Synthesis of 4-Thiouridine, 6-Thioinosine, and 6-Thioguanosine 3′,5′-*O*-Bisphosphates as Donor Molecules for RNA Ligation and Their Application to the Synthesis of Photoactivatable TMG-Capped U1 snRNA Fragments

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4-Thiouridine, 6-thioguanosine, and 6-thioinosine 3′,5′-bisphosphates (**9**, **20**, and **28**) were synthesized in good yields by considerably improved methods. In the former two compounds, uridine and 2-*N*-phenylacetylguanosine were converted via transient O-trimethylsilylation to the corresponding 4- and 6-*O*-benzenesulfonyl intermediates (**2** and **13**), which, *in turn*, were allowed to react with 2-cyanoethanethiol in the presence of *N*-methylpyrrolidine to give 4-thiouridine (**3**) and 2-*N*-phenylacetyl-6-thioguanosine derivatives (**14**), respectively. In situ dimethoxytritylation of these thionucleoside derivatives gave the 5′-masked products **4** and **15** in high overall yields from **1** and **11**. 6-*S*-(2-Cyanoethyl)-5′-*O*-(4,4′-dimethoxytrityl)-6-thioinosine (**23**) was synthesized via substitution of the 5′-*O*-tritylated 6-chloropurine riboside derivative **22** with 2-cyanoethanethiol. These *S*-(2-cyanoethyl)thionucleosides were converted to the 2′-*O*-(*tert*-butyldimethylsilyl)ribonucleoside 3′-phosphoramidite derivatives **7**, **18**, and **26** or 3′,5′-bisphosphate derivatives **8**, **19**, and **27**. Treatment of **8**, **19**, and **27** with DBU gave thionucleoside 3′,5′-bisphosphate derivatives **9**, **20**, and **28**, which were found to be substrates of T4 RNA ligase. These thionucleoside 3′,5′-bisphosphates were examined as donors for ligation with $m_3^{2,2,7}$ $G^{5'}$pppAmUmA, i.e., the 5′-terminal tetranucleotide fragment of U1 snRNA. The 4-thiouridine 3′,5′-bisphosphate derivative **9** was found to serve as the most active substrate of T4 RNA ligase with a reaction efficiency of 96%.

Introduction

Thio-substituted nucleotides are useful for various purposes in molecular biology. Particularly, 4-thiouridine, 6-thioinosine, and 6-thioguanosine have been incorporated into oligonucleotides and utilized as functional nucleosides for postsynthetic modification.[1] The inherent photo-cross-linking ability of thionucleoside-containing oligonucleotides has been widely used to study three-dimensional interaction between RNA−RNA or RNA−proteins at the atomic level.[2] In the photochemical activation of 4-thiopyrimidine or 6-thiopurine nucleotides by long wavelength UV light (330−350 nm), there is no detrimental effect on the common nucletides A, G, C, and U, such as is observed in photocrosslinking using short wavelength UV (250−280 nm). Since the distance between the photoactivatable thiocarbonyl function and the target molecule is nearly zero, the recognition site in nucleotide-nucleotide interaction can be definitively determined. Therefore, such detailed studies made it possible to construct the plausible 3D models of functional molecules such as hammerhead ribozymes,[3] U snRNA,[4] and rRNA.[5] In addition, it was also reported that oligonucleotides incorporating thionucleotides were available for photocrosslinking assay of interaction between proteins and nucleic acids.[6] These thionucleotides as cross-linking reagents are apparently powerful tools not only for studies of interaction of RNA−RNA[3−5] but also for determination of specific structural elements in recognition domains of RNA−protein complexes.[6]

Lührmann and co-workers have recently reported that the 5′-terminal 2,2,7-trimethylguanosine (TMG)-cap structure of U1 snRNA plays an important role as a signal to transport U1 snRNA from the cytoplasm to the nucleus.[7] They also isolated and identified a transport factor

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protein named Snurportin 1, which has proved to bind to the TMG-cap structure of U1 snRNA.[8] In their work, $m_3^{2,2,7}$ $G^{5'}$pppAmUmApdCp-Hx-Biotin (Hx = hexane-1,6-diyl group), which was derivatized from the 5′-terminal fragment of U1 snRNA synthesized by us,[9] was utilized for affinity column chromatography to isolate such a TMG-binding protein. At the next stage of the continuing studies on the mechanism of U snRNA transport, it is highly desirable to determine the binding site of the TMG cap in Snurportin 1. Therefore, it is of great importance to incorporate a thionucleoside into the 5′-terminal region of U1 snRNA.

Incorporation of thionucleotides into oligonucleotides by chemical[1a,3a,5b,10] and enzymatic[4a,11] methods has been extensively studied. In the chemical method, various oligonucleotides having thio-substituted nucleosides have been prepared by automated synthesis by use of phosphoramidite units[12] in which the thiol function was appropriately protected to avoid side reactions during the 3′-phosphitylation and the oxidation by iodine after each coupling cycle.

On the other hand, the previously known enzymatic methods required some thionucleoside derivatives. Thionucleoside 5′-triphosphates have been used as substrates of DNA-dependent RNA polymerases to obtain RNA transcripts incorporating thionucleosides at the 3′-terminal site or at random sites.[11] The dinucleoside monophosphate derivative ^{4s}UG was also used as a primer in RNA extension reaction using T7 RNA polymerase to introduce ^{4s}U into an RNA at the 5′-terminal site.[4a] In this case, the 5′-terminal site of the ^{4s}UG-RNA fragment thus obtained was phosphorylated enzymatically and subjected to RNA ligation with another RNA′ to obtain RNA′-^{4s}UG-RNA.[4a] However, this method is not generally applicable to any site-specifically modified oligonucleotides because of the requirement of the restricted sequence of ^{4s}UG.

Another enzyme widely used for RNA ligation is T4 RNA ligase. It joins the terminal 5′-phosphate of donor oligonucleotides with the 3′-terminal hydroxyl group of acceptor oligonucleotides. If thionucleoside 3′,5′-bisphosphates (p^sNp) are available as the donor substrates for RNA ligation, it will be possible to obtain any site-specifically modified RNA sequence using a two-step ligation. The first-step ligation provides oligonucleotides incorporating thionucleoside at the 3′-terminal site. The oligonucleotides would be useful not only as the substrate of the second-step enzymatic ligation but also as the substrate of photo-cross-linking reactions.

In this paper, we report improved methods for the chemical synthesis of 4-thiouridine, 6-thioinosine and 6-thioguanosine 3′,5′-diphosphates p^sNp and T4 RNA ligase mediated site-specific incorporation of thionucleosides into the 3′-terminal site of a 5′-terminal U1 snRNA tetramer, m_3 $^{2,2,7}G^{5'}$pppAmUmA, containing the unique TMG-cap structure.

Results and Discussion

Synthesis of 4-Thiouridine 3′,5′-*O*-Bisphosphate (8). For the synthesis of 4-thiouridine, 6-thioguanosine and 6-thioinosine derivatives, the 2-cyanoethyl[13] or pivaloxymethyl[10d] group has been used as the protecting group of these thiocarbonyl functions. These protecting groups were removed under basic conditions.[10]

In general, *S*-(2-cyanoethyl)thionucleosides have been prepared by multistep reactions involving protection-deprotection manipulation of the hydroxyl groups of the ribose residues and substitution of appropriate leaving groups at the C-4 (for pyrimidine nucleosides) or C-6 (for purine nucleosides) position with 2-cyanoethanethiol.

In this study, we explored a more straightforward method for the synthesis of 4-*S*-(2-cyanoethyl)-5′-*O*-(4,4′-dimethoxytrityl)-4-thiouridine (**4**) in situ from uridine (**1**) without extensive purification. A trisilylated species of **1**, obtained by transient silylation with hexamethyldisilazane, was allowed to react with 2,4,6-triisopropylbenzenesulfonyl chloride in CH_2Cl_2−0.2 M Na_2CO_3 in the presence of a catalytic amount of Bu_4NBr.[14] This phase-transfer reaction gave the 4-*O*-triisopropylbenzenesulfonyl derivative (**2**),[15] which was treated with 2-cyanoethanethiol in the presence of *N*-methylpyrrolidine. It turned out that this process did not affect the fragile trimethylsilyl groups so that the fully protected *S*-(2-cyanoethyl) ether derivative could be easily extracted and converted by treatment with trifluoroacetic acid to give the product **3**. Further in situ 5′-dimethoxytritylation of **3** gave the 5′-masked product **4** in 63% overall yield from **1**. The same compound has been obtained in a lower yield starting from uridine via a 6-step reaction, each step of which required column chromatography except for the first reaction.[10j] It should be emphasized that our route to **4** requires only one-time separation at the last stage. The 2′-*O*-silylation of **4** with *tert*-butyldimethylsilyl chloride in the presence of imidazole gave the 2′,5′-protected thionucleoside **5** in 40% yield. The 3′-phosphitylation of **5** with chloro(2-cyanoethoxy)(diisopropylamino)phosphine gave the amidite unit **7** in 79% yield (Scheme 1).

The 3′,5′-bisphosphorylated compound **8** was synthesized as follows. When the 5′-detritylation of **5** was carried out by treatment with 1% TFA in CH_2Cl_2, the

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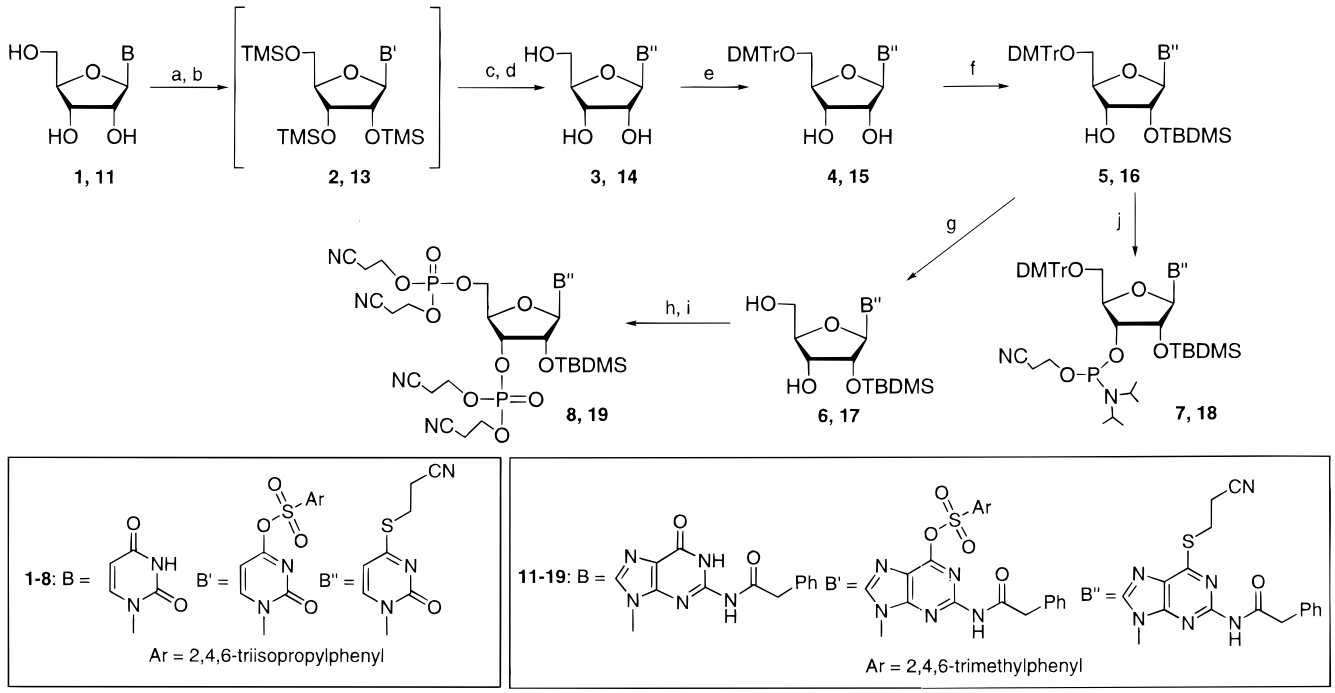
Scheme 1[a]



[a] Key: (a) hexamethyldisilazane, CH_3CN, reflux; (b) $ArSO_2Cl$, aq Na_2CO_3 or NEt_3-DMAP; (c) $HSCH_2CH_2CN$, *N*-methylpyrrolidine, CH_2Cl_2; (d) TFA, MeOH; (e) 4,4′-dimethoxytrityl chloride, pyridine; (f) TBDMSCl, imidazole; (g) 3% DCA in CH_2Cl_2; (h) 1*H*-tetrazole, bis(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine, CH_3CN; (i) *tert*-butyl hydroperoxide in hexane; (j) chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine, collidine, *N*-methylimidazole.

2′-3′ silyl migration occurred to give the desired 2′-*O*-TBDMS derivative contaminated with a small amount (ca. 5%) of its 3′-*O*-TBDMS isomer. However, it was found that the use of 3% DCA gave only the 2′-*O*-silylated uridine derivative **6** in a nearly quantitative yield. The 3′,5′-bisphosphitylation of the product **6** with bis (2-cyanoethoxy)(diisopropylamino)phosphine in the presence of 1*H*-tetrazole followed by the oxidation gave the bisphosphorylated compound **8** in 89% yield.

It is well-known that the cyanoethyl group can be removed easily by DBU treatment from $NCCH_2CH_2OP(O)(OR)_2$ but cannot from $NCCH_2CH_2OP(O)(OR)(O^-)$.[16] Therefore, we employed our previous effective method of the simultaneous removal of two 2-cyanoethyl groups from $(NCCH_2CH_2O)_2P(O)(OR)$ by the combined use of DBU and bis(trimethylsilyl)acetamide (BSA) for deprotection of all the 2-cyanoethyl groups in **8**.

This method gave the completely decyanoethylated product within 1.5 h. After the extraction, the 2′-*O*-TBDMS group was removed by treatment with $Et_3N{\cdot}3HF$ to give $p^{4s}Up$ (**9**) which was isolated in 67% yield by anion-exchange column chromatography using DEAE Sephadex A-25.

Synthesis of 6-Thioguanosine 3′,5′-*O*-Bisphosphate (20). 6-*S*-(2-Cyanoethyl)-2-*N*-phenylacetyl-6-thioguanosine (**14**) was synthesized by a combined use of the transient 2′,3′,5′-tri-*O*-TMS protection method and the direct displacement of a 6-*O*-sulfonylated species with 2-cyanoethanethiol from a practical point of view: The hydroxyl groups of 2-*N*-phenylacetylguanosine (**11**) were transiently protected by treatment with HMDS. After the 2′,3′,5′-*O*-trisilylated compound **12** was sulfonylated at the 6-*O* position with mesitylenesulfonyl chloride (MsCl) in the presence of DMAP and Et_3N, reaction of the resulting 6-*O*-sulfonylated species **13** with 2-cyanoethanethiol in the presence of *N*-methylpyrrolidine gave **14** in 91% overall yield from **11**. The 5′-tritylation of **14** gave the 5′-protected derivative **15** in 86% yield. The 2′-*O*-silylation of **15** was carried out in a manner similar to that described in the synthesis of **5** to give the desired product **16** in 28% yield. The usual phosphitylation of **16** gave the amidite unit **18** in 77% yield. On the other hand, the 3′,5′-bisphosphoylated compound **19** was obtained in 78% yield in a manner similar to that described in the synthesis of **8**. In the case of $p^{6s}Gp$ (**20**), decyanoethylation and desilylation were done under conditions similar to those described above, and finally the 2-*N*-phenylacetyl group was removed by treatment with NH_3. Thus, 6-thioguanosine 3′,5′-*O*-bisphosphate (**20**) was obtained from **19** in 59% yield.

Synthesis of 6-Thioinosine 3′,5′-*O*-Bisphosphate (28). In the previous synthesis of 6-thioinosine, introduction of the thiocarbonyl group into the C-6 position has been performed by reaction of inosine derivatives with Lawesson's reagent or by displacement of a 6-triazolylpurine riboside derivative with hydrogen sulfide.[10i] To prepare building units for incorporation of such thionucleosides into oligonucleotides, the thiocarbonyl group was further protected via *S*-alkylation with pivaloxymethyl chloride or cyanoethyl bromide under basic conditions. Thus, these synthetic routes could not directly give 6-*S*-protected thioinosine derivatives. Therefore, we tried to introduce an *S*-(2-cyanoethyl) function into the C-6 position via a 6-*O*-sulfonylinosine derivative. When 6-*O*-sulfonylation of inosine derivatives by using 2,4,6-triisopropylbenzensufonyl chloride (TPS) was carried out, N^1-sulfonylation occurred simultaneously. The yields of the

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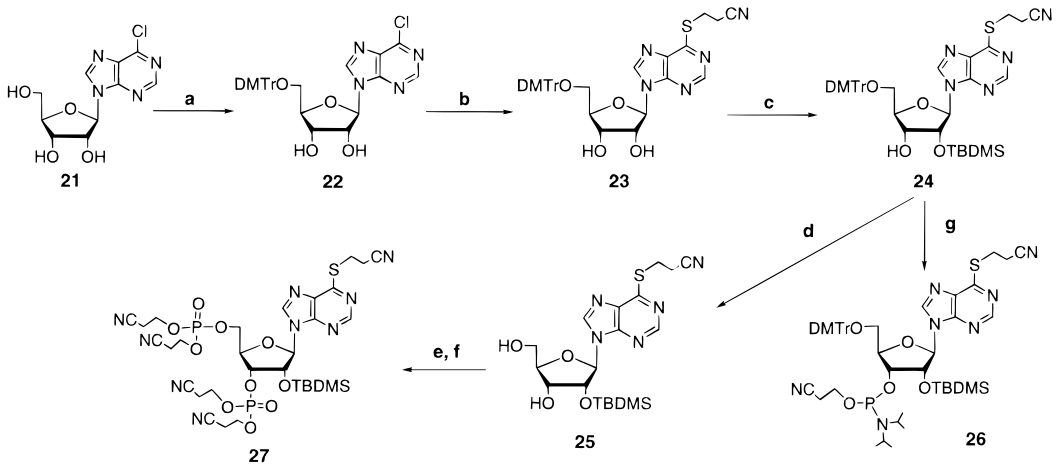
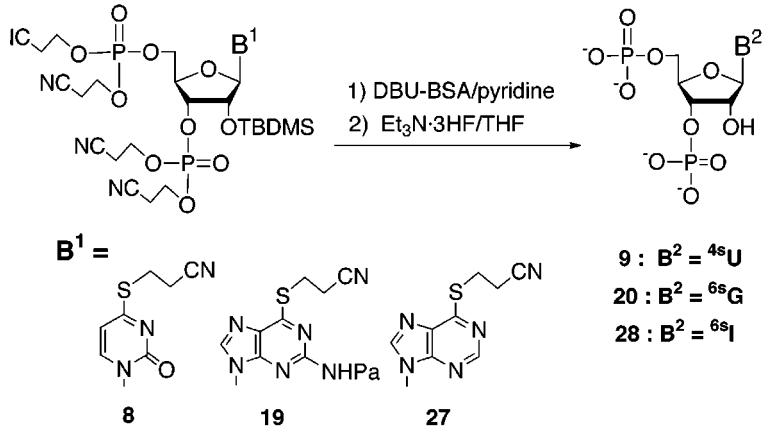
Scheme 2[a]



[a] Key: (a) 4,4′-dimethoxytrithyl chloride, pyridine; (b) $HSCH_2CH_2CN$, *N*-methylpyrrolidine, CH_2Cl_2; (c) TBDMSCl, imidazole, DMF; (d) 3% DCA in CH_2Cl_2; (e) 1*H*-tetrazole, bis(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine, CH_3CN; (f) *tert*-butyl hydroperoxide in hexane; (g) chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine, collidine, *N*-methylimidazole.

Scheme 3



N^1- and 6-*O*-TPS inosine derivatives were 56% and 31%, respectively. Thus, direct introduction of the 2-cyanoethylthio group into the C-6 position via 6-*O*-sulfonylation resulted in poorer yields of the desired product. Therefore, we chose 6-chloropurine riboside (**21**) as a starting material. After 5′-*O*-dimethoxytrityl-6-chloropurine riboside (**22**) was synthesized in 94% yield from **21**, reaction of **22** with 2-cyanoethanethiol in the presence of *N*-methylpyrrolidine produced the 6-*S*-(2-cyanoethyl)-5′-*O*-dimethoxytrityl-6-thioinosine (**23**) in 96% yield. In a manner similar to those described for uridine and guanosine, the synthetic unit **26** and 3′,5′-bisphosphoylated 6-thioinosine derivative **27** were synthesized in 76 and 70% yields, respectively (Scheme 2).

Site-Specific Incorporation of 4-Thionucleotide 3′,5′-Bisphosphate with T4 RNA Ligase. Oligonucleotides containing ^{4s}U have been prepared by use of ^{4s}UTP and appropriate polymerases such as T7 RNA polymerase in the presence of DNA templates and primers. Since ^{4s}UTP is incorporated four to five times more slowly than UTP in this system, ^{4s}U is randomly incorporated at all the available positions confronting all the adenosines on the templates.[4c]

For the site-specific introduction of ^{4s}U into RNA oligomers at a definite position, a dinucleotide ^{4s}UG has often been used as the primer in T7 RNA polymerase reaction in the presence of GTP. This procedure initially produces RNA oligomers starting from ^{4s}UG at the 5′-terminal site. Therefore, an additional enzyme reaction is necessary to join the RNA oligomers with 5′-upstream RNA fragments using T4 DNA ligase in the presence of DNA templates. This method always requires guanylic acid 3′-downstream from ^{4s}U because of the enzyme's inherent specificity.

Cross-linking experiments using U1 snRNA fragments incorporating ^{4s}U are highly useful to clarify TMG-proteins interaction in connection with the splicing mechanism.[17] Our target sequence, the 5′-terminal TMG-capped RNA trimer ($m_3^{2,2,7}G^{5'}$pppAmUmA) of U1 snRNA, does not have such a G nucleotide. Therefore, the above strategy using ^{4s}UpG as a primer cannot be applied to the modification of the 5′-terminal sequence of U1 snRNA. Therefore, we used p^{4s}Up for the site-specific incorporation of ^{4s}U into the 5′-terminal 10mer of U1 snRNA using T4 RNA ligase.

The enzymatic synthesis of the U1 snRNA oligomers containing ^{4s}U, ^{6s}I and ^{6s}G, respectively, were illustrated in Scheme 4. The U1 snRNA tetramer ($m_3^{2,2,7}G^{5'}$pppAmUmA) was synthesized according to the

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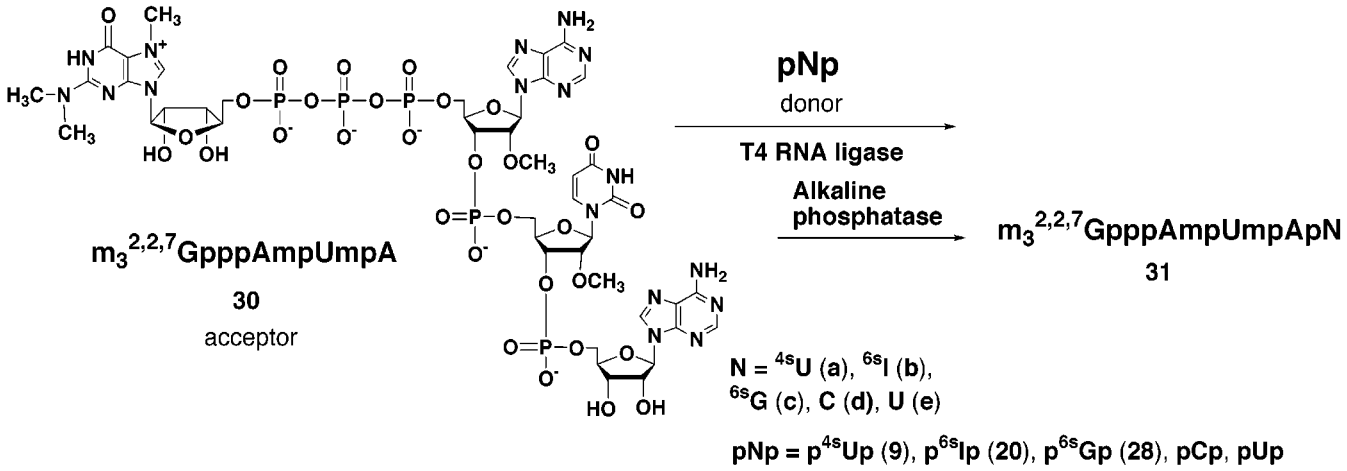
Scheme 4



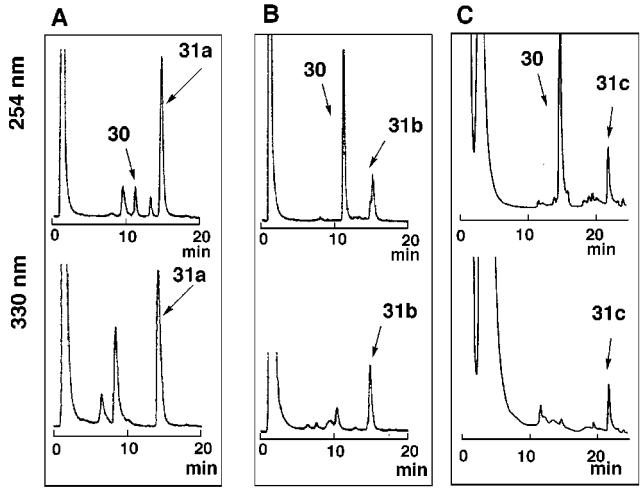


Figure 1. Anion-exchange HPLC profiles of the reaction mixtures obtained by the RNA ligations as shown in entries 1 (**A**), 2 (**B**), and 4 (**C**) of Table 1. The HPLC conditions described as method A (see the Experimental Section) were used for the analysis for A and B. Those described as method B were used for C.

Table 1. Efficiency of Thionucleoside 3′5′-Bisphospates 9, 20, and 28 as Donors in Ligation with $m_3^{2,2,7}G^{5'}$pppAmUmA 30 by T4 RNA Ligase

entry	donor	acceptor	ligation product[a] (%)	isolated yield (%)
1	p^{4s}Up **9**	**30**	**31a** (94)	81
2	p^{6s}Ip **20**	**30**	**31b** (27)	19
3	p^{6s}Gp **28**	**30**	**31c** (10)	6
4	p^{6s}Gp **28**	**30**	**31c** (23)	7
5	pCp	**30**	**31d** (96)	74
6	pUp	**30**	**31e** (88)	60

[a] The yield is estimated by HPLC analysis. All the reactions were carried out for 48 h except for entry 4 (9 days).

procedure reported previously by us[9] and used as an acceptor for the T4 RNA ligase reaction.[18] For example, the ligation reaction of $m_3^{2,2,7}G^{5'}$pppAmUmA (0.2 mM) with p^{4s}Up (2 mM) was performed using T4 RNA ligase (175 units to donor 1 μmol) in the presence of 8 mM ATP, 20 mM $MgCl_2$, and 5 mM DTT in 50 mM Tris-HCl buffer (pH 8.3) at 8 °C for 48 h. The conditions to incorporate the other nucleotides are described in the Experimental Section. The 3′-terminal phosphate group of the ligation products, thus obtained, were removed by treatment with calf intestinal alkaline phosphatase and the dephosphorylated pentamers were analyzed and purified by anion exchange HPLC (Figure 1). The ligation efficiency of p^{4s}Up toward $m_3^{2,2,7}G^{5'}$pppAmUmA was 94% at a level comparable to those of the reference materials pUp (88%) or pCp (96%). In contrast, the ligation efficiency of a purine nucleotide donors such as p^{6s}Ip and p^{6s}Gp were relatively low (27% and 10%, respectively), as shown in Table 1. The isolation yields of the ligation products are also described in the same table. The structure of the purified ligation products were characterized by the UV spectra and the composition analysis after enzymatic digestion with nuclease P1. The UV spectra of these products showed λmax peaks at 330 nm which supported the presence of thionucleotides (Figure 2). Furthermore, the respective HPLC profiles cleary showed the appearance of $m_3^{2,2,7}G^{5'}$pppAm, pUm, pA detected at 254 nm and a thio-substituted nucleotide detected at 330 nm after the nuclease P1 digestion (Figure 3). These observations made us confirm the successful incorporation of the thio-substituted nucleotides.

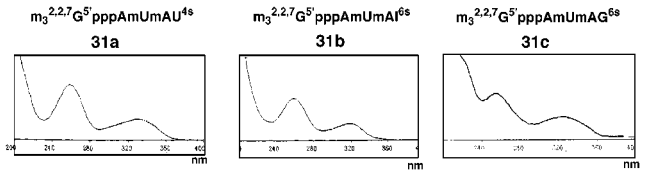


Figure 2. UV spectra of the peaks **31a**, **31b**, and **31c** shown in Figure 1.

Conclusion

In this study, we have established the hitherto most practical route to synthesize thio-substituted nucleosides and their derivatives. 4-*S*-(2-Cyanoethyl)-5′-*O*-(4,4′-dimethoxytrityl)-4-thiouridine **4**, a key intermediate for the synthesis of 4-thiouridine 3′, 5′-bisphosphate, was synthesized in a five-step reaction scheme which required only one-time column chromatography separation at the last stage. Similar reaction scheme, namely the transient protection and 6-*O*-selective sulfonylation followed by the direct displacement of the sulfonyloxy group with 2-

(18) (a) Uhlenbeck, O. C.; Cameron, V. *Nucleic Acids Res.* **1977**, *4*, 85−98. (b) England, T. E.; Uhlenbeck, O. C. *Nucleic Acids Res.* **1978**, *17*, 2069−2076. (c) Iwase, R.; Maeda, M.; Fujii, T.; Sekine, M.; Hata, T. *Nucleic Acids Res.* **1992**, *20*, 1643−1648.

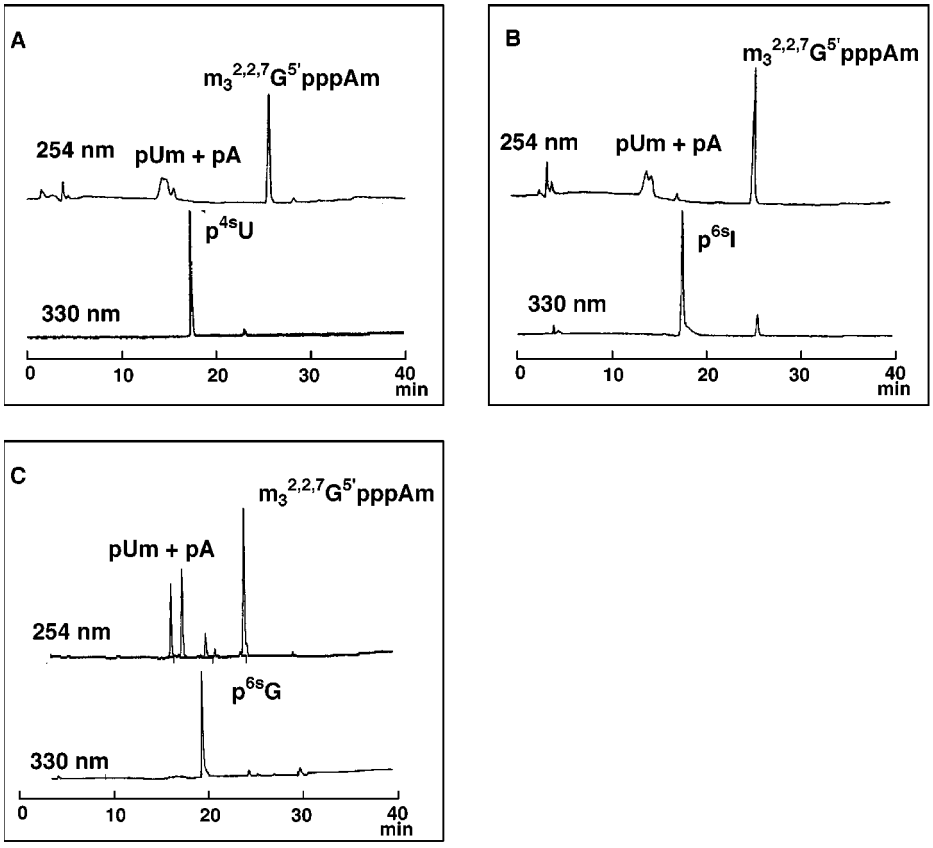


Figure 3. Anion-exchange HPLC profiles after nuclease P1 treatment of **31a** (A), **31b** (B), and **31c** (C).

cyanoethanethiol, was also effective to synthesize 6-thioguanosine derivatives. On the other hand, 6-*S*-(2-cyanoethyl)-6-thioinosine derivative **23** was obtained in a higher yield when commercially available 6-chloropurine riboside was used as a starting material. Three kinds of pNp donors **9**, **20**, and **28** (Scheme 3) required for RNA ligation were successfully synthesized. These donors have proved to be useful for incorporation of thionucleotides onto the 3′-terminal site of the TMG-capped RNA tetramer by using T4 RNA ligase. The RNA oligomers modified with a thionucleotide will be useful as a powerful tool for elucidation of RNA-RNA or RNA-protein interaction by photocrosslinking methods. Especially, the TMG capped RNA fragment **31a** incorporating ^{4s}U would be used for determination of the TMG-cap binding site of Snurportin 1.[8] These studies are now under investigation. Moreover, our preliminary result showed that an *E. coli* rRNA mimic 25mer (α-sarcin/ricin domain), which has ^{6s}G incorporated by use of p^{6s}Gp **20**, proved to crosslink to the binding site of another RNA fragment (thiostrepton domain) of the same rRNA molecule upon UV irradiation.[19] This fact also demonstrates a potential utility of **20** in studies related to molecular biology.

Experimental Section

^{1}H and ^{13}C NMR spectra were measured at 270 and 67.8 MHz, respectively, with TMS as the internal reference. ^{31}P NMR spectra were measured at 109.4 MHz with 80% phosphoric acid as the external reference. Column chromatography was performed with silica gel C-200 and C-300 purchased from Wako Pure Chemical Industries, Ltd. and a minipump for a goldfish bowl was conveniently used to attain sufficient pressure for rapid chromatographic separation. TLC was performed on precoated TLC plates of silica gel 60 F-254 (Merck). Anion-exchange HPLC was performed on a Gen-Pak Fax column (4.6 × 100 mm, waters) for the oligonucleotides or Whatman Partisil 10 SAX WCS analytical column (4.6 × 250 mm) for the enzymatic digestion. The analytical conditions used were a 10−63% (method A) or 1−30% (method B) linear gradient of solvent A (1.0 M NaCl in 25 mM NaH_2PO_4, pH 6.0) in solvent B (25 mM NaH_2PO_4, pH 6.0) for 30 min for the Gen-Pak Fax column and 0−30% linear gradient of solvent C (20% CH_3CN in 0.5 M KH_2PO_4) in solvent D (20% CH_3CN in 0.005 M KH_2PO_4) for 30 min for Whatman Partisil 10 SAX WCS analytical column. The flow rate was 1.0 mL/min and the column temperature was 50 °C. Ribonucleosides were purchased from Yamasa Co., Ltd. Pyridine was distilled two times from *p*-toluenesulfonyl chloride and from calcium hydride and stored over molecular sieves 4A. Elemental analyses were performed by the Microanalytical Laboratory, Tokyo Institute of Technology, at Nagatsuta.

4-*S*-(2-Cyanoethyl)-5′-*O*-(4,4′-dimethoxytrityl)-4-thiouridine (4). To a suspension of uridine **1** (3.66 g, 15 mmol) in dry acetonitrile (150 mL) was added hexamethyldisilazane (16 mL, 75 mmol). After being refluxed for 2 h, the mixture was cooled and ethanol (20 mL) was added. The solvent was removed under reduced pressure and coevaporated with dry toluene. The residue was dissolved in CH_2Cl_2 (300 mL). To the solution were added 2,4,6-triisopropylbenzenesulfonyl chloride (5.91 g, 19.5 mmol) and tetrabutylammonium bromide (193 mg, 0.6 mmol). To the mixture was added 0.2 M Na_2CO_3 (600 mL), and the mixture was stirred vigorously at room temperature for 16 h. The organic layer was washed three times with water, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (150 mL), and 2-cyanoethanethiol (3.0 mL, 33.8 mmol) and *N*-methylpyrrolidine (4.78 mL, 47 mmol) were added. After being stirred at room temperature for 30 min, the mixture was

(19) Morishita, R.; Matsumoto, H.; Madin, K.; Sawasaki, T.; Uchiumi, T.; Sekine, M.; Endo, Y. *Nucleic Acids Symposium Ser.* **1998**, *39*, 157−158.

diluted with CH_2Cl_2 and washed three times with 1 M KH_2PO_4. The organic layer was collected, dried over Na_2SO_4, filtered and evaporated under reduced pressure. To the residue was added 2% trifluoroacetic acid in methanol (150 mL). After being stirred at room temperature for 30 min, the reaction was quenched by adding pyridine (50 mL). The solution was evaporated under reduced pressure, and the residue was diluted with water−pyridine (400 mL, 3:1,v/v). The aqueous solution was washed three times with Et_2O. Every time the ethereal layer was back-extracted with water-pyridine (300 mL, 2:1, v/v) which was put in another separatory funnel. After extraction was performed, the two aqueous layers were combined and evaporated under reduced pressure. The residue was rendered anhydrous by coevaporation three times with dry pyridine and dissolved in dry pyridine (150 mL). To the solution was added 4, 4′-dimethoxytrityl chloride (5.08 g, 15 mmol). After being stirred at room temperature for 20 h, the mixture was diluted with CH_2Cl_2 (250 mL). The organic solution was washed three times with sat. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (180 g) eluted with CH_2Cl_2−MeOH (98.5:1.5−97.5: 2.5, v/v) to give **4** (5.81 g, 63%): ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.91 (2 H, m), 3.21 (1 H, br), 3.35−3.47 (4 H, m), 3.80 (6 H, s), 4.38−4.39 (3 H, m), 5.40 (1 H, br), 5.78 (1 H, d, J = 2.97 Hz), 6.04 (1 H, d, J = 6.83 Hz), 6.80−6.84 (4 H, m), 7.16−7.29 (9 H, m), 8.02 (1 H, d, J = 7.26 Hz); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 18.06, 25.27, 55.15, 61.80, 70.03, 75.98, 84.49, 86.88, 92.51, 113.17, 118.04, 125.18, 127.01. 140.83, 144.13, 154.61, 158.56, 175.79. Anal. Calcd for $C_{33}H_{33}N_3O_7S\cdot H_2O$: C, 60.82; H, 5.72; N, 6.45; S, 4.92. Found: C, 60.51; H, 5.09; N, 6.43; S, 6.67.

4-*S*-(2-Cyanoethyl)-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(4,4′-dimethoxytrityl)-4-thiouridine (5). Compound **4** (5.81 g, 9.43 mmol) was rendered anhydrous by coevaporation three times each with dry pyridine and dry toluene and dissolved in dry DMF (94 mL). To the solution were added imidazole (963 mg, 14.2 mmol) and TBDMSCl (1.7 g, 11.3 mmol). The mixture was stirred at room temperature for 36 h. The solvent was removed under reduced pressure and the residue was dissolved in CH_2Cl_2 (250 mL). The solution was washed three times with sat. $NaHCO_3$. The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (150 g) eluted with hexanes−EtOAc (7:3, v/v) to give **5** (2.72 g, 40%): ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.26, 0.38 (6 H, s), 0.98 (9 H, s), 2.91 (2 H, t, J = 6.6 Hz), 3.40 (2 H, m), 3.61 (2 H, m, 5′-H), 4.13 (1 H, d, J = 8.25 Hz), 4.34 (1 H, d, J = 4.29 Hz), 4.44 (1 H, m) 5.78 (1 H, d, J = 7.26 Hz), 5.83 (1 H, s), 6.88 (4 H, d, J = 8.58 Hz) 7.18−7.45 (9 H, m), 8.39 (1 H, d, $J_{5,6}$ = 6.93 Hz); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ -5.51, −4.45, 17.95, 18.17, 25.18, 25.75, 55.13, 60.81, 68.59, 76.37, 82.95, 86.92, 90.85, 103.18, 113.19, 118.04, 125.16, 127.03, 127.91, 128.07, 128.90, 130.01, 130.05, 134.95, 135.26, 141.01, 144.17, 153.46, 158.58, 175.27. Anal. Calcd for $C_{39}H_{47}N_3O_7SSi\ H_2O$: C, 62.63; H, 6.60; N, 5.62; S, 4.29. Found: C, 62.31; H, 6.25; N, 5.52; S, 4.77.

4-*S*-(2-Cyanoethyl)-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(4,4′-dimethoxytrityl)-4-thiouridine 3′-*O*-(2-Cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (7). Compound **5** (1.46 g, 2 mmol) was rendered anhydrous by coevaporation three times each with dry pyridine, dry toluene, and dry THF and dissolved in dry THF (6 mL). To the solution under argon atmosphere were added *N*-methylimidazole (80 μL, 1 mmol), collidine (1.59 mL, 12 mmol), and chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine (870 μL, 4 mmol). After being stirred at room temperature for 1 h, the mixture was diluted with CH_2Cl_2 (100 mL) and washed three times with sat. $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (60 g) eluted with hexanes−EtOAc-pyridine (70:30:0.5, v/v/v) to give **7** (1.47 g, 79%): ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.16, 0.29 (6H, s), 0.91−1.19 (2H, m), CH_3 of *i*Pr) 2.41 ⟨2.58⟩ (2 H, t, J = 6.27 Hz), 2.90 (2 H, m) 3.31−3.86 (8, H, m), 3.80 (6 H, m) 4.33−4.38 (3 H, m), 5.59 ⟨5.71⟩ (1 H, d, J = 7.26 Hz) 5.27 ⟨5.79⟩ (1 H, s), 6.84 (4 H, m), 7.16−7.45 (9 H, m), 8.34 ⟨8.40⟩ (1 H, d, J = 7.26 Hz); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ -5.37, −4.63, −4.58 (Si $(CH_3)_2$), 17.77, 17.95, 19.88, 19.98, 20.07, 20.18, 21.15, 24.19, 24.28, 24.40, 24.51, 24.98, 25.59, 42.68, 42.81, 43.00, 54.95, 57.40, 57.54, 57.68, 57.85, 60.45, 60.63, 74.68, 75.31, 86.99, 86.70, 86.83, 91.30, 91.59, 103.02, 112.92, 117, 14, 117.23, 118, 124.99, 126.86, 127.62, 127.91, 128.10, 128.18, 128.72, 130.06, 134.75, 134.90, 135.08, 137.48, 140.90, 143.85, 144.04, 153.37, 153.42, 158.44, 174.61, 174.81; ^{31}P NMR (67.8 MHz, $CDCl_3$) δ 149.29, 151.44 (1:1). Anal. Calcd for $C_{48}H_{64}N_5O_8SSiP$: C, 61.98; H, 6.93; N, 7.53; S, 3.45. Found: C, 61.87; H, 7.18; N, 7.38; S, 2.71.

4-*S*-(2-Cyanoethyl)]-2′-*O*-(*tert*-butyldimethylsilyl)-4-thiouridine (6). To a solution of compound **5** (150 mg, 0.2 mmol) in CH_2Cl_2 (9.8 mL) was added dichloroacetic acid (250 μL). After being stirred at room temperature for 2 min, the mixture was washed three times with sat. $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (5 g) eluted with $CHCl_3$−MeOH (99:1−98:2, v/v) to give **6** (85 mg, 99%): ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.14, 0.17 (6 H, s), 0.91 (9 H, s), 2.89 (2 H, t, J = 6.60 Hz), 3.42 (2 H, m), 3.86 (1 H, m), 4.05 (1 H, m), 4.15−4.22 (2 H, m), 4.64 (1 H, t, J = 3.96 Hz), 5.53 (1 H, d, J = 3.30 Hz), 6.27 (1 H, d, J = 6.27 Hz), 7.91 (1 H, J = 6.27 Hz); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ -5.53, −4.89, 17.74, 17.86, 25.00, 25.48, 60.18, 68.79, 75.24, 84.57, 92.40, 103.50, 117.90, 142.05, 153.64, 175.62. Anal. Calcd for $C_{18}H_{29}N_3O_5SSi$: C, 50.56; H, 6.84; N, 9.83; S, 7.50. Found: C, 50.42; H, 6.50; N, 9.85; S, 6.85.

Tetrakis(2-cyanoethyl) Ester of 4-*S*-(2-Cyanoethyl)-2′-*O*-(*tert*-butyldimethylsilyl)-4-thiouridine 3′,5′-Bisphosphate (8). Compound **6** (214 mg, 0.5 mmol) was rendered anhydrous by coevaporation three times each with dry pyridine, dry toluene and dry CH_3CN and finally dissolved in dry CH_3CN (5 mL). To the solution were added bis(2-cyanoethyl)-(*N*,*N*-diisopropyl)phosphoramidite (405 mg, 1.5 mmol) and 1*H*-tetrazole (160 mg, 2.25 mmol). The mixture was stirred under argon atmosphere at room temperature for 1 h. *tert*-Butyl hydroperoxide (1.5 mL, 15 mmol) was added and the resulting mixture was stirred at room temperature for 30 min. The mixture was diluted with CH_2Cl_2, and the solution was washed twice each with sat. $NaHCO_3$ (50 mL) and water. The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (10 g) eluted with CH_2Cl_2−MeOH (98:2, v/v) to give **8** (354 mg, 89%): ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.17, 0.26 (6 H, s), 0.94 (9 H, s) 2.76−2.91 (10 H, m), 3.40 (2 H, t, J = 7.30 Hz) 4.29−4.59 (12 H, m), 4.74 (1 H, m), 5.69 (1 H, s), 6.33 (1 H, d, J = 6.93 Hz), 7.83 (1 H, d, J = 7.26 Hz); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ -5.33, −4.76, 17.95, 18.08, 19.57, 19.68, 25.16, 25.54, 62.63, 62.70, 62.75, 62.82, 65.36, 73.05, 74.07, 77.18, 79.14, 92.29, 103.65, 116.39, 115.51, 115.62, 118.08, 140.04, 153.26, 175.97; ^{31}P NMR (67.8 MHz, $CDCl_3$) δ −1.81, −1.59. Anal. Calcd for $C_{30}H_{43}N_{57}O_{11}SSiP_2\cdot 5H_2O$: C, 40.49; H, 6.00; N, 11.02; S, 3.60. Found: C, 40.33; H, 5.01; N, 10.66; S, 4.43.

4-Thiouridine 3′,5′-Bisphosphate (9). To a solution of Compound **8** (37 mg, 0.05 mmol) in dry pyridine (5 mL) were added DBU (45 mL, 0.3 mmol) and BSA (460 mL, 1.88 mmol). After being stirred at room temperature for 1.5 h, the mixture was diluted with water. The aqueous solution was washed three times with Et_2O. The aqueous phase was collected and evaporated under reduced pressure. The residue was rendered anhydrous by coevaporation three times each with dry pyridine and dry toluene. To the anhydrous mixture was added triethylamine trihydrofluoride (403 mg, 2.5 mmol). After being stirred at room temperature for 20 h, the mixture was added dropwise to 2 M TEAB buffer (2 mL). The reaction mixture was purified by DEAE Sephadex A-25 (1 M ammonium bicarbonate). The eluate was lyophilized to give **9** (915 A_{330}, 67%): ^{1}H NMR (270 MHz, D_2O) δ 3.95−3.96 (2H, m), 4.41−4.45 (2 H, m), 4.54 (1 H, m), 6.33 (1 H, d, J = 6.93 Hz), 7.83 (1 H, d, J = 7.26 Hz); ^{13}C NMR (67.8 MHz, D_2O) δ −5.33, −4.76, 17.95, 18.08, 19.57, 19.68, 25.16, 25.54, 62.63, 62.70, 62.75, 62.82, 65.36, 73.05, 74.07, 77.18, 79.14, 92.29, 103.65, 116.39,

115.51, 115.62, 118.08, 140.04, 153.26, 175.97; ^{31}P NMR (67.8 MHz, $CDCl_3$) δ 4.42, 4.60; FAB calcd for $C_9H_{11}N_2Na_4O_{11}P_2S$ *m*/*z* 508.92, obsd, 508.92; UV (H_2O) λ_{max} 243 nm, 330.5 nm, λ_{min} 226.5 nm, 274.5 nm.

6-*S*-(2-Cyanoethyl)-2-*N*-phenylacetyl-6-thioguanosine (14). To a suspension of compound **11** (803 mg, 2 mmol) in dry acetonitrile was added hexamethyldisilazane (2.1 mL, 10 mmol). After being refluxed for 2 h, the mixture was cooled, and ethanol (2 mL) was added. The solvent was removed under reduced pressure, and the residue was coevaporated with dry toluene and dissolved in CH_2Cl_2 (300 mL). To the mixture were added mesitylenesulfonyl chloride (525 mg, 2.4 mmol), triethylamine (1.12 mL, 8 mmol), and 4-(dimethylamino)pyridine (12 mg, 0.1 mmol). After being stirred at room temperature for 1 h, the mixture which was cooled at 0 °C. To the mixture was added *N*-methylpyrrolidine (2.08 mL, 20 mmol). After 30 min, 2-cyanoethanethiol (2.0 mL, 20 mmol) was added, and the mixture was stirred at 0 °C for 30 min. The mixture was diluted with CH_2Cl_2 and washed three times with 1 M KH_2PO_4. The organic layer was collected, dried over Na_2SO_4, filtered and evaporated under reduced pressure. To the residue was added 2% trifluoroacetic acid/methanol (50 mL). After being stirred at room temperature for 20 min, the mixture was quenched by addition of pyridine (20 mL). The organic solution was evaporated under reduced pressure, and the residue was chromatographed on a column of silica gel (30 g) eluted with CH_2Cl_2-MeOH (96:4 v/v). After the eluent was evaporated under reduced pressure, the residue was diluted with CH_2Cl_2−pyridine (2:1, v/v) and the solution was washed three times with water. Every time the aqueous layer was back-extracted three times with CH_2Cl_2−pyridine (1:1, v/v) which was put in another separatory funnel. After the extraction, the two organic layers were combined and evaporated under reduced pressure to give compound **14** (943 mg, 91%): ^{1}H NMR (270 MHz, DMSO) δ 3.15 (2 H, t, J = 7.26 Hz), 3.55 (2 H, t, J = 6.92 Hz), 3.55−3.64 (2 H, m), 3.82 (2 H, s), 3.95 (1 H, m), 4.19 (1 H, m), 4.54 (1 H, dd), 4.99 (1 H, t), 5.17 (1 H, d, J = 4.29 Hz), 5.49 (1 H, d, J = 5.61 Hz), 5.93 (1 H, d, J = 5.28 Hz), 7.26−7.34 (5 H, m), 8.58 (1 H, s), 10.86 (1 H, bs); ^{13}C NMR (67.8 MHz, DMSO) δ 15.89, 22.28, 41.24, 59.25, 68.36, 71.95, 83.69, 85.19, 117.34, 124.55, 125.77, 126.29, 127.40, 133.68, 140.38, 147.80, 149.97, 156.66, 167.12. Anal. Calcd for $C_{21}H_{22}N_6O_5S$: C, 53.61; H, 4.71; N, 17.86; S, 6.81. Found: C, 52.62; H, 4.80; N, 17.34; S, 7.94.

6-*S*-(2-Cyanoethyl)-5′-*O*-(4,4′-dimethoxytrityl)-2-*N*-phenylacetyl-6-thioguanosine (15). Compound **14** (613 mg, 1.3 mmol) was rendered anhydrous three times with dry pyridine and dissolved in dry pyridine (13 mL). To the mixture was added 4,4′-dimethoxytrityl chloride (485 mg, 1.43 mmol). After being stirred at room temperature for 18 h, the mixture was diluted with CH_2Cl_2. The CH_2Cl_2 solution was washed three times with sat. $NaHCO_3$. The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (20 g) eluted with CH_2Cl_2−MeOH (100:0−99:1) to give **15** (863 mg, 86%): ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.80 (2 H, t, J = 7.25 Hz), 3.18, 3.35 (2 H, m), 3.53 (2 H, m), 3.75 (6 H, s), 3.82 (2 H, s), 4.39 (1 H, m), 4.48 (1 H, m), 4.96 (1 H, m), 5.91 (1 H, d, J = 6.27 Hz), 6.67 (4 H, m), 7.06−7.43 (18 H, m), 8.14 (1 H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 18.71, 24.51, 40.05, 55.11, 63.78, 73.80, 86.54, 87.01, 92.06, 112.99, 118.17, 123.74, 125.23, 126.77, 127.69, 127.82, 128.14, 128.57, 128.95, 129.20, 129.40, 129.79, 129.85, 133.41, 135.26, 135.29, 141.24, 144.12, 148.30, 149.65, 150.89, 158.38, 158.42, 160.20, 169.16. Anal. Calcd for $C_{42}H_{40}N_6O_7S$: C, 65.27; H, 5.22; N, 10.87; S, 4.51. Found: C, 65.70; H, 5.44; N, 10.12; S, 4.21.

6-*S*-(2-Cyanoethyl)-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(4,4′-dimethoxytrityl)-2-*N*-phenylacetyl-6-thioguanosine (16). A mixture of compound **15** (1.55 g, 2 mmol) and imidazole (200 mg, 3 mmol) was rendered anhydrous by coevaporation three times each with dry pyridine and dry toluene. The mixture was dissolved in dry DMF (20 mL) and TBDMSCl (360 mg, 2.4 mmol) was added. After being stirred at room temperature for 16 h, the mixture was diluted with EtOAc. The EtOAc solution was washed three times with satd $NaHCO_3$. The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was chromatographed on a column of high mesh silica gel (WAKO−C300) (100 g) eluted with hexanes−Et_2O (45:55, v/v) to give **16** (493 mg, 28%). The 3′-silylated isomer was eluted with hexanes−Et_2O (40:60, v/v) to give **16′** (799 mg, 45%): Analytical data for **16**: ^{1}H NMR (270 MHz, $CDCl_3$) δ −0.21, −0.01 (6 H, s), 0.84 (9 H, s), 3.10 (2 H, t, J = 6.76 Hz), 3.24−3.32 (2 H, m), 3.53 (2 H, m), 3.76 (6 H, s), 3.77 (2 H, s), 4.23 (1 H, m), 4.33 (1 H, m), 5.05 (1 H, t, J = 5.61 Hz), 5.88 (1 H, d, J = 5.94 Hz), 6.80 (4 H, m), 7.11−7.53 (14 H, m), 8.05 (1 H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ −5.23, −5.07, 17.72, 18.37, 25.03, 25.39, 43.90, 55.10, 63.43, 71.16, 75.01, 77.18, 84.21, 86.43, 88.09, 113.19, 118.42, 126.98, 127.17, 127.91, 128.70, 129.31, 129.90, 133.84, 135.49, 135.67, 141.60, 144.71, 149.43, 151.45, 158.54, 160.34, 168.61. Anal. Calcd for $C_{48}H_{54}N_6O_7SSi$: C, 64.99; H, 6.14; N, 9.47; S, 3.61. Found: C, 65.49; H, 6.43; N, 9.03; S, 3.88. Analytical data for **16′**: ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.00, 0.08 (6 H, s), 0.87 (9 H, s), 2.94 (2 H, t, J = 6.9 Hz), 3.23 (1 H, d, J = 10.2 Hz), 3.40 (1 H, d, J = 8.6 Hz),3.50 (2 H, t, J = 6.9 Hz), 3.69 (2 H, s), 3.75 (6 H, s), 4.17 (1 H, m), 4.48 (1 H, m), 4.69 (1 H, m), 5.91 (1 H, d, J = 5.6 Hz), 6.80 (4 H, m), 7.11−7.38 (14 H, m), 7.95 (1 H, s), 8.10 (1 H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ −4.85, −4.78, 18.06, 18.58, 24.82, 25.50, 25.72, 44.48, 55.20, 62.12, 72.59, 74.86, 85.55, 86.56, 89.76, 113.15, 118.29, 126.94, 127.49, 127.87, 128.03, 128.75, 128.97, 129.43, 129.94, 133.85, 135.51, 135.60, 141.62, 144.38, 149.18, 151.39, 148.54. Anal. Calcd for $C_{48}H_{54}N_6O_7SSi\cdot 5/2H_2O$: C, 61.85; H, 6.38; N, 9.02. Found: C, 61.33; H, 5.83; N, 8.70.

6-*S*-(2-Cyanoethyl)]-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(4′,4′′-dimethoxytrityl)-2-*N*-phenylacetyl-6-thioguanosine 3′-*O*-(2-Cyanoethyl)(*N*,*N*-diisopropyl)phosphoramidite (18). Compound **16** (177 mg, 0.2 mmol) was rendered anhydrous by coevaporation three times each with dry pyridine, dry toluene and dry THF, and finally dissolved in dry THF (0.6 mL). To the solution were added *N*-methylimidazole (8 ul, 0.1 mmol), collidine (159 μL, 1.2 mmol) and chloro(2-cyanoethyl)(*N*,*N*-diisopropylamino)phosphine (87 μL, 0.4 mmol) under argon atmosphere. After being stirred at room temperature for 40 min, the mixture was diluted with CH_2Cl_2 (25 mL) and washed three times with sat. $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (10 g) eluted with hexanes−EtOAc−Et_3N (70:30:1, v/v/v) to give **18** (217 mg, 77%): ^{1}H NMR (270 MHz, $CDCl_3$) δ −0.26, −0.05, −0.01 (6H, s), 0.76 (9 H, s), 0.95−1.32 (12 H, m), 2.18 (2 H, m), 2.61 (2 H, m), 3.09−4.02 (16 H, m), 4.24−4.38 (2 H, m), 5.01 ⟨5.14⟩ (1 H, m), 5.84 ⟨5.99⟩ (1 H, d, J = 7.26 Hz), 6.77−7.64 (14 H, m), 8.08 ⟨8.10⟩ (1 H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ −5.26, −4.83, −4.77, 17.70, 17.81, 18.39, 19.80, 19.91, 20.11, 20.18, 24.42, 24.53, 24.62, 25.11, 25.34, 25.43, 25.48, 29.15, 45.59, 42.79, 43.18, 43.36, 43.51, 43.79, 55.13, 58.73, 58.94, 63.04, 63.24, 72.04, 72.26, 75.72, 84.17, 84.64, 86.34, 86.67, 87.19, 88.05, 113.24, 117.11, 117.70, 118.55, 127.04, 127.82, 128.00, 128.49, 128.57, 128.81, 129.38, 129.83, 129.88, 129.99, 133.89, 134.14, 135.31, 135.42, 135.62, 135.87, 141.15, 141.89, 144.49, 144.85, 149.70, 151.41, 151.64, 158.56, 160.14, 160.25, 169.00; ^{31}P NMR (67.8 MHz, $CDCl_3$) δ 149.44, 151.84. Anal. Calcd for $C_{57}H_{71}N_8O_8SSiP$: C, 62.96; H, 6.58; N, 10.31. Found: C, 63.24; H, 6.70; N,10.14.

6-*S*-(2-Cyanoethyl)-2′-*O*-(*tert*-butyldimethylsilyl)-2-*N*-phenylacetyl-6-thioguanosine (17). To a solution of compound **16** (0.2 mmol, 177 mg) in CH_2Cl_2 was added dichloroacetic acid (250 μL). After being stirred at room temperature for 2 min, the mixture was washed three times with sat. $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (5 g) eluted with $CHCl_3$-MeOH (99:1−98:2, v/v) to give **17** (106 mg, 91%): ^{1}H NMR (270 MHz, $CDCl_3$) δ −0.35, −0.13 (6 H, s), 0.81 (9 H, s), 2.99 (2 H, t, J = 6.93 Hz), 3.50 (2 H, m), 3.71−3.94 (2 H, m), 3.84 (2 H, s), 4.30 (1 H, s), 4.36 (1 H, d, J = 4.95 Hz). 5.96 (1 H, dd, J = 6.93 Hz, J = 4.95 Hz), 5.74 (1 H, d, J = 7.26 Hz), 7.32−7.45 (5 H, m), 7.89 (1 H, s), 7.91 (1 H, bs); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ −5.39, −5.35, 17.67, 18.33, 24.85, 25.38, 44.78,

62.55, 71.97, 74.46, 77.18, 86.69, 90.42, 118.22, 127.57, 129.06, 129.31, 129.38, 133.68, 142.61, 148.48, 150.99, 160.99, 168.48. Anal. Calcd for $C_{27}H_{36}N_6O_5SSi$: C, 55.46; H, 6.20; N, 14.37; S, 5.48. Found: C, 55.22; H, 6.17; N, 14.31; S, 5.30.

Tetrakis(2-cyanoethyl) Ester of 6-*S*-(2-Cyanoethyl)-2′-*O*-(*tert*-butyldimethylsilyl)-6-thioguanosine 3′,5′-Bisphosphates (19). Compound **17** (58 mg, 0.1 mmol) was rendered anhydrous by coevaporation three times each with dry pyridine, dry toluene and dry CH_3CN. The residue was dissolved in dry CH_3CN (1 mL). To the solution were added bis(2-cyanoethyl) (*N*,*N*-diisopropyl) phosphoramidite (81 mg, 0.3 mmol) and 1*H*-tetrazole (32 mg, 0.45 mmol). The mixture was stirred at room temperature for 1 h and then *tert*-butyl hydroperoxide (0.3 mL, 3 mmol) was added. After 30 min, the solution was diluted with CH_2Cl_2 and washed with sat. $NaHCO_3$. The organic layer was dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (3 g) eluted with CH_2Cl_2-MeOH (97:3, v/v) to give **19** (75 mg, 78%): ^{1}H NMR (270 MHz, $CDCl_3$) δ −0.02, −0.00 (6 H, s), 0.78 (9 H, s), 2.76−2.81 (8 H, m), 3.04−3.12 (2 H, m), 3.46−3.76 (2 H, m), 3.84 (2 H, s), 4.29−4.38 (8 H, m), 4.50−4.58 (3 H, m), 5.18 (1 H, m), 5.40 (1 H, m), 5.81 (1 H, d, J = 5.94 Hz), 7.29−7.41 (5 H, m), 7.92 (1 H, s), 9.35 (1 H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ −5.37, −5.08, 17.76, 18.44, 19.34, 19.63, 24.89, 25.30, 25.54, 44.44, 62.28, 62.36, 62.61, 62.66, 66.92, 71.75, 77.18, 80.99, 89.33, 116.48, 116.55, 116.64, 118.53, 127.12, 128.68, 129.27, 134.65, 142.62, 149.00, 151.79, 160.50, 168.84; ^{31}P NMR (109.4 MHz, $CDCl_3$) δ −2.55, −1.98.

6-Thioguanosine 3′,5′-Bisphosphates (20). To a solution of compound **19** (75 mg, 0.078 mmol) in dry pyridine (7.8 mL) were added DBU (70 μL, 0.47 mmol) and BSA (723 μL, 2.93 mmol). After being stirred at room temperature for 1.5 h, the mixture was diluted with H_2O. The aqueous solution was washed three times with Et_2O. The aqueous phase was collected and evaporated under reduced pressure. The residue was treated with 25% ammonia-EtOH (40 mL 3:1,v/v) at 55 °C for 3 h. After the solution was evaporated under reduced pressure, the residue was diluted with water, washed three times with CH_2Cl_2, and evaporated under reduced pressure. The residue was rendered anhydrous by coevaporations three times each with dry pyridine and dry toluene. To the anhydrous mixture was added triethylamine trihydrofluoride (629 mg, 3.9 mmol). After being stirred at room temperature for 16 h, the mixture was added dropwise to 2 M TEAB buffer (2 mL). The reaction mixture was purified by DEAE Sephadex A-25 (1 M ammonium bicarbonate). The elution followed by lyophilization gave **20** (1144 $A_{338.5}$, 59%); ^{1}H NMR (270 MHz, D_2O) δ 3.94 (2 H, m), 4.45 (1 H, m), 4.66 (2 H, m), 4.77 (1 H, m), 5.93 (1 H, d, J = 6.93 Hz), 8.28 (1 H, s); ^{13}C NMR (67.8 MHz, D_2O) δ 63.91, 73.81, 73.90, 84.42, 86.25, 129.01, 138.77, 148.30, 157.24, 176.69; ^{31}P NMR (67.8 MHz, $CDCl_3$) δ 4.70, 4.88; FAB calcd for $C_{10}H_{12}N_5Na_4O_{10}P_2S$ m/z 547.94, obsd for $C_{10}H_{12}N_5Na_4O_{10}P_2S$ m/z 547.95; UV (H_2O) λ_{max} 254 nm, 338.5 nm, λ_{min} 240.5 nm, 288.5 nm.

6-Chloro-5′-*O*-(4,4′-dimethoxytrityl)purine Riboside (22). Compound **21** (880 mg, 3.07 mmol) was rendered anhydrous by coevaporation three times with dry pyridine. The residue was dissolved in dry pyridine (30 mL), and DMTrCl (1.25 g, 3.68 mmol) was added. After being stirred at room temperature for 6 h, the mixture was diluted with CH_2Cl_2 and washed three times with satd Na_2CO_3. The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (30 g) eluted with CH_2Cl_2−MeOH (99.5:0.5−99:1) to give **22** (1.69 g, 94%): ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.33 (1 H, dd, J = 3.30 Hz, J = 10.89 Hz), 3.46 (1 H, dd, J = 3.63 Hz, J = 10.53 Hz), 3.77 (6 H, s), 4.42−4.48 (2 H, m), 4.88 (1 H, t, J = 5.28 Hz), 6.05 (1 H, d, J = 5.61 Hz), 6.74 (4 H, m), 7.15−7.31 (9 H, m), 8.38 (1 H, s), 8.73 (1 H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 55.17, 63.34, 72.09, 75.49, 85.63, 86.72, 90.50, 113.14, 123.94, 125.25, 126.95, 127.84, 127.91, 128.18, 128.99, 129.90, 135.31, 136.46, 143.90, 144.21, 149.24, 150.94, 151.63, 151.96, 158.54. Anal. Calcd for $C_{31}H_{29}N_4O_6SCl$: C, 63.21; H, 4.96; N, 9.51; Cl, 6.02. Found: C, 64.44; H, 5.24; N, 9.08; Cl, 5.71.

6-*S*-(2-Cyanoethyl)-5′-*O*-(4,4′-dimethoxytrityl)-6-thioinosine (23). To a solution of **22** (1.29 g, 2 mmol) in CH_2Cl_2 (40 mL) were added *N*-methylpyrrolidine (1.8 mL, 20 mmol) and 2-cyanoethanethiol (2.04 mL, 20 mmol). After being stirred at room temperature for 2 h, the mixture was diluted with CH_2Cl_2 (150 mL). The CH_2Cl_2 solution was washed three times with 1 M KH_2PO_4 buffer. The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (60 g) eluted with CH_2Cl_2−MeOH (99:1−98:2) to give **23** (2.47 g, 96%): ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.91 (2 H, t, J = 7.26), 3.31 (1 H, dd, J = 3.30 Hz, J = 10.56 Hz), 3.45 (1 H, dd, J = 3.30 Hz, J = 10.56 Hz), 3.61 (2 H, t, J = 7.26), 3.76 (6 H, s), 4.40−4.46 (2 H, m), 4.83 (1 H, t, J = 5.28 Hz), 6.02 (1 H, d, J = 5.61), 6.72−6.75 (4 H, m), 7.16−7.28 (9 H, m), 8.24 (1 H, s), 8.66 (1 H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 18.42, 21.30, 24.28, 53.55, 55.06, 63.24, 71.63, 75.15, 85.09, 86.47, 89.70, 113.01, 117.97, 123.92, 125.12, 126.79, 127.71, 127.87, 128.05, 128.86, 129.83, 131.41, 135.31, 141.85, 144.21, 147.82, 148.81, 151.39, 158.38, 159.10. Anal. Calcd for $C_{34}H_{33}N_5O_6S$: C, 63.84; H, 5.20; N, 10.95; S, 5.06. Found: C, 65.18; H, 5.39; N, 10.20; S, 4.64.

6-*S*-(2-Cyanoethyl)-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(4,4′-dimethoxytrityl)-6-thioinosine (24). A mixture of compound **23** (1.29 g, 2 mmol) and imidazole (204 mg, 3 mmol) was rendered anhydrous by coevaporation three times each with dry pyridine and dry toluene, and finally dissolved in dry DMF (20 mL). To the mixture was added TBDMSCl (361 mg, 2.4 mmol). After being stirred at room temperature for 24 h, the mixture was diluted with ethyl acetate (150 mL), and the solution was washed three times with sat. $NaHCO_3$. The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (WAKO C-300, 100 g) eluted with hexanes−Et_2O (50:50, v/v) to give **24** (414 mg, 27%). The 3′-silylated isomer (**24′**) was eluted with hexanes−Et_2O (30:70−20:80, v/v) to give **24′** (614 mg, 41%): Analytical data for **24**: ^{1}H NMR (270 MHz, $CDCl_3$) δ −0.13, 0.02 (6 H, s), 0.86 (9 H, s), 2.96 (2 H, t, J = 7.09 Hz), 3.41 (1 H, dd, J = 3.96 Hz, J = 10.56 Hz), 3.53 (1 H, dd, J = 2.97 Hz, J = 10.56 Hz), 3.63 (2 H, t, J = 6.16), 3.80 (6 H, s), 4.28 (1 H, m), 4.38 (1 H, m), 5.01 (1 H, t, J = 4.95 Hz), 6.09 (1 H, d, J = 5.28 Hz), 6.82 (4 H, d, J = 8.91 Hz), 7.22−7.46 (9 H, m), 8.22 (1 H, s), 8.64 (1 H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ −5.30, −5.10, 17.70, 18.48, 24.28, 25.38, 55.02, 63.16, 71.34, 75.53, 84.08, 86.51, 88.23, 113.05, 117.95, 125.77, 127.73, 127.91, 129.88, 131.63, 135.38, 141.87, 144.37, 148.46, 151.75, 158.40, 158.83. Anal. Calcd for $C_{40}H_{47}N_5O_6SSi\ H_2O$: C, 62.23; H, 6.40; N, 9.07; S, 4.25. Found: C, 61.95; H, 6.78; N, 9.80; S, 4.29. Analytical data for **24′**: ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.00, 0.16 (6 H, s), 0.89 (9 H, s), 2.93 (2 H, t, J = 7.26 Hz), 3.14 (1 H, d, J = 6.59 Hz) 3.25 (1 H, d,J = 6.94 Hz), 3.50 (1 H, d, J = 7.59 Hz), 3.61 (2 H, t, J = 7.26 Hz), 3.78 (6 H, s), 4.19 (1 H, m), 4.59 (1 H, m), 4.74 (1 H, m), 6.03 (1 H, d, J = 4.62 Hz), 6.80 (4 H, d, J = 8.59 Hz), 7.22−7.46 (9 H, m), 8.24 (1 H, s), 8.68 (1 H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ −4.75, −4.59, 18.11, 18.77, 24.54, 25.64, 25.78, 55.32, 62.76, 72.12, 74.69, 84.67, 86.70, 88.70, 89.41 113.27, 127.05, 127.97, 128.17, 130.07, 132.09, 135.59, 135.64, 142.22, 144.41, 148.63, 152.00, 158.68, 159.13. Anal. Calcd for $C_{40}H_{47}N_5O_6SSi\cdot{}^1/_2H_2O$: C, 62.97; H, 6.34; N, 9.18. Found: C, 62.91; H, 6.22; N, 8.93.

6-*S*-(2-Cyanoethyl)-2′-*O*-(*tert*-butyldimethylsilyl)-6-thioinosine 3′-*O*-(2-Cyanoethyl)(*N*,*N*-diisopropyl)phosphoramidite (26). Compound **24** (60 mg, 0.08 mmol) was rendered anhydrous by coevaporation three times each with dry pyridine, dry toluene and dry THF, and finally dissolved in dry THF (0.24 mL). To the solution were added *N*-methylimidazole (3 μL, 0.04 mmol), collidine (64 μL, 0.48 mmol) and chloro(2-cyanoethoxy) (*N*,*N*-diisopropylamino)phosphine (35 μL, 0.16 mmol) under argon atmosphere. The resulting mixture was stirred at room temperature for 40 min. The mixture was diluted with CH_2Cl_2 (25 mL) and washed three times with satd $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (5 g) eluted with hexanes−EtOAc−Et_3N (80:20:0.5, v/v/v) to give **26** (76

mg, 76%): ^{1}H NMR (270 MHz, $CDCl_3$) δ −0.22 ⟨−0.21⟩, −0.04 ⟨−0.02⟩ (6 H, s), 0.75 (9 H, s), 1.03−1.25 (9 H, m), 2.29 ⟨2.65⟩ (2 H, t, J = 6.27 Hz), 2.95 (2 H, t, J = 7.26), 3.29−3.96 (14 H, m), 4.35−4.43 (2 H, m), 5.05 (1 H, m), 6.02 ⟨6.08⟩ (1 H, d, J = 6.26 Hz), 6.79−7.48 (13 H, m), 8.21 ⟨8.24⟩ (1 H, s), 8.60 ⟨8.24⟩ (1 H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ −5.17, −4.67, 17.81, 17.86, 18.67, 19.98, 20.09, 20.36, 20.45, 22.54, 24.44, 24.58, 24.67, 24.76, 25.43, 25.54, 29.22, 31.70, 42.81, 42.98, 43.25, 43.45, 53.73, 55.20, 57.40, 57.72, 58.87, 63.09, 63.25, 72.54, 72.76, 73.21, 73.35, 74.48, 75.22, 77.20, 83.81, 84.22, 86.56, 86.72, 88.11, 88.39, 89.02, 113.12, 113.17, 117.23, 117.54, 118.13, 126.92, 127.30, 127.85, 127.89, 128.03, 128.16, 130.01, 130.06, 130.10, 131.84, 131.91, 135.42, 135.47, 135.62, 135.67, 1142.19, 142.30, 144.40, 144.53, 148.75, 151.84, 158.51, 158.71; ^{31}P NMR (109.4 MHz, $CDCl_3$) δ 149.69, 151.64. Anal. Calcd for $C_{49}H_{64}N_7O_7SSiP$: C, 61.68; H, 6.76; N, 10.28; S, 3.36. Found: C, 61.73; H, 7.14; N, 9.67; S, 3.64.

6-*S*-(2-Cyanoethyl)-2′-*O*-(*tert*-butyldimethylsilyl)-6-thioinosine (25). To a solution of compound **24** (151 mg, 0.2 mmol) in CH_2Cl_2 (9.75 mL) was added dichloroacetic acid (250 μL). After being stirred at room temperature for 2 min, the mixture was washed three times with sat. $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (3 g) eluted with $CHCl_3$−MeOH (99.5:0.5−99:1, v/v) to give **25** (85 mg, 92%): ^{1}H NMR (270 MHz, $CDCl_3$) δ −0.18, −0.42 (6 H, s), 0.79 (9 H, s), 2.95 (2 H, t, J = 7.26 Hz), 3.63 (2 H, m), 3.78, 3.96 (2 H, m), 4.34−4.36 (2 H, m), 5.08 (1 H, dd, J = 7.26 Hz, J = 4.95 Hz), 5.81 (1 H, d, J = 7.26 Hz), 8.01 (1 H, s), 8.71 (1 H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ −5.59, −5.48, 17.59, 18.44, 24.33, 25.29, 62.95, 72.38, 74.39, 87.26, 90.91, 117.81, 132.70, 143.22, 147.21, 151.11, 160.49. Anal. Calcd for $C_{19}H_{29}N_5O_4SSi\cdot{}^1/_2H_2O$: C, 49.54; H, 6.56; N, 15.20; S, 6.96. Found: C, 49.45; H, 6.11; N, 14.89; S, 6.84.

Tetrakis(2-cyanoethyl) Ester of 6-*S*-(2-Cyanoethyl)-2′-*O*-(*tert*-butyldimethylsilyl)-6-thioinosine 3′,5′-Bisphosphate (27). Compound **25** (138 mg, 0.3 mmol), which was rendered anhydrous three times each with dry pyridine, dry toluene and dry CH_3CN, was dissolved in dry CH_3CN (5 mL). To the solution were added bis(2-cyanoethoxy) (*N*,*N*-diisopropyl)phosphoramidite (244 mg, 0.9 mmol) and 1*H*-tetrazole (95 mg, 1.35 mmol). The mixture was stirred at room temperature for 1 h and *tert*-butyl hydroperoxide (0.9 mL, 9 mmol) was added. After 30 min, the solution was diluted with CH_2Cl_2 (50 mL) and washed with sat. $NaHCO_3$. The organic layer was evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (10 g) eluted with CH_2Cl_2−MeOH (97:3, v/v) to give **27** (174 mg, 70%): ^{1}H NMR (270 MHz, $CDCl_3$) δ −0.07, 0.06 (6 H, s), 0.83 (9 H, s), 2.76−2.85 (4 H, m), 3.62 (2 H, m), 4.28−4.41 (8 H, m), 4.48−4.61 (3 H, m), 5.11−5.15 (2 H, m), 6.03 (1 H, d, J = 4.29), 8.28 (1 H, s), 8.73 (1 H, s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ -5.39, −5.19, 17.69, 18.46, 19.41, 19.52, 24.19, 25.23, 25.43, 62.50, 62.57, 62.68, 62.73, 65.99, 73.32, 75.22, 77.20, 80.36, 88.84, 116.46, 116.60, 118.06, 131.79, 141.98, 148.12, 151.77, 159.03; ^{31}P NMR (109.4 MHz, $CDCl_3$) δ −1.85, −1.94. Anal. Calcd for $C_{31}H_{43}N_9O_{10}SSiP_2\cdot 2H_2O$: C, 43.30; H, 5.51; N, 14.66; S, 3.73. Found: C, 43.73; H, 5.33; N, 13.17; S, 5.09.

6-Thioinosine 3′,5′-Bisphosphate (28). To a solution of compound **27** (83 mg, 0.1 mmol) in dry pyridine (10 mL) were added DBU (90 μL, 0.6 mmol) and BSA (930 μL, 3.75 mmol). After being stirred at room temperature for 1.5 h, the mixture was diluted with H_2O. The aqueous solution was washed three times with Et_2O. The aqueous layer was collected and evaporated under reduced pressure. The residue was rendered anhydrous by coevaporation three times each with dry pyridine and dry toluene. To the anhydrous mixture was added triethylamine trihydrofluoride (806 mg, 5 mmol). After being stirred at room temperature for 16 h, the mixture was added dropwise to 2 M TEAB buffer (2 mL). The reaction mixture was purified by DEAE Sephadex A-25 (1 M ammonium bicarbonate). The eluate was lyophilized to give **28** (1467 A_{311}, 75%): ^{1}H NMR (270 MHz, D_2O) δ 4.00 (2 H, m), 4.49 (1 H, m), 4.70−4.78 (2 H, m), 6.12 (1 H, d, J = 6.27 Hz), 8.29 (1 H, s), 8.67 (1 H, s); ^{13}C NMR (67.8 MHz, D_2O) 63.80 ($J_{(COP)}$ = 3.66 Hz), 73.55 ($J_{(COP)}$ = 3.66 Hz), 74.62, 84.57 ($J_{(CCOP)}$ = 6.54 Hz), 87.33, 134.93, 142.15, 144.56, 146.23, 175.09; ^{31}P NMR (109.4 MHz, $CDCl_3$) δ 3.77, 4.30; FAB calcd for $C_{10}H_{11}N_4Na_4O_{10}P_2S$ m/z 532.93, obsd 532.93; UV (H_2O) λ_{max} 228.5 nm, 311 nm, λ_{min} 213 nm, 256 nm.

Ligation of $m_3^{2,2,7}G^{5'}$pppAmUmA with Thionucleoside 3′,5′-Bisphosphates Using T4 RNA Ligase. Ligation acceptor **30** (1.7A_{259}) and donor pNp [N = ^{4s}U (6.8 A_{330}), ^{6s}I (7.84 A_{330}), ^{6s}G (9.92 A_{330}), C (3.64 A_{259}), or U (4.0 A_{259})] were dissolved in 50 mM Tris-HCl (pH 8.0, 200 μL) containing 20 mM $ZnCl_2$, 5 mM DTT and 8 mM ATP. T4 RNA ligase (70 units, 10 unit/μL) in glycerin-water (1:1,v/v) was added, and the resulting mixture was incubated at 8 °C. After 48 h, the reaction mixture was heated at 100 °C for 3 min and cooled to room temperature. Calf intestinal alkaline phosphatase (50 unit, 1 unit/μL) was added, and the resulting solution was incubated at 37 °C. After being incubated for 2 h, the mixture was heated at 90 °C for 2 min. The mixture was chromatographed on anion exchange HPLC, and the peak of $m_3^{2,2,7}G^{5'}$pppAmUmAN was collected. The fractions collected were lyophilized, and the contaminated salts were removed by gel filtration using Sephadex G-15 (17 mm × 100 cm). The eluent was lyophilized to give the desired ligated product. The yields of the ligated products **31a**−**e** are listed in Table 1.

Enzymatic Digestion of $m_3^{2,2,7}G^{5'}$pppAmUmAN (31a, 31b, 31c) with Nuclease P1. The ligation products (**31a**:0.76, **31b**:0.18, **31c**:0.30 A_{259}) was dissolved in 20 mM AcOH−AcONa (pH 5.3, 100 μL) containing 0.1 mM $ZnCl_2$. Nuclease P1 (4 units, 1 unit/μL) in glycerin−water (1:1, v/v) was added and the resulting mixture was incubated at 50 °C. After being incubated for 5 h, the mixture was heated at 100 °C for 3 min. The mixture was analyzed by anion-exchange HPLC. The HPLC profiles are shown in Figure 3.

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