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Synthesis of 5'-Thio-3'-O-Ribonucleoside Phosphoramidites

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Abstract

The chemical synthesis of phosphoramidite derivatives of all four 5'-deoxy-5'-thioribonucleosides is described. These phosphoramidites contained either trityl (A, G, C and U), dimethoxytrityl (A, G) or *tert*-butyldisulfanyl (G) as the 5'-*S* protecting group. The application of several of these phosphoramidites for solid-phase synthesis of oligoribonucleotides containing a 2'-*O*-photocaged 5'-*S*-phosphorothiolate linkage or 5'-thiol labeled RNAs is also further investigated.

Graphical Abstract

R = Tr, B = G(CHNMe₂)
R = DMT, B = G(CHNMe₂)
R = *t*-BuS, B = G(CHNMe₂)
R = Tr, B = A^{Bz}
R = DMT, B = A^{Bz}
R = Tr, B = C(COCH₂OPh)
R = Tr, B = U

INTRODUCTION

The 5'-*S*-phosphorothiolate (5'-PS) oligonucleotides (Figure 1) including oligodeoxynucleotides (ODNs) and oligoribonucleotides (ORNs) containing a 5'-*S*-phosphorothiolate linkage have received much attention due to their potential therapeutic applications and as biological probes to investigate the catalytic mechanisms of protein and RNA enzymes.^{1,2} The linear ODNs containing a site-specific 5'-*S*-phosphorothiolate DNA linkage have been prepared by solid-phase synthesis through coupling to 2'-deoxy-5'-thionucleoside phosphoramidites.^{3,4} These 5'-PS ODNs (Figure 1, I) have been used as suicide substrates for the studies of DNA site-specific recombinases,^{5–8} and DNA Topoisomerase.^{5,9–14} Additionally cyclic 5'-PS ODNs have been chemically synthesized and their stability and structural conformations were investigated.^{15–20} The cyclic d(GA-ps-GA) and d(A-ps-G) molecules containing a 5'-PS linkage were investigated as antiviral agents¹⁶ and Ricin Toxin A-chain inhibitors.²¹ The ODNs containing a RNA/DNA dinucleotide 5'-PS linkage (Figure 1, II) have been prepared by solid-phase synthesis with

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Supporting Information

¹H NMR and ³¹P NMR of phosphoramidites **26a–26g**, MALDI-TOF MS of **28**, **28a**, **29** and **30**, ¹H NMR and ¹³C NMR spectra of all other new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

coupling to 2'-deoxy-5'-thionucleoside phosphoramidites and has been used to investigate the catalytic mechanism of the hammerhead ribozyme.^{22–26} Oligonucleotides containing photocaged RNA/DNA dinucleotide 5'-PS linkage (Figure 1, II*) have been synthesized with coupling to 2'-deoxy-5'-thioguanosine phosphoramidite, followed by coupling to 2'-photocaged cytidine phosphoramidite, and used to reveal the general acid mechanism of HDV ribozyme catalyzed reaction.²⁷ For the preparation of photocaged RNA oligonucleotides containing a 5'-PS linkage (Figure 1, III) we reported a general and efficient approach by a two-step enzymatic ligation method starting from a caged 5'-PS dinucleotide (rB₁)-ps-(rB₂).²⁸ These caged RNAs have proven to be valuable substrates to investigate the mechanisms of the Varkud Satellite (VS) and hairpin ribozyme catalyzed reactions.^{29,30} In order to develop an efficient synthesis of ORNs containing a 2'-*O*-photocaged 5'-*S*-phosphorothiolate linkage (Figure 1, III), here we report the synthesis of 5'-*S*-tritylthio-3'-*O*-ribonucleoside phosphoramidites and their incorporation into ORNs containing a 5'-PS linkage by phosphoramidite chemistry.

RESULTS AND DISCUSSION

1. Synthesis of 5'-thio-2'-*O*-TBS guanosine derivatives

The DMT and trityl groups have been successfully utilized for the protection of 5'-thiol group during the preparation and incorporation of 2'-deoxy-5'-thionucleoside phosphoramidites into ODNs containing a 5'-PS DNA dinucleotide (dB₁)-ps-(dB₂) (Figure 1, I) by solid phase synthesis.^{3,4} Accordingly we chose trityl and DMT as protective groups for the synthesis of 5'-thio-3'-*O*-ribonucleoside phosphoramidites. 5'-Iodo-5'-deoxy-2',3'-*O*-isopropylideneguanosine (**1**) could be prepared by iodination of 2',3'-*O*-isopropylideneguanosine.³¹ Substitution of **1** with tritylthiol yielded 5'-tritylthio-5'-deoxy-2',3'-*O*-isopropylideneguanosine (**2**) in 65% yield (Scheme 1). Treatment of **2** with 40% formic acid removed the isopropylidene group and generated 5'-tritylthio-5'-deoxyguanosine (**3**) in 97% yield. Protection of the 2-NH₂ group of **3** with *N,N*-dimethylformamide dimethyl acetal generated **4** in 67% yield. Silylation of **4** with TBSOTf in the presence of triethylamine yielded the corresponding desired 2'-*O*-TBS isomer **5a** in 23% yield along with the undesired 3'-*O*-TBS isomer **5b** in 63% yield. Fortunately, the undesired 5'-tritylthio-3'-*O*-TBS isomer **5b** could be partially isomerized to the 2'-*O*-TBS isomer **5a** (30% yield) in refluxing methanol. It could also be converted into the 5'-DMT-thio-2'-*O*-TBS derivative **6a** in four steps (13% overall yield) as shown in Scheme 1.

Starting from guanosine, a straightforward method for the synthesis of **6a** was developed (Scheme 2). 5'-Iodo-5'-deoxyguanosine (**7**) prepared from guanosine by the reaction with iodine/Ph₃P³² could be converted into *N*²-dimethylaminomethylene-5'-DMT-thioguanosine (**8**) in two steps (73% yield). Silylation of **8** with TBSOTf in the presence of triethylamine yielded the corresponding desired 2'-*O*-TBS derivative **6a** (44% yield) along with the undesired 3'-*O*-TBS derivative **6b** (44% yield).

In addition to the synthesis of 5'-*S*-tritylthio- and 5'-DMT-thio-2'-*O*-TBS-5'-deoxyguanosine derivatives (**5a** and **6a**), we also developed an efficient method to synthesize 5'-*tert*-butyldisulfanyl-2'-*O*-TBS guanosine derivative (Scheme 3). Substitution of 5'-iodo-5'

5'-deoxy-2',3'-*O*-isopropylidene-2-thioadenosine (**1**) with potassium thioacetate yielded the corresponding 5'-acetylthio derivative (**9**).²⁸ After removal of the acetyl group and subsequent reaction with *tert*-butylthiol in the presence of 2,2'-dithiobis(5-nitropyridine), compound **9** was converted into the 5'-*tert*-butyldisulfanyl guanosine derivative (**10**) in 72% yield. Treatment of **10** with 50% formic acid removed the isopropylidene group. Protection of the 2-NH₂ group of guanosine with *N,N*-dimethylformamide dimethyl acetal generated **11** in 59% yield. Silylation of **11** with TBSOTf in the presence of triethylamine yielded the corresponding desired 5'-*tert*-butyldisulfanyl-2'-*O*-TBS derivative **12a** (19% yield) along with the undesired 5'-disulfide-3'-*O*-TBS derivative **12b** (54% yield). Similarly, the undesired 5'-disulfide-3'-*O*-TBS isomer **12b** could be partially isomerized to the 5'-disulfide-2'-*O*-TBS isomer **12a** (50% yield) in refluxing methanol.

2. Synthesis of 5'-thio-2'-*O*-TBS adenosine derivatives

5'-Acetylthio-5'-deoxy-2',3'-*O*-isopropylideneadenosine (**14**) was prepared from commercially available 2',3'-*O*-isopropylideneadenosine (**13**) and thioacetic acid under Mitsunobu conditions in quantitative yield according to Pignot et al.'s procedure (Scheme 4).³³ Treatment of **14** with 80% formic acid at 40 °C for 18 hours yielded 5'-acetylthio-5'-deoxyadenosine (**15**) in 87% yield. Compound **15** could be converted to the 5'-tritylthioadenosine derivative (**16a**) and the 5'-DMT-thioadenosine derivative (**16b**) in three steps (deacetylation, 5'-*S*-trityl protection and *N*⁶-protection) with 62%, and 39% yields, respectively. Silylation of **16a** and **16b** with TBSOTf yielded the corresponding desired 2'-*O*-TBS derivatives **17a** and **18a** in 36–40% yields along with the undesired 3'-*O*-TBS derivatives **17b** and **18b** in 36–47% yields (Scheme 4).

3. Synthesis of 5'-thio-2'-*O*-TBS cytidine derivative

5'-Chloro-5'-deoxycytidine (**19**) could be prepared by the direct halogenation of cytidine with SOCl₂ in 69% yield (Scheme 5).³⁴ *S*_N2 reaction of **19** with triphenylmethanethiol under basic condition generated 5'-tritylthio-5'-deoxycytidine (**20**) in 97% yield. Transient protection of the cytosine amino group of **20** with phenoxyacetyl group gave compound **21** in 71% yield.³⁵ Silylation of **21** with TBSOTf yielded the corresponding desired 2'-*O*-TBS derivatives **22a** in 21% yield along with the undesired 3'-*O*-TBS derivatives **22b** in 38% yield.

4. Synthesis of 5'-thio-2'-*O*-TBS uridine derivative

5'-Chloro-5'-deoxyuridine (**23**) could be prepared by the 5'-halogenation of uridine with carbon tetrachloride and triphenylphosphine in pyridine in 99% yield (Scheme 6).³⁶ *S*_N2 reaction of **23** with triphenylmethanethiol under basic condition generated 5'-tritylthio-5'-deoxyuridine (**24**) in 99% yield. Unfortunately, attempts to prepare the 2'-*O*-TBS derivatives **25a** by the reaction of **24** with TBSOTf failed. However, silylation of **24** with TBSCl in pyridine successfully gave the corresponding desired 2'-*O*-TBS derivatives **25a** in 62% yield along with the undesired 3'-*O*-TBS derivatives **25b** in 21% yield.

The RF value of 2'-*O*-TBS isomer on TLC is higher than that of 3'-*O*-TBS isomer. The structure was further confirmed by the COSY spectra (See supporting information for the cosy of **17a** and **17b**).

5. Synthesis of 5'-thio-3'-*O*-ribonucleoside phosphoramidites

3'-Phosphitylation of the 5'-thio 2'-*O*-TBS-ribonucleosides (**5a**, **6a**, **12a**, **17a**, **18a**, **22a** and **25a**) generated the corresponding 5'-thio-2'-*O*-TBS-3'-*O*-ribonucleoside phosphoramidites **26a-26g** in 65–97% yields (Scheme 7).

6. Solid-phase synthesis

As an example for application of these 5'-thio-3'-*O*-ribonucleoside phosphoramidites, we utilized both 5'-tritylthio-3'-*O*-guanosine phosphoramidites **26a** and 2'-photocaged cytidine phosphoramidite **27**³⁷ to prepare an 11 mer ORN containing a 5'-PS linkage: 5'-UUC_{2'-*O*-NBn}G_{5'-S}GGUCGGC-3' (**28**) successfully (Scheme 8). We modified the protocol to include double coupling for modified phosphoramidites. After standard solid phase synthesis to the residue immediately preceding the 5'-thionucleoside and subsequent double coupling to **26a**, the 5'-trityl group was removed by the treatment of aqueous silver nitrate. After further double coupling to phosphoramidite **27**, the synthesis was continued for the rest of the designed RNA. The synthesized RNA was deprotected and purified by dPAGE. The desired RNA containing a PS linkage (**28**) was obtained and the structure was confirmed by the MALDI-TOF MS. Attempts to extend this solid-phase synthetic method to prepare ORN containing an internal 5'-PS linkage (5'-GCGCG_{2'-*O*-NBn}A_{5'-S}AGGCGUC-3') for the mechanism studies of VS catalyzed reaction was unsuccessful. No desired length of RNA containing a 5'-PS linkage was isolated. We surmised that the failed synthesis could reflect inefficient removal of the 5'-S protecting group or inefficient coupling of the 5'-SH to the 2'-photocaged guanosine phosphoramidite. To investigate further, we carried out an additional synthesis of an oligonucleotide containing the sequence: 5'-UUUUG_{2'-*O*-NBn}U_{5'-S}UUUU-3'. After careful analysis of trityl yields during synthesis and HPLC and MS data of the resulting crude product mixture, we determined that coupling of the 5'-thiol group to the 2'-*O*-photocaged phosphoramidite occurred with poor efficiency. The trityl yield decreased to about 30% after coupling between 5'-tritylthioU-CE and 2'-*O*-photocaged G-CE phosphoramidite. The reverse phase HPLC profile of the crude synthesis showed that two major products, 5'-TrSUUUUU-3' and 5'-HSUUUUU-3', (confirmed by MS), form in a ratio of 54:46, indicating that the silver nitrate mediated detritylation occurred with only about 50% efficiency. Although the apparent trityl yield suggested that the modified phosphoramidites possibly couple with 30% efficiency, we could detect no desired 5'-UUUUG_{2'-*O*-NBn}U_{5'-S}UUUU-3'. We have also attempted to make other RNAs containing 5'-PS linkages including 5'-GGCAAGGAGGUAAAAUGUA_{2'-*O*-NBn}G_{5'-S}AAAAACAAU-3' (30 mer), 5'-ACGUU_{2'-*O*-NBn}A_{5'-S}ACGU-3' (10 mer), and 5'-GCCGUC_{2'-*O*-NBn}C_{5'-S}CCCG-3' (11 mer). However, according to MALDI-TOF MS, only 5'-GCCGUC_{2'-*O*-NBn}C_{5'-S}CCCG-3' containing a C_{2'-*O*-NBn}C_{5'-S} linkage (**28a**) formed in low yield. We conclude that at present only the 2'-*O*-photocaged cytidine phosphoramidite **27** couples to the 5'-thiol group with sufficient yield to give the corresponding RNA containing PS linkage. Further work will be

needed to identify strategies to improve 5'-thiol coupling efficiencies for the 2'-*O*-photocaged phosphoramidites of G, A, and U.

We also used **26c** to prepare the (5'-*tert*-butyldisulfanyl)-GG dinucleotide **29** by solid phase synthesis. Although the *tert*-butyldisulfanyl group of **29** is quite stable, as expected, the disulfide bond of **29** can be efficiently broken by DTT/Et₃N overnight to give free 5'-HS-GG dinucleotide (**30**) (Scheme 9). 5'-Thiol-tagged oligonucleotides have been received much attention due to their efficiency to conjugate to important biomolecules or nanoparticles through reaction with an α,β -unsaturated carbonyl groups or thiol group. They can easily react with cysteines in proteins to form disulfide bonds and or bind to gold nanoparticles.³⁸⁻⁴²

Conclusion

We have developed synthetic methods to prepare all of four 5'-thio-3'-*O*-ribonucleoside (guanosine, adenosine, cytidine and uridine) phosphoramidites. These phosphoramidites are useful for synthesis of RNAs that contain a 5'-thiol terminus. In addition, although coupling to the 5'-S occurs with low efficiency in some cases, the phosphoramidites enable synthesis of short RNAs containing and internal PS linkage. It is complementary to our enzymatic two ligation step method for the construction of long RNAs containing an internal 5'-PS PS linkage.²⁸

Experimental

5'-Deoxy-2',3'-*O*-isopropylidene-5'-tritylthioguanosine (**2**)

To the solution of 5'-deoxy-5'-iodo-2',3'-*O*-isopropylideneguanosine (**1**)³¹ (1.55 g, 3.58 mmol) and Ph₃CSH (1.09 g, 3.94 mmol) in DMF (15 mL), 1,1,3,3-tetramethylguanidine (454 mg, 3.94 mmol) was added. The mixture was stirred at room temperature for 3 h. TLC indicated the reaction was complete. The solvent was removed, the residue was isolated by silica gel chromatography, eluting with 5% methanol in chloroform to give **2** as a white foam: 1.35 g (65% yield). ¹H NMR (DMSO-*d*₆) δ 10.70 (brs, 1H), 7.80 (s, 1H), 7.35–7.20 (m, 15H), 6.50 (brs, 2H), 5.91 (s, 1H), 5.20 (m, 1H), 4.89 (dd, 1H, *J* = 3.2, 6.0 Hz), 3.77 (m, 1H), 2.41 (d, 2H, *J* = 6.52 Hz), 1.43 (s, 3H), 1.25 (s, 3H); ¹³C NMR (DMSO-*d*₆) δ 156.8, 153.6, 150.4, 144.2, 136.5, 129.1, 128.0, 126.8, 117.0, 113.1, 88.2, 85.3, 83.6, 83.3, 66.2, 34.6, 26.9, 25.3; HRMS (TOF, ESI/APCI) calcd for C₃₂H₃₂N₅O₄S [MH⁺] 582.2175, found 582.2169.

5'-Deoxy-5'-tritylthioguanosine (**3**)

5'-Deoxy-2',3'-*O*-isopropylidene-5'-tritylthioguanosine (**2**) (0.390 g, 0.67 mmol) was treated with 60% HCO₂H (50 mL) at room temperature overnight, TLC indicated that the reaction was not complete. The reaction was then heated at 40 °C for 4 h, which resulted in successful reaction completion as indicated by TLC. The solvent was removed and the residue was co-evaporated with ethanol until no formic acid was left. The residue was purified by silica gel chromatography, eluting with 10% methanol in chloroform to give **3** as a white foam: 0.351 g (97% yield). ¹H NMR (DMSO-*d*₆) δ 10.59 (brs, 1H), 7.85 (s, 1H), 7.34–7.23 (m, 15H), 6.48 (brs, 2H), 5.60 (d, 1H, *J* = 5.6 Hz), 5.47 (d, 1H, *J* = 5.6 Hz), 5.15

(d, 1H, $J = 5.2$ Hz), 4.45 (m, 1H), 3.85 (m, 1H), 3.62 (m, 1H), 2.55 (dd, 1H, $J = 5.9, 12.8$ Hz), 2.35 (dd, 1H, $J = 7.6, 12.8$ Hz); ^{13}C NMR (DMSO- d_6) δ 156.8, 153.8, 151.5, 144.4, 135.7, 129.2, 128.1, 126.9, 116.7, 86.4, 82.5, 72.70, 72.66, 66.4, 34.6; HRMS (TOF, ESI/APCI) calcd for $\text{C}_{29}\text{H}_{28}\text{N}_5\text{O}_4\text{S}$ $[\text{MH}^+]$ 542.1862, found 542.1854.

5'-Deoxy- N^2 -[(dimethylamino)methylene]-5'-tritylthioguanosine (**4**)

The mixture of 5'-deoxy-5'-tritylthioguanosine (**3**) (1.989 g, 3.67 mmol) and N,N -dimethylformamide dimethyl acetal (4.87 mL, 36.7 mmol) in a mixed solvent of anhydrous dichloromethane (30 mL) and anhydrous methanol (30 mL) was stirred at room temperature for 22 h. TLC indicated reaction was complete. The solvent was removed, and the residue was purified by silica gel chromatography, eluting with 10% methanol in chloroform to give **4** as a white foam: 1.47 g (67% yield). ^1H NMR (DMSO- d_6) δ 8.52 (brs, 1H), 7.98 (s, 1H), 7.33–7.20 (m, 16H), 5.71 (d, 1H, $J = 5.5$ Hz), 5.46 (d, 1H, $J = 5.9$ Hz), 5.23 (d, 1H, $J = 5.3$ Hz), 4.51 (dd, 1H, $J = 5.5, 11.0$ Hz), 3.96 (dd, 1H, $J = 5.0, 9.5$ Hz), 3.61 (m, 1H), 3.11 (s, 3H), 3.03 (s, 3H), 2.59 (dd, 1H, $J = 5.8, 12.8$ Hz), 2.36 (dd, 1H, $J = 7.8, 12.8$ Hz); ^{13}C NMR (DMSO- d_6) δ 157.9, 157.6, 157.3, 150.0, 144.4, 137.1, 129.2, 128.1, 126.9, 119.8, 86.9, 82.5, 73.0, 72.7, 66.3, 40.7, 34.7, 34.6; HRMS (TOF, ESI/APCI) calcd for $\text{C}_{32}\text{H}_{33}\text{N}_6\text{O}_4\text{S}$ $[\text{MH}^+]$ 597.2284, found 597.2272.

2'-*O*-*tert*-Butyldimethylsilyl-5'-deoxy- N^2 -[(dimethylamino)methylene]-5'-tritylthioguanosine (**5a**) and 3'-*O*-*tert*-Butyldimethylsilyl-5'-deoxy- N^2 -[(dimethylamino)methylene]-5'-tritylthioguanosine (**5b**)

To the solution of 5'-deoxy- N^2 -[(dimethylamino)methylene]-5'-tritylthioguanosine (**4**) (0.744 g, 1.25 mmol) in dichloromethane (35 mL) at 0 °C was added triethylamine (0.87 mL, 6.2 mmol), followed by the slow addition of TBSOTf (0.64 mL, 2.79 mmol). The mixture was stirred at room temperature for 4 h. TLC showed the reaction was complete. The mixture was washed with saturated aqueous sodium bicarbonate, brine. The solvent was removed and the residue was isolated by silica gel chromatography, eluting with 2% methanol in chloroform to give 2'-*O*-TBS isomer **5a** (the up spot on TLC): 0.202 g (23% yield) and 3'-*O*-TBS isomer **5b** (the bottom spot on TLC): 0.562 g (63% yield) as white foams.

5a: ^1H NMR (CDCl_3/TMS) δ 9.66 (brs, 1H), 8.55 (s, 1H), 7.67 (s, 1H), 7.45–7.20 (m, 15H), 5.78 (d, 1H, $J = 4.4$ Hz), 4.50 (m, 1H), 3.95 (m, 1H), 3.88 (m, 1H), 3.11 (s, 3H), 3.09 (s, 3H), 2.75 (dd, 1H, $J = 5.2, 13.0$ Hz), 2.59 (d, 1H, $J = 5.6$ Hz), 2.52 (dd, 1H, $J = 6.4, 13.0$ Hz), 0.84 (s, 9H), -0.03 (s, 3H), -0.12 (s, 3H); ^{13}C NMR (CDCl_3) δ 158.3, 158.0, 156.9, 150.2, 144.5, 136.4, 129.6, 128.1, 126.9, 120.8, 88.1, 87.8, 82.6, 75.9, 67.1, 41.4, 35.3, 34.6, 25.7, 18.0, -4.8 , -5.1 ; HRMS (TOF, ESI/APCI) calcd for $\text{C}_{38}\text{H}_{47}\text{N}_6\text{O}_4\text{SSi}$ $[\text{MH}^+]$ 711.3149, found 711.3138.

5b: ^1H NMR (CDCl_3/TMS) δ 9.15 (brs, 1H), 8.55 (s, 1H), 7.74 (s, 1H), 7.45–7.20 (m, 15H), 5.87 (d, 1H, $J = 5.0$ Hz), 4.42 (m, 1H), 4.09 (m, 1H), 4.00 (m, 1H), 3.24 (d, 1H, $J = 6.0$ Hz), 3.12 (s, 3H), 3.09 (s, 3H), 2.65 (dd, 1H, $J = 5.5, 12.7$ Hz), 2.44 (dd, 1H, $J = 6.4, 12.7$ Hz), 0.90 (s, 9H), 0.08 (s, 3H), 0.03 (s, 3H); ^{13}C NMR (CDCl_3) δ 158.2, 158.1, 156.8, 150.3, 144.4, 136.6, 129.6, 128.1, 127.0, 120.7, 88.5, 83.2, 74.4, 74.3, 67.1, 41.5, 35.2, 34.6,

25.8, 18.1, -4.6; HRMS (TOF, ESI/APCI) calcd for $C_{38}H_{47}N_6O_4SSi$ [MH^+] 711.3149, found 711.3139.

Conversion of 5b to 5a—The solution of **5b** (0.787 g, 1.10 mmol) in methanol (30 mL) was heated to reflux for 12 h. After it was cooled down, the mixture was treated with *N,N*-dimethylformamide dimethyl acetal (1.47 mL, 11.0 mmol) and stirred at room temperature for 24 h. The solvent was removed, the residue was isolated by silica gel chromatography, eluting with 2% methanol in chloroform to give **5a** as a white foam (the up spot on TLC): 0.237 g (30% yield) along with the recovered starting **5b** as a white foam (bottom TLC): 0.550 g (70% yield).

3'-*O*-*tert*-Butyldimethylsilyl-5'-deoxy-*N*²-[(dimethylamino)methylene]-5'-dimethoxytritylthioguanosine (6b)

To the solution of **5b** (0.578 g, 0.81 mmol) in THF/MeOH (3:1, 16 mL) was added a solution of $AgNO_3$ (275 mg, 2.0 eq.) in water (1.0 mL) and MeOH (5.0 mL). After 2 min, the solids were recovered by centrifugation. To the pellet was added THF/MeOH (1:1, 40 mL) and DTT (500 mg), and the mixture was stirred for 5 min. The yellow solid was removed by filtration through a short silica gel column, which was washed with THF/MeOH (1:1). The solution was evaporated under reduced pressure and the residue was washed with water. The solid was recovered by filtration through a pad of sand. The product was eluted from sand by washing with THF. The solvent was removed by rotary evaporation and co-evaporated with toluene. The residue was dissolved into pyridine (15 mL). To the resulting solution, DMTCl (549 mg, 1.62 mmol) and DMAP (198 mg, 1.62 mmol) were added. The mixture was stirred at room temperature for 72 h. The solvent was removed, the residue was isolated by silica gel chromatography, eluting with 3% methanol in chloroform to give **6b** as a pale-yellow foam: 0.297 g (47% yield). 1H NMR ($CDCl_3/TMS$) δ 9.87 (brs, 1H), 8.48 (s, 1H), 7.73 (s, 1H), 7.40–7.15 (m, 10H), 6.79 (d, 4H, J = 8.4 Hz), 5.85 (d, 1H, J = 4.8 Hz), 4.54 (m, 1H), 4.09 (m, 1H), 3.98 (m, 1H), 3.77 (s, 6H), 3.07 (s, 3H), 3.02 (s, 3H), 2.68 (dd, 1H, J = 5.2, 12.8 Hz), 2.44 (dd, 1H, J = 6.4, 12.8 Hz), 0.89 (s, 9H), 0.08 (s, 3H), 0.03 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 158.1, 158.0, 157.9, 150.2, 144.9, 136.58, 136.56, 130.5, 129.2, 127.8, 126.6, 120.4, 113.9, 88.3, 83.2, 74.1, 66.0, 55.1, 41.3, 35.0, 34.5, 25.6, 17.9, -4.78, -4.79; HRMS (TOF, ESI/APCI) calcd for $C_{40}H_{51}N_6O_6SSi$ [MH^+] 771.3360, found 771.3355.

2'-*O*-*tert*-Butyldimethylsilyl-5'-deoxy-*N*²-[(dimethylamino)methylene]-5'-dimethoxytritylthioguanosine (6a)

The solution of **6b** (0.179 g, 1.10 mmol) in methanol (10 mL) was heated to reflux overnight. After it was cooled down, the mixture was treated with *N,N*-dimethylformamide dimethyl acetal (0.477 mL, 3.59 mmol) and stirred at room temperature for 2 h. The solvent was removed, the residue was isolated by silica gel chromatography, eluting with 3% methanol in chloroform to give 2'-*O*-TBS guanosine derivative **6a** (the up spot on TLC): 51 mg (28% yield) and to recover the starting material **6b** (the low spot on TLC): 84 mg (47% yield). **6a**: 1H NMR ($CDCl_3/TMS$) δ 9.79 (brs, 1H), 8.55 (s, 1H), 7.70 (s, 1H), 7.40–7.15 (m, 10H), 6.79 (d, 4H, J = 8.5 Hz), 5.79 (d, 1H, J = 5.0 Hz), 4.52 (t, 1H, J = 5.0 Hz), 3.98 (m, 1H), 3.94 (m, 1H), 3.78 (s, 6H), 3.11 (s, 3H), 3.09 (s, 3H), 2.75 (dd, 1H, J = 5.5, 13.0

Hz), 2.64 (brs, 1H), 2.54 (dd, 1H, $J = 6.0, 13.0$ Hz), 0.84 (s, 9H), -0.03 (s, 3H), -0.12 (s, 3H); ^{13}C NMR (CDCl_3) δ 158.1, 157.9, 156.8, 150.1, 145.0, 136.72, 136.70, 136.3, 130.6, 129.3, 127.9, 126.7, 120.7, 113.2, 88.0, 82.7, 75.8, 73.0, 66.1, 55.2, 41.2, 35.2, 34.6, 25.5, 17.9, -4.92 , -5.22 ; HRMS (TOF, ESI/APCI) calcd $\text{C}_{40}\text{H}_{51}\text{N}_6\text{O}_6\text{SSi}$ $[\text{MH}^+]$ 771.3360, found 771.3369.

5'-Deoxy-*N*²-[(dimethylamino)methylene]-5'-dimethoxytritylthioguanosine (**8**)

5'-Dimethoxytrityl thioacetate (DMTSAc) (10.0 mmol) was prepared quantitatively by the reaction of DMTCI (3.38 g, 10.0 mmol) with potassium thioacetate (2.28 g, 20.0 mmol) in anhydrous dichloromethane (50 mL) at room temperature for 1 h. The solvent was removed and the residue was then purified by silica gel chromatography, eluting with 10% ethyl acetate in hexane. ^1H NMR (CDCl_3) δ 7.46 (brs, 5H), 7.38 (m, 4H), 7.02 (m, 4H), 4.00 (s, 6H), 2.46 (s, 3H); ^{13}C NMR (CDCl_3) δ 193.7, 158.3, 144.3, 136.2, 130.8, 129.6, 127.7, 127.0, 113.0, 69.7, 55.1, 30.5.

To the solution of 5'-deoxy-5'-iodoguanosine (**7**)³² (2.83 g, 7.2 mmol) and DMTSAc (3.27 g, 8.6 mmol) in DMF (50 mL), 1,1,3,3-tetramethylguanidine (1.35 mL, 10.75 mmol) was added. The mixture was stirred at room temperature for 24 h. DMF was removed under reduced pressure, and the residue was dissolved into dichloromethane. The solution was washed with water, saturated aqueous NaHCO_3 and brine. The solvent was removed and the residue was isolated by silica gel chromatography, eluting with 5–15% methanol in chloroform to give 5'-deoxy-5'-dimethoxytritylthioguanosine. The mixture of 5'-deoxy-5'-dimethoxytritylthioguanosine and *N,N*-dimethylformamide dimethyl acetal (11.4 mL, 85.6 mmol) in a mixed solvent of anhydrous dichloromethane (50 mL) and anhydrous methanol (50 mL) was stirred at room temperature for 20 h. TLC showed the reaction was complete. The solvent was removed, the residue was purified by silica gel chromatography, eluting with 10% methanol in chloroform to give **8** as a pale-yellow foam: 3.44 g (73% yield for two steps). ^1H NMR ($\text{DMSO}-d_6$) δ 11.3 (s, 1H), 8.52 (brs, 1H), 7.99 (s, 1H), 7.27 (m, 5H), 7.19 (m, 4H), 6.85 (m, 4H), 5.74 (d, 1H, $J = 8.0$ Hz), 5.46 (d, 1H, $J = 4.0$ Hz), 5.23 (d, 1H, $J = 4.0$ Hz), 4.52 (m, 1H), 3.98 (m, 1H), 3.73 (s, 6H), 3.67 (m, 1H), 3.11 (s, 3H), 3.03 (s, 3H), 2.61 (dd, 1H, $J = 4.0, 12.0$ Hz), 2.39 (m, 1H); ^{13}C NMR ($\text{DMSO}-d_6$) δ 158.2, 158.03, 157.96, 157.6, 150.4, 145.5, 137.4, 136.88, 136.85, 130.6, 129.4, 128.3, 127.0, 120.2, 113.6, 87.2, 82.9, 73.4, 73.1, 65.7, 55.4, 41.0, 35.0; HRMS (TOF, APCI) calcd for $\text{C}_{32}\text{H}_{33}\text{N}_6\text{O}_4\text{S}$ $[\text{MCl}^-]$ 691.2111, found 691.2100.

Synthesis of **6a** and **6b**

Both **6a** (0.959 g, 44% yield) and **6b** (0.953 g, 44% yield) were prepared by the reaction of **8** (1.837 g, 2.80 mmol) with TBSOTf (1.47 mL, 6.4 mmol) in presence of triethylamine (1.95 mL, 14.0 mmol) in dichloromethane (50 mL) according to the procedure for the synthesis of **5a** and **5b**.

5'-*tert*-Butyldisulfanyl-5'-deoxy-2',3'-*O*-isopropylideneguanosine (**10**)

Under argon a solution of 5'-aceylthio-5'-deoxy-2',3'-*O*-isopropylideneguanosine (**9**)²⁸ (0.902 g, 2.36 mmol) in a mixed solvent of THF and methanol (90 mL, v/v: 1/1) was saturated with ammonia at 0 °C for 30 min. The solution was stirred at 0 °C for additional 30

min. The solvent was removed, and the residue was dried over vacuum. Under argon the dried residue was dissolved into anhydrous DMF (50 mL). To the resulting solution 2,2'-dithiobis(5-nitropyridine) (805 mg, 2.60 mmol) and 2-methyl-2-propanethiol (2.66 mL, 2.36 mmol) were added and the mixture was stirred at room temperature overnight. The solvent was removed, and the residue was isolated by silica gel chromatography, eluting with 5–10% methanol in chloroform to give **10** as a white foam: 0.729 g (72% yield). ¹H NMR (DMSO-*d*₆) δ 10.78 (brs, 1H), 7.84 (s, 1H), 6.58 (brs, 2H), 5.97 (d, 1H, *J* = 1.6 Hz), 5.31 (dd, 1H, *J* = 6.4, 1.6 Hz), 5.06 (dd, 1H, *J* = 6.4, 3.2 Hz), 4.24 (m, 1H), 3.00 (dd, 1H, *J* = 6.8, 13.6 Hz), 2.90 (dd, 1H, *J* = 7.2, 13.6 Hz), 1.47 (s, 3H), 1.29 (s, 3H), 1.19 (s, 9H); ¹³C NMR (DMSO-*d*₆) δ 157.2, 153.9, 150.8, 137.0, 117.2, 113.5, 89.1, 85.9, 83.9, 83.5, 48.1, 42.8, 29.7, 27.1, 25.4; HRMS (TOF, ESI/APCI) calcd for C₁₇H₂₆N₅O₄S₂ [MH⁺] 428.1421, found 428.1431.

5'-*tert*-Butyldisulfanyl-5'-deoxy-*N*²-[(dimethylamino)methylene]guanosine (**11**)

5'-*tert*-Butyldisulfanyl-5'-deoxy-2',3'-*O*-isopropylideneguanosine (**10**) (0.665 g, 1.55 mmol) was treated with 50% HCO₂H (30 mL) at room temperature for 17 h and then heated to 40 °C for 2 h. The solvent was removed, the residue was dissolved into methanol, then basified with triethylamine. The solvent was removed, and the residue was co-evaporated with toluene to remove the trace amount of formic acid and then dried over vacuum. The dried residue was dissolved in methanol (20 mL). To the resulting solution *N,N*-dimethylformamide dimethyl acetal (2.06 mL, 15.5 mmol) was added and the mixture was stirred at room temperature overnight. The solvent was removed, and the residue was isolated by silica gel chromatography, eluting with 15% methanol in chloroform to give **11** as a white foam: 0.407 g (59% yield). ¹H NMR (DMSO-*d*₆) δ 11.33 (brs, 1H), 8.52 (s, 1H), 7.99 (s, 1H), 5.77 (d, 1H, *J* = 4.8 Hz), 5.56 (brs, 1H), 5.46 (brs, 1H), 4.62 (m, 1H), 4.13 (m, 1H), 4.05 (m, 1H), 3.13–3.00 (m, 2H), 3.14 (s, 3H), 3.01 (s, 3H), 1.25 (s, 9H); ¹³C NMR (DMSO-*d*₆) δ 158.3, 158.1, 157.6, 150.4, 137.8, 120.0, 87.3, 83.0, 73.2, 72.6, 48.1, 43.6, 41.1, 35.0, 29.8; HRMS (TOF, ESI/APCI) calcd for C₁₇H₂₇N₆O₄S₂ [MH⁺] 443.1530, found 443.1532.

2'-*O*-*tert*-Butyldimethylsilyl-5'-*tert*-butyldisulfanyl-5'-deoxy-*N*²-[(dimethylamino)methylene]guanosine (**12a**) and 3'-*O*-*tert*-Butyldimethylsilyl-5'-*tert*-butyldisulfanyl-5'-deoxy-*N*²-[(dimethylamino)methylene]guanosine (**12b**)

To the solution of **11** (0.448 g, 1.01 mmol) in dichloromethane (35 mL) at 0 °C was added triethylamine (0.70 mL, 5.1 mmol), followed by the slow addition of TBSOTf (0.46 mL, 2.02 mmol). The mixture was warmed up and stirred at room temperature for 4 h. TLC showed the reaction was complete. The mixture was washed with saturated sodium bicarbonate, brine. The solvent was removed, the residue was isolated by silica gel chromatography, eluting with 2–5% methanol in chloroform to give 2'-*O*-TBS isomer **12a** (the up spot on TLC): 0.105 g (19% yield) and 3'-*O*-TBS isomer **12b** (the low spot on TLC): 0.304 g (54% yield) as white foams.

12a: ¹H NMR (CDCl₃/TMS) δ 10.04 (brs, 1H), 8.58 (s, 1H), 7.77 (s, 1H), 5.83 (d, 1H, *J* = 4.0 Hz), 4.70 (m, 1H), 4.31 (m, 2H), 3.30–3.05 (m, 2H), 3.17 (s, 3H), 3.11 (s, 3H), 2.99 (brs, 1H), 1.32 (s, 9H), 0.83 (s, 9H), -0.03 (s, 3H), -0.14 (s, 3H); ¹³C NMR (CDCl₃) δ 158.2, 157.9, 156.8, 150.2, 136.5, 120.7, 87.8, 82.9, 75.3, 72.3, 48.2, 44.2, 41.3, 35.2, 29.7, 25.5,

17.8, -5.1, -5.3; HRMS (TOF, ESI/APCI) calcd for C₂₃H₄₁N₆O₄S₂Si [MH⁺] 557.2395, found 557.2401.

12b: ¹H NMR (CDCl₃/TMS) δ 9.95 (brs, 1H), 8.46 (s, 1H), 7.66 (s, 1H), 5.82 (d, 1H, *J* = 6.0 Hz), 4.79 (m, 1H), 4.43 (m, 1H), 4.25 (m, 2H), 3.20–2.90 (m, 2H), 3.13 (s, 3H), 3.02 (s, 3H), 1.28 (s, 9H), 0.93 (s, 9H), 0.18 (s, 3H), 0.17 (s, 3H); ¹³C NMR (CDCl₃) δ 158.1, 156.7, 150.2, 137.2, 120.7, 88.7, 83.8, 73.7, 73.4, 48.2, 43.6, 41.5, 35.2, 29.8, 25.9, 18.2, -4.50, -4.54; HRMS (TOF, ESI/APCI) calcd for C₂₃H₄₁N₆O₄S₂Si [MH⁺] 557.2395, found 557.2396.

Conversion of 12b to 12a—The solution of 3'-*O*-*tert*-butyldimethylsilyl-5'-*tert*-butyldisulfanyl-5'-deoxy-*N*²-[(dimethylamino)methylene]guanosine (**12b**) (0.165 g, 0.296 mmol) in methanol (10 mL) was heated to reflux for 3 h. After it was cooled down, the mixture was treated with *N,N*-dimethylformamide dimethyl acetal (0.395 mL, 2.96 mmol) and stirred at room temperature overnight. The solvent was removed, the residue was isolated by silica gel chromatography, eluting with 2% methanol in chloroform to give **12a** (the up spot on TLC): 82.5 mg (50% yield) along with the recovered starting **12b** (the low spot on TLC): 70.5 mg (43% yield).

5'-Acetylthio-5'-deoxy-2',3'-*O*-isopropylideneadenosine (**14**)

Under argon to a solution of triphenylphosphane (7.55 g, 28.8 mmol) in anhydrous THF (40 mL) at 0 °C, diethyl azodicarboxylate (4.53 mL, 28.8 mmol) was added over 5 min. After stirring for 30 min, 2',3'-*O*-isopropylideneadenosine (**13**) (4.00 g, 13.0 mmol) was added and stirring was continued for 30 min. To the resulted yellow suspension, a solution of thioacetic acid (2.06 mL, 28.8 mmol) in THF (5.0 mL) was slowly added and stirring was continued for another 1 h at 0 °C. During this time, the yellow suspension cleared, and an orange solution was obtained. The solvent was removed under reduced pressure and the residue was purified by silica gel chromatography, eluting with chloroform/THF (4:1, v/v), followed by 5–10% CH₃OH in chloroform to give **14**³³ as an oil: 4.76 g (100% yield). ¹H NMR (CDCl₃/TMS) δ 8.35 (s, 1H), 7.96 (s, 1H), 7.34 (brs, 2H), 6.12 (d, 1H, *J* = 1.8 Hz), 5.55 (dd, 1H, *J* = 6.3, 1.9 Hz), 5.01 (dd, 1H, *J* = 6.3, 3.0 Hz), 4.36 (m, 1H), 3.30 (dd, 1H, *J* = 13.8, 7.3 Hz), 3.19 (dd, 1H, *J* = 13.8, 6.6 Hz), 2.35 (s, 3H), 1.60 (s, 3H), 1.39 (s, 3H); ¹³C NMR (CDCl₃) δ 194.4, 156.0, 152.9, 148.8, 139.6, 120.0, 114.2, 90.6, 85.9, 84.0, 83.5, 31.1, 30.4, 26.9, 25.2.

5'-Acetylthio-5'-deoxyadenosine (**15**)

To the flask containing 5'-acetylthio-5'-deoxy-2',3'-*O*-isopropylideneadenosine (**14**) (1.10 g, 3.00 mmol), a mixture of formic acid (10 mL) and water (2.5 mL) was added. The reaction mixture was heated and stirred at 40 °C for 18 h. TLC showed the reaction was complete. The solvent was removed under reduced pressure, and the trace amount of formic acid was removed by co-evaporating with ethanol. The product was purified by silica gel chromatography, eluting with 10% methanol in chloroform to give **15**³³ 0.844 g (87% yield). ¹H NMR (DMSO-*d*₆) δ 8.36 (s, 1H), 8.17 (s, 1H), 7.33 (brs, 2H), 5.89 (d, 1H, *J* = 5.8 Hz), 5.56 (d, 1H, *J* = 5.4 Hz), 5.42 (d, 1H, *J* = 3.9 Hz), 4.80 (m, 1H), 4.11 (m, 1H), 3.93 (m,

1H), 3.36 (dd, 1H, $J = 13.8, 5.7$ Hz), 3.18 (dd, 1H, $J = 13.8, 7.5$ Hz), 2.35 (s, 3H); ^{13}C NMR (DMSO- d_6) δ 194.9, 156.1, 152.7, 149.4, 140.0, 119.2, 87.5, 82.9, 72.63, 72.56, 31.3, 30.5.

N⁶-Benzoyl-5'-deoxy-5'-tritylthioadenosine (16a)

Under argon 5'-acetylthio-5'-deoxyadenosine (**15**) (1.122 g, 3.45 mmol) in anhydrous methanol (60 mL) was saturated with ammonia at 0 °C for 30 min and kept at 0 °C for additional 30 min. TLC showed the reaction was complete. The solvent was removed, and the residue was dried over vacuum for 15 min. Under argon the dried residue was dissolved into dry pyridine (40 mL). To this solution, DMAP (42 mg, 0.35 mmol), triethylamine (1.34 mL, 9.66 mmol) and trityl chloride (2.32 g, 8.30 mmol) were added. After the reaction mixture was stirred at room temperature for 18 h, TLC showed reaction was complete. To this reaction mixture trimethylsilyl chloride (2.53 mL, 20.0 mmol) was slowly added and stirred at 0 °C for 15 min. Benzoyl chloride (2.32 mL, 20.0 mmol) was then added and the mixture was stirred at room temperature for 16 h. The reaction mixture was chilled in an ice bath, and added cold water (8 mL), followed after 5 min by concentrated aqueous ammonia (8 mL). After 30 min, the solvent was removed, the residue was dissolved into dichloromethane, washed with water, saturated NaHCO₃ and brine. The solvent was removed, the residue was purified by silica gel chromatography, eluting with 3% methanol in chloroform to give **16a** as a yellow foam: 1.349 g (62% yield). ^1H NMR (CDCl₃/TMS) δ 9.53 (brs, 1H), 8.43 (s, 1H), 8.11 (s, 1H), 7.91 (d, 2H, $J = 7.5$ Hz), 7.50–7.10 (m, 18H), 5.94 (d, 1H, $J = 5.5$ Hz), 4.72 (m, 1H), 4.08 (m, 1H), 4.04 (m, 1H), 2.66 (dd, 1H, $J = 5.9, 12.7$ Hz), 2.50 (dd, 1H, $J = 6.0, 12.7$ Hz); ^{13}C NMR (CDCl₃) δ 165.1, 152.0, 151.0, 148.9, 144.2, 142.1, 133.0, 132.8, 129.4, 128.6, 127.9, 126.3, 122.6, 88.9, 83.6, 74.2, 73.1, 66.8, 34.5; HRMS (TOF, ESI/APCI) calcd for C₃₆H₃₂N₅O₄S [MH⁺] 630.2175, found 630.2196.

N⁶-Benzoyl-5'-deoxy-5'-dimethoxytritylthioadenosine (16b)

Under argon 5'-acetylthio-5'-deoxyadenosine (**15**) (1.06 g, 3.26 mmol) in anhydrous methanol (40 mL) was saturated with ammonia at 0 °C for 30 min and kept at 0 °C for additional 30 min. The solvent was removed, and the residue was dried over vacuum for 15 min to give white foam. Under argon the dried residue was dissolved into dry pyridine (30 mL). To this solution, DMAP (40 mg, 0.33 mmol), triethylamine (0.64 mL, 4.6 mmol) and DMTCl (1.33 g, 3.92 mmol) were added. After the reaction mixture was stirred at room temperature for 5 h, TLC showed reaction was complete. To this reaction mixture at 0 °C trimethylsilyl chloride (2.06 mL, 16.3 mmol) was slowly added and stirred for 30 min. Benzoyl chloride (1.89 mL, 16.3 mmol) was then added and the mixture was stirred at room temperature for 3 h. The reaction mixture was chilled in an ice bath, and added cold water (5 mL), followed after 5 min by concentrated aqueous ammonia (5 mL). After 30 min, the solvent was removed, the residue was dissolved into dichloromethane, washed with water, saturated NaHCO₃ and brine. The solvent was removed, the residue was purified by silica gel chromatography, eluting with 3% methanol in chloroform to give **16b** as a yellow foam: 0.875 g (39% yield). ^1H NMR (CDCl₃/TMS) δ 9.55 (brs, 1H), 8.45 (s, 1H), 8.14 (s, 1H), 7.92 (d, 2H, $J = 7.5$ Hz), 7.50–7.10 (m, 12H), 6.76 (d, 4H, $J = 9.0$ Hz), 5.96 (d, 1H, $J = 5.5$ Hz), 4.74 (m, 1H), 4.12 (m, 1H), 4.09 (m, 1H), 3.71 (s, 6H), 2.68 (dd, 1H, $J = 6.0, 12.5$ Hz), 2.52 (dd, 1H, $J = 6.0, 12.5$ Hz); ^{13}C NMR (CDCl₃) δ 165.0, 158.0, 152.0, 151.0, 148.9, 144.9, 142.1, 136.6, 133.0, 132.8, 130.5, 129.2, 128.6, 128.2, 127.8, 126.6, 122.5, 113.1,

88.9, 83.7, 74.2, 73.1, 65.9, 55.1, 34.6; HRMS (TOF, ESI/APCI) calcd for $C_{38}H_{35}N_5O_6SNa$ [MNa^+] 712.2206, found 712.2197.

***N*⁶-Benzoyl-2'-*O*-*tert*-butyldimethylsilyl-5'-deoxy-5'-tritylthioadenosine (17a) and *N*⁶-Benzoyl-3'-*O*-*tert*-butyldimethylsilyl-5'-deoxy-5'-tritylthioadenosine (17b)**

To *N*⁶-benzoyl-5'-deoxy-5'-tritylthioadenosine (**16a**) (579 mg, 0.92 mmol) in dichloromethane (20 mL) was added triethylamine (0.64 mL, 4.6 mmol), followed by TBSOTf (250 μ L, 1.10 mmol). The mixture was stirred at room temperature for 1 h. TLC showed reaction was not complete. To the mixture was added additional TBSOTf (125 μ L, 0.55 mmol), and the mixture was stirred at room temperature overnight. The reaction was quenched with methanol (1 mL). The mixture was washed with saturated sodium bicarbonate, brine. The solvent was removed, the residue was isolated by silica gel chromatography, eluting with 1% methanol in chloroform to give 2'-*O*-TBS isomer **17a** (the up spot on TLC): 0.276 g (40% yield) and 3'-*O*-TBS isomer **17b** (the low spot on TLC): 0.319 g (47% yield).

17a: 1H NMR ($CDCl_3/TMS$) δ 9.14 (brs, 1H), 8.73 (s, 1H), 8.09 (s, 1H), 8.03 (d, 2H, J = 7.6 Hz), 7.60 (t, 1H, J = 7.3 Hz), 7.52 (t, 2H, J = 7.5 Hz), 7.45–7.20 (m, 15H), 5.87 (d, 1H, J = 4.6 Hz), 4.93 (m, 1H), 4.04 (m, 1H), 3.90 (m, 1H), 2.80 (dd, 1H, J = 6.0, 12.8 Hz), 2.61 (dd, 1H, J = 6.7, 12.8 Hz), 2.57 (d, 1H, J = 5.1 Hz), 0.83 (s, 9H), –0.053 (s, 3H), –0.17 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 164.5, 152.7, 151.2, 149.6, 144.3, 142.0, 133.6, 132.8, 129.5, 128.8, 127.9, 127.8, 126.8, 123.5, 89.5, 83.3, 74.6, 73.1, 67.0, 34.2, 25.5, 17.8, –5.0, –5.2; HRMS (TOF, ESI/APCI) calcd for $C_{42}H_{45}N_5O_4SSiNa$ [MNa^+] 766.2854, found 766.2891.

17b: 1H NMR ($CDCl_3/TMS$) δ 9.58 (brs, 1H), 8.61 (s, 1H), 8.13 (s, 1H), 7.98 (d, 2H, J = 7.3 Hz), 7.50–7.15 (m, 18H), 5.93 (d, 1H, J = 4.6 Hz), 4.68 (m, 1H), 4.26 (m, 1H), 4.04 (m, 1H), 3.47 (d, 1H, J = 5.6 Hz), 2.65 (dd, 1H, J = 5.4, 12.5 Hz), 2.55 (dd, 1H, J = 8.6, 12.5 Hz), 0.89 (s, 9H), 0.070 (s, 3H), 0.036 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 164.7, 152.1, 151.2, 149.5, 144.1, 142.1, 133.4, 132.4, 129.3, 128.5, 127.8, 126.6, 123.6, 89.4, 83.6, 74.1, 73.9, 66.8, 34.2, 25.5, 17.8, –4.77, –4.96; HRMS (TOF, ESI/APCI) calcd for $C_{42}H_{45}N_5O_4SSiNa$ [MNa^+] 766.2854, found 766.2833.

***N*⁶-Benzoyl-2'-*O*-*tert*-butyldimethylsilyl-5'-deoxy-5'-dimethoxytritylthioadenosine (18a) and *N*⁶-Benzoyl-3'-*O*-*tert*-butyldimethylsilyl-5'-deoxy-5'-dimethoxytritylthioadenosine (18b)**

To the solution of *N*⁶-benzoyl-5'-deoxy-5'-dimethoxytritylthioadenosine (**16b**) (837 mg, 1.21 mmol) in dichloromethane (25 mL) at 0 °C was added triethylamine (0.84 mL, 6.1 mmol), followed by the slow addition of TBSOTf (306 μ L, 1.33 mmol). The mixture was stirred at room temperature overnight. TLC showed reaction was not complete. To the mixture was added additional TBSOTf (278 μ L, 1.21 mmol), and the mixture was stirred at room temperature for 1 h. TLC showed reaction was almost complete. The reaction was quenched with methanol (1.0 mL). The mixture was washed with saturated sodium bicarbonate, brine. The solvent was removed, the residue was isolated by silica gel chromatography, eluting with 1% methanol in chloroform to give 2'-*O*-TBS isomer **18a** (the up spot on TLC): 0.351 g (36% yield) and 3'-*O*-TBS isomer **18b** (the low spot on TLC): 0.352 g (36% yield).

18a: ^1H NMR (CDCl_3/TMS) δ 9.17 (brs, 1H), 8.72 (s, 1H), 8.10 (s, 1H), 8.02 (d, 2H, $J=7.3$ Hz), 7.58 (t, 1H, $J=7.3$ Hz), 7.48 (t, 2H, $J=7.2$ Hz), 7.40–7.18 (m, 9H), 6.78 (m, 4H), 5.88 (d, 1H, $J=4.7$ Hz), 4.93 (m, 1H), 3.95 (m, 1H), 3.76 (m, 6H), 2.78 (dd, 1H, $J=6.0$, 12.8 Hz), 2.59 (dd, 1H, $J=6.6$, 12.8 Hz), 0.81 (s, 9H), -0.068 (s, 3H), -0.19 (s, 3H); ^{13}C NMR (CDCl_3) δ 164.4, 157.9, 152.5, 151.1, 149.5, 144.8, 141.9, 136.6, 136.5, 133.4, 132.6, 130.4, 129.2, 128.6, 127.7, 127.6, 126.5, 123.3, 113.0, 89.3, 83.3, 74.5, 73.0, 65.9, 55.0, 34.2, 25.3, 17.6, -5.2 , -5.4 ; HRMS (TOF, ESI/APCI) calcd for $\text{C}_{44}\text{H}_{49}\text{N}_5\text{O}_6\text{SSiNa}$ [MNa^+] 826.3071, found 826.3066.

18b: ^1H NMR (CDCl_3/TMS) δ 9.45 (brs, 1H), 8.69 (s, 1H), 8.19 (s, 1H), 8.02 (d, 2H, $J=7.4$ Hz), 7.56 (t, 1H, $J=7.2$ Hz), 7.49 (t, 2H, $J=7.7$ Hz), 7.39 (d, 1H, $J=7.6$ Hz), 7.35–7.15 (m, 7H), 6.79 (d, 4H, $J=8.8$ Hz), 5.94 (d, 1H, $J=4.8$ Hz), 4.67 (m, 1H), 4.28 (m, 1H), 4.05 (m, 1H), 3.77 (m, 6H), 3.21 (brs, 1H), 2.66 (dd, 1H, $J=5.4$, 12.7 Hz), 2.56 (dd, 1H, $J=6.8$, 12.7 Hz), 0.90 (s, 9H), 0.08 (s, 3H), 0.04 (s, 3H); ^{13}C NMR (CDCl_3) δ 164.9, 158.2, 152.5, 151.2, 149.7, 145.0, 142.0, 136.8, 133.7, 132.9, 130.7, 129.4, 128.9, 128.04, 127.99, 126.8, 123.8, 113.3, 89.8, 84.0, 74.38, 74.36, 66.2, 55.3, 34.6, 25.8, 18.1, -4.5 , -4.8 ; HRMS (TOF, ESI/APCI) calcd for $\text{C}_{44}\text{H}_{49}\text{N}_5\text{O}_6\text{SSiNa}$ [MNa^+] 826.3071, found 826.3062.

5'-Chloro-5'-deoxycytidine (19)

According to the procedure described by Kikugawa and Ichino,³⁴ 5'-chloro-5'-deoxycytidine (3.74 g, 69% yield) was prepared by the direct halogenation of cytidine (5.00 g, 20.6 mmol) with thionyl chloride (8.60 mL, 118 mmol) in HMPA (6 mL) at room temperature for 24 h. The product **19** was recrystallized from hot water and obtained as white needle crystals. ^1H NMR ($\text{DMSO}-d_6$) δ 7.59 (d, 1H, $J=7.6$ Hz), 7.28 (brs, 1H), 7.18 (brs, 1H), 5.81 (d, 1H, $J=4.8$ Hz), 5.76 (d, 1H, $J=7.6$ Hz), 5.43 (d, 1H, $J=5.6$ Hz), 5.31 (d, 1H, $J=5.2$ Hz), 4.03 (m, 1H), 3.96 (m, 1H), 3.91 (m, 1H), 3.87 (m, 1H), 3.79 (dd, 1H, $J=11.6$, 6.0 Hz); ^{13}C NMR ($\text{DMSO}-d_6$) δ 165.7, 155.5, 141.7, 94.7, 89.6, 82.5, 73.1, 70.9, 45.2.

5'-Tritylthio-5'-deoxycytidine (20)

To the solution of 5'-chloro-5'-deoxycytidine (**19**) (3.72 g, 15.3 mmol) and Ph_3CSH (7.69 g, 27.8 mmol) in DMSO (50 mL), 1,1,3,3-tetramethylguanidine (1.94 g, 16.8 mmol) was added. After the reaction mixture was stirred at room temperature for 18 h, water (100 mL) was then added. The precipitated solid was collected by filtration and further purified by silica gel chromatography, eluting with 8% methanol in chloroform to give **20** as a white foam: 7.48 g (97% yield). ^1H NMR ($\text{DMSO}-d_6$) δ 7.45 (d, 1H, $J=7.5$ Hz), 7.33 (m, 15H), 5.76 (d, 1H, $J=7.5$ Hz), 5.67 (d, 1H, $J=4.5$ Hz), 5.33 (d, 1H, $J=5.5$ Hz), 5.07 (d, 1H, $J=5.5$ Hz), 3.91 (m, 1H), 3.68–3.53 (m, 2H), 2.45 (dd, 1H, $J=4.8$, 12.8 Hz), 2.45 (dd, 1H, $J=7.6$, 12.8 Hz); ^{13}C NMR ($\text{DMSO}-d_6$) δ 165.5, 155.3, 144.4, 141.8, 129.2, 128.2, 127.0, 94.6, 90.0, 81.4, 73.0, 72.7, 66.3, 34.6; HRMS (TOF, ESI) calcd for $\text{C}_{28}\text{H}_{27}\text{N}_3\text{O}_4\text{SNa}$ [MNa^+] 524.1620, found 524.1615.

N-Phenoxyacetyl-5'-tritylthio-5'-deoxycytidine (21)

Under argon 5'-tritylthio-5'-deoxycytidine (**20**) (7.48 g, 14.9 mmol) was suspended in dry pyridine (90 mL). Chlorotrimethylsilane (15.2 mL) was added to the suspension at 0 °C.

After the mixture was stirred at room temperature for 45 min, a solution of phenoxyacetyl chloride (3.09 mL, 22.4 mmol) and 1,2,4-triazole (1.55 g, 22.4 mmol) in pyridine-acetonitrile (60 mL, 1:1) was slowly added. After the mixture was stirred at room temperature for 2.5 h, the reaction was quenched by addition of H₂O (15 mL). After stirring for 5 min, concentrated aqueous NH₄OH (6.7 mL) was added at 0 °C, and the mixture was stirred for 30 min. The solution was concentrated to remove pyridine and the residue was redissolved into water (20 mL), extracted with dichloromethane (3 × 40 mL). The organic layers were combined and evaporated, the residue was then purified by silica gel chromatography, eluting with 5 % MeOH in CH₂Cl₂ to give **21** (6.68 g, 71% yield) as a yellow foam. ¹H NMR (CDCl₃/TMS) δ 9.35 (brs, 1H), 8.05 (d, 1H, *J* = 7.6 Hz), 7.54 (d, 1H, *J* = 7.6 Hz), 7.45–6.91 (m, 20H), 5.68 (d, 1H, *J* = 4.8 Hz), 4.65 (s, 2H), 4.20 (m, 1H), 4.00 (m, 1H), 3.86 (dd, 1H, *J* = 3.6, 5.2 Hz), 2.56 (dd, 1H, *J* = 4.8, 12.8 Hz), 2.33 (dd, 1H, *J* = 7.2, 12.8 Hz); ¹³C NMR (CDCl₃) δ 168.6, 161.7, 156.69, 156.66, 144.6, 144.3, 130.0, 129.6, 128.2, 127.1, 122.7, 114.7, 96.9, 93.4, 84.6, 76.6, 73.7, 67.4, 67.2, 34.6; HRMS (TOF, ESI) calcd for C₃₆H₃₄N₃O₆S [MH⁺] 636.2168, found 636.2167.

2'-*O*-*tert*-Butyldimethylsilyl-*N*⁴-phenoxyacetyl-5'-tritylthio-5'-deoxycytidine (22a) and 3'-*O*-*tert*-Butyldimethylsilyl-*N*⁴-phenoxyacetyl-5'-tritylthio-5'-deoxycytidine (22b)

To the solution of **21** (1.00 g, 1.57 mmol) in dichloromethane (35 mL) at 0 °C was added triethylamine (1.09 mL, 7.85 mmol), followed by the slow addition of TBSOTf (0.54 mL, 2.36 mmol). The mixture was warmed up and stirred at room temperature for 3 h. TLC showed reaction was not complete. To the mixture were added additional triethylamine (1.09 mL, 7.85 mmol) and TBSOTf (0.54 mL, 2.36 mmol), and the mixture was stirred at room temperature for 1.5 h. TLC showed the reaction was complete. The mixture was neutralized with 1 N HCl, washed with brine and dried over MgSO₄. The solvent was removed, and the residue was isolated by silica gel chromatography, eluting with 25–50% ethyl acetate in hexane to give 2'-*O*-TBS isomer **22a** (the up spot on TLC): 0.256 g (21% yield) and 3'-*O*-TBS isomer **22b** (the low spot on TLC): 0.448 g (38% yield).

22a: ¹H NMR (CDCl₃/TMS) δ 9.54 (brs, 1H), 7.79 (d, 1H, *J* = 7.6 Hz), 7.50–6.80 (m, 21H), 5.64 (s, 1H), 4.65 (s, 2H), 4.15 (d, 1H, *J* = 4.8 Hz), 3.84 (m, 1H), 3.58 (m, 1H), 2.77 (dd, 1H, *J* = 3.6, 13.2 Hz), 2.49 (dd, 1H, *J* = 8.0, 13.2 Hz), 2.25 (d, 1H, *J* = 9.6 Hz), 0.91 (s, 9H), 0.23 (s, 3H), 0.12 (s, 3H); ¹³C NMR (CDCl₃) δ 168.7, 161.7, 156.7, 154.8, 144.6, 144.4, 129.9, 129.6, 128.1, 127.0, 122.6, 114.6, 96.3, 92.2, 82.3, 76.0, 72.8, 67.4, 67.2, 34.4, 25.9, 18.1, -4.3, -5.4; HRMS (TOF, ESI) calcd for C₄₂H₄₈N₃O₆SSi [MH⁺] 750.3033, found 750.3023.

22b: ¹H NMR (CDCl₃/TMS) δ 9.54 (brs, 1H), 7.97 (d, 1H, *J* = 7.6 Hz), 7.50–6.80 (m, 21H), 5.73 (d, 1H, *J* = 3.6 Hz), 4.64 (s, 2H), 4.05–3.95 (m, 2H), 3.88 (m, 1H), 3.42 (brs, 1H), 2.52 (dd, 1H, *J* = 4.4, 13.2 Hz), 2.39 (dd, 1H, *J* = 7.6, 12.8 Hz), 2.25 (d, 1H, *J* = 9.6 Hz), 0.83 (s, 9H), 0.001 (s, 3H), -0.05 (s, 3H); ¹³C NMR (CDCl₃) δ 168.8, 161.8, 156.8, 155.6, 145.4, 144.4, 130.0, 129.6, 128.2, 127.1, 122.6, 114.7, 96.7, 93.1, 83.4, 75.7, 74.4, 67.5, 34.6, 25.9, 18.2, -4.6, -4.7; HRMS (TOF, ESI) calcd for C₄₂H₄₈N₃O₆SSi [MH⁺] 750.3033, found 750.3028.

5'-Chloro-5'-deoxyuridine (23)

According to the procedure described by Anisuzzaman and Whistler,³⁶ 5'-chloro-5'-deoxyuridine (1.30 g, 99% yield) was prepared by the 5'-halogenation of uridine (1.22 g, 5.00 mmol) with carbon tetrachloride (0.48 mL, 5.0 mmol) and triphenylphosphane (2.62 g, 10.0 mmol) in dry pyridine (50 mL) at room temperature for 16 h. The reaction was quenched with methanol (10 mL), the solvent was removed and the residue was isolated by silica gel chromatography, eluting with 5% methanol in dichloromethane to give product as a white foam. ¹H NMR (CD₃OD/TMS) 7.73 (d, 1H, *J* = 8.0 Hz), 5.90 (d, 1H, *J* = 4.8 Hz), 5.79 (d, 1H, *J* = 8.0 Hz), 4.26 (m, 1H), 4.24–4.15 (m, 2H), 3.93 (dd, 1H, *J* = 12.4, 3.6 Hz), 3.85 (dd, 1H, *J* = 12.4, 4.4 Hz); ¹³C NMR (CD₃OD) δ 165.9, 152.2, 142.2, 103.0, 90.8, 84.1, 74.7, 71.8, 45.4.

5'-Tritylthio-5'-deoxyuridine (24)

To the solution of 5'-chloro-5'-deoxyuridine (**23**) (1.30 g, 4.95 mmol) and Ph₃CSH (2.07 g, 7.50 mmol) in DMSO (15 mL), 1,1,3,3-tetramethylguanidine (633 mg, 5.50 mmol) was added. After the reaction mixture was stirred at room temperature overnight, water (30 mL) was added. The precipitated solid was collected by filtration and further purified by silica gel chromatography, eluting with 5% methanol in chloroform to give **24**: 2.47 g (99% yield) as a white foam. ¹H NMR (CDCl₃/TMS) δ 10.65 (brs, 1H), 7.40 (d, 6H, *J* = 7.6 Hz), 7.34 (d, 1H, *J* = 8.0 Hz), 7.26 (t, 6H, *J* = 7.6 Hz), 7.18 (t, 3H, *J* = 7.2 Hz), 5.77 (d, 1H, *J* = 4.4 Hz), 5.65 (d, 1H, *J* = 8.0 Hz), 4.07 (m, 1H), 3.93 (m, 1H), 3.85 (m, 1H), 2.62 (dd, 1H, *J* = 4.0, 12.4 Hz), 2.42 (dd, 1H, *J* = 7.2, 12.8 Hz); ¹³C NMR (CDCl₃) δ 163.5, 150.5, 144.0, 139.9, 129.1, 127.7, 126.5, 102.1, 89.6, 82.0, 73.7, 72.1, 66.5, 34.4; HRMS (TOF, ESI) calcd for C₂₈H₂₆N₂O₅SNa [MNa⁺] 525.1460, found 525.1455.

2'-O-*tert*-Butyldimethylsilyl-5'-tritylthio-5'-deoxyuridine (25a) and 3'-O-*tert*-Butyldimethylsilyl-5'-tritylthio-5'-deoxyuridine (25b)

To the solution of **24** (503 mg, 1.00 mmol) in dry pyridine (2 mL) at room temperature, TBSCl (453 mg, 3.0 mmol) was added. The mixture was stirred at room temperature for 72 h. The solvent was removed, the residue was dissolved into dichloromethane, washed with saturated aqueous sodium bicarbonate, brine and dried over MgSO₄. The solution was removed, and the residue was isolated by silica gel chromatography, eluting with 25% ethyl acetate in hexane to give 2'-O-TBS isomer: **25a** as a white foam (the up spot on TLC): 0.385 g (62% yield) and 3'-O-TBS isomer: **25b** (the low spot on TLC): 0.135 g (21% yield).

25a: ¹H NMR (CDCl₃/TMS) δ 9.99 (brs, 1H), 7.46 (m, 6H), 7.36 (d, 1H, *J* = 8.0 Hz), 7.30 (m, 6H), 7.23 (m, 3H), 5.76 (d, 1H, *J* = 7.2 Hz), 5.71 (d, *J* = 3.2 Hz), 4.17 (dd, 1H, *J* = 3.6, 5.2 Hz), 3.81 (dd, 1H, *J* = 6.4, 10.4 Hz), 3.70 (t, 1H, *J* = 5.6 Hz), 2.72 (dd, 1H, *J* = 4.4, 13.2 Hz), 2.51 (dd, 1H, *J* = 7.0, 13.2 Hz), 0.91 (s, 9H), 0.14 (s, 3H), 0.10 (s, 3H); ¹³C NMR (CDCl₃) δ 163.7, 150.2, 144.3, 140.0, 129.6, 128.0, 126.9, 102.7, 90.2, 82.4, 75.3, 72.6, 67.1, 34.4, 25.7, 18.0, -4.7, -5.2; HRMS (TOF, ESI) calcd for C₃₄H₄₀N₂O₅SSiNa [MNa⁺] 639.2325, found 639.2329.

25b: ¹H NMR (CDCl₃/TMS) δ 9.85 (brs, 1H), 7.45–7.35 (m, 7H), 7.29 (m, 6H), 7.25 (m, 3H), 5.75 (d, 1H, *J* = 8.0 Hz), 5.67 (d, 1H, *J* = 4.4 Hz), 4.10 (m, 1H), 4.00 (m, 1H), 3.94 (m,

1H), 2.54 (dd, 1H, $J = 4.0, 12.8$ Hz), 2.44 (dd, 1H, $J = 6.8, 12.8$ Hz), 0.87 (s, 9H), 0.04 (s, 3H), -0.03 (s, 3H); ^{13}C NMR (CDCl_3) δ 163.6, 150.5, 144.3, 141.1, 129.5, 128.1, 127.0, 102.7, 91.6, 83.0, 74.1, 73.8, 67.0, 34.6, 25.8, 18.0, $-4.6, -4.8$; HRMS (TOF, ESI) calcd for $\text{C}_{34}\text{H}_{40}\text{N}_2\text{O}_5\text{SSiNa}$ $[\text{MNa}^+]$ 639.2325, found 639.2322.

3'-O-*tert*-Butyldimethylsilyl-5'-deoxy-*N*²-[(dimethylamino)methylene]-5'-tritylthioguanosine 3'-*N,N*-Diisopropyl(cyanoethyl)phosphoramidite (26a)

To the solution of **5a** (0.298 g, 0.42 mmol) and *i*-Pr₂NEt (365 μL , 2.10 mmol) in anhydrous dichloromethane (15 mL) at 0 °C, CIP(NPr-*i*)OCH₂CH₂CN (187 μL , 0.84 mmol) was added, followed by the addition of 1-methylimidazole (18 μL , 0.22 mmol). After the reaction mixture was stirred at room temperature for 1 h, the reaction was quenched with methanol (1.0 mL). The solvent was removed, the residue was purified by silica gel chromatography, eluting with 2% CH₃COCH₃ in CH₂Cl₂ containing 0.5% Et₃N to give product **26a** as white foam: 0.372 g (97% yield, >95% purity). ^1H NMR (CD_3CN) δ 9.80 (brs, 1H), 8.53 (s, 0.5H), 8.52 (s, 0.5H), 7.67 (s, 1H), 7.45–7.20 (m, 15H), 5.78 (m, 1H), 4.75 (m, 1H), 4.40–3.20 (m, 6H), 3.04 (m, 6H), 2.80–2.40 (m, 4H), 0.78 (m, 9H), -0.01 – -0.20 (m, 6H); ^{31}P NMR (CD_3CN) δ 152.3, 151.6; HRMS (TOF, ESI/APCI) calcd for $\text{C}_{47}\text{H}_{64}\text{N}_8\text{O}_5\text{PSSi}$ $[\text{MH}^+]$ 911.4227, found 911.4206.

3'-O-*tert*-Butyldimethylsilyl-5'-deoxy-*N*²-[(dimethylamino)methylene]-5'-dimethoxytritylthioguanosine 3'-*N,N*-Diisopropyl(cyanoethyl)phosphoramidite (26b)

To the solution of **6a** (50 mg, 0.065 mmol) and *i*-Pr₂NEt (87 μL , 0.50 mmol) in anhydrous dichloromethane (5 mL) at 0 °C, CIP(NPr-*i*)OCH₂CH₂CN (45 μL , 0.20 mmol) was added, followed by the addition of 1-methylimidazole (2.6 μL , 0.03 mmol). After the reaction mixture was stirred at room temperature for 1 h, the reaction was quenched with methanol (1.0 mL). The solvent was removed, the residue was purified by silica gel chromatography, eluting with 2% CH₃COCH₃ in CH₂Cl₂ containing 0.5% Et₃N to give product **26b** as white foam: 55 mg (87% yield, >95% purity). ^1H NMR (CD_3CN) δ 9.20 (brs, 1H), 8.52 (s, 0.6H), 8.51 (s, 0.4H), 7.65 (s, 1H), 7.35–7.20 (m, 9H), 6.83 (m, 4H), 5.78 (m, 1H), 4.75 (m, 1H), 4.40–3.20 (m, 6H), 3.76 (s, 6H), 3.04 (m, 6H), 2.80–2.40 (m, 4H), 0.79 (m, 9H), -0.01 – -0.20 (m, 6H); ^{31}P NMR (CD_3CN) δ 150.1, 149.3; HRMS (TOF, ESI/APCI) calcd for $\text{C}_{49}\text{H}_{68}\text{N}_8\text{O}_7\text{PSSi}$ $[\text{MH}^+]$ 971.4439, found 971.4442.

3'-O-*tert*-Butyldimethylsilyl-5'-*tert*-butyldisulfanyl-5'-deoxy-*N*²-[(dimethylamino)methylene]guanosine 3'-*N,N*-Diisopropyl(cyanoethyl)phosphoramidite (26c)

To the solution of **12a** (84 mg, 0.15 mmol) and *i*-Pr₂NEt (196 μL , 1.13 mmol) in anhydrous dichloromethane (5 mL) at 0 °C, CIP(NPr-*i*)OCH₂CH₂CN (100 μL , 0.45 mmol) was added, followed by the addition of 1-methylimidazole (5.6 μL , 0.07 mmol). After the reaction mixture was stirred at room temperature for 1 h, the reaction was quenched with methanol (1.0 mL). The solvent was removed, the residue was purified by silica gel chromatography, eluting with 1% CH₃OH in CH₂Cl₂ containing 0.5% Et₃N to give product **26c**: 100 mg (87% yield, 95% purity) as a white foam. ^1H NMR (CD_3CN) δ 9.83 (brs, 1H), 8.57 (s, 1H), 7.76 (s, 1H), 5.85 (m, 1H), 4.92 (m, 1H), 4.60–3.20 (m, 6H), 3.18–3.07 (m, 6H), 2.80–2.60 (m,

4H), 1.34 (s, 4.9H), 1.32 (s, 5.1H), 0.78 (m, 9H), -0.01--0.20 (m, 6H); ^{31}P NMR (CD_3CN) δ 150.6, 149.4; HRMS (TOF, ESI/APCI) calcd for $\text{C}_{32}\text{H}_{58}\text{N}_8\text{O}_5\text{PS}_2\text{Si}$ [MH^+] 757.3473, found 757.3479.

***N*⁶-Benzoyl-2'-*O*-*tert*-butyldimethylsilyl-5'-deoxy-5'-tritylthioadenosine 3'-*N,N*-Diisopropyl(cyanoethyl)phosphoramidite (26d)**

To the solution of **17a** (0.142 g, 0.19 mmol) and *i*-Pr₂NEt (330 μL , 1.90 mmol) in anhydrous dichloromethane (10 mL) at 0 °C, CIP(NPr-*i*)OCH₂CH₂CN (170 μL , 0.76 mmol) was added, followed by the addition of 1-methylimidazole (8.0 μL , 0.10 mmol). The reaction mixture was stirred at room temperature for 1 h. TLC showed the reaction was complete. The reaction was quenched with methanol (1.5 mL). The solvent was removed, the residue was purified by silica gel chromatography, eluting with 2% CH₃COCH₃ in CH₂Cl₂ containing 0.5% Et₃N to give **26d** as white foam: 0.174 g (97% yield, >95% purity). ^1H NMR (CD_3CN) δ 9.50 (brs, 1H), 8.54 (s, 0.4H), 8.53 (s, 0.6H), 8.22 (s, 1H), 8.00 (d, 2H, J = 7.5Hz), 7.70–7.20 (m, 18H), 5.92 (m, 1H), 5.11–4.90 (m, 1H), 4.40–3.20 (m, 6H), 2.85–2.40 (m, 4H), 0.73 (m, 9H), -0.04--0.25 (m, 6H); ^{31}P NMR (CD_3CN) δ 152.8, 151.4; HRMS (TOF, ESI/APCI) calcd for $\text{C}_{51}\text{H}_{63}\text{N}_7\text{O}_5\text{PSSi}$ [MH^+] 944.4118, found 944.4119.

***N*⁶-Benzoyl-2'-*O*-*tert*-butyldimethylsilyl-5'-deoxy-5'-dimethoxytritylthioadenosine 3'-*N,N*-Diisopropyl(cyanoethyl)phosphoramidite (26e)**

To the solution of **18a** (0.100 g, 0.124 mmol) and *i*-Pr₂NEt (109 μL , 0.625 mmol) in anhydrous dichloromethane (10 mL) at 0 °C, CIP(NPr-*i*)OCH₂CH₂CN (55 μL , 0.25 mmol) was added, followed by the addition of 1-methylimidazole (5.3 μL , 0.067 mmol). The reaction mixture was stirred at room temperature for 1 h. TLC showed the reaction was complete. The reaction was quenched with methanol (1.0 mL). The solvent was removed, the residue was purified by silica gel chromatography, eluting with 2% CH₃COCH₃ in CH₂Cl₂ containing 0.5% Et₃N to give **26e** as white foam: 81 mg (65% yield, >95% purity). δ 9.30 (brs, 1H), 8.55 (m, 1H), 8.22 (s, 1H), 8.00 (d, 2H, J = 7.5Hz), 7.70–7.20 (m, 12H), 6.82 (m, 4H), 5.92 (m, 1H), 5.11–4.95 (m, 1H), 4.40–3.20 (m, 6H), 3.75 (s, 6H), 2.85–2.45 (m, 4H), 0.77 (m, 9H), -0.04--0.20 (m, 6H); ^{31}P NMR (CD_3CN) δ 153.5, 152.1; HRMS (TOF, ESI/APCI) calcd for $\text{C}_{53}\text{H}_{67}\text{N}_7\text{O}_7\text{PSSi}$ [MH^+] 1004.4330, found 1004.4323.

***N*⁴-Phenoxyacetyl-2'-*O*-*tert*-butyldimethylsilyl-5'-deoxy-5'-tritylthiocytidine 3'-*N,N*-Diisopropyl(cyanoethyl)phosphoramidite (26f)**

To the solution of **22a** (317 mg, 0.42 mmol) and *i*-Pr₂NEt (365 μL , 2.1 mmol) in anhydrous dichloromethane (10 mL) at 0 °C, CIP(NPr-*i*)OCH₂CH₂CN (187 μL , 0.84 mmol) was added, followed by the addition of 1-methylimidazole (18 μL , 0.22 mmol). The reaction mixture was stirred at room temperature for 1 h. TLC showed the reaction was complete. The reaction was quenched with methanol (1.0 mL). The solvent was removed, the residue was purified by silica gel chromatography, eluting with 2% acetone in CH₂Cl₂ containing 0.2% Et₃N to give **26f** as white foam: 340 mg (85% yield, >95% purity). δ 10.21 (brs, 1H), 7.81 (m, 1H), 7.50–6.90 (m, 21H), 5.61 (m, 1H), 4.81 (s, 2H), 4.40–4.25 (m, 1H), 4.20–4.00 (m, 1H), 3.80–3.40 (m, 5H), 2.85–2.30 (m, 4H), 1.15–0.98 (m, 12H), 0.91 (m, 9H), 0.16–

0.05 (m, 6H); ^{31}P NMR (CD_3CN) δ 150.4, 149.0; HRMS (TOF, APCI) calcd for $\text{C}_{51}\text{H}_{65}\text{N}_5\text{O}_7\text{PSSi}$ [MH^+] 950.4112, found 950.4109

2'-O-*tert*-Butyldimethylsilyl-5'-deoxy-5'-tritylthiouridine 3'-*N,N*-Diisopropyl(cyanoethyl)phosphoramidite (**26g**)

To the solution of **25a** (155 mg, 0.25 mmol) and *i*-Pr₂NEt (218 μL , 1.25 mmol) in anhydrous dichloromethane (5.0 mL) at 0 °C, CIP(NPr-*i*)OCH₂CH₂CN (112 μL , 0.50 mmol) was added, followed by the addition of 1-methylimidazole (10.0 μL , 0.125 mmol). The reaction mixture was stirred at room temperature for 1 h. TLC showed the reaction was complete. The reaction was quenched with methanol (1.0 mL). The solvent was removed, the residue was purified by silica gel chromatography, eluting with 4% acetone in CH_2Cl_2 containing 0.5% Et₃N to give **26g** as white foam: 190 mg (93% yield, >95% purity). δ 7.50–7.20 (m, 18H), 5.80–5.60 (m, 2H), 4.40–3.40 (m, 7H), 2.85–2.30 (m, 4H), 1.30–0.98 (m, 12H), 0.89 (m, 9H), 0.15–0.06 (m, 6H); ^{31}P NMR (CD_3CN) δ 149.1, 148.7; HRMS (TOF, APCI) calcd for $\text{C}_{43}\text{H}_{58}\text{N}_4\text{O}_6\text{PSSi}$ [MH^+] 817.3584, found 817.3583.

Synthesis of Olig 5'-UUC_{2'-o-NBn}G_{5'-s}GGUCGGC-3' (**28**) and 5'-GCCGUC_{2'-o-NBn}C_{5'-s}CCCCG-3' (**28a**)

The standard RNA 1 μmol protocol was modified for the double coupling to phosphoramidite **26a** (75 mg in 1.0 mL CH_3CN). After coupling to **26a**, oxidation and capping, the column was removed from instrument, washed with water, and treated with aqueous AgNO_3 (50 mM, 3 mL) for 60 min in dark. The column was rinsed thoroughly with water, treated with DTT (50 mM, 3 mL) for 15 min. The column was washed with acetonitrile, dried over vacuum desiccator for 15 min. After double coupling to phosphoramidite **27** (75 mg in 0.75 mL CH_3CN), the synthesis was continued to finish the rest of synthesis. After the solid support was treated with concentrated ammonium hydroxide/ethanol (3:1, v/v) at 55 °C for 2–4 h and desilylation with triethylamine trihydrofluoride at 65 °C for 25 min, dPAGE gel purification gave desired RNA **28** (~20 nmol). MALDI-TOF MS calcd: 3649.0, found: 3648.7. Similarly oligo 5'-GCCGUC_{2'-o-NBn}C_{5'-s}CCCCG-3' (**28a**) (~30 nmol) could be prepared from phosphoramidites **26f** and **27**. MALDI-TOF MS calcd: 3568.1, found: 3568.2.

Synthesis of 5'-HS-GG dinucleotide (**30**)

After standard detritylation of *i*-Pr-Pac-G-RNA-CPG column with 3% TCA in dichloromethane, it was manually coupled to **26C** (33 mg in 0.30 mL dry acetonitrile) with activator (0.50 mL, 0.45 M tetrazole in acetonitrile) at room temperature for 20 min, then manually oxidized with 10% *tert*-BuOOH in decane/acetonitrile (1:5, v/v) (0.5 mL) for 1 min. Deprotection was achieved by treatment of the solid support with concentrated ammonium hydroxide/ethanol (3:1, v/v) at 55 °C for 2 h, followed by desilylation with triethylamine trihydrofluoride at 65 °C for 25 min.⁴³ The solvent was removed, the residue was extracted with water, rinsed with chloroform (3 $\times$ 0.3 mL). The crude 5'-*t*-BuS-SGG dinucleotide (**29**) was purified by Sep-Pack C₁₈ column and further purified by RP HPLC. MALDI-TOF MS calcd for M^+ : 733.16, Found: 733.23; Treatment of 5'-*t*-ButylSS-GG

dinucleotide with DTT in the presence of triethylamine yielded the corresponding 5'-HS-GG (30). MALDI-TOF MS calcd for M⁺: 645.12; Found: 645.44.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structures of oligonucleotides containing a site-specific 5'-*S*-phosphorothiolate linkage. **I**: ODN with a 5'-PS DNA dinucleotide (dB₁)-ps-(dB₂); **II**: ODN with 5'-PS RNA/DNA dinucleotide (rB₁)-ps-(dB₂); **III**: ORN with a 5'-PS RNA dinucleotide (rB₁)-ps-(rB₂).

Scheme 1.

Scheme 2.

Scheme 3.

Scheme 4.

Scheme 5.

Scheme 6.

Scheme 7.

Scheme 8.

Scheme 9.