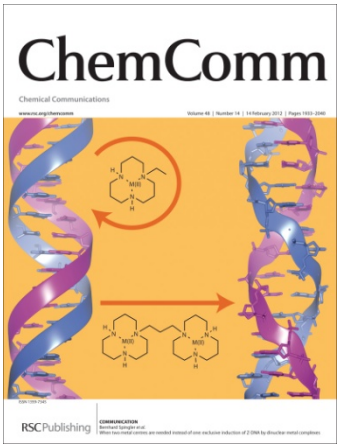

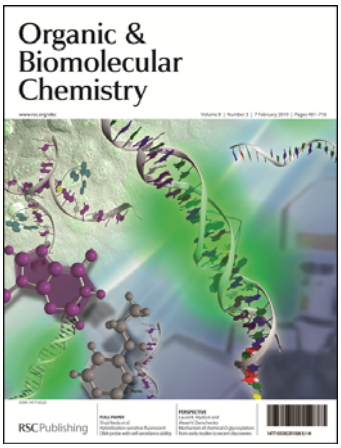

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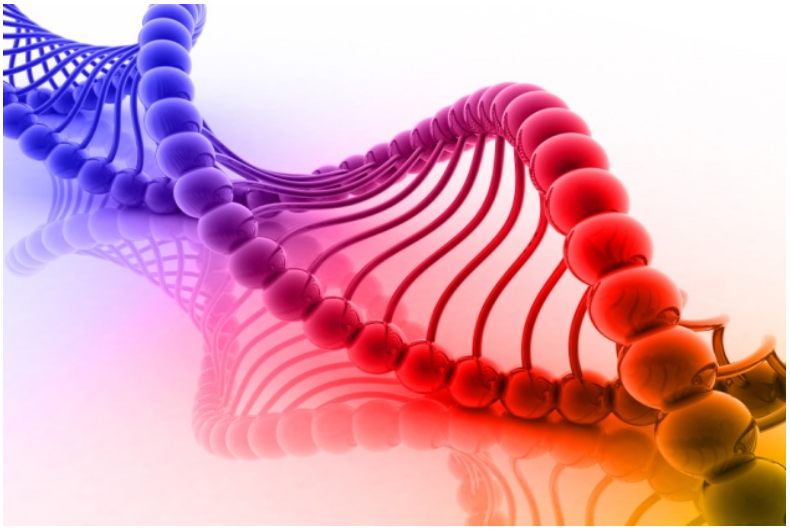

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Synthesis and duplex-forming ability of oligonucleotides containing 4′-carboxythymidine analogs†

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Oligonucleotides containing 4′-carboxy-, 4′-methoxycarbonyl-, 4′-carbamoyl-, and 4′-methylcarbamoyl-thymidines, and their 2′-methoxy, 2′-amino or 2′-acetamido analogs were prepared. Their duplex-forming ability with DNA and RNA complements was evaluated by UV melting experiments. Interestingly, 4′-carboxythymidine existing in the *S*-type sugar conformation was found to lead to an increase in the stability of the duplex formed with RNA complements compared to natural thymidine.

Introduction

Modified oligonucleotides with high binding affinity to complementary single-stranded nucleic acids are promising materials applicable to various nucleic acid-based technologies. In particular, those with high affinity for RNA complements are essential for applications in RNA-targeted therapies like antisense methodology and siRNA therapy.[1,2] To date, many artificial oligonucleotides have been developed towards practical use and to acquire increased RNA-binding affinity by introduction of substituents at the 2′-position like 2′-alkoxy groups and bridged chains like 2′,4′-BNA/LNA.[1] This increased binding affinity is due to preorganization of the sugar conformation to that in RNA duplexes, namely the *N*-type sugar conformation. However, in some cases, oligonucleotides, including several modified nucleotides, likely existing in *S*-type conformations, were also observed to increase the stability of duplexes formed with RNA complements. For instance, 4′-methoxymethyl- or 4′-aminomethyl-thymidine units could increase the melting temperature (T_m) value by up to 0.6 °C or 0.5 °C per modification, respectively, depending on the number and position of the introduction.[3] In contrast, 4′-hydroxymethylthymidine[4] and 4′-methylthymidine,[5] an analog without any functional group on the 4′-methyl group, seem to lead to a decrease in the stability. Given this background, we were interested in the effect of thymidines with highly functionalized methyl units at the 4′-position on the thermal stability of the formed duplexes. Here, the synthesis of oligonucleotides bearing 4′-carboxy-, 4′-methoxycarbonyl-, 4′-carbamoyl- and 4′-methylcarbamoyl-thymidines and their 2′-substituted analogs as shown in Fig. 1 was performed and their duplex formation with DNA and RNA complements was studied.

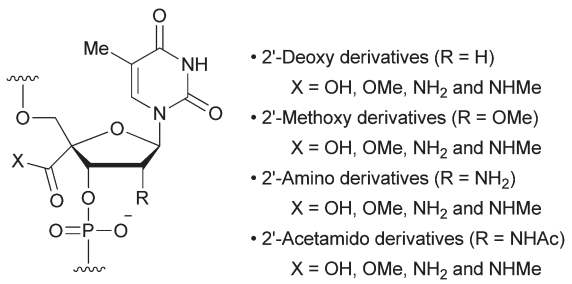

Fig. 1 Structures of 4′-carboxythymidine analogs used in this study.

Results and discussion

Synthesis

We planned to convert 4′-methoxycarbonylthymidines into analogs with the desired 4′-carboxylic acid equivalents by base treatment after oligonucleotide synthesis. Thus, the phosphoramidites **1**–**4** of 4′-methoxycarbonylthymidines were synthesized as shown in Scheme 1. The diol **5**[6] previously reported by Wengel's group was oxidized with a catalytic amount of TEMPO and $PhI(OAc)_2$, and the resulting carboxylic acid was reacted with $TMSCHN_2$ in MeOH to give the methyl ester **6** in 72% over 2 steps. Deoxygenation of **6** afforded the 2′-deoxy compound **8** *via* thiocarbonate **7**. By treatment of **8** with cyclohexene in the presence of $Pd(OH)_2$-C, 4′-methoxycarbonylthymidine **9**[7] was obtained in 45% yield.

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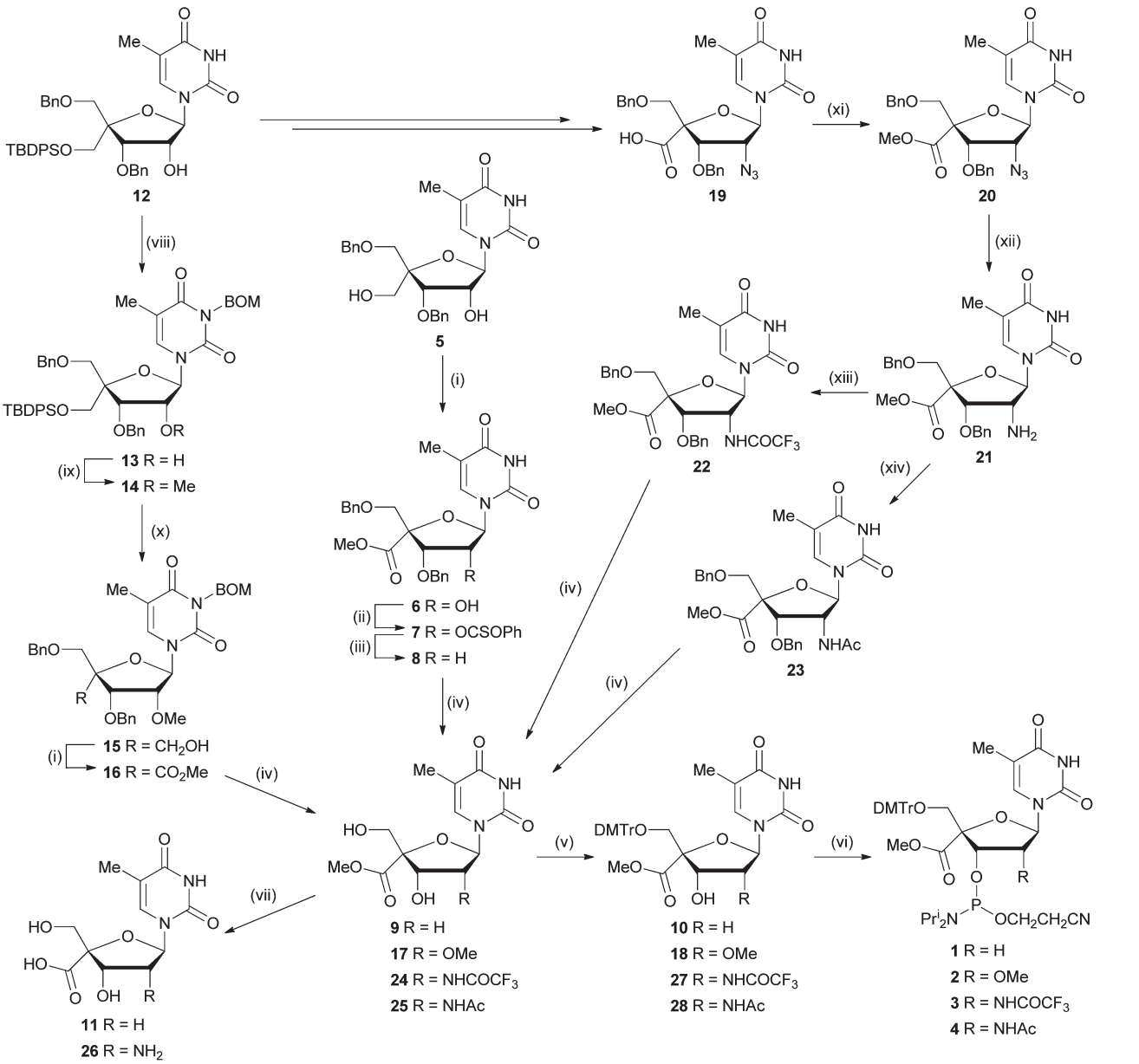
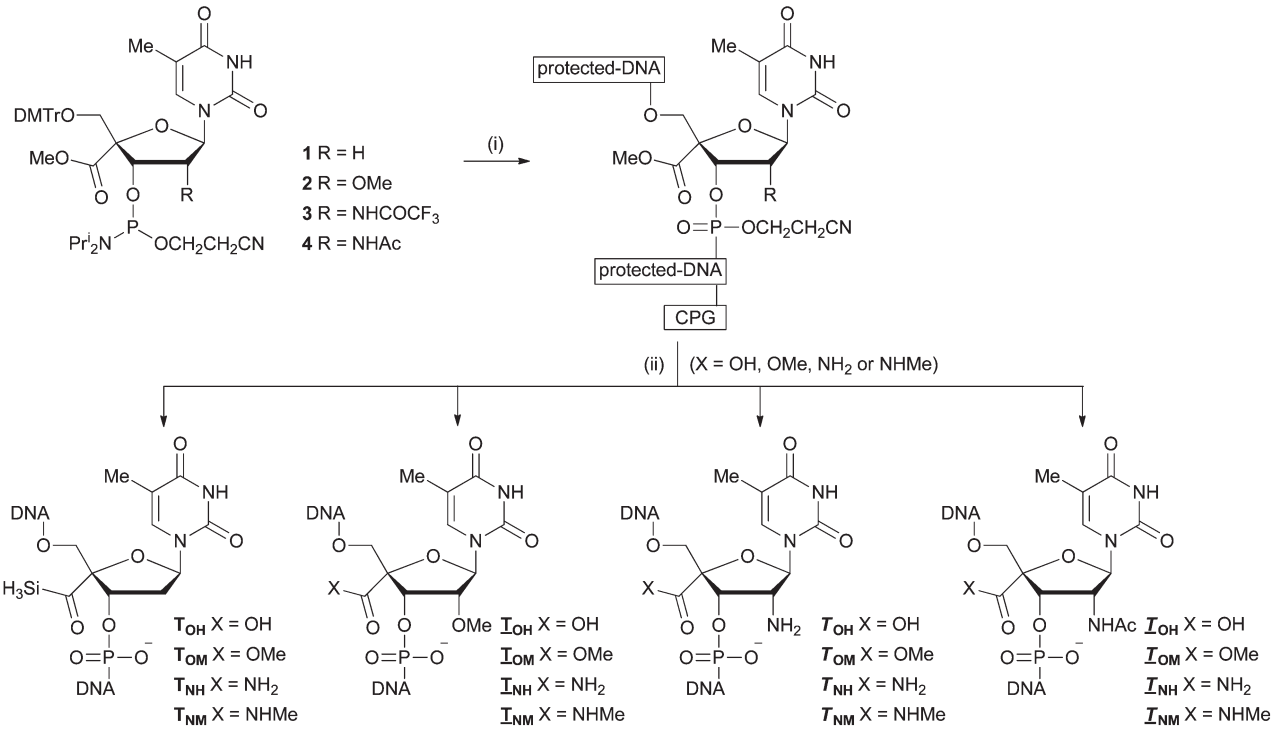
†Electronic supplementary information (ESI) available: Additional experimental procedures, NMR spectra of all new compounds, and HPLC and MALDI-TOF mass spectra of the synthesized oligonucleotides. See DOI: 10.1039/c2ob26712h



Scheme 1 *Reagents and conditions*: (i) TEMPO, $PhI(OAc)_2$, MeCN–H_2O (1 : 1), rt, 12 h, then $TMSCHN_2$, MeOH, rt, 0.5 h, 52–72% for 2 steps; (ii) PhOCSCl, DMAP, CH_2Cl_2, rt, 0.5 h, 79%; (iii) AIBN, *n*-Bu_3SnH, toluene, reflux, 2 h, 55%; (iv) 20% $Pd(OH)_2$-C, cyclohexene, MeOH, reflux, 10–12 h, 45%-quant.; (v) DMTrCl, pyridine, rt, 1–4 h, 83%-quant.; (vi) $Pr^i_2NP(Cl)OCH_2CH_2CN$, DIPEA, CH_2Cl_2, rt, 2–4 h, 56–95%; (vii) BOMCl, DBU, THF, rt, 0.5 h, 86%; (viii) NaH, MeI, DMF, rt, 1 h, 58%; (ix) TBAF, THF, rt, 11 h, quant.; (x) $TMSCHN_2$, MeOH, rt, 0.5 h, 89%; (xi) $NaBH_4$, $NiCl_2$, MeOH–THF (1 : 1), rt, 0.5 h, 89%; (xii) CF_3CO_2Et, Et_3N, MeOH, rt, 1 h, 92%; (xiii) Ac_2O, pyridine, rt, 1.5 h, quant.

Dimethoxytritylation of the 5′-hydroxyl group in **9** followed by phosphitylation of **10**[7] gave the desired phosphoramidite **1**, a building block for oligonucleotide synthesis. The synthesis of the 2′-methoxy analog **2** was achieved from **11**[8] prepared according to our previous report. After BOM-protection of the nitrogen at the 3-position of **11**, the resulting **12** was methylated to afford **13** by alkoxide formation, followed by treatment with MeI. Desilylation of **13** with TBAF produced **14**, which was converted into methyl ester **15** as before. Removal of benzyl and BOM groups by hydrogenolysis of **15** led to 2′-methoxy monomer **16** quantitatively. Analogous to **1**, the phosphoramidite **2** was prepared from **16** *via* compound **17**. 2′-Amino and 2′-acetamido analogs were synthesized from **18**[9] prepared from **11** according to our previous report. Methyl esterification of **18** using $TMSCHN_2$ yielded **19**, which was reduced by a combination of $NiCl_2$ and $NaBH_4$ to produce **20** in 89% yield. Trifluoroacetylation and acetylation of **20** gave **21** and **22** in high yields, respectively. These compounds **21** and **22** were hydrogenolyzed to afford monomers **23**, protected as the 2′-amino analog, and **24** as the 2′-acetamido one. Dimethoxytritylated **25** and **26**, and subsequently phosphoramidites **3** and **4**, were prepared as before. As expected, it was



Scheme 2 *Reagents and conditions*: (i) oligonucleotide synthesis on an automated DNA synthesizer; (ii) 50 mM NaOH aq., rt, 1.5 h then 55 °C, 12 h (for X = OH), 50 mM K_2CO_3 in MeOH, rt, 2 h (for X = OMe), 28% NH_3 aq., rt, 1.5 h then 55 °C, 12 h (for X = NH_2) or 40% MeNH aq., rt, 1.5 h then 55 °C, 12 h (for X = NHMe).

found from large $J_{1'2'}$ values (6.5–9.0 Hz) obtained by ^{1}H NMR measurements that the monomers **9**, **16**, **23** and **24** existed in *S*-type conformations.[10]

Next, introduction of the phosphoramidites **1–4** into oligonucleotides was performed on an automated DNA synthesizer using standard phosphoramidite chemistry (Scheme 2). For the synthesis of 4′-methoxycarbonyl-modified oligonucleotides, commercially available ultra-mild phosphoramidites‡ as other phosphoramidites were used. After completion of the synthesis on the DNA synthesizer, four base treatments (50 mM NaOH aq., 50 mM K_2CO_3 in MeOH, 28% NH_3 aq. and 40% $MeNH_2$ aq.) afforded cleavage from the resin, deprotection of nucleobases and phosphates, and conversion of 4′-methoxycarbonyl moieties to give the corresponding oligonucleotides containing 4′-carboxy, 4′-methoxycarbonyl, 4′-carbamoyl and 4′-methylcarbamoyl derivatives, respectively. The removal of the standard isobutyryl protecting group of the 2-amino moiety in G was completed by treatment with 50 mM NaOH aq. at 55 °C for 12 h. The list of the synthesized oligonucleotides is shown in Table 1. In all cases, they were obtained in moderate to good yields with high purities and their molecular weights were confirmed by MALDI-TOF-MS.

Table 1 Sequence of the synthesized oligonucleotides[a]

Oligonucleotide	Sequence
ON1a	5′-GCGTTTT$\mathbf{T_{OH}}$TTGCT-3′
ON1b	5′-GCGTTTT$\mathbf{T_{OM}}$TTGCT-3′
ON1c	5′-GCGTTTT$\mathbf{T_{NH}}$TTGCT-3′
ON1d	5′-GCGTTTT$\mathbf{T_{NM}}$TTGCT-3′
ON2a	5′-GCGTTTT$\mathbf{\underline{T}_{OH}}$TTGCT-3′
ON2b	5′-GCGTTTT$\mathbf{\underline{T}_{OM}}$TTGCT-3′
ON2c	5′-GCGTTTT$\mathbf{\underline{T}_{NH}}$TTGCT-3′
ON2d	5′-GCGTTTT$\mathbf{\underline{T}_{NM}}$TTGCT-3′
ON3a	5′-GCGTTTT$\mathbf{T_{OH}}$TTGCT-3′
ON3b	5′-GCGTTTT$\mathbf{T_{OM}}$TTGCT-3′
ON3c	5′-GCGTTTT$\mathbf{T_{NH}}$TTGCT-3′
ON3d	5′-GCGTTTT$\mathbf{T_{NM}}$TTGCT-3′
ON4a	5′-GCGTTTT$\mathbf{\underline{T}_{OH}}$TTGCT-3′
ON4b	5′-GCGTTTT$\mathbf{\underline{T}_{OM}}$TTGCT-3′
ON4c	5′-GCGTTTT$\mathbf{\underline{T}_{NH}}$TTGCT-3′
ON4d	5′-GCGTTTT$\mathbf{\underline{T}_{NM}}$TTGCT-3′
ON5a	5′-GCGTT$\mathbf{T_{OH}T_{OH}T_{OH}}$TGCT-3′
ON6a	5′-GCGT$\mathbf{T_{OH}TT_{OH}TT_{OH}}$GCT-3′

[a] Bold letters: Modified thymidines (see Scheme 2).

Evaluation of duplex-forming ability

UV melting experiments of the synthesized **ON1–4** with single modification and DNA or RNA complement, 5′-d-(AGCAAAAAACGC)-3′ or 5′-r(AGCAAAAAACGC)-3′, were carried out and the results are summarized in Table 2. In general, the binding affinity of singly modified **ON1–4** against complementary DNA and RNA was greatly affected by the 2′-substituent and almost no effect by the 4′-modification was observed. The order of stabilization was 2′-deoxy (**ON1a–d**), 2′-methoxy (**ON2a–d**), 2′-amino (**ON3a–d**) and 2′-acetamido (**ON4a–d**) groups. 2′-Amino and 2′-acetamido modifications led to an especially large decrease in the duplex stability compared to that of the unmodified DNA/DNA duplex (T_m = 51 °C). It is

‡Purchased from Glen Research.

Table 2 UV melting experiments with DNA and RNA complements[a,b]

Oligonucleotide	DNA complement T_m (°C)	DNA complement ΔT_m/mod.[c,d] (°C)	RNA complement T_m (°C)	RNA complement ΔT_m/mod.[c,d] (°C)
ON1a	51	0	47	+1
ON1b	51	0	46	0
ON1c	51	0	46	0
ON1d	51	0	46	0
ON2a	48	−3	44	−2
ON2b	48	−3	43	−3
ON2c	48	−3	43	−3
ON2d	48	−3	43	−3
ON3a	38	−13 (−2)	41	−5 (+3)
ON3b	37	−14 (−3)	37	−9 (−1)
ON3c	38	−13 (−3)	38	−8 (0)
ON3d	38	−13 (−3)	37	−9 (−1)
ON4a	35	−16	35	−11
ON4b	35	−16	35	−11
ON4c	35	−16	35	−11
ON4d	35	−16	34	−12

[a] Conditions: 10 mM sodium phosphate buffer (pH 7.2) and 100 mM NaCl. The final concentration of each oligonucleotide used was 4 μM. The sequences of DNA and RNA complements were 5′-d(AGCTTTTTTCGC)-3′ and 5′-r(AGCTTTTTTCGC)-3′, respectively. [b] T_m values of unmodified DNA/DNA and DNA/RNA duplexes were 51 °C and 46 °C, respectively. [c] The changes in T_m per modification compared with the unmodified duplexes are shown. [d] In parentheses, the changes in T_m per modification compared with the duplexes by 5′-GCGTT***T***TTTGCT-3′ (***T*** = 2′-aminothymidine) are shown. The T_m values of the duplexes with DNA and RNA complements were 40 °C and 38 °C, respectively.

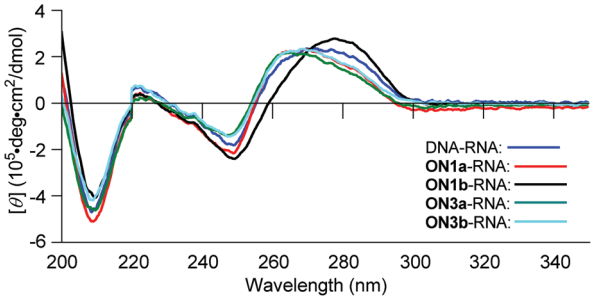


Fig. 2 CD spectra of duplexes with RNA complements by DNA, **ON1a**, **ON1b**, **ON3a** and **ON3b**.

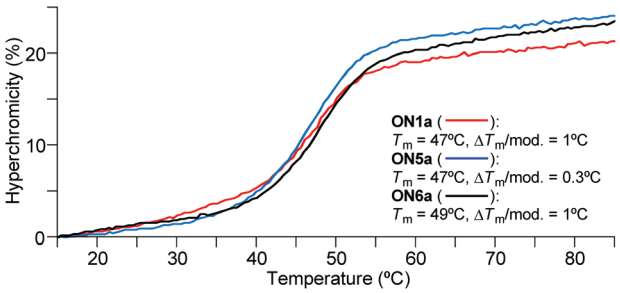


Fig. 3 UV melting profiles of duplexes with RNA complements by **ON1a**, **ON5a** and **ON6a**.

known that 2′-amino modification drastically destabilizes duplexes[11] and a UV melting experiment of 2′-aminothymidine-modified oligonucleotide, 5′-GCGTT***T***TTTGCT-3′ (***T*** = 2′-aminothymidine), under the same conditions showed decreased T_m values of 40 °C and 38 °C by 11 °C and 8 °C compared to those of unmodified DNA/DNA and DNA/RNA duplexes, respectively (see ESI† for the preparation of 2′-aminothymidine-modified oligonucleotide). From these points, the decreased binding affinity of 2′-amino-modified **ON3a–d** was considered to be reasonable. In the case of 2′-acetamido modification (**ON4a–d**), the destabilization of the formed duplex was likely caused by the steric bulkiness of the 2′-acetamido group.

It was very interesting that 4′-carboxy modifications of thymidine and 2′-aminothymidine (**ON1a** and **ON3a**) had some apparent effect on the duplex stability with RNA complements. The T_m values of **ON1a** and **ON3a** were increased by 1 °C and 3 °C compared to those of the 4′-unmodified oligonucleotides, 46 °C for a natural DNA/RNA duplex and 37 °C for the duplex between 2′-amino-modified oligonucleotide and RNA complement, respectively, though the T_m value of **ON3a** was much lower than that of **ON1a** due to destabilization by the 2′-amino modification.[11] Very recently, Leumann's group reported that the existence of a carboxy group at the C6′-position of tricyclo-DNA did not perturb the pairing affinity of tricyclo-DNA with an RNA complement and in their case, carboxy and carbamoyl modifications apparently increased stability of the duplexes formed with RNA.[12] However, in our case, only thymidine and 2′-aminothymidine with modifications by 4′-carboxy groups stabilized the duplex with RNA compared to their 4′-unmodified ones. To study global conformational changes in duplexes of **ON1a**, **ON1b**, **ON3a** and **ON3b** with RNA complements, circular dichroism (CD) spectroscopy was measured (Fig. 2). As a result, no major change of the global conformations was observed although in the case of **ON1b** containing 4′-methoxycarbonylthymidine the positive band at around 260–280 nm was red-shifted.

Duplex-forming ability of oligonucleotides **ON5a** and **ON6a** including three 4′-carboxythymidine units consecutively and alternately led to the highest stabilization of the duplex with an RNA complement. Under the same conditions shown in Table 2, the ability was compared to singly modified **ON1a** and the UV melting profiles are displayed in Fig. 3. The T_m value of **ON5a** with consecutive modifications was comparable to that of **ON1a** with a single modification. This indicated that consecutive modifications led to a significant decrease in ΔT_m/mod. (+1 °C → +0.3 °C), perhaps due to slight electrostatic repulsion by contiguous carboxylate ions. On the other hand, the T_m value of **ON6a** with alternate modifications was 49 °C. Its ΔT_m/mod. was 1 °C, almost the same as that of **ON1a**, and it formed a stable duplex with an RNA complement. The ^{1}H NMR spectrum of 4′-carboxythymidine[13] exhibited a $J_{1'2'}$ value of 6.7 Hz, meaning that it preferentially adopted the *S*-type conformation.[10] These results could indicate that 4′-carboxythymidine significantly stabilized the duplex with an RNA complement compared to 4′-methoxymethylthymidine (ΔT_m/

mod. = up to 0.6 °C) or 4′-aminomethylthymidine (ΔT_m/mod. = up to 0.5 °C), which also adopted *S*-type conformations.

Experimental

General

All moisture-sensitive reactions were carried out in well-dried glassware under a N_2 atmosphere. ^{1}H, ^{13}C and ^{31}P spectra were recorded on JEOL JNM-EX300 and JEOL JNM-EX400 spectrometers. Chemical shifts are reported in parts per million referenced to tetramethylsilane (δ = 0.00 ppm) for ^{1}H NMR spectra, $CDCl_3$ (δ = 77.0 ppm) and CD_3OD (δ = 49.0 ppm) for ^{13}C NMR spectra, and phosphoric acid (δ = 0.00 ppm) for ^{31}P NMR spectra. IR spectra were recorded on a JASCO FT/IR-4200 spectrometer. Optical rotations were recorded on a JASCO P-2200 polarimeter. FAB mass spectra were measured on JEOL JMS-600 or JEOL JMS-700 mass spectrometers. MALDI-TOF mass spectra were recorded on a Bruker Daltonics Autoflex II TOF/TOF mass spectrometer. Fuji Silysia silica gel PSQ-60B (0.060 mm) and FL-60D (0.060 mm) were used for flash column chromatography. For HPLC, SHIMADZU LC-10AT$_{VP}$, SHIMADZU SPD-10A$_{VP}$ and SHIMADZU CTO-10$_{VP}$ instruments were used.

3′,5′-Di-*O*-benzyl-4′-methoxycarbonyl-5-methyluridine (6). PhI(OAc)$_2$ (3.86 g, 12.0 mmol) and TEMPO (170 mg, 1.09 mmol) were added to a solution of compound **5**[6] (2.55 g, 5.45 mmol) in MeCN–H_2O (1 : 1, 30 mL) at room temperature. The reaction mixture was stirred for 12 h at room temperature. The resulting mixture was concentrated *in vacuo*, and the residue (5.98 g) was co-evaporated with anhydrous MeCN three times, and dissolved in anhydrous MeOH (30 mL). $TMSCHN_2$ (2 M in *n*-hexane, 3.0 mL, 6.0 mmol) was added to the solution at room temperature. The reaction mixture was stirred for 0.5 h at room temperature. The resulting mixture was concentrated *in vacuo* and the residue (6.11 g) was purified by column chromatography (silica gel, 150 g, *n*-hexane–EtOAc = 2 : 3) to give compound **6** (1.94 g, 72% for 2 steps from **5**) as a white foam.

Mp: 62–64 °C. $[\alpha]_D^{23}$ −35.4 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3449, 3177, 3065, 3033, 2952, 2870, 1713, 1472, 1455, 1373, 1279, 1240 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.59 (d, *J* = 1.0 Hz, 3H), 3.72 (s, 3H), 3.76–3.82 (m, 2H), 4.05 (d, *J* = 10.0 Hz, 1H), 4.55–4.74 (m, 5H), 6.21 (d, *J* = 6.5 Hz, 1H), 7.25–7.41 (m, 11H), 9.05 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.09, 52.74, 71.83, 73.86, 74.36, 75.28, 80.24, 89.08, 89.20, 111.51, 127.61, 128.02, 128.25, 128.26, 128.53, 128.69, 135.59, 136.59, 136.82, 150.76, 163.54, 169.58. MS (FAB): *m*/*z* = 497 $[M + H]^+$. HRMS (FAB): calcd for $C_{26}H_{29}N_2O_8$ $[M + H]^+$, 497.1924, found, 497.1923.

3′,5′-Di-*O*-benzyl-4′-methoxycarbonyl-2′-*O*-phenoxythiocarbonyl-5-methyluridine (7). Under a nitrogen atmosphere, DMAP (493 mg, 4.04 mmol) and PhOCSCl (0.33 mL, 2.42 mmol) were added to a solution of compound **6** (1.00 g, 2.02 mmol) in anhydrous CH_2Cl_2 (20 mL) at 0 °C. The reaction mixture was stirred at room temperature for 0.5 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with CH_2Cl_2. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (1.52 g) was purified by column chromatography (silica gel, 30 g, *n*-hexane–EtOAc = 1 : 1) to give compound **7** (1.01 g, 79%) as a white foam.

Mp: 58–61 °C. $[\alpha]_D^{23}$ −47.6 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3182, 3033, 2952, 2868, 2614, 2484, 1954, 1867, 1715, 1591, 1489, 1470, 1361, 1277 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.57 (d, *J* = 1.0 Hz, 3H), 3.70 (s, 3H), 3.86 (d, *J* = 10.0 Hz, 1H), 4.10 (d, *J* = 10.0 Hz, 1H), 4.59 (d, *J* = 11.5 Hz, 1H), 4.61 (d, *J* = 10.5 Hz, 1H), 4.68 (d, *J* = 10.5 Hz, 1H), 4.68 (d, *J* = 11.0 Hz, 1H), 4.80 (d, *J* = 6.0 Hz, 1H), 5.79 (dd, *J* = 6.0, 7.5 Hz, 1H), 6.66 (d, *J* = 7.5 Hz, 1H), 6.96–7.40 (m, 16H), 8.80 (brs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.06, 52.69, 71.83, 73.91, 75.47, 78.70, 81.50, 86.38, 89.67, 111.84, 115.28, 121.55, 126.79, 127.71, 127.85, 128.05, 128.25, 128.38, 128.66, 129.57, 135.75, 136.74, 136.85, 150.26, 153.28, 163.47, 168.82, 194.42. MS (FAB): *m*/*z* = 633 $[M + H]^+$. HRMS (FAB): calcd for $C_{33}H_{33}N_2O_9S$ $[M + H]^+$, 633.1907, found, 633.1920.

3′,5′-Di-*O*-benzyl-4′-methoxycarbonylthymidine (8). Under a nitrogen atmosphere, *n*-Bu_3SnH (0.81 mL, 3.00 mmol) and AIBN (33.1 mg, 0.202 mmol) were added to a solution of compound **7** (1.00 g, 1.58 mmol) in anhydrous toluene (20 mL) at room temperature. The reaction mixture was refluxed for 2 h. The resulting mixture was concentrated *in vacuo* and the residue (2.00 g) was purified by column chromatography (silica gel, 60 g, *n*-hexane–EtOAc = 1 : 1) to give compound **8** (424 mg, 55%) as a white foam.

Mp: 72–74 °C. $[\alpha]_D^{23}$ +24.6 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3183, 3065, 3034, 2952, 2920, 2871, 1695, 1496, 1454, 1434, 1399, 1365, 1281, 1207 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.56 (d, *J* = 1.0 Hz, 3H), 2.13–2.22 (m, 1H), 2.53–2.61 (m, 1H), 3.75 (s, 3H), 3.88 (d, *J* = 10.0 Hz, 1H), 4.08 (d, *J* = 10.0 Hz, 1H), 4.48–4.61 (m, 5H), 6.58 (t, *J* = 6.5 Hz, 1H), 6.96–7.40 (m, 10H), 7.52 (d, *J* = 1.0 Hz, 1H), 9.27 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.03, 37.71, 52.42, 70.98, 72.42, 73.74, 79.59, 85.70, 89.90, 111.08, 127.41, 127.48, 127.62, 127.87, 128.11, 128.39, 128.48, 128.58, 135.81, 136.98, 137.06, 150.24, 163.84, 169.80. MS (EI): *m*/*z* = 480 (M^+, 14.3), 355 (11.8), 261 (13.6), 248 (14.2), 217 (85.5), 181 (29.5), 157 (13.8), 127 (16.3), 91 (100). HRMS (EI): calcd for $C_{26}H_{28}N_2O_7$ $[M^+]$, 480.1897, found, 480.1900.

4′-Methoxycarbonylthymidine (9).[7] Under a nitrogen atmosphere, a solution of compound **8** (420 mg, 0.874 mmol) in MeOH (15 mL) and cyclohexene (8.8 mL, 87 mmol) were added to a suspension of 20% $Pd(OH)_2$ on carbon (307 mg, 0.437 mmol) in MeOH (5.0 mL) at room temperature. The reaction mixture was refluxed for 12 h. The resulting mixture was filtered and the filtrate was concentrated *in vacuo*. The residue (360 mg) was purified by column chromatography (silica gel, 10 g, $CHCl_3$–MeOH = 20 : 1 to 7 : 1) to give compound **9** (118 mg, 45%) as a white foam.

^{1}H NMR (400 MHz, CD_3OD) δ: 1.87 (d, *J* = 1.0 Hz, 3H), 2.32–2.36 (m, 2H), 3.75 (s, 3H), 3.92 (d, *J* = 12.0 Hz, 1H), 3.96 (d, *J* = 12.0 Hz, 1H), 4.57 (dd, *J* = 5.5, 6.5 Hz, 1H), 6.48 (t, *J* = 6.5 Hz, 1H), 7.74 (d, *J* = 1.0 Hz, 1H). ^{13}C NMR (75 MHz, CD_3OD)

δ: 12.47, 40.56, 52.55, 64.25, 73.33, 87.12, 93.37, 111.70, 138.43, 152.26, 166.40, 172.39.

5′-*O*-(4,4′-Dimethoxytrityl)-4′-methoxycarbonylthymidine (10).[7] Under a nitrogen atmosphere, DMTrCl (120 mg, 0.355 mmol) was added to a solution of compound **9** (71.0 mg, 0.236 mmol) in anhydrous pyridine (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (210 mg) was purified by column chromatography (silica gel, 10 g, $CHCl_3$–MeOH = 15 : 1) to give compound **10** (118 mg, 83%) as a white foam.

^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.38 (d, J = 1.0 Hz, 3H), 2.37–2.56 (m, 2H), 3.58 (d, J = 10.0 Hz, 1H), 3.70 (d, J = 10.0 Hz, 1H), 3.73 (s, 3H), 3.78 (s, 6H), 4.77 (dd, J = 3.0, 6.5 Hz, 1H), 6.65 (dd, J = 6.0, 8.0 Hz, 1H), 6.81–7.40 (m, 13H), 7.55 (d, J = 1.0 Hz, 1H), 9.51 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 11.60, 39.99, 52.59, 55.17, 65.03, 73.62, 85.66, 87.17, 91.55, 111.36, 113.19, 127.15, 127.95, 128.06, 130.08, 134.77, 134.89, 136.29, 143.91, 150.34, 158.61, 158.63, 163.93, 170.71.

3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-4′-methoxycarbonylthymidine (1). Under a nitrogen atmosphere, DIPEA (0.17 mL, 0.97 mmol) and i-Pr_2NP(Cl)-OCH_2CH_2CN (52 μL, 0.23 mmol) were added to a solution of compound **10** (117 mg, 0.194 mmol) in anhydrous CH_2Cl_2 (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with CH_2Cl_2. The combined organic layer was washed with sat. $NaHCO_3$, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (166 mg) was chromatographed (silica gel, 10 g, *n*-hexane–EtOAc = 2 : 3) to give **1** with a small amount of impurity (99.8 mg), which was reprecipitated from *n*-hexane–$CHCl_3$ to give compound **1** (86.7 mg, 56%) as a white powder.

Mp: 88–90 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.05–1.19 (m, 12H), 1.32 (d, J = 1.5 Hz, 1.5H), 1.33 (d, J = 1.5 Hz, 1.5H), 2.41–2.65 (m, 4H), 3.50–3.80 (m, 15H), 4.84–4.91 (m, 0.5H), 4.98–5.06 (m, 0.5H), 6.61 (t, J = 8.0 Hz, 0.5H), 6.65 (t, J = 8.0 Hz, 0.5H), 6.82–7.41 (m, 13H), 7.59 (d, J = 1.5 Hz, 0.5H), 7.62 (d, J = 1.5 Hz, 0.5H), 9.26 (brs, 1H). ^{31}P NMR (161 MHz, $CDCl_3$) δ: 149.89, 150.02. MS (FAB): m/z = 803 $[M + H]^+$. HRMS (FAB): calcd for $C_{42}H_{52}N_4O_{10}P$ $[M + H]^+$, 803.3421, found, 803.3430.

3-*N*-Benzyloxymethyl-3′,5′-di-*O*-benzyl-4′-*tert*-butyldiphenylsiloxymethyl-5-methyluridine (12). Under a nitrogen atmosphere, DBU (0.71 mL, 4.8 mmol) and BOMCl (0.61 mL, 4.4 mmol) were added to a solution of compound **11**[8] (2.60 g, 3.67 mmol) in anhydrous THF (15 mL) at 0 °C. The reaction mixture was stirred at room temperature for 0.5 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (3.40 g) was purified by column chromatography (silica gel, 60 g, *n*-hexane–EtOAc = 3 : 1) to give compound **12** (2.62 g, 86%) as a white foam.

Mp: 40–42 °C. $[\alpha]_D^{24}$ −6.8 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3409, 3065, 3031, 2930, 2858, 1711, 1668, 1496, 1455, 1428, 1362, 1265, 1209 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.06 (s, 9H), 1.62 (d, J = 1.0 Hz, 3H), 3.76–3.82 (m, 4H), 4.64 (d, J = 11.0 Hz, 1H), 4.68 (s, 2H), 4.76 (d, J = 11.0 Hz, 1H), 5.45 (d, J = 9.5 Hz, 1H), 5.49 (d, J = 9.5 Hz, 1H), 6.00 (d, J = 2.0 Hz, 1H), 7.19–7.68 (m, 26H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.76, 19.02, 26.75, 64.11, 70.47, 71.83, 72.10, 73.66, 73.86, 74.50, 78.06, 88.20, 92.17, 110.90, 127.53, 127.60, 127.67, 127.80, 127.87, 127.96, 128.03, 128.09, 128.22, 128.52, 128.55, 129.92, 129.95, 132.06, 132.09, 135.24, 135.62, 137.18, 137.25, 137.97, 151.18, 163.46. MS (FAB): m/z = 827 $[M + H]^+$. HRMS (FAB): calcd for $C_{49}H_{55}N_2O_8Si$ $[M + H]^+$, 827.3728, found, 827.3726.

3-*N*-Benzyloxymethyl-3′,5′-di-*O*-benzyl-4′-*tert*-butyldiphenylsiloxymethyl-2′-*O*-methyl-5-methyluridine (13). Under a nitrogen atmosphere, NaH (127 mg, 3.17 mmol) was added to a solution of compound **12** (2.50 g, 3.02 mmol) in anhydrous DMF (15 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h. MeI (0.20 mL, 3.2 mmol) was added to the above mixture at 0 °C, and the mixture was further stirred at room temperature for 1 h. After the addition of H_2O at 0 °C, the mixture was extracted with Et_2O. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (3.21 g) was purified by column chromatography (silica gel, 50 g, *n*-hexane–EtOAc = 3 : 1) to give compound **13** (1.48 g, 58%) as a colorless oil.

$[\alpha]_D^{24}$ +15.4 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3068, 3031, 2955, 2930, 2858, 1707, 1668, 1463, 1428, 1389, 1362, 1298, 1254, 1211 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.05 (s, 9H), 1.45 (d, J = 1.0 Hz, 3H), 3.36 (s, 3H), 3.68 (d, J = 11.0 Hz, 1H), 3.71 (dd, J = 3.5, 5.5 Hz, 1H), 3.92 (d, J = 11.0 Hz, 1H), 4.02 (d, J = 11.0 Hz, 1H), 4.11 (d, J = 11.0 Hz, 1H), 4.27 (d, J = 5.5 Hz, 1H), 4.45 (d, J = 12.0 Hz, 1H), 4.50 (d, J = 12.0 Hz, 1H), 4.58 (d, J = 12.0 Hz, 1H), 4.66 (d, J = 12.0 Hz, 1H), 4.69 (s, 2H), 5.46 (d, J = 9.5 Hz, 1H), 5.50 (d, J = 9.5 Hz, 1H), 5.82 (d, J = 3.5 Hz, 1H), 7.20–7.71 (m, 26H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.52, 19.24, 26.88, 58.61, 64.47, 70.36, 70.67, 72.06, 73.97, 73.53, 75.55, 83.23, 87.75, 87.88, 109.66, 127.53, 127.59, 127.61, 127.64, 127.70, 127.87, 127.98, 128.20, 128.34, 128.47, 128.53, 129.56, 129.69, 132.94, 133.40, 134.73, 135.56, 135.80, 137.40, 137.51, 138.00, 150.72, 163.46. MS (FAB): m/z = 841 $[M + H]^+$. HRMS (FAB): calcd for $C_{50}H_{57}N_2O_8Si$ $[M + H]^+$, 841.3884, found, 841.3882.

3-*N*-Benzyloxymethyl-3′,5′-di-*O*-benzyl-4′-hydroxymethyl-2′-*O*-methyl-5-methyluridine (14). TBAF (1 M in THF, 1.8 mL, 1.8 mmol) were added to a solution of compound **13** (1.40 g, 1.66 mmol) in THF (20 mL) at room temperature. The reaction mixture was stirred at room temperature for 11 h. The reaction was concentrated *in vacuo* and the residue (2.54 g) was purified by column chromatography (silica gel, 50 g, *n*-hexane–EtOAc = 3 : 1 to 1 : 1) to give compound **14** (1.07 g, quant.) as a colorless oil.

$[\alpha]_D^{19}$ +78.3 (*c* 1.10, $CHCl_3$). IR: ν_{max} (KBr): 3499, 3064, 3030, 2941, 2867, 1708, 1668, 1496, 1455, 1362, 1274, 1209 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.48 (d, J = 1.0 Hz, 3H), 2.75 (t, J = 7.0 Hz, 1H), 3.59 (s, 3H), 3.68 (d, J = 10.5 Hz, 1H), 3.75–3.87 (m, 4H), 4.40 (d, J = 6.0 Hz, 1H), 4.47 (d, J = 11.0 Hz, 1H), 4.48

(d, J = 11.5 Hz, 1H), 4.53 (d, J = 11.5 Hz, 1H), 4.68 (s, 2H), 4.75 (d, J = 11.0 Hz, 1H), 5.44 (d, J = 10.0 Hz, 1H), 5.47 (d, J = 10.0 Hz, 1H), 6.08 (d, J = 2.5 Hz, 1H), 7.19–7.38 (m, 15H), 7.63 (d, J = 1.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.48, 59.14, 63.59, 70.30, 70.33, 72.04, 73.00, 73.48, 75.86, 83.28, 87.21, 89.28, 109.82, 127.50, 127.56, 127.60, 127.66, 127.99, 128.15, 128.48, 128.51, 134.52, 137.08, 137.14, 137.86, 150.59, 163.30. MS (EI): m/z = 602 (M^+, 14.0), 511 (16.3), 496 (96.4), 249 (10.2), 235 (20.5), 181 (34.0), 140 (23.5), 111 (24.1), 99 (37.4), 91 (100). HRMS (EI): calcd for $C_{34}H_{38}N_2O_8$ [M^+], 602.2628, found, 602.2623.

3-*N*-Benzyloxymethyl-3′,5′-di-*O*-benzyl-4′-methoxycarbonyl-2′-*O*-methyl-5-methyluridine (15). $PhI(OAc)_2$ (1.23 g, 3.80 mmol) and TEMPO (53.9 mg, 0.345 mmol) were added to a solution of compound **14** (1.04 g, 1.73 mmol) in MeCN–H_2O (1 : 1, 20 mL) at room temperature. The reaction mixture was stirred for 12 h at room temperature. The resulting mixture was concentrated *in vacuo*, and the residue (1.88 g) was co-evaporated with anhydrous MeCN three times, and dissolved in anhydrous MeOH (10 mL). $TMSCHN_2$ (2M in *n*-hexane, 0.95 mL, 1.9 mmol) was added to this solution at room temperature. The reaction mixture was stirred for 0.5 h at room temperature. The resulting mixture was concentrated *in vacuo* and the residue (1.26 g) was purified by column chromatography (silica gel, 30 g, *n*-hexane–EtOAc = 3 : 1 to 2 : 1) to give compound **15** (566 mg, 52% for 2 steps from **14**) as a colorless oil.

$[\alpha]_D^{21}$ +9.7 (c 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3064, 3031, 2952, 2927, 2867, 1762, 1713, 1673, 1497, 1454, 1363, 1278, 1239, 1210 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.62 (d, J = 1.0 Hz, 3H), 3.40 (s, 3H), 3.69 (s, 3H), 3.80 (d, J = 10.5 Hz, 1H), 3.96 (dd, J = 5.5, 6.0 Hz, 1H), 4.05 (d, J = 10.5 Hz, 1H), 4.35 (d, J = 5.5 Hz, 1H), 4.57 (d, J = 11.5 Hz, 1H), 4.60 (d, J = 11.5 Hz, 1H), 4.62 (d, J = 11.5 Hz, 1H), 4.68 (s, 2H), 4.79 (d, J = 11.5 Hz, 1H), 5.46 (d, J = 9.5 Hz, 1H), 5.49 (d, J = 9.5 Hz, 1H), 6.40 (d, J = 6.0 Hz, 1H), 7.23–7.38 (m, 15H), 7.42 (d, J = 1.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.78, 52.52, 58.87, 70.47, 71.48, 72.03, 73.77, 74.22, 78.38, 82.81, 87.96, 88.78, 110.63, 127.50, 127.53, 127.56, 127.65, 127.67, 127.82, 128.18, 128.26, 128.54, 128.62, 134.44, 136.87, 137.30, 137.83, 151.01, 163.24, 169.33. MS (EI): m/z = 630 (M^+, 3.6), 539 (10.8), 524 (52.8), 247 (8.3), 91 (100). HRMS (EI): calcd for $C_{35}H_{38}N_2O_9$ [M^+], 630.2577, found, 630.2559.

4′-Methoxycarbonyl-2′-*O*-methyl-5-methyluridine (16). Under a nitrogen atmosphere, a solution of compound **15** (550 mg, 0.872 mmol) in MeOH (8.0 mL) and cyclohexene (4.4 mL, 44 mmol) were added to a suspension of 20% $Pd(OH)_2$ on carbon (306 mg, 0.436 mmol) in MeOH (2.0 mL) at room temperature. The reaction mixture was refluxed for 12 h. The resulting mixture was filtered and the filtrate was concentrated *in vacuo*. The residue (350 mg) was purified by column chromatography (silica gel, 10 g, $CHCl_3$–MeOH = 15 : 1 to 7 : 1) to give compound **16** (295 mg, quant.) as a white foam.

Mp: 112–114 °C. $[\alpha]_D^{21}$ −16.4 (c 1.00, CH_3OH). IR: ν_{max} (KBr): 3506, 3285, 3050, 2955, 2833, 1702, 1471, 1378, 1277, 1218 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD) δ: 1.88 (d, J = 1.0 Hz, 3H), 3.39 (s, 3H), 3.75 (s, 3H), 3.81 (d, J = 12.0 Hz, 1H), 4.00 (d, J = 12.0 Hz, 1H), 4.05 (dd, J = 5.0, 7.5 Hz, 1H), 4.45 (d, J = 5.0 Hz, 1H), 6.20 (d, J = 7.5 Hz, 1H), 7.75 (d, J = 1.0 Hz, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ: 12.45, 52.72, 58.50, 65.14, 72.05, 83.28, 88.44, 92.48, 112.13, 138.22, 152.41, 166.17, 171.68. MS (FAB): m/z = 331 [M + H]$^+$. HRMS (FAB): calcd for $C_{13}H_{19}N_2O_8$ [M + H]$^+$, 331.1141, found, 331.1149.

5′-*O*-(4,4′-Dimethoxytrityl)-4′-methoxycarbonyl-2′-*O*-methyl-5-methyluridine (17). Under a nitrogen atmosphere, DMTrCl (380 mg, 1.12 mmol) was added to a solution of compound **16** (285 mg, 0.863 mmol) in anhydrous pyridine (5.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (710 mg) was purified by column chromatography (silica gel, 15 g, $CHCl_3$–MeOH = 30 : 1) to give compound **17** (464 mg, 85%) as a white foam.

Mp: 81–91 °C. $[\alpha]_D^{22}$ +4.8 (c 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3469, 3224, 3065, 2953, 2936, 2837, 1697, 1607, 1580, 1509, 1464, 1387, 1252 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.40 (d, J = 1.0 Hz, 3H), 3.10 (d, J = 5.0 Hz, 1H), 3.54 (s, 3H), 3.56 (d, J = 12.0 Hz, 1H), 3.73 (d, J = 12.0 Hz, 1H), 3.74 (s, 3H), 3.78 (s, 6H), 4.05 (t, J = 5.0 Hz, 1H), 4.60 (t, J = 5.0 Hz, 1H), 6.26 (d, J = 5.0 Hz, 1H), 6.83–7.40 (m, 13H), 7.48 (d, J = 1.0 Hz, 1H), 9.96 (brs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 11.61, 52.52, 55.11, 58.67, 64.76, 71.39, 82.33, 87.11, 87.20, 88.47, 111.44, 113.20, 123.67, 127.12, 127.92, 128.00, 130.02, 134.62, 134.83, 135.19, 136.07, 143.85, 149.41, 150.37, 158.64, 163.96, 169.91. MS (FAB): m/z = 655 [M + Na]$^+$. HRMS (FAB): calcd for $C_{34}H_{36}N_2NaO_{10}$ [M + Na]$^+$, 655.2268, found, 655.2286.

3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-4′-methoxycarbonyl-2′-*O*-methyl-5-methyluridine (2). Under a nitrogen atmosphere, DIPEA (0.63 mL, 3.5 mmol) and i-$Pr_2NP(Cl)OCH_2CH_2CN$ (0.18 mL, 0.84 mmol) were added to a solution of compound **17** (444 mg, 0.702 mmol) in anhydrous CH_2Cl_2 (5.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with CH_2Cl_2. The combined organic layer was washed with sat. $NaHCO_3$, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (639 mg) was chromatographed (silica gel, 10 g, *n*-hexane–EtOAc = 1 : 1) to give **2** with a small amount of impurity (511 mg), which was reprecipitated from *n*-hexane–$CHCl_3$ to give compound **2** (450 mg, 77%) as a white powder.

Mp: 86–88 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.04–1.19 (m, 12H), 1.37 (d, J = 1.0 Hz, 2.1H), 1.38 (d, J = 1.0 Hz, 0.9H), 2.42–2.47 (m, 1.4H) 2.61–2.65 (m, 0.6H), 3.51–3.80 (m, 18H), 4.04–4.13 (m, 1H), 4.65–4.72 (m, 1H), 6.29 (d, J = 5.5 Hz, 0.7H), 6.32 (d, J = 6.0 Hz, 0.3H), 6.81–7.41 (m, 13H), 7.45 (d, J = 1.0 Hz, 0.3H), 7.51 (d, J = 1.0 Hz, 0.7H), 8.15–8.21 (m, 1H). ^{31}P NMR (161 MHz, $CDCl_3$) δ: 151.02, 151.56. MS (FAB): m/z = 833 [M + H]$^+$. HRMS (FAB): calcd for $C_{43}H_{54}N_4O_{11}P$ [M + H]$^+$, 833.3527, found, 833.3551.

(2′*R*)-2′-Azido-3′,5′-di-*O*-benzyl-4′-methoxycarbonylthymidine (19). Under a nitrogen atmosphere, $TMSCHN_2$ (2.0 M in *n*-hexane, 0.13 mL, 0.26 mmol) was added to a solution of compound

18[9] (107 mg, 0.211 mmol) in anhydrous THF–MeOH (1 : 1, 2.0 mL) at room temperature. The reaction mixture was stirred at ambient temperature for 0.5 h. The resulting mixture was concentrated *in vacuo* and the residue (113 mg) was purified by column chromatography (silica gel, 5 g, *n*-hexane–EtOAc = 2 : 1) to give compound **19** (97.5 mg, 89%) as a white foam.

Mp: 159–161 °C. $[\alpha]_D^{31}$ −50.9 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3179, 3066, 3031, 2954, 2927, 2878, 2110, 1755, 1691, 1664, 1455, 1376, 1274 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ: 1.67 (d, *J* = 1.0 Hz, 3H), 3.67 (s, 3H), 3.77 (dd, *J* = 6.0, 8.5 Hz, 1H), 3.79 (d, *J* = 10.0 Hz, 1H), 4.06 (d, *J* = 10.0 Hz, 1H), 4.41 (d, *J* = 6.0 Hz, 1H), 4.57 (d, *J* = 11.5 Hz, 1H), 4.62 (d, *J* = 11.5 Hz, 1H), 4.62 (d, *J* = 11.0 Hz, 1H), 4.81 (d, *J* = 11.0 Hz, 1H), 6.49 (d, *J* = 8.5 Hz, 1H), 7.28–7.39 (m, 11H), 8.31 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.29, 52.78, 63.82, 71.81, 74.04, 75.40, 81.50, 86.14, 89.81, 111.96, 127.75, 127.76, 128.22, 128.24, 128.27, 128.46, 128.48, 128.82, 128.84, 134.93, 136.45, 136.69, 150.39, 163.59, 168.95. MS (FAB): *m*/*z* = 522 $[M + H]^+$. HRMS (FAB): calcd for $C_{26}H_{28}N_5O_7$ $[M + H]^+$, 522.1998, found, 522.1973.

(2′*R*)-2′-Amino-3′,5′-di-*O*-benzyl-4′-methoxycarbonylthymidine (20). Under a nitrogen atmosphere, $NiCl_2$ (9.2 mg, 0.0709 mmol) and $NaBH_4$ (80.5 mg, 2.13 mmol) were added to a solution of compound **19** (370 mg, 0.709 mmol) in anhydrous THF–MeOH (1 : 1, 2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 0.5 h. The resulting mixture was filtrated and the filtrate was concentrated *in vacuo*. The residue (550 mg) was purified by column chromatography (silica gel, 20 g, $CHCl_3$–MeOH = 40 : 1) to give compound **20** (313 mg, 89%) as a white powder.

Mp: 75–78 °C. $[\alpha]_D^{28}$ −27.0 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3372, 3178, 3066, 3031, 2948, 2923, 2867, 1759, 1703, 1496, 1472, 1454, 1394, 1364, 1278 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ: 1.60 (d, *J* = 1.0 Hz, 3H), 3.58 (dd, *J* = 6.0, 8.5 Hz, 1H), 3.69 (s, 3H), 3.80 (d, *J* = 10.0 Hz, 1H), 4.09 (d, *J* = 10.0 Hz, 1H), 4.21 (d, *J* = 6.0 Hz, 1H), 4.58 (d, *J* = 11.5 Hz, 1H), 4.63 (d, *J* = 11.0 Hz, 1H), 4.66 (d, *J* = 11.5 Hz, 1H), 4.68 (d, *J* = 11.0 Hz, 1H), 6.14 (d, *J* = 8.5 Hz, 1H), 7.26–7.41 (m, 12H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.21, 52.63, 58.75, 72.46, 73.96, 75.56, 82.39, 89.12, 89.83, 111.31, 127.76, 128.08, 128.23, 128.27, 128.53, 128.59, 128.81, 130.79, 135.58, 137.01, 137.03, 151.00, 163.80, 169.85. MS (FAB): *m*/*z* = 496 $[M + H]^+$. HRMS (FAB): calcd for $C_{26}H_{30}N_3O_7$ $[M + H]^+$, 496.2086, found, 496.2070.

(2′*R*)-3′,5′-Di-*O*-benzyl-4′-methoxycarbonyl-2′-trifluoroacetamidothymidine (21). Under a nitrogen atmosphere, Et_3N (12 μL, 0.085 mmol) and CF_3CO_2Et (24 μL, 0.19 mmol) were added to a solution of compound **20** (84.4 mg, 0.170 mmol) in anhydrous MeOH (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1 h. The resulting mixture was concentrated *in vacuo* and the residue (134 mg) was purified by column chromatography (silica gel, 5 g, *n*-hexane–EtOAc = 1 : 1) to give compound **21** (92.3 mg, 92%) as a white foam.

Mp: 75–77 °C. $[\alpha]_D^{22}$ −19.8 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3235, 3070, 2928, 2863, 1709, 1556, 1469, 1386, 1276 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 1.89 (d, *J* = 0.5 Hz, 3H), 3.71 (s, 3H), 3.83 (d, *J* = 10.0 Hz, 1H), 4.03 (d, *J* = 10.0 Hz, 1H), 4.48 (d, *J* = 6.5 Hz, 1H), 4.51 (d, *J* = 11.0 Hz, 1H), 4.57 (d, *J* = 11.0 Hz, 1H), 4.59 (d, *J* = 11.5 Hz, 1H), 4.64 (d, *J* = 11.5 Hz, 1H), 4.71 (dt, *J* = 6.5, 8.0 Hz, 1H), 6.43 (d, *J* = 8.0 Hz, 1H), 7.25–7.41 (m, 11H), 7.68 (d, *J* = 8.0 Hz, 1H), 9.44 (brs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.16, 52.87, 55.66, 71.21, 73.96, 75.66, 79.59, 87.18, 90.06, 112.26, 115.50 (q, *J* = 286 Hz), 127.82, 127.86, 128.35, 128.40, 128.58, 128.69, 128.84, 128.95, 134.99, 136.19, 136.75, 150.84, 157.75 (q, *J* = 38 Hz), 163.63, 169.38. MS (FAB): *m*/*z* = 592 $[M + H]^+$. HRMS (FAB): calcd for $C_{28}H_{29}F_3N_3O_8$ $[M + H]^+$, 592.1908, found, 592.1886.

(2′*R*)-4′-Methoxycarbonyl-2′-trifluoroacetamidothymidine (23). Under a nitrogen atmosphere, a solution of compound **21** (95.5 mg, 0.162 mmol) in MeOH (4.0 mL) and cyclohexene (0.80 mL, 8.0 mmol) were added to a suspension of 20% $Pd(OH)_2$ on carbon (57.0 mg, 0.0810 mmol) in MeOH (1.0 mL) at room temperature. The reaction mixture was refluxed for 10 h. The resulting mixture was filtered and the filtrate was concentrated *in vacuo*. The residue (55.6 mg) was purified by column chromatography (silica gel, 10 g, $CHCl_3$–MeOH = 15 : 1) to give compound **23** (40.8 mg, 62%) as a white foam.

Mp: 108–110 °C. $[\alpha]_D^{22}$ −5.7 (*c* 1.00, MeOH). IR: ν_{max} (KBr): 3481, 3261, 3068, 2957, 2841, 1710, 1552, 1471, 1388, 1276 cm^{-1}. ^1H NMR (400 MHz, CD_3OD) δ: 1.90 (d, *J* = 1.0 Hz, 3H), 3.77 (s, 3H), 3.84 (d, *J* = 12.0 Hz, 1H), 4.08 (d, *J* = 12.0 Hz, 1H), 4.45 (d, *J* = 6.0 Hz, 1H), 4.75 (dd, *J* = 6.0, 8.5 Hz, 1H), 6.32 (d, *J* = 8.5 Hz, 1H), 7.81 (d, *J* = 1.0 Hz, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ: 12.48, 52.78, 56.83, 65.09, 72.89, 87.99, 93.60, 112.52, 117.20 (q, *J* = 285 Hz), 137.77, 152.68, 159.21 (q, *J* = 38 Hz), 166.17, 171.46. MS (FAB): *m*/*z* = 412 $[M + H]^+$. HRMS (FAB): calcd for $C_{14}H_{17}F_3N_3O_8$ $[M + H]^+$, 412.0969, found, 412.0966.

(2′*R*)-5′-*O*-(4,4′-Dimethoxytrityl)-4′-methoxycarbonyl-2′-trifluoroacetamidothymidine (25). Under a nitrogen atmosphere, DMTrCl (80.0 mg, 0.238 mmol) was added to a solution of compound **23** (61.1 mg, 0.149 mmol) in anhydrous pyridine (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (152 mg) was purified by column chromatography (silica gel, 10 g, $CHCl_3$–MeOH = 50 : 1) to give compound **25** (95.7 mg, 90%) as a white foam.

Mp: 127–130 °C. $[\alpha]_D^{22}$ −3.4 (*c* 0.66, $CHCl_3$). IR: ν_{max} (KBr): 3414, 3271, 3066, 2955, 2839, 1703, 1608, 1509, 1466, 1386, 1293, 1255 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 1.40 (d, *J* = 1.0 Hz, 1H), 3.08 (d, *J* = 4.0 Hz, 1H), 3.58 (d, *J* = 10.0 Hz, 1H), 3.69, (d, *J* = 10.0 Hz, 1H), 3.78 (s, 3H), 3.80 (s, 6H), 4.64 (dd, *J* = 4.0, 5.0 Hz, 1H), 4.39 (dt, *J* = 5.0, 8.5 Hz, 1H), 6.39 (d, *J* = 8.5 Hz, 1H), 6.86 (d, *J* = 8.5 Hz, 1H), 6.85–7.38 (m, 13H), 7.53 (d, *J* = 1.0 Hz, 1H), 8.06 (brs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 11.49, 52.97, 55.30, 55.72, 65.43, 72.78, 85.91, 88.01, 91.04, 112.44, 113.49, 115.57 (q, *J* = 286 Hz), 127.50, 128.27, 130.28, 134.49, 134.69, 135.63, 135.65, 143.58, 151.36, 157.38, 157.95 (q, *J* = 38 Hz), 158.94, 158.97, 164.39, 169.85. MS (FAB): *m*/*z* = 714 $[M + H]^+$, HRMS (FAB): calcd for $C_{35}H_{35}F_3N_3O_{10}$ $[M + H]^+$, 714.2276, found, 714.2247.

(2′*R*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-4′-methoxycarbonyl-2′-trifluoroacetamidothymidine (3). Under a nitrogen atmosphere, DIPEA (0.11 mL, 0.62 mmol) and i-$Pr_2NP(Cl)OCH_2CH_2CN$ (30 μL, 0.14 mmol) were added to a solution of compound **25** (88.7 mg, 0.124 mmol) in anhydrous CH_2Cl_2 (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 2 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with CH_2Cl_2. The combined organic layer was washed with sat. $NaHCO_3$, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (122 mg) was chromatographed (silica gel, 10 g, $CHCl_3$–MeOH = 50 : 1) to give **6** with a small amount of impurity (119 mg), which was reprecipitated from *n*-hexane–$CHCl_3$ to give compound **3** (102 mg, 95%) as a white powder.

Mp: 85–88 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.07–1.16 (m, 12H), 1.40 (d, J = 1.0 Hz, 1.5H), 1.43 (d, J = 1.0 Hz, 1.5H), 2.36–2.80 (m, 2H), 3.45–3.80 (m, 15H), 4.86–5.08 (m, 2H), 6.35 (d, J = 6.0 Hz, 0.5 H), 6.40 (d, J = 6.0 Hz, 0.5H), 6.86–7.52 (m, 15H), 7.95–7.99 (m, 1H). ^{31}P NMR (160 MHz, $CDCl_3$) δ: 151.96, 153.04. MS (FAB): m/z = 914 $[M + H]^+$. HRMS (FAB): calcd for $C_{44}H_{52}F_3N_5O_{11}P$ $[M + H]^+$, 914.3355, found, 914.3348.

(2′*R*)-2′-Acetamido-3′,5′-di-*O*-benzyl-4′-methoxycarbonylthymidine (22). Under a nitrogen atmosphere, Ac_2O (80 μL, 0.85 mmol) was added to a solution of compound **20** (352 mg, 0.710 mmol) in anhydrous pyridine (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1.5 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (438 mg) was purified by column chromatography (silica gel, 15 g, $CHCl_3$–MeOH = 30 : 1) to give compound **22** (408 mg, quant.) as a white foam.

Mp: 85–88 °C. $[\alpha]_D^{24}$ −31.4 (c 1.10, $CHCl_3$). IR: ν_{max} (KBr): 3317, 3204, 3067, 3033, 2952, 2927, 2867, 1762, 1683, 1543, 1455, 1374, 1283 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.56 (d, J = 1.0 Hz, 3H), 1.84 (s, 3H), 3.67 (s, 3H), 3.81 (d, J = 10.0 Hz, 1H), 4.03 (d, J = 10.0 Hz, 1H), 4.39 (d, J = 6.0 Hz, 1H), 4.53 (d, J = 11.5 Hz, 1H), 4.57 (d, J = 11.5 Hz, 1H), 4.60 (d, J = 12.0 Hz, 1H), 4.68 (d, J = 12.0 Hz, 1H), 4.77 (dt, J = 6.0, 8.5 Hz), 6.41 (d, J = 8.5 Hz, 1H), 6.98 (d, J = 8.5 Hz), 7.26–7.39 (m, 10H), 7.51 (d, J = 1.0 Hz, 1H), 9.84 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.16, 22.76, 52.62, 55.45, 71.72, 73.85, 75.48, 80.68, 86.61, 90.07, 111.83, 127.75, 128.11, 128.14, 128.18, 128.21, 128.45, 128.50, 128.75, 135.46, 136.89, 137.00, 151.15, 163.83, 169.42, 171.02. MS (FAB): m/z = 538 $[M + H]^+$. HRMS (FAB): calcd for $C_{28}H_{32}N_3O_8$ $[M + H]^+$, 538.2191, found, 538.2194.

(2′*R*)-2′-Acetamido-4′-methoxycarbonylthymidine (24). Under a nitrogen atmosphere, a solution of compound **22** (380 mg, 0.707 mmol) in THF–MeOH (1 : 1, 8.0 mL) and cyclohexene (3.6 mL, 35 mmol) were added to a suspension of 20% $Pd(OH)_2$ on carbon (199 mg, 0.283 mmol) in THF–MeOH (1 : 1, 2.0 mL) at room temperature. The reaction mixture was refluxed for 10 h. The resulting mixture was filtered and the filtrate was concentrated *in vacuo*. The residue (330 mg) was purified by column chromatography (silica gel, 10 g, $CHCl_3$–MeOH = 7 : 1 → 4 : 1) to give compound **24** (181 mg, 72%) as a white powder.

Mp: 162–163 °C. $[\alpha]_D^{27}$ −15.2 (c 0.85, MeOH). IR: ν_{max} (KBr): 3483, 3288, 3066, 2961, 2833, 1682, 1530, 1471, 1439, 1377, 1291 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD) δ: 1.89 (s, 3H), 1.94 (s, 3H), 3.75 (s, 3H), 3.81 (d, J = 11.5 Hz, 1H), 4.05 (d, J = 11.5 Hz, 1H), 4.34 (d, J = 6.0 Hz, 1H), 4.70 (dd, J = 6.0, 9.0 Hz, 1H), 6.20 (d, J = 9.0 Hz, 1H), 7.81 (s, 1H). ^{13}C NMR (75 MHz, CD_3OD) δ: 12.48, 22.37, 52.68, 56.35, 65.30, 73.23, 88.12, 93.46, 112.11, 138.04, 152.82, 166.22, 171.67, 173.82. MS (FAB): m/z = 358 $[M + H]^+$. HRMS (FAB): calcd for $C_{14}H_{20}N_3O_8$ $[M + H]^+$, 358.1252, found, 358.1252.

(2′*R*)-2′-Acetamido-5′-*O*-(4,4′-dimethoxytrityl)-4′-methoxycarbonylthymidine (26). Under a nitrogen atmosphere, DMTrCl (186 mg, 0.550 mmol) was added to a solution of compound **24** (65.5 mg, 0.183 mmol) in anhydrous pyridine (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (315 mg) was purified by column chromatography (silica gel, 10 g, $CHCl_3$–MeOH = 20 : 1) to give compound **26** (123 mg, quant.) as a white foam.

Mp: 142–144 °C. $[\alpha]_D^{27}$ −19.5 (c 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3489, 3323, 3188, 3063, 3017, 2953, 2835, 1694, 1607, 1579, 1509, 1465, 1378, 1291 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.34 (d, J = 1.0 Hz, 3H), 1.99 (s, 3H), 3.51 (d, J = 10.0 Hz, 1H), 3.69 (d, J = 10.0 Hz, 1H), 3.64 (d, J = 4.5 Hz, 1H), 3.73 (s, 3H), 3.78 (s, 6H), 4.57 (dd, J = 4.5, 5.5 Hz, 1H), 5.02 (dt, J = 5.5, 8.5 Hz, 1H), 6.36 (d, J = 8.5 Hz, 1H), 6.64 (d, J = 8.5 Hz, 1H), 6.84–7.42 (m, 13H), 7.55 (d, J = 1.0 Hz, 1H), 9.16 (brs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 11.56, 23.00, 52.84, 54.95, 55.26, 65.56, 72.71, 85.99, 87.74, 90.75, 112.05, 113.40, 127.33, 128.14, 128.25, 130.23, 130.25, 134.55, 134.77, 135.29, 143.63, 151.26, 158.85, 163.63, 170.21, 171.11. MS (FAB): m/z = 660 $[M + H]^+$. HRMS (FAB): calcd for $C_{35}H_{38}N_3O_{10}$ $[M + H]^+$, 660.2559, found, 660.2538.

(2′*R*)-2′-Acetamido-3′-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-4′-methoxycarbonylthymidine (4). Under a nitrogen atmosphere, DIPEA (0.15 mL, 0.86 mmol) and i-$Pr_2NP(Cl)OCH_2CH_2CN$ (45 μL, 0.21 mmol) were added to a solution of compound **26** (113 mg, 0.127 mmol) in anhydrous CH_2Cl_2 (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 4 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with CH_2Cl_2. The combined organic layer was washed with sat. $NaHCO_3$, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (210 mg) was chromatographed (silica gel, 10 g, $CHCl_3$–MeOH = 20 : 1) to give **4** with a small amount of impurity (113 mg), which was reprecipitated from *n*-hexane–$CHCl_3$ to give compound **4** (103 mg, 68%) as a white powder.

Mp: 92–95 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.08–1.18 (m, 12H), 1.35 (s, 1.8H), 1.37 (s, 1.2H), 2.00 (s, 1.8H), 2.03 (s, 1.2H), 2.40–2.64 (m, 2H), 3.51–3.80 (m, 15H), 4.68–4.76 (m, 1H), 4.99–5.07 (m, 1H), 6.23–6.41 (m, 2H), 6.83–7.43 (m, 13H), 7.53 (m, 1H), 8.15–8.21 (m, 1H). ^{31}P NMR (160 MHz,

$CDCl_3$) δ: 152.39, 152.99. MS (FAB): m/z = 860 $[M + H]^+$. HRMS (FAB): calcd for $C_{44}H_{55}N_5O_{11}P$ $[M + H]^+$, 860.3637, found, 860.3608.

Synthesis of oligonucleotides

Phosphoramidites **1–4** were used and the 0.2 μmol scale synthesis of oligonucleotides was performed on an automated DNA synthesizer (Applied Biosystems Expedite™ 8909) using a standard phosphoramidite protocol (DMTr-ON mode). **ON1–6** were prepared by cleavage from the CPG supports, deprotection of nucleobase and phosphate moieties, and conversion of 4′-carboxyl units by treatment with the corresponding base [50 mM NaOH aq., rt, 1.5 h then 55 °C, 12 h (for **ON1a–6a**); 50 mM K_2CO_3 in MeOH, rt, 2 h (for **ON1b–4b**); 28% NH_3 aq., rt, 1.5 h then 55 °C, 12 h (for **ON1c–4c**); or 40% MeNH aq., rt, 1.5 h then 55 °C, 12 h (for **ON1d–4d**)]. After neutralization with 1% HCl aq. for **ON1a–6a** and **ON1b–4b**, the solvent was concentrated *in vacuo*. For **ON1a–6a** and **ON1b–4b**, removal of ammonia or methylamine was carried out *in vacuo*. The crude **ON1–6** were purified with Sep-Pak® Plus C18 cartridges (Waters) followed by reversed-phase HPLC (Waters XBridge® MS C_{18} 2.5 μm, 10 × 50 mm). The composition of the **ON1–6** was confirmed by MALDI-TOF mass analysis. Yields and MALDI-TOF-MS data ($[M - H]^-$) for **ON1–6**; **ON1a**, 43% yield, found 3676.23 (calcd 3676.37); **ON1b**, 30% yield, found 3690.24 (calcd 3690.40); **ON1c**, 30% yield, found 3674.55 (calcd 3675.39); **ON1d**, 28% yield, found 3689.01 (calcd 3689.51); **ON2a**, 38% yield, found 3706.23 (calcd 3706.40); **ON2b**, 33% yield, found 3720.98 (calcd 3720.42); **ON2c**, 32% yield, found 3705.91 (calcd 3705.41); **ON2d**, 29% yield, found 3719.61 (calcd 3719.44); **ON3a**, 43% yield, found 3690.69 (calcd 3691.39); **ON3b**, 51% yield, found 3706.06 (calcd 3705.41); **ON3c**, 40% yield, found 3689.73 (calcd 3690.40); **ON3d**, 61% yield, found 3704.78 (calcd 3704.43); **ON4a**, 36% yield, found 3732.43 (calcd 3733.42); **ON4b**, 50% yield, found 3746.94 (calcd 3747.45); **ON4c**, 52% yield, found 3731.93 (calcd 3732.44); **ON4d**, 28% yield, found 3746.03 (calcd 3746.46); **ON5a**, 29% yield, found 3765.21 (calcd 3764.39); **ON6a**, 49% yield, found 3764.75 (calcd 3764.39).

UV melting experiments

UV melting experiments were carried out on SHIMADZU UV-1650 and SHIMADZU UV-1800 spectrophotometers equipped with T_m analysis accessory. **ON1–6** and ssDNA or ssRNA were dissolved in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl to give a final concentration of each strand of 4 μM. The samples were annealed by heating at 100 °C followed by slow cooling to 15 °C. The melting profiles were recorded at 260 nm from 15 °C to 85 °C at a scan rate of 0.5 °C min^{-1}. The two-point average method was employed to obtain the T_m values and the final ones were determined by averaging three independent measurements which were accurate to within 1 °C.

CD measurements

Under the same conditions as those of UV melting experiments, CD measurements were carried out from 350 nm to 200 nm at 4 °C on a JASCO J-720W spectropolarimeter.

Conclusions

The synthesis of oligonucleotides including 16 kinds of 4′-carboxythymidines was achieved by base treatment after oligonucleotide synthesis. Evaluation of their duplex-forming ability with DNA or RNA complements found that 4′-carboxythymidine led to an increase in stability of the duplex formed with RNA. In the recent report by Leumann's group, tc-DNA with a hexadodecyloxycarbonyl unit enhanced cellular uptake.[12] Therefore, the carboxyl group of 4′-carboxythymidine that we synthesized in this work might be practically useful as a prodrug functional group. Thus, 4′-carboxythymidine was considered to be a potent candidate for targeting RNA.

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