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Synthesis and hybridization properties of L-oligodeoxynucleotide analogues fixed in a low *anti* glycosyl conformation †

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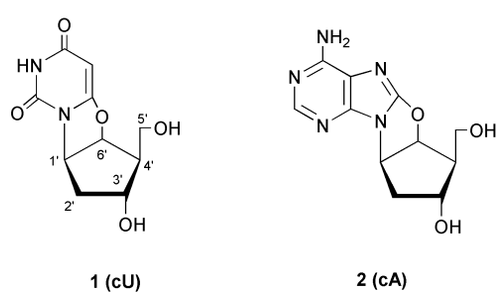
Received 7th October 2003, Accepted 1st December 2003
First published as an Advance Article on the web 10th December 2003

We have synthesized L-type enantiomers (cU and cA) of nucleoside analogues, whose glycosyl bonds are fixed in a low *anti* conformation (*ap* glycosyl conformation, $\chi \approx 180°$), and incorporated them into oligonucleotides to evaluate the hybridization ability with natural DNA and RNA sequences. Although the incorporation of the modified nucleosides into oligonucleotides decreased the hybridization ability with unmodified complementary DNA sequences, the fully-substituted 12mers (cU_{12} and cA_{12}) still retained the hybridization ability with the complementary unmodified DNA 12mers, regardless of their unnatural L-chirality. In contrast, cU_{12} and cA_{12} showed different hybridization behavior with complementary unmodified RNA 12mers. cU_{12} forms a more stable duplex with rA_{12} than the corresponding natural 12mer (dT_{12}), whereas cA_{12} cannot hybridize with rU_{12}. Based on the model structure of cU_{12}–rA_{12}, we discuss these experimental results.

Introduction

Recent progress of nucleic acid chemistry has enabled the application of oligonucleotides to medical treatment. Antigene and antisense oligonucleotides inhibit the expression of specific genes by hybridizing with the corresponding target DNA and RNA sequences to form a triplex and a duplex, respectively.[1–3] Since unmodified oligonucleotides have insufficient metabolic stability in biological fluids to maintain sufficient concentration for several hours for such purposes,[4] antisense oligonucleotides are required to have biological stability together with hybridization ability with their target sequences for medical applications. In this context, phosphorothioate analogues of oligonucleotides were utilized as a first generation antisense molecule due to the convenience of their synthesis and the resistance to nucleases.[5,6] However, phosphorothioate analogues were found to have some undesirable properties, such as binding to some proteins in a non-sequence specific manner[7,8] and possession of asymmetric phosphorus atoms. The former causes non-antisense effects leading to side effects, and the latter means a phosphorothioate oligonucleotide existing as a mixture of a large number of stereoisomers. For example, a 20-mer oligonucleotide, which has 19 phosphorothioate linkages, is a mixture of 2^{19} isomers. These stereoisomers were shown to have different abilities in hybridization with the target sequence.[9]

To improve such disadvantages of phosphorothioates, many other antisense molecules have been developed, such as N3′ → P5′ phosphoramidates,[10,11] BNA (LNA),[12–14] which is a conformationally locked nucleotide by bridging by the methylene group between the sugar O-2′ and C-4′ atoms to fix the sugar conformation in a 3′-*endo* mode. These oligonucleotide analogues have achiral phosphodiester linkages, and show a superior affinity toward target RNA sequences, but they do not have enough nuclease resistance against certain nucleases and serum.[15] Several research groups have focused on the nearly complete nuclease resistance of L-DNA, which consists of unnatural L-deoxyribose.[16–18] Although there are some conflicting reports for the hybridization properties of L-DNA with natural DNA and RNA,[19–21] L-DNA was not thought to hybridize with natural nucleic acids.[22] We found that the L-nucleotide residue of a heterochiral oligonucleotide, which consists of both D- and unnatural L-nucleotides, retains the Watson–Crick base-pairing and adopts an unusual low *anti* glycosyl conformation (*ap* glycosyl conformation, $\chi \approx 180°$) to form a right-handed duplex.[23] The results were also supported by another research group.[24] This unusual glycosyl conformation should make the L-nucleotide capable of forming a right-handed helix. On the basis of the structure of the heterochiral DNA duplex, we have designed novel L-nucleoside analogues with their glycosyl bonds fixed in the low *anti* conformation (Fig. 1)[25] and have already reported their racemic synthesis.[26,27] In a preliminary report, we have reported that annealing of the dodecadeoxynucleotides consisting of optically active **1** and **2** provides the formation of a stable right-handed B-form like duplex, regardless of their unnatural L-type chirality.[28] In this paper, we report the synthesis of an optically active form of **1** and **2**, and hybridization properties of modified oligonucleotides containing **1** and **2** with the complementary DNA and RNA.



1 (cU) **2 (cA)**

Fig. 1 Structures and numbering system[40] of L-type enantiomers of modified nucleoside analogues with a glycosyl bond fixed in a low *anti* conformation.

Results and discussion

Synthesis

The (−)-epoxide **3**, which was synthesized from cyclopentadiene in three steps,[29,30] was reacted with uracil to afford the 6′-α-hydroxy derivative **4** in a regioselective manner. After

† Electronic supplementary information (ESI) available: The characterization data of **1** and **4**–**12**. See http://www.rsc.org/suppdata/ob/b3/b312276j/

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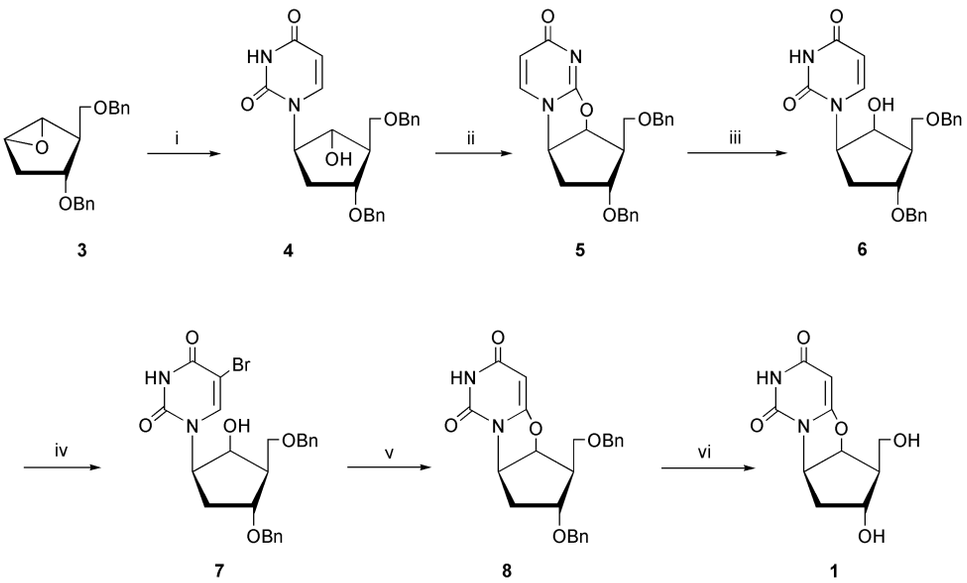
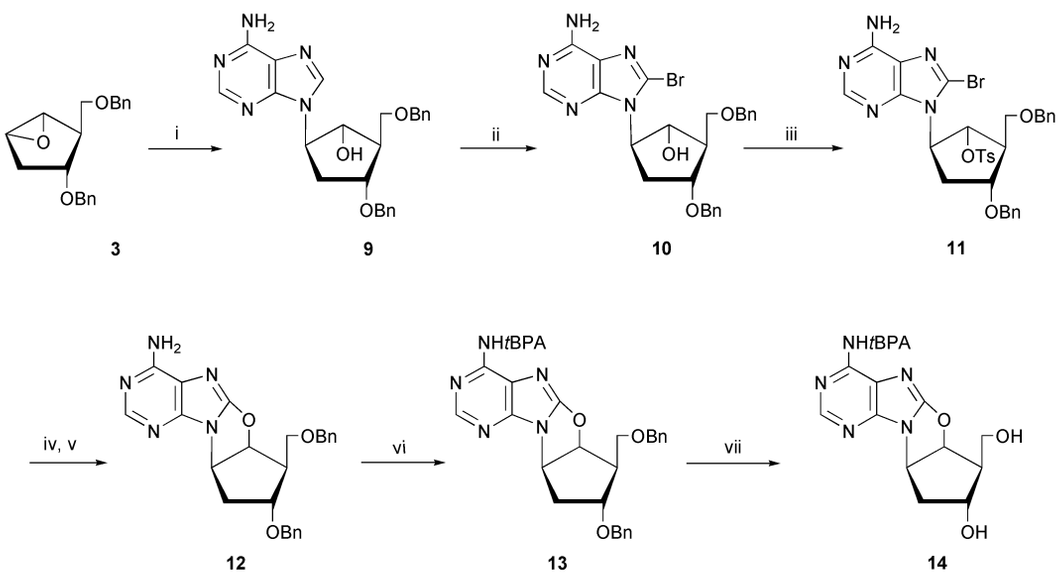


Scheme 1 *Reagents and conditions*: i, uracil, DBU, DMF, reflux, 53.6%; ii, DEAD, Ph_3P, THF, rt, 85.2%; iii, aq. NaOH, reflux, 93.4%; iv, NBS, NaN_3, 1,2-dimethoxyethane, rt, 91.4%; v, DBU, dioxane, reflux, 96.8%; vi, 20% $Pd(OH)_2/C$, cyclohexene, EtOH, reflux, 72.1%.



Scheme 2 *Reagents and conditions*: i, adenine, adenine sodium salt, DMF, 140 °C, 27 h, 73.7%; ii, Br_2, dioxane–0.5 M sodium acetate (pH 5.0), rt, 10 h, 78.3%; iii, TsCl, DMAP, CH_2Cl_2, rt, 96.7%; iv, sodium acetate, Ac_2O, AcOH, reflux, crude; v, NH_3/MeOH, 60 °C, 7 h, 74.3%; vi, *t*BPA-Cl, pyridine, rt, 86.6%; vii, 20% $Pd(OH)_2/C$, cyclohexene, DMF, 90 °C, 74.1%.

conversion into the 2,6′-*O*-anhydro derivative under Mitsunobu conditions, the anhydro bond was cleaved by treatment with aqueous sodium hydroxide to afford the 6′-β-isomer. The C5 position of the uracil ring was brominated with *N*-bromosuccinimide and sodium azide, and then the 6,6′-*O*-anhydro derivative **8** was obtained by treatment with DBU. Finally, reductive debenzylation of **8** afforded target compound **1** (Scheme 1).[27] Synthesis of the base protected adenosine derivative **14** is outlined in Scheme 2. Ring opening of **3** by the adenine sodium salt proceeded regioselectively to yield the 6′-α-hydroxy derivative **9**. After bromination at C-8 and subsequent tosylation at O-6′ of **9**, intramolecular cyclization gave the 8,6′-*O*-anhydro derivative **12**.[26] The anhydro linkage was expected to be labile under alkaline deprotecting conditions of oligonucleotides, and in fact we observed substantial decomposition of compound **12** by treatment with saturated methanolic ammonia at 55 °C for 8 h. Thus, *tert*-butylphenoxyacetyl (*t*BPA) group[31] was employed for the protection of the 6-amino group of the adenine ring. Debenzylation of **13** by catalytic hydrogenation afforded **14**. After protection of the 5′-hydroxy group with the 4,4′-dimethoxytrityl group, the L-type nucleoside analogues **1** and **14** were converted into the phosphoramidite derivatives **16** and **18**, respectively (Scheme 3). Compounds **16** and **18** were incorporated into oligonucleotides (Table 1) according to conventional phosphoramidite chemistry. Modified oligonucleotides containing **1** and **2** were characterized by MALDI-TOF MS spectra.

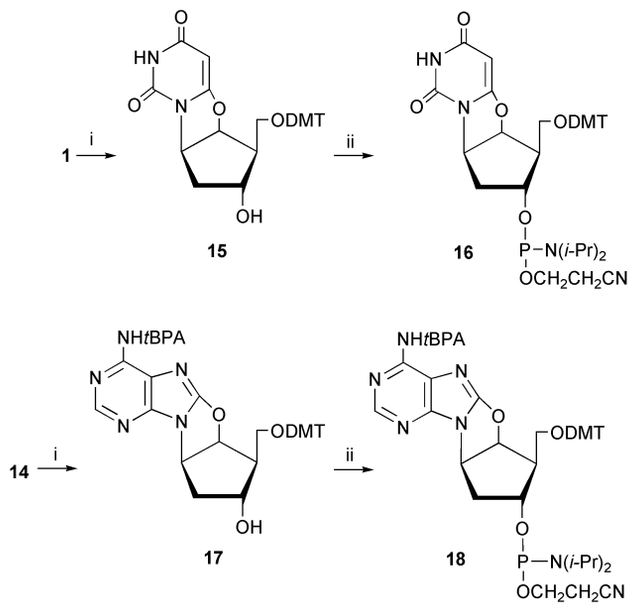
Nuclease resistance

To evaluate the biochemical stability of the carbocyclic L-type oligonucleotides, their susceptibility toward 3′-exonuclease was tested. L-DNA is known to be outstanding for its resistance to a variety of nucleases. Among them, snake venom phosphodiesterase (SVPDE) was reported to possess some activity to degrade L-nucleic acids.[18,32] Thus, we used SVPDE for such experiments. The results are shown in Fig. 2, which shows the time course of the absorbance of the 12mers after addition of SVPDE. Natural 12mers (dT_{12} and dA_{12}) show a sharp increase of their absorbance during 3 min. In contrast, cU_{12} and cA_{12} do not show any increase at all. These results indicate that the fully modified 12mers (cU_{12} and cA_{12}) have marked resistance toward

Table 1 Sequences of oligonucleotides used in this study[a]

Oligonucleotide	Sequence
$\mathbf{dT_{12}}$	d(TTTTTTTTTTTT)
$\mathbf{cU_1}$	d(TTTTTT(cU)TTTTT)
$\mathbf{cU_2}$	d(TTTTT(cUcU)TTTTT)
$\mathbf{cU_{12}}$	d(cUcUcUcUcUcUcUcUcUcUcU)
$\mathbf{dA_{12}}$	d(AAAAAAAAAAAA)
$\mathbf{cA_1}$	d(AAAAA(cA)AAAAA)
$\mathbf{cA_2}$	d(AAAAA(cAcA)AAAAA)
$\mathbf{cA_{12}}$	d(cAcAcAcAcAcAcAcAcAcAcA)
$\mathbf{rU_{12}}$	r(UUUUUUUUUUUU)
$\mathbf{rA_{12}}$	r(AAAAAAAAAAAA)

[a] cU and cA represent compounds **1** and **2**, respectively.



Scheme 3 *Reagents and conditions*: i, DMT-Cl, pyridine; ii, 2-cyanoethyl tetraisopropylphosphorodiamidite, diisopropylammonium tetrazolide, CH_2Cl_2.

SVPDE. In the case of the partly modified 12mers, cU_1 shows a biphasic increase of the absorbance, and this means that the enzyme quickly degrades the five thymidine residues of cU_1 from the 3′-end, whereas the cU residue is hydrolyzed very slowly. The 12mer containing two consecutive cU residues (cU_2) does not show biphasic degradation, suggesting that two consecutive cU residues are necessary to provide complete resistance toward SVPDE. This is in marked contrast to cA_1, whose single cA residue has enough resistance toward the enzyme.

UV melting studies of duplexes

The cU_{12}–cA_{12} duplex has been shown to form a B-form-like right-handed structure, regardless of its L-type chirality.[28] The aim of this work is to evaluate their hybridization ability with complementary DNA and RNA sequences, since unmodified L-DNA is reported to be unable to hybridize with natural DNA and RNA.[22] Fig. 3 shows the effects of introduction of cU (Fig. 3a) and cA (Fig. 3b) residues on the melting behavior of the dT_{12}–dA_{12} duplex. The duplex stability decreases as the number of modified nucleotide residues (cU or cA) increases. However, the duplex structure is still retained even in the dT_{12}–cA_{12} and cU_{12}–dA_{12} duplexes, although all of the nucleotide residues of cU_{12} and cA_{12} have L-type chirality. These results indicate that the modified 12mers (cU_{12} and cA_{12}) are able to hybridize with natural DNA sequences although the affinity is significantly decreased compared with the corresponding natural 12mers (dT_{12} and dA_{12}). This is in marked contrast to

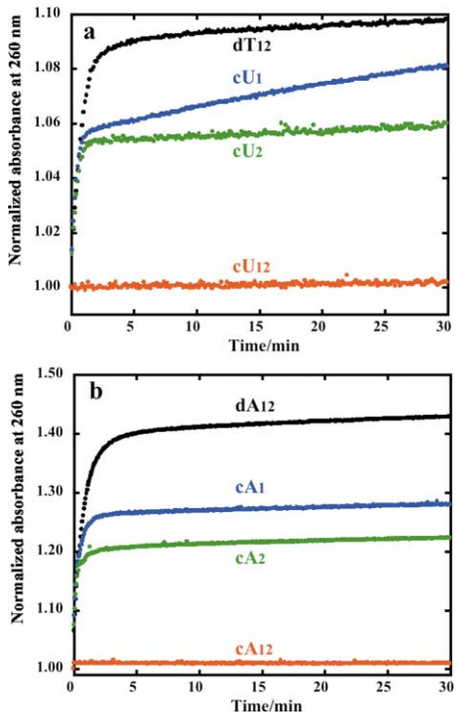


Fig. 2 Time course of the digestion of dT_{12}, cU_1, cU_2 and cU_{12} (a), and dA_{12}, cA_1, cA_2 and cA_{12} (b) with SVPDE. Absorbance was measured at 260 nm as a function of time after addition of SVPDE into the quartz cell containing each 12mer in 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.0 at 37 °C.

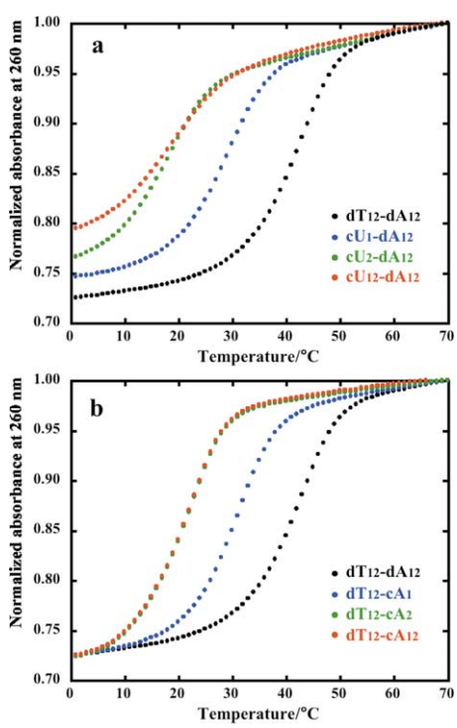


Fig. 3 Melting profiles of 12mers containing cU (a) and cA (b) with complementary DNA sequences dA_{12} and dT_{12}, respectively.

the disability of L-DNA to hybridize with the complementary DNA.[19,22] This result means that the fixation of the glycosyl bond in the low *anti* conformation makes the L-oligonucleotides capable of hybridizing with natural DNA strands. Fig. 4 shows the duplex stability of the modified 12mers with complementary unmodified RNA strands. The duplex stability of rU_{12}–dA_{12} also decreases as the number of substitution with cA increases, and rU_{12}–cA_{12} no longer forms the duplex (Fig. 4b). In the case of introduction of cU into dT_{12}–rA_{12}, the

Table 2 Hybridization data[a]

Oligonucleotide	T_m/°C	
	Complementary DNA[b]	Complementary RNA[c]
dT_{12}	42	36
cU_1	29	22
cU_2	19	17
cU_{12}	19	38
dA_{12}	42	27
cA_1	31	20
cA_2	22	19
cA_{12}	22	–

[a] T_m values were obtained by the first derivative plots of the melting curves. Experimental conditions are described in Materials and Methods. [b] Complementary DNA is dA_{12} or dT_{12}. [c] Complementary RNA is rA_{12} or rU_{12}.

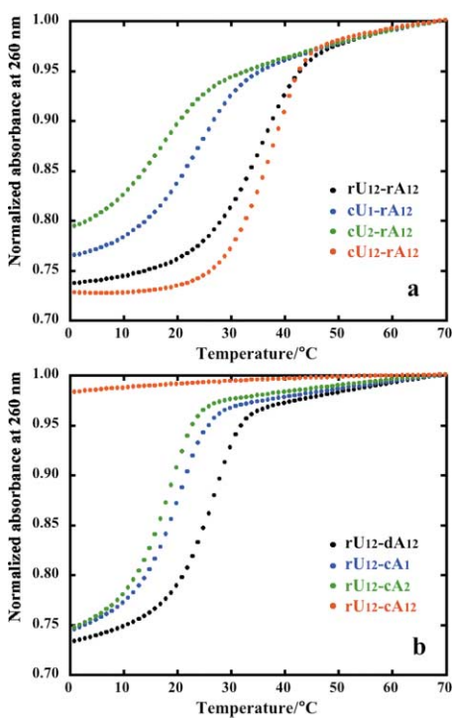


Fig. 4 Melting profiles of 12mers containing cU (a) and cA (b) with complementary RNA sequences rA_{12} and rU_{12}, respectively.

substitution of one or two dT residue with cU leads to the destabilization of the duplex as well, whereas complete substitution (cU_{12}) results in the formation of a more stable duplex than the parental duplex (dT_{12}–rA_{12}) (Fig. 4a). Thus, the modified L-type 12mers (cU_{12} and cA_{12}) primarily hybridize with natural DNA sequences, but the duplex formation with the RNA counterparts seems to lack generality. It may be plausible that the modified 12mers (cU_{12} and cA_{12}) are basically unable to form a duplex with natural RNA sequences, whereas the cU_{12}–rA_{12} duplex has a unique structural feature to stabilize the duplex. The hybridization selectivity of the modified 12mers to possess higher affinity toward DNA rather than RNA is compatible with the findings that cU_{12}–cA_{12} forms the B-form like right-handed duplex.[28] Melting temperatures (T_ms) of the duplexes are summarized in Table 2.

Then, we focused on the structural element stabilizing the cU_{12}–rA_{12} duplex. The duplex structures of the 12mers were analyzed by circular dichroism (CD) experiments (Fig. 5). The dT_{12}–rA_{12} duplex shows a typical CD spectrum for a DNA/RNA hybrid duplex (A-form). The spectrum of cU_{12}–cA_{12} duplex is very similar to that of the dT_{12}–dA_{12} duplex (B-form) as reported,[28] and the spectrum of the cU_{12}–dA_{12} duplex is also classified into the B-form. In contrast, although the cU_{12}–rA_{12}

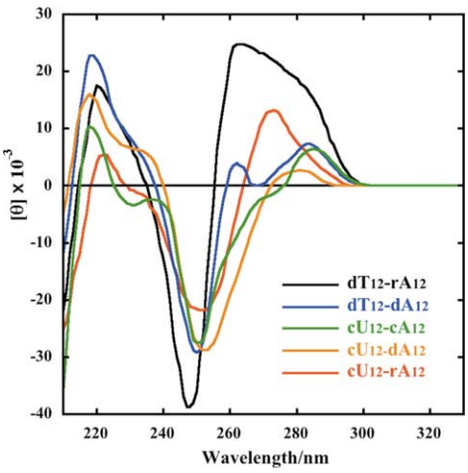


Fig. 5 CD spectrum of each 12mer duplex. Duplex concentration is 4 μM in 1 M NaCl, 10 mM sodium phosphate, pH 7.0 at 1 °C.

duplex should be classified as the B-form rather than the A-form, it is difficult to exactly specify the duplex as the A- or B-form. The distinct structural feature of the cU_{12}–rA_{12} duplex from the other duplexes can be also corroborated from the melting behavior. In addition, we found the cU_{12}–rA_{12} duplex to have an unusual salt concentration dependence on the duplex stability. The T_m of the cU_{12}–rA_{12} duplex is 38 °C in the presence of 1 M NaCl, however distinct duplex formation is no longer observed under low salt (0.1 M NaCl) conditions (Fig. 6). This is in marked contrast to the duplex of the unmodified dT 14mer with complementary oligo(dA), which is destabilized by only 14 °C with decreasing salt concentration from 0.8 M to 0.1 M.[33] This suggests that the cU_{12}–rA_{12} duplex has a unique structural feature to be highly stabilized by high salt conditions. To discuss the unusual salt concentration dependence of the duplex stability, the model structure of the cU_{12}–rA_{12} duplex was constructed (Fig. 7). The cU_{12}–rA_{12} duplex model was built starting from the standard B-DNA structure by energy-minimization with the continuum solvent model GB/SA,[34,35] which presents a hypothetical structure under low salt concentrations. Although we cannot directly discuss the mechanism for the stabilization of the cU_{12}–rA_{12} duplex under high salt concentrations using this model, a possible clue to the salt concentration dependence was found in this model. In the major groove of the model structure, the N7 imine group of adenine in the rA_{12} strand was positioned close to the 2′-hydroxy group of its 5′-neighboring residue (*ca.* 3.7 Å). This relative spatial position of the two functional groups that have partial electronegativity may be suitable for making an electrostatic interaction with one sodium cation by direct bridging, leading to stabilization of the duplex. For the rU_{12}–cA_{12} duplex, the uracil residues in the rU_{12} strand do not

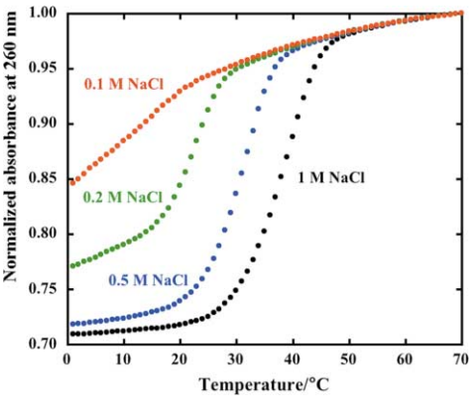


Fig. 6 Salt concentration dependence of thermal denaturation of the cU_{12}–rA_{12} duplex. Duplex concentration is 4 μM in 10 mM sodium phosphate, pH 7.0.

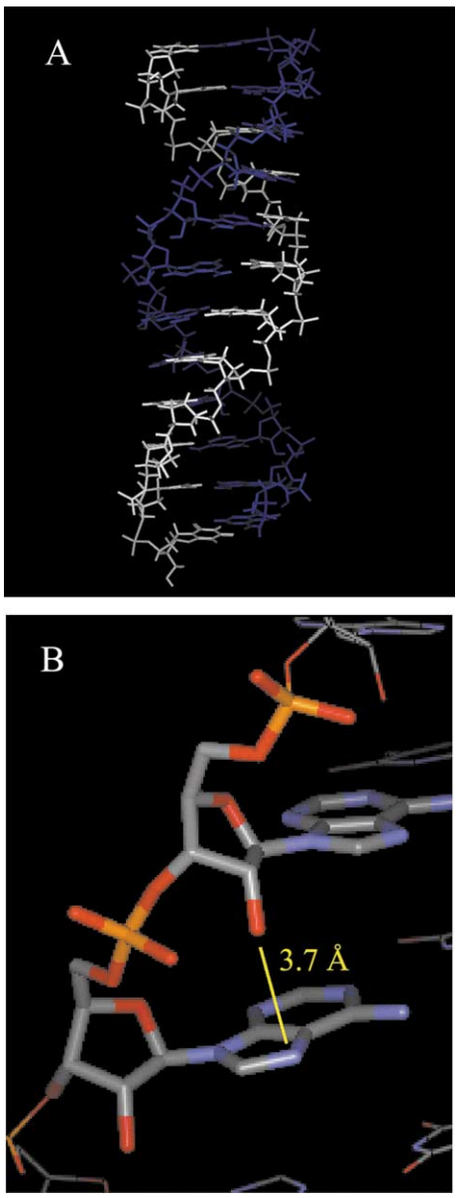


Fig. 7 Energy-minimized structure of the cU_{12}–rA_{12} duplex. (A) The cU_{12} and rA_{12} strands are drawn in white and blue, respectively. (B) The inter-residual distance between the N7 atom and the 5′-neighboring 2′-OH group in the rA_{12} strand is depicted by a yellow line. Hydrogen atoms are omitted for clarity.

have any hetero functionalities that can play geometrically the role in interacting with the sodium cation in this manner. There is no evidence of a bridging interaction with the sodium cation under high salt concentrations but it could be plausible because the duplex stabilization is highly dependent on salt concentration.

Conclusion

We have synthesized an optically active form (L-type) of the nucleoside analogues whose glycosyl bonds are fixed in the low *anti* conformation, and successfully incorporated them into oligonucleotides. The hybridization ability of cU_{12} and cA_{12} with the complementary natural nucleic acids is not sufficient for application to antigene and antisense methodologies, whereas the 12mers were found to form a duplex selectively with DNA rather than RNA, although cU_{12} showed superior hybridization ability to unmodified dT_{12} for oligo(rA). Our design of the conformationally fixed L-nucleotides based on the structure of the heterochiral DNA duplex enabled the helical control of L-DNA, namely a left-handed to right-handed helical switching, although the hybridization ability of the modified oligonucleotides (cU_{12} and cA_{12}) with natural DNA is not sufficient. This rationale would be useful for design of novel functional nucleic acids and regulation of helical sense of helix-forming molecules.

Experimental

Reagents for the DNA/RNA synthesizer were purchased from Applied Biosystems Japan (Tokyo, Japan) and Glen Research Co. (Sterling, VA, USA). Snake venom phosphodiesterase was purchased from Roche Diagnostics (Mannheim, Germany). TLC analyses were carried out on Merck silica gel $60F_{254}$ plates, which were visualized by UV illumination at λ = 254 nm. Column chromatography was performed by using Wakogel FC-40 or C-200 silica gel (Wako Pure Chemical Industries). The elution was carried out by increasing amounts of methanol in chloroform unless otherwise noted. ^{1}H NMR spectra were measured on a Varian Gemini 200 or Mercury 300 spectrometer, with *tert*-butyl alcohol for D_2O (1.23 ppm from DSS) or tetramethylsilane for other solvents as the internal standard. ^{31}P NMR spectra were measured on a Varian Mercury 300 spectrometer at 121.4 MHz, with trimethyl phosphate as the external standard. Mass spectra were obtained on a Hitachi M-4000H spectrometer. Specific rotations and CD spectra were measured on JASCO DIP-1000 and JASCO J-820 spectropolarimeters, respectively. HPLC analyses were performed on a Shimadzu LC-10A system. A μBondasphere C18 5 μm 100 Å column (3.9 × 150 mm, Waters Corporation, Milford, MA, USA) was used with a linear gradient of acetonitrile in 50 mM triethylammonium acetate (TEAA, pH 7.0).

The protected (−)-epoxide **3** was synthesized from cyclopentadiene *via* asymmetric hydroboration according to the literature procedure for the corresponding (+)-isomer.[29,30] Compounds **1** and **4**–**12** were synthesized by the same methods as the corresponding racemic compounds.[26,27] The characterization data of **1** and **4**–**12** are shown in electronic supplementary information. †

(6′a*S*,7′*S*,8′*R*,9′a*R*)-*N*-(8′-Benzyloxy-7′-benzyloxymethyl-7′,8′,9′,9′a-tetrahydro-6′a*H*-cyclopenta[4,5]oxazolo[3,2-*e*]-purin-4′-yl)-2-(4-*tert*-butylphenoxy)acetamide (13)

To a solution of **12** (444 mg, 1.0 mmol) in anhydrous pyridine (10 ml) was added *tert*-butylphenoxyacetyl chloride (272 mg, 1.2 mmol), and the mixture was stirred at room temperature for 1.5 h. After addition of H_2O, the mixture was extracted with $CHCl_3$ (70 ml) and the organic layer was washed with saturated aqueous $NaHCO_3$ (50 ml × 2). After the organic layer was dried with Na_2SO_4 and concentrated, the residue was purified by column chromatography on silica gel to give **13** (549 mg, 86.6%, as a colorless foam): $[\alpha]_D^{27}$ −131.8 (*c* 0.51 in $CHCl_3$); δ_H ($CDCl_3$) 1.31 (9H, s, 3 × CH_3), 2.19 (1H, dt, *J* 7.7 and 14.0, 2′a-H), 2.62–2.71 (2H, m, 2′b-H and 4′-H), 3.76–3.98 (3H, m, 3′-H, 5′a-H and 5′b-H), 4.39–4.56 (4H, m, 2 × Ar–CH_2), 4.76 (2H, s, ArOCH_2), 5.14–5.19 (1H, m, 1′-H), 6.01 (1H, t, *J* 6.9, 6′-H), 6.91–7.00 (2H, m, *t*BPA-Ha), 7.22–7.38 (12H, m, *t*BPA-Hb and 2 × ArCH_2), 8.60 (1H, s, 2-H), 9.06 (1H, br, NH); *m*/*z* (EI) 633.2946 (M^+. $C_{37}H_{39}N_5O_5$ requires 633.2949).

(6′a*S*,7′*S*,8′*R*,9′a*R*)-2-(4-*tert*-Butylphenoxy)-*N*-(7′,8′,9′,9′a-tetrahydro-8′-hydroxy-7′-hydroxymethyl-6′a*H*-cyclopenta[4,5]-oxazolo[3,2-*e*]purin-4′-yl)acetamide (14)

A mixture of **13** (545 mg, 0.86 mmol), 20% $Pd(OH)_2$/C (136 mg), cyclohexene (7 ml) in DMF (14 ml) was heated at 90 °C for 2 h. The catalyst was removed by filtration and was washed with hot DMF. The filtrate was concentrated to dryness and the residue was coevaporated with *m*-xylene several times, and the residue was purified by column chromatography on

silica gel to give **14** (289 mg, 74.1%, as a colorless solid): $[a]_D^{26}$ −172.3 (*c* 0.50 in $CHCl_3$); δ_H (DMSO-d_6) 1.25 (9H, s, 3 × CH_3), 1.85–1.95 (1H, m, 2′a-H), 2.08–2.18 (1H, m, 4′-H), 2.31–2.37 (1H, m, 2′b-H), 3.57–3.80 (3H, m, 3′-H, 5′a-H and 5′b-H), 4.75 (1H, t, *J* 5.2, OH), 4.87 (2H, s, ArOCH_2), 5.14–5.18 (2H, m, 1′-H and OH), 5.95 (1H, t, *J* 6.9, 6′-H), 6.85–6.90 (2H, m, *t*BPA-Ha), 7.28–7.33 (2H, m, *t*BPA-Hb), 8.47 (1H, s, 2-H), 10.50 (1H, br, NH); *m*/*z* (EI) 453.2011 (M^+. $C_{23}H_{27}N_5O_5$ requires 453.2010).

(5a*R*,7*R*,8*S*,8a*S*)-8-(4,4′-Dimethoxytrityloxymethyl)-5a,7,8,8a-tetrahydro-7-hydroxy-2*H*,6*H*-cyclopenta[4,5]oxazolo[3,2-*c*]-pyrimidine-2,4(3*H*)-dione (15)

To a solution of **1** (528 mg, 2.2 mmol) in anhydrous pyridine (10 ml) was added 4,4′-dimethoxytrityl chloride (894 mg, 2.2 mmol), and the mixture was stirred at room temperature for 2 h. After addition of EtOH, the solvent was evaporated under reduced pressure. The residue was extracted with $CHCl_3$ (70 ml) and the organic layer was washed with saturated aqueous $NaHCO_3$ (50 ml × 2). After the organic layer was dried with Na_2SO_4 and concentrated, the residue was purified by column chromatography on silica gel. After evaporation of the solvent, the residue was dissolved with small amounts of $CHCl_3$, and the solution was added dropwise to *n*-hexane : diethyl ether = 3 : 1 (v/v, 200 ml) to give **15** (1.21 g, 94.1%, as a colorless powder): δ_H ($CDCl_3$) 1.88–1.99 (1H, m, 2′a-H), 2.21–2.31 (1H, m, 4′-H), 2.55–2.63 (1H, m, 2′b-H and OH), 3.45–3.60 (2H, m, 5′a-H and 5′b-H), 3.80 (6H, s, 2 × OCH_3), 4.00–4.09 (1H, m, 3′-H), 4.86 (1H, t, *J* 7.1, 1′-H), 4.94 (1H, d, *J* 1.7, 5-H), 5.32 (1H, dd, *J* 5.5 and 7.1, 6′-H), 6.82–6.87 (4H, m, DMT-Ha), 7.22–7.43 (9H, m, DMT-Hb), 8.07 (1H, br, NH); *m*/*z* (EI) 542.2045 (M^+. $C_{31}H_{30}N_2O_7$ requires 542.2051).

(5a*R*,7*R*,8*S*,8a*S*)-7-[2-Cyanoethoxy(diisopropylamino)-phosphinoxy]-8-(4,4′-dimethoxytrityloxymethyl)-5a,7,8,8a-tetrahydro-2*H*,6*H*-cyclopenta[4,5]oxazolo[3,2-*c*]pyrimidine-2,4(3*H*)-dione (16)

To a solution of **15** (542 mg, 1 mmol) in anhydrous dichloromethane (5 ml) was added 2-cyanoethyl tetraisopropyl phosphorodiamidite (0.476 ml, 1.5 mmol) and diisopropylammonium tetrazolide (86 mg, 0.5 mmol), and the mixture was stirred at room temperature for 3 h. The mixture was extracted with CH_2Cl_2 (70 ml) and the organic layer was washed with saturated aqueous $NaHCO_3$ (50 ml × 2). After the organic layer was dried with Na_2SO_4 and concentrated, the residue was purified by column chromatography on silica gel [0–2% MeOH in benzene : triethylamine (99 : 1)]. After evaporation of the solvent, the residue was dissolved with small amounts of CH_2Cl_2, and the solution was added to *n*-pentane (100 ml). After decantation, the precipitate was dissolved in CH_2Cl_2 and evaporated to give **16** (716 mg, 96.3%, as a colorless foam): δ_P ($CDCl_3$) 146.32, 146.63; *m*/*z* (SIMS) 743.3209 (M^+ + 1. $C_{40}H_{48}N_4O_8P$ requires 743.3206).

(6′a*S*,7′*S*,8′*R*,9′a*R*)-2-(4-*tert*-Butylphenoxy)-*N*-[7′-(4,4′-dimethoxytrityloxymethyl)-7′,8′,9′,9′a-tetrahydro-8′-hydroxy-6′a*H*-cyclopenta[4,5]oxazolo[3,2-*e*]purin-4′-yl]acetamide (17)

Yield 98.3%; $[a]^{26}$ −76.3 (*c* 0.51 in $CHCl_3$); δ_H ($CDCl_3$) 1.30 (9H, s, 3 × CH_3), 2.00–2.11 (1H, m, 2′a-H), 2.29–2.39 (1H, m, 4′-H), 2.67–2.74 (1H, m, 2′b-H), 2.92 (1H, d, *J* 2.2, OH), 3.56 (1H, dd, *J* 8.0 and 9.6, 5′a-H), 3.73 (1H, dd, *J* 6.0 and 9.6, 5′b-H), 3.79 (6H, s, 2 × OCH_3), 4.15–4.24 (1H, m, 3′-H), 4.74 (2H, s, ArOCH_2), 5.13 (1H, dd, *J* 6.9 and 7.1, 1′-H), 5.86 (1H, dd, *J* 6.0 and 6.9, 6′-H), 6.06 (1H, br, NH), 6.81–6.98 (6H, m, DMT-Ha and *t*BPA-Ha), 7.20–7.41 (11H, m, DMT-Hb and *t*BPA-Hb), 8.60 (1H, s, 2-H); *m*/*z* (EI) 755.3324 (M^+. $C_{44}H_{45}N_5O_7$ requires 755.3317).

(6′a*S*,7′*S*,8′*R*,9′a*R*)-2-(4-*tert*-Butylphenoxy)-*N*-{8′-[2-cyanoethoxy(diisopropylamino)phosphinoxy]-7′-(4,4′-dimethoxytrityloxymethyl)-7′,8′,9′,9′a-tetrahydro-6′a*H*-cyclopenta[4,5]oxazolo[3,2-*e*]purin-4′-yl}acetamide (18)

Yield 89.7%; δ_P ($CDCl_3$) 145.54, 145.79; *m*/*z* (SIMS) 956.4478 (M^+ + 1. $C_{53}H_{63}N_7O_8P$ requires 956.4472).

Synthesis of oligonucleotides

LCAA-CPG carrying a cU or cA nucleoside unit was synthesized according to the procedure reported by Damha *et al*.[36] Oligoribo- and oligodeoxyribonucleotides were synthesized on an Applied Biosystems Model 392 DNA/RNA synthesizer with the 1.0 μmol scale. Deprotection of oligonucleotides were carried out according to the manufacturer's instructions apart from deprotection and cleavage from the solid support of modified oligonucleotides, which were treated with 28% aqueous ammonia at room temperature for 2 h. The crude oligonucleotides were purified with reversed phase HPLC. Finally, the purified oligonucleotides were desalted on a Sep-Pak Plus cartridge (Waters Corporation, Milford, MA, USA)

MALDI-TOF MS Analysis of modified oligonucleotides

1 OD unit of purified oligonucleotides was dissolved in 15 μl of distilled water. Aliquots (1 μl) of each solution were mixed with 1 μl of saturated aqueous solution of 3-hydroxy-2-picolinic acid as a matrix. TOF MS spectra were obtained by a Perkin-Elmer Applied Biosystems Voyager-Linear DE spectrometer in negative ion mode.

cU$_1$: *m*/*z*: 3585.40; $C_{120}H_{154}N_{24}O_{82}P_{11}$ $(M - 1)^-$ requires 3585.36. **cU$_2$**: *m*/*z*: 3583.34; $C_{120}H_{152}N_{24}O_{82}P_{11}$ $(M - 1)^-$ requires 3583.34. **cU$_{12}$**: *m*/*z*: 3563.64; $C_{120}H_{132}N_{24}O_{82}P_{11}$ $(M - 1)^-$ requires 3563.19. **cA$_1$**: *m*/*z*: 3707.26; $C_{121}H_{144}N_{60}O_{58}P_{11}$ $(M - 1)^-$ requires 3707.55. **cA$_2$**: *m*/*z*: 3719.59; $C_{122}H_{144}N_{60}O_{58}P_{11}$ $(M - 1)^-$ requires 3719.56. **cA$_{12}$**: *m*/*z*: 3839.67; $C_{132}H_{144}N_{60}O_{58}P_{11}$ $(M - 1)^-$ requires 3839.67.

Snake venom phosphodiesterase digestion

0.8 OD units of oligonucleotides were dissolved in 3 ml of 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0). The solution was transferred into a quartz cell (1 cm path length) and the temperature was maintained at 37 °C. After addition of snake venom phosphodiesterase (2 μl, 2 mg ml^{-1}, Boehringer Mannheim), absorbance at 260 nm was measured for 30 min.

Measurements of melting curves

The concentrations of oligonucleotide solutions were calculated by using equation and coefficients described by Borer.[37] The coefficients of the modified 12mers were calculated on the assumption that modified dimers have the same hypochromicity as the corresponding unmodified dimers. Molar coefficients (ε_{260}) of cU and cA were determined experimentally as 14500 and 14200 L mol^{-1} cm^{-1}. Each pair of 12mers was mixed and dissolved in a buffer containing 1 M NaCl, 10 mM sodium phosphate (pH 7.0) at duplex concentrations of 4 μM. After annealing, the solution was transferred to a quartz cell (1 cm path length), and melting curves were measured at least twice at 260 nm on a JASCO Ubest-55 spectrophotometer. The temperature was raised at a rate of 0.5 °C min^{-1}, and the T_m values were obtained by the first-derivative plots of the melting curves.

Measurements of CD spectra

The same samples (duplex concentration; 4 μM in 1 M NaCl, 10 mM sodium phosphate, pH 7.0) as used for UV melting experiments were employed for CD experiments. Measurements were carried out on a JASCO J-820 spectropolarimeter at 1 °C.

Modeling of energy-minimized structure of cU_{12}–rA_{12} duplex

All the modeling works were done on O2 R5000 (Silicon Graphics, Inc., Mountain View, USA) using the molecular modeling suite, Maestro 4.1 (Schroedinger, Portland, USA). The energy-minimization was done by the BatchMin molecular mechanics engine [38] with the MMFF94 parameter for the force-field and atomic charges.[39] The all-atom model was used for the study.

The initial structure of the cU_{12}–rA_{12} duplex was built using the dT_{12}–dA_{12} structure with the standard right-handed B-form duplex geometry as a template. The rA_{12} strand was prepared from the dA_{12} strand by replacing a hydrogen atom with a hydroxy group at each 2′-position of deoxyribose. A 3′-phosphoryl cU monomer was manually built by modifying a base-ribose structure of a dT nucleoside with C2′-*endo* pucker, and added to a phosphate group at the 3′-hydroxy group. Using this 3′-phosphoryl cU monomer, the cU_{12} strand was built by superimposing the base of cU on the base position of the dT residue in the dT_{12} strand and linking 3′–5′ phosphodiester bonds between the 3′- and 5′-cU residues. Then, the initial B-form structure of the cU_{12}–rA_{12} duplex was modeled from the cU_{12} and rA_{12} strands. To relax the steric crash in the initial structure, restricted minimization was first done under the condition of base positions fixed. Subsequently, the energy-minimized structure of the cU_{12}–rA_{12} duplex was calculated by the conjugated gradient method with the 0.01 kJ $Å^{-1}$ gradient convergent criterion with improper dihedral angle constraints of 1000 kJ mol^{-1} weight to maintain the plane of base-pairs and continuous solvation model GB/SA.[35,36]

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.

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