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PCR Amplification of 4′-ThioDNA Using 2′-Deoxy-4′-thionucleoside 5′-Triphosphates

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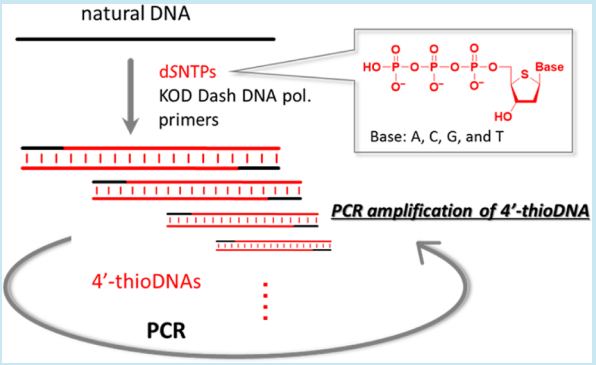
Supporting Information

ABSTRACT: 2′-Deoxy-4′-thioribonucleic acid (4′-thioDNA) having a sulfur atom instead of an oxygen atom in the furanose ring has a nuclease resistance and hybridization ability higher than that of natural DNA. Despite its great potential for various biological applications, a long 4′-thioDNA having all four kinds of 2′-deoxy-4′-thionucleosides has not been reported. In this study, we describe systematic analysis of the incorporation of 2′-deoxy-4′-thionucleoside 5′-triphosphates (dSNTPs) using various DNA polymerases. We found that family B DNA polymerases, which do not have 3′→5′ exonuclease activity, could efficiently incorporate dSNTPs via single nucleotide insertion and primer extension. Moreover, 104-mer PCR product was obtained even under the conditions in the presence of all four kinds of dSNTPs when KOD Dash DNA polymerase was used. The resulting PCR product was converted into a natural dsDNA by using PCR with dNTPs, and sequencing of the natural dsDNA revealed that the PCR cycle successfully proceeded without losing the sequence information of the template. To the best of our knowledge, this is the first example of accurate PCR amplification of highly modified DNA in the presence of only unnatural dNTPs.



KEYWORDS: *2′-deoxy-4′-thionucleoside 5′-triphosphate, 4′-thioDNA, PCR amplification*

Thus far, a large number of chemically modified nucleoside derivatives have been incorporated into oligonucleotides (ONs) for use in biological, bioengineering, and therapeutic applications. In general, these ONs are prepared via chemical methods using the corresponding phosphoramidite units. This method seems to be the most reliable for incorporating a modified unit into the desired position in the ON sequences. However, it is unsuitable for preparing long chain sequences. There is a possibility that an alternative method involving enzymatic synthesis using the corresponding modified nucleoside 5′-triphosphates (NTPs) could be applied. Since this method affords much longer sequences from readily available natural DNA templates, if successful, it would be a versatile method for use in a variety of applications. Therefore, intense efforts are currently underway toward efficient enzymatic synthesis of modified ONs by using not only primer extension and PCR amplification using DNA polymerases but also *in vitro* transcription with RNA polymerases.[1−10] A typical example of this research area is the investigation of the evolution of aptamers.[11−17] To the best of our knowledge, there are very few examples in which PCR proceeds in the presence of all four kinds of chemically modified dNTPs instead of natural dNTPs. Andreola et al. reported PCR in the presence of all four kinds of α-phosphorothioate dNTPs (α-PS-dNTPs); however, the incorporation efficiency of the full length product was very low.[18] Furthermore, no PCR examination has been successful using all four kinds of sugar-modified dNTP analogues. Quite recently, two groups have individually reported attractive results concerning selection of aptamers. Thus, Yu et al. succeeded to isolate fully modified aptamers consist of (3′,2′)-α-L-threose nucleic acid (TNA) using all four kinds of TNA triphosphates,[19,20] while Pinherio et al. isolated fully modified 1,5-anhydrohexitol nucleic acid (HNA) aptamers using all four kinds of HNA triphosphates,[21] respectively. However, these outstanding results demonstrated were based on a combination of DNA-polymerase-mediated primer extension and reverse-transcription, but not PCR, which is generally used for *in vitro* selection. Therefore, there is no example of a highly modifed nucleic acid analogue that can be amplified by direct PCR amplification. Since the resulting fully modified DNA analogues are expected to be synthetic genetic polymers,[19−21] and also to isolate functionalized aptamers, the development of chemically modified dNTPs, which can be amplified by PCR is highly desirable.

Our group has been developing a series of 4′-thionucleic acids, made up of 4′-thioribonucleosides[22] and 2′-deoxy-4′-

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thionucleosides.[23] We expected that the resulting 4′-thionucleic acids, that is, 4′-thioRNA and 4′-thioDNA, would exhibit not only high hybridization abilities and nuclease resistance arising from chemical modification but also biological equivalency with natural RNA and DNA because sulfur and oxygen atoms belong to the same group in the periodic table. In fact, these chemically modified ONs showed such favorable properties.[24−27] and therefore were utilized in gene silencing via RNAi machinery.[28−30] In addition, together with these successful results obtained by using a chemical approach, we have reported the isolation of partially modified 4′-thioRNA aptamers by using test tube evolution, since the 4′-thiopyrimidine nucleoside 5′-triphosphates (*S*UTP and SCTP) were good substrates for *in vitro* transcription by using T7 RNA polymerase.[31] Concerning 4′-thioDNA, we could amplify a partially modified 4′-thioDNA containing 2′-deoxy-4′-thiothymidine and 2′-deoxy-4′-thiocytidine units by using PCR in the presence of 2′-deoxy-4′-thiothymidine and 2′-deoxy-4′-thiocytidine 5′-triphosphates (d*S*TTP and d*S*CTP, Figure 1) with natural purine dNTPs

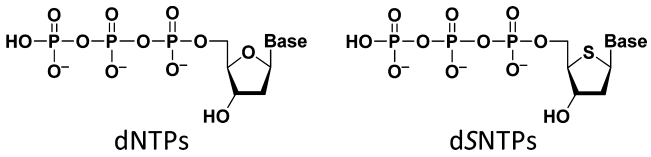


Figure 1. Chemical structures of 2′-deoxyribonucleoside 5′-triphosphates (dNTPs) and 2′-deoxy-4′-thioribonucleoside 5′-triphosphate (d*S*NTPs). Base = A, C, G, or T.

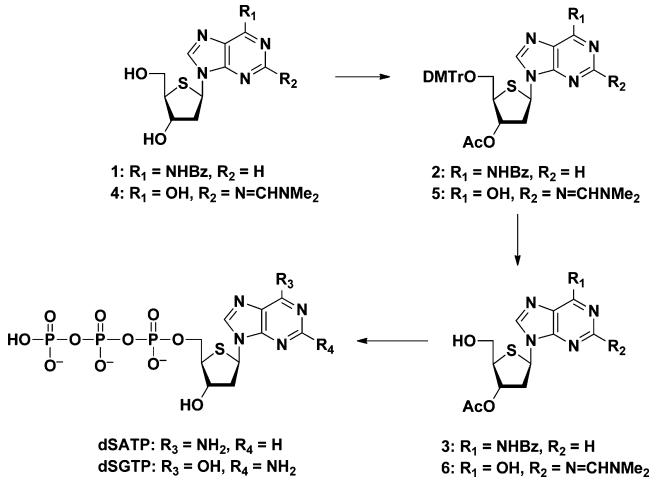
(dATP and dGTP). The amplified partially modified 4′-thioDNAs were used as templates for transcription to give RNA sequences *in vitro* and in mammalian cells.[32] This was the first example that 2′-deoxy-4′-thiopyrimidine nucleoside 5′-triphosphates as natural congeners could act as substrates for DNA polymerases and that the resulting partially modified 4′-thioDNAs were highly useful materials (synthesis and enzymatic recognition of 2′-deoxy-5-ethyl-4′-thiouridine 5′-triphosphate, which is not a 4′-S congener of natural dNTPs, are reported as one exception; see ref 33). These observations strongly suggested that 4′-thioDNAs as well as 4′-thioRNAs would be good candidates for not only chemically modified aptamers but also synthetic genetic polymers, if fully modified 4′-thionucleic acids could be freely obtained. Thus, we synthesized additional triphosphates, i.e., dSATP and dSGTP (Figure 1), and investigate their enzymatic recognition along with d*S*TTP and d*S*CTP by using DNA polymerases.

In this work, we first screened DNA polymerases by using primer extension in the presence of all four kinds of d*S*NTPs. As a result, polymerases belonging to family A, such as the Klenow fragment and Taq DNA polymerase, and reverse transcriptases (RTs) did not incorporate d*S*NTPs efficiently, whereas those belonging to family B, such as Therminator and KOD Dash DNA polymerases, effectively incorporated d*S*NTPs. Next, using family B DNA polymerases, PCR amplification in the presence of d*S*NTP(s) was examined, and we finally succeeded in the enzymatic synthesis of a 104-mer 4′-thioDNA by PCR in the presence of all four kinds of d*S*NTPs. Details of primer extension reactions and PCR amplifications in the presence of d*S*NTP(s) are described in this paper.

RESULTS AND DISCUSSION

Chemistry. The syntheses of dSATP and dSGTP are shown in Scheme 1. Starting with *N*-benzoyl-2′-deoxy-4′-thioadenosine (**1**),[23] two hydroxyl groups were successively protected with dimethoxytrityl (DMTr) and acetyl groups to give **2**. The DMTr group of **2** was then removed by treatment with 80% aqueous AcOH to give **3**, a substrate for the subsequent phosphitylation reaction. Following the efficient method reported by Ludwig and Eckstein,[34] **3** was treated with 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, followed by bis-(tri-*n*-butylammonium)pyrophosphate. Then the resulting cyclotriphosphite intermediate was oxidized with 1% iodine in pyridine−H_2O, and successive treatment with NH_4OH gave the desired dSATP in 50% yield after DEAE Sephadex column chromatographic purification. In the same manner, **4** was converted into **6** via fully protected **5**, and dSGTP was prepared from **6** by using the one-pot reaction described above.

Scheme 1. Syntheses of 2′-Deoxy-4′-thioadenosine 5′-Triphosphate (dSATP) and 2′-Deoxy-4′-thioguanosine 5′-Triphosphate (dSGTP)



Primer Extension with dSNTPs Using a Series of DNA Polymerases. We have previously reported that Therminator and KOD Dash DNA polymerases are the best for incorporating d*S*TTP and d*S*CTP under PCR conditions.[32] To further investigate the differences in the recognition of d*S*NTPs, we first carried out primer extension assays in the presence of d*S*NTPs using a series of DNA polymerases (Figure 2). The natural 5′-FITC labeled primer (20-mer) and template I (27-mer) were used in this experiment (Figure 2A). Thirteen DNA polymerases in total were chosen from families A, B, and Y DNA polymerases and reverse transcriptase (RT) for these assays. As shown in Figure 2B, almost no extension was observed when family A DNA polymerases and RT (lanes 2−5, 12 and 13) were used, although Tth DNA polymerase (lane 4) and M-MLV RT (lane 12) gave partial extension products. Dpo4 DNA polymerase (family Y) (lanes 14 and 15) gave better results than family A and RT ones did, although a strong band was observed at the position where three nucleotides were incorporated. On the other hand, the DNA polymerases belonging to family B (lanes 6−11) showed stronger activity than those belonging to families A and Y DNA polymerases or RT did. Of these polymerases, Vent (exo$^-$) (lane 6), Deep Vent (exo$^-$) (lane 7), Therminator (lane 10), and KOD Dash (lane 11) DNA polymerases showed notable

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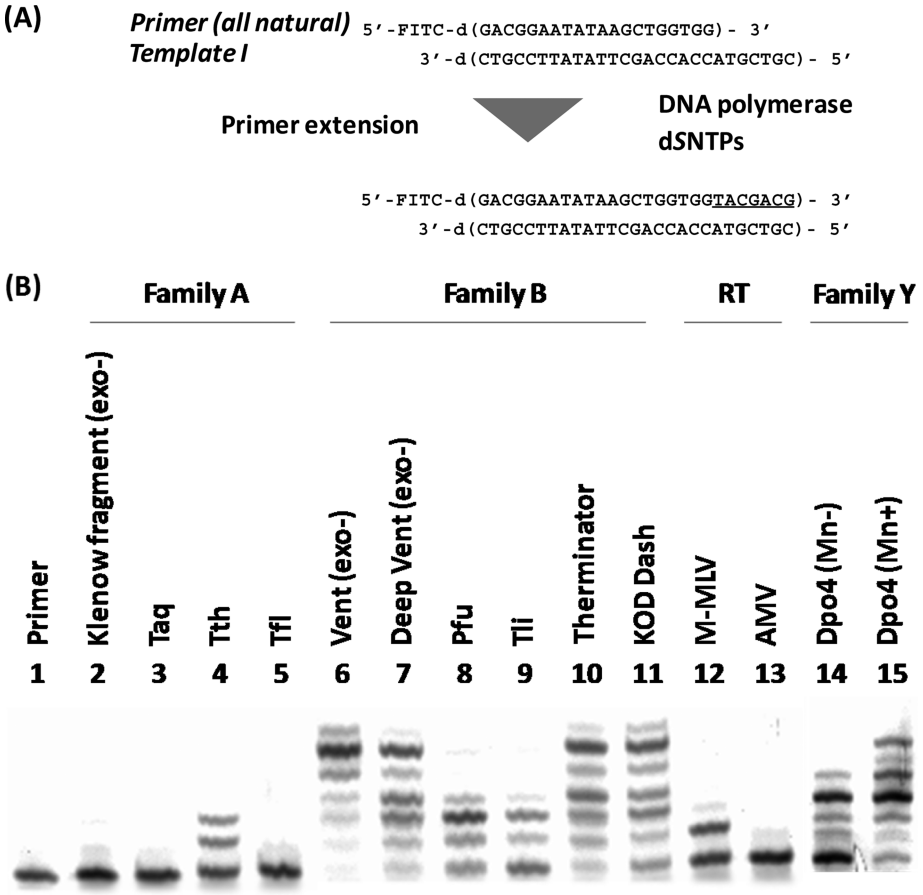

Figure 2. Primer extension by various DNA polymerases with dSNTPs and natural DNA template. (A) Sequences of DNA templates and primer. Underlined are extended nucleotides. (B) Denaturing polyacrylamide gel image of the products. Annealed 5′-FITC labeled primer (0.8 μM) and template I (1.0 μM) mixture, dSNTP (40 μM), and DNA polymerases (0.01−0.4 U/μL) in a reaction buffer (25 μL) were incubated at 37 or 74 °C.

efficiency for primer extension. It has been reported that family B DNA polymerases can be utilized to incorporate not only base-modified nucleotides but also sugar-modified nucleotides.[1,7−17] Our results indicated that family B DNA polymerases were considerably tolerant to dSNTPs. The Pfu (lane 8) and Tli (lane 9) DNA polymerases showed less activity than other family B DNA polymerases did. This is probably due to their higher fidelity to dNTPs caused by 3′→5′ exonuclease activity.[35] The family Y DNA polymerase Dpo4 (lane 14) showed activity comparable to that of the family B DNA polymerases especially when manganese ion was added (lane 15).[36] Family Y DNA polymerases have an ability to incorporate damaged dNTPs, such as 8-hydroxy-dGTP and 2-hydroxy-dATP.[37] This low fidelity to dNTPs of Dpo4 DNA polymerase could make it possible to incorporate sugar-modified dNTP analogues such as dSNTPs. From these results, there was a significant difference in the potential to incorporate dSNTPs among the families of DNA polymerases.

Kinetic Studies of the Incorporation of dSNTPs via Single Nucleotide Insertion. From the primer extension assays, we found that the DNA polymerases belonging to family B gave better results than those of families A and Y DNA polymerases or RT did. Next, we carried out single nucleotide insertion assays using Therminator DNA polymerase (family B) or the Klenow fragment (exo$^-$) (family A) to further investigate the efficiencies and selectivities of dSNTP incorporation by using steady-state kinetics. Templates I−IV were prepared to investigate each d(*S*)NTP incorporation (Table 1). The kinetic parameters (K_m = the Michaelis constant, V_{max} = the maximum rate of the enzyme reaction, and V_{max}/K_m = the insertion efficiency) were determined for each dSNTP with each primer-template set at various concentrations of triphosphates, and then the incorporation efficiencies relative to those of the corresponding natural dNTPs were calculated.

As shown in Table 1, the kinetic parameters for dTTP and dSTTP in the presence of a natural primer were compared. The relative efficiencies of the incorporations of dSNTPs (35−140) by using Therminator were comparable with those of dNTPs (100) (Table 1A). In contrast, the same experiment with the Klenow fragment showed that the relative efficiencies for dSNTPs incorporation were from 1 to 5 since the K_m values for dSNTPs (1.5−12 μM) were significantly higher than those for dNTPs (0.077−0.15 μM), as shown in Table 1B. This result indicates that Therminator incorporates dSNTP more efficiently than the Klenow fragment (exo$^-$) does because of its higher affinity for dSNTPs.

Li et al. have solved the atomic-level structures of complexes of the Klenow fragment with dNTPs and have revealed that the phenylalanine at position 667 (Phe667) is highly conserved and forms a platform, which is nearly parallel to the sugar rings of the incoming dNTPs.[38] In addition, it has been shown that the side chain of Phe667 is critical in distinguishing dNTPs from other sugar motifs as substrates.[39] Since the conformation of the sugar ring significantly changes upon substituting 4′-oxygen with 4′-sulfur due to the differences in the atomic radii (oxygen

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Table 1. Steady-State Kinetic Parameters for Insertion of Single Nucleotides into Template-Natural Primer Duplexes by Therminator DNA Polymerase (A) and Klenow Fragment (exo$^-$) (B)

Primer (natural) 5′-FITC-d(GACGGAATATAAGCTGGTGG)- 3′

Template I (for d(S)TTP) 3′-d(CTGCCTTATATTCGACCACC A TGCTGC)- 5′
Template II (for d(S)CTP) 3′-d(CTGCCTTATATTCGACCACC G TGCTGC)- 5′
Template III (for d(S)ATP) 3′-d(CTGCCTTATATTCGACCACC T TGCTGC)- 5′
Template IV (for d(S)GTP) 3′-d(CTGCCTTATATTCGACCACC C TGCTGC)- 5′

d(*S*) NTP	K_m (μM)	V_{max} (% min^{-1})	efficiency V_{max}/K_m (% min^{-1} M^{-1})	relative efficiency
		(A) Therminator		
dTTP	0.044 ± 0.03	0.36 ± 0.03	8.1 × 10^6	100
d*S*TTP	0.011 ± 0.013	0.12 ± 0.03	11 × 10^6	140
dCTP	0.18 ± 0.03	0.28 ± 0.06	1.6 × 10^6	100
d*S*CTP	0.25 ± 0.01	0.27 ± 0.04	1.1 × 10^6	69
dATP	0.042 ± 0.021	0.24 ± 0.06	5.7 × 10^6	100
d*S*ATP	0.030 ± 0.022	0.21 ± 0.07	7.0 × 10^6	120
dGTP	0.075 ± 0.014	0.38 ± 0.07	5.1 × 10^6	100
d*S*GTP	0.18 ± 0.04	0.26 ± 0.03	1.8 × 10^6	35
		(B) Klenow Fragment (exo$^-$)		
dTTP	0.15 ± 0.06	0.15 ± 0.02	1.0 × 10^6	100
d*S*TTP	8.5 ± 1.8	0.18 ± 0.03	2.1 × 10^4	2
dCTP	0.077 ± 0.027	0.33 ± 0.05	4.4 × 10^6	100
d*S*CTP	12 ± 6	0.34 ± 0.07	2.9 × 10^4	1
dATP	0.13 ± 0.05	0.33 ± 0.03	2.5 × 10^6	100
d*S*ATP	1.5 ± 0.9	0.19 ± 0.04	1.3 × 10^5	5
dGTP	0.15 ± 0.05	0.47 ± 0.01	3.1 × 10^6	100
d*S*GTP	12 ± 5	0.47 ± 0.02	4.1 × 10^4	1

60 pm vs sulfur 100 pm), bond lengths (C−O 1.428 Å vs C−S 1.839 Å), and torsion angles (C−O−C 114.2° vs C−S−C 94.6°), the steric barrier caused by Phe667 could lead to a loss of recognition of d*S*NTPs by the Klenow fragment (exo$^-$). Meanwhile, Yang et al. have studied the structure of RB69 DNA polymerase, which belongs to family B.[40] They have shown that the equivalent position of Phe667 in the Klenow fragment (exo$^-$) is substituted with asparagine (Asn564). The amide side chain of Asn564 forms a hydrogen bond via a water molecule with the nonbridging oxygen of the β-phosphate of the incoming dNTP, and steric hindrance around the sugar ring of d*S*NTPs has little effect. Since the asparagine is highly conserved around this site in family B DNA polymerases, it should be possible to incorporate d*S*NTPs as a substrate without a decrease in the affinities. These results are consistent with reports from other groups, which show that family B DNA polymerases can incorporate sugar-modified nucleoside 5′-triphosphates better than family A ones do.[1,7,9−17]

As described above, the efficiency of single nucleotide insertion of d*S*NTPs was comparable to that of natural dNTPs when natural primer was used. In order to further evaluate the efficiency of d*S*NTP incorporation, we next carried out similar assays using the natural-thio chimera primer and the thio primer (Table 2). The natural-thio chimera primer contained five consecutive 2′-deoxy-4′-thionucleotides at the 3′-end, and the thio primer consisted of only 2′-deoxy-4′-thionucleotides. As a result, the relative efficiencies of d*S*NTP incorporation significantly decreased when the natural-thio chimera primer was used. Interestingly, the relative efficiencies of d*S*NTP incorporation improved when the thio primer was used, except in the case of d*S*CTP. Thus, the relative efficiencies followed the trend of natural primer > thio primer > natural-thio chimera primer. These results suggest that the elongation of d*S*NTPs by using Therminator is relatively slow in the initial phase and recovers in a later phase after incorporation of several nucleotides (all of the information on steady-state kinetic parameters are presented in Supplementary Table S1).

As described earlier, a large number of sugar-modified nucleoside triphosphates have been prepared, and their single nucleotide insertion, followed by primer extension, has been investigated extensively. In many cases, the relative efficiencies of single nucleotide insertion are comparable to that of natural dNTP when natural primer is used. However, the efficiencies

Table 2. Steady-State Kinetic Parameters for Insertion of Single Nucleotides into Template-Natural, Natural-Thio, and Thio Primer Duplexes by Therminator DNA Polymerase[a]

Primer (natural) 5′-FITC-d(GACGGAATATAAGCTGGTGG)- 3′
Primer (natural-thio) 5′-FITC-d(GACGGAATATAAGCTGGTGG)- 3′
Primer (thio) 5′-FITC-d(GACGGAATATAAGCTGGTGG)- 3′

Template I (for d(S)TTP) 3′-d(CTGCCTTATATTCGACCACC A TGCTGC)- 5′
Template II (for d(S)CTP) 3′-d(CTGCCTTATATTCGACCACC G TGCTGC)- 5′
Template III (for d(S)ATP) 3′-d(CTGCCTTATATTCGACCACC T TGCTGC)- 5′
Template IV (for d(S)GTP) 3′-d(CTGCCTTATATTCGACCACC C TGCTGC)- 5′

d*S*NTP	primer	K_m (μM)	V_{max} (% min^{-1})	efficiency V_{max}/K_m (% min^{-1} M^{-1})	Relative efficiency
d*S*TTP	natural	0.011 ± 0.013	0.12 ± 0.03	11 × 10^6	100
	natural-thio	0.11 ± 0.09	0.025 ± 0.010	2.2 × 10^5	2
	thio	0.092 ± 0.036	0.25 ± 0.01	2.7 × 10^6	25
d*S*CTP	natural	0.25 ± 0.01	0.27 ± 0.04	1.1 × 10^6	100
	natural-thio	0.034 ± 0.017	0.014 ± 0.002	4.0 × 10^5	36
	thio	1.7 ± 0.12	0.35 ± 0.01	2.1 × 10^5	20
d*S*ATP	natural	0.030 ± 0.022	0.21 ± 0.07	7.0 × 10^6	100
	natural-thio	0.12 ± 0.07	0.021 ± 0.005	1.8 × 10^5	3
	thio	0.041 ± 0.016	0.15 ± 0.01	3.6 × 10^6	51
d*S*GTP	natural	0.18 ± 0.04	0.26 ± 0.03	1.8 × 10^6	100
	natural-thio	0.054 ± 0.017	0.043 ± 0.019	8.0 × 10^5	44
	thio	0.067 ± 0.001	0.11 ± 0.00	1.7 × 10^6	94

[a]Underlined sequences indicate 4′-thio nucleotides.

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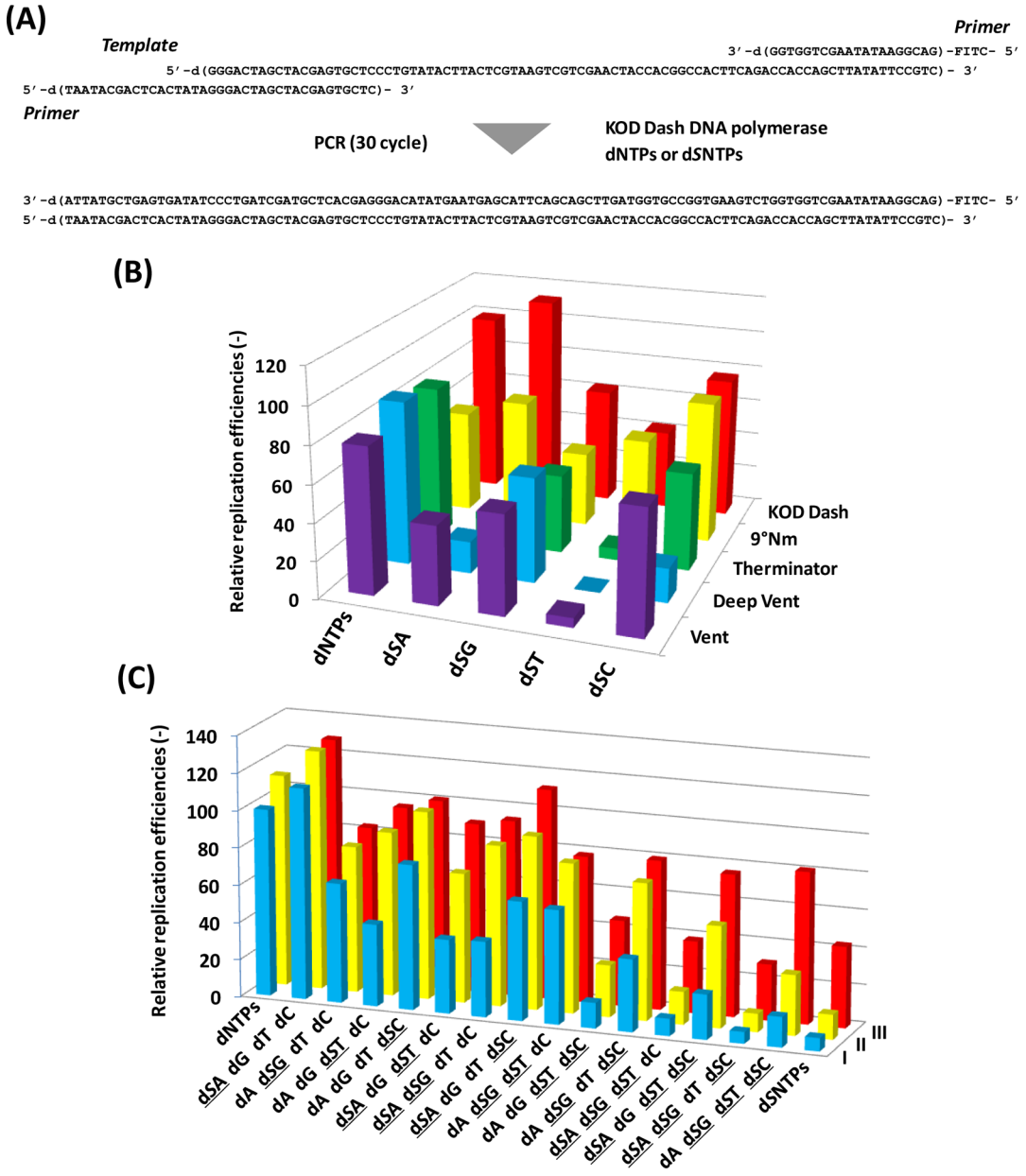

Figure 3. PCR in the presence of d*S*NTPs. (A) Sequences of template and primers for PCR. (B) Comparison of the yields of PCR products using KOD Dash, Vent, Deep Vent, Therminator, 9°Nm, and Pfu DNA polymerase in the presence of all natural dNTPs or dNTPs containing one kind of d*S*NTPs. *Y*-axis indicates the replication yields relative to that KOD Dash DNA polymerase in the presence of natural dNTPs. (C) Relative replication efficiencies of PCR. *Y*-axis indicates the replication yields relative to that with natural dNTPs in case of condition I. Reactions were performed in 20 μL of KOD Dash buffer, 0.5 μM primers, 0.2 mM corresponding d(*S*)NTPs, and 0.1 pmol of DNA template containing 1 U of KOD Dash DNA polymerase (**I**), 2 U of KOD Dash DNA polymerase (**II**), or 2 U of KOD dash DNA polymerase and 2% DMSO (**III**). The extension time for the elongation in PCR was 30 s (**I** and **II**) or 10 min (**III**).

are remarkably lower if the primer already includes modified nucleotide unit(s) on its 3′-end. This is similar to our single nucleotide insertion using a natural-thio chimera primer, and these data agree with those of other sugar-modified nucleoside triphosphates.[1,10] It is not clear why the relative efficiencies of d*S*NTP incorporation are higher when a thio primer is used. Further investigations to explain these results are needed and are being performed.

Optimization of the Conditions for the PCR with d*S*NTPs. We next studied PCR amplification of 4′-thioDNA containing all four kinds of 2′-deoxy-4′-thionucleosides in the presence of d*S*NTP(s). In our previous communication,[32] we have reported the PCR amplification with d*S*TTP and d*S*CTP in the presence of dATP and dGTP using KOD Dash DNA polymerase. The PCR amplification to give partially modified 4′-thioDNAs under the conventional conditions (condition I: primer extension at 72 °C for 30 s, 1 U of KOD Dash DNA polymerase) in the presence of one kind of d*S*NTP using a variety of PCR enzymes was further investigated with additional d*S*ATP and d*S*GTP. The template DNA and primers used in this experiment were natural oligonucleotides. The sequences of the 87-mer template and primers used to afford the 104-mer amplified dsDNA are shown in Figure 3A. The PCR products were quantified by measuring the fluorescence intensities of the corresponding bands. When Vent, Deep Vent, or Therminator DNA polymerase was used, PCR was inefficient in the presence

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of dSTTP. On the other hand, 9°Nm or KOD Dash DNA polymerases afforded the desired 4′-thioDNA regardless of the conditions (Figure 3B). Since KOD Dash DNA polymerase afforded the best results in the above experiments, further conditions, i.e., in the presence of more than two dSNTPs, were examined. The amount of PCR products remarkably decreased with an increase in the ratio of dSNTPs (Figure 3C, condition I). In order to improve PCR efficiency, we first increased the amount of the polymerase. As a result, some improvement was observed when the amount of the enzyme was increased from 1 U to 2 U (condition II: primer extension at 72 °C for 30 s, 2 U of KOD Dash DNA polymerase). However, the incorporations were insufficient especially in the case where all dNTPs were substituted to dSNTPs even under condition II. Therefore, we tested the addition of DMSO[41] or betaine,[42] which are known to be effective additives for PCR, or Mn^{2+}, which is known to increase the tolerance of polymerases for the incorporation of nucleotides with modifications in the furanose ring, to the reaction mixtures.[43] However, no significant improvement was observed when betaine or Mn^{2+} was added to the reaction mixture, whereas the addition of DMSO slightly improved the incorporation of dSNTPs. Accordingly, we increased the extension time for PCR from 30 s to 10 min to improve the incorporation of dSNTPs. Finally, the optimum conditions for PCR in the presence of dSNTPs were determined to be the following: primer extension at 72 °C for 10 min, 2 U of KOD Dash DNA polymerase, and 2% DMSO in the reaction mixture (condition III). The yield of the full-length PCR product was 43.3% when all four kinds of dSNTPs were used under condition III relative to that when only dNTPs were used under condition I (Figure 3C and Supplementary Figure S1; 13.4% under condition I and 7.1% under condition II, respectively).

In order to check the accuracy of the PCR in the presence of dSNTPs, the resulting PCR products from all four kinds of dSNTPs were sequenced. The modified PCR products were first converted into natural dsDNA by using PCR with dNTPs because 4′-thioDNA could not be directly subjected to the sequencing reaction (Supplementary Figure S2). We found that the dSNTPs were incorporated during the PCR cycle without losing the sequence information of the template. As described earlier, there is only one example in which PCR has been carried out in the presence of all four modified dNTPs (α-PS-dNTPs).[18] In that experiment, however, the sequence analysis of the resulting highly modified PCR product showed that there was a large deletion in the central region of the amplified fragment, indicating that incorporation of phosphorothioate dNTPs with a phosphorothioate template was inefficient. Accordingly, the results of PCR experiments is of great value because this is the first report on PCR amplification of unnatural oligomer using all four kinds of unnatural triphosphates with high accuracy.

Conclusion. In summary, we systematically analyzed the incorporation of dSNTPs using DNA polymerases. Primer extension assays using a natural DNA template indicated that family B DNA polymerases effectively incorporated dSNTPs, while families A and Y DNA polymerases and RT did not. Using KOD dash DNA polymerase, sufficient amounts of PCR product, that is, a highly modified 4′thioDNA, was obtained even in the presence of only dSNTPs by optimizing the conditions. Sequence analysis of the resulting PCR product after conversion into the natural dsDNA revealed that replication of 4′-thioDNA took place with a high accuracy. Since this is the first example of accurate PCR amplification of highly modified DNA in the presence of only unnatural dNTPs, the resulting 4′-thioDNA would be a good candidate for not only chemically modified aptamers but also synthetic genetic polymers.

METHODS

Synthesis of dSNTPs. All information on the organic synthesis is in the Supporting Information; analytical data of dSATP and dSGTP follows:

2′-Deoxy-4′-thioadenosine 5′-Triphosphate (dSATP). ^{1}H NMR (D_2O) δ 8.68 (s, 1 H), 8.28 (s, 1 H), 6.30 (t, 1 H, J = 6.6 Hz), 4.83 (m, 1 H), 4.35−4.23 (m, 2 H), 3.80 (m, 1 H), 2.74 (m, 2 H); ^{31}P NMR (D_2O) δ −6.3, −11.0, −21.8; ESI-LRMS m/z 505.9608 $[M\text{-}H]^-$; ESI-HRMS calcd for $C_{10}H_{15}N_5O_{11}P_3S$ $[M\text{-}H]^-$; 505.9702, found 505.9697.

2′-Deoxy-4′-thioguanosine 5′-Triphosphate (dSGTP). ^{1}H NMR (D_2O) δ 8.21 (s, 1 H), 6.13 (t, 1 H, J = 6.5 Hz), 4.80 (m, 1 H), 4.29 (m, 2 H), 3.76 (m, 1 H), 2.75 (m, 1 H), 2.63 (m, 1 H); ^{31}P NMR (D_2O) δ −5.3, −10.6, −19.9; ESI-LRMS m/z 521.9624 $[M\text{-}H]^-$; ESI-HRMS calcd for $C_{10}H_{15}N_5O_{12}P_3S$ $[M\text{-}H]^-$; 521.9651, found 521.9624.

Primer Extension. For the screening of DNA polymerase, the following enzymes were used: Taq (Takara), M-MLV (Takara), KOD Dash (TOYOBO), Vent (exo$^-$) (NEB), Deep Vent (exo$^-$) (NEB), Therminator (NEB), Klenow fragment (exo$^-$) (Promega), Tth (Promega), Tfl (Promega), Tli (Promega), Pfu (Promega), AMV (Promega), and Dpo4 (Trevigen). Annealed 5′-FITC labeled primer (0.8 μM) and template I (1.0 μM) mixture, dSNTP (40 μM), and DNA polymerases (0.01−0.4 U/μL) in a reaction buffer (25 μL) were incubated at 37 or 74 °C. After 10 min, 10-μL aliquots of the reaction mixture were sampled and added into the loading solution (10 μL) containing 10 M urea, 5 mM EDTA, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene cyanol. The samples were analyzed by 20% denaturing polyacrylamide gel electrophoresis (PAGE) containing 8 M urea, which was visualized and analyzed with a FLA-2000 (FUJIFILM).

Single Nucleotide Insertion and Steady-State Kinetics. Insertion reactions were initiated by adding dNTP (0.01−10 μM) or dSNTP (0.1−5 μM) to the reaction mixture containing a mixture of duplex consisting of 5′-FITC-labeled primer (0.8 μM) and DNA template I−IV (1.0 μM), 0.02 U/μL of the Klenow Fragment (exo$^-$) in a buffer containing 50 mM Tris-HCl (pH 7.2), 10 mM $MgSO_4$, and 0.1 mM DDT, or 0.003 U/μL of Therminator DNA polymerase in a ThermoPol buffer. Reaction time was adjusted for each combination to give extents of reaction of 25% or less. The reaction was quenched by adding 8 μL of the stop buffer. The extents were analyzed by 20% denaturing PAGE containing 8 M urea, which was quantitated by a FLA-2000 (FUJIFILM). Reaction velocities were calculated as the yield of reaction divided by reaction time. The kinetic parameters (K_m and V_{max}) were determined by linear regression analysis of a Hanes−Woolf plot with an average of three independent experiments.

PCR with dSNTP. For the first screening of DNA polymerase for PCR, KOD Dash, Vent (exo$^-$), Deep Vent (exo$^-$), Therminator, and 9°Nm were used. The PCR was performed in 20 μL of corresponding buffer containing DNA polymerase (0.05 U/μL), 87-mer DNA template (5.0 nM), 200 μM each of dNTPs or dSNTPs, and 0.5 μM primers. KOD DNA polymerase was utilized for the further optimization. The PCR mixture was prepared in a total volume of 20 μL by

adding 2 μL of 10× KOD DNA polymerase buffer, KOD Dash DNA polymerase (condition I ; 0.05 U/μL, conditions II and III ; 0.10 U/μL), 87-mer DNA template (5.0 nM), 200 μM each of dNTPs or dSNTPs, 0.5 μM primers, and 2% DMSO (only in condition III). The reaction mixtures were gently vortexed and then amplified using a thermal cycler (TaKaRa). PCR was performed under the condition I, II [94 °C, 15 s; (94 °C, 15 s; 50 °C, 15 s; 72 °C, 30 s) × 30 cycles; 72 °C, 5 min] or condition III [94 °C, 15 s; (94 °C, 15 s; 50 °C, 15 s; 72 °C, 10 min) × 30 cycles; 72 °C, 5 min]. PCR products were analyzed by 6.4% PAGE and were quantified by measuring the fluorescence intensities of the corresponding bands with a Molecular Imager FX Pro (Bio-Rad). Prior to a second PCR with dNTPs, the products were purified with High Pure PCR Product Purification Kit (Roche). After the second PCR with dNTPs, the products were repurified with the kit (Roche) and then were sequenced with each of PCR primer by a ABI PRISM 3100 Genetic Analyzer.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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