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Effects of Halogenated WNA Derivatives on Sequence Dependency for Expansion of Recognition Sequences in Non-Natural-Type Triplexes

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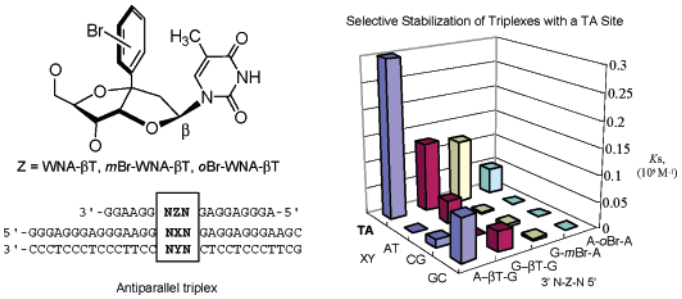

Triplex-forming oligonucleotides (TFOs) are sequence-specific DNA-binding agents, but their target duplexes are limited to homopurine/homopyrimidine sequences because of interruption of the pyrimidines bases in the purine region. This problem has not been fully solved despite a wide variety of studies. Recently, we have developed a bicyclic system as a novel scaffold for nucleoside analogues (WNA, W-shaped nucleoside analogues) and determined two useful compounds, WNA-βT (**2**) and WNA-βC (**5**), for highly stable and selective triplex formation at a TA and a CG interrupting site, respectively. However, subsequent investigations have shown that the triplex formation using WNA is dependent on the neighboring bases of the TFOs. In this study, we have synthesized new WNA derivatives having halogenated recognition bases or benzene rings and evaluated the effects of the modifications on the triplex stability as well as selectivity. It has been found that the WNA-βT analogues holding 5-halogenated pyrimidine bases (WNA-β^{Br}U (**3**) and WNA-β^{F}U (**4**)) exhibit high CG-selectivity. On the other hand, the WNA-βT derivatives having the bromo-substituted benzene ring (*m*Br-WNA-βT (**10**) and *o*Br-WNA-βT (**11**)) have shown high selectivity to a TA interrupting site with high stability in the sequences to which the original WNA-βT do not bind. Thus, sequence-dependency has been overcome by the sequence-dependent use of WNA-βT, *m*Br-WNA-βT, and *o*Br-WNA-βT.

Introduction

Triplex-forming oligonucleotides (TFOs) are sequence-specific DNA-binding agents and have shown potential ability as tools for modulation of gene expression,[1−9] sequence-selective cleavage,[10] gene recombination, and repair.[11−13] Purine-rich TFOs bind in antiparallel orientation to the homopurine/homopyrimidine sequences in the major groove of duplex DNA

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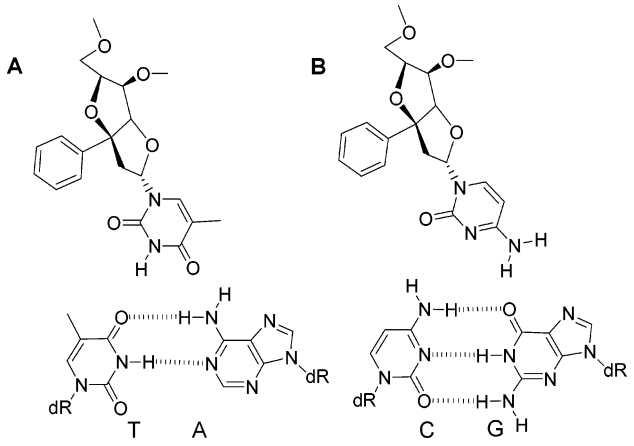

FIGURE 1. Speculated structure of WNA-βT/TA (A) and WNA-βC combinations (B).

by reverse Hoogsteen hydrogen bonds in a sequence-specific manner (G/GC, A/AT).[14−16] However, the most stable triplex DNA is hampered by the presence of one pyrimidine base in the homopurine sequence, as the pyrimidine bases present one hydrogen bonding site in the major groove. Therefore, pyrimidine/purine inversion sites (a TA and a CG base pair) are called interrupting sites and are a major limitation to formation of the triplex DNA. If this limitation is overcome by the development of artificial nucleoside bases, the TFOs will play more significant roles in genomic research. Despite a wide variety of approaches, this problem has not been fully solved.[17−20] We have previously reported that novel nucleosides analogues, WNA-βT and WNA-βC, can recognize a TA and a CG interrupting site to form triplexes with high stability and selectivity, respectively (Figure 1).[21,22] However, subsequent investigations have shown that the triplex formation using the WNA is dependent on the neighboring bases of the TFOs.[23] A similar sequence-dependency of a non-natural ribonucleoside observed in the parallel-type triplex formation[24,25] has been explained in terms of interaction of the nucleoside analogue.[26,27] In our case, it was found that a base recognition part and an aromatic ring of the WNA separately contributed to triplex selectivity and stability and that a minor modification caused drastic effects on triplex stabilization.[22] In our continuous study to overcome such sequence-dependency, a systematic modification of WNA-βT and WNA-βC (Figure 2) has been undertaken. This article describes in detail the synthesis and the evaluation of triplex stabilization of new halogenated derivatives of WNA.

Results

Synthesis. We have designed the WNA having 5-substituted uracil (**1**−**4**), 5-substituted cytosine (**5**−**8**), and a bromobenzene (**9**−**14**). The compounds having a halogenated base (**3**, **4**, **7**, **8**) were synthesized from the bicyclic intermediate (**15**) by a similar method as previously reported (Scheme 1).[22] Lewis acid catalyzed glycosidic bond formation and following separation of the β-isomers produced the corresponding WNA derivatives (**16a**−**19a**). The stereochemistry of the isolated isomer was determined by ^{1}H COSY and NOESY spectra. As the α,β-isomers of WNA-βU (**1**) that were obtained by a similar glycosidation with **15** were not separated, its derivative (**21a**) was obtained by deamination of WNA-βC (**20**) by using $NaHSO_3$.[28] Subsequently, these derivatives were deprotected (**16b**−**19b**, **21b**) and converted to the corresponding amidite precursors (**16c**−**19c**, **21c**).

The WNA derivatives having a bromobenzene were synthesized starting from 5-*O*-TBDPS and 2,3-*O*-isopropylidene protected D-ribonolactone (**22**) (Scheme 2).[22] 1,4- or 1,3-Dibromobenzene was treated with *n*BuLi in THF at −78 °C followed by the reaction with **22** to produce the corresponding adduct in two isomers, **23(*p*)** or **24(*m*)**, respectively.[29,30] In the case of the synthesis of the *o*-bromobenzene adduct **25(*o*)**, 1,2-dibromobenzene was treated with *n*BuLi in 1/1 THF/ether at −110 °C followed by the addition of **22** to produce **25(*o*)** in good yield.[31] These addition products were converted to the corresponding bicyclic intermediate (**26(*p*)**, **27(*m*)**, **28(*o*)**) as described previously without separation of the stereoisomers. After *N*-glycosidation to each bicyclic intermediate with thymine or *N*-benzoylcytosine,[32,33] the desired β-isomers were separated and their stereochemistry was determined by 2D-NMR spectra (**29a(*p*)**−**34a(*o*)**). Finally, all derivatives were converted to the corresponding amidite precursors (**29c(*p*)**−**34c(*o*)**).

The phosphoramidites described in Schemes 1 and 2 were incorporated into the 18-mer TFOs by using an automated DNA

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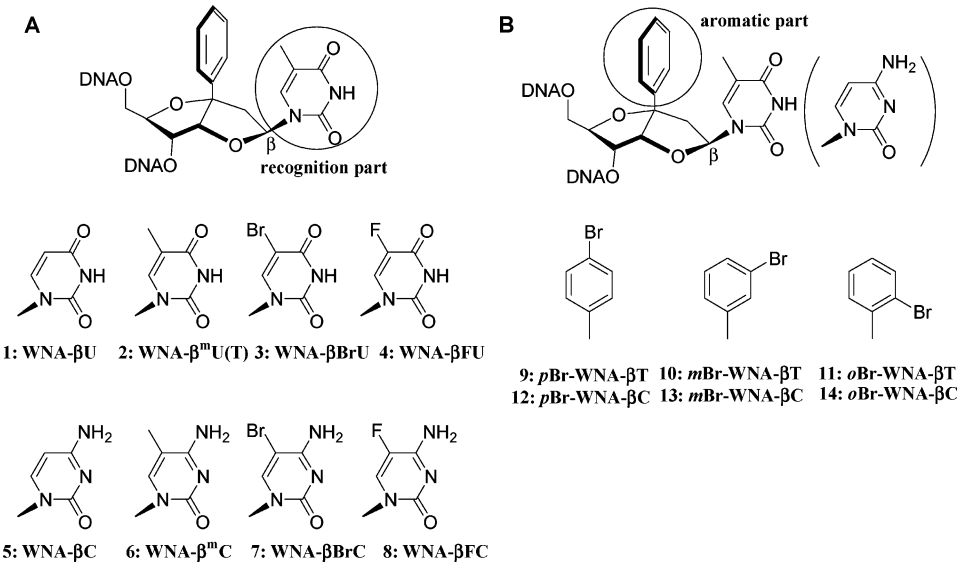
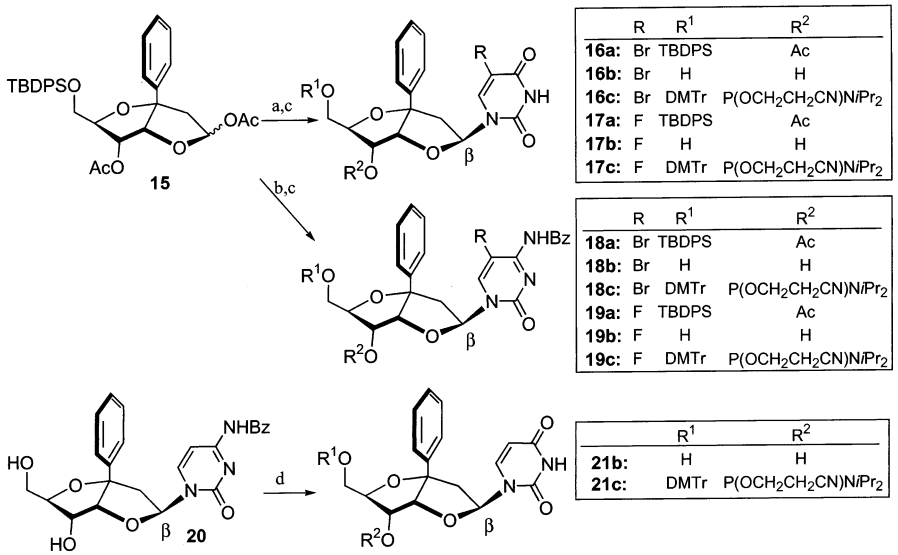


FIGURE 2. Structures of new WNA analogues containing halogenated recognition base (A) or aromatic ring (B).

SCHEME 1. Synthesis of WNA Analogues Having 5-Subsutituted Pyrimidine Nucleoside Bases[a]



	R	R^1	R^2
16a:	Br	TBDPS	Ac
16b:	Br	H	H
16c:	Br	DMTr	$P(OCH_2CH_2CN)NiPr_2$
17a:	F	TBDPS	Ac
17b:	F	H	H
17c:	F	DMTr	$P(OCH_2CH_2CN)NiPr_2$

	R	R^1	R^2
18a:	Br	TBDPS	Ac
18b:	Br	H	H
18c:	Br	DMTr	$P(OCH_2CH_2CN)NiPr_2$
19a:	F	TBDPS	Ac
19b:	F	H	H
19c:	F	DMTr	$P(OCH_2CH_2CN)NiPr_2$

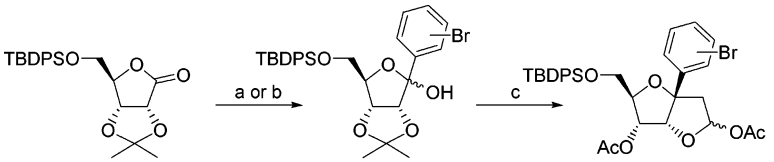
	R^1	R^2
21b:	H	H
21c:	DMTr	$P(OCH_2CH_2CN)NiPr_2$

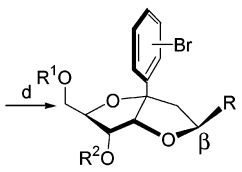
[a] Reagents and conditions. (a) **16a**: 5-bromouracil, BSA, TMSOTf, CH_3CN, 52%; **17a**: 5-fluorouracil, BSA, TMSOTf, CH_3CN, 59%. (b) **18a**: *N*-benzoylcytosine, BSA, SnCl, CH_3CN, 50%; **19a**: 5-bromo-*N*-benzoylcytosine, BSA, TMSOf, CH_3CN, 52%; **20a**: 5-fluoro-*N*-benzoylcytosine, BSA, TMSOf, CH_3CN, 33%; **21a**: 5-methyl-*N*-benzoylcytosine, BSA, SnCl, CH_3CN, 53%. (c) (i) nBu$_4$NF, THF; (ii) aqueous NaOH, MeOH, THF; (iii) DMTrCl, pyridine; (iv) $iPr_2NP(Cl)OCH_2CH_2CN$, iPr_2NEt, CH_2Cl_2. (d) (i) $NaHSO_3$, Na_2SO_3, dioxane/H_2O (1:1), 80 °C, 96%; (ii) DMTrCl, pyridine; (iii) $iPr_2NP(Cl)OCH_2CH_2CN$, iPr_2NEt, CH_2Cl_2.

synthesizer. The crude products were cleaved from the resin with 28% NH_4OH at 55 °C for 5 h and followed by purification by reverse-phase HPLC. Subsequently, deprotection of the DMTr group was accomplished with 10% acetic acid, and the resulting DMTrOH group was removed by washing with Et_2O. Structure and purity of the synthesized TFOs were confirmed by MALDI-TOF MS measurement. The sequences of the TFO and the duplex DNA are shown in Figure 3.

Evaluation of Triplex-Forming Ability of TFOs Containing the New WNA Derivative. The triplex-formation of all combinations of TFOs (**TFO1**−**4**, Z = 1−14) and their duplex targets was evaluated by gel shift assay with 15% nondenatured polyacrylamide gel at 10 °C using the ^{32}P-labeled TFO as a tracer. The triplex formation is performed by using 10 nM TFO and different concentrations of the target duplex (0−100 nM) in the buffer containing 20 mM Tris-HCl, 5 mM $MgCl_2$, 2.5 mM spermidine, and 10% sucrose at pH 7.5, and the triplex DNA is observed as the slower migration band relative to the single-stranded TFOs. Among the results of the gel-shift assay, successful examples of the gel-electrophoresis are shown in Figure 4. Figure 4A has demonstrated a successful result, in which the **TFO3** containing Z = *m*Br-WNA-βT exhibits specificity to a TA interrupting site in the TFO sequence 3′-GZA-5′. In addition, it is also shown in Figure 4B that the **TFO4** containing Z = *o*Br-WNA-βT can recognize a TA interrupting site in the TFO sequence 3′-AZA-5′ with high selectivity. All TFOs were evaluated by the same method, and equilibrium association constants (K_s) were calculated by quantification of these bands using the equation of K_s = [triplex]/([duplex]-

SCHEME 2. Synthesis of WNA Analogues Having Bromo-benzene Rings[a]





	-Br	R^1	R^2	R
29a(*p*)	p	TBDPS	Ac	
29b(*p*)	p	H	H	
29c(*p*)	p	DMTr	$P(OCH_2CH_2CN)NiPr_2$	
30a(*m*)	m	TBDPS	Ac	
30b(*m*)	m	H	H	
30c(*m*)	m	DMTr	$P(OCH_2CH_2CN)NiPr_2$	
31a(*o*)	o	TBDPS	Ac	
31b(*o*)	o	H	H	
31c(*o*)	o	DMTr	$P(OCH_2CH_2CN)NiPr_2$	
32a(*p*)	p	TBDPS	Ac	
32b(*p*)	p	H	H	
32c(*p*)	p	DMTr	$P(OCH_2CH_2CN)NiPr_2$	
33a(*m*)	m	TBDPS	Ac	
33b(*m*)	m	H	H	
33c(*m*)	m	DMTr	$P(OCH_2CH_2CN)NiPr_2$	
34a(*o*)	o	TBDPS	Ac	
34b(*o*)	o	H	H	
34c(*o*)	o	DMTr	$P(OCH_2CH_2CN)NiPr_2$	

[a] Reagents and conditions: (a) 1,4-dibromobenzene (or 1,3-dibromobenzene), *n*-BuLi, THF, −78 °C, 80% (87%); (b) 1,2-dibromobenzne, *n*-BuLi, ether/THF = 1:1, −110 °C, quantitative; (c) (i) allyltrimethylsilane, $ZnBr_2$, CH_3NO_2; (ii) OsO_4, $NaIO_4$, pyridine; (iii) 5% H_2SO_4, THF; (iv) Ac_2O, pyridine; (d) (i) thymine (or Bz-cytosine), BSA, $SnCl_4$ (or TMSOTf), CH_3CN; (ii) *n*-Bu_4NF, THF; (iii) aqueous NaOH, MeOH, THF; (iv) DMTrCl, pyridine; (v) $iPr_2NP(Cl)OCH_2CH_2CN$, iPr_2NEt, CH_2Cl_2.

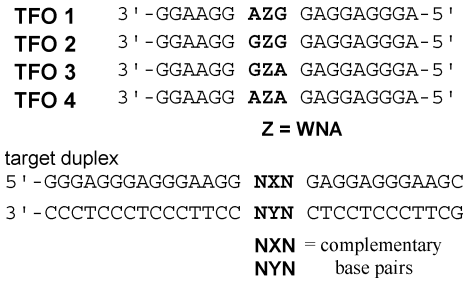


FIGURE 3. Sequences of TFO and the target duplexes used in this study.

[ssTFO]) and are summarized in Table 1. Data in Table 1 are compared in the 3D bar graph in Figure 5.

As previously reported, **TFO1** consisting of A at the 3′- and G at the 5′-side of WNA-βT forms a stable triplex with high selectivity to a TA interrupting site. This stabilization effect of WNA-βT on a TA site is quite remarkable when it is incorporated into **TFO1** and **TFO2** (3′-GZG-5′); however, it is completely lost in the sequence of either **TFO3** or **TFO4**. Surprisingly, drastic changes were observed in triplex formation with the use of WNA derivatives having uracil (U), 5-bromouracil (BrU), or 5-fluorouracil (FU), in that selectivity was changed to a CG site with **TFO1** having WNA-BrU or FU, and the triplex-stabilizing effect was lost with **TFO2**−**TFO4** having these analogues.

In the case of the WNA-βC, a stabilizing effect on a CG site was observed only in the sequence of **TFO1**. Substitution at the 5-position of cytosine of WNA-βC caused decrease in triplex stability (^{m}C, BrC, or FC). Considering that the abasic-type WNA derivative showed a nonselective stabilization effect in the sequence of **TFO1** and **TFO2** (WNA-H, Figure 6), the substituted cytosine unit apparently produced a destabilizing

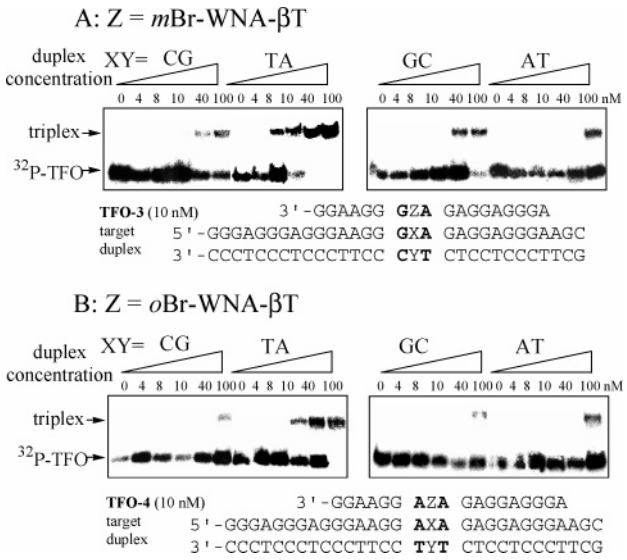


FIGURE 4. Gel-shift assay for determination of triplex formation. Triplex formation was done for 12 h at 22 °C in the buffer containing 20 mM Tris-HCl, 5 mM $MgCl_2$, 2.5 mM spermidine, and 10% sucrose at pH 7.5; 10 nM TFO containing ^{32}P-labeled one as a tracer and different duplex concentrations ranging from 0 to 100 nM were used. Electrophoresis was carried out at 10 °C with 15% nondenatured gel. (A) TFO-3 containing *m*Br-WNA-βT. (B) TFO-4 containing *o*Br-WNA-βT.

effect in triplex formation. Loss of triplex-stabilization effect with the WNA derivatives having substituted U and C has suggested that a pyrimidine part of WNA is located in a limited space of the triplex. We have already found that a larger (i.e., naphthalene) or smaller aromatic ring (i.e., thiophene) leads to loss of triplex-forming ability of WNA analogues.[23] Thus, we next investigated substitution effects of the benzene ring.

TABLE 1. Equilibrium Association Constants (K_s) of Triplex ($M^{-1} \times 10^7$) with TFO Containing WNA[a]

	3′-A**Z**G-5′(**TFO1**) 5′-A**X**G-3′; 3′-T**Y**C-5′ XY =				3′-G**Z**G-5′ (**TFO2**) 5′-G**X**G-3′; 3′-G**Y**G-5′ XY =				3′-G**Z**A-5′ (**TFO3**) 5′-G**X**A-3′; 3′-C**Y**T-5′ XY =				3′-A**Z**A-5′ (**TFO4**) 5′-A**X**A-3′; 3′-T**Y**T-5′ XY=			
Z[b]	TA	AT	CG	GC	TA	AT	CG	GC	TA	AT	CG	GC	TA	AT	CG	GC
dG	0.4	0.8	0.8	**8.6**	*c*	0.8	*c*	**6.6**	0.1	0.6	0.1	**6.9**	0.1	0.3	0.1	**5.8**
U	*c*	*c*	1.1	0.8	*c*	2.2	*c*	2.4	0.3	0.5	0.1	1.1	*c*	*c*	*c*	*c*
T	**30**	*c*	1.5	8.2	13	4.2	*c*	3.7	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
BrU	2.5	0.1	**4.0**	3.1	0.1	0.7	0.1	3.5	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
FU	0.7	2.7	**7.4**	2.7	0.1	0.2	0.1	2.4	0.4	0.4	0.6	0.7	*c*	*c*	*c*	*c*
C	*c*	2.5	**11.5**	4.7	0.3	1.4	0.2	0.8	0.2	0.2	0.4	0.4	*c*	0.6	*c*	0.3
*m*C	2.1	*c*	1.8	0.6	0.1	0.5	0.2	0.5	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
BrC	*c*	1.5	4.1	2.9	*c*	*c*	*c*	1.1	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
FC	*c*	0.6	4.8	2.2	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
*p*Br, T	0.8	*c*	*c*	1.8	*c*	3.1	1.4	3.5	*c*	0.2	1.7	1.1	*c*	*c*	*c*	1.0
*m*Br, T	*c*	*c*	*c*	1.0	*c*	1.9	*c*	1.9	**12**	0.2	0.4	0.4	*c*	*c*	*c*	*c*
*o*Br, T	5.8	2.3	2.9	3.3	3.8	3.9	0.5	4.0	0.5	0.8	1.0	1.4	**5.0**	0.1	0.1	0.1
*p*Br, C	0.6	*c*	2.3	3	*c*	*c*	0.1	0.9	0.4	*c*	0.6	*c*	*c*	*c*	*c*	*c*
*m*Br, C	3.5	0.1	0.4	3.3	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
*o*Br, C	*c*	*c*	0.3	1.9	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*

[a] 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; 10 nM TFO containing ^{32}P-label as a tracer and different duplex concentrations ranging from 0 to 100 nM were used. Electrophoresis was carried out at 10 °C with 15% nondenatured gel, and radioactive bands corresponding to the single strand TFO and those in the triplex were quantified to give the equilibrium association constants (K_s). K_s = [triplex]/([duplex][TFO]). [b] WNA are shown by abbreviations such that U and *p*Br, T represent WNA-βU and *p*BrWNA-βT, respectively. [c] Equilibrium association constant (K_s) is less than 0.1×10^7 (M^{-1}).

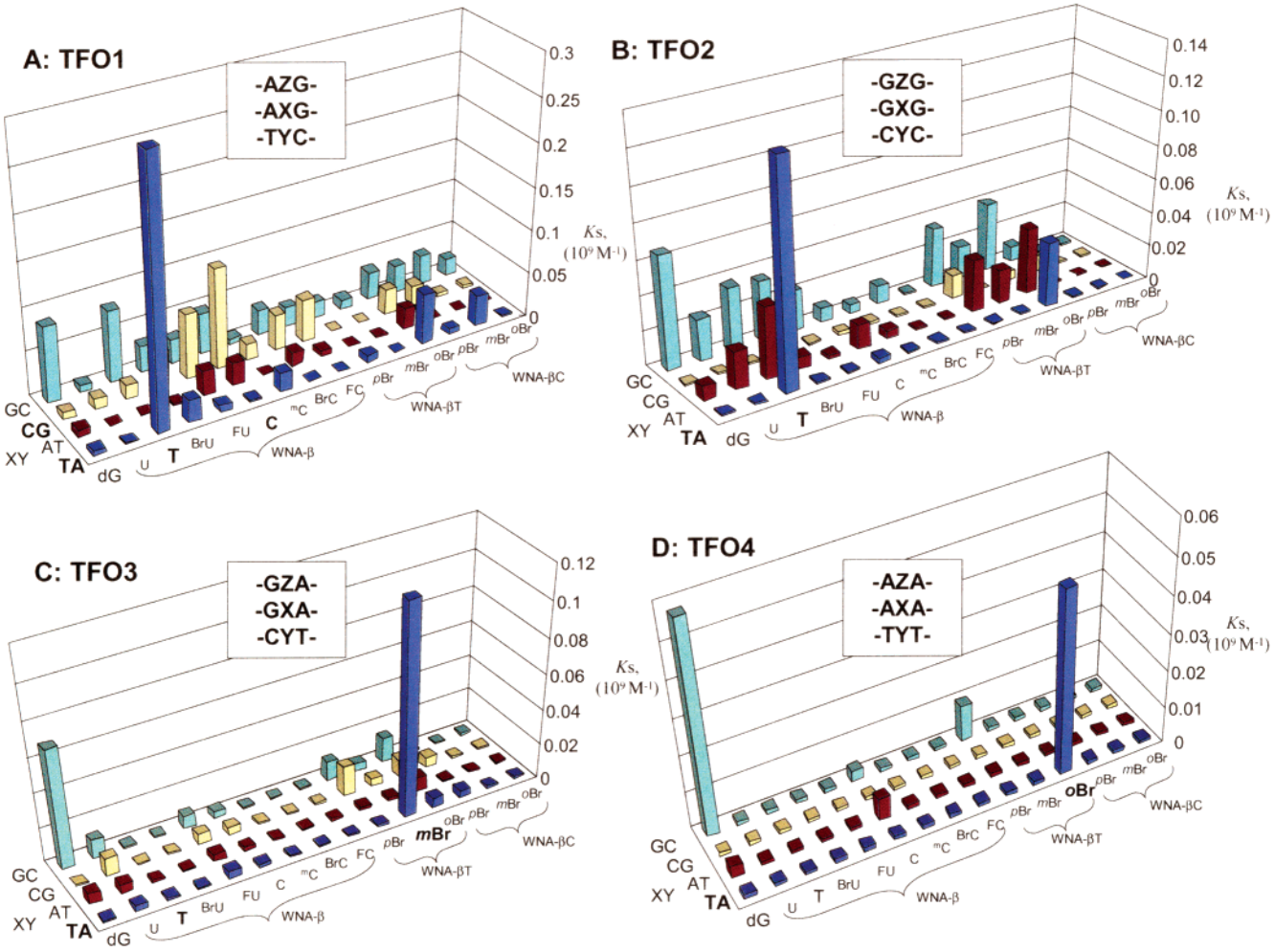


FIGURE 5. Comparison of triplex stability formed with **TFO1**−**TFO4** Incorporating a different WNA analogue: (A) **TFO1**, (B) **TFO2**, (C) **TFO3** and (D) **TFO4**. Data in Table 1 are shown as 3D bar graphs.

Bromo-substitution of WNA-βC caused almost complete loss of the stabilization effect regardless of its substitution position (*p*Br-, *m*Br-, or *o*Br-WNA-βC). On the other hand, bromo-substitution of WNA-βT showed an interesting effect on triplex

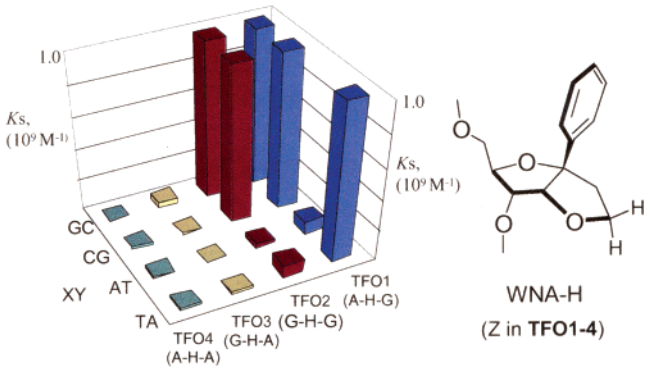


FIGURE 6. Comparison of triplex stability formed with WNA-H. Binding assay was performed under the same condition described for Table 1, except that a 20 mM concentration of Mg^{2+} was used in this experiment.

stability. *para*-Substitution resulted in loss of the stabilization effect in all TFO sequences. In the case of *meta*-substitution, *m*Br-WNA-βT exhibited a highly selective stabilization effect on a TA site only in the sequence of **TFO3** (3′-GZA-5′). Interestingly, *ortho*-substituted derivative *o*Br-WNA-βT achieved specific stabilization to a TA site only in the sequence of **TFO4** (3′-AZA-5′). Selective stabilization of WNA-βT in the sequence of **TFO1** and **TFO2**, *m*Br-WNA-βT in the sequence of **TFO3**, and *o*Br-WNA-βT in the sequence of **TFO4** is clearly shown in Figure 5. Finally, stable triplexes having a TA interrupting site can be formed with high selectivity by the use of TFO incorporating WNA-βT, *m*Br-WNA-βT, or *o*Br-WNA-βT, depending on the neighboring bases of the TFO. Determination of the triplex structure is now ongoing to reveal the specific effect of substitution of the benzene ring on triplex stabilization.

Conclusion

The formation of stable triplexes at any predetermined sequence is a major challenge for the general use of the antigene triplex strategy. In our previous study, it was reported that the TFO containing WNA-βT or WNA-βC stabilizes a TA or a CG interrupting site, respectively, with high selectivity and stability. In the present work, we attempted to overcome sequence-dependency in triplex formation with the WNA by synthetic study of substitution on the thymine or the cytosine unit or on the benzene ring. As a result, we have identified two new analogues for a TA interrupting site; *m*Br-WNA-βT in the TFO sequence of 3′-GZA-5′ (**TFO3**) and *o*Br-WNA-βT in the TFO sequence of 3′-AZA-5′ (**TFO4**). Thus, formation of stable triplexes having a TA interrupting site has been achieved with the WNA, regardless of the neighboring bases, by the sequence-dependent use of WNA-βT in the TFO sequence of 3′-GZG-5′ and 3′-AZG-5′, *m*Br-WNA-βT in the TFO sequence of 3′-GZA-5′, and *o*Br-WNA-βT in the TFO sequence of 3′-AZA-5′.

Experimental Section

Glycosidation. (1′*S*,3′*R*,4′*R*,5′*R*,7′*S*)-1-{4′-Acetoxy-3′-(*tert*-butyldiphenylsilyoxymethyl)-1′-phenyl-2′,6′-dioxabicyclo[3.3.0]oct-7′-yl}-5-bromouracil (WNA-βBrU, 16a). *N*,*O*-Bis(trimethylsilyl)-acetamide (BSA; 424 μL, 1.74 mmol) was added to a suspension of 5-bromouracil (166 mg, 0.871 mmol) in CH_3CN (4.0 mL). TMSOTf (189 μL, 1.05 mmol) and a solution of **15** (400 mg, 0.697 mmol) in CH_3CN (4.0 mL) were added to the above mixture. The reaction mixture was stirred for 1 h. The mixture was diluted with EtOAc and successively washed with saturated $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated. The isomers were separated by flash chromatography (silica gel, $CHCl_3$/EtOAc = 7:1) to give the desired β-isomer as a colorless foam in 52% yield. ^{1}H NMR (400 MHz, $CDCl_3$/TMS) δ 8.69 (bs, 1H), 7.75 (s, 1H), 7.66 (d, 2H, J = 7.2 Hz), 7.61 (d, 2H, J = 7.2 Hz), 7.56 (d, 2H, J = 8.2 Hz), 7.45−7.29 (m, 9H), 6.27 (dd, 1H, J = 8.2, 5.5 Hz), 5.15 (d, 1H, J = 4.1 Hz), 5.09 (dd, 1H, J = 8.8, 4.1 Hz), 4.25 (ddd, 1H, J = 8.8, 3.9, 3.3 Hz), 4.00 (dd, 1H, J = 11.5, 3.3 Hz), 3.74 (dd, 1H, J = 11.5, 3.9 Hz), 2.98 (dd, 1H, J = 13.7, 5.5 Hz), 2.52 (dd, 1H, J = 13.7, 8.2 Hz), 2.06 (s, 3H), 1.01 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.1, 158.7, 149.2, 139.3, 139.1, 135.6, 132.8, 129.8, 128.7, 128.0, 127.7, 125.2, 97.4, 92.3, 88.8, 88.7, 80.7, 73.0, 62.4, 48.6, 26.7, 20.7, 19.2. FTIR (film) 3192, 3071, 2930, 2856, 1693, 1622, 1448, 1427 cm^{-1}. HRMS (ESIMS) *m*/*z* calcd for $C_{35}H_{37}N_2O_7SiBrNa$ $(M + Na)^+$ 727.1446, 729.1433, found 727.1464, 729.1396.

Deprotection. General Procedure. A THF solution of the above compound and TBAF (1.0 M THF solution, 2 equiv to the compound.) was stirred for 1−2 h at room temperature, and the reaction mixture was diluted with AcOEt. The organic layer was successively washed with water and brine, dried over Na_2SO_4, and evaporated. The residue was purified by flash chromatography (silica gel, $CHCl_3$/CH_3OH) to give the corresponding compound, which was further deacetylated in a 9/1 THF/methanol solution containing 0.2 M NaOH (2 equiv) at 0 °C. After stirring for 45−60 min at 0 °C, the reaction mixture was quenched with acetic acid and diluted with MeOH, and then the solvent was evaporated. The residue was purified by flash chromatography (silica gel, $CHCl_3$/CH_3OH).

(1′*S*,3′*R*,4′*R*,5′*R*,7′*S*)-1-{4′-Hydroxy-3′-hydroxymethyl-1′-phenyl-2′,6′-dioxabicyclo[3.3.0]oct-7′-yl}-5-bromouracil (WNA-βBrU, 16b). A colorless foam (64%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.25 (s, 1H), 7.69 (d, 2H, J = 7.7 Hz), 7.35 (dd, 2H, J = 7.7, 7.3 Hz), 7.26 (t, 1H, J = 7.3 Hz), 6.22 (dd, 1H, J = 8.2, 6.2 Hz), 4.87 (d, 1H, J = 3.6 Hz), 4.01 (ddd, 1H, J = 8.8, 5.8, 2.6 Hz), 3.90−3.86 (m, 2H), 3.67 (dd, 1H, J = 12.2, 5.8 Hz), 2.80 (dd, 1H, J = 13.9, 6.2 Hz), 2.73 (dd, 1H, J = 13.9, 8.2 Hz). ^{13}C NMR (125 MHz, CD_3OD) δ 161.7, 151.4, 142.9, 141.5, 129.4, 128.7, 126.6, 97.3, 93.2, 91.2, 90.8, 84.6, 73.7, 63.5, 49.0. FTIR (KBr) 3400, 3184, 3066, 2948, 2814, 1683, 1653, 1506, 1277, 1056 cm^{-1}. HRMS (ESIMS) *m*/*z* calcd for $C_{17}H_{17}N_2O_6BrNa$ $(M + Na)^+$ 447.0162, 449.0144, found 447.0121, 449.0182.

General Procedure of the Synthesis of the β-Cyanoethylphosphoramidite Precursors of WNA. DMTrCl (1.5 equiv) was added to a solution of the dihydroxyl derivative of WNA in pyridine, and the mixture was stirred for 1 h. The mixture was diluted with EtOAc and successively washed with water and brine. The organic layer was dried over Na_2SO_4 and evaporated, and then the residue was purified by flash chromatography (silica gel, $CHCl_3$/CH_3OH containing 0.5% pyridine) to produce the corresponding DMTr-protected WNA. iPr$_2$NP(Cl)OC$_2$H$_4$CN (6 equiv) was added to a solution of the above DMTr derivative of WNA and iPr$_2$NEt (3 equiv) in dry CH_2Cl_2 at 0 °C. After stirring for 60 min at the same temperature, the reaction mixture was quenched with saturated $NaHCO_3$ solution and extracted with AcOEt. The organic layer was separated, dried over Na_2SO_4, and evaporated. The residue was purified by flash chromatography (silica gel, hexane/AcOEt) to give the purified material, which was crystallized in hexane at −78 °C. The hexane was removed by decantation, and the solid material was dried in a vacuum for several hours.

(1′*S*,3′*R*,4′*R*,5′*R*,7′*S*)-1-{4′-(2-Cyanoethyl-*N*,*N*-diisopropylphosphoramidityloxy)-3′-dimethoxytrithyloxymethyl-1′-phenyl-2′,6′-dioxabicyclo[3.3.0]oct-7′-yl}-5-bromouracil (WNA-βBrU, 16c). A white powder (85%). ^{1}H NMR (400 MHz, $CDCl_3$/TMS) δ 7.97 (s, 0.4H), 7.79 (s, 0.6H), 7.62 (d, 1.2H, J = 7.4 Hz), 7.60 (d, 0.8H, J = 8.5 Hz), 7.45 (d, 0.8H, J = 8.3 Hz), 7.43 (d, 1.2H, J = 7.1 Hz), 7.35−7.18 (m, 10H), 6.82 (d, 1.6H, J = 9.0 Hz), 6.81 (d, 2.4H, J = 9.0 Hz), 6.34 (dd, 0.4H, J = 8.1, 5.9 Hz), 6.20 (d, 0.6H, J = 7.8, 5.9 Hz), 5.09 (d, 0.4H, J = 3.6 Hz), 4.93 (d, 0.6H, J =

3.6 Hz), 4.41−4.12 (m, 2H), 3.80 (s, 3.6H), 3.79 (s, 2.4H), 3.78−3.64 (m, 1H), 3.62−3.39 (m, 4H), 3.24−3.19 (m, 1H), 3.10−3.05 (m, 1H), 2.66−2.29 (m, 3H), 1.14 (d, 2H, J = 6.9 Hz), 1.08 (d, 4H, J = 6.9 Hz), 0.91 (d, 2H, J = 6.9 Hz), 0.88 (d, 4H, J = 6.9 Hz). ^{31}P NMR (161 MHz, $CDCl_3$) δ 150.2, 149.5. FTIR (film) 3184, 3067, 2966, 2835, 1704, 1693, 1607, 1510, 1447 cm^{-1}. ESIMS (*m*/*z*) 949, 951 $[M + Na]^+$.

Synthesis of *p*Br-WNA-βT (Scheme 2) (1*RS*,2*R*,3*R*,4*R*)-1-*p*-Bromophenyl-5-*O*-(*tert*-butyldiphenylsilyl)-2,3-*O*-isopropylideneribose (23(*p*)). A solution of *n*-BuLi (1.6 M in hexane, 9.35 mL, 15.0 mmol) was added slowly to a solution of *p*-dibromobenzene (3.5 g, 15.0 mmol) in THF (30 mL) at −78 °C in portions. After stirring for 1 h at −78 °C, a solution of **22** (4.3 g, 10.0 mmol) in THF (30 mL) was added to the mixture. The reaction mixture was stirred for 2 h, allowed to warm to 0 °C, quenched with saturated NH_4Cl solution, and extracted with AcOEt. The organic layer was successively washed with water and brine, dried over Na_2SO_4, and evaporated. The residue was purified by flash chromatography (silica gel, hexane/AcOEt = 9:1) to give **23(*p*)** as a colorless oil (5.1 g, 8.7 mmol, 87%). ^{1}H NMR (400 MHz, $CDCl_3$/TMS) δ 7.71−7.65 (m, 4H), 7.48−7.40 (m, 10H), 4.90 (d, 0.7H, J = 5.6 Hz), 4.84 (d, 0.3H, J = 6.7 Hz), 4.46 (d, 1H, J = 6.7 Hz), 4.49−4.31 (m, 1H), 3.94 (dd, 0.7H, J = 11.2, 3.4 Hz), 3.84 (dd, 0.3H, J = 10.8, 4.9 Hz), 3.76 (dd, 0.7H, J = 11.2, 3.4 Hz), 3.70 (dd, 0.3H, J = 10.8, 4.9 Hz), 1.37 (s, 3H), 1.24 (s, 3H), 1.12 (s, 9H). FTIR (film) 3350 cm^{-1}. HRMS (ESIMS) *m*/*z* calcd for $C_{30}H_{34}O_4BrSi$ $(M - OH)^+$ 565.1404, 567.1389, found 565.1404, 567.1353.

(1*R*,3*R*,4*R*,5*R*,7*RS*)-1-*p*-Bromophenyl-3-(*tert*-butyldiphenylsilyloxymethyl)-4,7-diacetoxy-2,6-dioxabicyclo[3.3.0]octane (26(*p*)). A solution of **25(*p*)** (5.0 g, 8.6 mmol) and allyltrimethylsilane (3.33 mL, 20.5 mmol) in CH_3NO_2 (24 mL) was added to a suspension of zinc bromide (6.6 g, 29 mmol) in CH_3NO_2 (24 mL) at 0 °C. After stirring for 2 h at room temperature, the reaction mixture was quenched with saturated $NaHCO_3$ solution and extracted with EtOAc. The organic layer was successively washed with water and brine, dried over Na_2SO_4, and then evaporated. The residue was purified by flash chromatography (silica gel, hexane/EtOAc = 9:1) to give the corresponding allyated product as a colorless oil (α- and β-isomer mixtures, 4.59 g, 7.57 mmol, 88%). Aqueous solutions of OsO_4 (0.131 M, 9.2 mL, 1.21 mmol) and $NaIO_4$ (0.6 M, 50.5 mL, 30.3 mmol) were added to a solution of the colorless oil (4.59 g, 7.57 mmol) in pyridine (50 mL), and the reaction mixture was stirred for 30 h at room temperature. The reaction mixture was diluted with AcOEt and successively washed with water and brine, dried over Na_2SO_4, and evaporated. A solution of the residue in THF (100 mL)/5% H_2SO_4 (30 mL) was stirred for 12 h at 60 °C, quenched by the addition of saturated $NaHCO_3$ solution, and extracted with AcOEt. The organic layer was successively washed with water and brine, dried over Na_2SO_4, and evaporated. The residue was purified by flash chromatography (silica gel, $CHCl_3$/AcOEt = 5:1) to give a colorless foam (908 mg, 1.59 mmol, 21%, for two steps). Acetic anhydride (0.25 mL, 2.8 mmol) was added to a solution of the colorless foam (400 mg, 0.7 mmol) in pyridine (3.4 mL) at 0 °C and stirred for 12 h at room temperature. The reaction mixture was diluted with EtOAc and successively washed with water and brine, dried over Na_2SO_4, and evaporated. The residue was purified by flash chromatography (silica gel, hexane/AcOEt = 6:1) to give **26(*p*)** as a colorless foam (375 mg, 0.57 mmol, 82%). ^{1}H NMR (400 MHz, $CDCl_3$/TMS) δ 7.72−7.63 (m, 5H), 7.54 (d, 1H, J = 8.6 Hz), 7.48−7.31 (m, 8H), 6.63 (dd, 0.7H, J = 5.7, 1.6 Hz), 6.50 (d, 0.3H, J = 5.8 Hz), 5.02 (dd, 1H, J = 9.6, 4.3 Hz), 4.89 (d, 0.3H, J = 4.9 Hz), 4.78 (d, 0.7H, J = 4.3 Hz), 4.52−4.50 (m, 0.3H), 4.23−4.19 (m, 0.7H), 4.07 (dd, 1H, J = 11.8, 2.4 Hz), 3.74 (dd, 1H, J = 11.8, 3.0 Hz), 2.84 (dd, 0.7H, J = 15.3, 5.7 Hz), 2.70 (dd, 0.3H, J = 15.0, 5.8 Hz), 2.59 (dd, 1H, J = 15.3, 1.6 Hz), 2.14 (s, 2H), 2.04 (s, 4H), 1.05 (s, 7H), 1.02 (s, 2H). FTIR (film) 2931, 2858, 1752, 1747, 1693, 1622, 1448, 1427 cm^{-1}. HRMS (ESIMS) *m*/*z* calcd for $C_{33}H_{37}O_7SiBrNa$ $(M + Na)^+$ 675.1384, 677.1370, found 675.1352, 677.1335.

(1′*S*,3′*R*,4′*R*,5′*R*,7′*S*)-{4′-Acetoxy-1′-*p*-Bromophenyl-3′-(*tert*-butyldiphenylsilyloxymethyl)-2′,6′-dioxabicyclo[3.3.0]oct-7′-yl}-thymine (*p*Br-WNA-βT, (29a(*p*)). *N*,*O*-Bis(trimethylsilyl)acetamide (BSA; 0.4 mL, 1.66 mmol) and TMSOTf (0.15 mL, 0.83 mmol) were added to a suspension of thymine (105 mg, 0.83 mmol) in CH_3CN (5 mL). A solution of **26(*p*)** (360 mg, 0.55 mmol) in CH_3CN (5 mL) was added to the above mixture. The reaction mixture was stirred at room temperature for 1 h, quenched with saturated $NaHCO_3$, and extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated. The isomers were separated by flash chromatography (silica gel, CH_3Cl/hexane/acetone = 2:4:1) to give each isomer in total 98% yield. *p*Br-WNA-βT **(29a(*p*))** as a colorless foam (215 mg, 0.30 mmol, 55%). ^{1}H NMR (400 MHz, $CDCl_3$/TMS) δ 8.46 (bs, 1H), 7.69−7.56 (m, 4H), 7.48−7.28 (m, 10H), 7.18 (s, 1H), 6.23 (dd, 1H, J = 8.4, 5.8 Hz), 5.08 (d, 1H, J = 4.1 Hz), 4.27−4.22 (m, 1H), 4.00 (dd, 1H, J = 11.6, 3.0 Hz), 3.71 (dd, 1H, J = 11.6, 3.4 Hz), 2.86 (dd, 1H, J = 14.0, 5.8 Hz), 2.55 (dd, 1H, J = 14.0, 8.4 Hz), 2.04 (s, 3H), 1.97 (s, 3H), 1.01 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.1, 163.3, 138.7, 135.8, 135.6, 133.0, 132.8, 131.7, 129.8, 127.8, 127.8, 127.1, 122.0, 111.5, 92.1, 88.8, 86.4, 80.8, 72.8, 62.3, 48.0, 26.8, 20.7, 19.1, 12.6. FTIR (film) 2931, 1682, 1674, 1651 cm^{-1}. HRMS (ESIMS) *m*/*z* calcd for $C_{36}H_{40}N_2O_7SiBr$ $(M + H)^+$ 719.1783, 721.1770, found 719.1734, 721.1807.

(1′*S*,3′*R*,4′*R*,5′*R*,7′*S*)-(1′-*p*-Bromophenyl-4′-hydroxy-3′-hydroxymethyl-2′,6′-dioxabicyclo[3.3.0]oct-7′-yl)-thymine (*p*Br-WNA-βT, 29b(*p*)). A colorless oil (72%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.70 (bs, 1H), 7.69 (s, 1H), 7.64 (d, 2H, J = 8.6 Hz), 7.49 (d, 2H, J = 8.6 Hz), 6.25 (dd, 1H, J = 8.0, 6.5 Hz), 4.01−3.98 (m, 1H), 3.91−3.87 (m, 2H), 3.70−3.61 (m, 2H), 2.73−2.72 (m, 2H), 1.92 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 166.4, 152.2, 141.1, 139.0, 132.4, 128.8, 122.5, 111.7, 92.8, 90.6, 90.4, 84.7, 73.4, 63.1, 49.2, 12.3. FTIR (film) 3430, 1660, 1633, 1550 cm^{-1}. ESIMS (*m*/*z*) 439, 441 $(M + H)^+$, HRMS (ESIMS) *m*/*z* calcd for $C_{18}H_{20}N_2O_6Br$ $(M + H)^+$ 439.0449, 441.0481, found 439.0458, 441.0507.

(1′*S*,3′*R*,4′*R*,5′*R*,7′*S*)-{1′-*p*-Bromophenyl-3′-dimethoxytrithylloxymethyl-4′-*O*-(*N*,*N*-diisopropyl-β-cyanoethylphosphoramidyl)-2′,6′-dioxabicyclo[3.3.0]oct-7′-yl}-thymine (*p*Br-WNA-βT, 29c(*p*)). A white powder (46%). ^{1}H NMR (400 MHz, $CDCl_3$/TMS) δ 8.21 (bs, 1H), 7.65−7.41 (m, 6H), 7.35−7.15 (m, 8H), 6.81 (d, 4H, J = 6.0 Hz), 6.33 (t, 0.5H, J = 8.2 Hz), 6.11 (t, 0.5H, J = 7.3 Hz), 5.03 (d, 0.5H, J = 3.2 Hz), 4.92 (d, 0.5H, J = 3.2 Hz), 4.36−4.34 (m, 0.5H), 4.32−4.25 (m, 1H), 4.13−4.11 (m, 4H), 3.81 (s, 3H), 3.80 (s, 3H), 3.79−3.71 (m, 2H), 3.57−3.44 (m, 4H), 3.20 (dd, 1H, J = 10.7, 4.3 Hz), 2.97−2.90 (m, 1H), 2.59−2.50 (m, 1H), 2.39−2.31 (m, 1H), 2.00 (s, 1.5H), 1.96 (s, 1.5H), 1.13 (d, 4H, J = 6.7 Hz), 1.10−1.05 (m, 6H), 0.92 (d, 2H, J = 6.7 Hz). ^{31}P NMR (161.9 MHz, $CDCl_3$) δ 150.0, 149.2. FTIR (film) 2359, 1693, 1681, 1674, 1564, 1486 cm^{-1}. ESIMS (*m*/*z*) 941, 943 $(M + H)^+$.

Synthesis of the TFO Containing the WNA Analog. The triplex-forming oligodeoxynucleotides incorporating the WNA analogue were synthesized by using an automated DNA synthesizer (Applied Biosystems 394 DNA/RNA synthesizer) according to the standard protocol except for the use of DCI as the activator. Cleavage and deprotection of the synthesized oligomer were done in 28% NH_4OH at 55 °C for 5 h. HPLC conditions: column, Nacalai Tesque COSMOSIL5C18-AR-II; buffer A, 0.1 M TEAA, B, CH_3CN. B: 10% to 40%/20 min, 40% to 100%/30min, linear gradient; flow rate, 4 mL/min. A peak appeared at around t_R = 16 min and was collected and freeze-dried. The DMTr protecting group was cleaved in 10% aqueous acetic acid at room temperature for 30 min, the resulting DMTr-OH was removed by washing with ether, and the solvents were lyophilized. Structure and purity of the synthesized TFO were confirmed by MALDI-TOF MS measurements (Table 2).

TABLE 2. MALDI-TOF MS (Negative Mode) of TFO Containing WNA Analogue (M^{-1} *m/z*)

Z = WNA	calcd	found	Z = WNA	calcd	found
TFO1 (AZG)			**TFO3** (AZA)		
WNA-βU	5844.03	5842.26	WNA-βU	5844.03	5843.78
WNA-βT	5858.29	5857.91	WNA-βT	5858.29	5857.72
WNA-βBrU	5921.94	5921.04	WNA-βBrU	5921.94	5922.41
WNA-βFU	5862.02	5866.30	WNA-βFU	5862.02	5860.52
WNA-βC	5843.04	5843.44	WNA-βC	5843.04	5844.84
WNA-β^mC	5857.06	5859.97	WNA-β^mC	5857.06	5853.65
WNA-βBrC	5920.95	5920.50	WNA-βBrC	5920.95	5918.61
WNA-βFC	5861.03	5861.15	WNA-βFC	5861.03	5859.10
*p*Br-WNA-βT	5935.95	5937.14	*p*Br-WNA-βT	5935.95	5938.08
*m*Br-WNA-βT	5935.95	5933.81	*m*Br-WNA-βT	5935.95	5933.81
*o*Br-WNA-βT	5935.95	5938.71	*o*Br-WNA-βT	5935.95	5935.56
*p*Br-WNA-βC	5921.94	5921.34	*p*Br-WNA-βC	5921.94	5918.89
*m*Br-WNA-βC	5921.94	5925.35	*m*Br-WNA-βC	5921.94	5923.99
*o*Br-WNA-βC	5921.94	5918.95	*o*Br-WNA-βC	5921.94	5923.44
TFO2 (GZG)			**TFO4** (AZA)		
WNA-βU	5860.02	5860.15	WNA-βU	5828.04	5826.65
WNA-βT	5874.28	5875.76	WNA-βT	5842.30	5841.49
WNA-βBrU	5937.93	5936.79	WNA-βBrU	5905.95	5902.48
WNA-βFU	5878.01	5875.63	WNA-βFU	5846.03	5846.71
WNA-βC	5859.02	5857.84	WNA-βC	5827.05	5825.48
WNA-β^mC	5873.05	5870.04	WNA-β^mC	5841.07	5843.50
WNA-βBrC	5936.94	5935.96	WNA-βBrC	5904.96	5904.55
WNA-βFC	5877.02	5877.23	WNA-βFC	5845.04	5844.31
*p*Br-WNA-βT	5951.94	5951.99	*p*Br-WNA-βT	5919.96	5922.69
*m*Br-WNA-βT	5951.94	5950.96	*m*Br-WNA-βT	5919.96	5915.59
*o*Br-WNA-βT	5951.94	5955.02	*o*Br-WNA-βT	5919.96	5919.44
*p*Br-WNA-βC	5937.93	5936.57	*p*Br-WNA-βC	5905.95	5903.39
*m*Br-WNA-βC	5937.93	5940.96	*m*Br-WNA-βC	5905.95	5908.38
*o*Br-WNA-βC	5937.93	5935.97	*o*Br-WNA-βC	5905.95	5902.85

Purification of the Target Duplex. A mixture containing equal amounts of the complementary oligodeoxynucleotides was heated at 95 °C for 10 min, 55 °C for 30 min, 40 °C for 30 min, and 25 °C for 30 min. The duplex was purified by HPLC (column, ZORBAX Oligo Column (6.2 mm i.d. × 80 mm, 5 μm); buffer A, 20% CH_3CN, 80% 0.02 M sodium phosphate (pH = 7.0); B, A + 1.0 M NaCl. B: 40% to 80%/15 min, 80% to 100%/20 min, linear gradient; flow rate, 1.0 mL/min) and by ethanol precipitation for desalination.

Gel-Shift Assay. TFOs were 5′ end-labeled by using [γ-^{32}P]-ATP (4000 Ci/mmol, ICN Biomedicals, Inc.) and T4 polynucleotide kinase (500 U, TAKARA Bio, Inc.), in T4 kinase buffer according to the standard protocol. After incubating for 45 min at 37 °C, 250 mM EDTA and TEN 100 buffer were added to the mixture, and then the mixture was purified with DE52 and DOWEX50. The purity of labeled TFO was checked by 15% denatured polyacrylamide gel in the presence of 10 M urea. The mixture of the TFO (10 nM) containing the corresponding ^{32}P-labeled TFO (40000 cpm) and target duplex (0−100 nM) was incubated in a buffer containing 20 mM Tris-HCl (pH = 7.5), 20 mM (or 5 mM) $MgCl_2$, 2.5 mM spermidine, and 10% sucrose for 12−15 h at 22 °C. The mixture was analyzed by electrophoresis with 15% nondenatured polyacrylamide gel at 10 °C for 6−7 h at 110 V. Gels were visualized by BAS2500 and each band was quantified. The stability constants (K_s) for each TFO were then calculated using the equation K_s = [triplex]/([duplex][TFO]), and averaged data from those obtained by multiple experiments are shown in Table 1.

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Supporting Information Available: Experimental detail. COSY and NOESY NMR spectra for determination of stereochemistry of glycosidic bond of WNA derivatives **16a**−**19a** and **29a**−**34a**. ^{1}H, ^{13}C spectra of new compounds **16a,b**−**19a,b**, **21b**, **29a,b**−**34a,b**. ^{31}P NMR spectra of amidite precursors **16c**−**19c**, **21c**, **29c**−**34c**. This material is available free of charge via the Internet at http://pubs.acs.org.

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