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J. Am. Chem. Soc., **2008**, 130 (14), 4886-4896 • DOI: 10.1021/ja710342q

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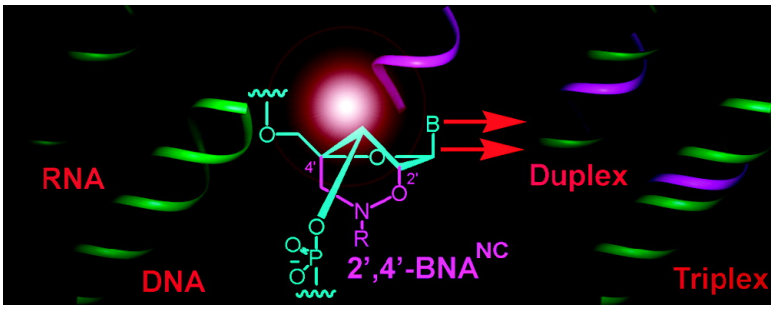

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Design, Synthesis, and Properties of 2′,4′-BNA^{NC}: A Bridged Nucleic Acid Analogue

S. M. Abdur Rahman,[†,§] Sayori Seki,[†] Satoshi Obika,[†,‡] Haruhisa Yoshikawa,[†] Kazuyuki Miyashita,[†] and Takeshi Imanishi*[,†]

Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan, and PRESTO, JST, Sanbancho Building, 5-Sanbancho, Chiyodaku, Tokyo 102-0075, Japan

Received November 22, 2007; E-mail: imanishi@phs.osaka-u.ac.jp

Abstract: The novel bridged nucleic-acid analogue 2′,4′-BNA^{NC} (2′-*O*,4′-*C*-aminomethylene bridged nucleic acid), containing a six-membered bridged structure with an N−O linkage, was designed and synthesized efficiently, demonstrating a one-pot intramolecular NC bond-forming key reaction to construct a perhydro-1,2-oxazine ring (**11** and **12**). Three monomers of 2′,4′-BNA^{NC} (2′,4′-BNA^{NC}[NH], [NMe], and [NBn]) were synthesized and incorporated into oligonucleotides, and their properties were investigated and compared with those of 2′,4′-BNA (LNA)-modified oligonucleotides. Compared to 2′,4′-BNA (LNA)-modified oligonucleotides, 2′,4′-BNA^{NC} congeners were found to possess: (i) equal or higher binding affinity against an RNA complement with excellent single-mismatch discriminating power, (ii) much better RNA selective binding, (iii) stronger and more sequence selective triplex-forming characters, and (iv) immensely higher nuclease resistance, even higher than the S_p-phosphorthioate analogue. 2′,4′-BNA^{NC}-modified oligonucleotides with these excellent profiles show great promise for applications in antisense and antigene technologies.

Introduction

Recently, the regulation of gene expression by chemically modified nucleic acids has attracted a great deal of attention from both chemists and biologists, and the use of chemically modified oligonucleotides in various gene silencing technologies such as antisense,[1] antigene,[2] and RNA interference[3] is growing steadily.[2,4,5] The first antisense drug, phosphorothioate (PS) oligonucleotide Vitravene (ISIS-2922), was approved by the FDA and appeared on the market in 1998 for the treatment of cytomegalovirus-induced retinitis;[6,7] currently phase III clinical trails are being conducted with several nucleic-acid analogues.[8] Apart from their use in therapy, modified oligonucleotides are also increasingly utilized in nucleic-acid nanotechnology,[9,10] oligonucleotide-based diagnostics,[11] gene-function determination, and drug-target validation.[12]

Despite the large number of chemically modified oligonucleotides developed over the last few decades,[4,13,14] the search for new molecules continues because most oligonucleotides developed to date have failed to give the desired response. A dramatic improvement in this area was achieved by including bridged nucleic acids (BNAs)[15] (often represented as locked nucleic acids[16,17]), in which the sugar conformation of the nucleotide(s) is locked by bridging. Among the structural analogues and configurational isomers of BNA developed to date,[15,17−31]

[†] Osaka University.
[‡] PRESTO, JST.
[§] On leave from Faculty of Pharmacy, University of Dhaka.

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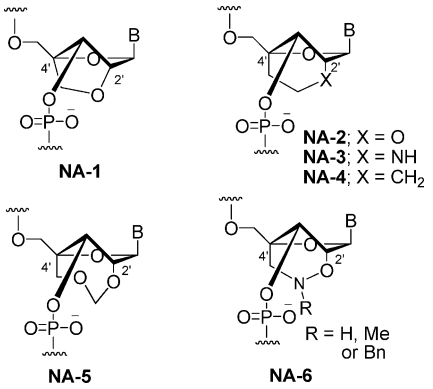

Figure 1. Structures of 2′,4′-linkage bridged nucleic acids.

2′-*O*,4′-*C*-methylene bridged nucleic acid (2′,4′-BNA,[18,19] also called LNA[16,17,20]), (**NA-1**, Figure 1), independently developed by Wengel et al. and us, has become extremely useful in nucleic-acid-based technologies.[32−43] The utility of this compound is due to its unprecedented hybridizing affinity for complementary strands (RNA and DNA), its sequence selectivity, its aqueous solubility, and its improved biostability compared to that of natural oligonucleotides. Because of its utility, 2′,4′-BNA (LNA) is now commercially available, and 2′,4′-BNA (LNA)-modified antisense oligonucleotides are entering human clinical trials.[44,45] Although genomics investigations using this molecule are vigorously expanding in a wide range of molecular biological technologies, it is clear that there is need for further development because: (i) the nuclease resistance of 2′,4′-BNA (LNA), although somewhat better than that of natural DNA, is significantly lower than that obtained by the PS oligonucleotide,[23,46] (ii) oligonucleotides either with consecutive 2′,4′-BNA (LNA) units or fully modified by this analogue are very rigid,[47,48] resulting in inefficient (or total failure of) triplex formation, and (iii) a kind of 2′,4′-BNA (LNA)-modified antisense oligonucleotides is shown to be hepatotoxic.[49]

In spite of these drawbacks, 2′,4′-BNA (LNA) remains the most promising bridged nucleic-acid derivative to date, although several similar modifications such as ENA (**NA-2**),[46,50] Aza-ENA (**NA-3**),[31] and **NA-4**[51] have been reported. We recently reported another BNA modification, 2′,4′-BNA^{COC} (**NA-5**), which dramatically improved nuclease resistance at the expense of decreased hybridizing ability.[52] Our continued efforts to engineer the BNA structure has resulted in the development of a novel BNA analogue, 2′,4′-BNA^{NC} (**NA-6**), which is more potent than 2′,4′-BNA (LNA).[53] Three structural analogues of 2′,4′-BNA^{NC}, namely, 2′,4′-BNA^{NC}[NH], [NMe], and [NBn], have been synthesized, and their biophysical properties and nuclease resistance were investigated. We recently communicated preliminary results regarding highly stable triplex formation by 2′,4′-BNA^{NC}[NH][53] and RNA-selective hybridization with the 2′,4′-BNA^{NC}[NMe] analogue.[54] Herein, we report full details regarding the development of all of the 2′,4′-BNA^{NC} analogues and compare their overall properties with those of 2′,4′-BNA (LNA).

Results and Discussion

1. Design of 2′,4′-BNA^{NC}. As described above, 2′,4′-BNA (LNA) having a five-membered bridged structure is insufficiently resistant to nucleases,[23,46] and fully modified 2′,4′-BNA (LNA) oligonucleotides do not have the flexibility required for efficient triplex formation.[47,48] To overcome these problems, BNA analogues with increased steric bulk and less conformational restriction were developed. 2′,4′-BNA^{COC} has a seven-membered bridged structure and exhibits dramatically improved nuclease resistance.[52] However, this modification conversely affects duplex stability (i.e., duplexes formed with this nucleic-acid analogue are less stable than those formed by 2′,4′-BNA (LNA)). On the other hand, ENA, which has a six-membered bridged structure, has slightly lower duplex-forming ability and significantly higher nuclease resistance than 2′,4′-BNA (LNA).[46,50] Triplex formation with ENA provided variable results compared to that of 2′,4′-BNA (LNA).[53,55] Taking into account the effect of the length of the bridged moiety, we designed a novel BNA, 2′,4′-BNA^{NC}, which has a six-membered bridged structure with a unique structural feature (N−O bond) in the sugar moiety. The bridged moiety was designed to have a nitrogen atom, which has proven importance in DNA chemistry such as: (i) acting as a conjugation site[56] and (ii) improvement of duplex and triplex stability by lowering repulsions between the negatively charged backbone phosphates.[21,30,57−60] Being a

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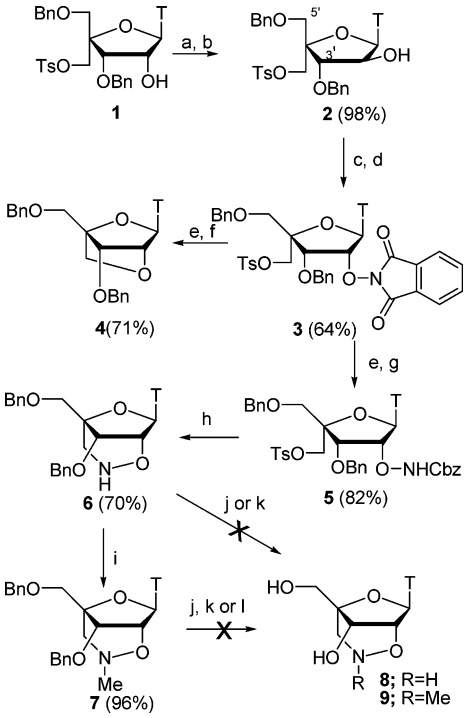
Scheme 1[a]

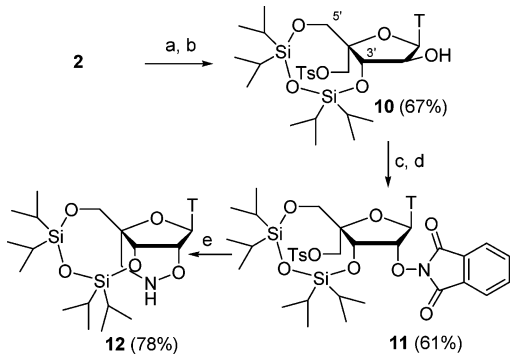


[a] Reaction Conditions: (a) MsCl, pyridine, room temperature, 1 h; (b) 1M NaOH, EtOH, room temperature, 1 h; (c) Tf_2O, DMAP, pyridine, room temperature, 4 h; (d) *N*-hydroxyphthalimide, DBU, MeCN, room temperature, 4 h; (e) $H_2NNH_2{\cdot}H_2O$, EtOH, 10 min; (f) 60% NaH, THF, 3 h; (g) Cbz-Cl, sat. $NaHCO_3$, CH_2Cl_2, 30 min; (h) 60% NaH, THF, 18 h; (i) PPTS, MeOH, HCHO, $NaBH_3CN$, 1 h; (j) $Pd(OH)_2$-C, cyclohexene, EtOH, reflux, 33 h; (k) 1M BCl_3/hexane, CH_2Cl_2, −78 °C; (l) 2,3-dichloro-5,6-dicyanobezoquinone (DDQ), CH_2Cl_2, reflux, 24 h. T = thymin-1-yl.

conjugation site, the nitrogen atom on the bridge can be functionalized by hydrophobic and hydrophilic groups, by steric bulk, or by appropriate functional moieties to: (i) control affinity toward complementary strands, (ii) regulate resistance against nuclease degradation, and (iii) allow synthesis of functional molecules designed for specific applications in genomics. In addition to the above possibilities, the N−O bond could be cleaved under appropriate reductive conditions to modulate the hybridizing properties of 2′,4′-BNA^{NC}.

2. Synthesis of 2′,4′-BNA^{NC}. 2.1. Synthesis of 2′,4′-BNA^{NC} Monomers and Phosphoroamidites. Our initial attempt to synthesize 2′,4′-BNA^{NC} monomers **8** and **9** through di-*O*-benzyl intermediate **6** was shown in Scheme 1. Nucleoside derivative **1**, synthesized by a modification of reported methods[61] (Supporting Information), was converted to a methyl sulfonic acid derivative, which was treated with an alkali to give alcohol **2** with an inverted stereochemical configuration in excellent yield. Alcohol **2** was reacted with trifluoromethanesulphonic anhydride (Tf_2O) in the presence of pyridine and dimethylaminopyridine (DMAP) to afford a triflate, which without purification, was subjected to S_N2 reaction with *N*-hydroxyphthalimide in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to afford **3**. Reaction of **3** with hydrazine monohydrate in ethanol gave free amine, the cyclization of which with 60% NaH unexpectedly yielded 2′,4′-BNA (LNA) nucleoside **4** exclusively instead of the desired perhydro-1,2-oxazine ring formation. On the other hand, the crude amine obtained from **3** was initially converted to the benzyl carbamate derivative **5**, which was then treated with NaH to furnish the desired cyclized product **6**. Debenzylation of **6** and its N−Me congener **7** (obtained by reductive methylation from **6**) under various conditions (i. $Pd(OH)_2$-C, cyclohexene, EtOH; ii. BCl_3, CH_2Cl_2, MeOH;[62] iii. DDQ, $CH_2Cl_2-H_2O$[63]) resulted in the failure to produce the desired respective monomers, **8** and **9**.

Scheme 2[a]



[a] Reaction Conditions: (a) 20% $Pd(OH)_2$-C, cyclohexene, EtOH, reflux, 22 h; (b) $TIPDSCl_2$, imidazole, DMF, room temperature, 5.5 h; (c) Tf_2O, DMAP, pyridine, room temperature, 7.5 h; (d) *N*-hydroxyphthalimide, DBU, MeCN, room temperature, 12 h; (e) H_2NNH_2, DABCO, pyridine, 40 h.

On the basis of these negative results, the *O*-benzyl groups at the 3′ and 5′ positions of **2** were replaced by the tetraisopropyldisiloxy group (**10** in Scheme 2). This was achieved by removing the benzyl groups by catalytic hydrogenolysis over $Pd(OH)_2$-C and silylating the C5′ and C3′ hydroxyls of the resultant triol selectively to give **10** in good yield. The 2′-hydroxyl of **10** was converted to a triflate by Tf_2O, pyridine, and DMAP, and subsequently transformed to phthalimide derivative **11** by the S_N2 reaction. Previously, we described the synthesis of 2′,4′-BNA^{NC}[NH] and 2′,4′-BNA^{NC}[NMe] monomers by deprotecting **11** to give the free amine,[53,54,64] cyclization of which could not be achieved directly to furnish **12**, and, therefore, we had to demonstrate different routes for the

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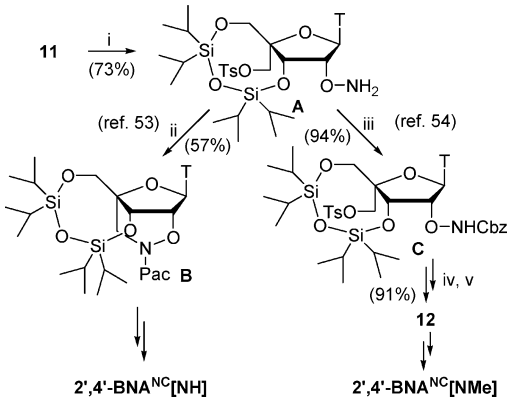
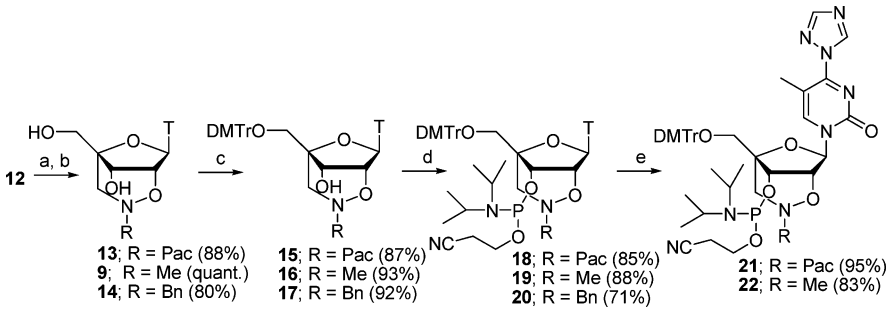
(64) Our initial synthetic routes for 2′,4′-BNA^{NC}[NH][53] and [NMe][54] analogues are shown here. Treatment of **11** with hydrazine monohydrate delivered the aminoxy derivative **A**, which was transformed to 2′,4′-BNA^{NC}[NH] intermediate **B** by a one-pot acylation/cyclization method or to benzyl carbamate derivative **C** for the synthesis of 2′,4′-BNA^{NC}[NMe] analogue. Reaction conditions: (i) $H_2NNH_2{\cdot}H_2O$, EtOH, rt, 15 min; (ii) $PhOCH_2COCl$ (PacCl), Et_3N, CH_2Cl_2, rt, 2 h; (iii) BnOCOCl(CbzCl), sat. $NaHCO_3$ (aq.), CH_2Cl_2, 0 °C; (iv) 60% NaH, THF, rt, 5 h; (v) BCl_3, CH_2Cl_2, 0 °C, 1 h.



Scheme 3[a]



[a] Reagents and Conditions: (a) PacCl, Et_3N, CH_2Cl_2, room temperature, 0.5 h (for synthesis of **13**)/20% aq. HCHO, $NaBH_3CN$, PPTS, MeOH, 0 °C, 1 h (for **9**)/BnBr, Et_3N, CH_2Cl_2, 9 h (for **14**); (b) TBAF, THF, room temperature, 5 to 10 min; (c) DMTrCl, pyridine, room temperature, 3.5 to 12 h; (d) $(^iPr_2N)_2PO(CH_2)_2CN$, dicyaniimidazole, MeCN, room temperature, 4 to 7 h; (e) 1,2,4-triazole, Et_3N, MeCN, 0 °C to room temperature, 5 to 8 h.

synthesis of 2′,4′-BNA^{NC}[NH][53] and [NMe] derivatives.[54,64] Whereas the synthesis of 2′,4′-BNA^{NC}[NH] intermediate (structure **B** in ref 64) was achieved in moderate yield by simultaneous acylation/cyclization using triethylamine and phenoxyacetyl chloride (PacCl), the synthesis of 2′,4′-BNA^{NC}[NMe] required a longer route via a benzyl carbamate derivative.[54,64] Gratifyingly, when **11** was treated with hydrazine monohydrate for a longer time, deprotection and cyclization occurred simultaneously to provide the desired **12** in low yield (Supporting Information). This one-pot reaction was further optimized by the addition of 1,4-diazabicyclo[2.2.2]octane (DABCO) and pyridine to give **12** from **11** directly in 78% yield (Scheme 2, and also see the Supporting Information for the optimized reaction conditions). It is noteworthy that this one-pot methodology greatly simplifies the synthesis of 2′,4′-BNA^{NC}, allowing its preparation on the multigram scale.

The synthesis of 2′,4′-BNA^{NC}[NH], [NMe], and [NBn] monomers and phosphoroamidites is shown in Scheme 3. For the successful incorporation into oligonucleotides, the 2′,4′-BNA^{NC}[NH] monomer was synthesized with the easily removable phenoxyacetyl (Pac) protecting group (**13**). Monomer **13** was synthesized in excellent yield from **12** by trapping the secondary nitrogen with PacCl and removing the silyl groups with tetrabutylammonium fluoride (TBAF). On the other hand, methylation of the nitrogen in **12** under reductive alkylation conditions and the same desilylation procedure furnished 2′,4′-BNA^{NC}[NMe] monomer **9** in quantitative yields. The structure of **9** was confirmed by ^{1}H NMR spectroscopy and X-ray crystallography (Figure 2 and Experimental Section). 2′,4′-BNA^{NC}[NBn] monomer **14** was synthesized from **12** via benzylation and desilylation. Because reductive alkylation and benzylation proceeded smoothly, it was anticipated that a wide range of *N*-substitution could be possible. To incorporate these synthetic 2′,4′-BNA^{NC} monomers (**13**, **9**, and **14**) into oligonucleotides, the primary hydroxyls of **13**, **9**, and **14** were selectively protected with the 4,4′-dimethoxytrityl group to give the trityl derivatives **15**, **16**, and **17**, respectively, in very good yields. The remaining secondary hydroxyls were then phosphitylated with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite to afford the 2′,4′-BNA^{NC}[NH]-, [NMe]-and [NBn]-thymine phosphoroamidites **18**, **19**, and **20**, respectively, each as a mixture of two stereoisomers. A portion of thymine phosphoroamidites **18** and **19** were converted to the triazole derivatives **21** and **22** (the precursors of the 2′,4′-BNA^{NC}[NH]- and [NMe]-5-methylcytosine phosphoroamidites), respectively, by the treatment of 1,2,4-triazole in the presence of triethylamine and phosphoryl chloride.[65]

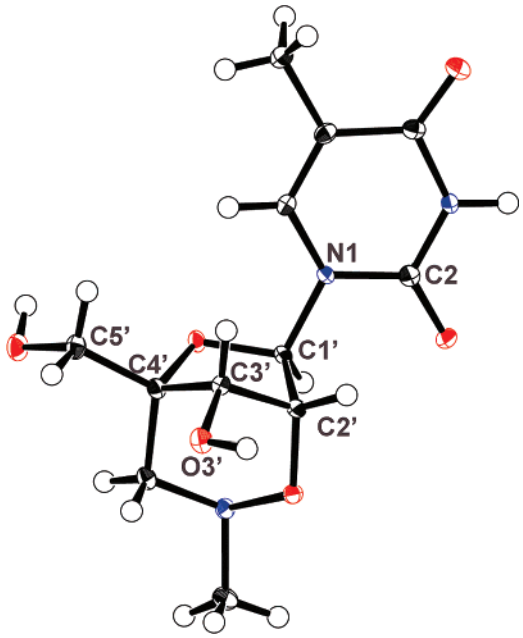


Figure 2. X-ray structure of **9**.

2.2. Conformational Analysis of 2′,4′-BNA^{NC}. The conformation of the sugar in 2′,4′-BNA^{NC} can be ascertained from the ^{1}H NMR spectra of **12**−**17**, as well as the ^{1}H NMR spectrum and crystal structure of 2′,4′-BNA^{NC}[NMe] monomer **9** (Figure 2, see also the Experimental Section). Non-observable coupling between the hydrogens at the C1′ and C2′ positions ($J_{1',2'} = 0$) in the ^{1}H NMR spectra of **9** and **12**−**17** strongly suggested the North-type (N-type) sugar pucker, as observed in 2′,4′-BNA (LNA).[18] The pseudorotation phase angle (*P*)[66] in the crystal structure of **9** was calculated to be 23.1°, which is close to those of 2′,4′-BNA (LNA), ENA, and 2′,4′-BNA^{COC} (17.4, 15.1, and 16.9°, respectively),[18,46,52] as compared in Table 1. This result indicates that the sugar puckering of 2′,4′-BNA^{NC} exists in the N-conformation and the *P* values of the above BNAs also fall in the C3′-endo conformation among the N-conformations.[66] The torsion angle (δ) and the maximum torsion angle or out of plane pucker (ν_{max}) of **9** ($\delta = 75.0°$, $\nu_{max} = 48.6°$), which are

(65) Scherr, M.; Klebba, C.; Häner, A.; Ganser, A.; Engels, J. W. *Biorg. Med. Chem. Lett.* **1997**, *7*, 1791.
(66) Saenger, W. *Principles of Nucleic Acid Structure*; Springer-Verlag: New York, 1984.

Table 1. Pseudorotaion Phase Angles (*P*) and Torsion Angles (δ and ν_{max}) of Different Bicyclic Nucleosides with 2′,4′-Linkage and Natural RNA

nucleosides	*P*	δ	ν_{max}
2′,4′-BNA (LNA) (**NA-1**)	17.4°	66.2°	56.6°
ENA (**NA-2**)	15.1°	75.7°	47.7°
2′,4′-BNA^{COC} (**NA-5**)	16.9°	77.7°	38.0°
2′,4′-BNA^{NC}[NMe] (**9**)	23.1°	75.0°	48.6°
A-type RNA	14°	83°	38°

5′-d(GCGTTTTTTGCT)-3′ (**23**)

5′-d(GCGTT**T**TTTGCT)-3′ (**24a -24d**)

5′-d(GCG**T**T**T**T**T**TGCT)-3′ (**25a -25d**)

5′-d(GCGTT**TTT**TGCT)-3′ (**26a -26d**)

5′-d(GCG**TTTTTT**GCT)-3′ (**27a -27d**)

5′-d(TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT)-3′ (**28**)

5′-d(TTTTT^{m}CT**T**T^{m}CT^{m}CT^{m}CT)-3′ (**29a - 29d**)

5′-d(TTTT**T**^{m}CT**T**T^{m}C**T**^{m}CT^{m}CT)-3′ (**30a - 30d**)

5′-d(TTTTT^{m}C**TTT**^{m}CT^{m}CT^{m}CT)-3′ (**31a - 31d**)

5′-d(**T**TTT**T**^{m}CTT**T**^{m}CT^{m}C**T**^{m}CT)-3′ (**32a**, **32b**, **32d**)

5′-d(T**T**TT**T**^{m}CT**T**T^{m}C**T**^{m}CT^{m}**C**T)-3′ (**33a**, **33b**, **33d**)

5′-d(**TTTT**^{m}C**T**TT^{m}C**T**^{m}C**T**^{m}CT)-3′ (**34a**, **34b**, **34d**)

5′-d(**TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT**)-3′ (**35a**, **35b**, **35d**)

5′-d(TTTTTTTT**T**T)-3′ (**36a** – **36d**)

Figure 3. Sequences of oligonucleotides used in this study. Underlined bold characters indicate the modified residues. Series **a**, **b**, **c**, and **d** represent 2′,4′-BNA^{NC}[NH]-, 2′,4′-BNA^{NC}[NMe]-, 2′,4′-BNA^{NC}[NBn]-, and 2′,4′-BNA (LNA)-modified oligonucleotides, respectively.

known to affect the helical structure of the duplex formed by oligonucleotides, are in the range of those of 2′,4′-BNA (LNA) (δ = 66.2°, ν_{max} = 56.6°),[18] ENA (δ = 75.7°, ν_{max} = 47.7°),[46] and 2′,4′-BNA^{COC} (δ = 77.7°, ν_{max} =38.0°).[52] These values further indicate that the conformation of **9** is restricted to the N-form seen in typical A-type RNA duplexes (P = 14°, δ = 83°, ν_{max} = 38°).[66] The observed variations of δ and ν_{max}, which indirectly indicate the degree of conformational restriction, are clearly related to the length of the bridged moiety.

2.3. Synthesis of Oligonucleotides 23−36d. Following a conventional phosphoroamidite protocol, a variety of modified oligonucleotides were synthesized using 2′,4′-BNA^{NC}-phosphoroamidites **18**−**22** and natural DNA amidite building blocks in an automated DNA synthesizer. A set of 2′,4′-BNA (LNA)-oligonucleotides with sequences identical to those of 2′,4′-BNA^{NC}-modified oligonucleotides was synthesized to allow direct comparison of their properties. The oligonucleotide sequences synthesized for the present study are provided in Figure 3. The coupling time for the modified oligonucleotides was 5−6 min, and 1*H*-terazole and activator-42 (purchased from Proligo) were used as activators. Coupling efficiency was determined by trityl monitors and was found to be 95 to 100%. Typical ammonia treatment cleaved the oligonucleotides from the solid support and removed the phenoxyacetyl groups (**24a**−**36a**). Simultaneously, the triazole group of (**33a**,**b** and **35a**,**b**) was converted to an amino group to give 2′,4′-BNA^{NC}-^{m}C-modified oligonucleotides. The oligonucleotides were purified by reverse phase HPLC (RP-HPLC) and characterized by MALDI-TOF mass spectroscopy (mass spectral data and yields of the oligonucleotides are provided in the Supporting Information).

3. Properties of 2′,4′-BNA^{NC}. 3.1. Duplex Formation and Thermal Stability of the Duplex Formed by 2′,4′-BNA^{NC}. Formation of stable duplexes with complementary single-stranded RNA (ssRNA) and single-stranded DNA (ssDNA) is essential for antisense and diagnostic applications. Duplex formation and the stability of duplexes formed by 2′,4′-BNA^{NC}-modified oligonucleotides **24a**−**c** to **27a**−**c** against complementary ssRNA **37** and ssDNA **38** (Tables 2 and 3) were examined by UV melting experiments (T_m measurement), and the results were compared with those obtained with natural and 2′,4′-BNA (LNA)-modified oligonucleotides **24d**-**27d**. The T_m values of the duplex formed between the complementary 12-mer ssRNA target (**37**) and oligonucleotides containing a single 2′,4′-BNA^{NC}[NH], [NMe] or [NBn] modification increased by 6 °C (2′,4′-BNA^{NC}[NH]) or 5 °C (2′,4′-BNA^{NC}[NMe] and [NBn]; Table 1) compared to that of natural oligonucleotide **23**. Further incremental increases in T_m were observed upon an increasing the number of modifications (oligonucleotides **25a**−**c** to **27a**−**c**). The increase in T_m per modification (ΔT_m/mod.) of 2′,4′-BNA^{NC}[NH], [NMe], and [NBn] ranges from 5.3 to 6.3 °C, 4.7 to 6.0 °C, and 4.7 to 6.0 °C, respectively, which is similar to that of 2′,4′-BNA (LNA) (ΔT_m/mod. = +5.0 to +7.0 °C). Interestingly, the T_m values of duplexes formed by 2′,4′-BNA^{NC}-[NH] oligonucleotides containing three or more modifications (**25a** to **27a**) are higher than those exhibited by the corresponding 2′,4′-BNA (LNA)-modified oligonucleotides (**25d**−**27d**). In addition, the T_m values of the duplexes formed with 2′,4′-BNA^{NC}[NMe] and [NBn] oligonucleotides, which have sterically hindered methyl or benzyl substituents (**25b**,**c** to **27b**,**c**), are similar to those obtained with 2′,4′-BNA (LNA). These results indicate that further modification of oligonucleotides with 2′,4′-BNA^{NC} should produce very stable duplexes with ssRNA and that oligonucleotides fully modified with 2′,4′-BNA^{NC}[NH] might form duplexes more stable than those obtained with fully modified 2′,4′-BNA (LNA) oligonucleotides.[16,17]

Affinity to the complementary 12-mer ssDNA target (**38**) was also investigated; the T_m values are summarized in Table 3. A single modification with 2′,4′-BNA^{NC}[NH] (**24a**) increased affinity to ssDNA only slightly (ΔT_m = +1 °C), whereas a single modification with 2′,4′-BNA^{NC}[NMe] and [NBn] (oligonucleotides **24b** and **24c**, respectively) decreased T_m by 1.0 and 2.0 °C, respectively, indicating that 2′,4′-BNA^{NC} is highly RNA-selective. By increasing the number of modifications to two 2′,4′-BNA^{NC}[NMe] units,[54] or three 2′,4′-BNA^{NC}[NMe] or [NBn] units (oligonucleotide **25b**,**c** and **26b**,**c**), affinity to ssDNA further decreased or remain unchanged, whereas modification with 2′,4′-BNA^{NC}[NH] and 2′,4′-BNA (LNA) units (oligonucleotides **25b**,**d** and **26b**,**d**) increased T_m compared to that of natural oligonucleotide **23**. The oligonucleotide with six 2′,4′-BNA^{NC}[NH] units (**27a**) showed a substantially increased T_m value (T_m = 73 °C; ΔT_m/mod. = +3.8 °C) presumably due to additional factors such as the effect of NH on stabilizing the duplex.[57−60]

The RNA selective binding affinity of 2′,4′-BNA^{NC} analogues was calculated from Tables 2 and 3, and the results are summarized in Table 4. The duplex formed by natural oligonucleotide **23** with the complementary ssRNA had a T_m value 5 °C lower than that obtained with the ssDNA complement. In contrast, increasing the number of 2′,4′-BNA^{NC} modifications gradually increased affinity for RNA, as shown by the ΔT_m

Table 2. T_m Values of Duplexes Formed by 2′,4′-BNA^{NC}- and 2′,4′-BNA (LNA)-Modified Oligonucleotides with Complementary ssRNA[a,b]

		T_m (ΔT_m/mod.) (°C)			
oligonucleotides	T =	2′,4′-BNA^{NC}[NH]	2′,4′-BNA^{NC}[NMe]	2′,4′-BNA^{NC}[NBn]	2′,4′-BNA (LNA)
d(GCGTTTTTTGCT) (**23**)		45	45	45	45
d(GCGTT**T**TTTGCT)(**24a**−**24d**)		51 (+6.0)	50 (+5.0)	50 (+5.0)	52 (+7.0)
d(GCG**TTTTT**GCT) (**25a**−**25d**)		64 (+6.3)	63 (+6.0)	60 (+5.0)	62 (+5.7)
d(GCGTT**TTT**GCT)(**26a**−**26d**)		61 (+5.3)	59 (+4.7)	59 (+4.7)	60 (+5.0)
d(GCG**TTTTTT**GCT) (**27a**−**27d**)		83 (+6.3)	80 (+5.8)	78 (+5.5)	80 (+5.8)

[a] Target ssRNA: 5′-r(AGCAAAAAACGC)-3′ (**37**). [b] Conditions: 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl; strand concentration = 4 μM.

Table 3. T_m Values of Duplexes Formed by 2′,4′-BNA^{NC}- and 2′,4′-BNA (LNA)-Modified Oligonucleotides with Complementary ssDNA[a,b]

		T_m (ΔT_m /mod.) (°C)			
oligonucleotides	T =	2′,4′-BNA^{NC}[NH]	2′,4′-BNA^{NC}[NMe]	2′,4′-BNA^{NC}[NBn]	2′,4′-BNA (LNA)
d(GCGTTTTTTGCT) (**23**)		50	50	50	50
d(GCGTT**T**TTTGCT)(**24a -24d**)		51 (+1.0)	49 (−1.0)	48 (−2.0)	53 (+3.0)
d(GCG**TTTTT**GCT) (**25a -25d**)		55 (+1.7)	51 (+0.3)	44 (−2.0)	56 (+2.0)
d(GCGTT**TTT**GCT)(**26a -26d**)		57 (+2.3)	50 (+0.0)	45 (−1.3)	54 (+1.3)
d(GCG**TTTTTT**GCT) (**27a -27d**)		73 (+3.8)	61 (+1.8)	58 (+1.3)	67 (+2.8)

[a] Target ssDNA: 5′-d(AGCAAAAAACGC)-3′ (**38**). [b] Conditions: 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl; strand concentration = 4 μM.

Table 4. Selective Binding Affinity to RNA by 2′,4′-BNA^{NC}- and 2′,4′-BNA (LNA)-Modified Oligonucleotides[a,b]

		$\Delta T_m = T_{m\,(ssRNA)} - T_{m\,(ssDNA)}$ (°C)			
oligonucleotides	T=	2′,4′-BNA^{NC}[NH]	2′,4′-BNA^{NC}[NMe]	2′,4′-BNA^{NC}[NBn]	2′,4′-BNA (LNA)
d(GCGTTTTTTGCT) (**23**)		−5	−5	−5	−5
d(GCGTT**T**TTTGCT)(**24a -24d**)		+0	+1	+2	−1
d(GCG**TTTTT**GCT) (**25a -25d**)		+9	+12	+16	+6
d(GCGTT**TTT**GCT)(**26a -26d**)		+4	+9	+14	+6
d(GCG**TTTTTT**GCT) (**27a -27d**)		+10	+19	+20	+13

[a] Target strands = RNA **37** and DNA **38**. [b] Conditions: 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl; strand concentration = 4 μM.

values (difference between affinities to RNA and DNA complements). Both 2′,4′-BNA^{NC}[NMe] and [NBn] displayed promising RNA selective binding affinity, superior to that of 2′,4′-BNA (LNA). Interestingly, the most prominent RNA selectivity was achieved with the 2′,4′-BNA^{NC}[NBn] analogue (RNA selectivity of 2′,4′-BNA^{NC}[NBn] is even higher than that of 2′,4′-BNA^{NC}[NMe][54]). Thus, modification with sterically hindered 2′,4′-BNA^{NC} analogues improves RNA selectivity, which could be very useful in antisense applications.[4] This increased affinity for RNA over DNA by 2′,4′-BNA^{NC} results from restricting the sugar conformation to the N-form,[15] as well as from unfavorable steric interactions with DNA strands.

3.2. Mismatch Discrimination Studies with 2′,4′-BNA^{NC}-Modified Oligonucleotides. Because 2′,4′-BNA^{NC}-modified oligonucleotides exhibited high-affinity sequence selective hybridization with ssRNA, their ability to discriminate bases was evaluated using single-mismatch ssRNA strands (Table 5). As indicated by the ΔT_m values in Table 5, any mismatched base in the target RNA strand resulted in a substantial decrease in the T_m of duplexes formed with 2′,4′-BNA^{NC}-modified oligonucleotides. For example, the ΔT_m values of duplexes formed with 2′,4′-BNA^{NC}[NH]-modified oligonucleotide **24a** having T−U, T−G, and T−C arrangements are −14, −5, −17 °C, respectively, which are lower than those of the corresponding natural DNA−RNA duplexes (ΔT_m = −12, −3, and −15 °C, respectively) and similar to those exhibited by duplexes formed with 2′,4′-BNA (LNA) oligonucleotide **24d** (ΔT_m = −13, −5, and −17 °C, respectively). The mismatch discrimination profiles of 2′,4′-BNA^{NC}[NMe] and [NBn] were also similar to that of 2′,4′-BNA (LNA), except in the case of the T−G arrangement for the 2′,4′-BNA^{NC}[NMe] derivative, as shown in Table 5. Thus, it appears that 2′,4′-BNA^{NC} not only exhibits high-affinity RNA selective hybridization, but is also highly selective in recognizing bases.

Table 5. T_m Values of Duplexes Formed by 2′,4′-BNA^{NC}- and 2′,4′-BNA (LNA)-Modified Oligonucleotides with Complementary ssRNA Containing a Single-Mismatch Base[a,b]

		T_m ($\Delta T_m = T_{m\,(mismatch)} - T_{m\,(match)}$) (°C)			
T (oligonucleotide)	X =	A (match)	U	G	C
natural (**23**)		45	33 (−12)	42 (−3)	30 (−15)
2′,4′-BNA^{NC}[NH] (**24a**)		51	37 (−14)	46 (−5)	34 (−17)
2′,4′-BNA^{NC}[NMe] (**24b**)		50	37 (−13)	48 (−2)	34 (−16)
2′,4′-BNA^{NC}[NBn] (**24c**)		50	36 (−14)	46 (−4)	33 (−17)
2′,4′-BNA (LNA) (**24d**)		52	39 (−13)	47 (−5)	35 (−17)

[a] Modified oligonucleotides, 5′-d(GCGTT**T**TTTGCT)-3′; Target ssRNA strand, 3′-r(CGCAA**X**AAACGA)-5′. [b] Conditions: 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl; strand concentration = 4 μM.

3.3. Helical Structure of 2′,4′-BNA^{NC}-Modified Oligonucleotide Duplex. To understand the helical structure of duplexes formed by 2′,4′-BNA^{NC}-modified oligonucleotides, duplexes containing 2′,4′-BNA^{NC}[NMe] and 2′,4′-BNA^{NC}[NBn] (Supporting Information) oligonucleotides were analyzed by CD spectroscopy and the spectra were compared with those of natural RNA/RNA (**39**/**37**), DNA/RNA (**23**/**37**), RNA/DNA (**39**/**38**), and DNA/DNA (**23**/**38**) duplexes (Figure 4 and Figure SI-1 in the Supporting Information). Generally, natural RNA/RNA duplexes are A-form duplexes exhibiting a positive cotton effect

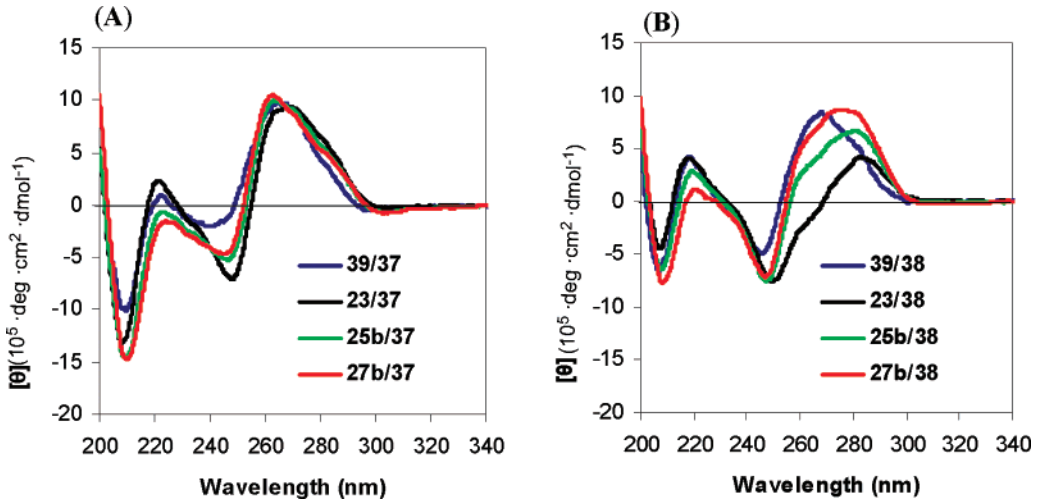


Figure 4. CD Spectra of duplexes formed by 2′,4′-BNA^{NC}[NMe]-modified oligonucleotides **25b** and **27b** with complementary RNA (**37**, part A) and DNA (**38**, part B). The spectra were compared with those of natural RNA duplex (**39**(5′-GCGUUUUUUGCT-3′)/**37**), DNA/RNA duplex (**23**/**37**), RNA/DNA duplex (**39**/**38**), and DNA duplex (**23**/**38**). Duplex concentration, 4μM in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl.

Table 6. T_m Values of Triplexes Formed by 2′,4′-BNA^{NC}- and 2′,4′-BNA (LNA)-Modified Oligonucleotides with Complementary dsDNA (in the Absence of Mg^{2+})[a,b]

oligonucleotides	T=	T_m (ΔT_m /mod.) (°C)			
		2′,4′-BNA^{NC}			2′,4′-BNA (LNA)
		[NH]	[NMe]	[NBn]	
d(TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT) (**28**)		33	33	33	33
d(TTTTT^{m}CT**T**T^{m}CT^{m}CT^{m}CT) (**29a - 29d**)		44 (+11.0)[c]	38 (+5.0)	33 (+0.0)	44 (+11.0)[c]
d(TTTTT^{m}CTT^{m}C**T**^{m}CT^{m}CT) (**30a - 30d**)		60 (+9.0)[c]	47 (+4.6)	32 (−0.3)	59 (+8.7)[c]
d(TTTTT^{m}C**TTT**^{m}CT^{m}CT^{m}CT) (**31a - 31d**)		59 (+8.7)[c]	42 (+3.0)	31 (−0.7)	52 (+6.3)[c]
d(**T**TTTT^{m}CTTT^{m}CT^{m}C**T**^{m}CT) (**32a**, **32b**, **32d**)		58 (+6.3)[c]	45 (+3.0)		57 (+6.0)[c]
d(**T**TTT**T**^{m}CTT^{m}CT^{m}CT^{m}CT) (**33a**, **33b**, **33d**)		64 (+6.2)[c]	50 (+3.4)		65 (+6.4)[c]
d(**TTTTT**^{m}CTTT^{m}C**T**^{m}CT^{m}CT) (**34a**, **34b**, **34d**)		78 (+6.4)[c]	59 (+3.7)		67 (+4.9)[c]
d(**TTTTT**^{m}CTTT^{m}CT^{m}CT^{m}CT) (**35a**, **35b**, **35d**)		80 (+3.1)[c]	<5 (<−2)		<5 (<−2)[c]

[a] Target dsDNA: 5′-d(GCTAAAAAGAAAGAGAGATCG)-3′/3′-d(CGATTTTTCTTTCTCTCTAGC)-5′; underlined portion indicates the target site for triplex formation. [b] Conditions: 7 mM sodium phosphate buffer (pH 7.0) containing 140 mM KCl; strand concentration = 1.5 μM. [c] Data from ref 53.

between 260 and 280 nm and a negative cotton band around 210 nm.[67] In contrast, natural DNA/DNA duplexes have a B-form helix with positive cotton bands around 280 and 220 nm and a negative cotton band around 240 nm. As described above, a control RNA/RNA duplex showed a typical A-form spectrum (part A of Figure 4) and a DNA/DNA duplex provided a B-form spectrum (part B of Figure 4). Minimal spectral changes were observed in the duplex formed by 2′,4′-BNA^{NC}-[NMe]-modified oligonucleotides **25b** and **27b** with RNA **37** compared with a natural RNA/RNA (**39**/**37**) duplex. Both **25b** and **27b** produced similar spectra, although the intensity of the cotton band at 220 nm was a little lower than that of the RNA/RNA duplex. Compared with DNA/RNA duplex **23**/**37**, the cotton bands near 220 and 240 nm were slightly decreased in intensity, similar to that observed for the A-form RNA duplex (**39**/**37**).

In contrast, there is a remarkable spectral change in duplexes formed with DNA complement (part B of Figure 4). The intensity of the cotton band at 220 nm was decreased, whereas that of the 280 nm band was increased, similar to that observed for natural RNA/DNA duplex (**39**/**38**). A larger effect was observed with oligonucleotide **27b** containing six 2′,4′-BNA^{NC}-[NMe] units. These observations indicate that the introduction of 2′,4′-BNA^{NC} modifications change a typical B-form helical structure of DNA/DNA (**23**/**38**) duplex to an A-form helix, that is, the A-form helical character is strengthened by the introduction of 2′,4′-BNA^{NC}-modified oligonucleotides. This change in helical conformation is similar to that obtained with 2′,4′-BNA (LNA),[19] which results from the conformational restriction of the sugar moiety to the N-form. The tendency of 2′,4′-BNA^{NC} duplexes to adopt the A-form is the primary reason for the high affinity of 2′,4′-BNA^{NC} oligonucleotides for RNA over DNA.

3.4. Triplex Formation and Triplex Stability. Applications in antigene[2] and gene repair technologies[68,69] require the formation of stable triplexes at physiological pH. In our preliminary report,[53] we showed that 2′,4′-BNA^{NC}[NH] has superior triplex-forming characteristics compared to those of 2′,4′-BNA (LNA) and ENA. We here disclose full details of triplex formation by the three 2′,4′-BNA^{NC} analogues (2′,4′-BNA^{NC}[NH], [NMe], and [NBn]) in the absence and presence of divalent metal (Mg^{2+}). The triplex-forming ability of the 2′,4′-BNA^{NC} analogues against a 21 bp double-stranded DNA (dsDNA) in the absence of divalent metal is summarized in Table 6. A single modification of the triplex-forming oligonucleotide (TFO) **28** with 2′,4′-BNA^{NC}[NH] (TFO **29a**) increased the T_m of the triplex by 11 °C, which is equal to that

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Table 7. T_m Values of Triplexes Formed by 2′,4′-BNA^{NC}- and 2′,4′-BNA (LNA)-Modified Oligonucleotides with Complementary dsDNA in the Presence of Mg^{2+} [a,b]

oligonucleotides	T=	T_m (ΔT_m/mod.) (°C)			
		2′,4′-BNA^{NC}			2′,4′-BNA (LNA)
		[NH]	[NMe]	[NBn]	
d(TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT) (**28**)		43	43	43	43
d(TTTTT^{m}CT**T**T^{m}CT^{m}CT^{m}CT) (**29a - 29d**)		55 (+12.0)	49 (+6.0)	44 (+1.0)	55 (+12.0)
d(TTTT**T**^{m}CT**T**T^{m}C**T**^{m}CT^{m}CT) (**30a - 30d**)		73 (+10.0)	61 (+6.0)	45 (+0.7)	72 (+9.7)
d(TTTTT^{m}C**TTT**^{m}CT^{m}CT^{m}CT) (**31a - 31d**)		71 (+9.3)	54 (+3.6)	44 (+0.3)	64 (+7.0)

[a] Target dsDNA: 5′-d(GCT*AAAAAGAAAGAGAGA*TCG)-3′/3′-d(CGATTTTTCTTTCTCTCTAGC)-5′; underlined portion indicates the target site for triplex formation. [b] Conditions: 7 mM sodium phosphate buffer (pH 7.0) containing 140 mM KCl and 10 mM $MgCl_2$; strand concentration = 1.5 μM.

exhibited by the triplex of 2′,4′-BNA (LNA)-modified TFO **29d**. The T_m of the triplex formed by the corresponding 2′,4′-BNA^{NC}-[NMe]-TFO **29b** is 5 °C higher than that of natural-TFO **28**, and that of 2′,4′-BNA^{NC}[NBn]-TFO **29c** is equal to that of the natural oligonucleotide. In the case of 2′,4′-BNA^{NC}[NH], increasing the number of modifications (TFOs **30a**−**34a**) greatly enhanced triplex thermal stability (ΔT_m/mod. = +6.2 to +9 °C), which is similar to or even higher than that of the corresponding 2′,4′-BNA (LNA)-TFOs (**30d**−**34d**; ΔT_m/mod. = +4.9 to +8.7 °C). The thermal stability of the triplex formed by the corresponding 2′,4′-BNA^{NC}[NMe]-TFOs (**30b**−**34b**), although significantly higher than that obtained with natural DNA-TFO **28**, is lower than that of 2′,4′-BNA^{NC}[NH]- and 2′,4′-BNA (LNA)-TFOs (**31a**,**d**−**35a**,**d**). Modification with 2′,4′-BNA^{NC}-[NBn] units (**29c**−**31c**) did not affect the thermal stability of the triplex; that is, the thermal stability was similar to that of natural DNA-TFO, **28**. The lower stability of the 2′,4′-BNA^{NC}-[NMe] and [NBn] triplexes might be due to unfavorable steric interaction with dsDNA, induced by the methyl or benzyl groups. Although this steric bulk is well tolerated in duplex formation, it appears to be unsuitable in the triplex, where the combined structural features are more complex and the accommodation of steric bulk is difficult.[68] The most striking behavior shown by 2′,4′-BNA^{NC}[NH] is that consecutive modification (as in TFO **31a**) with 2′,4′-BNA^{NC}[NH] does not affect triplex stability, as shown by the T_m of the triplex formed by **31a** (T_m value 59 °C, which is as high as that obtained with TFO **30a** containing interrupted modifications). In contrast, there is significant reduction in the T_m value of the triplex of 2′,4′-BNA (LNA)-modified TFO **31d** compared to the triplex of TFO **30d**, which has modifications separated by natural DNA units. A previous report described the more drastic reduction in T_m value of a 2′,4′-BNA (LNA)-TFO having four consecutive 2′,4′-BNA (LNA) modifications (ΔT_m/mod. = +1 °C only) compared to that obtained by alternating 2′,4′-BNA (LNA)-substitutions (ΔT_m/mod. = +4.5 °C).[70] Not only the consecutively modified TFO **31a** but also the extensively modified 2′,4′-BNA^{NC}[NH]-TFO **34a** showed a T_m value 11 °C higher than that of the corresponding 2′,4′-BNA (LNA)-TFO **34d**. In fact, in the case of 2′,4′-BNA (LNA), increasing the number of modifications from five (TFO **33d**) to seven (TFO **34d**) did not improve triplex stability significantly (T_m = 65 and 67 °C for TFOs **33d** and **34d**, respectively), consistent with our previous observations with 2′,4′-BNA (LNA).[71] In contrast, T_m improved markedly (14 °C) by increasing the number of modifications from five to seven with 2′,4′-BNA^{NC}[NH] residue (compare T_m values of TFOs **33a** and **34a**). Most interestingly, a fully modified 2′,4′-BNA^{NC}[NH]-TFO (**35a**) formed a stable triplex with a T_m value as high as 80 °C, whereas the corresponding 2′,4′-BNA (LNA)- and 2′,4′-BNA^{NC}[NMe]-TFOs (**35d** and **35b**, respectively) were unable to interact with the dsDNA. The same negative result was obtained previously with fully modified 2′,4′-BNA (LNA) oligonucleotides.[47,70] The inability of fully modified 2′,4′-BNA^{NC}[NMe] to form a stable triplex is probably due to steric factors, and in the case of 2′,4′-BNA (LNA) it is possibly due to the over-rigidity of its overall structure.[47,48,70] It is therefore noteworthy that modifications with 2′,4′-BNA^{NC}[NH] eliminate the limitations of placing alternating DNA monomers (using one 2′,4′-BNA (LNA) for every two or three DNA nucleotides)[70] for optimum triplex stability.

As it is well-known that the triplex is stabilized by multivalent metal cations (such as Mg^{2+}),[68] and T_m values in the presence of physiological concentrations of Mg^{2+} were measured (Table 7). Further incremental increases in T_m values were observed, as shown in Table 7. As expected, a similar variation in triplex stability was observed in triplexes formed by TFOs **31a** and **31d** with three consecutive 2′,4′-BNA^{NC}[NH] and 2′,4′-BNA (LNA) residues, respectively.

The extraordinarily high triplex stability exhibited by 2′,4′-BNA^{NC}[NH]-TFOs prompted us to find out possible reasons for its improved hybridization. Stabilization of the duplex and triplex structures by modified oligonucleotides containing amino nitrogen was reported to be accomplished by forming positively charged nitrogen atoms via protonation.[2,57−60] This positively charged nitrogen can stabilize duplexes and triplexes either by electrostatic interactions with the negatively charged phosphate strands or by reducing the repulsion between the phosphate backbone. To understand whether protonation of the amino nitrogen of 2′,4′-BNA^{NC}[NH] is possible or not at the experimental pH (pH 7.0−7.2), we conducted pH titration experiments for measuring the pKa of 2′,4′-BNA^{NC}[NH] monomer with a free N−H group on the bridged moiety.[72] The pKa was found to be less than 3.0 (Supporting Information for pH titration curves), which is even lower than that reported for the simple methylhydroxylamines.[73] This means that protonation of the

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(71) In our previous investigation, TFOs modified with five or seven 2′,4′-BNA (LNA) residues showed essentially similar T_m values. See, for example, Torigoe, H.; Hari, Y.; Sekiguchi, M.; Obika, S.; Imanishi, T. *J. Biol. Chem.* **2001**, *276*, 2354.

(72) The 2′,4′-BNA^{NC}[NH] monomer with the free N−H group (not the monomer **13** containing a Pac group, used for oligomerization) was synthesized from **12** by desilylation with 46% HF in acetonitrile.

(73) For usual pKa values of amines, hydroxylamines, and methylhydroxylamines, see, for example, Bissot, T. C.; Parry, R. W.; Campbell, D. H. *J. Am. Chem Soc.* **1957**, *79*, 796.

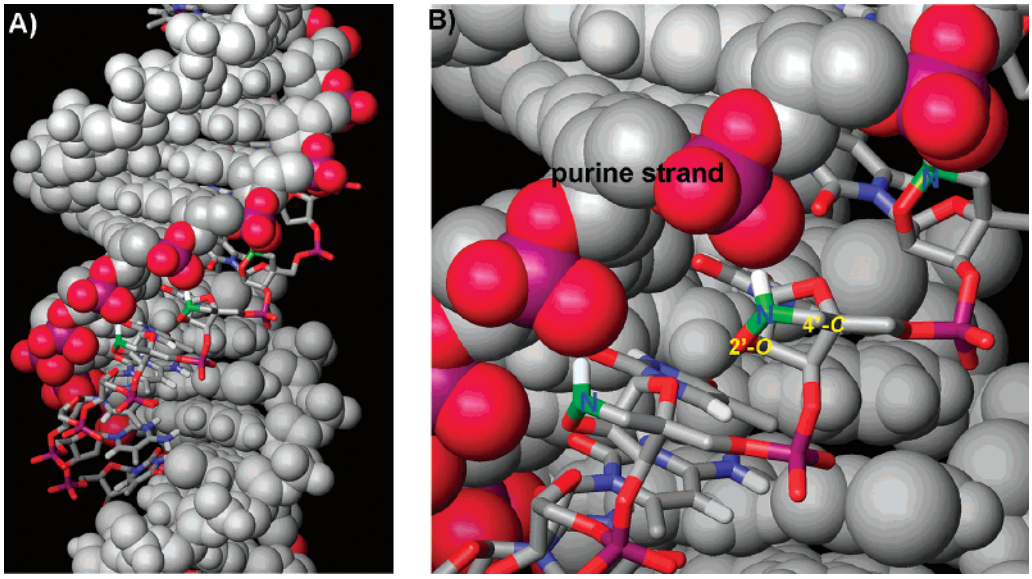

Figure 5. Molecular modeling of a parallel-motif triplex formed by 2′,4′-BNA^{NC}[NH]-TFO **31a** with dsDNA. (A) Overall view of the triplex: the dsDNA is shown as a gray CPK model with the phosphate backbone of the purine strand colored red and purple. TFO **31a** is shown as a colored tube model with three 2′,4′-BNA^{NC} nitrogens represented in green. (B) Expanded view of the area of the triplex containing three consecutive 2′,4′-BNA^{NC}[NH] residues, showing that the 2′,4′-BNA^{NC} nitrogens are very close to the phosphate moiety of the purine strand.

Table 8. Sequence-Specific Triplex Formation by 2′,4′-BNA^{NC}-Modified TFOs[a,b]

	T_m ($\Delta T_m = T_{m\,(mismatch)} - T_{m\,(match)}$) (°C)			
T (oligonucleotide)	**X**:**Y** = A:**T** (match)	G:C	C:G	T:G
natural (**28**)	43	21 (−22)	25 (−18)	18 (−25)
2′,4′-BNA^{NC}[NH] (**29a**)	55	30 (−25)	28 (−27)	15 (−40)
2′,4′-BNA^{NC}[NMe] (**29b**)	49	24 (−25)	22 (−27)	14 (−35)
2′,4′-BNA(LNA) (**29d**)	55	31 (−24)	35 (−20)	16 (−39)

[a] Modified TFO: 5′-d(TTTTTCT**T**TCTCTCT)-3′; target dsDNA: 5′-d(GCTAAAAAGA**X**AGAGAGATCG)-3′; 3′-d(CGATTTTTCT**Y**TCTCTC-TAGC)-5′. [b] Conditions: 7 mM sodium phosphate buffer (pH 7.0) containing 140 mM KCl and 10 mM $MgCl_2$; strand concentration = 1.5 μM.

amino nitrogen of 2′,4′-BNA^{NC}[NH] is unlikely at the experimental pH, and, hence, enhanced stabilization of the triplexes might not be happening via protonation. We speculate that stabilization of the triplexes might be governed by different reasons such as additional hydrogen bonding between the N−H hydrogen and the phosphate oxygen of dsDNA and/or conformational bias of the six-membered bridged structure, as seen in the case of ENA.[55,70]

To see the possibility of additional hydrogen bonding between nitrogen of the bridged moiety and the phosphate of the dsDNA strand, molecular modeling of a triplex formed by dsDNA and 2′,4′-BNA^{NC}[NH]-TFO with three consecutive 2′,4′-BNA^{NC}-[NH] residues (TFO **31a**) was performed (Figure 5). The structure was based on the A-form triplex geometry, and energy minimization was accomplished after the incorporation of three 2′,4′-BNA^{NC}[NH] residues. It was found that the nitrogens (green atoms) and *N*-hydrogens of the 2′,4′-BNA^{NC}-bridged structure are very close to the phosphodiester linkage (red and purple atoms in the purine strand), implying that there could be hydrogen bonding between *N*-hydrogens and phosphate oxygens of the purine strand.[2]

Next, sequence specificity or mismatch discrimination studies were performed using 2′,4′-BNA^{NC}-TFOs **29a** and **29b**; the results are summarized in Table 8. It was found that the T_m values of triplexes formed by 2′,4′-BNA^{NC}-TFOs with mismatched dsDNAs having G:C, C:G, and T:A arrangements decreased significantly compared to matched DNA (A:T arrangement). For example, against a dsDNA target having G:C arrangement at the center of the target, the T_m value decreased by 25 °C for both 2′,4′-BNA^{NC}[NH] and [NMe] oligonucleotides **29a** and **29b**, respectively. This is larger than the decrease by natural-TFO **28** and similar to that exhibited by 2′,4′-BNA-TFO **29d**. To the mismatched targets with C:G and T:A arrangements, again a very large decrease in T_m value was observed compared to matched (A:T) bases ($\Delta T_m = -27$ °C for both 2′,4′-BNA^{NC}-[NH] and [NMe] against C:G arrangement and −40 and −35 °C, respectively, for 2′,4′-BNA^{NC}[NH] and [NMe] against T:A arrangement). These values are significantly larger than that obtained with natural-TFO **28** ($\Delta T_m = -18$ and −25 °C for C:G and T:A arrangements, respectively). These results show that 2′,4′-BNA^{NC}-TFOs form triplexes with very high sequence specificity. The mismatch discriminating power of 2′,4′-BNA^{NC} analogues to C:G arrangements is even higher than that of 2′,4′-BNA (LNA) ($\Delta T_m = -20$ °C), whereas its discrimination for T:A arrangements is similar to that of 2′,4′-BNA (LNA). Because of its low triplex-forming ability, 2′,4′-BNA^{NC}[NBn] may not be suitable for triplex approaches, and therefore its sequence specificity would be meaningless.

3.5. Nuclease Resistance of 2′,4′-BNA^{NC} Oligonucleotides. The rapid degradation of oligonucleotides by nucleases must be overcome if oligonucleotides are to be used in in vivo applications. Although the backbone of PS oligonucleotides has improved resistance to nuclease degradation, their nuclease resistance is still suboptimal.[49,74] Additional nuclease resistance is therefore required. We examined the resistance of oligonucleotides modified with a single 2′,4′-BNA^{NC} unit (oligonucleotides **36a**−**c**) toward 3′-exonuclease (*Crotalus adamanteus* venom phosphodiesterase, CAVP, Pharmacia) degradation and compared it with natural oligonucleotide **40**, 2′,4′-BNA (LNA)-, and PS-modified oligonucleotides **36d** and **41**, respec-

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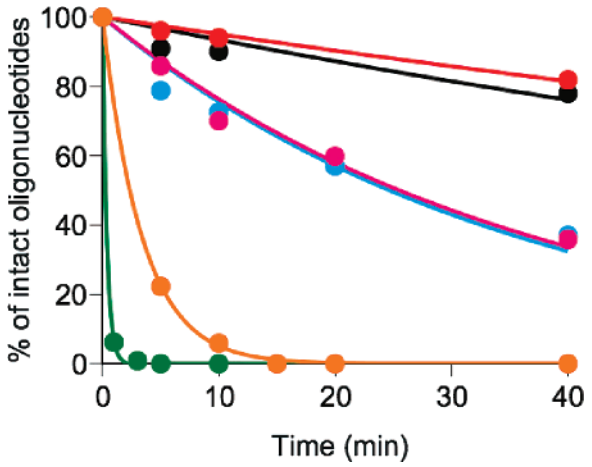

Figure 6. Nuclease resistance of T_8**X**T oligonucleotides against CAVP; **X** = natural-T (**40**) (green), phosphorthioate-T (**41**) (black), 2′,4′-BNA-T (**36d**) (orange), 2′,4′-BNA^{NC}[NH]-T (**36a**) (blue), 2′,4′-BNA^{NC}[NMe]-T (**36b**) (red), 2′,4′-BNA^{NC}[NBn]-T (**36c**) (magenta). Hydrolysis of the oligonucleotides (10 μg) was carried out at 37 °C in buffer (200 μL) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and CAVP (0.25 μg). Curve fitting equation: $y = 100 \times e^{-ax}$ (for parameters and errors, Experimental Section).

tively (Figure 6). Our preliminary report[54] showed that 2′,4′-BNA^{NC}[NMe]-modified oligonucleotides are much more resistant to degradation by snake venom phosphodiesterase (SVPDE, Boehringer Mannheim) than are 2′,4′-BNA (LNA)-modified oligonucleotides and are even more resistant than the more stable S_p-isomer of PS oligonucleotide.[75] In the present study, we examined the nuclease resistance of all of the 2′,4′-BNA^{NC} analogues (2′,4′-BNA^{NC}[NH], [NMe], and [NBn] analogues) using CAVP and compared the results with those obtained for 2′,4′-BNA (LNA) and PS oligonucleotides. Nuclease resistance was evaluated by incubating the specified oligonucleotides with CAVP and analyzing the percentage of intact oligonucleotides at several time points by RP-HPLC (Experimental Section). The results are shown in Figure 6. The natural and 2′,4′-BNA (LNA)-modified oligothymidylates (**40** and **36d**, respectively) were completely digested within 3 and 15 min, respectively. In marked contrast, 2′,4′-BNA^{NC}-modified oligothymidylates were stable under identical reaction conditions. 2′,4′-BNA^{NC}[NH]-modified oligonucleotide **36a** is remarkably stable under these conditions: about 40% survived after 40 min of exposure. Among the 2′,4′-BNA^{NC} analogues, 2′,4′-BNA^{NC}[NMe] was found to be the more stable analogue, with 82% of the oligonucleotides remaining intact after 40 min exposure at 37 °C, and 77% survived even after 90 min (data not shown). The overall nuclease resistance of 2′,4′-BNA^{NC}[NMe] is higher than that of S_p-PS oligonucleotide, consistent with our previous observation.[54] Unexpectedly, the nuclease resistance of 2′,4′-BNA^{NC}[NBn] (oligonucleotide **36c**) is similar to that of 2′,4′-BNA^{NC}[NH], showing that benzyl substitution does not improve nuclease resistance in comparison to that of 2′,4′-BNA^{NC}[NH]. The excellent resistance of 2′,4′-BNA^{NC}[NMe] oligonucleotide **36b** to CAVP is probably due to steric hindrance of the phosphodiester linkage exerted by the methyl-substituted six-membered bridged moiety. From these results, it can be anticipated that oligonucleotides with multiple 2′,4′-BNA^{NC} substitutions will be largely resistant toward nuclease degradation.

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Conclusion

We have synthesized three 2′,4′-BNA^{NC} nucleotides (2′,4′-BNA^{NC}[NH], [NMe], and [NBn]) with a six-membered bridged structure containing an amino nitrogen. The synthesis of the nucleotides was greatly simplified and improved, as demonstrated by the one-pot conversion of **11** to **12** in very good yield. The nitrogen on the bridge was easily functionalized with alkyl or benzyl groups, suggesting that a wide range of functional molecules can be synthesized by appropriate functionalization. The nucleotides were successfully incorporated into a variety of oligonucleotides for various studies.

2′,4′-BNA^{NC}-modified oligonucleotides show very high target affinity, similar to or even higher than that of 2′,4′-BNA (LNA). Following hybridization to a complementary RNA, the increase in T_m per modification was very high, that is, 5 to 6 °C, similar to that observed with 2′,4′-BNA (LNA). The T_m values of oligonucleotides with an increased number of 2′,4′-BNA^{NC}[NH] modifications exceed that of 2′,4′-BNA (LNA). In addition, 2′,4′-BNA^{NC}[NMe] and [NBn] along with their high RNA affinity comparable to that of 2′,4′-BNA (LNA), showed better RNA selectivity than 2′,4′-BNA (LNA). Triplex formation is greatly enhanced by 2′,4′-BNA^{NC}[NH] modifications. The triplex stability of 2′,4′-BNA^{NC}[NH]-modified oligonucleotides with consecutive or extensive modifications are consistently higher than those of 2′,4′-BNA (LNA) and ENA,[53] and a fully modified 2′,4′-BNA^{NC}[NH]-TFO formed a highly stable triplex at neutral pH. 2′,4′-BNA^{NC} modifications dramatically improve resistance to nuclease degradation, which is far higher than that of 2′,4′-BNA (LNA). The nuclease resistance of 2′,4′-BNA^{NC}[NMe] is even slightly higher than that of PS oligonucleotide. These characteristics indicate that all of the 2′,4′-BNA^{NC}[NH], [NMe] and [NBn] analogues have significant potential for antisense applications. 2′,4′-BNA^{NC}[NMe] and [NBn] will be particularly suitable for antisense approaches, whereas 2′,4′-BNA^{NC}[NH] might be more useful in antigene applications, but also for antisense approaches.

In summary, because of its pronounced affinity for target strands and extraordinarily high nuclease resistance, 2′,4′-BNA^{NC} is clearly a highly suitable BNA analogue for powerful applications in genomics. Moreover, instead of the common practice of functionalizing at the base (which might decrease the hybridizing affinity of a nucleic acid), appropriate functionalization of the nitrogen on the bridged structure might be possible without hampering hybridizing affinity,[76] allowing the generation of a wide range of molecules with improved nuclease resistance[54] or cellular uptake,[77] or with characteristics appropriate for a variety of applications such as fluorescence probes,[78] DNA cleavage activators,[79] etc.). In addition, cleavage of the N−O bond might modulate the hybridizing properties of 2′,4′-BNA^{NC} oligonucleotides, enabling their possible use in DNA nanotechnology.[80]

Experimental Section

Synthesis and Characterization of Compounds: General Aspects and Instrumentation. Melting points are uncorrected. All of the

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moisture-sensitive reactions were carried out in well-dried glassware under a N_2 atmosphere. Dichloromethane, ethanol, triethylamine, and pyridine were distilled from CaH_2. Dry MeCN was used as purchased. ^{1}H NMR (270, 300, or 500 MHz), ^{13}C NMR (67, 75, and 125 MHz) and ^{31}P NMR spectra (202 MHz) were recorded on JEOL EX-270, JEOL-AL-300, and JEOL GX-500 spectrometers, respectively. Chemical shifts are reported in parts per million downfield from internal tetramethylsilane for ^{1}H, $CHCl_3$ ($\delta = 77.0$) for ^{13}C NMR, and 85% H_3PO_4 ($\delta = 0$) for ^{31}P spectra. IR spectra were recorded on a JASCO FTIR-200 spectrometer. Optical rotations were recorded on a JASCO DIP-370 instrument. Mass spectra were measured on JEOL JMS-600 or JMS-700 mass spectrometers. For column chromatography, silica gel FL 100D was used. Full experimental details and characterization data for all of the new compounds are described in the Supporting Information.

X-ray Crystallography. Crystallographic data for **9** have been deposited at the Cambridge Crystallographic Data Center. CCDC-647092 contains the supplementary crystallographic data for **9**. This data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, U.K.; fax: +44 1223 336033; or deposit@ccdc.cam.ac.uk).

Oligonucleotide Synthesis. Synthesis of oligonucleotides **23**−**36d** was performed on a 0.2 μmol scale using an Expedite (TM) 8909 Nucleic Acid Synthesis System according to a standard phosphoroamidite protocol with 1-*H* tetrazole and activator-42 as the activators. All of the reagents were assembled, and the oligonucleotides were synthesized according to the standard synthesis cycle (trityl off mode) with the exception of a prolonged coupling time (5−6 min) for 2′,4′-BNA^{NC} monomers. For fully modified oligonucleotides **35a**, **35b**, and **35d**, and the universal solid support (Glen Research) was used, whereas for all other oligonucleotides the standard CPG-solid supports from Glen Research were used. After synthesis, the solid supported oligonucleotides were treated with concentrated ammonium hydroxide solution (1 mL) at room temperature for 1.5 h and then at 55 °C for 16−24 h. The ammonia solutions were then concentrated, and the crude oligonucleotides were initially purified by NAP 10 columns (Amersham Biosciences) followed by further purification by RP-HPLC with a Wako Wakopack^R WS-DNA (10 × 250 mm^2) or Waters X-Terra (10 × 50 mm^2) column using 4−32% MeCN in 0.1 M triethylammonium acetate buffer (pH 7.0). The oligonucleotides were analyzed for purity by HPLC and characterized by MALDI-TOF mass spectroscopy (for MALDI-TOF mass data and isolated yields, see the Supporting Information).

UV Melting Experiment. UV melting experiments were carried out on a Beckman DU-650 spectrometer equipped with a T_m analysis accessory. For the duplex formation study, equimolar amounts of the target RNA/DNA strand and oligonucleotide were dissolved in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl to provide final-strand concentrations of 4 μM. For the triplex formation study, equimolar amounts of target dsDNA and oligonucleotide were added in 7 mM Na_2HPO_4 buffer (pH 7.0) containing 140 mM KCl to give final strand concentrations of 1.5 μM. The oligonucleotide/target samples were then annealed by heating at 90 °C for 5 min, followed by slow cooling to room temperature. The samples were then stored at 4 °C for 1 h prior to T_m measurements. The melting profiles were recorded at 260 nm from 10 to 90 °C at a scan rate of 0.5 °C/min. T_m was calculated as the temperature at which the duplexes were half dissociated, determined by taking the first derivative of the melting curve.

CD Spectra. CD spectra were recorded on a JASCO J-720W spectropolarimeter. A solution of the duplex (4 μM) in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl was placed in the cell and the spectra were measured from 350 to 200 nm at 20 °C.

Nuclease Resistance Study. CAVP (0.25 μg) was added to a solution of oligonucleotides (1.5 nmol) **36a**−**36d**, **40**, and **41** in 50 mM Tris·HCl buffer (pH 8.0) containing 10 mM $MgCl_2$. The cleavage reaction was carried out at 37 °C. At several time points, a portion of each reaction mixture was removed and heated to 90 °C for 2 min to inactivate the nuclease. The amount of intact oligonucleotide remaining was evaluated by RP-HPLC. The percentages of intact oligonucleotides were then plotted against the time of exposure to get the oligonucleotide degradation curve with time. The fitted line for each oligonucleotide degradation was obtained by the model equation, $y = 100 \times e^{-ax}$. The parameters (a) and the errors (R^2) of the fitting curve of each oligonucleotide are as follows: **36a**, $a = 2.81 \times 10^{-2}$ min^{-1}, $R^2 = 0.974$; **36b**, $a = 5.12 \times 10^{-3}$ min^{-1}, $R^2 = 0.986$; **36c**, $a = 2.72 \times 10^{-2}$ min^{-1}, $R^2 = 0.984$; **36d**, $a = 2.97 \times 10^{-1}$ min^{-1}, $R^2 = 1.000$; **40**, $a = 2.75$ min^{-1}, $R^2 = 1.000$; **41**, $a = 6.84 \times 10^{-3}$ min^{-1}, $R^2 = 0.866$.

Acknowledgment. This work was partially supported by PRESTO and the Creation and Support Program for Start-ups from the Universities of the Japan Science and Technology Agency (JST). Generous help from Dr. Takuya Yoshida (Graduate School of Pharmaceutical Sciences, Osaka University) for measuring the pKa of 2′,4′-BNA^{NC}[NH] monomer is gratefully acknowledged. S.M.A.R. thanks the Japan Society for the Promotion of Science (JSPS) for financial support.

Supporting Information Available: Experimental details and synthesis of **1**, full experimental procedures for the synthesis of **2**−**22**, and the characterization data for all new compounds, table of optimized reaction conditions for the synthesis of **12**, ^{1}H and ^{13}C spectra of all new compounds (**3**−**7**, benzyl derivative of **12**, and **14** and **17**,) ^{31}P NMR spectra of **18**−**22**, MALDI-TOF mass data and yields of oligonucleotides **24a**−**d** to **36a**−**d**, CD spectra of duplexes formed by 2′,4′-BNA^{NC}-[NBn]-modified oligonucleotides, UV melting curves, pH titration curves for pKa measurement, complete ref 32, and crystallographic information files (CIF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA710342Q