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. For practical use in antisense strategy, chemical modification of oligonucleotides 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)\(^{7}\)/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\(^{3,4}\) 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\(^{3}\) vs. ENA\(^{7}\), aza-ENA\(^{8}\), 2',4'-BNA\(^{NC,9}\), 2',4'-BNA\(^{OC,10}\), PrNA\(^{11}\), etc\(^{6,6}\)). 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\(^{OC,10}\) 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\(^{OC,10}\)), 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 trifluoromethanesulfonfyl chloride in the presence of DMAP to afford a 2,2'-anhydro intermediate, which was then converted to an arabinino-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.](image-url)
Table 1. The DNA ON and RNA strands was evaluated via characterized by MALDI-TOF mass spectra (Table S1, ESI (Scheme 1) were purified by reverse phase HPLC and 7–11 than 95% from a trityl monitor. Modified ONs chemistry except for a prolonged coupling time of 40 min with automated DNA synthesizer using standard phosphoramidite N
2-cyanoethyl THF–CH3CN, rt, 45%.

Reagents and conditions: (i) TfCl, DMAP, CH2Cl2, 0 °C; (ii) 1 M NaOH aq., 1,4-dioxane, rt; (iii) Tf2O, pyridine, CH2Cl2, rt, 37% over 3 steps; (iv) Na2S2O7, DMF, rt, quant.; (v) Me3P, THF–H2O, rt; (vi) p-nitrophenyl chloroformate, Et3N, CH2Cl2, rt, 60% over 2 steps; (vii) H2, 20% Pd(OH)2/C, THF, rt, 94%; (viii) DMTrCl, pyridine, rt, 76%; (ix) 4,5-dicyanomimidazole, (Pr2N)2PO(CH2)2CN, THF–CH3CN, 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 510 was confirmed to be N-type by means of 1H-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′-tetraisopropylphosphorodiamidite and 4,5-dicyanomimidazole.

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-1H-tetrazole as an activator. The coupling efficiency of the phosphoramidite 6 was estimated to be more than 95% from a trityl moni. 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′-(GGGTZTTTTGCT)-3′. The UV melting profiles are shown in Fig. S1 and S2 (ESI†), and the Tm values are summarized in Table 1. The Tm 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 Tm values (ΔTm) ranged from +1 °C to +14 °C, which are comparable to those for 2′,4′-BNA C oc 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 ΔTm values with RNA (ΔTm (RNA)) and those with DNA (ΔTm (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 ONs8–10,18–21 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 adamanteus 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 C oc-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 digested 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 C oc-modified ON 19. These results clearly demonstrate the enhanced resistance...
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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Table 1  
$T_m$ values (°C) of duplexes formed by ONs 7–10 with their complementary strands

<table>
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<tr>
<th>ONs</th>
<th>RNA</th>
<th>DNA</th>
<th>RNA</th>
<th>DNA</th>
<th>RNA</th>
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<td></td>
<td>$T_m$</td>
<td>$\Delta T_m$</td>
<td>($\Delta T_m$/mod.)</td>
<td>$T_m$</td>
<td>$\Delta T_m$</td>
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</tr>
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<td>—</td>
<td>52</td>
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<td>49</td>
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<td>48</td>
<td>—</td>
<td>(—)</td>
</tr>
<tr>
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<td>51</td>
<td>+3</td>
<td>(+1.5)</td>
<td>45</td>
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<td>(—)</td>
</tr>
<tr>
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<td>(+2.3)</td>
<td>46</td>
<td>—</td>
<td>(—)</td>
</tr>
<tr>
<td>10</td>
<td>62</td>
<td>+14</td>
<td>(+2.3)</td>
<td>49</td>
<td>—</td>
<td>(—)</td>
</tr>
</tbody>
</table>

*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'-t(AGCAAAAAACGC)-3' or 5'-d(AGCAAAAAACGCG)-3'. Final concentration of each ON was 4 μM.*

Notes and references

15. A part of this work was presented at the 6th International Symposium on Nucleic Acids Chemistry (Takayama, Japan); A. Yahara, M. Nishida, T. Baba, T. Kodama, T. Imanishi and S. Obika, *Chem. Commun.*, 2006, 128, 15173.
17. Energy-minimized structure of 5'-d(TTTTTTTTTT)-3' against *Crotalus adamanteus* venom phosphodiesterase (CAPP, Pharmacut.), X = 2',4'-BNA with cyclic urea structure-T (open diamond) (ON 11); natural DNA-T (closed square) (ON 13); 2',4'-BNA(A/LNA)-T (closed triangle) (ON 14); 2',4'-BNA[COC][NMe]-T (closed diamond) (ON 15); ENA-T (open circle) (ON 16); 2',4'-BNA[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).

Fig. 2 Enzymatic stability of 5'-d(TTTTTTTTTT)-3' against *Crotalus adamanteus* venom phosphodiesterase (CAVP, Pharmacut.). X = 2',4'-BNA with cyclic urea structure-T (open diamond) (ON 11); natural DNA-T (closed square) (ON 13); 2',4'-BNA(A/LNA)-T (closed triangle) (ON 14); 2',4'-BNA[COC][NH]-T (closed diamond) (ON 15); ENA-T (open circle) (ON 16); 2',4'-BNA[NMe]-T (open square) (ON 17); phosphorothioate-T (closed circle) (ON 18) and 2',4'-BNA[COC]-T (open triangle) (ON 19).