N-Methyl substituted 2′,4′-BNA^{NC}: a highly nuclease-resistant nucleic acid analogue with high-affinity RNA selective hybridization†

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Received (in Cambridge, UK) 16th May 2007, Accepted 28th June 2007
First published as an Advance Article on the web 9th July 2007
DOI: 10.1039/b707352f

Oligonucleotides modified with a novel BNA analogue, 2′,4′-BNA^{NC}[N–Me], were synthesized, and in comparison to 2′,4′-BNA (LNA), have similarly high RNA affinity, better RNA selectivity and much higher resistance to nuclease degradation, suggesting that the novel BNA analogue may be particularly useful for antisense approaches.

For practical use in antisense approaches, chemically modified oligonucleotides should possess: (i) high resistance to nuclease degradation and (ii) high affinity to the target RNA strand, along with excellent RNA selectivity.[1] Although during the past few decades several chemically modified oligonucleotides with robust ability to bind RNA have been developed, most also have an increased affinity to complementary DNA.[2] Conformationally restricted amide-linked nucleic acids[3] and 2′,5′-linked DNA[4] have been reported to have selective binding affinity for complementary RNAs. However, in most cases, their hybridizing ability to complementary RNAs was similar to or less than that observed for natural DNA, which greatly restricts their usefulness for practical applications, such as in antisense technology and for diagnostic purposes. Recently, α-LNA[5] and α-L-LNA[6] have been reported to have excellent RNA specific binding affinity *via* parallel motif hybridization. Nevertheless, it was concluded that the thermal stability of parallel duplexes is lower than those of corresponding antiparallel duplexes. Backbone extended pyrrolidine peptide nucleic acids (*bep*-PNA)[7] and thioacetamido nucleic acids (TANA)[8] also possessed promising RNA selective binding affinity. However, their mode of RNA targeting was achieved *via* triplex formation, which requires the use of two folds of antisense nucleic acids. In addition to that, PNA has its own problems that restrict its usefulness, including limited aqueous solubility, poor cellular uptake[9] and ambiguity in binding complementary DNA/RNA in both parallel and antiparallel orientations.[10] Our aim is to develop an antisense oligonucleotide that is sufficiently stable in physiological conditions and binds well to RNA complements while at the same time, showing lower affinity to DNA complements. Oligonucleotides which meet these criteria are likely to be much more useful for *in vivo* antisense applications.

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† Electronic supplementary information (ESI) available: Full experimental procedures, characterization data of all new compounds, and UV melting profiles. See DOI: 10.1039/b707352f
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Several years ago, our group and Wengel and co-workers independently discovered 2′-*O*,4′-*C*-methylene bridged nucleic acids (2′,4′-BNA[11] or LNA[12]) (Fig. 1), which possesses higher nuclease resistance than natural DNA and exhibit unprecedented ability to hybridize with complementary RNA and DNA. This nucleic acid analogue has applications in therapeutics and genomics[13] and is now commercially available. However, inadequate RNA selectivity and nuclease resistance of 2′,4′-BNA (LNA) modified oligonucleotides might present obstacles to developing ideal antisense molecules.[14] Development of ethylene bridged nucleic acid (ENA) resulted in improved nuclease resistance to some extent but affinity to RNA was lowered slightly and RNA selectivity of ENA is similar to that of 2′,4′-BNA (LNA).[15] Therefore, we focused our attention on fine-tuning the BNA structure. As a result of our continued investigation, we report here the development of another bridged nucleic acid analogue; namely, *N*-methyl substituted 2′,4′-BNA^{NC} or 2′,4′-BNA^{NC}[N–Me]),[16] which displays high-affinity RNA selective hybridizing ability[17] and excellent nuclease resistance.

As shown in Scheme 1, 2′,4′-BNA^{NC}[N–Me] monomer **5**, thymine phosphoroamidite **6** and oligonucleotides **7–10** were synthesized from nucleoside derivative **1**.[16] The compound **1** was treated with hydrazine and the resulting free amino group[18] was reacted with benzyl chloroformate to give **2**. Base mediated cyclization of **2** proceeded smoothly to afford the tricyclic compound **3**, which was subjected to boron trichloride in dichloromethane to furnish the desired cyclized product **4** in good yield. 2′,4′-BNA^{NC}[N–Me] monomer **5** was then obtained by reductive methylation and subsequent desilylation. The structure of **5** was confirmed by ^{1}H NMR spectroscopy and X-ray crystallography.[19] In order to synthesize phosphoroamidite, the primary hydroxyl group of **5** was protected with a 4,4′-dimethoxytrityl group and the secondary hydroxyl was phosphitylated with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite, forming the 2′,4′-BNA^{NC}[N–Me]-thymine phosphoroamidite **6**. The 2′,4′-BNA^{NC}[N–Me]-modified oligonucleotides **7–10** were then synthesized in an automated DNA synthesizer according to a

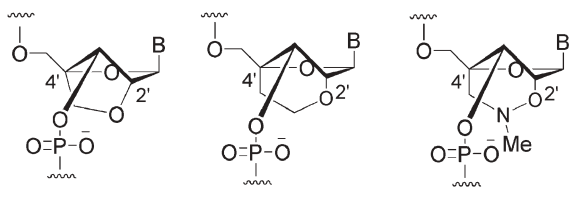
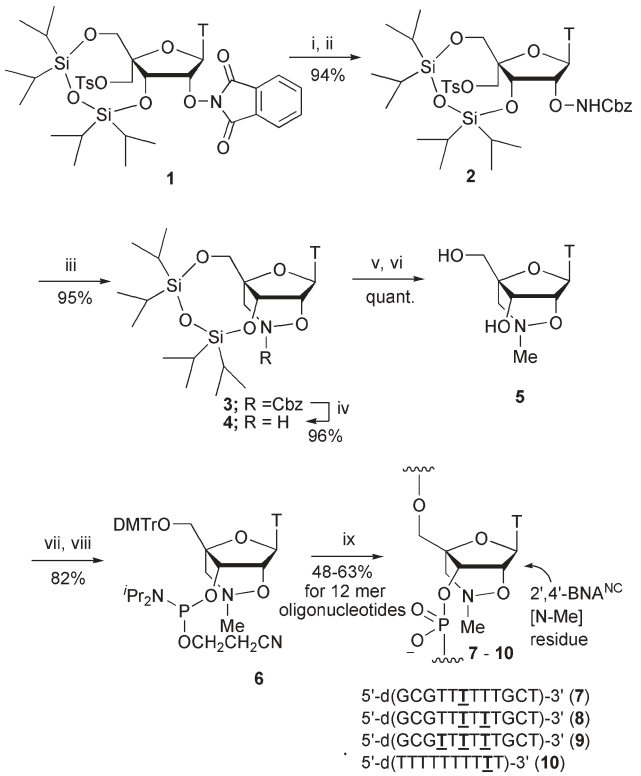


Fig. 1 Structures of 2′,4′-BNA (LNA), ENA and 2′,4′-BNA^{NC}[N–Me].



Scheme 1 *Reagents and conditions*: (i) $H_2NNH_2{\cdot}H_2O$, EtOH, rt, 10 min; (ii) CbzCl, sat. $NaHCO_3$, CH_2Cl_2, 0 °C; (iii) NaH, THF, rt, 5 h; (iv) BCl_3, CH_2Cl_2, 0 °C, 1 h; (v) 20% HCHO, $NaBH_3CN$, PPTS, MeOH, 0 °C, 1 h; (vi) TBAF, THF, rt, 5 min; (vii) DMTrCl, pyridine, rt, 12 h; (viii) $(^iPr_2N)_2PO(CH_2)_2CN$, dicyanoimidazole, MeCN, rt, 4 h; ix) DNA synthesizer (AB Expedite™ 8909). **T** = 2′,4′-BNA^{NC}[N–Me] residue.

standard phosphoroamidite protocol. The coupling efficiency of the modified oligonucleotides was 96–99% and the isolated yields were 48 to 63%. For comparison, a set of 2′,4′-BNA (LNA)-modified oligonucleotides **11–13** (Fig. 2) and **14** (Fig. 3) were synthesized, accordingly. The oligonucleotides were purified by HPLC and characterized by MALDI-TOF mass spectra.§

The ability of 2′,4′-BNA^{NC}[N–Me] oligonucleotides to hybridize to complementary RNA and DNA strands was evaluated *via* UV melting experiments and compared with that of 2′,4′-BNA (LNA)-modified oligonucleotides. The change in melting temperatures (ΔT_m) of the duplexes formed by 2′,4′-BNA^{NC}[N–Me]- and 2′,4′-BNA (LNA)-modified oligonucleotides (**7–9** and **11–13**, respectively) as compared with duplexes formed by natural DNA are summarized in Fig. 2.[20] It was found that the T_m values for hybrids formed by 2′,4′-BNA^{NC}[N–Me] oligonucleotides with RNA complements were high and comparable to T_m values for hybrids formed by 2′,4′-BNA (LNA)-modified oligonucleotides, **11–13**. In contrast, there is a great difference in their abilities to form a duplex with complementary DNA. Whereas 2′,4′-BNA (LNA)-modified oligonucleotides (**11–13**) exhibited a gradually increasing affinity for DNA complements upon increase of the number of modifications, the corresponding 2′,4′-BNA^{NC}[N–Me]-modified oligonucleotides showed no change or slightly decreased affinity towards target DNA; that is, T_m values decreased relative to that exhibited by the natural DNA **15**.[21] This interesting property (*i.e.* decreased affinity to complementary DNA while

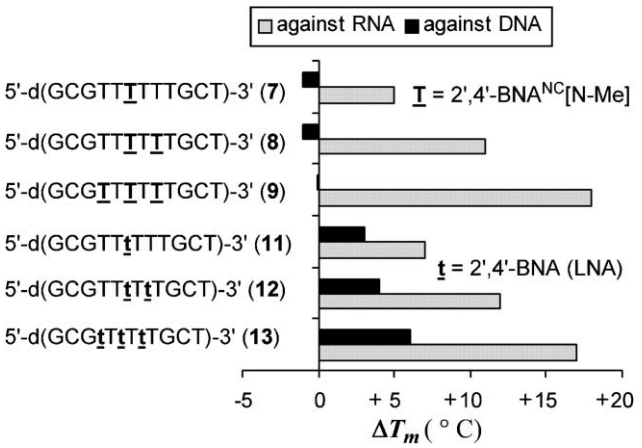


Fig. 2 The change in melting temperature (ΔT_m) of modified oligonucleotides (**7–9** and **11–13**) relative to the reference oligonucleotide **15**, 5′-d(GCGTTTTTTGCT)-3′. The T_m values of the duplexes formed by **15** with complementary DNA and RNA were 50 and 45 °C, respectively. T_m values were obtained from the maxima of the first derivatives of the melting curves (at 260 nm). *Conditions*: 4 μM strands solution in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl. Target sequences: DNA, 5′-d(AGCAAAAAACGC)-3′ and RNA, 5′-r(AGCAAAAAACGC)-3′.

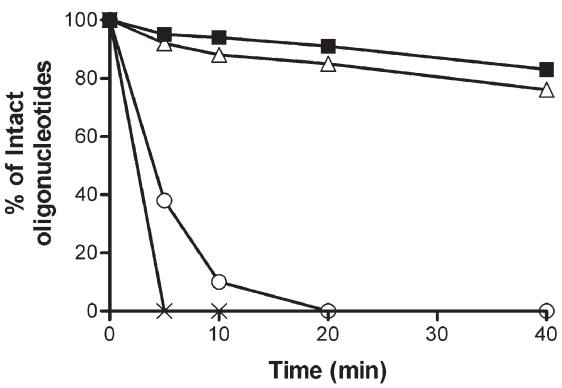


Fig. 3 Nuclease resistance of T_8**X**T oligonucleotides against SVPDE. **X** = natural-T (×) (**16**), 2′,4′-BNA-T (○) (**14**), phosphorthioate-T (△) (**17**), and 2′,4′-BNA^{NC}[N–Me]-T (■) (**10**). Hydrolysis of the oligonucleotides (10 μg) was carried out at 37 °C in 320 μl of a buffer containing 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$ and SVPDE (0.2 μg).

retaining a very strong ability to hybridize with complementary RNA) of 2′,4′-BNA^{NC}[N–Me]-modified oligonucleotides not only differs from what has been observed for 2′,4′-BNA (LNA)-modified oligonucleotides but also contrasted with the property of *N*-alkyl 2′-amino-LNA molecules having similar structural features.[22] Unfavorable steric interactions between methyl groups and the DNA strand may be a cause of the decreased affinity.

Resistance of a decamer oligonucleotide with a single 2′,4′-BNA^{NC}[N–Me] residue (**10**) to a 3′-exonuclease (snake venom phosphodiesterase, SVPDE) was assayed and the results were compared with those obtained by the corresponding natural oligonucleotide **16**, and with 2′,4′-BNA (LNA)-modified (**14**) and phosphorthioate-modified (**17**) oligonucleotides. After incubation of oligonucleotide solutions with SVPDE, the reaction mixtures were analyzed at several time points by reversed-phase HPLC to

monitor the percentage of intact oligonucleotides (Fig. 3). Under experimental conditions, the natural and 2′,4′-BNA (LNA)-modified oligothymidylates were completely digested within 5 and 20 min, respectively. In contrast, the 2′,4′-BNA^{NC}[N–Me]-modified oligonucleotide was remarkably stable; about 85% of the oligonucleotide survived after 40 min. The nuclease resistance of 2′,4′-BNA^{NC}[N–Me] was also slightly better than that of the phosphorthioate-modified oligonucleotide **17**.[23] The excellent resistance of **10** to SVPDE might result from steric hindrance around the phosphodiester linkage exerted by the six-membered bridged moiety with a methyl substituent.

In conclusion, we have synthesized a novel bridged nucleic acid 2′,4′-BNA^{NC}[N–Me] and shown that it has high-affinity hybridization similar to that of 2′,4′-BNA (LNA) against an RNA complement. Moreover, the nucleic acid analogue displayed RNA selectivity superior to that of 2′,4′-BNA (LNA) and other structural analogues of 2′,4′-BNA (LNA). Nuclease resistance of this nucleic acid analogue is abundantly higher than that of 2′,4′-BNA (LNA) and also slightly higher than that of phosphorthioate. Interestingly, the hydrophobic methyl substituent on the backbone might present an additional advantage in cellular uptake of the oligonucleotides.[24] All of these phenomena are essential for antisense applications and research in this direction is currently in progress.

Notes and references

§ MALDI-TOF-MS data: **7** [M − H]$^-$ 3688.5 (calc. 3689.5); **8** [M − H]$^-$ 3746.9 (calc. 3746.5); **9** [M − H]$^-$ 3804.9 (calc. 3803.6); **10** [M − H]$^-$ 3036.4 (calc. 3036.1); **11** [M − H]$^-$ 3660.8 (calc. 3660.4); **12** [M − H]$^-$ 3688.7 (calc. 3788.4); **13** [M − H]$^-$ 3716.9 (calc. 3716.4); **14** [M − H]$^-$ 3007.1 (calc. 3007.0); **15** [M − H]$^-$ 3632.6 (calc. 3632.4).

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18 Direct cyclization of the aminoxy derivative employing various bases resulted in failure.
19 The pseudorotation phase angle (*P*) was 23° indicating that the sugar conformation existed in a typical N-form (C3′-*endo*). CCDC 647092 for compound **5**. For crystallographic data in CIF or other electronic format see DOI: 10.1039/b707352f.
20 The T_m profiles of the duplexes formed by the oligonucleotides **7**–**9** and **12**, **13** are provided in the ESI†.
21 The T_m values of duplexes containing single-mismatch RNA strands also decreased significantly indicating that this nucleic acid analogue also has excellent sequence selectivity. For example, the T_m value of duplex formed by **7** with 3′-r(CGCAAUAAACGA)-5′ decreased by 13 °C.
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