Synthesis and Enzymatic Incorporation of an Azide-Modified Uridine Triphosphate Analogue

A thesis submitted towards partial fulfillment of

5 year integrated BS-MS programme

by

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Under the guidance of

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Certificate

This is to certify that this dissertation entitled "Synthesis and Enzymatic Incorporation of an Azide-Modified Uridine Triphosphate Analogue" towards the partial fulfillment of the 5 year integrated BS-MS programme at the Indian Institute of Science Education and Research Pune, represents original research carried out by Harita Rao at Indian Institute of Science Education and Research Pune under the supervision of Dr. S. G. Srivatsan.

Supervisor	**Head Bio / Chem/ Phys/ Math Sciences**
Date:	**Date:**
Place:	**Place:**

Dedicated to my father..

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TABLE OF CONTENTS

1. Introduction..... 6

2. Aim and Objectives..... . 16

3. Results and Discussion..... 16

4. Conclusion..... 23

5. Experimental Section..... 24

6. Appendix (Selected Spectrum)..... 33

7. References.....43

Introduction

Ribonucleic acid is one of the most essential biopolymers participating in diverse biological functions in all life forms. Of the various roles of RNAs in a cellular system the most familiar is that of a genetic information carrier, which directs the synthesis of proteins by the process termed as translation. In retrovirus RNA alone exists as genetic information, e.g. HIV.[1] Over the past few decades RNAs have been found to be involved in many other cellular functions. In contemporary biology, RNA is known to function as a genetic material, catalyst, and regulatory motif. After the discovery of ribozymes, catalytic RNA, RNA interference by micro RNAs (miRNA) and small interfering RNAs (siRNA) has been considered as one of the seminal contributions. MiRNAs and siRNAs are short (~22 bases) oligoribonucleotides, which can upregulate or downregulate gene expression by annealing to mRNA sequences. [2] Recently, noncoding regions of mRNA called riboswitches, which regulate protein expression by binding to small molecule metabolites were discovered.[3-4] The binding of the metabolite to the riboswitch occurring in a concentration dependent fashion enforces a structural reorganization of the riboswitch leading to a down regulation of the protein responsible for the biosynthesis of the metabolite itself.[5-6] Most riboswitches discovered so far occur in bacteria, and a very few occur in certain fungi and plant. Hence, riboswitches are being seriously considered as potential targets for anti-bacterial drug discovery.[6] Bacterial ribosomal RNA is one of the oldest and well understood RNAs, which is a target of a class of antibiotics, aminoglycosides.[7] Aminoglycosides bind to the bacterial ribosomal decoding aminoacyl site (A-site) of 16S rRNA leading to codon-anticodon misreading. This misreading results in improper protein translation, and hence, cell death.[8] Similarly, certain viral-specific RNA motifs, which are very important for the life cycle of the virus are targets for drug designing.

RNA performs its function by binding to protein, nucleic acid or small molecule. This functional diversity of RNA originates from its flexibility to fold into a number of secondary and tertiary structures, which provide binding pockets for biomolecules.[9] As a result, numerous biophysical methods have been employed to fabricate assays to understand interactions of RNA with small molecules, proteins or oligonucleotides.[10] RNA labelling with the help of a variety of methods has served unparalleled role for such studies with great sensitivity. RNA molecules have been tagged with radio labels, which have been useful for various purposes like quantification of RNA, identifying the 5′ and 3′ ends of RNA and

mapping the position of introns.[11] However, fluorescence-based methods using fluorescence properties such as fluorescence lifetime, fluorescence intensity, and fluorescence polarization, and fluorescence phenomenon like resonance energy transfer (FRET) have provided information regarding the structure and function of nucleic acids in real time with remarkable sensitivity. There are several examples of DNA and RNAs labelled with fluorescent dyes such as 5-carboxy-fluorescein, tetramethyrhodamine, nitrobenzoxadiazole and fluorescein isothiocyanate, to name a few (Figure 1).

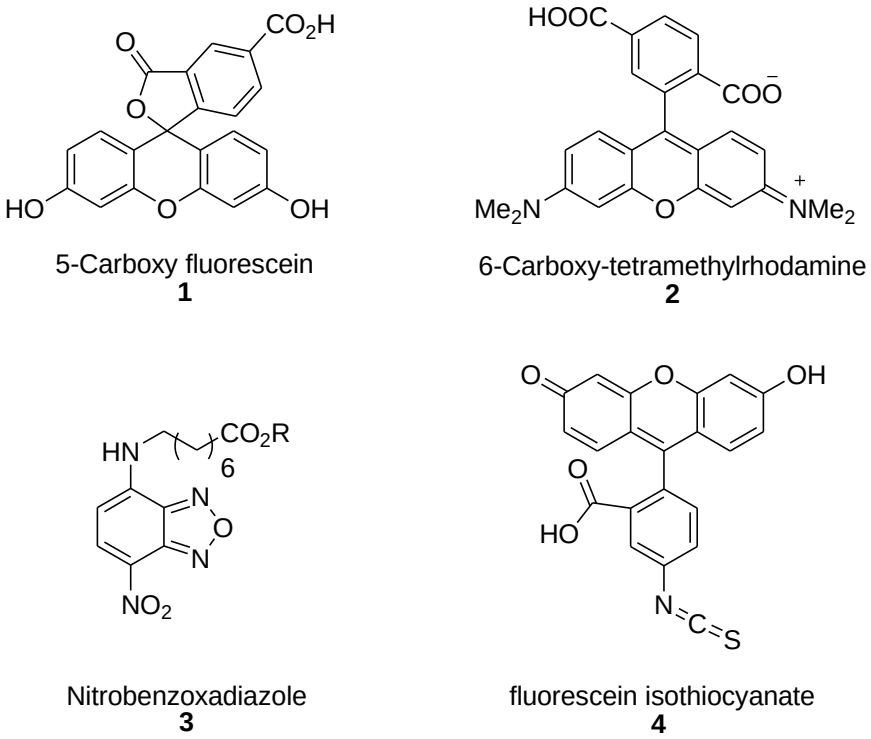


Figure 1. Commonly used fluorescent tags

Fluorescent nucleoside analogues have become important tools in studying RNA as their structure is similar to the native nucleosides (Figure 2). Most interesting feature of these analogues is that they can be very specifically placed at the point of examination with minimal structural disruption. These are extremely sensitive to the changes in microenvironment which enables the study of solvent effects on structure, dynamics and folding in RNA/DNA.[12] Another important class of non radioactive labels are affinity tags. Biotin has been exploited for its high affinity for streptavidine (Figure 3). Biomolecules are labelled with biotin tags quite frequently in order to immobilize them on strepavidin support which has also been used in affinity chromatography. Biotin labelled DNA and RNA sequences have been used as affinity probes for detection and isolation of oligonucleotides.

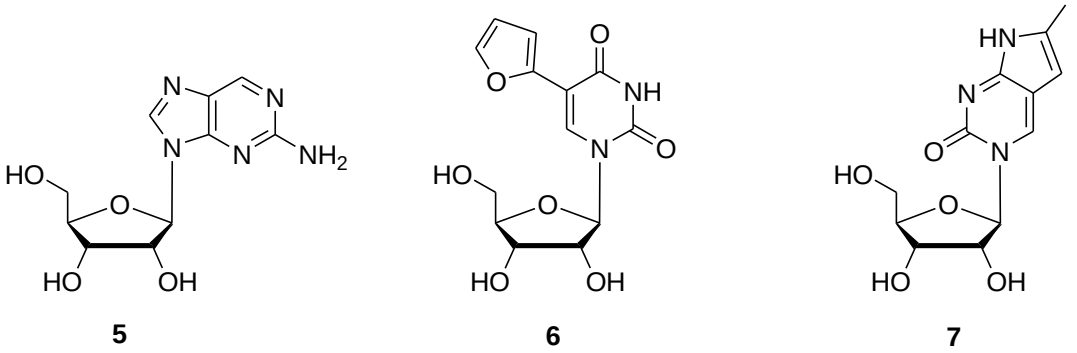


Figure 2: Examples of fluorescent ribonucleoside analogues.[13]

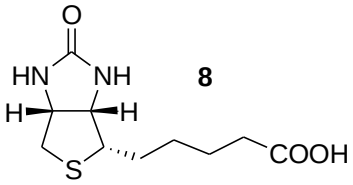


Figure 3: Biotin (affinity tag).

Caging of biomolecules is another appealing route which allows spatiotemporal control of biological processes (Figure 4). A biomolecule is said to be caged when it is attached to a photo labile protecting group which makes it inactive. The activity of the molecule is restored upon irradiation with a light of appropriate frequency.[14] Some nucleosides have been found useful in photo cross linking to form nucleoprotein adduct which have been further utilized for crystallographic studies Figure (4). Similarly, replacement of oxygen with selenium on nucleic acids has been proved to be give excellent in X-ray crystallography.[15] Selenium substituted nucleic acids offer selective derivatization at a variety of positions on the nucleic acid which is practically not possible with halogen substitution Figure (5).

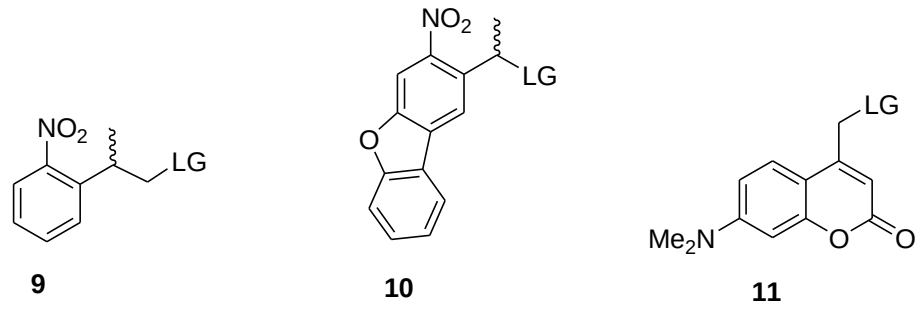


Figure 4: Examples of photo-labile protecting groups; LG= leaving group.

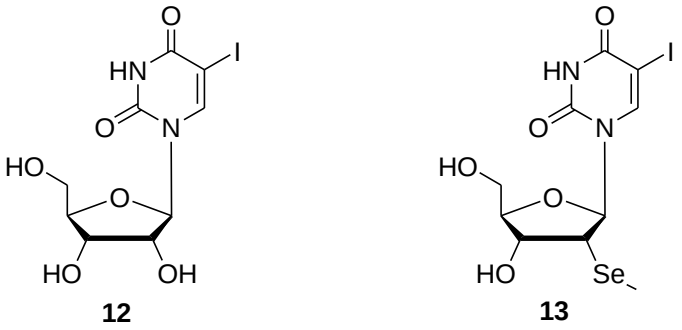


Figure 5: Nucleosides used for X-ray crystallographic studies.

NMR spectroscopy provides a highly efficient method to study the structure and dynamics of short RNA motifs. It is has used to determine hydrogen bonding patterns, long range coupling and also orientation of the helical domain.[15] Nucleosides have been site specifically labelled with all possible isotopes like ^{2}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P (Figure 6). Electron paramagnetic is resonance is another important spectroscopic tool that enabled detailed study RNA molecules. With the help of this spin labelling of nucleosides it has been possible to understand the geometry of metal binding pockets of RNA (Figure 7).[16]

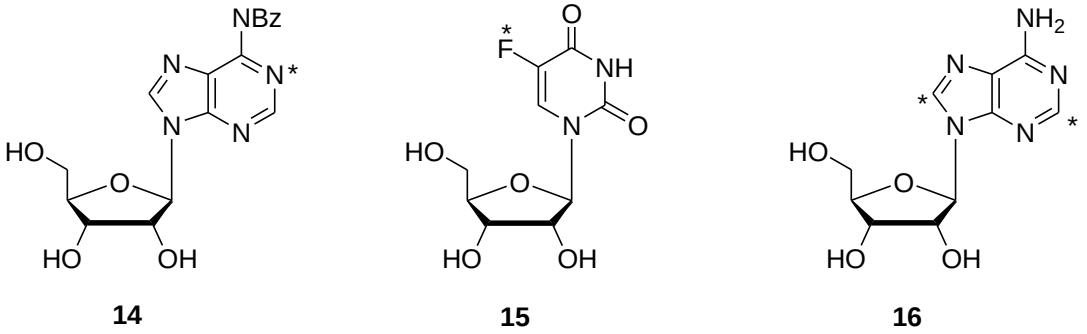


Figure 6: Nucleosides labelled with ^{15}N, ^{19}F, ^{13}C.

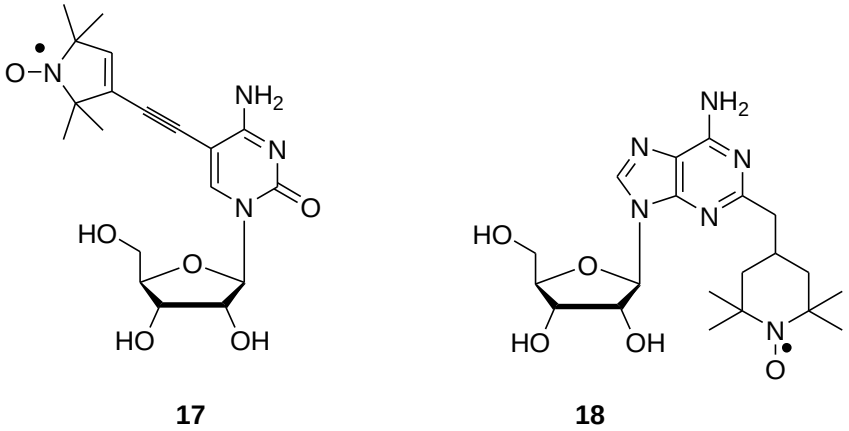


Figure 7: Spin labelled nucleosides.

Although these labels have been extensively used in understanding the structure, dynamics and recognition features of nucleic acids, they have some limitations owing to their large size or toxicity. Due to their large size, these labels are mostly attached to the 5´ or 3´-end of the oligonucleotides, and hence, are far removed from the actual interaction site. In order to label nucleic acids in a nonperturbing fashion, it is important that the label has similar size and shape as that of the native nucleosides, and also possess special function that can be utilized in studying the functions of RNA molecules. In this regard, nucleoside analogue have provided excellent systems for the development of bioanalytical assays to advance the understanding of nucleic acid structure and recognition properties.

Methods to label RNA oligonucleotides: Automated solid phase synthesis offers a rapid and economic method to generate site specifically labelled oligonucleotides of any desired sequence.[17] The easiest and the most widely used method to construct an oligonucleotide is to use phosphoramitide substrates (*Bis*-(2-cynoethyl)-N,N-diisopropylaminophosphine) as building blocks (Figure 9). In the presence of mild acids like tetrazole or benzothio tetrazole the phosphoramidite substrates are activated and become prone to nucleophilic attack. Solid phase synthesis of RNA is similar to the synthesis of DNA. However, additional effort has to taken for the protection of 2´-OH of ribonucleoside with a suitable protecting group. The 5´-OH is protected using dimethoxytrityl (DMT) group which can be deprotected in the presence an acid. The 2´-OH is protected using *tert*-butyldimethylsilyl (TBDMS) or triisopropylsilyloxymethyl (TOM) which can be deprotected in the presence of fluorides. Thereafter, the free amines on the nucleobase are protected with the help of acyl or benzoyl group, which are stable under acidic and mild basic conditions employed during solid phase synthesis. The first step involves DMT deprotection of the 5´-OH of the first nucleoside which is chemically hooked to the solid support. This free –OH attacks the phosphoramidite substrates introduced in the into the solid phase synthesis cycle in presence of a mild acid, benzothio tetrazole. The attack the hydroxyl is followed by an oxidation step and then 5´ end capping of the unreacted compound. The phosphoramidite substrates are sequentially introducing into the cycle following the same protocol. The last step is global deprotection of the bases which also cleaves the oligonucleotide from the solid support. This method however, is only suitable for such substrates which can sustain the harsh conditions employed in the solid phase synthesis cycle. Figure (8).[18]

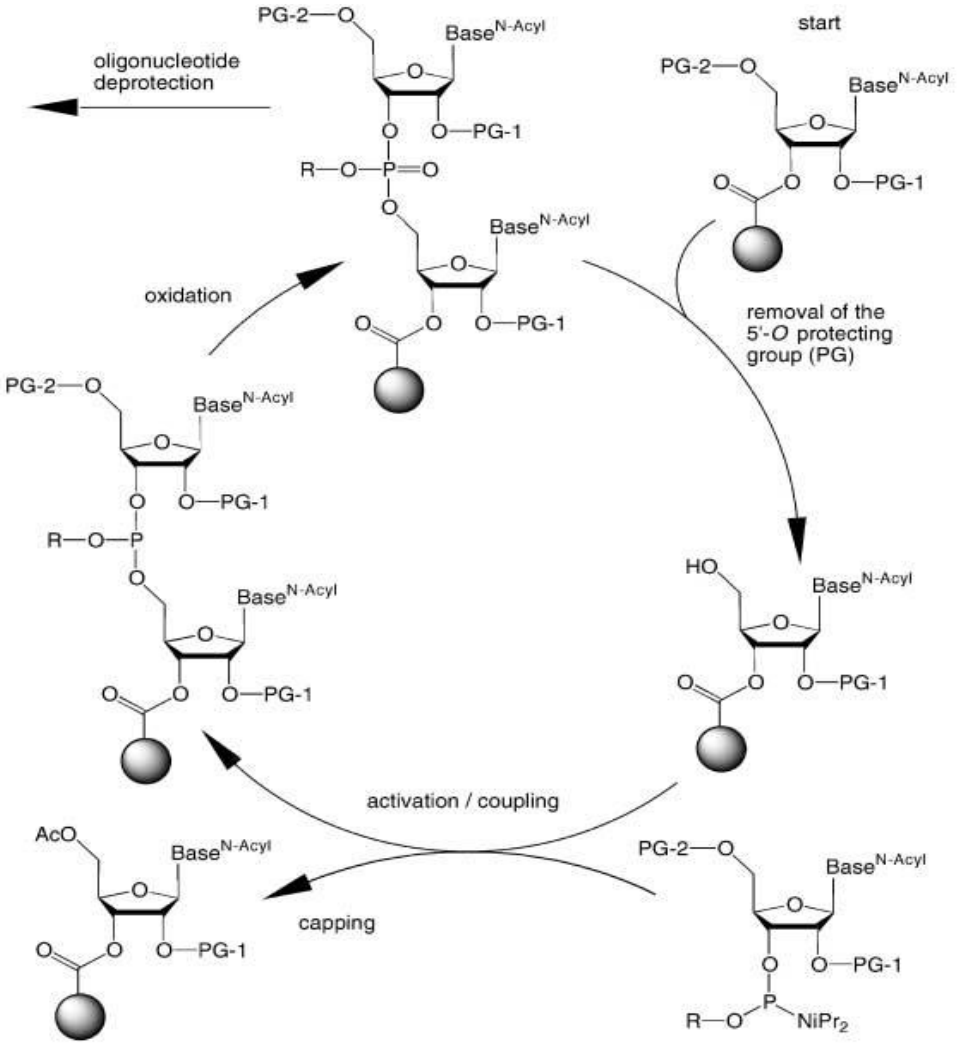
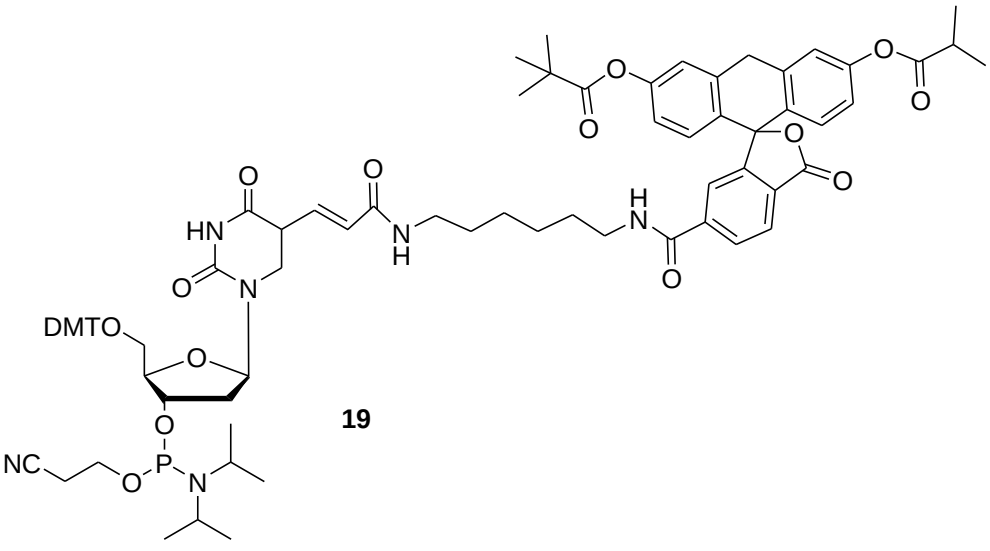


Figure 8: Schematic diagram of solidphase oligoribonucleotide synthesis using phosphoramidite approach.[18]



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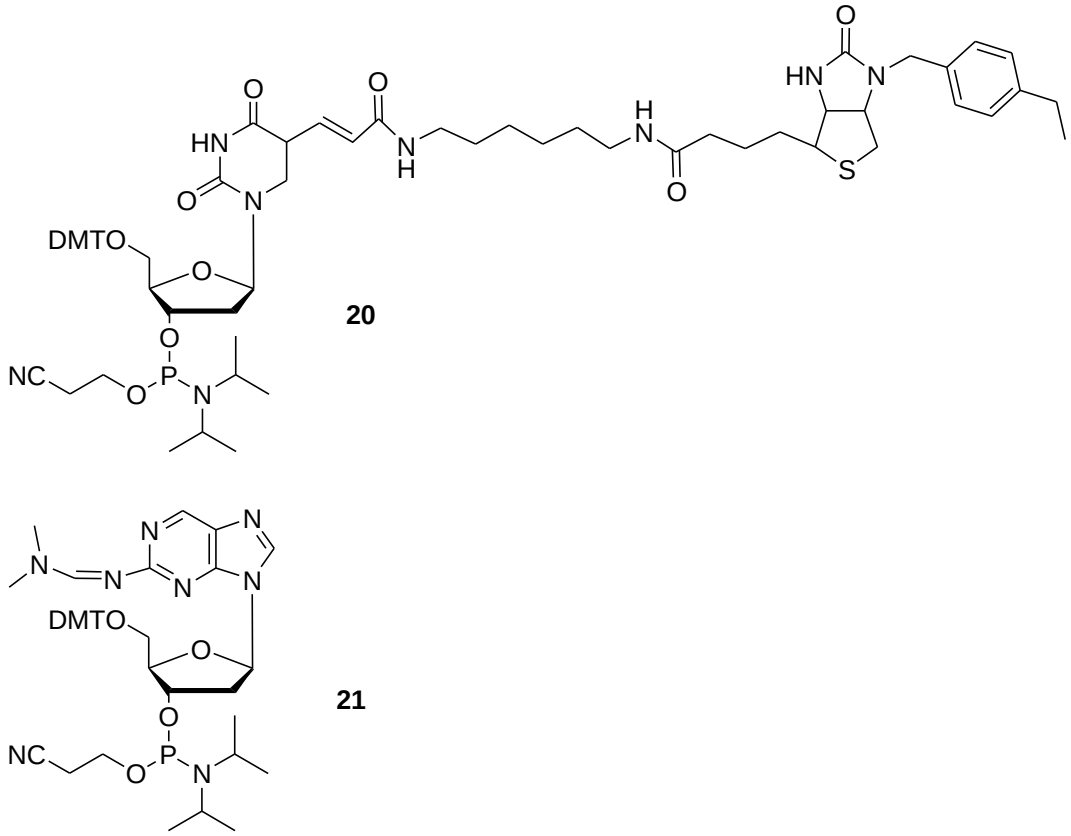


Figure 9: Fluorescein dT phosphoramidite **(19)**, biotin dT phosphoramidite **(20)**, 2-aminopurine phosphoramidite **(21)**

Alternative method for the synthesis of oligoribonucleotide employs extremely mild conditions by enzymatic incorporation of ribonucleoside triphosphates with the help of RNA polymerases. This method requires a double stranded oligonucleotide made of a synthetic template and a promoter DNA strand, RNA polymerase and ribonucleoside triphosphates (Figure 10). The RNA polymerase consensus promoter sequence is annealed to the DNA template, which is recognized by the RNA polymerase. The template then directs the incorporation of ribonucleoside triphosphate substrates into oligoribonucleotide in the 5' to 3' direction. Enzymatic incorporation offers synthesis of large quantities of oligoribonucleotide by performing transcription reaction with a very small amount ribonucleoside triphosphate substrate as well as numerous modified RNA transcripts can be synthesized by modifying the DNA template sequence. However, this method cannot be used for site-specific labeling of the oligoribonucletide as there is no control over the site specific enzymatic incorporation. Apart from this, only certain modifications are accepted by the enzyme and efficiently incorporated into the oligoribonucletide transcript.[19]

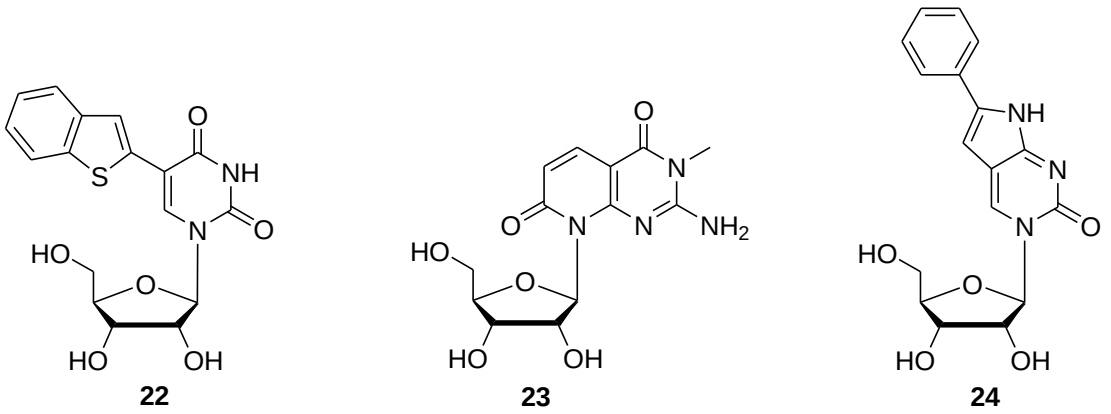


Figure 10: Modified ribonucleosides for enzymatic incorporation in RNA.

Modification of RNA with an azide group: Performing a chemical reaction in a biological sample is an extremely difficult task. Presence of multiple functionalities in complex biomolecules makes it impossible to achieve specific chemical reactions. One of the biggest challenges faced by chemical biologists today is to develop chemical methods to bioorthogonally functionalize biomolecules under biological conditions. The azide moiety is a unique functional group, which is known to undergo chemoselective bioorthogonal reactions, namely staudinger reduction, staudinger ligation and click reaction (Figure 11). These reactions facilitate the synthesis of RNA labelled with affinity tags or fluorophores, which can in turn be utilized for studying RNA-biomolecule interactions. Click reaction makes use of a copper catalysed 1,3-dipolar cycloaddition reaction between an azide and a terminal alkyne (Figure 12).[20-21] On the other hand, staudinger reduction and staudinger ligation are chemoselective reactions which take place between an azide and a phosphosphine without use any additional reagents (Figure 13,14).[22-23]

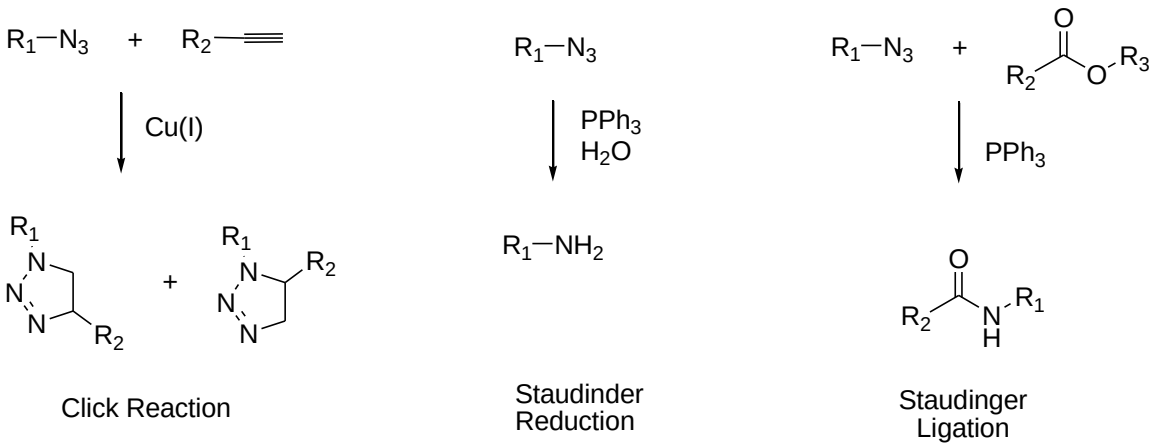


Figure 11: Bioorthogonal reactions involving azides.

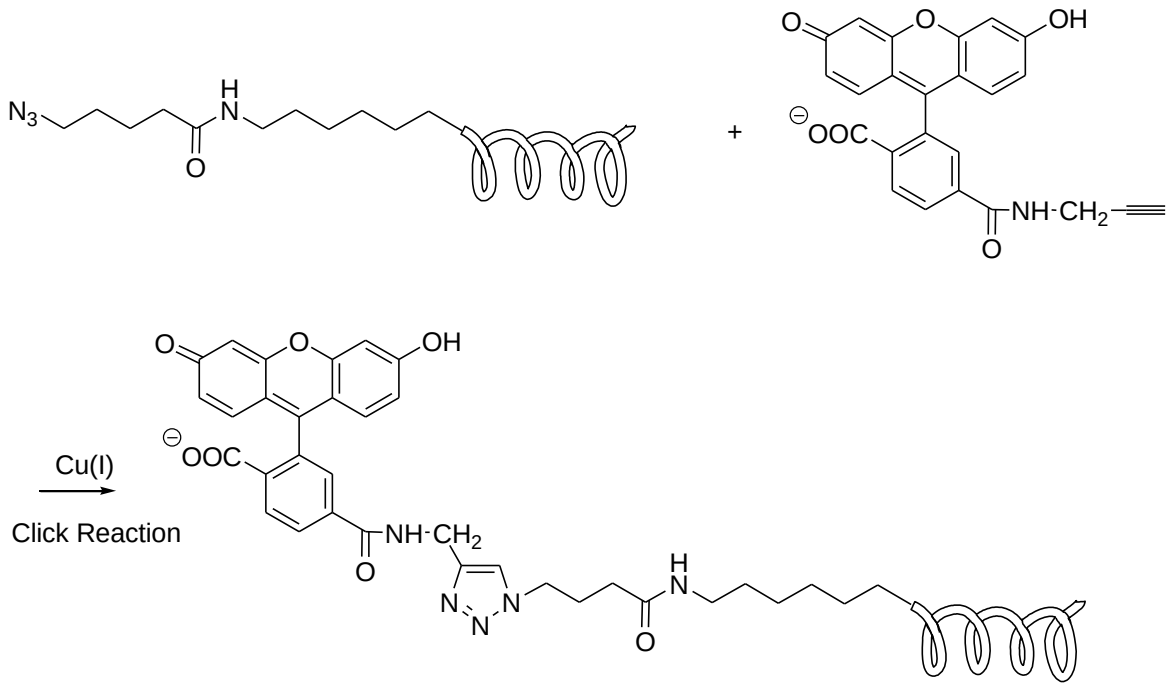


Figure 12: Click reaction between an azide labelled RNA and alkyne-modified fluorescein.[24]

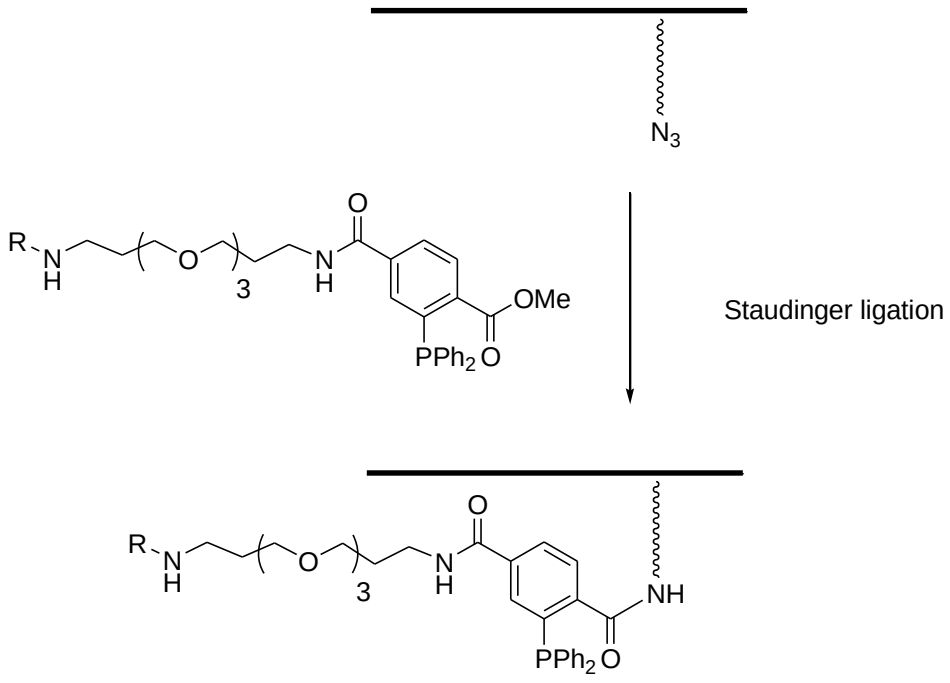
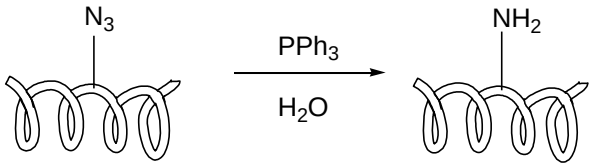


Figure 13: Staudinger ligation reaction between an azide labelled DNA and triphenylphosphine ester.[25]



Staudinger Reduction

Figure 14: Staudinger reduction of an azide to amine.

Although azide is a versatile functionality for bioorthogonal reactions, it is incompatible with solid phase synthesis protocol. The azide group can potentially react with trivalent phosphorus of the phosphoramitide substrate during the elongation step similar to that of Staudinger reaction.[26] Therefore, in order to incorporate azide functionality into oligonucleotides it is essential to develop incorporation methods, which will be mild as well as compatible with the azide modification. Recently, Marx and co-workers have reported enzymatic incorporation of azide substituted deoxynucleotide into DNA oligonucleotides by polymerase chain reaction.[25] Drawing inspiration from this report, we sought to develop a new method for enzymatic incorporation of azide functionalized ribonucleotide into oligoribonucleotide by transcription reaction. Such an effort would equip oligoribonucleotides with the azide functionality, which could be further modified by posttranscription chemical reactions by employing Staudinger ligation, Staudinger reduction and click chemistry (Figure 11).

Here, we report the synthesis of azide-modified ribonucleoside analogue, 5-(3-azidopropyl)-uridine **35** and its corresponding ribonucleoside triphosphate **36**. The trriphosphate has been effectively incorporated into oligoribonucleotide transcripts by T7 RNA polymerase in transcription reactions. Interestingly, the RNA polymerase prefers both natural and modified UTPs in a transcription reaction containing both the substrates in equimolar concentration. This feature can be exploited in labelling RNA in vivo by nucleoside salvage pathway, and can be further functionalized with report-like moieties by posttranscriptional chemical modification. We also report the efforts undertaken to effect posttranscriptional reduction of the azide group by Staudinger reduction.

Aim of the project:

The aim of the project is to synthesize azide-modified ribonucleoside analogue, and enzymatically incorporate the corresponding triphosphate into RNA oligonucleotides for posttranscriptional chemical modification.

Objectives:

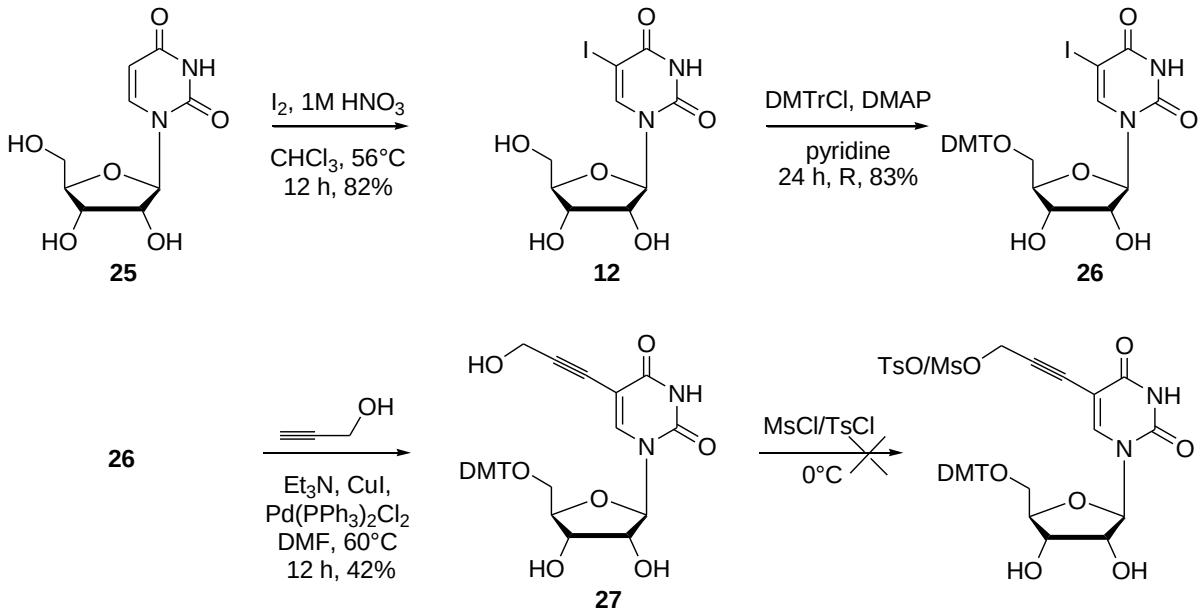
a) Synthesis of azide-modified uridine analogue
b) Synthesis of corresponding ribonucleoside triphosphate.
c) Enzymatic incorporation of ribonucleoside triphosphate using T7 RNA polymerase.
d) Characterization of the azide-modified RNA transcript.
e) Posttranscriptional chemical modification of azide-modified RNA transcripts using bio-orthogonal chemical reactions.
    - Staudinger ligation
    - Staudinger reduction
    - Click Reaction

Results and discussion

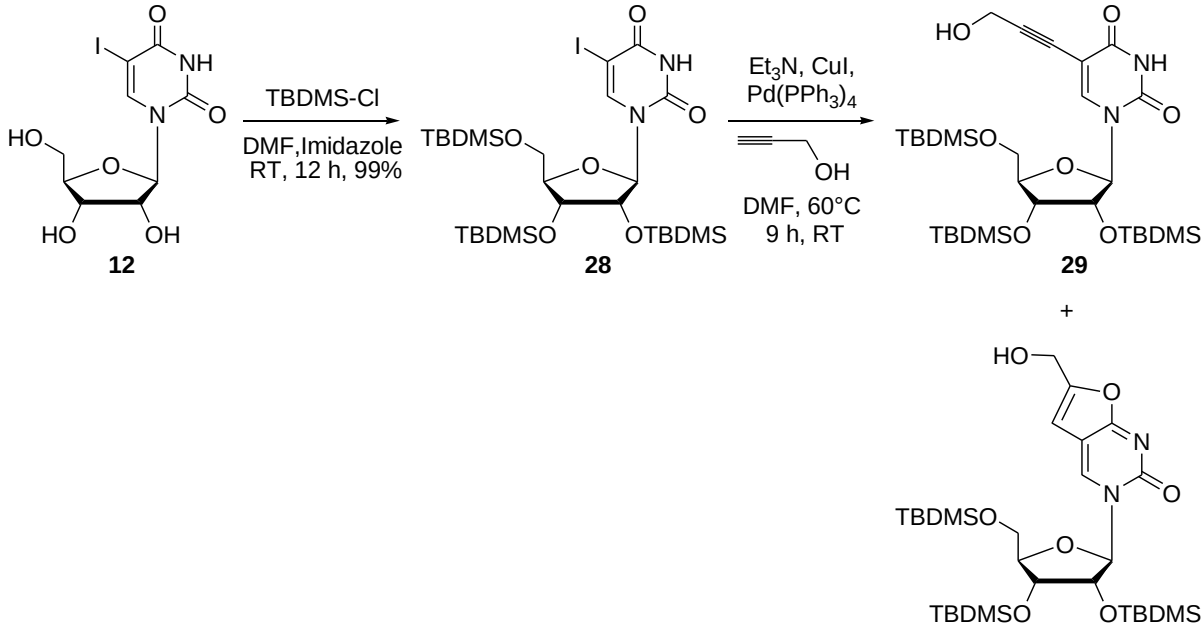
Synthesis: Palladium catalysed sonogashira reaction was employed for the synthesis of 5-(3-azidopropyl)-uridine **35**. Initial approach involved dimethoxytrityl (DMT) protection of 5'-OH of 5-iodouridine **12** to afford 5′-*O*-dimethoxytrityl-5-iodouridine **26** (Scheme 1). Sonogashira coupling of the product with propargyl alcohol in the presence $Pd(PPh_3)_4$ catalyst and CuI gave moderate yield of 5-(3-hydroxypropynl)-modified uridine **27**. However, the product upon reaction with tosyl chloride (TsCl) or mesyl chloride (MsCl) yielded multiple products, due the presence of 2′ and 3′ free –OH on the ribose sugar. Moreover, the stability of propargyl carbocation promotes the nucleophilic attack of the free chloride ion present in the reaction mixture leading to the formation of corresponding halo substituted product as the major product.

In order to overcome the difficulties faced in the previous method an alternate strategy was implemented, wherein 5-Iodouridine was protected with *tert*-butyldimethylsilyl chloride (TBDMS) to yield 2′,3′,5′-*O*-Tris*(tert*-butyldimethylsilyl*)*-5-iodouridine (**28**) (scheme 2). Subsequent Songashira coupling with propargyl alcohol in the presence of $Pd(PPh_3)_4$ and CuI yielded low yields of 2′,3′,5′-Tris*(tert*-butyldimethylsilyl*)*-5-(3-

hydroxypropynl)-uridine **29**. A fluorescent side product was also formed, which could be possibly the cyclised furano compound. Such cyclization products have been previously reported for coupling reactions with propargyl derivatives. Robins and co-workers had reported similar kind of reactions for a variety of substrates.[27] The separation of the two compounds was quite difficult as there was no significant difference between the polarities. Therefore, to avoid the cyclization reaction, the protection of N-H group of the base was necessary.

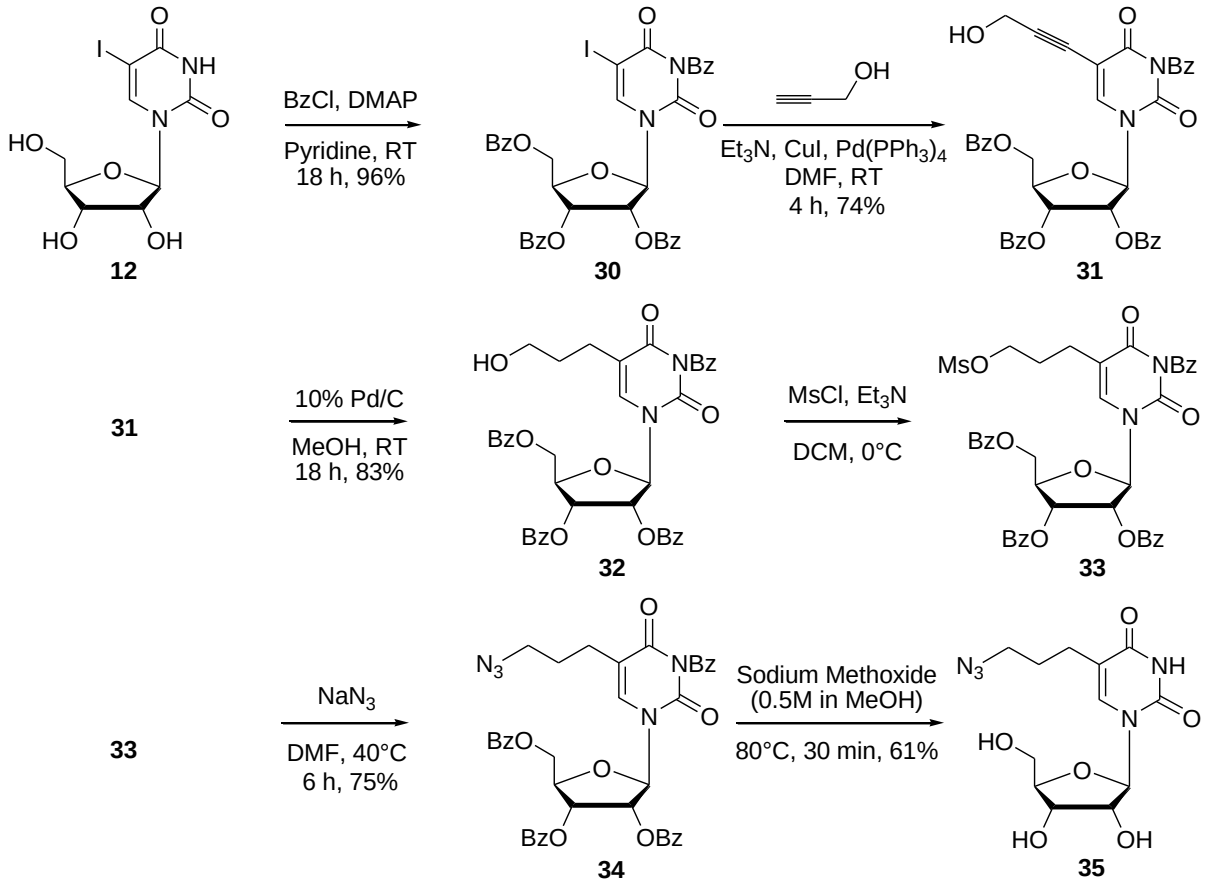


Scheme1. Synthesis of 5-propargyl substituted uridine using DMT protected nucleoside.

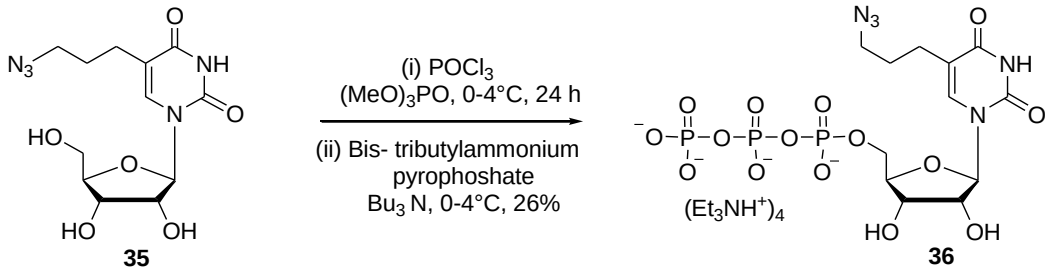


Scheme2. Synthesis of 5-propargyl substituted uridine using tri-TBDMS protected nucleoside.

The final strategy was designed such that it dealt with both the problems faced in the previous two methods (Scheme 3). In this method, 5-iodourine **12** was per-benzoylated by reaction with benzoyl chloride to yield the tetrabenzoyl-5-iodouridine product **30**. Sonogashira coupling of the per-benzoylated uridine with propargyl alcohol in the presence of $Pd(PPh_3)_4$ catalyst and CuI gave good yield of protected-5-(3-hydroxypropynl)uridine **31**. Catalytic hydrogenation of **31** followed by mesylation afforded the mesyl-protected nucleoside **32** in good yield. The reaction of **32** with sodium azide gave the azide substituted product. However, during the reaction, done for 6 h at 40^{o}C, the benzoyl group protecting the nitrogen on the base got deprotected. Further deprotection was accomplished by reaction with sodium methoxide leading to the formation of the azide modified ribonucleoside, 5-(3-azidopropyl)-uridine(**35**). The modified ribonucleoside triphosphate **36** was obtained by reacting of **35** with freshly distilled $POCl_3$, which was followed by reaction with bistributylammonium pyrophosphate (scheme 4).[28] The triphosphate was extensively purified by ion exchange column chromatography and reverse phase flash chromatography. MALDI-TOF, ^{1}H, ^{13}C and ^{31}P NMR were is used for characterization.



Scheme3. Synthesis of 5-azido substituted uridine using tetra-benzoyl protected nucleoside.



Scheme 4. Synthesis of ribonucleotide triphosphate for enzymatic incorporation into RNA.

Enzymatic incorporation of ribonucleoside triphosphate: .In vitro transcription reactions are performed in the presence of NTPs, promoter-template duplex and T7 RNA polymerase in Tris-HCl buffer (Figure 15). Several templates containing one or two dA residue have been used to direct the incorporation of azide-modified UTP **36** at a single or at multiple positions. These templates also contain a dT residue at the 5´-end to facilitate the incorporation of complementary base, adenosine at the 3´-end of each transcript. Therefore, reactions performed in the presence of α-^{32} P ATP will result in RNA transcripts containing the ^{32}P label at the 3´-end. These labelled transcripts can then be resolved on a denaturing polyacrylamide gel electrophoresis (PAGE), and imaged.

T7 promoter 5' TAA TAC GAC TCA CTA TAG 3'
Template **S1** 3' ATT ATG CTG AGT GAT ATC GCG GC**A** CGT 5'

↓ T7 RNA polymerase
GTP, CTP, ATP, UTP/**36**
α–^{32}P ATP

5' $_{ppp}$GCG CCG UGC A 3' **37**
5' $_{ppp}$GCG CCG **35**GC A 3' **38**

Template		Transcript
S2	3' ATT ATG CTG AGT GAT ATC G**A**G GCC CGT 5'	5' $_{ppp}$GC**35** CCG GGC A 3'
S3	3' ATT ATG CTG AGT GAT ATC GC**A** GCG CGT 5'	5' $_{ppp}$GCG **35**CG CGC A 3'
S4	3' ATT ATG CTG AGT GAT ATC GCG G**AA** CGT 5'	5' $_{ppp}$GCG CC**35** **35**GC A 3'
S5	3' ATT ATG CTG AGT GAT ATC GCG GC**A** C**A**T 5'	5' $_{ppp}$GCG CCG **35**G**35** A 3'

Figure 15. Schematic explaining the incorporation of ribonucleotide triphosphate by transcription reactions.

The transcription reaction performed in the presence of the template **S1** consisting of a dA residue at the +7 position away from the promoter led to the formation of the full length 10-mer transcript **38** (Figure 16, lane 2). The modified UTP **36** was incorporated with excellent efficiency of 98% with respect to a transcription reaction in the presence of natural UTP (Figure 16, compare lanes 1 and 2). A control transcription reaction performed in the absence of natural as well as modified UTP **36** did not produce any full length transcripts

(Figure 16, lane 3). However, when the reaction is performed in the presence of equimolar concentration of UTP and **36**, the enzyme incorporates both the natural and modified UTP (Figure 16, lane 4). Transcription performed in the presence of templates **S2** and **S3** in which the dA residues are present near the promoter sequence at +3 and +4 positions, the incorporation of **36** is reasonably good with 81% and 89% efficiency, respectively (Figure 16, lanes 6 and 8). In the presence of **S4** and **S5** wherein the RNA polymerase incorporates **36** at successive positions (+6 & +7) and alternating positions (+7 & +9), the incorporation efficiency is also appreciably high (79% and 81% respectively, Figure 16, lanes 10 and 12). In summary, T7 RNA polymerase incorporates the azide-modified UTP into RNA oligonucleotides with excellent efficiency.

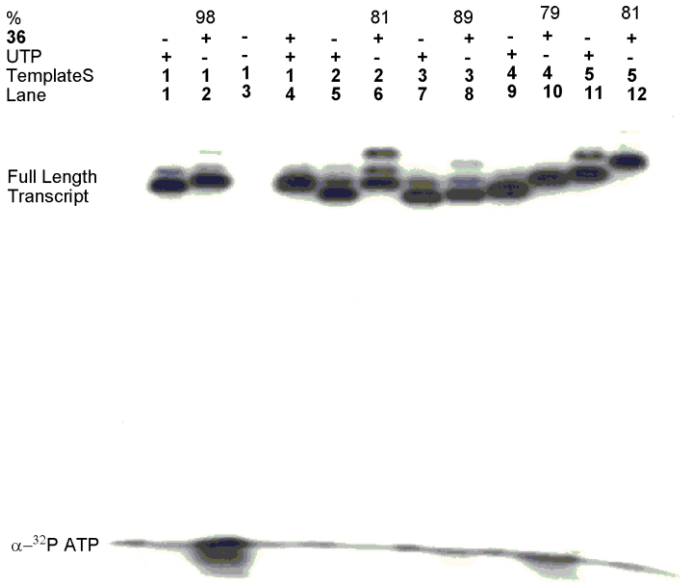


Figure 16. In vitro transcription reactions using natural and modified UTP **36** with template **S1-S5.** % incorporation of **36** is reported in comparison to the reaction performed in presence of NTPs.

Characterization of azide-modified transcript: Large scale transcription was performed with DNA template **S1**, followed by purification using denaturing PAGE. MALDI-TOF mass measurement of the purified transcript ensured the presence of full length modified transcript **38** containing the ribonucleoside **36** (Figure 17). Thereafter, enzymatic digestion of the transcript **38** was done in the presence of snake venom phosphodiesterase I, calf intestine alkaline phosphatase, RNase A and RNase T1. HPLC analysis of the ribonucleoside mix obtained from the digested RNA sample clearly showed the presence of modified ribonucleoside **36** in the transcript stochiometry (Figure 18). MADLI-TOF measurement of

individual fractions collected in HPLC further verified the presence of natural and modified ribonucleosides present in the transcript.

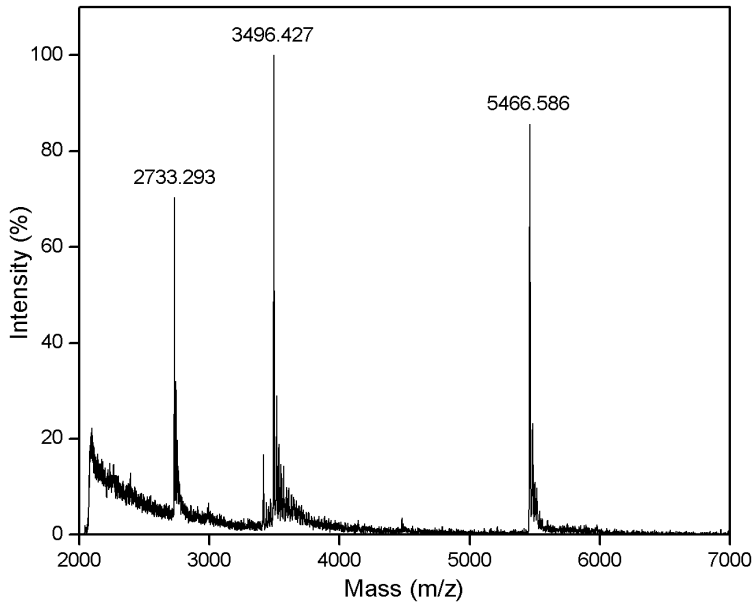


Figure 17. MALDI-TOF spectrum of azide functionalized RNA transcript **38** calibrated relative to a 18mer internal deoxynucleotide standard (m/z = 5466.586). Calculated mass [M]= 3497.424; observed mass= 3496.427.

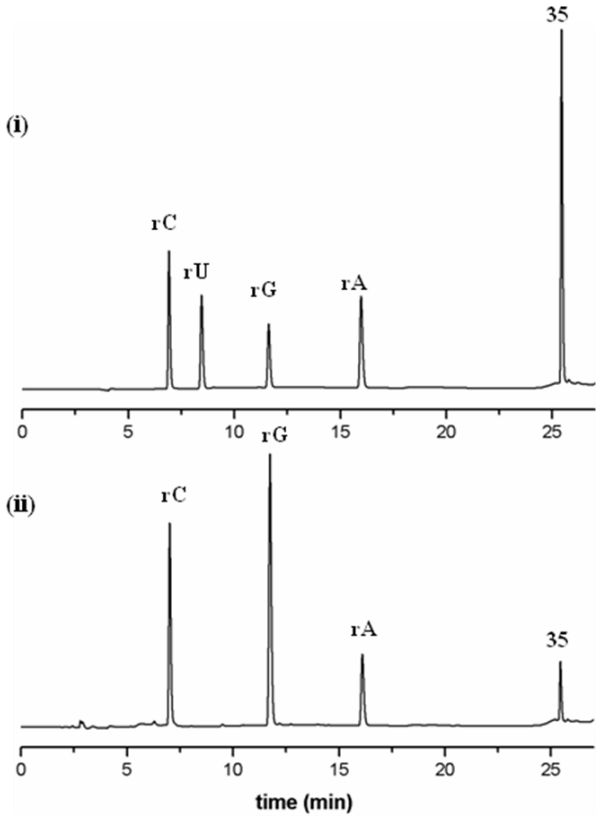


Figure 18. HPLC analysis profile for enzymatic digestion reaction at 260 nm. (i) Natural nucleosides samples and modified nucleoside **35**. (ii) Digestion of modified transcript **38**.

Thermal denaturation studies: UV-thermal denaturation studies were performed to ascertain the effect of azide-modification on the duplex stability. A control unmodified and modified duplexes were constructed by annealing control transcript **37** and modified transcript **38** to a perfect complementary oligoribonucleotide strand, respectively. Thermal melting analysis revealed marginal destabilization of the duplex due to the modification (Figure 19).

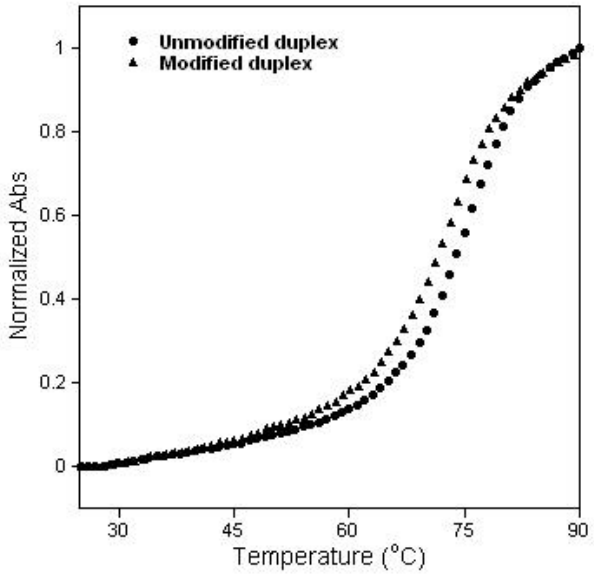


Figure 19. UV-thermal melting analysis of control unmodified and azide-modified duplexes (1 μM) in cacodylate buffer containing 500 mM NaCl. Tm for control duplex 74.7 ± 1 ^{o}C; Tm for modified duplex 74.0 ± 1 ^{o}C.

Staudinger reduction of azide modified RNA transcript:

As a proof of concept, we have reduced azide modified RNA transcript **38** using water soluble triphenylphosphine reducing agent (TPPTS, Figure 20). The reaction proceeded to partial conversion of azide to amine. The fractions isolated by HPLC purification of the reaction mixture were analysed by MALDI-TOF mass spectroscopy. One of the fraction corresponded to the unreacted azide, while the other corresponded to the desired amine-containing oligoribonucleotide (Figure 21).

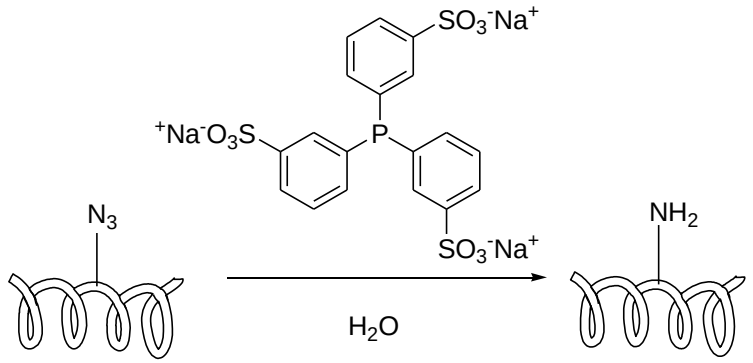


Figure 20. Reaction of azide functionalized RNA transcript **38** with water soluble TPPTS.

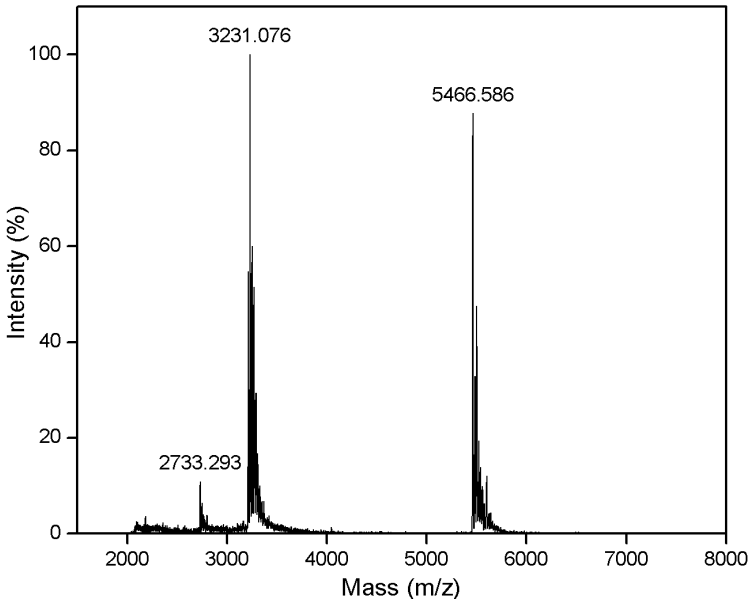


Figure 21. MALDI-TOF spectrum of azide functionalized RNA transcript 38 calibrated relative to a 18mer internal deoxynucleotide standard (m/z = 5466.586). Calculated mass [M]= 3232.048; observed mass= 3231.076.

Conclusions

We have been successful in synthesizing an azide-modified uridine triphosphate analogue that is stable under enzymatic incorporation conditions. The T7 RNA polymerase effectively incorporates the azide-modified triphosphate into RNA oligonucleotides with excellent efficiencies by transcription reactions. The incorporation of modified ribonucleoside into RNAs has been thoroughly characterized by radioactive labelling technique, enzymatic digestion and MALDI-TOF MS analysis of the transcript. As a proof of utility of this labelling method, we have been able to perform posttranscriptional Staundinger reduction to

produce the amino-modified RNA, which can be further labelled using N-hydroxysuccinamide ester method. Taken together, our results demonstrate that the transcription reaction is a viable method to functionalize RNA with azide group, a versatile functionality suitable for bioorthogonal chemical modification. Efforts are currently under investigation to label RNA with a variety of functionality by posttranscriptional chemical modification.

Experimental Section

Materials: The materials obtained from the suppliers were used without further purification. Tetrakis(triphenylphosphine)Palladium(0), 10% Pd/C, sodium azide were obtained from Sigma-Aldrich. Triphenylphosphine-3,3´,3´´-trisulfonic acid trisodium salt (TPPTS) was obtained from Alpha Aesar. DNA oligonucleotides were either purchased from Integrated DNA Technologies, Inc. Or from Sigma-Aldrich. Oligonucleotides were purified by gel electrophoresis under denaturing condition and desalted on Sep-Pak Classic C18 cartridges (Waters Corporation). Custom synthesized RNA oligonucleotides purchased from Dharmacon RNAi Technologies were deprotected according to the supplier's procedure, PAGE-purified and desalted on Sep-Pak Classic C18 cartridges. T7 RNA polymerase, ribonuclease inhibitor (Riboblock) and NTPs were obtained from Fermentas Life Science. Radiolabelled α-^{32} P ATP (2000 Ci/mmol) was obtained from the Board of Radiation and Isotope Technology, Government of India. Chemicals for preparing buffer solutions were purchased from Sigma-Aldrich (BioUltra grade). Autoclaved water was used in all biochemical reactions.

Instrumentation: NMR spectra was recorded on 400MHz Jeol ECS-400. All MALDI-MS measurements were recorded on Applied Biosystems 4800 Plus MALDI TOF/TOF analyser. Absorption spectra were recorded on a PerkinElmer, Lambda 45 UV-Vis spectrophotometer. UV-thermal melting analyses of oligonucleotides were performed on a Cary 300Bio UV-Vis spectrophotometer. Reversed-phase flash chromatographic (C18 Redi*SepRf* column) purifications were carried out using Teledyne ISCO, Combi Flash Rf.

Synthesis

5-Iodouridine (12): Uridine **25** (10 g, 40.98 mmol) and iodine (10.2 g, 40.82 mmol) were taken in a round bottom flask and were dissolved in a mixture of 1 M HNO_3 (50 mL) and $CHCl_3$ (28 mL). The reaction mixture was refluxed at 56^oC for 12 h and then cooled and kept in the refrigerator overnight which led to the formation of white solid residue. Thereafter the reaction mixture was brought to room temperature and allowed to stir for 15 minutes followed by filtration on a buckner funnel. The residue was dissolved in minimum amount of DMF and concentrated on the rotary vapour. DCM was added to the resulting syrup which again gave the solid residue. The residue was again filtered and the whole procedure was repeated until the colour of iodine was removed to yielded 5-Iodouridine 1**2** (12.3 g, 82%) white powdery solid. TLC ($CHCl_3$: MeOH = 70:30) *Rf* = 0.77; ^{1}H NMR (400 MHz, *d6*-DMSO): δ (ppm) 11.64 – 11.61 (m, 1H), 8.44 – 8.40 (m, 1H), 5.68 - 5.64 (m, 1H), 5.37 (s, 1H), 5.21 (s, 1H), 5.02 (s, 1H), 3.98 – 3.92 (m, 2H), 3.81 (s, 1H), 3.63 (d, *J* = 10.8 Hz, 1H), 3.52 (d, *J* = 10.0 Hz, 1H); ^{13}C NMR (100 MHz, *d6*-DMSO): δ (ppm) 160.54, 150.40, 145.17, 88.30, 84.72, 73.97, 69.39, 60.20; MALDI-TOF MS (m/z): Calculated for $C_9H_{11}IN_2O_6$ [M] = 370.098, found $[M+Na]^+$ = 393.016, $[M+K]^+$ = 408.991.

5´-*O*-Dimethoxytrityl-5-Iodouridine (26): 5-Iodouridine **12** (2 g, 5.4 mmol, 1 equiv) was added dissolved in 30 mL of anhydrous pyridine. Dimethoxytrityl chloride (2.38 g, 7.03 mmol) was added to the solution and stirred for 24 h at room temperature. Thereafter pyridine was removed in rotary vapour and then co-evaporated with toluene for azeotropic removal of traces of pyridine. The resulting syrup was dissolved in EtOAc and then washed with 5% $NaHCO_3$ followed by saturated solution of sodium chloride. The organic layer was dried over sodium sulphate and concentrated in rotary vapour. Purification was done by column chromatography on silica gel using 100:1→ 100:5 CH_2Cl_2/MeOH yielded 5´-*O*-dimethoxytrityl-5-Iodouridine **26 (**3.05 g, 84%)**,** a white foamy solid. TLC (CH_2Cl_2:MeOH = 95:5 + few drops of Et_3N) *Rf* = 0.28; ^{1}H NMR (400 MHz, CHLOROFORM-D): δ (ppm) 8.11 (s, 1H), 7.42 – 7.38 (m, 2H), 7.34 – 7.28 (m, 6H), 7.22 – 7.16 (m, 1H), 6.83 (d, *J* = 8.2 Hz, 4H), 5.92 (d, *J* = 5.2 Hz, 1H), 4.43 (t, *J* = 5.3 Hz, 1H), 4.39 (dd, *J* = 5.2, 3.3 Hz, 1H), 4.23 (q, *J* = 2.6 Hz, 1H), 3.77 (s, 6H), 3.36 (qd, *J* = 11.0, 2.8 Hz, 2H); MALDI-TOF MS (m/z): Calculated for $C_{30}H_{29}IN_2O_8$ [M] = 672.464, $[M+Na]^+$ = 695.183, $[M+K]^+$ = 711.1526.

5´-*O*-Dimethoxytrityl -5-(3-hydroxypropynl)-uridine (27): 26 (0.6 g, 0.9 mmol, 1 equiv), CuI (17 mg, 0.09 mmol, 0.1 equiv), $Pd(OAc)_2$ (4 mg, 0.018 mmol, 0.02 equiv) and PPh_3 (24

mg, 0.09 mmol, 0.1 eq) dissolved in 1 mL of degassed Et_3N and 2 mL degassed DMF in a round bottom flask. The round bottom flask is covered with aluminium foil to protect the reaction mixture from light. To the mixture propargyl alcohol (0.15 mL, 2.68 mmol, 3 equiv) was added and the reaction mixture was stirred at room temperature for 12 h. DMF and Et_3N were removed on the rotary vapour and then saturated $NaHCO_3$ was added to the resulting syrup which was extracted with EtOAc (3 x 100 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4 and concentrated in rotary vapour. Purification was done by column chromatography on silica gel using 100:1→ 100:4 CH2Cl2/MeOH along with a few drops of Et_3N yielded 5´-*O*-dimethoxytrityl-5-(3-hydroxypropynl)-uridine **27** (0.23 g, 42%)**,** a white foamy solid. TLC ($CHCl_3$:MeOH = 95:5 + few drops of Et_3N) *Rf* = 0.38; 8.79 (s, 1H), 7.37 – 7.34 (m, 2H), 7.28 (d, *J* = 1.6 Hz, 1H), 7.26 – 7.22 (m, 5H), 6.83 – 6.76 (m, 5H), 5.97 (d, *J* = 2.1 Hz, 1H), 4.59 – 4.55 (m, 1H), 4.51 (s, 2H), 4.45 – 4.42 (m, 1H), 4.36 – 4.32 (m, 1H), 3.77 – 3.76 (m, 6H), 3.52 – 3.47 (m, 2H); MALDI-TOF MS (m/z): Calculated for $C_{30}H_{29}IN_2O_8$ [M] = 600.615, $[M+Na]^+$ = 623.074, $[M+K]^+$ = 639.046.

2´,3´,5´-*O*-Tris*(tert*-butyldimethylsilyl*)*- 5-iodouridine (28): 5-Iodouridine **12**(1 g, 2.7 mmol, 1 equiv) and imidazole (1.84 g, 27 mmol, 10 equiv) were dissolved in 12 mL of dry DMF and the solution was cooled to 0 ^oC in an ice bath. To the solution *tert*-Butyldimethylsilyl chloride (3.26 g, 21.6 mmol, 8 equiv) was added. The reaction mixture was allowed to come to room temperature and was stirred for 12 h. DMF was removed in the rotary vapour and to the resulting syrup CH_2Cl_2 and water were added. The water layer was extracted with DCM (3 x 50 mL), the combined organic extracts were washed with saturated aqueous solution of NH_4Cl, saturated aqueous solution of NaCl, dried over Na_2SO_4 and concentrated on the rotary vapour. Purification was done by column chromatography on silica gel using 100:1→ 100:5 Hexane/EtOAc yielded 2´,3´,5´-*O*-Tris*(tert*-butyldimethylsilyl*)*- 5-iodouridine **28** (1.9 g, 98.5%)**,** a white foamy solid. TLC ($CHCl_3$:MeOH = 95:5 + few drops of Et_3N) *Rf* = 0.88; 8.32 (s, 1H), 8.04 (s, 1H), 6.00 (s, 1H), 4.11 – 4.07 (m, 1H), 4.03 (d, *J* = 3.3 Hz, 2H), 3.89 (d, *J* = 11.5 Hz, 1H), 3.72 (d, *J* = 11.8 Hz, 1H), 0.97 (s, 9H), 0.90 (s, 9H), 0.84 (s, 9H), 0.18 (s, 6H), 0.07 (d, *J* = 8.7 Hz, 6H), 0.01 (s, 3H), -0.07 (s, 3H); MALDI-TOF MS (m/z): Calculated for $C_{27}H_{53}IN_2O_6Si_3$ [M] = 712.226, found $[M+Na]^+$ = 735.333, $[M+K]^+$ = 751.292.

2´,3´,5´-*O*-Tris*(tert*-butyldimethylsilyl*)*-5-(3-hydroxypropynl)-uridine (29): 28 (1 g, 6.5, mmol, 1 equiv), CuI (11 mg, 0.056 mmol, 0.04 equiv), $Pd(PPh_3)_4$ (32.5 mg, 0.028 mmol, 0.02 equiv), Et_3N (0.4 mL, 2.81 mmol, 2 equiv) were dissolved in 5 mL of degassed DMF in

a round bottom flask. The round bottom flask is covered with aluminium foil to protect the reaction mixture from light. To the mixture propargyl alcohol (0.25 mL, 4.21 mmol, 3 equiv) was added. The reaction mixture was stirred at room temperature for 9 h. DMF and Et_3N were removed in the rotary vapour and saturated $NaHCO_3$ was added to the reaction mixture which was then extracted with EtOAc (3 x 100 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4 and concentrated in the rotary vapour to small volume. This solution was applied to a silica gel column and column was eluted using 100:5→ 100:25 Hexane/EtOAc . Evaporation of appropriate earlier fractions yielded 2´,3´,5´-*O*-Tris*(tert*-butyldimethylsilyl*)*-5-(3-hydroxypropynl)-uridine **29** (0.35 g, 39%) TLC (hexane: EtOAc = 50:50) *Rf* = 0.75; 8.72 (s, 1H), 8.03 (d, *J* = 8.2 Hz, 1H), 5.86 (d, *J* = 3.4 Hz, 1H), 5.68 (d, *J* = 8.1 Hz, 1H), 4.10 – 4.08 (m, 1H), 4.06 (d, *J* = 3.4 Hz, 2H), 3.99 (dd, *J* = 11.6, 1.5 Hz, 1H), 3.75 (d, *J* = 11.4 Hz, 1H), 0.94 (s, 9H), 0.90 (s, 9H), 0.89 (s, 9H), 0.12 (d, *J* = 4.5 Hz, 6H), 0.08 (s, 6H), 0.07 (d, *J* = 3.7 Hz, 6H).,); MALDI-TOF MS (m/z): Calculated for $C_{30}H_{56}N_2O_7Si_3$ [M] = 640.340, found $[M+Na]^+$ = 663.453, $[M+K]^+$ = 679.421.

Latter fractions were combined together and concentrated to yield cyclised furano-compound (0.27 g, 30.1%). TLC (hexane: EtOAc = 50:50) *Rf* = 0.61; 7.96 (s, 1H), 5.96 (d, *J* = 5.6 Hz, 1H), 4.11 (dd, *J* = 6.2, 3.5 Hz, 1H), 4.04 (d, *J* = 2.7 Hz, 2H), 3.93 (dd, *J* = 11.6, 1.3 Hz, 1H), 3.78 (t, *J* = 6.0 Hz, 2H), 3.75 – 3.72 (m, 1H), 0.98 – 0.95 (m, 9H), 0.89 (d, *J* = 2.8 Hz, 9H), 0.85 (d, *J* = 2.6 Hz, 9H), 0.17 (t, *J* = 3.4 Hz, 6H), 0.09 – 0.06 (m, 6H), 0.02 (s, 3H), -0.02 (d, *J* = 2.9 Hz, 3H) ; MALDI-TOF MS (m/z): Calculated for $C_{30}H_{56}N_2O_7Si_3$ [M] = 640.340, found $[M+Na]^+$ = 663.453, $[M+K]^+$ = 679.421.

***N*^{3},2´,3´,5´-*O*-Tetrabenzoyl-5-iodouridine (30):** Benzoyl Chloride (15.33 mL, 132.13 mmol, 15 equiv) and DMAP (123 mg) were added to a solution of 5-iodouridine **12** in pyridine (75mL). The reaction mixture was allowed to stir at room temperature for 18 h. Thereafter pyridine was removed in the rotary vapour, toluene was added to the residual mixture and co-evaporated two times to remove traces of pyridine. The resulting syrup was extracted with EtOAc (3 x 100 mL), the combined organic extracts were washed thoroughly with saturated aqueous solution of $NaHCO_3$ followed by saturated aqueous solution of NaCl, dried over sodium sulphate and concentrated on the rotary vapour. Purification was done by column chromatography on silica gel using 100:10→ 100:35 Hexane/EtOAc yielded *N*^{3},2´,3´,5´-*O*-Tetrabenzoyl-5-iodouridine **30** (6.7 g, 96%) pale yellow solid. TLC (hexane:EtOAc = 70:30) *Rf* = 0.35; ^{1}H NMR (400 MHz, CHLOROFORM-D): δ (ppm) 8.16 – 8.13 (m, 2H), 8.00 (d, *J* = 9.6 Hz, 3H), 7.89 (t, *J* = 8.2 Hz, 4H), 7.65 – 7.57 (m, 3H), 7.52 (t,

J = 7.6 Hz, 3H), 7.41 (dd, *J* = 10.8, 4.6 Hz, 4H), 7.32 (t, *J* = 7.8 Hz, 2H), 6.38 (d, *J* = 6.4 Hz, 1H), 5.91 (dd, *J* = 6, 3.2 Hz, 1H), 5.76 (t, *J* = 6.2 Hz, 1H), 4.83 (dd, *J* = 13.6, 3.8 Hz, 1H), 4.77 – 4.72 (m, 2H); ^{13}C NMR (100 MHz, CHLOROFORM-D): δ (ppm) 167.335, 166.221, 165.654, 165.442, 158.616, 149.183, 143.710, 135.374, 134.042, 133.940, 130.877, 130.715, 130.031, 129.954, 129.897, 129.303, 129.158, 129.083, 128.769, 128.676, 128.634, 128.223, 81.282, 74.150, 71.624, 69.641, 63.948; MALDI-TOF MS (m/z): Calculated for $C_{37}H_{27}IN_2O_{10}$ [M] = 786.07, found $[M+Na]^+$ = 809.133, $[M+K]^+$ = 825.052.

***N*^{3},2´,3´,5´-*O*-Tetrabenzoyl-5-(3-hydroxypropynl)-uridine (31):** Compound **30** (5.11 g, 6.5mmol, 1equiv), CuI (0.124 g, 0.65 mmol, 0.1 equiv), $Pd(PPh_3)_4$ (0.375 g, 0.325 mmol, 0.05 equiv), Et_3N (1.9 mL, 13 mmol, 2 equiv) were dissolved in 15 mL of degassed DMF in a round bottom flask. The round bottom flask is covered with aluminium foil to protect the reaction mixture from light. To the mixture propargyl alcohol (0.76 mL, 13 mmol, 2 equiv) was added. The reaction mixture was stirred at room temperature for 4 h. DMF and Et_3N were removed in the rotary vapour and saturated $NaHCO_3$ was added to the reaction mixture which was then extracted with EtOAc (3 x 100 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4 and concentrated in the rotary vapour. Purification was done by column chromatography on silica gel using 100:5→ 100:30 Hexane/EtOAc yielded *N*^{3},2´,3´,5´-*O*-Tetrabenzoyl-5-(3-hydroxypropynl)-uridine **31** (3.436 g, 74%) pale yellow solid. TLC (hexane: EtOAc = 50:50) *Rf* = 0.60; ^{1}H NMR (400 MHz, CHLOROFORM-D): δ (ppm) 8.13 – 8.10 (m, 2H), 8.00 – 7.98 (m, 2H), 7.91 (dd, *J* = 8.2, 1.0 Hz, 2H), 7.88-7.84 (m, 3H), 7.63 – 7.56 (m, 3H), 7.54 – 7.48 (m, 3H), 7.41 (td, *J* = 7.8, 2.4 Hz, 4H), 7.30 (t, *J* = 7.8 Hz, 2H), 6.35 (d, *J* = 6.0 Hz, 1H), 5.90 (dd, *J* = 5.8, 3.0 Hz, 1H), 5.78 (t, *J* = 6.0 Hz, 1H), 4.80-4.76 (m, 1H), 4.75 – 4.71 (m, 2H), 4.21 (d, *J* = 2.4 Hz, 2H), 2.33 (t, *J* = 4.8 Hz, 1H);); ^{13}C NMR (100 MHz, CHLOROFORM-D): δ (ppm) 167.43, 166.21, 165.63, 165.41, 160.45, 148.42, 142.00, 135.38, 134.00, 133.75, 130.95, 130.71, 129.99, 129.94, 129.81, 129.27, 129.06, 128.99, 128.74, 128.64, 128.21, 101.02, 93.35, 88.29, 81.11, 75.36, 74.26, 71.54, 63.99, 51.32; MALDI-TOF MS (m/z): Calculated for $C_{40}H_{30}N_2O_{11}$ [M] = 714.185, found $[M+Na]^+$ = 737.339, $[M+K]^+$ = 753.311.

***N*^{3},2´,3´,5´-*O*-Tetrabenzoyl-5-(3-hydroxypropyl) (32):** To a solution of **31** (2.516 g, 3.52 mmol) in 100 mL dry MeOH was added 10% Pd/C (1.132 g) in three parts at interval of 10 minutes in inert atmosphere under ice cold condition. The reaction mixture was done at room under H_2 atmosphere. The pressure of the system was maintained with the help of a H_2 bladder. The reaction was allowed to stir for 18 h. Thereafter the reaction mixture was

filtered over celite pad to remove Pd/C and the filtrate was concentrated in the rotary vapour. Purification was done by column chromatography on silica gel using 100:10→ 100:50 hexane/EtOAc yielded N^3,2´,3´,5´-*O*-Tetrabenzoyl-5-(3-hydroxypropyl) **32** (2.09 g, 83%) pale yellow solid. TLC (hexane: EtOAc = 20:80) *Rf* = 0.65.

The reaction was also tried on H-cube setup in a smaller scale wherein **31** (0.5 g, 0.7 mmol) was dissolved in EtOAc(100 mL). The reduction was done using 10% Pd/C cartridge at 40 psi. The organic (EtOAc) fraction obtained after reduction was concentrated in vacuum. Purification was done by column chromatography on silica gel using 100:10→ 100:50 hexane/EtOAc N^3,2´,3´,5´-*O*-Tetrabenzoyl-5-(3-hydroxypropyl) **32** (0.474 g, 94%) pale yellow solid. TLC (hexane: EtOAc = 20:80) *Rf* = 0.65. This procedure however did not work for large scale reactions.

^{1}H NMR (400 MHz, CHLOROFORM-D): δ (ppm) 8.15 (d, *J* = 7.6 Hz, 2H), 8.00 (d, *J* = 7.6 Hz, 2H), 7.89 (t, *J* = 6.4 Hz, 4H), 7.66-7.55 (m, 3H), 7.52 (t, *J* = 7.4 Hz, 3H), 7.41 (t, *J* = 7.4 Hz, 4H), 7.35 – 7.31 (m, 3H), 6.42 (d, *J* = 6.8 Hz, 1H), 5.93 (dd, *J* = 5.2, 3.2 Hz, 1H), 5.78 (t, *J* = 6.0 Hz, 1H), 4.89 (d, *J* = 12.0 Hz, 1H), 4.72 (s, 1H), 4.68 (dd, *J* = 12.0, 4.2 Hz, 1H), 3.50 (t, *J* = 5.6 Hz, 2H), 2.30 – 2.14 (m, 2H), 2.04 (s, 1H), 1.60-1.52 (m, 2H); ^{13}C NMR (100 MHz, CHLOROFORM-D): δ (ppm) 168.05, 165.91, 165.38, 165.20, 162.49, 149.07, 135.39, 134.90, 133.73, 133.70, 133.63, 131.04, 130.23, 129.73, 129.63, 129.52, 128.96, 128.93, 128.74, 128.44, 128.37, 127.97, 115.34, 86.92, 80.58, 73.23, 71.23, 63.66, 60.66, 31.19, 22.67; MALDI-TOF MS (m/z): Calculated for $C_{40}H_{34}N_2O_{11}$ [M] = 718.216, found $[M+Na]^+$ = 741.228, $[M+K]^+$ = 757.208.

***N*^{3},2´,3´,5´-*O*-Tetrabenzoyl-5-(3-methanesufonatepropyl)-uridine(33):** To a solution of **32** in dry CH_2Cl_2, Et_3N (1.17 mL, 8.4 mmol) was added and the solution was cooled to 0^{o}C in an ice bath. MsCl (0.66 mL, 8.4 mmol, 3 equiv) was added to mixture drop wise for 15 minutes. The reaction was allowed to stir for 2 h at 0^{o}C. To quench the reaction saturated solution of ammonium chloride was added to the reaction mixture and was extracted in ether (3 x 50 mL), dried over Na_2SO_4 and concentrated in the rotary vapour. The crude product (2.62 g) was used directly without further purification. TLC (hexane: EtOAc = 30:70) *Rf* = 0.65; ^{1}H NMR (400 MHz, CHLOROFORM-D): δ (ppm) 8.15 (dt, *J* = 8.6, 1.6 Hz, 2H), 8.03 – 7.97 (m, 2H), 7.92 – 7.87 (m, 4H), 7.66 – 7.56 (m, 3H), 7.55 – 7.49 (m, 3H), 7.45 – 7.38 (m, 5H), 7.32 (t, *J* = 7.8 Hz, 2H), 6.40 (d, *J* = 6.5 Hz, 1H), 5.92 (dd, *J* = 6.0, 3.2 Hz, 1H), 5.82 (t, *J* = 6.2 Hz, 1H), 4.87 (dd, *J* = 13.8, 4.6 Hz, 1H), 4.74 – 4.68 (m, 2H), 4.22 – 4.14 (m,

2H), 2.98 (s, 3H), 2.29 (t, *J* = 7.1 Hz, 2H), 1.86 (p, *J* = 6.6 Hz, 2H); MALDI-TOF MS (m/z): Calculated for $C_{41}H_{36}N_2O_{13}S$ [M] = 796.194, found $[M+Na]^+$ = 819.174, $[M+K]^+$ = 835.144.

2´,3´,5´-*O*-Tribenzoyl- 5-(3-azidopropyl)-uridine(34): The crude product obtained from the previous step, **33** (2.62 g, 3.3 mmol, 1 equiv) and NaN_3 (1.4 g, 2 1.4 mmol, 6.5 equiv) were dissolved in dry DMF (25 mL). The reaction mixture was stirred at 40^{o}C for 6 h. Thereafter the DMF was evaporated under reduced pressure. Saturated $NaHCO_3$ solution was added to the residue and was extracted with EtOAc (75 x 3 mL), dried over Na_2SO_4 and then concentrated in the rotary vapour. Purification was done by column chromatography on silica gel using 100:5→ 100:25 hexane/EtOAc yielded *2´,3´,5´-O-Tetrabenzoyl* protected 5-(3-azidopropyl)-uridine **34** (1.552 g, 75% for two step combined together) pale yellow solid. TLC (hexane: EtOAc = 20:80) *Rf* = 0.65; ^{1}H NMR (400 MHz, CHLOROFORM-D): δ (ppm) 8.76 (s, 1H), 8.17 – 8.13 (m, 2H), 8.02 – 7.98 (m, 2H), 7.95 (dd, *J* = 8.2, 1.14 Hz, 2H), 7.66 - 7.54 (m, 3H), 7.54 - 7.50 (m, 2H), 7.41 - 7.39 (m , 2H), 7.38 - 7.34 (m, 2H), 7.19 (s, 1H), 6.41 (d, *J* = 6.5 Hz, 1H), 5.92 (dd, *J* = 5.8, 3.4 Hz, 1H), 5.78 (t, *J* = 6.4 Hz, 1H), 4.88 (dd, *J* = 12.2, 2.6 Hz, 1H), 4.71 (dd, *J* = 6.2, 3.4 Hz, 1H), 4.65 (dd, *J* = 12.0, 3.6 Hz, 1H), 3.13 (t, *J* = 6.6 Hz, 2H), 2.17 – 2.01 (m, 2H), 1.63 – 1.55 (m, 2H); ^{13}C NMR (100 MHz, CHLOROFORM-D): δ (ppm) 166.09, 165.54, 165.49, 163.00, 150.32, 135.81, 133.95, 133.91, 133.85, 130.06, 129.96, 129.78, 129.34, 128.98, 128.70, 128.66, 128.40, 114.89, 87.21, 80.79, 73.39, 71.60, 64.21, 50.64, 27.49, 24.40; MALDI-TOF MS (m/z): Calculated for $C_{33}H_{29}N_5O_9$ [M] = 639.611, found $[M+Na]^+$ = 662.188, $[M+K]^+$ = 678.143.

5-(3-azidopropyl)-uridine(35): 34 (1.5 g, 2.018 mmol, 1equiv) was taken a round bottom flask equipped with stir bar. To it dry sodium methoxide (0.5 M in MeOH, 200 mL) solution was added drop wise. The reaction was stirred for 30 min at 60^{o}C. Thereafter the reaction mixture was cooled to room temperature and then quenched by pouring 1N HCl, adjusting the pH to nearly neutral (6.5-7.5). The solvent was removed in the rotary vapour. Purification was done by column chromatography on silica gel using 100:1→ 100:5 DCM /MeOH yielded 5-(3-azidopropyl)-uridine **35** (400 mg, 61%) white solid. TLC (DCM: MeOH = 85:15) *Rf* = 0.49; ^{1}H NMR (400 MHz, *d6*-DMSO): δ (ppm) 11.32 (s, 1H), 7.77 (s, 1H), 5.77 (d, *J* = 5.2 Hz, 1H), 5.36 (d, *J* = 5.6 Hz, 1H), 5.13 (t, *J* = 5.2 Hz, 1H), 5.07 (d, *J* = 5.2 Hz, 1H), 4.04 (dd, *J* = 10.8, 5.2 Hz, 1H), 3.97 (dd, *J* = 9.4, 5.0 Hz, 1H), 3.83 (q, *J* = 3.2 Hz, 1H), 3.65 (ddd, *J* = 12.0, 5.2, 3.2 Hz, 1H), 3.55 (ddd, *J* = 12.0, 4.8, 3.2 Hz, 1H), 3.34 (dd, *J* = 4.0, 2.8 Hz, 2H), 2.32 – 2.19 (m, 2H), 1.69 (p, *J* = 7.2 Hz, 2H) ; ^{13}C NMR (100 MHz, *d6*-DMSO): δ (ppm) 163.87, 151.13, 137.29, 112.80, 88.24, 85.21, 73.99, 70.29, 61.27, 50.70,

27.65, 24.23; MALDI-TOF MS (m/z): Calculated for $C_{12}H_{17}N_5O_6$ [M] = 327.293, found $[M+Na]^+$ = 350.006, $[M+K]^+$ = 365.97 .

5-(3-azidopropyl)-uridine-5´-triphosphate (36): To an ice cold solution of ribonucleoside (62.8 mg, 0.192 mmol, 1 equiv) in trimethyl phosphate (1.2 mL) was added freshly distilled $POCl_3$ (36 μL, 0.384 mmol, 2.5 equiv). The solution was stirred at 24 h at 0-4 °C. A solution of *bis*- tributylammonium pyrophosphate (0.5 M in DMF, 2 mL, 5.2 equiv) and tributylamine (0.51 mL, 2.11 mmol, 11 equiv) was rapidly added under ice cold condition. The reaction was quenched after 30 min with 1 M triethylammonium bicarbonate buffer (TEAB, pH 7.5, 15 mL) and was extracted with ethyl acetate (20 mL). The aqueous layer was evaporated and the residue was purified first on DEAE sephadex- A25 anion exchange column (10 mM-1 M TEAB buffer, pH 7.5) followed reverse phase flash column chromatography (C18 Redi*SepRf*, 0-40% acetonitrile in 100 mM triethylammonium actetate buffer, pH 7.2, 40 min). Appropriate fractions were lyophilized to afford the desired triphosphate product 5-(3-azidopropyl)-uridine-5´-triphosphate as a tetratriethylammonium salt (57 mg, 26%); ^{1}H NMR (400 MHz, D_2O): δ (ppm) 7.76 (s, 1H), 5.97 (d, *J* = 5.6 Hz, 1H), 4.41 – 4.38 (m, 2H), 4.24 - 4.19 (m , 3H), 3.34 (t, *J* = 6.6 Hz, 2H), 7.76 (s, 1H), 5.97 (d, *J* = 5.3 Hz, 1H), 4.44 – 4.37 (m, 2H), 4.22 (dd, *J* = 12.6, 4.7 Hz, 3H), 3.34 (t, *J* = 6.7 Hz, 2H), 2.43 (td, *J* = 7.2, 2.5 Hz, 2H), 1.81 - 1.74 (m, 2H); ; ^{13}C NMR (100 MHz, D_2O): δ (ppm); 165.85, 151.88, 137.75, 114.78, 87.79, 83.55, 73.42, 69.88, 65.15, 50.48, 27.00, 23.73 ; ^{31}P NMR (162 MHz, D_2O): δ (ppm) -9.43 (br, P_γ), -11.17 (br, P_α), -22.52 (br, P_β); ; MALDI-TOF MS (m/z) negative mode: Calculated for $C_{12}H_{20}N_5O_{15}P_3$ [M] = 567.235, found $[M-H]^-$ = 566.115.

Enzymatic incorporation of azide functionalized ribonucleotide tri-phosphate into RNA using α-^{32}P ATP[12]: The promoter-template duplexes were annealed in equimolar ratio (5 μM final concentration) by heating the T7 RNA polymerase consensus promoter (18mer) DNA sequence and the DNA template (**S1-S5)** in TE buffer (10mM Tris-HCl, 1mM EDTA, 100mM NaCl, pH 7.8) at 90^{o}C for 3 mins. The solution was allowed to come to room temperature slowly and then placed in an ice bath for 20 mins, stored at -40^{o}C for further use. The transcription reaction was carried out at 37^{o}C in 40 mM Tris-HCl buffer (pH 7.9) containing 250 nM annealed promoter-template duplexes, 10 mM $MgCl_2$, 10 mM of NaCl, 10 mM of dithiothreitol (DTT), 2mM spermidine, 1 U/μL RNase inhibitor (Riboblock), 1mM GTP, 1mM CTP, 1mM UTP and or 1mM modified UTP **36**, 20 μM ATP, 5 μCi α-^{32}P ATP

and 3 U/μL T7 RNA in a total volume 20 μL. The reaction was quenched after 3.5 h by adding 20 μL of loading buffer (7 M urea in 10 mM EDTA, 0.05% bromophenol blue, pH 8), heated for 3 mins at 75^{o}C followed by cooling in an ice bath. Polyacrylamide gel electrophoresis was performed using a denaturing gel (18%). The gel was exposed to x-ray sheet for about 30 mins. The sheet was developed, fixed and dried to give the image of the radio-labelled products. Quantification software (GeneTools from Syngene) was used to quantify the product bands.

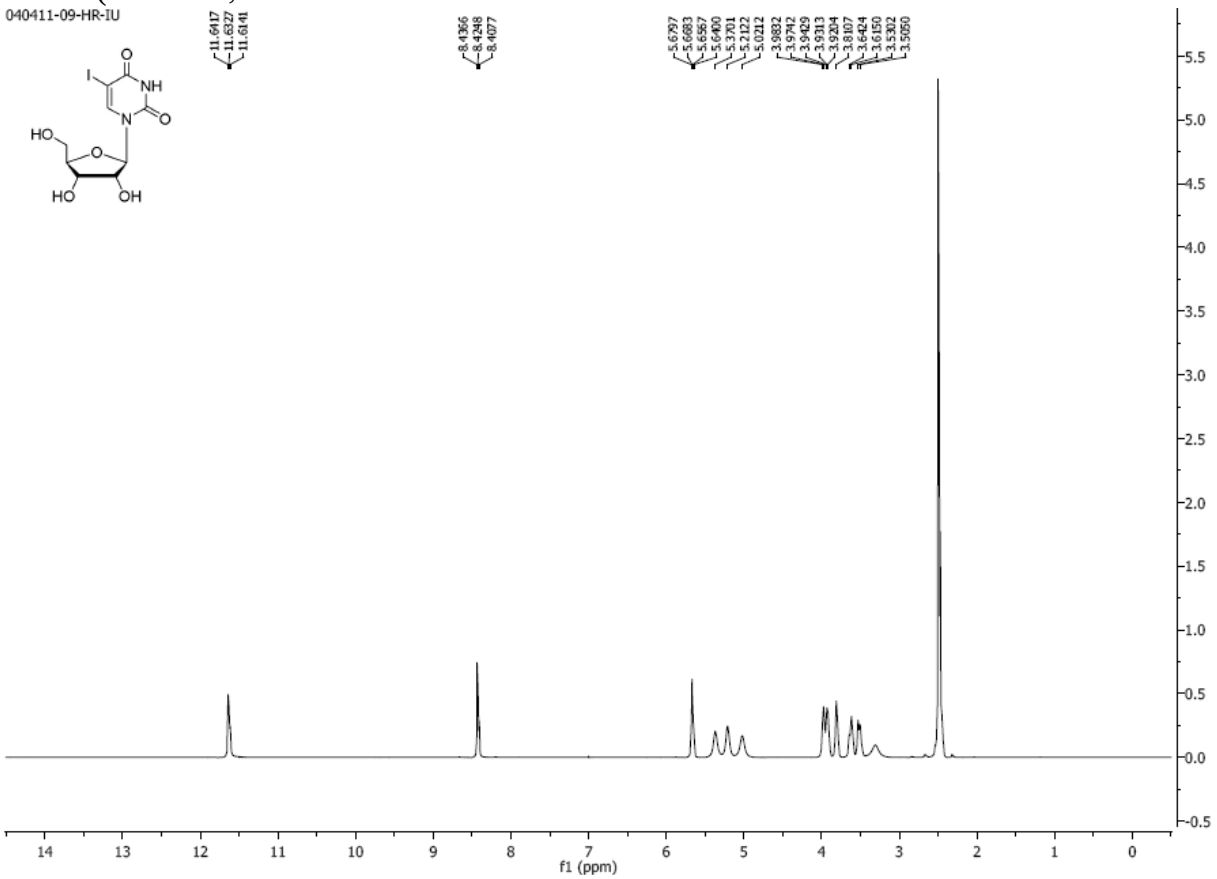
Large- scale transcription reaction: Large scale transription was performed in a 250 μL using **S1** DNA template. The reaction consisted of 2 mM GTP, 2 mM CTP, 2 mM ATP, 2 mM of modified UTP **36**, 20 mM $MgCl_2$, 0.4 U/μL RNase inhibitor (Riboblock), 300 nM of annealed template and 800 units T7 RNA polymerase. After incubating for 12 h at 37^{o}C, the volume was reduced to about 60 μL by speed vac. The reaction mixture was heated for 3 mins at 75^{o}C and subsequently cooled in an ice bath. A preparatory gel electrophoresis using 20% denaturing polyacrylamide gel was performed. After the gel was UV shadowed, relevant bands were cut out, crushed, eluted in 0.3 M sodium acetate and desalted using Sep-Pack cartridge. The yield of transcription reaction was 19-20 nmol. **38**, $\varepsilon_{260} = 91095\ M^{-1}\ cm^{-1}$.

Characterization by MALDI-TOF: Applied Biosystems 4800 Plus MALDI TOF/TOF analyser was used to determine the molecular mass of transcript. 1μL of ~198 μM stock solution transcript, 1μL of 100mM ammonium acetate buffer, 1 μL of a 100 μM DNA standard and 4 μL of HPA matrix (3-hydropiccolinic acid) were mixed to together. The resulting solution after desalting using on an ion exchange resin (dowex 50W-X8, 100-200 mesh) was used for MALDI analysis. The resulting spectrum was calibrated relative to an internal 18-mer DNA standard.

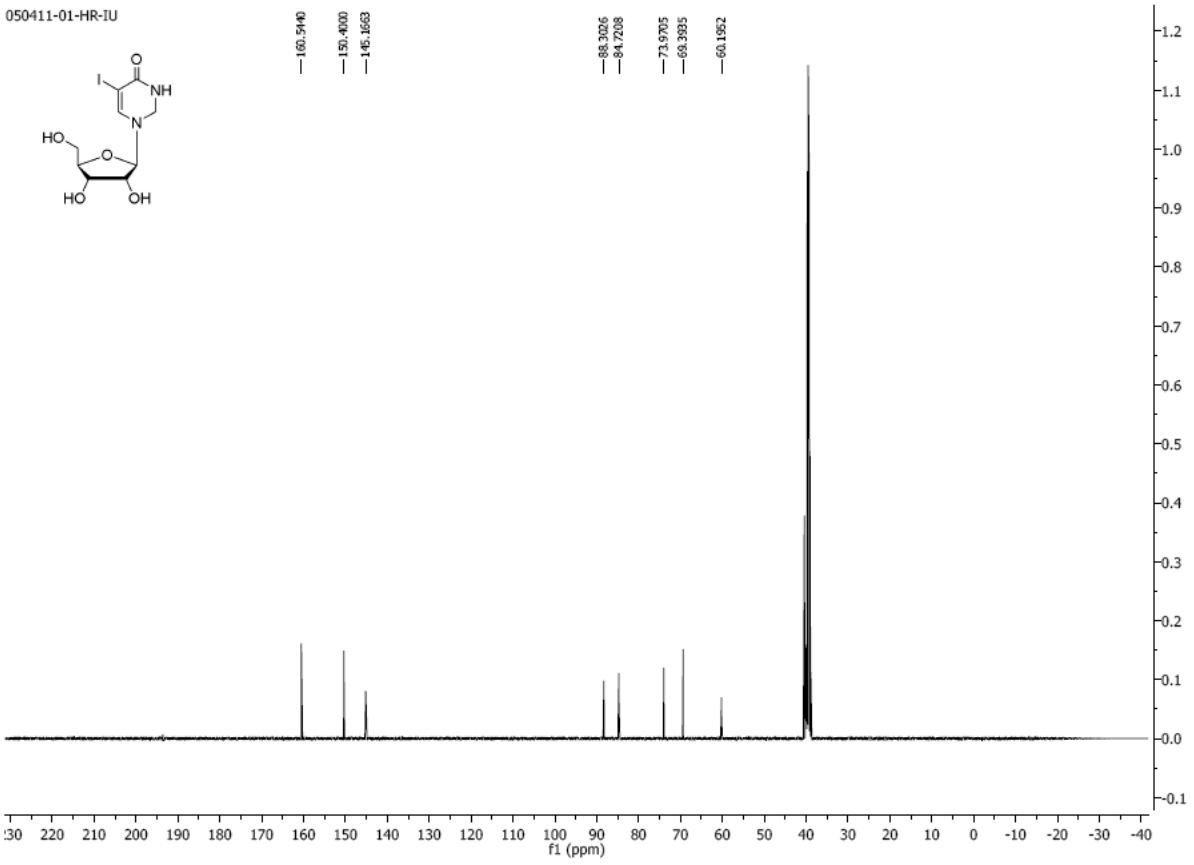
Characterization by enzymatic digestion: Enzymatic digestion of the transcript required 20 μL of dephosphoryation buffer, 40 μL of $MgCl_2$ (200 mM), 10 μL of RNase A (0.5 μg/μL), 20 μL of calf intestine alkaline phosphatise (1 U/μL), 20 μL of phosphatephosphodiesterase (0.4 U/400 μL) and 40 μL of the transcript (197 μM). The reaction mixture was incubated for 12 h at 37^{o}C. HPLC was performed to resolve the constituent nucleoside monomers.

Appendix: Selected Spectrum

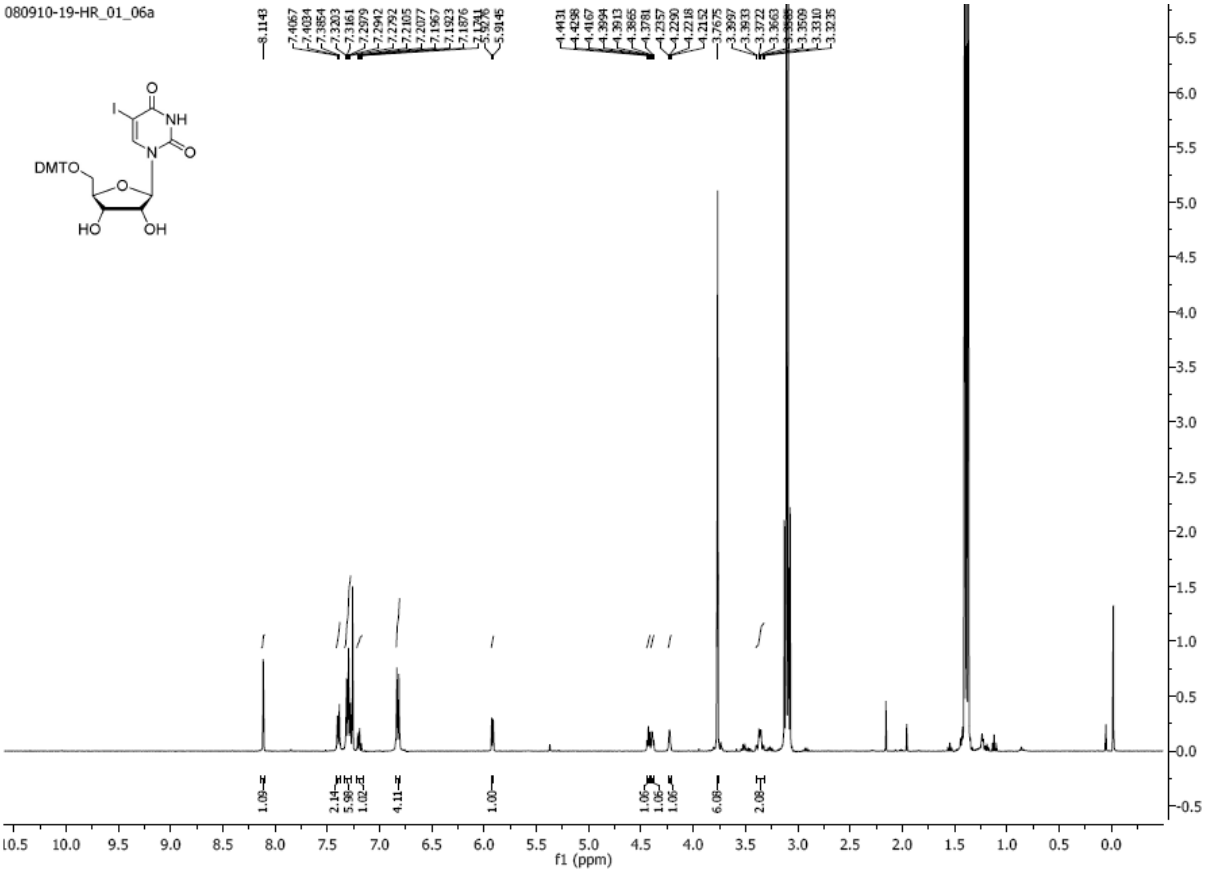
^{1}H NMR (400 MHz, *d6*-D



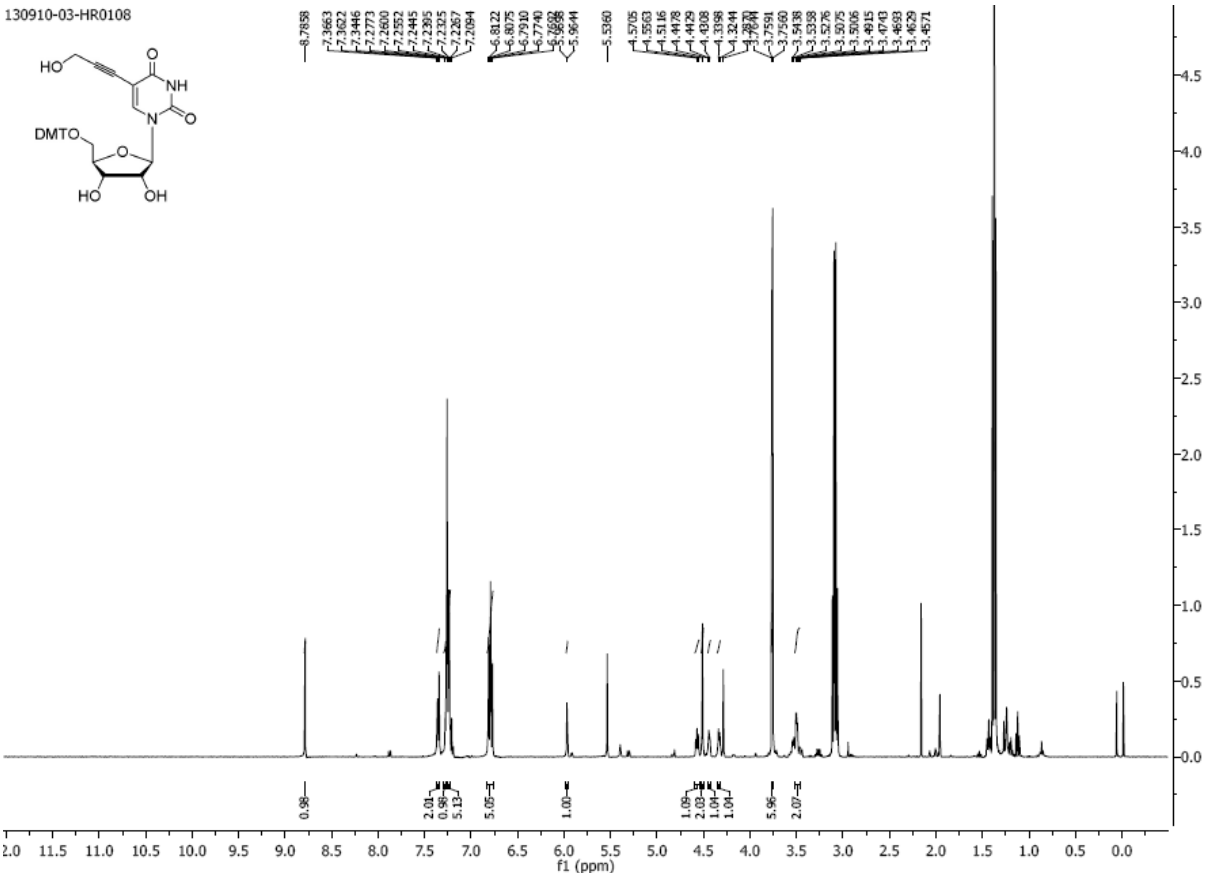
^{13}C NMR (100 MHz, *d6*-DMSO)



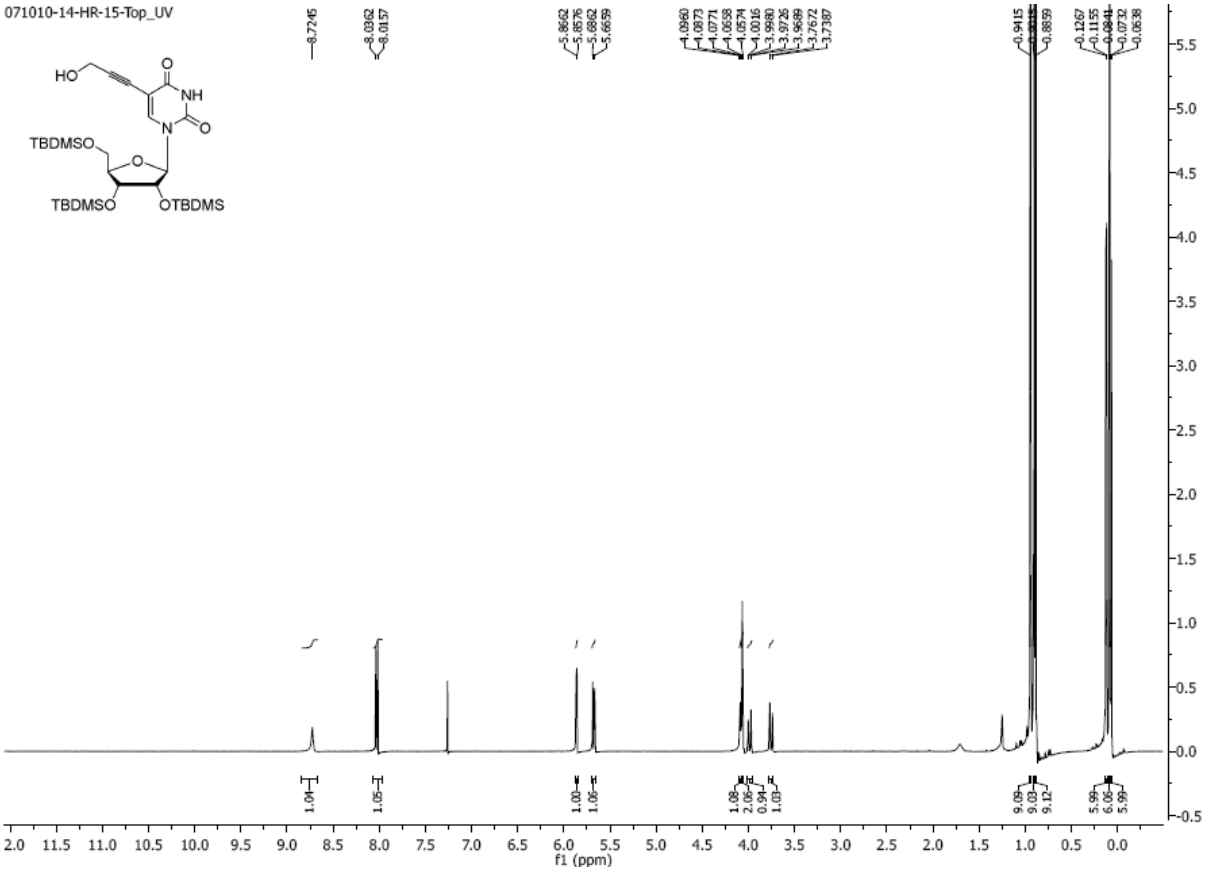
^{1}H NMR (400 MHz, *d6*-DMSO)



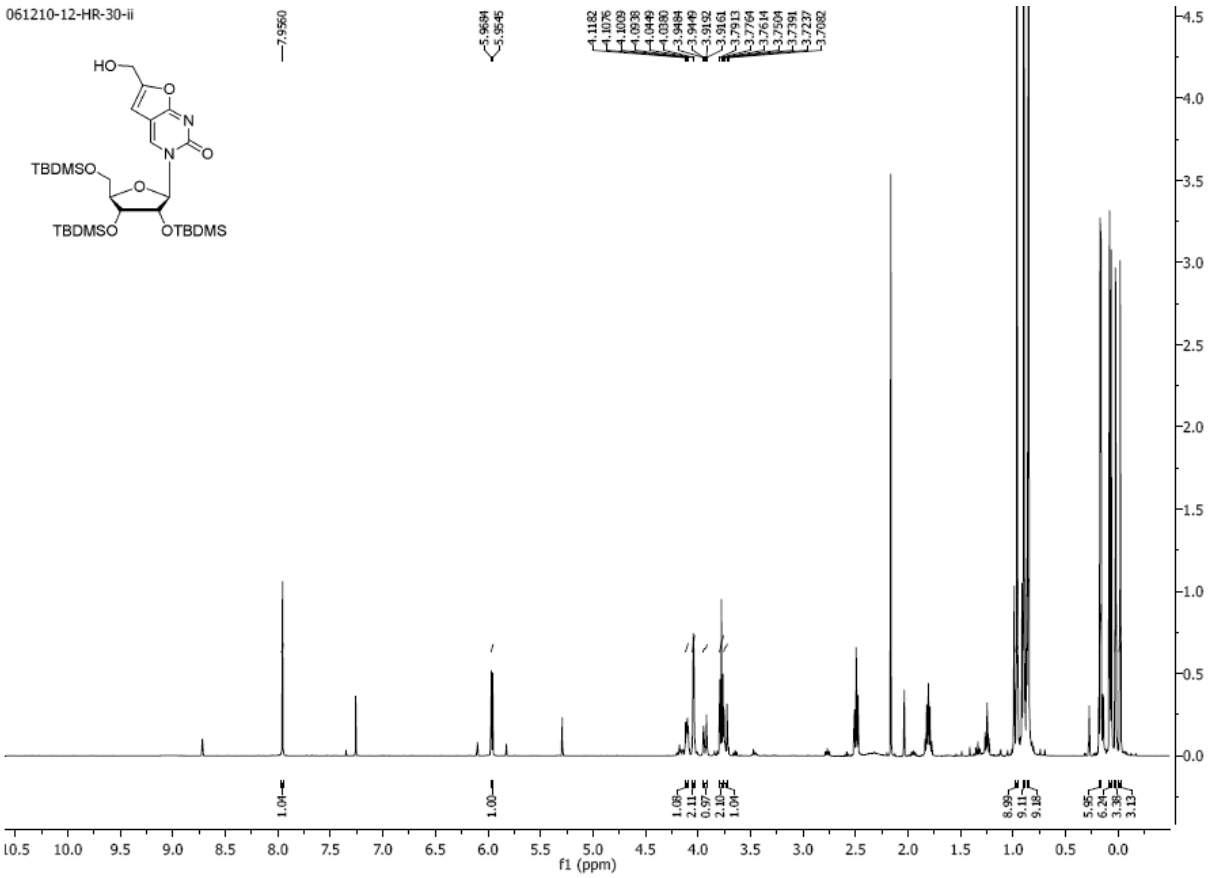
^{1}H NMR (100 MHz, *d6*-DMSO)



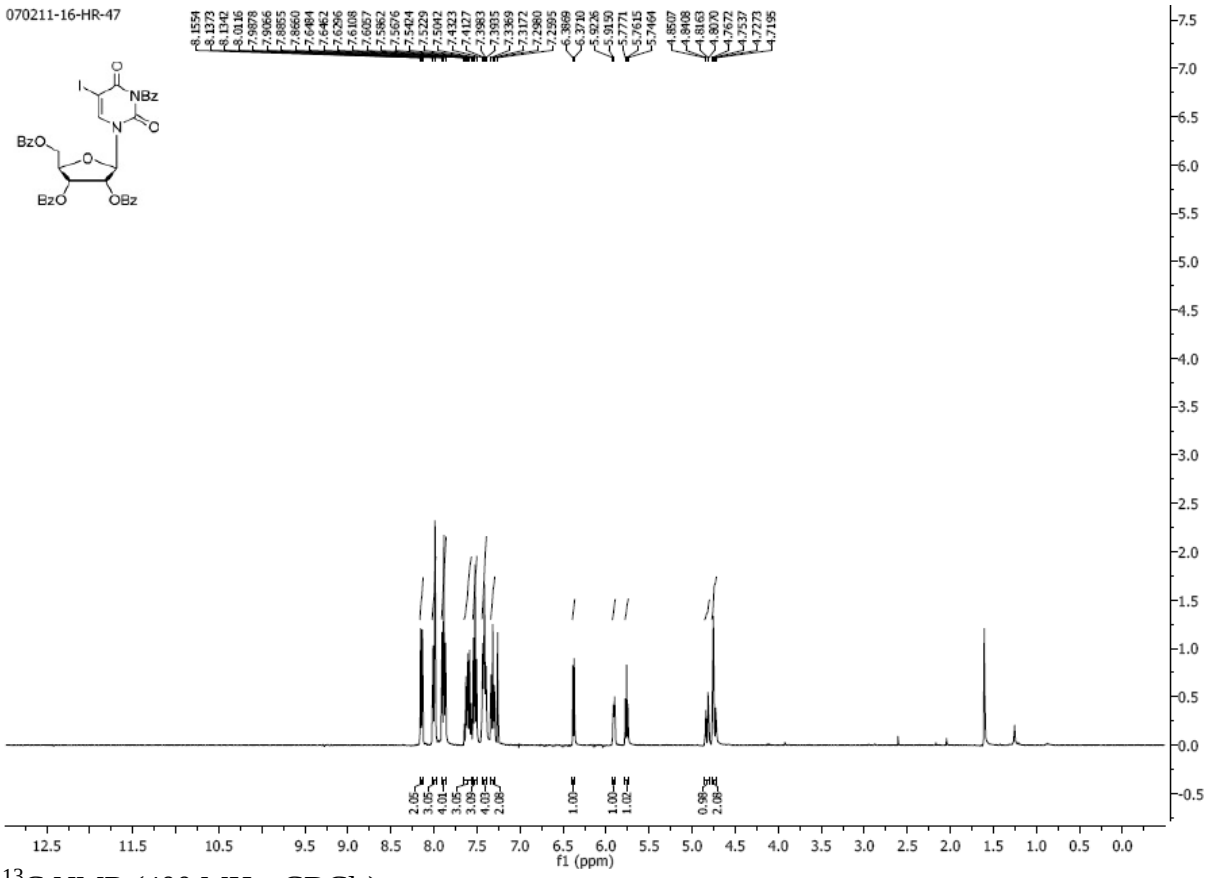
^{1}H NMR (400 MHz, $CDCl_3$)



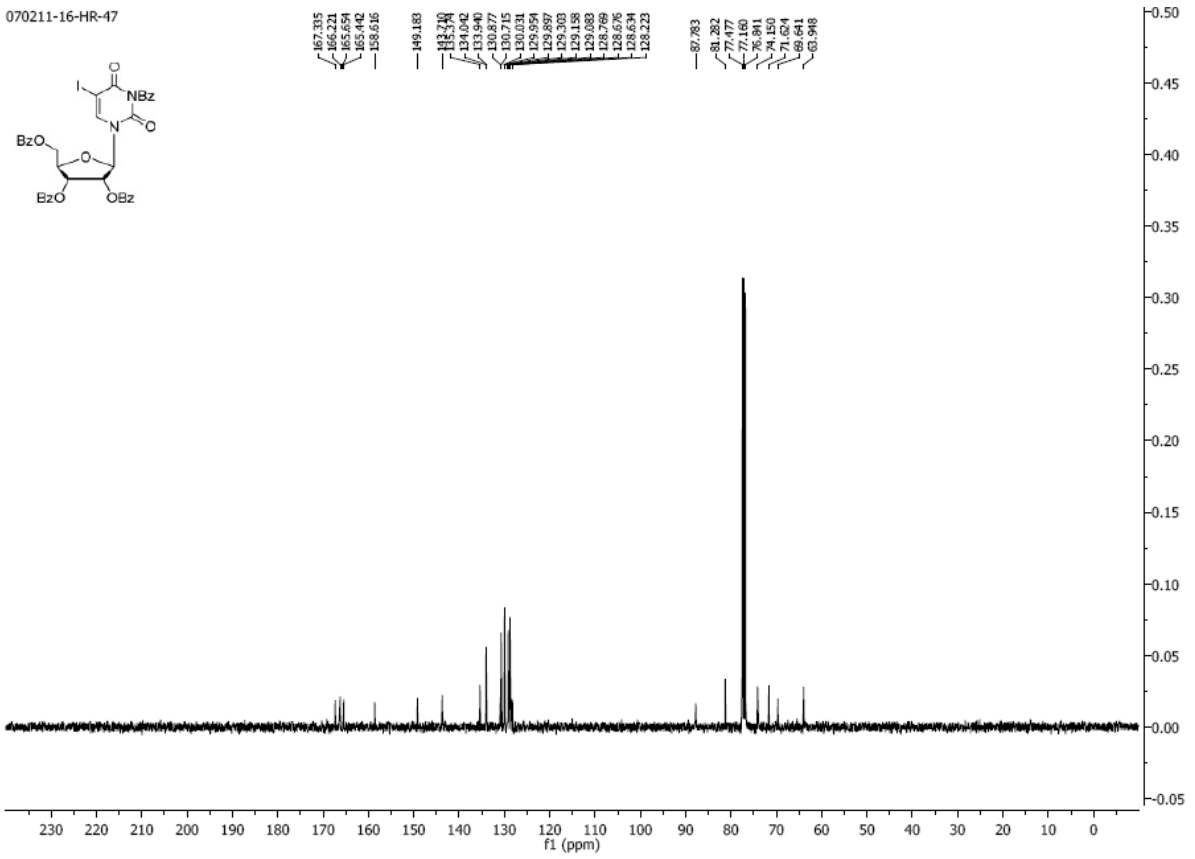
^{1}H NMR (400 MHz, $CDCl_3$)



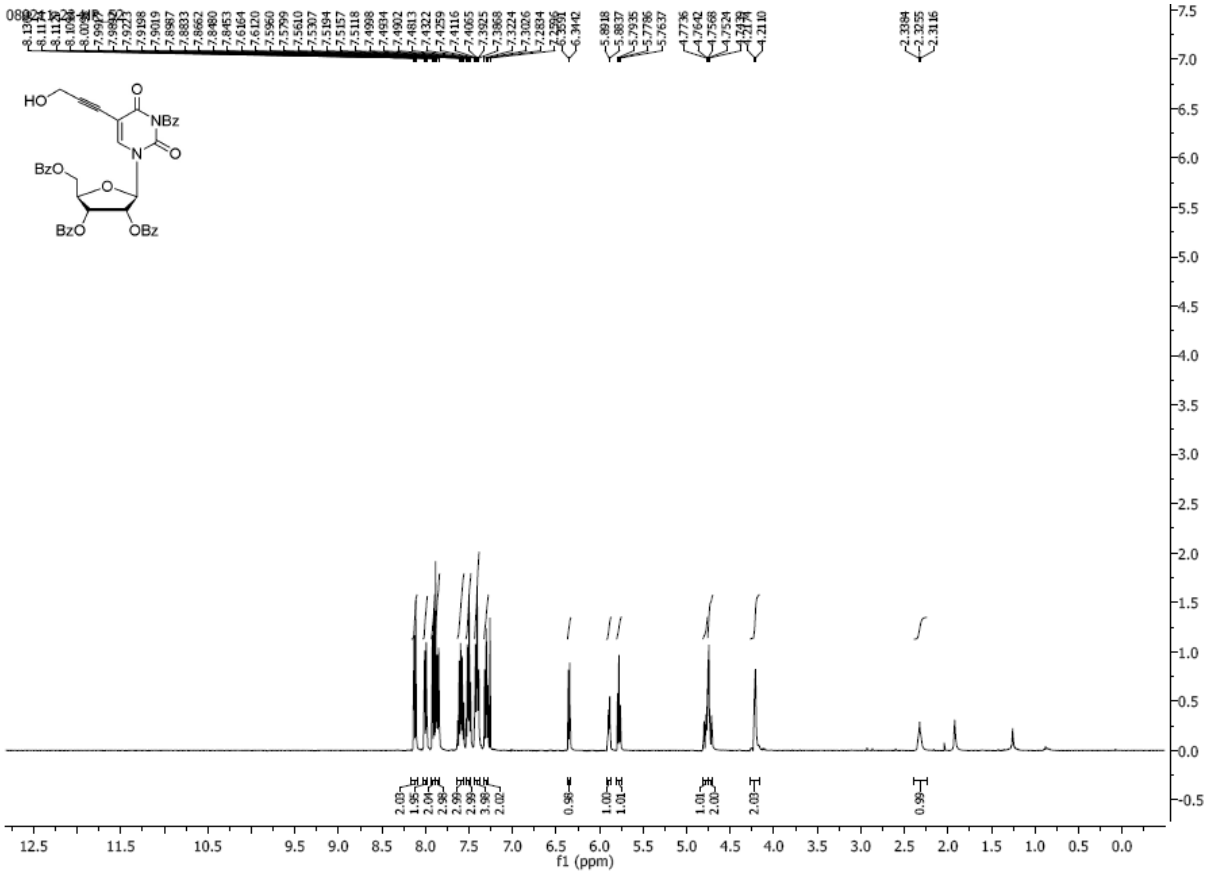
^{1}H NMR (400 MHz, $CDCl_3$)



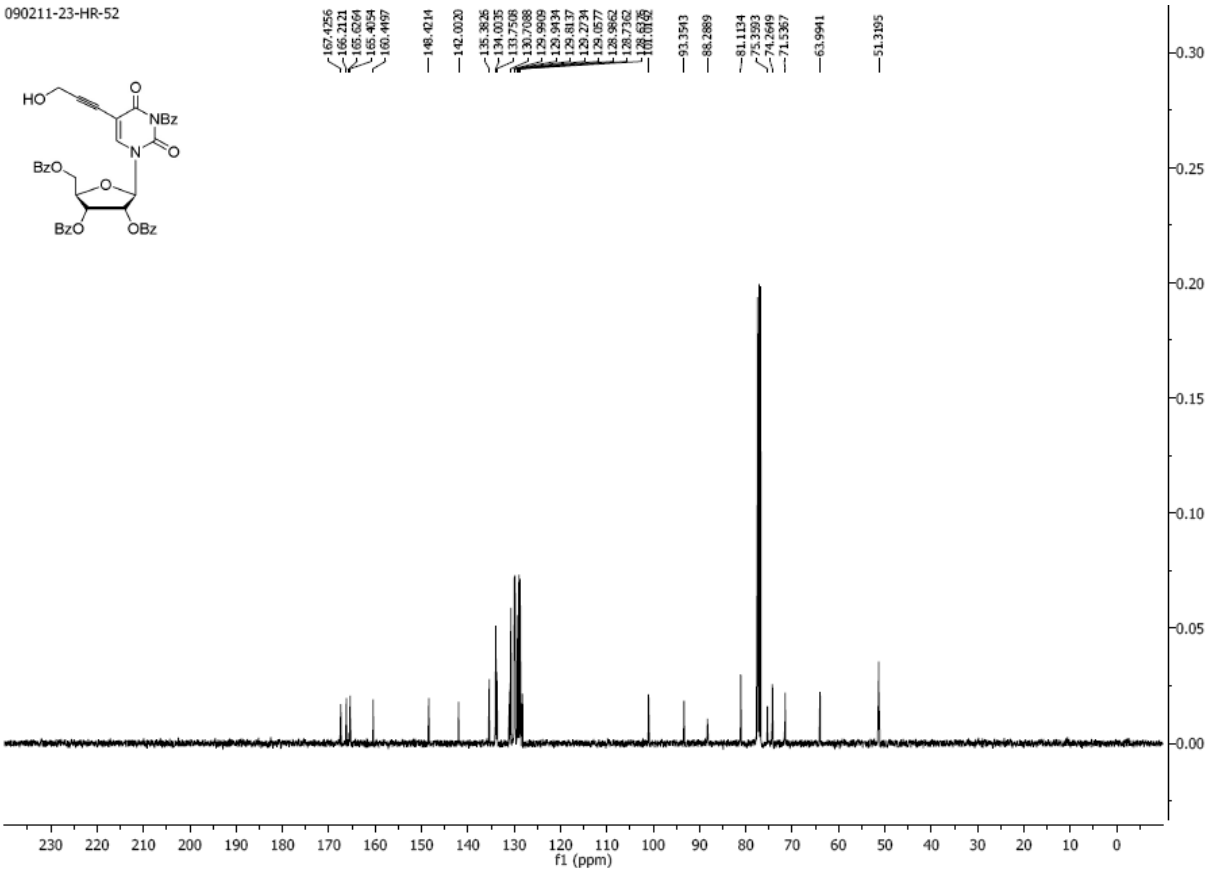
^{13}C NMR (400 MHz, $CDCl_3$)



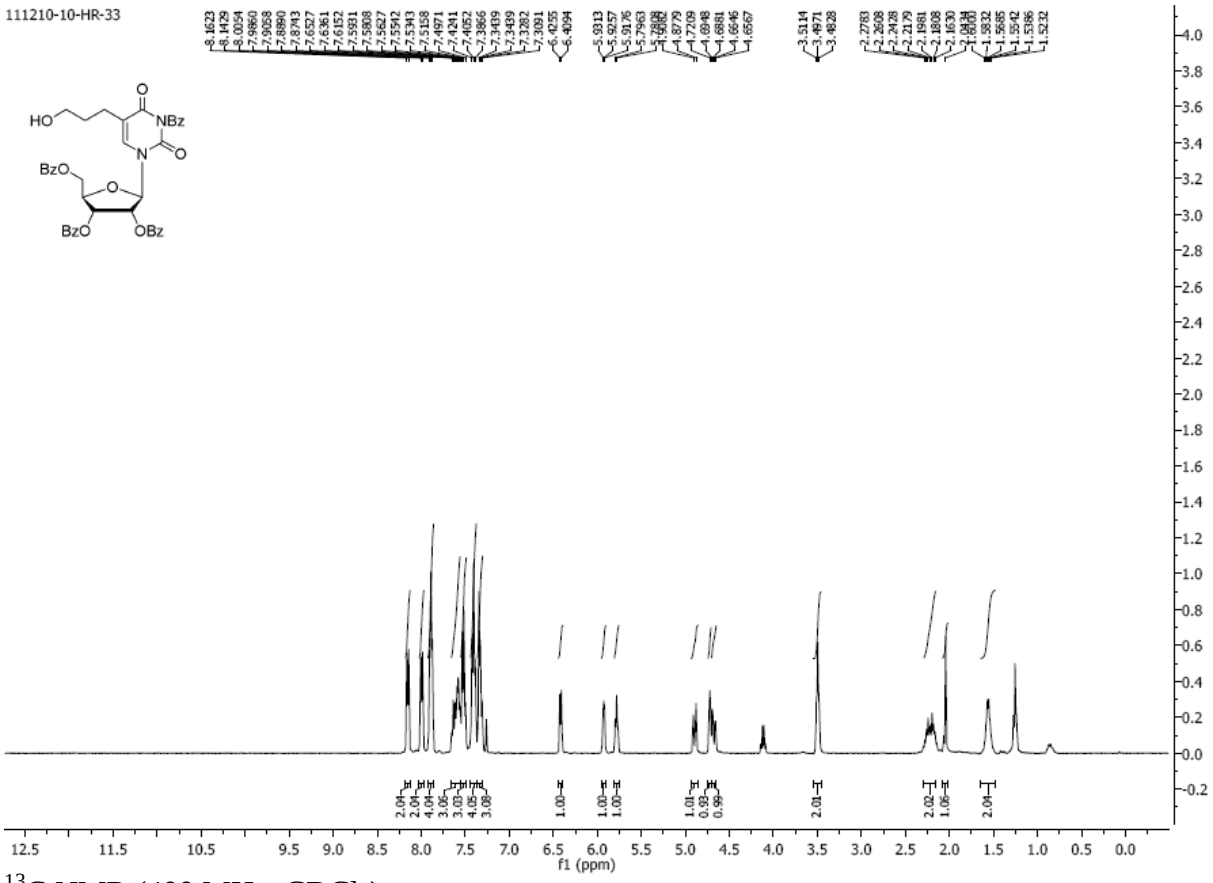
^{1}H NMR (400 MHz, $CDCl_3$)



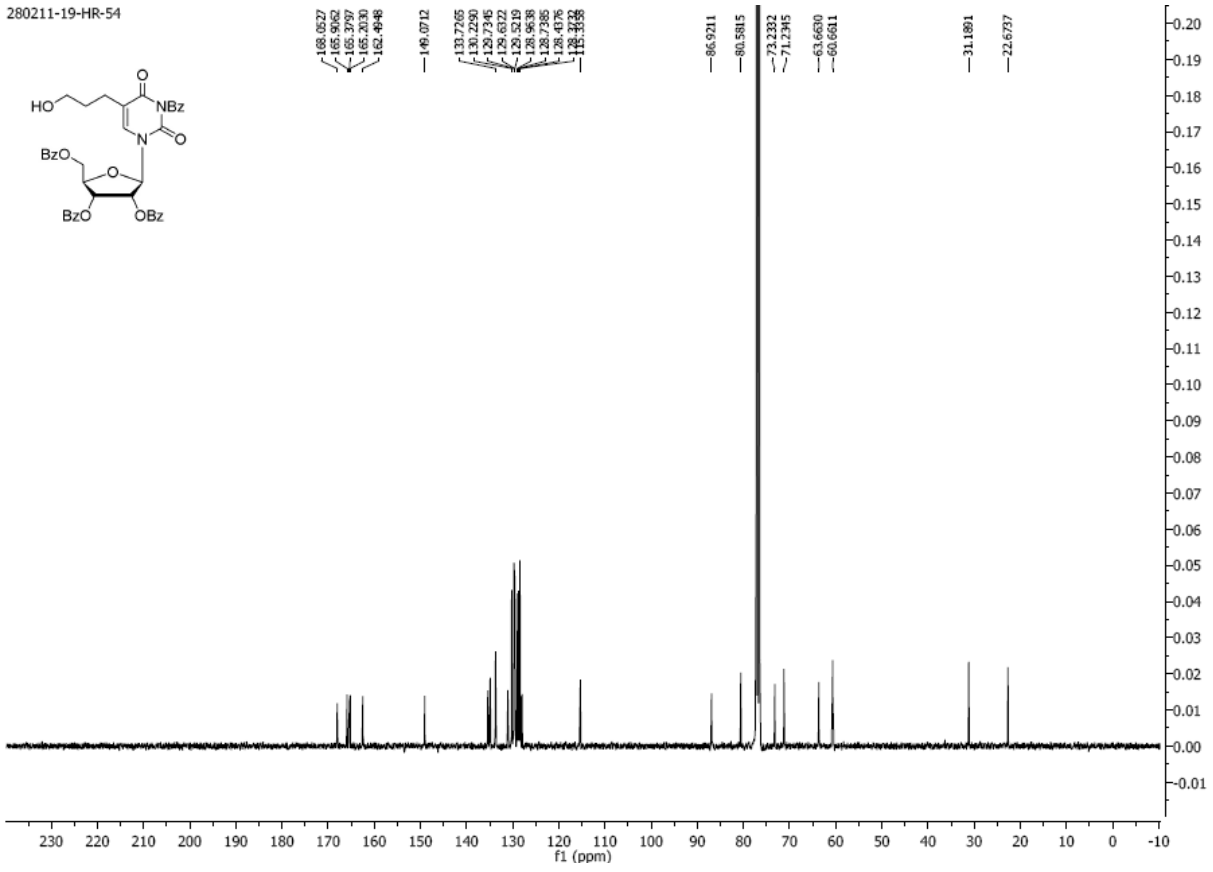
^{13}C NMR (400 MHz, $CDCl_3$)



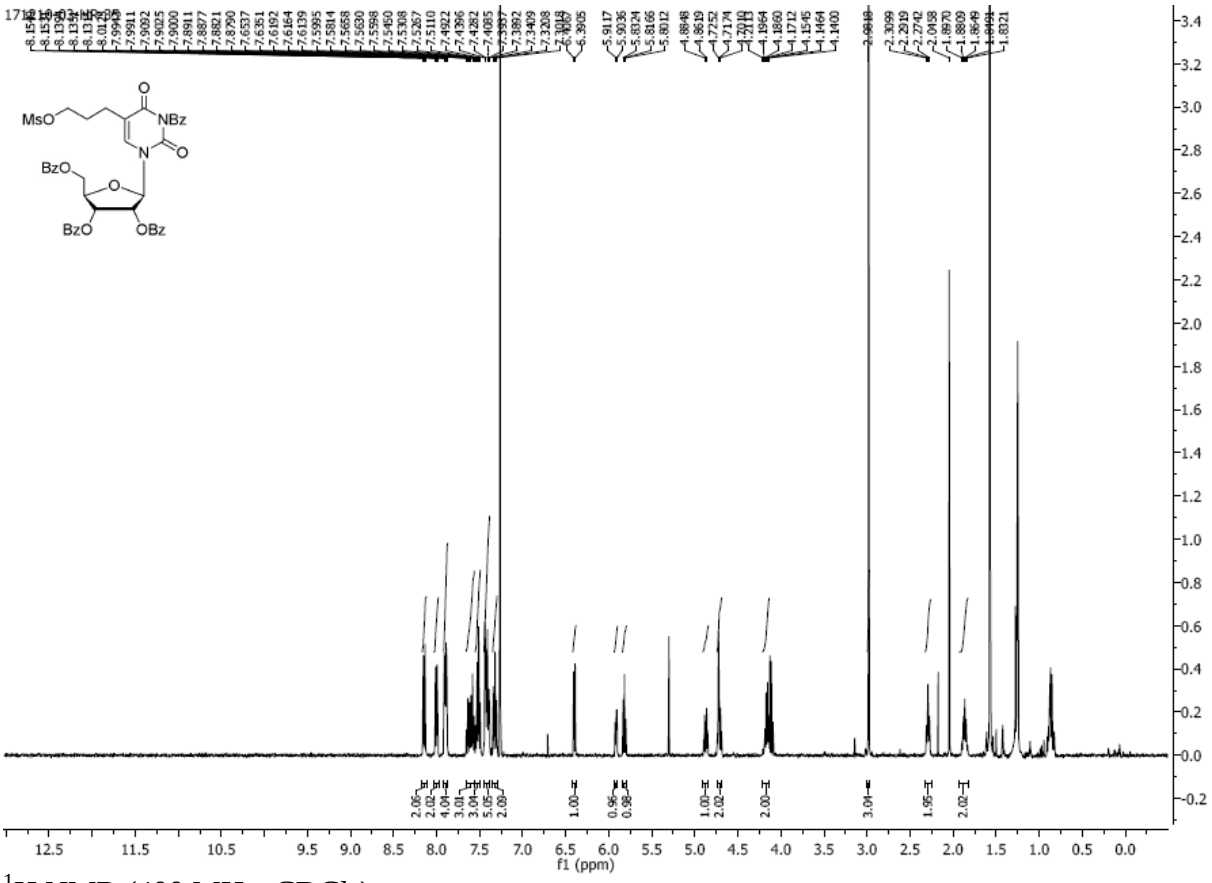
^{1}H NMR (400 MHz, $CDCl_3$)



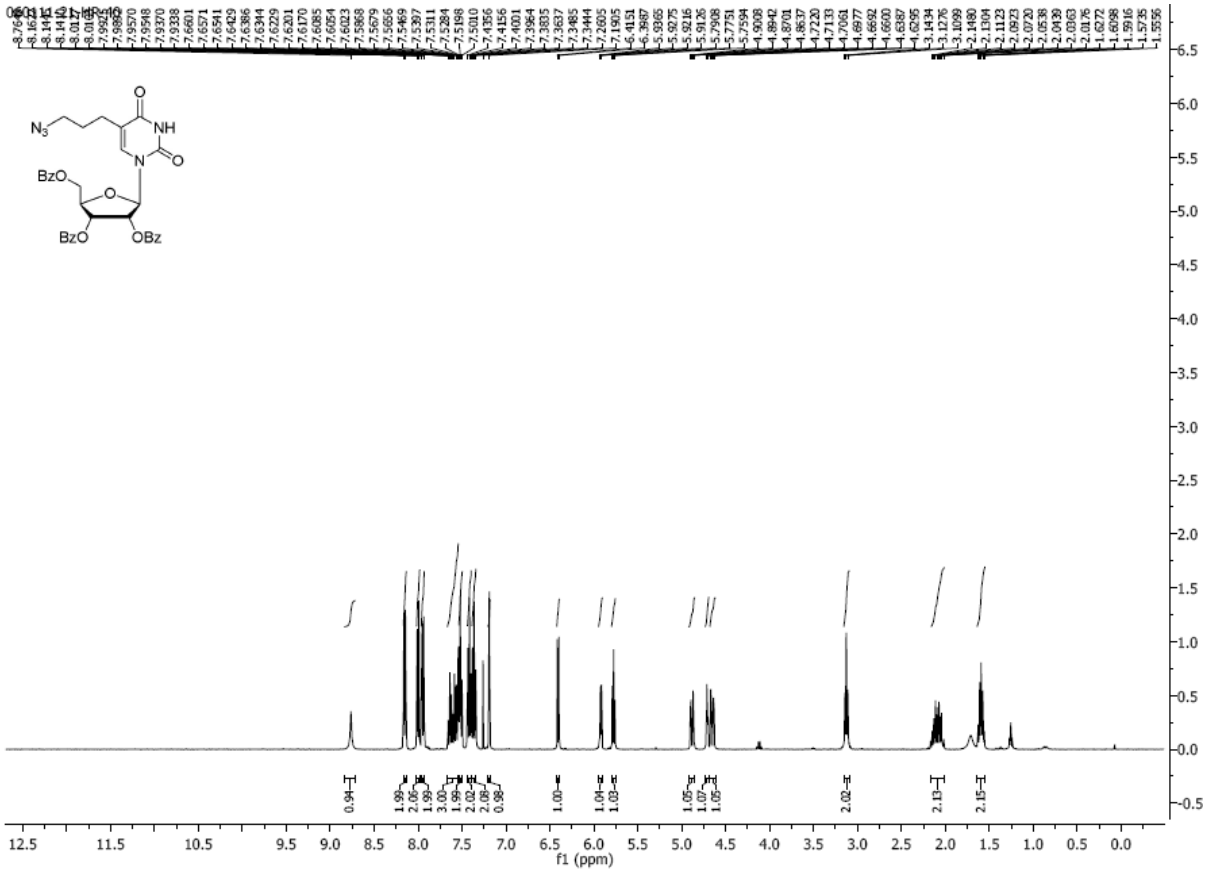
^{13}C NMR (400 MHz, $CDCl_3$)



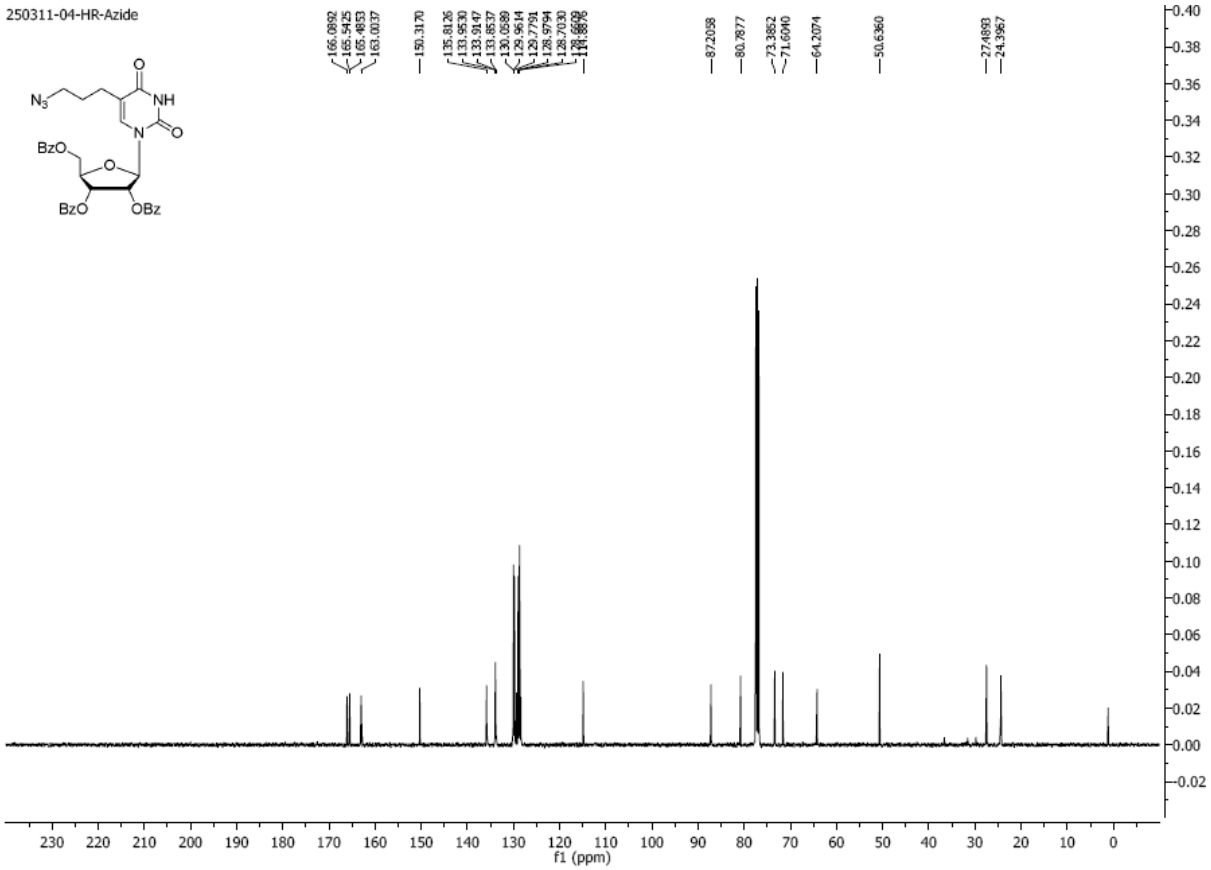
^{1}H NMR (400 MHz, $CDCl_3$)



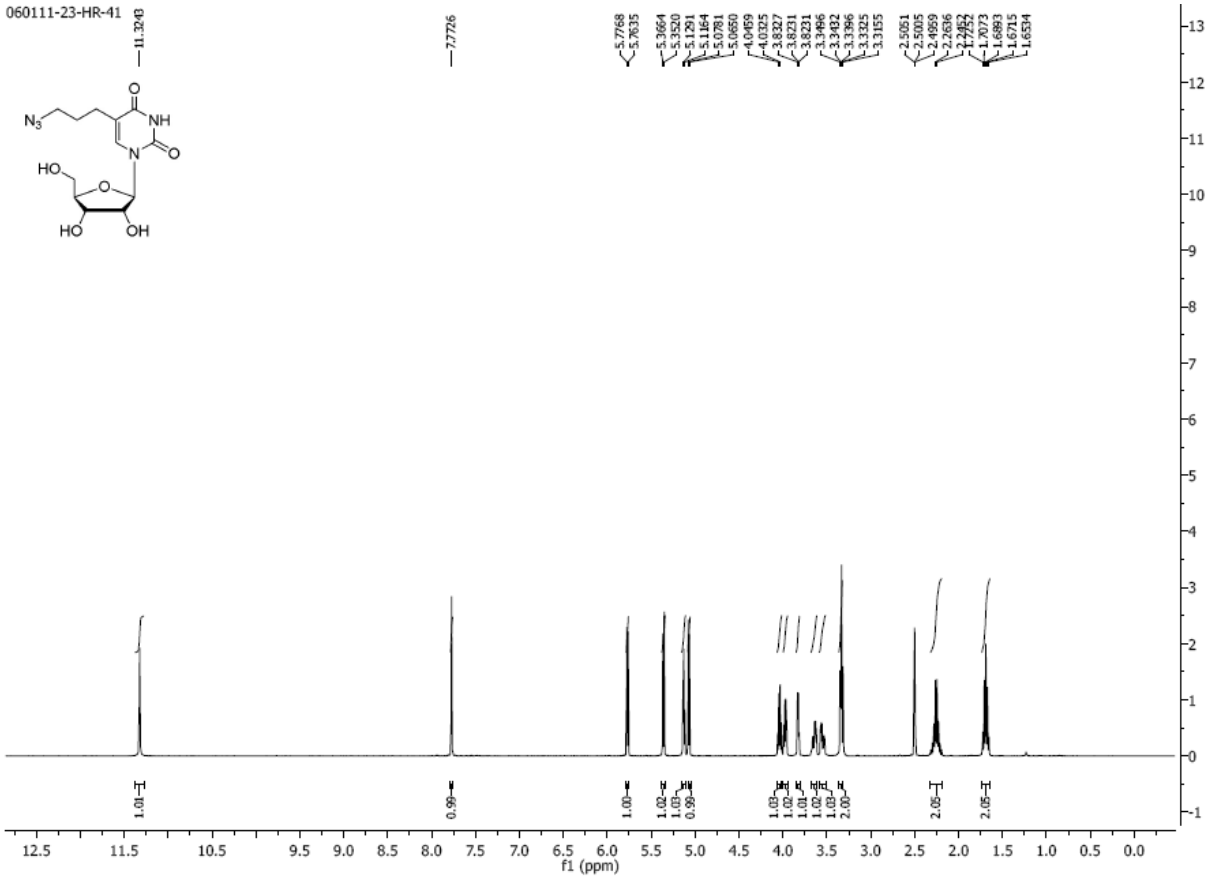
^{1}H NMR (400 MHz, $CDCl_3$)



^{13}C NMR (400 MHz, $CDCl_3$)



^{1}H NMR (400 MHz, *d6*-DMSO)



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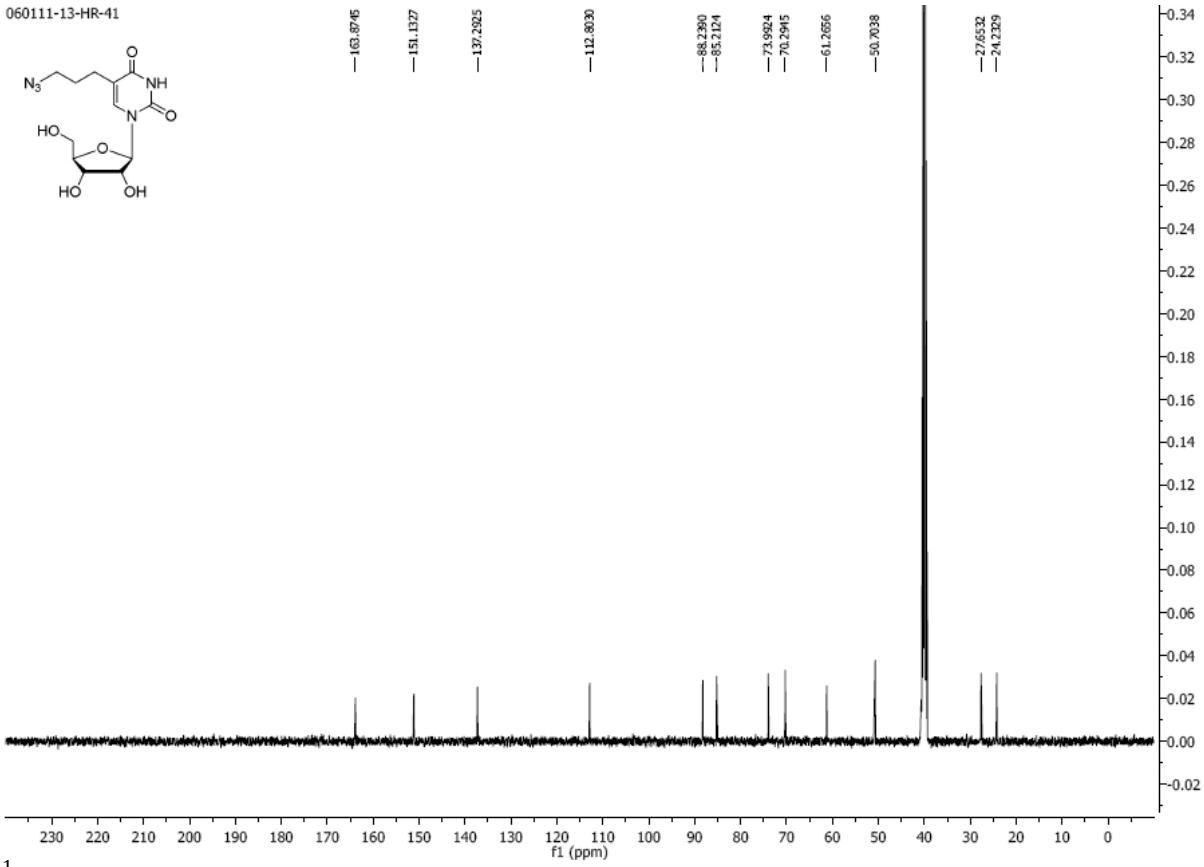
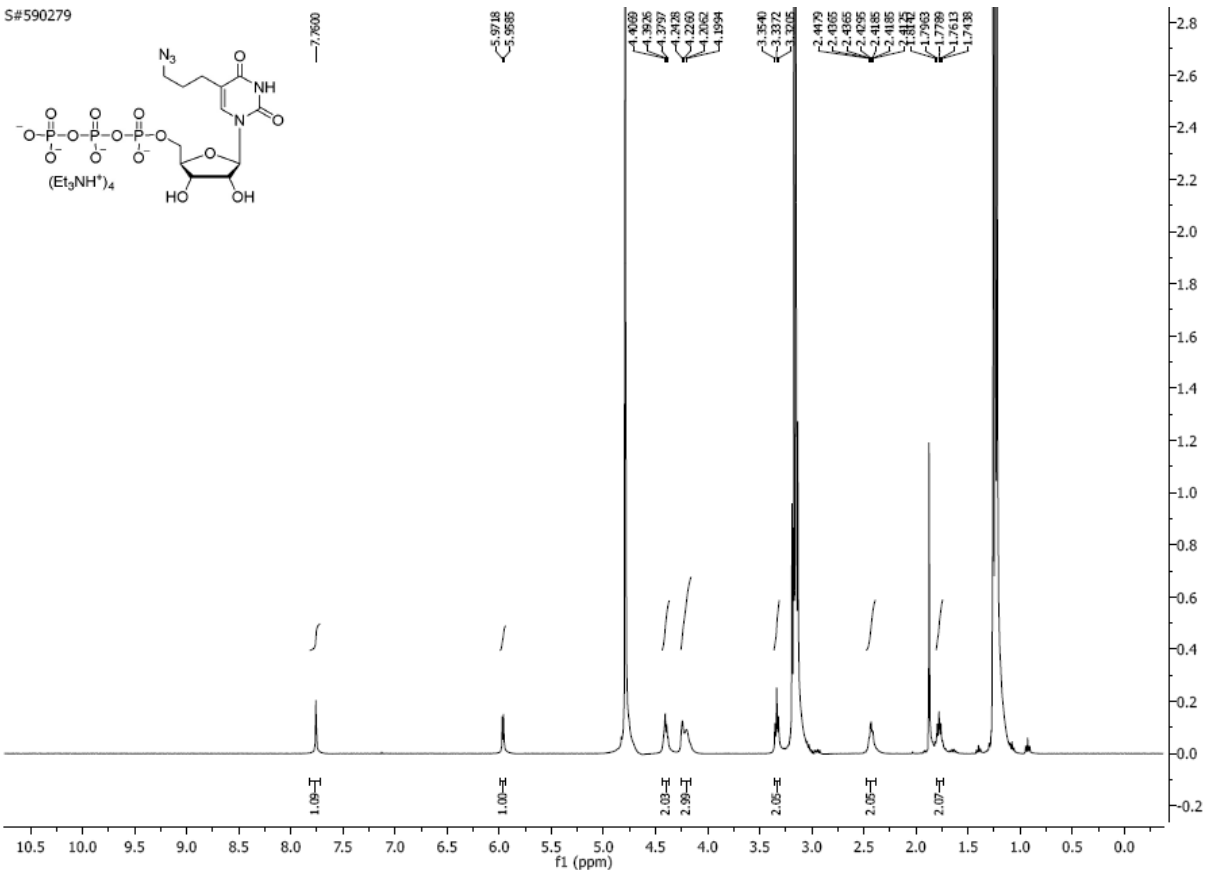


^{1}H NMR (400 MHz, D_2O)



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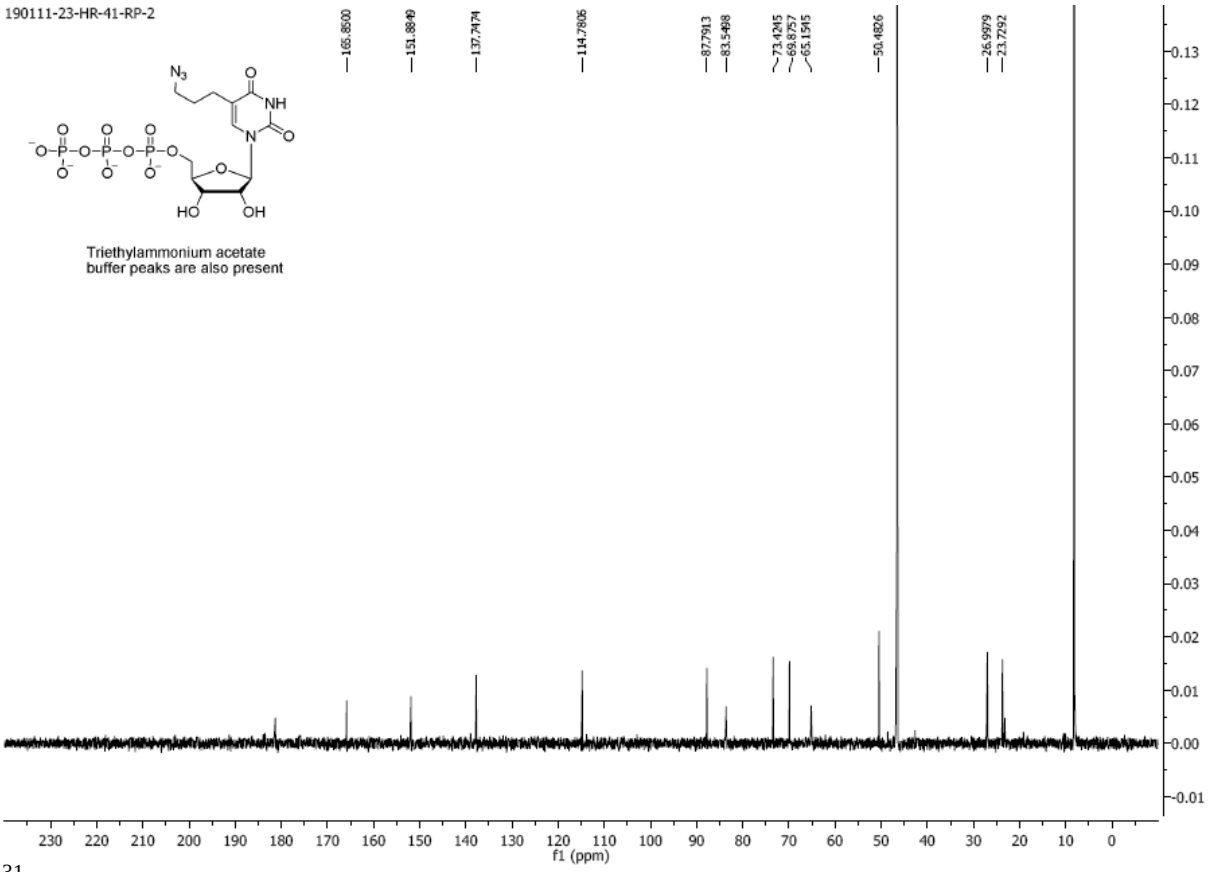
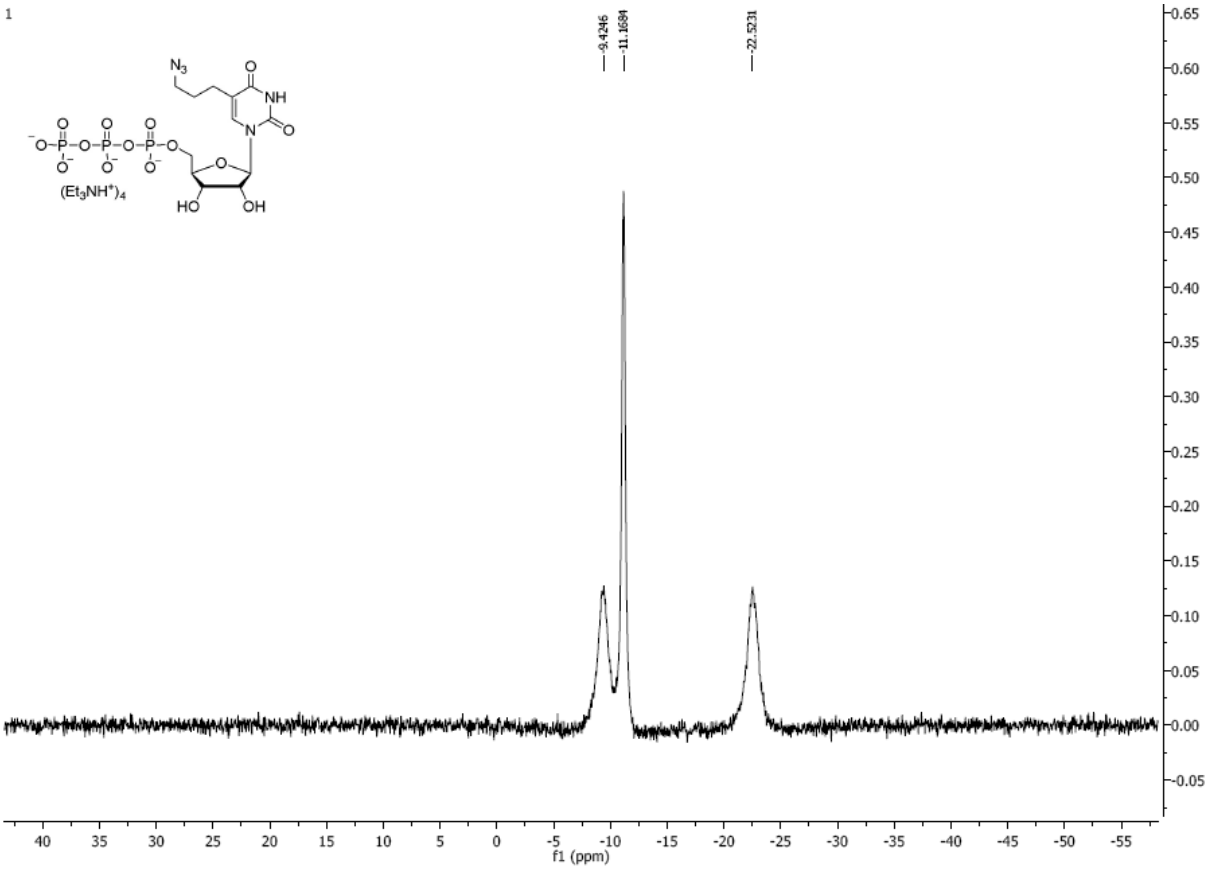
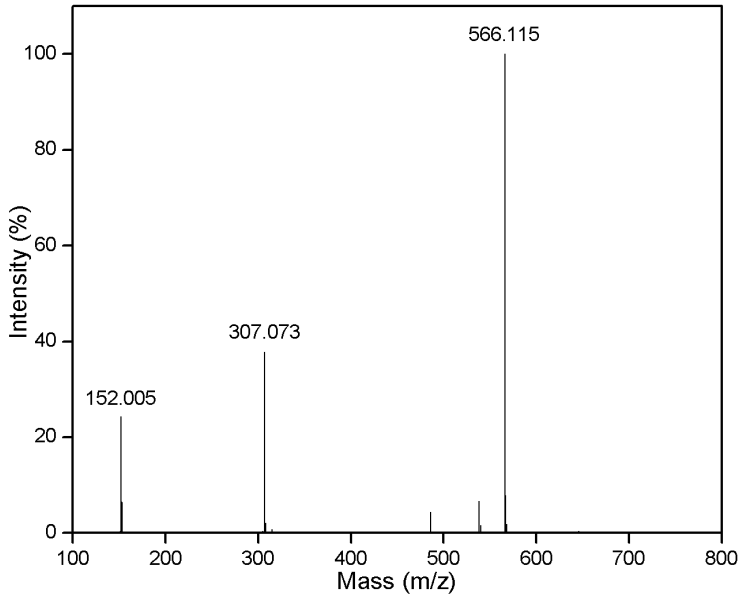


^{31}P NMR (400 MHz, D_2O)





MALDI-TOF MS spectrum of azide-modified uridine triphosphate **36** in negative mode. Calculated for $C_{12}H_{20}N_5O_{15}P_3$ [M] = 567.235, found $[M-H]^-$ = 566.115.

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