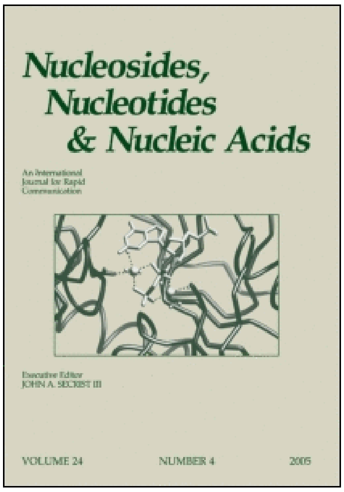

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SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDE BEARING 2'-*S*-ALKYL RESIDUE AND ITS EFFECT ON THE DUPLEX STABILITY

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ABSTRACT: 2'-Deoxy-2'-*S*-hexyluridine derivative was synthesized from 2,2'-anhydrouridine and 1-hexanethiol and incorporated into an oligodeoxyribonucleotide. The thermal stability of the duplexes formed by the 2'-*S*-hexyl modified ODN with either the complementary DNA or RNA strand was decreased compared to the unmodified counterparts.

There is growing interest in the use of modified oligodeoxyribonucleotides (ODNs) in antisense methods and for the structural study of nucleic acids. Many kinds of modified ODNs have been reported to date and several functional groups were incorporated into ODNs, so as to locate the group in the major or minor groove of helixes. Recently, modification of the sugar 2'-position, which was located in the minor groove of a double helix, has received much attention as an improved second-generation antisense compound.[1] The 2'-modified oligonucleotides promote increase in duplex stability and nuclease resistance.[2] In addition, the 2'-position of the sugar moiety was used for introducing functional groups such as reactive groups,[3] fluorescence groups,[4] lipophilic pendants,[5] or intercalating groups.[6]

Most of the modified groups were bound to the 2'-position of the sugar through an ether bond. However, there has been no report of the 2'-modified group bound through a thioether bond on DNA. It was reported that the sugar conformation depends on the 2'-substituent group and the electronegativity of the substituent is proportional to the ratio of 3'-endo and 2'-endo conformations.[7] Since the electronegativity of sulfur is smaller than that of oxygen, the proportion of 3'-endo conformation will be smaller.[8] The synthesis

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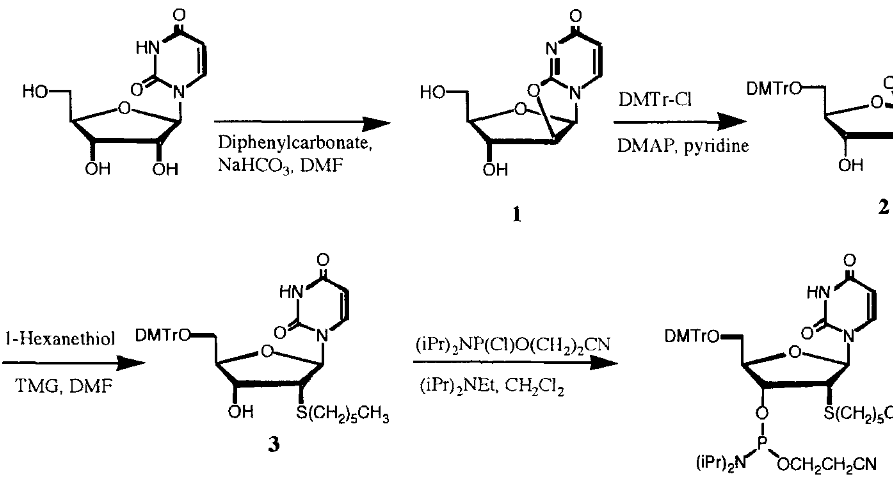
and conformation of 2'-*S*-methyl substituted derivatives were described by Fraser *et al.*[9] and they expressed the 2'-*S*-methyl nucleoside preferred a high 2'-endo conformation. However, the effect of an alkylthio group at the 2' position on the duplex stability has not been reported.

2'-Modification by a nucleophilic opening of an anhydro nucleoside was attempted in this study. This chemistry was reported in the early and pioneering studies of Brown *et al.*[10] and Divakar and Reese subsequently reported the synthesis of 2'-deoxy-2'-*S*-alkyluridine by the reaction of 2,2'-anhydrouridine with alkanethiol.[11] We applied this chemistry to the synthesis of a 2'-modified nucleoside. Here, we report the synthesis of 2'-*S*-hexyl-2'-deoxyuridine and its incorporation into ODN and the thermodynamic properties of the duplexes of the ODN with complementary oligodeoxyribonucleotide or oligoribonucleotide.

RESULTS AND DISCUSSION



Scheme

DMTr=dimthoxytrityl, DMAP=dimethylaminopyridine
TMG=tetramethylguanidine

2,2'-Anhydrouridine (**1**) was prepared by the treatment of uridine with diphenyl carbonate and sodium bicarbonate by the previously described methods.[12] The 2,2'-anhydrouridine reacted with 4,4'-dimethoxytrityl chloride in pyridine containing dimethylaminopyridine to protect the 5'-OH group (yield of 5'-dimethoxytrityl-2,2'-anhydrouridine, **2**; 85%). Compound **2** was allowed to react with 1-hexanethiol in DMF containing *N,N,N',N'*-tetramethylguanidine (TMG). This reaction proceeded and 5'-dimethoxytrityl-2'-deoxy-2'-*S*-hexyluridine **3** was obtained in 44% yield. The obtained **3** was converted to a protected nucleoside 3'-phosphoramidite derivative (**4**) by the reaction with chloro-(2-cyanoethyl)-diisopropylaminophosphine in the usual manner in moderate

TABLE 1. Tm's and thermodynamic parameters of 2'-modified- and unmodified ODN (ODN-2S and N-ODN) with DNA[a)] or RNA[b)]

	Tm /°C	ΔTm /°C	ΔH /kJ mol^{-1}	ΔΔH /kJ mol^{-1}	ΔS /J mol^{-1} K^{-1}	ΔΔS /J mol^{-1} K^{-1}
N-ODN/DNA	50.6		-496 (-500)		-1420 (-1440)	
ODN-2S/DNA	44.9	-5.7	-441 (-447)	55	-1270 (-1290)	150
N-ODN/RNA	51.2		-400		-1120	
ODN-2S/RNA	46.7	-4.5	-371	29	-1040	80

Melting curve was measured at 2 μM of ODN(each strand) in 150 mM sodium chloride/10 mM sodium phosphate (pH 7.0) for DNA/DNA duplex and in 150 mM sodium chloride/10 mM sodium phosphate (pH 7.0) containing 40 μM EDTA for DNA/RNA duplex. Rate of increasing temperature, 0.5 °C/min. The error in Tm values is estimated to be ± 0.5 °C. Thermodynamic parameters were calculated from a curve-fitting method as described in the text.

a) Complementary DNA sequence; 5'-TAG GCA TCT CCT ATG-3'

b) Complementary RNA sequence; 5'-UAG GCA UCU CCU AUG-3'

* The values in parentheses show the thermodynamic parameters determined by Tm^{-1} *vs.* log(C_T/4) plot, where C_T was total oligonucleotide concentration.

yield (51%). The 2'-modified nucleoside analog **4** was incorporated into ODN 15mer (5'-CAT AGG AGA XGC CTA-3', X = 2'-deoxy-2'-*S*-hexyluridine) on a DNA synthesizer. The coupling yield of **4** was 90% even in longer coupling time, 360 sec (for normal nucleoside phosphoramidite, >98%, 60 sec). These moderate yields for the phosphitylation and coupling reaction are probably due to the steric hindrance of a 2' substituent group in the nucleoside analog. The synthesized ODNs were purified by reversed-phase HPLC and gel filtration chromatography.

UV melting curves of the duplexes of the 2'-modified ODN 15mer (**ODN-2S**) with a complementary DNA or RNA strand were measured and the melting behavior was compared with that of the duplex of the corresponding unmodified strand (**N-ODN**). The results are summarized in TABLE 1. Their thermodynamic parameters were determined by the curve fitting for each melting curve, using a nonlinear, latest squares calculation program "TMSPEC", developed by Kodama *et al.* .[13] We confirmed that the thermodynamic parameters obtained from this program are consistent with those determined from Tm^{-1} *vs.* log(C_T/4) plots for the DNA/DNA duplexes (shown in parentheses in TABLE 1). [14] The introduction of the 2'-*S*-hexyl group decreased the Tm values of both ODN/DNA and ODN/RNA duplexes. This destabilization is attributable to both enthalpic and entropic contributions. This result suggests that local conformational

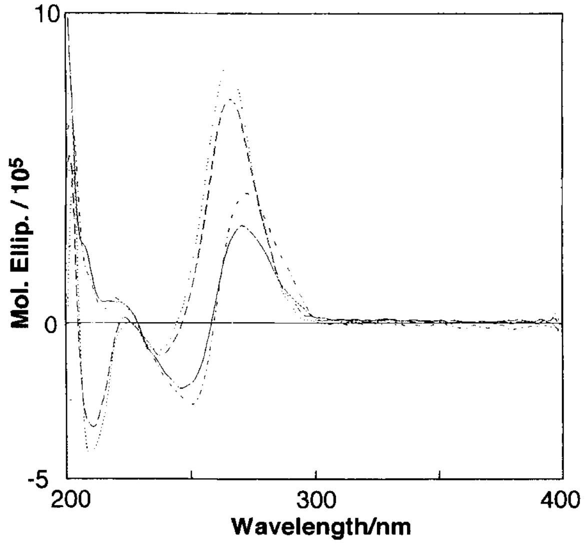


FIG. 1. CD spectra of unmodified and modified ODN duplexes. Solid line, **N-ODN**/DNA; dot-dash line, **ODN-2S**/DNA; broken line, **N-ODN**/RNA; dotted line, **ODN-2S**/RNA. Measurement conditions are the same as in TABLE 1 except that the measurement temperature was 20 °C.

change (H-bond deformation) and the displacement of the bound water in the minor groove give rise to the destabilization of the duplexes.

The CD spectra for the unmodified and the modified ODN duplexes formed with DNA or RNA are shown in FIG. 1. Spectra for both ODN/DNA duplexes are characteristic of B-form helices and the spectra for both ODN/RNA duplexes are characteristic of A-form like helices. The modification of ODN at the 2'-position shows slightly enhanced Cotton effects for ODN/DNA and ODN/RNA.

To know the sugar puckering of 2'-deoxy-2'-*S*-hexyluridine, its NMR study was carried out in deuterated methanol. The ratio 3'-endo (*N* conformer) and 2'-endo (*S* conformer) was calculated by the method of Altona and Sundaralingam[15] from the coupling constants $J_{1'2'}$ and $J_{3'4'}$. The obtained ratio is 20 : 80 for 3'-endo : 2'-endo of 2'-deoxy-2'-*S*-hexyluridine. A previous report by Uesugi *et al.* indicated that the ratio of the 3'-endo increases linearly with the electronegativity of the 2'-substituent. The 2'-sulfur substituent leads to a decrease of the 3'-endo conformer compared with the 2'-hydroxyl substituent, because sulfur has smaller electronegativity than oxygen. 2'-Endo conformation may destabilize the A-helix of the DNA/RNA heteroduplex, where the nucleosides are predominantly in 3'-endo form. In this study, the 2'-modified ODN/RNA duplex is largely destabilized (ΔTm = -4.5 °C) compared with 2'-*O*-substitution in a previous paper[16] which reported that the average ΔTm per substitution of 2'-*O*-nonyl is ca. -2 °C and that of 2'-*O*-pentyl is ca. -0.9 °C. This large destabilization is due to the unsuitable sugar conformation for A- helixes in addition to the steric hindrance of the alkyl group. On the other hand, the ODN-2S/DNA duplex is also largely destabilized (ΔTm = -5.7 °C) compared with the unmodified DNA duplex although the sugar conformation (2'-endo) is suited for the B-helix of the DNA duplex. This is likely caused by the large steric hindrance of the 2'-substituent in the narrow minor groove of the B-helix of the DNA duplex compared with that of A form-like helix of DNA/RNA duplex.

In conclusion, we have synthesized an uridine derivative bearing an *S*-hexyl group at the 2'-position by the nucleophilic addition to 2,2'-anhydrouridine, and the 2'-modified ODN was prepared by the incorporation of 2'-deoxy-2'-*S*-hexyluridine during the chemical synthesis of ODN. The alkylthio modification affected both enthalpic and entropic contributions to duplex stability . This relatively large negative effect on duplex formation will be due to the predominant 2'-endo sugar conformation of 2'-deoxy-2'-*S*-hexyluridine in addition to steric constraints in the minor groove.

EXPERIMENTAL SECTION

General. Thin-layer chromatography (TLC) was performed on Kieselgel 60F$_{254}$ (Art. 5554, E. Merck). Silica-gel column chromatography was performed on Silica gel 60 (63-200 μm, Merck). High-performance liquid chromatography (HPLC) was carried out on a Wakosil 5C18 column (4 mmϕ x 250 mm, Wako). The eluent was a linear gradient from 2.1% to 37.1% acetonitrile in 50 mM triethylammonium acetate (TEAA, pH 7.0) within 35 min for the unprotected ODNs. ^{1}H NMR spectra and ^{31}P NMR spectra were obtained with a Varian Gemini 200 or a JEOL α-500 spectrometer. ^{1}H NMR spectra were recorded relative to internal tetramethylsilane and ^{31}P NMR spectra were recorded relative to external 85% H_3PO_4. Mass spectra were measured by a Perkin Elmer Sciex API-100 instrument in ESI mode. Oligodeoxyribonucleotides were synthesized by phosphoramidite chemistry on an Applied Biosystems 381A DNA synthesizer. An oligoribonucleotide was purchased from Genset Corp. and purified by a Mono Q HR5/5 anion-exchange column. Normal nucleoside phosphoramidites were purchased from Glen Research. Chloro-(2-cyanoethoxy)-diisopropylaminophosphine[17] and 2,2'-anhydrouridine[11] (**1**) were prepared by the described method. All other reagents were purchased from Wako or Kanto Chemical Co.. All organic solvents for reactions were dried and distilled in the usual manner.

5'-*O*-(4,4'-Dimethoxytrityl)-2,2'-anhydrouridine (2). Compound **1** (1.070 g, 4.73 mmol) was reacted with 4,4'-dimethoxytrityl chloride (DMTr-Cl, 3.23 g, 9.54 mmol) in pyridine containing 4-dimethylaminopyridine (15 mg, 0.12 mmol) overnight at room temperature. The reaction mixture was poured into 5% aq. sodium bicarbonate solution and extracted with dichloromethane. After the organic layer was dried with anhydrous sodium sulfate, the solvent was removed by evaporation and coevaporation with toluene. The product was purified by silica-gel column chromatography using 5% methanol in dichloromethane containing 0.5% triethylamine as an eluent. The appropriate fractions were collected, evaporated, and precipitated with a small amount of dichloromethane into hexane to give **2** (white precipitates). Yield of **2** was 2.10 g (83.9%). ^{1}H NMR ($CDCl_3$) δ 7.34-6.75 (m, 14H, H6 and Ar), 6.16 (d, J=5.8 Hz, 1H, H1'), 5.96 (d, J=7.5 Hz, 2H, H5), 5.31 (d, J=5.8 Hz, 1H, H2'), 4.47-4.39 (m, 2H, H3' and H4'), 3.75 (s, 6H, CH_3O-), 3.12-2.92 (m, 2H, H5'); Mass (ESI-MS) *m/z* 527.0 ([M - H]$^-$, 527.18 calcd for $C_{30}H_{27}N_2O_7$).

5'-*O*-(4,4'-Dimethoxytrityl)-2'-deoxy-2'-*S*-hexyluridine (3). 1-Hexanethiol (0.572 g, 4.84 mmol) and *N,N,N',N'*-tetramethylguanidine (TMG, 0.556 g, 4.83 mmol) were added to a solution of **2** (0.512 g, 0.967 mmol) in DMF (4 mL) under N_2 atmosphere and the reaction mixture was stirred at 100 °C for 3h. Ethyl acetate was added to the reaction mixture; the solution was washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The residue was dissolved in a small amount of CH_2Cl_2; the solution was added dropwise to hexane, and the resulting precipitate was collected by filtration. The crude product was purified by silica gel column chromatography using 4% methanol in dichloromethane containing 0.5% triethylamine as an eluent to give **3** in 44% yield. ^{1}H NMR ($CDCl_3$) δ 8.42 (s, 1H, imido-N^{3}H), 7.82 (d, J=8.2 Hz, 1H, H6), 7.36 - 6.83 (m, 13H, Ar), 6.07 (d, J=7.9 Hz, 1H, H1'), 5.35 (d, J=8.2 Hz, 1H, H5), 4.39 (m, 1H, H3'), 4.19 (m, 1H, H4'), 3.80 (s, 6H, -O-CH_3), 3.54 (m, 1H, H2'), 3.50 (m, 2H, H5'), 2.92 (d, J=3.1 Hz, 1H, 3'-OH), 2.63 (t, J=7.5 Hz, 2H, -S-CH_2-), 1.25 - 1.63 (m, 8H, -$(CH_2)_4$-), 0.88 (t, J=7.0 Hz, 3H, CH_3). Mass (ESI-MS) m/z 645.6 ([M - H]$^-$, 645.27 calcd for $C_{36}H_{41}N_2O_7S$. Anal. Found: C, 65.78; H, 6.57; N, 4.38. Calcd for $C_{36}H_{42}N_2O_7S$: C, 66.85; H, 6.54; N, 4.33.

3'-*O*-[(2-Cyanoethyl)(diisopropylamino)]phosphino-5'-*O*-(4,4'-dimethoxy-trityl)-2'-deoxy-2'-*S*-hexyluridine (4). Chloro-(2-cyanoethoxy)-diisopropylaminophosphine (489 mg, 2.07 mmol) was added dropwise to a solution of **3** (268 mg, 0.414 mmol) in dry dichloromethane (5 mL) containing *N*-ethyl-*N,N*-diisopropylamine (433 μL, 2.48 mmol) under Ar atmosphere at room temperature. After this mixture was stirred for 3 h, dry methanol was added to the reaction mixture and the reaction mixture was poured into cold ethyl acetate (pre-washed with 5% aq. sodium hydrogencarbonate solution). The solution was washed with 5% aq. sodium hydrogencarbonate solution and water, dried with anhydrous sodium sulfate, and evaporated to dryness. The crude product was purified by silica-gel column chromatography using 3% methanol in dichloromethane containing 1% triethylamine as an eluent. The appropriate fractions were collected, evaporated, and precipitated with a small amount of dichloromethane into hexane to give precipitates. Yield of **4** was 0.198 g (51.2%). ^{1}H NMR ($CDCl_3$) δ 8.19 (s, 1H, imido-N^{3}H), 7.86, 7.81 (d, *J*=8.1 Hz, 1H, H6), 7.38 - 6.83 (m, 13H, Ar), 6.20 (t, *J*=7.1 Hz, 1H, H1'), 5.32, 5.29 (d, *J*=8.1 Hz, 1H, H5), 4.63 (m, 1H, H3'), 4.29, 4.21 (m, 1H, H4'), 4.03-3.69 (m, 2H, -CH_2-O-(βCN)), 3.80 (s, 6H, -O-CH_3), 3.63-3.41 (m, 5H, H2', H5', -CH- (iPr)), 2.66, 2.40 (t, *J*=6.1 Hz, 2H, -S-CH_2-), 2.60 (m, 2H, NC-CH_2-), 1.59 - 1.04 (m, 18H, -$(CH_2)_3$-, -CH_3 (iPr)), 0.87 (t, *J*=7.0 Hz, 3H, CH_3). ^{31}P NMR ($CDCl_3$) δ 150.65 and 151.23 ppm.

Oligodeoxyribonucleotide Synthesis. Oligodeoxyribonucleotides and their analogs were prepared using normal phosphoramidite coupling procedure on a DNA synthesizer. The oligodeoxyribonucleotides bearing the 2'-substituted 2'-thiouridine in place of thymidine were synthesized along with the normal and complementary oligodeoxyribonucleotides. The modified nucleoside phosphoramidite was incorporated into the oligodeoxyribonucleotides at the appropriate position by using the normal synthetic cycle except that the reaction time for the coupling step was 6 min. The deprotection and cleavage from CPG support was carried out by the treatment with conc. aq. ammonia solution at 55 °C for 13h. All modified oligodeoxyribonucleotides with 5'-(4,4'-dimethoxytrityl) group were isolated by reversed-phase HPLC on a Wakosil 5C18 column (4 mmϕ x 250 mm length) using 100 mM TEAA (pH 7.0) with a gradient of acetonitrile concentration. The isolated compound was treated with 10% acetic acid by the usual procedure to remove a 4,4'-dimethoxytrityl group followed by desalting on a Sephadex G-25 column. The modified oligodeoxyribonucleotides were further purified by reversed-phase HPLC after deprotection of the 5'-(4,4'-dimethoxytrityl) group. Isolated yields: **ODN-2S**, 10%; **N-ODN**, 29%; **C-ODN**, 57%. **C-ODN** is a complementary strand (DNA) to **N-ODN**.

Tm Measurements. UV absorbance was measured with a Hitachi UV-3000 spectrophotometer equipped with a Hitachi Temperature Controller SPR-10. The solution temperature in a cuvette was measured directly with a temperature data collector AM-7002 (Anritsu Meter Co., Ltd.). Absorbance and temperature data were recorded on NEC personal computer PC-9821. The rate of heating or cooling was 0.5 °C/min. Tm values were obtained in 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride for the duplex with DNA or in 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride and 0.01 mM EDTA for the duplex with RNA at a duplex concentration of 2 x 10^{-6} M.

CD Spectra. CD spectra were recorded on a JASCO J-720 spectropolarimeter equipped with a PTC-343 temperature controller, using the same solution for the Tm measurements at 20 ℃.

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