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PAPER

Bridged nucleic acid conjugates at 6′-thiol: synthesis, hybridization properties and nuclease resistances†

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The bridged nucleic acid (BNA) containing a thiol at the 6′-position in the bridged structure was synthesized from the disulfide-type BNA and conjugated with various functional molecules *via* the thioether or the disulfide linkage post-synthetically and efficiently in solution phase. The disulfide-linked conjugate was cleaved under reductive conditions derived from glutathione and an oligonucleotide bearing a free thiol was released smoothly. Conjugated functional molecules had great effects on duplex stability with the DNA complement. In contrast, the molecules little influenced the stability with the RNA complement. Moreover, the oligonucleotides with functional groups at the 6′-position had as high or higher resistances against 3′-exonuclease than phosphorothioate oligonucleotide (S-oligo).

Introduction

Selective inhibition of gene expression resulting from binding of oligonucleotides (ONs) to the target sequences shows promise as an attractive chemotherapy,[1] but there have been few clinical successes to date.[2] This is mainly due to the fact that the natural ONs do not have enough target specificity, resistance toward the nucleases, and ability to penetrate the cell membrane.[2] In order to improve their properties, nucleic acids have been chemically modified by a wide variety of approaches, for example, restriction of sugar conformation to an appropriate form[3,4] or conjugation with functional groups and molecules.[5] Numerous 2′,4′-bridged nucleic acid (BNA)[6]/LNA[7] analogues have been developed by our group[3] or other groups.[4] Their sugar moieties are restricted to North-type (N-Type) conformation by bridging between C2′- and C4′-positions. They have high duplex forming ability for complementary RNA because of the conformations similar to the ribonucleotides of the RNA duplex, and have high resistance against enzymatic degradation.

Conjugation of functional groups and molecules to ONs is frequently used for numerous applications, such as therapeutics, diagnostics and nanotechnology, because it can easily improve the existing ON's properties; moreover, it can provide brand new properties.[8,9] ON conjugates are prepared by some condensation reactions with an amino, a hydroxy, or a thiol group.[10] Above all, a thiol group is very useful since it reacts chemoselectively and has two major types of reaction, nucleophilic reaction and thiol–disulfide exchange.[8,10] As the disufide linkage is reversible, it is often used for DDS (Drug Delivery Systems).[11] The disulfide linkage is cleaved in the intracellular reducing environment and the ON is selectively released into the cell.[12] 5′- or 3′-termini are mostly selected as conjugation sites due to their easy accesibility.[8] The 1′-,[13] 2′-[14–16] and 4′-positions[17] of the sugar moiety, nucleobase,[18] and internucleotidic phosphodiester bond[19] are also used for conjugation. However, the three dimensional position of a functional molecule varies depending on the conjugation site and this has an important consequence for its effect.[15,20]

In this paper, we present the design and synthesis of a novel BNA bearing a thiol group in the bridged structure, conjugation of various molecules to the ON including the BNA, and evaluation of their thermal stabilities and nuclease resistances. The C6′-position has not often been used as conjugation site[21] and the effect of conjugation at this position has not been investigated enough. We report here the efficiency and the effect of conjugation at this position. Post-synthetic conjugation (solution phase conjugation) basically needs large equivalents of reagents because of the high diluted conditions. In this study, ONs were conjugated *via* a thiol group by a solution phase coupling approach because it is a simple and rapid method. Owing to the bridged structure, the three dimensional position of additional groups and molecules will be highly controlled and ON conjugates will display functions which are different from flexible analogs.

Results and discussion

Design and synthesis of phosphoramidite and preparation of oligonucleotides

We previously synthesized a BNA monomer bearing a disulfide bridged structure (disulfide-type BNA, **1**).[22] It was expected that

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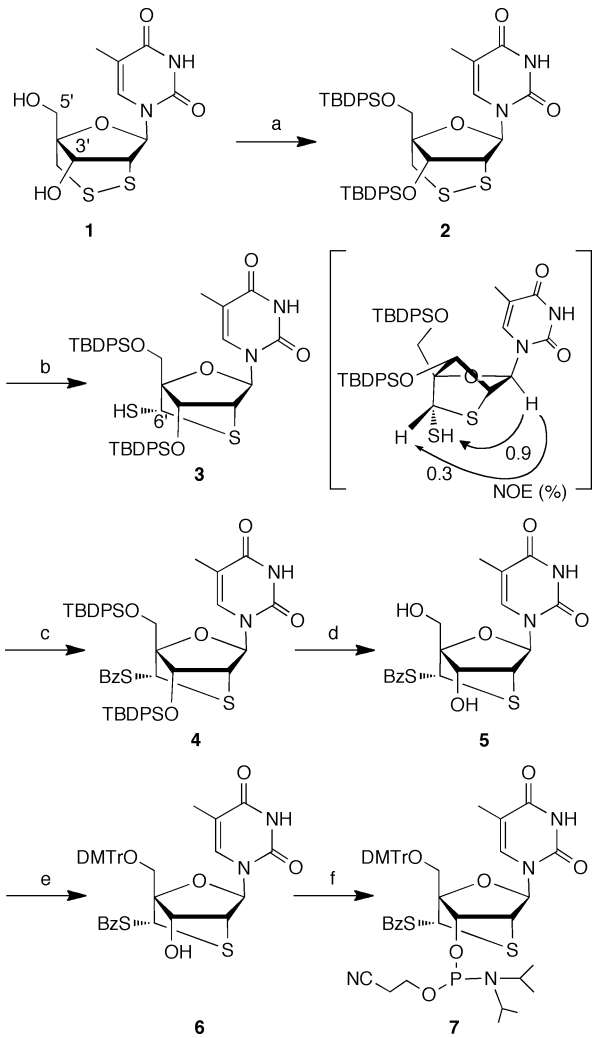
† Electronic supplementary information (ESI) available: HPLC and MALDI data for ON conjugates, NMR spectra of new compounds, and UV melting profiles. See DOI: 10.1039/c1ob05469d

this disulfide-type BNA with a 1,2-dithiane structure would be converted to a BNA bearing a thiol at the 6′-position by photoirradiation,[23] and various molecules were conjugated at the 6′-position *via* the thiol. Since this thiol-containing BNA has the skeleton of 2′-thio-LNA, which has high duplex-forming ability for complementary RNA and high enzymatic stability,[24] the thiol-containing BNA was also anticipated to have similar characters in those regards.

The thiol-containing BNA was synthesized from disulfide-type BNA monomer **1**[22] as shown in Scheme 1. First, the nucleoside **1**, reported previously by us, was treated with *tert*-butyldiphenylsilyl chloride (TBDPSCl) in the presence of *N*,*N*-dimethyl-4-aminopyridine to protect the 3′- and 5′-hydroxy groups. Then, the protected disulfide-type BNA monomer **2** was photoirradiated[23] to yield a thiol-containing BNA monomer **3** effectively and diastereoselectively. The configuration at the C6′ atom was determined by NOE (nuclear Overhauser effect) experiment. This diastereoselectivity is most likely explained by steric hindrance between the thiol and bulky 3′-*O*-TBDPS group. Next, the crude product **3** was subjected to benzoyl chloride in order to protect the chemically labile hemidithioacetal motif. Subsequently, the 3′- and 5′-*O*-TBDPS groups were removed by tetrabutylammonium fluoride to give nucleoside **5**. Tritylation at the 5′-hydroxy group with 4,4′-dimethoxytrityl chloride and phosphitylation at the 3′-hydroxy group with *N*,*N*-diisopropylamino-2-cyanoethylphosphino-chloridite afforded the desired phosphoramidite building block **7**.



Scheme 1 *Reagents and conditions*: (a) TBDPSCl, DMAP, DMF, 100 °C, 15 h, 70%; (b) hν, CH_2Cl_2, rt, 30 min; (c) BzCl, pyridine, rt, 1 h, 81% (over 2 steps); (d) TBAF, AcOH, THF, rt, 24 h, 70%; (e) DMTrCl, pyridine, rt, 3 h, 79%; (f) iPr$_2$NP(Cl)O(CH$_2$)$_2$CN, DIPEA, CH_3CN, 0 °C, 1.5 h, 66%. TBDPS = *tert*-butyldiphenylsilyl, Bz = benzoyl, TBAF = tetra-n-butylammonium fluoride, DMTr = 4,4′-dimethoxytrityl, DIPEA = *N*,*N*-diisopropylethylamine.

Compound **7** was introduced into ONs using an automated DNA synthesizer. The sequences were the same as those of our previous studies.[25] The concentration of phosphoramidite **7** was 0.10 M and its coupling time was 20 min, while the concentration of each unmodified phosphoramidite was 0.067 M and their coupling times were 90 s. 5-[3,5-Bis(trifluoromethyl)phenyl]-1*H*-tetrazole was used as an activator for both modified and unmodified phosphoramidite. Coupling yields were checked by trityl monitoring and were estimated to be over 95%. Synthesized ONs were cleaved from the solid supports and deprotected in the presence of 2,2′-dithiodipyridine (DTDP) to prevent a thiol group from reacting with released acrylonitrile and to preactivate the thiol with the pyridinesulfenyl group, which acts as a good leaving group in the presence of free thiol (Fig. 1).[26] The obtained ON **8** and ON **9** were purified by reverse-phase HPLC (RP-HPLC) and characterized by MALDI-TOF mass spectrometry.

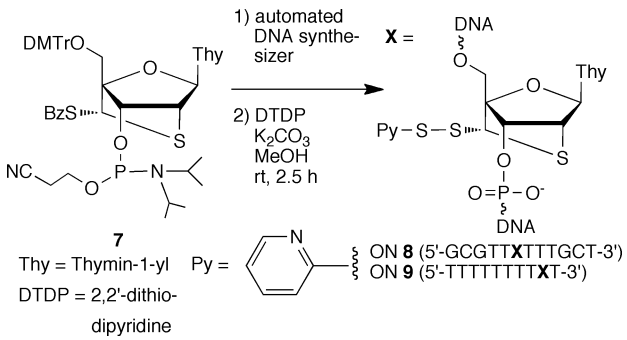


Fig. 1 Conversion of phosphoramidite **7** to ON **8**, **9**.

Solution-phase conjugation

Various molecules were conjugated to ON **8** or **9** *via* thioether or disulfide linkage. First, ON **8** or **9** was treated with dithiothreitol (DTT) dissolved in sodium phosphate buffer (pH = 8.0) to give ON **10** or **11**, then without any purification of ON **10** or **11**, acetic acid was added to acidify the solution and avoid side reactions (*e.g.*, alkylation of amino groups on nucleobases). Subsequently, 1 M primary halogenoalkyl derivative in DMF was added to the solution to afford thioether-linked conjugates (Fig. 2A). The reactions were analyzed by RP-HPLC. After completing the reactions, ONs were precipitated by adding 5 volumes of ethanol at 0 °C. The obtained ON conjugates were purified by RP-HPLC and characterized by MALDI-TOF mass spectrometry. Conjugation yields (HPLC yield and isolation yield) are shown in Fig. 2C. For most modifiers, clean conversions to new ONs were observed. For example, Fig. 3A shows the RP-HPLC profile of a representative

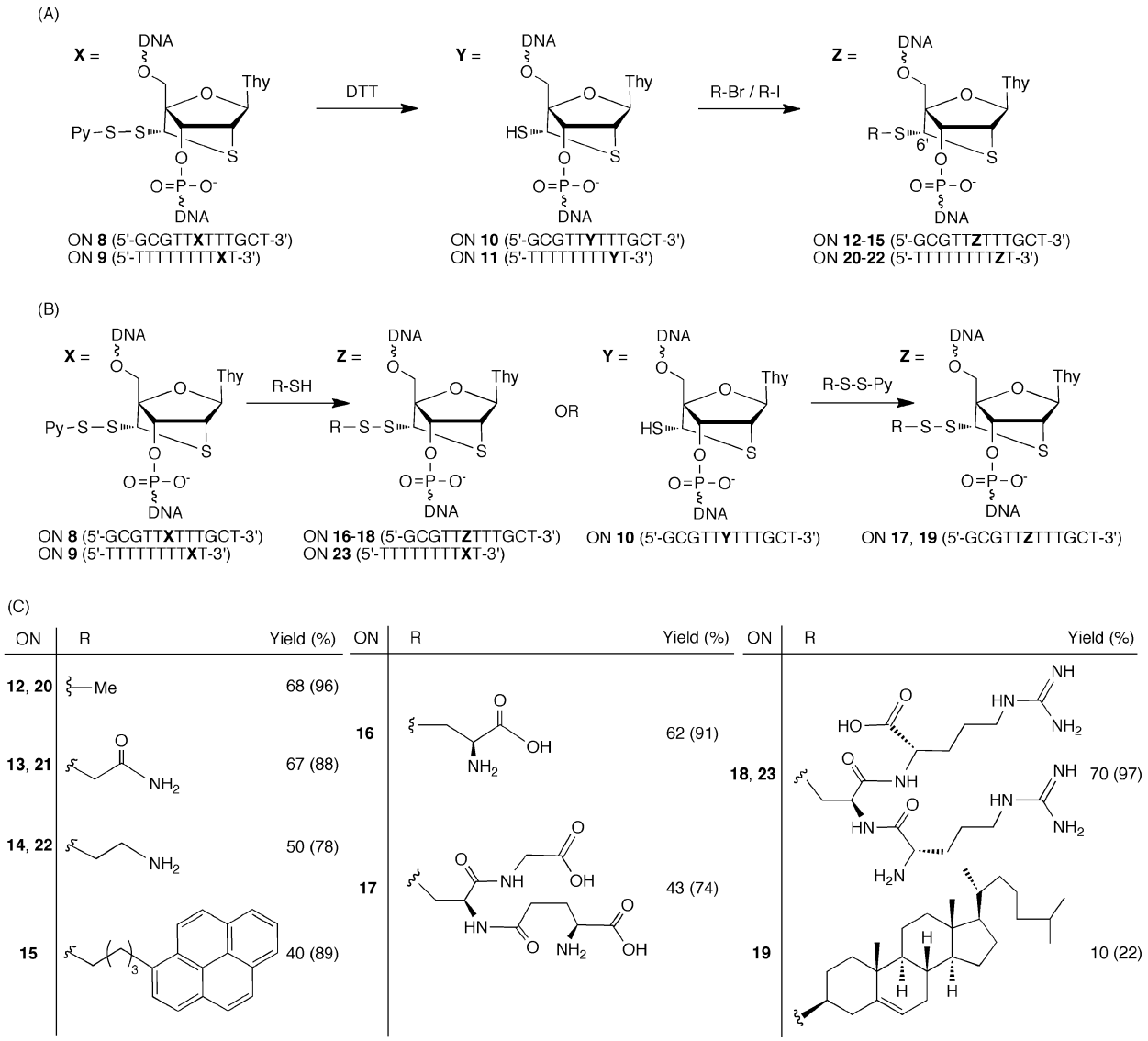


Fig. 2 Synthesis of thioether-linked conjugates and disulfide-linked conjugates. (A) Thioether-linked conjugation by *S*-alkylation, (B) disulfide-linked conjugation by disulfide exchange reaction, and (C) their conjugation yields. HPLC yield in parenthesis was determined by RP-HPLC of reaction solution, and the isolation yield was based on the UV absorption at 260 nm of purified ON.

synthesis of ON **13** from ON **8** *via* ON **10**. When treating ON **8** with DTT for 2 h, the peak corresponding to ON **8** (retention time = 28 min) disappeared, and ON **10** (retention time = 20 min) was generated. Furthermore, 24 h after adding 2-bromoacetamide, ON **10** was transformed into ON **13** (retention time = 16 min). For further synthetic details, see experimental section and the ESI.† These nucleophilic conjugations proceed in good yields, but the yield of ON **15** was slightly lower because it was more lipophilic than other ONs and was lost to some extent in ethanol precipitation.

The disulfide-linked conjugates were generally prepared by disulfide exchange reactions between a free thiol in reaction partners and a pyridinesulfenyl-activated ON **8** or **9** (Fig. 2B). In disulfide-linked conjugation, as in the case of thioether-linked conjugation, good conversions to ON conjugates were observed. For example, Fig. 3B shows the RP-HPLC profile of a typical synthesis of ON **16** from ON **8**. Twenty four hours after treating ON **8** with L-cysteine, the peak corresponding to ON **8** diminished and ON **16** (retention time = 14 min) appeared (see the experimental section and ESI for further synthesis).† Each conjugation yield was good, except ON **19**. When ON **8** was treated with glutathione, the yield of ON **17** was very low owing to the reducing ability of glutathione (ON **17** was reduced by glutathione to give ON **10**). In this case, the yield was improved by treating ON **10** with *S*-(2-thiopyridyl)glutathione.[27] Since thiocholesterol was not reacted with ON **8** due to its insolubility, the conjugation was performed by mixing 2-pyridyl-3-cholesteryl disulfide[28] with ON **10**. Nonetheless, the yield of ON **19** was still low because of its low solubility.

These data show that the C6′ thiol group has high reactivity and chemoselectivity except for the conjugation with a portion of highly lipophilic molecules. This BNA is expected to conjugate with a huge variety of molecules easily in solution phase.

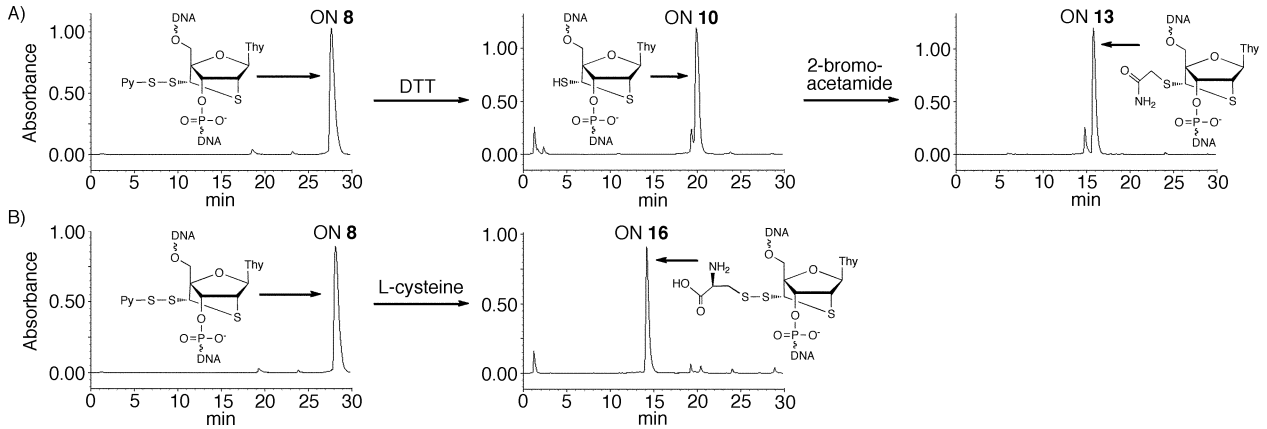


Fig. 3 RP-HPLC analyses of solution phase conjugations with a linear gradient of MeCN 6 to 12% over 30 min. (A) Thioether-linked conjugation with acetamide and (B) disulfide-linked conjugation with L-cysteine.

Evaluation of thermal stabilities

We evaluated the affinity of the synthesized ONs with complementary single-stranded RNA (ssRNA) and DNA (ssDNA) through UV melting experiments. The UV melting profiles are shown in Fig. S1 and S2 (ESI†), and the thermal denaturation temperatures (T_m values) are summarized in Table 1.

The T_m value of the ssDNA–ON **10** (containing naked thiol) duplex was lower than that of the natural DNA duplex (ΔT_m = −3 °C), but conjugation with a cationic group (ON **14**) led to improvement of the duplex stability (ΔT_m = −1 °C, $\Delta\Delta T_m$ = +2 °C). On the contrary, in the case of an anionic molecule conjugation, negative charge of carboxylic acid (ON **17**) destabilized its duplex with ssDNA (ΔT_m = −8 °C, $\Delta\Delta T_m$ = −5 °C). For the cationic group conjugate,[29] it reduced the electrostatic repulsion between the phosphodiester anions in contrast to the anionic group conjugate which increased it.[30] A pyrene conjugate also showed high duplex forming ability for ssDNA because of stacking effects or interaction between the pyrene moiety and hydrophobic nucleobases[31] (ΔT_m = +6 °C, $\Delta\Delta T_m$ = +9 °C). These data indicate that in DNA–DNA duplexes, conjugated groups and molecules directed toward the minor groove of duplexes and influence those hybridization properties.

ON **10** formed a stable duplex with complementary ssRNA (ΔT_m = +3 °C) in a similar manner to previous BNAs[3] because the bridged structure restricted the sugar conformation to N-type.[24] Surprisingly, in the DNA–RNA hybrid duplex, almost all conjugated molecules had no effect on the hybridization properties ($\Delta\Delta T_m$ = 0 °C to −1 °C) unlike double stranded DNA. This suggests that attached molecules are directed toward the outside of the helix in DNA–RNA hybrid duplexes and locked the positions by the rigid sugar conformation. The results have exhibited that, using this BNA, a wide variety of functional molecules can be attached to the center of the sequence, not 5′- or 3′-termini, without taking into account the affinity for target RNA. Generally, this characteristic is advantageous for antisense delivery technology.

Table 1 Evaluation of thermal denaturation temperatures (T_m values)

	DNA complement			RNA complement		
ON	T_m	ΔT_m[a]	$\Delta\Delta T_m$[b]/°C	T_m	ΔT_m[a]	$\Delta\Delta T_m$[b]/°C
natural	51	—	—	47	—	—
10	48[c]	−3	—	50[c]	+3	—
12	47	−4	−1	50	+3	0
13	48	−3	0	50	+3	0
14	50	−1	+2	49	+2	−1
15	57	+6	+9	47	0	−3
16	47	−4	−1	49	+2	−1
17	43	−8	−5	49	+2	−1

Each strand sequence: 5′-GCGTTXTTTGCT-3′ (X = modified monomer), target strand sequence: 5′-AGCAAAAAACGC-3′. Thermal denaturation studies' conditions: 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl; each strand concentration = 4 μM; scan rate of 0.5 °C min^{-1} at 260 nm. The number is the average of three independent measurements.[a] ΔT_ms are calculated relative to T_m values of unmodified DNA–DNA and DNA–RNA duplexes. [b] $\Delta\Delta T_m$s are calculated relative to T_m values of ON **10**–DNA and ON **10**–RNA duplexes. [c] Thermal denaturation study's condition: 10 mM sodium phosphate buffer (pH = 7.4) containing 100 mM NaCl and 400 μM TCEP; each strand concentration = 4 μM; scan rate of 0.5 °C min^{-1} at 260 nm.

Effect of functional groups on nuclease resistances

We examined the resistance of decathymidilate derivatives involving a single thiol-containing BNA structure or its conjugates toward 3′-exonuclease (*Crotalus Adamanteus* Venom Phosphodiesterase, CAVP)[32] degradation and compared those with natural ssDNA and a phosphorothioate-modified ON (S-oligo). After incubation of each ON solution at 37 °C in the presence of CAVP, the ratio of intact ONs was analyzed at several time points by RP-HPLC (Fig. 4). The natural ssDNA was completely digested within 2 min, whereas ON **11** bearing a free thiol and its conjugates were considerably stable under these conditions; 90% of intact ON **11** survived after 40 min. In the same manner as other BNAs, 3′-phosphodiester linkages of these BNAs were hardly digested by the 3′-exonuclease, probably due to the sterically hindered environment around the 3′-phosphodiester linkage.[25] Although the nuclease resistance of natural DNA was the same either in the presence or absence of DTT (0.75 mM) and DTDP (3.8 μM), the reducing agent could not be excluded as the cause of the high enzymatic stability of ON **11** (free thiol).[32] Conjugation of some functional groups significantly enhanced the stability: acetamide-conjugate (ON **21**) and ethylamine-conjugate (ON **22**) were more stable than phosphorothioate-modified ON. This result is opposed

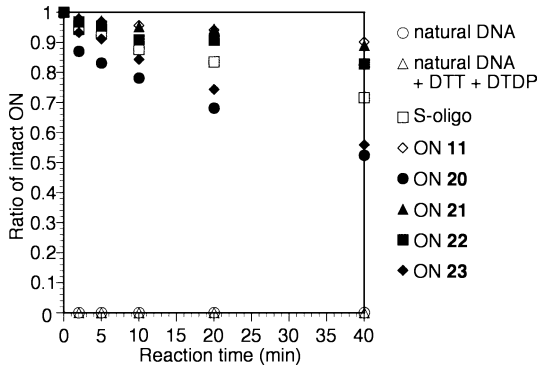


Fig. 4 Nuclease resistance of ONs against *Crotalus Adamanteus* Venom Phosphodiesterase (CAVP). Hydrolysis of the ONs (7.5 μM) was carried out at 37 °C in buffer (100 μL) containing 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, and CAVP (0.35 μg). ON **11** was prepared by treating ON **9** with DTT and used without any purification.

to Chattopadhyaya's work;[33] introduction of amide or amine substitution lead to lowered enzymatic stability. Even though ON **20** (–Me) and ON **23** (–S-cationic tripeptide) were more degraded than ON **11**, these ONs were significantly more stable than natural ON. Since the enzymatic stabilities were not reflected in bulkiness of the ON conjugates, these results are attributed to other effects, not only to the steric effect.[34] However, the conjugation effects regarding the resistance to enzymatic degradation are not clear yet. We will soon elucidate the relevance between substituents at the C6′ position and nuclease resistances.

Response of disulfide linkage to glutathione

In order to make the best use of the disulfide linkage, it is necessary that the disulfide bond is cleaved. It is known that the disulfide bond is cleaved under intracellular reductive conditions.[12] But, it is possible that the disulfide bond is not cleaved because of the sterically hindered environment around the linkage. Thus, we assessed the response of the disulfide linkage in ON **18** to glutathione. Fig. 5 shows the RP-HPLC profile of conversion from ON **18** (disulfide form) to ON **10** (free thiol form). When treating ON **18** with glutathione (1 mM to 10 mM) for 30 min, the peak corresponding to ON **18** disappeared, and ON **10** was generated. This result demonstrates that the disulfide-linked conjugates will be cleaved on intracellular reductive conditions and the ONs with free thiol-containing BNA monomers be released in cells.

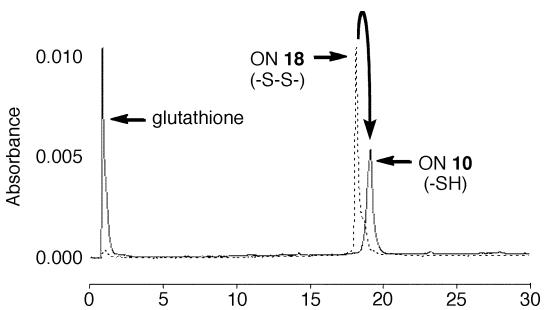


Fig. 5 RP-HPLC analysis of conversion from ON **18** to ON **10**. ON **18** in the absence of glutathione (dashed line) and in the presence of 10 mM glutathione (solid line).

Conclusions

We successfully synthesized a novel BNA bearing a thiol group in its bridged structure and conjugated functional molecules to it at the 6′-thiol group post-synthetically and efficiently in solution phase. Conjugated functional molecules had great effects on duplex stability with the DNA complement. In contrast, the molecules did not influence the stability with the RNA complement so much. Moreover, the ONs have as high or higher resistances against 3′-exonuclease than phosphorothioate ON (S-oligo).

Thus, this thiol-containing BNA is expected to have a wide field of application, for example, ON labeling, DNA detection, RNA-directed therapeutics, and DDS.

Experimental section

General

Dichloromethane, DMF and pyridine were distilled from CaH_2. ^{1}H NMR (400 and 300 MHz), ^{13}C NMR (100.5 and 75.5 MHz) and ^{31}P NMR (161.8 MHz) were recorded on JEOL JNM-ECS-400 or JNM-AL-300 spectrometers. Chemical shifts are reported in parts per million referenced to internal tetramethylsilane (0.00 ppm) and residual $CHCl_3$ (7.26 ppm) and methanol (3.31 ppm) for ^{1}H NMR, and chloroform-d_1 (77.0 ppm) and methanol-d_4 (49.0 ppm) for ^{13}C NMR. Relative to 85% H_3PO_4 as external standard for ^{31}P NMR. IR spectra were recorded on a JASCO FT/IR-4200 spectrometers. Optical rotations were recorded on a JASCO DIP-370 instrument. Mass spectra were measured on JEOL JMS-700 mass spectrometers. MALDI-TOF mass spectra were recorded on a Bruker Daltonics Autoflex II TOF/TOF mass spectrometer. For column chromatography, Fuji Silysia PSQ-100B or FL-100D silica gel was used. For high performance liquid chromatography (HPLC), SHIMADZU LC-6AD, SPD-10AV_{VP} and CTO-10A_{VP} were used.

1-{2-Thio-3,5-di-*O*-(*tert*-butyldiphenylsilyl)-2-*S*,4-*C*-(1-thia-ethylene)-β-D-ribofuranosyl}thymine (2). To a solution of compound **1**[22] (50 mg, 0.16 mmol) and *N*,*N*-dimethyl-4-aminopyridine (96 mg, 0.79 mmol) in DMF (0.20 mL) was added *tert*-butyldiphenylsilyl chloride (0.14 mL, 0.55 mmol) and the resultant mixture was stirred at 100 °C for 15 h under a N_2 atmosphere. After addition of CH_3OH, the reaction mixture was diluted with Et_2O, washed with H_2O, dried over $MgSO_4$, and concentrated. The crude product was purified by column chromatography (n-hexane/AcOEt = 3/1) to give compound **2** (88 mg, 70%) as a white foam; $[\alpha]_D^{25}$ –21.2 (*c* 1.0, CH_2Cl_2); IR ν_{max} (KBr): 1690 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.00 (9 H, s), 1.10 (9 H, s), 1.43 (3 H, s), 2.77 (1 H, d, J = 13 Hz), 2.81 (1 H, d, J = 6 Hz), 3.38 (1 H, d, J = 12 Hz), 3.60 (1 H, d, J = 13 Hz), 3.71 (1 H, d, J = 12 Hz), 3.89 (1 H, d, J = 6 Hz), 6.58 (1 H, s), 6.77 (1 H, s), 7.16–7.63 (20 H, m), 7.98 (1 H, brs); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 12.2, 19.3, 19.3, 26.8, 27.0, 31.6, 51.6, 66.7, 68.3, 84.9, 89.2, 110.2, 127.8, 127.8, 127.9, 130.0, 130.2, 130.4, 132.2, 132.3, 132.6, 134.4, 135.4, 135.4, 135.6, 135.8, 149.6, 163.6; MS (FAB) *m*/*z* 795 (M + H$^+$); HRMS (FAB): Calcd for $C_{43}H_{51}N_2O_5S_2Si_2$ (M + H$^+$): 795.2778. Found: 795.2782.

1-[2-Thio-3,5-di-*O*-(*tert*-butyldiphenylsilyl)-2-*S*,4-*C*-{(*S*)-sulfanylmethylene}-β-D-ribofuranosyl]thymine (3). Compound **2** (200 mg, 0.25 mmol) was dissolved in CH_2Cl_2 (100 mL) at room temperature. The solution was stirred and photoirradiated by a high pressure mercury lamp at ambient temperature for 30 min. The solvent was removed to give compound **3** as a white foam. The product was used without purification; $[\alpha]_D^{25}$ +21.2 (*c* 1.0, CH_2Cl_2); IR ν_{max} (KBr): 1589, 1686, 1898, 1961, 2857, 2930, 3071, 3178 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.08–1.12 (21 H, m), 2.23 (1 H, d, *J* = 12 Hz), 3.01 (1 H, d, *J* = 2 Hz), 4.02 (1 H, d, *J* = 11 Hz), 4.45 (1 H, d, *J* = 12 Hz), 4.48 (1 H, d, *J* = 2 Hz), 4.57 (1 H, d, *J* = 11 Hz), 5.80 (1 H, s), 7.26–7.70 (20 H, m), 8.27 (1 H, brs); ^{13}C NMR (100.5 MHz, $CDCl_3$) δ 11.7, 19.5, 20.2, 27.2, 27.8, 49.8, 54.2, 60.9, 74.1, 90.3, 91.3, 110.1, 128.1, 128.3, 128.4, 130.4, 130.4, 130.6, 130.8, 132.5, 133.3, 134.4, 135.6, 135.9, 136.2, 149.8, 164.0; MS (FAB) *m/z* 795 (M + H^+); HRMS (FAB): Calcd for $C_{43}H_{51}N_2O_5S_2Si_2$ (M + H^+): 795.2778. Found: 795.2776.

1-[2-Thio-3,5-di-*O*-(*tert*-butyldiphenylsilyl)-2-*S*,4-*C*-{(*R*)-(benzoylsulfanyl)methylene}-β-D-ribofuranosyl]thymine (4). To a solution of compound **3** (200 mg, 0.25 mmol) in pyridine (2.5 mL) was added benzoyl chloride (0.088 mL, 0.76 mmol) and the resultant mixture was stirred at room temperature for 1 h under a N_2 atmosphere. After addition of H_2O, the reaction mixture was diluted with AcOEt, dried over Na_2SO_4, and concentrated. The crude product was purified by column chromatography ($CHCl_3/CH_3OH$ = 60/1) to give compound **4** (184 mg, 81%, over 2 steps) as a white foam; $[\alpha]_D^{23}$ +43.4 (*c* 1.0, CH_2Cl_2); IR ν_{max} (KBr): 1689, 2858, 2891, 2933, 3050, 3069 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.05– 1.14 (21 H, m), 3.07 (1 H, d, *J* = 2 Hz), 4.22 (2 H, s), 4.48 (1 H, s), 5.45 (1 H, s), 5.83 (1 H, s), 7.23–7.90 (26 H, m); ^{13}C NMR (100.5 MHz, $CDCl_3$) δ 11.4, 19.4, 20.0, 27.0, 27.4, 53.6, 54.0, 60.8, 74.0, 90.3, 91.7, 109.9, 127.5, 128.0, 128.0, 128.1, 128.1, 128.5, 128.9, 130.1, 130.2, 130.3, 130.4, 130.6, 132.1, 132.6, 133.1, 134.0, 134.3, 135.4, 135.8, 136.1, 149.6, 164.0, 191.2; MS (FAB) *m/z* 899 (M + H^+); HRMS (FAB): Calcd for $C_{50}H_{55}N_2O_6S_2Si_2$ (M + H^+): 899.3040. Found: 899.3073.

1-[2-Thio-2-*S*,4-*C*-{(*R*)-(benzoylsulfanyl)methylene}-β-D-ribofuranosyl]thymine (5). To a solution of compound **4** (64 mg, 0.071 mmol) in THF (0.71 mL) was added tetrabutylammonium fluoride (1.0 M in THF, 0.21 mL, 0.21 mmol) and acetic acid (0.061 mL, 1.1 mmol) and the resultant mixture was stirred at room temperature for 23 h. Furthermore, tetrabutylammonium fluoride (1.0 M in THF, 0.14 mL, 0.14 mmol) and acetic acid (0.041 mL, 0.7 mmol) was added to the mixture and the mixture was stirred at room temperature for 25 h. The reaction mixture was concentrated and the crude product was purified by column chromatography ($CHCl_3/CH_3OH$ = 20/1 to 10/1) and purified further by column chromatography ($AcOEt/CH_3OH$ = 30/1) to give compound **5** (21 mg, 70%) as a white foam; $[\alpha]_D^{25}$ +192.9 (*c* 1.0, MeOH); IR ν_{max} (KBr): 1580, 1597, 1685, 2099, 2320, 2925, 3063, 3373 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 1.85 (3 H, s), 3.65 (1 H, s), 3.85 (1 H, d, *J* = 12 Hz), 3.91 (1 H, d, *J* = 12 Hz), 4.59 (1 H, d, *J* = 3 Hz), 5.33 (1 H, s), 5.88 (1 H, s), 7.47– 7.51 (2 H, m), 7.61– 7.65 (1 H, m), 7.92– 7.94 (2 H, m), 8.12 (1 H, s); ^{13}C NMR (100.5 MHz, CD_3OD) δ 12.7, 54.7, 58.5, 72.6, 91.4, 92.8, 110.1, 128.3, 130.0, 135.1, 137.3, 137.6, 152.0, 166.5, 192.3; MS (FAB) *m/z* 423 (M + H^+); HRMS (FAB): Calcd for $C_{18}H_{19}N_2O_6S_2$ (M + H^+): 423.0685. Found: 423.0681.

1-[2-Thio-5-*O*-(4,4′-dimethoxytrityl)-2-*S*,4-*C*-{(*R*)-(benzoylsulfanyl)methylene}-β-D-ribofuranosyl]thymine (6). To a solution of compound **5** (260 mg, 0.61 mmol) in pyridine (3.1 mL) was added 4,4′-dimethoxytrityl chloride (310 mg, 0.92 mmol) and the resultant mixture was stirred at room temperature for 2.5 h under a N_2 atmosphere. Furthermore 4,4′-dimethoxytrityl chloride (210 mg, 0.61 mmol) was added to the mixture and the mixture was stirred at room temperature for 30 min. After addition of H_2O, the reaction mixture was diluted with CH_2Cl_2, dried over Na_2SO_4, and concentrated. The crude product was purified by column chromatography (0.5% triethylamine in n-hexane/AcOEt = 1/1 to AcOEt only) to give compound **6** (350 mg, 79%) as a white foam; $[\alpha]_D^{24}$ −100.4 (*c* 1.0, CH_2Cl_2); IR ν_{max} (KBr): 1508, 1580, 1608, 1685, 1973, 2044, 2321, 2835, 2933, 3066, 3412 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.01–1.14 (21 H, m), 3.08 (1 H, d, *J* = 2 Hz), 4.22 (2 H, s), 4.48 (1 H, d, *J* = 2 Hz), 5.45 (1 H, s), 5.83 (1 H, s), 7.26–7.96 (26 H, m); ^{13}C NMR (100.5 MHz, $CDCl_3$) δ 12.3, 54.0, 54.0, 55.4, 59.8, 73.3, 87.5, 90.0, 91.1, 110.5, 113.5, 127.3, 127.5, 128.2, 128.3, 128.9, 130.2, 134.2, 134.8, 135.0, 135.1, 136.1, 144.1, 150.2, 158.8, 158.8, 164.1, 191.0; MS (FAB) *m/z* 747 (M + Na^+); HRMS (FAB): Calcd for $C_{39}H_{36}N_2NaO_8S_2$ (M + Na^+): 747.1811. Found: 747.1821.

1-[2-Thio-3-*O*-{2-cyanoethoxy(diisopropylamino)phosphino}-5-*O*-(4,4′-dimethoxytriryl)-2-*S*,4-*C*-{(*R*)-(benzoylsulfanyl)methylene}-β-D-ribofuranosyl]thymine (7). To a solution of compound **6** (350 mg, 0.48 mmol) in acetonitrile (8.8 mL) was added *N*,*N*-diisopropylethylamine (0.34 mL, 1.9 mmol) and *N*,*N*-diisopropylamino-2-cyanoethylphosphino chloridite (0.22 mL, 0.97 mmol) and the resultant mixture was stirred at 0 °C for 2 h under a N_2 atmosphere. The reaction mixture was concentrated and the crude product was purified by column chromatography (0.5% triethylamine in n-hexane/AcOEt = 1/1 to 1/2) to give compound **7** (290 mg, 66%) as a white foam; ^{31}P NMR (161.8 MHz, $CDCl_3$) δ 150.0, 150.4; MS (FAB) *m/z* 925 (M + H^+); HRMS (FAB): Calcd for $C_{48}H_{54}N_4O_9PS_2$ (M + H^+): 925.3070. Found: 925.3076.

ON synthesis

Synthesis of the thiol-containing BNA-modified ONs was performed on an Applied Biosystems Expedite™ 8909 Nucleic Acid Synthesis System on a 1.0 μmol scale using a phosphoramidite coupling protocol and 5-[3,5-bis(trifluoromethyl)phenyl]-1*H*-tetrazole as the activator. The concentration of phosporamidite **7** was 0.10 M and its coupling time was 20 min, while the concentration of each unmodified phosporamidite was 0.067 M and their coupling times were 90 s. The solid-supported ONs (DMTr-off) were treated with 50 mM K_2CO_3 and 100 mM 2,2′-dithiodipyridine in CH_3OH at room temperature for 2.5 h, then neutralized by 5% acetic acid and concentrated. The crude ONs were roughly purified with a GE Healthcare Nap 10 column, and then carefully by RP-HPLC on a Waters XBridge™ OST C18 2.5 μm (10 × 50 mm) using MeCN in 0.1 M triethylammonium acetate buffer (pH = 7.0). The purity of the ONs was analyzed by RP-HPLC on a Waters XBridge™ Shield RP 18 2.5 μm (4.6 × 50 mm) and characterized by MALDI-TOF mass spectrometry. The overall yields were 8.4% for ON **8** and 16.6% for ON **9** calculated from the UV absorbance at 260 nm.

General procedure for solution-phase conjugation of ONs

Thioether-linked conjugation. 500 μM ON **8** or **9** (12 μL, 6 nmol) was treated with 10 mM dithiothreitol (DTT) dissolved in 100 mM sodium phosphate buffer (pH = 8.0) (60 μL, 0.6 μmol) at room temperature for 2 h to give ON **10** or **11**, then, 5% acetic acid (3.4 μL) was added, and 1 M primary halogenoalkyl derivative in DMF (1.8 μL, 1.8 μmol) was added to the solution to afford a thioether-linked conjugate. The reaction was analyzed by RP-HPLC. After completing the reaction, the ON was precipitated by adding 5 volumes of ethanol. The mixture was kept at 0 °C for 30 min, centrifuged at 13 200 rpm for 15 min at 4 °C, and the resulting supernatant solution was removed. The obtained ON conjugates were purified by RP-HPLC and characterized by MALDI-TOF mass spectrometry. Each conjugation yield was calculated from UV absorption at 260 nm of the ON conjugate.

Disulfide-linked conjugation. A solution of 500 μM ON **8** or **9** (10 μL, 5 nmol) in 0.1 mM TEAA buffer (pH = 7.0, 22 μL) was treated with 10 mM thiol compound in DMF (1.25 μL, 1.25 μmol) at room temperature for 24 h to give a disulfide-linked conjugate. The method of reaction analysis, purification, characterization, and yield calculation was the same as thioether-linked conjugates.

Thermal denaturation experiments. Thermal denaturation experiments were carried out on SHIMADZU UV-1650 and UV-1800 spectrometers equipped with a T_m analysis accessory. For the duplex formation study, equimolecular amounts of the target ssDNA/ssRNA and ONs were dissolved in 10 mM sodium phosphate buffer (pH = 7.2) containing 100 mM NaCl to give a final strand concentration of 4.0 μM. The ON/target samples were then annealed by heating at 90 °C followed by slow cooling to room temperature. The melting profile was recorded at 260 nm from 5 to 90 °C at a scan rate of 0.5 °C/min. T_m values for ON **10** were measured on the condition that ON **8** and target ssDNA/ssRNA were dissolved in 11 mM sodium phosphate buffer (pH = 7.4) containing 111 mM NaCl, annealed by heating at 90 °C followed by slow cooling to room temperature, and then 4 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) aqueous solution was added to the mixtures to give a final strand concentration of 4.0 μM, pH 7.2.[35]

Nuclease resistance study. CAVP (Amersham Pharmacia Biotech, NJ, United States) (0.35 μg) was added to a solution of ON (750 pmol) in 50 mM Tris-HCl buffer (pH = 8.5) containing 10 mM $MgCl_2$. ON **11** was prepared by treating ON **9** with DTT (100 eq.) and used without any purification. The cleavage reaction was carried out at 37 °C. At several time points, a portion of each reaction mixture was removed and heated to 90 °C for 2 min to inactivate the nuclease. The amount of intact ON was then plotted against the exposure time to obtain the ON degradation curve with time. The fitted line for each ON degradation was obtained.

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35 A reducing agent TCEP-HCl was added in order to reduce ON **8** and generate ON **10**. Acidic TCEP-HCl aqueous solution was added into the annealed mixtures, therefore the final pH and the concentrations of sodium phosphate buffer and NaCl conditions for the UV melting experiment were equal to the other experiments.