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Synthesis and properties of 3′-amino-2′,4′-BNA, a bridged nucleic acid with a N3′→P5′ phosphoramidate linkage

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ABSTRACT

The synthesis and properties of a bridged nucleic acid analogue containing a N3′→P5′ phosphoramidate linkage, 3′-amino-2′,4′-BNA, is described. A heterodimer containing a 3′-amino-2′,4′-BNA thymine monomer, and thymine and methylcytosine monomers of 3′-amino-2′,4′-BNA and their 5′-phosphoramidites, were synthesized efficiently. The dimer and monomers were incorporated into oligonucleotides by conventional 3′→5′ assembly, and 5′→3′ reverse assembly phosphoramidite protocols, respectively. Compared to a natural DNA oligonucleotide, modified oligonucleotides containing the 3′-amino-2′,4′-BNA residue formed highly stable duplexes and triplexes with single-stranded DNA (ssDNA), single-stranded RNA (ssRNA), and double-stranded DNA (dsDNA) targets, with the average increase in melting temperature (T_m) against ssDNA, ssRNA and dsDNA being +2.7 to +4.0 °C, +5.0 to +7.0 °C, and +5.0 to +11.0 °C, respectively. These increases are comparable to those observed for 2′,4′-BNA-modified oligonucleotides. In addition, an oligonucleotide modified with a single 3′-amino-2′,4′-BNA thymine residue showed extraordinarily high resistance to nuclease degradation, much higher than that of 2′,4′-BNA and substantially higher even than that of 3′-amino-DNA and phosphorothioate oligonucleotides. The above properties indicate that 3′-amino-2′,4′-BNA has significant potential for antisense and antigene applications.

1. Introduction

Recently much attention has been focused on chemically modified oligonucleotides due to their potential application in antisense and antigene technologies,[1–3] diagnostics,[4] and nucleic acid nanotechnology.[5,6] In order to be effective and practically useful in the above technologies, modified oligonucleotides must have specific characteristics such as high target-binding affinity, sequence specificity and nuclease resistance. Although numerous modified oligonucleotides have been developed in the past few decades, most do not exhibit properties satisfying the above criteria, thus severely restricting their utility in oligonucleotide-based therapy and nucleic acid nanotechnology.

In 1994, Gryaznov et al. reported N3′→P5′ phosphoramidate linked oligonucleotides (3′-amino-DNA, Fig. 1)[7] in which the 3′-O was replaced by a 3′-N atom. A number of structural analogues of N3′→P5′ phosphoramidate were synthesized, and all were shown to possess excellent nuclease resistance along with superior hybridizing properties with complementary ssDNA, ssRNA and dsDNA.[7–11] The increased hybridization characteristics of these analogues result from their RNA-like structures. The resemblance to RNA is due to the dominant N-type sugar pucker generated by the electronegativity of the nitrogen atom,[12,13] which is further enforced by 2′-substitutions (2′-fluoro or 2′-OH, a ribo sugar). As a result of these promising properties, N3′→P5′ oligonucleotides act as potent antisense molecules in cells and in vivo.[14]

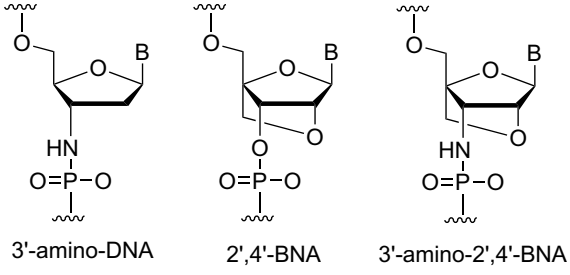

Figure 1. Structures of 3′-amino-DNA, 2′,4′-BNA and 3′-amino-2′,4′-BNA.

Several years ago, our group and Wengel et al. independently discovered a novel modified nucleic acid, 2′-*O*,4′-*C*-bridged nucleic acid (2′,4′-BNA, also named locked nucleic acid, or LNA)[15–17]

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which has a fixed N-type sugar-conformation. This nucleic acid derivative shows unprecedented hybridizing ability with complementary strands of ssRNA, ssDNA and dsDNA,[15–26] and somewhat increased resistance to nuclease degradation compared to natural DNA.[21,27,28] Due to these interesting properties, 2′,4′-BNA/LNA is one of the most promising and popular nucleic acid analogues currently being utilized in various genomic technologies,[29–31] and is now commercially available. However, this nucleic acid is not sufficiently resistant to nucleases, and is significantly less resistant than phosphorothioate oligonucleotide.[32,33] Hence, the nuclease resistance ability of phosphorothioate oligonucleotide is even considered as suboptimal,[34] further enhancement of nuclease resistance of 2′,4′-BNA/LNA is required for practical in vivo use.

To optimize the nuclease resistance and hybridizing properties of 2′,4′-BNA, we aimed to develop a bridged nucleic acid (BNA) with a N3′→P5′ phosphoramidite linkage, 3′-amino-2′,4′-BNA (Fig. 1). In order to incorporate 3′-amino-2′,4′-BNA into oligonucleotides easily by the conventional phosphoramidite protocols, we initially synthesized a heterodimer unit of 3′-amino-2′,4′-BNA.[35] However, the synthesis of 3′-amino-2′,4′-BNA oligonucleotides via the heterodimer approach (Fig. 2)[35] has some serious drawbacks: (i) only limited oligonucleotide sequences can be synthesized by heterodimer unit **10**, and oligonucleotides representing all sequence combinations require the separate synthesis of 16 different heterodimers; (ii) oligonucleotides containing consecutive modifications cannot be synthesized via the dimer approach. Due to these limitations, we could not investigate the hybridizing characteristics of 3′-amino-2′,4′-BNA in detail. To overcome these problems, we planned a synthetic approach based on the 5′→3′ reverse assembly protocol shown in Fig. 2, and developed a facile synthetic route for 5′-phosphoramidites of 3′-amino-2′,4′-BNA thymine and methyl cytosine monomer analogues. These compounds were incorporated into a variety of oligonucleotides. In our preliminary report, we communicated the synthesis of 3′-amino-2′,4′-BNA oligonucleotides via a dimer unit, showed one example of hybridization with DNA and RNA, and presented preliminary data showing the nuclease resistance of the BNA oligonucleotides.[35] Herein, we report full details of the synthesis of 3′-amino-2′,4′-BNA phosphoramidites via both approaches, the utilization of these compounds in the synthesis of various oligonucleotides, and give a detailed account of their hybridization and nuclease resistance profiles.

2. Results and discussion

2.1. Synthesis of 3′-amino-2′,4′-BNA heterodimer unit 10

Since 3′-amino-2′,4′-BNA contains a N3′→P5′ phosphoramidate linkage instead of an O3′→P5′ linkage, conventional synthesis via the 3′-phosphoramidite approach is not possible because the P–N bond in the 3′-phosphorodiamidite group would be simultaneously cleaved by the common activators used for DNA synthesis. To overcome this problem, we initially synthesized heterodimer **10** for use in typical phosphoramidite protocols. The synthesis of **10** was accomplished from the known compound, 3-azido-3-deoxy-furanose **1**,[36] as shown in Scheme 1. Removal of the benzoyl groups of **1** by base-mediated hydrolysis afforded diol **2**, which was transformed to compound **3** via selective silylation with *tert*-butyldiphenylsilyl chloride (TBDPSCl), followed by tosylation with *p*-toluenesulphonyl chloride (TsCl) in the presence of 4-dimethylaminopyridine (DMAP) and triethylamine (Et_3N). Acetolysis of **3** with acetic acid, acetic anhydride and concd sulfuric acid provided an anomeric mixture of diacetate intermediate[37] which was coupled with thymine in the presence of *N,O*-bis(trimethylsilyl)acetamide (BSA) and trimethylsilyl triflate (TMSOTf) to give the nucleoside analogue **4** in very good yield. Treatment of **4** with potassium carbonate yielded the desired bicyclic nucleoside **5** quantitatively. Desilylation by tetrabutyl ammonium fluoride (TBAF) furnished a known nucleoside derivative[38] (Supplementary Data), the hydroxyl group of which was tritylated by 4,4′-dimethoxytrityl chloride (DMTrCl) to afford the trityl derivative **6**. Then, the 3′-azido group was reduced to an amino group by the action of triphenylphosphine (PPh_3), pyridine and NH_4OH to give compound **7** in excellent yield. Following reported procedures,[8,39] compound **7** was coupled with the methyl phosphonate derivative **8**[39] to give the heterodimer **9**. Desilylation followed by phosphitylation in the presence of 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphorodiamidite and disopropylammonium tetrazolide provided the desired heterodimer phosphoramidite **10**.

2.2. Synthesis of 3′-amino-2′,4′-BNA monomers 5′-phosphoramidites

As the synthesis of a variety of 3′-amino-2′,4′-BNA oligonucleotides via the heterodimer approach (Fig. 2) is problematic, synthesis via 5′→3′ reverse assembly was undertaken. Synthesis of

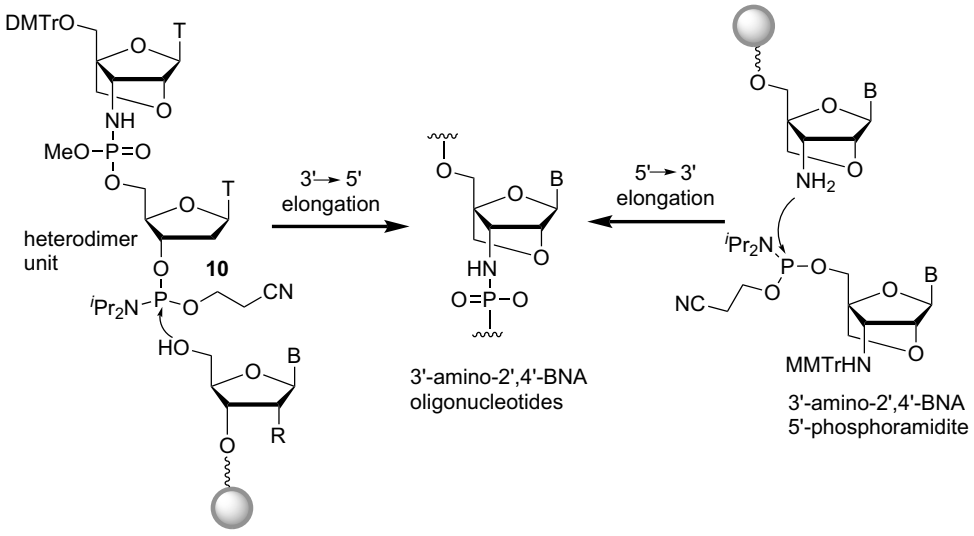
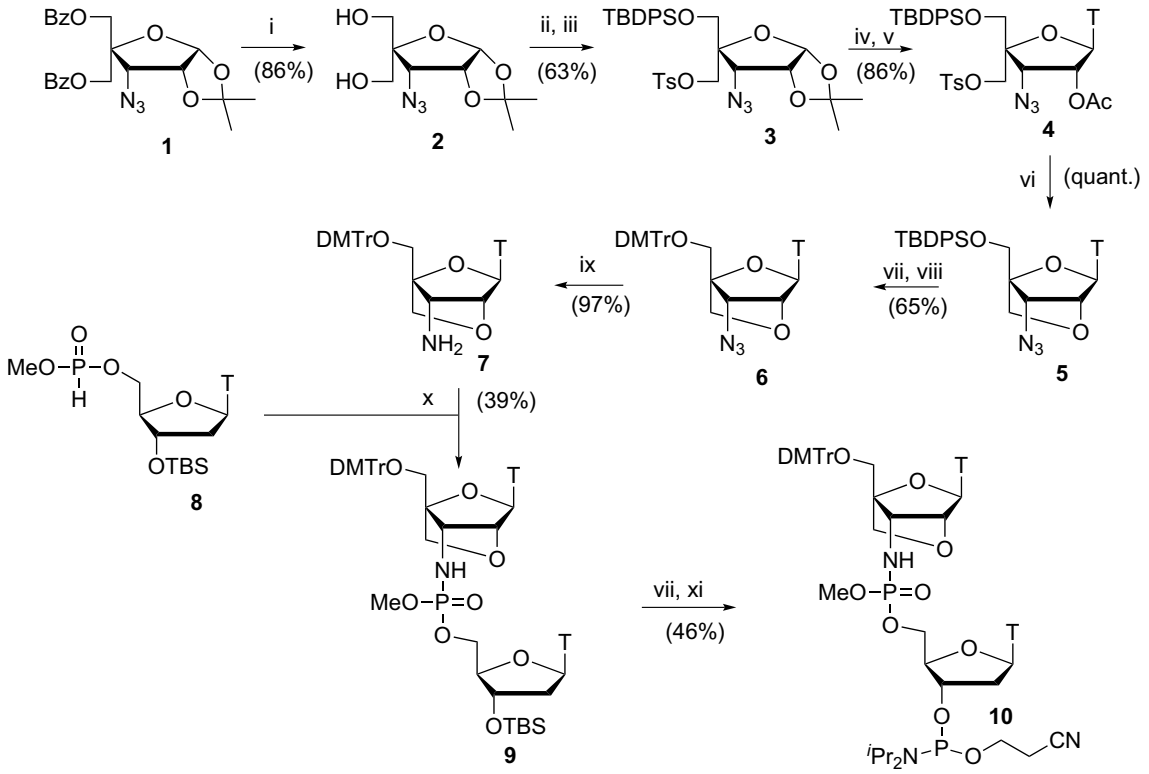


Figure 2. Synthetic strategies for 3′-amino-2′,4′-BNA oligonucleotides.



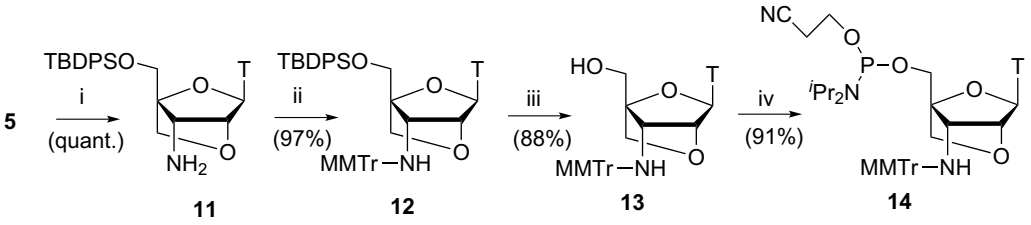
Scheme 1. Reagents and conditions: (i) K_2CO_3, MeOH, 0 °C; (ii) TBDPSCl, Et_3N, CH_2Cl_2, rt; (iii) TsCl, DMAP, Et_3N, CH_2Cl_2, rt; (iv) concd H_2SO_4, Ac_2O, AcOH, rt; (v) thymine, BSA, TMSOTf, DCE, 80 °C; (vi) K_2CO_3, MeOH, rt; (vii) TBAF, THF, rt; (viii) DMTrCl, DMAP, pyridine, rt; (ix) PPh_3, pyridine, rt, then NH_4OH aq rt; (x) CCl_4, Et_3N, MeCN, rt; (xi) $(^iPr_2N)_2POCH_2CH_2CN$, diisopropylammonium tetrazolide, MeCN-THF, rt. TBDPSCl, *tert*-butyldiphenylsilyl chloride; DMAP, 4-dimethylaminopyridine, BSA, *N,O*-bis(trimethylsilyl)acetamide; DCE, dichloroethane; DMTrCl, 4,4′-dimethoxytrityl chloride.

oligonucleotides following this approach requires 5′-phosphoramidites instead of the conventional 3′-phosphoramidites; this process is called phosphoramidite transfer reaction.[9,10,40] In order to apply this reverse assembly strategy to the synthesis of 3′-amino-2′,4′-BNA oligonucleotides, we synthesized 3′-amino-2′,4′-BNA -5′-phosphoramidites derivatives **14** and **20** (Schemes 2 and 3). The synthesis of 3′-amino-2′,4′-BNA-thymine 5′-phosphoramidite **14** is shown in Scheme 2. The azido group of intermediate **5** was reduced to an amino group quantitatively to give the 3′-amino derivative **11**. Protection of the amino functionality with 4′-monomethoxytrityl chloride (MMTrCl) in the presence of pyridine afforded compound **12** in excellent yield. Desilylation with TBAF produced alcohol **13** smoothly, which was phosphitylated to give the desired 5′-phosphoramidite **14** in 91% yield.

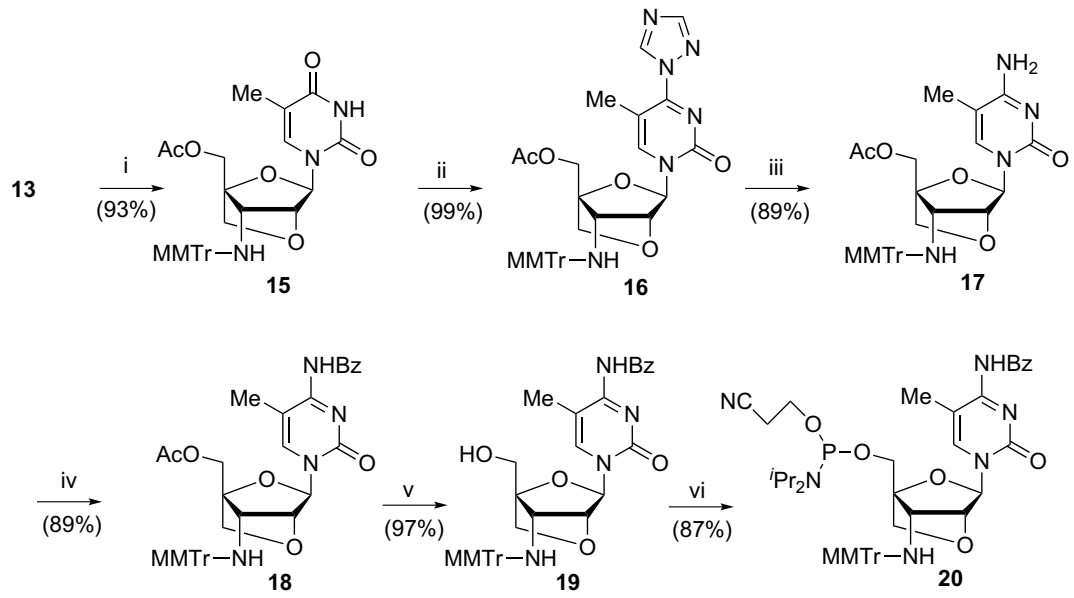
The synthesis of 3′-amino-2′,4′-BNA-5-methylcytosine 5′-phosphoramidite **20** is outlined in Scheme 3. The primary hydroxyl group of **13** was protected with an acetyl group to afford compound **15** in 93% yield. Compound **15** was transformed to the triazole derivative **16** by the treatment of 1*H*-triazole, phosphoryl chloride and triethylamine[41] in nearly quantitative yield. Exposure to aqueous ammonia delivered amine **17**, whose amino group was protected with a benzoyl group via conventional benzoylation to give compound **18** in good yield. Deacetylation by lithium hydroxide followed by phosphitylation furnished the desired 5′-phosphoramidite **20** in good yield.

2.3. Synthesis of oligonucleotides

Using the phosphoramidites **10, 14**, and **20**, and natural DNA building blocks, a number of different 3′-amino-2′,4′-BNA-modified oligonucleotides (oligonucleotides **21–27**, Table 1) were synthesized using an automated DNA synthesizer (see Experimental). A set of 12-mer oligonucleotides **21**–**24**, 15-mer polypyrimidine oligonucleotides **25** and **26**, and 10-mer oligothymidylate **27**, were synthesized in order to study duplex and triplex forming properties and nuclease resistance properties, respectively. Heterodimer phosphoramidite **10** was used for the synthesis of oligonucleotides **21, 25**, and **27**, and monomer phosphoramidites **14** and **20** were used for the synthesis of the remaining oligonucleotides. The oligonucleotides were purified by RP-HPLC and



Scheme 2. Reagents and conditions: (i) PPh_3, pyridine, rt, then NH_4OH, rt; (ii) MMTrCl, pyridine, rt; (iii) TBAF, THF, rt; (iv) $(^iPr_2N)_2POCH_2CH_2CN$, diisopropylammonium tetrazolide, MeCN-THF, rt.



Scheme 3. Reagents and conditions: (i) Ac_2O, pyridine, rt; (ii) 1*H*-triazole, $POCl_3$, Et_3N, MeCN, 0 °C; (iii) NH_4OH aq, 1,4-dioxane, rt; (iv) BzCl, Et_3N, CH_2Cl_2, rt; (v) LiOH–H_2O (2:1), rt; (vi) $(^iPr_2N)_2POCH_2CH_2CN$, diisopropylammonium tetrazolide, MeCN-THF, rt.

Table 1
3′-Amino-2′,4′-BNA oligonucleotides and MALDI-TOF mass data[a]

Oligonucleotides (5′-d……-3′)	Mass [M-H]$^-$ Found/Calcd.
GCGTT**T**TTGCT (**21**)	3024.6/3024.1
GCG**T**T**T**T**T**TGCT (**22**)	3717.2/3714.4
GCGTT**TTT**TGCT (**23**)	3716.6/3714.4
GCG**TTTTTT**GCT (**24**)	3799.8/3795.5
TTTTT^{m}CT**T**T^{m}CT^{m}CT^{m}CT (**25**)	4523.3/4523.1
TTT**T**T$^{\mathbf{m}}$**C**T**T**T$^{\mathbf{m}}$**C**T^m**C**T^{m}CT (**26**)	4634.4/4631.2
TTTTTTTT**T**T (**27**)	3010.7/3009.5

[a] **T** = 3′-amino-2′,4′-BNA-T, $^{\mathbf{m}}$**C** = 3′-amino-2′,4′-BNA-^{m}C.

characterized and verified by their MALDI-TOF mass spectra. MALDI-TOF mass data are summarized in Table 1.

2.4. Duplex formation and duplex thermal stability

The ability of 3′-amino-2′,4′-BNA-modified oligonucleotides to form duplexes in physiological salt and buffer conditions, and the thermal stability of duplexes formed with ssDNA and ssRNA complements, was studied. Thermal stability was evaluated by melting temperature (T_m). The results obtained with natural DNA and the 2′,4′-BNA-modified analogues are compared in Table 2. Modification of natural DNA oligonucleotide **28** by a single 3′-amino-2′,4′-BNA residue (oligonucleotide **21**) led to an increase in T_m of 4 and 7 °C against ssDNA and ssRNA, respectively. Duplex thermal stability further improved upon increasing the number of modifications. Incorporating three 3′-amino-2′,4′-BNA residues either consecutively or separated by natural DNA units (oligonucleotides **22** and **23**, respectively) resulted in duplexes with very high thermal stability. The most prominent enhancement in thermal stability was observed in duplexes formed with complementary ssRNA. The increase in T_m per modification (ΔT_m/mod.) was +6.0 and +5.3 °C for **22** and **23**, respectively. Against ssDNA, the ΔT_m/mod. was found to be +2.0 °C for both **22** and **23**, implying that 3′-amino-2′,4′-BNA oligonucleotides have preferential RNA-binding affinity. A modified oligonucleotide containing six consecutive 3′-amino-2′,4′-BNA residues (oligonucleotide **24**) also formed duplexes with ssRNA and ssDNA with remarkably improved thermal

Table 2
T_m values of duplexes formed by 3′-amino-2′,4′-BNA oligonucleotides with ssDNA and ssRNA[a,b]

Oligonucleotides (5′-d……-3′)	T_m (ΔT_m/mod.) (°C)	
	ssDNA	ssRNA
GCGTTTTTTGCT (**28**)	47	45
GCGTT**T**TTTGCT (**21**)	51 (+4.0)	52 (+7.0)
GCG**T**T**T**T**T**TGCT (**22**)	53 (+2.0)	63 (+6.0)
GCGTT**TTT**TGCT (**23**)	53 (+2.0)	61 (+5.3)
GCG**TTTTTT**GCT (**24**)	63 (+2.7)	75 (+5.0)
GCGTT**t**TTTGCT (**29**)	53 (+6.0)	52 (+7.0)[c]
GCG**t**T**t**T**t**TGCT (**30**)	56 (+3.0)	62 (+5.7)[c]
GCGTT**ttt**TGCT (**31**)	54 (+2.3)	60 (+5.0)[c]
GCG**tttttt**GCT (**32**)	67 (+3.3)	80 (+5.8)[d]

[a] Targets: ssDNA, 5′-d(AGCAAAAAACGC)-3′; ssRNA, 5′-r(AGCAAAAAACGC)-3′.
[b] Conditions: 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl; strand concentration = 4 μM. **T** = 3′-amino-2′,4′-BNA-T, **t** = 2′,4′-BNA-T.
[c] Data from Ref. 42.
[d] Data from Ref. 21.

stability (T_m values of 63 and 75 °C for ssDNA and ssRNA, respectively). Compared with 2′,4′-BNA-modified oligonucleotides **29** to **32**,[21,42] the 3′-amino-2′,4′-BNA-modified oligonucleotides showed slightly decreased affinity towards ssDNA, but affinity to ssRNA was similar to that of 2′,4′-BNA except for oligonucleotide **24** (compare the T_m value of **24** with that of **32**). These results indicate that 3′-amino-2′,4′-BNA is more RNA-selective than 2′,4′-BNA. The equal or comparable RNA-binding affinity of 3′-amino-2′,4′-BNA to 2′,4′-BNA indicates that N-type conformational characteristics are already optimized by the 2′-*O*,4′-*C* bridge, and that 3′-N does not contribute to the restriction of sugar puckering, in contrast to findings using non-bridged natural nucleic acids.[7–11] The relatively lower affinity of oligonucleotide **24** compared to 2′,4′-BNA oligonucleotide **32** indicates that consecutive backbone modifications reduce duplex stability of bridged nucleic acids.

2.5. Triplex-forming properties

Next, the triplex forming ability of 3′-amino-2′,4′-BNA-modified triplex-forming oligonucleotides (TFOs) was investigated under

neutral conditions with or without 10 mM $MgCl_2$ (Table 3). Two dsDNA targets (targets A and B) were used for singly and multiply modified TFOs (TFOs **25** and **26**, respectively). The reason for using two different targets is that the target A duplex has a T_m value (ca. 57 °C) substantially lower than the T_m of the triplex formed with **26**. Therefore, a hairpin DNA (target B) was used for TFO **26**, and for direct comparison with the 3′-amino-2′,4′-BNA-TFOs, we measured the T_ms of triplexes formed by the natural TFO **33** against both targets. As shown in Table 3, replacement of only a single natural DNA-nucleotide of **33** by 3′-amino-2′,4′-BNA-nucleotide (TFO **25**) resulted in significant stabilization of the triplex (T_m = 44 and 55 °C in the absence and presence of $MgCl_2$, respectively; ΔT_m = +11 °C in both cases). The incorporation of five 3′-amino-2′,4′-BNA nucleotides of thymine and methyl cytosine bases (TFO **26**) resulted in the formation of a triplex with thermal stability 27 °C (−$MgCl_2$) and 32 °C (+$MgCl_2$) higher (ΔT_m/mod. = +5.4 and +6.4 °C, respectively) than that obtained for natural TFO **33** with the same target (target B). These increased T_ms are comparable to those found for the corresponding 2′,4′-BNA-modified TFOs **34** and **35** (compare T_ms of **25** and **26** with **34** and **35**, respectively).

Mismatch discrimination by 3′-amino-2′,4′-BNA oligonucleotide **25** was examined against dsDNA targets containing a mismatched base in the homopurine strand. The results are summarized in Table 4. It was found that the T_m values of triplexes formed by 3′-amino-2′,4′-BNA-TFO **25** with mismatched dsDNAs having G:C, C:G, and T:A arrangements decreased significantly compared to matched DNA (A:T arrangement). Against dsDNA targets containing central G:C, C:G, and T:A arrangements, the T_m values decreased by 24, 23, and 39 °C, respectively. Except in the case of the G:C arrangement, these decreases are larger than those exhibited by natural DNA-TFO **33**. These values are also comparable to those found for 2′,4′-BNA-TFO **34**. These results show that, like 2′,4′-BNA, 3′-amino-2′,4′-BNA also has excellent mismatch discrimination ability.

Table 3
T_m Values of triplexes formed by 3′-amino-2′,4′-BNA oligonucleotides with dsDNA[a,b]

Oligonucleotides (5′-d……-3′)	Targets	T_m (ΔT_m/mod.) (°C)	
		−$MgCl_2$	+$MgCl_2$[b]
TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT (**33**)	A	33	44
TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT (**33**)	B	32	39
TTTTT^{m}CT**T**T^{m}CT^{m}CT^{m}CT (**25**)	A	44 (+11)	55 (+11)
TTT**T**T^{m}**C**T**T**^{m}**C**T^{m}**C**T^{m}CT (**26**)	B	59 (+5.4)	71 (+6.4)
TTTTT^{m}CT**t**T^{m}CT^{m}CT^{m}CT (**34**)	A	44 (+11)	57 (+13)
TTT**t**T^{m}**c**T**t**T^{m}**c**T^{m}**c**T^{m}CT (**35**)	B	60 (+5.6)	72 (+6.6)

[a] Target dsDNAs: Target A = 5′-d(GCTAAAAAGAAAGAGAGATCG)-3′/3′-d(CGATTTTCTTTCTCTCTAGC)-5′, Target B = 5′-d(CGATCTCTCTTTCTTTTTAGCCCCCGCTAAAAAGAAAGAGAGATCG)-3′; underlined portion indicates the target site for triplex formation.

[b] Conditions: 7 mM sodium phosphate buffer (pH 7.0) containing 140 mM KCl in the absence or the presence of $MgCl_2$ (10 mM); strand concentration = 1.5 μM. **T** = 3′-amino-2′,4′-BNA-T, **^{m}C** = 3′-amino-2′,4′-BNA-^{m}C, **t** = 2′,4′-BNA-T, **^{m}c** = 2′,4′-BNA-^{m}C.

Table 4
Sequence-specific triplex formation by 3′-amino-2′,4′-BNA-modified TFOs[a]

T (TFO)	T_m ($\Delta T_m = T_{m\,(mismatch)} - T_{m\,(match)}$) (°C)			
	X:Y = A:T (match)	G:C	C:G	T:A
Natural (**33**)	44	20 (−24)	25 (−19)	17 (−25)
3′-Amino-2′,4′-BNA (**25**)	55	31 (−24)	32 (−23)	16 (−39)
2′,4′-BNA (**34**)	57	31 (−26)	35 (−22)	16 (−41)

Modified TFO: 5′-d(TTTTT^{m}CT**T**T^{m}CT^{m}CT^{m}CT)-3′.
Target dsDNA: 5′-d(GCTAAAAAGA**X**AGAGAGATCG)-3′.
3′-d(CGATTTTTCT**Y**TCTCTCTAGC)-5′.

[a] Conditions: 7 mM sodium phosphate buffer (pH 7.0) containing 140 mM KCl and 10 mM $MgCl_2$; strand concentration = 1.5 μM.

2.6. Nuclease resistance properties

Resistance to degradation by nucleases is extremely important for the in vivo application of oligonucleotides. Our preliminary report showed the superior nuclease resistance of the 3′-amino-2′,4′-BNA oligonucleotide over natural DNA, 2′,4′-BNA and phosphorothiate oligonucleotides at low nuclease (snake venom phosphodiesterase or SPVDE, Boehringer Mannheim) concentration.[35] In the present study, in order to understand the relative nuclease resistance of 3′-amino-2′,4′-BNA with N3′→P5′-natural DNA (or 3′-amino-DNA) and other structurally related analogues, we conducted nuclease resistance studies at high nuclease concentration using *Crotalus admanteus* venom phosphodiesterase (CAVP) (Pharmacia). Figure 3 shows the collective nuclease resistance profiles of 3′-amino-2′,4′-BNA oligonucleotide **27**, 3′-amino-DNA oligonucleotide **39**, and other nucleic acid analogues (oligonucleotides **36**–**38**). Under the experimental conditions (see Experimental), the natural oligothymidylates **36** and 2′,4′-BNA oligonucleotide **37** were completely digested within 3 and 10 min, respectively, upon exposure to 0.8 μg of CAVP (Fig. 3A). Phosphorothioate oligonucleotide **38** degraded gradually (about 50% decomposed after 60 min) and 3′-amino-DNA-oligonucleotide **39** degraded only slightly. On the other hand, 3′-amino-2′,4′-BNA oligonucleotide **27** remained intact

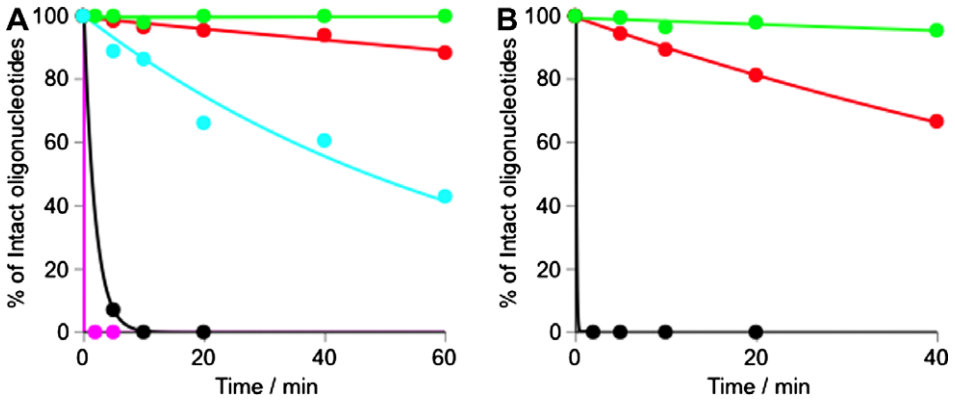


Figure 3. Nuclease resistance of T$_8$**X**T oligonucleotides against CAVP; **X** = natural-T (**36**) (magenta), 2′,4′-BNA-T (**37**) (black), phosphorthioate-T (**38**) (cyan), 3′-amino-DNA-T (**39**) (red) and 3′-amino- 2′,4′-BNA-T (**27**) (green). Hydrolysis of the oligonucleotides (3 nmol) was carried out at 37 °C in buffer (400 μl) containing 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$ and CAVP (0.8 μg for experiment A and 1.6 μg for experiment B).

even after 60 min exposure. Next, the CAVP concentration was increased two fold (1.6 μg for 3 nmol oligonucleotide, Fig. 3B) and resistance to nuclease degradation was examined. The 2′,4′-BNA-modified oligonucleotide **37** decomposed quantitatively within 2 min, and 3′-amino-DNA oligonucleotide **39** also decomposed at a constant rate depending on the time of exposure. In marked contrast, 3′-amino-2′,4′-BNA oligonucleotide **27** was essentially intact even at that very high nuclease concentration. This result clearly indicates that 3′-amino-2′,4′-BNA has extremely high nuclease resistance, much higher than that of natural and 2′,4′-BNA-modified oligonucleotides, and substantially higher even than that of phosphorothioate oligonucleotides and 3′-amino-DNA oligonucleotide bearing the same N3′→P5′-linkage. The extraordinarily high nuclease resistance of 3′-amino-2′,4′-BNA results from the combined effects of the N3′→P5′ linkage and the 2′-*O*,4′-*C*-bridged moiety.

3. Conclusions

In conclusion, we have synthesized 3′-amino-2′,4′-BNA dimer and thymine and methyl cytosine monomer residues and incorporated them into oligonucleotides via conventional 3′→5′ assembly (for dimer residue) and 5′→3′ reverse assembly (for monomer residues) phosphoramidate protocols. The synthesis via the dimer residue has some limitations, such as oligonucleotides with consecutive modifications or containing residues with a variety of nucleobase combinations, cannot be synthesized. On the other hand, the synthetic protocol via the monomer residues eliminates these shortcomings and a variety of oligonucleotides, including a consecutively modified oligonucleotide, were synthesized efficiently. 3′-Amino-2′,4′-BNA-modified oligonucleotides exhibit very strong binding affinity for ssDNA, ssRNA, and dsDNA targets, comparable to that of 2′,4′-BNA. Moreover, 3′-amino-2′,4′-BNA shows better RNA selective binding affinity than 2′,4′-BNA. The mismatch discrimination of 3′-amino-2′,4′-BNA is similar to that of 2′,4′-BNA. The nuclease resistance of 3′-amino-2′,4′-BNA is excellent, much higher than that of natural and 2′,4′-BNA oligonucleotides, and substantially higher even than that of 3′-amino-DNA and phosphorothioate oligonucleotides. Given these superior properties, 3′-amino-2′,4′-BNA oligonucleotide has significant potential for practical antisense and antigene applications. Biological application of 3′-amino-2′,4′-BNA-modified oligonucleotides and synthesis of the 3′-amino-2′,4′-BNA-adenine and -guanine monomers are now in progress.

4. Experimental

4.1. Synthesis and characterization of compounds: general aspects and instrumentation

Melting points are uncorrected. All moisture-sensitive reactions were carried out in well-dried glassware under a N_2 atmosphere. Dichloromethane, dichloroethane, acetonitrile, triethylamine, and pyridine were distilled from CaH_2. Tetrahydrofuran (THF) was dried by CaH_2 and $LiAlH_4$. ^1H NMR (270 or 300 MHz), ^{13}C NMR (67 or 75 MHz), and ^{31}P NMR spectra (202 MHz) were recorded on a JEOL EX-270, JEOL-AL-300, and JEOL GX-500 spectrometer, respectively. Chemical shifts are reported in parts per million downfield from internal tetramethylsilane for ^1H spectra, $CHCl_3$ (δ = 77.0 ppm) or methanol (49.0) for ^{13}C NMR, and 85% H_3PO_4 (δ = 0 ppm) for ^{31}P spectra. IR spectra were recorded on a JASCO FT/IR-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 100 D was used. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra were recorded on an Applied Biosystems Voyager DE mass spectrometer.

Full experimental details and characterization data for compounds **2**–**10** are described in Supplementary Data. The experimental procedures and data for compounds **11**–**20** are given below:

4.1.1. 3′-Amino-5′-*O*-*tert*-butyldiphenylsilyl-3′-deoxy-5-methyl-2′-*O*,4′-*C*-methyleneuridine (11)

Triphenylphosphine (5.41 g, 29.6 mmol) was added to a solution of compound **5** (5.49 g, 10.3 mmol) (for details of the synthesis of **5**, see Supplementary Data) in pyridine (90.7 ml) and the mixture was stirred at room temperature for 6 h. After compound **5** disappeared, 28% ammonium solution (227 ml) was added and the resulting mixture was stirred at room temperature for 19 h. The solvent was evaporated under reduced pressure, and the residue was purified by column chromatography using ethyl acetate and *n*-hexane (2:1, v/v) as eluents to give compound **11** (5.26 g, quant.) as a white foam: mp 89–92 °C. $[\alpha]_D^{21}$ +49.51 (c = 1.00, $CDCl_3$). IR ν_{max} (KBr): 3175, 3046, 2955, 2858, 1700, 1467, 1430 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.05 (9H, s), 1.62 (3H, s), 3.27 (1H, s), 3.75, 3.68 (2H, AB, *J* = 9 Hz), 3.88, 4.00 (2H, AB, *J* = 12 Hz), 4.25 (1H, s), 5.54 (1H,s), 7.31–7.46 (6H, m), 7.61–7.66 (4H, m), 8.19 (1H, br). ^{13}C NMR ($CDCl_3$): δ 12.4, 19.5, 27.0, 53.6, 59.1, 70.9, 77.2, 80.5, 86.7, 90.2, 110.1, 127.9, 130.0, 130.1, 132.3, 132.6. MS (FAB): *m/z* 508 $(M+H)^+$. *Anal.* Calcd for $C_{27}H_{33}N_3O_5Si{\cdot}1/10\ H_2O$: C, 63.65; H, 6.56; N, 8.25. Found: C, 63.36; H, 6.58; N, 8.03.

4.1.2. 5′-*O*-*tert*-Butyldiphenylsilyl-3′-deoxy-3′-(4-methoxytriphenylmethylamino)-5-methyl-2′-*O*,4′-*C*-methyleneuridine (12)

4-Methoxytriphenylmethyl chloride (MMTrCl) (15.0 g, 48.5 mmol) was added to a solution of compound **11** (16.4 g, 32.3 mmol) in pyridine (191 ml). The reaction mixture was stirred at room temperature for 11 h. After the mixture was quenched with saturated $NaHCO_3$ (aq), the combined organic layers were extracted with dichloromethane, washed with water and brine, dried over sodium sulfate, and concentrated under reduced pressure. The residue was purified by column chromatography by eluting with ethyl acetate and *n*-hexane (1:2, v/v) to yield compound **12** (24.3 g, 97%) as a white foam: mp 102–105 °C. $[\alpha]_D^{22}$ +20.03 (*c* = 1.00, CH_3COCH_3). IR ν_{max} (KBr): 3168, 3064, 2934, 2250, 1687, 1507, 1464 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.13 (9H, s), 1.62 (3H, s), 1.94 (1H, d, *J* = 9.7 Hz), 2.47 (1H, s), 2.74 (1H, d, *J* = 10 Hz), 3.73 (3H, s), 3.82, 3.90 (2H, AB, *J* = 8.6 Hz), 4.25, 4.35 (2H, AB, *J* = 12 Hz), 5.36 (1H, s), 6.68–7.74 (10H, m), 8.63 (1H, br). ^{13}C NMR ($CDCl_3$): δ 12.2, 19.6, 27.1, 55.2, 56.9, 60.7, 70.5, 72.2, 77.2, 77.7, 86.4, 89.3, 109.5, 113.3, 126.7, 127.8, 128.0, 129.4, 130.3, 132.6, 134.0, 135.5, 137.2, 145.5, 145.6, 148.9, 158.1, 163.5. MS (FAB): *m/z* 780 $(M+H)^+$. Anal. Calcd for $C_{47}H_{49}N_3O_6Si{\cdot}3/4H_2O$: C, 71.14; H, 6.41; N, 5.30. Found: C, 71.21; H, 6.53; N, 5.05.

4.1.3. 3′-Deoxy-3′-(4-methoxytriphenylmethylamino)-5-methyl-2′-*O*,4-*C*-methyleneuridine (13)

To a solution of compound **12** (24.3 g, 31.2 mmol) in THF (282 ml) was added TBAF (1M in THF) (34.3 ml, 34.3 mmol) and the mixture was stirred at room temperature for 4 h. The reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography, eluting with ethyl acetate and *n*-hexane (3:1, v/v) to give compound **13** (14.7 g, 88%) as white foam: mp 136–141 °C. $[\alpha]_D^{22}$ +40.69 (*c* = 1.00, MeOH). IR ν_{max} (KBr): 3479, 3188, 3025, 1687, 1508, 1465, 1254 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.68 (3H, s), 2.01 (1H, s), 2.22 (1H, s), 2.95 (1H, s), 3.69, 3.92 (2H, AB, *J* = 8.1 Hz), 3.77 (3H, s), 4.13, 4.27 (2H, AB, *J* = 12 Hz), 5.28 (1H, s), 6.77 (2H, d, *J* = 8.6 Hz), 7.12–7.31 (8H, m), 7.47–7.49 (4H, m), 7.94 (1H, s). ^{13}C NMR ($CDCl_3$): δ 12.4, 55.7, 57.3, 58.2, 71.8, 72.9, 78.4, 87.6, 90.9, 110.1, 114.1, 127.6, 128.8,

129.3, 129.5, 130.9, 136.6, 138.8, 147.0, 147.6, 151.1, 159.8. MS (FAB): *m/z* 542 $(M+H)^+$. Anal. Calcd for $C_{31}H_{31}N_3O_6{\cdot}1H_2O$: C, 66.53; H, 5.94; N, 7.51. Found: C, 66.20; H, 5.96; N, 7.42.

4.1.4. 5′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-3′-dexoy-3′-(4-methoxytriphenylmethylamino)-5-methyl-2′-*O*,4′-*C*-methyleneuridine (14)

2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (70.7 μl, 2.23 mmol) was added to a suspension of compound **13** (500 mg, 928 μmol) and diisopropylammonium tetrazolide (261 mg, 1.3 mmol) in acetonitrile (6 ml) and THF (2 ml). The mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography using ethyl acetate and *n*-hexane (2:3→1:1, v/v) to give compound **14** (624 mg, 91%) as a white solid: mp 101–103 °C. ^{31}P NMR ($CDCl_3$): δ 148.8, 148.9.

4.1.5. 5′-*O*-Acetyl-3′-deoxy-3′-(4-methoxytriphenylemethylamino)-5-methyl-2′-*O*,4′-*C*-methyleneuridine (15)

Acetic anhydride (1.56 ml, 16.6 mmol) was added to a solution of compound **13** (8.11 g, 15.0 mmol) in pyridine (150 ml) and the mixture was stirred for 28 h at room temperature. The reaction mixture was quenched with 28% NH_4OH, then concentrated under reduced pressure and extracted with ethyl acetate. The combined organic layers were washed with water, brine, and then dried over sodium sulfate. After the solvent was removed, the residue was purified by column chromatography by eluting with ethyl acetate and *n*-hexane (1:1, v/v) to give compound **15** (15.1 g, 93%) as a white solid: mp 136–139 °C. $[\alpha]_D^{22}$ +46.66 (*c* = 0.50, $CHCl_3$). IR ν_{max} (KBr): 3193, 3060, 2251, 1746, 1687, 1606, 1507, 1254 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.52 (3H, s), 1.91 (1H, d, *J* = 10 Hz), 2.05 (3H, s), 2.33 (1H, s), 2.80 (1H, d, *J* = 11 Hz), 3.71 (3H, s), 3.73, 3.86 (2H, AB, *J* = 9 Hz), 4.50, 4.72 (2H, AB, *J* = 12 Hz), 5.28 (1H, s), 6.68 (2H, d, *J* = 9 Hz), 7.09–7.25 (8H, m), 7.34–7.37 (4H, m), 8.01 (1H, d, *J* = 5 Hz). ^{13}C NMR ($CDCl_3$): δ 2.6, 21.0, 55.3, 56.7, 60.0, 70.5, 71.7, 77.2, 77.3, 86.2, 86.8, 109.3, 113.3, 126.8, 127.9, 128.0, 129.4, 134.1, 137.0, 145.2, 145.4,148.8, 158.1, 163.3, 169.9. MS (EI): *m/z* 583 (M^+, 0.7), 372 (100). Anal. Calcd for $C_{33}H_{33}N_3O_7{\cdot}1/2H_2O$: C, 66.88; H, 5.78; N, 7.09. Found: C, 66.73; H, 5.91; N, 6.80.

4.1.6. 5′-*O*-Acetyl-3′-deoxy-3′-(4-methoxytriphenylmethylamino)-4-(1,2,4-triazol-1-yl)-5-methyl-2′-*O*,4′-*C*-methyleneuridine (16)

Triethylamine (25.5 ml, 184 mmol) was added dropwise to a stirred mixture of 1*H*-1,2,4-triazole (11.7 g, 169 mmol) and phosphoryl chloride (3.48 ml, 37.3 mmol) in acetonitrile (50.0 ml) at 0 °C. A solution of **15** (1.67 g, 2.38 mmol) in acetonitrile (19 ml) was then added to the mixture at 0 °C. The resulting mixture was stirred at room temperature for 6 h. The reaction mixture was quenched by the addition of $NaHCO_3$ (aq), and was concentrated under reduced pressure. The residue was diluted with dichloromethane, washed with saturated $NaHCO_3$ (aq), water and brine, and dried over sodium sulfate. The solvent was evaporated under reduced pressure. The residue was purified by column chromatography using ethyl acetate and *n*-hexane (1:1→2:1, v/v) to give compound **16** (1.79 g, 99%) as a yellow foam: ^{1}H NMR ($CDCl_3$): δ 2.03 (1H, d, *J* = 11 Hz), 2.14 (3H, s), 2.34 (3H, s), 2.65 (1H, s), 2.83 (1H, d, *J* = 10 Hz), 3.77 (3H, s), 3.87, 3.99 (2H, AB, *J* = 9 Hz), 4.58, 4.84 (2H, AB, *J* = 12 Hz), 5.52 (1H, s), 6.70 (2H, d, *J* = 9 Hz), 7.18–7.29 (8H, m), 7.37–7.40 (4H, m), 7.78 (1H, s), 8.16 (1H, s) 9.33 (1H, s).

4.1.7. 5′-*O*-Acetyl-3′-deoxy-3′-(4-methoxytriphenylmethylamino)-5-methyl-2′-*O*,4′-*C*-methylenecytidine (17)

Compound **16** (954 mg, 1.51 mmol) was dissolved in a mixture of 28% ammonium solution and dioxane (35.0 ml, 1:6, v/v) and stirred at room temperature for 1.5 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography using ethyl acetate and methanol (10:1, v/v) to yield compound **17** (781 mg, 89%) as a white solid: mp 154–158 °C. $[\alpha]_D^{22}$ +120.62 (*c* = 0.52, $CHCl_3$). IR ν_{max} (KBr): 3087, 2250, 1745, 1666, 1610, 1488, 1250 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.80 (3H, s), 2.01 (1H, d, *J* = 10 Hz), 2.10 (3H, s), 2.44 (1H, s), 2.85 (1H, d, *J* = 8.0 Hz), 3.78 (3H, s), 4.03, 4.10 (2H, AB, *J* = 8.0 Hz), 4.86, 4.90 (2H, AB, *J* = 13 Hz), 5.34 (1H, s), 6.73 (2H, d, *J* = 9.0 Hz), 7.18–7.30 (8H, m), 7.42–7.44 (4H, m), 8.35 (1H, d, *J* = 7.0 Hz). ^{13}C NMR ($CDCl_3$): δ 13.5, 21.1, 55.2, 56.5, 60.0, 70.5, 71.7, 77.2, 77.3, 86.5, 86.8, 101.0, 113.3, 126.8, 127.9, 128.0, 129.3, 136.7, 137.1, 145.2, 145.4, 155.1, 158.1, 165.4, 170.0.

4.1.8. 5′-*O*-Acetyl-*N*^{4}-benzoyl-3′-deoxy-3′-(4-methoxytriphenylmethylamino)-5-methyl-2′-*O*,4′-*C*-methylenecytidine (18)

Trimethylamine (324 μl, 2.34 mmol) and benzoyl chloride (136 μl, 1.17 mmol) were added to a solution of compound **17** (575 mg, 987 μmol) in dichloromethane (10 ml). The mixture was stirred at room temperature for 12 h. The reaction was quenched by the addition of saturated $NaHCO_3$ (aq), and the mixture was extracted with ethyl acetate. The combined organic layer was washed with water, then brine, and dried over sodium sulfate. Evaporation to dryness under reduced pressure, followed by column chromatography using ethyl acetate and *n*-hexane (1:2, v/v), gave compound **18** (581 mg, 89%) as a white solid: mp 107–110 °C. $[\alpha]_D^{21}$ +156.11 (c = 0.57, $CHCl_3$). IR ν_{max} (KBr): 3074, 2054, 1706, 1565, 1247 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.99 (3H, s), 2.03 (1H, d, *J* = 10 Hz), 2.15 (3H, s), 2.44 (1H, s), 2.88 (1H, d, *J* = 11 Hz), 3.78 (3H, s), 3.82, 3.94 (2H, AB, *J* = 8.0 Hz), 4.59, 4.82 (2H, AB, *J* = 13 Hz), 5.40 (1H, s), 6.74 (2H, d, *J* = 9.0 Hz), 7.09–7.25 (8H, m), 7.34–7.37 (4H, m), 8.35 (1H, d, *J* = 7.0 Hz). ^{13}C NMR ($CDCl_3$): δ 13.6, 21.2, 55.3, 56.6, 70.5, 71.7, 77.1, 77.2, 86.5, 86.9, 110.5,113.3, 126.9, 127.9, 128.0, 128.1, 129.3, 129.8,132.5, 135.3, 136.9, 145.1, 146.6, 158.2, 159.5, 169.9,179.5. Anal. Calcd for $C_{40}H_{38}N_4O_7{\cdot}1/3H_2O$: C, 69.35; H, 5.63; N, 8.09. Found: C, 69.28; H, 5.64; N, 8.00.

4.1.9. *N*^{4}-Benzoyl-3′-deoxy-3′-(4-methoxytriphenylmethylamino)-5-methyl-2′-*O*,4′-*C*-methylenecytidine (19)

Lithium hydroxide (210 mg, 4.99 mmol) was added to a solution of compound **18** (685 mg, 999 μmol) in a mixture of THF and water (9.53 ml, 2:1, v/v), and the mixture was stirred for 4 h at room temperature. After dilution with water, the mixture was extracted with dichloromethane, the organic phase was washed with water, then brine, and dried over sodium sulfate. The solvents were removed under reduced pressure and the residue was purified by column chromatography by eluting with ethyl acetate and *n*-hexane (1:1, v/v) to provide compound **19** (623 mg, 97%) as a white solid: mp 215–218 °C. $[\alpha]_D^{21}$ +110.68 (c = 0.55, $CHCl_3$). IR ν_{max} (KBr): 3468, 3065, 2954, 2250, 1702, 1644, 1565 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.90 (3H, s), 2.33 (1H, s), 2.95 (1H, s), 2.85 (1H, d, *J* = 8.0 Hz), 3.75 (3H, s), 3.71, 3.94 (2H, AB, *J* = 9.0 Hz), 4.15, 4.30 (2H, AB, *J* = 12 Hz), 5.35 (1H, s), 6.74 (2H, d, *J* = 9.0 Hz), 7.12–7.29 (8H, m), 7.46–7.48 (4H, m), 8.25 (2H, br). ^{13}C NMR ($CDCl_3$): δ 13.6, 55.3, 56.4, 58.5, 70.6, 71.7, 77.2, 77.3, 86.5, 89.1, 110.7, 113.4, 127.0, 127.9, 128.1, 129.3, 129.8, 132.5, 135.6, 136.9,137.0, 145.3, 145.4, 146.7, 158.3, 159.6. Anal. Calcd for $C_{38}H_{36}N_4O_6{\cdot}1$AcOEt: C, 68.84; H, 6.05; N, 7.64. Found: C, 68.57; H, 5.87; N, 7.97.

4.1.10. *N*^{4}-Benzoyl-5′-*O*-[2-cyanoethoxy(diisopropylamino)-phosphino]-3′-deoxy-3′-(4-methoxytriphenylmethylamino)-5-methyl-2′-*O*,4′-*C*-methylenecytidine (20)

2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (59.5 μl, 1.87 mmol) was added to a suspension of compound **19** (502 mg, 780 μmol) and diisopropylammonium tetrazolide

(220 mg, 1.09 mmol) in acetonitrile (6 ml) and THF (2 ml). The mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography using ethyl acetate and *n*-hexane (1:2, v/v) to give compound **20** (575 mg, 87%) as a white solid: mp 96–98 °C. ^{31}P NMR ($CDCl_3$): δ 148.7, 148.8.

4.2. Oligonucleotide synthesis and purification

Synthesis of 3′-amino-2′,4′-BNA-modified oligonucleotides **21**–**27** was performed on a 0.2 μmol scale following the 5′→3′ reverse assembly protocol using phosphoramidites **14** and **20** (and/or the 3′–5′ conventional phosphoramidite assembly protocol using heterodimer **10** (for oligonucleotides **21** and **25** only)) on an Expedite (TM) 8909 Nucleic Acid Synthesis System. All 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 for 3′-amino-2′,4′-BNA monomers (320 s instead of 80 s used for natural monomers). Dicyanoimidazole was found to be a more efficient activator than 1-*H* tetrazole. Standard CPG-solid supports from Glen Research were used for the synthesis. Using a trityl monitor, the coupling efficiency of 3′-amino-2′,4′-BNA monomers was estimated to be 91–94%. After synthesis, the solid-supported oligonucleotides were treated with 28% ammonium hydroxide solution (1 ml) at room temperature for 1.5 h, then at 55 °C for 17–20 h. The ammonia solutions were then concentrated, and the crude oligonucleotides were initially purified by NAP^{TM} 10 columns (Amersham Biosciences) followed by further purification by reverse phase high performance liquid chromatography (RP-HPLC) on a Waters X-Terra (10 × 50 mm) column using 6–14% MeCN in 0.1 M triethylammonium acetate buffer (pH 7.0) at 50 °C. The oligonucleotides were analyzed for purity by HPLC and characterized by MALDI-TOF mass spectroscopy.

4.3. UV melting experiments

UV melting experiments were carried out on Beckman DU-650 and Shimadzu UV-1650PC spectrometers equipped with a T_m analysis accessory. For the duplex formation study, equimolar amounts of the target RNA/DNA strand and each 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 to 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.

4.4. Nuclease resistance studies

Crotalus admanteus venom phosphodiesterase (CAVP) (0.8 μg for experiment A and 1.6 μg for experiment B, Fig. 3) was added to a solution of oligonucleotides **27** and **36–39**, (3 nmol each) 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, equal portions of each reaction mixture were collected and heated to 90 °C for 3 min in order to inactivate the nuclease. The amount of intact oligonucleotide remaining was evaluated by RP-HPLC.

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Supplementary data

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