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A practical post-modification synthesis of oligodeoxynucleotides containing 4,7-diaminoimidazo[5′,4′:4,5]pyrido[2,3-*d*]pyrimidine nucleoside

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ABSTRACT

We describe herein the practical post-modification synthesis of oligodeoxynucleotides (ODNs) containing 4,7-diaminoimidazo[5′,4′:4,5]pyrido[2,3-*d*]pyrimidine nucleoside (ImN^N). Since the ImN^N nucleoside unit possessing tribenzoyl groups on its exocyclic amino groups as the protecting group was quite unstable under acidic conditions, cleavage of its glycosidic linkage in ODN has been suggested throughout the conditions of solid-phase synthesis. As an alternative approach, we investigated a post-modification synthesis of the desired ODNs containing the ImN^N unit. Starting with protected 4-amino-7-chloro-1-(2-deoxy-β-D-ribofuranosyl)imidazo[5′,4′:4,5]pyrido[2,3-*d*]pyrimidine derivative **1**, conversion into the corresponding phosphoramidite unit was examined. The *p*-bromobenzoyl group (*p*-BrBz) was the best protecting group of 4-amino group of **1** to give the phosphoramidite unit **9** for the post-modification synthesis. After carrying out the ODN synthesis linked to the controlled pore glass (CPG) support, the support was treated with ammonium hydroxide at 55 °C to remove the protecting groups, detach the ODN form the CPG support, and convert the 7-chloro group into a desired amino group. As a result, the desired ODNs containing ImN^N were obtained in good yield.

1. Introduction

Thus far, a large number of chemically modified nucleoside derivatives have been incorporated into oligodeoxynucleotides (ODNs) with the aim of biological, bioengineering, and therapeutic applications. However, the desired ODN synthesis has sometimes failed because of instability of modified nucleoside units in ODN throughout the conditions of solid-phase synthesis. If this is the case, a post-modification synthesis would be an effective alternative approach to prepare the desired ODNs.[1–3] In this method, a labile structure is substituted to the stable form, which is converted into the desired structure in the last stage of ODN synthesis.[4]

Among our studies, we have been investigating the design and synthesis of artificial base-pairing motifs beyond the Watson–Crick base pairs.[5–10] We had previously reported the synthesis of a series of nucleoside derivatives possessing an imidazo[5′,4′:4,5]pyrido[2,3-*d*]pyrimidine (Im) and a 1,8-naphthyridine (Na) moieties, which were expected to form base pairs (a series of Im:Na pairs) with four hydrogen bonds (H-bonds) when they were introduced into ODNs. In fact, an ImO^N:NaN^O pair (Fig. 1A) in an ODN, for example, markedly stabilized DNA duplexes thermally and thermodynamically.[6] Furthermore, we demonstrated that the ImO^N:NaN^O base pair was selectively recognized as a complementary base by DNA polymerases due to the four H-bonds and the shape complementarity of the pair with the purine:pyrimidine base pair.[11,12] Quite recently, we designed a new Im:Na pair, that is, the ImN^N:NaO^O pair (Fig. 1B) consisting of a DAAD:ADDA H-bonding pattern (D = donor and A = acceptor), and demonstrated the ImN^N:NaO^O pair was more thermally and thermodynamically stable because of a favorable secondary interaction due to the arrangement of H-bonding pattern.[10] In order to utilize this artificial base pair for a variety of applications, including a study of enzymatic recognition by DNA polymarases and structural analysis by NMR and X-ray, preparation of more ODNs containing ImN^N and NaO^O was required. However, the overall yield of ODN synthesis containing

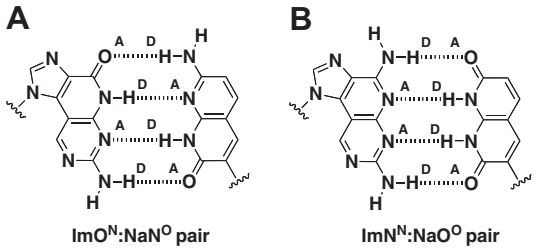

Figure 1. Structures of Im:Na base pairs.

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ImN^N was quite low, unlike that of ODN synthesis containing other Im and Na nucleoside derivatives including NaO^O.

In this work, we investigated why incorporation of ImN^N unit into ODN resulted in low yield, and found that multiple electron-withdrawing groups such as benzoyl group on the nucleobase accelerate cleavage of the glycosidic linkage. Accordingly, we developed an efficient post-modification synthesis of ODN containing ImN^N. Herein, we present the experimental details of the results of this research.

2. Results and discussion

As described in our previous papers, we prepared ODNs containing ImN^N using a phosphoramidite unit **5** (Scheme 1). In accordance with our previous method,[5] we planned to prepare a new 30-mer ODN containing an ImN^N unit (5′-GTGGGCAAGImN^NGTGCGCTGACCATCCAGAAC-3′). After carrying out the usual procedure for ODN synthesis, followed by C-18 cartridge column purification, the resulting solution was analyzed by reversed-phase high-performance liquid chromatography (HPLC) using a C-18 column. As a result, this analysis afforded a complex HPLC profile (Fig. 2A), distinct from the case of ODN synthesis containing NaO^O, and isolation yield of the desired ODN was quite low (the peak corresponding to the desired 30-mer ODN is marked by asterisk). In the solid-phase synthesis of ODN using DNA synthesizer, the elongated ODN on controlled pore glass (CPG) support is exposed by acidic (3% trichloroacetic acid (TCA) in CH_2Cl_2), coupling (1*H*-tetrazole in CH_3CN), capping (Ac_2O in THF/pyridine), and oxidation (0.02 M I_2 in THF/H_2O/pyridine) conditions until the desired length is reached. Among these conditions, it is known that the acidic conditions often cause a cleavage of the glycosidic linkage, especially in purine nucleoside units.[13] Since the phosphoramidite unit **5** has three benzoyl groups on its nucleobase moiety, the cleavage of the glycosidic linkage under the acidic conditions was suspected. To confirm this assumption, UV spectra of **4** in CH_2Cl_2 (neutral) and 3% TCA in CH_2Cl_2 (acidic) solutions were compared. Consequently, the resulting two UV spectra had no inflection point (compare the spectra 1 and 2), which suggested a structural change other than protonation on its nucleobases, that is, the cleavage of the glycosidic linkage of **4** had occurred. Since the spectrum under acidic conditions did not change even after 1 h (compare the spectra 2 and 3), this undesired cleavage was presumed to occur immediately under the acidic conditions (Fig. 3A).[14] On the other hand, UV spectra of unprotected ImN^N derivative **3** under the neutral and acidic conditions had an inflection point around 340 nm (compare the spectra 1 and 2), suggesting the structural change from neutral to protonation form. The spectrum under the acidic conditions then gradually changed (compare the spectra 2 and 3), arising from the cleavage of the glycosidic linkage. (Fig. 3B, the inflection point of this alteration was observed at 360 nm).[14] These results suggested that the ImN^N derivative possessing tribenzoyl groups on its exocyclic amino groups was labile, giving abasic site under the acidic conditions arising from a protonation of the N3 position (Scheme 2). To stabilize its glycosidic linkage, we examined preparation of other protected ImN^N units, such as dibenzoate and dibutylaminomethylene derivatives. However, none of the attempts afforded the desired compounds in good yield (data not shown).

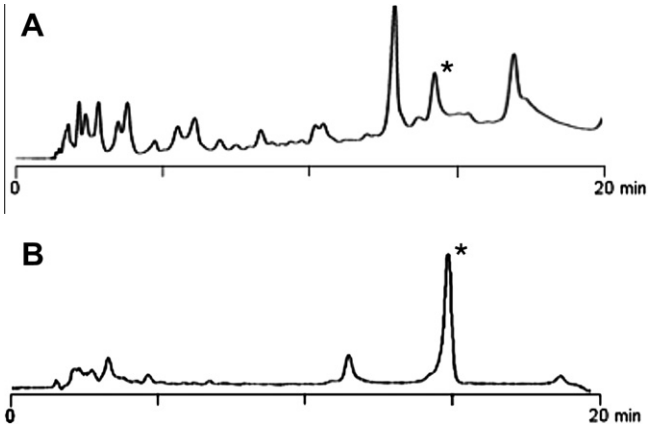
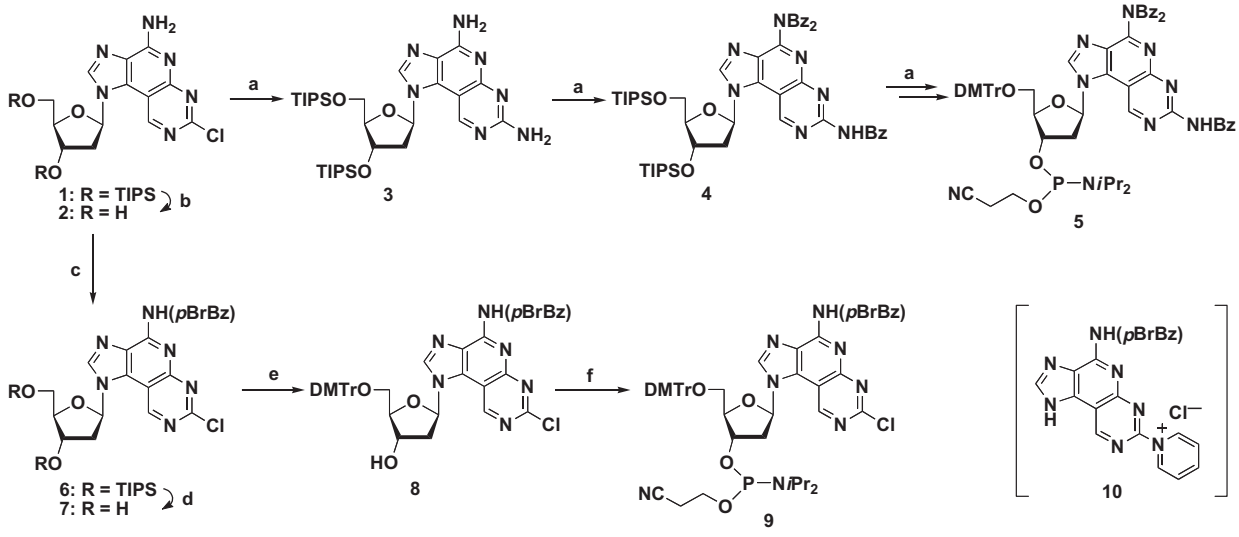


Figure 2. HPLC profiles of ODN synthesis (A) using phosphoramidite **5**; (B) using **9**, after C-18 cartridge column purification. The peak marked with asterisk is the desired 30-mer.

As described in the Introduction, the post-modification synthesis is an effective alternative approach for ODN synthesis. Accordingly, we planned to adopt this approach for ODN synthesis containing the ImN^N. As shown in Scheme 1, ImN^N derivative **3** was prepared via 4-amino-7-chloro derivative **1**[5] by treatment with a mixture of ammonium hydroxide–1,4 dioxane (1:1) at



Scheme 1. Reagents: (a) Ref. 5; (b) NH_4F in MeOH, reflux; (c) *p*-BrBzCl, Hunig's base in CH_2Cl_2, then NaOEt in EtOH–THF (1:1); (d) TBAF in THF; (e) DMTrCl, Et_3N in DMF; (f) 2-cyanoetyl *N*,*N*-diisopropylchlorophosphoramidite, iPr_2NEt, DMAP in CH_2Cl_2.

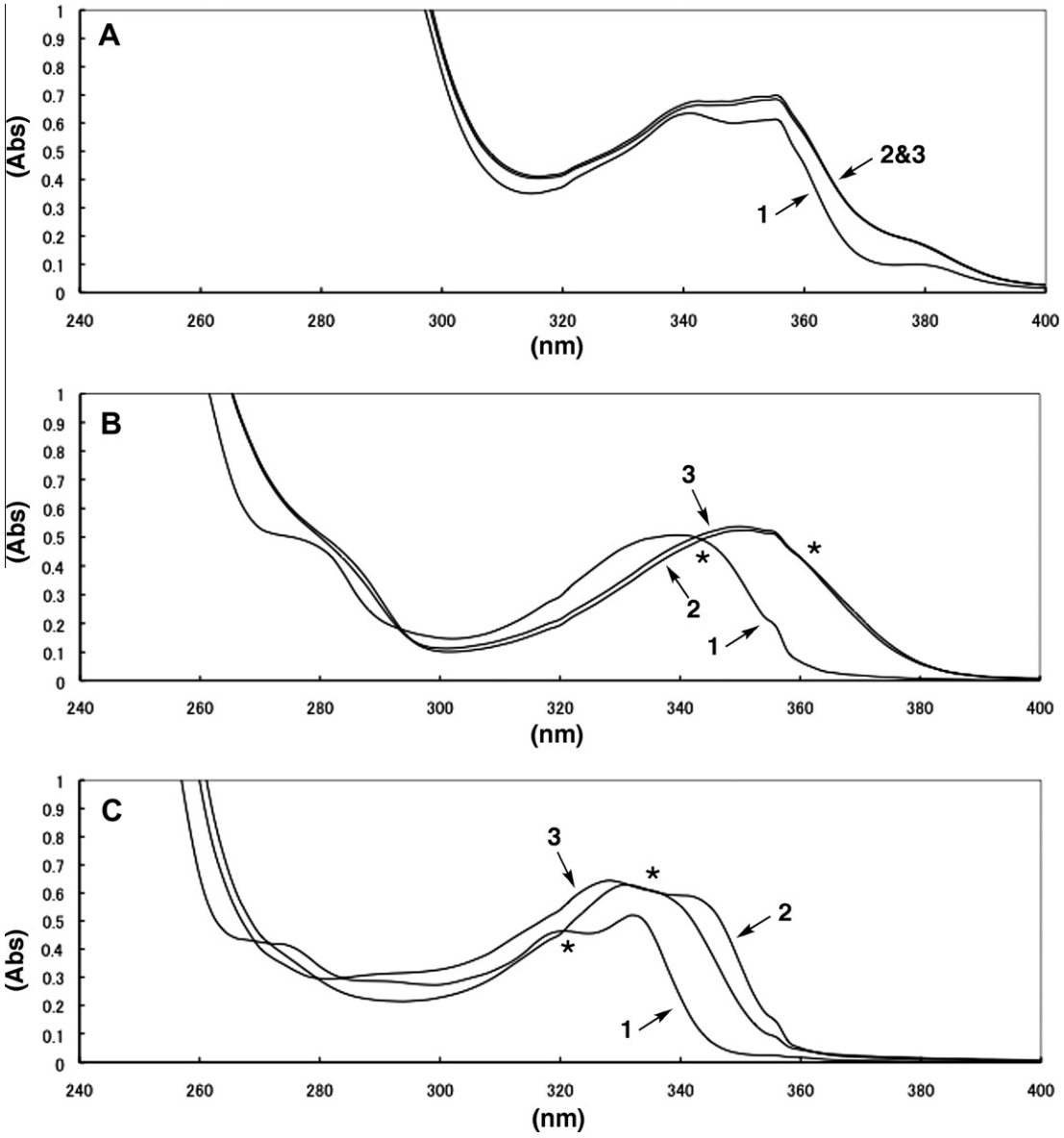
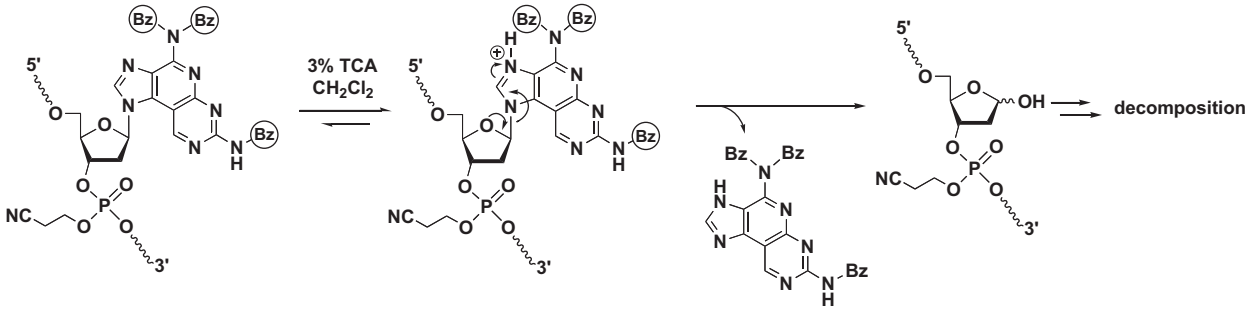


Figure 3. UV spectra of (A) compound **4**; (B) compound **3**; (C) compound **6**, in CH_2Cl_2 (neutral) and 3% TCA in CH_2Cl_2 (acidic). Spectra **1** were UV absorption under neutral conditions, and **2** (0 sec) and **3** (after 1800 or 3600 s) were those under acidic conditions. The inflection points were marked with asterisk.



Scheme 2. Proposed mechanism of the cleavage of glycosidic linkage.

100 °C. If this nucleophilic substitution occurs under milder conditions, a 4-amino-7-chloroimidazopyridopyrimidine skeleton like **1** would be a suitable structure for the post-modification synthesis. To confirm this strategy, compound **2** prepared form **1** was treated with ammonium hydroxide at 55 °C for 17 hr, which are the usual conditions for ODN synthesis. As a result, complete conversion of **2** into the corresponding ImN^N derivative was confirmed by HPLC analysis (see the Supplementary data, Fig. S2). Since the alternative

route has been suggested, protection of the excyclic amino group on 4-position of **1** with a benzoyl group was examined. However, the resulting monobenzoate was a rather polar compound compared with the corresponding tribenzoate derivative, and thus this protecting group was unsuitable for further conversion, especially in the chromatographic purification of the corresponding phosphoramidite unit. In order to decrease the polarity and to ease the chromatographic purification of the resulting compounds, a *p*-bromobenzoyl (*p*-BrBz) group was chosen as the protecting group. The desired protection functioned well when **1** was treated with *p*-bromobenzoyl chloride in CH_2Cl_2 in the presence of Hunig's base,[15] followed by NaOEt in a mixture of EtOH and THF (1:1) to give monoacylated product **6** in 74% yield by two steps. The resulting **6** was then converted into the free nucleoside **7** by treatment with tetrabutylammonium fluoride in THF. When **7** was treated with dimethoxytrityl chloride (DMTrCl) in pyridine,[15] a polar spot was observed in TLC analysis and no desired **8** was obtained. The resulting polar compound had no signal corresponding to the sugar portion, and the ^{1}H NMR analysis indicated this could be 7-pyridinium derivative **10** (see the Supplementary data, Fig. S3). Since pyridine was found to be unsuitable as reaction solvent, dimethoxytritylation of **7** was carried out in DMF in the presence of triethylamine[16] to give **8** in 78% yield. Treatment of **8** with *N*,*N*-diisopropylchlorophosphoramidite in CH_2Cl_2 in the presence of Hunig's base afforded the phosphoramidite unit **9**.

Prior to the ODN synthesis, stability of the glycosidic linkage was evaluated using **6** by monitoring of UV absorption. As can be seen in Figure 3C, the UV spectra of **6** under the neutral and the acidic conditions had the inflection point around 320 nm (compare the spectra 1 and 2), and the spectrum under the acidic conditions gradually changed (compare the spectra 2 and 3), which suggested the cleavage of the glycosidic linkage (the inflection point of this alteration was observed at 340 nm).[14] The velocity of the cleavage reaction was rather slow, and the $t_{1/2}$ of **6** was estimated to be 350 sec, which was longer than not only that of **4** ($t_{1/2}$ = <30 s) but also that of silylated *N*-benzoyladenine ($t_{1/2}$ = 300 s). This result indicates that the 4-amino-7-chloroimidazopyridopyrimidine nucleoside unit possessing a *p*-BrBz group such as **9** in ODN can tolerate ODN synthesis.

Once the conditions had been fixed, synthesis of the desired 30 mer was tried again. After carrying out the usual procedure for ODN synthesis using the phosphoramidite **9**, the ODN supported on CPG was treated with ammonium hydroxide at 55 °C for 17 h, and the reaction mixture after C-18 cartridge column purification was analyzed by HPLC using a C-18 column. As can be seen in Figure 2B, the HPLC profile of the mixture was quite simple, distinct from the previous experiment (Fig. 2A), giving the desired sequence as a major product. Further purification of the peak corresponding to the desired sequence was carried out to give pure ODN. To confirm the nucleoside composition, the resulting ODN was treated with a mixture of nuclease P1, snake venom phosphodiesterase, and alkaline phosphatase, and the reaction mixture was analyzed by HPLC. The peak corresponding to the ImN^N, confirmed by co-elution with an authentic sample, was clearly observed, and the nucleoside composition calculated from the areas of the peaks supported the sequence of ODN (Fig. 4). Since no peak corresponding to **2** was observed in the HPLC profile, this post-modification synthesis via nucleophilic substitution of 7-chloro group into 7-amino group worked well. Using this synthetic method, we tried the synthesis of another sequence (5′-CGAAImN^NAACC-3′) for structural analysis by NMR study. This attempt also afforded the desired 9-mer in good yield.

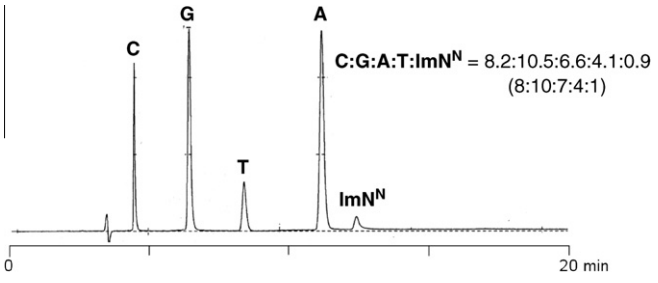


Figure 4. HPLC profile of the 30-mer ODN after complete hydrolysis.

In conclusion, we investigated why incorporation of ImN^N unit in ODN does not result in high yield, and revealed that the cause arises from the instability of the glycosidic linkage of ImN^N derivative possessing tribenzoyl groups on its exocyclic amino groups under acidic conditions. Accordingly, post-modification synthesis of the desired ODN was examined as an alternative approach. Starting with 4-amino-7-chloro derivative **1**, which is a precursor of 4,7-diamino derivative, conversion into the corresponding phosphoramidite unit was examined. The *p*-BrBz group was the best protecting group of 4-amino group of **1** for further chemical conversion. Since dimethoxytritylation of **7** in pyridine afforded unexpected **10**, compound **8** was prepared by treatment of **7** with DMTrCl in DMF in the presence of triethylamine. The resulting **8** was then converted into the phosphoramidite unit **9** for the post-modification synthesis. After carrying out the ODN synthesis linked to the CPG support, the support was treated with ammonium hydroxide at 55 °C for 17 hr to remove the protecting groups, detach the ODN form the CPG support, and convert 7-chloro group into the amino group. As a result, the desired ODNs containing ImN^N were obtained in good yield. Using these ODNs, a study of enzymatic recognition by DNA polymarases and structural analysis of the ImN^N:NaO^O pair is now under investigation. The results will be reported in due course.

3. Experimental section

3.1. General methods

Physical data were measured as follows: Melting points are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded at 400 or 500 MHz and 100 or 125 MHz instruments (Bruker FT-NMR AV400 or AV500) in $CDCl_3$ or DMSO-d_6 as the solvent with tetramethylsilane as an internal standard. Chemical shifts are reported in parts per million (δ), 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 KANTO CHEMICAL Silica Gel 60 (spherical) 63–210 or Silica Gel 60 N (spherical, neutral) 63–210.

3.2. 4-Amino-7-chloro-1-(2-deoxy-β-D-ribofuranosyl)imidazo-[5′,4′:4,5]pyrido[2,3-*d*]pyrimidine (2)

A solution of **1**[3] (97 mg, 0.15 mmol) in MeOH (5 mL) containing ammonium fluoride (56 mg, 1.5 mmol) was heated under reflux for 50 h. The reaction mixture was cooled and concentrated in vacuo. The residue was purified by a short silica gel column, eluted with MeOH in $CHCl_3$ (10–40%), to give **2** (36 mg, 72%) as a white solid: ^{1}H NMR (DMSO-d_6) δ 9.25 and 8.64 (each s, each 1H), 8.02 (br s, 2H, exchangeable with D_2O), 6.64 (dd, 1H, J = 5.5 Hz), 5.43 (m, 1H, exchangeable with D_2O), 4.98 (m, 1H, exchangeable with D_2O), 4.40 (m, 1H), 3.96 (m, 1H), 3.51 (dd, 1H, J = 4.0, 12.0 Hz), 3.43 (dd, 1H, J = 4.5, 12.0 Hz), 2.79 (m, 1H), 2.52 (m, 1H); ^{13}C NMR (DMSO-d_6) δ 159.34, 157.78, 157.41, 154.37, 141.04, 131.05, 128.42, 107.03, 88.15, 85.89, 69.82, 60.90; ESIMS-LR m/z = 359

(MNa$^+$); ESIMS-HR Calcd for $C_{13}H_{13}ClN_6O_3Na$ 359.0635, found 359.0660.

3.3. N^4-*p*-Bromobenzoylamino-7-chloro-1-(2-deoxy-3,5-di-*O*-triisopropylsilyl-β-D-ribofuranosyl)imidazo[5′,4′:4,5]pyrido[2,3-*d*]pyrimidine (6)

To a solution of **1** (1.07 g, 1.6 mmol) in CH_2Cl_2 (16 mL) including *N*,*N*-diisopropylethylamine (1.66 mL, 9.8 mmol) was added *p*-BrBzCl (1.07 g, 4.9 mmol), and the reaction mixture was stirred for 12 h at room temperature. The reaction was quenched by addition of ice and the reaction mixture was partitioned between $CHCl_3$ and H_2O. The separated organic layer was washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried (Na_2SO_4) and concentrated in vacuo. Then, the residue was dissolved in a mixture of EtOH–THF (32 mL each) and NaOEt (2.66 mL of 20% EtOH solution) was added at 0 °C. After being stirred for 10 min, the reaction mixture was neutralized by 1 N HCl. The solvent was removed in vacuo, and the residue was partitioned between $CHCl_3$ and H_2O. The separated organic layer was washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried (Na_2SO_4) and concentrated in vacuo, and the residue was purified by a silica gel column (neutralized), eluted with MeOH in $CHCl_3$ (0–5%), to give **6** (1.07 g, 80%) as a white solid: ^{1}H NMR (DMSO-d_6) δ 11.45 (s, 1H, exchangeable with D_2O), 9.77 and 8.70 (each s, each 1H), 7.98 and 7.77 (each d, each 2H, J = 8.5 Hz), 6.87 (dd, 1H, J = 5.3 Hz), 4.76 (m, 1H), 4.09 (m, 1H), 3.78 (dd, 1H, J = 3.8, 11.4 Hz), 3.65 (dd, 1H, J = 3.9, 11.4 Hz), 3.15 (m, 1H), 2.69 (ddd, 1H, J = 5.9, 13.3 Hz), 1.12–0.97 (m, 42H); ^{13}C NMR (DMSO-d_6) δ 165.19, 158.08, 157.35, 156.07, 152.86, 141.65, 133.83, 132.68, 132.37, 131.55, 130.62, 126.47, 108.53, 87.87, 85.56, 70.71, 62.07, 17.83, 17.51, 11.56, 11.16; ESIMS-LR m/z = 853 (MNa$^+$); ESIMS-HR Calcd for $C_{38}H_{56}BrClN_6O_4Si_2Na$ 853.2671, found 853.2660.

3.4. N^4-*p*-Bromobenzoylamino-7-chloro-1-(2-deoxy-β-D-ribofuranosyl)imidazo[5′,4′:4,5]pyrido[2,3-*d*]pyrimidine (7)

To a solution of **6** (400 mg, 0.48 mmol) in THF was added TBAF (1 M in THF, 1.0 mL, 1 mmol) at 0 °C. After being stirred for 40 min at room temperature, the the reaction was quenched by addition of acetic acid (60 μL, 1.0 mmol). The solvent was removed in vacuo, and the residue was purified by a silica gel column, eluted with MeOH in $CHCl_3$ (0–15%), to give **7** (250 mg, quant) as a white solid: ^{1}H NMR (DMSO-d_6) δ 11.47 (br s, 1H, exchangeable with D_2O), 9.75 and 8.82 (each s, each 1H), 7.99 and 7.77 (each d, each 2H, J = 8.5 Hz), 6.80 (m, 1H), 5.46 (d, 1H, J = 5.0 Hz, exchangeable with D_2O), 4.88 (t, 1H, J = 5.2 Hz, exchangeable with D_2O), 4.46 (m, 1H), 3.99 (m, 1H), 3.53 (ddd, 1 H, J = 4.3, 4.9, 11.8 Hz), 3.45 (m, 1 H), 2.90 (m, 1H), 2.58 (m, 1H); ^{13}C NMR (DMSO-d_6) δ 157.34, 142.23, 131.55, 130.68, 126.46, 88.20, 85.98, 69.56, 60.66; ESIMS-LR m/z = 540 (MH$^+$); ESIMS-HR Calcd for $C_{20}H_{16}BrClN_6O_4$ 541.0003, found 540.9855.

3.5. N^4-*p*-Bromobenzoylamino-7-chloro-1-[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl]imidazo[5′,4′:4,5]pyrido-[2,3-*d*]pyrimidine (8)

To a solution of 7 (571 mg, 1.1 mmol) in DMF (10 mL) containing triethylamine (0.91 mL, 6.6 mmol) was added DMTrCl (1.11 g, 3.3 mmol), and the reaction mixture was stirred for 22 h at room temperuture. The reaction was quenched by addition of ice and the reaction mixture was partitioned between $CHCl_3$ and H_2O. The separated organic layer was washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried (Na_2SO_4) and concentrated in vacuo, and the residue was purified by a silica gel column (neutralized), eluted with MeOH in $CHCl_3$ (0–13%), to give 8 (704 mg, 78%) as a white solid: ^{1}H NMR (DMSO-d_6) δ 11.46 (s, 1H, exchangeable with D_2O), 9.97 and 8.87 (each s, each 1H), 7.92 and 7.73 (each d, each 2H, J = 8.3 Hz), 7.11–6.58 (m, 13 H), 6.56 (m, 1 H), 5.50 (d, 1H, J = 5.8 Hz, exchangeable with D_2O), 4.54 (m, 1H), 4.08 (m, 1H), 3.65 (s, 6H), 3.25 (m, 1H), 3.13 (dd, 1H, J = 2.5, 10.6 Hz), 2.78 (dd, 1H, J = 4.7, 10.6 Hz), 2.63 (m, 1H); ^{13}C NMR (DMSO-d_6) δ 165.31, 158.48, 157.84, 157.81, 157.48, 156.18, 152.88, 144.50, 141.95, 135.28, 135.20, 133.89, 132.71, 132.31, 131.50, 130.61, 129.32, 129.27, 129.18, 127.56, 127.50, 127.31, 126.45, 112.83, 108.52, 85.80, 85.25, 84.92, 79.15, 68.90, 62.06, 54.89, 37.88; ESIMS-LR m/z = 844 (MNa$^+$); ESIMS-HR Calcd for $C_{41}H_{34}BrClN_6O_6$ 843.1278, found 843.1309.

Physical data for N^4-*p*-Bromobenzoylamino-7-pyridiniumimidazo[5′,4′:4,5]pyrido[2,3-*d*]pyrimidine chloride (**10**): ^{1}H NMR (DMSO-d_6) δ 12.16 (br s, 1H, exchangeable with D_2O), 10.28 and 8.78 (each s, each 1H), 10.18 (d, 2H, J = 5.7 Hz), 8.96 (t, 1H, J = 7.5 Hz), 8.45 (m, 2 H), 8.10 and 8.82 (each d, each 2H).

3.6. N^4-*p*-Bromobenzoylamino-7-chloro-1-[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl]imidazo[5′,4′:4,5]pyrido-[2,3-*d*]pyrimidine 2-cyanoethyl *N*,*N*′-diisopropylphosphoramidite (9)

To a mixture of 8 (205 mg, 0.25 mmol), *N*,*N*-diisopropylethylamine (174 μL, 1.0 mmol), and dimethylaminopyridine (5 mg) in CH_2Cl_2 (5 mL) was added 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (61 μL, 0.28 mmol) at 0 °C. After the mixture was stirred for 1 h at room temperature, the mixture was diluted with $CHCl_3$. The mixture was washed with saturated aqueous $NaHCO_3$, followed by brine. The separated organic layer was dried (Na_2SO_4), and concentrated in vacuo. The residue was purified by a silica gel column (neutralized), eluted with hezane/AcOEt (4:1 to 1:2), to give 9 (148 mg, 58%) as a white foam: ESIMS-LR m/z 1044 (MNa$^+$); ESIMS-HR Calcd. for $C_{50}H_{51}BrClN_8O_7PNa$ 1045.2367, found 1045.2405; ^{31}P NMR ($CDCl_3$) δ: 149.63, 149.07.

3.7. Synthesis and characterization of ODNs containing ImN^N using the post-modification method

ODNs were synthesized on a DNA synthesizer (NTS H-6, Nihon Techno Service) using the phosphoramidite unit **9**, and commercially available deoxyribonucoside phosphoramidite units (benzoyl protected dA, acetyl protected dC, and isobutyryl protected dG) at 0.2 μmol scale following the standard procedure. For the incorporation of **9** into the ODNs, a 0.11 M solution in dry CH_3CN, and coupling time of 10 min was used. After completion of synthesis, the CPG support was then treated with concentrated NH_4OH (2 mL) at 55 °C for 17 h, and 0.2 M TEAA buffer (2 mL, pH 7.0) was added to the reaction mixture. The whole mixture was pored onto a C-18 cartrige column (YMC dispoSPE, f 0.6 × 1.6 cm), and the column was washed with 10% CH_3CN in 0.1 M TEAA buffer (pH 7.0) (2 mL), followed by H_2O (3 mL). Then, a solution of 3% aq TFA (4 mL) was eluted to remove the DMTr group. After being washed with H_2O (3 mL), 0.1 M TEAA buffer (2 mL), and H_2O (3 mL), the ODN was eluted with 20% and 50% aq CH_3CN (3 mL each). An aliquot of eluate was analyzed 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 25 min) in 0.1 M TEAA buffer (pH 7.0). The resulting ODN was further purified by reverse-phase HPLC under the same conditions to give a highly purified ODNs. The structure of ODNs containing ImNN were confirmed by MALDI–TOF/MS: 5′-GTGGGCAAGImN^{N}GTGCGCTGACCATCCAGAAC-3′, calculated mass: $C_{295}H_{367}N_{124}O_{174}P_{29}$ 9331.0 (MH$^-$), observed mass: 9336.2; 5′-CGAAImN^{N}AACC-3′, calculated mass: $C_{90}H_{110}N_{41}O_{47}P_8$ 2764.54 (MH$^-$), observed mass: 2766.52.

The former sequence was further confirmed by complete hydrolysis to give nucleoside units. The purified 30-mer ODN (0.25 OD units at 260 nm) was incubated with snake venom phosphodiesterase (6 μL, 68 Unit/mL), nuclease P1 (10 μL, 0.5 U/mL), and alkaline phosphatase (5 μL, 0.1 U/mL) in a buffer containing 100 mM Tris-HCl (pH 7.6) and 2 mM $MgCl_2$ (total 500 μL) at 37 °C for 18 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: $\in_{ODN}$ = the sum of $\in_{nucleoside}$/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; ImN^N, 26,200. 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 25% over 30 min) in 0.1 M TEAA buffer (pH 7.0). The results were shown in Fig. 4.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.10.035.

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14. Full data of UV spectrum monitoring were shown in the Supplementary data.
15. When the reaction with *p*-bromobenzoyl chloride was carried out in pyridine, unexpected cleavege of the glycosidic linkage occurred to give a product like compound **10**. Since no decomposition has occurred when **1** was stirred in pyridine, acylation of the exocyclic amino group seemed to be necessary for such cleavage reaction.
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