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Synthesis and properties of microenvironment-sensitive oligonucleotides containing a small fluorophore, 3-aminobenzonitrile or 3-aminobenzoic acid

Hiroaki Ozaki [a, *], Takeshi Kawai [a], Masayasu Kuwahara [b]

[a] Graduate School of Science and Technology, Gunma University, 4-2 Aramaki, Maebashi, Gunma 371-8510, Japan
[b] Graduate School of Science and Technology, Gunma University, 1-5-1 Tenjin-Cho, Kiryu, Gunma 376-8515, Japan

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ABSTRACT

Two C-nucleosides bearing small fluorescent groups as a base were synthesized by Heck-type coupling reaction and incorporated into DNA. They exhibited environment-sensitive fluorescence and opposite solvatochromic properties. The modified DNAs containing 3-aminobenzonitrile or 3-aminobenzoic acid retained the duplexes and their fluorescence reflected the microenvironment.

Fluorescent nucleic acids are attractive molecules for reading genetic records and an important analytical tool for studying structures of nucleic acids and intermolecular interactions with other molecules. Many fluorescent nucleic acid probes have been developed so far. Especially, fluorescent nucleoside analogs have been used for the labeling at arbitrary positions in nucleic acids. These fluorophores were attached at various positions to the nucleoside, such as C5-position of pyrimidine nucleoside,[1] C2 of purine nucleoside,[2] and 2′ of deoxyribose.[3] They were used for the incorporation of fluorophores into DNAs or RNAs. Moreover, nucleoside analogs, which were replaced by nucleic acid bases in fluorophores, have been reported.[4] Okamoto et al. developed a microenvironment-sensitive fluorescent probe using the Neil Red nucleoside.[4a] Kool et al. reported nucleosides bearing polycyclic aromatics as bases, such as naphthalene, phenanthrene, and pyrene.[5,6] A small fluorescent group is preferred as the modified group in DNA because it is not expected to disrupt the DNA duplexes significantly.

3-Aminobenzonitrile (*m*-cyanoaniline) was reported as a fluorescent molecule that is sensitive to several parameters of the surrounding environment, such as polarity and water content.[7] 3-Aminobenzoic acid is also a fluorescent molecule, in which the fluorescence of the molecule depends on the polarity of the solvent and the pH of its aqueous solutions.[8] The molecular size, like that of a fluorescent molecule, is relatively small. The modified amino acid was derived using 3-amonobenzonitrile derivatives to produce a novel fluorescent probe for studying living systems.[9] We also reported a fluorescent C-nucleoside bearing 3-aminobenzonitrile as the nucleobase.[10] The fluorescence intensity and emission wavelength of this nucleoside analog were found to be strongly dependent on the polarity of the solvents. These nucleoside analogs are expected to not form a Watson–Crick base pair with specific natural bases, but the oligodeoxyribonucleotide bearing these nucleoside analogs can be expected to form duplexes since the bases are small. Such bases will be equally affected by four natural bases. When the bases have an environmental sensitive fluorescent property, they will be responsive to perturbation raised from the surrounding environment. Such a fluorescent base is useful for the analysis of any single-base mutations.[11]

Here, we report the synthesis of fluorescent C-nucleoside analogs containing a relatively small and microenvironment-sensitive fluorophore, their incorporation into DNA, and their fluorescent properties. We describe in detail the synthesis and fluorescence properties of two of these nucleoside analogs, which have 3-aminobenzonitrile or 3-aminobenzoic acid as a nucleobase, as

* Corresponding author.
E-mail address: h-ozaki@gunma-u.ac.jp (H. Ozaki).

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shown in Fig. 1. These nucleosides are incorporated into DNA, and their properties, such as the stability and fluorescence in the duplexes, are studied.

1. Results and discussion

Fluorescent aromatic groups were attached to glycal by a Heck-type coupling reaction to produce C-nucleosides. For the coupling reaction, iodophenyl derivatives were prepared by iodination of the corresponding aniline derivatives. The 3-aminobenzonitrile derivative, which is the nucleobase moiety of compound **1**, and the 3-aminobenzoic acid derivative, which is the nucleobase moiety of compound **2**, were prepared as shown in Scheme 1. The 3-aminobenzonitrile derivative was synthesized by diazotization and the Sandmeyer reaction of 2-amino-5-nitrobenzonitrile to obtain 2-iodo-5-nitrobenzonitrile (**3**), and then reduced to give 5-amino-2-iodobenzonitrile (**4**). The 3-aminobenzoic acid derivative was synthesized by diazotization and the Sandmeyer reaction of 2-amino-5-nitrobenzoic acid to obtain 2-iodo-5-nitrobenzoic acid sodium salt (**5**), and then subjected to esterification and reduction to give methyl 3-amino-6-iodobenzoate (**7**) in good yield. The amino group of **7** was protected by a phenoxyacetyl (Pac) group before coupling with glycal. The coupling reaction of a halogenated aromatic compound with glycal has been reported in the literature.[12] Both iodo-derivated 3-aminobenzonitorile (**4**) and protected 3-aminobenzoic acid (**8**) were coupled by a Heck-type reaction with protected glycal, as shown in Scheme 2. The reaction proceeded by palladium-catalyzed couplings followed by deprotection of silyl groups and the reduction in moderate yields totally. A part of compound **10** was deprotected in the amino group to obtain compound **2** for the measurement of its fluorescent spectrum. The anomeric configurations of compound **1** and compound **2** were determined by coupling constants of H1′ and H2′ protons. Ren et al. reported that H1′− H2′coupling constants were 6–8 Hz for α-C-nucleosides, and 5 and 10 Hz for β-C-nucleosides.[13] Experimental H1′− H2′coupling constants of compound **1** and compound **2** were J = 5.4 and 10.5 Hz, and J = 5.9 Hz and 10.0 Hz, respectively. Therefore, both compounds were assigned to be β-anomers. Compound **1** and compound **10** were derived to protected nucleosides to be incorporated into DNA. The amino group of compound **1** was protected by the Pac group. Initially, the amino group was protected by the acetyl group, but the protection group was not efficiently removed after the oligonucleotide assembling. Therefore, the Pac group was used as an amine-protecting group since it could be easily removed by mild conditioning. To assemble oligonucleotides containing fluorescent C-nucleoside analogs, protected C-nucleoside 3′-phosphoramidites were synthesized, as shown in Scheme 3. The 5′-hydroxy groups of compounds **9** and **10** were protected with a dimethoxytrityl (DMTr) group (**11** and **12**), and then, the 3′-hydroxy group was derived to cyanoethyldiisopropylphosphoramidite (**13** and **14**). The compounds were characterized by their melting temperatures, ^{1}H NMR, ^{13}C NMR, and ESI mass spectrometry. Also, compound **1** and compound **2** were characterized by their specific rotations. In addition, 5′-DMTr-nucleoside 3′-phosphoroamidite analogs, **13** and **14**, were identified by ^{31}P NMR and high-resolution mass spectroscopy.

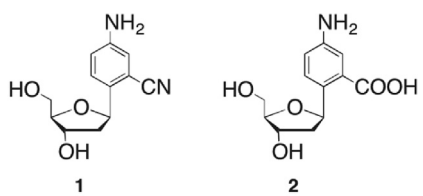
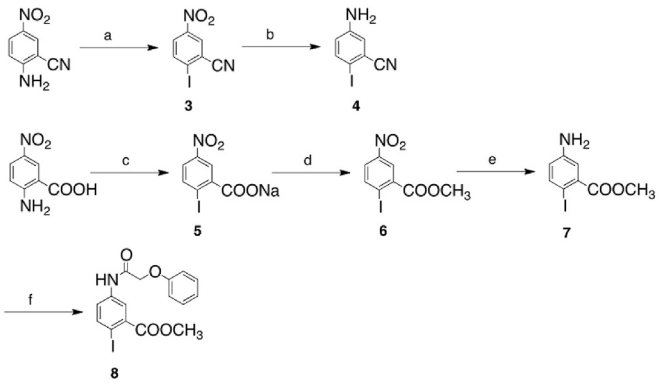
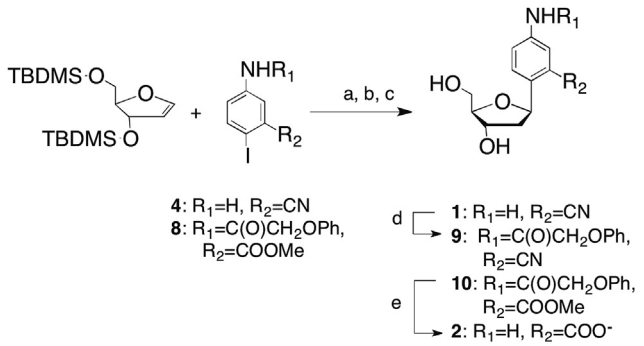


Fig. 1. Structures of fluorescence C-nucleosides in this study.



Scheme 1. Reagents and conditions: (a) (i) $NaNO_2$, conc. HCl, (ii) KI; (b) $SnCl_2$, EtOH; (c) (i) $NaNO_2$, conc. HCl, (ii) KI; (d) (i) $SOCl_2$, (ii) MeOH; (e) $SnCl_2$, EtOH; (f) $(Pac)_2O$, DMF.



Scheme 2. Reagents and conditions: (a) $Pd(OAc)_2$, $As(Ph)_3$, n-Bu_3N, DMF, 90 °C; (b) AcOH, n-TBAF; (c) $NaBH(OAc)_3$, CH_3CN, AcOH; (d) $(Pac)_2O$, DMF; (e) 1 M NaOH aq.

These modified oligodeoxyribonucleotides (ODNs) and other unmodified ODNs were synthesized by the solid-phase phosphoramidite method using a DNA synthesizer. The modified C-nucleoside phosphoramidites were coupled with prolonged coupling time (600 s). These synthesized ODNs were deprotected and purified in an ordinary manner; their sequences and abbreviations are listed in Table 1 **mDNA-1** and **mDNA-2** contain nucleoside **1** and nucleoside **2**, respectively. Both nDNA and cDNA are natural oligodeoxyribonucleotides. nDNA has the same sequence as that of mDNA, but contains thymidine instead of a modified nucleoside. cDNA is a complementary ODN against mODN and nODN, and A, G, C, or T is contained as a complementary base to the modified nucleoside.

It was reported that 3-aminobenzonitrile exhibited a shorter fluorescence wavelength in a hydrophobic than that in a hydrophilic solvent and fluorescence quenching in hydrogen-bond forming solvents, especially water.[7] In addition, the fluorescence of 3-aminobenzoic acid depended on the solvent and solution pH.[8] Fluorescence spectra of their attached C-nucleosides were measured for different solvents and pH values (Fig. 2). Nucleoside **1** showed strong emission in hydrophobic solvents and poor emission in water, similarly to 3-aminobenzonitrile. The pH of the solutions did not affect the fluorescence intensity at pH 4–9. On the other hand, nucleoside **2** showed strong emission in hydrophilic solvents and poor emission in hydrophobic solvents. Also, as the pH increased from 4 to 9, the fluorescence intensity increased, markedly changing between pH 4 and 5, corresponding the pKa of the 3-aminobenzoic acid moiety. This suggested that fluorescence properties depended on the dissociation of the carboxy group.

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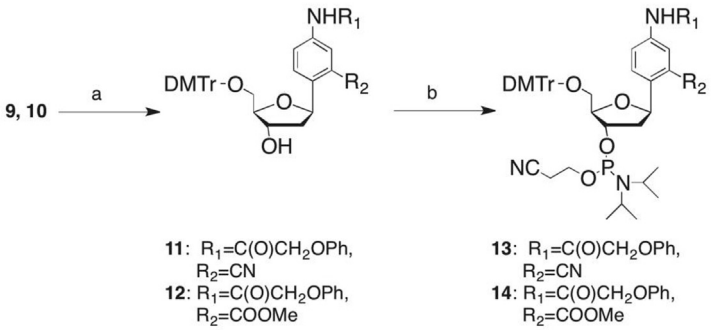

Scheme 3. Reagents and conditions: (a) DMTrCl, pyridine, rt; (b) 2-cyanoethyldiisopropylchlorophosphoramidite, *i*-Pr_2NEt, pyridine, CH_2Cl_2, rt.

The stabilities of their duplexes were investigated through a UV melting experiment. The melting curves for all duplexes were S-shaped, indicating the formation of double helixes. The melting temperatures (Tm) obtained from the UV melting curves are summarized in Table 2. Incorporation of nucleoside **1** or **2** resulted in a decrease in Tm for any complementary bases, compared to the A-T base pairs (**nDNA-T**/**cDNA-A** in Table 2). This result shows that the pairing stability of both nucleoside analogs is lower than that of the Watson-Click base pair. In addition, the Tm values of the duplexes that have a purine base as a complementary base of the nucleoside analogs was 2.2–4.6 °C higher than that of duplexes having a pyrimidine base. Purine base stabilized the duplexes because they had a larger stacking interaction with its neighbor base rather than with the pyrimidine base. However, any base as a complementary base was not discriminated because the differences of Tm were within 4.6 °C.

Table 1
Oligonucleotides used in this study.

#	Sequences, 5′ to 3′
modified ODN	
mDNA-1	5′-CAT AGG AGA **1**GC CTA-3′
mDNA-2	5′-CAT AGG AGA **2**GC CTA-3′
unmodified ODN	
nDNA-T	5′-CAT AGG AGA **T**GC CTA-3′
complementary ODN	
cDNA-A	5′-TAG GC**A** TCT CCT ATG-3′
cDNA-G	5′-TAG GC**G** TCT CCT ATG-3′
cDNA-C	5′-TAG GC**C** TCT CCT ATG-3′
cDNA-T	5′-TAG GC**T** TCT CCT ATG-3′
cDNA-D	5′-TAG GC_ TCT CCT ATG-3′

The duplexes' fluorescence spectra appear in Fig. 3. In a single strand, **mDNA-1** bearing 3-aminobenzonitrile showed larger fluorescence than the duplexes. Moreover, no wavelength shift was observed by the duplex formations. These results suggest that the

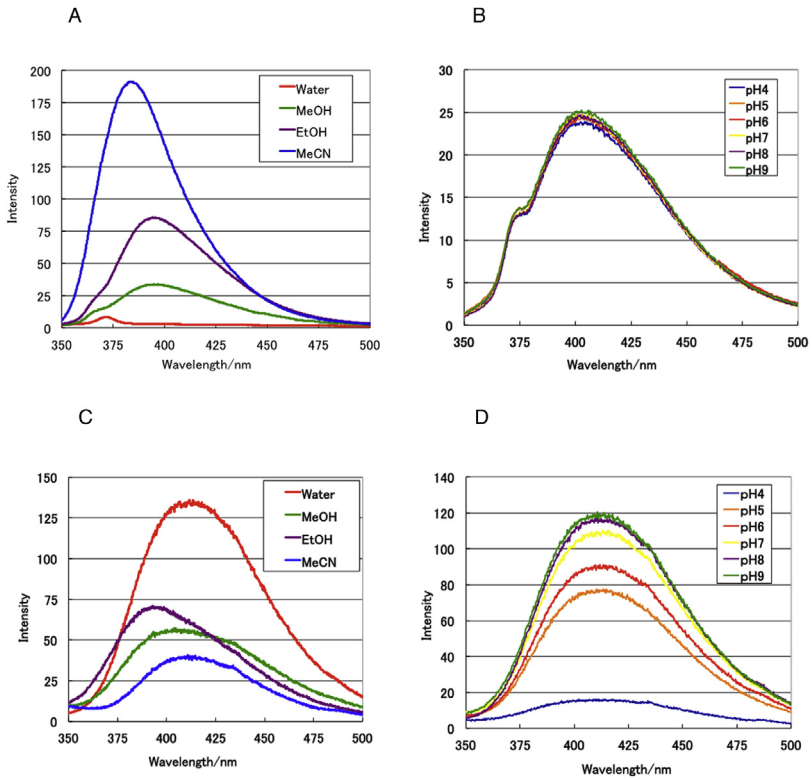

Fig. 2. Fluorescence spectra of nucleosides. Panels A & B, nucleoside **1**; Panels C & D, nucleoside **2**. Conc. of nucleoside; A, C, and D, 2 μM; B, 60 μM: Ex = 328 nm for A and B; Ex = 290 nm for C and D.

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Table 2
Melting temperatures of modified DNA/DNA duplexes.

Duplex	Tm/°C
mDNA-1/cDNA-A	42.0
mDNA-1/cDNA-G	42.1
mDNA-1/cDNA-C	37.5
mDNA-1/cDNA-T	38.9
mDNA-2/cDNA-A	40.6
mDNA-2/cDNA-G	41.6
mDNA-2/cDNA-C	38.3
mDNA-2/cDNA-T	38.4
nDNA-T/cDNA-A	53.9

Conc. of DNA, 2 μM; Buffer,150 mM NaCl, 10 mM Sodium phosphate (pH 7.0).

fluorescence moiety in the single strand form, when placed in a hydrophobic environment, excluded water molecules through an intramolecular interaction because nucleoside **1** exhibited higher fluorescence intensity in hydrophobic solvents. On the other hand, the fluorescence moiety in the duplexes may not form a base pair with its complementary base, but will be placed near the sugar moiety such as in groove, in which the water molecule accesses the fluorescence moiety more easily. **mDNA-2** showed lower and similar fluorescence intensity in the single strand than that in the duplexes. The fluorescence of 3-aminobenzoic acid in **mDNA-2** was opposite to that of 3-aminobenzonitrile, that is, the fluorescence intensity in water was greater than that in a hydrophobic solvent. These results suggest that the fluorescence moiety in the single strand form placed in a hydrophobic environment was excluded from water molecules, as in the case of **mDNA-1**. The fluorescence intensities of the duplexes depended on the complementary base, but the differences were small.

It was reported that the neighboring nucleobase, particularly guanine, can quench the fluorescence of fluorophore; science guanine had the lowest oxidation potential in the natural four bases.[14] **mDNA-1** and **mDNA-2** had guanine as the neighboring nucleobase at the 3′-side of the modified nucleoside. To know the effect on fluorescent properties, the modified ODN, which had adenine, had been prepared (**mDNA-1A** and **mDNA-2A**, see Supplementary data). Adenine, similar to guanine, is a purine nucleobase and had the next lowest oxidation potential of guanine in bases. The modified ODN (**mDNA-1A** and **mDNA-2A**) showed similar fluorescence properties even though it exhibited slightly higher fluorescence intensity than that of the guanine-containing modified ODN (Supplementary data). These results show that the neighboring guanine at the 3′-side of the modified nucleoside had little effect on the fluorescence properties of the modified ODN. The modified nucleoside moiety was not near the neighboring base pair, and this could also be a reason for abovementioned phenomenon.

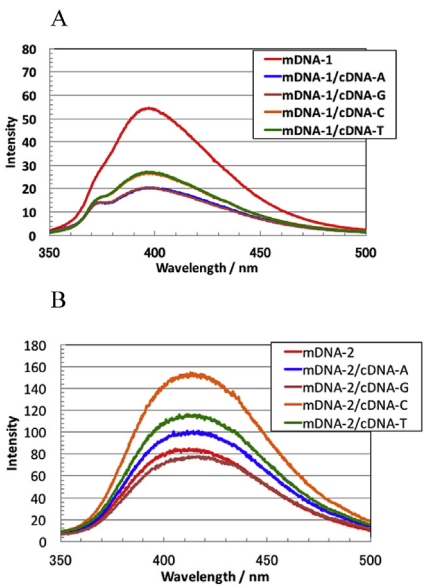

Fig. 3. Fluorescence spectra of modified DNA. A, **mDNA-1**; B, **mDNA-2**. Conc. of duplex, 2 μM; Buffer, 150 mM NaCl, 10 mM sodium phosphate (pH 7); Temperature, 20 °C; Ex = 290 nm.

It was confirmed as to where the modified nucleosides in the duplexes were placed by the collisional quenching study. Collisional quenching by small molecules such as acrylamide is widely used for assessing the solvent accessibility of a fluorophore.[15] We used acrylamide quenching here to monitor the fluorescent nucleosides' exposure to the solvent. We thought that the modified bases in the duplex might not have been placed outside the duplex. For the model in which the modified nucleosides were placed outside of the duplex, we prepared 14-mer DNA (**cDNA-D**), the complementary strand to the modified nucleotide that do not contain a complementary nucleotide, to form a bulge. In the bulged duplex mDNA-#/cDNA-D, the modified bases are expected to be extraherical.[16] Stern-Volmer quenching constants by acrylamide are shown in Table 3.

In all duplexes with **mDNA-1** and **mDNA-2**, the bulged duplexes had higher quenching constants than other duplexes. This indicates that the modified bases in bulged duplexes were exposed to the surrounding solvent. On the other hand, the Tm data of other duplexes showed that the modified nucleosides did not form base pairs via hydrogen bond. These results suggested that the modified bases would be placed near a duplex such as in groove. The fluorescence intensities and the quenching constants of the duplexes slightly depended on the complementary base, though the modified bases did not form base pairs. This indicate that the modified bases detected a small change in the duplex structure caused by the difference in the complementary base. Also, the quenching constants for the duplexes containing **mDNA-1** were smaller than those for **mDNA-2**. This reflects the stronger interaction between the carboxyl group of the modified nucleoside in **mDNA-2** and acrylamide.

Two novel fluorescent C-nucleosides were synthesized by a Heck-type coupling reaction, which exhibited environment-sensitive fluorescence properties. Both nucleosides had opposite solvatochromic properties, and were incorporated into DNA. The fluorescence spectrum of the modified DNA slightly reflected their microenvironment depending on the complementary bases. These properties may be useful for analyzing gene polymorphism, such as SNPs. Furthermore, since these modified DNAs retain duplexes,

Table 3
Stern-Volmer quenching constants.

Sample	K_{SV}/M^{-1}
mDNA-1/cDNA-D	1.59
mDNA-1/cDNA-A	0.53
mDNA-1/cDNA-G	0.64
mDNA-1/cDNA-C	0.34
mDNA-1/cDNA-T	0.65
mDNA-2/cDNA-D	24.6
mDNA-2/cDNA-A	13.2
mDNA-2/cDNA-G	12.1
mDNA-2/cDNA-C	19.8
mDNA-2/cDNA-T	18.2

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even though Tm is low, and the fluorescence reflects the microenvironment, these nucleosides might be useful in the study of interactions between DNA and other molecules such as proteins.

2. Experimental

2.1. Synthesis of nucleoside derivatives

2.1.1. 2-Iodo-5-nitrobenzonitrile (**3**)

A solution of sodium nitrite (2.88 g, 41.7 mmol) in water (10 mL) was added dropwise to a solution of 2-amino-5-nitrobenzonitrile (5.10 g, 31.2 mmol) in conc. hydrogen chloride aqueous solution (40 mL) at 0 °C under N_2 gas, and stirred for 30 min at 0 °C. A solution of potassium iodide (33.1 g, 199 mmol) in water (50 mL) was added dropwise to the solution. After stirring at 0 °C for 3 h and then overnight at room temperature, the product was extracted with dichloromethane. The organic layer was washed with 10% sodium hydroxide aq. solution, 5% sodium hydrogen carbonate aq. solution, and water. The organic layer was dried with sodium sulfate and concentrated in vacuo. The crude product was purified by silica gel column chromatography (dichloromethane:hexane = 2:1 v/v) to obtain the desired product **3** as a pale yellow solid (6.32 g, 73.9%): mp 122–123 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.43 (d, J = 2.4 Hz, 1H), 8.18 (d, J = 8.7 Hz, 1H), 8.11 (dd, J = 2.6 and 8.8 Hz, 1H); ^{13}C NMR (125.65 MHz, $CDCl_3$) δ 147.66, 141.08, 128.70, 127.68, 122.41, 117.53, 106.97; ESI-MS (POS) m/z $(M+Na)^+$: Calcd for $C_7H_3N_2O_2INa$: 296.9, found: 297.0.

2.1.2. 3-Amino-6-iodonitrobenzonitrile (**4**)

A mixture of compound **3** (5.03 g, 18.3 mmol), tin (II) chloride dihydrate (20.7 g, 91.7 mmol), and ethanol (40 mL) was stirred under N_2 gas at room temperature for 90 min. Satl. sodium hydrogen carbonate aq. solution was added to the reaction mixture for neutralization and the product was extracted with ethyl acetate. The organic layer was washed with brain and treated by activated charcoal. The solution was dried with sodium sulfate and concentered in vacuo to obtain the desired product **4** as a brown solid (3.70 g, 82.9%): mp 119–121 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.59 (d, J = 8.4 Hz, 1H), 6.91 (d, J = 2.7 Hz, 1H), 6.60 (dd, J = 2.9 and 8.4 Hz, 1H); ^{13}C NMR (125.65 MHz, $CDCl_3$) δ 146.55, 139.86, 120.65, 119.88, 119.47, 118.88, 82.43; ESI-MS (POS) m/z $(M+Na)^+$: Calcd for $C_7H_5N_2INa$: 266.9, found: 266.9.

2.1.3. Sodium 2-iodo-5-nitrobenzoate (**5**)

A solution of sodium nitrite (2.21 g, 32.1 mmol) in water (15 mL) was added dropwise to a solution of 2-amino-5-nitrobenzoic acid (4.50 g, 24.7 mmol) in conc. hydrochloric acid (40 mL) at 0 °C under Ar gas, and stirred for 30 min at 0 °C. A solution of potassium iodide (13.10 g, 78.9 mmol) in water (40 mL) was added dropwise to the solution. After stirring at 0 °C for 3 h and then overnight at room temperature, the solution was neutralized with 10% sodium hydroxide and the product was extracted with ethyl acetate. The solution was washed with satl. sodium hydrogen carbonate aq. solution, dried with sodium sulfate and concentrated in vacuo. The crude product was purified by silica gel column chromatography (ethyl acetate:hexane = 2:1v/v) to obtain the desired product **5** as a pale yellow solid (5.64 g, 72.5%): mp 197–198 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.80 (d, J = 2.4 Hz, 1H), 8.29 (d, J = 8.7 Hz, 1H), 8.03 (d, J = 2.7 and 8.7 Hz, 1H); ^{13}C NMR (125.65 MHz, CD_3OD) δ 167.92, 149.21, 144.17, 139,07, 127.17, 125.87, 103.09; ESI-MS (POS) m/z $(M+Na)^+$: Calcd for $C_7H_4NO_4INa_2$: 337.9, found: 338.4.

2.1.4. Methyl 2-iodo-5-nitromenzoate (**6**)

A mixture of sodium 2-iodo-5-nitrobenzoate (5.64 g, 17.9 mmol) and thionyl chloride (18 mL) was refluxed for 90 min. The volatile components were removed by reduced pressure, and the remaining residue was dissolved in methanol (40 mL). The solution was then refluxed for 120 min. The solution was evaporated to dryness and the residue was dissolved in ethyl acetate. The solution was washed with satl. sodium hydrogen carbonate aq. solution, dried with sodium sulfate, and concentrated in vacuo to obtain the desired product **6** as a pale yellow solid (5.63 g, quantitative): mp 158–159 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.63 (d, J = 2,7 Hz, 1H), 8.22 (d, J = 8.7 Hz, 1H), 7.98 (dd, J = 2.7 and 8.7 Hz, 1H), 4.00 (s, 3H); ^{13}C NMR (125.65 MHz, $CDCl_3$) δ 165.61, 143.14, 142.25, 135.49, 128.83, 124.68, 95.71, 52.80; ESI-MS (POS) m/z $(M+Na)^+$: Calcd for $C_8H_6NO_4INa$: 329.92, found: 329.9.

2.1.5. Methyl 3-amino-6-iodobenzoate (**7**)

A mixture of compound **6** (5.63 g, 18.3 mmol), tin (II) chloride dihydrate (12.4 g, 55.0 mmol), and ethanol (40 mL) was stirred under Ar gas at room temperature for 90 min. Satl. sodium hydrogen carbonate aq. solution was added to the reaction mixture for neutralization at 0 °C, and the product was extracted with ethyl acetate. The organic layer was treated by activated charcoal, dried with sodium sulfate, and concentered in vacuo to obtain the desired product **7** as an orange gum (4.72 g, 93.1%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.67 (d, J = 8.4 Hz, 1H), 7.13 (d, J = 2.7 Hz, 1H), 6.51 (d, J = 2.7 and 8.4 Hz, 1H), 3.90 (s, 3H); ^{13}C NMR (125.65 MHz, $CDCl_3$) δ 167.07, 146.38, 141.57, 135.41, 129.27, 119.61, 78.69, 52.40; ESI-MS (POS) m/z $(M+Na)^+$: Calcd for $C_8H_8NO_2INa$: 300.0, found: 300.1.

2.1.6. Methyl N-phenoxyacetyl-3-amino-6-iodobenzoate (**8**)

Phenoxyacetyl anhydride (2.45 g, 8.56 mmol) was added to a solution of compound **7** (1.98 g, 7.15 mmol) in DMF (15 mL) under Ar gas at 0 °C. After stirring under Ar gas at room temperature for 120 min, the reaction mixture was evaporated to dryness. The crude product was purified by silica gel column chromatography (ethyl acetate:hexane = 1:9 to 4:6 v/v) to obtain the desired product **8** as a light brown solid (2.42 g, 82.3%): mp 103–105 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.36 (brs, 1H), 7.99 (s, J = 2.4 Hz, 1H), 7.95 (d, J = 8.4 Hz, 1H), 7.58 (dd, J = 2.7 and 8.7 Hz, 1H), 7.36 (t, J = 8.0 Hz, 2H), 7.08 (t, J = 7.4 Hz, 1H), 6.99 (d, J = 8.1 Hz, 2H), 4.62 (s, 2H), 3.94 (s, 3H); ^{13}C NMR (125.65 MHz, $CDCl_3$) δ 166.60, 156.72, 141.93, 136.02, 135.45, 129.98, 124.11, 122.66, 122.23, 122.13, 114.79, 87.96, 67.45, 52.69; ESI-MS (POS) m/z $(M+Na)^+$: Calcd for $C_{16}H_{14}NO_4INa$: 434.0, found: 434.1.

2.1.7. 6-(2-Deoxy-β-D-ribofranosyl)-3-aminobenzonitrile (**1**)

A solution of palladium (II) acetate (0.11 g, 0.45 mmol) and triphenylarsine (0.34 g, 0.93 mmol) in degassed DMF (5 mL) was stirred under Ar gas at room temperature for 30 min. A solution of compound **4** (1.14 g, 4.67 mmol), 1,4-anhydro-3,5-bis-*O*-(*tert*-butyldimethylsilyl)-2-deoxy-D-*erythro*-pent-1-enitol[17] (2.01 g, 5.83 mmol), and tributylamine (0.74 mL) in degassed DMF was added to the solution under Ar gas. The mixture was stirred at 90 °C for 5 h and then at 0 °C for 30 min. Acetic acid (2.0 mL) and 1 M tetrabuthylammoniumfluoride in THF (10.0 mL) were added to the reaction mixture, and then the resulting mixture was stirred overnight at room temperature under Ar gas. Satl. sodium hydrogen carbonate aq. solution was added to the reaction mixture for neutralization at 0 °C, and the product was extracted with ethyl acetate. The organic layer was dried with sodium sulfate and concentrated in vacuo. The crude product was purified by silica gel column chromatography (ethyl acetate:hexane = 1:1 to 9:1 v/v) to obtain 6-(2R-5R-5-hydroxymethyl-4-oxo-tetrahydrofuran-2-yl)-3-aminobnzonitrile (0.584 g, 53.8%).

A part of the obtained compound (0.580 g, 2.50 mmol) was dissolved in acetonitrile (3 mL) and acetic acid (2 mL), and stirred at 0 °C for 15 min. Sodium triacetoxyborohydrate (1.16 g, 0.550 mmol)

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was added to the solution, and the resulting mixture was stirred at room temperature for 1 h under Ar gas. Satl. sodium hydrogen carbonate aq. solution was added to the reaction mixture for neutralization at 0 °C, and the product was extracted with ethyl acetate. The organic layer was dried with sodium sulfate and concentrated in vacuo. The crude product was purified by silica gel column chromatography (ethyl acetate:methanol = 100:0 to 99.7:0.3 v/v) to obtain the desired product **1** as a brown gum (0.155 g, 26.5%): $[\alpha]^{22}_D$ = +75 (*c* = 0.20, CH_3OH); ^1H NMR (300 MHz, CD_3OD) δ 7.30 (d, *J* = 8.4 Hz, 1H), 6.81 (dd, *J* = 2.4 and 8.7 Hz, 1H), 6.80 (s, 1H), 5.19 (dd, *J* = 5.4 and 10.5 Hz, 1H), 4.22 (m, 1H), 3.82 (m, 1H), 3.57 (d, *J* = 9.6 Hz, 1H), 2.12 (m, 1H), 1.85 (m, 1H); ^{13}C NMR (125.65 MHz, CD_3OD) δ 148.21, 132.47, 127.69, 119.08, 117.55, 116.81, 110.28, 87.65, 77.81, 72.88, 62.38, 42.72; ESI-MS (POS) *m*/*z* $(M+Na)^+$: Calcd for $C_{12}H_{14}N_2O_3Na$: 257.1, found: 257.1.

2.1.8. 6-(2-Deoxy-β-D-ribofranosyl)-N-phenoxyacetyl-3-aminobenzonitrile (**9**)

Phenoxyacetyl dehydrate (0.112 g, 0.426 mmol) was added to a solution of compound **1** (0.121 g, 0.478 mmol) in DMF (3.5 mL) under Ar gas at 0 °C. After stirring under Ar gas at room temperature for 2 h, the reaction mixture was concentrated in vacuo to dryness. The crude product was purified by silica gel column chromatography (ethyl acetate:hexane = 95:5 to 100:0 v/v) to obtain the desired product **9** as a brown solid (0.105 g, 59.6%): mp 143–146 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 8.43 (s, 1H), 8.04 (d, *J* = 7.2 Hz,1H), 7.77 (d, *J* = 8.7 Hz, 1H), 7.54 (d, *J* = 9.0 Hz, 1H), 7.36 (t, *J* = 7.7 Hz, 2H), 7.08 (t, *J* = 7.5 Hz, 1H), 6.99 (d, *J* = 8.7 Hz, 2H), 5.36 (dd, *J* = 5.4 and 10.5 Hz, 1H), 4.64 (s, 2H), 4.54 (m, 1H), 4.06 (m, 1H), 3.83 (m, 2H), 2.39 (m, 1H), 2.03 (m, 1H); ^{13}C NMR (150.91 MHz, CD_3OD) δ 169.86, 159.21, 142.86, 139.07, 130.72, 128.57, 126.32, 125.11, 122.98, 122.47, 118.15, 115.94, 111.43, 89.46, 79.05, 74.34, 68.58, 63.82, 44.38; ESI-MS (POS) *m*/*z* $(M+Na)^+$: Calcd for $C_{20}H_{20}N_2O_5Na$: 391.1, found: 391.2.

2.1.9. Methyl 6-(2-deoxy-β-d-ribofranosyl)-(N-phenoxyacetyl-)3-aminobenzoate (**10**)

A solution of palladium (II) acetate (0.063 g, 0.28 mmol) and triphenylarsine (0.19 g, 0.062 mmol) in degassed DMF was stirred under Ar gas at room temperature for 20 min. A solution of compound **8** (0.980 g, 2.38 mmol) and 1,4-anhydro-3,5-bis-*O*-(*tert*-butyldimethylsilyl)-2-deoxy-D-*erythro*-pent-1-enitol (1.15 g, 3.34 mmol) in degassed DMF and then tributylamine (0.60 mL, 4.27 mmol) were sequentially added to the solution under Ar gas. The mixture was stirred at 90 °C for 210 min and then at 0 °C for 15 min. Acetic acid (2.0 mL) and 1 M tetrabuthylammoniumfluoride in THF (3.0 mL) were added to the reaction mixture at 0 °C, and the resulting mixture was stirred overnight at room temperature under Ar gas. The reaction mixture was diluted with ethyl acetate, washed with satl. sodium hydrogen carbonate aq. solution, dried with sodium sulfate, and concentrated in vacuo. The crude product was purified by silica gel column chromatography (ethyl acetate:hexane = 3:7 to 10:0 v/v) to obtain methyl 6-(2R-5R-5-hydroxymethyl-4-oxo-tetrahydrofuran-2-yl)-(*N*-phenozyacetyl-) 3-aminobnzoate (0.299 g, 31.5%).

A part of the obtained compound (0.276 g, 0.691 mmol) was dissolved in acetonitrile (10 mL) and acetic acid (4 mL), and then stirred at 0 °C for 15 min. Sodium triacetoxyborohydrate (0.920 g, 4.34 mmol) was added to the solution, and the resulting mixture was stirred at room temperature for 3 h under Ar gas. The reaction mixture was diluted in ethyl acetate. The solution was washed with satl. sodium hydrogen carbonate aq. solution, dried with sodium sulfate, and concentrated in vacuo. The crude product was purified by silica gel column chromatography (ethyl acetate:methanol = 100:0 to 95:5 v/v) to obtain the desired product **10** as a white solid (0.225 g, 81.1%): mp 177–178 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 8.14 (d, *J* = 2.4 Hz, 1H), 7.82 (m, 2H), 7.31 (m, 2H), 7.01 (m, 3H), 5.73 (dd, *J* = 5.7 and 9.6 Hz, 1H), 4.67 (s, 2H), 4.25 (m, 1H), 3.91 (m, 1H), 3.89 (s, 3H), 3.86 (d, *J* = 7.5 Hz, 1H), 2.46 (m, 1H), 1.78 (m, 1H); ^{13}C NMR (125.65 MHz, CD_3OD) δ165.01, 154.54, 136.88, 133.28, 126.02, 125.24, 123.43, 120.84, 118.23, 114.84, 111.23, 103.86, 84.00, 73.80, 69.50, 63.86, 59.21, 48.02, 40.20; ESI-MS (POS) *m*/*z* $(M+Na)^+$: Calcd for $C_{21}H_{23}NO_7Na$: 424.1, found: 424.2.

2.1.10. 6-(2-Deoxy-β-D-ribofranosyl)-3-aminobenzoic acid (**2**)

Compound **10** (11 mg, 0.027 mmol) was incubated in 1 M sodium hydroxide (3 mL) at 55 °C for two days. The reaction mixture was neutralized by acetic acid and triethylamine. The crude product was purified by reversed-phase HPLC to obtain the desired product as a yellow gum of a triethylammonium salt (4.5 mg, 47%): $[\alpha]^{22}_D$ = +12 (*c* = 0.14, CH_3OH); ^1H NMR (300 MHz, CD_3OD) δ 7.31 (d, *J* = 8.4 Hz, 1H), 6.82 (d, J = 2,4 Hz, 1H), 6.71 (dd, *J* = 8.4 Hz, 2.4 Hz, 1H), 5.53 (dd, *J* = 5.9 and 10.0 Hz, 1H), 4.21 (m, 1H), 3.83 (m, 1H), 3.67 (d, *J* = 5.1 Hz, 2H), 3.13 (q, *J* = 7.3 Hz, 6H, -$\underline{CH_2}CH_3$-), 2.36–2.30 (m, 1H), 1.89–1.81 (m, 1H), 1.27 (t, *J* = 7.4 Hz, 9H, $CH_2\underline{CH_3}$); ^{13}C NMR (150.91 MHz, CD_3OD) δ 177.74, 147.57, 140.88, 129.86, 127.92, 117.19, 115.00, 88.48, 79.24, 74.51, 64.22, 47.63, 45.09, 9.21; ESI-MS (POS) *m*/*z* $(M+H)^+$: Calcd for $C_{18}H_{31}N_2O_5$: 355.2, found: 355.3.

2.1.11. 5′-Dimethoxytrityl-6-(2-deoxy-β-D-ribofranosyl)-(N-phenoxyacetyl-)3-aminobenzonitrile (**11**)

A solution of dimethoxytrityl chloride (0.111 g, 0.325 mmol) and triethylamine (0.028 mL) in pyridine (1 mL) was added dropwise to a solution of compound **9** (0.075 g, 0.204 mmol) and dimethylaminopyridine (0.005 g) in pyridine (2 mL) under Ar gas. After stirring at room temperature for 2 h, 0.1 mL of methanol was added to the reaction mixture. The solution was diluted with dichloromethane, washed with satl. sodium hydrogen carbonate aq. solution, dried with magnesium sulfate, and concentrated in vacuo. The crude product was purified by silica gel column chromatography (dichloromethane:hexane = 7:3 to 10:0 v/v) to obtain the desired product **11** as a white foam (0.100 g, 73.1%): ^1H NMR (300 MHz, $CDCl_3$) δ 8.35 (s, 1H), 8.03 (s, 1H), 7.71–7.63 (m, 2H), 7.46–6.81 (m, 18H), 5.48 (dd, *J* = 6.0 and 9.3 Hz, 1H), 4.63 (s, 1H), 4.45 (sbr, 1H), 4.10 (sbr, 1H), 3.79 (s, 6H), 3.36 (sbr, 1H), 2.52–2.45 (m, 1H), 2.02–1.93 (m, 1H); ^{13}C NMR (150.91 MHz, $CDCl_3$) δ 166.57,158.52, 156.74, 144.73, 142.41, 136.40, 135.90, 130.07, 130.01, 128.16, 127.88, 127.19, 126.88, 124.42, 123.52, 122.73, 116.82, 114.82, 113.16, 110.71, 86.34, 86.29, 74.30, 67.46, 64.03, 55.25, 43.44; ESI-MS (POS) *m*/*z* $(M+Na)^+$: Calcd for $C_{41}H_{38}N_2O_7Na$: 693.3, found: 693.3.

2.1.12. 5′-Dimethoxytrityl-6-(2-deoxy-β-D-ribofranosyl)-(N-phenoxyacetyl-)-methyl-3-aminobenzoate (**12**)

A solution of dimethoxytrityl chloride (0.175 g, 0.517 mmol) and triethylamine (0.1 mL) in pyridine (1 mL) was added dropwise to a solution of compound **10** (0.123 g, 0.306 mmol) and dimethylaminopyridine (0.01 g) in pyridine (2 mL) under Ar gas. After stirring at room temperature for 2 h, 0.1 mL of methanol was added to the reaction mixture. The solution was diluted with dichloromethane, washed with satl. sodium hydrogen carbonate aq. solution, dried with magnesium sulfate, and concentrated in vacuo. The crude product was purified by silica gel column chromatography (hexane:ethyl acetate = 7:3 to 5:5 v/v) to obtain the desired product **12** as a white solid (0.196 g, 91.0%): mp 86–87 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 8.34 (s, 1H), 8.08 (d, *J* = 2.4 Hz, 1H), 7.84 (d, *J* = 8.4 Hz, 1H), 7.75 (dd, *J* = 2.4 and 8.7 Hz, 1H), 7.49–7.21 (m, 11H), 7.07 (t, *J* = 7.4 Hz, 1H), 7.00 (d, *J* = 7.8 Hz, 2H), 6.84–6.82 (m, 4H), 5.81 (dd, *J* = 6.3 Hz, 8.5 Hz, 1H), 4.62 (s, 2H), 4.36 (m, 1H), 4.05 (m, 1H), 3.89 (s, 3H), 3.83 (s, 6H), 3.39 (m, 2H), 2.53 (m, 1H), 1.91 (m, 1H); ^{13}C

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NMR (125.65 MHz, $CDCl_3$) δ 166.74, 158.42, 156.83, 144.79, 141.09, 135.97, 135.52, 130.04, 129.92, 128.30, 128.13, 127.83, 127.18, 126.77, 124.01, 122.50, 121.53, 119.75, 114.76, 113.09, 86.20, 85.48, 74.20, 67.45, 64.15, 55.19, 52.25, 50.86, 43.83, 30.94; ESI-MS (POS) *m/z* $(M+Na)^+$: Calcd for $C_{42}H_{41}NO_9Na$: 726.3, found: 726,2.

2.1.13. 3′-O-[Cyanoethoxy-(N,N-diisopropylamino)phosphino]-5′-O-dimethoxytrityl-6-(2-deoxy-β-D-ribofranosyl)-(N-phenoxyacetyl-)3-aminobenzonitrile (**13**)

Diisopropyldiethylamine (0.053 mL, 0.306 mmol) and 2-cyanoethyl-*N,N*-iisopropylchlorophosphoramidite (0.062 mL, 0.277 mmol) were added to a solution of compound **11** (0.093 g, 0.139 mmol) in dichloromethane (1.5 mL) at 0 °C under Ar gas. After stirring at room temperature for 30 min, a small amount of methanol was added to the reaction mixture and the reaction mixture was diluted with ethyl acetate. The solution was washed with 5% sodium hydrogen carbonate aq. solution, dried with magnesium sulfate, and concentrated in vacuo. The crude product was purified by silica gel column chromatography (dichloromethane:triethylamine = 9:1 v/v). The appropriate fraction was collected, evaporated, and precipitated with a small amount of dichloromethane into hexane to obtain the desired product **11** as a white foam (0.051 g, 42%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.44 (s, 1H), 8.05 (s, 1H), 7.73 (d, *J* = 8.4 Hz, 1H), 7.67 (d, *J* = 8.4 Hz, 1H), 7.48–7.22 (m, 11H), 7.07 (t, *J* = 8.4 Hz, 1H), 6.99 (d, *J* = 8.4 Hz, 2H), 6.84–6.80 (m, 4H), 5.47 (dd, *J* = 3.3 Hz, 9.0 Hz, 1H), 4.64 (s, 2H), 4.52 (m, 1H), 4.25 (m, 1H), 3.79 (s, 6H), 3.65–3.54 (m, 4H), 3.39–3.29 (m, 2H), 2.64 (t, *J* = 6.3 Hz, 1H), 2.59 (m, 1H), 2.44 (t, *J* = 6.3 Hz, 1H), 1.91 (m 1H), 1.17–1.08 (m 12H); ^{13}C NMR (125.65 MHz, $CDCl_3$) δ166.54, 158.41, 156.71, 144.67, 142.17, 136.43, 135.86, 130.04, 129.94, 128.20, 128.12, 127.79, 127.18, 126.81, 124.41, 123.43, 122.61, 117.78, 117.48, 116.77, 114.75, 113.05, 110.78, 86.13, 85.94, 75.44, 67.37, 63.57, 58.48, 58.34, 58.21, 58.07, 55.19, 52.82, 43.16, 42.84, 42.68, 24.61, 24.55, 24.50, 24.45, 20.41, 20.35, 20.15, 20.10; ^{31}P NMR ($CDCl_3$) δ 148.2 and 149.0; HRMS (ESI, NEG) *m/z* $(M-H)^-$: Calcd for $C_{50}H_{54}N_4O_8P$: 869.3685, found: 869.3693.

2.1.14. 3′-O-[Cyanoethoxy-(N,N-diisopropylamino)phosphino]-5′-O-dimethoxytrityl-6-(2-deoxy-β-D-ribofranosyl)-(N-phenoxyacetyl-)methyl-3-aminobenzoate (**14**)

Diisopropyldiethylamine (0.083 mL, 0.475 mmol) and 2-cyanoethyl-*N,N*-iisopropylchlorophosphoramidite (0.082 mL, 0.366 mmol) were added to a solution of compound **12** (0.139 g, 0.198 mmol) in dichloromethane (2 mL) at 0 °C under Ar gas. After stirring at room temperature for 30 min, a small amount of methanol was added to the reaction mixture, which was diluted with ethyl acetate. The solution was washed with 5% sodium hydrogen carbonate aq. solution, dried with magnesium sulfate, and concentrated in vacuo. The crude product was purified by silica gel column chromatography (dichloromethane:triethylamine = 92:8 to 98:2 v/v). The appropriate fraction was collected, evaporated, and precipitated with a small amount of dichloromethane into hexane to obtain the desired product **11** as a white foam (0.146 g, 81.6%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.34 (s, 1H), 8.07 (m, 1H), 7.90 (t, *J* = 9.0 Hz, 1H), 7.76 (m, 1H), 7.51–7.20 (m, 11H), 7.07 (t, *J* = 7.8 Hz, 1H), 6.99 (d, *J* = 7.8 Hz, 2H), 6.85–6.80 (m, 4H), 5.79 (dd, *J* = 6.6 Hz, 15.9 Hz, 1H), 4.62 (s, 2H), 4.45 (m, 1H), 4.19 (m, 1H), 3.89 (s, 3H), 3.79 (s, 6H), 3.64–3.59 (m, 4H), 3.40–3.29 (m, 2H), 2.67 (t, *J* = 6.5 Hz, 1H), 2.61–2.54 (m, 1H), 2.42 (t, *J* = 6.7 Hz, 1H), 1.94–1.81 (m 1H), 1.18–1.04 (m 12H); ^{13}C NMR (125.65 MHz, $CDCl_3$) δ 166.86, 158.87, 157.33, 145.28, 141.35, 136.53, 136.02, 130.57, 130.42, 128.69, 128.28, 127.85, 127.28, 127.25, 124.42, 123.00, 121.99, 117.78, 117.57, 115.27, 113.53, 107.74, 86.55, 85.55, 76.77, 67.94, 64.37, 59.09, 58.95, 55.70, 53.34, 52.73, 43.70, 43.60, 43.50, 25.07, 25.01, 24.95, 24.90, 20.85, 20.80, 20.63, 20.57; ^{31}P NMR ($CDCl_3$) δ 148.9 and 149.8; HRMS (ESI, NEG) *m/z* $(M-H)^-$: Calcd for $C_{51}H_{57}N_3O_{10}P$: 902.3787, found: 902.3799.

2.2. Syntheses of ODNs and modified ODNs

Oligodeoxyribonucleotides analogs were prepared using the normal phosphoramidite coupling procedure on a DNA synthesizer. The modified nucleoside phosphoramidite (**13** or **14**) was incorporated into an oligodeoxyribonucleotides at the appropriate position by using the normal synthetic cycle, except that the reaction time for the coupling step was 600 s. For the synthesis of normal ODNs, **mDNA-1**, the CPG support was treated with conc. aq. ammonia at 55 °C for 18 h. For the synthesis of **mDNA-2**, the CPG support was treated with 0.4 M sodium hydroxide at 55 °C for 18 h. All modified oligodeoxyribonucleotides with the 5′-(4,4′-dimethoxytrityl) group were isolated by reversed-phase HPLC on a Wakosil 5C18 column (10 mm × 250 mm length) using 50 mM TEAA (pH 7.0) with a gradient of 15.0–40.0% acetonitrile in 25 min. The isolated compound was treated with 80% acetic acid by the usual procedure to remove a 4,4′-dimethoxytrityl group, followed by desalting on a Sephadex G-25 column. The modified oligodeoxyribonucleotides were further purified by reversed-phase HPLC after deprotection of the 5′-(4,4′-dimethoxytrityl) group. Isolated yields: **mODN-1**, 12.0%; **mODN-2**, 12.6%. ESI-MS (reconstructed): **mODN-1**, Calcd. for $[M^{-14}+13H^++Na^+]$ 4612.8; found 4612.7; **mODN-2**, Calcd. for $[M^{-14}+13H^++Na^+]$ 4630.7; found 4630.1.

2.3. General

UV spectra were measured with a Shimadzu UV-2550 spectrometer. The UV-thermal denaturation behavior of the association complex of the DNA was measured with a Shimadzu UV-2550 equipped with a thermal controller and the Tm values were determined with a TMSPC-8. Fluorescence spectra were recorded using a Shimadzu RF-5300C equipped with a thermal controller. Specific rotations were measured on JASCO DIP-1000. ESI mass spectra were measured with a Perkin Elmer Sciex API-100. High resolution mass spectra were obtained on Thermo Fisher Scientific Exactive spectrometer (ESI). NMR spectra were measured with a JEOL JMN-AL-300, α-500, or ECA600. High-pressure liquid chromatography on an ODS-silica gel column (4.6 mm × 250 mm) was carried out with a linear gradient elution (0–25%) of acetonitrile in 50 mM triethylammonium acetate (pH 7.0) over 35 min at a flow rate of 1.0 mL/min.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.tet.2017.10.062.

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