Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 2933–2939

BIOORGANIC & MEDICINAL CHEMISTRY

Nucleosides and Nucleotides. Part 214: Thermal Stability of Triplexes Containing 4′α-*C*-Aminoalkyl-2′-deoxynucleosides†

Naoko Atsumi, Yoshihito Ueno,‡ Makiko Kanazaki, Satoshi Shuto and Akira Matsuda*

Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan

Received 8 March 2002; accepted 17 April 2002

Abstract—In order to develop novel antigene molecules forming thermally stable triplexes with target DNAs and having nuclease resistance properties, we synthesized oligodeoxynucleotides (ODNs) with various lengths of aminoalkyl-linkers at the 4′α position of thymidine and the aminoethyl-linker at the 4′α position of 2′-deoxy-5-methylcytidine. Thermal stability of triplexes between these ODNs and a DNA duplex was studied by thermal denaturation. The ODNs containing the nucleoside **2** with the aminoethyl-linker or the nucleoside **3** with the aminopropyl-linker thermally stabilized the triplexes, whereas the ODNs containing the nucleoside **1** with the aminomethyl-linker or the nucleoside **4** with the 2-[*N*-(2-aminoethyl)carbamoyl]oxy]ethyl-linker thermally destabilized the triplexes. The ODNs containing **2** were the most efficient at stabilizing the triplexes with the target DNA. The ODNs containing 4′α-*C*-(2-aminoethyl)-2′-deoxy-5-methylcytidine (**5**) also efficiently stabilized the triplexes with the target DNA. Stability of the ODN containing **5** to nucleolytic hydrolysis by snake venom phosphodiesterase (a 3′-exonuclease) was studied. It was found that the ODN containing **5** was more resistant to nucleolytic digestion by the enzyme than an unmodified ODN. In a previous paper, we reported that the ODNs containing **2** were more resistant to nucleolytic digestion by DNase I (an endonuclease) than the unmodified ODNs. Thus, it was found that the ODNs containing 4′α-*C*-(2-aminoethyl)-2′-deoxynucleosides were good candidates for antigene molecules.

Introduction

Triplex forming oligodeoxynucleotides (ODNs) have attracted a great deal of attention because of their potential use in gene therapy.[2] In intermolecular triplexes, a third strand of ODN binds to the major groove of the DNA. To date, two major classes of triplexes have been identified based on the orientation of the third strand. When the third strand consists mainly of pyrimidines, Hoogsteen-type Py•PuPy base triplets (T•AT and C^+•GC) are formed, in which the third strand is parallel to the purine strand of the target duplex.[3,4] When the third strand is predominantly purines, Pu•PuPy-type base triplets (G•GC and A•AT) are formed, where the third strand is antiparallel to the purine strand of the target duplex.[5,6] However, in general, the binding of a third-strand ODN to a target DNA duplex is thermodynamically weaker than duplex formation itself. Thus, much effort has been made to increase the affinity of the third strand for its target.

On the other hand, naturally occurring polyamines, such as spermidine and spermine, are known to bind strongly to DNA[7–9] and stabilize duplex[10,11] and triplex formation,[12–14] although the precise mode of binding is not clear. Their enhanced thermal stability can be explained by the reduction of the anionic electrostatic repulsion between the phosphate moieties by the cationic polyamines. Consequently, ODN analogues carrying various polyamines have been synthesized,[15–21] some of which have been shown to increase the thermal stability of duplexes and triplexes and to be more resistant to nucleases than unmodified ODNs.

Recently, Cuenoud et al. reported a synthesis of ODNs containing 2′-*O*-aminoethyl-5-methyluridine and 2′-*O*-aminoethyl-5-methylcytidine.[22–24] They showed that the ODNs containing the 2′-*O*-aminoethylnucleosides thermally stabilized triplexes with target DNAs. The thermal stabilization of the triplexes by the

*Corresponding author. Tel.: +81-11-706-3228; fax: +81-11-706-4980; e-mail: matuda@pharm.hokudai.ac.jp

†For Part 213 of this series, see ref 1.

‡Present address: Faculty of Engineering, Gifu University, Yanagido 1-1, Gifu 501–1193, Japan.

0968-0896/02/$ - see front matter
PII: S0968-0896(02)00141-4

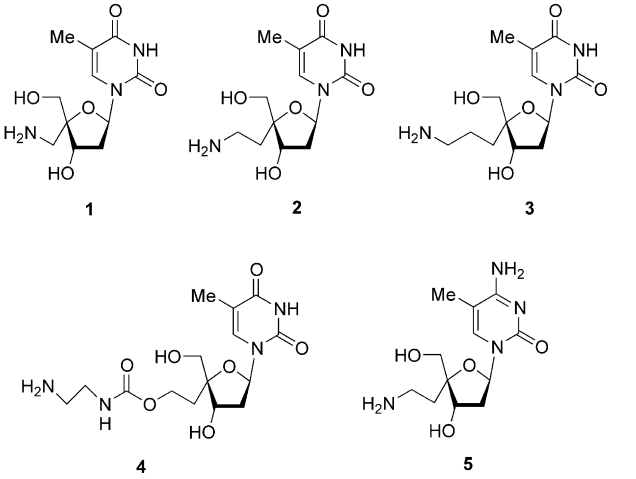


Figure 1. Structures of the modified nucleoside analogues.

2′-*O*-aminoethylnucleosides can be explained by an electrostatic interaction between the positively charged aminoethyl residue of the nucleosides and a *pro-R* oxygen of a negatively charged phosphate at the second strand of the target DNA. Recently, we also synthesized ODNs containing 4′α-*C*-aminoalkylthymidines **1**–**4** (Fig. 1).[25–27] We found that the ODNs containing 4′α-*C*-aminoethylthymidine formed thermally stable duplexes with the complementary DNA and RNA, and were much more resistant to nucleolytic hydrolysis by snake venom phosphodiesterase (a 3′-exonuclease) and DNase I (an endonuclease) than the unmodified parent ODNs. On the basis of this background information and our own experimental results, we envisaged that if the distal ammonium cation of the aminoalkyl-linkers of the 4′α-*C*-aminoalkyl-2′-deoxynucleosides interacted with the phosphate group of the target DNA in the triplex, as in the 2′-*O*-aminoethyl-nucleoside, and thermally stabilized the triplex, the ODNs containing 4′α-*C*-aminoalkylthymidines would be good candidates for antigene molecules.

In this paper, we report thermal stabilities of triplexes between the ODNs containing the 4′α-*C*-aminoalkylthymidines **1**–**4** and 4′α-*C*-aminoethyl-2′-deoxy-5-methylcytidine (**5**) and the target DNA. The resistance of the ODNs containing 4′α-*C*-aminoethyl-2′-deoxy-5-methylcytidine (**5**) to nucleolytic hydrolysis by snake venom phosphodiesterase was also studied.

Results

Thermal stability of triplexes containing 4′α-*C*-aminoalkylthymidines

To study the thermal stability of triplexes consisting of ODNs containing 4′α-*C*-aminoalkylthymidines at various positions and in different numbers and the target DNA, 5′-[AG(TC)$_9$C(T)$_5$AG(GA)$_9$CT]-3′ (**27**) (Table 1), the ODNs containing 4′α-*C*-aminomethyl (**1**), 4′α-*C*-(2-aminoethyl) (**2**), 4′α-*C*-(3-aminopropyl) (**3**), and 4′α-*C*-[2-[*N*-(2-aminoethyl)carbamoyl]oxy]ethyl]thymidine (**4**) (Fig. 1) were synthesized according to the methods reported previously.[25,26] The sequences of the synthesized ODNs are shown in Table 1. The thermal stability of the triplexes between these ODNs and the target duplex **27** was studied by thermal denaturation in a buffer containing 0.5 M NaCl at pH 7.0. Two transitions were observed in the melting profile of each triplex (data not shown): the transition with the higher T_m was due to melting of the target duplex **27** (88 °C), and the transition with the lower T_m corresponded to dissociation of the third strand from the triplex. The T_ms are listed in Table 2. The stability of the triplexes was dependent on the number and length of the aminoalkyl-linkers of the modified nucleosides. The T_m of the control triplex was 44.1 °C. The ODNs containing **2** or **3**

Table 1. Sequences of ODNs used in this study[a]

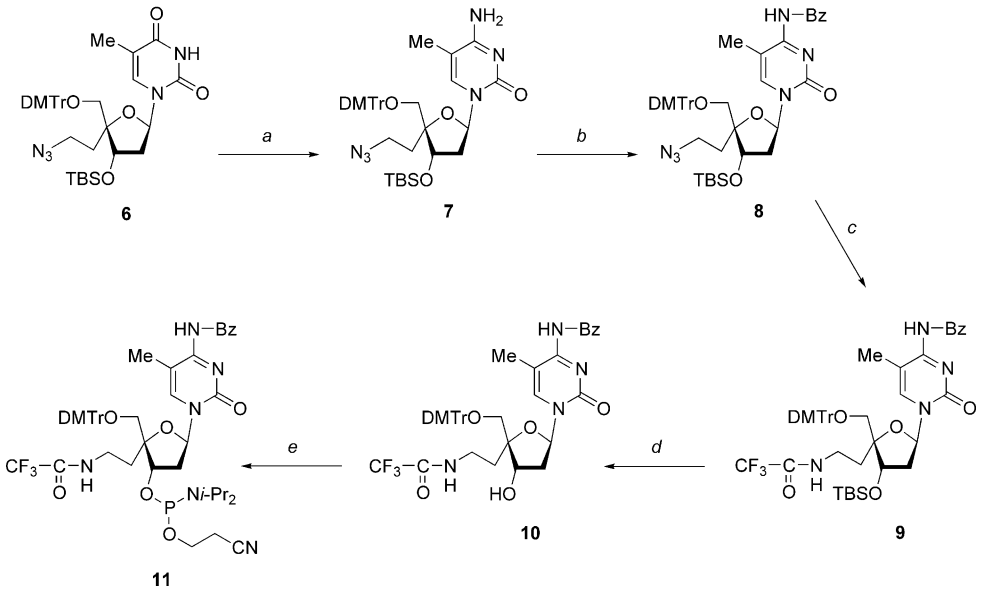
ODNs	
12	5′-MTMTMTMTMTMTMTMTMT-3′
13-1, -2,-3, or **-4**	5′-M***X***MTMTMTMTMTMTMTMT-3′
14-1, -2, -3, or **-4**	5′-MTMTMTMTMTMTMTMTM***X***-3′
15-1, -2, -3, or **-4**	5′-MTMTMTMTM***X***MTMTMTMT-3′
16-1, -2, -3, or **-4**	5′-MTMTM***X***MTMTMTM***X***MTMT-3′
17-1, -2, -3, or **-4**	5′-M***X***MTMTMTM***X***MTMTMTM***X***-3′
18-1, -2, -3, or **-4**	5′-M***X***MTM***X***MTMTM***X***MTMTM***X***-3′
19-1, -2, -3, or **-4**	5′-M***X***MTM***X***MTM***X***MTM***X***MTM***X***-3′
20	5′-**5**TMTMTMTMTMTMTMT-3′
21	5′-MTMTMTMTMTMTMTMT**5**T-3′
22	5′-MTMTMTMT**5**TMTMTMTMT-3′
23	5′-MTMT**5**TMTMTMTMT**5**TMT-3′
24	5′-MT**5**TMTMT**5**TMTMT**5**TMT-3′
25	5′-MT**5**TMT**5**TMT**5**TMT**5**TMT-3′
26	5′-MT**5**T**5**TMT**5**TMT**5**TMT**5**T-3′
27	5′-AG(TC)$_9$C(T)$_5$AG(GA)$_9$CT-3′

[a]**1** series (***X***=**1**); **2** series (***X***=**2**); **3** series (***X***=**3**); **4** series (***X***=**4**); T, thymidine; M, 2′-deoxy-5-methylcytidine.

Table 2. Hybridization data[a]

ODNs	T_m (°C)	Δ T_m (°C)	ODNs	T_m (°C)	Δ T_m (°C)
12	44.1	—			
13-1	42.3	−1.8	**13-4**	43.6	−0.5
14-4	42.8	−1.3	**14-4**	44.9	+0.8
15-1	41.3	−2.8	**15-4**	44.6	+0.5
16-1	41.3	−2.8	**16-4**	43.6	−0.5
17-1	43.8	−0.3	**17-4**	41.8	−2.3
18-1	42.9	−1.2	**18-4**	42.1	−2.0
19-1	44.0	−0.1	**19-4**	41.4	−2.7
13-2	45.2	+1.1	**20**	44.2	+0.1
14-2	44.4	+0.3	**21**	44.3	+0.2
15-2	44.2	+0.1	**22**	44.5	+0.4
16-2	46.5	+2.4	**23**	44.7	+0.6
17-2	47.2	+3.1	**24**	45.4	+1.3
18-2	47.9	+3.8	**25**	47.2	+3.1
19-2	46.7	+2.6	**26**	46.5	+2.4
13-3	44.2	+0.1			
14-3	44.6	+0.5			
15-3	45.3	+1.2			
16-3	45.4	+1.3			
17-3	46.0	+1.9			
18-3	46.0	+1.9			
19-3	46.3	+2.2			

[a]Experimental conditions are described in the Experimental.



Scheme 1. Conditions: (a) (1) 2,4,6-triisopropylbenzenesulfonyl chloride, DMAP, Et_3N, CH_3CN, rt, 1 h; (2) concd NH_4OH, 0 °C–rt, 2 h, 75%; (b) Bz_2O, pyridine, rt, 24 h, 93%; (c) H_2, Pd/C, $EtOCOCF_3$, Et_3N, EtOAc, rt, 3 h, 56%; (d) TBAF, THF, rt, 4 h, 95%; (e) *i*-$Pr_2NP(Cl)O(CH_2)_2CN$, *i*-Pr_2NEt, CH_2Cl_2, rt, 10 min, 62%.

thermally stabilized the triplexes, whereas the ODNs containing **1** or **4** thermally destabilized the triplexes. The triplexes containing **2** or **3** became more stable as the number of the modified nucleosides increased except for the triplex containing five molecules of **2**. When the same numbers of the modified nucleosides were incorporated into the ODNs at the same positions, the order of ΔT_m values [T_m (each ODN) $-T_m$ (the control ODN **12**)] for the ODNs was as follows: the ODNs containing **2** > the ODNs containing **3** > the ODNs containing **1** > the ODNs containing **4** except for the ODNs containing one residue of the modified nucleosides at their 3′-end or center. ΔT_m values for the ODNs containing four molecules of **2** (ODN **18**–**2**) or five molecules of **3** (ODN **19**–**3**) were +3.8 and +2.2 °C, respectively. Therefore, it was found that the ODNs containing **2** with the aminoethyl-linker were the most efficient at stabilizing the triplexes with the target DNA **27**.

Synthesis of ODNs containing 4′α-*C*-(2-aminoethyl)-2′-deoxy-5-methylcytidine

When the various lengths of aminoalkyl-linkers were incorporated into the ODNs at the 4′α-position of the thymidine residues, the thymidine analogue **2** with the aminoethyl-linker stabilized the triplexes very efficiently. The parallel-type triplexes are formed by Hoogsteen-type T·AT and C^+·GC base triplets.[2] Thus, we next planned to synthesize a cytidine analogue with the aminoethyl-linker at its 4′α-position. Formation of the parallel-type triplexes requires conditions of low pH (pH <6), because unmodified cytosine residues, if present in the third strand, must be protonated to bind with guanine of the G/C duplex.[2,28] It is known that the ODNs containing 2′-deoxy-5-methylcytidine form stable parallel-type triplexes under neutral conditions.[29] Thus, we synthesized 4′α-*C*-(2-aminoethyl)-2′-deoxy-5-methylcytidine (**5**). A phosphoramidite of **5** was synthesized as follows: 4′α-*C*-azidoethyl-3′-*O*-*tert*-butyldimethylsilyl-2′-deoxy-5′-*O*-dimethoxytritylthymidine (**6**), which was prepared according to the reported method,[26] was treated with 2,4,6-triisopropylbenzenesulfonyl chloride in the presence of DMAP and Et_3N, followed by NH_4OH, to afford the 2′-deoxy-5-methylcytidine derivative **7** in 75% yield. After protection of the *exo*-amino function of **7** with benzoyl group, the azide group in **8** was hydrogenated with Pd/C in the presence of ethyl trifluoroacetate to give the *N*-protected 4′α-*C*-aminoethyl-2′-deoxy-5-methylcytidine derivative **9** in 56% yield. After removal of the TBS group, **10** was phosphitylated by a standard procedure[30] to give the corresponding nucleoside phosphoramidite **11** in 62% yield (Scheme 1).

The ODNs containing **5** were prepared on a DNA synthesizer by the phosphoramidite method.[31] The sequences of the synthesized ODNs are shown in Table 1. The ODNs obtained were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, and the observed molecular weights supported their structures (see Experimental).

Thermal stability of the triplexes containing 4′α-*C*-(2-aminoethyl)-2′-deoxy-5-methylcytidine

The thermal stability of the triplexes between the ODNs (**20**–**26**) containing 4′α-*C*-(2-aminoethyl)-2′-deoxy-5-methylcytidine (**5**) at various positions and in different numbers and the target duplex **27** was also studied by thermal denaturation in the buffer containing 0.5 M NaCl at pH 7.0. Two transitions were observed in the melting profile of each triplex (data not shown): the transition with the higher T_m was due to melting of the target duplex, and the transition with the lower T_m corresponded to dissociation of the third strand from the triplex. The T_ms are summarized in Table 2. The stability of the triplexes was dependent on the position and

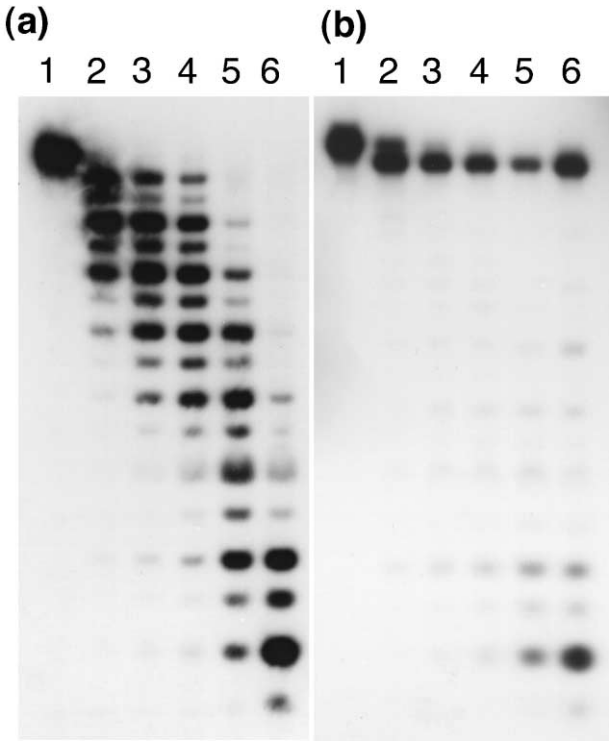


Figure 2. Polyacrylamide gel electrophoresis of 5′-^{32}P-labeled ODNs hydrolyzed by snake venom phosphodiesterase: (a) **12**; (b) **21**. ODNs were incubated with snake venom phosphodiesterase for 0 min (lane 1), 10 min (lane 2), 20 min (lane 3), 30 min (lane 4), 60 min (lane 5), and 120 min (lane 6). Experimental conditions are described in the Experimental.

number of the modified nucleoside **5**. The ODNs containing **5** thermally stabilized the triplexes with the target DNA relative to the control ODN **12**. The ODN, which contained one residue of **5** at the center, stabilized the triplex more than the ODNs containing one residue of **5** at its 5′-end or near its 3′-end. The triplexes containing **5** became thermally more stable as the number of the modified nucleosides increased except for the triplex containing five molecules of **5**. The ΔT_m values for the ODNs containing four molecules of **5** (ODN **25**) was $+3.1\,^{\circ}C$.

Nuclease resistance of the ODN containing 4′α-*C*-(2-aminoethyl)-2′-deoxy-5-methylcytidine

Recently, we found that the ODN containing **2** was more resistant to nucleolytic digestion both by snake venom phosphodiesterase (a 3′-exonuclease) and DNase I (an endonuclease) than the unmodified ODN.[26] We next examined the stability of the ODNs containing **21** against snake venom phosphodiesterase. The ODN **21** containing **5** was labeled at the 5′-end with ^{32}P and incubated with the enzyme, and the reactions were analyzed by polyacrylamide gel electrophoresis under denaturing conditions. The control ODN **12** was hydrolyzed randomly by the enzyme after 120 min of incubation (Fig. 2a). In contrast, the phosphodiester linkage at the 5′-side of **5** was highly resistant to the enzyme, and the phosphodiester linkage at the 3′-side of **5** was also slightly resistant to the enzyme (Fig. 2b). Thus, it was found that the ODN containing **5** was more resistant to nucleolytic digestion by snake venom phosphodiesterase than the unmodified ODN.

Discussion

The ODNs with the various lengths of the aminoalkyl-linkers at the 4′α position of thymidine and the aminoethyl-linker at the 4′α position of 2′-deoxy-5-methylcytidine were synthesized using the phosphoramidite method. The thermal stability of the triplexes between these ODNs and the target duplex was studied by thermal denaturation. It was found that the stability of the triplexes was dependent on the number and length of the aminoalkyl-linkers of the modified nucleosides. The ODNs containing **2** with the aminoethyl-linker or **3** with the aminopropyl-linker thermally stabilized the triplexes, whereas the ODNs containing **1** with the aminomethyl-linker or **4** with 2-[*N*-(2-aminoethyl)carbamoyl]-oxy]ethyl-linker thermally destabilized the triplexes. The ODNs containing **2** with the aminoethyl-linker most efficiently stabilized the triplexes with the target DNA.

Recently, Cuenoud et al. reported that the ODNs containing the 2′-*O*-aminoethylnucleosides also enhanced thermal stability of triplexes with DNA duplexes.[22–24] Based on the molecular dynamics (MD) simulation and an NMR study, they concluded that the enhanced thermal stability of the triplexes upon incorporation of 2′-*O*-aminoethylnucleosides was due to an electrostatic interaction between the positively charged aminoethyl chain of the nucleoside and a *pro-R* oxygen of a negatively charged phosphate of the second strand of the target DNA. To understand the effect of the 4′α-*C*-(2-aminoethyl)thymidine **2** on the triplex formation, we also performed an MD simulation of the triplex containing **2**. The model of the triplex containing **2** derived from the MD simulation showed that the ammonium ion at the end of the aminoethyl chain of **2** could form an intermolecular ionic bond with the *pro-R*-oxygen atom of the phosphate group of the second strand of the target duplex (data not shown). Thus, we reasoned a priori that the aminomethyl-linker of **1** was too short to stabilize the triplexes while the 2-[[*N*-(2-aminoethyl)carbamoyl]oxy]ethyl-linker of **4** was too long to stabilize the triplexes.

The 4′α-*C*-(2-aminoethyl)thymidine (**2**) and 4′α-*C*-(2-aminoethyl)-2′-deoxy-5-methylcytidine (**5**) thermally stabilized the triplexes with the target DNAs. However, the stabilization of the triplexes by the 4′α-*C*-(2-aminoethyl)-2′-deoxynucleosides seems to be less efficient than the stabilization by the 2′-*O*-aminoethylnucleoside. An increase of the T_m value of $+0.95\,^{\circ}C$ per modification was observed when four molecules of **2** were incorporated into the ODN, whereas an increase of T_m value of the $+0.78\,^{\circ}C$ per modification was observed when four molecules of **5** were incorporated into the ODN. On the other hand, the ODNs containing 2′-*O*-aminoethylnucleosides stabilize the triplexes by an increase of the T_m value of $+3.5\,^{\circ}C$ per modification.[22] It has been reported that an ODN consisting of a nucleoside adopting an *N-type* sugar conformation thermally stabilizes a triplex with a DNA duplex.[32–34] The 2′-*O*-aminoethylnucleoside is known to adopt predominantly an *N-type* sugar conformation,[23,24] whereas the 4′α-*C*-(2-aminoethyl)-2′-deoxynucleoside adopts predominantly an *S-type*

sugar conformation.[26] Thus, the difference in their sugar conformation should influence the thermal stability of the triplexes with the DNA duplex.

The triplexes containing 4′α-*C*-(2-aminoethyl)thymidine (**2**) and 4′α-*C*-(2-aminoethyl)-2′-deoxy-5-methylcytidine (**5**) became thermally more stable as the number of the modified nucleosides increased until four molecules of **2** or **5** were incorporated into the ODNs. However, the thermal stabilities of the triplexes containing five molecules of **2** or **5** decreased relative to those of the triplexes containing four molecules of **2** or **5**. This phenomenon might be due to the sequence of the ODNs used, in which 2′-deoxy-5-methylcytidine derivatives were alternatively incorporated into the ODNs. When the parallel-type triplexes are formed, the N3 position of the cytosine residues, if present in the third strand, must be protonated to bind with the guanine of the G/C duplex. Therefore, the triplexes of this sequence might already be stabilized by positive charges in the M^+/G/C base triplets; thus, additional positive charges can not further stabilize them.

Resistance of the ODNs to nucleolytic hydrolysis by nucleases is an important factor in antigene studies.[2] Thus, the stability of the ODNs containing 4′α-*C*-(2-aminoethyl)-2′-deoxy-5-methylcytidine (**5**) to nucleolytic hydrolysis by snake venom phosphodiesterase (a 3′-exonuclease) was studied. It was found that the ODN containing **5** was more resistant to nucleolytic digestion by the enzyme than the unmodified ODN. In our previous paper, we reported that ODNs containing 4′α-*C*-(2-aminoethyl)thymidine (**2**) were more resistant to nucleolytic digestion by DNase I (an endonuclease) than the unmodified ODN.[26] Thus, it was found that the ODNs containing 4′α-*C*-(2-aminoethyl)nucleosides were resistant against both 3′-exo and endo nucleases.

In conclusion, we have found that the ODNs containing 4′α-*C*-(2-aminoethyl)thymidine (**2**) and 4′α-*C*-(2-aminoethyl)-2′-deoxy-5-methylcytidine (**5**) thermally stabilized the triplexes with the DNA duplex, and that the ODNs containing **5** were more resistant to nucleolytic digestion by snake venom phosphodiesterase than the unmodified ODN. Thus, the ODNs containing 4′α-*C*-(2-aminoethyl)-2′-deoxynucleosides appear to be good candidates for antigene molecules.

Experimental

General remarks

NMR spectra were recorded at 400 MHz (^{1}H), at 100 MHz (^{13}C) and at 202 MHz (^{31}P). Chemical shifts (δ) are reported in ppm downfield from TMS or 85% H_3PO_4. *J* values are given in Hertz. Mass spectra were obtained by fast atom bombardment (FAB) method. Thin-layer chromatography was done on Merck coated plates $60F_{254}$. The silica gel or the neutralized silica gel used for column chromatography were Merck silica gel 5715 or ICN silica 60A, respectively.

4′α-C-Azidoethyl-3′-*O*-*tert*-butyldimethylsilyl-2′-deoxy-5′-*O*-dimethoxytrityl-5-methylcytidine (7). A solution of 4′α-*C*-azidoethyl-3′-*O*-*tert*-butyldimethylsilyl-2′-deoxy-5′-*O*-dimethoxytritylthymidine (**6**)[26] (1.6 g, 2.2 mmol), Et_3N (1.2 mL, 8.8 mmol), DMAP (0.81 g, 6.6 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (1.33 g, 4.4 mmol) in CH_3CN (100 mL) was stirred at room temperature for 1 h. The mixture was cooled in an ice-bath. Concentrated NH_4OH (25%, 160 mL) was added, and the mixture was stirred at room temperature for 2 h. The mixture was evaporated, and the residue was partitioned between EtOAc and H_2O. The organic layer was washed brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (SiO_2, 1–4% EtOH in $CHCl_3$) to give **7** (1.2 g, 75% as a yellow foam): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (br s, 2H), 7.20 (s, 1H), 7.41–6.82 (m, 13H), 6.21 (dd, 1H, *J* = 5.9, 6.3), 4.60 (m, 1H), 3.80 (s, 6H), 3.42–3.35 (m, 2H), 3.26 (m, 1H), 3.07 (d, 1H, *J* = 10.3), 2.45 (m, 1H), 2.19 (m, 1H), 1.98 (m, 1H), 1.68 (m, 1H), 1.44 (s, 3H), 0.83 (s, 9H), 0.02, −0.05 (each s, each 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.46, 158.60, 144.04, 137.93, 135.11, 130.04, 130.00, 128.10, 127.87, 127.07, 113.17, 113.13, 101.37, 87.23, 86.79, 84.75, 72.29, 65.12, 55.25, 46.98, 41.37, 31.14, 25.70, 17.93, 12.61, −4.55, −5.07. Anal. calcd for $C_{39}H_{50}N_6O_6Si\cdot 1/2H_2O$: C, 64.10; H, 7.43; N, 10.69. Found: C, 64.20; H, 7.17; N, 10.58.

***N*^{4}-Benzoyl-4′α-*C*-azidoethyl-3′-*O*-*tert*-butyldimethylsilyl-2′-deoxy-5′-*O*-dimethoxytrityl-5-methylcytidine (8).** A solution of **7** (86 mg, 0.12 mmol) and Bz_2O (54 mg, 0.24 mmol) in pyridine (5 mL) was stirred at room temperature for 24 h. The mixture was partitioned between EtOAc and aqueous saturated $NaHCO_3$. The organic layer was washed with H_2O and then brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (SiO_2, 4–13% EtOAc in hexane) to give **8** (93 mg, 93% as a white foam): FAB-MS *m*/*z* 831 (MH^+); ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.3 (br s, 1H), 8.29 (m, 2H), 7.77 (s, 1H), 7.41–6.84 (m, 16H), 6.24 (dd, 1H, *J* = 6.3, 6.6), 4.63 (dd, 1H, *J* = 4.6, 6.8), 3.80 (s, 6H), 3.42–3.36 (m, 2H), 3.26 (m, 1H), 3.12 (d, 1H, *J* = 10.3), 2.40 (ddd, 1H, *J* = 4.6, 6.3, 13.7), 2.27 (ddd, 1H, *J* = 6.6, 6.8, 13.7), 2.00 (m, 1H, *J* = 14.6), 1.70 (m, 1H, *J* = 14.6), 1.65 (s, 3H), 0.86 (s, 9H), 0.05, −0.01 (each s, each 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.28, 159.59, 158.65, 147.75, 143.91, 137.00, 136.57, 134.97, 134.93, 132.26, 129.98, 129.73, 128.04, 127.97, 127.92, 127.15, 113.22, 113.19, 111.85, 87.84, 87.04, 84.47, 72.74, 65.39, 55.23, 46.91, 41.19, 31.28, 25.70, 17.96, 12.98, −4.58, −5.04; HRMS calcd for $C_{46}H_{55}O_7N_6Si$: 831.3901. Found: 831.3918.

***N*^{4}-Benzoyl-3′-*O*-*tert*-butyldimethylsilyl-2′-deoxy-5′-*O*-dimethoxytrityl-5-methyl-4′α-*C*-(2-*N*-trifluoroacetyl)aminoethylcytidine (9).** A solution of **8** (0.415 g, 0.5 mmol), Et_3N (0.49 mL, 3.5 mmol), ethyl trifluoroacetate (0.30 mL, 2.5 mmol) and Pd/C (10%, 85 mg) in EtOAc (45 mL) was stirred under atmospheric pressure of H_2 at room temperature for 3 h. The catalyst was filtered off with Celite, and the filtrate was evaporated. The residue was purified by column chromatography (a neutralized SiO_2, 15% EtOAc in hexane) to give **9** (0.25 g, 56% as a

white foam): FAB-MS *m/z* 901 (MH^+); ^1H NMR (400 MHz, $CDCl_3$) δ 13.3 (br s, 1H), 8.29 (m, 2H), 7.61 (s, 1H), 7.54–7.24 (m, 12H), 6.93 (m, 1H), 6.86–6.84 (m, 4H), 6.28 (dd, 1H, $J=6.3$, 6.8), 4.55 (m, 1H), 3.79 (m, 8H), 3.50 (m, 1H), 3.38 (m, 1H), 3.31 (d, 1H, $J=10.3$), 3.17 (d, 1H, $J=10.3$), 2.34 (ddd, 1H, $J=3.9$, 6.3, 13.7), 2.26 (m, 1H), 2.03 (m, 1H), 1.72 (m, 1H), 1.71 (s, 3H), 0.86 (s, 9H), 0.05, 0.01 (each s, each 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.91, 179.12, 162.51, 162.13, 161.78, 161.46, 159.44, 158.61, 143.77, 136.70, 135.06, 134.74, 132.34, 130.34, 130.23, 129.96, 129.62, 129.19, 129.02, 128.91, 128.03, 127.88, 127.13, 116.38, 113.19, 113.16, 111.96, 88.24, 87.04, 84.80, 72.74, 64.83, 55.13, 39.87, 36.01, 31.03, 30.95, 30.88, 30.79, 25.52, 17.79, 12.82, −4.89, −5.26; HRMS calcd for $C_{48}H_{56}F_3N_4O_8Si$: 901.3820. Found: 901.3817.

***N*^{4}-Benzoyl-2′-deoxy-5′-*O*-dimethoxytrityl-5-methyl-4′α-*C*-(2-*N*-trifluoroacetyl) aminoethylcytidine (10).** A solution of **9** (0.254 g, 0.28 mmol), TBAF (1.0 M in THF, 0.56 mL, 0.56 mmol) in THF (15 mL) was stirred at room temperature for 4 h. The mixture was evaporated, and the residue was purified by column chromatography (SiO_2, 20–50% EtOAc in hexane) to give **10** (0.21 g, 95% as a white foam): FAB-MS *m/z* 786 (MH^+); ^1H NMR (400 MHz, $CDCl_3$) δ 13.3 (br s, 1H), 8.25 (m, 2H), 7.69 (s, 1H), 7.60 (br s, 1H), 7.54–6.83 (m, 16H), 6.32 (dd, 1H, $J=5.9$, 7.3), 4.55 (m, 1H), 3.77 (m, 7H), 3.43–3.33 (m, 2H), 3.29 (t, 2H, $J=10.5$), 2.51 (ddd, 1H, $J=2.9$, 5.9, 13.7), 2.43 (m, 1H), 2.10–1.95 (m, 2H), 1.71 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.29, 179.41, 159.56, 158.65, 157.03, 148.19, 143.86, 136.72, 134.88, 134.79, 132.49, 129.96, 129.81, 129.67, 128.06, 127.98, 127.20, 115.75, 113.27, 112.24, 88.60, 87.39, 84.94, 84.87, 73.54, 73.41, 66.47, 55.27, 55.21, 40.98, 35.75, 30.76, 13.06; HRMS calcd for $C_{42}H_{42}F_3N_4O_8$: 787.2955. Found: 787.2969.

***N*^{4}-Benzoyl-2′-deoxy-5′-*O*-dimethoxytrityl-5-methyl-4′α-*C*-(2-*N*-trifluoroacetyl)aminoethylcytidine 3′-*O*-(2-cyanoethyl) *N,N*-diisopropylphosphoramidite (11).** A solution of **10** (0.844 g, 1.1 mmol), *N,N*-diisopropylethylamine (0.77 mL, 4.4 mmol) and chloro(2-cyanoethoxy)(*N,N*-diisopropylamino)phosphine (0.49 mL, 2.2 mmol) in CH_2Cl_2 (35 mL) was stirred at room temperature for 10 min. The mixture was partitioned between $CHCl_3$ and aqueous saturated $NaHCO_3$. The organic layer was washed with H_2O and then brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (a neutralized SiO_2, 10–30% EtOAc in hexane) to give **11** (0.67 g, 62% as a white foam): FAB-MS *m/z* 987 (MH^+); ^{31}P NMR (202 MHz, $CDCl_3$) δ 150.44, 149.92; HRMS calcd for $C_{51}H_{59}F_3N_6O_9P$: 987.4033. Found: 987.4017.

Synthesis of ODNs

ODNs were synthesized on a DNA synthesizer (Applied Biosystem Model 392) by the phosphoramidite method.[31] The fully protected ODNs were then deblocked and purified by the same procedure as for the purification of normal ODNs.[35] That is, each ODN linked to the resin was treated with concentrated NH_4OH at 55 °C for 16 h, and the released ODN protected by a DMTr group at the 5′-end was chromatographed on a C-18 silica gel column (1 × 10 cm, Waters) with a linear gradient of CH_3CN (from 0 to 30%) in 0.1 M TEAA buffer (pH 7.0). The fractions were concentrated, and the residue was treated with aqueous 80% AcOH at room temperature for 20 min, then the solution was concentrated, and the residue was coevaporated with H_2O. The residue was dissolved in H_2O and the solution was washed with Et_2O, then the H_2O layer was concentrated to give a deprotected ODN. The ODN was further purified by reversed-phase HPLC, using a J'sphere ODN M80 column (4.6 × 150 mm, YMC) with a linear gradient of CH_3CN (from 10 to 35% over 20 min) in 0.01 M TEAA buffer (pH 7.0) to give a highly purified ODNs **20** (6), **21** (7), **22** (7), **23** (4), **24** (4), **25** (4), and **26** (2). The yields are indicated in parentheses as OD units at 260 nm starting from 1 μmol scale.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Spectra were obtained on a Voyager Elite reflection time-of-flight mass spectrometry (PerSeptive Biosystems, Inc., Framingham, MA, USA) equipped with a nitrogen laser (337 nm, 3-ns pulse) in the negative ion mode. 3-Hydroxypicolinic acid (HPA), dissolved in H_2O to give a saturated solution at room temperature, was used as the matrix. Time-to-mass conversion was achieved by calibration by using the peak representing the C^+ cation of the charged derivative to be analyzed. ODN **20**: calculated mass, 5444.1; observed mass, 5446.2. ODN **21**: calculated mass, 5444.1; observed mass, 5445.9. ODN **22**: calculated mass, 5444.1; observed mass, 5444.5. ODN **23**: calculated mass, 5487.1; observed mass, 5486.5. ODN **24**: calculated mass, 5530.1; observed mass, 5530.3. ODN **25**: calculated mass, 5573.2; observed mass, 5573.7. ODN **26**: calculated mass, 5616.2; observed mass, 5615.9.

Thermal denaturation

The solution, that contained the oligodeoxynucleotide (3 μM) and the target duplex (3 μM) in a buffer of 0.01 M sodium cacodylate (pH 7.0) and 0.5 M NaCl, was heated at 60 °C for 5 min and then cooled gradually to an appropriate temperature, and used for the thermal denaturation study. Thermal-induced transitions of each mixture were monitored at 260 nm on a Beckman DU 650 spectrophotometer. Sample temperature was increased 0.5 °C/min.

Partial hydrolysis of ODN with snake venom phosphodiesterase

Each ODN labeled with ^{32}P at the 5′-end (5 pmol) was incubated with snake venom phosphodiesterase (0.32 μg) in the presence of Torula RNA (0.15 OD units at 260 nm) in a buffer containing 37.5 mM Tris–HCl (pH 8.0) and 7.5 mM $MgCl_2$ (total 20 μL) at 37 °C. At appropriate periods, aliquots of the reaction mixture were separated and added to a solution of EDTA

(5 mM, 10 μL), then mixture were heated for 3 min at 90 °C. The solutions were analyzed by electrophoresis on 20% polyacrylamide gel containing 7 M urea.[36]

Acknowledgements

This investigation was supported in part by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science, Sports, and Culture of Japan and a Grant from 'Research for the Future' Program of the Japan Society for the Promotion of Science (JSPS-RFTF97I00301). We thank Y. Misawa (Hokkaido University) for technical assistance.

References and Notes

1. Kodama, T.; Shuto, S.; Ichikawa, S.; Matsuda, A. Submitted for publication.
2. Thuong, N. T.; Hélène, C. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 666.
3. Moser, H. E.; Dervan, P. B. *Science* **1987**, *238*, 645.
4. Rajagopal, P.; Feigon, J. *Nature* **1989**, *339*, 637.
5. Beal, P. A.; Dervan, P. B. *Science* **1991**, *251*, 1360.
6. Pilch, D. S.; Levenson, C.; Shafer, R. H. *Biochemistry* **1991**, *30*, 6081.
7. Tabor, C. W.; Tabor, H. *Ann. Rev. Biochem.* **1976**, *45*, 285.
8. Tabor, C. W.; Tabor, H. *Ann. Rev. Biochem.* **1984**, *53*, 749.
9. Etter, M. C. *Acc. Chem. Res.* **1990**, *23*, 120.
10. Tabor, H. *Biochemistry* **1962**, *1*, 496.
11. Thomas, T. J.; Bloomfield, V. A. *Biopolymers* **1984**, *23*, 1295.
12. Hampel, K. J.; Crosson, P.; Lee, J. S. *Biochemistry* **1991**, *30*, 4455.
13. Thomas, T.; Thomas, T. J. *Biochemistry* **1993**, *32*, 14068.
14. Musso, M.; Van Dyke, M. W. *Nucleic Acids Res.* **1995**, *23*, 2320.
15. Tung, C.-H.; Breslauer, K. J.; Stein, S. *Nucleic Acids Res.* **1993**, *21*, 5489.
16. Prakash, T. P.; Barawkar, D. A.; Kumar, V.; Ganesh, K. N. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1733.
17. Barawkar, D. A.; Kumar, V.; Ganesh, K. N. *Biochem. Biophys. Res. Comm.* **1994**, *205*, 1665.
18. Schmid, N.; Behr, J.-P. *Tetrahedron Lett.* **1995**, *36*, 1447.
19. Nara, H.; Ono, A.; Matsuda, A. *Bioconjugate Chem.* **1995**, *6*, 54.
20. Sund, C.; Puri, N.; Chattopadhyaya, J. *Tetrahedron* **1996**, *52*, 12275.
21. Haginoya, N.; Ono, A.; Nomura, Y.; Ueno, Y.; Matsuda, A. *Bioconjugate Chem.* **1997**, *8*, 271.
22. Cuenoud, B.; Casset, F.; Hüsken, D.; Natt, F.; Wolf, R. M.; Altmann, K.-H.; Martin, P.; Moser, H. E. *Angew. Chem., Int. Ed.* **1998**, *37*, 1288.
23. Blommers, M. J. J.; Natt, F.; Jahnke, W.; Cuenoud, B. *Biochemistry* **1998**, *37*, 17714.
24. Carlomagno, T.; Blommers, M. J. J.; Meiler, J.; Cuenoud, B.; Griesinger, C. *J. Am. Chem. Soc.* **2001**, *123*, 7364.
25. Ueno, Y.; Nagasawa, Y.; Sugimoto, I.; Kojima, N.; Kanazaki, M.; Shuto, S.; Matsuda, A. *J. Org. Chem.* **1998**, *63*, 1660.
26. Kanazaki, M.; Ueno, Y.; Shuto, S.; Matsuda, A. *J. Am. Chem. Soc.* **2000**, *122*, 2422.
27. Ueno, Y.; Tomino, K.; Sugimoto, I.; Matsuda, A. *Tetrahedron* **2000**, *56*, 7903.
28. Singleton, S. F.; Dervan, P. B. *Biochemistry* **1992**, *31*, 10995.
29. Povsic, T. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 3059.
30. Sinha, N. D.; Biernat, J.; Köster, H. *Tetrahedron Lett.* **1983**, *24*, 5843.
31. Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859.
32. Roberts, R. W.; Crothers, D. M. *Science* **1992**, *258*, 1463.
33. Han, H.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3806.
34. Shimizu, M.; Konishi, A.; Shimada, Y.; Inoue, H.; Ohtsuka, E. *FEBS Lett.* **1992**, *302*, 155.
35. Gait, M. J., Ed. *Oligonucleotides Synthesis: A Practical Approach*; IRL: Oxford, UK, 1984.
36. Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor University Press: Cold Spring Harbor, NY, 1982.