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Triplex-forming enhancement with high sequence selectivity by single 2′-*O*,4′-*C*-methylene bridged nucleic acid (2′,4′-BNA) modification

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Abstract

Triplex-forming ability of the oligonucleotides containing one 2′-*O*,4′-*C*-methyleneribonucleic acid (2′,4′-BNA) unit was investigated by measurement of the melting temperature (T_m), and the 2′,4′-BNA modification promoted the marked triplex stabilization in a highly sequence-selective manner. © 2000 Elsevier Science Ltd. All rights reserved.

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Pyrimidine oligodeoxyribonucleotides are able to bind to homopurine sequences in dsDNA to form triplex DNA.[1] During the past few years, interest in the possibility of regulation of gene expression by triplex-forming oligonucleotides (TFOs) has grown because of their widely usable application as molecular biological tools and as a direct way to treat serious diseases, such as cancer and genetic disorders. Since the binding affinity of the natural pyrimidine oligonucleotides towards dsDNA is relatively low under physiological conditions, extensive efforts have been directed towards developing novel DNA and RNA analogues for the practical use of the antigene technologies and for clarification of the triplex structure.[2] However, only a few modified TFOs with sufficient binding affinity under physiological conditions have been reported.[2] It occurred to us that restriction of the oligonucleotide conformation in a suitable form would be quite effective for triplex formation, as well as binding with complementary ssDNA or ssRNA.[3] Despite their importance, there is only limited information available on the triplex-forming ability of the conformationally restricted or locked oligonucleotides.[3–5] Recently, we have achieved the first synthesis of a novel nucleoside with a fixed *N*-type conformation, 2′-*O*,4′-*C*-methyleneribonucleic acids (2′-*O*,4′-*C*-methylene bridged nucleic acid; 2′,4′-BNA) (**1**)[6]

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(see Fig. 1) and found that the BNA has extraordinary hybridizing ability towards complementary natural nucleic acids, especially towards complementary RNA.[7,8] In addition, we also found that partial 2′,4′-BNA modification of pyrimidine oligonucleotides promotes stable triplex formation at neutral pH.[4] Now, we deal with the triplex-forming ability and sequence selectivity of the oligonucleotides containing only one BNA monomer unit, and as a result, *N*-form sugar puckering was shown to play an important role in stable triplex formation.

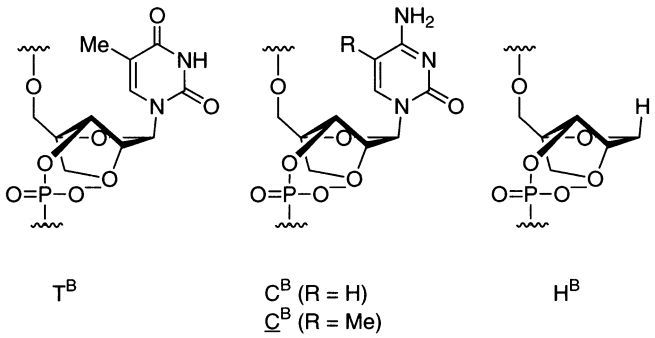
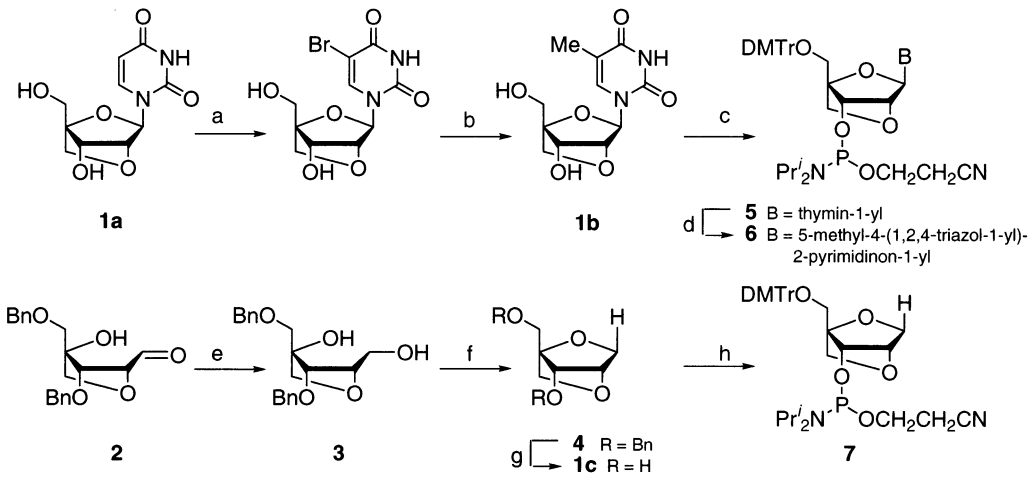


Figure 1. Structure of 2′,4′-BNA (2′-*O*,4′-*C*-methylene bridged nucleic acid)

As shown in Scheme 1, 2′-*O*,4′-*C*-methyleneuridine (**1a**)[6] was effectively converted to its 5-methyl congener **1b**† via 5-bromination and subsequent Pd-catalyzed cross-coupling reaction with trimethylaluminum.[11] As a control unit to investigate the effects of the fixed sugar moiety



Scheme 1. (a) NBS, NaN_3, 1,2-dimethoxyethane, 20°C, 84%. (b) (i) $(NH_4)_2SO_4$, 1,1,1,3,3,3-hexamethyldisilazane, reflux; (ii) $PdCl_2$, Ph_3P, Me_3Al, THF, reflux; (iii) NH_4Cl, MeOH–H_2O, reflux, 57%. (c) (i) DMTrCl, DMAP, pyridine, 20°C, 95%; (ii) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, diisopropylammonium tetrazolide, MeCN–THF, 20°C, 99%. (d) 1,2,4-Triazole, $POCl_3$, Et_3N, MeCN, 0°C, 91%. (e) $NaBH_4$, MeOH, 20°C, 95%. (f) 1,1′-azobis(*N*,*N*-Dimethylformamide), *n*-Bu_3P, CH_2Cl_2, 20°C, 95%. (g) 20% $Pd(OH)_2$–C, cyclohexene, EtOH, reflux, 68%. (h) (i) DMTrCl, pyridine, 20°C, 99%; (ii) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, diisopropylammonium tetrazolide, MeCN–THF, 20°C, 86%

† Recently, Wengel et al. reported the synthesis of **1b** from 4-*C*-hydroxymethylribofuranose derivative.[8,9] Independently, we also achieved the synthesis of **1** in a similar manner.[10]

on triplex formation, abasic analogue **1c** was also synthesized according to the literature[12] with some modifications. The aldehyde **2**[13,14] was reduced with $NaBH_4$ to afford the diol **3**, which was then cyclized under the Mitsunobu conditions to yield **4**. The abasic analogue **1c** was readily obtained by the hydrogenolysis of **4**.

The phosphoramidites **5** and **7**, suitable building blocks for DNA synthesis, were prepared by dimethoxytritylation and subsequent phosphitylation of **1b** and **1c**. According to Xu's procedure,[15] the amidite **5** was transformed into the 4-triazolo derivative **6**, which was easily converted into the 5-methylcytidine derivative by treatment with conc. ammonia after oligonucleotide synthesis. TFOs I and II containing one 2′,4′-BNA monomer were effectively prepared by the standard phosphoramidite protocol on a DNA synthesizer.‡

The triplex-forming property of TFOs I and II towards the target duplex 5′-d(GCTAAAAA-GAYAGAGAGATCG)-3′/3′-d(CGATTTTTCTZTCTCTCTAGC)-3′, in which Y·Z are all four natural base pairs, was studied by an analysis of the UV melting curve. At first, T_m measurements for TFO I were carried out in 7 mM sodium phosphate buffer (pH 6.0) containing 140 mM potassium chloride and 0.5 mM magnesium chloride (Table 1).[16] The T_m values for the modified TFOs I containing one BNA thymine monomer (T^B) or one BNA cytidine monomer (C^B) were compared with those for the TFO I having one BNA abasic monomer (H^B) or the unmodified reference sequences. The incorporation of BNA monomer T^B or C^B showed a significant enhancement of triplex-stability towards a matched duplex sequence with the ΔT_m values of +10 and +7°C [T_m values of triplexes were 54°C (X·Y·Z = T^B·A·T) and 44°C (T·A·T); 57°C (C^B·G·C) and 50°C (C·G·C)]. Furthermore, the sufficient sequence selectivity of the

Table 1
T_m values (°C) of triplexes involving TFO I[a]

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    5'-d(TTTTTCTXTCTCTCT)-3'    TFO I
5'-d(GCTAAAAAGAYAGAGAGATCG)-3'
3'-d(CGATTTTTCTZTCTCTCTAGC)-5'
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	Y·Z			
X	A·T	G·C	T·A	C·G
T^B	**54**	30	17	36
C^B	22	**57**	15	34
H^B	17	21	21	25
T	44	21	17	27
C	18	50	16	26

[a] UV melting profiles were measured in 7 mM sodium phosphate buffer (pH 6.0) containing 140 mM KCl and 0.5 mM $MgCl_2$ at a scan rate of 0.5°C/min at 260 nm. The first derivative was calculated from the UV melting profile, and the peak temperatures in the first derivative curve were designated as the melting temperature, T_m. The oligonucleotide concentration used was 1.5 μM for each strand.

‡ The BNA containing 2′-*O*,4′-*C*-methylenecytidine was prepared as described earlier.[7] The BNAs were synthesized on the DNA synthesizer (Gene Assembler® Plus, Pharmacia, 0.2 μmol scale, 5′-dimethoxytrityl on). After treatment with conc. ammonia, removal of the 5′-dimethoxytrityl group and purification were performed on NENSORB™ PREP reversed-phase columns. The purity of the BNAs was verified using reversed-phase HPLC and the compositions were determined by MALDI-TOF-MS.

modified TFOs I containing a BNA monomer was also confirmed from an observation of a large decrease in the T_m value when they hybridized with mismatched duplex sequences.

On the other hand, the T_m values of TFOs II, in which the cytosine nucleobases were replaced with 5-methylcytosine, were also elucidated in 7 mM sodium phosphate buffer (pH 7.0) containing 140 mM potassium chloride and 10 mM magnesium chloride. All T_m data are summarized in Table 2, and selected melting profiles are shown in Fig. 2. In analogy with the results of TFOs I, it was found that the incorporation of a BNA monomer shows remarkable triplex-stabilizing ability with sequence selectivity. Especially, T^B·A·T triad increased the T_m

Table 2
T_m values (°C) of triplexes involving TFO II[a]

5'-d(TTTTTC̲TXTC̲TC̲TC̲T)-3' TFO II
5'-d(GCTAAAAAGAYAGAGAGATCG)-3'
3'-d(CGATTTTTCTZTCTCTCTAGC)-5'

	Y·Z			
X	A·T	G·C	T·A	C·G
T^B	**57**	31	16	35
C^B	27	**53**	15	33
H^B	16	20	20	24
T	44	20	17	25
C̲[b]	18	43	16	25

[a] UV melting profiles were measured in 7 mM sodium phosphate buffer (pH 7.0) containing 140 mM KCl and 10 mM $MgCl_2$.

[b] C̲ = 2′-deoxy-5-methylcytidine.

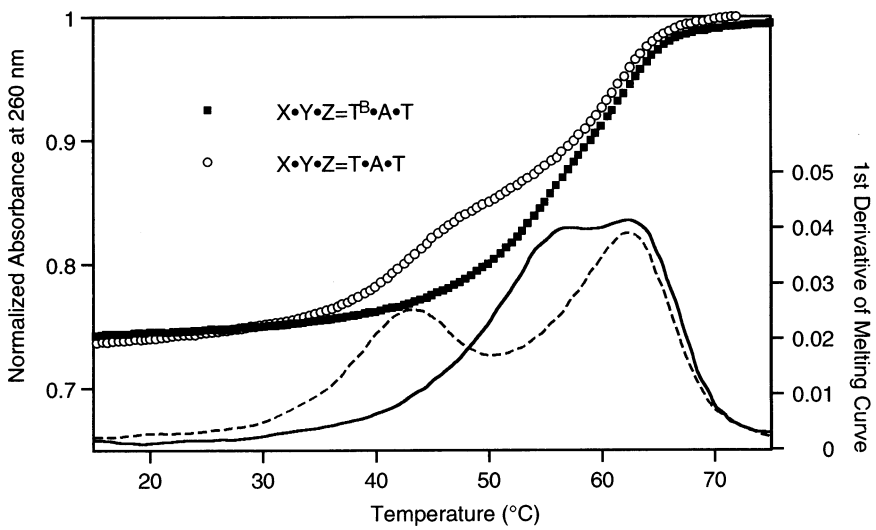

Figure 2. UV melting profiles (260 nm) for the triplexes involving TFO II (X·Y·Z = T^B·A·T, closed square; T·A·T, open circle) and first derivative of the melting curves (X·Y·Z = T^B·A·T, solid line; T·A·T, dashed line). The triplexes were melted at a scan rate of 0.5°C/min with detection at 260 nm. The normalized absorbance was obtained simply by dividing the observed absorbance by the absorbance at 80°C

value by +13 and +41°C, compared with T·A·T and H^B·A·T triads, respectively, which was an unprecedented result of triplex stabilization by an oligonucleotide analogue. This clearly indicates that both the bridged sugar moiety and a nucleobase were required for the efficient triplex stabilization, the same as for duplex stabilization.[12]

In conclusion, incorporation of single BNA unit in TFOs significantly enhanced triplex-forming ability with high sequence selectivity, which reveal that the BNA is a promising candidate not only for an antisense molecule[7,8,17] but also for a novel and practical antigene molecule.

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