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Two-step synthesis of 5′-deoxy-5′-thioguanosine-5′-monophosphorothioate and its incorporation efficiency into 5′-terminus of RNA for preparation of thiol-functionalized RNA

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ABSTRACT

Several 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. While phosphorothioate modification is one of the most popular methods for functionalizing the 5′-terminus of RNA by a transcription or kinase reaction, conjugation of terminal phosphorothioates with fluorophores has been achieved only with a low efficiency. To overcome this limitation, we have developed a two-step synthetic method for 5′-deoxy-5′-thioguanosine-5′-monophosphorothioate by combining two known reactions and measured its incorporation efficiency into the 5′-terminus of RNA by in vitro transcription using T7 RNA polymerase that requires guanosine to efficiently initiate transcription, followed by treatment of alkaline phosphatase, yielding a terminal sulfhydryl group at the 5′-termini of RNA molecules. Since the sulfhydryl group can be used as an alternative to phosphorothioates, our method may provide a useful route to efficiently introduce reporters, such as fluorophores, into the 5′-terminus of RNA via a stable thio-linker, or to tether the oligomer to a solid support.

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Although the modern biological systems are based on DNA genomes and protein enzymes, RNA plays important roles in many fundamental cellular processes including regulation of protein biosynthesis, RNA splicing, and retroviral replication, with remarkable features: (1) it contains only four different 'building blocks' that share similar chemical properties, (2) it can fold into various tertiary structures that are highly tolerant of sequence variations, and (3) it is easily soluble in water.[1] In addition, the discovery of the self-splicing pre-rRNA in *Tetrahymena* and the cleavage of tRNA precursor by the RNA component within a ribonucleoprotein complex demonstrated the existence of catalytic RNAs, the *ribozymes*.[2] From this point of view, site-specific substitution and derivatization of RNA can provide powerful tools for studying RNA structure and function.[3] Although the conventional solid-phase synthetic method can be used to introduce functional groups at any specific position of oligonucleotides shorter than approximately 40 nucleotides (nt),[4] there are a limited number of methodologies for site-specific modification and substitution of larger RNA molecules.

While several 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.[5] Fluorophores are the most attractive probes for RNA structure, but conjugating terminal phosphorothioates with fluorophores has been achieved with only a low efficiency.[6,7] The sulfhydryl group is another special reactive group that can be incorporated into nucleic acids as an alternative to the use of phosphorothioates,[8] because the thiol-reactive functional groups include haloacetamides, maleimides, benzylic halides, and bromomethyl ketones,[9] and the thiol group demonstrates a unique property of the thiol-disulfide exchange reaction. A free thiol group can be chemically introduced into the 5′-termini of RNA using carbodiimide and cysteamine, but the phosphoramidate linkage is not very stable.[10]

To overcome these limitations, we have developed a two-step synthetic method for 5′-deoxy-5′-thioguanosine-5′-monophosphorothioate (GSMP) and measured its incorporation efficiency into the 5′-terminus of RNA by in vitro transcription using T7 RNA polymerase that requires guanosine to efficiently initiate transcription, followed by treatment of alkaline phosphatase for introduction of a terminal sulfhydryl group into the 5′-termini of RNA molecules.[11]

GSMP was synthesized via a two-step reaction by simply combining two known reactions (Scheme 1).[12,13] In brief, iodine was added over 5 min to a magnetically stirred suspension of

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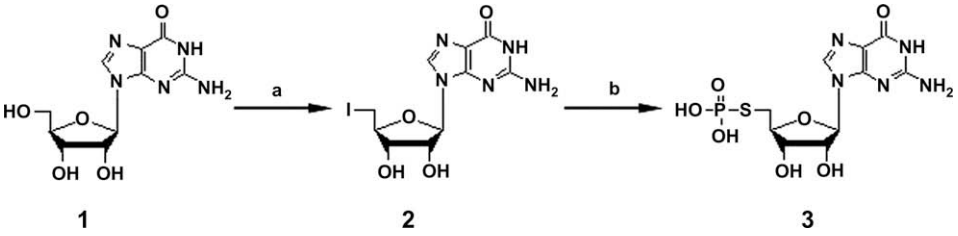
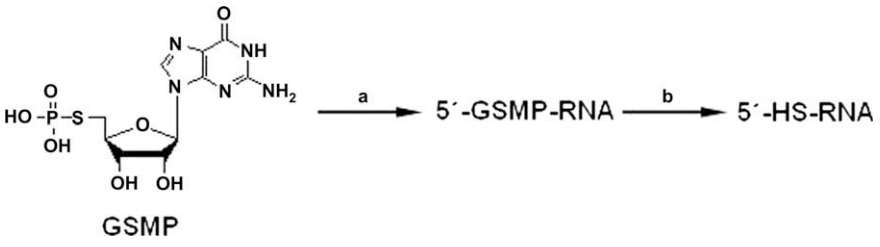
guanosine hydrate, triphenylphosphine, and imidazole in *N*-methyl-2-pyrrolidinone at room temperature. After 3 h the solution was diluted with dichloromethane and water, yielding a white crystalline solid **2**.[12a] Iodinated guanosine **2** was then reacted with trisodium thiophosphate in water for 3 days, yielding the desired product **3**.[11,12b] GSMP **3** was characterized by proton NMR and then quantitatively tested as a substrate for in vitro transcription (Scheme 2), since GSMP **3** had already been suggested that it could be used to introduce a terminal sulfhydryl group into the 5′-termini of RNA molecules.[11,14]

A 97-mer single-stranded DNA containing a T7 promoter at the 5′-end (5′-CAG GAC TGC TCT CAC TCT CAC GCA CCA AGA AGC TGC CAT TGA TCC CGC TGC TCA GCA GAT ACT CAG CGG CCC CCC CTA TAG TGA GTC GTA TTA GTC C-3′) was used as the template, and the 71-nt 5′-GSMP-RNA was synthesized by runoff transcription in the presence of GSMP **3** with molar ratios of GSMP:GTP:ATP:CTP:UTP = 0:1:1:1:1, 4:1:1:1:1, 8:1:1:1:1, 10:1:1:1:1, 20:1:1:1:1, and 50:1:1:1:1 mM, respectively. Figure 1(a) shows that GSMP can be used as a substrate for T7 RNA polymerase that requires guanosine to efficiently initiate in vitro transcription, as reported,[11,15] although a comparison of these incorporation efficiencies with published reports using similar initiator nucleotides is complicated by the facts that each laboratory prefers slightly different transcription conditions and that the nucleotide concentrations frequently vary among studies.

This result, however, clearly demonstrates that the in vitro transcription can be used to incorporate a sulfhydryl moiety to 5′-end of RNA molecule, since an enzymatic step using alkaline phosphatase can be applied to convert 5′-GSMP-RNA to 5′-HS-RNA.[14] Therefore, each of the 5′-GSMP-RNA obtained was dephosphorylated, according to the literature,[11] by incubation with 10 units of alkaline phosphatase in buffer 3 (New England Biolab, MA) at 37 °C for 2 h and stopped by incubation with 10 μL of 200 mM EGTA for 10 min at 65 °C. The resulting RNA was recovered by ethanol precipitation, and tested for quantitation of the thiol group using thiol and sulfide quantitation kit (Molecular Probes, OR) according to the manufacturer's manual (see Table 1 for percentage of the sulfhydryl group existent at 5′-end of RNA molecules).[14]



Scheme 1. Synthetic scheme for 5′-deoxy-5′-thioguanosine-5′-monophosphorothioate **3** (GSMP). Reagents and conditions: (a) $P(Ph)_3$, I_2, imidazole, *N*-methyl-2-pyrrolidinone; (b) trisodium thiophosphate, water.



Scheme 2. Schematic diagram for preparation of thiol-functionalized RNA at 5′-end. Reagents and conditions: (a) T7 RNA polymerase, GSMP:GTP:ATP:CTP:UTP; (b) alkaline phosphatase.

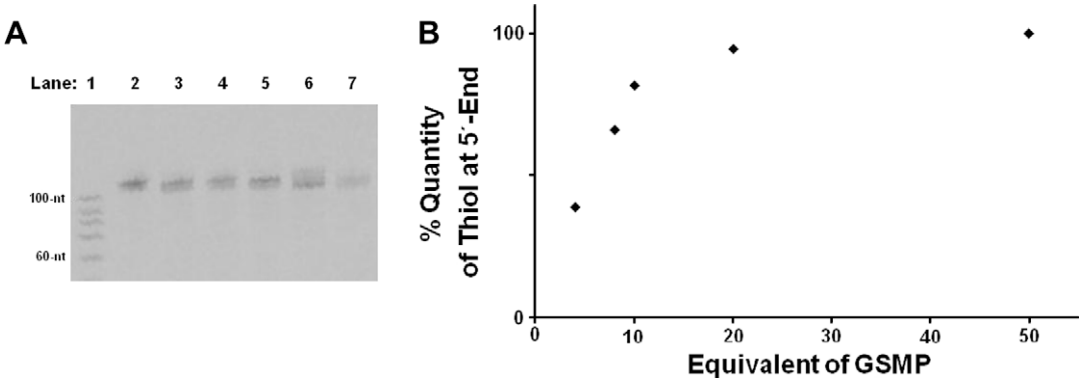


Figure 1. (a) Image of T7 RNA polymerase transcripts from the template to compare the in vitro transcription efficiency. Lane 1, ssDNA ladder (IDT, IA); lane 2, 5′-GTP-RNA resulting from the transcript without GSMP; lanes 3–7, mixtures of 5′-GTP-RNA and 5′-GSMP-RNA resulting from 4, 8, 10, 20, and 50 equiv of GSMP, respectively. The image of the RNA products was obtained using Gel Doc 2000 Gel Documentation System (Bio-Rad, CA), following denaturing 7.5 M urea/8% polyacrylamide gel electrophoresis and EtBr staining; (b) Quantitation of the sulfhydryl group existent at 5′-end of RNA molecule, resulting from alkaline phosphatase treatment to convert 5′-GSMP-RNA to 5′-HS-RNA.

Table 1
Percentage of the sulfhydryl group existent at 5′-end of RNA molecule, resulting from alkaline phosphatase treatment to convert 5′-GSMP-RNA to 5′-HS-RNA

[GSMP]:[GTP]:[ATP]:[CTP]:[UTP]	Percentage quantity of thiol at 5′ end
4:1:1:1:1	38.9 (3.44 μg)
8:1:1:1:1	66.0 (3.10 μg)
10:1:1:1:1	81.5 (3.45 μg)
20:1:1:1:1	94.6 (2.80 μg)
50:1:1:1:1	100 (2.26 μg)

The total amount of each alkaline phosphatase-treated RNA used for percentage measurement of the sulfhydryl group existent at 5′-end of RNA molecule is presented in the parenthesis.

Figure 1b shows a saturation graph for the percent quantity of the sulfhydryl group at 5′-end of RNA molecule. When the ratio of GSMP:GTP was 4:1, approximately 39% of the nascent transcript were initiated with GSMP. The percent of transcripts initiated with GSMP increased to 66%, 81%, 95%, and 100% as the GSMP:GTP ratio was varied to 8:1, 10:1, 20:1, and 50:1, respectively, although 50 times excess of GSMP appeared to slightly inhibit transcription by T7 RNA polymerase (Fig. 1a). These thiol-containing RNAs generated by the transcription/dephosphorylation reactions were successfully conjugated to maleimide-activated nanoparticles (data not shown).

To summarize, we report that GSMP, an initiator for the in vitro transcription, was synthesized via a two-step reaction by combining two known reactions and used according to the literature[11] as a substrate for T7 RNA polymerase, which was followed by an additional step of dephosphorylation of 5′-GSMP-RNA in order to produce 5′-HS-RNA. Our method may provide a useful route to efficiently introduce reporters, such as fluorophores, into the 5′-terminus of RNA via a stable thio-linkage, or to tether the oligomer to a solid support.

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11. It should be noted that it was reported that GSMP could be prepared via a 4-step synthesis and used to introduce the sulfhydryl group at the 5′-terminus of RNA (Zhang, L.; Sun, L.; Cui, Z.; Gottlieb, R. L.; Zhang, B. *Bioconjugate Chem.* **2001**, *12*, 939–948).
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13. *General methods*: Unless otherwise noted, reagents were obtained from commercial suppliers and were used without further purification. Depc-treated deionized water was used whenever necessary. ^{1}H NMR spectra were carried out on Bruker 400 MHz spectrometer and TMS was used as an internal reference for ^{1}H. All experiments were performed in duplicate.
GSMP synthesis: GSMP was synthesized via a two-step reaction (Scheme 1 and see Ref.[12]). In brief, iodine (4.02 g, 15.8 mmol) was added over 5 min to a magnetically stirred suspension of guanosine hydrate (1.5 g, 5 mmol), triphenylphosphine (4.32 g, 16.5 mmol), and imidazole (2.25 g. 33.1 mmol) in *N*-methyl-2-pyrrolidinone (20 ml) at room temperature. During the addition complete dissolution occurred and the solution warmed to 60 °C. The solution cooled back to room temperature and after 3 h was diluted with dichloromethane (200 ml) and water (60 ml). A white crystalline solid separated from solution and was collected by filtration to give 1.4 g (71.2%) of **2**. Trisodium thiophosphate (0.48 g, 2.6 mmol) was added to a suspension of iodinated guanosine **2** (0.283 g, 0.72 mmol) in 1.4 ml of water. 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 10 ml of water and precipitated by addition of 20 ml of methanol. After removing the precipitate by filtration, filtrate was evaporated and dissolved in small amount of water and applied to reverse-phase chromatography. The desired product was collected and dried by lyophilizer (68% yield from **2**). 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 (ddd, 2H), 2.83 (m, 2H).
14. *In vitro transcription and thiol quantitation*: A 97-mer single-stranded DNA containing a T7 promoter at the 5′-end (5′-CAG GAC TGC TCT CAC TCT CAC GCA CCA AGA AGC TGC CAT TGA TCC CGC TGC TCA GCA GAT ACT CAG CGG CCC CCC CTA TAG TGA GTC GTA TTA GTC C-3′) was used as the template. Transcription reactions were carried out with 50 units of T7 RNA polymerase in the presence of 0.2 mM each GTP, ATP, CTP, and UTP, 12 μg of DNA template, 2 mM spermidine, 10 mM dithiothreitol, 6 mM $MgCl_2$, and 40 mM Tris buffer (pH 7.9) at 37 °C in a total of 0.2 mL solution. The 71-nt 5′-GSMP-RNA was synthesized by runoff transcription in the presence of GSMP **3** with a ratio of GSMP:GTP:ATP:CTP:UTP = 0:1:1:1:1, 4:1:1:1:1, 8:1:1:1:1, 10:1:1:1:1, 20:1:1:1:1, and 50:1:1:1:1 mM, respectively. Each of the GSMP-labeled RNAs was purified by denaturing 7.5 M urea/8% polyacrylamide gel electrophoresis, and the resulting 5′-GSMP-RNA was dephosphorylated by incubation with 10 units of alkaline phosphatase in buffer 3 (New England Biolab, MA) 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, and tested for quantitation of the thiol group using thiol and sulfide quantitation kit (Molecular Probes, OR) according to the manufacturer's manual.
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