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# '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 ($ac^4C$) 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-deazaadenine ($ac^6az^8c^7A$). It was found that PDP containing $ac^4C$ and $ac^6az^8c^7A$ 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

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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 $Bu_4NF$ 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 ($ac^4C$) 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 $ac^4C$ since it has already been reported by us that the acetyl group of $ac^4C$ 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*-acetyldeoxycytidine is oriented to the 5-vinyl hydrogen via a unique hydrogen bond between the 5-proton and the carbonyl oxygen, and the acetyl

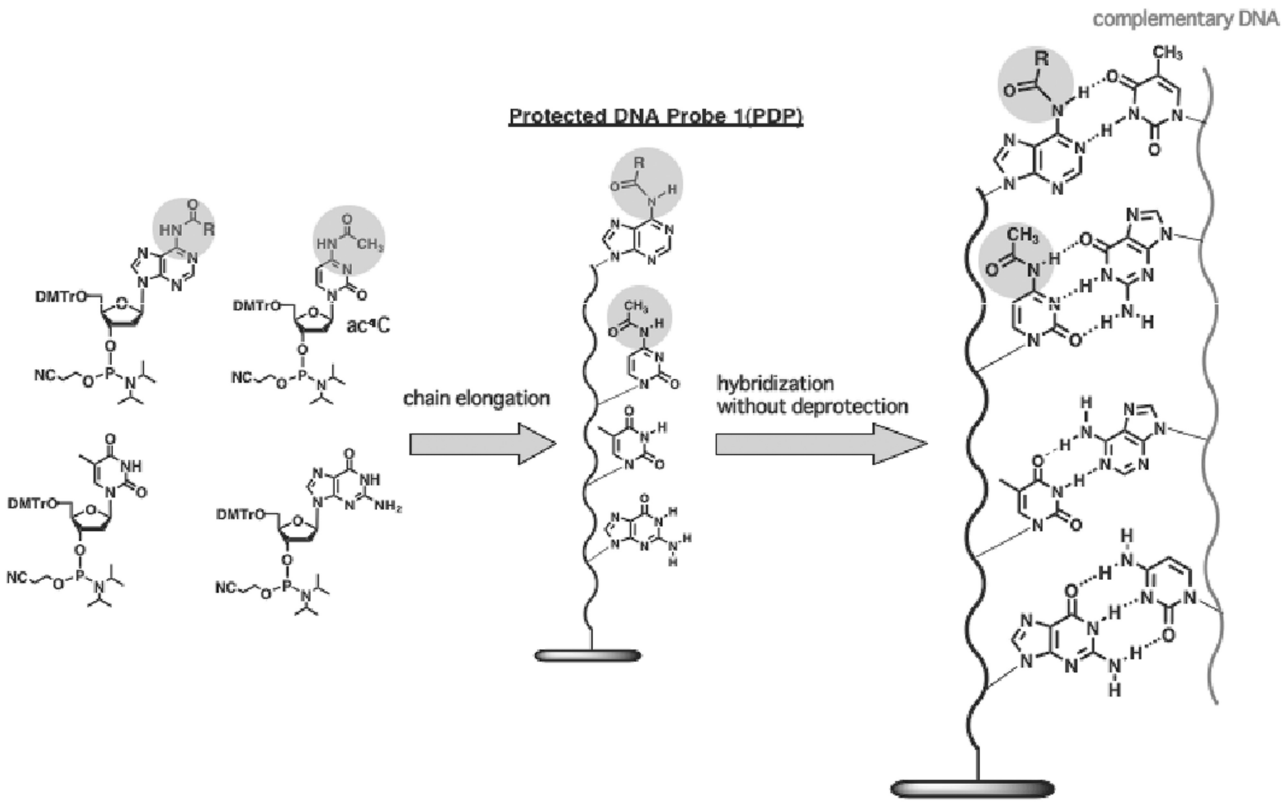



**Figure 1.** Schematic representation of the PDP method.

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

$^{1}H$, $^{13}C$ and $^{31}P$ NMR spectra were recorded at 270, 68 and 109 MHz, respectively. The chemical shifts were measured from tetramethylsilane for $^{1}H$ NMR spectra, $CDCl_3$ (77 p.p.m.) for $^{13}C$ NMR spectra and 85% phosphoric acid (0 p.p.m.) for $^{31}P$ 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 IEEE1394 (Hamamatsu photonics). The fluorescence images were analysed by using QuantArray ver 3.0 (GSI Lumonics) or Hamamatsu photonics AQUA-Lite. High performance liquid chromatography (HPLC) was performed using the following systems: reversed-exchange HPLC was done on a Waters Aliance system with a Waters 3D UV detector and an Waters XTerra MS C18 column (4.6×150 mm); a linear gradient (0–30%) of solvent I [0.1 M ammonium acetate buffer (pH 7.0)] in solvent II ($CH_3CN$) 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 Mariner™ (PerSeptive Biosystems Inc.). MALDI-TOF mass was performed by using Bruker Daltonics [Matrix: 3-hydroxypicolinic acid (100 mg/ml) in $H_2O$—diammoniumhydrogen citrate (100 mg/ml) in $H_2O$ (10 : 1, v/v)]. Highly cross-linked polystyrene (HCP) was purchased from ABI. Porous glass was prepared according to our previous method (30) and cut by 1 mm thickness to obtaine disc-type CPG plates. Fluorescein, T or $ac^4$-dC phosphoramidite units were purchased from Glen Research. The *N*-unprotected dG phosphoramidite unit was prepared by deprotection of the corresponding *N*-isobutyryl-dG phosphoramidite unit.

### Synthesis of 6-*N*-acetyl-5′-*O*-[bis(4-methoxyphenyl) phenylmethyl]-2′-deoxyadenosine 3′-[2-cyanoethyl *N*,*N*-bis(1-methylethyl)phosphoramidite] 3

Compound **2** (753 mg, 1 mmol) was rendered anhydrous by repeated coevaporation with dry $CH_3CN$ (3 ml×3) and dissolved in dry THF (10 ml). To the mixture was added $EtNiPr_2$ (608 µl, 4.4 mmol) and AcCl (157 µl, 2.2 mmol). After the mixture was stirred at room temperature for 1 h, $H_2O$ (2 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 $Na_2SO_4$, 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-$CHCl_3$ (70 : 30–0 : 100, v/v) containing 1% $Et_3N$ 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 $CHCl_3$ to remove the last traces of pyridine to give 3 (516 mg, 65%): $^{1}H$ NMR ($CDCl_3$) 1.01–1.10 (m, 12 H), 2.36 (t, 1H, $J$ = 6.3 Hz), 2.47–2.52 (m, 5H), 2.84–2.89 (m, 1H), 3.26–3.67 (m, 12H), 4.21 (d, 1H, $J$ = 3.2 Hz), 4.70 (t, 1H, $J$ = 3.2 Hz), 6.34 (d, 1H, $J$ = 3.5 Hz), 6.66 (2d, 4H, $J$ = 8.6 Hz), 7.05–7.30 (m, 9H), 8.16 (d, 1H, $J$ = 5.1 Hz), 8.52 (s, 1H), 9.46 (brs, 1H). $^{13}C$ NMR ($CDCl_3$) δ 19.9, 20.05 20.11, 24.2, 24.3, 24.4, 24.5, 25.4, 38.9, 39.0, 42.9, 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, 141.8, 144.3, 149.6, 150.8, 151.9, 158.3, 170.6. $^{31}P$ NMR ($CDCl_3$) δ 149.2, 149.4. HRMS (ESI) m/z (M + H) calcd for $C_{42}H_{51}N_7O_7P^+$: 796.3587; found: 796.3591.

### Synthesis of 6-*N*-acetyl-5′-*O*-[bis(4-methoxyphenyl) phenylmethyl]-7-deaza-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 pyridine (25 ml). To the mixture was added TMSCl (531 µl, 7.5 mmol). After the mixture was stirred at room temperature for 30 min, AcCl (935 µl, 7.5 mmol) was added to the mixture. After the mixture was stirred at room temperature for 4 h, 28% ammonia solution (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 $Na_2SO_4$, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (20 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 **9**. 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 9 (1.4 g, 95%). $^{1}H$ NMR ($CDCl_3$) 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). $^{13}C$ NMR ($CDCl_3$) 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 $C_{34}H_{35}N_4O_6^+$: 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-methylethyl)phosphoramidite] 10

Compound **9** (1.4 g, 2.4 mmol) was rendered anhydrous by repeated coevaporation with dry $CH_3CN$ (3 ml×3) and dissolved in dry $CH_2Cl_2$ (20 ml). To the mixture was added ethyldiisopropylamine (575 µl, 3.5 mmol) and 2-cyanoethoxy[*N*,*N*-di(1-metylethyl)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 $Na_2SO_4$, filtered and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (20 g) with hexane-$CHCl_3$ (50 : 50–0 : 100, v/v) containing 1% $Et_3N$ and then $CHCl_3$-MeOH (100 : 0–97 : 3, v/v) containing 1% $Et_3N$ to give the fractions containing **10**. 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 **10** (1.7 g, 91%). $^1$H NMR ($CDCl_3$) δ 1.01–1.12 (m, 12 H), 2.24 (s, 3H), 2.36 (t, 1H, $J$ = 3.8 Hz), 2.49–2.54 (m, 3H), 3.23–3.27 (m, 2H), 3.50–3.70 (m, 10H), 4.14–4.18 (m, 1H), 4.57–4.72 (m, 1H), 6.77 (d, 4H, $J$ = 8.9 Hz), 6.81 (d, 1H, $J$ = 3.2 Hz), 7.10–7.36 (m, 10H), 8.43 (s, 1H), 9.37 (brs, 1H). $^{13}$C NMR ($CDCl_3$) δ 20.1, 20.2, 20.3, 20.4, 22.8, 24.5, 24.6, 24.7, 29.7, 39.8, 43.1, 43.3, 55.2, 58.1, 58.2, 58.4, 58.5, 63.4, 63.6, 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. $^{31}$P NMR ($CDCl_3$) δ 149.2, 149.4. HRMS (ESI) m/z (M + H) calcd for $C_{43}H_{51}N_6O_7P^+$: 795.3635; found: 795.3621.

### Synthesis of 8-aza-5′-*O*-[bis(4-methoxyphenyl) phenylmethyl]-7-deaza-2′-deoxyadenosine 12

Compound **11** (6.0 g, 5.1 mmol) was dissolved in saturated $NH_3$/MeOH (60 ml). The reaction vessel was sealed. After being stirred at 60°C for 3 days, the mixture was cooled and evaporated under reduced pressure. The residue was partitioned between ethyl acetate (50 ml) and water (50 ml). The aqueous phase was collected and evaporated under reduced pressure. The residue was rendered anhydrous by repeated coevaporation with dry pyridine (×3) and dissolved in dry pyridine (40 ml). To the mixture was successively added $Et_3N$ (710 µl, 5.1 mmol), $CHCl_2COOH$ (420 µl, 5.1 mmol) and DMTrCl (2.6 g, 7.7 mmol). After the mixture was stirred at room temperature for 4 h, water (5 ml) was added to the mixture. After being stirred at room temperature for 5 min, the mixture was partitioned between $CHCl_3$ (200 ml) and brine (150 ml). The organic phase was collected, dried over $Na_2SO_4$, 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 **12**. 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 12 (2.2 g, 78%). $^1$H 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). $^{13}$C NMR ($CDCl_3$) δ 21.2, 38.0, 54.9, 64.2, 72.3, 77.2, 84.0, 85.6, 86.0, 100.9, 112.8, 123.8, 125.1, 126.5, 127.5, 127.6, 127.7, 128.0, 128.0, 129.0, 129.9, 132.0, 135.9, 136.3, 144.7, 149.0, 153.8, 155.3, 157.4, 158.1, 158.2. δ 158.3. 149.2, 149.4. HRMS (ESI) m/z (M + Na) calcd for $C_{31}H_{31}N_5NaO_5^+$: 576.2223; found: 576.2275.

### Synthesis of 6-*N*-acetyl-8-aza-5′-*O*-[bis(4-methoxyphenyl) phenylmethyl]-7-deaza-2′-deoxyadenosine 13

Compound **12** (2.0 g, 3.6 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine (3 ml×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 $Na_2SO_4$, 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%). $^1$H NMR ($CDCl_3$) δ 2.30 (s, 3H), 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). $^{13}$C NMR ($CDCl_3$) δ 24.6, 38.1, 55.2, 55.3, 64.2, 73.2, 77.2, 84.0, 85.3, 86.3, 104.2, 113.0, 113.1, 126.6, 127.6, 127.7, 128.0, 129.0, 129.9, 135.9, 137.3, 139.3, 144.6, 151.0, 154.4, 155.3, 158.2. HRMS (ESI) m/z (M + Na) calcd for $C_{33}H_{33}N_5NaO_6^+$: 618.2329; found: 618.2329.

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

Compound **13** (1.2 g, 2.0 mmol) was rendered anhydrous by repeated coevaporation with dry $CH_3CN$ (3ml×3) and

dissolved in dry $CH_2Cl_2$ (20 ml). To the mixture was added diisopropylamine (170 µl, 1.2 mmol), 2-cyanoethoxy[*N*,*N*-di(1-metylethyl)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 $Na_2SO_4$, 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% $Et_3N$ and then $CHCl_3$-MeOH (100 : 0–97 : 3, v/v) containing 1% $Et_3N$ 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, 129.9, 135.8, 135.9, 137.2, 144.6, 151.3, 154.2, 155.2, 158.0, 158.1, 168.3. $^{31}$P NMR ($CDCl_3$) δ 149.2, 149.4. HRMS (ESI) m/z (M + Na) calcd for $C_{42}H_{50}N_7NaO_7P^+$: 818.3407; found: 818.3408.

### 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 $CH_3CN$ (500 µl) at room temperature for 1 min. Then, the mixture containing oligoDNAs was released from the resin by treatment with a solution of $Et_3N{\cdot}3HF$ (0.20 M) and $Et_3N$ (0.40 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*: $ac^6A$) MALDI-TOF Mass (M + H) calcd for $C_{128}H_{164}N_{45}O_{76}P_{12}^+$ 3920.61, found 3919.16.

Oligonucleotide **6**: TACCTAA*ATCCAT (A*: $bz^6A$) MALDI-TOF Mass (M + H) calcd for $C_{133}H_{166}N_{45}O_{76}P_{12}^+$: 3982.68; found: 3987.16.

Oligonucleotide **15**: TACCTAA*ATCCAT (A*: $ox^8ac^6A$) MALDI-TOF Mass (M + H) calcd for $C_{128}H_{164}N_{45}O_{77}P_{12}^+$: 3936.61; found: 3933.83.

Oligonucleotide **16**: TACCTAA*ATCCAT (A*: $ac^6c^7A$) MALDI-TOF Mass (M + H) calcd for $C_{129}H_{165}N_{44}O_{76}P_{12}^+$: 3919.62; found: 3919.56.

Oligonucleotide **17**: TACCTAA*ATCCAT (A*: $ac^6az^8c^7A$) MALDI-TOF Mass (M + H) calcd for $C_{128}H_{164}N_{45}O_{76}P_{12}^+$: 3920.61; found: 3921.27

Oligonucleotide **18**: TACCTA*A*A*TCCAT (A*: $ac^6az^8c^7A$) MALDI-TOF Mass (M + H) calcd for $C_{132}H_{168}N_{45}O_{78}P_{12}^+$: 4002.74; found: 3997.33

Oligonucleotide **19**: TA*CCTAA*ATCCA*T (A*: $ac^6az^8c^7A$) MALDI-TOF Mass (M + H) calcd for $C_{132}H_{168}N_{45}O_{78}P_{12}^+$: 4002.74; found: 3995.09

PDP **20**: TA*C*C*TA*A*A*TC*C*A*T (A*: $ac^6az^8c^7A$, C*: $ac^4C$), MALDI-TOF Mass (M + H) calcd for $C_{144}H_{180}N_{45}O_{84}P_{12}^+$: 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 for 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$^{TM}$, 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 oligodeoxyribonucleotide **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 $CH_3CN$ (500 µl) at room temperature for 1 min. Then, the oligomer was released from the resin by treatment with a solution of $Et_3N{\cdot}3HF$ (0.2 M) and $Et_3N$ (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.

PDP **23**: 5′-$H_2N$-$(CH_2)_6$-pTTTTT-GC*C*TC*C*GG TTC*A*T-3′; MALDI-TOF Mass (M + H) calcd for $C_{193}H_{253}N_{54}O_{123}P_{12}^+$: 5854.89; found: 5854.23.

PDP **25**: 5′-$H_2N$-$(CH_2)_6$-pTTTTT-GC*C*TC*TGGTT C*A*T-3′; MALDI-TOF Mass (M + H) calcd for $C_{192}H_{252}N_{53}O_{123}P_{18}^+$: 5827.86; found: 5832.28.

PDP **26**: 5′-$H_2N$-$(CH_2)_6$-pTTTTT-GC*C*TC*C*A*GT TC*A*T-3′; MALDI-TOF Mass (M + H) calcd for $C_{195}H_{255}N_{54}O_{123}P_{18}^+$: 5880.92; found: 5882.44,

On other hand, unmodified oligonucleotide **21**–**23** were purchased from Sigma Genosys.

Oligonucleotide **21**: 5′-$H_2N$-$(CH_2)_6$-pTTTTT-GCCTCC GGTTCAT-3′

Oligonucleotide **22**: 5′-$H_2N$-$(CH_2)_6$-pTTTTT-GCCTCT GGTTCAT-3′

Oligonucleotide **23**: 5′-$H_2N$-$(CH_2)_6$-pTTTTT-GCCTCC AGTTCAT-3′

These oligonucleotides **21**–**26** were spotted and immobilized on activated ester-coated glass plates by Kaken Geneqs Inc.

### 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/cm$^2$, 16-hydroxyhexadodecanoyl 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/$CH_3CN$ (500 μl) for 1 min.

Sequence of PDPs **28**–**30**

**28**: 5′-d(GA*TA*C*A*TTGA*C*C*TTTTTTT)
**29**: 5′-d(GA*TA*C*C*TTGA*C*C*TTTTTTT)
**30**: 5′-d(GA*TA*C*A*C*TGA*C*C*TTTTTTT)

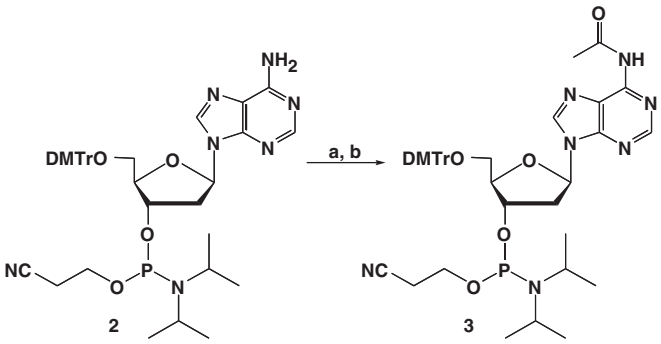



**Figure 2.** Synthesis of ac$^6$A phosphoramidite unit **3**. Reagents and conditions: (**a**) AcCl, $EtNiPr_2$, THF, rt,1 h; (**b**) pyridine–$NH_3$ aq. (2 : 1, v/v), rt, 10 min.

### Match/mismatch discrimination by use of PDPs on CPG plates

A porous glass plate (10 mg) having a PDP was added to a 2 μM solution of target oligoDNA having a fluorescence group in 100 mM sodium phospate buffer (270 μl, 1 M NaCl, pH 7.0). The mixture was incubated at 60°C for 13 h. Next, washing of the glass plate was performed with 100 mM sodium phospate buffer (500 μl, 100 mM NaCl, pH 7.0) at 60°C for 1 h. After drying of the CPG plate, the fluorescence strength of the plate was measured by fluorescence microscopy.

## 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 (ac$^6$A) and 6-*N*-benzoyladenine (bz$^6$A), to examine their base pairing properties. The phosphoramidite unit **3** of ac$^6$A 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 bz$^6$A 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 $CH_3CN$ for 1 min and the successive release of the 5′-terminal DMTr group was carried out by treatment with 3% trichloroacetic acid in $CH_2Cl_2$. Finally, the modified DNA oligomers **5** and **6** were released from the resin by treatment with 0.2 M $Et_3N$-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.

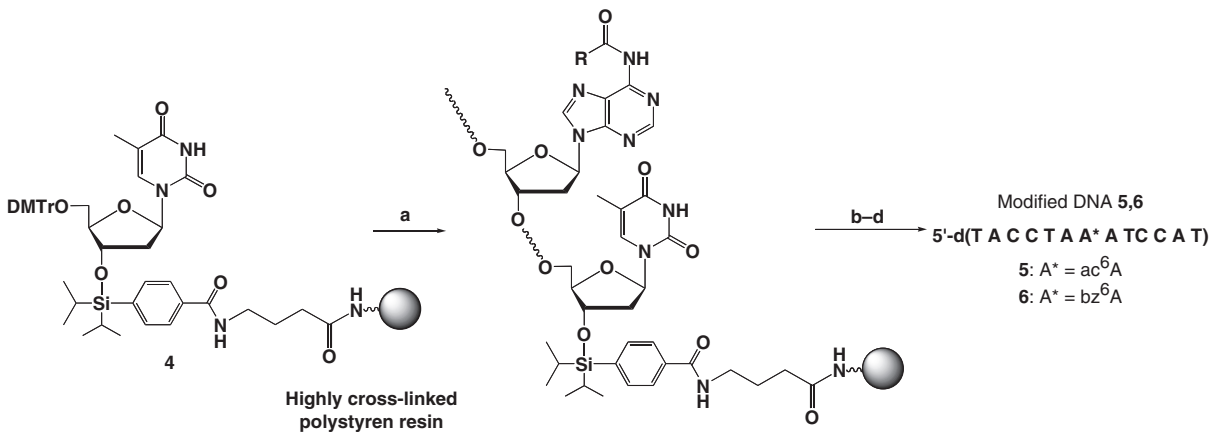



**Figure 3.** Synthesis of modified DNA **5** and **6**. Reagents and conditions: (**a**) protocol of the activated phosphite method on ABI392 DNA synthesizer; (**b**) DBU, $CH_3CN$, rt, 1 min; (**c**) 3% $CCl_3COOH$, $CH_2Cl_2$, rt, 1 min; (**d**) $EtNH_3$-3HF, THF, rt, 4 h.

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 $ac^6A$ and $bz^6A$ decreased the stability of the resulting duplex [$T_m$: 40.3°C ($ac^6A$), 37.1°C ($bz^6A$) 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 ($ac^6A$), −2.5°C ($bz^6A$) 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, $ac^6A$ and $bz^6A$

| | |
|---|---|
| Complementary DNA | **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 A T C C A T)** |
| Modified DNA **5–6** | **5′-d(T A C C T A A*A T C C A T)** |

| | Unmodified DNA | | Modified DNA 5 A* = $ac^6A$ | | Modified DNA 6 A* = $bz^6A$ | |
|---|---|---|---|---|---|---|
| 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 | 44.5 | – | 40.3 | – | 37.1 | – |
| G | 31.8 | −12.7 | 33.6 | −6.7 | 34.6 | −2.5 |
| C | 26.0 | −18.5 | 25.8 | −14.5 | 25.8 | −11.3 |
| A | 28.8 | −15.7 | 28.1 | −12.2 | 29.1 | −8.0 |

[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.

### Synthesis of DNA oligomers having *N*-acylated 8-oxoadenine and 7-deazaadenine derivatives

Next, we designed three adenine mimic bases, i.e. 6-*N*-acetyl-8-oxoadenine ($ac^6ox^8A$), 6-*N*–acetyl-7-deazaadenine ($ac^6c^7A$), and 6-*N*-acetyl-8-aza-7-deazaadenine ($ac^6az^8c^7A$), to fix the acyl group in a manner that it does not interfere with the Watson–Crick base pairing formation. The *ab initio* MO calculation of these adenine mimics at the MP2//HF/6-31G* level indicated that the structures having an intramolecular hydrogen bond via the 7-membered ring between the acetyl group and the C–H bond at the 7-position are more stable than the other structures without the hydrogen bond, as shown in Figure 4. The distances of these hydrogen bonds are 2.04, 2.25 and 2.27 Å. This computer modeling indicated that these modified bases can exist in such structures so that the W–C base pair site becomes available.

We prepared the phosphoramidite derivatives of these adenine mimics to incorporate them into DNA oligomers. The phosphoramidite unit of $ac^6ox^8A$ was synthesized according to Essigmann's procedure (33). The synthesis of the phosphoramidite building block **10** of $ac^6c^7A$ is shown in Figure 5. First, *O*-selective tritylation of compound **7** (34) with DMTrCl in the presence of $Et_3N$ and $CHCl_2COOH$ (35) gave compound **8** in 68% yield. Treatment of **8** with AcCl in the presence of TMSCl followed by hydrolysis gave the *N*-acetylated compound **9** in 95% yield. Finally, phosphitylation of **9** with $ClP(OCE)N(iPr)_2$ afforded the desired phosphoramidite **10** of $ac^6c^7A$ in 91% yield.

For the synthesis of the phosphoramidite derivative **14** of $ac^6az^8c^7A$, *O*-selective tritylation was carried out to give compound **12** after treatment of compound **11** (36)

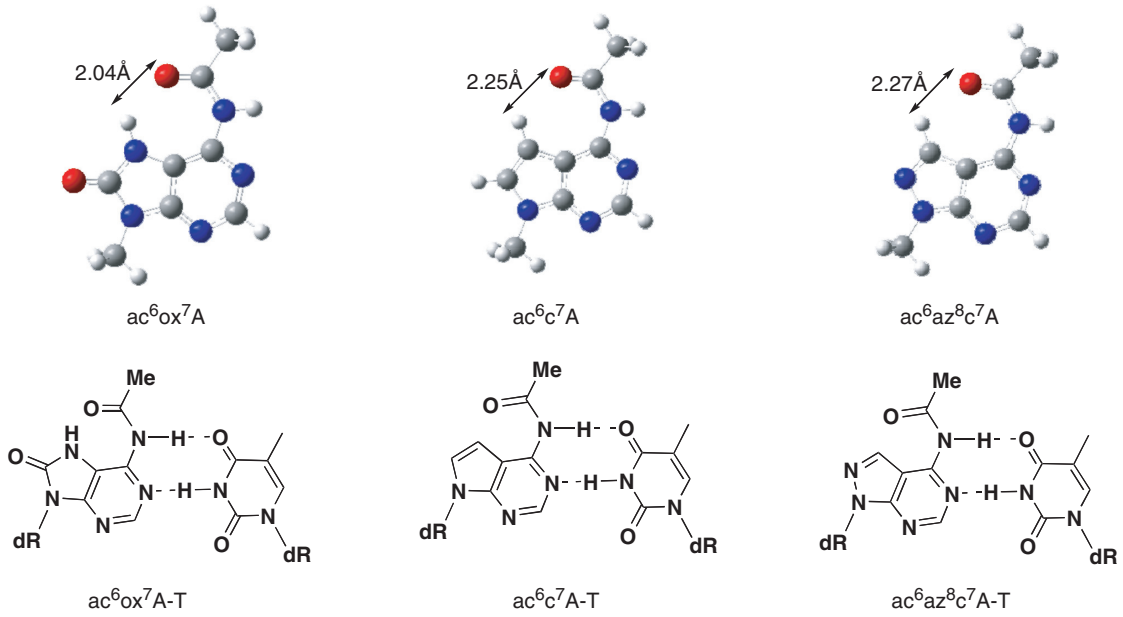



**Figure 4.** The most stable structures of $ac^6ox^8A$, $ac^6c^7A$ and $ac^6az^8c^7A$ by *ab initio* MO calculation.

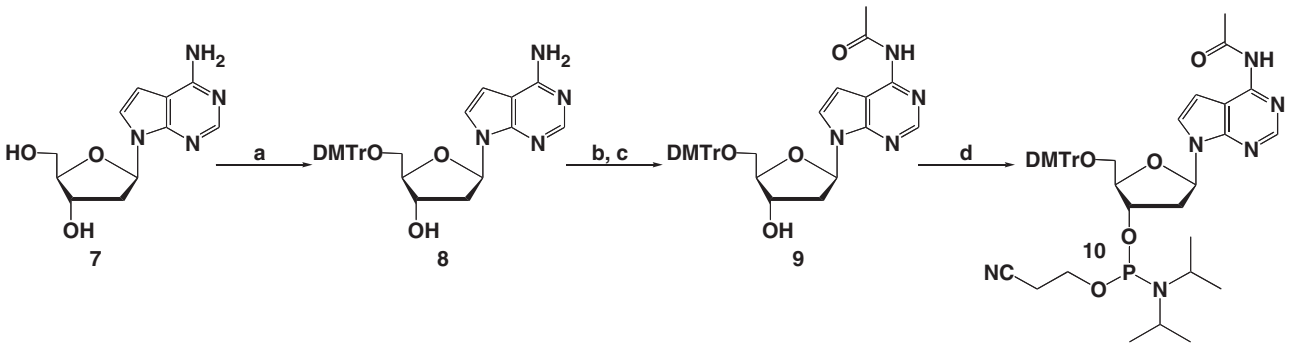



**Figure 5.** Synthesis of the $ac^6c^7A$ phosphoramidite unit **10**. Reagents and conditions: (**a**) DMTrCl, $Et_3N$, $CHCl_2COOH$, pyridine, rt, 4 h; (**b**) TMSCl, pyridine, rt, 30 min, then AcCl, rt, 4 h; (**c**) pyridine-concentrated $NH_3$ aq. (2 : 1, v/v), rt, 10 min; (**d**) $ClP(OCE)NiPr_2$, $EtNiPr_2$, $CH_2Cl_2$, rt, 30 min.

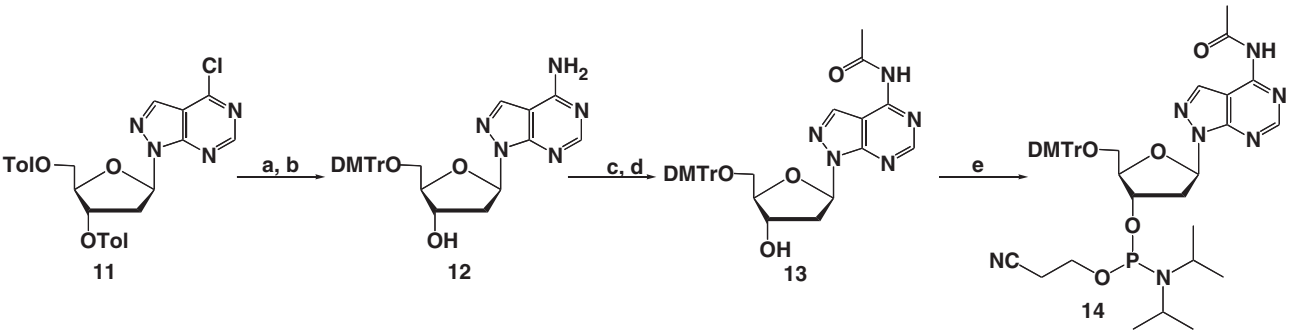



**Figure 6.** Synthesis of $ac^6az^8c^7A$ phosphoramidite unit **14**. Reagents and conditions: (**a**) $NH_3$, MeOH, 60°C, 3 d; (**b**) DMTrCl, $Et_3N$, $CHCl_2COOH$, pyridine, rt, 4 h; (**c**) TMSCl, pyridine, rt, 30 min, then AcCl, rt, 4 h; (**d**) pyridine–concentrated $NH_3$ aq. (2:1, v/v), rt, 10 min; (**e**) $CEOP(NiPr_2)_2$, $HNiPr_2$, 1*H*-tetrazole, $CH_2Cl_2$, rt, 12 h.

with saturated $NH_3$/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 $CEOP(NiPr_2)_2$ to the target phosphoramidite unit **14** in 72% yield.

Based on the X-ray structural analysis of $ac^4C$, Parthasarathy and coworkers reported that the distance between the carbonyl 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 $ac^4C$ 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 $ac^6c^7A$ and $ac^6az^8c^7A$ was smaller (only 0.3–0.4 p.p.m.) than that of $ac^4C$. As far as the possibility of the intramolecular hydrogen bonds suggested by the *ab initio* MO calculation of $ac^6c^7A$ and $ac^6az^8c^7A$ is concerned, further studies are needed.

The three modified DNA oligomers **15**–**17** incorporating $ac^6ox^8A$, $ac^6c^7A$ or $ac^6az^8c^7A$ were synthesized and isolated in 44, 25 and 24% yields, respectively, using the activated phosphite method without base protection described above.

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

The hybridization affinity and base recognition ability of the DNA 13mers containing $ac^6ox^7A$ (**15**), $ac^6c^7A$ (**16**) and $ac^6az^8c^7A$ (**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 $ac^6ox^8A$ were lower than those of the unmodified DNA 13mer, as shown in Table 2 ($T_m$ 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 $ac^6c^7A$ ($T_m$ 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 $ac^6c^7A$ as the proton donor might decrease compared with the unmodified adenine because the acidity of the amide proton of $ac^6c^7A$ 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 $ac^6az^8c^7A$ were similar to those of unmodified DNA 13mer ($T_m$ of X = T: 44.7°C versus 44.5°C, base recognition ability: −13.5°C versus −12.7°C). The $T_m$ values of DNA 13mer **18** containing three consecutive $ac^6az^8c^7As$ and **19** containing three discontinuous

**Table 2.** $T_m$ values for DNA 13mer duplexes containing A, $ac^6ox^8A$, $ac^6$ $c^7A$ and $ac^6az^8c^7A$

| | |
|---|---|
| Complementary DNA | **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 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)** |
| Modified DNA **18** | **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)** |

| | Unmodified DNA | | Modified DNA **15** A* = $ac^6ox^8A$ | | Modified DNA **16** A* = $ac^6c^7A$ | | Modified DNA **17** A* = $ac^6az^8c^7A$ | | Modified DNA **18** A* = $ac^6az^8c^7A$ | | Modified DNA **19** A* = $ac^6az^8c^7A$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 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] | $T_m$ (°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 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 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′** |
| Complementary RNA | **r(A U G G A U X U A G G U A)-5′** |
| Unmodified DNA | **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)** A* = $ac^6az^8c^7A$ C* = $ac^4C$ |

| | 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)[c] | $\Delta T$m(°C)[b] | $T_m$ (°C)[c] | $\Delta T$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 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 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 10 mM sodium phosphate (pH 7.0), 100 mM NaCl, 0.1 mM EDTA and 2 μM duplex.

$ac^6az^8c^7A$s 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 $ac^6az^8c^7A$s 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 $ac^6az^8c^7A$ 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 $ac^6az^8c^7A$s and $ac^4C$s, which were substituted for all A and C bases in these sequences, were synthesized using four phosphoramidite building blocks involving the *N*-unprotected dG and T phosphoramidite units. Previously, we reported that the hybridization affinity of oligonucleotides incorporating an $ac^4C$ 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 $ac^6az^8c^7A$ 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: 51.3°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

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

| | SNPs site |
|---|---|
| DNA 20mer **27** having a wild-type sequence of p53 | 5′-Cy3-d(G G C A T G A A C C G G A G G C C C A T) |
| wild-type unmodified probe **21** | 3′-d(T A C T T G G C C T C C G)-$T_5$ |
| HSC-4 unmodified probe **22** | 3′-d(T A C T T G G T C T C C G)-$T_5$ |
| Ca9-22 unmodified probe **23** | 3′-d(T A C T T G A C C T C C G)-$T_5$ |
| wild-type PDP **24** | 3′-d(TA*C*T T G G C* C*T C*C*G)-$T_5$ |
| HSC-4 PDP **25** | 3′-d(TA*C*T T G G T C*T C*C*G)-$T_5$ |
| Ca9-22 PDP **26** | 3′-d(TA*C*T T G A*C* C*T C*C*G)-$T_5$ |

A* = $ac^6az^8c^7A$ C* = $ac^4C$ slide glass

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**.

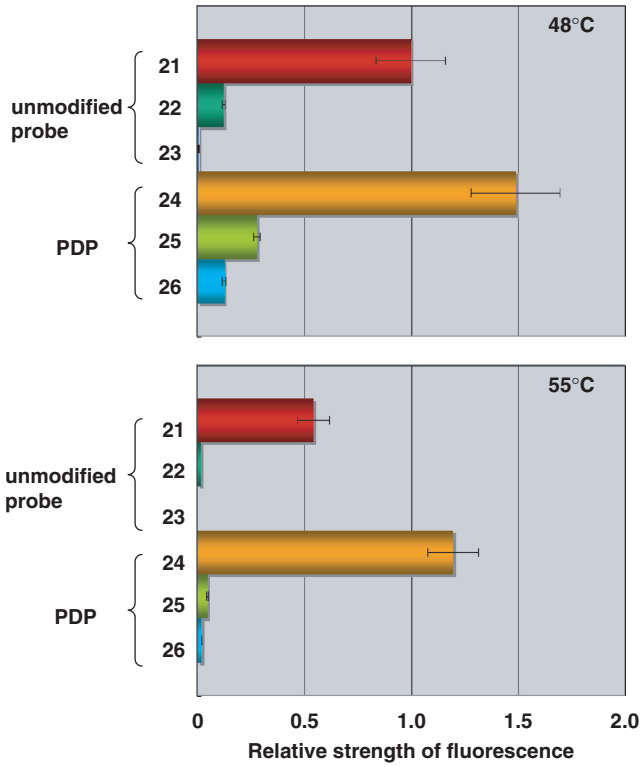
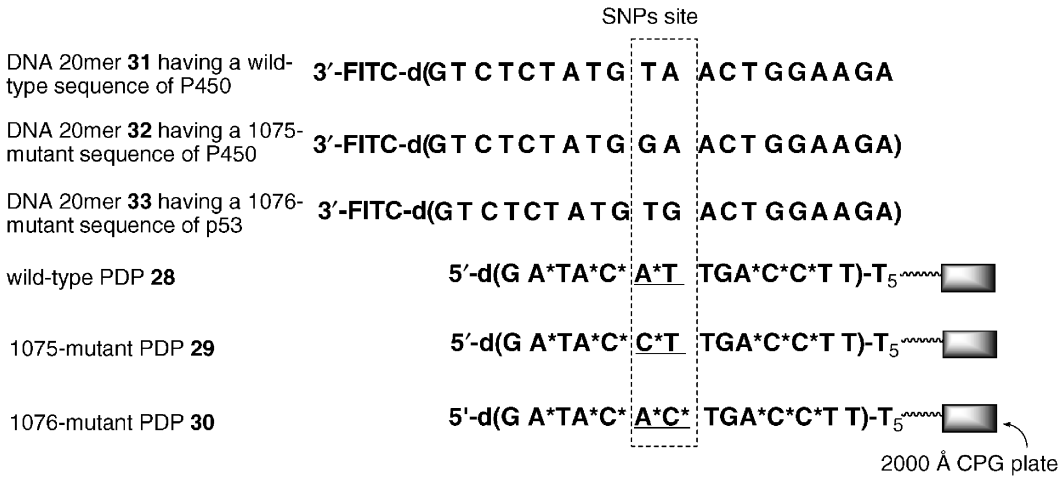



**Figure 7.** Match/mismatch discrimination of target DNA **27** by unmodified probes **21**–**23** and PDPs **24**–**26**. The fluorescence of Cy3 was measured using a fluorescence imager (Laser power 40, PM Gain 70). The hybridization was performed for 16 h at (**a**) 48°C and (**b**) 55°C on a slide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

**Table 5.** Sequences of 3′-FITC -labeled targets **31**–**33** and PDPs **28**–**30**




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. The intensity of the perfectly matched duplex, wild-type probe **21**/sample **27**, was 2.2 times smaller than that of PDP **24**/sample **27** at 55°C, although the base recognition of the unmodified probe was 1.2 times higher than that of PDP at 55°C. These results indicated that on-chip hybridization using PDP could increase sequence selectivity without a decrease in fluorescence intensity compared with that of the unmodified probe.

Similarly, we also examined the probability of failure of detection of the wild-type target **27** by PDP **26** having a sequence complementary to the Ca-9 mutant. As a result, it was found that the selectivity of the PDP was sufficiently high to distinguish the matched base pair from the C–A mismatched base pair (the intensity was 60 times smaller than that of the perfectly matched base pair), although the base recognition of unmodified probe was higher than that of the PDP 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 $CH_3CN$ (1 min) and 3% TCA in $CH_3CN$ (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**.

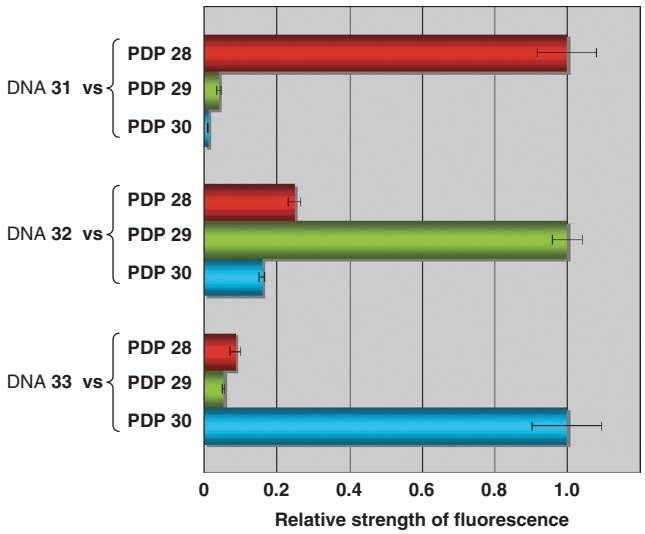



**Figure 8.** Match/mismatch discrimination using PDPs. The hybridization was performed for 12 h at 60°C on CPG plates. The fluorescence of FITC was measured using fluorescence microscopy.

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 $ac^4C$ 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 ($ac^6az^8c^7A$), 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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## REFERENCES

1. Stec,W.J., Zon,G. and Egan,W. (1984) Automated solid-phase synthesis, separation, and stereochemistry of phosphorothioate analogs of oligodeoxyribonucleotides. *J. Am. Chem. Soc.*, **106**, 6077–6079.
2. Miller,P.S., Agris,C.H., Murakami,A., Reddy,M.P., Spitz,S.A. and Ts'o,P. O. (1983) Preparation of oligodeoxyribonucleoside methylphosphonates on a polystyrene support. *Nucleic Acids Res.*, **11**, 6225–6242.
3. Inoue,H., Hayase,Y., Imura,A., Iwai,S., Miura,K. and Ohtsuka,E. (1987) Synthesis and hybridization studies on two complementary nona(2′-O-methyl)ribonucleotides. *Nucleic Acids Res.*, **15**, 6131–6148.
4. Nielsen,P.E., Egholm,M., Berg,R.H. and Buchardt,O. (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science*, **254**, 1497–1500.
5. Herrlein,M.K., Nelson,J.S. and Letsinger,R.L. (1995) A covalent lock for self-assembled oligonucleotide conjugates. *J. Am. Chem. Soc.*, **117**, 10151–10152.
6. Gartner,Z.J., Kanan,M.W. and Liu,D.R. (2002) Multistep small-molecule synthesis programmed by DNA templates. *J. Am. Chem. Soc.*, **124**, 10304–10306.
7. Stein,C.A. & Krieg,A.M. (eds) (1998) *Applied Antisense Oligonucleotide Technology*, Wiley-Liss (New York).
8. Crooke,S.T. (ed.) (2001) *Antisense Drug Technology-Principles, Strategies and Application*, Marcel Dekker (New York).
9. Gewirtz,A. M. (ed.) (2004) *Nucleic Acid Therapeutics in Cancer*, Hamana Press (Totowa, N. J.).
10. Manoharan,M. (2004) RNA interference and chemically modified small interfering RNAs. *Curr. Opin. Chem. Biol.*, **8**, 570–579.
11. Amarzguioui,M., Holen,T., Babarie,E. and Prydz,H. (2003) Tolerance for mutations and chemical modifications in a siRNA. *Nucleic Acids Res.*, **31**, 589–595.
12. Schena,M., Shalon,D., Davis,R.W. and Brown,P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*, **270**, 467–470.
13. Lockhart,D.J., Dong,H., Byrne,M.C., Follettie,M.T., Gallo,M.V., Chee,M.S., Mittmann,M., Wang,C., Kobayashi,M. *et al.* (1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotechnol.*, **14**, 1675–1680.
14. Kwiatkowski,R.W., Lyamichev,V., Arruda,M. and Neri,B. (1999) Clinical, genetic, and pharmacogenetic applications of the invader assay. *Mol. Diagn.*, **4**, 353–364.
15. Yamane,,A. (2002) MagiProbe: a novel fluorescence quenching-based oligonucleotide probe carrying a fluorophore and an intercalator. *Nucleic Acids Res.*, **30**, e97.
16. Nakatani,K., Sando,S. and Saito,I. (2001) Scanning of guanine-guanine mismatches in DNA by synthetic ligands using surface plasmon resonance. *Nat. Biotechnol.*, **19**, 51–55.

17. Kerman,K., Saito,M., Morita,Y., Takamura,Y., Ozsoz,M. and Tamiya,E. (2004) Electrochemical coding of single-nucleotide polymorphisms by monobase-modified gold nanoparticles. *Anal. Chem.*, **76**, 1877–1884.
18. Beaucage,S.L. and Caruthers,M.H. (1981) Deoxynucleoside phosphoramidites-A new class of key intermediates for deoxypolynucleotide synthesis. *Tetrahedron Lett.*, **22**, 1859–1862.
19. Parey,N., Baraguey,C., Vasseur,J.-J. and Debart,F. (2006) First evaluation of acyloxymethyl or acylthiomethyl groups as biolabile 2′-O-protections of RNA. *Org. Lett.*, **8**, 3869–3872.
20. Parthasarathy,R., Ginell,S.L., De,N.C. and Chheda,G.B. (1978) Conformation of N4-acetylcytidine, a modified nucleoside of tRNA, and stereochemistry of codon-anticodon interaction. *Biochem. Biophys. Res. Commun.*, **83**, 657–663.
21. Wada,T., Kobori,A., Kawahara,S. and Sekine,M. (2001) Synthesis and hybridization ability of oligodeoxyribonucleotides incorporating N-acyldeoxycytidine derivatives. *Eur. J. Org. Chem.*, **24**, 4583–4593.
22. Ohkubo,A., Sakamoto,K., Miyata,K., Taguchi,H., Seio,K. and Sekine,M. (2005) Convenient synthesis of *N*-unprotected deoxynucleoside 3′-phosphoramidite building blocks by selective deacylation of N-acylated species and their facile conversion to other *N*-functionalized derivatives. *Org. Lett.*, **7**, 5389–5392.
23. LeProust,E., Zhang,H., Yu,P., Zhou,X. and Gao,X. (2001) Characterization of oligodeoxyribonucleotide synthesis on glass plates. *Nucleic Acids Res.*, **29**, 2171–2180.
24. Ohkubo,A., Seio,K. and Sekine,M. (2007) A new hydrophobic linker effective for the in situ synthesis of DNA-CPG conjugates as tools for SNP analysis. *Tetrahedron Lett.*, **48**, 5147–5150.
25. Vainrub,A. and Pettitt,B.M. (2003) Sensitive quantitative nucleic acid detection using oligonucleotide microarrays. *J. Am. Chem. Soc.*, **125**, 7798–7799.
26. Gryaznov,S.M. and Letsinger,R.L. (1991) Synthesis of oligonucleotides via monomers with unprotected bases. *J. Am. Chem. Soc.*, **113**, 5876–5877.
27. Ohkubo,A., Seio,K. and Sekine,M. (2004) A new strategy for the synthesis of oligodeoxynucleotides directed towards perfect O-selective internucleotidic bond formation without base protection. *Tetrahedron Lett.*, **45**, 363–366.
28. Ohkubo,A., Seio,K. and Sekine,M. (2004) O-selectivity and utility of phosphorylation mediated by phosphite triester intermediates in the *N*-unprotected phosphoramidite method. *J. Am. Chem. Soc.*, **126**, 10884–10896.
29. Kobori,A., Miyata,K., Ushioda,M., Seio,K. and Sekine,M. (2002) A new silyl ether-type linker useful for the automated synthesis of oligonucleotides having base-labile protective groups. *Chem. Lett.*, **1**, 16–17.
30. Tsukahara,T. and Nagasawa,H. (2004) Probe-on-carriers for oligonucleotide microarrays (DNA chips). *Sci. Tech. Adv. Mat.*, **5**, 359–362.
31. Hayakawa,Y., Kataoka,M. and Noyori,R. (1996) Benzimidazolium triflate as an efficient promoter for nucleotide synthesis via the phosphoramidite method. *J. Org. Chem.*, **61**, 7996–7997.
32. McCollum,C. and Andrus,A. (1991) An optimized polystyrene support for rapid, efficient oligonucleotide synthesis. *Tetrahedron Lett.*, **32**, 4069–4072.
33. Wood,M.L., Esteve,A., Morningstar,M.L., Kuziemko,G.M. and Essigmann,J.M. (1992) Genetic effects of oxidative DNA damage: comparative mutagenesis of 7,8-dihydro-8-oxoguanine and 7,8-dihydro-8-oxoadenine in Escherichia coli. *Nucleic Acids Res.*, **20**, 6023–6032.
34. Seal,F. and Kehne,A. (1985) Oligomers with alternating thymidine and 2′-deoxytubercidin: duplex stabilization by a 7-deazapurine base. *Biohemistry*, **24**, 7556–7561.
35. Hayakawa,Y. and Kataoka,M. (1998) Facile synthesis of oligodeoxyribonucleotides via the phosphoramidite method without nucleoside base protection. *J. Am. Chem. Soc.*, **120**, 12395–12401.
36. Seela,K. and Kaiser,K. (1988) 8-Aza-7-deazaadenine N8- and N8-(b-D-2′-deoxyribofuranosides): building blocks for automated DNA synthesis and properties of oligodeoxyribonucleotides. *Helv. Chem. Acta.*, **71**, 1813–1823.
37. Wada,T., Kobori,A., Kawahara,S. and Sekine,M. (1998) Synthesis and properties of oligodeoxyribonucleotides containing 4-*N*-acetylcytosine bases. *Tetrahedron Lett.*, **39**, 6907–6910.
38. Jia,L.-Q., Osada,M., Ishioka,C., Gamo,M., Ikawa,S., Suzuki,T., Shimodaira,H., Niitani,T., Kudo,T. *et al.* (1997) Screening the p53 status of human cell lines using a yeast functional assay. *Mol. Carcinogenesis*, **19**, 243–253.
39. Imai,J., Ieiri,I., Mamiya,K., Miyahara,S., Fruumi,H., Nanba,E., Yamane,M., Fukumaki,Y., Ninomiya,H. *et al.* (2000) Polymorphism of the cytochrome P450 (CYP) 2C9 gene in Japanese epileptic patients: genetic analysis of the CYP2C9 locus. *Pharmacogenetics*, **10**, 85–89.
40. Prive,G.G., Heinemann,U., Chandrasegaran,S., Kan,L.-S., Kopka,M.L. and Dickerson,R.E. (1987) Helix geometry, hydration, and G.A mismatch un a B-DNA decamer. *Science*, **238**, 498–504.
41. Chou,S.-H, Zhu,L. and Reid,B.R. (1997) Sheared purine-purine pairing in biology. *J. Mol. Biol.*, **267**, 1055–1067.