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Site-specific introduction of functional groups into phosphodiester oligodeoxynucleotides and their thermal stability and nuclease-resistance properties

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ABSTRACT

We report here the site-specific introduction of functional groups into phosphodiester oligodeoxynucleotides (ODNs). ODNs containing both 5-(*N*-aminohexyl)-carbamoyl-2′-deoxyuridine (H), which serves as a tether for the further conjugation of functional groups, and 5-(*N,N*-dimethylaminohexyl)carbamoyl-2′-deoxyuridine (D), which contributes to the thermal stability of the duplex and to the resistance to nucleolytic hydrolysis by nucleases, were synthesized. Functional groups such as folic acid and palmitic acid were site-specifically introduced into the terminus of the aminohexyl-linker of H. The thermal stability and resistance toward nuclease digestion of the modified ODNs were studied. We found that ODNs containing D and H formed stable duplexes with both the complementary DNA and RNA strands even when a bulky functional group such as folic acid, palmitic acid or cholesterol was attached to the terminus of the amino-linker. We also found that ODN analogues which contained D were more resistant to nucleolytic degradation by exo- and endonuclease than the unmodified ODN. Furthermore, duplexes formed by ODNs containing D and the complementary RNA could elicit RNase H activity.

INTRODUCTION

An antisense oligodeoxynucleotide (ODN) is a short piece of synthetic DNA which is complementary to a segment of mRNA. It hybridizes to mRNA by Watson–Crick base-pairing and inhibits translation of the mRNA in a sequence-specific manner (1). For ODNs to be effective as antisense molecules, they need to form stable Watson–Crick hybrids with complementary target RNAs and be sufficiently resistant to degradation by ubiquitous nucleases (1). Since a natural phosphodiester linkage in DNA is a good substrate for ubiquitous nucleases, several chemically-modified ODNs have been synthesized and used in antisense studies to improve this property (1–4).

It is well known that bacteriophage ϕW-14 DNA contains up to 50% α-putrescinylthymine in place of thymine, and is more resistant to DNase I and snake venom phosphodiesterase than unmodified DNAs (5–9). In addition, this modification results in a higher melting temperature (T_m) than that expected for unmodified DNA based on the GC content (8,9). However, short synthetic ODNs containing α-putrescinylthymine were found to reduce the thermal stability of duplexes compared with their parent ODNs in our laboratory (10). 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 developed a new and convenient post-synthetic modification method for the synthesis of ODNs with various amino-linkers using 5-methoxycarbonyl-2′-deoxyuridine (**3**) as a convertible nucleoside to prevent tedious protection–deprotection processes in preparing monomer units containing such amino-linkers (11,12). When ODNs containing **3** at various positions are treated with several diaminoalkanes, the desired ODNs containing 5-(*N*-aminoalkyl)carbamoyl-2′-deoxyuridines can be readily obtained. Using this method, we found that ODNs containing 5-(*N*-aminohexyl)carbamoyl-2′-deoxyuridines (**H**s) not only form stable Watson–Crick duplexes with both the corresponding complementary DNA and RNA strands, but also show good resistance to nucleolytic hydrolysis by snake venom phosphodiesterase, and are stable in medium containing 10% fetal calf serum (12). These properties could make these ODNs potentially useful as antisense molecules.

Besides the above-mentioned requirement for antisense molecules, antisense ODNs also need to be able to penetrate cellular membranes to reach their site of action (1,13). Recently, it has been reported that the cellular uptake of ODNs was improved by covalent attachment of lipophilic groups such as cholesterol (14–19), phospholipid (20,21), palmitic acid (16,22,23) and vitamin E (24) that interact specifically with cell membranes.

The terminal amino groups of **H**s in ODNs can be readily conjugated with such lipophilic groups. However, not all of the amino-linkers of **H**s should be modified by such lipophilic groups, since the terminal amino groups of the linkers play important roles in stabilizing duplex formation and in preventing nucleolytic hydrolysis by nucleases, probably by interacting with phosphate anions of the duplexes and nucleases. Therefore, we designed 5-(*N,N*-dimethylaminohexyl)carbamoyl-2′-deoxyuridine (**D**), instead of **H**, to impart thermal stability and to prevent against nucleolytic degradation by nucleases, while **H** was used as an amino tether which could be conjugated with lipophilic groups.

In this paper, we describe the site-specific introduction of functional groups into phosphodiester ODNs containing **D** and **H** by this novel method, which is outlined in Scheme 1. Functional groups such as folic acid (25–27) and palmitic acid were introduced into ODNs in a site-specific manner. The thermal stability of ODNs containing functional groups with complementary

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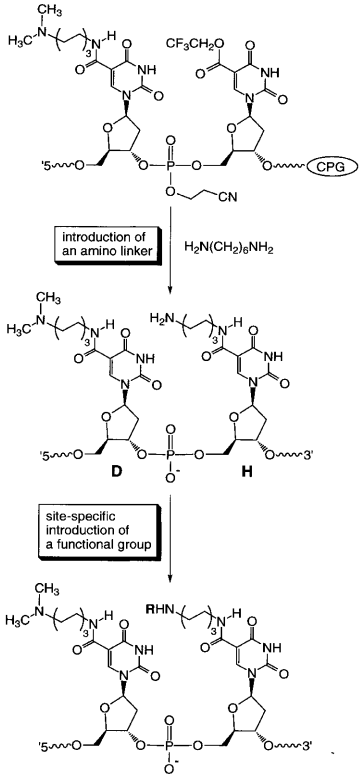
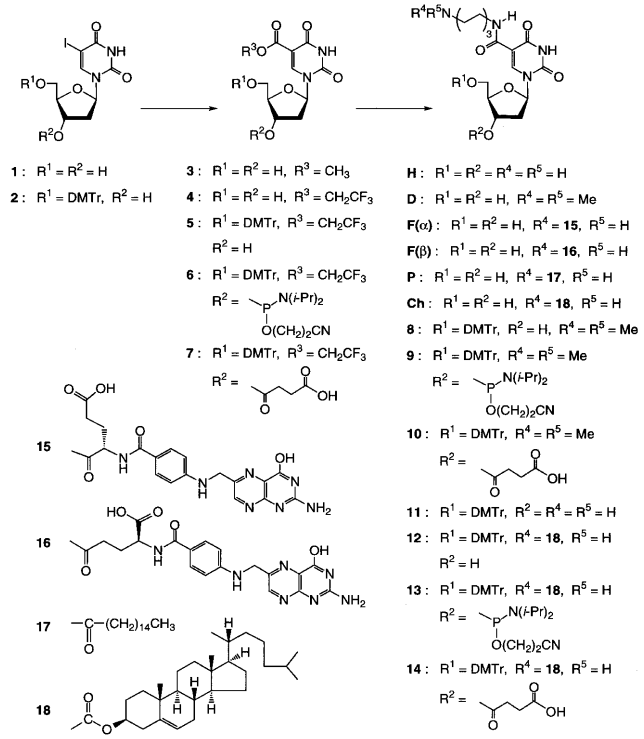


Scheme 1.



Scheme 2.

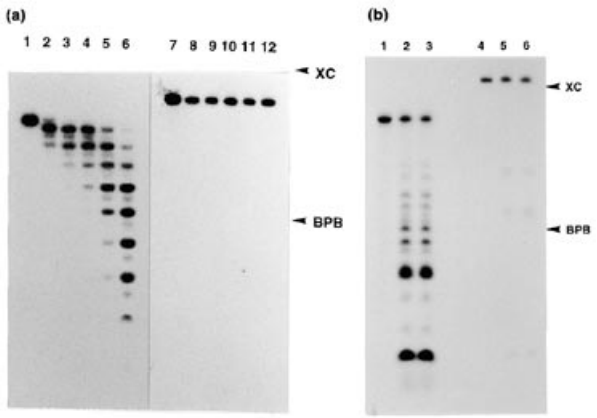


Figure 1. Polyacrylamide gel electrophoresis of 5′-^{32}P-labeled ODNs hydrolyzed by nucleases. (**a**) **21** (lanes 1–6) and **23** (lanes 7–12) were incubated with snake venom phosphodiesterase at 37°C for 0 min (lanes 1 and 7), 10 min (lanes 2 and 8), 20 min (lanes 3 and 9), 30 min (lanes 4 and 10), 60 min (lanes 5 and 11) and 120 min (lanes 6 and 12). (**b**) **36** (lanes 1–3) and **37** (lanes 4–6) were incubated with nuclease P1 at 10°C for 0 min (lanes 1 and 4), 10 min (lanes 2 and 5) and 20 min (lanes 3 and 6).

DNA and RNA strands, the nuclease-resistance properties of ODNs containing **D**, and the ability of ODNs to elicit RNase H activity were also examined.

RESULTS AND DISCUSSION

Synthesis

In a previous study, we used **3** as a convertible nucleoside (11,12). Although this nucleoside is sufficiently reactive against diaminoalkanes in MeOH at ambient temperature, we found that **3** in ODNs, especially in those which contained several **3** per ODN molecule, showed somewhat low reactivity against diaminoalkanes, and the yields of the desired ODNs containing 5-(*N*-aminoalkyl)carbamoyluracil were reduced (data not shown). Therefore, to simplify the conversion of a convertible nucleoside to the desired nucleoside in the ODN, we designed 5-trifluoroethoxycarbonyl-2′-deoxyuridine (**4**) as a new convertible nucleoside. Palladium-catalyzed carbonylation of 5′-*O*-dimethoxytrityl-5-iodo-2′-deoxyuridine (**2**) with carbon monoxide in CF_3CH_2OH gave **5** in 85% yield. To examine the chemical reactivity of the trifluoroethoxycarbonyl group with diaminoalkane, **5** was deprotected to give **4**. The chemical reactivity of **4** with a large excess of 1,6-diaminohexane in CF_3CH_2OH was then compared with that of **3**. The reactions were monitored by reverse-phase HPLC and obeyed pseudo-first-order kinetics (data not shown). We found that **4** was more reactive with 1,6-diaminohexane than **3** ($k_{trifluoroethyl}/k_{methyl}$ = 98). Therefore, we used **4** as a new convertible nucleoside. Nucleoside **5** was then phosphitylated by a standard protocol (28) to give phosphoramidite unit **6**. To synthesize **D**, **5** was treated with *N*,*N*-dimethyl-1,6-diaminohexane (29) in pyridine to give **8**, which was phosphitylated by a standard method (28) to give phosphoramidite unit **9**. To incorporate **4** and **D** into the 3′-end of ODNs, **5** and **8** were further derivatized to their 3′-succinates **7** and **10**, which were then reacted with controlled pore glass (CPG) to give solid supports containing **4** (21.9 μmol/g) and **D** (31.8 μmol/g), respectively.

It was difficult to introduce cholesterol into ODNs in reactions with ODNs containing **H** due to its low solubility and reactivity. Therefore, cholesterol-modified nucleoside analogue **12** was synthesized. Cholesterol was converted into carbonylimidazolide, and it was then reacted with 5-aminohexylcarbamoyl-2′-deoxyuridine derivative **11** to give cholesterol-modified nucleoside

analogue **12**, which was phosphitylated by a standard procedure (28) to give the corresponding nucleoside 3′-phosphoramidite **13**. To incorporate a cholesterol-modified nucleoside unit into the 3′-end of the ODNs, **12** was further derivatized to 3′-succinate **14**, which was then reacted with a controlled pore glass (CPG) to give a solid support containing 5-(*N*-cholesteryloxycarbonylaminohexyl) carbamoyl-2′-deoxyuridine (**Ch**) (45.1 μmol/g).

In this study, the nucleoside analogues **D**, **H** and **Ch** were first incorporated into ODNs with a model sequence, $(TM)_8T$, where T is thymidine and M is 5-methy-2′-deoxycytidine, to compare the thermal stabilities of duplexes composed of ODNs and the complementary DNA or RNA strands. ODNs were synthesized by the phosphoramidite method (30) on a DNA synthesizer. The coupling yield of the nucleoside phosphoroamidite unit **9** was 99%. The fully protected ODNs (1 μmol) linked to the solid support were treated with concentrated NH_4OH or a large excess of 1,6-diaminohexane in MeOH at 55°C overnight, followed by C-18 column chromatography; de-tritylation gave ODNs **21–31**. Furthermore, we also synthesized ODNs **36–40**, which contain the complementary sequence of the second initiation codon of the *nef* gene of human immunodeficiency virus type-1 (HIV-1) (31). The reader is referred to Scheme 2 for structures of ODNs **1–14**, **H**, **D**, **F(α)**, **F(β)**, **P** and **Ch**. The synthesized ODNs are listed in Table 1.

Introduction of functional groups into ODNs

The introduction of folic acid into ODNs was examined. ODN **29** (5.0 OD_{260} units), which contains one **H** and three **D**, was treated with folic anhydride in 50 mM $NaHCO_3$–Na_2CO_3 buffer (pH 10.3) at room temperature. Analysis of the reaction mixture by anion-exchange HPLC after 12 h showed the disappearance of the starting material and the formation of a slower-eluting product (**33**). ODN **33** was obtained in 0.49 OD_{260} units. To confirm the introduction of 5-(*N*-folylaminohexyl)carbamoyl-2′-deoxyuridine (**F**), **33** was hydrolyzed by a mixture of nuclease P1 and alkaline phosphatase to the corresponding nucleosides, and the nucleoside composition was analyzed by HPLC. Peaks corresponding to the modified nucleosides, confirmed by coelution with authentic samples, were observed, and the composition of the nucleosides calculated from the area of the peaks was close to the theoretical value. The two peaks, which corresponded to the α-carboxylate and γ-carboxylate folate derivatives, **F**(α) and **F**(γ), were detected not only at 260 nm but also at 350 nm. Furthermore, the ODN (**40**) containing **F** was analyzed by electrospray ionization (ESI) mass spectrometry and the observed molecular weight supported its structure.

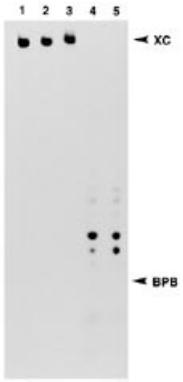


Figure 2. Polyacrylamide gel electrophoresis of 5′-^{32}P-labeled RNA **43** hydrolyzed by *E.coli* RNase H in the presence of complementary strands: lane 1, **43**; lane 2, **43** + enzyme; lane 3, **43** + **36**; lane 4, **43** + **36** + enzyme; lane 5, **43** + **37** + enzyme.

Table 1. Synthesized ODNs and hybridization data[a]

	Sequence[b]	T_m (°C) DNA[c]	ΔT_m (°C)	T_m (°C) RNA[d]	ΔT_m (°C)
21	5′-T M T M T M T M T M T M T M T M T-3′	49		69	
22	5′-T M T M T M T M **D** M T M T M T M T-3′	52	+3	71	+2
23	5′-T M T M T M T M T M T M T M T M **D**-3′	54	+5	69	0
24	5′-**D** M T M T M T M **D** M T M T M T M **D**3′	61	+12	73	+4
25	5′-**D** M T M T M **D** M T M **D** M T M T M **D**-3′	62	+13	73	+4
26	5′-**D** M T M T M T M **D** M T M T M T M **H**-3′	57	+8	72	+3
27	5′-**H** M T M T M T M **D** M T M T M T M **D**-3′	59	+10	72	+3
28	5′-**D** M T M T M **D** M T M **D** M T M T M **H**-3′	59	+10	73	+4
29	5′-**H** M T M T M **D** M T M **D** M T M T M **D**-3′	62	+13	72	+3
30	5′-**D** M T M T M **D** M T M **D** M T M T M **Ch**-3′	57	+8	73	+4
31	5′-**Ch** M T M T M **D** M T M **D** M T M T M **D**-3′	57	+8	72	+3
32	5′-**D** M T M T M **D** M T M **D** M T M T M **F**-3′	57	+8	72	+3
33	5′-**F** M T M T M **D** M T M **D** M T M T M **D**-3′	56	+7	70	+1
34	5′-**D** M T M T M **D** M T M **D** M T M T M **P**-3′	61	+12	73	+4
35	5′-**P** M T M T M **D** M T M **D** M T M T M **D**-3′	56	+7	71	+2
36	5′-G C T G G C T C A G C T C G T C T C A T-3′				
37	5′-G C **D** G G C **D** C A G C **D** C G T C **D** C A **D**-3′				
38	5′-**Ch** G G C **D** C A G C **D** C G T C **D** C A **D**-3′				
39	5′-**P** G G C **D** C A G C **D** C G T C **D** C A **D**-3′				
40	5′-**F** G G C **D** C A G C **D** C G T C **D** C A **D**-3′				

[a]Experimental conditions are described in Materials and Methods.
[b]M, 5-methyl-2′-deoxycytidine. **H**, **D**, **Ch**, **P** and **F**, see Scheme 2.
[c]The complementary DNA: 5′-TG$(GA)_9$GGT-3′ (**41**).
[d]The complementary RNA: 5′-UG$(GA)_9$GGU-3′ (**42**) or 5′-AGAAUGAGACGAGCUGAGCCAGCAGC-3′ (**43**).

In a similar manner, palmitic acid was introduced into ODNs. ODN **29** (0.5 OD_{260} units) was treated with *N*-succinimidyl palmitate in 50 mM $NaHCO_3$–Na_2CO_3 buffer (pH 10.3) at room temperature. After 2 h, by collecting the newly appeared main peak, ODN **35** was obtained in 0.29 OD_{260} units. These experiments showed that palmitic acid and folic acid were successfully conjugated with the terminal amino group of **H** in ODNs in a site-specific manner by this method.

Thermal stability

The stability of duplexes formed by these ODNs and a target complementary DNA, 5′-TG(GA)$_9$GGT-3′ (**41**), or RNA, 5′-UG(GA)$_9$GGU-3′ (**42**), was studied by thermal denaturation. One-transition curves that were similar to each other were observed for all of the melting profiles (data not shown). Melting temperatures (T_ms) are listed in Table 1. However, T_ms of duplexes of ODNs **36–40** with a complementary RNA, 5′-AGAAUGA-GACGAGCUGAGCCAGCAGC-3′ (**43**), were not available due to the complicated profiles of their thermal denaturation curves.

With DNA (**41**) as a complementary strand, the T_ms of duplexes between **41** and **22–25** were significantly higher than that of the control duplex formed by **41** and **21** (T_m = 49°C). The stability of the duplexes depended on the number of modified nucleosides **D**. The duplexes became more stable as the number of **D** increased. This result is consistent with our previous results for duplexes containing **H** (11,12). A duplex between **41** and **23** with a linker group at its 5′-end, was slightly more stable than that between **41** and **22** with a linker group at its center. When **H** was introduced into ODNs instead of **D**, duplexes **26–29** with **41** became slightly less stable than the corresponding duplexes (**41–24**, **41–25**) containing **D**. The T_ms of duplexes containing **H** at their 5′-end were slightly higher than those of duplexes containing **H** at their 3′-end.

On the other hand, when bulky functional groups such as cholesterol, folic acid and palmitic acid were attached to the terminus of the amino-linker of **H**, duplexes **30–35** with **41** became slightly less stable than those (**41–24**, **41–25**) containing three or four **D**s, but were still significantly more stable than the control duplex. The stability of the duplexes depended on the position and the nature of the functional groups. The T_ms of duplexes with folic acid and palmitic acid at their 5′-end were lower than those of duplexes with functional groups at their 3′-end.

When RNA (**42**) was used as a complementary strand, the T_ms with these ODNs followed a trend similar to the T_ms of the corresponding DNA–DNA duplexes, although ΔT_ms by modification were less than those of the DNA–DNA duplexes. When ODN analogues form duplexes with **42**, the amino-linkers should be accommodated in the major groove, since the 5-position of 2′-deoxyuridine is in the major groove. The major groove of A-type duplexes (DNA–RNA) is narrower than that of B-type duplexes (DNA–DNA) (32). Therefore, linker groups which include functional groups may not be well-accommodated in the narrower major groove of DNA–RNA duplexes, compared with DNA–DNA duplexes. However, it is worth noting that the T_ms of duplexes **30–35** with **42** were higher than that of the control duplex, even though bulky functional groups such as cholesterol, folic acid and palmitic acid were attached to the terminus of the amino-linkers.

Partial digestion of ODNs with snake venom phosphodiesterase and nuclease P1

The stability of ODNs containing **D** against nucleolytic digestion was examined, since resistance to nucleolytic hydrolysis by nucleases is an important factor in antisense studies (1). Two kinds of nucleases, snake venom phosphodiesterase (a 3′-exonuclease) and nuclease P1 (an endonuclease), were used in this study. ODNs **23** and **37** were labeled at the 5′-end with ^{32}P and incubated with an appropriate nuclease. The reactions were then analyzed by polyacrylamide gel electrophoresis under denaturation conditions (33). The control ODN **21** was hydrolyzed randomly by snake venom phosphodiesterase after 120 min of incubation, while the phosphodiester linkage of ODNs with **D** at the 3′-end was resistant to this nuclease (Fig. 1a). The phosphodiester linkages around the nucleoside analogue **D** were also more resistant to nuclease P1 than those beside thymidines (Fig. 1b). These data were similar to those for ODNs containing **H**, although the half-lives of the ODNs were not compared.

Degradation of target RNA by RNase H

It has been postulated that the antisense activity of ODNs is due, at least in part, to cleavage of the RNA strand of a DNA–RNA duplex by RNase H (1). Therefore, we examined whether the DNA–RNA duplex between **37** and **43** elicits RNase H activity. Duplexes consisting of **37** and **43** labeled with ^{32}P at the 5′-end were incubated with *Escherichia coli* RNase H, and the products were analyzed by polyacrylamide gel electrophoresis. As shown in Figure 2, **43** in the **37–43** duplex (lane 4) and the **36–43** duplex (lane 5) was hydrolyzed similarly by the enzyme. Therefore, the DNA–RNA duplex of ODN containing **D** is a substrate for *E.coli* RNase H.

Recently, Uchiyama *et al.* reported that *E.coli* RNase H interacts with the duplex in the minor groove (34). Therefore, the enzyme cleaves the RNA strand of the duplex almost equally when the complementary ODN contains either thymidine or 5-phenyl-2′-deoxyuridine, in which the 5-phenyl group is accommodated in the major groove of the duplex. Moreover, it has also been shown that ‘chimeric’ ODNs composed of regions containing modified sugar derivatives and unmodified regions can elicit RNase H activity (35). Therefore, duplexes formed by RNA and ODNs containing **D**, in which the amino-linkers are accommodated in the major groove of the duplex, could be a substrate for the enzyme.

CONCLUSION

In this paper, we reported a novel method for the site-specific introduction of functional groups into phosphodiester ODNs carrying amino linkers. Using this method, folic acid and palmitic acid could be efficiently introduced into ODNs. ODNs with amino-linkers at the 5-position of 2′-deoxyuridine formed stable duplexes with both the complementary DNA and RNA strands, even when a bulky functional group is attached to the terminus of the amino-linker. We also found that ODN analogues containing **D** are more resistant to nucleolytic degradation by exo- and endonuclease than the unmodified ODN. Furthermore, duplexes formed by ODNs containing **D** and the complementary RNA could elicit RNase H activity. Applications of these ODNs as antisense ODNs, especially the membrane permeability of ODNs containing **Ch**, **F** and **P**, are currently being studied.

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 chromatography were Merk silica gel 5715 or ICN silica 60A, respectively. Melting points were measured on a Yanagimoto MP-3 micromelting point apparatus (Yanagimoto) and are uncorrected. 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. UV absorption spectra were recorded with a Shimadzu UV-240 spectrophotometer (Shimadzu Co.).

5′-*O*-Dimethoxytrityl-5-iodo-2′-deoxyuridine (2)

After successive coevaporation with pyridine, 5-iodo-2′-deoxyuridine (5.00 g, 14.1 mmol) was dissolved in pyridine (50 ml). DMTrCl (5.26 g, 15.5 mmol) was added to the solution, and the mixture was stirred at room temperature. After 3 h, EtOH (5 ml) was added to the mixture, and the whole was further stirred for 10 min. The solvent was concentrated, and then the residue was dissolved in $CHCl_3$. The solution was washed with aqueous saturated $NaHCO_3$ and then brine, dried (Na_2SO_4), and concentrated. The residue was chromatographed over a silica gel column (4.6 × 12 cm) with 0–5% MeOH in $CHCl_3$ containing 0.2% pyridine to give **2** (9.28 g, quant. as a white foam): FAB-MS *m/z* 656 (M^+); ^{1}H-NMR ($CDCl_3$) δ 8.60 (s, 1H, N*H*-3), 8.14 (s, 1H, H-6), 7.35–6.84 (m, 13H, DMTr), 6.32 (t, 1H, H-1′, $J_{1',2'a} = J_{1',2'b}$ = 6.3 Hz), 4.57–4.55 (m, 1H, H-3′), 4.09 (q, 1H, H-4′, $J_{4',3'} = J_{4',5'a} = J_{4',5'b}$ = 3.0 Hz), 3.81 (s, 6H, CH_3O), 3.40 (dd, 2H, H-5′a,b, $J_{5'a,4'} = J_{5'b,4'}$ = 3.0, $J_{5'a,5'b}$ = 10.7 Hz), 2.49, 2.30 (each ddd, each 1H, H-2′a,b, $J_{2'a,1'} = J_{2'b,1'}$ = 6.3, $J_{2'a,3'} = J_{2'b,3'}$ = 2.8, $J_{2'a,2'b}$ = 13.7 Hz), 1.62 (bs, 1H, HO-3′); FAB exact MS calcd for $C_{30}H_{29}IN_2O_7$ 656.1021, found 656.1046.

5′-*O*-Dimethoxytrityl-5-trifluoroethoxycarbonyl-2′-deoxyuridine (5)

A mixture of **2** (4.22 g, 6.43 mmol), bis(benzonitrile)palladium dichloride (50 mg, 0.129 mmol), Et_3N (2.12 ml, 12.9 mmol), and CF_3CH_2OH (4.61 ml, 64.3 mmol) in CH_3CN (50 ml) was heated at 60°C for 11 h under CO atmosphere. The reaction mixture was filtered through a Celite pad and washed with EtOH. The combined filtrate and washings were concentrated, and then the residue was dissolved in $CHCl_3$. The solution was washed with aqueous saturated $NaHCO_3$ and then brine, dried (Na_2SO_4) and concentrated. The residue was chromatographed over a silica gel column (3.0 × 13 cm) with 0–6% MeOH in $CHCl_3$ to give **5** (3.60 g, 85% as a yellow foam): FAB-MS *m/z* 656 (M^+); ^{1}H-NMR ($CDCl_3$) δ 8.61 (s, 1H, H-6), 7.40–6.81 (m, 13H, DMTr), 6.16 (t, 1H, H-1′, $J_{1',2'a} = J_{1',2'b}$ = 6.2 Hz), 4.35 (q, 2H, CF_3CH_2CO, $J_{F,H}$ = 8.5 Hz), 4.18 (dt, 1H, H-3′, $J_{3',4'}$ = 4.0, $J_{3',2'a} = J_{3',2'b}$ = 4.4 Hz), 4.05 (q, 1H, H-4′, $J_{4',3'} = J_{4',5'a} = J_{4',5'b}$ = 4.0 Hz), 3.79 (s, 6H, CH_3O), 3.49–3.43 (m, 2H, H-5′a,b), 2.55, 2.26 (each ddd, each 1H, H-2′a,b, $J_{2'a,1'} = J_{2'b,1'}$ = 6.2, $J_{2'a,3'} = J_{2'b,3'}$ = 4.4, $J_{2'a,2'b}$ = 13.3 Hz); FAB exact MS calcd for $C_{33}H_{31}F_3N_2O_9$ 656.1981, found 656.1979.

5-Trifluoroethoxycarbonyl-2′-deoxyuridine (4)

Aqueous 80% AcOH (2 ml) containing **5** (50 mg, 76 µmol) was stirred at room temperature for 30 min, and then the solution was concentrated. The residue was chromatographed over a silica gel column (2.1 × 14.5 cm) with 0–10% MeOH in $CHCl_3$ to give **4** (23 mg, 85% as a white powder): mp 186–187°C; FAB-MS *m/z* 354 (M^+); UV λ_{max} (H_2O) 276 nm; λ_{max} (H^+) 276 nm; λ_{max} (OH^-) 272 nm; ^{1}H-NMR (DMSO-*d*$_6$) δ 11.61 (s, 1H, N*H*-3), 8.84 (s, 1H, H-6), 6.08 (t, 1H, H-1′, $J_{1',2'a} = J_{1',2'b}$ = 6.3 Hz), 5.27 (d, 1H, HO-3′, *J* = 4.3 Hz), 4.99 (t, 1H, HO-5′, *J* = 4.7 Hz), 4.82 (q, 2H, CF_3CH_2CO, *J* = 9.0 Hz), 4.24–4.23 (m, 1H, H-3′), 3.87 (dt, 1H, H-4′, $J_{4',3'} = J_{4',5'a} = J_{4',5'b}$ = 3.3 Hz), 3.62–3.48 (m, 2H, H-5′a,b), 2.25–2.20 (m, 2H, H-2′a,b). Anal. calcd for $C_{12}H_{13}F_3N_2O_7$: C, 40.69; H, 3.70; F, 16.09; N, 7.91. Found: C, 40.84; H, 3.61; F, 15.67; N, 7.73.

5′-*O*-Dimethoxytrityl-5-trifluoroethoxycarbonyl-3′-*O*-[(2-cyanoethyl)(*N*,*N*-diisopropylamino)phosphinyl]-2′-deoxyuridine (6)

After successive coevaporation with pyridine, **5** (584 mg, 0.889 mmol) was dissolved in CH_2Cl_2 (10 ml) containing *N*,*N*-diisopropylethylamine (0.23 ml, 1.33 mmol). 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.297 ml, 1.33 mmol) was added to the solution and the reaction mixture was stirred for 20 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 chromatographed over a neutral silica gel column (2.9 × 7 cm) with 66% EtOAc in hexane to give **6** (502 mg, 66% as a white foam): ^{31}P-NMR ($CDCl_3$) δ 150.05, 149.57 (85% H_3PO_4 as an internal standard); FAB exact MS calcd for $C_{42}H_{48}F_3N_4O_{10}P$ 856.3060, found 857.3140.

5′-*O*-Dimethoxytrityl-5-trifluoroethoxycarbonyl-3′-*O*-succinyl-2′-deoxyuridine (7)

A solution of **5** (1.54 g, 2.35 mmol), succinic anhydride (470 mg, 4.70 mmol), and 4-(*N*,*N*-dimethylamino)pyridine (DMAP) (47 mg, 0.385 mmol) in pyridine (3 ml) was stirred at room temperature for 2 days. H_2O (1 ml) was added to the mixture, and the whole was further stirred for 10 min. The solvent was concentrated. The residue was diluted with $CHCl_3$, which was washed with aqueous saturated KH_2PO_4, and then brine, dried (Na_2SO_4) and evaporated. The residue was chromatographed on a silica gel column (2.7 × 7 cm) with 0–20% MeOH in $CHCl_3$ to give **7** (796 mg, 51% as a white foam) and **5** (644 mg, 36% as a white foam) was recovered. Data for **7**: FAB-MS *m/z* 756 (M^+); ^{1}H-NMR ($CDCl_3$) δ 8.75 (s, 1H, H-6), 7.50–6.83 (m, 13H, DMTr), 6.20 (t, H-1′, $J_{1',2'a} = J_{1',2'b}$ = 5.7 Hz), 5.29 (m, 1H, H-3′), 4.24 (dq, 2H, CF_3CH_2CO, $J_{F,H}$ = 8.5 Hz, $J_{H,H}$ = 4.3 Hz), 4.27 (m, 1H, H-4′), 3.80 (s, 6H, CH_3O), 3.43 (m, 2H, H-5′a,b), 2.74–2.62, 2.40–2.30 (m, 2H, H-2′a,b), 2.60 (m, 4H, 3′-OCOCH_2CH_2OCO-); FAB exact MS calcd for $C_{37}H_{35}F_3N_2O_{12}$ 756.2142, found 756.2135.

5′-*O*-Dimethoxytrityl-5-(*N*,*N*-dimethylaminohexyl)carbamoyl-2′-deoxyuridine (8)

N,*N*-Dimethyl-1,6-diaminohexane (13 mg, 91 µmol) (29) was added to a solution of **5** (50 mg, 76 µmol) in pyridine (3 ml), and the mixture was stirred overnight at room temperature. The solution was concentrated, and then the residue was dissolved in

$CHCl_3$. The whole was washed with aqueous saturated $NaHCO_3$ and then brine, dried (Na_2SO_4) and concentrated. The residue was chromatographed over a silica gel column (1.8 × 5 cm) with 0–10% MeOH in $CHCl_3$ containing 0.5% Et_3N to give **8** (50 mg, 94% as a white foam): FAB-MS *m/z* 701 (M^++1); ^{1}H-NMR ($CDCl_3$) δ 8.65 (t, 1H, -N*H*CO, *J* = 5.6 Hz), 8.53 (s, 1H, H-6), 7.41–6.81 (m, 13H, DMTr), 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′), 4.06–3.90 (m, 1H, H-4′), 3.77 (s, 6H, CH_3O), 3.39–3.31 (m, 2H, H-5′a,b), 2.78–2.74 (m, 2H, -C*H*$_2$NHCO-), 2.44 (ddd, 1H, H-2′a, $J_{2'a,1'}$ = 6.5, $J_{2'a,3'}$ = 4.3, $J_{2'a,2'b}$ = 13.7 Hz), 2.36 (dt, 2H, -C*H*$_2$NMe$_2$, *J* = 7.7 Hz), 2.26 (s, 6H, -NMe$_2$), 2.19 (ddd, 1H, H-2′b, $J_{2'a,1'}$ = 6.5 Hz, $J_{2'a,2'b}$ = 13.7 Hz, $J_{2'a,3'}$ = 4.3 Hz), 1.58–1.41 (m, 4H, -C*H*$_2$CH$_2$NH-, Me$_2$NCH$_2$C*H*$_2$-), 1.35–1.33 (m, 4H, -(CH$_2$)$_2$-); FAB exact MS calcd for $C_{39}H_{47}N_4O_8$ 699.3394, found 699.3368.

5-(*N,N*-Dimethylaminohexyl)carbamoyl-2′-deoxyuridine (D)

Aqueous 80% AcOH (2 ml) containing **8** (219 mg, 0.314 mmol) was stirred at room temperature for 30 min, and then the solution was concentrated, and the residue coevaporated with water. The residue was chromatographed over a silica gel column (2.1 × 7.0 cm) with 0–20% MeOH in $CHCl_3$ containing 0.5% Et_3N to give **D** (100 mg, 80% as white crystals): m.p. 164–167°C; FD-MS *m/z* 399 (M^++1); UV λ_{max} (H_2O) 276 nm; λ_{max} (H^+) 277 nm; λ_{max} (OH^-) 277 nm; ^{1}H-NMR (DMSO-d_6) δ 8.65 (t, 1H, -N*H*CO, *J* = 5.8 Hz), 8.67 (s, 1H, H-6), 6.12 (t, 1H, H-1′, $J_{1',2'a} = J_{1',2'b}$ = 6.5 Hz), 4.25–4.21 (m, 1H, H-3′), 3.85 (dt, 1H, H-4′, $J_{4',3'} = J_{4',5'a} = J_{4',5'b}$ = 3.5 Hz), 3.57–3.55 (m, 2H, H-5′a,b), 3.24 (dt, 2H, -C*H*$_2$NHCO-, *J* = 5.8 Hz), 2.25–2.05 (m, 2H, H-2′a,b), 2.19 (t, 2H, -C*H*$_2$NMe$_2$, *J* = 6.9 Hz), 2.12 (s, 6H, -NMe$_2$), 1.49–1.44 (m, 2H, -C*H*$_2$CH$_2$NH-), 1.41–1.33 (m, 2H, Me$_2$NCH$_2$C*H*$_2$-), 1.28–1.27 (m, 4H, -(CH$_2$)$_2$-); FAB exact MS calcd for $C_{18}H_{30}N_4O_6$ 398.2165, found 399.2273.

5′-*O*-Dimethoxytrityl-5-(*N,N*-dimethylaminohexyl)carbamoyl-3′-*O*-[(2-cyanoethyl)(*N,N*-diisopropylamino)phosphinyl]-2′-deoxyuridine (9)

Compound **8** (1.99 g, 2.83 mmol) was phosphitylated as described in the preparation of **6** to give **9** (1.63 g, 63% as a white foam): FAB-MS *m/z* 901 (M^++1); ^{31}P-NMR ($CDCl_3$) δ 149.87, 149.48 (85% H_3PO_4 as an internal standard); FAB exact MS calcd for $C_{48}H_{64}N_6O_9P$ 899.4472, found 901.4644.

5′-*O*-Dimethoxytrityl-5-(*N,N*-dimethylaminohexyl)carbamoyl-3′-*O*-succinyl-2′-deoxyuridine (10)

Compound **8** (200 mg, 0.286 mmol) was succinylated as described in the preparation of **7** to give **10** (168 mg, 73% as a white foam): FAB-MS m/z 801 (M^+); ^{1}H-NMR ($CDCl_3$) δ 8.76 (s, 1H, H-6), 8.66 (t, 1H, -N*H*CO-5, *J* = 5.6 Hz), 7.46–6.87 (m, 13H, DMTr), 6.15 (t, H-1′, $J_{1',2'a} = J_{1',2'b}$ = 6.1 Hz), 5.20 (m, 1H, H-3′), 4.25–4.24 (m, 1H, H-4′), 3.82 (s, 6H, CH_3O), 3.48–3.42 (m, 4H, H-5′a,b, -C*H*$_2$NHCO-), 2.98–2.92 (m, 2H, MeNC*H*$_2$-), 2.73 (s, 6H, Me$_2$N-), 2.67–2.34 (m, 6H, H-2′a,b, 3′-OCOC*H*$_2$C*H*$_2$OCO-), 1.81–1.78 (m, 2H, -C*H*$_2$CH$_2$NHCO-5), 1.61–1.52 (m, 2H, Me$_2$NCH$_2$C*H*$_2$-), 1.49–1.35 (m, 4H, -(CH$_2$)$_2$-); FAB exact MS calcd for $C_{39}H_{45}N_4O_8$ 800.3632, found 801.3687.

5-(*N*-Cholesteryloxycarbonylaminohexyl)carbamoyl-5′-*O*-dimethoxytrityl-2′-deoxyuridine (12)

Triethylamine (0.106 ml, 0.648 mmol) and TMSCl (0.080 ml, 0.648 mmol) were added to a solution of **11** (324 mg, 0.483 mmol) in DMF (5 ml) and the mixture was stirred at room temperature for 1 h. *N,N*′-Carbonyldiimidazole (78 mg, 0.483 mmol) was separately added to a solution of cholesterol (187 mg, 0.483 mmol) in CH_2Cl_2 (5 ml), and the mixture was stirred at room temperature for 1 h. It was added to the above solution and the mixture was stirred at room temperature for 7 days. The solvent was concentrated. The residue was diluted with $CHCl_3$, which was washed with aqueous saturated $NaHCO_3$, and then brine, dried (Na_2SO_4) and evaporated. The residue was chromatographed on a silica gel column (2.8 × 9 cm) with 0–30% MeOH in $CHCl_3$ to give **12** (184 mg, 36% as a white foam): FAB-MS *m/z* 1084 (M^+); ^{1}H-NMR ($CDCl_3$:CD_3OD = 3:1, v/v) δ 8.87 (t, 1H, -N*H*COO-, *J* = 5.5 Hz), 8.65 (s, 1H, H-6), 7.45–6.87 (m, 13H, DMTr), 6.25 (t, 1H, H-1′, $J_{1',2'a} = J_{1',2'b}$ = 6.5 Hz), 5.58 (t, 1H, -CON*H*-, *J* = 5.6 Hz), 5.43 (d, 1H, cholesteryloxy-H-6, *J* = 4.4 Hz), 4.53–4.48 (m, 1H, H-3′), 4.14 (dt, 1H, H-4′, $J_{4',3'} = J_{4',5'a} = J_{4',5'b}$ = 4.0 Hz), 3.85 (s, 6H, CH_3O), 3.42–3.38 (m, 5H, H-5′a,b, -C*H*$_2$NHCOO-, cholesteryloxy-H-3), 3.15 (dt, 2H, -CONHC*H*$_2$-, $J_{N,H}$ = 5.6 Hz, $J_{H,H}$ = 6.4 Hz), 2.54, 2.28 (each ddd, each 1H, H-2′a,b, $J_{2'a,1'} = J_{2'b,1'}$ = 6.5 Hz, $J_{2'a,3} = J_{2'b,3'}$ = 3.5 Hz, $J_{2'a,2'b}$ = 13.6 Hz), 2.38 (d, 1H, cholesteryloxy-H-7a, *J* = 4.4 Hz), 1.42–1.40 (m, 4H, -C*H*$_2$CH$_2$NH-, -NHCH$_2$C*H*$_2$-), 1.24–1.15 (m, 4H, -(CH$_2$)$_2$-), 2.12–0.75 (m, 41H, cholesteryloxy-H).

5-(*N*-Cholesteryloxycarbonylaminohexyl)carbamoyl-5′-*O*-dimethoxytrityl-3′-*O*-[(2-cyanoethyl)(*N,N*-diisopropylamino)phosphinyl]-2′-deoxyuridine (13)

Compound **12** (300 mg, 0.277 mmol) was phosphitylated as described in the preparation of **6** to give **13** (318 mg, 92% as a white foam): FAB-MS *m/z* 1284 (M^+); ^{31}P-NMR ($CDCl_3$) δ 149.93, 149.51 (85% H_3PO_4 as an internal standard).

5-(*N*-Cholesteryloxycarbonylaminohexyl)carbamoyl-5′-*O*-dimethoxytrityl-3′-*O*-succinyl-2′-deoxyuridine (14)

Compound **12** (84 mg, 77 µmol) was succinylated as described in the preparation of **7** to give **14** (61 mg, 67% as a white foam): FAB-MS *m/z* 1185 (M^+); FAB exact MS calcd for $C_{69}H_{91}N_4O_{13}$ 1183.6582, found 1185.6820.

5′-*O*-Dimethoxytrityl-5-(*N*-folylaminohexyl)carbamoyl-2′-deoxyuridine (19)

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCI) (36 mg, 0.298 mmol) and *N*-hydroxysuccinimide (34 mg, 0.298 mmol) were added to a solution of folic acid dihydrate (71 mg, 0.149 mmol) in DMF (3 ml), and the mixture was stirred at room temperature in the dark. After 12 h, **11** (100 mg, 0.149 mmol) was added to the solution and the mixture was stirred at room temperature for 24 h. The solvent was concentrated and the residue was suspended in MeOH. The precipitates were collected to give **19** (18 mg, 11% as a yellow powder): FAB-MS *m/z* 1095 (M^+); ^{1}H-NMR (DMSO-d_6) δ 11.93 (bs, 1H, folate-COO*H*), 11.45 (bs, 1H, folate-O*H*), 8.68 (t, 1H, -CON*H*-5, *J* = 5.4 Hz), 8.65 (s, 1H, folate-CH), 8.44 (s, 1H, H-6), 7.97 (d, 1H, folate-CON*H*-, *J* = 7.4 Hz), 7.78 (m, 1H, -CON*H*-), 7.66 (d, 2H, folate-phenyl, *J* = 8.6 Hz), 7.38–7.29 (m, 3H, folate-N*H*$_2$,

folate-CH_2NH), 7.28–6.84 (m, 13H, DMTr), 6.64 (d, 2H, folate-phenyl, J = 8.6 Hz), 6.08 (t, 1H, H-1′, $J_{1',2'a} = J_{1',2'b}$ = 6.1 Hz), 5.34 (d, 1H, HO-3′, J = 4.4 Hz), 4.49 (dt, 2H, -CH_2NH-, J = 5.4 Hz), 4.31–4.28 (m, 1H, -CONHC*H*-), 4.11–4.09 (m, 1H, H-3′), 3.94–4.91 (m, 1H, H-4′), 3.74 (s, 6H, CH_3O), 3.27–3.22 (m, 2H, H-5′a,b), 3.20–3.18 (m, 2H, -CH_2NHCO-), 3.04–2.97 (m, 2H, -CONHCH_2-), 2.46–2.09 (m, 4H, folate-$(CH_2)_2$-), 2.07–1.83 (m, 2H, H-2′a,b), 1.47–1.16 (m, 8H, -$(CH_2)_4$-).

5-(*N*-Folylaminohexyl)carbamoyl-2′-deoxyuridine [F(α) and F(γ)]

Aqueous 80% AcOH (2 ml) containing **19** (158 mg, 0.144 mmol) was stirred at room temperature for 20 min, and then the solution was concentrated. The residue was washed with MeOH to give **F** (39 mg, 34% as a yellow powder): FAB-MS *m/z* 793 (M^+); ^{1}H-NMR (DMSO-d_6) δ 8.70 (t, 1H, -CON*H*-5, J = 5.6 Hz), 8.67 (s, 1H, folate-CH), 8.64 (s, 1H, H-6), 7.94 (d, 1H, folate-CON*H*-, J = 7.6 Hz), 7.77 (m, 1H, -CON*H*-, J = 5.5 Hz), 7.65 (d, 2H, folate-phenyl, J = 8.6 Hz), 6.92–6.87 (m, 3H, folate-NH_2, folate-CH_2NH), 6.64 (d, 2H, folate-phenyl, J = 8.6 Hz), 6.12 (t, 1H, H-1′, $J_{1',2'a} = J_{1',2'b}$ = 6.5 Hz), 5.28 (m, 1H, HO-3′), 5.00 (m, 1H, HO-5′), 4.48 (dt, 2H, -CH_2NH-, J = 5.7 Hz), 4.23 (m, 2H, -CONHC*H*-, H-3′), 3.85 (q, 1H, H-4′, J = 3.4 Hz), 3.55 (m, 2H, H-5′a,b), 3.22 (m, 2H, -CH_2NHCO-), 3.00 (m, 2H, -CONHCH_2-), 2.25–2.13 (m, 4H, folate-$(CH_2)_2$-), 2.08–1.85 (m, 2H, H-2′a,b), 1.84–1.18 (m, 8H, -$(CH_2)_4$-).

Synthesis of the controlled pore glass support containing 4, D and Ch

Aminopropyl controlled pore glass (400 mg, 34.3 µmol, 85.7 µmol/g, CPG Inc., NJ) was added to a solution of **7** (104 mg, 0.137 mmol) and WSCI (27.4 mg, 0.137 mmol) in anhydrous DMF (3 ml) and the mixture was kept for 2 days at room temperature. After the resin was washed with anhydrous pyridine, a capping solution (3 ml, 0.1 M DMAP in pyridine:Ac_2O = 9:1) was added and the whole was kept for 2 days at room temperature. The resin was washed with EtOH and acetone and was dried under vacuum. The amount of loaded nucleoside **4** to the solid support is 21.9 µmol/g from calculation of released dimethoxytrityl cation by a solution of 70% $HClO_4$:EtOH (3:2, v/v). In a similar manner, the solid supports containing **D** and **Ch** were obtained in 31.8 µmol/g and 45.1 µmol/g of loading amounts, respectively.

Synthesis of ODNs

ODNs were synthesized on a DNA synthesizer (Applied Biosystem Model 381A, CA) by the phosphoramidite method (30). For the incorporation of **Ch**, a 0.1 M solution of **13** in anhydrous CH_2Cl_2 was used, and an additional CH_2Cl_2 column wash before and after the coupling step was included in the synthesis cycle (16). Each ODN (1 µmol) linked to the resin was treated with concentrated NH_4OH (2 ml) or 1,6-hexanediamine (400 mg) in MeOH (2 ml) for 16 h at 55°C. After filtration of the resin, the filtrate was concentrated under reduced pressure. The residue obtained from the reaction with concentrated NH_4OH was purified by C-18 or C-8 silica gel column chromatography (1 × 10 cm) with a linear gradient of CH_3CN in 0.1 M TEAA buffer (pH 7.0). The residue obtained from the reaction with 1,6-hexanediamine was chromatographed on Sephadex G-25 (1.7 × 27 cm) with 0.1 M TEAA buffer (pH 7.0), before further purification on the C-18 column. Fractions were concentrated, and the residue was treated with 80% AcOH for 20 min at room temperature. 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 sample was further purified on a DEAE cellulose column (1 × 9 cm) with a linear gradient from 0.1 to 1.0 M TEAB buffer (pH 8), and then purified by HPLC with a C-18 silica gel column (Inertsil ODS-2, GL Science Inc.) using a linear gradient of CH_3CN in 0.1 M TEAA buffer (pH 7.0), when necessary.

Incorporation of folic acid into ODNs

A solution of folic acid (5 mg) and WSCI (5 mg) in DMSO (500 µl) was added to a solution of each ODN (2–9 OD_{260} units) in 0.05 M Na_2CO_3–$NaHCO_3$ (500 µl, pH 10.3), and the mixture was kept for 12 h at room temperature. The mixture was chromatographed on Sephadex G-25 column (1.6 × 22 cm) with 0.1 M TEAA buffer (pH 7.0), and purified on a DEAE cellulose column (1 × 9 cm) with a linear gradient from 0.1 to 1.0 M TEAB buffer (pH 8). Fractions were concentrated, and the residue was further purified by anion-exchange HPLC (TSK-GEL DEAE-2SW, Tosoh Co.) with a linear gradient of ammonium formate in 20% aqueous CH_3CN, and then purified on NAP-5 column (Pharmacia Biotech) with H_2O as an eluent.

N-Succinimidyl palmitate (20)

N-Hydroxysuccinimide (45 mg, 0.4 mmol) and WSCI (76 mg, 0.4 mmol) were added to a solution of palmitic acid (51 mg, 0.2 mmol) in DMF (5 ml) and the whole was stirred for 2 h at room temperature. The solvent was evaporated, the residue was dissolved in $CHCl_3$, and the solution was successively washed with water, aqueous saturated $NaHCO_3$ and then brine, dried (Na_2SO_4) and concentrated. A mixture of AcOEt and acetone was added to the residue and the resulting precipitate was collected to give **20** (66 mg, 94%): EI-MS *m/z* 353 (M^+).

Incorporation of palmitic acid into ODNs

A solution of **20** (1 mg) in DMF (70 µl) was added to a solution of each ODN (0.5–2 OD_{260} units) in 0.05 M Na_2CO_3–$NaHCO_3$ (70 µl, pH 10.3), and the mixture was kept for 2 h at room temperature. The solvent was removed *in vacuo*, and the residue was purified by anion-exchange HPLC (TSK-GEL DEAE-2SW, Tosoh Co.) with a linear gradient of ammonium formate in 20% aqueous CH_3CN, and then purified on NAP-5 column (Pharmacia Biotech) with H_2O as an eluent.

Electrospray ionization mass spectrometry

Spectra were obtained on a Quattro II (Micromass, Manchester, UK) triple quadrupole mass spectrometer equipped with an ESI source in the negative ion mode. The HPLC-purified ODN samples were dissolved in aqueous 50% 2-propanol containing 1% triethyl amine (10 pmol ODN/µl) and introduced into the ion source through a loop injector with a carrier solvent, 33% aqueous methanol, flowing at 10 µl/min flow rate. About 15 scans were acquired in ~1 min periods and combined to obtain smoothed spectra. All molecular masses of the ODNs were calculated from the multiple-charge negative-ion spectra. The observed average molecular masses of **38**, **39** and **40** were 6607.1, 6433.0 and 6618.1, respectively, and fit the calculated molecular weights

(theoretical average molecular masses) for these compounds, i.e., 6607.3 (for **38**, $C_{240}H_{343}N_{70}O_{117}P_{17}$), 6433.1 (for **39**, $C_{228}H_{329}N_{70}O_{116}P_{17}$) and 6618.1 (for **40**, $C_{231}H_{316}N_{77}O_{120}P_{17}$) within a commonly accepted error range of ESI MS, 0.01%.

Thermal denaturation

Each sample was heated at 80°C for 10 min, cooled gradually to an appropriate temperature, and used for the thermal denaturation study. Thermal-induced transitions of each mixture of ODNs were monitored at 260 nm on a Perkin Elmer Lambda 2S. Sample temperature was increased 1°C/min. Each sample contained appropriate ODNs (3 μM) and **41** (3 μM) in a buffer of 0.01 M sodium cacodylate (pH 7.0) containing 0.01 M NaCl, or ODNs (3 μM) and **42** (3 μM) in a buffer of 0.01 M sodium cacodylate (pH 7.0) containing 0.1 M NaCl.

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