Bioorganic & Medicinal Chemistry 17 (2009) 1398–1403

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis of oligodeoxynucleotides incorporating 2-*N*-carbamoylguanine and evaluation of the hybridization properties

Takeshi Sasami, Yoko Odawara, Akihiro Ohkubo, Mitsuo Sekine *, Kohji Seio *

Department of Life Science, Tokyo Institute of Technology, 4259 Nagatsuta, Yokohama 226-8501, Japan

ARTICLE INFO

Article history:
Received 29 October 2008
Revised 14 November 2008
Accepted 15 November 2008
Available online 24 November 2008

Keywords:
Modified guanosine
Decarbamoylation
DNA duplex stability
Base discrimination

ABSTRACT

Previously, we reported 2-*N*-carbamoylguanine (cmG) as a guanine analog. We further studied the synthetic protocol and hybridization properties of oligodeoxynucleotides (ODNs) incorporating cmG. These ODNs were synthesized using the phosphoramidite of cmG without protection of the 6-*O* position. However, the isolated products were contaminated with deacylated products having guanine in place of cmG. The detailed analysis of the synthetic process suggested that the deacylation resulted from the reaction of the carbamoyl moiety with capping reagents. Protection of the 6-*O* position suppressed the side reaction. The thermal stability of the DNA duplexes incorporating cmG was analyzed. An analysis of T_m values revealed that the base discrimination ability of cmG was comparable to or higher than that of the canonical guanine depending on the flanking bases.

1. Introduction

Oligodeoxynucleotides (ODNs) form double strands not only using canonical Watson–Crick base pairs but also using stable mismatch base pairs.[1] Among the mismatch base pairs, G-T,[2] G-A,[3,4] and G-G[5,6] mismatch base pairs are rather stable in canonical B-type duplexes. In the case of technologies utilizing DNA–DNA hybridization, such as DNA microarrays, accurate hybridization to the complementary sequence must proceed without forming stable mismatch base pairs.[7,8] To avoid formation of these mismatch base pairs, several research groups have reported modified nucleic acids capable of selective base discrimination.[9–11]

As one of such modified nucleobases, we have reported 2-*N*-carbamoyl guanine (cmG).[10] The base-pairing properties of cmG were evaluated by analyzing the T_m of 5′-(CGGC[cmG]AGGAG)-3′/3′-(GCCG[Y]TCCTC)-5′ where Y was C, G, T, or A opposite cmG. The cmG formed the most stable base pair when Y was C and the most stable mismatch base pair when Y was T. In comparison to the canonical guanine, the cmG formed a stable base pair with C as Y whereas the Wobble-type cmG-T mismatch was destabilized. Thus, it was suggested that the cmG had more selective base recognition ability than the canonical guanine.

In this study, we first attempted to improve the synthetic route to the cmG phosphoramidite derivative and the original conditions for the synthesis of ODNs incorporating cmG. The new route tested in this study included using phosphoramidite **2** lacking the protection of the 6-*O* position of the guanine ring by a diphenylcarbamoyl (Dpc) group[12] in place of the previously reported phosphoramidite **1** (Fig. 1). However, as discussed later, the detailed synthetic study revealed that the 6-*O*-protection by Dpc was necessary to prevent the side reaction of the carbamoyl moiety with an acid anhydride used as the capping reagent. We also evaluated the base-pairing properties of cmG in various sequences to clarify the sequence dependence of the base pairing of cmG. The results shown in this paper are important for the future utilization of ODNs incorporating cmG as hybridization probes[13,14] and nucleic acid drugs[15,16] from the view point of ODN synthesis and DNA hybridization.

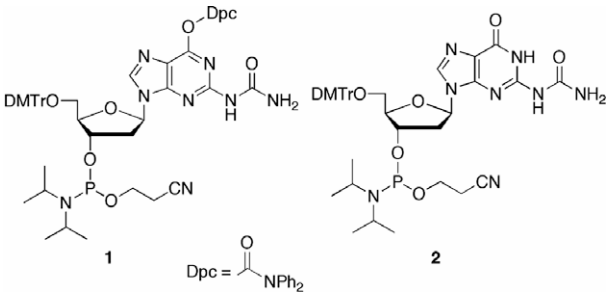
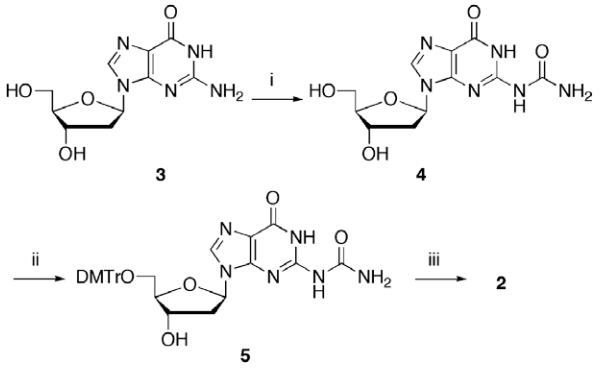


Figure 1. Previously reported phosphoramidite **1** and 6-*O*-unprotected **2**.

* Corresponding authors. Tel.: +81 45 924 5706; fax: +81 45 924 5772.
E-mail addresses: msekine@bio.titech.ac.jp (M. Sekine), kseio@bio.titech.ac.jp (K. Seio).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.11.042



Scheme 1. Reagents and conditions: (i) a—TMSCl (5.0 equiv), pyridine, rt, 1 h; b—phenyl chloroformate (1.5 equiv), rt, 5 h; c—aqueous ammonia (10.0 equiv), pyridine, rt, 1 h, 55%; (ii) DMTrCl (1.5 equiv), pyridine (5.0 equiv), DMF, rt, 4 h, 84%; (iii) (CEO)P[N(*i*-Pr)$_2$]$_2$ (1.3 equiv), 1*H*-tetrazole (0.6 equiv), (*i*-Pr)$_2$NH (0.6 equiv), CH_2Cl_2, rt, 2 h, 90%.

2. Results and discussion

2.1. Synthesis of the 2-*N*-carbmoyl guanine phosphoramidite 2

The synthesis of phosphoramidite **2** from deoxyguanosine (**3**) is shown in Scheme 1. First the hydroxyl group of **3** was transiently protected[17,18] with the trimethylsilyl group, and the acylation of the 3′,5′-*O*-disilylated guanine intermediate derivative was carried out using phenyl chloroformate (1.5 equiv). Subsequently, the 2-*N*-phenoxycarbonyldeoxyguanosine derivative was converted to the carbamoyl derivative **4** without purification by treatment with aqueous ammonia (10 equiv). The reaction of **4** with DMTrCl (1.5 equiv) in DMF formed the 5′-DMTr derivative **5** in 84% yield. DMF was used as the solvent because of the poor solubility of **4** in pyridine. The 5′-DMTr derivative **5** was further converted to phosphoramidite **2** according to the usual phosphitylation procedure.[19] Thus, cmG phosphoramidite **2** was successfully prepared in only three purification steps without 6-*O*-protection.

2.2. ODN synthesis using phosphoramidite 2

We synthesized **ODN1**, 5′-d(TACTA[cmG]CTCAT)-3′, using **2** on an automated DNA synthesizer. The chain elongation cycle consisted of (i) detritylation, (ii) coupling, (iii) capping with phenoxyacetic anhydride (Pac_2O), and (iv) iodine oxidation.[20] After the final chain elongation, the ODN having the terminal DMTr group was cleaved from the solid support by treatment with aqueous ammonia, and the base- and phosphate-protecting groups were removed in the same solution. Figure 2 shows the anion-exchange HPLC profile of the crude product obtained after the ammonia treatment. The desired **ODN1** incorporating cmG was obtained as the major product showing the retention time of 21 min (Fig. 2A). However, a significant amount of side product was also observed at the retention time of 23 min. The side product was proved to be 5′-d(TACTA G CTCAT)-3′, which lacked the carbamoyl moiety, on the basis of coinjection with the standard sample synthesized independently (Fig. 2D). Because we previously reported the synthesis of the same ODN containing cmG without such a side reaction,[10] the unexpected removal of the carbamoyl moiety could not result from its simple ammonolysis. The only difference between the previously reported synthetic protocol and the current one is the structure of the phosphoramidites, that is, the 6-*O*-protected derivative **1** in the former protocol and the unprotected species **2** in the current protocol. Therefore, we expected that the removal of the carbamoyl moiety could include some side reactions that occur only in the absence of 6-*O*-protection, and tried to clarify the side reactions.

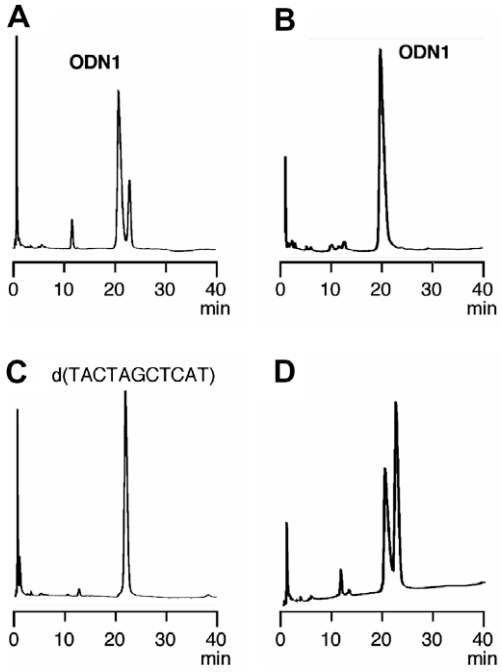
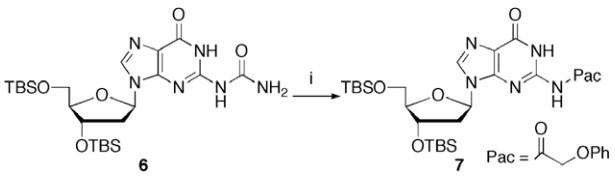


Figure 2. Anion-exchange HPLC analysis; 30 °C, 0.75 ml/min 20–22% B in 40 min. (A) Crude **ODN1** synthesized using **2**, (B) **ODN1** synthesized using 6-*O*-protected **1**, (C) 5′-d(TACTAGCTCAT)-3′, and (D) mixture of **ODN1** synthesized from **2** and 5′-d(TACTAGCTCAT)-3′. The peak near 0 min is the injection peak.

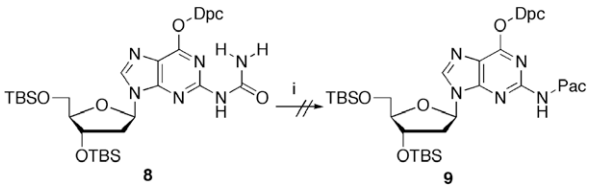
2.3. Side reaction of the 2-*N*-carbmoyl guanine moiety resulting from acylated regents

It is well known that the amino group of the carbamoyl moiety can react with some acylating agents such as acyl chloride[21,22] and acid anhydride.[23] Therefore, the most plausible side reaction is the reaction between the acylating agent used in the capping step and the amino group of the carbamoyl moiety. We then examined the reactivity of Pac_2O toward the carbamoyl moiety at the nucleoside level.

The 3′,5′-*O*-disilylated 2-*N*-carbmoyl guanine derivative **6** was treated with 4.0 equiv of phenoxyacetic anhydride in pyridine (Scheme 2). The TLC analysis of the reaction mixture indicated the formation of a new product, which was purified after 10 h. Structure assignment by ^{1}H NMR revealed the unexpected formation of the 2-*N*-phenoxyacetyldeoxyguanosine derivative **7** contaminated with phenoxyacetamide, which could not be separated by column chromatography. This result explains the previously mentioned removal of the carbamoyl moiety from the ODN, because if a similar exchange of the carbamoyl moiety with a Pac group should occur in the ODN synthesis, the Pac group attached to the 2-amino group would be removed by the subsequent ammonia treatment to produce the unmodified guanine residue. In addition, we carried out a similar experiment using the 3′,5′-*O*-disilylated 6-*O*-diphenylcarbamoyl derivative **8**, and confirmed that the Dpc-derivative did not react even after 48 h (Scheme 3).



Scheme 2. Reagents and conditions: (i) Pac_2O (4.0 equiv), pyridine, rt, 10 h.



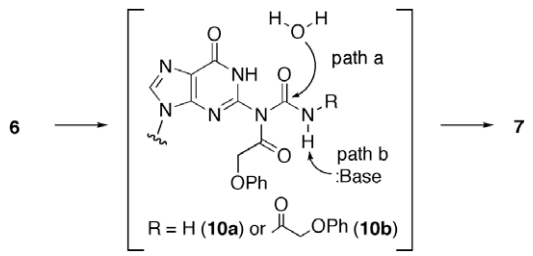
Scheme 3. Reagents and conditions: (i) Pac_2O (4.0 equiv), pyridine, rt, 2 d, no reaction.

The plausible mechanism of the acyl exchange reaction is shown in Scheme 4. The reaction of the 2-*N*-carbamoylguanine moiety with Pac_2O could yield the 2-*N*-Pac derivatives **10a** and **10b** having an additional Pac group at the amino group of the carbamoyl moiety. This acylation of the 2-*N* position changed the original property of the 2-amino group of the guanine moiety to a good leaving group for the subsequent hydrolysis (path a) or elimination (path b). Sugai et al. have reported a similar *N*-decarbamoylation by which *N*-carbamoylamino acylates were converted to *N*-acylated derivatives.[23] The first acylation of the 2-amino group is similar to the reaction reported by Greenberg et al. in which the conversion of the Pac protecting group at the amino group of guanine was exchanged with the acetyl group during the capping process.[24]

The 6-*O*-Dpc group of **8** could contribute to the suppression of side reactions in several ways. First, the amino group of the carbamoyl moiety could form an intramolecular hydrogen bond at the N1 or N3 position. Such a hydrogen bond might reduce the reactivity of this amino group toward Pac_2O, and the elimination by path b might be suppressed. In addition, the presence of the electron-withdrawing Dpc group could reduce the reactivity at the 2-*N* position toward the first acylation.

2.4. Synthesis of d(cmGT)

The previously mentioned liquid-phase experiment indicated the possibility of elimination of the carbamoyl moiety during the capping reaction. Next, we tested this possibility in the solid phase by synthesizing a dimer composed of 2-*N*-carbamoylguanine and thymidine, 5′-d(cmGT)-3′, on a DNA synthesizer. First, 6-*O*-protected cmG phosphoramidite **1** or the 6-*O*-unprotected counterpart **2** was coupled to the thymidine residues on the solid supports. After the standard capping, oxidation, and detritylation steps, the dimer was subjected to additional 10 or 20 cycles of capping, oxidation, and detritylation. The capping regent used here was a mixture of solutions A and B, where solution A was 5% (w/v) phenoxyacetic anhydride and 10% (v/v) pyridine in tetrahydrofuran, and solution B was 16% (v/v) 1-methylimidazole in tetrahydrofuran. The volume ratio of solutions A and B is determined by the hardware of the DNA synthesizer and is not known. The capping reaction time was set up to be 1 min. After the 10 and 20 cycles, the products were released from the solid supports by treatment with aqueous ammonia at ambient temperature for 8 h. For comparison, the dimer was also synthesized using phosphoramidite **2** without the additional capping-oxidation-detritylation cycles. The crude product was analyzed by reversed-phase HPLC (Fig. 3).



Scheme 4. Two plausible pathways for the removal of the carbamoyl moiety.

The dimer, d(cmGT), synthesized using **2** without additional capping–oxidation–detritylation cycles was observed at a retention time of 19 min with high purity (Fig. 3A). On the other hand, the dimer synthesized using **2** with an additional 20 cycles (Fig. 3C) was contaminated with d(GT), the structure of which was confirmed by coinjection with the standard sample of d(GT) (Fig. 3D) synthesized independently. Therefore, it was confirmed that the side reaction with phenoxyacetic anhydride occurred in both the liquid- and solid-phase conditions.

For comparison, we analyzed d(cmGT) synthesized using 6-*O*-peotected phosphoramidite **1** under the same conditions, including 20 additional capping–oxidation–detritylation cycles. The use of **1** suppressed the side reaction by the capping regents (Fig. 3B); this result is agreement with that of the liquid-phase experiment described previously.

2.5. Synthesis of oligonucleotides using phosphoramidite 1

The previously mentioned experiments clearly suggested that 6-*O*-protected phosphoramidite **1** was more suitable for the synthesis of ODNs incorporating cmG. Thus, the ODNs incorporating cmG were synthesized on an automated DNA synthesizer with phosphoramidite **1**. Previously, we had synthesized an ODN having 5′-d(CGGC[cmG]AGGAG)-3′ sequence and had studied the thermal stability of cmG-C, cmG-A, cmG-G, and cmG-T base pairs.[10] In short, the base recognition of cmG was somewhat higher than that of G, and the base-pairing ability of cmG was almost the same as G. The mismatched cmG-T pair was destabilized compared with the G-T Wobble mismatch. However, it is well known that the stability of a base pair is affected by the nearest neighbor base pairs,[2] and more T_m data

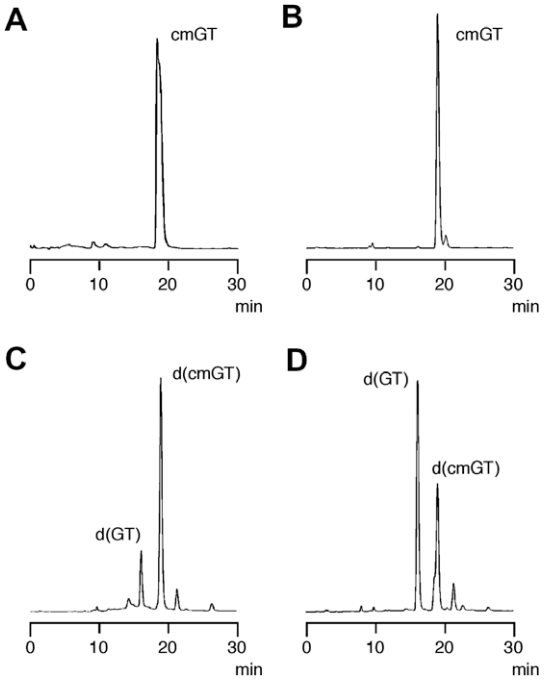


Figure 3. Reversed phase of (A) d(cmGT) using **2** without the additional cycles, (B) d(cmGT) using **1** with 20 additional cycles, (C) d(cmGT) using **2** with 20 additional cycles, (D) mixture of the crude products shown in (C) and d(GT).

on the duplexes having different nearest neighbor bases flanking cmG was necessary to clarify the general properties of the base-pairing property of cmG. Therefore, we synthesized **ODN1**: 5′-d(TACTA[cmG]CTCAT)-3′, **ODN2**: 5′-d(TACTA[cmG]ATCAT)-3′, **ODN3**: 5′-d(TACTC[cmG]CTCAT)-3′ and **ODN4**: 5′-d(TACTC[cmG]ATCAT)-3′ having 5′-A[cmG]C-3′,5′-A[cmG]A-3′,5′-C[cmG]C-3′, and 5′-C[cmG]A-3′ sequences, respectively, near the center of the strands. We chose these sequences considering the stabilities of the G-T mismatch predictable by using the nearest neighbor parameters.[2] As described in Ref. 2, the general trend for the nucleotide at the 5′ side of the G base of a G-T mismatch in order of decreasing stability is C > G > T ⩾ A. On the 3′ side of the G base, the stability order is C ⩾ G > T ⩾ A.[2] Thus the above ODNs are designed to contain the most stable nearest neighbor combination **ODN3**: 5′-C[cmG]C-3′ and the least stable **ODN2:** 5′-A[cmG]A-3′**.**

2.6. Stability of the single mismatches in the DNA duplexes

We measured the thermal stability of the DNA duplexes containing **ODN1–ODN4** by UV melting analysis and evaluated the stabilities of the cmG-C and cmG-T base pairs using the complementary strands of **ODN1–ODN4** and the counter strands that form a single cmG-T mismatch. For reference, we measured the T_m of the duplexes having a canonical guanine in place of cmG. The stability of the Watson–Crick base pairs and the GT mismatches in the duplexes are shown in Table 1.

In the case of the duplexes containing **ODN1** that have a –A[cmG]C–/–TYG– sequence, the cmG base was paired with cytosine (Y = C) with a T_m of 40.2 °C, which was smaller by 1.0 °C than the T_m of the canonical G-C pair (41.2 °C). Similarly, the cmG of **ODN1** formed cmG-T with a T_m of 24.0 °C, while the canonical G-T base pair showed a T_m of 25.2 °C, which was higher by 1.2 °C. These results suggested that the cmG base showed base-pairing properties very similar to those of the canonical guanine base in the –A[cmG]C–/–TYG– sequence context.

To compare the base-pairing properties of cmG more quantitatively, we calculated ΔT_m as the difference between the T_m of the Watson–Crick base pair and that of the mismatch base pair. In addition, we also calculated $\Delta\Delta T_m$ as the difference between the ΔT_m of cmG and that of G. As a result, ΔT_m, which corresponds to the base selectivity of cmG in **ODN1** at 16.2 °C and that of G, at 15.9 °C. These values suggested almost identical base selectivity of cmG and G, as indicated by the very small $\Delta\Delta T_m$ of 0.3 °C.

The cmG base in **ODN3** having a –C[cmG]C–/–GYG– sequence also showed base-pairing properties similar to those of G, as indicated by the small $\Delta\Delta T_m$ of 0.5 °C.

On the other hand, the base-pairing properties of cmG in **ODN2** and **ODN4** are slightly different from those of **ODN1** and **ODN3**. For example, in the case of **ODN2**, the cmG base was paired with cytosine (X = C) with a T_m of 32.3 °C, which was lower by 2.1 °C than that of the corresponding canonical G-C base pair (T_m = 34.4 °C). By contrast, the cmG base of **ODN2** formed the cmG-T base pair with T_m = 12.9 °C, which was lower than that of the canonical G-T base pair (T_m = 16.4 °C) by 3.5 °C. These results indicated that the destabilization of the Watson–Crick base pair and the Wobble base pair by the carbamoyl modification became more significant in **ODN2** than in **ODN1** and **ODN3**. As a result, the $\Delta\Delta T_m$ of **ODN3** was calculated to be 1.4 °C, which was larger than those of **ODN1** (0.3 °C) and **ODN3** (0.5 °C). Interestingly, the base-pairing properties of cmG in **ODN4** were similar to those of **ODN2**, and were characterized by the rather large decrease in the cmG-C and cmG-T base pair stabilities, as well as the large $\Delta\Delta T_m$ of 2.2 °C.

The comparison of the bases flanking cmG in **ODN2** and **ODN4** indicated that the duplexes that showed a larger T_m decrease of the cmG-T base pair shared the 5′-cmGA-3′/3′-TT-5′ sequence. Because previously reported ^{1}H NMR studies[25] have suggested that the guanine residue of the G-T mismatch and the 3′-downstream base pair is well-stacked in 5′-GA-3′/3′-TT-5′, one of the reasons for the larger destabilization of the 5′-cmGA-3′/3′-TT-5′ sequence seemed to be the steric hindrance between the rather large carbamoyl moiety and the 3′-downstream bases.

Table 1
T_m values (°C) and the standard deviations[a] of 5′-d(TACTN^{1}XN^{2}TCAT)-3′/3′-d(ATGA-M^{1}YM^{2}AGTA)-5′

–N^{1}XN^2–/–M^{1}YM^2–	X	Y = C	Y = T	ΔT_m[b]	$\Delta\Delta T_m$[c]
–AXC–/–TYG–	cmG	40.2	24.0	16.2	0.3
	ODN1	(0.4)	(0.2)		
	G	41.2	25.2	15.9	—
		(0.3)	(0.2)		
–AXA–/–TYT–	cmG	32.3	12.9	19.4	1.4
	ODN2	(0.5)	(0.1)		
	G	34.4	16.4	18.0	
		(0.3)	(0.3)		
–CXC–/–GYG–	cmG	46.9	31.6	15.3	0.5
	ODN3	(0.4)	(0.2)		
	G	47.6	32.8	14.8	
		(0.5)	(0.1)		
–CXA–/–GYT–	cmG	41.8	24.1	17.7	2.2
	ODN4	(0.4)	(0.2)		
	G	43.7	28.2	15.5	
		(0.3)	(0.5)		

[a] In parentheses.
[b] ΔT_m was calculated as (T_m of Y = C) – (T_m of Y = T).
[c] $\Delta\Delta T_m$ was calculated as (ΔT_m of X = cmG) – (ΔT_m of X = G).

3. Conclusions

The 6-*O*-unprotected 2-*N*-carbamoyldeoxyguanosine 3′-phosphoramidite derivative was synthesized from 2′-deoxyguanosine via only three isolation steps. However, the 6-*O*-unprotected cmG residue was found to react with the phenoxyacetic anhydride used in the capping reaction. The side reaction was confirmed in the model experiments that were carried out both in the liquid phase and the solid phase in which the 2-*N*-carbamoyl guanine moiety was converted to the 2-*N*-phenoxyacetyl guanine moiety. However, use of the previously reported 6-*O*-protected phosphoramidite derivative suppressed the reaction with phenoxyacetic anhydride. Thus, although more chemical steps are required for the synthesis, the previously reported 6-*O*-protected phosphoramidite is more useful in practice than the 6-*O*-unprotected one.

The stabilities of the Wobble-type cmG-T and Watson–Crick-type cmG-C base pairs were evaluated in the duplexes incorporating cmG by UV melting experiments using oligonucleotides having four different sequences. Although the carbamoyl modification decreased the stability of the Watson–Crick and Wobble base pairs in all sequences tested, the decrease in the Wobble-type base pair was more significant in the sequence having a 5′-cmGA-3′/3′-TT-5′ sequence, and selectivity of the base recognition indicated by the $\Delta\Delta T_m$ values was relatively large in these sequences. This might be due partly to the steric hindrance between the carbamoyl moiety and the lower base pairs, which lowered the stability of the cmG-T base pair.

In conclusion, the results shown in this study provided the details of the synthesis and hybridization properties of the ODNs incorporating cmG, which are expected to be useful for the application of this modified nucleoside in oligonucleotide probes and nucleic acid drugs.

4. Experimental procedures

4.1. General procedures

The dry solvents were purchased and stored over molecular sieves 4A. ^{1}H, ^{13}C and ^{31}P NMR spectra were obtained at 500, 126 and 203 MHz, respectively. The chemical shifts were measured from tetramethylsilane (0.0 ppm) or DMSO-d_6 (2.49 ppm) for ^{1}H NMR, $CDCl_3$ (77.0 ppm), DMSO-d_6 (39.7 ppm) for ^{13}C NMR and 85% phosphoric acid (0.0 ppm) for ^{31}P NMR. MALDI-TOF and ESI-TOF mass spectra were obtained in the positive ion mode.

4.2. Synthesis of phosphoramidite unit

4.2.1. 2-*N*-Carbamoyl deoxyguanosine (4)

Deoxyguanosine **3** (1.0 g, 3.5 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine. The residue was suspended in dry pyridine (30 ml) was added trimethylsilyl chloride (1.8 ml, 21 mmol). The solution was stirred at ambient temperature 1 h, and then added phenyl chloroformate (660 μl, 5.3 mmol). The solution was stirred at ambient temperature 5 h. The solution was diluted with ethyl acetate (150 ml) and washed twice with brine (150 ml). The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was diluted with pyridine and added aqueous ammonia (5.0 ml, 35 mmol). The solution was concentrated under reduced pressure. The residue was purified by the precipitation from methanol (5 ml) to give the compound **4** (600 mg, 55%). ^{1}H NMR (DMSO-d_6) 2.22–2.27 (1H, m), 2.52–2.57 (1H, m), 3.49–3.56 (2H, m), 3.80 (1H, dd, J = 4.6 Hz, J = 7.6 Hz), 4.35 (1H, t, J = 2.6 Hz), 4.92 (1H, s), 5.29 (1H, s), 6.15 (1H, t, J = 6.7 Hz), 6.44 (1H, s br), 7.23 (1H, s br), 8.12 (1H, s), 10.01 (1H, s br), 11.99 (1H, s br): ^{13}C NMR (DMSO-d_6) 61.5, 70.5, 83.1, 87.7, 119.3, 137.0, 148.8, 148.9, 155.2, 156.2: MS m/z Calcd for $C_{11}H_{15}N_6O_5^+$: 311.1104, found 311.1088.

4.2.2. 2-*N*-Carbamoyl-5′-*O*-(4,4′-dimethoxytrityl) deoxyguanosine (5)

2-*N*-Carbamoyldeoxyguanosine **4** (1.4 g, 4.6 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine. The residue was suspended in dry DMF (35 ml). To this mixture was added pyridine (1.9 ml, 23 mmol) and 4,4′-dimethoxytrityl chloride (2.4 g, 7.0 mmol), and the mixture was stirred at ambient temperature for 12 h. The solution was diluted with ethyl acetate (250 ml) and washed twice with brine (200 ml) and once with saturated $NaHCO_3$aq (200 ml). The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by the precipitation from ethyl acetate (5 ml) and *n*-hexane (5 ml) to give the compound **5** (2.4 g, 84%). ^{1}H NMR (DMSO-d_6) 2.31–2.35 (1H, m), 2.66–2.71 (1H, m), 3.10–3.17 (2H, m), 3.71 (6H, d, J = 1.7 Hz), 3.92 (1H, m), 4.39 (1H, dd, J = 5.3 Hz, J = 10.5 Hz), 5.33 (1H, s br), 6.20 (1H, t, J = 6.2 Hz), 6.77–6.82 (4H, m), 7.16–7.38 (9H, m), 7.99 (1H, s): ^{13}C NMR (DMSO-d_6) 55.0, 55.0, 64.0, 70.3, 83.0, 85.4, 85.8, 113.0, 113.1, 119.5, 126.6, 127.4, 127.7, 129.7, 129.1, 129.7, 135.5, 135.6, 136.9, 144.9, 148.8, 148.9, 156.2, 158.0, 158.0: MS m/z Calcd for $C_{32}H_{33}N_6NaO_7^+$: 635.2230, found 635.2229.

4.2.3. 2-*N*-Carbamoyl-5′-*O*-(4,4′-dimethoxytrityl)-deoxyguanosine 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (2)

Compound **5** (1.0 g, 1.6 mmol) was rendered anhydrous by coevaporation three times with dry pyridine and toluene and acetonitrile and finally dissolved in dry CH_2Cl_2 (16 ml). To this solution were added diisopropylamine (137 μl, 0.98 mmol), 1*H*-tetrazole (69 mg, 0.98 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (678 μl, 2.1 mmol). The solution was stirred at ambient temperature for 2 h, and then dissolved in ethyl acetate (150 ml), washed three times with $NaHCO_3$ (150 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 chloroform–methanol containing 0.5% triethylamine (97:3–96:4) to give **2** (1.2 g, 90%). ^{1}H NMR ($CDCl_3$-d_1) 1.05–1.32 (14H, m), 2.42 (1H, m), 2.56–2.67 (3H, m), 2.91 (4H, m), 3.25–3.37 (2H, m), 3.51–3.87 (8H, m), 4.20–4.23 (1H, m), 4.69 (1H, m), 5.81 (2H, br), 6.32 (1H, m), 6.70–6.78 (4H, m), 7.12–7.37 (9H, m), 7.80 (1H, m), 8.89 (1H, br) 10.00–12.00 (1H, br): ^{13}C NMR ($CDCl_3$-d_1) 18.6, 19.5, 20.4, 20.5, 20.6, 20.8, 23.3, 24.7, 24.8, 29.5, 29.9, 40.4, 43.5, 45.7, 47.1, 55.4, 55.5, 55.5, 58.3, 58.5, 58.6, 63.6, 63.7, 83.7, 85.6, 85.8, 86.7, 86.7, 113.2, 113.4, 117.9, 118.1, 120.0, 120.1, 127.2, 128.1, 128.4, 128.5, 130.3, 135.8, 135.8, 135.8, 135.9, 136.0, 144.7, 149.2, 156.8, 158.7, 158.8: ^{31}P NMR ($CDCl_3$-d_1) 149.9: MS m/z Calcd for $C_{41}H_{50}N_8O_8P^+$: 813.3489, found 813.3712.

4.3. 3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-2-*N*-phoxyacetyl-deoxyguanosine (7)

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-2-*N*-carbamoyldeoxyguanosine **6** (200 mg, 0.37 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine. The residue was suspended in dry pyridine (3.7 ml). To this mixture was added phenoxyacetic anhydride (425 mg, 1.5 mmol), and the mixture was stirred at ambient temperature for 24 h. The solution was diluted with ethyl acetate (50 ml) and washed twice with brine (50 ml) and once with saturated $NaHCO_3$aq (50 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 (70:30) to give mixture of **6** and phenoxyacetamide (180 mg, 69%, molar ration of **6** and phenoxyacetylamide was 2:1). ^{1}H NMR (DMSO-d_6) 0.03 (6H, s), 0.10 (6H, s), 0.86 (9H, s), 0.88 (9H, s), 2.29–2.34 (1H, m), 2.69–2.75 (1H, m), 3.31–3.73 (2H, m), 3.84 (1H, d, J = 6.3 Hz), 4.51 (1H, d, J = 2.3 Hz), 4.84 (2H, s), 6.20 (1H, d, J = 6.6 Hz), 6.93–6.99 (3H, m), 7.27–7.32 (2H, m), 8.21 (1H, s), 11.78 (1H, br): ^{13}C NMR (DMSO-d_6) −5.5, −5.5, −5.0, −4.8 17.7, 18.0, 25.7, 25.9, 62.6, 66.3, 72.0, 82.7, 87.3, 114.6, 120.5, 121.4, 129.6, 137.4, 147.3, 148.3, 154.9, 157.6, 170.9: MS m/z Calcd for $C_{30}H_{48}N_5O_6Si_2^+$: 630.3143, found 630.3193.

4.4. Oligonucleotide synthesis

The ODNs incorporating a 2-*N*-carbamoyl deoxyguanosine was synthesized on an Applied Biosystems 394 automated DNA/RNA synthesizer using the standard 1.0 mol phosphoramidite cycle of detritylation, coupling, capping and iodine oxidation. The phosphoramidites **1** and **2** were dissolved in dry acetonitrile so that the final concentration became 0.1 M. The oligonucleotides were cleaved from the CPG supports and the protecting groups were removed by treatment with aqueous ammonia for 8 h. The solution containing the DMTr-ON oligonucleotides thus obtained were placed on the C 18 cartridge column and the failure sequences were eluted by use of 10% CH_3CN/0.1 M ammonium acetate as an eluent. After being washed with 0.1 M ammonium acetate and water, the column was treated with aqueous 2% TFA to remove DMTr group, washed with 0.1 M ammonium acetate and water. The target oligonucleotide was eluted by use of 20% CH_3CN/water. Pure material was obtained by use of the C18 cartridge column to give the pure oligonucleotide after being lyophilized to dryness. The yield of the pure material was calculated by assuming the molar extinction coefficients. The structures were confirmed by MALDI-TOF mass spectroscopy.

4.5. HPLC analysis of the oligonucleotides

Analysis of the dimer nucleotides was performed by reverse phase chromatography on the C18 column (4.6 × 150 mm) at 30 °C. Flow rate: 1 ml/min; eluant A: 30 mM ammonium acetate buffer; B: CH_3CN; gradient: 0–10% B in A within 30 min; UV detection at 254 nm. Analysis of the 11 mer oligonucleotides was performed by anion-exchange chromatography on the Gen-Pak FAX column (4.6 × 100 mm) at 30 °C. Flow rate: 0.75 ml/min; eluant A: 20 mM sodium phosphate buffer, 10% CH_3CN (v/v); eluant A: 0.1 M NaCl and 20 mM sodium phosphate buffer, 10% CH_3CN (v/v); gradient: 20–20% B in A within 40 min; UV detection at 254 nm.

4.6. UV measurement

Each duplex 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 solution was separated into quartz cells (10 mm) and incubated at 80 °C. After 1100 s, the solution was cooled to 10 °C at the rate of 0.5 °C/min and heated to 80 °C at the same rate. During this annealing and melting, the absorption at 260 nm was recorded at every 1 °C and used to draw UV-melting curves. The T_m value was calculated as the temperature that gave the maximum of the first derivative of the UV-melting curve.

Acknowledgments

This study was supported by Industrial Technology Research Grant Program in '05 from the New Energy and Industrial Technology Development Organization (NEDO) of Japan. This study was also supported in by a Grant-in-Aid for Scientific Research (B) 20350074, and in part from Research for Promoting Technological Seeds of JST. This work was supported by a Grant-in-Aid for JSPS Fellows (20009483), and in part from Japan Society for the Promotion of Science (JSPS).

References and notes

1. SantaLucia, J., Jr.; Allawi, H. T.; Seneviratne, P. A. *Biochemistry* **1996**, *35*, 3555.
2. Allawi, H. T.; SantaLucia, J., Jr. *Biochemistry* **1997**, *36*, 10581.
3. Allawi, H. T.; SantaLucia, J., Jr. *Biochemistry* **1998**, *37*, 2170.
4. Ebel, S.; Lane, A. N.; Brown, T. *Biochemistry* **1992**, *31*, 12083.
5. Skelly, J. V.; Edwards, K. J.; Jenkins, T. C.; Neidle, S. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 804.
6. Faibis, V.; Cognet, J. A. H.; Boulard, Y.; Sowers, L. C.; Fazakerley, G. V. *Biochemistry* **1996**, *35*, 14452.
7. Timofeev, E.; Mirzabekov, A. *Nucleic Acids Res.* **2001**, *29*, 2626.
8. Kurian, K. M.; Watson, C. J.; Wyllie, A. H. *J. Pathol.* **1999**, *187*, 267.
9. Seio, K.; Sasami, T.; Ohkubo, A.; Ando, K.; Sekine, M. *J. Am. Chem. Soc.* **2007**, *129*, 1026.
10. Sasami, T.; Odawara, Y.; Ohkubo, A.; Sekine, M.; Seio, K. *Tetrahedron Lett.* **2007**, *48*, 5325.
11. Shoda, K.; Okamoto, I.; Wada, T.; Seio, K.; Sekine, M. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1795.
12. Kamimura, T.; Tsuchiya, K.; Urakami, K.; Koura, K.; Sekine, M.; Shinozuka, K.; Miura, K.; Hata, T. *J. Am. Soc. Chem.* **1984**, *106*, 4552.
13. Nelson, B. P.; Grimsrud, T. E.; Liles, M. R.; Goodman, R. M.; Corn, R. M. *Anal. Chem.* **2001**, *73*, 1.
14. Palecek, E.; Fojta, M.; Jelen, F. *Biochemistry* **2002**, *56*, 85.
15. Kurreck, J.; Wyszko, E.; Gillen, C.; Erdmann, V. A. *Nucleic Acids Res.* **2002**, *30*, 1911.
16. Agrawal, S. *Biochim. Biophys. Acta* **1999**, *1489*, 53.
17. Ti, G. S.; Gaffney, B. L.; Jones, R. A. *J. Am. Chem. Soc.* **1982**, *104*, 1316.
18. Fan, Y.; Gaffney, B. L.; Jones, R. A. *Org. Lett.* **2004**, *6*, 2555.
19. Sinha, N. D.; Biernat, J.; Koster, H. *Tetrahedron Lett.* **1983**, *24*, 5843.
20. Beaucage, S. L.; Bergstrom, D. E.; Glick, G. D.; Jones, R. A. John Wiley & Sons, Inc.: New York, 2000.
21. Vansdadia, R. N.; Roda, K. P.; Parekh, H. *J. Inst. Chem.* **1989**, *61*, 65.
22. Heinicke, G.; Hung, T. V.; Prager, R. H.; Ward, A. D. *Aust. J. Chem.* **1984**, *37*, 831.
23. Suzuki, M.; Nagasawa, C.; Sugai, T. *Tetrahedron* **2001**, *57*, 4841.
24. Zhu, Q.; Delaney, M. O.; Greenberg, M. M. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1105.
25. Allawi, H. T.; SantaLucia, J., Jr. *Nucleic Acids Res.* **1998**, *26*, 4925.