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Synthesis of a dA-dT Base Pair Analogue and Its Effects on DNA-Ligand Binding[1]

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Two nucleoside derivatives containing the base analogues 3-deazaadenine and 3-methyl-2-pyridone have been prepared as analogues of dA and dT, respectively. After conversion into the appropriately protected phosphoramidites, DNA sequences were prepared with site-specifically placed analogues. When present in a duplex DNA sequence, the analogues result in the deletion of one or both of the hydrogen bonding functional groups (the N3-nitrogen of dA and the O2-carbonyl of dT) present in the minor groove. Binding by two ligands, 4′,6-diamidine-2-phenyl indole (DAPI) and Hoechst 33258 in the minor groove has been probed using a variety of DNA sequences. These sequences contain a $d(GAATTC)_2$ core with analogue nucleosides substituted for one or more of the dA and dT residues. DAPI bound strongly to any sequence that contained both O2-carbonyls of the central two dT residues. The presence of a dc^3A residue did in some cases enhance binding. With one of the central O2-carbonyls deleted, the binding was noticeably reduced, and with both absent, no significant binding could be detected. Similar although less dramatic results were observed with Hoechst 33258 binding to analogue sequences.

Key Words: DNA; 2-pyridone; 3-deazaadenine; minor groove; base analogue; ligand binding; DAPI; Hoechst 33258.

INTRODUCTION

A variety of small molecule ligands bind within the minor groove structure of dA–dT rich sequences of B-form DNA. Crystallographic structures suggest that in many cases these ligands make hydrogen bonding contacts with the functional groups on the “floor” of the minor groove, namely the N3-nitrogens of the dA residues and the O2-carbonyls of the dT residues. One approach to probe these interactions requires the preparation of appropriate dA and dT analogues from which these two functional groups are absent. Site-specific placement of the analogues within a target sequence will permit the disruption of specific inter-complex hydrogen bonds and allow dissection of the critical binding interactions. The dA analogue, 3-deaza-2′-deoxyadenosine

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(dc^3A) provides a purine-like base residue lacking the functional group of interest, the N3-nitrogen. This derivative has been incorporated into DNA sequences *(1–4)* to permit studies involving base pairing *(5,6)*, ligand binding *(6)*, protein recognition *(3,4)*, and DNA curvature *(7,8)*. This derivative has also been used in enzymatic methods to prepare DNA polymers *(9)*. The dc^3A derivative appears to base pair normally with dT, but the observed T_M values for sequences containing this analogue vary with pH *(6)* and this effect may result from the increased pKa of the heterocycle *(9)*. CD spectra of sequences containing this analogue suggest that they adopt normal B-form helices *(3,7)*.

The dT analogue required is that lacking the O2-carbonyl. The pyrimidine derivative was found to be unacceptable since the tautomeric form of the N3-nitrogen is altered from a hydrogen bond donor to a hydrogen bond acceptor; duplexes containing this analogue are severely destabilized *(10)*. Instead we chose a 2-pyridone heterocycle. 2-Pyridones prefer the *keto* rather than the *enol* tautomeric form based upon UV studies *(11)*. Preliminary studies with this derivative indicate that it will base pair effectively with dA or dc^3A *(12)*.

A variety of ligands including distamycin *(13)*, netropsin *(14)*, Hoechst 33258 *(15)*, and 4′,6-diamidine-2-phenyl indole (DAPI) *(16)* are known to bind within the minor groove of dA–dT rich sequences of DNA. X-ray structures of these DNA ligand complexes suggest that the ligands fit tightly within the minor groove and make contacts with the purine N3-nitrogens and pyrimidine O2-carbonyls on the floor or the groove. In many cases the hydrogen bonding interactions are bifurcated or three centered. To probe the contributions from those interactions involving functional groups in the minor groove we have prepared the sequences containing various dc^3A and dm^32P residues and determined the effects of ligand binding by both DAPI and Hoechst 33258 on the stability of these DNA complexes.

MATERIALS AND METHODS

NMR spectra were obtained on a Varian spectrometer (400 MHz). HRMS, LRMS (FAB) mass spectra were obtained from the Mass Spectrometry Laboratory, School of Chemical Science, University of Illinois (Urbana, IL). Rotary evaporations were performed under reduced pressure with Buchi systems. Thin layer chromatography (TLC) was performed on Silica Gel 60 F254 precoated on aluminum sheets (EM Separations Technology) using typically dichloromethane:methanol 9:1. Anhydrous solvents and starting materials were purchased from the Aldrich Chemical Company and used without further purification unless otherwise specified. UV scans and absorbances were obtained by using a Beckman DU 640 spectrophotometer.

2-Amino-5-iodo-3-methyl-pyridine ***(2)***

A mixture of 2-amino-3-picoline (**1**) (5.5 g, 50 mmol), periodic acid dihydrate (2.28 g, 10 mmol), and iodine (5.1 g, 20 mmol) was heated in a solution of acetic acid (30 ml), water (6 ml) and sulfuric acid (0.9 ml) at 80°C for 4 hours. It was then poured into a solution of 10% $Na_2S_2O_4$ to remove any unreacted iodine and the resulting mixture was extracted with dichloromethane. The organic extract was washed with aqueous 10% NaOH, dried with Na_2SO_4, and concentrated *in vacuo*. The residue

was isolated by column chromatography on silica gel eluting with ether and dichloromethane (1:1) to give a colorless solid of **2** (73%, 8.5 g), R_f: 0.40 [Et_2O: CH_2Cl_2 (1:1)]; UV-Vis λmax = 237 and 296 nm; ^{1}H NMR ($CDCl_3$): δ = 8.08 (1H, s, ArH), 7.52 (1H, s, ArH), 4.54 (2H, br, NH_2), 2.06 (3H, s, CH_3) ppm.

HRMS (EI) calc: 233.9657, found: 233.9654.

5-Iodo-3-methyl-2-pyridone ***(3)***

2-Amino-5-iodo-3-methyl-pyridine **2** (8.3 g, 36.9 mmol) dissolved in 50 ml concentrated sulfuric acid was stirred and cooled and then treated with sodium nitrite (44.3 mmol, 3.05 g). The resulting mixture was stirred at 60°C for 15 min. After cooling, the solution was poured into crushed ice. Boric acid (73.8 mmol, 4.56 g) was then added and the solution was heated quickly to 100°C. The solution was then neutralized with aqueous NH_4OH after it was cooled to ambient temperature. The solvent was evaporated *in vacuo* followed by the addition of methanol, and the suspension was removed by filtration. The filtrate was evaporated to dryness, the residue was isolated by column chromatography on silica gel, eluting with methanol in dichloromethane (5:95). A dark oil **3** (75%, 5.97 g) was obtained. R_f: 0.10 [CH_3OH:CH_2Cl_2(5:95)]. ^{1}H NMR ($CDCl_3$): δ = 7.52 (1H, s, ArH), 7.49 (1H, s, ArH), 2.13 (3H, s, CH_3) ppm.

HRMS (FAB) calc: 235.9578, found: 235.9572.

5-Iodo-3-methyl-2-[2-(4-nitrophenyl)ethoxy]pyridine ***(4)***

5-Iodo-3-methyl-2-pyridone **3** (1 g, 4.63 mmol) was washed with 1,4-dioxane twice and then was dissolved in 50 ml of 1,4-dioxane followed by the addition of triphenylphosphine (17.1 mmol, 4.50 g) and *p*-nitrophenylethanol alcohol (17.1 mmol, 2.86 g). Diethyl azodicarboxylate (DEAD) (17.1 mmol, 2.69 ml) was added to the stirred solution dropwise. The mixture was stirred under argon at ambient temperature for 1.5 h. The solvent was evaporated and the residue was isolated by column chromatography on silica gel eluting with ether and petroleum ether (1:1) to give white solid **4** (71%, 1.11 g). R_f: 0.78 [CH_3OH:CH_2Cl_2 (5:95)]. ^{1}H NMR ($CDCl_3$): δ = 8.11 (2H, d, Ar-H), 8.05 (1H, s, ArH), 7.57 (1H, s, ArH), 7.38 (2H, d, ArH), 4.47 (2H, t, CH_2), 3.12 (2H, t, CH_2), 2.01 (3H, s, CH_3) ppm.

HRMS (FAB) calc: 385.0049, found: 385.0049.

5′-O-(4,4′-Dimethoxytrityl)thymidine ***(5)***

Thymidine (5 g, 20.6 mmol) was dissolved in 40 ml pyridine (after being coevaporated from pyridine twice) and 4,4′-dimethoxytrityl chloride (7.70 g, 22.7 mmol) was added to the solution. The mixture was stirred under argon at ambient temperature for 2 hours. The solvent was evaporated and the residue was purified by column chromatography on silica gel eluting with methanol in dichloromethane (1:99) to give white foam **5** (DMT-thymidine) (85%, 9.5 g). R_f: 0.27 [CH_3OH:CH_2Cl_2(5:95)]; ^{1}H NMR ($CDCl_3$): δ = 7.56 (1H, s, ArH), 7.25, 6.81 (13H, m, ArH), 6.42 (1H, t, $H_{1'}$), 4.58 (1H, m, $H_{3'}$), 4.03 (1H, d, $H_{4'}$), 3.79 (6H, s, OCH_3), 3.40 (2H, m, $H_{5'}$), 2.34 (2H, m, $H_{2'}$), 1.41 (3H, s, CH_3) ppm.

*3′-O-(*t*-Butyldiphenylsilyl)-5′-O-(4,4′-dimethoxytrityl)thymidine* ***(6)***

5′-O-DMT-thymidine **5** (8 g, 14.7 mmol) and imidazole (2.5 g, 36.7 mmol) were dissolved in 30 ml of DMF and *t*-butyl-diphenylsilyl chloride (4.2 ml, 16.2 mmol)

was added dropwise while the solution was cooled externally with ice. The mixture was stirred under argon at room temperature for 12 h, the solvent was evaporated and 300 ml dichloromethane was added. Brine was added to remove the imidazole. The organic layer was collected, evaporated, and the residue was purified by column chromatography on silica gel eluting with ether and dichloromethane (1:1) to give white foam **6** (5′-DMT-3′-*t*BDPS-thymidine) (92%, 10.56 g). R_f: 0.70 [CH_3OH:CH_2Cl_2(5:95)]; ^{1}H NMR ($CDCl_3$): δ = 7.70–6.80 (24H, m, ArH), 6.52 (1H, t, $H_{1'}$), 4.55 (1H, s, $H_{3'}$), 4.08 (1H, s, $H_{4'}$), 3.35 (2H, d, $H_{5'}$), 2.40 2.10 (2H, m, $H_{2'}$), 1.40 (3H, s, CH_3), 1.01(9H, s, *t*-butyl) ppm.

*3′-O-(*t*-butyldiphenylsilyl)thymidine **(7)***

5′-DMT-3′-*t*-BDPS-thymidine **6** (5 g, 6.4 mmol) was added to a solution of 3.6 g *p*-toluenesulfonic acid, 364 ml dichloromethane, and 156 ml methanol, and the mixture was stirred on ice for 30 min, TLC showed reaction to be complete and 1.35 g Na_2CO_3 was added to neutralize the solution. The solvent was evaporated and dichloromethane was added and the suspension was filtered. The filtrate was evaporated, residue was isolated by column chromatography on silica gel eluting with methanol in dichloromethane (1:99) to give a white foam **7** (3′-*t*-BDPS-thymidine) (80%, 2.23 g). R_f: 0.32 [CH_3OH:CH_2Cl_2(5:95)]; ^{1}H NMR ($CDCl_3$): δ = 7.70, 7.42 (10H, m, ArH), 6.28 (1H, t, $H_{1'}$), 4.45 (1H, s, $H_{3'}$), 4.00 (1H, s, $H_{4'}$), 3.45 (2H, d, $H_{5'}$), 2.20 (2H, d, $H_{2'}$), 1.82 (3H, s, CH_3), 1.11 (9H, s, *t*-butyl) ppm.

*1,2-Dehydro-3-*O*-(*t*-butyldiphenylsilyl)-5-hydroxymethyl-furan **(9)***

3′-*t*-BDPS-thymidine **8** (2.14 g, 4.8 mmol) was dissolved in 25 ml of 1,1,1,3,3,3-hexamethydisilazane (HMDS) and ammonium sulfate (0.158 g, 1.2 mmol) was added. The mixture was refluxed under argon for 2 h. After evaporation of the solvent, the residue was dissolved in 22 ml dichloromethane and 30 ml brine was added. The organic layer was collected and evaporated, and to the residue was added a solution of 0.66 g K_2CO_3 (4.8 mmol) in 50 ml methanol and the mixture was stirred on ice for 30 min. The solvent was evaporated and the residue was redissolved in 300 ml dichloromethane to which saturated $NaHCO_3$ solution was added. The organic layer was separated, the solvent was evaporated, and the residue was purified by column chromatography on silica gel eluting with methanol in dichloromethane (5:95) to give light yellow oil **9** (70%, 1.19 g). R_f: 0.36 [CH_3OH:CH_2Cl_2(5:95)]; ^{1}H NMR ($CDCl_3$): δ = 7.73, 7.44 (10H, m, ArH), 6.47(1H, d, $H_{1'}$), 4.95 (1H, d, $H_{2'}$), 4.77 (1H, d, $H_{3'}$), 4.46 (1H, t, $H_{4'}$), 3.22 (2H, m, 5′-H), 1.12 (3H, s, *t*-butyl) ppm.

*2-[2-(4-Nitrophenyl)ethoxy]-5-{(2′*R*)-*cis*-3-[2′,3′-dehydro-3′-(*t*-butyl-diphenylsilyloxy)]-5′-hydroxymethyl-2′-furanyl]}-3-methylpyridine **(10)***

A mixture of bis(dibenzylideneacetone) palladium(0) (120 mg, 0.23 mmol) and 1,3-bis(diphenylphosphino)propane (94.8 mg, 0.23 mmol) in dry acetonitrile was stirred under argon at ambient temperature for 20 min. This mixture was then transferred by syringe to a solution of protected heterocycle (**4**) (710 mg, 2.3 mmol), the sugar (**9**) (820 mg, 2.53 mmol), and tri-*n*-butylamine (2 ml, 0.7 mmol) in 100 ml of dry acetonitrile. The resulting yellow orange solution was stirred under argon at 80°C for 8 h. The reaction mixture was then filtered through celite and the volatiles were

removed by rotary evaporation. The residue was purified by column chromatography (petroleum ether: ether (2:1)) to give 1.02 g (73%) of **10**. R_f: 0.40 [petroleum ether:ether(1:1)]; ^{1}H NMR ($CDCl_3$): δ = 8.18 (2H, d, ArH), 7.82 (2H, d, ArH), 7.74, 7.45 (10H, m, ArH), 5.50 (1H, d, $H_{1'}$), 4.74 (1H, m, $H_{2'}$), 4.51 (2H, t, CH_2), 4.25 (1H, s, $H_{4'}$), 3.86 (2H, m, $H_{5'}$), 3.15 (2H, t, CH_2), 2.02 (3H, s, CH_3), 1.09 (9H, s, *t*-butyl) ppm.

HRMS (FAB) calc: 611.2580, found: 611.2577.

*2-[2-(4-Nitrophenyl)ethoxy]-5-(β-*D-glycero-*pentofuran-3′-ulos-1′*yl)*-3-methylpyridine* ***(11)***

To a solution of compound **10** (146 mg, 0.25 mmol) in THF (5 ml) at 0°C was added acetic acid (0.05 ml, 1 mmol) followed by 0.37 ml of 1.0 M solution of tetra-*n*-butyl-ammonium fluoride in THF. The desilylation reaction was completed in 10 min based on TLC analysis. The volatiles were removed by rotary evaporation, and the residue was purified by column chromatography (ether-dichloromethane, 1:4) to afford 44.8 mg of compound **11** (80%). R_f: 0.28 [ether: CH_2Cl_2(1:1)]; NMR ($CDCl_3$): δ = 8.10 (2H, d, Ar-H), 8.01 (1H, s, ArH), 7.50 (1H, s, ArH), 7.45 (2H, d, ArH), 5.08 (1H, m, $H_{1'}$), 4.59 (2H, t, CH_2), 4.01 (1H, s, $H_{4'}$), 3.98 (2H, s, $H_{5'}$), 3.20 (2H, t, CH_2), 2.64 (2H, d, $H_{2'}$), 2.10 (3H, s, CH_3) ppm.

HRMS (FAB) calc: 373.1401, found: 373.1399.

*2-[2-(4-Nitrophenyl)ethoxy]-5-(1′,2′-dideoxy-β-*D-erythro-*pentofuranosyl)-3-methylpyridine* ***(12)***

To a solution of compound **11** (139 mg, 0.62 mmol) in acetonitrile (5 ml) and acetic acid (4 ml) at 0°C was added sodium triacetoxyborohydride (328 mg, 1.55 mmol). The reaction was complete within 10 minutes based on TLC analysis. Volatiles were then removed, and the residue was purified by column chromatography eluting with methanol-dichloromethane 7.5:92.5 to give compound **12** (77%, 106 mg). R_f: 0.13 [MeOH: CH_2Cl_2(5:95)]; NMR ($CDCl_3$): δ = 8.15 (2H, d, ArH), 7.90 (1H, s, ArH), 7.41(2H, d, ArH), 7.34 (1H, s, ArH), 5.07 (1H, m, $H_{1'}$), 4.54 (2H, t, CH_2), 4.42 (1H, m, $H_{3'}$), 3.98 (1H, s, $H_{4'}$), 3.74 (2H, d, $H_{5'}$), 3.18 (2H, t, CH_2), 2.10 (3H, s, CH_3), 2.08 (2H, d, $H_{2'}$) ppm.

HRMS (FAB) calc: 375.1559, found: 375.1556.

*2-[2-(4-Nitrophenyl)ethyl]-5-[5′-*O*-(4,4′-dimethoxytrityl)-1′,2′-dideoxy-β-*D-erythro-pentofuranosyl)-3-methylpyridine ***(13)***

To compound **12** (72.4 mg, 0.17 mmol) dissolved in 2.3 ml of dry pyridine was added dimethylaminopyridine (41.5 mg, 0.34 mmol) and triethylamine (0.023 ml, 0.17 mmol). 4,4′-Dimethoxytrityl chloride (115.2 mg, 0.34 mmol) was added to this solution and the mixture was stirred under argon at ambient temperature for 48 h. The solvent was then evaporated and the residue was separated by column chromatography on silica gel eluting with TEA: MeOH: CH_2Cl_2 (1:1:98) to give white foam **13** (62%, 66 mg). R_f: 0.38 [CH_3OH: CH_2Cl_2 (5:95)]; NMR ($CDCl_3$): δ = 8.10 (2H, d, ArH), 7.91(1H, s, ArH), 7.45, 7.24, 6.79 (16H, m, ArH), 5.08 (1H, m, $H_{1'}$), 4.53

(2H, t, CH_2), 4.41 (1H, d, $H_{3'}$), 4.00 (1H, m, $H_{4'}$), 3.78 (6H, s, OCH_3), 3.25 (2H, d, $H_{5'}$), 3.15 (2H, t, CH_2), 2.10 (2H, d, $H_{2'}$), 2.02 (3H, s, CH_3) ppm.

HRMS (FAB) calc: 677.2864, found: 677.2863.

*2-[2-(4-Nitrophenyl)ethoxy]-5-[5′-*O*-(4,4′-dimethoxytrityl)-1′,2′-dideoxy-3′-O-[(2-cyanoethoxy)diisopropylaminophosphino]-β-*D*-erythro-pentofuranosyl)-3-methylpyridine **(14)***

Compound **13** (160 mg, 0.256 mmol) was dissolved in 9 ml dry CH_2Cl_2 and followed by the addition of *N,N*-diisopropanylethylamine (DIEA) (0.27 ml, 1.534 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.171 ml, 0.767 mmol) with external cooling by ice. The resulting solution was stirred under argon at ambient temperature for 1.5 h. The reaction was stopped with adding a few drops of water, the solvents were then evaporated and the residue was precipitated by adding a few drops of CH_2Cl_2 and large amount of hexane. This step was performed three times and the compound was dried *in vacuo* to give 160 mg (71%) of **14**. $\mathbf{R}_f$: 0.8 [MeOH:CH_2Cl_2 (5:95)]; ^{31}P NMR ($CDCl_3$): δ = 150.280, 150.233.

DNA Synthesis

The native and modified 12-mers were prepared by solid phase DNA synthesis and deprotected using standard protocols; the only exception being a slightly different methodology for removing the *p*-nitrophenylethyl group from dm^{3}2P. This protecting group was removed as the first step after sequence assembly by treating the solid support with a solution of 40% TEA/pyridine for 2 h, then 0.5 M 1,8-diazabicyclo[5.4.0] undec-7-ene (DBU) in anhydrous pyridine for 8–48 h, and finally by washing with acetonitrile three times. After evaporation of the volatiles, ammonia treatment was performed in the conventional manner.

Purification of the oligonucleotides employed fast flow HPLC (4.6 × 120 mm, reverse phase C18 column, trityl on); 100% A for 1 min, then using a linear gradient 0–50% B over 4.5 min (A: 50 mM triethylammonium acetate; B: 50 mM triethylammonium acetate in 70% acetonitrile, pH 7.0). The DMT protected 12-mers had retention times of about 5.0 min. The collected fractions were reduced in volume and detritylated with 80% aqueous acetic acid (60 min, on ice). The resulting solutions were then desalted (Sephadex G-10), and stored at −20°C.

Nucleoside Analysis

Small amounts of oligonucleotides containing modified bases were digested with snake venom phosphodiesterase and calf intestinal alkaline phosphatase into monomeric units: a 50 μl reaction mixture contained 0.5 A_{260} unit of oligonucleotide was incubated with snake venom phosphodiesterase and alkaline phosphatase before analysis by HPLC.

Thermal Denaturation Studies

Thermal denaturation studies were performed in a solution of 20 mM NaH_2PO_4 pH 7.0 and 200 mM NaCl. Absorbance and temperature values were measured with an AVIV 14DS UV/Visible spectrophotometer equipped with digital temperature control. The temperature of the cell compartment increased in 1.0°C steps (from

10–95°C). When equilibrium was reached, temperature and absorbance data were collected. The T_M values were determined manually from the absorbance vs temperature plots. Ligands when present were adjusted to a ratio of 1:1 relative to the target DNA.

RESULTS AND DISCUSSION

The analogue base pair chosen for this study was composed of two nucleosides. One was 3-deaza-2′-deoxyadenosine (dc^3A, Fig. 1), lacking the N3-nitrogen normally found in the minor groove of a B-form duplex DNA. This derivative has been synthesized and used in a number of other studies *(1–4)*. The choice for the pyrimidine analogue, that lacking the O2-carbonyl, proved to be a more difficult decision. Simply eliminating the O2-carbonyl from the pyrimidine ring system results in a 4-pyrimidinone, but in order for this derivative to maintain ring planarity a double bond is introduced between the N3 nitrogen and C2 carbon. The N3-nitrogen in that derivative no longer functions as a hydrogen bond donor and significant DNA helix destabilization is observed *(10)*, even with a single substitution by this derivative. The necessary analogue was designed by using a C-nucleoside that contained a 3-methyl-2-pyridone heterocycle (dm^32P, Fig. 1). 2-Pyridones prefer the *keto* tautomer and thus result in a derivative with interstrand hydrogen bonding properties with dA (or dc^3A) that should mimic those in native dA–dT base pairs.

Syntheses. As noted above, the dc^3A analogue has been prepared previously *(24)* and converted to an appropriately protected phosphoramidite derivative for incorporation into DNA sequences using standard solid-phase based protcocols. For the dm^32P derivative we chose to prepare a protected version of a 5-iodo-2-pyridone derivative (**4**, Scheme 1) and using a Heck reaction *(17)*, couple the heterocycle to the 1,2-dideoxy glycan (**9**). The glycan itself was prepared from dT with a bulky silyl protecting group on the 3′-hydroxyl (**7**, Scheme 2) and then the requisite double bond was introduced by elimination of the heterocycle (→ **9**) in refluxing HMDS (Scheme 2)—similar to a previously described procedure *(18)*. The bulky 3′-protecting group ensured that the heterocycle was added by a Heck reaction in such a manner that only the β-nucleoside (**10**) resulted (Scheme 1). It was then necessary to remove the silyl protecting group (→ **11**), reduce the resulting carbonyl (→ **12**), and protect the carbohydrate in the conventional manner (→ **13**). The final step generated the desired phosphoramidite derivative **14** suitable for DNA synthesis. DNA syntheses proceeded

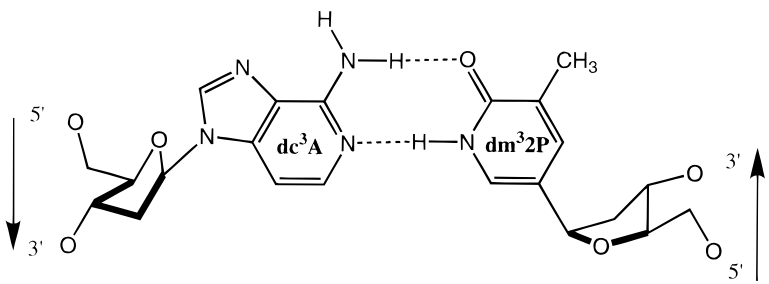
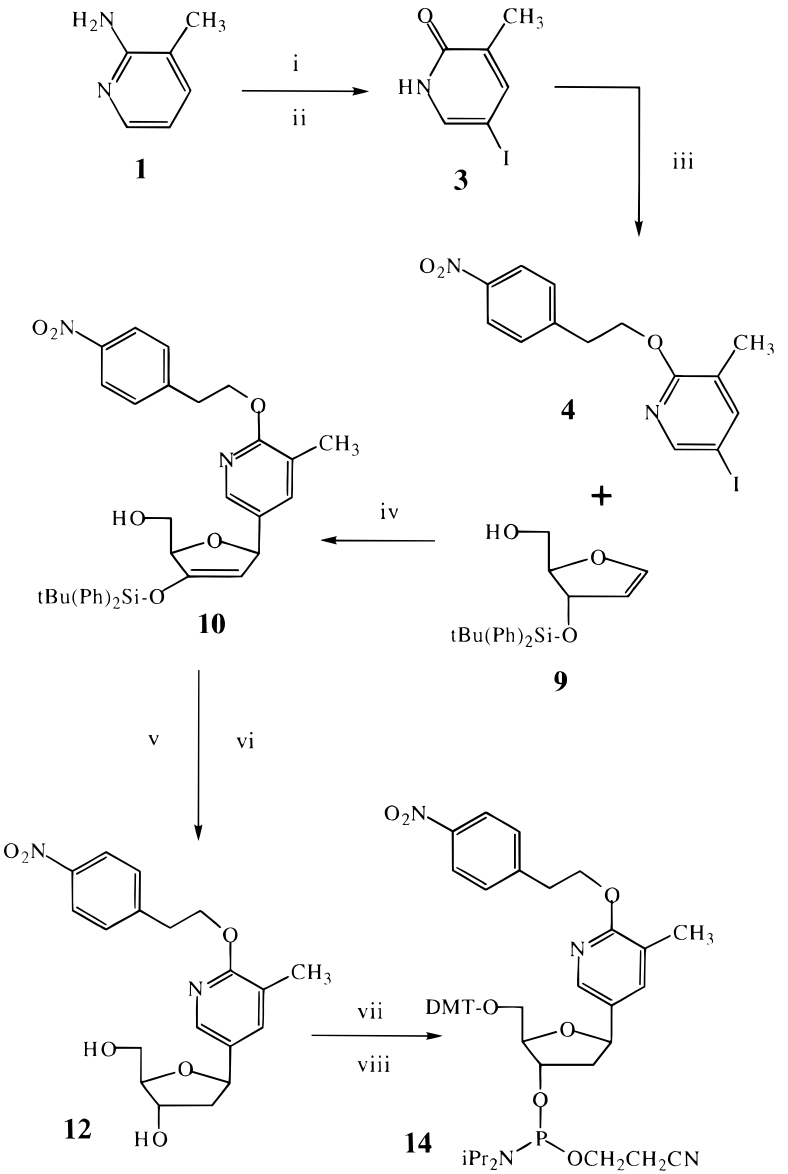
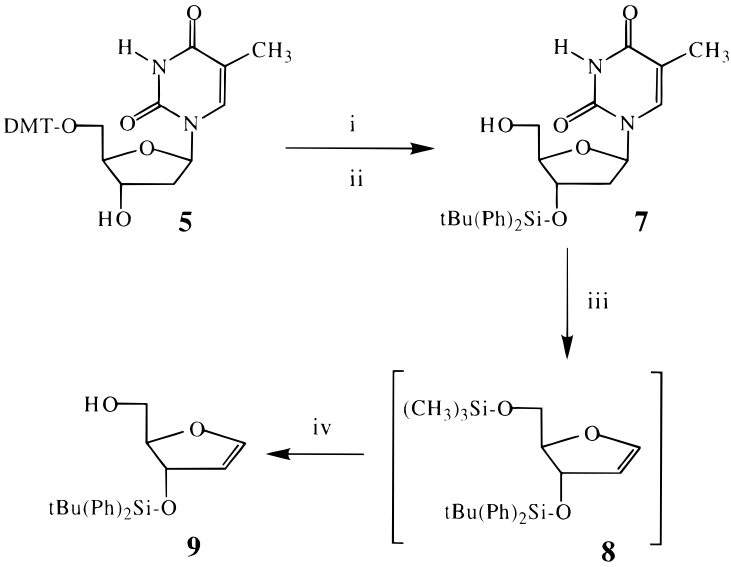


FIG. 1. Structure of the base pair involving dc^3A and dm^32P.



i $HIO_4/I_2/CH_3COOH$, ii $H_2SO_4/NaNO_2$, iii p-nitrophenylethanol/Ph_3P/DEAD, iv $(dba)_2Pd^0/nBu_3N/Ph_2PCH_2CH_2CH_2PPh_2$, v nBu_4N^+-F, vi $Na(OAc)_3BH/CH_3CHOOH$, vii DMT-Cl/DMAP/pyridine, vii Cl-$P(iPr)_2NOCH_2CH_2CN$/DIEA

SCHEME 1.



i = $tBu(Ph)_2Si$-Cl/DMF/imidazole, ii = pTSA/methanol/methylene chloride, iii = HMDS, $(NH_4)_2SO_4$ 80 ^{0}C, iv = K_2CO_3/CH_3OH

SCHEME 2.

in the normal manner and the isolated oligonucleotides were characterized by nucleoside digestion and MALDI-TOF analyses *(19)*.

Ligand binding studies. We have examined the interactions of two ligands (i) 4′, 6-diamidine-2-phenyl indole (DAPI) and Hoechst 33258 at stoichiometries of 1:1 with both native and modified DNA duplexes containing the target sequence $d(GAATTC)_2$. Single crystal *X*-ray analysis *(16)* indicates that specific contacts are made between the indole N-H and the base and sugar residues, as well as between the two terminal amidines and the base and sugar residues located within the minor groove of the dA–dT rich sequence (Fig. 2a). Based upon the crystal structure analysis, the indole N-H makes a bifurcated hydrogen bond with the two O2-carbonyls of the central two dT residues; one of the terminal amidines interacts with two O4′-oxygens of the sugar residues while the second interacts with an N3-nitrogen of a central dA residue and the O2-carbonyl of the dT residue of the adjacent base pair (Fig. 2a). Similar types of contacts have been proposed for the interaction of Hoechst 33258 with the same core sequence *(15)*. As illustrated in Fig. 2b for Hoechst 33258, bifurcated hydrogen bonds are formed with one of the imidazole nitrogens of each benzimidazole ring. One involves the O2-carbonyls of the two central dT residues while the second involves the N3-nitrogen of one of the central dA residues and the O2-carbonyl of the adjacent dT.

The binding of ligands to the minor groove has been reported to result in enhanced

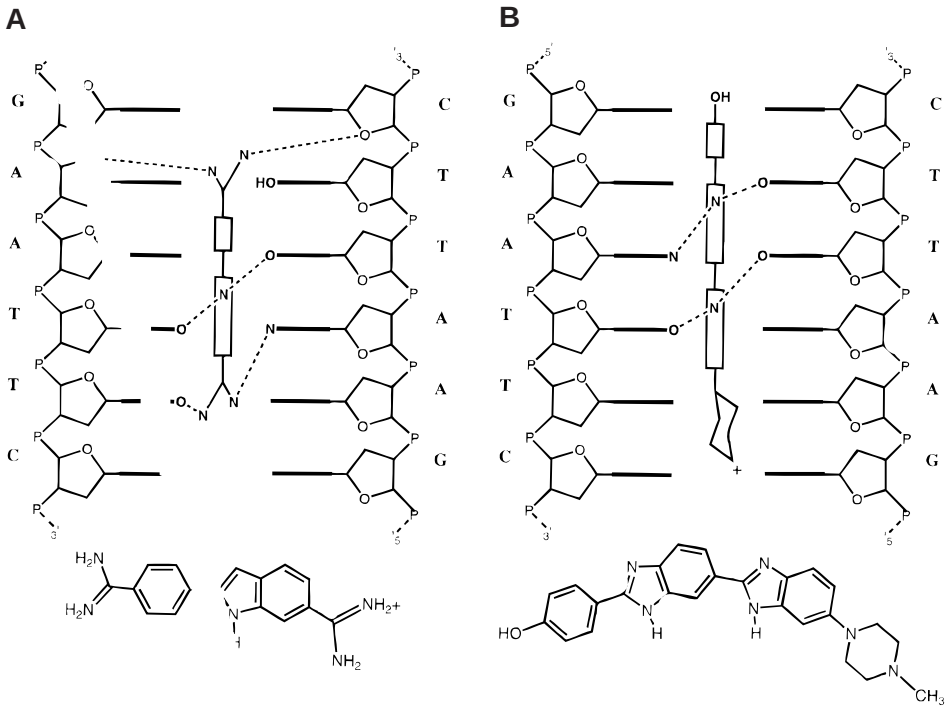


FIG. 2. (a) Schematic of the interactions between the minor groove binding ligand DAPI and the target DNA. (b) Schematic of the interactions between the minor groove binding ligand Hoechst 33258 and the target DNA.

T_M values for the ligand DNA complex *(6)* and has been used to assess the binding of distamycin A to sequences containing dc^3A. We have used this property to obtain relative complex stabilities for DAPI or Hoechst 33258 and a series of analogue DNA duplexes. At a 1:1 ratio of DNA dodecamer and DAPI the T_M for the complexed was observed to increase by 8.5°C (Fig. 3a). In the presence of either nucleoside analogue, some structural changes to the minor groove can be expected and if dramatic in nature could themselves be responsible for changes in binding effects. In cases where the structural perturbations are severe, some loss in ligand binding stability, as measured by T_M enhancements, are likely to occur. In general the substitution of one or two nucleoside analogues has not altered the essential B-form conformation of the duplex based upon CD analyses (data not shown). With the two central dA–dT base pairs of the dodecamer sequence fully modified, the CD spectra is altered somewhat from that of the conventional B-form conformation suggesting some change in base stacking or groove structure.

With the substitution of a single dc^3A residue (noted as "da" in sequences) in the center of the sequence (aT–TA) and additional +0.7°C increase in T_M was observed. This result is consistent with a previous report suggesting that some hydrophobic interactions are likely to contribute to minor grove–ligand interactions *(14)*. By

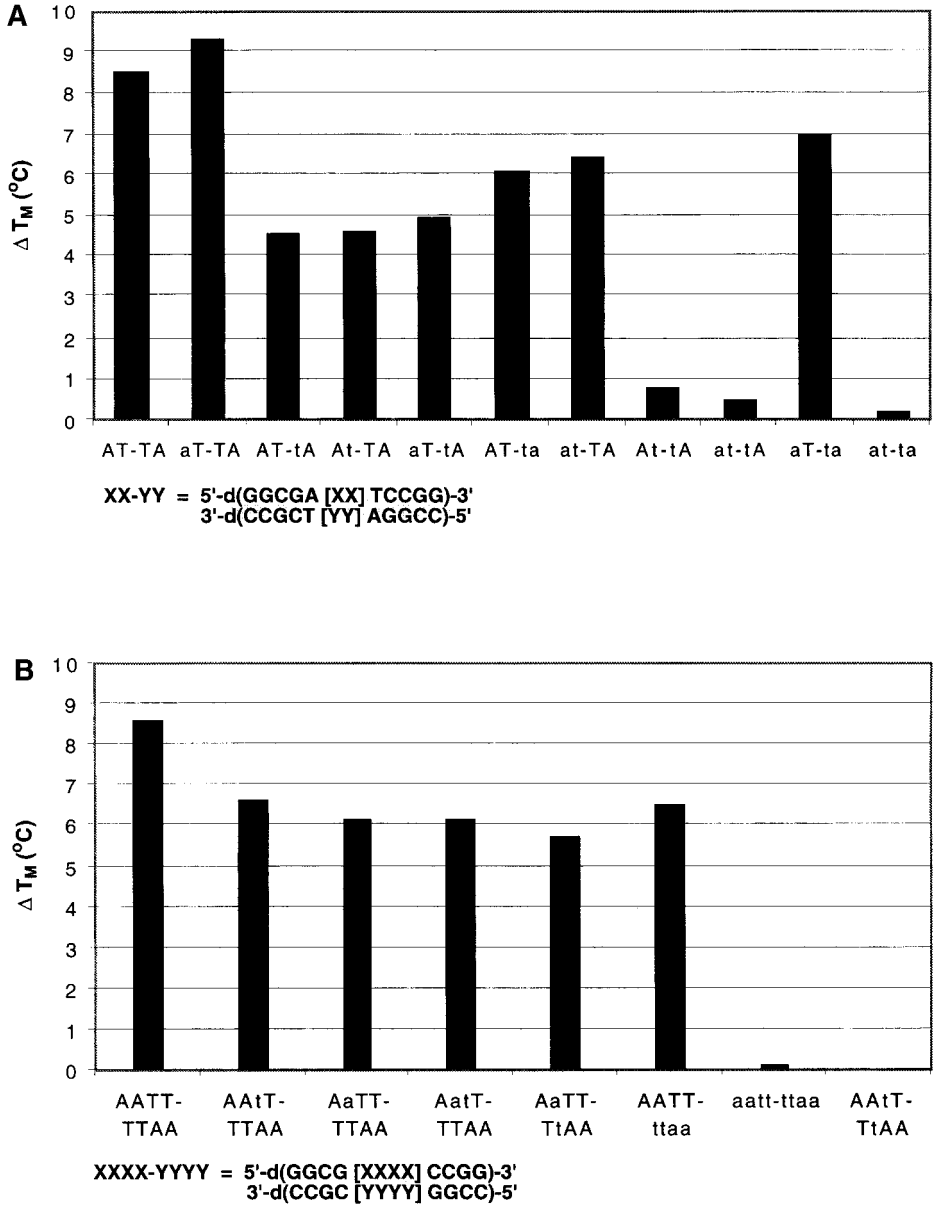


FIG. 3. (a) Change in T_M values for the target DNA sequence in the presence of one equivalent of DAPI. (b) Change in T_M values for the target DNA sequence in the presence of one equivalent of Hoechst 33258.

comparison, the addition of a single dm^{3}2P (noted as "dt" in sequences) to one of the central base pair positions (AT–tA) results in roughly a 50% decrease in the T_M enhancement. A similar result is obtained with a single dt substitution at the other central dT residue. Sequences having both a da and a dt residue (aT–tA, AT–ta, at–TA) tend to have intermediate effects, suggesting the presence of a mild hydrophobic effect moderating the loss of stability due to the missing O2-carbonyl. When both central dT residues are replaced by dt (At–tA, at–tA) the T_M enhancement is essentially lost suggesting no significant binding to these sequences. If the central core sequence contains all analogue residues except for a single dT (aT–ta) then an intermediate enhancement of T_M is observed (+6°C), but if the last dT residue in this sequence is replaced by dt (at–ta) then binding is again lost with no T_M enhancement. These results suggest that the central bifurcated hydrogen bond between DAPI and the dA–dT rich minor groove is critical for effective binding by the ligand to the target DNA sequence. With one member of the three centered interaction absent, the T_M enhancement falls to roughly 50% of that observed with the native duplex (~+4.5°C), and with both members absent, no significant enhancement is present. The presence of da residues can slightly enhance the T_M effects in most cases. For example, the sequence aT–ta exhibits a +7°C T_M enhancement, but after substitution of the last dT residue and forming at–ta, essentially no enhancement effect is present suggesting the absence of complex formation. This lack of binding for the at–ta sequence may in part result from conformational changes observed for this duplex *(19)*.

Although there are some moderating effects by the presence of da residues as noted above, these effects are not dramatic even though one of the terminal amidines should interact with the N3-nitrogen of one of the central dA residues (Fig. 2a). In many of the single da-substituted sequences it is possible for the DAPI ligand to adopt the reverse orientation such that the single amidine-N3-nitrogen hydrogen bond can be maintained. However, when both central dA residues are replaced by da, ligand binding is still effective providing that a single dT residue is present (aT–ta), suggesting that the hydrogen bonding interaction involving the indole N-H and the O2-carbonyls contributes more to complex stability that does the amidine interaction to the N3-nitrogen.

At a 1:1 ratio of DNA dodecamer and Hoechst 33258 an increase in T_M of 8.6°C was obtained (Fig. 3b). As with the DAPI complex, the deletion of some minor groove functional groups resulted in a moderation in the observed T_M enhancement. Many of these complexes resulted in T_M enhancements of 6–6.5°C, somewhat smaller than that of the native complex. With each of these complexes one of the functional groups involved in either of the two bifurcated hydrogen bonds is absent. The sequence AATT–ttaa (Fig. 3b) resulted in a complex with a T_M enhancement of 6.5°C, slightly reduced from that of the native case. In this complex one functional group from each of the two bifurcated hydrogen bonds is absent suggesting that effective complex formation will occur as long as one of the two H-bond acceptors for each of the bifurcated interactions is present. In the aatt–ttaa complex all the functional groups involved in the two bifurcated hydrogen bonding interactions are absent, and as a consequence, no T_M enhancement is present, suggesting no complex is formed in this case. However, in this sequence with the fully modified core the duplex undergoes a conformational change to form a more A-like helix as suggested by recent CD

conformational analyses *(19)*. The A-like helix may be bound only poorly by the Hoechst ligand. No Hoechst 33258–DNA complex is formed with the sequence (AAtT–TtAA) lacking just the two central O2-carbonyls, a sequence that remains essentially B-form in conformation (data not shown). Even though the second bifurcated H-bond can still be formed in this case, the disruption of the interactions to the two central dT residues is sufficient to eliminate effective complex formation.

These studies suggest that in spite of a number of interactions between DAPI or Hoechst 33258 and the minor groove functional groups of the AATT–TTAA duplex, the two O2-carbonyls in the center of the sequence, and those involved in each case in a bifurcated hydrogen bond, are critical for effective complex formation. In the absence of one of the O2-carbonyls, complex formation can still take place, but based upon T_M enhancement studies, complex stability is somewhat reduced. In the absence of both O2-carbonyls, complex formation does not appear to occur. Previous work *(6)* with dc^3A substituted sequences and distamycin indicated that there was no significant difference in the T_M enhancements for the native and analogue sequences. This observation is consistent with the results of the present work since it is the carbonyls of the dT residues rather than the nitrogens of the dA residues that appear critical for binding.

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