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Synthesis and hybridization properties of oligodeoxynucleotides incorporating 2-*N*-carbamoylguanine derivatives as guanine analogs

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Abstract—2-*N*-Carbamoyldeoxyguanosine and its derivatives were synthesized and incorporated into ODNs. The T_m analyses revealed higher selective base recognition ability of 2-*N*-carbamoylguanine than that of guanine. The new guanine analog must be useful for the development of functional oligodeoxynucleotides capable of precise base recognition.

The highly selective hybridization of oligodeoxynucleotides (ODNs) to the complementary ODN is the most important property which can be applied to hybridization-based technologies such as antisense DNA,[1] DNA chips,[2] and PCR techniques.[3] However, it is well known that guanine can form not only the Watson–Crick G–C base pair but also the rather stable wobble G–T, G–A and G–G mismatch base pairs in canonical B-type duplexes.[4] Such mismatch formation could be the reason for the inaccuracy of hybridization-based technologies. Therefore, if the selective hybridization could be achieved by use of modified nucleobases instead of natural ones, ODN probes incorporating such modified bases must improve the accuracy of DNA microarray detection, and must be useful for the above-mentioned techniques.

We have studied oligoribonucleotides having 3-deazaguanine (c^3G) in place of the canonical guanine base.[5] The studies of hybridization affinity of 2′-*O*-methylated RNA 10mers incorporating c^3G for the target DNA or RNA oligomers revealed that incorporation of c^3G could destabilize the sheared-type G–A mismatch[6] because of the absence of the hydrogen-bond accepter at the position 3. Unfortunately, the Watson–Crick base pair formed between c^3G and C was less stable than the canonical G–C pair, and c^3G could not be used as the modified base for the synthesis of the functional oligonucleotides.[5] Interestingly, it was also found by us that modification of the 2-amino function of c^3G gave a new modified guanine base, 2-acetyl-3-deazaguanine (a^2c^3G), which can form a Watson–Crick base pair as stable as the canonical G–C pair. Moreover, the sheared-type a^2c^3G–A mismatch and the wobble-type a^2c^3G–U mismatch were significantly destabilized so that the total base discrimination ability of a^2c^3G became higher than that of the canonical guanine.[7] Although these results indicated the usefulness of a^2c^3G as an artificial base applicable to the functional nucleic acids, simpler modified nucleosides which can be prepared readily from canonical nucleosides must be preferable because the synthesis of a^2c^3G nucleosides requires multi-step chemical reactions.[8–13]

In this Letter, we designed 2-*N*-carbamoylguanine derivatives as new guanine analogs having high base discrimination ability assuming that the acyl-type substitution at the amino group can improve the base discrimination ability of the guanine base as in the case of a^2c^3G. We chose carbamoyl-type substituents because they can be readily introduced to the amino group of guanine and are stable toward the aqueous ammonia treatment, which is necessary for removal of the protecting groups

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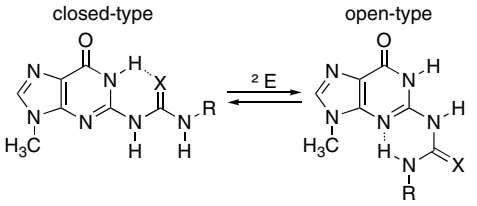


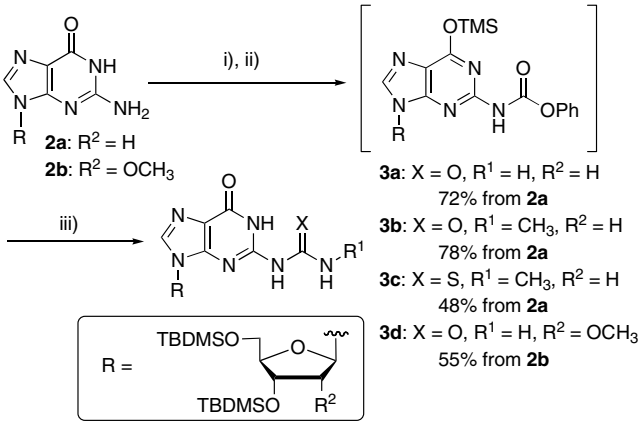
Figure 1. Structure of 9-methyl-cmG, X = O, R = H (**1a**), -mcmG, X = O, R = CH_3 (**1b**) and -mscmG X = S, R = CH_3 (**1c**).

in oligonucleotide synthesis.[14] In addition, intramolecular hydrogen bond between the amino group of the carbamoyl group and the nitrogen atom at position 3 of guanine can stabilize the 'open-type' conformation which can form a base pair (Fig. 1). It should be noted that without such an intramolecular hydrogen bond, the 'closed-type' conformation, which inhibits the formation of the Watson–Crick base pair, must be predominant. A similar conformational change by an intramolecular hydrogen bond was also proposed in the case of a^2c^3G. In addition to the simplest 2-*N*-carbamoyldeoxyguanosine (cmG), 2-*N*-(*N*-methylcarbamoyl)deoxyguanosine (mcmG) and 2-*N*-(*N*-methylthiocarbamoyl)deoxyguanosine (mscmG), we also designed 2-*N*-carbamoyl-2′-*O*-methylguanosine (cmG_m).

First of all, we carried out theoretical studies on the open-type and closed-type conformation of the cmG and its derivatives. 9-Methyl-cmG (**1a**), 9-methyl-mcmG (**1b**), and 9-methyl-mscmG (**1c**) were used as the models of their nucleoside derivatives. The structures of the open-type and closed-type conformers were optimized at the HF/6-31G** level. The energies of the optimized structures were evaluated with single-point calculations at the MP2/6-31G** level.[15,16]

For both 9-methyl-cmG and 9-methyl-mcmG, the closed-type conformers were found to be slightly more stable than the open-type conformers by 1.56 and 1.31 kcal/mol, respectively, than the corresponding open-type conformers. In contrast, the open-type conformer of mscmG was more stable by 3.31 kcal/mol than the closed-type one. Although these data suggested that the closed-type conformers of 9-methyl-cmG and -mcmG were more stable than their open-type conformers, it should be noted that Zimmerman and co-workers[17,18] previously reported that 2-*N*-[*N*-(*n*-butyl)carbamoyl]guanine could form stable supramolecular complexes having four hydrogen bonds in open-type conformation at least at the nucleoside level. These theoretical results together with the experimental data by Zimmerman et al. indicated that all of cmG and mcmG and mscmG can form base pairs in open-type conformation when incorporated in DNA and RNA duplexes.

The synthesis of 2-*N*-carbamoylguanosine derivatives and 2-*N*-thiocarbamoylguanosine derivatives **3a**–**3d** is outlined in Scheme 1. Acylation of the 3′,5′-*O*-disilylated derivatives **2a** and **2b** with phenyl chloroformate (1.5 equiv) followed by treatment of the resulting 2-*N*-phenoxycarbonylguanosine intermediate with aqueous



Scheme 1. Reagents and conditions: (**3a**, **3b**, **3d**) (i) TMSCl (1.5 equiv), pyridine, rt, 1 h; (ii) PhOC(=O)Cl (1.5 equiv), pyridine, rt, 4 h; (iii) For **3a** 28% NH_3aq (5.0 equiv), pyridine, rt, 2 h (from **2a**, 3 steps, 68%). For **3b** 40% $MeNH_2$/methanol (5.0 equiv), pyridine, rt, 2 h (from **2a**, 3 steps, 78%). For **3d** 28% NH_3aq (5.0 equiv), pyridine, rt, 2 h (from **2b**, 3 steps, 55%). (**3c**) (i) NaH (1.3 equiv), DMF, 70 °C, 1 h; (ii) methyl thioisocyanate (3.0 equiv), DMF, 70 °C, 36 h, (from **2a**, 3 steps, 48%).

ammonia (5.0 equiv) and methylamine (5.0 equiv) gave compounds **3a** and **3b**, respectively. Compound **3c** was obtained by the base-selective reaction of the 3′,5′-*O*-disilylated deoxyguanosine with methyl thioisocyanates (3.0 equiv) in the presence of sodium hydride (1.3 equiv) according to the procedure of Zimmerman for the methyl carbamoyl modification of the guanine ring (Scheme 1).[18]

Nucleosides **3a**–**3c** were converted to their phosphoramidite derivatives **7a**–**7c** (Fig. 2 and Scheme 2) by use of the standard phosphytilation procedure.[19] To increase the solubility of the deoxyribonucleosides, the carbonyl group of each deoxyguanosine derivative was protected by the diphenylcarbamoyl (dpc) group.[20] On the other hand, 2′-*O*-methyl phosphoramidite **7d** was synthesized by removal of the TBDMS groups of **3d** in 81% followed by the successive treatments with DMTrCl (64%) and phosphytilation (58%) under the conditions same as those shown in the step (ii), (iii) and (iv) in Scheme 2. In this case, the protection of the 6-*O* position was not necessary because of the higher solubility of **7d** than **7a**–**7c**.

The solid-phase synthesis of ODNs and a 2′-*O*-methyl-RNA containing 2-*N*-carbamoylguanine derivatives was carried out in a DNA synthesizer by use of the standard phosphoramidite method. The ODNs were released from the polymer supports and deprotected by treatment with conc. NH_3aq for 8 h at ambient temperature. The 2-*N*-carbamoyl groups were stable under such basic conditions. The products were purified on a C18 cartridge column by the DMTr-ON purification method and analyzed by use of anion-exchange HPLC. The products were characterized by MALDI-TOF mass.[21]

Next, the hybridization properties of these modified ODNs, 5′-d(CGGCXAGGAG)-3′ where X is G, cmG, mcmG or mscmG, were studied by measuring the T_m

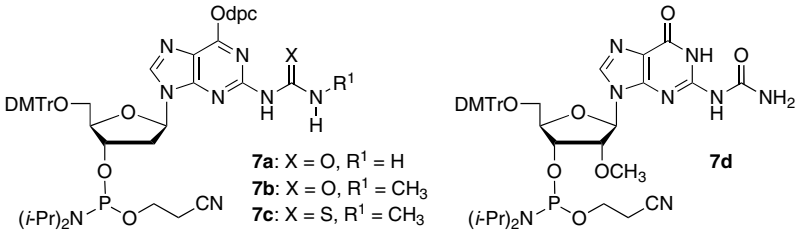
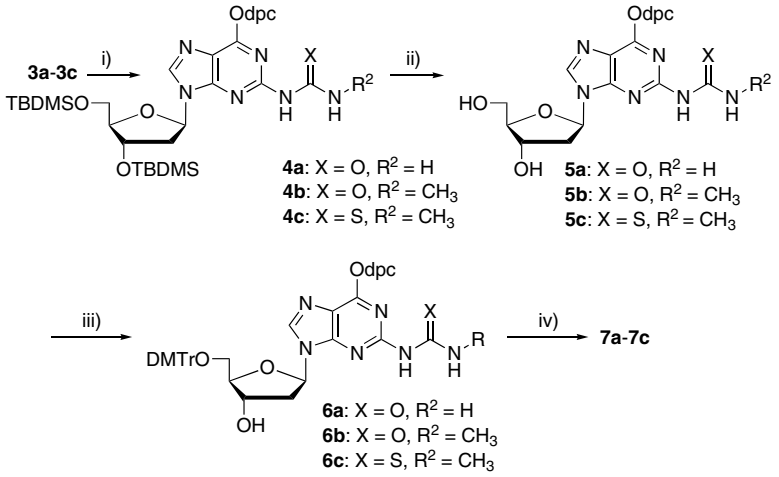


Figure 2. Phosphoramidite units **7a**–**7c**.



Scheme 2. Reagents and conditions: (i) DpcCl (1.3 equiv), $(i\text{-Pr})_2$NEt (2.0 equiv), pyridine, rt, 30 min, (**4a**: 91%, **4b**: 94%, **4c**: 81%); (ii) $3HF{\cdot}NEt_3$ (3.0 equiv), THF, rt, 10 h, (**5a**: quant, **5b**: 81%, **5c**: 80%); (iii) DMTrCl (1.2 equiv), pyridine, rt, 2 h, (**6a**: 76%, **6b**: 87%, **6c**: 78%); (iv) (a) (CEO)P[N(i-Pr)$_2$]$_2$ (1.1 equiv), 1*H*-tetrazole (0.5 equiv), $(i\text{-Pr})_2$NH (0.5 equiv), CH_2Cl_2, rt, 2 h (**7a**: 75%); (b) Cl(i-Pr$_2$N)P(OCE) (2.0 equiv), $(i\text{-Pr})_2$NEt (2.5 equiv), CH_2Cl_2, rt, 2 h (**7b**: 68%); (c) Cl-(i-Pr$_2$N)P(OCE) (1.3 equiv), $(i\text{-Pr})_2$NEt (1.6 equiv), CH_2Cl_2, rt, 2 h (**7c**: 47%).

values of the duplexes with the target ODN, 3′-d(GCCGYTCCTC)-5′ where Y is C, A, G or T. As shown in Table 1, the Watson–Crick base pairs formed between the canonical G and C were as strong as that between the cmG and C, $T_m = 51.5$ and 50.8 °C, respectively. Interestingly, the cmG–T mismatch base pair was more destabilized by than the G–T mismatch pair by $\Delta\Delta T_m = -3.6$ °C. Although the cmG–G mismatch base pair was stabilized in comparison to the G–G mismatch base pair ($\Delta\Delta T_m = 2.0$ °C), the total selectivity of cmG

Table 1. T_m values (°C)[a] for 10mer duplexes containing 2-*N*-carbamoylguanine derivatives 5′-d(CGGCXAGGAG)-3′/3′-d(GCCGYTCCTC)-5′

	X = G T_m (ΔT_m)[b]	cmG T_m (ΔT_m)[b] {$\Delta\Delta T_m$}[c]	mcmG T_m (ΔT_m)[b] {$\Delta\Delta T_m$}[c]	mscmG T_m (ΔT_m)[b] {$\Delta\Delta T_m$}[c]
Y = C	51.5 ± 0.01	50.8 ± 0.31	50.6 ± 0.14	49.6 ± 0.34
A	36.7 ± 0.30 (−14.8 ± 0.31)	35.5 ± 0.84 (−15.3 ± 0.54) {−0.5}	36.0 ± 0.53 (−14.6 ± 0.41) {+0.2}	35.8 ± 0.78 (−13.8 ± 0.43) {+1.0}
G	34.8 ± 0.47 (−16.7 ± 0.46)	36.1 ± 0.44 (−14.7 ± 0.14)[d] {+2.0}	37.3 ± 0.28 (−13.3 ± 0.20)[d] {+3.4}	34.9 ± 0.18 (−14.7 ± 0.32) {+2.0}
T	38.6 ± 0.38 (−12.9 ± 0.44)[d]	34.3 ± 0.21 (−16.5 ± 0.15) {−3.6}	37.1 ± 0.42 (−13.5 ± 0.40) {−0.6}	38.7 ± 0.40 (−10.9 ± 0.25)[d] {+2.0}

[a] The averages and the standard deviations of three to four experiments were obtained under the following conditions: 2 μM duplexes, 10 mM sodium phosphate buffer, 0.1 M NaCl, 0.1 mM EDTA, pH 7.0.
[b] The ΔT_m is the difference of T_m of a X–C pair and the corresponding modified X–Y pair.
[c] The $\Delta\Delta T_m$ is the difference of ΔT_m of a X–Y pair and the corresponding modified G–Y pair.
[d] The selectivity index defined as the $\Delta\Delta T_m$ of the smallest absolute value.

(−14.7 °C) was higher than that of G (−12.9 °C), as shown by the selectivity index.

The mcmG was similar to cmG in the base pairing profile such as the base selectivity and the base pair stabilities. In this case, the matched base pair (X = C) was as stable as that of the canonical guanine (T_m = 50.6 °C), and the most stable mismatch pair was the mcmG–G pair which was stabilized in comparison to the G–G pair ($\Delta\Delta T_m$ = +3.4 °C). As the result, the selectivity was proved to be 13.3 °C, which was between those of cmG and G.

It should be noted for mcmG that each of the mismatches including mcmG was more stable than the corresponding mismatch pair incorporating cmG. These results suggest the possibility that cmG could perform more precise base recognition than mcmG. In contrast to cmG and mcmG, the Watson–Crick base pairs of mscmG–C were less stable than that of the canonical G–C base pair (T_m = 49.6 °C). In terms of the selectivity, mscmG was the worst of all the four modified and unmodified guanines as suggested by the selectivity index of −10.9 °C.

The hybridization properties of the duplex of 2′-*O*-methyl-RNA incorporating cmG_m and the target ODN were analysed. As shown in Table 2, the base discrimination of cmG_m was qualitatively similar to but quantitatively more significant than that in DNA shown in Table 2. The cmG_m–dC (T_m = 59.9 °C) base pair was as strong as the canonical G_m–dC (T_m = 60.2 °C) and the cmG_m–T mismatch base pair was more destabilized by $\Delta\Delta T_m$ = −4.3 °C than the G_m–T mismatch pair. The cmG_m–dG mismatch base pair was stabilized in comparison to the G_m–dG mismatch base pair ($\Delta\Delta T_m$ = +1.8 °C), the selectivity of cmG_m (14.0 °C) was higher than that of G_m (9.7 °C), as shown by the selectivity index.

In this study, we reported new oligonucleotides incorporating 2-*N*-carbamoylguanine base analogs such as cmG, mcmG, mscmG, and cmG_m. Deoxynucleosides having these modified bases could be readily synthesized from deoxyguanosine. Therefore, unlike the previously reported a^2c^3G, cmG and its derivatives are more convenient for large-scale synthesis. The base pairing profile of cmG and mcmG was characterized by the stable Watson–Crick pairing with C, the destabilized mismatch base pair with T, and the stabilized mismatch base pair with G. This trend was also observed in 2′-*O*-methyl-RNA/DNA duplexes. The tendency was quite similar to that of the previously reported a^2c^3G in the 2′-*O*-methyl-RNA structures.[7] Because a^2c^3G, cmG and mcmG derivatives have the acyl-type substituents on the 2-amino group, the base pairing profiles observed in these studies might be common to 2-*N*-acylated guanine derivatives. The mechanism of the destabilization of the cmG–T and mcmG–T mismatch is not clear. One plausible explanation is that cmG and mcmG are in the 'closed-type' conformation in the cmG–T and mcmG–T base pair, and the formation of wobble type hydrogen bonds is blocked by the carbamoyl groups. The hypothesis is supported by the fact that mscmG, whose 'open-type' conformation is much more stable than the 'closed-type' conformation, did not show such base pair selectivity. It is unclear why the stability of the cmG–G mismatches was increased. The G–G base pair in the 5′-CGA-3′/3′-GGT-5′ sequence was reported to exist in G(*anti*)-G(*anti*) conformation.[22] Therefore, the carbamoyl group might participate in the base pairing in a unique manner. The structural studies together with more detailed thermodynamic studies are under way to clarify such a dynamic mechanism and the generality in various sequences of the higher base recognition ability of the 2-*N*-acylated guanines. These results shown in this Letter indicated the potential usefulness of oligodeoxynucleotides incorporating these modified bases as artificial hybridization probes capable of highly accurate sequence recognition.

Table 2. T_m values (°C)[a] for 10mer duplexes containing 2-*N*-carbamoylguanine 5′-($C_mG_mG_mC_mXA_mG_mG_mA_mG_m$)-3′/3′-d(GCCGYTCCTC)-5′[a]

	X = G_m T_m (ΔT_m)	cmG_m T_m (ΔT_m) {$\Delta\Delta T_m$}
Y = C	60.2 ± 0.87	59.9 ± 0.60
A	40.5 ± 0.28 (−19.7 ± 0.76)	38.4 ± 0.25 (−21.5 ± 0.70) {−1.8}
G	41.7 ± 1.25 (−18.5 ± 0.26)	44.1 ± 0.54 (−15.8 ± 0.65) {+2.7}
T	50.5 ± 0.09 (−9.7 ± 0.66)[b]	45.9 ± 0.19 (−14.0 ± 0.32)[b] {−4.3}

[a] The N_m shows a 2′-*O*-methyl-nucleoside residue.
[b] The selectivity index defined as the $\Delta\Delta T_m$ of the smallest absolute value.

Acknowledgments

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19. Spectroscopic and mass analysis data of compound **7a**–**7d**. Compound **7a**: ^{1}H NMR ($CDCl_3$) δ 1.11–1.33 (14H, m), 2.46 (1H, m), 2.55–2.74 (3H, m), 3.32–3.40 (2H, m), 3.57–3.87 (4H, m), 3.75 (6H, m), 4.26–4.32 (1H, m), 4.67–4.72 (1H, m), 5.36 (1H, s, br), 6.35 (1H, m), 6.79 (4H, m), 7.17–7.50 (19H, m), 8.07 (1H, m), 8.53 (1H, s, br); ^{13}C NMR ($CDCl_3$) δ 20.6, 20.7, 20.8, 20.9, 24.9, 25.0, 25.0, 40.2, 43.6, 43.6, 43.7, 43.7, 55.6, 55.6, 58.5, 58.6, 58.6, 58.7, 63.7, 63.8, 73.9, 74.0, 74.4, 74.5, 84.7, 84.8, 86.2, 86.2, 86.4, 86.9, 113.6, 117.8, 117.9, 120.5, 127.3, 127.4, 128.3, 128.4, 128.5, 129.6, 130.3, 130.4, 130.4, 135.8, 135.8, 135.9, 135.9, 142.1, 144.7, 150.4, 153.1, 154.8, 154.9, 155.0, 156.2, 159.0; ^{31}P NMR ($CDCl_3$) δ 149.9. MS *m*/*z* calcd for $C_{54}H_{59}N_9NaO_9P^+$: 1030.3993, found 1030.4001. Compound **7b**: ^{1}H NMR ($CDCl_3$) δ 1.13–1.27 (14H, m), 2.43 (1H, m), 2.58–2.76 (3H, m), 2.94 (3H, m), 3.35 (2H, m), 3.61–3.84 (4H, m), 3.73 (6H, m), 4.29–4.33 (1H, m), 4.71 (1H, m), 6.35 (1H, m), 6.80 (4H, m), 7.18–7.43 (19H, m), 7.63 (1H, m), 8.09 (1H, m), 8.61 (1H, s); ^{13}C NMR ($CDCl_3$) δ 20.1, 20.2, 20.3, 20.4, 24.5, 24.5, 24.6, 26.7, 39.5, 39.7, 43.2, 43.3, 55.1, 55.1, 58.1, 58.1, 58.2, 58.3, 63.3, 63.4, 73.4, 73.6, 74.0, 74.1, 84.4, 85.7, 85.8, 86.0, 86.5, 113.1, 117.4, 117.5, 119.8, 126.9, 126.9, 127.8, 128.0, 128.0, 129.2, 129.9, 130.0, 130.0, 135.4, 135.5, 141.7, 144.4, 150.1, 153.0, 154.4, 154.5, 155.6, 155.6, 158.5; ^{31}P NMR ($CDCl_3$) δ 150.0, 150.3. MS *m*/*z* calcd for $C_{55}H_{60}N_9NaO_9P^+$: 1044.4149, found 1044.3683. Compound **7c**: ^{1}H NMR ($CDCl_3$) δ 1.12–1.29 (14H, m), 2.46 (1H, m), 2.57–2.75 (3H, m), 2.25 (3H, m), 3.30–3.38 (2H, m), 3.58–3.88 (4H, m), 3.75 (6H, m), 4.27–4.33 (1H, m), 4.67 (H, m), 6.32 (1H, m), 6.79 (4H, m), 7.16–7.43 (19H, m), 8.10 (11H, m), 8.52 (1H, s), 10.59 (1H, m); ^{13}C NMR ($CDCl_3$) δ 20.3, 20.4, 20.6, 20.6, 24.7, 24.7, 24.8, 32.6, 39.8, 43.4, 43.4, 43.5, 55.3, 58.2, 58.3, 58.4, 58.4, 63.4, 63.6, 73.6, 74.1, 74.2, 84.8, 84.8, 86.1, 86.1, 86.3, 86.7, 113.3, 113.3, 117.5, 117.6, 120.2, 120.3, 127.1, 127.1, 128.0, 128.2, 128.2, 129.4, 130.1, 130.1, 130.2, 135.5, 135.6, 135.7, 141.7, 142.4, 144.5, 150.0, 151.8, 158.7, 158.7, 180.0; ^{31}P NMR ($CDCl_3$) δ 150.0, 150.2. MS *m*/*z* calcd for $C_{55}H_{60}N_9NaO_8PS^+$: 1060.3921, found 1060.3889. Compound **7d**: ^{1}H NMR ($CDCl_3$) δ 1.02–1.31 (14H, m), 2.36 (1H, m), 2.62–2.65 (3H, m), 3.30–3.38 (2H, m), 3.31–3.77 (4H, m), 3.71 (6H, m), 4.12–4.19 (1H, m), 4.32 (1H, m), 4.56 (H, m), 6.00–6.02 (1H, m), 6.79–6.83 (4H, m), 7.20–7.43 (9H, m), 7.86–7.82 (1H, m); ^{13}C NMR ($CDCl_3$) δ 9.1, 10.1, 23.4, 24.4, 25.4, 26.4, 43.2, 44.2, 45.0, 46.2, 47.3, 53.9, 55.0, 56.2, 57.3, 59.3, 83.0, 84.2, 87.0, 87.2, 112.9, 114.2, 114.2, 117.8, 120.3, 120.4, 126.7, 127.6, 127.6, 127.9, 128.0, 128.0, 128.9, 129.2, 129.2, 129.3, 129.9, 129.9, 131.0, 131.1, 131.2, 134.7, 135.7, 135.8, 135.9, 136.4, 144.7, 144.8, 144.8, 149.3, 157.0, 159.0; ^{31}P NMR ($CDCl_3$) δ 151.3, 152.2. MS *m*/*z* calcd for $C_{42}H_{51}N_8NaO_9P^+$: 865.3414, found 865.3468.
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21. Sequence, yield and MALDI-TOF mass analyses of ODNs containing 2-*N*-carbamoylguanine derivatives, 5′-d(CGGC[$_{cm}$G]AGGAG)-3′; yield 23%, MALDI-TOF mass $[M-H]^+$ calcd for 3158.4, found 3158.6, 5′-d(CGGC[$_{mcm}$G]AGGAG)-3′; yield 26%, MALDI-TOF mass $[M-H]^+$ calcd for 3172.5, found 3172.6, 5′-d(CGGC[$_{mscm}$G]AGGAG)-3′; yield 7%, MALDI-TOF mass $[M-H]^+$ calcd for 3188.8, found 3188.6, 2′-*O*-methyl 5′-(CGGC[$_{cm}$G$_m$]AGGAG)-3′; yield 27%, MALDI-TOF mass $[M-H]^+$ calcd for 3460.7, found 3460.1.
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