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Synthesis of 1′-phenyl-2′-OMe ribose analogues connecting the thymine base at the 1′ position through a flexible linker for the formation of a stable anti-parallel triplex DNA

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ABSTRACT

We have previously developed the innovative bicyclic nucleoside analogues (WNA) for the formation of the triplex DNA. The WNA analogue consists of an aromatic ring and a recognition base on the bicyclic skeleton, and the recognition of the CG or TA interrupting sites has been achieved by the WNA analogues. However, the stabilization ability of the WNA analogue is dependent on its neighboring nucleobases within the TFO. We hypothesized that the sequence dependency might arise from the fixed conformation of the bicyclic ring of the WNA. Thus, it was expected that an open-linker between the sugar part and the nucleobase might produce the flexibility and improve the stabilizing effect of the nucleobase analogues. We now report the design and synthesis of a new nucleoside analogue as an open-form of WNA-βT, the 1′-phenyl-2′-OMe-ribose derivative, connecting the thymine base to the ribose part through a methylene linker (**1**) or an ethylene linker (**2**). TFO containing the 3′-dA-**1**-dG context recognized the CG interrupting site, and that with the 3′-dG-**1**-dG context recognized the GC site. In contrast, **2** displayed a stabilizing effect on all four base pairs with some preferences for the TFO containing 3′-dA-**2**-dG and 3′-dG-**2**-dG. These results suggested that a flexible linker between the nucleobase and the ribose part may improve the sequence dependency for the triplex formation.

1. Introduction

The triplex forming oligonucleotide (TFO) is the one of the binding molecules to the major groove of DNA, and has the potential to be a biological tool for gene targeting and regulation.[1,2] A parallel triplex DNA is formed with the pyrimidine-rich TFO and the homopurine–homopyrimidine duplex based on the stabilization by the two Hoogsteen hydrogen bonds between the protonated dC (dC^+) and the GC base pair and those between the T and AT base pair. Due to the contribution of dC^+, the parallel triplex DNA is stable under acidic conditions. On the other hand, the anti-parallel triplex is formed by the interaction between the purine-rich TFO and purine bases in the homopurine–homopyrimidine duplex, in which two reverse Hoogsteen hydrogen bonds of the dG to GC base pair and those of the dA to AT base pair are responsible for its high stability under neutral conditions. However, the pyrimidine nucleotide insertions in the homopurine strand, dC and T, inhibit the stable triplex formation.[3,4] Thus, the CG and TA base pairs are called interrupting sites. In order to expand the triplex recognition code, we developed innovative bicyclic nucleoside analogues called the W-shaped nucleoside analogues (WNAs). The WNA analogue has a benzene ring as the stacking part and a nucleobase as the recognition part as shown by WNA-βT in Fig. 1. We have already

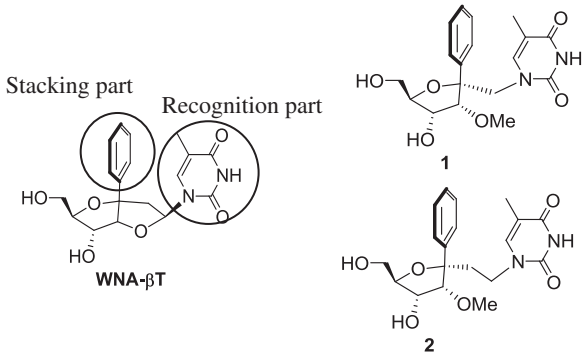

Fig. 1. Basic structure of WNA analogues (WNA-βT, left), and the structures of 1′-phenyl-2′OMe derivatives, **1** (right-top) and **2** (right-down), in this study.

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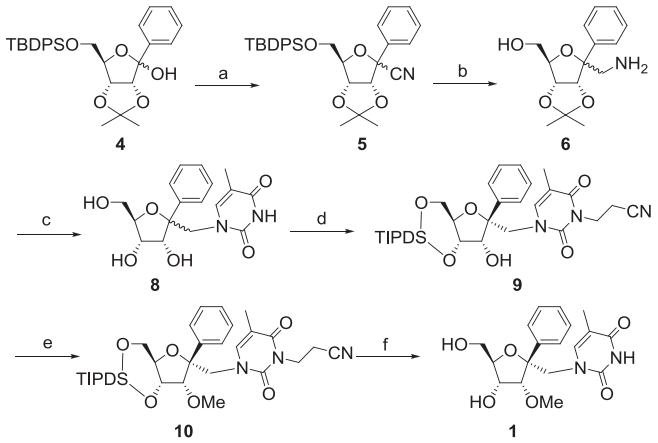
revealed that TFOs containing the WNA analogues could recognize the CG or TA interrupting sites with a high selectivity and high stability in an anti-parallel triplex formation.[5] Recently, we have demonstrated that TFO containing WNA-βT inhibited the cell proliferation and induced a caspase-dependent apoptosis by the inhibition of the oncogene expression.[6] Therefore, these artificial nucleoside analogues, which are able to stabilize the triplex DNA, have the potential to become a gene targeting agent. Unfortunately, the stabilization effect of the WNA-containing TFO is dependent on its neighboring nucleobases within TFO.[7] The sequence dependency of the TFO has been partially solved by the use of a variety of WNA analogues. Nevertheless, the development of the nucleoside analogues for the formation of a stable triplex DNA without a sequence dependency is a challenging theme.

We speculated that the sequence dependency of the WNA analogues might arise from the rigid conformation of the bicyclic ring of the WNA skeleton, although it contributed to the specific recognition for the CG or TA interrupting sites. Previously we synthesized the 1′-phenyl substituted nucleoside analogues whose recognition base was directly connected to the sugar part at the 1′ position without a bicyclic sugar ring.[8] However, these 1′-phenyl branched nucleoside analogues were unstable under strong acid or basic solution conditions, and thereby it was impossible to incorporate them into oligonucleotides. Therefore, in order to clarify the role of the bicyclic ring of the WNA skeleton for the formation of the triplex DNA, we designed new 1′,1′-disubstituted nucleoside analogues with a flexible linker between the recognition base and the sugar part. Thus, the 1′-phenyl-2′-OMe-ribose derivatives connecting the thymine base to the ribose part through a methylene linker (**1**) and that with an ethylene linker (**2**) were designed (Fig. 1). In this paper, we describe the synthesis and evaluation of the triplex forming ability of the new nucleoside analogues (**1** and **2**).

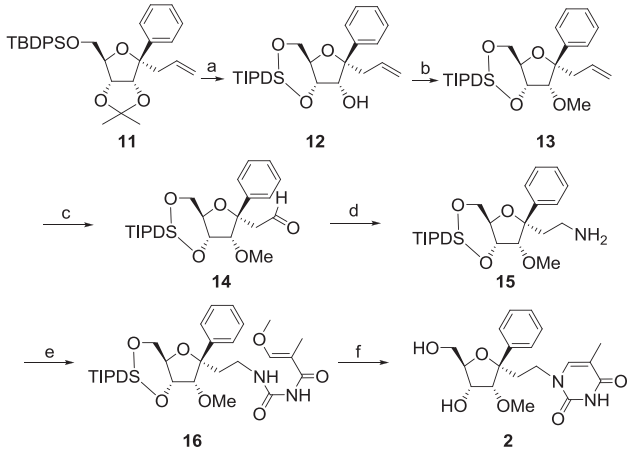
2. Results and discussion

The synthesis of the 1′,1′-disubstituted nucleoside analogues was started with the intermediates (**4** and **11**), which were previously reported by our group.[5] The synthesis of **1** is shown in Scheme 1. Compound **4** was subjected to cyanidation by the treatment with TMSCN and $ZnBr_2$ to produce **5** in a good yield as a mixture of stereoisomers. The cyano group of **5** was converted to the corresponding amino group by reduction with $LiAlH_4$, and then the TBDPS group was removed to form **6**. The amino group of **6** was reacted with 3-methoxy-2-methylacryloylisocyanate **7**[9] in the presence of TMSCl and $AgNO_3$.[10] During this reaction, the 5′-OH group of **6** was silylated with TMSCl for protection during the reaction with the isocyanate **7** to convert the amino group to the corresponding ureido derivative. The ureido compound was treated in 1 M H_2SO_4 in H_2O to affect cyclization together with the deprotection of the acetonide group to afford the triol intermediate **8**. The N3 position of the thymine base and the 5′- and 3′-hydroxyl groups of the ribose part were protected using acrylonitrile and $TIPDSCl_2$, respectively. The two isomers of the resulting compounds **9** were separated by column chromatography. Their stereochemistries were determined by analysis of their ^{1}H-COSY and NOESY spectra. Subsequently, the 2′-hyrodoxyl group of the desired β-phenyl substituted analogue was methylated using Meerwein reagent[11] to yield **10**. The TIPDS group and the cyanoethyl group of **10** were then removed by a sequential treatment with TBAF followed by *t*-BuOK to produce the diol compound **1** in a good yield.



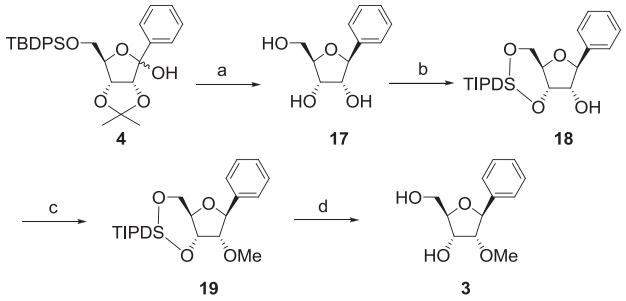
Scheme 1. Reagents and conditions: (a) TMSCN, $ZnBr_2$, CH_3NO_2, 0 °C, (97%). (b) $LiAlH_4$, THF, reflux, (90%). (c) (i) TMSCl, $AgNO_3$, Et_3N, 3-methoxy-2-methylacryloylisocyanate (**7**), benzene, (ii) 1 M H_2SO_4 aq, 90 °C, (81% for two steps). (d) (i) acrylonitrile, TBAH, pyridine, reflux, (ii) $TIPDSCl_2$, pyridine, (α-Ph 66%, β-Ph (**9**) 28% for two steps). (e) Me_3OBF_4, CH_2Cl_2, (99%). (f) (i) TBAF, THF, (ii) *t*-BuOK, *t*-BuOH, (75% for two steps).

The synthesis of **2** is shown in Scheme 2. Compound **11** was converted to the corresponding triol by the reaction with TBAF and followed by DOWEX (H^+), which was protected with TIPDS to produce compound **12**. The 2′-hydroxyl group of **12** was methylated by MeI and NaHMDS to form **13**. The allyl group of **13** was converted to the corresponding ethylamino group (**15**) by a sequence of reactions. The vinyl group of **13** was subjected to an oxidative cleavage reaction using OsO_4 and $NaIO_4$ to form the aldehyde **14**. The aldehyde **14** was then transformed into the corresponding oxime, followed by reduction with $NaBH_4/NiCl_2$ to produce the amino compound **15**.[12] The amino group of **15** was reacted with the isocyanate **7**[9] to form the corresponding ureido compound **16**, which was treated with TBAF for the deprotection of TIPDS followed by cyclization in 1 M H_2SO_4 to form the thymine base, then the desired diol compound **2** was obtained.



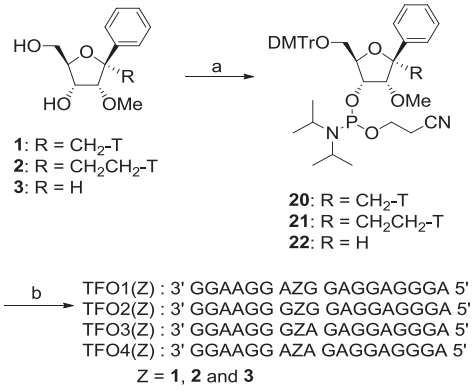
Scheme 2. Reagents and conditions: (a) (i) TBAF, THF (87%), (ii) DOWEX (H^+), MeOH (92%), (iii) $TIPDSCl_2$, pyridine (97%). (b) MeI, NaHMDS, THF, 0 °C (71%). (c) OsO_4, $NaIO_4$, pyridine, H_2O (74%). (d) (i) NH_2OH–HCl, MeOH (90%). (ii) $NaBH_4$, $NiCl_2$, MeOH, (74%). (e) (i) **7**, benzene (90%). (f) (i) TBAF, THF (90%) (ii) 1 M H_2SO_4 aq, 90 °C (72%).

The 1′-phenyl substituted compound (**3**), as a control compound, was synthesized from **4**. The 1′-hydroxyl group of **4** was reduced by Et_3SiH and TMSOTf, and then the stereoisomer at the 1′ position was separated to produce the undesired α-phenyl substituted compound (11%) and a desired β-phenyl substituted compound (66%) by column chromatography. These isomers were determined by an analysis ^{1}H-COSY and NOESY spectra. The protecting groups of the β-phenyl compound were removed using TBAF and DOWEX (H^+) in a good yield. The resulting triol compound **17** was transformed into **3** by sequential reactions as described for the synthesis of **1** and **2** (Scheme 3).



Scheme 3. Reagents and conditions: (a) (i) Et_3SiH, $BF_3 \cdot Et_2O$, CH_2Cl_2, −78 °C to −40 °C (77%, α-Ph 11%, β-Ph 66%), (ii) TBAF, THF, (iii) DOWEX (H^+), MeOH, (82% for two steps). (b) $TIPDSCl_2$, pyridine (92%). (c) MeI, NaHMDS, THF, 0 °C, (77%). (d) TBAF, THF, (68%).

Finally, these diol compounds (**1**, **2**, and **3**) were converted into the corresponding amidite precursors (**20**, **21**, and **22**, respectively) by conventional methods (Scheme 4). They were incorporated into **TFO1**–**4** with four different sequences using an automated DNA synthesizer. The **TFO1** in the sequence 3′-AZG-5′ containing **1** at the position of Z was called **TFO1**(**1**). After purification by HPLC, the purity and the structure were identified by MALDI-TOF MS measurements. These TFOs were then evaluated for their triplex forming ability by the gel-shift assay, and the association constant, *Ks*, was calculated from the intensity of the radioactive bands, as previously described.[5] The *Ks* values are summarized in Fig. 2 as a bar graph.



Scheme 4. Reagents and conditions: (a) (i) DMTrCl, pyridine, (ii) *i*-$Pr_2NP(Cl)OCH_2CH_2CN$, DIPEA, CH_2Cl_2, 0 °C, (63–73% for two steps). (b) DNA automated synthesizer.

Regarding the stabilization ability of the methylene-linked nucleoside **1**, **TFO1**(**1**) displayed a selective stabilization to the CG-having duplex (Fig. 2A), while **TFO2**(**1**) was selective to the GC-having duplex (Fig. 2B), while no stable triplexes were formed with **TFO3**(**1**) and **TFO4**(**1**) (Fig. 2C and D). In comparison to the control compound **3**, **1** exhibited a stabilization effect in the sequence of **TFO1** and **TFO2**, while none or a destabilizing effect was observed in the sequence of **TFO3** and **TFO4**, respectively. These results indicated that the phenyl group within **TFO4**(**3**) was stacked with the neighboring nucleobase in the sequence of 3′-GZA-5′, and **1** hampered the triplex formation without the hydrogen bonding formation. Therefore, the methylene linker may not produce enough flexibility to overcome the rigidity of the WNA structure.

The ethylene-linked nucleoside **2** showed a different feature during the triplex formation. **TFO1**(**2**) and **TFO2**(**2**) formed stable triplexes with all four target duplexes without remarkable selectivity. Interestingly, the stabilities of these triplexes were higher than the natural triplexes containing the G–GC triplet (Fig. 2A and B). Although the stability was relatively low, **TFO3**(**2**) and **TFO4**(**2**) formed triplexes with all the duplex targets (Fig. 2C and D), demonstrating the unique property of **2**. It is postulated from the non-selective stabilizing effect of **2** that the ethylene linker is flexible enough to avoid steric repulsive interactions with adjacent nucleoside, which are supposed to be a problem of the rigid conformed WNA analogues as sequence dependency.

3. Conclusion

In conclusion, we synthesized a new nucleoside analogue as an open-form of WNA-βT, 1′-phenyl-2′-OMe-ribose derivative with the thymine base through the flexible linker, i.e., the methylene-linked **1** and ethylene-linked **2** in order to avoid steric repulsion of bicyclic WNA structure. The results of the triplex forming ability have shown that **TFO1**(**1**) and **TFO2**(**1**) stabilize the triplex DNA having the CG site and GC site, respectively. Furthermore, it has been shown that **TFO1**–**4**(**2**) has an ability to form the non-selective triplexes with all duplex targets without sequence dependency. Consequently, the adjustment of the linker flexibility between the recognition base and sugar skeleton of the nucleoside analogues has the potential to enhance the stability and selectivity of triplex DNA formation as a general sequence. New nucleoside analogues have been developed based on this result.

4. Experimental

4.1. General

The ^1H NMR (400 MHz), ^{13}C (125 MHz), and ^1H-COSY, NOESY spectra were recorded by Varian UNITY-400 and INOVA-500 spectrometers. The ^{31}P NMR (162 MHz) spectrum was recorded using 10% phosphoric acid in D_2O as the internal standard at 0 ppm. The IR spectra were obtained using a Perkin–Elmer FTIR-spectrumOne. The high-resolution mass spectra were recorded by an Applied Biosystems Mariner System 5299 spectrometer.

4.2. Synthesis of 1

*4.2.1. (1R,5R,6RS,8R)-6-Cyano-3,3-dimethyl-6-phenyl-8-(t-butyldiphenylsilyloxymethyl)-2,4,7-trioxabicyclo[3.3.0]octane (**5**).* To a suspension of $ZnBr_2$ (2.7 g, 11.9 mmol) in CH_3NO_2 (100 mL) was added a solution of **4** (3.0 g, 5.9 mmol) in CH_3NO_2 (20 mL) and TMSCN (19.8 mL, 0.15 mol) at 0 °C. After stirring for 2 h, the reaction mixture was diluted with Et_2O. The organic layer was washed with satd $NaHCO_3$ solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc=10/1) to give compound **5** as a colorless oil (3.0 g, 5.8 mmol, 97%). λ_{max} (neat) 2932, 1738 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 7.71–7.29 (15H, m), 5.08 (1H, d *J* 5.6 Hz), 4.94 (1H, t, *J* 4.4 Hz), 4.93 (1H, d, *J* 5.6 Hz), 4.59 (1H, d, *J* 6.4 Hz), 4.53 (1H, d, *J* 6.0, 12.2 Hz), 4.04–3.89 (2H, m), 1.10 (9H, s), 1.07 (3H, s), 1.03 (3H, s); δ_C (125 MHz, $CDCl_3$) 136.9, 135.8, 135.7, 135.6, 135.5, 134.6, 132.9, 132.8, 129.9, 129.2, 128.9, 128.8, 128.1, 127.8, 126.4, 125.0, 120.6, 117.8, 117.3, 114.0, 89.1, 87.1, 86.4, 84.8, 84.0, 83.1, 81.1, 63.2, 63.1, 28.0, 27.0, 26.9, 26.8, 26.0, 25.5, 25.2, 25.0, 19.2; HRMS (ESI-TOF): $M+Na^+$, found 536.2219. $C_{31}H_{35}NO_4SiNa$ requires 536.2228.

*4.2.2. (1R,5R,6RS,8R)-6-Aminomethyl-3,3-dimethyl-8-hydroxymethyl-6-phenyl-2,4,7-trioxabicyclo[3.3.0]octane (**6**).* To a solution of **5** (2.3 g, 4.5 mmol) in THF (90 mL) was added a solution of $LiAlH_4$ in THF (1.0 M, 23 mL, 22.4 mmol) at 0 °C. The reaction mixture was refluxed for 2 h. After cooling to 0 °C, water was continuously added to the reaction along with an aqueous 10% sodium hydroxide solution. The mixture was filtered through a Celite545 pad, and then the filtrate was extracted with Et_2O. The organic layer was washed with a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography ($CHCl_3$:MeOH=10/1) to give compound **6** as a pale yellow oil (1.1 g,

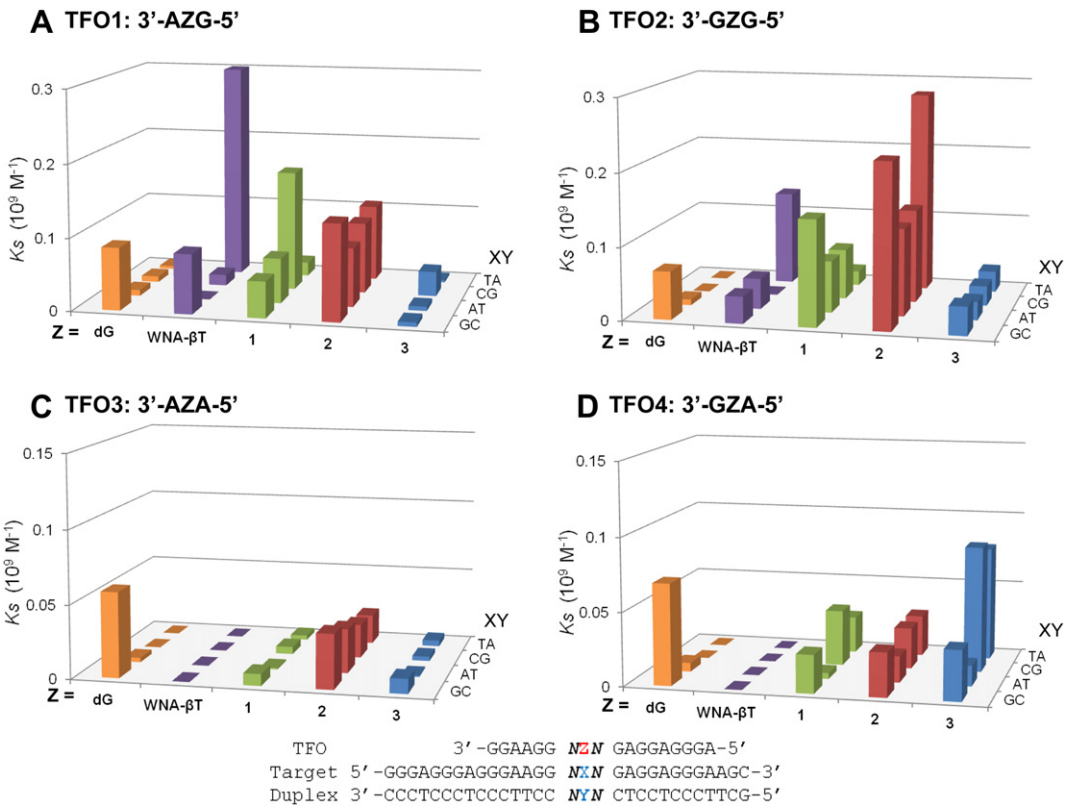


Fig. 2. Bar graphs of the association constants (*Ks*) of the TFOs. These values were determined from the results of the gel-shift assay. Triplex formation was done for 12 h at 22 °C in the buffer containing 20 mM Tris–HCl, 5 mM $MgCl_2$, 2.5 mM spermidine, and 10% sucrose at pH 7.5. Electrophoresis was done at 10 °C with a non-denatured polyacrylamide gel. Ten nano molar TFO containing the ^{32}P-labeled one as the tracer was used. The association constant was calculated using the concentration of each component and the following equation: *Ks*=[Triplex]/[Duplex][TFO]. (A) TFO1: 3′AZG5′, (B) TFO2: 3′GZG5′, (C) TFO3: 3′AZA5′, (D) TFO4: 3′GZA5′, Z=dG, WNA analogue, or new 1′-phenyl-2′OMe ribose derivatives (**1**, **2**, **3**) as shown at the bottom of the graph.

4.1 mmol, 90%). λ_{max} (neat) 3366, 2923, 2854 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 7.67–7.20 (5H, m), 4.92–4.85 (1.7H, m), 4.69 (0.3H, dd, *J* 4.0, 7.0 Hz), 4.47 (0.7H, dd, *J* 2.1, 2.4 Hz), 4.24–4.18 (0.3H, m), 4.00 (0.7H, dd, *J* 2.4, 12.2 Hz), 3.80 (0.3H, dd, *J* 3.7, 11.9 Hz), 3.70 (0.7H, dd, *J* 2.4, 12.2 Hz), 3.65 (0.3H, dd, *J* 3.7, 11.9 Hz), 3.24–2.99 (2H, m), 1.59 (0.9H, s), 1.36 (0.9H, s), 1.24 (2.1H, s), 1.22 (2.1H, s); δ_C (125 MHz, $CDCl_3$) 142.4, 128.5, 128.4, 127.5, 125.4, 125.3, 114.9, 88.1, 87.6, 82.7, 82.0, 63.2, 50.6, 47.0, 26.1, 24.8; HRMS (ESI-TOF): $M+H^+$, found 280.1588. $C_{15}H_{22}NO_4$ requires 280.1543.

*4.2.3. (1RS,2R,3R,4R)-1-Phenyl-1-(thymin-1-yl)methylribose (**8**).* To a solution of **6** (2.1 g, 7.5 mmol) in benzene (77 mL) and triethylamine (11 mL) was added $AgNO_3$ (6.8 g, 38.1 mmol) and TMSCl (1.94 mL, 15.3 mmol). After stirring for 1 h, the solution of **7**[9] in benzene (1.0 M, 15 mL, 15 mmol) was added to a reaction mixture. After stirring for 3 h, the reaction was quenched with a satd $NaHCO_3$ solution and filtered through a Celite545 pad, and then the filtrate was extracted with EtOAc. The organic layer was washed with a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was suspended in a 1 M H_2SO_4 solution (75 mL) and heated at 90 °C. After stirring for 1.5 h, the reaction mixture was neutralized by a satd $NaHCO_3$ solution at room temperature, and then extracted with EtOAc. The organic layer was dried over Na_2SO_4, and then evaporated under reduced pressure. The residue was purified by flash column chromatography ($CHCl_3$:MeOH=15/1 to 10/1) to give compound **8** as a colorless oil (2.2 g, 6.2 mmol, 81% for two steps). λ_{max} (neat) 3374, 2920, 1673 cm^{-1}; δ_H (400 MHz, DMSO) 11.09 (0.6H, s), 10.85 (0.4H, s), 7.43–7.13 (6H, m), 5.43 (0.4H, d, *J* 5.8 Hz), 5.02 (0.4H, d, *J* 5.5 Hz), 4.81 (0.6H, t, *J* 5.8 Hz), 4.72 (0.4H, t, *J* 5.2 Hz), 4.70 (0.6H, d, *J* 6.4 Hz), 4.65 (0.6H, d, *J* 6.4 Hz), 4.37 (0.6H, d, *J* 14.6 Hz), 4.20 (0.6H, t, *J* 4.9 Hz), 4.08–3.76 (3.8H, m), 3.68 (0.6H, ddd, *J* 4.6, 11.9 Hz), 3.52–3.42 (1.4H, m), 1.64 (1.8H, s), 1.62 (1.2H, s); δ_C (125 MHz, CD_3OD) 166.5, 153.5, 144.2, 144.0, 140.6, 129.0, 128.8, 128.5, 128.2, 126.8, 110.0, 89.9, 84.6, 84.2, 79.7, 75.8, 72.8, 72.3, 64.0, 62.4, 56.0, 52.1, 49.3, 49.1, 48.9, 48.8, 48.6, 12.1, 12.0; HRMS (ESI-TOF): $M+H^+$, found 349.1400. $C_{17}H_{21}N_2O_6$ requires 349.1394.

*4.2.4. (1R,2R,3S,4R)-1-{3-(2-Cyanoethyl)thymine-1-yl}-methyl-1-phenyl-3,5-O-(1,1,3,3-tetraisopropyl-1,3-disiloxyl)ribose (**9**).* To a solution of **8** (2.0 g, 5.7 mmol) in pyridine (72 mL) was added acrylonitrile (0.38 mL, 5.7 mmol) and tetrabutylammonium hydroxide in MeOH (1.0 M, 172 μL, 0.17 mmol). After stirring for 22 h at 90 °C, the reaction mixture was diluted with CH_2Cl_2 at room temperature. The organic layer was washed with a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography ($CHCl_3$:MeOH=50/1 to 10/1) to give a white solid (1.7 g, 4.3 mmol). The white solid was then dissolved in pyridine (43 mL) and $TIPDSCl_2$ (3.4 mL, 10.9 mmol) at 0 °C. After stirring for 5 h at room temperature, the reaction mixture was diluted with EtOAc. The organic layer was continuously washed with water and a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc=5/1 to 1/1) to give compound **9** as a colorless foam (775 mg, 1.2 mmol, 28%) and α-Ph isomer (1.8 g, 2.8 mmol, 66%). λ_{max} (neat) 2260, 1690, 1660, 1638 cm^{-1}; δ_H (400 MHz, CD_3OD) 7.43 (2H, d, *J* 7.0 Hz), 7.28–7.21 (4H, m), 4.62 (1H, d, *J* 14.7 Hz), 4.42 (1H, d, *J* 4.3 Hz), 4.21 (1H, dt, *J* 8.5, 3.1, 2.4 Hz), 4.13 (1H, d, *J* 14.7 Hz), 4.14–3.40 (5H, m), 3.95–3.89 (1H, m), 2.46 (2H, m), 1.77 (3H, s), 1.09–0.72 (28H, m); δ_C (125 MHz, $CDCl_3$) 163.0, 151.1, 140.3, 140.0, 128.1, 127.8, 125.9, 117.1, 108.5, 86.8, 81.0, 78.3, 71.7, 62.3, 51.5, 36.5, 14.4, 17.3, 17.1, 17.0, 16.9,

15.7, 13.3, 13.1, 12.8, 12.6; HRMS (ESI-TOF): M+H$^+$, found 644.3192. $C_{32}H_{50}N_3O_7Si_2$ requires 644.3182.

*4.2.5. (1R,2R,3R,4R)-1-{3-(2-Cyanoethyl)thymine-1-yl}methyl-2-O-methyl-1-phenyl-3,5-O-(1,1,3,3-tetraisopropyl-1,3-disiloxyl)ribose (**10**).* To a solution of **9** (340 mg, 0.53 mmol) in CH_2Cl_2 (5.2 mL) was added a proton-sponge (1.7 g, 7.9 mmol) and Me_3OBF_4 (935 mg, 6.3 mmol) at 0 °C. After stirring for 5 h at room temperature, the reaction mixture was quenched by a 5% citrate solution and diluted with EtOAc. The mixture was filtered through a Celite545 pad, and then the filtrate was continuously washed with 5% citrate solution and satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc=5/1 to 3/2) to give compound **10** as a colorless foam (344 mg, 0.52 mmol, 99%). λ_{max} (neat) 2946, 2249, 1707, 1662, 1651 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 7.39 (2H, d, *J* 7.3 Hz), 7.23–7.14 (4H, m), 4.63 (1H, d, *J* 14.6 Hz), 4.17–3.98 (8H, m), 3.69 (3H, s), 2.30 (2H, dd, *J* 6.4, 5.8 Hz), 1.80 (3H, s), 1.05–0.73 (28H, m); δ_C (125 MHz, $CDCl_3$) 170.2, 163.0, 150.9, 140.5, 139.8, 127.9, 127.5, 126.0, 117.1, 108.4, 88.1, 86.6, 80.6, 70.9, 60.7, 60.5, 51.8, 43.1, 36.5, 17.5, 17.3, 17.1, 17.0, 15.7, 13.4, 13.1, 12.9, 12.7, 12.6; HRMS (ESI-TOF): M+H$^+$, found 658.3315. $C_{33}H_{52}N_3O_7Si_2$ requires 658.3338.

*4.2.6. (1R,2R,3R,4R)-2-O-Methyl-1-phenyl-1-(thymine-1-yl)methylribose (**1**).* To a solution of **10** (220 mg, 0.34 mmol) in THF (3.4 mL) was added a solution of TBAF in THF (1.0 M, 840 μL, 0.84 mmol). After stirring for 45 min, the reaction mixture was diluted with a satd $NaHCO_3$ solution and EtOAc. The organic layer was washed with a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography ($CHCl_3$:MeOH=100/1 to 25/1) to give the desired compound as a colorless foam (127 mg, 0.31 mmol, 92%). This colorless foam (110 mg, 0.27 mmol) was dissolved in a solution of potassium *t*-butoxide (89.1 mg, 0.79 mmol) in *t*-butanol (5.3 mL). After stirring for 2 h, the reaction mixture was treated with ammonium chloride, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography ($CHCl_3$:MeOH=50/1) to give compound **1** as a colorless foam (77.3 mg, 0.21 mmol, 81%). λ_{max} (neat) 3385, 2923, 1674 cm^{-1}; δ_H (400 MHz, CD_3OD) 77.48 (2H, d, *J* 7.0 Hz), 7.29–7.19 (3H, m), 7.08 (1H, s), 4.43 (1H, d, *J* 14.6 Hz), 4.31 (1H, d, *J* 14.6 Hz), 4.24 (1H, dd, *J* 4.9, 4.6 Hz), 4.13 (1H, t, *J* 4.9 Hz), 3.86 (1H, d, *J* 5.2 Hz), 3.70–3.59 (5H, m), 1.69 (3H, s); δ_C (125 MHz, CD_3OD) 166.6, 152.9, 144.0, 129.0, 128.5, 126.6, 110.0, 89.8, 87.3, 85.7, 71.8, 63.8, 60.0, 51.5, 12.1; HRMS (ESI-TOF): M+H$^+$, found 363.1579. $C_{18}H_{23}N_2O_6$ requires 363.1551.

4.3. Synthesis of 2

*4.3.1. (1′S,2′R,3′R,4′R)-1′-Allyl-1′-phenyl-3′,5′-O-tetraisopropyl-disiloxyribose (**12**).* To a solution of **11** (7.1 g, 13 mmol) in a THF (200 mL) was added TBAF in THF solution (1 M, 20 mL, 20 mmol). After stirring for 2 h, the reaction mixture was diluted with EtOAc. The organic layer was continuously washed with water and a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc=3/2) to give a colorless oil (3.24 g, 11 mmol, 87%). This oil was dissolved in MeOH (50 mL). After the addition of DOWEX™ 50WX8-100 (1.15 g), the mixture was stirred for 26 h. The reaction mixture was then filtered off. The filtrate was then evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc=2/3) to give the triol compound as a white solid (2.55 g, 10 mmol, 92%). Subsequently, this white solid was dissolved in pyridine (100 mL), then this solution was cooled to 0 °C. The 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (3.82 mL, 12 mmol) was dropwise added to the reaction mixture at the same temperature. After stirring for 3 h at room temperature, the reaction was quenched by water and extracted with EtOAc. The organic layer was continuously washed with water and a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc=6/1) to give compound **12** as a colorless oil (4.78 g, 9.7 mmol, 97%). λ_{max} (neat) 3503, 2945, 2868, 1464 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 7.43 (3H, d, *J* 7.3 Hz), 7.17–7.30 (2H, m), 5.50–5.60 (1H, m), 4.91 (2H, t, *J* 8.9 Hz), 4.25 (1H, t, *J* 6.1 Hz), 4.03–4.11 (3H, m), 3.93 (1H, dd *J* 6.0, 10.1 Hz), 3.00 (1H, s), 2.81 (1H, dd, *J* 7.0, 14.5 Hz), 2.70 (1H, dd, *J* 7.0, 14.5 Hz), 0.88–1.07 (24H, m); δ_C (125 MHz, $CDCl_3$) 144.0, 133.3, 127.9, 126.7, 125.4, 117.4, 86.7, 81.4, 78.2, 72.6, 63.6, 39.1, 17.5, 17.4, 17.3, 17.2, 17.1, 17.0, 16.9, 13.3, 13.2, 12.9, 12.7, 12.6; HRMS (ESI-TOF): M+H$^+$, found 493.2784. $C_{26}H_{45}O_5Si_2$ requires 493.2800.

*4.3.2. (1′S,2′R,3′R,4′R)-1′-Allyl-2′-methoxy-1′-phenyl-3′,5′-O-tetraisopropyldisiloxyribose (**13**).* To a solution of **12** (4.78 g, 9.7 mmol) in THF (100 mL) was added methyliodide (6.1 mL, 98 mmol) and NaHMDS in a THF solution (1.9 M, 15 mL, 29 mmol). After stirring for 2 h, the reaction mixture was quenched by a satd NH_4Cl solution and extracted with EtOAc. The organic layer was continuously washed with water and a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc=15/1) to give compound **13** as a colorless oil (3.5 g, 6.9 mmol, 71%). λ_{max} (neat) 2945, 2868, 1465 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 7.43 (2H, d, *J* 2.1 Hz), 7.22–7.28 (2H, m), 7.14–7.18 (1H, m), 5.46–5.53 (1H, m), 4.85 (1H, d, *J* 8.2 Hz), 4.22 (1H, dd, *J* 4.1, 8.4 Hz), 4.02–4.11 (2H, m), 3.96 (1H, dd, *J* 2.1, 12.5 Hz), 3.62 (3H, s), 2.78 (1H, dd, *J* 7.0, 14.5 Hz), 2.63 (1H, dd, *J* 7.0, 14.5 Hz), 0.85–1.04 (24H, m); δ_C (125 MHz, $CDCl_3$) 144.6, 133.8, 127.8, 126.5, 125.6, 117.3, 88.1, 86.6, 80.5, 71.1, 61.5, 40.6, 17.6, 17.4, 17.3, 17.2, 17.1, 17.0, 13.4, 13.1, 12.8, 12.7; HRMS (ESI-TOF): M+Na$^+$, found 529.2803. $C_{27}H_{46}NaO_5Si_2$ requires 529.2776.

*4.3.3. (1′S,2′R,3′R,4′R)-1′-(2-Formylethyl)-2′-methoxy-1′-phenyl-3′,5′-O-tetraisopropyldisiloxyribose (**14**).* To a solution of **13** (3.5 g, 6.9 mmol) in pyridine (80 mL) was added a solution of OsO_4 in water (0.131 M, 12.7 mL, 1.7 mmol) and $NaIO_4$ in water (0.6 M, 70 mL, 41.5 mmol). After stirring for 3 h, the reaction mixture was diluted with EtOAc. The organic layer was continuously washed with water and a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc=10/1) to give compound **14** as a colorless oil (2.6 g, 5.5 mmol, 74%). λ_{max} (neat) 2943, 2868, 1723, 1465 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 9.61 (1H, s, *J* 1.8 Hz), 7.48–7.51 (2H, m), 7.29–7.32 (1H, m), 7.19–7.24 (2H, m), 4.28 (1H, dd, *J* 3.4, 9.2 Hz), 4.21 (1H, dd, *J* 1.2, 13.0 Hz), 4.08–4.13 (1H, m), 3.98 (1H, dd, *J* 2,6, 13.0 Hz), 3.76 (1H, dd, *J* 4.0), 3.60 (1H, s), 2.95 (1H, dd, *J* 3.7, 16.8 Hz), 2.65 (1H, dd, *J* 1.7, 16.7 Hz), 0.77–1.06 (30H, m); δ_C (125 MHz, $CDCl_3$) 170.8, 142.5, 128.4, 128.2, 127.7, 125.1, 125.0, 88.2, 85.6, 81.0, 60.7, 51.2, 42.6, 17.5, 17.4, 17.3, 17.2, 17.1, 17.0, 13.4, 13.3, 12.8, 12.7; HRMS (ESI-TOF): M+Na$^+$, found 531.2545. $C_{26}H_{44}NaO_6Si_2$ requires 531.2569.

*4.3.4. (1′S,2′R,3′R,4′R)-1′-(2-Aminoethyl)-2′-methoxy-1′-phenyl-3′,5′-O-tetraisopropyldisiloxyribose (**15**).* To a solution of **14** (2.6 g, 5.5 mmol) in ethanol (30 mL) was added a solution of NH_2OH–HCl in pyridine (5 mL, 656 mg, 9.44 mmol), then the reaction mixture was heated at 70 °C for 16 h. The reaction mixture was diluted with EtOAc, continuously washed with water and a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc=6/1) to give a colorless oil (2.6 g, 5.0 mmol, 90%). This colorless oil (554 mg, 1.06 mmol) was dissolved in methanol (13 mL). $NiCl_2$ (503 mg, 2.12 mmol) and $NaBH_4$ (400 mg, 10.6 mmol) were added to this mixture at −78 °C. After stirring for 2 h at the

same temperature, the reaction mixture was warmed up to room temperature. After stirring for 2 h, the reaction mixture was quenched by a satd $NaHCO_3$ solution and extracted with $CHCl_3$. The organic layer was continuously washed with water and a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography ($CHCl_3$:MeOH=20/1) to give compound **15** as a colorless oil (400 mg, 0.79 mmol, 74%). λ_{max} (neat) 2944, 2868, 1465 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 7.19–7.32 (5H, m), 4.52–4.58 (1H, m), 4.12 (1H, d *J* 4.1 Hz), 3.68 (1H, d, *J* 4.0 Hz), 3.33 (3H, s), 3.18 (1H, s), 2.67–2.70 (1H, m), 2.56–2.60 (1H, m), 2.10 (1H, dd, *J* 7.0, 14.3 Hz), 1.88 (1H, dd, *J* 7.0, 14.3 Hz), 0.86–1.24 (30H, m); δ_C (125 MHz, $CDCl_3$) 144.3, 128.1, 126.8, 125.4, 88.6, 87.1, 80.2, 70.4, 60.8, 60.4, 37.8, 37.1, 17.5, 17.4, 17.3, 17.1, 17.0, 13.4, 13.1, 12.8, 12.7; HRMS (ESI-TOF): $M+H^+$, found 510.3033. $C_{26}H_{48}NO_5Si_2$ requires 510.3066.

*4.3.5. (1′S,2′R,3′R,4′R)-1′-[(N-3-Methoxy-2-methylacryoyl)-ureido]-2′-methoxy-1′-phenyl-3′,5′-O-tetraisopropyldisiloxy-ribose (**16**).* To a solution of **15** (370 mg, 0.73 mmol) in benzene (15 mL) was added a solution of **7** in benzene (excess amount). After stirring for 5 h, the reaction mixture was quenched by a satd NH_4Cl solution and extracted with EtOAc. The organic layer was continuously washed with water and a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography ($CHCl_3$:MeOH=99/1) to give compound **16** as a colorless oil (463 mg, 0.71 mmol, 90%). λ_{max} (neat) 2943, 2865, 1684, 1543 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 8.67 (1H, s), 8.06 (1H, s), 7.46 (1H, d, *J* 7.3 Hz), 7.24 (1H, t, *J* 7.1 Hz), 7.17 (1H, t, *J* 7.2 Hz), 4.58 (1H, dd, *J* 2.0, 13.0 Hz), 4.48 (1H, dd, *J* 2.0, 13.0 Hz), 4.10–4.15 (1H, m), 3.90–4.00 (1H, m), 3.78 (1H, d, *J* 4.0 Hz), 3.40 (3H, s), 3.00 (1H, dd, *J* 5.2, 16.2 Hz), 2.81 (1H, dd, *J* 5.2, 16.2 Hz), 2.70 (1H, dd, *J* 3.7, 16.8 Hz), 2.90–3.30 (1H, m), 2.18–2.38 (1H, m), 0.85–1.25 (30H, m); δ_C (125 MHz, $CDCl_3$) 168.8, 157.9, 154.0, 144.2, 128.1, 126.7, 125.4, 107.4, 88.7, 86.5, 80.4, 70.5, 61.3, 61.1, 60.2, 35.4, 35.1, 17.6, 17.4, 17.3, 17.2, 17.1, 17.0, 13.4, 13.1, 12.8, 12.7, 8.8; HRMS (ESI-TOF): $M+Na^+$, found 673.3286. $C_{32}H_{54}N_2NaO_8Si_2$ requires 673.3311.

*4.3.6. (1′S,2′R,3′R,4′R)-1′-[(3,4-Dihydro-2,4-dioxo-5-methyl-1(2H) pyrimidinyl)]-2′-methoxy-1′-phenylribose (**2**).* To a solution of **16** (430 mg, 0.66 mmol) in THF (5 mL) was added a solution of TBAF in THF (1.59 mL, 1.59 mmol). After stirring for 2 h, the reaction mixture was diluted with EtOAc. The organic layer was continuously washed with water and a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography ($CHCl_3$:MeOH=99/1) to give a colorless oil (244 mg, 0.60 mmol, 90%). This colorless oil (220 mg, 0.54 mmol) was dissolved in a solution of H_2SO_4 (1 M, 8 mL), then the mixture was stirred for 3 h at 90 °C. The reaction mixture was neutralized by a satd $NaHCO_3$ solution and extracted with EtOAc. The organic layer was continuously washed with water and a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography ($CHCl_3$:MeOH=99/1) to give compound **2** as a colorless foam (127 mg, 0.34 mmol, 72%). λ_{max} (neat) 3414, 2931, 2836, 1673, 1471, 1447 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 8.86 (1H, s), 7.43 (2H, d, *J* 7.3 Hz), 7.32 (2H, t, *J* 7.3 Hz), 7.23 (1H, t, *J* 7.5 Hz), 6.63 (1H, s), 4.12 (2H, d *J* 3.6 Hz), 3.87–3.93 (1H, m), 3.66–3.81 (3H, m), 3.61 (1H, d *J* 3.6 Hz), 3.56 (3H, s), 3.35–3.45 (1H, m), 2.44–2.51 (1H, m), 2.32–2.39 (1H, m), 2.12 (2H, s), 1.75 (3H, m); δ_C (125 MHz, $CDCl_3$) 164.1, 150.7, 143.5, 141.0, 128.6, 127.4, 109.8, 89.1, 85.4, 83.8, 70.5, 63.4, 60.2, 44.9, 33.0, 12.1; HRMS (ESI-TOF): $M+Na^+$, found 399.1522. $C_{19}H_{24}N_2NaO_6$ requires 399.1527.

4.4. Synthesis of 3

*4.4.1. (1S,2R,3S,4R)-1-Deoxy-1-phenylribose (**17**).* To a mixture of **4** (10.1 g, 20.1 mmol) in CH_2Cl_2 (67 mL) and Et_3SiH (3.9 mL, 24.1 mmol) was added $BF_3{\cdot}Et_2O$ (3.8 mL, 30.1 mmol) at −78 °C. After stirring for 40 min at −40 °C, the reaction mixture was diluted with EtOAc, washed with a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc=30/1) to give the α-phenyl compound as a colorless oil (1.08 g, 2.2 mmol, 11%), and the β-phenyl compound as a colorless oil (6.48 g, 13.3 mmol, 66%). The desired β-phenyl compound (4.93 mmol, 10.1 mmol) was dissolved in THF (40 mL), and then TBAF·$3H_2O$ (3.82 g, 12.1 mmol) was added to the reaction mixture. After stirring for 2 h, the reaction mixture was evaporated under reduced pressure. The residue was dissolved in MeOH (37 mL), and then DOWEX-50WX8-100 (3.12 g) was added to the reaction mixture. After stirring for 40 h, the reaction mixture was filtered off, and the filtrate was evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc=1/1) to give compound **17** as a colorless foam (1.42 g, 6.76 mmol, 82% for two steps). λ_{max} (neat) 3346, 3272, 3201 cm^{-1}; δ_H (400 MHz, CD_3OD) 7.44–7.25 (5H, m), 4.70 (1H, d, *J* 7.2 Hz), 4.03 (1H, d, *J* 4.0 Hz), 3.98 (1H, q, *J* 4.0 Hz), 3.87 (1H, t, *J* 6.0 Hz), 3.80 (1H, dd, *J* 3.6, 12.0), 3.74 (1H, dd, *J* 5.2, 12.0 Hz); δ_C (125 MHz, $CDCl_3$) 141.9, 129.2, 128.7, 127.4, 86.3, 85.6, 79.1, 72.9, 63.7; HRMS (ESI-TOF): $M+Na^+$, found 233.0804. $C_{11}H_{14}O_4Na$ requires 233.0784.

*4.4.2. (1S,2R,3S,4R)-1-Deoxy-3,5-O-(1,3-disiloxanediyl-1,1,3,3-tetraisopropyl)-1-phenylribose (**18**).* To a solution of **17** (1.0 g, 4.76 mmol) in pyridine (48 mL) was added $TIPDSCl_2$ (1.8 mL, 5.72 mmol) at 0 °C. After stirring for 1 h at room temperature, the reaction mixture was diluted with EtOAc, washed with a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc=15/1 to 10/1) to give compound **18** as a colorless oil (1.99 g, 4.40 mmol, 92%). λ_{max} (neat) 3518, 2945, 2868 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 7.40 (2H, d, *J* 7.3 Hz), 7.32 (2H, t, *J* 7.3 Hz), 7.25 (1H, t, *J* 7.3 Hz), 4.82 (1H, d, *J* 3.7 Hz), 4.36 (1H, d, *J* 6.1 Hz), 4.13–3.92 (3H, m), 2.93 (1H, t, *J* 3.7 Hz), 1.10–0.93 (28H, m); δ_C (125 MHz, $CDCl_3$) 140.1, 128.4, 127.62, 125.8, 85.5, 82.4, 71.7, 62.6, 17.5, 17.4, 17.3, 17.1, 17.1, 17.0, 13.4, 13.2, 12.9, 12.7; HRMS (ESI-TOF): $M+H^+$, found 453.2531. $C_{23}H_{41}O_5Si_2$ requires 453.2487.

*4.4.3. (1S,2R,3S,4R)-1-Deoxy-3,5-O-(1,3-disiloxanediyl-1,1,3,3-tetraisopropyl)-2-O-methyl-1-phenylribose (**19**).* To a solution of **18** (1.98 g, 4.37 mmol) in THF (44 mL) was added MeI (2.7 mL, 43.7 mmol) and NaHMDS (2.07 M in THF, 6.4 mL, 13.1 mmol) at 0 °C. After stirring for 1 h, the reaction was quenched by a satd NH_4Cl solution, extracted with EtOAc, and then the organic layer was washed with a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc=20/1) to give compound **19** as a colorless oil (1.57 g, 3.36 mmol, 77%). λ_{max} (neat) 2946, 2866 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 7.42 (2H, d, *J* 7.3 Hz), 7.31 (2H, t, *J* 7.6 Hz), 7.23 (1H, t, *J* 7.3 Hz), 4.95 (1H, s), 4.36 (1H, dd, *J* 3.6, 4.9 Hz), 4.19 (1H, dd, *J* 13.1, 2.4 Hz), 3.57 (3H, s), 3.56 (1H, t, *J* 4.9 Hz), 1.10–0.88 (28H, m); δ_C (125 MHz, $CDCl_3$) 141.1, 128.3, 127.4, 125.7, 87.2, 84.6, 80.9, 70.6, 60.7, 58.9, 17.5, 17.4, 17.4, 17.3, 17.2, 17.0, 13.5, 13.1, 12.9, 12.6; HRMS (ESI-TOF): $M+H^+$, found 467.2660. $C_{24}H_{43}O_5Si_2$ requires 467.2644.

*4.4.4. (1S,2R,3S,4R)-1-Deoxy-2-O-methyl-1-phenylribose (**3**).* To a solution of **19** (1.54 g, 3.30 mmol) in THF (33 mL) was added a solution of TBAF in THF (8.25 mL, 8.25 mmol). After stirring for 1 h, the reaction mixture was diluted with EtOAc. The organic layer was continuously washed with water and a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc=2/1) to give the compound **3** as a colorless oil (505.6 mg, 2.25 mmol, 68%).

λ_{max} (neat) 3406, 2931 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 7.37–7.26 (5H, m), 4.83 (1H, d *J* 5.5 Hz), 4.18 (1H, t, *J* 5.5 Hz), 3.99 (1H, dd, *J* 4.3, 3.7 Hz), 3.91 (1H, dd, *J* 3.6, 12.2 Hz), 3.77 (1H, dd, *J* 3.6, 12.2 Hz), 3.62 (1H, dd, *J* 5.5 Hz), 3.41 (3H, s); δ_C (125 MHz, $CDCl_3$) 139.82, 128.59, 128.01, 126.01, 86.49, 84.46, 82.87, 70.55, 62.85, 58.40; HRMS (ESI-TOF): $M+H^+$, found 225.1128. $C_{12}H_{17}O_4$ requires 225.1121.

4.5. General procedure for synthesis of amidite precursor

To a solution of the corresponding diol compound (**1**, **2**, and **3**) in pyridine was added DMTrCl (2 equiv). After stirring for 1 h, the reaction mixture was diluted with EtOAc. The organic layer was continuously washed with water and a satd NaCl solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography ($CHCl_3$:MeOH solvent system) to give the DMTr-protected compound as the colorless foam. The DMTr-protected compound was dissolved in CH_2Cl_2 and diisopropylethylamine (6.0 equiv) at 0 °C. After stirring for 15 min at the same temperature, *N*,*N*-diisopropylchlorophosphoramidite (3.0 equiv) was added to the reaction mixture, which was stirred for 1 h at room temperature. The reaction mixture was diluted with EtOAc, and the organic layer was washed with a satd $NaHCO_3$ solution, dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash column chromatography (Hexane:EtOAc solvent system) to give the colorless foam. This foam was dissolved in CH_2Cl_2 and poured into hexane at −78 °C. The resulting white powder was collected and dried under vacuum to give the corresponding amidite precursor.

Compound **20**: as a white powder (73% for two steps): δ_H (400 MHz, $CDCl_3$) 7.61–7.17 (14H, m), 6.84 (1H, s), 6.76–6.73 (4H, m), 6.51–3.81 (5H, m), 3.76 (6H, s), 3.56 (3H, s), 3.44–3.08 (2H, m), 2.58–2.22 (4H, m), 1.73 (3H, s), 1.29–0.85 (14H, m); d_P (162 MHz, $CDCl_3$) 150.7, 150.1; ESI-TOF-MS: $M+Na^+$, found 887.3707. $C_{48}H_{57}N_4O_9PNa$ requires 887.3755.

Compound **21**: as a white powder (64% for two steps): λ_{max} (neat) 2969, 2345, 1509 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 8.01 (1H, s), 7.58 (1H, d, *J* 7.3 Hz), 7.52 (1H, d, *J* 7.3 Hz), 7.17–7.39 (12H, m), 6.78 (4H, s), 6.65 (1H, d, *J* 11.0 Hz), 4.83 (0.2H, s), 4.70 (0.2H, s), 4.43 (0.5H, s), 4.20–4.33 (1H, m), 3.76 (7H, s), 3.45 (6H, d, *J* 5.9 Hz), 3.17–3.24 (1H, m), 2.64 (1H, t, *J* 6.3 Hz), 2.56 (1H, t, *J* 6.3 Hz), 2.20–2.54 (2H, m), 2.12–2.29 (1H, m), 1.72 (5H, d, *J* 8.9 Hz), 1.62 (1H, s), 1.19–1.37 (14H, m), 1.12 (2H, d, *J* 7.7 Hz), 0.95 (4H, d, *J* 6.7 Hz), 0.82 (8H, t, *J* 6.6 Hz); d_P (162 MHz, $CDCl_3$) 151.3, 151.1; HRMS (ESI-TOF): $M+Na^+$, found 901.3902. $C_{49}H_{59}N_4NaO_9P$ requires 901.3912.

Compound **22**: as a white powder (63% for two steps): δ_H (400 MHz, $CDCl_3$) 7.50–7.18 (14H, m), 6.80 (4H, t, *J* 8.5 Hz), 4.88 (1H, dd, *J* 3.6, 7.2 Hz), 4.37–4.24 (2H, m), 3.94–3.21 (15H, m), 2.64 (2H, t, *J* 6.7), 2.30 (2H, t, *J* 6.8 Hz), 1.16–0.98 (12H, m); d_P (162 MHz, $CDCl_3$) 150.6, 150.1; HRMS (ESI-TOF): $M+H^+$, found 727.3510. $C_{42}H_{52}N_2O_7P$ requires 727.3507.

4.6. MALDI-TOF MS result of synthesized TFOs

TFO1(**1**): calcd for 5860.06, found 5859.80. **TFO2**(**1**): calcd for 5876.05, found 5875.34. **TFO3**(**1**): calcd for 5860.06, found 5859.51. **TFO4**(**1**): calcd for 5844.07, found 5843.88. **TFO1**(**2**): calcd for 5874.07, found 5873.64. **TFO2**(**2**): calcd for 5890.06, found 5889.00. **TFO3**(**2**): calcd for 5874.07, found 5873.49. **TFO4**(**2**): calcd for 5858.08, found 5873.49. **TFO1**(**3**): calcd for 5722.01, found 5721.14. **TFO2**(**3**): calcd for 5738.00, found 5737.89. **TFO3**(**3**): calcd for 5722.01, found 5721.04. **TFO4**(**3**): calcd for 5706.02, found 5705.13.

4.7. Gel-shift assay

Triplex formation was done for 12 h at 22 °C in the buffer containing 20 mM Tris–HCl, 5 mM $MgCl_2$, 2.5 mM spermidine, and 10% sucrose at pH 7.5. Electrophoresis was done at 10 °C with a non-denatured polyacrylamide gel. 10 nM TFO containing the ^{32}P-labeled one as the tracer was used. The association constant was calculated using the concentration of each component and the following equation; *Ks*=[Triplex]/[Duplex][TFO].

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