chromatography (*n*-hexane/ethyl acetate = 9:1) to afford **3** as a clear oil (23.8 g, quant). $[\alpha]^{25}_D$ = +84.8 (*c* 1.00, $CHCl_3$). IR (KBr): 1457, 1372, 1105, 1025, cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ_H 1.07 (9H, s), 1.29 (6H, s), 3.67 (1H, d, *J* = 10.2 Hz), 3.76 (1H, d, *J* = 10.2 Hz), 4.09 (1H, d, *J* = 11.1 Hz), 4.14 (1H, d, *J* = 11.1 Hz), 4.33 (1H, d, *J* = 5.1 Hz), 4.46–4.59 (4H, m), 4.79 (1H, d, *J* = 12.3 Hz), 5.78 (1H, d, *J* = 3.9 Hz), 7.27–7.76 (20H, m). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C 19.2, 26.2, 26.5, 26.7, 64.6, 71.9, 72.2, 73.5, 76.6, 78.1, 79.5, 87.5, 104.1, 113.1, 127.4, 127.5, 127.5, 127.5, 127.6, 128.1, 128.2, 129.4, 133.1, 133.4, 134.7, 135.6, 135.7, 137.8, 138.0. MS (FAB): *m/z* 661 (M + Na^+). Anal. Calcd for $C_{39}H_{46}O_6Si$: C, 73.32; H, 7.26. Found: C, 73.44; H, 7.32.

2′-O-Acetyl-3′,5′-di-O-benzyl-4′-C-tert-butyldiphenylsiloxymethyl-5-methyluridine (4). To a stirring solution of compound **3** (23.0 g, 36.0 mmol) in acetic acid (26.0 mL, 460 mmol) were added acetic anhydride (45.0 mL, 480 mmol) and concd sulfuric acid (200 μL) at 0 °C. The reaction mixture was stirred at room temperature for 5 h. After completion of the reaction, the solution was neutralized with satd $NaHCO_3$, and the product was extracted with ethyl acetate. The organic phase was washed with water and brine solution and dried (Na_2SO_4). After concentration, the crude product (25.7 g) was obtained as yellow syrup which was used for the next reaction without purification.

The crude product (25.7 g, 37.6 mmol) was dissolved in acetonitrile (200 mL), and thymine (14.2 g, 110 mmol) and bis(trimethylsilyl)-acetamide (BSA) (47.0 mL, 190 mmol) were added. The solution was heated at 40 °C until all the substrate dissolved and then was cooled to 0 °C. TMS-triflate (10.5 mL, 57.7 mmol) was added dropwise, and the reaction mixture was stirred at room temperature for 1 h. After completion of the reaction, ice–water was added, and the product was extracted with dichloromethane. The organic phase was washed with water and brine solution, and dried (Na_2SO_4). The solution was concentrated to afford the crude product which was purified by column chromatography (*n*-hexane/ethyl acetate = 1:1) to afford **4** as a white solid (20.0 g, 74%, two steps). Mp: 55–59 °C. $[\alpha]_D^{24}$ = −11.7 (*c* 0.800, $CHCl_3$). IR (KBr): 1747, 1693, 1232, 1113 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ_H 1.03 (9H, s), 1.52 (3H, s), 1.96 (3H, s), 3.68 (1H, d, *J* = 10.8 Hz), 3.71 (1H, d, *J* = 10.5 Hz), 3.75 (1H, d, *J* = 10.5 Hz), 3.94 (2H, d, *J* = 10.8 Hz), 4.40 (1H, d, *J* = 5.7 Hz), 4.55 (2H, m), 5.37 (1H, t, *J* = 6.0 Hz), 6.15 (1H, d, *J* = 6.0 Hz), 7.18–7.62 (20H, m), 7.87 (1H, s). ^{13}C NMR (75 MHz, $CDCl_3$): 12.0, 19.2, 20.6, 26.9, 63.8, 72.2, 73.7, 74.6, 74.9, 77.7, 85.5, 87.8, 111.3, 127.6, 127.7, 127.7, 127.7, 127.8, 128.1, 128.3, 128.6, 129.7, 129.8, 132.6, 132.9, 135.5, 135.7, 135.7, 137.2, 137.5, 150.4, 163.6, 170.2. MS (FAB): *m/z* 749 (M + H^+). Anal. Calcd for $C_{43}H_{48}N_2O_8Si$: C, 68.96; H, 6.46; N, 3.74. Found: C, 68.92; H, 6.45; N, 3.74.

3′,5′-Di-O-benzyl-4′-C-tert-butyldiphenylsiloxymethyl-5-methyluridine (5). To a solution of compound **4** (20.0 g, 26.7 mmol) in THF (100 mL) was added 40% aqueous methylamine solution (62.1 mL, 800 mmol) and stirred for 30 min at room temperature. After completion of the reaction, the product was extracted with ethyl acetate. The organic phase was washed with water and brine solution and dried (Na_2SO_4). The product was purified by column chromatography (*n*-hexane/ethyl acetate = 1:1) to afford **5** as a white solid (18.3 g, quant). Mp: 61–63 °C. $[\alpha]^{25}_D$ = −12.2 (*c* 0.750, $CHCl_3$). IR (KBr): 3403, 3175, 1688, 1468, 1272, 1113 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.05 (9H, s), 1.60 (3H, s), 3.55 (1H, d, *J* = 10.4 Hz), 3.63 (1H, d, *J* = 10.4 Hz), 3.75 (1H, d, *J* = 10.8 Hz), 3.81 (1H, d, *J* = 10.8 Hz), 3.84 (1H, d, *J* = 10.4 Hz), 4.30 (1H, d, *J* = 5.6 Hz,) 4.41 (1H, ddd, *J* = 4.8 Hz, 5.6 Hz, 10.8 Hz), 4.49 (2H, s), 4.64 (1H, d, *J* = 11.2 Hz), 4.75 (1H, d, *J* = 11.2 Hz), 5.95 (1H, d, *J* = 5.0 Hz), 7.21–7.66 (20H, m), 9.04 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$): 12.1, 19.0, 26.8, 64.2, 72.2, 73.6, 74.1, 74.5, 78.5, 87.9, 90.9, 110.9, 127.6, 127.8, 127.8, 127.8, 128.9, 128.0, 128.0, 128.5, 129.9, 132.2, 132.2, 135.6, 136.5, 137.2, 137.2, 150.5, 163.8. MS (FAB): *m/z* 707 (M + H^+). Anal. Calcd for $C_{41}H_{46}N_2O_7Si$: C, 69.66; H, 6.56; N, 3.96. Found: C, 69.59; H, 6.59; N, 3.93.

2,2′-Anhydro-3′,5′-di-O-benzyl-4′-C-tert-butyldiphenylsiloxymethyl-5-methyluridine (6). To a solution of compound **5** (17.0 g, 24.0 mmol) in dichloromethane (250 mL) was added DMAP (11.7 g, 95.7 mmol). The reaction mixture was placed in an ice bath, TfCl (7.6 mL, 71.2 mmol) was added dropwise, and the reaction mixture was stirred at room temperature for 1 h. After completion of the reaction, the reaction was quenched with ice-cold water, and the product was extracted with dichloromethane. The organic phase was washed with water and brine solution, and dried (Na_2SO_4). The product was purified by column chromatography (*n*-hexane/ethyl acetate = 1:1) to afford **6** as white solid (16.5 g, quant.). Mp: 51–54 °C. $[\alpha]^{26}_D$ = −33.5 (*c* 1.00, $CHCl_3$). IR (KBr): 1667, 1650, 1563, 1482, 1112 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ: 1.04 (9H, s), 1.98 (3H, s), 3.33 (2H, m), 3.70 (1H, d, *J* = 10.8 Hz), 3.83 (1H, d, *J* = 10.8 Hz), 4.31 (1H, d, *J* = 12 Hz), 4.32 (1H, d, *J* = 8.8 Hz), 4.38 (1H, d, *J* = 12 Hz), 4.60 (1H, d, *J* = 11.5 Hz), 4.60 (1H, d, *J* = 11.5 Hz), 5.52 (1H, dd, *J* = 5.2, 8 Hz), 6.27 (1H, d, *J* = 6.0 Hz), 7.09–7.67 (21H, m). ^{13}C NMR (75 MHz, $CDCl_3$): 13.9, 18.9, 26.7, 63.9, 69.4, 73.4, 83.9, 87.1, 88.7, 89.9, 118.9, 127.4, 127.6 127.7, 127.8, 128.1, 128.3, 128.4, 128.5, 129.8, 130.1, 131.9, 132.3, 132.3, 135.3, 135.5, 136.4 136.9, 159.1, 172.3. MS (FAB): *m/z* 689 (M + H^+). Anal. Calcd for $C_{41}H_{44}N_2O_6Si$: C, 71.48; H, 6.44; N, 4.07. Found: C, 71.38; H, 6.49; N, 4.08.

3′,5′-Di-O-benzyl-4′-C-tert-butyldiphenylsiloxymethyl-5-methyl-arabinouridine (7). To a solution of compound **6** (16.5 g, 23.4 mmol) in THF (200 mL) was added 1 N NaOH solution (70.0 mL, 70.0 mmol), and the mixture was stirred at room temperature for 16.5 h. The solution was neutralized with NH_4Cl solution, and the product was extracted with dichloromethane. The organic phase was washed with water and brine solution, dried (Na_2SO_4), and concentrated. The crude product thus obtained was purified by column chromatography (*n*-hexane/ethyl acetate = 1:1) to afford **7** as white solid (16.4 g, 97%). Mp: 67–70 °C. $[\alpha]^{26}_D$ = +24.5 (*c* 0.840, $CHCl_3$). IR (KBr): 3347, 3184, 1690, 1471 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.99 (9H, s), 1.54 (3H, s), 3.42 (1H, d, *J* = 10.0 Hz), 3.52 (1H, d, *J* = 10.5 Hz), 3.60 (1H, d, *J* = 10.0 Hz), 3.79 (1H, d, *J* = 10.5 Hz), 4. 34 (1H, d, *J* = 6.4 Hz), 4.43 (1H, d, *J* = 11.6 Hz) 4.51 (1H, d, *J* = 11.6 Hz), 4.62 (1H, d, *J* = 11.6 Hz), 4.90 (1H, d, *J* = 6.4 Hz), 4.93 (1H, d, *J* = 11.6 Hz), 5.10 (1H, dd, *J* = 6.4, 12.4 Hz), 6.36 (1H, d, *J* = 6.0 Hz), 7.19–7.68 (20H, m), 7.77 (1H,s), 9.95 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$): 12.1, 18.9, 26.5, 64.1, 69.6, 72.6, 73.7, 75.4, 81.7, 85.4, 85.5, 109.7, 127.6, 127.8, 127.8, 127.8, 127.9, 128.1, 128.3, 128.5, 129.6, 129.7 132.5, 135.6, 135.7, 137.1, 137.4, 138.2, 151.4, 164.7. MS (FAB): *m/z* 707 (M + H^+). Anal. Calcd for $C_{41}H_{46}N_2O_7Si$: C, 69.66; H, 6.56; N, 3.96. Found: C, 69.42; H, 6.54; N, 3.97.

3′,5′-Di-O-benzyl-4′-C-tert-butyldiphenylsiloxymethyl-2′-O-(1,3-dihyro-1,3-dioxo-2H-isoindol-2-yl)-5-methyluridine (9). To a solution of compound **7** (3.00 g, 4.24 mmol) in dichloromethane (15 mL) were added pyridine (1.70 mL, 21.2 mmol) and trifluoromethanesulfonic anhydride (1.50 mL, 8.91 mmol) at 0 °C. The reaction mixture was stirred in an ice bath for 40 min. After completion of the reaction, ice-cold water was added, and the product was extracted with dichloromethane. The organic phase was washed with water and brine, dried (Na_2SO_4), and concentrated. The crude product **8** (4.10 g) was used for the next reaction without further purification.

The crude triflate **8** (4.60 g. 5.48 mmol) was dissolved in acetonitrile (20 mL), and *N*-hydroxyphthalimide (4.00 mg, 24.5 mmol) and DBU (3.70 mL, 24.7 mmol) were added. The reaction mixture was stirred at room temperature for 24 h. After completion of the reaction, the solution was diluted with dichloromethane, and water was added. The product was extracted with dichloromethane. The organic phase was washed with water and brine, dried (Na_2SO_4), and concentrated. The product was purified by column chromatography (*n*-hexane/ethyl acetate = 2:1) to produce **9** as a white amorphous solid (2.80 g, 79% two steps). Mp: 78–80 °C. $[\alpha]^{25}_D$ = +43.0 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3188, 3067, 2934, 2862, 1791, 1730, 1692, 1465, 1427, 1421, 1366, 1267, 1189, 1106, 973 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.08 (9H, s), 1.38 (3H, s), 3.65 (1H, d, *J* = 10.4 Hz), 4.02 (1H, d, *J* = 11.6 Hz), 4.09 (1H, d, *J* = 10.4 Hz), 4.22 (1H, d, *J* = 11.6 Hz), 4.48 (1H, d, *J* = 11.2 Hz), 4.52 (1H, d, *J* = 5.2 Hz), 4.55 (1H, d, *J* = 11.2 Hz), 4.73 (1H, d, *J* = 11.2 Hz), 4.85 (1H, dd, *J* = 3.2 Hz, 2.8 Hz), 5.13 (1H, d, *J* = 11.2 Hz), 6.40 (1H, d, *J* = 3.2 Hz), 7.16 – 7.86 (26H, m), 8.37 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 11.8, 19.3, 26.9, 64.6, 70.5, 73.1, 73.6, 75.1, 87.2, 88.3, 88.5, 110.4,

123.6, 127.6, 127.7, 127.8, 128.3, 128.5, 129.6, 129.6, 132.9, 133.3, 134.5, 135.6, 135.7, 137.2, 137.4, 150.0, 163.1. MS (FAB) m/z 852 (M + H^+). HRMS (FAB) calcd for $C_{49}H_{49}N_3O_9Si$ (M + H^+): 852.3316, found 852.3284.

*3′,5′-Di-O-benzyl-2′-O-(1,3-dihyro-1,3-dioxo-2H-isoindol-2yl)-4′-C-hydroxymethyl-5-methyluridine (**12**).* To a solution of compound **9** (2.80 g, 3.28 mmol) in THF (15 mL) was added TEA·3HF (5.60 mL, 34.3 mmol), and the solution was refluxed for 18 h. The reaction mixture was then cooled and ice-cold water was added. The product was extracted with ethyl acetate, washed with satd sodium hydrogencarbonate and brine, dried (Na_2SO_4), and concentrated. The product was purified by column chromatography (n-hexane/ethyl acetate = 1:1) to produce **12** as white amorphous solid (1.80 g, 90%). Mp: 96–98 °C. $[\alpha]^{25}_D$ = +39.1 (c 1.00, $CHCl_3$). IR ν_{max} (KBr): 3504, 3181, 3062, 2881, 1789, 1733, 1689, 1466, 1375, 1272, 1187, 1105, 1057, 974 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.36 (3H, s), 2.64 (1H, br s), 3.83 (1H, d, J = 10.4 Hz), 3.92 (1H, d, J = 7.2 Hz), 3.96 (1H, d, J = 10.4 Hz), 4.12 (1H, d, J = 7.2 Hz), 4.49 (1H, d, J = 11.2 Hz), 4.54 (1H, d, J = 11.2 Hz), 4.63 (1H, d, J = 6 Hz), 4.74 (1H, d, J = 12 Hz), 4.93 (1H, dd, J = 6 Hz, 1.6 Hz), 5.16 (1H, d, J = 11.6 Hz), 6.34 (1H, d, J = 1.2 Hz), 7.15–7.84 (15H, m), 8.59 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 11.7, 64.1, 70.0, 73.0, 73.6, 75.3, 87.2, 87.8, 88.6, 110.4, 123.7, 127.7, 128.0, 128.1, 128.3, 128.5, 128.6, 128.6, 134.6, 135.5, 136.8, 137.2, 149.8, 163.4. MS (FAB): m/z 614 (M + H^+). HRMS (FAB): calcd for $C_{33}H_{31}N_3O_9$ (M + H^+) 614.2138, found 614.2155.

*3′,5′-Di-O-benzyl-4′-carboxyl-2′-O-(1,3-dihyro-1,3-dioxo-2H-isoindol-2yl)-5-methyluridine (**13**).* To a solution of compound **12** (1.80 g, 2.93 mmol) in DMF (20 mL) was added 4A molecular sieves (2.00 g), and the mixture was stirred for 10 min. To the solution was added PDC (11.6 g, 30.8 mmol), and the mixture was stirred at room temperature for 16 h. The reaction was quenched with ice-cold water. The product was extracted with ethyl acetate, washed with water, and purified by column chromatography (n-hexane/ethyl acetate = 1:1 to ethyl acetate/methanol 10:1). The product **13** was obtained as white amorphous solid (1.40 g, 75%). Mp: 137–139 °C. $[\alpha]^{26}_D$ = +23.9 (c 1.00, $CHCl_3$). IR ν_{max} (KBr): 3178, 3066, 3032, 2873, 1790, 1736, 1468, 1376, 1275, 1187, 1125, 967 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.59 (3H, s), 3.90 (1H, d, J = 10.4 Hz), 4.10 (1H, d, J = 10.4 Hz), 4.59 (1H, d, J = 11.6 Hz), 4.63 (1H, d, J = 11.6 H z), 4.68 (1H, d, J = 4.4 Hz), 4.91 (1H, d, J = 11.2 Hz), 5.06 (1H, t, J = 5.6 Hz) 5.27 (1H, d, J = 11.2 Hz), 6.70 (1H, d, J = 6.4 Hz), 7.27 - 7.86 (16 H, m), 9.16 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 11.9, 71.4, 73.9, 74.6, 74.6, 74.7, 78.1, 87.7, 88.4, 89.0, 111.4, 123.7, 127.6, 127.7, 127.8, 128.1, 128.2, 128.3, 128.4, 128.5, 134.8, 136.2, 136.3, 136.7, 150.6, 162.9, 164.0, 170.6. MS (FAB) m/z 628 (M + H^+). HRMS (FAB): calcd for $C_{33}H_{29}N_3O_{10}$ (M + H^+) 628.1931, found 628.1938.

*2′-O-Amino-3′,5′-di-O-benzyl-4′-carboxyl-5-methyluridine (**14**).* To a solution of compound **13** (400 mg, 0.637 mmol) in ethanol (2 mL) was added hydrazine monohydrate (40.0 μL, 0.823 mmol), and the mixture was stirred at room temperature for 10 min. After completion of the reaction, the reaction solution was concentrated and ethyl acetate was added. The precipitate was filtered, and the filtrate was extracted with ethyl acetate, washed with water and brine, and dried (Na_2SO_4). The crude compound **14** (380 mg) was used for the next reaction without further purification.

*(1S,5R,6R,8S)-3-Aza-8-benzyloxy-1-benzyloxymethyl-6-(thymin-1-yl)-4,7-dioxabicyclo[3.2.1]octan-2-one. (**15**).* The crude key intermediate **14** (320 mg, 0.643 mmol) was dissolved in DMF (5 mL), and EDCI·HCl (150 mg, 0.782 mmol) and HOBt·H_2O (106 mg, 0.784 mmol) were added at room temperature. The reaction mixture was stirred at room temperature for 11 h, and then ice-cold water was added. The product was extracted with ethyl acetate. The organic phase was washed with water and brine and dried (Na_2SO_4). The product was purified by column chromatography (n-hexane/ethyl acetate = 2:1 to 1:1) to yield **15** as a white solid (240 mg, 77% two steps). Mp: 96–98 °C. $[\alpha]^{23}_D$ = +89.53 (c 1.00, $CHCl_3$). IR ν_{max} (KBr): 3190, 3064, 3033, 2926, 2877, 1699, 1494, 1455, 1392, 1362, 1268, 1203, 1109, 1053, 1018, 984, 916 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.19 (3H, s), 3.98 (1H, d, J = 11 Hz), 4.28 (1H, d, J = 11 Hz), 4.51 (1H, d, J = 11 Hz), 4.57 (1H, s), 4.58 (1H, d, J = 11 Hz), 4.73 (1H, d, J = 12.4 Hz), 4.88 (1H, d, J = 12.4 Hz), 5.18 (1H, s), 6.25 (1H, s), 7.21–7.38 (10 H, m), 7.57 (1H, s), 7.75 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 11.8, 64.4, 69.3, 72.2, 73.7, 78.2, 80.1, 87.6, 110.3, 127.8, 127.9, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 135.1, 137.5, 137.6 150.8, 163.3, 167.9. MS (FAB): m/z 480 (M + H^+). HRMS (FAB): calcd for $C_{25}H_{25}N_3O_7$ (M + H^+) 480.1771, found 480.1779.

*(1S,5R,6R,8S)-3-Aza-8-hydroxy-1-hydroxymethyl-6-(thymin-1-yl)-4,7-dioxabicyclo[3.2.1]octan-2-one (**16**).* To a solution of compound **15** (110 mg, 0.229 mmol) in ethanol/chloroform 5:1 (5 mL) was added palladium hydroxide on carbon (110 mg). The reaction vessel was degassed several times with hydrogen gas, and the reaction mixture was stirred at room temperature under a hydrogen atmosphere overnight. After completion of the reaction, the solution was filtered, and the filtrate was concentrated. The product was further purified by column chromatography (n-hexane/ethyl acetate = 1:1 to 100% ethyl acetate) to produce **16** as a white solid (55.0 mg, 80%). Mp: 261–263 °C dec. $[\alpha]^{24}_D$ = +31.81 (c 1.00, MeOH). IR ν_{max} (KBr): 3474, 3406, 3237, 3056, 2979, 2932, 2819, 1693, 1481, 1422, 1386, 1359, 1282, 1207, 1104, 1052, 997, 967, 916 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ_H 1.86 (3H, s), 3.88 (1H, d, J = 12.8 Hz), 4.20 (1H, d, J = 12.8 Hz), 4.54 (1H, J = 3.6 Hz), 4.67 (1H, J = 3.6 Hz), 6.07 (1H, s), 7.94 (1H, d, J = 1.4 Hz). ^{13}C NMR (100 MHz, DMSO): δ_C 12.4, 48.6, 55.3, 61.9, 79.8, 80.5, 86.9, 108.6, 135.0, 149.9, 163.8, 167.1. MS (FAB): m/z 300 (M + H^+). HRMS (FAB): calcd for $C_{11}H_{13}N_3O_7$ (M + H^+) 300.0831, found 300.0830.

*3′,5′-Di-O-benzyl-4′-carboxyl-2′-O-(N-methyleneamino)-5-methyluridine (**17**).* Formalin (37 wt % in H_2O, 80.0 μL, 0.986 mmol) was added to a stirring solution of compound **14** (380 mg, 0.763 mmol) in methanol (5 mL). The reaction mixture was stirred at room temperature for 2 h. The solution was concentrated, and then water was added. The product was extracted with ethyl acetate, washed with water and brine, dried (Na_2SO_4), and concentrated. The product was purified by column chromatography (n-hexane/ethyl acetate = 1:1 to 1:2) to produce **17** as a white solid (250 mg, 65% two steps). Mp: 81–83 °C. $[\alpha]^{26}_D$ = −29.3 (c 1.00, $CHCl_3$). IR ν_{max} (KBr): 3172, 3064, 2944, 2872, 1699, 1469, 1366, 1274, 1127, 1070, 916 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.53 (3H, s), 3.77 (1H, d, J = 10.8 Hz), 4.07 (1H, d, J = 10.8 Hz), 4.45 (1H, d, J = 4.8 Hz), 4.54 (1H, d, J = 11.2 Hz), 4.60 (1H, d, J = 12 Hz), 4.63 (1H, d, J = 12 Hz), 4.69 (1H, J = 11.2 Hz), 5.04 (1H, dd, J = 4.4 Hz, 3.2 Hz), 6.44 (1H, d, J = 7.2 Hz), 6.53 (1H, d, J = 8 Hz), 7.03 (1H, d, J = 7.2 Hz), 7.19–7.34 (10H, m), 7.52 (1H, s), 9.56 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 12.0, 72.0, 73.9, 74.7, 79.8, 83.6, 86.2, 89.7, 111.8, 125.2, 127.7, 127.7, 127.7, 127.8, 128.2, 128.3, 128.7, 128.9, 136.2, 136.6, 137.2, 139.9, 150.7, 164.2, 170.8. MS (FAB): m/z 510 (M + H^+). HRMS (FAB): calcd for $C_{26}H_{27}N_3O_8$ (M + H^+) 510.1876. found 510.1880.

*3′,5′-Di-O-benzyl-4′-carboxyl-2′-O-(N-methylamino)-5-methyluridine (**18**).* To a solution of compound **17** (250 mg, 0.488 mmol) in a methanolic solution of pyridinium p-toluenesulfonate (1M, 4.90 mL, 4.89 mmol) was added sodium cyanoborohydride (62.0 mg, 0.986 mmol) at 0 °C, and the mixture was stirred for 10 min. Then the reaction mixture was allowed to come to room temperature and stirred for 2 h. After completion of the reaction, the solvent was evaporated, and the product was diluted with ethyl acetate. The product was washed with water and brine, dried (Na_2SO_4), and concentrated. The crude product **18** (235 mg) was used for the next reactions without further purification.

*(1S,5R,6R,8S)-3-Aza-8-benzyloxy-1-benzyloxymethyl-3-methyl-6-(thymin-1-yl)-4,7-dioxabicyclo[3.2.1]octan-2-one (**19**).* To a solution of compound **18** (235 mg, 0.459 mmol) in dimethylformamide (4 mL) were added EDCI·HCl (105 mg, 0.547 mmol) and HOBt·H_2O (75.0 mg, 0.555 mmol). The reaction mixture was stirred at room temperature overnight. After completion of the reaction, water was added, and the product was extracted with ethyl acetate, washed with water and brine, dried (Na_2SO_4), and concentrated. The compound was purified by column chromatography (n-hexane/ethyl acetate = 4:1 to 1:1) to produce **19** as a white solid (190 mg, 80% two steps). Mp: 90–92 °C. $[\alpha]^{26}_D$ = +62.3 (c 1.00, $CHCl_3$). IR ν_{max} (KBr): 3164, 3029,

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2926, 2878, 1698, 1456, 1392, 1362, 1274, 1215, 1155, 1094, 1065, 983 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.41 (3H, s), 3.25 (3H, s), 3.95 (1H, d, J = 11.6 Hz), 4.25 (1H, d, J = 11.6 Hz), 4.34 (1H, d, J = 3.2 Hz), 4.56 (1H, d, J = 10.8 Hz), 4.59–4.64 (3H, m), 4.73 (1H, d, J = 10.8 Hz), 6.08 (1H, s), 7.23–7.37 (10 H, m), 7.62 (1H, d, J = 1.6 Hz), 9.22 (1H, br s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 11.9, 33.7, 64.7, 69.5, 72.6, 73.9, 78.5, 80.2, 87.9, 110.8, 127.6, 127.9, 128.2, 128.3, 128.5, 134.5, 136.5, 137.2, 149.9, 163.9. MS (FAB): m/z 494 ($M + H^+$). HRMS (FAB): calcd for $C_{26}H_{27}N_3O_7$ ($M + H^+$) 494.1927, found 494.1931.

*(1S,5R,6R,8S)-3-Aza-8-hydroxy-1-hydroxymethyl-3-methyl-6-(thymin-1-yl)-4,7-dioxabicyclo[3.2.1]octan-2-one (**20**).* To a solution of compound **19** (190 mg, 0.385 mmol) in ethanol/chloroform 5:1 (5 mL) was added palladium hydroxide on carbon (190 mg). The reaction vessel was degassed several times with hydrogen gas, and the reaction mixture was stirred at room temperature under a hydrogen atmosphere overnight. After completion of the reaction, the solution was filtered, and the filtrate was concentrated. The product was further purified by column chromatography (n-hexane/ethyl acetate = 1:1 to 100% ethyl acetate) to produce **20** as a white solid (105 mg, 90%). Mp: 232–234 °C dec; $[\alpha]^{26}_D$ = +14.9 (c 1.00, EtOH). IR ν_{max} (KBr): 3444, 3226, 3070, 2941, 1678, 1469, 1412, 1281, 1199, 1078,988 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ_H 1.86 (3H, s), 3.22 (3H, s), 3.87 (1H, d, J = 12.8 Hz), 4.18 (1H, d, J = 12.8 Hz), 4.53 (1H, J = 3.6 Hz), 4.71 (1H, J = 3.6 Hz), 6.04 (1H, s), 7.95 (1H, d, J = 1.2 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ_C 12.6, 33.7, 57.3, 63.9, 82.1, 82.8, 89.1, 111.0, 136.9, 151.8, 166.4. MS (FAB): m/z 314 ($M + H^+$). HRMS (FAB): calcd for $C_{12}H_{15}N_3O_7$ ($M + H^+$) 314.0988, found 314.0981.

*(1S,5R,6R,8S)-3-Aza-1-(4,4′-dimethoxytrityloxymethyl)-8-hydroxy-3-methyl-6-(thymin-1-yl)-4,7-dioxabicyclo[3.2.1]octan-2-one (**21**).* To a stirring solution of compound **20** (50.0 mg, 0.159 mmol) in pyridine (3 mL) were added DMTrCl (65 mg, 0.191 mmol) and triethylamine (100 μL, 0.727 mmol). The reaction mixture was stirred at room temperature for 8 h. Saturated $NaHCO_3$ was added, and the product was extracted with ethyl acetate. The organic layer was washed with water and brine, dried (Na_2SO_4), and concentrated. The product was purified by column chromatography (1% triethylamine in n-hexane/ethyl acetate = 2:1 to 100% ethyl acetate) to produce **21** as a yellowish white solid (58.0 mg, 60%). Mp: 136–138 °C. $[\alpha]^{28}_D$ = −21.1 (c 1.00, $CHCl_3$). IR ν_{max} (KBr): 3339, 3189, 3062, 2926, 2850, 1693, 1608, 1509, 1464, 1395, 1253, 1177, 1080, 1033, 978 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ_H 1.36 (3H, s), 3.18 (3H, s), 3.72 (1H, d, J = 12 Hz), 3.76 (6H, s), 3.95 (1H, d, J = 12 Hz), 4.70 (1H, d, J = 3.6 Hz), 4.76 (1H, d, J = 3.6 Hz), 6.03 (1H, s), 6.81–6.84 (4H, m), 7.21 – 7.42 (9H, m), 7.71 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 12.0, 33.7, 55.2, 57.9, 64.5, 81.0, 87.3, 87.5, 111.3, 113.4, 127.1, 127.3, 127.7, 127.8, 128.0, 128.1, 129.1, 130.1, 130.1, 134.2, 135.0, 139.4, 144.1, 149.7, 158.7, 163.7. MS (FAB): m/z 638 ($M + Na^+$). HRMS (FAB): calcd for $C_{33}H_{33}N_3O_9Na$ ($M + Na^+$) 638.2109, found 638.2097.

*(1S,5R,6R,8S)-3-Aza-8-(2-cyanoethoxy(diisopropylamino)-phosphinoxy)-1-(4,4′-dimethoxytrityloxymethyl)-3-methyl-6-(thymin-1-yl)-4,7-dioxabicyclo[3.2.1]octan-2-one (**22**).* To a stirring solution of compound **21** (50.0 mg, 0.081 mmol) in anhydrous acetonitrile were added 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphorodiamidite (30.0 μL, 0.094 mmol) and 4,5-dicyanoimidazole (10.0 mg, 0.058 mmol). The reaction mixture was stirred at room temperature for 7 h. After completion of the reaction, satd $NaHCO_3$ was added, and the product was extracted with ethyl acetate. The organic layer was washed with water and brine, dried (Na_2SO_4), and concentrated. The product was purified by column chromatography (1% triethylamine in n-hexane/ethyl acetate = 2:1 to 1:1) to produce **22** as a white solid (46.0 mg, 70%). Mp: 117–119 °C. ^1H NMR (400 MHz, $CDCl_3$): δ_H 0.97–1.26 (15H, m), 2.39 (1H, t, J = 6 Hz), 2.40–2.63 (1H, m), 3.18 (3H, s), 3.53–3.62 (4H, m), 3.79 (6H, s), 3.95–4.04 (1H, m), 4.88–4.99 (2H, m), 6.08 (1H, s), 6.80–6.85 (4H, m), 7.23–7.43 (9H, m), 7.78 (1H, s), 8.66 (1H, br s). ^{31}P NMR (161 MHz, $CDCl_3$): δ 150.9, 151.5. MS (FAB): m/z 816 ($M + H^+$). HRMS (FAB): calcd for $C_{42}H_{50}N_5O_{10}P$ ($M + H^+$) 816.3373, found 816.3376.

Oligonucleotide Synthesis. Oligonucleotides **23–27** were synthesized on a 0.2 μmol scale using an Expedite 8909 Nucleic Acid Synthesis System according to the standard phosphoramidite protocol. 5-[3,5-Bis(trifluoromethyl)phenyl]-1*H*-tetrazole (Activator 42: Act42) was used as the activator, and Cap mix A (10% acetic acid in tetrahydrofuran) and Cap mix B (10% 1-methylimidazole in tetrahydrofuran/pyridine) were used as the capping agents. The standard synthesis cycle (trityl off mode) was used for assembly of the reagents and synthesis of the oligonucleotides, except that the coupling time was extended to 30–45 min for the HxNA monomers. The synthesized HxNA-phosphoramidite was dissolved in anhydrous acetonitrile. Standard CPG-solid supports from Glen Research were used. After synthesis, the synthesized oligonucleotides were cleaved from the solid support by treatment with 50 mM K_2CO_3 in methanol solution at room temperature for 90 min. The extract was treated with 1 M triethylamine acetic acid (TEAA), and the oligonucleotides were purified by Nap-10 column and reversed-phase HPLC (RP-HPLC) and then characterized by MALDI-TOF mass spectrometry.

UV Melting Experiments. The UV melting experiments were carried out on Shimadzu UV-1800 and Shimadzu UV-1650 instruments. To determine the T_m of the duplexes, equimolar amounts of target RNA/DNA strands and modified oligonucleotides were dissolved in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl to provide a final strand concentration of 4 μM. The samples were annealed at 90 °C and slowly cooled to room temperature. The melting experiment was monitored at 260 nm from 0 to 80 °C at a scan rate of 0.5 °C/min. T_m was calculated as the temperature at which the duplexes were half dissociated, determined by taking the first derivative of the melting curve.

Nuclease Resistance Study. The sample solutions were prepared by dissolving 0.75 μmol of oligonucleotides in 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$. In each sample solutions, 0.175 μL CAVP was added and the cleavage reaction was carried out at 37 °C. A portion of each reaction mixture was removed at timed intervals and heated to 90 °C for 5 min to deactivate the nuclease. Aliquots of the timed samples were analyzed by RP-HPLC to evaluate the amount of intact oligonucleotides remaining. The percentage of intact oligonucleotide in each sample was calculated and plotted against the digestion time to obtain a degradation curve with time.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

MALDI-TOF-MS data and yields of oligonucleotides **23** to **27a**, and MALDI-TOF-MS data of oligonucleotides **28** to **31** with an unlocked modification, ^1H and ^{13}C spectra of all new compounds (**3–7**, **9**, **12**, **13**, **15–17**, **19–21**), ^1H and ^{31}P NMR spectrum of **22**, UV melting curves for the duplexes between oligonucleotides **23–26** and DNA or RNA complement, CD spectra of duplexes formed by HxNA-modified oligonucleotides, Job plot experiment of **24** and **32**, HPLC profiles of enzymatic degradation of singly (**24**), doubly (**25**), and triply (**26**) modified HxNA oligonucleotides with respect to time, and MALDI-TOF-MS spectra for **24–31**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Zamecnik, P. C.; Stephenson., M. L. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 280−284.
(2) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543−584.
(3) Cohen, J. S. *Trends Biotechnol.* **1992**, *10*, 87−91.
(4) Opalinska, J. B.; Gewirtz, A. M. *Nat. Rev. Drug Discov.* **2002**, *1*, 503−514.
(5) Stein, A.; Cheng, Y. C. *Science* **1993**, *261*, 1004−1012.
(6) Gewirtz, A. M.; Sokol, D. L.; Ratajczak, M. Z. *Blood* **1998**, *92*, 712−736.
(7) Buchini, S.; Leumann, C. J. *Curr. Opin. Chem. Biol.* **2003**, *7*, 717−726.
(8) Crooke, S. T. *Annu. Rev. Med.* **2004**, *55*, 61−95.
(9) Yamamoto, T.; Nakatani, M.; Narukawa, K.; Obika, S. *Future Med. Chem.* **2011**, *3*, 339−365.
(10) Agrawal, S.; Kandimalla, E. R. *Mol. Med. Today* **2000**, *6*, 72−81.
(11) Herdewijn, P. *Liebigs Ann.* **1996**, 1337−1348.
(12) (a) Tarköy, M.; Bolli, M.; Schweizer, B.; Leumann, C. *Helv. Chim. Acta* **1993**, *76*, 481−510. (b) Stauffiger, A.; Leumann, C. J. *Eur. J. Org. Chem.* **2009**, 1153−1162. (c) Scheidegger, S. P.; Leumann, C. J. *Chem.—Eur. J.* **2006**, *12*, 8014−8023. (d) Ittig, D.; Gerber, A.-B.; Leumann, C. J. *Nucleic Acids Res.* **2011**, *39*, 373−380.
(13) (a) Saneyoshi, H.; Deschamps, J. R.; Marquez, V. E. *J. Org. Chem.* **2010**, *75*, 7659−7669. (b) Terrazas, M.; Ocampo, S. M.; Perales, J. C.; Marquez, V. E.; Eritja, R. *ChemBioChem* **2011**, *12*, 1056−1065.
(14) (a) Christensen, N. K.; Petersen, M.; Nielsen, P.; Jacobsen, J. P.; Olsen, C. E.; Wengel, J. *J. Am. Chem. Soc.* **1998**, *120*, 5458−5463. (b) Kvaerno, L.; Wengel, J. *J. Org. Chem.* **2001**, *66*, 5498−5503.
(15) (a) Wang, G.; Gunic, E.; Girardet, J. L.; Stoisavljevic, V. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1147−1150. (b) Wang, G. *Tetrahedron Lett.* **1999**, *40*, 6343−6346.
(16) (a) Obika, S.; Nanbu, D.; Hari, Y.; Morio, K.; In, Y.; Ishida, T.; Imanishi, T. *Tetrahedron Lett.* **1997**, *38*, 8735−8738. (b) Obika, S.; Nanbu, D.; Hari, Y.; Andoh, J.; Doi, T.; Imanishi, T. *Tetrahedron Lett.* **1998**, *39*, 5401−5404.
(17) Imanishi, T.; Obika, S. *Chem. Commun.* **2002**, 1653−1659.
(18) (a) Singh, S. K.; Nielsen, P.; Koshkin, A. A.; Wengel, J. *Chem. Commun.* **1998**, 455−456. (b) Koshkin, A. A.; Singh, S. K.; Nielsen, P.; Rajwanshi, V. K.; Kumar, R.; Meldgaard, M.; Olsen, C. E.; Wengel, J. *Tetrahedron* **1998**, *54*, 3607−3630. (c) Wengel, J. *Acc. Chem. Res.* **1999**, *32*, 301−310.
(19) (a) Singh, S. K.; Wengel, J. *Chem. Commun.* **1998**, 1247−1248. (b) Koshkin, A. A.; Nielsen, P.; Meldgaard, M.; Rajwanshi, V. K.; Singh, S. K.; Wengel, J. *J. Am. Chem. Soc.* **1998**, *120*, 13252−13253. (c) Birte, Vester; Wengel, J. *Biochemistry* **2004**, *43*, 13233−13241.
(20) Wahlestedt, C.; Salmi, P.; Good, L.; Kela, J.; Johnsson, T.; Hökfelt, T.; Broberger, C.; Porreca, F.; Lai, J.; Ren, K.; Ossipov, M.; Koshkin, A.; Jakobsen, N.; Skouv, J.; Oerum, H.; Jacobsen, M. H.; Wengel, J. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5633−5638.
(21) (a) Elmén, J.; Lindow, M.; Schütz, S.; Lawrence, M.; Petri, A.; Obad, S.; Lindholm, M.; Hedtjärn, M.; Hansen, H. F.; Berger, U.; Gullans, S.; Kearney, P.; Sarnow, P.; Straarup, E. M.; Kauppinen, S. *Nature* **2008**, *452*, 896−899. (b) Straarup, E. M.; Fisker, N.; Hedtjärn, M.; Lindholm, M. W.; Rosenbohm, C.; Aarup, V.; Hansen, H. F.; Ørum, H.; Hansen, J. B. R.; Koch, T. *Nucleic Acids Res.* **2010**, *38*, 7100−7111. (c) Gupta, N.; Fisker, N.; Asselin, M.-C.; Lindholm, M.; Rosenbohm, C.; Ørum, H.; Elmén, J.; Seidah, N. G.; Straarup, E. M. *PLoS One* **2010**, *5*, No. e10682.
(22) Morita, K.; Takagi, M.; Hasegawa, C.; Kaneko, M.; Tsutsumi, S.; Sone, J.; Ishikawa, T.; Imanishi, T.; Koizumi, M. *Bioorg. Med. Chem.* **2003**, *11*, 2211−2226.
(23) (a) Kumar, R.; Singh, S. K.; Koshkin, A. A.; Rajwanshi, V. K.; Meldgaard, M.; Wengel, J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2219−2222. (b) Rosenbohm, C.; Christensen, S. M.; Sørensen, M. D.; Pedersen, D. S.; Larsen, L. E.; Wengel, J.; Koch, T. *Org. Biomol. Chem.* **2003**, *1*, 655−663.
(24) Albæk, N.; Petersen, M.; Nielsen, P. *J. Org. Chem.* **2006**, *71*, 7731−7740.
(25) (a) Srivastava, P.; Barman, J.; Pathmasiri, W.; Plashkevych, O.; Wenska, M.; Chattopadhyaya, J. *J. Am. Chem. Soc.* **2007**, *129*, 8362−8379. (b) Varghese, O. P.; Barman, J.; Pathmasiri, W.; Plashkevych, O.; Honcharenko, D.; Chattopadhyaya, J. *J. Am. Chem. Soc.* **2006**, *128*, 15173−15187. (c) Zhou, C.; Plashkevych, O.; Chattopadhyaya, J. *Org. Biomol. Chem.* **2008**, *6*, 4627−4633.
(26) Prakash, T. P.; Siwkowski, A.; Allerson, C. R.; Migawa, M. T.; Lee, S.; Gaus, H. J.; Black, C.; Seth, P. P.; Swayze, E. E.; Bhat, B. *J. Med. Chem.* **2010**, *53*, 1636−1650.
(27) (a) Hari, Y.; Obika, S.; Ohnishi, R.; Eguchi, K.; Osaki, T.; Ohishi, H.; Imanishi, T. *Bioorg. Med. Chem.* **2006**, *14*, 1029−1038. (b) Mitsuoka, Y.; Kodama, T.; Ohnishi, R.; Hari, Y.; Imanishi, T.; Obika, S. *Nucleic Acids Res.* **2009**, *37*, 1225−1238. (c) Miyashita, K.; Rahman, S. M. A.; Seki, S.; Obika, S.; Imanshi, T. *Chem. Commun.* **2007**, 375−3767. (d) Rahman, S. M. A.; Seki, S.; Obika, S.; Yoshikawa, H.; Miyashita, K.; Imanishi, T. *J. Am. Chem. Soc.* **2008**, *130*, 4886−4896. (e) Nishida, M.; Baba, T.; Kodama, T.; Yahara, A.; Imanishi, T.; Obika, S. *Chem. Commun.* **2010**, 5283−5285.
(28) (a) Rahman, S. M. A.; Imanishi, T.; Obika, S. *Chem. Lett.* **2009**, *38*, 512−517. (b) Obika, S.; Rahman, S. M. A.; Fujisaka, A.; Kawada, Y.; Baba, T.; Imanishi, T. *Heterocycles* **2010**, *81*, 1347−1392.

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Design, Synthesis, and Properties of Boat-Shaped Glucopyranosyl Nucleic Acid

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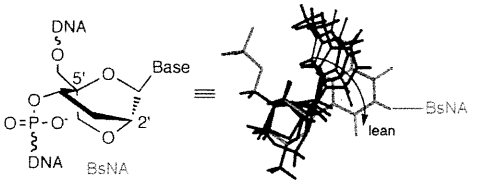
ABSTRACT



A boat-shaped glucopyranosyl nucleic acid (BsNA) was synthesized to investigate the possibility that the lean of a nucleobase is a factor affecting duplex-forming ability of oligonucleotides. From the crystal structure of a BsNA nucleoside and the thermal stability of duplex oligonucleotides, it was found that not only the lean of the base but also the rotation angle of the glycosidic bond axis were important factors in a stable duplex formation.

Much effort to develop nucleic acid therapeutics has been made to date.[1] Natural oligonucleotides (ONs) are not appropriate for therapeutic applications because they do not have enough target specificity, resistance toward nucleases, or cell membrane permeability. To improve their properties, nucleic acids have been chemically modified, and several clinical trials are currently being conducted with these artificial nucleic acids.[1] In addition to use in therapy, chemically modified ONs are also used in many other areas, such as nanotechnology,[2] diagnostics,[3] and drug target validation and gene function determination.[4] For these reasons, chemically modified ONs have attracted increasing attention.

We and other groups have developed numerous 2′,4′-bridged nucleic acid (2′,4′-BNA)[5]/locked nucleic acid (LNA)[6] analogues[7,8] whose sugar moieties are fixed in the North-type (C3′-*endo*) conformation, similar to a nucleotide in an A-type RNA duplex, by a bridge between the C2′- and C4′-positions. Because of this structural preorganization,[9] these analogues have high duplex-forming ability for complementary RNA. We have attempted to create additional artificial nucleic acids that form stable duplexes with complementary strands. However, there have been few BNA analogues[8b,c,e,11] that have affinities for RNA as high as the original 2′,4′-BNA/LNA, which remains the most promising BNA derivative even now. 2′,4′-BNA^{COC}, which has the sugar conformation closest

(1) (a) Chen, X.; Dudgeon, N.; Shen, L.; Wang, J. H. *Drug Discovery Today* **2005**, *10*, 587. (b) Juliano, R.; Bauman, J.; Kang, H.; Ming, X. *Mol. Pharmaceutics* **2009**, *6*, 686. (c) Yamamoto, T.; Nakatani, M.; Narukawa, K.; Obika, S. *Future Med. Chem.* **2011**, *3*, 339. (d) Shukla, S.; Sumaria, C. S.; Pradeepkumar, P. I. *ChemMedChem* **2010**, *5*, 328.

(2) (a) Bandy, T. J.; Brewer, A.; Burns, J. R.; Marth, G.; Nguyen, T.; Stulz, E. *Chem. Soc. Rev.* **2011**, *40*, 138. (b) Endo, M.; Sugiyama, H. *ChemBioChem* **2009**, *10*, 2420.

(3) (a) Singh, R. P.; Oh, B. K.; Choi, J. W. *Bioelectrochemistry* **2010**, *79*, 153. (b) Cerqueira, L.; Azevedo, N. F.; Almeida, C.; Jardim, T.; Keevil, C. W.; Vieira, M. J. *Int. J. Mol. Sci.* **2008**, *9*, 1944. (c) MacAskill, A.; Crawford, D.; Graham, D.; Faulds, K. *Anal. Chem.* **2009**, *81*, 8134.

(4) Ravichandran, L. V.; Dean, N. M.; Marcusson, E. G. *Oligonucleotides* **2004**, *14*, 49.

(5) Obika, S.; Nanbu, D.; Hari, Y.; Morio, K.; In, Y.; Ishida, T.; Imanishi, T. *Tetrahedron Lett.* **1997**, *38*, 8735.

(6) Singh, S. K.; Nielsen, P.; Koshkin, A. A.; Wengel, J. *Chem. Commun.* **1998**, 455.

(7) For the BNA or LNA analogues reported before 2010, see the following review. Obika, S.; Rahman, S. M. A.; Fujisaka, A.; Kawada, Y.; Baba, T.; Imanishi, T. *Heterocycles* **2010**, *81*, 1347.

(8) For the BNA or LNA analogues not included in ref 7, see the following references. (a) Zhou, C.; Liu, Y.; Andaloussi, M.; Badgujar, N.; Plashkevych, O.; Chattopadhyaya, J. *J. Org. Chem.* **2009**, *74*, 118. (b) Prakash, T. P.; Siwkowski, A.; Allerson, C. R.; Migawa, M. T.; Lee, S.; Gaus, H. J.; Black, C.; Seth, P. P.; Swayze, E. E.; Bhat, B. *J. Med. Chem.* **2010**, *53*, 1636. (c) Seth, P. P.; Vasquez, G.; Allerson, C. A.; Berdeja, A.; Gaus, H.; Kinberger, G. A.; Prakash, T. P.; Migawa, M. T.; Bhat, B.; Swayze, E. E. *J. Org. Chem.* **2010**, *75*, 1569. (d) Nishida, M.; Baba, T.; Kodama, T.; Yahara, A.; Imanishi, T.; Obika, S. *Chem. Commun.* **2010**, *46*, 5283. (e) Seth, P. P.; Allerson, C. R.; Berdeja, A.; Siwkowski, A.; Pallan, P. S.; Gaus, H.; Prakash, T. P.; Watt, A. T.; Egli, M.; Swayze, E. E. *J. Am. Chem. Soc.* **2010**, *132*, 14942. (f) Mori, K.; Kodama, T.; Baba, T.; Obika, S. *Org. Biomol. Chem.* **2011**, *9*, 5272.

(9) Kool, E. T. *Chem. Rev.* **1997**, *97*, 1473.

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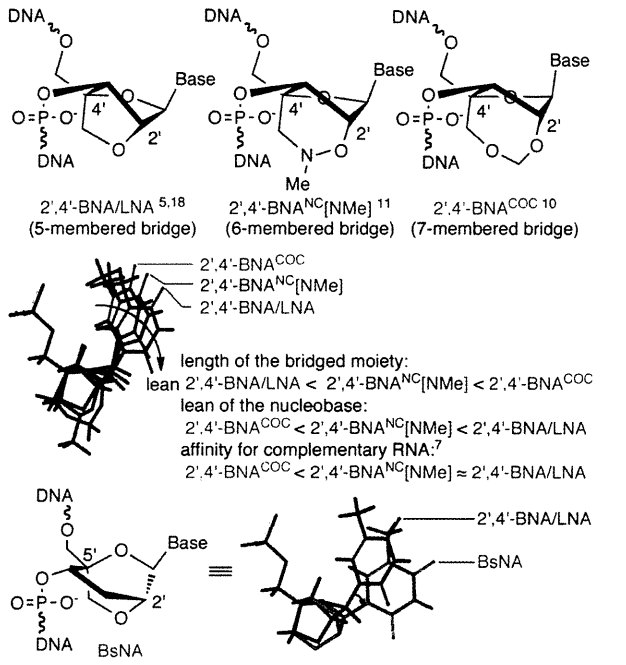
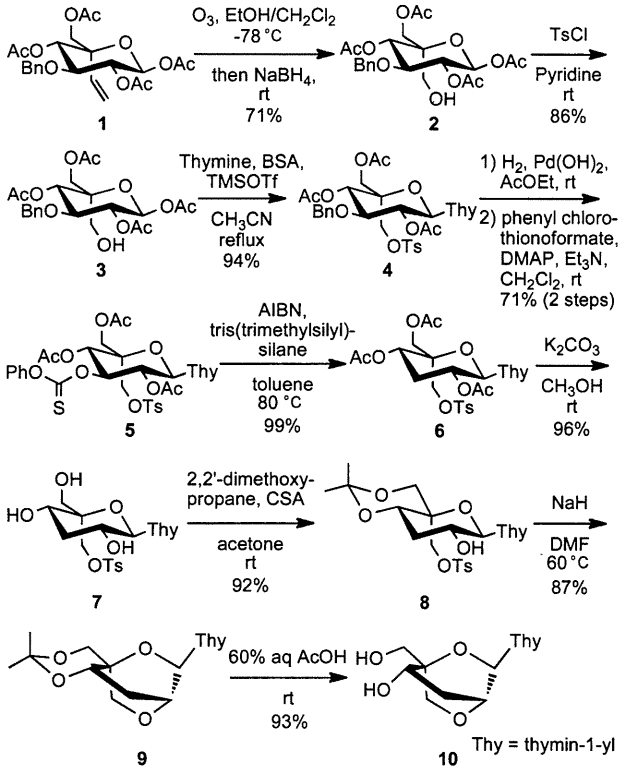


Figure 1. Relevance between the leans of nucleobases of BNA analogues, their binding affinities for ssRNA, and the molecular model of BsNA.

Scheme 1. Synthesis of Nucleoside **10**



to a typical A-type RNA duplex in the BNA analogues developed by us, forms a stable duplex with complementary RNA, but its affinity is not as high as that of LNA.[10] Thus, there is a need to develop a new type of artificial nucleic acid based on a brand new concept. We investigated all aspects of the structural properties of BNAs to determine the factors that affect the duplex-forming ability of ONs.

When internucleotidic phosphodiester bonds are kept immobile, the nucleobases of BNA analogues lean in the direction of the arrow in Figure 1 with decreasing numbers of atoms forming the bridge. In BNA analogues, the binding affinity for complementary RNA tends to become higher with decreasing size of the bridge and is the highest when the bridge is composed of 5 or 6 atoms. We suspected the lean of the nucleobases could be a factor impacting the duplex-forming ability and wondered what the binding affinity would be if the nucleobase leaned at still larger angles than that of LNA. We newly designed a boat-shaped glucopyranosyl nucleic acid (BsNA[12]), which had a pyranose ring as the basic skeleton. The nucleobase will lean more than that of LNA owing to this unusual skeleton.[13] We report here the synthesis of BsNA and the thermal stability of the ONs including BsNA.

BsNA was synthesized from known glucopyranoside **1**[14] as shown in Scheme 1. First, ozonolysis of glucopyranoside **1** followed by sodium borohydride reduction yielded alcohol **2**. Then, the tosylation of alcohol **2** was carried out with *p*-toluenesulfonyl chloride in pyridine, and the resulting compound **3** was coupled with a silylated thymine by Vorbrüggen's method[15] to give compound **4**. Next, **4** was subjected to hydrogenolysis conditions using palladium hydroxide and the resultant alcohol was treated with phenyl chlorothionoformate in the presence of *N*,*N*-dimethyl-4-aminopyridine to yield compound **5**. Subsequently, **5** was deoxygenated smoothly using tris(trimethylsilyl)silane and azobisisobutyronitrile,[16] and the obtained compound **6** was deacetylated to give triol **7**. The 4′- and 6′-hydroxy groups of triol **7** were protected as a isopropylidene ketal, and the resulting compound **8** was subjected to sodium hydride under moderate heating conditions to form the bridge between the C2′- and C5′-positions. Finally, removal of the isopropylidene group with aqueous acetic acid furnished desired nucleoside **10**.

Tritylation of **10** at the 6′-hydroxy group with 4,4′-dimethoxytrityl chloride and phosphitylation at the

(10) (a) Hari, Y.; Obika, S.; Ohnishi, R.; Eguchi, K.; Osaki, T.; Ohishi, H.; Imanishi, T. *Bioorg. Med. Chem.* **2006**, *14*, 1029. (b) Mitsuoka, Y.; Kodama, T.; Ohnishi, R.; Hari, Y.; Imanishi, T.; Obika, S. *Nucleic Acids Res.* **2009**, *37*, 1225.

(11) Rahman, S. M. A.; Seki, S.; Obika, S.; Yoshikawa, H.; Miyashita, K.; Imanishi, T. *J. Am. Chem. Soc.* **2008**, *130*, 4886.

(12) BsNA was also independently synthesized by the ISIS Pharmaceuticals group and was reported very recently. Migawa, M. T.; Seth, P. P.; Swayze, E. E.; Ross, B. S.; Song, Q.; Han, M. *PCT Int. Appl.* 2011, WO 2011/017521 A2.

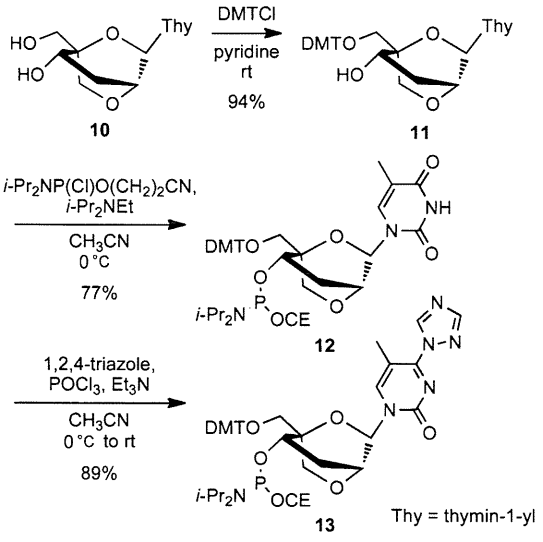
(13) Zhou, C.; Chattopadhyaya, J. *ARKIVOC* **2009**, *iii*, 171.

(14) Blériot, Y.; Vadivel, S. K.; Herrera, A. J.; Greig, I. R.; Kirby, A. J.; Sinay, P. *Tetrahedron* **2004**, *60*, 6813.

(15) Vorbrüggen, H.; Bennua, B. *Chem. Ber.* **1981**, *114*, 1279.

(16) Barton, D. H. R.; McCombie, S. W. *J. Chem. Soc., Perkin Trans. 1* **1975**, 1574.

Scheme 2. Synthesis of Phosphoramidite Building Blocks



4′-hydroxy group with 2-cyanoethyl *N*,*N*-diisopropylaminochlorophosphoramidite afforded the desired phosphoramidite building block **12** (Scheme 2). A portion of the thymine phosphoramidite **12** was converted to the triazolyl derivative **13**, which is a convertible phosphoramidite, by the treatment of 1,2,4-triazole in the presence of triethylamine and phosphoryl chloride.[17]

The conformation of the sugar in BsNA was determined from the crystal structure of compound **10**[19] (Figure 2). Compared to the X-ray structure of 2′,4′-BNA analogues,[10a,11,18] the thymine base of **10** leans more, as we designed; C5′/C6′–C1′–N1 angles, e.g., of 2′,4′-BNA^{COC}, 2′,4′-BNA^{NC}[NMe], 2′,4′-BNA/LNA, and **10** were 106°, 111°, 112°, and 125°, respectively.

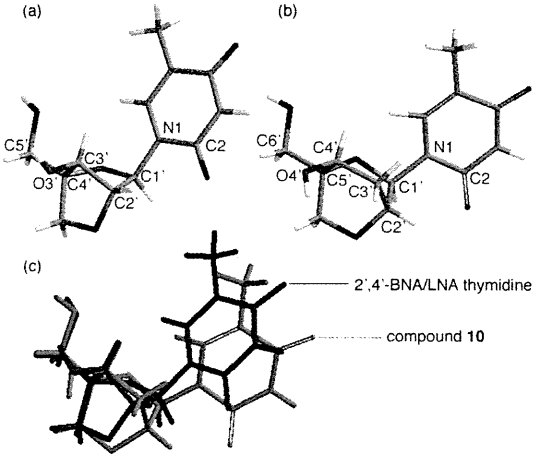


Figure 2. X-ray structures of 2′,4′-BNA/LNA thymidine (a)[18] and **10** (b),[19] and their superimposed images (c).

BsNA-phosphoramidites **12** and **13** were introduced into ONs using an automated DNA synthesizer (Table 1).

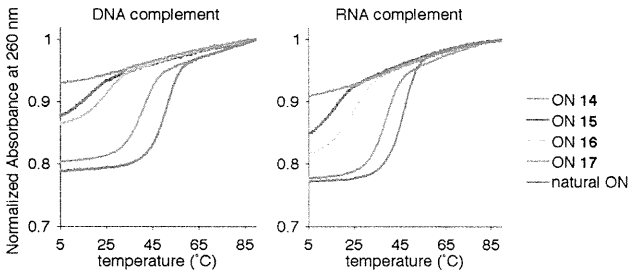
Table 1. Yields and MALDI-TOF-MS Data for the Oligonucleotides

oligonucleotide[a]	yield (%)[b]	calcd [M-H]$^-$	found [M-H]$^-$
5′-d(GCGTTTTTTGCT)-3′ (**14**)	46	3675.4	3676.2
5′-d(GCGTTTTTTGCT)-3′ (**15**)	55	3759.5	3755.6
5′-d(GCGTTTTTTGCT)-3′ (**16**)	29	3759.5	3756.7
5′-d(GCGTTTTTTGCT)-3′ (**17**)	34	3885.6	3883.9
5′-d(TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT)-3′ (**18**)	14	4539.0	4536.9
5′-d(TTTTT^{m}CTTT^{m}CT^{m}CT^{m}CT)-3′ (**19**)	6	4539.0	4539.0

[a] Underlined bold characters indicate the modified residues. Superscript m shows that the following C is a 5-methylcytidine derivative. [b] The isolation yields for ON **14**–**19** were calculated from the UV absorbance at 260 nm.

The sequences were the same as those in our previous studies, and cytidines of ONs **18** and **19** were replaced by 5-methylcytidines for the stable triplex formation.[7] Each coupling reaction of modified monomers was accomplished using 5-[3,5-bis(trifluoromethyl)phenyl]-1*H*-tetrazole as an activator over 6 min. Coupling yields were checked by trityl monitoring and were estimated to be over 95%. Synthesized ONs were cleaved from the solid supports and deprotected by treatment with concentrated ammonium hydroxide solution. Simultaneously, the triazole group of ON **19** was converted to an amino group to give a BsNA-5-methylcytosine-modified oligonucleotide.

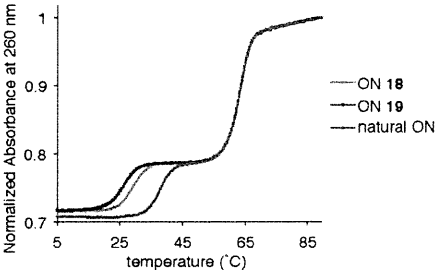
Table 2. Evaluation of Thermal Denaturation Temperatures (T_m Values) of Duplexes[a]



	T_m (°C)[b]	
oligonucleotide	DNA complement	RNA complement
Natural	51	48
14	41	39
15	ND[c]	ND[c]
16	25	24
17	ND[c]	ND[c]

[a] UV melting curves for the duplexes formed by ONs and the target strand, 5′-AGCAAAAAACGC-3′, were measured under the following 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. [b] T_m was determined by taking the first derivative of the melting curve. The number is the average of three independent measurements. [c] ND = not detected.

Table 3. Evaluation of Thermal Denaturation Temperatures (T_m Values) of Triplexes[a]



oligonucleotide	T_m (°C)[b]
Natural	37
18	29
19	26

[a] UV melting curves for the triplexes formed by ONs and the target strand, 5′-d(GCT*AAAAAGAAAGAGAGA*TCG)-3′/3′-d(CGA*TTTTTC-TT-TCTCTCT*AGC)-5′, were measured under the following conditions: 7 mM sodium cacodylate buffer (pH 7.0) containing 140 mM KCl and 10 mM $MgCl_2$; each strand concentration = 1.5 μM; scan rate of 0.5 °C min^{-1} at 260 nm. The italic portions indicate the target site for triplex formation. [b] T_m was determined by taking the first derivative of the melting curve. The number is the average of three independent measurements.

We evaluated the affinity of the synthesized ONs with complementary single-stranded RNA (ssRNA) and DNA (ssDNA) and double-stranded DNA (dsDNA) through UV melting experiments. The UV melting profiles and thermal denaturation temperatures (T_m values) are summarized in Tables 2 and 3. BsNA formed unstable duplexes with ssRNA and ssDNA and a triplex with dsDNA. When a larger number of BsNA monomers were introduced into ONs, a smaller hyperchromicity was observed. This is perhaps explained by steric repulsion or destabilization of the hydrogen bonds between the base pairs due to a too large lean of the BsNA nucleobase. This indicates that the lean of the nucleobase may be an important factor in the duplex-forming ability.

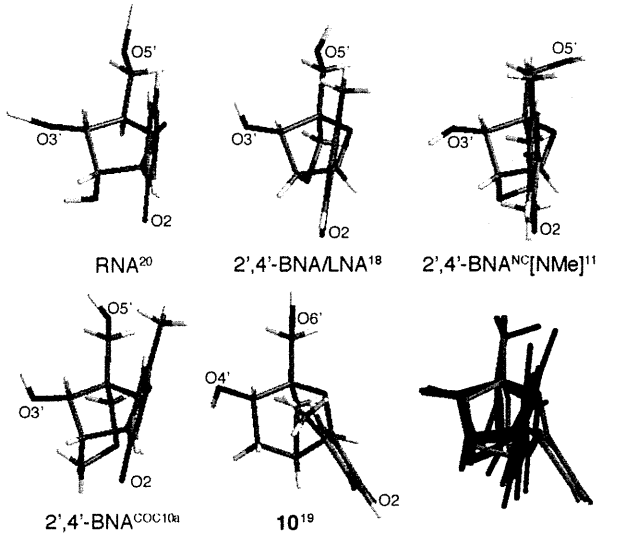


Figure 3. Nucleobase orientations of a typical A-type RNA duplex, BNA analogues, and **10**, and their superimposed images. Hydrogen atoms are omitted in the superimposed images.

However, other factors can be attributed to the destabilization of the duplex and triplex. As shown in Figure 3, the nucleobase orientation of **10** (magenta) differs from those of a typical A-type RNA duplex or other BNA analogues (black), and moreover, the rotation of the C1′–N1 bond axis in **10** may be restricted. Therefore, in the hybridization, the nucleobase orientation of the target strand perhaps needs to be altered to form hydrogen bonds, which is an unfavorable process. In addition, the axial H3′ has the potential to inhibit π-stacking between neighboring bases. Investigation of these possibilities is currently underway in our laboratory.

Acknowledgment. This work was supported by a Grant-in-Aid for Challenging Exploratory Research (22651076) from JSPS, for Young Scientists (B) (20790010) and for Scientific Research on Innovative Area (22136006) from MEXT, and the Program for Promotion of Fundamental Studies in Health Sciences of NIBIO.

Supporting Information Available. Experimental details and characterization data for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

(17) (a) Scerr, M.; Klebba, C.; Hanner, A.; Ganser, A.; Engels, J. W. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1791. (b) Sung, W. L. *J. Chem. Soc., Chem. Commun.* **1981**, 1089.

(18) Morita, K.; Takagi, M.; Hasegawa, C.; Kaneko, M.; Tsutsumi, S.; Sone, J.; Ishikawa, T.; Imanishi, T.; Koizumi, M. *Bioorg. Med. Chem.* **2003**, *11*, 2211.

(19) Crystallographic data for **10** can be found in the Supporting Information. Deposition no. CCDC 840597.

(20) RNA structure was obtained using Spartan '06, Wavefunction, Inc., Irvine, CA.

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Benzylidene Acetal Type Bridged Nucleic Acids: Changes in Properties Upon Cleavage of the Bridge Triggered by External Stimuli

Kunihiko Morihiro, Tetsuya Kodama, and Satoshi Obika*[a]

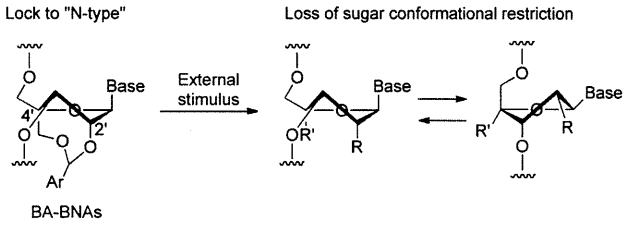
Abstract: Four classes of benzylidene acetal type bridged nucleic acids (BA-BNAs) were designed with 2′,4′-bridged structures that cleaved upon exposure to appropriate external stimuli. Cleavage of 6-nitroveratrylidene and 2-nitrobenzylidene acetal type BNA bridges occurred upon photoirradiation and subsequent treatment with thiol caused changes in secondary structure to afford 4′-*C*-hydroxymethyl RNA. Benzylidene and 4-nitrobenzylidene acetal type BNA responded to acids and reducing agents, respectively, resulting in hydrolysis of the acetal-bridged structure. Cleavage of the bridge removed sugar conformational restrictions and changed the duplex- and triplex-forming properties of the BNA-modified oligonucleotides. Moreover, oligonucleotides incorporating a single BA-BNA modification had considerably improved stability toward 3′-exonuclease, which was lost upon cleavage of the bridge. Thus, these new BNAs may be useful as therapeutic and detection tools by sensing various environments.

Keywords: DNA · nuclease resistance · nucleic acids · oligonucleotides · RNA

Introduction

The ability to respond to an external stimulus is an important requirement for molecular tools aimed at regulating biological phenomena or acting as sensing systems for nanodevices. The regulation of nucleic acid properties is a particularly attractive research area due to its diverse applications, such as gene regulation,[1] molecular diagnostics,[2] catalysts[3] or functional nanoscale materials.[4] Various external stimuli, such as light,[5] pH,[6] temperature,[7] change in redox potential,[8] or small molecules,[9] have been used for this purpose.

Previously, we[10a] and Wengel's[10b] group independently synthesized 2′-*O*,4′-*C*-methylene-bridged nucleic acid (2′,4′-BNA/LNA)[10] with an N-type locked sugar conformation imposed by the bridged structure, and demonstrated that oligonucleotides (ONs) containing 2′,4′-BNA/LNA have high affinity for complementary single-stranded RNA (ssRNA)[11] and double-stranded DNA (dsDNA).[12] Since these characteristics of 2′,4′-BNA/LNA are due to the fixed N-type sugar conformation, we were inspired to design benzylidene acetal type BNAs (BA-BNAs) that have a labile 2′,4′-bridged structure (Scheme 1). The sugar conformation of BA-BNAs is locked in the N-type conformation, but upon exposure to an appropriate external stimulus, the bridge is cleaved and the sugar conformational restriction is lost. This causes changes in the properties of the nucleic acid, including its ability to form duplexes or triplexes, and allows control of various molecular systems. We recently communicated the synthesis and properties of a light-responsive BNA that contained a photolabile 6-nitroveratryl group in the acetal-bridged structure (Scheme 2, 6-NV^B).[13] The bridged structure of 6-NV^B can be cleaved upon photoirradiation (form **II**) and subsequent treatment with glutathione (GSH) causes secondary structure changes to afford 4′-*C*-hydroxymethyl RNA (form **III**).[14] With these two steps required to change the structure of 6-NV^B, the binding affinity of ONs containing 6-NV^B for complementary ssRNA can be changed in two stages. Nucleic acids that respond to both light and GSH may have potential in therapeutics, diagnostics, or as detection agents by sensing GSH concentration.



Scheme 1. Cleavage of the bridged structure and loss of sugar conformational restriction triggered by external stimulus.

The introduction of other aryl groups into the bridged structure could provide BNAs that lose their locked sugar conformation in response to other external stimuli. Therefore, we designed three BA-BNAs containing either a 2-nitrobenzyl (2-NB^B), a benzyl (B^B), or a 4-nitrobenzyl (4-NB^B) group in the bridged structure (Scheme 2). 2-NB^B responds to light in the same manner as 6-NV^B, but the secon-

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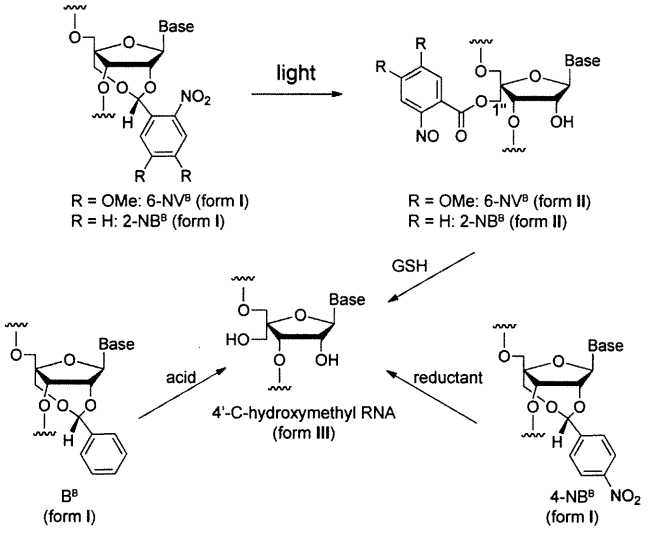
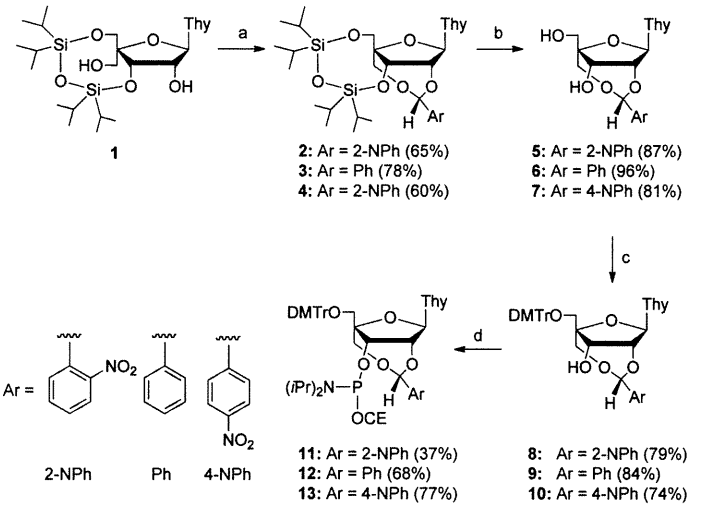
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201100541.



Scheme 2. Change in structure of BA-BNAs responding to various external stimuli.



Scheme 3. Synthesis of BA-BNA phosphoramidites. Reagents and conditions: a) 2-nitrobenzaldehyde, $ZnCl_2$, toluene, RT, 37 h (**2**); benzaldehyde, $ZnCl_2$, RT, 14 h (**3**); 4-nitrobenzaldehyde, $ZnCl_2$, HFIP, RT, 18 h (**4**); b) TBAF, THF, 0°C; c) DMTrCl, pyridine, RT, 15 h (**8**); 11 h (**9**); 21 h (**10**); d) $(iPr_2N)_2PO(CH_2)_2CN$, 4,5-dicyanoimidazole, CH_3CN, RT, 17 h (**11**); 40 h (**12**); 17 h (**13**). CE = cyanoethyl, DMTr = 4,4'-dimethoxytrityl, HFIP = 1,1,1,3,3,3-hexafluoroisopropanol, Thy = thymin-1-yl, TBAF = tetra-*n*-butylammonium fluoride.

dary reaction with GSH is predicted to proceed faster than that of 6-NV^B because of the increased electrophilicity of the 1''-*O*-carbonyl group. B^B has an electron-donating phenyl group at the bridged moiety, and the acetal structure is easily degraded under acidic conditions to form 4'-*C*-hydroxymethyl RNA. In contrast, 4-NB^B has a 4-nitrophenyl group at the bridged structure. The electron-withdrawing nitro group of 4-NB^B can be reduced to an electron-donating amino group by treatment with a reducing agent, upon which the acetal-bridged structure becomes very unstable and can be hydrolyzed even under neutral conditions. Thus, B^B has the potential to be a sensor of acidic conditions and 4-NB^B has the potential to be a sensor of reducing conditions. Herein, we report the synthesis of these new BA-BNAs and describe the changes in structure and properties of these BA-BNAs, including 6-NV^B.

Results

Synthesis of BA-BNAs phosphoramidites: BA-BNAs were synthesized from nucleoside derivative **1**,[13] as shown in Scheme 3.[15] When the 3'- and 5'-hydroxyl groups of the nucleoside are modified with the 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl (TIPDS) moiety, the sugar conformation is prelocked in the N-type conformation,[16] similar to that of 2',4'-BNAs. Compound **1**, therefore, has advantages for the construction of the 2',4'-bridged moiety. The 2',4'-bridged structures were constructed by treating diol **1** with three types of aldehydes and ring-closed compounds **2–4** were obtained as single diastereomers. The configuration at the acetal carbon atom was determined from NOESY spectra (Figures S18 and S19 in Supporting Information and references [13] and [15]). The NOESY spectrum showed no correlation between acetal-H/1'-H or a correlation between acetal-H/TIPDS-H. It should not be possible to show such a NOESY spectrum for the other diastereomer. A plausible transition state model for this reaction is described in Supporting Information (Figure S21). Desilylation was carried out by using TBAF to afford the corresponding nucleoside analogues **5–7**. Tritylation at the primary hydroxyl group with DMTrCl and phosphitylation at the secondary hydroxyl group yielded phosphoramidites **11–13**. Phosphoramidites **11–13** and the 6-NV^B amidite building block were introduced into ONs **14–18** on an automated DNA synthesizer (Figure 1, details are given in the Supporting Information).

5'-d(GCGTT**X**TTTGCT)-3' (**14a–d**)
5'-d(GCG**X**T**X**T**X**TGCT)-3' (**15a–d**)
5'-d(TTTTT^{m}CT**X**T^{m}CT^{m}CT^{m}CT)-3' (**16a–d**)
5'-d(TTTT**X**^{m}CT**X**T^{m}C**X**^{m}CT^{m}CT)-3' (**17a–d**)
5'-d(TTTTTTTT**X**T)-3' (**18a–d**)

Figure 1. Sequences of ONs used in this study. **X**s indicate the modified residues. Series **a**, **b**, **c**, and **d** represent 6-NV^B-, 2-NB^B-, B^B- and 4-NB^B-modified ONs, respectively.

Change in structure of light-responsive BNA: We evaluated the cleavage of the 2-NB^B bridge upon photoirradiation. A UV-LED (365 nm) lamp was used as the light source. ON **14b** (form **I**), which contains one 2-NB^B unit, was photoirradiated at 365 nm for 5 s and the resulting products were analyzed by reverse-phase HPLC (RP-HPLC) and MALDI-TOF mass spectrometry (Figure 2B). The signals corresponding to the bridge-closed form (form **I**) disappeared and the bridge-opened form (form **II**) was generated. Furthermore, when ON **14b** was photoirradiated for 5 s and then treated with GSH for 60 min, 2-NB^B was transformed into 4'-*C*-hydroxymethyl RNA (form **III**; Figure 2C).

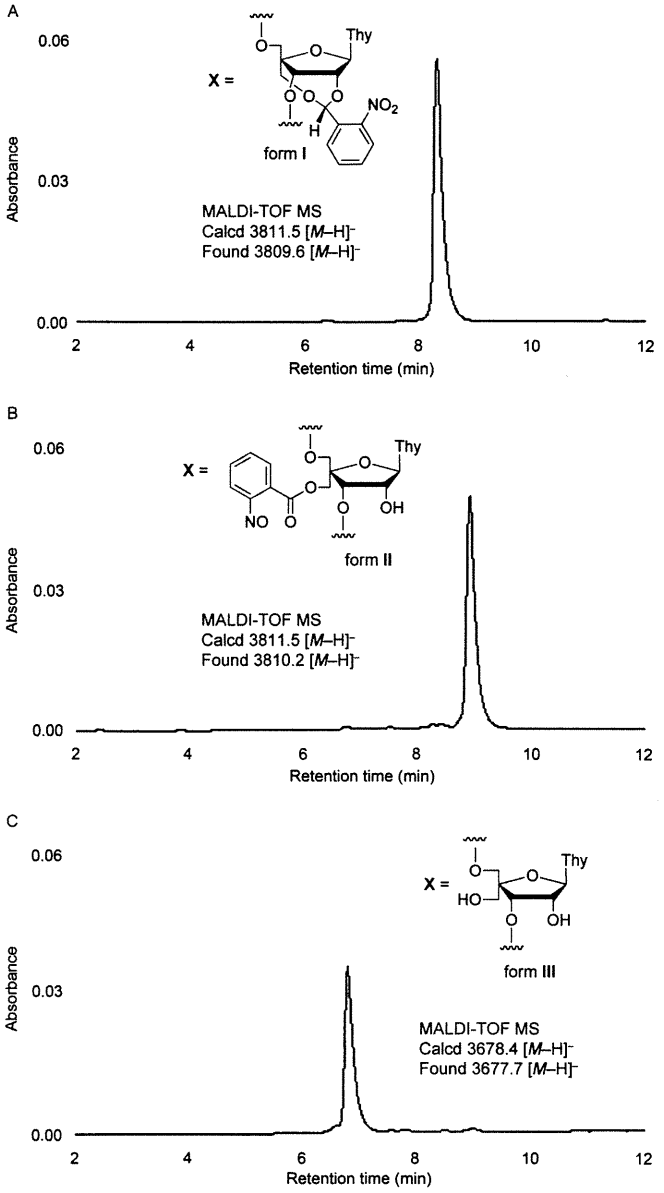


Figure 2. RP-HPLC analysis of ON **14a**. A) ON **14a** (form **I**) (10 μM) before photoirradiation. B) ON **14a** (10 μM) after irradiation at 365 nm for 5 s. C) ON **14a** (10 μM) after irradiation at 365 nm for 5 s and subsequent reaction with GSH (10 μM) for 60 min. The reaction mixture was analyzed by RP-HPLC by using an XTerra MS C18 column (4.6×50 mm) with a linear gradient of CH_3CN (6 to 24% over 15 min) in 0.1 M triethylammonium acetate (pH 7.0).

We compared the reaction efficiency of 2-NB^B with 6-NV^B against GSH. Following photoirradiation of ON **14a** and **14b** for 5 s (form **II**), the products were treated with GSH and the remaining intact ON was quantified by RP-HPLC (Figure 3). As expected, the reaction of 2-NB^B (form **II**) with GSH proceeded faster than that of 6-NV^B (form **II**) ($t_{1/2}$=6 vs. >200 min). The efficient reaction of 2-NB^B (form **II**) with GSH prompted us to evaluate the reactivity with glutamate (Glu), cysteine (Cys), and glycine (Gly), which are constituent amino acids of GSH. The results showed that 2-NB^B (form **II**) reacted only with Cys and was converted into 4′-*C*-hydroxymethyl RNA (form **III**). Interestingly, the reaction rate with Cys was faster than that with GSH (Figure 3; $t_{1/2}$=<1 vs. 6 min), indicating that 2-NB^B (form **II**) could work not only as a good GSH senser, but also as a remarkable Cys sensor. These results indicate that the thiol group is important for the reaction of 6-NV^B and 2-NB^B (form **II**) with GSH.

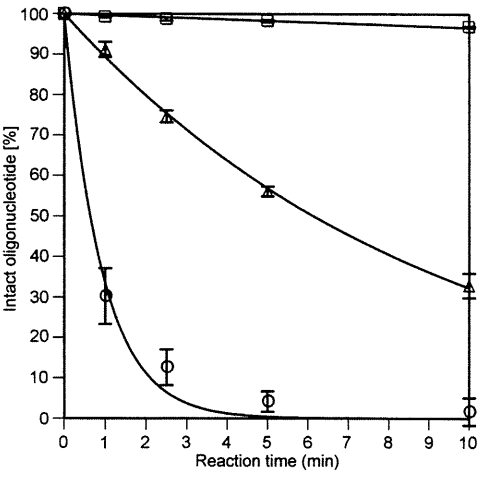


Figure 3. Time course of the reaction of ON **14a** (form **II**) with GSH (□; $t_{1/2}$>200 min), ON **14b** (form **II**) with GSH △; $t_{1/2}$=6 min) and ON **14b** (form **II**) with Cys (○; $t_{1/2}$<1 min). Error bars indicate standard deviation (n=3). Conditions: 25 mM sodium phosphate buffer (pH 7.2), 10 μM ON **14a** or ON **14b**, and 10 μM GSH or Cys; reaction temperature=37°C.

Change in structure of acid-responsive BNA: The change in the structure of B^B under acidic conditions was evaluated in a similar way. ON **14c** (form **I**), which contains one B^B unit, was placed in a solution of citrate phosphate buffer at pH 3.0 for 60 min and analyzed by RP-HPLC (Figure 4). The acetal-bridged structure was hydrolyzed and 4′-*C*-hydroxymethyl RNA (form **III**) was generated.

We next examined the efficiency of the hydrolysis of the acetal-bridged structure of B^B at pH 3.0–5.0 (Figure 5) and found that the hydrolysis rate decreased as the pH of the solution increased. This means that cleavage of the bridged structure of B^B is dependent on the pH under acidic conditions, suggesting that B^B holds promise for development as a pH sensor.

Change in structure of reductant-responsive BNA: The reaction of 4-NB^B with the reducing agents sodium ascorbate,[17] potassium ferrocyanide,[18] dithiothreitol,[19] GSH,[20] sodium thiosulfate,[21] and sodium dithionite[22] was examined. Of these reducing agents, only sodium dithionite could reduce the nitro group of 4-NB^B (Figure 6). The signal corresponding to the 4-NB^B-modified ON (form **I**) disappeared within 10 min, but 4′-*C*-hydroxymethyl RNA (form **III**) appeared somewhat later, indicating that reduction of the nitro group proceeded rapidly, but hydrolysis of the acetal-bridged structure proceeded at a slower rate. In contrast, the initial reduction of ON **14b** (form **I**) containing 2-NB^B occurs much more slowly and there are no traces of subse-

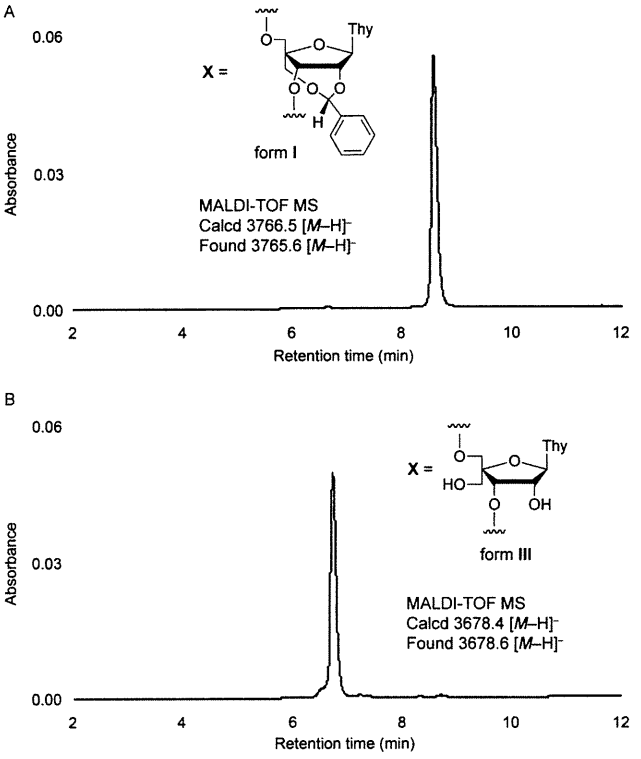


Figure 4. RP-HPLC analysis of ON **14b**. A) ON **14b** (10 μM) before treatment with acid. B) ON **14b** (10 μM) after reaction in a solution of citrate phosphate buffer at pH 3.0 for 60 min at 37 °C. The reaction mixture was analyzed by RP-HPLC by using an XTerra MS C18 column (4.6×50 mm) with a linear gradient of CH_3CN (6 to 24% over 15 min) in 0.1 M triethylammonium acetate (pH 7.0).

quent hydrolysis even after 10 min (Figure S24 in the Supporting Information).

Duplex- and triplex-forming abilities: Next, the ability of the synthesized BA-BNAs to form duplexes and triplexes was evaluated. These BNAs reacted with each stimulus orthogonally and lost their sugar conformational restriction. These structural changes caused changes in the properties of the ONs, such as their duplex- and triplex-forming abilities. The duplex-forming abilities of BA-BNA-modified ONs were evaluated against complementary ssDNA/RNA by measuring T_m values (Table 1). The duplex-forming abilities of ONs **14a–d**, which contain one BA-BNA unit, changed only slightly upon bridge cleavage. The T_m values of duplex-

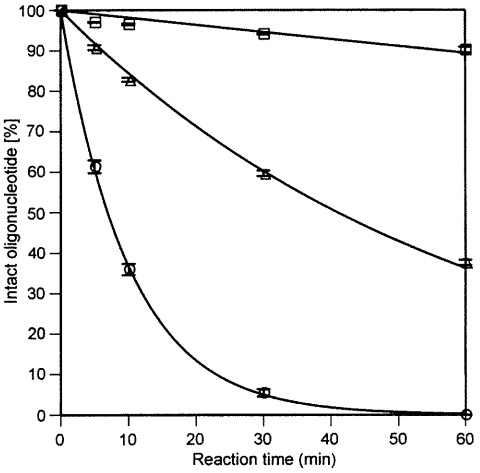


Figure 5. Time course of the reaction of ON **14c** in a solution at pH 3.0 (○; $t_{1/2}$ = 7 min), 4.0 (△; $t_{1/2}$ = 41 min), and 5.0 (□; $t_{1/2}$ > 300 min). Error bars indicate standard deviation (n = 3). Conditions: 25 mM citrate phosphate buffer and 10 μM ON **14c**; reaction temperature = 37 °C.

es formed between ONs containing three BA-BNA units and ssDNA or ssRNA were dramatically changed by external stimuli because the synergistic effects of bulky aryl moieties on the duplex stability[23] were canceled upon bridge cleavage. Figure 7 showed the ΔT_m values of each ON (form **II** and **III**) relative to that of form **I**. ONs **15a–d** (form **III**) showed remarkably higher binding affinity to their DNA complement than form **I** (Figure 7A; T_m values are at most 12 °C higher). With ssRNA, the binding affinity of form **III** was much higher than form **I** in the case of **15a**, whereas they were almost identical in case of **15b–d**. ON **15b** (form **II**) showed much lower T_m values than form **III** (Figure 7B).

We also evaluated the triplex-forming ability of ONs containing BA-BNA with target dsDNA (Table 2). BA-BNAs (form **I**) showed low binding affinity, especially if they had three modifications (T_m value < 20 °C). In addition, no triplex formation was observed with 6-NV^B and 2-NB^B after photoirradiation (form **II**), whereas a stable triplex was formed (T_m of 40 °C) after conversion into 4′-*C*-hydroxymethyl RNA (form **III**).

Nuclease resistance of BA-BNA-modified ONs: We examined the resistance of ONs modified with a single BA-BNA unit (ON **18a–d**) toward 3′-exonuclease (*Crotalus adamanteus* venom phosphodiesterase (CAVP)) degradation and compared it with natural ONs. ONs were incubated with CAVP and the percentage of intact ONs was analyzed at several time points by RP-HPLC (Figure 8). The natural oligothymidylates were essentially digested within 20 min, whereas BA-BNA-modified ONs (ON **18a–d**; form **I**) were remarkably stable under these conditions, with about 95% of each

Table 1. T_m Values [°C] of duplexes formed by BA-BNA-modified ONs with complementary ssDNA/RNA.[a]

Duplexes **X**=	T_m [°C] 6-NV^B (**I**)	6-NV^B (**II**)	2-NB^B (**I**)	2-NB^B (**II**)	B^B (**I**)	4-NB^B (**I**)	4′-*C*-hydroxymethyl RNA (**III**)
14a–d/ssDNA	41	39	43	42	45	44	44
15a–d/ssDNA	–[b]	–[b]	23	–[b]	–[b]	24	32
14a–d/ssRNA	39	37	43	39	45	45	43
15a–d/ssRNA	–[b]	–[b]	38	–[b]	34	41	37

[a] Target strand: 5′-AGCAAAAAACGC-3′. Conditions: 10 mM sodium phosphate buffer solution (pH 7.2) containing 100 mM NaCl; each strand concentration = 4 μM; scan rate of 0.5 °C min^{-1} at 260 nm. **X** = BA-BNA. The number is average of three independent measurements. [b] T_m < 20 °C or not detectable.

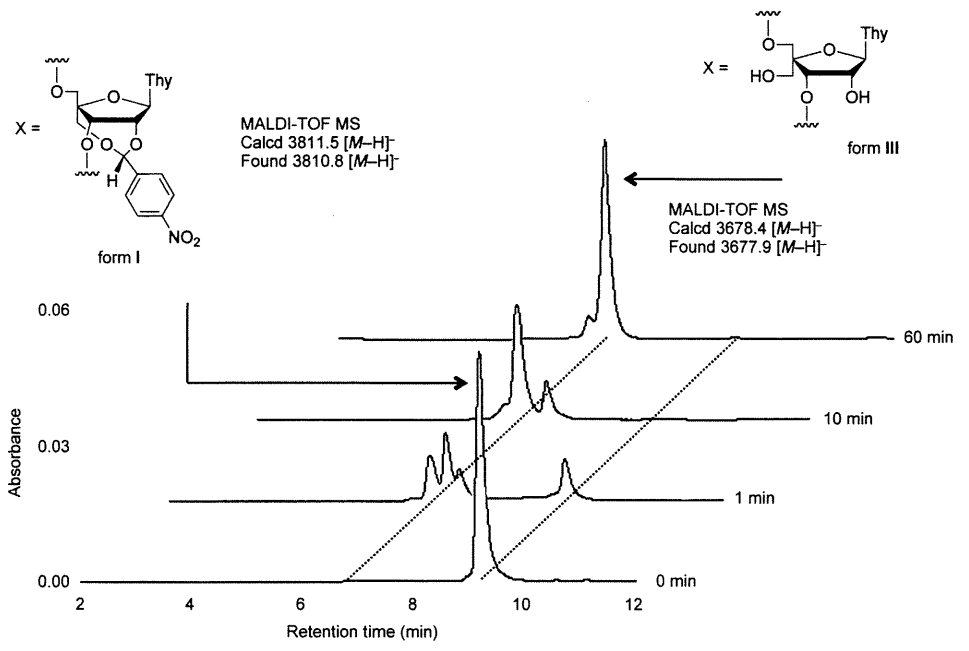


Figure 6. RP-HPLC analysis of ON **14c**. ON **14c** (10 μM) was treated with sodium dithionite (25 mM) for a given time at 37 °C. The reaction mixture was analyzed by RP-HPLC using an XTerra MS C18 column (4.6 × 50 mm) with a linear gradient of CH_3CN (6% to 24% over 15 min) in 0.1 M triethylammonium acetate (pH 7.0).

Table 2. T_m Values [°C] of triplexes formed by BA-BNA-modified ONs with target dsDNA.[a]

Oligonucleotides X=	T_m [°C] 6-NV^B (**I**)	6-NV^B (**II**)	2-NB^B (**I**)	2-NB^B (**II**)	B^B (**I**)	4-NB^B (**I**)	4′-*C*-hydroxymethyl RNA (**III**)
16a–d	24	26	32	27	25	35	41
17a–d	_[b]	_[b]	_[b]	_[b]	_[b]	_[b]	40

[a] Target strand: 5′-d(GCT*AAAAAGAAAGAGAGA*TCG)-3′/3′-d(CGA*TTTTTCTTTCTCTCT*ATC)-5′; the italic portion indicates the target site for triplex formation. Conditions: 7 mM sodium phosphate buffer solution (pH 7.0) containing 140 mM KCl and 10 mM $MgCl_2$; each strand concentration = 1.5 μM; scan rate of 0.5 °C min^{-1} at 260 nm. **X** = BA-BNA; ^mC = 2′-deoxy-5-methylcytidine. The number is average of three independent measurements. [b] T_m < 20 °C or not detectable.

ON remaining intact after 20 min of exposure (form **I**: $t_{1/2}$ > 30 h). The nuclease resistance of ON **18a** and **18b** decreased drastically upon photoirradiation (form **II**; $t_{1/2}$ = 24 min). Conversion to 4′-*C*-hydroxymethyl RNA (form **III**) also decreased nuclease resistance, but form **III** was still more stable than natural ON ($t_{1/2}$ = 11 min *vs* 5 min), in agreement with the report by Wengel et al.[14]

Discussion

Orthogonal reactivity of BA-BNAs to each stimulus: The synthesized BA-BNAs respond to each stimulus (light, acid, or reductant) orthogonally by undergoing cleavage of the bridge according to the type of aryl moiety introduced into the acetal-bridged structure. 6-NV^B and 2-NB^B have a photoreactive 6-nitroveratryl[24] and 2-nitrobenzyl group,[25] respectively. Because an aryl moiety containing an *ortho*-nitro group is required for photoreactivity,[26] the bridge in B^B and 4-NB^B could not be cleaved by light (Figure S22 in Supporting Information). Moreover, a nitro group on the aryl moiety also affects the stability of the acetal bridge against acid. Benzylidene acetal is more sensitive to acid than methylene acetal because of the presence of the electron-donating phenyl group;[27] therefore, B^B loses its bridge under mild acidic conditions. In addition, it was impossible, in our hands, to synthesize ONs with a BA-BNA that had a 2,5-dimethoxybenzyl group because the acetal bridge was degraded by the acid treatment required for detritylation by the automated DNA synthesizer (data not shown). In contrast, all of the other BNAs were stable under these acidic conditions because the introduction of a nitro group into the aryl moiety reduced the electron density of the acetal bridge (Figure S23 in the Supporting Information). Furthermore, the position of the nitro group on the aryl moiety affected the reactivity with the reducing agent. 4-NB^B could effectively react with sodium dithionite and the resulting 4-aminobenzylidene acetal-bridged structure was easily hydrolyzed under neutral conditions. However, reduction of the nitro group in 2-NB^B under the same conditions was less effective (Figure S24 in the Supporting Information). It has been reported that the less-hindered *para*-nitro group in 2,4-dinitroaryl derivatives was preferentially reduced by some reducing agents;[28] therefore, reduction of the *para*-nitro group by sodium dithionite proceeded faster than that of the *ortho*-nitro group.

Change in hybridization properties of each BA-BNA-modified ONs: The hybridizing abilities of ONs **15a–d**, which have three BA-BNAs, to ssDNA were dramatically increased by converting form **I** into form **III**. In general, ONs containing a BNA with a seven-membered bridged ring had low hybridization affinities for their DNA complement.[29] In addition to the seven-membered bridge, BA-BNAs have a bulky aryl moiety in the minor groove, which may substantially destabilize the duplex structure. Transformation from form **I** to form **III** did not affect the hybridizing abilities of these ONs with ssRNA, except for 6-NV^B. Since the sugar

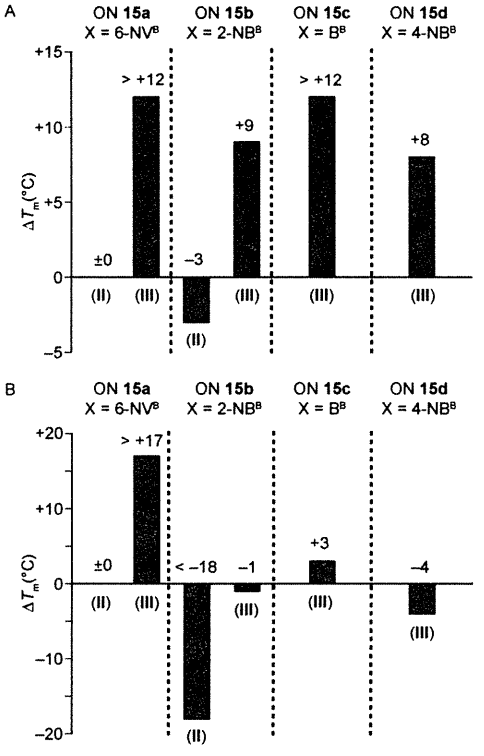


Figure 7. ΔT_m values of ONs **15a–d** (form **II** and **III**) relative to that of form **I** for A) ssDNA and B) ssRNA. ON **15** sequence: 5′-d(GCG**X**T**X**T**X**TGCT)-3′.

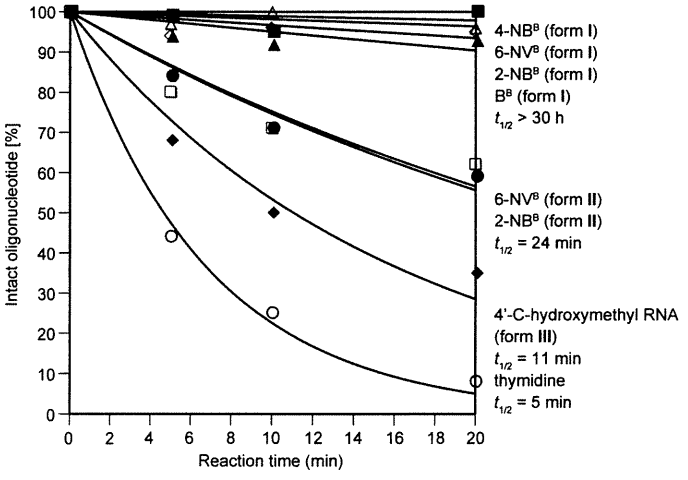


Figure 8. Nuclease resistance of 5′-d(T_8**X**T)-3′ against CAVP (Amersham Pharmacia Biotech); **X** = thymidine (○), 6-NV^B (form **I**) (△), 6-NV^B (form **I**) (□), 2-NB^B (form **I**) (◇), 2-NB^B (form **II**) (■), B^B (▲), 4-NB^B (■), 4′-*C*-hydroxymethal RNA (◆). Hydrolysis of the oligonucleotides (7.5 μM) was carried out at 37 °C in buffer (100 μL) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and CAVP (0.02 μg).

conformation of BA-BNA (form **I**) is restricted to N-type, which is the major conformation in the A-form of the RNA duplex structure,[30] these compounds can bind to RNA despite the presence of a large hydrophobic group in the minor groove.[31] 6-NV^B (form **I**) has a methoxy group at the 3-position of the aryl moiety that may hinder formation of a stable duplex due to steric repulsion (based on a modeling study; Figure S26 in the Supporting Information). Form **III** retained the ability to bind to ssRNA despite a flexible sugar conformation due to a reduction in steric hindrance. 6-NV^B and 2-NB^B (form **II**) lack sugar conformational restriction, yet have bulky substituents; hence, ONs containing form **II** are unable to bind to ssRNA. These results demonstrate that ONs with 6-NV^B and 2-NB^B will have different behavior following photoirradiation and subsequent thiol treatment in two stages.

The hybridizing abilities of all BA-BNAs to target dsDNA were changed upon exposure to the appropriate stimulus. Molecular modeling of triplex ON units helped clarify this change in affinity (Figure S27 in the Supporting Information) and showed that the aryl moiety of BA-BNAs (form **I** and **II**) in a triplex is positioned near the phosphodiester linkage. The resulting steric hindrance would decrease their ability to hybridize with dsDNA. Form **III** has a high triplex-forming ability due to its reduced steric hindrance.

Nuclease resistance of BA-BNA-modified ONs: Previous studies have shown that the substituent on the bridge structure, and the bridge size, contribute to the nuclease resistance of 2′,4′-BNA/LNA analogues and sterically hindered structures increase the nuclease resistance of modified ONs.[29a,b,d,32] In this study, ONs modified with BA-BNA (form **I**) showed high nuclease resistance against CAVP, probably due to steric hindrance of the aryl-substituted seven-membered bridged moiety. On the other hand, ONs with forms **II** and **III**, which were generated upon cleavage of the bridge, showed decreased nuclease resistance.

General overview: Several properties of ONs containing BA-BNAs can be regulated by various external stimuli, suggesting that BA-BNAs may be applicable to a variety of biotechnological applications. For example, the binding affinities of ONs containing 6-NV^B or 2-NB^B for ssRNA could be changed in two stages: first, by light and then with a thiol. Interestingly, the behavior of an ON could be changed according to which BNA is introduced (Figure 9A). These BA-BNAs could be useful for trapping or releasing functional ssRNA (mRNA, miRNA, or ribozyme) in a low thiol concentration or high thiol concentration environment at the desired time.[33] ONs modified with B^B or 4-NB^B would be able to bind ssRNA regardless of sugar conformation restriction by the bridge, although nuclease resistance is destroyed upon cleavage of the bridge (Figure 9B). These characteristics have a potential application as a molecular system in degradable devices, the properties of which could change in a controlled fashion at a specific location in the molecule.[34]

Many nucleic acid materials have been developed by focusing on base-pair hydrogen-bonding interactions,[35] stacking within DNA helix,[36] the inversion of helicity,[37] the activity of the 2′-hydroxyl group,[38] and recognition of the

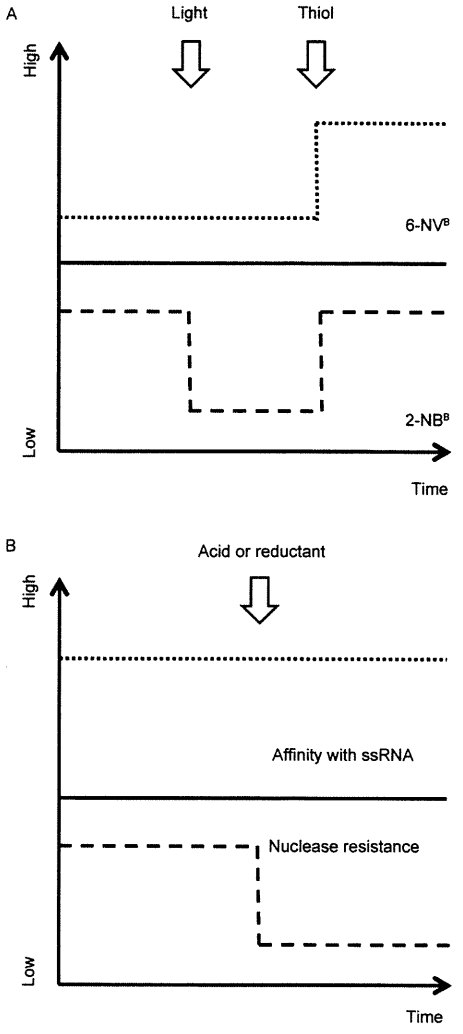


Figure 9. Basic concept behind changing the properties of BA-BNA-modified ONs by external stimuli. A) Change in the affinity of 6-NV^B- or 2-NB^B-modified ONs for ssRNA. B) Change in the affinity of B^B- or 4-NB^B-modified ONs for ssRNA and the nuclease resistance of B^B- or 4-NB^B-modified ONs.

phosphodiester linkage.[39] In this study, we have demonstrated that regulation of sugar conformational restriction is also an effective strategy for controlling the properties of nucleic acids. Molecular devices that exhibit similar responses to different stimuli are unique and attractive candidates for new types of molecular tools. Furthermore, the development of various types of BA-BNAs, in which each responds to a different stimulus, could be easily designed and simply achieved by altering the type of aryl moiety in the bridged structure.

Conclusion

We have synthesized BA-BNAs with a cleavable 2′,4′-bridged structure containing an aryl moiety. Each BA-BNA responds orthogonally to a specific external stimulus. The bridged structures of 6-NV^B and 2-NB^B were cleaved by photoirradiation and subsequent treatment with thiol triggered a change in the secondary structure to generate 4′-*C*-hydroxymethyl RNA. In contrast, the bridged structure of B^B was hydrolyzed under acidic conditions and that of 4-NB^B was hydrolyzed under reducing conditions. The sugar conformational restriction of these BA-BNAs was lost upon cleavage of the bridge. In conclusion, the duplex- and triplex-forming abilities and nuclease resistance of these BA-BNA-modified ONs could be changed at will in response to an appropriate external stimulus.

Experimental Section

Synthesis and characterization of compounds: General aspects and instrumentation: All moisture-sensitive reactions were carried out in well-dried glassware under an N_2 atmosphere. Acetonitrile, pyridine, and toluene were distilled from CaH_2. 6-NV^B and 2-NB^B were stable under ambient light conditions and did not need to be specially protected from light. ^{1}H, ^{13}C, ^{31}P, NOESY, and H–H COSY spectra were recorded on JEOL JNM-EX270, JEOL JNM-ECS400, and JEOL JNM-LA500 spectrometers. Chemical shifts are reported in parts per million referenced to $CHCl_3$ ($\delta=7.26$ ppm) and CH_3OH ($\delta=3.30$ ppm) for ^{1}H NMR spectra, and $CDCl_3$ ($\delta=77.0$ ppm) and CD_3OD ($\delta=49.0$ ppm) for ^{13}C NMR spectra. IR spectra were recorded on JASCO FT/IR-200 and JASCO FT/IR-4200 spectrometers. Optical rotations were recorded on a JASCO DIP-370 instrument. FAB mass spectra were measured on JEOL JMS-600 or JEOL JMS-700 mass spectrometers. MALDI-TOF mass spectra were recorded on a Bruker Daltonics Autoflex II TOF/TOF mass spectrometer. Fuji Silysia silica gel PSQ-100B (0.100 mm) and FL-100D (0.100 mm) were used for column chromatography, and silica gel PSQ-60B (0.060 mm) and FL-60D (0.060 mm) were used for flash column chromatography. For HPLC, SHIMADZU LC-10AT$_{VP}$, SHIMADZU SPD-10A$_{VP}$, and SHIMADZU CTO-10$_{VP}$ instruments were used.

Compound 2: Under an N_2 atmosphere, a solution of 2-nitrobenzaldehyde (3.8 g, 25 mmol) in anhydrous toluene (5 mL) and zinc chloride (150 mg, 1.1 mmol) were added to **1**[13] (500 mg, 0.94 mmol) and the resultant mixture was stirred at room temperature for 37 h. After the addition of a saturated aqueous solution of $NaHCO_3$ at 0 °C, the reaction mixture was diluted with AcOEt, washed with water and brine, dried over Na_2SO_4, and concentrated. The crude product was purified by column chromatography (*n*-hexane/AcOEt 2/1) to give **2** as a white foam (400 mg, 65 %). M.p. 140–143 °C; $[\alpha]_D^{23}=-47.0$ ($c=1.00$ in $CHCl_3$); ^{1}H NMR (270 MHz, $CDCl_3$): $\delta=8.12$ (brs, 1H; N3-H), 8.02 (t, $J=8$ Hz, 1H; Ar-H), 7.89 (t, $J=8$ Hz, 1H; Ar-H), 7.66 (t, $J=8$ Hz, 1H; Ar-H), 7.58 (s, 1H; H6), 7.49 (t, $J=8$ Hz, 1H; Ar-H), 6.91 (s, 1H; acetal-H), 6.15 (s, 1H; H1′), 4.65 (d, $J=6$ Hz, 1H; H3′), 4.41 (d, $J=6$ Hz, 1H; H2′), 4.03 (d, $J=13$ Hz, 1H; H5′), 4.00 (d, $J=13$ Hz, 1H; H1″), 3.71 (d, $J=13$ Hz, 1H; H1″), 3.67 (d, $J=13$ Hz, 1H; H5′), 1.92 (s, 3H; CH_3), 1.13–1.08 ppm (m, 28H; TIPDS-H); ^{13}C NMR (67.8 MHz, $CDCl_3$): $\delta=164.2$, 150.1, 148.0, 134.7, 133.6, 133.3, 129.4, 128.1, 124.2, 110.2, 98.1, 91.9, 89.3, 79.3, 70.6, 68.2, 59.6, 17.3, 17.3, 17.2, 17.1, 17.1, 17.0, 16.9, 12.7, 12.6, 12.4 ppm; IR (KBr): $\tilde{\nu}_{max}=1274$, 1465, 1528, 1681, 2947 cm^{-1}; MS (FAB): *m/z*: 664 $[M+H]^+$; HRMS (FAB): *m/z* calcd for $C_{30}H_{46}N_3O_{10}Si_2$ $[M+H]^+$: 664.2722; found: 664.2728.

Compound 4: Under an N_2 atmosphere, a solution of 4-nitrobenzaldehyde (4.3 g, 28 mmol) in anhydrous HFIP (12 mL) and zinc chloride (130 mg, 0.94 mmol) were added to **1** (500 mg, 0.94 mmol) and the resultant mixture was stirred at room temperature for 18 h. After the addition of a saturated aqueous solution of $NaHCO_3$ at 0 °C, the reaction mixture was diluted with AcOEt, washed with water and brine, dried over Na_2SO_4, and concentrated. The crude product was purified by column chromatography (*n*-hexane/AcOEt 9/1→7/3) to give **4** as a yellow foam (370 mg, 60 %). M.p. 118–120 °C; $[\alpha]_D^{23}=-5.4$ ($c=1.00$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=9.27$ (brs, 1H; N3-H), 8.21 (t, $J=8$ Hz, 2H; Ar-H), 7.69 (t, $J=8$ Hz, 2H; Ar-H), 7.54 (s, 1H; H6), 6.27 (s, 1H;

acetal-H), 6.14 (s, 1H; H1′), 4.67 (d, J=6 Hz, 1H; H3′), 4.42 (d, J=6 Hz, 1H; H2′), 4.06 (d, J=13 Hz, 1H; H5′), 3.93 (d, J=13 Hz, 1H; H1″), 3.80 (d, J=13 Hz, 1H; H1″), 3.69 (d, J=13 Hz, 1H; H5′), 1.91 (s, 3H; CH_3), 1.12–1.07 ppm (m, 28H; TIPDS-H); ^{13}C NMR (100.5 MHz, $CDCl_3$): δ= 163.6, 149.7, 148.1, 145.5, 135.1, 127.3, 123.5, 110.3, 102.2, 92.4, 88.9, 79.3, 77.2, 70.8, 68.7, 60.1, 17.3, 17.3, 17.2, 17.2, 17.1, 17.1, 16.9, 13.4, 12.7, 12.6, 12.4 ppm; IR (KBr): $\tilde{\nu}_{max}$=1347, 1525, 1695, 2946 cm^{-1}; MS (FAB): m/z: 664 $[M+H]^+$; HRMS (FAB): m/z calcd for $C_{30}H_{46}N_3O_{10}Si_2$ $[M+H]^+$: 664.2722; found: 664.2712.

Compound 5: TBAF (1.0 M in THF, 1.2 mL, 1.2 mmol) was added to a solution of **2** (370 mg, 0.60 mmol) in THF (17 mL) at 0°C and the mixture was stirred at 0°C for 1 h. The reaction mixture was concentrated and the crude product was purified by column chromatography (AcOEt/MeOH 30/1) to give **5** as a white powder (220 mg, 87%). M.p. 153–156°C; $[\alpha]_D^{27}$=−59.9 (c=1.00 in MeOH); ^{1}H NMR (270 MHz, CD_3OD): δ=8.04 (d, J=8 Hz, 1H; Ar-H), 8.02 (d, J=1 Hz, 1H; H6), 7.86 (dd, J= 1, 8 Hz, 1H; Ar-H), 7.70 (dt, J=1, 8 Hz, 1H; Ar-H), 7.56 (dt, J=1, 8 Hz, 1H; Ar-H), 6.91 (s, 1H; acetal-H), 6.14 (s, 1H; H1′), 4.60 (d, J=6 Hz, 1H; H3′), 4.37 (d, J=6 Hz, 1H; H2′), 3.95 (d, J=12 Hz, 1H; H1″), 3.76 (d, J=12 Hz, 1H; H1″), 3.72 (d, J=12 Hz, 1H; H5′), 3.70 (d, J=12 Hz, 1H; H5′), 1.87 ppm (d, J=1 Hz, 3H; CH_3); ^{13}C NMR (75.5 MHz, CD_3OD): δ=166.5, 152.2, 150.0, 137.7, 135.0, 133.8, 130.6, 128.9, 125.0, 110.8, 99.2, 93.1, 90.6, 81.4, 72.0, 69.1, 60.8, 12.5 ppm; IR (KBr): $\tilde{\nu}_{max}$= 1272, 1356, 1471, 1528, 1692, 3378 cm^{-1}; MS (FAB): m/z: 422 $[M+H]^+$; HRMS (FAB): m/z calcd for $C_{18}H_{20}N_3O_9$ $[M+H]^+$: 422.1220; found: 422.1171.

Compound 7: TBAF (1.0 M in THF, 0.96 mL, 0.96 mmol) was added to a solution of **4** (320 mg, 0.48 mmol) in THF (12 mL) at 0°C and the resultant mixture was stirred at 0°C for 1 h. The reaction mixture was concentrated and the crude product was purified by column chromatography (AcOEt/MeOH 30/1) to give **7** as a yellow powder (160 mg, 81%). M.p. 164–167°C; $[\alpha]_D^{24}$=+59.9 (c=1.00 in MeOH); ^{1}H NMR (400 MHz, CD_3OD): δ=8.24 (d, J=8 Hz, 2H; Ar-H), 7.97 (s, 1H; H6), 7.76 (d, J= 8 Hz, 2H; Ar-H), 6.46 (s, 1H; acetal-H), 6.21 (s, 1H; H1′), 4.62 (d, J= 6 Hz, 1H; H3′), 4.35 (d, J=6 Hz, 1H; H2′), 4.02 (d, J=12 Hz, 1H; H1″), 3.85 (d, J=12 Hz, 1H; H1″), 3.78 (d, J=12 Hz, 1H; H5′), 3.71 (d, J= 12 Hz, 1H; H5′), 1.88 ppm (s, 3H; CH_3); ^{13}C NMR (100.5 MHz, CD_3OD): δ=166.5, 152.2, 149.4, 148.0, 137.8, 128.6, 124.3, 110.8, 103.0, 93.1, 90.6, 81.4, 71.9, 69.2, 60.9, 12.6 ppm; IR (KBr): $\tilde{\nu}_{max}$=1349, 1522, 1693, 3396 cm^{-1}; MS (FAB): m/z: 422 $[M+H]^+$; HRMS (FAB): m/z calcd for $C_{18}H_{20}N_3O_9$ $[M+H]^+$: 422.1220; found: 422.1171.

Compound 8: Under an N_2 atmosphere, DMTrCl (200 mg, 0.58 mmol) was added to a solution of **5** (190 mg, 0.44 mmol) in anhydrous pyridine (11 mL) and the resultant mixture was stirred at room temperature for 15 h. After the addition of a saturated aqueous solution of $NaHCO_3$, the reaction mixture was diluted with AcOEt, washed with water and brine, dried over Na_2SO_4, and concentrated. The crude was purified by column chromatography (0.5% triethylamine in n-hexane/AcOEt 1/2) to give **8** as a white foam (250 mg, 79%). M.p. 140–142°C; $[\alpha]_D^{26}$=−24.0 (c=1.00 in MeOH); ^{1}H NMR (270 MHz, $CDCl_3$): δ=9.27 (s, 1H; N3-H), 7.97 (d, J=8 Hz, 1H; Ar-H), 7.76 (d, J=8 Hz, 1H; Ar-H), 7.54 (t, J=8 Hz, 1H; Ar-H), 7.47–6.76 (m, 15H), 6.10 (s, 1H; H1′), 4.74 (t, J=5 Hz, 1H; H3′), 4.46 (d, J=6 Hz, 1H; H2′), 3.93 (d, J=13 Hz, 1H), 3.79–3.72 (m, 2H), 3.72 (s, 6H; OCH_3×2), 3.36–3.26 (m, 3H), 1.47 ppm (s, 3H; CH_3); ^{13}C NMR (67.8 MHz, $CDCl_3$): δ=163.7, 158.2, 149.6, 147.4, 143.7, 135.1, 134.7, 134.6, 133.2, 132.8, 129.5, 128.9, 127.6, 127.5, 126.7, 123.6, 112.9, 109.9, 98.0, 92.2, 88.1, 86.4, 79.1, 70.9, 69.1, 61.4, 54.7, 11.7 ppm; IR (KBr): $\tilde{\nu}_{max}$=1252, 1693, 3008 cm^{-1}; MS (FAB): m/z: 746 $[M+Na]^+$; HRMS (FAB): m/z calcd for $C_{39}H_{37}N_3NaO_{11}$ $[M+Na]^+$: 746.2326; found: 746.2325.

Compound 9: Under an N_2 atmosphere, DMTrCl (150 mg, 0.45 mmol) was added to a solution of **6**[15] (130 mg, 0.35 mmol) in anhydrous pyridine (9 mL) and the resultant mixture was stirred at room temperature for 11 h. After the addition of a saturated aqueous solution of $NaHCO_3$, the reaction mixture was diluted with AcOEt, washed with water and brine, dried over Na_2SO_4, and concentrated. The crude product was purified by column chromatography (0.5% triethylamine in n-hexane/AcOEt 1/1→1/2) to give **9** as a white foam (200 mg, 84%). M.p. 165–168°C; $[\alpha]_D^{26}$=+7.80 (c=1.00 in MeOH); ^{1}H NMR (270 MHz, $CDCl_3$): δ=8.52 (brs, 1H; N3-H), 7.52–6.83 (m, 19H), 6.27 (s, 1H; acetal-H), 6.23 (s, 1H; H1′), 4.79 (dd, J=4, 6 Hz, 1H; H3′), 4.50 (d, J=6 Hz, 1H; H2′), 3.97 (d, J=13 Hz, 1H; H1″), 3.87 (d, J=13 Hz, 1H; H1″), 3.79 (s, 6H; OCH_3× 2), 3.43 (d, J=11 Hz, 1H; H5′), 3.34 (d, J=11 Hz, 1H; H5′), 2.77 (d, J= 4 Hz, 1H; OH), 1.64 ppm (s, 3H; CH_3); ^{13}C NMR (67.8 MHz, $CDCl_3$): δ=164.3, 158.6, 150.0, 144.2, 139.3, 136.3, 135.3, 135.2, 130.0, 128.8, 128.3, 128.0, 127.1, 126.1, 113.3, 110.3, 103.8, 92.6, 88.3, 86.7, 79.1, 71.1, 69.9, 62.3, 60.4, 55.2, 21.0, 14.1, 12.1 ppm; IR (KBr): $\tilde{\nu}_{max}$=1252, 1691, 2930, 3062, 3382 cm^{-1}; MS (FAB): m/z: 701 $[M+Na]^+$; HRMS (FAB): m/z calcd for $C_{39}H_{39}N_2O_9$ $[M+H]^+$: 679.2656; found: 679.2692.

Compound 10: Under an N_2 atmosphere, DMTrCl (150 mg, 0.45 mmol) was added to a solution of **7** (140 mg, 0.34 mmol) in anhydrous pyridine (9 mL) and the resultant mixture was stirred at room temperature for 21 h. After the addition of a saturated aqueous solution of $NaHCO_3$, the reaction mixture was diluted with AcOEt, washed with water and brine, 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 give **10** as a yellow foam (180 mg, 74%). M.p. 160–162°C; $[\alpha]_D^{24}$=+5.2 (c=1.00 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ=9.70 (s, 1H; N3-H), 8.06 (d, J=8 Hz, 2H; Ar-H), 7.55 (d, J=8 Hz, 2H; Ar-H), 7.37–7.12 (m, 9H), 6.74 (d, J=8 Hz, 4H; Ar-H), 6.24 (s, 1H; acetal-H), 6.03 (s, 1H; H1′), 4.76 (s, 1H; H3′), 4.45 (d, J=6 Hz, 1H; H2′), 3.88 (s, 2H; H1″), 3.67 (s, 6H; OCH_3×2), 3.32 (t, J=12 Hz, 2H; H5′), 2.15 (s, 1H; OH), 1.45 ppm (s, 3H; CH_3); ^{13}C NMR (100.5 MHz, $CDCl_3$): δ=164.5, 158.6, 150.1, 147.9, 145.8, 144.2, 136.3, 135.2, 135.0, 130.0, 127.98, 128.01, 127.3, 127.1, 123.5, 113.3, 110.4, 102.0, 93.5, 88.4, 86.8, 79.3, 77.2, 71.3, 69.9, 62.2, 55.2, 12.1 ppm; IR (KBr): $\tilde{\nu}_{max}$=1348, 1511, 1691, 3211 cm^{-1}; MS (FAB): m/z: 746 $[M+Na]^+$; HRMS (FAB): m/z calcd for $C_{39}H_{37}N_3NaO_{11}$ $[M+Na]^+$: 746.2326; found: 746.2312.

Compound 11: Under an N_2 atmosphere, 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphane (0.29 mL, 0.90 mmol) and 4,5-dicyanoimidazole (0.25 M in CH_3CN, 1.3 mL, 0.33 mmol) were added to a solution of **8** (220 mg, 0.30 mmol) in anhydrous CH_3CN (4 mL) and the resultant mixture was stirred at room temperature for 17 h. After the addition of a saturated aqueous solution of $NaHCO_3$, the reaction mixture was diluted with AcOEt, washed with water and brine, dried over Na_2SO_4, and concentrated. The crude was purified by column chromatography (0.5% triethylamine in n-hexane/AcOEt 1/1) followed by the precipitation from n-hexane/AcOEt to give **11** as a white foam (102 mg, 37%). M.p. 113–117°C; ^{31}P NMR (202 MHz, $CDCl_3$): δ=150.7, 150.2 ppm; MS (FAB): m/z: 924 $[M+H]^+$; HRMS (FAB): m/z calcd for $C_{48}H_{55}N_5O_{12}P$ $[M+H]^+$: 924.3585; found: 924.3575.

Compound 12: Under an N_2 atmosphere, 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphane (0.35 mL, 1.1 mmol) and 4,5-dicyanoimidazole (0.25 M in CH_3CN, 1.6 mL, 0.40 mmol) were added to a solution of **9** (250 mg, 0.36 mmol) in anhydrous CH_3CN (5 mL) and the resultant mixture was stirred at room temperature for 40 h. After the addition of a saturated aqueous solution of $NaHCO_3$, the reaction mixture was diluted with AcOEt, washed with water and brine, dried over Na_2SO_4, and concentrated. The crude was purified by column chromatography (0.5% triethylamine in n-hexane/AcOEt 1/1) followed by precipitation from n-hexane/AcOEt to give **12** as a white foam (220 mg, 68%). M.p. 116–118°C; ^{31}P NMR (202 MHz, $CDCl_3$): δ=151.2, 150.5 ppm; MS (FAB): m/z: 879 $[M+H]^+$; HRMS (FAB): m/z calcd for $C_{48}H_{56}N_4O_{10}P$ $[M+H]^+$: 879.3734; found: 879.3712.

Compound 13: Under an N_2 atmosphere, 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphane (0.16 mL, 0.51 mmol) and 4,5-dicyanoimidazole (0.25 M in CH_3CN, 0.80 mL, 0.20 mmol) were added to a solution of **8** (130 mg, 0.17 mmol) in anhydrous CH_3CN (3 mL) and the resultant mixture was stirred at room temperature for 15 h. After the addition of a saturated aqueous solution of $NaHCO_3$, the reaction mixture was diluted with AcOEt, washed with water and brine, dried over Na_2SO_4, and concentrated. The crude product was purified by column chromatography (0.5% triethylamine in n-hexane/AcOEt 1/1) followed by precipitation from n-hexane/AcOEt to give **13** as a white foam (120 mg, 77%). M.p. 109–111°C; ^{31}P NMR (161.8 MHz, $CDCl_3$): δ=151.2, 150.6 ppm; MS

(FAB): *m/z*: 924 $[M+H]^+$; HRMS (FAB): *m/z* calcd for $C_{48}H_{55}N_5O_{12}P$ $[M+H]^+$: 924.3585; found: 924.3585.

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[1] a) C. Hélène, J. J. Toulmé, *Biochim. Biophys. Acta Gene Struct. Expression.* **1990**, *1049*, 99–125; b) E. Uhlmann, A. Peyman, *Chem. Rev.* **1990**, *90*, 543–584; c) L. J. Maher III, *Cancer Invest.* **1996**, *14*, 66–82; d) G. J. Hannon, *Nature* **2002**, *418*, 244–251.

[2] a) S. A. E. Marras, S. Tyagi, F. R. Kramer, *Clin. Chim. Acta* **2006**, *363*, 48–60; b) M. Famulok, J. S. Hartig, G. Mayer, *Chem. Rev.* **2007**, *107*, 3715–3743.

[3] a) D. Sen, C. R. Geyer, *Curr. Opin. Chem. Biol.* **1998**, *2*, 680–687; b) L. Q. Sun, M. J. Cairns, E. G. Saravolac, A. Baker, W. L. Gerlach, *Phamacol. Rev.* **2000**, *52*, 325–347.

[4] a) S. Jäger, G. Rasched, H. Kornreich-Leshem, M. Engeser, O. Thum, M. Famulok, *J. Am. Chem. Soc.* **2005**, *127*, 15071–15081; b) F. E. Alemdaroglu, A. Herrmann, *Org. Biomol. Chem.* **2007**, *5*, 1311–1320.

[5] a) G. Mayer, A. Heckel, *Angew. Chem.* **2006**, *118*, 5020–5042; *Angew. Chem. Int. Ed.* **2006**, *45*, 4900–4921; b) X. Tang, I. J. Dmochowski, *Mol. Biosyst.* **2007**, *3*, 100–110; c) D. D. Young, A. Deiters, *Org. Biomol. Chem.* **2007**, *5*, 999–1005; d) J. P. Casey, R. A. Bildner, W. T. Monroe, *Mol. Pharm.* **2009**, *6*, 669–685; e) H. M. Lee, D. R. Larson, D. S. Lawrence, *ACS Chem. Biol.* **2009**, *4*, 409–427; f) H. Yu, J. Li, D. Wu, Z. Qiu, Y. Zhang, *Chem. Soc. Rev.* **2010**, *39*, 464–473.

[6] a) T. Ohmichi, Y. Kawamoto, P. Wu, D. Miyoshi, H. Karimata, N. Sugimoto, *Biochemistry* **2005**, *44*, 7125–7130; b) J. A. Lee, M. C. DeRosa, *Chem. Commun.* **2010**, *46*, 418–420.

[7] R. Tashiro, H. Sugiyama, *J. Am. Chem. Soc.* **2005**, *127*, 2094–2097.

[8] A. Hatano, S. Makita, M. Kirihara, *Tetrahedron* **2005**, *61*, 1723–1730.

[9] T. Wada, N. Minamimoto, Y. Inaki, Y. Inoue, *J. Am. Chem. Soc.* **2000**, *122*, 6900–6910.

[10] a) S. Obika, D. Nanbu, Y. Hari, K. Morio, Y. In, T. Ishida, T. Imanishi, *Tetrahedron Lett.* **1997**, *38*, 8735–8738; b) S. K. Singh, P. Nielsen, A. Koshkin, J. Wengel, *Chem. Commun.* **1998**, 455–456.

[11] S. Obika, D. Nanbu, Y. Hari, J. Ando, K. Morio, T. Doi, T. Imanishi, *Tetrahedron Lett.* **1998**, *39*, 5401–5404.

[12] S. Obika, Y. Hari, T. Sugimoto, M. Sekiguchi, T. Imanishi, *Tetrahedron Lett.* **2000**, *41*, 8923–8927.

[13] a) K. Morihiro, T. Kodama, M. Nishida, T. Imanishi, S. Obika, *ChemBioChem* **2009**, *10*, 1784–1788; b) K. Morihiro, T. Kodama, M. Nishida, T. Imanishi, S. Obika, *Nucleic Acids Symp. Ser.* **2009**, *53*, 187–188.

[14] K. D. Nielsen, F. Kirpekar, P. Roepstorff, J. Wengel, *Bioorg. Med. Chem.* **1995**, *3*, 1493–1502.

[15] We previously reported the syntheses of compound **3** and **6** in the following manuscript: Y. Kasahara, S. Kitadume, K. Morihiro, M. Kuwahara, H. Ozaki, H. Sawai, T. Imanishi, S. Obika, *Bioorg. Med. Chem. Lett.* **2010**, *20*, 1626–1629.

[16] R. Buff, H. S. Evans, J. Hunziker, *Acta Crystallogr. Sect. C* **1998**, *54*, 1860–1862.

[17] A. Bendich, L. J. Machlin, O. Scandura, G. W. Burton, D. D. M. Wayner, *Adv. Free. Radical Biol. Med.* **1986**, *2*, 419–444.

[18] E. S. Krol, J. L. Bolton, *Chem.-Biol. Interact.* **1997**, *104*, 11–27.

[19] W. W. Cleland, *Biochemistry* **1964**, *3*, 480–482.

[20] W. Guoyao, Y-Z. Fang, S. Yang, J. R. Lupton, N. D. Turner, *J. Nutr.* **2004**, *134*, 489–492.

[21] M. J. Matteucci, W. J. Reed, D. A. Tanen, *Acad. Emeerg. Med.* **2003**, *10*, 299–302.

[22] K. K. Park, C. H. Oh, W. K. Joung, *Tetrahedron Lett.* **1993**, *34*, 7445–7446.

[23] M. Sekine, Y. Oeda, Y. Iijima, H. Taguchi, A. Ohkubo, K. Seio, *Org. Biomol. Chem.* **2011**, *9*, 210–218.

[24] a) A. Patchornik, B. Amit, R. B. Woodward, *J. Am. Chem. Soc.* **1970**, *92*, 6333–6335; b) B. Amit, U. Zehavi, A. Patchornik, *J. Org. Chem.* **1974**, *39*, 192–196.

[25] a) J. A. Barltrop, P. J. Plant, P. Schofield, *Chem. Commun. (London)* **1966**, 822–823; b) Y. V. Il'ichev, M. A. Schwörer, J. Wirz, *J. Am. Chem. Soc.* **2004**, *126*, 4581–4595.

[26] V. N. R. Pillai, *Synthesis* **1980**, 1–26.

[27] P. G. W. Wuts, T. W. Greene, *Greene's Protective Groups in Organic Synthesis*, 4th ed., Wiley, New York, **2006**, pp. 322–323.

[28] a) M. O. Terpko, R. F. Heck, *J. Org. Chem.* **1980**, *45*, 4992–4993; b) K. Mukkanti, Y. V. S. Rao, B. M. Choudary, *Tetrahedron Lett.* **1989**, *30*, 251–252; c) C. Bourget, E. Trevisiol, I. Bridon, M. Kotera, J. Lhomme, A. Laayoun, *Bioorg. Med. Chem.* **2005**, *13*, 1453–8176.

[29] a) K. Morita, M. Takagi, C. Hasegawa, M. Kaneko, S. Tsutsumi, J. Sone, T. Ishikawa, T. Imanishi, M. Koizumi, *Bioorg. Med. Chem.* **2003**, *11*, 2211–2226; b) Y. Hari, S. Obika, R. Ohnishi, K. Eguchi, T. Osaki, H. Ohishi, T. Imanishi, *Bioorg. Med. Chem.* **2006**, *14*, 1029–1038; c) Y. Mitsuoka, T. Kodama, R. Ohnishi, Y. Hari, T. Imanishi, S. Obika, *Nucleic Acids Res.* **2009**, *37*, 1225–1238; d) M. Nishida, T. Baba, T. Kodama, A. Yahara, T. Imanishi, S. Obika, *Chem. Commun.* **2010**, *46*, 5283–5285.

[30] a) C. Altona, M. Sundaralingam, *J. Am. Chem. Soc.* **1972**, *94*, 8205–8212; b) S. Arnott, D. W. L. Hukins, S. D. Dover, *Biochem. Biophys. Res. Commun.* **1972**, *48*, 1392–1399.

[31] T. Wu, K. Nauwelaerts, A. Vam Aerschot, M. Froeyen, E. Lescrinier, P. Herdewijn, *J. Org. Chem.* **2006**, *71*, 5423–5431.

[32] a) J. J. Sørensen, J. T. Nielsen, M. Petersen, *Nucleic Acids Res.* **2004**, *32*, 6078–6085; b) S. M. A. Rahman, S. Seki, S. Obika, H. Yoshikawa, K. Miyashita, T. Imanishi, *J. Am. Chem. Soc.* **2008**, *130*, 4886–4896; c) J. Xu, Y. Liu, C. Dupouy, J. Chattopadhyaya, *J. Org. Chem.* **2009**, *74*, 6534–6554; d) C. Zhou, J. Chattopadhyaya, *J. Org. Chem.* **2010**, *75*, 2341–2349.

[33] a) A. Meister, M. E. Anderson, *Annu. Rev. Biochem.* **1983**, *52*, 711–760; b) R. R. Perry, J. Mazetta, M. Levin, S. C. Barranco, *Cancer* **1993**, *72*, 783–787; c) S. C. Barranco, R. R. Perry, M. E. Duem, M. Quraishi, A. L. Werner, S. G. Gregorcyk, P. Kolm, *Dis. Colon Rectum* **2000**, *43*, 1133–1140.

[34] a) D. F. Bainton, *J. Cell Biol.* **1981**, *91*, 66s-76s; b) V. M. Arlt, H. G. Glatt, E. Muckel, U. Pabel, B. L. Sorg, A. Seidel, H. Frank, H. H. Schmeiser, D. H. Phillips, *Int. J. Cancer* **2003**, *105*, 583–592; c) Y. Chen, L. Hu, *Med. Res. Rev.* **2009**, *29*, 29–64.

[35] L. Kröck, A. Heckel, *Angew. Chem.* **2005**, *117*, 475–477; *Angew. Chem. Int. Ed.* **2005**, *44*, 471–473.

[36] H. Asanuma, T. Ito, T. Yoshida, X. G. Liang, M. Komiyama, *Angew. Chem.* **1999**, *111*, 2547–2549; *Angew. Chem. Int. Ed.* **1999**, *38*, 2393–2395.

[37] E. Yashima, K. Maeda, Y. Okamoto, *Nature* **1999**, *399*, 449–451.

[38] S. G. Chaulk, A. M. MacMillan, *Nucleic Acids Res.* **1998**, *26*, 3173–3178.

[39] P. Ordoukhanian, J.-S. Taylor, *J. Am. Chem. Soc.* **1995**, *117*, 9570–9571.

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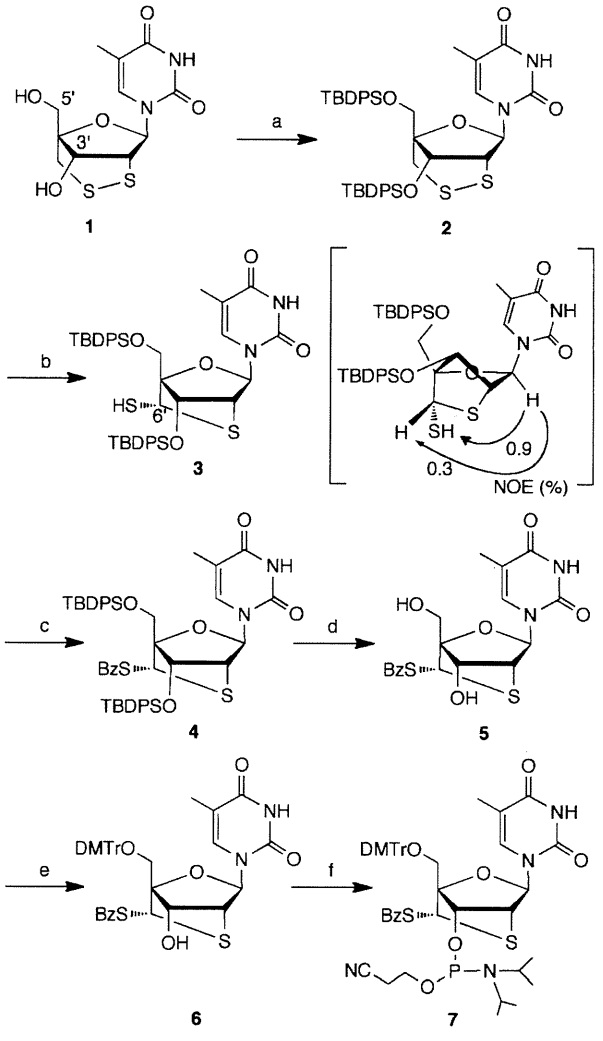
† Electronic supplementary information (ESI) available: HPLC and MALDI data for ON conjugates, NMR spectra of new compounds, and UV melting profiles. See DOI: 10.1039/c1ob05469d

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.

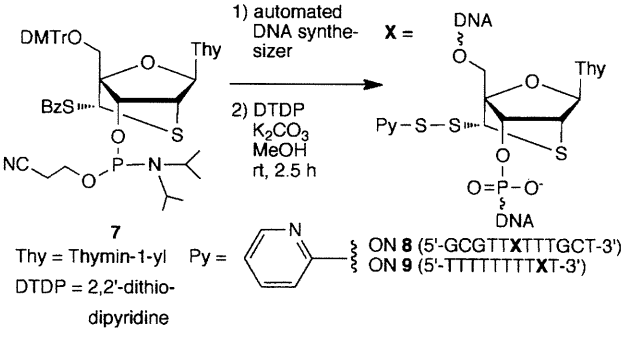


Fig. 1 Conversion of phosphoramidite **7** to ON **8**, **9**.

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

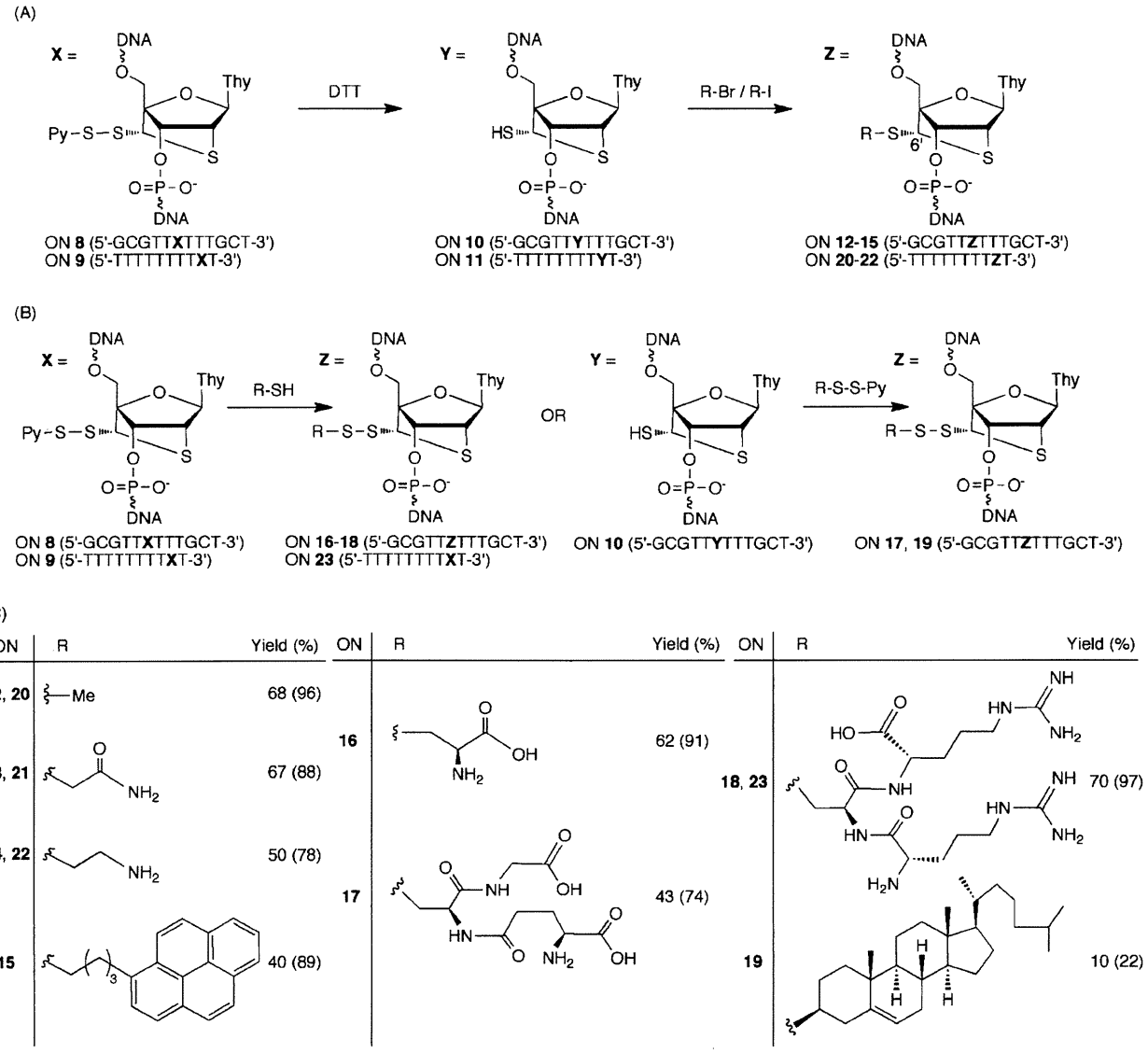


Fig. 2 Synthesis of thioether-linked conjugates and disulfide-linked conjugates. (A) Thioether-linked conjugation by *S*-alkylation, (B) disulfide-linked conjugation by disulfide exchange reaction, and (C) their conjugation yields. HPLC yield in parenthesis was determined by RP-HPLC of reaction solution, 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.