Synthesis, duplex-forming ability, and enzymatic stability of oligonucleotides modified with amide-linked dinucleotides containing a 3′,4′-tetrahydropyran-bridged nucleic acid

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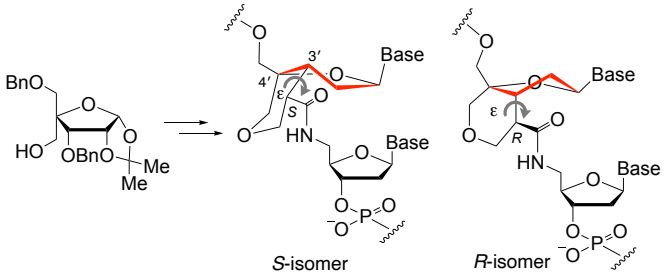


ABSTRACT: Replacement of a phosphodiester linkage with an amide linkage can improve the binding affinity of oligonucleotides to complementary RNA and their stability toward nucleases. In addition, restricting the conformation of the sugar moiety and the phosphate backbone in oligonucleotides effectively improves duplex stability. In this study, we designed amide-linked dinucleotides containing a 3′,4′-tetrahydropyran-bridged nucleic acid (3′,4′-tpBNA) with a constrained sugar conformation as well as a torsion angle ε. Phosphoramidites of the designed dinucleotides were synthesized and incorporated into oligonucleotides. Conformational analysis of the synthesized dinucleotides showed that the sugar conformation of the *S*-isomer of the amide-linked dinucleotide containing 3′,4′-tpBNA was N-type, which has the same conformation as that of the RNA duplex, while that of another *R*-isomer was S-type. T_m analysis indicated that the oligonucleotides containing the synthesized *S*-isomer showed RNA-selective hybridizing ability, although their duplex-forming ability was slightly inferior to that of natural oligonucleotides. Interestingly, the stability of the oligonucleotides toward endonucleases was significantly improved by modification with the two types of amide-linked dinucleotides developed in this study.

Introduction

In recent years, rapid progress has been made in the development of oligonucleotide-based therapeutics, such as antisense oligonucleotides and small interfering ribonucleic acids (siRNAs).[1–5] The oligonucleotide-based therapeutics developed so far have been generally modified using artificial nucleic acids,[6] because natural DNA and

RNA have insufficient ability to hybridize with complementary RNA and stability against nucleases for practical application as oligonucleotide therapeutics. To address these issues, various chemically modified nucleic acids have been developed.[7–9] For instance, chemical modification of the phosphate backbone can improve the nuclease resistance of oligonucleotides. A typical backbone modification is performed using phosphorothioate (PS), which can significantly enhance the potency of oligonucleotide-based therapeutics because oligonucleotides with PS modifications show excellent nuclease resistance. However, PS-modifications have recently been found to result in nonspecific binding to proteins, which causes hepatotoxicity.[10] In addition, a disadvantage of PS-modification is that the sulfur atom in the PS-linkage introduces a chiral phosphorus atom with two different stereochemical configurations, resulting in numerous diastereomers of oligonucleotides that are difficult to isolate and purify. Therefore, the development of an alternative to PS backbone modifications is necessary. Replacement of a phosphodiester linkage in oligonucleotides by an amide linkage can increase their binding affinity with RNA.[11] Moreover, oligonucleotides, which have partial amide linkages, are resistant to degradation by nuclease compared to unmodified DNA; therefore, antisense oligonucleotides and siRNAs with amide linkages have already been applied for some *in vitro* and *in vivo* studies.[12–18]

Restricting the sugar conformation in oligonucleotides is an effective approach to improving the thermal stability of duplexes. For example, 2′,4′-BNA[19,20] or LNA[21,22] has the locked N-type sugar conformation and exhibits predominant duplex-forming ability with RNA. In addition, 2′,4′-BNA/LNA modification can enhance the nuclease resistance of oligonucleotides, probably due to the steric effect of the incorporated bridge structure. Thus, 2′,4′-BNA/LNA-modified antisense oligonucleotides have already been applied in clinical trials.[23] Restricting the torsion angles of the phosphate backbone in oligonucleotides can also improve their duplex-forming ability. Bicyclo-DNAs and tricyclo-DNAs, which are nucleic acid derivatives with a constrained torsion angle γ, can form thermally stable duplexes with complementary RNA.[24–27] Moreover, constrained nucleic acid (CNA) derivatives have also been developed, in which the conformation of the phosphate backbone is fixed by a six-membered chair-form ring.[28–37] Based on these strategies, restriction of both the sugar conformation and phosphate backbone is considered to be an effective approach for enhancing RNA-binding ability. In fact, tricyclic-LNA derivatives, which have a constrained N-type sugar and torsion angle γ, show excellent RNA-binding ability.[38]

We have previously developed several 3′,4′-bridged nucleic acids (3′,4′-BNAs, **Figure 1**).[39–43] The sugar conformation of all 3′,4′-BNAs and *trans*-3′,4′-BNAs is an S-type. Shaikh *et al.* developed a bicyclic 2′-deoxyribonucleoside with a 3′,4′-bridge structure, and X-ray crystal structural analysis revealed that the sugar conformation of the nucleoside was, interestingly, an N-type.[44] In short, a 3′,4′-*cis*-fused bicyclic sugar can restrict the conformation of the sugar moiety toward the N-form or the S-form. Using this information, we designed amide-linked dinucleotides containing a 3′,4′-tetrahydropyran-bridged nucleic acid (3′,4′-tpBNA, **Figure 1**). We also studied the sugar conformation of these bicyclic nucleosides and the effect of the restriction of the dihedral angle ε by the introduction of this 3′,4′-*cis*-fused ring on the properties of oligonucleotides. In this study, amide-linked dinucleotides containing 3′,4′-tpBNA (**Figure 1**) were synthesized, incorporated into oligonucleotides, and their properties

evaluated.

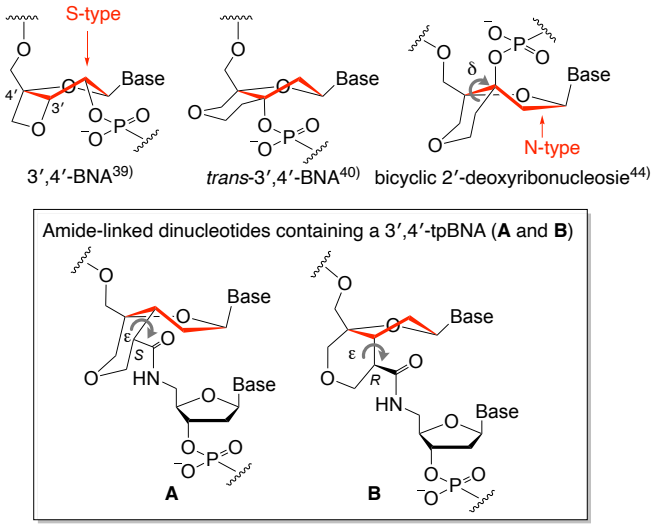
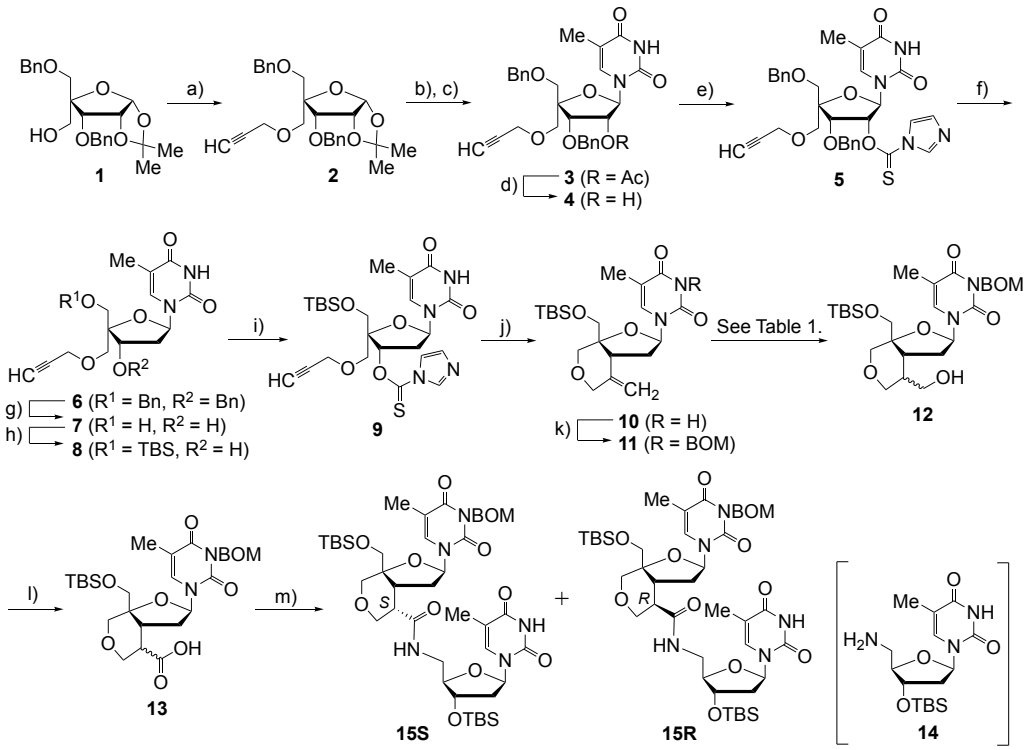


Figure 1. Structures of artificial nucleosides with a 3′,4′-bridge structure

Results and discussion

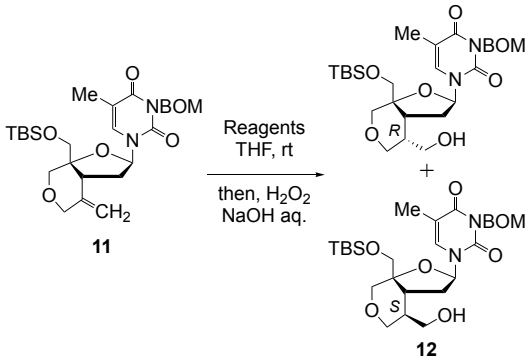
Synthesis of amide-linked dinucleotides containing a 3′,4′-tpBNA. First, the bicyclic structure in 3′,4′-BNA was constructed (**Scheme 1**). The primary alcohol in compound **1**[45] was propargylated to produce compound **2**. Compound **2** was converted into 5-methyluridine derivative **3** in 82% yield using a two-step glycosylation procedure. The acetyl group in **3** was removed by base treatment to yield **4** almost quantitatively. Secondary alcohol **4** was treated with 1,1′-thiocarbonyldiimidazole (TCDI). Then, radical deoxygenation was performed using AIBN and *n*-Bu_3SnH to give compound **6** in moderate yield. The benzyl groups were removed using BCl_3 to give 4′-*C*-propargyloxymethylthymidine **7** in 79% yield. To construct the bicyclic sugar moiety by intramolecular radical cyclization, the primary alcohol in compound **7** was protected by TBS. This was followed by the treatment of compound **8** with TCDI and DMAP to produce radical precursor **9**. Then, radical cyclization from **9** using AIBN and *n*-Bu_3SnH was investigated successfully to give 3′,4′-BNA **10** in 47% yield. The 3-position of the thymine nucleobase was protected by the BOM group to yield compound **11** in good yield. Hydroboration and oxidation of the exocyclic methylene group in **11** were investigated (**Table 1**). Thexylborane ($ThxBH_2$) was used as a hydroboration reagent to successfully give the desired hydroxymethylated product **12** in 71% yield with a diastereomeric ratio of *ca.* 1:1 (entry 1). The stereochemistry of the asymmetric carbon produced by this reaction was determined after dimerization and separation of each diastereomer. In this reaction, the structure of the *cis*-fuced bicyclic structure of compound **11** might be attributed to its diastereoselectivity. Bulky reagents can generally access convex faces more easily than concave faces. Thus, hydroboration with the bulky 9-BBN was investigated, which improved the diastereoselectivity from 1:1 to 3:1, although the isolated yield of **12** decreased to 51% (entry 2). However, the treatment of compound **11** with the very bulky $lcpBH_2$ did not give the desired product **12** (entry 3). The obtained diastereomixture of an alcohol **12** (*dr*: *ca.* 3:1, entry 1 in **Table 1**) was oxidized to give carboxylic acid **13** (*dr*: ca. 3:1), followed by amide

bond formation using HBTU, HOBt, and amine **14**[46] to yield the amide-linked 3′,4′-BNAs **15S** (67%) and **15R** (21%), respectively. These dimers, i.e., **15S** and **15R,** were separated by column chromatography using *n*-hexane/acetone (3/2) as the eluent.



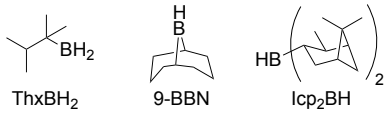
Scheme 1. a) NaH, propargyl bromide, THF, rt, 25 h, 75%; b) Ac_2O, H_2SO_4, AcOH, rt, 0.5 h; c) thymine, BSA, TMSOTf, MeCN, reflux, 2 h, 82% (2 steps from **2**); d) K_2CO_3, MeOH, rt, 4 h, 98%; e) TCDI, DMAP, CH_2Cl_2, rt, 4 h, 88%; f) AIBN, *n*-Bu_3SnH, toluene, 90°C, 3 h, 51%; g) BCl_3, CH_2Cl_2, –78°C, 1.5 h, 79%; h) TBSCl, pyridine, 60°C, 20 h, 94%; i) TCDI, DMAP, CH_2Cl_2, rt, 3 h, 96%; j) AIBN, *n*-Bu_3SnH, toluene, 90°C, 0.5 h, 47%; k) BOMCl, DBU, THF, rt, 4 h, 68%; l) TEMPO, $PhI(OAc)_2$, MeCN/H_2O (1/1), rt, 17 h, 84% (*dr*: *ca.* 3:1); m) **14**, HOBt, HBTU, DIPEA, CH_2Cl_2, rt, 24 h, 67% (**15S**) and 21% (**15R**).

Table 1. Hydroboration and oxydation of compound **11**



Entry	Reagent	Time	Yield (*R*:*S*)

1	$ThxBH_2$ (3 eq)	20 h	71% (1:1)
2	9-BBN (3 eq)	18 h	51% (3:1)
3	Icp_2BH (3 eq)	24 h	0%



To determine the stereochemistry of asymmetric carbon produced by hydroboration and oxidation reactions, the structural analysis of the 3′,4′-bridge structure in **15S** and **15R** was performed by computational methods. In this computational structural analysis, the model structures of amide-linked 3′,4′-tpBNAs (**15S′** and **15R′**) were used to simplify conformational analysis of 3′,4′-bridge structures (**Figure 2**), and the calculation was carried out using density functional theory (DFT) with the basis function 6-31G*. In this analysis, the possible conformations of **15S'** and **15R'** were firstly derived, then the energies of all the obtained conformations were calculated and compared to determine their most stable conformation. As the results, the model *S*-isomer (**15S'**) was found to have an N-type sugar conformation, whereas the model *R*-isomer (**15R'**) had an S-type sugar conformation. In addition, the sugar conformations of **15S** and **15R** were analyzed using ^{1}H NMR measurements (**Figure 3**).[47] The coupling constants of **15S** ($^3J_{H1'H2'}$ = 0, 7.0 Hz) indicated that the sugar conformation of the isolated compound **15S** was restricted to an N-type conformation. On the other hand, the coupling constants $^3J_{H1'H2'}$ of **15R** were 5.0 Hz and 9.0 Hz, which suggested that the sugar conformation of **15R** was predominantly S-type. Because the *S*-isomer of amide-linked 3′,4′-BNA has a condensed ring skeleton similar to that in the bicyclic 2′-deoxyribonucleoside with a 3′,4′-bridge structure developed by Shaikh *et al*.,[44] the sugar conformation of the *S*-isomer is the N-type. In contrast, the preferable S-type sugar conformation of the *R*-isomer resulted from 1,3-diaxial steric repulsion between the carbamoyl group and the 5′-carbon (**Figure 3**). From these structural analysis results, we presumed that compound **15S** was the *S*-isomer and compound **15R** was the *R*-isomer.

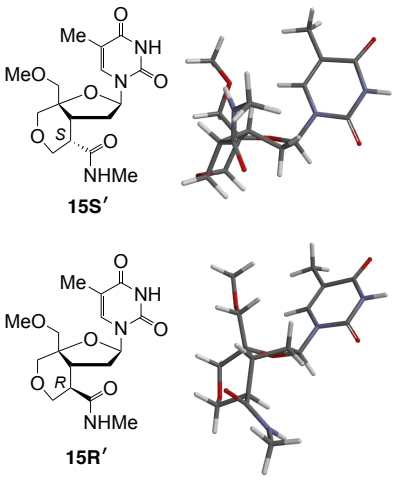


Figure 2. The computational structural analysis of synthesized 3′,4′-tpBNAs (theoretical calculation was carried out at DFT ωB97XD/6-31G* level)

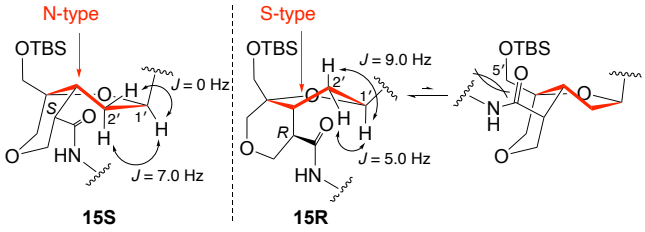
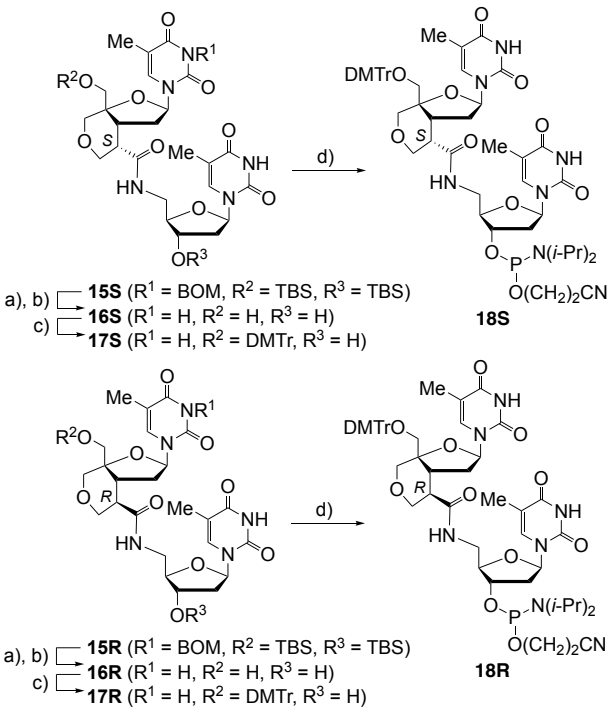


Figure 3. Conformational analysis of **15S** and **15R** by ^{1}H NMR measurement

The synthesis of phosphoramidites from isolated dimers **15S** and **15R** was performed according to **Scheme 2**. The BOM groups of **15S** and **15R** were removed by catalytic hydrogenation, followed by the removal of the TBS groups using TBAF to give compounds **16S** and **16R**, respectively. Finally, the primary alcohol at the 5′ position was protected by the DMTr group, followed by phosphitylation to produce the desired phosphoramidites, **18S** and **18R**. The synthesis of oligonucleotides (**ON1–6**) was performed on an automated DNA synthesizer using a common phosphoramidite chemistry with a prolonged coupling time (2 min) for the introduction of the amide-linked 3′,4′-tpBNAs. This extension of the coupling time allowed for almost quantitative incorporation of phosphoramidites **18S** and **18R** into the oligonucleotides. **ON1** and **ON2** were prepared as a control for **ON3–6** without the 3′,4′-bridge structure by using phosphoramidite **19** (**Figure 4**).[46] After purification of the oligonucleotides synthesized by reversed-phase HPLC, the purity and molecular weights of **ON1–6** were confirmed by analytical HPLC and mass spectrometric analysis, respectively (**Table 2**).



Scheme 2. a) $Pd(OH)_2$, THF/H_2O (1/1), rt, 4 h; b) TBAF, THF, rt, 24 h, 94% (**16S** for 2 steps from **15S**) and quant. (**16R** for 2 steps from **15R**); c) DMTrCl, pyridine, rt, 22 h, 71% (**17S**) and 84% (**17R**); d) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2, 3 h, 65% (**18S**) and 82% (**18R**).

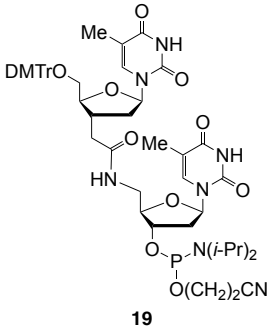
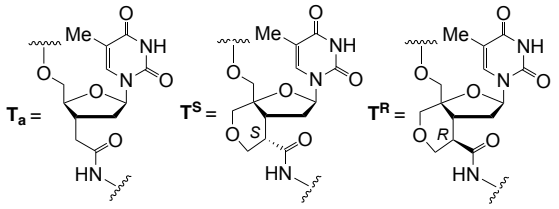


Figure 4. Phosphoramidite **19** for incorporation of amide-linked dinucleotide without 3′,4′-bridged structure

Table 2. Synthesized oligonucleotides

	Sequence (5′-3′)	Yield (%)	Calcd. $[M–H]^-$	Found $[M–H]^-$
ON1	GCGTTT$_a$TTTGCT	9	3594.5	3593.2
ON2	GCGT$_a$TT$_a$TT$_a$TGCT	18	3516.6	3515.9
ON3	GCGTTT^{S}TTTGCT	26	3636.5	3635.6
ON4	GCGT^{S}TT^{S}TT^{S}TGCT	5	3642.7	3643.2

ON5	GCGTTT^{R}TTTGCT	32	3636.5	3636.9
ON6	GCGT^{R}TT^{R}TT^{R}TGCT	19	3642.7	3642.2



Properties of amide-linked dinucleotides containing a 3′,4′-tpBNA. The duplex-forming ability of the modified oligonucleotides (**ON1**–**6**) with complementary ssDNA and ssRNA was evaluated by UV melting experiments and compared with that of the natural oligonucleotide **ON7**. The results are summarized in **Table 3,** and the UV melting data are shown in **Figure S1** in ESI. For duplexes formed with both ssRNA and ssDNA, the T_m values of **ON1**, which included one amide linkage, were slightly lower than those of **ON7**. The T_m values of **ON3**–**6**, which include 3′,4′-tpBNAs, were compared with those of **ON1** and **ON2** to evaluate the effect of the 3′,4′-bridge structure on the duplex stability. The duplexes formed with ssRNA, such as **ON3**, which contain the *S*-isomer of amide-linked 3′,4′-tpBNA, showed almost the same duplex-forming ability as **ON1**. In contrast, the duplex formed between **ON3** and ssDNA was less stable than the duplex formed between **ON1** and ssDNA. This tendency was also observed in the comparison of **ON2** and **ON4**, in which three phosphodiester bonds were replaced with amide bonds. These results show that the *S*-isomer of amide-linked 3′,4′-tpBNA has an RNA-selective hybridizing property, probably because the sugar conformation of the *S*-isomer is N-type (**Figure 2** and **Figure 3**), which is the same conformation as that of the RNA duplex. Interestingly, **ON5** and **ON6**, which contain the *R*-isomer of amide-linked 3′,4′-tpBNA, showed much lower hybridizing ability with both ssRNA and ssDNA compared to **ON1**–**4**.

Table 3. T_m values of duplexes formed by **ON1**–**7**

		DNA	RNA
	Sequence (5′-3′)	T_m (ΔT_m) (°C)	T_m (ΔT_m) (°C)
ON7	GCGTTTTTTGCT	52	47
ON1	GCGTTT$_a$TTTGCT	49 (–3)	45 (−2)
ON2	GCGT$_a$TT$_a$TT$_a$TGCT	45 (–7)	46 (−1)
ON3	GCGTTT^{S}TTTGCT	46 (–6)	44 (−3)
ON4	GCGT^{S}TT^{S}TT^{S}TGCT	38 (–14)	44 (−3)
ON5	GCGTTT^{R}TTTGCT	43 (–9)	39 (−8)
ON6	GCGT^{R}TT^{R}TT^{R}TGCT	30 (–22)	28 (−19)

Conditions:10 mM phosphate buffer (pH 7.2), 100 mM NaCl, and 4 μM oligonucleotides. The T_m values are the averages of three measurements. The sequences of complementary ssDNA and ssRNA are 5′-

d(AGCAAAAAACGC)-3′ and 5′-r(AGCAAAAAACGC)-3′, respectively. ΔT_m: change in T_m value compared to that of natural DNA (**ON7**).

To clarify why the introduction of the *R*-isomer into oligonucleotides significantly decreased the stability of duplexes, the CD spectra of the oligonucleotides **ON2**, **ON4**, **ON6**, and **ON7** and the duplexes formed by them were measured (**Figure 5**). Similar spectra were obtained for all single-stranded oligonucleotides, that is, **ON2**, **ON4**, **ON6**, and **ON7**, suggesting that the introduction of artificial nucleic acids had little effect on the structure of single-stranded oligonucleotides. The CD spectra of all DNA/DNA duplexes showed that, like the natural **ON7**/DNA duplex, the modified oligonucleotides **ON2**, **ON4**, and **ON6** also formed B-type DNA duplexes. The CD spectrum of the natural **ON7**/RNA duplex showed a positive Cotton band at 270 nm and a negative Cotton band at 210 nm, which are generally observed in the CD spectra of A-type RNA/RNA duplexes. In addition, **ON2,** which has an amide linkage, formed an A-type duplex with complementary RNA, which is consistent with a previous report,[48] and the structures of the **ON4**/RNA and **ON6**/RNA duplexes were similar to those of the A-type, as observed in the CD spectra. However, as a characteristic result of **ON6** modification by the *R*-isomer, both the positive Cotton band around 280 nm and the negative Cotton band around 250 nm in the CD spectrum of the **ON6**/DNA duplex, which is characteristic of the B-type DNA duplex, were weakened compared to those of other DNA/DNA duplexes. Moreover, the positive Cotton band at approximately 270 nm in the **ON6**/RNA duplex was weaker than that in other DNA/RNA duplexes. These results indicate that the structure of the duplex formed by **ON6** was slightly different from that of common natural duplexes, probably because the torsion angles of the most stable conformation of the *R*-isomer obtained by computational structural analysis (**Figure 2**) were completely different from those of natural oligonucleotides as well as oligonucleotides modified with amide linkages (**Table S1** in the ESI)[49]. From these findings, the dramatic decrease in the stability of the duplexes due to the introduction of the *R*-isomer probably results from the energetically unfavorable structure of the duplex formed by **ON5** and **ON6**.

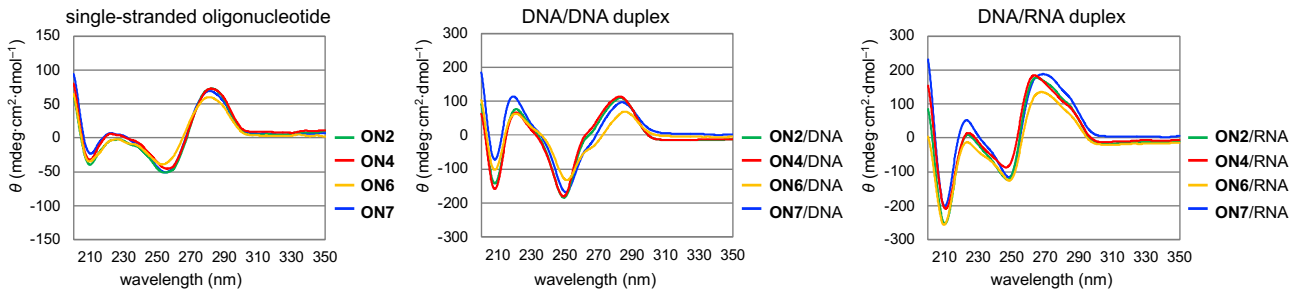


Figure 5. CD spectra of single-stranded oligonucleotides (**ON2**, **ON4**, **ON6**, and **ON7**) and duplexes formed by **ON2**, **ON4**, **ON6**, and **ON7**[a]

[a]Conditions: 10 mM phosphate buffer (pH 7.2), 100 mM NaCl, and 4 μM oligonucleotide at 10 °C. The sequences of complementary ssDNA and ssRNA are 5′-d(AGCAAAAAACGC)-3′ and 5′-r(AGCAAAAAACGC)-3′, respectively.

The enzymatic stabilities of the modified oligonucleotides **ON2**, **ON4**, and **ON6**, in which three phosphodiester bonds were replaced with amide bonds by the introduction of amide-linked dinucleotides, were evaluated and compared with those of the natural oligonucleotide **ON7** and PS-modified oligonucleotide **ON8**. Nuclease S1 was used as an endonuclease that catalyzed the specific degradation of single-stranded DNA and RNA to 5'-mononucleotides. After enzymatic degradation, the oligonucleotides were analyzed by reverse-phase HPLC, and the ratio of residual oligonucleotides is summarized in **Figure 6**. Approximately 10% of **ON2,** which has three amide linkages, remained after 120 minutes, whereas most of the natural **ON7** was degraded within 20 minutes, which is consistent with a previous report.[14] The stability of **ON4** and **ON6** modified with the 3′,4′-tpBNA developed in this study was almost the same as that of **ON2**, which indicates that the greater stability of **ON4** and **ON6** than natural **ON7** might be attributed to the effect of replacing the phosphodiester bond with an amide bond. The enzymatic stability of the 12-mer oligonucleotides **ON2**, **ON4**, and **ON6** was slightly lower than that of fully PS-modified **ON8**. Surprisingly, characteristic peaks were observed in the HPLC charts after the enzymatic degradation of the oligonucleotides substituted with amide linkages (**ON2**, **ON4**, and **ON6**). The retention times of the characteristic peaks were close to those of the intact oligonucleotides (**Figure S2** in the ESI), suggesting that partially degraded oligonucleotides may have remained for **ON2**, **ON4**, and **ON6**. Thus, the major products generated by the enzymatic degradation of **ON2**, **ON4**, and **ON6** were isolated by reverse-phase HPLC purification, and their masses were measured by LC/MS (**Figure S3** in ESI). All obtained products were identified as 10-mer oligonucleotides with 5′-phosphate (sequence: 5′-GTTTTTTGCT-3′) lacking two nucleotides at the 5′-end. However, in the HPLC chart of **ON8** after enzymatic treatment, no peak of the 10-mer oligonucleotide was observed, probably because **ON8** was randomly degraded at the thiophosphate bond by the S1 nuclease. Based on these results, the ratio of the remaining 10-mer and 12-mer oligonucleotides was included to re-evaluate the enzyme resistance for **ON2**, **ON4**, and **ON6**. Interestingly, the ratio of intact 10-mer oligonucleotides after treatment with S1 nuclease for 120 min was nearly 50%, which indicates that the 10-mer oligonucleotides generated from **ON2**, **ON4**, and **ON6** were more stable under digestion conditions by endonuclease compared to the fully PS-modified **ON8**. This implies that the phosphodiester bonds that are susceptible to enzymatic cleavage may be stabilized by partially replacing the phosphodiester bonds in the oligonucleotides with amide bonds. Interestingly, both diastereomers of the amide-linked 3′,4′-tpBNA confer similar stability against nuclease-mediated degradation, but this is not the case for sequences containing a single chiral PS modification; the resistance to enzymatic degradation of PS-modified DNA depends on the stereochemistry of the phosphorus atom in PS.[50] In addition, although an oligonucleotide with PS modification is highly resistant to nucleases, oligonucleotides bind non-specifically to proteins due to PS modification, which leads to hepatotoxicity.[10] Therefore, replacement of the phosphodiester linkage with an amide linkage may be an effective strategy for reducing the toxicity of oligonucleotides derived from PS modification. In particular, the *S*-isomer of the amide-linked dinucleotide containing a 3′,4′-tpBNA might be a promising alternative to PS-modification for the practical application of oligonucleotide-based medicines because the *S*-isomer has an RNA-selective hybridizing ability, as

shown in **Table 3**.

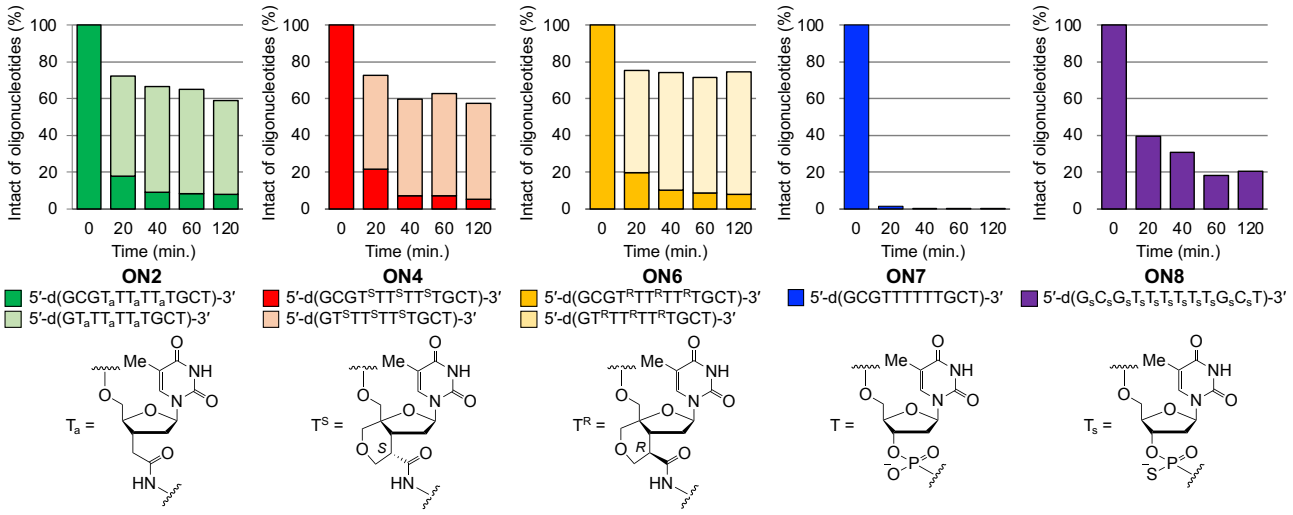


Figure 6. Endonuclease resistance of the modified oligonucleotides[a]

[a]Conditions: 30 unit/mL S1 nuclease, 30 mM sodium acetate (pH 4.6), 0.28 M NaCl, 1.0 mM $ZnSO_4$, and 15 μM of each oligonucleotide at 37°C.

Conclusion

In this study, two types of amide-linked dinucleotides containing a 3′,4′-tpBNA based on bicyclic 2′-deoxyribonucleoside were designed, synthesized, and incorporated into oligonucleotides. Conformational analysis of the amide-linked dinucleotides containing 3′,4′-tpBNA showed that the sugar conformation of the *S*-isomer was N-type, while that of the *R*-isomer was S-type. UV-melting experiments revealed that the duplex-forming ability of oligonucleotides with the *S*-isomer of amide-linked 3′,4′-tpBNA showed an RNA-selective binding affinity. Interestingly, endonuclease digestion experiments showed that the incorporation of an *S*- or *R*-isomer of amide-linked 3′,4′-tpBNA into oligonucleotides could significantly enhance the stability, despite not introducing a PS-modification. Furthermore, the 10-mer oligonucleotides produced by the enzymatic digestion of 12-mer oligonucleotides, in which phosphodiester bonds were partially substituted with amide bonds, were more stable than a fully PS-modified oligonucleotide. The results of this study imply that an amide-linked dinucleotide containing 3′,4′-tpBNA, especially its *S*-isomer, is a promising molecule for the practical application of oligonucleotide-based therapeutics.

Experimental Section

General. All moisture-sensitive reactions were carried out in well-dried glassware under a N_2 atmosphere. The progress of the reactions was monitored by analytical thin layer chromatography (TLC) on glass plates (TLC silica gel 60 F_{254}) and the products were visualized by UV light. For reactions that require heating, an oil bath was used. For column chromatography, PSQ-100B silica gel (Fuji Silysia Chemical Ltd.) was used. NMR experiments were performed on JEOL JNM-ECS300, JNM-ECS400, and JNM-ECA500. ^{1}H NMR spectra were recorded at 300, 400,

and 500 MHz. ^{13}C NMR spectra were recorded at 76, 101, and 126 MHz. ^{31}P NMR spectra were recorded at 202 MHz. Chemical shifts (δ) are expressed in ppm relative to internal tetramethylsilane (0.00 ppm), residual CD_3OD (3.31 ppm) and $CHCl_3$ (7.26 ppm) for ^{1}H NMR spectra, internal tetramethylsilane (0.00 ppm), residual CD_3OD (49.0 ppm) and $CHCl_3$ (77.2 ppm) for ^{13}C NMR spectra, and 85% H_3PO_4 (δ = 0.00 ppm) for ^{31}P NMR spectra. MALDI-TOF mass spectra of new compounds except for **18S** and **18R** were recorded on JEOL SpiralTOF JMS-S3000 and ESI-MS spectra of **18S** and **18R** were recorded on JMS-T100LP. Gene Design nS-8 Oligonucleotides Synthesizer was used as an automated DNA synthesizer (for 0.2- and 1.0-μmol scale). For high performance liquid chromatography (HPLC), SHIMADZU CBM-20A, DGU-20A$_3$, LC-20AT, CTO-20A, SPD-20A and FRC-10A were used. Waters XBridge® OST C18 2.5 μm (10 × 50 mm) was used for preparative HPLC, and Waters XBridge® OST C18 2.5 μm (4.6 × 50 mm) was used for analytical HPLC. For liquid chromatography-mass spectrometry (LC/MS), Waters ACQUITY RDa Detector coupled to the ACQUITY UPLC H-Class System and TUV were used., and ACQUTIY UPLC® Oligonucleotide BEH C18 1.7 μm (2.1 × 100 mm) was used. MALDI-TOF mass spectra of all new oligonucleotides were recorded on Bruker Daltonics Autoflex maX TOF/TOF mass spectrometer. ESI-TOF-MS spectra of all oligonucleotides used in the enzymatic degradation experiments were recorded on Waters ACQUITY RDa Detector. The UV melting experiments were performed on SHIMADZU UV-1650PC and UV-1800 spectrometers that were equipped with T_m analysis accessory quartz cuvettes of 1-cm optional path lengths. CD spectra were measured using a spectrophotometer, JASCO J-720W.

3,5-Di-O-benzyl-1,2-O-isopropylidene-4-C-propargyloxymethyl-α-D-erythro-pentofuranose (2). Under N_2 atmosphere, sodium hydride (0.93 g, 23 mmol) and propargyl bromide (9.2 M in toluene, 4.8 mL, 44 mmol) were added to a solution of compound **1**[45] (8.90 g, 22 mmol) in anhydrous THF (100 mL) at 0 °C. The resultant mixture was stirred for 25 h at room temperature. After addition of H_2O, the mixture was extracted with AcOEt. The organic layer was washed with brine and dried over Na_2SO_4, and then concentrated under reduced pressure. The residue (11.6 g) was purified by silica gel column chromatography (*n*-hexane/AcOEt = 1:1) to give compound **2** (7.30 g, 75%) as a white form. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.34–7.24 (10H, m), 5.76 (1H, d, *J* = 3.5 Hz), 4.74 (1H, d, *J* = 12.0 Hz), 4.61 (1H, t, *J* = 4.5 Hz), 4.57–4.53 (2H, m), 4.46 (1H, d, *J* = 12.0 Hz), 4.27–4.17 (3H, m), 4.06 (1H, d, *J* = 11.0 Hz), 3.79 (1H, d, *J* = 11.0 Hz), 3.66 (1H, d, *J* = 10.0 Hz), 3.52 (1H, d, *J* = 10.0 Hz), 2.38 (1H, t, *J* = 2.5 Hz), 1.65 (3H, s), 1.34 (3H, s). ^{13}C{^{1}H} NMR (76 MHz, $CDCl_3$) δ: 138.0, 137.6, 128.10, 128.06, 127.6, 127.3, 113.2, 104.0, 86.4, 79.8, 79.1, 77.9, 74.3, 73.3, 72.3, 71.3, 69.9, 60.1, 58.6, 26.6, 26.0, 20.8, 13.9. HRMS (MALDI-TOF) *m*/*z*: $[M+Na]^+$ calcd for $C_{26}H_{30}O_6Na$, 461.1940; found, 461.1935.

2'-O-Acetyl-3',5'-di-O-benzyl-4'-C-propargyloxymethyl-5-methyluridine (3). Under N_2 atmosphere, acetic anhydride (17.2 mL, 18 mmol) and concd sulfuric acid (0.097 mL, 1.8 mmol) were added to a solution of compound ***3′*** (8.0 g, 18 mmol) in acetic acid (20 mL) at 0 °C. The reaction mixture was stirred for 0.5 h at room temperature. After completion of the reaction, the solution was neutralized with saturated aqueous $NaHCO_3$ and the product was extracted with AcOEt. The organic layer was washed with brine and dried over Na_2SO_4, and then concentrated under reduced pressure. The obtained residue (9.23 g) was dissolved in anhydrous MeCN (100 mL), and thymine (2.75 g,

22 mmol) and BSA (17.8 mL, 73 mmol) were added. The solution was refluxed until all the substrate dissolved and then was cooled to 0 °C. TMSOTf (2.0 mL, 11 mmol) was added, and the reaction mixture was refluxed for 2 h. After completion of the reaction, the solution was cooled to room temperature, added saturated aqueous $NaHCO_3$ and extracted with AcOEt. The organic layer was washed with brine and dried over Na_2SO_4. The residue (10.1 g) was concentrated under reduced pressure and purified by silica gel column chromatography (*n*-hexane/AcOEt = 1:1) to give compound **3** (8.2 g, 82%, 2 steps) as a white form. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.06 (1H, s), 7.50 (1H, s), 7.37–7.32 (10H, m), 6.32 (1H, d, *J* = 7.0 Hz), 5.41 (1H, t, *J* = 6.5 Hz), 4.62–4.55 (4H, m), 4.43 (1H, d, *J* = 5.5 Hz), 4.18–4.08 (2H, m), 3.85 (1H, d, *J* = 10.5 Hz), 3.74 (1H, d, *J* = 9.5 Hz), 3.68 (1H, d, *J* = 3.0 Hz), 3.66 (1H, d, *J* = 2.5 Hz), 2.42 (1H, t, *J* = 2.5 Hz), 2.06 (3H, s), 1.51 (3H, s). ^{13}C{^{1}H} NMR (76 MHz, $CDCl_3$) δ: 170.4, 163.9, 150.8, 137.6, 137.3, 135.8, 128.8, 128.2, 128.1, 127.9, 127.7, 111.6, 87.1, 85.4, 79.2, 78.4, 75.2, 75.03, 74.98, 73.8, 72.5, 69.8, 58.9, 20.8, 12.1. HRMS (MALDI-TOF) *m*/*z*: $[M+Na]^+$ calcd for $C_{30}H_{32}N_2O_8Na$, 571.2056; found, 571.2051.

3',5'-Di-O-benzyl-4'-C-propargyloxymethyl-5-methyluridine (4). Under N_2 atmosphere, potassium carbonate (3.10 g, 22 mmol) was added to a solution of **3** (12.2 g, 22 mmol) in anhydrous MeOH (200 mL) and the reaction mixture was stirred for 4 h. After completion of the reaction, saturated aqueous NH_4Cl was added, and the product was extracted with AcOEt. The organic layer was washed with brine and dried over Na_2SO_4. The residue (11.8 g) was concentrated under reduced pressure and purified by silica gel column chromatography (*n*-hexane/AcOEt = 1:2) to give compound **5′** (11.0 g, 98%) as a white form. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.00 (1H, s), 7.39–7.27 (11H, m), 5.91 (1H, d, *J* = 5.0 Hz), 4.72 (1H, d, *J* = 11.5 Hz), 4.68 (1H, d, *J* = 11.5 Hz), 4.57 (1H, d, *J* = 11.5 Hz), 4.53 (1H, d, *J* = 11.5 Hz), 4.43–4.38 (1H, m), 4.26 (1H, d, *J* = 6.0 Hz), 4.19 (2H, t, *J* = 2.5 Hz), 3.76–3.66 (4H, m), 3.33 (1H, d, *J* = 11.0 Hz), 2.44 (1H, t, *J* = 2.0 Hz), 1.60 (3H, s). ^{13}C{^{1}H} NMR (76 MHz, $CDCl_3$) δ: 164.2, 151.1, 137.6, 137.4, 136.6, 128.7, 128.6, 128.1, 128.0, 127.8, 127.7, 111.1, 90.4, 87.1, 79.0, 78.7, 75.4, 74.3, 73.8, 72.5, 70.3, 59.0, 12.2. HRMS (MALDI-TOF) *m*/*z*: $[M+Na]^+$ calcd for $C_{28}H_{30}N_2O_7Na$, 529.1951; found, 529.1945.

3',5'-Di-O-benzyl-4'-C-propargyloxymethyl-2'-O-imidazolylthiocarbonyl-5-methyluridine (5). Under N_2 atmosphere, thiocarbonyldiimidazole (0.70 g, 3.9 mmol) and DMAP (0.24 g, 2.0 mmol) were added to a solution of compound **4** (1.00 g, 2.0 mmol) in anhydrous CH_2Cl_2 (20 mL). The reaction mixture was stirred at room temperature for 4 h. The resulting mixture was purified by silica gel column chromatography (*n*-hexane/AcOEt = 1:1) to give compound **5** (1.08 g, 88%) as a light yellow form. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.34 (1H, s), 8.28 (1H, s), 7.54 (1H, s), 7.48 (1H, d, *J* = 1.0 Hz), 7.43 (2H, d, *J* = 7.0 Hz), 7.39–7.15 (8H, m), 7.02 (1H, s), 6.58 (1H, d, *J* = 8.0 Hz), 5.90 (1H, dd, *J* = 8.0, 5.5 Hz), 4.71–4.67 (3H, m), 4.60 (1H, d, *J* = 11.5 Hz), 4.49 (1H, d, *J* = 11.5 Hz), 4.17 (2H, dd, *J* = 9.0, 2.5 Hz), 3.89 (1H, d, *J* = 10.5 Hz), 3.83 (2H, dd, *J* = 10.0, 7.5 Hz), 3.66 (1H, d, *J* = 10.0 Hz), 2.46 (1H, t, *J* = 2.5 Hz), 1.52 (3H, d, *J* = 1.5 Hz). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ: 182.9, 163.9, 150.1, 137.5, 137.0, 136.9, 135.1, 131.0, 128.6, 128.4, 128.2, 128.0, 127.7, 117.8, 112.0, 87.5, 84.5, 81.8, 78.8, 78.2, 75.6, 75.4, 73.9, 73.3, 69.6, 58.8, 12.1. HRMS (MALDI-TOF) *m*/*z*: $[M+Na]^+$ calcd for $C_{32}H_{32}N_4O_7NaS$, 639.1889; found, 639.1884.

3',5'-Di-O-benzyl-4'-C-propargyloxymethylthymidine (6). Under N_2 atmosphere, *n*-Bu_3SnH (0.096 mL, 0.27 mmol) and AIBN (5 mg, 0.035 mmol) were added to a solution of compound **5** (110 mg, 0.18 mmol) in anhydrous toluene

(2 mL) at room temperature. The reaction mixture was refluxed for 3 h. The resulting mixture was concentrated under reduced pressure and the residue (250 mg) was purified by silica gel column chromatography (*n*-hexane/AcOEt = 1:1) to give compound **6** (45 mg, 51%) as a white form. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.21 (1H, s), 7.59 (1H, s), 7.37–7.28 (10H, m), 6.39 (1H, t, *J* = 6.5 Hz), 4.63 (1H, d, *J* = 12.0 Hz), 4.56 (2H, s), 4.47 (1H, d, *J* = 12.0 Hz), 4.35 (1H, d, *J* = 3.0 Hz), 4.21 (1H, d, *J* = 15.5 Hz), 4.15 (1H, d, *J* = 15.5 Hz), 3.83 (1H, d, *J* = 10.5 Hz), 3.73 (2H, s), 3.67 (1H, d, *J* = 10.5 Hz), 2.65–2.60 (2H, m), 2.53–2.48 (1H, m), 2.42 (1H, d, *J* = 2.0 Hz), 2.26–2.20 (1H, m), 1.62 (3H, s). ^{13}C{^{1}H} NMR (76 MHz, $CDCl_3$) δ: 164.0, 150.5, 137.5, 137.3, 135.9, 128.5, 128.4, 128.3, 127.8, 127.7, 127.44, 127.38, 127.3, 110.1, 87.4, 84.4, 79.4, 79.2, 74.8, 73.5, 72.3, 69.9, 58.7, 37.9, 12.0. HRMS (MALDI-TOF) *m*/*z*: $[M+Na]^+$ calcd for $C_{28}H_{30}N_2O_6Na$:, 513.2002; found, 513.1996.

4'-C-Propargyloxymethylthymidine (7). Under N_2 atmosphere, BCl_3 (1 M in hexane, 15 mL, 15 mmol) was added to a solution of compound **6** (1.2 g, 2.5 mmol) in anhydrous CH_2Cl_2 (25 mL) at –78 °C. The reaction mixture was stirred at –78 °C for 1.5 h. Anhydrous MeOH was added, the reaction mixture was warmed to room temperature and concentrated under reduced pressure. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH = 9:1) to give compound **7** (0.60 g, 79%) as a white form. ^{1}H NMR (400 MHz, CD_3OD) δ: 7.83 (1H, s), 6.31 (1H, t, *J* = 6.4 Hz), 4.62 (1H, s), 4.49 (1H, t, *J* = 5.6 Hz), 4.20 (2H, d, *J* = 2.0 Hz), 3.70 (2H, d, *J* = 2.4 Hz), 3.68 (2H, d, *J* = 3.6 Hz), 3.34 (1H, s), 3.31–3.30 (1H, m), 2.86 (1H, t, *J* = 2.4 Hz), 2.34 (2H, t, *J* = 6.0 Hz), 1.88 (3H, s). ^{13}C{^{1}H} NMR (101 MHz, CD_3OD) δ: 152.4, 138.4, 111.5, 89.9, 85.9, 80.5, 76.1, 72.8, 70.8, 64.4, 59.6, 41.5, 12.5. HRMS (MALDI-TOF) *m*/*z*: $[M+Na]^+$ calcd for $C_{14}H_{18}N_2O_6Na$, 333.1063; found, 333.1057.

5'-O-tert-Butyldimethylsilyl 4'-C-propargyloxymethylthymidine (8). Under N_2 atmosphere, TBSCl (600 mg, 4.0 mmol) was added to a solution of compound **7** (420 mg, 1.4 mmol) in anhydrous pyridine (15 mL). The reaction mixture was stirred at 60 °C for 20 h. Dry MeOH was added and concentrated under reduced pressure. The residue (1.06 g) was extracted with $CHCl_3$ and the organic layer was washed with H_2O and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH = 9:1) to give compound **8** (540 mg, 94%) as a white form. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.18 (1H, s), 7.54 (1H, d, *J* = 1.2 Hz), 6.44 (1H, dd, *J* = 5.6, 9.2 Hz), 4.48–4.45 (1H, m), 4.26–4.14 (2H, m), 3.87 (1H, d, *J* = 10.8 Hz), 3.82 (1H, d, *J* = 9.2 Hz), 3.74 (1H, d, *J* = 10.8 Hz), 3.54 (1H, d, *J* = 9.2 Hz), 2.68 (1H, d, *J* = 4.4 Hz), 2.52 (1H, t, *J* = 2.8 Hz), 2.40–2.35 (1H, m), 2.27–2.19 (1H, m), 1.92 (3H, d, *J* = 0.9 Hz), 0.94 (9H, s), 0.13 (3H, s), 0.13 (3H, s). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ: 163.8, 150.3, 135.5, 110.9, 88.2, 84.7, 78.6, 75.7, 73.7, 69.8, 66.2, 58.8, 40.9, 25.9, 18.7, 12.5, –5.4, –5.5. HRMS (MALDI-TOF) *m*/*z*: $[M+Na]^+$ calcd for $C_{20}H_{32}N_2O_6NaSi$, 447.1927; found, 447.1922.

5'-O-tert-Butyldimethylsilyl-4'-C-propargyloxymethyl-3'-O-imidazol-1-ylthiocarbonylthymidine (9). Under N_2 atmosphere, thiocarbonyldiimidazole (1.70 g, 9.5 mmol) and DMAP (0.581 g, 4.8 mmol) were added to a solution of compound **8** (2.02 g, 4.8 mmol) in anhydrous CH_2Cl_2 (50 mL). The reaction mixture was stirred at room temperature for 3 h. The resulting mixture was purified by silica gel column chromatography (*n*-hexane/AcOEt = 1:1) to give compound **9** (2.44 g, 96%) as a light yellow form. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 9.31 (1H, s), 8.42 (1H, s), 7.68 (1H, s), 7.52 (1H, s), 7.08 (1H, s), 6.45 (1H, t, *J* = 6.5 Hz), 6.21 (1H, d, *J* = 6.0 Hz), 4.16 (2H, d, *J* = 1.0 Hz),

3.92 (1H, d, J = 10.5 Hz), 3.89 (1H, d, J = 11.0 Hz), 3.71 (2H, s), 2.67–2.63 (1H, m), 2.58–2.52 (1H, m), 2.43 (1H, d, J = 2.0 Hz), 1.94 (3H, s), 0.95 (9H, s), 0.16 (6H, s). $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$) δ: 182.3, 163.6, 150.3, 137.0, 135.0, 131.0, 118.1, 111.5, 87.6, 84.5, 83.0, 78.4, 76.7, 69.5, 58.9, 38.8, 25.8, 18.3, 12.5, –5.4, –5.5. HRMS (MALDI-TOF) m/z: $[M+Na]^+$ calcd for $C_{24}H_{34}N_4O_6NaSiS$, 557.1861; found, 557.1861.

(1R,6R,8R)-1-tert-Butyldimethylsiloxymethyl-5-methylene-8-(thymin-1-yl)-3,9-dioxabicyclo[4.3.0]nonane (10). Under N_2 atmosphere, n-Bu_3SnH (0.135 mL, 0.37 mmol) and AIBN (11 mg, 0.070 mmol) were added to a solution of compound **9** (125 mg, 0.23 mmol) in anhydrous toluene (6 mL) at room temperature. The reaction mixture was refluxed for 0.5 h. The resulting mixture was concentrated under reduced pressure and the residue (490 mg) was purified by silica gel column chromatography (n-hexane/AcOEt = 1:1) to give compound **10** (45 mg, 47%) as a white form. ^1H NMR (400 MHz, $CDCl_3$) δ: 7.69 (1H, d, J = 0.9 Hz), 6.15 (1H, dd, J = 7.3, 2.3 Hz), 5.00 (2H, d, J = 8.7 Hz), 4.12 (2H, s), 3.83 (2H, d, J = 2.3 Hz), 3.52 (1H, d, J = 12.4 Hz), 3.38–3.28 (2H, m), 2.80–2.72 (1H, m), 2.22 (1H, ddd, J = 14.3, 9.0, 2.6 Hz), 1.95 (3H, d, J = 1.4 Hz), 0.94 (9H, s), 0.12 (3H, s), 0.11 (3H, s). $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$) δ: 164.0, 150.4, 140.0, 126.6, 114.0, 110.2, 85.8, 84.7, 68.4, 68.0, 63.9, 40.6, 37.5, 26.0, 18.6, 12.7, –5.2, –5.4. HRMS (MALDI-TOF) m/z: $[M+Na]^+$ calcd for $C_{20}H_{32}N_2O_5NaSi$, 431.1973; found, 431.1973.

(1R,6R,8R)-8-(N^3-Benzyloxymethylthymin-1-yl)-1-tert-butyldimethylsiloxymethyl-5-methylene-3,9-dioxabicyclo[4.3.0]nonane (11). Under N_2 atmosphere, BOMCl (0.070 mL, 0.50 mmol) and DBU (0.082 mL, 0.55 mmol) were added to a solution of compound **10** (170 mg, 0.42 mmol) in anhydrous THF (5 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 AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue (350 mg) was purified by silica gel column chromatography (n-hexane/AcOEt = 1:1) to give compound **11** (150 mg, 68%) as a colorless oil. ^1H NMR (500 MHz, $CDCl_3$) δ: 7.68 (1H, s), 7.39–7.24 (5H, m), 6.12 (1H, dd, J = 7.0, 2.5 Hz), 5.49 (2H, s), 5.00 (2H, d, J = 9.0 Hz), 4.71 (2H, s), 4.12 (2H, s), 3.84 (2H, d, J = 5.0 Hz), 3.51 (1H, d, J = 12.0 Hz), 3.37 (1H, d, J = 12.5 Hz), 3.26 (1H, dd, J = 11.0, 9.5 Hz), 2.79–2.71 (1H, m), 2.22–2.17 (1H, m), 1.94 (3H, s), 0.94 (9H, s), 0.11 (6H, s). $^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$) δ: 163.4, 150.9, 140.1, 138.1, 134.4, 128.6, 127.7, 127.6, 127.0, 114.0, 109.4, 85.9, 85.4, 72.2, 68.5, 68.1, 64.0, 40.5, 37.6, 26.0, 18.6, 13.4, –5.2, –5.3. HRMS (MALDI-TOF) m/z: $[M+Na]^+$ calcd for $C_{28}H_{40}N_2O_6NaSi$, 551.2548; found, 551.2548.

(1R,6R,8R)-8-(N^3-Benzyloxymethylthymin-1-yl)-1-tert-butyldimethylsiloxymethyl-5-hydroxymethyl-3,9-dioxabicyclo[4.3.0]nonane (12). Under N_2 atmosphere, 9-BBN (0.5 M in THF, 13.5 mL, 6.8 mmol) was added to a solution of compound **11** (1.19 g, 2.3 mmol) in anhydrous THF (25 mL). The reaction mixture was stirred at room temperature for 17 h. Then, NaOH (1 M, 13 mL, 13 mmol) and H_2O_2 (0.1 M, 1.3 mL, 13 mmol) were added to a reaction mixture, and the mixture was stirred at room temperature for 30 minutes. After addition of sat. $NaHCO_3$, the mixture was extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue (2.41 g) was purified by silica gel column chromatography (n-hexane/AcOEt = 1:1) to give compound **12** (629 mg, 51%) as a mixture of diasteromers (R:S = 3:1). ^1H NMR (400 MHz, $CDCl_3$) δ: 7.68 (0.75H, s), 7.53 (0.25H, s), 7.39–7.25 (5H, m), 6.12 (0.25H, dd, J = 8.5, 5.5 Hz), 6.04 (0.75H, d, J = 8.5 Hz),

5.49 (2H, d, *J* = 4.5 Hz), 4.71 (2H, s), 3.93 (1.5H, s), 3.87 (1H, dd, *J* = 14.5, 6.0 Hz), 3.83–3.77 (0.5H, m), 3.73–3.67 (1H, m), 3.61–3.42 (3.5H, m), 3.28 (0.75H, t, *J* = 14.5 Hz), 3.20 (0.75H, d, *J* = 14.5 Hz), 2.79–2.72 (0.75H, m), 2.69–2.61 (1H, m), 2.54–2.49 (0.25H, m), 2.24–2.13 (1H, m), 2.05–2.00 (1H, m), 1.93 (3H, s), 0.95–0.94 (9H, m), 0.14–0.12 (6H, m). $^{13}C\{^{1}H\}$ NMR (101 MHz, $CDCl_3$) δ: 163.6, 150.9, 139.7, 139.6, 138.0, 134.4, 128.3, 127.7, 127.63, 127.59, 109.2, 105.2, 85.1, 84.5, 72.2, 70.3, 69.1, 65.0, 63.5, 62.4, 37.1, 34.1, 33.0, 32.4, 26.01, 25.93, 18.6, 13.5, –5.2, –5.36, –5.39. HRMS (MALDI-TOF) *m*/*z*: $[M+Na]^+$ calcd for $C_{28}H_{42}N_2O_7NaSi$, 569.2659; found, 569.2653.

(1R,6R,8R)-8-(N^3-Benzyloxymethylthymin-1-yl)-1-tert-butyldimethylsiloxymethyl-5-carboxyl-3,9-dioxabicyclo[4.3.0]nonane (13). Under N_2 atmosphere, $PhI(OAc)_2$ (286 mg, 0.89 mmol) and TEMPO (13 mg, 0.088 mmol) were added to a solution of compound **12** (162 mg, 0.30 mmol) in MeCN/H_2O (1:1, 6 mL) at room temperature. The reaction mixture was stirred at room temperature for 17 h. The mixture was extracted with $CHCl_3$ and the organic layer was washed with H_2O and brine. The residue was dried over Na_2SO_4, concentrated under reduced pressure and purified by silica gel column chromatography ($CHCl_3$/MeOH = 14:1) to give compound **13** (140 mg, 84%) as a white form. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.62 (0.75H, d, *J* = 2.5 Hz), 7.56 (0.25H, d, *J* = 1.5 Hz), 7.38–7.25 (5H, m), 6.07–6.15 (1H, m), 5.48 (2H, d, *J* = 7.5 Hz), 4.69 (2H, s), 4.04–4.00 (1H, m), 3.90 (1H, d, *J* = 3.5 Hz), 3.68–3.47 (3H, m), 3.24–3.20 (1H, m), 3.14–3.05 (1H, m), 2.96–2.88 (1H, m), 2.75–2.64 (1H, m), 2.27–2.13 (1H, m), 1.92 (3H, d, *J* = 1.0 Hz), 0.95–0.92 (9H, m), 0.14–0.09 (6H, m). $^{13}C\{^{1}H\}$ NMR (126 MHz, $CDCl_3$) δ: 176.5, 175.3, 163.50, 163.49, 150.9, 137.9, 137.8, 134.2, 134.0, 128.2, 127.7, 127.6, 127.6, 109.7, 109.6, 86.0, 84.7, 83.7, 83.1, 72.2, 70.4, 70.3, 69.3, 67.7, 65.8, 63.2, 63.0, 42.4, 39.6, 38.3, 36.3, 33.2, 27.6, 26.0, 25.9, 18.5, 18.3, 13.4, –5.3, –5.4, –5.6. HRMS (MALDI-TOF) *m*/*z*: $[M+Na]^+$ calcd for $C_{28}H_{40}N_2O_8NaSi$, 583.2452; found, 583.2446.

(1R,5S,6R,8R)-8-(N^3-Benzyloxymethylthymin-1-yl)-1-tert-butyldimethylsiloxymethyl-5-(3'-O-tert-butyldimethyl-5'-deoxythymidin-5'-ylaminocarbonyl)-3,9-dioxabicyclo[4.3.0]nonane (15S) and (1R,5R,6R,8R)-8-(N^3-Benzyloxymethylthymin-1-yl)-1-tert-butyldimethylsiloxymethyl-5-(3'-O-tert-butyldimethyl-5'-deoxythymidin-5'-ylaminocarbonyl)-3,9-dioxabicyclo[4.3.0]nonane (15R). Under N_2 atmosphere, HOBt (105 mg, 0.78 mmol), HBTU (323 mg, 0.85 mmol) and DIPEA (0.247 ml, 1.4 mmol) were added to a solution of compound **13** (398 mg, 0.71 mmol) in anhydrous CH_2Cl_2 (10 mL) after azeotropic with anhydrous toluene. The reaction mixture was stirred at room temperature for 10 min and amine **14**[46] in anhydrous CH_2Cl_2 (5 ml) was added to the reaction mixture. The reaction mixture was stirred at room temperature for 24 h and the resulting mixture was concentrated under reduced pressure. The residue (1.99 g) was purified by silica gel column chromatography (*n*-hexane/Acetone =3:2) to give a compound **15S** (457 mg, 67%) and **15R** (132 mg, 21%) as a white form. Compound **15S**: ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.88 (1H, s), 7.53 (1H, d, *J* = 1.5 Hz), 7.38–7.23 (5H, m), 7.04 (1H, s), 6.50 (1H, dd, *J* = 6.5, 4.0 Hz), 6.12 (1H, d, *J* = 7.0 Hz), 5.77 (1H, t, *J* = 7.0 Hz), 5.48 (2H, dd, *J* = 12.5 Hz, 9.5 Hz), 4.69 (2H, s), 4.31–4.28 (1H, m), 3.94–3.86 (4H, m), 3.69 (1H, t, *J* = 11.5 Hz), 3.64–3.59 (1H, m), 3.52 (1H, d, *J* = 12.0 Hz), 3.44–3.39 (1H, m), 3.26 (1H, d, *J* = 12.5 Hz), 2.98–2.88 (2H, m), 2.80–2.76 (1H, m), 2.54–2.49 (1H, m), 2.21–2.16 (2H, m), 1.93 (3H, d, *J* = 1.5 Hz), 1.91 (3H, d, *J* = 1.0 Hz), 0.94 (9H, s), 0.87 (9H, s), 0.14 (3H, s), 0.13 (3H, s), 0.06 (3H, s), 0.03 (3H, s). $^{13}C\{^{1}H\}$ NMR (126 MHz, $CDCl_3$) δ: 176.5, 175.3, 163.5, 163.4, 150.8, 150.1, 138.5, 138.0, 134.4, 128.3, 127.7, 127.6, 111.1,

109.5, 89.9, 85.2, 84.8, 84.5, 72.2, 70.4, 67.7, 64.0, 63.5, 41.7, 40.5, 69.3, 38.6, 34.2, 33.3, 26.1, 25.7, 18.6, 17.9, 13.4, 12.3, –4.75, –4.84, –5.2. HRMS (MALDI-TOF) *m/z*: $[M+Na]^+$ calcd for $C_{44}H_{67}N_5O_{11}NaSi_2$, 920.4273; found, 920.4268. Compound **15R**: ^1H NMR (500 MHz, $CDCl_3$) δ: 10.07 (1H, s), 7.64 (1H, s), 7.33–7.19 (5H, m), 7.06 (1H, d, *J* = 7.0 Hz), 6.92 (1H, s), 6.52 (1H, dd, *J* = 9.0, 5.0 Hz), 5.61–5.56 (2H, m), 5.46 (1H, dd, *J* = 9.0, 4.0 Hz), 4.76–4.69 (2H, m), 4.58–4.54 (1H, m), 4.00–3.88 (4H, m), 3.66 (1H, d, *J* = 10.5 Hz), 3.60 (1H, d, *J* = 10.5 Hz), 3.34–3.37 (2H, m), 3.31 (1H, d, *J* = 13.5 Hz), 2.72 (2H, dd, *J* = 10.5, 6.5 Hz), 2.64 (1H, dd, *J* = 12.5, 5.0 Hz), 2.60–2.49 (2H, m), 2.30–2.18 (2H, m), 1.94 (3H, s), 1.90 (3H, s), 0.94 (9H, s), 0.87 (9H, s), 0.14 (3H, s), 0.13 (3H, s), 0.06 (6H, s). $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$) δ: 171.8, 163.5, 163.1, 151.5, 150.7, 139.0, 138.2, 134.2, 128.2, 127.5, 127.4, 111.1, 110.9, 91.5, 86.1, 84.6, 82.2, 72.2, 71.1, 70.4, 70.4, 68.1, 67.8, 45.8, 40.2, 39.0, 38.9, 37.4, 26.0, 25.7, 18.3, 17.9, 13.3, 12.3, –4.8, –4.9, –5.3, –5.4. HRMS (MALDI-TOF) *m/z*: $[M+Na]^+$ calcd for $C_{44}H_{67}N_5O_{11}NaSi_2$, 920.4273; found, 920.4268.

(1S,5S,6R,8R)-8-(thymin-1-yl)-1-hydroxymethyl-5-(5'-deoxythymidin-5'-ylaminocarbonyl)-3,9-dioxabicyclo[4.3.0]nonane (16S). Under H_2 atmosphere, $Pd(OH)_2$ (210 mg, 1.2 mmol) was added to a solution of compound **15S** (420 mg, 0.47 mmol) in MeOH/THF (1:1, 6 mL). The reation mixture was stirred at room temperature for 4 h. The mixture was filtered by celite and concentrated under reduced pressure. The crude product (315 mg) was obtained which was used for the next reaction without purification. The crude product (315 mg) was dissolved in anhydrous THF (6 mL), and TBAF (1 M in THF, 0.89 mL, 0.89 mmol) was added. The reaction mixture was stirred at room temperature for 24 h and concentrated under reduced pressure. The residue (0.410 g) was purified by silica gel chromatography ($CHCl_3$/MeOH = 9:1) to give compound **16S** (240 mg, 94%, 2 steps) as a white form. ^1H NMR (500 MHz, CD_3OD) δ: 7.98 (1H, d, *J* = 1.0 Hz), 7.44 (1H, d, *J* = 1.0 Hz), 6.11 (1H, t, *J* = 6.5 Hz), 6.06 (1H, d, *J* = 7.0 Hz), 4.20–4.18 (1H, m), 3.87–3.77 (4H, m), 3.69 (1H, t, *J* = 11.5 Hz), 3.47 (1H, d, *J* = 12.0 Hz), 3.43–3.37 (2H, m), 3.31 (1H, d, *J* = 12.5 Hz), 3.00–2.95 (1H, m), 2.91–2.83 (2H, m), 2.23–2.19 (2H, m), 1.97–2.02 (1H, m), 1.86 (3H, d, *J* = 1.5 Hz), 1.81 (3H, d, *J* = 1.0 Hz). $^{13}C\{^1H\}$ NMR (101 MHz, CD_3OD) δ: 173.6, 166.5, 166.3, 152.3, 152.2, 138.44, 138.40, 111.8, 110.8, 87.2, 86.1, 85.9, 84.8, 73.0, 68.8, 64.8, 63.1, 42.29, 42.26, 42.0, 36.0, 33.8, 12.6, 12.4. HRMS (MALDI-TOF) *m/z*: $[M+Na]^+$ calcd for $C_{24}H_{31}N_5O_{10}Na$, 572.1969; found, 572.1963.

(1S,5R,6R,8R)-8-(thymin-1-yl)-1-hydroxymethyl-5-(5'-deoxythymidin-5'-ylaminocarbonyl)-3,9-dioxabicyclo[4.3.0]nonane (16R). Under H_2 atmosphere, $Pd(OH)_2$ (25 mg, 0.14 mmol) was added to a solution of compound **15R** (49 mg, 0.054 mmol) in MeOH/THF (1:1, 2 mL). The reation mixture was stirred at room temperature for 1.5 h. The mixture was filtered by celite and concentrated under reduced pressure. The crude product (40 mg) was obtained which was used for the next reaction without purification. The crude product (40 mg) was dissolved in anhydrous THF (1 mL), and TBAF (1 M in THF, 0.10 mL, 0.10 mmol) was added. The reaction mixture was stirred at room temperature for 24 h and concentrated under reduced pressure. The residue (41 mg) was purified by silica gel chromatography ($CHCl_3$/MeOH = 9:1) to give compound **16R** (31 mg, quant., 2 steps) as a white form. ^1H NMR (500 MHz, CD_3OD) δ: 7.96 (1H, s), 7.47 (1H, s), 6.21 (1H, t, *J* = 6.5 Hz), 6.11 (1H, t, *J* = 7.0 Hz), 4.31–4.28 (1H, m), 3.93–3.90 (2H, m), 3.82 (1H, d, *J* = 13.0 Hz), 3.67–3.51 (6H, m), 3.45 (1H, d, *J* = 14.0, 4.0 Hz), 2.84–2.80 (1H,

m), 2.68–2.64 (1H, m), 2.46–2.41 (1H, m), 2.38–2.29 (2H, m), 2.26–2.22 (1H, m), 1.89 (3H, s), 1.88 (3H, s). $^{13}C\{^{1}H\}$ NMR (101 MHz, CD_3OD) δ: 174.7, 166.42, 166.37, 152.4, 152.2, 139.0, 138.0, 111.6, 111.2, 87.9, 86.8, 86.1, 84.3, 72.8, 70.5, 68.0, 66.0, 45.6, 42.3, 39.8, 38.3, 37.9, 12.6, 12.4. HRMS (MALDI-TOF) *m*/*z*: $[M+Na]^+$ calcd for $C_{24}H_{31}N_5O_{10}Na$, 572.1969; found, 572.1963.

(1R,5S,6R,8R)-1-(4,4'-Dimethoxytrityl)oxymethyl-8-(thymin-1-yl)-5-(5'-deoxythymidin-5'-ylaminocarbonyl)-3,9-dioxabicyclo[4.3.0]nonane (17S). Under N_2 atmosphere, DMTrCl (107 mg, 0.32 mmol) was added to a solution of compound **16S** (145 mg, 0.26 mmol) in anhydrous pyridine (3 mL) after azeotropic with anhydrous pyridine. The reaction mixture was stirred at room temperature for 22 h. MeOH was added and concentrated under reduced pressure. The residue (205 mg) was purified by silica gel column chromatography ($CHCl_3$/MeOH = 9:1) to give compound **17S** (160 mg, 71%) as a white form. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 9.81 (1H, s), 9.58 (1H, s), 7.67 (1H, s), 7.43 (2H, d, *J* = 7.5 Hz), 7.34–7.19 (7H, m), 7.10 (1H, s), 6.88–6.81 (5H, m), 6.00 (1H, d, *J* = 6.5 Hz), 5.90 (1H, s), 4.30–4.20 (1H, m), 3.97–3.96 (1H, m), 3.86–3.39 (12H, m), 3.41 (1H, d, *J* = 11.0 Hz), 3.24–3.15 (2H, m), 2.89–2.85 (1H, m), 2.59–2.57 (1H, m), 2.36–2.27 (3H, m), 1.84 (3H, s), 1.45 (3H, s). $^{13}C\{^{1}H\}$ NMR (101 MHz, $CDCl_3$) δ: 171.3, 164.5, 164.1, 158.54, 158.51, 150.7, 147.3, 144.4, 139.4, 135.4, 130.1, 129.1, 128.1, 127.9, 127.8, 127.7, 127.0, 113.2, 113.1, 111.0, 110.2, 86.5, 83.5, 81.4, 72.0, 67.6, 63.5, 55.2, 41.0, 38.9, 35.4, 33.0, 29.7, 12.2, 12.1. HRMS (MALDI-TOF) *m*/*z*: $[M+Na]^+$ calcd for $C_{45}H_{49}N_5O_{12}Na$, 874.3275; found, 874.3270.

(1R,5R,6R,8R)-1-(4,4'-Dimethoxytrityl)oxymethyl-8-(thymin-1-yl)-5-(5'-deoxythymidin-5'-ylaminocarbonyl)-3,9-dioxabicyclo[4.3.0]nonane (17R). Under N_2 atmosphere, DMTrCl (44 mg, 0.13 mmol) was added to a solution of compound **16R** (60 mg, 0.11 mmol) in anhydrous pyridine (1 mL) after azeotropic with anhydrous pyridine. The reaction mixture was stirred at room temperature for 17 h. MeOH was added and concentrated under reduced pressure. The residue (144 mg) was purified by silica gel column chromatography ($CHCl_3$/MeOH = 9:1) to give compound **17R** (78 mg, 84%) as a white form. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 10.23 (1H, s), 10.14 (1H, s), 8.07 (1H, s), 7.41 (2H, d, *J* = 7.5 Hz), 7.32–7.24 (7H, m), 6.84–6.85 (5H, m), 6.50 (1H, dd, *J* = 8.5, 5.5 Hz), 5.47 (1H, s), 4.53–4.48 (1H, m), 3.99 (2H, d, *J* = 12.5 Hz), 3.84–3.82 (2H, m), 3.78 (6H, s), 3.55 (1H, d, *J* = 13.0 Hz), 3.39 (1H, t, *J* = 11.0 Hz), 3.30 (1H, d, *J* = 14.0 Hz), 3.14–3.02 (3H, m), 2.95–2.88 (1H, m), 2.59 (2H, dd, *J* = 12.5, 5.5), 2.32–2.26 (2H, m), 2.01–1.96 (1H, s), 1.81 (3H, s), 1.51 (3H, s). $^{13}C\{^{1}H\}$ NMR (126 MHz, $CDCl_3$) δ: 172.6, 164.3, 164.0, 158.7, 151.2, 150.0, 144.2, 139.4, 135.4, 135.3, 135.0, 130.1, 128.1, 128.1, 127.2, 113.3, 111.6, 110.1, 90.1, 87.1, 85.6, 84.7, 81.9, 70.2, 69.8, 68.3, 66.7, 55.5, 55.3, 55.4, 44.9, 39.0, 38.6, 38.5, 36.2, 12.2, 12.1. HRMS (MALDI-TOF) *m*/*z*: $[M+Na]^+$ calcd for $C_{45}H_{49}N_5O_{12}Na$, 874.3275; found, 874.3248.

(1R,5S,6R,8R)-1-(4,4'-Dimethoxytrityl)oxymethyl-8-(thymin-1-yl)-5-(3'-O-2-cyanoethyl-N,N-diisopropylaminophosphinyl-5'-deoxythymidin-5'-ylaminocarbonyl)-3,9-dioxabicyclo[4.3.0]nonane (18S). Under N_2 atmosphere, DIPEA (0.060 mL, 0.35 mmol) and *N*,*N*-diisopropylamino-2-cyanoethylphosphino chloridite (0.046 mL, 0.21 mmol) were added to a solution of compound **17S** (148 mg, 0.17 mmol) in anhydrous CH_2Cl_2 (3 mL) after azeotropic with anhydrous toluene. 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 AcOEt. The organic layer was washed with brine, dried over

Na_2SO_4, and concentrated under reduced pressure. The residue (230 mg) was purified by silica gel column chromatography ($CHCl_3$/MeOH = 9:1) to give compound **18S** (118 mg, 65%) as a white form. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.61 (1H, s), 8.47 (1H, s), 7.70 (1H, d, *J* = 9.0 Hz), 7.45 (2H, d, *J* = 7.5 Hz), 7.35–7.21 (8H, m), 7.18 (0.5H, s), 7.03 (0.5H, d, *J* = 1.5 Hz), 6.90 (0.5H, dd, *J* = 7.5, 2.5 Hz), 6.86–6.84 (4H, m), 6.68 (0.5H, dd, *J* = 7.5, 3.5 Hz), 6.12 (1H, d, *J* = 7.0 Hz), 5.91 (0.5H, dd, *J* = 7.5, 6.0 Hz), 5.73 (0.5H, t, *J* = 7.5 Hz), 4.43–4.37 (1H, m), 4.26–4.23 (0.5H, m), 3.54–3.64 (14H, m), 3.43 (1H, dd, *J* = 11.0, 2.5 Hz), 3.33 (1H, d, *J* = 15.0 Hz), 3.23–3.15 (2H, m), 2.97–2.86 (1H, m), 2.61–2.51 (3.5H, m), 2.47–2.32 (1.5H, m), 2.26–2.18 (1H, m), 1.90 (3H, d, *J* = 10.0 Hz), 1.52 (3H, d, *J* = 1.5 Hz), 1.19–1.16 (12H, m). ^{31}P{^{1}H} NMR (202 MHz, $CDCl_3$) δ: 150.2 (s), 149.0 (s). HRMS (ESI) *m*/*z*: $[M+Na]^+$ calcd for $C_{54}H_{66}N_7O_{13}NaP$, 1074.4354; found, 1074.4354.

(1R,5R,6R,8R)-1-(4,4′-Dimethoxytrityl)oxymethyl-8-(thymin-1-yl)-5-(3′-O-2-cyanoethyl-N,N-diisopropylaminophosphinyl-5′-deoxythymidin-5′-ylaminocarbonyl)-3,9-dioxabicyclo[4.3.0]nonane (18R). Under N_2 atmosphere, DIPEA (0.041 mL, 0.23 mmol) and *N*,*N*-diisopropylamino-2-cyanoethylphosphino chloridite (0.031 mL, 0.14 mmol) were added to a solution of compound **17R** (100 mg, 0.12 mmol) in anhydrous CH_2Cl_2 (1 mL) after azeotropic with anhydrous toluene. 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 AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue (300 mg) was purified by silica gel column chromatography ($CHCl_3$/MeOH = 9:1) to give compound **18R** (101 mg, 82%) as a white form. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 9.81 (1H, s), 7.61 (1H, s),7.44–7.25 (11H, m), 6.94 (1H, s), 6.85 (4H, d, *J* = 7.0 Hz), 6.52–6.51 (1H, m), 5.55–5.56 (1H, m), 4.64–4.59 (1H, m), 4.07–3.95 (3H, m), 3.91–3.76 (9H, m), 3.62–3.51 (2H, m), 3.50–3.36 (3H, m), 3.17 (1H, dd, *J* = 9.5, 3.5 Hz), 3.10 (1H, d, *J* = 9.5 Hz), 2.98–2.90 (1H, m), 2.83–2.56 (5H, m), 2.47–2.41 (0.5H, m), 2.38–2.32 (0.5H, m), 2.18–2.12 (0.5H, m), 2.09–2.04 (0.5H, m), 1.88 (3H, s), 1.48 (3H, d, *J* = 10.0 Hz), 1.21–1.14 (12H, m). ^{31}P{^{1}H} NMR (202 MHz, $CDCl_3$) δ:150.1 (s), 149.7 (s). HRMS (ESI) *m*/*z*: $[M+Na]^+$ calcd for $C_{54}H_{66}N_7O_{13}NaP$, 1074.4354; found, 1074.4341.

Conformational analysis of amide-linked dinucleotides containing a 3′,4′-tpBNA by *ab initio* calculation. The theoretical calculation was performed using the Spartan'20 program. The calculation was carried out at density functional theory (DFT) with the basis function 6-31G* (ωB97XD/6-31G*) by using model compounds **15S′** and **15R′** shown in **Figure 2**.

Synthesis of oligonucleotides ON1–8. Compounds **18S**, **18R**, and **19**,[46] dT-phosphoramidite (Sigma), Bz-dC-phosphoramidite (Sigma), and iBu-dG-phosphoramidite (Sigma) were dissolved in anhydrous MeCN to a final concentration of 0.1 M. The synthesis of oligonucleotides (**ON1**–**8**) was performed on a 0.2 μmol scale by using an automated DNA synthesizer (Gene Design nS-8 Oligonucleotides Synthesizer) with 0.25 M 5-Ethylthio-1*H*-tetrazole (ETT) in MeCN as an activator. **18S** and **18R** were incorporated into oligonucleotides at a prolonged coupling time of 2 min. The oligonucleotides synthesized in trityl-on mode were cleaved from the GPG resin by treatment with 28% aqueous NH_3 at room temperature for 1.5 h. All protecting groups of oligonucleotides were removed by treatment with 28% aqueous NH_3 at 55 °C for 16 h (for **ON1–6**). Removal of NH_3 was carried out *in vacuo*. The

crude oligonucleotides were purified by Sep-Pak® Plus C18 Cartridge (Waters) with the 5′-DMTr group being removed during purification using 1% (v/v) aqueous trifluoroacetic acid. The separated oligonucleotides were further purified by reversed-phase HPLC (Waters XBridge® OST C18 Column 2.5 μm, 10 × 50 mm) using 0.1 M TEAA buffer (pH 7.0) and MeCN (for **ON1–7**) or H_2O/HFIP/TEA (100/1/0.1) solution and MeOH (for **ON8**). The compositions of new oligonucleotides (**ON1**–**6** and **ON8**) were confirmed by MALDI-TOF mass analysis. The overall yields were shown in **Table 2** calculated from the UV absorbance at 260 nm. Measurement method and conditions in reverse-phase HPLC are shown below.

Confirmation of oligonucleotides composition. The composition of the synthesized ONs was confirmed using MALDI-TOF mass. A mixture of 10 mg/mL of 3- hydroxypicolinic acid and 1 mg/mL of diammonium hydrogen citrate in MeCN: 0.1% TFA = 1:1 (v/v) solution was used as matrix. The matrix (1.5 μL) was dried on the measurement plate. Then, 1.5 μL of ON dissolved in distilled water was placed on the dried matrix and left to dry before measurement. Molecular weight measurements were performed in negative reflector mode, and oligothymidylic acids (7 mer, 9 mer, 11 mer, 13 mer) were used as an external standard.

Quantification of oligonucleotides. The synthesized ONs were quantified by measuring the UV external absorption at 260 nm using nanodrop. The absorbance was calculated using the following values (dT = 8600, dC = 7100, dG = 12100) and the same absorbance was used for the modified oligonucleotides.

Thermal denaturation experiments. For UV-melting experiments using the duplexes formed by **ON1**–**7** and ssRNA, oligonucleotides were dissolved in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl to give a final concentration of 4.0 μM for each strand. The samples were annealed by heating at 100 °C followed by slow cooling down to room temperature. The melting profiles were recorded at 260 nm from 10 °C to 90 °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. For UV-melting experiments using the duplexes formed by **ON1**–**7** and ssDNA, oligonucleotides were dissolved in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl to give a final concentration of 4.0 μM for each strand. The samples were annealed by heating at 100 °C followed by slow cooling down to room temperature. The melting profiles were recorded at 260 nm from 10 °C to 90°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.

CD (Circular Dichroism) spectrum measurements. These spectra were recorded at 10 °C in the quartz cuvette of 1 cm optical path length. The samples were prepared in the same manner as described in the UV melting experiments. The molar ellipticity was calculated from the equation $[\theta] = \theta/cl$, where θ indicates the relative intensity, c is the sample concentration, and l means the cell path length in centimeters.

Enzymatic degradation experiments. Enzymatic degradation experiments were conducted using 30 unit/mL S1 nuclease (Worthington Biochemical Co.), 1.0 mM $ZnSO_4$, 30 mM sodium acetate (pH 4.6), 0.28 M NaCl, and 15 μM each oligonucleotide **ON2**, **ON4**, and **ON6**–**8** at 37 °C. 60 μL of each reaction solution was prepared, then, the cleavage reaction was carried out at 37 °C. 10 μL of the reaction solution was transfered to a 0.2-mL tube at each

time points (20, 40, 60, and 120 min), and S1 nuclease was inactivated to heat under 90°C for 1.5 h. Each solution was diluted with 20 μL of H_2O/HFIP/TEA (100/1/0.1) solution, and the remained amounts of each intact (12-mer) and 10-mer oligonucleotides were analyzed by HPLC.

Author Contributions

T.O. designed the experiments. T.A. performed experiments. T.O., T.A., and S.O. co-wrote the paper. S.O. supervised the project. All authors have given approval to the final version of the article.

Data Availability Statement

The data underlying this study are available in the published article and the Supporting Information.

Supporting Information

The supporting information is available free of charge at https://xxxxxxxxxx.

NMR spectra, UV melting data, structural analysis data, and HPLC, MALDI-TOF-MS, and LC/MS charts (PDF)

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Abbreviations Used

PS, phosphorothioate; 2′,4′-BNA, 2′,4′-bridged nucleic acid; LNA, locked nucleic acid; CNA, constrained nucleic acid

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