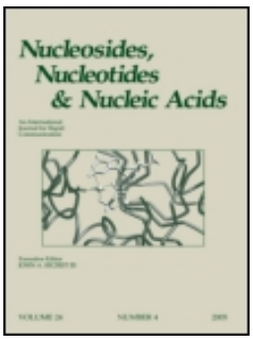

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SYNTHESIS AND SUBSTRATE EVALUATION OF (*E*)-5-[(3-SELENOPHENE-2-CARBOXAMIDO)PROP-1-EN-1-YL]-URIDINE-5′-*O*-TRIPHOSPHATE FOR RNA POLYMERASE

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□ *Design, synthesis and T7 RNA polymerase substrate evaluation of* (E)*-5-[(3-selenophene-2-carboxamido)prop-1-en-1-yl]-uridine-5′-O-triphosphate is reported. The title compound is shown to be a good substrate for RNA polymerase by RNA labeling through* in vitro *transcription. pTRI-plasmid DNA with β-actin gene sequence (∼300 base pairs) with T7 promoter was used as a template for the* in vitro *transcription. Transcribed product is characterized for incorporation by gel assay and for integrity, full length and size by bioanalyzer. The title compound will be very useful in biophysical techniques to obtain information on dynamics and recognition properties in real time as well as 3D structure of nucleic acids.*

Keywords Modified AA-UTP; selenophene containing UTP; In Vitro transcription

INTRODUCTION

Ribonucleic acid involves in several key cellular processes such as catalysis, and regulation of genetic information by interacting with proteins, nucleic acids and small molecule metabolites. These interactions are governed by intrinsic conformational dynamics resulting from diverse secondary and tertiary structures.[1] To mention a few, techniques such as fluorescence, nuclear magnetic resonance, electron paramagnetic resonance and electrophoresis are commonly used to advance our understanding on the structural dynamics and recognition properties of nucleic acids.[2] In addition, high resolution X-ray structure of functional RNA motifs offers fundamental understanding on correlation of RNA structure with their function at molecular level.[3] Three-dimensional structural determination of nucleic

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acids using X-ray crystallography has heavily relied on heavy atom derivatization of nucleic acids for phase determination. Single-wavelength anomalous dispersion (SAD) phasing or multi-wavelength anomalous dispersion (MAD) phasing are the conventional techniques in structural determination which involves the anomalous scattering from a bromine or iodine atom of brominated or iodinated nucleic acids.[4] Halogenated oligonucleotides, being light sensitive, undergo dehalogenation upon X-ray exposure causing failures in phasing. To overcome this issue, the use of selenium atom that displays superior anomalous scattering property in protein X-ray crystallography has also been extended to nucleic acid X-ray crystallography.[5] Huang et al used Se-MAD phasing technique successfully to report the x-ray structure of selenium modified DNA and RNA oligonucleotides.[6] These selenium heavy atom derivatization methodologies have also been successfully used in the X-ray structural determination of nucleic acids,[7] nucleic acid-protein and nucleic acid-small molecule complexes.[8] To broaden our understanding on how RNA structure complements its function, it will be attractive to design a dual function label that allows obtaining information on dynamics and recognition properties in real time as well as 3D structure of nucleic acids at the same time. For example, an anomalous scattering heavy atom (selenium) containing conformation-sensitive fluorescent selenophene label on the nucleosides would facilitate such structure-function correlation analysis by both X-ray crystallography and fluorescence.[9] Aminoallyl-RNA can be obtained by the incorporation of 5-(3-aminoallyl)-uridine-5'-triphosphate into RNA by T7 RNA polymerase. The aminoallyl functionality in the aminoallyl nucleotides serves as an anchor point to install various labels, tags and probe molecules such as biotin, fluorescent dyes etc by conjugation via either prelabeling of aminoallyl nucleotides or post-labeling of product RNA.[10] Commonly, these labeled aminoallyl probes are used in microarrays for simultaneous analysis of multiple gene expression levels.[11] In this manuscript, we describe the utilization of the aminoallyl handle present in (*E*)-5-[3-Aminoallyl]-uridine-5′-triphosphate, AA-UTP to attach a dual function selenophene label to obtain (*E*)-5-[(3-selenophene-2-carboxamido)prop-1-en-1-yl]-uridine-5′-O-triphosphate. This selenophene labeled AA-UTP will find applications in biophysical techniques to obtain information on dynamics and recognition properties in real time as well as 3D structure of nucleic acids.

RESULTS AND DISCUSSION

An anomalous scattering heavy atom (selenium) containing conformation-sensitive fluorescent selenophene label on the nucleosides would facilitate structure-function correlation analysis by both X-ray crystallography and fluorescence. Pawar *et al.*[9] showed usefulness of selenophene

label as a fluorescence probe by synthesizing UTP containing (selenophen-2-yl)prymidine core for monitoring binding of aminoglycoside antibiotics to the bacterial ribosomal decoding A-site. Selenophene-conjugated uridine analogue was shown to display emission in the visible region and very good fluorescence solvatochromism.[9] This work is essentially based on the earlier development of fluorescent assay involving nucleoside analogues bearing a furan moiety at 5-position to detect the presence of DNA abasic sites and the binding of aminoglycoside antibiotics to RNA.[12] The advantage of replacing furan with selenophene is the incorporation of anomalous heavy atom for 3D structural determination in addition to generating minimally perturbing emissive nucleobase analogue. Selenium containing nucleobases were shown to be the good substrates for polymerases.[13] Aminoallyl nucleotides aid enzymatic introduction of functional groups into nucleic acid targets. Aminoallyl-RNA can be easily obtained by the incorporation of (*E*)-5-(3-aminoallyl)-uridine-5'-triphosphate (AA-UTP) by RNA polymerases. The aminoallyl handle present in (*E*)-5-[3-Aminoallyl]-uridine-5′-triphosphate, AA-UTP **4** allows facile attachment of dual biophysical functional selenophene label to obtain (*E*)-5-[(3-selenophene-2-carboxamido)prop-1-en-1-yl]-uridine-5′-O-triphosphate **5**.

In view of above facts and in continuation of our efforts in synthesizing various biologically useful phosphorylated nucleosides,[14] we are much interested to synthesize and evaluate the selenophene labeled AA-UTP as substrate for RNA polymerase. The reaction pathway leading to selenophene tagged AA-UTP, (*E*)-5-[(3-selenophene-2-carboxamido)prop-1-en-1-yl]-uridine-5′-O-triphosphate is depicted in **Scheme 1**. The chemistry essentially involves coupling of activated ester, 2,5-dioxopyrrolidin-1-yl selenophene-2-carboxylate **3** to aminoallyl moiety of AA-UTP **4**. The required selenophene-2-carboxylic acid N-hydroxy succinimidyl activated ester was synthesized in two steps starting from selenophene-2-carbaldehyde **1**. The first step is the oxidation of carbaldehyde to carboxylic acid and the second step is the coupling of N-hydroxysuccinimide to the carboxylic acid. Though there are many reagents available for the oxidation step, silver (I) oxide was reported to work very well for heterocycle carbaldehyde oxidation.[15] Silver (I) oxide mediated oxidation of selenophene-2-carbaldehyde **1** in aqueous sodium hydroxide solution resulted in the formation of selenophene-2-carboxylic acid **2** in 97% yield. DCC mediated coupling of N-hydroxysuccinimide to selenophene-2-carboxylic acid in dry acetonitrile as the solvent afforded 2,5-dioxopyrrolidin-1-yl selenophene-2-carboxylate **3** in 96% yield. This step does not require any purification other than filtering to remove the precipitated side product dicyclohexyl urea. As the activated ester of selenophene-2-carboxylic acid is ready, the next step would be the preparation of the coupling partner AA-UTP **4**. The common and popular method to make aminoallyl nucleoside involves mercuration of uridine triphosphates using mercuric acetate and subsequent

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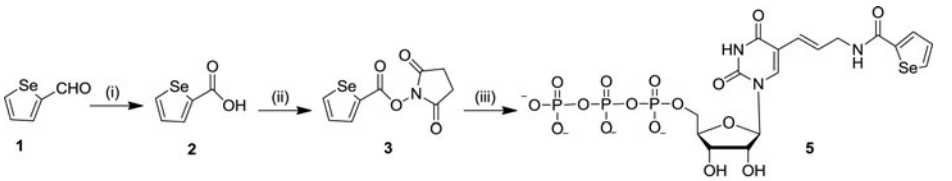
palladium-catalyzed reaction with allylamine.[16] This method heavily use no greener, toxic and hazardous mercuric acetate and obtaining mercury-free allylamino nucleoside triphosphates is challenging. Hence we synthesized AA-UTP **4** via palladium-catalyzed Heck coupling of 5-iodouridine triphosphates with allylamine, a highly concise and mercury free method recently reported.[17–19] With AA-UTP in hand, it is then coupled with 2,5-dioxopyrrolidin-1-yl selenophene-2-carboxylate **3** in the presence of triethyl amine as the base and anhydrous dimethylformamide as the solvent. The reaction mixture was reduced under vacuum, reconstituted in 0.1 M aqueous triethylammonium bicarbonate (TEAB) buffer, pH 7.8, pH adjusted to 6.5 using dilute acetic acid and loaded on a DEAE Sepharose column. The desired product was eluted using a linear gradient of 0–1 M TEAB and the fractions containing the product was pooled, evaporated and coevaporated with water. The triethylammonium salt of (*E*)-5-[(3-selenophene-2-carboxamido)prop-1-en-1-yl]-uridine-5′-O-triphosphate **5** thus obtained was subjected to ion-exchange with sodium perchlorate in acetone for twice to afford the sodium salt of **5** in 76% yield with 98% purity by HPLC. Fluorescence of 100 μmolar aqueous solution of selenophene labeled AA-UTP **5** was compared with the same concentrations of other NTPs (C, G, A and U) commonly used in the polymerase mediated *in vitro* transcription reactions. Selenophene labeled AA-UTP **5** displays strong fluorescence signal (~3 order) at its emission maximum (454 nm) upon excitation at 375 nm where as CTP, GTP, UTP and ATP do not display any fluorescence at the same measurement conditions. This observation is in accordance with earlier similar observations on UTP probe having (selenophen-2-yl)pyrimidine core.[9]



Reagents and conditions: (i) Ag_2O, NaOH, H_2O, 50°C, 97%; (ii) N-hydroxysuccinimide, DCC, CH_3CN, rt, 96%; (iii) AA-UTP **4**, $(C_2H_5)_3N$, DMF, rt, 76%

SCHEME 1 Synthesis of (*E*)-5-[(3-selenophene-2-carboxamido)prop-1-en-1-yl]-uridine-5′-O-triphosphate **5**.

Modified RNA oligonucleotides can be obtained by enzymatic methods using RNA polymerases and ligases. Here we performed *in vitro* transcription reactions in the presence of SP6, T3 and T7 phage RNA polymerases enzyme mixture to incorporate (*E*)-5-[(3-selenophene-2-carboxamido)prop-1-en-1-yl]-uridine-5′-O-triphosphate **5** into RNA by using MAXIscript$^{(TM)}$ kit (Life Technologies Corporation, Carlsbad, CA, USA) following manufacturer's protocol. The RNA polymerase enzyme mixture includes ribonuclease inhibitor protein and hence protects the newly transcribed RNA from

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degradation by RNases, if any. Transcription reactions have been carried out in the presence of promoter pTRI-β-actin-Mouse plasmid DNA template having ~300 base pairs. The template contains several adenosine residues to facilitate the incorporation of selenophene labeled AA-UTP. RNA polymerase first binds to its double-stranded DNA promoter, then it separates the two DNA strands, and uses the 3′ to 5′ strand as a template to synthesize a complementary 5′ to 3′ at the end of the DNA template. All transcription reactions were performed in triplicates with following components: pTRI-β-actin-Mouse plasmid DNA template; reaction buffer, 1X; T7 RNA polymerase enzyme mix; 0.5 mM of each ATP, CTP and GTP; and selenophene labeled AA-UTP and/or UTP (total final concentration 0.5 mM). In control transcription reaction, no selenophene labeled AA-UTP **5** was added. Two other reaction were set up with the selenophene labeled AA-UTP at different ratios as below indicated. In another reaction, a mixture of 0.2 mM of selenophene labeled AA-UTP and 0.3 mM UTP (ratio of 40:60) was added. This reaction was designated as "Mixed". In third reaction, 0.5 mM of selenophene labeled AA-UTP was added (represnting 100% for UTP) and no unlabeled UTP was added. This reaction was designated as "Labeled". Transcription reactions in triplicate were incubated at 37°C. In order to hydrolyze the remaining plasmid DNA, turbo DNase was added and the reaction mixture was further incubated at 37°C for 15 min. Purifications of the RNA from these transcription reactions were done by using the MEGAclear$^{(TM)}$ kit (Life Technologies Corporation, Carlsbad, CA, USA) as per manufacturer's protocol. The transcription yield of RNA was calculated by measuring absorbance at 260 nm. The transcript yields indicates that (*E*)-5-[(3-selenophene-2-carboxamido)prop-1-en-1-yl]-uridine-5′-O-triphosphate **5** is a good substrate for SP6, T3 and T7 phage RNA polymerases. Assessing the yield of all three reactions, shows control transcription reaction with no selenophene labeled AA-UTP **5** and the mixed reaction (40:60) have similar yield. This indicates the T7 Polymerase efficiency is not reduced by the presence of selenophene labeled AA-UTP **5** in *in vitro* Transcription reaction. However, in the reaction that contains 100% selenophene labeled AA-UTP **5** showed a 20% reduction in the yield. Low yield of *in vitro* transcription reaction, in which 100% selenophene labeled AA-UTP 5 could be due to fidelity and recognization of T7 RNA polymerase enzyme with the modified compound **5**.[20,21]

The products of transcription reactions were analyzed by gel analysis. Because the total yield of RNA produced is high in non-limiting NTP concentration conditions, unlabelled, and nonisotopically labeled transcripts can be visualized in gels. To perform gel assay, aliquot of transcription reactions were mixed with gel loading buffer II containing formaldehyde. 10 μL of this mixture were loaded on to 2% agarose gel precasted with Ethidium bromide stain. Gel bands were visualized using AlphaImager (**Figure 2**). The gel picture clearly indicates the incorporation of the probe in the RNA transcript as seen in the lane B and C, Irrespective of all three transcripts having

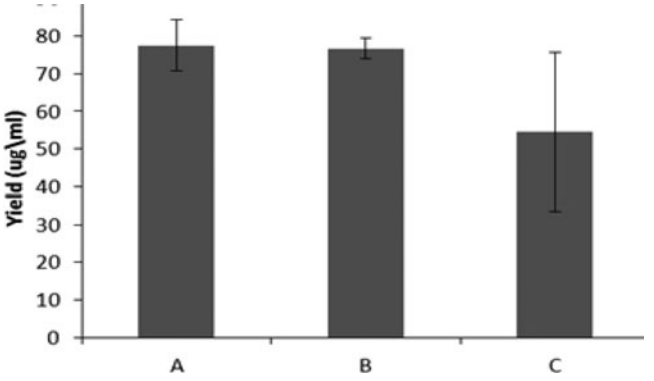

FIGURE 1 SP6, T3 and T7 phage RNA polymerases transcription yields (quantified at A260 nm by using NanoDrop): A) control transcription reaction, no selenophene labeled AA-UTP **5,** but 0.5 mM UTP was used; B) Labeled transcription reaction, 0.2 mM of selenophene labeled AA-UTP **5** and 0.3 mM UTP was used (mixed in ratio of 40:60); C) labeled transcription reaction, 0.5 mM of selenophene labeled AA-UTP **5** (ratio of 100%) and no UTP was used.

the same size, the labeled reactions in the lane B and C had slower movement compared to the control sample in lane A. This is due the molecular weight difference between unmodified and modified selenophene labeled AA-UTP **5,** which retards the shift as compared to control lane A. Additionally, due to complete incorporation of selenophene labeled AA-UTP **5** and removal of unincorporated NTPs from the *in vitro* transcription reactions, there is no additional bands observed in the gel. Transcript RNAs from all the transcription reactions were analyzed for their integrity, full length and size using Bioanalyzer (Agilent). The promoter pTRI-β-actin-Mouse plasmid DNA template used in the transcript reaction has ~300 base pairs. The control, mixed and labeled transcript reactions resulted in the full length RNA transcript formation (with high integrity) with ~ 339, 328, and 330 base pairs respectively. Bioanlayzer profile indicates the incorporation of the selenophene labelled AA-UTP **5** in the RNA transcript. The selenophene labeled AA-UTP **5** labeled generate signficantly stronger signal due to the additional fluorescence from the selenophene labeled AA-UTP **5.** This can be accessed from the bioanalyzer profile for the selenophene labeled AA-UTP **5,** both mixed

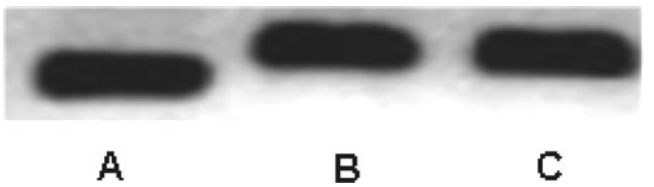

FIGURE 2 Gel analysis of transcription reactions. A) control transcription reaction, no selenophene labeled AA-UTP **5,** but 0.5 mM UTP was used; B) mixed transcription reaction, 0.2 mM of selenophene labeled AA-UTP **5** and 0.3 mM UTP was used; C) labeled transcription reaction, 0.5 mM of selenophene labeled AA-UTP **5** and no UTP was used.

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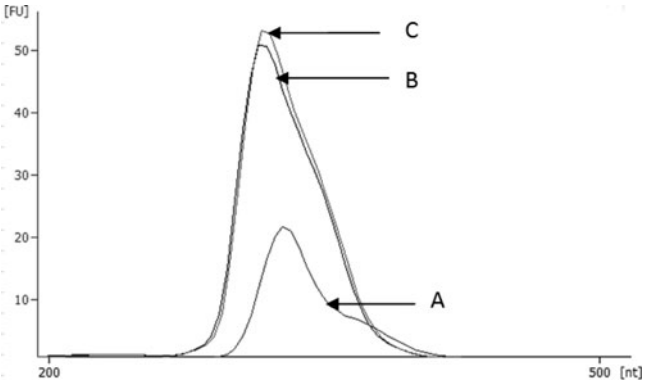

FIGURE 3 Integrity and size anlaysis (using Bioanalyzer) of product transcript RNA from A) control transcription reaction, no selenophene labeled AA-UTP **5,** but 0.5 mM UTP was used; B) mixed transcription reaction, 0.2 mM of selenophene labeled AA-UTP **5** and 0.3 mM UTP was used; C) labeled transcription reaction, 0.5 mM of selenophene labeled AA-UTP **5** and no UTP was used. (FU-Fluoresecent Units, nt-nucleotides)

and labeled have twice the fluorescent units compared to the unlabeled control reaction.[22] (**Figure 3**).

In summary, (*E*)-5-[(3-selenophene-2-carboxamido)prop-1-en-1-yl]-uridine-5′-O-triphosphate **5** synthesized from AA-UTP serves as a good substrate for RNA polymerases. This observation hold great promise on the design of this probe molecule because selenophene label can be attached later also to aminoallyl modified RNA in a post-labeling fashion using 2,5-dioxopyrrolidin-1-yl selenophene-2-carboxylate **3**. Selenophene labeled AA-UTP **5** will find applications in biophysics filed as this probe contains both anomalous scattering heavy atom (selenium) as well as conformation-sensitive fluorescent selenophene label in its structure. Selenophene derivatization of nucleic acids is of great importance because of its dual probe nature and could provide new avenues to study structure-function relationships in biomacromolecules.

EXPERIMENTAL

All of the commercial reagents and solvents are used as such without further purification. Nucleosides were purchased from ChemGenes Corporation. (Wilmington, MA, USA). ^{1}H NMR spectra were recorded in D_2O on a Bruker 400 MHz and ^{31}P NMR were recorded on a Bruker 162 MHz. Chemical shifts are reported in ppm, and signals are described as s (singlet), d (doublet), t(triplet), q (quartet), and m (multiplet). ESI mass was recorded on Applied Biosystems/Sciex MDX API 150 model. HPLC was run on a Waters 2996 (Waters Corporation, Milford, MA, USA) using Hypersil SAX column. FPLC (fast protein liquid chromatography) was performed on a

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AKTA purifier (GE Healthcare, Piscataway, NJ, USA) using DEAE Sepharose column.

Synthesis of selenophene-2-carboxylic acid (2) To a solution of NaOH (1.95 g, 48.75 mmol) in water (20 mL) was added Ag_2O (4.37 g, 18.86 mmol) in water (20 mL) at room temperature. After stirring for 15 min, selenophene-2-carbaldehyde **1** (2 g, 12.57 mmol) was added and the reaction mixture was stirred at room temperature for 0.5 h, and at 50°C for 4 h. A crystalline precipitate formed was filtered off and washed with water. The filtrate and washings were combined, poured into chilled 10% HCl (10 mL) and extracted with ethyl acetate. The ethyl acetate extract was washed successively with water and brine; and dried over anhydrous sodium sulfate. The organic solution was evaporated under reduced pressure to give selenophene-2-carboxylic acid **2** in 97% yield (2.14 g, 12.2 mmol). The characterization data for **2** was in agreement with that reported earlier.[23] ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 8.24–8.27 (m, 1H), 8.02–8.05 (m, 1H), 7.35–7.38 (m, 1H).

Synthesis of 2,5-dioxopyrrolidin-1-yl selenophene-2-carboxylate (3) A mixture of selenophene-2-carboxylic acid **2** (2 g, 11.4 mmol), N-hydroxy succinimide (1.32 g, 11.4 mmol) and N,N′-dicyclohexyl carbodimide (DCC) (2.35 g, 11.4 mmol) in dry acetonitrile (50 mL) was stirred at room temperature for 14 h. The reaction mixture was filtered to remove dicyclohexyl urea and the filtrate was evaporated and dried under vacuum to afford 2,5-dioxopyrrolidin-1-yl selenophene-2-carboxylate **3** in 96% yield (2.98 g, 10.94 mmol). The product was used as such for the next step.

Synthesis of (*E*)-5-[(3-selenophene-2-carboxamido)prop-1-en-1-yl]-uridine-5′-O-triphosphate (5) A mixture of 2,5-dioxopyrrolidin-1-yl selenophene-2-carboxylate **3** (1 g, 3.66 mmol), (*E*)-5-[3-Aminoallyl]-uridine-5′-triphosphate, AA-UTP **4** (2.16 g, 3.66 mmol) and triethylamine (2 mL, 14.33 mmol) in anhydrous dimethylformamide (50 mL) was stirred at room temperature for 15 h. The reaction mixture was reduced under vacuum, reconstituted in 0.1 M aqueous triethylammonium bicarbonate (TEAB) buffer, pH 7.8 (200 mL), pH adjusted to 6.5 using dilute acetic acid and loaded on a DEAE Sepharose column. The desired product was eluted using a linear gradient of 0–1 M triethylammonium biocarbonate (TEAB) and the fractions containing the product was pooled, evaporated and coevaporated with water (3 × 100 mL). The triethylammonium salt of (*E*)-5-[(3-selenophene-2-carboxamido)prop-1-en-1-yl]-uridine-5′-O-triphosphate **5** thus obtained was subjected to ion-exchange with sodium perchlorate (2 g) in acetone (50 mL) for twice to afford the sodium salt of **5** in 76% yield (2.08 g, 2.78 mmol). ^{1}H NMR (D_2O, 400 MHz) δ (ppm) 8.42–8.44 (m, 1H), 7.99–8.03 (m, 1H), 7.45–7.48 (m, 1H), 6.54–6.60 (m, 1H), 6.41–6.45 (m, 1H), 6.02–6.04 (d, J = 5.2 Hz, 1H), 4.47–4.49(m, 2H), 4.29–4.33 (m, 3H), 4.11–4.13 (m, 1H), 1.66–1.69 (m, 1H), 1.36–1.42 (m, 1H); ^{31}P NMR (D_2O, 162 MHz) δ −6.93 (d, J = 19.8 Hz, 1P), −10.34 (d, J = 20.9 Hz, 1P),

−21.33 (t, J = 20.3 Hz, 1P); MS (ESI, m/z): calc. for $C_{17}H_{22}N_3O_{16}P_3Se$ $[M\text{-}H]^-$: 695.9, found 696.5.

RNA Labeling Assay Through *In vitro* Transcription

RNA polymerase *in vitro* transcription was performed by using MAXIscript(TM) kit (Life Technologies Corporation, Carlsbad, CA, USA) following manufacturer's protocol. All transcription reactions were performed in a 20 μL final volume at the following final concentrations of components: pTRI-β-actin-Mouse plasmid DNA template, 0.5 mg/mL (1 μg total); reaction buffer, 1X; RNA polymerase enzyme mix,T7 15 U/μL; 0.5 mM of each ATP, CTP and GTP; and selenophene labeled AA-UTP and/or UTP (total final concentration 0.5 mM). In control reaction, no selenophene labeled AA-UTP was added. In another reaction, a mixture of 0.2 mM of selenophene labeled AA-UTP and 0.3 mM UTP was added. In third reaction, 0.5 mM of selenophene labeled AA-UTP (and no UTP) was added. Transcription reactions in triplicate were incubated at 37°C for 4 hours. In order to hydrolyze the remaining plasmid DNA, 1 μL of turbo DNase was added and the reaction mixture was further incubated at 37°C for 15 min. Purifications of the RNA from these transcription reactions were done by using the MEGAclear(TM) kit (Life Technologies Corporation, Carlsbad, CA, USA) as per manufacturer's protocol. The transcription yield of RNA was assessed using NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA) by measuring absorbance at 260 nm. To perform gel assay, 10 μL of transcription reactions were mixed with 10 μL of gel loading buffer II (contains formaldehyde). 10 μL of this mixture were loaded in 2% agarose gel [precast Ethidium bromide gel; Reliant™ GELS (2%SKG,TBE, EB,2×12W) (Lonza, Basel, Switzerland)] and quantified using AlphaImager (ProteinSimple, Santa Clara, California, USA).

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