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Effects of 5-(*N*-aminohexyl)carbamoyl-2′-deoxyuridine on endonuclease stability and the ability of oligodeoxynucleotide to activate RNase H

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ABSTRACT

To evaluate an endonuclease resistance property of oligodeoxynucleotides (ODNs) containing 5-(*N*-aminohexyl)carbamoyl-2′-deoxyuridines (Hs) and to elucidate whether a duplex consisting of the ODN analogue and its complementary RNA induces RNase H activity, the ODNs containing the deoxyuridine analogues, Hs, at intervals of one, two, three, four and five natural nucleosides were synthesized. From partial hydrolysis of these ODNs with nuclease S1 (an endonuclease), it was found that the ODNs became more stable towards nucleolytic hydrolysis by the enzyme as the number of H increased. Furthermore, to examine whether the duplexes composed of the ODNs containing Hs and their complementary RNAs are substrates for RNase H or not, the duplexes of these ODNs and their complementary RNA strands were treated with *Escherichia coli* RNase H. It was found that cleavage of the RNA strands by the enzyme was kinetically affected by the introduction of Hs into the duplexes.

INTRODUCTION

Antisense oligodeoxynucleotides (ODNs) have been applied extensively to the regulation of cellular and viral gene expression (1–3). They hybridize to mRNA targets by Watson–Crick base-pairing and inhibit translation of the mRNA in a sequence-specific manner. One of the major problems encountered when using naturally occurring phosphodiester ODNs as antisense molecules is their rapid degradation by nucleases found in cell culture media and inside cells (1–3). To overcome this problem, several types of backbone-modified ODNs such as methylphosphonates, phosphoramidates and phosphorothioates have been synthesized and used for antisense studies (1–5).

Bacteriophage ϕW-14 DNA is known to contain up to 50% α-putrescinylthymine in place of thymine, and to be more resistant to DNase I and snake venom phosphodiesterase than unmodified DNAs (6–10). This modification also causes a higher melting temperature (T_m) than that expected for unmodified DNA on the basis of the GC content (9,10). However, short synthetic ODNs containing α-putrescinylthymine were found to reduce the thermal stability of duplexes as compared with their parent ODNs in our laboratory (11). We thought that the length of the amino-linkers and the distal position of the amino group would be important in protecting against nucleolytic hydrolysis by nucleases and in stable duplex formation.

Recently, we have developed a new and convenient post-synthetic modification method for the synthesis of ODNs having various amino-linkers using 5-methoxycarbonyl-2′-deoxyuridine (**1**) or 5-trifluoroethoxycarbonyl-2′-deoxyuridine (**2**) as a convertible nucleoside (12–15). On treatment of the ODNs containing **1** or **2** at specific positions with several diaminoalkanes, the desired ODNs containing 5-(*N*-aminoalkyl)carbamoyl-2′-deoxyuridines can be readily obtained. By this method, we can find an optimum length of the linker for a desired function without synthesizing a number of mononucleoside units with a variety of linker lengths separately. Consequently, we found that the ODNs containing 5-(*N*-aminohexyl)carbamoyl-2′-deoxyuridines (**H**s) effectively stabilized duplex formation with both the complementary DNA and RNA strands. Furthermore, the ODNs containing **H**s showed good resistance to nucleolytic hydrolysis by snake venom phosphodiesterase (3′-exonuclease) and was also stable in medium containing 10% fetal calf serum (FCS) (13). These properties make the ODNs containing **H**s leading to a candidate as a novel antisense molecule.

It is known that ribonuclease H (RNase H) catalyzes the hydrolysis of the RNA in a DNA–RNA heteroduplex in the presence of divalent cations such as Mg^{2+} or Mn^{2+} (16). This enzyme is widely present in various organisms and plays an important role in DNA replication (16). Although the role of RNase H in the antisense strategy is not clearly understood, it has been postulated that antisense activity of antisense ODNs is due, at least in part, to cleavage of the RNA strand in a DNA–RNA duplex by RNase H (1–3). Furthermore, it has been suggested that the phosphodiester ODNs are degraded more rapidly inside cells by endonucleases than the corresponding backbone-modified ODNs (3,17,18).

As a part of a continuing study of the ODNs containing **H**s as antisense molecules, in this paper we examine the endonuclease-resistance properties of the ODNs containing **H**s and their ability to elicit RNase H activity. For this, **H**s were introduced into heptadecanucleotides at intervals of one, two, three, four and five natural nucleosides (Fig. 1). The thermal and thermodynamic stabilities of duplexes composed of these ODNs and their complementary RNA strands, the resistance of the ODNs to nucleolytic hydrolysis by nuclease S1 (endonuclease), and whether

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control: 5'-CTTGTATTTGTTTTTCT-3'
  gap 5: 5'-CTHGTATTHGTTTTHCT-3'
  gap 4: 5'-CHTGTAHTTGTHTTTCH-3'
  gap 3: 5'-CHTGHATTHGTTHTTCH-3'
  gap 2: 5'-CHTGHATHTGHTTHTCH-3'
  gap 1: 5'-CTHGHAHTHGHTHTHCH-3'
target RNA: 5'-AGAAAAACAAAUACAA-3'
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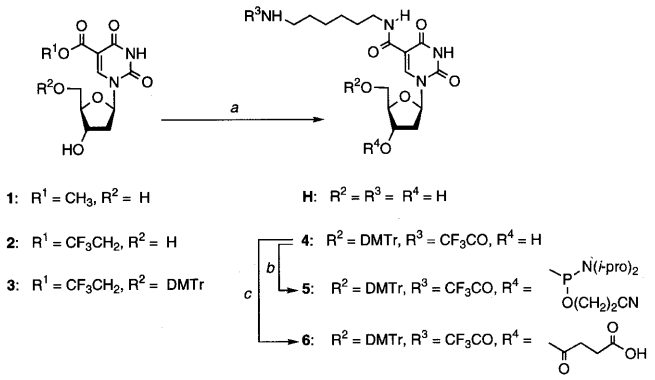
Figure 1. Sequences of ODNs synthesized. H = 5-(*N*-aminohexyl)carbamoyl-2′-deoxyuridine.



Scheme 1. (**a**) (1) $NH_2(CH_2)_6NH_2$, pyridine, room temperature, (2) CF_3COOEt, Et_3N, DMF, room temperature; (**b**) 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloride, *N*,*N*-diisopropylethylamine, CH_2Cl_2, room temperature; (**c**) succinic anhydride, DMAP, pyridine, room temperature.

the duplexes of the ODNs containing **H**s with their complementary RNA strands elicit RNase H activity were examined.

RESULTS AND DISCUSSION

Synthesis

The ODNs containing **H**s were synthesized with a DNA synthesizer using a suitably protected 5-(*N*-aminohexyl)carbamoyl-2′-deoxyuridine phosphoramidite, **5**. The synthesis of **5** is shown in Scheme 1. 5′-*O*-Dimethoxytrityl-5-trifluoroethoxycarbonyl-2′-deoxyuridine (**3**), prepared by a previously described method (14,15), was easily reacted with 1,6-diaminohexane. Without purification of the product, the amino group was protected with trifluoroacetyl group to give **4**. The modified nucleoside **4** was converted to the protected nucleoside phosphoramidite **5** by the standard method of ODN synthesis (19–21). To incorporate **H** into the 3′-end of the ODNs, **4** was further derivatized to its 3′-succinate **6**, which was then linked to controlled pore glass (CPG) to give a solid support containing **4** (36.0 µmol/g).

Unmodified DNA heptadecamer (control) and heptadecamers containing **H**s which replaced natural nucleosides at intervals of one, two, three, four and five nucleosides, were synthesized on a DNA synthesizer. The average coupling yield of **5** was 96% using a 0.12 M solution of the amidite derivative in CH_3CN and a 360 s coupling time. The fully protected ODNs linked to the solid support were deprotected and purified by the same procedures as for the purification of natural ODNs. Each ODN produced showed a single peak in reversed phase HPLC analysis. The nucleoside composition of the modified ODNs was analyzed by HPLC after complete digestion with a mixture of snake venom phosphodiesterase and calf intestine alkaline phosphatase described previously (13). The identity of the peak corresponding to the modified nucleoside was confirmed by coelution with an authentic sample. The nucleoside composition calculated from the peak's area was close to the predicted value (data not shown).

Thermal and thermodynamic stability

Stable duplex formation with mRNA is one of the most important factors in antisense study. Therefore, stability of duplexes formed by these ODNs and their complementary RNA, 5′-AGAAAAACAAAUACAA-3' (target RNA), was studied by thermal denaturation. The UV melting profiles of all the duplexes exhibited helix to coil transitions whose shapes were similar to that of the unmodified duplex. The thermodynamic parameters (ΔH°, ΔS° and ΔG°) of the duplexes were determined by calculations based on the slope of a $1/T_m$ versus $\ln(C_T/4)$ plot, where C_T is total concentration of single strands. T_ms and the parameters are listed in Table 1. The T_m and negative free energy ($-\Delta G^\circ_{37}$) values of all the modified ODNs containing **H**s were greater than those of the corresponding natural ODN. The values of T_m and $-\Delta G^\circ_{37}$ became greater as the number of the modified nucleoside increased. From these results, it was found that the duplexes containing **H**s were, both thermally and thermodynamically, more stable than the corresponding unmodified duplex.

Circular dichroism

To study the conformation of the duplexes, CD spectra of the duplexes composed of the ODNs containing **H**s and their complementary RNA strands were measured. As shown in Figure 2, all the spectra showed a positive band at 260 nm and negative bands at 210 and 245 nm. The shapes of all spectra were similar. However, the spectra of the duplexes containing the modified nucleosides, **H**s, showed slightly enhanced cotton effects in both negative and positive bands as compared with the spectra of the corresponding unmodified duplex. This suggests that the aminohexyl groups introduced into the 5-positions of the 2′-deoxyuridine residues might slightly affect the conformation of the duplexes.

Table 1. Melting temperatures (T_ms) and thermodynamic parameters of ODN–RNA duplexes

ODNs	T_m (3 µM) (°C)	ΔH° (kcal/mol)	ΔS° (cal/Kmol)	ΔG°_{37} (kcal/mol)
gap 1	57.8	–121	–339	–15.9
gap 3	51.8	–125	–356	–14.4
gap 5	50.8	–115	–327	–13.3
control	42.8	–149	–446	–11.1

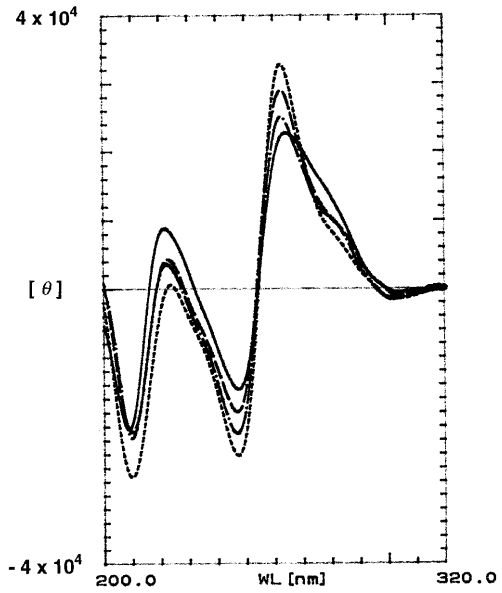


Figure 2. CD spectra of ODN-RNA duplexes at 10°C in 0.01 M NaCl, 0.01 M sodium cacodylate (pH 7.0). (—) control-RNA; (- - - -) gap 1-RNA; (– –) gap 3-RNA; (– · –) gap 5-RNA.

Nuclease resistance of modified ODNs

In a previous study, we found that the ODNs containing **H**s were more resistant to nucleolytic hydrolysis by snake venom phosphodiesterase (3′-exonuclease) and more stable in the presence of 10% FCS than the unmodified ODNs (13). Therefore, in this study we examined the resistance of the heptadecamers to nucleolytic digestion by nuclease S1 (an endonuclease). The modified heptadecamers and a control, referred to as gap 1, gap 2, gap 3, gap 4, gap 5 and control, were labeled with ^{32}P at the 5′-end and incubated with nuclease S1. The reactions were analyzed by denaturing polyacrylamide gel electrophoresis (Fig. 3). The half-lives of these ODNs are summarized in Figure 4. The values of the half-lives increased with the number of the modified nucleosides. The ODN containing eight molecules of **H**s ($T_{1/2}$ = 25 min) was seven times more stable than the unmodified ODN ($T_{1/2}$ = 3.5 min). From these results, it was found that the ODNs containing **H**s were more stable not only towards the nucleolytic hydrolysis by 3′-exonuclease but also by endonuclease than the corresponding unmodified ODN.

Degradation of the target RNA by RNase H

It has been postulated that the antisense activity of antisense ODNs is due, at least in part, to cleavage of the RNA strand of a DNA–RNA duplex by RNase H (1–3). Therefore, we next examined whether the duplexes of the ODNs containing **H**s and their complementary RNA strands induce RNase H activity. The duplexes consisting of each ODN containing **H**s and its complementary RNA labeled with ^{32}P at the 5′-end were incubated with *Escherichia coli* RNase H. The products were analyzed by denaturing polyacrylamide gel electrophoresis (Figs 5 and 6). When 6.0 U RNase H was used in the reactions, the RNAs in the duplexes containing **H**s and the control duplex were similarly hydrolyzed by the enzyme (Fig. 5). On the other hand, when only 0.5 U RNase H was used, the initial rate of RNA cleavage in the duplexes containing **H**s was evidently lower than that of the control duplex (Fig. 6). The rate of RNA cleavage became lower as the number of the modified nucleoside **H**s increased.

Escherichia coli RNase H has been crystallized (22–24), and the substrate specificity and kinetic properties of the enzyme have been studied (25–27). Its preferred substrate is a DNA–RNA heteroduplex. The presence of both the 2′-hydroxyl groups and phosphate groups in the RNA strand is essential to its activity (28,29). 2′-Methoxy modifications of the DNA strand have been reported to inactivate the enzyme (25,30,31), while substitution of an uncharged methylphosphonate for the phosphate group produces a substrate that is not cleaved by the enzyme (28). The minimum length of a DNA–RNA hybrid required for cleavage by the enzyme is four nucleosides from the study using ‘chimeric’ ODNs (30,31).

Furthermore, the active site of the enzyme is thought to make contact with the minor groove of its substrate duplex (32). The aminohexyl-linkers in the ODN analogues should be accommodated in the major groove when the analogues form duplexes with complementary RNAs, since the 5-position of the 2′-deoxyuridine in a duplex is located in the major groove. Therefore, the enzyme should not interact with the aminohexyl-linkers of the substrate duplex directly.

Inspection of the CD spectra showed that the conformation of duplex was slightly affected by the introduction of **H**s into the ODNs. Therefore, cleavage of the RNA strand by RNase H might be affected by minute changes in the conformation of the duplex.

Site-directed mutagenesis studies suggested that the handle region of *E.coli* RNase H (residues 84–99), a region rich in basic amino acid residues, is necessary for effective binding to the substrate (33). This suggests that electrostatic interaction between the phosphate backbone of the nucleotide and this cluster of positive charges is involved in substrate binding. Therefore, cleavage of RNA strand by RNase H might be influenced by an interaction between the amino groups of the aminohexyl-linkers and the phosphate anion in the phosphate backbone in the duplex.

CONCLUSION

ODNs containing deoxyuridine analogue **H**s at intervals of one, two, three, four and five natural nucleosides were synthesized to study the resistance to endonuclease activity, and to determine whether or not a duplex consisting of the ODN analogue and its complementary RNA induces RNase H activity. Duplexes that contained **H**s were more stable, both thermally and thermodynamically, than the corresponding unmodified duplex. When ODNs containing **H**s were treated with nuclease S1 (an endonuclease), it was found that the ODN analogues were more stable to nucleolytic hydrolysis by the enzyme than the corresponding unmodified ODN. Furthermore, it was found that the kinetics of cleavage of the RNA strand by the enzyme were affected by the introduction of **H**s into duplexes.

MATERIALS AND METHODS

General experimental data

Thin-layer chromatography was done on Merk coated plates $60F_{254}$. The silica gel or the neutralized silica gel used for column

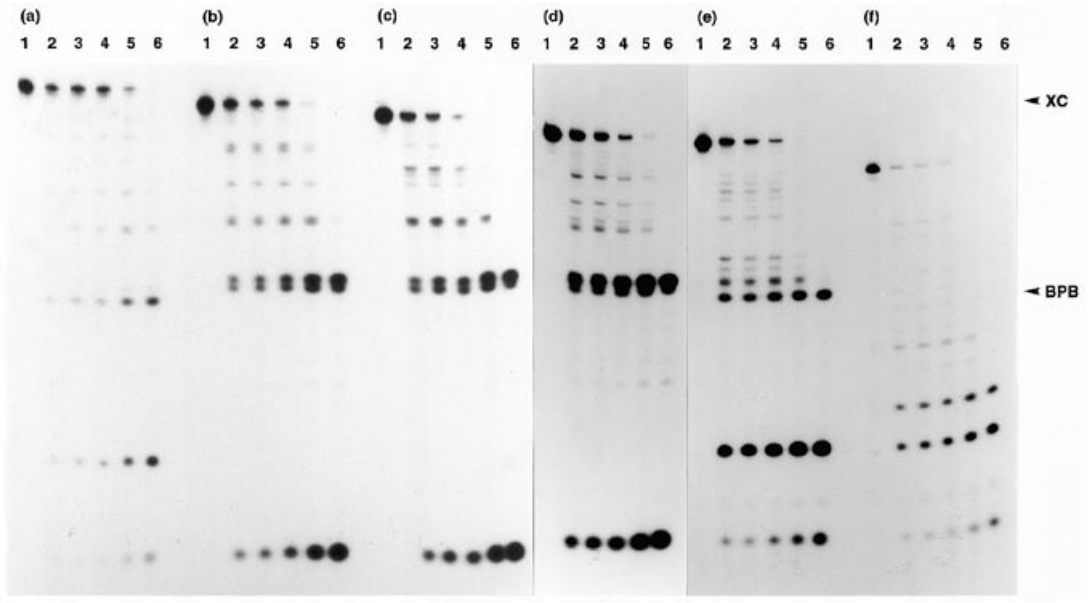


Figure 3. Polyacrylamide gel electrophoresis of 5′-^{32}P-labeled ODNs hydrolyzed by nuclease S1: (**a**) gap 1; (**b**) gap 2; (**c**) gap 3; (**d**) gap 4; (**e**) gap 5; (**f**) control. ODNs were incubated with nuclease S1 for 0 min (lane 1), 10 min (lane 2), 20 min (lane 3), 30 min (lane 4), 60 min (lane 5), and 120 min (lane 6). Experimental conditions are described under Materials and Methods.

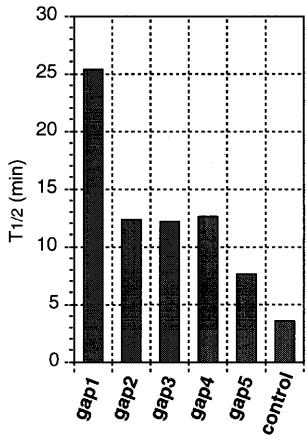


Figure 4. Half-lives of ODNs treated with nuclease S1.

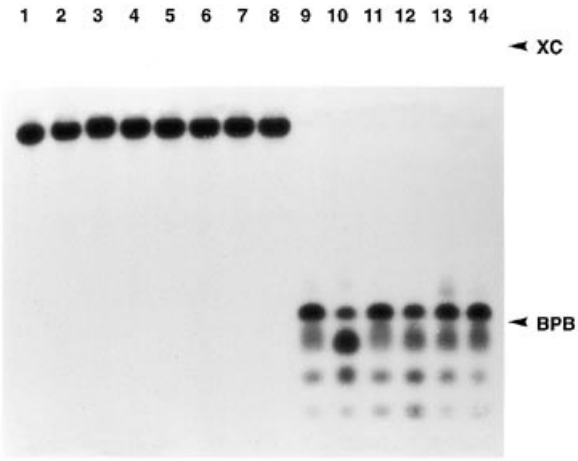


Figure 5. Polyacrylamide gel electrophoresis of 5′-^{32}P-labeled RNA hydrolyzed by *E.coli* RNase H (6.0 U). ODN–RNA duplexes were incubated with *E.coli* RNase H for 20 min at 30°C: lane 1, RNA; lane 2, RNA + enzyme; lane 3, RNA + gap 1; lane 4, RNA + gap 2; lane 5, RNA + gap 3; lane 6, RNA + gap 4; lane 7, RNA + gap 5; lane 8, RNA + control; lane 9, RNA + gap 1 + enzyme; lane 10, RNA + gap 2 + enzyme; lane 11, RNA + gap 3 + enzyme; lane 12, RNA + gap 4 + enzyme; lane 13, RNA + gap 5 + enzyme; lane 14, RNA + control + enzyme. Experimental conditions are described under Materials and Methods.

chromatography were Merk silica gel 5715 or ICN silica 60A, respectively. The ^{1}H-NMR spectra were recorded with a JEOL EX-270 or a Bruker ARX-500 spectrometer with tetramethylsilane as an internal standard. Chemical shifts are reported in parts per million (δ), and signals are expressed as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). All exchangeable protons were detected by the addition of D_2O. CD spectra were measured by a JASCO J720 Spectropolarimeter.

5′-O-Dimethoxytrityl-5-(N-trifluoroacetylaminohexyl)carbamoyl-2′-deoxyuridine (4). 1,6-Diaminohexane (536 mg, 4.61 mmol) was added to a solution of 5′-*O*-dimethoxytrityl-5-trifluoroethoxycarbonyl-2′-deoxyuridine (14,15) (1.01 g, 1.54 mmol) in pyridine (15 ml), and the mixture was stirred overnight at room temperature. The mixture was concentrated *in vacuo* and taken in $CHCl_3$, which was washed with aqueous saturated KH_2PO_4 and brine. The separated organic phase was dried (Na_2SO_4), and concentrated to dryness. The residue was coevaporated with toluene and dissolved in DMF (15 ml). Triethylamine (640 µl, 4.62 mmol) and ethyl trifluoroacetate (550 µl, 4.62 mmol) were added to the solution, and the mixture was stirred at room temperature. After 1 h, further amounts of triethylamine (1.28 ml) and ethyl trifluoroacetate (1.1 ml) were added to the solution, and the mixture was stirred at room temperature. After 1 h, the

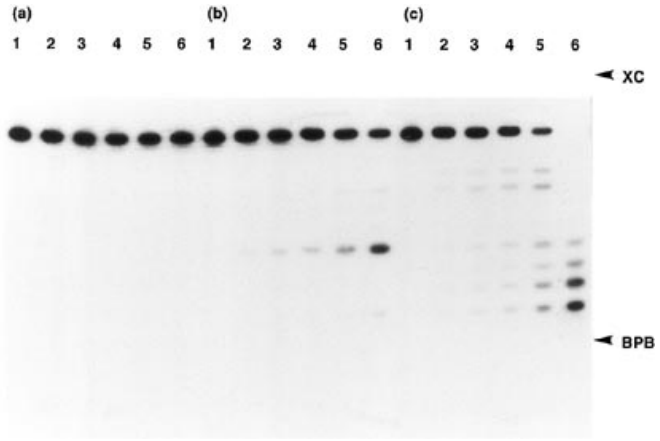


Figure 6. Polyacrylamide gel electrophoresis of 5′-^{32}P-labeled RNA hydrolyzed by *E.coli* RNase H (0.5 U) in the presence of complementary ODN strands: (**a**) gap 4; (**b**) gap 5; (**c**) control. ODN–RNA duplexes were incubated with *E.coli* RNase H (0.5 U) for 0 min (lanes 1), 10 min (lanes 2), 20 min (lanes 3), 30 min (lanes 4), 60 min (lanes 5) and 120 min (lanes 6). Experimental conditions are described under Materials and Methods.

mixture was concentrated *in vacuo* and taken in EtOAc, which was washed with aqueous saturated $NaHCO_3$ and brine. The separated organic phase was dried (Na_2SO_4), and concentrated to dryness. The residue was purified on a neutral silica gel column (2.8 × 16 cm) with 0–5% MeOH in $CHCl_3$ to give **4** (890 mg, 75%, as a white foam): FAB-MS *m/z* 769 (M^+); ^{1}H-NMR ($CDCl_3$) δ 8.71 (br s, 1H, NH-3), 8.61 (s, 1H, H-6), 8.54 (t, 1H, $CF_3CONHCH_2$-, J = 6.4 Hz), 7.42–6.82 (m, 13H, DMTr), 6.56–6.53 (m, 1H, -CH_2NHCO-), 6.16 (t, 1H, H-1′, $J_{1',2'a} = J_{1',2'b}$ = 6.5 Hz), 4.34–4.29 (m, 1H, H-3′), 3.98 (dt, 1H, H-4′, $J_{4',5'a} = J_{3',4'}$ = 4.5, $J_{4',5'b}$ = 5.4 Hz), 3.80 (s, 6H, -OCH_3), 3.49 (dd, 1H, H-5′a, $J_{5'a,5'b}$ = 10.3, $J_{4',5'a}$ = 4.5 Hz), 3.42 (dd, 1H, H-5′b, $J_{5'a,5'b}$ = 10.3, $J_{4',5'b}$ = 5.4 Hz), 3.39 (q, 2H, $CF_3CONHCHa_2CHb_2$-, $J_{a,NH} = J_{a,b}$ = 6.4 Hz), 3.29 (q, 2H, -CHc_2CHd_2NHCO-, $J_{d,CH} = J_{c,d}$ = 6.6 Hz), 2.47 (ddd, 1H, H-2′a, $J_{2'a,2'b}$ = 13.6, $J_{1',2'a}$ = 6.5, $J_{2'a,3'}$ = 4.1 Hz), 2.26 (dd, 1H, H-2′b, $J_{2'a,2'b}$ = 13.6, $J_{1',2'b}$ = 6.5 Hz), 1.58–1.35 (m, 8H, -$(CH_2)_4$-).

3′-O-[(2-Cyanoethyl)(N,N-*diisopropylamino)phosphinyl]-5′-O-dimethoxytrityl-5-(*N-*trifluoroacetylaminohexyl)carbamoyl-2′-deoxyuridine (**5**).* After successive coevaporation with pyridine, **4** (910 mg, 1.19 mmol) was dissolved in CH_2Cl_2 (15 ml) containing *N,N*-diisopropylethylamine (410 µl, 2.38 mmol). 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite (390 µl, 1.78 mmol) was added to the solution, and the reaction mixture was stirred for 30 min at room temperature. The mixture was diluted with $CHCl_3$ and washed with aqueous saturated $NaHCO_3$ and brine. The separated organic phase was dried (Na_2SO_4) and concentrated. The residue was purified on a neutral silica gel column (3.7 × 10 cm) with 25–50% EtOAc in hexane to give **5** (0.88 g, 76%, as a white foam): ^{31}P-NMR ($CDCl_3$) δ 150.36, 149.61 (85% H_3PO_4 as an internal standard).

*5′-O-Dimethoxytrityl-3′-O-succinyl-5-(*N-*trifluoroacetylamino-hexyl)carbamoyl-2′-deoxyuridine (**6**).* A solution of **4** (200 mg, 0.26 mmol), succinic anhydride (52 mg, 0.52 mmol), and 4-(*N,N*-dimethylamino)pyridine (DMAP) (6 mg, 0.05 mmol) in pyridine (3 ml) was stirred overnight at room temperature. H_2O (10 ml) was added to the mixture, and the whole was further stirred for 10 min. The mixture was concentrated and taken in $CHCl_3$, which was washed with aqueous saturated KH_2PO_4 and brine. The separated organic phase was dried (Na_2SO_4) and concentrated. The residue was purified on a silica gel column (1.7 × 12 cm) with 0–8% EtOH in $CHCl_3$ to give **6** (193 mg, 85%, as a white foam): FAB-MS *m/z* 867 (M^++1); ^{1}H-NMR ($CDCl_3$) δ 9.52 (br s, 1H, NH-3), 8.74 (s, 1H, H-6), 8.54–8.50 (m, 1H, $CF_3CONHCH_2$-), 7.41–6.81 (m, 13H, DMTr), 6.54–6.52 (m, 1H, -CH_2NHCO-), 6.06 (dd, 1H, H-1′, $J_{1',2'a}$ = 5.7, $J_{1',2'b}$ = 7.9 Hz), 5.33–5.11 (m, 1H, H-3′), 4.21–4.19 (m, 1H, H-4′), 3.79 (s, 6H, -OCH_3), 3.46–3.44 (m, 2H, H-5′a,b), 3.38 (q, 2H, $CF_3CONHCHa_2CHb_2$-, $J_{a,NH} = J_{a,b}$ = 7.0 Hz), 3.26 (dt, 2H, -CHc_2CHd_2NHCO-, $J_{d,CH}$ = 6.7, $J_{c,d}$ = 6.3 Hz), 2.72–2.55 (m, 4H, 3′-$OCOCH_2CH_2COO$-), 2.37–2.11 (m, 2H, H-2′a,b), 1.41–1.29 (m, 8H, -$(CH_2)_4$-).

Synthesis of the controlled pore glass support containing 4

Aminopropyl controlled pore glass (312 mg, 26.7 µmol, 85.7 µmol/g, CPG Inc., NJ) was added to a solution of **6** (93 mg, 0.104 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (21 mg, 0.104 mmol) in anhydrous DMF (1 ml), and the mixture was kept for 25 h at room temperature. After the resin was washed with anhydrous pyridine, a capping solution (1 ml, 0.1 M DMAP in pyridine:Ac_2O = 9:1) was added, and the whole was kept for 2 h at room temperature. The resin was washed with EtOH and acetone, and dried under vacuum. The amount of loaded nucleoside **4** to the solid support is 36.0 µmol/g from calculation of released dimethoxytrityl cation by a solution of 70% $HClO_4$:EtOH (3:2, v/v).

Synthesis of ODNs

ODNs were synthesized on a DNA/RNA synthesizer (Applied Biosystem Model 392) by the phosphoramidite method (20,21). The fully protected ODNs were then deblocked and purified by the same procedure as for the purification of normal ODNs. That is, each ODN linked to the resine 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 water. The residue was dissolved in water, and the solution was washed with Et_2O, then the H_2O layer was concentrated to give a deprotected ODN gap 1 (8.1), gap 2 (12.6), gap 3 (44.7), gap 4 (38.6), and gap 5 (44.0). The yields are indicated in parentheses as OD units at 260 nm starting from 1 µmol.

Thermal denaturation and CD spectroscopy

Each sample containing an appropriate ODN and a complementary RNA in a buffer of 0.01 M sodium cacodylate (pH 7.0) containing 0.01 M NaCl was heated at 80°C for 10 min, then cooled gradually to room temperature, and used for the thermal denaturation study. Thermally induced transitions were monitored at 260 nm on a Perkin Elmer Lambda2S. Sample temperature was increased 1°C/min. Thermodynamic parameters were derived by the reported method (35). Samples for CD

spectroscopy were prepared by the same procedure used in the thermal denaturation study, and spectra were measured at 10°C. The ellipticities of duplexes were recorded from 200 to 320 nm in a cuvette with a path length of 1 mm. CD data were converted into mdeg·mol of residues^{-1}·cm^{-1}.

Partial hydrolysis of ODN with nuclease S1

Each ODN labeled with ^{32}P at the 5′-end (10 pmol) was incubated with nuclease S1 (0.2 U, Takara Shuzo Co., Ltd) in the presence of torula RNA (0.27 OD units at 260 nm) in a buffer containing 30 mM sodium acetate (pH 4.6), 0.28 M NaCl, and 10 mM $ZnSO_4$ (total 20 µl) at 37°C. At appropriate periods, aliquots of the reaction mixture (4 µl) were separated and added to a solution of EDTA (5 mM, 10 µl), then this mixture was heated for 5 min at 90°C. The solutions were analyzed by electrophoresis on 20% polyacrylamide gel containing 8 M urea (34). Densities of radioactivity of the gel were visualized by a Bio-imaging Analyzer (Bas 2000, Fuji Co., Ltd) and the cleavage rates were determined.

Hydrolysis of duplexes with *E.coli* RNase H

A mixture of RNA labeled with ^{32}P at the 5′-end (10 pmol) and unlabeled RNA (40 pmol) was incubated with *E.coli* RNase H (Takara Shuzo Co., Ltd) in the presence of the complementary ODN (50 pmol) in a buffer containing 10 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 0.01% bovine serum albumin (total 20 µl) at 30°C. At appropriate periods, aliquots of the reaction mixture (4 µl) were separated and added to a solution of 9 M urea in 2× TBE (5 µl). The mixtures were analyzed by electrophoresis on 20% polyacrylamide gel containing 8 M urea (34).

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