Published online July 19, 2006

3476–3483 *Nucleic Acids Research, 2006, Vol. 34, No. 12*
doi:10.1093/nar/gkl491

Synthesis and properties of 4′-ThioDNA: unexpected RNA-like behavior of 4′-ThioDNA

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Received May 23, 2006; Revised June 24, 2006; Accepted June 26, 2006

ABSTRACT

The synthesis and properties of fully modified 4′-thioDNAs, oligonucleotides consisting of 2′-deoxy-4′-thionucleosides, were examined. In addition to the known literature properties (preferable hybridization with RNA and resistance to endonuclease hydrolysis), we also observed higher resistance of 4′-thioDNA to 3′-exonuclease cleavage. Furthermore, we found that fully modified 4′-thioDNAs behaved like RNA molecules in their hybridization properties and structural aspect, at least in the case of the 4′-thioDNA duplex. This observation was confirmed by experiments using groove binders, in which a 4′-thioDNA duplex interacts with an RNA major groove binder, lividomycin A, but not with DNA groove binders, to give an increase in its thermal stability. Since a 4′-thioDNA duplex competitively inhibited the hydrolysis of an RNA duplex by RNase V_1, it was not only the physical properties but also this biological data suggested that a 4′-thioDNA duplex has an RNA-like structure.

INTRODUCTION

Synthetic short oligonucleotides (ONs), especially RNA molecules, are assuming an increasingly important role in chemistry because of their potential utility in antisense, ribozyme, RNA aptamer and RNA interference applications. Thus far, a variety of modified nucleoside units have been synthesized and incorporated into ONs with the aim of developing useful applications (1,2). Among the nucleoside derivatives developed, sugar-modified nucleosides, including 2′-*O*-alkylated nucleosides, 2′,4′-bridged nucleosides and 2′-deoxy-2′-fluoronucleosides, seem to be the most promising units for developing functional ONs because the corresponding ONs show high hybridization properties and nuclease resistance. In our group, we have been working on developing 4′-thionucleic acids as functional ONs. These ONs consist of 4′-thionucleosides, a different mode of sugar-modified nucleoside analogue from the above mentioned sugar-modified nucleosides (3–7). We have already reported the synthesis and properties of 4′-thioRNA, which is made up of 4′-thioribonucleosides (8–11). Since 4′-thioRNAs exhibited high hybridization ability, nuclease resistance and structural similarity to A-form RNA duplexes, applications for isolating the 4′-thioRNA aptamers by SELEX and developing modified short interfering RNAs containing 4′-thioribonucleosides are currently under study (12–14). As part of our research project, we became interested in the properties of 4′-thioDNAs, which consist of 2′-deoxy-4′-thionucleosides (Figure 1). Although Walker and his coworkers had investigated the synthesis and properties of 4′-thioDNA, the ONs prepared were only partially modified with 2′-deoxy-4′-thiopyrimidine nucleosides (4–7). However, their preliminary results showing high hybridization to the complementary RNA and endonuclease (nuclease S1) resistance of 4′-thioDNA seemed promising for the development of functional ONs (6). Thus while carrying out further investigations on 4′-thioDNAs, we discovered some unexpected properties of 4′-thioDNA.

Herein we describe the first synthesis of fully modified 4′-thioDNAs consisting of four kinds of 2′-deoxy-4′-thionucleosides, the practical synthesis of which was recently reported by us (15), and the properties of these 4′-thioDNAs, including unexpected RNA-like behavior in hybridization and structural aspects, based on CD spectra and enzymatic recognition.

MATERIALS AND METHODS

General Materials and Methods

Experimental procedures and physical data for phosphoramidite units and CPG supports of 2′-deoxy-4′-thionucleosides

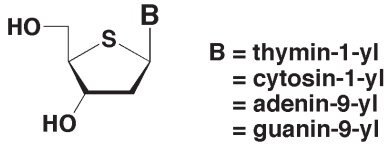

Figure 1. Structure of 2′-deoxy-4′-thionucleosides.

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were given in Supplementary Data. DNase I was purchased from TaKaRa. SVPD was from MP Biomedicals, Inc. RNase V_1 was from Ambion. [γ-^{32}P]ATP was purchased from PerkinElmer. DNA1, DNA2, DNA3, DNA4 and DNA5 were purchased from SIGMA Genosys.

Synthesis of 4′-thioDNA

Support bound 4′-thioDNAs were synthesized on an Applied Biosystem 3400 DNA synthesizer using 2′-deoxy-4′-thionucleoside phosphoramidite units at a 1.0 μmol scale following the standard procedure. Each of phosphoramidite units was used at a concentration of 0.1 M in dry acetonitrile, and the coupling time was extended to 10 min for each step. After completion of the synthesis, the CPG support was treated with concentrated NH_4OH at 55°C for 16 h, and the support was filtered off. The filtrate was concentrated and the oligodeoxynucleotide protected by a DMTr group at the 5′ end was chromatographed on a C-18 silica gel column with a linear gradient of acetonitrile (from 5 to 40%) in 0.1 N triethylammonium acetate (TEAA) buffer (pH 7.0). The fractions were combined and concentrated. The residue was treated with aqueous acetic acid (70%) for 15 min at room temperature. The solution was concentrated, and the residue was purified on reversed phase high performance liquid chromatography (HPLC), using a J'sphere ODS-M80 column (4.6 × 150 mm; YMC) with a linear gradient of acetonitrile (from 5 to 25%) in 0.1 N TEAA buffer (pH 7.0). The structure of each thioDNA was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass (MALDI-TOF/MS) spectrometry on a Voyager-DE pro. thioDNA1, calculated mass: $C_{146}H_{184}N_{55}O_{73}P_{14}S_{15}$ 4788.5 (M–H), observed mass: 4789.2; thioDNA2, calculated mass: $C_{147}H_{184}N_{57}O_{73}P_{14}S_{15}$ 4828.5 (M–H), observed mass: 4833.0; thioDNA3, calculated mass: $C_{116}H_{146}N_{46}O_{58}P_{11}S_{12}$ 3835.4 (M–H), observed mass: 3833.5; thioDNA4, calculated mass: $C_{150}H_{180}N_{75}O_{58}P_{14}S_{15}$ 4872.6 (M–H), observed mass: 4872.5; thioDNA5, calculated mass: $C_{150}H_{195}N_{30}O_{88}P_{14}S_{15}$ 4737.4 (M–H), observed mass: 4738.5.

UV melting experiment

Thermally induced transitions were monitored at 260 nm on a Beckman DU 650 spectrophotometer. Samples were prepared as follows.

(i) Duplex formation: a solution containing an appropriate oligonucleotide and a complementary sequence (3 μM each) in a buffer of 10 mM sodium cacodylate (pH 7.0) containing 100 mM NaCl was heated at 90°C for 5 min, then cooled gradually to room temperature and used for the thermal denaturation study. The sample temperature was increased at a rate of 0.5°C/min.

(ii) Effect of distamycin A for thermal stability: a solution containing an appropriate oligonucleotide, a complementary sequence (3 μM each) and distamycin A (3 μM) in a buffer of 10 mM sodium cacodylate (pH 7.0) containing 100 mM NaCl was heated at 90°C for 5 min, then cooled gradually to room temperature and used for the thermal denaturation study. The sample temperature was increased at a rate of 0.5°C/min.

(iii) Effect of methyl green or lividomycin A for thermal stability: a solution containing an appropriate oligonucleotide, a complementary sequence (3 μM each) and methyl green or lividomycin A (4.3 μM) in a buffer of 2.5 mM sodium cacodylate (pH 7.0) containing 25 mM NaCl was heated at 90°C for 5 min, then cooled gradually to room temperature and used for the thermal denaturation study. The sample temperature was increased at a rate of 0.5°C/min.

CD measurements

CD spectra were obtained at 25°C on a Jasco J720. The solution containing samples in a buffer of 10 mM Na cacodylate (pH 7.0) containing 100 mM NaCl was prepared, and the sample spectra were subtracted from the buffer spectrum. The molar ellipticity was calculated from the equation $[\theta] = \theta/cl$, where θ is the relative intensity, c the sample concentration and l the cell path length in centimetres.

Assay for DNase I stability

Each DNA sample labeled with ^{32}P at the 5′ end (at 0.5 μM) was mixed with the corresponding unlabeled DNA (at 50 μM). The DNA sample was incubated in DNase I buffer supplemented with DNase I (0.05 U/μl) at 37°C. At each time point, 2 μl aliquots of the reaction mixture were taken, and the reactions were terminated by addition of 8 μl loading buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, 7 M urea, 0.05% bromophenol blue and 0.05% xylene cyanol). Samples were subjected to electrophoresis on 20% polyacrylamide gel containing 7 M urea. After electrophoresis, the radioactivity of each band was estimated by a Bio-imaging analyzer (Bas 2500, Fuji Co., Ltd). In the case of duplex stability, DNA1:DNA2 and thioDNA1:thioDNA2 were used in the experiment.

Assay for SVPD stability

Each DNA sample labeled with ^{32}P at the 5′ end (at 0.5 μM) was mixed with the corresponding unlabeled DNA (at 25 μM). The DNA sample was incubated in a buffer (40 mM Tris–HCl, 8 mM $MgCl_2$ and 5 mM DTT, pH 7.5) supplemented with SVPD (3.4 mU/μl) at 37°C. As in the case of the assay for DNase I stability, aliquots of the reaction mixture were analyzed.

Assay for human serum stability

Each DNA sample labeled with ^{32}P at the 5′ end (at 0.5 μM) was mixed with the corresponding unlabeled DNA (at 50 μM). The DNA sample was incubated in phosphate-buffered saline (PBS) supplemented with 90% human serum at 37°C. As in the assay for DNase I stability, aliquots of the reaction mixture were analyzed.

Inhibition assay of DNase I digestion

DNA1 labeled with ^{32}P at the 5′ end (at 0.025 μM) was mixed with the corresponding unlabeled DNA1 (at 0.25 μM). The DNA sample was incubated in DNase I buffer supplemented with thioDNA1 (at 0.5 μM) or without thioDNA1. Then DNase I (0.013 U/μl) was added at 37°C. As in the assay for DNase I stability, aliquots of the reaction mixture were analyzed. In the case of the experiment for the duplex, DNA1:DNA2 was used as a substrate and thioDNA3 as an inhibitor.

5′-d(AGTCCGAATTCACGT)-3′ : DNA1 and thioDNA1
3′-d(TCAGGCTTAAGTGCA)-5′ : DNA2 and thioDNA2
5′-r(AGUCCGAAUUCACGU)-3′ : RNA1
3′-r(UCAGGCUUAAGUGCA)-5′ : RNA2

5′-d(CGCGAATTCGCG)-3′ : DNA3 and thioDNA3
5′-r(CGCGAAUUCGCG)-3′ : RNA3

5′-d(AAAAAAAAAAAAAAA)-3′ : DNA4 and thioDNA4
3′-d(TTTTTTTTTTTTTTT)-5′ : DNA5 and thioDNA5
5′-r(AAAAAAAAAAAAAAA)-3′ : RNA4
3′-r(UUUUUUUUUUUUUUU)-5′ : RNA5

Figure 2. Sequences of DNA, 4′-thioDNA and RNA.

Table 1. Thermal stability of duplexes and effect of distamycin A, methyl green and lividomycin A[a]

Duplex	UV melting	Distamycin A	Methyl green	Lividomycin A
	T_m(°C)	ΔT_m(°C)	ΔT_m(°C)	ΔT_m(°C)
DNA1:DNA2	55.7 ± 0.2	9.5	4.0	1.3
RNA1:RNA2	66.2 ± 0.2	0.2	0.9	10.2
thioDNA1:thioDNA2	65.2 ± 0.3	0.2	1.0	4.4
RNA1:DNA2	51.6 ± 0.2	−0.1	0.6	10.3
thioDNA1:DNA2	48.3 ± 0.2	3.1	1.5	3.2
thioDNA1:RNA2	64.6 ± 0.2	0.7	1.0	6.1
DNA3	44.3 ± 0.7	14.4	3.6	1.5
RNA3	63.2 ± 0.2	−0.3	1.0	16.7
thioDNA3	56.5 ± 1.5	1.1	1.8	5.3
DNA4:DNA5	38.0 ± 0.5	2.5	N.D.[b]	N.D.[b]
RNA4:RNA5	29.3 ± 0.3	0.4	−0.6	1.9
thioDNA4:thioDNA5	24.5 ± 0.3	0.4	0.1	1.5

[a]Errors reflect standard deviation from three independent experiments.
[b]No obvious transition was observed.

Inhibition assay of RNase V_1 digestion

RNA1 labeled with ^{32}P at the 5′ end (at 0.025 μM) was mixed with the corresponding unlabeled RNA1:RNA2 (at 0.25 μM). The RNA sample was incubated in RNase V_1 buffer supplemented with DNA3 (or thioDNA3) (at 0.5 μM), or without DNA3 (or thioDNA3). Then RNase V_1 (0.5 mU/μl) was added at 37°C. As in the case of the assay for DNase I stability, aliquots of the reaction mixture were analyzed. The kinetic analyses of the reactions were obtained using Hanes–Woolf plots. The analyzed concentrations of each RNA1:RNA2 was 0.075, 0.1 and 0.25 μM. The concentration of each inhibitor (DNA3 or thioDNA3) was 0.3 μM.

RESULTS AND DISCUSSION

Duplex stability and structural aspects of 4′-thioDNAs

The phosphoramidite units and CPG supports for 4′-thioDNA synthesis were prepared according to the usual method, which is given in the Supplementary Data. We first synthesized a series of ONs1 (DNA1, thioDNA1 and RNA1) followed by a series of the complementary ONs2 (DNA2, thioDNA2 and RNA2) (Figure 2). These sequences are the same as those used in our previous study for 4′-thioRNA (11). Thermal stabilities of the complementary duplexes were measured

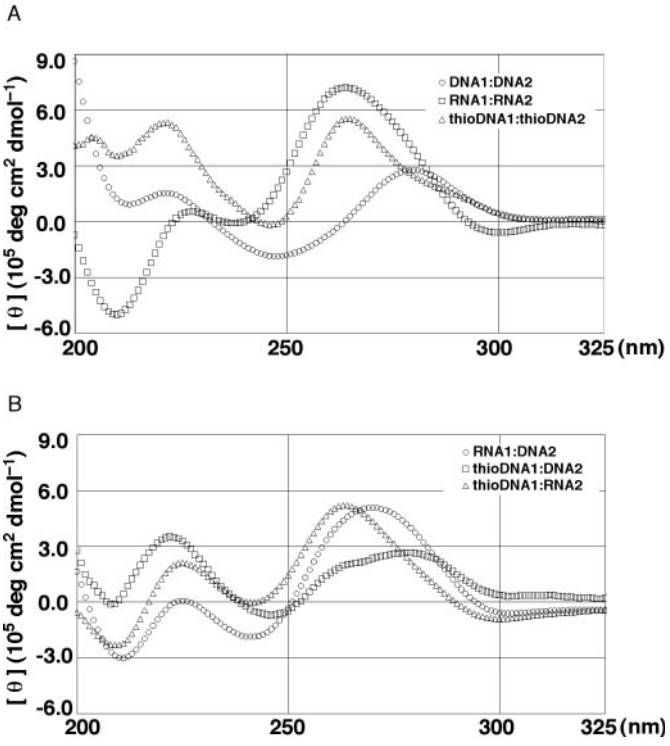

Figure 3. CD spectra of duplexes consist of ON1:ON2.

by ultraviolet melting experiments in a buffer of 10 mM sodium cacodylate (pH 7.0) containing 100 mM NaCl (Table 1) (thermodynamic parameters measured by differential scanning calorimetry are presented in the Supplementary Data). Homoduplexes consisting of natural DNA and RNA, namely DNA1:DNA2 and RNA1:RNA2, had T_m values of 55.7 ± 0.2 and 66.2 ± 0.2°C, respectively. A duplex of thioDNA1:thioDNA2 showed a higher T_m value (65.2 ± 0.2°C) than that of DNA1:DNA2, and it was similar to that of RNA1:RNA2. T_m measurements were also carried out for heteroduplexes. The heteroduplex RNA1:DNA2 (T_m = 51.6 ± 0.2°C) was less thermally stable than the corresponding homoduplexes (DNA1:DNA2 and RNA1:RNA2). When RNA1 was changed to thioDNA1, the corresponding T_m value was nearly the same (thioDNA1:DNA2 = 48.3 ± 0.2°C) as that of the RNA1:DNA2 heteroduplex. In contrast, thioDNA1 formed a thermally stable duplex with RNA2 (thioDNA1:RNA2) to give a T_m value of 64.6 ± 0.2°C, which is similar to that of RNA1:RNA2. The overall order of thermal stabilities for these six duplexes was RNA1:RNA2 ≈ thioDNA1:thioDNA2 ≈ thioDNA1:RNA2 > DNA1:DNA2 > RNA1:DNA2 ≈ thioDNA1:DNA2. These results led us to believe that 4′-thioDNA may behave as an RNA-like molecule despite the absence of the 2′-hydroxyl groups.

To confirm our speculation, CD spectra of each duplex were measured. As can be seen in Figure 3A, the duplex DNA1:DNA2 showed a typical B-form spectrum (having a positive band near 280 nm), while that of RNA1:RNA2 was characteristic of an A-form spectrum (having a positive band near 260 nm). The CD spectrum of thioDNA1:thioDNA2 had a positive band near 260 nm though a small shoulder was observed near 280 nm, and

thus an A-form of the duplex was suggested. Figure 3B shows the CD spectra of the heteroduplexes, RNA1:DNA2, thioDNA1:DNA2 and thioDNA1:RNA2. The CD spectrum of RNA1:DNA2 was characteristic of an A-like form, while thioDNA1:DNA2 did not show an obvious spectral pattern, and seemed to adopt an intermediate form between the A- and B-forms. When DNA2 was substituted for RNA2, a spectrum of thioDNA1:RNA2 convincingly exhibited the A-form.

In the CD spectra of nucleic acids, spectra at the longer wavelengths will be affected by the number of bases per helical turn and the inclination of the bases with respect to the helical axis (16). The results discussed above focused on the spectra at the longer wavelengths and suggested an RNA-like behavior of 4′-thioDNA in the stacking mode of the nucleobases. On the other hand, spectra at the shorter wavelengths, which represent the conformation of the phosphate–sugar backbone (17), were fairly scattered in each duplex. Since 4′-thioDNA consists of 2′-deoxy-4′-thionucleosides, in which the furanose ring oxygen was substituted by a sulfur atom, the conformational change of the phosphate-sugar backbone is not surprising. Walker and his coworkers reported CD spectra and crystal structures of ONs partially containing 4′-thiothymidine (4,18). Their results indicate that the ONs fundamentally form a B-shaped helix and differ from our observation. However these results come from partial modification (only one or two residues) of the self-complementary 12mer DNA sequence, and not a full modification, as in our case. Therefore, we next investigated the interaction between the duplex and some groove binders.

Groove binders recognize and bind tightly to their own favorite helical structures of nucleic acid duplexes (19,20). Therefore, the topology of the helix grooves could possibly be determined by an examination of the interaction with the groove binders. As specific groove binders, commercially available distamycin A (a DNA minor groove binder that recognizes an AATT sequence), methyl green (a DNA major groove binder) and lividomycin A (an RNA major groove binder) were chosen (no selective RNA minor groove binder is known). The differences between T_m values (ΔT_ms) in the presence and absence of the groove binder are listed in Table 1. The DNA groove binders increased the T_m values of DNA1:DNA2 by 9.7 (for distamycin A) and 4.0°C (for methyl green), respectively, while no obvious change of the T_m value was observed in the presence of the RNA major groove binder, lividomycin A ($\Delta T_m = 1.3$°C). On the other hand, lividomycin A, but not distamycin A and methyl green, solely increased the T_m value of RNA1:RNA2 by 10.2°C. These results indicated that each binder distinguished the topology of each groove in its sequence context. When the experiment was carried out for thioDNA1:thioDNA2, the DNA binders did not increase the T_m values, while lividomycin A increased the T_m value by 4.4°C. Although the degree of thermal stabilization was smaller than that with RNA1:RNA2, the interaction of thioDNA1:thioDNA2 with the groove binders was similar to that of RNA1:RNA2, but not that of DNA1:DNA2. We carried out further experiments using the groove binders for the heteroduplexes. The T_m values of RNA1:DNA2 were not changed in the presence of the DNA binders, while lividomycin A increased the T_m value by 10.3°C, as in the case of RNA1:RNA2. The CD spectrum suggested that RNA1:DNA2 adopted an A-like form and thus the topology of this heteroduplex would tend to interact with the RNA groove binder. The results obtained with thioDNA1:DNA2 are somewhat ambiguous. The heteroduplex seemed to bind with both distamycin A and lividomycin A, although the interactions were rather small. Since the CD spectrum of thioDNA1:DNA2 (Figure 3B) showed intermediate characteristics between the A- and B-forms, these results may very well agree with its structural aspect, as suggested by the CD spectrum. For the interaction with thioDNA1:RNA2 which gave the A-form spectrum, only lividomycin A increased the T_m value by 6.1°C.

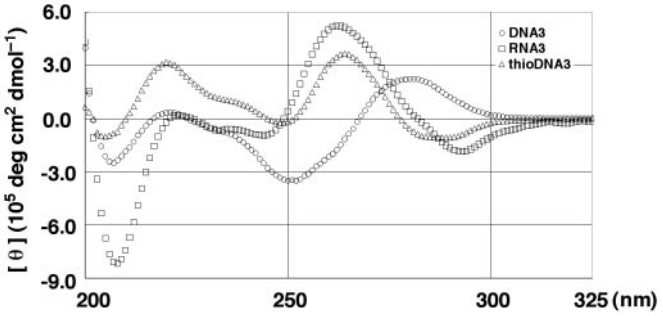

Figure 4. CD spectra of self-complementary duplexes consist of ON3.

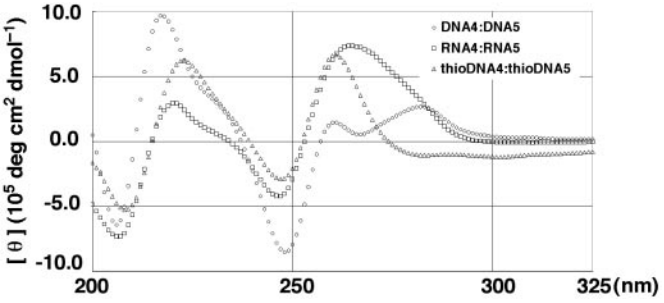

Figure 5. CD spectra of duplexes consist of ON4:ON5.

To further confirm the RNA-like behavior of 4′-thioDNA, we prepared a series of ONs3 consisting of a self-complementary Dickerson sequence (DNA3, thioDNA3 and the corresponding RNA3), and a series of homosequences ONs4 and ONs5 (Figure 2). The results of the T_m measurements and the groove binder experiments are also shown in Table 1, and their CD spectra are shown in Figures 4 and 5, respectively. For the series ONs3, thioDNA3, like RNA3, formed a more thermally stable self complementary duplex than DNA3, and the resulting duplex showed a preference for interacting with the RNA groove binder, lividomycin A (Table 1). As can be seen in Figure 4, the CD spectrum of thioDNA3 closely resembled that of RNA3 showing an A-form structure. Differing from the results of the series of ONs3, the duplex consisting of DNA4:DNA5 showed a T_m of 38.0 ± 0.5°C, while the corresponding RNA4:RNA5 formed a less stable duplex (T_m of 29.3 ± 0.3°C). In this sequence, however, the T_m value of thioDNA4:thioDNA5, like RNA4:RNA5, was decreased relative to that of DNA4:DNA5. Although the experiment using groove binders showed no significant enhancement of T_m

Table 2. Enzymatic stability of single- and double-stranded DNA and 4′-thioDNA[a,b]

Nuclease	Half life DNA1	thioDNA1	DNA1:DNA2	thioDNA1:thio DNA2
DNase I	1.5 ± 0.1 min	not digested[c]	3.3 ± 1.2 min	not digested[d]
SVPD	2.8 ± 0.4 min	>8 h	N.D.[e]	N.D.[e]
90% human serum	40 ± 1.3 min	190 ± 12 min	N.D.[e]	N.D.[e]

[a]Errors reflect standard deviation from three independent experiments.
[b]The results of PAGE analysis are presented in the Supplementary Data.
[c]The experiment was carried out for up to 12 h.
[d]The experiment was carried out for up to 24 h.
[e]Not determined.

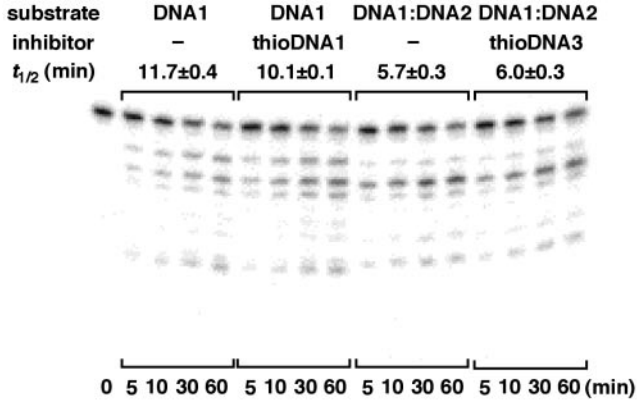

Figure 6. Inhibition assay of DNase I digestion. Errors reflect standard deviation from three independent experiments.

values, neither DNA4:DNA5 and RNA4:RNA5 nor thioDNA4:thioDNA5, the CD spectrum of thioDNA4:thioDNA5 was markedly different from that of DNA4:DNA5, and rather similar to that of RNA4:RNA5 (Figure 5). Since duplexes consisting of poly[dA]:poly[dT] are known to adopt a different helical structure from the typical B-form (21), the results obtained in the groove binder experiments may not be so surprising.

Nuclease sensitivity of 4′-thioDNAs

Walker and his coworkers reported nuclease sensitivity of a 4′-thiothymidine-containing ON separated by one unmodified residue at the 3′ end using snake venom phosphodiesterase (SVPD; a 3′-exonuclease) and nuclease S1 (an endonuclease). According to their report, the modified ON showed high resistance to hydrolysis by nuclease S1, while no significant resistance to hydrolysis by SVPD (6). Since these results came from a poly-T sequence, in which the ON also contained a natural thymidine unit on its 3′ end and therefore may have been unsuitable for the investigation of degradation by SVPD the nuclease sensitivity was reinvestigated using a fully modified 4′-thioDNA. The nuclease sensitivity of 4′-thioDNA was examined using DNase I, an endonuclease hydrolyzing both single- and double-stranded DNA, SVPD and 90% human serum. The DNA1 and thioDNA1 were labeled at the 5′ end with ^{32}P and incubated under appropriate conditions in the presence of nuclease or serum (see Materials and Methods). The reactions were then analyzed by PAGE under denaturing conditions (see Supplementary Data), and the resulting half-lives ($t_{1/2}$s) calculated based on the ratio of full-length ON at each time point are listed in Table 2. The single-stranded thioDNA1 was completely intact in the presence of DNase I for up to 12 h under conditions in which DNA1 was readily hydrolyzed ($t_{1/2}$ = 1.5 ± 0.1 min), and these results agreed with those previously reported for nuclease S1 (6). In addition, the thioDNA1:thioDNA2 duplex was also intact in the presence of the same enzyme, while the DNA1:DNA2 duplex afforded a $t_{1/2}$ of 3.3 ± 1.2 min. For sensitivity to SVPD, our experiment afforded different results from the previous data (6). The thioDNA1 was slowly hydrolyzed by SVPD ($t_{1/2}$ = >8 h) under conditions in which the DNA1 was readily hydrolyzed ($t_{1/2}$ = 2.8 ± 0.4 min). This result may come from the difference between the fully modified thioDNA1 and that containing one natural thymidine unit on its 3′ end. On further investigation of sensitivity in 90% human serum, the thioDNA1 was hydrolyzed by the 3′-exonuclease (see Supplementary Data) (22) and $t_{1/2}$ was calculated to be 190 ± 12 min. Under the same conditions, the DNA1 afforded a $t_{1/2}$ of 40 ± 1.3 min, and thus, it can be concluded that the 4′-thioDNA showed significant resistance to both endonuclease(s) and exonuclease(s).

We have suggested that the 4′-thioDNA duplex is structurally closer to an RNA duplex than to a DNA duplex. As mentioned above, the 4′-thioDNA duplex was completely resistant to DNase I, and this may be attributed to structural differences. Accordingly, we next examined whether the 4′-thioDNA duplex is recognized by DNase I, which would furnish further structural evidence. To that end, an inhibition assay of the nuclease hydrolysis of a DNA duplex by the 4′-thioDNA duplex was carried out. As described above, DNA1:DNA2 was hydrolyzed by DNase I with a $t_{1/2}$ of 5.7 ± 0.3 min (Figure 6). When a 2-fold amount of the thioDNA3 homoduplex, which was used to avoid interstrand exchange, was added as an inhibitor, no obvious elongation of the $t_{1/2}$ value was observed. Similar to the double-stranded ON, the single-stranded thioDNA1 also did not inhibit the hydrolysis of DNA1 ($t_{1/2}$ = 11.7 ± 0.4 min vs. $t_{1/2}$ = 10.1 ± 0.1 min). Contrary to the case of DNase I, the thioDNA1 was slowly hydrolyzed by SVPD (Table 1), and this ON inhibited the SVPD hydrolysis of the DNA1 (data not shown). Weston *et al*. reported the X-ray structure of a DNase I–d(GGTATACC)$_2$ complex, and suggested the importance of the minor groove width and depth of a B-form DNA for their interaction (23). This strict recognition

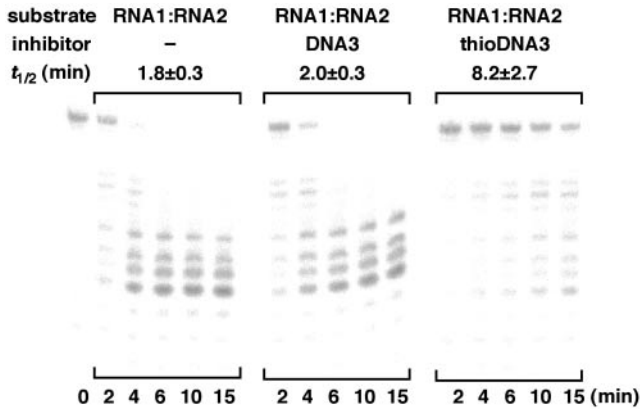

Figure 7. Inhibition assay of RNase V_1 digestion. Errors reflect standard deviation from three independent experiments.

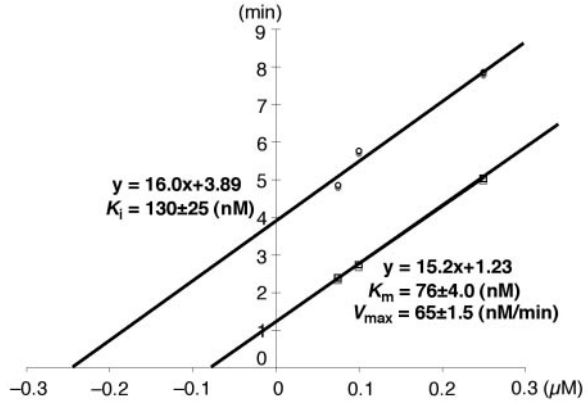

Figure 8. Hanes–Woolf plots of RNase V_1 and RNA1:RNA2 without (square) and with thioDNA3 (circle). Errors reflect standard deviation from three independent experiments.

of the helical structure by DNase I would be responsible for the 4′-thioDNA duplex not being an inhibitor. For the interaction with a single-stranded DNA, it is difficult to define the structure of a single-stranded DNA, and little is known of the interaction with either DNase I or SVPD. In addition, SVPD hydrolyzes not only single-stranded DNA analogs but also RNA analogs (24). Therefore, a detailed discussion must await further structural evidence between these nucleases and the single-stranded ONs.

Since the inhibition assay using DNase I showed that the conformation of 4′-thioDNA duplex differed from the usual B-form structure, we next investigated the interaction with RNase V_1, which is an RNA duplex-specific endonuclease (25). As shown in Figure 7, the duplex of RNA1:RNA2 was readily hydrolyzed by RNase V_1, and the $t_{1/2}$ was calculated to be 1.8 ± 0.3 min. When a 2-fold amount of the DNA3 homoduplex was added as an inhibitor, no obvious inhibition of the RNA duplex hydrolysis was observed. This result indicates that RNase V_1 does not recognize the B-form DNA duplex as the substrate. In contrast, addition of the thioDNA3 homoduplex apparently inhibited the RNA hydrolysis and the $t_{1/2}$ was elongated to 8.2 ± 2.7 min. Under the same reaction conditions, the thioDNA3 homoduplex alone was completely resistant to hydrolysis by RNase V_1 (data not shown). Since the thioDNA3 homoduplex acted as an inhibitor of RNase V_1, which recognizes the RNA duplex, a kinetic study was carried out for the interaction with the enzyme. As shown in Figure 8, K_m and V_{max} values of the RNA1:RNA2 hydrolysis by RNase V_1, analyzed by Hanes–Woolf plots, were estimated to be 76 ± 4.0 nM and 65 ± 1.5 nM/min, respectively. In the presence of the thioDNA3 duplex as an inhibitor, the secondary plot afforded the same slope as that with no inhibitor, and thus it was concluded that the thioDNA3 duplex is a competitive inhibitor of RNase V_1. The K_i value of the thioDNA3 duplex was estimated to be 130 ± 25 nM. Since the 4′-thioDNA duplex has some affinity to RNase V_1, but not to DNase I, this too suggests an RNA-like behavior of the 4′-thioDNA duplex in its interaction with the enzyme. Although the X-ray structure of RNase V_1 has not been solved, Auron *et al*. suggested that the enzyme interacts with the minor groove of the RNA duplex (26). Accordingly, the shape of the minor groove of the 4′-thioDNA duplex would be close to that of the RNA duplex, while the similarity of the major groove is suggested from the results of the RNA major groove binder, lividomycin A. In addition, Wyatt and Walker suggested that interaction of RNase V_1 with a 2′-hydroxyl group in RNA is necessary for efficient cleavage but not for binding (27). Our results indicating that the 4′-thioDNA duplex was not hydrolyzed by RNase V_1 and that it inhibited this enzyme competitively with RNA duplex are in good agreement with their previous report.

CONCLUSION

In this article, we have presented the first preparation of fully modified 4′-thioDNAs consisting of four kinds of 2′-deoxy-4′-thionucleosides. In addition to the known literature properties (preferable hybridization with RNA and resistance to endonuclease digestion), we pointed out that 4′-thioDNA also exhibits higher resistance to 3′-exonuclease hydrolysis. Furthermore, we found that fully modified 4′-thioDNA behaved as an RNA-like molecule in its hybridization properties and structural aspects, at least in the 4′-thioDNA:4′-thioDNA homoduplex. The experiments using groove binders and nucleases also supported this unexpected behavior. It is known that the B-form DNA duplex is forced to change to the A-form helical structure under dehydrating conditions (16), while our results suggested that the 4′-thioDNA duplex adopted the A-form in aqueous buffer at neutral pH and moderated salt conditions. The most typical change of the 4′-thioDNA is the substitution of the furanose ring oxygens (termed O4′) by the more hydrophobic sulfur atoms (termed S4′). Since the O4′ oxygens play a critical role in stabilizing minor groove hydration spines via a series of hydrogen bonds (28), the substitution of S4′ sulfurs would disturb this type of hydration, and give a dehydrated environment. Consequently, the 4′-thioDNA duplex may adopt an A-form in aqueous buffer conditions. Thus far, oligonucleotide N3′ → P5′phosphoramidates (29) and ONs containing 3′-methylene backbones (3′-methylene phosphonate backbone, methylene methylimino backbone and amide backbone) (30–32) composed of 2′-deoxyfuranose-based nucleosides are known RNA-like molecules. The nucleoside

units of those modified ONs prefer the 3′-endo sugar conformation in solution (31,33), while that of 4′-thioDNA adopts a 2′-endo sugar conformation in solution and crystals (34). It is nevertheless worth noting that, despite such structural differences in nucleoside units, 4′-thioDNA can adopt an RNA-like conformation. In addition, since 4′-thioDNA possesses the phosphodiester linkages and is available via standard ON synthesis, 4′-thioDNA should be a new type of RNA-like molecule. Further investigation into its structural aspects and the applications of 4′-thioDNA are in progress.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors would like to thank Ms Y. Misawa (Hokkaido University) for technical assistance and Ms S. Oka [Center for Instrumental Analysis, Hokkaido University) for technical assistance with the manuscript. This article constitutes part 242 of Nucleosides and Nucleotides [for part 241 in this series, see Ref. (35)]. This work was supported in part by Grant-in-Aid for Scientific Research from the Japan Society for Promotion of Science (No. 15 209 003 and No. 18 109 001). Funding to pay the Open Access publication charges for this article was provided by the Japan Society of Promotion of Science.

Conflict of interest statement. None declared.

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