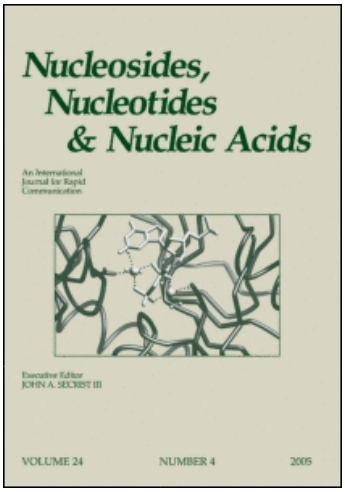

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Synthesis and Properties of Oligodeoxyribonucleotides Bearing a Polyamino Group at the 2' Position via 2'-*O*-Carbamoylmethyl and 2'-*S*-Carbamoylmethyl groups

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SYNTHESIS AND PROPERTIES OF OLIGODEOXYRIBONUCLEOTIDES BEARING A POLYAMINO GROUP AT THE 2′ POSITION VIA 2′-*O*-CARBAMOYLMETHYL AND 2′-*S*-CARBAMOYLMETHYL GROUPS

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□ *An oligodeoxyribonucleotide containing 2′-O-methoxycarbonylmethyluridine was synthesized and converted into several 2′-modified oligodeoxyribonucleotides by a postsynthetic modification method. Using this method, oligodeoxyribonucleotides bearing a polyamine at the 2′ position were easily prepared, which could form duplexes with either complementary DNA or RNA.*

Keywords 2′-Modified oligonucleotides; postsynthetic modification; duplex stability

INTRODUCTION

Many modified DNAs have been prepared and their properties investigated. Modification of the major groove has been studied by many researchers. We studied the modifications at the C5 position of pyrimidine in the major groove. Furthermore, the 2′ position of the nucleoside in DNA is located in the minor groove, which is the binding site of DNA-binding proteins such as nucleases, and is an interesting position for the introduction of functional groups. The catalytic domain of RNase works in the minor groove of RNA to cleave the strand.[1] It has been reported the preparation of an oligodeoxyribonucleotide (ODN) bearing a functional group, which acts as an acid-base catalysis, at the 2′ position to obtain an artificial nuclease.[2] A 2′-modified ODN has also been reported as a second-generation antisense compound. 2′-*O*-[2-(Methoxy)ethyl]-modification[3] and 2′-*O*-(3-aminopropyl)-modification[4] of ODN improved both the

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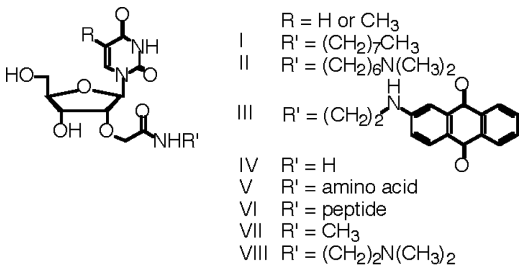


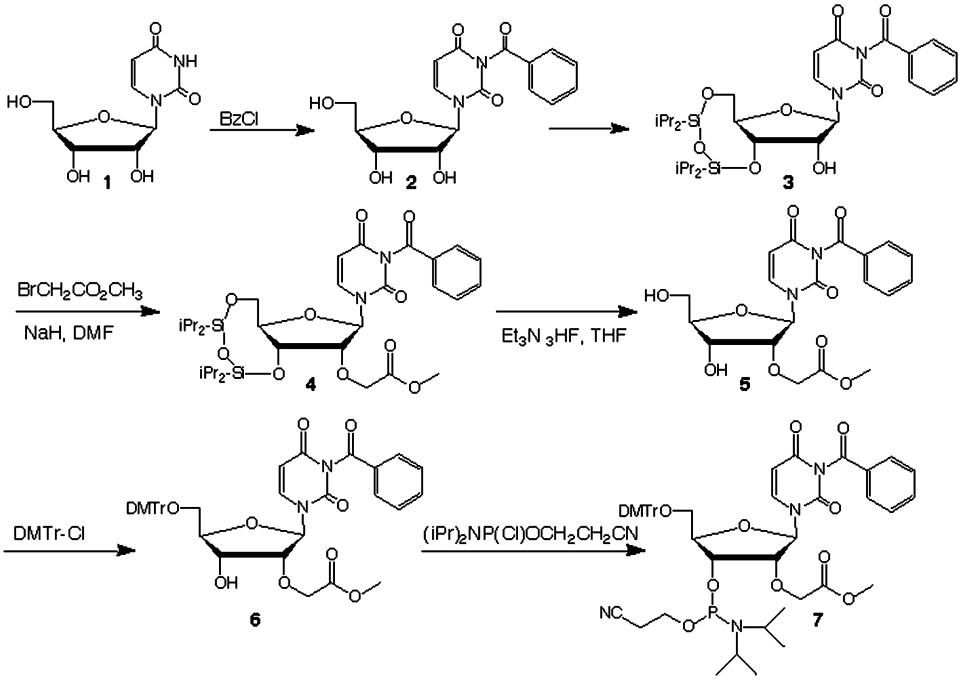
FIGURE 1 Structures of reported 2′-modified nucleoside analogues.

stability of the ODN/RNA duplex and nuclease resistance. Series of 2′-*O*-(2-aminoethyl)ribonucleosides and 2′-*O*-(2-meraptoethyl)ribonucleosides has been synthesized for site-specific introduction of functional groups into RNA.[5] Recently, 2′-*O*-(2-aminoethoxymethyl)ribonucleosides and 2′-*O*-(3-aminopropyoxymethyl)ribonucleosides has been synthesized and incorporated into DNA for postsynthetic functionalization.[6] The 2′-position has been used for introducing functional groups such as reactive groups,[7] fluorescence groups,[8] lipophilic pendants,[9] or intercalating groups.[10] Figure 1 shows the structures of reported 2′-modified nucleosides. Häner et al. introduced several reporter groups via a carbamoylmethyl group at the 2′ position of the nucleoside (I, II, and III) and studies the effect on the ODNs affinity for RNA.[11,12] Sproat et al. showed that 2′-*O*-carbamoylmethyl modification (IV) increased the stability of the duplex with RNA.[13] Recently, Stetsenko et al. reported the synthesis of an ODN bearing the carboxymethoxy group at the 2′-position and the conjugation of peptides (V and VI) to the ODN.[14–16] Manoharan and Egli et al. demonstrated the crystal structure of the ODN containing 2′-*O*-(*N*-methylcarbamoyl)methyl-modified thymidine (VII); this substitution did not result in steric hindrance.[17] Praksh et al. synthesized ODNs bearing 2′-*O*-carbamoylmethyl (IV), 2′-*O*-*N*-methylcarbamoylmethyl (VII), and 2′-*O*-(*N*-dimethylaminoethyl)carbamoylmethyl (VIII) modifications by postsynthetic modification method. These reports suggest that modification at the 2′-position is a suitable for the modification of ODNs without distortion.

In the previous study, we synthesized an ODN containing 2′-methoxycarbonylmethylthio-2′-deoxyuridine for introducing a variety of functional groups at the 2′ position.[18] The ODN was easily functionalized by postsynthetic modification method to produce an ODN containing 2′-*S*-modified 2′-deoxyuridine. In the present article, we describe the synthesis of an ODN containing 2′-*O*-methoxycarbonylmethyluridine and its postsynthetic modification to obtain a 2′-*O*-modified ODN containing an amine. The stabilities of these ODN/DNA or ODN/RNA duplexes were measured by a UV melting experiment.

RESULTS AND DISCUSSION

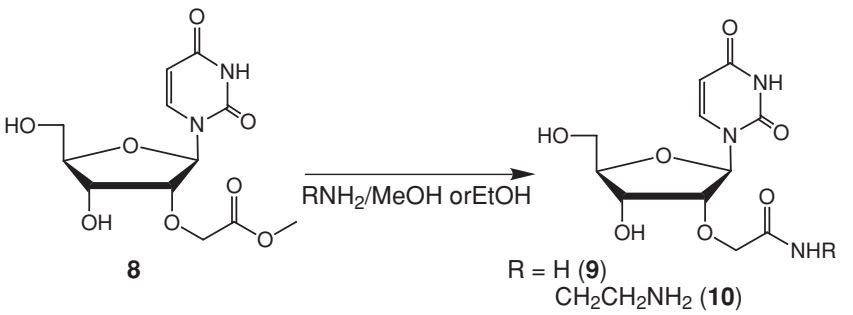
The 2′-hydroxy group of uridine **1** was alkylated by methyl bromoacetate in the presence of sodium hydride in DMF after blocking the 3-imino and the 3′- and 5′-hydoxy groups as represented in Scheme 1. The 3-imino group of uracil was protected with a benzoyl group to prevent *N*-alkylation. The protected 2′-*O*-methoxycarbonylmethyluridine (**5**) was converted to 5′-dimethoxytrityl-protected 2′-*O*-methoxycarbonylmethyluridine 3′-phosphoramidite (**7**) for incorporation into ODN.



SCHEME 1

First, we investigated the reactivity of the methoxycarbonylmethyl group; 2′-*O*-Methoxycarbonylmethyluridine **8** derived from N^3-benzoyl-2′-*O*-methoxycarbonylmethyluridine **5** was treated with ammonia or diaminoethane at 37°C for 12 hours as shown in Scheme 2. The reaction products were purified by HPLC and analyzed using ESI-MS. The structures of the designed compounds **9** and **10** were confirmed by the ESI-MS data. This result indicates that these amines are capable of reacting with the 2′-methoxycarbonyl group in the ODN by an ester–amide exchange reaction.

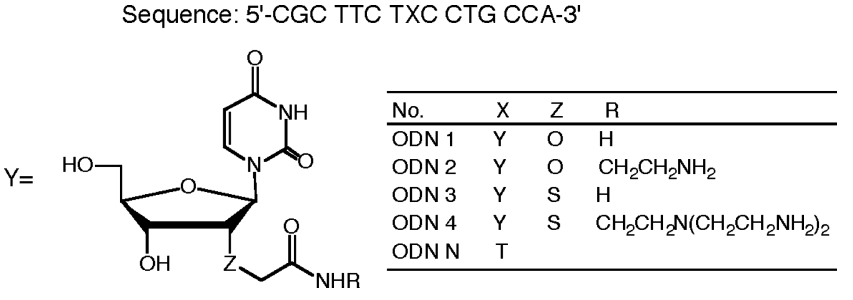
ODNs containing the 2′-*O*-modified uridine (Figure 2) were synthesized by an ester–amide exchange reaction on a solid support after incorporation of N^3-benzoyl-2′-*O*-methoxycarbonylmethyluridine **5** into the ODN. An ODN containing **5** (5′-CGC TTC TXC CTG CCA-3′, X = **5**) was synthesized using the protected nucleoside phosphoramidite **7** by a DNA synthesizer.



SCHEME 2

The coupling yield of **7** in the DNA synthesizer was 95% based on a dimethoxytrityl cation assay with a longer coupling time (360 seconds) and double coupling. The ODN was treated with amines followed by methanolic ammonia on CPG support. The products (**ODN1** and **ODN2**) were purified by reversed-phase HPLC. The ODNs obtained were characterized by nuclease digestion. The compositions of the nucleosides were in agreement with those of the desired sequences as shown in Figure 3. The 2′-*S*-modified ODNs (**ODN3** and **ODN4**) were prepared as described previously.[18]

The binding affinity of these ODNs to complementary DNA or RNA was determined for the UV melting curves of the corresponding duplexes. The melting temperatures are summarized in Table 1 together with the melting temperatures of the unmodified ODN (**ODNN**) and the 2′-*S*-modified ODNs (**ODN3** and **ODN4**). The melting temperature of each modified ODN with complementary DNA was by 4.6–6.0°C lower than that of the unmodified **ODNN**. The lowering of melting temperature was independent of the substituent atom (oxygen or sulfur) at the 2′ position and was slightly dependent on the number of amino groups present on the substituent group. On the other hand, the melting temperature of the 2′-*O*-modified ODNs/RNA (ΔT, approximately −4.2°C to −4.3°C) were higher than those of the 2′-*S*-modified ODNs (ΔT, approximately −5.7°C to −6.4°C).



No.	X	Z	R
ODN 1	Y	O	H
ODN 2	Y	O	$CH_2CH_2NH_2$
ODN 3	Y	S	H
ODN 4	Y	S	$CH_2CH_2N(CH_2CH_2NH_2)_2$
ODN N	T		

FIGURE 2 Structure of the ODNs containing 2′-modified 2′-deoxyuridine.

TABLE 1 Melting temperatures of ODN/DNA and ODN/RNA

	DNA (°C)		RNA (°C)	
ODN	Tm	ΔTm	Tm	ΔTm
ODNN	61.5	—	68.8	—
ODN1	55.5	−6.0	64.6	−4.2
ODN2	56.9	−4.6	64.5	−4.3
ODN3	55.7	−5.8	63.1	−5.7
ODN4	56.4	−5.1	62.4	−6.4

ΔTm is difference between Tm of modified and unmodified duplex.

We considered that the 2′-*O*-modified nucleoside preferred the C3′-endo conformation, which fitted in the A-type DNA/RNA duplex, rather than 2′-*S*-modified nucleoside.[19,20] The stability of the duplex also depended on the substituent group. It was reported that lipophilic modifications such as an n-octyl group, decreased duplex stability but intercalating substituents, such as 2-aminoanthraquinone and positively charged *N,N*-dimethylamino residues, increased duplex stability.[12] In the present work, the number of the amino groups present on the substituent group did not affect the stability of the duplexes. These results suggest that the substituent groups in **ODN1** and **ODN2** could not interact directly with the DNA backbone.

The 2′-*O*-carbamoylmethyl group caused some perturbations in the duplexes but the effect was smaller than that of 2′-*S*-carbamoylmethyl modification. Also, the methyl ester group of the methoxycarbonylmethyl substituent is useful in postsynthetic functionalization. The modification of ODN via the 2′-*O*-carbamoylmethyl group is suitable for the preparation of a variety of 2′-modified oligodeoxyribonucleotides.

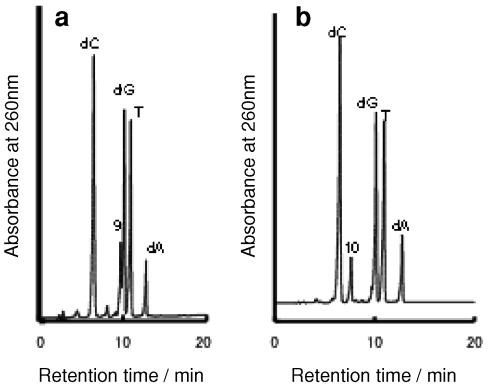


FIGURE 3 HPLC analysis of the digested **ODN1 (a)** and **ODN2 (b)** by snake venom phosphodiesterase and alkaline phosphatase. HPLC condition: column, Wakosil 5C18 (4 mm i.d. × 250 mm); eluent, A, 50 mM TEAA (pH 7.2), B, 50 mM TEAA in 70% CH_3CN; gradient, 3–53%B in 35 minutes; flow rate, 1.0 mL/min.

MATERIAL AND METHODS

General

Thin-layer chromatography (TLC) was performed on Kieselgel $60F_{254}$ (Art. 5554, E. Merck). Silica-gel column chromatography was performed on Silica gel 60 (63–200 μm or 40–63 μm, Merck). High-performance liquid chromatography (HPLC) was carried out on Wakosil 5C18 columns (4 mm × 250 mm length or 10 mm × 250 mm length, Wako, Osaka, Japan). The eluent was acetonitrile gradient in 50 mM triethylammonium acetate (TEAA, pH 7.0). ^{1}H NMR spectra and ^{31}P NMR spectra were obtained with a JEOL AL-300 or a JEOL λ-500 spectrometer (JEOL Co., Akishima, Japan). Mass spectra were measured by a Perkin Elmer Sciex API-100 instrument (MDS/Sciex, Concord, ON, Canada) in ESI mode. Oligodeoxyribonucleotides were synthesized by a phosphoramidite chemistry on an Applied Biosystems 392 DNA synthesizer (Applied Biosystems, Foster City, CA, USA). Normal nucleoside phosphoramidites were purchased from Glen Research (Sterling, VA, USA). Dichloro(2-cyanoethoxy)phosphine and chloro(diisopropylamino)-2-cyanoethoxyphosphine were prepared by the described method.[9] Snake venom phosphodiesterase(SVPD) was purchased from Worthinton (Lakewood, NJ, USA). Nuclease P1 was purchased from Yamasa Co (Chiba, Japan). Alkaline phosphatase(AP) was purchased from Boehringer Mannheim BmbH (Mannheim, Germany). All other reagents were purchased from Wako (Osaka, Japan) or Kanto Chemical Co., Inc. (Tokyo, Japan). All organic solvents for reactions were dried and distilled in the usual manner.

N^3-Benzoyl-2′-*O*-methoxycarbonylmethyl-1′,3′-*O*-(1,1,3,3-tetraisopropyldisiloxane)uridine (4)

Sodium hydride (0.03 g, 1.08 mmol) and methyl bromoacetate (0.65 mL, 6.86 mmol) were added to the solution of N^3-Benzoyl-1′,3′-*O*- (1,1,3,3-tetraisopropyldisiloxane)uridine[21] (**3**, 0.58 g, 7.20 mmol) in DMF under Ar at 0°C. After stirring at 0°C for 6.5 hours, a small amount of methanol was added to the reaction solution and the solution was evaporated to dryness. The residue was dissolved in ethyl acetate (50 mL) and washed with saturated sodium bicarbonate solution and saturated sodium chloride solution. The organic layer was dried over magnesium sulfate and evaporated to dryness. The product was purified by silica gel column chromatography using 2% methanol in dichloromethane as an eluent. Yield of **3** was 88% (0.57 g).

^{1}H NMR ($CDCl_3$) δ 8.01 (1H, d, *H6*, J = 8.0 Hz), 7.93–7.42 (5H, m, *N-3-benzoyl*), 5.86 (1H, d, *H5*, J = 8.4 Hz), 5.67 (1H, d, *H1′*, J = 5.1 Hz), 4.53 (1H, dd, *H2′*, J = 5.1 and 9.0 Hz), 4.28 (1H, d, *H3′*, J = 13.5 Hz), 4.17 (1H,

d, *H4′*, $J = 8.1$ Hz), 4.13–4.02 (2H, m, *H5′*), 3.74 (3H, s, *OCH_3*), 2.56 (2H, d, *2′-CH_2-*, $J = 5.4$ Hz), 1.12–0.97 (28H, m, *isopropyl*). Mass m/z 685.4 (MNa^+, 685.3 calcd. for $C_{31}H_{46}N_2O_{10}NaSi_2$).

N^3-Benzoyl-2′-*O*-methoxycarbonylmethyluridine (5)

A mixture of N^3-benzoyl-2′-*O*-methoxycarbonylmethyl-1′,3′-*O*- (1,1,3,3-tetraisopropyldisiloxane)uridine (**4**, 0.46 g, 0.69 mmol) and triethylamine trihydrofluoride (0.57 mL, 3.47 mmol) in THF (7 mL) was stirred overnight at room temperature. A small amount of methanol was added to the reaction mixture and the solution was evaporated to dryness. The residue was dissolved in ethyl acetate (50 mL) and washed with saturated sodium bicarbonate solution and saturated sodium chloride solution The organic layer was dried over magnesium sulfate and evaporated to dryness. The product was purified by silica gel column chromatography using 1% methanol in dichloromethane as an eluent. Yield of **3** was 62% (0.18 g). ^{1}H NMR ($CDCl_3$) δ 7.48–7.97 (6H, m, *N-3-benzoyl and H6*), 5.84 (1H, d, *H5*, $J = 8.1$ Hz), 5.75 (1H, d, *H1′*, $J = 3.3$ Hz), 4.46–4.15 (3H, m, *H2′, H3′ and H4′*), 4.03–3.80 (2H, m, *H5′*), 3.76 (3H, s, *OCH_3*), 3.47 (2H, s, *2′-CH_2-*).

N^3-Benzoyl-5′-*O*-dimethoxytrityl-2′-*O*-methoxycarbonylmethyluridine (6)

5 (0.8 g, 1.90 mmol) was react with 4,4′-dimethoxytrityl chloride (0.77 g, 2.28 mmol) in pyridine (5 mL) under Ar at room temperature for 17 hours. A small amount of methanol was added to the reaction mixture and the solution was evaporated to dryness. The residue was dissolved in ethyl acetate (30 mL) and washed with saturated sodium bicarbonate solution and saturated sodium chloride solution The organic layer was dried over magnesium sulfate and evaporated to dryness. The product was purified by silica gel column chromatography using 5% methanol in dichloromethane as an eluent. Yield of **3** was 89% (1.12 g). ^{1}H NMR ($CDCl_3$) δ 7.95–7.39 (6H, m, *N-3-benzoyl and H6*), 7.34–6.81 (13H, m, *DMTr*), 5.86 (1H, s, *H1′*), 5.37 (1H, d, *H5*, $J = 8.1$ Hz), 4.54–4.48 (2H, m, *H3′ and H4′*), 4.48–4.33 (1H, m, *H2′*), 4.15–4.06 (2H, m, *H5′*), 3.78 (6H, s, *OCH_3*), 3.73 (3H, s, *OCH_3*), 3.59 (2H, s, *2′-CH_2-*).

3′-*O*-[Cyanoethoxy-(*N,N*-diisopropylamino)phosphino]-N^3-benzoyl-5′-*O*-dimethoxytrityl-2′-*O*-methoxycarbonylmethyluridine (7)

2-Cyanoethyl-*N,N*-iisopropylchlorophosphoramidite was added to **6** (0.22 g, 0.30 mmol) in dichloromethane (3.7 mL) under Ar at room temperature. After stirring at room temperature for 2 hours, a small amount

of methanol was added to the reaction mixture and the solution was evaporated to dryness. The residue was dissolved in ethyl acetate (20 mL) and washed twice with saturated sodium bicarbonate solution and saturated sodium chloride solution. The organic layer was dried over magnesium sulfate and evaporated to dryness. The product was purified by silica gel column chromatography using 50% ethyl acetate in dichloromethane containing 5% triethylamine as an eluent. The appropriate fraction was collected, evaporated, and precipitated with a small amount of dichloromethane into hexane to give white precipitates. Yield of **6** was 89% (0.25 g). ^{31}P NMR ($CDCl_3$) δ 151.44 and 151.07.

Reaction of 2′-Methoxycarbonylmethyluridine (8) with Amines

Compound **8** was derived from of N^3-benzoyl-2′-*O*-methoxycarbonylmethyluridine (**5**). **8** was treated with methanolic ammonia or 50% 1,2-diaminoetrhane in ethanol at 37°C for 12 hours. The products were analyzed and purified by reversed-phase HPLC. These yields which estimated by HPLC analyses, were about 60%. **9:** Mass m/z 324.1 (MNa^+, 323.9 calcd. for $C_{11}H_{15}N_3O_7Na$). **10:** Mass m/z 345.1 (MH^+, 345.1 calcd. for $C_{13}H_{21}N_4O_7$).

Syntheses of ODNs and Modified ODNs

Oligodeoxyribonucleotides analogs were prepared using normal phosphoramidite coupling procedure on a DNA synthesizer. The oligodeoxyribonucleotides bearing the 2′-*O*-modified uridine in place of thymidine were synthesized along with the normal oligodeoxyribonucleotides. The modified nucleoside phosphoramidite (**7**) was incorporated into the oligodeoxyribonucleotides at the appropriate position by using the normal synthetic cycle except that the reaction time for the coupling step was 5 minutes and the coupling was performed twice. For the synthesis of an oligodeoxyribonucleotide containing the modified base (**ODN-1** or **ODN-2**), the CPG support was treated with methanolic ammonia at 37°C for 24 hours or 50% 1,2-diaminoethane in ethanol at 37°C for 23 hours, then concentrated aqueous ammonia at 55°C for 4 hours, reapectively. The deprotection and cleavage from CPG support for nomal ODNs was carried out by treatment with concentrated aqueous ammonia solution at 50°C for 18 hours. All modified oligodeoxyribonucleotides with 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% to 40.0% acetonitrile in 25 minutes. The isolated compound was treated with 10% 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 5′-(4,4′-dimethoxytrityl) group. Isolated yields: **ODN-1**, 7.5%; **ODN-2**, 25.1%.

Nuclease Digestion of Oligodeoxyribonucleotides Containing the Modified Base

The modified oligomers (ca. 0.2 $OD_{260\,nm}$) were treated overnight with nuclease P1 (ca. 4 units) in 0.18 M sodium acetate (pH 5.3) at 37°C. To the reaction mixtures, 500 mM Tris-HCl(pH 9.0) containing 10 mM $MgCl_2$, snake venom phosphodiesterase (0.25 units) and alkaline phosphatase (2 units) were added, then the reaction mixtures were incubated overnight at 37°C. The reaction mixtures were analyzed by reversed-phase HPLC. The nucleoside composition ratios were calculated from areas of the peaks in the HPLC chart.

Tm Measurements

UV absorbance was measured with a Hitachi UV-3000 spectrophotometer equipped with a Hitachi Temperature Controller SPR-10 and a temperature data collector AM-7002 (Anritsu Meter Co., Ltd., Tokyo, Japan). The rate of heating or cooling was 0.5°C/min. Tm values were obtained in 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride and 10 μM EDTA at a duplex concentration of 2×10^{-6} M. Alternatively, Tm was measured with Shimazu UV-2550 spectrophotometer equipped with TMSPC-8 Tm analysis system.

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