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

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ABSTRACT

2′-*O*-Methyl-RNAs incorporating 3-deazaguanine (c^3G) were synthesized by use of *N,N*-diphenylcarbamoyl and *N,N*-dimethylaminomethylene as its base protecting groups to suppress sheared-type 5′-GA-3′/5′-GA-3′ tandem mismatched base pairing which requires the N^3 atom. These modified RNAs hybridized more weakly with the complementary and single mismatch-containing RNAs than the unmodified RNAs. The T_m experiments were performed to clarify the effects of replacement of the fifth G with c^3G on stabilization of 2′-*O*-methyl-(5′-CGGCGAGGAG-3′)/5′-CUCCGAGCCG-3′ and 2′-*O*-methyl-(5′-CGGGGACGAG-3′)/5′-CUCGGACCCG-3′ duplexes, which form sheared-type and face-to-face type 5′-GA-3′/5′-GA-3′ tandem mismatched base pairs, respectively. Consequently, this replacement led to more pronounced destabilization of the former duplex that needs the N^3 atom for the sheared-type base pair than the latter that does not need it for the face-to-face type base pair. A similar tendency was observed for 2′-*O*-methyl-RNA/DNA duplexes. These results suggest that the N^3 atom of G plays an important role in stabilization of the canonical G/C base pair as well as the base discrimination and its loss suppressed formation of the undesired sheared-type mismatched base pair. Computational studies based on *ab initio* calculations suggest that the weaker hydrogen bonding ability and larger dipole moment of c^3G can be the origin of the lower T_m.

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 O^6-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 N^3 atom of the guanine residue on the catalytic activity of hammerhead ribozymes (13). Despite these pioneering studies related to 3-deazaguanosine and 3-deazadeoxyguanosine 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 other mismatch base pairs. For example, oligoribonucleotides incorporating 2-thiothymidine or 2-thiouridine, which has a 2-thiocarbonyl group in place of the 2-carbonyl group, stabilized duplexes with DNA and RNA and this modified base recognized adenine more precisely than guanine preventing the formation of a thermodynamically stable G/U (T) wobble base pair (17–20). This improved molecular recognition of

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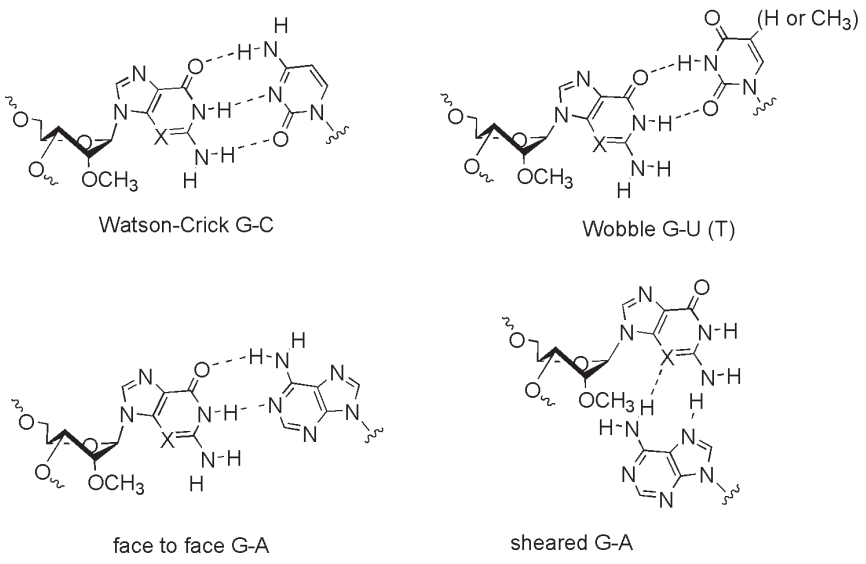


Figure 1. Hydrogen bonding modes related to this study (X = N or CH).

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 been often 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 set 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 N^3 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

^{1}H, ^{13}C and ^{31}P 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-d_6 (2.49 p.p.m.) for ^{1}H NMR, $CDCl_3$ (77.0 p.p.m.), DMSO-d_6 (39.7 p.p.m.) for ^{13}C NMR and 80% phosphoric acid for ^{31}P 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 CH_3CN as a solvent. TLC was performed with Merck silica gel 60 (F_{254}) 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 CH_3CN (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 × 100 mm) with the linear gradient of buffer A: 25 mM sodium phosphate (pH 6.0) and 10% CH_3CN; and buffer B: 25 mM sodium phosphate, 1 M NaCl (pH 6.0) and 10% CH_3CN. 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 dimethylacetal (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%). ^{1}H NMR (DMSO-d_6, 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,

J = 6.1 Hz), 6.06 (1H, s), 7.97 (1H, s), 8.01 (1H, s), 10.57 (1H, br); ^{13}C NMR (DMSO-d_6, δ): 34.12, 61.16, 70.08, 74.13, 77.22, 85.49, 88.29, 126.98, 137.83, 141.05, 150.91, 154.93, 157.33. ESI-MS $[M+H]^+$ calcd. for $C_{14}H_{20}N_5O_5$ 338.1464, found 338.1462.

2-*N*-(*N*,*N*-Dimethylaminomethylene)-3′, 5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-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-tetraisopropyldisiloxane (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 $NaHCO_3$. 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 $MgSO_4$, 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%). ^{1}H NMR (DMSO-d_6, 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); ^{13}C NMR (DMSO-d_6): 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 $[M+H]^+$ calcd. for $C_{26}H_{46}N_5O_6Si_2$ 580.2987, found 580.2920.

2-*N*-(*N*,*N*-Dimethylaminomethylene)-*O*-6-(*N*, *N*-diphenylcarbamoyl)-3′, 5′-O-(1,1,3,3-tetraisopropyldisiloxane-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 $NaHCO_3$ 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 $MgSO_4$, filtered and concentrated under reduced pressure. The residue was chromatographed on a silica gel column with hexane-ethyl acetate (25:75, v/v) to give **3** (270 mg, 72%). ^{1}H NMR (DMSO-d_6, 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 (2H, m), 7.40–7.45 (8H, m), 8.24 (1H, s), 8.40 (1H, s); ^{13}C-NMR (DMSO-d_6, δ): 11.97, 12.29, 12.45, 16.78, 16.82, 16.89, 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 $[M+H]^+$ calcd. for $C_{39}H_{55}N_6O_7Si_2$ 775.3671, found 775.3661.

2-*N*-(*N*,*N*-Dimethylaminomethylene)-*O*-6-(*N*, *N*-diphenylcarbamoyl)-2′-*O*-methyl-3′, 5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl) -3-deazaguanosine (4)

Compound **3** (440 mg, 0.57 mmol) was rendered anhydrous by repeated co-evaporation with dry toluene, and then dissolved in dry DMF (9 ml). To this solution was added CH_3I (180 μl, 2.8 mmol) and NaH (41 mg, 1.7 mmol) at −20°C and the resulting mixture was stirred at −20°C for 2 h. The mixture was poured into sodium phosphate (pH 7, 30 ml) and the materials were extracted with ethyl acetate (30 ml). The organic layer was washed twice with saturated aqueous NaCl, dried over $MgSO_4$, 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 **4** (347 mg, 77%). ^{1}H-NMR (DMSO-d_6, δ): 0.95–1.06 (28H, m), 2.97 (3H, s), 3.07 (3H, s), 3.51 (3H, s), 3.93–3.99 (1H, m), 4.10 (1H, dd, J = 2.7, 12.7 Hz), 4.19 (1H, m), 4.53 (1H, dd, J = 5.4, 8.1 Hz), 6.01 (1H, d, J = 2.0 Hz), 6.86 (1H, s), 7.28 (2H, m), 7.40–7.46 (8H, m), 8.28 (1H, s), 8.39 (1H, s); ^{13}C-NMR (DMSO-d_6, δ): 12.01, 12.31, 12.44, 12.80, 16.79, 16.89, 16.90, 17.01, 17.18, 17.21, 17.24, 34.21, 58.56, 60.27, 69.88, 81.26, 81.84, 88.00, 96.37, 126.79, 127.59, 129.24, 142.18, 142.26, 142.42, 147.02, 151.66, 151.12, 155.18. ESI-MS $[M+H]^+$ calcd. for $C_{40}H_{57}N_6O_7Si_2$ 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%). ^{1}H-NMR (DMSO-d_6, 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, dd, 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 (2H, m, Ar-H), 7.41–7.47 (8H, m, Ar-H), 8.37 (1H, s), 8.46 (1H, s,); ^{13}C-NMR (DMSO-d_6, 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 $[M+H]^+$ calcd. for $C_{28}H_{31}N_6O_6$ 547.2305, found 547.2278.

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 $NaHCO_3$ (10 ml × 2) and then twice with saturated aqueous NaCl (10 ml × 2). The organic layer

was dried over $MgSO_4$, 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%).

^{1}H NMR ($CDCl_3$, 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).^{13}C ($CDCl_3$, 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, 135.79, 135.83, 141.27, 142.75, 143.49, 144.73, 148.06, 152.63, 156.12, 156.20, 158.84. ESI-MS $[M+H]^+$ calcd. for $C_{49}H_{49}N_6O_8$ 849.3612, found 849.3605.

5′-*O*-(4, 4′-Dimethoxytrityl) -2-(*N*,*N*-dimethylaminomethylene)-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 CH_2Cl_2 (13 ml), and to this solution were added *N*,*N*-diisopropylethylamine (260 μL, 1.3 mmol) and chloro(2-cyanoethosy)(*N*,*N*-diisopropylamino)phosphine (233 μl, 1.1 mmol). The mixture was stirred at ambient temperature for 1.5 h. The reaction was quenched by adding water (1 ml). The mixture was diluted with CH_2Cl_2 (20 ml) and then washed twice with 5% aqueous Na_2CO_3. The organic layer was dried, filtered and concentrated under reduced pressure. The residue was purified with recycle HPLC to give **7** (572 mg, 62%). ^{1}H NMR ($CDCl_3$, 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, CH_2CN), 3.01 (6H, m, N-CH_3), 3.31.36 (1H, m), 3.40 (3, OCH_3), 3.41–3.50 (1H, m), 3.51–3.65 (4H, m), 3.76 (6H, m, OCH_3), 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, s, J = 6.4, 6.6 Hz), 6.80–6.83 (4H, m), 6.93 (1H, s), 7.19–7.49 (19H, m), 7.99, 8.04 (1H, s, s), 8.42 (1H, s); ^{13}C NMR (DMSO-d_6, 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 $C_{58}H_{66}N_8O_9P$ 1049.4690, found 1049.4668.

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 $C_{26}H_{26}N_5O_7$ 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 $C_{26}H_{27}N_6O_6$ 519.1992, found 519.1963.

*O-6-(N,N-Diphenylcarbamoyl)-2′-O-methyl -3-deazaguanosine (**10**)*. ESI-MS $[M+H]^+$ calcd. for $C_{25}H_{26}N_6O_6$ 492.1883, found 492.1857.

*2-N-(Aminomethylene)- 2′-O-methyl -3-deazaguanosine (**11**)*. ESI-MS $[M+H]^+$ calcd. for $C_{13}H_{18}N_5O_5$ 324.1308, found 324.1296.

*2-N-Formyl-2′-O-methyl -3-deazaguanosine (**12**)*. ESI-MS $[M+H]^+$ calcd. for $C_{13}H_{17}N_4O_6$ 325.1148, found 325.1192.

*2′-O-Methyl-3-deazaguanosine (**13**)*. ESI-MS $[M+H]^+$ calcd. for $C_{12}H_{17}N_4O_5$ 297.1199, found 297.1220.

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

The oligonucleotides incorporating 2′-*O*-methyl-3-deazaguanosine, M1, 5′-CGGC[c^3G]AGGAG-2′, and M3, 5′-CGGG[c^3G]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 oligonucleotide 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 $C_{109}H_{141}N_{45}O_{66}P_9$ 3414.7, found 3418.0
M2: MALDI-TOF mass $[M-H]^-$ calcd for $C_{109}H_{141}N_{45}O_{66}P_9$ 3414.7, found 3415.2

T_m 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 T_m 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 T_m 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 ε_{260} is identical to that of RNA. The oligonucleotide concentration incorporating 3-deazaguanosine was determined on the assumption that the ε_{260} is identical to that of guanosine.

Table 1. The hybridization properties (T_m [°C]) of 2′-*O*-methyl-RNAs **M1** and **M2** with their complementary RNA (**R1**) and DNA (**D1**), and single mismatched RNA (**R2**–**R4**) and DNA (**D2**–**D4**) targets

Complementary strand	2'-*O*-methyl-RNA/RNA m(5'-CGGCXAGGAG-3') M1 or M2 3'-GCCGYUCCUC-5' R1, R2, R3 or R4			2'-*O*-methyl-RNA/DNA m(5'-CGGCXAGGAG-3') M1 or M2 3'-d(GCCGYTCCTC)-5' D1, D2, D3 or D4		
	2'-*O*-methyl-RNA					
	M1 (X = c^3G)		ΔT_m^{c3G}	**M2** (X = G)	ΔT_m^{G}	ΔT_m^{M2-M1}
	X/Y	T_m^{c3G}		X/Y	ΔT_m^{G}	
RNA						
R1 (Y = C)	c^3G/C	65	—	G/C	71	—	6
R2 (Y = A)	c^3G/A	49	−16	G/A	50	−21	1
R3 (Y = G)	c^3G/G	52	−13	G/G	54	−17	2
R4 (Y = U)	c^3G/U	58	−7	G/U	62	− 9	4
DNA						
D1 (Y = C)	c^3G/C	51	—	G/C	59	—	8
D2 (Y = A)	c^3G/A	36	−15	G/A	40	−19	4
D3 (Y = G)	c^3G/G	37	−14	G/G	41	−18	4
D4 (Y = T)	c^3G/T	46	−5	G/T	50	− 9	4

ΔT_m^{c3G}, $\Delta T_m^{G} = T_m$(mismatch) - T_m(match)

RESULTS AND DISCUSSION

Synthesis of 2′-*O*-methyl-3-deazaguaosine 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 N^2 positions, respectively. Previously, Seela *et al*. (13) reported the synthesis of oligoribonucleotides incorporating 3-deazaguanine by use of the phenoxyacetyl (pac) group (36) for the N^2 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-tetraisopropyldisiloxane-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 CH_3I 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

Table 2. The thermal stability of the duplexes containing a tandem G/A mismatch

Complementary strand	2'-*O*-methyl-RNA/RNA m(5'-CGGCXAGGAG-3' M1 or M2 3'-GCCGAGCCUC-5' R5 or D5 (T) Sheared-type tandem mismatch: X = G				2'-*O*-methyl-RNA/DNA m(5'-CGGCXAGGAG-3' M1 or M2 3'-GCCGAGCCUC-5' R5 or D5 (T) Sheared-type tandem mismatch: X = G		
	2'-*O*-methyl-RNA						
	M1 X = c^3G X/A	T_m^{c3G}	M2 X = G X/A	T_m^{G}	ΔT_m^{M2-M1}		
RNA (R5)	c^3G/A	44	G/A	50	6		
DNA (D5)	c^3G/A	27	G/A	36	9		
Complementary strand	2'-*O*-methyl-RNA/DNA m(5'-CGGGXACGAG-3' M3 or M4 3'-GCCCAGGCUC-5' R6 or D6 (T) Face-to-face type tandem mismatch: X = G				2'-*O*-methyl-RNA/DNA m(5'-CGGGXACGAG-3' M3 or M4 3'-GCCCAGGCUC-5' R6 or D6 (T) Face-to-face type tandem mismatch: X = G		
	2'-*O*-methyl-RNA						
	M3 X = c^3G X/A	T_m^{c3}	M4 X = G X/A	T_m^{G}	ΔT_m^{M4-M3}		
RNA (R6)	c^3G/A	50	G/A	53	3		
DNA (D6)	c^3G/A	36	G/A	40	4		

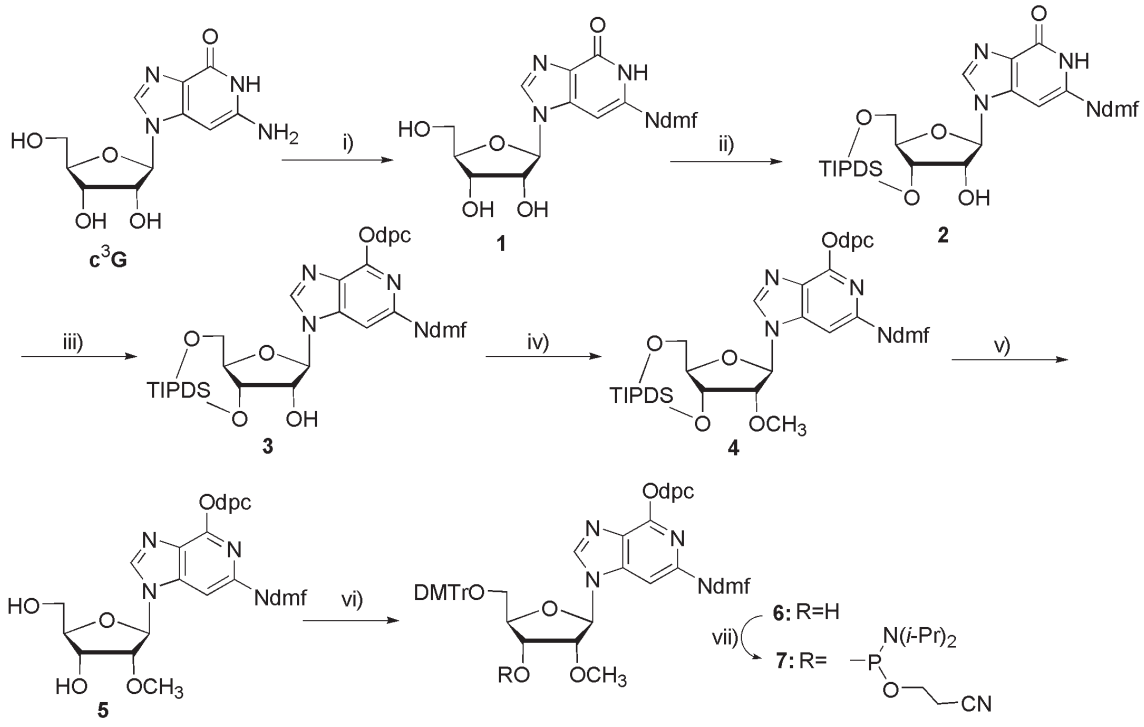
of these protecting groups was carefully checked by use of the diol **5**. Compound **5** was dissolved in aqueous NH_3 and the mixture was kept at 50°C. The reaction was monitored by reversed phase HPLC and the products were analyzed by ESI-MS. After 25 min we observed five intermediates **8**–**12** 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 (c^3G). 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. **M1** and **M3** are 2′-*O*-methyl-RNAs incorporating a 3-deazaguanine. **M2** and **M4** are the derivatives of **M1** and **M3**, respectively, having the canonical guanine base in place of 3-deazaguanine. **M1** and **M3** differ in their bases flanking the c^3G base,



Scheme 1. (i) $(MeO)_2CH(NMe_2)$ (5 equiv.), DMF, room temperature, 5 h, 91%; (ii) $TIPDSCl_2$ (1.1 equiv.), pyridine, room temperature, 4 h, 70%; (iii) $(i\text{-}Pr)_2N\text{-}Et$ (2.0 equiv.), dpcCl (1.2 equiv.), pyridine, room temperature, 4.5 h, 72%; (iv) NaH (3.0 equiv.), CH_3I (5.0 equiv.), DMF, −20°C, 77%; (v) NEt_3-3HF (65 equiv.), THF, room temperature, 6 h, 80%; (vi) DMTrCl (1.2 equiv.), pyridine, room temperature, 80%; (vii) Cl-P(OCE)(N-iPr$_2$) (1.2 equiv.), $(i\text{-}Pr)_2N\text{-}Et$ (1.5 equiv.), CH_2Cl_2, room temperature, 1.5 h, 62%.

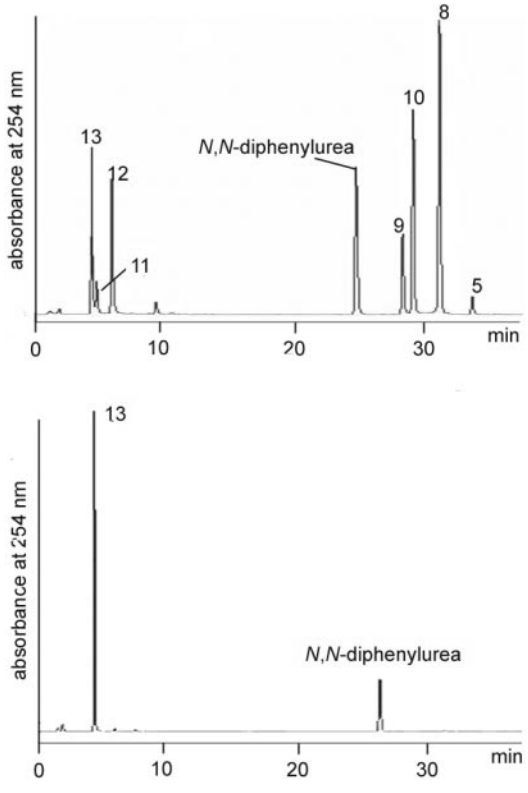
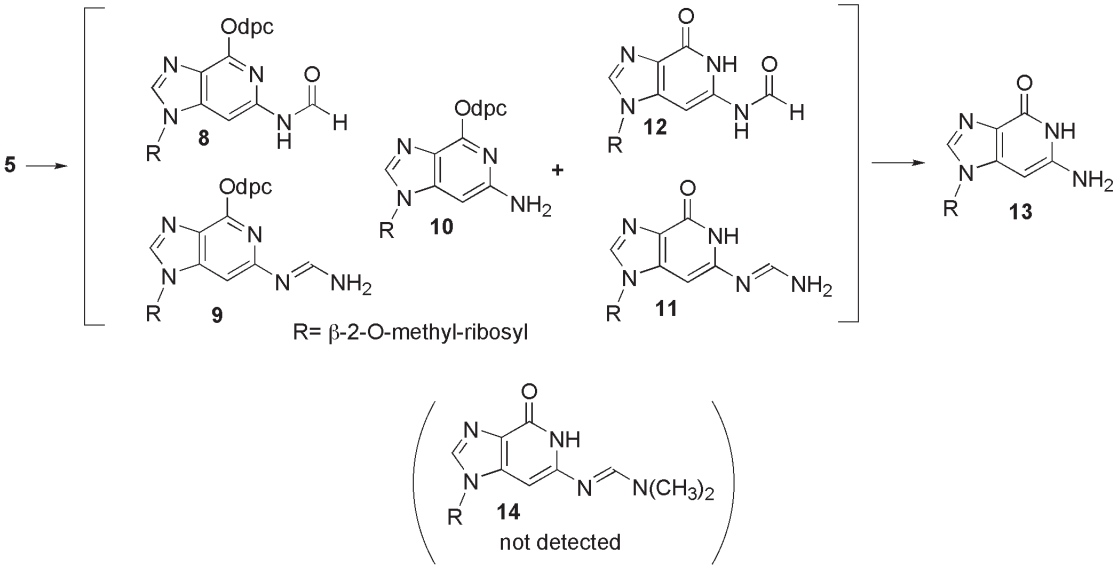


Figure 2. Reversed-phase HPLC profile obtained after treatment of **5** with aqueous ammonia for 25 min at 50°C.

5′-C[c^{3}G]AG-3′ for **M1** and 5′-G[c^{3}G]AC-3′ for **M3**. 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 c^{3}G 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 c^{3}G 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 T_m values of the two tandem mismatches, **M1**/**R5** having 5′-CXAG-3′/3′-GAGC-5′ (X = G or c^{3}G) and **M3**/**R6** having 5′-GXAC-3′/3′-CAGG-5′ (X = G or c^{3}G), would be interesting.

These oligonucleotides having a c^{3}G 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 NH_3 at 50°C for 12 h.



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

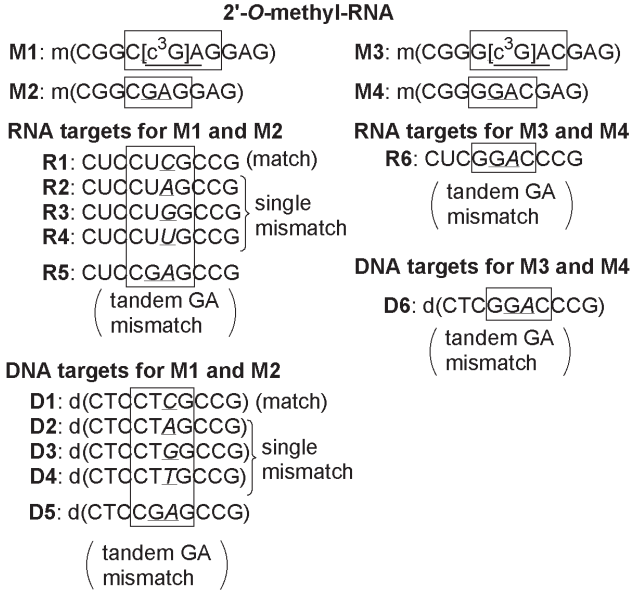


Figure 3. 2′-*O*-methyl-RNA, RNA and DNA strands used in this study.

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 c^3G represented by $\Delta T_m^{c^3G}$ in Table 1 became smaller than those of G represented by ΔT_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 c^3G 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′/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′/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′/3′-GAGC-5′ sequences than in 5′-GXAC-3′/3′-CAGG-5′ sequences ($\Delta T_m^{M4-M3} = 3$ and 4°C). It is well known that, in the case of RNA/RNA, the tandem G/A mismatches in 5′-CGAG-3′/3′-GAGC-5′ predominantly form sheared-type G/A base pairs which involve a hydrogen bond of the N^3 of the guanine and the amino proton of adenine. Therefore, more significant destabilization of the tandem mismatches in 5′-CXAG-3′/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 N^3 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.

Computational studies of energy and structure of c^3G/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 (m^9G)/1-methylcytosine (m^1C) and 3-deaza-9-methylguanine (m^9c^3G)/m^1C revealed that the (m^9c^3G)/m^1C Watson–Crick base pair (hydrogen bond energy: $E_{HB} = -23.21$ kcal/mol) was less stable by 1.40 kcal/mol than the m^9G/m^1C pair ($E_{HB-} = -24.61$ 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 c^3G.

In addition, we also examined the stacking interactions of c^3G 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 m^9c^3G 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 m^9G, 6.48 debye, calculated by the same procedure. These values indicated the presence of larger dipole–dipole interactions between the neighboring bases and c^3G 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 examined to reach unambiguous conclusion, these results *in vacuo* indicated that the very polar electronic distribution of c^3G might be another intrinsic factor that destabilized the duplex incorporating c^3G.

CONCLUSION

In this study, we clarified for the first time the hybridization and base discrimination properties of several 2′-*O*-methyl-RNAs incorporating c^3G. 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 c^3G on two-types of tandem G/A mismatches in the 5′-GXAC-3′/3′-CAGG-5′ and 5′-CXAG-3′/3′-GAGC-5′ (X = G or c^3G) sequences were examined. The incorporation of c^3G 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′/3′-GAGC-5′ sequence. It is well known that the sheared-type G/A mismatch, which is stabilized by the hydrogen bond between the N^3 of guanine and the amino group of adenine, predominantly forms in RNA duplexes having a 5′-CGAG-3′/3′-GAGC-5′ sequence. Therefore, the large destabilization of the 5′-CGAG-3′/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 c^3G 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 T_m decrease was most significant for the c^3G/C base pair.

Computational studies revealed that the T_m decrease by incorporation of c^3G could be partly attributed to both the weaker hydrogen bonds of c^3G/C pair and possibly weakened stacking ability of c^3G due to the increased electrostatic repulsion resulted from the larger dipole moment of c^3G.

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 c^3G 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-deazaguanosine 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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Conflict of interest statement. None declared.

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