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Synthesis and properties of oligodeoxynucleotides containing 5-carboxy-2′-deoxycytidines

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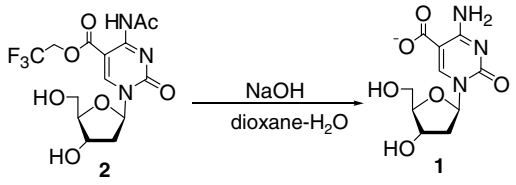
Abstract—5-Carboxy-2′-deoxycytidine (dC^{COO^-}) was synthesized as an anion-carrier to seek a new possibility of modified oligodeoxynucleotides capable of stabilization of duplexes and triplexes. The base pairing properties of this compound were evaluated by use of ab initio calculations. These calculations suggest that the Hoogsteen-type base pair of dC^{COO^-}-G is less stable than that of the canonical C^+-G pair and the Watson–Crick-type base pair of dC^{COO^-}-G is slightly more stable than the natural G-C base pair. The modified cytosine base showed a basicity similar to that of cytosine (pK_a 4.2). It turned out that oligodeoxynucleotides 13mer and 14mer incorporating dC^{COO^-} could form duplexes with the complementary DNA oligomer, which were more stable than the unmodified duplex. In contrast, it formed a relatively unstable triplex with the target ds DNA.

Sequence selective double helix formation of DNA is an essential step in various techniques of gene analysis such as DNA chip/microarrays,[1] molecule beacon,[2] and TaqMan PCR,[3] and in the gene regulation such as antisense and siRNA strategies.[4] Moreover, the triplex formation of DNA is another useful property of DNA which can be used in the antigene strategy and gene targeting techniques.[5–9] Over the past two decades, a considerable number of studies have been reported on the synthesis of artificial DNAs incorporating modified nucleosides that can stabilize such supramolecular structures of DNA. One of the strategies to stabilize DNA duplexes and triplexes is to introduce cationic substituents into the base or sugar moieties to reduce the electrostatic repulsions between their internucleotidic phosphate groups. On the other hand, few papers have appeared on the synthesis of oligonucleotide derivatives with anionic substitutions probably because it has been considered that such a modification destabilized duplexes due to additional electrostatic repulsion between the phosphate group and the anionic substituent.[10–12] In this paper, we report the synthesis and properties of oligodeoxynucleotides incorporating 5-carboxy-2′-deoxycytidine (**1**: dC^{COO^-}) which was expected to exist in a mono-anionic form at pH 7.0.

Matsuda et al. have previously reported the hybridization properties of oligodeoxynucleotides with *N*-alkylcarbamoyldeoxycytidine. They synthesized a key phosphoramidite building block via 5-(trifluoroethoxycarbonyl)-2′-deoxycytidine **2** for the synthesis of these modified oligomers.[13] To estimate the acidity of compound **1**, compound **2** synthesized according to Matsuda's procedure was hydrolyzed by treatment with 0.1 M NaOH in dioxane–H_2O (1:1, v/v) for 2 h at room temperature (Scheme 1).[14] The acidic and basic properties of **1** were examined by monitoring the pH dependence of its UV absorbance at 300 nm, as shown in Figure 2. The UV-titration curve showed a biphasic transition between the mono-anionic species **1** (dC^{COO^-}) and the zwitterion species **3** ($dC^{COO^-}+$) (Fig. 1) in the pH range of 2.0–9.0. Thus, compound **1**



Scheme 1. The structures of 5-carboxy-2′-deoxycytitidine **1** and its precursor **2**.

Keywords: Modified oligodeoxynucleotide; 5-Substituted cytosine; Triplex formation; Duplex formation; T_m experiments.
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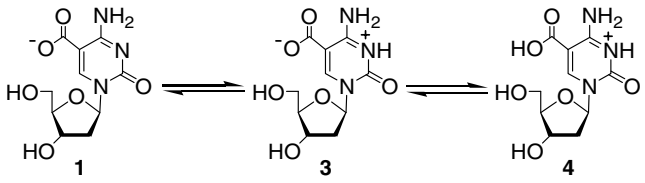

Figure 1. Possible ionic species **3**–**4** of compound **1**.

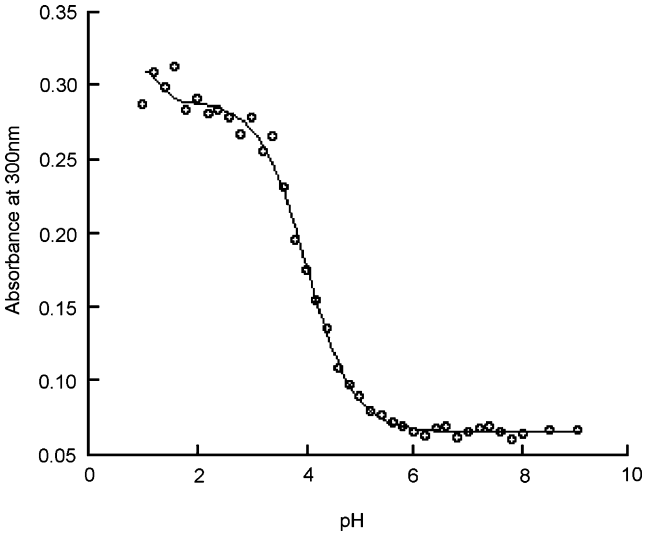

Figure 2. pK_a value measured under the following condition; 10 mM citric acid, 250 mM NaCl, 10 mM $MgCl_2$ (pH 1.0–4.8) and 10 mM cacodylate, 250 mM NaCl, 10 mM $MgCl_2$ (pH 5.0–9.0).

was estimated to have a pK_a value of 4.0 which corresponds to that of the N^3-protonated species **3**. The zwitterion species **3** exists at around pH 2.0, although the protonated cationic form **4** (dC^{COOH}+) could not be observed at the pH range measured but might be present at a lower pH region (<1.0).[15–17]

It is well known that the amino group at the *ortho* position of benzoic acid, for example in the case of anthranilic acid, decreases the pK_a value of the original carboxylic acid by 2 units.[15] Because the pK_a of the cytosine N^3 position of **1** observed here was almost identical to that of 2′-deoxycytidine,[18,19] it seems that the carboxylate function at the 5 position of the cytosine ring does not affect the basicity of the cytosine ring.

The stability of the Watson–Crick base pair between the dissociated anionic form of 5-carboxy-1-methylcytosine ($m^1C^{COO^-}$) and 9-methylguanine (m^9G) was theoretically estimated by MO calculations at the MP2/6-31++G(2d, p)//HF/6-31G(d) level and compared with that of canonical base pair of 1-methylcytosine (m^1C) and m^9G. The hydrogen bond energy of the Watson–Crick-type base pair of $m^1C^{COO^-}$-m^9G (Fig. 3B) was calculated to be −30.05 kcal/mol, while the naturally occurring G-C base pair (Fig. 3A) shows the hydrogen bond energy of −27.07 kcal/mol. These results suggested that the modified base pair was more stable than the canonical base pair by 3.0 kcal/mol.

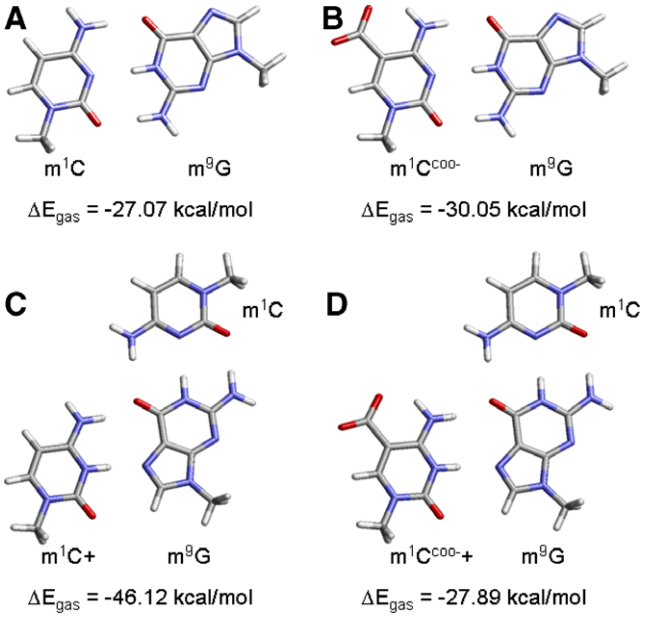

Figure 3. Calculated by Gaussian at the MP2/6-31++G(2d,p)//HF/6-31G(d) level.

The hydrogen bond energies of the Hoogsteen-type base pairs of the N^3-protonated m^1C (m^1C+) and $m^1C^{COO^-}$ ($m^1C^{COO^-}$+) with the m^1C-m^9G pair (Fig. 3C and D) were also estimated similarly. As a result, the formation of the natural-type C+ · G-C pair was accompanied by marked stabilization of −46.12 kcal/mol. The very large stabilization of natural-type C+ · G-C pair can be attributable to the molecular cation–dipole interactions.[20] In contrast, the zwitterionic form of C^{COO^-}+ could form the Hoogsteen base pair with G of G-C with the much smaller stabilization energy of −27.89 kcal/mol probably because of the cancellation of the stabilization effect of the positive charge at N^1 position of the cytosine by the carboxylate anion.

The theoretical analysis described here indicates that if dC^{COO^-} could be incorporated into a DNA duplex and the third strand of a DNA triplex, the modified nucleoside could stabilize the former and destabilize the latter as far as the base pairing modes were concerned.

Table 1. T_m and ΔT_m values (°C) for a DNA 14mer triplex

TFO (ODN **5**)
5′-TTTTTTXTTTCTTT-3′
5′-CAAAAAAGAAAGAAA C T T
3′-GTTTTTTCTTTCTTT G T T
hairpin duplex

pH	X = dC	X = dC^{COO^-} (ODN **5**)	ΔT_m
7.6	24	19	−5
7.0	28	24	−4
6.4	36	31	−5
5.8	43	39	−4

T_m values were measured under the following conditions; 10 mM sodium cacodylate (pH 5.8, 6.4, 7.0, and 7.6), 500 mM NaCl, 10 mM $MgCl_2$. ODNs 2 μM and 2 μM hairpin duplex. ΔT_m = (T_m of the modified triplex) − (T_m of the natural-type triplex).

In order to clarify the properties of oligodeoxynucleotides incorporating the modified deoxynucleoside **1**, the phosphoramidite building block was synthesized by a modification of the procedure reported by Matsuda et al.[13] and used for the synthesis of an ODN 14mer **5**: 5′-d(TTTTTT[C^{COO^-}]TTTCTTT)-3′ and ODN 13mers **7**–**9**: 5′-d(TA<u>X</u>GAC<u>X</u>CGT<u>X</u>AA)-3′ (<u>X</u> = C^{COO^-} or C) using a DNA synthesizer. Instead of the conventional treatment with aqueous ammonia, the fully protected oligonucleotide synthesized on highly cross-linked polystyrene beads was treated with 0.1 M NaOH in dioxane–H_2O (1:1, v/v) at room temperature for 5 h for removal of the protecting groups and for the cleavage from the solid supports. The completion of the deprotection was confirmed by anion-exchange HPLC (data not shown). The structures of ODNs **5**, **7**–**9** were characterized by MALDI-TOF-mass spectrometry.[21]

We carried out the thermal denaturation experiments of the modified ODN **5** as the TFO and the hairpin duplex (Table 1). The stabilities of the triplex were evaluated in cacodylate buffer of pH 5.8–7.6. For comparison, the unmodified triplex was also analyzed. As shown in Table 1, the T_m values of the unmodified triplex (X = dC) changed from 24 to 43 °C as the pH decreased. On the other hand, the modified triplex gave lower T_m values of 19–39 °C in the same pH range. At each pH, the modified triplex (X = dC^{COO^-}) was uniformly less stable than the unmodified one by 4–5 °C. Because the pK_a of the cytosine ring of dC^{COO^-} is almost identical to that of dC as described above, the T_m difference between the unmodified triplex and the modified triplex incorporating **1** could not be explained in terms of the basicity of the cytosine rings (Fig. 2). Therefore, the significant destabilization of the triplex should be due to the unexpected low hydrogen bond energy of the Hoogsteen-type base pair of the protonated form **4** of **1** and G of G-C, as shown in Figure 3.

Next, we carried out UV-melting experiments by use of modified ODN **5** and the complementary strand: 5′-d(AAAGAAAGAAAAAA)-3′. Three kinds of 10 mM sodium cacodylate buffers (pH 7.0) of different salt concentrations such as buffer 1 (1 M NaCl), buffer 2 (100 mM NaCl + 10 mM $MgCl_2$), and buffer 3 (100 mM NaCl) were tested. The results are shown in Table 2. The duplex incorporating **1** an additional anionic charge of the carboxylate anion at the range of pH 4.0–9.0 was as stable as or slightly more stable than the unmodified duplex under the experimental conditions tested. For example, in buffer 1 containing 1 M NaCl, the modified duplex's T_m (51 °C) was slightly higher than that (49 °C) of unmodified one by 2 °C. A similar trend was also observed in buffer 2 and buffer 3, although the T_m values were smaller than that observed in the 1 M NaCl buffer. As far as the T_m data obtained from the buffer 2 and buffer 3 solutions were compared, it seemed unlikely that the divalent cation, Mg^{2+}, has any unique effect other than the general salt effects. It should be noted that the difference in thermodynamic stability between the dC^{COO^-}-G pair and the canonical dC-dG pair is well correlated with the above-mentioned abinitio calculations which indicated higher stability of the dC^{COO^-}-G pair than the dC-G pair.

Next, we carried out UV-melting experiments by use of three kinds of modified ODNs **7**–**9** and the complementary strand: 5′-d(TTGACGGGTCGTA)-3′. These results are shown in Table 3. The one-point modified ODN **7** was more stable by 2 °C than the unmodified duplex under the experimental conditions tested. The two-point modified ODN **8** was more stable by 4 °C than the unmodified duplex. A similar addition effect with an increase of 7 °C was observed for the three-point ODN **9**. It turned out that the unmodified duplex was linearly stabilized to a degree of 2–3 °C per one modification. It should be noted that replacement of the G-C base pair with the anionic dC^{COO^-}-G base pair resulted in significant stabilization of the duplex.

We successfully introduced 5-carboxy-2′-deoxycytidine into the ODNs **7**–**9**. We found the modified TFO, ODN **5**, showed slightly lower affinity than the unmodified one toward the hairpin target. It seems that, since the triplex is more anionic than the duplex, the triplex was destabilized due to the anionic repulsion. In contrast, the ODNs **5**, **7**–**9** formed more stable duplexes than the unmodified counterpart with the complementary strand despite the presence of an additional anionic charge of the carboxylate residue. Such different behavior of ODN **5** in the DNA triplex and the duplex could be qualitatively predicted by the ab initio calculations of the Hoogsteen-type and Watson–Crick-type base pairs. Although there have been reported many chemical modifications aiming at the increase of the duplex stabilization, very few attempts have been made on the anionic substitutions in oligonucleotides. However, the results

Table 2. T_m and ΔT_m values (°C) for a DNA 14mer duplex

5′-TTTTTTXTTTCTTT-3′
3′-AAAAAAGAAAGAAA-5′

	X = dC	X = dC^{COO^-} (ODN **5**)	ΔT_m
buffer 1	49	51	+2
buffer 2	43	45	+2
buffer 3	25	26	+1

T_m values were measured under the following conditions: modified ODN 2 μM and 2 μM complementary ODN in 10 mM sodium cacodylate buffer (pH 7.0) containing 1 M NaCl (buffer 1), 100 mM NaCl + 10 mM $MgCl_2$ (buffer 2) or 100 mM NaCl (buffer 3). ΔT_m = (T_m of the modified duplex) − (T_m of the natural-type duplex).

Table 3. T_m and ΔT_m values (°C) for a DNA 13mer duplex

5′-TAXGACXCGTXAA-3′
3′-ATGCTGGGCAGTT-5′

	X = dC^{COO-}	T_m	ΔT_m
ODN **6**	5′-TACGACCCGTCAA-3′	65	—
ODN **7**	5′-TACGAC<u>X</u>CGTCAA-3′	67	+2
ODN **8**	5′-TA<u>X</u>GACCCGT<u>X</u>AA-3′	69	+4
ODN **9**	5′-TA<u>X</u>GAC<u>X</u>CGT<u>X</u>AA-3′	72	+7

T_m values were measured under the following conditions: ODN in 10 mM sodium cacodylate buffer (pH 7.0) containing 1 M NaCl (buffer 1). ΔT_m = (T_m of the modified duplex) − (T_m of the natural-type duplex).

in this study unexpectedly suggested that the dC^{COO-} introduced into polyanionic oligodeoxynucleotides did not destabilize the DNA duplexes. The conclusion reached in this study is that the appropriately designed anionic modification of oligonucleotides should not be necessarily avoided as far as the stable duplex formation is concerned, and is very informative for the design of artificial nucleic acids useful for various gene analysis techniques.

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14. *Spectroscopic and mass analysis data of compound* ***1.*** Compound**1**: ^{1}H NMR (DMSO) δ: 1.98–2.04 (1H, m), 2.22–2.27 (1H, m), 3.52–3.60 (2H, m), 3.84–3.86 (1H, q), 4.19–4.20 (1H, t), 4.99 (1H, s), 5.22 (1H, s), 6.06–6.08 (1H, d), 8.76 (1H, s), 13.0 (1H, br); ^{13}C NMR(DMSO) δ: 41.1, 61.0, 70.2, 86.2, 87.8, 95.1, 148.3, 153.4, 163.4, 166.6; MS (*m*/*z*) calcd for 272.0877 (M^++H), found 272.0876.
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