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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