extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=5:1) to give a ca. 1:1 anomeric mixture **17** (660 mg, 48% from **4**) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.92 (0.5H, ddd, J=5.9, 11.0, 13.2 Hz), 2.05 (0.5H, ddd, J=6.2, 8.4, 12.8 Hz), 2.31 (0.5H, dd, J=5.1, 13.2 Hz), 2.59 (0.5H, td, J=6.6, 12.8 Hz), 3.55–3.69 (2H, m), 4.15–4.17 (0.5H, m), 4.23–4.36 (1.5H, m), 4.46–4.64 (6H, m), 5.00–5.09 (1H, m), 5.26–5.30 (1H, m), 5.37–5.43 (1H, m), 5.99–6.11 (1H, m), 6.85–6.89 (2H, m), 7.26–7.34 (12H, m). HRMS (FAB) *m/z* calcd for $C_{29}H_{31}O_4$ $[M+H]^+$: 431.2217; found 431.2224.

4.15. 4-(2-Deoxy-3,5-di-*O*-benzyl-D-ribofuranosyl)-1-hydroxybenzene (18)

Under a nitrogen atmosphere, $Pd(PPh_3)_4$ (207 mg, 0.179 mmol) was added to a solution of compound **17** (385 mg, 0.895 mmol) in anhydrous THF (10 mL) and the mixture was stirred until solids of $Pd(PPh_3)_4$ dissolved completely. Then, $NaBH_4$ (41 mg, 1.07 mmol) was added and the mixture was stirred at room temperature for 17 h. After addition of 3% HCl aq, the mixture was filtered through a pad of Celite®. The filtrate was extracted with Et_2O. The extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography ($CHCl_3$ to $CHCl_3$/MeOH=60:1) to give a ca. 1:1 anomeric mixture **18** (318 mg, 91%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.91 (0.5H, ddd, J=5.9, 10.8, 13.2 Hz), 2.04 (0.5H, ddd, J=6.4, 8.8, 12.7 Hz), 2.31 (0.5H, ddd, J=1.0, 4.9, 13.2 Hz), 2.59 (0.5H, td, J=6.4, 12.7 Hz), 3.56–3.68 (2H, m), 4.15–4.17 (0.5H, m), 4.23–4.30 (1H, m), 4.33 (0.5H, td, J=4.9, 9.3 Hz), 4.47–4.62 (4H, m), 5.01 (0.5H, dd, J=6.4, 8.8 Hz), 5.07 (0.5H, dd, J=4.9, 10.8 Hz), 5.12 (0.5H, br s), 5.14 (0.5H, br s), 6.71–6.75 (2H, m), 7.20–7.35 (12H, m). HRMS (FAB) *m/z* calcd for $C_{25}H_{26}NaO_4$ $[M+Na]^+$: 413.1723; found 413.1742.

4.16. 1-Benzoyloxy-4-(2-deoxy-3,5-di-*O*-benzyl-β-D-ribofuranosyl)benzene (19) and 1-benzoyloxy-4-(2-deoxy-3,5-di-*O*-benzyl-α-D-ribofuranosyl)benzene (20)

Under a nitrogen atmosphere, BzCl (129 μL, 1.11 mmol) was added to a solution of compound **18** (318 mg, 0.923 mmol) and Et_3N (154 μL, 1.11 mmol) in anhydrous CH_2Cl_2 (10 mL) at room temperature and the mixture was stirred for 1.5 h. After addition of water, the mixture was extracted with Et_2O. The extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=20:1) to give a separable mixture of **19** and **20** (365 mg, 91%, **19**/**20**=ca. 1:1) as a colorless oil. Compound **19**: $[\alpha]_D^{22}$ +14.7 (*c* 1.06, $CHCl_3$). IR ν_{max} (KBr) 2860, 1739, 1263, 1078 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.94 (1H, ddd, J=6.0, 10.5, 13.0 Hz), 2.38 (1H, ddd, J=1.5, 5.5, 13.0 Hz), 3.60 (1H, dd, J=5.0, 10.0 Hz), 3.67 (1H, dd, J=5.0, 10.0 Hz), 4.18 (1H, m), 4.28 (1H, ddd, J=2.5, 5.0, 5.0 Hz), 4.58 (2H, d, J=14.0 Hz), 4.60 (2H, d, J=14.0 Hz), 5.18 (1H, dd, J=5.5, 10.5 Hz), 7.20 (2H, ddd, J=1.0, 1.0, 8.0 Hz), 7.25–7.38 (10H, m), 7.42 (2H, ddd, J=1.0, 1.0, 8.0 Hz), 7.51 (2H, ddd, J=1.0, 8.0, 8.0 Hz), 7.63 (1H, ddd, J=1.0, 1.0, 8.0 Hz), 8.20 (2H, ddd, J=1.0, 1.0, 8.0 Hz). ^{13}C NMR ($CDCl_3$) δ 41.3, 71.1, 71.1, 73.5, 80.0, 81.5, 83.9, 121.5, 127.2, 127.6, 127.7, 128.4, 128.4, 128.5, 129.5, 130.2, 133.6, 138.1, 138.2, 139.4, 150.2, 165.2. HRMS (FAB) *m/z* calcd for $C_{32}H_{30}NaO_5$ $[M+Na]^+$: 517.1985; found 517.2005. Compound **20**: $[\alpha]_D^{24}$ +7.4 (*c* 0.87, $CHCl_3$). IR ν_{max} (KBr) 2864, 1739, 1264, 1078 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 2.08 (1H, ddd, J=6.0, 7.5, 13.5 Hz), 2.65 (1H, ddd, J=7.0, 7.0, 13.5 Hz), 3.59–3.67 (2H, m), 4.28 (1H, ddd, J=4.5, 6.5, 6.5 Hz), 4.38 (1H, ddd, J=4.5, 4.5, 4.5 Hz), 4.49 (1H, d, J=12.0 Hz), 4.51 (1H, d, J=12.0 Hz), 4.59 (1H, d, J=12.0 Hz), 4.61 (1H, d, J=12.0 Hz), 5.12 (1H, dd, J=7.0, 7.5 Hz), 7.18 (2H, d, J=8.0 Hz), 7.24–7.38 (10H, m), 7.46 (2H, d, J=8.0 Hz), 7.51 (2H, dd, J=8.0, 8.0 Hz), 7.64 (1H, dd, J=8.0, 8.0 Hz), 8.20 (2H, d, J=8.0 Hz). ^{13}C NMR ($CDCl_3$) δ 41.5, 70.8, 71.7, 73.5, 79.8, 80.6, 82.9, 121.5, 127.2, 127.6, 127.6, 127.7, 128.4, 128.5, 130.1, 133.5, 138.0, 138.2, 140.3, 150.1, 165.2. HRMS (FAB) *m/z* calcd for $C_{32}H_{30}NaO_5$ $[M+Na]^+$: 517.1985; found 517.1974.

4.17. 1-Benzoyloxy-4-(2-deoxy-β-D-ribofuranosyl)benzene (21)

A solution of compound **19** (170 mg, 0.343 mmol), 20% $Pd(OH)_2$/C (100 mg), and cyclohexene (3.5 mL, 34.3 mmol) in EtOH (7 mL) was stirred at 70 °C for 2 h. The mixture was filtered through a pad of Celite® and the filtrate was concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=2:1) to give compound **21** (89 mg, 82%) as a white solid. $[\alpha]_D^{22}$ +34.8 (*c* 1.05, $CHCl_3$). IR ν_{max} (KBr) 3390, 2932, 1737, 1508, 1270, 1200, 1079 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 2.05 (1H, ddd, J=6.0, 10.5, 13.0 Hz), 2.14 (1H, br s), 2.19 (1H, br s), 2.26 (1H, ddd, J=1.5, 5.5, 13.0 Hz), 3.76 (2H, m), 4.01 (1H, ddd, J=4.5, 4.5, 4.5 Hz), 4.42 (1H, m), 5.20 (1H, dd, J=5.5, 10.5 Hz), 7.20 (2H, ddd, J=1.0, 1.0, 8.0 Hz), 7.41 (2H, ddd, J=1.0, 1.0, 8.0 Hz), 7.51 (2H, ddd, J=1.0, 8.0, 8.0 Hz), 7.65 (1H, dddd, J=1.0, 1.0, 8.0, 8.0 Hz), 8.20 (2H, dd, J=1.0, 8.0 Hz). ^{13}C NMR ($CDCl_3$) δ 44.0, 63.4, 73.7, 79.6, 87.3, 121.7, 127.2, 128.6, 129.4, 130.2, 133.7, 138.8, 150.4, 165.3. HRMS (FAB) *m/z* calcd for $C_{18}H_{18}NaO_5$ $[M+Na]^+$: 337.1046; found 337.1035.

4.18. 1-Benzoyloxy-4-[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl]benzene (22)

Under a nitrogen atmosphere, DMTrCl (106 mg, 0.313 mmol) was added to a solution of compound **21** (82 mg, 0.260 mmol) in anhydrous pyridine (3 mL) at room temperature and the mixture was stirred for 1 h. After addition of water, the mixture was extracted with Et_2O. The extracts were washed with water, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=3:1) to give compound **22** (145 mg, 91%) as a white solid. $[\alpha]_D^{23}$ +13.8 (*c* 1.06, $CHCl_3$). IR ν_{max} (KBr) 3454, 3004, 2933, 2837, 1739, 1607, 1508, 1263, 1079 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.87 (1H, br s), 2.05 (1H, ddd, J=5.5, 10.0, 13.0 Hz), 2.25 (1H, J=1.5, 5.5, 13.0 Hz), 3.28 (1H, dd, J=4.5, 10.0 Hz), 3.36 (1H, dd, J=5.0, 10.0 Hz), 3.78 (6H, s), 4.07 (1H, m), 4.30 (1H, m), 5.20 (1H, dd, J=5.5, 10.0 Hz), 6.82 (4H, d, J=9.0 Hz), 7.15–7.55 (15 H, m), 7.63 (1H, dd, J=7.5, 7.5 Hz), 8.20 (1H, d, J=7.5 Hz). ^{13}C NMR ($CDCl_3$) δ 43.9, 55.2, 64.4, 74.6, 79.5, 86.2, 86.3, 113.1, 121.5, 126.8, 127.1, 127.8, 128.2, 128.6, 129.5, 130.1, 130.1, 133.6, 136.0, 139.5, 144.8, 150.2, 158.4, 165.2. HRMS (FAB) *m/z* calcd for $C_{39}H_{36}NaO_7$ $[M+Na]^+$: 639.2353; found 639.2359.

4.19. 1-Benzoyloxy-4-{3-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl}benzene (3)

Under a nitrogen atmosphere, *i*-$Pr_2NP(Cl)OCH_2CH_2CN$ (36 μL, 0.161 mmol) was added to a solution of compound **22** (66 mg, 0.108 mmol) and *i*-Pr_2NEt (96 μL, 0.554 mmol) in anhydrous CH_2Cl_2 (1 mL) at room temperature and the mixture was stirred for 25 min. After addition of satd $NaHCO_3$ aq, the mixture was extracted with Et_2O. The extracts were washed with water, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=5:1) to give compound **3** (84 mg, 95%) as a white solid. ^{1}H NMR ($CDCl_3$) δ 1.09 (3H, d, J=6.8 Hz), 1.16–1.20 (9H, m), 2.00–2.09 (1H, m), 2.32–2.49 (2H, m), 2.63 (1H, dd, J=6.3, 6.9 Hz), 3.23–3.36 (2H, m), 3.53–3.89 (4H, m), 3.79 (3H, s), 3.79 (3H, s), 4.24–4.25 (1H, m), 4.51–4.55 (1H, m), 5.17–5.22 (1H, m), 6.80–6.84 (4H, m), 7.17–7.30

(5H, m), 7.35–7.38 (4H, m), 7.47–7.53 (6H, m), 7.62–7.66 (1H, m), 8.19–8.21 (2H, m). ^{31}P NMR ($CDCl_3$) δ 147.8, 148.0. HRMS (FAB) *m/z* calcd for $C_{48}H_{53}N_2NaO_8P$ $[M+Na]^+$: 839.3422; found 839.3443.

4.20. 1-Allyloxy-2-(3,5-di-*O*-benzyl-2-*O*,4-*C*-methylene-β-D-ribofuranosyl)benzene (27)

Under a nitrogen atmosphere, a solution of compound **26**[8] (1.95 g, 5.69 mmol) in anhydrous THF (50 mL) was added to a solution of 2-allyloxyphenylmagnesium iodide [prepared from 2-allyloxyphenyl iodide (5.92 g, 22.8 mmol) and *i*-PrMgCl·LiCl (1.3 M in THF, 17.5 mL, 22.8 mmol) in anhydrous THF (180 mL) at −40 °C], and the mixture was stirred at −40 °C for 11 h. After addition of satd NH_4Cl aq, the mixture was extracted with AcOEt. The extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=3:1 to 1:1) to give an appropriate compound (R_f=ca. 0.5, *n*-hexane/AcOEt=1:1), which was dissolved in anhydrous CH_2Cl_2 (50 mL); TMAD (1.11 g, 6.47 mmol) and *n*-Bu_3P (1.61 mL, 6.47 mmol) were added at room temperature and the mixture was stirred for 2 h. The mixture was concentrated under reduced pressure and the residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=5:1) to give compound **27** (1.48 g, 57% from **26**) as a white powder. $[\alpha]_D^{21}$ +60.3 (*c* 1.36, $CHCl_3$). IR ν_{max} (KBr) 3063, 3031, 2876, 1596, 1490, 1454, 1239, 1102, 1030 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 3.87, 3.88 (2H, AB, *J*=11 Hz), 4.02 (1H, s), 4.08, 4.12 (2H, AB, *J*=8 Hz), 4.41 (2H, s), 4.40, 4.50 (2H, AB, *J*=12 Hz), 4.67 (2H, s), 5.26 (1H, dd, *J*=1, 11 Hz), 5.40 (1H, dd, *J*=1, 17 Hz), 5.35 (1H, s), 5.94–6.08 (1H, m), 6.80 (1H, d, *J*=8 Hz), 6.94 (1H, t, *J*=8 Hz), 7.18–7.40 (11H, m), 7.54 (1H, d, *J*=8 Hz). ^{13}C NMR ($CDCl_3$) δ 66.5, 68.3, 71.8, 73.3, 73.5, 78.0, 79.0, 80.5, 85.4, 110.7, 117.0, 120.5, 126.7, 127.3, 127.4, 127.4, 127.5, 127.5, 128.1, 128.2, 128.2, 132.8, 137.5, 138.0, 154.2. Mass (EI) *m/z* 458 (M^+, 2.5). Anal. Calcd for $C_{29}H_{30}O_5$: C, 75.96; H, 6.59; found: C, 75.94; H, 6.63.

4.21. 2-(3,5-Di-*O*-benzyl-2-*O*,4-*C*-methylene-β-D-ribofuranosyl)-1-hydroxybenzene (29)

Under a nitrogen atmosphere, $Pd(PPh_3)_4$ (370 mg, 0.32 mmol) was added to a solution of compound **27** (1.46 g, 3.18 mmol) in anhydrous THF (30 mL) and the mixture was stirred until solids of $Pd(PPh_3)_4$ dissolved completely. Then, $NaBH_4$ (361 mg, 9.55 mmol) was added and the mixture was stirred at room temperature for 19 h. After addition of 3% HCl aq, the mixture was filtered through a pad of Celite®. The filtrate was extracted with AcOEt. The extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=5:2) to give compound **29** (1.07 g, 80%) as a white powder. $[\alpha]_D^{21}$ +14.0 (*c* 1.19, $CHCl_3$). IR ν_{max} (KBr) 3336, 3031, 2877, 1496, 1456, 1100, 1029 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 3.72 (2H, s), 3.97, 4.04 (2H, AB, *J*=8 Hz), 4.31 (2H, d, *J*=2 Hz), 4.50, 4.59 (2H, AB, *J*=11 Hz), 4.66 (2H, d, *J*=2 Hz), 5.42 (1H, s), 6.80–6.91 (3H, m), 7.13–7.37 (11H, m), 8.55 (1H, s). ^{13}C NMR ($CDCl_3$) δ 64.1, 72.3, 72.8, 73.6, 77.8, 82.1, 86.5, 118.0, 119.5, 120.5, 126.2, 127.5, 127.7, 127.8, 128.3, 128.4, 128.9, 137.2, 137.3, 155.3. HRMS (MALDI-TOF) *m/z* calcd for $C_{26}H_{26}NaO_5$ $[M+Na]^+$: 441.1678; found, 441.1676.

4.22. 1-Benzoyloxy-2-(3,5-di-*O*-benzyl-2-*O*,4-*C*-methylene-β-D-ribofuranosyl)benzene (30)

Under a nitrogen atmosphere, BzCl (42 μL, 0.36 mmol) was added to a solution of compound **29** (112 mg, 0.28 mmol) and Et_3N (50 μL, 0.36 mmol) in anhydrous CH_2Cl_2 (2 mL) at room temperature and the mixture was stirred for 3 h. After addition of water and concentrated under reduced pressure, the mixture was extracted with AcOEt. The extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=7:1) to give compound **30** (130 mg, 93%) as a colorless oil. $[\alpha]_D^{21}$ +52.1 (*c* 1.14, $CHCl_3$). IR ν_{max} (KBr) 3063, 3032, 2877, 1737, 1489, 1452, 1259, 1203, 1029 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 3.86 (2H, s), 4.01, 4.07 (2H, AB, *J*=8 Hz), 4.09 (1H, s), 4.34 (1H, s), 4.44, 4.53 (2H, AB, *J*=12 Hz), 4.66, 4.67 (2H, AB, *J*=12 Hz), 5.29 (1H, s), 7.18–7.39 (13H, m), 7.48–7.54 (2H, m), 7.61–7.67 (2H, m), 8.14–8.20 (2H, m). ^{13}C NMR ($CDCl_3$) δ 66.3, 72.1, 73.4, 73.6, 77.7, 79.5, 80.1, 85.7, 122.1, 126.1, 127.3, 127.4, 127.5, 127.6, 128.2, 128.3, 128.4, 128.7, 128.9, 133.8, 137.4, 138.0, 147.0, 164.7. Mass (EI) *m/z* 522 (M^+, 1.3). Anal. Calcd for $C_{33}H_{30}O_6$: C, 75.84; H, 5.79; found: C, 75.49; H, 5.88.

4.23. 1-Benzoyloxy-2-(2-*O*,4-*C*-methylene-β-D-ribofuranosyl) benzene (31)

A solution of compound **30** (263 mg, 0.503 mmol), 20% $Pd(OH)_2$/C (100 mg), and cyclohexene (5.0 mL, 50.3 mmol) in EtOH (5 mL) was refluxed for 1 h. The mixture was filtered through a pad of Celite® and the filtrate was concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=1:2 to 1:5) to give compound **31** (153 mg, 89%) as a white solid. $[\alpha]_D^{22}$ +4.2 (*c* 0.94, CH_3OH). IR ν_{max} (KBr) 3379, 2947, 1736, 1452, 1264, 1200, 1173, 1026 cm^{-1}. ^{1}H NMR (CD_3OD) δ 3.87, 3.98 (2H, AB, *J*=8 Hz), 3.90, 3.93 (2H, AB, *J*=11 Hz), 4.13 (1H, s), 4.13 (1H, s), 5.11 (1H, s), 7.19–7.23 (1H, m), 7.31–7.38 (2H, m), 7.54–7.60 (2H, m), 7.68–7.73 (2H, m), 8.17–8.21 (2H, m). ^{13}C NMR (CD_3OD) δ 59.3, 71.5, 73.6, 80.9, 83.7, 88.3, 123.4, 127.2, 128.3, 129.6, 129.9, 130.2, 131.0, 132.7, 135.1, 148.5, 166.1. Mass (EI) *m/z* 342 (M^+, 0.3). Anal. Calcd for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30; found: C, 66.34; H, 5.33.

4.24. 1-Benzoyloxy-2-[5-*O*-(4,4′-dimethoxytrityl)-2-*O*,4-*C*-methylene-β-D-ribofuranosyl]benzene (32)

Under a nitrogen atmosphere, DMTrCl (85 mg, 0.25 mmol) was added to a solution of compound **31** (58 mg, 0.17 mmol) in anhydrous pyridine (1 mL) at room temperature and the mixture was stirred for 3 h. After addition of satd $NaHCO_3$ aq, the mixture was extracted with AcOEt. The extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=2:1) to give compound **32** (121 mg, 94%) as a white solid. $[\alpha]_D^{22}$ −13.5 (*c* 1.00, $CHCl_3$). IR ν_{max} (KBr) 3458, 3007, 2949, 2836, 1737, 1606, 1508, 1451, 1254, 1175, 1068, 1030 cm^{-1}. ^{1}H NMR (acetone-d_6) δ 3.46, 3.54 (2H, AB, *J*=11 Hz), 3.79 (6H, s), 3.91 (2H, s), 4.16 (1H, s), 4.35 (1H, s), 5.19 (1H, s), 6.89–6.92 (1H, m), 7.22–7.46 (10H, m), 7.57–7.66 (4H, m), 7.74–7.79 (1H, m), 7.91–7.94 (1H, m), 8.23–8.26 (2H, m). ^{13}C NMR (acetone-d_6) δ 55.6, 61.0, 71.8, 73.6, 80.8, 82.9, 86.9, 86.9, 113.9, 123.5, 127.0, 127.6, 128.4, 128.6, 129.0, 129.4, 129.8, 130.1, 130.8, 131.0, 131.0, 133.1, 134.9, 136.7, 136.9, 146.2, 148.3, 159.5, 165.3. HRMS (FAB) *m/z* calcd for $C_{40}H_{36}NaO_8$ $[M+Na]^+$: 667.2308; found, 667.2304.

4.25. 1-Benzoyloxy-2-{3-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-5-*O*-(4,4′-dimethoxytrityl)-2-*O*,4-*C*-methylene-β-D-ribofuranosyl}benzene (23)

Under a nitrogen atmosphere, *i*-$Pr_2NP(Cl)OCH_2CH_2CN$ (46 μL, 0.206 mmol) was added to a solution of compound **32** (33 mg, 0.051 mmol) and *i*-Pr_2NEt (71 μL, 0.408 mmol) in anhydrous CH_2Cl_2 (1 mL) at room temperature and the mixture was stirred for 1 h. After addition of satd $NaHCO_3$ aq, the mixture was extracted with AcOEt. The extracts were washed with satd

$NaHCO_3$ aq, water, and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=4:1) to give compound **23** (40 mg, 92%) as a white solid. ^{1}H NMR ($CDCl_3$) δ 0.87 (3H, d, *J*=6.4 Hz), 0.94 (3H, d, *J*=6.9 Hz), 1.07–1.10 (6H, m), 2.29–2.32 (1H, m), 2.43–2.47 (1H, m), 3.39–3.56 (5H, m), 3.65–3.71 (1H, m), 3.81 (3H, s), 3.82 (3H, s), 3.86–3.90 (1H, m), 4.01–4.07 (1H, m), 4.31–4.45 (2H, m), 5.33 (0.5H, s), 5.36 (0.5H, s), 6.84–6.88 (4H, m), 7.22–7.46 (10H, m), 7.52–7.58 (4H, m), 7.65–7.69 (1H, m), 7.88–7.96 (1H, m), 8.19–8.24 (2H, m). ^{31}P NMR (acetone-d_6) δ 146.9, 147.7. HRMS (FAB) *m/z* calcd for $C_{49}H_{54}N_2O_9P$ $[M+H]^+$: 845.3567; found, 845.3565.

4.26. 2-[5-*O*-(4,4′-Dimethoxytrityl)-2-*O*,4-*C*-methylene-β-D-ribofuranosyl]-1-hydroxybenzene (33)

Under a nitrogen atmosphere, K_2CO_3 (103 mg, 0.75 mmol) was added to a solution of compound **32** (120 mg, 0.19 mmol) in MeOH (5 mL) at room temperature and the mixture was stirred for 5 min. After removal of MeOH under reduced pressure and the mixture was extracted with AcOEt. The extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=3:1) to give compound **33** (96 mg, 96%) as a white solid. $[\alpha]_D^{22}$ −2.3 (*c* 0.57, $CHCl_3$). IR ν_{max} (KBr) 3338, 3006, 2949, 1606, 1507, 1457, 1296, 1250, 1030 cm^{-1}. ^{1}H NMR (acetone-d_6) δ 2.76 (1H, br s), 3.32, 3.39 (2H, AB, *J*=10 Hz), 3.66 (6H, s), 3.82 (2H, s), 4.09 (1H, s), 5.10 (1H, s), 6.70–6.80 (6H, m), 6.97–7.03 (1H, m), 7.08–7.23 (3H, m), 7.28–7.33 (4H, m), 7.44–7.54 (3H, m). ^{13}C NMR (acetone-d_6) δ 55.6, 61.0, 71.8, 73.6, 80.8, 82.9, 86.9, 86.9, 113.9, 123.5, 127.0, 127.6, 128.4, 128.6, 129.0, 129.4, 129.8, 130.1, 130.8, 131.0, 131.0, 133.1, 134.9, 136.7, 136.9, 146.2, 148.3, 159.5, 165.3. HRMS (FAB) *m/z* calcd for $C_{33}H_{32}NaO_7$ $[M+Na]^+$: 563.2046; found, 563.2050.

4.27. 1-Allyloxy-2-[5-*O*-(4,4′-dimethoxytrityl)-2-*O*,4-*C*-methylene-β-D-ribofuranosyl]benzene (34)

Under a nitrogen atmosphere, K_2CO_3 (27 mg, 0.20 mmol) and allyl bromide (17 μL, 0.20 mmol) were added to a solution of compound **33** (96 mg, 0.18 mmol) in anhydrous acetone (16 mL) at room temperature and the mixture was stirred for 14 h. After addition of water, acetone was removed under reduced pressure. The mixture was extracted with AcOEt. The extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=3:1) to give compound **34** (90 mg, 87%) as a white solid. $[\alpha]_D^{22}$ +5.1 (*c* 0.93, $CHCl_3$). IR ν_{max} (KBr) 3431, 3007, 2943, 2836, 1605, 1508, 1453, 1298, 1249, 1178, 1078, 1031 cm^{-1}. ^{1}H NMR (acetone-d_6) δ 3.47, 3.53 (2H, AB, *J*=10 Hz), 3.77 (6H, s), 3.96 (2H, s), 4.21 (1H, s), 4.64 (2H, s), 5.25 (1H, s), 5.26 (1H, d, *J*=10 Hz), 5.47 (1H, d, *J*=17 Hz), 6.05–6.15 (1H, m), 6.88–7.02 (6H, m), 7.20–7.35 (4H, m), 7.44–7.47 (4H, m), 7.59–7.62 (2H, m), 7.75–7.78 (1H, m). ^{13}C NMR (acetone-d_6) δ 55.6, 61.3, 69.1, 72.1, 73.5, 79.3, 81.1, 82.5, 86.7, 86.9, 112.2, 113.9, 117.1, 121.4, 127.6, 127.9, 128.6, 129.1, 129.3, 129.3, 131.0, 131.1, 134.5, 136.8, 137.0. HRMS (FAB) *m/z* calcd for $C_{36}H_{36}NaO_7$ $[M+Na]^+$: 603.2359; found 603.2357.

4.28. 1-Allyloxy-2-{3-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-5-*O*-(4,4′-dimethoxytrityl)-2-*O*,4-*C*-methylene-β-D-ribofuranosyl}benzene (24)

Under a nitrogen atmosphere, $(i\text{-}Pr_2N)_2POCH_2CH_2CN$ (64 μL, 0.20 mmol) was added to a solution of compound **34** (69 mg, 0.12 mmol) and DIHT (24 mg, 0.12 mmol) in anhydrous MeCN/THF (2 mL, 3:1) at room temperature. After being stirred for 4 h, the mixture was concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=2:1) to give compound **24** (75 mg, 81%) as a white solid. ^{1}H NMR ($CDCl_3$) δ 0.82 (3H, d, *J*=6.6 Hz), 0.90 (3H, d, *J*=6.6 Hz), 1.04–1.08 (6H, m), 2.26–2.31 (1H, m), 2.37–2.42 (1H, m), 3.36–3.55 (5H, m), 3.64–3.71 (1H, m), 3.80 (3H, s), 3.80 (3H, s), 3.88–3.94 (1H, m), 4.05–4.11 (1H, m), 4.21 (0.5H, d, *J*=7.4 Hz), 4.34 (0.5H, d, *J*=9.9 Hz), 4.42 (0.5H, s), 4.50 (0.5H, s), 4.56–4.59 (2H, m), 5.24–5.31 (1H, m), 5.37–5.40 (1.5H, m), 5.43–5.47 (0.5H, m), 5.97–6.12 (1H, m), 6.81–6.99 (6H, m), 7.21–7.34 (4H, m), 7.41–7.46 (4H, m), 7.54–7.57 (2H, m), 7.71–7.73 (0.5H, m), 7.77–7.79 (0.5H, m). ^{31}P NMR (acetone-d_6) δ 146.9, 147.7. HRMS (FAB) *m/z* calcd for $C_{45}H_{54}N_2O_8P$ $[M+H]^+$: 781.3618; found, 781.3638.

4.29. 1-Allyloxy-3-(3,5-di-*O*-benzyl-2-*O*,4-*C*-methylene-β-D-ribofuranosyl)benzene (28)

Under a nitrogen atmosphere, a solution of compound **26** (1.38 g, 4.03 mmol) in anhydrous THF (20 mL) was added to a solution of 3-allyloxyphenylmagnesium iodide [prepared from 2-allyloxyphenyl iodide (4.19 g, 16.1 mmol) and *i*-PrMgCl·LiCl (1.3 M in THF, 12.4 mL, 16.1 mmol) in anhydrous THF (50 mL) at room temperature], and the mixture was stirred for 15 h. After addition of satd NH_4Cl aq, the mixture was extracted with AcOEt. The extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=3:1 to 1:1) to give an appropriate compound (R_f=ca. 0.5, *n*-hexane/AcOEt=1:1), which was dissolved in anhydrous CH_2Cl_2 (30 mL); TMAD (616 mg, 3.58 mmol) and *n*-Bu_3P (0.89 mL, 3.58 mmol) were added at room temperature and the mixture was stirred for 2 h. After addition of water, the mixture was extracted with Et_2O. The extracts were washed with water, brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=5:1) to give compound **28** (1.30 g, 70% from **26**) as a colorless oil. $[\alpha]_D^{26}$ +7.1 (*c* 1.45, $CHCl_3$). IR ν_{max} (KBr) 3030, 2878, 1600, 1449, 1281, 1105, 1031 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 3.86 (2H, s), 4.06 (1H, s), 4.07, 4.10 (2H, AB, *J*=8 Hz), 4.22 (1H, s), 4.43, 4.49 (2H, AB, *J*=12 Hz), 4.62 (2H, s), 4.65 (2H, s), 5.15 (1H, s), 5.25 (1H, d, *J*=11 Hz), 5.36 (1H, d, *J*=17 Hz), 5.92–6.06 (1H, m), 6.79 (1H, d, *J*=8 Hz), 6.85 (1H, d, *J*=8 Hz), 6.92 (1H, s), 7.19–7.35 (11H, m). ^{13}C NMR ($CDCl_3$) δ 66.5, 68.6, 72.0, 73.5, 73.6, 77.5, 80.9, 83.7, 85.7, 111.5, 113.7, 117.5, 127.4, 127.5, 127.5, 127.6, 128.2, 128.2, 129.3, 133.1, 137.3, 137.9, 140.7, 158.5. Mass (EI) *m/z* 458 (M^+, 12.5). Anal. Calcd for $C_{29}H_{30}O_5$: C, 75.96; H, 6.59; found: C, 75.66; H, 6.62.

4.30. 3-(3,5-Di-*O*-benzyl-2-*O*,4-*C*-methylene-β-D-ribofuranosyl)-1-hydroxybenzene (35)

Under a nitrogen atmosphere, $Pd(PPh_3)_4$ (162 mg, 0.14 mmol) was added to a solution of compound **28** (640 mg, 1.40 mmol) in anhydrous THF (150 mL) and the mixture was stirred until solids of $Pd(PPh_3)_4$ dissolved completely. Then, $NaBH_4$ (159 mg, 4.19 mmol) was added and the mixture was stirred at room temperature for 17 h. After addition of 3% HCl aq, the mixture was filtered through a pad of Celite®. The filtrate was extracted with AcOEt. The extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=5:2) to give compound **35** (594 mg, quant.) as a colorless oil. $[\alpha]_D^{25}$ +11.7 (*c* 1.36, $CHCl_3$). IR ν_{max} (KBr): 3311, 3030, 2879, 1595, 1453, 1362, 1206, 1105, 1032 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 3.85, 3.88 (2H, AB, *J*=11 Hz), 4.08 (1H, s), 4.07, 4.11 (2H, AB, *J*=8 Hz), 4.22 (1H, s), 4.38, 4.49 (2H, AB, *J*=12 Hz), 4.66 (2H, s), 5.12 (1H, s), 5.59 (1H, s), 6.70 (1H, d, *J*=8 Hz), 6.80 (1H, d, *J*=8 Hz), 6.78 (1H, s), 7.13–7.34 (11H, m). ^{13}C NMR

($CDCl_3$) δ 66.3, 72.1, 73.5, 73.7, 77.4, 80.9, 83.6, 85.8, 112.2, 114.4, 117.2, 127.5, 127.6, 127.6, 127.7, 128.2, 128.3, 129.6, 137.3, 137.9, 140.8, 155.9. HRMS (MALDI-TOF) *m/z* calcd for $C_{26}H_{26}NaO_5$ $[M+Na]^+$: 441.1678; found, 441.1677.

4.31. 1-Benzoyloxy-3-(3,5-di-*O*-benzyl-2-*O*,4-*C*-methylene-β-D-ribofuranosyl)benzene (36)

Under a nitrogen atmosphere, BzCl (200 μL, 1.70 mmol) was added to a solution of compound **35** (594 mg, 1.42 mmol) and Et_3N (240 μL, 1.70 mmol) in anhydrous CH_2Cl_2 (15 mL) at room temperature and the mixture was stirred for 17 h. After addition of satd $NaHCO_3$ aq, the mixture was extracted with AcOEt. The extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=3:1) to give compound **36** (730 mg, 95%) as a colorless oil. $[\alpha]_D^{25}$ +28.3 (*c* 1.49, $CHCl_3$). IR ν_{max} (KBr) 3423, 3030, 2867, 1599, 1490, 1450, 1260, 1097 cm^{-1}. ^1H NMR ($CDCl_3$) δ 3.85, 3.87 (2H, AB, *J*=11 Hz), 4.07 (1H, s), 4.09, 4.12 (2H, AB, *J*=8 Hz), 4.25 (1H, s), 4.46, 4.52 (2H, AB, *J*=11 Hz), 4.64 (2H, s), 5.20 (1H, s), 7.11–7.42 (14H, m), 7.48–7.54 (2H, m), 7.62–7.67 (1H, m), 8.19 (2H, d, *J*=7 Hz). ^{13}C NMR ($CDCl_3$) δ 68.7, 69.5, 71.9, 73.7, 74.5, 75.2, 81.7, 94.8, 89.1, 113.2, 114.3, 117.5, 119.5, 127.6, 127.8, 128.2, 128.4, 129.3, 133.1, 137.3, 137.4, 142.0, 158.5. HRMS (MALDI-TOF) *m/z* calcd for $C_{33}H_{30}NaO_6$ $[M+Na]^+$: 545.1940; found, 545.1936.

4.32. 1-Benzoyloxy-3-(2-*O*,4-*C*-methylene-β-D-ribofuranosyl) benzene (37)

A solution of compound **36** (730 mg, 1.40 mmol), 20% $Pd(OH)_2/C$ (200 mg), and cyclohexene (14 mL, 140 mmol) in EtOH (14 mL) was refluxed for 1 h. The mixture was filtered through a pad of Celite® and the filtrate was concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=1:2 to 1:5) to give compound **37** (452 mg, 95%) as a white solid. $[\alpha]_D^{26}$ −17.4 (*c* 0.97, $CHCl_3$). IR ν_{max} (KBr) 3381, 2947, 1733, 1591, 1487, 1447, 1264, 1146, 1030 cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.69 (1H, br s), 3.05 (1H, br s), 3.95 (2H, s), 3.97, 4.04 (2H, AB, *J*=8 Hz), 4.16 (1H, s), 4.23 (1H, s), 5.13 (1H, s), 7.08–7.18 (3H, m), 7.26 (1H, s), 7.35–7.41 (1H, m), 7.47–7.53 (2H, m), 7.61–7.66 (1H, m), 8.17 (2H, d, *J*=7 Hz). ^{13}C NMR ($CDCl_3$) δ 59.0, 71.0, 72.3, 82.9, 83.6, 86.7, 118.6, 120.9, 122.6, 128.5, 129.1, 129.6, 130.1, 133.7, 140.5, 151.0, 165.2. Mass (FAB) *m/z* 343 ($M+H^+$). Anal. Calcd for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30; found: C, 66.55; H, 5.33.

4.33. 1-Benzoyloxy-3-[5-*O*-(4,4′-dimethoxytrityl)-2-*O*,4-*C*-methylene-β-D-ribofuranosyl]benzene (38)

Under a nitrogen atmosphere, DMTrCl (175 mg, 0.515 mmol) was added to a solution of compound **37** (147 mg, 0.429 mmol) in anhydrous pyridine (4 mL) at room temperature and the mixture was stirred for 12 h. After addition of satd aq $NaHCO_3$ solution, the mixture was extracted with AcOEt. The extracts were washed with water, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=3:1 to 1:1) to give compound **38** (252 mg, 91%) as a white solid. $[\alpha]_D^{22}$ −12.8 (*c* 0.85, $CHCl_3$). IR ν_{max} (KBr): 3478, 3006, 2950, 1736, 1606, 1508, 1447, 1254, 1178, 1072, 1032 cm^{-1}. ^1H NMR (acetone-d_6) δ 2.85 (1H, s), 3.41, 3.55 (2H, AB, *J*=11 Hz), 3.74 (3H, s), 3.75 (3H, s), 3.98, 4.00 (2H, AB, *J*=8 Hz), 4.18 (1H, s), 4.27, 4.41 (2H, AB, *J*=5 Hz), 5.15 (1H, s), 6.84–6.89 (4H, m), 7.11–7.31 (4H, m), 7.38–7.61 (11H, m), 7.68–7.75 (1H, m), 8.13–8.16 (2H, m). ^{13}C NMR ($CDCl_3$) δ 55.2, 60.2, 71.7, 72.9, 82,8, 83.3, 85.9, 86.3, 113.1, 118.6, 120.8, 122.7, 126.7, 127.8, 128.0, 128.4, 129.3, 129.4, 129.9, 130.0, 130.1, 133.5, 135.6, 135.6, 141.0, 144.5, 151.0, 158.3, 165.0. HRMS (EI) *m/z* calcd for $C_{40}H_{36}O_8$ $[M]^+$: 644.2410; found: 644.2410.

4.34. 1-Benzoyloxy-3-{3-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-5-*O*-(4,4′-dimethoxytrityl)-2-*O*,4-*C*-methylene-β-D-ribofuranosyl}benzene (25)

Under a nitrogen atmosphere, *i*-$Pr_2NP(Cl)OCH_2CH_2CN$ (45 μL, 0.20 mmol) was added to a solution of compound **38** (32 mg, 0.05 mmol) and *i*-Pr_2NEt (87 μL, 0.50 mmol) in anhydrous CH_2Cl_2 (1 mL) at 0 °C and the mixture was stirred at room temperature for 1 h. After addition of satd $NaHCO_3$ aq, the mixture was extracted with AcOEt. The extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt=3:1) to give compound **25** (30 mg, 71%) as a white solid. ^1H NMR (400 MHz, $CDCl_3$) δ 0.88 (3H, d, *J*=6.9 Hz), 0.96 (3H, d, *J*=6.8 Hz), 1.06–1.09 (6H, m), 2.30–2.33 (1H, m), 2.42–2.45 (1H, m), 3.40–3.49 (5H, m), 3.66–3.69 (1H, m), 3.75–3.76 (6H, m), 3.92–3.95 (1H, m), 4.10–4.15 (1H, m), 4.21 (0.5H, d, *J*=7.4 Hz), 4.28 (0.5H, s), 4.35 (0.5H, d, *J*=9.3 Hz), 4.39 (0.5H, s), 5.26 (1H, s), 6.80–6.83 (4H, m), 7.14–7.17 (2H, m), 7.24–7.27 (3H, m), 7.39–7.53 (10H, m), 7.60–7.64 (1H, m), 8.12–8.16 (2H, m). ^{31}P NMR ($CDCl_3$) δ 148.2, 148.4. HRMS (FAB) *m/z* calcd for $C_{49}H_{54}N_2O_9P$ $[M+H]^+$: 845.3567; found, 845.3573.

4.35. Synthesis of TFOs

The 0.2 μmol scale synthesis of TFOs **39–43** was performed on an automated DNA synthesizer (Applied Biosystems Expedite™ 8909 or Genedesign nS-8) using a standard phosphoramidite protocol. By treatment with 28% NH_3 aq at room temperature for 4 h, TFOs synthesized on DMTr-ON mode were cleaved from the CPG resin and all protecting groups on TFOs were removed. The crude TFOs **39–41** and **43** obtained were purified with NENSORB™ PREP or Sep-Pak® Plus C18 cartridges followed by reversed-phase HPLC (ChemcoPak® CHEMCOSORB 300-5C18, 4.6 mm×250 mm or Waters XBridge® MS C_{18} 2.5 μm, 10 mm×50 mm). For TFO **42**, before reversed-phase HPLC purification, treatment with $NaBH_4$ (2.2 mg, 58.2 μmol) and $Pd(PPh_3)_4$ (2.1 mg, 1.9 μmol) in H_2O (200 μL) at room temperature for 3 h was performed to remove an allyl group. The composition of the TFOs was confirmed by MALDI-TOF-MS analysis. MALDI-TOF-MS data ($[M-H]^-$) for TFOs **39–43**; **39**, found 4464.44 (calcd 4463.98); **40**, found 4463.89 (calcd 4463.98); **41**, found 4464.43 (calcd 4463.98); **42**, found 4491.60 (calcd 4492.00); **43**, found 4492.34 (calcd 4492.00).

4.36. UV melting experiments

UV melting experiments were performed on SHIMADZU UV-1650 and SHIMADZU UV-1800 spectrophotometers equipped with T_m analysis accessory. The TFOs **39–43** and hairpin dsDNA targets were dissolved in 10 mM sodium cacodylate buffer (pH 6.8) containing 140 mM KCl and 50 mM $MgCl_2$ to give a final concentration of each strand of 1.89 μM. The samples were annealed in boiling water followed by slow cooling to 5 °C. The melting profiles were recorded at 260 nm from 5 °C to 90 °C at a scan rate of 0.5 °C/min. The two-point average method was used to obtain the T_m values and the final values were determined by averaging three independent measurements, which were accurate to within 1 °C.

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Synthesis and properties of thymidines with six-membered amide bridge

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ABSTRACT

Artificial thymidine monomers possessing amide or N-methylamide bridges were designed, synthesized, and introduced into oligonucleotides. UV-melting experiments showed that these oligonucleotides preferred single-stranded RNA (ssRNA) to single-stranded DNA (ssDNA) in duplex formation. Both amide- and N-methylamide-modified oligonucleotides led to a significant increase in the binding affinity to ssRNA by up to +4.7 and +3.7 °C of the T_m value per modification, respectively, compared with natural oligonucleotide. In addition, their oligonucleotides showed high stability against 3′-exonuclease.

1. Introduction

Artificial nucleic acids that possess strong and sequence-selective binding affinities with single-stranded RNA (ssRNA) and/or high nuclease-resistant properties are promising tools for nucleic acid-based technologies such as the antisense method.[1,2] In fact, a large number of nucleic acid derivatives have been developed to date.[1] The breakthrough was the discovery by us[3] and Wengel's group[4] of 2′,4′-BNA/LNA with a 2′-*O*,4′-*C*-methylene-bridged structure in the sugar moiety, (Fig. 1). The 2′,4′-BNA/LNA modification of oligonucleotides leads to a great improvement in hybridizing ability with ssRNA and an increase in nuclease resistance compared with natural oligonucleotides. Since 2′,4′-BNA/LNA was reported, the development of artificial nucleic acids with additional 2′,4′-bridged structures has attracted much attention. In particular, ring-enlargement from a five-membered bridge to a six-membered one in 2′,4′-bridged structures would be a useful modification. For example, ENA, the six-membered analog of 2′,4′-BNA/LNA, shows the same level of duplex-hybridizing ability with ssRNA as does 2′,4′-BNA/LNA, and improved enzymatic stability as compared to 2′,4′-BNA/LNA (Fig. 1).[5,6] Recently, we developed AmNA with five-membered amide bridge, the duplex-forming ability of which was comparable to that of 2′,4′-BNA/LNA, and the nuclease resistance of which was superior to that of 2′,4′-BNA/LNA.[7] Against this background, we were interested in the properties of the ring-enlarged analog of AmNA shown in Figure 2. In addition, various substituents could be introduced into the nitrogen in the amide bridge to improve the functions of the oligonucleotides. In this study, two thymidines with a six-membered amide bridge, that is, NH and NMe analogs, were synthesized, and the duplex-forming abilities and the nuclease resistances of their oligonucleotides were evaluated.

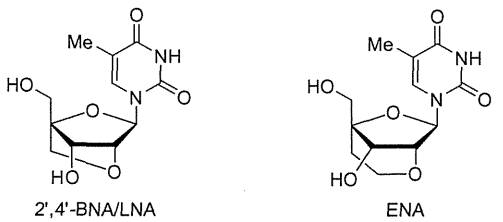

Figure 1. Structures of 2′,4′-BNA/LNA and ENA monomers.

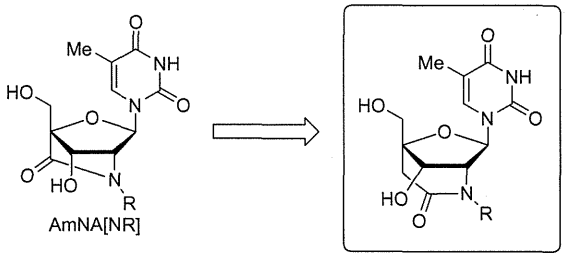

Figure 2. Design of nucleic acid monomers (R = H or Me) used in this study.

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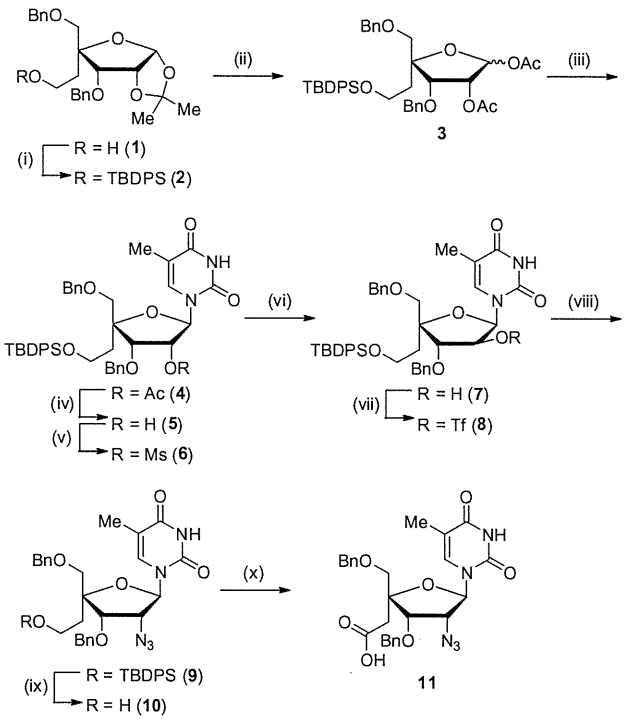


Scheme 1. Reagents and conditions: (i) TBDPSCl, DMAP, CH_2Cl_2, rt, 16 h, quant.; (ii) Ac_2O, AcOH, concd H_2SO_4, rt, 2 h, 91%; (iii) thymine, BSA, TMSOTf, MeCN, reflux, 3.5 h, 84%; (iv) K_2CO_3, MeOH, rt, 1 h, 95%; (v) MsCl, Et_3N, CH_2Cl_2, 0 °C, 1.5 h, 91%; (vi) NaOH, EtOH, rt, 12 h, 89%; (vii) Tf_2O, pyridine, CH_2Cl_2, 0 °C, 0.5 h, 83%; (viii) NaN_3, DMF, rt, 13 h, quant.; (ix) TBAF, THF, rt, 17 h, quant.; (x) PDC, MS4Å, DMF, rt, 12 h, 94%.

2. Results and discussion

2.1. Synthesis

The silylation of known compound **1**[5] using TBDPSCl gave **2** in quantitative yield (Scheme 1); **2** was converted to diacetate **3**. The reaction of **3** with silylated thymine, prepared in situ from thymine and *N,O*-bis(trimethylsilyl)acetamide (BSA), in the presence of TMSOTf produced the desired β-isomer **4**. Next, introduction of a nitrogen atom at the 2′-position was performed using a double-stereoinversion approach. After deacetylation of **4** on exposure to K_2CO_3 in MeOH, stereoinversion of the 2′-hydroxyl group was achieved by mesylation of the resulting **5**, followed by treatment with NaOH. Then, **7** underwent reaction with Tf_2O to give **8** in 83% yield, and compound **9**, with a nitrogen atom at the 2′-position, was obtained by azidation. Desilylation of **9**, followed by oxidation of **10** with PDC in DMF, efficiently produced carboxylic acid **11**.

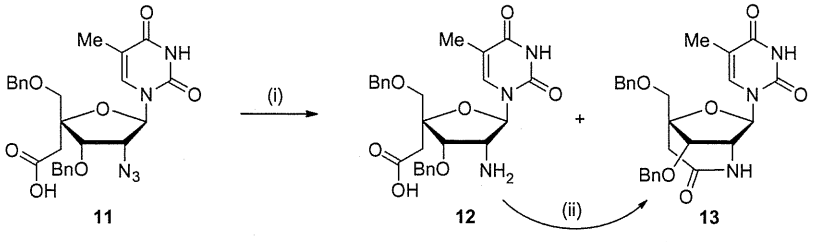
Next, reduction of the azide group in **11** was examined (Scheme 2). Reduction by $NaBH_4$ in *i*-PrOH gave the corresponding amine **12** in 55% yield. Fortunately, under Staudinger conditions using Me_3P, ring-closed **13** was produced together with **12**. Compound **12** was converted to the desired **13** using EDC or MsCl; the yields were 72% and 86%, respectively.

The synthesis of phosphoramidite **14** was carried out in three steps from **13** (Scheme 3). Diol **15** was prepared by hydrogenolysis of **13**. Then, dimethoxytritylation of **15**, followed by phosphitylation, yielded the desired phosphoramidite **14**. Concerning the *N*-methyl congener **17**, protection of the imide nitrogen of thymine in **13**, and successive methylation of **18** gave **19**, which was hydrogenolyzed to produce diol **20**. Using a similar synthetic route to **14**, **20** was converted into the desired phosphoramidite **17** with an N-methylamide bridge via dimethoxytritylated **21**. The phosphoramidites **14** and **17** obtained were used to synthesize modified oligonucleotides **22–29** on an automated DNA synthesizer (see the Section 4 for details). These amide bridges were stable under conventional conditions, that is, aqueous ammonia or methanolic K_2CO_3, for cleavage from the resin and removal of β-cyanoethyl groups on the phosphates.

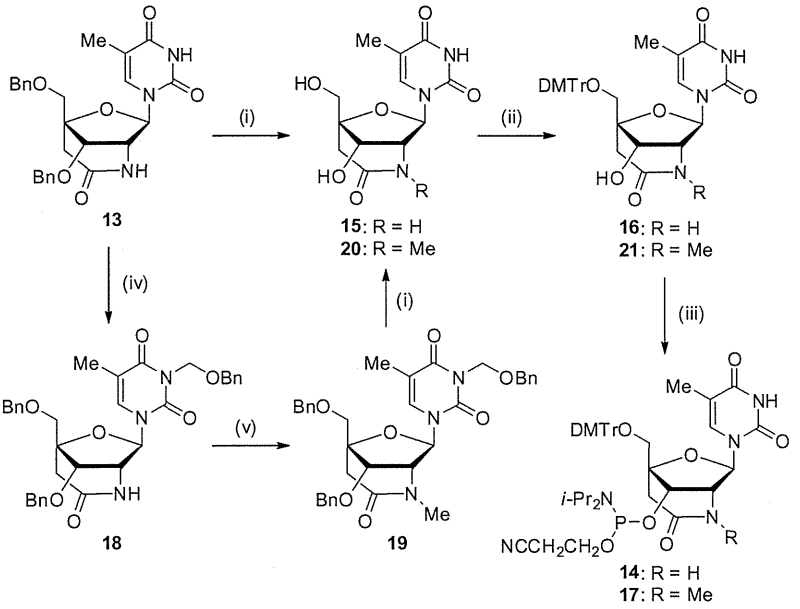
The duplex-forming abilities of oligonucleotides **22–27** with ssDNA or ssRNA were evaluated and the results are summarized in Table 1. Regardless of whether it was the NH or NMe analog, the modified oligonucleotides generally showed a significantly decreased affinity for ssDNA compared with natural oligonucleotide **30**, although in the case of **24**, possessing three NH analogs, the affinity was increased by +1.7 °C per modification. Concerning ssRNA, the single-modified oligonucleotides **22** and **25** had almost the same affinities as natural oligonucleotide **30**. However, by multiple modifications, the binding affinity was greatly increased and the changes in T_m value per modification (ΔT_m/mod.) of the NH (**24**) or NMe (**27**) analogs were +4.7 and +3.7 °C, respectively. These results demonstrate that oligonucleotides containing these thymidines with six-membered amide bridges formed stable duplexes with ssRNA and recognized ssRNA more selectively than ssDNA in the duplex formation.

The binding affinities with ssDNA and ssRNA of the oligonucleotides modified by AmNA[NH] or AmNA[NMe] with a five-membered amide bridge, shown in Figure 2, and those of the oligonucleotides modified by HxNA[NMe] with a six-membered hydroxamate bridge, shown in Figure 3, were evaluated in our previous reports.[7,8] Although these analogs showed lower duplex-forming abilities with ssRNA than AmNA[NH] or AmNA[NMe], one of these analogs, the NH analog, was more stably bound to ssRNA compared with HxNA[NMe] with a six-membered bridge similar to these amide bridges.

The stabilities of oligonucleotides **28** and **29** including these analogs against 3′-exonuclease were determined and compared with those of AmNA[NH]-, AmNA[NMe]-, and HxNA[NMe]-modified oligonucleotides **31-33** and natural **30** (Fig. 4 and the sequences of oligonucleotides used are shown in the legend of Fig. 4). Under conditions where natural **30** decomposed completely within 5 min, over 50% and 60% of **28** and **29** remained after 40 min. The NMe analog (**29**) showed higher stability against nuclease than the NH analog (**28**) did. This is probably because ac-



Scheme 2. Reagents and conditions: (i) $NaBH_4$, *i*-PrOH, reflux, 1 h, 55% (**12**) or Me_3P, THF/H_2O (5:1), rt, 14 h, ca 35% (**12**) and 43% (**13**); (ii) EDC, DMAP, CH_2Cl_2, rt, 24 h, 72% or MsCl, Et_3N, CH_2Cl_2, 0 °C to rt, 17 h, 86%.



Scheme 3. Reagents and conditions: (i) H_2, 20% $Pd(OH)_2$-C, MeOH, rt, 24 h, 61% (**15**) or 20% $Pd(OH)_2$-C, cyclohexene, EtOH, reflux, 2 h, quant. (**20**); (ii) DMTrCl, pyridine, rt, 3–17 h, 81% (**16**) and 69% (**21**); (iii) $(i\text{-}Pr_2N)_2POCH_2CH_2CN$, 1*H*-tetrazole, MeCN/THF (3:1), rt, 12 h, 42% (**14**) or $i\text{-}Pr_2NP(Cl)OCH_2CH_2CN$, $i\text{-}Pr_2NEt$, CH_2Cl_2, rt, 2 h, 73% (**17**); (iv) $BnOCH_2Cl$, DBU, DMF, 0 °C, 0.5 h, 86%; (v) NaH, MeI, DMF, 0 °C, 2 h, 60%.

Table 1
T_m values (°C) of duplexes formed between oligonucleotides and ssDNA or ssRNA[a,b]

Oligonucleotide	ssDNA	ssRNA
5′-TTTTTTTTTT-3′ (**30**)	21	19
5′-TTTTTTTTTT-3′ (**22**)	16 (−5.0)	20 (+1.0)
5′-TTTTTTTTTT-3′ (**23**)	19 (−1.0)	27 (+4.0)
5′-TTTTTTTTTT-3′ (**24**)	26 (+1.7)	33 (+4.7)
5′-TTTTTTTTTT-3′ (**25**)	15 (−6.0)	20 (+1.0)
5′-TTTTTTTTTT-3′ (**26**)	13 (−4.0)	26 (+3.5)
5′-TTTTTTTTTT-3′ (**27**)	18 (−1.0)	30 (+3.7)

[a] Conditions: 10 mM sodium phosphate buffer (pH 7.2) and 100 mM NaCl. The final concentration of each oligonucleotide used was 4 μM. The sequences of ssDNA and ssRNA are 5′-d(AAAAAAAAAA)-3′ and 5′-r(AAAAAAAAAA)-3′, respectively. *T* and T indicate NH analog and NMe analog, respectively.

[b] The change in T_m value per modification is shown in parentheses.

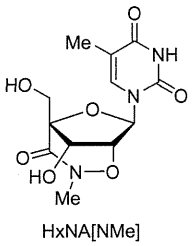


Figure 3. Structure of HxNA[NMe] monomer.

cess of the nuclease to the phosphodiester linkage was prevented by the more bulky N-methylamide bridge. In a comparison of **28** and **29** with **31** and **32**, respectively, no significant improvement in nuclease resistance as a result of enlargement of the amide bridge was observed. However, oligonucleotides **28** and **29** were quite stable compared with **33**, including an HxNA[NMe] with the same six-membered ring size. In contrast, the six-membered hydroxamate bridge of HxNA was opened under the nuclease treatment conditions, and, consequently, the 5′-phosphate moiety of HxNA showed high resistance against nuclease.[8] However, in the

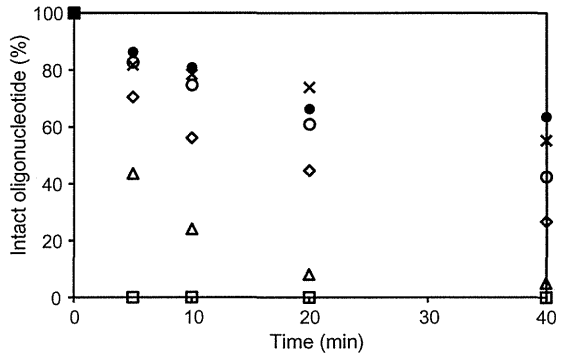


Figure 4. Enzymatic stability of oligonucleotides **28** and **29**. Conditions: 1.75 μg/mL Crotalus admanteus venom phosphodiesterase (CAVP), 10 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.0), and 7.5 μM each oligonucleotide at 37 °C. The sequence of oligonucleotides used was 5′-TTTTTTTTTT-3′. T = NH analog (open circle, oligonucleotide **28**), NMe analog (closed circle, oligonucleotide **29**), AmNA[NH] (open diamond, oligonucleotide **31**), AmNA[NMe] (cross, oligonucleotide **32**), HxNA[NMe] (open triangle, oligonucleotide **33**) and natural (open square, oligonucleotide **30**).

cases of these NMe and NH analogs bearing a six-membered amide bridge, as well as AmNA[NH] and AmNA[NMe] bearing a five-membered amide bridge, the 5′-phosphate moieties were easily degradable and such a phenomenon was not observed. These results suggest that resistance against nuclease was greatly affected by not only the bridge size but also by the composition of the bridge, for example, whether there is any substituent and/or heteroatom, or the location of the substituent and/or heteroatom.

3. Conclusion

Two thymidines bearing six-membered bridges, that is, amide and N-methylamide bridges, were successfully synthesized and introduced into oligonucleotides, using an automated DNA synthesizer. These oligonucleotides formed stable duplexes with ssRNA, but the duplexes with ssDNA were destabilized. They also had high nuclease resistance, which were slightly better than those of

AmNAs bearing five-membered amide bridges. Moreover, their nuclease resistances were much higher than that of HxNA[NMe] with the same bridge size. These findings demonstrated that the composition of the bridge moiety is a key factor in the development of bridged nucleic acids. We believe that the accumulation of properties such as duplex-forming ability and nuclease resistance through the development of various bridged nucleic acids will contribute to developing ideal useful tools for nucleic acid-based technologies.

4. Experimental

4.1. General

All chemicals were purchased from chemical suppliers. For column chromatography, Fuji Silysia silica gel PSQ-100B and FL-100D were used. All melting points were measured on a Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra were recorded on a JEOL ECS400 spectrometer. 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. Mass spectra were measured on a JEOL JMS-600 or JEOL JMS-700 mass spectrometer. MALDI-TOF mass spectra were recorded on a Bruker Daltonics Autoflex II TOF/TOF mass spectrometer.

4.2. 3,5-Di-*O*-benzyl-4-(2-*tert*-butyldiphenylsiloxyethyl)-1,2-*O*-isopropylidene-α-D-ribofuranose (2)

Under nitrogen atmosphere, DMAP (350 mg, 2.87 mmol), Et_3N (4.0 mL, 29 mmol), and TBDPSCl (4.0 mL, 15 mmol) were added to a solution of alcohol **1**[5] (3.96 g, 9.56 mmol) in CH_2Cl_2 (100 mL) at 0 °C. The reaction mixture was stirred at room temperature for 16 h. The reaction was then quenched with satd $NaHCO_3$ aq and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (8.82 g) was purified by column chromatography (silica gel 200 g, *n*-hexane/EtOAc = 10:1) to give silyl ether **2** (6.28 g, quant.) as a colorless oil.

$[\alpha]_D^{25}$ +16.3 (*c* 1.00, $CHCl_3$). IR (KBr): 3068, 3031, 2932, 2857, 1496, 1472, 1454, 1428, 1383, 1312, 1256, 1209 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.00 (s, 9H), 1.30 (s, 3H), 1.50 (s, 3H), 1.87 (ddd, *J* = 7.0, 8.0, 14.5 Hz, 1H), 2.43 (ddd, *J* = 5.0, 6.5, 14.5 Hz, 1H), 3.29 (d, *J* = 10.5 Hz), 1H, 3.70 (d, *J* = 10.5 Hz, 1H), 3.82 (ddd, *J* = 5.0, 7.0, 12.0 Hz, 1H), 3.94 (ddd, *J* = 6.5, 8.0, 12.0 Hz, 1H), 4.21 (d, *J* = 5.5 Hz, 1H), 4.35 (d, *J* = 12.5 Hz, 1H), 4.47 (d, *J* = 12.5 Hz, 1H), 4.53 (d, *J* = 12.0 Hz, 1H), 4.60 (dd, *J* = 4.0, 5.5 Hz, 1H), 4.74 (d, *J* = 12.0 Hz, 2H), 5.74 (d, *J* = 4.0 Hz, 1H), 7.19–7.67 (m, 20H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 19.08, 26.15, 26.59, 26.80, 34.93, 59.78, 72.41, 73.37, 77.88, 79.26, 86.65, 104.16, 113.01, 127.50, 127.52, 127.57, 127.72, 127.80, 128.29, 128.32, 129.45, 133.87, 135.57, 138.08, 138.21. MS (FAB): *m*/*z* 675 (MNa^+). HRMS (FAB): calcd for $C_{40}H_{48}O_6SiNa$ (MNa^+) 675.3118, found 675.3115.

4.3. 1,2-Di-*O*-acetyl-3,5-di-*O*-benzyl-4-(2-*tert*-butyldiphenylsiloxyethyl)-D-ribofuranose (3)

Under nitrogen atmosphere, Ac_2O (10 mL, 110 mmol) and H_2SO_4 (0.1% in AcOH, 4.0 mL) were added to a solution of silyl ether **2** (5.86 g, 8.98 mmol) in AcOH (30 mL) at room temperature. The reaction mixture was stirred at room temperature for 2 h. The reaction was then quenched with satd $NaHCO_3$ aq and extracted with EtOAc. The combined organic layer was washed with satd $NaHCO_3$ aq, water and brine, dried over Na_2SO_4 and concentrated. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (6.18 g) was purified by column chromatography (silica gel 200 g, *n*-hexane/EtOAc = 5:1) to give diacetate **3** as a colorless oil (5.67 g, 91%).

$[\alpha]_D^{24}$ −16.3 (*c* 1.00, $CHCl_3$). IR (KBr): 3069, 3031, 2931, 2857, 1748, 1496, 1472, 1455, 1428, 1369, 1309, 1219 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.02 (s, 9H), 1.83 (s, 3H), 1.93 (s, 3H), 1.96–2.13 (m, 2H), 3.39 (d, *J* = 10.0 Hz, 1H), 3.50 (d, *J* = 10.0 Hz, 1H), 3.81–3.93 (m, 2H), 4.36–4.44 (m, 3H), 4.46 (d, *J* = 12.0 Hz, 1H), 4.57 (d, *J* = 12.0 Hz, 1H), 5.28 (d, *J* = 5.5 Hz, 1H), 6.03 (s, 1H), 7.21–7.67 (m, 20H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 19.09, 20.63, 20.93, 26.81, 35.75, 59.70, 73.16, 73.18, 73.44, 74.68, 78.07, 86.66, 97.61, 127.44, 127.49, 127.55, 127.57, 127.70, 127.75, 128.23, 128.32, 129.47, 133.87, 133.91, 135.53, 137.80, 138.15, 169.37, 169.70. MS (FAB): *m*/*z* 719 (MNa^+). HRMS (FAB): calcd for $C_{41}H_{48}O_8SiNa$ (MNa^+) 719.3016, found 719.2999.

4.4. 2′-*O*-Acetyl-3′,5′-di-*O*-benzyl-4′-(2-*tert*-butyldiphenylsiloxyethyl)-5-methyluridine (4)

Under nitrogen atmosphere, thymine (1.41 g, 11.2 mmol) and BSA (6.8 mL, 28 mmol) were added to a solution of diacetate **3** (4.36 g, 11.0 mmol) in MeCN (20 mL) at room temperature. The reaction mixture was refluxed for 1.5 h. TMSOTf (1.9 mL, 10 mmol) was added to the resulting mixture at 0 °C. The reaction mixture was refluxed for 3.5 h. The reaction was then quenched with satd $NaHCO_3$ aq and extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (5.71 g) was purified by column chromatography (silica gel 150 g, *n*-hexane/EtOAc = 3:2) to give acetate **4** as a white foam (5.14 g, 84%).

Mp: 45–48 °C. $[\alpha]_D^{25}$ +10.2 (*c* 1.00, $CHCl_3$). IR (KBr): 3172, 3068, 2930, 2857, 1747, 1693, 1470, 1428, 1372, 1234 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.01 (s, 9H), 1.48 (s, 3H), 1.76–1.83 (m, 1H), 2.04 (s, 3H), 2.03–2.11 (m, 1H), 3.41 (d, *J* = 10.5 Hz, 1H), 3.71–3.83 (m, 2H), 3.89 (d, *J* = 10.5 Hz, 1H), 4.35 (d, *J* = 6.5 Hz, 1H), 4.38 (d, *J* = 11.5 Hz, 1H), 4.39 (d, *J* = 9.5 Hz, 1H), 4.44 (d, *J* = 9.5 Hz, 1H), 4.57 (d, *J* = 11.5 Hz, 1H), 5.35 (dd, *J* = 5.0, 6.5 Hz, 1H), 6.08 (d, *J* = 5.0 Hz, 1H), 7.20–7.41 (m, 16H), 7.49 (s, 1H), 7.60–7.65 (m, 4H), 7.83 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.96, 19.06, 20.82, 26.82, 35.09, 59.27, 73.10, 73.38, 74.22, 75.24, 77.43, 86.03, 87.35, 111.21, 127.58, 127.66, 127.85, 128.01, 128.42, 128.59, 129.67, 133.47, 133.51, 135.51, 135.69, 137.32, 137.49, 150.28, 163.58, 170.05. MS (FAB): *m*/*z* 763 (MH^+). HRMS (FAB): calcd for $C_{44}H_{51}N_2O_8Si$ (MH^+) 763.3415, found 763.3402.

4.5. 3′,5′-Di-*O*-benzyl-4′-(2-*tert*-butyldiphenylsiloxyethyl)-5-methyluridine (5)

K_2CO_3 (450 mg, 330 mmol) was added to a solution of compound **4** (5.04 g, 6.61 mmol) in MeOH (10 mL) at room temperature. The reaction mixture was stirred at room temperature for 1 h. The reaction was then quenched with satd NH_4Cl aq and extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (4.76 g) was purified by column chromatography (silica gel 100 g, *n*-hexane/EtOAc = 4:3) to give alcohol **5** as a white foam 4.54 g, 95%).

Mp: 62–64 °C. $[\alpha]_D^{26}$ −10.3 (*c* 1.00, $CHCl_3$). IR (KBr): 3423, 3179, 3066, 2928, 2856, 1695, 1471, 1428, 1391, 1362, 1270 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.06 (s, 9H), 1.56 (s, 3H), 1.77 (ddd, *J* = 6.5, 8.0, 14.5 Hz, 1H), 2.14 (dt, *J* = 5.0, 14.5 Hz, 1H), 2.83 (d, *J* = 8.0 Hz, 1H), 3.45 (d, *J* = 10.5 Hz, 1H), 3.73–3.88 (m, 2H), 3.85 (d, *J* = 10.5 Hz, 1H), 4.14 (d, *J* = 6.0 Hz, 1H), 4.30 (m, 1H), 4.46 (d, *J* = 12.0 Hz, 1H), 4.49 (d, *J* = 12.0 Hz, 1H), 4.56 (d, *J* = 11.0 Hz, 1H), 4.63 (d, *J* = 11.0 Hz, 1H), 5.82 (d, *J* = 6.0 Hz, 1H), 7.22–7.65 (m,

21H), 8.12 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.04, 19.05, 26.83, 35.36, 59.36, 73.49, 74.05, 74.57, 75.02, 79.41, 87.03, 88.43, 111.93, 127.50, 127.64, 127.66, 128.01, 128.06, 128.25, 128.57, 128.60, 129.65, 129.68, 133.51, 135.52, 135.89, 137.02, 137.33, 150.80, 163.69. MS (FAB): *m/z* 721 (MH^+). HRMS (FAB): calcd for $C_{42}H_{49}N_2O_7Si$ (MH^+) 721.3309, found 721.3320.

4.6. 3′,5′-Di-*O*-benzyl-4′-(2-*tert*-butyldiphenylsiloxyethyl)-2′-*O*-methanesulfonyl-5-methyluridine (6)

Under nitrogen atmosphere, Et_3N (4.0 mL, 29 mmol) and MsCl (0.70 mL, 9.0 mmol) were added to a solution of alcohol **5** (3.26 g, 4.52 mmol) in CH_2Cl_2 (40 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1.5 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with CH_2Cl_2. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (3.80 g) was purified by column chromatography (silica gel 100 g, *n*-hexane/EtOAc = 4:3) to give mesylate **6** as a white foam (3.29 g, 91%).

Mp: 57–62 °C. $[\alpha]_D^{22}$ +214.8 (*c* 1.00, $CHCl_3$). IR (KBr): 3168, 3068, 3031, 2932, 2857, 1694, 1538, 1471, 1428, 1361, 1272 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.02 (s, 9H), 1.44 (s, 3H), 1.85 (ddd, *J* = 6.5, 8.0, 14.5 Hz, 1H), 2.10 (dt, *J* = 5.0, 14.5 Hz, 1H), 3.07 (s, 3H), 3.44 (d, *J* = 6.5 Hz, 1H), 3.70–3.86 (m, 2H), 3.95 (d, *J* = 6.5 Hz, 1H), 4.33 (d, *J* = 5.0 Hz, 1H), 4.37 (d, *J* = 12.0 Hz, 1H), 4.42 (d, *J* = 12.0 Hz, 1H), 4.45 (d, *J* = 12.0 Hz, 1H), 4.83 (d, *J* = 12.0 Hz, 1H), 5.26 (dd, *J* = 3.0, 5.0 Hz, 1H), 6.01 (d, *J* = 3.0 Hz, 1H), 7.18–7.63 (m, 21H), 8.29 (br s, 1H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 11.90, 19.08, 26.85, 35.11, 38.83, 59.13, 72.65, 73.40, 73.78, 76.39, 80.30, 86.74, 87.83, 111.27, 127.60, 127.68, 127.70, 128.11, 128.12, 128.47, 128.64, 129.70, 129.73, 133.42, 133.46, 135.28, 135.53, 137.11, 137.22, 150.46, 163.44. MS (FAB): *m/z* 799 (MH^+). HRMS (FAB): calcd for $C_{43}H_{48}N_2O_9F_3SiS$ (MH^+) 799.3085, found 799.3085.

4.7. 3′,5′-Di-*O*-benzyl-4′-(2-*tert*-butyldiphenylsiloxyethyl)-5-methylarabinouridine (7)

1 M NaOH aq (12 mL, 12 mmol) was added to a solution of mesylate **6** (3.16 g, 3.95 mmol) in EtOH (40 mL) at room temperature. The reaction mixture was stirred at room temperature for 12 h. The reaction was then quenched with satd NH_4Cl aq at 0 °C and extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (2.72 g) was purified by column chromatography (silica gel 100 g, *n*-hexane/EtOAc = 1:1) to give alcohol **7** as a white foam (2.55 g, 89%).

Mp: 56–58 °C. $[\alpha]_D^{26}$ +43.5 (*c* 1.00, $CHCl_3$). IR (KBr): 3357, 3181, 3068, 3031, 2929, 2856, 1695, 1472, 1455, 1428, 1390, 1362, 1281 1206 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.02 (s, 9H), 1.71 (d, *J* = 1.0 Hz, 3H), 1.88–1.93 (m, 2H), 3.52 (d, *J* = 10.5 Hz, 1H), 3.72–3.86 (m, 2H), 3.91 (d, *J* = 10.5 Hz, 1H), 4.14 (d, *J* = 4.0 Hz, 1H), 4.26 (d, *J* = 8.0 Hz, 1H), 4.39 (ddd, *J* = 4.0, 5.0, 8.0 Hz, 1H), 4.45 (d, *J* = 12.0 Hz, 1H), 4.49 (d, *J* = 11.5 Hz, 1H), 4.51 (d, *J* = 11.5 Hz, 1H), 4.74 (d, *J* = 12.0 Hz, 1H), 5.96 (d, *J* = 5.0 Hz, 1H), 7.21–7.65 (m, 20H), 7.48 (d, *J* = 1.0 Hz, 1H), 9.01 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.27, 19.09, 26.86, 35.16, 59.59, 72.65, 73.18, 73.70, 74.66, 83.80, 84.65, 85.07, 109.27, 127.66, 127.83, 127.93, 128.33, 128.39, 128.70, 129.69, 129.72, 133.44, 135.50, 136.62, 137.40, 137.55, 150.67, 163.99. MS (FAB): *m/z* 721 (MH^+). HRMS (FAB): calcd for $C_{42}H_{49}N_2O_7Si$ (MH^+) 721.3309, found 721.3300.

4.8. 3′,5′-Di-*O*-benzyl-4′-(2-*tert*-butyldiphenylsiloxyethyl)-5-methyl-2′-*O*-(trifluoromethanesulfonyl)arabinouridine (8)

Under nitrogen atmosphere, pyridine (1.9 mL, 17 mmol) and Tf_2O (1.4 mL, 8.6 mmol) were added to a solution of alcohol **7** (2.07 g, 2.87 mmol) in CH_2Cl_2 (30 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 0.5 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with CH_2Cl_2. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (2.56 g) was purified by column chromatography (silica gel 100 g, *n*-hexane/EtOAc = 2:1) to give triflate **8** as a white foam (2.03 g, 83%).

Mp: 37–40 °C. $[\alpha]_D^{25}$ +37.1 (*c* 1.00, $CHCl_3$). IR (KBr): 3190, 3069, 2930, 2858, 1695, 1455, 1426, 1245, 1215, 1143 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.03 (s, 9H), 1.67 (s, 3H), 1.81–1.95 (m, 2H), 3.36 (d, *J* = 10.5 Hz, 1H), 3.69 (d, *J* = 10.5 Hz, 1H), 3.72–3.86 (m, 2H), 4.38 (d, *J* = 12.0 Hz, 1H), 4.43 (d, *J* = 12.0 Hz, 1H), 4.48 (d, *J* = 11.5 Hz, 1H), 4.57 (d, *J* = 4.5 Hz, 1H), 4.72 (d, *J* = 11.5 Hz, 1H), 5.35 (dd, *J* = 4.5, 5.5 Hz, 1H), 6.19 (d, *J* = 5.5 Hz, 1H), 7.19–7.63 (m, 21H), 8.10 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.21, 19.00, 26.77, 34.04, 59.04, 71.23, 73.32, 73.47, 80.48, 81.51, 84.37, 87.53, 110.98, 118.24 (q, *J* = 318 Hz), 127.64, 127.67, 127.69, 127.98, 128.01, 128.33, 128.45, 128.53, 129.72, 129.79, 133.26, 133.28, 135.47, 135.49, 136.41, 137.12, 150.13, 163.48. MS (FAB): *m/z* 853 (MH^+). HRMS (FAB): calcd for $C_{43}H_{48}N_2O_9F_3SiS$ (MH^+) 853.2802, found 853.2813.

4.9. (2′*R*)-2′-Azido-3′,5′-di-*O*-benzyl-4′-(2-*tert*-butyldiphenylsiloxyethyl)thymidine (9)

Under nitrogen atmosphere, NaN_3 (464 mg, 7.14 mmol) was added to a solution of triflate **8** (2.03 g, 2.38 mmol) in DMF (20 mL) at room temperature. The reaction mixture was stirred at room temperature for 13 h. The resulting mixture was added to water and extracted with Et_2O. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (2.20 g) was purified by column chromatography (silica gel 150 g, *n*-hexane/EtOAc = 2:1) to give azide **9** as a white foam (1.80 g, quant.).

Mp: 46–49 °C. $[\alpha]_D^{25}$ −5.1 (*c* 1.00, $CHCl_3$). IR (KBr): 3179, 3068, 2929, 2857, 2109, 1694, 1470, 1428, 1362, 1268 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.03 (s, 9H), 1.58 (d, *J* = 1.0 Hz, 3H), 1.78 (ddd, *J* = 6.0, 8.0, 14.5 Hz, 1H), 2.15 (ddd, *J* = 5.0, 5.5, 14.5 Hz, 1H), 3.44 (d, *J* = 11.0 Hz, 1H), 3.70–3.85 (m, 2H), 3.91 (d, *J* = 11.0 Hz, 1H), 3.97 (t, *J* = 6.0 Hz, 1H), 4.28 (d, *J* = 6.0 Hz, 1H), 4.43 (d, *J* = 11.5 Hz, 1H), 4.49 (d, *J* = 11.5 Hz, 1H), 4.49 (d, *J* = 11.5 Hz, 1H), 4.79 (d, *J* = 11.5 Hz, 1H), 5.99 (d, *J* = 6.0 Hz, 1H), 7.20–7.64 (m, 20H), 7.49 (d, *J* = 1.0 Hz, 1H), 7.93 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.10, 19.04, 26.82, 35.22, 59.26, 65.17, 73.41, 73.47, 74.28, 79.06, 86.00, 87.57, 111.09, 127.55, 127.64, 127.66, 128.05, 128.12, 128.21, 128.51, 128.64, 129.66, 129.71, 133.43, 133.44, 135.24, 135.50, 136.90, 137.13, 150.25, 163.66. MS (FAB): *m/z* 746 (MH^+). HRMS (FAB): calcd for $C_{42}H_{48}N_5O_6Si$ (MH^+) 746.3374, found 746.3404.

4.10. (2′*R*)-2′-Azido-3′,5′-di-*O*-benzyl-4′-(2-hydroxyethyl) thymidine (10)

Under nitrogen atmosphere, TBAF (1.0 M in THF, 2.6 mL, 2.6 mmol) was added to a solution of azide **9** (1.78 g, 2.38 mmol) in THF (20 mL) at room temperature. The reaction mixture was stirred at room temperature for 17 h. The resulting mixture was added to water and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (2.20 g) was purified by column chromatography (silica gel 75 g, *n*-hexane/EtOAc = 1:1) to give compound **10** as a white foam (1.22 g, quant.).

Mp: 45–50 °C. $[\alpha]_D^{25}$ +10.2 (*c* 1.00, $CHCl_3$). IR (KBr): 3441, 3182, 3063, 2927, 2109, 1693, 1496, 1469, 1455, 1364, 1267 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.56 (s, 3H), 1.66 (br s, 1H), 1.76 (dt, *J* = 6.0, 15.0 Hz, 1H), 2.21 (dt, *J* = 6.0, 15.0 Hz, 1H), 3.44 (d,

J = 10.5 Hz, 1H), 3.75 (t, J = 6.0 Hz, 2H), 3.82 (d, J = 10.5 Hz, 1H), 4.04 (t, J = 6.0 Hz, 1H), 4.30 (d, J = 6.0 Hz, 1H), 4.47 (d, J = 11.5 Hz, 1H), 4.53 (d, J = 11.5 Hz, 1H), 4.55 (d, J = 12.0 Hz, 1H), 4.83 (d, J = 12.0 Hz, 1H), 6.10 (d, J = 6.0 Hz, 1H), 7.22–7.38 (m, 10H), 7.44 (s, 1H), 8.34 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.12, 34.83, 58.28, 64.83, 73.32, 73.66, 74.50, 79.56, 86.30, 87.81, 111.36, 127.67, 128.11, 128.27, 128.37, 128.61, 128.72, 135.19, 136.72, 136.96, 150.22, 163.39. MS (FAB): m/z 508 (MH^+). HRMS (FAB): calcd for $C_{26}H_{30}N_5O_6$ (MH^+) 508.2196, found 508.2204.

4.11. (2′*R*)-2′-Azido-3′,5′-di-*O*-benzyl-4′-(2-carboxymethyl) thymidine (11)

MS4Å (3.0 g) and PDC (10.9 g, 28.8 mmol) were added to a solution of compound **10** (1.22 g, 2.38 mmol) in DMF (20 mL) at room temperature. The reaction mixture was stirred at room temperature for 12 h. The resulting mixture was added to water and AcOH and extracted with EtOAc. The combined organic layer was washed with 0.4 M $(COOH)_2$ aq, 0.2 M $(COONH_4)_2$ aq, and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (3.20 g) was purified by column chromatography (silica gel 75 g, *n*-hexane/EtOAc/AcOH = 50:50:1) to give carboxylic acid **11** as a white foam (1.18 g, 94%).

Mp: 78–81 °C. $[\alpha]_D^{25}$ −29.4 (c 1.00, $CHCl_3$). IR (KBr): 3510, 3179, 3033, 2929, 2108, 1705, 1470, 1455, 1269, 1213 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.56 (s, 3H), 2.71 (d, J = 16.5 Hz, 1H), 2.88 (d, J = 16.5 Hz, 1H), 3.77 (d, J = 10.0 Hz, 1H), 3.94 (d, J = 10.0 Hz, 1H), 3.99 (dd, J = 5.5, 7.0 Hz, 2H), 4.37 (d, J = 5.5 Hz, 1H), 4.49 (d, J = 11.0 Hz, 1H), 4.53 (d, J = 10.5 Hz, 1H), 4.61 (d, J = 10.5 Hz, 1H), 4.88 (d, J = 11.0 Hz, 1H), 6.22 (d, J = 7.0 Hz, 1H), 7.20–7.48 (m, 11H), 9.65 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.14, 38.05, 64.99, 73.80, 73.99, 75.30, 80.26, 85.30, 86.36, 111.74, 127.81, 128.06, 128.19, 128.46, 128.52, 128.89, 135.59, 136.81, 137.02, 150.67, 164.38, 175.09. MS (FAB): m/z 522 (MH^+). HRMS (FAB): calcd for $C_{26}H_{28}N_5O_7$ (MH^+) 522.1989, found 522.1979.

4.12. (2′*R*)-2′-Amino-3′,5′-di-*O*-benzyl-4′-(2-carboxymethyl) thymidine (12) and (2′*R*)-3′,5′-Di-*O*-benzyl-2′,4′-(2-oxo-iminoethano)thymidine (13)

Reduction by $NaBH_4$: Under nitrogen atmosphere, $NaBH_4$ (56.2 mg, 1.49 mmol) was added to a solution of carboxylic acid **11** (155 mg, 0.297 mmol) in *i*-PrOH (3.0 mL) at 0 °C. The reaction mixture was refluxed for 1 h. The reaction was then quenched with acetone and the resulting mixture was concentrated. The obtained crude residue (220 mg) was purified by column chromatography (silica gel 5.0 g, $CHCl_3$/MeOH = 20:1) to give compound **12** as a white foam (81.3 mg, 55%).

Staudinger reaction: Under nitrogen atmosphere, Me_3P (1.0 M in toluene, 0.55 mL, 0.55 mmol) was added to a solution of carboxylic acid **11** (241 mg, 0.462 mmol) in THF/H_2O (5:1, 6.0 mL) at room temperature. The reaction mixture was stirred at room temperature for 24 h. The resulting mixture was concentrated. The obtained crude residue (293 mg) was purified by column chromatography (silica gel 10 g, $CHCl_3$/MeOH = 30:1) to give lactam **13** as a white foam (106 mg, 43%) together with compound **12** (81 mg, ca. 35%) including a small amount of impurity.

Condensation using EDC: Under nitrogen atmosphere, EDC (157 mg, 0.820 mmol) and DMAP (10.0 mg, 0.0820 mmol) were added to a solution of amino acid **12** (81.3 mg, 0.164 mmol) in CH_2Cl_2 (3.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 24 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with CH_2Cl_2. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (65.4 mg) was purified by column chromatography (silica gel 3.0 g, $CHCl_3$/MeOH = 20:1) to give lactam **13** as a white foam (56.6 mg, 72%).

Condensation using MsCl: Under nitrogen atmosphere, Et_3N (0.32 mL, 2.25 mmol) and MsCl (70 μL, 0.0820 mmol) were added to a solution of amino acid **12** (223 mg, 0.450 mmol) in CH_2Cl_2 (5.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 17 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with CH_2Cl_2. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (300 mg) was purified by column chromatography (silica gel 10 g, $CHCl_3$/MeOH = 20:1) to give lactam **13** as a white foam (185 mg, 86%).

Compound **12**: Mp: 45–48 °C. $[\alpha]_D^{26}$ −12.2 (c 1.00, $CHCl_3$). IR (KBr): 3426, 3197, 3033, 2925, 2878, 1697, 1497, 1473, 1455, 1390, 1274, 1213 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD): δ 1.65 (s, 3H), 2.84 (s, 2H), 3.84 (d, J = 9.5 Hz, 1H), 3.92 (d, J = 9.5 Hz, 1H), 4.19–4.20 (m, 1H), 4.61–4.85 (m, 5H), 6.22 (d, J = 8.0 Hz, 1H), 7.25–7.42 (m, 10H), 7.55 (s, 1H), 7.90 (br s, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ 12.48, 38.19, 57.92, 74.79, 75.07, 76.89, 81.36, 86.89, 89.23, 112.37, 128.66, 128.79, 128.97, 129.10, 129.19, 129.72, 136.82, 138.01, 138.86, 152.64, 165.92, 173.68. MS (FAB): m/z 496 (MH^+). HRMS (FAB): calcd for $C_{26}H_{30}N_3O_7$ (MH^+) 496.2084, found 496.2081.

Compound **13**: Mp: 112–114 °C. $[\alpha]_D^{21}$ +78.9 (c 1.00, $CHCl_3$). IR (KBr): 3500, 3169, 3063, 2926, 1685, 1454, 1367, 1273, 1211 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.37 (d, J = 1.0 Hz, 3H), 2.40 (d, J = 18.0 Hz, 1H), 2.58 (d, J = 18.0 Hz, 1H), 3.60 (d, J = 11.0 Hz, 1H), 3.72 (d, J = 11.0 Hz, 1H), 4.23 (dd, J = 4.0, 6.0 Hz, 2H), 4.32 (d, J = 4.0 Hz, 1H), 4.54 (d, J = 11.5 Hz, 1H), 4.56 (d, J = 11.5 Hz, 1H), 4.60 (d, J = 11.5 Hz, 1H), 4.71 (d, J = 11.5 Hz, 1H), 5.83 (s, 1H), 7.25–7.35 (m, 11H), 7.94 (d, J = 1.0 Hz, 1H), 9.40 (br s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.96, 38.60, 55.75, 68.71, 70.57, 71.96, 73.57, 83.78, 90.10, 109.61, 127.56, 127.89, 127.97, 128.23, 128.43, 128.66, 135.54, 136.96, 137.07, 150.72, 164.25, 169.56. MS (FAB): m/z 478 (MH^+). HRMS (FAB): calcd for $C_{26}H_{28}N_3O_6$ (MH^+) 478.1978, found 478.1983.

4.13. (2′*R*)-2′,4′-(2-Oxo-iminoethano)thymidine (15)

Lactam **13** (50.0 mg, 0.105 mmol) in MeOH (2.0 mL) was added to a suspension of 20% $Pd(OH)_2$ on carbon (50.0 mg, 0.462 mmol) in MeOH (1.0 mL) at room temperature under nitrogen atmosphere. The reaction mixture was stirred at room temperature for 24 h under hydrogen atmosphere. The resulting mixture was filtered and concentrated. The obtained crude residue (25.4 mg) was purified by recrystallization from MeOH to give nucleoside **15** as a white solid (19.1 mg, 61%).

Mp: 187–188 °C. $[\alpha]_D^{23}$ +36.5 (c 0.200, CH_3OH); IR (KBr): 3449, 3318, 3175, 3060, 2926, 2819, 1707, 1651, 1469, 1386, 1272, 1215 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ 1.85 (s, 3H), 2.29 (d, J = 18.0 Hz, 1H), 2.46 (d, J = 18.0 Hz, 1H), 3.68 (d, J = 12.5 Hz, 1H), 3.75 (d, J = 12.5 Hz, 1H), 3.82 (d, J = 4.0 Hz, 1H), 4.33 (d, J = 4.0 Hz, 1H), 5.68 (s, 1H), 8.35 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ 12.55, 38.80, 60.02, 61.82, 64.42, 85.99, 91.01, 110.08, 137.78, 152.07, 166.63, 173.32. MS (FAB): m/z 298 (MH^+). HRMS (FAB): calcd for $C_{26}H_{28}N_3O_6$ (MH^+) 298.1039, found 298.1044.

4.14. (2′*R*)-5′-*O*-(4,4′-Dimethoxytrityl)-2′,4′-(2-oxo-iminoethano)thymidine (16)

Under nitrogen atmosphere, DMTrCl (95.7 mg, 0.283 mmol) was added to a solution of nucleoside **15** (28.0 mg, 0.0941 mmol) in pyridine (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (159 mg) was purified by column chromatography (silica gel 5.0 g, $CHCl_3$/MeOH = 20:1) to give compound **16** as a white foam (45.9 mg, 81%).

Mp: 204–206 °C. $[\alpha]_D^{23}$ +80.7 (*c* 1.00, $CHCl_3$). IR (KBr): 3563, 3341, 3062, 2933, 2838, 1705, 1657, 1608, 1509, 1466, 1384, 1254 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ 1.20 (d, J = 1.0 Hz, 3H), 2.37 (d, J = 18.0 Hz, 1H), 2.44 (d, J = 18.0 Hz, 1H), 3.33 (d, J = 11.0 Hz, 1H), 3.37 (d, J = 11.0 Hz, 1H), 3.74 (s, 6H), 3.94 (d, J = 4.0 Hz, 1H), 4.56 (d, J = 4.0 Hz, 1H), 5.66 (s, 1H), 6.83–7.47 (m, 13H), 7.86 (d, J = 1.0 Hz, 1H). ^{13}C NMR (75 MHz, CD_3OD): δ 12.28, 39.32, 55.72, 59.82, 64.37, 65.71, 85.55, 88.06, 91.70, 110.67, 114.28, 128.19, 129.03, 129.44, 131.45, 136.38, 136.59, 136.87, 145.75, 152.01, 160.32, 160.35, 166.52, 173.00. MS (FAB): *m/z* 600 (MH^+). HRMS (FAB): calcd for $C_{26}H_{28}N_3O_6$ (MH^+) 600.2346, found 600.2347.

4.15. (2′*R*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′,4′-(2-oxo-iminoethano)thymidine (14)

Under nitrogen atmosphere, 1*H*-tetrazole (10.1 mg, 0.144 mmol) and $(i\text{-}Pr_2N)_2POCH_2CH_2CN$ (46 μL, 0.14 mmol) were added to a solution of compound **16** (72.0 mg, 0.120 mmol) in THF/MeCN (3:1, 2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 12 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with CH_2Cl_2. The combined organic layer was washed with satd $NaHCO_3$ aq, water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (99.0 mg) was purified by column chromatography (silica gel 3.0 g, $CHCl_3$/MeOH = 20:1) to give **14** as a white foam (70.2 mg, 73%).

Mp: 127–129 °C. ^1H NMR (400 MHz, $CDCl_3$): δ 0.99 (d, J = 7.0 Hz, 1.8H), 1.09 (d, J = 7.0 Hz, 4.2 H), 1.10 (d, J = 7.0 Hz, 4.2H), 1.16 (d, J = 7.0 Hz, 1.8H), 1.23 (s, 0.9H), 1.26 (s, 2.1H), 2.36–2.64 (m, 4H), 3.24–3.81 (m, 12H), 4.18 (dd, J = 4.0, 6.0 Hz, 0.3H), 4.32 (dd, J = 4.0, 5.5 Hz, 0.7H), 4.62 (dd, J = 4.0, 7.0 Hz, 0.3H), 4.76 (dd, J = 4.0, 7.0 Hz, 0.7H), 5.74 (s, 0.7H), 5.76 (s, 0.3H), 6.28 (d, J = 5.5 Hz, 0.7H), 6.33 (d, J = 6.0 Hz, 0.3H), 6.82–7.47 (m, 13H), 7.83 (s, 1H), 8.27 (br s, 1H). ^{31}P NMR (160 MHz, $CDCl_3$): δ 148.70, 150.53. MS (FAB): *m/z* 800 (MH^+), HRMS (FAB): calcd for $C_{42}H_{51}N_5O_9P$ (MH^+) 800.3424, found 800.3406.

4.16. (2′*R*)-3-Benzyloxymethyl-3′,5′-di-*O*-benzyl-2′,4′-(2-oxo-iminoethano)thymidine (18)

Under nitrogen atmosphere, DBU (0.12 mL, 0.79 mmol) and BOMCl (55 μL, 0.39 mmol) were added to a solution of lactam **13** (94.0 mg, 0.197 mmol) in DMF (2.0 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 0.5 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (112 mg) was purified by column chromatography (silica gel 5.0 g, *n*-hexane/EtOAc = 1:1) to give compound **18** as a white foam (101 mg, 86%).

Mp: 66–68 °C. $[\alpha]_D^{28}$ +79.7 (*c* 1.00, $CHCl_3$). IR (KBr): 3070, 3032, 2924, 2819, 1703, 1661, 1496, 1454, 1364, 1274, 1212 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.43 (d, J = 1.0 Hz, 3H), 2.45 (d, J = 17.5 Hz, 1H), 2.60 (d, J = 17.5 Hz, 1H), 3.59 (d, J = 10.5 Hz, 1H), 3.76 (d, J = 10.5 Hz, 1H), 3.96 (dd, J = 4.0, 5.5 Hz, 1H), 4.19 (d, J = 4.0 Hz, 1H), 4.51 (d, J = 11.0 Hz, 1H), 4.55 (d, J = 11.0 Hz, 1H), 4.60 (d, J = 11.0 Hz, 1H), 4.63 (d, J = 11.0 Hz, 1H), 4.69 (s, 2H), 5.43 (d, J = 9.5 Hz, 1H), 5.46 (d, J = 9.5 Hz, 1H), 5.74 (s, 1H), 6.15 (d, J = 5.5 Hz, 1H), 7.25–7.38 (m, 15H), 7.92 (d, J = 1.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.53, 38.68, 56.09, 68.73, 70.27, 70.57, 72.27, 72.37, 73.69, 83.76, 90.53, 109.48, 127.55, 127.64, 127.71, 127.91, 128.30, 128.34, 128.58, 128.73, 133.97, 136.59, 136.88, 137.82, 150.79, 163.29, 169.26. MS (FAB): *m/z* 598 (MH^+). HRMS (FAB): calcd for $C_{34}H_{36}N_3O_7$ (MH^+) 598.2553, found 598.2529.

4.17. (2′*R*)-3-Benzyloxymethyl-3′,5′-di-*O*-benzyl-2′,4′-(*N*-methyl-2-oxo-iminoethano)thymidine (19)

Under nitrogen atmosphere, 60% NaH (in mineral oil, 10.4 mg, 0.260 mmol) was added to a solution of compound **18** (130 mg, 0.218 mmol) in DMF (2.0 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 0.5 h. MeI (68 μL, 1.1 mmol) was added to the resulting mixture at 0 °C. The reaction mixture was stirred at 0 °C for 2 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with Et_2O. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (139 mg) was purified by column chromatography (silica gel 5.0 g, *n*-hexane/EtOAc = 3:2) to give *N*-Me lactam **19** as a white foam (79.6 mg, 60%).

Mp: 48–50 °C. $[\alpha]_D^{28}$ +67.8 (*c* 0.760, $CHCl_3$). IR (KBr): 3065, 3031, 2925, 2819, 1707, 1651, 1496, 1453, 1365, 1279, 1212 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.45 (d, J = 1.0 Hz, 3H), 2.42 (d, J = 17.0 Hz, 1H), 2.60 (d, J = 17.0 Hz, 1H), 3.07 (s, 3H), 3.68 (d, J = 12.0 Hz, 1H), 3.74 (d, J = 12.0 Hz, 1H), 3.82 (d, J = 4.0 Hz, 1H), 4.16 (d, J = 4.0 Hz, 1H), 4.54–4.61 (m, 4H), 4.70 (s, 2H), 5.43 (d, J = 9.5 Hz, 1H), 5.47 (d, J = 9.5 Hz, 1H), 5.66 (s, 1H), 7.23–7.39 (m, 15H), 7.91 (d, J = 1.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.55, 34.17, 38.77, 63.11, 68.51, 70.20, 71.19, 72.27, 72.72, 73.64, 84.24, 88.87, 109.38, 127.52, 127.66, 127.70, 127.89, 128.27, 128.30, 128.32, 128.62, 128.69, 133.93, 136.69, 136.92, 137.81,150.68, 163.35, 167.29. MS (FAB): *m/z* 612 (MH^+). HRMS (FAB): calcd for $C_{35}H_{38}N_3O_7$ (MH^+) 612.2710, found 612.2712.

4.18. (2′*R*)-2′,4′-(*N*-Methyl-2-oxo-iminoethano)thymidine (20)

Under nitrogen atmosphere, *N*-Me lactam **19** (79.6 mg, 0.130 mmol) in EtOH (8.0 mL) and cyclohexene (1.3 mL, 13 mmol) were added to a suspension of 20% $Pd(OH)_2$ on carbon (91.0 mg) in EtOH (2.0 mL) at room temperature. The reaction mixture was refluxed for 2 h. The resulting mixture was filtered and concentrated. The obtained crude residue was purified by column chromatography (silica gel 2.0 g, $CHCl_3$/MeOH = 10:1) to give nucleoside **20** as a white powder (40.8 mg, quant.).

Mp: >300 °C. $[\alpha]_D^{28}$ +85.0 (*c* 1.40, CH_3OH). IR (KBr): 3430, 3301, 3175, 2925, 1705, 1473, 1386, 1273, 1215 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 1.77 (d, J = 1.0 Hz, 3H), 2.21 (d, J = 18.0 Hz, 1H), 2.39 (d, J = 18.0 Hz, 1H), 3.02 (s, 3H), 3.57 (d, J = 12.5 Hz, 1H), 3.64 (d, J = 12.5 Hz, 1H), 3.80 (d, J = 4.0 Hz, 1H), 4.23 (d, J = 4.0 Hz, 1H), 5.60 (s, 1H), 8.18 (d, J = 1.0 Hz, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ 12.55, 34.92, 39.01, 61.60, 64.86, 67.68, 86.54, 89.33, 110.19, 137.47, 152.07, 166.65, 170.81. MS (FAB): *m/z* 312 (MH^+). HRMS (FAB): calcd for $C_{13}H_{18}N_3O_6$ (MH^+) 312.1196, found 312.1207.

4.19. (2′*R*)-5′-*O*-(4,4′-Dimethoxytrityl)-2′,4′-(*N*-methyl-2-oxo-iminoethano)thymidine (21)

Under nitrogen atmosphere, DMTrCl (175 mg, 0.567 mmol) was added to a solution of nucleoside **20** (26.8 mg, 0.0861 mmol) in pyridine (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 17 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with CH_2Cl_2. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (320 mg) was purified by column chromatography (silica gel 5.0 g, $CHCl_3$/MeOH = 25:1) to give compound **21** as a white foam (36.6 mg, 69%).

Mp: 155–157 °C. $[\alpha]_D^{26}$ +6.1 (*c* 2.10, $CHCl_3$). IR (KBr): 3350, 3198, 3087, 3006, 2933, 2836, 1694, 1651, 1508, 1464, 1392, 1350, 1254 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 1.30 (s, 3H), 2.44 (d, J = 17.5 Hz, 1H), 2.54 (d, J = 17.5 Hz, 1H), 3.09 (s, 3H), 3.33 (d, J = 11.0 Hz, 1H), 3.38 (d, J = 11.0 Hz, 1H), 3.75 (s, 3H), 3.76 (s, 3H),

3.94 (d, J = 4.0 Hz, 1H), 4.28 (br s, 1H), 4.49 (br s, 1H), 5.61 (s, 1H), 6.80–7.42 (m, 13H), 7.75 (s, 1H), 7.90 (br s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.91, 34.51, 38.54, 55.20, 62.87, 65.43, 65.98, 84.96, 86.88, 88.60, 110.39, 111.34, 127.20, 128.07, 130.10, 134.92, 135.08, 144.05, 152.35, 158.69, 164.37, 168.32. MS (FAB): m/z 614 (MH^+). HRMS (FAB): calcd for $C_{34}H_{36}N_3O_8$ (MH^+) 614.2502, found 614.2496.

4.20. (2′*R*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′,4′-(*N*-methyl-2-oxo-iminoethano)thymidine (17)

Under nitrogen atmosphere, DIPEA (10.1 mg, 0.144 mmol) and i-$Pr_2NP(Cl)OCH_2CH_2CN$ (46 μL, 0.14 mmol) were added to a solution of compound **21** (72.0 mg, 0.120 mmol) in CH_2Cl_2 (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 2 h. The reaction was then quenched with satd $NaHCO_3$ aq at 0 °C and extracted with CH_2Cl_2. The combined organic layer was washed with satd $NaHCO_3$ aq, water, and brine, dried over Na_2SO_4, and concentrated. The obtained crude residue (99.0 mg) was purified by column chromatography (silica gel 3.0 g, $CHCl_3$/MeOH = 20:1) to give **17** as a white foam (70.2 mg, 73%).

Mp: 109–111 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.99 (d, J = 7.0 Hz, 3.0H), 1.07 (d, J = 7.0 Hz, 3.0H), 1.11 (d, J = 7.0 Hz, 3.0H), 1.16 (d, J = 7.0 Hz, 3.0H), 1.26 (s, 1.5H), 1.29 (s, 1.5H), 2.36–2.64 (m, 4H), 3.17 (s, 3H), 3.22–3.88 (m, 12H), 4.10 (d, J = 4.5 Hz, 0.5H), 4.27 (d, J = 4.5 Hz, 0.5H), 4.60 (dd, J = 4.5, 7.0 Hz, 0.5H), 4.75 (dd, J = 4.0, 6.5 Hz, 0.5H), 5.67 (s, 0.5H), 5.69 (s, 0.5H), 6.82–7.47 (m, 13H), 7.83 (s, 1H), 8.27 (br s, 1H). ^{31}P NMR (400 MHz, $CDCl_3$): δ 148.86, 149.97. MS (FAB): m/z 814 (MH^+). HRMS (FAB): calcd for $C_{43}H_{53}N_5O_9P$ (MH^+) 814.3581, found 814.3599.

4.21. Oligonucleotide synthesis

Phosphoramidites **14** and **17** were used and the 0.2 μmol scale synthesis of oligonucleotides was performed on an automated DNA synthesizer (Gene Design nS-8) using a standard phosphoramidite protocol (DMTr-ON mode). Oligonucleotides **22–29** were prepared by cleavage from the CPG supports and deprotection of the phosphate moieties (28% NH_4 aq, rt, 1.5 h or 50 mM K_2CO_3 in MeOH, rt, 2 h). Removal of ammonia was carried out in vacuo. In the case of K_2CO_3 treatment, after neutralization with 1% HCl aq, the solvent was concentrated in vacuo. The crude **22–29** were purified with Sep-Pak® Plus C18 cartridges (Waters), followed by reversed-phase HPLC (Waters XBridge® MS C_{18} 2.5 μm, 10 × 50 mm). The compositions of **22–29** were confirmed by MALDI-TOF mass analysis. MALDI-TOF mass analysis. MALDI-TOF MS data ($[M-H]^-$) for **22–29**: **22**, found 3033.70 (calcd 3034.00); **23**, found 3090.36 (calcd 3089.03); **24**, found 3145.24 (calcd 3144.07); **25**, found 3048.53 (calcd 3048.02); **26**, found 3117.32 (calcd 3117.08); **27**, found 3187.85 (calcd 3186.25); **28**, found 3033.23 (calcd 3034.00); **29**, found 3048.78 (calcd 3048.02).

4.22. UV-melting experiments

UV-melting experiments were carried out using SHIMADZU UV-1650 and SHIMADZU UV-1800 spectrophotometers equipped with a T_m analysis accessory. Oligonucleotides and ssDNA or ssRNA were dissolved in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl to give a final concentration of each strand of 4 μM. The samples were annealed by heating at 100 °C followed by slow cooling to 15 °C. The melting profiles were recorded at 260 nm from 15 to 85 °C at a scan rate of 0.5 °C/min. The two-point average method was employed to obtain the T_m values and the final values were determined by averaging three independent measurements which were accurate to within 1 °C.

4.23. Enzymatic degradation experiments

Enzymatic degradation experiments were carried out under conditions of 1.75 μg/mL Crotalus admanteus venom phosphodiesterase (CAVP), 10 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.0) and 7.5 μM each oligonucleotide at 37 °C. The amount of intact oligonucleotides was determined by reversed-phase HPLC (Waters XBridge® MS C_{18} 2.5 μm, 10 × 50 mm).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.04.049.

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Nucleic Acid Modifications

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Selenomethylene Locked Nucleic Acid Enables Reversible Hybridization in Response to Redox Changes**

*Kunihiko Morihiro, Tetsuya Kodama, Kentefu, Yoshihiro Moai, Rakesh N. Veedu, and Satoshi Obika**

DNA and RNA play important roles not only for the storage and flow of genetic information but also for the modulation of gene expression within an organism. For example, non-coding RNAs regulate many biological processes such as cell proliferation, cell death, and cell development.[1–3] It has also been suggested that guanine-rich DNA sequences regulate a variety of gene expression through G-quadruplex structures.[4,5] Moreover, epigenetic DNA modifications, including 5-methylcytosine, have recently been reported to participate in various diseases.[6–8] These gene-regulation systems, which are said to be natural nucleic acid switches, are controlled by changes in biochemical environments and gene-expression levels within cells.

Recently, chemically modified nucleotides that can reversibly change their properties by sensing differences in the surrounding environment have attracted attention. Such artificial nucleotides show promise as nucleic acid switches regulated by biological functions, which are impossible for natural nucleic acid switches. Several external-stimulus-responsive nucleic acids have been developed by regulating hydrogen-bonding interactions between nucleobases,[9] stacking within the DNA helices,[10] or the inversion of helicity.[11] Herein, we designed and synthesized a new redox-responsive nucleotide focusing on the reversible oxidation/reduction of selenium, as well as incorporated it into an oligonucleotide (ON) and characterized its properties as a nucleic acid switch.

Locked nucleic acid (LNA)[12]/2′,4′-bridged nucleic acid (2′,4′-BNA)[13] **1** has a methylene bridge between the 2′-oxygen and 4′-carbon atoms of the ribose sugar, which locks it in the C3′-*endo* conformation (Figure 1a). ONs containing LNA show strong binding affinity against complementary DNA and RNA.[12,14] Since the initial synthesis, a number of

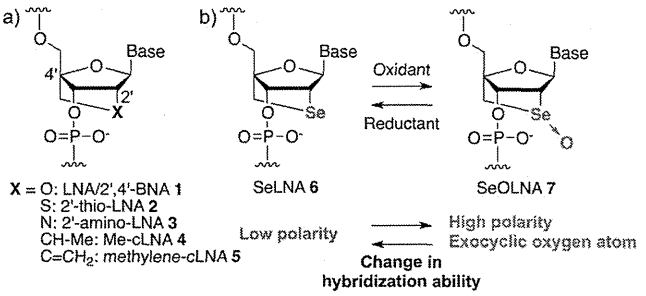

Figure 1. a) Formulas of 2′-substituted LNA analogues. b) Reversible structural change between SeLNA (**6**) and SeOLNA (**7**) by oxidant and reductant.

LNA analogues have been developed.[15] For example, 2′-thio-LNA (**2**)[16,17] and 2′-amino-LNA (**3**)[17,18] have the oxygen atom in the bridge replaced with sulfur and nitrogen atoms, respectively. These analogues show high binding affinity against complementary strands, similar to LNA, and we anticipated that the type of heteroatom at the 2′-position would have little influence on the binding properties of LNA analogues. On the other hand, it is known that the substituent on the bridged structure influences the hybridization property of LNA analogues because it interacts electrostatically and sterically with the minor groove of the duplexes. For example, Chattopadhyaya and co-workers reported that Me-cLNA (**4**), where the 2′-oxygen atom of LNA was replaced with a methyl-substituted carbon atom, shows reduced binding affinity for complementary RNA relative to LNA,[19] while Seth et al. reported that the replacement of the 2′-oxygen atom in LNA with an exocyclic methylene group does not affect the hybridization ability of modified ON [methylene-cLNA (**5**)].[20] These substituents in LNA analogues are located in the minor groove and thus can influence the duplex stability depending on their polarity and orientation.

Given this background, we designed a new LNA analogue possessing a low polaraity selenium atom at the 2′-position [SeLNA (**6**), Figure 1b]. Micura and co-workers reported the reversible oxidation/reduction of 2′-methylselenoguanosine in RNA;[21] therefore, a reversible structural change between SeLNA and its selenoxide-bridged analogue SeOLNA (**7**) should be possible. SeOLNA would have a highly polar selenoxide group in the bridge and the exocyclic oxygen atom

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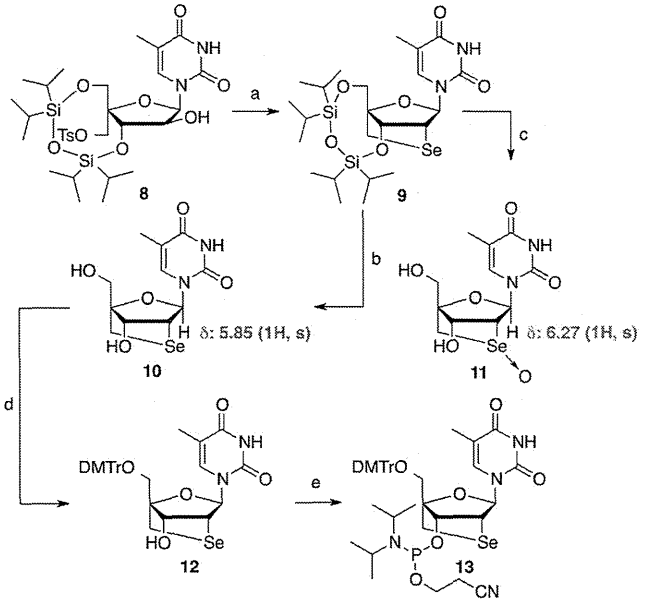
would be located in the minor groove of the duplex, hence the hybridization ability of SeOLNA is expected to be strikingly different from that of SeLNA.

The synthesis of SeLNA phosporamidite is summarized in Scheme 1. An arabino nucleoside derivative **8**[22] was converted into the triflate, which was treated immediately with sodium selenide[23] in EtOH/tetrahydrofuran (THF) to afford



Scheme 1. Synthesis of SeLNA phosphoramidite **13**: a) 1) Tf_2O, pyridine, CH_2Cl_2, 0°C to RT; 2) Se, $NaBH_4$, EtOH, THF, 60°C, 44% yield (2 steps); b) TBAF, THF, 0°C, 67% yield; c) 1) *m*CPBA, CH_2Cl_2, 0°C; 2) TBAF, THF, 0°C (2 steps); d) DMTrCl, pyridine, RT, 77% yield; e) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, DIPEA, MeCN, RT, 82% yield. Tf = trifluoromethanesulfonyl, TBAF = tetra-*n*-butylammonium fluoride, DMTr = 4,4′-dimethoxytrityl, DIPEA = *N*,*N*-diisopropylethylamine.

the desired cyclized product **9**. Desilylation with tetra-*n*-butylammonium fluoride (TBAF) was carried out to give the corresponding nucleoside **10**. Alternatively, **9** was oxidized by *meta*-chloroperoxybenzoic acid (*m*CPBA) and converted into SeOLNA nucleoside **11** as a single diastereomer. The C3′-*endo* sugar conformation of **10** and **11** was confirmed by ^{1}H NMR spectroscopy where the H1′ signal was observed as a singlet.[24] We also examined the stereochemistry of the selenoxide group based on the change in the chemical shifts ($\Delta\delta$ values) induced by oxidation. Hartree–Fock (HF) and density functional theory (DFT) calculations of the (R_{Se})-isomer **S1** and (S_{Se})-isomer **S2** (see Supporting Information for formulas of **S1** and **S2**) clearly indicated that the experimental $\Delta\delta$ values of H1′, H6′α, and H6′β induced by oxidation were in very good agreement with the calculated ones for the (R_{Se})-isomer; therefore, we confirmed the stereochemistry of the selenium center of selenoxide **11** as the (R_{Se})-configuration (Supporting Information, Table S1). Tritylation at the primary hydroxy group of **10** with DMTrCl and phosphitylation at the secondary hydroxy group yielded phosphoramidite **13**. The amidite **13** was incorporated into ONs using conventional solid-phase phosphoramidite synthesis. The ON sequences used in this study are shown in Figure 2.

There have been reports of the oxidation of 2′-methylseleno RNA by iodine treatment during solid-phase synthesis;[21,25–28] however, we did not observe such oxidation of

```
14     5'-d(GCGTTTTTTGCT)-3'
15     5'-r(GCGUUUUUUGCU)-3'
16a-c  5'-d(GCGTTTTTTGCT)-3'
17a-c  5'-d(GCGTTTTTTGCT)-3'
18a-c  5'-d(GCGTTTTTTGCT)-3'
19a-c  5'-d(GCGTTTTTTGCT)-3'
20     5'-d(AGCAAAAAACGC)-3'
21     5'-r(AGCAAAAAACGC)-3'
MBa-b  5'-FAM-d(TTTTTTCTTTTGTCCCTTCTT-
                AAAAAA)-BHQ1-3'
```

Figure 2. ON sequences used in this study. Underlined bold characters indicate modified residues. Series **a** = SeLNA, **b** = SeOLNA, and **c** = LNA modifications in the ONs.

SeLNA-containing ONs. Moreover, SeLNA was found to be stable in air (Figure S3). This may be because SeLNA is stable against oxidants or that deoxygenation of SeOLNA occurs during DNA synthesis. Therefore, we investigated the redox properties of SeLNA in ONs. Initially, ON **16a** having one SeLNA unit was treated with hydrogen peroxide, and the resulting product was analyzed by reverse-phase (RP) HPLC at several reaction times (Figure 3a). The signal corresponding to ON **16a** completely disappeared within 12 hours and ON **16b** with SeOLNA was generated. Further oxidation to the selenone-bridged analogue was not observed in the MALDI-TOF mass spectra. We then reduced SeOLNA back to SeLNA using dithiothreitol (DTT) as a reducing agent; reduction of SeOLNA was complete within 12 hours. We also evaluated the concentration dependence of the redox reagents and confirmed that other redox systems could also

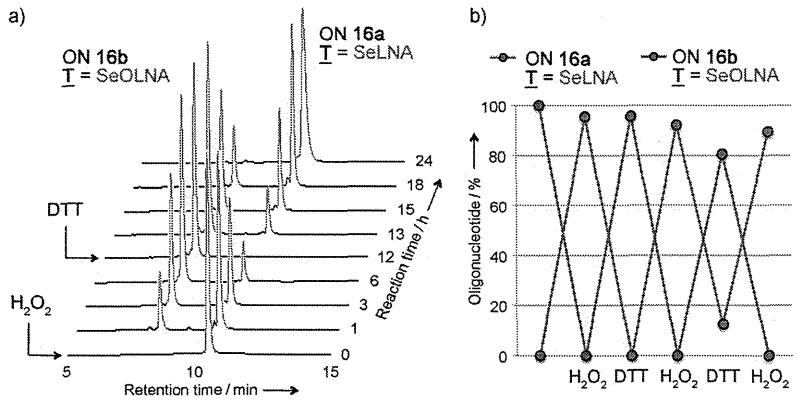


Figure 3. a) Reversible redox reaction of ON **16a** by H_2O_2/DTT observed by RP-HPLC. Conditions: ON **16a** (10 μM), sodium phosphate buffer (25 mM, pH 7.2), H_2O_2 (10 mM), DTT (10 mM), 37°C. b) Repetitive redox reaction of ON **16a**. The percentages of ON **16a** and ON **16b** were obtained from the HPLC peak areas.

change the oxidation state of SeLNA (Figure S2). Moreover, redox reactions of ON **16a** using H_2O_2 and DTT were repeated, showing that the reaction was reversible at least five times in a row (Figure 3b). Thus, SeLNA has the potential to work as a redox switch for various biomolecules. There have been few reports on nucleosides or nucleotides that can reversibly change their structures and properties in response to the surrounding redox conditions.[29]

The hybridization ability of SeLNA- and SeOLNA-modified ONs to complementary DNA and RNA was evaluated by UV melting experiments and compared with LNA-modified ONs (Table 1). SeLNA- and SeOLNA-modified ONs formed more stable duplexes with an RNA complement than with a DNA complement, similar to other LNA analogues that were previously reported. As the number of modifications increased, the difference in hybridization ability between SeLNA and SeOLNA increased. Notably, ON **19a** with six consecutive SeLNA units showed a T_m value that was over 20°C higher than that of ON **19b** with six consecutive SeOLNA units (DNA: 71°C versus 44°C; RNA: 84°C versus 64°C). To clarify the factors affecting the differences in hybridization ability between SeLNA and SeOLNA, we studied the thermodynamic parameters by concentration-dependent melting experiments (Table 2; Tables S4,S5). Thermodynamic data indicated that the introduction of an SeLNA unit to an ON caused gains in entropy and losses in enthalpy compared to the natural duplexes. Especially, the high stability of duplexes formed between ON **19a** containing six consecutive SeLNAs and complementary DNA or RNA was due to the large entropy gain that more than compensated for the loss of enthalpy. On the other hand, duplex formation of ON **19b** with the complementary strand had a heavy disadvantage in terms of entropy. Egli, Rozners, and co-workers revealed that a 2′-fluoro-modified RNA duplex was dramatically dehydrated relative to an unmodified RNA duplex owing to the low hydrogen-bonding ability of the 2′-fluorine atom with water molecules.[30] Consecutive low-polarity selenium atoms in ON **19a** could contribute to partial dehydration of the minor groove during the transition from single strands to duplexes, resulting in a decrease in entropy loss as compared to SeOLNA and LNA. Additionally, decreased Watson–Crick H-bonding strength from the less electronegative 2′-selenium atom might lead to a disadvantage in terms of enthalpy.[31]

Table 1: Melting temperatures of SeLNA-, SeOLNA-, and LNA-modified duplexes.[a]

Duplex		T_m (ΔT_m per modification) [°C][b]		
	T=	SeLNA	SeOLNA	LNA
16a–c/20		49 (−2.0)	46 (−5.0)	52 (+1.0)
17a–c/20		50 (−0.5)	43 (−4.0)	53 (+1.0)
18a–c/20		53 (+0.7)	44 (−2.3)	55 (+1.3)
19a–c/20		71 (+3.3)	44 (−1.2)	66 (+2.5)
16a–c/21		50 (+4.0)	48 (+2.0)	52 (+6.0)
17a–c/21		56 (+5.0)	50 (+2.0)	56 (+5.0)
18a–c/21		65 (+6.3)	56 (+3.3)	63 (+5.7)
19a–c/21		84 (+6.3)	64 (+3.0)	79 (+5.5)

[a] UV melting profiles were measured using a solution containing each oligonucleotide at a concentration of 4.0 μM in 100 mM NaCl and 10 mM sodium phosphate buffer at pH 7.2. [b] The T_m value given is the average of three independent measurements. ΔT_m values are calculated relative to the T_m values of unmodified DNA **14**/DNA **20** (51°C) or DNA **14**/RNA **21** (46°C) duplexes.

Table 2: Thermodynamic data for duplexes.[a]

Duplex	$\Delta H°$ [kcal mol^{-1}]	$\Delta S°$ [cal K^{-1} mol^{-1}]	$\Delta G°_{310K}$ [kcal mol^{-1}]
14/20	−84.6	−235	−11.6
19a/20	−65.2	−164	−14.5
19b/20	−96.0	−278	−9.9
19c/20	−80.3	−211	−15.0
14/21	−98.4	−282	−10.9
19a/21	−76.3	−187	−18.2
19b/21	−125.1	−346	−17.9
19c/21	−101.5	−262	−20.3

[a] These values were determined by van't Hoff plots with six data points (0.89–10.9 μM).

Interestingly, ON **19a** containing six consecutive SeLNA modifications showed much higher hybridization ability than ON **19c** containing six consecutive LNAs (DNA: 71°C versus 66°C; RNA: 84°C versus 79°C). Such excellent hybridization was also observed in ONs modified with six consecutive 2′,4′-BNA^{NC} modifications, which have a six-membered bridging structure.[22] Modeling studies suggest that the torsion angle (δ) and the maximum out-of-plane pucker (ν_{max}) of the SeLNA nucleoside was closer to that of 2′,4′-BNA^{NC} than that of LNA (Table S2). SeLNA has a five-membered bridge; however, the large selenium atom expands the size of the bridge and affects the hybridization properties.

Circular dichroism (CD) spectroscopy of SeLNA- and SeOLNA-modified duplexes were also performed to investigate their structural preferences (Figure S7). The spectrum of SeLNA-modified ON **19a** with complementary DNA **20** was very similar to that of a natural RNA/DNA (**15/20**) duplex. Moreover, minimal spectral differences were observed in the duplex formed between ON **19a** with RNA **21** compared with a natural RNA/RNA (**15/21**) duplex. These observations indicate that SeLNA formed duplexes with DNA and RNA in the same manner as natural RNA did. On the other hand, the CD spectra of SeOLNA-modified duplexes were different from that of natural DNA/DNA (**14/20**), DNA/RNA (**14/21**), RNA/DNA (**15/20**), and RNA/RNA (**15/21**) duplexes, suggesting that the duplexes modified with SeOLNA might not be typical A- and B-form duplexes, likely owing to local conformational alterations. However, it is possible that the CD spectra do not reflect the structure of SeOLNA-modified duplexes, because CD spectra may be affected by the existence of the UV-absorbing selenoxide group.

Measuring the T_m values revealed that there were great differences in hybridization ability between the SeLNA-modified ON and SeOLNA-modified ON when six consecutive modifications were introduced. This observation prompted us to use SeLNA in a nucleic acid switch to sense changes in the surrounding redox environment. We designed and synthesized a molecular-beacon-type DNA probe bearing

six consecutive SeLNA modifications in the stem region labeled with FAM at the 5′ end and BHQ1 at the 3′ end (Figure 4a, **MBa**).[32] We measured the change in the fluorescence intensity over time of **MBa** following addition of H_2O_2 (Figure 4b). The fluorescence intensity of **MBa** was gradually recovered in accordance with the reaction time, showing

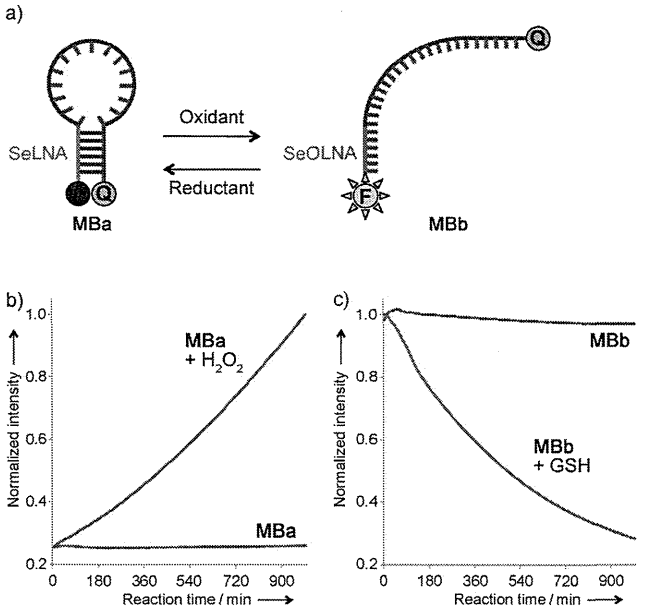


Figure 4. a) Scheme of the structure and fluorescent changes of an SeLNA-modified molecular-beacon-type probe (**MB**) in response to oxidant and reductant. b) Changes in the fluorescence intensity of **MBa** without (red) and **MBa** with (blue) H_2O_2. c) Changes in fluorescence intensity of **MBb** without (blue) and **MBa** with (red) GSH. Conditions: **MB** (0.1 μM), H_2O_2 or GSH (1000 μM), NaCl (100 mM), sodium phosphate buffer (10 mM, pH 7.2), 37°C.

dissociation of the hairpin structure. On the other hand, the addition of a reductant, glutathione (GSH), to the solution of **MBb** caused a decrease in the fluorescence intensity (Figure 4c). H_2O_2 is known as a marker for oxidative stress[33] and GSH as an important redox scavenger of reactive oxygen species.[34] Thus, **MB** could be used as a method for sensing the redox environment within a cell.

In conclusion, we synthesized a novel LNA analogue with a selenomethylene-bridged moiety, SeLNA. The selenium atom in the bridge of SeLNA could be converted into a selenoxide moity by treatment with an oxidant and reverted back to the selenide by treatment with a reductant. SeLNA-modified ONs showed high duplex-forming ability. Six consecutive SeLNA modifications imparted superior hybridizing ability compared to normal LNA modifications. This duplex-forming ability was disrupted by oxidation to SeOLNA. Finally, we demonstrated that a SeLNA-modified molecular-beacon-type probe could be used to sense changes in the surrounding redox environment. Recently, we reported the synthesis of SeLNA triphosphate and the enzymatic incorporation of an SeLNA nucleotide.[35] Further applications, such as the development of SeLNA-modified antisense agents targeting RNA overexpressed in oxidative environments and the evolution of SeLNA-modified aptamers towards the elucidation of aptamer structures by X-ray crystallography, are now in progress in our laboratory.

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