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Synthesis and properties of cross-linkable DNA duplex using 4-amino-2-oxo-6-vinyl-1,3,5-triazine

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ABSTRACT

We synthesized the DNA oligonucleotide containing a new cross-linkable 4-amino-2-oxo-6-vinyltriazine (AOVT) nucleobase analogue (Et-AOVT) and evaluated these properties. Our results of the cross-link assay and thermal denaturing assay of duplexes containing AOVT demonstrated that the additional aza of AOVT has an impact on the duplex stability and crosslink properties. Our data suggests that the additional 5-aza of AOVT is involved in the hydrogen bonding with the complementary guanine, and this hydrogen bonding system successfully flipped the reactive vinyl group out to the major groove of the duplex demonstrating a new paradigm of a “cross-linkable duplex”.

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1. Introduction

Various RNAs, such as mRNAs, pre-mRNAs, and non-coding RNAs, are currently recognized as a potential drug target to regulate genetic diseases.[1–4] Synthetic oligonucleotides (ONs) are one of the promising druggable candidates in that the ONs can directly interact with gene-coding DNAs, mRNAs and functional non-coding RNAs in a sequence-specific manner.[5–9] Various potent ONs that perform the efficient inhibition of translation were developed by improving their chemical stability, binding affinity and selectivity toward the target DNA/RNAs.[10–13] In addition to single, double and higher-order structuring nucleic acids, nucleic acid binding proteins (NABPs) can also be targeted by using decoy-ONs and nucleic acid aptamers.[14,15] In all these techniques, the strong binding affinity with the targets is a pivotal factor to compete with other target-binding molecules. To further improve an affinity with the target nucleic acids and NABPs, cross-link formation is one of the robust approaches as it forms a stiff covalent linkage to targets.[16–22] Apart from the DNA-crosslinking natural products, such as cisplatin[23] and mitomycin,[24] Rokita's groups reported artificial inter-strand cross-linkers using a cross-linkable small molecule.[25,26] In addition, various crosslink-forming oligonucleotides (CFOs) have also been developed and various attractive approaches of stimulus-responsible CFOs by using photo,[27] UV[28–30], or oxidative[31,32] activation have been reported. We have focused on developing the methodology of ON-based gene regulations using cross-linkable artificial nucleobase, and have also reported several CFOs containing cross-linkable 2-amino-6-vinylpurine (2-AVP)[33–35] and 4-amino-6-oxo-2-vinylpyrimidine (AOVP)[36–38] targeting DNAs and RNAs. One of important benefits of our cross-linking nucleobase is *the requirement of no external stimuli* such as UV-irradiation which potentially is toxic to biological tissues,[39] but just requiring the *Michael acceptor (crosslinker) - donor (target) proximity effect* acquired by Watson-Crick base pairing. Furthermore, the crosslinking property was provided by minimal structural changes to the canonical nucleobases which is potentially compatible within natural nucleic acid-related intracellular biological reactions. In a context of efforts on studying our CFOs, we designed a new cross-linkable nucleobase, 4-amino-2-oxo-6-vinyl-1,3,5-triazine (AOVT), in which the structure contains an electron-deficient triazine ring.

We hypothesized for this molecule that (i) the electron-withdrawing effect of the triazine increases the reactivity of the 6-vinyl group of AOVT than that of the pyrimidine-type AOVP, and also that (ii) the additional aza moiety effectively reduces the reactivity of AOVT with guanine (G) by forming a stable base pairing via a three hydrogen bonding system (Fig. 1). Using such a unique molecular interaction with G, we assumed that our reactive cross-linking vinyl group can be encapsulated in the oligo-duplex remaining intact from the reaction with complementary bases,

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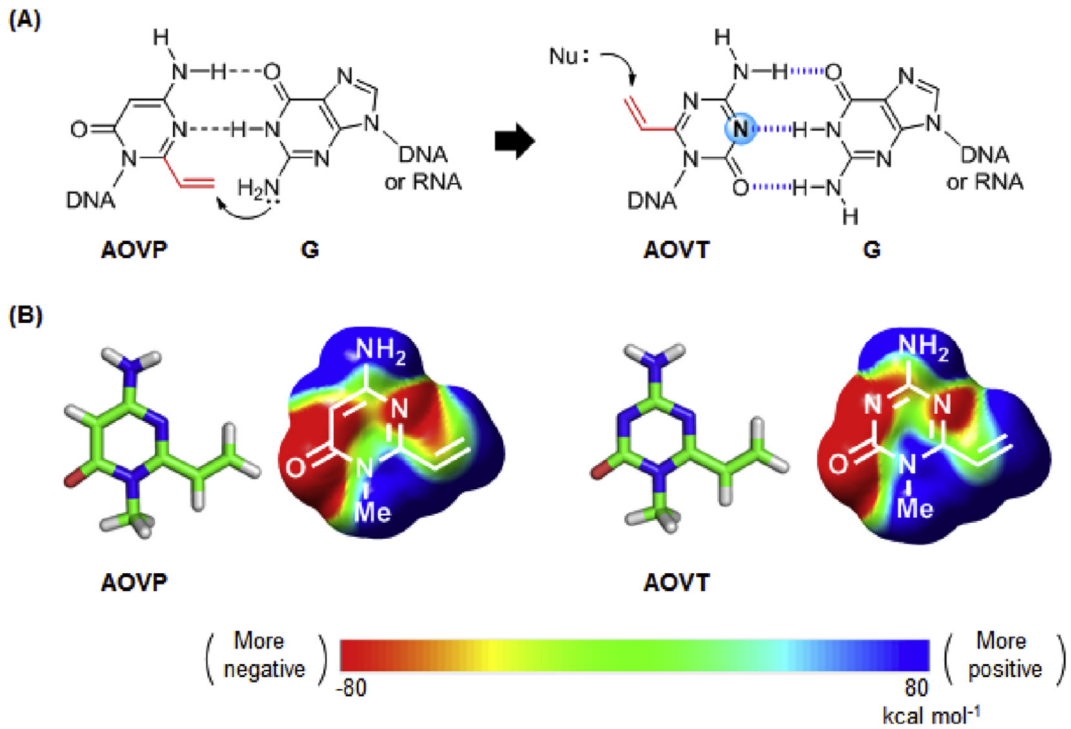

Fig. 1. Alteration of AOVP to a new cross-linkable AOVT having a reduced reactivity with guanine. (A) Additional 3-aza of AOVT enables forming of a stable AOVT-G base pair and to crosslink to endogenous nucleophiles. (B) Optimized structure and difference in electron density maps of me-AOVP and me-AOVT.

which may be able to target nucleophiles of the oligo-duplex binding species, such as NABPs. We now report the synthesis of CFOs having AOVT and its crosslink properties to complementary base and also report structural preferences of AOVT in the form of AOVT-G base pair.

2. Results and discussion

2.1. Synthesis of 6-(octylthio)ethyl 5-azacytidine derivative

We initially designed 6-vinyl-decitabine derivative (**1**) as a cross-linkable AOVT precursor (Fig. 2). Our previous study suggested that the direct glycosylation of the 6-octylthioethyl-substituted 5-azacytosine derivative with the ribose-sugar was difficult to obtain the desired compound. We planned to synthesize the C6-substituted 5-azacytosine by ring opening of the 5-azacytosine moiety forming guanylurea and subsequent ring closing reaction[40] using the orthoester, 1,1,1-trimethoxy-3-(octylthio) propane (**6**), which was synthesized in 3 steps from 3-bromopropionitrile (**3**) (Scheme S-1).[41–43] The glycosylation of the silylated 5-azacytosine (**8**) with 1,3,5-tri-*O*-acetyl-2-deoxy-D-ribose was carried out to yield the α/β anomeric mixture of 3′,5′-O-acetyl-5-azacytidine (**9**) (Scheme 1).[44] The 5-azacytosine moiety of **9** was transiently converted to the guanylurea (**10**) by ammonia treatment, and then **10** was condensed with orthoester **6**. However, the desired ring-closed product **11** was not obtained. We next modified the scheme of which the 3′,5′-O-TIPDS -protected intermediate (**12**) was prepared from **10**, then carried out the condensation of **12** with the orthoester (**6**). As a result, the C-6 substituted 5-azacytidine derivative (**13**) was obtained as an anomeric mixture in 43% yield. However, rapid cleavage of the glycosidic bond of **13** occurred in 10 min by the treatment with 3% TCA/CH_2Cl_2, which is one of the typical deprotection conditions of the DMTr group during solid-phase oligonucleotide synthesis (data not shown). These results suggested the difficulty in the incorporation of the AOVT nucleoside into oligonucleotides by a general solid-phase synthesis.

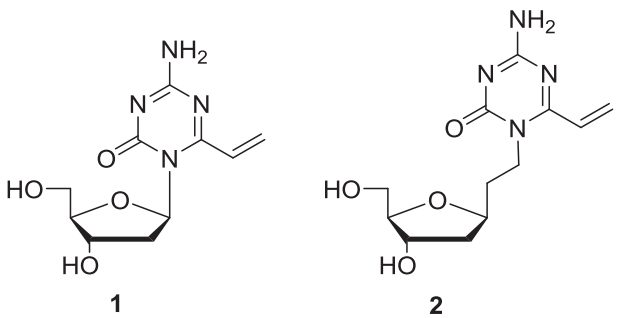

Fig. 2. Structures of cross-linkable decitabine analogues **1** and **2.**

2.2. Synthesis of phosphoramidite 25 and oligonucleotide modified with Et-AOVT nucleoside

The ethyl-bridged compound (Et-AOVT nucleoside, **2**) was next designed to avoid the glycosidic bond cleavage (Fig. 2), and the ethyl spacer between the pentose sugar and 5-azacytosine moiety allows decoupling the 1-*N*-neighboring 4′-O group participation and to avoid release of the nucleobase.[45–47] The sugar part (**16**) was synthesized in several steps from 5-*O*-Tr-2-deoxy-D-ribose (**15**) according to a previously reported method (Scheme 2).[48] After the reduction of the ethylester of **16**, the mesyl group was introduced to the hydroxyl group of **17** to yield **18**. For the glycosylation, the sodium salt of the commercially available 5-azacytosine (**7**) was coupled with **18** in the presence of $CsCO_3$ and the desired 5-azacytidine analogue (**19**) was successfully synthesized in 55% yield. The formation of the glycosidic bond at the N1 position of 5-azacytosine was confirmed by HMQC and HMBC analyses (Figs. S1,

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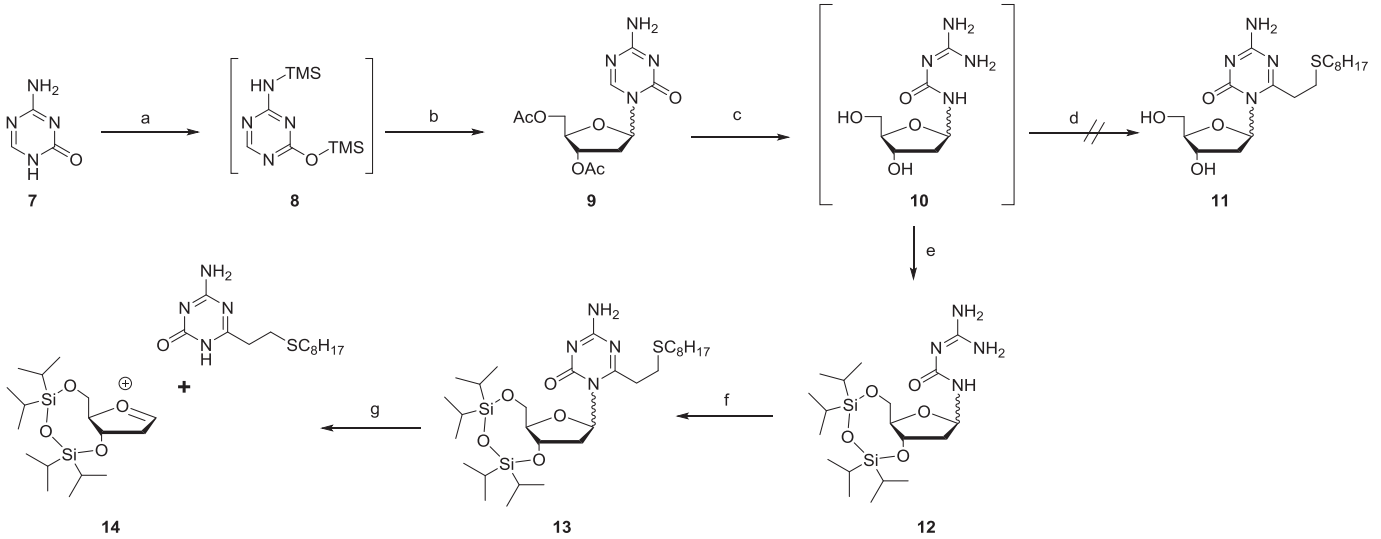

Scheme 1. Synthesis of 6-(octylthio)ethyl 5-azacytidine derivative. [a]Reagents and conditions: (a) BSA, CH_3CN, 50 °C, 2 h; (b) 1,3,5-tri-*O*-acetyl-2-deoxy-*D*-ribose, CH_3CN, 76% (anomeric mixture); (c) conc. NH_4OH-MeOH-1,4-dioxane (2:1:1, v/v/v), 50 °C, 2 h; (d) 1,1,1-trimethyl-3-(octylthio)propane **6**, DMF, 120 °C, 2 h; (e) $TIPDSCl_2$, pyridine, r.t., 24 h, quant (2 steps); (f) orthoester **6**, DMF, 120 °C, 2 h, 43%; (g) 3% trichloroacetic acid/CH_2Cl_2, r.t. 10 min.

S2). For the derivatization of **19** at the C6 position of the 5-azacytosine moiety, **19** was converted to the guanylurea intermediate (**20**) and condensed with the orthoester (**6**) affording the desired C6-octylthioethyl-5-azacytidine derivative (**21**) in 54% yield (2 steps). After 4-N-acetylation and the deprotection of the 5′-O-Tr and 3′-O-MOM groups by BF_3OEt_2,[49,50] the 5′-hydroxyl group was selectively protected with the DMTr group, then the 3′-hydroxyl group was phosphitylated to yield the Et-AOVT nucleoside phosphoramidite (**25**).

The DNA oligonucleotide 5′-O-DMTr-d[CCGCGTXTCGCCG]-3′ (X = Et-AOVT) was synthesized using the Et-AOVT phosphoramidite (**25**) and other building units suitable for ultra-mild oligonucleotide synthesis (*N*-Tac-protected phosphoramidite for dC, dG, and dA) in an automated DNA synthesizer. The synthesized oligonucleotide was treated with 45 mM K_2CO_3/MeOH containing 10 mM 1-octanethiol at room temperature for 4 h to cleave from the resin. After the DMTr-ON purification by reverse-phase (RP) HPLC, detritylation was carried out in an aqueous 10% AcOH solution at room temperature for 30 min. The obtained oligonucleotide was further purified by RP-HPLC, and produced ODN**1** (C6-protected) was characterized by a MALDI-TOF MS analysis. To deprotect the C6-vinyl group of AOVT, the sulfide of the C6-octylthioethyl group was oxidized to the sulfoxide by the treatment of magnesium monoperoxyphthalate (MMPP) yielding ODN**2**, which was followed by β-elimination of 1-octanesulfenic acid in an aqueous 0.5 M NaOH solution. However, a significant decomposition of AOVT to guanylurea was observed by RP-HPLC and characterized by the corresponding MALDI-TOF MS peak at 3906.2 $[M-H]^-$ (calcd for guanylurea, *m*/*z* 3905.6). As an alternate procedure for the generation of the C6-vinyl group, subsequent treatment with aqueous 10% AcOH after oxidation was carried out and the desired ODN**3** containing C6-vinyl-AOVT was quantitatively detected by RP-HPLC (Fig. S3) and MALDI-TOF MS analyses: found 3941.5, calcd 3941.6 $[M-H]^-$ (for more detailed analysis of ODN3, see Fig. S6).

2.3. Crosslink reactivity of ODN3 with complementary DNAs or RNAs

Next, The alkylating properties of ODN**3** were first evaluated under neutral conditions (pH 7.0) containing the FAM-labelled complementary DNAs 5′-FAM-d[CGGCGA**Y**ACGCGG]-3′ (**Y** = T, G, A, or C) (cDNA-T, cDNA-G, cDNA-A, or cDNA-C, respectively) or RNAs 5′-FAM-r[CGGCGA**Y**ACGCGG]-3′ (**Y** = U, G, A, or C) (cRNA-U, cRNA-G, cRNA-A, or cRNA-C, respectively). The reaction mixture was analyzed by 20% polyacrylamide gel electrophoresis (PAGE) containing 7 M urea (Fig. 3) and the yields of the cross-linked product were calculated based on the intensity of fluorescence of each bands observed on the gels. When ODN**3** formed a duplex with cDNA-C, cDNA-T and cDNA-A, a relatively high yield of the cross-linked product was observed (52–96% in 24 h). On the other hand, when ODN**3** formed the duplex with cDNA-G, the cross-linking efficiency remained low (21% in 24 h). This trend was more prominent in the DNA/RNA heteroduplex system in that AOVT-ODN**3** cross-linked with cRNA-G in less than a 5% yield even after a 24 h incubation. Contrary to this result, AOVT-ODN**3** cross-linked with cRNA-U and C in very high yields (96% with cRNA-U and 88% with cRNA-C in 24 h). Compared with the target base selectivity of the AOVP-containing DNA oligonucleotide previously reported, AOVT also showed a relatively high reactivity with thymine, and drastically increased the reactivity with the cytosine base of cDNA, which was the most reactive complementary base among the four bases. For the reaction with cRNA, AOVT reacted with uracil as seen in the case of AOVP and also with cytosine, which reactivity to cytosine is due to the AOVT-specific character. This interesting result that only one nitrogen addition on AOVP drastically changes the crosslink preferences could be attributed to the fact that the additional hydrogen bond acceptor enabled forming of the AOVT-cytosine wobble base pair similar to the cytosine-cytosine wobble-like base pair (Fig. S8).

2.4. Identification of crosslinked base of cRNA-U and cRNA-C

To identify the position of the cross-linking site of AOVT to the complementary RNA strand, the alkali-hydrolysis based footprinitng of the cross-linked (CL) duplex of ODN**3**:cRNA-U (CL-rU) and ODN**3**:cRNA-C (CL-rC), which showed a very high crosslink yield among all the tested duplexes, was carried out. CL-rU and CL-rC were synthesized and purified by RP-HPLC and their purities were confirmed by PAGE and MALDI-TOF MS (Figs. S8-S10). The purified CL-rU and CL-rC were incubated in a 45 mM K_2CO_3-MeOH

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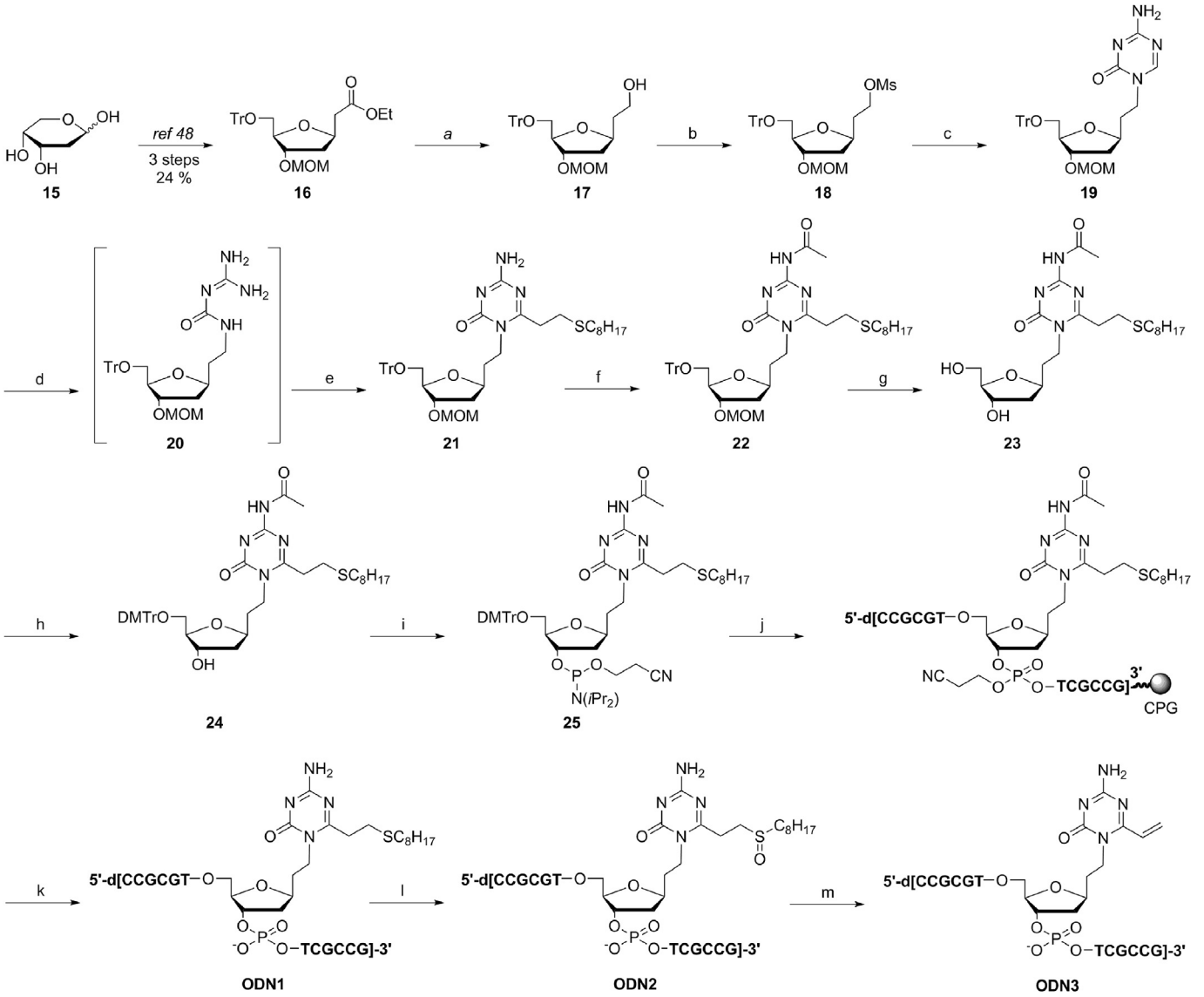

Scheme 2. Synthesis of Et-AOVT nucleoside phosphoramidite **25**, and oligonucleotide synthesis. [a]Reagents and conditions: (a) 3 M $LiBH_4$-THF, rt, 12 h, 95%; (b) MsCl, Et_3N, CH_2Cl_2, 0 °C, 1 h, 94%; (c) 5-azacytosine sodium salt, $CsCO_3$, DMF, 90 °C, 5 h, then 120 °C, 2 h, 55%; (d) conc. NH_4OH-MeOH-1,4-dioxane (2:1:1, v/v/v), 50 °C, 12 h; (e) orthoester **6**, DMF, 120 °C, 1.5 h, 54% (2 steps); (f) AcCl, pyridine, rt, 1 h, 80%; (g) BF_3·OEt, CH_2Cl_2, 0 °C, 1.5 h, 57%; (h) DMTrCl, pyridine, 0 °C, 1 h, 61%; (i) 2-Cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite, DIPEA, CH_2Cl_2, 0 °C, 45 min, 61%. (j) DNA synthesizer; (k) 45 mM K_2CO_3-MeOH, 10 mM 1-Octanethiol, rt, 4 h; (l) 70 μM MMPP (2 eq), 1.9 mM carbonate buffer (pH 10), 26 °C, 30 min; (m)10% aq. AcOH, 26 °C, 2 h.

(5% H_2O) solution at room temperature because the more basic 0.1 M aq. NaOH solution produced a significant cleavage of the cross-linkage quantitatively yielding the guanylurea-converted AOVT-ODN (AOVT*-ODN) (Figs. S11-14). In this alkali cleavage experiment, only the complementary RNA strand was selectively cleaved as it contains 2′-OH that facilitate the alkali cleavage of the RNA strand by attacking the neighboring 3′-phosphodiester bond,[51,52] and the N mer of the cleaved FAM-labelled RNA strands (N = 1,2,3, …6) from the CL-duplex can be observed if the AOVT cross-linked to the complementary base (7 position from 5′-end) of the 13 mer of the cRNAs. In addition, the cleaved FAM-labelled RNA strands (N = 7, 8 … 12) will not appear because products of the alkali-cleavage beyond the cross-link site are very large because attaching the DNA 13 mer to AOVT-ODN is stable under mild basic conditions. Our gel showed a prominent disruption of the ladder cleaved strands compared with the control experiment conducted with the non-crosslinked cRNA-U and C. By comparison with the cleaved 6 mer RNA [5′-FAM-r(CGGCGA)p-3′], the footprint of both the cleaved CL-rU and CL-rC generated the cleaved 1–6 mer of the RNA strand whereas did not generate the 7–12 mer of the cleaved products, suggesting that AOVT clearly cross-linked to rU of cRNA-U and rC of cRNA-C (Fig. 4).

2.5. Thermal denaturing analysis of duplexes containing AOVT^{et}

We next carried out a thermal denaturing study of the non-reactive AOVT-containing duplex to study the mechanism for the lowest reactivity with ODN**3** to guanine of the DNA (and RNA). The non-reactive ODN**3** (ODN**3**^{et}**)** was prepared by the reduction using $NaBH_4$ to avoid formation of a cross-linkage during the assay (Figs. S15-17).[38] In this assay, the unmodified ODN**4** 5′-d[CCGCGTCTCGCCG]-3′, and previously reported non-reactive AOVP^{et}-ODN 5′-d[CCGCGTXTCGCCG]-3′ (X = vinyl reduced AOVP^{et}) were used for comparison.[53]

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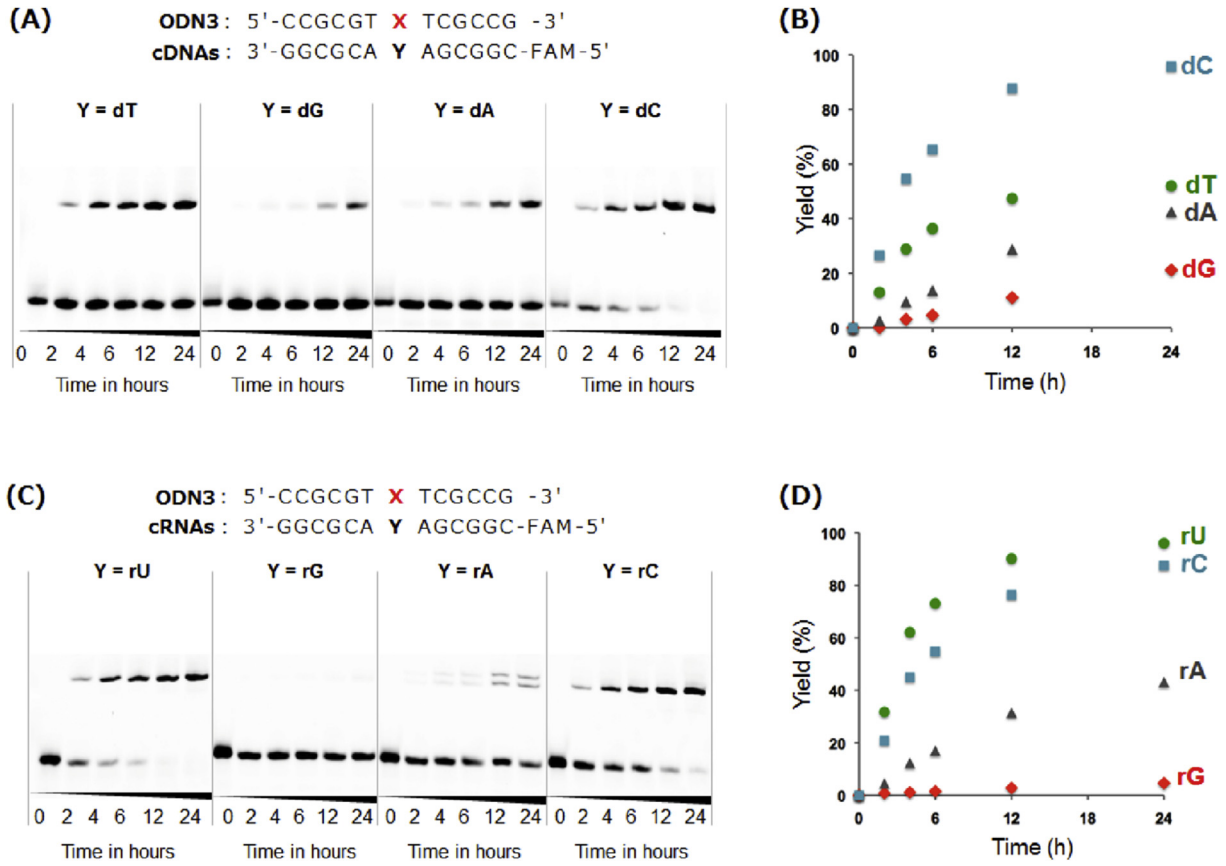

Fig. 3. Cross-linking properties of ODN**3** for the target cDNAs and cRNAs. (A) Denaturing gel electrophoresis of crosslink reaction to cDNAs (B) Quantification of DNA/DNA crosslinked product on the gel at each time point. (C) Denaturing gel electrophoresis of crosslink reaction to cRNAs. (D) Quantification of DNA/RNA crosslinked product on the gel at each time point. The reaction was performed in 50 mM MES-buffer (pH 7.0) containing 100 mM NaCl, 5 μM ODN**3** and 2 μM cDNAs (or cRNAs) at 37 °C.

The ODN**3**^{et}:cDNA-G (or cRNA-G) duplex showed lower T_m values (T_m = 56 °C and 52 °C, respectively) than that of the unmodified ODN**4**:cDNA-G (or cRNA-G) (T_m = 67 °C and 66 °C, respectively) (Fig. 5). However, it should be noted that the ODN**3**^{et}-duplexes still have a sufficient thermo-stability to form a stable duplex under the stated reaction conditions. A previous study reported that AOVP^{et}-ODN showed approximately similar T_m values (0 to +4 °C of ΔT_m with cDNAs and cRNAs). In comparison to that, the T_m values of ODN**3**^{et}:cDNA-G and cRNA-G were distinctive in that ODN**3**^{et} showed a +10 to +17 °C ΔT_m compared to other all the duplexes containing the other AOVP- or AOVT-base pairing. Taken together, our results of cross-link assay and T_m measurement strongly suggest that AOVT forms stable triple hydrogen bonds with guanine thus acquiring higher thermo-stable ODN**3**^{et}:DNA (or RNA) duplexes than those of other duplexes, and thus the C6-vinyl group of AOVT strongly favored orienting toward the opposite side of complementary bases, thus the conformational effect reduced the crosslinking with guanine (Fig. 6).

2.6. Reactivity of AOVT with exogenous nucleophile in the duplex form

To further investigate the possible "cross-linkable duplex" using the *vinyl-intact* AOVT-G base pairing system, ODN**3**:cRNA-G in the duplex form was further reacted with exogenous nucleophiles. The 1 μM of ODN**3** (C6-vinyl) and 1 μM of cRNA-G were mixed in the buffer and pre-incubated at 37 °C for 10 min, then a high concentration (250 μM) of 4-*tert*-butylbenzenethiol as an exogenous nucleophile was added. The reaction was monitored by RP-HPLC, and the peak of ODN**3** (Rt = 9 min) mostly disappeared on the HPLC profile and a new major peak appeared (Rt = 16 min) after 2 h of incubation (Fig. 7). The MALDI-TOF MS analysis for this major new peak was conducted and found to be AOVT-ODN bearing the thiol-adduct. This result indicates that the remaining C6-vinyl group of AOVT in the duplex is still cross-linkable to the exogenous nucleophiles even in the duplex. We consider that this AOVT-G base pairing system should provide a new "cross-linkable duplex" material and could expand the possibility of cross-linking oligonucleotides to target not only the single stranded DNA or RNA, but also nucleic acid binding proteins (NABPs).

3. Conclusions

We have developed a new cross-linkable nucleic acid derivative, Et-AOVT nucleoside, which was successfully incorporated into the DNA oligonucleotide by solid-phase synthesis. ODN**3** was found to have a relatively high reactivity with the pyrimidine nucleobases of the complementary DNAs or RNAs under physiological conditions. A very interesting behavior of AOVT was also observed when the complementary base was guanine. Thermal denaturing analyses implied that AOVT formed Watson-Crick-like base pairing as seen in the canonical C-G base pair. This molecular interaction should allow the C6-vinyl group of AOVT to orient toward the major groove of the duplex, which allowed the significantly disturbed AOVT to react with the complementary guanine. We actually found a certain correlation of this property with the results obtained in our cross-link assay where AOVT showed a significantly low reactivity with guanine. The high reactivity of the C6-vinyl group of the AOVT-G

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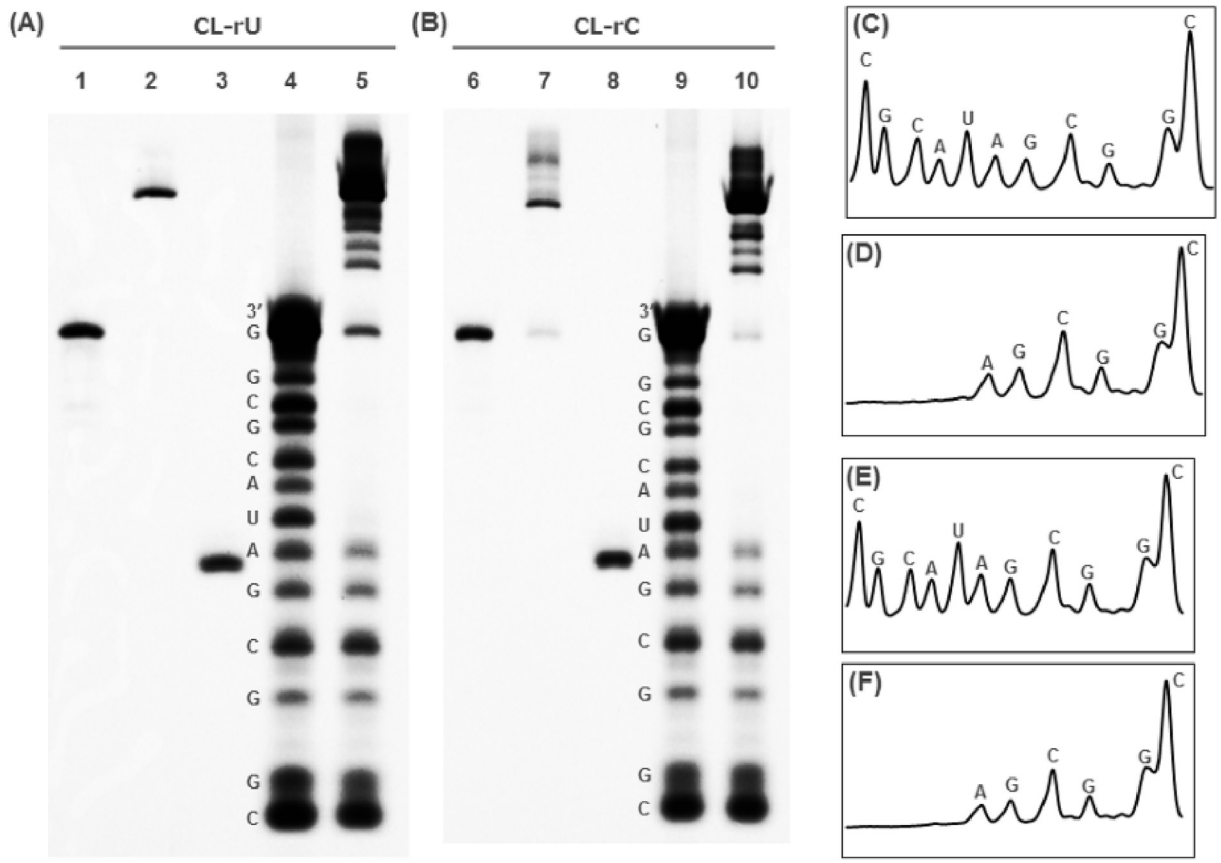

Fig. 4. Alkali-footprinting of cross-linked CL-rU and CL-rC. FAM-labelled full-length and shortened oligonucleotides were visualized by 20% formamide/20% denatured polyacrylamide gel electrophoresis. (A) Lanes 1, 2, and 3 are cRNA-U, CL-rU, and 5′-FAM-r[CGGCGAp]-3′, respectively. Lanes 4 and 5 are gels of alkali cleaved products of cRNA-U and CL-rU. (B) Lanes 6, 7, and 8 are cRNA-C, CL-rC, and 5′-FAM-r[CGGCGAp]-3′, respectively. Lanes 9 and 10 are gels of alkali cleaved products of cRNA-C and CL-rC. (C ~ F) Quantification of bands in lanes 4, 5, 9 and 10, respectively. Alkali RNA cleavage was conducted in 45 mM K_2CO_3-MeOH (5% H_2O) at 26 °C for 4 h. FAM-labelled fragments were visualized on a 20% formamide/20% acrylamide denaturing gels, and the fluorescent intensity of each product was quantitatively measured by a fluoro-image analyzer.

pair with exogenous thiol-nucleophiles suggested the formation of a cross-linkable oligo-duplex. This unique property of AOVT inspires a further possibility of this kind of cross-linkable nucleobase analogue as a robust tool to form covalent linkages with duplex-binding components such as cysteine or lysine residues of nucleic acid binding proteins.

4. Experimental section

4.1. General materials and methods

The NMR spectra were recorded using Brucker 400 or 600 MHz spectrometer. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded at 600 MHz (^{1}H NMR, 600 MHz; ^{13}C NMR, 150 MHz; ^{31}P NMR, 162 MHz) or 400 MHz (^{1}H NMR, 400 MHz; ^{13}C NMR, 100 MHz). The chemical shifts were measured from tetramethylsilane (0 ppm), $CDCl_3$ (7.26 ppm) or DMSO-d_6(2.49 ppm) for ^{1}H NMR spectra, $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.7 ppm) for ^{13}C NMR spectra, and 85% H_3PO_4 for ^{31}P NMR spectra as external standards. The electrospray ionization (ESI) mass spectra were recorded on BioTOF II (Bruker Daltonics). We did not measure IR spectra, because ^{1}H NMR, ^{13}C NMR, HMQC, HMBC, HRMS analysis was enough for characterization of nucleoside derivatives. The MALDI-TOF mass spectra were recorded on Autoflex speed mass spectrometer and the laser at 337 nm by negative or positive mode using 3-hydroxypicolinic acid as a matrix. Rf values on thin-layer chromatography (TLC) analysis were determined using E. Merck Silica gel 60 F_{254} pre-coated glass plates. Column chromatography was performed with Kanto Chemical silica gel 60 N (spherical, neutral, 100–210 μm). Flash chromatography was performed with Kanto Chemical silica gel 60 N (spherical, neutral, 40–50 μm). The synthesis of modified oligonucleotides was performed using an automated DNA synthesizer 392 (Applied Biosystem). The unmodified oligonucleotides were purchased from JBioS, Co Ltd. HPLC was performed using Nacalai Tesque COSMOSIL 5C18-MS-II (4.6 or 10 × 250 mm) as a column. Anhydrous methanol, DMF, THF, CH_2Cl_2, 1,4-dioxane, pyridine, DMSO, toluene, and acetonitrile were purchased from Wako Pure Chemical Industries Ltd.

4.2. Synthesis of nucleoside derivatives

4.2.1. Synthesis of 3-octylthiopropioimidate (**5**)

To a **4** (9.72 g, 48.8 mmol) was added anhydrous CH_3OH (11.8 mL, 293 mmol) and then, was cooled to 0 °C. To this solution was dropwisely added AcCl (10.4 mL, 146 mmol). The resulting mixture was stirred at 0 °C for 20 h (carefully monitored not to exceed 5 °C). The solvent was then removed at 0–20 °C with vacuum pump to give **5** (12.0 g, 92%) as a white powder: Rf = 0.31 (AcOEt-Hexane, 1:6 with 0.5% Et_3N); ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.70 (br, 1H), 11.79 (br, 1H), 4.31 (s, 3H), 3.08 (t, 2H, J = 6.8 Hz), 2.88 (t, 2H, J = 6.8 Hz), 2.59 (t, 2H, J = 7.2 Hz), 1.57 (quint, 2H, J = 7.2 Hz), 1.39–1.26 (m, 10H), 0.87 (t, 3H, J = 6.8 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 178.5, 60.9, 33.3, 31.9, 29.34, 29.26, 29.24, 28.83, 27.2, 22.7, 14.2; HRMS (ESI) calcd. for $C_{12}H_{26}NOS^+$ $[M+H]^+$ m/z

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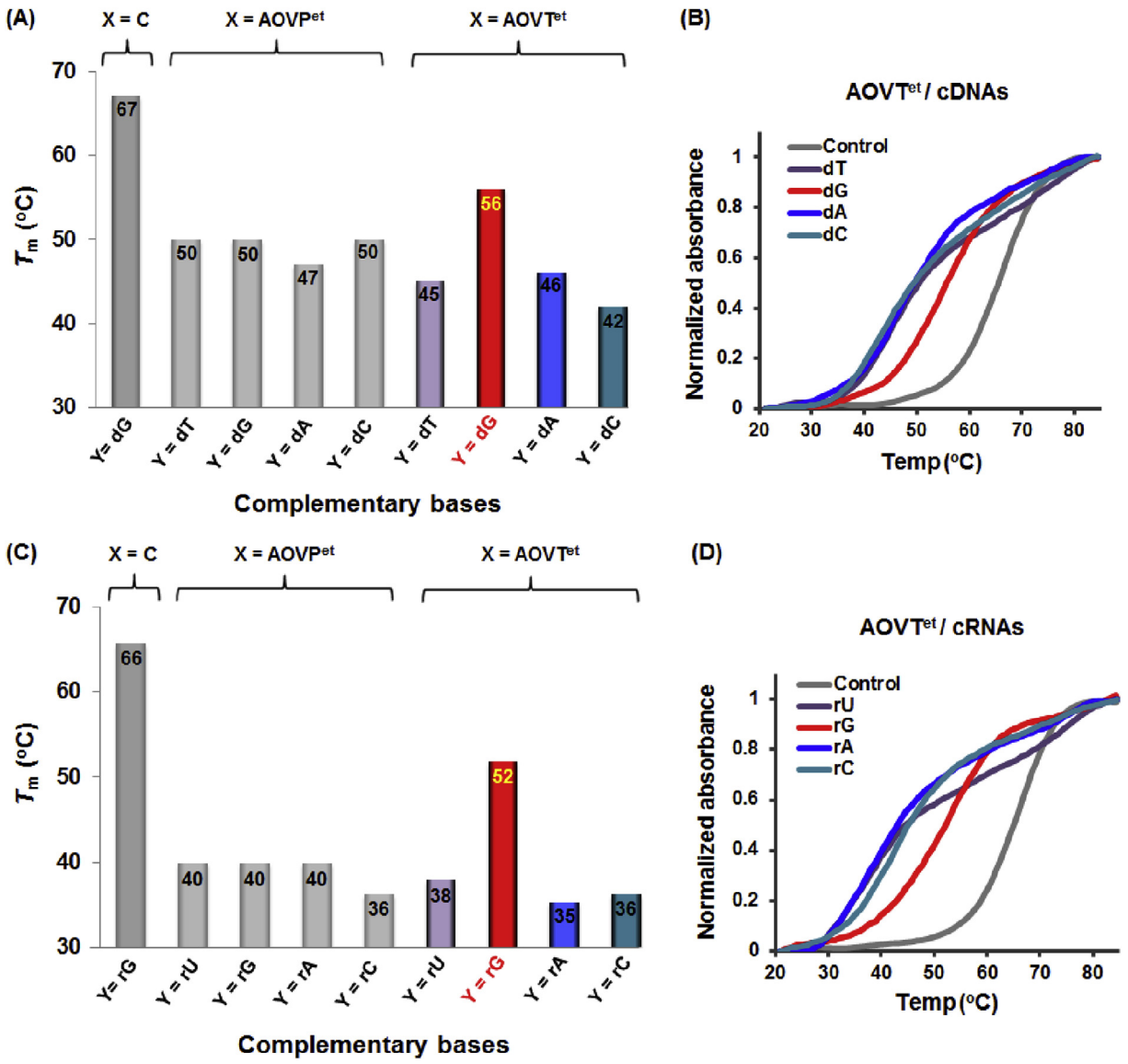

Fig. 5. Comparative thermal denaturing analysis of duplexes containing ODN**3**^{et}. (A) Comparative studies of T_m values of ODN**3**^{et}, AOVP^{et}-ODN, and unmodified ODN**4** with DNA templates. (B) Sigmoidal melting curves of ODN**3**^{et}/cDNAs and ODN**4**/cDNA-G (C) Comparative studies of T_m values of ODN**3**^{et}., AOVP^{et}-ODN, and unmodified ODN**4** with RNA templates. (D) Sigmoidal melting curves of ODN**3**^{et}/cRNAs and ODN**4**/cRNA-G. T_m values were measured in 50 mM MES buffer (pH 7.0) containing 100 mM NaCl. Sequences of ODNs: 5′-d[CCGCGTXTCGCCG]-3′ (X = AOVT^{et}, AOVP^{et} or C for ODN**3**^{et} AOVP^{et}-ODN, or ODN**4**, respectively). Sequence of cDNAs: 5′-FAM-d[CGGCGAYACGCGG]-3′ (Y = T, G, A, or C). Sequence of cRNAs 5′-FAM-r[CGGCGAYACGCGG]-3′ (Y = U, G, A, or C).

232.1730, found *m/z* 232.1759.

*4.2.2. Synthesis of 3-octylthiopropioimidate (**6**)*

To a suspension of **5** (7.82 g, 29.3 mmol) in anhydrous hexane (29 mL) was added anhydrous CH_3OH (11.8 mL, 293 mmol). The mixture was stirred at room temperature for 24 h. The resulting suspension containing NH_4Cl was diluted by hexane (50 mL). The affording white precipitate (NH_4Cl and amide) was removed by filtration. The resulting solution was evaporated, and then was diluted with hexane (50 mL). The white precipitates was removed by filtration again, and then this procedure was repeated 2 times again to afford **6** (5.56 g, 68%, purity = 91%) as a colorless solution: Rf = 0.76 (AcOEt/Hexane, 1:6); ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.23 (s, 9H), 2.50 (t, 2H, J = 7.8 Hz), 2.49 (1H, d, J = 17.4 Hz), 2.49 (1H, ddd, J = 5.4, 5.4, 1.8 Hz), 2.01 (1H, d, J = 17.4 Hz), 2.01 (1H, ddd, J = 5.4, 5.4, 1.8 Hz), 1.57 (quint, 2H, J = 7.2 Hz), 1.37–1.26 (m, 10H), 0.86 (t, 3H, J = 7.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 115.2, 49.7, 32.6, 32.0, 31.3, 29.8, 29.39, 29.38, 29.1, 25.7, 22.8, 14.3; HRMS (ESI) calcd. for $C_{13}H_{27}O_2S^+$ $[M - OCH_3]^+$ *m/z* 247.1726, found *m/z* 247.1729, calcd. for $C_{14}H_{30}NaO_3S^+$ $[M+Na]^+$ *m/z* 301.1808, found *m/z* 301.1810.

*4.2.3. Synthesis of 3′,5′-O-acetyl-2′-deoxy-5-azacytidine (**9**)*

5-azacytosine (0.980 g, 7.40 mmol) was rendered anhydrous by repeated co-evaporation with anhydrous acetonitrile and was dissolved in anhydrous acetonitrile (40 mL) under argon atmosphere. To a solution was added BSA (4.92 mL, 20.0 mmol) and the mixture was stirred at 50 °C for 2 h. After the solution became colorless, an anhydrous acetonitrile solution (3 mL) containing 1,3,5-tri-*O*-acetyl-2-deoxy-D-ribose (2.50 g, 9.60 mmol) was added to the reaction mixture, followed by addition of TMSOTf (1.74 mL, 9.60 mmol) After the resulting orange-colored solution was stirred at ambient temperature for 30 min, the mixture was diluted with CH_2Cl_2 (250 mL), and then poured to aqueous sat. $NaHCO_3$ (150 mL). The mixture was extracted with CH_2Cl_2 and aqueous layer

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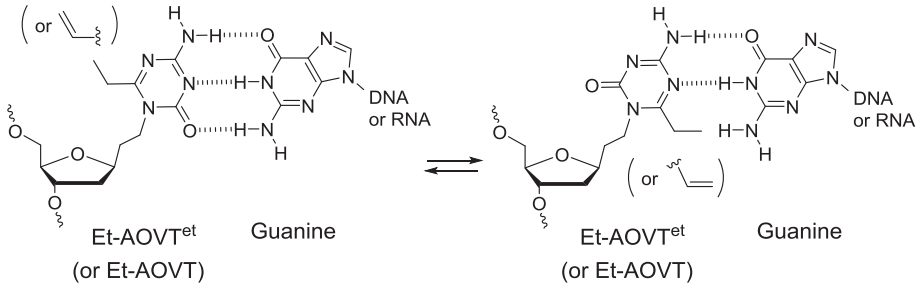

Fig. 6. Possible equilibrium in AOVT^{et} (or AOVT)-G base pair.

was repeatedly extracted with CH_2Cl_2. The organic layer was combined, dried over Na_2SO_4, filtered, and evaporated to afford 9 (1.75 g, 76%) as a white solid of an anomeric mixture: Rf = 0.41 ($CH_3OH/CHCl_3$, 1:10); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.31 (s, 1H),8.30 (s, 1H), 7.60 (br, 2H), 7.50 (br, 2H), 6.02–5.99 (m, 2H), 5.21 (dd, 1H, J = 6.0, 3.6 Hz), 5.11 (d, 1H, J = 6.4 Hz), 4.79 (dd, 1H, J = 4.4, 4.4 Hz) 4.28–4.19 (m, 3H), 4.11 (dddd, 2H, J = 12.0, 12.0, 12.0, 4.4 Hz), 2.73 (ddd, 1H, J = 15.2, 6.4, 6.4 Hz), 2.53 (ddd, 1H, J = 14.4, 14.4, 7.2 Hz), 2.37 (ddd, 1H, J = 14.4, 14.4, 14.4, 2.8 Hz), 2.21 (d, 1H, J = 15.2 Hz), 2.05–1.92 (m, 12 H),; ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.68, 170.65, 170.57, 170.3, 166.7, 166.3, 156.7, 155.9, 153.9153.3, 87.9, 86.4, 84.4, 82.3, 74.62, 74.57, 64.2, 63.9, 37.9, 37.0, 21.3, 21.1, 21.0; HRMS (ESI) calcd. for $C_{12}H_{16}N_4NaO_6^+$ $[M+Na]^+$ m/z 335.0962, found m/z 335.0962.

*4.2.4. Synthesis of N-(aminoiminomethyl)-N′-(2-deoxy-β-D-erythro-pentofuranosyl) urea (**10**)*

To a solution of **9** (1.09 g, 3.50 mmol) in dioxane-MeOH (17.5 mL, 1:1, v/v) was added 28% NH_4OH (17.5 mL), and then was sealed tightly with glass-stopper. The mixture was stirred at 50 °C for 2 h. The resulting solution was evaporated under reduced pressure at 43 °C. The resulting wet solid was co-evaporated with ethanol three times and CH_3CN three times to afford **10**. The product was directly used without further purification; HRMS (ESI) calcd. for $C_7H_{15}N_4O_4^+$ $[M+H]^+$ m/z 219.1088, found m/z 219.1087.

*4.2.5. Synthesis of 3,5-O-[1,1,3,3-tetrakis(1-methylethyl)-1,3-disiloxanediyl]-N-(aminoiminomethyl)-N′-(2-deoxy-β-D-erythro-pentofuranosyl) urea (**12**)*

To a solution of **10** (200 mg, 0.917 mmol) in pyridine (1.83 mL) was dropwisely added $TIPDSCl_2$ (315 μL, 1.01 mmol). The resulting mixture was stirred at room temperature for 24 h. The solution was diluted with Et_2O, and washed with water three times. The organic layer was dried over Na_2SO_4, filtered and evaporated to afford **12** (420 mg, quant.) as a yellow oil. The product was directly used without further purification: Rf = 0 ($CH_3OH/CHCl_3$, 1:8); HRMS

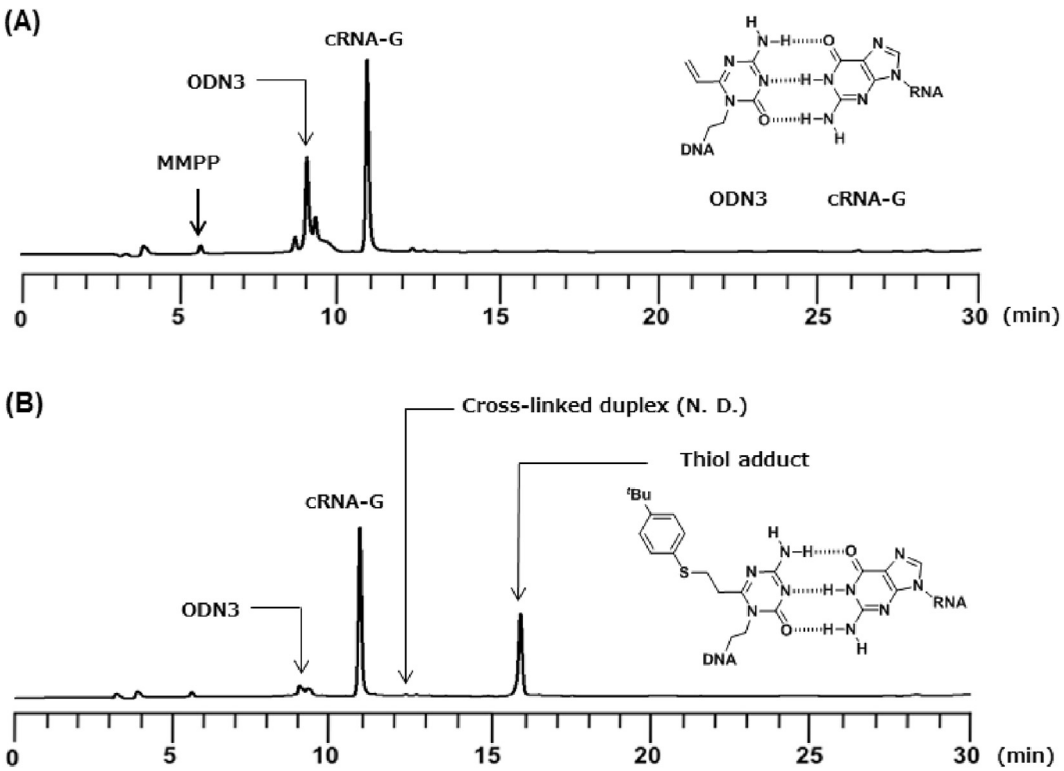

Fig. 7. Reactivity of vinyl group of ODN**3** with exogenous thiol in the form of duplex. (A) RP-HPLC profile after pre-incubation of ODN**3** and cRNA-G in 50 mM MES (pH 7.0) containing 100 mM NaCl at 37 °C for 10 min (B) RP-HPLC profile after the subsequent addition of thiol and incubation at 37 °C for 2 h. HPLC conditions: COSMOSIL 5C18-MS-II (4.6 × 250 mm), solvent A: 0.1 M TEAA buffer (pH 7.0), B: CH_3CN, B: 5–20%/30 min.

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(ESI) calcd. for $C_{19}H_{41}N_4O_5Si_2^+$ $[M+H]^+$ *m/z* 461.2610, found *m/z* 461.2653.

4.2.6. Synthesis of 3′,5′-O-[1,1,3,3-tetrakis (1-methylethyl)-1,3-disiloxanediyl]-5-aza-(2-octylthioethyl)cytidine (**13**)

To a solution of **12** (43.0 mg, 93.5 μmol) in DMF (470 μL) was added orthoester **6** (163 mg, 468 μmol, purity = 80%), and then was stirred at 120 °C for 2 h. After the solution became yellow solution, the mixture was diluted with Et_2O. The mixture was washed with NH_4Cl aq., $NaHCO_3$ aq., and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated. The crude product was chromatographed on silica gel, eluting with $CHCl_3$ then, $CH_3OH/CHCl_3$, 1:150 then, $CH_3OH/CHCl_3$, 1:100 then, $CH_3OH/CHCl_3$, 1:70 to afford **13** (25.6 mg, 43%) as a mixture of two anomers, yellow oil: Rf = 0.43 ($CH_3OH/CHCl_3$, 1:15); ^1H NMR (400 MHz, $CDCl_3$) δ 6.23 (dd, 1H, *J* = 9.2, 6.4 Hz), 6.09 (dd, 1H, *J* = 8.4, 5.2 Hz), 5.25 (br, 1H), 4.89–4.84 (m, 1H), 4.59–4.52 (m, 1H), 4.34 (dddd, 1H, *J* = 4.4, 4.4, 4.4, 4.4 Hz), 4.20–3.85 (m, 7H), 3.12–2.78 (m, 8H), 2.60–2.50 (m, 4H), 2.10–2.06 (m, 1H), 1.40–1.30 (m, 10H), 1.16–0.89 (m, 49H); HRMS (ESI) calcd. for $C_{30}H_{58}N_4NaO_5SSi_2^+$ $[M+Na]^+$ *m/z* 665.3559, found *m/z* 665.3563.

4.2.7. Synthesis of 9-[2-(2′-deoxy-3′-methoxymethyl-5′-O-trityl-β-D-ribofuranosyl)ethyl]-6-(2-octylthioethyl)-5-azacytosine (**18**)

Compound **17** (2.67 g, 5.94 mmol) was rendered anhydrous by repeated coevaporation with anhydrous acetonitrile, and then was dissolved in anhydrous CH_2Cl_2 (32 mL). Et_3N (661 μL, 4.74 mmol) was added to a solution and then MsCl (271 μL, 3.48 mmol) was added dropwisely at 0 °C. The resulting mixture was stirred at 0 °C for 1 h and the reaction mixture was diluted with CH_2Cl_2. The solution was washed with sat. NH_4Cl aq. followed by brine. The organic layer was dried over Na_2SO_4, filtered and evaporated. The crude product was purified by silica gel column chromatography (eluent: 25% EtOAc/75% hexane) to afford **18** (2.96 mg, 94%) as a colorless oil: Rf = 0.55 (EtOAc/Hexane, 1:1); ^1H NMR (600 MHz, $CDCl_3$) δ 7.45–7.44 (m, 6H), 7.30 (dd, 6H, *J* = 7.2, 7.2 Hz), 7.24 (tt, 3H, *J* = 7.2, 1.2 Hz), 4.62 (d, 1H, *J* = 10.8 Hz), 4.61 (d, 1H, *J* = 10.8 Hz), 4.41–4.35 (m, 2H), 4.25–4.20 (m, 2H), 4.03 (ddd, 1H, *J* = 7.2, 7.2, 2.4 Hz), 3.14 (quint, 2H, *J* = 13.8, 13.8, 13.8, 4.2 Hz), 2.11–2.05 (m, 2H), 1.97–1.91 (m, 1H), 1.71 (ddd, 1H, *J* = 16.2, 6.0, 6.0 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 144.1, 128.9, 128.0, 127.3, 95.5, 86.8, 84.4, 79.0, 74.9, 67.7, 64.6, 55.6, 38.9, 37.4, 35.3; HRMS (ESI) calcd. for $C_{29}H_34NaO_7S^+$ $[M+Na]^+$ *m/z* 549.1917, found *m/z* 549.1917, calcd. for $C_{19}H_{15}^+$ $[Tr]^+$ *m/z* 243.1168, found *m/z* 243.1162.

4.2.8. Synthesis of 9-[2-(2′-deoxy-β-D-ribofuranosyl)ethyl]-5-azacytosine (**19**)

5-azacytosine/Na salt (829 mg, 6.19 mmol) was added to the **18** (2.96 g. 5.63 mmol) in anhydrous DMF (56 mL). The resulting mixture was stirred at 90 °C for 5 h and then, was added Cs_2CO_3 (ca. 4.0 mg). The mixture was further stirred at 120 °C for 2 h. The resulting mixture was diluted with AcOEt (300 mL). The mixture was washed with NH_4Cl aq. followed by brine. The aqueous layer was extracted with AcOEt (50 mL × 4). The organic layer was combined, washed with brine, dried over Na_2SO_4, filtered, and evaporated. The crude product was purified by silica gel column chromatography (eluent: 97% $CHCl_3$/2% CH_3OH/1% Et_3N) to afford **19** (1.68 g, 55%) as a white form: Rf = 0.53 ($CH_3OH/CHCl_3$, 1:10); ^1H NMR (600 MHz, $CDCl_3$) δ 7.98 (s, 1H), 7.45–7.43 (m, 6H), 7.30 (dd, 6H, *J* = 7.2, 7.2 Hz), 7.24 (tt, 3H, *J* = 7.8, 1.8 Hz), 4.61 (d, 1H, *J* = 10.2 Hz), 4.59 (d, 1H, *J* = 10.2 Hz), 4.22–4.21 (m, 1H), 4.05–4.00 (m, 3H), 3.84 (ddd, 1H, *J* = 15.0, 9.0, 9.0 Hz), 3.15 (dd, 1H, *J* = 10.2, 4.2 Hz), 3.09 (dd, 1H, *J* = 10.2, 4.2 Hz), 2.24–2.19 (m, 1H), 2.02 (ddd, 1H, *J* = 13.2, 5.4, 1.8 Hz), 1.75–1.65 (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 144.1, 128.9, 128.0, 127.3, 95.5, 86.8, 84.4, 79.0, 74.9, 67.7, 64.6, 55.6, 38.9, 37.4, 35.3; HRMS (ESI) calcd. for $C_{31}H_{35}N_4O_5^+$ $[M+H]^+$ *m/z* 543.2602, found *m/z* 543.2610, calcd. for $C_{31}H_{34}N_4NaO_5^+$ $[M+Na]^+$ *m/z* 565.2421, found *m/z* 565.2431, calcd. for $C_{62}H_{69}N_8O_{10}^+$ [2 M + H]$^+$ *m/z* 1085.5131, found *m/z* 1085.5176, calcd. for $C_{62}H_{68}N_8NaO_{10}^+$ [2 M + Na]$^+$ *m/z* 1107.4951, found *m/z* 1107.5000.

4.2.9. Synthesis of 5-O-Tr-3-O-MOM-N-(aminoiminomethyl)-N′-(2-deoxy-β-D-erythro-pentofuranosyl) urea (**20**)

To a solution of **19** (38.1 mg, 70.2 μmol) in dioxane-methanol (2.0 mL, 1/1, v/v) was added 28% NH_4OH (2.0 mL), and then was sealed with a glass stopper. The mixture was stirred at 50 °C for 12 h. The resulting solution was evaporated under vacuum at 43 °C. The resulting moist solid was co-evaporated with ethanol three times, CH_3CN three times and Et_2O to afforded **20** as a white form. The product directly used for the next step without further purification: Rf = 0 ($CH_3OH/CHCl_3$, 1:10); HRMS (ESI) calcd. for $C_{30}H_{37}N_4O_5^+$ $[M+H]^+$ *m/z* 533.2758, found *m/z* 533.2760, calcd. for $C_{30}H_{36}N_4NaO_5^+$ $[M+Na]^+$ *m/z* 555.2578, found *m/z* 555.2579.

4.2.10. Synthesis of 9-[2-(2′-deoxy-β-D-ribofuranosyl)ethyl]-6-(2-octylthioethyl)-5-azacytosine (**21**)

To a solution of **20** (70.2 μmol) in anhydrous DMF (350 μL) was added orthoester **6** (108 mg, 351 μmol, purity = 90%), and then was stirred at 120 °C for 90 min. After the solution became yellow solution, the mixture was diluted with Et_2O. The solution was washed with NH_4Cl aq., $NaHCO_3$ aq., and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated. The crude product was purified by silica gel column chromatography (eluent $CH_3OH/CHCl_3$, 1:300 with 1% Et_3N) to afford **21** (27.2 mg, 54% in 2 steps) as a pale yellow form: Rf = 0.44 ($CH_3OH/CHCl_3$, 1:15 with 1% Et_3N); ^1H NMR (600 MHz, $CDCl_3$) δ 7.45 (d, 6H, *J* = 7.2 Hz), 7.30 (dd, 6H, *J* = 7.2, 7.2 Hz), 7.24 (dd, 3H, *J* = 7.2, 7.2 Hz), 5.49 (br, 1H), 5.13 (br, 1H), 4.60 (d, 1H, *J* = 11.4 Hz), 4.59 (d, 1H, *J* = 11.4 Hz), 4.19–4.18 (m, 1H), 4.08–4.01 (m, 4H), 3.17 (dd, 1H, *J* = 9.6, 4.8 Hz), 3.08 (dd, 1H, *J* = 9.6, 4.8 Hz), 2.93 (ddd, 1H, *J* = 16.2, 7.2, 7.2 Hz), 2.84–2.82 (m, 2H), 2.44 (dd, 1H, *J* = 7.2, 7.2 Hz), 2.20–2.15 (m, 1H), 2.03–2.00 (m, 1H), 1.75–1.69 (m, 1H), 1.66 (ddd, 1H, *J* = 13.2, 13.2, 6.0 Hz), 1.50 (quint, 2H, *J* = 7.2 Hz), 1.30–1.24 (m, 10H), 0.88 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 144.1, 128.9, 128.0, 127.3, 95.5, 86.8, 84.4, 79.0, 74.9, 67.7, 64.6, 55.6, 38.9, 37.4, 35.3; HRMS (ESI) calcd. for $C_{41}H_{55}N_4O_5S^+$ $[M+H]^+$ *m/z* 715.3888, found *m/z* 715.3952, calcd. for $C_{41}H_{54}N_4NaO_5S^+$ $[M+Na]^+$ *m/z* 737.3707, found *m/z* 737.3712.

4.2.11. Synthesis of 9-[2-(2′-deoxy-3′-O-methoxymethyl-5′-O-trityl-β-D-ribofuranosyl)ethyl]-6-(2-octylthioethyl)-4-N-acetyl-5-azacytosine (**22**)

To a solution of **21** (44.0 mg, 61.5 μmol) in pyridine (0.31 mL) was slowly added AcCl (8.55 μL, 92.3 μmol). After addition, the mixture was warmed to room temperature, stirred for 1 h, and then the mixture was diluted with Et_2O (25 mL). The mixture was washed with NH_4Cl aq. (× 2), sat. $NaHCO_3$ aq., and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated. The crude product was purified by silica gel column chromatography (eluent: $CH_3OH/CHCl_3$, 1:300) to afford **22** (37.4 mg, 80%) as a pale yellow form: Rf = 0.56 ($CH_3OH/CHCl_3$, 1:15); ^1H NMR (600 MHz, $CDCl_3$) δ 7.58 (br, 1H), 7.43 (d, 6H, *J* = 7.8 Hz), 7.30 (dd, 6H, *J* = 7.8, 7.8 Hz), 7.24 (dd, 3H, *J* = 7.8, 7.8 Hz), 4.60 (d, 1H, *J* = 12.0 Hz), 4.59 (d, 1H, *J* = 12.0 Hz), 4.25–4.19 (m, 1H), 4.13–4.01 (m, 4H), 3.29 (s, 3H), 3.18–3.09 (m, 3H), 3.04–2.98 (m, 1H), 2.85–2.83 (m, 2H), 2.58 (s, 3H), 2.45 (t, 2H, *J* = 7.2 Hz), 2.22–2.18 (m, 1H), 2.03 (dd, 1H, *J* = 13.2, 4.2 Hz), 1.78–1.54 (m, 4H), 1.50 (t, 2H, *J* = 7.2 Hz), 1.31–1.22 (m, 10H), 0.88 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.2, 171.1, 161.7, 155.1, 144.0, 128.9, 128.1, 127.3, 95.5, 86.9, 84.6, 78.9, 75.8, 68.4, 64.5, 55.6, 43.0, 38.7, 34.4, 33.7,

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32.8, 32.0, 30.5, 29.7, 29.4, 29.0, 28.0, 22.9, 14.3; HRMS (ESI) calcd. for $C_{43}H_{57}N_4O_6S^+$ $[M+H]^+$ *m/z* 757.3993, found *m/z* 757.3999, calcd. for $C_{43}H_{56}N_4NaO_6S^+$ $[M+Na]^+$ *m/z* 779.3813, found *m/z* 779.3823.

4.2.12. Synthesis of 9-[2-(2′-deoxy-β-D-ribofuranosyl)ethyl]-6-(2-octylthioethyl)-4-N-acetyl-5-azacytosine (**23**)

To a chilled solution of **22** (185 mg, 0.245 mmol) and dimethylsulfide (3.18 mL, 43.4 mmol) in anhydrous CH_2Cl_2 (6.1 mL) was slowly added borontrifluoride-diethylether complex (696 μL, 5.64 mmol) at 0 °C and was stirred at 0 °C for 1.5 h. The reaction mixture was poured into a mixture of CH_2Cl_2 (70 mL) and sat $NaHCO_3$ aq. (40 mL) with stirring at 0 °C. Organic layer of the mixture was washed with $NaHCO_3$ aq. (× 3), and brine (× 2). The aqueous layer was extracted with CH_2Cl_2 (× 5), and then these organic layers were all combined. The organic layer was dried over Na_2SO_4, filtered and evaporated. The crude product was purified by silica gel column chromatography (eluent: $CH_3OH/CHCl_3$, 1:40 with 1% Et_3N) to give **23** (65.6 mg, 57%) as a yellow oil: Rf = 0.26 ($CH_3OH/CHCl_3$, 1:9 with 1% Et_3N); ^1H NMR (600 MHz, $CDCl_3$) δ 7.74 (s, 1H), 4.36 (br, 1H), 4.19–4.07 (m, 3H), 3.86 (dd, 1H, *J* = 4.2, 4.2 Hz), 3.75–3.72 (m, 1H), 3.65–3.62 (m, 1H), 3.12–3.07 (m, 4H), 2.90 (ddd, 2H, *J* = 7.2, 7.2, 1.2 Hz), 2.61 (s, 2H), 2.56 (t, 2H, *J* = 7.2, 7.2 Hz), 2.24–2.14 (m, 2H), 2.03–2.02 (m, 1H), 2.00 (dd, 1H, *J* = 6.0, 2.4 Hz), 1.82–1.75 (m, 2H), 1.59 (quint, 2H, *J* = 7.8 Hz), 1.41 (dd, 2H, *J* = 7.2, 7.2 Hz), 1.39–1.24 (m, 10H), 0.88 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.2, 170.1, 161.8, 158.7, 155.2, 87.3, 75.6, 75.6, 73.3, 63.2, 46.0, 42.7, 41.6, 34.4, 34.1, 33.0, 32.0, 29.8, 29.4, 29.1, 28.1, 26.1, 22.9, 14.3, 8.9, 0.2; HRMS (ESI) calcd. for $C_{22}H_{39}N_4O_5S^+$ $[M+H]^+$ *m/z* 471.2636, found *m/z* 471.2635, calcd. for $C_{22}H_{38}N_4NaO_5S^+$ $[M+Na]^+$ *m/z* 493.2455, found *m/z* 493.2454.

4.2.13. Synthesis of 9-[2-(2′-deoxy-5′-O-DMTr-β-D-ribofuranosyl) ethyl]-6-(2-octylthioethyl)-4-N-acetyl-5-azacytosine (**24**)

To a chilled solution of **23** (30.0 mg) in pyridine (640 μL) was added DMTrCl (32.4 mg, 95.7 μmol) in two portion over 5 min at 0 °C. After addition, the mixture was stirred at 0 °C for 1 h. The mixture was diluted with CH_2Cl_2, washed with $NaHCO_3$ aq. (× 2), and brine (× 2). The organic layer was dried over Na_2SO_4, filtered and evaporated. The crude product was co-evaporated with toluene, and then was purified by silica gel column chromatography (eluent: $CH_3OH/CHCl_3$, 1:200 with 1% Et_3N) to give **24** (30.2 mg, 61%) as a yellow oil: Rf = 0.60 ($CH_3OH/CHCl_3$, 1:10 with 1% Et_3N); ^1H NMR (600 MHz, $CDCl_3$) δ 7.61 (br, 1H), 7.41 (d, 2H, *J* = 8.4 Hz), 7.29 (ddd, 6H, *J* = 8.4, 8.4, 1.8 Hz), 7.21 (dd, 1H, *J* = 7.2, 7.2 Hz), 6.83 (d, 4H, *J* = 8.4 Hz), 4.29–4.27 (m, 1H), 4.14–4.11 (m, 1H), 4.10 (dd, 2H, *J* = 7.2, 7.2 Hz), 3.91–3.88 (m, 1H), 3.79 (s, 6H), 2.58 (s, 3H), 2.46 (t, 2H, *J* = 7.2 Hz), 3.23 (dd, 1H, *J* = 9.6, 4.8 Hz), 3.15 (ddd, 1H, *J* = 16.8, 7.2, 7.2 Hz), 3.09 (dd, 1H, *J* = 9.6, 6.0 Hz), 2.99 (ddd, 1H, *J* = 16.8, 6.6, 6.6 Hz), 2.89–2.81 (m, 2H), 2.21–2.15 (m, 1H), 1.96 (ddd, 1H, *J* = 12.6, 5.4, 1.8 Hz), 1.81–1.73 (m, 3H), 1.51 (quint, 2H, *J* = 7.2 Hz), 1.31–1.24 (m, 10H), 0.88 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.2, 171.1, 161.7, 158.7, 155.1, 144.9, 136.08, 136.05, 130.2, 128.3, 128.1, 127.1, 113.3, 86.4, 86.1, 75.4, 74.6, 64.6, 55.5, 46.1, 42.9, 40.9, 34.3, 33.8, 32.9, 32.0, 29.7, 29.4, 29.1, 28.0, 26.1, 22.9, 14.3; HRMS (ESI) calcd. for $C_{43}H_{57}N_4O_7S^+$ $[M+H]^+$ *m/z* 773.3942, found *m/z* 773.3947, calcd. for $C_{43}H_{56}N_4NaO_7S^+$ $[M+Na]^+$ *m/z* 795.3762, found *m/z* 795.3784, calcd. for $C_{21}H_{19}O_2^+$ $[DMTr]^+$ *m/z* 303.1380, found *m/z* 303.1397.

4.2.14. Synthesis of 9-[2-(2′-deoxy-3′-O-N,N-diisopropylcyanoethylphosphoramidyl-5′-O-DMTr-β-D-ribofuranosyl)ethyl]-6-(2-octylthioethyl)-4-N-acetyl-5-azacytosine (**25**)

To a solution of **24** (48.1 mg, 62.2 μmol) and DIPEA (63.3 μL, 373 μmol) in anhydrous CH_2Cl_2 (1.24 mL) was cooled to 0 °C, and then was added 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (41.6 μL, 186 μmol). After the mixture was stirred at 0 °C for 45 min, the solution was diluted with CH_2Cl_2, washed with $NaHCO_3$ aq., and washed with brine. The organic layer was dried over Na_2SO_4, filtered and evaporated. The crude product was co-evaporated with toluene, and then was purified by silica gel column chromatography (eluent: AcOEt/hexane, 1:1 with 1% Et_3N). The resulting product was diluted with Et_2O. The organic layer was washed with $NaHCO_3$ aq., and distilled water. The organic layer was dried over Na_2SO_4, filtered and evaporated to give **25** (30.1 mg, 61%) as a mixture of two diastereomer at phosphorus, white form: Rf = 0.53 ($CH_3OH/CHCl_3$, 1:20 with 1% Et_3N); ^1H NMR (600 MHz, $CDCl_3$) δ 7.59 (br, 2H), 7.42 (dd, 4H, *J* = 8.0, 8.0 Hz), 7.32–7.27 (m, 12H), 7.22 (dd, 2H, *J* = 7.2, 7.2 Hz), 6.82 (dd, 8H, *J* = 8.0, 8.0 Hz), 4.38 (dd, 2H, *J* = 8.4, 8.4 Hz), 4.13–4.04 (m, 8H), 3.793, 3.788 (2s, 12H), 3.74–3.49 (m, 10H), 3.18–3.09 (m, 6H), 3.00 (ddd, 2H, *J* = 16.8, 7.2, 7.2 Hz), 2.84 (t, 4H, *J* = 7.2 Hz), 2.60 (dd, 1H, *J* = 6.6, 6.6 Hz), 2.58 (s, 6H), 2.43 (ddd, 6H, *J* = 7.8, 7.8, 7.8 Hz), 2.23–2.17 (m, 4H), 2.13 (dd, 1H, *J* = 13.2, 5.4 Hz), 2.02 (ddd, 1H, *J* = 13.2, 4.8 Hz), 1.83–1.75 (m, 5H), 1.49 (quint, 4H, *J* = 7.2 Hz), 1.32–1.21 (m, 29H), 1.56 (dd, 14H, *J* = 6.6, 6.6 Hz), 1.17 (d, 4H, *J* = 6.6 Hz), 1.05 (d, 4H, *J* = 6.6 Hz), 0.87 (t, 6H, *J* = 7.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.2, 171.1, 161.7, 158.68, 158.66, 155.1, 145.0, 136.2, 136.1, 130.30, 130.26, 128.42, 128.37, 128.0, 127.04, 127.00, 113.3, 86.2, 75.9, 75.6, 75.4, 64.2, 64.1, 58.49, 58.46, 58.4, 58.3, 55.5, 55.4, 43.4, 43.34, 43.32, 43.26, 43.0, 42.9, 40.2, 34.4, 33.7, 32.89, 32.87, 32.0, 31.2, 29.7, 29.4, 29.0, 28.0, 26.1, 24.83, 24.79, 24.76, 24.70, 24.65, 23.1, 22.9, 22.7, 20.61, 20.57, 20.41, 20.36, 14.3; ^{31}P NMR (162 MHz, $CDCl_3$) δ 148.2, 147.9; HRMS (ESI) calcd. for $C_{52}H_{74}N_6O_8PS^+$ $[M+H]^+$ *m/z* 973.5026, found *m/z* 973.5016, calcd. for $C_{52}H_{73}N_6NaO_8PS^+$ $[M+Na]^+$ *m/z* 995.4840, found *m/z* 995.4827.

4.3. Oligonucleotide synthesis

4.3.1. Synthesis of ODN1

The synthesis of DNA oligonucleotide having Et-AOVT nucleotide was performed on ABI 392 automated DNA synthesizer. The 5′-DMTr-ON synthesis was conducted standard 1.0 μmol scale phosphoramidite approach, which consists of (i) detritylation, (ii) coupling, (iii) capping, and (iv) iodine oxidation. 5-ethylthiotetrazole was used for activating reagent, 3% dichloroacetic acid in CH_2Cl_2 was used for detritylation. 5% $(Tac)_2O$ in THF and 16% *N*-methylimidazole in THF were used for capping reaction. 0.02 M I_2 in THF-pyridine-H_2O (7:2:1, v/v/v) was used for oxidation. All phosphoramidite including AOVT-nucleoside phosphoramidite were prepared as 0.1 M CH_3CN solution and coupled for 110s. After the chemical chain elongation, deprotection and cleavage from the solid support was conducted by 45 mM K_2CO_3/MeOH solution containing 10 mM 1-octanethiol at room temperature for 4 h. Purification of 5′-DMTr-ON oligonucleotide was conducted on Nacalai Tesque COSMOSIL 5C18-MS-II RP-HPLC column (10 × 250 mm) at a flow rate of 4 mL/min with a gradient mobile phase from 10% CH_3CN in 0.1 M TEAA buffer (pH 7.0) to 40% CH_3CN for 20 min. HPLC profiles were obtained by monitoring UV absorption at 254 nm. Collected oligonucleotide was freeze-dried and treated with aqueous 10% AcOH for 30 min at room temperature, and then centrifuged by centrifugation. Resulting oligonucleotide was further purified on Nacalai Tesque COSMOSIL 5C18-MS-II RP-HPLC column (10 × 250 mm) at a flow rate of 4 mL/min with a gradient mobile phase from 5% CH_3CN in 0.1 M TEAA buffer (pH 7.0) to 40% CH_3CN for 25 min. Collected pure ODN**1** was dried by freeze-dry and characterized by corresponding MALDI-TOF MS peak at 4085.4 $[M-H]^-$ (calcd *m/z* 4087.9).

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4.3.2. Synthesis of cross-linked oligonucleotides

2 μM of ODN**3** and 2 μM of cRNA-U (or C) were incubated in 50 mM MES buffer (pH 7.0) containing 100 mM NaCl at 37 °C for 48 h. Obtained cross-linked duplex was purified on Nacalai Tesque COSMOSIL 5C18-MS-II RP-HPLC column (4.5 × 250 mm) at a flow rate of 1 mL/min with a gradient mobile phase from 5% CH_3CN in 0.1 M TEAA buffer (pH 7.0) to 40% CH_3CN for 25 min. Collected cross-linked duplexes were dried by freeze-dry and characterized by corresponding MALDI-TOF MS peak at 8675.1 $[M+H]^+$ (calcd *m/z* 8675.7 for ODN**3**:cRNA-U) and 8676.4 $[M+H]^+$ (calcd *m/z* 8674.7 for ODN**3**:cRNA-C), respectively. The purity of purified cross-linked duplexes were confirmed on denaturing 20% polyacrylamide gel electrophoresis (7 M urea).

4.3.3. Synthesis of AOVT^{et}-ODN

After ODN**1** was converted to ODN**3**, the oligonucleotide was incubated in aqueous 5.7 mM $NaBH_4$ (50 eq.) solution at 26 °C for 2 h. Obtained crude product was purified on Nacalai Tesque COSMOSIL 5C18-MS-II RP-HPLC column (4.5 × 250 mm) at a flow rate 1 mL with a gradient mobile phase from 5% CH_3CN in 0.1 M TEAA buffer (pH 7.0) to 40% CH_3CN for 25 min. Collected AOVT^{et}-ODN was dried by freeze-dry and characterized by corresponding MALDI-TOF MS peak at 3945.5 $[M+H]^+$ (calcd. *m/z* 3945.7).

4.4. Alkali-footprinting of cross-linked CL-rU and CL-rC

0.1 M of cross-linked duplex was incubated in 45 mM K_2CO_3-MeOH (5% H_2O) for 4 h at 26 °C, and then neutralized using 1 M HCl and 0.5 M TEAA buffer (pH 7.0). After evaporating the solution, the residual was desalted by Zip-tip C18 prior to analysis on denaturing 20% polyacrylamide containing 20% formamide and 7 M urea. Bands of the RNA fragments on gel were scanned by fluorescent image analyzer (FLA-5100, FujiFilm).

4.5. Crosslinking reaction and PAGE analysis of reaction products

The unmasking of 6-vinyl group of ODN**1** was conducted in a step-wise manner. First, the 6-octylthioethyl group of 70 μM ODN**1** was oxidized by the treatment with 70 μM MMPP in 1.9 mM carbonate buffer (pH 10) at 26 °C for 30 min, and then the reaction mixture was subsequently added 3.4 times volume of 10% aq. AcOH. After the incubation at 26 °C for 2 h, the solution was concentrated by centrifugation under reduced pressure and right after completion of evaporation, resulting ODN**3** in active vinyl-form was used for crosslinking reaction without further purification. The obtained ODN**3** was characterized by corresponding MALDI-TOF MS peak at 3941.5 $[M-H]^-$ (caldcd *m/z* 3941.6). In the cross-link assay the active 5 μM ODN**3** and 2 μM 5′-FAM labelled cDNAs or cRNAs were incubated at 37 °C in 50 mM MES buffer (pH 7.0) containing 100 mM NaCl. Aliquots of reaction mixture were taken and quenched by adding 95% formamide containing 20 mM EDTA at each time point 2, 4, 6, 12, and 24 h. The yielding cross-linked products at each time point was visualized on denaturing 20% polyacrylamide gels containing 7 M urea, and quantitated by using a fluorescent image analyzer (FLA-5100, FujiFilm). The yield of crosslink reaction was calculated from the ratio of the cross-linked product and remaining cDNAs or cRNAs.

4.6. UV melting temperature analysis

All duplexes containing AOVT^{et}-ODN and cDNAs (or cRNAs) were annealed by heating at 90 °C for 1 min and cooled down gradually to room temperature. T_m measurement was performed with temperature controller. Both the heating and cooling curves were measured over a temperature range of 20 °C to 85 °C at 1.0 °C/min in three times. The absorbance at 260 nm was recorded at every temperature points.

4.7. The reactivity of AOVT in the duplex with exogenous nucleophile

1 μM ODN**3** and 1 μM 5′-FAM-labelled cRNA-G were pre-incubated for 10 min at 37 °C in a solution containing 50 mM MES buffer (pH 7.0) and 100 mM NaCl. To a solution was added the 100 μM 4-*tert*-butylbenzenethiol and incubated at 37 °C. Aliquots were taken at 2 h, diluted with 0.1 M TEAA, and analyzed on Nacalai Tesque COSMOSIL 5C18-MS-II RP-HPLC column (4.6 × 250 mm) at a flow rate 1 mL with a gradient mobile phase from 5% CH_3CN in 0.1 M TEAA buffer (pH 7.0) to 40% CH_3CN for 25 min. The observed new major peak (Rt = 16 min) was collected and characterized as a thiol adduct by MALDI-TOF MS peak at 4110.9 $[M-H]^-$ (calcd *m/z* 4109.7).

4.8. Quantum mechanical calculations

Optimized structure of me-AOVP and me-AOVT were obtained by ab-initio MO calculation at RB3LYP/6-311G+ (d,p) level of theory using the program Gaussian 09.[54] Electrostatic potential (ESP) map was drawn on gauss view.

Conflict of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2017.01.043.

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