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Synthesis and characterization of oligodeoxynucleotides containing a novel tetraazabenzo[*cd*]azulene:naphthyridine base pair

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ABSTRACT

The synthesis and thermal stability of oligodeoxynucleotides (ODNs) containing 4-amino-2,3,5,6-tetraazabenzo[*cd*]azulen-7-one nucleosides **5** (BaO^N) with the aim of developing new base pairing motif is described. The tricyclic nucleoside **5** was prepared starting with the 7-deaza-7-iodopurine derivative **1** via a palladium catalyzed cross-coupling reaction with methyl acrylate, followed by an intramolecular cyclization. The resulting nucleoside was incorporated into ODNs, and the base pairing property of the BaO^N:NaN^O (2-amino-7-hydroxy-1,8-naphthyridine nucleoside) pair in the duplex was evaluated by a thermal denaturation study. The melting temperature (T_m) of the duplex containing the BaO^N:NaN^O pair showed a higher value than that of the duplexes containing the adenine:thymine (A:T) and the guanine:cytosine (G:C) pairs, however it was lower than that of the ImO^N:NaN^O (ImO^N = 7-amino-imidazo[5′,4′:4,5]pyrido[2,3-*d*]pyrimidin-4(5*H*)-one nucleoside) pair. A temperature-dependent ^{1}H NMR study revealed that the H-bonding ability of BaO^N was lower than that of ImO^N, which would explain why the BaO^N:NaN^O pair was less thermally stable than the ImO^N:NaN^O pair.

1. Introduction

In a right-handed double helix of DNA, nucleobases form Watson–Crick base pairs of adenine:thymine (A:T) and guanine:cytosine (G:C) via two and three hydrogen bonds (H-bonds), respectively. These selective base pairs play a critical role in not only conserving and transmitting genetic information but also maintaining the stability of the helical structure. In general, three factors are thought to contribute to its stability: first, the interaction via H-bonds between the complementary bases; second, the aromatic Π–Π interaction between stacking base pairs (stacking interaction); and third, the size of the base pairs (shape complementarity) since Watson–Crick base pairs consist of a combination of purine and pyrimidine bases.

Thus far, a number of noncanonical base paring motifs have been designed based on the aforementioned factors in order to apply them to bioorganic chemistry, biotechnology, and medicinal chemistry.[1–5] Although the end goal of each base pairing motif is different, stable and specific interactions between the bases are indispensable for the development of a new base paring motif. In order to stabilize and regulate DNA structure, we have been working on a project to develop new base paring motifs consisting of four H-bonds (Fig. 1). As the first generation of such base pairing motifs, we have designed complementary pairs consisting of the imidazopyridopyrimidine (Im) bases such as ImO^N:ImN^O (Fig. 1a).[6,7] Although this base pair was demonstrated to stabilize the helical structure by means of four H-bonds and increased stacking interaction due to the expanded aromatic surface (positive effects), distortion of the helical structure arising from extension of the intrastrand C1′–C1′ distance (a negative effect) was also suggested. Since the ImO^N:ImN^O pair was thought to not satisfy the shape complementarity, new *C*-nucleoside derivatives possessing 1,8-naphthyridines (Na), which are complementary to the Im bases, were designed (Fig. 1b). The resulting base pairs, such as ImO^N:NaN^O, markedly stabilized the duplexes thermally, independent of the mode of incorporation of the pairs into the oligodeoxynucleotides (ODNs) (ca. +8 °C per pair relative to the A:T pair).[7–9] Furthermore, we have succeeded in demonstrating a selective recognition of the Im:Na pairs by DNA polymerases.[10,11] Therefore, the Im:Na base pair appears to be promising as an alternative and highly thermally stable base pair beyond the Watson–Crick base pairs. However, the question has been raised as to whether the Im:Na pair has the best geometry in the double helix from the standpoint of shape complementarity. Thus, the Im bases can be considered as ring-expanded analogs of purine toward the minor groove direction, while the Na bases are ring-expanded analogs of pyrimidine toward the major groove direction, which may cause a distortion of the helical structure arising from a shift of the intrastrand C1′–C1′ position. In order to avoid this possible

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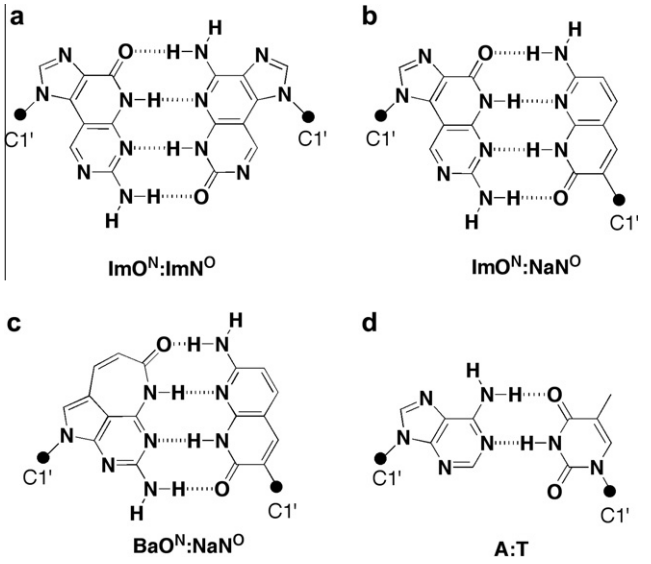


Figure 1. Base pairing motifs of: (a) ImO^N:ImN^O, (b) ImO^N:NaN^O, (c) newly designed BaO^N:NaN^O, and (d) A:T. To clarify the intrastrand C1′–C1′ position, the C1′ positions of each base pair were marked with dot.

distortion, we envisioned a new base paring motif in which both bases are expanded toward the major groove direction. As one of the candidates, we designed a nucleoside derivative possessing a 4-amino-2,3,5,6-tetraazabenzo[*cd*]azulen-7-one (BaO^N),[7] which is expected to form a base pair with NaN^O with four H-bonds (Fig. 1c). As can be seen by comparison with a natural base pair such as A:T (Fig. 1d), the proposed base pair, BaO^N:NaN^O, is expected to have a similar intrastrand C1′–C1′ position.

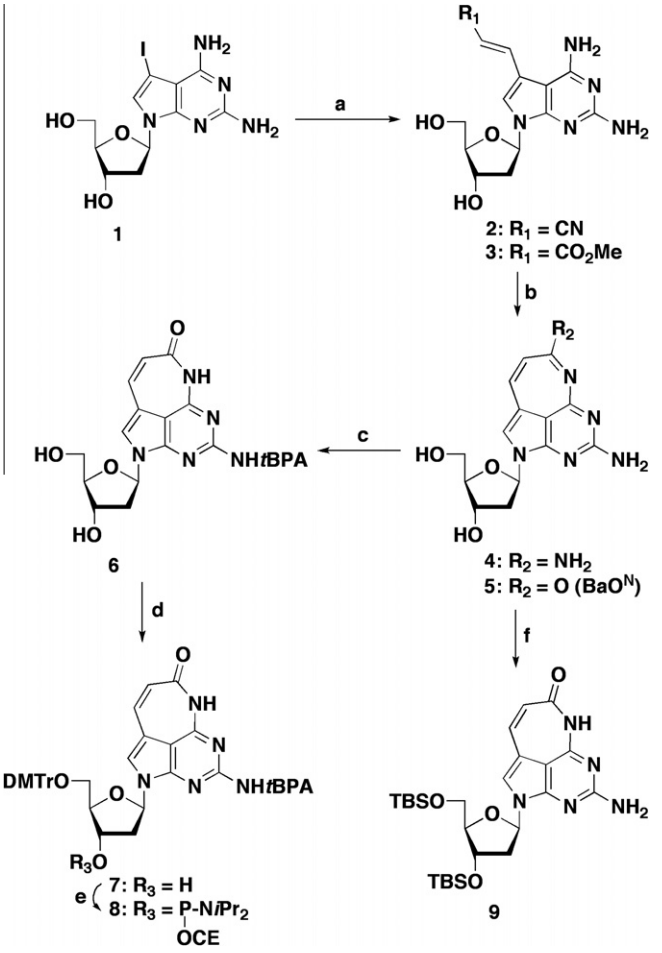
In this work, we describe the synthesis of the nucleoside unit **5** (BaO^N) and the thermal stabilities of the duplex containing the newly designed base pairing motif, BaO^N:NaN^O.

2. Results and discussion

2.1. Chemistry

We recently reported the synthesis of the 4,7-amino-2,3,5,6-tetraazabenzo[*cd*]azulene nucleoside **4** from **1** via **2**, and its spontaneous hydrolysis to the 4-amino-2,3,5,6-tetraazabenzo[*cd*]azulen-7-one nucleoside (BaO^N) **5**, the desired compound in this study.[12] To prepare **5** more conveniently, we decided to synthesize it via **3** (Scheme 1). Thus, the palladium catalyzed cross-coupling reaction of the 7-iodo derivative **1** with methyl acrylate was first investigated. The reaction was carried out in DMF at 70 °C in the presence of a Pd catalyst [$Pd(PPh_3)_4$, $(dba)_3Pd_2{\cdot}CHCl_3$, $(PhCN)_2PdCl_2$, $Pd(OAc)_2$, or $(PPh_3)_2PdCl_2$] with or without CuI. The best result was obtained when $(PPh_3)_2PdCl_2$ was used without CuI to give **3** in 62% yield. The resulting **3** was subsequently treated with NaOMe in MeOH to give **5** in 60% yield, the spectral data of which were identical with those reported in our previous paper.[12]

Thus far, only studies directed at the synthesis of nucleoside derivatives possessing the 2,3,5,6-tetraazabenzo[*cd*]azulene skeleton as a nucleobase have been reported.[13,14] However, no reports on ODNs containing such tricyclic artificial nucleobases have appeared. Since **5** has a lactam moiety, the stability of **5** under the conditions of ODN synthesis was examined prior to ODN synthesis. In the general protocol for ODN synthesis, the elongating ODN on a controlled pore glass (CPG) support is exposed to acidic and oxidative conditions, the former to remove the DMTr group and the latter to oxidize the phosphorus atom. The resulting ODN is then treated with basic conditions to remove all the protecting groups and detach the ODN from the CPG support. Thus, **5** was treated



Scheme 1. Reagents and conditions: (a) methyl acrylate, Et_3N, $(PPh_3)_2PdCl_2$, DMF, 70 °C, 62%; (b) 0.1 M NaOMe in MeOH, 70 °C, 60%; (c) TMSCl, pyridine then *t*-butylphenoxyacetyl chloride, 93%; (d) DMTrCl, pyridine, 88%; (e) 2-cyanoetyl *N*,*N*-diisopropylchlorophosphoramidite, iPr_2NEt, CH_2Cl_2, 78%; (f) TBSCl, imidazole, DMF, 69%.

under the above conditions to check its stability, and it was shown to be stable against both 80% aqueous AcOH and 0.03 M I_2 in THF/pyridine/H_2O (8:1:1) (data not shown).[15] On the other hand, **5** was found to be sensitive under the basic conditions used in the usual ODN synthesis. Therefore, **5** was treated with concentrated NH_4OH at 55 °C, and its stability was analyzed by HPLC. As a result, decomposition of **5** was observed and its half-life ($t_{1/2}$) was estimated to be 40 min (Fig. 2a). Even when the reaction temperature was decreased to room temperature, the estimated $t_{1/2}$ (5 h) was still insufficient because treatment for approximately 12 h under these conditions is required for ODN synthesis (Fig. 2b). Treatment with 0.05 M K_2CO_3 in MeOH is also applicable to ODN synthesis if more labile protecting groups are used for the protection of exocyclic amino groups of adenine, guanine, and cytosine moieties (termed ultramild phosphoramidites). To our delight, **5** was sufficiently stable under these conditions (Fig. 2c).

In order to incorporate **5** into ODNs, the exocyclic amino group of **5** must be protected with an appropriate protecting group, which can be removed with 0.05 M K_2CO_3 in MeOH. Sinha et al. reported using a *t*-butylphenoxyacetyl (*t*BPA) group for the protection of the exocyclic amino groups, which was removed under the aforementioned conditions.[16] Hence, we planned to introduce this protecting group on **5**. Compound **5** was first treated with trimethylsilyl chloride (TMSCl) in pyridine for selective protection of the 3′ and 5′-hydroxyl groups. Treatment with *t*BPA-Cl followed by

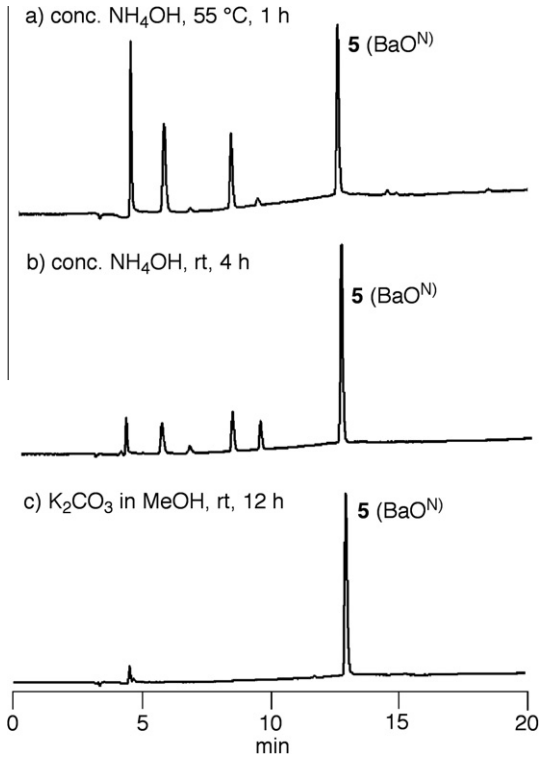


Figure 2. HPLC profiles of treatment of **5** with various basic conditions.

aqueous workup provided **6** in 93% yield. Since it is necessary to check whether the *t*BPA group can be removed without decomposition, **6** was treated with 0.05 M K_2CO_3 in MeOH, and the *t*BPA group was removed smoothly without decomposition of the lactam moiety (see Supplementary data). Protection of the 5′-hydroxyl group with a dimethoxytrityl (DMTr) group and subsequent treatment of the resulting **7** with *N,N*-diisopropylchlorophosphoramidite in the presence of Hünig's base afforded the phosphoramidite unit **8** (Scheme 1).

2.2. Synthesis of oligodeoxynucleotides

We next prepared ODNs on a DNA/RNA synthesizer. In one series in our studies, we used three sets of complementary 17mers (ODN I:ODN II, ODN III:ODN IV, and ODN V:ODN VI) (Table 1) to investigate the thermal stability of the duplexes containing new base pairing motifs.[8] Thus, the BaO^Ns were incorporated in the X position of ODNs I, III, and V. In general, the phosphoramidite units were dissolved in acetonitrile to be loaded on to the DNA/RNA synthesizer. However, an acetonitrile solution of **8** was found to be too viscous, necessitating use of dichloromethane as solvent. The fully protected ODNs (1 μmol scale) linked to the CPG support were prepared by the usual phosphoramidite method. Then, the resulting ODNs were treated with 0.05 M K_2CO_3 in MeOH for 12 h at room temperature, followed by C-18 column chromatography. The ODNs were detritylated with 80% aqueous AcOH, and each ODN obtained showed a single peak on reverse-phase HPLC. Each ODN was completely hydrolyzed to the corresponding nucleosides by a mixture of nuclease P1, snake venom phosphodiesterase, and alkaline phosphatase, and the nucleoside composition was analyzed by HPLC. In the case of ODN I and ODN III (X = BaO^N), the peak corresponding to the tricyclic nucleoside **5**, confirmed by co-elution with an authentic sample, was clearly observed, and the nucleoside composition calculated from the areas of the peaks supported the structure of each (Supplementary data). On the other hand, no peak corresponding to **5** was observed when the resulting ODN V′ (which was not desired ODN V and termed as ODN V′) was hydrolyzed. The matrix-assisted laser desorption/ionization time-of-flight mass (MALDI-TOF/MS) spectrum of ODN V′ suggested that three *t*BPA groups still remained on its structure (data not shown). In the case of ODNs I and III, the *t*BPA groups were removed by treatment with 0.05 M K_2CO_3 in MeOH at room temperature. However, the *t*BPA groups on three consecutive BaO^N residues like ODN V′ were resistant under the same conditions. Therefore, the ODN linked to the solid support was treated with 0.05 M K_2CO_3 in MeOH at 55 °C for 8 h, and the resulting ODN V was hydrolyzed and analyzed by HPLC. The peak corresponding to **5** was observed, and the nucleoside composition calculated from the areas of the peaks supported the structure of ODN V.

Table 1
Sequences of ODNs and hybridization data[a]

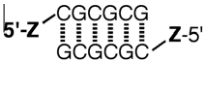
	Duplex	X	Y	T_m[a] (°C)	ΔT_m[b] (°C)
		A	T	47.2	–
ODN I	5′-GCACCGAAXAAACCACG-3′	G	C	48.5	+1.3
ODN II	3′-CGTGGCTTYTTTGGTGC-5′	ImO^N	NaN^O	55.3	+8.1
		BaO^N	NaN^O	52.0	+4.8
		G	C	55.1	+7.9
ODN III	5′-GCXCGAAXAAACCXCG-3′	ImO^N	NaN^O	79.8	+32.6
ODN IV	3′-CGYGGCTTYTTTGGYGC-5′	BaO^N	NaN^O	65.2	+18.0
		G	C	55.2	+5.0
ODN V	5′-GCACCGAXXXAACCACG-3′	ImO^N	NaN^O	77.7	+30.5
ODN VI	3′-CGTGGCTYYYTTGGTGC-5′	BaO^N	NaN^O	65.7	+18.5

[a] Experimental conditions are described in the Section 3. The data presented are average of triplicates.
[b] The ΔT_m values were obtained by subtracting data for the T_m possessing X:Y = A:T from that for each duplex.

The thermal stability of the duplexes formed by ODN I:ODN II (containing one X:Y pair), ODN III:ODN IV (containing three nonconsecutive X:Y pairs), and ODN V:ODN VI (containing three consecutive X:Y pairs) was measured by thermal denaturation in a buffer of 10 mM sodium cacodylate (pH 7.0) containing 1 mM NaCl. Each profile of the thermal denaturation showed a single transition corresponding to a helix-to-coil transition to give a melting temperature (T_m). For comparison, T_m values of DNA duplexes containing natural A:T and G:C pair(s) and noncanonical ImO^N:NaN^O pair(s) along with those of BaO^N:NaN^O pair(s) were measured under the same conditions. The resulting T_ms and ΔT_ms were calculated based on the T_m of the duplex (X:Y = A:T, common to ODN I:ODN II, ODN III:ODN IV, and ODN V:ODN VI) and are listed in Table 1. When one A:T pair of ODN I:ODN II was replaced with a G:C pair, the T_m value was increased to 48.5 °C from 47.2 °C (ΔT_m = 1.3 °C), which arises from a two to three increase in H-bonds. The substitution of X:Y into the ImO^N:NaN^O pair remarkably increased the T_m value to 55.3 °C (ΔT_m = 8.1 °C) as reported

2.3. Thermal stability of the DNA duplexes containing BaO^N:NaN^O pairs

Table 2
Measurement of stacking abilities of each nucleobase

Duplex	Z	T_m[a] (°C)	ΔT_m[b] (°C)	$\Delta T_m/Z$[b] (°C)
	None	40.8	–	–
	T	49.1	+8.3	+4.2
	A	54.9	+14.1	+7.1
	Im-O^N	57.4	+16.6	+8.3
	Ba-O^N	61.1	+20.3	+10.2

[a] Experimental conditions are described in Section 3. The data presented are averages of triplicates.
[b] The ΔT_m values were obtained by subtracting data for the T_m possessing Z = none from that for each duplex.

previously.[8] This elevation was thought to be due to the effects of: (1) an increase of H-bonds from two to four; (2) an increase of stacking ability of the expanded aromatic surface; and (3) a suitable distance between the base pair at the C1′ (purine:pyrimidine type). As explained in the Introduction, we designed a BaO^N:NaN^O pair to enhance the duplex stability by adopting a better geometry of the intrastrand C1′–C1′ position than an ImO^N:NaN^O pair. Contrary to our expectation, the T_m value of ODN I:ODN II containing a BaO^N:NaN^O pair was lower than that of the ImO^N:NaN^O pair (52.0 °C vs. 55.3 °C), although the BaO^N:NaN^O pair was more stable than the A:T (ΔT_m = 4.8 °C) and G:C (DT_m = 3.5 °C) pairs. The same tendency was observed in the results of ODN III:ODN IV and ODN V:ODN VI. Hence, the newly designed base pairing motif BaO^N:NaN^O stabilized the duplex via four H-bonds irrespective of the mode of incorporation,[17] although its stabilizing effect was smaller than that of the ImO^N:NaN^O pair. In order to understand why the BaO^N:NaN^O pair is less thermally stable than the ImO^N:NaN^O pair in the DNA duplex, two experiments were carried out, as discussed in the next section.

2.4. Considerations as to why the BaO^N:NaN^O pair is less thermally stable than the ImO^N:NaN^O pair

As one of the possible explanations of the above results, the BaO^N may have a weaker stacking ability than the ImO^N in the DNA duplex. To examine this possibility, the stacking ability of BaO^N was estimated. According to the method reported by Guckian et al.,[18] a series of self-complementary ODN VII, where Z was added at the 5′-end of each paired duplex (dangling end), were prepared, and the T_m values were determined in a buffer of 0.01 M sodium phosphate (pH 7.0) containing 1 M NaCl (Table 2). As a comparison, the T_m data for non-dangling and natural bases (T and A) at the dangling end are listed (Table 2). The two unpaired Ts and As added +8.3 °C (+4.2 °C/base) and +14.1 °C (+7.1 °C/base) to the T_ms, respectively, relative to the control duplex (Z = none). These increments of T_m values can be estimated as their own stacking ability of the nucleobases. When ImO^N was introduced into the Z position, the T_m value was increased to 57.4 °C (ΔT_m = 8.3 °C/base), which was higher than those of the natural nucleobases.[6] Contrary to our speculation, the duplex possessing BaO^N in the Z position added +10.2 °C/base of T_m, which was the highest among the nucleobases examined. Accordingly, it was revealed that the lower thermal stability of a BaO^N:NaN^O pair compared with that of an ImO^N:NaN^O pair was not due to their stacking ability.

As an alternative explanation, we considered the difference of H-bonding ability between BaO^N and ImO^N. In our previous paper,[6] we showed that ImO^N formed an anti-parallel homodimer via four H-bonds in a nucleoside level.[19] Since BaO^N is also expected to form an anti-parallel homodimer via four H-bonds, we compared its ability to form the homodimer to that of ImO^N by a temperature-dependent ^{1}H NMR study. The results of ImO^N are shown in Figure 3a.[6] Thus, the amide proton was observed at around 12.6 ppm as a broad singlet, and the amino protons were observed at around 7.0 ppm as a single coalesced signal at 60 °C. These results showed that ImO^N prefers to exist as a monomer at that temperature. On the other hand, the amide proton signal was shifted downfield with decreasing temperature. In addition, the amino protons were split into two sets of broad singlets. These spectral changes indicated formation of the expected anti-parallel homodimer, which had already been observed at 0 °C and more clearly at −60 °C. Based on these observations, a temperature-dependent ^{1}H NMR study of BaO^N was carried out. First, the hydroxyl groups of **5** were silylated with *tert*-butyldimethylsilyl chloride to give **9** (Scheme 1). ^{1}H NMR measurements of **9** were conducted at variable temperatures in $CDCl_3$ at 25 mM concentrations to observe the anti-parallel homodimer formation. As can be seen in Figure 3b, the amide proton was observed at around 9.0 ppm as a broad singlet, and the amino protons were observed at around 5.0 ppm as a single coalesced signal at 60 °C. These results indicated the existence of BaO^N as a monomer, as was the case for ImO^N. In addition, the amide and amino proton signals were shifted downfield with decreasing temperature. Finally, the amino protons were split

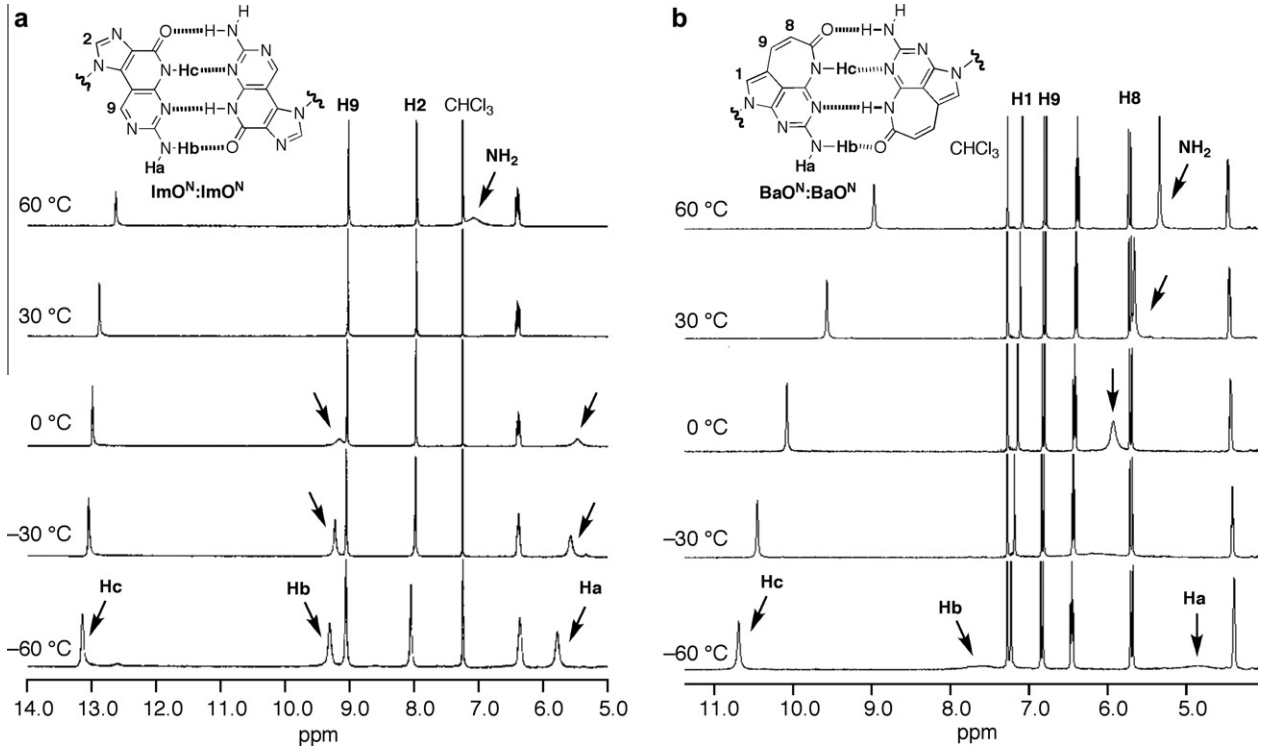


Figure 3. Partial ^{1}H NMR spectra obtained in the course of carrying out a temperature-dependent measurements of: (a) ImO^N and (b) BaO^N at 25 mM in $CDCl_3$.

into two sets of broad singlets at around 8.5 and 5.0 ppm at −60 °C. From these results, it was confirmed that BaO^N also formed an antiparallel homodimer in $CDCl_3$ via four H-bonds. However, clear differences between ImO^N and BaO^N were observed in the spectra at 30, 0, and −30 °C. For example, the homodimer formation of ImO^N has already been observed at 0 °C as described above. In contrast, BaO^N existed preferentially as a monomer at the same temperature, because the amino protons were still observed at around 6.0 ppm as a single coalesced signal. Since the ImO^N ring system has fourteen electrons and satisfies Hückel's rule, this ring system would be planer. In contrast, the BaO^N ring system has thirteen electrons, which does not satisfy Hückel's rule. Therefore, the BaO^N ring system would probably not be planer. These results and considerations indicated that the H-bonding ability of BaO^N would be weaker than that of ImO^N and would support the lesser thermal stability of a BaO^N:NaN^O pair than an ImO^N:NaN^O pair.

In conclusion, we have designed a new base pairing motif consisting of a BaO^N:NaN^O pair to thermally stabilize the DNA duplex. The corresponding BaO^N nucleoside was prepared starting from the 7-deaza-7-iodopurine derivative **1** via a palladium catalyzed cross-coupling reaction with methyl acrylate, followed by intramolecular cyclization. After the incorporation of BaO^N into the ODNs, the thermal stability of the resulting duplex containing the BaO^N:NaN^O pair(s) was evaluated. The BaO^N:NaN^O pair thermally stabilized the duplexes better than the A:T and G:C pairs, however the stabilizing effect was less than that of the ImO^N:NaN^O pair, which we previously developed.[8] A temperature-dependent ^1H NMR study revealed that the H-bonding ability of BaO^N was weaker than that of ImO^N, which would explain why the BaO^N:NaN^O pair was less thermally stable than the ImO^N:NaN^O pair. In order to clarify whether the BaO^N and NaN^O units form base pairs with four H-bonds in the duplex and whether the resulting BaO^N:NaN^O pair adopts the intrastrand C1′–C1′ position similar to natural A:T pair, an NMR study is required, which is now under investigation.

3. Experimental section

3.1. General methods

Physical data were measured as follows: melting points are uncorrected. ^1H and ^{13}C NMR spectra were recorded at 270 or 500 MHz and 67.5 or 125 MHz instruments in $CDCl_3$ or DMSO-d_6 as the solvent with tetramethylsilane as an internal standard. Chemical shifts are reported in parts per million (d), and signals are expressed as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). All exchangeable protons were detected by addition of D_2O. TLC was done on Merck Kieselgel F254 precoated plates. Silica gel used for column chromatography was YMC Gel 60A (70–230 mesh). Iatrobeads used for column chromatography was 6RS-8090 (Mitsubishi Chemical Medience Co.). Aminosilica gel was Chromatorex NH-DM1020 (Fuji Silysia Chemical Ltd).

3.2. 2,4-Diamino-5-[(*E*)-1-(methoxycarbonyl)-2-ethenyl]-7-(2-deoxy-β-D-*erythro*-pentofuranosyl)pyrrolo[2,3-*d*]pyrimidine (3)

To a solution of **1** (391 mg, 1.0 mmol) in DMF (10 mL) including Et_3N (0.28 mL, 2.0 mmol) and $(PPh_3)_2PdCl_2$ (70 mg, 0.1 mmol) was added methyl acrylate (1.8 mL, 20 mmol), and the reaction mixture was heated at 70 °C for 5 h. The solvent was removed in vacuo, and the residue was purified by an aminosilica gel column, eluted with MeOH in $CHCl_3$ (3%), to give **3** (215 mg, 62%) as a yellow solid. An analytical sample was crystallized from MeOH to give pale yellow crystals: mp 195–196 °C; FAB-LRMS *m/z* 350 [$(M+H)^+$]; ^1H NMR (DMSO-d_6) δ 7.82 (d, 1H, J = 15.5 Hz), 7.71 (s, 1H, H-6), 6.36 (dd, 1H, J = 5.7 and 8.3 Hz), 6.36 (br s, 2H, exchangeable with D_2O), 6.30 (d, 1H, J = 15.5 Hz), 5.82 (br s, 2H, exchangeable with D_2O), 5.22 (d, 1H, J = 3.5 Hz, exchangeable with D_2O), 5.02 (t, 1H, J = 5.8 Hz, exchangeable with D_2O), 4.31 (m, 1H), 3.77 (m, 1H), 3.68 (s, 3H), 3.55 and 3,49 (each m, each 1H), 2.38 (ddd, 1H, J = 8.3, 5.5, and 13.2 Hz), 2.09 (ddd, 1H, J = 5.7, 2.9, and 13.2 Hz); ^{13}C NMR (DMSO-d_6) δ 167.1, 160.0, 158.5, 154.3, 133.1, 120.8, 114.0, 112.1, 92.4, 87.2, 82.3, 71.0, 62.0, 51.1, 39.5; Anal. Calcd for $C_{15}H_{19}N_5O_5$: C, 51.57; H, 5.48; N, 20.05. Found: C, 51.71; H, 5.42; N, 20.02.

3.3. 4-Amino-2-(2-deoxy-β-D-*erythro*-pentofuranosyl)-2,6-dihydro-7*H*-2,3,5,6-tetraazabenzo[*cd*]azulen-7-one (5)[12]

A solution of **3** (255 mg, 0.73 mmol) in 0.1 M NaOMe in MeOH (73 mL) was heated at 70 °C for 12 h. The reaction mixture was cooled to 0 °C, and the resulting precipitate was corrected and washed with MeOH to give **5** (89 mg, 38%) as yellow solid. The filtrate was removed in vacuo, and the residue was purified by an aminosilica gel column, eluted with MeOH in $CHCl_3$ (5–25%), to give additional **5** (50 mg, 22%). The physical data of **5** were identical with those of reported previously: UV λ_{max} (H_2O) 392 nm (ε = 1170), 301 nm (ε = 8830), 242 nm (ε = 33,300); λ_{max} (0.5 M HCl) 311 nm (ε = 6820), 279 nm (ε = 10,600), 243 nm (ε = 33,000); λ_{max} (0.5 M NaOH) 243 nm (ε = 22,100); ^1H NMR (DMSO-d_6) δ 10.08 (d, 1H, J = 1.2 Hz, exchangeable with D_2O), 7.34 (s, 1H), 6.93 (d, 1H, J = 12.0 Hz), 6.29 (dd, 1H, J = 5.9 and 7.9 Hz), 6.25 (br s, 2H, exchangeable with D_2O), 5.58 (d, 1H, J = 11.6 Hz), 5.26 (d, 1H, J = 3.6 Hz, exchangeable with D_2O), 5.02 (t, 1H, J = 5.8 Hz, exchangeable with D_2O), 4.30 (m, 1H), 3.77 (m, 1H), 3.49 (m, 2H), 2.49 (m, 1H), 2.12 (ddd, 1H, J = 6.1, 2.3, and 12.8 Hz).

3.4. 4-(4-*tert*-Butylphenoxyacetylamino)-2-(2-deoxy-β-D-*erythro*-pentofuranosyl)-2,6-dihydro-7*H*-2,3,5,6-tetraazabenzo[*cd*]azulen-7-one (6)

To a suspension of **5** (400 mg, 1.26 mmol) in pyridine (25 mL) was added chlorotrimethylsilane (0.97 mL, 7.6 mmol), and the reaction mixture was stirred at room temperature. After being stirred for 1 h, *tert*-butylphenoxyacetyl chloride (0.39 mL, 1.9 mmol) was added to the mixture, and the whole was stirred for additional 1 h. The reaction was quenched by addition of H_2O, and the solvent was removed in vacuo. The residue was purified by a silica gel column, eluted with MeOH in $CHCl_3$ (2–5%), to give **6** (595 mg, 93%) as a yellow solid. An analytical sample was crystallized from MeOH to give pale yellow crystals: mp 153 °C (decomp.); FAB-LRMS *m/z* 508 [$(M+H)^+$]; ^1H NMR (DMSO-d_6) δ 10.74 (br s, 1H, exchangeable with D_2O), 10.31 (br s, 1H, exchangeable with D_2O), 7.66 (s, 1H), 7.28 (d, 2H, J = 8.6 Hz), 7.04 (d, 1H, J = 12.2 Hz), 6.83 (d, 2H, J = 8.6 Hz), 6.40 (dd, 1H, J = 6.1 and 7.9 Hz), 5.68 (d, 1H, J = 12.2 Hz), 5.31 (d, 1H, J = 4.3 Hz, exchangeable with D_2O), 5.08 (s, 2H), 4.92 (t, 1H, J = 4.3 Hz, exchangeable with D_2O), 4.34 (m, 1H), 3.82 (m, 1H), 3.52 (m, 2H), 2.44 (ddd, 1H, J = 7.9, 5.5, and 13.4 Hz), 2.20 (ddd, 1H, J = 6.1, 3.0, and 13.4 Hz), 1.23 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 166.8, 156.0, 154.3, 153.2, 153.0, 143.5, 134.2, 126.4, 122.9, 121.0, 114.3, 113.2, 102.2, 87.7, 83.2, 71.1, 67.9, 62.0, 39.9, 34.1, 31.6; Anal. Calcd for $C_{26}H_{29}N_5O_6 \cdot 0.5H_2O$: C, 60.46; H, 5.85; N, 13.56. Found: C, 60.20; H, 5.74; N, 13.40.

3.5. 4-(4-*tert*-Butylphenoxyacetylamino)-2-(2-deoxy-5-*O*-4,4′-dimethoxytrityl-β-D-*erythro*-pentofuranosyl)-2,6-dihydro-7*H*-2,3,5,6-tetraazabenzo[*cd*]azulen-7-one (7)

To a solution of **6** (600 mg, 1.2 mmol) in dry pyridine (6 mL) was added DMTrCl (1.8 mmol, 610 mg), and the reaction mixture

was stirred for 1 h at room temperature. The reaction was quenched by addition of ice. The mixture was concentrated in vacuo. The residue was diluted with AcOEt, which was washed with H_2O and saturated aqueous $NaHCO_3$, followed by brine. The organic layer was dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by a silica gel column, eluted with $CHCl_3$/acetone (19:1–9:1), to give **7** (840 mg, 88%) as a yellow solid: FAB-LRMS *m/z* 810 [(M+H)$^+$]; FAB-HRMS calcd for $C_{47}H_{47}N_5O_8$ 809.3425, found 810.3509 [(M+H)$^+$]; ^{1}H NMR ($CDCl_3$) δ 9.25 (br s, 1H, exchangeable with D_2O), 8.95 (br s, 1H, exchangeable with D_2O), 7.41 (d, 2H, *J* = 7.3 Hz), 7.31-7.20 (m, 10H), 6.88 (d, 2H, *J* = 8.6 Hz), 6.68 (d, 4H, *J* = 9.2 Hz), 6.68 (t, 1H, *J* = 6.4 Hz), 6.45 (d, 1H, *J* = 11.6 Hz), 5.72 (dd, 1H, *J* = 11.6 and 1.8 Hz), 4.77 (m, 1H), 4.63 (br s, 2H), 4.19 (m, 1H), 3.76 (s, 6H), 3.51 (br s, 1H, exchangeable with D_2O), 3.45 (dd, 1H, *J* = 3.7 and 10.4 Hz), 3.40 (dd, 1H, *J* = 3.7 and 10.4 Hz), 2.59 (m, 2H), 1.28 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 166.8, 158.7, 155.1, 153.6, 153.0, 152.8, 147.4, 145.0, 144.7, 139.6, 135.8, 135.7, 133.8, 130.3, 129.3, 128.4, 128.0, 128.0, 127.9, 127.2, 127.1, 126.6, 122.7, 120.7, 114.5, 113.5, 113.3, 103.3, 86.8, 86.3, 83.9, 77.4, 72.4, 68.1, 63.9, 55.4, 55.4, 41.3, 34.3, 31.6.

3.6. 4-(4-*tert*-Butylphenoxyacetylamino)-2-{2-deoxy-3-*O*-[(*N*,*N*-diisopropylamino)-2-cyanoethoxyphosphino]-5-*O*-4,4′-dimethoxytrityl-β-D-*erythro*-pentofuranosyl}-2,6-dihydro-7*H*-2,3,5,6-tetraazabenzo[*cd*]azulen-7-one (8)

To a solution of **7** (700 mg, 0.86 mmol) in dry CH_2Cl_2 (9 mL) were added *N*,*N*-diisopropylethylamine (600 mL, 3.5 mmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (580 mL, 2.6 mmol), and the reaction mixture was stirred for 30 min at room temperature. The reaction was quenched by addition of ice. The reaction mixture was diluted with AcOEt, which was washed with H_2O (twice) and saturated brine. The organic layer was dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by a silica gel column, eluted with hexane/AcOEt (3:2–1:1), to give **8** (680 mg, 78%) as a yellow solid: FAB-LRMS *m/z* 1010 [(M+H)$^+$]; FAB-HRMS calcd for $C_{56}H_{64}N_7O_9P$ 1009.4503, found 1010.4598 [(M+H)$^+$]; ^{31}P NMR ($CDCl_3$) δ 149.3 and 149.1.

3.7. 4-Amino-2-(2-deoxy-3,5-di-*O*-*tert*-butyldimethylsilyl-β-D-*erythro*-pentofuranosyl)-2,6-dihydro-7*H*-2,3,5,6-tetraazabenzo[*cd*]azulene-7-one (9)

To a solution of **5** (93 mg, 0.29 mmol) in DMF (3 mL) containing imidazole (120 mg, 1.8 mmol) was added *tert*-butyldimethylsilyl chloride (160 mg, 0.88 mmol), and the reaction mixture was stirred for 3 h at 50 °C. The reaction was quenched by addition of MeOH and the solvent was removed in vacuo. The residue was dissolved in a mixture of $CHCl_3$ and MeOH (9:1), and the organic solution was washed with 1 N HCl, saturated aqueous $NaHCO_3$, and saturated brine. The organic layer was dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by a silica gel column, eluted with hexane/AcOEt (3:1–2:1), to give **9** (110 mg, 69%) as a yellow solid. An analytical sample was crystallized from MeOH to give yellow crystals: mp 147–148 °C; EI-LRMS *m/z* 545 (M$^+$); ^{1}H NMR ($CDCl_3$) δ 9.81 (br s, 1H, exchangeable with D_2O), 7.11 (s, 1H), 6.81 (d, 1H, *J* = 11.5 Hz), 6.43 (m, 1H), 5.88 (br s, 2H, exchangeable with D_2O), 5.75 (d, 1H, *J* = 11.5 Hz), 4.54 (m, 1H), 3.93 (m 1H), 3.82 (dd, 1H, *J* = 11.2 and 3.6 Hz), 3.75 (dd, 1H, *J* = 11.2 and 3.0 Hz), 2.33 (m, 2H), 0.94 and 0.92 (each s, each 9H), 0.10 (s, 12H); ^{13}C NMR ($CDCl_3$) δ 169.0, 162.4, 155.0, 153.8, 134.9, 120.3, 119.9, 114.3, 99.6, 87.7, 83.5, 72.2, 63.2, 41.6, 26.3, 26.1, 18.8, 18.4, −4.3, −4.5, −5.0, −5.1; Anal. Calcd for $C_{26}H_{43}N_5O_4Si_2$: C, 57.21; H, 7.94; N, 12.83. Found: C, 57.15; H, 7.82; N, 12.53.

3.8. Stability of 5 under basic conditions (experiments shown in Fig. 2)

Compound of **5** (ca. 1 mg) was dissolved in concentrated NH_4OH (2 mL) in a vial tube, and the solution was heated at 55 °C (or kept at room temperature). An aliquot of the reaction mixture (25 mL) at the appropriate time was diluted with 0.1 M TEAA buffer (pH 7.0) (450 mL), and this solution was analyzed by HPLC, using a J'sphere ODN M80 column (4.6 × 250 mm, YMC) with a linear gradient of acetonitrile (from 9.5% to 41% over 20 min) in 0.1 M TEAA buffer (pH 7.0). In the case of basic conditions shown in Figure 3c, an aliquot of the reaction mixture (25 mL) was diluted with 0.1 M TEAA buffer (pH 7.0)(450 mL) and 0.05 M aqueous AcOH (25 mL), and this solution was analyzed by HPLC under the same conditions.

3.9. Synthesis of ODNs I, III, V and VII containing BaO^N

ODNs were synthesized on a DNA synthesizer (Applied Biosystem Model 3400) using BaO^N phosphoramidite unit **8**, and commercially available deoxyribonucoside phosphoramidite units (phenoxyacetyl protected dA, acetyl protected dC, and isopropylphenoxyacetyl protected dG) at 1 μmol scale following the standard procedure. For the incorporation of BaO^N into the ODNs, a 0.12 M solution in dry CH_2Cl_2, and coupling time of 10 min was used. After completion of synthesis, the CPG support was then treated with 0.05 M K_2CO_3 in MeOH for 12 h at room temperature. In the case of ODN V (containing three consecutive BaO^N), the CPG support was treated with 0.05 M K_2CO_3 in MeOH at 55 °C for 8 h. After filtration of the CPG support, the released ODN protected by a DMTr group at the 5′-end was chromatographed on a C-18 silica gel column (1 × 13 cm) with a linear gradient of acetonitrile (from 0 to 50%) in 0.1 M TEAA buffer (pH 7.0). The fractions were concentrated, and the residue was treated with aqueous 80% AcOH at room temperature for 10 min, then the solution was concentrated, and the residue was coevaporated with H_2O. The residue was dissolved in H_2O and the solution was washed with AcOEt, then the H_2O layer was concentrated to give a deprotected ODN. The ODN was further purified by reverse-phase HPLC, using a J'sphere ODN M80 column (4.6 × 150 mm, YMC) with linear gradient of acetonitrile (from 5 to 20% over 20 min) in 0.1 M TEAA buffer (pH 7.0) to give a highly purified ODNs.

3.10. Hyperchromicity, extinction coefficients and nucleoside composition of the ODNs

Each ODN (0.5 OD units at 260 nm) was incubated with snake venom phosphodiesterase (6 μL, 1 mg/0.5 mL), nuclease P1 (12 μL, 1 mg/mL), and alkaline phosphatase (10 μL, 0.1 U/mL) in a buffer containing 100 mM Tris–HCl (pH 7.7) and 2 mM $MgCl_2$ (total 528 mL) at 37 °C for 8 h. Hyperchromicity of each ODN was determined by comparing UV absorbencies at 260 nm of the solutions before and after hydrolysis. The extinction coefficients (at 260 nm) of each ODN were determined using the following equation: ε_{ODN} = the sum of $\varepsilon_{nucleoside}$/hyperchromicity. The extinction coefficients (at 260 nm) of nucleosides used for calculations were as follows: dA, 15,400; dG, 11,700; dT, 8800; dC, 7300; BaO^N, 13,900. After the reaction mixture was heated in boiling water for 5 min, the enzymes were removed from the reaction mixture by filtration with Micropure®-EZ device (Millipore), and the filtrate was concentrated. Nucleoside composition was determined by analysis of the residue with reverse-phase HPLC, using a J'sphere ODN 80 column (4.6 × 250 mm, YMC) with a linear gradient of acetonitrile (from 5% to 80% over 30 min) in 0.1 M TEAA buffer (pH 7.0). The results were shown in the Supplementary data (Fig. S2 and Table S1).

3.11. Thermal denaturation

Each sample containing an appropriate duplex (6 μM for the experiments in Table 1 and 5 μM for the experiments in Table 2) in a buffer solution (10 mM sodium cacodylate (pH 7.0) containing 1 mM NaCl for the experiments in Table 1 and 10 mM sodium cacodylate (pH 7.0) containing 1 M NaCl for the experiments in Table 2) was heated at 95 °C for 5 min, cooled gradually to an appropriate temperature, and used for the thermal denaturation study. Thermal-induced transitions of each mixture of ODNs were monitored at 260 nm on a Beckman DU 650 spectrophotometer. The sample temperature was increased 0.5 °C/min.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.11.023.

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