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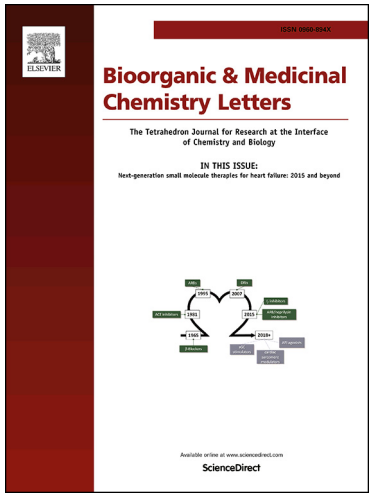

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Insight into the Recognition Mechanism of DNA Cytosine-5 Methyltransferases (DNMTs) by Incorporation of Acyclic 5-Fluorocytosine (^{F}C) Nucleosides into DNA

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ABSTRACT

DNA cytosine-5 methyltransferase (DNMT) catalyzes methylation at the C5 position of cytosine in the CpG sequence in double stranded DNA to give 5-methylCpG (mCpG) in the epigenetic regulation step in human cells. The entire reaction mechanism of DNMT is divided into six steps, which are scanning, recognition, flipping, loop locking, methylation, and releasing. The methylation and releasing mechanism are well-investigated; however, few reports are known about other reaction steps. To obtain insight into the reaction mechanism, we planned the incorporation of acyclic nucleosides, which make it easy to flip out the target nucleobase, into oligodeoxynucleotides (ODNs) and investigated the interaction between the ODN and DNMT. Here, we describe the design and synthesis of ODNs containing new acyclic 5-fluorocytosine nucleosides and their physiological and biological properties, including their interactions with DNMT. We found that the ODNs containing the acyclic 5-fluorocytosine nucleoside showed higher flexibility than those that contain 5-fluoro-2′-deoxycytidine. The observed flexibility of ODNs is expected to influence the scanning and recognition steps due to the decrease in helicity of the B-form.

DNA methylation has a profound effect on gene expression, particularly in gene promoter regions.[1,2] There are a number of repetitive DNA sequences, and some genes are hypermethylated, leading to silencing of the gene.[3-5] Unusual DNA methylation patterns are found in tumor cells, and their inhibition could be a promising target in anticancer drug development. Methylation at the C5 position of cytosine in a CpG sequence in double stranded DNA is catalyzed by DNA cytosine-5 methyltransferase (DNMT) to give 5-methylCpG (mCpG), using *S*-adenosyl-L-methionine (SAM) as a methyl group donor. The whole reaction of DNMT is shown in Figure 1A.[6,7] Genome DNA is associated with DNMT in a sequence independent manner and scans its recognition site (a: scanning). After finding its specific recognition site, DNMT forms a complex (b: recognition) and then makes a transition to flip out the complex (c: flipping). The resulting complex causes an induced fit to the loop locking conformation for the methylation reaction (d: loop locking). Then, the methylation reaction and releasing of the methylated DNA occur (e and f: methylation and releasing). The methylation and releasing mechanism have been investigated well, as shown in Figure 1B. First, the Glu residue in the catalytic pocket of DNMT protonates the nitrogen in the cytosine, and a thiol of the Cys residue in the active site acts as a nucleophile and attacks the C6 position of the cytosine to form an enzyme-linked intermediate. Then, the C5 position of the cytosine is methylated by SAM. Upon deprotonation of the C5 position, β-elimination of the added Cys residue occurs to complete the reaction. Contrary to the methylation and releasing steps, little is known about other reaction steps, which are scanning, recognition, flipping and loop locking (Figure 1A(a) to (d)).[8] Ring-opening of the ribose of the nucleoside and its incorporation into an oligodeoxynucleotide (ODN) increases the flexibility at the modification site and decreases the thermal stability of duplex.[9-11] The flexibility at the acyclic nucleotide site would easily flip out its nucleotide by DNMT (Figure 1A(c)), and the decrease in thermal stability of the modified ODN would influence the interaction of ODN and DNMT by the conformational change of ODN (Figure 1A(a) and (b)). To eliminate the discussion of the releasing step (Figure 1A(f)), which could cause concern with thermal stability of the duplex, 5-fluorocytosine (^{F}C) is used as a nucleobase in our study; 5-fluorocytosine works as one of the mechanism-based inhibitors of DNMT,[12,13] and the proposed mechanism is illustrated in Figure 1C. Methylation will occur in the same manner as natural cytosine methylation steps, the releasing step is blocked, and the corresponding intermediate is trapped. It is easy to discuss the complicated reaction steps by its simplification and detect the covalent-bonding ODN-DNMT complex using the difference of the molecular weight by

polyacrylamide gel electrophoresis (PAGE) and/or mass spectrum. Therefore, we planned the incorporation of new acyclic nucleosides, which make it easy to flip out from double stranded DNA, into ODNs and investigate the interaction between the ODN and DNMT. Here, we describe the synthesis of new acyclic nucleosides containing 5-fluorocytosine, the interaction between ODNs containing acyclic nucleosides and DNMT, and the physiological properties of modified ODNs. We also discuss the scanning, recognition and flipping mechanism

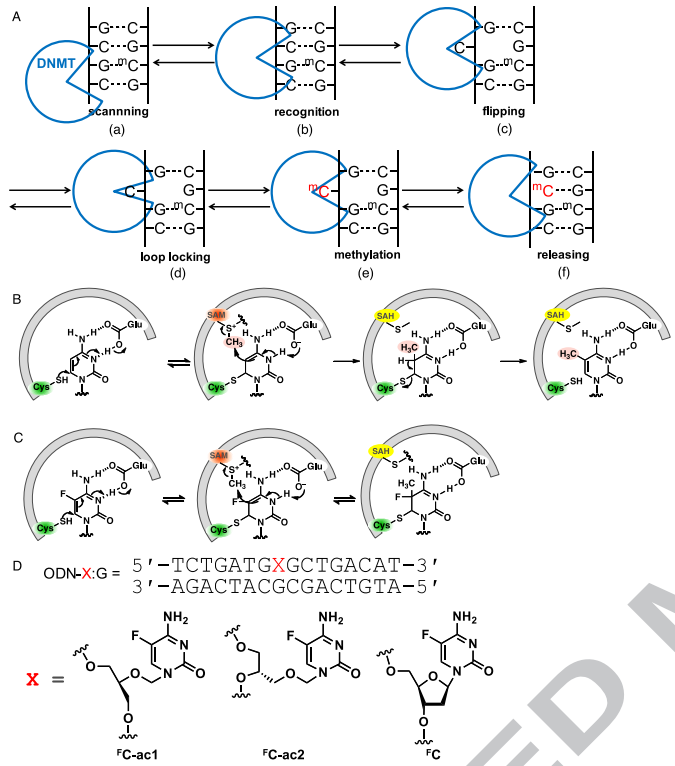


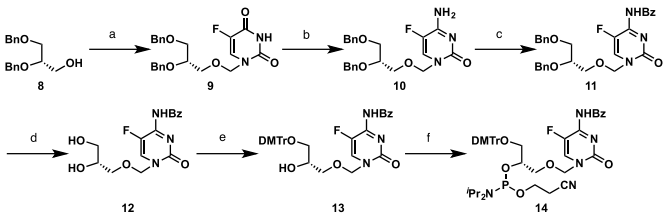
Figure 1. A) Models for the interactions between DNA and DNMT during methylation reaction. B) Proposed mechanism of cytosine methylation by DNMTs. C) Proposed mechanism of inhibition of DNMTs by 5-fluorocytosine (^{F}C). D) Sequences of synthesized ODNs containing ^{F}C-ac1, ^{F}C-ac2, and ^{F}C.

(Figure 1A(a), (b) and (c)) of DNMT.

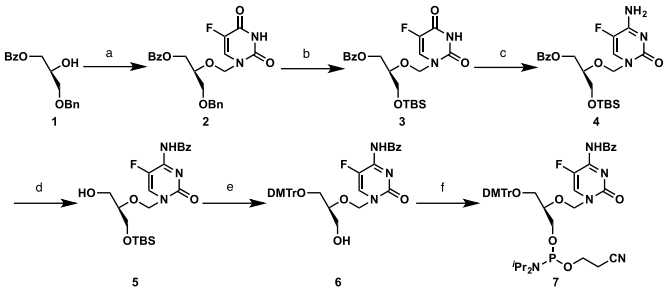
Results and Discussion

We designed ^{F}C-ac1 and 2 (Figure 1D) to investigate the recognition by DNMT based on the difference of the glycerol skeleton. ^{F}C-ac1 is a ganciclovir-type 2′-deficient acyclic nucleoside[14], which has the same number of bonds between 5′ and 3′, and a glycerol skeleton containing ^{F}C as a nucleobase for trapping DNMT. On the other hand, ^{F}C-ac2 is an analogue of 2′-nor-2′-deoxynucleoside[15], which has a short internucleotide linkage and a longer linker from the backbone to ^{F}C. These compounds were synthesized as presented in Schemes 1 and 2. The hydroxyl group of the protected glycerol derivative **1**[16] was chloromethylated, and then 5-fluorouracil derivative **2** was synthesized by Herdewijn's method.[17] The benzyl group of **2** was removed by Pd black and 1,4-cyclohexadiene under microwave irradiation. After protection of the resulting hydroxy group by the TBS group, the 5-fluorouracil moiety was converted to 5-fluorocytosine by the conventional method[18] to give **4**. The N^4 amino group of **4** was protected by the benzoyl group, and then selective removal of the benzoyl protection of hydroxyl group was carried out by NaOH in THF-H_2O to yield **5**. The primary alcohol of **5** was protected by the 4,4′-dimethoxytrityl (DMTr) group to produce **6**, and then **6** was converted to a phosphoramidite unit **7**. The phosphoramidite **14** was prepared as shown in Scheme 2 in a manner similar to the synthesis of **7**. Namely, the hydroxyl group of dibenzyloxy glycerol derivative **8**[19] was chloromethylated, and then 5-fluorouracil derivative **9** was synthesized. The 5-fluorouracil moiety was converted to 5-fluorocytosine to give **10**, and the N^4 amino group of **10** was protected by the benzoyl group to give **11**, and the benzyl groups were removed under the same conditions in Scheme 1 to yield **12**. The primary alcohol of **12** was protected by the 4,4′-dimethoxytrityl (DMTr) group to produce **13**, and then **13** was converted to phosphoramidite unit **14**. The control compound 5-fluoro-2′-deoxycytidine (d^{F}C) phosphoramidite unit was also synthesized using a previous method.[20]

The ODNs containing ^{F}C-ac1 and ^{F}C-ac 2 (ODN-^{F}C-ac1 and ODN-^{F}C-ac2) were prepared using DNA synthesizer (Figure 1D).



Scheme 2. Synthesis of phosphoramidite unit of ^{F}C-ac2 (**14**). Reaction conditions: a) i) $(CH_2O)_n$, 4 M HCl/dioxane, 0 °C ii) *N*,*O*-bis(trimethylsilyl)acetamide, $SnCl_4$, MeCN, 2 steps 56%; b) TPSCl, Et_3N, DMAP, MeCN, then *aq*. NH_4OH, 82%; c) BzCl, Et_3N, DMAP, CH_2Cl_2, quant; d) 1,4-cyclohexadiene, Pd black, MeCN, microwave 120 °C, 37%; e) DMTrCl, pyridine, 60%; f) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, *i*Pr_2NEt, DMAP, CH_2Cl_2, 80%.



Scheme 1. Synthesis of phosphoramidite unit of ^{F}C-ac1 (**7**). Reaction conditions: a) i) $(CH_2O)_n$, 4 M HCl/dioxane, 0 °C, ii) *N*,*O*-bis(trimethylsilyl)acetamide, $SnCl_4$, MeCN, 2 steps 58%; b) i) 1,4-cyclohexadiene, Pd black, MeOH, microwave 120 °C, ii) TBSCl, imidazole, CH_2Cl_2, 2 steps 88%; c) TPSCl, Et_3N, DMAP, MeCN, then *aq*. NH_4OH, 96%, d) i) BzCl, Et_3N, HOAt, CH_2Cl_2, ii) NaOH, THF, H_2O, 2 steps 35%; e) i) DMTrCl, pyridine, ii) TBAF, THF, 2 steps 60%; f) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, *i*Pr_2NEt, DMAP, CH_2Cl_2, 44%.

ODN-^{F}C was used for control experiments, and its complement ODN-G was also synthesized. To ascertain whether the methylation steps of ODN-^{F}C-ac1 and ODN-^{F}C-ac2 were the same as that of ODN-^{F}C, we first studied the interaction between ODN-^{F}C-ac1 or ODN-^{F}C-ac2 and a bacterial DNMT, M.HhaI, from *Haemophilus haemolyticus*. M.HhaI typically methylates the internal cytosine in the target sequence 5′-G**C**GC-3′; in our studies, ^{F}C-ac1 and ^{F}C-ac2 were substituted in place of the cytosine. Each double stranded ODN (ODN-^{F}C-ac1:G, ODN-^{F}C-ac2:G, and ODN-^{F}C:G) was incubated with 4 equiv. of M.HhaI at 37 °C for 120 min in the presence or absence of SAM. It was reported that ODNs containing ^{F}C instead of cytosine in a CpG sequence had a covalent complex formation with M.HhaI in the presence of SAM (Figure 1C).[21] ODN-^{F}C-ac1 and ODN-^{F}C-ac2 formed a stable complex with M.HhaI, mainly in the presence of SAM in denaturing polyacrylamide gel (Figure 2; lanes 3, 6 and 9), as well as ODN-^{F}C. Moreover, to investigate the mechanism of ODN-^{F}C-ac1 and ODN-^{F}C-ac2, the reversibility assay of the ODN-M.HhaI complex was carried out in Figure S1. The complexes were not disrupted by the addition of 100 equiv. of

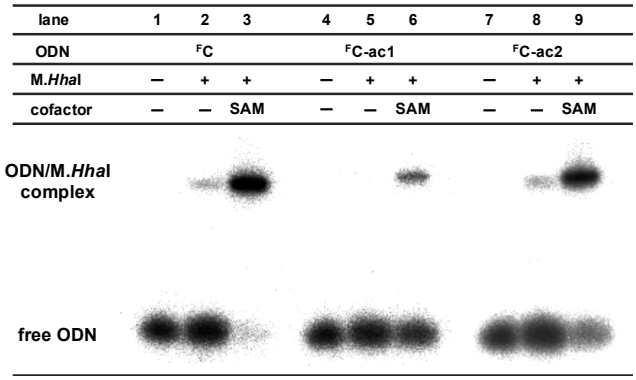


Figure 2. Electrophoretic mobility shift assay of ODN-^{F}C:G, ODN-^{F}C-ac1:G, and ODN-^{F}C-ac2:G with or without SAM: The reactions were performed with a 25 nM ODN and 240 μM SAM by incubating at 37 °C for 2 h with 100 nM M.HhaI in 50 mM Tris-HCl (pH 7.5), 10 mM EDTA and 5 mM β-mercaptoethanol, and analyzed by denaturing PAGE (8%, 140 V, 40 min). Lanes 1, 4 and 7; free ODN (control), lanes 2, 5 and 8; ODN without SAM, lanes 3, 6 and 9; ODN with SAM.

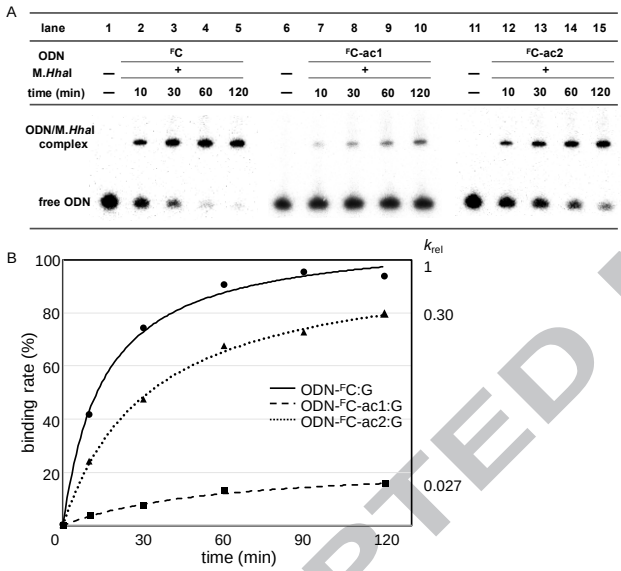


Figure 3. Electrophoretic mobility shift assay of ODN-^{F}C:G, ODN-^{F}C-ac1:G, and ODN-^{F}C-ac2:G: A) The reactions were performed with 25 nM ODN and 240 μM SAM by incubating at 37 °C for 0-120 min with 100 nM M.HhaI in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM NaCl, 5 mM β-mercaptoethanol, 5% glycerol, and analyzed by denaturing PAGE (8%, 140 V, 1 h). Lanes 1, 6 and 11; 0 min, lanes 2, 7 and 12; 10 min, lanes 3, 8 and 13; 30 min, lanes 4, 9 and 14; 60 min, lanes 5 10 and 15; 120 min. B) Time course of binding rate between ODN and M.HhaI and relative rate constants (k_{rel}); ODN-^{F}C:G (solid), ODN-^{F}C-ac1:G (dashed), ODN-^{F}C-ac2:G (dot).

non-labeled ODN after 24 h of incubation. These results showed that ODN-^{F}C-ac1 and ODN-^{F}C-ac2 formed a covalent complex with M.HhaI, which demonstrated the same finding as previously known for ODN-^{F}C. Therefore, ODN-^{F}C-ac1 and ODN-^{F}C-ac2 containing an acyclic nucleoside would form the covalent complex by the same reaction mechanism as ODN-^{F}C. From these results, ODN-^{F}C, ODN-^{F}C-ac1, and ODN-^{F}C-ac2 would share the same mechanism in methylation step (Figure 1A (e) and Figure 1C) and eliminate the discussion of releasing step (Figure 1A (f) and Figure 1C). The reactivities of ODN-^{F}C-ac1 and ODN-^{F}C-ac2 were lower than ODN-^{F}C. To assess the difference of their reactivities, the detailed time course of the ODN-M.HhaI complex formation was investigated in Figure 3. The reaction with ODN-^{F}C:G showed rapid and efficient formation of an ODN-M.HhaI complex, giving greater than 90% yield after 120 min reaction time (Figure 3A, lane 5). On the other hand, a band corresponding to a complex with M.HhaI was observed in 15% yield after 120 min for ODN-^{F}C- ac1:G (Figure 3A, lane 10), and in 79% yield after 120 min for ODN-^{F}C-ac2:G (Figure 3A, lane 15). The relative rate constant (k_{rel}) values of complex formation are shown in Figure 3B. ODN-^{F}C:G revealed the fastest complex formation, and ODN-^{F}C-ac1:G had a 37.0 times lower k_{rel} value (0.027). On the other hand, the k_{rel} value of ODN-^{F}C-ac2:G (0.30) was 11.1 times greater than that of ODN-^{F}C-ac1:G, and the value was still 3.3 times lower than ODN-^{F}C:G. To confirm what the difference of k_{rel} values depends on, we investigated the flexibility and the structure of ODN-^{F}C-ac1:G and ODN-^{F}C-ac2:G.

Table 1. T_m values and thermodynamic parameters of ODN-^{F}C:G, ODN-^{F}C-ac1:G and ODN-^{F}C-ac2:G.

5′-TCTGATGXGCTGACAT-3′
3′-AGACTACGCGACTGTA-5′

ODN	T_m (°C)	DT_m (°C)	$-DH°$(kJ/mol)	$-TDS°$(kJ/mol)	$-DG°$(kJ/mol)
ODN-^{F}C:G	66.1	—	414	331	82.9
ODN-^{F}C-ac1:G	61.4	– 4.7	340	271	69.5
ODN-^{F}C-ac2:G	62.1	– 4.0	379	303	75.5

To evaluate the flexibility of ^{F}C-ac1 and 2 in ODNs, T_m was measured (Table 1). ODN-^{F}C-ac1:G and ODN-^{F}C-ac2:G showed 61.4 and 62.1 °C as the T_m value, respectively. These values are 4.7 and 4.0 °C lower than those of ODN-^{F}C:G (66.1 °C). These results suggested that the flexibility of an acyclic nucleoside modification influenced the thermal stability of ODN duplexes entirely, and ODN-^{F}C-ac1 was suggested to be more flexible than ODN-^{F}C-ac2. The $-\Delta H°$, $-T\Delta S°$, and $-\Delta G°$ values of the unmodified ODN-^{F}C:G (414, 331 and 82.9 kJ/mol, respectively) were the highest of all. On the other hand, the $-T\Delta S°$ values of the ODN-^{F}C-ac1:G and ODN-^{F}C-ac2:G were 271 and 303 kJ/mol, respectively, which were lower than those of the unmodified one. The $-\Delta H°$ and $-\Delta G°$ values were also lower, and the results implied that the lower $-T\Delta S°$ was due to the flexibility of ODN compensating the unfavorable disadvantage in $-\Delta H°$. When comparing ODN-^{F}C-ac1:G with ODN-^{F}C-ac2:G, ODN-^{F}C-ac1:G had the lower $-\Delta H°$, $-T\Delta S°$, and $-\Delta G°$. The thermodynamic parameters indicated that the flexibility of ODN-^{F}C-ac1:G is higher than that of ODN-^{F}C-ac2:G.

To study the influences of the flexibility on the duplex structure, we measured CD spectra of the duplex of ODN-^{F}C-ac1:G, ODN-^{F}C-ac2:G, and ODN-^{F}C:G at 25 °C (Figure 4). The observed CD spectra of ODN-^{F}C-ac1:G, ODN-^{F}C-ac2:G, and ODN-^{F}C:G consisted of a positive cotton effect (an increase at 275 nm and a decrease at 245 nm) due to the nucleobase stacking and the helicity of the duplex similar to a conventional B-form duplex.[22] ODN-^{F}C-ac2:G has a lower increase at 275 nm, and ODN-^{F}C-ac1:G has a lower increase and decrease showing not only at 275 nm but also at 245 nm compared with ODN-^{F}C:G. These results suggested that both of the ODNs containing acyclic nucleosides revealed a weaker nucleobase stacking effect due to the increase in flexibility at the acyclic modification site, and ODN-^{F}C-ac1:G had a decrease of helicity of the B-form duplex. The differences in the CD spectra corresponded to the difference of thermodynamic parameters, especially a lower $-T\Delta S°$.

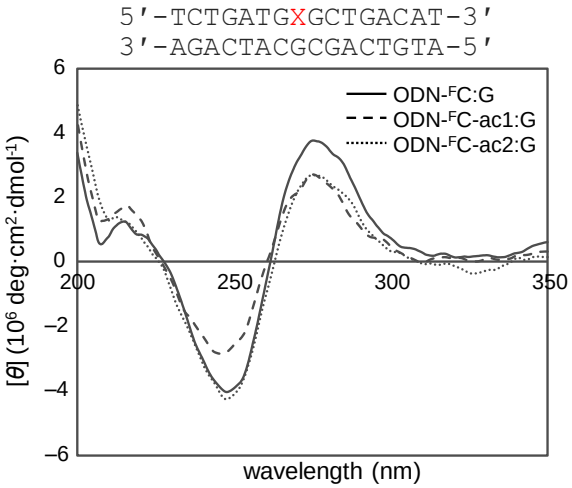


Figure 4. CD spectra of ODNs containing ^{F}C-ac1, ^{F}C-ac2, and ^{F}C; ODN-^{F}C:G (solid), ODN-^{F}C-ac1:G (dashed), ODN-^{F}C-ac2:G (dot). The duplex (1.5 μM) at 25 °C in a buffer of 10 mM Na cacodylate (pH 7.0) with 150 mM NaCl was used for each measurement.

ODN-^{F}C, ODN-^{F}C-ac1, and ODN-^{F}C-ac2 would share the same mechanism in the methylation step (Figure 1A (e) and Figure 1C); however, the k_{rel} values of ODN-^{F}C-ac1:G and ODN-^{F}C-ac2:G are lower than ODN-^{F}C:G, and a negative correlation was observed between the flexibility and ODN-DNMT complex formation. These results suggested that not only the methylation reaction step but also the scanning, recognition, and flipping steps (Figure 1A (a), (b) and (c)) are very important for the entire reaction of DNMT. X-ray structure of the cocrystal between ODN containing ^{F}C, DNMT, and *S*-adenosyl-L-homocysteine (SAH) had a characteristic conformation of the B-form duplex, and all the nucleobase specific interactions occurred in the major groove of ODN.[23] The first contact between ODN and DNMT was independent from the sequence of ODN; therefore the higher helicity of B-form duplex is especially important (scanning; Figure 1A(a)). Next, the recognition step occurs in a sequence specific manner in the major groove. The width and depth of the grooves are characteristic of each conformation of DNA/RNA. Therefore, nucleobase specific interactions in the major groove are also influenced from the higher helicity of the B-form (recognition; Figure 1A(b)). This consideration can explain the negative correlation between the flexibility and ODN-DNMT complex formation. On the other hand, in the flipping step, the local flexibility at the target cytosine site may increase the flipping of the target acyclic nucleoside because of the weaker nucleobase stacking effect (Figure 4). To investigate the flipping step in detail, other modified ODNs, which have various penalty in stacking, are necessary.

In summary, to obtain insight into the recognition mechanism of DNMT, we designed and synthesized acyclic nucleosides containing a ^{F}C nucleobase, ^{F}C-ac1, and ^{F}C-ac2. The ODNs containing ODN-^{F}C-ac1 and ODN-^{F}C-ac2 showed a higher flexibility than that of ODN ^{F}C, and ODN-^{F}C-ac1 was more flexible than ODN-^{F}C-ac2 from the data obtained by the thermodynamic parameters and CD spectra. A negative correlation was observed between the flexibility and ODN-DNMT complex formation. A weaker nucleobase stacking effect due to the increase in flexibility at the acyclic modification site may provide a higher flip out effect (Figure 1A(c)); however, the local flexibility would influence the scanning and recognition steps (Figure 1A(a) and (b)) due to the decrease in helicity of the entire shape of the B-form duplex shown in our CD spectra (Figure 4). Further investigations are necessary in our lab to clarify the recognition mechanism of DNMT and to develop other modified ODNs, which make minor structural changes in the helicity of the B-form.

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Supplementary Material

Supplementary data associated with this article can be found in the online version.

Design and synthesis of new acyclic nucleosides into ODNs and investigate the interaction between the ODN and DNMT.

The synthesized ODNs showed a higher flexibility from the data obtained by the thermodynamic parameters and CD spectra.

A negative correlation was observed between the flexibility and ODN-DNMT complex formation.