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Synthesis and properties of a novel bridged nucleic acid with a P3′ → N5′ phosphoramidate linkage, 5′-amino-2′,4′-BNA†

Satoshi Obika, Osamu Nakagawa, Akiko Hiroto, Yoshiyuki Hari and Takeshi Imanishi*

Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: imanishi@phs.osaka-u.ac.jp; Fax: +81 6 6879 8204; Tel: +81 6 6879 8200

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5′-Amino-2′,4′-BNA, a novel analogue of BNA series compounds, was successfully synthesized, and its incorporated oligonucleotides showed potent duplex- and triplex-forming ability and resistance against snake venom phosphodiesterase.

In the last decade the synthesis and evaluation of various nucleic acid analogues have been developed for their practical antisense and antigene applications.[1–5] One promising chemical derivative of natural oligonucleotides is involved in the internucleoside linkage modifications. N3′ → P5′ phosphoramidate linked oligonucleotides were known to show superior duplex- and triplex-forming affinity because of the dominant N-type sugar puckering which decreases the *gauche* effect between 3′-nitrogen and 4′-oxygen by replacing from 3′-oxygen to nitrogen, as well as excellent nuclease resistant ability.[6–9] On the other hand, more easily preparable P3′ → N5′ phosphoramidate can be cleaved at its phosphoramidate linkage under mild acidic conditions,[10] and this was applied to a novel technology of DNA sequence-determination.[11] However, oligonucleotide P3′ → N5′ phosphoramidates were reported to have only weak binding affinity for complementary nucleic acid strands, probably due to the difference in preferential sugar conformation (Fig. 1).[12–16]

Recently, we achieved the synthesis of a novel nucleoside with a fixed N-type conformation, 2′-*O*,4′-*C*-methylene bridged nucleic acid (2′,4′-BNA)[17] and found that 2′,4′-BNA modified oligonucleotides exhibited strong hybridizing ability with complementary strands of RNA and DNA.[18–20] Moreover, these 2′,4′-BNA oligonucleotides appeared to possess high affinity for dsDNA.[19–24]

Therefore, we were interested in a combination of 2′,4′-BNA and P3′ → N5′ phosphoramidate linkage to develop P3′ → N5′ phosphoramidate linked oligonucleotides having N-type sugar puckering. We report here the synthesis, hybridizing properties and enzymatic stability of novel oligonucleotide analogues with a 5′-amino-2′,4′-BNA monomer unit (Fig. 1).

The synthesis of the 5′-amino-2′,4′-BNA modified oligonucleotides is shown in Scheme 1. Starting material **1**[25] was coupled with silylated thymine to give **2**. On exposure of **2** to K_2CO_3, bicyclic nucleoside **3** was obtained. Desilylation of **3** followed by mesylation provided **4**, which underwent catalytic reduction to afford **5**. Reaction of **5** with NaN_3, and reduction of an azide group gave the desired 5′-amino-2′,4′-BNA **6**; without further purification, **6** was tritylated into **7**, which was phosphitylated to give amidite **8**. By using **8** and natural amidite building blocks, 5′-amino-2′,4′-BNA modified oligonucleotides **9–15** were successfully prepared according to a standard phosphoramidite protocol.‡

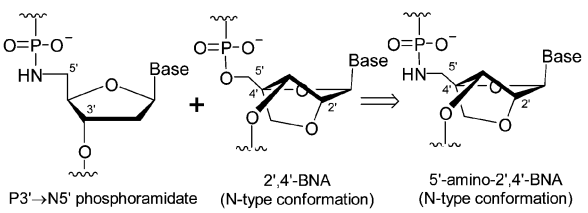
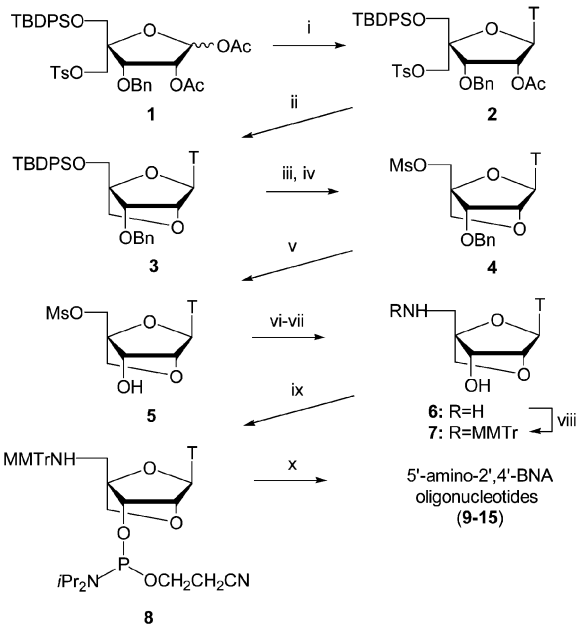


Fig. 1 Structures of the modified oligonucleotides.

The duplex-forming ability of 5′-amino-2′,4′-BNA modified oligonucleotides **9–11** with complementary ssDNA and ssRNA was evaluated under near-physiological conditions by means of melting temperature (T_m) experiments (Table 1). Replacement of natural nucleotides by 5′-amino-2′,4′-BNA resulted in significant stabilization of the duplexes formed with both complementary DNA and RNA, and increases in T_m per modification by +2 to +5 °C towards DNA and +4 to +5 °C towards RNA were observed. This increase in T_m is roughly comparable to that of the corresponding 2′,4′-BNA modified oligonucleotides **16–18**. Note that oligonucleotides containing a 2′,4′-BNA unit and a P3′ → N5′ phosphoramidate linkage showed great affinity for complementary RNA and DNA, in contrast with the decreasing stability of the duplex in the case of P3′ → N5′ phosphoramidate linked oligonucleotides having no 2′,4′-BNA framework.[12]

Next, the binding affinity of 5′-amino-2′,4′-BNA modified oligonucleotides for homopurine–homopyrimidine dsDNA was studied under neutral conditions with or without $MgCl_2$. Although the T_m values of 5′-amino-2′,4′-BNA modified oligonucleotides **12–14** were slightly lower than those of 2′,4′-BNA modified oligonucleotides **19–21** (Table 2), the ΔT_m



Scheme 1 *Reagents and conditions*: i, 2TMS-T, TMSOTf, $ClCH_2CH_2Cl$, reflux, 97%; ii, K_2CO_3, MeOH, r.t., 94%; iii, TBAF, THF, r.t., 92%; iv, MsCl, pyridine, r.t., 99%; v, $Pd(OH)_2$/C, cyclohexene, EtOH, reflux, 98%; vi, NaN_3, DMF, 100 °C, quant.; vii, PPh_3, pyridine, r.t., then NH_4OH aq., r.t.; viii, MMTrCl, pyridine, r.t., 87% over 2 steps; ix, (*i*-$Pr_2N)_2POCH_2CH_2CN$, diisopropylammonium tetrazolide, MeCN–THF, r.t., 90%; x, DNA synthesizer (AB Expedite™ 8909).

† Electronic supplementary information (ESI) available: acid-mediated cleavage of modified or natural oligonucleotides. See http://www.rsc.org/suppdata/cc/b3/b307290h/

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Table 1 T_m values of 5′-amino-2′,4′-BNA modified oligonucleotides with complementary DNA and RNA[a]

Oligonucleotide	T_m (ΔT_m/modification)/°C	
	DNA	RNA
5′-GCGTTTTTTGCT-3′	47	45
5′-GCGTTT**T**TTTGCT-3′(**9**)	52 (+5)	50 (+5)
5′-GCGTTT**T**TT**T**GCT-3′(**10**)	52 (+3)	54 (+5)
5′-GCG**T**T**T**T**T**GCT-3′(**11**)	53 (+2)	58 (+4)
5′-GCGTT**t**TTTGCT-3′(**16**)	53 (+6)	52 (+7)
5′-GCGTT**t**T**t**TGCT-3′(**17**)	54 (+4)	57 (+6)
5′-GCG**t**T**t**T**t**TGCT-3′(**18**)	56 (+3)	62 (+6)

[a] UV melting profiles measured in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl at a scan rate of 0.5 °C min^{-1} at 260 nm. The concentration of oligonucleotide used was 4 μM for each strand. The sequence of target DNA or RNA complements is 5′-AGCAAAAAACGC-3′. **T**: 5′-amino-2′,4′-BNA with thymine. **t**: 2′,4′-BNA with thymine.

values per modification of **12–14** were over 7 °C higher than those of the natural oligonucleotide.

Table 2 T_m values of 5′-amino-2′,4′-BNA modified oligonucleotides with dsDNA[a]

Oligonucleotide	T_m (ΔT_m/modification)/°C	
	−$MgCl_2$	+10 mM $MgCl_2$
5′-TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT-3′	32	42
5′-TTTTT^{m}CT**T**T^{m}CT^{m}CT^{m}CT-3′ (**12**)	40 (+8)	50 (+8)
5′-TTTTT^{m}CT**T**T^{m}C**T**^{m}CT^{m}CT-3′ (**13**)	45 (+7)	57 (+8)
5′-TTTT**T**^{m}CT**T**T^{m}C**T**^{m}CT^{m}CT-3′ (**14**)	52 (+7)	64 (+7)
5′-TTTTT^{m}CT**t**T^{m}CT^{m}CT^{m}CT-3′ (**19**)	42 (+10)	52 (+10)
5′-TTTTT^{m}CT**t**T^{m}C**t**^{m}CT^{m}CT-3′ (**20**)	48 (+8)	60 (+9)
5′-TTTT**t**^{m}CT**t**T^{m}C**t**^{m}CT^{m}CT-3′ (**21**)	56 (+8)	70 (+9)

[a] UV melting profiles were 7 mM sodium phosphate buffer (pH 7.0) containing 140 mM KCl at a scan rate of 0.5 °C min^{-1} at 260 nm. The concentration of oligonucleotide used was 1.5 μM for each strand. The sequence of target dsDNA is 5′-GCTGCTAAAAAGAAAGAGA-GATCGTCG-3′/3′-CGACGATTTTTCTTTCTCTCTAGCAGC-5′. **T**: 5′-amino-2′,4′-BNA with thymine. **t**: 2′,4′-BNA with thymine. ^{m}C: 2′-deoxy-5-methylcytidine.

The nuclease-resistance of 5′-amino-2′,4′-BNA modified oligonucleotide **15** against 3′-exonuclease (snake venom phosphodiesterase, SVPDE) was compared with that of natural oligothymidilate **22** and 2′,4′-BNA modified oligonucleotide **23** (Fig. 2). The degradation of oligonucleotides was analyzed by reversed-phase HPLC analysis, and was determined by the percentage of full-length oligonucleotides at several time points. Although natural oligonucleotide **22** was immediately digested within 5 min, 50% of 5′-amino-2′,4′-BNA modified oligonucleotide **15** was unchanged after 40 min (Fig. 2). In addition, the nuclease-resistance of 5′-amino-2′,4′-BNA modified oligonucleotide **15** was found to be superior to that of 2′,4′-BNA oligonucleotide **23**.

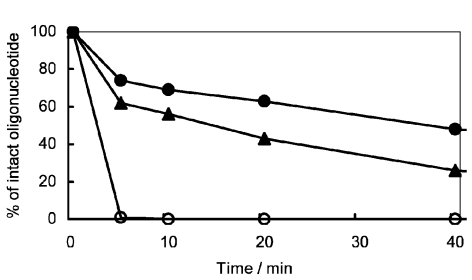

Fig. 2 Enzymatic stability of modified oligonucleotides (5′-TTTTTTTTTX-3′, X: natural T (**22**) (open circles), 2′,4′-BNA T (**23**) (triangles) or 5′-amino-2′,4′-BNA T (**15**) (closed circles)) against SVPDE. Hydrolysis of the oligonucleotides (10 μg) was carried out at 37 °C in a buffer (320 μl) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ and SVPDE (0.05 μg).

5′-Amino-2′,4′-BNA modified oligonucleotide **15** was readily cleaved under mild acidic condtions, while the corresponding natural (**22**) and 2′,4′-BNA modified (**23**) oligonucleotides were quite stable under the same conditions (ESI†).

In conclusion, the synthesis of 5′-amino-2′,4′-BNA monomer and its modified oligonucleotides was successfully accomplished. The modified oligonucleotides showed great properties of hybridization to ssDNA, ssRNA and dsDNA, along with resistance to enzymatic degradation. We also confirmed that 5′-amino-2′,4′-BNA modified oligonucleotide can be cleaved under mild acidic condtions. These properties of 5′-amino-2′,4′-BNA modified oligonucleotides would be useful not only for antisense/antigene applications but also for many novel technologies in the post-genome-sequencing era.

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Notes and references

‡ The coupling yields for attachment of amidite **8**, determined by a trityl monitor, were >94%. The synthesized oligonucleotides were purified by reversed-phase HPLC, and the compositions were determined by MALDI-TOF-MS: **9** [M − H]$^-$ 3659.50 (calc. 3659.45); **10** [M − H]$^-$ 3686.56 (calc. 3686.47); **11** [M − H]$^-$ 3713.70 (calc. 3713.50); **12** [M − H]$^-$ 4523.63 (calc. 4523.09); **13** [M − H]$^-$ 4550.33 (calc. 4550.11); **14** [M − H]$^-$ 4577.95 (calc. 4577.14); **15** [M − H]$^-$ 3006.37 (calc. 3006.08).

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