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THE ABILITY OF 1-ARYLTRIAZOLE-CONTAINING NUCLEOBASES TO RECOGNIZE A TA BASE PAIR IN TRIPLEX DNA

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Dedicated to Professor Victor Snieckus on the occasion of his 77th birthday

Abstract – Phosphoramidites bearing propargyl and (*N*-propargylcarbamoyl)methyl groups at the C1-position of deoxyribose were synthesized and introduced into oligonucleotides by using an automated DNA synthesizer. Copper-catalyzed alkyne-azide 1,3-dipolar cycloaddition of the oligonucleotides with various aryl azides led to triplex-forming oligonucleotides (TFOs) possessing the corresponding aryltriazole-containing nucleobases. The triplex-forming ability of TFOs with double-stranded DNA (dsDNA) was evaluated through UV-melting experiments, and it was demonstrated that *m*-hydroxy or *m*-ureido derivatives in the (1-aryltriazol-4-yl)methyl nucleobases likely interacted with a TA base pair in dsDNA.

INTRODUCTION

Triplex formation of double-stranded DNA (dsDNA) by an oligonucleotide (TFO: triplex-forming oligonucleotide) is applicable in various dsDNA-targeting technologies. In triplex formation, TFOs consisting of pyrimidine sequences can sequence-selectively and stably recognize dsDNA via Hoogsteen hydrogen bond formation with AT and GC base pairs in dsDNA by T and C in TFO, respectively. Since no natural nucleic acid specifically recognizes a CG or TA base pair in dsDNA, many studies have been conducted to develop artificial nucleic acids capable of recognizing these base pairs.[1-4] However, targeting of a TA base pair is difficult because access to the 4-carbonyl oxygen in T is sterically hindered

by the 5-methyl group in T. Therefore, reports on the development of artificial nucleic acids recognizing a TA base pair are scarce.[2-4]

We considered that facile and efficient syntheses of various derivatives of a nucleobase structure were necessary for implementing detailed and rational designs of nucleobases for CG or TA base pair recognition, and we have used post-elongation modification (PEM) methods, namely, modification methods after oligonucleotide synthesis, to synthesize derivatives.[2,5,6] We used a copper-catalyzed alkyne-azide cycloaddition (CuAAC) reaction[7] as a PEM method and evaluated the various derivatives synthesized; 2-(1-*m*-carbonylaminophenyl-1,2,3-triazol-4-yl)ethyl nucleobases were recently predicted to interact with the A base of a TA base pair (Figure 1a).[2] In addition, replacement of the ethylene unit by a methylenoxy unit led to a significant decrease in the binding affinity to a TA base pair. This may be due to the high flexibility of the methyleneoxy unit. Thus, based on unit length and suppression of the unit flexibility, nucleobases possessing a methylene unit would be of interest (Figure 1a). However, Guianvarc'h *et al.* reported the nucleobase S, which is the N-H group in the amide moiety thought to interact with the 4-carbonyl group in T of a TA base pair (Figure 1b).[3] Therefore, nucleobases possessing an amide unit may also be effective as a scaffold for screening nucleobases to recognize a TA base pair (Figure 1b).

Based on previous studies, we synthesized TFOs containing various derivatives of two types of nucleobases, as shown in Figure 1. The TA base pair-recognition ability of the nucleobases was examined by UV melting experiments of triplexes formed with dsDNA.

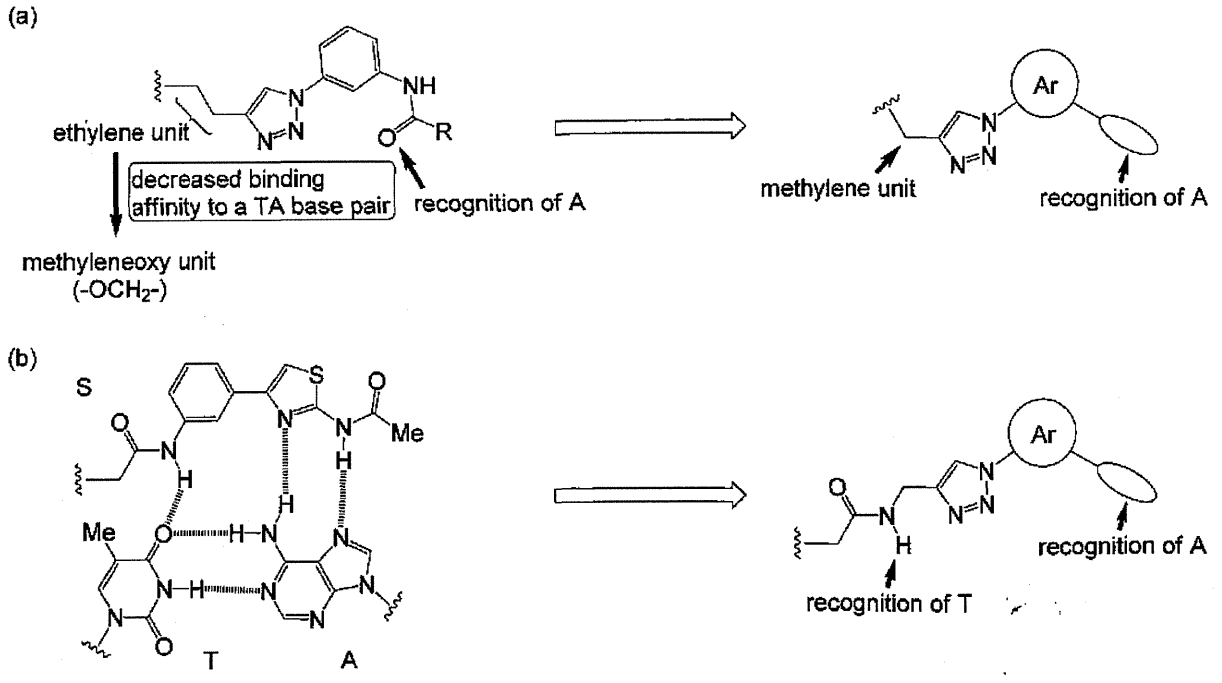
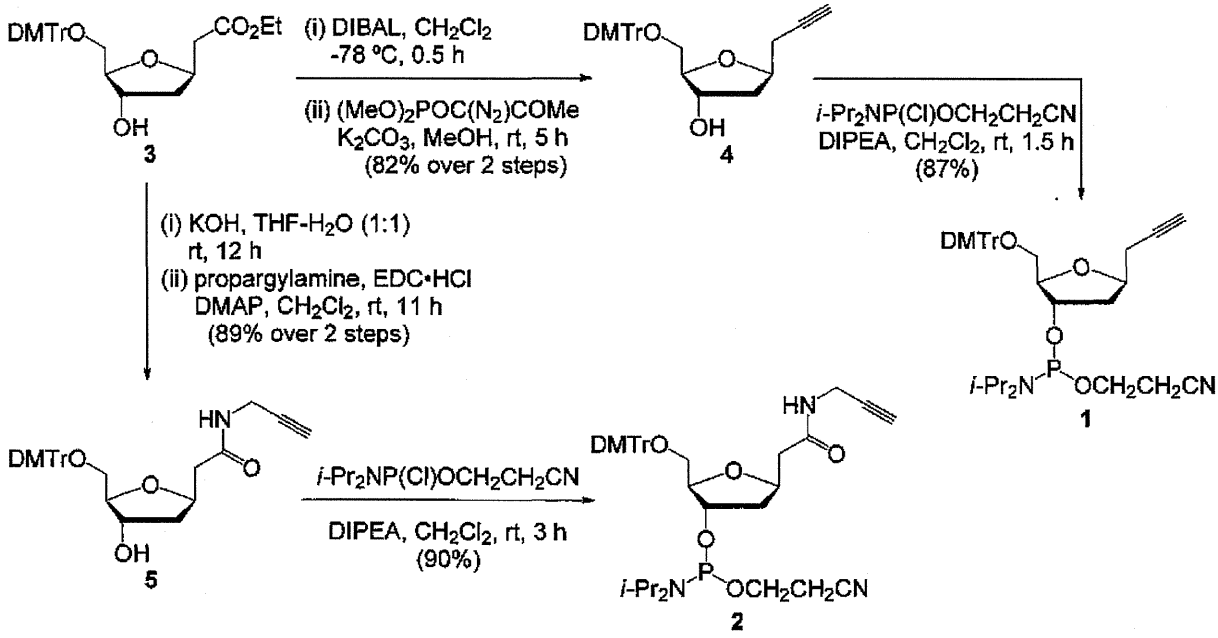


Figure 1. Nucleobases designed based on our previous results (a) and reports by another group (b)

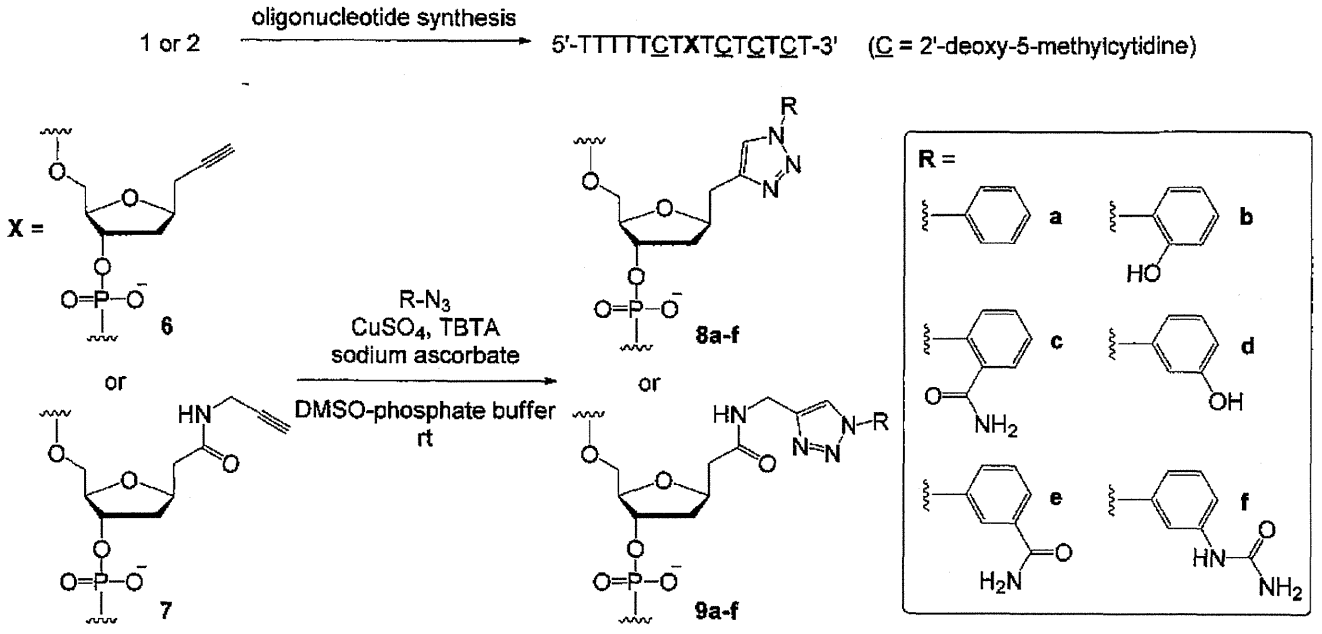
RESULTS AND DISCUSSION

As shown in Scheme 1, phosphoramidites **1** and **2** possessing ethynyl units to be converted into desired nucleobases after oligonucleotide synthesis were synthesized. Reduction of **3**[8] with DIBAL followed by treatment with Ohira-Bestmann reagent, $(MeO)_2POC(N_2)COMe$,[9] afforded **4** in 82% yield over 2 steps, which was phosphitylated to give the desired **1**. For synthesis of **2**, hydrolysis of **3** under alkaline conditions and condensation with propargylamine in the presence of EDC•HCl and DMAP furnished **5** in 89% yield over 2 steps. The desired phosphoramidite **2** was prepared in 90% yield in the same procedure as **1**.



Scheme 1. Synthesis of the desired phosphoramidites **1** and **2**

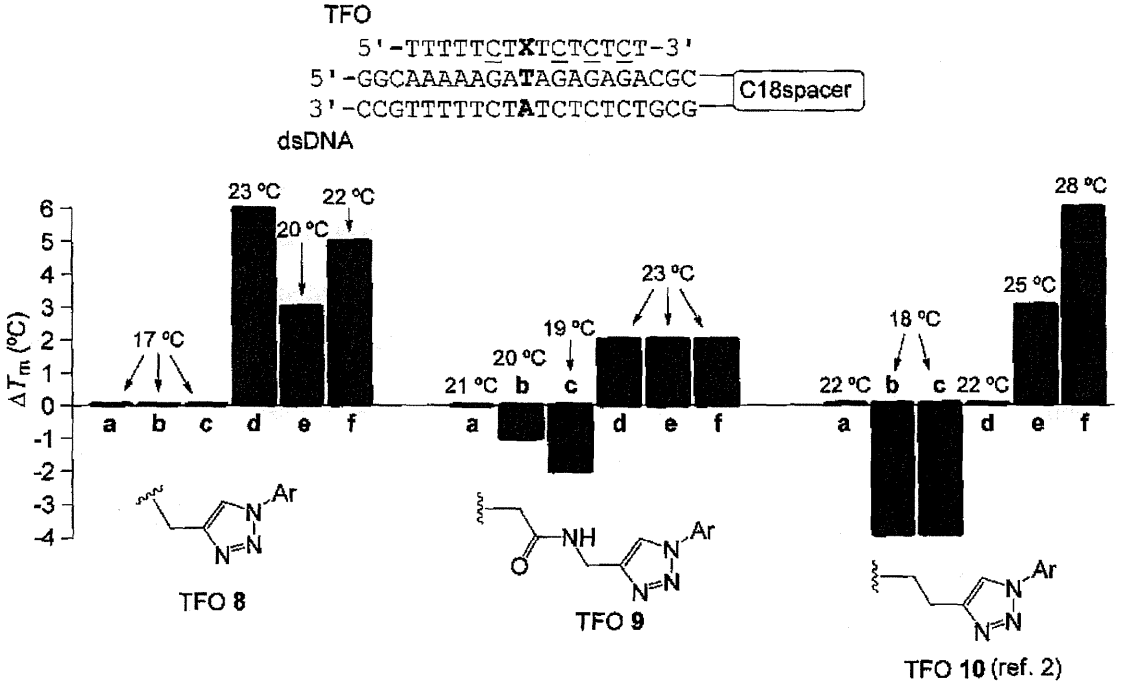
Introduction of the synthesized phosphoramidites **1** and **2** into oligonucleotides was performed using an automated DNA synthesizer under conditions of general phosphoramidite chemistry; singly-modified oligonucleotides **6** and **7** as substrates of CuAAC reactions for PEM were successfully synthesized. Through the CuAAC reactions of **6** and **7** with aryl azides[10] under optimized conditions [$CuSO_4$, sodium ascorbate, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA)[11] and azides in 30% DMSO-phosphate buffer (pH 7.0)],[12] TFOs **8a–f** and **9a–f** bearing the corresponding aryltriazole moieties were obtained, respectively. The purity and molecular weight of all oligonucleotides synthesized were confirmed by reversed-phase HPLC and MALDI-TOF-MS, respectively.



Scheme 2. Synthesis of TFOs **8** and **9** using CuAAC reactions

The ability of non-natural nucleobases to recognize a TA base pair was evaluated by UV melting experiments of triplexes formed from TFOs **8** and **9** with a dsDNA target, and the results were compared with those of TFO **10** reported in our previous study.[2] The obtained T_m values and the difference (ΔT_m) in the T_m values of TFOs possessing any substitution (**b–f**) on the benzene ring from those of unsubstituted congeners (**a**) are summarized in Figure 2. TFO **8a** possessing an unsubstituted phenyl group showed a T_m value of 17 °C, which was drastically lower than that (22 °C) of TFO **10a**. The (1-phenyltriazol-4-yl)methyl unit in TFO **8a** may be spatially incompatible with this dsDNA target in triplex formation because these nucleobases in TFOs **8a** and **10a** likely have no positive interaction with a TA base pair. Based on the results of TFOs **8a–c**, the decrease in T_m values was not observed for the *o*-substituent, unlike TFOs **10a–c**. In contrast, *m*-substituents (**8d–f**) generally increased in the binding affinity to a TA base pair, and hydroxy (**8d**) and ureido (**8f**) groups at the *m*-position led to significantly increased T_m values of +6 °C and +5 °C compared with unsubstituted **8a**, respectively. These results suggest that these analogs positively interact with the TA base pair, for example, via formation of a hydrogen bond with A of a TA base pair. In contrast to the significant stabilization by **8d**, **10d** containing the same *m*-hydroxy group did not stabilize the triplex at all, which is of interest, and further investigation involving a computational study will be required to clarify the reason for this observation. For TFO **8** bearing (1-aryltriazol-4-yl)methyl nucleobases, remarkable changes in T_m values were observed, which were thought to be caused by the lower flexibility of the methylene unit, as expected (Figure 1a). However, the T_m value of TFO **9a** possessing an unsubstituted phenyl group was higher than that of **8a**, but nearly the same as that of **10a**. This result implies that the amide N-H in **9a** does not recognize the T

of a TA base pair, unlike the S found by Sun's group[3] (Figure 1b). TFOs **9b–c** with *o*-substituents showed slightly decreased affinity to the TA base pair, while **9d–f** with *m*-substituents showed increased T_m values of +2 °C compared with that of **9a**. However, changes of T_m values were globally low, which may have been caused by the high flexibility of the four-atom length-chain, -$CH_2CONHCH_2$-.



Conditions: 10 mM sodium cacodylate buffer (pH 6.8), 100 mM KCl, 50 mM $MgCl_2$, and 1.9 μM of TFOs and dsDNA. **X** and C indicate nucleotides bearing non-natural nucleobases and 2'-deoxy-5-methylcytidine, respectively. Structures of **a-f** are shown in Scheme 2.

Figure 2. Summary of T_m and ΔT_m values obtained by UV melting experiments

In conclusion, two phosphoramidites with ethynyl units were synthesized and introduced into the oligonucleotides. Moreover, through CuAAC reactions of the oligonucleotides, the facile synthesis of TFOs bearing 1-aryltriazole-containing nucleobases was fulfilled. UV melting experiments of synthesized TFOs demonstrated that [1-(*m*-hydroxy- or 1-*m*-ureido-phenyl)triazol-4-yl]methyl nucleobases likely interacted with a TA base pair. Based on this finding and our previous results, the appropriate spacer between aryltriazole and deoxyribose moieties is likely of one- or two-atom length and less flexible. In future studies, we will structurally optimize phenyltriazole and spacer moieties to set the functional group at a suitable position to recognize the TA base pair.

EXPERIMENTAL

All moisture-sensitive reactions were carried out in thoroughly dried glassware under a nitrogen atmosphere. ^1H, ^{13}C and ^{31}P spectra were recorded on a JEOL JNM-AL300 or JEOL JNM-EX400

spectrometer. Chemical shifts are reported in parts per million referenced to tetramethylsilane (δ = 0.00 ppm) for ^{1}H NMR spectra, $CDCl_3$ (δ = 77.0 ppm) and CD_3OD (δ = 49.0 ppm) for ^{13}C NMR spectra, and phosphoric acid (δ = 0.00 ppm) for ^{31}P NMR spectra. IR spectra were recorded on a JASCO FT/IR-4200 spectrometer. Specific rotations were recorded on a JASCO P-2200 polarimeter. EI and FAB 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 or JEOL JMS-S3000 mass spectrometer. Fuji Silysia silica gel PSQ-60B (0.060 mm) and FL-60D (0.060 mm) were used for flash column chromatography. For HPLC, SHIMADZU LC-10AT$_{VP}$, SHIMADZU SPD-10A$_{VP}$ and SHIMADZU CTO-10$_{VP}$ instruments were used. EYELA Cute Mixer CM-1000 was used as a shaker.

1,2-Dideoxy-5-*O*-(4,4'-dimethoxytrityl)-1-(prop-2-ynyl)-β-D-ribofuranose (4)

Under a nitrogen atmosphere, DIBAL (5.1 mL, 4.74 mmol) was added to a solution of **3**[8] (1.2 g, 2.37 mmol) in anhydrous CH_2Cl_2 (10 mL) at -78 °C, and the mixture was stirred for 0.5 h. After addition of saturated $NaHCO_3$ aq., the mixture was extracted with CH_2Cl_2. The organic extracts were washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt = 1:2) to give the aldehyde (1.0 g, 92%). This compound was not subjected to further purification and a portion of this was used in the next step. Under a nitrogen atmosphere, $(MeO)_2POC(N_2)COMe$ (250 mg, 1.30 mmol) and K_2CO_3 (450 mg, 3.24 mmol) were added to a solution of aldehyde (500 mg, 1.08 mmol) in anhydrous MeOH (10 mL) at rt. After being stirred for 5 h, the mixture was concentrated under reduced pressure. The residue was extracted with AcOEt. The organic 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 **4** (420 mg, 82% for 2 steps) as a colorless oil. $[\alpha]_D^{24}$ +8.4 (*c* 1.06, $CHCl_3$); IR ν_{max} (KBr) 2932, 1607, 1509, 1462, 1444, 1301, 1251, 1177, 1085, 1035 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.92–2.02 (3H, m), 2.43–2.49 (2H, m), 3.09 (1H, dd, *J* = 6.0, 10.0 Hz), 3.24 (1H, dd, *J* = 4.5, 10.0 Hz), 3.77 (6H, s), 3.93–3.94 (1H, m), 4.29–4.34 (2H, m), 6.81–6.83 (4H, m), 7.22–7.44 (9H, m); ^{13}C-NMR ($CDCl_3$) δ 25.0, 39.7, 55.1, 64.5, 70.0, 74.4, 76.3, 80.2, 85.9, 86.1, 113.0, 126.7, 127.7, 128.1, 130.0, 136.0, 144.8, 158.4; MS (EI) *m/z* 458 (M^+, 100); HRMS (EI) *m/z* Calcd for $C_{29}H_{30}O_5$: 458.2093. Found 458.2089.

3-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-1,2-dideoxy-5-*O*-(4,4'-dimethoxytrityl)-1-(prop-2-ynyl)-β-D-ribofuranose (1)

Under a nitrogen atmosphere, 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (120 μL, 0.539 mmol) was added to a solution of compound **4** (190 mg, 0.414 mmol) and *N*,*N*-diisopropylethylamine (210 μL, 0.539 mmol) in anhydrous CH_2Cl_2 (2 mL) at 0 °C, and the mixture was stirred at rt for 1.5 h.

After addition of water, the solvent was removed under reduced pressure and the residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt = 5:1) to give compound **1** (237 mg, 87%) as a colorless syrup. ^{1}H NMR ($CDCl_3$) δ 1.08 (3H, d, J = 7.0 Hz), 1.13–1.18 (9H, m), 1.91–1.96 (2H, m), 2.06–2.21 (1H, m), 2,43–2.46 (1H, m), 2.50–2.55 (2H, m), 2.60 (1H, t, J = 6.5 Hz) 3.11–3.16 (2H, m), 3.52–3.81 (10H, m), 4.10–4.15 (1H, m), 4.30–4.33 (1H, m), 4.44–4.48 (1H, m), 6.79–6.83 (4H, m), 7.19–7.36 (7H, m), 7.44–7.47 (2H, m); ^{31}P-NMR ($CDCl_3$) δ 147.1, 147.7; MS (FAB) *m/z* 681 $[M+Na]^+$; HRMS (FAB) *m/z* Calcd for $C_{38}H_{47}N_2O_6P$ $[M+Na]^+$: 681.3069. Found 681.3093.

2-[1,2-Dideoxy-5-*O*-(4,4'-dimethoxytrityl)-β-D-ribofuranos-1-yl]-*N*-(prop-2-ynyl)ethanamide (5)

KOH (220 mg, 3.95 mmol) was added to a solution of **3**[8] (1.0 g, 1.97 mmol) in THF/H_2O (1:1, 10 mL) at 0 °C and the mixture was stirred at rt for 12 h. After addition of 2N HCl aq., the mixture was extracted with AcOEt. The organic extracts were washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt = 1:6) to give carboxylic acid (970 mg, 96%). This compound was not subjected to further purification and a portion of this was used in the next step. Under a nitrogen atmosphere, propargylamine (27 μL, 0.426 mmol), EDC·HCl (82 mg, 0.426 mmol) and DMAP (9 mg, 0.0711 mmol) were added to a solution of carboxylic acid (170 mg, 0.355 mmol) in anhydrous CH_2Cl_2 (3 mL) at rt and the mixture was stirred for 11 h. After addition of water, the mixture was extracted with CH_2Cl_2. The organic 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 = 1:3) to give compound **5** (170 mg, 89% for 2 steps) as a colorless syrup. $[\alpha]_D^{22}$ -13.8 (*c* 1.12, $CHCl_3$); IR n_{max} (KBr) 3288, 2931, 1656, 1607, 1509, 1444, 1300, 1251, 1082, 1034 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.72 (1H, d, J = 3.5 Hz), 1.83 (1H, ddd, J = 5.5, 9.5, 13.0 Hz), 2.00 (1H, ddd, J = 2.0, 4.0, 13.0 Hz), 2.13 (1H, t, J = 2.5 Hz), 2.44 (1H, dd, J = 8.5, 15.5 Hz), 2.55 (1H, dd, J = 3.0, 15.5 Hz), 3.16 (1H, dd, J = 5.5, 10.0 Hz), 3.24 (1H, dd, J = 5.0, 10.0 Hz), 3.80 (6H, s), 3.98 (1H, ddd, J = 2.5, 5.0, 5.0 Hz), 4.31–4.36 (1H, m), 4.41–4.51 (1H, m), 6.70 (1H, brs), 6.81–6.86 (4H, m), 7.22–7.44 (9H, m); ^{13}C-NMR ($CDCl_3$) δ 28.9, 40.7, 42.0, 55.2, 64.3, 71.3, 73.8, 74.7, 79.6, 86.2, 86.4, 113.1, 126.8, 127.8, 128.1, 130.0, 135.8, 144.7, 158.5, 170.5; MS (EI) *m/z* 515 (M^+, 100); HRMS (EI) *m/z* Calcd for $C_{31}H_{33}NO_6$: 515.2308. Found 515.2307.

2-{3-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-1,2-dideoxy-5-*O*-(4,4'-dimethoxytrityl)-β-D-ribofuranos-1-yl}-*N*-(prop-2-ynyl)ethanamide (2)

Under a nitrogen atmosphere, 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (52 μL, 0.233 mmol) was added to a solution of compound **5** (100 mg, 0.194 mmol) and *N*,*N*-diisopropylethylamine (100 μL, 0.582 mmol) in anhydrous CH_2Cl_2 (2 mL) at 0 °C, and the mixture was stirred at rt for 3 h. After addition of saturated $NaHCO_3$ aq., the solvent was removed under reduced pressure and the residue was purified

by flash silica gel column chromatography (*n*-hexane/AcOEt = 1:1) to give compound **2** (125 mg, 90%) as a colorless syrup. ^{1}H NMR ($CDCl_3$) δ 1.08 (4H, d, J = 7.0 Hz), 1.12–1.18 (8H, m), 1.75–1.84 (1H, m), 2.05–2.21 (2H, m), 2.40–2.48 (2H, m), 2.56–2.60 (2H, m), 3.14–3.20 (2H, m), 3.51–3.88 (10H, m), 3.95–4.00 (2H, m), 4.10–4.13 (1H, m), 4.40–4.45 (2H, m), 6.68 (1H, brs), 6.81–6.85 (4H, m), 7.20–7.34 (7H, m), 7.42–7.45 (2H, m); ^{31}P-NMR ($CDCl_3$) δ 147.7, 148.2; HRMS (MALDI-TOF) *m/z* Calcd for $C_{40}H_{50}N_3NaO_7P$ $[M+Na]^+$: 738.3279. Found 738.3297.

Synthesis of oligonucleotides

The syntheses of **6** and **7** were performed on a 0.2-μmol scale or 1.0-μmol scale on an automated DNA synthesizer (Gene Design nS-8) using the common phosphoramidite protocol. TFOs synthesized on DMTr-ON mode were cleaved from the CPG resin by treatment with 28% NH_3 aq. at rt for 1.5 h and all the protecting groups on TFOs were removed by treatment with 28% NH_3 aq. at 55 °C for 12 h. The obtained crude TFOs were purified on Sep-Pak® Plus C18 cartridges (Waters) followed by reversed-phase HPLC (Waters XBridge® OST C18 column 2.5 μm, 10 mm × 50 mm). The composition of the TFOs was confirmed by MALDI-TOF-MS analysis. MALDI-TOF-MS data ($[M-H]^-$) for **6** and **7**: **6**, found 4409.26 (calcd. 4409.93). **7**, found 4467.01 (calcd. 4466.99).

Click chemistry: General procedure

A solution of azide compound (5 mM in DMSO, 8 μL) was added to a mixture of $CuSO_4$ (2 mM in H_2O, 4 μL), TBTA (2 mM in DMSO, 4 μL), sodium ascorbate (10 mM in H_2O, 4 μL), **6** or **7** [0.2 mM in phosphate buffer (pH 7.0), 20 μL] in a 1.5-mL Eppendorf tube. The mixture was shaken at rt using a shaker (1000 rpm) until the reaction was complete. The entire product was purified by reversed-phase HPLC [column: Waters XBridge® OST C18 column 2.5 μm, 4.6 mm × 50 mm; eluent: gradient system of MeCN/0.1 M triethylammonium acetate buffer (pH 7.0); flow rate: 1.0 mL/min] to give the desired TFO **8** or **9**. Yields and MALDI-TOF-MS data ($[M-H]^-$) for TFOs **8a-f** and **9a-f**: **8a**, 77% yield, found 4529.21 (calcd. 4529.06); **8b**, 71% yield, found 4545.93 (calcd. 4545.01); **8c**, 65% yield, found 4571.01 (calcd. 4572.03); **8d**, 78% yield, found 4545.11 (calcd. 4545.01); **8e**, 84% yield, found 4572.12 (calcd. 4572.03); **8f**, 71% yield, found 4588.02 (calcd. 4587.05); **9a**, 75% yield, found 4586.41 (calcd. 4586.11); **9b**, 73% yield, found 4630.72 (calcd. 4630.12); **9c**, 74% yield, found 4628.68 (calcd. 4629.14); **9d**, 81% yield, found 4602.54 (calcd. 4602.11); **9e**, 80% yield, found 4629.49 (calcd. 4629.14); **9f**, 89% yield, found 4644.31 (calcd. 4644.15).

UV-Melting experiments

UV-Melting experiments were carried out on SHIMADZU UV-1650 and SHIMADZU UV-1800 spectrometers equipped with T_m analysis accessory. Equimolecular amounts of the target hairpin-loop dsDNA and TFOs were dissolved in 10 mM sodium cacodylate buffer (pH 6.8) containing 100 mM KCl

and 50 mM $MgCl_2$ to give a final strand concentration of 1.9 μM. The samples were annealed by heating at 100 °C 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.

ACKNOWLEDGEMENTS

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REFERENCES AND NOTES

1. Reviews: S. O. Doronina and J.-P. Behr, *Chem. Soc. Rev.*, 1997, 63; I. Luyten and P. Herdewijn, *Eur. J. Med. Chem.*, 1998, **33**, 515; D. M. Gowers and K. R. Fox, *Nucleic Acids Res.*, 1999, **27**, 1569; K. R. Fox, *Curr. Med. Chem.*, 2000, **7**, 17; J. Robles, A. Grandas, E. Pedroso, F. J. Luque, R. Eritja, and M. Orozco, *Curr. Org. Chem.*, 2002, **6**, 1333; M. G. M. Purwanto and K. Weisz, *Curr. Org. Chem.*, 2003, **7**, 427; S. Buchini and C. J. Leumann, *Curr. Opin. Chem. Biol.*, 2003, **7**, 717; K. R. Fox and T. Brown, *Quart. Rev. Biophys.*, 2005, **38**, 311; D. A. Rusling, V. J. Broughton-Head, T. Brown, and K. R. Fox, *Curr. Chem. Biol.*, 2008, **2**, 1; V. Malnuit, M. Duca, and R. Benhida, *Org. Biomol. Chem.*, 2011, **9**, 326; K. R. Fox and T. Brown, *Biochem. Soc. Trans.*, 2011, **39**, 629; Y. Hari, S. Obika, and T. Imanishi, *Eur. J. Org. Chem.*, 2012, 2875.
2. Y. Hari, M. Nakahara, and S. Obika, *Bioorg. Med. Chem.*, 2013, **21**, in press (doi: 10.1016/j.bmc.2013.05.034).
3. D. Guianvarc'h, R. Benhida, J.-L. Fourrey, R. Maurisse, and J.-S. Sun, *Chem. Commun.*, 2001, 1814; D. Guianvarc'h, J.-L. Fourrey, R. Maurisse, J.-S. Sun, and R. Benhida, *Bioorg. Med. Chem.*, 2003, **11**, 2751.
4. S. Verma and P. S. Miller, *Bioconjugate Chem.*, 1996, **7**, 600; O. A. Amosova and J. R. Fresco, *Nucleic Acids Res.*, 1999, **27**, 4632; D. Guianvarc'h, J.-L. Fourrey, R. Maurisse, J.-S. Sun, and R. Benhida, *Org. Lett.*, 2002, **4**, 4209; Y. Wang, D. A. Rusling, V. E. C. Powers, O. Lack, S. D. Osborne, K. R. Fox, and T. Brown, *Biochemistry*, 2005, **44**, 5884; S. Buchini and C. J. Leumann, *Eur. J. Org. Chem.*, 2006, 3152; S. Obika, H. Inohara, Y. Hari, and T. Imanishi, *Bioorg. Med. Chem.*, 2008, **16**, 2945; Y. Hari, S. Kashima, H. Inohara, S. Ijitsu, T. Imanishi, and S. Obika, *Tetrahedron*, 2013, **69**, 6381.
5. Y. Hari, M. Nakahara, J. Pang, M. Akabane, T. Kuboyama, and S. Obika, *Bioorg. Med. Chem.*, 2011, **19**, 1162.

6. Y. Hari, M. Akabane, Y. Hatanaka, M. Nakahara, and S. Obika, *Chem. Commun.*, 2011, **47**, 4424; Y. Hari, M. Akabane, and S. Obika, *Chem. Commun.*, 2013, **49**, in press (doi: 10.1039/c3cc44030c).
7. Reviews: H. C. Kolb, M. G. Finn, and K. B. Sharpless, *Angew. Chem. Int. Ed.*, 2001, **40**, 2004; H. C. Kolb and K. B. Sharpless, *Drug Discov. Today*, 2003, **8**, 1128.
8. J. Hovinen and H. Salo, *J. Chem. Soc., Perkin Trans. 1*, 1997, 3013.
9. S. Ohira, *Synth. Commun.*, 1989, **19**, 561; S. Müller, B. Liepold, G. J. Roth, and H. J. Bestmann, *Synlett*, 1996, 521.
10. All azides used in this study were prepared according to the previous reports.[2,5,13]
11. T. R. Chan, R. Hilgraf, K. B. Sharpless, and V. V. Fokin, *Org. Lett.*, 2004, **6**, 2853.
12. M. Nakahara, T. Kuboyama, A. Izawa, Y. Hari, T. Imanishi, and S. Obika, *Bioorg. Med. Chem. Lett.*, 2009, **19**, 3316.
13. H. Tomioka, T. Matsushita, S. Murata, and S. Koseki, *Liebigs Ann.*, 1996, 1971; J. Andersen, U. Madsen, F. Björkling, and X. Liang, *Synlett*, 2005, 2209.

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Synthesis and Properties of 2′-*O*,4′-*C*-Ethyleneoxy Bridged 5-Methyluridine

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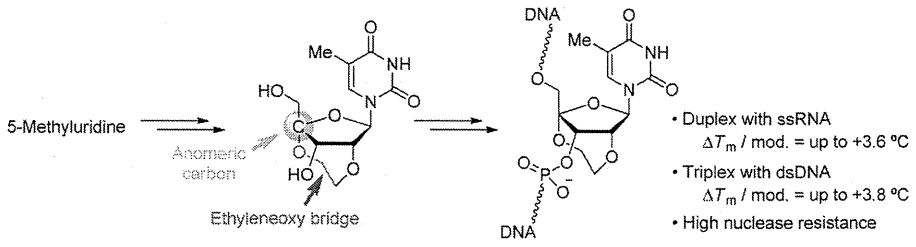
ABSTRACT



2′-*O*,4′-*C*-Ethyleneoxy bridged 5-methyluridine (EoNA-T), possessing a seven-membered linkage and an anomeric 4′-carbon, was synthesized and introduced into oligonucleotides by using an automated DNA synthesizer. The EoNA-modified oligonucleotides significantly stabilized the duplexes with single-stranded RNA and triplexes with double-stranded DNA relative to the natural oligonucleotide and oligonucleotides modified by another seven-membered bridged 5-methyluridine, 2′,4′-BNA^{COC}-T. In addition, EoNA-T showed excellent nuclease resistance.

Artificial nucleic acids that stabilize complexes with target nucleic acids are useful materials for various nucleic acid technologies such as gene therapy and genetic diagnosis. Among numerous analogs developed to date, nucleic acids bridged between the 2′- and 4′-positions generally lead to an increased affinity to single-stranded RNA (ssRNA) or double-stranded DNA (dsDNA), or both.[1−6] Moreover, the bridged nucleic acids have increased resistance to nuclease degradation when compared with a natural nucleic acid. The bridge size between the 2′- and 4′-positions is considered to crucially affect the binding affinity and nuclease resistance. 2′,4′-Methylene-bridged nucleic acid (BNA/LNA) with a five-membered bridge has outstanding high-binding affinities to ssRNA and dsDNA, as well as improved

(1) Reviews: Kaur, H.; Babu, B. R.; Maiti, S. *Chem. Rev.* **2007**, *107*, 4672. Obika, S.; Rahman, S. M. A.; Fujisaka, A.; Kawada, Y.; Baba, T.; Imanishi, T. *Heterocycles* **2010**, *81*, 1347.

(2) (a) Obika, S.; Nanbu, D.; Hari, Y.; Morio, K.; In, Y.; Ishida, T.; Imanishi, T. *Tetrahedron Lett.* **1997**, *38*, 8735. (b) Nielsen, P.; Wengel, J. *Chem. Commun.* **1998**, 455. (c) Koshkin, A. A.; Singh, S. K.; Nielsen, P.; Rajwanshi, V. K.; Kumar, R.; Meldgaard, M.; Olsen, C. E.; Wengel, J. *Tetrahedron* **1998**, *54*, 3607. (d) Obika, S.; Nanbu, D.; Hari, Y.; Andoh, J.; Morio, K.; Doi, T.; Imanishi, T. *Tetrahedron Lett.* **1998**, *39*, 5401. (e) Obika, S.; Hari, Y.; Sugimoto, T.; Sekiguchi, M.; Imanishi, T. *Tetrahedron Lett.* **2000**, *41*, 8923. (f) Obika, S.; Uneda, T.; Sugimoto, T.; Nanbu, D.; Minami, T.; Doi, T.; Imanishi, T. *Bioorg. Med. Chem.* **2001**, *9*, 1001. (g) Torigoe, H.; Hari, Y.; Sekiguchi, M.; Obika, S.; Imanishi, T. *J. Biol. Chem.* **2001**, *276*, 2354.

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

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

(5) Nishida, M.; Baba, T.; Kodama, T.; Yahara, A.; Imanishi, T.; Obika, S. *Chem. Commun.* **2010**, *46*, 5283.

(6) For other nucleic acids bridged between 2′- and 4′-positions, see: (a) Kumar, R.; Singh, S. K.; Koshkin, A. A.; Rajwanshi, V. K.; Meldgaard, M.; Wengel, J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2219. (b) Singh, S. K.; Kumar, R.; Wengel, J. *J. Org. Chem.* **1998**, *63*, 10035. (c) Varghese, O. P.; Barman, J.; Pathmasiri, W.; Plashkevych, O.; Honcharenko, D.; Chattopadhyaya, J. *J. Am. Chem. Soc.* **2006**, *128*, 15173. (d) Morita, K.; Hasegawa, C.; Kaneko, M.; Tsutsumi, S.; Sone, J.; Ishikawa, T.; Imanishi, T.; Koizumi, M. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 73. (e) Rahman, S. M. A.; Seki, S.; Obika, S.; Yoshikawa, H.; Miyashita, K.; Imanishi, T. *J. Am. Chem. Soc.* **2008**, *130*, 4886. (f) Seth, P. P.; Siwkowski, A.; Allerson, C. R.; Vasquez, G.; Lee, S.; Prakash, T. P.; Wancewicz, E. V.; Witchell, D.; Swayze, E. E. *J. Med. Chem.* **2009**, *52*, 10. (g) Seth, P. P.; Vasquez, G.; Allerson, C. A.; Berdeja, A.; Gaus, H.; Kinberger, G. A.; Prakash, T. P.; Migawa, M. T.; Bhat, B.; Swayze, E. E. *J. Org. Chem.* **2010**, *75*, 1569. (h) Seth, P. P.; Allerson, C. R.; Berdeja, A.; Siwkowski, A.; Pallan, P. S.; Gaus, H.; Prakash, T. P.; Watt, A. T.; Egli, M.; Swayze, E. E. *J. Am. Chem. Soc.* **2010**, *132*, 14942. (i) Shrestha, A. R.; Hari, Y.; Yahara, A.; Osawa, T.; Obika, S. *J. Org. Chem.* **2011**, *76*, 9891. (j) Yahara, A.; Shrestha, A. R.; Yamamoto, T.; Hari, Y.; Osawa, T.; Yamaguchi, M.; Nishida, M.; Kodama, T.; Obika, S. *ChemBioChem* **2012**, *13*, 2513. (k) Hari, Y.; Osawa, T.; Kotobuki, Y.; Shrestha, A. R.; Yahara, A.; Obika, S. *Bioorg. Med. Chem.* **2013**, *21*, 4405.

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nuclease resistance.[2] In contrast, seven-membered bridge analogs such as PrNA,[3] 2′,4′-BNA^{COC},[4] and urea-BNA[5] have excellent nuclease resistance because of the sterically large bridge structure, although they lack the high binding affinities probably due to fixation of their sugar conformation being incomplete (Figure 1).

4′-Alkoxynucleosides tend toward the N-type sugar conformation presumably because of the anomeric effect on the 4′-carbon atom.[7,8] Recently, Rosenberg's group reported that oligonucleotides modified by 4′-alkoxythymidines increased the T_m value of duplexes with ssRNA by approximately 1 °C per modification.[8] Thus, we designed a 2′-*O*,4′-*C*-ethyleneoxy bridged nucleic acid (EoNA) with a seven-membered bridged structure between the 2′- and 4′-positions and an anomeric carbon at the 4′-position. Its sugar conformation is anticipated to be sufficiently restricted to the N-form by the additional anomeric effect despite the large, seven-membered bridge (Figure 1). This can contribute not only to high nuclease resistance but also to acquisition of high duplex- or triplex-forming ability. Furthermore, as far as we know, there are no reports on 2′,4′-bridged nucleic acids with an additional heteroatom on the 4′-carbon atom, which motivated us to conduct the present study. Here, 2′-*O*,4′-*C*-ethyleneoxy bridged 5-methyluridine (EoNA-T) was synthesized, and evaluation of its oligonucleotides was carried out.

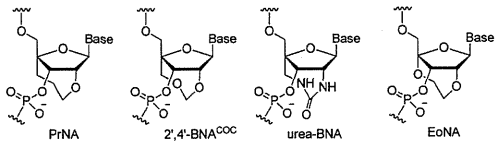
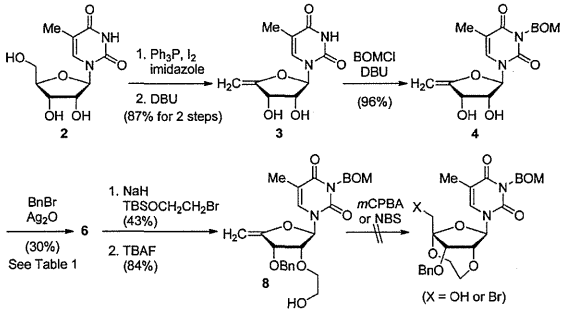


Figure 1. Structures of PrNA, 2′,4′-BNA^{COC}, urea-BNA, and EoNA designed in the present study.

The synthesis of EoNA-T phosphoramidite **1** was examined (Schemes 1 and 3). After conversion from commercially available 5-methyluridine **2** into *exo*-olefin **3**, **4** was obtained by BOM-protection. Benzylation of **4** was examined (Table 1). A conventional method using NaH and BnBr was used for the preferential 2′-*O*-benzylation reaction leading to the 7:1 separable mixture of 2′-*O*-benzylated **5** and 3′-*O*-benzylated **6**. Dibenzylated compound **7** was also obtained in 30% yield. In contrast, a reaction system using Bu_2SnO/BnBr or Ag_2O/BnBr proceeded in a monobenzylation reaction without regioselectivity. Consequently, in a reaction using Ag_2O/BnBr, the desired product **6** was isolated in 30% yield after purification by silica gel column chromatography (Scheme 1).

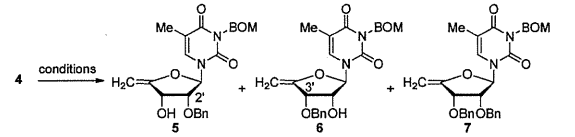
After bonding of the 2-hydroxyethyl unit to 2′-oxygen of **6** via two steps, **8** was treated with *m*CPBA or NBS to

Scheme 1. Synthesis of Intermediates and Attempted Bridge Construction



construct the bridged structure.[9] In the former reaction, 3-benzyloxymethylthymine (BOM-T) was the only isolated product (83% yield). The reaction using NBS afforded dioxane **9** in 24% yield via attack of hydroxyl group on the 1′-carbon, as shown in Scheme 2. In both cases, no desired product was detected at all. These results imply that construction of the bridged structure by the attack on the 4′-carbon is difficult.

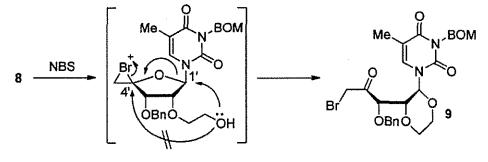
Table 1. Benzylation of **4**[a]



conditions	yield of **5** and **6** (**5**:**6**)	yield of **7**
NaH, BnBr, DMF, 0 °C, 0.5 h	45% (7:1)	30%
Bu_2SnO, BnBr, DMF, 80 °C, 19 h	47% (8:10)	3%
Ag_2O, BnBr, CH_2Cl_2, rt, 16 h	75% (9:10)	6%

[a] Recovery yields of starting material were 19% in NaH/BnBr, 28% in Bu_3SnO/BnBr, or 17% in Ag_2O/BnBr.

Scheme 2. Possible Reaction Mechanism



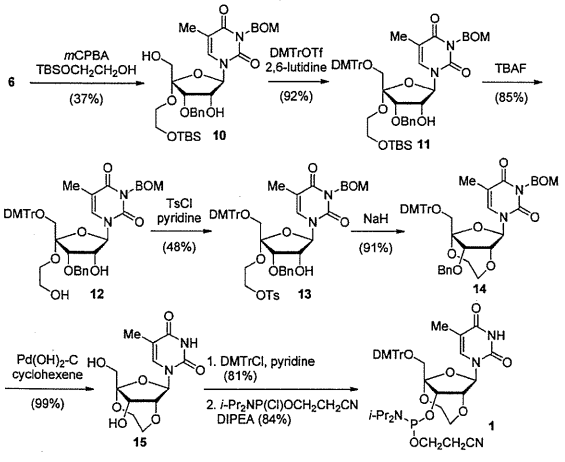
As an alternative route, construction of the bridged structure after introduction of the 2-hydroxyethoxy unit

(7) Tong, W.; Agback, P.; Chattopadhyaya, J. *Acta Chem. Scand.* **1993**, *47*, 145.

(8) Liboska, R.; Snášel, J.; Barvík, I.; Buděšínský, M.; Pohl, R.; Točík, Z.; Páv, O.; Rejman, D.; Novák, P.; Rosenberg, I. *Org. Biomol. Chem.* **2011**, *9*, 8261.

(9) For examples of synthesis of 4′-substituted nucleosides from 4′,5′-unsaturated nucleosides, see: (a) Verheyden, J. P.; Moffatt, J. G. *J. Am. Chem. Soc.* **1975**, *97*, 4386. (b) Maag, H.; Rydzewski, R. M.; McRoberts, M. J.; Crawford-Ruth, D.; Verheyden, J. P. H.; Prisbe, E. J. *J. Med. Chem.* **1992**, *35*, 1440. (c) Haraguchi, K.; Takeda, S.; Tanaka, H. *Org. Lett.* **2003**, *5*, 1399. (d) Haraguchi, K.; Sumino, M.; Tanaka, H. *J. Org. Chem.* **2006**, *71*, 4433.

Scheme 3. Synthesis of Phosphoramidite **1**



on the 4′-carbon was investigated (Scheme 3). Treatment of **6** with *m*CPBA and an excess amount of siloxyethanol in a solvent-free system led to compound **10** as the sole isolated product. This stereoselectivity might be caused by the less hindered β-face attack of *m*CPBA on the olefin. Protection of the primary alcohol of **10** by 4,4′-dimethoxytrityl triflate (DMTrOTf)[10] followed by desilylation of **11** afforded diol **12**. Although no ring closure of **12** proceeded under Mitsunobu conditions using TMAD and Bu_3P,[11] the tosylated compound **13** prepared from **12** was treated with NaH to give the desired product **14** in 91% yield. All protecting groups were removed by hydrogenolysis to obtain EoNA-T monomer **15**. The ^{1}H NMR measurement demonstrated that the $J_{1',2'}$ and $J_{2',3'}$ values of **15** were 0 and 6 Hz, respectively, which coincided with those of 2′,4′-BNA^{COC}-T monomer.[4a,12] Afterward, the desired phosphoramidite **1** was obtained according to common methods to prepare a suitable building block for oligonucleotide synthesis. The oligonucleotide synthesis was performed on an automated DNA synthesizer using common phosphoramidite chemistry with a prolonged coupling time (20 min) for the introduction of the analog **1**.[13] Concerning the oligonucleotide **18** with three consecutive modifications shown in Table 1, successful synthesis was achieved using double-coupling cycles[14] together with the prolonged coupling time.[13]

The duplex- and triplex-forming abilities of the modified oligonucleotides **17**–**20** with ssDNA, ssRNA, and dsDNA were evaluated by UV melting experiments and compared with those of the corresponding natural counterparts **16** and 2′,4′-BNA^{COC}-modified oligonucleotides **21**–**24** (Table 2; see Figure 1 for the structure of 2′,4′-BNA^{COC}). The duplex-forming ability of **17**–**20** with ssDNA and ssRNA showed the same tendency as that of the 2′,4′-BNA^{COC}-modified congeners **21**–**24**; the duplexes with ssDNA were destabilized relative to that of **16**, and the duplexes with ssRNA were stabilized. However, this modification rather than the 2′,4′-BNA^{COC} modification enabled the stable formation of the duplexes with ssRNA. Interestingly, stabilization by this modified nucleic acid was apparently synergistic, and the quintuple-modified oligonucleotide **20** stabilized the duplex with ssRNA by +3.6 °C per modification, the T_m value of which was 69 °C. In triplexes formed with dsDNA, 2′,4′-BNA^{COC}-modified oligonucleotides **21**–**24** showed almost no stabilization. In contrast, oligonucleotides **17**–**20** showed significant stabilization of the triplexes formed, and up to +3.8 °C per modification was observed. These results may imply that the N-type sugar conformation constrained by not only the bridge structure but also an anomeric effect contributes to significant stabilization of the duplexes and triplexes formed with ssRNA and dsDNA, as expected.

Table 2. Duplex- and Triplex-Forming Abilities of Modified Oligonucleotides with ssDNA, ssRNA, and dsDNA[a]

oligonucleotide	ssDNA T_m (°C)	ssRNA T_m (°C)	dsDNA T_m (°C)
5′-TCTTCTTTTTCTCT-3′ (**16**)	50	51	31
5′-TCTTCTTTTTCTCT-3′ (**17**)	48 (−2.0)	52 (+1.0)	32 (+1.0)
5′-TCTTCTTTTTCTCT-3′ (**18**)	47 (−1.0)	59 (+2.7)	37 (+2.0)
5′-TCTTCTTTTTCTCT-3′ (**19**)	47 (−1.0)	60 (+3.0)	41 (+3.3)
5′-TCTTCTTTTTCTCT-3′ (**20**)	48 (−0.4)	69 (+3.6)	50 (+3.8)
5′-TCTTCTTTTTCTCT-3′ (**21**)	48 (−2.0)	52 (+1.0)	31 (0)
5′-TCTTCTTTTTCTCT-3′ (**22**)	45 (−1.7)	57 (+2.0)	31 (0)
5′-TCTTCTTTTTCTCT-3′ (**23**)	45 (−1.7)	57 (+2.0)	33 (+0.7)
5′-TCTTCTTTTTCTCT-3′ (**24**)	42 (−1.6)	62 (+2.2)	33 (+0.4)

[a] Conditions: 10 mM sodium cacodylate buffer (pH 7.2), 140 mM KCl, and 4 μM of each oligonucleotide for duplex; and 10 mM sodium cacodylate buffer (pH 7.2), 140 mM KCl, 5 mM $MgCl_2$, and 1.5 μM of each oligonucleotide for triplex. **T** = EoNA-T. *T* = 2′,4′-BNA^{COC}-T. C = 2′-deoxy-5-methylcytidine. The sequences of ssDNA, ssRNA, and dsDNA are 5′-d(AGAGAAAAAGAAGA)-3′, 5′-r(AGAGAAAAAGAAGA)-3′, and 5′-d(GGCAGAAGAAAAAGAGACGC)-spacer18-d(GCGTCTTCTTTTTCTCTGCC)-3′, respectively.

The enzymatic stability of the modified oligonucleotides was evaluated using 3′-exonuclease. A comparison of oligonucleotides **25**–**29** is shown in Figure 2a. Although the 2′,4′-BNA/LNA-modified compound **28** and the natural compound **29** were quickly degraded, **25**, which had this analog, showed high resistance against the nuclease, as we expected. The ability was comparable to that observed by 2′,4′-BNA^{COC} modification (**26**) and was better than that of **27**, which had a chiral phosphorothioate linkage, i.e., an S_p-isomer possessing a highly nuclease-resistant property.[15] Moreover, examination of the oligonucleotides modified at the 3′-terminus demonstrated that modification by this analog significantly suppressed degradation of

(10) Tärkoy, M.; Bolli, M.; Leumann, C. *Helv. Chim. Acta* **1994**, *77*, 716.
(11) Tsunoda, T.; Otsuka, J.; Yamamiya, Y.; Itô, S. *Chem. Lett.* **1994**, 539.
(12) The $J_{1',2'}$ value (0 Hz) indicates that the sugar conformation of EoNA-T monomer **15** adopts the N-form. Altona, C.; Sundaralingam, M. *J. Am. Chem. Soc.* **1973**, *95*, 2333.
(13) By means of monitoring the DMTr cation, the average coupling yield for incorporation of **1** into oligonucleotides was estimated to be 90–95%.
(14) A double-coupling and waiting cycle was carried out prior to the oxidation step.
(15) Burgers, P. M. J.; Eckstein, F. *Biochemistry* **1979**, *18*, 592.

the 5′-site by the nuclease compared with 2′,4′-BNA^{COC}, although the reason for this suppression is unclear (Figure 2b).

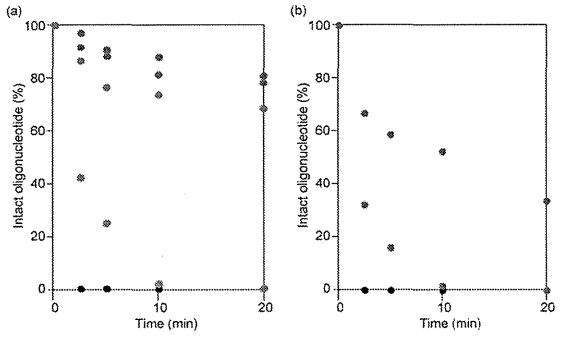


Figure 2. Degradation experiments by nuclease. Conditions: 2 μg/mL *Crotalus admanteus* venom phosphodiesterase (CAVP), 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0), 7.5 μM each oligonucleotide at 37 °C. (a) 5′-TTTTTTTTTT-3′ [T = EoNA-T (**25**, red), 2′,4′-BNA^{COC}-T (**26**, blue), 3′-S_p-phosphorothioate-T (**27**, green), 2′,4′-BNA/LNA-T (**28**, pink), and natural T (**29**, black)]. (b) 5′-TTTTTTTTTT-3′ [T = EoNA-T (**30**, red), 2′,4′-BNA^{COC}-T (**31**, blue), and natural T (**29**, black)].

In conclusion, we designed and synthesized a 2′-*O*,4′-*C*-ethyleneoxy bridged 5-methyluridine. The synthetic process for the desired phosphoramidite was short (12 steps from 5-methyluridine). The modified oligonucleotides showed stabilization of complexes with ssRNA and dsDNA and increased stability against nuclease degradation. These properties were superior to those of 2′,4′-BNA^{COC} that are most excellent among a series of seven-membered bridged nucleic acids. These modified oligonucleotides are advantageous in the development of applications in which mRNA and genomic DNA are targeted. Moreover, this result suggests that the design concept of addition of a heteroatom at the 4′-carbon atom in the bridged structure is valuable to the development of prominent tools for nucleic acid based technologies.

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Supporting Information Available. Full experimental details, representative UV melting data, representative HPLC data of nuclease experiments, ^1H, ^{13}C, and ^{31}P spectra of all new compounds, and HPLC charts and MALDI-TOF-MS spectra of new oligonucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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2′,4′-BNA bearing a chiral guanidinopyrrolidine-containing nucleobase with potent ability to recognize the CG base pair in a parallel-motif DNA triplex†

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In order to expand the target sequence used in triplex DNA formation, seven novel nucleotide analogues were synthesized and incorporated into triplex-forming oligonucleotides by post-elongation modification approaches. Among them, GP^{B}, equipped with a suitable restricted conformation of sugar and nucleobase moieties, was found to have the highest sequence-selectivity and affinity towards CG base pairs within double-stranded DNA.

The triplex-forming oligonucleotide (TFO) can sequence-specifically bind to double-stranded DNA (dsDNA) to form triplex DNA. Thus, TFOs can be used for genomic DNA-targeting technologies *in vitro* and *in vivo*.[1] However, practical use of this technology is difficult because the sequence of dsDNA that can be targeted by TFO is limited to homopurine tracts. For example, while T and protonated C in TFO can recognize adenine and guanine bases of AT and GC base pairs in dsDNA *via* two Hoogsteen hydrogen bonds, respectively, no natural nucleic acid can recognize pyrimidine–purine base pairs, such as CG and TA, with high sequence-selectivity and affinity. Thus, to overcome this sequence limitation, researchers have attempted to synthesize nucleoside or nucleotide analogues that can sequence-specifically bind to CG and TA in dsDNA.[2]

By using post-elongation modification (PEM) approaches, we have so far tried to develop a nucleobase capable of recognizing a pyrimidine–purine base pair in a parallel motif triplex.[3,4] PEM allows synthesis of a wide variety of nucleobase derivatives, including substrates that are labile to oligonucleotide synthetic processes, because the chemical modification is carried out after the oligonucleotide synthesis. Consequently, rational and detailed investigation of the nucleobase structure would be achieved. Recently, using the PEM strategy, a 4-[(3*R*,4*R*)-3,4-dihydroxypyrrolidino]pyrimidine (**diHP**) nucleobase was found to be capable of recognizing CG base pairs with high sequence-selectivity and moderate affinity.[3] We believe that the chiral cyclic alcohol unit in this nucleobase is responsible for recognizing the guanine base of the targeted CG base pair (Fig. 1).[5] On the other hand, Fox's[6] and Seidman's[7] groups reported that the guanidine unit is a promising counterpart of guanine in the CG base pair. A molecular dynamics (MD) snapshot showed that the guanidine might form two hydrogen bonds with the opposite G.[7] Under such a background, we were interested in studying a combination of the chiral pyrrolidine unit and the guanidine unit. The guanidine unit could be oriented in a suitable position for the opposite G recognition by the conformational restriction imposed by the pyrrolidine structure. On the other hand, sugar modifications adopting the N-form sugar conformation, such as 2′,4′-BNA,[8] 2′-methoxy[9] and 2′-aminoethoxy[10] modifications, stabilize the triplex. Thus, in this study, the base pair recognition ability of the chiral guanidinopyrrolidine derivatives prepared using the PEM approach was evaluated, and then, modifications of both nucleobase and sugar moieties were investigated. The new nucleic acid (**GP^{B}**), which was modified at the level of nucleobase and sugar moieties, was found to possess potent binding affinity towards CG base pairs within dsDNA in the formation of triplex DNA.

The structures of the new modified nucleotides in TFOs are shown in Fig. 2. TFOs containing **GP**, **GP′**, **diGP**, **mGP**, **GE**, **GP^{OMe}**, and **GP^{B}** were synthesized by treatment with the corresponding amines after introduction of known triazolylated phosphoramidites according to our previous procedure[3] (Table S1, ESI†). The guanidine unit was synthesized through

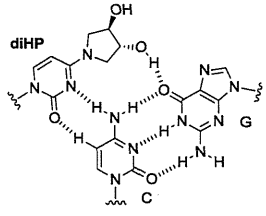

Fig. 1 Possible mode of recognition of a CG base pair by a diHP nucleobase.

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† Electronic supplementary information (ESI) available: Full experimental procedures, characterization data of all new compounds, and HPLC and MALDI-TOF mass spectral data of the newly synthesized oligonucleotides. See DOI: 10.1039/c3cc44030c

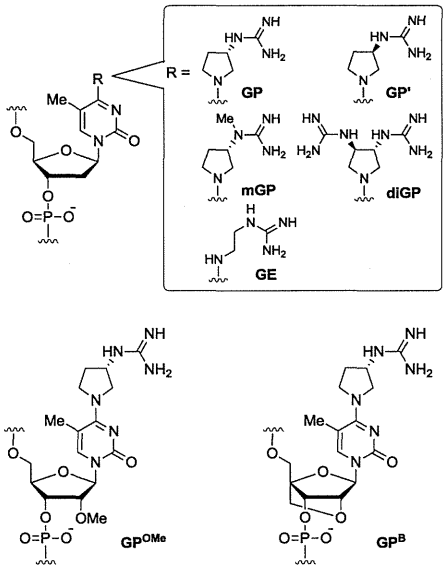


Fig. 2 Structures of the new modified nucleotides used in this study.

the PEM approach by introducing an amino group, which was followed by amidation of this amino group by pyrazole-1-formamidine in 2 steps.[7,11] However, we found that the desired guanidine derivatives could be directly synthesized by treatment with amine compounds bearing a protection-free guanidine. As a representative example, the structure of **GP^{B}** was confirmed by an alternative synthesis of TFO from a monomer bearing the modified nucleobase (Scheme S2, ESI†).

UV-melting of the triplexes formed between the singly modified TFOs and the dsDNA targets was carried out, and the results of these experiments are summarized in Table 1. As targets, hairpin dsDNAs linked to a hexa(ethylene glycol) unit (C18-spacer) were used to stabilize the duplex and prevent transition of the duplex into a single strand from overlapping with that of the duplex into the triplex. The affinity (T_m = 36 °C) and the selectivity (ΔT_m = ≥17 °C) of **GP** for a CG base pair were

Table 1 T_m values (°C) of triplexes between TFOs and hairpin dsDNA targets[a]

TFO 5'-TTTTTCTXTCTCTCT-3'

hairpin dsDNA 5'-GGCAAAAAGAYAGAGAGACGC-C18-spacer
3'-CCGTTTTTCTZTCTCTCTGCG-C18-spacer

		YZ			
X	ΔT_m[c]	CG	TA	GC	AT
GP	≥17 (CG)	36	19	18	16
GP'	≥11 (CG)	30	19	16	16
mGP	≥11 (CG)	29	18	16	15
diGP	≥15 (CG)	26	21	17	17
GE	≥15 (CG)	32	20	17	16
GP^{OMe}	≥17 (CG)	34	17	16	15
GP^{B}	≥26 (CG)	44	18	17	14
P^{B}[b]	≥10 (CG)	37	18	22	27
T	≥15 (AT)	29	20	23	44

[a] Conditions: 10 mM sodium cacodylate buffer (pH 6.8), 100 mM KCl and 50 mM $MgCl_2$. The concentration of each oligonucleotide used was 1.89 μM. C indicates 2′-deoxy-5-methylcytidine. [b] 2′,4′-BNA bearing 2-pyridone as a nucleobase.[12] [c] Difference between the T_m value in targeting base pairs shown in parentheses and those in targeting other base pairs.

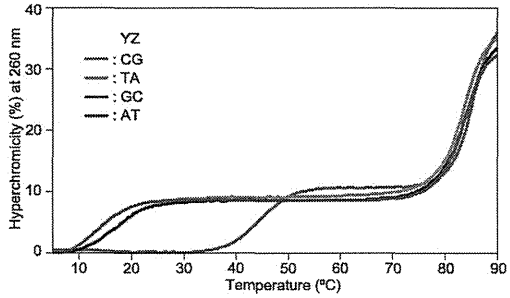


Fig. 3 UV-melting profiles of triplexes containing **GP^{B}**-YZ triplets.

superior to those of **diHP** (T_m = 33 °C and ΔT_m = ≥15 °C).[3] Since the diastereomer **GP′** had low affinity for the CG base pair, the stereochemistry of the guanidine unit was essential as we expected. Methylated GP (**mGP**) also showed low affinity, which strongly suggested that the guanidine unit interacts with the G base of the CG base pair. On the other hand, a C_2-symmetric bis-guanidine (**diGP**) showed quite a low T_m value (26 °C) for the triplex with dsDNA (YZ = CG), which was probably due to improper conformational orientation caused by steric and/or electrostatic repulsion between the two guanidine groups. In comparison with **GE**, a nucleobase developed by Seidman's group,[7] **GP** had better affinity and selectivity for the CG base pair. This result suggests that the guanidine unit in **GP** can adopt a suitable orientation for recognition of the G base in a CG base pair because of the conformationally restricted pyrrolidine ring. Next, a combination of the **GP** base and sugar modification was tested. Although no improvement was observed when the 2′-MeO derivative **GP^{OMe}** was used, **GP^{B}** with 2′,4′-BNA modification led to dramatic increases in not only the affinity (T_m = 44 °C) for a CG base pair but also the selectivity (ΔT_m = ≥26 °C) (Fig. 3). The ability of **GP^{B}** to recognize CG base pairs was much higher than that of the 2′,4′-BNA coupled with 2-pyridone nucleobase (**P^{B}**)[12] previously developed by us (T_m = 37 °C and ΔT_m = ≥10 °C). The triplex containing a **GP^{B}**-CG base triplet showed the same stability as that of the canonical triplex (X-YZ = T-AT), whereas the CG selectivity of **GP^{B}** was much higher than the AT selectivity of T.

In general, the nearest neighboring residues influence the recognition ability of the nucleobases.[13] Therefore, the effect of base triplets adjacent to a **GP^{B}**-CG base triplet was examined and compared to that of a T-CG base triplet,[14] the most stable natural triplet formed with a CG base pair (Table 2). The results showed

Table 2 T_m values (°C) of triplexes with different base pairs adjacent to a **GP^{B}**-CG base triplet[a]

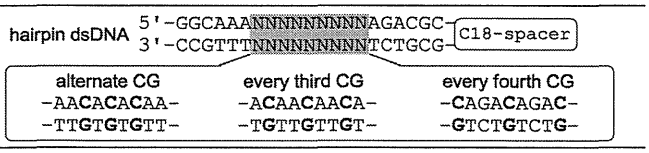
hairpin dsDNA 5'-GGCAAAAANNCNNAGAGACGC-C18-spacer
3'-CCGTTTTTNNGNNTCTCTGCG-C18-spacer

5′-GC	-AGCAG- / -TCGTC-	3′-GC	-GACGA- / -CTGCT-	3′,5′-GCs	-AGCGA- / -TCGCT-

Sequences of TFO	dsDNA targets	X = GP^{B}	X = T
5′-TTTTTTCXTCTCTCT-3′	5′-GC	26	23
5′-TTTTTCTXCTTCTCT-3′	3′-GC	34	29
5′-TTTTTTCXCTTCTCT-3′	3′,5′-GCs	25	21

[a] The conditions are listed in the footnote of Table 1.

Table 3 T_m values (°C) of triplexes containing three GP^B-CG base triplets[a]



Sequences of TFO	dsDNA targets[b]	X = GP^B	X = T
5′-TTTTTXTXTXTCTCT-3′	Alternate CG	21	nd[b]
5′-TTTTXTTXTTXTTCT-3′	Every third CG	~65	nd[b]
5′-TTTXTCTXTCTXTCT-3′	Every fourth CG	49	nd[b]

[a] The conditions are listed in the footnote of Table 1. [b] Not detected.

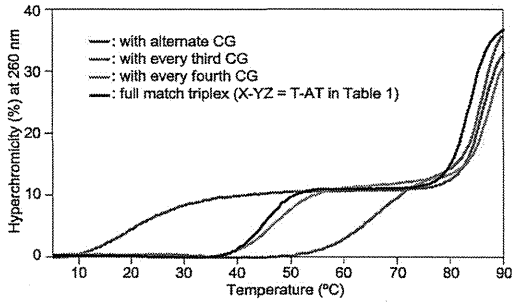


Fig. 4 UV-melting profiles of triplexes containing three GP^B-CG triplets.

that the triplexes containing GP^B-CG were more stable than those containing T-CG, regardless of the presence of the adjacent C-GC base triplet. However, when a C-GC base triplet was at the 5′-flanking site of a GP^B-CG base triplet, the T_m values diminished remarkably. The decrease in the T_m values might be caused by the electrostatic repulsion between the contiguous positive-charges of protonated C and GP^B in TFO.

Furthermore, we investigated whether multiple CG base pairs within dsDNA could be recognized. Based on the results reported in Tables 1 and 2, the dsDNAs in which each CG base pair was sandwiched between AT base pairs were used (Table 3 and Fig. 4). In all the cases, formation of triplexes containing three T-CG base triplets was not observed; nonetheless, use of GP^B enabled triplex formation. Although the destabilizing effect caused by the deformation induced by crowding of 2′,4′-BNA modifications was considered, TFOs possessing three GP^B exhibited a T_m value of 21 °C in the triplex formed with dsDNA (alternate CG).[15] When TFOs contained three GP^B modifications at every third and every fourth CG base pair, very stable triplexes were formed with the corresponding T_m value of approximately 65 °C and 49 °C, respectively, which is higher than that of the natural canonical triplex (T_m = 44 °C) presented in Table 1.[16]

In this study, we achieved the facile synthesis of guanidine-containing nucleobases by using PEM approaches and developed the 2′,4′-BNA bearing 4-[(3*S*)-3-guanidinopyrrolidino]-5-methylpyrimidin-2-one nucleobase (GP^B) as a partner of the CG base pair in triplex formation. Regarding the affinity and base-pair discrimination, the ability of GP^B to recognize a CG base pair is similar to or higher than that of T to recognize a AT base pair and form a stable T-AT triplet. However, the recognition ability was significantly affected by the adjacent base triplets, which in practice should have been T-AT triplets. Thus, to develop an artificial nucleic acid to overcome this problem, efforts to synthesize various derivatives by using the powerful PEM approach, as well as evaluation of these derivatives, are underway.

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Notes and references

1 Reviews: D. Praseuth, A. L. Guieysse and C. Hélène, *Biochim. Biophys. Acta*, 1999, **1489**, 181; M. M. Seidman and P. M. Glazer, *J. Clin. Invest.*, 2003, **112**, 487; S. Buchini and C. J. Leumann, *Curr. Opin. Chem. Biol.*, 2003, 7, 717; M. Duca, P. Vekhoff, K. Oussedik, L. Halby and P. B. Arimondo, *Nucleic Acids Res.*, 2008, **36**, 5123.

2 Reviews: D. M. Gowers and K. R. Fox, *Nucleic Acids Res.*, 1999, **27**, 1569; K. R. Fox, *Curr. Med. Chem.*, 2000, 7, 17; M. G. M. Purwanto and K. Weisz, *Curr. Org. Chem.*, 2003, 7, 427; K. R. Fox and T. Brown, *Q. Rev. Biophys.*, 2005, **38**, 311; V. Malnuit, M. Duca and R. Benhida, *Org. Biomol. Chem.*, 2011, **9**, 326; Y. Hari, S. Obika and T. Imanishi, *Eur. J. Org. Chem.*, 2012, 2875.

3 Y. Hari, M. Akabane, Y. Hatanaka, M. Nakahara and S. Obika, *Chem. Commun.*, 2011, **47**, 4424.

4 Y. Hari, M. Nakahara, J. Pang, M. Akabane, T. Kuboyama and S. Obika, *Bioorg. Med. Chem.*, 2011, **19**, 1162; Y. Hari, M. Nakahara and S. Obika, *Bioorg. Med. Chem.*, 2013, **21**, DOI: 10.1016/j.bmc.2013.05.034.

5 The 2-carbonyl oxygen of **diHP** might weakly interact with C–H of the target C. J. Marfurt and C. Leumann, *Angew. Chem., Int. Ed.*, 1998, **37**, 175; S. Buchini and C. J. Leumann, *Tetrahedron Lett.*, 2003, **44**, 5065; R. T. Ranasinghe, D. A. Rusling, V. E. C. Powers, K. R. Fox and T. Brown, *Chem. Commun.*, 2005, 2555.

6 S. R. Gerrard, M. M. Edrees, I. Bouamaied, K. R. Fox and T. Brown, *Org. Biomol. Chem.*, 2010, **8**, 5087.

7 A. Semenyuk, E. Darian, J. Liu, A. Majumdar, B. Cuenoud, P. S. Miller, A. D. MacKerell, Jr. and M. M. Seidman, *Biochemistry*, 2010, **49**, 7867.

8 S. Obika, Y. Hari, T. Sugimoto, M. Sekiguchi and T. Imanishi, *Tetrahedron Lett.*, 2000, **41**, 8923; H. Torigoe, Y. Hari, M. Sekiguchi, S. Obika and T. Imanishi, *J. Biol. Chem.*, 2001, **276**, 2354; S. Obika, T. Uneda, T. Sugimoto, D. Nanbu, T. Minami, T. Doi and T. Imanishi, *Bioorg. Med. Chem.*, 2001, **9**, 1001; B.-W. Sun, B. R. Babu, M. D. Sørensen, K. Zakrzewska, J. Wengel and J.-S. Sun, *Biochemistry*, 2004, **43**, 4160.

9 M. Shimizu, A. Konishi, Y. Shimada, H. Inoue and E. Ohtsuka, *FEBS Lett.*, 1992, **302**, 155; J. L. Asensio, R. Carr, T. Brown and A. N. Lane, *J. Am. Chem. Soc.*, 1999, **121**, 11063.

10 B. Cuenoud, F. Cassert, D. Hüsken, F. Natt, R. M. Wolf, K.-H. Altmann, P. Martin and H. E. Moser, *Angew. Chem., Int. Ed.*, 1998, **37**, 1288; M. J. J. Blommers, F. Natt, W. Jahnke and B. Cuenoud, *Biochemistry*, 1998, **37**, 17714.

11 M. A. Maier, I. Barber-Peoc'h and M. Manoharan, *Tetrahedron Lett.*, 2002, **43**, 7613.

12 S. Obika, Y. Hari, M. Sekiguchi and T. Imanishi, *Angew. Chem., Int. Ed.*, 2001, **40**, 2079; S. Obika, Y. Hari, M. Sekiguchi and T. Imanishi, *Chem.–Eur. J.*, 2002, **8**, 4796; H. Torigoe, Y. Hari, S. Obika and T. Imanishi, *Nucleosides, Nucleotides Nucleic Acids*, 2003, **22**, 1097.

13 Review: M. D. Frank-Kamenetskii and S. M. Mirkin, *Annu. Rev. Biochem.*, 1995, **64**, 65.

14 K. Yoon, C. A. Hobbs, J. Koch, M. Sardaro, R. Kuntny and A. L. Weis, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 3840; I. Radhakrishnan and D. J. Patel, *J. Mol. Biol.*, 1994, **241**, 600.

15 No triplex possessing three consecutive GP^B-CG or T-CG base triplets was observed under the same conditions. Previously, it was reported that consecutive T-CG base triplets imparted a drastic decrease in stability of triplexes. D. M. Gowers and K. R. Fox, *Nucleic Acids Res.*, 1997, **25**, 3787.

16 Extremely high stability of the triplex with every third CG might be caused by the low number of C-GC triplets, *i.e.*, less electrostatic repulsion between positive-charges of protonated C and GP^B in TFO.

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Synthesis of novel polyesteramine dendrimers by divergent and convergent methods

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ABSTRACT

Novel dendrimers having an adamantane structure as a core were synthesized such that even low generation dendrimers had a globular structure. Moreover we tried to give them biodegradable function by using ester bonds. Synthesis of the dendrimers, particularly at higher generations, proved difficult via a stepwise procedure, and thus a convergent route was used in which the adamantane core is coupled to the dendritic segments in the final step. We achieved the synthesis of two separate dendrimers with convergent methods till the third generations. The convergent dendrimers were synthesized in good yields compared with divergent one and both dendrimers were found to have narrow polydispersities by GPC analysis.

1. Introduction

Dendrimers are very interesting macromolecules with highly branched structures and globular shapes. Molecular sizes of dendrimers are increased stepwise via repeated reaction sequences. Since the first dendrimers were synthesized by Tomalia et al. [1–3], many kinds of dendrimers have been synthesized [4], used not only for chemical applications but also for biomedical applications [5–11]. For example, commercially available polyamidoamine (PAMAM) and polypropylenimine (PPI) dendrimers are widely used as drugs [6], gene delivery systems [7], and MRI contrast agents [9]. Additionally, these dendrimers provide a high gene transfer efficiency into mammalian cells [12–15]. This transfer efficiency is considered to be a result of the many interior tertiary amines, which exist in the dendrimer, leading to an effect known as a proton sponge [16]. Moreover, these dendrimers have many functional groups such as amino groups and hydroxyl groups on their periphery [12–15,17], and modification of these surface groups with various molecules offers the chance for other potential applications [18–23]. However, for medical applications, dendrimers must be less toxic and more biodegradable than such dendrimers. Recently polyester dendrimers called 'biodendrimers' have been reported [24–26], which have building blocks known to be biocompatible or degradable to natural metabolites in vivo. Other types of polyester dendrimers have been synthesized [27–29] and have shown an antitumor effect [29]. Furthermore, a robust and biodegradable PEGylated dendrimer based on a polyester-polyamide hybrid core has been synthesized and biodistribution and chemotherapy study in tumored mice have been evaluated [30]. However, there are few reports on polyester dendrimers including primary and tertiary amines. To form complexes with plasmid DNA, antisense oligonucleotide or siRNA and other biological molecules, it is necessary for the dendrimers to have primary amines. These amino groups would not only allow complex formation, but would also interact with cellular membranes and enable conjugation with various ligands.

In this study, we designed novel polyester dendrimers **X**–**Z** named 'polyesteramine dendrimers' (Fig. 1).

As the core of the dendrimer, we selected an adamantane structure. Typically, planar or linear molecules, such as ammonia, ethylenediamine, 1,4-diaminobutane, benzene derivatives, lactic acid, succinic acid, adipic acid, and ethylene glycol, have been used for the dendrimer core [4,24–26,28]. These dendrimers maintain a planar structure in lower generations. Adamantane, on the other hand, has a three-dimensional structure, and dendrimers having an adamantane core are expected to have a more globular structure than dendrimers such as PAMAM even in lower generations. In terms of synthesis, PAMAM dendrimers are synthesized by a typical stepwise and iterative two-step reaction sequence [1–3], consisting of amidation of methyl acrylate with ethylenediamine and Michael

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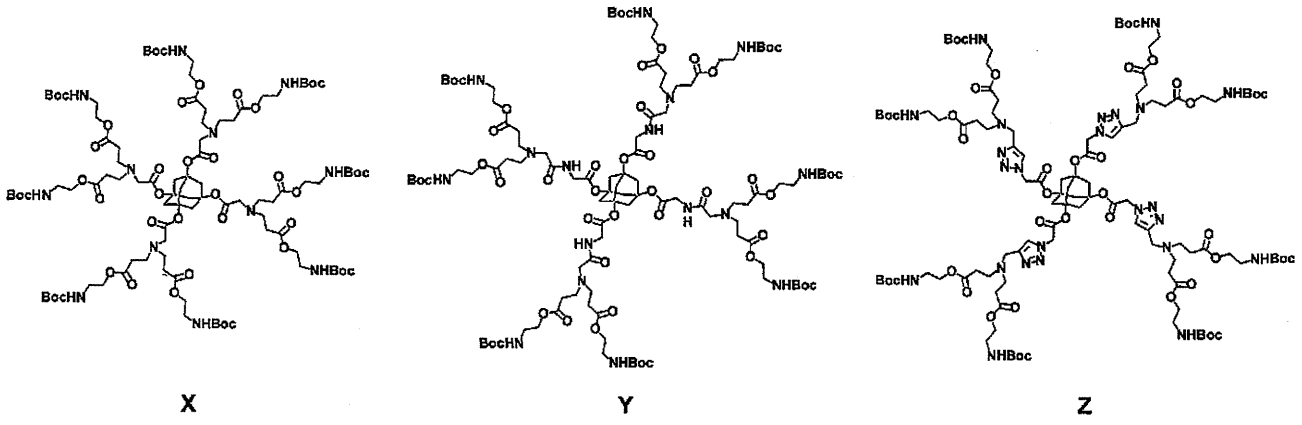


Fig. 1. Structures of polyester dendrimers **X**, **Y**, and **Z**.

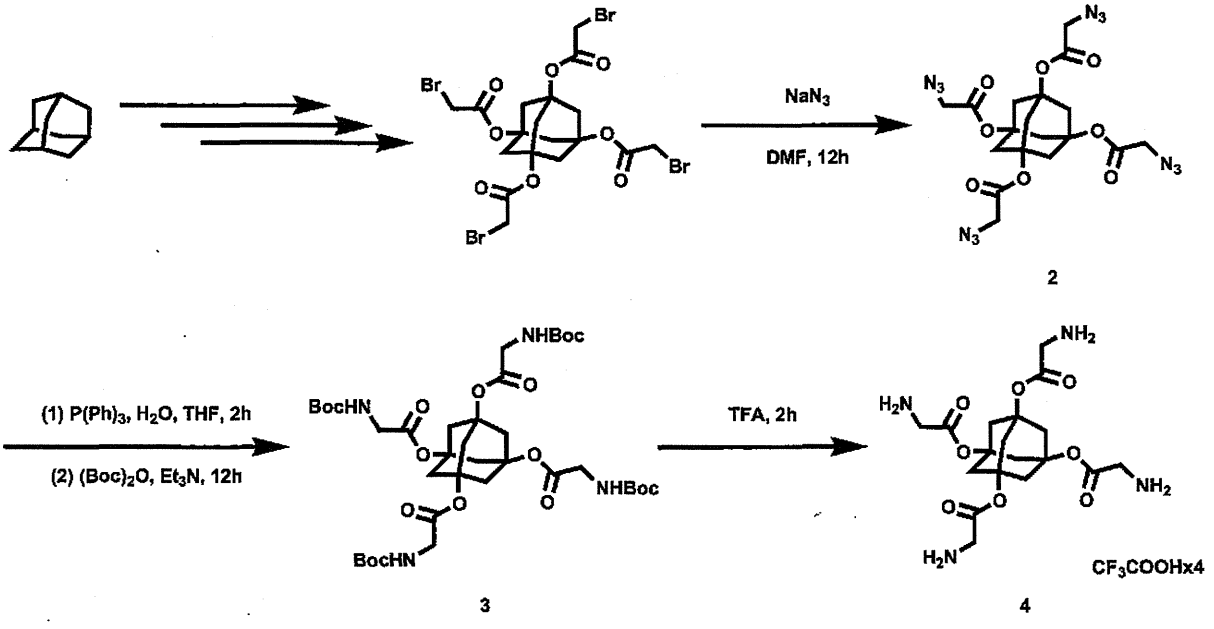
addition of primary amines with methyl acrylate. But it is known that this method sometimes leads to a lot of structural defects and also requires a long reaction time, which is a critical impediment for obtaining dendrimers with a uniform molecular weight, particularly in higher generations [1,2]. Separation of dendrimers having primary amines in the periphery is also a difficult task. In order to resolve these problems, we designed a novel dendrimer having a three-dimensional adamantane core, and synthesized dendrimers via two separate convergent routes employing amidation and Huisgen [3+2] cycloaddition reaction as the key coupling reactions, respectively.

2. Results

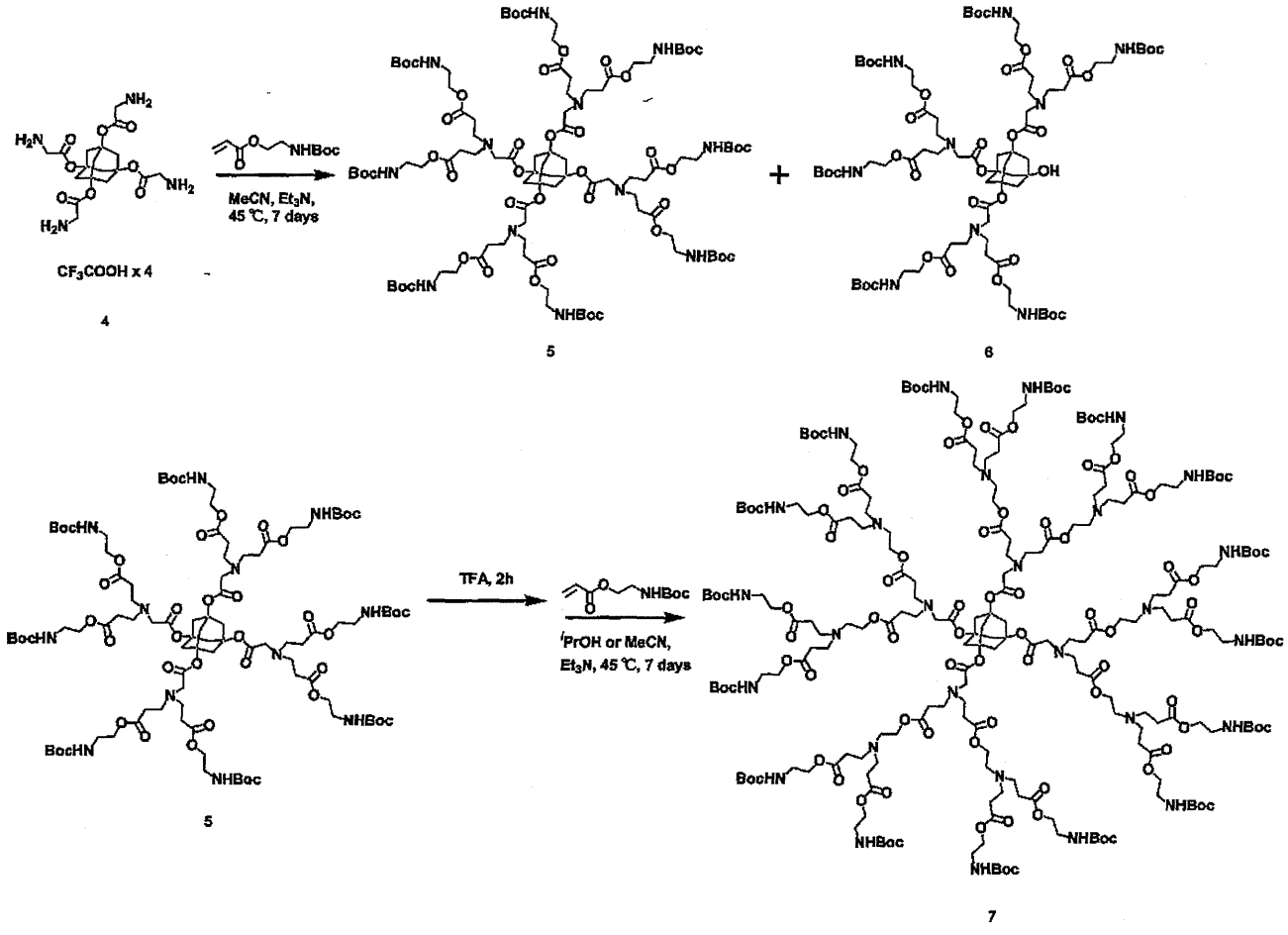
1,3,5,7-Tetrakis(aminoacetoxy)adamantane core **4** was synthesized as shown in Scheme 1. 1,3,5,7-Tetrakis(bromoacetoxy)adamantane, prepared according to a literature procedure, was treated with NaN_3 to give azidoacetoxy derivative **2** in 73% yield. Although several attempts to obtain **4** by direct reduction of **2** resulted in a complex mixture, Boc-protected derivative **3** was successfully obtained by reduction of **2** using triphenylphosphine and simultaneous Boc-protection in 76% yield. Deprotection of the Boc group of **3** by treatment with trifluoroacetic acid (TFA) smoothly took place, and the desired glycinoyloxy derivative **4** was isolated as a tetratrifluoroacetate salt in 91% yield.

At first, we examined the usual stepwise elongation method for the synthesis of dendrimer **7** as shown in Scheme 2. Michael reaction of **4** with 2-Boc-aminoethylacrylate **1**, prepared from 2-Boc-aminoethanol and acryloyl chloride, proceeded to give the first generation dendrimer **5** in 32% yield accompanied by the deacylated product **6** in 32% yield. Although we examined the reaction under various conditions, it was not possible to prevent formation of the deacylated product **6**. Next, deprotection of dendrimer **5** by TFA followed by Michael reaction was carried out. However, unfortunately, a complex mixture was given. MALDI-TOF-MS analysis of the crude product showed the existence of a number of incompletely reacted products (Fig. 2). The desired second generation dendrimer **7** was also detected by the spectrum, but could not be isolated from the mixture.

These results indicated that it was going to be difficult to obtain higher generation dendrimers having a uniform molecular weight by the present stepwise method. Therefore, we selected a convergent method for the synthesis of the higher generation dendrimers. We planned for the adamantane core to be coupled with dendritic



Scheme 1. Synthesis of two types of adamantane core.



Scheme 2. Synthesis of novel dendrimers by divergent method.

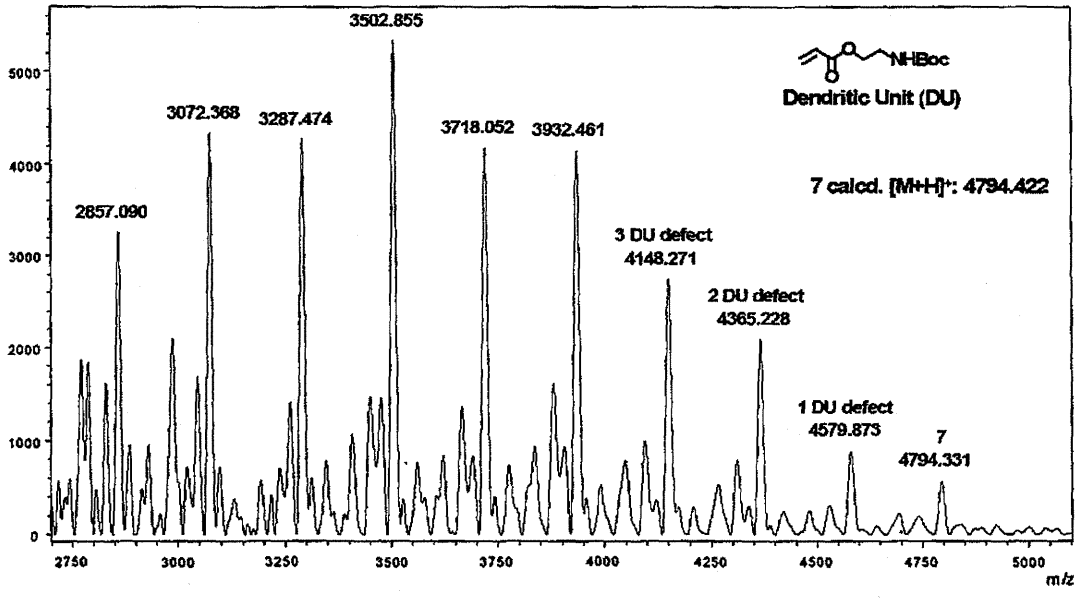


Fig. 2. MALDI-TOF-MS spectra of crude Boc-G2 (**7**).

segments by amide formation. The segments were to be synthesized according to a usual procedure (deprotection and Michael reaction) as shown in Scheme 3. Michael reaction of Gly–OBn with acrylate **1** afforded bisadduct **8**, which corresponds to the segment for the first generation, in 93% yield. The segment **9** for the second generation was prepared from **8** by deprotection of the Boc group followed by Michael reaction in 37% yield. Similarly, the segment **10** for the third generation was obtained in 22% yield.