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Synthesis and properties of thymidines with six-membered amide bridge

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ABSTRACT

Artificial thymidine monomers possessing amide or N-methylamide bridges were designed, synthesized, and introduced into oligonucleotides. UV-melting experiments showed that these oligonucleotides preferred single-stranded RNA (ssRNA) to single-stranded DNA (ssDNA) in duplex formation. Both amide- and N-methylamide-modified oligonucleotides led to a significant increase in the binding affinity to ssRNA by up to +4.7 and +3.7 °C of the T_m value per modification, respectively, compared with natural oligonucleotide. In addition, their oligonucleotides showed high stability against 3′-exonuclease.

1. Introduction

Artificial nucleic acids that possess strong and sequence-selective binding affinities with single-stranded RNA (ssRNA) and/or high nuclease-resistant properties are promising tools for nucleic acid-based technologies such as the antisense method.[1,2] In fact, a large number of nucleic acid derivatives have been developed to date.[1] The breakthrough was the discovery by us[3] and Wengel's group[4] of 2′,4′-BNA/LNA with a 2′-*O*,4′-*C*-methylene-bridged structure in the sugar moiety, (Fig. 1). The 2′,4′-BNA/LNA modification of oligonucleotides leads to a great improvement in hybridizing ability with ssRNA and an increase in nuclease resistance compared with natural oligonucleotides. Since 2′,4′-BNA/LNA was reported, the development of artificial nucleic acids with additional 2′,4′-bridged structures has attracted much attention. In particular, ring-enlargement from a five-membered bridge to a six-membered one in 2′,4′-bridged structures would be a useful modification. For example, ENA, the six-membered analog of 2′,4′-BNA/LNA, shows the same level of duplex-hybridizing ability with ssRNA as does 2′,4′-BNA/LNA, and improved enzymatic stability as compared to 2′,4′-BNA/LNA (Fig. 1).[5,6] Recently, we developed AmNA with five-membered amide bridge, the duplex-forming ability of which was comparable to that of 2′,4′-BNA/LNA, and the nuclease resistance of which was superior to that of 2′,4′-BNA/LNA.[7] Against this background, we were interested in the properties of the ring-enlarged analog of AmNA shown in Figure 2. In addition, various substituents could be introduced into the nitrogen in the amide bridge to improve the functions of the oligonucleotides. In this study, two thymidines with a six-membered amide bridge, that is, NH and NMe analogs, were synthesized, and the duplex-forming abilities and the nuclease resistances of their oligonucleotides were evaluated.

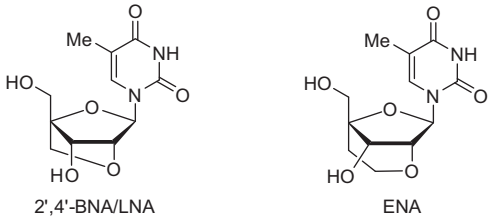

Figure 1. Structures of 2′,4′-BNA/LNA and ENA monomers.

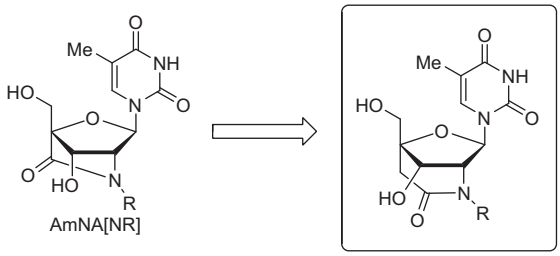

Figure 2. Design of nucleic acid monomers (R = H or Me) used in this study.

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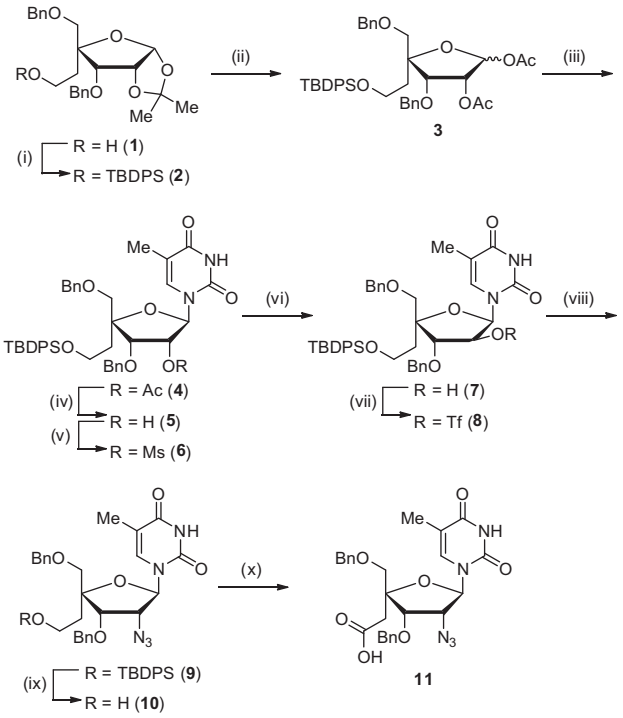


Scheme 1. Reagents and conditions: (i) TBDPSCl, DMAP, CH_2Cl_2, rt, 16 h, quant.; (ii) Ac_2O, AcOH, concd H_2SO_4, rt, 2 h, 91%; (iii) thymine, BSA, TMSOTf, MeCN, reflux, 3.5 h, 84%; (iv) K_2CO_3, MeOH, rt, 1 h, 95%; (v) MsCl, Et_3N, CH_2Cl_2, 0 °C, 1.5 h, 91%; (vi) NaOH, EtOH, rt, 12 h, 89%; (vii) Tf_2O, pyridine, CH_2Cl_2, 0 °C, 0.5 h, 83%; (viii) NaN_3, DMF, rt, 13 h, quant.; (ix) TBAF, THF, rt, 17 h, quant.; (x) PDC, MS4Å, DMF, rt, 12 h, 94%.

2. Results and discussion

2.1. Synthesis

The silylation of known compound **1**[5] using TBDPSCl gave **2** in quantitative yield (Scheme 1); **2** was converted to diacetate **3**. The reaction of **3** with silylated thymine, prepared in situ from thymine and *N,O*-bis(trimethylsilyl)acetamide (BSA), in the presence of TMSOTf produced the desired β-isomer **4**. Next, introduction of a nitrogen atom at the 2′-position was performed using a double-stereoinversion approach. After deacetylation of **4** on exposure to K_2CO_3 in MeOH, stereoinversion of the 2′-hydroxyl group was achieved by mesylation of the resulting **5**, followed by treatment with NaOH. Then, **7** underwent reaction with Tf_2O to give **8** in 83% yield, and compound **9**, with a nitrogen atom at the 2′-position, was obtained by azidation. Desilylation of **9**, followed by oxidation of **10** with PDC in DMF, efficiently produced carboxylic acid **11**.

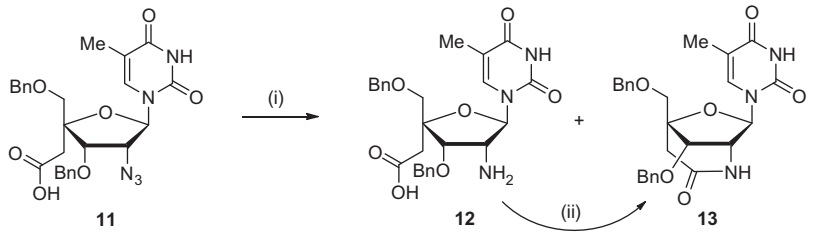
Next, reduction of the azide group in **11** was examined (Scheme 2). Reduction by $NaBH_4$ in *i*-PrOH gave the corresponding amine **12** in 55% yield. Fortunately, under Staudinger conditions using Me_3P, ring-closed **13** was produced together with **12**. Compound **12** was converted to the desired **13** using EDC or MsCl; the yields were 72% and 86%, respectively.

The synthesis of phosphoramidite **14** was carried out in three steps from **13** (Scheme 3). Diol **15** was prepared by hydrogenolysis of **13**. Then, dimethoxytritylation of **15**, followed by phosphitylation, yielded the desired phosphoramidite **14**. Concerning the *N*-methyl congener **17**, protection of the imide nitrogen of thymine in **13**, and successive methylation of **18** gave **19**, which was hydrogenolyzed to produce diol **20**. Using a similar synthetic route to **14**, **20** was converted into the desired phosphoramidite **17** with an N-methylamide bridge via dimethoxytritylated **21**. The phosphoramidites **14** and **17** obtained were used to synthesize modified oligonucleotides **22**–**29** on an automated DNA synthesizer (see the Section 4 for details). These amide bridges were stable under conventional conditions, that is, aqueous ammonia or methanolic K_2CO_3, for cleavage from the resin and removal of β-cyanoethyl groups on the phosphates.

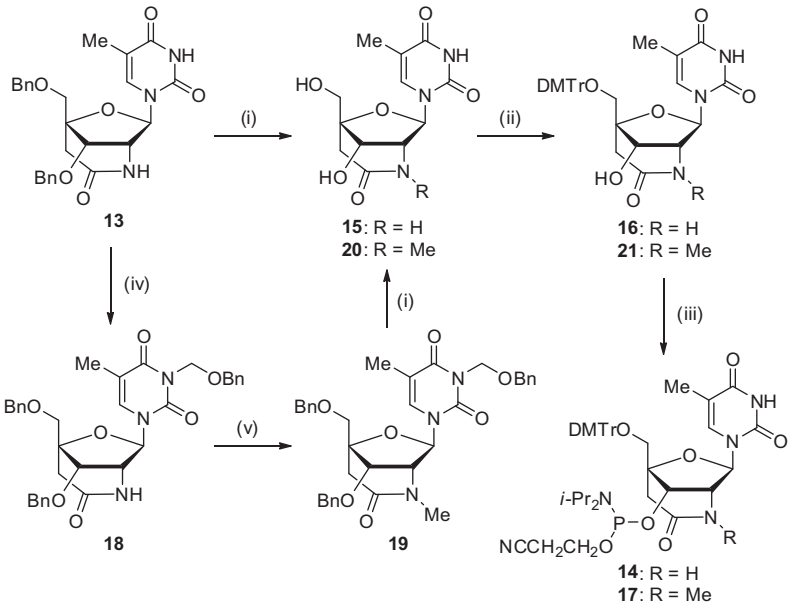
The duplex-forming abilities of oligonucleotides **22**–**27** with ssDNA or ssRNA were evaluated and the results are summarized in Table 1. Regardless of whether it was the NH or NMe analog, the modified oligonucleotides generally showed a significantly decreased affinity for ssDNA compared with natural oligonucleotide **30**, although in the case of **24**, possessing three NH analogs, the affinity was increased by +1.7 °C per modification. Concerning ssRNA, the single-modified oligonucleotides **22** and **25** had almost the same affinities as natural oligonucleotide **30**. However, by multiple modifications, the binding affinity was greatly increased and the changes in T_m value per modification (ΔT_m/mod.) of the NH (**24**) or NMe (**27**) analogs were +4.7 and +3.7 °C, respectively. These results demonstrate that oligonucleotides containing these thymidines with six-membered amide bridges formed stable duplexes with ssRNA and recognized ssRNA more selectively than ssDNA in the duplex formation.

The binding affinities with ssDNA and ssRNA of the oligonucleotides modified by AmNA[NH] or AmNA[NMe] with a five-membered amide bridge, shown in Figure 2, and those of the oligonucleotides modified by HxNA[NMe] with a six-membered hydroxamate bridge, shown in Figure 3, were evaluated in our previous reports.[7,8] Although these analogs showed lower duplex-forming abilities with ssRNA than AmNA[NH] or AmNA[NMe], one of these analogs, the NH analog, was more stably bound to ssRNA compared with HxNA[NMe] with a six-membered bridge similar to these amide bridges.

The stabilities of oligonucleotides **28** and **29** including these analogs against 3′-exonuclease were determined and compared with those of AmNA[NH]-, AmNA[NMe]-, and HxNA[NMe]-modified oligonucleotides **31**-**33** and natural **30** (Fig. 4 and the sequences of oligonucleotides used are shown in the legend of Fig. 4). Under conditions where natural **30** decomposed completely within 5 min, over 50% and 60% of **28** and **29** remained after 40 min. The NMe analog (**29**) showed higher stability against nuclease than the NH analog (**28**) did. This is probably because ac-



Scheme 2. Reagents and conditions: (i) $NaBH_4$, *i*-PrOH, reflux, 1 h, 55% (**12**) or Me_3P, THF/H_2O (5:1), rt, 14 h, ca 35% (**12**) and 43% (**13**); (ii) EDC, DMAP, CH_2Cl_2, rt, 24 h, 72% or MsCl, Et_3N, CH_2Cl_2, 0 °C to rt, 17 h, 86%.



Scheme 3. Reagents and conditions: (i) H_2, 20% $Pd(OH)_2$-C, MeOH, rt, 24 h, 61% (**15**) or 20% $Pd(OH)_2$-C, cyclohexene, EtOH, reflux, 2 h, quant. (**20**); (ii) DMTrCl, pyridine, rt, 3–17 h, 81% (**16**) and 69% (**21**); (iii) $(i\text{-}Pr_2N)_2POCH_2CH_2CN$, 1*H*-tetrazole, MeCN/THF (3:1), rt, 12 h, 42% (**14**) or $i\text{-}Pr_2NP(Cl)OCH_2CH_2CN$, $i\text{-}Pr_2NEt$, CH_2Cl_2, rt, 2 h, 73% (**17**); (iv) $BnOCH_2Cl$, DBU, DMF, 0 °C, 0.5 h, 86%; (v) NaH, MeI, DMF, 0 °C, 2 h, 60%.

Table 1
T_m values (°C) of duplexes formed between oligonucleotides and ssDNA or ssRNA[a,b]

Oligonucleotide	ssDNA	ssRNA
5′-TTTTTTTTTT-3′ (**30**)	21	19
5′-TTTTTTTTTT-3′ (**22**)	16 (−5.0)	20 (+1.0)
5′-TTTTTTTTTT-3′ (**23**)	19 (−1.0)	27 (+4.0)
5′-TTTTTTTTTT-3′ (**24**)	26 (+1.7)	33 (+4.7)
5′-TTTTTTTTT-3′ (**25**)	15 (−6.0)	20 (+1.0)
5′-TTTTTTTTT-3′ (**26**)	13 (−4.0)	26 (+3.5)
5′-TTTTTTTTT-3′ (**27**)	18 (−1.0)	30 (+3.7)

[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 ssDNA and ssRNA are 5′-d(AAAAAAAAAA)-3′ and 5′-r(AAAAAAAAAA)-3′, respectively. *T* and T indicate NH analog and NMe analog, respectively.

[b] The change in T_m value per modification is shown in parentheses.

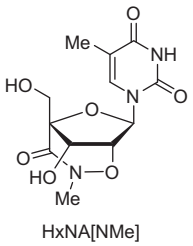


Figure 3. Structure of HxNA[NMe] monomer.

cess of the nuclease to the phosphodiester linkage was prevented by the more bulky N-methylamide bridge. In a comparison of **28** and **29** with **31** and **32**, respectively, no significant improvement in nuclease resistance as a result of enlargement of the amide bridge was observed. However, oligonucleotides **28** and **29** were quite stable compared with **33**, including an HxNA[NMe] with the same six-membered ring size. In contrast, the six-membered hydroxamate bridge of HxNA was opened under the nuclease treatment conditions, and, consequently, the 5′-phosphate moiety of HxNA showed high resistance against nuclease.[8] However, in the

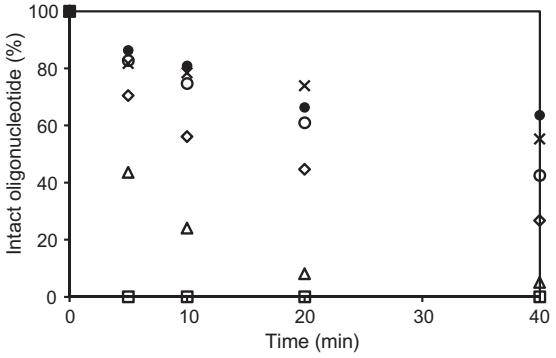


Figure 4. Enzymatic stability of oligonucleotides **28** and **29**. Conditions: 1.75 μg/mL Crotalus admanteus venom phosphodiesterase (CAVP), 10 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.0), and 7.5 μM each oligonucleotide at 37 °C. The sequence of oligonucleotides used was 5′-TTTTTTTTT-3′. T = NH analog (open circle, oligonucleotide **28**), NMe analog (closed circle, oligonucleotide **29**), AmNA[NH] (open diamond, oligonucleotide **31**), AmNA[NMe] (cross, oligonucleotide **32**), HxNA[NMe] (open triangle, oligonucleotide **33**) and natural (open square, oligonucleotide **30**).

cases of these NMe and NH analogs bearing a six-membered amide bridge, as well as AmNA[NH] and AmNA[NMe] bearing a five-membered amide bridge, the 5′-phosphate moieties were easily degradable and such a phenomenon was not observed. These results suggest that resistance against nuclease was greatly affected by not only the bridge size but also by the composition of the bridge, for example, whether there is any substituent and/or heteroatom, or the location of the substituent and/or heteroatom.

3. Conclusion

Two thymidines bearing six-membered bridges, that is, amide and N-methylamide bridges, were successfully synthesized and introduced into oligonucleotides, using an automated DNA synthesizer. These oligonucleotides formed stable duplexes with ssRNA, but the duplexes with ssDNA were destabilized. They also had high nuclease resistance, which were slightly better than those of

AmNAs bearing five-membered amide bridges. Moreover, their nuclease resistances were much higher than that of HxNA[NMe] with the same bridge size. These findings demonstrated that the composition of the bridge moiety is a key factor in the development of bridged nucleic acids. We believe that the accumulation of properties such as duplex-forming ability and nuclease resistance through the development of various bridged nucleic acids will contribute to developing ideal useful tools for nucleic acid-based technologies.

4. Experimental

4.1. General

All chemicals were purchased from chemical suppliers. For column chromatography, Fuji Silysia silica gel PSQ-100B and FL-100D were used. All melting points were measured on a Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra were recorded on a JEOL ECS400 spectrometer. IR spectra were recorded on JASCO FT/IR-200 and JASCO FT/IR-4200 spectrometers. Optical rotations were recorded on a JASCO DIP-370 instrument. Mass spectra were measured on a JEOL JMS-600 or JEOL JMS-700 mass spectrometer. MALDI-TOF mass spectra were recorded on a Bruker Daltonics Autoflex II TOF/TOF mass spectrometer.

4.2. 3,5-Di-*O*-benzyl-4-(2-*tert*-butyldiphenylsiloxyethyl)-1,2-*O*-isopropylidene-α-D-ribofuranose (2)

Under nitrogen atmosphere, DMAP (350 mg, 2.87 mmol), Et_3N (4.0 mL, 29 mmol), and TBDPSCl (4.0 mL, 15 mmol) were added to a solution of alcohol **1**[5] (3.96 g, 9.56 mmol) in CH_2Cl_2 (100 mL) at 0 °C. The reaction mixture was stirred at room temperature for 16 h. The reaction was then quenched with satd $NaHCO_3$ aq and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (8.82 g) was purified by column chromatography (silica gel 200 g, *n*-hexane/EtOAc = 10:1) to give silyl ether **2** (6.28 g, quant.) as a colorless oil.

$[\alpha]_D^{25}$ +16.3 (*c* 1.00, $CHCl_3$). IR (KBr): 3068, 3031, 2932, 2857, 1496, 1472, 1454, 1428, 1383, 1312, 1256, 1209 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.00 (s, 9H), 1.30 (s, 3H), 1.50 (s, 3H), 1.87 (ddd, *J* = 7.0, 8.0, 14.5 Hz, 1H), 2.43 (ddd, *J* = 5.0, 6.5, 14.5 Hz, 1H), 3.29 (d, *J* = 10.5 Hz), 1H, 3.70 (d, *J* = 10.5 Hz, 1H), 3.82 (ddd, *J* = 5.0, 7.0, 12.0 Hz, 1H), 3.94 (ddd, *J* = 6.5, 8.0, 12.0 Hz, 1H), 4.21 (d, *J* = 5.5 Hz, 1H), 4.35 (d, *J* = 12.5 Hz, 1H), 4.47 (d, *J* = 12.5 Hz, 1H), 4.53 (d, *J* = 12.0 Hz, 1H), 4.60 (dd, *J* = 4.0, 5.5 Hz, 1H), 4.74 (d, *J* = 12.0 Hz, 2H), 5.74 (d, *J* = 4.0 Hz, 1H), 7.19–7.67 (m, 20H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 19.08, 26.15, 26.59, 26.80, 34.93, 59.78, 72.41, 73.37, 77.88, 79.26, 86.65, 104.16, 113.01, 127.50, 127.52, 127.57, 127.72, 127.80, 128.29, 128.32, 129.45, 133.87, 135.57, 138.08, 138.21. MS (FAB): *m*/*z* 675 (MNa^+). HRMS (FAB): calcd for $C_{40}H_{48}O_6SiNa$ (MNa^+) 675.3118, found 675.3115.

4.3. 1,2-Di-*O*-acetyl-3,5-di-*O*-benzyl-4-(2-*tert*-butyldiphenylsiloxyethyl)-D-ribofuranose (3)

Under nitrogen atmosphere, Ac_2O (10 mL, 110 mmol) and H_2SO_4 (0.1% in AcOH, 4.0 mL) were added to a solution of silyl ether **2** (5.86 g, 8.98 mmol) in AcOH (30 mL) at room temperature. The reaction mixture was stirred at room temperature for 2 h. The reaction was then quenched with satd $NaHCO_3$ aq and extracted with EtOAc. The combined organic layer was washed with satd $NaHCO_3$ aq, water and brine, dried over Na_2SO_4 and concentrated. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (6.18 g) was purified by column chromatography (silica gel 200 g, *n*-hexane/EtOAc = 5:1) to give diacetate **3** as a colorless oil (5.67 g, 91%).

$[\alpha]_D^{24}$ −16.3 (*c* 1.00, $CHCl_3$). IR (KBr): 3069, 3031, 2931, 2857, 1748, 1496, 1472, 1455, 1428, 1369, 1309, 1219 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.02 (s, 9H), 1.83 (s, 3H), 1.93 (s, 3H), 1.96–2.13 (m, 2H), 3.39 (d, *J* = 10.0 Hz, 1H), 3.50 (d, *J* = 10.0 Hz, 1H), 3.81–3.93 (m, 2H), 4.36–4.44 (m, 3H), 4.46 (d, *J* = 12.0 Hz, 1H), 4.57 (d, *J* = 12.0 Hz, 1H), 5.28 (d, *J* = 5.5 Hz, 1H), 6.03 (s, 1H), 7.21–7.67 (m, 20H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 19.09, 20.63, 20.93, 26.81, 35.75, 59.70, 73.16, 73.18, 73.44, 74.68, 78.07, 86.66, 97.61, 127.44, 127.49, 127.55, 127.57, 127.70, 127.75, 128.23, 128.32, 129.47, 133.87, 133.91, 135.53, 137.80, 138.15, 169.37, 169.70. MS (FAB): *m*/*z* 719 (MNa^+). HRMS (FAB): calcd for $C_{41}H_{48}O_8SiNa$ (MNa^+) 719.3016, found 719.2999.

4.4. 2′-*O*-Acetyl-3′,5′-di-*O*-benzyl-4′-(2-*tert*-butyldiphenylsiloxyethyl)-5-methyluridine (4)

Under nitrogen atmosphere, thymine (1.41 g, 11.2 mmol) and BSA (6.8 mL, 28 mmol) were added to a solution of diacetate **3** (4.36 g, 11.0 mmol) in MeCN (20 mL) at room temperature. The reaction mixture was refluxed for 1.5 h. TMSOTf (1.9 mL, 10 mmol) was added to the resulting mixture at 0 °C. The reaction mixture was refluxed for 3.5 h. The reaction was then quenched with satd $NaHCO_3$ aq and extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (5.71 g) was purified by column chromatography (silica gel 150 g, *n*-hexane/EtOAc = 3:2) to give acetate **4** as a white foam (5.14 g, 84%).

Mp: 45–48 °C. $[\alpha]_D^{25}$ +10.2 (*c* 1.00, $CHCl_3$). IR (KBr): 3172, 3068, 2930, 2857, 1747, 1693, 1470, 1428, 1372, 1234 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.01 (s, 9H), 1.48 (s, 3H), 1.76–1.83 (m, 1H), 2.04 (s, 3H), 2.03–2.11 (m, 1H), 3.41 (d, *J* = 10.5 Hz, 1H), 3.71–3.83 (m, 2H), 3.89 (d, *J* = 10.5 Hz, 1H), 4.35 (d, *J* = 6.5 Hz, 1H), 4.38 (d, *J* = 11.5 Hz, 1H), 4.39 (d, *J* = 9.5 Hz, 1H), 4.44 (d, *J* = 9.5 Hz, 1H), 4.57 (d, *J* = 11.5 Hz, 1H), 5.35 (dd, *J* = 5.0, 6.5 Hz, 1H), 6.08 (d, *J* = 5.0 Hz, 1H), 7.20–7.41 (m, 16H), 7.49 (s, 1H), 7.60–7.65 (m, 4H), 7.83 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.96, 19.06, 20.82, 26.82, 35.09, 59.27, 73.10, 73.38, 74.22, 75.24, 77.43, 86.03, 87.35, 111.21, 127.58, 127.66, 127.85, 128.01, 128.42, 128.59, 129.67, 133.47, 133.51, 135.51, 135.69, 137.32, 137.49, 150.28, 163.58, 170.05. MS (FAB): *m*/*z* 763 (MH^+). HRMS (FAB): calcd for $C_{44}H_{51}N_2O_8Si$ (MH^+) 763.3415, found 763.3402.

4.5. 3′,5′-Di-*O*-benzyl-4′-(2-*tert*-butyldiphenylsiloxyethyl)-5-methyluridine (5)

K_2CO_3 (450 mg, 330 mmol) was added to a solution of compound **4** (5.04 g, 6.61 mmol) in MeOH (10 mL) at room temperature. The reaction mixture was stirred at room temperature for 1 h. The reaction was then quenched with satd NH_4Cl aq and extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (4.76 g) was purified by column chromatography (silica gel 100 g, *n*-hexane/EtOAc = 4:3) to give alcohol **5** as a white foam 4.54 g, 95%).

Mp: 62–64 °C. $[\alpha]_D^{26}$ −10.3 (*c* 1.00, $CHCl_3$). IR (KBr): 3423, 3179, 3066, 2928, 2856, 1695, 1471, 1428, 1391, 1362, 1270 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.06 (s, 9H), 1.56 (s, 3H), 1.77 (ddd, *J* = 6.5, 8.0, 14.5 Hz, 1H), 2.14 (dt, *J* = 5.0, 14.5 Hz, 1H), 2.83 (d, *J* = 8.0 Hz, 1H), 3.45 (d, *J* = 10.5 Hz, 1H), 3.73–3.88 (m, 2H), 3.85 (d, *J* = 10.5 Hz, 1H), 4.14 (d, *J* = 6.0 Hz, 1H), 4.30 (m, 1H), 4.46 (d, *J* = 12.0 Hz, 1H), 4.49 (d, *J* = 12.0 Hz, 1H), 4.56 (d, *J* = 11.0 Hz, 1H), 4.63 (d, *J* = 11.0 Hz, 1H), 5.82 (d, *J* = 6.0 Hz, 1H), 7.22–7.65 (m,

21H), 8.12 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.04, 19.05, 26.83, 35.36, 59.36, 73.49, 74.05, 74.57, 75.02, 79.41, 87.03, 88.43, 111.93, 127.50, 127.64, 127.66, 128.01, 128.06, 128.25, 128.57, 128.60, 129.65, 129.68, 133.51, 135.52, 135.89, 137.02, 137.33, 150.80, 163.69. MS (FAB): *m*/*z* 721 (MH^+). HRMS (FAB): calcd for $C_{42}H_{49}N_2O_7Si$ (MH^+) 721.3309, found 721.3320.

4.6. 3′,5′-Di-*O*-benzyl-4′-(2-*tert*-butyldiphenylsiloxyethyl)-2′-*O*-methanesulfonyl-5-methyluridine (6)

Under nitrogen atmosphere, Et_3N (4.0 mL, 29 mmol) and MsCl (0.70 mL, 9.0 mmol) were added to a solution of alcohol **5** (3.26 g, 4.52 mmol) in CH_2Cl_2 (40 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1.5 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with CH_2Cl_2. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (3.80 g) was purified by column chromatography (silica gel 100 g, *n*-hexane/EtOAc = 4:3) to give mesylate **6** as a white foam (3.29 g, 91%).

Mp: 57–62 °C. $[\alpha]_D^{22}$ +214.8 (*c* 1.00, $CHCl_3$). IR (KBr): 3168, 3068, 3031, 2932, 2857, 1694, 1538, 1471, 1428, 1361, 1272 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.02 (s, 9H), 1.44 (s, 3H), 1.85 (ddd, *J* = 6.5, 8.0, 14.5 Hz, 1H), 2.10 (dt, *J* = 5.0, 14.5 Hz, 1H), 3.07 (s, 3H), 3.44 (d, *J* = 6.5 Hz, 1H), 3.70–3.86 (m, 2H), 3.95 (d, *J* = 6.5 Hz, 1H), 4.33 (d, *J* = 5.0 Hz, 1H), 4.37 (d, *J* = 12.0 Hz, 1H), 4.42 (d, *J* = 12.0 Hz, 1H), 4.45 (d, *J* = 12.0 Hz, 1H), 4.83 (d, *J* = 12.0 Hz, 1H), 5.26 (dd, *J* = 3.0, 5.0 Hz, 1H), 6.01 (d, *J* = 3.0 Hz, 1H), 7.18–7.63 (m, 21H), 8.29 (br s, 1H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 11.90, 19.08, 26.85, 35.11, 38.83, 59.13, 72.65, 73.40, 73.78, 76.39, 80.30, 86.74, 87.83, 111.27, 127.60, 127.68, 127.70, 128.11, 128.12, 128.47, 128.64, 129.70, 129.73, 133.42, 133.46, 135.28, 135.53, 137.11, 137.22, 150.46, 163.44. MS (FAB): *m*/*z* 799 (MH^+). HRMS (FAB): calcd for $C_{43}H_{48}N_2O_9F_3SiS$ (MH^+) 799.3085, found 799.3085.

4.7. 3′,5′-Di-*O*-benzyl-4′-(2-*tert*-butyldiphenylsiloxyethyl)-5-methylarabinouridine (7)

1 M NaOH aq (12 mL, 12 mmol) was added to a solution of mesylate **6** (3.16 g, 3.95 mmol) in EtOH (40 mL) at room temperature. The reaction mixture was stirred at room temperature for 12 h. The reaction was then quenched with satd NH_4Cl aq at 0 °C and extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (2.72 g) was purified by column chromatography (silica gel 100 g, *n*-hexane/EtOAc = 1:1) to give alcohol **7** as a white foam (2.55 g, 89%).

Mp: 56–58 °C. $[\alpha]_D^{26}$ +43.5 (*c* 1.00, $CHCl_3$). IR (KBr): 3357, 3181, 3068, 3031, 2929, 2856, 1695, 1472, 1455, 1428, 1390, 1362, 1281 1206 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.02 (s, 9H), 1.71 (d, *J* = 1.0 Hz, 3H), 1.88–1.93 (m, 2H), 3.52 (d, *J* = 10.5 Hz, 1H), 3.72–3.86 (m, 2H), 3.91 (d, *J* = 10.5 Hz, 1H), 4.14 (d, *J* = 4.0 Hz, 1H), 4.26 (d, *J* = 8.0 Hz, 1H), 4.39 (ddd, *J* = 4.0, 5.0, 8.0 Hz, 1H), 4.45 (d, *J* = 12.0 Hz, 1H), 4.49 (d, *J* = 11.5 Hz, 1H), 4.51 (d, *J* = 11.5 Hz, 1H), 4.74 (d, *J* = 12.0 Hz, 1H), 5.96 (d, *J* = 5.0 Hz, 1H), 7.21–7.65 (m, 20H), 7.48 (d, *J* = 1.0 Hz, 1H), 9.01 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.27, 19.09, 26.86, 35.16, 59.59, 72.65, 73.18, 73.70, 74.66, 83.80, 84.65, 85.07, 109.27, 127.66, 127.83, 127.93, 128.33, 128.39, 128.70, 129.69, 129.72, 133.44, 135.50, 136.62, 137.40, 137.55, 150.67, 163.99. MS (FAB): *m*/*z* 721 (MH^+). HRMS (FAB): calcd for $C_{42}H_{49}N_2O_7Si$ (MH^+) 721.3309, found 721.3300.

4.8. 3′,5′-Di-*O*-benzyl-4′-(2-*tert*-butyldiphenylsiloxyethyl)-5-methyl-2′-*O*-(trifluoromethanesulfonyl)arabinouridine (8)

Under nitrogen atmosphere, pyridine (1.9 mL, 17 mmol) and Tf_2O (1.4 mL, 8.6 mmol) were added to a solution of alcohol **7** (2.07 g, 2.87 mmol) in CH_2Cl_2 (30 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 0.5 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with CH_2Cl_2. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (2.56 g) was purified by column chromatography (silica gel 100 g, *n*-hexane/EtOAc = 2:1) to give triflate **8** as a white foam (2.03 g, 83%).

Mp: 37–40 °C. $[\alpha]_D^{25}$ +37.1 (*c* 1.00, $CHCl_3$). IR (KBr): 3190, 3069, 2930, 2858, 1695, 1455, 1426, 1245, 1215, 1143 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.03 (s, 9H), 1.67 (s, 3H), 1.81–1.95 (m, 2H), 3.36 (d, *J* = 10.5 Hz, 1H), 3.69 (d, *J* = 10.5 Hz, 1H), 3.72–3.86 (m, 2H), 4.38 (d, *J* = 12.0 Hz, 1H), 4.43 (d, *J* = 12.0 Hz, 1H), 4.48 (d, *J* = 11.5 Hz, 1H), 4.57 (d, *J* = 4.5 Hz, 1H), 4.72 (d, *J* = 11.5 Hz, 1H), 5.35 (dd, *J* = 4.5, 5.5 Hz, 1H), 6.19 (d, *J* = 5.5 Hz, 1H), 7.19–7.63 (m, 21H), 8.10 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.21, 19.00, 26.77, 34.04, 59.04, 71.23, 73.32, 73.47, 80.48, 81.51, 84.37, 87.53, 110.98, 118.24 (q, *J* = 318 Hz), 127.64, 127.67, 127.69, 127.98, 128.01, 128.33, 128.45, 128.53, 129.72, 129.79, 133.26, 133.28, 135.47, 135.49, 136.41, 137.12, 150.13, 163.48. MS (FAB): *m*/*z* 853 (MH^+). HRMS (FAB): calcd for $C_{43}H_{48}N_2O_9F_3SiS$ (MH^+) 853.2802, found 853.2813.

4.9. (2′*R*)-2′-Azido-3′,5′-di-*O*-benzyl-4′-(2-*tert*-butyldiphenylsiloxyethyl)thymidine (9)

Under nitrogen atmosphere, NaN_3 (464 mg, 7.14 mmol) was added to a solution of triflate **8** (2.03 g, 2.38 mmol) in DMF (20 mL) at room temperature. The reaction mixture was stirred at room temperature for 13 h. The resulting mixture was added to water and extracted with Et_2O. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (2.20 g) was purified by column chromatography (silica gel 150 g, *n*-hexane/EtOAc = 2:1) to give azide **9** as a white foam (1.80 g, quant.).

Mp: 46–49 °C. $[\alpha]_D^{25}$ −5.1 (*c* 1.00, $CHCl_3$). IR (KBr): 3179, 3068, 2929, 2857, 2109, 1694, 1470, 1428, 1362, 1268 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.03 (s, 9H), 1.58 (d, *J* = 1.0 Hz, 3H), 1.78 (ddd, *J* = 6.0, 8.0, 14.5 Hz, 1H), 2.15 (ddd, *J* = 5.0, 5.5, 14.5 Hz, 1H), 3.44 (d, *J* = 11.0 Hz, 1H), 3.70–3.85 (m, 2H), 3.91 (d, *J* = 11.0 Hz, 1H), 3.97 (t, *J* = 6.0 Hz, 1H), 4.28 (d, *J* = 6.0 Hz, 1H), 4.43 (d, *J* = 11.5 Hz, 1H), 4.49 (d, *J* = 11.5 Hz, 1H), 4.49 (d, *J* = 11.5 Hz, 1H), 4.79 (d, *J* = 11.5 Hz, 1H), 5.99 (d, *J* = 6.0 Hz, 1H), 7.20–7.64 (m, 20H), 7.49 (d, *J* = 1.0 Hz, 1H), 7.93 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.10, 19.04, 26.82, 35.22, 59.26, 65.17, 73.41, 73.47, 74.28, 79.06, 86.00, 87.57, 111.09, 127.55, 127.64, 127.66, 128.05, 128.12, 128.21, 128.51, 128.64, 129.66, 129.71, 133.43, 133.44, 135.24, 135.50, 136.90, 137.13, 150.25, 163.66. MS (FAB): *m*/*z* 746 (MH^+). HRMS (FAB): calcd for $C_{42}H_{48}N_5O_6Si$ (MH^+) 746.3374, found 746.3404.

4.10. (2′*R*)-2′-Azido-3′,5′-di-*O*-benzyl-4′-(2-hydroxyethyl) thymidine (10)

Under nitrogen atmosphere, TBAF (1.0 M in THF, 2.6 mL, 2.6 mmol) was added to a solution of azide **9** (1.78 g, 2.38 mmol) in THF (20 mL) at room temperature. The reaction mixture was stirred at room temperature for 17 h. The resulting mixture was added to water and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (2.20 g) was purified by column chromatography (silica gel 75 g, *n*-hexane/EtOAc = 1:1) to give compound **10** as a white foam (1.22 g, quant.).

Mp: 45–50 °C. $[\alpha]_D^{25}$ +10.2 (*c* 1.00, $CHCl_3$). IR (KBr): 3441, 3182, 3063, 2927, 2109, 1693, 1496, 1469, 1455, 1364, 1267 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.56 (s, 3H), 1.66 (br s, 1H), 1.76 (dt, *J* = 6.0, 15.0 Hz, 1H), 2.21 (dt, *J* = 6.0, 15.0 Hz, 1H), 3.44 (d,

J = 10.5 Hz, 1H), 3.75 (t, *J* = 6.0 Hz, 2H), 3.82 (d, *J* = 10.5 Hz, 1H), 4.04 (t, *J* = 6.0 Hz, 1H), 4.30 (d, *J* = 6.0 Hz, 1H), 4.47 (d, *J* = 11.5 Hz, 1H), 4.53 (d, *J* = 11.5 Hz, 1H), 4.55 (d, *J* = 12.0 Hz, 1H), 4.83 (d, *J* = 12.0 Hz, 1H), 6.10 (d, *J* = 6.0 Hz, 1H), 7.22–7.38 (m, 10H), 7.44 (s, 1H), 8.34 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.12, 34.83, 58.28, 64.83, 73.32, 73.66, 74.50, 79.56, 86.30, 87.81, 111.36, 127.67, 128.11, 128.27, 128.37, 128.61, 128.72, 135.19, 136.72, 136.96, 150.22, 163.39. MS (FAB): *m*/*z* 508 (MH^+). HRMS (FAB): calcd for $C_{26}H_{30}N_5O_6$ (MH^+) 508.2196, found 508.2204.

4.11. (2′*R*)-2′-Azido-3′,5′-di-*O*-benzyl-4′-(2-carboxymethyl) thymidine (11)

MS4Å (3.0 g) and PDC (10.9 g, 28.8 mmol) were added to a solution of compound **10** (1.22 g, 2.38 mmol) in DMF (20 mL) at room temperature. The reaction mixture was stirred at room temperature for 12 h. The resulting mixture was added to water and AcOH and extracted with EtOAc. The combined organic layer was washed with 0.4 M $(COOH)_2$ aq, 0.2 M $(COONH_4)_2$ aq, and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (3.20 g) was purified by column chromatography (silica gel 75 g, *n*-hexane/EtOAc/AcOH = 50:50:1) to give carboxylic acid **11** as a white foam (1.18 g, 94%).

Mp: 78–81 °C. $[\alpha]_D^{25}$ −29.4 (*c* 1.00, $CHCl_3$). IR (KBr): 3510, 3179, 3033, 2929, 2108, 1705, 1470, 1455, 1269, 1213 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.56 (s, 3H), 2.71 (d, *J* = 16.5 Hz, 1H), 2.88 (d, *J* = 16.5 Hz, 1H), 3.77 (d, *J* = 10.0 Hz, 1H), 3.94 (d, *J* = 10.0 Hz, 1H), 3.99 (dd, *J* = 5.5, 7.0 Hz, 2H), 4.37 (d, *J* = 5.5 Hz, 1H), 4.49 (d, *J* = 11.0 Hz, 1H), 4.53 (d, *J* = 10.5 Hz, 1H), 4.61 (d, *J* = 10.5 Hz, 1H), 4.88 (d, *J* = 11.0 Hz, 1H), 6.22 (d, *J* = 7.0 Hz, 1H), 7.20–7.48 (m, 11H), 9.65 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.14, 38.05, 64.99, 73.80, 73.99, 75.30, 80.26, 85.30, 86.36, 111.74, 127.81, 128.06, 128.19, 128.46, 128.52, 128.89, 135.59, 136.81, 137.02, 150.67, 164.38, 175.09. MS (FAB): *m*/*z* 522 (MH^+). HRMS (FAB): calcd for $C_{26}H_{28}N_5O_7$ (MH^+) 522.1989, found 522.1979.

4.12. (2′*R*)-2′-Amino-3′,5′-di-*O*-benzyl-4′-(2-carboxymethyl) thymidine (12) and (2′*R*)-3′,5′-Di-*O*-benzyl-2′,4′-(2-oxo-iminoethano)thymidine (13)

Reduction by $NaBH_4$: Under nitrogen atmosphere, $NaBH_4$ (56.2 mg, 1.49 mmol) was added to a solution of carboxylic acid **11** (155 mg, 0.297 mmol) in *i*-PrOH (3.0 mL) at 0 °C. The reaction mixture was refluxed for 1 h. The reaction was then quenched with acetone and the resulting mixture was concentrated. The obtained crude residue (220 mg) was purified by column chromatography (silica gel 5.0 g, $CHCl_3$/MeOH = 20:1) to give compound **12** as a white foam (81.3 mg, 55%).

Staudinger reaction: Under nitrogen atmosphere, Me_3P (1.0 M in toluene, 0.55 mL, 0.55 mmol) was added to a solution of carboxylic acid **11** (241 mg, 0.462 mmol) in THF/H_2O (5:1, 6.0 mL) at room temperature. The reaction mixture was stirred at room temperature for 24 h. The resulting mixture was concentrated. The obtained crude residue (293 mg) was purified by column chromatography (silica gel 10 g, $CHCl_3$/MeOH = 30:1) to give lactam **13** as a white foam (106 mg, 43%) together with compound **12** (81 mg, ca. 35%) including a small amount of impurity.

Condensation using EDC: Under nitrogen atmosphere, EDC (157 mg, 0.820 mmol) and DMAP (10.0 mg, 0.0820 mmol) were added to a solution of amino acid **12** (81.3 mg, 0.164 mmol) in CH_2Cl_2 (3.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 24 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with CH_2Cl_2. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (65.4 mg) was purified by column chromatography (silica gel 3.0 g, $CHCl_3$/MeOH = 20:1) to give lactam **13** as a white foam (56.6 mg, 72%).

Condensation using MsCl: Under nitrogen atmosphere, Et_3N (0.32 mL, 2.25 mmol) and MsCl (70 μL, 0.0820 mmol) were added to a solution of amino acid **12** (223 mg, 0.450 mmol) in CH_2Cl_2 (5.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 17 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with CH_2Cl_2. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (300 mg) was purified by column chromatography (silica gel 10 g, $CHCl_3$/MeOH = 20:1) to give lactam **13** as a white foam (185 mg, 86%).

Compound **12**: Mp: 45–48 °C. $[\alpha]_D^{26}$ −12.2 (*c* 1.00, $CHCl_3$). IR (KBr): 3426, 3197, 3033, 2925, 2878, 1697, 1497, 1473, 1455, 1390, 1274, 1213 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD): δ 1.65 (s, 3H), 2.84 (s, 2H), 3.84 (d, *J* = 9.5 Hz, 1H), 3.92 (d, *J* = 9.5 Hz, 1H), 4.19–4.20 (m, 1H), 4.61–4.85 (m, 5H), 6.22 (d, *J* = 8.0 Hz, 1H), 7.25–7.42 (m, 10H), 7.55 (s, 1H), 7.90 (br s, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ 12.48, 38.19, 57.92, 74.79, 75.07, 76.89, 81.36, 86.89, 89.23, 112.37, 128.66, 128.79, 128.97, 129.10, 129.19, 129.72, 136.82, 138.01, 138.86, 152.64, 165.92, 173.68. MS (FAB): *m*/*z* 496 (MH^+). HRMS (FAB): calcd for $C_{26}H_{30}N_3O_7$ (MH^+) 496.2084, found 496.2081.

Compound **13**: Mp: 112–114 °C. $[\alpha]_D^{21}$ +78.9 (*c* 1.00, $CHCl_3$). IR (KBr): 3500, 3169, 3063, 2926, 1685, 1454, 1367, 1273, 1211 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.37 (d, *J* = 1.0 Hz, 3H), 2.40 (d, *J* = 18.0 Hz, 1H), 2.58 (d, *J* = 18.0 Hz, 1H), 3.60 (d, *J* = 11.0 Hz, 1H), 3.72 (d, *J* = 11.0 Hz, 1H), 4.23 (dd, *J* = 4.0, 6.0 Hz, 2H), 4.32 (d, *J* = 4.0 Hz, 1H), 4.54 (d, *J* = 11.5 Hz, 1H), 4.56 (d, *J* = 11.5 Hz, 1H), 4.60 (d, *J* = 11.5 Hz, 1H), 4.71 (d, *J* = 11.5 Hz, 1H), 5.83 (s, 1H), 7.25–7.35 (m, 11H), 7.94 (d, *J* = 1.0 Hz, 1H), 9.40 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.96, 38.60, 55.75, 68.71, 70.57, 71.96, 73.57, 83.78, 90.10, 109.61, 127.56, 127.89, 127.97, 128.23, 128.43, 128.66, 135.54, 136.96, 137.07, 150.72, 164.25, 169.56. MS (FAB): *m*/*z* 478 (MH^+). HRMS (FAB): calcd for $C_{26}H_{28}N_3O_6$ (MH^+) 478.1978, found 478.1983.

4.13. (2′*R*)-2′,4′-(2-Oxo-iminoethano)thymidine (15)

Lactam **13** (50.0 mg, 0.105 mmol) in MeOH (2.0 mL) was added to a suspension of 20% $Pd(OH)_2$ on carbon (50.0 mg, 0.462 mmol) in MeOH (1.0 mL) at room temperature under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 24 h under hydrogen atmosphere. The resulting mixture was filtered and concentrated. The obtained crude residue (25.4 mg) was purified by recrystallization from MeOH to give nucleoside **15** as a white solid (19.1 mg, 61%).

Mp: 187–188 °C. $[\alpha]_D^{23}$ +36.5 (*c* 0.200, CH_3OH); IR (KBr): 3449, 3318, 3175, 3060, 2926, 2819, 1707, 1651, 1469, 1386, 1272, 1215 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ 1.85 (s, 3H), 2.29 (d, *J* = 18.0 Hz, 1H), 2.46 (d, *J* = 18.0 Hz, 1H), 3.68 (d, *J* = 12.5 Hz, 1H), 3.75 (d, *J* = 12.5 Hz, 1H), 3.82 (d, *J* = 4.0 Hz, 1H), 4.33 (d, *J* = 4.0 Hz, 1H), 5.68 (s, 1H), 8.35 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ 12.55, 38.80, 60.02, 61.82, 64.42, 85.99, 91.01, 110.08, 137.78, 152.07, 166.63, 173.32. MS (FAB): *m*/*z* 298 (MH^+). HRMS (FAB): calcd for $C_{26}H_{28}N_3O_6$ (MH^+) 298.1039, found 298.1044.

4.14. (2′*R*)-5′-*O*-(4,4′-Dimethoxytrityl)-2′,4′-(2-oxo-iminoethano)thymidine (16)

Under nitrogen atmosphere, DMTrCl (95.7 mg, 0.283 mmol) was added to a solution of nucleoside **15** (28.0 mg, 0.0941 mmol) in pyridine (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (159 mg) was purified by column chromatography (silica gel 5.0 g, $CHCl_3$/MeOH = 20:1) to give compound **16** as a white foam (45.9 mg, 81%).

Mp: 204–206 °C. $[\alpha]_D^{23}$ +80.7 (*c* 1.00, $CHCl_3$). IR (KBr): 3563, 3341, 3062, 2933, 2838, 1705, 1657, 1608, 1509, 1466, 1384, 1254 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 1.20 (d, *J* = 1.0 Hz, 3H), 2.37 (d, *J* = 18.0 Hz, 1H), 2.44 (d, *J* = 18.0 Hz, 1H), 3.33 (d, *J* = 11.0 Hz, 1H), 3.37 (d, *J* = 11.0 Hz, 1H), 3.74 (s, 6H), 3.94 (d, *J* = 4.0 Hz, 1H), 4.56 (d, *J* = 4.0 Hz, 1H), 5.66 (s, 1H), 6.83–7.47 (m, 13H), 7.86 (d, *J* = 1.0 Hz, 1H). ^{13}C NMR (75 MHz, CD_3OD): δ 12.28, 39.32, 55.72, 59.82, 64.37, 65.71, 85.55, 88.06, 91.70, 110.67, 114.28, 128.19, 129.03, 129.44, 131.45, 136.38, 136.59, 136.87, 145.75, 152.01, 160.32, 160.35, 166.52, 173.00. MS (FAB): *m*/*z* 600 (MH^+). HRMS (FAB): calcd for $C_{26}H_{28}N_3O_6$ (MH^+) 600.2346, found 600.2347.

4.15. (2′*R*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′,4′-(2-oxo-iminoethano)thymidine (14)

Under nitrogen atmosphere, 1*H*-tetrazole (10.1 mg, 0.144 mmol) and $(i\text{-}Pr_2N)_2POCH_2CH_2CN$ (46 μL, 0.14 mmol) were added to a solution of compound **16** (72.0 mg, 0.120 mmol) in THF/MeCN (3:1, 2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 12 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with CH_2Cl_2. The combined organic layer was washed with satd $NaHCO_3$ aq, water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (99.0 mg) was purified by column chromatography (silica gel 3.0 g, $CHCl_3$/MeOH = 20:1) to give **14** as a white foam (70.2 mg, 73%).

Mp: 127–129 °C. ^1H NMR (400 MHz, $CDCl_3$): δ 0.99 (d, *J* = 7.0 Hz, 1.8H), 1.09 (d, *J* = 7.0 Hz, 4.2 H), 1.10 (d, *J* = 7.0 Hz, 4.2H), 1.16 (d, *J* = 7.0 Hz, 1.8H), 1.23 (s, 0.9H), 1.26 (s, 2.1H), 2.36–2.64 (m, 4H), 3.24–3.81 (m, 12H), 4.18 (dd, *J* = 4.0, 6.0 Hz, 0.3H), 4.32 (dd, *J* = 4.0, 5.5 Hz, 0.7H), 4.62 (dd, *J* = 4.0, 7.0 Hz, 0.3H), 4.76 (dd, *J* = 4.0, 7.0 Hz, 0.7H), 5.74 (s, 0.7H), 5.76 (s, 0.3H), 6.28 (d, *J* = 5.5 Hz, 0.7H), 6.33 (d, *J* = 6.0 Hz, 0.3H), 6.82–7.47 (m, 13H), 7.83 (s, 1H), 8.27 (br s, 1H). ^{31}P NMR (160 MHz, $CDCl_3$): δ 148.70, 150.53. MS (FAB): *m*/*z* 800 (MH^+), HRMS (FAB): calcd for $C_{42}H_{51}N_5O_9P$ (MH^+) 800.3424, found 800.3406.

4.16. (2′*R*)-3-Benzyloxymethyl-3′,5′-di-*O*-benzyl-2′,4′-(2-oxo-iminoethano)thymidine (18)

Under nitrogen atmosphere, DBU (0.12 mL, 0.79 mmol) and BOMCl (55 μL, 0.39 mmol) were added to a solution of lactam **13** (94.0 mg, 0.197 mmol) in DMF (2.0 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 0.5 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (112 mg) was purified by column chromatography (silica gel 5.0 g, *n*-hexane/EtOAc = 1:1) to give compound **18** as a white foam (101 mg, 86%).

Mp: 66–68 °C. $[\alpha]_D^{28}$ +79.7 (*c* 1.00, $CHCl_3$). IR (KBr): 3070, 3032, 2924, 2819, 1703, 1661, 1496, 1454, 1364, 1274, 1212 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.43 (d, *J* = 1.0 Hz, 3H), 2.45 (d, *J* = 17.5 Hz, 1H), 2.60 (d, *J* = 17.5 Hz, 1H), 3.59 (d, *J* = 10.5 Hz, 1H), 3.76 (d, *J* = 10.5 Hz, 1H), 3.96 (dd, *J* = 4.0, 5.5 Hz, 1H), 4.19 (d, *J* = 4.0 Hz, 1H), 4.51 (d, *J* = 11.0 Hz, 1H), 4.55 (d, *J* = 11.0 Hz, 1H), 4.60 (d, *J* = 11.0 Hz, 1H), 4.63 (d, *J* = 11.0 Hz, 1H), 4.69 (s, 2H), 5.43 (d, *J* = 9.5 Hz, 1H), 5.46 (d, *J* = 9.5 Hz, 1H), 5.74 (s, 1H), 6.15 (d, *J* = 5.5 Hz, 1H), 7.25–7.38 (m, 15H), 7.92 (d, *J* = 1.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.53, 38.68, 56.09, 68.73, 70.27, 70.57, 72.27, 72.37, 73.69, 83.76, 90.53, 109.48, 127.55, 127.64, 127.71, 127.91, 128.30, 128.34, 128.58, 128.73, 133.97, 136.59, 136.88, 137.82, 150.79, 163.29, 169.26. MS (FAB): *m*/*z* 598 (MH^+). HRMS (FAB): calcd for $C_{34}H_{36}N_3O_7$ (MH^+) 598.2553, found 598.2529.

4.17. (2′*R*)-3-Benzyloxymethyl-3′,5′-di-*O*-benzyl-2′,4′-(*N*-methyl-2-oxo-iminoethano)thymidine (19)

Under nitrogen atmosphere, 60% NaH (in mineral oil, 10.4 mg, 0.260 mmol) was added to a solution of compound **18** (130 mg, 0.218 mmol) in DMF (2.0 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 0.5 h. MeI (68 μL, 1.1 mmol) was added to the resulting mixture at 0 °C. The reaction mixture was stirred at 0 °C for 2 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with Et_2O. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (139 mg) was purified by column chromatography (silica gel 5.0 g, *n*-hexane/EtOAc = 3:2) to give *N*-Me lactam **19** as a white foam (79.6 mg, 60%).

Mp: 48–50 °C. $[\alpha]_D^{28}$ +67.8 (*c* 0.760, $CHCl_3$). IR (KBr): 3065, 3031, 2925, 2819, 1707, 1651, 1496, 1453, 1365, 1279, 1212 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.45 (d, *J* = 1.0 Hz, 3H), 2.42 (d, *J* = 17.0 Hz, 1H), 2.60 (d, *J* = 17.0 Hz, 1H), 3.07 (s, 3H), 3.68 (d, *J* = 12.0 Hz, 1H), 3.74 (d, *J* = 12.0 Hz, 1H), 3.82 (d, *J* = 4.0 Hz, 1H), 4.16 (d, *J* = 4.0 Hz, 1H), 4.54–4.61 (m, 4H), 4.70 (s, 2H), 5.43 (d, *J* = 9.5 Hz, 1H), 5.47 (d, *J* = 9.5 Hz, 1H), 5.66 (s, 1H), 7.23–7.39 (m, 15H), 7.91 (d, *J* = 1.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.55, 34.17, 38.77, 63.11, 68.51, 70.20, 71.19, 72.27, 72.72, 73.64, 84.24, 88.87, 109.38, 127.52, 127.66, 127.70, 127.89, 128.27, 128.30, 128.32, 128.62, 128.69, 133.93, 136.69, 136.92, 137.81,150.68, 163.35, 167.29. MS (FAB): *m*/*z* 612 (MH^+). HRMS (FAB): calcd for $C_{35}H_{38}N_3O_7$ (MH^+) 612.2710, found 612.2712.

4.18. (2′*R*)-2′,4′-(*N*-Methyl-2-oxo-iminoethano)thymidine (20)

Under nitrogen atmosphere, *N*-Me lactam **19** (79.6 mg, 0.130 mmol) in EtOH (8.0 mL) and cyclohexene (1.3 mL, 13 mmol) were added to a suspension of 20% $Pd(OH)_2$ on carbon (91.0 mg) in EtOH (2.0 mL) at room temperature. The reaction mixture was refluxed for 2 h. The resulting mixture was filtered and concentrated. The obtained crude residue was purified by column chromatography (silica gel 2.0 g, $CHCl_3$/MeOH = 10:1) to give nucleoside **20** as a white powder (40.8 mg, quant.).

Mp: >300 °C. $[\alpha]_D^{28}$ +85.0 (*c* 1.40, CH_3OH). IR (KBr): 3430, 3301, 3175, 2925, 1705, 1473, 1386, 1273, 1215 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 1.77 (d, *J* = 1.0 Hz, 3H), 2.21 (d, *J* = 18.0 Hz, 1H), 2.39 (d, *J* = 18.0 Hz, 1H), 3.02 (s, 3H), 3.57 (d, *J* = 12.5 Hz, 1H), 3.64 (d, *J* = 12.5 Hz, 1H), 3.80 (d, *J* = 4.0 Hz, 1H), 4.23 (d, *J* = 4.0 Hz, 1H), 5.60 (s, 1H), 8.18 (d, *J* = 1.0 Hz, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ 12.55, 34.92, 39.01, 61.60, 64.86, 67.68, 86.54, 89.33, 110.19, 137.47, 152.07, 166.65, 170.81. MS (FAB): *m*/*z* 312 (MH^+). HRMS (FAB): calcd for $C_{13}H_{18}N_3O_6$ (MH^+) 312.1196, found 312.1207.

4.19. (2′*R*)-5′-*O*-(4,4′-Dimethoxytrityl)-2′,4′-(*N*-methyl-2-oxo-iminoethano)thymidine (21)

Under nitrogen atmosphere, DMTrCl (175 mg, 0.567 mmol) was added to a solution of nucleoside **20** (26.8 mg, 0.0861 mmol) in pyridine (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 17 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with CH_2Cl_2. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (320 mg) was purified by column chromatography (silica gel 5.0 g, $CHCl_3$/MeOH = 25:1) to give compound **21** as a white foam (36.6 mg, 69%).

Mp: 155–157 °C. $[\alpha]_D^{26}$ +6.1 (*c* 2.10, $CHCl_3$). IR (KBr): 3350, 3198, 3087, 3006, 2933, 2836, 1694, 1651, 1508, 1464, 1392, 1350, 1254 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 1.30 (s, 3H), 2.44 (d, *J* = 17.5 Hz, 1H), 2.54 (d, *J* = 17.5 Hz, 1H), 3.09 (s, 3H), 3.33 (d, *J* = 11.0 Hz, 1H), 3.38 (d, *J* = 11.0 Hz, 1H), 3.75 (s, 3H), 3.76 (s, 3H),

3.94 (d, J = 4.0 Hz, 1H), 4.28 (br s, 1H), 4.49 (br s, 1H), 5.61 (s, 1H), 6.80–7.42 (m, 13H), 7.75 (s, 1H), 7.90 (br s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.91, 34.51, 38.54, 55.20, 62.87, 65.43, 65.98, 84.96, 86.88, 88.60, 110.39, 111.34, 127.20, 128.07, 130.10, 134.92, 135.08, 144.05, 152.35, 158.69, 164.37, 168.32. MS (FAB): m/z 614 (MH^+). HRMS (FAB): calcd for $C_{34}H_{36}N_3O_8$ (MH^+) 614.2502, found 614.2496.

4.20. (2′*R*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′,4′-(*N*-methyl-2-oxo-iminoethano)thymidine (17)

Under nitrogen atmosphere, DIPEA (10.1 mg, 0.144 mmol) and *i*-$Pr_2NP(Cl)OCH_2CH_2CN$ (46 μL, 0.14 mmol) were added to a solution of compound **21** (72.0 mg, 0.120 mmol) in CH_2Cl_2 (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 2 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with CH_2Cl_2. The combined organic layer was washed with satd $NaHCO_3$ aq, water, and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (99.0 mg) was purified by column chromatography (silica gel 3.0 g, $CHCl_3$/MeOH = 20:1) to give **17** as a white foam (70.2 mg, 73%).

Mp: 109–111 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.99 (d, J = 7.0 Hz, 3.0H), 1.07 (d, J = 7.0 Hz, 3.0H), 1.11 (d, J = 7.0 Hz, 3.0H), 1.16 (d, J = 7.0 Hz, 3.0H), 1.26 (s, 1.5H), 1.29 (s, 1.5H), 2.36–2.64 (m, 4H), 3.17 (s, 3H), 3.22–3.88 (m, 12H), 4.10 (d, J = 4.5 Hz, 0.5H), 4.27 (d, J = 4.5 Hz, 0.5H), 4.60 (dd, J = 4.5, 7.0 Hz, 0.5H), 4.75 (dd, J = 4.0, 6.5 Hz, 0.5H), 5.67 (s, 0.5H), 5.69 (s, 0.5H), 6.82–7.47 (m, 13H), 7.83 (s, 1H), 8.27 (br s, 1H). ^{31}P NMR (400 MHz, $CDCl_3$): δ 148.86, 149.97. MS (FAB): m/z 814 (MH^+). HRMS (FAB): calcd for $C_{43}H_{53}N_5O_9P$ (MH^+) 814.3581, found 814.3599.

4.21. Oligonucleotide synthesis

Phosphoramidites **14** and **17** were used and the 0.2 μmol scale synthesis of oligonucleotides was performed on an automated DNA synthesizer (Gene Design nS-8) using a standard phosphoramidite protocol (DMTr-ON mode). Oligonucleotides **22**–**29** were prepared by cleavage from the CPG supports and deprotection of the phosphate moieties (28% NH_4 aq, rt, 1.5 h or 50 mM K_2CO_3 in MeOH, rt, 2 h). Removal of ammonia was carried out in vacuo. In the case of K_2CO_3 treatment, after neutralization with 1% HCl aq, the solvent was concentrated in vacuo. The crude **22**–**29** 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 compositions of **22**–**29** were confirmed by MALDI-TOF mass analysis. MALDI-TOF mass analysis. MALDI-TOF MS data ($[M-H]^-$) for **22**–**29**: **22**, found 3033.70 (calcd 3034.00); **23**, found 3090.36 (calcd 3089.03); **24**, found 3145.24 (calcd 3144.07); **25**, found 3048.53 (calcd 3048.02); **26**, found 3117.32 (calcd 3117.08); **27**, found 3187.85 (calcd 3186.25); **28**, found 3033.23 (calcd 3034.00); **29**, found 3048.78 (calcd 3048.02).

4.22. UV-melting experiments

UV-melting experiments were carried out using SHIMADZU UV-1650 and SHIMADZU UV-1800 spectrophotometers equipped with a T_m analysis accessory. Oligonucleotides 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 to 85 °C at a scan rate of 0.5 °C/min. The two-point average method was employed to obtain the T_m values and the final values were determined by averaging three independent measurements which were accurate to within 1 °C.

4.23. Enzymatic degradation experiments

Enzymatic degradation experiments were carried out under conditions of 1.75 μg/mL Crotalus admanteus venom phosphodiesterase (CAVP), 10 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.0) and 7.5 μM each oligonucleotide at 37 °C. The amount of intact oligonucleotides was determined by reversed-phase HPLC (Waters XBridge® MS C_{18} 2.5 μm, 10 × 50 mm).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.04.049.

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