Synthesis of 2′-O-methyl-RNAs incorporating a 3-deazaguanine, and UV melting and computational studies on its hybridization properties

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INTRODUCTION

The synthesis and properties of 3-deazaguanosine and 3-deaza-2′-deoxyguanosine derivatives have been widely studied in the development of anti-bacterial, anti-viral and anti-tumor agents (1–9). The usefulness of 3-deaza-6-O-methyldeoxyguanosine in studying the reaction mechanism of O6-alkylguanine-DNA alkyltransferase was also reported (10). Besides such studies on the biological activities of 3-deazaguanine nucleosides, Seela and co-workers (11,12) have reported studies on the synthesis and physicochemical properties of oligonucleotides incorporating 3-deazaguanine, focusing on the thermal stability of DNA duplexes and the importance of the N³ atom of the guanine residue on the catalytic activity of hammerhead ribozymes (13). Despite these pioneering studies related to 3-deazaguanosine and 3-deaza-2′-deoxyguanosine derivatives, no papers have been reported about the effect of the 3-deazaguanine base on the base-discriminating ability toward the opposite nucleobases of RNA–RNA or RNA–DNA duplexes upon its incorporation into RNA.

Recently, much attention has been paid to new strategies for more precise detection of DNA or RNA sequences to improve essentially the accuracy of gene diagnosis and mRNA expression analysis as well as gene regulation (14–16). To this end, there have been reported several studies in an attempt to increase the base discrimination ability of the canonical nucleobases by rational molecular design on the basis of hydrogen bonding patterns of Watson–Crick and base pairs. Computational studies based on ab initio calculations suggest that the weaker hydrogen bonding ability and larger dipole moment of c⁵G can be the origin of the lower Tₘ.

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2-thiouridine has been considered favorable to increase the selectivity of antisense oligonucleotides (21) and molecular probes (22–24).

It is well known that guanine can form a stable G/U(T) mismatch with uracil (thymine) and a stable G/A mismatch with adenine, depending on the neighboring sequences, as shown in Figure 1. Particularly, the G-A mismatch has often been observed when 4 nt sequences such as 5'-GGAC-3'/3'-CAGG-5' and 5'-CGAG-3'/3'-GAGC-5' having a tandem 5'-GA-3'/3'-AG-5' mismatch at the central position is involved in DNA or RNA (25–29). The former involves two sets of face-to-face base pairs between G and A, while the latter 5' has two sets of sheared-type base pairs between G and A. Formation of the partial duplex structure of 5'-CGAG-3'/3'-GAGC-5' requires the N3 nitrogen in the guanine bases in both strands, since this site is used for the hydrogen bonding. In this study, our interests were focused on the suppression of formation of the sheared-type mismatched base pairs by use of 3-deazaguanine in place of guanine.

In this paper, we report the synthesis, hybridization and base discrimination properties of 2'-O-methylated oligoribonucleotides incorporating 3-deazaguanine as our continuous study on base-discriminating oligoribonucleotides as probes directed toward development of new RNA-type probes and chips (19,30). We selected 2'-O-methylated RNA species focusing on its favorable properties as RNA drugs and molecular probes targeting RNA (31). The detailed UV melting experiments revealed that the formation of a sheared-type G/A mismatch in 2'-O-methyl-RNA/RNA and 2'-O-methyl-RNA/DNA duplexes could be suppressed by replacing guanine with 3-deazaguanine.

**MATERIALS AND METHODS**

**General methods**

1H, 13C and 31P NMR spectra were obtained on a Varian unity INOVA apparatus at 500, 126 and 203 MHz, respectively. The chemical shifts were measured from tetramethylsilane (0 p.p.m.) or DMSO-d6 (2.49 p.p.m.) for 1H NMR, CDCl3 (77.0 p.p.m.), DMSO-d6 (39.7 p.p.m.) for 13C NMR and 80% phosphoric acid for 31P NMR. Column chromatography was performed with Wako silica gel C-200. Recycle high-performance liquid chromatography (HPLC) was performed on JALGEL GS-310 column by use of CH3CN as a solvent. TLC was performed with Merck silica gel 60 (F254) plates. ESI mass spectra were measured on Mariner™, UV spectra were measured by a Biospec-mini spectrophotometer. UV melting curves were obtained by a Pharmaspec UV-1700 spectrophotometer equipped with TMSPC-8 temperature controller. Reversed-phase HPLC was performed on Atlantis C-18 column with the linear gradient of CH3CN (0–30%, 1%/min) in 0.1 M ammonium acetate (pH 7.0). Anion exchange HPLC was performed by use of GENPAK Fax column (waters, 4.6 x 100 mm) with the linear gradient of buffer A: 25 mM sodium phosphate (pH 6.0) and 10% CH3CN; and buffer B: 25 mM sodium phosphate, 1 M NaCl (pH 6.0) and 10% CH3CN. The oligonucleotides without the 3-deazaguanine modification were purchased from Greiner and Sigma–Aldrich Japan Co., Ltd, and used after purification by anion exchange HPLC if necessary.

**2-N-((N,N-Dimethylaminomethylene)-3-deazaguanosine (1)**

3-Deazaguanosine (1.1 g, 3.9 mmol) was rendered anhydrous by repeated co-evaporation with DMF, and then dissolved in anhydrous DMF (20 ml). To this solution was added N,N-dimethylformamide dimethylacetel (2.6 ml, 20 mmol) and the resulting solution was stirred for 5 h. The reaction was quenched by adding excess methanol (5 ml) and the solution was concentrated under reduced pressure. To this residue was added methanol (20 ml) and the resulting precipitate was collected by filtration to give 1 (1.2 g, 91%). 1H NMR (DMSO-d6, δ): 2.93 (3H, s), 3.04 (3H, s), 3.58 (1H, m), 3.62 (1H, m), 3.90 (1H, m), 4.07 (1H, br), 4.26 (1H, br), 5.08 (1H, t), 5.18 (1H, br), 5.44 (1H, br), 5.61 (1H, d,

![Figure 1. Hydrogen bonding modes related to this study (X = N or CH).](image-url)
2-N-(N,N-Dimethylaminomethylene)-3', 5'-O-(1,1,3,3-tetraisopropylsiloxane-1,3-diyl)-3-deazaguanosine (2)

Compound 1 (3.2 g, 9.5 mmol) was rendered anhydrous by repeated co-evaporation with dry pyridine. The residue was dissolved in dry pyridine (95 ml), and to this solution was added 1,3-dichloro-1,1,3,3-tetraisopropylsiloxane (3.2 ml, 10.2 mmol). The reaction mixture was stirred at ambient temperature for 4 h. To this solution was added water (10 ml) and saturated NaHCO₃. The resulting solution was concentrated under reduced pressure and the residue was dissolved in ethyl acetate (50 ml), washed with saturated NaCl (50 ml × 2). The organic layer was collected, dried over MgSO₄, filtered, and concentrated under reduced pressure. The residue was chromatographed on a silica gel column with chloroform-methanol (100:2–100:4.5, v/v) to give 2 (3.9 g, 70%).

**1H-NMR** (DMSO-d₆, δ): 1.05 (28H, m), 2.92 (3H, s), 3.04 (3H, s), 3.93 (1H, dd, J = 3.8, 13.2 Hz), 4.03 (1H, m), 4.13 (1H, dd, J = 2.7, 13.2 Hz), 4.19 (1H, m), 4.32 (1H, m), 5.67 (1H, d, J = 1.5 Hz), 5.77 (1H, br), 5.91 (1H, s), 7.88 (1H, s), 7.96 (1H, s), 10.67 (1H, br); **13C-NMR** (DMSO-d₆): 11.96, 12.33, 12.44, 12.78, 16.75, 16.82, 16.87, 16.92, 17.15, 17.19, 17.36, 34.09, 60.50, 69.15, 73.84, 77.19, 81.13, 89.68, 127.06, 136.40, 140.21, 151.15, 154.95, 157.31. ESI-MS [M+H]⁺ calcld. for C₁₂H₁₄N₂O₂Si₂ 580.2987, found 580.2920.

2-N-(N,N-Dimethylaminomethylene)-O-6-(N, N-diphenylcarbamoyl)-3', 5'-O-(1,1,3,3-tetraisopropylsiloxane-1,3-diyl)-3-deazaguanosine (3)

Compound 2 (282 mg, 0.49 mmol) was rendered anhydrous by repeated co-evaporation with dry pyridine, and then dissolved in dry pyridine (5.8 ml). To this solution was added ethyldiisopropylamine (89 ml, 0.98 mmol) and N,N-diphenylcarbamoyl chloride (135 mg, 0.6 mmol). The reaction mixture was stirred for 4.5 h. The reaction was quenched with saturated aqueous NaHCO₃ (10 ml). The mixture was extracted with ethyl acetate (15 ml), and the organic layer was washed twice with saturated aqueous NaCl (10 ml). The organic layer was dried over MgSO₄, filtered, and concentrated under reduced pressure. The residue was chromatographed on a silica gel column with hexane-ethyl acetate (1:1–3:7, v/v) to give 3 (270 mg, 72%).

**1H-NMR** (DMSO-d₆, δ): 0.96–1.17 (28H, m), 2.97 (3H, s), 3.07 (3H, s), 3.95 (1H, dd, J = 2.7, 12.9 Hz), 4.01–4.06 (3H, m), 4.14 (1H, dd, J = 3.4, 12.9 Hz), 4.29 (1H, m), 4.36 (1H, dd, J = 5.4, 7.8 Hz), 5.77 (1H, br), 5.83 (1H, d, J = 2.2 Hz), 6.93 (1H, s), 7.26–7.29 (29H, m), 7.40–7.45 (8H, m), 8.24 (1H, s), 8.40 (1H, s); **13C-NMR** (DMSO-d₆, δ): 11.97, 12.29, 12.45, 16.78, 16.82, 16.93, 17.20, 17.24, 17.38, 60.67, 69.28, 73.21, 81.45, 90.26, 96.41, 126.76, 127.62, 129.22, 141.79, 142.18, 142.44, 146.93, 151.66, 155.02, 155.06. ESI-MS [M+H]⁺ calcld. for C₃₉H₅₅N₆O₇Si₂ 789.3827, found 789.3843.

2-N-(N,N-Dimethylaminomethylene)-O-6-(N, N-diphenylcarbamoyl)-2'-O-methyl-3'-deazaguanosine (5)

Compound 4 (998 mg, 1.3 mmol) was rendered anhydrous by repeated co-evaporation with dry THF. The residue was dissolved in dry THF (15 ml). To this solution was added triethylamine trihydrofluoride (680 μl, 84 mmol) at 0°C. The reaction mixture was warmed to ambient temperature and stirred for 6 h. Toluene (30 ml) was added and the solvents were removed under reduced pressure. The residue was chromatographed on a silica gel column with ethyl acetate-methanol (100:4–100:4.5, v/v) to give 5 (553 mg, 80%).

**1H-NMR** (DMSO-d₆, δ): 2.96 (3H, s), 3.06 (3H, s), 3.62–3.65 (2H, m), 3.98 (1H, d, J = 3.4 Hz), 4.16 (1H, dd, J = 5.1, 6.1 Hz), 4.31 (1H, dd, J = 3.2 Hz), 5.15 (1H, d, J = 5.1 Hz), 5.27 (1H, d, J = 5.4 Hz), 5.94 (1H, d, J = 6.1 Hz), 7.00 (1H, s, CH), 7.27–7.29 (29H, m, Ar-H), 7.41–7.47 (8H, m, Ar-H), 8.37 (1H, s), 8.46 (1H, s); **13C-NMR** (DMSO-d₆, δ): 34.28, 57.44, 61.06, 68.38, 82.43, 86.23, 86.74, 96.58, 126.77, 127.02, 127.56, 129.25, 142.18, 143.00, 143.11, 146.93, 151.73, 155.10, 155.15. ESI-MS [M+H]⁺ calcld. for C₄₀H₅₇N₆O₇Si₂ 857.4301, found 857.4306.

5'-O-(4, 4'-Dimethoxytrityl)-2-N-(N,N-dimethylaminomethylene)-O-6-(N, N-diphenylcarbamoyl)-2'-O-methyl-3'-deazaguanosine (6)

Compound 5 (60 mg, 0.11 mmol) was rendered anhydrous by repeated co-evaporation with dry pyridine. The residue was dissolved in dry pyridine (660 μl). To this solution was added 4,4'-dimethoxytrityl chloride (45 mg, 0.13 mmol) and the resulting solution was stirred for 3.5 h. Chloroform (5 ml) was added and the solution was washed twice with saturated aqueous NaHCO₃ (10 ml × 2) and then twice with saturated aqueous NaCl (10 ml × 2). The organic layer was...
was dried over MgSO₄, filtered and concentrated under reduced pressure. The residual pyridine was removed by co-evaporation with toluene and the residue was chromatographed on a silica gel column with hexane-ethyl acetate-triethylamine (40:60:0.5, v/v) to give 6 (75 mg, 80%).

1H NMR (CDCl₃, δ): 3.01 (3H, s), 3.03 (3H, s), 3.39 (1H, dd, J = 4.4, 10.7 Hz), 3.42 (3H, s), 3.47 (1H, dd, J = 3.2, 10.7 Hz), 3.75 (6H, s), 4.09 (1H, dd, J = 5.1 Hz), 4.19 (1H, m), 4.34 (1H, m), 5.84 (1H, d, J = 5.1 Hz), 6.81 (4H, d, J = 8.5 Hz), 6.91 (1H, s), 7.19-7.49 (19 H, m), 8.02 (1H, s), 8.44 (1H, s). 13C (CDCl₃, δ): 34.92, 40.95, 55.47, 59.27, 63.51, 70.07, 84.10, 84.31, 86.97, 87.52, 96.59, 113.54, 127.22, 128.24, 128.38, 128.83, 129.15, 130.32, 130.52, 130.60, 130.79, 135.75, 135.84, 135.95, 141.29, 142.90, 144.00, 144.06, 144.73, 147.49, 153.52, 157.28, 160.32.

5'-O-(4, 4'-Dimethoxytrityl)-2'-O-(N,N-dimethylaminomethyl)6-O-(N,N-diphenylcarbamoyl)-2'-O-methyl-3-deazaguanosine 3'-[2-(cyanoethyl N,N-diisopropylphosphoramidite)] (7)

Compound 6 (746 mg, 0.88 mmol) was rendered anhydrous by repeated co-evaporation with dry toluene. The residue was dissolved in dry CH₂Cl₂ (13 ml), and to this solution was added N,N-diisopropylethylamine (260 mg, 2.35 mmol). The solution was cooled to 0°C and then the solution was added to a solution of the triethylamine (40:60:0.5, v/v) to give 7 (572 mg, 62%). 1H NMR (CDCl₃, δ): 1.03-1.07 (4H, m, isopropyl), 1.17-1.23 (8H, m, isopropyl), 2.35 (1H, m, N-CH), 2.65 (2H, m, CH₂CN), 3.01 (6H, m, N-CH₃), 3.31-3.36 (1H, m), 3.40 (3, OCH₃), 3.41-3.50 (1H, m), 3.51-3.65 (4H, m), 3.76 (6H, m, OCH₃), 3.84-3.93 (1H, m), 4.09-4.15 (1H, m), 4.30-4.36 (1H, br × 2), 4.44 (1H, m), 5.82, 5.86 (1H, s, J = 6.4, 6.6 Hz), 6.80-6.81 (4H, m), 6.93 (1H, s), 7.19-7.49 (19H, m), 7.99, 8.04 (1H, s, s), 8.42 (1H, s). 13C NMR (CDCl₃, δ): 20.75, 20.80, 24.93, 27.99, 35.03, 41.05, 43.53, 43.63, 43.76, 43.86, 55.61, 58.71, 59.30, 63.37, 63.63, 84.12, 84.33, 84.60, 84.09, 87.09, 87.19, 87.62, 98.99, 113.67, 118.06, 127.38, 128.37, 128.49, 128.60, 128.91, 129.26, 130.42, 130.46, 130.52, 135.75, 135.84, 135.95, 141.29, 142.90, 144.00, 144.06, 144.74, 144.83, 148.08, 152.78, 156.19, 156.27, 159.00. ESI-MS [M-H][+] calcd. for C₃₀H₂₉N₆O₈ 849.3612, found 849.3605.

Removal of the dpc and dmf group from 5

Compound 5 (1.1 mg) was dissolved in aqueous ammonia (1 ml) and the solution was incubated at 50°C. The aliquot was removed from the reaction mixture and analyzed by reversed phase HPLC and the compounds corresponding to each peak were analyzed by ESI-mass spectrometry, described below.

2-N-Formyl-6-O-(N,N-diphenylcarbamoyl)-2'-O-methyl-3-deazaguanosine (8). ESI-MS [M+H][+] calcd. for C₃₆H₃₈N₆O₇ 520.1832, found 520.1815.

2-N-(Aminomethylene)-6-O-(N,N-diphenylcarbamoyl)-2'-O-methyl-3-deazaguanosine (9). ESI-MS [M+H][+] calcd. for C₃₆H₂₇N₆O₇ 519.1992, found 519.1963.

O-6-(N,N-Diphenylcarbamoyl)-2'-O-methyl-3-deazaguanosine (10). ESI-MS [M+H][+] calcd. for C₂₅H₂₆N₆O₆ 492.1883, found 492.1857.

2-N-(Aminomethylene)-2'-O-methyl-3-deazaguanosine (11). ESI-MS [M+H][+] calcd. for C₁₃H₁₈N₄O₅ 324.1308, found 324.1296.

2'-O-Methyl-3-deazaguanosine (13). ESI-MS [M+H][+] calcd. for C₁₂H₁₂N₄O₅ 297.1199, found 297.1200.

Synthesis of 2'-O-methyl-RNA incorporating 3-deazaguanine, M1 and M3

The oligonucleotides incorporating 2'-O-methyl-3-deazaguanosine, M1, 5'-CGGG[C₅G]AGGAG-2', and M3, 5'-CGGG[C₅G]ACGAG-2', were synthesized on an Applied Biosystems 394 automated DNA/RNA synthesized using the standard 1.0 μmol phosphoramidite cycle of detritylation, coupling, capping and iodine oxidation. The cleavage of the synthesized oligonucleotides from the solid support and the deprotection of the nucleobases were carried out by treatment with 28% aqueous ammonia at 50°C for 12 h. The oligonucleotides were purified by anion exchange HPLC and desalted by use of disposable C18 cartridge column. Structure of synthesized oligonucleotides was confirmed by MALDI-TOF Mass spectroscopy.

M1: MALDI-TOF mass [M-H][+] calcd. for C₁₀₉H₁₄₁N₄₅O₆₆P₉ 3414.7, found 3414.08

M2: MALDI-TOF mass [M-H][+] calcd. for C₁₀₉H₁₄₁N₄₅O₆₆P₉ 3414.7, found 3415.2

Tₘ measurement

Each oligonucleotide was dissolved in 10 mM sodium phosphate (pH 7.0) containing 100 mM NaCl and 0.1 mM EDTA so that the final concentration of each oligonucleotide became 2 μM. The solutions were separated into quartz cells (10 mm) and incubated at 85°C. After 10 min the solutions were cooled to 5°C at 0.5°C/min and then heated until the temperature reached 85°C at the same rate. During this annealing and melting, the absorptions at 260 nm were recorded and used to draw UV melting curves. The Tₘ values were calculated as the temperature that gave maximum first derivatives of the UV melting curves. The same experiment was repeated four times and the average of the Tₘ values was given in Tables 1 and 2. The oligonucleotide concentrations of DNA and RNA were determined as described in the literature (32). The concentration of 2'-O-methyl-RNA was determined on the assumption that the ε₂₆₀ is identical to that of guanosine. The oligonucleotide concentration incorporating 3-deazaguanosine was determined on the assumption that the ε₂₆₀ is identical to that of guanosine.
RESULTS AND DISCUSSION

Synthesis of 2'-O-methyl-3-deazaguanosine
3'-phosphoramidite unit

The phosphoramidite unit of 2'-O-methyl-3-deazaguanosine 7 was synthesized starting from 3-deazaguanosine, as shown in Scheme 1. To perform the selective methylation at the 2'-hydroxyl group, appropriate protection of the base moiety and the 3'- and 5'-hydroxyl groups was necessary. We chose N,N-diphenylcarbamoyl (dpc) (13,33–34) and N,N-dimethylaminomethylene (dmf) (35) as the protecting groups of the 6-O and N2 positions, respectively. Previously, Seela et al. (13) reported the synthesis of oligoribonucleotides incorporating 3-deazaguanine by use of the phenoxycetyl (pac) group (36) for the N2 position. We chose the dmf group in place of the pac group since the latter was found to be labile under the somewhat basic conditions required for the 2'-O-alkylation, as described below.

3-Deazaguanosine was synthesized according to the literature (2). The amino group of 3-deazaguanosine was protected with the dmf group to give compound 1, and the 3'- and 5'-hydroxyl functions were simultaneously blocked by the 1,3-tetraisopropylsiloxane-1,3-diyl (TIPDS) group (37) to give compound 2. Subsequently, compound 2 was converted to the 6-O-acylated product 3 by treatment with N,N-diphenylcarbamoyl chloride. The reaction of 3 with CH3I in the presence of NaH gave the 2'-O-methylated product 4. It should be noted that, in contrast to the methylation of the 2'-hydroxyl group of guanosine derivatives, the methylation of 3 could be carried out in good yield without using expensive organic bases (38) or a special silyl protecting group (39) which is more stable to basic conditions.

The TIPDS group was removed by treatment with triethylamine tri(hydrogen fluoride) (40) to give the diol 5. The usual dimethoxytritylation of 5 followed by the 3'-phosphitylation gave the phosphoramidite derivative 7.

Deprotection of the dpc and dmf group

To the best of our knowledge, this is the first example of the use of the combination of the dpc and dmf groups for protection of the 3-deazaguanine base. Therefore, the deprotection
of these protecting groups was carefully checked by use of the diol 5. Compound 5 was dissolved in aqueous NH₃ and the mixture was kept at 50°C. The reaction was monitored by reversed phase HPLC and the products were analyzed by ESI-MS. After 25 min we observed five intermediates 8–12 besides the starting material 5 and the fully deprotected product 13 (Figure 2 and Scheme 2).

These intermediates can be classified into two groups. One includes a group of compounds 8–10 having the dpc group and the other includes a group of compounds 11 and 12 without the dpc group. Previously, Seela et al. (13) reported that the \( t_{1/2} \) (time required for completion) for removal of the dpc group from 2-N-phenoxyacetyl-6-O-(N,N-diphenylcarbamoyl)-3-deazaguanosine was only 4 min. On the contrary, in our case, the intermediates having the dpc group were observed even after 25 min and the complete disappearance of these intermediates required 10 h. This observation suggested that the dpc group attached to the 6-O-position of the 3-deazaguanosine derivative became much more stable when the amino group was protected by the amidine-type protecting group. Moreover, it was also revealed that a significant portion of the dmf group was cleaved via the formyl intermediates 8 and 12 or the aminomethylene intermediates 9 and 11. The formation of the former two and the latter two can be explained by the nucleophilic attack of the carbon center of the dmf group by a hydroxide ion and ammonia, respectively. Interestingly, Lebeau and co-workers (41) proposed previously a mechanism whereby the deprotection of dmf group introduced to guanine proceeded without the formation of an \( N \)-formyl intermediate. These results indicated that the amino group of 5 was a poorer leaving group than that of guanine. This difference can be attributed to the loss of the electron-withdrawing nitrogen atom at position 3 which made the amino group a poorer leaving group. The lower leaving group ability of the amino group of 3-deazaguanine was also suggested by the increased stability of the acyl-type protecting group (12,13).

Hybridization properties of a 2′-O-methyl-RNA incorporating 3-deazaguanine

Next, we examined the hybridization properties of 2′-O-methyl-RNA incorporating 3-deazaguanine (cG). Considering the application of the modified 2′-O-methyl-RNA to antisense technology or gene expression analysis, the hybridization of the modified RNA with RNA and DNA was studied. The sequences used in these studies are shown in Figure 3.

The sequences were designed as follows. \textbf{M1} and \textbf{M3} are 2′-O-methyl-RNAs incorporating a 3-deazaguanine. \textbf{M2} and \textbf{M4} are the derivatives of \textbf{M1} and \textbf{M3}, respectively, having the canonical guanine base in place of 3-deazaguanine. \textbf{M1} and \textbf{M3} differ in their bases flanking the cG base,
RNA targets R1–R4 having C or its one-point mutation at the sixth position from the 5’ end were designed to compare the base discrimination ability of c3G with that of guanine. DNA oligomers D1–D4 are the DNA counterparts of R1–R4. RNA oligomers R5 and R6 were designed to clarify the effects of the c3G on the tandem G/A mismatches. It is well known that tandem G/A mismatches can be stabilized in two different base pairing modes of the face-to-face-type and the sheared-type (Figure 1). In the latter geometry, the nitrogen at position 3 of guanine participates in a hydrogen bond. Therefore, the replacement of the nitrogen atom by a carbon atom is expected to destabilize selectively the sheared-type base pair. The NMR studies revealed that the tandem G/A mismatches in a RNA duplex block 5’-CGAG-3’/3’-GAGC-5’ are in the sheared-type base pair (28), and those of 5’-GGAC-3’/3’-CAGG-5’ are in the face-to-face-type base pair (42). The molecular dynamics simulation also supported these models (43). Therefore, the comparison of the Tm values of the two tandem mismatches, M1/R5 having 5’-CXAG-3’/3’-GAGC-5’ (X = G or c3G) and M3/R6 having 5’-GXAC-3’/3’-CAGG-5’ (X = G or c3G), would be interesting.

These oligonucleotides having a c3G residue were synthesized by use of commercially available 2’-O-methylribonucleoside phosphoramidite derivatives and phosphoramidite 7. The synthesis was performed according to the standard procedure for the synthesis of 2’-O-methyl-RNAs and the deprotection was carried out by use of aqueous NH3 at 50°C for 12 h.

Figure 2. Reversed-phase HPLC profile obtained after treatment of 5 with aqueous ammonia for 25 min at 50°C.
The hybridization properties of the 2′-O-methyl-RNAs to the complementary or mismatch-containing oligoribonucleotides were clarified by measuring the UV melting curves. These results are summarized in Tables 1 and 2.

As shown in Table 1, the \( T_m \) values of the 2′-O-methyl-RNA/RNA and 2′-O-methyl-RNA/DNA decreased by the introduction of 3-deazaguanine in all cases. Particularly, the duplexes with the complementary strand, M1/R1 and M1/D1, were destabilized more than the other duplexes containing mismatches. For example, the \( T_m \) value of the M1/R1 was lower by 6°C than that of M2/R1, whereas the \( T_m \) value of the single G/A mismatch-containing M1/R2 was lower by only 1°C than that of the corresponding M2/R2. Similar trends were observed when the results of the hybridization of M1 and M2 with R3–R4 and D1–D4 were compared. As the result of the specific destabilization of the Watson–Crick base pair, the base discrimination ability of c3G represented by \( \Delta T_m^{c3G} \) in Table 1 became smaller than those of G represented by \( \Delta T_m^G \) in all cases. These results indicate that the nitrogen atom at position 3 of guanine is important to enhance the base discrimination ability of guanine by stabilizing the Watson–Crick base pair with cytosine.

Next, we examined the effect of the incorporation of c3G on the thermal stability of tandem G/A mismatches by measuring the \( T_m \) of the duplexes containing a tandem G/A mismatch. The results are shown in Table 2. As revealed by the comparison of M2/R5 and M1/R5, and M2/D5 and M1/D5 duplexes, the introduction of a 3-deazaguanine decreased the stability of the tandem G/A mismatch in the 5′-CXAG-3′-GAGC-5′ sequences by 6°C (\( \Delta T_m^{M2-M1} = T_m^{M2} - T_m^{M1} \)) for RNA target and 9°C for DNA target, respectively. Similarly, the stability of the tandem mismatches in 5′-GXAC-3′-CAGG-5′ sequences was also decreased by 3 and 4°C as shown by M3/R6 and M4/R6, and M3/D6 and M4/D6 duplexes, respectively. Interestingly, the destabilization was more significant (\( \Delta T_m^{M2-M1} = 6 \) and 9°C) in 5′-CXAG-3′-GAGC-5′ sequences than in 5′-GXAC-3′-CAGG-5′ sequences (\( \Delta T_m^{M2-M3} = 3 \) and 4°C). It is well known that, in the case of RNA/RNA, the tandem G/A mismatches in 5′-GXAC-3′-CAGG-5′ sequences predominantly form sheared-type G/A base pairs which involve a hydrogen bond of the N3 of the guanine and the amino proton of adenine. Therefore, more significant destabilization of the tandem mismatches in 5′-CXAG-3′-GAGC-5′ sequences shown in Table 2 might be attributed to the inhibition of the sheared-type G/A base pairing by the replacement of N3 with C(3)H. It should also be noted that these results suggested the possibility of formation of the sheared-type G/A mismatches in 2′-O-methyl-RNA/RNA and 2′-O-methyl-RNA/DNA duplexes despite the presence of a bulky 2′-O-methyl group in the minor groove.

Scheme 2. The structures of intermediates and products found in the deprotection of the dpc and dmf groups of 5.

Figure 3. 2′-O-methyl-RNA, RNA and DNA strands used in this study.
Computational studies of energy and structure of c3G/C base pair in duplex

As shown in Table 1, the replacement of the nitrogen atom at position 3 of guanine by the carbon atom destabilized most significantly in the case of the base pair with cytosine.

The previously reported molecular orbital calculations of 9-methylguanine (m9G)/1-methylcytosine (m1C) and 3-deaza-9-methylguanine (m9c3G)/m1C revealed that the (m9c3G/m1C Watson–Crick base pair (hydrogen bond energy: EHB = −23.23 kcal/mol) was less stable by 1.40 kcal/mol than the m9G/m1C pair (EHB = −24.61 kcal/mol) (44). Therefore, the difference in the hydrogen bond energy between the modified and unmodified base pairs seems to be one of the factors that destabilized the duplex incorporating c3G.

In addition, we also examined the stacking interactions of c3G in the duplexes. Previous computational studies have revealed the importance of the dipole–dipole interaction, which is equivalent to the electrostatic interactions in partial atomic charge model, in the stacking of nucleobases (45–47). We calculated the dipole moment of m9c3G by use of Gaussian 03 program (48) at MP2/6-31G**(0.25)//MP2/6-31G* level (45–47) to be 8.19 debye which was much larger than the dipole moment of m9G, 6.48 debye, calculated by the same procedure. These values indicated the presence of larger dipole–dipole interactions between the neighboring bases and c3G in the duplex. It should be noted that in general, the dipole–dipole interactions intrinsically contribute to the destabilization of base–base stacking (49). Although many other factors such as solvent effects (49,50) should be considered, the very polar electronic distribution of c3G might be another intrinsic factor that destabilized the duplex incorporating c3G.

CONCLUSION

In this study, we clarified for the first time the hybridization and base discrimination properties of several 2′-O-methyl-RNAs incorporating c3G. It turned out that the N,N-diphenylcarbamoyl (dpc) and N,N-dimethylaminomethylene (dmf) group were successfully used as base protecting groups. It was revealed that the dmf group was cleaved by treatment with ammonia accompanying the formation of N-formyl and N-aminomethylene compounds as intermediates. The effects of c3G on two-types of tandem G/A mismatches in the 5′-GXAC-3′/′CAGG-5′ and 5′-CXAG-3′/′GAGC-5′ (X = G or c3G) sequences were examined. The incorporation of c3G into 2′-O-methyl-RNAs decreased the stability of both of the tandem mismatches in comparison with guanine. However, the decrease of the duplex stability was more significant for the 5′-CXAG-3′/′GAGC-5′ sequence. It is well known that the sheared-type G/A mismatch, which is stabilized by the hydrogen bond between the N1 of guanine and the amino group of adenine, predominately forms in RNA duplexes having a 3′-CGAG-3′/′GAGC-5′ sequence. Therefore, the large destabilization of the 5′-CGAG-3′/′GAGC-5′ type tandem G/A mismatch by the 3′-deaza modification could be explained by the absence of the hydrogen bond between the CH(3) of c3G and the amino group of adenine.

2′-O-methyl-RNAs incorporating a 3-deazaguanine hybridized less strongly to the complementary and single mismatch-containing RNAs than the RNA counterparts, and the base-discriminating properties became lower than that of guanine because the Tm decrease was most significant for the c3G/C base pair.

Computational studies revealed that the Tm decrease by incorporation of c3G could be partly attributed to both the weaker hydrogen bonds of c3G/C pair and possibly weakened stacking ability of c3G due to the increased electrostatic repulsion resulted from the larger dipole moment of c3G.

These results suggested a new design strategy of artificial nucleobases having a 3-deazaguanine skeleton. To improve the duplex stability and base discrimination ability, such new c3G derivatives should be able to form stronger Watson–Crick hydrogen bonds and have lower dipole moments.

For example the computational studies by Kawahara et al. (44) predicted that the Watson–Crick type base pair could be stabilized by modified guanine bases such as 2-N-formylguanine, 8-oxoguanine and 8-azaguanine. Therefore, readily obtainable 3-deazaguanosines derivatives such as 2-N-formyl-3-deazaguanine (12) or other N-acyl-3-deazaguanine derivatives might be useful modified bases capable of improved single mismatch and tandem G/A mismatch recognition. Work along these lines is currently in progress in our laboratory.

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REFERENCES

1. Gupta,P.K., Robins,R.K. and Revankar,G.R. (1985) A new synthesis of certain 7-(β-ribofuranosyl) and 7-(2-deoxy-β-ribofuranosyl) derivatives of 3-deazaguanine via the sodium salt glycosylation procedure. Nucleic Acids Res., 13, 5341–5352.
2. Cook,P.D. and Robins,R.K. (1978) Synthesis of 7- and 9-β-d-ribofuranosides of 3-deaza-6-thioguanine and 3-deaza-2,6-diaminopurine by a novel ring closure of 4(5)-cyano-5(4)-cyanomethylimidazole β-D-ribofuranosides. J. Org. Chem., 43, 289–293.
3. Liu,M.-C., Luo,M.-Z., Mozdziesz,D.E., Lin,T.-S., Dutschman,G.E., Gullen,E.A., Cheng,Y.-C. and Sartorelli,A.C. (2001) Synthesis of halogen-substituted 3-deazaadenosine and 3-deazaadenosine analogues as potential antitumor/antiviral agents. Nucleosides Nucleotides Nucleic Acids, 20, 1975–2000.
4. Liu,M.-C., Luo,M.-Z., Mozdziesz,D.E., Lin,T.-S., Dutschman,G.E., Cheng,Y.-C. and Sartorelli,A.C. (1999) Synthesis of 2′-methylene-substituted 5-azacytidine, 6-azacytidine, and 3-deazaadenine nucleoside analogues as potential antitumor/antiviral agents. Nucleosides Nucleotides Nucleic Acids, 18, 55–72.
5. Minakawa,N. and Matsuda,A. (1993) Nucleosides and nucleotides. 116. Convenient synthesis of 3-deazaadenosine, 3-deazaguanosine, and 3-deazainosine via ring closure of 5-ethynyl-1-(3-oxo-1,3-oxazol]-2-yl)ethyne by condensation with ammonia accompanying the formation of N-formyl and N-aminomethylene compounds as intermediates. The effects of c3G on two-types of tandem G/A mismatches in the 5′-GXAC-3′/′CAGG-5′ and 5′-CXAG-3′/′GAGC-5′ (X = G or c3G) sequences were examined. The incorporation of c3G into 2′-O-methyl-RNAs decreased the stability of both of the tandem mismatches in comparison with guanine. However, the decrease of the duplex stability was more significant for the 5′-CXAG-3′/′GAGC-5′ sequence. It is well known that the sheared-type G/A mismatch, which is stabilized by the hydrogen bond between the N1 of guanine and the amino group of adenine, predominately forms in RNA duplexes having a 3′-CGAG-3′/′GAGC-5′ sequence. Therefore, the large destabilization of the 5′-CGAG-3′/′GAGC-5′ type tandem G/A mismatch by the 3′-deaza modification could be explained by the absence of the hydrogen bond between the CH(3) of c3G and the amino group of adenine.
riboflavonosylimidazo-4-carboxamide or -carbonitile. Tetrahedron, 49, 557–570.

6. Minakawa,N., Kojima,N. and Matsuda,A. (1999) Nucleosides and Nucleotides 184, Synthesis and conformational investigation or anti-fixed 3-deaza-3-halopurine ribonucleosides. J. Org. Chem., 64, 7138–7172.

7. Revankar,G.R., Gupta,P.K., Adams,A.D., Dalley,N.K., McKerman,P.A., Cook,P.D., Canonico,P.G. and Robins,R.K. (1984) Synthesis and antiviral/antitumor activities of certain 3-deazaguanine nucleosides and nucleotides. J. Med. Chem., 27, 1389–1396.

8. Lucas,D.L., Chiang,P.K., Webster,H.K., Robins,R.K., Wiesmann,W.P. and Wright,D.G. (1984) Effects of 3-deazaguanine and 3-deazauracil on the growth and maturation of the human promyelocytic leukemia cell line, HL-60. Adv. Exp. Med. Biol., 165, 321–325.

9. Page,T., Jacobsen,S.J., Smejkal,R.M., Scheele,J., Nyhan,W.L., Mangum,J.H. and Robins,R.K. (1985) Studies on the mechanism of cytotoxicity of 3-deazaguanine in human cancer cells. Cancer Chemother. Pharmacol., 15, 59–62.

10. Spratt,T.E. and de los Santos,H. (1992) Reaction of O\textsuperscript{6}-alkylguanine-DNA alkytransferase with O\textsuperscript{6}-methylguanine analogues: evidence that the oxygen of O\textsuperscript{6}-methylguanine is protonated by the protein to effect protein transfer. Biochemistry, 31, 3688–3694.

11. Seela,F., Debelak,H., Andrews,L. and Beigelman,L. (2003) Synthesis and properties of 2\textsuperscript{-}amino-2-thiothymine act as selectively binding complementary agents.

12. Vainrub,A. and Pettitt,B.M. (2004) Theoretical aspects of genomic variation screening using DNA microarrays. Nucleic Acids Res., 26, 2224–2229.

13. Hemmer,D. (1979) Tetraazopropylsiloxane-1,3-diyli diol for group selection of 3\textsuperscript{-} and 5\textsuperscript{-}hydroxy function of nucleosides. J. Chem. Res., (St), 24–25.

14. Grott,M., Douglas,M., Beijer,B., Garcia,R.G., Eritja,R. and Sproat,B. (1997) Protection of the guanine residue. J. Org. Chem., 62, 937–941.

15. Markiewicz,W.T. (1979) Tetraazopropylsiloxane-1,3-diyli diol for group selection of 3\textsuperscript{-} and 5\textsuperscript{-}hydroxy function of nucleosides. J. Chem. Res., (St), 24–25.

16. Westman,E. and Stromberg,R. (1994) Removal of O\textsuperscript{6}-methylguanosine protection in RNA-synthesis. Tetrahedron Lett., 35, 2775–2778.

17. Vlahoulis,J.C. and Teoule,R. (1987) The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. Nucleic Acids Res., 15, 397–416.

18. Vlahoulis,J.C. and Teoule,R. (1987) The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. Nucleic Acids Res., 15, 397–416.

19. Vlahoulis,J.C. and Teoule,R. (1987) The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. Nucleic Acids Res., 15, 397–416.

20. Vlahoulis,J.C. and Teoule,R. (1987) The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. Nucleic Acids Res., 15, 397–416.

21. Vlahoulis,J.C. and Teoule,R. (1987) The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. Nucleic Acids Res., 15, 397–416.

22. Vlahoulis,J.C. and Teoule,R. (1987) The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. Nucleic Acids Res., 15, 397–416.

23. Vlahoulis,J.C. and Teoule,R. (1987) The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. Nucleic Acids Res., 15, 397–416.

24. Vlahoulis,J.C. and Teoule,R. (1987) The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. Nucleic Acids Res., 15, 397–416.

25. Vlahoulis,J.C. and Teoule,R. (1987) The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. Nucleic Acids Res., 15, 397–416.

26. Vlahoulis,J.C. and Teoule,R. (1987) The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. Nucleic Acids Res., 15, 397–416.

27. Vlahoulis,J.C. and Teoule,R. (1987) The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. Nucleic Acids Res., 15, 397–416.

28. Vlahoulis,J.C. and Teoule,R. (1987) The final deprotection step in oligonucleotide synthesis is reduced to a mild and rapid ammonia treatment by using labile base-protecting groups. Nucleic Acids Res., 15, 397–416.
characterization of 10 stacked base dimers. Comparison of stacked and H-bonded base pairs. J. Phys. Chem., 100, 5590–5596.

47. Seio, K., Ukawa, H., Shohda, K. and Sekine, M. (2005) Computational evaluation of intermolecular interactions of a universal base 3-nitropyrole in stacked dimers and DNA duplexes. J. Biomol. Struct. Dyn., 22, 735–746.

48. Frisch, M.J., Trucks, G.W., Schlegel, H.B., Scuseria, G.E., Robb, M.A., Cheeseman, J.R., Montgomery, J.A., Jr, Vreven, T., Kudin, K.N., Burant, J.C. et al. (2004) Gaussian 03, Revision C.02. Gaussian, Inc., Wallingford, CT.

49. Sponer, J., Florian, J., Ng, H.-L., Sponer, J. and Spackova, N. (2000) Local conformational variations observed in B-DNA crystals do not improve base stacking: computational analysis of base stacking in a d(CATGGGCCCATG)2 B—A intermediate crystal structure. Nucleic Acids Res., 28, 4893–4902.

50. Florian, J., Sponer, J. and Warshel, A. (1999) Thermodynamic parameters for stacking and hydrogen bonding of nucleic acid bases in aqueous solution: ab initio/langevin dipoles study. J. Phys. Chem. B, 103, 884–892.