid tetraplex (1104.98 + ~ 4.4) (Figure 5.15 B) with mass difference of ~ 4.4 units. These mass peaks strongly confirm the formation of hybrid *aep*-C_5:dC_5 (1:1) i-motif structure under acidic conditions.


Intens.
16092016_NKS_GC_dc5_pc5+ve.d: +MS, 0.1-0.6min #(3-33)
A
(2MP+ 2Md +10H)= 5527.92 /5
= 1105.584
1104.9805
1105.1808
1104.7750
1105.3797
1105.5794
1105.7776
1104.5781
1104.1532
1104.3526
0.2
m/z


Intens.
+MS, 0.1-0.6min #(3-33)
Sodium adducts
B
1104.9805
1109.3762
1113.7721
1118.3689
1122.7642
1127.1618
1131.3577
1135.7541
1140.1510
1144.7479
4.4
m/z


Intens.
+MS, 0.1-0.5min #(5-32)
C
(MP+ 2Md +5H)=4147.24/4 = 1036.81
1036.8039
1037.0584
1037.3096
1036.5571
1037.5601
1037.8035
1038.0661
1038.7744
1036.0846
1036.3460
0.25
m/z

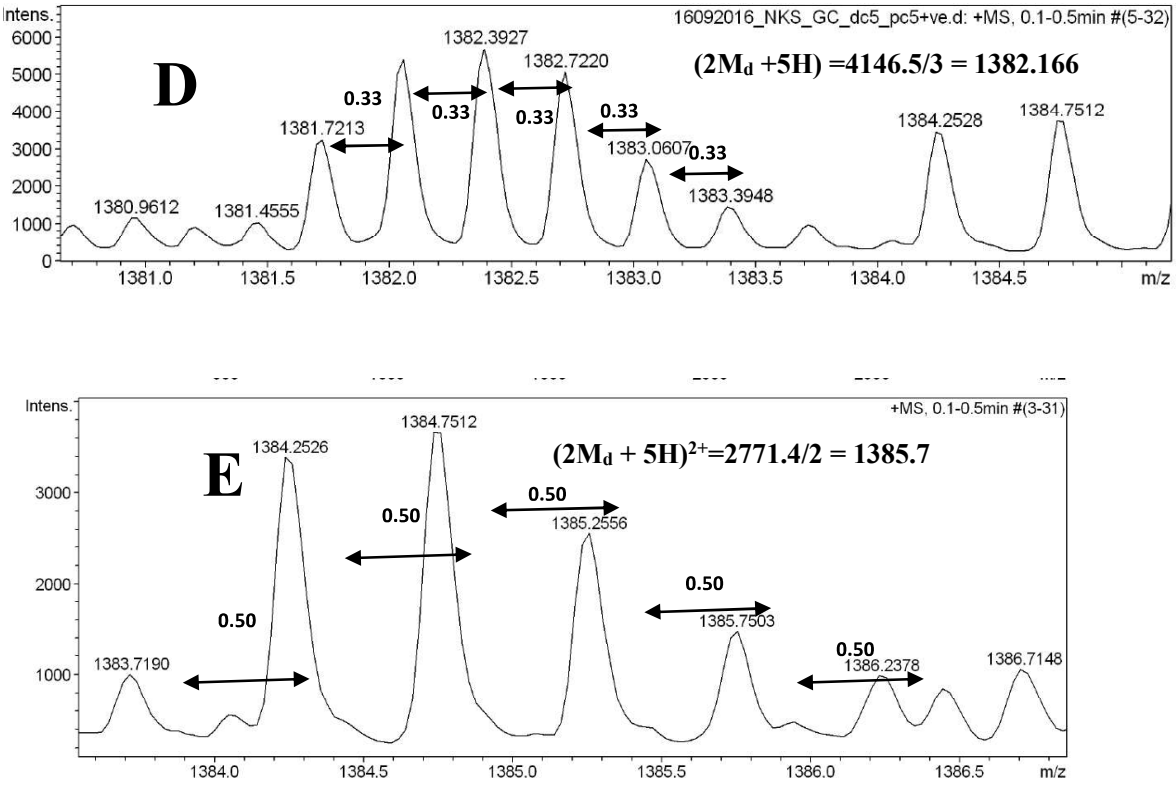


Figure 5. 15 .ESI-MS of hybrid i-motif dC$_5$:*aep*-C$_5$ (1:1) and associated species

the mass spectra of annealed *aep*-C$_5$ (positive mode) exhibit mass peaks as triply charged dimer as m/z 918.4700 (Figure 5.16 C) with isotopic pattern separated by m/z 0.33$[2M_p+4H]^{3+}$,a tetra charged trimer as m/z 1033.2809 (Figure 5.16 B) with isotopic pattern separated by m/z 0.25$[3M_p+5H]^{4+}$, and a quintuple charged tetramer as m/z 1102.1630 (Figure 5.16 A) with isotopic pattern separated by m/z 0.2 $(4M_p+8H)^{5+}$. These mass peaks indicate that *aep*-C$_5$ forms *self-duplex*, *triplex* and *tetraplex (as trace)* under acidic conditions. These complex structures are apparently formed due to the hydrogen bonding between cytosine and hemi protonated cytosine of *aep* -C$_5$. Although *tetraplex* structure as i-motif in *aep*-C$_5$ were not observed in CD and UV studies because of probably two reasons: (i) poor stability and (ii) very less

quantities. The poor stability of *aep*-C_5-*tetraplex* is probably due to the strong repulsion between the positively charged prolyl backbones of *aep*-C_5.All the prominent mass peaks are summarized in the table 5.2.

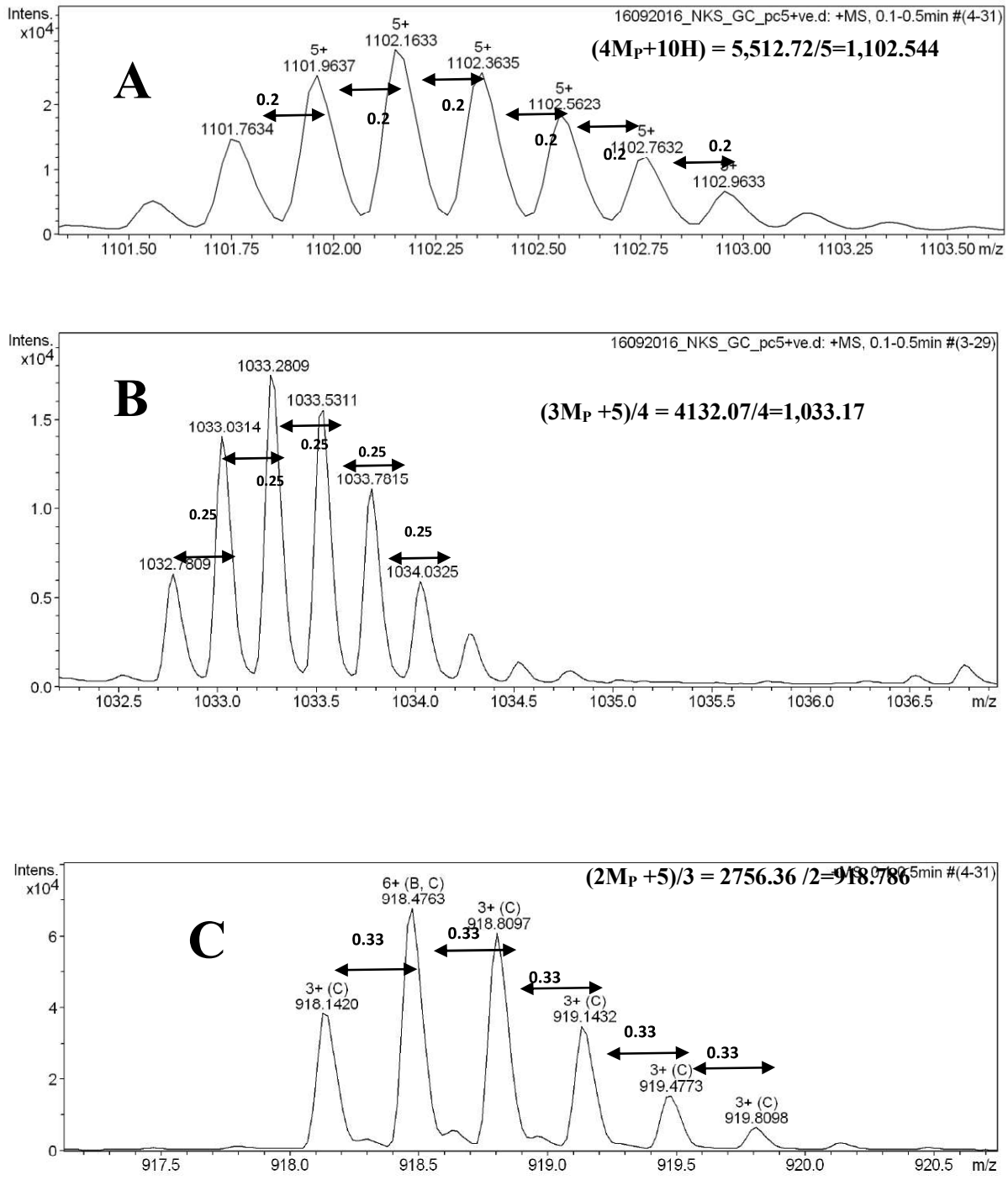


Figure 5. 16 .ESI-MS of hybrid i-motif dC_5: *aep*-C_5 (1:1) and associated species

Table 5. 2 ESI-Mass analysis of *aep*-C_5/dC_5/hybrid dC_5:*aep*-C_5

Entry	*aep*-C_5/hybrid *aep*-C_5:dC_5 (calculated Mass)	Mass (Observed)*
1.	dC_5 (M_d =1383.2760)	1382.0566 $(M_d\text{-}H)^{-*}$
2.	*aep*-C_5 (M_p=1375.6900)	1376.7100 $(Mp+H)^{+}$
3.	Annealed-*aep*-C_5, (pH4.5)	918.4700 $(2M_p+4H)^{3+}$; 1033.2809 $(3M_p+5H)^{4+}$; 1102.1630 $(4M_p+8H)^{5+}$
4.	Annealed-*aep*-C_5:dC_5 (1:1); (pH 4.5)	1036.8030 $(2M_d+M_p+5H)^{4+}$; 1104.9805 $(2M_p+2M_d+10H)^{5+}$;1382.3900$(2M_d+M_p+5H)^{3+}$,1384.75$(2M_d+5H)^{2+}$

*M_d: Molecular mass of DNA dC_5 ; M_p: Molecular mass of PNA *aep*-C_5

5.2.6 FE-SEM studies.

We here compared the morphologies of annealed-*aep*-C_5/dC_5/hybrid *aep*-C_5:dC_5, under acidic (pH 4.5) conditions, by FE-SEM imaging technique. The FE-SEM images (at 200nm scale) of annealed *aep*-C_5/dC_5/hybrid dC_5:*aep*-C_5 (1:1), under pH 4.5, are depicted (Figure 5.17), while their SEM images at other scales and EDAX are provided in the Supporting Information. The SEM image of dC_5 shows small round shaped crystals (nm size) (Figure 5.17A), while the SEM-image of *aep*-C_5 shows cuboid shaped crystals (μm-nm size) (Figure 5.16B). Importantly the SEM-images

hybrid *aep*-C_5:dC_5 (1:1) shows increased large (µm) sized symmetrical brick shaped. crystals (Figure 5. 17 C&D).The

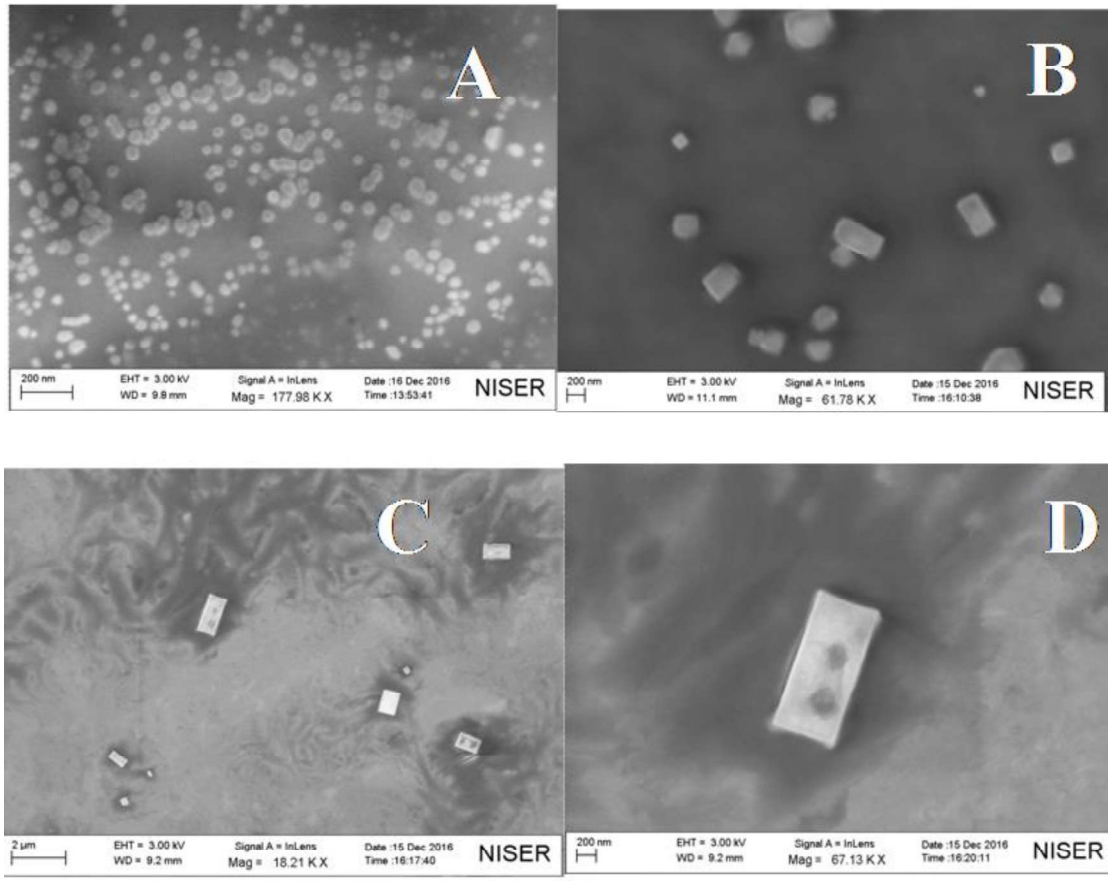


Figure 5. 17 FE-SEM images of annealed (at pH 4.5) samples: a. dC_5 (A); b, *aep*-C_5 (B); (c) hybrid dC_5:*aep*-C_5 (1:1) (C& D)

increase in size of structure could be possibly due to the ion-ion attraction forces between positively charged aep-PNA back bone and negatively charged DNA phosphate back bone. These SEM-images comparisons strongly support the interaction of dC_5 with *aep*-$C_5$by ion-ion interaction and formation of hybrid *aep*-C_5:dC_5 i-motif

5.2.7 RNA-*aep* PNA Hybrid i-motif formation

After studying the hybrid i-motif formation from aep-PNA and DNA we planned to study the RNA-*aep* PNA hybrid i-motif formation. We designed an analogous sequence rU_2C_5 to study the hybrid i-motif formation and as control also. To form i-motif 50 µm of rU_2C_5 and 50 µm of hybrid i-motif (25 µm each of *aep*-C_5 and rU_2C_5) were annealed in sodium acetate buffer (100mM pH 4.5) and recorded CD spectra at 10 ° C. The CD spectrum of RNA shows CD maxima at 279 nm. Hybrid i-motif also shows similar spectrum with CD maxima at 285 nm. (Figure 5.18)

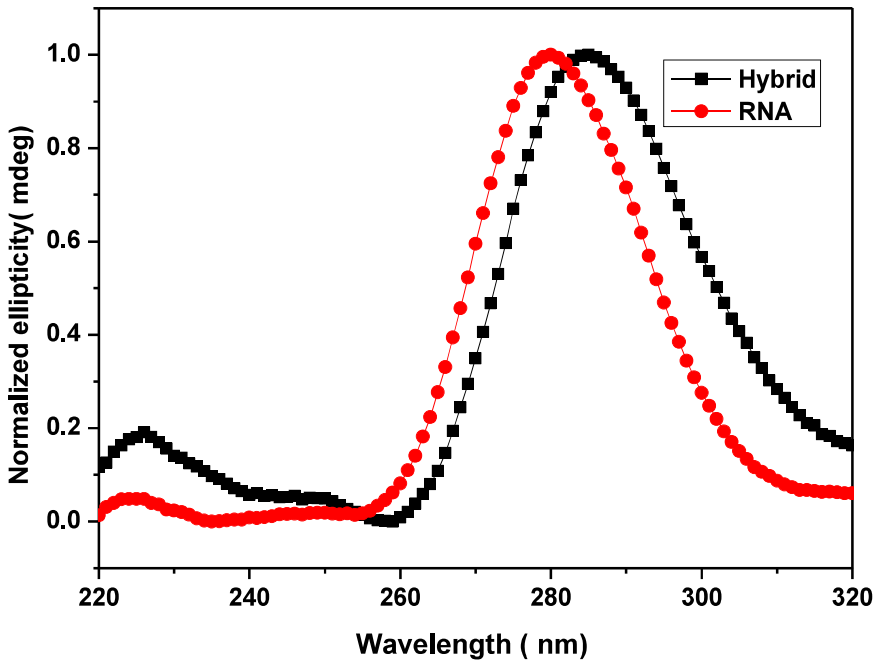


Figure 5. 18 i-motif formation by RNA and Hybrid RNA-PNA

5.2.8 CD-spectra of aep PNA-RNA hybrid i-motif

To study the stability of hybrid i-motif formed by RNA and PNA we recorded temperature dependent CD spectra of hybrid i-motif along with the control RNA. The experiments performed from 10ºC to 90 ºC with an interval of 10ºC .We observed a depletion of intensity of i-motif peak at 279 nm with increase in temperature of RNA i-motif. (Figure 5.19) Similar results observed for the hybrid i-motif .The intensity of peak at 287 nm depleted and shifted to 276 nm with rise in temperature (Figure 5.20). We plotted the CD maxima verses temperature (Figure 5.21). We observed an inverse sigmoidal curve with smooth transition for hybrid i-motif but for RNA i-motif we could not observe a smooth transition. This experiments support that the hybrid – imotif formed by PNA-RNA is more stable than the control RNA i-motif.

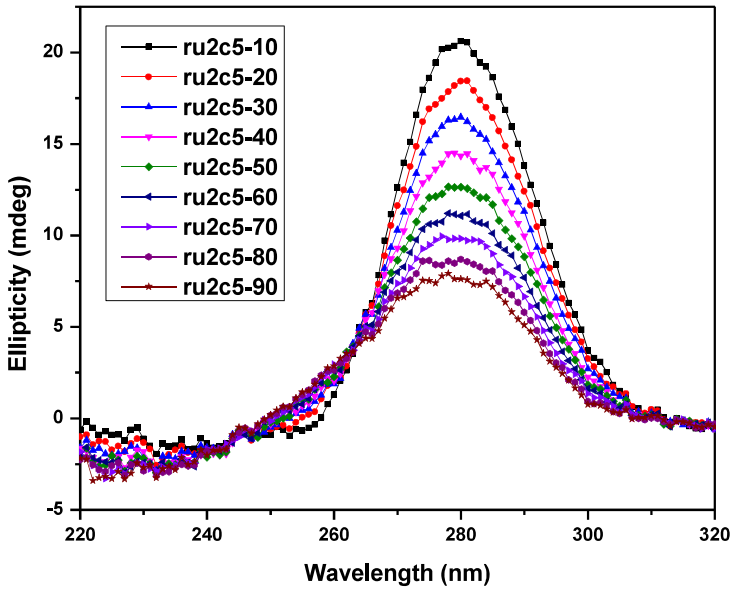


Figure 5. 19 Temperature dependent CD-Spectra of RNA i-motif

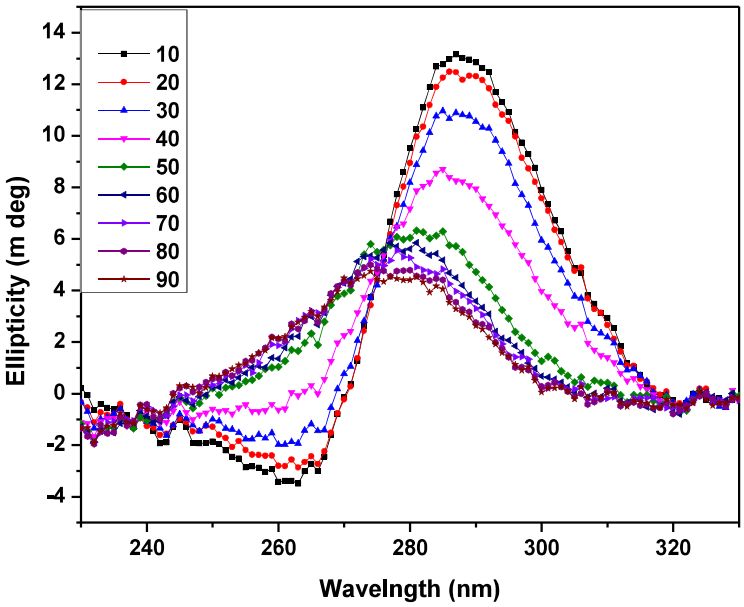


Figure 5. 20 Temperature dependent CD-Spectra of hybrid (RNA-PNA) i-motif

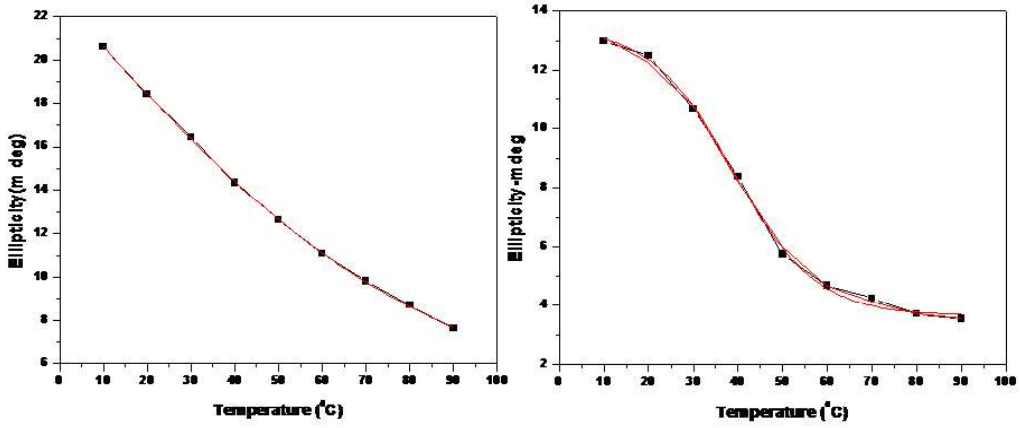


Figure 5. 21 CD melting profoile of RNA and hybrid (RNA-PNA) i-motif

As the i-motif structure is sensitive to pH changes we were interested to study the effect of pH on i-motif .To study the i-motif formation at different pH we prepared samples of RNA and hybrid in different pH (100 mM sodium acetate) ranging from 4.0 to 6.0 We observed the formation of i-motif by RNA at pH 4.0 ,4.5 and 5.0 with CD maxima at 279 nm at pH 5.5 the peak at 281 nm shifted to lower wavwlength 277 nm , at pH6.0 and 6.5 also the CD maxima of RNA observed at 277 nm. .(Figure 5.22) We also recorde the similar CD spectra form thehybrid i-motif .We observed similar spectra like RNA .The CD maxima of hybrid i-motif remains at 287 nm from pH 4.0 to 5.0 , shifted to 279 nm at pH 5.5 , further on increasing the pH to 6.0 and 6.5 the CD maxima shifted to 275 nm. .(Figure 5.23) From this experiments it is clear that the stability of i-motif is depend on the pH .RNA forms i-motif upto pH5.0 and cannot form imotif beyond pH5.0 hybrid i-motif also forms i-motif upto pH 5.0 and a transition at pH 5.5 and no i-motif formation after 5.5 pH not observed.

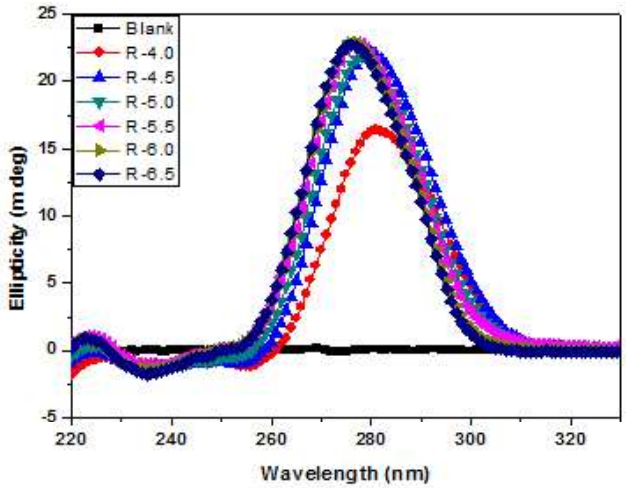


Figure 5. 22 pH dependent CD spectra of RNA i-motif

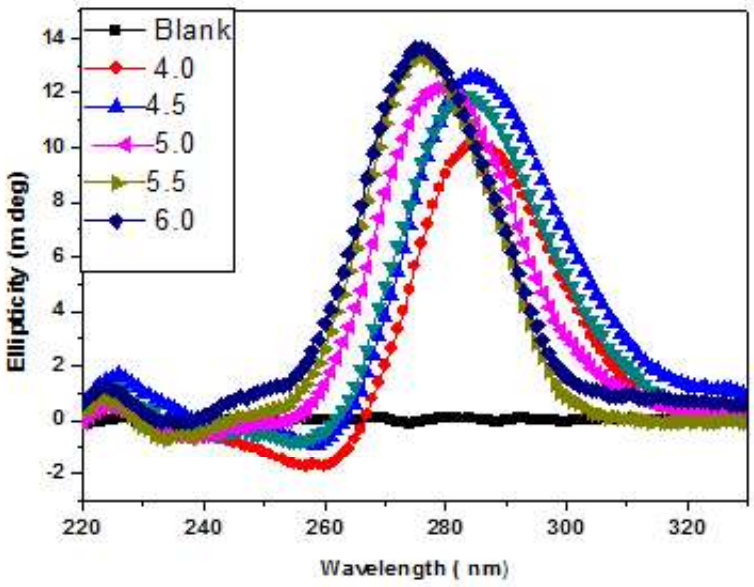


Figure 5. 23 pH dependent CD spectra of Hybrid i-motif

5.3 Conclusions

In summary we have synthesized the aep-PNA cytosine pentamer (*aep*-C_5) and characterized. The *aep*-C_5 successfully employed in the formation of hybrid i-motif with DNA and studied by using UV/CD/NMR/ESI-MS/FE-SEM .The hybrid i-motif is vaery stable owing to the stabilization by ion-ion interaction. This is the first report of hybrid i-motif where ion-ion attractions used to stabilize the i-motif. This structure is the most stable hybrid i-motif .The RNA-PNA hybrid i-motif also studied by CD spectroscopy. The RNA-PNA hybrid i-motif is more stable than the native RNA i-motif. In conclusion we have developed two novel i-motif structures and studied their biophysical studies by various techniques. The resulting structures can be used in nanotechnology to form stable i-motif for various applications.

5.4 Experimental section

Methods and materials

All reagents were purchased from commercial sources. DMF and DCM dried over calcium hydride and stored on 4 Å molecular sieves. DIPEA dried over anhydrous potassium hydroxide and stored on 4 Å molecular sieves. Deoxy oligonucleotides purchased from IDT. HPLC performed on Waters semi-preparative HPLC by using the sunfire column (C-18, 4.6 x150 mm, 5µm).ESI-MS experiments performed on Bruker ESI-MicroTOF-QII. NMR spectra recorded on Bruker-700 MHz or Bruker-400 MHz. UV experiments and melting experiments performed on JASCO-V730 with a peltier system. Melting experiments were performed at a rate of $1^{O}C$ per minute increment at 295 nm. The melting data processed by origin, the melting curves fitted in sigmoidal plot and T_m determined by differentiating first derivative.CD experiments were performed on JASCO-J 1500 all CD experiments were performed at $5^{O}C$ otherwise mentioned. Temperature controlled by peltier system.CD experiments performed by subtracting buffer as blank. All CD spectra recorded 2 scans and averaged, CD spectra recorded from 330-220 nm or 400-200 nm. Melting experiments performed at a rate of $3.3^{O}C$ per minute at an interval of $10^{O}C$.

Experimental procedure

Synthesis and purification of aep-PNA C_5: aep-PNA cytosine monomer synthesized starting from 4-Hydroxy proline (**1**) following similar method reported literature.[1,2]

*4-Hydroxy proline ester hydrochloride (**2**)*: 4-Hydroxy proline (10.0 g, 76.33 m mol) dissolved in dry methanol (150 ml) and stirred for 10 minutes under nitrogen atmosphere. The reaction mixture cooled to 0^{o}C by ice bath and thionyl chloride (152.6 mmol, 11.07 ml) added dropwise under nitrogen atmosphere .The ice bath removed and the reaction mixture refluxed for six hours. The solvents were removed under reduced pressure to obtain a white solid which was dissolved in methanol and again evapourated to dryness to yield 13.54 g of the title compound in 98% yield. ^{1}H NMR,(400 M Hz-DMSO-D_6): δ ppm 9.81 (2H br, s),5.60 (1H br, s), 4.5 (2H ,m), 3.75 (3H , s), 3.35 (1H ,m), 3.06 (1H , d, 12Hz), 2.22-2.17 (1H ,m), 2.12-2.05 (1H ,m).^{13}C NMR, (100 M Hz-DMSO-D_6) δ ppm 169.07,68.44, 57.48, 53.06, 36.99, HRMS: (ESI-Tof) Calcd for $C_6H_{11}O_3N$ 146.0812,Found 146.0821

*2-N-Boc-aminoethylbromide (**4**):* 2-Bromo ethylamine hydrobromide (10.0 g, 49 mmol) dissolved in dry methanol (100 ml) and stirred under nitrogen atmosphere for 10 minutes. Boc anhydride (22.62g, 98.5 m mol) and triethyl amine (13.7 ml, 98.5 mmol) dissolved in drymethanol and added to the reaction mixture dropwise for 30 minutes and stirred overnight under nitrogen atmosphere. The reaction mixture evapourated to dryness and dissolved in water and extracted with dichloromethane .The organic layer washed with brine and water, dried with anhydrous sodium sulphate and concentrated in roto followed by purification on silcagel with dichloromethane obtained 8.79 g of title compound as colourless syrup in 80% yield.

TLC 15% EtOAc/DCM R_f =0.76 ^{1}H NMR (700 MHz, $CDCl_3$) δ ppm 4.93(1H, s,br), 3.54-3.53(2H, m,br), 3.47-3.46 (2H, m, br)1.55 ma,1.45 mi(9H,s), ^{13}C NMR, (175 MHz, $CDCl_3$) δ ppm 146.81, 85.26, 42.41, 32.89, 28.41, 27.40 , HRMS: Calcd. ($C_7H_{14}BrNO_2$ +Na) 246.0100 & 248.0080 (M & M+2) Found 246.0070 & 248.0050.

N-Boc-aminoethyl-4-(R)-hydroxy-2-(S)-proline methyl ester (*aep*) (***5***): 4-Hydroxy proline ester hydrochloride(5.0 g, 27.6 mmol) and 2-Bromo boc ethyl amine(6.16 g, 27.6 mmol) , potassium carbonate(3.8 g, 27.6 mmol) and diisopropyl ethyl amine(9.6 ml, 55.2 mmol) were dissolved in dry dimethyl formamide (50 ml) and stirred at 60ºC overnight .The reaction mixture concentrated to dryness and purified by column chromatography on silica gel using dichloromethane and methanol as solvents to obtain 4.37 g of title compound as brown oil in 55% yield. TLC 40% EtOAc/DCM R_f =0.45 ^{1}H NMR,(400 MHz-$CDCl_3$): δ ppm 5.34(1H,br,s), 4.46(1H, m),3.67(3H, s), 3.56(1H, t,8Hz),3.37-3.33 (1H, m), 3.1(2H, m),2.77-2.71(1H, m),2.66-2.63(1H, m),2.48-2.45(1H, m), 2.10-2.07 (2H, m),1.39 (9H, s), ^{13}C NMR, (100 M Hz- CD Cl_3) δ ppm 174.55, 156.3, 79.13, 70.23, 64.28,61.22,54.03,53.51,51.76,39.40,39.21, 28.43 HRMS: (ESI-Tof) Calcd for $C_{13}H_{24}O_5N_2$ 289.1758, Found 289.1718.

O-Mesyl N-Boc-aminoethyl-4-(R)-hydroxy-2-(S)-proline methyl ester (***6***): Compound **5** (4.0 g, 13.88 mmol) dissolved in dry dichloromethane (50 ml) and triethyl amine (3.87 ml, 27.7 mmol) was added and, the reaction mixture cooled to 0ºC and stirred under nitrogen atmosphere for 10 minutes .Mesyl chloride (1.94 ml, 27.7 mmol) was added dropwise and the reaction mixture stirred two hours at room temperature. After completion of the reaction dichloromethane was added to the reaction mixture and extracted with water. The organic layer was washed with sodium bicarbonate and brine solutions, dried with anhydrous sodium sulphate concentrated to obtain 4.47 g of title compound as brown syrup in 88% yield. TLC 15% EtOAc/DCM R_f =0.61 ^{1}H NMR,(400 M Hz-$CDCl_3$): δ ppm , 5.18-5.15(2H, m) 3.67(3H, s),3.58 (1H, t 8Hz),3.44-3.48(1H, m), 3.12-3.13(1H, m,br),2.98 (3H, s)2.78-2.82(1H,m), 2.76-2.72 (1H,m),2.69-2.65 (1H,m), 2.38-2.25 (1H,m),1.38 (9H, s), ^{13}C NMR, (100 M Hz-

$CDCl_3$) δ ppm 173.41,156.06, 79.22, 76.84, 63.86, 58.43, 53.55, 52.10, 39.02, 38.38, 36.78, 28.40. HRMS: HRMS: (ESI-Tof) Calcd for $C_{14}H_{26}O_7N_2S$+Na 1389.1353, Found 389.1303

1-(N-Boc-aminoethyl)-4-(S)-(N^4 -Acetyl-cytosin-1-yl)-2-(S)-proline methyl ester (7): The mesylate derivative (**6**) (3.0 g, 8.19 mmol), N^4-Acetyl cytosine (2.5 g, 16.3 mmol), potassium carbonate (4.5g, 32.7mmol), 18-Crown-6 (0.43g, 1.63mmol) dissolved in dry dimethyl formamide, stirred 24 hours at 70ºC .The reaction mixture concentrated to dryness and purified by silica gel chromatography using dichloromethane and methanol as solvents to obtain 0.866 g title compound as white solid in 25% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 8.67 ma,8.42 mi (1H, d 8Hz) 8.0 (1 H s,br) , 7.40 mi,7.31 ma (1H, d 8Hz), 5.34-5.20(1H, m),3.74 ma.3.69 mi(3H, s),3.66-3.64(1H, m), 3.25-3.23 (2H, m), 3.13-3.05(2H, m), 2.85-2.77(1H, m),2.70-2.54 (1H, m),2.35-2.31(1H, m),2.21 mi,2.18 ma (3H, s),2.04-1.99 (1H, m),1.93-1.97(1H, m), 1.45 ma,1.42 mi(9H, s), (^{13}C NMR, 100 MHz, $CDCl_3$) δ ppm 173.76, 73.44, 172.0, 717.02, 162.3, 161.97, 157.83, 156.19, 155.73, 148.27, 147.0,114.1, 97.22, 96.13, 79.5, 65.36, 64.81, 58.28, 57.55,54.26,53.55, 52.43,52.26, 50.44,39.0, 38.85, 37.02, 36.22, 31.93, 30.33, 29.36, 28.45, 24.8, 24.48, 22.69, 21.23.HRMS: Calcd. $C_{19}H_{29}N_5O_6$ 424.1291, Found 424.1261

The *aep*-C monomer hydrolysed using 1N lithium hydroxide in THF and water and neutralized by Dowex resin (H^+ form). The hydrolysis was confirmed by TLC and mass spectrometry the acid directly used in the solid phase synthesis without any further purification. The *aep*-Homo oligomer *aep*-PNA C_5 were synthesized by

following solid phase peptide synthesis methods with MBHA resin under Boc chemistry conditions. The PNA was cleaved from resin under acidic conditions by treatment with cocktail of TFA:TIPS:Water (95:2.5:2.5) for 2 hours. The reaction mixture was filtered using sintered funnel and then resin washed twice with TFA. The filtrate was concentrated under reduced pressure, and then re-dissolved in dry methanol. This methanolic solution was precipitated with dry diethyl ether and separated after centrifugation. The precipitate of *aep*-C_5 was dissolved in Milli- Q water and employed for the gel-filtration using Sephadex G-15 column with eluting solvent 0.1 % TFA in water. The fractions containing a*ep*-PNA were lyophilized and purified by HPLC under following conditions 0-22 min 15% B,22-24 min 70% B, 24-26 min 70% B,26-27.5 min 100% A,27.5-30.0 min 100%A at a flow rate 1 ml/min. A=0.1%TFA in H_2O, B=0.1%TFA in CH_3CN t_R =13.94 min .The retention time (t_R) of PNA C_5 is 13.94 min.The purified *aep*-C_5 PNA was dissolved in nuclease free water as stock solution (3mM) and used for biophysical studies.

UV and CD experiments: All UV and CD experiments were performed with 45μM strand concentration in 100 mM sodium acetate buffer pH4.5 otherwise mentioned. For hybrid i-motif formation dC_5 (22.5 μM) and *aep*-C_5 (22.5 μM) were mixed, and then annealed. The annealing was performed as following: the sample was heated to 90^{O}C for 10 minutes then allowed to reach room temperature, and further cooled to 4 ^{O}C for 5 hours before the experiments. The pH dependent CD experiments were performed in 100mM sodium acetate of different pH with total strand concentration of 45μM.

NMR Experiments: 0.3mM of each dC_5 and *aep*-C_5 were mixed in 100mM sodium acetate pH 4.5 with 0.1mM EDTA. Hybrid i-motif was made by mixing 0.2mM each dC_5 and *aep*-C_5 annealed and stored at 4^{O}C for 24 hours before experiments. Ten

percent D_2O was added efore the experiments. ^1H 1D experiments were performed with water suppression. All NMR experiments were performed at 25^OC.

ESI-MS experiments: 0.1mM of each dC_5 and *aep*-C_5 were mixed in 35mM sodium acetate of pH 4.5 and annealed as described earlier and samples were stored at 4^oC for 8 hours before the experiments. An aliquot was diluted to 20 times in methanol and water solution (1:1 with 0.1 % formic acid) and subjected to ESI-MS. The source temperature maintained at 50^OC during the experiments.

5.5 Reffernces

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5.6 Appendix

1. NMR and HRMS spectra

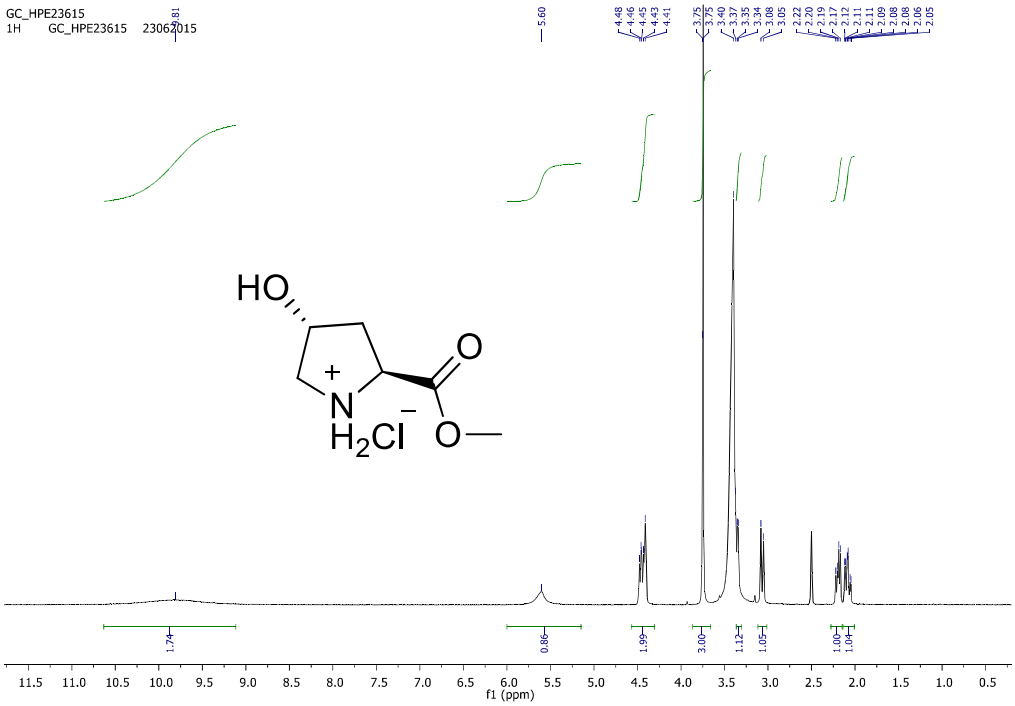


Figure A1. ^{1}H NMR of compound **2**

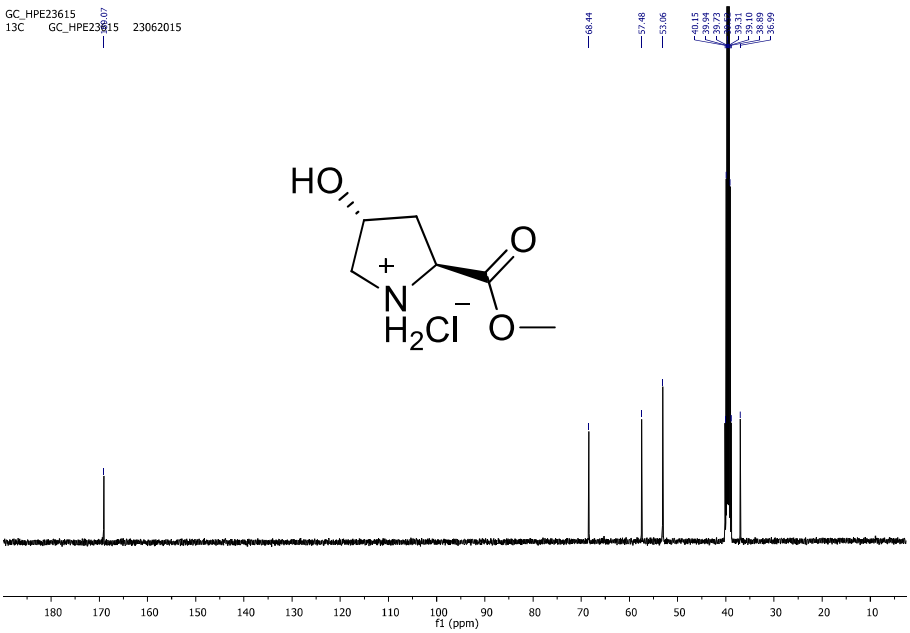


Figure A2. ^{13}C NMR of compound **2**

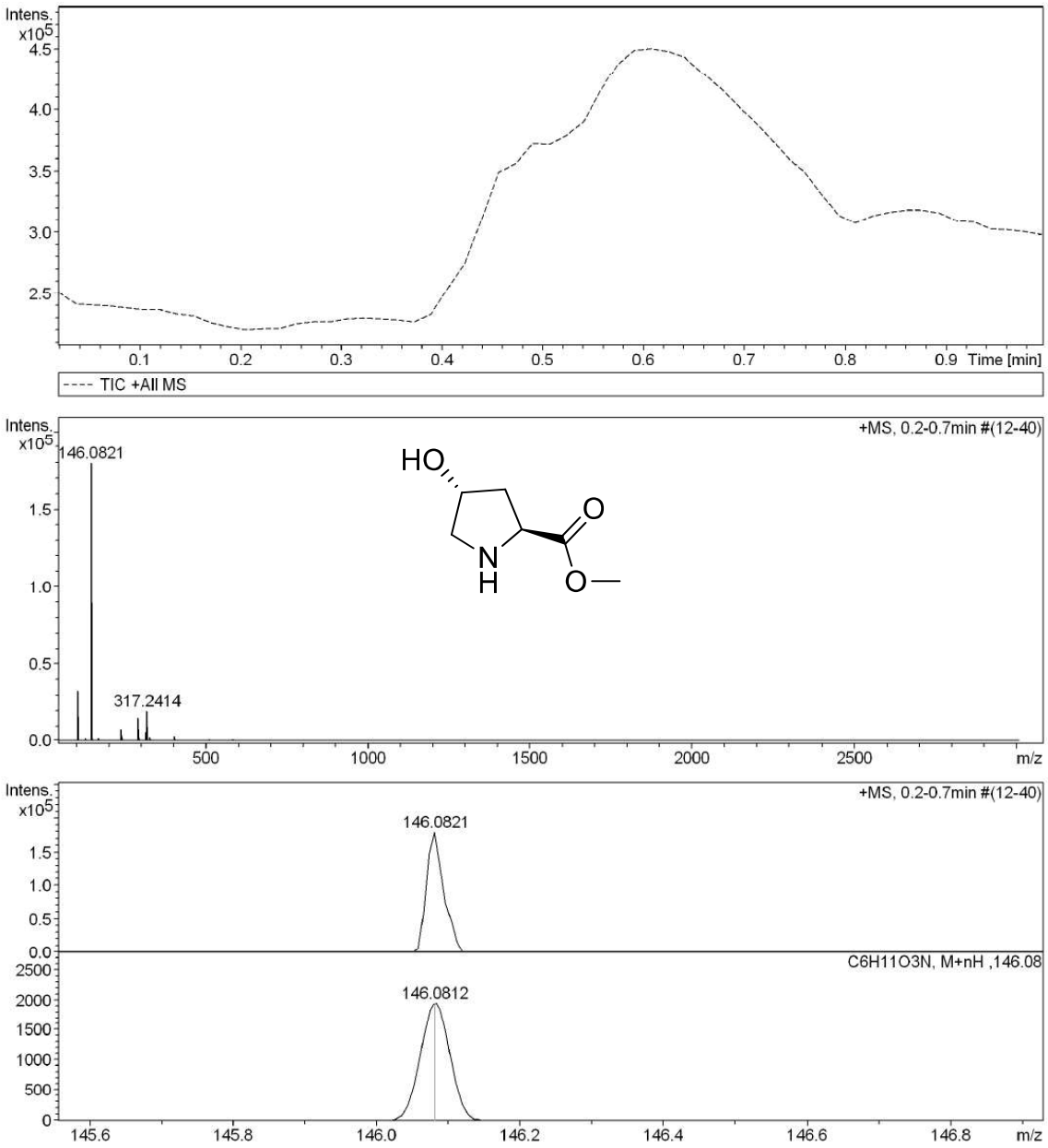


Figure A3. HRMS of compound **2**

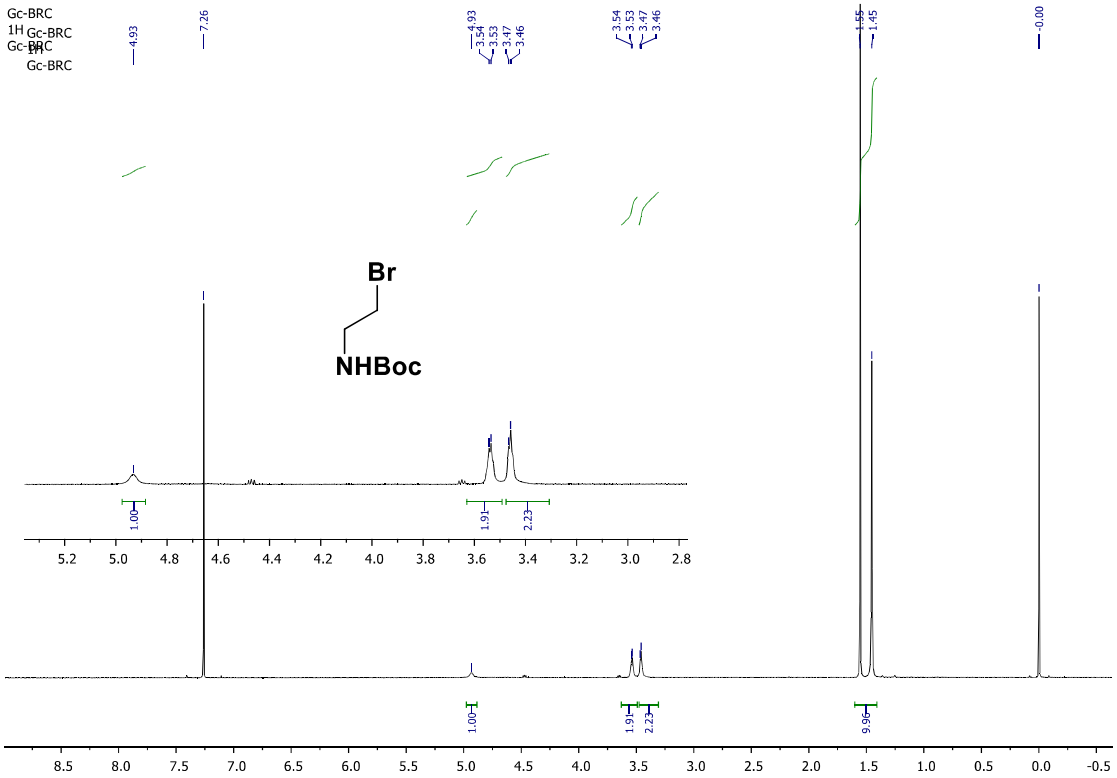


Figure A4. ^{1}H NMR of compound **4**

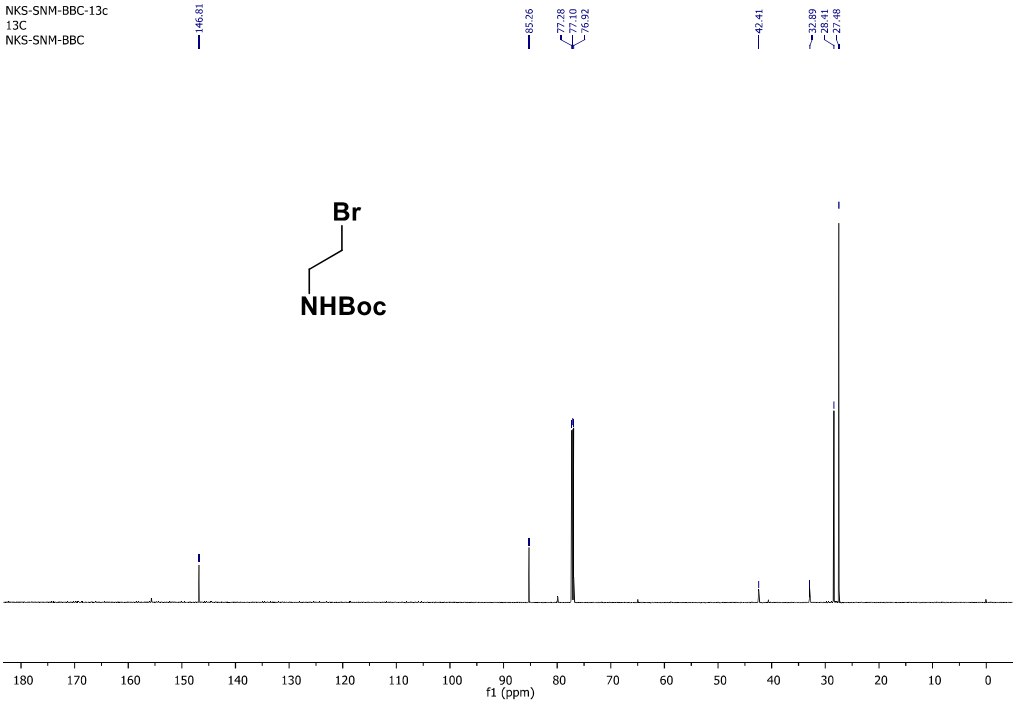


Figure A5. ^{13}C NMR of compound **4**

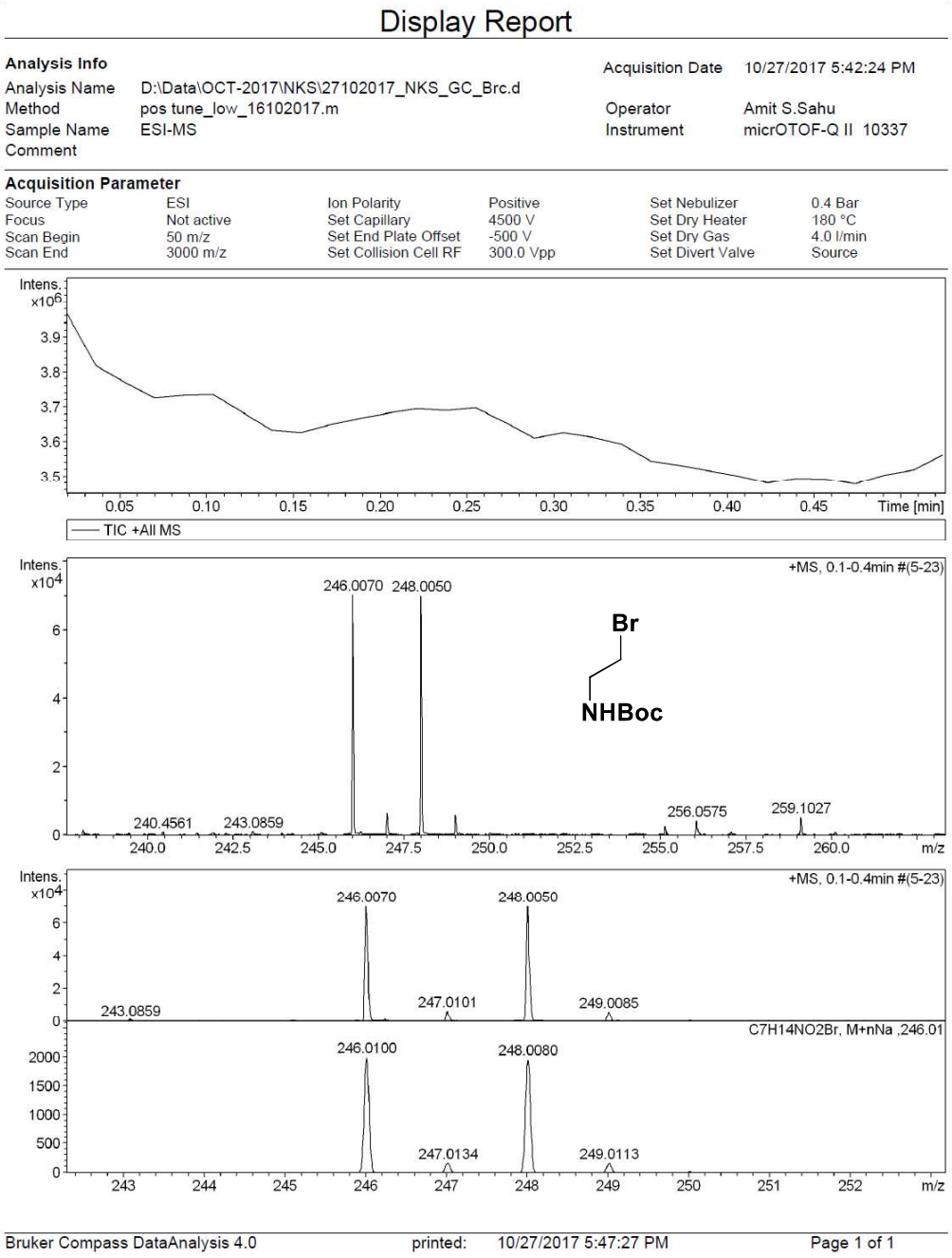


Figure A6. HRMS of compound **4**

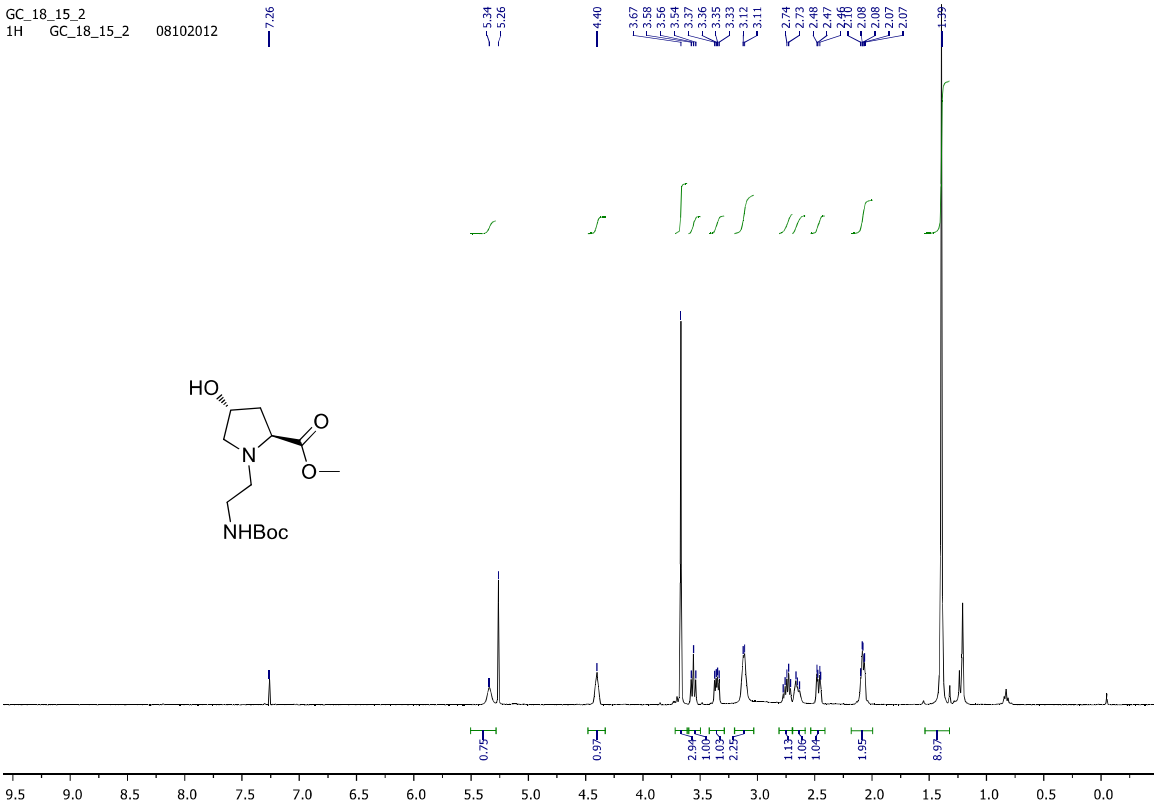


Figure A7. ^{1}H NMR of compound **5**

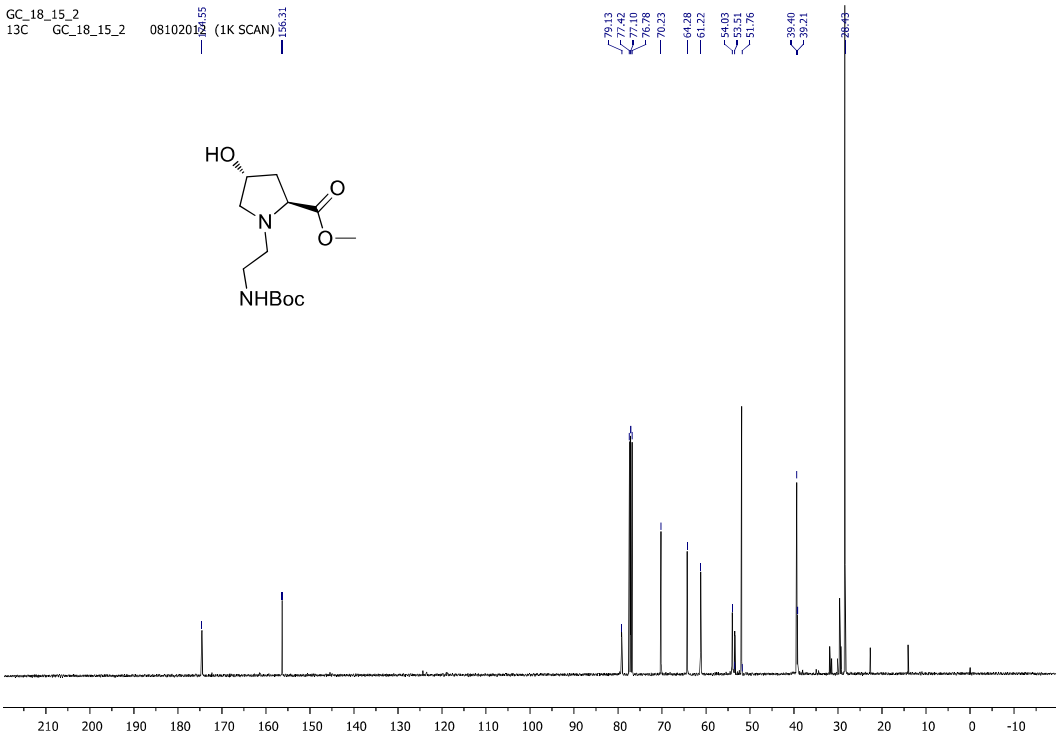


Figure A8. ^{13}C NMR of compound **5**

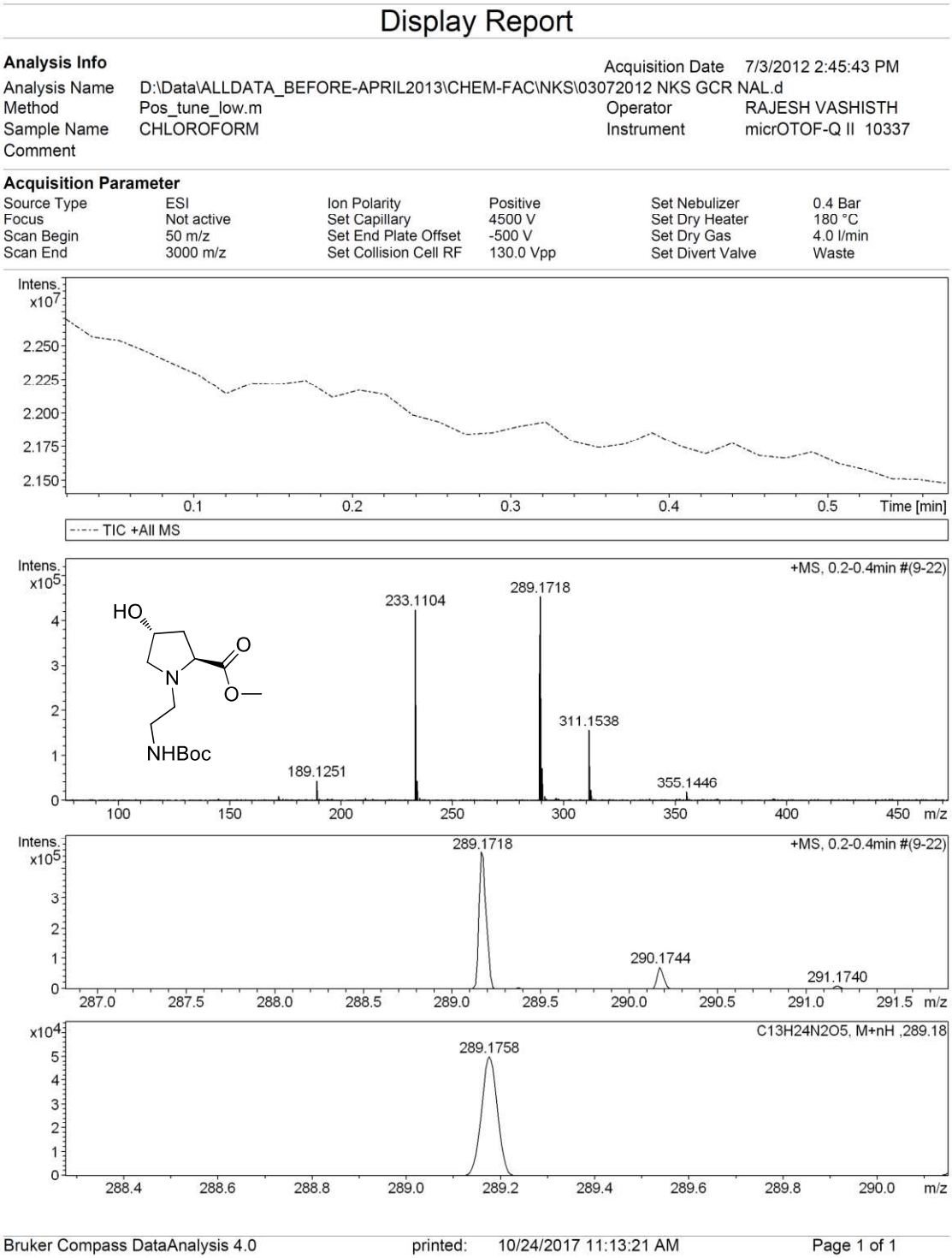


Figure A9. HRMS of compound **5**

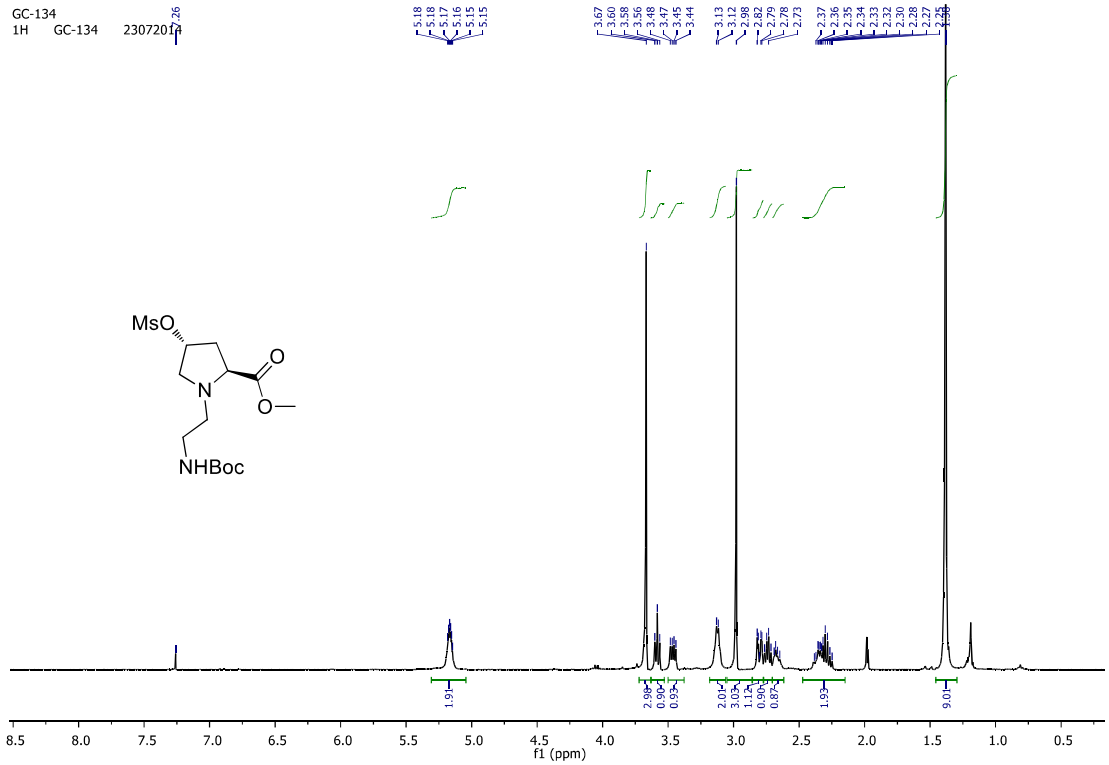


Figure A10. . ^{1}H NMR of compound **6**

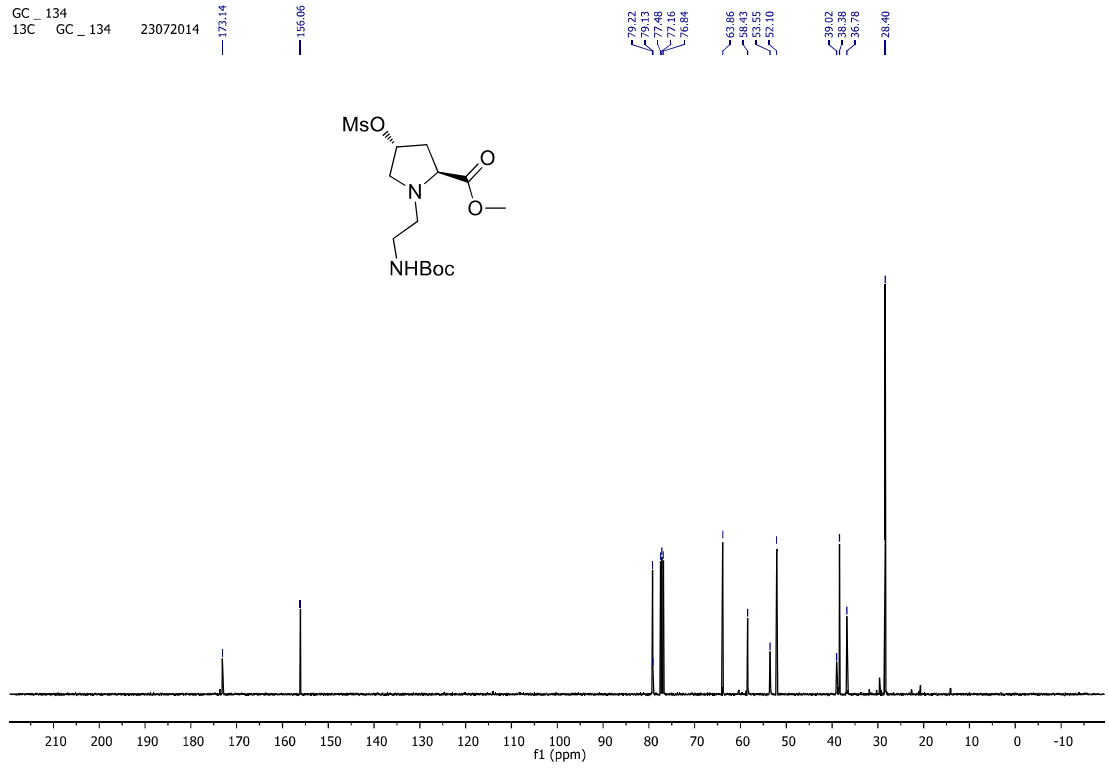


Figure A11.^{13}C NMR of compound **6**

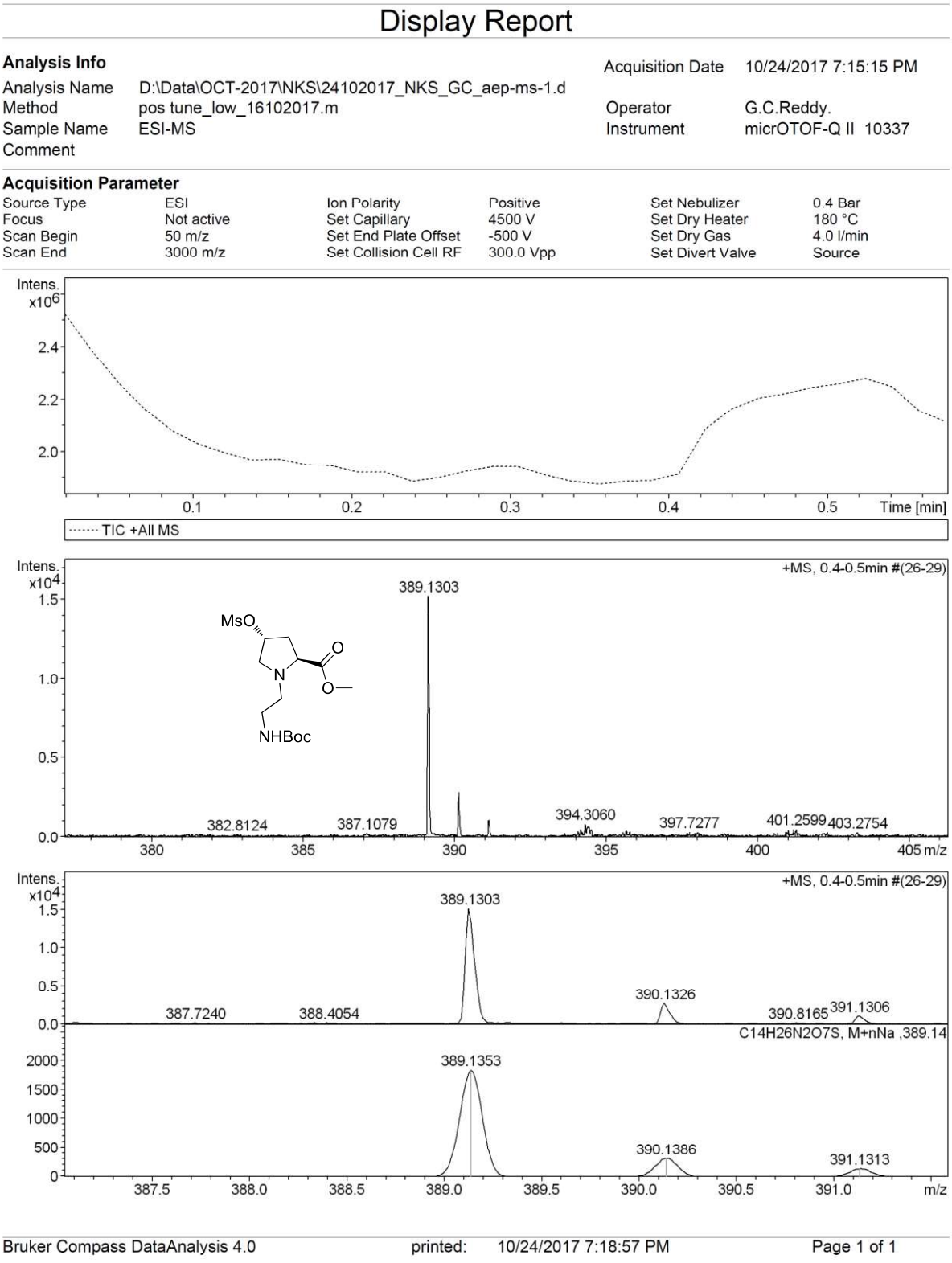


Figure A12. HRMS of compound **6**

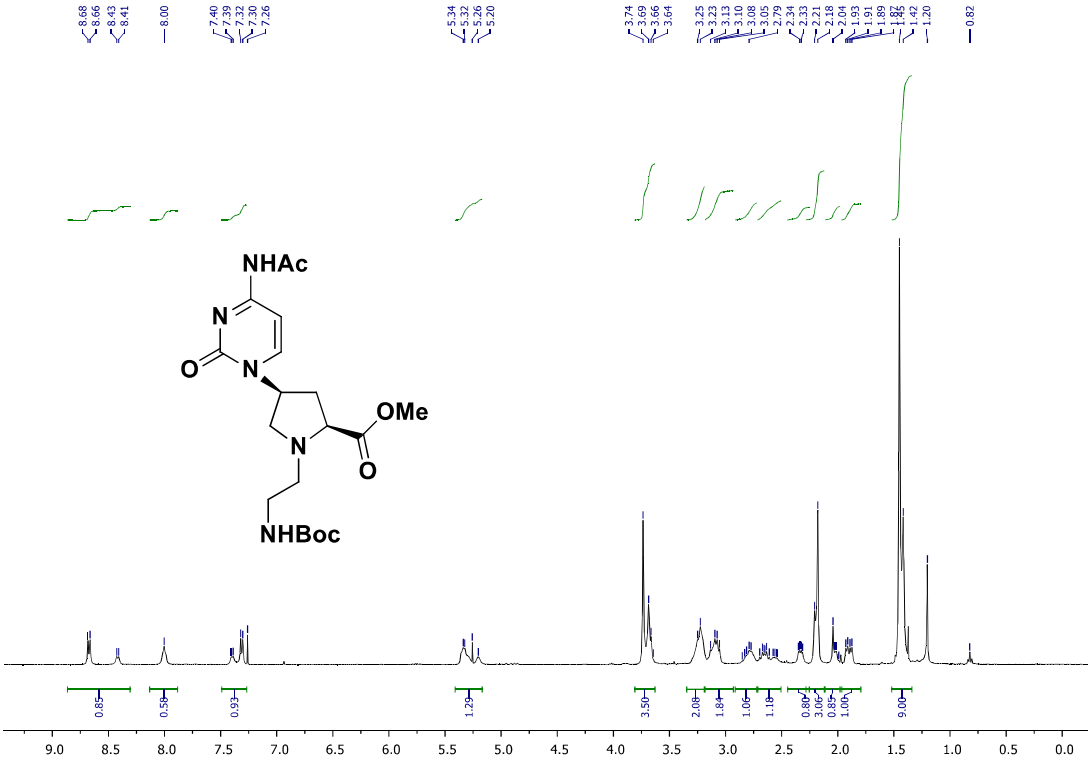


Figure A13. ^{1}H NMR of compound **7**

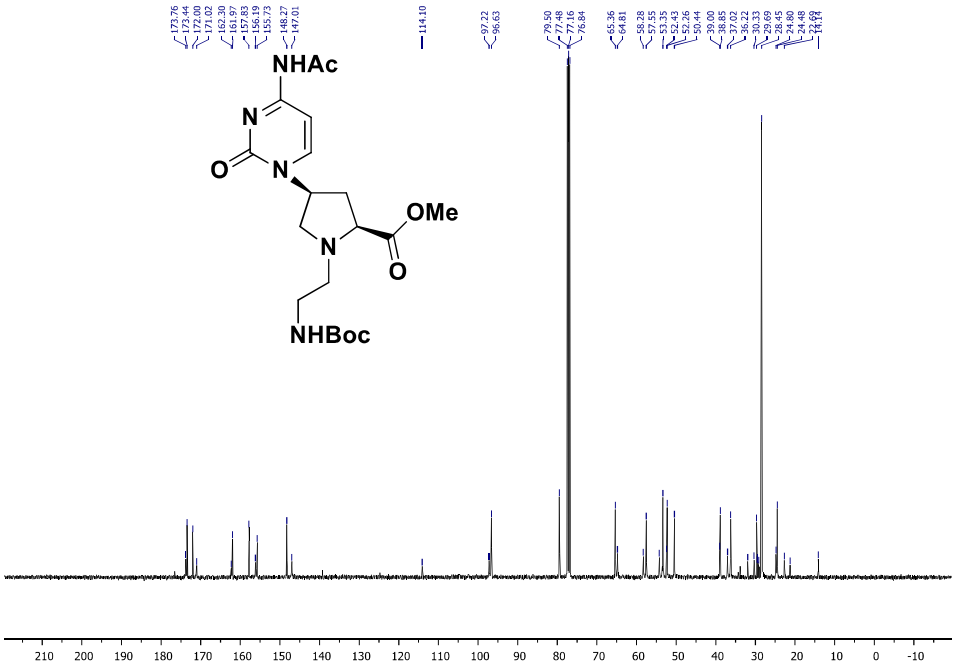


Figure A14. ^{13}C NMR of compound **7**

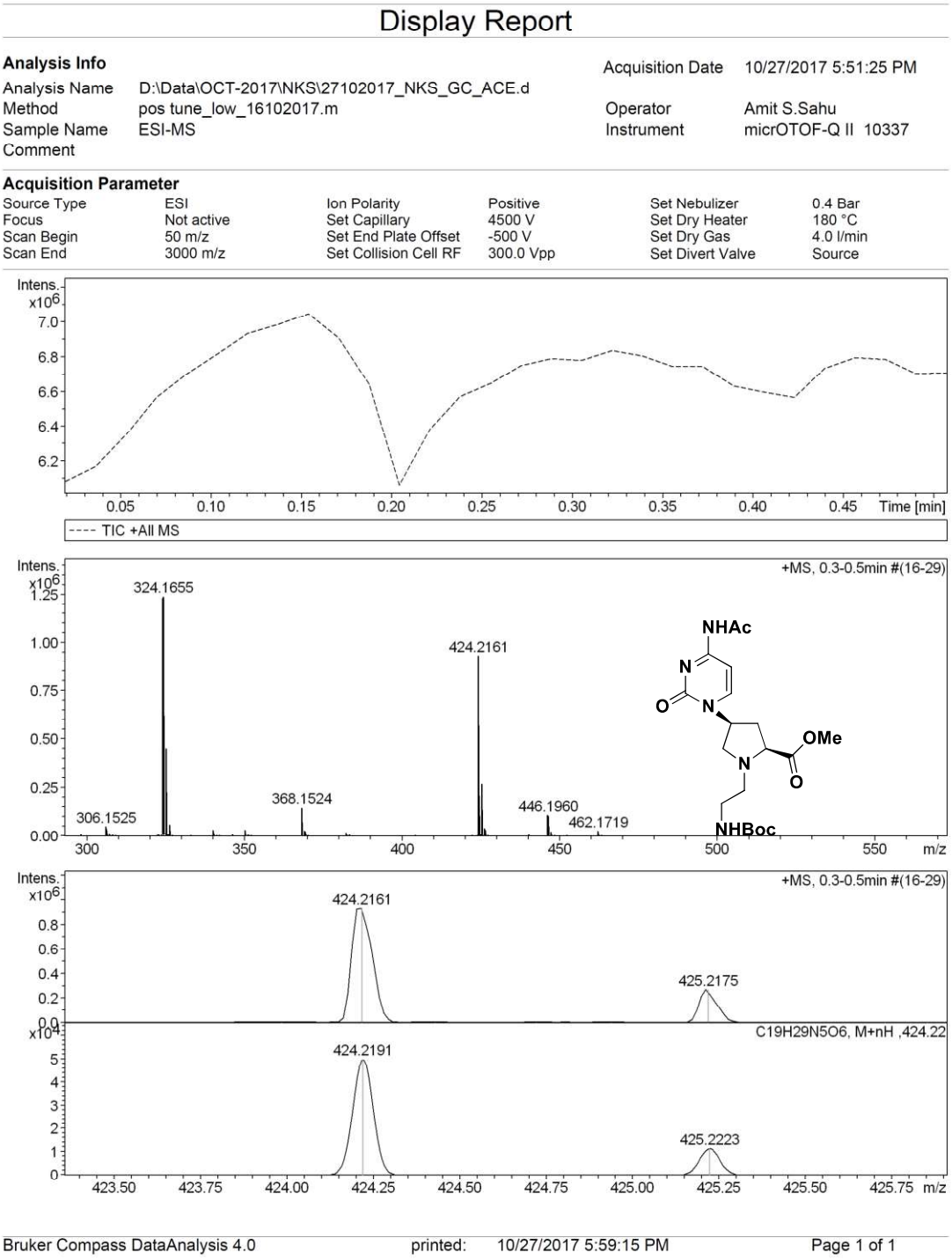


Figure A15. HRMS of compound **7**

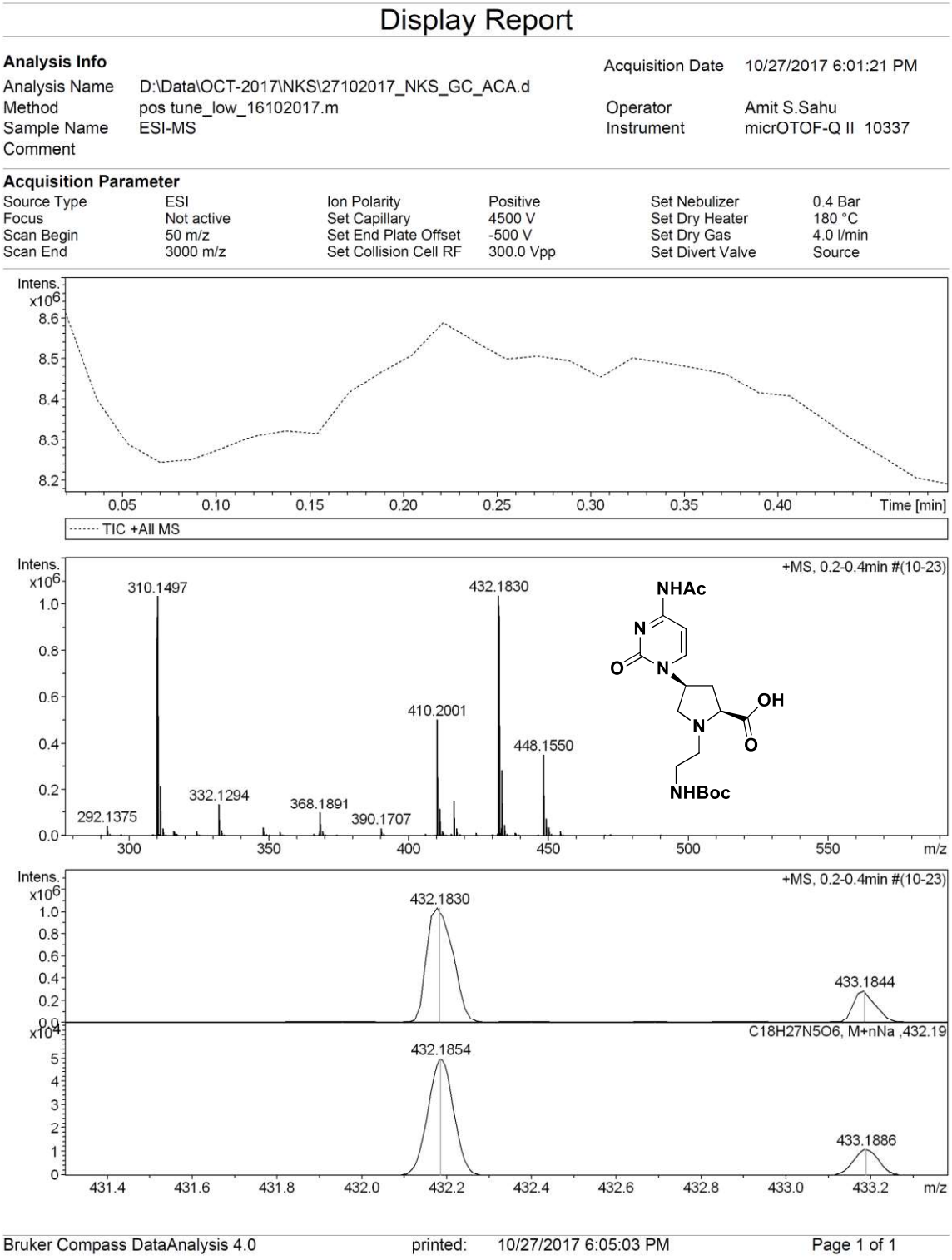
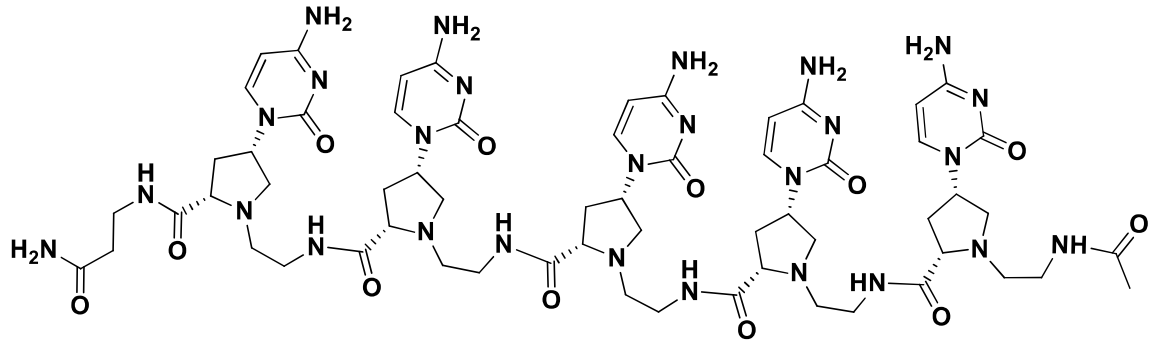


Figure A16. HRMS of *aep*-C acid

2. Analytical HPLC of *aep*-PNA C_5 (aep-C5)



aep-PNA C_5 (pC_5)

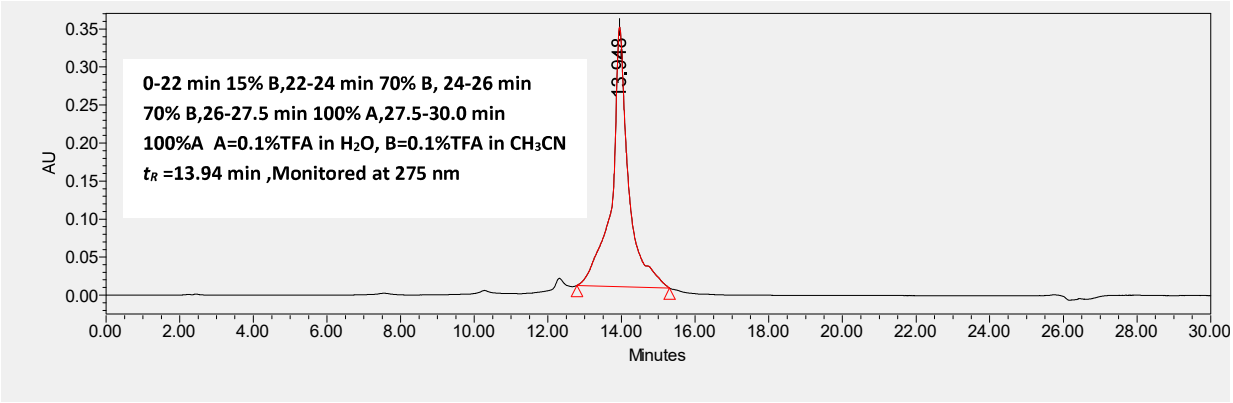
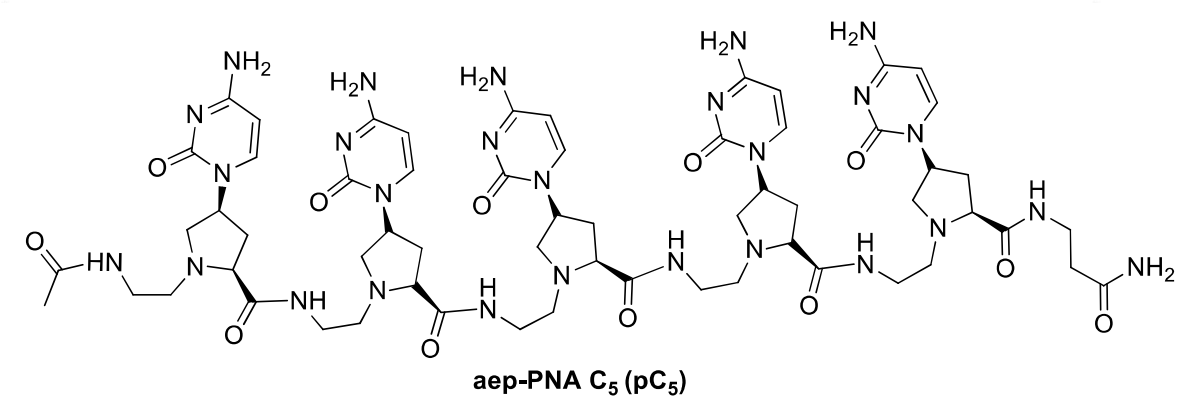


Figure A17. Chromatogram of analytical HPLC of *aep*-C_5

3. Mass spectrum of *aep*-C_5



aep-PNA C_5 (pC_5)

Chemical Formula: $C_{60}H_{85}N_{27}O_{12}$
Exact Mass: 1375.687

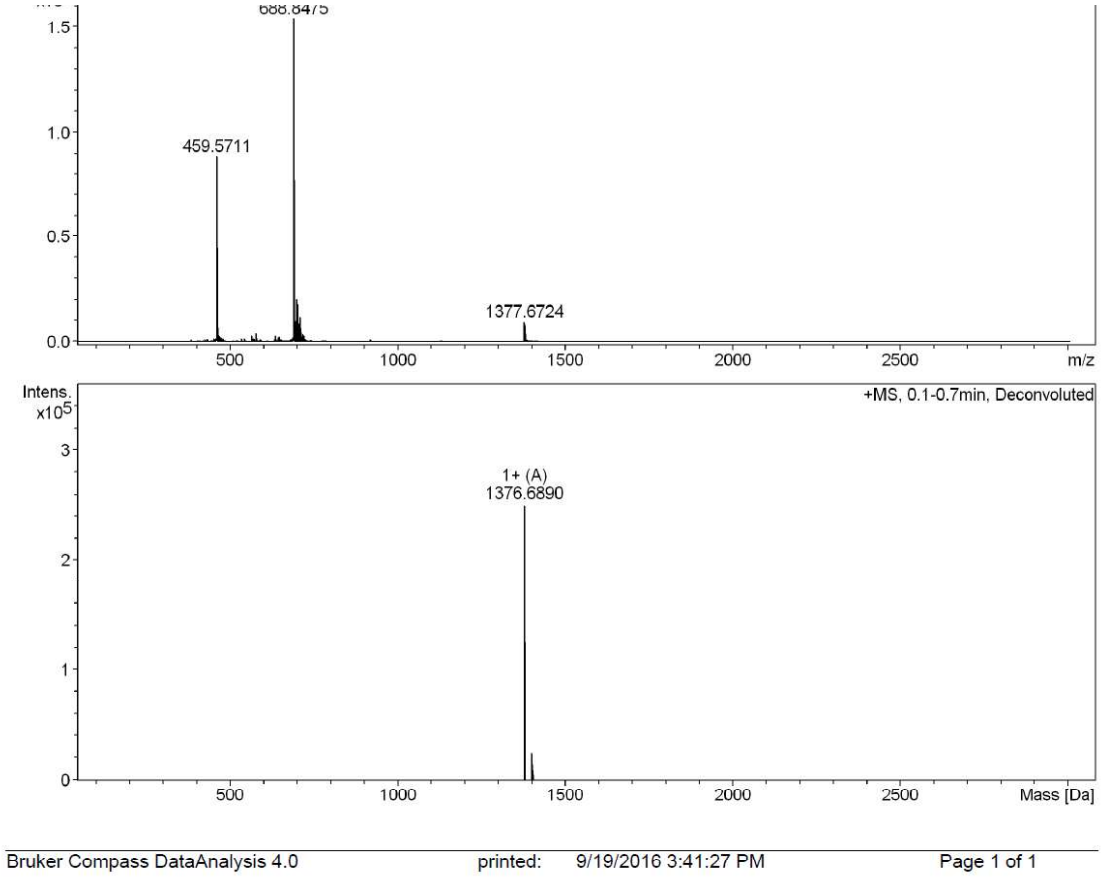


Figure A18. Mass spectrum of *aep*-C_5

4. Mass spectrum of dC_5

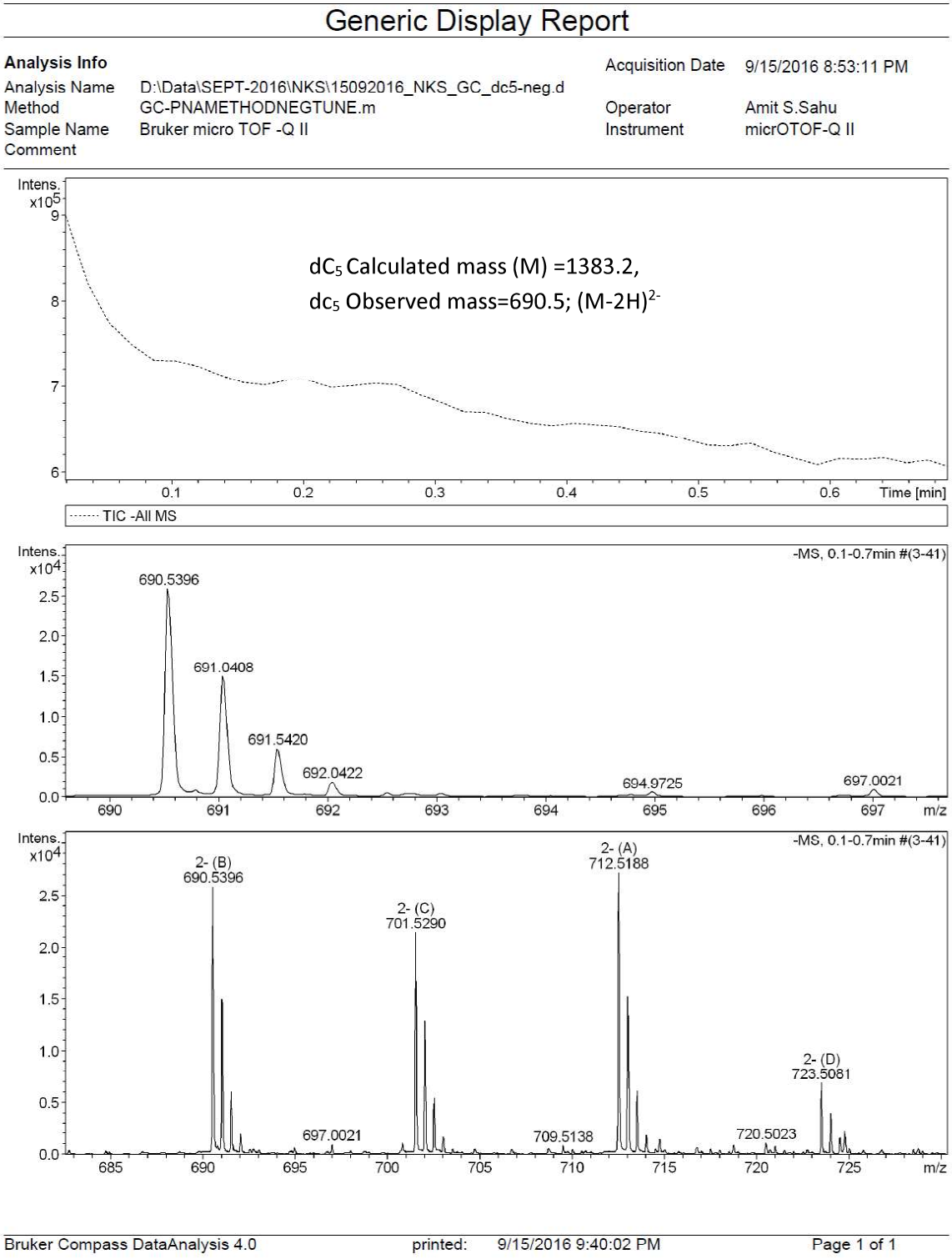


Figure A19. Mass spectrum of dC_5

5. ESI-MS spectra of annealed dC_5

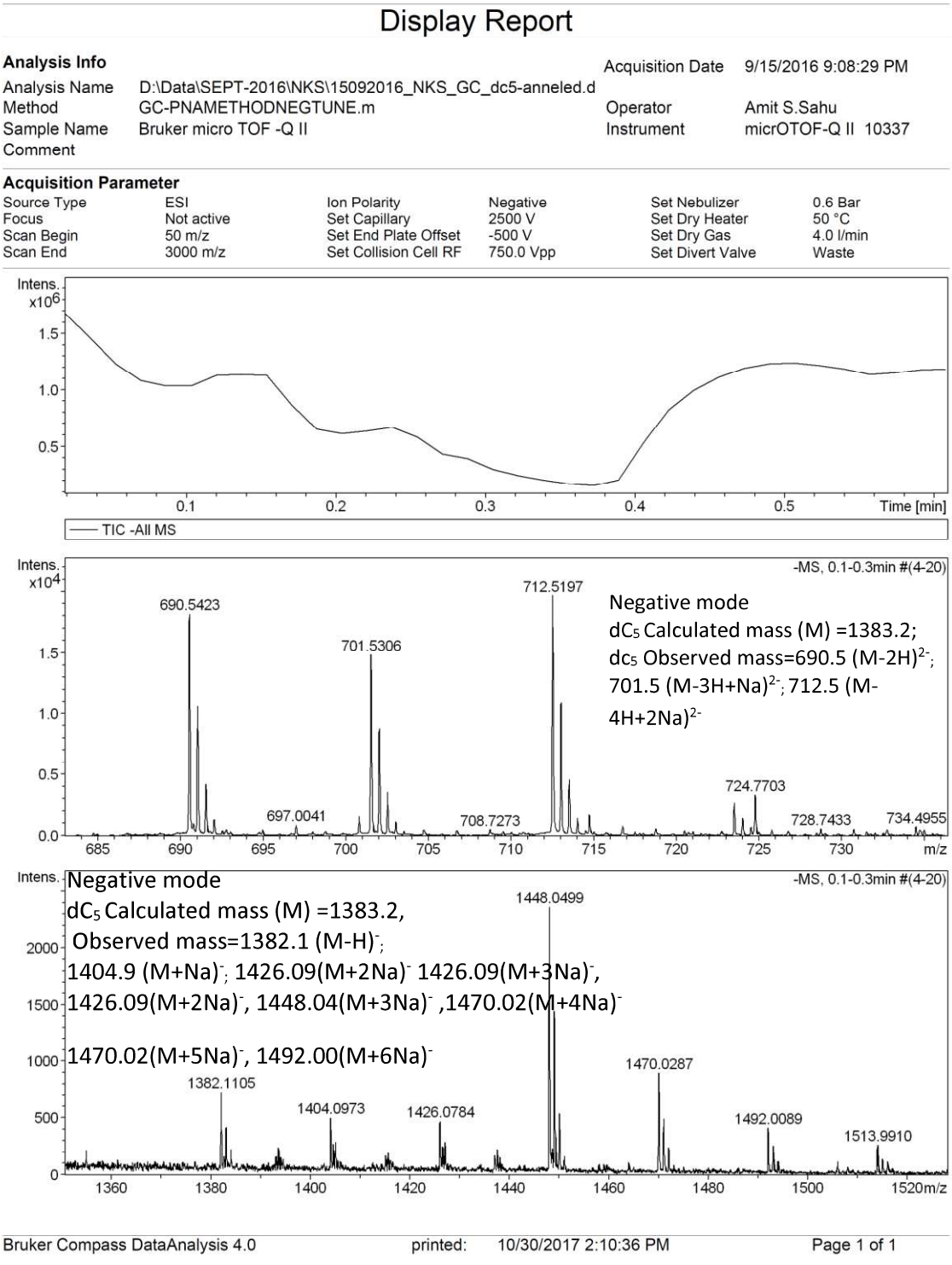


Figure A20. ESI-MS spectra of dC_5 in negative mode at pH 4.5

Chapter 6

Synthesis and Spectroscopic Studies of Berberine Immobilized Modified Cellulose Material

Chapter 6

Synthesis and Spectroscopic Studies of Berberine Immobilized Modified Cellulose Material

Contents

6.1 Introduction

Cellulose is major constituent of plant cell walls which bind with proteoglycan material for their survival. Cellulose is the most abundant natural homopolymer containing long chains of anhydroglucose units linked together by β-1,4-glycosidic bond.[1,2] Since cellulose is considered as the most abundant renewable biopolymer containing free hydroxyl functional groups which provide opportunities to prepare a varieties of functional biocompatible polymers for industrial and medical applications.[3] Recently grafted cellulose polymers have been considered as carrier of therapeutic drugs and other bioactive synthetic/natural molecules.[4-6] Recently, amino acids has been immobilized at cellulose to control the morphology of cellulose based materials. For an example, Gly/Ser amino acid increase the hydrophilicity at cellulose surface, Ala/Val/Leu/Ile amino acids enhance the hydrophobicity, and Phe/Tyr/Trp make UV active cellulose based materials (Figure 6. 1).[7,8] Further cellulose materials are

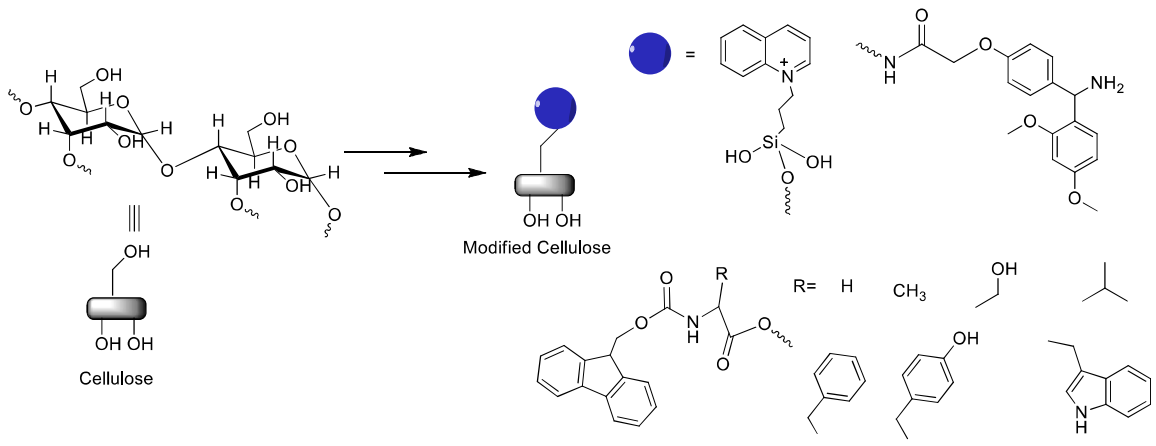


Figure 6. 1 Chemical structure of cellulose and immobilization of biomolecules/ fluorescent molecules on cellulose surface.

employed as solid support for peptide synthesis.[9] Peptide, short sequences, containing cellulose materials reportedly control the interaction of cellulose with specific cells. Kato and co-workers have shown that cellulose provides a useful surface for peptide-array-based experiments for analyses of cell-adhesive peptides.[10] The modified surface of polymeric materials are considered for enhancing the selective cell-adhering properties. Moreover another bioactive molecules as *Chalcones* and its deivative are immobilized on cellulose surface and explored in screening of Chalocones' antibacterial activities.[11] As growing demand of cellulose based materials, the fluorescence labelled cellulose materials are developed by immobilization of fluorescent molecules, 5-(4, 6-dichlorotriazinyl) amino derivatives, at cellulose surface.[12,13] The cellulose based modified polymer are examined as potential therapeutic drugs candidates.[14] Covalently attached flourophores on cellulose surface are synthesized and used as flouorescent labels.[15]

In recent time, the immobilizations of bioactive natural products on cellulose polymers are emerging area of research in preparedness of biocompatible functional materials for screening of the most appropriate drugs. In recent years cellulose derived nanomaterials are prepared by grafting biomolecules or fluorophores at cellulose surface. These cellulose materials are considered as potential candidates for many biocompatible applications such as sensors, drug delivery agents and bio imaging.[16] Citric acid/cysteine modified cellulosed based materials are prepared and employed in sensing of chloride ion, ant counterfeiting and UV Shielding applications.[17] Fluorescein grafted cellulose nanocrystals are prepared and examined as effective pH sensors.[18] Amino functionalized carbon quantum dots are coupled with oxidized cellulose to synthesize fluorescent quantum dot loaded cellulose nanomaterial. These

materials have properties such as cytocompatiblity and permeability which make them suitable for *in vitro* bioimaging techniques.[19] Cellulose nanocrystals containing fullerene (CNC-C_{60}) are synthesized by grafting C_{60} with amine functionalized cellulose nanocrystals which show antiradical effects on scavenging hydroxyl radicals in vitro.[20]

6.2 Rational of present works

Since Berberine, bioactive isoqunoline based natural product, is reported as DNA G-quadruplex binding agents and considered as potential *anti*-tumor/*anti*-cancer therapeutic drugs.[21-23] Importantly, Berberine and its derivatives have high color index and fluorescence properties, and being routinely used Indian leather/textiles industries.

6.2.1 Hypothesis and objectives

We hypothesized to immobilize Berberine moiety on biocompatible solid support as cellulose for screen of G-quadruplex forming DNA. This chapter describes the synthesis of Berberine immobilized cellulose materials and their spectroscopic studies by UV/Fluorescence/IR, powder X-ray studies. Finally this chapter also describe studies of their morphology by confocal/SEM imaging techniques.

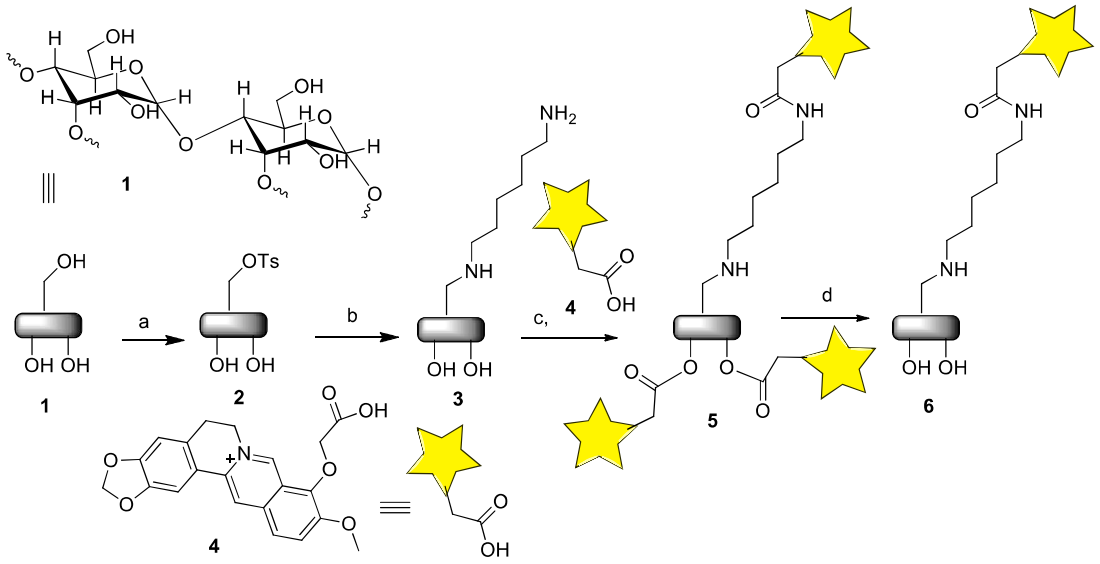
6.3 Results and discussion

6.3.1 Synthesis of Berberine immobilized cellulose

The synthetic scheme of Berberine immobilized cellulose material is described in scheme 6.1. The hydroxy groups of cellulose (**1**) was tosylated as compound (**2**) with tosylchloride in presence of organic base Et_3N. This tosylated cellulose material was derived into amine functionality by treatment with hexamethyldiamine under basic conditions. The formation of amino functionalized cellulose materials was qualitatively established by well-known Ninhydrine test. In next, commercially

available Berberine hydrochloride was modified into carboxylate group with two steps: *O*-alkylation with ethyl bromoacetate at high temperature under vacuum conditions, and then ester hydrolysis of *O*-alkylated Berberine derivative under alkaline conditions.

Scheme 6. 1 Synthesis of Berberine immobilized cellulose



Reaction and condition: (a) TsCl/Et_3N; b. Hesamethyldiamine/DMF/; (c) Compound **4**, EDC/DMF/ DIEA; (d) aq. NaOH (0.1 N).

This Berberine carboxylate was coupled with freshly synthesized amino functionalized cellulose material via amide bond under peptide coupling reaction conditions using EDC and DIEA. The progress of the reaction was monitored by ninhydrine test. The Berberine immobilized cellulose material was purified by gentle aqueous washing methods which removed the excess unreacted reagents, and then characterized by IR and Powder X-ray techniques. The purified Berberine

immobilized cellulose material was employed for spectroscopic and other properties as morphology studies.

6.3.2 Powder X-ray diffraction studies

Powder X-ray diffraction is one of the important technique which used for characterization of micro crystalline bulk compounds. Herein the Berberine immobilized cellulose materials and intermediates were characterized by comparison with unmodified cellulose using well-known powder X-ray diffractometer and X-ray diffraction pattern of native cellulose (**1**) and modified cellulose (**2-6**) and native are depicted in the Figure 6.2. The X-ray diffracted peaks for native cellulose were observed at angle (2θº) = 22.6º, 16.3 º, and 14.8 º in diffraction plane (002), which are characteristic for reported cellulose data (**1**).[24] The X-ray diffraction pattern of modified

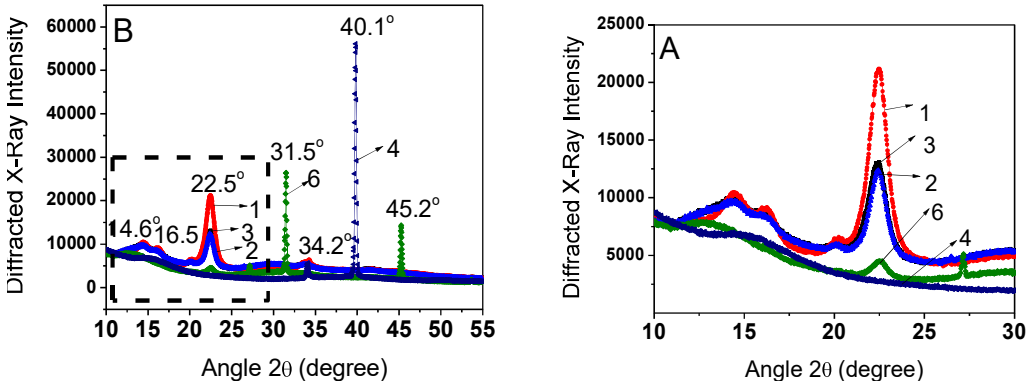


Figure 6. 2 X-Ray diffraction pattern of native cellulose (**1**), tosylate derivative **(2**); amine derivative **(3);** Berberine derivative **(4)** and Berberine immobilized cellulose material **(6)** Full range (A) and Extended region (B).

cellulose materials **2** & **3** were depleted at angle 22.6 º, which support the partial modification of native cellulose material which support the hexamethylenediamine

group, good nucleophile, replaced the tosyl group, good leaving group tosyl group. The powder X-ray pattern shows peaks at angle (2θ) 40.1 º (strong) and 34.2 º (very weak) for Berberine derivative **4**. The Berberine modified cellulose material 6 exhibits peaks at angle (2θ) 45.2 º (strong), 31.5 º (strong), 22.5 º (weak). The appearance of low intensity peaks at angle (2θ) 22.5º , and broad hump at 14-16 º in difractograms of modified cellulose material **6** are equivalent to peak angle of cellulose materials (**1-3**). These results support the immobilization of Berberine are occurred at cellulose surface via amide bond through linker.

6.3.3 FT-IR Studies

Here solid state FT-IR technique was employed for characterization of Berberine grafted cellulose materials. The FT-IR spectra of cellulose materials (**1-6)** were recorded from respective dry powder using solid state probe, and their IR spectra are depicted in Figure 6.3. In case of native cellulose powder (**1**), FT-IR frequencies are appeared at 3000-3600 cm^{-1} which reportedly belong to the characteristic OH stretching band of cellulose, and FT-IR frequencies at 1650 cm^{-1} belong to the bending of adsorbed water molecules at cellulose surface. In case of modified cellulose powder, their FT-IR spectra exhibit the appearance of peaks at 3000-3600cm^{-1} with significantly decreased intensity as compare to the native cellulose. Additional new peaks are appeared at frequencies region 1200-1400 cm^{-1} for compound **2** and **3** which strongly support the modification of cellulose with tosyl and amine groups. The FT-IR spectra of lone Berberine carboxylate derivatives (**4**) shows peak at frequencies 3415cm^{-1} (stong) which presumably belongs to O-H stretching of its carboxylic acid functional group. The FT-IR spectra of compound **6** display new peaks at 1600-1700cm^{-1}, probably for amide bending, and IR peaks in region 1000-

1450cm^{-1} are disappeared. These IR results further support the partial modification of native cellulose surface with Berberine derivative via hexamethyldiamine linker.

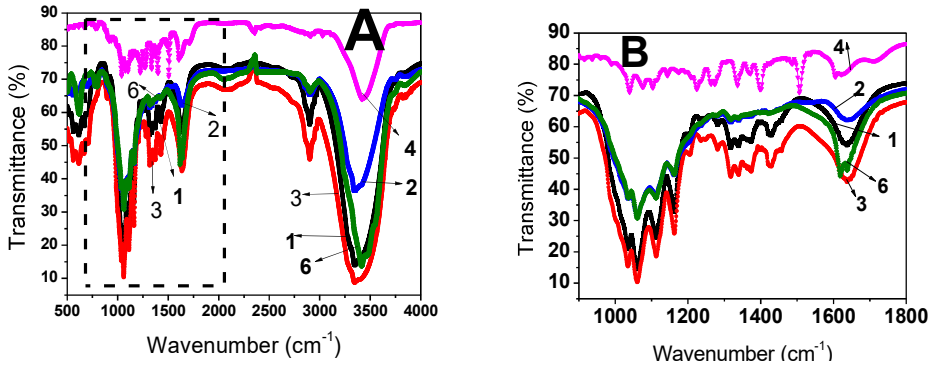


Figure 6. 3 FT-IR spectra (solid probe) of 2/3/4/6 full rage (A); extended range (B).

Further we attempted to characterize immobilization of Berberine on cellulose surface by NMR and ES-MS techniques. However $^{1}H/^{13}C$ NMR and mass spectra of modified cellulose material were similar as those of unmodified cellulose. This is possibly due to partial grafting of Berberine at cellulose surface. The detection of partial modification of cellulose surface became extremely difficult by NMR and mass studies because their signals are masked by unmodified cellulose. Finally the surface morphology of modified cellulose are studied by known scanning electron microscopy (SEM) techniques.[25]

6.3.4 Scanning electron microscopy (SEM)

The morphology of surface depends on the physiochemical nature of expose surface by SEM-imaging of thin layer film at silicon surface. The thin layers of samples of cellulose materials **1/3//4/5/6** were prepared from by dropping DMSO solution of these samples on silicon wafer, and then we recorded the their SEM-images. Their SEM images at nano-scales, at selective resolution (~1-2μm) are illustrated in

Figure 6. 4A-4E. The SEM images of unmodified cellulose powder (1) and were observed as crystalline type morphologies (Figure 4A), while SEM of image of amino functionalized cellulose material (**2**) are different from native cellulose as less crystalinality features (Figure 6.4B). The SEM image of lone Berberine derivative (**4**) shows cubic crystalline type of surface morphology (Figure. 6.4C), while the SEM images of Berberine immobilized, dark yellow, cellulose material **(5)** shows the

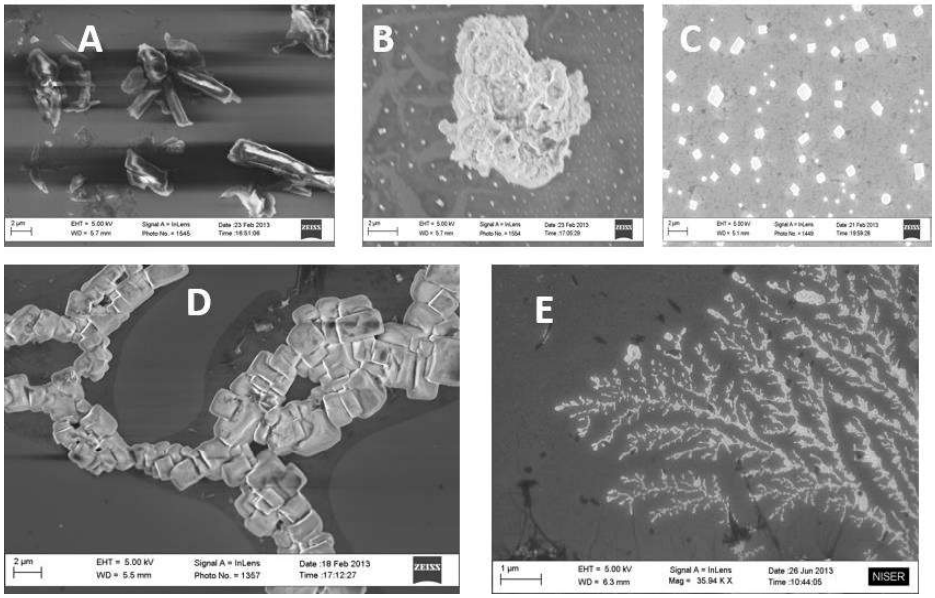


Figure 6. 4 SEM images: A. native cellulose (**1**) with 2µm size; B. amino derivatized cellulose (**3**); C. Berberine carboxylate (**4**); D Berberine immobilized cellulose (**5**); E. NaOH treated Berberine immobilized cellulose material (**6**).

densely grafted Berberine derivative on (Figure 6.4 D). The SEM image of pale yellow colored hydrolyzed cellulose materials **6** shows the lightly grafted Berberine derivative at cellulose surface (Figure 6.4 E). These SEM results strongly support the immobilization of cellulose materials at cellulose surface presumably by linker.

6.3.5 UV and fluorescence studies

Since Berberine and its derivatives are fluorescent dye molecules so Berberine carboxylate (**4**) immobilized cellulose materials could show fluorescent properties. We recorded the UV and fluorescence spectra Berberine containing cellulose materials in both phases: solution & solid.

Solid phase. Both UV and fluorescence spectra (solid/solution phase) are provided in Figure 6.5. The absorption spectra of solid powder samples of **4/6** are depicted in Figure 5A, while their emissions are given in Figure 6.5B. The UV absorption spectra of Berberine immobilized cellulose material **6** exhibits an absorbance peaks at λ_{max} 442nm as similar to control sample, free Berberine derivative **4**. The fluorescence

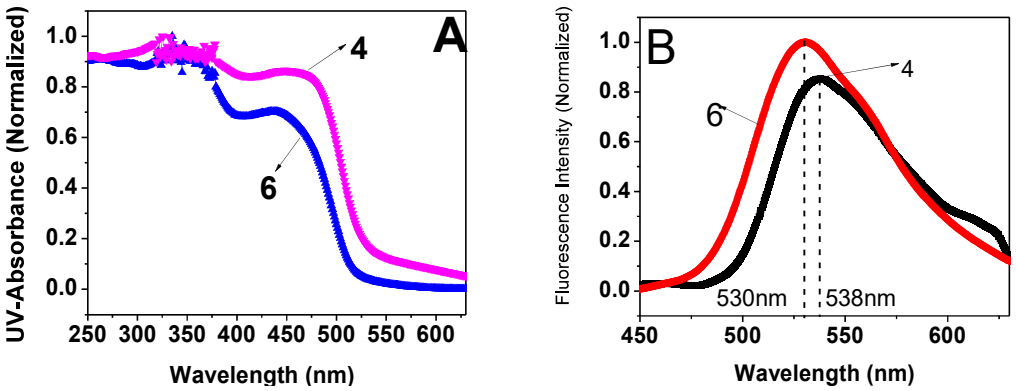


Figure 6. 5 UV spectra (with *solid probe*) Berberine carboxylate derivative (**4**) and Berberine immobilized modified cellulose (**6**); Fluorescence spectra (with solid Probe) Berberine carboxylate derivative (**4**) and Berberine immobilized modified cellulose (**6**).

spectra of modified cellulose **4/6** (powder form) show an emission peaks (λ_{em}) at ~530 nm and 538nm for Berberine grafted cellulose (**6**) and Berberine derivative (**4**) respectively at same excitation wavelength (λ_{ex}) 342nm (Figure 6.5B). The

comparative UV studies strongly support the immobilization of Berberine at functionalized cellulose surface.

Solution phase. The UV and fluorescence spectra of Berberine immobilized cellulose material and Berberine derivative were also recorded in solution phase. The dissolution of cellulose materials **6** along control sample Berberine derivative (**4**) were performed in aqueous solution of LiOH:Urea by following the reported procedure.[26] The alkaline condition aq. LiOH:Urea could also hydrolysed the ester bonds of cellulose derivative **5**, so only modified cellulose material **6** was employed for dissolution in LiOH:Urea conditions. The UV and fluorescence spectra of **1/4/6** were recorded with solution phase probe. The normalized UV spectra of materials **1/4/6** are given in Figure 6A, while their normalized fluorescence spectra are depicted in Figure 6B. The UV spectrum of lone Berberine carboxylate (**4)** exhibits absorbance at wavelengths (λ_{abs}) 267nm, 345nm, and 430nm (Figure 6A), which are also appeared in UV spectrum of Berberine immobilized cellulos (**6**), however, the peak intensities are significantly gone down. However there is no absorbance peak, as per expectation for control sample, unmodified cellulose (**1**). These UV/fluorescence results strongly support the presence of Berberine at surface of cellulose (**6**). In next, the fluorescence spectra of both Berberine (**4**) and Berberine immobilized material (**6**), exhibit emission peaks at wavelength 446 nm at similar excitation wavelength (λ_{ex}) 345nm. The fluorescence spectra of cellulose materials **4** & **6** exhibit emission peaks at 517nm at same λ_{ex} 424nm. Though the nature of emission is slightly different in Berberine cellulosee derivative **6** as compare to derivative lone Berberine derivative **4**. These UV/fluorescence studies further support the immobilization of Berberine at cellulose polymer.

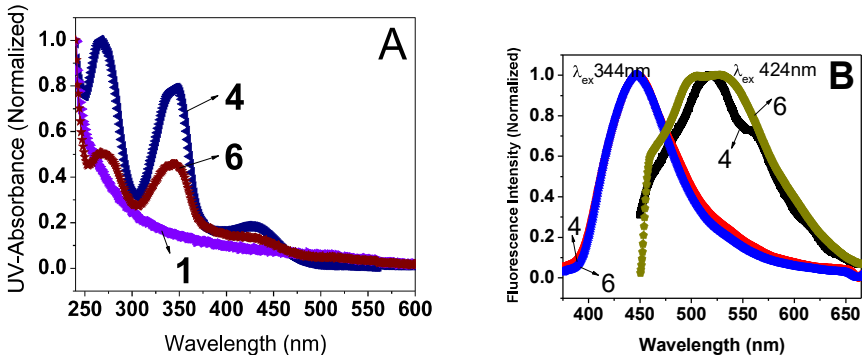


Figure 6. 6 UV spectra of native cellulose, Berberine carboxylate derivative (**4**) and Berberine immobilized modified cellulose (**6**) solution in LiOH:Urea; Fluorescence spectra (with solid Probe) Berberine carboxylate derivative (**4**) and Berberine immobilized modified cellulose (**6**) solution in LiOH:Urea.

In understanding the nature of fluorescence, photoluminescence (PL) spectra of free Berberine derivative (**4**) and Berberine immobilized material (**6**), were also recorded

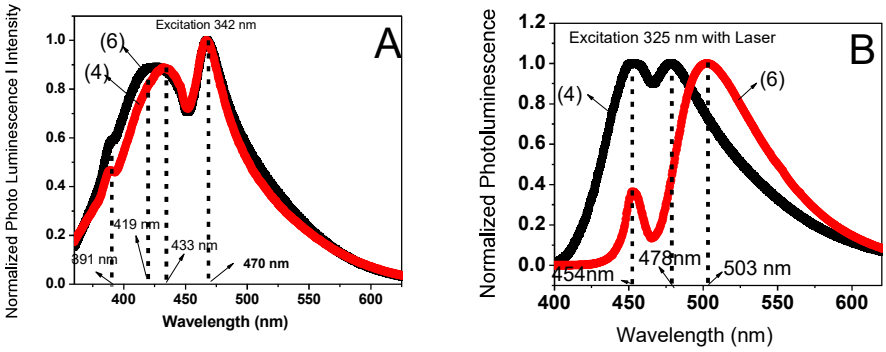


Figure 6. 7 Photoluminescence spectra of Berberine acid (**4**) and Berberine modified cellulose (**6**): without laser (A); and with laser (B)

by using laser source at two different λ_{ex}. The PL spectra of cellulose **4/6** with λ_{ex} 342nm and 325nm are depicted in Figure 6.7A & 7B. The PL spectra of control material **4** with λ_{ex} 342nm (Figure 6.7A), exhibits two strong emission peaks at wavelengths (λ_{em}) 433nm and 470nm, while PL spectra of modified cellulose material

(**6**) show one extra shoulder peaks at λ_{em} 391nm along with strong peaks (λ_{em} 419nm), slightly blue shift (~25nm) with control sample (**4**). In Figure.6.7B, the PL spectra of cellulose **4** with λ_{ex} 325nm demonstrate two types of emission peaks at wavelengths λ_{em} 454 nm and λ_{em} 478 nm with same peak intensity. Both λ_{em} peaks of control samples (**4**) are responsible for being florescent. The PL spectra of Berberine immobilized cellulose material (**6**) also exhibit two emission peaks at 454 nm and 503 nm but their intensities are quite different. From comparison studies, Berberine immobilized cellulose material (**6**) has shown red shift with ~25 nm over control sample (**4**) with significant dropped down in peak intensity at λ_{em} 503nm. These PL results reveal that the majority of fluorescence properties are exhibited from λ_{em} 503nm unlike to control sample (**4**). Interestingly, UV/ Flourescence/PL studies results strongly suggest the immobilization of Berberine at cellulose materials. These fluorescent cellulose materials would be applicable in the development of light sensitive functional materials.

6.3.6 Fluorescence microscopy and confocal microscopy studies

In repertoire of biocompatible functional materials, the role of newly synthesized florescent material was examined in screening of Berberine interacting biomacromolecules by using bioimaging techniques such as fluorescence microscope and confocal.

Fluorescence microscope. Free Beberine derivative (**4**) (Figure6.8) and Berberine immobilized cellulose (**6**)(Figure 6.9) were visualized with fluorescence microscope within different colour channels. Fluorescence intensity of control sample (**4**) in all color channels is very low compare to that of new functionalized material (**6**) at same

scale of visualization. The florescence microscopic image of control samples' images as oval shaped and that of modified cellulose (**6**) as rod shaped are observed. These studies further support strongly immobilization of Berberine at cellulose.

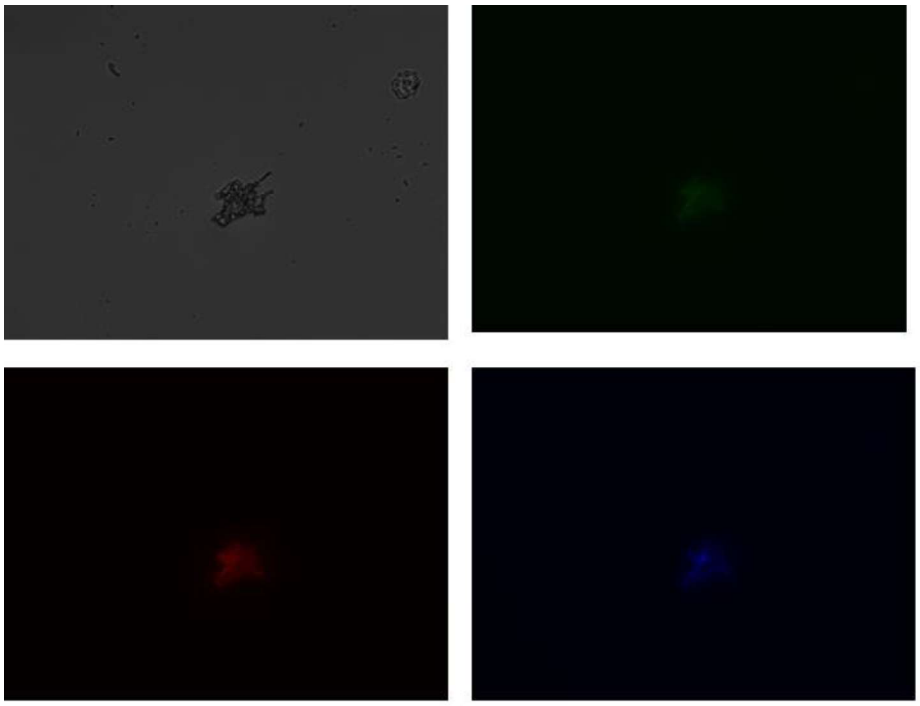


Figure 6. 8 Berberine acid fluorescence microscope images

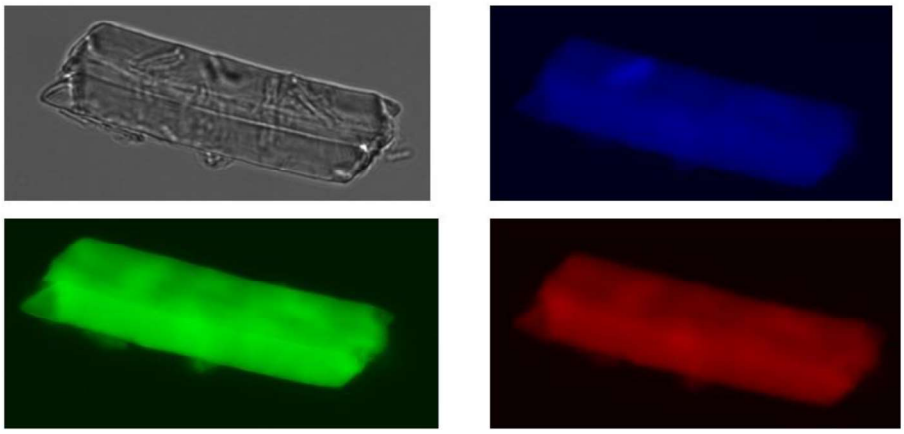


Figure 6. 9 Cellulose Berberine fluorescence microscopic images

Confocal. In end, the confocal studies were performed with Berberine immobilized cellulose material (**6**) by using confocal microscope using green channel. The confocal images of Berberine immobilized cellulose (**6**) with resolution of 5µm are given in Figure 6.10. The confocal image of Berberine cellulose (**6**) were observed as green color fiber in green channel. These studies strongly support the immobilization of Berberine derivative at cellulose surface.

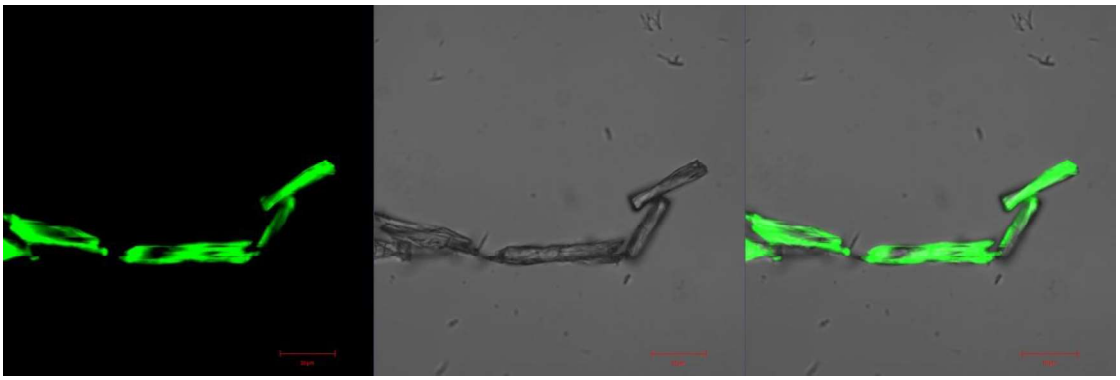


Figure 6. 10 Confocal microscope images of Berberine modified cellulose

6.4 Conclusion

We have successfully immobilized the Berberine on cellulose surface which produced fluorescent labelled cellulose material. Their photophysical properties, here, are studied using UV-Visible, fluorescence and photoluminescence techniques. The labelled fluorescent material are visualized by fluorescent microscope and confocal microscope. Finally SEM images of modified cellulose materials are strongly supported the immobilization of berberine at cellulose surface. This novel fluorescent material could be used in the screening DNA G-quadrplex structures.

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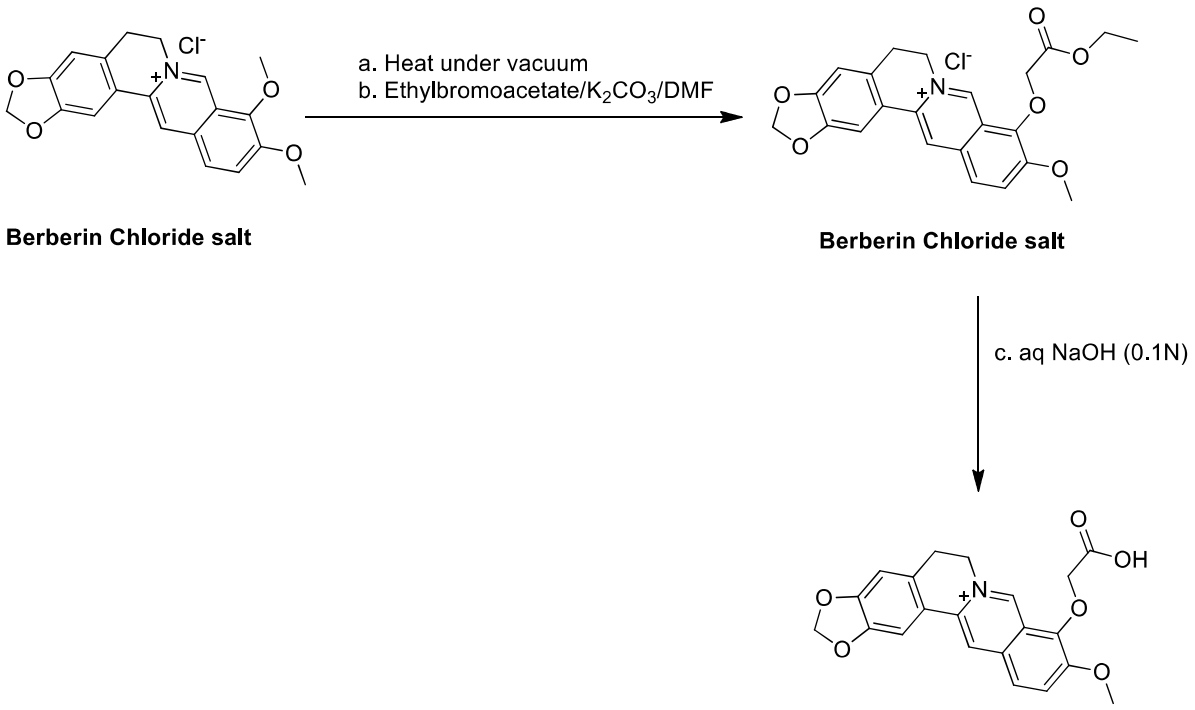
6.6 Appendix

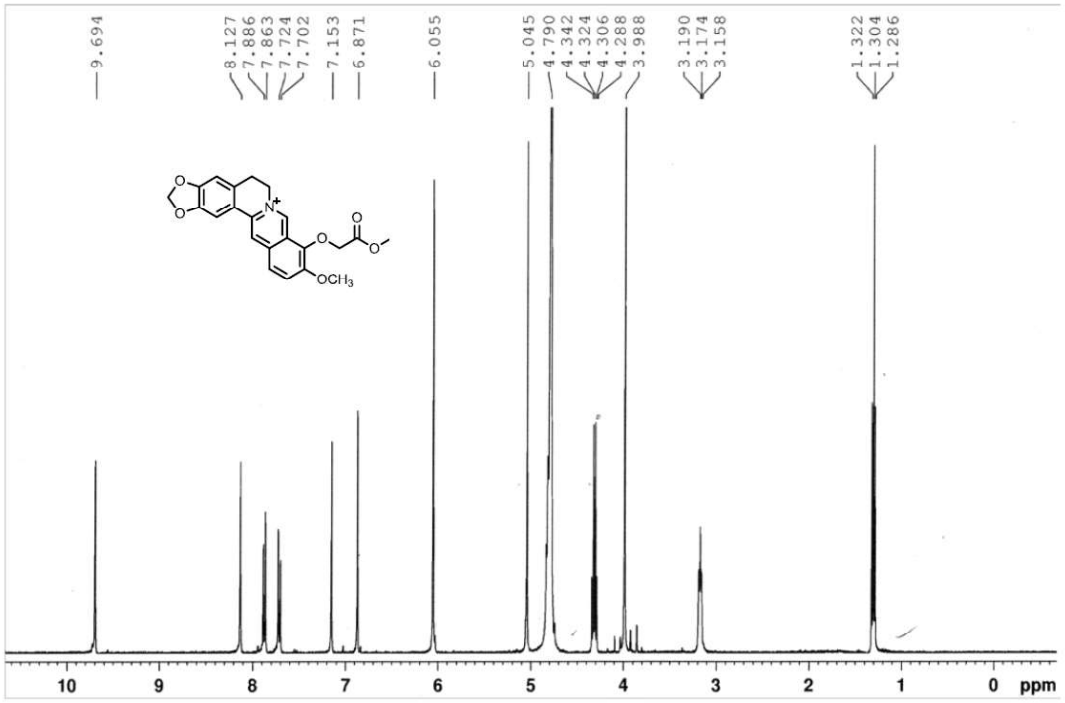
Contents

1. Synthesis of Berberine derivative*

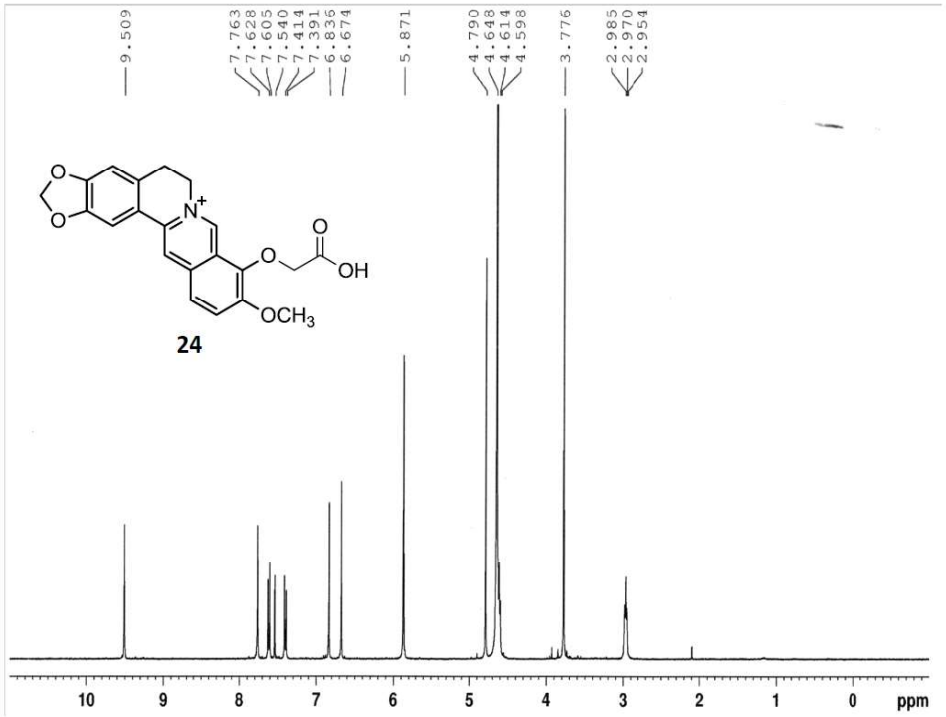


*W.-J. Zhang et al. *Bioorg. Med. Chem.* **2007**, *15*, 5493–5501

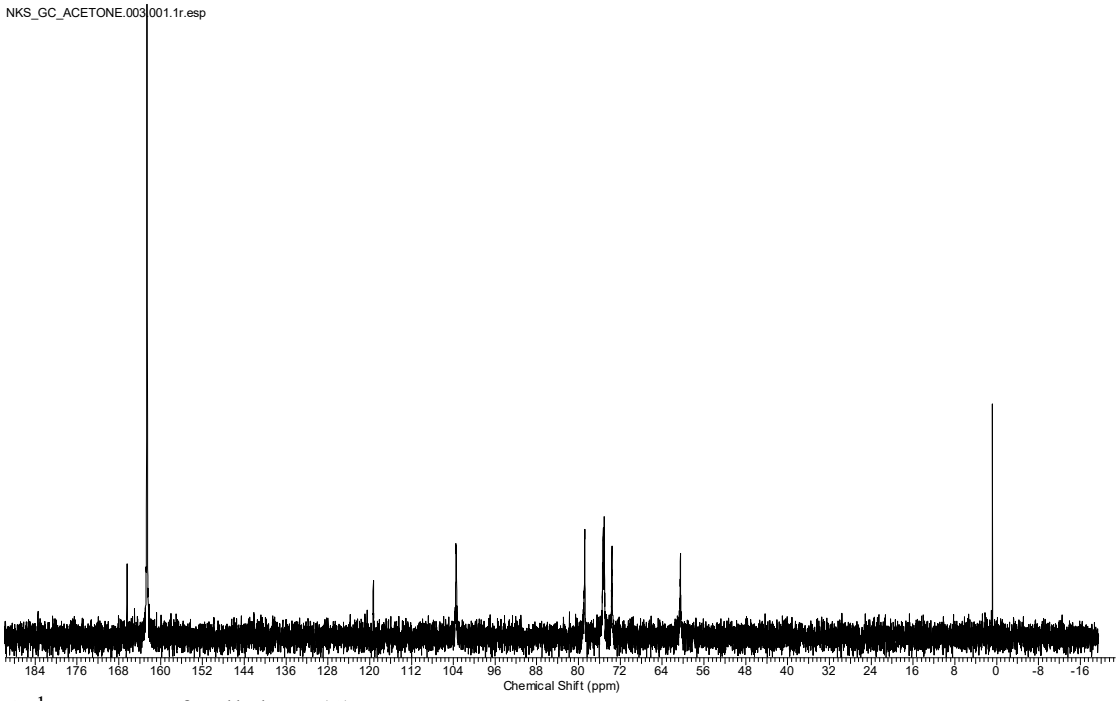
2. ^{1}H-NMR of Berberine ester derivative ester



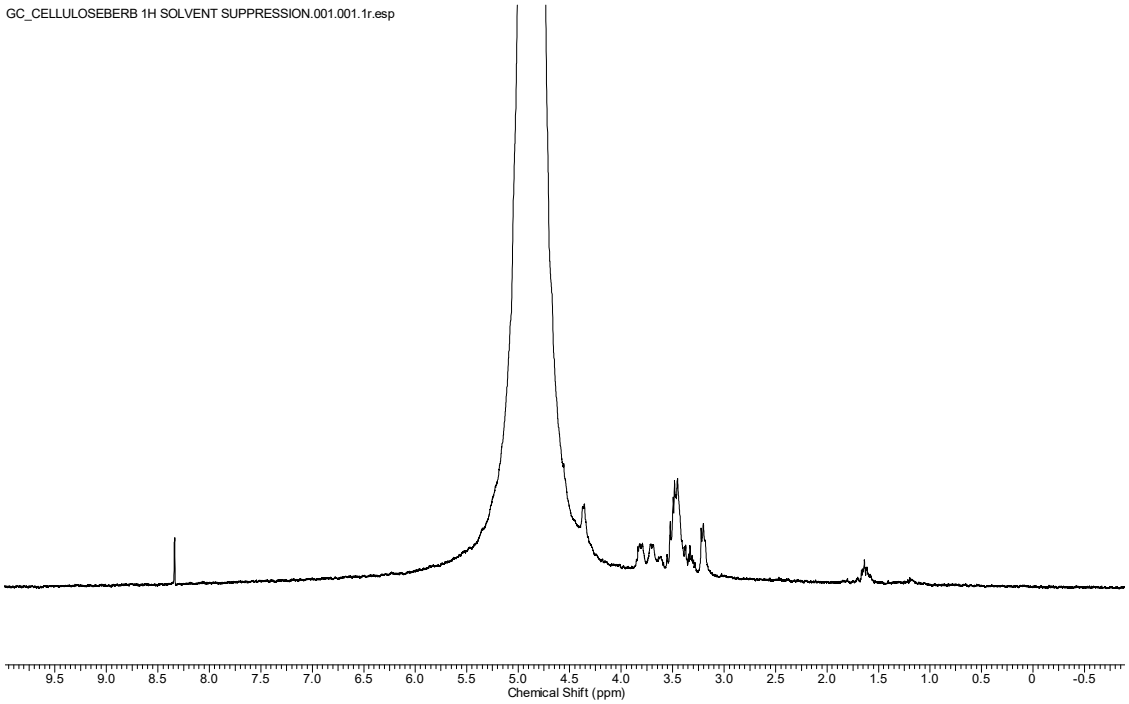
3. ^{1}H-NMR of Berberine acid derivative (4)

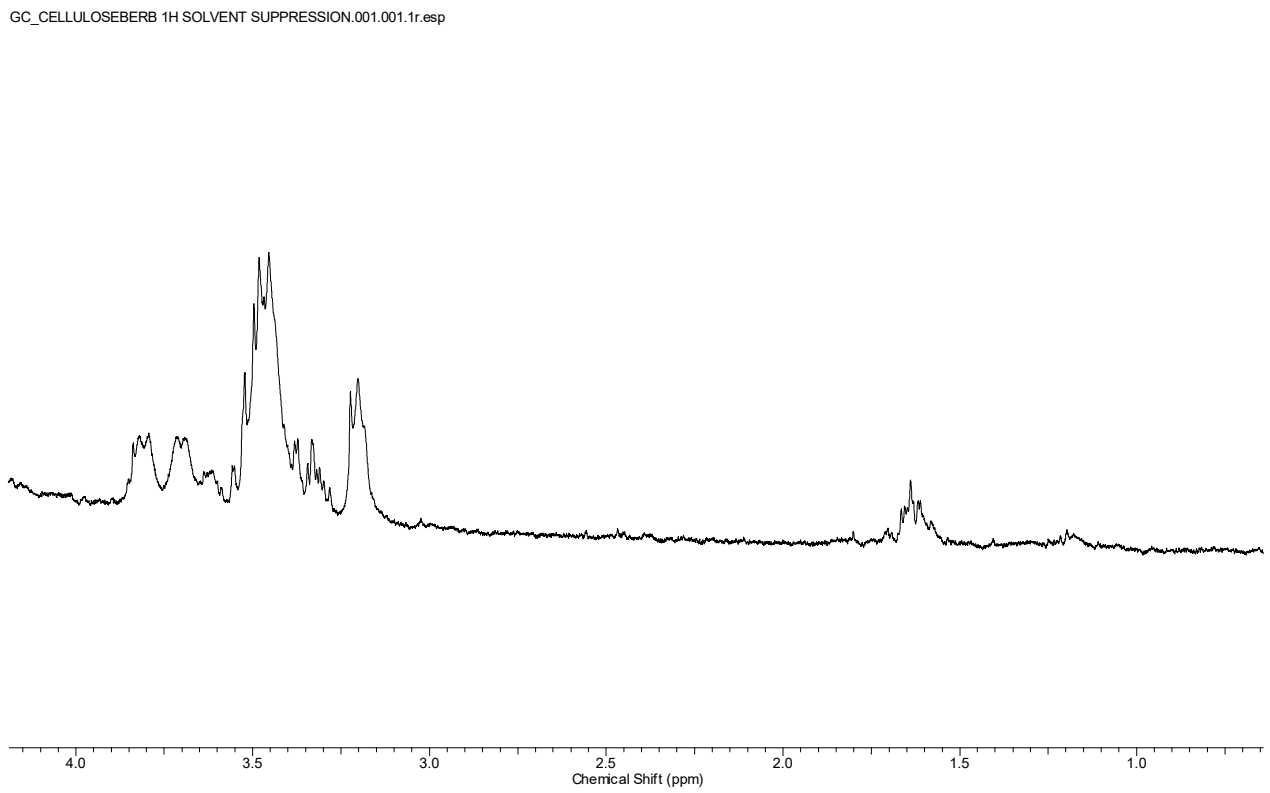


3. ^{13}C-NMR of cellulose (**1**)



4. ^{1}H-NMR of cellulose (**1**)

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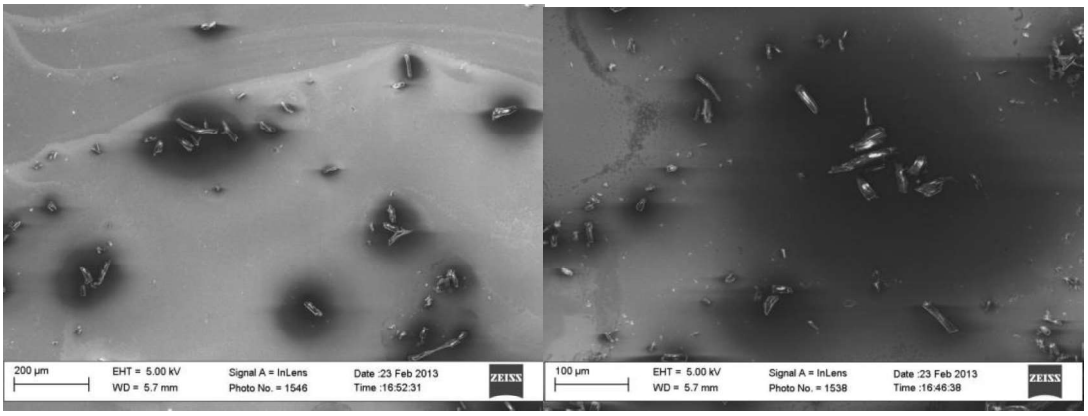
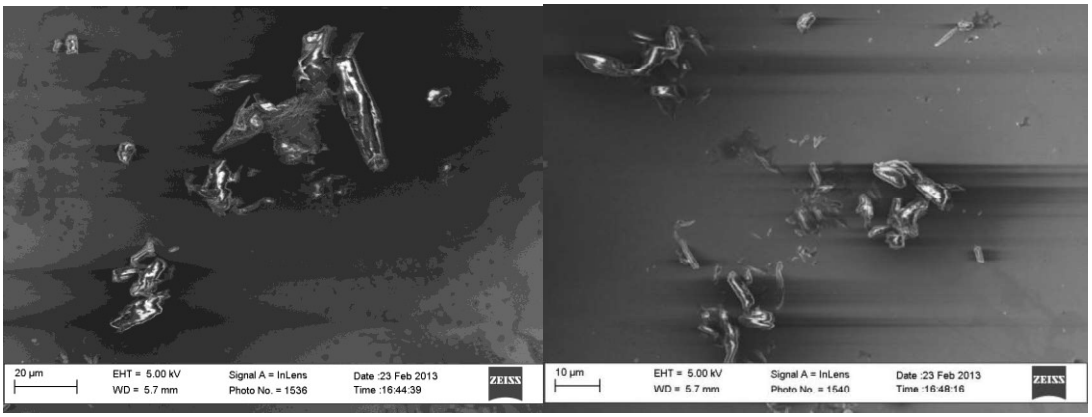
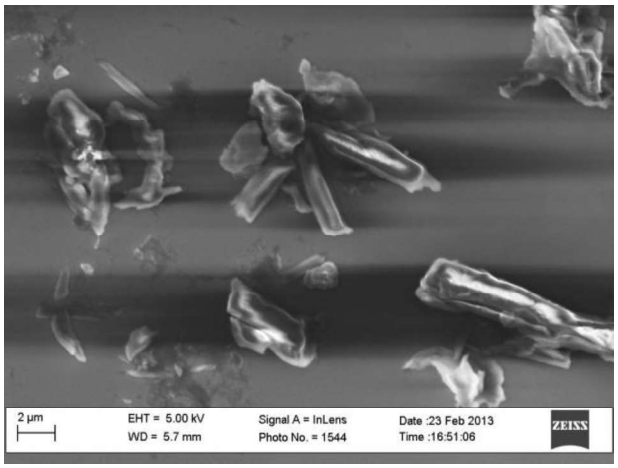
3.5
3.0
2.5
2.0
1.5
1.0
Chemical Shift (ppm)

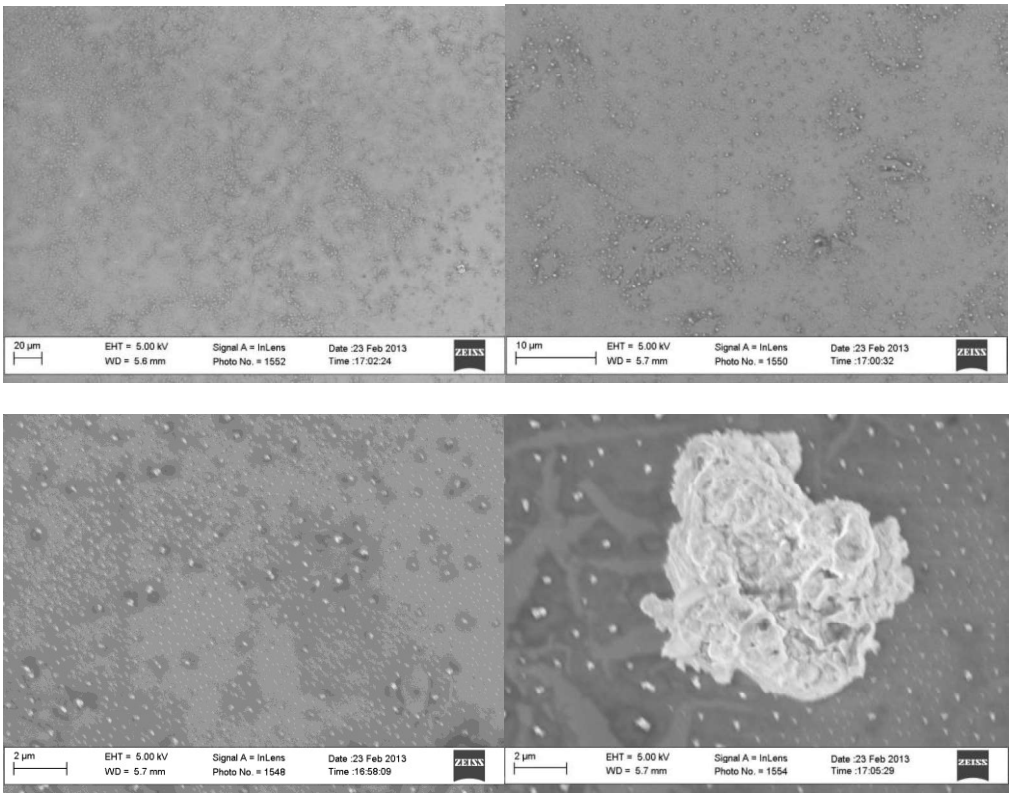
5. SEM image of native cellulose **(1)**



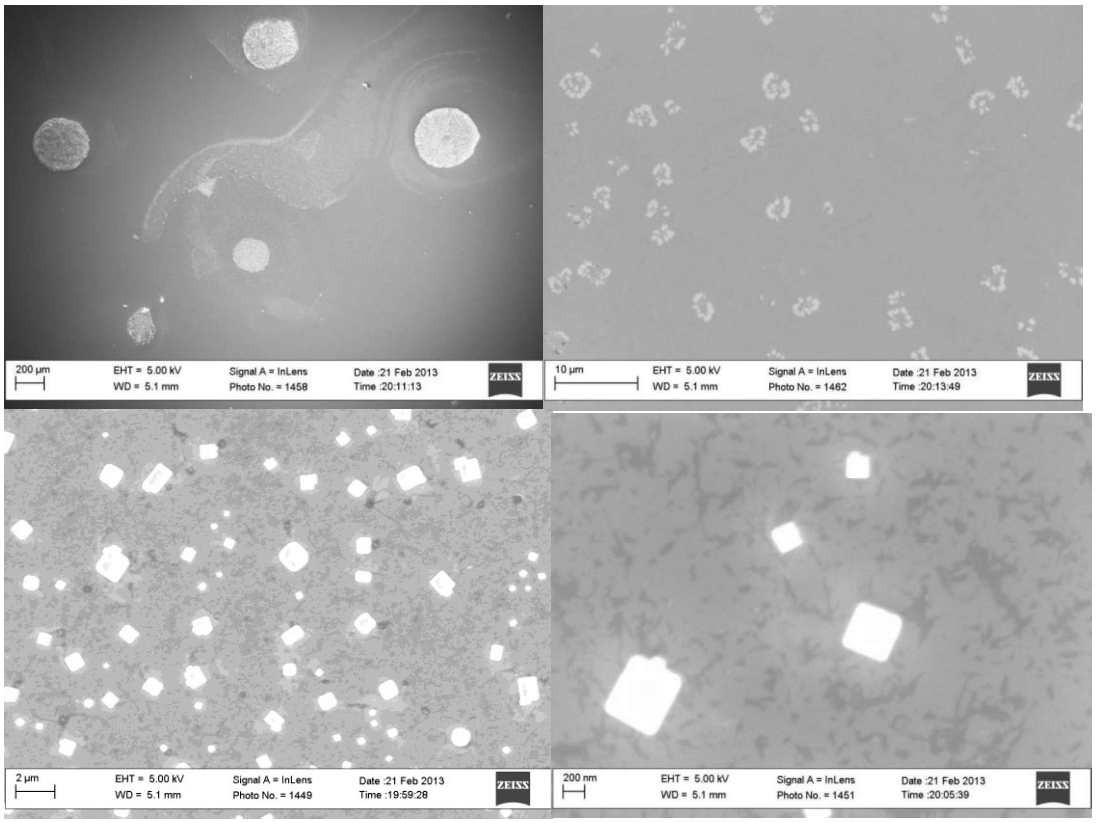


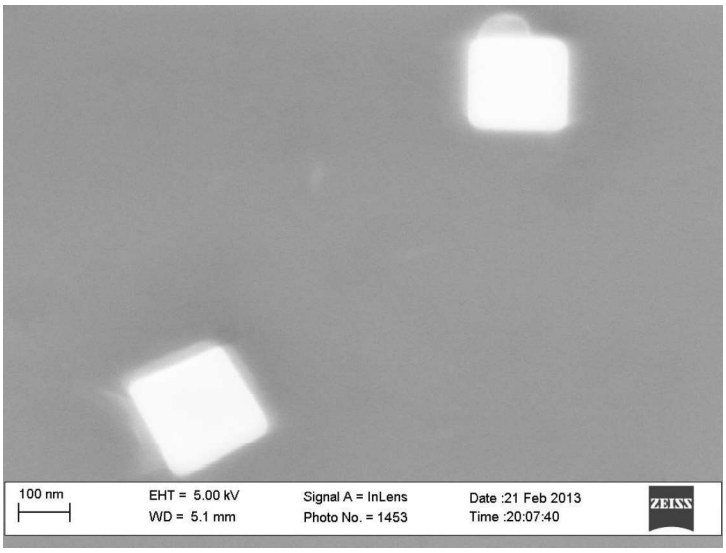


6. Amino functionalized modified cellulose (**3**)

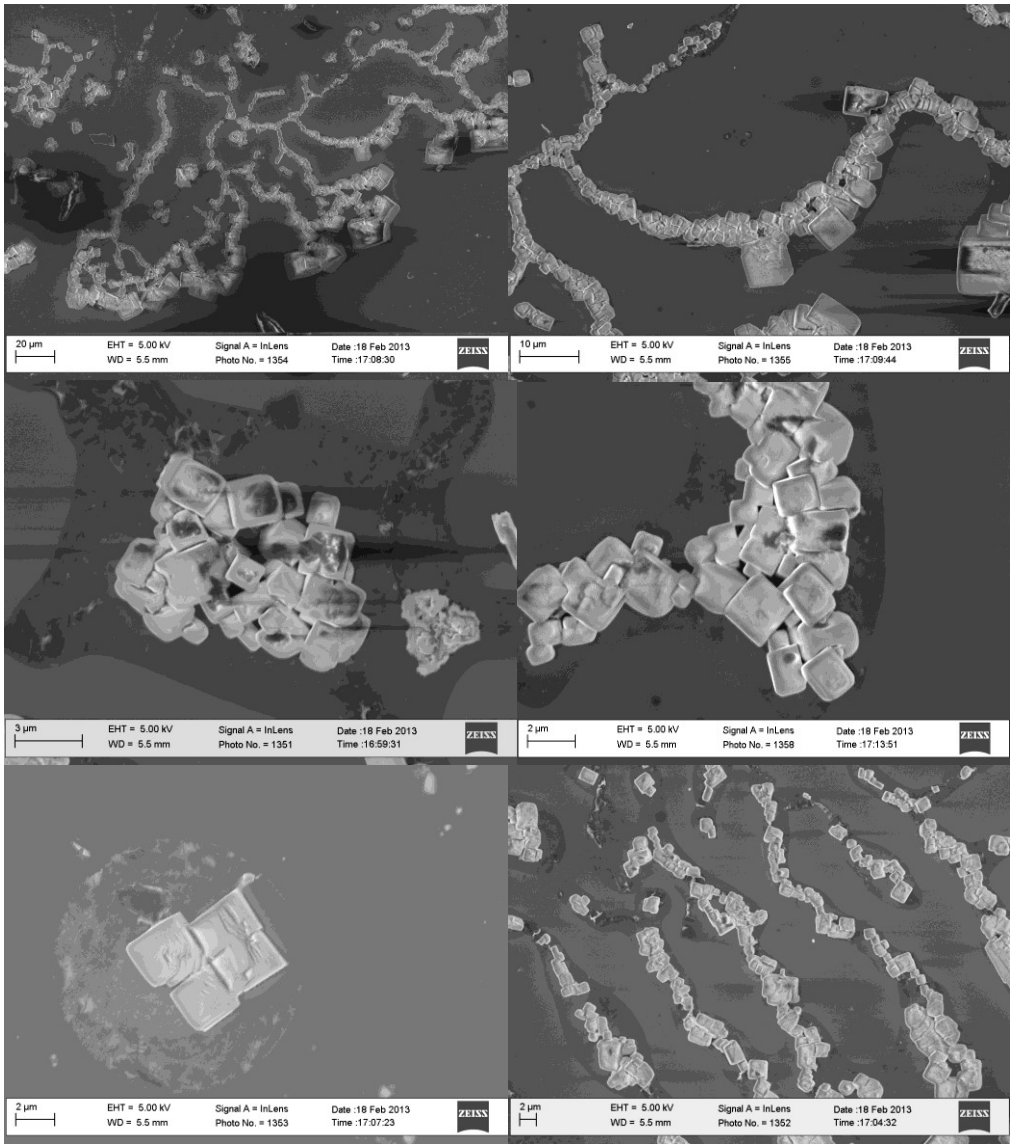


7. Acid functionalized Berbarine derivative **(4)**

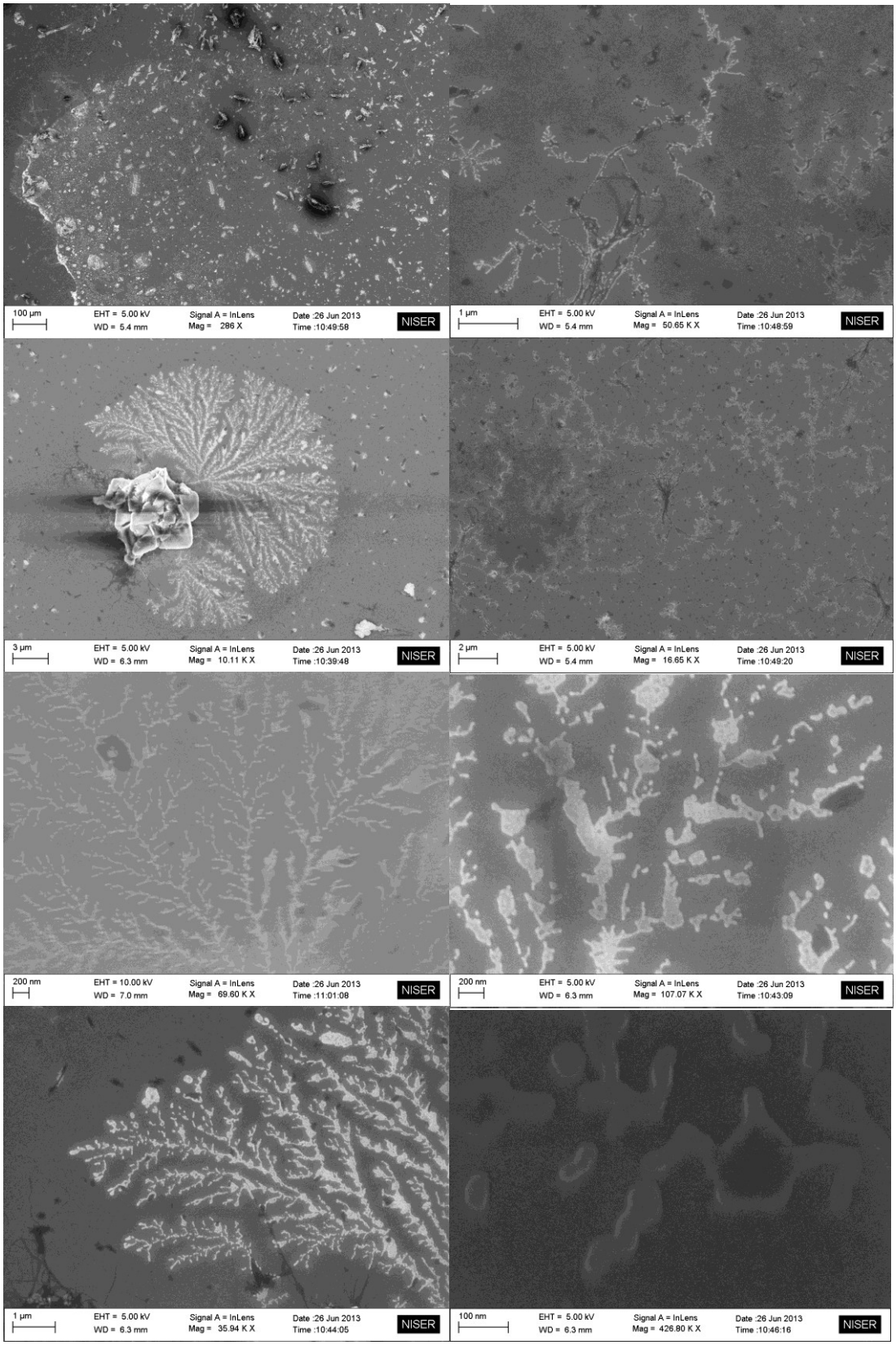


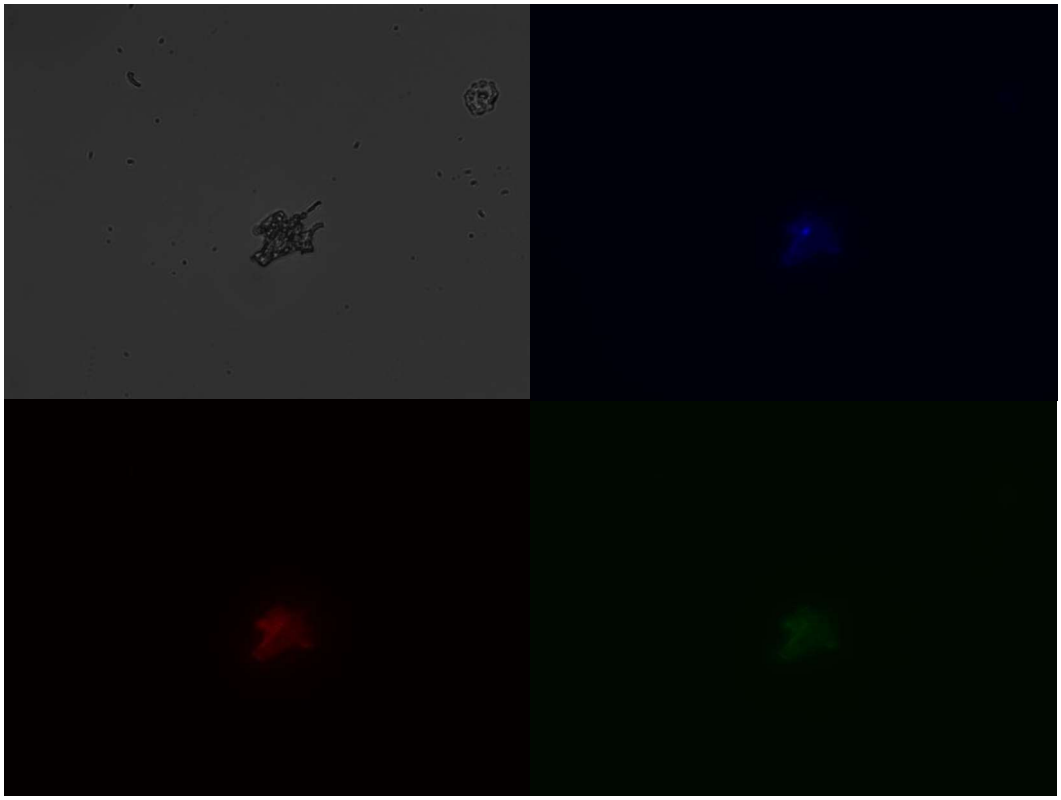


8. Berberine immobilized modified cellulose derivative **(5)**

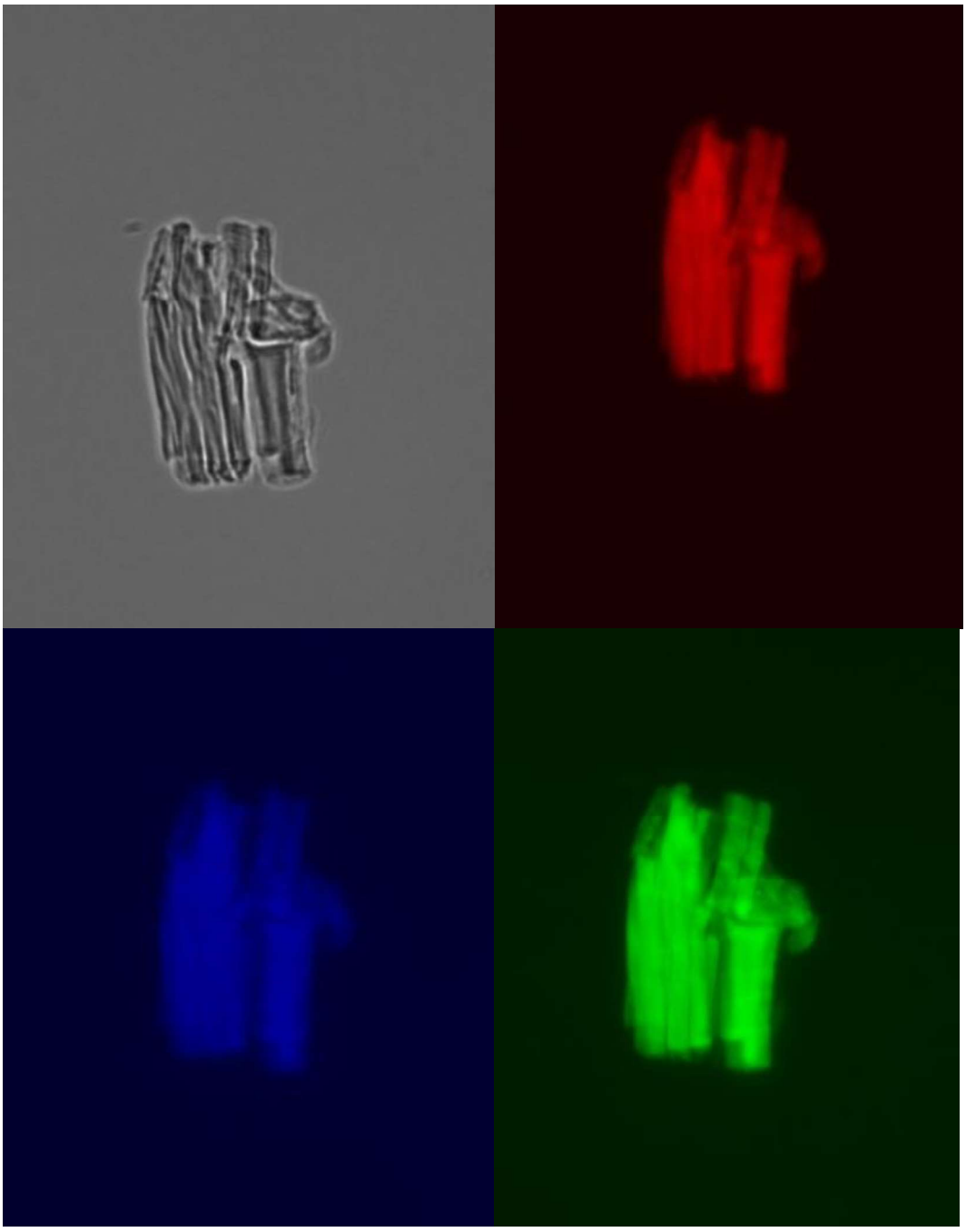


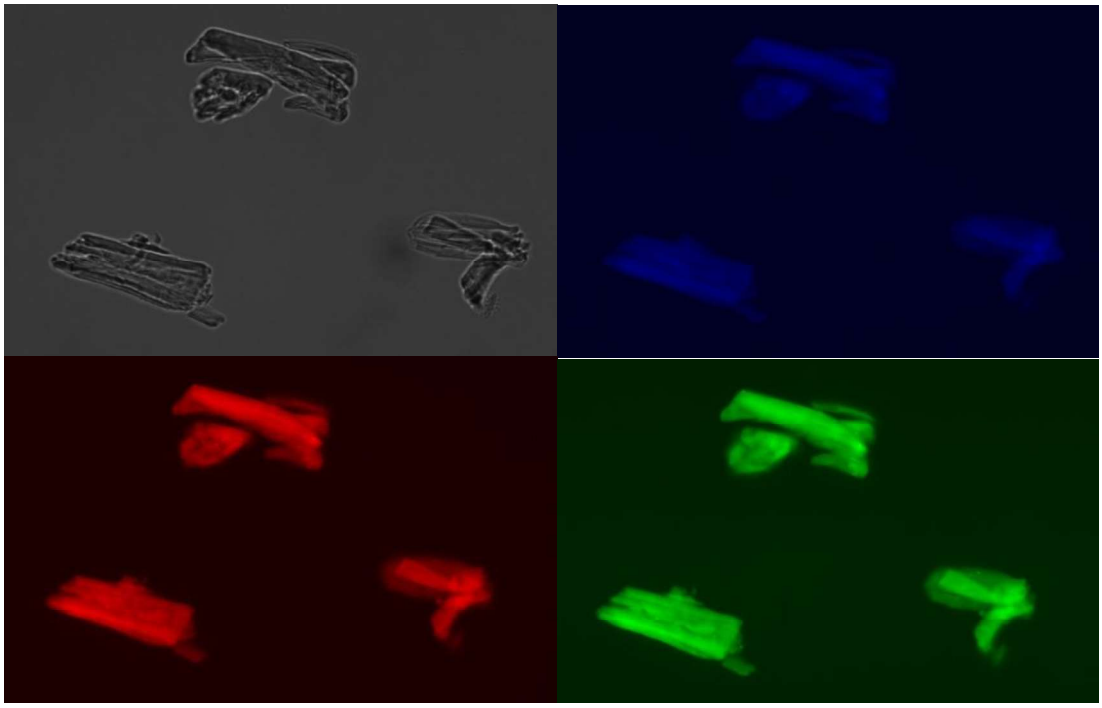
9. Modified Berberine immobilized cellulose (**6**)

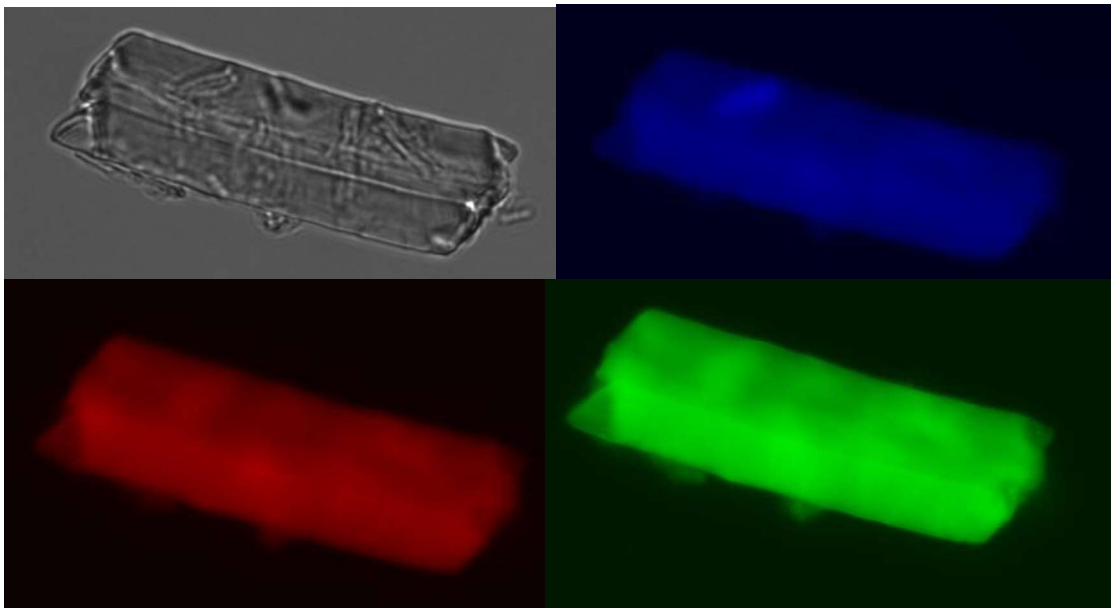




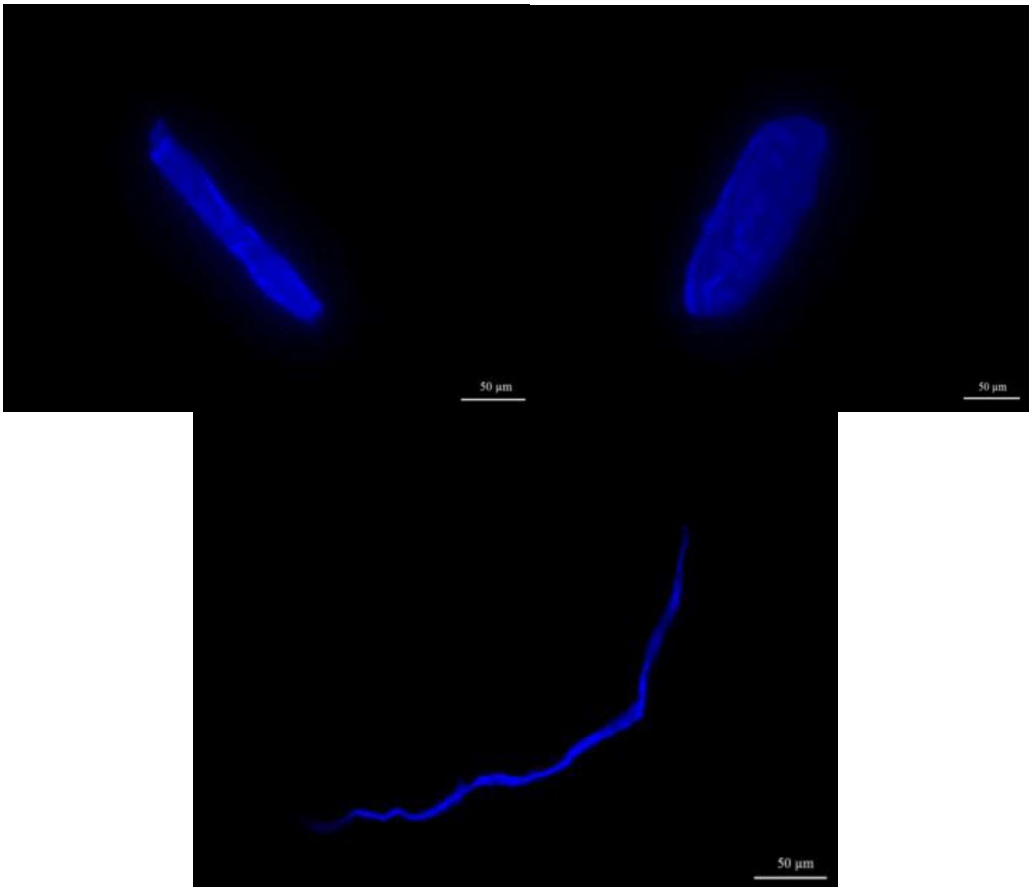
11. Fluorescence microscopic images of Berberine immobilized cellulose **(6)**



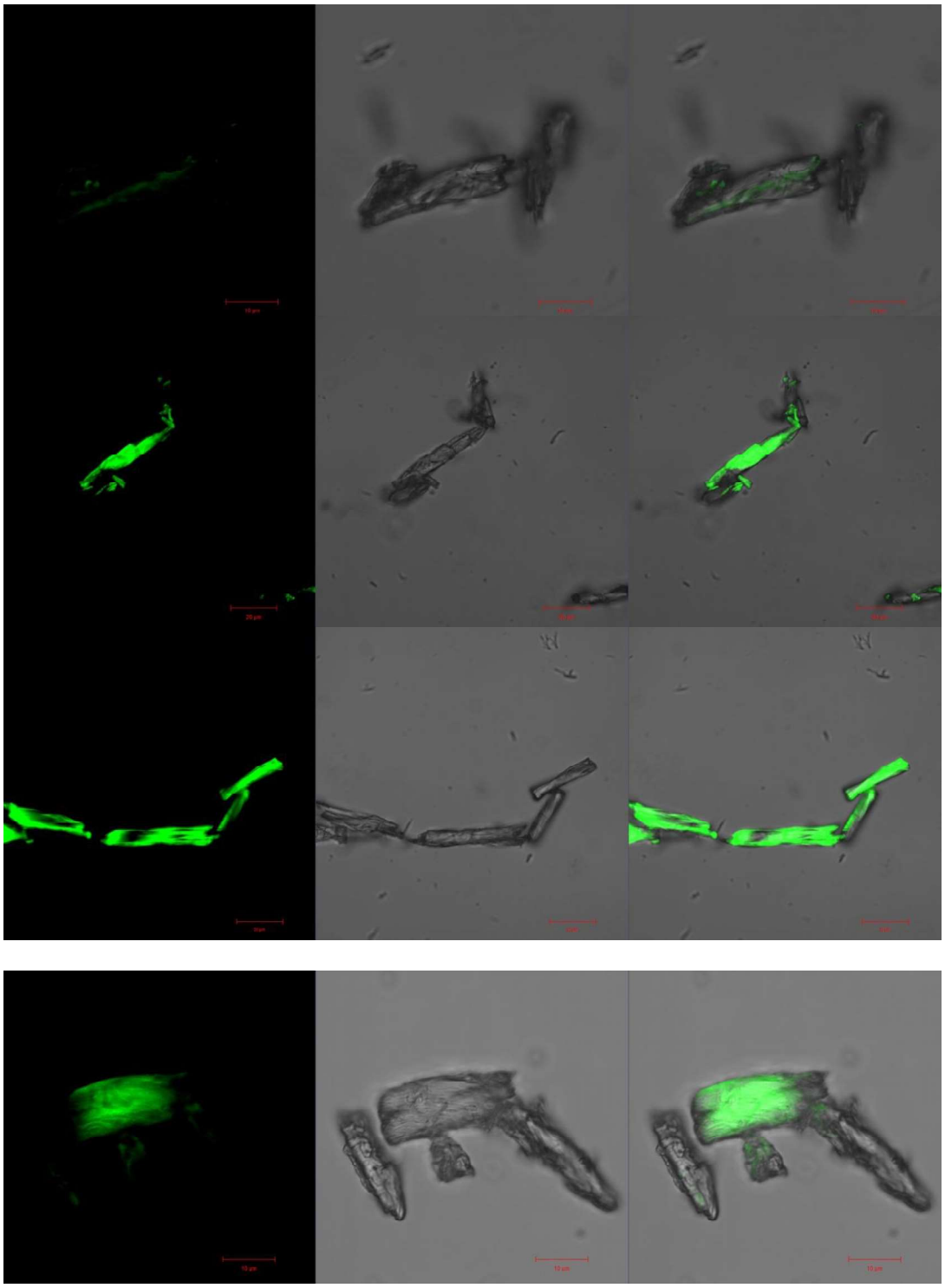


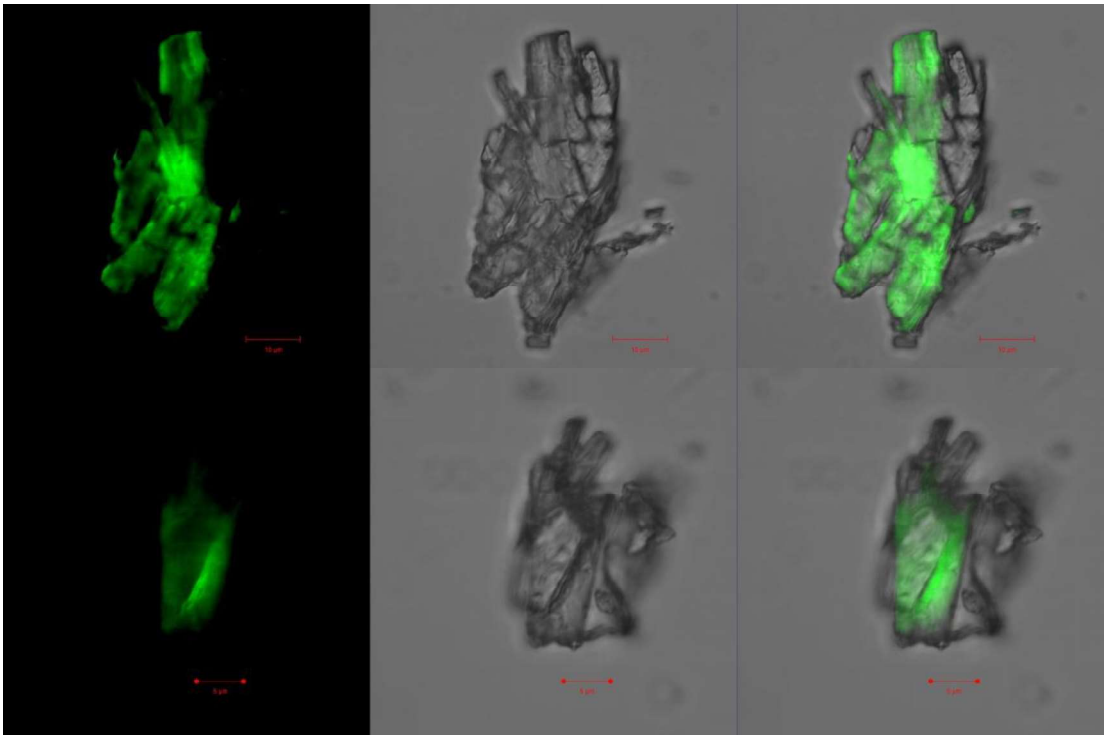


12. Fluorescence microscopic images of Berberine immobilized cellulose (**6**)



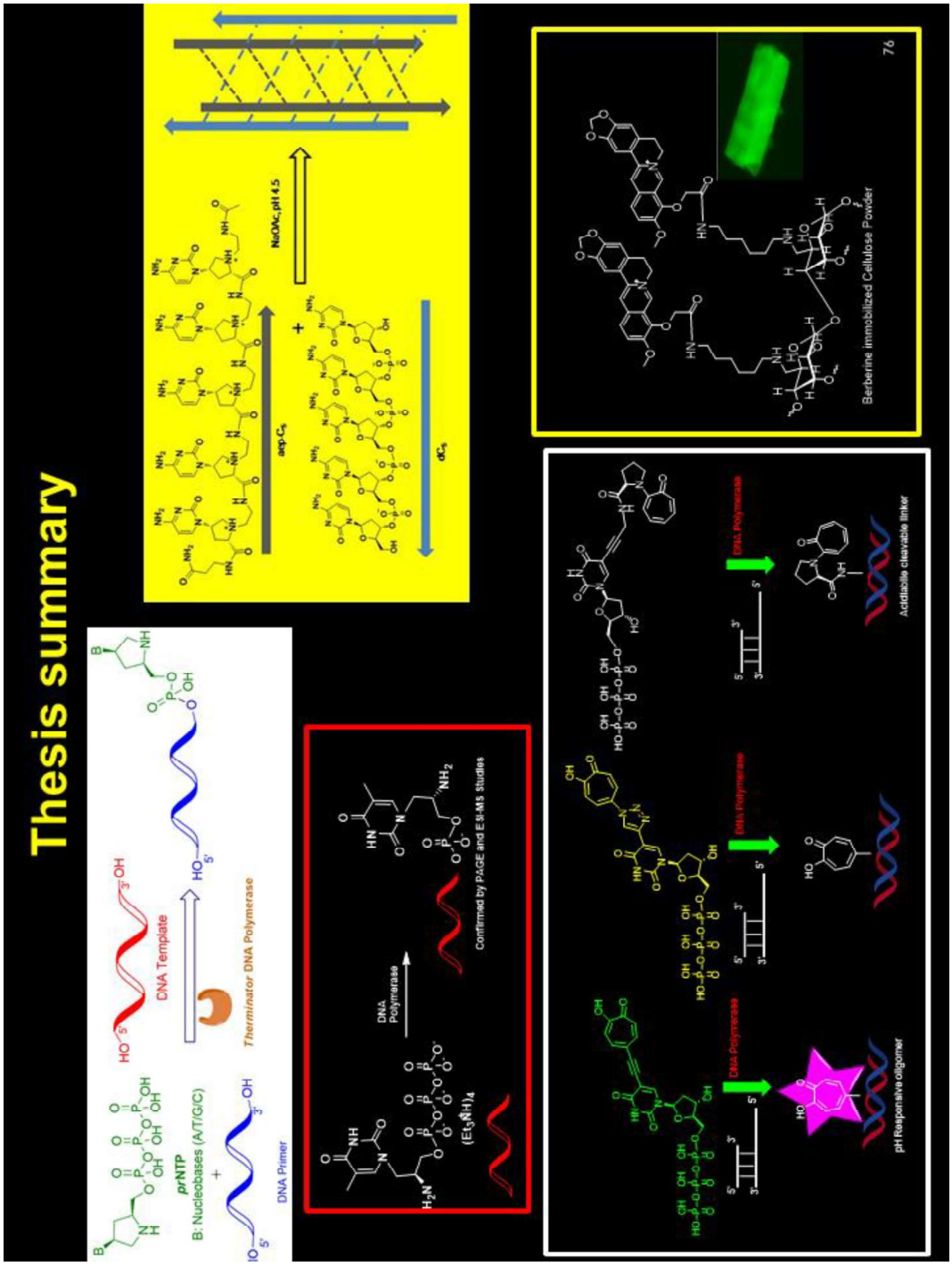
13. Confocal image of Berberine immobilized cellulose **(6)**





Thesis summary

Title of the Thesis: “Synthesis and Biochemical Evaluation of Nucleoside Triphosphates and Nucleic Acid Analogues”



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Hybrid DNA i-motif: Aminoethylprolyl-PNA (pC_5) enhance the stability of DNA (dC_5) i-motif structure

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ABSTRACT

This report describes the synthesis of C rich sequence, cytosine pentamer, of *aep* PNA and its biophysical studies for the formation of hybrid DNA:*aep*-PNAi-motif structure with DNA cytosine pentamer (dC_5) under acidic pH conditions. Herein, the CD/UV/NMR/ESI-Mass studies strongly support the formation of stable hybrid DNA i-motif structure with *aep*-PNA even near acidic conditions. Hence *aep*-PNA C-rich sequence cytosine could be considered as potential DNA i-motif stabilizing agents *in vivo* conditions.

Since after discovery of DNA duplex structure, DNA are also known to form *triplex*, *tetraplex*, and other many more DNA polymorphs.[1] Importantly, DNA forms two type of *tetraplex* structures from the repeated G-/C-rich sequences: (i) the G-quadruplex (G_4), which encompasses of stacked guanine tetrads stabilized by monovalent cations (Na^+/K^+); and (ii) the i-motif, a cytosine-rich structure comprising of two hemi-protonated parallel duplexes intercalated in an antiparallel orientation (Fig. 1).[2,3] The role of those DNA tetraplexes structures are reportedly considered in the maintenance of telomere and gene regulation. Recently, the occurrence of i-motif are noticed in regulatory regions of the human genome such as centromeres, telomeres, and oncogene promoter regions.[4] These structures are also considered as potential materials for nanotechnology research.[5,6]

Structurally, the DNA i-motif is reportedly a compact structure with short base-pairing distances (~3.1 Å), helical twist (~12–16°) between adjacent C:C$^+$H base pairs and close sugar-sugar contacts, C—H—O interactions (Fig. 1).[3] However, the repulsive interactions also exist between the charged *C-imino* protons and phosphates groups of DNA strands, which intend the destabilization of that i-motif structure. *In vitro*, the formation of DNA i-motif structures are occurred only under acidic conditions, near acidic pH range (3.5–5.5) and the maximum stability are noticed at pH equal to the pKa of the cytosine N-3. Though DNA i-motifs are stable at neutral pH conditions *in vivo* presumably due to other cellular factors such as negative super helicity, cellular proteins and molecular crowding conditions.[7] Recently, the formation of DNA i-motif are noticed *in vitro*, at neutral pH conditions, in presence of metal ions (Cu^{2+}/Ag^+).[8] Furthermore, the stable i-motif structures have been achieved by sugar/phosphate backbone modified DNA analogues which are following-Thio-phosphoramidates,[9] RNA,[10] Locked nucleic acid (LNA),[11] and 2′-fluoro DNA analogue.[12]

Nevertheless the stabilization of DNA i-motifs are also explored by hybridization with similar C-rich sequence of DNA analogues, and with the sequence specific DNA binding small synthetic molecules.[13] In repertoire of structurally backbone modified DNA analogues, Peptide nucleic acid (PNA), consisting aminoethylglycinate (*aeg*) backbone, has emerged as a potential DNA analogue because of its promisable binding affinities with DNA duplex/triplex structures.[14] The C-rich sequence (TC_5/TC_8) of *aeg*-PNAs are also explored in the formation of stable i-motif.[15,16] Moreover modified PNA as Alanyl-PNA, alanine backbone instead of *aeg*, are reportedly known the formation of the stable i-motif from its TC_8 sequence.[17] Further, *aeg*-PNA are successfully employed for the formation of hybrid DNA (or RNA) i-motif structure which are more stable than respective DNA (or RNA) i-motif.[18,19] The stabilization of PNA:DNA (or PNA:RNA) hybrid i-motif are presumably considered because the hybridized *aeg*-PNA strand diminish the ion-ion repulsive interaction between negatively charged DNA's phosphate backbone in that hybrid i-motif.

These results inspired us to design the hybrid DNA i-motif from the positively charged backbone containing DNA or PNA analogue which could enhance the stability of DNA i-motif structure near

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Dideoxy nucleoside triphosphate (ddNTP) analogues: Synthesis and polymerase substrate activities of pyrrolidinyl nucleoside triphosphates (prNTPs)

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ABSTRACT

The dideoxynucleoside triphosphates (ddNTPs) terminate the bio-polymerization of DNA and become essential chemical component of DNA sequencing technology which is now basic tool for molecular biology research. In this method the radiolabeled or fluorescent dye labeled ddNTP analogues are being used for DNA sequencing by detection of the terminated DNA fragment after single labeled ddNTP incorporation into DNA under PCR conditions. This report describes the syntheses of rationally designed novel amino-functionalized ddNTP analogue such as Pyrrolidine nucleoside triphosphates (prNTPs), and their polymerase activities with DNA polymerase by LC–MS and Gel-electrophoretic techniques. The Mass and PAGE analyses strongly support the incorporation of prNTPs into DNA oligonucleotide with Therminator DNA polymerase as like control substrate ddNTP. As resultant the DNA oligonucleotide are functionalized as amine group by prNTP incorporation with polymerase. Hence prNTPs provide opportunities to prepare demandable conjugated DNA with other biomolecules/dyes/fluorescence molecule without modifying nucleobase structure.

1. Introduction

The dideoxynucleoside triphosphate (ddNTP) is structurally modified derivative of deoxynucleoside triphosphate (dNTP), and also known as Sanger's nucleotide which contains dideoxygenated ribose ring, nucleobase (A/T/G/C) and triphosphate residues (Fig. 1). In late seventies, Sanger et al. established the function of ddNTPs as termination of DNA polymerization after single incorporation, enzymatically, into DNA primer (at 3′-OH), and laid down the foundation of the DNA sequencing technology.[1] In DNA sequencing, the labeled ddNTP analogues have been employed to terminate the DNA synthesis from DNA primer under PCR condition, and make detectable primer with inclusion of one labeled-ddNTP residue. The labeling of ddNTPs are being performed by introducing the radioactive isotope or chromophores into the chemical structure of ddNTPs such as α-^{32}P-radioisotope for radioactive ddNTPs,[2] and big-dyes for fluorescent ddNTPs.[3] Due to cost and safety reasons, the radioactive labeling are not being encouraged for practical application in sequencing. The dye/fluorophore-labeled ddNTP, however, are appreciated to employ in DNA sequencing and their synthesis are accomplished by attaching dye/fluorophore, via linker, at the nucleobase region of modified ddNTP which are obtained by functionalization-such as amine or alcohol functional group-at purine/pyrimidine carbon atom of nucleobases. Mostly the purine 7-deazapurine and C-5-substituted pyrimidine containing ddNTP analogues have been used for functionalization with amino group which coupled with carboxylate group of dyes via amide bond. Recently, four different colors dyes are being employed to prepare four different colors of ddNTPs such as red-ddTTP, blue-ddATP, green-ddCTP, and yellow-ddGTP and then employed in DNA sequencing technologies.[4]

In addition, the 3′-hydroxyl group protected dNTP derivatives have also been synthesized, and explored their ability to terminate the DNA synthesis as like ddNTP (Fig. 1). Though only the unhindered protecting groups show remarkable characters in DNA sequencing as terminators. If 3′-OH protecting groups of dNTPs are linear and softly cleavable with physiological pH condition or UV-light then these dNTPs are being used as reversible terminators. A variety of 3′-OH protected/modified dNTP terminators, reversible or irreversible, have been explored in DNA sequencing under.[5–7] Moreover the sugar ring modified dNTPs as cyclopentane ring containing dNTPs are also explored and found as antiviral characters.[8] Recently pyrrolidine ring containing non-sugar nucleoside derivatives, including PNA, have also been studied.[9–11]

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Methylene Salicylicacidyl Hexamer (MSH) Has DNAse Activity

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Abstract Salicylic acid and formaldehyde form heterogenous methyl/methylene salicylicacidyl oligomers and polymers in presence of sulfuric acid (H_2SO_4) and sodium nitrite ($NaNO_2$). One of the oligomers as aurintricarboxylic acid (ATA), methelene bridged salicylic acid trimer, has been identified and explored in biochemical research, which is a potent inhibitor of many biological processes. A very few reports are also available on *dimer*, *trimer*, and *tetramer* of methelene bridged salicylic acids from same reaction mixture. Herein, we report the isolation and biochemical screening of partial purified low-molecular component as methylene salicylicacidyl hexamer (MSH) from the above reaction mixture. The interaction of methylene salicylicacidyl oligomer with DNA was studied by agarose and polyacrylamide gel electrophoresis, which suggest that methylene salicylicacidyl oligomer has DNAse activity. So far, no such significant reports are available on low-molecular oligomer of methelene bridged salicylic acids. In further, we also attempted to investigate the nature of nuclease activity, which clearly indicates DNA exonuclease type of activity. Further studies are needed to establish the mechanism of actions.

Keywords DNA · DNASE · Salicylic acid · Aurintricarboxylic acid (ATA)

Ankit Tiwari, Chandrasekhar Reddy Gade, Manjusha Dixit and Nagendra K. Sharma contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s12010-015-1678-z) contains supplementary material, which is available to authorized users.

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Synthesis and spectroscopic studies of berberine immobilized modified cellulose material†

Chandrasekhar Reddy Gade and Nagendra K. Sharma*

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This report describes the synthesis, characterization and spectroscopic studies of berberine immobilized modified cellulose materials, which could be a promising new biocompatible fluorescence material because berberine is a natural fluorescent molecule having important pharmacological aspects including selective binding with DNA G-quadruplex. Thus, berberine immobilized cellulose materials could be applicable in the screening of G-quadruplexing DNA sequence by bio-imaging techniques.

Cellulose is the most abundant natural biopolymer containing a long chain of anhydroglucose units, which are linked together by a β-1,4-glycosidic bond.[1,2] Cellulose and related polymers occur in nature as major components of plant cell walls and woody tissues, where these polymers bind to proteoglycan materials. Cellulose contains multiple hydroxyl functional groups at the surface. Thus, its chemical reactivities are almost like aliphatic alcohols, which are available for further transformation into other desired functional groups such as amino and carboxylate groups. By altering cellulose chemical functionality, many cellulose functional materials have been synthesized and studied in order to obtain a new type of functional material.[3–5] Recently, morphologies of cellulose surface have been easily achieved in a controlled approach with a gentle chemical reaction. For example, surface properties of cellulose such as aliphatic, aromatic and hydrophilic properties have been accomplished by incorporation of respective nature (aliphatic, aromatic and hydrophilic) of amino acids at cellulose *via* ester bond.[6,7] In spite of multiple hydroxyl groups, cellulose material is usually insoluble in most organic solvents and recently has been used as solid support for peptide synthesis.[8] Fascinatingly, cellulose-supported peptides have been synthesized and used in the development of peptide-array experiments for cell-adhesive peptides analysis.[9] Recently, bioactive natural products as chalcone analogues have been attached with cellulose in expansion of microarrays for screening of antibacterial activities.[10]

As in progress of new synthetic functional materials, biocompatible fluorescent materials such as fluorescent-labeled cellulose materials have also been generated efficiently.[11,12] Due to their biocompatibility and hydroxyl functionality, chemical syntheses of cellulose functional materials are an emerging area of research and have been exploited for industrial and medical applications including as drug delivery agents.[13] In the repertoire of new cellulose functional materials, we planned to immobilize natural bioactive fluorescent products such as berberine on cellulose material. As is well known, berberine is a potential anti-tumor and anti-cancer drug candidate and has been considered a potential therapeutic agent.[14–16] In addition, berberine and related compounds have high colour index and are being used as dyeing agents in textile and leather industries. Moreover, berberine and related derivatives are small fluorescent molecules which can easily be visualized, even at low concentration, by fluorescence spectrophotometry and other available bioimaging techniques. Herein, we report the synthesis; characterization; and photophysical properties of berberine immobilized modified cellulose material *via* hexamethyl linker with amide bond, which could be a potential fluorescent functional material for finding DNA G-quadruplex forming DNA sequences in the presence of other secondary structures (duplex and triplex) forming DNA sequences. By doing this, the off-target effect of berberine-related pharmacological properties can be minimized.

Results and discussions

The stepwise synthesis of modified cellulose materials is given in Scheme 1. The hydroxyl functional group of cellulose powder

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† Electronic supplementary information (ESI) available: ^{1}H-/^{13}C-NMR spectra of berberine derivatives (2) are provided. SEM image of berberine, cellulose, and berberine immobilized cellulose at different resolutions are given. See DOI: 10.1039/c4ra06015f