

JA990799i

Revised manuscript received

May 20, 1999

Privileged document

Communication

Supplementary Material

Selective Recognition of a dC-dG Base Pair by Oligonucleotide-Directed Triplex Formation Using a dC Residue Tethering an Intercalator

Diego A. Gianolio and Larry W. McLaughlin*

Department of Chemistry, Merkert Chemistry Center, Boston College,
Chestnut Hill, MA 02167

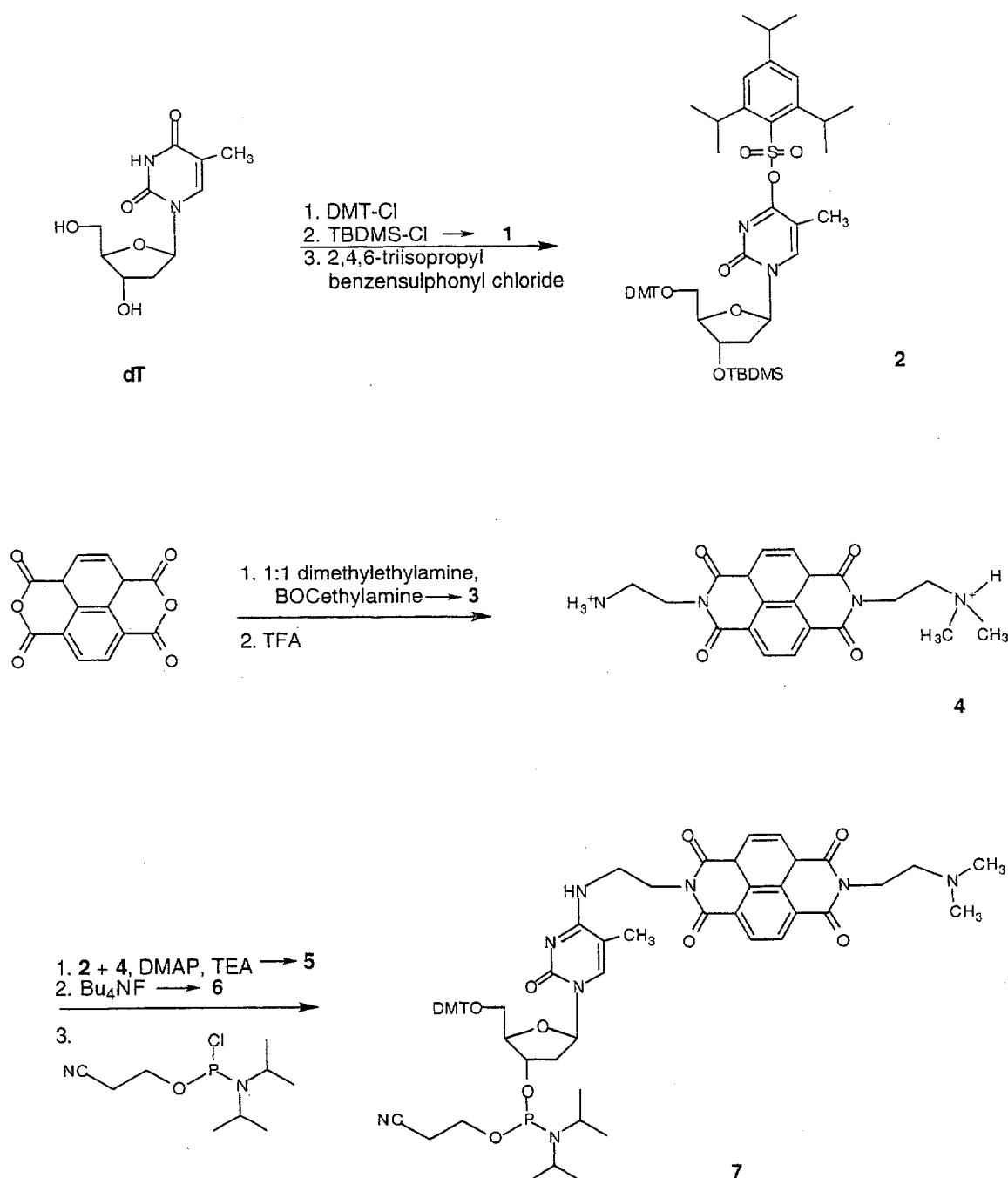
Experimental

Materials:

NMR spectra were obtained on a Varian spectrometer (400 MHz). HRMS, LRMS (FAB) and MALDI-Voyager mass spectra were obtained from the Mass Spectrometry Laboratory, School of Chemical Sciences, 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). Anhydrous solvents and starting materials were purchased from Aldrich Chemical Company and used without further purification unless otherwise specified. UV scans and absorbances were obtained using a Beckman DU 640 spectrophotometer. Oligodeoxynucleotides were synthesized on an Applied Biosystems 381A DNA Synthesizer. 2'-Deoxynucleotide phosphoramidites and 3' terminal nucleoside controlled pore glass support (CPG) were purchased from Glenn Research (Sterling, VA). High performance liquid chromatography (HPLC) was performed on a Beckman HPLC system using C-18 reversed-phase columns (ODS-Hypersil, 5mm particle size, 120A pore) with detection at 260 nm. Oligonucleotides were desalted with Econo-Pac 10DG disposable chromatography Columns (Bio Rad, Hercules, CA). T_m measurements were performed on an AVIV Spectrophotometer, Model 14DS UV/Vis.

Methods:

Synthetic scheme for the generation of the modified phosphoramidite derivative of m^5C :



5'-O-4,4'-Dimethoxytrityl-3'-O-*tert*butyldimethylsilyl thymidine (1)

To 1.71 g (3.1 mmol) of 5'-O-4,4'-dimethoxytrityl thymidine dissolved in 15 ml of freshly distilled pyridine was added 0.44 ml (3.1 mmol) of anhydrous triethylamine dropwise into the reaction flask and the mixture was stirred at ambient temperature for 10 minutes. To this mixture was added 1.42 g (9.4 mmol) of *t*-butyldimethylsilyl chloride and 0.86 g (12.6 mmol) of imidazole and the mixture stirred under a nitrogen atmosphere for 12 hours. The reaction was then quenched with 5 ml of methanol and the solvent was evaporated *in vacuo* until a solid residue was left. The desired product was then purified by silica gel column chromatography using a gradient of methanol in dichloromethane (3%). 1.96 g (2.9 mmol) of **1** were obtained. Yield 95 %.

R_f (methanol/dichloromethane, 1/19): 0.48; mp: 92 - 94 °C;

U.V. (methanol): $\lambda_{\text{max}} = 203, 233$ ($\epsilon = 3.95 \times 10^4 \text{ cm}^{-1}\text{M}^{-1}$), $\lambda_{\text{min}} = 267 \text{ nm}$.

¹H NMR (DMSO-d₆): $\delta = 0.02$ (s, 6H, Si-CH₃); 0.76 (s, 9H, *t*-butyl); 1.49 (s, 3H, base-CH₃); 2.10 - 2.31 (m, 2H, H2'2''); 3.11 - 3.24 (m, 2H, H5'5''); 3.71 (s, 6H, -OCH₃); 3.79 (m, 1H, H4'); 4.43 (m, 1H, H3'); 6.13 (t, $J = 6.8 \text{ Hz}$, 1H, H1'); 6.86 - 7.38 (m, 13H, Ar-H); 7.52 (s, 1H, Ar-H); 11.33 (s, 1H, N-H) ppm.

¹³C NMR (CDCl₃): $\delta = -4.9, -4.7, 11.9, 20.7, 25.7, 41.5, 55.2, 62.9, 72.1, 84.9, 86.8, 111.0, 113.2, 113.2, 127.1, 128.0, 128.1, 130.0, 130.0, 135.4, 135.6, 144.3, 150.1, 159.7$ ppm.

HRMS calculated for C₃₇ H₄₆ N₂ O₇ Si (M + H⁺): 658.3074, found: 658.3073.

5'-O-4,4'-Dimethoxytrityl-3'-O-*tert*butyldimethylsilyl-4-O-triisopropylphenylsulfonyl thymidine (2)

To 1.0 g (1.52 mmol) of **1** that had been dried overnight in a flask under high vacuum was added 15 ml of anhydrous dichloromethane under an atmosphere of nitrogen. To this mixture 0.85 ml (6.07 mmol) of anhydrous triethylamine was added dropwise and the flask was wrapped with aluminum foil prior to the addition of 0.827 g (2.73 mmol) of 2,4,6-triisopropylbenzenesulfonyl chloride and 46 mg (0.38 mmol) of dimethylaminopyridine. The mixture was then stirred at ambient temperature for 18 hours. The solution (now a crimson color) was quenched with 5 ml of methanol and evaporated *in vacuo* until an oily residue remained. The desired product was purified by silica gel column chromatography, using a gradient of hexane in dichloromethane. A yellowish colored solid was obtained that was

dried for few hours under high vacuum and immediately stored under nitrogen at 0 °C yielding 0.73 g (0.79 mmol) of **2**, 52 % yield.

R_f (methanol/dichloromethane, 1/19): 0.86; mp: 87 - 91 °C;

U.V.(methanol): λ_{\max} = 202, 233 (ϵ = 3.94×10^3 cm⁻¹M⁻¹), λ_{\min} = 282 nm.

¹H NMR (DMSO-d₆): δ = 0.12 - 0.24 (m, 6H, Si-CH₃); 0.72 (s, 9H, *t*-butyl); 1.18 - 1.22 (m, 18H, isopr.CH₃); 1.59 (s, 3H, base-CH₃); 2.24 (t, J = 6 Hz, 2H, H_{2'}H_{2''}); 2.94 (m, 1H, CH); 3.15 - 3.42 (m, 2H, H_{5'}H_{5''}); 3.71 (s, 6H, -OCH₃); 3.86 (m, 1H, H_{4'}); 4.15 (m, 2H, CH); 4.36 (m, 1H, H_{3'}); 5.96 (t, J = 6.0 Hz, 1H, H_{1'}); 6.84 - 7.30 (m, 13H, Ar-H); 7.32 (s, 2H, Ar-H); 8.17 (s, 1H, Ar-H) ppm.

¹³C NMR (CDCl₃): δ = -5.0, -4.7, 11.5, 17.8, 23.4, 23.5, 24.3, 24.6, 25.6, 29.6, 34.2, 42.1, 55.2, 61.8, 70.4, 86.7, 86.8, 87.1, 103.6, 113.2, 113.2, 124.0, 127.2, 128.0, 128.2, 130.0, 135.2, 143.2, 144.1, 151.1, 153.7, 154.3, 158.7, 166.3 ppm.

LRMS: 925 (M⁺), 303 (DMT⁺).

N-(2-*tert*Butoxycarbonylaminoethyl)-N'-[2-(N,N-dimethylamino)-ethyl]-1,4,5,8-naphthalenetetracarboxylic diimide (3)

The synthesis has been performed following a known procedure¹ starting from 712 mg (2.65 mmol) of 1,4,5,8-naphthalenetetracarboxylic dianhydride, 425 mg (2.65 mmol) of mono-*tert*-butoxycarbonylaminoethylamine and 0.29 ml (2.65 mmol) of N,N-dimethylethylenediamine. The desired product was then purified by silica gel column chromatography using a gradient of methanol in chloroform (2%), eluting as the second band in between the two symmetric secondary products roughly in a 1:2:1 ratio. 509 mg (1.06 mmol) of **3** were obtained, yield 40 %, as a yellow solid.

R_f (methanol/dichloromethane, 1/19): 0.19; m.p.: 177 °C d.;

U.V.(methanol): λ_{\max} = 234, 340 (sh), λ_{\min} = 357, 377 (ϵ = 2.50×10^4 cm⁻¹M⁻¹) nm.

¹H NMR (DMSO-d₆): δ = 1.19 (s, 9H, *t*-butyl); 2.22 (s, 6H, N-CH₃); 2.54 (m, 2H, CH₂); 3.26 (m, 2H, CH₂); 4.12 - 4.18 (m, 4H, CH₂); 8.66 (m, 4H, Ar-H).

¹³C NMR (DMSO-d₆): δ = 28.3, 37.6, 38.2, 45.6, 56.5, 77.7, 118.0, 118.0, 126.3, 130.5, 162.8, 163.0 ppm.

HRMS calculated for C₂₅H₂₉N₄O₆ (M + H⁺): 481.2087, found: 481.2089.

N-(2-Aminoethyl)-N'-[2-(N,N-dimethylamino)ethyl]-1,4,5,8-naphthalenetetracarboxylicdiimide dihydroditrifluoroacetate (4)

346 mg (0.72 mmol) of BOC-protected compound **3** was treated according to a known procedure.¹ The desired product was isolated in the bis-protonated form with trifluoroacetic counterions as an off-white solid. A total of 410 mg (0.67 mmol), yield 94 %, was obtained.

R_f (methanol/dichloromethane, 1/9): 0.15; m.p.: 190 °C d.;

U.V.(methanol): λ_{\max} = 233, 340 (sh), λ_{\min} = 357, 377 (ϵ = 1.47×10^4 cm⁻¹M⁻¹) nm.

¹H NMR (DMSO-d₆): δ = 2.91 (s, 6H, N-CH₃); 3.18 (m, 2H, CH₂); 3.46 (m, 2H, CH₂); 4.33 - 4.41 (m, 4H, CH₂); 7.87 (s, 3H, NH₃⁺); 8.72 (m, 4H, Ar-H); 9.28 (s, 1H, NH⁺).

¹³C NMR (DMSO-d₆): δ = 35.7, 37.6, 38.1, 42.9, 54.9, 118.0, 126.4, 126.4, 126.6, 126.8, 130.6, 130.7, 163.4, 163.4 ppm.

HRMS calculated for C₂₀ H₂₁ N₄ O₄ (M + H⁺): 381.1563, found: 381.1563.

N⁴-2-{[N-[2-(N,N-dimethylamino)ethyl]-1,4,5,8-naphthalene-tetracarboxylicdiimidyl]-N'-}ethyl-5'-O-4,4'-dimethoxytrityl-3'-O-tertbutyl-dimethylsilyl-5-methyl-2'-deoxycytidine (5)

To 0.38 g (0.63 mmol) of **4**, evaporated twice from freshly distilled pyridine and under nitrogen atmosphere, was added 8 ml of freshly distilled pyridine, followed by 0.44 ml (3.16 mmol) of anhydrous triethylamine dropwise and the mixture was stirred for 15 minutes at ambient temperature. To this solution was added 0.58 g (0.63 mmol) of **2** followed by 39 mg (0.32 mmol) of dimethylaminopyridine and the flask was then kept under nitrogen and stirred for 4 hours. The solvent was removed *in vacuo* until an oily residue was obtained, the desired product was subsequently purified by silica gel column chromatography, using a gradient of methanol in chloroform (3%) resulting in 0.58 g (0.57 mmol) of **5**, 91% yield, as a yellow solid.

R_f (methanol/dichloromethane, 1/9): 0.54; mp: 158 - 161 °C;

U.V.(methanol): λ_{\max} = 234, λ_{\min} = 276, 357, 378 (ϵ = 1.76×10^4 cm⁻¹M⁻¹) nm.

¹H NMR (DMSO-d₆): δ = 0.03 (s, 6H, Si-CH₃); 0.76 (s, 9H, *t*-butyl); 1.36 (s, 3H, base-CH₃); 2.03 - 2.05 (m, 2H, H_{2'}2''); 2.19 (s, 6H, N-CH₃); 2.48 (m, 4H, CH₂); 3.12 - 3.23 (m, 2H, H_{5'}5''); 3.71 - 3.76 (m, 7H, -OCH₃ + H_{4'}); 4.16 - 4.36 (m, 5H, CH₂ + H_{3'}); 6.02 (t, J = 6.4 Hz, 1H, H_{1'}); 6.88 - 7.40 (m, 15H, Ar-H + NH); 8.64 (s, 4H, Ar-H) ppm.

^{13}C NMR (DMSO-d_6): $\delta = -4.9, -4.6, 12.0, 17.8, 25.1, 25.8, 30.9, 38.2, 45.5, 55.3, 56.1, 62.9, 71.9, 84.3, 85.7, 113.5, 118.0, 118.0, 126.3, 126.9, 127.9, 128.1, 129.9, 130.4, 130.6, 135.6, 144.7, 158.4, 162.9, 163.2$ ppm.

HRMS calculated for $\text{C}_{57}\text{H}_{65}\text{N}_6\text{O}_{10}\text{Si}$ ($\text{M} + \text{H}^+$): 1021.4531, found: 1021.4525.

N⁴-2-{[N-[2-(N,N-dimethylamino)ethyl]-1,4,5,8-naphthalene-tetracarboxylicdiimidyl]-N'-}ethyl-5'-O-4,4'-dimethoxytrityl-5-methyl-2'-deoxycytidine (6)

To 0.570 g (0.56 mmol) of **5** dissolved in 4 ml of anhydrous THF was added 0.67 ml of a 1M solution of tetrabutylammonium fluoride dropwise. The mixture was allowed to stir at ambient temperature and after 1 hour the reaction was complete as monitored by TLC. The solvent was removed *in vacuo* and the desired product was purified by silica gel column chromatography, using a gradient of methanol in chloroform (6%) resulting in 0.462 g (0.51 mmol) of **6**, 91% yield, as a dark yellow solid.

R_f (methanol/dichloromethane, 1/9): 0.26;

U.V.(methanol): $\lambda_{\text{max}} = 234, 268, \lambda_{\text{min}} = 358, 378$ ($\epsilon = 1.26 \times 10^4 \text{ cm}^{-1}\text{M}^{-1}$) nm.

^1H NMR (DMSO-d_6): $\delta = 1.30$ (s, 3H, base-CH₃); 1.91 - 2.05 (m, 2H, H_{2'}2''); 2.24 (s, 6H, N-CH₃); 2.58 (m, 4H, CH₂); 3.14 (m, 2H, H_{5'}5''); 3.68 - 3.82 (m, 7H, -OCH₃ + H_{4'}); 4.18 - 4.28 (m, 5H, CH₂ + H_{3'}); 5.24 (d, $J = 4.4$ Hz, 1H, OH), 6.07 (t, $J = 6.8$, 1H, H_{1'}); 6.88 - 7.38 (m, 15H, Ar-H + NH); 8.63 (s, 4H, Ar-H) ppm.

^{13}C NMR (DMSO-d_6): $\delta = 12.9, 13.7, 19.4, 23.3, 38.2, 44.5, 45.6, 55.3, 55.8, 57.7, 63.9, 70.7, 84.5, 85.5, 86.0, 102.1, 113.5, 118.0, 126.4, 126.5, 126.9, 127.0, 127.8, 128.0, 128.1, 130.0, 130.3, 130.6, 135.6, 135.7, 136.9, 144.9, 154.9, 158.3, 163.1, 163.2, 163.3$ ppm.

HRMS calculated for $\text{C}_{51}\text{H}_{51}\text{N}_6\text{O}_{10}$ ($\text{M} + \text{H}^+$): 907.3667, found: 907.3664.

N⁴-2-{[N-[2-(N,N-dimethylamino)ethyl]-1,4,5,8-naphthalene-tetracarboxylicdiimidyl]-N'-}ethyl-5'-O-4,4'-dimethoxytrityl-3'-O-(2-cyano-ethoxy)diisopropylaminophosphino-5-methyl-2'-deoxycytidine (7)

To 50 mg (0.055 mmol) of **6** dissolved in 1 ml of anhydrous dichloromethane was added 0.019 ml (0.083 mmol) of 2-cyanoethyl diisopropylchlorophosphoramidite dropwise, followed by the addition of 0.039 ml (0.22 mmol) of diisopropylethylamine. The mixture was allowed to stir at room temperature for 15 minutes and then was quenched with 0.5 ml of

methanol and concentrated *in vacuo*. The desired product was precipitated from the solution by adding cold diethyl ether. The product was filtered and washed with additional cold diethyl ether to give 49 mg (0.044 mmol) of **7**, yield 80%, as a yellow solid.

Rf (methanol/dichloromethane, 1/9): 0.59;

^{31}P NMR (CDCl_3): δ = 147.38, 147.94 ppm.

N⁴-2-[[N-[2-(N,N-dimethylamino)ethyl]-1,4,5,8-naphthalene-tetracarboxylicdiimidyl]-N'-}ethyl-1,4,5,8-naphthalene-tetracarboxylicdiimidyl]-5-methyl-2'-deoxycytidine (HPLC standard)

To 50 mg (0.55 mmol) of **6** dissolved in 1 ml of dichloromethane, was added 0.1 ml of a solution of 3% trichloroacetic acid in dichloromethane, and the mixture was allowed to stir for 1 hour at room temperature. The solvent was removed *in vacuo* until an oily residue remained. The desired product was purified by silica gel preparative thin layer chromatography, using 20 % methanol in dichloromethane as eluent producing 8 mg (0.014 mmol), 25 % yield, of a yellow solid.

Rf (methanol/dichloromethane, 2/8): 0.26;

U.V.(methanol): λ_{max} = 234, λ_{min} = 281, 358, 378 (ϵ = $1.37 \times 10^4 \text{ cm}^{-1}\text{M}^{-1}$) nm.

^1H NMR (DMSO-d_6): δ = 1.64 (s, 3H, base- CH_3); 1.83 - 1.94 (m, 2H, $\text{H}_2'\text{H}_2''$); 2.20 (s, 6H, N- CH_3); 2.53 (t, J = 7.2, 2H, CH_2); 3.48 - 3.69 (m, 5H, CH_2 + $\text{H}_4'\text{H}_5'\text{H}_5''$); 4.16 - 4.25 (m, 5H, CH_2 + H_3'); 4.97 (m, 1H, 5' OH); 5.15 (m, 1H, 3' OH); 6.03 (t, J = 6.8, 1H, H_1'); 7.49 (s, 1H, Base-H); 8.64 (s, 4H, Ar-H) ppm.

^{13}C NMR (DMSO-d_6): δ = 13.3, 13.5, 14.1, 18.7, 29.2, 37.7, 45.1, 56.2, 61.5, 70.5, 84.7, 87.2, 96.4, 101.9, 118.0, 126.2, 126.8, 130.3, 130.6, 137.4, 155.1, 162.9, 163.1, 163.3 ppm.

HRMS calculated for $\text{C}_{30}\text{H}_{33}\text{N}_6\text{O}_8$ ($\text{M}+\text{H}^+$): 605.2360, found: 605.2361.

DNA synthesis

The native 25-mer target sequences and the 15-mer containing the added intercalation functionality, were prepared by solid phase DNA synthesis using standard protocols.² A slightly different methodology was applied when performing the deprotection and purification of the 15-mer. The naphthalene-modified cytosine proved to be unstable under concentrated ammonia deprotection conditions, an addition/elimination reaction probably

takes place at the N⁴ position displacing the naphthalene moiety and generating the more stable m⁵C nucleoside. The fast deprotecting cytidine phosphoramidite (Ac-dC) was therefore incorporated into the 15-mer, requiring milder deprotection conditions, a solution 0.05 M K₂CO₃ in methanol at room temperature for 2 hours. Under these conditions the modified nucleoside remained largely intact as could be confirmed by HPLC analysis. The pH of the mixture was then adjusted to 7.0 with 2% CH₃COOH in H₂O, filtered and then concentrated *in vacuo* until methanol was completely evaporated.

Purification of the oligonucleotides was accomplished by fast flow HPLC (4.6 x 120 mm column, trityl on), starting with 0% B for 1 minute, then using a linear gradient 0 - 50% B over 4.5 minutes (A: 50 mM triethylammonium acetate, adjusted to pH = 7.0 with glacial CH₃COOH, B: 50 mM triethylammonium acetate in 70% acetonitrile). The DMT protected 15-mer oligonucleotide had a retention time of about 4.9 minutes. The collected oligonucleotide was reduced in volume, and detritylated with 80% aqueous acetic acid (45 min, 0 °C). The resulting oligonucleotide was analyzed by HPLC (4.6 x 250 mm column) using a linear gradient 0 - 100% B over 60 minutes (A: 20 mM KH₂PO₄, adjusted to pH = 5.5 with KOH 2M, B: 20 mM KH₂PO₄ in 70% methanol). The 15-mer oligonucleotide had a retention time of about 28 minutes and eluted following a single smaller peak with a retention time of about 26 minutes possibly the sequence lacking the naphthalene intercalator, perhaps resulting from some unavoidable partial hydrolysis of the product. A second purification was therefore necessary at this stage. The isolated oligonucleotide was then desalted (Sephadex G-10), and stored at -20 °C. The unnatural nucleoside could be incorporated into the DNA strands with essentially the same coupling efficiencies as the common nucleosides.

MALDI-Voyager MS for 5'TTTCTTTTCTXTCTT3' (X = nucleoside containing naphthalene functionality): calculated for (15-mer⁺): 4819, found: 4820.

Nucleoside Analysis

The 25-mers were digested into monomeric units with a combination of snake venom phosphodiesterase and alkaline phosphatase: a 20 µl reaction mixture containing 0.5 A₂₆₀ unit of oligomer, 50 mM tris·HCl, pH 8.0, 100 mM MgCl₂, 1 unit of snake venom phosphodiesterase, 1 unit of alkaline phosphatase was incubated overnight at 37 °C. An aliquot of this mixture was

analyzed by HPLC (4.6 x 250 mm column) using a linear gradient 0 - 100% B over 60 minutes (A: 20 mM KH_2PO_4 , adjusted to pH = 5.5 with KOH 2M, B: 20 mM KH_2PO_4 in 70% methanol). The 15-mer was effectively digested with a combination of S1 nuclease and alkaline phosphatase: a 10 μl reaction mixture containing 0.5 A_{260} unit of oligomer, 200 mM NaCl, 5 mM MgCl_2 , 0.1 mM ZnSO_4 , 25 mM sodium acetate, pH 5.9, 1 unit S1 nuclease was incubated overnight at 37 °C. To this mixture was added 7 μl of 0.1 M Tris-HCl, pH 8.0, and 1 unit of calf intestinal alkaline phosphatase and the reaction was incubated for an additional 60 minutes at 37 °C. An aliquot of this mixture was also analyzed by HPLC (see Figure 1).

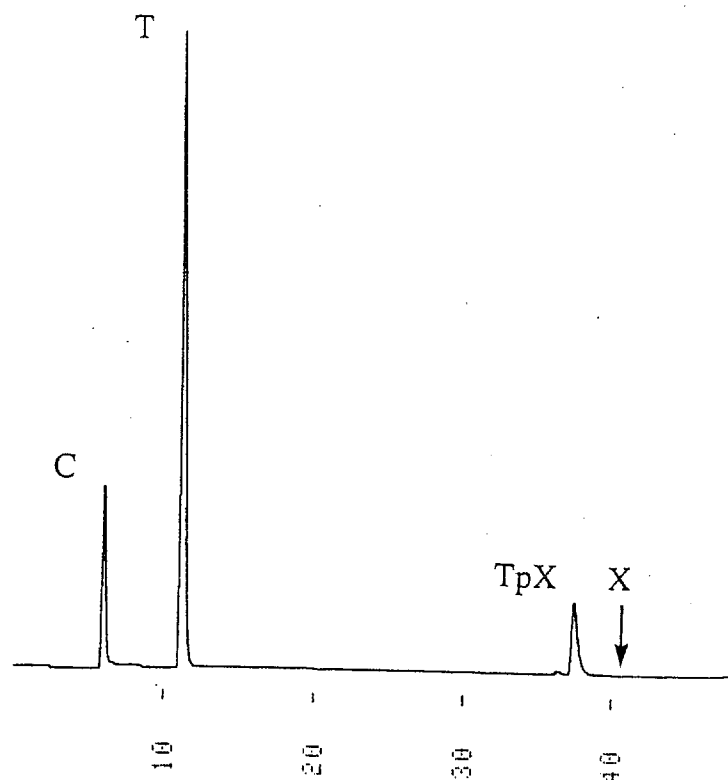


Figure 1. HPLC analysis of S1 nuclease/alkaline phosphatase digestion of a small quantity of the X-containing 15-mer and its resolution on a reverse phase C-18 column.

The digestion resulted in monomeric units of C, T and a later eluting peak with a retention time of about 38 minutes that did not correspond to the

modified nucleoside as expected, but to the dimer TpX. This correspondence was proved by making co-injections with standards of X (retention time about 40 minutes) or TpX and comparing the areas of each eluted peak. TpX proved to be refractory to enzymatic digestion.

MALDI-Voyager MS for 5'XpT3': calculated for (2-mer⁺): 909, found: 910.

Thermal Denaturation Studies

Thermal Denaturation Studies were performed in 10 mM PIPES pH 6.4 or 7.0, 10 mM HEPES pH 7.5 and 10 mM magnesium chloride, 50 mM sodium chloride at strand ratios of 1:1:1 at a concentration of 1 μ M. 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 $^{\circ}$ C steps (from 0 to 85 $^{\circ}$ C) and when thermal equilibrium was reached, temperature and absorbance data were collected. T_m values were determined from first-order derivatives of the Absorbance vs Temperature plots or graphically from the same plots.

Cooling curves were obtained in essentially the same manner with 1 $^{\circ}$ C decreases (from 85 to 0 $^{\circ}$ C). Examples of such cooling curves (dotted lines) overlaid on heating curves (solid lines) for the triplex containing C⁺-G-C and X-C-G base triplets are illustrated in Figures 2 and 3, respectively.

References

- (1) Lokey, R. S.; Kwok, Y.; Guelev, V.; Pursell, C. J.; Hurley, L. H.; Iverson, B. L. *J. Am. Chem. Soc.* **1997**, *119*, 7202-7210.
- (2) Matteucci, M. D.; Caruthers, M. H. *J. Am. Chem. Soc.* **1981**, *103*, 3185-3191.

Figure 2. Absorbance vs. Temperature plots for:

5' - TTTCCTTTCTCTCTT - 3'
 5' - GCGCGAAAGAAAAGAGAGAACCCGG - 3'
 3' - CCGGCTTCTTTTCTCTCTGGGCC - 5'

Solid line (heating curve), dashed line (cooling curve).

