Synthesis, RNA selective hybridization and high nuclease resistance of an oligonucleotide containing novel bridged nucleic acid with cyclic urea structure†

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A novel bridged nucleic acid bearing cyclic urea structure was successfully synthesized and introduced into oligonucleotide, displaying attractive characteristics of highly RNA selective hybridization ability and excellent resistance towards nuclease degradation.

Since the Human Genome Project was completed, much attention has been given to genome technologies to regulate target gene expression or function. The most simple and promising approach to down-regulate target gene expression in a living cell is antisense strategy, which prevents translation by hybridization of an oligonucleotide with its complementary RNA strand.[1] For practical use in antisense strategy, chemical modification of oligonulcleotides is essential to achieve high resistance towards nuclease degradation, high affinity to target mRNA with sequence specificity and RNA selectivity. 2′,4′-BNA (2′,4′-Bridged Nucleic Acid)[2]/LNA[3] whereby the sugar moiety is restricted to North-type (N-type) conformation was developed by our group and Wengel's group independently (Fig. 1). Oligonucleotides (ONs) containing 2′,4′-BNA confer moderate resistance against enzymatic degradation[3a,4] and strong affinity with their RNA complements,[3,5] but they still showed high affinity with their DNA complements.[3,5] On the other hand, other bridged nucleic acids bearing a different type of bridge structure between 2′- and 4′-positions have been developed to date (Fig. 1).[6–13] Depending on the ring size and/or the elements which compose the bridged structure, the modified ONs varied among properties such as enzymatic stability and RNA selectivity. In general, ONs containing bridged nucleosides with a large bridged ring size revealed higher resistance against enzymatic degradation (*i.e.*, 2′,4′-BNA/LNA *vs.* ENA,[7] aza-ENA,[8] 2′,4′-BNA^{NC},[9] 2′,4′-BNA^{COC},[10] PrNA,[11] *etc.*[6b]). In addition, incorporation of heteroatoms in an appropriate position of the bridged moiety showed a tendency to enhance hybridization ability (*i.e.*, PrNA[11] *vs.* 2′,4′-BNA^{COC},[10] or carbocyclic ENA[12,13] *vs.* 2′,4′-BNA^{NC} [9]) and in some cases RNA selectivity (*i.e.*, aza-ENA,[8] 2′,4′-BNA^{NC},[9] 2′,4′-BNA^{COC} [10]). However, it is still unclear how the bridged moiety itself affects the hybridization properties of ONs, and further evaluation of the relationship between the bridged structure and properties of the modified ONs is ongoing.

Here, we focus on a urea structure, containing both N–H and C=O groups as a proton donor and acceptor, respectively, and introduced it into the bridged moiety connecting the 2′- and 4′-positions of nucleoside to evaluate hybridization properties and enzymatic stability.[14]

As shown in Scheme 1, the phosphoramidite derivative of a novel bridged nucleic acid bearing a cyclic urea moiety was synthesized from known 4′-hydroxymethyl nucleoside derivative **1**.[3] At first, **1** was treated with trifluoromethanesulfonyl chloride in the presence of DMAP to afford a 2,2′-anhydro intermediate, which was then converted to an arabino-type nucleoside under alkaline conditions. Subsequent triflation at the 2′-hydroxy group afforded ditriflate **2**. Replacement of the two triflate groups by azide groups successfully proceeded to give diazide **3**. Reduction under Staudinger conditions,[15] followed by a ring-closure reaction with *p*-nitrophenyl chloroformate gave desired compound **4**.

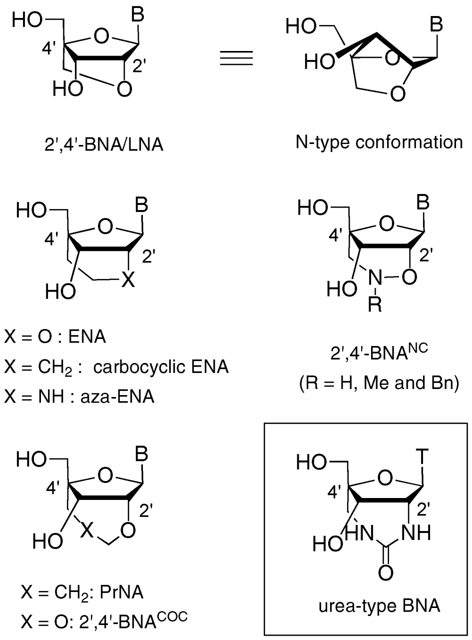

Fig. 1 Structure of 2′,4′-BNA/LNA and selected derivatives with a different type of bridge structure. B = nucleobase, T = thymin-1-yl.

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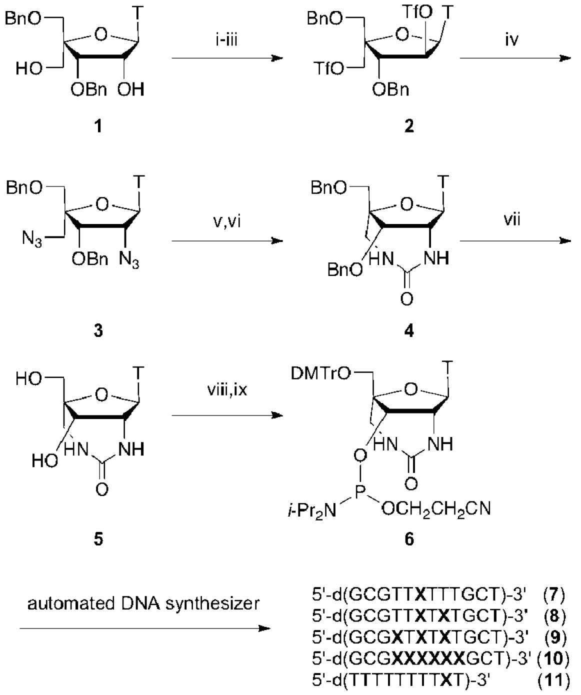

Scheme 1 *Reagents and conditions*: (i) TfCl, DMAP, CH_2Cl_2, 0 °C; (ii) 1 M NaOH aq., 1,4-dioxane, rt; (iii) Tf_2O, pyridine, CH_2Cl_2, rt, 37% over 3 steps; (iv) NaN_3, DMF, rt, quant.; (v) Me_3P, THF–H_2O, rt; (vi) *p*-nitrophenyl chloroformate, Et_3N, CH_2Cl_2, rt, 60% over 2 steps; (vii) H_2, 20% $Pd(OH)_2/C$, THF, rt, 94%; (viii) DMTrCl, pyridine, rt, 76%; (ix) 4,5-dicyanoimidazole, $(i\text{-}Pr_2N)_2PO(CH_2)_2CN$, THF–$CH_3CN$, rt, 45%. **X** denotes the bridged nucleoside with cyclic urea structure.

Benzyl protection groups of **4** were removed by hydrogenolysis with 20% $Pd(OH)_2/C$ to afford desired diol **5**. The sugar conformation of **5**[16] was confirmed to be N-type by means of ^{1}H-NMR spectroscopy where the H1′ signal was observed as a singlet.[2,17] Phosphoramidite **6** was obtained by protection of the 5′-hydroxyl group with 4,4′-dimethoxytrityl chloride and phosphitylation of the 3′-hydroxy group with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite and 4,5-dicyanoimidazole.

Phosphoramidite **6** was incorporated into ONs on an automated DNA synthesizer using standard phosphoramidite chemistry except for a prolonged coupling time of 40 min with 5-ethylthio-1*H*-tetrazole as an activator. The coupling efficiency of the phosphoramidite **6** was estimated to be more than 95% from a trityl monitor. Modified ONs **7–11** (Scheme 1) were purified by reverse phase HPLC and characterized by MALDI-TOF mass spectra (Table S1, ESI†).

The ability of ONs **7–10** to hybridize to complementary RNA and DNA strands was evaluated *via* UV melting experiments and compared with the corresponding natural DNA ON **12**, 5′-d(GCGTTTTTTGCT)-3′. The UV melting profiles are shown in Fig. S1 and S2 (ESI†), and the T_m values are summarized in Table 1. The T_m values for duplexes formed by modified ONs **7–10** with RNA complement were higher than that of the duplex formed by the unmodified DNA **12** and RNA complement. As the number of modifications increased, the duplex formed with RNA complement was well stabilized. Changes in T_m values (ΔT_m) ranged from +1 °C to +14 °C, which are comparable to those for 2′,4′-BNA^{COC} and better than those for PrNA.[16] In contrast, the thermal stability of the hybrids formed by modified ONs **7–10** with DNA complement was diminished compared with the duplex involving natural DNA ON **12**. In the case of ONs **8**, **9** and **10**, the differences in ΔT_m values with RNA (ΔT_m (RNA)) and those with DNA (ΔT_m (DNA)) were +10 °C, +13 °C and +17 °C, respectively, clearly indicating that modification of ON with 2′,4′-BNA bearing cyclic urea structure **5** enhances RNA selective hybridization ability. The RNA selective binding abilities of the modified ONs were also revealed in the recently developed modified ONs[8–10,18–21] and would be suitable for antisense strategy targeting mRNA or miRNA.

Helical structure of the duplexes formed by ONs **7–10** and RNA and DNA complements was evaluated by CD measurements, and the CD spectra were compared with those of the corresponding natural duplexes, DNA **12**/RNA complement and DNA **12**/DNA complement. As shown in Fig. S3 (ESI†) duplexes involving ONs **7–10** and their RNA complement exhibited almost identical CD spectra to that of the DNA **12**/RNA duplex, indicating that 2′,4′-BNA containing cyclic urea structure **5** completely adapted to the DNA **12**/RNA helical structure when it was incorporated into **12**. In contrast, incorporation of **5** into the DNA **12**/DNA duplex changed its CD profile (Fig. S4, ESI†). As the quantity of **5** increased, the intensity at 220 nm gradually decreased while that at 260 nm increased. This result suggests that the DNA **12**/DNA helix shifted from B to A-like helical structure by the incorporation of **5**, probably due to the rigid N-type (RNA-type) sugar conformation of **5**. The RNA selectivity of ONs containing the 2′,4′-BNA with cyclic urea structure **5** could be caused by such differences in the shift of helical structure revealed by the change of CD profile. In both cases, no evidence of unexpected distortion of the helical structure arising from a urea moiety containing hydrogen donor and acceptor sites was observed.

Enzymatic stability of decathymidylate derivative (ON **11**) involving a single 2′,4′-BNA with cyclic urea structure **5** was evaluated by using 3′-exonuclease (*Crotalus admanteus* venom phosphodiesterase, CAVP) and compared with the corresponding natural (**13**), 2′,4′-BNA(LNA)-modified (**14**) and 2′,4′-BNA^{NC}[NH]-modified (**15**), ENA-modified (**16**), 2′,4′-BNA^{NC}[NMe]-modified (**17**), phosphorothioate-modified (**18**) and 2′,4′-BNA^{COC}-modified (**19**) ONs. After incubation of each ON solution at 37 °C in the presence of CAVP, the reaction mixture was analyzed at several time points by reversed-phase HPLC, and the percentage of the remaining intact ONs was plotted in Fig. 2. Under the conditions used in this experiment, natural ON **13** was completely degraded within 2 min, and the 2′,4′-BNA(LNA) modified congener **14** was digested in 20 min. In contrast, modification by the 2′,4′-BNA with the cyclic urea structure **5** significantly enhanced the stability against CAVP; *ca.* 90% of ON **11** survived after 40 min, which was considerably better than 2′,4′-BNA^{NC}[NH]-modified ON **15**, ENA-modified ON **16**, and comparable to 2′,4′-BNA^{NC}[NMe]-modified ON **17**, phosphorothioate-modified ON **18**, and 2′,4′-BNA^{COC}-modified ON **19**. These results clearly demonstrate the enhanced resistance

Table 1 T_m values (°C) of duplexes formed by ONs **7–10** and **12** with their complementary strands[a]

	RNA			DNA			RNA selectivity
ONs	T_m	ΔT_m	$(\Delta T_m/\text{mod.})$	T_m	ΔT_m	$(\Delta T_m/\text{mod.})$	ΔT_m (RNA) − ΔT_m (DNA)
12	48	—	—	52	—	—	—
7	49	+1	(+1.0)	48	−4	(−4.0)	+5
8	51	+3	(+1.5)	45	−7	(−3.5)	+10
9	55	+7	(+2.3)	46	−6	(−2.0)	+13
10	62	+14	(+2.3)	49	−3	(−0.5)	+17

[a] The UV melting experiments were carried out 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 with target strand, 5′-r(AGCAAAAAACGC)-3′ or 5′-d(AGCAAAAAACGC)-3′. Final concentration of each ON was 4 μM.

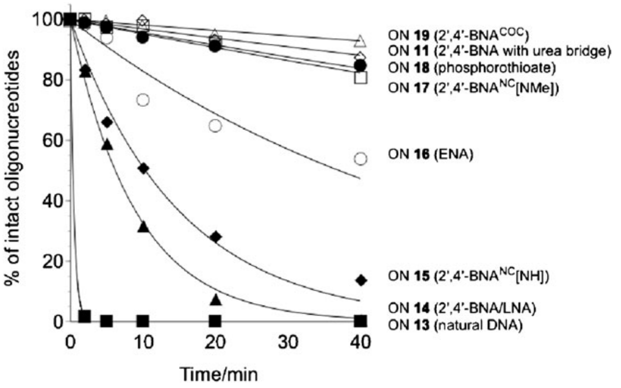

Fig. 2 Enzymatic stability of 5′-d(TTTTTTTTXT)-3′ against *Crotalus admanteus* venom phosphodiesterase (CAVP, Pharmacia Biotech). X = 2′,4′-BNA with cyclic urea structure-T (open diamond) (ON **11**); natural DNA-T (closed square) (ON **13**); 2′,4′-BNA(LNA)-T (closed triangle) (ON **14**); 2′,4′-BNA^{NC}[NH]-T (closed diamond) (ON **15**); ENA-T (open circle) (ON **16**); 2′,4′-BNA^{NC}[NMe]-T (open square) (ON **17**); phosphorothioate-T (closed circle) (ON **18**) and 2′,4′-BNA^{COC}-T (open triangle) (ON **19**). Experiments were performed at 37 °C in 100 μL of buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.75 nmol each ON and CAVP (0.175 μg).

of BNAs can be attributed to steric hindrance around the phosphodiester linkage rather than the elements composing the bridge structure.

In conclusion, we successfully synthesized novel bridged nucleic acid monomer **5** bearing cyclic urea structure and incorporated it into ONs. To the best of our knowledge, this is the first example of a nucleic acid analogue with a bridged structure between 2′- and 4′-positions containing a carbonyl group. Without any distortion of a helical structure brought about by the urea bridge, ONs containing **5** formed a stable duplex with RNA complement in a highly RNA selective manner. Nuclease resistance of this nucleic acid analogue is abundantly higher than that of natural DNA and 2′,4′-BNA(LNA) and is also slightly higher than that of phosphorothioate. The characteristics of this nucleic acid analogue are essential for application to antisense technology, and research in this direction is now in progress.

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