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W-shape nucleic acid (WNA) for selective formation of non-natural anti-parallel triplex including a TA interrupting site

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Abstract—Novel nucleoside analogs have been designed for selective formation of anti-parallel triplexes including a TA or a CG interrupting site. The new compounds are constructed of a W-shape bicyclic nucleic acid (WNA) bearing an aromatic ring as a stacking motif and a guanine for the formation of Hoogesteen hydrogen bonds, and are expected to effect triplex stabilization by both stacking and complementary hydrogen bonds. Purine-rich triplex-forming oligodeoxynucleotide (TFO) incorporating the new analog, **WNA-7βG**, formed a stable triplex with high selectivity to the TA site.

Since triplex formation within the major groove of duplex DNA has been proposed as a selective method for specific inhibition of gene expression at a predetermined sequence, a major concern has been to overcome the intrinsic limitation that triplexes are formed only toward homopurine–homopyrimidine sequences of the duplex.[1] In triplexes, pyrimidine oligonucleotides adopt parallel orientation and purine oligonucleotides tend to enjoy an anti-parallel orientation. In both cases, pyrimidine bases within the homopurine strand of the duplex inhibit triplex formation; therefore, efforts have been focused on the development of a non-natural base structure to stabilize triplexes at such interrupting sites.[2] Recently, interesting nucleoside analogs have been shown to stabilize triplexes by forming one hydrogen bond to the pyrimidine base at the interrupting site.[3] Nevertheless, triplex formation at any DNA sequence has remained a challenging theme.

In this approach, we focused on an anti-parallel triplex formed with purine-rich triplex-forming oligonucleotides (TFO's), because the anti-parallel triplexes are formed under physiological conditions with higher stability than the parallel ones with pyrimidine TFO's. A disadvantageous point of the anti-parallel triplex is that their formation is inhibited by physiological ionic con-

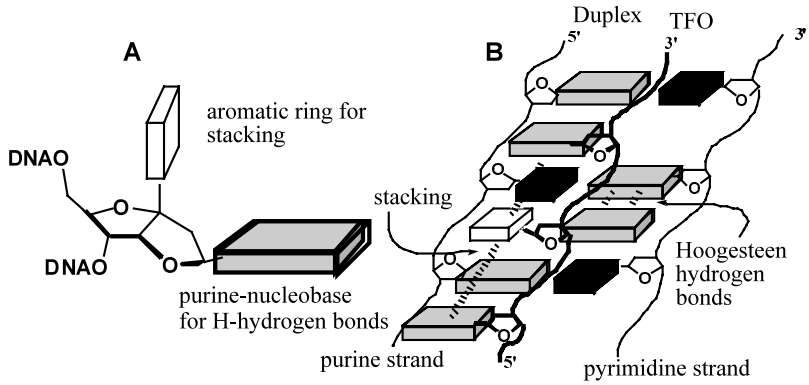

Figure 1. Schematic structure of the new bicyclic nucleoside analog, W-shape nucleoside analog (WNA); **A**, and expected role in the anti-parallel triplex; **B**.

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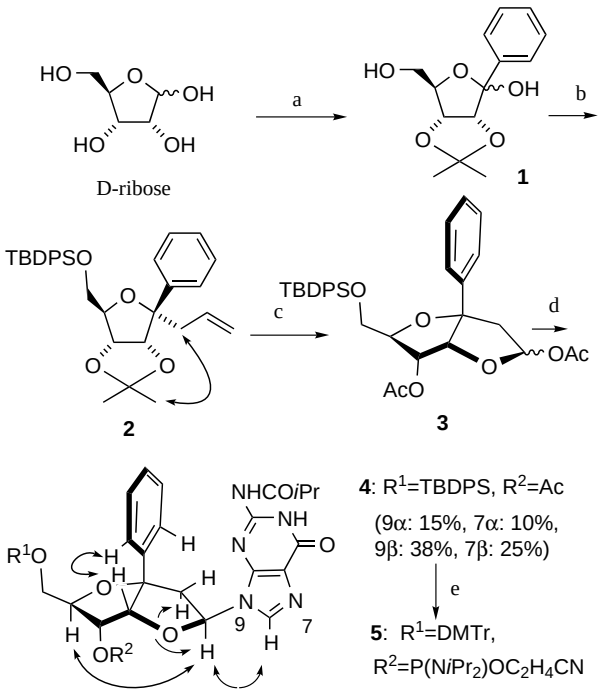
ditions, especially the presence of K^+;[4] nevertheless, purine-rich TFO's have been applied to a number of in vivo studies.[5]

We designed a new general structure of nucleoside analogs bearing an aromatic part for stacking and a purine base for Hoogesteen hydrogen bonds (Fig. 1A). The bicyclic structure might contribute to fix conformation of these two components, and was named the W-shaped nucleic acid (WNA) after its shape. In the anti-parallel orientation, the purine base is expected to form two Hoogesteen hydrogen bonds toward the target purine base within a pyrimidine strand of the duplex, and an aromatic may play a role to maintain the stacking interaction of the TFO continuously through the new nucleoside analog (Fig. 1B).

As the first concrete examples, we synthesized bicyclic nucleoside analogs bearing a benzene ring for the stacking part and guanine for the hydrogen bonding site (Scheme 1). The compound connecting a guanine at 9-*N* or 7-*N* with β-configuration is named WNA-9βG or WNA-7βG, respectively (**4**). The synthesis started with D-ribose (Scheme 1). Protection of the 2,3-dihydroxyl group with acetonide, acetylation of the residual 1,5-hydroxyl groups, selective deacetylation at the 1-position, oxidation of the 1-hydroxyl group to carbonyl, followed by addition of phenyllithium furnished



Scheme 1. (a) 1. Acetone, H^+, 2. Ac_2O, pyridine, 3. piperidine, THF (55%), 4. PCC, CH_2Cl_2, 5. PhLi, THF (53%); (b) 1. TBDPSCl, triazole, pyridine, 2. allyltrimethylsilane, $ZnBr_2$, CH_3NO_2, (44%, α-isomer in α:β = 7:6); (c) 1. OsO_4, $NaIO_4$, pyridine (45%), 2. 5% H_2SO_4, THF, 60°C, 3. Ac_2O, pyridine (57%); (d) 2*N*-isobutyryllguanine, BSA, TMSOTf, CH_3CN, 50°C; (e) 1. TBAF, THF, 2. 0.2 M NaOH, MeOH, 3. DMTrCl, pyridine, 4. *i*Pr_2NEt, CH_2Cl_2, ClP(N*i*Pr_2)-OC_2H_4CN, (57%).

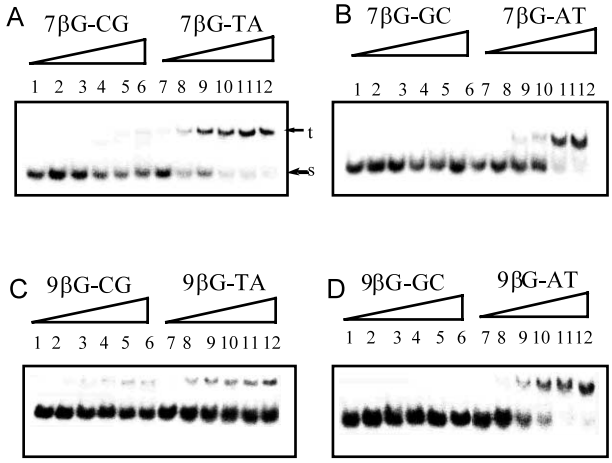


Figure 2. Gel shift assay for determination of triplex formation. Triplex formation was done for 12 h at 22°C in the buffer containing 20 mM Tris–HCl, 20 mM $MgCl_2$, 2.5 mM spermidine and 10% sucrose at pH 7.5. Electrophoresis was done at 10°C with 15% non-denatured polyacrylamide gel. A–D: 10 nM TFO containing the ^{32}P-labeled one as the tracer was used. The concentration of duplex was increased from lane 1 to 12 (nM); 10, 20, 40, 60, 80, 100, 10, 20, 40, 60, 80, 100. The combination of X-Y-Z is shown such as 7βG-CG.

TFO		3' GGAAGG A**X**G GAGGAGGGA 5'
	5'	GGGAGGGAGGGAAGG A**Y**G GAGGAGGGAAGC 3'
target duplex	3'	CCCGCCCTCCCTTCC T**Z**C CTCCTCCTCTCG 5'

introduction of the benzene ring (**1**). Protection of the 5-hydroxyl group and the following allylation at the 1-position gave **2** in a ratio of α:β = 7:6, from which the desired α-isomer was easily isolated. Subsequently, oxidative cleavage of the vinyl group and deprotection of 2,3-*O*-acetonide spontaneously provided hemiketal, then the formed hydroxyl groups were acetylated to afford **3**. *N*-Glycosidation with 2*N*-isobutyrylguanine produced a mixture of isomers in a ratio of 9α:7α:9β:7β = 17:11:44:28, which were easily isolated by flash chromatography.[6] The stereochemistry of **2** and WNA-9βG (**4**) was determined by ^{1}H COSY and NOESY as illustrated in Scheme 1.

The structures of other isomers were similarly determined. The phosphoramidite intermediates (**5**) were derived from **4** (WNA-9βG and WNA-7βG) by the conventional method, and applied to an automated DNA synthesizer. The oligodeoxynucleotide incorporating the WNA was cleaved from the resin with 30% NH_4OH and the DMTr-protected oligomer was purified by HPLC with an ODS column. As an ODS column is not suitable for purification of the non-protected TFO's used in this study, deprotection of DMTr and further purification was done by HPLC with a Poros column.[7] Incorporation of the WNA into the TFO was confirmed by MALDI-TOF MASS measurement.

The triplex-forming ability of TFO's incorporating the new WNA-9βG or 7βG was evaluated by gel shift assay

with non-denatured polyacrylamide gel (Fig. 2). As the purine-rich TFO tends to form disordered-aggregation,[8] the gel-shift assay with the labeled-duplex bands, in which relatively high concentration of the TFO is needed, did not produce reliable results. On the other hand, the gel-shift assay with the use of the labeled TFO at low concentrations gave reproducible results with better resolution between the single strand and the triplex.[8] The less-mobile bands were confirmed to those corresponding to triplexes. In this assay, selectivity of WNA-7βG and 9βG has been clearly demonstrated (Fig. 2A–D). The WNA-7βG exhibited selectivity to the TA pair, and WNA-9βG showed selective affinity to the AT pair. Affinity constants between the TFO and the duplex were obtained approximately by quantification of each band in Fig. 2 as follows; K_d (nM): 7βG-CG, >1000; **7βG-TA**, **25**, 7βG-GC, >1000; 7βG-AT, 65, in the series of WNA-7βG, and of 9βG-CG, >1000; 9βG-TA, >1000; 9βG-GC, >1000; **9βG-AT**, **55**, in the series of the **9β**-isomer. As the dissociation constant of $K_d = 1$ nM was obtained in the same gel shift assay with the natural triplex containing a G-GC combination at the XYZ site,[9] the triplex in the combination of 7βG-TA is slightly less stable than the natural triplex with G-GC. Thus, it has been demonstrated that the new nucleoside analog WNA-7βG exhibited selective triplex stabilization at a TA interrupting site with relatively high affinity.

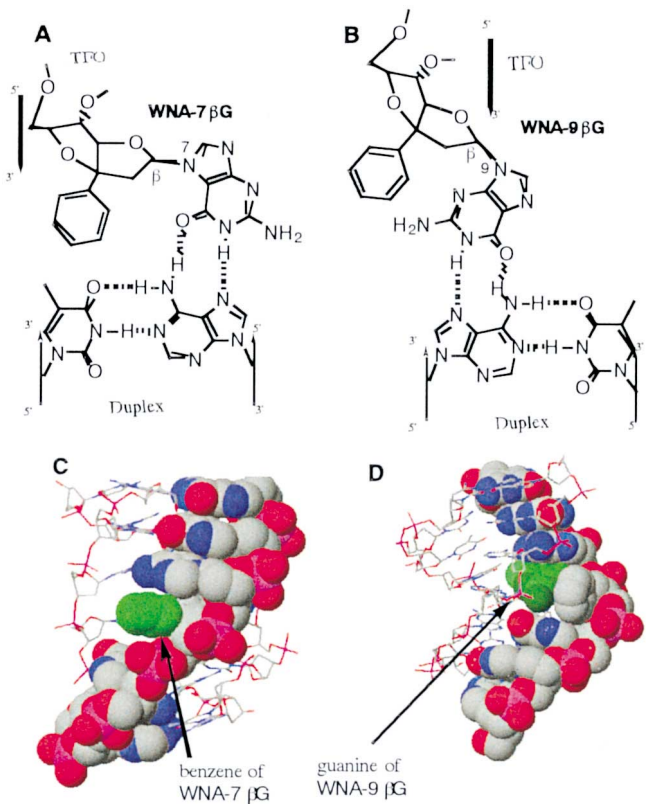


Figure 3. Plausible base triplet structures with WNA-7βG (A) and WNA-9βG (B). The limited region of triplex includine the WNA was subjected to MD calculation, and only the TFO is shown with Space-Filling Model in C and D. The benzene ring (in C) and the guanine (in D) are shown in green.

It has been suggested from a molecular modeling by MM and MD that the guanine base of WNA-7βG may form two hydrogen bonds with the adenine of the homopyrimidine strand within the duplex, and that the benzene ring may face the thymine as shown in Fig. 3A. In such a triplex structure, benzene was shown to be stacked by the two purine bases in the TFO to effect stacking interaction (Fig. 3C).[10] Stability and selectivity obtained with WNA-7βG may be due to the effect of both the hydrogen bonds and the stacking interaction. In a similar molecular modeling, the regioisomer WNA-9βG may form hydrogen bonds with adenine in the purine strand of the duplex (Fig. 3B). An MD calculation of the triplex containing WNA-9βG indicated that the guanine base rather than the benzene ring might be stacked between the other purine bases (Fig. 3D). The fact that different selectivity was obtained with the same WNA skeleton has suggested that the WNA skeleton would be useful as a common structure for the design of new nucleoside analogs.

In conclusion, we have designed new W-shaped nucleic acid derivatives (WNA) and discovered that WNA-7βG is a new candidate for the formation of non-natural type triplexes with high selectivity and affinity to a TA interrupting site. As the new structure of WNA would maintain stacking interaction with the benzene ring, its structure would provide a useful platform for the design of new nucleic acid analogs for the formation of triplexes at any sequence of the duplex.

Acknowledgements

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