‘Protected DNA Probes’ capable of strong hybridization without removal of base protecting groups

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ABSTRACT

We propose a new strategy called the ‘Protected DNA Probes (PDP) method’ in which appropriately protected bases selectively bind to the complementary bases without the removal of their base protecting groups. Previously, we reported that 4-N-acetylcytosine oligonucleotides (ac4C) exhibited a higher hybridization affinity for ssDNA than the unmodified oligonucleotides. For the PDP strategy, we created a modified adenine base and synthesized an N-acylated deoxyadenosine mimic having 6-N-acetyl-8-aza-7-deazadenine (ac6az8c7A). It was found that PDP containing ac4C and ac6az8c7A exhibited higher affinity for the complementary ssDNA than the corresponding unmodified DNA probes and showed similar base recognition ability. Moreover, it should be noted that this PDP strategy could guarantee highly efficient synthesis of DNA probes on controlled pore glass (CPG) with high purity and thereby could eliminate the time-consuming procedures for isolating DNA probes. This strategy could also avoid undesired base-mediated elimination of DNA probes from CPG under basic conditions such as concentrated ammonia solution prescribed for removal of base protecting groups in the previous standard approach. Here, several successful applications of this strategy to single nucleotide polymorphism detection are also described in detail using PDPs immobilized on glass plates and those prepared on CPG plates, suggesting its potential usefulness.

INTRODUCTION

Until date, a number of artificial oligonucleotides (1–6) containing functional groups have been reported as powerful tools for the suppression of specific genes (7–11), the exhaustive analysis of gene expression (12,13), and the detection of single nucleotide polymorphisms (SNPs) (14–17). However, when the standard phosphoramidite approach was used for these syntheses, base-labile functional groups could not be incorporated into DNA derivatives since ammonia treatment was required for removal of the base protecting groups and for release of DNA oligomers from the polymer supports (18). For example, RNA oligomers having 2-substituents such as acyloxymethyl or acylthiomethyl that can be hydrolyzed by esterases in cells, are very labile in concentrated ammonia solution although these RNA oligomers are expected to act as RNA interference drugs (19). Similarly, oligonucleotide derivatives having an N-acyl type substituent on dC (20,21), dA and dG (22) could not be synthesized. Therefore, for the development of such highly functionalized oligonucleotides, a new synthetic strategy should be explored, which does not include the problematic aqueous ammonia treatment. This strategy should be more important in DNA chip chemistry. Namely, a similar problem on the base-lability of the Si–O bond in linkers of DNA chips/microarrays has also arisen in the on-chip synthesis of oligonucleotide probes on glass plates (23,24). This is because most of the DNA oligomers were eliminated from the slide glasses by treatment with concentrated ammonia solution. The best density of DNA probes on glass plates must be controlled by a two-step procedure involving the on-chip synthesis of DNA probes and the ammonia-mediated elimination of the once-immobilized DNA.
probes. This two-step regulation increases a greater risk in the stable supply of DNA chips with constant probe density (25).

Therefore, our concern was focused on a unique method using N-unprotected deoxynucleoside 3’-phosphoramidite derivatives without base protection that does not require the ammonia treatment. In 1991, Gryaznov and Letsinger (26) first reported a phosphoramidite method without base protection. We have recently developed a new method, i.e. the activated phosphite method (27,28) for the synthesis of natural-type unmodified oligodeoxynucleotides using hydroxybenzotriazole derivatives as activators. This strategy enabled us to obtain oligodeoxynucleotides without treatment with concentrated ammonia solution. In fact, DNA oligomers containing base-labile functional groups have been efficiently synthesized without decomposition (28) using silyl linkers (29) that can be cleaved under mild conditions of Bu4NF in tetrahydrofuran (THF). The activated phosphite method may also be useful in the on-chip synthesis of DNA chips without use of the concentrated ammonia treatment.

For general use, however, the activated phosphite method incurs a minor problem in that the conditions for the conventional phosphoramidite protocols using DNA synthesizers should be modified to those optimized for this strategy. Therefore, we considered another new concept where the problematic concentrated ammonia treatment should be eliminated without changing the conventional phosphoramidite protocols so that such a method might be more useful for wider application.

In this paper, we propose a different strategy to eliminate the problematic concentrated ammonia treatment using adenine and cytosine analogs having an acyl group on their amino groups in place of the corresponding N-free natural nucleobases. The N-acylated adenine and cytosine analogs were designed in such a manner that the acyl groups can not only work as the protecting groups during the oligonucleotide synthesis but can also preserve the sites of Watson–Crick base pairing of these modified bases with thymine and guanine, respectively, even without removal of the acyl groups. To examine the possibility of our protected DNA probes (PDP) strategy, we used 4-N-acetylcytosine (ac4C) and 6-N-acylated adenine derivatives, as shown in Figure 1.

Since the base protecting groups of T and G have proved to be unnecessary in the usual phosphoramidite approach (26–28), they can be used in our strategy without base protection.

We chose ac4C since it has already been reported by us that the acetyl group of ac4C serves not only as the protecting group in DNA synthesis but also as a functional group that can increase hybridization affinity and have similar base recognition ability of the cytosine base (20,21). It has also been revealed that the acetyl group of 4-N-acetyldideoxycytidine is oriented to the 5-vinyl hydrogen via a unique hydrogen bond between the 5-proton and the carbonyl oxygen, and the acetyl.

Figure 1. Schematic representation of the PDP method.
group does not interfere with the formation of the base pair with G.

In this paper, we report an adenine mimic having an amino protecting group capable of formation of stable hydrogen bonds with thymine and also describe the high throughput synthesis and promising hybridization and base-recognition properties of the PDP incorporating such N-acylated adenine mimics.

MATERIALS AND METHODS

General remarks

1H, 13C and 31P NMR spectra were recorded at 270, 68 and 109 MHz, respectively. The chemical shifts were measured from tetramethylsilane for 1H NMR spectra, CDCl3 (77.9 ppm) for 13C NMR spectra and 85% phosphoric acid (0 ppm) for 31P NMR spectra. UV spectra were recorded on a U-2000 spectrometer. Column chromatography was performed with silica gel C-200 purchased from Wako Co. Ltd, and a minipump for a goldfish bowl was conveniently used to attain sufficient pressure for rapid chromatographic separation. The SNPs detection was performed by using Perkin Elmer ScanArray 5000 system or Olympus fluorescence microscopy system BX-FLA with ORCA IEEJ1394 (Hamamatsu photonics). The fluorescence images were analysed by using QuantArray ver. 3.0 (GSI Lumionics) or Hamamatsu photonics AQUA-Lite. High performance liquid chromatography (HPLC) was performed using the following systems: reversed-exchange HPLC was done on a Waters Alliance system with a Waters Aliance system with a Waters 3D UV detector and a Gen-PakTM LC10 AD VP following systems: reversed-exchange HPLC was done on a Shimadzu LC-10 AD VP with a Shimadzu 3D UV detector and an Waters XTerra MS C18 column (4.6×150 mm); a linear gradient (0–50%) of solvent IV [25 mM phosphate buffer (pH 6.0)] in solvent III [25 mM phosphate buffer (10–67%) of Solvent III [1 M NaCl in 25 mM phosphate buffer]] at a flow rate of 1.0 ml/min for 30 min; anion-exchange HPLC was done on a Shimadzu LC-10 AD VP with a Shimadzu 3D UV detector and an Waters XTerra MS C18 column (4.6×150 mm); a linear gradient (10–67%) of Solvent III [1 M NaCl in 25 mM phosphate buffer (pH 6.0)] in solvent IV [25 mM phosphate buffer (pH 6.0)] was used at 50°C at a flow rate of 1.0 ml/min for 30 min; anion-exchange HPLC was done on a Shimadzu LC-10 AD VP with a Shimadzu 3D UV detector and a Gen-PakTM FAX column (Waters, 4.6×100 mm); a linear gradient (10–67%) of Solvent III [1 M NaCl in 25 mM phosphate buffer (pH 6.0)] in solvent IV [25 mM phosphate buffer (pH 6.0)] was used at 50°C at a flow rate of 1.0 ml/min for 40 min. ESI mass was performed by use of MarinerTM (PerSeptive Biosystems Inc.). MALDI-TOF mass was performed by using Bruker Daltonics [Matrix: 3-hydroxyoxycylic acid (100 mg/ml) in H2O—diammoniumhydrogen citrate (100 mg/ml) in H2O (10 : 1, v/v)]. Highly cross-linked polystere (HCP) was purchased from ABI. Porous glass was prepared according to our previous method (30) and cut by 1 mm thickness to obtain discy type CPG plates. Fluorescein, T or ac4-dC phosphoramide units were purchased from Glen Research. The N-unprotected dG phosphoramide unit was prepared by deprotection of the corresponding N-isobutyl-dG phosphoramide unit.

Synthesis of 6-N-acetyl-5′-O-[bis(4-methoxyphenyl)phenylmethyl]-2′-deoxyadenosine 9

Compound 8 (1.4 g, 2.5 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine (3 ml×3) and dissolved in dry THF (10 ml). To the mixture was added EtNiPr2 (608 μl, 4.4 mmol) and AcCl (157 μl, 2.2 mmol). After the mixture was stirred at room temperature for 1 h, H2O (2 ml) was added to the mixture. After being stirred at room temperature for 10 min, the mixture was partitioned between CHCl3 (100 ml) and brine (100 ml). The organic phase was collected, dried over Na2SO4, filtered and evaporated under reduced pressure. Pyridine (5 ml) and 28% ammonia solution (5 ml) were added to the residue. After being stirred at room temperature for 10 min, the mixture was evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (20 g) with hexane-CHCl3 (70:30–0:100, v/v) containing 1% Et3N to give the fractions containing 3. The fractions were collected and evaporated under reduced pressure. The residue was finally evaporated by repeated coevaporation three times each with toluene and CHCl3 to remove the last traces of pyridine to give 3 (516 mg, 65%).

Synthesis of 6-phoramidite unit.

1H NMR (CDCl3) 1.01–1.10 (m, 12 H), 1.30–1.40 (m, 10H), 8.46 (s, 1H), 8.63 (brs, 1H). 13C NMR (CDCl3) δ 24.6, 24.7, 34.9, 35.0, 35.1, 43.1, 54.9, 55.0, 57.9, 58.0, 58.2, 58.3, 63.0, 63.2, 73.0, 73.2, 73.7, 84.5, 84.6, 85.7, 85.8, 86.2, 112.9, 117.3, 117.4, 122.2, 123.5, 126.1, 126.6, 127.9, 129.8, 135.4, 144.8, 149.6, 150.8, 151.9, 158.3, 170.6.

HRMS (ESI) m/z (M+H) calcd for C42H51N7O7P+: 796.3587; found: 796.3591.

Synthesis of 6-N-acetyl-5′-O-[bis(4-methoxyphenyl)phenylmethyl]-7-deaza-2′-deoxyadenosines 9

Compound 8 (1.4 g, 2.5 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine (3 ml×3) and dissolved in dry THF (25 ml). To the mixture was added TMSCI (531 μl, 7.5 mmol). After the mixture was stirred at room temperature for 30 min, AcCl (935 μl, 7.5 mmol) was added. After the mixture was stirred at room temperature for 30 min, 28% ammonia solution (5 ml) was added to the mixture. After being stirred at room temperature for 10 min, the mixture was partitioned between CHCl3 (150 ml) and brine (100 ml). The organic phase was collected, dried over Na2SO4, filtered and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (20 g) with hexane-CHCl3 (50:50–0:100, v/v) containing 1% pyridine and then CHCl3-MeOH (100:0–97:3, v/v) containing 1% pyridine to give the fractions containing 9. The fractions were collected and evaporated under reduced pressure. The residue was finally evaporated by repeated coevaporation three times each with toluene and CHCl3 to remove the last traces of pyridine to give compound 9 (1.4 g, 95%). 1H NMR (CDCl3) δ 2.29 (s, 3H), 2.39–2.59 (m, 2H), 3.33–3.39 (m, 2H), 3.70 (s, 6H), 4.05 (d, 1H, J = 4.1 Hz), 4.57–4.61 (m, 1H), 5.56 (brs, 2H), 6.77 (d, 4H, J = 8.6 Hz), 6.86 (d, 1H, J = 4.1 Hz), 7.05–7.34 (m, 10H), 8.46 (s, 1H), 8.63 (brs, 1H). 13C NMR (CDCl3) δ 24.6, 40.4, 55.2, 63.9, 72.7, 77.2, 83.1, 85.2, 86.6, 108.6,
113.2, 123.5, 126.9, 127.9, 128.1, 130.0, 135.6, 135.7, 144.5, 149.9, 150.3, 158.5. HRMS (ESI) m/z (M + H) calcd for C_{34}H_{35}N_{4}O_{6}: 595.2557; found: 595.2551.

**Synthesis of 6-N-acetyl-5'-O-[bis(4-methoxyphenyl)phenylmethyl]-7-deaza-2'-deoxyadenosine 3'-[2-cyanoethyl N,N-bis(1-methyl)phosphoramidite] 10**

Compound 9 (1.4 g, 2.4 mmol) was rendered anhydrous by repeated coevaporation with dry CH_{2}CN (3 ml x 3) and dissolved in dry CH_{2}Cl_{2} (20 ml). To the mixture was added ethyldiisopropylamine (575 µl, 3.5 mmol) and 2-cyanoethoxy[3N,N-di(1-methyl)amino]chlorophosphine (571 µl, 2.6 mmol). After the mixture was stirred at room temperature for 30 min, water (5 ml) was added to the mixture. After being stirred at room temperature for 10 min, the mixture was partitioned between CHCl_{3} (100 ml) and brine (100 ml). The organic phase was collected, dried over Na_{2}SO_{4}, filtered and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (30 g) with hexane-CHCl_{3} to remove the last traces of pyridine to give compound 10 (2.3 g, 78%). 1H NMR (CDCl_{3}) δ 2.35–2.49 (m, 1H), 3.02–3.11 (m, 1H), 3.22 (dd, 1H, J = 6.2 Hz, J = 9.2 Hz), 3.32 (dd, 1H, J = 5.1 Hz, J = 9.7 Hz), 3.79 (s, 6H), 4.03 (dd, 1H, J = 5.1 Hz, J = 11.1 Hz), 4.86 (dd, 1H, J = 6.1 Hz, J = 11.5 Hz), 5.55 (brs, 2H), 6.73 (d, 4H, J = 8.1 Hz), 6.76–6.83 (m, 1H), 7.16–7.34 (m, 9H), 7.39 (d, 2H, J = 1.6 Hz), 7.82 (s, 1H), 8.38 (s, 1H). 13C NMR (CDCl_{3}) δ 21.2, 28.0, 35.4, 43.1, 51.8, 51.9, 52.0, 52.1, 63.4, 63.5, 68.3, 73.3, 73.6, 73.9, 74.2, 77.3, 83.9, 84.9, 85.0, 85.1, 85.2, 86.4, 104.2, 109.0, 113.1, 117.4, 117.5, 123.7, 126.9, 127.8, 128.2, 130.1, 130.5, 135.7, 144.6, 150.2, 152.8, 158.5, 169.0. 31P NMR (CDCl_{3}) δ 149.2, 149.4. HRMS (ESI) m/z (M + Na) calced for C_{53}H_{51}N_{5}O_{5}: 576.2223; found: 576.2275.

**Synthesis of 6-N-acetyl-8-aza-5'-O-[bis(4-methoxyphenyl)phenylmethyl]-7-deaza-2'-deoxyadenosine 3'-[2-cyanoethyl N,N-bis(1-methyl)phosphoramidite] 13**

Compound 12 (2.0 g, 3.6 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine (3 ml x 3) and dissolved in dry pyridine (36 ml). To the mixture was added TMSCl (1.4 ml, 11.0 mmol). After the mixture was stirred at room temperature for 30 min, AcCl (765 µl, 11.0 mmol) was added to the mixture. After the mixture was stirred at room temperature for 4 h, conc. NH_{3} (12 ml) was added to the mixture. After being stirred at room temperature for 10 min, the mixture was partitioned between CHCl_{3} (150 ml) and brine (100 ml). The organic phase was collected, dried over Na_{2}SO_{4}, filtered and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (30 g) with hexane-CHCl_{3} (50:50–0:100, v/v) containing 1% pyridine and then CHCl_{3}-MeOH (100:0–97:3, v/v) containing 1% pyridine to give the fractions containing 13. The fractions were collected and evaporated under reduced pressure. The residue was finally evaporated by repeated coevaporation three times each with toluene and CHCl_{3} to remove the last traces of pyridine to give compound 13 (2.2 g, 62%). 1H NMR (CDCl_{3}) δ 2.38–2.48 (m, 1H), 3.01–3.10 (m, 1H), 3.20–3.35 (m, 2H), 3.76 (s, 6H), 4.06 (dd, 1H, J = 5.1 Hz, J = 11.3 Hz), 4.84 (m, 1H), 6.72–6.83 (m, 5H), 7.15–7.35 (m, 9H), 7.36 (d, 2H, J = 6.48 Hz), 8.22 (s, 1H), 8.56–8.65 (s, 2H). 13C NMR (CDCl_{3}) δ 24.6, 38.1, 51.2, 55.3, 64.2, 73.2, 77.2, 84.0, 85.3, 86.3, 104.2, 113.0, 113.1, 126.6, 127.6, 127.8, 128.0, 129.0, 132.9, 132.0, 135.9, 136.3, 144.7, 149.0, 153.8, 155.3, 157.4, 158.1, 158.2. HRMS (ESI) m/z (M + Na) calced for C_{33}H_{31}N_{5}O_{5}: 576.2223; found: 576.2275.

**Synthesis of 6-N-acetyl-8-aza-5'-O-[bis(4-methoxyphenyl)phenylmethyl]-7-deaza-2'-deoxyadenosine 3'-[2-cyanoethyl N,N-bis(1-methyl)phosphoramidite] 14**

Compound 13 (1.2 g, 2.0 mmol) was rendered anhydrous by repeated coevaporation with dry CH_{2}CN (3 ml x 3) and...
dissolved in dry CH$_2$Cl$_2$ (20 ml). To the mixture was added disopropylamine (170 µl, 1.2 mmol), 2-cyanethoxy[N,N-di(1-methylethyl)amino]chlorophosphine (760 µl, 2.4 mmol) and 1-H-tetrazole (84 mg, 1.2 mmol). After the mixture was stirred at room temperature for 12 h, water (5 ml) was added to the mixture. After being stirred at room temperature for 10 min, the mixture was partitioned between CHCl$_3$ (100 ml) and brine (100 ml). The organic phase was collected, dried over Na$_2$SO$_4$, filtered and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (30 g) with hexane-CHCl$_3$ (50 : 50–0 : 100, v/v) containing 1% Et$_3$N and then CHCl$_3$:MeOH (100 : 0–97 : 3, v/v) containing 1% Et$_3$N to give the fractions containing 14. The fractions were collected and evaporated under reduced pressure. The residue was finally evaporated by repeated coevaporation three times each with toluene and CHCl$_3$ to remove the last traces of pyridine to give compound 14 (1.2 g, 72%). $^1$H NMR (CDCl$_3$) δ 1.07–1.28 (m, 12H), 2.30 (s, 3H), 2.42–2.62 (m, 3H), 3.14–3.29 (m, 3H), 3.56–3.81 (m, 10H), 4.22 (s, 1H), 4.82–4.97 (m, 1H), 6.67–6.73 (m, 4H), 6.83 (t, 1H, J = 4.1 Hz), 7.12–7.37 (m, 11H), 8.22 (s, 1H), 8.56 (s, 1H), 8.58 (s, 1H). $^{13}$C NMR (CDCl$_3$) δ 20.1, 20.2, 20.3, 24.4, 24.5, 24.7, 29.6, 30.9, 37.3, 37.4, 43.1, 43.3, 55.1, 58.1, 58.3, 58.4, 58.6, 63.6, 63.7, 73.3, 73.7, 74.0, 77.2, 84.4, 85.2, 85.4, 85.9, 104.4, 112.8, 117.3, 117.4, 126.3, 126.4, 127.4, 128.0, 128.1, 129.8, 135.8, 135.9, 137.2, 144.6, 151.3, 154.2, 155.2, 158.0, 158.1, 168.3.

Synthesis of oligonucleotides 5–6, 15–19 and PDP 20
The synthesis of oligodeoxyribonucleotides 5–6 and 15–17 was carried out on an HCP resin having a silyl linker in an ABI 392 DNA synthesizer (26). The fully protected oligomer after chain elongation was deprotected by treatment with a 10% 1,8-diazabicycloundec-7-ene (DBU) solution in CH$_3$CN (500 µl) at room temperature for 1 min. Then, the mixture containing oligoDNAs was released from the resin by treatment with a solution of Et$_3$N (0.2 M) and Et$_3$N (0.4 M) in THF (500 µl) at room temperature for 4 h. The polymer support was removed by filtration and washed with 0.1 M ammonium acetate buffer (1 ml × 3). The filtrate was purified by anion-exchange HPLC to give oligonucleotides 5–6 and 15–17.

Oligonucleotide 5: TACCTAA*ATCCAT (A*: ac$_6^6$A) MALDI-TOF Mass (M + H) calculated for C$_{128}$H$_{164}$Na$_{18}$O$_{76}$P$_{12}^{+}$: 3920.61, found: 3919.16.

Oligonucleotide 6: TACCTAA*ATCCAT (A*: bz$_6^6$A) MALDI-TOF Mass (M + H) calculated for C$_{133}$H$_{166}$Na$_{19}$O$_{76}$P$_{12}^{+}$: 3982.68, found: 3987.16.

Oligonucleotide 15: TACCTAA*ATCCAT (A*: ox$_8^8$ac$_6^6$A) MALDI-TOF Mass (M + H) calculated for C$_{128}$H$_{164}$Na$_{18}$O$_{76}$P$_{12}^{+}$: 3936.61; found: 3933.83.

Oligonucleotide 16: TACCTAA*ATCCAT (A*: ac$_6^6$O$_2^2$A) MALDI-TOF Mass (M + H) calculated for C$_{129}$H$_{165}$Na$_{44}$O$_{76}$P$_{12}^{+}$: 3919.62; found: 3919.56.

Oligonucleotide 17: TACCTAA*ATCCAT (A*: ac$_6^6$az$_2^2$A) MALDI-TOF Mass (M + H) calculated for C$_{129}$H$_{164}$Na$_{44}$O$_{76}$P$_{12}^{+}$: 3920.61; found: 3921.27.

Oligonucleotide 18: TACCTAA*A*TCCAT (A*: ac$_6^6$az$_2^2$A) MALDI-TOF Mass (M + H) calculated for C$_{132}$H$_{168}$Na$_{44}$O$_{76}$P$_{12}^{+}$: 4002.74; found: 3997.33.

Oligonucleotide 19: TACCCTAA*A*TCCAA (A*: ac$_6^6$az$_2^2$A) MALDI-TOF Mass (M + H) calculated for C$_{132}$H$_{168}$Na$_{44}$O$_{76}$P$_{12}^{+}$: 4002.74; found: 3995.09.

PDP 20: TA*C*C*TAA*A*TCC*GC (A*: ac$_6^6$az$_2^2$A, C*: ac$_6^6$A), MALDI-TOF Mass (M + H) calculated for C$_{144}$H$_{180}$Na$_{44}$O$_{76}$P$_{12}^{+}$: 4256.91; found: 4254.82.

$T_m$ measurement
An appropriate oligonucleotide (2 µM) and its complementary 2 µM ssDNA 12mer or ssRNA 12mer were dissolved in a buffer consisting of 150 mM NaCl (RNA: 10 mM), 10 mM sodium phosphate and 0.1 mM EDTA adjusted to pH 7.0. The solution was kept at 80°C for 10 min to allow complete dissociation of the duplex to single strands, cooled at the rate of 1.0°C/min, and kept at 15°C for 10 min. After that, the melting temperatures ($T_m$) were determined at 260 nm using a UV spectrometer (Pharma Spec UV-1700 ת), Shimadzu) by increasing the temperature at the rate of 1.0°C/min.

Preparation of slide glass plates containing oligonucleotides 21–23 and PDPs 24–26
The synthesis of oligodeoxyribonucleotides 24–26 was carried out on an HCP resin having a silyl linker in ABI 392 DNA synthesizer (27). The oligomer after chain elongation was deprotected by treatment with a 10% DBU solution in CH$_3$CN (500 µl) at room temperature for 1 min. Then, the oligomer was released from the resin by treatment with a solution of Et$_3$N:3HF (0.2 M) and Et$_3$N (0.4 M) in THF (500 µl) at room temperature for 4 h. The polymer support was removed by filtration and washed with ammonium acetate buffer (1 ml × 3). The filtrate was purified by anion-exchange HPLC.
Match/mismatch discrimination by use of PDPs on slide glass plates

A slide glass plate having unmodified probes and PDP was added to a 0.02 μM solution of the target oligoDNA having a Cy3 residue at the 5’ position in 5×SSC buffer (pH 7.0) containing 0.2% SDDS. The mixture was incubated at 48°C or 55°C for 16 h. Next, the glass plate was washed with 5×SSC buffer (pH 7.0) containing 0.2% SDDS for 5 min. After drying of the glass plate, the fluorescence strength of the plate was measured by fluorescence imager (Perkin Elmer ScanArray 5000 system).

Synthesis of PDPs 28–30 on CPG plate

The protected oligonucleotides probes on porous glass (2.7 μmol/g, 11 pmol/cm², 16-hydroxyhexadecanoyl linker) were synthesized in ABI 392 synthesizer. The coupling efficiency was monitored by DMTr cation assay.

After chain elongation, the 2-cyanoethyl group of the protected oligomer was deprotected by treatment with 10% DBU/CH3CN (500 μl) for 1 min.

Sequence of PDPs 28–30

28: 5’-d(GA*TA*C*A*C*TTGA*C*C*TTTTTTTT)
29: 5’-d(GA*TA*C*A*C*TTGA*C*C*TTTTTTTT)
30: 5’-d(GA*TA*C*A*C*TTGA*C*C*TTTTTTTT)

RESULTS AND DISCUSSION

Synthesis and properties of DNA oligomers having an N-acylated adenine derivative

First, we carried out the synthesis of DNA oligomers having an N-acylated adenine, i.e. 6-N-acetyladenine (ac6A) and 6-N-benzoyladenine (bz6A), to examine their base pairing properties. The phosphoramidite unit of ac6A was synthesized in 65% yield by acetylation of the N-unprotected dA phosphoramidite with acetyl chloride (22), as shown in Figure 2. The phosphoramidite unit of bz6A was commercially available. We tried to synthesize these modified DNA oligomers using the activated phosphite method and a silyl linker (28), as shown in Figure 3. Each chain elongation was carried out using 1-hydroxy-6-nitrobenzotriazole in the presence of benzimidazolium triflate (BIT) (31) as an activator on thymidine-loaded HCP resins (4) (32). After chain elongation, the selective removal of the cyanoethyl groups of the internucleotidic phosphates was carried out by treatment with 10% DBU in CH3CN for 1 min and the successive release of the 5’-terminal DMTr group was carried out by treatment with 3% trichloroacetic acid in CH2Cl2. Finally, the modified DNA oligomers 5 and 6 were released from the resin by treatment with 0.2 M Et3N-3HF in THF at room temperature for 4 h. Purification of the crude products by anion-exchange HPLC gave the modified oligomers 5 and 6 in 33 and 41% yields, respectively.

Figure 2. Synthesis of ac6A phosphoramidite unit 3. Reagents and conditions: (a) AcCl, Et3NPr2, THF, rt, 1 h; (b) pyridine-NH3 aq. (2:1, v/v), rt, 10 min.

Figure 3. Synthesis of modified DNA 5 and 6. Reagents and conditions: (a) protocol of the activated phosphite method on ABI392 DNA synthesizer; (b) DBU, CH3CN, rt, 1 min; (c) 3% CC13COOH, CH3Cl2, rt, 1 min; (d) Et3N-3HF, THF, rt, 4 h.
Subsequently, $T_m$ experiments were carried out on the duplexes formed between these modified DNA oligomers and DNA oligomers having the complementary or single mismatch sequences, as shown in Table 1. These results showed that incorporation of a single $N$-acylated adenine base such as ac$^6$A and bz$^6$A decreased the stability of the resulting duplex [$T_m$: 40.3°C (ac$^6$A), 37.1°C (bz$^6$A) versus 44.5°C (unmodified DNA duplex)]. Moreover, it was found that the base recognition abilities of these acylated bases, which correspond to the difference ($\Delta T_m$) in the $T_m$ value between the matched and the most stable mismatched duplexes, were significantly lower than that of the unmodified DNA [$\Delta T_m$: −6.7°C (ac$^6$A), −2.5°C (bz$^6$A) versus −12.7°C (unmodified DNA)]. These results indicated that simple introduction of an acyl or benzoyl group into an adenine base decreases the stability of DNA because these protecting groups block the Watson–Crick base pairing site in the most stable conformer.

### Table 1. $T_m$ values for DNA 13mer duplexes containing A, ac$^6$A and bz$^6$A

| Complementary DNA | Unmodified DNA | Modified DNA 5 | Modified DNA 6 |
|-------------------|----------------|----------------|----------------|
|                   | $d(A T G G A T X T G G T A)$-5' | $5'-d(T A C C T A A T C C A A T)$ | $5'-d(T A C C T A A T C C A A T)$ |
| $T_m$ (°C)$^a$ | $\Delta T_m$ (°C)$^b$ | $T_m$ (°C)$^a$ | $\Delta T_m$ (°C)$^b$ |
| X                 |                | $A' = ac^6A$ | $A' = bz^6A$ |
| T                 |               | 40.3          | 37.1          |
| G                 | −12.7         | 33.6          | 24.6          |
| C                 | −18.5         | 25.8          | 25.8          |
| A                 | −15.7         | 28.1          | 29.1          |

$^a$The $T_m$ values are accurate within ±0.5°C. The $T_m$ measurements were carried out in a buffer containing 150 mM sodium phosphate (pH 7.0), 100 mM NaCl, 0.1 mM EDTA and 2 μM duplex.

$^b$ $\Delta T_m$ is the difference in the $T_m$ value between the duplex having a thymine and those having other bases in the X position.

**Figure 4.** The most stable structures of ac$^6$ox$^7$A, ac$^6$c$^7$A and ac$^6$az$^8$c$^7$A by *ab initio* MO calculation.
with saturated NH₃/MeOH, as shown in Figure 6. In a manner similar to that described in the synthesis of compound 9, treatment of 12 with AcCl in the presence of TMSCl followed by hydrolysis gave the N-acetylated compound 13 in 62% yield, which was further converted by reaction with CEOP(NiPr₂)₂ to the target phosphoramidite unit 14 in 72% yield.

Based on the X-ray structural analysis of ac6C, Parthasarathy and coworkers reported that the distance between the carbonoyl oxygen and the 5-vinyl proton was 2.14 Å, suggesting that the electron withdrawing ability of the acetyl group probably has polarized 5-H also sufficiently to take part in hydrogen bonding (20). Previously, we reported that the chemical shift of the 5-H proton of ac4C exhibited a downfield shift of 1.21 p.p.m. compared with that of dC in its 1H NMR spectrum because of an intramolecular hydrogen bond between the carbonyl oxygen atom and the 5-vinyl proton (21). However, the downfield shift of the 7-H protons of ac6c7A and ac6az8c7A was smaller (only 0.3–0.4 p.p.m.) than that of ac6C. As far as the possibility of the intramolecular hydrogen bonds suggested by the ab initio MO calculation of ac6c7A and ac6az8c7A is concerned, further studies are needed.

The three modified DNA oligomers 15–17 incorporating ac6ox8A, ac6c7A or ac6az8c7A were synthesized and isolated in 44, 25 and 24% yields, respectively, using the activated phosphate method without base protection described above.

**Hybridization and base recognition of DNA oligomers having N-acetylated 8-oxoadenine and 7-deazaadenine derivatives**

The hybridization affinity and base recognition ability of the DNA 13mers containing ac6ox8A (15), ac6c7A (16) and ac6az8c7A (17) were measured and the results are listed in Table 2. The hybridization affinity and base recognition ability of the DNA 13mer 15 containing an ac6ox8A was lower than those of the unmodified DNA 13mer, as shown in Table 2 (Tₘ of X=T: 42.4°C versus 44.5°C, base recognition ability: −11.3°C versus −12.7°C). A similar decrease in the hybridization affinity and base recognition ability was observed for the DNA 13mer 16 containing an ac6c7A (Tₘ of X=T: 43.8°C versus 44.5°C, base recognition ability: −11.7°C versus −12.7°C). The ability of the amide proton of ac6c7A as the proton donor might decrease compared with the unmodified adenine because the acidity of the amide proton of ac6c7A might become lower than that of the adenine base due to replacement of the electronegative nitrogen atom by a carbon atom at the 7-position. However, it was found that the hybridization affinity and base recognition ability of DNA 13mer 17 containing an ac6az8c7A were similar to those of unmodified DNA 13mer (Tₘ of X=T: 44.7°C versus 44.5°C, base recognition ability: −13.5°C versus −12.7°C). The Tₘ values of DNA 13mer 18 containing three consecutive ac6az8c7As and 19 containing three discontinuous
Table 2. $T_m$ values for DNA 13mer duplexes containing A, ac$^b$ox$^8$A, ac$^b$c$^7$A and ac$^b$az$^8$c$^7$A

| Complementary DNA | d(A T G G A T X T A G G T A)-5' | d(A T G G A T X T A G G T A)-5' |
|-------------------|----------------------------------|----------------------------------|
| Unmodified DNA    | 5'-d(T A C C T A A T C C A T)     | 5'-d(T A C C T A A T C C A T)     |
| Modified DNA 15-17| 5'-d(T A C C T A A A T C C A T)   | 5'-d(T A C C T A A A T C C A T)   |
| Modified DNA 18   | 5'-d(T A C C T A A A T C C A T)   | 5'-d(T A C C T A A A T C C A T)   |
| Modified DNA 19   | 5'-d(T A C C T A A A T C C A T)   | 5'-d(T A C C T A A A T C C A T)   |

Unmodified DNA  | Modified DNA 15 | Modified DNA 16 | Modified DNA 17 | Modified DNA 18 | Modified DNA 19 |
|----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| X   | $T_m$ (°C)$^a$ | $\Delta T_m$ (°C)$^b$ | $T_m$ (°C)$^a$ | $\Delta T_m$ (°C)$^b$ | $T_m$ (°C)$^a$ | $\Delta T_m$ (°C)$^b$ | $T_m$ (°C)$^a$ | $\Delta T_m$ (°C)$^b$ | $T_m$ (°C)$^a$ | $\Delta T_m$ (°C)$^b$ |
| T   | 44.5            | -               | 42.4            | -               | 43.8            | -               | 44.7            | -               | 52.3            | -               | 51.1            | -               |
| G   | 31.8            | -12.7           | 31.1            | -11.3           | 32.1            | -11.7           | 31.2            | -13.5           | 35.7            | -16.6           | 37.4            | -13.7           |
| C   | 26.0            | -18.5           | 25.5            | -16.9           | 24.2            | -19.6           | 25.8            | -18.9           | 32.6            | -19.7           | 35.7            | -15.4           |
| A   | 28.8            | -15.7           | 28.0            | -14.4           | 26.7            | -17.1           | 29.1            | -15.6           | 30.8            | -21.5           | 31.9            | -19.2           |

$^a$The $T_m$ values are accurate within ±0.5°C. The $T_m$ measurements were carried out in a buffer containing 150mM sodium phosphate (pH 7.0), 100mM NaCl, 0.1mM EDTA and 2μM duplex.

$^b$$\Delta T_m$ is the difference in the $T_m$ value between the duplex having a thymine and those having other bases at the X position.

Table 3. $T_m$ values for PDP 20 (recognition site of unmodified DNA and PDP: A and A')-DNA and PDP 20-RNA duplexes

| Complementary DNA | d(A T G G A T X T A G G T A)-5' | d(A U G G A U X U A G G U A)-5' |
|-------------------|----------------------------------|----------------------------------|
| Complementary RNA | 5'-d(T A C C T A A T C C A T)     | 5'-d(T A C C T A A A T C C A T)   |
| PDP 20            | 5'-d(T A C C T A A A T C C A T)   | 5'-d(T A C C T A A A T C C A T)   |

Unmodified DNA vs DNA | PDP 20 vs DNA | Unmodified DNA vs RNA | PDP 20 vs RNA

| X   | $T_m$ (°C)$^a$ | $\Delta T_m$ (°C)$^b$ | $T_m$ (°C)$^a$ | $\Delta T_m$ (°C)$^b$ | X   | $T_m$ (°C)$^a$ | $\Delta T_m$ (°C)$^b$ | $T_m$ (°C)$^a$ | $\Delta T_m$ (°C)$^b$ |
|-----|----------------|-----------------------|----------------|-----------------------|-----|----------------|-----------------------|----------------|-----------------------|
| T   | 44.5           | -                     | 59.7           | -                     | U   | 40.0           | -                     | 51.3           | -                     |
| G   | 31.8           | -12.7                 | 42.2           | -17.5                 | G   | 30.4           | -9.6                   | 41.8           | -9.5                   |
| C   | 26.0           | -18.5                 | 40.2           | -19.5                 | C   | 29.7           | -10.3                  | 42.2           | -9.1                   |
| A   | 28.8           | -15.7                 | 38.9           | -20.8                 | A   | 30.5           | -9.5                   | 41.6           | -9.7                   |

$^a$The $T_m$ values are accurate within ±0.5°C. The $T_m$ measurements were carried out in a buffer containing 150mM sodium phosphate (pH 7.0), 100mM NaCl, 0.1mM EDTA and 2μM duplex.

$^b$$\Delta T_m$ is the difference in the $T_m$ value between the duplex having a thymine and those having other bases at the X position.

$^c$The $T_m$ values are accurate within ±0.5°C. The $T_m$ measurements were carried out in a buffer containing 10mM sodium phosphate (pH 7.0), 100mM NaCl, 0.1mM EDTA and 2μM duplex.

ac$^a$az$^8$c$^7$As were also measured to study the additive effect of this adenine mimic. The hybridization affinity of DNA 18 was significantly higher by 7.8°C than that of unmodified DNA ($T_m$ of X=T: 52.3°C versus 44.5°C). Similarly, DNA 19 containing three discontinuous ac$^a$az$^8$c$^7$As showed strong hybridization affinity ($T_m$ of X=T: 51.1°C). Interestingly, the base recognition ability of DNA 18 increased by 3.9°C ($\Delta T_m$: -16.6°C versus -12.7°C) compared with the unmodified DNA, though the base recognition ability of DNA 19 ($\Delta T_m$: -13.7°C) was similar to that of unmodified DNA. These results indicated that ac$^a$az$^8$c$^7$A must be a good candidate adenine mimic useful for realization of the PDP strategy.

Hybridization and base recognition of PDP 20

To examine the utility of the PDP strategy, PDP 20 containing ac$^a$az$^8$c$^7$As and ac$^c$Cs, which were substituted for all A and C bases in these sequences, were synthesized using four phosphoramidite building blocks involving the N-protected dG and T phosphoramidite units. Previously, we reported that the hybridization affinity of oligonucleotides incorporating an ac$^c$ residue slightly increased compared with unmodified oligonucleotides (21,37). The effect of these modified bases on the hybridization affinity and base recognition ability of PDP 20 having an ac$^a$az$^8$c$^7$A at the recognition site was studied, as shown in Table 3. As a result, the $T_m$ value of the duplex between PDP 20 and the complementary DNA oligomer was significantly higher by 15.2°C than that of the unmodified duplex ($T_m$ of X=T: 59.7°C versus 44.5°C). The base recognition ability was also increased by 4.8°C ($\Delta T_m$: -17.5°C versus -12.7°C). These results showed that PDP 20 not only has high hybridization affinity but also has high recognition ability even in the presence of the nine modified bases in the DNA 13mer probe. Moreover, it was found that the hybridization affinity of PDP 20 for the complementary RNA oligomer was increased by 11.3°C ($T_m$ of X=T: 53.3°C versus 40.0°C) with similar base recognition ability compared with that of the unmodified DNA ($\Delta T_m$: -9.1°C versus -9.5°C).

In further study, we examined the $T_m$ values of other PDPs having different sequences to study the effect of the sequence on hybridization affinity and base
recognition ability, as shown in Tables S1–3 of Supplementary Data. These results also indicate that the hybridization affinity of PDPs increased not only toward the complementary DNA strand but also toward the complementary RNA strand without losing sequence selectivity.

### SNPs analysis using PDPs immobilized on glass plates by the post synthetic procedure

To see if PDPs are actually superior to unmodified DNA probes when they are immobilized on glass plates, we prepared glass plates having PDPs that target three kinds of oligonucleotides, i.e. wild-type, HSC-4 and Ca9-22 mutant, selected from the SNPs in the 947 and 966th regions of a human p53 gene (38), as shown in Table 4. The 5’-amino unmodified probes 21–23 and PDPs 24–26 complementary to the above three sequences were synthesized. They were attached to the activated ester-coated glass plates via an amide bond to give slide glass plates having unmodified probes 21–23 and PDPs 24–26.

Thus, hybridization experiments of a Cy3-labeled DNA 20mer 27 having the wild-type sequence of the p53 gene with PDPs 24–26 and unmodified probes 21–23 (21 and 24: wild-type probes, 22 and 25: HSC-4 mutant probes, 23 and 26: Ca9 mutant probes) were carried out, as shown in Figure 7. Their hybridization affinity at 48°C was evaluated by the strength of fluorescence remaining on the glass plates. The strength of fluorescence derived from the wild-type 27 that bound to the wild-type PDP 24 was 1.5 times higher than that observed in the case of the unmodified wild-type probe 21. This result indicated markedly higher binding affinity of PDP 24 for the fully matched target 27 than the unmodified probe 21.

### Table 4. Sequences of Cy3-labeled target 27, unmodified probes 21–23, and PDPs 24–26

| Target DNA 20mer 27 having a wild-type sequence of p53 | SNPs site |
|--------------------------------------------------------|-----------|
| 5’-Cy3-d(GGCA<TGACC>G-AAGGCCCAT)                     |           |
| wild-type unmodified probe 21                          | 3’-d(TATC<TTGCCTCCG)T5 | |
| HSC-4 unmodified probe 22                             | 3’-d(TATC<TTGCCTCCG)T5 | |
| Ca9-22 unmodified probe 23                            | 3’-d(TATC<TTGCCTCCG)T5 | |
| wild-type PDP 24                                       | 3’-d(TAT'C<TTGCCTCCG)T5 | |
| HSC-4 PDP 25                                          | 3’-d(TAT'C<TTGCCTCCG)T5 | |
| Ca9-22 PDP 26                                         | 3’-d(TAT'C<TTGCCTCCG)T5 | |

\[
A^\ast = \text{ac}^\ast \text{az}^\ast \text{c}^7 \text{A} \quad C^\ast = \text{ac}^\ast \text{C}
\]

slide glass

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Figure 7. Match/mismatch discrimination of target DNA 27 by unmodified probes 21–23 and PDPs 24–26. The fluorescence of Cy3 was measured using a fluorescence imager (Laser power 40, PM Gain 70). The hybridization was performed for 16 h at (a) 48°C and (b) 55°C on a slide glass.

Subsequently, we compared the probability of failure of detection of the wild-type target 27 by PDP 25 and the unmodified probe 22 both having a sequence complementary to the HSC-4 mutant. In the hybridization
of the HSC-probes 22 and 25 to wild-type 27, a G–T mismatch base pair should be formed. As a result, in the case of the PDP, the fluorescence intensity obtained in the combination of the HSC-4 mutant PDP 25 and the wide-type sample 27 was 0.28 that was 5.3 times smaller than that of the wild-type probe 24/wild-type sample 27. On the other hand, in the case of the unmodified probe, the fluorescence intensity of the HSC-4 mutant probe 22/sample 27 was found to be 0.12 that was 8.0 times smaller than the wild-type probe 21/sample 27. These results indicated that the unmodified probe was superior to PDP in terms of sequence selectivity for hybridizations carried out at 48°C. As shown in the example of PDP 20, the PDP tends to stabilize the DNA duplex in comparison to the unmodified DNA probe. Therefore, the optimized temperature for the hybridization could be higher in the system using PDP than that using unmodified DNA. Therefore, we tried to increase the hybridization temperature to 55°C. As expected, the ability of base recognition of the PDP greatly increased. The fluorescence intensity of the perfectly matched duplex, wild-type probe 24/sample 27, became 1.20, whereas the combination of the HSC-4 mutant probe 25/sample 27 having a G–T mismatch was 0.05, 24 times smaller than 1.20. It should be noted that, although the fluorescence intensity of the perfectly matched combination slightly decreased under this high-temperature condition, the intensity was still greater than that of the unmodified probe at 48°C and 55°C.

SNPs analysis using PDPs prepared on CPG plates by on-chip synthesis

Furthermore, to examine hybridization of in situ synthesized PDP, we carried out model experiments of SNP analysis using FITC-labeled DNA 20mers 31–33 having the wild-type 31, 1075-mutant 32, and 1076-mutant sequence 33 of a P450 gene (39,40) and PDP synthesized on uniformly flattened square discs of CPG (PDP–CPG discs) with thickness of 1 mm (30), as shown in Table 5. Three kinds of PDP–CPG discs 28–30 (28: wild-type probe, 29: 1075-mutant probe, 30: 1076-mutant probe) were prepared directly by the synthesis of the PDPs on these discs according to the standard phosphoramidite chemistry. Deprotection of the cyanoethyl groups and the 5'-terminal DMTr group was carried out using DBU in CH3CN (1 min) and 3% TCA in CH3CN (1 min), respectively, to give PDP–CPG discs 28–30. It should be noted that the usual treatment with ammonia is no longer required in this protocol, and the PDP could be immobilized to the CPG plates with quite high density (8 mol/g). The PDP–CPG discs 28–30 thus obtained were allowed to hybridize with the target DNA 20mers 31–33 having a fluoresceine residue at the 3' position. The hybridization affinity was estimated by the fluorescence strength on the disc, as shown in Figure 8. Similar to the experiments shown in Figure 7 the fluorescence intensity of each target DNA, for example, the DNA 20mer 31 captured by the matched PDP 28, was compared to those captured by the wrong PDPs 29 and 30.
In the detection of the wild-type sample 31, the use of the matched PDP 28 gave fluorescence intensities that were 24 times and 91 times larger than those obtained when the 1075-mutant PDP 29 and the 1076-mutant PDP 30 were used, respectively. These results indicated the satisfactory base discrimination of ac8C on the in-situ synthesized DNA chips. Similarly, in the detection of the 1075-mutant target 32 and the 1076-mutant 33, the in-situ synthesized PDP–CPG discs showed precise recognition of the target DNA by the corresponding PDP 29 and PDP 30, respectively. When the target site was GA (sample 32), the PDP–CPG discs 28–30 showed somewhat low recognition ability (at most 4-fold discrimination), probably because of the higher stability of the G–A mismatch (41), but the highly selective discrimination (more than 10-fold) between G–T matched and T–C* mismatched base pairs could be performed when the target site was TG (sample 33).

CONCLUSIONS

In summary, we successfully synthesized modified DNAs having an N-acylated adenine mimic, 6-N-acetyl-8-aza-7-deazaadenine (ac8az7A), with hybridization affinity superior to those having an unmodified adenine base. In addition, we have demonstrated that the protected oligonucleotide probes, PDP, attached to CPG discs could be easily synthesized by the conventional phosphoramidite approach without ammonia treatment and could be used as new tools capable of hybridization with DNA with high binding affinity and without a decrease in base recognition. The present strategy that is performed in a straightforward manner eliminates the time-consuming procedures for isolation of DNA probes as well as for deprotection of the base moieties and would be useful for development of new DNA chips. Further studies are now under way in this direction.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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