Molecular Probe Designs For Nucleic Acid Based Detection

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MOLECULAR PROBE DESIGNS FOR NUCLEIC ACID BASED DETECTION

by

MANINDAR KAUR

Under the direction of Dr. Zhen Huang

ABSTRACT

Nucleosides and nucleotides are powerful building units for creating functional molecules with novel properties. The structural and functional variations of these biomolecules have caused a renaissance of nucleic acid chemistry and biology, and paved the way for fresh avenues in nucleic acid research, including therapeutics, nucleic acid biology, structure-function studies, catalytic and mechanistic analysis, and material science and nanotechnology.

Modified nucleic acids are instrumental in discovering functional oligonucleotides as significant biochemical and therapeutic agents. A variety of synthetic strategies have been developed to design novel analogs with tunable physico-chemical properties, such as enhanced duplex stability, binding affinity, nuclease resistance, bioavailability, and base-pair fidelity. These engineered nucleic acids are useful structural, functional, and mechanistic probes for disease detection, molecular
sensing, and fundamental understanding of the structures and biological functions of nucleic acids (DNA and RNA).

The structural, functional and spectroscopic repertoire of the nucleic acids can be further enhanced by strategic substitution of the oxygen atoms with selenium atoms. Selenium derivatization of nucleic acids generates modified biopolymers with unique structural and functional features that make them strong contenders for biochemical and biophysical research. The substitution of oxygen with selenium in the nucleobases permits a search for novel aspects of nucleic acid base-pairing and stacking interactions at the atomic level. Huang and co-workers have demonstrated that the single-atom modified (SAM) nucleosides and nucleic acids, where a single oxygen atom is strategically replaced with a selenium atom, are yellow colored and have over a 100 nm red-shift in the absorption maximum (1-3). With minimal structural perturbation, SAM nucleosides and nucleotides could be of immense significance in the detection and visualization of nucleic acids. Selenium-derivatized nucleic acids (SeNAs) could also serve as imperative tools in the structural, functional and mechanistic studies of nucleic acids and their complexes with proteins, small molecules, and/or metal ions.

INDEX WORDS: Single-atom modification, Fluorescent nucleoside, Selenium derivatization, Biochemical and molecular probes, Visual detection, Nucleoside 5’-triphosphates, Thiol-modification
MOLECULAR PROBE DESIGNS FOR NUCLEIC ACID BASED DETECTION

by

MANINDAR KAUR

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December 2013
DEDICATION

This work is dedicated to my dad Bhupinder Singh, who has been the ultimate source of strength, my mom Rohin Kaur for all the effort she put in raising me, my husband Virendra Singh, for his never ending support and encouragement and my daughter Tamanna Singh, for understanding that mommy needs to work longer and harder.
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................................................... v
TABLE OF CONTENTS ................................................................................................................................................ vi
LIST OF TABLES ........................................................................................................................................................ x
LIST OF FIGURES ...................................................................................................................................................... xi
LIST OF SCHEMES .................................................................................................................................................... xvi
1 INTRODUCTION ..................................................................................................................................................... 1

1.1 Significance of Nucleic Acids .......................................................................................................................... 1

1.2 Single Atom Modification (SAM) in Nucleic Acids ....................................................................................... 2

1.3 Fluorescence Based Detection of Nucleic Acids ............................................................................................. 7

2 SYNTHESIS AND OPTICAL STUDY OF 6-SELENO-2’-DEOXYGUANOSINE ................................................. 10

2.1 Fluorescent Nucleoside Analogs: Early Examples ......................................................................................... 10

2.2 Fluorescent Nucleoside Analogs: Expanded Nucleobases ........................................................................... 11

2.3 Fluorescent Nucleoside Analogs: Extended Nucleobases ........................................................................... 13

2.4 Fluorescent Hydrocarbons ............................................................................................................................. 14

2.5 Metal Containing Fluorescent Nucleoside Analogs ..................................................................................... 15

2.6 Material and Methods ................................................................................................................................... 16

2.6.1 Synthesis of 6-Seleno-2’-Deoxyguanosine ................................................................................................. 16

2.6.2 Optical Studies of 6-Seleno-2’-Deoxyguanosine ......................................................................................... 19

2.6.3 HPLC Analysis and Purification of 6-Seleno-2’-Deoxyguanosine .............................................................. 19

2.7 Results and Discussion .................................................................................................................................... 20

2.7.1 Synthetic Design ......................................................................................................................................... 20

2.7.2 Absorption Properties of 6-Seleno-2’-Deoxyguanosine ......................................................................... 20

2.7.3 Emission behavior of 6-Seleno-2’-Deoxyguanosine: pH Effect .............................................................. 23
2.7.4 *Emission behavior of 6-Seleno-2’-Deoxyguanosine: Solvent Effect* ..........25

2.8 Conclusions ..................................................................................................................27

3 CHEMICAL SYNTHESIS AND FLUORESCENCE STUDY OF 6-SELENO-2’-DEOXYGUANOSINE DERIVATIZED OLIGODEOXYRIBONUCLEOTIDES ..................................................29

3.1 Synthetic Oligodeoxyribonucleotides .........................................................................29

3.2 Material and Methods ..................................................................................................30

3.2.1 *Solid-Phase Synthesis of 6-Seleno-2’-Deoxyguanosine Derivatized Oligodeoxyribonucleotides* ..........................................................................................................................30

3.2.2 *HPLC Analysis and Purification* .................................................................................32

3.2.3 *Desalting Using Sep-Pak C18 Vac Column* .................................................................32

3.2.4 *Optical Analysis of Se*dG ODNs* ................................................................................33

3.3 Results and Discussion ..................................................................................................33

3.3.1 *Synthetic Design* ......................................................................................................33

3.3.2 *UV-Vis Analysis* .......................................................................................................34

3.3.3 *HPLC Analysis* .........................................................................................................35

3.3.4 *Fluorescence Analysis* ...............................................................................................35

3.4 Conclusions ....................................................................................................................36

4 SYNTHESIS OF 6-SELENO-2’-DEOXYGUANOSINE-5’-TRIPHOSPHATE AS A BIO CHEMICAL PROBE .................................................................................................37

4.1 Synthetic Nucleotides ....................................................................................................37

4.2 Selenium Functionalized Nucleotides ............................................................................37

4.3 Fluorescent Nucleotide Substrates ...............................................................................38

4.4 Material and Methods ....................................................................................................39

4.4.1 *Synthesis of 6-Seleno-2’-Deoxyguanosine-5’-Triphosphate* ........................................39
4.4.2 HPLC Analysis and Purification ................................................................. 41

4.4.3 UV-Vis Absorption and Fluorescence Study .................................................. 41

4.4.4 Synthesis of Oligonucleotides ...................................................................... 42

4.4.5 Enzymatic Incorporation Studies .................................................................... 42

4.4.6 Nuclease Resistance Studies of dG-DNA and Se-dG-DNA ............................. 43

4.5 Results and Discussion ....................................................................................... 43

4.5.1 Synthetic Design .............................................................................................. 43

4.5.2 Absorption and Emission Properties ............................................................... 45

4.5.3 Enzymatic Synthesis of dG-DNA and Se-dG-DNA ........................................ 47

4.5.4 Nuclease Resistance Studies of dG-DNA and Se-dG-DNA ............................. 53

4.6 Conclusions ........................................................................................................ 54

5 SYNTHESIS OF 6-SELENOGUANOSINE-5’-TRIPHOSPHATE ............................... 55

5.1 Selenium Functionalized Ribonucleoside Triphosphates .................................. 55

5.2 Materials and Methods ....................................................................................... 56

5.2.1 Synthesis of N²-Phenoxyacetyl-6-(2-Cyanoethylseleno)-Guanosine .............. 56

5.2.2 Synthesis of 6-Selenoguanosine-5’-Triphosphate .......................................... 57

5.2.3 HPLC Purification of 2-Phenoxyacetyl-6-(2-Cyanoethylseleno)-Guanosine-5’-
Triphosphate ........................................................................................................ 59

5.3 Results and Discussion ....................................................................................... 59

5.3.1 Synthetic Design .............................................................................................. 59

5.3.2 HPLC Purification of 2-Phenoxyacetyl-6-(2-Cyanoethylseleno)-Guanosine-5’-
Triphosphate ........................................................................................................ 62
5.3.3 Proposed Study ........................................................................................................... 62

5.4 Conclusions and Future Prospects .................................................................................. 63

6 SYNTHESIS AND BIOCHEMICAL STUDY OF S-MERCAPTOPROPYL-5-(MERCAPTO-
METHYL)-2'-DEOXYURIDINE-5'-TRIPHOSPHATE .................................................................. 64

6.1 Introduction .................................................................................................................... 64

6.2 Materials and Methods .................................................................................................. 65

6.2.1 Synthesis of S-(3-(Acetylthio)propyl)-5-(Mercaptomethyl)-2'-Deoxyuridine ....... 65

6.2.2 Synthesis of S-Mercaptopropyl-5-(Mercaptomethyl)-2'-Deoxyuridine-5'-
Triphosphate ...................................................................................................................... 69

6.2.3 HPLC Analysis and Purification .................................................................................. 71

6.2.4 UV-Vis Absorption Study ........................................................................................ 71

6.2.5 Synthesis of Oligonucleotides .................................................................................. 71

6.2.6 Enzymatic Incorporation Studies .............................................................................. 72

6.3 Results and Discussion .................................................................................................. 72

6.3.1 Synthetic Design ....................................................................................................... 72

6.3.2 HPLC Purification and Analysis ................................................................................ 75

6.3.3 Absorption Properties ............................................................................................... 76

6.3.4 Enzymatic Incorporation of TTP, AcSTTP and HSTTP into DNA ....................... 77

6.3.5 Other Proposed Studies ............................................................................................. 81

6.4 Conclusions and Future Prospects ................................................................................ 81

REFERENCES ......................................................................................................................... 82

APPENDIX ............................................................................................................................... 97
LIST OF TABLES

Table 3.1 Sequences of $^{32}$dG-ODNs used for fluorescence measurements..............................33
Table 4.1 Sequences of DNA primers and templates used for the enzymatic incorporation of $^{32}$dGTP. The underlined bases are the sites for dGTP and $^{32}$dGTP incorporation.......................48
Table 6.1 Sequences of DNA primers and templates used for the enzymatic incorporation of TTP, $^{32}$TTP and $^{3}$TTP. The underlined bases are the sites for incorporation of TTP, $^{32}$TTP and $^{3}$TTP. ..................................................................................................................................................77
LIST OF FIGURES

Figure 1.1 Probable sites for chemical modifications in DNAs and RNAs................................................. 2
Figure 1.2 Atom-specific replacement of oxygen with S and Se on (a) the nucleobase and (b) the sugar and phosphate backbone. The asterisk (*) indicates the naturally occurring modifications. .................................................................................................................................................. 3
Figure 1.3 Atom-specific replacement of hydrogen with halogens (F, Cl, Br or I) on nucleobase and sugar.................................................................................................................................................................................. 6
Figure 1.4 Jablonski diagram: mechanism of fluorescence and phosphorescence. ......................... 7
Figure 2.1 Structures of naturally occurring fluorescent nucleosides. .............................................11
Figure 2.2 Structures of fluorescent nucleoside analogs with expanded nucleobases....................12
Figure 2.3 Structures of fluorescent nucleoside analogs with extended nucleobases....................14
Figure 2.4 Structures of fluorescent hydrocarbons. The respective quantum yields are reported in parenthesis....................................................................................................................................................................14
Figure 2.5 Metal containing fluorescent nucleoside analogs...............................................................15
Figure 2.6 Absorption spectra of dG (—), N2-tBPAc-6-CE-Se-dG (—), and Se-dG (—) in water at 25 °C. Inset: Se-dG (left): yellow; dG (right): colorless..................................................................................................................................................21
Figure 2.7 RP-HPLC analysis of dG and Se-dG [monitored at 260 nm (red) and 360 nm (black)]; (a) and (b) commercial dG (retention time: 13.9 min); (c) and (d) synthesized Se-dG (retention time: 14.8 min); (e) and (f) co-injection of commercial dG and synthesized Se-dG (retention times: 13.9 and 14.8 min, respectively). ...............................................................................................................................21
Figure 2.8 Tautomers of 6-seleno-2'-deoxyguanosine, Se-dG...............................................................22
Figure 2.9 UV-Vis spectra: absorption profiles of (A) Se-dG and (B) dG, as a function of pH..22
Figure 2.10 pKₐ titration plot: pH versus wavelength (nm) plot for Se-dG; the fitted curve yields the pKₐ value 7.57 (± 0.02). ........................................................................................................................................................................23
Figure 2.11 Fluorescence spectra of $^{Se}$dG in aqueous solutions with pH 1-12: (A) Excitation spectra of $^{Se}$dG as a function of pH at 25 °C; the emission wavelength was 390 nm. (B) Emission spectra of $^{Se}$dG as a function of pH at 25 °C, with excitation at 305 nm.

Figure 2.12 Fluorescence spectra of dG in aqueous solutions with pH 1-12: (A) Excitation spectra of dG as a function of pH at 25 °C; the emission wavelength was 395 nm. (B) Emission spectra of dG as a function of pH at 25 °C, with excitation at 258 nm.

Figure 2.13 Concentration-dependent fluorescence spectra of $^{Se}$dG at pH 6: (A) Excitation spectra of $^{Se}$dG at pH 6 and 25 °C; the emission wavelength was 390 nm. (B) Emission spectra of $^{Se}$dG at pH 6 and 25 °C; the excitation wavelength was 305 nm.

Figure 2.14 Concentration-dependent fluorescence spectra of $^{Se}$dG at pH 7.4: (A) Excitation spectra of $^{Se}$dG at pH 7.4 and 25 °C; the emission wavelength was 390 nm. (B) Emission spectra of $^{Se}$dG at pH 7.4 and 25 °C; the excitation wavelength was 305 nm.

Figure 2.15 UV-Vis spectra: absorption profile of $^{Se}$dG in different solvents at 25 °C.

Figure 2.16 Fluorescence spectra of $^{Se}$dG in different solvents: (A) Excitation spectra of $^{Se}$dG in different solvents at 25 °C; the emission wavelength was 405 nm. (B) Emission spectra of $^{Se}$dG in different solvents at 25 °C; the excitation wavelength was 320 nm.

Figure 2.17 Concentration-dependent fluorescence spectra of $^{Se}$dG in iso-propanol: (A) Excitation spectra of $^{Se}$dG at 25 °C; the emission wavelength was 395 nm. (B) Emission spectra of $^{Se}$dG at 25 °C; the excitation wavelength was 320 nm.

Figure 2.18 Concentration-dependent fluorescence spectra of $^{Se}$dG in ethanol: (A) Excitation spectra of $^{Se}$dG at 25 °C; the emission wavelength was 395 nm. (B) Emission spectra of $^{Se}$dG at 25 °C; the excitation wavelength was 320 nm.

Figure 3.1 Absorption spectra of $^{Se}$dG-modified oligodeoxyribonucleotides.

Figure 3.2 RP-HPLC analysis of A$^{Se}$GA and G$^{Se}$GG trimers [monitored at 260 nm (black), 330 nm (green) and 360 nm (red)]; A. Purified A$^{Se}$GA sample (retention time: 11.9 min for $^{Se}$dG
monomer and 13.7 min for $^{Se}dG$ dimer); B. Purified $^{Se}GG$ sample (retention time: 10.5 min for $^{Se}dG$ monomer and 13.1 min for $^{Se}dG$ dimer).

Figure 3.3 Emission spectra of $^{A}G$ and $^{G}G$ trimers in solution with pH 7.4: (A) excitation at 260 nm; (B) excitation at 305 nm.

Figure 4.1 Absorption spectra of dGTP (—), $^{N^2}$-tBPAc-6-CE-$^{Se}$dGTP (—), $^{Se}$dGTP (—), and $^{Se}$dGTP-dimer (—) in water at 25 °C.

Figure 4.2 RP-HPLC analysis of dGTP and $^{Se}$dGTP [monitored at 260 nm (black) and 360 nm (red)]: (a) and (b) commercial dGTP (retention time: 12.4 min); (c) and (d) synthesized $^{Se}$dGTP (retention time: 13.6 min); (e) and (f) co-injection of commercial dGTP and synthesized $^{Se}$dGTP (retention times: 12.4 and 13.6 min, respectively).

Figure 4.3 Fluorescence spectra of $^{Se}$dGTP in aqueous solutions: (A) Excitation spectra of $^{Se}$dGTP as a function of pH at 25 °C; the emission wavelength was 445 nm. (B) Emission spectra of $^{Se}$dGTP as a function of pH at 25 °C, with excitation at 380 nm.

Figure 4.4 Concentration-dependent fluorescence spectra of $^{Se}$dGTP at pH 7.3: (A) Excitation spectra of $^{Se}$dGTP at pH 7.3 and 25 °C; the emission wavelength was 450 nm. (B) Emission spectra of $^{Se}$dGTP at pH 7.3 and 25 °C; the excitation wavelength was 380 nm.

Figure 4.5 Enzymatic incorporation of a single nucleotide (dGTP and $^{Se}$dGTP) into DNA (T1): a) Enzymatic incorporation of dGTP and $^{Se}$dGTP by Klenow exo(-) on DNA template T1. b) Gel electrophoresis autoradiography of the polymerization reaction. Lanes 2 and 3 show single nucleotide incorporation forming 18-nucleotide long DNA.

Figure 4.6 MALDI-MS analysis for enzymatic incorporation of a single nucleotide (dGTP and $^{Se}$dGTP) into DNA (T1): MS spectra of dG-extended DNA (O-18-mer, calculated m/z for [M+H]$^+$: 5644, observed m/z for [M+H]$^+$: 5648); MS spectra of $^{Se}$dG-extended DNA (Se-18-mer, calculated m/z for [M+H]$^+$: 5708, observed m/z for [M+H]$^+$: 5712); T1: calculated m/z for [M+H]$^+$: 6322, observed m/z for [M+H]$^+$: 6329.
Figure 4.7 Time course of the incorporation of single nucleotide (dGTP and Se\text{dGTP}) into DNA (T1): a) Gel electrophoresis autoradiography of the incorporation reaction over time (120 min). b) Plot of the incorporation of dGTP and Se\text{dGTP} into DNA with respect to time.

Figure 4.8 Enzymatic incorporation of all natural dNTPs, dGTP and Se\text{dGTP} into DNA (T2): a) Enzymatic incorporation of all dNTPs, dGTP and Se\text{dGTP} by Klenow exo(-) on DNA template T2. b) Gel electrophoresis autoradiography of the polymerization reaction.

Figure 4.9 Time course of the incorporation of all natural dNTPs, dGTP and Se\text{dGTP} into DNA (T2): a) Gel electrophoresis autoradiography of the polymerization reaction over time (120 min). b) Plot of the incorporation of dGTP and Se\text{dGTP} (along with other dNTPs) into DNA with respect to time.

Figure 4.10 Time course of the enzymatic digestion of dGTP and Se\text{dGTP} incorporated DNA with Exonuclease III. a) Gel electrophoresis autoradiography; b) plot of digestion of dG-DNA and Se\text{dG}-resisitive DNA with respect to time.

Figure 5.1 RP-HPLC analysis of n-Pac-6CE-SeGTP, 6, monitored at 260 nm (black) and 305 nm (red) (retention time: 31.04 min).

Figure 6.1 RP-HPLC analysis of AcS\text{TTP and HS\text{TTP}: HS\text{TTP} (red curve, retention time: 24.75 min); AcS\text{TTP} (blue curve, retention time: 24.96 min) and co-injection of AcS\text{TTP and HS\text{TTP} (black curve, retention times: 24.75 and 24.96 min, respectively).}

Figure 6.2 Absorption spectra of TTP (—), AcS\text{TTP (—), and HS\text{TTP (—)} in water at 25 °C.

Figure 6.3 Enzymatic incorporation of a single nucleotide (TTP, AcS\text{TTP and HS\text{TTP}) into DNA (T1): a) Enzymatic incorporation of TTP, AcS\text{TTP and HS\text{TTP} by Klenow exo(-) on DNA template T1. Gel electrophoresis autoradiography of the polymerization reaction with (b) \(Kf = 0.0015 \text{U/µL and reaction time = 60 min for TTP and 90 min for AcS\text{TTP and HS\text{TTP), (c) Kf = 0.00075 \text{U/µL and reaction time = 60 min, (d) Kf = 0.0015 \text{U/µL and reaction time = 30 min, and (d) Kf = 0.00075 \text{U/µL and reaction time = 30 min.}}}}
Figure 6.4 Enzymatic incorporation of three consecutive bases (TTP, AcSTTP and HSSTTP) into DNA (T2): (a) Enzymatic incorporation of TTP, AcSTTP and HSSTTP by Klenow exo(-) on DNA template T2. (b) Gel electrophoresis autoradiography of the polymerization reaction with Kf = 0.0015 U/µL and reaction time = 5 min for TTP (lane 2) and 60 min for AcSTTP (lane 4) and HSSTTP (lane 3).

Figure 6.5 Enzymatic incorporation of all natural dNTPs, TTP, AcSTTP and HSSTTP into DNA (T3): a) Enzymatic incorporation of all dNTPs, TTP, AcSTTP and HSSTTP by Klenow exo(-) on DNA template T3; (b) Gel electrophoresis autoradiography of the polymerization reaction with Kf = 0.0015 U/µL and reaction time = 60 min for TTP (lane 3) and 90 min for AcSTTP (lane 5) and HSSTTP (lane 4).
LIST OF SCHEMES

Scheme 2.1 Synthesis of 6-seleno-2'-deoxyguanosine(SeG) ................................................................. 18
Scheme 3.1 Synthesis of 6-seleno-2'-deoxyguanosine modified oligonucleotides, 6 .................. 31
Scheme 4.1 Synthesis of 6-seleno-2'-deoxyguanosine-5'-triphosphate(SeGTP) ......................... 44
Scheme 5.1 Synthesis of 2-phenoxyacetyl-6-(2-cyanoethylseleno)-guanosine, 4 ............................ 60
Scheme 5.2 Synthesis of 6-selenoguanosine-5'-triphosphate(SeGTP) ............................................. 61
Scheme 6.1 Synthesis of S-(3(acetylthio)propyl)-5-(mercaptomethyl)-2'-deoxyuridine (7) ........ 74
Scheme 6.2 Synthesis of S-mercaptopropyl-5-(mercaptomethyl)-2'-deoxyuridine-5'-triphosphate, HS-TTP(10) .................................................................................................................. 75
1 INTRODUCTION

1.1 Significance of Nucleic Acids

Nucleosides, nucleotides and oligonucleotides are of paramount significance with multifaceted and intricate roles in cell biology. Nucleic acids are the fundamental bio-macromolecules that replicate, transcribe and translate genetic information. Virtually omnipresent, nucleic acids are the storehouse of genetic information. For several decades they have generated curiosity about the origin of life and nature’s preference for ribose- and deoxyribonucleic acids as the molecular basis of life’s genetic systems. Thus, a detailed structural and functional analysis of nucleic acids is essential to the knowledge of life. However, their potential as functional bio-molecules has been greatly undermined. Nucleosides and nucleotides are significant and powerful building blocks. They can be easily modeled and chemically functionalized at various positions, with diverse functional groups, for creating functional molecules with novel properties. Modified analogs, with tailored properties, are potential candidates for novel drug therapies and effective biological tools for the fundamental understanding of diseases at molecular and genetic level. Both chemical and enzymatic approaches have been utilized to synthesize functionalized nucleic acids. The modification of nucleic acids presents enormous opportunities for tailoring the properties and functions of these bio-macromolecules, escalating their potential in biochemical and biomedical applications.

It is amazing how only five elements, namely oxygen (O), nitrogen (N), phosphorus (P), carbon (C) and hydrogen (H) [rarely sulfur (S) and selenium (Se)], create nucleic acids. However, their functions have been limited by the combination of only four nucleotide building blocks. Therefore, chemical modifications help in creating nucleic acids with tunable physico-chemical properties for applications in catalysis, molecular binding and nucleic-acid labeling. The basic structure of a nucleotide consists of a base, a sugar and a phosphate group. All these three components have various active sites (figure 1.1) which were, and still are, prime targets for chemical modification.
Figure 1.1 Probable sites for chemical modifications in DNAs and RNAs.

1.2 Single Atom Modification (SAM) in Nucleic Acids

Single atom modification in nucleic acids involves the atom-specific substitution of a single nucleotide atom with another equivalent atom. Oxygen atoms are found in abundance in nucleic acids. Therefore, atom specific replacement of oxygen with sulfur, selenium and tellurium (other members of the chalcogen family), is a useful and successful chemical strategy to improve and tune the biophysical and biochemical properties of nucleic acids. Compared to oxygen (atomic radius: 0.73 Å), they have larger atomic sizes (atomic radius of S: 1.02 Å, Se: 1.16 Å and Te: 1.40 Å) and electron densities (21). These features offer distinctive and novel properties, without causing any significant structural perturbation. The modified nucleic acids act as good structural mimics of the corresponding oxo-nucleic acids. Figure 1.2 demonstrates the atom specific modification of oxygen with sulfur and selenium on the nucleobase, sugar and phosphate backbone.

Sulfur is an interesting element with divergent chemical properties. It is widely found in a broad array of natural biomolecules owing to its chemical and structural behavior. Sulfur is an essential element for all forms of life and is involved in various biochemical processes. In search of bioactive compounds, a variety of modified oligonucleotides containing a sulfur atom on the sugar, the nucleobase, or the phosphoryl group have been synthesized. Sulfur adds substantial functionality to nucleic acids, as a result of its structural and chemical versatility. Incorporation of sulfur in nucleosides, nucleotides and nucleic acids can afford exclusive biological properties like increased
metabolic stability and bioavailability, nuclease resistance, high hybridization, and decreased toxicity (22-32). These sulfur-derivatized oligonucleotides are valuable tools in understanding protein-nucleic acid interactions, nucleic acid structure-function relationship, and oligonucleotide therapy (17,33-39).

![Diagram of nucleobase and sugar modifications](image)

Figure 1.2 Atom-specific replacement of oxygen with S and Se on (a) the nucleobase and (b) the sugar and phosphate backbone. The asterisk (*) indicates the naturally occurring modifications.

Selenium is an essential element and a vital micronutrient for humans with potential antioxidant properties. It is present naturally in foods and soils, and is available via dietary supplements (40-42). Analogous to sulfur, natural occurrence of selenium in tRNAs has been observed in certain bacteria and mammals. The selenium nucleoside, identified as 5-[(methylamino)methyl]-2-selenouridine (mmnm^2-se^2U), is often found at the first or wobble position of the anticodon loop of tRNA (43,44). While the precise function of selenium at the C-2 position and the comprehensive utility of Se-derivatized tRNAs are not delineated, this modification is essential for catalysis and regulation and probably capable of augmenting translational efficiency and efficacy(45). Selenium derivatization of nucleic acids generates the modified biopolymers with novel properties. Huang
and co-workers have pioneered the synthesis and structure-function studies of selenium-derivatized (or -modified) nucleic acids (SeNA). Atom-specific selenium modification at different positions in the nucleobase, sugar, and phosphate group has been achieved by Huang’s research group along with other groups (46-65). Through different modifications in the nucleobase and sugar, it has been demonstrated that the presence of selenium atom facilitates crystallization and crystallographic phasing (46, 47, 66) to the extent that it has surpassed all other known approaches (like, heavy-atom soaking, co-crystallization, and halogen derivatization) for nucleic acid X-ray crystallography. The nucleosides, nucleotides and nucleic acids obtained by atom-specific substitution of oxygen with selenium serve as useful biophysical, structural, and mechanistic probes. The stability and compatibility of the selenium functionality, with chemical and enzymatic synthesis, has made the introduction and incorporation of selenium at different positions a reliable and useful technique.

Tellurium, a non-essential trace element, is toxic to humans and does not occur naturally in any biomolecules. Tellurium, with larger size, lower electronegativity (Te: 2.0; Se: 2.55; S: 2.58) and more metallic character, has distinctive chemical properties in comparison with sulfur and selenium (67). Owing to its exceptional physical, chemical and spectroscopic properties, tellurium compounds have recently garnered appreciable interest from the researchers in drug development and diagnostics and in structure determination of proteins and nucleic acids. Atom-specific incorporation of tellurium into nucleic acids was pioneered by Huang and co-workers (68-70). Tellurium functionality has been successfully introduced into different positions of the nucleobase and sugar (68-70). The thermal stability and X-ray crystallographic studies of the Te-DNA thus obtained reveals that the modified DNA is virtually identical to the native DNA in structure and stability. It is likely that the presence of an electron-rich tellurium atom in a DNA base facilitates electron delocalization by donation of electrons to the relatively electron-deficient DNA duplexes. This phenomenon helps in direct DNA imaging without significant structural perturbation. In scanning tunneling microscope (STM) imaging the Te-DNA duplexes exhibit higher visibility and conductivity and re-
veal stronger topographic and current peaks than the corresponding native duplexes. Thus, by the virtue of their unique optoelectronic properties, tellurium derivatized nucleic acids open a novel approach towards the imaging of nucleic acids and their complexes with proteins and small molecules.

In other examples of atom specific mutagenesis of nucleic acids, halogen atoms are considered equivalent to hydrogen atoms. Although, the options for halogen replacement on the nucleobase and sugar are limited (figure 1.3) compared with the sulfur and selenium modifications, halogen-derivatized nucleic acids analogs are well applied in nucleic acid structure-function studies and in drug discovery and development. Similar in size, fluorine is the closest mimic of the hydrogen atom when introduced in biomolecules. It offers minimal structural perturbation, along with improved stability contributed by the stronger C—F bond. With 100 % isotopic abundance, $^{19}$F qualifies for the ideal atomic label for probing the structure, conformation and dynamics of nucleic acids via fluorine labeling and $^{19}$F-NMR(71-73). $^{19}$F-NMR has been extensively utilized for the investigating several fluoro-nucleobases: 5-fluorouridine in U/G wobble pair investigation (74), dynamics of hammerhead ribozyme(75); 5-fluorocytosine in base-flipping in HhaI methyltransferase–DNA interactions (76); 2-fluoroadenosine in base-pairing interaction study (77). The 2'-F labels have been successfully used for exploring DNA motifs and RNA folding pathways (78-81). The 2'-'α-fluoro labeled siRNAs, with improved thermostability and resistance towards nucleases, are strong contenders in siRNA therapeutics (82,83). The 2'-β-fluoro labeled nucleosides have potential antiviral and anticancer activities (84-86).
The applications of other halogen (chlorine, bromine or iodine) derivatized nucleic acids are not as widespread as of fluoro-modified analogs. The most familiar chloro-modified analog, 2-chloroadenosine has been explored for nuclease resistance studies (87), along with other potential therapeutic applications (88-91). In general, bromine and iodine have been introduced into nucleic acids via C-5-position of pyrimidine, C-8-position of purine, and C-2-position of adenosine. Analogous to selenium, bromine and iodine serve as anomalous scattering centers in X-ray crystallography via MAD (multi-wavelength anomalous diffraction) and SAD (single-wavelength anomalous diffraction) and as isomorphous heavy atoms in MIR (multiple isomorphous replacement). The introduction of a single bromine or iodine-atom has resulted in several X-ray crystal structures (92-97). Nevertheless, the halogen (heavy-atom) modified nucleic acid analogs present their own disadvantages: (i) the total possible locations on the nucleoside for halogen-derivatization are limited; (ii) bromide and iodide being good leaving groups can be easily replaces when located at C-2’ of the sugar; (iii) halogen functionality is sensitive to X-ray irradiation, resulting in dehalogenation (Br and I) and failure in structure determination (98,99); (iv) placement of bromine/iodine at C-8 of purines causes rotation from anti- to syn-conformation resulting in structural perturbation (100-102). Significant structural perturbation can also occur if the halogen modification is placed in the
major-groove of the A-form double helix (103). Halogen-modified DNA/RNA also find application in therapeutics (104,105).

![Jablonski diagram: mechanism of fluorescence and phosphorescence.](image)

**Figure 1.4 Jablonski diagram: mechanism of fluorescence and phosphorescence.**

### 1.3 Fluorescence Based Detection of Nucleic Acids

Fluorescence is the light emitted from a molecule after it has absorbed light of a different/shorter wavelength. Fluorescence is an intrinsic property. Fluorescence is generally studied in highly conjugated polycyclic aromatic molecules. When the irradiating light is removed, fluorescence stops. But some molecules continue to emit longer-wavelength radiation, even after the removal of exciting radiation. This phenomenon is known as phosphorescence (106). Fluorescence from a compound is dependent on several factors like pH, temperature, impurity and polarity of solvent. In fluorescence spectroscopy, a photon of energy is absorbed by the fluorophore. This absorption of light by the molecule promotes an electron from its ground electronic state ($S_0$) to one of the vibrational levels in the excited electronic states ($S_1, S_2, S_3$, etc.). The fluorophore rapidly deactivate to the lowest vibrational state of the excited electronic state ($S_1$). This process is often visualized with a Jablonski diagram (figure 2.1). The molecule then drops down to one of the various vibrational levels of the ground electronic state again, emitting a photon in the process. The light thus emitted is known as fluorescence.
Fluorescence spectroscopy is one of the most sensitive analytical techniques. Fluorescence based tools have been valuable in studying the fundamental structure, folding, recognition, reactivity and dynamics of biomolecules in biological systems (107-113). As many of the biological macromolecules are inherently non-emissive, fluorescent analogs of the building blocks (carbohydrates, phospholipids, fatty acids, amino acids and nucleosides) of biomolecules have been designed and synthesized (114). Carbohydrates do not have any conjugated π-system and hence, lack any useful absorption/emission feature. Therefore, significant structural modifications are necessary to bestow useful photo-physical properties. The biological membrane is a complex constitution of a diverse variety of constituents. The plethora of fluorescent probes, labels and methodologies available are dictated largely by their function and largely include non-covalent fluorescent membrane probes (115-118), polar head group-labels (119-121) and lipophilic chain-labels (includes in-chain, chain-end and on-chain labeling) (122-125). These fluorescent probes have greatly contributed to our understanding of various functions, properties, interactions and dynamics of the biological membranes. Fluorescence spectroscopy has also been instrumental in detecting and understanding the structure, function and dynamics of proteins and nucleic acids, especially under physiological conditions (126-135). Proteins play a central role in modern biology. Their recognition, function and cell localization depends on their three-dimensional structure and susceptibility to environmental factors. This dependency on structure limits the structural diversity of the amino-acid probes. The chromophores for fluorescent amino-acid probes are designed for specific applications like exploring conformational behavior, studying structural and functional behavior in varied environments and, binding interactions with other molecules (114).

Although it has been overwhelmingly helpful in studying proteins, fluorescence spectroscopy has found limited use in nucleic acids due to their low fluorescence quantum yields. This presented a major challenge in the biophysical study of nucleic acids thereby initiating extensive search for emissive nucleoside/nucleotide analogs. The purine and pyrimidine bases present a fer-
tile ground for diverse chemical modification with minimal structural and electronic perturbations and varied photo-physical properties. With the advancement in the design and synthesis of fluorescent nucleoside mimics and surrogates, the contemporary landscape of fluorescent nucleic acids is very vast. Chapter 2 of this dissertation illustrates a few examples of the various designs and the resultant significance of fluorescent nucleoside analogs. The fluorescent analogs have a diverse range of applications. These analogs can be easily incorporated into the oligonucleotides via standard synthetic methods, without significant structural, chemical and electronic perturbations. The resultant oligonucleotides serve as sensitive probes for investigating the conformational changes and structural perturbations resulting from changes in the microenvironment. Due to their high sensitivity and selectivity, the fluorescent analogs have minimal interference with the biological system enabling an efficient visualization of DNA/RNA molecules, both in vivo and in vitro. This further helps in tracking the location, transportation and global conformational changes in nucleic acids and their complexes. All these advances have resulted in the widespread application of fluorescent mimics/probes in biophysical, biochemical and biomedical studies, disease diagnosis, and drug development.
2 SYNTHESIS AND OPTICAL STUDY OF 6-SELENO-2’-DEOXYGUANOSINE

Different nucleoside analogs, containing alternate or extended bases or covalently attached fluorescent labels, have been designed and used to study the native nucleic acids. Fluorescent analogs of nucleic acid bases afford sensitive probes for exploring the structure, interactions, and dynamics of nucleic acids. Discussion of all the fluorescent nucleoside analogs is beyond the scope of this dissertation. Therefore, this report includes only a few relevant examples.

2.1 Fluorescent Nucleoside Analogs: Early Examples

Nucleosides, the structural subunits of nucleic acids, are composed of a pentose sugar (ribose or deoxyribose) linked to a nucleobase (nitrogenous heterocyclic rings: purines and pyrimidines) via a beta-glycosidic bond. Naturally occurring nucleic acids are practically non-emissive in physiological conditions (136). However, strong acidic solutions (with pH as low as 1) can induce weak fluorescence in native nucleobases like adenine and guanine (137-139). But, such strong conditions are inapt for probing the nucleic acids and their interactions with other molecules. Such challenges propelled the nucleoside chemists to explore new emissive nucleoside analogs. Presently, the development of fluorescent nucleoside analogs is a rapidly evolving field.

The nucleobases (purines and pyrimidines) present a fertile ground for diverse chemical modifications where minimal molecular and electronic perturbation can dramatically alter the photo-physical characteristics such as molar absorptivity, quantum yield, Stokes shift, lifetime, and response to external stimuli (114,140-147). Adenosine analogs formycin (1), 2-aminopurine ribonucleoside (2) and 2, 6-diaminopurine ribonucleoside (3), are amongst the first reported naturally occurring fluorescent nucleobases (figure 2.1) (148). These analogs have characteristic red-shifted absorption (280 – 303 nm) and emission (340 – 370 nm) bands. A constitutional isomer of adenine, 2-aminopurine is one of the most widely used fluorescent nucleoside because of its enhanced photo-physical properties, high quantum yield ($\Phi = 0.68$) at physiological pH, low excitation
energy, sensitivity to the micro-environment and ability to form WC-like base pairs with uridine and thymidine.

Figure 2.1 Structures of naturally occurring fluorescent nucleosides.

2.2 Fluorescent Nucleoside Analogs: Expanded Nucleobases

Since the first report on fluorescence in nucleobases, a variety of fluorescent nucleosides, derived from structural modification of 2-aminopurine, have been reported (149). Another remarkable fluorophore, 8-vinyl-deoxyadenosine (1, figure 2.2), was obtained by substituting the hydrogen at 8 position of adenine with a vinyl moiety (147,150,151). This modification produces remarkable photo-physical properties, like high quantum yield (Φ = 0.66) and pH insensitivity, making it a potential probe for exploring duplex structure and stability. Tor and co-workers were able to design highly emissive nucleosides by incorporating a furan ring at the 8-position of purines (2 and 3) (152). They also developed a series of fluorescent thymidine and cytosine analogs by incorporating a furan/thiophene ring or fusing aromatic five-membered heterocyclic rings at the 5-position of pyrimidines (4 - 6) (153-157). Hirao and co-workers synthesized a series of fluorescent purine analogs, for the expansion of the genetic alphabet by the unnatural base-pair systems, by incorporating a thiophene or thiazole ring at the 6-position (7 - 9) (158-162). Such a substitution results in red-shifted absorption bands (~355 nm) with strong emission in the visible region (~450 nm). These fluorophores have been helpful in site-specific RNA labeling.

Extended fluorescent nucleoside analogs can also be designed by tethering a fluorophore to the natural nucleobase via flexible or rigid linkers. This method of connecting known
chromophores to purines and pyrimidines via electronically non-conjugating linkers spawn new fluorophores/chromophores with unique photo-physical characteristics. In pursuit of base-discriminating fluorosides capable of detecting single nucleotide polymorphism (SNP), Saito and co-workers designed pendant fluorophore-labeled deoxyuridine (10) and cytosine (11) analogs. These nucleosides retain their hydrogen bonding face, have isolated absorption at ~335 nm and intense emission at ~400 nm (Φ = ~0.2) (163).

Figure 2.2 Structures of fluorescent nucleoside analogs with expanded nucleobases.
Seela et al. have meticulously investigated the photo-physical characteristics of adenosine analogs by attaching functionalized alkenes and alkynes to the 7-position (12) (149,164). Unusual 2-substituted adenosine analogs (13), which displayed increased emission quantum efficiencies upon incorporation in RNA, have also been reported (165). Xanthene-type fluorophores (14) were investigated by Burgess and co-workers (166). The fluorophore is conjugated to the base via an ethynyl linkage. An increase in extinction coefficient was observed upon extending the rigid linker by a phenyl or phenyl-ethynyl moiety. The chemical structures for the discussed fluorophores 1 - 14 are shown in figure 2.2.

### 2.3 Fluorescent Nucleoside Analogs: Extended Nucleobases

Extended conjugation of natural nucleobases, resulting from the fusion of additional aromatic rings onto the purine/pyrimidine core, also facilitates exciting and favorable photo-physical properties, with high quantum efficiencies and emission in the visible range. In the early 1970s Leonard and co-workers investigated the etheno- (1,) and benzo- (2) derivatives of adenine and cytosine (figure 2.3) (167,168). These extended heterocycles display improved structural and optical properties.

These nucleosides have found unique fluorescence based applications in structural and functional exploration of DNAs and RNAs (169,170). Godde et al. have reported fluorescent, naptho-substituted analogs of thymidine (3) and cytosine (4) (figure 2.3) (171,172). These fluorophores have largely red-shifted absorption (360 – 370 nm) and emission (434 – 456 nm) bands with respect to the native nucleobases. With high fluorescence quantum yields (Φ = 0.82 for 3 and Φ = 0.62 for 4), these pH-responsive nucleosides and their derivatives have been successfully introduced into oligonucleotides to get more insight about the double- and triple-stranded structures. Saito and co-workers were able to gain attractive fluorescent properties by fusing benzo- and naptha-rings to the nucleobases (5 – 8, figure 2.4) (173-175). The cytidine analog 6 forms stable base pairs: wobble base-pair with A and Watson-Crick base-pair with G. But this benzo-analog 6 has a
lower quantum efficiency (Φ = 0.04) compared with its naphtho-analog 5 (Φ = 0.26) (174). The adenosine (7) and inosine (8) analogs, with modest quantum yields (Φ = 0.12), act as acceptable pyrimidine-discriminating fluorescent nucleosides.

![Structures of fluorescent nucleoside analogs with extended nucleobases.](image1)

**2.4 Fluorescent Hydrocarbons**

Another simple procedure to generate fluorescent nucleosides is based on the replacement of natural nucleobases with established fluorophores like polycyclic aromatic hydrocarbons. These base analogs lack the Watson-Crick hydrogen bonding face, but facilitate selective excitation with isolated absorption bands around and above 345 nm (141). Some selected examples are shown in figure 2.4 with their respective names and quantum yields (114). Some of these nucleosides have been beneficial in exploring the environment and dynamics of oligonucleotides (176-179).

![Structures of fluorescent hydrocarbons. The respective quantum yields are reported in parenthesis.](image2)
2.5 Metal Containing Fluorescent Nucleoside Analogs

Various metal containing emissive nucleosides have also been reported. Figure 2.5 shows the chemical structures of some of these nucleosides. The electrochemistry and luminescent properties of Ru\textsuperscript{II}-complexes of 7-deazaadenine 2'-deoxyribonucleosides bearing bipyridine, phenanthroline or terpyridine ligands (1, 2), linked to 7-position of deoxyadenosine via an acetylene or phenylene tether, was investigated by Hocek and co-workers (180). They also studied the corresponding 2'-deoxyadenosine derivatives obtained by linking the spacer to the 8-position (3) (181). The modified nucleosides exert significant cytostatic and antiviral effects. Tor an co-workers have investigated the electrochemical and photo-physical properties nucleosides containing [(bpy)\textsubscript{2}M(3-ethynyl-1,10-phenanthroline)]\textsuperscript{2+} (M = Ru\textsuperscript{II}, Os\textsuperscript{II}) (4) metal center attached covalently to the 5-position in 2'-deoxyuridine (182-185). They observed that the Ru\textsuperscript{II}-based nucleosides displayed stronger fluorescence (Φ = 0.137 at 629 nm) compared to the Os\textsuperscript{II}-based nucleosides (Φ = 0.0003 at 749 nm). It was also found that upon incorporation in oligonucleotides, these nucleosides cause minimal structural perturbation. These metal containing nucleosides have been used to synthesize a diverse variety of metal-containing nucleoside triphosphates which act as good substrates for vent (exo-) and Pwo polymerases. The modified oligonucleotides thus obtained have found use in detecting single nucleotide polymorphism via the luminescence generated by the fluorescent nucleoside (182-185).

![Figure 2.5 Metal containing fluorescent nucleoside analogs.](image-url)
Unfortunately, these modifications are often bulky and usually result in colorless fluorophores. Thus, such types of modifications are not ideal for probing the structure, function and dynamics of nucleic acids. However, it is very challenging to design and discover minimally-modified nucleosides which are colored and exhibit significant fluorescence. Herein, we report the first observation of fluorescence in a nucleoside with a single atom substitution. We have chemically synthesized 6-seleno-2'-deoxyguanosine (6-Se dG) nucleoside by replacing oxygen atom at 6-position with a selenium atom. This single atom modification not only imparts a strong yellow color to the otherwise colorless 2'-deoxyguanosine (dG) nucleoside, but also makes it fluorescent under physiological conditions. Since the label is small, it has significant advantages over classical fluorescent-labels with large functional groups. This new class of “atom-modified fluorescent labels” can be very useful for luminescence based detection of nucleic acid and protein-nucleic acid complexes.

2.6 Material and Methods

2.6.1 Synthesis of 6-Seleno-2'-Deoxyguanosine

The synthesis of 6-seleno-2'-deoxyguanosine was achieved from commercially available starting material N2-[2-(4-tert-butylphenoxy)acetyl]-5'-O-(4,4'-dimethoxytri-phenylmethyl)-2'-deoxyguanosine (1) (scheme 2.1). Compound 2 was synthesized from compound 1 using published procedure (2): a solution of 1 (500mg, 0.66 mmol), 4-dimethylaminopyridine (15 mg, 0.12 mmol, DMAP) and triethylamine (0.18 mL, 1.32 mmol, 2 eq., TEA) was prepared in methylene chloride (2 mL, CH₂Cl₂) under argon. To this solution 2,4,6-(triisopropylbenzene)sulfonyl chloride (300 mg, 1 mmol, 1.5 eq., TIBS), dissolved in CH₂Cl₂ (1 mL), was added. The contents were stirred at room temperature for 15 min. The progress of the reaction was monitored by silica gel thin layer chromatography (TLC; 5% methanol in CH₂Cl₂). After the completion of reaction (as monitored by TLC), the mixture was injected into sodium selenide (NCCH₂CH₂SeNa) solution. For preparing the sodium selenide solution, di-(2-cyanoethyl) diselenide (700 mg, 2.64 mmol, 8 eq.) was dissolved in ethanol
(15 mL) on an ice bath. A suspension of NaBH₄ (150 mg) was prepared in ethanol (4 mL) and injected into the diselenide solution to furnish sodium selenide. The progress of selenium incorporation reaction was monitored by TLC (5% methanol in CH₂Cl₂). After the completion of reaction, water (10 mL) was added to the reaction flask. The solution was extracted with CH₂Cl₂ (3 x 20 mL) and dried over anhydrous MgSO₄. The solution was filtered and the solvent was evaporated under reduced pressure. The crude product was purified by a silica gel column equilibrated with CH₂Cl₂ and eluted with a step-wise gradient of MeOH/CH₂Cl₂ mixtures (CH₂Cl₂, 1.0, 2.0, 3.0, 4.0% MeOH in CH₂Cl₂, 200 mL each) to afford bright yellow N²-[2-(4-tert-butyl-phenoxy)acetyl]-6-(2-cyanoethyl)seleno-5'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxyguanosine (2, 460 mg, 80% yield over two steps). The compound was used as such without any further characterization.

To deprotect the 5'-dimethoxytrityl (DMTr) group, N²-[2-(4-tert-butylphenoxy)acetyl]-6-(2-cyanoethyl)seleno-5'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxyguanosine (2) (160 mg, 0.183 mmol) was dissolved in acetic acid (80%, 1 mL) and stirred at room temperature for 1 h. The progress of the reaction was monitored by TLC (5% methanol in CH₂Cl₂). After the completion of reaction, the solution was neutralized with TEA and the solvent was evaporated under reduced pressure. The crude compound 3 was then purified by a silica gel column equilibrated with methylene chloride and eluted with a step-wise gradient of methanol/ methylene chloride mixtures (CH₂Cl₂, 1.0, 2.0, 3.0, 4.0% MeOH in CH₂Cl₂, 200 mL each) to afford pale yellow N²-[2-(4-tert-butyl-phenoxy)acetyl]-6-(2-cyanoethyl)seleno-2'-deoxyguanosine (3, 95 mg, 91%). HRMS (ESI-TOF): molecular formula, C₂₅H₃₀N₆O₅Se; [M+H⁺]⁺: 575.1508 (calc. 575.1516), [M+Na⁺]⁺: 597.1351 (calc. 597.1335); ¹H NMR (400 MHz, CD₃SOCD₃) δ: 1.25 (s, 9H, 3x CH₃-tBu), 2.30-2.35 and 2.69-2.76 (2x m, J₁⁻₂= 6.4 Hz, 2H, H-2'), 3.19 (t, J= 6.4 Hz, 2H, Se-CH₂-CH₂-CN), 3.53-3.62 (m, 2H, Se-CH₂-CH₂-CN), 3.53-3.62 (m, 2H) and 3.87-3.88 (br, 1H) (H-3' and H-5'), 4.43 (br, 1H, H-4'), 4.95 (s, 2H, CH₂-O), 6.35 (t, J₁⁻₂= 6.4 Hz, 1H, H-1'), 6.86-6.88 (d, J = 8.64 Hz, 2H, CH-arom), 7.30-7.32 (d, J = 8.72 Hz, 2H, CH-arom), 8.59 (s, 1H, H-8), 10.82 (s, 1H, NH); ¹³C NMR (100 MHz, CD₃SOCD₃) δ: 18.37 and 18.89
(Se-CH2-CH2-CN), 31.22 (CH3-tBu), 33.68 (C-tBu), 61.35 (C-5'), 67.12 (CH2-O), 70.39 (C-3'), 83.31 (C-4'), 87.85 (C-1'), 113.83 and 125.99 (CH-arom), 119.84 (CN), 130.25 (C-5), 142.51 (C-8), 143.03 and 157.24 (C-arom), 148.46 (C-6), 151.57 (C-4), 155.53 (C-2), 167.23 (C=O).

Scheme 2.1 Synthesis of 6-seleno-2'-deoxyguanosine (6-SeG).

Compound 3 (10 mg, 0.017 mmol) was then treated with 0.05 M K2CO3 in methanol (4 mL; pH = 10-11) at room temperature for 2 h to remove the cyanoethyl and (4-tert-butyl-phenoxy)acetyl protecting groups. The crude product was neutralized with HCl and purified by reverse phase high performance liquid chromatography (RP-HPLC). The 6-seleno-2'-deoxyguanosine nucleoside was eluted at 355 nm, using a combination of two solvents at varying proportions: buffer A (2.5 mM TEAAc in water) and buffer B (2.5 mM TEAAc, 50% water and 50% acetonitrile). The fractions were lyophilized to afford bright yellow 6-seleno-2'-deoxyguanosine (4, 2.5 mg, 42%).

HRMS (ESI-TOF): molecular formula, C10H13N5O3Se; [M-H-]: 330.0103 (calc. 330.0111); 1H NMR (400 MHz, CD3OD) δ: 2.34-2.40 and 2.62-2.71 (2x m, J=6.4 Hz, 2H, H-2'), 3.70-3.80 (quat. of d, 2H) and 3.98 (br, 1H) (H-3' and H-5'), 4.51 (br, 1H, H-4'), 6.26 (t, J=6.4 Hz, 1H, H-1'), 8.23 (s, 1H, H8); 13C NMR (100 MHz, CD3OD) δ: 41.38 (C-2'), 63.28 (C-5'), 72.67 (C-3'), 85.68 (C-4'), 89.39 (C-1'), 120.91 (C-5), 133.90 (C-8), 141.37 (C-4), 147.55 (C-2), 174.51 (C=O).
2.6.2 Optical Studies of 6-Seleno-2'-Deoxyguanosine

All absorption and emission measurements were made in 10 mM sodium phosphate buffer. UV-Vis spectra were recorded with respect to pure solvent/buffer reference. The pH of the buffer was adjusted using HCl or NaOH solutions. For all the fluorescence measurements (unless mentioned) sample concentrations were fixed at 5 µM, and the excitation and emission slit widths were fixed at 10 nm. The baseline was subtracted at all times. Both dG and Se-dGstock solutions were prepared in methanol for all measurements. The absorption and emission spectra were recorded at every 1.0 pH unit (from pH 1 to 12) to study the effect of pH on dG and Se-dG (figures 2.9, 2.11 and 2.12). A pH titration curve was obtained for Se-dG by measuring the absorption every 0.1 pH unit between pH 7 – 9 and every 0.5 – 1.0 pH unit between pH 4 – 7 and pH 9 – 12. Figure 2.10 shows a plot of the titration data obtained.

2.6.3 HPLC Analysis and Purification of 6-Seleno-2'-Deoxyguanosine

The crude 6-seleno-2'-deoxyguanosine was purified and analyzed by reversed-phase high performance liquid chromatography (RP-HPLC). The purification was performed on a Welchrom C18-XB, 10 µm, 21.2*250 mm column using Shimadzu SPD-10A VP liquid chromatography with a flow-rate of 6 mL/min [buffer A: 2.5 mM triethylammoniumacetate (TEAAc, pH 7.1) in water; buffer B: 2.5 mM TEAAc (pH7.1) in 50% acetonitrile] and a linear gradient from 100 % buffer A to 100 % buffer B in 15 min. The desired peak was collected and the buffer was removed by lyophilization. All samples were analyzed on Welchrom C18-XB column (4.6*250 mm) and measured at a flow rate of 1.0 mL/min and a linear gradient of 0 to 25% buffer B in 20 min [buffer A: 20 mM TEAAc (pH 7.1) in water; buffer B: 20 mM TEAAc (pH 7.1) in 50% acetonitrile].
2.7 Results and Discussion

2.7.1 Synthetic Design

6-Seleno-2’-deoxyguanosine (4, scheme 2.1) was synthesized using N\textsuperscript{2}_[2-(4-tert-butylnaphenoxo)acetyl]-5’-O-(4,4’-dimethoxytri-phenylmethyl)-2’-deoxyguanosine (1). The synthesis of 2 has been previously reported (2). Compound 2 was detritylated using acid solution. The cyanophenyl (CE) and (4-tert-butyl-phenoxy)acetyl (t-BPAc) are labile protecting groups. These can be deprotected under ultra-mild conditions (0.05 M K\textsubscript{2}CO\textsubscript{3} in methanol). The de-protection, as discussed below, can also be monitored by UV-Vis due to the unique absorption profiles of compounds 3 and 4, resulting from the selenium-functionality (figure 2.7).

2.7.2 Absorption Properties of 6-Seleno-2’-Deoxyguanosine

Native 2’-deoxyguanosine (dG) has an absorption maximum at 254 nm (figure 2.6). Introduction of selenium yields an interesting absorption profile to the nucleoside. The absorption spectrum of N\textsuperscript{2}_[tBPAc]-6-(2-cyanoethyl)seleno-2’-deoxy-5’-guanosine (N\textsuperscript{2}-tBPAc-6-CE-Se-dG, 3) shows discreet bands at 256 nm and 308 nm (figure 2.6) in aqueous solution. When the protecting groups are completely removed, the resulting \textsuperscript{Se}dG (4) shows an isolated absorption maximum at 357 nm and is bright yellow in color (inset: figure 2.6). Thus, a single atom replacement not only imparts a strong color to the otherwise colorless nucleoside, but also results in over 100 nm red-shift in the UV absorption. This observation was also confirmed by the HPLC analysis comparison of commercial dG with synthesized \textsuperscript{Se}dG at 260 nm and 360 nm (figure 2.7). \textsuperscript{Se}dG absorbs at both 260 nm (slightly) and 360 nm (strongly), whereas native dG has no absorbance at 360 nm.
Figure 2.6 Absorption spectra of dG (---), N²-tBPAC-6-CE-Se-dG (--), and 6dG (---) in water at 25 °C. Inset: 6dG (left): yellow; dG (right): colorless.

Figure 2.7 RP-HPLC analysis of dG and 6dG [monitored at 260 nm (red) and 360 nm (black)]; (a) and (b) commercial dG (retention time: 13.9 min); (c) and (d) synthesized 6dG (retention time: 14.8 min); (e) and (f) co-injection of commercial dG and synthesized 6dG (retention times: 13.9 and 14.8 min, respectively).

X-ray crystallographic analysis and theoretical calculations, have confirmed the existence of 6-thioguanine in different tautomeric forms (186-188). Tautomerism in 6-selenoguanine has also been investigated using high-level ab initio calculations (189). Of the different tautomers possible, the 6-selenol form was found to be the most stable in gas phase, whereas the 6-selenone form was predicted to be the most stable in aqueous solution (figure 2.8). The introduction of selenium-functionality also influences the pKₐ of N-1 imino proton in guanosine; deprotonation generates the more stable 6-selenolic form (189,190). Interestingly, during the pKₐ measurements on 6dG, we observed that the 6-selenolic form has a distinct UV-absorption profile (figure 2.9 A). The protona-
tion-deprotonation of $^\text{Se}dG$ and $dG$ (for comparison) was monitored by UV spectrophotometry in solutions with pH values ranging from 1 to 12 (figure 2.9 A and B respectively). In basic solutions, the absorption maximum of $^\text{Se}dG$ shifts to ~330 nm, whereas, the absorption maximum is red-shifted to ~357 nm under neutral and acidic conditions. Under highly acidic conditions (pH 1), the nucleobase is completely protonated and absorbs around 367 nm (figure 2.9 A). Taking advantage of the strong dependence of absorption on solution pH, we calculated the $pK_a$ of $^\text{Se}dG$ by recording the UV-Vis spectra at different pH values. The $pK_a (7.57 \pm 0.02)$ of $^\text{Se}dG$ was obtained from the fitted titration plot (figure 2.10).

Figure 2.8 Tautomers of 6-seleno-2'-deoxyguanosine, $^\text{Se}dG$.

Figure 2.9 UV-Vis spectra: absorption profiles of (A) $^\text{Se}dG$ and (B) $dG$, as a function of pH.
2.7.3 Emission behavior of 6-Seleno-2'-Deoxyguanosine: pH Effect

Guanine and its nucleosides/nucleotides are known to fluoresce in extreme pH conditions (pH ~1) (136,139). The low pH luminescence is dictated by the electron distribution at the guanine base in such conditions. Thus, it is meaningful to investigate the impact of introducing selenium-functionality into the guanosine base; especially the fluorescence of Se-dG as a function of pH since the electron-rich Se atom alters the dG electron distribution. Therefore, we measured the fluorescence of Se-dG in solutions with pH values ranging from 1 to 12 (figure 2.11). For the direct comparison, fluorescence from dG was also measured (figure 2.12). The pH-dependent fluorescence profile obtained for dG is consistent with data presented in some early reports (136,139). With an excitation wavelength of 258 nm (same as the absorption maximum), dG has maximum fluorescence emission at 395 nm. However, the pH dependant emission from Se-dG narrates a different story: with an excitation wavelength of 305 nm (which is not the same as the absorption maximum of ~ 360 nm) Se-dG has maximum fluorescence emission at 395 nm. It is probably due to the fluorescence quenching by solvent (water) molecules that no emission is observed on excitation of Se-dG at 360 nm. Unlike dG, Se-dG is practically non-emissive under strong acid conditions (pH 1-2). Se-dG is fluorescent in solutions with other pH, with maximum emission at pH 6.0. Our experiments also indi-
cate that \textsuperscript{5}dG is fluorescent under physiological pH 7.4. The concentration-dependent fluorescence profiles, at pH 6 and pH 7.4, are presented in figures 2.13 and figure 2.14, respectively.

Figure 2.11 Fluorescence spectra of \textsuperscript{5}dG in aqueous solutions with pH 1-12: (A) Excitation spectra of \textsuperscript{5}dG as a function of pH at 25 °C; the emission wavelength was 390 nm. (B) Emission spectra of \textsuperscript{5}dG as a function of pH at 25 °C, with excitation at 305 nm.

Figure 2.12 Fluorescence spectra of dG in aqueous solutions with pH 1-12: (A) Excitation spectra of dG as a function of pH at 25 °C; the emission wavelength was 395 nm. (B) Emission spectra of dG as a function of pH at 25 °C, with excitation at 258 nm.
2.7.4 Emission behavior of 6-Seleno-2'-Deoxyguanosine: Solvent Effect

Solvent polarity is a significant parameter when considering any environmental effect on the absorption and emission spectra (147,191-193). Thus, we measured the UV-Vis absorption of SdG in different solvents and observed that SdG nucleoside exhibits interesting solvatochromicity (figure 2.15). In general, the UV-absorption maximum is red-shifted to approximately 370 nm in polar solvents. A detailed analysis on the fluorescence of SdG, in solvents with different polarities, was also performed (figure 2.16). Excitingly, SdG is noticeably emissive in iso-propanol and has little or no fluorescence in other solvents used in this study, which suggests that the solvent polarity
can significantly affect the emission profile. Such polarity-dependent emission is probably due to ground state interactions between the nucleoside and the solvent molecules. The excitation spectra of Se-dG in different solvents were monitored at 405 nm. We found that the fluorescence excitation maxima were slightly red-shifted to 320 nm in the organic solvents. Concentration-dependent fluorescence profiles for Se-dG in isopropanol (figure 2.17) and ethanol (figure 2.18) were also obtained.

![Figure 2.15 UV-Vis spectra: absorption profile of Se-dG in different solvents at 25 °C.](image)

![Figure 2.16 Fluorescence spectra of Se-dG in different solvents: (A) Excitation spectra of Se-dG in different solvents at 25 °C; the emission wavelength was 405 nm. (B) Emission spectra of Se-dG in different solvents at 25 °C; the excitation wavelength was 320 nm.](image)
Figure 2.17 Concentration-dependent fluorescence spectra of $^{\text{Se}}$dG in iso-propanol: (A) Excitation spectra of $^{\text{Se}}$dG at 25 °C; the emission wavelength was 395 nm. (B) Emission spectra of $^{\text{Se}}$dG at 25 °C; the excitation wavelength was 320 nm.

Figure 2.18 Concentration-dependent fluorescence spectra of $^{\text{Se}}$dG in ethanol: (A) Excitation spectra of $^{\text{Se}}$dG at 25 °C; the emission wavelength was 395 nm. (B) Emission spectra of $^{\text{Se}}$dG at 25 °C; the excitation wavelength was 320 nm.

2.8 Conclusions

We have developed a simple strategy for synthesizing 6-seleno-2'-deoxyguanosine ($^{\text{Se}}$dG). Although structurally similar to 2'-deoxyguanosine (dG), $^{\text{Se}}$dG exhibits distinctive optical properties. Unlike dG, the single atom modified $^{\text{Se}}$dG is a bright yellow solid and is fluorescent under physiological conditions. To our knowledge, this is the first observation of a fluorescent nucleoside obtained by single-atom alteration. We also found that the absorption and emission of $^{\text{Se}}$dG are sensitive to pH and solvent polarity. Thus, the Se-modified nucleoside has great potential as a useful visual and fluorescent probe for to investigate protein-nucleic acid interactions and further contribute to mo-
molecular understanding of the protein dynamics. In particular, this fluorescent Se-nucleoside would be very useful in studying the structure and dynamics of trans-membrane transporter proteins for understanding the mechanisms of active transportation in biological membranes at the molecular level and identifying the molecular basis of the action of drugs that target the transporter proteins.
3 CHEMICAL SYNTHESIS AND FLUORESCENCE STUDY OF 6-SELENO-2'-DEOXYGUANOSINE DERIVATIZED OLIGODEOXYRIBONUCLEOTIDES

3.1 Synthetic Oligodeoxyribonucleotides

The rational design of drugs and therapeutic agents has become more plausible with our increasing understanding of biochemistry and molecular and cellular biology. Chemically modified nucleic acids, with their diverse functionalities, have drawn widespread attention from multidisciplinary research teams. Ample growth has occurred in developing optimized and automated methods for the preparation of modified nucleic acids, which serve as significant tools in fundamental research as structure-and-function probes, chemical probes, and mechanistic probes. Synthetic oligonucleotides have been useful probes in exploring the biochemical processes and pathways. Availability of diverse variety of modified nucleosides, development of functionalized phosphoramidites, and proficient methods for the solid-phase as well as enzymatic synthesis, have augmented the utility of such oligonucleotides. Various reports have underscored the synthesis and application of synthesis oligonucleotides with modifications in the sugar, purine/pyramidine heterocyclic base, and the phosphodiester backbone (15,30,167,194-201).

Selenium-modified nucleic acids (SeNAs) are valuable derivatives for crystallographic phase determination (49,55,63,66,202). SeNA are promising candidate for crystallographic phasing and other biological applications. Huang and coworkers have developed diverse building units for the incorporation of 2-Se-thymidine (Se²T) (57), 4-Se-thymidine (Se⁴T) (1), 6-Se-guanosine (Se⁶G) (2,50), and 2-Se-uridine (Se²U)(51,53) into DNA and RNA by solid-phase synthesis. Crystallographic studies with the corresponding selenium-modified oligonucleotides demonstrate improved stability and binding affinity, with insignificant structural perturbation. Structurally these are very similar to the native oligonucleotides. Interestingly, the atom-specific replacement of oxygen with selenium in the nucleobases also imparts a bright yellow color to the otherwise colorless nucleoside (hence, the corresponding nucleotide and oligonucleotide). For instance, relative to the native dG (UV λmax= 254
nm, $\varepsilon = 1.22 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$), the absorption maximum of Se$_{6}\text{dG}$ (UV $\lambda_{\text{max}} = 360$ nm, $\varepsilon = 2.3 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$) is red-shifted by 100 nm, resulting in yellow DNA(2). This large shift in the absorption maximum is likely due to the greater delocalization of selenium electrons, thereby lowering the excitation energy of the nucleotide.

Herein we report the solid-phase synthesis of short oligodeoxyribonucleotides (trimers) functionalized with 6-seleno-deoxyguanosine nucleoside. The purified and characterized oligos were analyzed for their optical behavior.

### 3.2 Material and Methods

#### 3.2.1 Solid-Phase Synthesis of 6-Seleno-2'-Deoxyguanosine Derivatized Oligodeoxyribonucleotides

Synthesis of $^6\text{dG}$ phosphoramidite: N$^2$-[2-[(4-tert-butyl-phenoxy)acetyl]-6-(2-cyanoethyl)seleno-2'-deoxyguanosine phosphoramidite (5, scheme 3.1) was synthesized from N$^2$-[2-[(4-tert-butyl-phenoxy)acetyl]-6-(2-cyanoethyl)seleno-5'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxyguanosine (2, scheme 2.1) using a previously published procedure (2): Compound 2(250 mg, 0.29 mmol) and 5-(benzylthio)-1H-tetrazole (BTT, 27 mg, 0.15 mmol) were weighed out in a 15 mL flask and dried over nighton a high vacuum. The compounds were then dissolved in anhydrous methylene chloride (1 mL) under argon. After this 2-cyanoethyl N,N,N,N-tetraisopropylphosphorodiimid (phosphoramidite, 103 mg, 0.34 mmol, 1.2 eq.) was injected into the reaction flask and the solution was allowed to stir at room temperature for 30 min under argon. The progress of the reaction was monitored by recording TLC (30% ethyl acetate in CH$_2$Cl$_2$): a mixture of two diastereomers is obtained. After the complete consumption of starting compound (as indicated by TLC) the reaction was quenched with saturated sodium bicarbonate (NaHCO$_3$, 3 mL), stirred for 5 min. and extracted with CH$_2$Cl$_2$ (3 x 5 mL). The combined organic layer was dried over anhy. MgSO$_4$, filtered and the solvent was evaporated under reduced pressure. The crude product
(mixture of two diastereomers) was purified on a silica gel column equilibrated with CH₂Cl. The product was eluted with (30% ethyl acetate in CH₂Cl₂). The product obtained from the column was further dissolved in CH₂Cl₂ (1 mL) and precipitated from pentane (200 mL) as a pale green solid (230 mg, 75 % yield).

Scheme 3.1 Synthesis of 6-seleno-2'-deoxyguanosine modified oligonucleotides, 6.

**Synthesis of Se-dG Oligodeoxyribonucleotides (6):** an automated Applied BioSystem 394 DNA Synthesizer (Applied Biosystems, Foster City, CA) was used to synthesize all the modified oligodeoxyribonucleotides (ODNs), employing standard β-cyanoethylphosphoramidite chemistry (1 μmol scale). Only ultra-mild native nucleoside phosphoramidite reagents (Glen Research) were used in the synthesis. Oligonucleotides were synthesized in DMTr-on mode. The synthesized ODNs were cleaved from the beads and simultaneously deprotected (cyanoethyl and (4-tert-butylphenoxyl)-acetyl protecting groups) by treating with 0.05 M K₂CO₃/methanol solution at room temperature for 12 h. The solution was then neutralized with 2 M TEAAc buffer (pH 7.1) and purified.
by RP-HPLC. For 5’-DMTr deprotection, the lyophilized ODNs were acidified to pH 5 using acetic acid and heated at 45 °C for 1 h. After complete detritylation (monitored by analysis on HPLC), the samples were neutralized with 2 M TEAAc buffer (pH 7.1) and re-purified by RP-HPLC. The samples were then desalted using Sep-Pak C18 3 cc Vac column.

### 3.2.2 HPLC Analysis and Purification

The DMTr-on modified ODNs were eluted at 260 nm, with a flow-rate of 6 mL/min [buffer A: 10 mM TEAAc (pH 7.1) in water; buffer B: 10 mM TEAAc (pH 7.1) in 50% acetonitrile] and a linear gradient from 10 % buffer B to 100 % buffer B in 15 min. The desired peaks were collected and the buffer was removed by lyophilization. All the samples were analyzed on Welchrom C18-XB column (4.6*250 mm) at 260 nm and 360 nm, and measured at a flow rate of 1.0 mL/min and a linear gradient of 5 % to 60 % buffer B in 8 min, reaching 100 % buffer B in 12 min [buffer A: 20 mM TEAAc (pH 7.1) in water; buffer B: 20 mM TEAAc (pH 7.1) in 50% acetonitrile]. The DMTr-off modified ODNs were also eluted at 260 nm, with a flow-rate of 6 mL/min [buffer A: 10 mM TEAAc (pH 7.1) in water; buffer B: 10 mM TEAAc (pH 7.1) in 50% acetonitrile] and a linear gradient from 5 % buffer B to 70 % buffer B in 15 min, reaching 100 % buffer B in 20 min. The desired peaks were collected and the buffer was removed by lyophilization. All the samples were analyzed on Welchrom C18-XB column (4.6*250 mm) at 260 nm and 360 nm, and measured at a flow rate of 1.0 mL/min and a linear gradient of 5 % to 40 % buffer B in 15 min, reaching 100 % buffer B in 17 min [buffer A: 20 mM TEAAc (pH 7.1) in water; buffer B: 20 mM TEAAc (pH 7.1) in 50% acetonitrile].

### 3.2.3 Desalting Using Sep-Pak C18 Vac Column

The DMTr-off ODNs were desalted using Sep-Pak C18 3 cc Vac column: the column was equilibrated by eluting pure acetonitrile (1 mL), followed by water (1 mL) and TEAAc buffer (2 M, pH 7.1, 1 mL). The detritylated sample (dissolved in 0.5 mL water) was loaded on the column. The sample was desalted by washing with water (3 X 1 mL) and eluting desalted ODNs with 40 % ace-
tonitrile in water (0.6 mL). All the fractions were analyzed by UV-Vis. The product containing fraction was lyophilized to dryness. The samples were then quantified by UV-Vis analysis and used for fluorescence measurements.

### 3.2.3 Optical Analysis of $^6$Se$^6$dG ODNs

All absorption measurements were made in de-ionized water (unless mentioned). The emission measurements were made in 10 mM sodium phosphate buffer (of specified pH). The pH of the buffer was adjusted using HCl or NaOH solutions. For all the fluorescence measurements (unless mentioned) sample concentrations were fixed at 10 µM, and the excitation and emission slit widths were fixed at 10 nm. The baseline was subtracted at all times. The stock solutions were prepared in de-ionized water for all measurements.

### 3.3 Results and Discussion

#### 3.3.1 Synthetic Design

A list of 6Se-dG-modified oligodeoxyribonucleotides used in this study is presented in table 3.1. The $^6$Se$^6$dG derivatized ODNs were synthesized using ultramild phosphoramidites. Strong basic conditions, like ammonia treatment, for the deprotection of the nucleobase cause de-selenization of the modified $^6$Se$^6$dG nucleobase. Thus, ultramild amine protecting group [([4-tert-butyl-phenoxy)acetyl]] was used. These ultramild protecting groups can be removed under ultramild deprotection conditions, i.e. 0.05 M K$_2$CO$_3$ in methanol (r.t., 12 h). The ODNs could be synthesized either in DMT$_{r}$-on or DMT$_{r}$-off mode.

Table 3.1 Sequences of $^6$Se$^6$G-ODNs used for fluorescence measurements.

1. d-(A$^6$SeGA)
2. d-(G$^6$SeGG)
### 3.3.2 UV-Vis Analysis

The DMTr-off and desalted modified ODNs were analyzed by UV-Vis spectrophotometry. All the Se\textsuperscript{d}G-derivatized ODNs are yellow in color and exhibit two discreet absorption bands: one at ~260 nm corresponding to absorption by native nucleobases and, other at 360 nm arising from the selenium modification in the nucleobase. The Se\textsuperscript{d}G derivatized ODNs have a strong tendency to dimerize. The dimer has a blue-shifted absorption maximum at ~330 nm. Thus, a broad absorption band in UV-Vis spectrum, stretching from 330-360 nm, indicates the presence of both monomer and dimer species in the sample solution. Figure 3.1 presents the UV-Vis analysis of the synthesized and purified ODNs. The UV-Vis analysis of A\textsuperscript{Se}GA in pH 6 and pH 9 is also shown. It clearly demonstrates conversion to a dimeric species, with absorption ~ 330 nm, at pH 9 whereas at pH 6 it still has a strong absorption at 360 nm, indicating the presence of a 6Se-dG monomer. The extinction coefficient for Se\textsuperscript{d}G at 260 nm (5.3 x 10\textsuperscript{3} M\textsuperscript{-1}cm\textsuperscript{-1}) and 360 nm (2.3 x 10\textsuperscript{4} M\textsuperscript{-1}cm\textsuperscript{-1}) have been previously reported (2). The extinction coefficient for Se\textsuperscript{d}G at 260 nm was used to calculate the quantity of the ODNs obtained.

![Absorption spectra of Se\textsuperscript{d}G-modified oligodeoxyribonucleotides.](image)
3.3.3 HPLC Analysis

The HPLC analysis of the synthesized and purified \( \text{Se}d\text{G} \)-derivatized ODNs further supported the dimerization of \( \text{Se}d\text{G} \) under experimental conditions. The RP-HPLC profiles for \( A^{\text{Se}GA} \) and \( G^{\text{Se}GG} \) trimers, at wavelengths 260 nm, 330 nm and 360 nm, are shown in figure 3.2. The \( A^{\text{Se}GA} \) trimer, has two peaks with retention times of 11.9 min and 13.7 min (figure 3.2 A). The peak at 11.9 min is from the trimer with \( \text{Se}d\text{G} \) in the form of a monomer, as the signal corresponding to 330 nm is very weak and 360 nm is strong. The peak with a retention time of 13.7 min is from the trimer where \( \text{Se}d\text{G} \) has already dimerized as the signal corresponding to 330 nm is stronger and 360 nm is weaker. A similar pattern is also observed for \( G^{\text{Se}GG} \) trimer (figure 3.2 B). The retention time for the peak corresponding to \( \text{Se}d\text{G} \) monomer is 10.5 min whereas the peak illustrating dimerized \( \text{Se}d\text{G} \) has a retention time of 13.1 min.

![Figure 3.2](image)

Figure 3.2 RP-HPLC analysis of \( A^{\text{Se}GA} \) and \( G^{\text{Se}GG} \) trimers [monitored at 260 nm (black), 330 nm (green) and 360 nm (red)]; A. Purified \( A^{\text{Se}GA} \) sample (retention time: 11.9 min for \( \text{Se}d\text{G} \) monomer and 13.7 min for \( \text{Se}d\text{G} \) dimer); B. Purified \( G^{\text{Se}GG} \) sample (retention time: 10.5 min for \( \text{Se}d\text{G} \) monomer and 13.1 min for \( \text{Se}d\text{G} \) dimer).

3.3.4 Fluorescence Analysis

The emission behavior of \( A^{\text{Se}GA} \) and \( G^{\text{Se}GG} \) trimers was analyzed under physiological pH (7.4). The samples were excited at different wavelengths to get different emission profiles. But it appears that the fluorescence of \( \text{Se}d\text{G} \) is quenched in these ODNs. The strongest emission was observed with excitation at 260 nm and 305 nm (figure 3.3). The emission from \( A^{\text{Se}GA} \) is stronger
compared to emission from G^SeGG trimer. This indicates that the neighboring nucleobases have a significant effect on the fluorescence arising from ^Se_dG.

Figure 3.3 Emission spectra of A^SeGA and G^SeGG trimers in solution with pH 7.4: (A) excitation at 260 nm; (B) excitation at 305 nm.

3.4 Conclusions

We have demonstrated that 6-seleno-2′-deoxyguanosine derivatized oligodeoxyribonucleotides can be successfully synthesized using the strategy we developed recently (2). The synthesized ODNs are yellow in color and structurally similar to the native ODNs with only a single atom modification. Like the ^Se_dG nucleoside, ^Se_dG-modified ODNs also have interesting absorption profile with two discreet absorption bands at ~ 260 nm and 360 nm. Unlike the ^Se_dG nucleoside, the fluorescence from ^Se_dG-modified ODNs appears to be significantly quenched. The emission from ^Se_dG-modified ODNs is also dependant on the neighboring bases of ^Se_dG. But being strongly yellow in color, the Se-derivatized ODNs have great potential as a useful visual probe for nucleic acid transportation, diagnosis and dynamics.
SYNTHESIS OF 6-SELENO-2'-DEOXYGUANOSINE-5’-TRIPHOSPHATE AS A BIOCHEMICAL PROBE

4.1 Synthetic Nucleotides

Nucleotides play crucial role in several metabolic processes like DNA polymerization, RNA transcription and protein synthesis. Nucleotides are powerful building units for creating functional molecules with novel properties. They can be easily modeled and functionalized at various positions with diverse functional groups. The basic structure of a nucleotide consists of three components: a base, a sugar and a phosphate group. All these three components have various active sites which were and still are prime targets for chemists for various modifications. Various modifications have helped scientists to have an in-depth understanding about nucleic acid structures, functions and dynamics like the factors responsible for stronger Watson-Crick base pairing, helical backbone, thermodynamic stability of DNA/RNA duplexes, efficient cross-pairing and improved binding and catalysis (203-209). Nucleoside triphosphates serve as direct precursors for chemical and enzymatic synthesis of modified (unnatural) nucleic acids. Synthetic nucleotides also serve as efficient substrates to a variety of DNA and RNA polymerases (210-215) and have been helpful in probing the ability and specificity of DNA polymerases (216) and binding and catalytic ac. The modification of nucleic acids presents enormous opportunities for tailoring the properties and functions of these biomolecules, escalating their potential in biochemical and biomedical applications (10-20).

4.2 Selenium Functionalized Nucleotides

Selenium derivatization of nucleic acids generates modified biopolymers with novel properties. In search of bioactive compounds, a variety of modified nucleosides and nucleotides, with selenium functionalization at different positions in the nucleobase, sugar, and phosphate group, have been successfully achieved by Huang’s research group, along with other groups (46,47,49,50,53,55,57-66). Selenium-derivatized nucleic acids entail unique structural and func-
tional features like, stability and compatibility with chemical and enzymatic synthesis, which make
them strong contenders for biochemical and biophysical research. The synthesis of nucleoside tri-
phosphate analogues with selenium at the non-bridging α-position of phosphate has been success-
fully demonstrated by Huang and co-workers (217,218). Owing to the chirality of the seleno-
phosphate, the synthesis results in the formation of $R_p$ and $S_p$ diastereomers. As a typical example,
both the diastereomers obtained from the synthesis of thymidine 5'-($\alpha$-P-seleno)-triphosphate ($\alpha$-
Se-TTP) were subjected to enzymatic synthesis of $\text{PSe-DNAs}$ (217). It was observed that the DNA
polymerase is capable of recognizing both the diastereomers to the same capacity as the natural
TTP. The $S_p$ and $R_p$ diastereomers of the Se-derivatized nucleotides are instrumental for the funda-
mental understanding of the structure and biophysical chemistry of the polymerases, nuclease,
kinases and phosphatases. Synthesis of a yellow 4-Se-thymidine-5'-triphosphate ($\text{Se}$TTP) from the 4-
Se-thymidine nucleoside was also successfully achieved by the Huang group (3). This modified
$\text{Se}$TTP acts as a good substrate for DNA polymerase and is recognized with virtually the same effi-
ciency as the native TTP. The enzymatic incorporation of $\text{Se}$TTP also results in yellow DNA. These
colored Se-DNAs could serve as unique visual probes for enzymatic assays. Thus, the nucleotides
and nucleic acids obtained by atom-specific substitution of oxygen with selenium, could serve as
useful biophysical, structural, and mechanistic probes (1,2,49-51,55,57,66).

4.3 Fluorescent Nucleotide Substrates

Fluorescent nucleotides, with well analyzed fluorescence behavior and which are good sub-
strates to the polymerases, can be used to a great advantage in a variety of studies including the dy-
namics and mechanism of substrate binding and transformation. With such potential significance,
considerable efforts have been made towards the development of fluorescent nucleotides. Several
fluorescent nucleotide analogs have been reported in literature (114,219-227). These modified
fluorophores also find use in diverse applications as molecular probes and nucleic acid reporters.
But most of the reported fluorescent nucleotides have severe limitations: some are neither sub-
strates nor inhibitors of polymerases (219,228), some have low quantum yields, while some are structurally perturbing or incompatible. Most of the reported fluorescent nucleotides are colorless, thus making them unsuitable for probing the structure and dynamics of nucleic acids. On the other hand, selenium derivatization of the nucleobase imparts a strong yellow color to the otherwise colorless nucleotides and nucleic acids, resulting in strongly colored DNAs and RNAs. Incorporation of a single Se atom in the nucleobases can cause over a 100 nm red-shift in the absorption maximum of the nucleobases (1-3). This distinctive property is very useful in nucleic acid-based detection and visualization. With their unique spectroscopic properties, the Se-DNAs have great potential in detection, visualization, and profiling of nucleic acids and their complexes. Herein, we report the first chemical synthesis of a fluorescent nucleotide 6-Se-2'-deoxyguanosine-5'-triphosphate (Se-dGTP) obtained by single atom replacement of C6 oxygen with selenium. With discrete optical properties, the selenium-modified triphosphate is bright yellow and measurably fluorescent under physiological conditions. It is a good substrate for DNA polymerase and results in nuclease-resistant yellow DNA. All these unique features make Se-dGTP a strong candidate for an effective nucleic acid visualization probe.

4.4 Material and Methods

4.4.1 Synthesis of 6-Seleno-2'-Deoxyguanosine-5'-Triphosphate

N²-tBPAc-6-CE-Se-dG (3, scheme 2.1, 10 mg, 0.0178 mmol) was weighed in a 10 mL r.b. flask and dried overnight under vacuum. 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (7.2 mg, 2 eq.) was dried in a flask for 10 min. Tri-n-butylammonium pyrophosphate (25 mg, 3 eq.) was dried overnight in a separate flask, under high vacuum. The pyrophosphate was dissolved in DMF (0.3 mL) with additional TBA (0.1 mL) and transferred into the flask containing 2-chloro-4H-1,3,2-benzodioxoa-phosphorin-4-one, dissolved in DMF (0.2 mL). The mixture was flushed with argon and allowed to stir at room temperature. After 30 min. the mixture was transferred into the flask con-
taining vacuum dried compound 3 dissolved in dioxane (0.2 mL) and DMF (0.1 mL). The combined contents were allowed to stir at room temperature for 4 h to yield the cyclic phosphite intermediate 8 (scheme 4.1). The cyclic-phosphite 8 was then oxidized by adding iodine solution (0.02% I₂/THF/Pyridine/H₂O, 1 mL). The mixture was stirred for 1 h and then hydrolyzed with water (3 mL) and allowed to stir at room temperature for 4 h to ensure complete hydrolysis. The sample was precipitated using NaCl (0.3 M)/ ethanol (3 volumes). The precipitate was analyzed by recording a TLC (5:3:2 of iso-ProOH:NH₃·H₂O:H₂O). The crude N²-[2-(4-tert-butyl-phenoxy)acetyl]-6-(2-cyanoethyl)seleno-2'-deoxyguanosine-5'-triphosphate, N²-tBPAc-6-CE-Se-dGTP, 9 was then purified by reverse phase HPLC. HRMS (ESI-TOF): molecular formula, C₃₅H₃₃N₆O₁₄P₃Se; [M-H⁻]: 813.0338 (calc. 813.0360); ¹H NMR (400 MHz, D₂O) δ: 1.19(s, 9H, 3x CH₃-tBu), 2.58-2.63 and 2.81-2.88 (2x m, 2H, H-2'), 3.11(t, J = 6.76 and 6.92 Hz, 2H, Se-CH₂-CH₂-CN), 3.51(t, J = 6.36 and 6.88 Hz, 2H, Se-CH₂·CH₂-CN), 4.17-4.23 (m, 2H) and 4.30 (br, 1H) (H-3' and H-5'), 6.54 (t, J = 6.66 Hz, 1H, H-1'), 6.89-6.92 (d, J = 8.68 Hz, 2H, CH-arom), 7.30-7.33 (d, J = 8.64 Hz, 2H, CH-arom), 8.59 (s, 1H, H-8); ¹³C NMR(100 MHz, D₂O)δ: 19.46 and 19.85 (Se-CH₂-CN), 31.52 (CH₃-tBu), 34.85 (C-tBu), 39.85 (C-2'), 63.33 (C-5'), 66.32 (CH₂-O), 71.85 (C-3'), 84.47 (C-4'), 86.81 (C-1'), 114.94 and 127.36 (CH-arom), 121.46 (CN), 131.06 (C-5), 143.57 (C-8), 146.08 and 159.30 (C-arom), 148.88 (C-6), 151.91 (C-4), 155.37 (C-2), 170.55 (C=O); ³¹P NMR (162 MHz, D₂O) δ: -22.56 (t, J₈₋₆ = 19.88 and 19.94 Hz, 1P, P-β), -10.98 (d, J₆ = 19.76 Hz, 1P, P-α), -9.43 (d, J₇ = 19.70 Hz, 1P, P-γ).

HPLC purified N²-tBPAc-6-CE-Se-dGTP (9) was treated with 0.05 M K₂CO₃ (4:1 = MeOH: H₂O; 5 mL) at room temperature for 12 h, to remove the 2-cyanoethyl and (4-tert-butyl-phenoxy)-acetyl protecting groups. The pure 6-seleno-2'-deoxyguanosine-5'-triphosphate (Se-dGTP, 10) was recovered as a bright yellow solid by NaCl (0.3 M)/ ethanol (3 volumes) precipitation. The precipitates were analyzed by UV-Vis, HPLC and TLC (5:3:2 of iso-ProOH: NH₃·H₂O: H₂O). No further purification was carried out. HR-MS (ESI-TOF): molecular formula, C₁₅H₁₆N₅O₁₂P₃Se; [M-H⁻]: 569.9087 (calc. 569.9101); ¹H NMR (400 MHz, D₂O) δ: 2.52-2.56 and 2.83 -2.29 (2x m, 2H, H-2'), 4.24-4.29 (m, 3H,
H-3’ and H-5’), 6.36 (t, 1H, $J_{1'-2'} = 6.68$ and $6.8$ Hz, H-1’), 8.35 (s, 1H, H8); $^{13}$C NMR (100 MHz, CD$_3$OD) δ: 35.98 (C-2’), 62.96 (C-5’), 68.45 (C-3’), 83.35 (C-4’), 83.41 (C-1’), 138.75 (C-5), 139.51 (C-8), 148.74 (C-4), 157.37 (C-2); $^{31}$P NMR (162 MHz, D$_2$O) δ: -21.60 (t, $J_{\beta} = 19.05$ and 20.07 Hz, 1P, P-β), -10.87 (d, $J_{\alpha} = 18.95$ Hz, 1P, P-α), -6.22 (d, $J_{\gamma} = 19.67$ Hz, 1P, P-γ).

### 4.4.2 HPLC Analysis and Purification

The crude N$^2$-tBPAc-6-CE-Se-dGTP (9) was purified and analyzed by reversed-phase high performance liquid chromatography (RP-HPLC). The N$^2$-tBPAc-6CE-Se-dGTP was eluted at 305 nm, with a flow-rate of 6 mL/min [buffer A: 20 mM TEAc (pH 7.1) in water; buffer B: 20 mM TEAc (pH 7.1) in 50% acetonitrile] and a linear gradient from 5% buffer B to 100% buffer B in 30 min. The desired peak was collected and the buffer was removed by lyophilization. All samples were analyzed on Welchrom C18-XB column (4.6*250 mm) and measured at a flow rate of 1.0 mL/min and a linear gradient of 0 to 40% buffer B in 25 min [buffer A: 20 mM TEAc (pH 7.1) in water; buffer B: 20 mM TEAc (pH 7.1) in 50% acetonitrile]. The yellow precipitates of $^{s_e}$dGTP (7) were analyzed at 260 and 360 nm wavelengths with a flow rate of 1.0 mL/min and a linear gradient of 0 to 25% buffer B in 20 min (figure 4.2).

### 4.4.3 UV-Vis Absorption and Fluorescence Study

All absorption spectra were recorded in de-ionized water (figures 4.1). All the emission measurements were made in 10 mM sodium phosphate buffer (figures 4.3 and 4.4). The pH of the buffer was adjusted using HCl or NaOH solutions. For all the fluorescence measurements (unless mentioned) sample concentrations were fixed at 5 µM, and the excitation and emission slit widths were fixed at 15 nm. The baseline was subtracted at all times. The $^{s_e}$dGTP stock solution was prepared in water for all measurements.
**4.4.4 Synthesis of Oligonucleotides**

The DNA promoter (P) and template (T) sequences (table 4.1) were synthesized on an automated Applied BioSystem 394 DNA Synthesizer (Applied Biosystems, Foster City, CA) employing standard β-cyanoethylphosphoramidite chemistry (1 μmol scale). Oligonucleotides were synthesized in DMTr-off mode, with average coupling efficiency greater 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, crushed and soaked in water. The oligonucleotides were 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.

**4.4.5 Enzymatic Incorporation Studies**

DNA primers were labeled at the 5'-terminus by incubating in the presence of γ-[^32]P]-ATP (Perkin Elmer, Boston, MA) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA) for 1 h at 37 °C. Polymerase reactions were carried out by adding klenow exo-minus DNA polymerase (Epicentre Biotechnologies, Madison, WI) to a premixed solution containing DNA primer(s) (P1 and P2, table 3.1) and DNA template(s) (T1 and T2, table 3.1), dNTPs (dATP, dCTP and TTP, Epicentre Biotechnologies, Madison, WI), dNTP substrate (dGTP/SeGTP) and DTT in klenow exo-minus DNA polymerase 10X reaction buffer at 37 °C. For the full length primer extension experiments, the reaction mixtures were incubated at 37 °C for 2 hr. and quenched by the addition of the gel loading dye solution (5 μL each, containing 50 % glycerol, 0.60 % xylene cyanol, 0.60 % bromphenol blue, 20 mL of 1 mM EDTA, saturated urea). The analysis was performed on 19%, polyacrylamide gel electrophoresis (PAGE). For the time course polymerization reactions, aliquots (5 μL) were removed at designated time points, and combined with gel loading buffer (5 μL). Reaction progress was monitored over time by 19% denaturing gel electrophoresis. For single nucleotide enzymatic incorporation reactions, no other dNTPs, except for substrate dNTPs (dGTP/SeGTP), were added to the reac-
tion mixture. The reaction mixtures were incubated at 37 °C for 1 hr. and analyzed on 19%, polyacrylamide gel. Polymerase reactions contained primer (1.5 μM), template (5 μM), dNTPs (0.1 mM each), DTT (5 mM), and 10X reaction buffer (1X) in a final reaction volume of 5 μL.

4.4.6 Nuclease Resistance Studies of dG-DNA and 5eG-DNA

Enzymatic digestion of dGTP and 5eGTP, by Exonuclease III (Promega Corporation, Madison, WI), was studied over time. Primer (P2) was 5’-32P-labeled using T4 polynucleotide kinase and [γ-32P]-ATP. The DNA polymerization was performed using primer P2 (1.5 μM), template T2 (5 μM), dGTP / 5eGTP and other dNTPs (0.1 mM), DTT (5 mM), and Klenow exo(-) DNA polymerase (0.02 U/μL) at 37 °C for 2 h. The polymerized DNA was extracted by NaCl (0.3 M)/ ethanol (3 volumes) precipitation. The dG-DNA and 5eG-DNA were dissolved in Exonuclease III 10X Reaction Buffer (660mM Tris-HCl (pH 8.0) and 6.6mM MgCl2; final concentration: 1X) and were enzymatically digested using Exonuclease III (0.0002 U/μL) at 37 °C. Aliquots of the reaction mixture were taken at the indicated time points and quenched with gel loading buffer and analyzed by 19% polyacrylamide gel electrophoresis (figure 4.10).

4.5 Results and Discussion

4.5.1 Synthetic Design

6-Se-2'-deoxyguanosine-5'-triphosphate (10, 5eGTP, scheme 4.1) was synthesized via a “new synthetic methodology” developed in our laboratory for protection-free triphosphate synthesis (229,230). In this strategy, 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one and tri-n-butylammonium pyrophosphate are used to form a triphosphite intermediate which reacts with the nucleoside to form P2, P3-dioxo-P1-5'-nucleosidycyclotriphosphite (8). The synthesis of nucleoside N2-tBPAC-6-CE-Se-dG (3) is outlined in scheme 2.1. The nucleoside 3 was selectively phosphitylated by a mild phosphitylating reagent generated in situ from the reaction of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one and tri-n-butylammonium pyrophosphate. The cyclic phosphite in-
Intermediate (8) so obtained was oxidized and hydrolyzed to generate $N^2$-tBPAC-6-CE-Se-dGTP (9). The cyanoethyl and (4-tert-butyl-phenoxy)-acetyl protecting groups were removed under ultra mild conditions (0.05 M $K_2CO_3$ in 4:1 methanol/water) to generate 6-Se-2'-deoxyguanosine-5'-triphosphate (10). The Se dGTP was purified by HPLC, analyzed by UV-Vis and HPLC and characterized using mass spectrometry and NMR ($^1H$, $^{13}C$, $^{31}P$) spectroscopy.

Scheme 4.1 Synthesis of 6-seleno-2'-deoxyguanosine-5'-triphosphate (Se dGTP).
4.5.2 Absorption and Emission Properties

The native 2'-deoxyguanosine-5'-triphosphate (dGTP) shows an absorption maximum at 252 nm. The \( \text{N}^2\text{-}[2\text{-}(4\text{-tert-butyl-phenoxy})\text{acetyl}]\text{-6-}\text{(2-cyanoethyl)seleno-2'}\text{-deoxyguanosine-5'}\text{-triphosphate} \) (9) shows two discreet absorption bands at 256 nm and 305 nm (figure 3.1). When the protecting groups are completely removed, the resulting 6-Se-2'-deoxyguanosine-5'-triphosphate (10) shows absorption maximum at 357 nm (~ 105 nm red shift compared to the native dGTP). Under basic conditions, 6-Se-2'-deoxyguanosine-5'-triphosphate deprotonates and forms a dimer. The triphosphate dimer shows an absorption maximum around 330 nm. The dimer can be reduced by using \( \text{B}_2\text{H}_6\)-tetrahydrofuran complex or dimer formation can be avoided by keeping the solution pH slightly acidic (< 6.5). HPLC analysis of commercial dGTP and synthesized \( \text{s}^\text{dGTP} \) (10), at 260 nm and 360 nm, further supported our observation (figure 4.2). \( \text{s}^\text{dGTP} \) absorbs at both 260 nm (slightly) and 360 nm (strongly), whereas native dGTP has no absorbance at 360 nm.

![Figure 4.1 Absorption spectra of dGTP, N^2-tBPAc-6-CE-Se-dGTP, s^dGTP, and s^dGTP-dimer in water at 25 °C.](image)
Motivated by the exciting emission behavior of the 6-Se-2'-deoxyguanosine (4), we decided to investigate the fluorescence properties of 6-Se-2'-deoxyguanosine-5'-triphosphate (10). The triphosphate 10 is fluorescent at physiological pH 7.3. Unlike the nucleoside 4 (which has maximum emission at 390 nm with an excitation at 305 nm, figure 2.12), the triphosphate 10 has a red shifted emission at 445 nm and the excitation wavelength of 380 nm is close to its absorption maximum (~360 nm) (figure 4.3). In general the fluorescence emission from $^{57}$dGTP is low compared to the emission from $^{57}$dG, but it is measureable. A pH dependant emission profile for $^{57}$dGTP was obtained using solutions with pH 1 – 11 and it was observed that the triphosphate has maximum fluorescence at pH 7.3 (figure 4.3). Concentration-dependent fluorescence profile of $^{57}$dGTP at pH 7.3 is presented in figure 4.4. On further analysis, compound 9 was found to be non-emissive under all the experimental conditions.

Figure 4.2 RP-HPLC analysis of dGTP and $^{57}$dGTP [monitored at 260 nm (black) and 360 nm (red)]: (a) and (b) commercial dGTP (retention time: 12.4 min); (c) and (d) synthesized $^{57}$dGTP (retention time: 13.6 min); (e) and (f) co-injection of commercial dGTP and synthesized $^{57}$dGTP (retention times: 12.4 and 13.6min, respectively).
**Figure 4.3** Fluorescence spectra of $^{34}$dGTP in aqueous solutions: (A) Excitation spectra of $^{34}$dGTP as a function of pH at 25 °C; the emission wavelength was 445 nm. (B) Emission spectra of $^{34}$dGTP as a function of pH at 25 °C, with excitation at 380 nm.

**Figure 4.4** Concentration-dependent fluorescence spectra of $^{34}$dGTP at pH 7.3: (A) Excitation spectra of $^{34}$dGTP at pH 7.3 and 25 °C; the emission wavelength was 450 nm. (B) Emission spectra of $^{34}$dGTP at pH 7.3 and 25 °C; the excitation wavelength was 380 nm.

### 4.5.3 Enzymatic Synthesis of dG-DNA and $^{34}$dG-DNA

To investigate the incorporation efficiency of 6-Se-2'-deoxyguanosine-5'-triphosphate (10) into DNA, polymerization reactions were performed in the presence of klenow exo minus DNA polymerase (Kf-). A list of DNA primers and templates used for the primer extension reactions is presented in table 4.1.
Sequences of DNA primers and templates used for the enzymatic incorporation of s^dGTP. The underlined bases are the sites for dGTP and s^dGTP incorporation.

**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 CCC TGG -5'
- **T2** (55-mer) 3'-d-GGC ATT ATG CTG AGT GAT ATC CGT TGG ACT CGT TCCGG GTT TGC ATG T -5'

Single nucleotide incorporation of the dGTP and s^dGTP into DNA was studied using a short DNA template (T1). The DNA polymerization was performed with primer (1.5 μM), template (5 μM), dGTP / s^dGTP (0.1 mM), and Klenow exo(-) DNA polymerase (0.002 U/μL) and analyzed by 19% polyacrylamide gel electrophoresis. Like dGTP, s^dGTP also acts as a good substrate for DNA polymerase (figure 4.5). From the intensity of the product DNA band it appears that the s^dGTP is well recognized by the DNA polymerase and the single selenium-atom substitution does not have a significant structural impact. The single nucleotide incorporation was confirmed by MALDI-MS analysis (figure 4.6). A 64 Dalton mass difference between the resultant dG-DNA and s^dG-DNA indicates the incorporation of Se-modified guanosine base.

![Figure 4.5 Enzymatic incorporation of a single nucleotide (dGTP and s^dGTP) into DNA (T1): a) Enzymatic incorporation of dGTP and s^dGTP by Klenow exo(-) on DNA template T1. b) Gel electrophoresis autoradiography of the polymerization reaction. Lanes 2 and 3 show single nucleotide incorporation forming 18-nucleotide long DNA.](image-url)
Figure 4.6 MALDI-MS analysis for enzymatic incorporation of a single nucleotide (dGTP and $^\text{Se}$dGTP) into DNA (T1): MS spectra of dG-extended DNA (O-18-mer, calculated m/z for [M+H]$^+$: 5644, observed m/z for [M+H]$^+$:5648); MS spectra of $^\text{Se}$dG-extended DNA (Se-18-mer, calculated m/z for [M+H]$^+$: 5708, observed m/z for [M+H]$^+$: 5712); T1: calculated m/z for [M+H]$^+$:6322, observed m/z for [M+H]$^+$:6329.

We also performed a time-course experiment, using P1/T1 for single nucleotide incorporation, to compare the efficiency of incorporation (and recognition) of $^\text{Se}$dGTP with native dGTP. The single nucleotide incorporation was performed with primer (1.5 μM), template (5 μM), dGTP/$^\text{Se}$dGTP (0.1 mM), and Klenow exo(-) DNA polymerase (0.0002 U/μL) at 37 °C and analyzed by 19% polyacrylamide gel electrophoresis. The experiment was monitored over a period of 120 min. Aliquots of the reaction mixture were taken at the indicated time points and quenched with gel loading buffer. The results are presented as gel electrophoresis autoradiography in figure 4.7 (a). It was observed that $^\text{Se}$dGTP is more selective in controlling the reaction at single nucleotide incorporation, whereas native dGTP shows extended n+2 DNA product (probably as a result of formation of G/T wobble pair) along with the desired n+1 DNA. Thus, under these conditions, the polymerization efficiency of dGTP and $^\text{Se}$dGTP appear similar (figure 4.7 (b)); the single atom selenium modification inhibits G/T wobble pair formation.
Figure 4.7 Time course of the incorporation of single nucleotide (dGTP and $^{32}$dGTP) into DNA (T1): a) Gel electrophoresis autoradiography of the incorporation reaction over time (120 min). b) Plot of the incorporation of dGTP and $^{32}$dGTP into DNA with respect to time.
a) $5'\cdot32P\cdot$ dGCG TAA TAC GAC TCA CTA TAG-3'

3'-dGCG ATT ATG CTG AGT GAT ATC CGT TGG ACT ACT CGG GCT TTC CGG CTT TGG ATG T-5'

\[
\text{dGTP/SeGTP}\quad\text{Other dNTPs}\quad\text{Kf, 37 °C, 1 h}
\]

$5'\cdot32P\cdot$ dGCG TAA TAC GAC TCA CTA TAG GCA ACC TGA TGA GGC CGA AAG GCC GAA ACG TAC A-3'

3'-dGCG ATT ATG CTG AGT GAT ATC CGT TGG ACT ACT CGG GCT TTC CGG CTT TGG ATG T-5'

b)

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Figure 4.8 Enzymatic incorporation of all natural dNTPs, dGTP and SeGTP into DNA (T2): a) Enzymatic incorporation of all dNTPs, dGTP and SeGTP by Klenow exo(-) on DNA template T2. b) Gel electrophoresis autoradiography of the polymerization reaction.

We also studied the efficiency of incorporation of more Se-modified guanosine nucleotides using a longer DNA template (T2). The incorporation was carried out by extending a 21 nucleotide primer along a 55 nucleotide template. The DNA polymerization was performed with primer (1.5 μM), template (5 μM), dGTP /SeGTP and other dNTPs (0.1 mM), and Klenow exo(-) DNA polymerase (0.04 U/μL) and analyzed by 19% polyacrylamide gel electrophoresis. Figure 4.8 shows the primer extension using T2 template. This polymerization involved the incorporation of ten SeGTP, and in two locations along the template, two SeGTP had to be incorporated consecutively. The full extension SeG-DNA product migrated faster compared with the product band for dG-DNA, probably due to the electronic effect of selenium atom. This difference in DNA mobility is more pronounced for the 55 nucleotide long product, which involves the incorporation of ten SeGTP, than for the 18 nucleotide product obtained by extending a 17 nucleotide primer along a 21 nucleotide
template, involving the incorporation of only one $^\text{Se}d\text{GTP}$ (figure 4.5 (b)). Further experiments are needed to confirm/demonstrate the efficient incorporation of multiple $^\text{Se}d\text{GTP}$s.

a)

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<th>$^\text{Se}d\text{GTP}$</th>
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Figure 4.9 Time course of the incorporation of all natural dNTPs, dGTP and $^\text{Se}d\text{GTP}$ into DNA (T2): a) Gel electrophoresis autoradiography of the polymerization reaction over time (120 min). b) Plot of the incorporation of dGTP and $^\text{Se}d\text{GTP}$ (along with other dNTPs) into DNA with respect to time.

Through these enzymatic incorporation studies we observed that $^\text{Se}d\text{GTP}$ was tolerated as a substrate for DNA polymerase. We performed a time-course experiment for this primer-template set as well to compare the efficiency of incorporation of multiple modified bases. The DNA polymerization was performed with primer (1.5 μM), template (5 μM), dGTP /$^\text{Se}d\text{GTP}$ and other dNTPs (0.1 mM), and Klenow exo(-) DNA polymerase (0.0015 U/μL for dGTP and 0.015 U/μL for $^\text{Se}d\text{GTP}$ ) at 37 °C and analyzed by 19% polyacrylamide gel electrophoresis. Aliquots of the reaction mixture were taken at the indicated time points and quenched with gel loading buffer. The results are presented as gel electrophoresis autoradiography in figure 4.9 (a). Under the experimental conditions used, the incorporation of dGTP was more efficient yielding the 55 nucleotide dG-DNA in ~ 5 min. On the
other hand, the polymerization involving $^\text{Se}$dGTP was stalled for $\sim$ 5 min. at some mid-point but gave full length 55-mer $^\text{Se}$dG-DNA product after 90 min. of incubation. Figure 4.9 (b) shows a plot of the incorporation of dGTP and $^\text{Se}$dGTP (along with other dNTPs) into the DNA T2 with respect to time. This study indicated that single atom modification in $^\text{Se}$dGTP is somewhat less tolerated as a substrate by the DNA polymerase compared with the native dGTP while extending longer DNAs.

### 4.5.4 Nuclease Resistance Studies of dG-DNA and $^\text{Se}$dG-DNA

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<th>t/min</th>
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<th>dGTP</th>
<th>$^\text{Se}$dGTP</th>
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![Gel electrophoresis autoradiography](image)

![Plot of digestion](image)

Figure 4.10 Time course of the enzymatic digestion of dGTP and $^\text{Se}$dGTP incorporated DNA with Exonuclease III. a) Gel electrophoresis autoradiography; b) plot of digestion of dG-DNA and $^\text{Se}$dG-resistant DNA with respect to time.

We also conducted some experiments to study the resistance of the $^\text{Se}$dG-DNA, in comparison to the native dG-DNA, towards nuclease. For this, we synthesized the DNA along the 55 nucleotide template (T2) and purified it by NaCl/ethanol precipitation (to remove any unconsumed dNTPs and short fragments). The precipitated 55 nucleotide long DNAs, dG-DNA with ten dGTP and $^\text{Se}$dG-DNA with ten $^\text{Se}$dGTP, were treated with exonuclease III and the reaction was monitored over a period of 25 min. Aliquots of the reaction mixture were taken at the indicated time points and
quenched with gel loading buffer. The gel electrophoresis autoradiography and plot of digestion of dG-DNA and Se\textsuperscript{2}dG-DNA with respect to time are shown in figure 4.10. It was observed that dG-DNA started fragmenting within 5 minutes of treatment with nuclease, whereas the Se\textsuperscript{2}dG-DNA offered more resistance to the nuclease, losing only few nucleotides over the course of the experiment.

4.6 Conclusions

Herein, we report the first chemical synthesis of 6-Se-2'-deoxyguanosine-5'-triphosphate (Se\textsuperscript{2}dGTP). We have demonstrated that the simple, one-pot and protection-free method for triphosphate synthesis developed in Dr. Huang’s laboratory could be successfully used for the synthesis of modified triphosphates. The synthesized Se\textsuperscript{2}dGTP has distinct optical properties. With only a single atom modification, this Se-modified triphosphate is bright yellow (like the nucleoside Se\textsuperscript{2}dG) and measurably fluorescent at physiological pH 7.3. It displays a discreet absorption band at ~ 360 nm. In general the fluorescence emission from Se\textsuperscript{2}dGTP is low compared to the emission from Se\textsuperscript{2}dG, but it is measureable. With an excitation wavelength of 380 nm, Se\textsuperscript{2}dGTP displays a weak fluorescence emission at 445 nm. The synthetic Se-DNA is significantly nuclease-resistant compared to the native DNA, and is efficiently recognized by DNA polymerase. Since the Se\textsuperscript{2}dGTP is yellow in color and measurably fluorescent at physiological pH, it can used as an effective biochemical probe for exploring the dynamics of interactions of nucleotides with proteins and small molecules. The Se\textsuperscript{2}dG-DNA being yellow in color, also offers potential applications as deoxyribonucleic acid (DNA) visualization probe.
5 SYNTHEISIS OF 6-SELENOGUANOSINE-5'-TRIPHOSPHATE

5.1 Selenium Functionalized Ribonucleoside Triphosphates

Selenium, in the form of selenocysteine (Sec), has been found in several charged tRNAs such as tRNA (Sec). This Se-containing tRNA is the longest characterized mammalian tRNA (90 nt in length) and its anticodon is complementary to the stop codon UGA (231,232). The secondary structures of tRNA (Sec) differ among the three domains of life: bacteria, eukarya and archaea (233,234). The selenium nucleoside, identified as 5-[(methylamino)methyl]-2-selenouridine (mnm$^5$se$_2$U), is often found at the first or wobble position of the anticodon loop of tRNA (43,44). While the precise function of selenium at the C-2 position and the comprehensive utility of Se-derivatized tRNAs are not delineated, this modification is essential for catalysis and regulation and probably capable of augmenting translational efficiency and efficacy (45). The substitution of oxygen with selenium in the nucleobases permits a search for novel aspects of nucleic acid base-pairing and stacking interactions at the atomic level. Huang and coworkers have developed diverse building units like, 6-seleno-guanosine (Se$_6$G) (50) and 2-seleno-uridine (Se$_2$U) (53), for their incorporation into RNA by solid-phase synthesis. The 2-seleno-modification has a pronounced ability to discriminate wobble base-pairing. Structural and biophysical studies on 2-seleno-uridine containing oligonucleotides highlight the increased fidelity of Se$_2$U/A base pairs. Huang's research group was the first to develop the enzymatic synthesis of stereo-defined phosphoroselenoates (PSe) (217,218). This enzymatic synthesis is based on the recognition of the $S_P$ and/or $R_P$ diastereomers of $\alpha$-Se-dNTPs or $\alpha$-Se-NTPs by the polymerases. Only the $S_P$ diastereoisomer of $\alpha$-Se-ATP is efficiently recognized by T7 RNA polymerase, enabling synthesis of diastereomerically-pure PSe-RNAs (218). Later, the $\alpha$-Se-NTPs ($\alpha$-Se-ATP, $\alpha$-Se-GTP, $\alpha$-Se-CTP and $\alpha$-Se-UTP) were used to transcribe hammerhead ribozyme to generate diastereomerically-pure PSe-RNAs for structural and mechanistic studies (235). Recently, Huang and coworkers have developed a novel, efficient, one-pot synthesis of $\alpha$-Se-NTPs without protecting the nucleosides and used this method for the large-scale synthesis of $\alpha$-Se-NTPs for PSe-
RNA transcription (48). Moreover, Se-modification renders the PSe-RNAs resistant to nucleases, thereby making them potential therapeutic agents. Recently, we have demonstrated that the synthesized SeUTP is stable and recognizable by T7 RNA polymerase (51). Under the optimized conditions, the transcription yield of SeU-RNA can reach up to 85% of the corresponding native RNA. Furthermore, the transcribed SeU-hammerhead ribozyme has the similar activity as the corresponding native, which suggests usefulness of SeU-RNAs in function and structure studies of non-coding RNAs.

Herein, we report the synthesis of 6-selenoguanosine-5'-triphosphate (SeGTP). With only a single atom modification, this Se-modified triphosphate is bright yellow in color. It could potentially be used for exploring the structure and dynamics of RNA and its complexes with proteins and small molecules/ligands.

5.2 Materials and Methods

5.2.1 Synthesis of N²-Phenoxyacetyl-6-(2-Cyanoethylseleno)-Guanosine

N²-phenoxyacetyl-6-(2-cyanoethylseleno)-guanosine (4, scheme 5.1) was synthesized from commercially available starting material N²-phenoxyacetyl-2'-(tert-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)guanosine, 1. 2'-O-(tert-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)-2-phenoxyacetyl-6-(2-cyanoethyl)-selenoguanosine nucleoside (2) was synthesized from compound 1 using a published procedure (50). N²-phenoxyacetyl-6-(2-cyanoethylseleno)-guanosine (4) was obtained from compound 2 by selectively removing the 2'-O-(tert-butyldimethylsilyl) and 5'-O-(4,4'-dimethoxytrityl) protecting groups (scheme 5.1). For deprotecting the 2'-TBDMS group TEA.3HF was used. Compound 2 was weighed in a 25 mL r.b. flask (2 g, 2.11 mmol) and dissolved in THF (5 mL). The desilylation reagent TEA.3HF (1 mL) was then added to the reaction mixture. The reaction was allowed to stir overnight at 40 °C. During, this time the reaction (desilylation) was about 90 % complete (as monitored on TLC in 10 % methanol in dichloromethane). The reaction mixture was neutralized and the solvent was evaporated under reduced pressure. No further puri-
fication was performed and the crude product was used as such for detritylation reaction. For deprotecting the 5'-DMTr group, dichloroacetic acid (3 %, 1 mL) was added to this crude reaction mixture and stirred at room temperature. The trityl cation was quenched with methanol (1 mL). More dichloroacetic acid was added. Reaction progress was monitored by TLC (10 % methanol in dichloromethane). The reaction mixture was neutralized and the solvent was evaporated under reduced pressure to yield the crude product 4. The mixture was diluted with ethyl acetate and washed with saturated NaCl (3 x 10 mL). The organic layer was dried over anhydrous MgSO₄, filtered, and concentrated to yield crude product. The crude product was purified via silica gel column chromatography (solvent gradient from pure dichloromethane up to 7 % methanol in dichloromethane) to give N²-phenoxyacetyl-6-(2-cyanoethylseleno)-guanosine (4) as a dirty white solid (0.8886 g, 79 %). HRMS (ESI-TOF): molecular formula, C₂₁H₂₂N₆O₆Se; [M+H]⁺: 535.0848 (calc. 535.0839); ¹H NMR (400 MHz, CD₃SOCD₃) δ: 3.17-3.21 (m, 3H) and 3.54-3.57 (m, 3H)[Se-CH₂-CH₂-CN, Se-CH₂-CH₂-CN and H-5'), 3.94 (m, 1H, H-4'), 4.17 (br, 1H, H-3'), 4.57 (br, 1H, H-2'), 4.99 [(2H, CH₂-O), (1H, 5'-OH)], 5.23 and 5.53 (b, 2 H, 2'-OH and 3'-OH), 5.93-5.95 (d, J₁⁻₂= 8 Hz, 1H, H-1'), 6.94-6.97 (m, 3H, CH-arom), 7.31 (t, J = 8 Hz, 2H, CH-arom), 8.64 (s, 1H, H-8), 10.88 (s, 1H, NH); ¹³C NMR (100 MHz, CD₃SOCD₃) δ: 18.48 and 19.03 (Se-CH₂-CH₂-CN), 61.17 (C-5'), 67.12 (CH₂-O), 70.24 (C-3'), 73.81 (C-2'), 85.65 (C-4'), 87.25 (C-1'), 114.49 120.94 and 129.50 (CH-arom), 119.97 (CN), 130.32 (C-5), 142.72 (C-8), 148.93 (C-6), 151.77 (C-4), 157.51 (C-2), 157.87 (C-arom), 167.28 167.23 (C=O).

5.2.2 Synthesis of 6-Selenoguanosine-5'-Triphosphate

N²-phenoxyacetyl-6-(2-cyanoethylseleno)-guanosine (4, 25 mg, 0.047 mmol) was weighed in a 10 mL r.b. flask and dried overnight under vacuum. 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (19 mg, 2 eq.) was dried in a flask for 10 min. Tri-n-butylammonium pyrophosphate (108 mg, 5 eq.) was dried overnight in a separate flask, under high vacuum. The pyrophosphate was dissolved in DMF (0.3 mL) with additional TBA (0.1 mL) and transferred into the flask containing 2-
chloro-4H-1,3,2-benzodioxaphosphorin-4-one, dissolved in dioxane (0.4 mL). The mixture was flushed with argon and allowed to stir at room temperature. After 45 min. the mixture was transferred into the flask containing vacuum dried compound 4 dissolved in dioxane (0.4 mL) and DMF (0.15 mL). The combined contents were allowed to stir at room temperature for 4 h to yield the cyclic phosphite intermediate 5 (scheme 5.2). The cyclic phosphite 5 was then oxidized by adding iodine solution (0.02% I$_2$/THF/Pyridine/H$_2$O, 1 mL). The mixture was stirred for 1 h and then hydrolyzed with water (3 mL) and allowed to stir at room temperature for 4 h to ensure complete hydrolysis. The sample was precipitated using NaCl (0.3 M)/ ethanol (3 volumes). The precipitate was analyzed by recording a TLC (5:3:2 of iso-PrOH:NH$_3$.H$_2$O:H$_2$O).

The crude N$_2$-phenoxyacetyl-6-(2-cyanoethyl)selenoguanosine-5'-triphosphate, N$_2$-n-PAc-6-CE-SeGTP, 6 was then purified by reverse phase HPLC. HRMS (ESI-TOF): molecular formula, C$_{21}$H$_{25}$N$_6$O$_{15}$P$_3$Se; [M-H]+: 772.9684 (calc. 772.9683); $^1$H NMR (400 MHz, D$_2$O) δ: 3.54 (t, $J = 6.72$ and 6.76 Hz, 2H, Se-CH$_2$-CH$_2$-CN), 4.28 (b, 2H, H-5'), 4.42 (b, 1H, H-4'), 4.62 (b, 1H, H-3'), 4.89 (br, 3H, CH$_2$-O and H-2'), 6.16-6.18 (d, $J_{1'-2'} = 5.56$ Hz, 1H, H-1'), 6.97-7.02 (m, 3H, CH-arom), 7.29-7.33 (t, $J = 7.64$ and 7.72 Hz, 2H, CH-arom), 8.60 (s, 1H, H-8); $^{13}$C NMR (100 MHz, D$_2$O) δ: 19.54 and 19.88 (Se-CH$_2$-CH$_2$-CN), 66.14 (C-5'), 67.98 (CH$_2$-O), 71.24 (C-3'), 75.25 (C-2'), 84.97 (C-4'), 88.08 (C-1'), 115.21, 122.88 and 130.59 (CH-arom), 121.53 (CN), 131.03 (C-5), 143.35 (C-8), 149.13 (C-6), 151.94 (C-4), 157.50 (C-2), 180.68 (C=O); $^{31}$P NMR (162 MHz, D$_2$O) δ: -23.13 (t, $J_\beta = 17.82$ and 19.44 Hz, 1P, P-β), -11.48 (d, $J_\alpha = 19.44$ Hz, 1P, P-α), -10.46 (d, $J_\gamma = 19.70$ Hz, 1P, P-γ).

HPLC purified N$_2$-n-PAc-6-CE-SeGTP 6 was stored, as a dry pale green solid, in -80 °C refrigerator until further use. Only a small portion of the purified sample was treated with 0.05 M K$_2$CO$_3$ (4:1 = MeOH: H$_2$O; 5 mL) at room temperature for 12 h, to remove the 2-cyanoethyl and phenoxyacetyl protecting groups. The pure 6-selenoguanosine-5'-triphosphate (SeGTP, 7, scheme 5.2) was recovered as a bright yellow solid by NaCl (0.3 M)/ ethanol (3 volumes) precipitation.
molecular formation of $^{35}$-GTP was confirmed by MS analysis. HR-MS (ESI-TOF): molecular formula, $C_{10}H_{16}N_{5}O_{13}P_{3}Se$; [M-H$^{-}$]: 585.9030 (calc. 585.9050).

5.2.3 HPLC Purification of 2-Phenoxyacetyl-6-(2-Cyanoethylseleno)-Guanosine-5'-Triphosphate

The crude $N^2$-n-Pac-6-CE-SeGTP (6) was purified and analyzed by RP-HPLC. The $N^2$-n-Pac-6-CE-SeGTP was eluted at 305 nm, with a flow-rate of 6 mL/min [buffer A: 20 mM TEAAc (pH 7.1) in water; buffer B: 20 mM TEAAc (pH 7.1) in 50% acetonitrile] and a linear gradient from 5 % buffer B to 100 % buffer B in 30 min. The desired peak was collected and the buffer was removed by lyophilization. The pure fractions were analyzed on Welchrom C18-XB column (4.6*250 mm) and measured at a flow rate of 1.0 mL/min and a linear gradient of 0 to 40 % buffer B in 25 min [buffer A: 20 mM TEAAc (pH 7.1) in water; buffer B: 20 mM TEAAc (pH 7.1) in 50% acetonitrile]. The HPLC profile of the purified $N^2$-n-Pac-6-CE-SeGTP, at 260 nm and 305 nm wavelengths, is shown in figure 5.1.

5.3 Results and Discussion

5.3.1 Synthetic Design

$2'$-O-(tert-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)-2-phenoxyacetyl-6-(2-cyanoethyl)-selenoguanosine (2, scheme 5.1) was synthesized from compound 1 using a published procedure (50). $N^2$-phenoxyacetyl-6-(2-cyanoethyl)selenoguanosine nucleoside ($N^2$-n-Pac-6-CE-SeG, 4) was obtained from compound 2 by selective deprotection of the $2'$-TBDMS and 5'-DMTr groups. Since strong basic conditions can cause deselenization of the base, phenoxyacetyl (n-Pac) was used to protect the $2$-NH$_2$ of this guanosine nucleoside and 2-cyanoethyl was used to protect selenium (to avoid any oxidation under the experimental conditions). Both these groups can be deprotected under ultramild conditions (0.05 M K$_2$CO$_3$ in methanol).
Scheme 5.1 Synthesis of 2-phenoxyacetyl-6-(2-cyanoethylseleno)-guanosine, 4.

N²-n-Pac-6-CE-SeG was then used to synthesize N²-phenoxyacetyl-6-(2-cyanoethyl)selenoguanosine-5'-triphosphate (N²-n-Pac-6-CE-SeGTP, 6) via the “new synthetic methodology” developed in our laboratory for protection-free triphosphate synthesis (229,230). In this strategy, the triphosphate intermediate, formed by the reaction of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one with tri-n-butylammonium pyrophosphate, reacts with the nucleoside to form \( P^2 \), \( P^3 \)-dioxo-\( P^1 \)-5'-nucleosidylcyclotriposphite (5). The synthesis of nucleoside N²-n-Pac-6-CE-SeGTP (6) is outlined in scheme 5.2. The N²-n-Pac-6-CE-SeGTP was purified and analysed by HPLC, and characterized using mass spectrometry and NMR (\(^1\)H, \(^{13}\)C, \(^{31}\)P) spectroscopy.

This protected triphosphate will be further deprotected under ultra mild conditions (0.05 M K₂CO₃ in 4:1 methanol/water) to generate 6-selenoguanosine-5'-triphosphate (SeGTP, 7). The synthesized and purified SeGTP will be used as a substrate for RNA transcription using T7 RNA polymerase.
Scheme 5.2 Synthesis of 6-selenoguanosine-5'-triphosphate (\(^{79}\)GTP).
5.3.2 HPLC Purification of 2-Phenoxyacetyl-6-(2-Cyanoethylseleno)-Guanosine-5’-Triphosphate

The crude N²-n-Pac-6-CE-SeGTP (6) was purified and analyzed by RP-HPLC. The N²-n-Pac-6-CE-SeGTP was eluted at 305 nm, with a flow-rate of 6 mL/min [buffer A: 20 mM TEAAc (pH 7.1) in water; buffer B: 20 mM TEAAc (pH 7.1) in 50% acetonitrile] and a linear gradient from 5% buffer B to 100% buffer B in 30 min. The desired peak was collected and the buffer was removed by lyophilization. All the dry fractions were analyzed on Welchrom C18-XB column (4.6*250 mm) and measured at a flow rate of 1.0 mL/min and a linear gradient of 0 to 40% buffer B in 25 min [buffer A: 20 mM TEAAc (pH 7.1) in water; buffer B: 20 mM TEAAc (pH 7.1) in 50% acetonitrile (figure 5.1).

Figure 5.1 RP-HPLC analysis of n-Pac-6-CE-SeGTP, 6, monitored at 260 nm (black) and 305 nm (red) [retention time: 31.04 min].

5.3.3 Proposed Study

The synthesized and purified 6-selenoguanosine-5’-triphosphate (SeGTP, 7) will be enzymatically incorporated into RNA. It may or may not act as a good substrate for transcribing RNA using T7 RNA polymerase. Since G is the first base introduced in any transcription process, there might be a need for certain optimization of the experimental conditions to facilitate transcription. This study might help us better understand the fidelity of the T7 RNA polymerase. Some un-natural
RNA polymerases might also be used for transcribing $^{\text{Se}}$GTP into RNA. These experiments might also help in generating longer selenium derivatized RNAs for exploring the structure and dynamics of RNA and its complexes with proteins and small molecules/ligands.

5.4 Conclusions and Future Prospects

Herein, we report the first chemical synthesis of 6-selenoguanosine-5'-triphosphate ($^{\text{Se}}$GTP). We have demonstrated that the simple, one-pot and protection-free method for triphosphate synthesis developed in Dr. Huang’s laboratory could be successfully used for the synthesis of modified triphosphates. With only a single atom modification, this Se-modified triphosphate is bright yellow in color. It could potentially act as a good substrate for generating longer selenium derivatized RNAs for exploring the structure and dynamics of RNA and its complexes with proteins and small molecules/ligands. Since the single-atom selenium modification renders a strong yellow color to the synthetic $^{\text{Se}}$G-RNA (50), it could be potentially used as an RNA visualization probe.
SYNTHESIS AND BIOCHEMICAL STUDY OF S-MERCAPTOPROPYL-5-(MERCAPTO-METHYL)-2'-DEOXYURIDINE-5'-TRIPHOSPHATE

6.1 Introduction

The use of nanoparticles is evolving in present day therapeutics. They promise a bright future in photodiagnostics and photothermal therapy of cancers and other diseases (236,237). In particular, there has been more focus on the gold nanoparticles (AuNPs) in biochemistry and nanomedicine. A variety of ligands have been discovered to stabilize AuNPs, but the best proven stabilizers have been thiolates. Thiolates form robust AuNPS by the virtue of strong Au-S bond between soft acid Au and the soft thiolate base. Functional thiolates have also been used along this line. Oligonucleotides, peptides and PEGs are easily attached to AuNPs in this way (236). AuNPs have been successfully used as colorimetric biosensors which utilize the color change arising from the interparticle plasmon coupling during AuNP aggregation (red-to-purple or blue) or redispersion of an AuNP aggregate (purple-to-red) (238-244). This nature of gold nanoparticles has been applied for sensing enzymatic activity as well (245,246). Exploring enzymatic reactions involving nucleoside triphosphates as substrates is of vital significance in molecular biology.

The stability of non-modified AuNPs is sensitive to various factors. Hence, different methods have been designed to increase their stability, which can be accomplished by using different kinds of stabilizers like, surfactant, polymer, nucleoside or oligonucleotide. Nucleobases are capable of binding with the citrate-capped AuNPs by displacing the weakly bound citrate ions via metal-ligand interactions. It has also been demonstrated that the nucleotides (adenosine mono-, di- and triphosphates) are further capable of stabilizing the AuNPs in the order ATP> ADP> AMP (245). Cytidine triphosphate (CTP) stabilized AuNPs have been useful probes for detection of calcium ions (Ca²⁺) with high sensitivity and selectivity (247,248).

Herein, we report the synthesis of a novel thiol functionalized triphosphate, S-mercaptopropyl-5-(mercapto-methyl)-2'-deoxyuridine-5'-triphosphate (10, scheme 6.2). The modi-
fication has been introduced to increase the ability of TTP to stabilize AuNPs. The synthetic DNA obtained by the incorporation of this thiol-functionalized TTP will be further used to study the stabilization of gold nanoparticles with reference to the native DNA. The study will be based on the strong affinity of gold nanoparticles for thiol-functionality. The modified DNA will also find use and application in DNA/RNA microchip surface functionalization for rapid detection of various diseases and pathogens.

6.2 Materials and Methods

6.2.1 Synthesis of S-(3-(Acetylthio)propyl)-5-(Mercaptomethyl)-2′-Deoxyuridine

S-(3(acetylthio)propyl)-5-(mercaptomethyl)-2′-deoxyuridine was synthesized from thymidine in seven steps (scheme 6.1). 3’, 5’-bis-O-(tert-butyldimethylsilyl)-thymidine(1): Thymidine (2 g, 0.0083 mol), tert-butyldimethylsilyl chloride (TBDMSi-Cl) (2.74 g, 0.0182 mol) and imidazole (3.39 g, 0.0498 mol) were weighed out in a 100 mL flask and dissolved in anhydrous DMF (10 mL). The reaction mixture was stirred for 12 h. at room temperature. The reaction progress was monitored by recording TLC (20 % ethyl acetate in CH2Cl2). After the complete consumption of the starting material, the reaction mixture was poured into ethyl acetate (20 mL) and extracted with sodium chloride (NaCl) solution (sat., 3 x 20 mL). The organic phase was dried over MgSO4. The solvent was evaporated under reduced pressure. The pale green oily syrup was purified via silica gel flash column chromatography (1 % MeOH in CH2Cl2) to give 3’, 5’-bis-O-(tert-butyldimethylsilyl)-thymidine(1, 3.83 g, 98 %) as a white flaky solid. HRMS (ESI-TOF): molecular formula, C22H42N2O5Si2; [M+H+]+: 471.2710 (calc. 471.2705); [M-H+]+Na+: 493.2557 (calc. 493.2524); 1H NMR (400 MHz, CDCl3) δ: 0.08-0.11 (m, 12H, 4 x CH3-Si), 0.88-0.93 (m, 18H, 6 x CH3-tBu), 1.92-2.01 (m, 3H, H-2’ and CH3-Si), 2.25 (b, 1H, H-2’), 3.78-3.93 (b, m, 3H, H-3’ and H-5’), 4.40 (br, 1H, H-4’), 6.35 (b, 1H, H-1’), 7.47 (b, 1H, H-6); 13C NMR (100 MHz, CDCl3) δ: -5.35 to -4.39 (CH3-Si), 12.73 (C5-
3', 5'-Bis-O-(tert-butyl(dimethyl)silyl)-5-bromomethyl-2'-deoxyuridine (2): Compound, 1 (400 mg, 0.8497 mmol), N-bromo-succinimide (378 mg, 2.124 mmol), and benzoyl peroxide (6 mg, 0.0248 mmol) were weighed out in a 50 mL flask and dissolved in dry CCl₄ (25 mL). The reaction was heated to reflux for 1.5 h, during which time the solution changed from colorless to orange. The appearance of orange color is indicative of formation of bromine in the reaction. The reaction progress was monitored by recording TLC (15 % ethyl acetate in CH₂Cl₂). The reaction was allowed to cool and filtered to remove insoluble succinimide. The solvent was evaporated under reduced pressure to yield crude 3', 5'-bis-O-(tert-butyl(dimethyl)silyl)-5-bromomethyl-2'-deoxyuridine (2) as a yellow solid. The crude compound 2 was used as such, without any further purification, for the next step (249). HRMS (ESI-TOF): molecular formula, C₂₂H₄₁BrN₂O₅Si₂; [M+H⁺]: 549.1806 (calc. 549.1810).

S-acetyl-3',5'-bis-O-(tert-butyl(dimethyl)silyl)-5-(mercaptomethyl)-2'-deoxyuridine(3): Potassium thioacetate (AcOSK, 388 mg, 3.3972 mmol) was weighed out in the flask containing crude compound 2 and dried under vacuum for about 15 min. Anhydrous DMF (2 mL) was added to the reaction flask, under argon atmosphere. The reaction was heated at 70 °C for 2 h. The reaction progress was monitored by recording TLC (15 % ethyl acetate in CH₂Cl₂). The reaction mixture was diluted with ethyl acetate (10 mL) and washed with NaCl solution (sat., 5 x 20 mL). The organic layer was dried using anhydrous MgSO₄. The solvent was evaporated under reduced pressure to yield dark brown oily syrup of crude compound 3. The crude product 3 was purified using preparative TLC plate (12 % EtOAc in CH₂Cl₂) to yield a brown product. HRMS (ESI-TOF): molecular formula, C₂₂H₄₄N₂O₆SSi₂; [M-H⁻]: 543.2379(calc. 543.2386); ¹H NMR (400 MHz, CDCl₃) δ: 0.05-0.08 (2 × s, 12H, 4 × CH₃-Si), 0.86-0.89 (2 × s, 18H, 6 × CH₃-tBu), 2.00 (b, 1H, H-2'), 2.26 (b, 4H, H-2' and CH₃-CO), 3.72 (s, 1H, C₅-CH₂-S-), 3.79 (s, 2H, H-5'), 3.90 (s, 1H, H-3'), 4.41 (s, 1H, H-4'), 6.25 (t, 1H, H-1').
$7.71$ (s, $1$H, H-6), $9.94$ (s, $1$H, NH); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$: -5.34 to -4.58 (CH$_3$-Si), 18.09-18.53 [tBuC-(CH$_3$)$_3$], 25.87-26.08 (CH$_3$-tBu), 30.58 (C5-C$_2^\equiv$), 41.07 (C-2'), 63.14 (C-5'), 72.22 (C-3'), 85.32 (C-4'), 87.93 (C-1'), 110.92 (C-5), 138.52 (C-6), 150.33 (C$_{\equiv}$=O), 163.13 (C$_{\equiv}$=O), 195.73 (CH$_3$-C=O).

3',5'-Bis-O-(tert-butyldimethylsilyl)-5-(mercaptomethyl)-2'-deoxyuridine (4): Compound 3 was dissolved in MeOH (3 mL) and hydrolyzed using 1 M NaOH/MeOH solution (1 mL). The reaction progress was monitored by recording TLC (20 % ethyl acetate in CH$_2$Cl$_2$). The reaction completed in 2 h at room temperature. The reaction mixture was neutralized with acetic acid (AcOH) and the solvent was evaporated under reduced pressure to yield dark brown solid. The crude compound 4 was used as such for the next step.

S-(3-bromopropyl)-3',5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptomethyl)-2'-deoxyuridine (5): Potassium carbonate (K$_2$CO$_3$, 470 mg, 3.4006 mmol) and dithiothreitol (DTT, 262 mg, 1.6985 mmol) were weighed out in the flask containing crude compound 4, and dried under vacuum for about 15 min. Anhydrous DMF (2 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 (15 % EtOAc in CH$_2$Cl$_2$). 1,3-Dibromopropane (0.8 mL, 0.0078 mmol) was then added dropwise, with vigorous stirring, to the reaction mixture, under argon atmosphere. The reaction was stirred at room temperature for 12 h. Reaction progress was monitored by TLC (15 % EtOAc in CH$_2$Cl$_2$). After the complete consumption of the intermediate formed by the reaction of DTT with compound 4, the reaction mixture was diluted with ethyl acetate (10 mL) and washed with NaCl solution (sat., 5 X 20 mL). The organic layer was dried using anhydrous MgSO$_4$. The solvent was evaporated under reduced pressure to yield brown oily compound 5. The crude product 5 was purified using preparative TLC plate (12 % EtOAc in CH$_2$Cl$_2$) to yield a golden-brown oily product. HRMS (ESI-TOF): molecular formula, C$_{25}$H$_{47}$BrN$_2$O$_5$SSi$_2$; [M+H$^+$]$: 623.2030$ (calc. 623.2000); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 0.04-0.06 (2 x s, 12H, 4 x CH$_3$-Si), 0.86-0.89 (2 x s, 18H, 6 x CH$_3$-tBu), 1.97 (m, 1H, H-2'), 2.29 (m, 1H, H-2'), 2.19 (m, 2H, Br-CH$_2$-CH$_2$-CH$_2$-S-), 3.42-3.51 (m, 6H, C$_5$-CH$_2$-S- and
Br-CH₂-CH₂-CH₂-S_), 3.74-3.83 (b, 2H, H-3' and H-5'), 3.93 (m, 1H, H-5'), 4.40 (m, 1H, H-4'), 6.30 (t, 1H, H-1'), 7.61 (s, 1H, H-6'); 13C NMR (100 MHz, CDCl₃) δ: -5.09 to -4.46 (CH₃-Si), 18.15-18.58 [Bu(CH₃)₃], 25.94 and 26.17 (CH₃-tBu), 30.68 (C5-CH₂-), 32.14 and 32.46 (Br-CH₂-CH₂-CH₂-S-), 36.24 (Br-CH₂-CH₂-CH₂-S-), 41.51 (C-2'), 63.25 (C-5'), 72.48 (C-3'), 85.37 (C-4'), 88.09 (C-1'), 112.26 (C-5), 137.45 (C-6), 150.25 (C₂=O), 163.20 (C₄=O).

S-(3(acetythio)propyl)-3',5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptomethyl)-2'-deoxyuridine (6): Potassium thioacetate (388 mg, 3.3972 mmol) and compound 5 were weighed out in a 25 mL flask and dried under vacuum for about 15 min. Anhydrous DMF (2 mL) was added to the reaction flask, under argon atmosphere. The reaction was heated at 70 °C for 3 h. Reaction progress was monitored by TLC (20 % EtOAc in CH₂Cl₂). The reaction mixture was diluted with ethyl acetate (10 mL) and washed with NaCl solution (sat., 5 X 20 mL). The organic phase was dried using MgSO₄. The solvent was evaporated in vacuo to yield brown oily compound 6. The crude product 6 was purified using preparative TLC plate (12 % EtOAc in CH₂Cl₂) to yield a golden brown oily product. HRMS (ESI-TOF): molecular formula, C₂₇H₅₀N₂O₆S₂Si₂; [M+H]⁺: 619.2733 (calc. 619.2727); 1H NMR (400 MHz, CDCl₃) δ: 0.08-0.12 (2 x s, 12H, 4 x CH₃-Si), 0.90-0.93 (2 x s, 18H, 6 x CH₃-tBu), 1.85-1.89 (m, 1H, H-2'), 1.97-2.04 (m, 1H, H-2'), 2.32-2.35, 2.58-2.63 and 2.93-2.99 (m, 9H, CO-CH₂-CH₂-CH₂-S- and CH₃-CO-), 3.41-3.47 (m, 2H, C5-CH₂-S-) 3.74-3.83 (m, 2H, H-3' and H-5'), 3.93-3.95 (m, 1H, H-5'), 4.41 (b, 1H, H-4'), 6.31 (t, 1H, H-1'), 7.61 (s, 1H, H-6); 13C NMR (100 MHz, CDCl₃) δ: -4.99 to -4.33 (CH₃-Si), 18.30-18.74 [Bu(CH₃)₃], 26.07-26.29 (CH₃-tBu), 27.90, 29.44, 30.95 and 31.60 (CH₃-CO-CH₂-CH₂-CH₂-S-CH₂-C5), 41.59 (C-2'), 63.41 (C-5'), 72.67 (C-3'), 85.50 (C-4'), 88.23 (C-1'), 112.45 (C-5), 137.50 (C-6), 150.22 (C₂=O), 163.20 (C₄=O), 195.76 (CH₃-CO-).

S-(3(acetythio)propyl)-5-(mercaptomethyl)-2'-deoxyuridine (7): Compound 6 (26.7 mg, 0.0431 mmol) was dissolved in THF (1 mL). Triethylamine trihydrofluoride (30 μL, 0.688 mmol) was added to the reaction flask, under argon atmosphere. The reaction was heated at 45 °C for 5 h. Reaction progress was monitored by TLC (10 % MeOH in CH₂Cl₂). The solvent was evaporated un-
der reduced pressure and the crude product 7 was purified using preparative TLC plate (10% MeOH in CH₂Cl₂) to yield 7 (1.5 mg, 9%) as a pale colored oily product. HRMS (ESI-TOF): molecular formula, C₁₅H₂₂N₂O₆S₂; [M-H]⁻: 389.0847 (calc. 389.0847); ¹H NMR (400 MHz, MeOD) δ: 1.81-1.85 (m, 2H, H-2'), 2.31 (s, 3H, CH₃), 2.25-2.30 (m, 2H, -CO-S-CH₂-CH₂-S-), 2.54 (t, 2H, -CO-S-CH₂-S-CH₂-S-), 2.95 (t, 2H, -CO-S-CH₂-CH₂-S-), 3.41 (s, 2H, C₅C₆), 3.76-3.80 and 3.95 (m, 3H, H-3' and H-5'), 4.43 (m, 1H, H-4'), 6.29 (t, J₁-₂ = 8 Hz, 1H, H-1'), 8.01 (s, 1H, H-6); ¹³C NMR (100 MHz, MeOD) δ: 28.47, 28.94, 30.33, 30.74 and 31.45 (C₅C₆), 41.54 (C-2'), 62.95 (C-5'), 72.34 (C-3'), 86.63 (C-4'), 89.01 (C-1'), 112.92 (C-5), 139.38 (C-6), 152.15 (C₂=O), 165.11 (C₃=O), 197.60 (CH₃-C=O).

6.2.2 Synthesis of S-Mercaptopropyl-5-(Mercaptomethyl)-2'-Deoxyuridine-5'-Triphosphate

Synthesis of S-(3(acetylthio)propyl)-5-(mercaptomethyl)-2'-deoxyuridine-5'-triphosphate, i.e. TTP (9, scheme 6.2): S-(3(acetylthio)propyl)-5-(mercaptomethyl)-2'-deoxyuridine [(7, scheme 6.1) 5 mg, 0.013 mmol] was weighed in a 10 mL r.b. flask and dried overnight under vacuum. 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (5 mg, 2 eq.) was dried in another flask for 10 min. Tri-n-butylammonium pyrophosphate (30 mg, 5 eq.) was dried overnight in a separate flask, under high vacuum. The pyrophosphate was dissolved in DMF (0.3 mL) with additional TBA (0.1 mL). The 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one was dissolved in DMF (0.2 mL) and transferred into the flask containing pyrophosphate under argon. A lot of argon was flushed through the reaction flask as the mixture was allowed to react at room temperature. The mixture was allowed to stir at room temperature. After 45 min the mixture was transferred into the flask containing vacuum dried compound 7 dissolved in DMF (0.2 mL) and toluene (0.1 mL). The combined contents were allowed to stir at room temperature for 4 h to yield the cyclic phosphite intermediate 8 (scheme 6.2). The cyclic-phosphite 8 was then oxidized by adding iodine solution (0.02% I₂/THF/Pyrıdine/H₂O, 1 mL). The mixture was stirred for 1 h and then hydrolyzed with water (5
mL) and allowed to stir at room temperature for 4 h to ensure complete hydrolysis. The sample was precipitated using NaCl (0.3 M)/ethanol (3 volumes). The precipitate was analyzed by recording a TLC (5:3:2 of iso-ProOH::NH₃:H₂O:H₂O). The crude S-(3(acetylthio)propyl)-5-(mercaptomethyl)-2'-deoxyuridine-5'-triphosphate, AcS-TTP, 9 was then purified by reverse phase HPLC. HR-MS (ESI-TOF): molecular formula, C₁₅H₂₃N₂O₁₅P₃S₂; [M-2H+Na⁺]: 650.9647 (calc. 650.9656); ¹H NMR (400 MHz, D₂O) δ: 1.82-1.86 (m, 2H, H-2'), 2.34 (s, 1H, CH₂), 2.37-2.40 (m, 2H, -CO-S-CH₂-CH₂-CH₂-S-), 2.59-2.63 (t of d, 2H, -CO-S-CH₂-CH₂-CH₂-S-), 2.92-2.96 (t of d, 2H, -CO-S-CH₂-CH₂-CH₂-S-), 3.53(s, 2H, C₅-CH₂-S), 4.19-4.20 (m, 3H, H-3' and H-5'), 4.67 (br, 1H, H-4'), 6.34 (t, J₁-₂ = 6.72 and 7.0 Hz, 1H, H-1'), 7.90 (s, 1H, H-6); ¹³C NMR (100 MHz, D₂O) δ: 28.50, 28.59 and 28.90 (-CO-S-CH₂-CH₂-CH₂-S-), 30.66 and 30.86 (CH₃-CO-S- and C₅-CH₂-S-), 39.59 (C-2'), 66.29 (C-5'), 71.67 (C-3'), 86.03 (C-4'), 86.51 (C-1'), 112.98 (C-5), 139.33 (C-6), 152.34 (C₂=O), 165.66 (C₆=O), 202.28 (CH₃-C=O); ³¹P NMR (162 MHz, D₂O) δ: -23.39 (t, J₀ = 19.44 Hz, 1P, P-β), -11.84 (d, J₀ = 21.06 Hz, 1P, P-α), -10.97 (d, J₀ = 19.44 Hz, 1P, P-γ).

Synthesis of S-mercaptopropyl-5-(mercaptomethyl)-2'-deoxyuridine-5'-triphosphate, H²S-TTP (10, scheme 6.2): S-(3(acetylthio)propyl)-5-(mercaptomethyl)-2'-deoxyuridine-5'-triphosphate, AcS-TTP was treated with NH₃ (1% in MeOH) for 12 h at room temperature to depoect the thiol protecting acetate group to yield S-mercaptopropyl-5-(mercaptomethyl)-2'-deoxyuridine-5'-triphosphate, H²S-TTP (10, scheme 6.2). The sample was purified by precipitating using NaCl (0.3 M)/ethanol (3 volumes). ¹H NMR (400 MHz, D₂O) δ: 1.90 (b, 2H, H-2'), 2.41 (b, 2H, -CO-S-CH₂-CH₂-CH₂-S-), 2.67 and 2.74 (b, 4H, -CO-S-CH₂-CH₂-CH₂-S-), 3.55 (s, 2H, C₅-CH₂-S), 4.19 and 4.22 (b, 3H, H-3' and H-5'), 4.68 (br, 1H, H-4'), 6.31 (b, 1H, H-1'), 7.91 (s, 1H, H-6); ¹³C NMR (100 MHz, D₂O) δ: 28.06, 29.66 and 36.45 (HS-CH₂-CH₂-CH₂-S-), 38.65 (C-2'), 65.13 (C-5'), 70.24 (C-3'), 85.23-85.57 (C-4' and C-1'), 112.19 (C-5), 138.59 (C-6), 151.49 (C₂=O), 164.87 (C₆=O); ³¹P NMR (162 MHz, D₂O) δ: -21.76 (t, J₀ = 18.8 and 20 Hz, 1P, P-β), -11.38 (d, J₀ = 19.3 Hz, 1P, P-α), -6.14 (d, J₀ = 19.8 Hz, 1P, P-γ).
6.2.3 HPLC Analysis and Purification

The crude \(\text{Ac}^5\text{TTP}\) was purified and analyzed by reversed-phase high performance liquid chromatography (RP-HPLC). The \(\text{Ac}^5\text{TTP}\) was eluted at 260 nm, with a flow-rate of 6 mL/min [buffer A: 20 mM TEAAc (pH 7.1) in water; buffer B: 20 mM TEAAc (pH 7.1) in 50% acetonitrile] and a linear gradient from 5 % buffer B to 40 % buffer B in 20 min, reaching 100 % buffer B in 30 min. The desired peak was collected and the buffer was removed by lyophilization. All samples were analyzed on Welchrom C18-XB column (4.6*250 mm) and measured at a flow rate of 1.0 mL/min and a linear gradient of 0 to 25 % buffer B in 20 min [buffer A: 20 mM TEAAc (pH 7.1) in water; buffer B: 20 mM TEAAc (pH 7.1) in 50% acetonitrile]. The colorless precipitates of \(\text{HS}^5\text{TTP}\) were also analyzed 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 6.1).

6.2.4 UV-Vis Absorption Study

All absorption spectra were recorded in de-ionized water (figure 6.2). The baseline was subtracted at all times.

6.2.5 Synthesis of Oligonucleotides

The DNA primer (P) and template (T) sequences (table 6.1) were synthesized on an automated Applied BioSystem 394 DNA Synthesizer (Applied Biosystems, Foster City, CA) employing standard β-cyanoethylphosphoramidite chemistry (1 μmol scale). Oligonucleotides were synthesized in DMTγ-off mode, with average coupling efficiency greater than 99%. The oligonucleotides were de-protected in concentrated \(\text{NH}_4\text{OH}\) (55 °C, 16 h), and purified by 19% denaturing polyacrylamide gel electrophoresis. The gel was visualized under UV light, crushed and soaked in water. The oligonucleotides were 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.

6.2.6 Enzymatic Incorporation Studies

DNA primers were labeled at the 5'-terminus by incubating in the presence of γ-[32P]-ATP (Perkin Elmer, Boston, MA) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA) for 1 h at 37 °C. Polymerase reactions were carried out by adding klenow exo-minus DNA polymerase (Epicentre Biotechnologies, Madison, WI) to a premixed solution containing DNA primer(s) (P1 or P2, table 6.1) and DNA template(s) (T1, T2 or T3, table 6.1), dNTPs (dATP, dCTP and dGTP, Epicentre Biotechnologies, Madison, WI), dNTP substrate (TTP/ AcSTTP/ HSSTTP) and DTT in klenow exo-minus DNA polymerase 10X reaction buffer at 37 °C. The reaction mixtures were incubated at 37 °C and quenched by the addition of 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). The analysis was performed on 19%, polyacrylamide gel electrophoresis (PAGE). For single nucleotide enzymatic incorporation reactions, no other dNTPs, except for substrate dNTPs (TTP/ AcSTTP/ HSSTTP), were added to the reaction mixture. The reaction mixtures were incubated at 37 °C for desired time and analyzed on 19%, polyacrylamide gel. 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.

6.3 Results and Discussion

6.3.1 Synthetic Design

S-(3'-S-acetylpropyl)-5-(mercaptopmethyl)-2’-deoxyuridine was synthesized from thymidine in seven steps as shown in scheme 6.1. Compounds 1, 2 and 3 were synthesized by using literature reported methodology (249). The free 3'- and 5'- hydroxy groups on thymidine were protected with
tert-butyldimethylsilyl protecting group. It was a clean and efficient reaction yielding 98% of pure 3', 5'-bis-O-(tert-butyldimethylsilyl)-thymidine. 3', 5'-bis-O-(tert-butyldimethylsilyl)-5-bromomethyl-2'-deoxyuridine (2) was obtained by radical bromination at benzylic position using N-bromosuccinimide under reflux conditions, in the presence of catalytic amounts of benzoyl peroxide. Residual insoluble succinimide was removed by filtration. Compound 2 was used immediately without any purification. S-acetyl-3', 5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptopmethyl)-2'-deoxyuridine (3) was obtained by the nucleophilic substitution of bromide with thioacetate. Alkaline hydrolysis of compound 3, using NaOH in MeOH, afforded 3',5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptopmethyl)-2'-deoxyuridine (4). Since naked/un-protected thiol-compounds have a strong tendency to dimerize, any characterization or purification on compound 4 was difficult. So the sample was used as such for the next step. The dimer of compound 4 was reduced in situ using dithiothreitol (DTT). The reaction was performed in an oxygen free, argon rich atmosphere. Alkylation of the free thiol anion thus generated, using 1, 3-dibromopropane, afforded S-(3'-bromopropyl)-3', 5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptopmethyl)-2'-deoxyuridine (5). Compound 5 is a stable intermediate. S-(3(acetylthio)propyl)-3', 5'-bis-O-(tert-butyldimethylsilyl)-5-(mercaptopmethyl)-2'-deoxyuridine (6) was obtained by the nucleophilic substitution of bromide with thioacetate. Selective 3', 5'-bis-O-desilylation of compound 6, using triethylamine trihydrofluoride (TEA.3HF) in THF, yielded S-(3(acetylthio)propyl)-5-(mercaptopmethyl)-2'-deoxyuridine (7).

S-(3(acetylthio)propyl)-5-(mercaptopmethyl)-2'-deoxyuridine (7) was used to synthesize S-(3(acetylthio)propyl)-5-(mercaptopmethyl)-2'-deoxyuridine-5'-triphosphate (AcS<sub>TTP</sub>, 9, scheme 6.2).<sup>11</sup> As shown in figure 14.3. It was observed that the addition of toluene to the nucleoside 7 is crucial as earlier attempts to synthesize triphosphate, by dissolving 7 in DMF or DMF/1,4-dioxane (1:1) mixture, failed. Only when the nucleoside was dissolved in DMF/toluene (2:1) mixture, triphosphate formation was observed. We believe that the toluene interacts with the bulky base and
helps it to stack better, making 5'-OH more available for phosphorylation yielding cyclic phosphate intermediate 8. S-mercaptopropyl-5-(mercaptomethyl)-2'-deoxyuridine-5'-triphosphate (HS'TTP, 10) was obtained by deprotection of the thiol-protecting acetate group with ammonia.

Scheme 6.1 Synthesis of S-(3(acetylthio)propyl)-5-(mercaptomethyl)-2'-deoxyuridine (7)
6.3.2 HPLC Purification and Analysis

The crude $^\text{Ac}$TTP (9) was purified and analyzed by RP-HPLC. The $^\text{Ac}$TTP was eluted at 260 nm, with a flow-rate of 6 mL/min [buffer A: 20 mM TEAAc (pH 7.1) in water; buffer B: 20 mM TEAAc (pH 7.1) in 50% acetonitrile] and a linear gradient from 5 % buffer B to 40 % buffer B in 20 min. The desired peak was collected and the buffer was removed by lyophilization. All the dry fractions of $^\text{Ac}$TTP and precipitated $^\text{HS}$TTP were analyzed on Welchrom C18-XB column (4.6*250 mm) and measured at a flow rate of 1.0 mL/min and a linear gradient of 0 to 25 % buffer B in 20 min [buffer A: 20 mM TEAAc (pH 7.1) in water; buffer B: 20 mM TEAAc (pH 7.1) in 50% acetonitrile (figure 5.1). The compounds being structurally similar have a small, but measureable, difference in
their retention times on the column. A RP-HPLC analysis of purified $Ac^5TTP$ and $H^5TTP$, at 260 nm wavelength, is presented in figure 6.1.

![Figure 6.1 RP-HPLC analysis of $Ac^5TTP$ and $H^5TTP$](image)

Figure 6.1 RP-HPLC analysis of $Ac^5TTP$ and $H^5TTP$: $Ac^5TTP$ (red curve, retention time: 24.75 min); $Ac^5TTP$ (blue curve, retention time: 24.96 min) and co-injection of $Ac^5TTP$ and $H^5TTP$ (black curve, retention times: 24.75 and 24.96 min, respectively).

### 6.3.3 Absorption Properties

The native thymidine-5'-triphosphate (TTP) shows an absorption maximum at ~267 nm. The modification at the C5-methyl of the thymine base does not alter the absorption properties of the resultant nucleobase. Hence, S-(3(acetylthio)propyl)-5-(mercaptomethyl)-2'-deoxyuridine-5'-triphosphate ($Ac^5TTP$, 9) and S-mercaptopropyl-5-(mercaptomethyl)-2'-deoxyuridine-5'-triphosphate, ($H^5TTP$, 10) have similar absorption profiles (figure 6.2).
6.3.4 Enzymatic Incorporation of TTP, AcS\text{^\text{\textregistered}}TTP and HS\text{^\textregistered}TTP into DNA

To investigate the incorporation efficiency of \textit{S}-\{3(acyethylthio)propyl\}-5-(mercaptomethyl)-2'-deoxyuridine-5'-triphosphate (AcS\text{^\textregistered}TTP, 9) and \textit{S}-mercaptopropyl-5-(mercaptomethyl)-2'-deoxyuridine-5'-triphosphate, (HS\text{^\textregistered}TTP, 10) into DNA, polymerization reactions were performed in the presence of klenow exo minus DNA polymerase (Kf-). A list of DNA primers and templates used for the primer extension reactions is presented in table 6.1.

Table 6.1 Sequences of DNA primers and templates used for the enzymatic incorporation of TTP, AcS\text{^\textregistered}TTP and HS\text{^\textregistered}TTP. The underlined bases are the sites for incorporation of TTP, AcS\text{^\textregistered}TTP and HS\text{^\textregistered}TTP.

**Primers:**

- **P1** (17-mer) 5'-d-TAG CGG GTT GCT GGT GG-3'
- **P2** (21-mer) 5'-d-GGG TAA TAC GAC TCA CTA TAG-3'

**Templates:**

- **T1** (21-mer) 3'-d-ATC GCC CAA CGA CCA CCA ATGG-5'
- **T2** (23-mer) 3'-d-ATC GCC CAA CGA CCA CCAAAAT GG-5'
- **T3** (55-mer) 3'-d-CGC ATT ATG CTG AGT GAT ATC CGT TGG ACT ATG CCG GCT TTC CGG CCT TGC ATG T-5'

Figure 6.2 Absorption spectra of TTP (—), AcS\text{\textregistered}TTP (—), and HS\text{\textregistered}TTP (—) in water at 25 °C.
Figure 6.3 Enzymatic incorporation of a single nucleotide (TTP, AcSTTP and HSSTTP) into DNA (T1): (a) Enzymatic incorporation of TTP, AcSTTP and HSSTTP by Klenow exo(-) on DNA template T1. Gel electrophoresis 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 HSSTTP, (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.

The DNA polymerization was performed with primer (1.5 µM), template (3 µM), TTP/ AcSTTP/ HSSTTP (0.1 mM), and Klenow exo(-) DNA polymerase (varying amounts) and analyzed by 19% polyacrylamide gel electrophoresis (figures 6.3, 6.4 and 6.5). Like TTP, AcSTTP and HSSTTP also act as fine substrates for DNA polymerases. Single nucleotide incorporation of the TTP, AcSTTP and HSSTTP into DNA was studied using a short, 21 nt long DNA template (T1) and the results are shown in figure 6.3. As evident from the autoradiographs obtained, AcSTTP acts as a better substrate than
by TTP and $^{13}TTP$: under different reaction times and with different enzyme concentrations, $^{13}TTP$ always terminates polymerization after the incorporation of a single nucleotide ($n + 1$ DNA, figure 6.3, lane 4) whereas TTP and $^{13}TTP$ either result in the formation of $n + 2$ DNA product or a mixture of desired $n + 1$ DNA along with some $n + 2$ DNA (figure 6.3, lanes 2 and 3, respectively). From the intensity of the product DNA band it appears that both, $^{13}TTP$ and $^{13}TTP$, are well recognized by the DNA polymerase. With the modified DNA moving slower than the native DNA, there is a measureable structural impact of the modification on the product mobility. The single nucleotide incorporation will be further confirmed by MALDI-MS analysis.

The DNA template T2 was designed to study the efficiency of incorporation of three consecutive modified bases. The 17 nt long primer P1 was enzymatically extended to a 20 nt DNA product by incorporating three consecutive thymidine bases over the template T2. The DNA polymerization was performed with primer (1.5 μM), template (3 μM), TTP/ $^{13}TTP$/ $^{13}TTP$ (0.1 mM), and Klenow exo(-) DNA polymerase (0.0015 U/μL) and analyzed by 19% polyacrylamide gel electrophoresis (figure 6.4). The reaction was incubated for 5 min with TTP as a substrate (figure 6.4, lane 2) whereas the reactions with $^{13}TTP$ and $^{13}TTP$ as substrates, the incubation time was 60 min (figure 6.4, lanes 3 and 4, respectively). Under these experimental conditions, $^{13}TTP$ appears to be the best substrate for DNA polymerase.

We also studied the efficiency of incorporation using a longer DNA template (T3). The incorporation was carried out by extending a 21 nucleotide primer along a 55 nucleotide template. The DNA polymerization was performed with primer (1.5 μM), template (5 μM), TTP/ $^{13}TTP$/ $^{13}TTP$ and other dNTPs (0.1 mM), and Klenow exo(-) DNA polymerase (0.04 U/μL) and analyzed by 19% polyacrylamide gel electrophoresis. Figure 3.8 shows the primer extension using T2 template. This polymerization involved the incorporation of three TTP/ $^{13}TTP$/ $^{13}TTP$ (figure 6.5).
Figure 6.4 Enzymatic incorporation of three consecutive bases (TTP, AcTTP and HsTTP) into DNA (T2): (a) Enzymatic incorporation of TTP, AcTTP and HsTTP by Klenow exo(-) on DNA template T2. (b) Gel electrophoresis autoradiography of the polymerization reaction with Kf = 0.0015 U/µL and reaction time = 5 min for TTP (lane 2) and 60 min for AcTTP (lane 4) and HsTTP (lane 3).

(a) 5'-32P-dTAG CGG GTT GCT GTT GG-3'  
3'-dATC GCC CAA CGA CCA CCAA TGG-5'  

\[
\text{TTP/AcTTP/HsTTP} \quad \text{Kf, 37 °C}
\]

5'-32P-dTAG CGG GTT GCT GTT GGT-3'  
3'-dATC GCC CAA CGA CCA CCAA TGG-5'  

(b)  

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Figure 6.5 Enzymatic incorporation of all natural dNTPs, TTP, AcTTP and HsTTP into DNA (T3): (a) Enzymatic incorporation of all dNTPs, TTP, AcTTP and HsTTP by Klenow exo(-) on DNA template T3; (b) Gel electrophoresis autoradiography of the polymerization reaction with Kf = 0.0015 U/µL and reaction time = 60 min for TTP (lane 3) and 90 min for AcTTP (lane 5) and HsTTP (lane 4).
6.3.5 Other Proposed Studies

The synthesized and purified \( \text{AcS} \)-DNA and \( \text{HST} \)-DNA will be used to study the stabilization of gold nanoparticles with reference to the native DNA. The study will be based on the strong affinity of gold nanoparticles for thiol-functionality. \( \text{HST} \)-DNA will be used as such to reduce gold whereas \( \text{AcS} \)-DNA will be hydrolyzed in-situ with ammonia to generate free thiol-group for gold nanoparticle stabilization. The modified DNA will also find use and application in DNA/RNA microchip surface functionalization for rapid detection of pathogens.

6.4 Conclusions and Future Prospects

We have successfully synthesized a novel nucleoside, \( S-(3\text{acetylthio}propyl)-5-(\text{mercaptomethyl})-2'\text{-deoxyuridine} \), from thymidine. This modified nucleoside was successfully used to synthesize \( S-(3\text{acetylthio}propyl)-5-(\text{mercaptomethyl})-2'\text{-deoxyuridine-5'-triphosphate} \) and \( S\text{-mercaptopropyl-5-(mercaptomethyl)-2'-deoxyuridine-5'-triphosphate} \). Both the forms of the triphosphates are efficient substrates for DNA polymerases. The polymerase recognition and incorporation efficiency of the modified triphosphates, relative to the native, has also been effectively demonstrated by the use of different combination of DNA primers and templates. The modified DNA thus obtained will be further used for gold nanoparticle stabilization. The modified DNA will also find use and application in DNA/RNA microchip surface functionalization for rapid detection of pathogens.
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APPENDIX

STRUCTURAL CHARACTERIZATION DATA