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Synthesis and Biochemical Studies of a Novel Thiol Modified Nucleotide

Razieh Esmaeili
raz.esmaeili@gmail.com

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SYNTHESIS AND BIOCHEMICAL STUDIES OF A NOVEL THIOL MODIFIED NUCLEOTIDE

by

RAZIEH ESMAEILI

Under the Direction of Zhen Huang, PhD

ABSTRACT

Nucleic acids are important bio-macromolecules in living systems. They are involved in important functions like gene expression and regulation. Nucleoside triphosphates serve as precursors for biochemical synthesis of modified nucleic acids and nucleotide coenzymes. The modification of nucleic acids, particularly at nucleobases, can expand the function and chemical properties of nucleic acid. Herein, we report the chemical synthesis of a novel thiol-modified nucleoside $S$-(3-(acetyllthio)propyl)-5-(mercaptomethyl)-uridine and the corresponding nucleotide via a “new synthetic methodology” developed in our laboratory. The synthesized triphosphate was used for RNA transcription. The activity and nuclease resistance of the transcribed RNA is studied. The results showed that the properties of the nucleotide with thiol functionality are as good as the native. The modified RNA can be used for RNA/protein
complex structure studies and gold nanoparticles stabilizer. They can also serve as a probe in DNA/RNA microchip surface functionalization for detection of various diseases and pathogens.

INDEX WORDS: Modified nucleic acids, Nucleoside, Thiol-modification of nucleic acids, Transcription, Hammerhead ribozyme, Thiol-uridine, Nucleoside 5’-triphosphate
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RAZIEH ESMAEILI

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in the College of Arts and Sciences Georgia State University 2014
SYNTHESIS AND BIOCHEMICAL STUDIES OF A NOVEL THIOL MODIFIED NUCLEOTIDE

by

RAZIEH ESMAEILI

Committee Chair: Zhen Huang
Committee: Stuart A. Allison
Kathryn Grant

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
December 2014
DEDICATION

I dedicate this work to my dear husband, Ali Tabei, whose never ending support and love has been the strongest inspiration and encouragement to me during this journey.
ACKNOWLEDGEMENTS

All the work presented in this thesis was performed under the direction of my advisor Dr. Zhen Huang. I am sincerely thankful to him for offering me the opportunity to work in his research group. None of this work could be achieved without his criticism, encouragement and guidance. I deeply appreciate his continuous support and useful suggestions on my research throughout this period. His dedication to science has been always inspiring to me during my time in his group. My appreciation also goes to my thesis committee members, Dr. Stuart A. Allison and Dr. Kathryn Grant for their useful discussion and raising insightful questions to help me better understand my work and also for helping me edit my writing. I sincerely thank them. I cannot find any word to express my gratitude to my dear friend and mentor Dr. Manindar Kaur. She has always been a great friend, patient mentor inside and outside the lab. I deeply thank all her advice and instructive teachings and trainings that she provided to me. I am thankful to the other former and current lab members for all the helps and friendship they offered to me, Dr. Julianne Catton-Williams, Dr. Jozef Salon, Dr. Abdur Rob, Dr. Sibo Jiang, Dr. Wen Zhang, Dr. Huiyan Sun, Dr. Lilian Kamau, Edwin Ogbonna, Dominique Stephens, James Campbell, Travon Haynes, Chuilun Kong, Ziyuan Fang, Cen Chen and Yifei Wang. I learned a lot from them and sincerely appreciate them. The help and guidance extended by core facility staff and faculty of Department of Chemistry at Georgia State University is deeply appreciated. I extend my thankfulness to my best friends and family members, specially my dear husband for all the support, help and love they gave to me. I also thank the funding agencies, NSF and NIH, for financially supporting of this project.
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1 INTRODUCTION

1.1 Nucleic acids

Nucleic acids, which include deoxyribonucleic acids (DNA) and ribonucleic acid (RNA), are important bio-macromolecules with polymeric structure in living systems. They are essential for all known forms of life. Because of the specific structure of nucleic acids and their crucial role in genetic systems, fully understanding the chemistry of DNA and RNA constitutes a central element in our knowledge of any kind of life existing in the universe. The quest for such an understanding has been an initiation of many research areas in the nucleic acid field. For example, synthesis of chemically modified nucleic acid analogous and comparing them with the natural counterparts has been one of the most interesting strategy to investigate properties of nucleic acids.\textsuperscript{2,3} Nowadays, more and more attention has been put on the synthesis and modification of nucleic acid derivatives.\textsuperscript{4-7} Various modifications in nucleic acid structure have helped scientists to have an in-depth understanding about nucleic acid structures and functions.\textsuperscript{8,9}

Nucleoside triphosphates, which are the building block of nucleic acids, serve as direct precursors for chemical synthesis of modified unnatural nucleic acids and nucleotide coenzymes.\textsuperscript{10,11} Over the past few decades, nucleoside triphosphates (NTPs) and deoxy-nucleoside triphosphates (dNTPs) are finding widespread application in biochemistry and molecular biology as substrates for DNA and RNA polymerases for sequencing, mutagenesis, and the labeling of hybridization probes and also therapeutics.\textsuperscript{12-15} The emergence of RNA/DNA technology and unusual properties of DNA/RNA molecules has drawn the attention of researchers in RNA chemistry, biochemistry and molecular biology.\textsuperscript{16}
1.2 Thiol modified nucleosides and nucleotides

Medicinal problems have been concern for all societies. Nano-science is one of the most progressing areas in today’s research; therefore, it should be beneficial for human health and curing disease. Metal nanoparticles started having therapeutic application in 1970s. Among all metals, gold nanoparticles (AuNPs) have been proved to be superior to classic compounds.\textsuperscript{17}

There are many methods to prepare and stabilize medically useful AuNPs. Various stabilizers can be utilized to keep high quality of AuNPs such as: citrate, various ligands, polymers, dendrimers, surfactants and also biomolecules such as oligonucleotides and DNA and RNA. Thiolates (for instance oligonucleotides modified with a thiolate group) have been proved to be the best stabilizer for AuNPs.\textsuperscript{17-19} This is based on the fact that thiol functionality has a strong tendency to form self-assembled monolayers in gold surface by being chemisorbed. DNA and RNA can immobilize AuNPs if they are modified by thiol functionalities. Thiols can stabilize AuNPs by forming a strong Au—$S$ bond between the soft “Au” acid and the soft base “$S$”.\textsuperscript{20}

Herein, we report the synthesis of a novel thiol modified nucleoside and corresponding nucleotide and biochemical studies of the thiol-RNA/DNA. Our ultimate goal is to use this thiol-functionalized triphosphate and DNA/RNA to stabilize AuNPs. Our hypothesis is that incorporating multiple thiol functionality into the oligonucleotide could increase the stability of the S—Au bond; therefore, more stabilized AuNPs. These stabilized AuNPs will be further used in DNA/RNA microchip surface functionalization for detection of various diseases and pathogens. Moreover, the thiol-oligonucleotide thus formed can also serve as a probe for RNA/protein complex structure and functions studies.
2 EXPERIMENTAL

S-(3’-S-acetylpropyl)-5-(mercaptomethyl)-uridine was synthesized from 5-methyl uridine in seven steps. The purified nucleoside then was used to synthesize S-(3’-S-acetylpropyl)-5-(mercaptomethyl)-uridine-5′-triphosphate. The triphosphate thus formed was used as a substrate for T7 RNA polymerases. The stability of the transcribed thiol-RNA was tested by treating with different nuclease enzyme to investigate its resistance. The activity of the hammerhead ribozyme, product of the transcription, was tested as well. The 2’-deoxy version of this nucleotide was synthesized previously by us using similar procedure and it is reported as a part of dissertation of our former lab member. Herein, I am reporting again some of our result from 2’-deoxy version for the comparison purposes.

2.1 Materials and instrumentation

Most solvents and reagents were purchased from Sigma-Aldrich, Fisher Scientific and Fluka and were used as obtained, unless otherwise specified. Solid reagents were dried under high vacuum. The reactions were performed under argon gas with stirring. All solvents were dried and purged with argon before use. Solvent mixtures are indicated as volume/volume ratios. Analytical thin layer chromatography (TLC, Dynamic Adsorbents, Inc.) was conducted using alumina basic TLC plates with F$_{254}$ indicator (0.250 mm thick), and visualized under UV-light. Column chromatography was performed using Fluka silica gel (mesh size 0.040 – 0.063 mm). Transcription experiment was done using T7 RNA Polymerase and Transcription Kit from Epicenter Company. A DNA template (55 nt, 5’-TGTACGTTTTCGGCCTTTCGGCCTCATCAGGTTGCCATAGTGAGTCGTATTACGC-3’) was designed and synthesized on solid phase for in vitro transcription of the thiol-modified hammerhead ribozymes using the NTPs analogues. T7 RNA polymerase promoter (top strand DNA, 55-nt, 5’-GCGTAATACGACTCACT
ATAGGCAACCTGATGAGGCCGAAAGGCCGAAACGTACA-3’ and an RNA substrate (5’-GGUCAUCUUUCCUAC-CUGUACGUCGUUGCCUAA-3’) for activity study were purchased from Integrated DNA Technologies. RNase R was bought from Epicenter. Phosphodiesterase I from Crotalus Adamanteus venom and Fetal Bovine Serum (FBS) were purchased from Sigma-Aldrich and Life Technologies respectively. The DNA templates and primer used for enzymatic incorporation of the 2’-deoxy nucleotide was synthesized by solid phase synthesis.

NMR spectra were recorded on Bruker-400 MHz. All chemical shifts (δ) are in ppm relative to tetramethylsilane (TMS). High-resolution mass spectra (HR-MS) and MALDI analyses were performed at Georgia State University Mass Spectrometry Facility, Atlanta, Georgia.

2.2 Synthesis of the thiol-functionalized nucleoside

S-(3’-S-acetylpropyl)-5-(mercaptomethyl)-uridine was synthesized from 5-methyl uridine in seven steps (Scheme 2.1). The 2’-deoxy version was also synthesized using similar methodology. Herein, the focus of synthesis report is on the ribo version of the nucleoside.
Scheme 2.1 Synthesis of S-(3'-S-acetylpropyl)-5-(mercaptomethyl)-uridine, (7); The pink color shows the substituent atoms for the 2'-deoxy version of the nucleosides.

2.2.1 2', 5'-Bis-O-(tert-butyldimethylsilyl)-5-methyluridine, 1

5-methyl uridine (1 g, 0.0039 mol), tert-butyldimethylsilyl chloride (TBDMSi-Cl) (3.5 g, 0.0192 mol, 6 eq) and imidazole (1.6 g, 0.0192 mol, 6 eq) were dissolved in anhydrous dimethylformamide (DMF) (10 mL). The reaction mixture was stirred for 12 h at room temperature. The reaction progress was monitored by recording TLC (5% ethyl acetate in dichloromethane; Rf = 0.4). When starting material was consumed completely, the reaction mixture was poured into ethyl acetate (150 mL) and extracted with saturated sodium bicarbonate.
solution (8 X 50 mL). The organic phase was dried over anhydrous magnesium sulfate (MgSO₄). The solvent was evaporated under reduced pressure. The pale yellow oily syrup was purified via silica gel flash column chromatography (2% methanol in dichloromethane) to give compound 1 (3.2 g, 100% yield) as a colorless sticky liquid. HR-MS (ESI): chemical formula: C₂₂H₄₂N₂O₁₀Si₂, Observed mass [M-H]: 485.2499 (Calculated mass = 485.2509): Figure 3.1 Error! Reference source not found.; ¹H NMR (CDCl₃): Figure 3.2; ¹³C NMR (CDCl₃): Figure 3.3

2.2.2 2', 5'-Bis-O-(tert-butyldimethylsilyl)-5-bromomethyl-uridine, 2

2', 5'-Bis-O-(tert-butyldimethylsilyl)-uridine, 1 (800 mg, 1.64 mmol), N-bromo-succinimide (730 mg, 4.1 mmol, 2.5 eq.), and benzoyl peroxide (32 mg, 0.1312 mmol, 0.08 eq.) were dissolved in CCl₄ (25 mL). The reaction was heated to reflux for 1 h, during this time the solution changed from colorless to orange. Completion of the reaction was monitor by TLC, using 8% ethyl acetate in dichloromethane as eluent (Rₐ = 0.6). The reaction was allowed to cool and filtered to remove insoluble succinimide. The solvent was evaporated in vacuo to yield a crude yellow 2', 5'-bis-O-(tert-butyldimethylsilyl)-5-bromomethyl-uridine, 2. The crude compound 2 was used as such, without further purification for the next step.

2.2.3 S-acetyl-2', 5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptomethyl)-uridine, 3

Crude compound 2 and potassium thioacetate (AcSK) (470 mg, 4.1 mmol, 2.5 eq. of compound 1) were placed in a 50 mL flask and dried under vacuum for about 30 minutes. Anhydrous DMF (3 mL) was added to the reaction flask, under argon atmosphere. The reaction was heated at 70 °C for 1.5 h. The reaction mixture was diluted with ethyl acetate (90 mL) and washed with saturates NaHCO₃ aqueous solution (5 X 30 mL). The organic phase was dried using anhydrous MgSO₄. The solvent was evaporated in vacuo to yield dark brown oily syrup of crude compound 3. The crude product 3 was purified using gradient column chromatography (0,
1, 2, 3% EtOAc in CH$_2$Cl$_2$) to yield a brown product. $R_f = 0.4$ in 8% ethylacetate in CH$_2$Cl$_2$; HR-MS (Figure 3.4): Chemical formula: C$_{24}$H$_{44}$N$_2$O$_7$SSi$_2$. Observed mass [M-H]: 559.2339 (Calculated mass = 559.2330), $^1$H NMR (CDCl$_3$) (Figure 3.5) and $^{13}$C NMR (CDCl$_3$) (Figure 3.6).

2.2.4 2', 5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptomethyl)-uridine, 4

Compound 3 was dissolved in MeOH (3 mL) and hydrolyzed using 1 M NaOH/MeOH solution (1 mL). Based on TLC (using 8% ethyl acetate in CH$_2$Cl$_2$, $R_f = 0.2$) the reaction completed in 4 h at room temperature. The reaction mixture was neutralized with AcOH and solvent was evaporated under reduced pressure to yield dark brown solid. The crude compound 4 was used as such for the next step.

2.2.5 S-(3-bromopropyl)-2',5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptomethyl)-uridine, 5

Crude compound 4, K$_2$CO$_3$ (2 eq.) and DTT (1 eq.) were placed in a 50 mL flask and dried under vacuum for about 15 min. Anhydrous DMF (5 mL) was added to the reaction flask, under argon atmosphere. The reaction was stirred at room temperature for 1 h. Reaction progress was monitored by TLC (5 % EtOAc in CH$_2$Cl$_2$, $R_f = 0.2$). 1,3-Dibromopropane (10 eq.) was added drop wise to the reaction mixture, under argon atmosphere, with vigorous stirring. The reaction was stirred at room temperature for 12 h. The reaction mixture was diluted with ethyl acetate (60 mL) and washed with saturated aqueous NaHCO$_3$ solution (20 mL, five times). The organic phase was dried using anhydrous MgSO$_4$. The solvent was evaporated in a reduced pressure rotary evaporator to yield brown oily compound 5. The crude product 5 was purified using gradient column chromatography (0, 1, 2, 3% EtOAc in CH$_2$Cl$_2$) to yield a golden-brown oily product. $R_f = 0.45$ in 5% EtOAc in CH$_2$Cl$_2$; HR-MS (Figure 3.7): Chemical formula:
C_{25}H_{47}BrN_{2}O_{6}SSi_{2}, Observed mass [M-H]: 637.1819 (Calculated mass = 637.1877), \(^1\)H NMR (CDCl\(_3\)) (Figure 3.8) and \(^{13}\)C NMR (CDCl\(_3\)) (Figure 3.9).

2.2.6  \(S\)-(3(acetylthio)propyl)-3',5'-bis-O-(tert-butylidemethylsilyl)-5-mercaptomethyl-uridine, 6

Compound 5 and AcSK (2.5 eq.) were placed in a 50 mL flask and dried under vacuum for about 15 min. Anhydrous DMF (5 mL) was added to the reaction flask, under argon atmosphere. The reaction was heated at 70 °C for 1.5 h. The reaction mixture was diluted with ethyl acetate (60 mL) and washed with saturated NaHCO\(_3\) solution (5 X 20 mL). The organic phase was dried using MgSO\(_4\). The solvent was evaporated in vacuo to yield brown oily compound 6. The crude product 6 was purified using gradient column chromatography (0, 1, 2, 3 and 4% EtOAc in CH\(_2\)Cl\(_2\)) to yield a golden brown oily product. \(R_f = 0.5\) in 8% ethylacetate in CH\(_2\)Cl\(_2\); HR-MS (Figure 3.10): Chemical formula: C\(_{27}\)H\(_{50}\)N\(_{2}\)O\(_7\)S\(_2\)Si\(_2\) Observed mass [M-H]: 633.2547 (Calculated mass = 633.2525); \(^1\)H NMR (CDCl\(_3\)) (Figure 3.11) and \(^{13}\)C NMR (CDCl\(_3\)) (Figure 3.12).

2.2.7  \(S\)-(3(acetylthio)propyl)-5-(mercaptomethyl)-uridine, 7

Compound 6 (30 mg, 0.047 mmol) was dissolved in dry tetrahydrofuran (1 mL). Triethylamine-trihydrofluoride (20 \(\mu\)L, 0.47 mmol, 10 eq.) was added to the reaction flask, under argon atmosphere, while stirring. The reaction was heated at 45 °C for 12 h. Reaction progress was monitored by analytical TLC (eluent: 10% MeOH in CH\(_2\)Cl\(_2\)). The reaction mixture was neutralized by triethylamine. The solvent was evaporated under reduced pressure and the crude product 7 was purified using preparative TLC plate (10% MeOH in CH\(_2\)Cl\(_2\), \(R_f = 0.5\)) to yield 7 (2 mg, 10%) as a pale orange solid product. HR-MS (Figure 3.13): Chemical formula:
C_{15}H_{22}N_{2}O_{7}S_{2}, Observed mass [M-H]: 405.0787  (Calculated mass = 405.0868); $^1$H NMR (MeOD) (Figure 3.14) and $^{13}$C NMR (MeOD) (Figure 3.15).

2.3  Synthesis of the thiol-functionalized nucleoside-5’ triphosphate

2.3.1  S-(3(acetyltio)propyl)-5-(mercaptomethyl)-uridin-5’ triphosphate, 9

S-(3(acetyltio)propyl)-5-(mercaptomethyl)-uridine ([compound 7, Scheme 2.1],10 mg, 0.015 mmol) was weighed in a 10 mL round bottom flask and dried overnight under vacuum. 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (5 mg, 1.5 eq.) was dried in another flask for 5 minutes. Tributylammonium pyrophosphate (25 mg, 3 eq.) was dried overnight in a separate flask, under high vacuum. The 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one was dissolved in dry DMF (0.1 mL) . The pyrophosphate was also dissolved in dry DMF (0.3 mL) with additional tributylamine (TBA) (0.1 mL) and transferred by a syringe into the flask containing the 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one under argon atmosphere. A lot of argon was purged through the reaction flask as the mixture was allowed to react at room temperature. The mixture was allowed to stir at room temperature for 45 minutes to yield an inorganic triphosphate. During that time, the vacuum dried compound 7 was dissolve in dry DMF (0.3 mL) and an additional of dry toluene (0.1 mL). The prepared triphosphate solution was added to the flask containing compound 7. The combined contents were allowed to stir at room temperature for 2.5 h to yield the cyclic phosphite intermediate 8 (Scheme 2.2). Reaction was monitored at this step by TLC (10% MeOH in CH$_2$Cl$_2$; $R_f = 0.35$). The cyclic-phosphite 8 was then oxidized by adding iodine solution (0.1% I$_2$/THF/Pyridine/H$_2$O, 0.4 mL). The mixture was stirred for 40 min and then hydrolyzed with water (3 mL) and allowed to stir at room temperature for 12 h to ensure complete hydrolysis. The sample was precipitated using NaCl (0.3 M)/ ethanol (3 volumes). The precipitate was analyzed using analytical TLC (5:3:2 of iso-PrOH:30% NH$_3$.H$_2$O:H$_2$O; $R_f = 0.3$).
The crude S-(3(acetyltio)propyl)-5-(mercaptomethyl)-uridine-5'-triphosphate was desalted by a syringe filter to be prepared for HPLC purification. The sample was then purified and analyzed by reversed-phase high performance liquid chromatography (RP-HPLC). 5% yield; HR-MS (Figure 3.16): Chemical formula: \( \text{C}_{15}\text{H}_{25}\text{N}_{2}\text{O}_{16}\text{P}_{3}\text{S}_{2} \), Observed mass \([\text{M-H}]^–\): 644.9756 (Calculated mass = 644.9786); \(^1\text{H} \text{NMR} (\text{D}_2\text{O}) \) (Figure 3.17); \(^{13}\text{C} \text{NMR} (\text{D}_2\text{O}) \) (Figure 3.18); \(^{31}\text{P} \text{NMR} (\text{D}_2\text{O}) \) (Figure 3.19).

2.3.2 **S-mercaptopropyl-5-(mercaptomethyl)-uridin-5’ triphosphate, 10**

The pure triphosphate 9 was treated with ammonia in 5% ammonium hydroxide in methanol/water 1:1 (v/v) solution at room temperature and overnight to remove acetyl group from the thiol functionality in the side chain. Then ammonia was evaporated in vacuu-fuge. The pH of the sample was checked to make sure it is neutral. Then, it was precipitated in NaCl (0.3 M)/ ethanol (3 volumes). HR-MS (Figure 3.20): Chemical formula: \( \text{C}_{15}\text{H}_{25}\text{N}_{2}\text{O}_{16}\text{P}_{3}\text{S}_{2} \), Observed mass \([\text{M-H}]^–\): 644.9756 (Calculated mass = 644.9786); \(^1\text{H} \text{NMR} (\text{D}_2\text{O}) \) (Figure 3.21); \(^{13}\text{C} \text{NMR} (\text{D}_2\text{O}) \) (Figure 3.22); \(^{31}\text{P} \text{NMR} (\text{D}_2\text{O}) \) (Figure 3.23).
Scheme 2.2 Synthesis of S-mercaptopropyl-5-(mercaptopethyl)-uridine-5'-triphosphate (The pink color indicates the 2'-deoxy version)

2.4 RP-HPLC Analysis and Purification

The crude compound 9 (AcSUTP) was purified and analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC). The purification was performed on a Welchrom C18-XB (10 μm, 21.1 × 250 mm) preparative column, using Shimadzu SPD-10A VP liquid chromatography by monitoring under 260 nm, with a flow-rate of 6 mL/min [buffer A: 10 mM triethylammoniumacetate (TEA.Ac, pH 7.1) in water; buffer B: 10 mM TEA.Ac (pH 7.1) in 50% acetonitrile in water] and a linear gradient starting from 5% buffer B to 40% buffer B in 10 min, reaching 100% buffer B in 20 min and in 30 min going back to 5% buffer B. The desired peak was collected and the buffer was removed by lyophilization. All the dry fractions of compound 9 were analyzed on Welchrom C18-XB column (4.6 × 250 mm, 5 μ) and measured at
a flow rate of 1.0 mL/min and a linear gradient of 0 to 25% buffer B in 20 min, reaching to 100% buffer B in 22 min, staying at 100% buffer B for 5 minutes, then going back to 0% buffer B till the end of program, which was totally 36 min [buffer A: 20 mM TEA.Ac (pH 7.3) in water; buffer B: 20 mM TEA.Ac (pH 7.3) in 50% acetonitrile]. A RP-HPLC profile of crude compound 9 is shown in Figure 3.24. And RP-HPLC analysis of purified compound 9, at 260 nm wavelength, is presented in Figure 3.25. The white precipitates of compound 10 (HSUTP) were also analyzed by analytical RP-HPLC at 260 nm with a flow rate of 1.0 mL/min and a linear gradient of 0 to 25 % buffer B in 20 min (Figure 3.25).

2.5 UV-VIS property study

UV-VIS spectra were obtained by a reference to deionized water (Figures 3.27 and 3.28). The base line was subtracted from the recorded spectra all the time.

2.6 Synthesis of oligonucleotides

The DNA primer (P) and template (T) sequences (Table 3.1) were synthesized by solid phase synthesis on an automated Applied BioSystem 394 DNA Synthesizer (Applied Biosystems, Foster City, CA) using standard β-cyanoethylphosphoramidite chemistry (1 μmol scale). Oligonucleotides were synthesized in DMTr-off mode, with average coupling efficiency more than 99%. The oligonucleotides were de-protected in concentrated NH₄OH (55 °C, 16 h), and purified by 19% denaturing polyacrylamide gel electrophoresis. The gel was visualized under UV light. The desired band was cut off and transferred into a tube, crushed and soaked in water overnight. Water was removed by lyophilization and the residue of the gel was soaked again in order to maximize the extraction of oligonucleotide from the gel. The oligonucleotides were dissolved in less amount of water and then recovered from water solution by NaCl (0.3 M)/
ethanol (3 volumes) precipitation. Oligonucleotides were quantified by UV-Vis absorbance at 260 nm using standard molar extinction coefficients and characterized by MALDI-TOF MS.¹

2.7 Enzymatic studies

2.7.1 RNA transcription

Transcription experiment was performed by following the standard procedures from the manufacturer, Epicentre (AmpliScribe T7- Flash Transcription Kit).²¹ The materials for the transcription experiments were labeled using radioactive ATP Alpha³²P (Perkin Elmer, Boston, MA). Each transcription reaction (5 μL) contained ATP, CTP, GTP, and UTP or HSUTP or AcSUTP (0.5 mM each), linearized plasmid DNA template (1 μM) (T55: 5′-TGT ACG TTT CGG CCT TTC GGC CTC ATC AGG TTG CCT ATA GTG AGT CGT ATT ACG C-3′), the complementary 55-nt promoter, DTT (10 mM), transcription buffer (1×) for T7 RNA polymerase, T7 RNA polymerase (1 unit/μl), and RNase-free water. In order to minimize pipetting error, all the materials except the T7 RNA polymerase enzyme and uridine triophosphates (UTP, HSUTP and AcSUTP) were mixed together in a cocktail solution, then divided into three portions to be used for the three different substrates. The T7 RNA polymerase enzyme was added the last to each reaction vial. In the time-course experiments, a gel-loading dye (10 μl) saturated with urea and containing 100 mM EDTA was used to quench the reaction at each time point, then the reaction mixture was stored in dry ice until the end of experiment. It was followed by loading 5 μl of the reaction mixture containing dye on a 19% denaturing polyacrylamide gel electrophoresis and then autoradiography (Figure 3.29). The transcription experiment was further performed using three more different templates (Figure 3.30):

T G4 : 5′-TGT GCG TTT CGC CCT TTC GGC CTC ATC AGG TTG CCT ATA GTG AGT CGT ATT ACG C-3′
2.7.2 Ribozyme activity study

Transcription of the hammerhead ribozyme (5'-GGCAACCUGAUAGGGCCGAAAGG CCGAAACGUACA-3') for the catalytic activity experiments was carried out using the standard procedures described previously (150 μL reaction scale). For this transcription experiment, the 55-nt double stranded DNA (5'-TGTACGTTTCGGCCCTTTCCGCGCTCAGGTTGCCTAT AGTGAGTCGTATTACGC-3') and its complementary sequence was used. After the transcription, the native and thiol-modified ribozymes were precipitated in NaCl (0.3 M)/ ethanol (3 volumes) and adjusted to the same concentration, monitored by UV-VIS and using standard extinction coefficient for oligonucleotides. The RNA substrate (20 nt, 5'-ACCUGUACGUCC UUGCCUAA-3'; Integrated DNA Technology (IDT), Coralville, IA) was kinased with γ-32P-ATP at the 5’ end for the ribozyme digestion by one hour incubating the RNA with T4 polynucleotide kinase (Thermo Fisher Scientific, Waltham, MA) and 1x reaction buffer for T4 PNK at 37 °C, followed by NaCl (0.3 M)/ ethanol (3 volumes) precipitation. The digestion was completed in the buffer (10 mM Tris-HCl, 10 mM MgCl2, pH 7.6) and with 5'-32P-labeled RNA substrate (final concentration: 50 μM) at room temperature. Aliquots (5 μL each) were taken at the time intervals (0, 5, 10, 30, 60, and 120 min), and each was diluted with 10 μL gel loading dye containing EDTA (50 mM) and saturated urea solution (aqueous), followed by storing in dry ice to quench the digestion. The 5'-radio labeled RNA substrate was cleaved into the 9-nt fragment and the 5'-32P-RNA fragment (11-nt). Digestion of the 32P-labled RNA substrate was
monitored via denaturing 19% polyacrylamide gel electrophoresis and autoradiography. The
time-course results of the ribozyme digestion are shown in Figure 3.31.

2.7.3 *Ribozyme durability study*

The procedure of durability study was the same as that of ribozyme activity study
mentioned above. However, the concentration of the transcribed ribozyme was diluted to 1:10 of
that in activity study. Moreover, the time intervals were as: 0, 2, 4, 6, 24 and 72 h. The 19%
denaturing polyacrylamide gel electrophoresis of this study was then visualized by
autoradiography (Figure 3.32).

2.7.4 *Nuclease resistance experiment*

The ribozymes were transcribed as previously discussed. The transcribed ribozymes were
purified by centrifugation using a membrane (3000 Dalton cutoff). The concentrated samples
were adjusted to the same molar concentration, analyzed by UV-VIS. These modified and native
ribozymes were then digested with fetal bovine serum (FBS), RNase R and phosphodiesterase I
from Crotalus Adamanteus venom. The digestion time was set for 30 min, 1 and 2 h at 37°C. In
order to find the best resistance of the synthesized ribozymes, different concentration of the
nuclease enzymes were used. The reaction was quenched by adding 7 μL of gel loading dye
containing EDTA (100mM), saturated with urea. The digestion reactions were analyzed by 19%
PAGE and autoradiography (Figures 3.33, 3.34, 3.35 and 3.36).

2.7.5 *DNA polymerization of the 2’-deoxy version nucleotide1*

The 5’-end of the DNA primers were radio-labeled using γ-[32P]-ATP (Perkin Elmer,
Boston, MA) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and incubating
at 37 °C for one hour. The kinase reaction was quenched by heating up the reaction mixture to 70 °C for five minutes, followed by NaCl (0.3 M)/ ethanol (3 volumes) precipitation.

DNA primer(s) (P1 or P2, Table 3.1) and DNA template(s) (T1, T2 or T3, Table 3.1), dNTPs (dATP, dCTP and dGTP, Epicentre Biotechnologies, Madison, WI), dNTP substrate (TTP/ AcSTTP/ HSTTP) and DTT were premixed together in Klenow exo-minus DNA polymerase 10x reaction buffer. Klenow exo-minus DNA polymerase (Epicentre Biotechnologies, Madison, WI) was added to the prepared cocktail solution and incubated at 37 °C for the desired time. The reaction mixtures were quenched by adding the gel loading dye solution (10 μL each, containing 50 % glycerol, 0.60 % xylene cyanol, 0.60 % bromophenol blue, 20 mL of 1 mM EDTA, saturated urea) and analyzed on 19%, polyacrylamide gel electrophoresis (PAGE). For single nucleotide enzymatic incorporation reactions, no other dNTPs, except the T substrate (TTP/ AcSTTP/ HSTTP), were added to the reaction mixture. The reaction mixtures were incubated at 37 °C for desired time. Analysis was done on a 19%, polyacrylamide gel electrophoresis. Polymerase reactions contained primer (1.5 μM), template (3 μM), dNTPs (0.1 mM each), DTT (5 mM), and 10X reaction buffer (1X) in a final reaction volume of 5 μL.
3 RESULTS AND DISCUSSION

3.1 Synthetic route design and characterization of the synthesized compounds

As shown in Scheme 2.1, both S-(3’-S-acetylpropyl)-5-(mercaptomethyl)-uridine and S-(3’-S-acetylpropyl)-5-(mercaptomethyl)-2'-deoxyuridine were synthesized similarly from 5-methyluridine and thymidine, respectively, in seven steps. Compounds 1, 2 and 3 were synthesized based on literature reported methodology.15 The nucleoside 5’-triphosphates were synthesized using new methodology developed in our lab22 (Scheme 2.2) and then characterized successfully.

The free 2’- and 5’- hydroxy groups on 5-methyluridine, as well as the 3’ and 5’ –OH groups on thymidine were protected with tert-butyldimethylsilyl protecting group. The reaction was quite clean and efficient yielding nearly 100% of pure 2’, 5’-bis-O-(tert-butyldimethylsilyl)-5-methyluridine and 3’, 5’-bis-O-(tert-butyldimethylsilyl)-thymidine. Formation of compound 1 was confirmed by high resolution mass spectrometry (Figure 3.1), and the purity of the compound was analyzed by $^1$H (Figure 3.2) and $^{13}$C NMR (Figure 3.3).

Figure 3.1 ESI-MS analysis of 2’, 5’- Bis-O-(tert-butyldimethylsilyl)-uridine, 1, Chemical formula: C$_{22}$H$_{42}$N$_2$O$_6$Si$_2$, Observed mass [M-H]: 485.2499 (Calculated mass = 485.2509)
Figure 3.2 $^1$H NMR of 2', 5'-bis-O-(tert-butyldimethylsilyl)-5-methyluridine, 1.

Figure 3.3 $^{13}$C NMR of 2', 5'-bis-O-(tert-butyldimethylsilyl)-5-methyluridine, 1.
3', 5'-bis-O-(tert-butylimethylsilyl)-5- bromomethyl-uridine (2) was yielded by using N-bromosuccinimide to afford radical bromination at benzylic position under reflux conditions, in the presence of catalytic amounts of benzoyl peroxide. Using filtration, residual insoluble succinimide was then removed. Compound 2 was used instantly without any purification. Since this compound was difficult to purify, any characterization of it was difficult.

Nucleophilic substitution of bromide in compound 2 was accomplished by using thioacetate to obtain S-acetyl-2', 5'-bis-O-(tert-butylimethylsilyl)-5-(mercaptomethyl)-uridine (compound 3). The molecular formation of compound 3 was confirmed by ESI-MS (high resolution) analysis (Figure 3.4). The purity of compound 3 was confirmed by NMR analysis (Figures 3.5 and 3.6).

![Graph](image)

Figure 3.4 ESI-MS analysis of S-acetyl-2',5'-bis-O-(tert-butylimethylsilyl)-5-(mercaptomethyl)-uridine, 3, Chemical formula: C_{24}H_{44}N_{2}O_{7}SSi_{2}. Observed mass [M-H]$: 559.2339
Figure 3.6 $^1$H NMR of S-acetyl-2', 5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptomethyl)-uridine, 3.

Figure 3.5 $^{13}$C NMR of S-acetyl-3', 5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptomethyl)-2'-deoxyuridine, 3.
Compound 4 was afforded by alkaline hydrolysis of compound 3 using sodium hydroxide in methanol. Due to strong tendency of un-protected S-H group on compound 4 to form a dimer by making disulfide bond, any characterization or purification of it was difficult. Therefore, the sample was used as such for the next step.

The disulfide bind thus formed resulting from dimerization of compound 4 was reduced in situ with dithiothreitol (DTT). The reaction was done in an argon rich, oxygen free atmosphere. The free thiol anion [-S]⁻ thus generated, was alkylated using 1,3-dibromopropane to afford compound 5. Compound 5 is a stable intermediate and was purified by column chromatography and characterized by NMR (Figures 3.8 and 3.9). The molecular formation of compound 5 was confirmed by ESI-MS (high resolution) analysis (Figure 3.7).

![Figure 3.7 ESI-MS analysis of S-(3’-bromopropyl)-2’, 5’-bis-O-(tert-butyldimethylsilyl)-5-(mercaptomethyl)-uridine, 5. Chemical formula: C₂₅H₄₇BrN₂O₆SSi₂, Observed mass [M-H]: 637.1819 (Calculated mass = 637.1877).](image-url)
Figure 3.8 $^1$H NMR of S-(3'-bromopropyl)-2', 5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptomethyl)-uridine, 5

Figure 3.9 $^{13}$C NMR of S-(3'-bromopropyl)-2', 5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptomethyl)-uridine, 5
Nucleophilic substitution of bromide in compound 5 by thioacetate yielded compound 6.

The molecular formation of compound 6 was confirmed by ESI-MS (high resolution) analysis (Figure 3.10). The purity of compound 6 was analyzed by NMR (Figures 3.11 and 3.12).

Figure 3.10 ESI-MS analysis of S-(3(acetylthio)propyl)-2', 5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptomethyl)-uridine, 6, Chemical formula : C_{27}H_{50}N_{2}O_{7}S_{2}Si_{2}, Observed mass [M-H] : 633.2547 (Calculated mass = 633.2525).

Figure 3.11 $^1$H NMR of S-(3(acetylthio)propyl)-2', 5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptomethyl)-uridine, 6
De-protection of compound 6 using TEA.3HF and HF was initially challenging, and we had difficulty removing TBDMS group from 2’ and 5’ position of the ribo-nucleoside. To overcome this problem, we tried another protecting group (triisopropylsilyl (TIPS) ethers) to protect simultaneously 3’ and 5’ of 5-methyluridine at the first step. However the reaction didn’t work after step two. Therefore, we decided to use TBDMS again, but remove it from compound 5 first and then introduce thioacetate to it to obtain compound 7. This strategy was also challenging: having two triol compound to purify by column was time consuming and not very efficient. Other than using TEA.3HF and HF to remove TBDMS, we used another fluoride reagent and solvent system for de-protection. Pyridine.HF complex (70%) and EtOH was employed for 3’, 5’-bis-O-desilylation reaction. The reaction was successful. However, the yield

![Figure 3.12 13C NMR of S-(3(acetylthio)propyl)-2’, 5’-bis-O-(tert-butyldimethylsilyl)-5-(mercaptomethyl)-uridine, 6](image-url)
was not great. At the end, we were able to remove protecting groups using triethylamine trihydrofluoride (TEA.3HF) in very dry THF and obtain a higher yield. The molecular formation of compound 7 was confirmed by ESI-MS (high resolution) analysis (Figure 3.13) and then characterized by NMR (Figures 3.14 and 3.15).

Chemical formula: C\textsubscript{15}H\textsubscript{22}N\textsubscript{2}O\textsubscript{7}S\textsubscript{2}, Observed mass [M-H]: 405.0787 (Calculated mass = 405.0796).

Figure 3.13 ESI-MS analysis of S-(3(acetyltio)propyl)-5-(mercaptomethyl)-uridine, 7

Figure 3.14 \textsuperscript{1}H NMR of S-(3(acetyltio)propyl)-5-(mercaptomethyl)-uridine, 7
S-(3(acetylthio)propyl)-5-(mercaptomethyl)-uridine-5'-triphosphate (AcSUTP, compound 9, Scheme 2.2) was synthesized from S-(3(acetylthio)propyl)-5-(mercaptomethyl)-uridine (compound 7 in Scheme 2.1) using a relatively new methodology developed in our lab. Our earlier attempts to synthesize the 5'-triphosphate of the deoxy-nucleoside version by using DMF or DMF/1,4-dioxane (1:1) mixture as the solvent systems was not successful. Only after dissolving the nucleoside in DMF/toluene (3:1) mixture, formation of triphosphate was observed. Our hypothesis is that toluene can interact with the bulky base, providing better stacking and therefore, causing the 5' hydroxyl group more available to afford cyclic phosphate intermediate (compound 8, Scheme 2.2). This strategy was used for the ribo version as well. In order to obtain better yield and cleaner reaction, several optimization such as changing reaction time and the
equivalent of the reagents were tried. The best yield was observed when reaction time between nucleoside and triphosphate was 2 h (second step in Scheme 2.2) and the equivalent of tributylammonium pyrophosphate and 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-on to the nucleoside was 3 and 1.5 respectively. Compound 9 was characterized using HR-MS and $^1$H, $^{13}$C and $^{31}$P NMR analysis as shown in Figures 3.16 to 3.19.

Figure 3.17 ESI-MS analysis of S-(3(acetylthio)propyl)-5-(mercaptomethyl)-uridine-5’-triphosphate, 9, Chemical formula: C$_{15}$H$_{25}$N$_{2}$O$_{16}$P$_{3}$S$_{2}$, Observed mass [M-H]: 644.9756 (Calculated mass = 644.9786).

Figure 3.16 $^1$H NMR analysis of S-(3(acetylthio)propyl)-5-(mercaptomethyl)-uridine-5’-triphosphate, 9.
Figure 3.18 $^{13}$C NMR analysis of S-(3(acetylthio)propyl)-5-(mercaptomethyl)-uridine-5’-triphosphate, 9.

Figure 3.19 $^{31}$P NMR analysis of S-(3(acetylthio)propyl)-5-(mercaptomethyl)-uridine-5’-triphosphate, 9.
The acetate protecting group of the thiol functionality of compound 9 was removed in presence of ammonia, yielding S-mercaptopropyl-5-(mercaptomethyl)-uridine-5’-triphosphate (HSUTP, Compound 10, Scheme 2.2). Figures 3.20 to 3.23 represent HR-MS and NMR analysis of compound 10.

Figure 3.20 ESI-MS analysis of S-mercaptopropyl-5-(mercaptomethyl)-uridine-5’-triphosphate 10, Chemical formula: C_{13}H_{22}N_{2}O_{15}P_{3}S_{2}, Observed mass [M-H]: 602.9653 (Calculated mass = 602.9680).

Figure 3.21 $^1$H NMR of S-mercaptopropyl-5-(mercaptomethyl)-uridine-5’-triphosphate 10
Figure 3.22 $^{13}$C NMR of S-mercaptopropyl-5-(mercaptomethyl)-uridine-5’-triphosphate, 10

Figure 3.23 $^{31}$P NMR of S-mercaptopropyl-5-(mercaptomethyl)-uridine-5’-triphosphate, 10
3.2 RP-HPLC purification and analysis

The crude compound 9 (AcSUTP) was purified and analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC). Several HPLC programs were tried to get the best resolution. Only the program shown in Figure 3.24 afforded the best separation. Compound 9 (AcSUTP) eluted at 20.20 min under this condition. Compound 10 was purified by only NaCl/EtOH precipitation and then analyzed on RP-HPLC at flow rate of 1.0 mL/min under 260 nm. The HPLC profile of the crude compound 9 is shown in Figure 3.22. The desired peak had retention time of 20.20 min and it was stronger than other peaks, meaning the synthesis has been efficient and clean. All native uridine 5’-triphosphate and the purified thiol modified ones were analyzed on RP-HPLC (Figure 3.25). Since AcSUTP and HSUTP are structurally similar, the difference on their retention time on the column was small but measurable. The retention time difference was much smaller in the deoxy version. RP-HPLC analysis of the deoxy version is showed in Figure 3.26.

![Figure 3.24 RP-HPLC profile of crude compound 9, and the program used, (retention time of the desired peak: 20.20 min)](image-url)
Figure 3.25 RP-HPLC analysis of UTP, AcSUTP and HSUTP
UTP: **orange** curve, retention time: 11.48 min; AcSUTP: **blue** curve, retention time: 24.86 min; HSUTP: **green** curve, retention time: 23.25 min; Co-injection of all: **violet** curve, retention times: 11.48, 23.25 and 24.86

Figure 3.26 RP-HPLC analysis of AcSTTP and HSTTP\(^1\) HSTTP (**red** curve, retention time: 24.75 min); AcSTTP (**blue** curve, retention time: 24.96 min) and co-injection of AcSTTP and HSTTP (black curve, retention times: 24.75 and 24.96 min, respectively).
3.3 UV-VIS absorption properties

The maximum UV absorption of the native uridine 5’-triphosphosphate was measured at ~262 nm. The modified uridine 5’-triphosphate (AcSUTP & HSUTP) showed maximum absorption at ~267 nm, which is slightly different from that of the native UTP. The UV absorption profiles of AcSUTP and AcSUTP were similar (Figure 3.27). Unlike uridine, modification at the nucleobase of thymidine did not change the wavelength of the maximum absorption of the native TTP at all. All TTP, S-(3(acetylthio)propyl)-5-(mercaptomethyl)-2’-deoxyuridine-5’- triphosphate (AcSTTP) and S-mercaptopropyl-5-(mercaptomethyl)-2’-deoxyuridine-5’- triphosphate, (HSTTP) have similar UV absorption profiles (Figure 3.28).

Figure 3.27 UV absorption spectra of UTP (—), AcSUTP (—), and HSUTP (—) in water at room temperature.
3.4 Enzymatic studies

3.4.1 RNA in vitro transcription by T7 RNA polymerase

Transcription of the RNA for the native and the thiol-modified nucleotides were performed using T7 RNA polymerase and by following the manufacturer procedure (Epicentre, AmpliScribe T7-Flash Transcription Kit). A 55-mer DNA template and the complementary promoter for the T7 RNA polymerase were used in presence of all NTPs and radioactive α-[\textsuperscript{32}P]-ATP. The transcribed RNA’s were analyzed on a 19% PAGE. Autoradiogram of the time-course experiment of the transcription is shown in Figure 3.29. From the intensity of the bands, it can be determined that the modified nucleotides are recognizable by T7 RNA polymerase and can act as good substrates for the T7 enzyme. It was observed that transcription of AcSUTP substrate is
initially faster but the yield is lower than that of native. The transcription yield of HSUTP seems even better than the native UTP. Incorporation of the HSUTP & AcSUTP into RNA will be further confirmed by MALDI-MS.

Figure 3.29 Transcription time course experiment. a) Sequences of promoter and template used and the product of the reaction in presence of T7 RNA polymerase. b) Gel autoradiogram of the reaction (analyzed on 19% PAGE). c) Normalized curves, quantified by phosphorimager, intensities were measure by reference to the intensity of UTP in 120 min as 100%.
Transcription was also carried out using three other templates shown in Figure 3.30. These templates were designed in such a way that the ribozyme thus produced will have C instead of U in two positions. Therefore, in each sequence of these templates, one A has been replaced by G. The reaction time was 2 h, and 19% PAGE was used for analysis. The results showed that the modified nucleotides (AcSUTP & HSUTP) gave better yield compared to the native UTP.

a) 

P55 : 3’- ACA TGC AAA GCC GGA AAG CCG GAG TAG TCC AAC GGA TAT CAC TCA GCA TAA TGC C-3’

T55 : 5’ - TGT ACG TTT CGG CCT TTC GGC CTC ATC AGG TTG CCT ATA GTG AGT CGT ATT ACG C-3’

T55_A4G : 5’- TGT GCG TTT CGG CCT TTC GGC CTC ATC AGG TTG CCT ATA GTG AGT CGT ATT ACG C-3’

T55_A28G : 5’- TGT ACG TTT CGG CCT TTC GGC CTC GTC AGG TTG CCT ATA GTG AGT CGT ATT ACG C-3’

T55_A32G : 5’- TGT ACG TTT CGG CCT TTC GGC CTC ATC GGG TTG CCT ATA GTG AGT CGT ATT ACG C-3’

By T55 5’-GGCAACCUGAUAGGCGAAAAGGCGAAAACGUACA-3’

By T55_A4G 5’-GGCAACCUGAUAGGCGAAAAGGCGAAAACGUACA-3’

By T55_A28G 5’-GGCAACCUGAUAGGCGAAAAGGCGAAAACGUACA-3’

By T55_A32G 5’-GGCAACCUGAUAGGCGAAAAGGCGAAAACGUACA-3’

c) 

Figure 3.30 Transcription experiment using mutant templates; a) sequences of promoter and templates used; b) sequences of the ribozymes thus produced; c) gel autoradiogram of the experiment.
### 3.4.2 Ribozyme catalytic activity analysis

The activity of the transcribed hammerhead ribozyme was examined by incubating the 5′-32P labeled 20-nt substrate with the transcribed RNAs in presence of Tris-HCl/MgCl₂ buffer. The gel image (Figure 3.31) shows that the 5′-radio labeled RNA substrate was cleaved into the 9-nt fragment and the 5′-32P-RNA fragment (11-nt), suggesting that the thiol-modified ribozymes are as active as the native counterpart.

![Cleavage site diagram](image)

Figure 3.31 Catalytic activity of the modified ribozyme and the native counterpart.

- **a)** Secondary structure of the transcribed hammerhead ribozyme and its labeled substrate. The red letters indicated that the bases are highly conserved.
- **b)** Normalized curves of the modified ribozymes activity (red and green curves) by comparison to the cleavage of the substrate by the native U-ribozyme (120 min was defined as 1).
- **c)** Time-course experiment of the digestion of the labeled substrate at 5′-end by hammerhead native and modified ribozymes.
3.4.3 Ribozyme durability study

To examine the durability of the transcribed ribozyme, they were 10 fold diluted with respect to the concentrations in the activity study and were incubated with 5'-\textsuperscript{32}P-labeled RNA substrate at room temperature for longer time. The time intervals were 0, 2, 4, 6, 24 and 72 h. The autoradiograph of this experiment (Figure 3.32) indicates that the AcSU-modified ribozymes is as durable as the native one. However, HSU-ribozyme did not show durability like the native one.

![Gel autoradiogram of the ribozyme durability study](image)

Figure 3.32 Gel autoradiogram of the ribozyme durability study

3.4.4 Nuclease resistance experiment

The stability of the transcribed ribozymes was examined by incubating the transcribed RNAs with different nucleaseS including Fetal Bovine Serum (FBS), phosphodiesterase (PDE) I from Crotalus Adamanteus venom and RNase R (various amounts and different reaction times) and analyzed on 19% gel electrophoresis and autoradiography (Figures 3.33, 3.34, 3.35 and 3.36).
Figure 3.33 PAGE autoradiography of nuclease resistance experiment.
a) Digestion of Native U-RNA, b) digestion of HSU-RNA, c) digestion of AcSU-RNA, enzyme concentration used: 0.1 U/μL of RNase R, 0.04% of Fetal Bovine Serum (FBS) and $10^{-5}$ U/μL of phosphodiesterase (PDE), reaction mixtures were incubated at 37 °C for 30 min.

Figure 3.34 PAGE autoradiography of nuclease resistance experiment.
a) Digestion of Native U-RNA, b) digestion of HSU-RNA, c) digestion of AcSU-RNA, enzyme concentration used: 0.05 U/μL of RNase R, 0.02% of Fetal Bovine Serum (FBS) and $10^{-5}$ U/μL of phosphodiesterase (PDE), reaction mixtures were incubated at 37 °C for 30 min.
Figure 3.35 PAGE autoradiography of nuclease resistance experiment. 

a) Digestion of Native U-RNA, b) digestion of HSU-RNA, c) digestion of AcSU-RNA, enzyme concentration used: 0.1 U/μL of RNase R, 0.006% of Fetal Bovine Serum (FBS) and 0.3x10^{-5} U/μL of phosphodiesterase (PDE), reaction mixtures were incubated at 37 °C for 30 min.

Figure 3.36 PAGE autoradiography of nuclease resistance experiment. 

a) Digestion of Native U-RNA, b) digestion of HSU-RNA, c) digestion of AcSU-RNA, enzyme concentration used: 0, 0.1, 0.01, 0.001 U/μL of RNase R. Reaction mixtures were incubated at 37 °C for 1h.

The result shows that the modified RNAs are relatively resistant to the nuclease and their stability is similar to the native.
3.4.5 Incorporation of 2'-deoxy version of nucleotides into DNA

The incorporation efficiency of S-(3(acetylthio)propyl)-5-(mercaptomethyl)-2'-deoxyuridine-5'-triphosphate (AcSTTP, 9) and S-mercapto propyl-5-(mercaptomethyl)-2'-deoxyuridine-5'-triphosphate, (HSTTP, 10) into DNA was investigated by using different combination of DNA templates and primers (listed in Table 3.1) under Klenow exo minus DNA polymerase (Kf-).

Table 3.1 Sequences of the DNA primers and templates used for enzymatic incorporation of TTP/HSTTP/AcSTTP into DNA. The highlighted bases are where incorporation of “T” occurs.

**Primers:**
- P1 (17-mer) 5’-d-TAG CGG GTT GCT GGT GG -3’
- P2 (21-mer) 5’-d-GCG TAA TAC GAC TCA CTA TAG-3’

**Templates:**
- T1 (21-mer) 3’-d-ATC GCC CAA CGA CCA CCA CCA TGG- 5’
- T2 (23-mer) 3’-d-ATC GCC CAA CGA CCA CCA CCA AAAT GG- 5’
- T3 (55-mer) 3’-d-CGC ATT ATG CTG AGT GAT ATC CGT TGG ACT ACT CCG GCT TTC CGG CTT TGC ATG T -5’

The DNA polymerization was carried out with primer (1.5 μM), template (3 μM), all NTPs including TTP/AcSTTP/ HSTTP (0.1 mM each), and Klenow exo(-) DNA polymerase (various concentrations) and analyzed by 19% polyacrylamide gel electrophoresis (Figures 3.37, 3.39 and 3.40).

The results showed that AcSTTP and HSTTP act as good substrates for DNA polymerase, like TTP. A relatively short DNA template (T1, 21-nt) was used to study single nucleotide incorporation. As can be seen in Figure 3.37, AcSTTP is a better substrate for DNA polymerase: in different conditions (various enzyme concentration and reaction time), AcSTTP terminates DNA polymerization once single nucleotide incorporates into DNA (n+1 DNA),
while TTP and HSTTP cause formation of either n+1 DNA or a mixture of n+1 and n+2 DNA.

In addition, the intensity of the products on the gel autoradiogram indicates that DNA polymerase can recognize both HSTTP and AcSTTP well. Moreover, it appears that there is a measurable difference in the mobility of the products as a result of structural impact that modification causes. The single nucleotide incorporation of HSTTP is confirmed by MALDI-MS analysis (Figure 3.38). AcSTTP single nucleotide incorporation will be confirmed by MALDI-MS as well.

Figure 3.37 Single nucleotide enzymatic incorporation of TTP/AcSTTP/HSTTP into DNA (T1): (a) incorporation of TTP/AcSTTP/HSTTP by Klenow exo(-) into DNA template T1. PAGE autoradiography of the polymerization reaction with (b) Kf(-) = 0.0015 U/µL and reaction time = 60 min for TTP and 90 min for AcSTTP and HSTTP, (c) Kf(-) = 0.00075 U/µL and
reaction time = 60 min, (d) Kf(-) = 0.0015 U/µL and reaction time = 30 min, and (d) Kf(-) = 0.00075 U/µL and reaction time = 30 min.

Figure 3.38 MALDI-TOF MS analysis of the single nucleotide incorporation of HSTTP into DNA by Kf(-). Chemical Formula: C_{181}H_{229}N_{68}O_{111}P_{17}S_{2}, matrix: 3-hydroxypicolinic acid (3HPA); Observed mass: 5722.6 (Cal. mass: 5720.9)

In order to examine the incorporation efficiency of three consecutive modified thymidine 5’-triphosphates, template T2 was used. Primer P1 (17-nt) was extended to a 20-nt DNA using Kf(-) and by incorporation of three consecutive “T” bases on template T2. The DNA polymerization reaction was carried out in the presence of (1.5 µM), template (3 µM), TTP/AcSTTP/HSTTP (0.1 mM), and Klenow exo(-) DNA polymerase (0.0015 U/µL) and then was analyzed on a 19% PAGE (Figure 3.39). The incubation time for TTP was 5 min (Figure 3.39, lane 2) while AcSTTP and HSTTP were incubated for 60 min (Figure 3.39, lanes 3 and 4, respectively). In these conditions, AcSTTP seems to be the best substrate for DNA polymerase.
We also designed a longer DNA template (55-nt, T3) to further investigate the incorporation efficiency. The polymerization reaction was performed on extension of primer P2 (21-nt) over template T3 (55-nt). The reaction was carried out using primer (1.5 μM), template (5 μM), all dNTPs including TTP/ AcSTTP/TTP (0.1 mM), and Klenow exo(-) DNA polymerase (0.04 U/μL) followed by analyzing on a 19% PAGE. The results are shown in Figure 3.40. Three “T” were involved in this reaction

Figure 3.39 Enzymatic incorporation of three consecutive thymidine bases (TTP/AcSTTP/HSTTP) into DNA (template T2):
(a) Polymerization reaction of TTP, AcSTTP and HSTTP by Klenow exo(-) over DNA template T2. (b) Gel electrophoresis autoradiography of the incorporation reaction with Kf(-)= 0.0015 U/μL, incubation time = 5 min for TTP (lane 2) and 60 min for HSTTP (lane 3) and AcSTTP (lane 4).
Figure 3.40 a) DNA polymerization reaction in presence of all dNTPs including TTP, AcSTTP and HSTTP using template T3 and Klenow exo(-) b) Gel electrophoresis autoradiography of the experiment with Kf(-) = 0.0015 U/µL incubation time = 60 min for TTP (lane 3) and 90 min HSTTP (lane 4) and AcSTTP (lane 5).
4 CONCLUSIONS AND FUTURE PROSPECTS

We have successfully synthesized and characterized a novel nucleoside, S-(3’-S-acetylpropyl)-5-(mercaptomethyl)-uridine, from 5-methyl uridine. The purified nucleoside was used to synthesize S-(3’-S-acetylpropyl)-5-(mercaptomethyl)-uridine -5’-triphosphate and S-mercaptopropyl-5-(mercaptomethyl)-uridine -5’-triphosphate. Both triphosphates were characterized successfully. The triphosphates thus formed were used as a substrate for T7 RNA polymerase. A 55-nt dsDNA templates and promoter were used to incorporate the modified triphosphate into RNA. The experiments showed that the modified ribozymes transcribed are as active as the native counterpart and this modification at the nucleobase will not cause any deficiency in the catalytic activity of the ribozymes. Like the native RNA, the modified ones are relatively resistant to the nucleases. The 2’-deoxy version of the same nucleoside and nucleotide were also synthesized and characterized. It was observed that the 2’-doxy thiol-modified nucleotide can act as an efficient substrate for DNA polymerase. The modified RNA/DNA thus obtained will be used for gold nanoparticle stabilization. Since Au-nanoparticles have strong affinity for thiol-functionality, the study will be based on that. The modified RNA/DNA will also find use and application in DNA/RNA microchip surface functionalization for rapid detection of disease and pathogens and also, it will be utilized in studying nucleic acid/protein complexes structure and function.
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APPENDICES

Appendix A: ESI HR-Mass spectra
Appendix B: $^1$H NMR spectra
Appendix C: $^{13}$C NMR spectra
APENDIX D: $^{31}$P NMR spectra