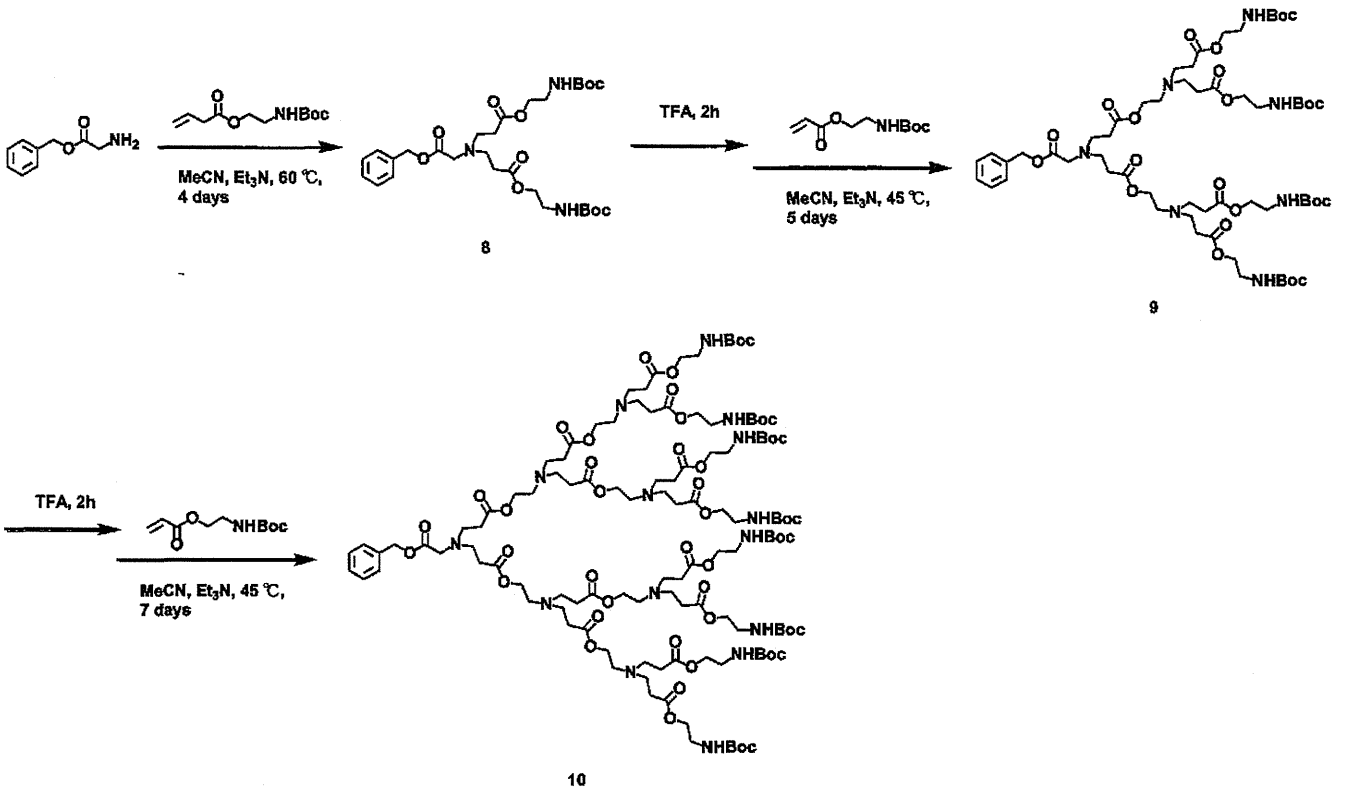


Scheme 3. Synthesis of benzyl ester glycine dendrons.

The coupling reaction between the adamantane core **4** with the segment was carried out as shown in Scheme 4. After removal of the benzyl ester of **8** under reductive conditions, the resultant carboxylic acid was coupled with the adamantane core **4** using PyBOP as a condensing agent to give **11** in 87% yield; PyBOP is known to be superior to DCC, TFFH, and a number of other commercially available peptide coupling reagents [31]. The segments for the higher generation dendrimers, **9** and **10**, were also coupled with **4** according to the same procedure to give **12** and **13** in 36% and 22% yields, respectively.

Recently, Huisgen [3+2] dipolar cycloaddition reaction has gained much attention in general synthetic chemistry [32], and has also been applied to the synthesis of dendrimers [33–38]. As it is obvious that azido-intermediate **2** would work as a substrate for the Huisgen reaction with a proper alkyne derivative, we synthesized novel segments having an alkyne structure as shown in Scheme 5. In this case, propargyl amine was employed for the starting material, which reacted quantitatively with acrylate **1** to give **14** as the first generation segment. Segment **15** for the second generation, and segment **16** for the third generation were also obtained in 24% and 14% yields, respectively, via sequential reactions (deprotection by TFA treatment and Michael reaction with acrylate).

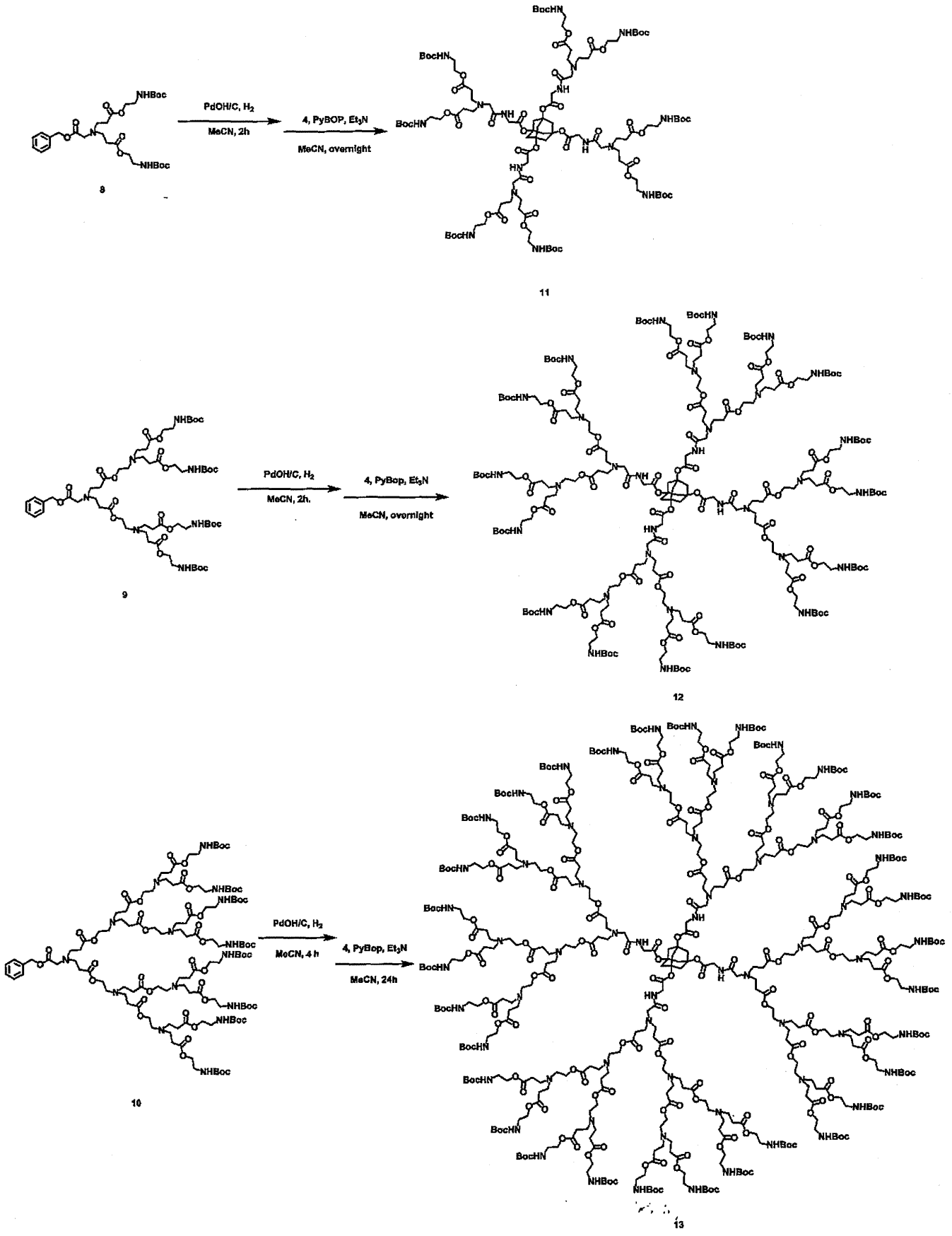
As shown in Scheme 6, dendrimers **17**, **18**, and **19**, were obtained by coupling of azido-derivative **2** with segments **14**, **15**, and **16**, respectively, under the typical conditions of Huisgen reaction. The yield for dendrimers of the higher generation decreased, but was nonetheless satisfactory.

Molecular weight data determined by MALDI-TOF-MASS and gel permeation chromatography (GPC) and polydispersity indices (PDIs) are summarized in Table 1. The mass spectrometry data agrees with the calculated molecular weights. All dendrimers show narrow PDIs, indicating that these compounds were obtained in pure form, thus confirming the effectiveness of a convergent route for the synthesis of dendrimers.

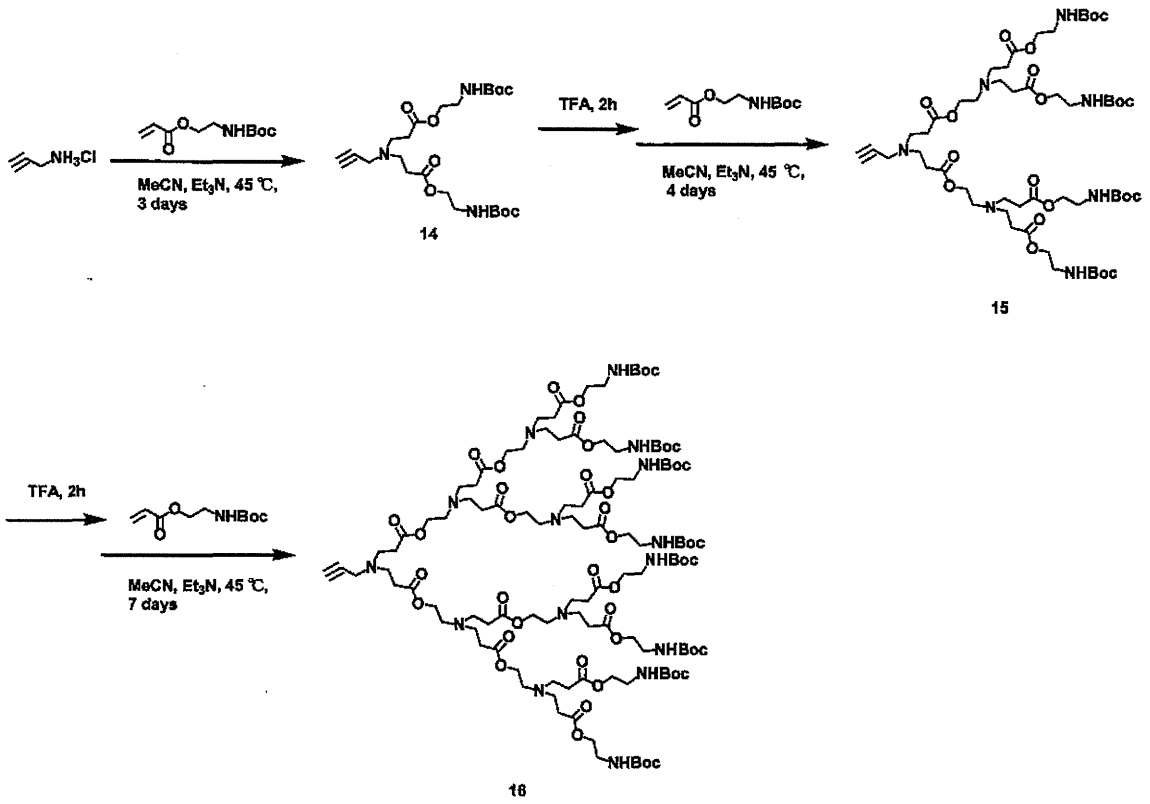
3. Discussion

We aimed to synthesize biodegradable polyester dendrimers including primary and tertiary amino groups for biochemical and medical applications. We chose adamantane as a dendrimer core to produce novel dendrimers that were more globular in lower generations than commercially available dendrimers and many other reported dendrimers. The number-average molecular weight (M_n) and weight-average molecular weight (M_w), which were determined by GPC measurements, deviate from the mass spectrometry data as the generation number increases (Table 1). This result is consistent with the previous data of dendrimers adopting a more globular structure [25,26]. In this study, amide glycine dendrimers **11**–**13** (AG Boc-G1–3) and click chemistry dendrimers **17**–**19** (CC Boc-G1–3) show less M_w than the expected one even at the low generation numbers (G2 and G3). These results suggest that the novel dendrimers possessing an adamantane core have a more globular structure even in lower generations. We constructed molecular models of G1, AG G1, and CC G1, which are compared with PAMAM G1 (Fig. 3). Although PAMAM G1 is planar, the novel G1, AG G1, and CC G1 dendrimers are more steric due to a tetrahedral core. We expect these novel dendrimers to grow up more spherically.

It is known that the transfection activity of PAMAM dendrimers increases with higher generations [12,13]. This enhanced activity is largely considered to be a result of the spherical structure of the dendrimers. That is, the more spherical the dendrimer structures, the better their ability as a gene carrier. However, it is also the case that the higher the dendrimer generation, the greater the toxicity of the dendrimer [8]. If the dendrimers can be degraded and metabolized at physiological conditions after drug delivery, they



Scheme 4. Synthesis of amide glycine dendrimers.



Scheme 5. Synthesis of click dendrons.

would have greater application as non-viral carriers. Recently, a variety of biodegradable polymers have been used as non-viral carriers for plasmid DNA delivery [39]. Park et al. reported that a poly(amino ester) including primary and tertiary amines and esters exhibited relatively slow biodegradability, as the DNA/polymer complex was maintained for 7 days [40]. By contrast, in synthesis of 1,3,5,7-tetrakis(aminoacetoxy)adamantane bearing free primary amines, generation of primary amino groups led to readily self-degradation. In the synthesis of divergent dendrimer Boc-G1 **5**, the generation of primary amino groups also led to the appearance of incomplete Boc-G1 **6**. Fife et al. reported that the existence of intramolecular neighboring amino groups effectively catalyzed ester hydrolysis [41]. Thus, this phenomenon would be due to high degradability of 1,3,5,7-tetrakis(aminoacetoxy)adamantane bearing neighboring amines. From these results, we considered that the emergence of free primary amino groups is an obstacle to synthesis of polyesteramine dendrimers because of degradation of the adamantane core. Thus, it is very important to limit generation of free primary amines in the synthesis of polyesteramine dendrimers. Using two separate convergent methods, we were able to produce polyesteramine dendrimers until the third generation. The yields of the two kinds of dendrimers synthesized by a convergent approach drop with higher generations, which is consistent with previous observations [31,36]. This could be due to significant retention of the polar dendrimers in the silica column, steric hindrance of the higher generation dendrons, and partial degradability.

4. Conclusion

In summary, we have presented divergent and convergent procedures for the synthesis of novel polyesteramine dendrimers. Convergent approaches were more efficient than the divergent method in synthesizing higher generation dendrimers. By monitoring Michael addition reactions in synthesis of the higher generation dendrons and dendrimers with MALDI-TOF-MASS, we found that they were not attained completely in higher generations. Though the low yields must be improved, these methods provide interesting new polyesteramine dendrimers and insight into possible modifications or changes of the periphery and core of such dendrimers. We are currently working toward the characterization of the dendrimers given here and the synthesis of a variety of surface-modified dendrimers. Polyesteramine dendrimers offer many potential platforms in areas such as medicine, catalysis, photonics, and nanotechnology.

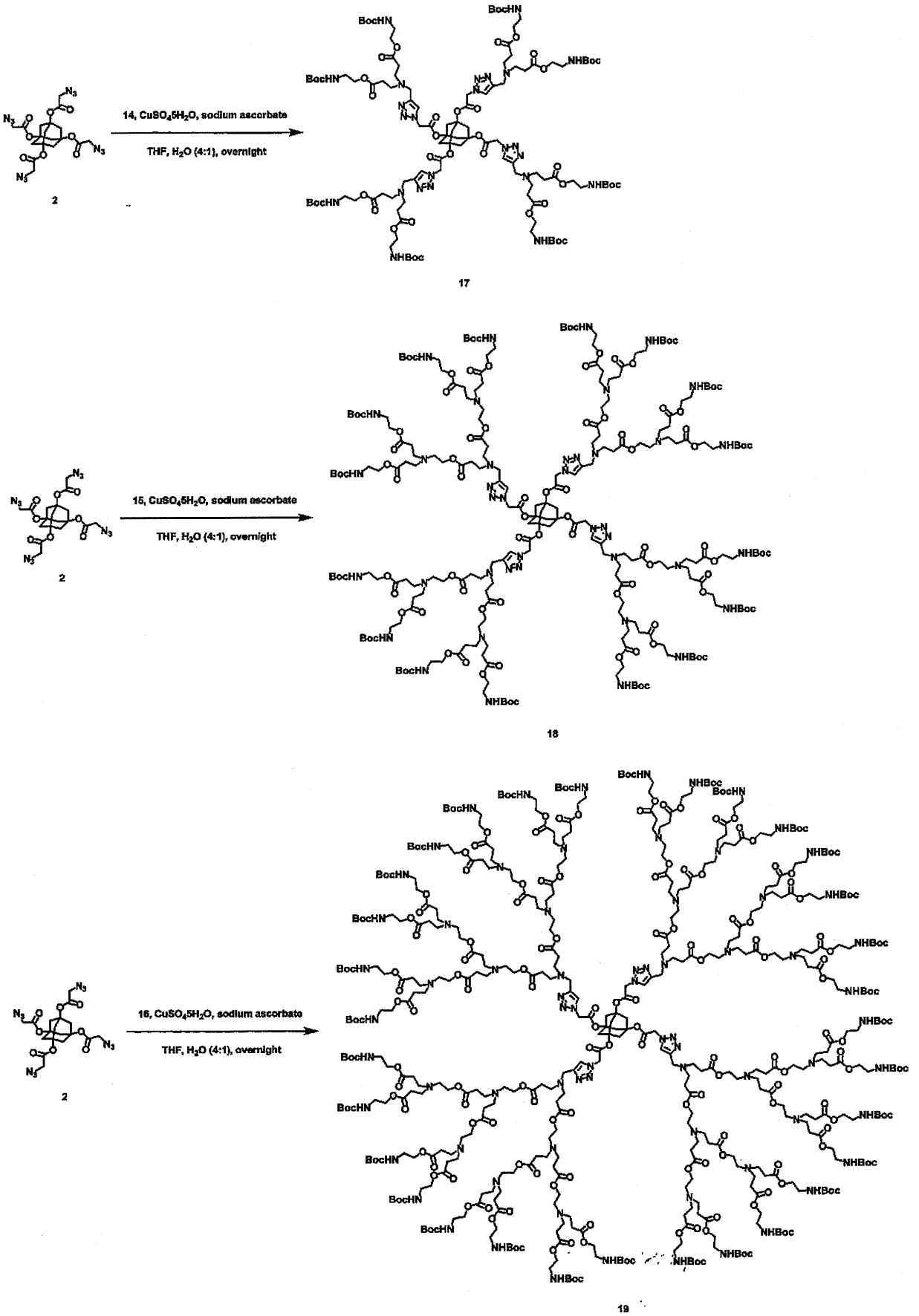
5. Experimental

5.1. General

All solvents were dried and freshly distilled prior to use. All chemicals were purchased from chemical suppliers. For column chromatography, Fuji Silysia silica gel PSQ-100B (0.100 mm) and FL-100D (0.100 mm) was used. ^{1}H NMR (270, 300 or 400 MHz) and ^{13}C NMR (67 or 75 MHz) spectra were recorded on JEOL JNM-EX270, JEOL JNM-AL300, and JEOL JNM-ECS400 spectrometers, respectively. IR spectra were recorded on a JASCO FT/IR-200 and JASCO FT/IR-4200 spectrometer. 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 mass spectrometer. Gel permeation chromatography (GPC) was performed using THF as the eluent on a SHIMADZU column and refractive index detector. Polystyrene standards (820, 2460, 4100, 12,400, and 18,100) were used for calibration.

5.2. Synthesis of 2-Boc-aminoethylacrylate 1

The dendritic unit **1** was prepared by modification of a published procedure [42]. 2-Aminoethanol (25.4 g, 0.41 mol) was



Scheme 6. Synthesis of click chemistry dendrimers.

Table 1
MALDI-TOF-MS and GPC data for novel polyesteramine dendrimers

Dendrimer	GPC		M_w/M_n	MALDI-TOF-MS	
	M_n	M_w		calcd M_w	Found
3	1167	1178	1.01	829	852 $[M+Na]^+$
5	2623	2689	1.03	2150	2151 $[M+H]^+$
11	2625	2701	1.03	2379	2379 $[M+H]^+$
12	4536	4745	1.05	5022	5022 $[M+H]^+$
13	8550	9066	1.06	10,308	10,304 $[M+H]^+$
17	2985	3150	1.06	2475	2475 $[M+H]^+$
18	5114	5486	1.07	5118	5119 $[M+H]^+$
19	8568	8912	1.04	10,404	10,404 $[M+H]^+$

(s, 8H). ^{13}C NMR ($CDCl_3$) δ=43.0, 50.5, 79.1, 167.0. MS (FAB): *m/z* 555 $[M+Na]^+$. HRMS (FAB): *m/z* calcd for $C_{18}H_{20}N_{12}O_8$ $[M+Na]^+$: 555.1527; found: 555.1417. Anal. Calcd for $C_{18}H_{20}N_{12}O_8$: C, 40.61; H, 3.79; N, 31.57. Found: C, 40.58; H, 3.78; N, 31.22.

5.3.2. Synthesis of 1,3,5,7-tetrakis(n-Boc-aminoacetoxy)adamantane **3**. The adamantane compound **2** (2.4 g, 4.43 mmol) was dissolved in a 3:1 solvent ratio of THF/H_2O (29.6 ml), and triphenylphosphine (5.1 g, 19.48 mmol) was added. After stirring for 2 h, $(Boc)_2O$ (4.3 g, 19.48 mmol) and triethylamine (3.7 ml, 26.57 mmol) were added to the reaction mixture and stirred overnight. The reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purifica-

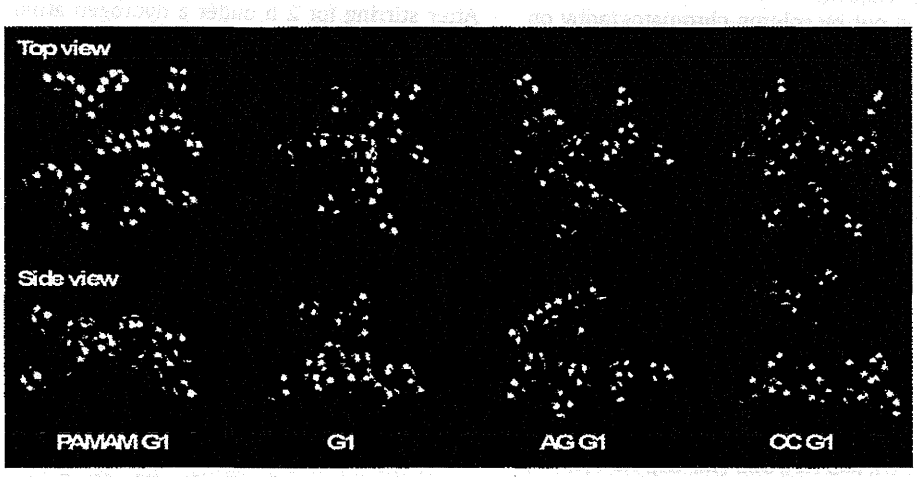


Fig. 3. Molecular models of PAMAM G1, G1, AG G1, and CC G1 constructed by Spartan '06.

dissolved in $CHCl_3$ (500 ml) in a flask equipped with a magnetic stirrer, and $(Boc)_2O$ (100.0 g, 0.45 mol) was added. After stirring for 30 min, the concentrated solution was diluted with CH_2Cl_2 (500 ml), and triethylamine (113.6 ml, 0.82 mol) and acryloyl chloride (44.5 g, 0.49 mol) were added. After stirring for 1 h under a nitrogen atmosphere, satd $NaHCO_3$ aq solution (200 ml) was added to the reaction mixture. The aqueous layer was extracted with CH_2Cl_2 and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and hexane as eluent. A pale yellowish oil; 74.9 g, 85%. FTIR: ν (cm^{-1}) 3371, 3095, 3043, 2983, 2938, 1986, 1706. ^1H NMR ($CDCl_3$): δ=1.37 (s, 9H), 3.33–3.38 (m, 2H), 4.15 (t, 2H, *J*=5 Hz), 4.98 (br, 1H), 5.78 (dd, 1H, *J*=1, 10 Hz), 6.06 (dd, 1H, *J*=10, 17 Hz), 6.36 (dd, 1H, *J*=1, 17 Hz). ^{13}C NMR ($CDCl_3$): δ=28.2, 39.5, 63.6, 79.3, 127.9, 131.1, 155.7, 165.9. MS (FAB): *m/z* 216 $[M+H]^+$. HRMS (FAB): *m/z* calcd for $C_{10}H_{17}N_1O_4$ $[M+H]^+$: 216.1158; found: 216.1226. Anal. Calcd for $C_{10}H_{17}NO_4$: C, 55.80; H, 7.96; N, 6.51. Found: C, 55.93; H, 7.87; N, 6.47.

5.3. Synthesis of adamantane core

5.3.1. Synthesis of 1,3,5,7-tetrakis(azidoacetoxy)adamantane **2**. 1,3,5,7-Tetrakis(bromoacetoxy)adamantane was synthesized from adamantane in three steps according to a literature method [43]. 1,3,5,7-Tetrakis(bromoacetoxy)adamantane (200.0 mg, 0.29 mmol) was dissolved in DMF (2.9 ml) and sodium azide (152.0 mg, 2.34 mmol) was added. After stirring for 2 h under a nitrogen atmosphere, water was added to the reaction mixture together with CH_2Cl_2. The aqueous layer was extracted with CH_2Cl_2 and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using $CHCl_3$. A white solid; 112.7 mg, 73%. FTIR: ν (cm^{-1}) 2923, 2208, 2108, 1739. ^1H NMR ($CDCl_3$) δ=2.62 (s, 12H), 3.80

tion of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and hexane as eluent. A white foam; 2.8 g, 76%. FTIR: ν (cm^{-1}) 3362, 2978, 2934, 1755, 1707. ^1H NMR (CD_3Cl) δ=1.45 (s, 36H), 2.53 (s, 12H), 3.82 (d, 8H, *J*=6 Hz), 4.93 (t, 4H, *J*=5 Hz). ^{13}C NMR ($CDCl_3$) δ=28.3, 42.8, 43.1, 78.6, 80.1, 155.6, 169.0. MS (FAB): *m/z* 851 $[M+Na]^+$. HRMS (FAB): *m/z* calcd for $C_{38}H_{60}N_4O_{16}$ $[M+Na]^+$: 851.4004; found: 851.3887. MS (MALDI): *m/z* calcd for $C_{38}H_{60}N_4O_{16}$ $[M+Na]^+$: 851.400; found: 851.746.

5.3.3. Synthesis of 1,3,5,7-tetrakis(aminoacetoxy)adamantane tetratrifluoroacetate **4**. Compound **3** (2.0 g, 2.38 mmol) was added to TFA (7.5 ml) and stirred for 2 h. The concentrated compound was reprecipitated with ethyl acetate and lyophilized. A white solid; 1.9 g, 91%. FTIR: ν (cm^{-1}) 3362, 2978, 2934, 1755, 1707. ^1H NMR (D_2O) δ=2.64 (s, 12H), 3.87 (s, 8H). ^{13}C NMR (CD_3OD) δ=41.5, 43.8, 80.9, 167.2. MS (FAB): *m/z* 429 $[M+H]^+$. HRMS (FAB): *m/z* calcd for $C_{18}H_{28}N_4O_8$ $[M+H]^+$: 429.1907; found: 429.1967. Anal. Calcd for $C_{26}H_{32}F_{12}N_4O_{16}\cdot H_2O$: C, 34.60; H, 3.80; N, 6.21. Found: C, 34.44; H, 3.97; N, 5.95.

5.4. Synthesis of dendrimers by divergent method

5.4.1. Synthesis of Boc-G1 **5**. Compound **4** (519.8 mg, 0.59 mmol) was dissolved in CH_3CN (5.9 ml), and **1** (5.1 g, 23.51 mmol) and triethylamine (650 μl, 4.70 mmol) were added. After stirring for 7 days at 45 °C under a nitrogen atmosphere, the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and hexane as eluent. A pale yellowish foam; 403.8 mg, 32%. FTIR: ν (cm^{-1}) 3380, 2977, 1713. ^1H NMR ($CDCl_3$) δ=1.36 (s, 72H), 2.36–2.43 (m, 28H),

2.89 (t, 16H, J=6 Hz), 3.26–3.32 (m, 24H), 4.06 (t, 16H, J=5 Hz), 5.14 (br, 8H). ^{13}C NMR ($CDCl_3$) δ=28.3, 33.4, 39.4, 43.3, 49.3, 54.8, 63.5, 78.0, 79.2, 155.6, 169.6, 171.9. MS (FAB): m/z 2150 $[M+H]^+$. MS (MALDI): m/z calcd for $C_{98}H_{164}N_{12}O_{40}$ $[M+H]^+$: 2150.117; found: 2150.650.

5.4.2. Synthesis of Boc-G2 **7**. Compound **5** (112 mg, 0.05 mmol) was added to TFA (2.0 ml) and stirred for 2 h. The concentrated compound was reprecipitated with ethyl acetate and lyopholized. The deprotected compound (100 mg, 0.04 mmol) was dissolved in ^{i}PrOH (0.4 ml), and **1** (1.5 g, 7.07 mmol) and triethylamine (123 µl, 0.88 mmol) were added. After stirring for 7 days at 45 °C under a nitrogen atmosphere, the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and acetone as eluent. A yellowish gum; trace.

5.5. Synthesis of benzyl ester glycine dendrons

5.5.1. Synthesis of BnGD Boc-G1 **8**. Glycine benzyl ester *p*-toluenesulfonate (2.0 g, 5.92 mmol) was dissolved in CH_3CN (10 ml), and **1** (3.8 g, 17.78 mmol) and triethylamine (1.6 ml, 11.86 mmol) were added. After stirring for 4 days at 60 °C under a nitrogen atmosphere, the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and hexane as eluent. A pale yellowish oil; 3.3 g, 93%. FTIR: ν (cm^{-1}) 3730, 3592, 3368, 3064, 2977, 1958, 1712. ^{1}H NMR ($CDCl_3$) δ=1.43 (s, 18H), 2.46 (t, 4H, J=7 Hz), 2.98 (t, 4H, J=7 Hz), 3.33–3.39 (m, 4H), 3.46 (s, 2H), 4.12 (t, 4H, J=5 Hz), 5.13 (m, 4H), 7.34 (s, 5H). ^{13}C NMR ($CDCl_3$) δ=28.4, 33.4, 39.6, 49.6, 54.5, 63.7, 66.3, 79.4, 128.2, 128.3, 128.5, 135.4, 155.7, 170.8, 172.1. MS (FAB): m/z 596 $[M+H]^+$. HRMS (FAB): m/z calcd for $C_{29}H_{45}N_3O_{10}$ $[M+H]^+$: 596.3105; found: 596.3177.

5.5.2. Synthesis of BnGD Boc-G2 **9**. Compound **8** (721 mg, 1.21 mmol) was added to TFA (3 ml) and stirred for 2 h. The concentrated compound was lyopholized. The deprotected compound (916 mg, 1.24 mmol) was dissolved in CH_3CN (14.1 ml), and **1** (6.1 g, 28.31 mmol) and triethylamine (1.2 ml, 8.49 mmol) were added. After stirring for 5 days at 45 °C under a nitrogen atmosphere, the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and hexane as eluent. A yellowish gum; 577 mg, 37%. FTIR: ν (cm^{-1}) 3730, 3372, 2976, 2011, 1957, 1715. ^{1}H NMR ($CDCl_3$) δ=1.37 (s, 36H), 2.39 (t, 12H, J=7 Hz), 2.64 (t, 4H, J=6 Hz), 2.75 (t, 8H, J=7 Hz), 2.92 (t, 4H, J=7 Hz), 3.27–3.33 (m, 8H), 3.40 (s, 2H), 4.02–4.08 (m, 12H), 5.06 (s, 2H), 5.17 (br, 4H), 7.28 (s, 5H). ^{13}C NMR ($CDCl_3$) δ=28.3, 32.7, 33.2, 39.4, 49.3, 49.6, 51.8, 54.4, 62.0, 63.5, 66.0, 79.2, 128.0, 128.0, 128.3, 135.3, 155.5, 170.6, 171.8, 171.9. MS (FAB): m/z 1256.6 $[M+H]^+$. HRMS (FAB): m/z calcd for $C_{59}H_{97}N_7O_{22}$ $[M+H]^+$: 1256.6687; found: 1256.6769. MS (MALDI): m/z calcd for $C_{59}H_{97}N_7O_{22}$ $[M+H]^+$: 1256.669; found: 1256.432.

5.5.3. Synthesis of BnGD Boc-G3 **10**. Compound **9** (535 mg, 0.43 mmol) was added to TFA (2 ml) and stirred for 2 h. The concentrated compound was lyopholized. The deprotected compound (631 mg, 0.38 mmol) was dissolved in CH_3CN (3.8 ml), and **1** (3.3 g, 15.24 mmol) and triethylamine (740 µl, 5.33 mmol) were added. After stirring for 7 days at 45 °C under a nitrogen atmosphere, the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and acetone as eluent. A brownish gum; 212 mg, 22%. FTIR: ν (cm^{-1}) 3726, 3593, 3371, 2977, 2837, 2013, 1729. ^{1}H NMR ($CDCl_3$) δ=1.35 (s, 72H), 2.38 (t, 28H, J=6 Hz), 2.63 (t, 12H, J=5 Hz), 2.74 (t, 24H, J=6 Hz), 2.89 (t, 4H, J=7 Hz), 3.27–3.32 (m, 16H), 3.37 (s, 2H), 4.01–4.07 (m, 28H), 5.04 (s, 2H), 5.19 (br, 8H), 7.27 (s, 5H). ^{13}C NMR ($CDCl_3$) δ=28.3, 32.5, 32.7, 33.2, 39.4, 49.3, 49.4, 49.6, 51.7, 51.8, 54.5, 62.0, 62.1, 63.4, 66.0, 79.1, 127.9, 128.0, 128.3, 135.4, 155.5, 170.6, 171.7, 171.8, 171.8. MS (MALDI): m/z calcd for $C_{119}H_{201}N_{15}O_{46}$ $[M+H]^+$: 2578.943; found: 2576.640.

5.6. Synthesis of amide glycine dendrimers

5.6.1. Synthesis of AG Boc-G1 **11**. Compound **8** (150 mg, 0.25 mmol) was dissolved in CH_3CN (2.5 ml) and PdOH/C (150 mg) was added. After stirring for 2 h under a hydrogen atmosphere, the reaction mixture was filtered. The concentrated compound was dissolved in CH_3CN (2.5 ml), and **4** (37 mg, 0.042 mmol), PyBOP (109 mg, 0.21 mmol), and triethylamine (47 ul, 0.34 mmol) were added. After stirring for 1 day at room temperature under a nitrogen atmosphere, the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and acetone as eluent. A pale yellowish foam; 87 mg, 87%. FTIR: ν (cm^{-1}) 3331, 2974, 2927, 1712. ^{1}H NMR ($CDCl_3$) δ=1.43 (s, 72H), 2.49–2.53 (m, 28H), 2.80 (t, 16H, J=6 Hz), 3.13 (s, 8H), 3.33–3.40 (m, 16H), 3.93 (d, 8H, J=5 Hz), 4.12 (t, 16H, J=5 Hz), 5.28 (br, 8H), 8.04 (t, 4H, J=6 Hz). ^{13}C NMR ($CDCl_3$) δ=28.5, 32.1, 39.5, 41.4, 43.1, 49.6, 58.3, 63.9, 78.5, 79.4, 155.8, 168.2, 171.3, 172.2. MS (MALDI): m/z calcd for $C_{106}H_{176}N_{16}O_{44}$ $[M+H]^+$: 2379.613; found: 2378.987.

5.6.2. Synthesis of AG Boc-G2 **12**. Compound **9** (188 mg, 0.15 mmol) was dissolved in CH_3CN (1.5 ml) and PdOH/C (190 mg) was added. After stirring for 2 h under a hydrogen atmosphere, the reaction mixture was filtered. The concentrated compound was dissolved in CH_3CN (1.5 ml), and **4** (22 mg, 0.025 mmol), PyBOP (97 mg, 0.19 mmol), and triethylamine (28 µl, 0.20 mmol) were added. After stirring overnight at room temperature under a nitrogen atmosphere, the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and acetone as eluent. A yellowish gum; 45 mg, 36%. FTIR: ν (cm^{-1}) 3364, 2926, 2854, 1713. ^{1}H NMR ($CDCl_3$) δ=1.42 (s, 144H), 2.47–2.51 (m, 60H), 2.74–2.85 (m, 64H), 3.14 (s, 8H), 3.33–3.38 (m, 32H), 3.91 (d, 8H, J=4 Hz), 4.12 (t, 48H, J=5 Hz), 5.26 (br, 16H), 7.96 (t, 4H, J=5 Hz). ^{13}C NMR ($CDCl_3$) δ=28.4, 31.7, 32.6, 39.5, 49.3, 49.8, 52.0, 57.9, 63.7, 78.5, 79.3, 155.7, 171.3, 172.0. MS (MALDI): m/z calcd for $C_{226}H_{384}N_{32}O_{92}$ $[M+H]^+$: 5022.628; found: 5021.661.

5.6.3. Synthesis of AG Boc-G3 **13**. Compound **10** (162 mg, 0.063 mmol) was dissolved in CH_3CN (0.63 ml) and PdOH/C (160 mg) was added. After stirring for 4 h under a hydrogen atmosphere, the reaction mixture was filtered. The concentrated compound was dissolved in CH_3CN (0.63 ml), and **4** (9 mg, 0.010 mmol), PyBOP (41 mg, 0.079 mmol), and triethylamine (12 µl, 0.083 mmol) were added. After stirring for 1 day at room temperature under a nitrogen atmosphere, the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and acetone as eluent. A brownish gum; 24 mg, 22%. FTIR: ν (cm^{-1}) 3364, 2925, 1701. ^{1}H NMR ($CDCl_3$) δ=1.43 (s, 288H), 2.46 (t, 124H, J=6 Hz), 2.72 (t, 48H, J=6 Hz), 2.82 (t, 112H, J=6 Hz), 3.15 (s, 8H), 3.33–3.39 (m, 64H), 3.91 (s, 8H), 4.09–4.14 (m, 112H), 5.23 (br, 32H), 7.94 (br, 4H). ^{13}C NMR ($CDCl_3$) δ=28.4, 32.7, 39.6, 49.7, 52.0, 62.2, 63.6, 79.4, 155.9, 172.3.

MS (MALDI): *m/z* calcd for $C_{466}H_{800}N_{64}O_{188}$ $[M+H]^+$: 10,308.657; found: 10,303.944.

5.7. Synthesis of click dendrons

5.7.1. Synthesis of CD Boc-G1 **14**. Propargyl amine hydrochloride (915 mg, 10.0 mmol) was dissolved in CH_3CN (10 ml), and **1** (6.5 g, 30.0 mmol) and triethylamine (2.8 ml, 20.00 mmol) were added. After stirring for 3 days at 45 °C under a nitrogen atmosphere, the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and hexane as eluent. A pale yellowish oil; 5.3 g, quant. FTIR: ν (cm^{-1}) 3955, 3728, 3592, 3356, 2976, 2104, 2013, 1706. ^1H NMR ($CDCl_3$) δ=1.34 (s, 18H), 2.16 (t, 1H, J=2 Hz), 2.38 (t, 4H, J=7 Hz), 2.74 (t, 4H, J=7 Hz), 3.23–3.30 (m, 4H), 3.35 (d, 2H, J=2 Hz), 4.04 (t, 4H, J=5 Hz), 5.21 (br, 2H). ^{13}C NMR ($CDCl_3$) δ=28.7, 33.2, 39.8, 41.9, 49.2, 63.8, 74.0, 77.9, 79.5, 156.0, 172.2. MS (FAB): *m/z* 486 $[M+H]^+$. HRMS (FAB): *m/z* calcd for $C_{23}H_{39}N_3O_8$ $[M+H]^+$: 486.2737; found: 486.2823.

5.7.2. Synthesis of CD Boc-G2 **15**. Compound **14** (1.2 g, 2.54 mmol) was added to TFA (5.0 ml) and stirred for 2 h. The concentrated compound was lyopholized. The deprotected compound (510 mg, 0.81 mmol) was dissolved in CH_3CN (8.1 ml), and **1** (3.5 g, 16.26 mmol) and triethylamine (676 μl, 4.87 mmol) were added. After stirring for 4 days at 45 °C under a nitrogen atmosphere, the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and hexane as eluent. A yellowish gum; 223 mg, 24%. FTIR: ν (cm^{-1}) 3725, 3372, 2976, 2103, 2014, 1711. ^1H NMR ($CDCl_3$) δ=1.40 (s, 36H), 2.19 (t, 1H, J=2 Hz), 2.43 (t, 12H, J=7 Hz), 2.69 (t, 4H, J=6 Hz), 2.79 (t, 12H, J=7 Hz), 3.31–3.37 (m, 10H), 4.06–4.12 (m, 12H), 5.14 (br, 4H). ^{13}C NMR ($CDCl_3$) δ=28.1, 32.6, 32.6, 39.3, 41.5, 48.6, 49.5, 51.7, 61.9, 63.4, 73.2, 77.6, 79.1, 155.4, 171.6, 171.8. MS (FAB): *m/z* 1146.6 $[M+H]^+$. HRMS (FAB): *m/z* calcd for $C_{53}H_{91}N_7O_{20}$ $[M+H]^+$: 1146.6319; found: 1146.6372. MS (MALDI): *m/z* calcd for $C_{53}H_{91}N_7O_{20}$ $[M+H]^+$: 1146.632; found: 1146.663.

5.7.3. Synthesis of CD Boc-G3 **16**. Compound **15** (196 mg, 0.17 mmol) was added to TFA (3.0 ml) and stirred for 2 h. The concentrated compound was lyopholized. The deprotected compound was dissolved in CH_3CN (1.6 ml), and **1** (1.4 g, 6.56 mmol) and triethylamine (318 μl, 2.30 mmol) were added. After stirring for 7 days at 45 °C under a nitrogen atmosphere, the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and acetone as eluent. A brownish gum; 55 mg, 14%. FTIR: ν (cm^{-1}) 3727, 3374, 2976, 2838, 2102, 2014, 1731. ^1H NMR ($CDCl_3$) δ=1.42 (s, 72H), 2.22 (t, 1H, J=2 Hz), 2.45 (t, 28H, J=7 Hz), 2.70 (t, 12H, J=6 Hz), 2.81 (t, 28H, J=7 Hz), 3.33–3.39 (m, 18H), 4.08–4.14 (m, 28H), 5.15 (br, 8H). ^{13}C NMR ($CDCl_3$) δ=28.4, 32.7, 32.9, 32.9, 39.6, 41.8, 48.9, 49.6, 49.8, 51.9, 52.0, 62.2, 62.4, 63.7, 73.5, 78.0, 79.4, 155.7, 171.9, 172.0, 172.0. MS (MALDI): *m/z* calcd for $C_{113}H_{195}N_{15}O_{44}$ $[M+H]^+$: 2468.832; found: 2468.015.

5.8. Synthesis of click chemistry dendrimers

5.8.1. Synthesis of CC Boc-G1 **17**. Compounds **2** (50 mg, 0.090 mmol) and **14** (201 mg, 0.41 mmol) were dissolved in a 4:1 solvent ratio of THF/H_2O (4.1 ml), and $CuSO_4 \cdot 5H_2O$ (21 mg, 0.082 mmol) and sodium ascorbate (33 mg, 0.17 mmol) were added. After stirring overnight, the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and acetone as eluent. A pale yellowish foam; 184 mg, 82%. FTIR: ν (cm^{-1}) 3367, 2928, 1701. ^1H NMR ($CDCl_3$) δ=1.34 (s, 72H), 2.41–2.45 (m, 28H), 2.69 (t, 16H, J=6 Hz), 3.23–3.29 (m, 16H), 3.75 (s, 8H), 4.03 (t, 16H, J=5 Hz), 5.06 (s, 8H), 5.30 (br, 8H), 7.58 (s, 4H). ^{13}C NMR ($CDCl_3$) δ=28.3, 32.5, 39.4, 42.6, 47.9, 48.7, 50.9, 53.8, 63.4, 79.1, 124.1, 144.0, 155.6, 164.8, 172.0. MS (MALDI): *m/z* calcd for $C_{110}H_{176}N_{24}O_{40}$ $[M+H]^+$: 2475.712; found: 2474.737.

5.8.2. Synthesis of CC Boc-G2 **18**. Compounds **2** (25 mg, 0.046 mmol) and **15** (318 mg, 0.28 mmol) were dissolved in a 4:1 solvent ratio of THF/H_2O (2.8 ml), and $CuSO_4 \cdot 5H_2O$ (14 mg, 0.055 mmol) and sodium ascorbate (22 mg, 0.11 mmol) were added. After stirring overnight, the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and acetone as eluent. A yellowish gum; 85 mg, 36%. FTIR: ν (cm^{-1}) 3371, 2976, 2110, 1731. ^1H NMR ($CDCl_3$) δ=1.42 (s, 144H), 2.42–2.53 (m, 60H), 2.70 (t, 16H, J=6 Hz), 2.81 (t, 48H, J=6 Hz), 3.33–3.39 (m, 32H), 3.82 (s, 8H), 4.08–4.13 (m, 48H), 5.13 (s, 8H), 5.22 (br, 16H), 7.64 (s, 4H). ^{13}C NMR ($CDCl_3$) δ=28.4, 32.5, 32.9, 39.6, 42.9, 48.1, 48.7, 49.8, 52.0, 62.2, 63.7, 79.4, 124.1, 155.7, 164.9, 172.1. MS (MALDI): *m/z* calcd for $C_{230}H_{384}N_{40}O_{88}$ $[M+H]^+$: 5118.727; found: 5118.790.

5.8.3. Synthesis of CC Boc-G3 **19**. Compounds **2** (8 mg, 0.014 mmol) and **16** (221 mg, 0.089 mmol) were dissolved in a 4:1 solvent ratio of THF/H_2O (890 μl), and $CuSO_4 \cdot 5H_2O$ (4 mg, 0.018 mmol) and sodium ascorbate (7 mg, 0.036 mmol) were added. After stirring overnight, the reaction mixture was extracted with ethyl acetate and dried over Na_2SO_4. Purification of the concentrated compound was carried out by column chromatography on silica gel using ethyl acetate and acetone as eluent. A brownish gum; 30 mg, 21%. FTIR: ν (cm^{-1}) 3363, 2925, 1703. ^1H NMR ($CDCl_3$) δ=1.43 (s, 288H), 2.42–2.54 (m, 124H), 2.71 (t, 48H, J=6 Hz), 2.82 (t, 112H, J=7 Hz), 3.34–3.40 (m, 64H), 3.83 (s, 8H), 4.09–4.15 (m, 112H), 5.14 (s, 8H), 5.24 (br, 32H), 7.64 (s, 4H). ^{13}C NMR ($CDCl_3$) δ=28.5, 32.7, 32.9, 39.6, 49.6, 49.8, 52.0, 62.2, 63.7, 79.4, 155.7, 172.1, 172.1. MS (MALDI): *m/z* calcd for $C_{470}H_{800}N_{72}O_{184}$ $[M+H]^+$: 10,404.756; found: 10,403.867.

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Bioorganic & Medicinal Chemistry 21 (2013) 5583–5588

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Triplex-forming ability of oligonucleotides containing 1-aryl-1,2,3-triazole nucleobases linked via a two atom-length spacer

Yoshiyuki Hari *, Motoi Nakahara, Satoshi Obika *

Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 1 May 2013
Revised 22 May 2013
Accepted 23 May 2013
Available online 31 May 2013

Keywords:
Artificial nucleobases
1,3-Dipolar cycloaddition
Triazoles
Triplex DNA
Triplex-forming oligonucleotides

ABSTRACT

Phosphoramidites containing 2-propynyloxy or 1-butyn-4-yl as nucleobase precursors were synthesized and introduced into oligonucleotides using an automated DNA synthesizer. Copper-catalyzed alkyne-azide 1,3-dipolar cycloaddition of the oligonucleotides with various azides gave the corresponding triazolylated oligonucleotides, triplex-forming ability of these synthetic oligonucleotides with double-stranded DNA targets was evaluated by UV melting experiments. It was found that nucleobases containing 2-(1-*m*-carbonylaminophenyl-1,2,3-triazol-4-yl)ethyl units likely interacted with A of a TA base pair in a parallel triplex DNA.

1. Introduction

A triplex-forming oligonucleotide (TFO) that can form a triplex DNA with double-stranded DNA (dsDNA) would be a promising tool for gene-targeting therapy and genetic diagnosis.[1] In general, triplex DNA can be sequence-selectively formed by recognition of AT and GC base pairs in dsDNA by T and C in TFO via Hoogsteen hydrogen bonds, respectively. The sequence of dsDNA targeted by TFO is limited to these purine–pyrimidine base pairs. Therefore, towards recognition of pyrimidine–purine base pairs such as CG and TA, a number of synthetic nucleotides have been developed to date.[2] Concerning CG base pair recognition, several promising nucleotides have already been synthesized. In contrast, there is almost no nucleotide recognizing a TA base pair with high selectivity and stability. The difficulty to find nucleotides for TA base pair recognition is considered to be caused by disturbance of hydrogen bonding interaction with the 4-carbonyl group due to the steric bulkiness of the 5-methyl group in T.

Recently, we have tried to develop nucleotides capable of recognizing a CG base pair by means of the post-elongation modification (PEM) methods such as copper-catalyzed alkyne-azide 1,3-dipolar cycloaddition[3] and nucleophilic substitution reaction[4] to construct artificial nucleobases. Since this PEM method allows us to efficiently synthesize various derivatives in a short period, the detailed molecular design of nucleobases based on the experimental results and their evaluation can be implemented. Therefore, we considered that the PEM method can be applied to investigate nucleobases for TA base pair recognition and this could bring useful information concerning molecular design for TA recognition. In this study, nucleobases with two atom-length spacers such as methyleneoxy and ethylene were designed to avoid the steric repulsion from 5-methyl group of T and with the recognition unit to bind to the opposite A. The recognition unit was synthesized by using copper-catalyzed alkyne-azide 1,3-dipolar cycloaddition as PEM method to investigate the fine structure (Fig. 1). The details of our results obtained are described.

2. Results and discussion

2.1. Synthesis

The synthesis of an oligonucleotide **1** bearing a 2-propynyloxy group was carried out according to Scheme 1. Chlorosugar **2**

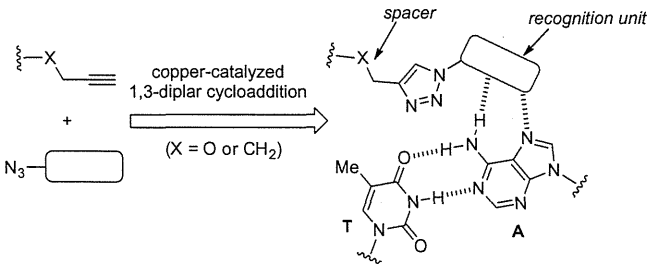

Figure 1. Nucleobases designed in this study.

* Corresponding authors. Tel./fax: +81 6 6879 8201 (Y. Hari); tel.: +81 6 6879 8200; fax: +81 6 6879 8204 (S. Obika).

E-mail addresses: hari@phs.osaka-u.ac.jp (Y. Hari), obika@phs.osaka-u.ac.jp (S. Obika).

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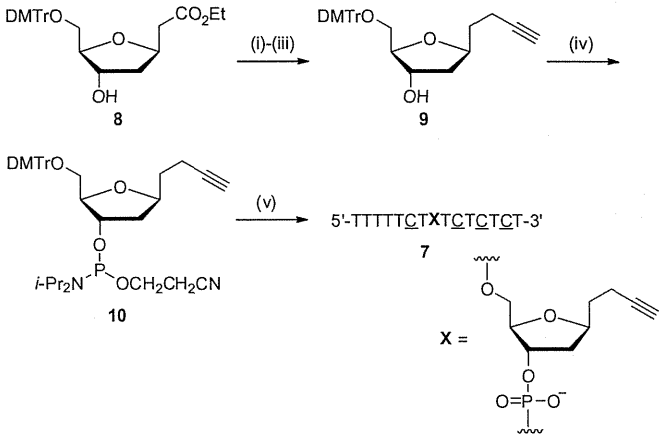
http://dx.doi.org/10.1016/j.bmc.2013.05.034

underwent reaction with propargyl alcohol in MeCN to give desired β-isomer **3** in 34% yield, the stereochemistry of which was determined by NOESY correlations of deprotected **4** shown in Scheme 1. The primary alcohol of **4** was protected by DMTrCl in the presence of pyridine and phosphitylation of **5** gave phosphoramidite **6**, a building block for oligonucleotide synthesis. The oligonucleotide precursor **1** for copper-catalyzed alkyne-azide 1,3-dipolar cycloaddition was prepared on an automated DNA synthesizer using standard phosphoramidite chemistry.

The synthesis of oligonucleotide **7** with the incorporation of 1-butyn-4-yl group was shown in Scheme 2. Reduction of compound **8** prepared according to the report[5] with $LiAlH_4$, tosylation followed by treatment with lithium acetylide–ethylenediamine complex was carried out in three-steps; this gave the compound **9** in 53% yield. Phosphoramidite **10** was obtained by phosphitylation of **9** and the yield was 97%.

Copper-catalyzed alkyne-azide 1,3-dipolar cycloaddition of oligonucleotides **1** and **7** with various azides was carried out according to the reaction conditions previously reported in our earlier study.[3a] This was performed to convert the oligonucleotides into TFOs (Scheme 3, and see experimental for the synthesis of azides). In all cases, the corresponding TFOs **11a–h** and **12a–j** were successfully obtained by reversed-phase HPLC purification after the reaction.

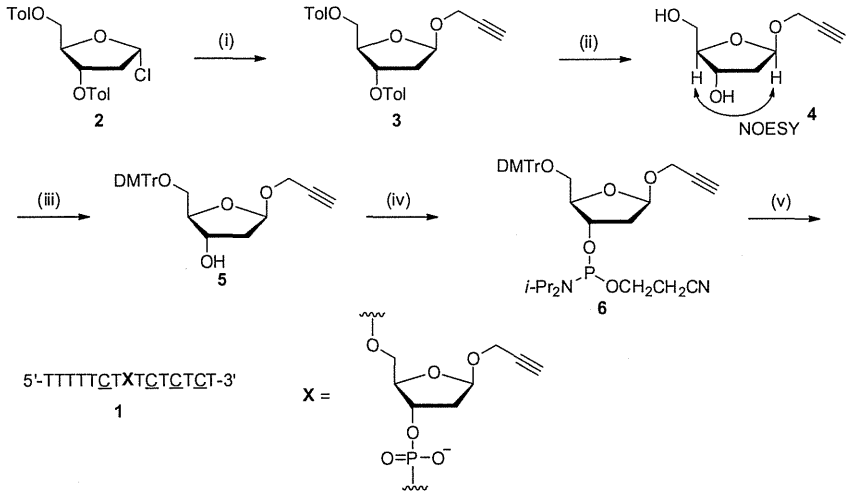


Scheme 2. Reagents and conditions: (i) $LiAlH_4$, THF, 0 °C, 4 h, (ii) TsCl, pyridine, rt, 12 h, (iii) lithium acetylide–ethylenediamine complex, THF, rt, 9 h, 53% for three-steps, (iv) *i*-$Pr_2NP(Cl)OCH_2CH_2CN$, *i*-Pr_2NEt, CH_2Cl_2, rt, 5 h, 97%, (v) DNA synthesis (C = 2′-deoxy-5-methylcytidine).

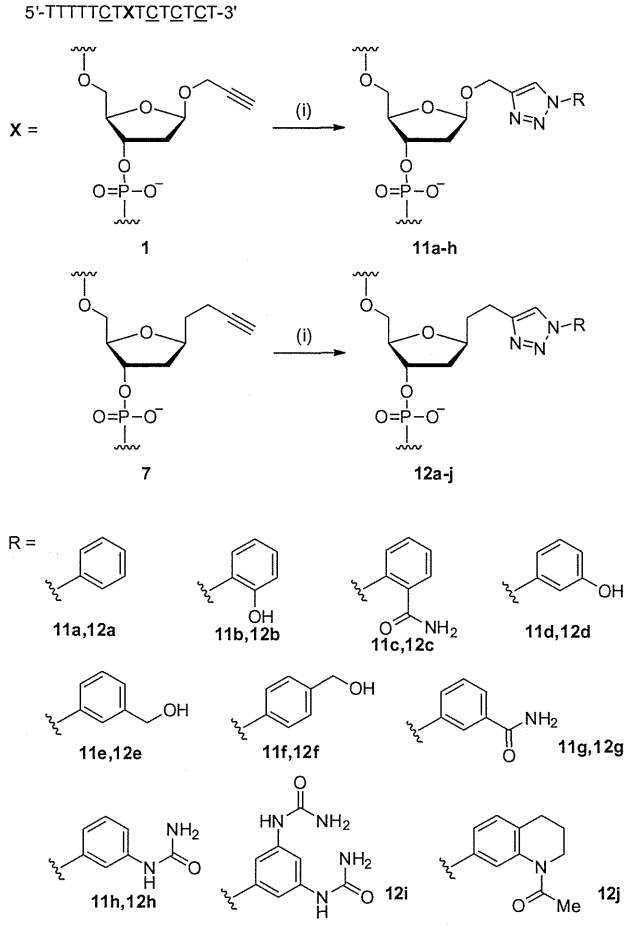
2.2. Evaluation

In triplex DNA between TFO and dsDNA, base pair-recognition ability of the nucleobases consisting of 1-aryl-1,2,3-triazol-4-yl unit and spacer unit in TFO was evaluated by UV-melting experiment. The results of TFOs **11** with methyleneoxy spacer and TFOs **12** with ethylene spacer are summarized in Tables 1 and 2, respectively. The measurement was carried out under neutral conditions and hairpin dsDNA targets containing four kinds of natural base pairs, TA, CG, GC and AT base pairs for YZ were used (For details see footnote of Table 1). Regarding TFOs **11** with methyleneoxy spacer, when substituent at 1-position of triazole was a simple phenyl group (**11a**) without any functional group for hydrogen bond formation, the range of T_m values against all four hairpin dsDNA targets was from 20 °C to 22 °C. *o*-Hydroxyphenyl derivative (**11b**) gave almost same T_m values as **11a**, while *o*-carbamoyl congener (**11c**) led to a decrease in T_m values against any base pairs likely due to the existence of bulky carbamoyl group at the *o*-position (Fig. 2). Among TFOs **11d–h** including *m*- or *p*-substituted phenyl analogs, only TFO **11e** with *m*-hydroxymethyl group and TFO **11h** with *m*-ureidophenyl one seem to stabilize the triplexes with dsDNA (YZ = TA) compared to that observed with TFO **11a** including an unsubstitued phenyl group though without stabilization selective to dsDNA (YZ = TA) (Table 1 and Fig. 2).

Among TFOs **12** in which an ethylene spacer was incorporated, unsubstitued phenyl analog (**12a**) increased the T_m value by +2 °C against dsDNA for either TA or CG base pair compared to the corresponding methyleneoxy spacer (**11a**). *o*-Substituents (**12b** and **12c**) decreased the stability of triplexes formed with all dsDNA targets (Fig. 3). This is likely due to the steric repulsion between the *o*-substituents and the target base pairs. No major changes were observed in TFOs **12d–f**. TFO **12g** containing *m*-carbamyl group stabilized the triplex with dsDNA (YZ = TA) and the ΔT_m value observed was +3 °C. Significant improvement was observed in TFO **12h** containing the *m*-ureido group. The ΔT_m value of **12h** against dsDNA with TA base pair was +6 °C, which was significantly higher than that observed (ΔT_m = +2 °C to +3 °C) against the other dsDNA targets.



Scheme 1. Reagents and conditions: (i) propargyl alcohol, MeCN, rt, 4 h, 34%, (ii) NaOMe, MeOH, rt, 5 h, quant., (iii) DMTrCl, pyridine, rt, 6 h, 98%, (iv) *i*-$Pr_2NP(Cl)OCH_2CH_2CN$, *i*-Pr_2NEt, CH_2Cl_2, rt, 15 h, 90%, (v) DNA synthesis (C = 2′-deoxy-5-methylcytidine).



Scheme 3. Reagents and conditions: (i) R-N_3, $CuSO_4$, sodium ascorbate, tris[(1-benzyl-1,2,3-triazol-4-yl)methyl]amine (TBTA), DMSO-phosphate buffer (10 mM, pH 7.0), rt, 0.5–12 h, 67–91%.

Table 1
T_m values (°C) of triplexes between TFOs **11** including a methyleneoxy spacer and four hairpin dsDNA targets[a,b]

TFO	YZ			
	TA	CG	GC	AT
11a	20	22	22	20
11b	20 (±0)	23 (+1)	20 (−2)	20 (±0)
11c	17 (−3)	21 (−1)	19 (−3)	18 (−2)
11d	21 (+1)	24 (+2)	21 (−1)	20 (±0)
11e	22 (+2)	25 (+3)	23 (+1)	23 (+3)
11f	19 (−1)	22 (±0)	19 (−3)	19 (−1)
11g	19 (−1)	23 (+1)	20 (−2)	19 (−1)
11h	23 (+3)	25 (+3)	23 (+1)	21 (+1)

[a] Conditions: 10 mM sodium cacodylate buffer (pH 6.8), 100 mM KCl and 50 mM $MgCl_2$. The final concentration of each oligonucleotide used was 1.89 μM. The sequence of hairpin dsDNA targets is as follows: 5′-GGCAAAAAGA**Y**AGAGAGACGC-hexaethyleneglycol-GCGTCTCT**Z**TCTTTTTGCC-3′ (YZ = TA, CG, GC or AT).
[b] ΔT_m: Difference in the T_m value from that of **11a** is shown in parenthesis.

In the above analysis on substituent in the phenyl ring (Tables 1 and 2), TFO **12** with ethylene spacer showed higher ΔT_m values than TFO **11** with methyleneoxy spacer (Figs. 2 and 3). In addition, a high ΔT_m value of TFO **12h** to dsDNA (YZ = TA) was observed when compared with TFO **11h** with the same *m*-ureidophenyl group. These results may imply that the ethylene spacer is less flexible than methyleneoxy spacer and consequently the

Table 2
T_m values (°C) of triplexes between TFOs **12** including a ethylene spacer and four hairpin dsDNA targets[a,b]

TFO	YZ			
	TA	CG	GC	AT
12a	22	24	20	21
12b	18 (−4)	22 (−2)	19 (−1)	20 (−1)
12c	18 (−4)	20 (−4)	19 (−1)	16 (−5)
12d	22 (±0)	22 (−2)	21 (+1)	21 (±0)
12e	23 (+1)	23 (−1)	20 (±0)	19 (−2)
12f	22 (±0)	23 (−1)	19 (−1)	19 (−2)
12g	25 (+3)	25 (+1)	22 (+2)	22 (+1)
12h	28 (+6)	27 (+3)	23 (+3)	23 (+2)
12i	27 (+5)	27 (+3)	23 (+3)	22 (+1)
12j	27 (+5)	26 (+2)	21 (+1)	21 (±0)

[a] Conditions are shown in the footnote of Table 1.
[b] ΔT_m: Difference in the T_m value from that of **12a** is shown in parenthesis.

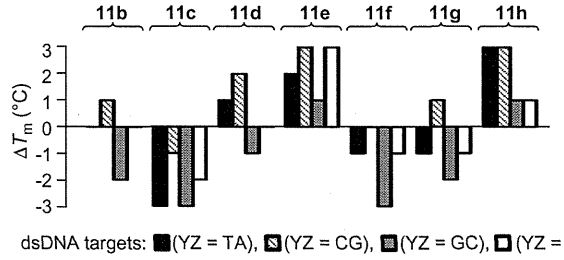


Figure 2. The difference in the T_m values of TFOs **11b–h** from that of TFO **11a**.

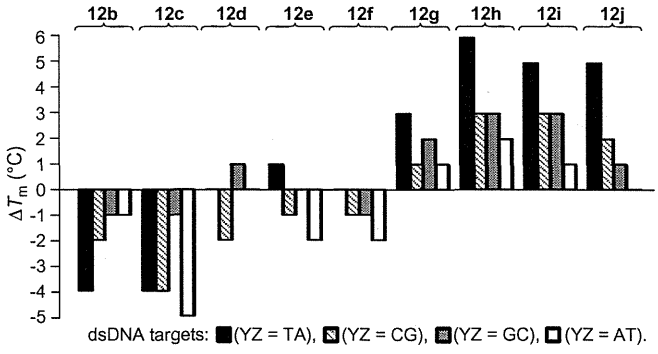


Figure 3. The difference in the T_m values of TFOs **12b–j** from that of TFO **12a**.

aryltriazole moiety attached to ethylene spacer lies in a suitable space for TA base pair recognition.

C_2-Symmetric 3,5-bisureidophenyl group (**12i**) designed with consideration of free rotation of bond between aryl moiety and triazole ring gave almost same result observed with the monoureido derivative (**12h**). Interestingly, ΔT_m value of *N*-acetyl-1,2,3,4-tetrahydroquinoline (**12j**) to the TA base pair was +5 °C. The results of TFOs **12h–j** strongly suggest that the carbonyl oxygen of the common 2-(1-*m*-carbonylaminophenyl-1,2,3-triazol-4-yl)ethyl unit forms hydrogen bond-mediated recognition with A (probably with 6-amino group in A) of the TA base pair, as shown in Figure 4. In fact, under same conditions, the T_m value of triplex of TFO **12h** and dsDNA (YZ = TA) was comparable to that (T_m = 29 °C) of triplex containing a T-CG base triplet forming a single hydrogen bond though this was lower than that (T_m = 32 °C) of triplex containing a G-TA base triplet.[6] The above results do not imply that TFOs **12h–j** formed a stable and selective triplex to only dsDNA (YZ = TA) among all four dsDNA targets. However, this result would provide

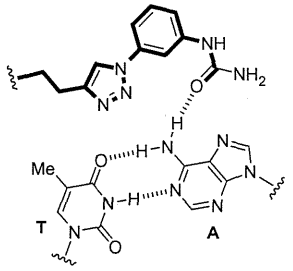


Figure 4. Plausible recognition mode of a TA base pair by 2-[1-(*m*-ureidophenyl)-1,2,3-triazol-4-yl]ethyl nucleobase in TFO **12h**. The moiety displayed in black is 2-(1-*m*-carbonylaminophenyl-1,2,3-triazol-4-yl)ethyl unit.

useful information on the design of nucleobase for TA base pair recognition in the formation of triplex DNA.

3. Conclusion

Oligonucleotides bearing two type of spacers attached to acetylene unit were synthesized and by the copper-catalyzed alkyne-azide 1,3-dipolar cycloaddition of them with various azides the preparation of oligonucleotides with the corresponding substituted triazoles was achieved. The evaluation of their triplex-forming ability with dsDNA demonstrated that 2-(1-*m*-carbonylaminophenyl-1,2,3-triazol-4-yl)ethyl unit could make a hydrogen bond to a TA base pair though the stability and sequence-selectivity of the triplex formed was not satisfactory as assessed by UV-melting experiments. Our results obtained could contribute considerably in finding of nucleobases to recognize a TA base pair at a practical level.

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 or JEOL AL300 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 or JEOL JMS-S3000 mass spectrometer. EYELA Cute Mixer CM-1000 was used as a shaker.

4.2. Prop-2-ynyl 2-deoxy-3,5-di-*O*-toluoyl-β-D-ribofuranoside 3

Under a nitrogen atmosphere, propargyl alcohol (360 μL, 9.25 mmol) was added to a solution of **2** (3.0 g, 7.71 mmol) in anhydrous CH_3CN (75 mL) at 0 °C and the mixture was stirred for 6 h at room temperature. After addition of water, the mixture was extracted with AcOEt. The organic extracts were washed with saturated $NaHCO_3$ 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 = 5:1) to give compound **3** (1.2 g, 39%) as a colorless oil. Compound **3**: $[\alpha]_D^{26}$ +138.0 (*c* 1.00, $CHCl_3$). IR ν_{max} (KBr) 1718, 1611, 1273, 1178, 1109, 1020 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.29 (1H, dd, *J* = 2.0, 14.5 Hz), 2.40–2.41 (7H, m), 2.54–2.61 (1H, m), 4.28 (1H, dd, *J* = 2.5, 15.5 Hz), 4.50–4.56 (2H, m), 4.62–4.66 (1H, m), 5.42–5.46 (1H, m), 5.51 (1H, d, *J* = 5.5 Hz), 7.21–7.24 (4H, m), 7.90–7.95 (4H, m). ^{13}C NMR (101 MHz, $CDCl_3$) δ 21.6, 21.7, 39.2, 54.2, 64.1, 74.2, 74.4, 81.5, 102.2, 122.4, 126.9, 127.0, 129.1, 129.1, 129.6, 129.8, 143.8, 143.9, 166.2, 166.4. MS (EI) *m*/*z* 408 (M^+, 100); HRMS (EI) *m*/*z* Calcd for $C_{24}H_{24}O_6$: 408.1573. Found 408.1580.

4.3. Prop-2-ynyl 2-deoxy-β-D-ribofuranoside 4

Under a nitrogen atmosphere, NaOMe (240 mg, 4.41 mmol) was added to a solution of **3** (600 mg, 1.47 mmol) in anhydrous MeOH (5 mL) at room temperature and the mixture was stirred for 5 h. The solvent was removed under reduced pressure and the residue was purified by flash silica gel column chromatography (*n*-hexane/AcOEt = 1:7) to give compound **4** (251 mg, quant) as a colorless oil. $[\alpha]_D^{26}$ +216.4 (*c* 1.00, CD_3OD); IR ν_{max} (KBr) 3284, 2928, 1086, 1034 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 1.78 (1H, ddd, *J* = 1.5, 3.5, 14.0 Hz), 2.26 (1H, ddd, *J* = 5.0, 8.0, 14.0 Hz), 2.70 (1H, t, *J* = 2.5 Hz), 3.48 (1H, dd, *J* = 5.0, 12.0 Hz), 3.57 (1H, dd, *J* = 3.5, 12.0 Hz), 3.80–3.84 (1H, m), 4.02–4.06 (1H, m), 5.25 (1H, dd, *J* = 1.5, 5.5 Hz); ^{13}C NMR (101 MHz, CD_3OD) δ 42.2, 54.7, 63.0, 72.2, 75.4, 80.5, 87.0, 103.1; MS (FAB) *m*/*z* 174 $[M+H]^+$; HRMS (FAB) *m*/*z* Calcd for $C_8H_{12}O_4$ $[M+H]^+$: 173.0814. Found 173.0809.

4.4. Prop-2-ynyl 2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranoside 5

Under a nitrogen atmosphere, 4,4′-DMTrCl (283, 0.836 mmol) was added to a solution of **4** (120 mg, 0.697 mmol) in anhydrous pyridine (5 mL) at room temperature and the mixture was stirred for 6 h. After addition of water, the mixture 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 **5** (324 mg, 98%) as a colorless oil. $[\alpha]_D^{23}$ +86.5 (*c* 1.03, $CDCl_3$); IR ν_{max} (KBr) 1607, 1509, 1444, 1301, 1251, 1177, 1084, 1034 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.07 (1H, d, *J* = 13.5 Hz), 2.25–2.31 (1H, m), 2.43 (1H, t, *J* = 2.5 Hz), 3.16 (2H, d, *J* = 4.5 Hz), 3.77 (6H, s), 4.19–4.24 (2H, m), 4.28 (1H, dd, *J* = 1.0, 2.5 Hz), 5.46 (1H, d, *J* = 5.0 Hz), 6.80–6.83 (4H, m), 7.17–7.32 (7H, m), 7.40–7.42 (2H, m). ^{13}C NMR (101 MHz, $CDCl_3$) δ 14.1, 41.1, 53.9, 55.1, 63.9, 73.2, 74.3, 79.3, 86.0, 86.9, 102.7, 113.0, 126.7, 127.7, 128.0, 130.0, 135.8, 135.9, 144.7, 158.4; MS (FAB) *m*/*z* 697 $[M+Na]^+$; HRMS (FAB) *m*/*z* Calcd for $C_{29}H_{30}NaO_6$ $[M+Na]^+$: 497.1935. Found 497.1944.

4.5. Prop-2-ynyl 3-*O*-[2-cyanoethoxy(diisopropylamino)-phosphino]-2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranoside 6

Under a nitrogen atmosphere, 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (40 μL, 0.177 mmol) was added to a solution of compound **5** (70 mg, 0.148 mmol) and *N*,*N*-diisopropylethylamine (75 μL, 0.443 mmol) in anhydrous CH_2Cl_2 (2 mL) at room temperature and the mixture was stirred for 15 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 = 4:1) to give compound **6** (90 mg, 90%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.00 (2H, d, *J* = 3.0 Hz), 1.11–1.16 (9H, m), 1.86–1.98 (3H, m), 2.52–2.62 (1H, m), 3.08–3.14 (1H, m), 3.28–3.41 (1H, m), 3.51–3.60 (3H, m), 3.78 (6H, s), 4.12–4.34 (4H, m), 5.43 (1H, m), 6.80–6.84 (4H, m), 7.19–7.35 (7H, m), 7.42–7.46 (2H, m); ^{31}P NMR (101 MHz, $CDCl_3$) δ 147.3, 148.2; MS (FAB) *m*/*z* 697 $[M+Na]^+$; HRMS (FAB) *m*/*z* Calcd for $C_{38}H_{47}N_2NaO_7P$ $[M+Na]^+$: 697.3019. Found 697.3049.

4.6. 1-(1-Butyn-4-yl)-1,2-dideoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranose 9

Under a nitrogen atmosphere, $LiAlH_4$ (360 mg, 9.47 mmol) was added to a solution of **8**[5] (1.2 g, 2.37 mmol) in anhydrous THF (20 mL) at room temperature and the mixture was stirred for 9 h. After addition of water, the mixture 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 = 5:1) to give compound **5** (1.1 g) as a colorless oil. This compound was not subjected to further purification and a portion of this was used in the next step. Under a nitrogen atmosphere, *p*-TsCl (246 mg, 1.29 mmol) was added to a solution of alcohol (500 mg, 1.08 mmol) in anhydrous pyridine (20 mL) at room temperature and the mixture was stirred for 12 h. After addition of water, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure. Flash silica gel column chromatography (*n*-hexane/AcOEt = 3:2) of the residue was performed to obtain appropriate compound [539 mg, Rf = 0.3 (*n*-hexane/AcOEt = 1:1)] as a colorless oil, 250 mg of this compound was dissolved in anhydrous THF (3 mL). Under a nitrogen atmosphere, lithium acetylide ethylenediamine complex (82 mg, 0.889 mmol) was added to the solution at room temperature and the mixture was stirred for 9 h. After addition of saturated NH_4Cl aq, the mixture 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 = 3:2) to give compound **9** (124 mg, 53% for three-steps) as a colorless oil. $[\alpha]_D^{24}$ +3.0 (*c* 0.31, $CHCl_3$); IR ν_{max} (KBr) 2933, 1607, 1509, 1445, 1301, 1251, 1177, 1074, 1034 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.73–1.80 (2H, m), 1.92–1.99 (3H, m), 2.27–2.36 (2H, m), 3.06 (1H, dd, *J* = 6.0, 10.0 Hz), 3.20 (1H, dd, *J* = 5.0, 10.0 Hz), 3.77 (6H, s), 3.86–3.90 (1H, m), 4.26–4.33 (2H, m), 6.80–6.83 (4H, m), 7.17–7.44 (9H, m); ^{13}C NMR (101 MHz, $CDCl_3$) δ 15.3, 34.5, 40.4, 55.1, 64.4, 68.5, 74.6, 83.9, 85.6, 86.1, 113.1, 126.7, 127.6, 128.1, 130.0, 136.0, 144.8, 158.4; HRMS (MALDI-TOF) *m/z* Calcd for $C_{30}H_{32}NaO_5$ $[M+Na]^+$: 495.2142. Found 495.2141.

4.7. 1-(1-Butyn-4-yl)-3-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-1,2-dideoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranose 10

Under a nitrogen atmosphere, 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (51 μL, 0.223 mmol) was added to a solution of compound **9** (90 mg, 0.190 mmol) and *N,N*-diisopropylethylamine (97 μL, 0.571 mmol) in anhydrous CH_2Cl_2 (2 mL) at room temperature and the mixture was stirred for 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 = 1:1) to give compound **10** (124 mg, 97%) as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$) δ 1.17 (3H, d, *J* = 7.0 Hz), 1.13–1.18 (9H, m), 1.74–1.89 (3H, m), 1.97–2.13 (2H, m), 2.30–2.46 (3H, m), 2,44 (1H, m), 3.07–3.18 (2H, m), 3.52–3.85 (10H, m), 4.05–4.11 (1H, m), 4.26–4.32 (1H, m), 4.40–4.47 (1H, m), 6.80–6.84 (4H, m), 7.16–7.46 (9H, m); ^{31}P NMR (162 MHz, $CDCl_3$) δ 147.5, 147.7; MS (FAB) *m/z* 673 $[M+H]^+$; HRMS (FAB) *m/z* Calcd for $C_{39}H_{50}N_2O_6P$ $[M+H]^+$: 673.3407. Found 673.3434.

4.8. Oligonucleotides 1 and 7

The synthesis of **1** and **7** was 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 and all the protecting groups on TFOs were removed by treatment with 28% NH_3 aq at room temperature for 3 h. The obtained crude TFOs were purified on Sep-Pak® Plus C18 cartridges (Waters) followed by reversed-phase HPLC (Waters XBridge® OST C18 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 **1** and 7:1, found 4423.01 (calcd 4423.94). **7**, found 4424.31 (calcd 4423.96).

4.9. Azide synthesis

Among azide reagents used for click chemistry, 2-azidobenzamide, 3-azidobenzamide, 1,1′-(5-azido-1,3-phenylene)diurea and *N*-acetyl-7-azide-1,2,3,4-tetrahydroquinoline were new compounds which were prepared according to the following procedure.

4.9.1. 2-Azidobenzamide

Under a nitrogen atmosphere, $SOCl_2$ (1 mL) was added to 2-azidobenzoic acid (200 mg, 1.23 mmol) and the mixture was refluxed for 2 h. The organic layer was evaporated. 10% aqueous NH_3 (3 mL) was added to the residue at 0 °C and the mixture was stirred for 0.5 h. The mixture was extracted with $CHCl_3$. 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 ($CHCl_3$/MeOH = 10:1) to give desired compound (185 mg, 93%) as yellow solids. Mp 130–131 °C. IR (KBr) 3368, 3168, 2130, 2103, 1655, 1620, 1599, 1574, 1483, 1452, 1403, 1230, 1163, 1127, 1084 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 7.22 (1H, dt, *J* = 1.5, 7.5 Hz), 7.33 (1H, dd, *J* = 1.5, 7.5 Hz), 7.50 (1H, dt, *J* = 1,5 and 7.5 Hz), 7.56 (1H, br s), 7.57 (1H, dd, *J* = 1.5 and 7.5 Hz), 7.73 (1H, br s). ^{13}C NMR (101 MHz, $CDCl_3$) δ 119.7, 124.8, 128.3, 129.7, 131.4, 136.6, 167.1. MS (EI) *m/z* 162 (M^+, 100); HRMS (EI) *m/z* Calcd for $C_7H_6N_4O$: 162.0542. Found 162.0550.

4.9.2. 3-Azidobenzamide

Under a nitrogen atmosphere, $SOCl_2$ (2 mL) was added to 3-azidobenzoic acid (120 mg, 0.88 mmol) and the mixture was refluxed for 2 h. The organic layer was evaporated. 10% aqueous NH_3 (3 mL) was added to the residue at 0 °C and the mixture was stirred for 0.5 h. The mixture was extracted with $CHCl_3$. 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 ($CHCl_3$/MeOH = 9:1) to give desired compound (100 mg, 83%) as brown solids. Mp 135–136 °C. IR ν_{max} (KBr) 3358, 3171, 2198, 2113, 1658, 1483, 1444, 1395, 1314, 1288, 1164, 1129 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6) δ 7.25 (1H, d, *J* = 7.5 Hz), 7.46 (1H, br s), 7.48 (1H, t, *J* = 7.5 Hz), 7.58 (1H, s), 7.68 (1H, d, *J* = 7.5 Hz), 8.06 (1H, br s). ^{13}C NMR (101 MHz, DMSO-d_6) δ 118.0, 121.9, 124.2, 130.0, 136.0, 139.6, 167.1. MS (EI) *m/z* 162 (M^+, 100); HRMS (EI) *m/z* Calcd for $C_7H_6N_4O$: 162.0542. Found 162.0546.

4.9.3. 1,1′-(5-Azido-1,3-phenylene)diurea

Under a nitrogen atmosphere, $SnCl_2$ (3.2 g, 1.70 mmol) was added to a solution of 5-iodo-1,3-dinitrobenzene (500 mg, 1.70 mmol) in anhydrous EtOH (10 mL) at room temperature and the mixture was stirred for 3 h at 70 °C. Ice (10 g) was added and the pH of the solution was controlled to approximately 9 by 10% NaOH aq. After the solids were removed by filtration through Celite®, the filtrate was extracted with AcOEt. The organic extracts were washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. Under a nitrogen atmosphere, 0.4 N HCl aq

(10 mL) and KNCO (340 mg, 4.08 mmol) were added to the residue and the mixture was stirred for 16 h at room temperature. The solids obtained by filtration was purified by flash silica gel column chromatography (AcOEt/MeOH = 30:1) to give 1,1′-(5-iodo-1,3-phenylene)diurea (105 mg, 19% from 5-iodo-1,3-dinitrobenzene) as light brown solids. Mp 135–138 °C. IR ν_{max} (KBr) 3292, 3195, 1672, 1585, 1544, 1442, 1347 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 5.92 (4H, br s), 7.78 (1H, t, J = 1.8 Hz), 7.48 (2H, d, J = 1.8 Hz), 8.81 (2H, br s). ^{13}C NMR (101 MHz, DMSO-d_6) δ 94.6, 105.6, 118.6, 142.2, 155.8. HRMS (MALDI) m/z Calcd for $C_8H_9IN_4NaO_2$: 342.9662. Found 342.9652.

According to the reaction conditions reported previously,[7] NaN_3 (26 mg, 0.41 mmol), CuI (7.7 mg, 41 μmol), sodium ascorbate (25 mg, 0.12 mmol) and *N,N′*-dimethylethylenediamine (4.4 μL, 0.81 mmol) were added to a solution of 1,1′-(5-iodo-1,3-phenylene)diurea (95 mg, 0.30 mmol) in DMSO-H_2O (5:1, 10 mL) and the mixture was stirred for 19 h at room temperature. After addition of brine, 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 (AcOEt/MeOH = 15:1) to give the desired compound (50 mg, 72%) as white powder. Mp >300 °C. IR ν_{max} (KBr) 3258, 2115, 1668, 1594, 1556 cm^{-1}. ^1H NMR (300 MHz, CD_3OD) δ 6.92 (2H, d, J = 1 Hz), 7.10 (1H, t-like, J = 1 Hz). ^{13}C NMR (76 MHz, CD_3OD) δ 104.3, 106.6, 142.3, 142.8, 159.1. HRMS (MALDI) m/z Calcd for $C_8H_9N_7NaO_2$: 258.0710. Found 258.0707.

4.9.4. *N*-Acetyl-7-azido-1,2,3,4-tetrahydroquinoline

Under a nitrogen atmosphere, *t*-BuONO (270 μL, 2.28 mmol) and $TMSN_3$ (240 μL, 1.51 mmol) was added to a solution of *N*-acetyl-7-amino-1,2,3,4-tetrahydroquinoline[8] (270 mg, 1.42 mmol) at 0 °C. The mixture was stirred for 9 h at room temperature. After addition of water, the mixture was extracted with AcOEt. The organic extracts were washed with saturated $NaHCO_3$ 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 = 1:1) to give the compound (280 mg, 91%) as light yellow solids. Mp 53–55 °C. IR ν_{max} (KBr) 2944, 2109, 2050, 1656, 1606, 1576, 1498, 1454, 1406, 1352, 1300, 1263, 1231, 1209, 1136, 1019 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 1.96 (2H, quint, J = 7.0 Hz), 2.25 (3H, s), 2.72 (2H, t., J = 7.0 Hz), 3.76 (2H, t, J = 7.0 Hz), 6.79 (1H, d, J = 7.0 Hz), 6.94 (1H, br s), 7.12 (1H, d, J = 7.0 Hz). ^{13}C NMR (101 MHz, $CDCl_3$) δ 23.2, 23.6, 26.3, 43.2 (br s), 115.1, 115.2, 129.4, 137.5, 139.9 (br s), 169.7. MS (FAB) m/z 217 $[M+H]^+$; HRMS (FAB) m/z Calcd for $C_{11}H_{13}N_4O$ $[M+H]^+$: 217.1084. Found 217.1087.

4.10. Click chemistry: general procedure

A solution of azide compound (10 mM in DMSO, 3 μL) was added to a mixture of $CuSO_4$ (2 mM in H_2O, 3 μL), TBTA (2 mM in DMSO, 6 μL), sodium ascorbate (10 mM in H_2O, 3 μL), **1** or **7** [0.9 mM in phosphate buffer (pH 7.0), 3.3 μL] and H_2O (8.7 μL) in a 1.5 mL Eppendorf tube. The mixture was shaken at room temperature using a shaker (1000 rpm) until the reaction was complete. The entire product was purified by reversed-phase HPLC [column: Waters XBridge® OST C18 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 **11** or **12**, the identification was confirmed by MALDI-TOF-MS analysis. Isolated yield and MALDI-TOF-MS data ($[M-H]^-$) for TFOs **11a–h** and **12a–j**: **11a**, 67%. Found 4545.85 (calcd 4545.06); **11b**, 74%. Found 4560.24 (calcd 4561.06); **11c**, 91%. Found 4588.03 (calcd 4588.09); **11d**, 85%. Found 4561.66 (calcd 4561.06); **11e**, 86%. Found 4575.27 (calcd 4575.09); **11f**, 74%. Found 4574.77 (calcd 4575.09); **11g**, 69%. Found 4587.89 (calcd 4588.09); **11h**, 79%. Found 4603.52 (calcd 4603.10); **12a**, 80%. Found 4543.77 (calcd 4543.09); **12b**, 83%. Found 4559.18 (calcd 4559.09); **12c**, 82%. Found 4586.26 (calcd 4586.11); **12d**, 83%. Found 4559.51 (calcd 4559.09); **12e**, 74%. Found 4573.72 (calcd 4573.11); **12f**, 82%. Found 4573.90 (calcd 4573.11); **12g**, 71%. Found 4586.99 (calcd 4586.11); **12h**, 77%. Found 4602.02 (calcd 4601.13); **12i**, 71%. Found 4659.83 (calcd 4659.17); **12j**, 90%. Found 4640.51 (calcd 4640.20).

4.11. UV melting experiments

UV melting experiments were performed on SHIMADZU UV-1650 and SHIMADZU UV-1800 spectrophotometers equipped with T_m analysis accessory. TFOs **11** and **12** and hairpin dsDNA targets were dissolved in 10 mM sodium cacodylate buffer (pH 6.8) containing 100 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. A 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.

Acknowledgments

This work was supported in part by the Program to Disseminate Tenure Tracking System of the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT), a Grant-in-Aid for Scientific Research on Innovative Areas (No. 22136006) from MEXT, a Grant-in-Aid for JSPS Fellows of Japan Society for the Promotion of Science (JSPS) and Mochida Memorial Foundation for Medical and Pharmaceutical Research.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.05.034.

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Tetrahedron 69 (2013) 6381–6391

Contents lists available at SciVerse ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Base-pair recognition ability of hydroxyphenyl nucleobases in parallel triplex DNA

Yoshiyuki Hari *, Satoshi Kashima, Hiroyasu Inohara, Shin Ijitsu, Takeshi Imanishi, Satoshi Obika *

Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 30 April 2013
Received in revised form 21 May 2013
Accepted 23 May 2013
Available online 30 May 2013

Keywords:
Artificial nucleobases
2′,4′-BNA
Parallel motif triplex
Triplex DNA
Triplex-forming oligonucleotides

ABSTRACT

Oligonucleotides containing 2′-deoxyribonucleotides bearing hydroxyphenyl nucleobases or their 2′,4′-BNA-modified analogs were synthesized, and the triplex-forming ability of their oligonucleotides with double-stranded DNA targets was evaluated by UV melting experiments. Results showed that 2′-deoxyribonucleotide bearing 2′-hydroxyphenyl nucleobase could be recognized by a dUA base pair while no affinity to a TA base pair was observed. The ,4′-BNA modification led to a further increase in the binding affinity to a dUA base pair. The ,4′-BNA bearing 3-hydroxyphenyl nucleobase showed moderate binding affinity to a TA base pair, but without selectivity.

1. Introduction

Triplex DNA is formed by interaction between double-stranded DNA (dsDNA) and an oligonucleotide, called a triplex-forming oligonucleotide (TFO). Triplex formation is useful for targeting dsDNA and can contribute to the development of various nucleic acid technologies.[1] However, there is the sequence limitation of target dsDNA as a problem of triplex formation. Although AT or GC base pairs within dsDNA are recognized by T or C in TFO in a parallel-oriented manner to form T-AT or protonated C-GC (C^+H-GC) base triplets, respectively (Fig. 1), it is difficult to target dsDNA containing pyrimidine–purine base pairs (TA or CG base pairs). Therefore, many artificial nucleic acids have been developed for recognizing TA or CG base pairs in triplex formation.[2] However, most of them target CG base-pair recognition and there are not many approaches for a TA base pair.

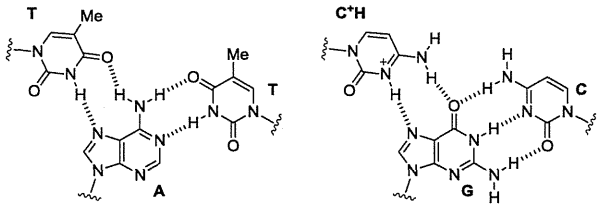


Fig. 1. Structures of T-AT and C^+H-GC base triplets.

On the basis of the structure of the T-CG base triplet,[3] a previous report described the design of a 2-pyridone nucleobase (P) for CG base-pair recognition and demonstrated that 2-pyridone bound to a CG base pair and that 2′,4′-BNA with P (P^B) effectively recognized a CG base pair (Fig. 2).[4] Hydrogen bond formation between the 2-carbonyl group in P and 4-amino group in C was considered as the recognition mode (Fig. 2). This finding suggests that a hydroxyphenyl nucleobase might be a promising candidate for a TA base pair and could hydrogen-bond with the 4-carbonyl group in T through the hydroxyl group, despite possible steric hindrance by the 5-methyl group in T (Fig. 2). Therefore, in this study, TFOs including 2′-deoxyribonucleotides with 2-, 3-, and 4-hydroxyphenyl nucleobases

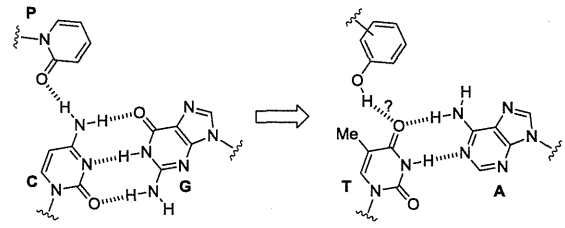


Fig. 2. Design of nucleobase for a TA base-pair recognition.

* Corresponding authors. Tel./fax: +81 6 6879 8201 (Y.H.); tel.: +81 6 6879 8200; fax: +81 6 6879 8204 (S.O.); e-mail addresses: hari@phs.osaka-u.ac.jp (Y. Hari), obika@phs.osaka-u.ac.jp (S. Obika).

0040-4020/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tet.2013.05.107

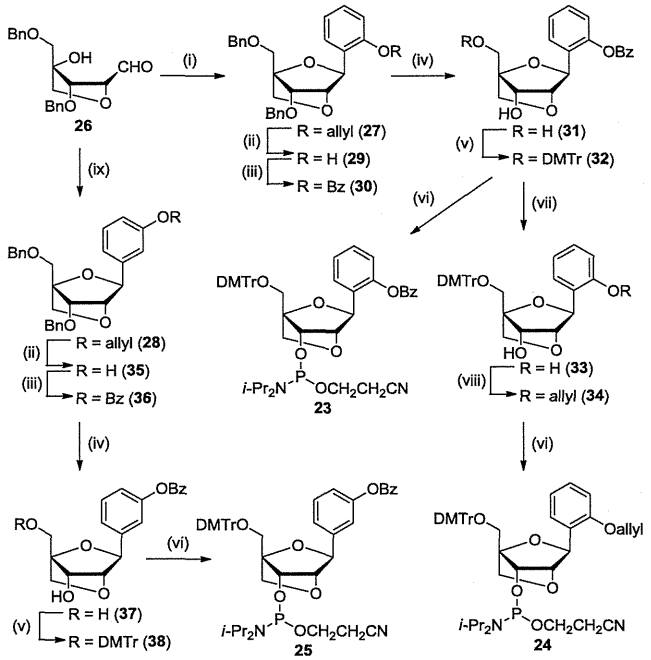
that have hydroxyl groups located at different positions, and TFOs including 2′,4′-BNA with 2- and 3-hydroxyphenyl nucleobases, were synthesized and the triplex-forming ability of their TFOs with dsDNA was evaluated using UV melting experiments.[5]

2. Results and discussion

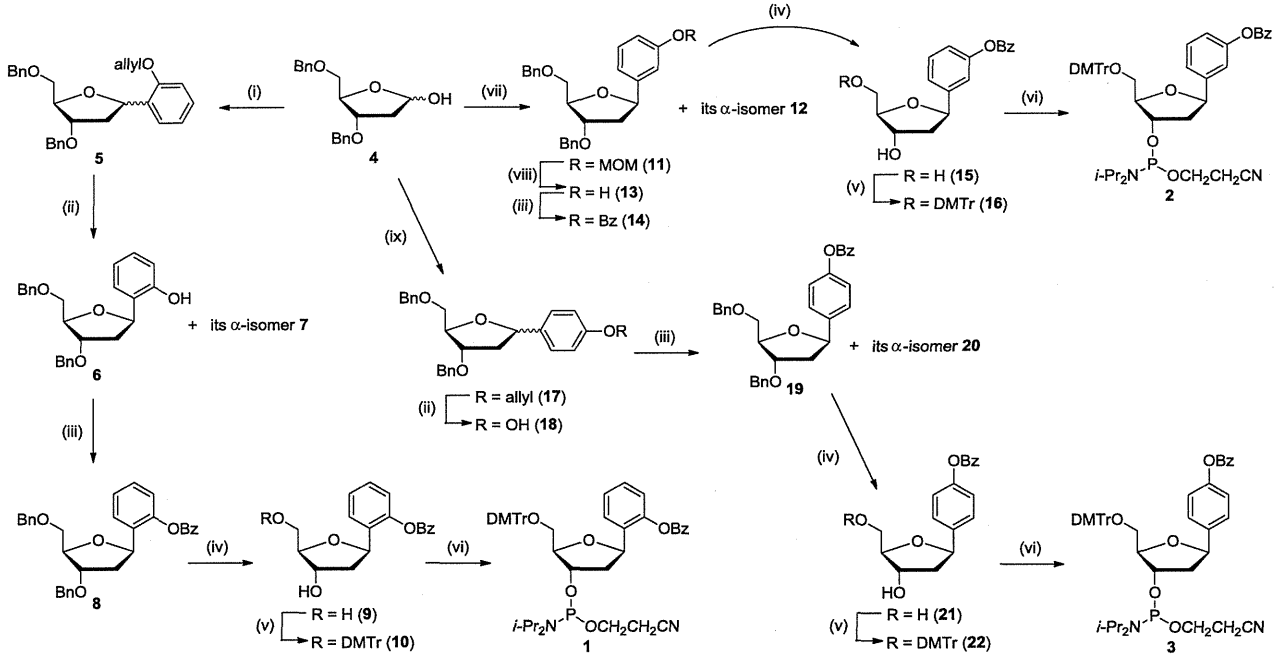
2.1. Synthesis

The synthesis of phosphoramidites **1**–**3** composed of a 2′-deoxyribose unit and hydroxyphenyl units is shown in Scheme 1. Coupling of compound **4**[6] and 2-allyloxyphenyllithium followed by the Mitsunobu reaction[7] using *N*,*N*,*N*′,*N*′-tetramethyl azodicarboxamide (TMAD) and *n*-Bu_3P gave **5** (β-isomer/α-isomer=ca. 1:1), which had its allyl group deprotected to afford the desired β-isomer **6** and its α-isomer **7**. A benzoyl group was chosen as the protecting group of the phenolic hydroxyl group in oligonucleotide synthesis. Reaction of **6** with BzCl in the presence of Et_3N gave **8** in 97% yield, which was converted into diol **9** by hydrogenolysis using $Pd(OH)_2$–C and cyclohexene. Dimethoxytritylation of **9** followed by phosphitylation afforded 2-hydroxyphenyl phosphoramidite **1**, a suitable building block for oligonucleotide synthesis. For the 3-hydroxyphenyl derivative, the β-isomer **11** was isolated in 28% yield in two steps via reaction of **4** with 3-methoxymethoxyphenyllithium and ring-closure. The methoxymethyl group (**11**) was converted into a benzoyl group (**14**) through a deprotection–protection process. Then, the phosphoramidite **2** was synthesized via three steps (debenzylation–dimethoxytritylation–phosphitylation). The synthesis of 4-hydroxyphenyl phosphoramidite **3** was achieved by a synthetic route similar to that of 2-hydroxyphenyl derivative **1**. Reaction of **4** with 4-allyloxyphenyllithium and ring-closure reaction yielded **17** as a 1:1 anomeric mixture. Palladium-catalyzed deallylation of **17** proceeded to give **18**, which was benzoylated to afford a separable mixture of β-isomer **19** and α-isomer **20**. Hydrogenolysis of the β-isomer **19** produced **21** in 82% yield, which underwent reaction with DMTrCl in pyridine to produce **22** in 91% yield. The desired phosphoramidite **3** was obtained via phosphitylation.

The 2′,4′-BNA-modified phosphoramidites **23**–**25** with 2- and 3-hydroxyphenyl nucleobase units were synthesized according to the synthetic route shown in Scheme 2. A previous study reported



Scheme 2. Reagents and conditions: (i) 2-allyloxyphenylmagnesium iodide, THF, −40 °C; TMAD, *n*-Bu_3P, CH_2Cl_2, rt, 57% over two steps; (ii) $NaBH_4$, $Pd(PPh_3)_4$, THF, rt, 80% (**29**) and quant. (**35**); (iii) BzCl, Et_3N, CH_2Cl_2, rt, 93% (**30**) and 98% (**36**); (iv) 20% $Pd(OH)_2$–C, cyclohexene, EtOH, reflux, 89% (**31**) and 95% (**37**); (v) DMTrCl, pyridine, rt, 94% (**32**) and 91% (**38**); (vi) $(i\text{-}Pr_2N)_2POCH_2CH_2CN$, DIHT, MeCN/THF, rt, 92% (**23**), 81% (**24**), and 71% (**25**); (vii) K_2CO_3, MeOH, rt, 5 min, 96%; (viii) allyl bromide, K_2CO_3, acetone, rt, 14 h, 87%; (ix) 3-allyloxyphenylmagnesium iodide, THF, rt; TMAD, *n*-Bu_3P, CH_2Cl_2, rt, 70% over two steps.



Scheme 1. Reagents and conditions: (i) 2-allyloxyphenyllithium, THF, −78 °C; TMAD, *n*-Bu_3P, CH_2Cl_2, rt, 38% (β-isomer/α-isomer= ca. 1:1) over two steps; (ii) $NaBH_4$, $Pd(PPh_3)_4$, THF, rt, 55% (**6**) and 45% (**7**), and 91% (**18**, β-isomer/α-isomer=ca. 1:1); (iii) BzCl, Et_3N, CH_2Cl_2, rt, 97% (**8**), 98% (**14**), and 91% (**19**/**20**=ca. 1:1); (iv) 20% $Pd(OH)_2$–C, EtOH, 70 °C, 94% (**9**), 96% (**15**), and 82% (**21**); (v) DMTrCl, pyridine, rt, 98% (**10**), 72% (**16**), and 91% (**22**); (vi) $i\text{-}Pr_2NP(Cl)OCH_2CH_2CN$, $i\text{-}Pr_2NEt$, CH_2Cl_2, rt, 58% (**1**), 58% (**2**), and 95% (**3**); (vii) 3-methoxymethoxyphenyllithium, THF, −78 °C; TMAD, *n*-Bu_3P, CH_2Cl_2, rt, 28% (**11**) and 24% (**12**) over two steps; (viii) 10% HCl aq, THF, rt, 2 days, 91%; (ix) 4-allyloxyphenyllithium, THF, −60 °C; TMAD, *n*-Bu_3P, CH_2Cl_2, rt; 48% (β-isomer/α-isomer=ca. 1:1) over two steps.

that reaction of aldehyde **26** with arylmagnesium reagents proceeded with high diastereoselectivity and that Mitsunobu reaction of the resulting products led predominantly to 2′,4′-BNA-modified C-nucleosides with β-configuration.[8] Thus, **26** was treated with excess 2- or 3-allyloxyphenylmagnesium bromide in THF to give coupling compounds; ring-closure using TMAD afforded the β-anomers **27** and **28** in 57% and 70% yield, respectively. Interestingly, in both cases, only the β-anomer was isolated; its α-isomer was not observed. Palladium-catalyzed reaction of 2-hydroxyphenyl derivative **27** with $NaBH_4$ gave deallylated compound **29**, which was benzoylated by BzCl in the presence of Et_3N to produce **30** in high yield. After debenzylation of **30** to produce **31**, compound **32** was obtained in 94% yield by dimethoxytritylation. Phosphitylation of **32** using $(i\text{-}Pr_2N)_2POCH_2CH_2CN$ and diisopropylammonium tetrazolide (DIHT) gave benzoyl-protected phosphoramidite **23** with a 2-hydroxyphenyl unit and 2′,4′-BNA modification in 92% yield. In addition, the allyl-protected congener **24** was prepared through debenzoylation of **32**, followed by allylation and phosphitylation. Analogous to synthesis of **23**, 2′,4′-BNA phosphoramidite **25** with a 3-hydroxyphenyl unit was synthesized. After conversion of the allyl group of **28** into a benzoyl group (**36**) in two steps, **37** was produced by the hydrogenolysis. The desired phosphoramidite **25** was obtained from **37** via dimethoxytritylated **38**.

Next, introduction of the phosphoramidites synthesized into oligonucleotides was investigated using an automated DNA synthesizer. The use of benzoyl-protected phosphoramidites **1**–**3** and **25**, with the exception of the 2′,4′-BNA-modified 2-hydroxy analog **23**, successfully led to the desired TFOs according to standard phosphoramidite chemistry. For **23**, two undesirable oligonucleotides having an approximately +17 mass unit difference from molecular weight of the desired TFO were obtained. This result implies that a structure constrained by the methylene linkage between the 2′-oxygen and 4′-carbon atoms might trigger a ring-opening reaction by conventional ammonia treatment as shown in Fig. 3. Eventually, the desired TFO **42** was obtained by oligonucleotide synthesis using allyl-protected phosphoramidite **24** followed by treatment with $NaBH_4$ and $Pd(PPh_3)_4$ to remove an allyl group. The sequence of TFOs prepared is listed in Fig. 4.

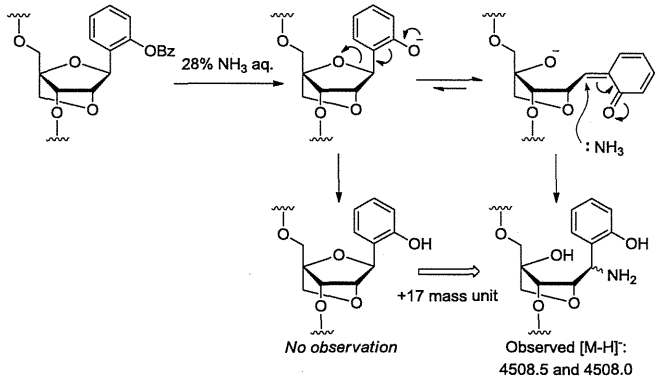


Fig. 3. Plausible mechanism of ring-opening reaction.

2.2. Evaluation

Triplex-forming ability of the TFOs **39**–**43** with dsDNA was evaluated through UV melting experiments at 10 mM sodium cacodylate (pH 6.8) plus 140 mM KCl and 50 mM $MgCl_2$. As targets, hairpin dsDNAs containing five types of base pairs (i.e., TA, its demethylated UA, CG, AT, and GC base pairs) at the same position were used. Results are summarized in Table 1. In general, TFOs **39**–**41** containing 2′-deoxy derivatives 2H, 3H, and 4H, did not sequence-specifically form stable triplexes with dsDNA (YZ=TA)

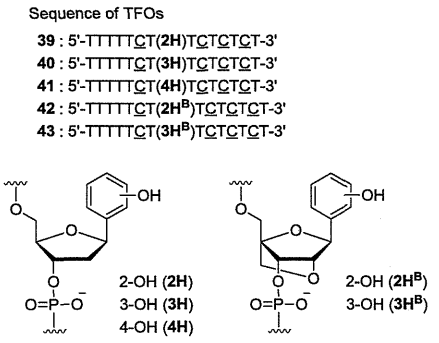


Fig. 4. TFOs synthesized in this study.

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

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

TFO (X)	YZ				
	TA	UA	CG	AT	GC
39 (2H)	23	28	27	27	31
40 (3H)	26	27	27	28	25
41 (4H)	24	25	25	30	27
42 ($2H^B$)	28	37	29	36	39
43 ($3H^B$)	34	35	34	33	33

[a] Conditions: 10 mM sodium cacodylate buffer (pH 6.8), 140 mM KCl, and 50 mM $MgCl_2$. The final concentration of each oligonucleotide used was 1.89 μM. C̲ indicates 2′-deoxy-5-methylcytidine.

[b] Triplexes containing T-AT and P^B-CG base triplets as X-YZ had T_m values of 44 °C and 37 °C, respectively. P^B indicates 2′,4′-BNA bearing 2-pyridone nucleobase.

and no significant sequence-selectivity toward a certain base pair was observed because the hydroxyl group could function not only as a hydrogen donor but also as a hydrogen acceptor. However, the T_m value of a triplex with dsDNA (YZ=UA) by TFO **39** was greater than that with dsDNA (YZ=TA) by +5 °C. In addition, TFO **39** provided nearly the same stability to TFO **41** (X=4H) in the triplex with dsDNA (YZ=TA), while TFO **39** formed a stable triplex with dsDNA (YZ=UA) compared to TFO **41**. These results suggest that a 2-hydroxyphenyl nucleobase forms a hydrogen bond with U of a UA base pair without forming any hydrogen bond with a TA base pair because the 5-methyl group in T prevents bond formation (Fig. 5). In contrast, TFO **40** (X=3H) significantly stabilized triplexes formed

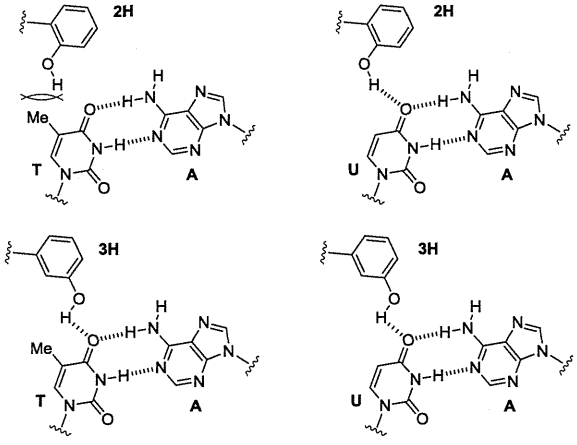


Fig. 5. Proposed structures of base triplets by **2H** and **3H**.

with dsDNAs (YZ=TA and UA) compared to TFO **41**. The T_m values of triplexes (X-YZ=3H-TA and 3H-UA) were comparable to that of triplex (X-YZ=2H-UA). This suggests that the 3-hydroxyphenyl nucleobase recognized not only a UA base pair, but also a TA base pair by effective avoidance of the bulky 5-methyl group in T, despite the lack of stability. When TFOs **42** and **43** containing 2′,4′-BNA with 2- or 3-hydroxyphenyl nucleobases were used, affinities to any dsDNA targets increased without any sequence-selectivity. TFO **42** (X=$2H^B$) formed a stable triplex with dsDNA (YZ=UA) rather than with dsDNA (YZ=TA), which was comparable to triplexes formed with TFOs, including 2′,4′-BNA (P^B) with a 2-pyridone nucleobase and dsDNA (YZ=CG).[9] As mentioned, P^B likely bonds to a CG base pair via a single hydrogen bond. Therefore, 2-hydroxyphenyl nucleobase in $2H^B$ forms a single hydrogen bond with an UA base pair and cannot recognize a TA base pair because of the 5-methyl group. In contrast, TFO **43** (X=$3H^B$) had T_m values of 34 °C and 35 °C against dsDNAs (YZ=UA and TA), respectively. This stability was significantly higher than that of the triplex (X-YZ=$2H^B$-TA), although it was slightly lower than that of triplex (X-YZ=$2H^B$-UA). These results suggest that 3-hydroxyphenyl nucleobase in $3H^B$ should recognize both UA and TA base pairs through a single hydrogen bond (Fig. 5). Results also indicated that 3-hydroxyphenyl nucleobase can recognize a TA base pair in a parallel triplex formation, although no sequence-selectivity was observed.

3. Conclusion

Syntheses of 2′-deoxyribonucleotides bearing 2-, 3-, and 4-hydroxyphenyl nucleobases and 2′,4′-BNA-modified nucleotides containing 2- and 3-hydroxyphenyl nucleobases were developed. Evaluation of triplex-forming ability of TFOs containing their hydroxyphenyl nucleobases with dsDNA showed 3-hydroxyphenyl nucleobase might recognize a TA base pair through a single hydrogen bond without any sequence selectivity. Recently, we succeeded in the facile synthesis of various triazole or pyrimidinone nucleobases on oligonucleotides using post-elongation modification (PEM) and found promising nucleobases for CG base-pair recognition.[10] Thus, the synthesis of various 3-hydroxyphenyl units using PEM could be effective for screening of nucleobases for TA base-pair recognition. Further investigation of nucleobases based on the 3-hydroxyphenyl structure is underway.

4. Experimental

4.1. General methods

All chemicals were purchased from chemical suppliers. For column chromatography, Fuji Silysia silica gel BW-300, 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 JEOL EX270, JEOL ECS400, and JEOL GX-500 spectrometers. 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 obtained using 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.

4.2. 1-Allyloxy-2-(2-deoxy-3,5-di-*O*-benzyl-D-ribofuranosyl) benzene (5)

Under a nitrogen atmosphere, a solution of 2-deoxy-3,5-di-*O*-benzyl-D-ribofuranose **4**[6] (1.47 g, 4.69 mmol) in anhydrous THF (6 mL) was added to a solution of 2-allyloxyphenyllithium [prepared from 2-allyloxyphenyl bromide (3.98 g, 18.7 mmol) and *n*-BuLi (2.67 M in hexane, 6.9 mL, 18.4 mmol) in anhydrous THF (20 mL) at −78 °C], and the mixture was stirred at −78 °C for 16 h. After addition of satd NH_4Cl aq, 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=5:1) to give the appropriate compounds (R_f=ca. 0.1), which were dissolved in CH_2Cl_2 (7 mL); TMAD (750 mg, 4.35 mmol) and *n*-Bu_3P (1.1 mL, 4.35 mL) were added at room temperature. After being stirred at room temperature for 6 h, water was added and 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=15:1) to give a ca. 1:1 anomeric mixture **5** (773 mg, 38% from **4**) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.77 (0.5H, ddd, J=7.4, 10.2, 13.2 Hz), 1.96 (0.5H, ddd, J=5.8, 7.8, 12.7 Hz), 2.56 (0.5H, ddd, J=1.0, 5.4, 13.2 Hz), 2.73 (0.5H, td, J=6.8, 12.7 Hz), 3.56–3.71 (2H, m), 4.12–4.13 (0.5H, m), 4.20–4.24 (0.5H, m), 4.27–4.31 (0.5H, m), 4.38–4.63 (6.5H, m), 5.22–5.25 (1H, m), 5.34–5.51 (1H, m), 5.96–6.06 (1H, m), 6.78–6.81 (1H, m), 6.91–6.98 (1H, m), 7.16–7.34 (11H, m), 7.52 (0.5H, d, J=7.4 Hz), 7.59 (0.5H, d, J=6.8 Hz). HRMS (FAB) m/z calcd for $C_{28}H_{30}NaO_4$ $[M+Na]^+$: 453.2036; found, 453.2049.

4.3. 2-(2-Deoxy-3,5-di-*O*-benzyl-β-D-ribofuranosyl)-1-hydroxybenzene (6) and 2-(2-deoxy-3,5-di-*O*-benzyl-α-D-ribofuranosyl)-1-hydroxybenzene (7)

Under a nitrogen atmosphere, $Pd(PPh_3)_4$ (290 mg, 0.253 mmol) was added to a solution of compound **5** (545 mg, 1.27 mmol) in anhydrous THF (10 mL) and the mixture was stirred until solids of $Pd(PPh_3)_4$ dissolved completely. Then, $NaBH_4$ (58 mg, 1.52 mmol) was added and the mixture was stirred at room temperature for 24 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=20:1 to 10:1) to give compound **6** (270 mg, 55%) as a colorless oil and compound **7** (221 mg, 45%) as a colorless oil. Compound **6**: $[\alpha]_D^{22}$ +54.0 (*c* 1.01, $CHCl_3$). IR ν_{max} (KBr) 3338, 3028, 2867, 1495, 1455, 1247, 1095, 1077 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 2.20–2.33 (2H, m), 3.55 (1H, dd, J=2.5, 10.0 Hz), 3.67 (1H, dd, J=3.0, 10.0 Hz), 4.18–4.20 (1H, m), 4.24–4.26 (1H, m), 4.47 (1H, d, J=12.0 Hz), 4.51 (1H, d, J=12.0 Hz), 4.54 (1H, d, J=12.0 Hz), 4.68 (1H, d, J=12.0 Hz), 5.20 (1H, dd, J=6.0, 10.5 Hz), 6.80 (1H, ddd, J=2.0, 8.0, 8.0 Hz), 6.89 (1H, dd, J=2.0, 8.0 Hz), 7.01 (1H, d, J=2.0, 8.0 Hz), 7.18 (1H, ddd, J=2.0, 8.0, 8.0 Hz), 7.26–7.36 (10H, m), 8.10 (1H, s). ^{13}C NMR ($CDCl_3$) δ 39.4, 69.5, 71.3, 73.5, 81.3, 82.6, 84.7, 117.4, 119.4, 123.8, 125.9, 127.7, 127.8, 127.9, 127.9, 128.1, 128.5, 129.3, 137.3, 137.8, 155.8. HRMS (FAB) m/z calcd for $C_{25}H_{26}NaO_4$ $[M+Na]^+$: 413.1723; found 413.1742. Compound **7**: $[\alpha]_D^{23}$ +6.6 (*c* 0.86, $CHCl_3$). IR ν_{max} (KBr) 3337, 3033, 2925, 2862, 1494, 1455, 1246, 1105, 1074 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 2.23 (1H, ddd, J=4.0, 8.0, 12.5 Hz), 2.65 (1H, ddd, J=7.0, 7.0, 12.5 Hz), 3.58–3.64 (2H, m), 4.27–4.32 (1H, m), 4.37 (1H, ddd, J=4.5, 4.5, 4.5 Hz), 4.49 (1H, d, J=12.0 Hz), 4.50 (1H, d, J=12.0 Hz), 4.55 (1H, d, J=12.0 Hz), 4.57 (1H, d, J=12.0 Hz), 5.23 (1H, dd, J=7.0, 8.0 Hz), 6.81 (1H, ddd, J=2.0, 8.0, 8.0 Hz), 6.88 (1H, dd, J=2.0, 8.0 Hz), 6.96 (1H, dd, J=2.0, 8.0 Hz), 7.17 (1H, ddd, J=2.0, 8.0, 8.0 Hz), 7.25–7.38 (10H, m), 8.09 (1H, s). ^{13}C NMR ($CDCl_3$) δ 39.4, 70.2, 71.6, 73.5, 79.9, 81.2, 82.9, 117.3, 119.5, 124.6, 127.5, 127.6, 127.7, 127.7, 127.8, 128.4, 129.1, 137.5, 137.5, 137.9, 155.4. HRMS (FAB) m/z calcd for $C_{25}H_{27}O_4$ $[M+H]^+$: 391.1904; found 391.1895.

4.4. 1-Benzoyloxy-2-(2-deoxy-3,5-di-*O*-benzyl-β-D-ribofuranosyl)benzene (8)

Under a nitrogen atmosphere, BzCl (88 μL, 0.753 mmol) was added to a solution of compound **6** (270 mg, 0.627 mmol) and Et_3N (105 μL, 0.753 mmol) in anhydrous CH_2Cl_2 (6 mL) 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 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=10:1) to give compound **8** (332 mg, 97%) as a colorless oil. $[\alpha]_D^{24}$ +57.1 (*c* 0.90, $CHCl_3$). IR ν_{max} (KBr) 3062, 3032, 2867, 1738, 1489, 1452, 1263, 1219, 1181, 1081 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.86 (1H, ddd, *J*=6.0, 10.5, 13.0 Hz), 2.38 (1H, ddd, *J*=1.5, 5.0, 13.0 Hz), 3.56 (1H, dd, *J*=5.0, 10.0 Hz), 3.64 (1H, dd, *J*=5.0, 10.0 Hz), 4.09–4.11 (1H, m), 4.20–4.23 (1H, m), 4.40 (2H, s), 4.57 (2H, s), 5.35 (1H, dd, *J*=5.0, 10.5 Hz), 7.14–7.36 (13H, m), 7.48 (2H, dd, *J*=8.0, 8.0 Hz), 7.63 (1H, dd, *J*=8.0, 8.0 Hz), 7.65 (1H, dd, *J*=8.0, 8.0 Hz), 8.20 (2H, d, *J*=8.0 Hz). ^{13}C NMR ($CDCl_3$) δ 39.8, 70.9, 73.4, 75.0, 81.2, 83.5, 122.1, 126.3, 126.7, 127.5, 127.5, 127.6, 127.6, 128.2, 128.3, 128.3, 128.128.7, 129.3, 130.1, 133.7, 134.2, 137.9, 138.1, 147.7, 165.0. HRMS (FAB) *m/z* calcd for $C_{32}H_{31}O_5$ $[M+H]^+$: 495.2166; found 495.2165.

4.5. 1-Benzoyloxy-2-(2-deoxy-β-D-ribofuranosyl)benzene (9)

A solution of compound **8** (191 mg, 0.386 mmol), 20% $Pd(OH)_2/C$ (120 mg), and cyclohexene (3.9 mL, 38.6 mmol) in EtOH (8 mL) was stirred at 70 °C for 1.5 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 ($CHCl_3$/MeOH=60:1) to give compound **9** (114 mg, 94%) as a colorless oil. $[\alpha]_D^{22}$ +35.0 (*c* 1.03, $CHCl_3$). IR ν_{max} (KBr) 3410, 2918, 1732, 1451, 1263, 1219, 1179, 1086 cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.01 (1H, ddd, *J*=6.5, 9.0, 13.5 Hz), 2.19 (1H, ddd, *J*=2.5, 6.0, 13.5 Hz), 2.33 (2H, s), 3.58–3.69 (2H, m), 3.84 (1H, ddd, *J*=4.0, 4.0, 4.0 Hz), 4.25–4.28 (1H, m), 5.31 (1H, dd, *J*=6.0, 9.0 Hz), 7.14 (1H, dd, *J*=1.0, 8.0 Hz), 7.28 (1H, ddd, *J*=1.0, 1.0, 8.0 Hz), 7.34 (1H, ddd, *J*=1.0, 8.0, 8.0 Hz), 7.50 (1H, ddd, *J*=1.0, 8.0, 8.0 Hz), 7.53 (2H, ddd, *J*=1.0, 8.0, 8.0 Hz), 7.64 (1H, ddd, *J*=1.0, 8.0, 8.0 Hz), 8.19 (2H, dd, *J*=1.0, 8.0 Hz). ^{13}C NMR ($CDCl_3$) δ 42.6, 63.1, 73.2, 74.9, 86.6, 122.5, 126.4, 126.5, 128.6, 128.7, 129.1, 130.1, 133.8, 148.0, 165.3. HRMS (MALDI-TOF) *m/z* calcd for $C_{18}H_{18}NaO_5$ $[M+Na]^+$: 337.1046; found 337.1052.

4.6. 1-Benzoyloxy-2-[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl]benzene (10)

Under a nitrogen atmosphere, DMTrCl (60 mg, 0.176 mmol) was added to a solution of compound **9** (46.0 mg, 0.146 mmol) in anhydrous pyridine (2 mL) 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 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 3:1) to give compound **10** (89 mg, 98%) as a white solid. $[\alpha]_D^{26}$ +30.2 (*c* 1.02, $CHCl_3$). IR ν_{max} (KBr) 3456, 2933, 1737, 1607, 1509, 1450, 1252, 1176, 1079 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.69 (1H, d, *J*=4.0 Hz), 2.03 (1H, ddd, *J*=6.5, 9.0, 13.0 Hz), 2.22 (1H, ddd, *J*=3.0, 6.5, 13.0 Hz), 3.28 (1H, dd, *J*=5.0, 10.0 Hz), 3.35 (1H, dd, *J*=5.0, 10.0 Hz), 3.79 (6H, s), 3.98 (1H, ddd, *J*=5.0, 5.0, 5.0 Hz), 4.32–4.35 (1H, m), 5.35 (1H, dd, *J*=6.5, 9.0 Hz), 6.82 (4H, d, *J*=9.0 Hz), 7.15–7.36 (10H, m), 7.44–7.53 (4H, m), 7.62–7.68 (2H, m), 8.19 (2H, d, *J*=8.0 Hz). ^{13}C NMR ($CDCl_3$) δ 42.8, 55.2, 64.3, 74.3, 74.6, 85.5, 86.2, 113.1, 122.2, 126.3, 126.7, 126.8, 127.8, 128.2, 128.2, 128.7, 129.2, 130.1, 130.2, 133.7, 134.6, 136.0, 144.8, 147.6, 158.5, 164.9. HRMS (FAB) *m/z* calcd for $C_{39}H_{36}NaO_7$ $[M+Na]^+$: 639.2353; found 639.2350.

4.7. 1-Benzoyloxy-2-{3-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl}benzene (1)

Under a nitrogen atmosphere, *i*-$Pr_2NP(Cl)OCH_2CH_2CN$ (22 μL, 0.098 mmol) was added to a solution of compound **10** (40 mg, 0.065 mmol) and *i*-Pr_2NEt (57 mL, 0.327 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 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=5:1) to give compound **1** (31 mg, 58%) as a white solid. ^1H NMR ($CDCl_3$) δ 0.90 (3H, d, *J*=6.8 Hz), 0.96 (3H, d, *J*=6.4 Hz), 1.06–1.09 (6H, m), 1.89–2.01 (1H, m), 2.20–2.43 (3H, m), 3.23–3.35 (2H, m), 3.38–3.48 (3H, m), 3.53–3.58 (1H, m), 3.79 (3H, s), 3.79 (3H, s), 4.15–4.16 (1H, m), 4.39–4.47 (1H, m), 5.29–5.36 (1H, m), 6.80–6.84 (4H, m), 7.14–7.38 (10H, m), 7.46–7.56 (4H, m), 7.64–7.74 (2H, m), 8.18–8.23 (2H, m). ^{31}P NMR ($CDCl_3$) δ 147.3, 148.3. HRMS (FAB) *m/z* calcd for $C_{48}H_{53}N_2NaO_8P$ $[M+Na]^+$: 839.3432; found 839.3421.

4.8. 3-(2-Deoxy-3,5-di-*O*-benzyl-β-D-ribofuranosyl)-1-(methoxymethoxy)benzene (11) and 3-(2-deoxy-3,5-di-*O*-benzyl-α-D-ribofuranosyl)-1-(methoxymethoxy)benzene (12)

Under a nitrogen atmosphere, a solution of compound **4** (180 mg, 0.573 mmol) in anhydrous THF (5 mL) was added to a solution of 3-methoxymethoxyphenyllithium [prepared from 3-methoxymethoxyphenyl bromide (690 mg, 3.18 mmol) and *n*-BuLi (1.65 M in hexane, 1.9 mL, 3.18 mmol) in anhydrous THF (20 mL) at −78 °C], and the mixture was stirred at −78 °C for 2 h. After addition of satd NH_4Cl aq, the mixture was extracted with AcOEt. The 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=1:1) to give appropriate compounds (R_f=ca. 0.5), which were dissolved in CH_2Cl_2 (6 mL); TMAD (131 mg, 0.763 mmol), and *n*-Bu_3P (190 μL, 0.763 mmol) were added at room temperature. After being stirred at room temperature for 2 h, water was added and the mixture was extracted with AcOEt. The 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=20:1) to give compound **11** (78 mg, 28%) as a colorless oil and compound **12** (66 mg, 24%) as a colorless oil. Compound **11**: $[\alpha]_D^{23}$ +26.2 (*c* 1.03, $CHCl_3$). IR ν_{max} (KBr) 2892, 1488, 1454, 1147, 1076 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.91 (1H, ddd, *J*=6.0, 10.5, 13.0 Hz), 2.36 (1H, ddd, *J*=1.0, 6.0, 13.0 Hz), 3.45 (3H, s), 3.58 (1H, dd, *J*=5.5, 10.5 Hz), 3.67 (1H, dd, *J*=4.5, 10.5 Hz), 4.16 (1H, m), 4.30 (1H, ddd, *J*=2.5, 4.5, 5.5 Hz), 4.56 (2H, d, *J*=12.0 Hz), 4.60 (2H, d, *J*=12.0 Hz), 5.12 (1H, dd, *J*=5.0, 10.5 Hz), 5.14 (2H, s), 6.94 (1H, ddd, *J*=1.0, 2.0, 8.0 Hz), 7.01 (1H, m), 7.06 (1H, m), 7.20–7.38 (11H, m). ^{13}C NMR ($CDCl_3$) δ 41.1, 55.9, 71.0, 71.1, 73.4, 80.2, 81.5, 83.8, 94.4, 114.0, 115.1, 119.6, 127.6, 127.6, 127.7, 128.3, 128.4, 129.3, 138.1, 138.2, 143.4, 157.3. HRMS (FAB) *m/z* calcd for $C_{27}H_{30}NaO_5$ $[M+Na]^+$: 457.1985; found 457.1985. Compound **12**: $[\alpha]_D^{23}$ +18.3 (*c* 1.24, $CHCl_3$). IR ν_{max} (KBr) 2894, 1487, 1454, 1151, 1077 cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.06 (1H, ddd, *J*=6.5, 8.0, 13.0 Hz), 2.62 (1H, ddd, *J*=6.5, 6.5, 13.0 Hz), 3.45 (3H, s), 3.62 (2H, m), 4.26 (1H, ddd, *J*=4.5, 6.6, 6.5 Hz), 4.36 (1H, ddd, *J*=4.5, 4.5, 4.5 Hz), 4.49 (2H, d, *J*=12.0 Hz), 4.59 (2H, d, *J*=12.0 Hz), 5.05 (1H, dd, *J*=6.5, 8.0 Hz), 5.16 (2H, s), 6.93 (1H, ddd, *J*=1.0, 2.0, 8.0 Hz), 7.03 (1H, m), 7.08 (1H, m), 7.22–7.37 (11H, m). ^{13}C NMR ($CDCl_3$) δ 40.9, 56.0, 70.8, 71.7, 73.5, 80.0, 80.6, 82.9, 94.4, 113.9,

115.1, 119.5, 127.6, 127.6, 128.3, 128.4, 129.4, 138.1, 144.5. HRMS (FAB) m/z calcd for $C_{27}H_{30}NaO_5$ $[M+Na]^+$: 457.1985; found 457.1988.

4.9. 3-(2-Deoxy-3,5-di-*O*-benzyl-β-D-ribofuranosyl)-1-hydroxybenzene (13)

10% HCl aq (7 mL) was added to a solution of compound **11** (286 mg, 0.657 mmol) in THF (10 mL), and mixture was stirred at room temperature for 2 days. The mixture was extracted with Et_2O and 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=5:1) to give compound **13** (234 mg, 91%) as a colorless oil. $[\alpha]_D^{23}$ +20.5 (c 0.96, $CHCl_3$). IR ν_{max} (KBr) 3331, 3032, 2861, 1591, 1456, 1077 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.89 (1H, ddd, J=5.5, 10.0, 13.0 Hz), 2.33 (1H, ddd, J=1.5, 5.0, 13.0 Hz), 3.58 (1H, dd, J=5.0, 10.0 Hz), 3.67 (1H, dd, J=4.5, 10.0 Hz), 4.15 (1H, m), 4.30 (1H, ddd, J=2.5, 4.5, 5.0 Hz), 4.55 (2H, s), 4.58 (2H, s), 5.08 (1H, dd, J=5.0, 10.0 Hz), 5.21 (1H, s), 6.67 (1H, ddd, J=1.0, 2.0, 8.0 Hz), 6.80 (1H, m), 6.86 (1H, m), 7.13 (1H, dd, J=8.0, 8.0 Hz), 7.24–7.36 (10H, m). ^{13}C NMR ($CDCl_3$) δ 41.1, 71.1, 73.4, 80.3, 81.4, 83.7, 112.9, 114.6, 118.4, 127.7, 127.7, 128.4, 128.4, 129.5, 138.0, 138.2, 143.4, 155.8. HRMS (FAB) m/z calcd for $C_{25}H_{26}NaO_4$ $[M+Na]^+$: 413.1723; found 413.1712.

4.10. 1-Benzoyloxy-3-(2-deoxy-3,5-di-*O*-benzyl-β-D-ribofuranosyl)benzene (14)

Under a nitrogen atmosphere, BzCl (90 μL, 0.779 mmol) was added to a solution of compound **13** (234 mg, 0.599 mmol) and Et_3N (109 μL, 0.779 mmol) in anhydrous CH_2Cl_2 (6 mL) at room temperature and the mixture was stirred for 30 min. 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 10:1) to give compound **14** (290 mg, 98%) as a colorless oil. $[\alpha]_D^{23}$ +12.7 (c 1.03, $CHCl_3$). IR ν_{max} (KBr) 2860, 1738, 1451, 1233, 1078 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.93 (1H, ddd, J=6.5, 10.0, 13.0 Hz), 2.40 (1H, ddd, J=1.0, 5.0, 13.0 Hz), 3.58 (1H, dd, J=5.0, 10.5 Hz), 3.67 (1H, dd, J=4.5, 10.5 Hz), 4.16 (1H, m), 4.31 (1H, ddd, J=2.0, 4.5, 5.0 Hz), 4.56 (2H, s), 4.58 (2H, s), 5.17 (1H, dd, J=5.0, 10.0 Hz), 7.12 (1H, ddd, J=1.0, 2.0, 8.0 Hz), 7.22–7.39 (13H, m), 7.50 (2H, dd, J=8.0, 8.0 Hz), 7.63 (1H, dd, J=8.0, 8.0 Hz), 8.20 (2H, d, J=8.0 Hz). ^{13}C NMR ($CDCl_3$) δ 41.2, 71.0, 73.4, 79.9, 81.5, 83.9, 119.2, 120.8, 123.5, 127.5, 127.6, 127.7, 128.3, 128.4, 128.5, 129.3, 129.6, 130.1, 133.5, 138.0, 138.1, 143.8, 151.0, 165.1. HRMS (FAB) m/z calcd for $C_{32}H_{30}NaO_5$ $[M+Na]^+$: 517.1985; found 517.2000.

4.11. 1-Benzoyloxy-3-(2-deoxy-β-D-ribofuranosyl)benzene (15)

A solution of compound **14** (230 mg, 0.466 mmol), 20% $Pd(OH)_2$/C (150 mg), and cyclohexene (4.7 mL, 46.6 mmol) in EtOH (10 mL) was stirred at 70 °C for 1.5 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 **15** (140 mg, 96%) as a colorless oil. $[\alpha]_D^{24}$ +19.1 (c 1.88, $CHCl_3$). IR ν_{max} (KBr) 3372, 2929, 1736, 1263 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.95 (1H, ddd, J=6.5, 10.0, 13.0 Hz), 2.22 (1H, ddd, J=2.0, 5.0, 13.0 Hz), 2.94 (1H, br s), 3.13 (1H, br s), 3.68 (2H, m), 3.96 (1H, m), 4.30 (1H, m), 5.16 (1H, dd, J=5.0, 10.0 Hz), 7.11 (1H, ddd, J=1.0, 2.0, 8.0 Hz), 7.21 (2H, m), 7.37 (1H, dd, J=8.0, 8.0 Hz), 7.49 (2H, dd, J=8.0, 8.0 Hz), 7.62 (1H, dd, J=8.0, 8.0 Hz), 8.17 (2H, dd, J=2.0, 8.0 Hz). ^{13}C NMR ($CDCl_3$) δ 43.6, 63.2, 73.4, 79.4, 87.4, 119.2, 120.9, 123.6, 128.6, 129.3, 129.5, 130.1, 133.7, 143.4, 151.0, 163.3. HRMS (MALDI-TOF) m/z calcd for $C_{18}H_{18}NaO_5$ $[M+Na]^+$: 337.1046; found 337.1058.

4.12. 1-Benzoyloxy-3-[2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl]benzene (16)

Under a nitrogen atmosphere, DMTrCl (49 mg, 0.145 mmol) was added to a solution of compound **15** (38 mg, 0.121 mmol) in anhydrous pyridine (1.5 mL) 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, 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 3:1) to give compound **16** (53 mg, 72%) as a white solid. $[\alpha]_D^{23}$ +23.2 (c 1.16, AcOEt). IR ν_{max} (KBr) 3454, 2932, 1736, 1607, 1508, 1252, 1176, 1080 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.76 (1H, d, J=4.0 Hz), 2.08 (1H, ddd, J=6.0, 10.0, 13.5 Hz), 2.28 (1H, ddd, J=2.0, 6.0, 13.5 Hz), 3.25 (1H, dd, J=4.5, 10.0 Hz), 3.35 (1H, dd, J=5.0, 10.0 Hz), 3.75 (6H, s), 4.06 (1H, ddd, J=4.5, 5.0, 7.0 Hz), 4.46 (1H, m), 5.20 (1H, dd, J=6.0, 10.0 Hz), 6.75–6.85 (4H, m), 7.10–7.40 (11H, m), 7.45 (2H, dd, J=1.0, 8.0 Hz), 7.48 (2H, dd, J=8.0, 8.0 Hz), 7.62 (1H, ddd, J=1.0, 8.0, 8.0 Hz), 8.20 (2H, dd, J=1.5, 8.0 Hz). ^{13}C NMR ($CDCl_3$) δ 44.0, 55.1, 64.5, 74.6, 79.4, 86.2, 86.4, 113.1, 119.2, 120.8, 123.4, 126.7, 127.8, 128.1, 128.5, 129.3, 129.5, 130.0, 130.1, 130.1, 133.5, 135.9, 136.0, 143.9, 144.8, 151.0, 158.4, 158.4, 165.1. HRMS (FAB) m/z calcd for $C_{39}H_{36}NaO_7$ $[M+Na]^+$: 639.2353; found 639.2376.

4.13. 1-Benzoyloxy-3-{3-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl}benzene (2)

Under a nitrogen atmosphere, i-$Pr_2NP(Cl)OCH_2CH_2CN$ (30 μL, 0.134 mmol) was added to a solution of compound **16** (41 mg, 0.067 mmol) and i-Pr_2NEt (58 μL, 0.335 mmol) in anhydrous CH_2Cl_2 (1 mL) at room temperature and the mixture was stirred for 30 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 4:1) to give compound **2** (32 mg, 58%) as a white solid. ^1H NMR ($CDCl_3$) δ 1.08 (3H, d, J=6.8 Hz), 1.15–1.19 (9H, m), 2.02–2.11 (1H, m), 2.34–2.47 (2H, m), 2.61 (1H, dd, J=6.3, 6.8 Hz), 3.23–3.34 (2H, m), 3.53–3.87 (4H, m), 3.74 (3H, s), 3.74 (3H, s), 4.24–4.25 (1H, m), 4.49–4.53 (1H, m), 5.18–5.22 (1H, m), 6.76–6.82 (4H, m), 7.12–7.18 (2H, m), 7.22–7.27 (2H, m), 7.31–7.41 (7H, m), 7.45–7.52 (4H, m), 7.59–7.63 (1H, m), 8.12–8.15 (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 839.3444.

4.14. 1-Allyloxy-4-(2-deoxy-3,5-di-*O*-benzyl-D-ribofuranosyl)benzene (17)

Under a nitrogen atmosphere, a solution of compound **4** (1.00 g, 3.18 mmol) in anhydrous THF (30 mL) was added to a solution of 4-allyloxyphenyllithium [prepared from 4-allyloxyphenyl bromide (2.70 g, 12.7 mmol) and s-BuLi (1.05 M in hexane and cyclohexane, 12.1 mL, 12.7 mmol) in anhydrous THF (130 mL) at −78 °C], and the mixture was stirred at −60 °C for 1 h. After addition of satd NH_4Cl aq, the mixture was extracted with CH_2Cl_2. The extracts were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (n-hexane/AcOEt=3:2) to give appropriate compounds (R_f=ca. 0.5), which were dissolved in CH_2Cl_2 (30 mL); TMAD (600 mg, 3.48 mmol) and n-Bu_3P (0.87 mL, 3.48 mL) were added at room temperature. After being stirred at room temperature for 19 h, water was added and the mixture was extracted with Et_2O. The