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SYNTHESIS OF OLIGONUCLEOTIDES WITH 6-FORMYL-2'-*O*-METHYLURIDINE AND THERMAL STABILITY OF THEIR DUPLEXES

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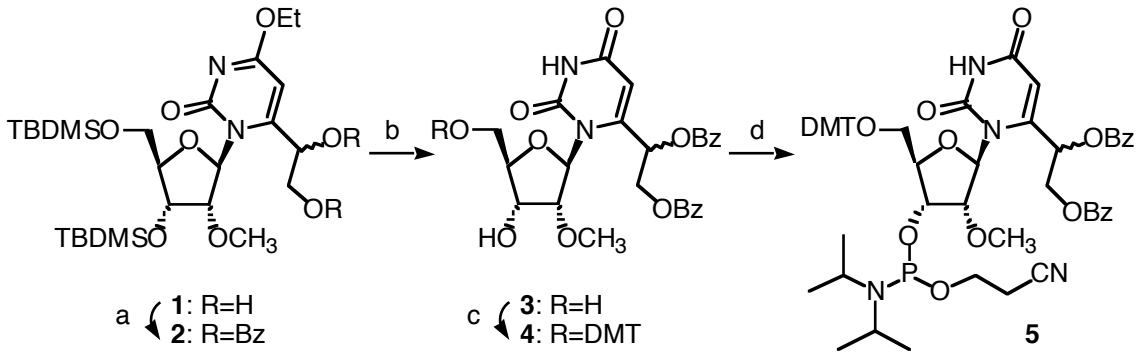
Abstract - Single 6-formyl-2'-*O*-methyluridine or 2'-*O*-methyluridine was introduced into oligonucleotides (9-23 mers), which have simple base-sequences, as a substituent for thymidine to compare the ability of duplex formation and the thermal stability of the resulting duplexes.

The 5-methyl group of thymidine exists in the major groove of DNA, and a modification at C5 of 2'-deoxyuridine and its incorporation into oligomers is a useful design target for the study of DNA-protein interactions.[1-3] However, the introduction of 6-substituted pyrimidine nucleosides into oligonucleotides has rarely been studied. We recently succeeded in introducing a 6-formyl-2'-*O*-methylcytidine unit into an oligonucleotide duplex (23 mers) containing the specific Myb binding sequence, 3'-TTGAC-5', based on computer modeling, as a substituent for the second thymidine in the binding sequence, to study the interaction between the 6-formyl group of the oligonucleotide and the guanidino group of Arg-190 in Myb.[4] Through the investigation of 6-modified pyrimidine oligonucleotides, we were interested in studying the stability of various oligonucleotide duplexes containing 6-

formyluridine derivatives.[5]

Synthesis of the oligonucleotide building block (**5**), which is a precursor of the 6-formyluridine derivative,[4,5] is shown in Scheme 1. Benzoylation of the known vicinal diol (**1**)[4] gave **2** in 94% yield, and the subsequent acidic hydrolysis proceeded in 82% yield to afford the 6-substituted uridine derivative (**3**). Dimethoxytritylation of the 5'-hydroxyl group provided **4** in 78% yield. The building block synthesis of the modified oligonucleotide was completed by 3'-*O*-phosphitylation in 95% yield.[6]



Scheme 1. *Reagents*: (a) BzCl (3 eq), DMAP (5 eq), CH_2Cl_2. (b) 1 M HCl-MeOH (1:10). (c) DMTCl (1.5 eq), py. (d) $[(i\text{Pr})_2\text{N}]_2\text{POCH}_2\text{CH}_2\text{CN}$ (1.8 eq), 4,5-dicyanoimidazole (0.7 eq), CH_2Cl_2.

The nucleoside phosphoramidite unit (**5**) obtained as above was incorporated into the six kinds of oligonucleotide sequences by an automated DNA synthesizer with a trityl-off mode (Scheme 2). After ammonolysis, these oligonucleotides were purified by polyacrylamide urea gel electorophoresis.

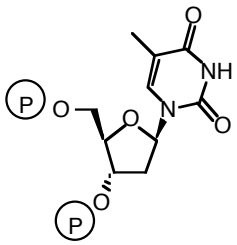
① 5'-CTT TXT TTG-3'
② 5'-CAA AXA AAG-3'
③ 5'-CTT TTT XTT TTT G-3'
④ 5'-TTT TTX TTT T-3'
⑤ 5'-CGC GAA XTC GCG-3'
⑥ 5'-AGA ATG TGT GT**C AGX T**AG GGT GT-3'

Scheme 2. Oligonucleotide sequences containing 6-substituted 2'-*O*-methyluridine at the X position. The strand ⑥ includes the modified Myb binding sequence (bald face), in which the natural sequence is 5'-CAGTT-3'.

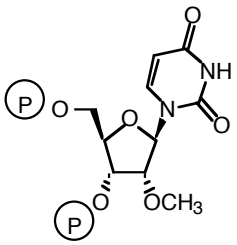
Each oligonucleotide, containing a 6-(1,2-dihydroxy)ethyl-2'-*O*-methyluridine unit, was treated with 100 equivalent of $NaIO_4$ in 50 mM HCO_2NH_4 buffer at 0 °C for 15 min to

produce the 6-formyl group at the X position (Scheme 2).[7] Excess amount of oxidant and inorganic salts were removed by Sep-Pak® (C_{18}) treatment.

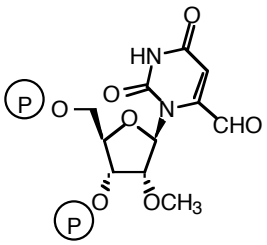
We also prepared the corresponding six oligonucleotides containing 2'-*O*-methyluridine (U1) at the same position (X) in Scheme 2 to evaluate the effect of the 2'-*O*-methyl group itself in the duplex formation. These modified oligomers were annealed with the natural complementary oligonucleotides. In the case of Dickerson-Drew type ⑤, X = U2, and its isomerical strand with 2'-*O*-methyluridine, X = U1, were annealed with themselves. The Tm values of the duplexes were measured in the Tm buffer [6 mM sodium phosphate buffer (pH 6.7), 120 mM NaCl, and 12 mM $MgCl_2$]. The results are shown in Table 1.



T



U1



U2

Table 1. Tm values of synthetic oligonucleotides containing U1, U2, or native T, in the Tm buffer: 6 mM sodium phosphate buffer (pH 6.7), 120 mM NaCl, and 12 mM $MgCl_2$.

duplexes	Tm (°C)	duplexes	Tm (°C)
① 5'-CTT TXT TTG-3'		④ 5'-TTT TTX TTT T-3'	
3'-GAA AAA AAC-5'		3'-AAA AAA AAA A-5'	
X = T	32	X = T	20
X = U1	23	X = U1	19
X = U2	n.d.[a]	X = U2	9
② 5'-CAA AXA AAA-3'		⑤ 5'-CGC GAA XTC GCG-3'	
3'-GTT TAT TTC-5'		3'-GCG CTX AAG CGC-5'	
X = T	28	X = T	64
X = U1	22	X = U1	40
X = U2	n.d.	X = U2	n.d.
③ 5'-CTT TTT XTT TTT G-3'		⑥ 5'-ACACCCT**AACTG**ACACACATTCT-3'	
3'-GAA AAA AAA AAA C-5'		3'-TGTGGGA**TXGAC**TGTGTGTAAGA-5'	
X = T	48	X = T	73
X = U1	41	X = U1	67
X = U2	35	X = U2	66

a) not detected

It has been reported that the Tm values of 2'-*O*-methyl oligonucleotides are almost same as those of natural DNA duplexes.[8] In our experiments using 9 to 23-mers, the Tm value of each oligonucleotide, which contained single 2'-*O*-methyluridine, was lower than the corresponding natural oligonucleotide. After the double modification at the 2'- and 6-positions, this tendency to destabilization intensified. Especially in the Dickerson-Drew dodecamer duplex of ⑤, the existence of the two 6-substituted nucleotide units at the proximal positions in the duplex causes a steep lowering of the Tm value.

In summary, we have demonstrated that oligonucleotide synthesis containing 6-formyl-2'-*O*-methyluridine in the six kinds of sequences (①-⑥) to investigate the thermal stability of their duplexes. In the length of 9 bases and in the Dickerson-Drew type dodecamer, the duplex formation could not be detected. Presumably, 6-formyl-2'-*O*-methyluridine exists in syn conformation in the oligonucleotides,[9] and this phenomenon would depress the stable duplex formation.

EXPERIMENTAL

General Information. ^{1}H NMR (400 MHz) spectra were measured on a JEOL GSX-400 spectrometer and ^{31}P NMR (240 MHz) spectra were measured on a JEOL ECP-600 spectrometer. Chemical shifts were reported in ppm on the d scale relative to the internal standard (Me_4Si) for ^{1}H NMR and the external standard of H_3PO_4 for ^{31}P NMR. MS spectra and HRMS spectra were measured on a JEOL JMS D-300 mass spectrometer in FAB mode (*m*-nitrobenzyl alcohol as a matrix). UV spectra were recorded on a JASCO Ubest-55 or a Shimadzu UV-1600 spectrophotometer. Elemental analyses were carried out in the Microanalytical Laboratory, School of Pharmaceutical Sciences, Showa University. A commercially available hexane solution of BuLi was titrated before use with diphenyl acetic acid in THF. THF was distilled from benzophenone ketyl. Column chromatography was carried out on silica gel (silica gel 60, Merck). TLC was performed on silica gel (precoated silica gel plate F_{254}, Merck). Preparative HPLC was carried out on a Shimadzu LC-6AD with a Shim-pack PREP-SIL(H)•KIT column (2×25 cm). Oligonucleotides were synthesized in the 30 nmol scale with a Beckman Oligo 1000M DNA

Synthesizer by the Beckman standard protocol.

1-[3,5-Bis-*O*-*tert*-butyldimethylsilyl-2-*O*-methyl-β-D-ribo-furanosyl]-6-(1,2-dibenzoyloxyethyl)-4-ethoxy-2-pyrimidinone(2). To the mixture of 1-[3,5-bis-*O*-*tert*-butyldimethylsilyl-2-*O*-methyl-β-D-ribo-furanosyl]-6-(1,2-dihydroxyethyl)-4-ethoxy-2-pyrimidinone[4] (**1**, 581.8 mg, 1.01 mmol) and DMAP (617.0 mg, 5.05 mmol) in CH_2Cl_2 (10 mL), BzCl (0.35 mL, 3.03 mmol) was added at 0 °C, and then the solution was stirred at rt for 1 h. The mixture was partitioned between EtOAc (300 mL) and H_2O (50 mL). The organic layer was washed with saturated aqueous $NaHCO_3$, H_2O, and brine (50 mL each), successively, and dried over Na_2SO_4. Silica gel column chromatography (10-20% EtOAc in hexane) afforded 742.3 mg of **2** as a white foam (94%). A portion of **2** (diastereomer mixture) was separated into two components, **2a** (retention time: t_R 9.9 min) and **2b** (t_R 12.8 min), by HPLC (25% EtOAc in hexane) for physical data. For **2a**: UV (MeOH) λ_{max} 276.5 and 229.5 nm, λ_{min} 255 nm. ^{1}H NMR ($CDCl_3$) δ 8.08 – 8.06 (2H, m), 8.01 – 7.99 (2H, m), 7.62 – 7.54 (2H, m), 7.48 – 7.40 (4H, m), 6.39 (1H, dd, *J* = 3.1 and 7.7 Hz), 6.03 (1H, s), 5.88 (1H, br s), 4.92 (1H, dd, *J* = 3.1 and 12.2 Hz), 4.75 (1H, dd, *J* = 7.7, 12.2 Hz), 4.74 – 4.69 (2H, m), 4.45 – 4.34 (2H, m), 3.98 (1H, m), 3.91 (1H, dd, *J* = 4.5 and 11.4 Hz), 3.74 (1H, dd, *J* = 5.7 and 11.4 Hz), 3.47 (3H, s), 1.32 (3H, t, *J* = 7.1 Hz), 0.93 and 0.84 (18H, each as s), 0.15 and 0.01 (12H, each as s). FAB MS (+NaI) *m/z* 805 $(M+Na)^+$, 767 $(M-Me)^+$, 725 $(M-{}^tBu)^+$. HRMS (FAB) calcd for $C_{40}H_{58}N_2O_{10}NaSi_2$: 805.3531, found: 805.3530. For **2b** UV (MeOH) λ_{max} 282 and 229 nm, λ_{min} 255 nm. ^{1}H NMR δ 8.09 – 8.07 (2H, m), 7.99 – 7.97 (2H, m), 7.62 – 7.54 (2H, m), 7.48 – 7.40 (4H, m), 6.37 (1H, dd, *J* = 3.4 and 6.2 Hz), 6.10 (1H, d, *J* = 5.6 Hz), 6.07 (1H, s), 4.86 (1H, dd, *J* = 3.4 and 12.2 Hz), 4.81 (1H, dd, *J* = 6.2 and 12.2 Hz), 4.79 (1H, t, *J* = 5.6 Hz), 4.56 (1H, dd, *J* = 4.3 and 5.6 Hz), 4.43 – 4.35 (2H, m), 3.99 (1H, dt, *J* = 4.3 and 5.6 Hz), 3.95 (1H, dd, *J* = 5.6 and 10.7 Hz), 3.77 (1H, dd, *J* = 4.3 and 10.7 Hz), 3.38 (3H, s), 1.31 (3H, t, *J* = 7.2 Hz), 0.90 and 0.87 (18H, each as s), 0.14, 0.13, 0.04, and 0.03 (12H, each as s). FAB MS (+NaI) *m/z* 805 $(M+Na)^+$, 725 $(M-{}^tBu)^+$. HRMS (FAB) calcd for $C_{40}H_{58}N_2O_{10}NaSi_2$: 805.3531, found: 805.3530.

2'-*O*-Methyl-6-(1,2-dibenzoyloxyethyl)uridine (3). Compound (**2**) (229.6 mg, 0.293 mmol) was dissolved in MeOH (4 mL) and 1 M HCl (0.40 mL) was added at 0 °C. The solution was stirred at rt for 8 h, and then concentrated *in vacuo*. The residue was purified by silica gel column chromatography (1% MeOH in a EtOAc-hexane (2 : 1) solution) to obtain 126.3 mg of **3** as a colorless wax (82%). This compound, diastereomer mixture, was used without further purification for the next step. ^{1}H NMR ($CDCl_3$) δ 9.23 (1H, br s), 8.08 – 8.05 (2H, m), 8.02 – 7.99 (2H, m), 7.65 – 7.55 (2H, m), 7.50 – 7.40 (4H, m), 6.38 and 6.32 (1H, each as dd, J = 2.5 and 7.3 Hz), 6.10 – 6.06 (1H, each as s), 6.03 (0.4 H, d, J = 6.6 Hz), 5.93 (0.6H, d, J = 4.9 Hz), 4.99 – 4.95 (1H, m), 4.86 (0.4H, t, J = 6.2 Hz), 4.71 – 4.54 (2.6H, m), 4.17 – 4.16 and 4.08 – 4.03 (1H, each as m), 3.90 – 3.84 (1H, m), 3.77 – 3.72 (1H, m), 3.54 and 3.47 (3H, each as s), 3.36 (0.6H, br), 3.10 (0.4H, d, J = 4.4 Hz), 2.99 (0.6H, d, J = 6.1 Hz), 1.92 (0.4H, br). FAB MS (+NaI) *m/z* 549 $(M+Na)^+$. HRMS (FAB) calcd for $C_{26}H_{26}N_2O_{10}Na$: 549.1488, found: 549.1486.

5'-*O*-(4,4'-Dimethoxytrityl)-2'-*O*-methyl-6-(1,2-dibenzoyloxyethyl)uridine (4). The mixture of **3** (150.5 mg, 0.286 mmol) and DMTCl (145.3 mg, 0.429 mmol) in dry pyridine (3 mL) was stirred at rt overnight. EtOH (0.5 mL) was added and the solution was concentrated *in vacuo*, and the residue was partitioned between EtOAc (30 mL) and saturated aqueous $NaHCO_3$ (10 mL). The organic layer was washed with H_2O and brine (10 mL each), and dried over Na_2SO_4. Silica gel column chromatography (20-50% EtOAc in hexane) gave 184.7 mg of **4** as a white powder (78%). A portion of **4** (diastereomer mixture) was separated into two components, **4a** (t_R 15.3 min) and **4b** (t_R 16.5 min), by HPLC (40% hexane in EtOAc) for physical data. For **4a**: UV (MeOH) λ_{max} 266 nm (ε 13000) and 232 nm (ε 51000), λ_{min} 257.5 nm (ε 12000). ^{1}H NMR ($CDCl_3$) δ 8.66 (1H, br), 8.08 – 8.01 (4H, m), 7.64 ~7.60 (1H, m), 7.58 – 7.53 (1H, m), 7.49 – 7.39 (6H, m), 7.33 – 7.31 (4H, m), 7.24 – 7.20 (2H, m), 7.17 – 7.13 (1H, m), 6.79 – 6.76 (4H, m), 6.38 (1H, dd, J = 2.9 and 7.7 Hz), 6.01 (1H, d, J = 2.4 Hz), 5.87 (1H, s), 5.03 (1H, dd, J = 2.9 and 12.2 Hz), 4.65 (1H, dd, J = 7.7 and 12.2 Hz), 4.56 – 4.50 (2H, m), 4.01 – 3.97 (2H, m), 3.74 (6H, s), 3.57 (3H, s), 3.40 (1H, dd, J = 3.7 and 10.3 Hz), 3.34 (1H, dd, J = 6.1 and 10.3 Hz), 2.65 (1H, br). HRMS (FAB) calcd for $C_{47}H_{44}N_2O_{12}Na$: 851.2792, found: 851.2788. Anal. Calcd for $C_{47}H_{44}N_2O_{12}\bullet H_2O$: C, 66.66; H, 5.48; N, 3.31.

Found: C, 66.90; H, 5.40; N, 3.19. The physical data for **4b** are as follows: UV (MeOH) λ_{max} 267.5 nm (ε 8000) and 232 nm (ε 48000), λ_{min} 258 nm (ε 11000). ^{1}H NMR ($CDCl_3$) δ 8.46 (1H, br), 8.07 – 8.05 (2H, m), 7.95 – 7.93 (2H, m), 7.65 – 7.60 (1H, m), 7.58 – 7.54 (1H, m), 7.49 – 7.40 (6H, m), 7.33 – 7.28 (4H, m), 7.23 – 7.19 (2H, m), 7.15 – 7.11 (1H, m), 6.78 – 6.73 (4H, m), 6.16 (1H, d, *J* = 4.6 Hz), 6.13 (1H, dd, *J* = 2.9 and 5.1 Hz), 6.03 (1H, d, *J* = 2.2 Hz), 4.76 – 4.70 (2H, m), 4.59 – 4.54 (2H, m), 3.94 – 3.90 (1H, m), 3.70 and 3.68 (6H, each as s), 3.55 (3H, s), 3.50 (1H, dd, *J* = 5.3 and 10.7 Hz), 3.39 (1H, dd, *J* = 3.2 and 10.7 Hz), 2.79 (1H, d, *J* = 7.3 Hz). HRMS (FAB) calcd for $C_{47}H_{44}N_2O_{12}Na$: 851.2792, found: 851.2789. Anal. Calcd for $C_{47}H_{44}N_2O_{12}\bullet H_2O$: C, 66.66; H, 5.48; N, 3.31. Found: C, 66.46; H, 5.39; N, 3.16.

5'-*O*-(4,4'-Dimethoxytrityl)-2'-*O*-methyl-6-(1,2-dibenzoyloxyethyl)uridine-3'-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (5). To the mixture of compound (**4**) (146.8 mg, 0.177 mmol) and 2-cyanoethyl tetraisopropylphosphorodiamidite (0.101 mL, 0.319 mmol) in CH_2Cl_2 (3 mL), the solution of 4,5-dicyanoimidazole (14.6 mg, 0.124 mmol) in MeCN (0.3 mL) was added. The mixture was stirred at rt for 4.5 h and diluted with CH_2Cl_2 (30 mL). This was washed with saturated aqueous $NaHCO_3$ (2×10 mL), and the organic layer was dried over Na_2SO_4. Silica gel column chromatography (1% Et_3N in 25-50% EtOAc in hexane) gave **5** as a colorless oil, which was dissolved in toluene (2 mL), and hexane (25 mL) was added to the solution with stirring. After decantation, 172.5 mg of **5** was obtained as a form (95%). This was used for oligonucleotide synthesis under the Beckman standard conditions. ^{1}H NMR ($CDCl_3$) δ 8.22 (1H, br), 8.08 – 8.05 (2H, m), 8.04 – 7.94 (2H, m), 7.63 – 7.52 (2H, m), 7.49 – 7.39 (6H, m), 7.34 – 7.15 (7H, m), 6.79 – 6.75 (4H, m), 6.38 – 6.33 (0.6H, m), 6.26 – 6.25 (0.4H, m), 6.02 and 5.87 (1H, each as d, *J* = 1.7 and 3.2 Hz), 6.01 and 6.00 (1H, each as s), 4.99 (0.6H, dd, *J* = 3.4 and 12.2 Hz), 4.81 – 4.51 (3.4H, m), 4.26 – 4.10 (1H, m), 3.94 – 3.79 (1H, m), 3.75, 3.74, 3.72, 3.71, and 3.70 (6H, each as s), 3.69 – 3.42 (4H, m), 3.52, 3.50, 3.48, and 3.45 (3H, each as s), 3.39 (0.4H, dd, *J* = 3.4 and 10.3 Hz), 3.27 (0.6H, dd, *J* = 5.9 and 10.3 Hz), 2.76 – 2.73 (0.4H, m), 2.66 – 2.58 (1H, m), 2.38 – 2.34 (0.6H, m), 1.29 – 1.01 (12H, m). ^{31}P NMR δ 154.0, 153.7, 153.3, 153.0. FAB MS (+NaI) *m/z* 1068 $(M+K)^+$, 1052 $(M+Na)^+$. HRMS (FAB) calcd for $C_{56}H_{61}N_4O_{13}NaP$: 1051.3870, found: 1051.3862.

Oligonucleotides including 6-formyluracil base.[4] Oligonucleotide ⑥ (X = U2), for example, was prepared from purified oligonucleotide ⑥ (X = 6-(1,2-dihydroxy)ethyluracil base as the precursor). The precursor ⑥ (10 nmol) was dissolved in 50 mM HCO_2NH_4 (600 μL), and 100 mM $NaIO_4$ (45.0 μL) was added at 0 °C. After 15 min of mixing, ethylene glycol (1 mg) was added, and the mixture was loaded on the Sep-Pak (C18), which was washed with H_2O, and formyl-oligonucleotide ⑥ (X = U2) was eluted with 60% MeOH (5 mL). The eluate was concentrated in vacuo and the amount of oligonucleotide was estimated by UV-absorption at 260 nm.

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5. In an initial attempt for synthesis of a precursor of 6-formyl-2'-deoxyuridine in an automated solid phase process, it was found that 2'-deoxy-6-(1,2-diacyloxy)ethyluridine was unstable even in storage at ambient temperature. Therefore, we used stable 6-(1,2-dibenzoyloxy)ethyl-2'-*O*-methyluridine as the precursor of the 6-formyl-2'-*O*-methyluridine unit for oligonucleotide synthesis. For a leading reference on 2'-deoxy-5-(1,2-diacetoxy)ethyluridine as a precursor of 2'-deoxy-5-formyluridine, see: H. Sugiyama, S. Matsuda, K. Kino, Q.-M. Zhang, S. Yonei, and I. Saito, *Tetrahedron Lett.*, 1996, **37**, 9067.
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7. Previously, we proved the formation of the 6-formyl group at the X position on the strand ⑥ (originally, X was 6-(1,2-dihydroxy)ethyl-2'-*O*-methylcytidine) under the same oxidative conditions; *i.e.*, 6-formyl-2'-*O*-methylcytidine was detected by HPLC after enzymatic hydrolysis of the resulting oligonucleotide ⑥, see ref. 4a.
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