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A Cytosine Analogue Containing a Conformationally Flexible Acyclic Linker for Triplex Formation at Sites with Contiguous G-C Base Pairs†,§

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Abstract

Two nucleoside derivatives of the pyrimidine bases T and $m^{5ox}C$ have been prepared with flexible acyclic carbohydrate linkers. A new procedure, beginning with (*R*)-(-)-2,2-dimethyl-1,3-dioxolane-4-methanol permits the preparation of the stereochemically pure acyclic derivatives of both protected nucleoside analogues without contamination by a problematic rearrangement product. By simply increasing the flexibility of the carbohydrate portion of the $am^{5ox}C$ nucleoside derivative, 15-mer triplexes containing five contiguous G-C base pairs exhibit a 7-8 °C increase in T_m value.

Introduction

It is generally difficult to effectively target polypurine sequences containing blocks of contiguous G-C base pairs with a parallel-stranded polypyrimidine sequence. The use of C or m^5C for bidentate recognition of G-C base pairs necessitates protonation of the N^3-nitrogen, and placing a number of protonated residues at neighboring sites results in charge-charge repulsion effects. Although triplex formation at duplex targets containing contiguous G-C base pairs has been described for two neutral pyrimidine analogues of a protonated C residue,[1,2] these triplexes do not appear to be as stable as those obtained with targets containing only a few isolated G-C residues imbedded within a series of A-T base pairs.[3,4] Analogues that are not based upon a pyrimidine ring system but are more purine-like, can also function effectively to target G-C base pairs from within the parallel-stranded motif.[5,6] These purine analogues are composed of a larger heterocyclic ring (relative to the pyrimidine), and presumably results in some change in the conformation and/or position of the sugar-phosphate backbone within the major groove. NMR studies have indicated that the sugar conformation for the third strand at C^+-G-C base triplets differs from that found in T-A-T base triplets.[7] Conformational preferences in the sugar-phosphate backbone may also account for the recent observation[8] that N^7-glycosylated guanine (N^7G) is very effective in triplex formation at sites with contiguous G-C base pairs, but is less effective than m^5C in targeting sequences containing isolated G-C base pairs.[9-11]

Recently, nucleoside derivatives of T and C have been prepared[12] such that the sugar conformation was restricted to the 2'-*endo*/1'-*exo* range by the introduction of a five-membered ring that included the C_3'- and C_5'-carbons. These two bicyclo-deoxynucleoside (bcd) derivatives have quite different effects upon DNA triplex formation; the presence of bcdT residues results in more stable DNA triplexes, while the presence of bcdC residues destabilizes these complexes. A related conformationally restricted riboacetal derivative of T was also effective in stabilizing DNA triplexes.[13] These observations, as well as those regarding the differential

† Abbreviations: M = m^5C = 2'-deoxy-5-methylcytidine, aT = acyclic thymidine = 1-{[2-hydroxy-1-(hydroxymethyl)ethyoxy]-methyl}thymdine, $am^{5ox}C$ = acyclic $m^{5ox}C$ = 4-amino-1-{[2-hydroxy-1-(benzyloxymethyl)ethoxy]-methyl}-5-methyl-2,6-[1*H*,3*H*]-pyrimidione.

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stabilities for analogues such as N^7-guanine targeting isolated or contiguous sequences of G-C base pairs, suggest that the sugar conformation necessary to effectively form various X-G-C base triplets, particularly with sequences of contiguous G-C base pairs, may differ from that preferred in the T-A-T base triplet.

To explore the role of sugar conformation in parallel-stranded DNA triplexes containing contiguous G-C base pairs, we prepared two nucleoside derivatives which contain flexible acyclic carbohydrate analogues, one corresponds to thymidine (aT) and one corresponds to a recently described[3] protonated C analogue, ($am^{5ox}C$). We have examined the stability of DNA triplexes containing these acyclic nucleosides in the third strand.

Experimental

Materials

HPLC grade solvents were obtained from Fisher Scientific (Fair Lawn, NJ), other reagents were from Aldrich Chemical Co. (Milwaukee, WI). 5'-Dimethoxytrityl nucleoside phosphoramidite monomers as well as all ancillary reagents for nucleic acid synthesis were obtained from Cruachem through Fisher Scientific or from Applied Biosystems, Inc. (Foster City, CA). Oligonucleotides were synthesized using nucleoside phosphoramidite derivatives and an Applied Biosystems 381A DNA synthesizer. High-performance liquid chromatography (HPLC) was carried out on ODS-Hypersil column (0.46 x 25 cm, Shandon Southern, England), using a Beckman HPLC system. ^1H NMR spectra were obtained at 300 or 500 MHz on Varian XL-300 or 500 multinuclear spectrometers. ^{31}P NMR spectra were obtained at 121 MHz on the Varian XL-300. Absorption spectra were recorded by a Perkin-Elmer Lambda 3B UV/Vis spectrophotometer. Mass spectra were obtained from the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois, Urbana, IL.

Methods

The synthesis of the derivatives of T and $m^{5ox}C$ containing acyclic carbohydrates is described below. We note here that compound **3** is commercially available, but at a prohibitive cost (~$200/g), therefore we have provided a two step procedure to generate the starting material **3** from the more reasonably priced **1** (~$18/g). Due to the availability of **3**, we have not provided full characterization of the intermediate product **2** or commercially available material **3**.

(R)-2,2-Dimethyl-4-benzoxymethyl-1,3-dioxolane (2)

To 5 g (37.8 mmol) of (R)-(-)-2,2-dimethyl-1,3-dioxolane-4-methanol dissolved in 150 ml of anhydrous THF was added 1.8 g (45.0 mmol) of 60% NaH in mineral oil. After a 10 min reaction period, 10 ml (84.1 mmol) of benzyl bromide was added portion wise. The mixture was stirred for 3h at ambient temperature and then 200 ml methylene chloride was added to the reaction mixture. The solid residue was removed by filtration and the filtrate was evaporated to dryness. Column chromatography (silica gel) with gradient methylene chloride in hexane yielded 8.06 g (96%) of the desired product.

R_f (methylene chloride) 0.22.

^1H NMR ($CDCl_3$) δ = 1.36 (s, 3H, CH_3), 1.44 (s, 3H, CH_3), 3.52 (dd,dd, 2H, CH_2), 3.75 and 4.05 (dd and dd, 2H, CH_2), 4.31 (m, 1H, CH), 4.57 (dd, 2H, CH_2), 7.34 (m, 5H, Ph).

^{13}C ($CDCl_3$) δ = 25.4, 26.8, 66.9, 71.1, 73.5, 74.7, 109.4, 127.7, 128.4 ppm.

(S)-3-Benzyloxy-1,2-propanediol (3)

To 8 g (36.0 mmol) of (R)-2,2-dimethyl-1,3-dioxolane-4-benzyloxymethyl (**2**) dissolved in 200 ml of 50% methanol in methylene chloride was added 5 ml of concentrated HCl and the solution stirred for 10 min. A solution of $NaHCO_3$ was added to neutralize the excess HCl and the solution was dried over sodium sulfate. After filtration, the solvent was removed to yield 6.57g (~99%) of the desired product.

^1H NMR ($CDCl_3$) δ = 2.15 (t, 1H, OH), 2.65 (d, 1H, OH), 3.56 (m, 2H, CH_2), 3.68 (m, 2H, CH_2), 3.90 (m, 1H, CH), 4.56 (s, 2H, CH_2), 7.32 (m, 5H, Ph) ppm.

Otherwise identical with the commercially available material.

(R)-1-Benzyloxy-3-O-*t*-butyldiphenylsilylpropanol (4)

To 5 g (27.4 mmol) of (S)-3-benzyloxy-1,2-propandiol (**3**) dissolved in 200 ml of anhydrous methylene chloride was added 0.25 g 4-dimethylaminopyridine and 6 ml of triethylamine followed by dropwise addition of 10 ml (37.7 mmol) of t-butyldiphenyl chlorosilane while stirring under argon. The reaction mixture was stirred overnight at ambient temperature and the mixture was then washed with saturated $NaHCO_3$ solution. The resulting solution was dried over sodium sulfate and evaporated to dryness. Column chromatography (silica gel) with gradient methanol in methylene chloride yielded 10.4 g (90%) of desired product as an oil.

R_f (methylene chloride/methanol 98.5/1.5): 0.32.

UV (methanol): λ_{max} = 206, 252, 259, 264, 270 nm, λ_{min} = 239, 256, 262, 268 nm.

HRMS(FAB): for $C_{26}H_{32}O_3SiNa$ ($M + Na^+$) calculated: 443.2018, found 443.2016

^1H NMR($CDCl_3$) δ = 1.06 (s, 9H, $3CH_3$), 2.48 (d, 1H, OH), 3.57 (m, 2H, CH_2), 3.73 (d, 2H, CH_2), 3.92 (m, 1H, CH), 4.53 (s, 2H, CH_2), 7.25-7.75 (m, 15H, ArH) ppm.

^{13}C NMR ($CDCl_3$) δ = 19.2, 26.8, 64.7, 70.8, 70.9, 73.4, 127.7, 127.8, 128.4, 129.78, 133.2, 135.6 ppm.

(R)-1-Benzyloxy-2-chloromethoxypropane-3-O-*t*-butyldiphenylsilylpropane (5)

To 4.21 g (10 mmol) of (R)-1-benzyloxy-3-O-t-butyldiphenylsilyl propanol (**4**) dissolved in 50 ml of anhydrous methylene chloride was added 1.39 g of paraformaldehyde. The mixture was cooled to -4°C and HCl gas was passed through the solution until all solid material had disappeared. The mixture was sealed and stored at 0°C overnight. The reaction mixture was dried over sodium sulfate and evaporated to dryness at low temperature (10°C). The crude product obtained in this manner was used directly in the following step.

R_f (methylene chloride/methanol 98.5/1.5): 0.35.

^1H NMR ($CDCl_3$) δ = 1.04 (s, 9H, $3CH_3$), 3.64 (m, 2H, CH_2), 3.79 (d, 2H, CH_2), 4.05 (m, 1H, CH), 4.53 (s, 2H, CH_2), 5.61 (dd, 2H, CH_2), 7.25-7.75 (m, 15H, ArH) ppm.

(R)-1-{[2-O-*t*-Butyldiphenylsilyl-1-(benzyloxymethyl)ethoxy]methyl}thymine (6)

To 1.26 g (10 mmol) of oven dried (100°C) thymine suspended in 150 ml of anhydrous acetonitrile was added 5 ml of N,O-bis(trimethylisilyl)acetamide. The mixture was stirred at room temperature under argon for 30 min at which point the solution had turned clear. The mixture was cooled in ice-water bath and the crude (R)-1-benzyloxy-2-chloromethoxypropane-3-O-*t*-butyldiphenylsilyl propane (**5**) from the previous step was added. To this cooled mixture was added dropwise 2 ml of $SnCl_4$. After 10 min, the ice-water bath was

removed and the mixture was stirred further for 1h. At this point, 200 ml of methylene chloride was added and the mixture was washed with saturated $NaHCO_3$ solution. The organic layer was dried over sodium sulfate and evaporated to dryness. Flash column chromatography (silica gel) with a 0 to 5% gradient methanol in methylene chloride yield 4.5 g (80%) of desired product.

R_f (methylene chloride/methanol, 95/5): 0.63.

UV (methanol): λ_{max} = 203 (ε = 37.9), 264 (ε = 8.65) nm; λ_{min} = 236 nm.

HRMS (FAB) for $C_{32}H_{40}N_2O_5Si$ ($M+H^+$) calculated 559.2621, found 559.2628.

^1H NMR ($CDCl_3$) δ = 1.03 (s, 9H, $3CH_3$), 1.80 (s, 3H, CH_2), 3.52 and 3.57 (dd and dd, 2H, CH_2), 3.70 (d, 2H, CH_2), 3.91 (m, 1H, CH), 4.48 (s, 2H, CH_2), 5.22 (dd, 2H, CH_2), 7.09 (s, 1H, ArH), 7.2-7.7 (15H, ArH), 8.61 (s, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) δ = 12.2, 19.2, 26.8, 63.7, 69.9, 73.4, 76.2, 78.8, 111.1, 127.6, 127.7, 128.4, 129.8, 133.1, 135.5, 137.9, 139.3, 150.9, 163.80 ppm.

(R)-1-{[2-O-*t*-Butyldiphenylsilyl-1-(hydroxymethyl)ethoxy]methyl}thymine (7)

To 560 mg (1.0 mmol) of (R)-1-{[2-O-t-butyldiphenylsily-1-(benzyloxymethyl)ethoxy]-methyl}-thymine (**6**) dissolved in 30 ml ethanol and 20 ml cyclohexene was added 0.6 g of 20% $Pd(OH)_2/C$ and the mixture was refluxed for 24 h. The solid residue was removed by filtration and the filtrate was evaporated to dryness to yield 462 mg (98%) of the desired product.

R_f (methylene chloride/methanol, 95/5): 0.39.

UV (methanol): λ_{max} = 204 (ε = 30.2), 220 (shoulder), 264 (ε = 8.70) nm; λ_{min} = 235 nm.

HRMS (FAB) for $C_{25}H_{33}N_2O_5Si$ ($M+H^+$) calculated 469.2159, found 469.2159.

^1H NMR ($CDCl_3$), δ = 1.06 (s, 9H, $3CH_3$), 1.86 (s, 3H, CH_3), 2.57 (s, 1H, OH), 3.6-3.9 (m, 5H, $2CH_2$ and CH), 5.21 (dd, 2H, CH_2), 7.05 (s, 1H, ArH), 7.35-7-70 (m, 10H, ArH's), 9.75 (b, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) δ = 12.3, 19.2, 26.8, 62.5, 64.0, 76.8, 80.2, 111.6, 127.8, 129.9, 132.9, 133.0, 135.6, 139.4, 151.3, 164.0 ppm.

(R)-1-{[2-O-*t*-Butyldiphenylsilyl-1-O-(4,4'-dimethoxytritylmethyl)ethoxy]methyl}thymine (8)

To 460 mg (0.98 mmol) of (R)-1-{[2-O-t-butyldiphenylsily-1-(hydroxymethyl)ethoxy]-methy}-thymine dissolved in anhydrous pyridine was added 500 mg (1.48 mmol) of 4,4'-dimethoxytriphenylmethyl chloride. The mixture was stirred at ambient temperature for 4h at which point the pyridine was removed by rotary evaporation. Column chromatography (silica gel) with gradient methanol in methylene chloride (0 to 2.5%) yielded 698 mg (92%) of desired product.

R_f (methylene chloride/methanol, 95/5): 0.52.

UV (methanol): λ_{max} = 202 (ε = 74.4), 235 (shoulder), 267 (ε = 19.2) nm; λ_{min} = 255 nm.

HRMS (FAB) for $C_{46}H_{51}N_2O_7Si$ ($M+H^+$) calculated 771.3466, found 771.3466.

^1H NMR ($CDCl_3$), δ = 0.97 (s, 9H, $3CH_3$), 1.80 (s, 3H, CH_3), 3.48 (m, 2H, CH_2), 3.66 (d, 2H, CH_2), 3.77 (s, 6H, $2CH_3$), 3.91 (m, 1H, CH), 5.24 (dd, 2H, CH_2), 6.75-7.65 (m, 24H, ArH's), 8.22 (s, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) δ = 12.2, 19.2, 26.7, 55.2, 63.6, 64.0, 76.3, 79.3, 86.2, 111.2, 113.1, 126.8, 127.7, 127.8, 128.1, 129.8, 130.0, 133.0, 135.5, 135.9, 139.2, 144.8, 150.7, 158.4, 163.7 ppm.

(S)-1-{[2-O-4,4'-Dimethoxytrityl-1-(hydroxymethyl)ethoxy]methyl}thymine (9)

To 500 mg (0.65 mmol) of (R)-1-{[2-O-*t*-butyldiphenylsily-1-O-(4,4'-dimethoxytrityloxymethyl)-ethoxy]methyl}thymine (**8**) dissolved in 50 ml of THF was added 2 ml of 1 M nBu_4NF (2 mmol) in THF. The reaction mixture stirred at room temperature for 5h. A few grams of silica gel was added to the reaction, the solvent was removed and solid material was added to the top of a freshly packed column of silica gel. Flash column chromatography (methylene chloride/methanol) yielded 319 mg (92%) of the desired product.

R_f (methylene chloride/methanol, 95/5): 0.40.

UV (methanol): λ_{max} = 206 (ε = 85.7), 234 (ε = 23.4), 267 (ε = 9.76) nm; λ_{min} = 223, 255 nm.

HRMS (FAB) for $C_{30}H_{33}N_2O_7$ ($M+H^+$) calculated 532.2209, found 532.2210.

^1H NMR ($CDCl_3$), δ = 1.87 (s, 3H, CH_3), 2.38 (b, 1H, OH), 3.21 (d, 2H, CH_2), 3.65 (m, 2H, CH_2), 3.78 (s, 6H, $2CH_3$), 3.84 (m, 1H, CH), 5.26 (dd, 2H, CH_2), 6.8-7.45 (14H, ArH's), 8.83 (b, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) δ = 12.3, 55.2, 63.0, 63.7, 76.8, 79.3, 86.6, 111.5, 113.2, 126.9, 127.9, 128.1, 130.0, 135.8, 139.3, 144.6, 151.1, 158.6, 163.7 ppm.

Specific rotations (literature value for **9**: -4.40°[37]): $[\alpha]^{20}_{546}$ = -4.50°

(R)-1-{[2-O-(2-Cyanoethoxy)-diisopropylaminophosphino-1-O-(4,4'-dimethoxytritylmethyl)ethoxy]methyl}thymine (10)

To 533 mg (1 mmol) of (S)-1-{[2-O-4,4'-dimethoxytrityl-1-(hydroxymethyl)ethoxy]methyl}-thymine (**9**) dissolved in 60 ml freshly distilled anhydrous methylene chloride was added 1 ml of anhydrous diisopropyl-ethylamine followed by 400 mg (1.69 mmol) of 2-cyanoethyl N,N-diisopropylchloro-phosphoramidite. The reaction mixture was stirred under argon for 1.5h after which 1 ml of methanol was added to stop the reaction. The mixture was washed with saturated $NaHCO_3$ and dried over sodium sulfate. The organic solution was evaporated to dryness and the residue was precipitated into hexane to yield 667 mg (91%) of desired product.

R_f (methylene chloride/methanol, 95/5): 0.55.

^{31}P NMR ($CDCl_3$) δ = 148.4, 148.6 ppm.

HRMS (FAB) for $C_{39}H_{50}N_4O_8P$ ($M+H^+$) calculated 733.3366, found 733.3366.

(R)-4-Amino-1-{[2-O-t-butyldiphenylsilyl-1-(benzyloxymethyl)ethoxy]methyl}-5-methyl-2,6-[1*H*,3*H*]-pyrimidione (12)

To 1 g (7 mmol) of oven dried (100°C) 6-aminothymine suspended in 100 ml of anhydrous acetonitrile was added 5 ml of N,O-bis(trimethylsilyl)acetamide. The mixture stirred at ambient temperature under argon for 30 min at which point it became clear. The mixture was cooled (ice-water bath) and (R)-1-benzyloxy-2-chloromethoxypropane-3-O-t-butyldiphenylsilylpropane (**5**) [prepared from 2.7 g (6.4 mmol) of (R)-1-benzyloxy-3-O-t-butyldiphenylsilyl propanol (**4**)] was added followed by 1.5 ml of $SnCl_4$ (dropwise). After 10 min, the ice-water bath was removed and the mixture stirred an additional 1h at ambient temperature. At this point, 200 ml of methylene chloride was added and the mixture was washed with saturated $NaHCO_3$ solution.

The organic layer was dried over sodium sulfate and evaporated to dryness. Flash column chromatography (silica gel) with a 0 to 5% gradient of methanol in methylene chloride yielded 1.77 g (48%) of the desired product (**12**) as the major product and 0.81 g (22%) of (**11**) as the minor product. Assignment of the identity of the two regioisomers (**11** and **12**) is described after the product characterizations:

Characterization of **12**:

R_f (methylene chloride/methanol, 95/5): 0.26.

UV (methanol): λ_{max} = 206, 273 nm; λ_{min} = 240 nm.

HRMS (FAB) for $C_{32}H_{40}N_3O_5Si$ ($M+H^+$) calculated 574.2731, found 574.2737.

^1H NMR (DMSO-d_6) δ = 0.95 (s, 9H, $3CH_3$), 1.66 (s, 3H, CH_3), 3.40-3.80 (m, 4H, $2CH_2$), 4.07 (m, 1H, CH), 4.45 (s, 2H, CH_2), 5.28 (s, 2H, CH_2), 6.08 (s, 2H, NH_2), 7.20-7.70 (m, 15H, ArH), 10.28 (s, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) δ = 7.8, 19.2, 26.8, 63.2, 69.8, 73.3, 78.7, 81.7, 127.5, 127.6, 127.7, 128.3, 128.4, 129.8, 133.1, 133.2, 135.5, 137.9, 150.0, 152.0, 163.1 ppm.

Partial characterization of **11**:

R_f (methylene chloride/methanol, 95/5): 0.42.

^1H NMR (DMSO-d_6) δ = 0.95 (s, 9H, $3CH_3$), 1.66 (s, 3H, CH_3), 3.52 (m, 2H, CH_2), 3.64 (m, 2H, CH_2), 3.96 (m, 1H, CH), 4.46 (s, 2H, CH_2), 5.46 (dd, 2H, CH_2), 6.31 (s, 2H, NH_2), 7.20-7.70 (m, 15H, ArH), 10.56 (s, 1H, NH) ppm.

Regioisomer assignment: Assignment of the structure of the two regioisomers is based upon the chemical shift of the exocyclic amine in DMSO as described for a series of simple alkylation compounds.[14]

(R)-4-Amino-4-N-[1-(dimethylamino)ethylidine]-1-{[2-O-t-butyldiphenylsilyl-1-(benzyloxymethyl)ethoxy]methyl}-5-methyl-2,6-[1*H*,3*H*]-pyrimidione (13)

To 1 g (1.74 mmol) of (R)-4-amino-1-{[2-O-t-butyldiphenylsilyl-1-(benzyloxy-methyl)ethoxy]-methyl}-5-methyl-2,6-[1*H*,3*H*]-pyrimidione (**12**) dissolved in anhydrous methylene chloride was added 2 ml of N,N-dimethylacetamide dimethyl acetal. The mixture was stirred at ambient temperature for 2h at which point 1 ml water was added to stop the reaction. The mixture was dried over sodium sulfate and evaporated to dryness. Column chromatography (silica gel) with 2.5% methanol in methylene chloride yielded 0.92 g (82%) of product.

R_f (methylene chloride/methanol, 95/5): 0.60.

UV (methanol): λ_{max} = 204 (ε = 63.2), 287 (ε = 17.5) nm; λ_{min} = 251 nm.

HRMS (FAB) for $C_{36}H_{47}N_4O_5Si$ ($M+H^+$) calculated 643.3305, found 643.3316.

^1H NMR ($CDCl_3$) δ = 1.02 (s, 9H,3H3), 1.65 (s, 3H, CH_3), 1.81 (s, 3H, CH_3), 2.98 (s, 6H, $2CH_3$), 3.60 (dd and dd, 2H, CH_2), 3.74 (dd and dd, 2H, CH_2), 4.19 (m, 1H, CH), 4.51 (s, 2H, CH_2), 5.54 (s, 2H, CH_2), 7.20-7.75 (m, 15H, ArH), 8.14 (s, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) δ = 9.9, 16.5, 19.2, 26.8, 63.8, 70.2, 73.1, 79.2, 93.4, 94.4, 127.3, 127.5, 127.6, 128.2, 129.5, 133.5, 135.6, 138.5, 151.6, 153.1, 159.7, 165.0 ppm.

(R)-4-Amino-4-N-[1-(dimethylamino)ethylidine]-1-{[2-O-*t*-butyldiphenylsilyl-1-(hydroxymethyl)ethoxy]methyl}-5-methyl-2,6-[1*H*,3*H*]-pyrimidione (14)

To 0.9 g (1.4 mmol) of (R)-4-amino-4-N-[1-(dimethylamino)ethylidine]-1-{[2-O-t-butyldiphenylsily-1-(benzyloxymethyl)ethoxy]-methyl}-5-methyl-2,6-[1*H*,3*H*]-pyrimidione (**13**) dissolved in 50 ml of ethanol and 35 ml cyclohexene was added 1.0 g of 20% $Pd(OH)_2/C$ and the mixture was refluxed for 50 h. The solid residue was removed by filtration and the filtrate was evaporated to dryness to yield 745 mg (96%) of product.
R_f (methylene chloride/methanol, 95/5): 0.35.
UV (methanol): λ_{max} = 202 (ε = 36.9), 219 (shoulder), 290 (ε = 16.6) nm; λ_{min} = 251 nm.
HRMS (FAB) for $C_{29}H_{41}N_4O_5Si$ ($M+H^+$) calculated 553.2834, found 553.2846.
^1H NMR ($CDCl_3$) δ = 1.04 (s, 9H, $3CH_3$), 1.65 (s, 3H, CH_3), 1.91 (s, 3H, CH_3), 3.03 (s, 6H, $2CH_3$), 3.60-3.84 (m, 4H, $2CH_2$), 3.92 (m, 1H, CH), 5.34 and 5.64 (d and d, 2H, CH_2), 7.35-7.75 (m, 10H, ArH), 7.92 (b, 1H, NH) ppm.
^{13}C NMR ($CDCl_3$) δ = 9.9, 16.7, 19.2, 26.8, 62.8, 63.6, 70.2, 80.0, 93.4, 94.1, 127.7, 129.7, 133.2, 135.6, 151.4, 153.4, 159.8, 164.96 ppm.

(R)-4-Amino-4-N-[1-(dimethylamino)ethylidine]-1-{[2-O-*t*-butyldiphenylsilyl-1-O-(4,4'-dimethoxytritylmethyl)ethoxy]methyl}-5-methyl-2,6-[1*H*,3*H*]-pyrimidione (15)

To 360 mg (0.65 mmol) of (R)-4-amino-4-N-[1-(dimethylamino)ethylidine]-1-{[2-O-*t*-butyldiphenylsilyl-1-(hydroxymethyl)-ethoxy]-methyl}-5-methyl-2,6-[1*H*,3*H*]-pyrimidione (**14**) dissolved in 20 ml anhydrous pyridine was added 300 mg (0.885 mmol) of DMT-Cl. The mixture was stirred at ambient temperature for 3h at which point the pyridine was removed. Column chromatography (silica gel) with a gradient of methanol in methylene chloride (0 to 2%) yielded 481 mg (86%) of the desired product.
Rf (methylene chloride/methanol, 95/5): 0.62.
UV (methanol): λ_{max} = 203 (ε = 77.8), 220 (shoulder), 234 (shoulder), 284 (ε = 14.0) nm; λ_{min} = 257 nm.
HRMS (FAB) for $C_{50}H_{59}N_4O_7Si$ ($M+H^+$) calculated 855.4153, found 855.4153.
^1H NMR ($CDCl_3$) δ = 0.94 (s, 9H, $3CH_3$), 1.66 (s, 6H, $2CH_3$), 2.96 (s, 6H, $2CH_3$), 3.12 (dd, 2H, CH_2), 3.64 (dd, 2H, CH_2), 3.76 (s, 6H, $2CH_3$), 4.35 (m, 1H, CH), 5.64 (dd, 2H, CH_2), 6.70-7.65 (m, 23H, ArH), 7.73 (s, 1H, NH),
^{13}C NMR ($CDCl_3$) δ = 10.0, 16.2, 19.1, 26.7, 55.2, 64.12, 70.7, 80.3, 85.7, 93.4, 113.0, 126.4, 127.6, 128.2, 129.5, 130.1, 133.4, 135.6, 136.3, 136.4, 145.2, 151.4, 153.1, 158.2, 159.8, 165.0 ppm.

(S)-4-Amino-4-N-[1-(dimethylamino)ethylidine]-1-{[2-O-4,4'-dimethoxytritylmethyl-1-(hydroxymethyl)ethoxy]methyl}-5-methyl-2,6-[1*H*,3*H*]-pyrimidione (16)

To 400 mg (0.47 mmol) of (R)-4-amino-4-N-[1-(dimethylamino)ethylidine]-1-{[2-O-t-butyldiphenylsilyl-1-O-(4,4'-dimethoxytritylmethyl) ethoxy]-methyl}-5-methyl-2,6-[1*H*,3*H*]-pyrimidione (**15**) dissolved in 30 ml THF was added 1.5 ml of 1M nBu4NF in THF. The reaction mixture was stirred at ambient temperature for 5h at which point a few grams of silica gel was added. The solvent was removed and the residue was applied to the top of a silica gel column. Chromatography (methylene chloride with a gradient of methanol) yielded 252 mg (87%) of the desired product.
Rf (methylene chloride/methanol, 95/5): 0.40.
UV (methanol): λ_{max} = 204 (ε = 52.5), 232 (shoulder), 285 (ε = 12.8) nm; λ_{min} = 258 nm.
HRMS (FAB) for $C_{34}H_{41}N_4O_7$ ($M+H^+$) calculated 617.2975, found 617.2975.

^{1}H NMR ($CDCl_3$) δ = 1.66 (s, 3H, CH_3), 1.87 (s, 3H, CH_3), 3.01 (s, 6H, $2CH_3$), 3.14 (m, 2H, CH_2), 3.63 (m, 2H, CH_2), 3.76 (3, 6H, $2CH_3$), 3.96 (m, 1H, CH), 5.40 and 5.68 (d and d, 2H, CH_2), 6.75-7.50 (m, 13H, ArH), 7.80 (b, 1H, NH) ppm.
^{13}C NMR ($CDCl_3$) δ = 9.9, 16.6, 55.2, 63.3, 63.6, 70.5, 79.3, 86.0, 93.4, 113.01, 126.7, 127.78, 128.2, 130.1, 136.0, 144.9, 151.5, 153.6, 158.4,, 159.8, 165.0 ppm.
$[\alpha]^{20}_{546}$: -4.06°

(R)-4-Amino-4-N-[1-(dimethylamino)ethylidine]-1-{[2-O-(2-cyanoethoxy)-diisopropylamino-phosphino-1-O-(4,4'-dimethoxytritylmethyl)ethoxy]-methyl}-5-methyl-2,6-[1*H*,3*H*]-pyrimidione (17)

To 154 mg (0.25 mmol) of (S)-4-amino-4-N-[1-(dimethylamino)ethylidine]-1-{[2-O-4,4'-dimethoxytritylmethyl-1-(hydroxymethyl)ethoxy]-methyl}-5-methyl-2,6-[1*H*,3*H*]-pyrimidione (**16**) dissolved in 15 ml of anhydrous methylene chloride was added 0.2 ml diisopropylethylamine. To this mixture, was added 120 mg (0.5 mmol) of 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite and the reaction mixture stirred at ambient temperature for 1h. The reaction was then stopped with 0.5 ml of methanol and the resulting mixture was washed with saturated $NaHCO_3$ and dried over sodium sulfate. The organic solution was evaporated to dryness and the residue was precipitated into hexane to yield 184 mg (90%) of desired product.
R_f (methylene chloride/methanol, 95/5): 0.51.
^{31}P NMR ($CDCl_3$) δ = 147.7, 147.6 ppm.
Mass spectral data yielded only fragments of the compound [i.e., 621, m - $(iPr)_2POCH_2CH_2CN$].

DNA Synthesis

The 15-mers containing acyclic derivatives (aT and $am^{5ox}C$) as well as the native 25-mers were prepared by solid phase DNA synthesis under standard conditions.[15] Both analogues could be incorporated into DNA strands with coupling efficiencies that were comparable to those of common nucleoside phosphoramidites.

Deprotection of the amidine protecting group present on oligonucleotides containing $am^{5ox}C$ was done under the commonly used conditions of conc. ammonia at 50 °C overnight (12h). Purification of the oligonucleotides was accomplished by HPLC (trityl on) using 50 mM triethylammonium acetate (pH 7.0) and a gradient of acetonitrile (20 - 65% over 40 min). The DMT-protected 15 mer oligonucleotides had retention times of about 20 min. The collected oligonucleotides were reduced in volume, detritylated with 80% aqueous acetic acid (30 min, 0 °C), desalted (Sephadex G-10), and stored at -20 °C. The resulting oligonucleotides were analyzed by HPLC (4.6 x 250 mm column of ODS-Hypersil) using 20 mM potassium phosphate, pH 5.5 with a gradient of methanol (0 to 100% over 60 min). The 15-mer oligonucleotides had retention times of about 20 min and eluted as single peaks.

Nucleoside Analysis

Oligomers containing acyclic T or acyclic $m^{5ox}C$ were effectively digested into monomeric units only with a combination of spleen phosphodiestease, S1 nuclease, and snake venom phosphodiesterase/calf intestinal alkaline phosphatase: a 10 µL reaction mixture containing 0.5 A_{260} unit of oligomer in 200 mM sodium

chloride/5 mM $MgCl_2$/0.1 mM $ZnSO_4$/25 mM sodium acetate, pH 5.5, 1 unit of spleen phosphodiesterase and 1 unit of S1 nuclease was incubated overnight at 37°C. To this mixture was added 5 µL of 0.1 M Tris-HCl, pH 8.0, and 1 unit of calf intestinal alkaline phosphatase and, 1 unit of snake venom phosphodiesterase and the reaction incubated for an additional 60 min at ambient temperature. An aliquot of this mixture was analyzed by HPLC (4.6 x 250 mm column of ODS-Hypersil, 50 mM triethylammonium acetate pH 7.0) and resulted in the elution of aT with a retention time of 11.3 min, and $am^{5ox}C$ with a retention time of 13.7 min (0 - 50% acetonitrile over 2 h).

Thermal Denaturation Studies

Thermal denaturation studies were performed in 25 mM PIPES pH 6.4 or pH 7.0, 25 mM HEPES, pH 7.5 or pH 8.0 and 10 mM magnesium chloride and 200 mM sodium chloride at triplex concentrations in the low micromolar range (~1 µM). Absorbance and temperature values were measured with an AVIV 14DS UV/Visible spectrophotometer equipped with digital temperature control. The temperature of the cell compartment was increased by 0.5 °C steps (from 0 to 95 °C) and when thermal equilibrium was reached, temperature and absorbance data were collected. T_m values were determined both from first-order derivatives and by graphical analysis of the Absorbance vs Temperature plots.

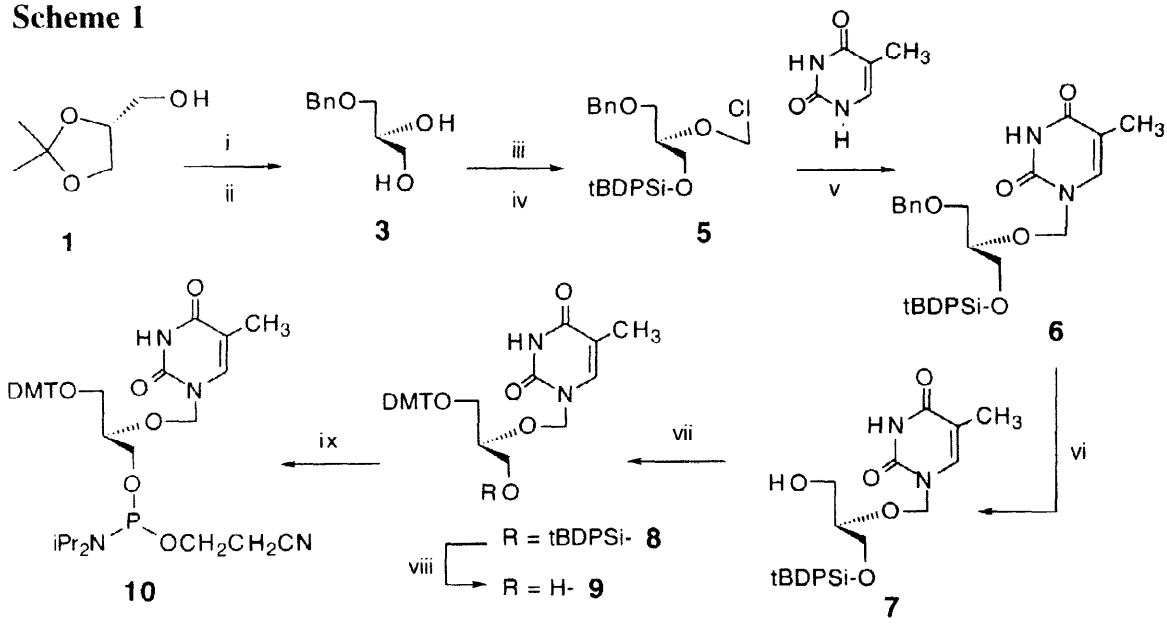
Results and Discussion

The synthesis of a number of acyclic nucleosides, those containing an acyclic or ring-opened carbohydrate residue, have been described using a variety of acyclic residues. Members of this class of agent can function as effective antivirals. For example the guanine derivative 9-[(2-hydroxyethoxy)-methyl]guanine (DHPG), has been approved for clinical use against cytomegalovirus,[16] and both DHPC and acylovir are active inhibitors of herpes simplex virus type 1.[17] In addition to their value as antiviral agents, a few of these derivatives have subsequently been incorporated into DNA sequences for examination as potential hybridization probes,[18-20] but the decrease in observed double-stranded helix stability when such derivatives are present has largely diminished further interest in these analogues. DNA containing acyclic nucleosides has not been examined to date in studies involving DNA triplexes.

Nucleoside Synthesis

The acyclic nucleosides chosen for study in DNA triplexes are those that can be viewed as having a ring-opened carbohydrate as the result of the elimination of the C-2' carbon. They have been prepared most simply by the glycosylation of the desired heterocycle with 2-O-(acetoxymethyl)-1,3-di-O-benzylgycerol.[18,21-23] Unfortunately, this approach generates a racemic mixture when the *meso* acyclic nucleoside is deprotected and converted to the DMT-protected phosphoramidite derivative necessary for incorporation into DNA by chemical synthesis protocols. Maintaining the C4'-chiral center in such derivatives can be accomplished by using the mono-benzyl derivative, (S)-3-(benzyloxy)1,2-propanediol (see **3**, Scheme 1),[18] but the exorbitant cost of this material makes the route using this starting material less attractive. Additionally, the use of the describedpivaloyl protecting group, in conjunction with the single benzyl group, maintains the integrity of the stereochemical center, but the synthesis is still complicated by the observation that the pivaloyl group undergoes migration[18] during the chloromethylation reaction. To eliminate these concerns, we chose an

Scheme 1



(i) NaH, Bn-Br, (ii) HCl, CH_3OH, (iii) tBDPSi-Cl, DMAP, (iv) $(CH_2O)_n$, HCl, (v) $SnCl_4$, (vi) $Pd(OH)_2/C$, cyclohexene (vii) DMT-Cl/pyridine, (viii) nBu_4NF, (ix) $iPr_2NP(Cl)OCH_2CH_2CN$.

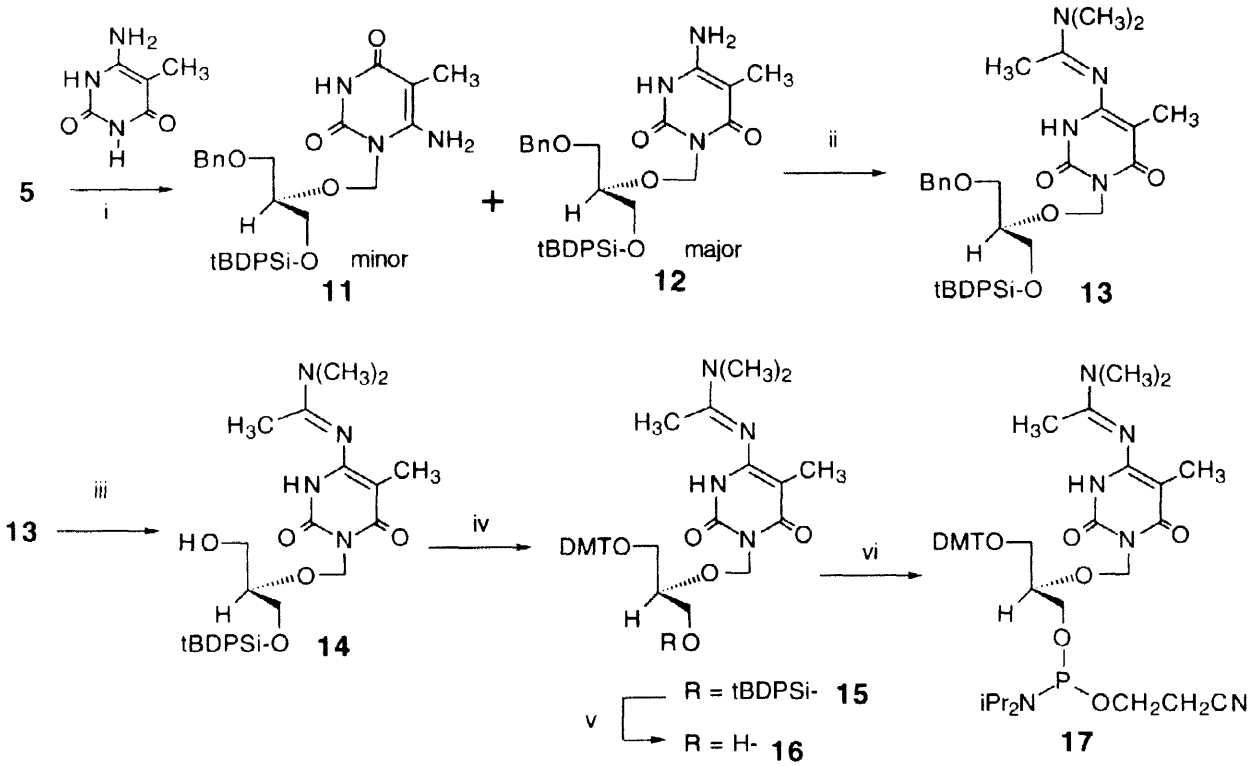
alternative route for the synthesis of the acyclic T derivative (aT) that begins with (*R*)-(-)-2,2-dimethyl-1,3-dioxolane-4-methanol (**1**, Scheme 1). Benzylation of the primary hydroxyl group followed by hydrolysis of the isopropylidene readily generates **3**. We replaced the pivaloyl group with the *t*-butyldiphenylsilyl protecting group (tBDPSi), which was stable to the conditions of chloromethylation, and did not undergo migration during the reaction. The chloromethyl derivative **5** could be used to glycosylate thymine to generate **6**. The fully protected acyclic nucleoside could then be deprotected in a step-wise manner in order to introduce both the DMT-group and the phosphoramidite moiety without loss of optical activity, and generate the aT phosphoramidite derivative **10**.

For a C-like acyclic nucleoside, we chose the heterocycle 6-aminothymine and glycosylated it with the acyclic carbohydrate **5** (Scheme 2). As with the 2'-deoxynucleoside,[2] glycosylation of 6-aminothymine produced two compounds, the major product (**12**) can be viewed as a 5-methyl-6-oxo-cytosine derivative ($am^{5ox}C$), while the minor product (**11**) is the 6-aminothymine acyclic nucleoside. The two regioisomers could be separated chromatographically, and the amino group of the desired cytosine derivative protected as the amidine derivative (**13**). Then in a manner similar to that described for aT, the carbohydrate could be deprotected in a step-wise manner and converted to the DMT-protected phosphoramidite without loss of stereochemical integrity.

DNA Synthesis

DNA sequences of 15 residues were prepared containing from two to six of the acyclic nucleoside analogues using standard phosphoramidite DNA synthesis protocols. The coupling yields for the acyclic nucleoside derivatives were indistinguishable from those of the common nucleoside derivatives based upon the intensity of the liberated DMT cation. After assembly, the oligonucleotides could be purified by HPLC. To

Scheme 2



i. BSA, $SnCl_4/CH_3CN$, 0 °C→25 °C; Chromatography; ii. $CH_3(OCH_3)_2N(CH_3)_2/CH_2Cl_2$; iii. $Pd(OH_2)/C$ in ethanol/cyclohexene 3/2, reflux; iv. DMT-Cl/pyridine; v. nBu_4N^+-F/THF; vi. $ClP(OCH_2CH_2CN)N(iPr)_2$/ $DIEA/CH_2Cl_2$.

confirm that the acyclic nucleoside were present in the sequences, a small portion was digested and analyzed by HPLC. A single nuclease was not sufficient to generate the nucleoside building blocks – it appears that the acyclic nucleosides can be refractory to some nucleases. Only in the presence of three enzymes (nuclease P1, S1 nuclease and snake venom phosphodiesterase) could the sequences be completely converted to the corresponding nucleosides.

DNA Triplex Studies

These 15-mers, containing from two to six acyclic residues were used to target a 25-mer DNA duplex containing a polypurine target sequence (see Table 1). The third strand contained T residues for the targeting of A-T base pairs, and m^5C or $m^{5ox}C$ base residues for the targeting of G-C base pairs. We initially examined a target sequence containing four G-C base pairs (and 11 A-T base pairs) arranged so that all of the G-C base pairs had A-T nearest neighbors. This design eliminated the problem of charge-charge repulsion at adjacent sites when targeting the G-C base pairs with 5-methyl-2'-deoxycytidine (M). Relative triplex stability was determined from thermally induced transitions at two pH values (with and without spermine) (Table 1). The substitution of three native T residues by the acyclic derivative (aT) in a triplex containing four isolated target G-C base pairs resulted in a dramatic reduction in the T_m value from 44 °C to 25 °C at pH 6.4 (compare entries 1 and 3, Table 1). A corresponding reduction in T_m from 35 °C to 14 °C was observed at pH 7.0. The presence of the polyamine spermine has been observed to enhance the stability of DNA triplexes, and in the presence of

Table 1. T_m Values for T- and aT-Containing DNA Triplexes[a]

```
          5' T T*T M T*T T*T M T M T M T T
5' G C G C G A A A G A A A A G A G A G A A C C C G G
3' C G C G C T T T C T T T T C T C T C T T G G G C C
```

entry	T*	[Spermine]	T_m (°C) pH 6.4	ΔT_m(°C)[b]	T_m (°C) pH 7.0	ΔT_m(°C)[b]
1	T	0	44	–	35	–
2	T	0.5 mM	55	–	43	–
3	aT	0	25	–19	14	–21
4	aT	0.5 mM	29	–26	16	–27

a Conditions: 10 mM PIPES (pH 6.4 or 7.0) 50 mM NaCl, 10 mM $MgCl_2$. M = m^5C, aT = the acyclic thymidine derivative.
b ΔT_m measures the change in T_m value between the T-containing and aT-containing DNA triplexes.

0.5 mM spermine, the T_m of the native T-containing 15-mer triplex was increased by 11 °C (compare entries 1 and 2, Table 1) at pH 6.4, and a similar increase of 9 °C was apparent at pH 7.0. However, corresponding increases in T_m values for the aT-containing triplex were more moderate. At pH 6.4 the aT-containing triplex exhibited an increase in T_m of only 4 °C (compare entries 3 and 4, Table 1) and an even smaller increase of 2 °C was observed at pH 7.0. These moderate increases in T_m values for the aT-containing sequence in the presence of spermine had the effects of reducing even further the corresponding ΔT_ms between the T-containing and aT-containing triplexes to –26 and –27 °C (compare entries 2 and 4, Table 1).

While the nucleoside derivative $m^{5ox}C$ can be used effectively, in a pH independent manner, to target isolated G-C base pairs in the formation of DNA triplexes, corresponding complexes containing contiguous G-C targets were quite unstable. Since this derivative functions as a bidentate hydrogen bond donor without the need for protonation, the charge-charge repulsion effects present with neighboring C^+-G-C (or M^+-G-C) base triplets should not be present. Two effects might contribute to the observed reduction in the stability of such complexes, (i) ineffective base stacking, or (ii) conformational preferences of the carbohydrate. In order to examine these possibilities, we prepared sequences containing 5-methyl-2'-deoxycytidine (M) and the acyclic nucleoside $am^{5ox}C$ for targeting a duplex containing five contiguous G-C base pairs. We recognized that the use of the flexible acyclic carbohydrate present in the $am^{5ox}C$ derivative may present an unfavorable entropy barrier to complex formation. On the other hand, the introduction of some flexibility into the backbone of the third strand might assist in optimizing the base-base interactions particularly at sites of contiguous G-C base pair targets (see Figure 1).

Triplexes of questionable stability were formed with a third strand composed of five contiguous m^5C residues over the pH range 6.4 - 8.5. In fact, only at pH 6.4 could any significant triplex transition be observed,and it exhibited only minimal hyperchromicity. Charge-charge repulsion by the protonated N^3-nitrogens is likely to limit hydrogen bonding and base stacking interactions at targets of contiguous G-C base pairs. Replacement of two of the M residues by the $am^{5ox}C$ 2'-deoxynucleoside derivative provides some

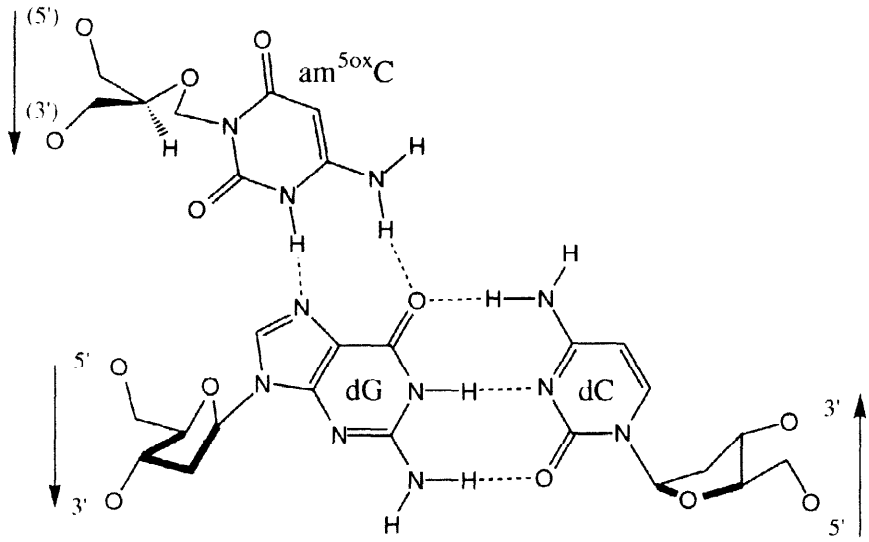


Figure 1. Base triplet involving $am^{5ox}C$ and a target dG-dC base pair.

separation of the charged M residues and results in triplex formation through a broader pH range, but the transitions occur at relatively low temperatures, particularly at neutral or slightly basic pH values (entries 1 and 2, Table 2, values in parentheses). The presence of minimal hyperchromicity in these transitions suggests less than optimal base stacking is present in such complexes. Replacement of these two $m^{5ox}C$ residues by the corresponding derivative containing a flexible acyclic linkage is a relatively moderate change. It simply introduces some added flexibility into the third strand, without altering the nature of the base-base interactions. Nevertheless, this alteration to the third strand results in T_m values that are increased by 7 - 8 °C over the pH range 7.0 - 8.5 (entries 1 and 2, Table 2). More moderate, but still positive effects (ΔT_m = +3 °C), were observed at pH 6.4 (Table 2). These transitions exhibit more favorable hyperchromicity, most notably at pH 6.4. With increasing pH, the hyperchromicity of the triplex transition decreases somewhat, presumably in relation to the extent of protonation of the m^5C residues. The presence of the polyamine spermine increased the T_m values by 2 - 3 °C in all cases. Complex formation for this triplex could be confirmed using a gel shift assay (data not shown).

A corresponding 15-mer was prepared in which three alternating M residues were replaced by the acyclic analogue (entries 3 and 4, Table 2). This triplex exhibited T_m values of roughly 20 °C, and these values were essentially independent of pH. Two more complexes were prepared in which additional m^5C residues were replaced by the $am^{5ox}C$ analogue (5 - 8, Table 1). Although these complexes exhibited T_m values that were slightly lower that those observed with the previous two complexes, well defined triplex transitions were present in all cases.

When all six G-C base pairs in the target duplex are targeted with the neutral, acyclic $am^{5ox}C$ residues the triplex transitions obtained at five different pH values (6.4, 7.0, 7.5, 8.0 and 8.5) were virtually identical (Figure 2). The hyperchromicity observed for the complex containing six $am^{5ox}C$ residues is reduced

Table 2. Effect of Acyclic Analogues on the T_m Values for m^5C and $m^{5ox}C$ Containing 15-mers Targeting Contiguous G-C Base Pairs[a]

```
5'        TTT Z TTTT X Y X Y X TT          3'
5'  GCGCGAAA G AAAA G G G G G AA CCCGG     3'
3'  CGCGCTTT C TTTT C C C C C TT GGGCC     5'
```

					pH Value				
entry	X[b]	Y[b]	Z[b]	[Spermine]	6.4	7.0	7.5	8.0	8.5
1	m^5C	$am^{5ox}C$	m^5C	-	22 (19)[c]	20 (13)	20 (12)	20 (12)	20 (12)
2	m^5C	$am^{5ox}C$	m^5C	0.5 mM	25 (28)	23 (15)	23 (15)	23 (16)	23 (nt)
3	$am^{5ox}C$	m^5C	m^5C	-	21	19	19	19	19
4	$am^{5ox}C$	m^5C	m^5C	0.5 mM	23	21	21	21	21
5	$am^{5ox}C$	$am^{5ox}C$	m^5C	-	16	16	16	16	16
6	$am^{5ox}C$	$am^{5ox}C$	m^5C	0.5 mM	18	18	18	18	18
7	$am^{5ox}C$	$am^{5ox}C$	$am^{5ox}C$	-	15 (nt)	15 (nt)	15 (nt)	15 (nt)	15 (nt)
8	$am^{5ox}C$	$am^{5ox}C$	$am^{5ox}C$	0.5 mM	17 (15)	17 (15)	17 (15)	17 (14)	17 (nt)

a. Buffer consists of 50 mM NaCl, 10 mM $MgCl_2$, and 25 mM PIPES (pHs 6.4 and 7.0) and 25 mM HEPES (pHs 7.5, 8.0 and 8.5).
b. "a" denotes the nucleoside derivative containing the acyclic carbohydrate linker and the base residue 5-methyl-cytosine (m^5C) or N^3-glycosylated 6-aminothymine ($m^{5ox}C$)
c. Values for the triplexes containing only native 2'-deoxyribose sugars (parentheses) were taken from ref. 2 (nt = no triplex).

somewhat (relative to complexes such as entry 1, Table 2), and this property likely reflects variations in base stacking effects for the modified cytosine base residues. The latter complexes, containing six acyclic linkers in the backbone of the third strand, are reduced somewhat in stability from those containing only 2 or 3 acyclic $am^{5ox}C$ residues (compare entry 7 with entries 1 and 3, Table 2). Nevertheless, these triplexes containing acyclic linkers are still much more stable than the (all) nucleoside complexes, either that containing five contiguous m^5C residues, or that employing five contiguous $m^{5ox}C$ residues - neither of which resulted in well-defined triplex transitions in the absence of spermine.

The triplexes reported here have employed an acyclic linker of known chirality that corresponds to that present in native nucleosides at C_4' of the deoxyribose ring as this seemed a prudent course of action. We have subsequently learned that triplexes prepared using the racemic $am^{5ox}C$ analogue, prepared using the procedures described in the experimental from the corresponding racemic starting material, exhibit T_m values that are virtually indistinguishable from those reported in Table 1.

Discussion

We have made two reciprocal observations with regard to the use of acyclic nucleosides in the formation of DNA triplexes. Replacing three deoxyribose residues of thymidine nucleosides in the third strand of an otherwise stable DNA triplex with the related acyclic derivative, results in a significant decrease in the T_m value for the complex ($\Delta T_m \sim -20\ ^oC$). By comparison, the introduction of two or three of the acyclic deoxyribose

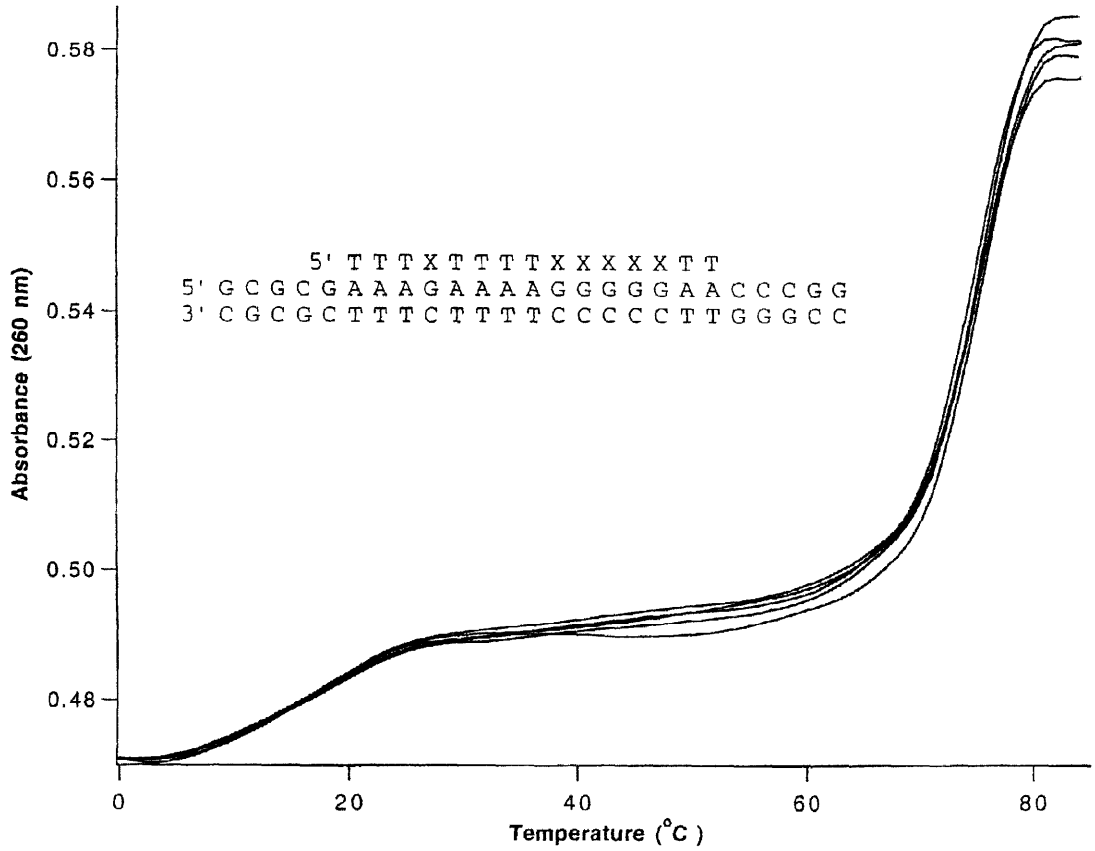


Figure 2. Overlay of absorbance vs. temperature plots for entry 7, Table 2 under the following conditions: 50 mM NaCl, 10 mM $MgCl_2$, and at pH values 6.4, 7.0, 7.5 and 8.0 M = m^5C, X = $am^{5ox}C$.

analogues into the $m^{5ox}C$ nucleoside at sites involving multiple target G-C base pairs results in a 7 - 8 °C increase in T_m. While these increases in T_m values appear to be moderate, we note that the cause of these effects, that of simply increasing the flexibility of the carbohydrate residue at two or three sites in the third strand, without altering base-base interactions, would not initially be expected to have any significant effects on the targeting of a DNA duplex by a third strand of DNA. These two observations suggest that increased flexibility in the DNA backbone does not assist in optimizing the base-base interactions in all cases, and most notably in the T-A-T base triplet. Presumably the favorable binding energy derived from the formation of two Hoogsteen hydrogen bonds and effective base stacking interactions cannot overcome the unfavorable entropy barrier necessary for the flexible linker to adopt the preferred conformation necessary for the base triplet to form. The more restricted cyclic deoxyribose residue may already confine the base residue to a more optimal orientation for base triplet formation. By comparison, the 7 - 8 °C increase in T_m for the triplexes containing the analogue cytosine base attached to the acyclic carbohydrate linker suggests that increased flexibility permits improved hydrogen bonding or base stacking interactions that results in enhanced stability at sites containing multiple G-C base pair targets. However, the effects from such acyclic linkers may be limited. As the number of acyclic residues present in the DNA triplexes studied here was increased from two to six, a slight decrease in

triplex T_m was observed, presumably as the unfavorable entropy effects overcome enhanced base stacking or hydrogen bonding interactions.

Previous studies[12] have indicated that the use of a *less* flexible bicyclic carbohydrate moiety for thymine in the third strand actually enhances binding for the T-A-T base triplet, presumably by limiting conformational flexibility of the carbohydrate linker to a range that is near optimal for base-base interactions. In the same study, reducing the conformational flexibility of the carbohydrate moiety of cytosine resulted in significant destabilization of a DNA triplex containing C^+-G-C base triplets, suggesting that the conformational preferences in that case were less than optimal. Restricted conformational effects in the carbohydrate moiety of the nucleosides are also present with the riboacetal linkage described for thymine and employed in DNA triplex studies.[13] Although in this latter study the conformational flexibility of the deoxyribose residue was restricted by making an analogue in which the internucleotide phosphate was also removed, control experiments suggested that the increased T_ms observed were primarily the result of the less flexible carbohydrate of thymine, and not simply due to the absence of the charged phosphodiester residue. The present work results in reciprocal observations regarding triplexes containing flexible acyclic linkers of thymine and cytosine analogues - added flexibility in the T-A-T base triplet is detrimental to complex stability, while added flexibility in the m^{5ox}C-G-C base triplet enhances triplex stability. More effective triplex formation should then result from the use of conformationally restricted carbohydrate moieties for thymine residues in conjunction with conformationally more flexible acyclic linkers for cytosine or cytosine analogues, particularly at sites of contiguous G-C base pair targets.

More effective targeting of the G-C base pairs by the analogue base may also reduce the pH dependence normally observed when C or m^5C residues are present in the third strand. With the described 15-mer, the substitution by only two acyclic linkers, optimally placed within the block of contiguous G-C base pair targets, resulted in a 7 - 8 ^oC increase in triplex T_m with no observable pH dependence, although in principle three m^5C^+-G-C base triplets should still be present in the complex. The lack of pH effects in these DNA triplexes suggests that protonation effects are not as critical to complex formation with the acyclic linkers as in the case of native nucleosides; it appears that base stacking effects predominate. The initial observation[24] that m^5C forms DNA triplexes with reduced pH dependence (relative to C) is generally thought to result from the enhanced base stacking ability of the methylated derivative,[25,26] rather than any real effect on the pKa of the N^3-nitrogen.[26] Similar effects have been noted for DNA duplexes containing T (vs. U)[27] or m^5C (vs. C).[28] Analogues designed to provide enhanced base stacking such as the propynyl T derivatives[29] also enhance triplex stability. While the introduction of a flexible acyclic linker does not add functionality to enhance base stacking interactions, the increased flexibility may permit more effective base stacking between adjacent base residues and thereby reduce the protonation effects normally present with m^5C^+-G-C base triplets.

We also note recent work involving the use of N^7-glycosylated guanine for the recognition of G-C base pairs in the parallel-stranded recognition motif.[8] The N^7G-G-C base triplets are significantly more stable in contiguous sequences than when present as isolated base triplets, although in either case, two hydrogen bonds should be present between the N^7G and the Hoogsteen functional groups of the target G residue. Such

differences can be explained as a result of variations in base stacking effects between adjacent N^7G residues vs. between N^7G and adjacent pyrimidines. Such effects are also likely to be mediated by the conformation of the carbohydrate residue linking adjacent base residues, particularly since the conformation will vary depending on whether two pyrimidines, two purines, or one of each is present. The carbohydrate conformation for adjacent N^7G residues is likely to differ from that present at alternating N^7G and T residues.

Although we have initially prepared derivatives that maintain a stereogenic center identical to that present at C4' of the native 2'-deoxyribonucleosides, more recent experiments indicate that the nature of this stereogenic center is relatively unimportant in the stability of these complexes. This observation re-enforces the suggestion that it is the flexibility of these carbohydrate analogues that permits more effective triplex formation, and not a specific conformation dictated by the presence of a single stereogenic site in the backbone of the acyclic analogue.

Conclusion

Parallel-stranded triplexes composed entirely of flexible linkers are unlikely to result in stable complexes since the stability of the T-A-T base triplet does not benefit from such flexibility. Nevertheless, particular sequence targets, including blocks of G-C sequences, or the effectiveness of specific base analogues employed to permit triplex formation at generalized sequence targets, may both benefit from the selected use of nucleoside analogues containing flexible acyclic linkers.

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