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Synthesis of 5′-Deoxy-5′-thioguanosine-5′-monophosphorothioate and Its Incorporation into RNA 5′-Termini

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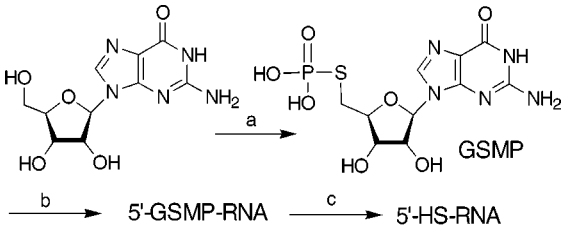
ABSTRACT



5′-Deoxy-5′-thioguanosine-5′-monophosphorothioate (GSMP) was synthesized in four steps with 35% overall yield. GSMP serves as a good substrate for in vitro transcription with T7 RNA polymerase to yield 5′-GSMP-RNA, which was converted to 5′-HS-RNA by dephosphorylation with alkaline phosphatase. The thiol-reactive agents can be efficiently introduced into the 5′-terminus of RNA.

RNA molecules play critical roles in many cellular processes. The development of methods for studying the molecular details of the complex interactions and essential functions of RNA in cellular metabolism is challenging. Site-specific substitution and derivatization provide powerful tools for studying RNA structure and function.[1] Although solid phase chemical synthesis can be used to introduce functional groups at any specific position of oligonucleotides shorter than approximately 40 nt,[2] investigation of larger RNA molecules faces a limited number of methodologies for site-specific modification and substitution. 5′-Modifications of RNA molecules have been shown to have broad applications in studying RNA structures, mapping RNA−protein interactions, and in vitro selection of catalytic RNAs. Phosphorothioate modification is one of the most popular methods for functionalizing the 5′-terminus of RNA by a transcription or kinase reaction.[3] However, labeling efficiency of terminal phosphorothioate with fluorophores is low,[4] and fluorophores are the most attractive probes for RNA structure.[5] The sulfhydryl group is a special reactive group that can be incorporated into nucleic acids.[6] The thiol-reactive functional groups are primarily alkylating reagents, including iodoacetamides, maleimides, benzylic halides, and bromomethyl ketones.[7] The thiol group demonstrates a unique property; that is, the thiol−disulfide exchange reaction. A pyridyl disulfide group is the most popular type of thiol−disulfide

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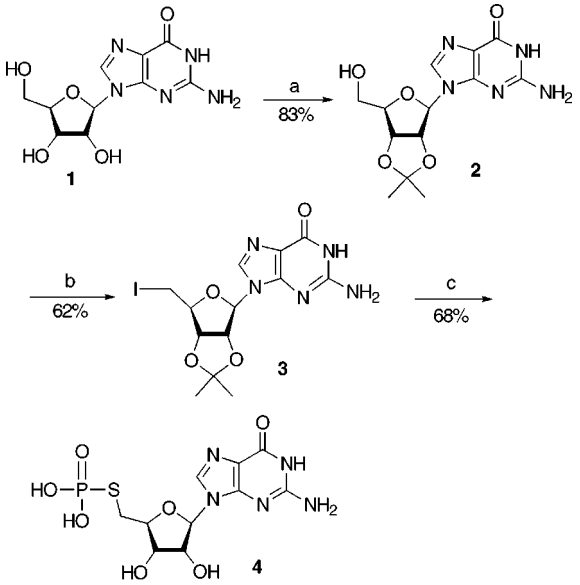
exchange functional group used in the construction of cross-linkers or modification reagents. A pyridyl disulfide will readily undergo an interchange reaction with a free sulfhydryl to yield a single mixed disulfide product. Once a disulfide linkage is formed, it may be cleaved using disulfide reducing agents (DTT, etc.). Although 5′-(guanosine-5′-monophosphorothioate)-RNA (5′-GMPS-RNA) can react with pyridyl disulfide to form a phosphorothioate sulfide compound (R-SSPO$_3$-RNA),[8] a limitation of phosphorothioate sulfide product is the low stability.[9] The free thiol groups can be introduced into the 5′-termini of RNA by chemically using carbodiimide and cysteamine,[10] but the phosphoramidate linkage is also not very stable. However, we report herein an enzymatic method for the introduction of 5′-terminal sulfhydryl group at the 5′-termini of large RNA molecules. The 5′-deoxy-5′-thioguanosine-5′-monophophorothioate (GSMP) **4** was synthesized as a substrate for T7 RNA polymerase that requires guanosine to efficiently initiate transcription.[11] The in vitro transcription was used to incorporate a sulfhydryl moiety to 5′-end of RNA molecule.

5′-Deoxy-5′-thioguanosine-5′-monophosphorothioate was synthesized shown on Scheme 1. Guanosine **1** was treated with acetone and 70% perchloric acid at room temperature for 70 min to give 2′,3′-isopropylideneguanosine **2** with 83% yield[12] and reacted with methyltriphenoxyphosphonium iodide[13] in THF to yield 2′,3′-isopropylidene-5′-deoxy-5′-iodoguanosine (**3**) with 62% yield. The deprotection of **3** with 50% aqueous formic acid for 2.5 days and subsequent reaction with trisodium thiophosphate yielded the desired product **4** (68% yield from **3**).[14] GSMP **4** was characterized by proton and phosphorus NMR and MS spectroscopy and tested as a substrate for in vitro transcription.

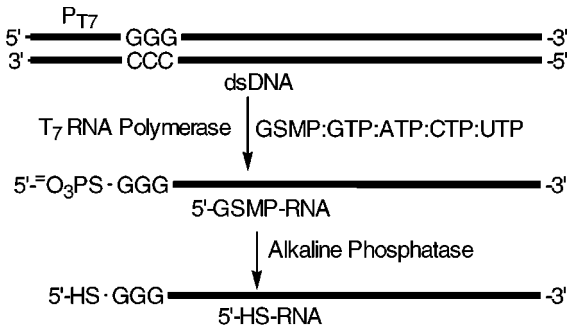
A 222-mer double-stranded (ds) DNA containing a T7 promoter was used as the template for in vitro transcription (Scheme 2). Transcription reactions were carried out with 20 units of T7 RNA polymerase in the presence of 1 mM each GTP, ATP, CTP and UTP, 10 μg of DNA template, 10 μCi α-^{32}P-ATP, 4 mM spermidine, 0.05% Triton X-100, 12 mM $MgCl_2$, and 40 mM Tris buffer (pH 7.5) at 37 °C in a total of 200 μL solution. The 196 nt 5′-GSMP-RNA was synthesized by runoff transcription in the presence of GSMP **4** with a ratio of GSMP:GTP:ATP:CTP:UTP = 8:1:1:1:1

Scheme 1. Synthesis of 5′-Deoxy-5′-thioguanosine-5′-monophosphorothioate **4**[a]



[a] (a) acetone, 70% $HClO_4$; (b) methyltriphenoxy-phosphonium iodide, THF; (c) (1) 50% HCOOH, (2) trisodium thiophosphate, water, 3 days.

Scheme 2. Schematic Diagram of Preparing Sulfhydryl Incorporated RNA



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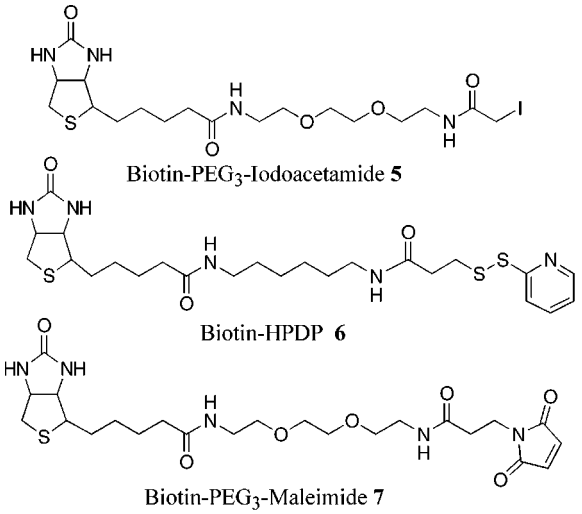
(14) Hampton, A.; Brox, L. W.; Bayer, M. *Biochemistry* **1969**, *8*, 2303−2311. **Preparation of compound 4.** To a suspension of 5′-deoxy-5′-iodoguanosine (2.83 g, 7.2 mmol) in 140 mL of water was added trisodium thiophosphate (4.8 g, 26 mmol). The reaction mixture was stirred for 3 days at room temperature under argon atmosphere. After filtration to remove any precipitate, the filtrate was evaporated under reduced pressure. The residue was dissolved in 100 mL of water and trisodium thiophosphate was precipitated by addition of 200 mL of methanol. After removal of the precipitate by filtration, the filtrate was evaporated and dissolved in a small amount of water and subjected to reverse phase chromatography (C_{18}). The desired product was collected and dried by lyophilizer (1.9 g, 68% for two steps). R_f = 0.36 (*i*-PrOH/NH_3/H_2O = 6:3:1); ^{1}H NMR (400 MHz, DMSO-d_6 + D_2O): δ 7.82 (s, 1H), 5.63 (d, J = 5.9 Hz, 1H), 4.28 (dd, J = 3.9 Hz 1H), 4.08 (m, 1H), 2.83 (m, 2H). ^{31}P NMR (DMSO-d_6 + D_2O): δ 16.4 ppm. MS (ESI) m/z found 378 [M − 1]$^-$ (calcd $C_{10}H_{14}N_5O_7PS$, 379).

mM. The body-labeled RNA was purified by denaturing 7.5 M urea/8% polyacrylamide gel electrophoresis. The 5′-GSMP-RNA was dephosphorylated by incubation with 10 units of alkaline phosphatase in buffer 3 (New England Biolab) at 37 °C for 2 h and stopped by incubation with 10 μL of 200 mM EGTA for 10 min at 65 °C. The RNA was recovered by ethanol precipitation. The dephosphorylation of 5′-GSMP-RNA can also be done by $AgNO_3$ or $HgCl_2$ plus DTT to produce 5′-HS-RNA (data not shown).

The 5′-GTP-RNA and 5′-HS-RNA were treated with Biotin-PEG_3-iodoacetamide (**5**), Biotin-HPDP (**6**), and Biotin-PEG_3-Maleimide (**7**) (Scheme 3) in 10 mM HEPES (pH 7.8), 300 mM NaCl, and 1 mM EDTA at room temperature for 2 h. The reaction mixtures were extracted with phenol/chloroform once and chloroform once and precipitated with ethanol. The RNA pellets were resuspended in 20 μL of pure water and stored at −20 °C. A 2 μL aliquot of RNAs was incubated with 15 μg of streptavidin in the binding buffer (20 mM HEPES, pH 7.4, 5.0 mM EDTA and 1.0 M NaCl) at room temperature for 20 min prior to mixing with 0.25 volumes of formamide loading buffer (90% formamide; 0.01% bromophenol blue and 0.025% xylene cyanol). The biotinylated RNA products were resolved by electrophoresis through 7.5 M urea/8% polyacrylamide gels. The biotinylated RNA can form a complex with streptavidin and the mobility of the 5′-biotin-RNA::streptavidin complex through the gel will be retarded relative to unbiotinylated RNA. The fraction of product formation relative to total RNA at each lane was quantitated with a Molecular Dynamics PhosphorImager.

Scheme 3. Structures of Thiol-Reactive Biotin Agents



The streptavidin gel-shift results of 5′-HS-RNA with **5**, **6**, and **7** are shown in Figure 1. When 5′-HS-RNA reacted with **5**, **6**, and **7**, the thiol-modified RNA molecules were biotinylated. They showed a band-shift in the presence of streptavidin upon gel electrophoresis that represented a streptavidin::RNA complex (lanes 4, 6, and 9, respectively), no retarded band was detected without streptavidin (lane 5, 7, and 10). When 5′-GTP-RNA was treated with **6**, **7**, and **8**, no biotinylated RNA was detected in the presence of streptavidin (lane 1, 2, and 3, respectively). The retarded band disappeared after treatment with DTT (lane 8), which indicated the thiol−disulfide exchange reaction of 5′-HS-RNA with Biotin-HPDP **7**. The results suggested that the biotin group was transferred to the terminal thiol of the 5′-HS-RNA and not to other groups of RNA.

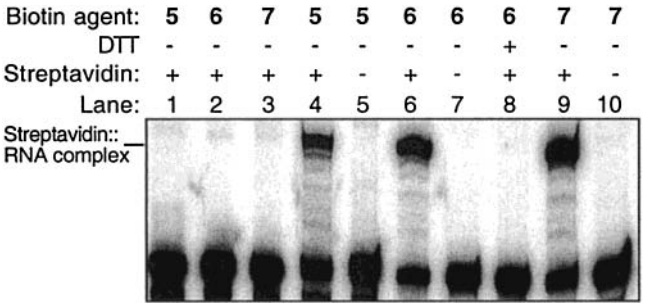


Figure 1. The autoradiogram of the streptavidin gel-shift analysis of transcription products (5′-GTP-RNA and 5′-GSMP-RNA) following the incubation with **5**, **6**, and **7**. Lane 1−3, 5′-GTP-RNA; lane 4−10, 5′-HS-RNA.

The overall yield (three steps) of biotinylated RNA is 57% with **6** and 60% with **7** for GSMP (lane 6 and 9, respectively). The average incorporation efficiency of GSMP with **6** and **7** is over 80% for each step with a ratio of GSMP:GTP = 8:1 for transcription. If the ratio of GSMP:NTP is increased to 16:1, the incorporating yield will be significantly increased (data not shown). The experiments demonstrated that GSMP **4** can serve as a good initiator for T7 RNA polymerase to introduce the sulfhydryl group into 5′-end of RNA.

The 5′-deoxy-5′-thioribonucleotide-5′-triphosphate and 5′-deoxy-5′-thio-2′-deoxyribonucleotide-5′-triphosphate used to form the internal thiophosphate bridge are not substrates for DNA-dependent RNA polymerase and DNA polymerase I from *E. coli*, but weak competitive inhibitors.[15] However, our result is consistent with the observation that the 5′-modified guanosines can serve as initiators for T7 RNA polymerase transcript and supports that T7 RNA polymerase does not interact with the 5′-phosphate of the initiating nucleotide.[16]

Furthermore, the 5′-GMPS-RNA was also prepared by a method similar to that described above with the modification that guanosine-5′-monophosphorothioate (GMPS) **8** replaced GSMP **4**. To compare the transcriptional incorporation efficiency of GSMP with GMPS, the 5′-GMPS-RNA and 5′-HS-RNA were coupled with **5** followed by analysis with a streptavidin gel-shift assay because 5′-GMPS-RNA can form stable biotin-RNA product with iodoacetamide **5** (Figure 2). In Figure 2, the overall yield of biotinylated RNA with **5** is 55% for GSMP in three steps (lane 2), which is

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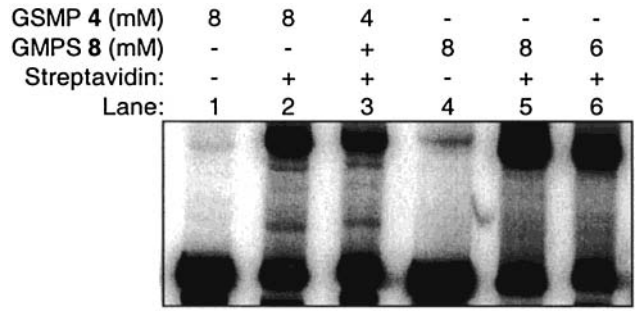

Figure 2. The autoradiogram of the streptavidin gel-shift assays of 5′-GMPS-RNA and 5′-HS-RNA with **5**.

almost the same overall yield (57%) as GMPS in two steps (lane 5). Thus, GSMP can serve as good an initiator for T7 RNA polymerase as GMPS.

However, the major advantage of the 5′-HS-RNA generated from 5′-GSMP-RNA is that it provides a stable disulfide linkage that is crucial for bioconjugation or immobilized binding studies. We observed that the Biotin-linker-SSPO$_3$-RNA is less stable than Biotin-SS-RNA (Figure 3). In **Figure**

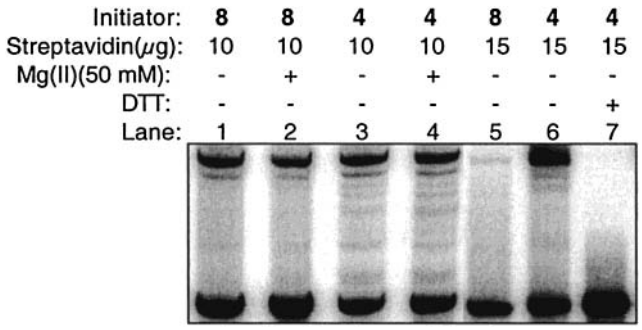

Figure 3. The autoradiogram of the stability analysis of 5′-Biotin-SSPO$_3$-RNA and 5′-Biotin-SS-RNA.

3, the freshly made 5′-Biotin-SSPO$_3$-RNA was detected by streptavidin gel-shift assay (lane 1). After the purification by denaturing 7.5 M urea/8% polyacrylamide gel electrophoresis, the 5′-Biotin-SSPO$_3$-RNA was completely decomposed (lane 5); but the 5′-Biotin-S-S-RNA remained stable without any detectable decomposition under identical conditions (lane 6). The 5′-Biotin-SSPO$_3$-RNA (lane 2) and 5′-Biotin-SS-RNA (lane 4) are stable when they were incubated with 50 mM Mg(II) at 37 °C for 2 h. Famulok et al.[9b] reported that the phosphorothioate sulfide RNA (R-SSPO$_3$-RNA) is slightly unstable compared to the alkyl-disulfide RNA under certain condition (50 mM K-MOPS buffer, 200 mM NaCl, room temperature, and pH 7.4), but it is unstable at pH > 7.5, temperature higher than 23 °C, or even low-salt conditions. Their results suggested that the metal ions can stabilize the phosphorothioate sulfide compounds.

We have also studied the derivatization of 5′-HS-RNA with other different thiol-reactive functional agents: phenylanaline-pyridyl disulfides, β-galactose-pyridyl disulfides, and pyrene-maleimide. The coupling of 5′-thiol-RNA with these reagents is almost quantitative.[17]

In conclusion, the results demonstrate that GSMP can serve as substrates for T7 RNA polymerase to introduce a sulfhydryl group into the 5′-end of RNA. This is the first report that a free thiol group can be incorporated into the 5′-terminus of RNA by in vitro transcription. This report provides a useful method to introduce reporters via a stable thio-linkage, such as fluorophores, into the 5′-terminus of RNA efficiently. This method may have potential applications in analysis and detection of RNA, mapping of RNA−protein interactions, in vitro selection of novel catalytic RNAs, and even gene array.

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Supporting Information Available: Experimental details and spectral/analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) The quantitative analysis for the coupling reactions of 5′-HS-RNA with pyridyl disulfide reagents was determined using the concentration of the pridine-2-thione released by measuring the absorbance at 343 nm; quantification for pyrene-maleimide was determined by the Molecular Dynamics PhosphorImager.