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Nucleic Acids Research, 1998, Vol. 26, No. 13 **3127–3135**

A comparative study of the thermal stability of oligodeoxyribonucleotides containing 5-substituted 2′-deoxyuridines

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Received April 2, 1998; Revised and Accepted May 6, 1998

ABSTRACT

Two series of modified oligonucleotides based on the self-complementary dodecamer d(CGCTAATTAGCG) were synthesized. The first contained the –C≡CCH$_2$R linker at C5 of deoxyuridine at position 4 (T*) of d(CGCT*AATTAGCG) and the second contained the –SR linker. The goal of the study was to evaluate and compare these two types of side chains for suitability as tethers for linking reporter groups to oligonucleotides. Our primary concern was how these tethers would effect duplex stability. The modified nucleosides were synthesized by palladium-mediated coupling reactions between the substituted alkyne and 5′-(4,4′-dimethoxytrityl)-5-iodo-2′-deoxyuridine and between a disulfide and 5-chloromercurio-2′-deoxyuridine. The C5 deoxyuridine side chains evaluated included C≡CCH$_3$, C≡CCH$_2$NHC(O)CH$_3$, C≡CCH$_2$N(CH$_3$)$_2$, C≡CCH$_2$NHC(O)C$_5$H$_4$N, C≡CCH$_2$NHC(O)C$_{10}$H$_{15}$, SCH$_3$, SC$_6$H$_5$ and SCH$_2$CH$_2$NHC(O)CH$_3$. The nucleosides containing these substituents were incorporated into oligodeoxyribonucleotides by standard phosphoramidite methodology. Melting studies demonstrated that the sequence containing the C≡CCH$_3$ side chain had the highest T_m value (59.1°C) in comparison with the control sequence (T_m = 55.2°C) and that any additional substituent on C3 of the propynyl group lowered the T_m value relative to propynyl. Nevertheless, even the most destabilizing substituent, adamantylcarbamoyl, yielded an oligodeoxyribonucleotide that dissociated with a T_m of 54°C, which is only 1.2°C less than the control sequence. In contrast, the thioether substituents led to lower T_m values, ranging from as low as 45.1°C for SPh up to 52.2°C for SMe. Replacing the methyl of the SMe substituent with a CH$_2$CH$_2$NHC(O)CH$_3$ tether led to no further reduction in melting temperature. The T_m value of the CH$_2$CH$_2$NHC(O)CH$_3$-containing oligonucleotide was less than the natural sequence by 1.6°C/substituent. This is sufficiently small that it is anticipated that the C5 thioether linkage may be as useful as the acetylenic linkage for tethering reporter groups to oligonucleotides. More importantly, the thioether linkage provides a means to position functional groups to interact specifically with opposing complementary (target) sequences.

INTRODUCTION

As a site for tethering molecular reporter devices to oligodeoxyribonucleotides, the C5 position of the pyrimidine nucleosides is nearly ideal, since groups of different sizes may be attached without adversely effecting DNA duplex formation. In recent years, a variety of specialized probe moieties, such as biotin (1–3), fluorophores (4–6), paramagnetic probes (7–9), pendant catalytic moieties (10–13) and cross linkers (14–17), have been coupled to deoxyuridine and then incorporated into nucleic acids either chemically or enzymatically through the corresponding triphosphates (1,4,18–20).

Despite the numerous reports on the application of C5-modified deoxyuridines (dU) as components of oligodeoxyribonucleotides, only a few scattered reports have appeared in the literature which address the binding stability of the individual duplexes in comparison with control sequences containing natural bases. Although the effect of C5 alkynyl and alkyl chain length on duplex thermal stability has been evaluated (21), there has been no comparative investigation on the effects of the size of alkyl-, alkenyl- or alkynyl-linked reporter groups on the thermal stability of nucleic acid duplexes. 5-Propynylpyrimidines have been reported to stabilize both duplex and triplex nucleic acids (22,23). Substitution of the methyl group of thymine with 1-alkynyl moieties in the d(A-T)$_{10}$ sequence resulted in higher T_m values for the duplexes containing 5-(1-propynyl)- to 5-(1-hexynyl)-2′-deoxyuridines. As the alkynyl chain length increases, the corresponding T_m values slightly decrease. The thermal stability of the duplexes containing 5-(1-heptynyl)- and 5-(octynyl)-2′-deoxyuridines were found to be lower than that of d(A-T)$_{10}$ (21). Other substituents may be added without compromising the stabilizing effect of the alkynyl group. Tolstikov *et al*. report that 5-(3-methoxypropynyl)-2′-deoxyuridine increases T_m values by ~1°C/substituent (24).

Our study was designed to serve two purposes. First, to allow us to gain more insight into the significance and effects of the size of the reporter groups tethered to the 5 position of dU using propargylamine as the linker arm and, second, to compare the C5 thioether tether with the C5 acetylenic tether. Deoxyuridine C5

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thioethers are relatively easy to synthesize by the palladium-mediated reaction of disulfides with 5-choromeruri-2′-deoxyuridine (25). However, the effect of this substitution on oligonucleotide duplex stability had not been previously evaluated. Three nucleoside analogs, 5-methylthio-, 5-phenylthio- and 5-(2-acetamido-ethylthio)-2′-deoxyuridine, were sufficient for this evaluation. In this report we focus on the synthesis and incorporation of the modified nucleosides into the oligodeoxyribonucleotide sequence d(CGCT*AATTGCG) and determination of the concentration-dependent thermal denaturation of the resulting duplexes.

MATERIALS AND METHODS

General procedures

5-Iodo-2′-deoxyuridine and propargylamine were purchased from Aldrich Chemical Co. 5′-*O*-(4,4′-Dimethoxytrityl)-3′-(*N*,*N*-diisopropylamino-β-cyanoethoxyphosphonyl)-5-(1-propynyl)-2′-deoxyuridine was obtained from Glen Research. NMR spectra were recorded on a Bruker AC250 or ARX300 or a Varian VXR-500S spectrometer. ^{1}H and ^{13}C signals were internally referenced to TMS unless otherwise stated, while 85% phosphoric acid was utilized as an external standard for all ^{31}P spectra. The individual extinction coefficients (ε) for the modified bases at 260 nm were determined experimentally from the slope of the plot of UV absorbance versus concentration ($\varepsilon = A/c$). Fast atom bombardment (FAB), chemical impact (CI) and electron impact ionization (EI), plasma desorption (PD) and matrix-assisted laser desorption ionization (MALDI) mass spectra were recorded by the mass spectrometry laboratories of the Department of Medicinal Chemistry and Molecular Pharmacology or the Department of Biochemistry, Purdue University. MALDI mass analysis were performed in 3-hydroxypicolinic acid as the matrix. Elemental analysis was performed by the Microanalysis Laboratory, Department of Chemistry, Purdue University. Analytical thin layer chromatography (TLC) was carried out on pre-coated Whatman 60F254 plates. Chromatotron preparative chromatography plates were prepared from silica gel 60 PF254 containing a gypsum binding agent manufactured by Merck. Anhydrous solvents were freshly distilled from appropriate drying agents or purchased from Aldrich Chemical; all other chemicals were reagent grade or better quality and used as received.

Synthetic procedures

*5′-(4,4′-Dimethoxytrityl)-5-iodo-2′-deoxyuridine (**2**).* 5-Iodo-2′-deoxyuridine (**1**) (295 mg, 0.83 mmol) was dissolved in pyridine (10 ml) and half of the solvent evaporated *in vaccuo*. 4,4′-Dimethoxytrityl chloride (0.340 g, 1.0 mmol) was added to the remaining solution and the mixture stirred overnight at room temperature. Then, cold water (15 ml) was added and the resulting mixture extracted with CH_2Cl_2 (2 × 20 ml). The organic layer was washed with water (10 ml) and dried over Na_2SO_4 and then the solvent evaporated. Chromatography on a chromatotron plate (2 mm, silica gel), eluted with CH_2Cl_2/CH_3OH (98:2), gave pure compound **2** (538 mg, 98.5%). R_f = 0.36 (CH_2Cl_2/CH_3OH 9:1 v/v); ^{1}H NMR 250 MHz ($CDCl_3$) δ 8.38 (s, N3-H, 1H), 8.13 (s, H-6, 1H), 7.46–7.23 (m, DMTr aromatic protons, 9H), 6.85 (d, J = 8.8 Hz, DMTr aromatic protons, 4H), 6.30 (dd, J_1 = 7.6 Hz, J_2 = 5.5 Hz, H-1′, 1H), 4.54 (m, H-3′, 1H), 4.08 (m, H-4′, 1H), 3.80 (s, OCH_3, 6H), 3.40 (m, H-5′, 2H), 2.33 and 2.44 (2 sets of multiplets, H-2′, 2H), 1.98 (broad s, 3′-OH, 1H); ^{13}C NMR 62.9 MHz ($CDCl_3$) δ 159.83, 158.66, 149.7, 144.26, 135.39, 135.28, 130.08, 128.11, 128.01, 127.10, 123.80, 113.37, 87.06, 86.45, 85.55, 72.46, 68.51, 63.41, 55.27, 41.46; MS-PD calculated for $C_{30}H_{29}IN_2O_7$ 656, found m/z 656 (M^+).

*5-(3-Acetamidopropyn-1-yl)-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyuridine (**4**).* Compound **2** (150 mg, 0.22 mmol) was dissolved in dry DMF (2 ml). The solution was degassed by repetitive evacuation of the container and purging the vacuum with an inert gas. Triethylamine (108 mg, 1.07 mmol, 150 µl), *N*-acetyl-propargylamine (64 mg, 0.66 mmol), $Pd(PPh_3)_4$ (25 mg, 0.022 mmol) and copper(I) iodide (9 mg, 0.047 mmol) were added and the mixture stirred at ambient temperature under nitrogen. After 8 h, a 5% disodium EDTA solution (5 ml) was added and the resulting mixture was extracted with ethyl acetate (3 × 30 ml). The extracts were combined and washed with water (10 ml), dried over Na_2SO_4 and concentrated under reduced pressure. The foamy residue was purified by chromatography on a chromatotron plate (1 mm, silica gel) eluted with CH_2Cl_2/MeOH/Et_3N (94:5:1). After combining the corresponding fractions and evaporating the solvent, compound **4** was obtained as a white foam (110 mg, 80%). R_f = 0.23 ($CH_2Cl_2/CH_3CN/Et_3N$ 49:49:2); R_f = 0.75 (methanol/EtOAc, 15:85 v/v); HRMS-FAB calculated for $C_{35}H_{35}N_3O_8$ 625.2424, found m/z 626.2502 $(MH)^+$; ^{1}H NMR 500 MHz (CH_3OH-d_4) δ 8.18 (s, H-6, 1H), 7.48–7.20 (m, DMTr aromatic protons, 9H), 6.89–6.87 (m, DMTr aromatic protons, 4H), 6.22 (t, J = 6.5 Hz, H-1′, 1H), 4.51–4.55 (m, H-3′, 1H), 4.04–4.07 (m, H-4′, 1H), 3.77–3.87 (m, H-5′, 2H), 3.78 (s, 2 $OC\underline{H}_3$, 6H), 2.33–2.45 (m, H-2′, 2H), 1.88 (s, $-COC\underline{H}_3$, 3H).

*5-(3-Acetamidopropyn-1-yl)-5′-O-(4,4′-dimethoxytrityl)-3′-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite)-2′-deoxyuridine (**9**).* In a small round bottom flask were combined compound **4** (0.100 g, 0.16 mmol) and diisopropylammonium tetrazolide (34 mg, 0.2 mmol). To this mixture, under dry nitrogen (dry box), was added CH_2Cl_2 (2 ml) followed by 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (26) (63 mg, 0.21 mmol). This solution was allowed to stand at ambient temperature with occasional gentle swirling for 1 h. Analysis of the crude reaction mixture by TLC (CH_2Cl_2/MeOH 9:1) showed the complete disappearance of the starting material and the appearance of a new component. The reaction mixture was transferred to a separatory funnel and diluted with 20 ml CH_2Cl_2. This solution was washed with water (3 × 10 ml) and then dried over anhydrous Na_2SO_4 and the solvent evaporated under reduced pressure at room temperature to an oil. The residue was purified by chromatography on a chromatotron plate (1 mm, silica gel) eluting with CH_2Cl_2/MeOH/Et_3N (94:5:1) to give **9** as a white foam after evaporation of the final fraction (120 mg, 91%). R_f = 0.42 ($CH_2Cl_2/CH_3CN/Et_3N$ 49:49:2); MS-FAB calculated for $C_{44}H_{52}N_5O_9P$ 825.89, found m/z 825.8 (M^+); ^{31}P NMR 121.5 MHz (acetone-d_6) δ 149.09 and 148.95 (phosphoramidite diastereomers).

*5-[3-(N,N-dimethylamino)propyn-1-yl]-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyuridine (**7**).* Compound **7** was prepared from **2** following the same procedure described above for the transformation of **2** to **4**. The product was purified by chromatography on a chromatotron plate (1 mm, silica gel) eluting with CH_2Cl_2/MeOH (9:1 v/v). After combining the corresponding fractions and evaporating the solvent, **7** was obtained as a white

foam (100 mg, 90%). R_f = 0.28 (CH_2Cl_2/MeOH 9:1); MS-PD calculated for $C_{35}H_{37}N_3O_7$ 611.26, found *m/z* $(MH)^+$ 612; ^1H NMR 250 MHz ($CDCl_3$) δ 7.99 (s, H-6, 1H), 7.44–7.21 (m, DMTr aromatic protons, 9H), 6.8 (d, *J* = ~10 Hz, DMTr aromatic protons, 4H), 6.29 (t, *J* = 6 Hz, H-1′, 1H), 4.48 (m, H-4′, 1H), 4.05 (m, H-3′, 1H), 3.77 (s, $OC\underline{H}_3$, 6H), 3.40–3.33 (m, 5′-H, 2H), 3.18 (s, $C\underline{H}_2$-N, 2H), 2.44–2.27 (2 sets of multiplets, H-2′ + 3′-O$\underline{H}$, 3H), 2.09 [s, $N(C\underline{H}_3)_2$, 6H]; ^{13}C NMR 62.9 MHz ($CDCl_3$) 29.83, 41.33, 43.75, 48.18, 55.20, 63.5, 72.0, 75.97, 85.04, 86.2, 86.85, 89.17, 100.26, 113.28, 126.94, 127.89, 128.01, 129.94, 144.50, 149.30, 158.56, 161.52; Analysis calculated for $C_{35}H_{37}N_3O_7$, C 68.72, H 6.10, N 6.87; found, C 68.45, H 6.05, N 6.72.

*5-[3-(N,N-dimethylamino)propyn-1-yl]-5′-O-(4,4′-dimethoxytrityl)-3′-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite)-2′-deoxyuridine (**12**)*. Compound **12** was prepared from **7** in 98% yield following the procedure described above for the transformation of **4** to **9**. R_f = 0.27 (CH_3OH/CH_2Cl_2 5:95); ^{31}P NMR 101.27 MHz (CH_3OH-d_4) δ 148.778, 148.567 (phosphoramidite diastereomers).

N-(2-Propyn-1-yl)nicotinamide. To a solution of nicotinic acid chloride (1.068 g, 6 mmol) in anhydrous pyridine (10 ml), was added Et_3N (0.7 g, 7 mmol, 0.5 ml) and the mixture stirred at room temperature under nitrogen. Once the acid chloride had dissolved, propargylamine (0.365 g, 6.6 mmol, 250 µl) was added dropwise to the reaction mixture. After 4 h, an analysis of the crude reaction mixture by TLC (5% methanol in CH_2Cl_2) showed the appearance of a new component in the mixture. Water (40 ml) was added to the reaction mixture and the aqueous solution was extracted with CH_2Cl_2 (3 × 40 ml). The combined organic solutions were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was purified by chromatography on a chromatotron plate (2mm, silica gel) and eluted with 4% methanol in CH_2Cl_2. The product, *N*-(2-propyn-1-yl)nicotinamide, was obtained as a white solid (748, mg 78%). R_f = 0.26 (CH_2Cl_2/CH_3OH 9:1 v/v); MS-EI calculated for $C_9H_8N_2O$ 160, found *m/z* 160 (M^+), 78; MS-CI 161 $(M+H)^+$; ^1H NMR 250 MHz ($CDCl_3$) δ 9.0 (d, *J* = 1.6 Hz, H-2 aromatic, 1H), 8.74 (m, H-6, 1H), 8.16 (m, H-5, 1H), 7.41 (m, H-4, 1H) 6.79 (broad singlet, $\underline{H}$-N, 1H), 4.28 (dd, J_1 = 5.2 Hz, J_2 = 2.5 Hz, N-$C\underline{H}_2$-, 2H), 2.32 (t, *J* = 2.5 Hz, $C\underline{C}\underline{H}$, 1H); ^{13}C NMR 62.9 MHz ($CDCl_3$) δ 29.90, 72.32, 79.0, 123.62, 135.26, 147.94, 152.61. Analysis calculated for $C_9H_8N_2O$, C 67.49, H 5.03, N 17.49; found, C 67.24, H 4.82, N 17.65.

*5-[3-(Nicotinamido)-1-propyn-1-yl]-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyuridine (**5**)*. Compound **5** was prepared from **2** following the procedure described above for the transformation of **2** to **4**. The crude product was purified by chromatography on a chromatotron plate (1 mm, silica gel) eluting with EtOAc/CH_2Cl_2/MeOH (50:50:5 v/v). Compound **5** was obtained as a white foam (86 mg, 62.5%). R_f = 0.39 (CH_3OH/CH_2Cl_2 1:9); R_f = 0.23 (CH_2Cl_2/EtOAc/CH_3OH 50:50:5); ^1H NMR 500 MHz ($CDCl_3$) δ 12.84 (s, N_3-$\underline{H}$, 1H) 8.895 (d, *J* = 1.5, H-2 pyridinyl ring, 1H), 8.675 (dd, J_1 = 4.5 Hz, J_2 = 2 Hz, H-4 pyridine, 1H), 8.223 (s, H-6 pyrimidine, 1H), 7.90 (m, H-6 pyridine, 1H), 7.65 (s, N$\underline{H}$-CO-, 1H), 7.47–7.17 (m, DMTr aromatic protons, H-5 pyridine, 10H), 6.80 (d, *J* = 7 Hz, DMTr aromatic protons, 4H), 6.14 (t, *J* = 10 Hz, H-1′, 1H), 4.585 (m, H-3′, 1H), 4.18 (m, -$C\underline{H}_2$N- and H-4′, 3H), 3.85 (s, $OC\underline{H}_3$, 6H), 3.35 (m, H-5′, 1H), 3.45 (m, H-5″, 1H), 2.56 (m, H-2′, 1H), 2.315 (m, H-2″, 1H); ^{13}C NMR 62.9 MHz ($CDCl_3$) δ 162.42, 158.30, 151.84, 149.61, 149.18, 148.47, 148.26, 142.75, 135.58, 135.40, 135.19, 134.80, 129.79, 129.50, 127.72, 126.66, 123.33, 123.23, 122.94, 122.54, 113.05, 86.70, 65.61, 70.83, 63.42, 54.95, 41.69, 30.32; MS-CI *m/z* 689 $(M+H)^+$; MS-PD calculated for $C_{39}H_{36}N_4O_8$ 688, found *m/z* 688.8 $(M)^+$, 303 $(DMTr)^+$. Analysis calculated for $C_{39}H_{36}N_4O_8$, C 68.01, H 5.21, N 8.13; found, C 67.72, H 5.26, N 8.31.

N-(2-Propyn-1-yl)adamantane-1-carboxamide. To a solution of 1-adamantanecarbonyl chloride (1.074 g, 6 mmol) in CH_2Cl_2 (10 ml) were added triethylamine (1 ml) and propargylamine (364 mg, 6.6 mmol). The solution was stirred overnight at ambient temperature. The reaction mixture was diluted with CH_2Cl_2 (20 ml) and the organic layer washed with water (2 × 20 ml) and dried over anhydrous Na_2SO_4. Following concentration under reduced pressure, the crude product was purified by column chromatography (silica gel) eluting with CH_2Cl_2/CH_3OH (95:5 v/v). The desired compound was obtained as a white solid (1.08 g, 83%). R_f = 0.65 (CH_2Cl_2/CH_3OH 95:5 v/v); MS-EI calculated for $C_{14}H_{19}NO$ 217.3, found *m/z* 217 (M^+), 135, 107, 93, 79; MS-CI found *m/z* 218 $(MH)^+$; ^1H NMR 300 MHz ($CDCl_3$) δ 5.75 (broad singlet, N$\underline{H}$, 1H), 3.73 (dd, J_1 = 5 Hz, J_2 = 2.5 Hz, -$C\underline{H}_2$-, 2H), 2.23 (t, J = 2.5 Hz, C ≡ C$\underline{H}$, 1H), 2.05 (m, adamantane -CH- protons, 3H), 1.86 (m, -$C\underline{H}_2$-, 6H), 1.73 (m, 3 × CH_2, 6H); ^{13}C NMR 75.47 MHz ($CDCl_3$) δ 177.61, 71.66, 40.67, 39.21, 36.56, 29.32, 28.15, 27.95. Analysis calculated for $C_{14}H_{19}NO$, C 77.38, H 8.81, N 6.45; found, C 77.22, H 9.02, N 6.47.

*5-[3-(Adamantane-1-carbamido)propyn-1-yl]-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyuridine (**6**)*. Compound **6** was prepared from **2** in 67% yield by a similar procedure described above for the transformation of **2** to **5**. Purification of the crude product was carried out by chromatography on a chromatotron plate (2 mm, silica gel) eluting with CH_2Cl_2/EtOAc/CH_3OH (49:49:2). R_f = 0.4 (CH_2Cl_2/EtOAc/CH_3OH 49:49:2); MS-PD calculated for $C_{44}H_{47}N_3O_8$ 745.33, found *m/z* 746.7 $(M+H)^+$; ^1H NMR 500 MHz ($CDCl_3$) δ 9.3 (broad s, N_3-H, 1H), 8.12 (s, H-6, 1H), 7.42–7.18 (m, DMTr aromatic protons, 9H), 6.83 (d, *J* = 9 Hz, 4H), 6.33 (dd, J_1 = 7.5 Hz, J_2 = 6 Hz, H-1′, 1H), 5.54 (t, *J* = ~6 Hz, N$\underline{H}$-CO-, 1H), 4.50 (m, H-3′, 1H), 4.06 (m, H-4′, 1H), 3.95 (m, $C\underline{H}_2$-N, 2H), 3.79 (s, $OC\underline{H}_3$, 6H), 3.41 (dd, J_1 = 11 Hz, J_2 = 3 Hz, H-5′, 1H), 3.36 (dd, J_1 = 11 Hz, J_2 = 4 Hz, H-5″, 1H), 2.5 (m, H-2′, 1H), 2.3 (m, H-2″, 1H), 1.956 (br s), 1.684 (d, *J* = 2.5 Hz), 1.62 and 1.60 (broad m, adamantane protons). Analysis calculated for $C_{44}H_{47}N_3O_8$, C 70.85, H 6.35, N 5.63′; found, C 68.59, H 6.42, N 5.53.

*5-[3-(Nicotinamido)-1-propyn-1-yl]-5′-O-(4,4′-dimethoxytrityl)-3′-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite)-2′-deoxyuridine (**10**)*. Compound **10** was prepared from **5** in 85% yield by a similar procedure described above for the transformation of **4** to **9**. The product was purified by chromatography on a chromatotron plate (1 mm, silica) eluting with $CH_2Cl_2/CH_3OH/Et_3N$ (90:9:1 v/v). R_f = 0.75 ($CH_2Cl_2/CH_3OH/Et_3N$ 90:9:1); ^{31}P NMR 121.5 MHz (CH_3OH-d_4) δ 149.657, 149.521 (two phosphoramidite diastereomers).

*5-[3-(Adamantane-1-carbamido)propyn-1-yl]-5′-O-(4,4′-dimethoxytrityl)-3′-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite)-2′-deoxyuridine (**11**)*. Compound **11** was prepared from **6** in 89% yield by a similar procedure described above for the transformation of **4** to **9**. ^{31}P NMR 121.5 MHz (acetone-d_6) δ 149.66, 149.28 (two phosphoramidite diastereomers).

N-Acetoxysuccinimide. To a solution of *N*-hydroxysuccinamide (345.3 mg, 3 mmol) in anhydrous THF (1 ml) under an inert atmosphere was added a solution of glacial acetic acid (174 µl, 3 mmol) in THF (1 ml). Dicyclohexylcarbodiimide (DCC, 620 mg, 3 mmol) was dissolved in THF (1 ml) and then added to the reaction mixture. The mixture was stirred overnight at room temperature. The resulting white precipitate (dicyclohexylurea) was removed by filtration and the filtrate was used in the following reaction without further purification. For characterization the *N*-acetoxysuccinimide was purified by chromatography on silica gel (EtOAc/MeOH, 9:1 v/v). R_f = 0.45 (CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.84 (s, C$\underline{H}_2$, 4H), 2.34 (s, C$\underline{H}_3$, 3H); MS-CI for $C_6H_7NO_4$ calculated 157; found *m/z* 158 $(M+H)^+$.

*5-(2-Acetamidoethylthio)-2′-deoxyuridine (**17**).* 5-(Trifluoroacetamidoethylthio)-2′-deoxyuridine (25) (**16**) (0.615 g, 1.5 mmol) was dissolved in methanol (10 ml). To this solution was added concentrated ammonium hydroxide (30 ml, 28.7%). The reaction container was capped and the mixture was stirred overnight. Excess ammonia was removed by bubbling nitrogen into the mixture and methanol evaporated under reduced pressure. The remaining solution was dried by lyophilization. The residue was dissolved in anhydrous ethanol (3 ml). To this solution was added a solution of *N*-acetoxysuccinimide (0.315 g, 2 mmol) and Et_3N (300 µl, 2.5 mmol) in THF (2 ml). After stirring for 4 h at room temperature, the solvent was evaporated and the residue purified by chromatography on silica gel (EtOAc to EtOAc/MeOH 80:20 gradient) to give **17** as a white solid (462 mg, 87%). R_f = 0.32 (EtOAc/MeOH 85:15 v/v); MS-FAB calculated for $C_{13}H_{19}N_3O_6S$ 345, found *m/z* 346 $(MH)^+$; ^{1}H NMR 300 MHz (CH_3OH-d_4) δ 8.33 (s, H-6, 1H), 6.25 (t, *J* = 7 Hz, H-1′), 4.41 (m, H-3′, 1H), 3.93 (m, H-4′, 1H), 3.78 (m, H-5′, 2H), 3.31 (m, SC$\underline{H}_2$-, 2H), 2.78 (t, *J* = 6 Hz, -C$\underline{H}_2$N-, 2H), 2.29 (m, H-2′, 2H), 1.95 (s, acetyl C$\underline{H}_3$, 3H); ^{13}C NMR 125 MHz (DMSO-d_6) 169.3, 161.65, 150.0, 142.55, 106.8, 87.54, 84.58, 70.23, 61.1, 39.94, 37.88, 32.1, 22.6; UV (methanol) λ_{max} 282.4, 202.0 nm. Analysis calculated for $C_{13}H_{19}N_3O_6S$, C 45.21, H 5.55, N 12.17, O 27.80, S 9.28; found C 44.88, H 5.37, N 12.27, S. 9.32.

5-(2-Acetamidoethylthio)-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyuridine. 5-(2-Acetamidoethylthio)-2′-deoxyuridine (**17**, 470 mg 1.08 mmol) was dried by repeated co-evaporation with dry pyridine (3 × 3 ml) *in vaccuo* and dissolved in anhydrous pyridine (5 ml). To this solution was added 4,4′-dimethoxytrityl chloride (430 mg, 1.27 mmol) and the mixture stirred overnight at room temperature. Then, cold water (25 ml) was added to the stirred reaction mixture. The aqueous solution was discarded and the residue dried *in vaccuo*. The final product was purified by chromatotron on a 1 mm thick layer of silica gel using a gradient solvent system (1–5% methanol in CH_2Cl_2). 5-(2-Acetamidoethylthio)-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine was obtained as a white foam (63%). R_f = 0.3 (CH_2Cl_2/MeOH); MS-PD calculated for $C_{34}H_{37}N_3O_8S$ 647.74, found *m/z* 671.7 $(M+H+Na)^+$, 647.8, (M^+); MS-CI *m/z* 303 $(DMTr^+)$; ^{1}H NMR 300 MHz (CH_3OH-d_4) δ 7.92 (s, H-6, 1H), 7.29–7.03 (m, aromatic DMTr, 9H), 6.68 (d, *J* = 9 Hz, aromatic DMTr, 4H), 6.06 (t, *J* = 6 Hz, H-1′, 1H), 4.30 (m, H-3′, 1H), 3.85 (m, H-4′, 1H), 3.60 (s, OC$\underline{H}_3$, 6H), 3.18 (m, H-5′, 2H), 2.96 (m, SC$\underline{H}_2$-, 2H), 2.40 (t, *J* = 7 Hz, -C$\underline{H}_2$-N, 2H), 2.22 (m, H-2′, 2H), 1.68 (s, COC$\underline{H}_3$, 3H); ^{13}C NMR 75 MHz (CH_3OH-d_4) 160.2, 151.9, 146.16, 145.85, 137.16, 137.0, 131.37, 131.32, 129.36, 128.9, 127.9, 114.2, 107.8, 88.0, 87.1, 72.66, 64.9, 55.7, 41.7, 39.9, 34.5, 22.6, 15.5.

*5-(2-Acetamidoethylthio)-5′-O-(4,4′-dimethoxytrityl)-3′-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite)-2′-deoxyuridine (**20**).* Compound **20** was obtained from **17** in 89% yield by the procedure described above for the transformation of **4** to **9**. R_f = 0.54 (CH_2Cl_2/CH_3OH/Et_3N 90:5:5); MS-FAB calculated for $C_{43}H_{54}N_5O_9PS$ 847.96, found *m/z* 870 $(M+Na)^+$, 847 $(M)^+$; MS-PD *m/z* 303 $(DMTr)^+$, 847 $(M)^+$; ^{31}P NMR 121.5 MHz (CH_3OH-d_4) δ 149.86 and 149.68 (phosphoramidite diastereomers).

*5-Methylthio-2′-deoxyuridine (**14**).* To 5-chloromercurio-2′-deoxyuridine (27) (0.926 g, 2 mmol) in a 100 ml round bottom flask was added a solution of Li_2PdCl_4 in methanol (0.1 M, 40 ml). Dimethyldisulfide (0.470 g, 5 mmol) was added and the resulting mixture stirred at room temperature for 18 h. H_2S was bubbled into the reaction mixture for 30 s and the mixture filtered by gravity filtration. The filtrate was then evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (15 cm, i.d. 2.5 cm) eluting with CH_2Cl_2/CH_3OH (87:13) to give 5-methylthio-2′-deoxyuridine as a white solid (228 mg, 41.5%). A sample of this compound was recrystallized from anhydrous alcohol. R_f = 0.54 (CH_2Cl_2/CH_3OH 85:15); ^{1}H NMR 250 MHz (DMSO-d_6) δ 11.56 (s, N_3-H, 1H), 7.86 (s, H-6, 1H), 6.16 (t, *J* = 6.5 Hz, H-1′, 1H), 5.23 (d, *J* = 6 Hz, 3′-OH, 1H), 5.11 (t, *J* = 6 Hz, 5′-OH, 1H), 4.26 (m, H-3′, 1H), 3.78 (m, H-4′, 1H), 3.57 (m, H-5′, 2H), 2.23 (s, SC$\underline{H}_3$, 3H), 2.12 (m, H-2′, 2H); ^{13}C NMR 62.9 MHz (DMSO-d_6) δ 161.26, 149.84, 136.95, 110.15, 87.49, 84.53, 70.32, 61.02, 40.01, 15.07; MS-PD calculated for $C_{10}H_{14}N_2O_5S$ 274, found *m/z* 275 $(M+H)^+$; MS-CI *m/z* 275 $(M+H)^+$, 159, 117, Analysis calculated for $C_{10}H_{14}N_2O_5S$, C 43.79, H 5.14, N 10.21, S 11.69; found, C 43.80, H 5.05, N 9.96, S 11.59.

*5-Phenylthio-2′-deoxyuridine (**15**).* Compound **15** was obtained in 43% yield by a similar procedure to that described above for the synthesis of **14**. The desired product **15** was purified by column chromatography (silica gel) eluting with CH_2Cl_2/CH_3OH (85:15). R_f = 0.64 (CH_2Cl_2/CH_3OH 85:15 v/v), MS-CI calculated for $C_{15}H_{16}N_2O_5S$ 336, found *m/z* 337 $(M+H)^+$ 319, 301, 221; MS-PD *m/z* 338; ^{1}H NMR 250 MHz (DMSO-d_6) δ 11.65 (s, N_3-H, 1H), 8.44 (s, H-6, 1H), 7.30–7.11 (m, 5-phenyl protons, 5H), 6.11 (t, *J* = 6.4 Hz, H-1′, 1H), 5.35 (d, *J* = 4.2 Hz, 3′-OH, 1H), 5.15 (t, *J* = 5 Hz, 5′-OH, 1H), 4.23 (m, H-3′, 1H), 3.78 (m, H-4′, 1H), 3.53 (m, H-5′, 2H), 2.21 (m, H-2′, 2H); ^{13}C NMR 62.9 MHz (DMSO-d_6) δ 161.32, 150.28, 136.40, 129.04, 126.42, 125.68, 103.39, 87.69, 85.04, 70.03, 60.84, 40.33. Analysis calculated for $C_{15}H_{16}N_2O_5S$, C 53.56, H 4.79, N 8.33, S 9.53; found, C 53.20, H 4.70, N 8.17, S 9.32.

5′-O-(4,4′-Dimethoxytrityl)-5-methylthio-2′-deoxyuridine. 5-Methylthio-2′-deoxyuridine (**14**) was transformed to the corresponding 5′-*O*-DMTr derivative in 93% yield by the procedure described above for the transformation of **1** to **2**. The title compound was purified by chromatography on a chromatotron plate (2 mm silica gel) eluting with CH_2Cl_2/CH_3OH (93:7 v/v). R_f = 0.45 (CH_2Cl_2/CH_3OH 93:7); ^{1}H NMR 300 MHz ($CDCl_3$) δ 8.63 (broad d, 1H), 8.4 (broad singlet, N_3-H, 1H), 7.88 (s, H-6, 1H), 7.42–7.15 (m, DMTr aromatic protons, 9H), 6.84 (m, DMTr aromatic protons, 4H), 6.30 (t, *J* = 5.7 Hz, H-1′, 1H), 4.5 (m, H-3′, 1H), 4.05 (m, H-4′, 1H), 3.79 (s, OC$\underline{H}_3$, 6H), 3.38 (m, H-5′, 2H), 2.45 (m, H-2′, 1H), 2.30 (m, H-2″, 1H), 2.10 (s, SC$\underline{H}_3$, 3H).

5′-O-(4,4′-Dimethoxytrityl)-5-phenylthio-2′-deoxyuridine. 5-Phenylthio-2′-deoxyuridine (**15**) was transformed to the

corresponding 5′-*O*-DMTr derivative, 5′-*O*-(4,4′-dimethoxytrityl)-5-phenylthio-2′-deoxyuridine, in 76% yield by the procedure described above for the transformation of **1** to **2**. The desired compound was purified by chromatography on a chromatotron silica gel plate (2 mm) eluting with CH_2Cl_2/CH_3OH (93:5 v/v). R_f = 0.29 (CH_2Cl_2/CH_3OH 93:5); ^{1}H NMR 300 MHz ($CDCl_3$) δ 8.8 (broad singlet, N_3-H, 1H), 8.61 (d, J = 4 Hz, 3′-OH, 1H), 8.17 (s, H-6, 1H), 7.4–7.1 (m, DMTr and phenylthio protons, 14H), 6.24 (dd, J_1 = 9 Hz, J_2 = 3 Hz, DMTr aromatic protons, 4H), 6.34 (dd, J_1 = 7.6 Hz, J_2 = 5.6 Hz, H-1′, 1H), 4.57 (m, H-3′, 1H), 4.06 (m, H-4′, 1H), 3.72 (s, $OC\underline{H}_3$, 6H), 3.25 (m, H-5′, 2H), 2.48 (m, H-2′, 1H), 2.32 (m, H-2″, 1H).

*5′-O-(4,4′-Dimethoxytrityl)-3′-O-(2-cyanoethyl-N,N-diisopropyl-phosphoramidite)-5-phenylthio-2′-deoxyuridine (**19**).* 5′-*O*-(4,4′-Dimethoxytrityl)-5-phenylthio-2′-deoxyuridine was transformed to the corresponding phosphoramidite derivative **19** in 89% yield by the procedure described above for the transformation of **4** to **9**. Compound **19** was purified by chromatography on a chromatotron silica gel plate (1 mm) eluted with $CH_2Cl_2/CH_3OH/Et_3N$ (94:5:1). R_f = 0.60 ($CH_2Cl_2/CH_3OH/Et_3N$ 94:5:1); ^{31}P NMR 101.25 MHz (acetone-d_6) 149.91, 149.75 (two phosphoramidite diastereomers). ^{1}H NMR 250 MHz (acetone-d_6) (^{1}H NMR spectrum of **19** contains two almost identical sets of sometimes overlapping signals as a result of the presence of the two diastereomers. Not all signals are reported reported here) δ 8.28 and 8.24 (singlets, H-6), 6.32 (two sets of triplets, H-1′), 4.73 (m, H-3′), 4.2 (m, H-4′), 3.72 (s, $OC\underline{H}_3$), 3.4 (m, H-5′), 2.57 (m, H-2′).

*5′-O-(4,4′-Dimethoxytrityl)-3′-O-(2-cyanoethyl-N,N-diisopropyl-phosphoramidite)-5-methylthio-2′-deoxyuridine (**18**).* 5′-*O*-(4,4′-Dimethoxytrityl)-5-methylthio-2′-deoxyuridine was transformed to the corresponding phosphoramidite derivative **18** in 77% yield by the procedure described above for the transformation of **4** to **9**. Compound **18** was purified by chromatography on a chromatotron silica gel plate (1 mm) eluted with $CH_2Cl_2/CH_3OH/Et_3N$ (94:5:1). R_f = 0.4 ($CH_2Cl_2/CH_3OH/Et_3N$ 94:5:1); ^{31}P NMR 101.25 MHz (acetone-d_6) 149.55, 149.41 (phosphoramidite diastereomers). ^{1}H NMR 250 MHz (acetone-d_6) δ 8.28 and 8.24 (singlets, H-6), 6.32 (two sets of triplets, H-1′), 4.73 (m, H-3′), 4.2 (m, H-4′), 3.72 (s, $OC\underline{H}_3$), 3.4 (m, H-5′), 2.57 (m, H-2′).

Synthesis and characterization of oligodeoxyribonucleotides. Oligodeoxyribonucleotides **I–IX** (Fig. 1) were prepared from commercially available dA, dC, dG and T phosphoramidites (Glen Research) on a 380B (Applied Biosystems) automated DNA synthesizer (1 μM scale) by standard solid phase phosphoramidite chemistry (28–30). After cleavage from the solid support and deprotection by aqueous ammonia at 50°C for 24 h, the oligonucleotides were purified using 20% polyacrylamide–8 M urea preparative gel electrophoresis. The desired oligodeoxyribonucleotides were extracted from the gels and desalted with Waters C18 SepPaks™ following the manufacturer's instructions. The purified oligonucleotides were evaporated to dryness at 45°C using a Speed Vac™ drying apparatus and stored at –10°C. Incorporation of the intact modified bases and the integrity of the resulting oligodeoxyribonucleotides were confirmed by MALDI-TOF mass spectrometry. The observed single cleavage product ions were all within 0.1% of the calculated formula weight for oligonucleotides **I–IX**.

Oligonucleotide	T*	R
I	21	CH_3
II	22	$C{\equiv}CCH_3$
III	23	$C{\equiv}CCH_2NHC(O)CH_3$
IV	24	$C{\equiv}CCH_2N(CH_3)_2$
V	25	$C{\equiv}CCH_2NHC(O)C_3H_4N$
VI	26	$C{\equiv}CCH_2NHC(O)C_{10}H_{15}$
VII	17	$SCH_2CH_2NHC(O)CH_3$
VIII	15	SC_6H_5
IX	14	SCH_3

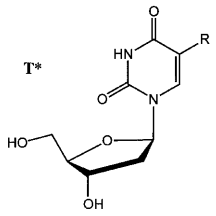


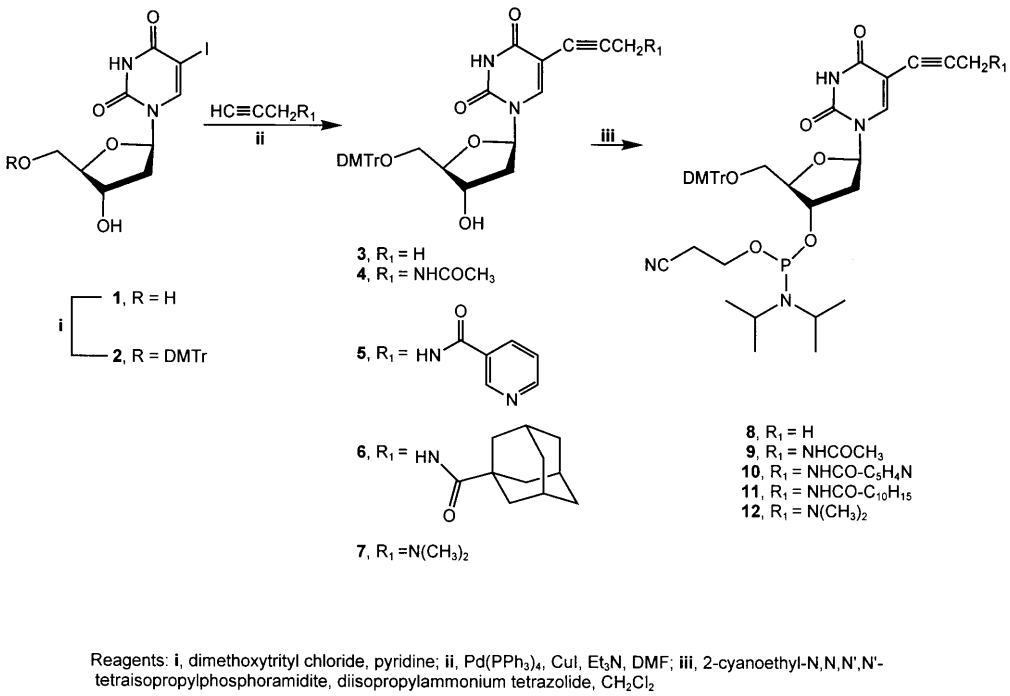
Figure 1. Structures of oligodeoxyribonucleotides 5′-d(CGC**T***AATTAGCG)-3′ **I–IX**.

Thermal denaturation studies

Solution preparation. The stock solution was prepared by dissolving each oligonucleotide in pH 7 buffer consisting of 1.0 M NaCl, 10 mM sodium phosphate and 0.1 mM EDTA. The concentration of oligonucleotides in the stock solution was determined by UV spectroscopy, based on the assumption that at high temperature oligonucleotides are unpaired and unstacked. An aliquot of the stock solution (50 μl) was diluted to 2.7 ml and the UV melting curve recorded. The upper baseline in each UV melting curve was fitted to a straight line ($y = a + bx$) using the graphing software Igor Pro™. The absorbance at 25°C was then calculated using this equation. The extinction coefficient of oligonucleotide at 25°C was taken as the sum of the individual mononucleotides in the strand at 260 nm. The extinction coefficient for 5-(3-acetamidopropyn-1-yl)-2′-deoxyuridine was measured to be 3818/mol/cm and was used as the extinction coefficient for all the 5-alkynyl uracil nucleosides. The extinction coefficients for nucleosides **14**, **15** and **17** were determined to be 5090, 9388 and 5090/mol/cm respectively at 260 nm. Beer's law was applied to calculate the concentration of oligonucleotide for each solution.

UV melting measurements. Absorbance versus temperature profiles were recorded in fused quartz cuvettes at 260 nm on a Cary 3TM UV-visible spectrophotometer equipped with a Peltier temperature controlling device and thermal software. All the samples were degassed in a vacuum desiccator before use. Thermal denaturing studies for each oligonucleotide were carried out at 6, 15, 30, 50, 80, 100, 130 and 160 μM concentrations. A layer of silicon oil (Dow 200 fluid, 100 CSTKS) was placed on the surface of the aqueous solution to prevent solvent evaporation. Dry nitrogen was continuously run through the measurement chamber to prevent condensation of water vapor at low temperatures. Prior to thermal denaturation studies, each sample was heated to 85–90°C and allowed to equilibrate at this temperature for at least 10 min. Absorbance versus temperature curves for the oligomers were obtained with both a cooling and heating ramp of 0.3°C/min. Absorbance readings were taken at 0.2°C intervals. Reversibility of each helix–coil transition was demonstrated, since the absorbance value of the melting curves (up ramp) were found to differ from the annealing curves (down ramp) by <2%.

Data analysis. The data from the Cary 3 spectrometer were analyzed and T_m values for each specific concentration were calculated as previously described (31). The melting temperatures of the duplexes derived from oligonucleotides **I–IX** were measured at eight or more different concentrations but are here reported only at 15 μM strand concentration. The enthalpy and entropy for each transition was calculated from the equation developed by Breslauer (32) [$1/T_m$ =



Scheme 1. Synthetic pathway for the preparation of 5′-dimethoxytrityl-5-alkynyl-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)-2′-deoxyuridines **9–12**.

$(R/\Delta H^\circ)\ln C_T + (\Delta S^\circ/\Delta H^\circ)]$ for a self-complementary sequence. Thus, a plot of $1/T_m$ versus $\ln C_T$ reveals the concentration dependence of the melting temperature. The slope of the resulting line provides a good measure of the van't Hoff enthalpy for transitions that proceed in a two-state manner. The entropy of the transition for each individual transition was calculated from the intercept of the plot of $1/T_m$ versus $\ln C_T$. This method for the determination of enthalpy is much less sensitive to the choice of baseline when analyzing experimental melting curves. This feature results from the fact that the slope of a $1/T_m$ versus $\ln C_T$ line results from differences in the T_m values obtained from several melting curves. The standard free energies for each duplex was calculated at room temperature (298 K) and a temperature near to the melting temperature (328 K) employing the general equation $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$.

RESULTS AND DISCUSSION

With the exception of the studies cited in the introduction, little effort has been made to determine the effect of larger and more complex alkynyl substitution on duplex stability. We have been particularly interested in determining what happens to duplex stability when one tethers a universal alkynyl linker arm, –C≡C-CH_2-NH-C(O)-R and incorporates R substituents differing substantially in size. A second goal of the study, which is described below, was to compare the alkynyl tether with a thioalkyl tether. The latter, like the alkynyl tethers, are relatively easy to synthesize by palladium-mediated chemistry.

Synthesis

Synthesis of 5-alkynyl-2′-deoxyuridine derivatives (Scheme 1) was initiated by 4,4′-dimethoxytrityl protection of readily available 5′-iodo-2′-deoxyuridine (**1**). Protection of the 5′-hydroxyl group with a 4,4′-dimethoxytrityl (DMTr) group was carried out following the standard procedure (28) in excellent yield. Tritylation of 5′-hydroxyl of 5-iodo-2′-deoxyuridine (**1**) prior to the coupling reaction eliminated the need for protection and deprotection of hydroxyl groups of the nucleosides, generally required to obtain good yields in palladium-catalyzed coupling reactions (33,34). Palladium-mediated coupling reactions were carried out in DMF following the procedure reported by Hobbs (35).

The thioether-linked deoxyuridine derivatives **14–16** were synthesized employing the methodology developed by Bergstrom and co-workers (Scheme 2; 25). Direct coupling of nucleoside **13** with *N*,*N*′-bisacetylcystamine in the presence of Li_2PdCl_4 yielded nucleoside **17** in poor yield, presumably because *N*,*N*′-bisacetylcystamine complexes with too high affinity to Pd(II). However, nucleoside **17** can be obtained indirectly by first preparing 5-(3-trifluoroacetamido)-2′-deoxyuridine (**16**) as previously described (25) and then removing the trifluoroacetyl group with ammonia followed by acetylation of the free amine with *N*-acetoxysuccinimide.

For incorporation into oligonucleotides, the nucleosides were converted to 5′-dimethoxytrityl-3′-phosphoramidites employing known procedures (28). In addition to the control sequence d(CGCTAATTAGCG), sequences **II–IX**, containing the modified deoxyribonucleosides **14**, **15**, **17** and **21–26** at position 4 were synthesized (Fig. 1). The self-complementary sequence d(CGC**T***AATTAGCG) (where T* is the C5-modified base) was chosen for this study because it is structurally well understood. Variants of the sequence containing matched, mismatched or other modified base pairs have been investigated extensively by X-ray crystallography (36), ^{1}H NMR (8,37,38), CD, UV melting and calorimetric methods (8,39,40). Melting temperature (T_m) studies have demonstrated that helix–coil transitions involving the modified dodecamers at high salt concentrations are bimolecular in nature (41).

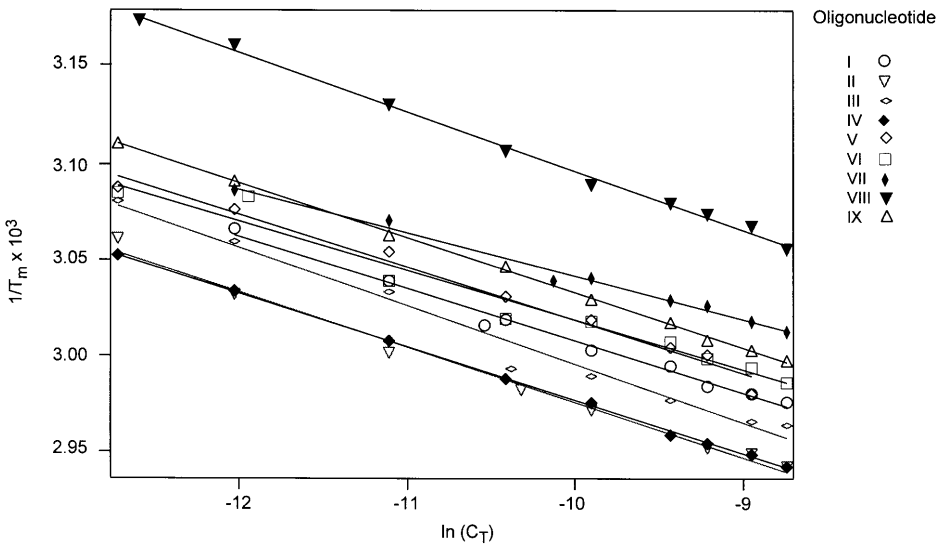


Figure 2. Plots of reciprocal melting temperature (T_m^{-1}) versus the natural logarithm of total strand concentration ($\ln C_T$) for oligodeoxyribonucleotides **I–IX**. The values of $\Delta H°$ and $\Delta S°$ resulting from the slopes and intercepts (respectively) of these lines are tabulated in Table 1.

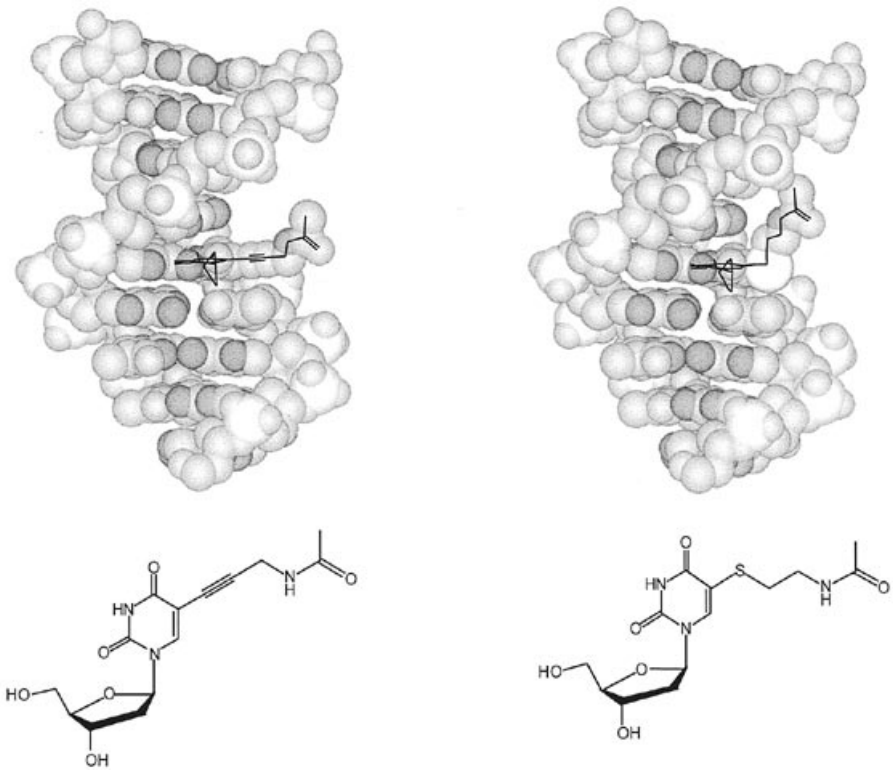
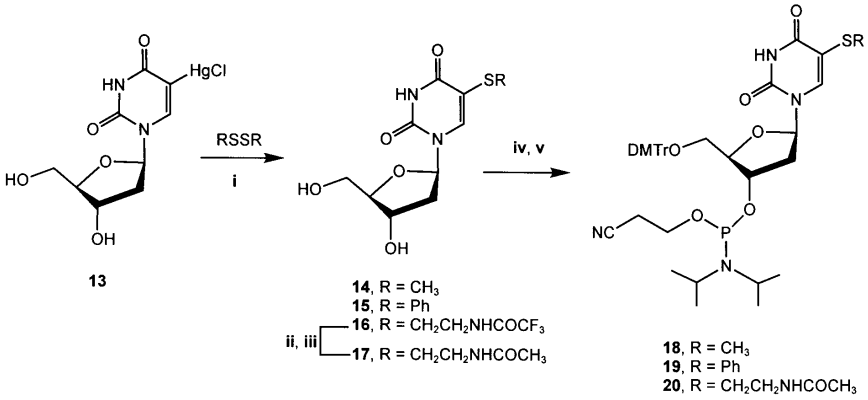


Figure 3. Model of the 9mer duplex, d(GAATT*CGCG)·d(CGCGAATTC) containing (2-acetamidoethylthio)-2′-deoxyuridine (**23**) and 3-acetamido-1-propynyl-2′-deoxyuridine (**17**).

Thermal denaturation studies

Melting studies were done and melting curves obtained as outlined in the experimental section. Most of the melting curve profiles resembled that obtained for the natural sequence. The thermodynamic parameters were obtained from the melting profiles of the concentration dependence of the melting temperature as described in the experimental section. The T_m^{-1} versus natural logarithm of total concentration plots are linear and the two-state model appears adequate for the data presented here (Fig. 2). The T_m and the thermodynamic values extracted from the concentration-dependent thermal denaturing studies are listed in Table 1. The data for the corresponding sequence containing the natural base pair are also listed. Free energies ($\Delta G°$) for duplex formation of the modified oligonucleotides, as well as the natural sequence, were calculated at 25 and 55°C (Table 1). Inspection of $\Delta G°_{55°}$ values in comparison with the corresponding T_m values revealed the expected correlation; larger T_m values corresponded to more negative ΔG values.



Reagents: **i**, Li_2PdCl_4, CH_3OH; **ii**, NH_4OH, CH_3OH; **iii**, succinimidylacetate, THF; **iv**, dimethoxytrityl chloride, pyridine; **v**, 2-cyanoethyl N,N,N',N'-tetraisopropylphosphoramidite, diisopropylammonium tetrazolide, CH_2Cl_2.

Scheme 2. Synthetic pathways for the preparation of 5-methylthio-, 5-phenylthio- and 5-(2-acetamidoethylthio)-2′-deoxyuridine phosphoramidites **18–20**.

Table 1. Melting temperatures (T_m) and thermodynamic parameters for helix–coil transitions of the oligonucleotide sequences 5′-d(CGC**T***AATTAGCG)-3′ containing C-5-modified nucleosides[a]

Oligodeoxynucleotide	I	II	III	IV	V	VI	VII	VIII	IX
T_m (°C)	55.2	59.1	55.8	55.9	58.3	54.0	52.1	45.1	52.2
$\Delta G_{55°}$ (kcal/mol)	–7.1	–7.9	–7.5	–7.3	–7.9	–7.1	–6.5	–5.3	–6.7
$\Delta G_{25°}$ (kcal/mol)	–14.3	–13.4	–12.8	–12.9	–13.5	–13.0	–12.9	–10.6	–12.3
ΔH (kcal/mol)	–85.3	–68.0	–65.8	–68.8	–69.3	–71.9	–76.8	–63.5	–67.8
ΔS (cal/mol K)	–238.3	–183.0	–177.7	–187.4	–187.1	–197.6	–214.3	–177.5	–186.4

[a]See Figure 1 for T* structures.
All solutions contained 1.0 M NaCl, 1 mM EDTA, 10 mM sodium phosphate, pH 7.0.

The C5 propynyl group is one of the most duplex stabilizing substituents known (22,23). Consequently, we used propynyl-dU as a standard for comparison with other C5 substituents. In our study, substitution of the thymine methyl by a propynyl group (Fig. 1 and Table 1, oligodeoxyribonucleotide **II**) resulted in an increase in T_m of ~2°C/substitution. In contrast, all of the amide-substituted propynyl-containing oligonucleotides (**III**, **V** and **VI**) melted at a lower temperature, but only the adamantyl-containing oligonucleotide had a lower T_m value (–0.6°C/substituent) than the control oligonucleotide. The dimethylamino substituent is a special case, since this side chain should be protonated in aqueous solution at neutral pH. Previous investigations on oligonucleotides containing C5-linked protonated amines have shown that an amino group placed on an alkyl tether stabilizes a nucleic acid duplex relative to a similar size alkyl without the amino group (42,43). Although the duplexes containing the propynyl group and acetamidopropynyl group melt at higher temperature than the natural duplex, the ΔH and ΔS values are substantially less negative.

The duplexes containing the SCH_3 and $SCH_2CH_2NHC(O)CH_3$ substituents (**VII** and **IX**) both melted 1.5°C/substituent lower than the control sequence. Since the SCH_3- and SCH_2CH_2-$NHC(O)CH_3$-containing sequences have nearly identical T_m values, it appears that there is not an additional penalty for the longer $SCH_2CH_2NHC(O)CH_3$ tether. Within the 5-thioether-linked 2′-deoxyuridine series, the 5-thiophenyl group proved to be particularly destabilizing. Oligonucleotide **VIII** showed a much lower T_m (45.1°C) than the control dodecamer **I** and oligonucleotides **VII** and **IX**. Molecular models show that the relatively large hydrophobic group (thiophenyl) would fit within the major groove only if the local helix is deformed.

The stabilizing effect of conjugated groups substituted at the C5 position of pyrimidine bases on the thermal stability of the resulting duplexes has been attributed to the enhanced stacking interactions (22). C5 substituents that increase stacking interactions by virtue of an extended conjugated π system appear to be particularly stabilizing. We anticipated that the thioether tether would be more stabilizing than an alkyl tether because of the polarizability of the sulfur atom, but needed to complete the study outlined here to determine if this effect could contribute as much to base stacking as an extended π system. Support for this prediction stemmed from the observation that 5-bromodeoxyuridine stabilizes duplexes relative to thymidine. Our results show that the thioether substituent is not as stabilizing as C5 bromo and indeed is even destabilizing relative to C5 methyl. At the same time, it appears that the thioether tether is more stabilizing than longer alkyl groups. A single C5 ethyl substituent in place of either of the C5 methyls in the 10mer d(GGAGATCTCC) lowers the T_m by 4°C (44) We observed only a 1.5°C drop in T_m per thioether substituent.

The main advantage of the thioether tether may be the access it provides C5-tethered substituents to complementary DNA sequences. Models of the duplex from the modified sequence d(GAATT*CGCG), where T* is either the deoxyribonucleoside dU-C≡$CCH_2NHC(O)CH_3$ or dU-$SCH_2CH_2NHC(O)CH_3$ (Fig. 3), illustrate striking differences in the way in which these two tethers position attached groups. In an energy minimized conformation, the end of the acetamidopropynyl group projects well out from

the major groove of the helix. The uracil C5 carbon and carbon atoms 1–3 of the propynyl group are constrained to a 180° angle. As a result, the $NHC(O)CH_3$ group cannot approach the opposing strand any closer than ~6 Å, unless one forces the side chain to adapt higher energy conformations containing destabilizing gauche interactions. On the other hand, the lower energy barrier for rotation (45) and the 90° angle of C-S-C bonds provides the opportunity for the C5 thioether tether to track up the major groove and closely approach sites on the opposing strand without the need to assume higher energy conformations of the tether. Models of the duplex in Figure 3 show that the methyl group of the acetamidoethylthio tether can approach within ~2.3 Å of the N4 nitrogen of a deoxycytidine residue in the opposing strand. This deoxycytidine lies 2 bp upstream from the C5-modified deoxyuridine. For applications in which one wishes to crosslink two oligonucleotides, cleave an opposing strand or position groups within the major groove in order to enhance binding, the thioether tether may provide an advantage. For example, we have constructed a deoxyuridine thioether-linked bipyridine which, when incorporated into an oligonucleotide, positions a transition metal in sufficiently close proximity to an opposing strand to facilitate efficient metal-mediated site-specific cleavage. (46).

Conclusion

The T_m values of the duplexes derived from oligonucleotide d(CGCT*AATTAGCG) containing the modified nucleosides **22** (dU-C≡CCH_3), **23** [dU-C≡$CCH_2NHC(O)CH_3$], **25** [dU-C≡$CCH_2NHC(O)C_5H_4N$] and **26** [dU-C≡$CCH_2NHC(O)C_{10}H_{15}$] were respectively 59.1, 55.8, 58.3 and 54°C. With the exception of oligonucleotide **VI**, which contained the large lipophilic adamantyl group, the T_m values are higher than the unmodified oligonucleotide **I**. It is clear from this data that one can attach a variety of groups via an amidopropynyl spacer without greatly compromising the stabilizing effect of the conjugated –C≡C– group. Although the amidopropynyl tether provides greater duplex stability than the amidoethylthio tether, the latter provides a means to position functional groups in the major groove. The T_m values and thermodynamic data obtained as part of this study serve as guides to facilitate rational design of optimally modified oligonucleotides for future applications.

ACKNOWLEDGEMENTS

The National Institutes of Health is gratefully acknowledged for support of this research through grants AI36601 and CA65930. We thank Purdue Research Foundation for a fellowship to M.A. Preliminary studies on the palladium mediated coupling reactions by Xiaoping Lin are also greatly appreciated.

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