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Synthesis and Characterization of DNA Duplexes Containing an $N^4C-Ethyl-N^4C$ Interstrand Cross-Link

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Abstract: Short DNA duplexes containing an $N^4C-ethyl-N^4C$ interstrand cross-link, C−C, were synthesized on controlled pore glass supports. Duplexes having two, three, or four A/T base pairs on either side of the C−C cross-link and terminating with a C_4 overhang at their 5′-ends were prepared. The cross-link was introduced using a convertible nucleoside approach. Thus, an oligonucleotide terminating at its 5′-end with O^4-triazoyl-2′-deoxyuridine was first prepared on the support. The triazole group of support-bound oligomer was displaced by the aminoethyl group of 5′-dimethoxytrityl-3′-*O*-*tert*-butyldimethylsilyl-N^4-(2-aminoethyl)deoxycytidine to give the cross-link. The dimethoxytrityl group was removed, and the upper and lower strands of the duplex were extended from two 5′-hydroxyl groups of the cross-link using protected nucleoside 3′-phosphoramidites. The *tert*-butyldimethylsilyl group of the resulting partial duplex was then removed, and the chain was extended in the 3′-direction from the resulting 3′-hydroxyl of the cross-link using protected nucleoside 5′-phosphoramidites. The cross-linked duplexes were purified by HPLC and characterized by enzymatic digestion and MALDI-TOF mass spectrometry. Duplexes with three or four A/T base pairs on either side of the C−C cross-link gave sigmoidal shaped A_{260} profiles when heated, a behavior consistent with cooperative denaturation of the A/T base pairs. Each cross-linked duplex could be ligated to an acceptor duplex using T4 DNA ligase, a result that suggests that the C−C cross-link does not interfere with the ligation reaction, even when it is located only two base pairs from the site of ligation. The ability to synthesize duplexes with a defined interstrand cross-link and to incorporate these duplexes into longer pieces of DNA should enable preparation of substrates that can be used for a variety of biophysical and biochemical experiments, including studies of DNA repair.

Introduction

A variety of bifunctional alkylating agents such as nitrogen mustards, cis-platinum, cyclophosphamide, mitomycin C, and photoreactive compounds such as psoralen react with DNA to form interstrand cross-links. Many of the bifunctional alkylators are used clinically to treat cancer, and the DNA interstrand cross-links formed are believed to be the primary lesion responsible for their effectiveness as therapeutic agents.[1−3] Interstrand cross-

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links can be repaired by the cell through mechanisms that are only now beginning to be characterized.[4−14] Such repair would be expected to reduce the therapeutic efficacy of these compounds. Thus, a better understanding of repair processes could potentially lead to the development of more effective therapeutic agents.

A serious limitation to studying repair of DNA interstrand cross-links is the lack of substrates of defined structure. Reaction of DNA with bifunctional alkylating agents usually results in the formation of many products, only a small percentage of which is the desired interstrand cross-link. This problem can be limited to some extent by carrying out reactions on short oligonucleotide duplexes and isolating the interstrand cross-linked duplex by gel or chromatography methods. This postsynthetic approach has been used successfully to prepare, for example, duplexes cross-linked with a nitrogen mustard,[15,16] bifunctional pyrroles,[17] mitomycin C,[18,19] or *N*,*N*′-bis(2-chloroethyl)nitrosourea.[20] In some cases, these duplexes have been ligated into plasmid DNAs which subsequently have been used as substrates for repair studies.[6,10,21]

A more attractive synthetic strategy is a procedure in which the cross-linked duplex is prepared directly on a solid support. For example, Hopkins and co-workers[22] have recently reported solid support-mediated syntheses of nitrous acid cross-linked duplexes. This direct synthetic approach has the potential advantage of producing relatively large quantities of well-defined compounds for use in both physical and biological studies.

In this paper we describe the direct, support-mediated syntheses of short DNA duplexes that contain a single N^4C-ethyl$-N^4C$ interstrand cross-link. These duplexes serve as simple models for interstrand cross-linked DNA and are potential substrates for DNA repair studies. In addition, we describe some preliminary physical studies on these cross-linked duplexes and their ligation into longer DNA duplexes.

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Experimental Section

Materials. 5′-*O*-Dimethoxytrityldeoxyribonucleoside-3′-*O*-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidites, 3′-*O*-dimethoxytritylthymidine-5′-*O*-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite, and protected deoxyribonucleoside-controlled pore glass supports were purchased from Glen Research, Inc., Sterling VA. Phosphoramidite solutions were prepared using HPLC grade acetonitrile that was dried and stored over calcium hydride. Reversed-phase HPLC was carried out using a Rainin Microsorb-C-18 column (0.46 cm × 15 cm), and strong anion-exchange (SAX) HPLC was carried out using a Rainin Dynamax II column (0.46 cm × 25 cm), both purchased from Varian Associates, Walnut Creek, CA. The C-18 column was eluted with a 20 mL linear gradient of acetonitrile in 50 mM sodium phosphate buffer (pH 5.8) at a flow rate of 1.0 mL/min. The SAX column was eluted with 18 mL of a linear gradient of ammonium sulfate in a buffer that contained 1 mM ammonium acetate (pH 6.2) in 20% acetonitrile at a flow rate of 0.6 mL/min. The columns were monitored at 260 nm for analytical runs and at 290 nm for preparative runs. Oligomers purified by HPLC were desalted on C-18 SEP PAK cartridges (Waters Inc.). The SEP PAK was pre-equilibrated by washing with 10 mL aliquots of acetonitrile; 50% aqueous acetonitrile and 2% acetonitrile in 50 mM sodium phosphate, pH 5.8 (buffer A). The oligonucleotide solution was diluted into buffer A to give a final acetonitrile concentration of 3%, and the solution was applied to the cartridge. The cartridge was washed with 10 mL of water, and the oligomer was eluted with 3 mL of 50% aqueous acetonitrile. Polyacrylamide gel electrophoresis was carried out on 20 cm × 20 cm × 0.75 cm gels containing 20% acrylamide and 7 M urea. The running buffer was TBE, which contained 89 mM Tris, 89 mM boric acid, and 0.2 mM ethylenediaminetetraacetate buffered at pH 8.0. The gel loading buffer contained 90% formamide, 0.05% xylene cyanol, and 0.05% bromophenol blue.

Synthesis of 5′-*O*-Dimethoxytrityl-2′-deoxyuridine. A stirred solution of 2′-deoxyuridine (5 g, 22 mmol) in 110 mL of anhydrous pyridine was treated with dimethoxytrityl chloride (8.91 g, 26.5 mmol) for 18 h at room temperature, after which an additional 500 mg of dimethoxytrityl chloride was added, and stirring was continued for 3 h. The reaction solution was diluted with 25 mL of 95% ethanol and after 15 min of stirring was evaporated on a rotary evaporator. The resulting syrup was dissolved in 300 mL of ethyl acetate, and the solution was extracted with two 300 mL portions of 5% sodium bicarbonate followed by 300 mL of saturated sodium chloride. The organic layer was dried over anhydrous sodium sulfate. Following filtration, the solution was evaporated, and the resulting foamy residue was dissolved in 100 mL of ethyl acetate. This solution was added dropwise to 500 mL of hexane with vigorous stirring. The resulting precipitate was collected by centrifugation and dried under vacuum to give a solid, white powder (10.9 g, 20.5 mmol, 93%). UV (methanol): λ_{max} 204 nm, 231 nm, 262 nm. ^{1}H NMR (DMSO-d_6): δ 2.18 (m, 2H, *H*2′/2″), 3.25 (m, 2H, *H*5′/5″), 3.78 (s, 6H, O−C*H*$_3$), 3.87 (q, 1H, *H*3′), 4.23 (q, 1H, *H*4′), 5.33 (d, 1H, 3′−O*H*), 5.37 (d, 1H, *H*6), 6.14 (t, 1H, *H*1′), 6.8−7.4 (m, 13H, Ph−*H*), 7.63 (d, 1H, *H*5), 11.33 (s, 0.3H, N*H*).

Synthesis of 5′-*O*-Dimethoxytrityl-3′-*O*-*tert*-butyldimethylsilyl-2′-deoxyuridine. A stirred solution of 5′-dimethoxytrityl-2′-deoxyuridine (10 g, 18.8 mmol) in 25 mL of anhydrous *N*,*N*-dimethylformamide was treated with *tert*-butyldimethylsilyl chloride (6.2 g, 41 mmol) and imidazole (5.6 g, 83 mmol) for 1 h at room temperature. A 10 mL aliquot of methanol was added, and stirring was continued for 5 min. The reaction solution was poured into 300 mL of ethyl acetate, and the solution was extracted with two 300 mL portions of 5% sodium bicarbonate followed by 300 mL of saturated sodium chloride. The organic layer was evaporated and the foamy residue dissolved in 25 mL of ethyl acetate. This solution was added dropwise to 1.5 L of vigorously stirred hexane. The hexane was decanted, and the precipitate was washed with 200 mL of hexane. The precipitate was dissolved in ethyl acetate and transferred to a round-bottom flask, and the solution was evaporated to yield a tan foam (8.03 g, 12.5 mmol, 67%). ^{1}H NMR (DMSO-d_6): δ 0.00 (s, 3H, Si−C*H*$_3$), 0.01 (s, 3H, Si−C*H*$_3$), 0.77 (s, 9H, −C*H*$_3$), 2.20 (m, 2H, *H*2′/2″), 3.17 (m, 2H, *H*5′/5″), 3.73 (s, 6H,

O−CH_3), 3.79 (q, 1H, *H*3′), 4.40 (q, 1H, *H*4′), 5.42 (d, 1H, *H*6), 6.12 (t, 1H, *H*1′), 6.88−7.38 (m, 14H, Ph−*H*), 7.70 (d, 1H, *H*5), 11.33 (s, 0.3H, N*H*).

Synthesis of 5′-*O*-Dimethoxytrityl-3′-*O*-*tert*-butyldimethylsilyl-4-(*N*-1-triazoyl)-2′-deoxyuridine (2). Phosphorous oxychloride (1.6 mL, 2.8 g, 17 mmol) was slowly added to a stirred suspension of 1,2,4-triazole (5.8 g, 8.4 mmol) in 100 mL of anhydrous acetonitrile at 0 °C. Anhydrous triethylamine (12 mL) was then added dropwise, and the solution was stirred for 30 min. A solution of 5′-*O*-dimethoxytrityl-3′-*O*-*tert*-butyldimethylsilyl-2′-deoxyuridine (1.4 g, 2.1 mmol) in 15 mL of anhydrous acetonitrile was then added dropwise, and stirring was continued for 1.5 h. The reaction was quenched by addition of 150 mL of 5% sodium bicarbonate, and the aqueous solution was extracted with ethyl acetate. The organic layer was washed with saturated sodium chloride and dried over anhydrous sodium sulfate. After filtration, the solvents were evaporated, and the product was purified by silica gel flash column chromatography using ethyl acetate as solvent. Evaporation of the solvents gave a white foam (1.0 g, 1.4 mmol, 68%). UV (95% ethanol): λ_{max} 266 nm, 231 nm, λ_{min} 255 nm. ^{1}H NMR (DMSO-d_6): δ 0.00 (s, 3H, Si−CH_3), 0.06 (s, 3H, Si−CH_3), 0.77 (s, 9H, −CH_3), 2.37 (m, 2H, *H*2′/2″), 3.35 (m, 1H, *H*5′/5″), 3.73 (s, 6H, −OCH_3), 3.93 (m, 1H, *H*4′), 4.40 (q, 1H, *H*3′), 6.11 (t, 1H, *H*1′), 6.67 (d, 1H, *H*6), 6.8−7.3 (m, 14H, Ph−*H*), 8.39 (s, 0.5H, triazole−*H*), 8.61 (d, 1H, *H*5), 9.45 (s, 0.5H, triazole−*H*).

Synthesis of 5′-*O*-Dimethoxytrityl-3′-*O*-*tert*-butyldimethylsilyl-N^4-(2-aminoethyl)-2′-deoxycytidine (3). A solution of **2** (1 g, 1.4 mmol) in 15 mL of anhydrous pyridine was added dropwise with stirring to a solution of ethylenediamine (3.6 g, 4.0 mL, 60 mmol) in 50 mL of anhydrous pyridine. Stirring was continued for 1 h, and the solvents were then evaporated. Residual pyridine was removed by coevaporation with 95% ethanol. The resulting foamy residue was dissolved in 20% methanol/1% triethylamine/chloroform (v/v) and the product purified by silica gel flash chromatography to give a glassy solid (960 mg, 1.4 mmol, 100%). UV (95% ethanol): λ_{max} 275 nm, 235 nm, λ_{min} 258 nm. ^{1}H NMR (DMSO-d_6): δ −0.015 (s, 3H, Si−CH_3), −0.073 (s, 3H, Si−CH_3), 0.77 (s, 9H, −CH_3), 2.18 (m, 2H, *H*2′/2″), 2.65 (t, 2H, −CH_2−CH_2−NH_2), 3.24 (t, 2H, −CH_2−CH_2−NH_2), 3.42 (m, 2H, *H*5′), 3.74 (s, 6H, −OCH_3), 3.79 (m, 1H, *H*3′), 4.08 (s, 0.9H, −NH_2), 4.39 (q, 1H, *H*4′), 5.62 (d, 1H, *H*6), 6.15 (t, 1H, *H*1′), 6.98−7.38 (m, 14H, Ph−*H*), 7.67 (d, 1H, *H*5).

Synthesis of 1,2-Bis-(N^4-2′-deoxycytidylyl)ethane (1). A solution of 5′-*O*-dimethoxytrityl-3′-*O*-*tert*-butyldimethylsilyl-4-(*N*-1-triazoyl)-2′-deoxyuridine (**2**, 1.3 mg, 2 μmol) and 5′-*O*-dimethoxytrityl-3′-*O*-*tert*-butyldimethylsilyl-N^4-(2-aminoethyl)-2′-deoxycytidine (**3**, 3.2 mg, 5 μmol) in 50 μL of anhydrous pyridine was incubated at room temperature for 16 h. Examination by silica gel TLC indicated that the reaction was complete. The solvents were evaporated, and the residue was treated with 200 μL of 0.1 N hydrochloric acid for 30 min at 65 °C. The solvents were evaporated, the residue was dissolved in 50% aqueous acetonitrile, and the solution was examined by C-18 reversed-phase HPLC using a 2−20% linear gradient of acetonitrile. Two compounds were observed:N^4-(2-aminoethyl)-2′-deoxycytidine [^{1}H NMR (D_2O): δ 0.70 (m, 2H, *H*2′), 1.41 (t, 2H, N^4−CH_2−CH_2−NH_2), 1.95 (t, 2H, N^4−CH_2−CH_2−NH_2), 2.17 (m, 2H, *H*5′), 2.43 (q, 1H, *H*4′), 2.82 (m, 1H, *H*3′) 4.26 (d, 1H, *H*5), 4.66 (t, 1H, *H*1′), 6.13 (d, 1H, *H*6)] and 1,2-bis-(N^4-deoxycytidylyl)ethane (**1**) [UV (50% aqueous acetonitrile): λ_{max} 274 nm, λ_{min} 240 nm. ^{1}H NMR (D_2O): 0.63 (m, 4H, *H*2′), 2.00 (s, 4H, N^4−CH_2−CH_2−N^4), 2.14 (m, 4H, *H*5′), 2.42 (q, 2H, *H*4′), 2.80 (m, 2H, *H*3′), 4.28 (d, 2H, *H*5), 4.62 (t, 2H, *H*1′), 6.05 (d, 2H, *H*6). The molar ratio of N^4-(2-aminoethyl)-2′-deoxycytidine to **1** was 1.47:1.

Syntheses of $N^4C-Ethyl-N^4C$ Cross-Linked Duplexes. 5′-d-*U*-$(T)_n$∼CPG (**5**), where $n = 2$, 3, or 4 and *U* is 4-triazoyl-2′-deoxyuridine, was prepared on the DNA synthesizer. The concentration of the protected nucleoside-3′-*O*-β-cyanoethyl-*N*,*N*-diisopropyl phosphoramidites was 0.15 M, the coupling time was 120 s, and the synthesizer was programmed to remove the 5′-terminal dimethoxytrityl group after the last coupling step. The oligomer-derivatized support was transferred to a 500 μL conical Reacti-Vial (Pierce Chemical Co.) and treated with 150 μL of a 0.1 M solution of nucleoside **3** in dry pyridine for 16 h at room temperature (**5**, $n = 2$) or 150 μL of 0.2 M of nucleoside **3** at 37 °C for 16 h (**5**, $n = 3$) or 48 h (**5**, $n = 4$). The excess solution was removed, the support (**6**) was transferred back to the synthesis column, and the support was washed with two 10 mL aliquots of acetonitrile. Chain extension was then continued in the 5′-direction after removal of the 5′-dimethoxytrityl group from **6**. After the final coupling step, the synthesizer was programmed to acetylate the 5′-ends of the partial cross-linked duplex to give **7**. The support was then treated with 0.8 mL of anhydrous triethylamine for 32 h at room temperature. The triethylamine was flushed from the column, and the support was washed with two 10 mL aliquots of acetonitrile and then dried under vacuum. The dry support was then treated with 1 mL of 1 M tetra-*n*-butylammonium fluoride (TBAF) in tetrahydrofuran for 60 min at room temperature. The TBAF solution was flushed from the support, and the support was washed with 10 mL of 50% aqueous acetonitrile followed by 10 mL of acetonitrile. Synthesis was then continued in the 3′-direction using a 0.14 M solution of 3′-*O*-dimethoxytritylthymidine-5′-*O*-β-cyanoethyl-*N*,*N*-diisopropylphosphoramidite. The coupling time was 180 s, and the 3′-terminal dimethoxytrityl group was removed at the end of the synthesis to give support-bound oligomer **8**.

Each support was treated with 400 μL of concentrated ammonium hydroxide for 5 h at 65 °C. The supernatant was removed from the support, and the support was washed with four 200 μL aliquots of 50% aqueous acetonitrile. The combined supernatant and washings were evaporated to dryness under vacuum at 37 °C. The cross-linked duplexes were purified by HPLC. An aliquot (13 A_{260} units) of crude oligomer **1827** was first purified on the C-18 reversed-phase column using a linear gradient of 2−50% acetonitrile. The product peak, which was contaminated with shorter oligonucleotides, was desalted on a C-18 SEP PAK cartridge and then further purified on the SAX column using a linear gradient of 0−0.5 M ammonium sulfate. The oligomer was desalted on a C-18 SEP PAK cartridge to give 0.74 A_{260} unit of **1827**. Crude oligomer **1828** (45 A_{260} units) and **1829** (45 A_{260} units) were each purified by SAX HPLC using a linear gradient of 0.001−0.8 M ammonium sulfate. A total of 5.8 A_{260} units of **1828** and 4.5 A_{260} units of **1829** were obtained after desalting on a SEP PAK cartridge. Oligomer **1828** was further purified by C-18 reversed-phase HPLC using a linear gradient of 2−20% acetonitrile. A total of 2.3 A_{260} units of **1828** were obtained after desalting on a SEP PAK cartridge.

The cross-linked oligomers (0.02 A_{260} unit each) were phosphorylated in 9.5 μL of solution that contained 70 mM Tris, pH 7.6, 10 mM magnesium chloride, 5 mM dithiothreitol, and 63 μM γ-[^{32}P]-ATP (specific activity 17 μCi/mmol). The reactions were initiated by addition of 0.5 μL (5 units) of T4 polynucleotide kinase and incubated at 37 °C for 3 h. Each cross-linked oligomer migrated as a single band on a 20% polyacrylamide gel run under denaturing conditions as shown in Figure 2.

The cross-linked oligomers were subjected to enzymatic digestion with a combination of snake venom phosphodiesterase (SVPD) and calf intestinal phosphatase (CIP). Oligomer **1827** (0.04 A_{260} unit), dissolved in 16 μL of a solution containing 10 mM Tris (pH 8.1) and 2 mM magnesium chloride, was treated with 3 μL (6 ng) of SVPD and 1 μL (10 units) of CIP for 16 h at 37 °C. Oligomers **1828** (0.1 A_{260} unit) and **1829** (0.1 A_{260} unit), each dissolved in 17 μL of enzyme buffer, were treated with 2 μL of SVPD and 0.5 μL of CIP for 16 h at 37 °C. A 10 μL aliquot of each reaction mixture was treated with an additional 2 μL of SVPD and 0.5 μL of CIP for 18 h at 37 °C. Each digest was analyzed by C-18 reversed-phase HPLC using a linear gradient of 2−20% acetonitrile. The nucleoside ratios were calculated after dividing the area of each peak by the appropriate extinction coefficient. The ϵ_{260} values used were as follow: dA, 14 100; dC, 7300; dT, 9000; dC−dC, 16 240. The ϵ_{260} values of dA, dC, dG, and dT were determined experimentally in 50 mM sodium phosphate buffer, pH 5.8, that contained 2% acetonitrile. The ϵ_{260} value of dC−dC was calculated using the extinction coefficient of N^4-methyl-2′-deoxycytidine (ϵ_{260} 8210).[23] The results are shown in Table 1. The molecular weights of each cross-linked oligomer were determined by MALDI-TOF mass spectrometry, and the results are shown in Table 1.

Thermal Denaturation Experiments. The cross-linked duplexes

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Table 1. Nucleoside Ratios and Mass Spectral Data for Cross-Linked Duplexes

cross-linked duplex		nucleoside ratio expected	nucleoside ratio found	mass expected	mass found
1827				5264	5269
	dA	2.00	1.87		
	dC	4.00	3.91		
	dT	2.00	2.15		
	dC−dC	1.00	1.00		
1828				6498	6497
	dA	1.00	0.92		
	dC	1.33	1.32		
	dT	1.00	1.00		
	dC−dC	0.33	0.33		
1829				7733	7735
	dA	1.00	1.07		
	dC	1.00	1.11		
	dT	1.00	1.00		
	dC−dC	0.25	0.28		

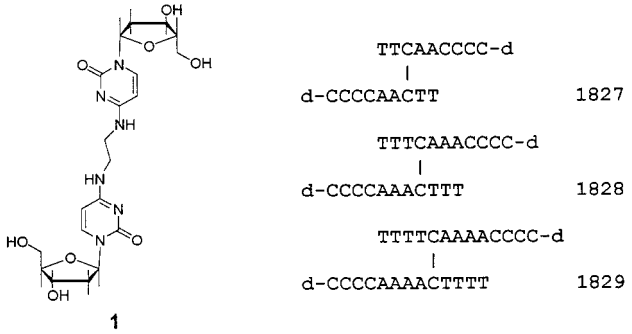


Figure 1. Structure of the N^4C−ethyl−N^4C cross-link and sequences of the C−C cross-linked duplexes.

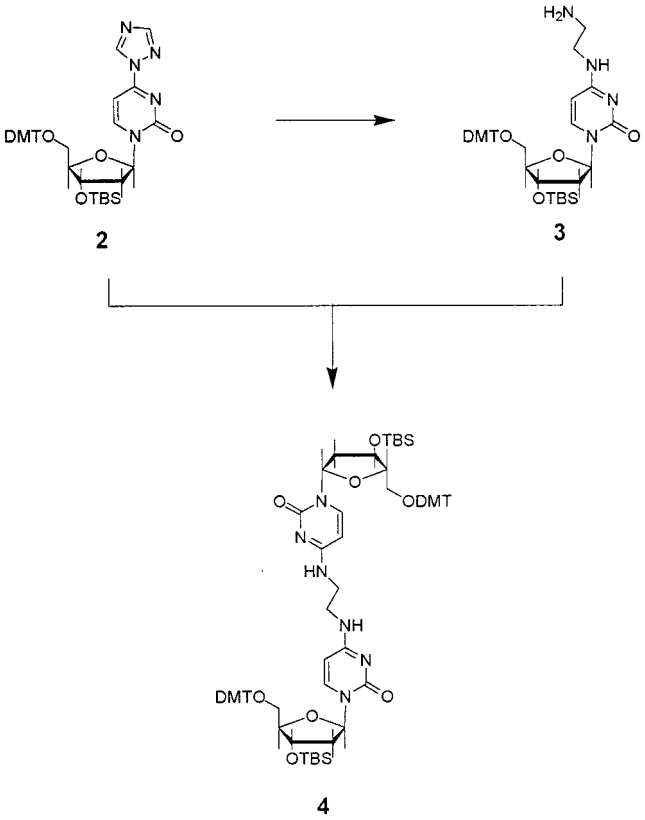
or their constituent strands were dissolved in a buffer containing 50 mM Tris, pH 7.2, 100 mM sodium chloride, and 0.5 mM ethylenediamine tetraacetate. The thermal denaturation experiments were carried out using a Cary 3E UV−vis spectrophotometer fitted with a thermostated sample holder and temperature controller as previously described.[24] Duplexes were heated from 0 to 70 °C at a rate of 0.5 °C/min, and the absorbance at 260 nm was recorded as a function of the temperature.

Ligation Reactions. Both 5′-hydroxyl groups of each cross-linked duplex were end-labeled using γ-[^{32}P]-ATP and T4 polynucleotide kinase as described above. Following the reaction, the kinase was inactivated by heating. A non-cross-linked control duplex, **1830**, was prepared by annealing d-[^{32}P]-pCCCCAAAACTTTT and d-pCCCCAAAAGTTTT. An acceptor duplex, **1831**, was prepared by annealing equimolar amounts of d-CGTCCTCACTCCTGG and d-pGGGGCCAGGAGTGAGGACG. Ligation reactions were carried out with 0.1 μM cross-linked duplex or control duplex **1830** and 0.4 μM acceptor duplex **1831** in a buffer that contained 50 mM Tris-HCl (pH7.5), 10 mM magnesium chloride, 100 mM sodium chloride, 10 mM dithiothreitol, 1 mM ATP, and 25 μg/mL bovine serum albumin. The reactions were initiated by the addition of 5 units of T4 DNA ligase, and the reaction mixtures were incubated at 16 °C for 30 min. The reactions were quenched by the addition of 90% formamide loading buffer. Samples were electrophoresed on a denaturing 20% polyacrylamide gel and visualized by autoradiography as shown in Figure 5.

Results and Discussion

Syntheses of Cross-Linked Duplexes. Oligodeoxyribonucleotide duplexes were synthesized that contain an N^4C−ethyl−N^4C cross-link (**1**) whose structure is shown in Figure 1. The sequences of these duplexes are also shown in Figure 1. Each duplex contains a central C−C cross-link flanked on either side by two (**1827**), three (**1828**), or four (**1829**) A−T base pairs. The C_4 overhangs at the 5′-ends of each duplex provide sites for ligation of the cross-linked duplexes into longer pieces of DNA.

Scheme 1



Examination of molecular models suggests that the ethyl group that links the two opposing cytosines can be accommodated in the major groove with minimal distortion of the B-form helix. As far as we know, this type of cross-link has not been observed when DNA is treated with alkylating agents. However, Romereo et al. recently reported isolation of an N^3C−N^3C cross-link that results from reaction of mechlorethamine with DNA that contains a C/C mismatch.[25]

The N^4C−ethyl−N^4C (C−C) cross-link itself can be synthesized readily in solution using a convertible nucleoside approach,[26−28] as shown in Scheme 1. Thus, in a series of test reactions, 5′-*O*-dimethoxytrityl-3′-*O*-*tert*-butyldimethylsilyl-4-(*N*-1-triazoyl)-2′-deoxyuridine (**2**) was converted to the protected N^4-(2-aminoethyl)deoxycytidine derivative (**3**) by reaction with ethylenediamine at room temperature in pyridine. Subsequent reaction in pyridine between **2** and an excess of aminoethyl nucleoside **3** at room temperature results in formation of the fully protected C−C cross-link, **4**. Displacement of the triazole group of **2** by the 2-amino group of **3** was complete after 16 h at room temperature, as judged from silica gel TLC. Treatment of **4** with 0.1 N hydrochloric acid removed the dimethoxytrityl and *tert*-butyldimethylsilyl groups to give **1**, which was characterized by ^{1}H NMR.

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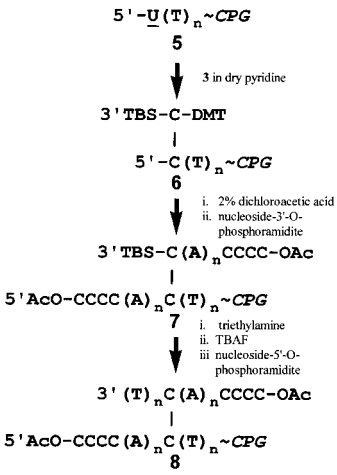
Scheme 2



The convertible nucleoside approach was combined with an orthogonal synthesis scheme to produce the C−C cross-linked duplexes as outlined in Scheme 2. Oligo-T terminated with a 4-triazoyl-2′-deoxyuridine (**5**) was prepared on a controlled pore glass support using the DNA synthesizer. The support was removed from the synthesizer and treated with a solution of 5′-*O*-dimethoxytrityl-3′-*O*-*tert*-butyldimethylsilyl-N^4-(2-aminoethyl)-2′-deoxycytidine (**3**) in pyridine at 37 °C for periods up to 48 h. To determine the extent of conversion to the cross-linked oligomer, portions of the support were deprotected by sequential treatment with concentrated ammonium hydroxide and 80% aqueous acetic acid, and the products were analyzed by reversed-phase HPLC. Unlike the result for the reaction in solution, the percent conversion did not exceed 70%, even after prolonged incubation. Failure to completely convert the 4-triazoyl-dU to the C−C cross-link was not due to hydrolysis of the 4-triazoyl-dU to deoxyuridine during the incubation. Thus, digestion of the crude oligonucleotide with a combination of snake venom phosphodiester and calf intestinal phosphatase did not yield deoxyuridine. It appears that a portion of support-bound oligomer **6** is inaccessible to reaction with the somewhat bulky aminoethyl nucleoside **3**. This may be due to steric blocking caused by adjacent oligomer chains on the surface of the support. Alternatively or additionally, some of the oligomer chains may be sequestered in pores on the support and may thus be unable to react with **3**.

The 5′-terminal dimethoxytrityl group of **6** was removed, and the upper and lower strands of the cross-link were extended in the 5′-direction. Because of the symmetry of the duplex, the upper and lower strands have the same sequence and can thus be synthesized simultaneously. To maximize coupling yields, the phosphoramidite concentration was increased from 0.1 to 0.15 M, and the coupling times were increased from 60 to 120 s. Under these conditions, coupling appeared to be almost quantitative, as judged from the intensity of the color of the dimethoxytrityl cation released after each coupling cycle. After the final detritylation, the 5′-hydroxyl groups on the upper and lower strands of the duplex were each capped with acetic anhydride to give the 5′-acetylated, support-bound oligomer **7**.

Selective removal of the *tert*-butyldimethylsilyl group from the 3′-position of the cross-link allows extension of 3′-end of the upper strand of the duplex. Braich and Damha have reported previously using a similar strategy to extend one chain of a branched RNA.[29] In our hands, initial attempts to remove the 3′-*O*-*tert*-butyldimethylsilyl group of oligomer **7** by treatment with a solution of tetra-*n*-butylammonium fluoride (TBAF) in tetrahydrofuran suggested that some degradation of the support-bound oligomer had occurred. Similar problems were reported during the syntheses of branched oligoribonucleotides[29,30] and during the syntheses of oligoribonucleotides carrying a 5′-*O*-silyl protecting group.[31] We reasoned that a possible cause of this degradation could be attack of the 3′-oxygen anion, produced during fluoride ion cleavage of the TBS group, on adjacent phosphotriester linkages. To test this hypothesis, a 15-mer, d-T_{15}, was prepared. Treatment of support-bound 15-mer with TBAF followed by cleavage of the oligomer from the support with ammonium hydroxide gave the same HPLC profile as material treated only with ammonium hydroxide. This result suggests that fluoride ion alone does not cleave the cyanoethylphosphotriester linkages of the oligomer. The 5′-hydroxyl of the support-bound 15-mer was converted to its *tert*-butyldimethylsilyl derivative. Treatment of this TBS-d-T_{15} with TBAF followed by ammonium hydroxide treatment gave rise to shorter $n-1$ and $n-2$ oligomers, as judged from the strong anion-exchange HPLC. In a separate experiment, the support-bound TBS-d-T_{15} was first treated with anhydrous triethylamine.[29,30] This treatment removes cyanoethyl phosphate protecting groups but does not cleave the oligomer from the support.[32] The triethylamine-treated oligomer was then reacted with TBAF, followed by ammonium hydroxide cleavage from the support. Examination by HPLC showed the absence of the shorter oligomers seen when TBS-d-T_{15} was treated with TBAF alone. TBAF treatment did not appear to cleave significant amounts of the oligomer from the support. Thus, 80−90% of the expected OD was recovered from the TBAF-treated oligomer.

Support-bound partial duplex **7** was pretreated with triethylamine followed by reaction with TBAF for 60 min at room temperature. In initial experiments, approximately 70% of the TBS groups were removed when the support was treated with commercial TBAF solution. Virtually quantitative removal could be achieved, however, if the TBAF solution was stored over molecular sieves prior to use. This treatment most likely removes traces of water that suppress the fluoride-mediated cleavage of the silyl ether. Subsequent experiments showed that brief (10 min) exposure to dry TBAF was sufficient to remove the *tert*-butyldimethylsilyl group, thus lessening the possibility of cleaving the oligomer from the support.

The partial duplex was then extended in the 3′-direction using 3′-*O*-dimethoxytritylthymidine-5′-*O*-phosphoramidite to give **8**. The coupling reactions appeared to proceed in >90% yield, as judged from the intensity of the color of the trityl cation released after each synthetic cycle. The 3′-dimethoxytrityl group was removed after the last coupling step, and the oligomer was deprotected and cleaved from the support by treatment with concentrated ammonium hydroxide solution. Following deprotection, each cross-linked duplex was purified by strong anion-exchange HPLC alone or in combination with C-18 reversed-phase HPLC.

The synthetic scheme described above is similar to that used by Harwood and co-workers to prepare duplexes that contain a G−G nitrous acid cross-link.[22] In our case, the C−C cross-link is introduced using the convertible nucleoside approach on the support-bound oligomer, whereas the G−G cross-link was introduced in the form of the protected phosphoramidite. For C−C cross-linked duplexes of the type described here, the

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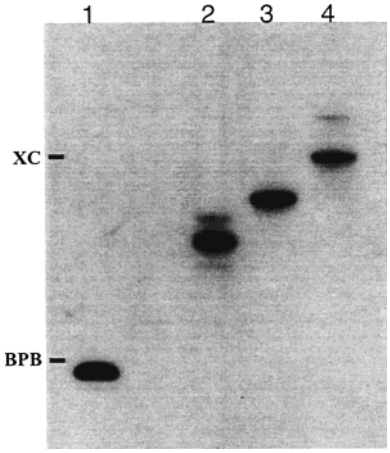

Figure 2. Polyacrylamide gel electrophoresis of cross-linked duplexes. A linear 9-mer, d-CCCCAACTT (lane 1), and cross-linked duplexes **1827** (lane 2), **1828** (lane 3), and **1829** (lane 4) were each 5′-end labeled with γ-[^{32}P]-ATP and subjected to electrophoresis on a 20% polyacrylamide gel run under denaturing conditions. XC and BPB are xylene cyanol and bromophenol blue dyes, respectively.

convertible nucleoside approach provides a versatile method for introducing cross-links of varying chain length into the duplex without the necessity of synthesizing individual protected cross-link phosphoramidites. Such duplexes could provide interesting substrates for physical studies and DNA repair studies.

Characterization of Cross-Linked Duplexes. The duplexes were phosphorylated using γ-[^{32}P]-ATP and polynucleotide kinase and analyzed by electrophoresis on a denaturing 20% polyacrylamide gel. As shown in Figure 2, each duplex migrated as a single band on the gel in accordance with the size of the duplex. Duplex **1829**, which contains 26 nucleotides, migrated with the xylene cyanol tracking dye. This mobility is similar to that of a linear 28-mer, which comigrates with xylene cyanol on this gel.[33]

The cross-linked duplexes could be hydrolyzed to their component nucleosides and C−C cross-link by treatment with a combination of snake venom phosphodiesterase, a 3′-exonuclease, and bacterial alkaline phosphatase. It appears that hydrolysis of phosphodiester linkages in the vicinity of the cross-link is slow. Thus, prolonged incubation, and in the cases of duplexes **1828** and **1829** addition of a second aliquot of snake venom phosphodiester, were required to effect complete degradation. The digests were analyzed by C-18 reversed-phase HPLC. A typical digest for oligomer **1829** is shown in Figure 3. The C−C cross-link produced in the digests had a retention time identical to that of C−C synthesized in solution. As shown in Table 1, the ratios of the component nucleosides and the cross-link were consistent with the structure of each duplex.

In addition to enzymatic digestion, the duplexes were also characterized by MALDI-TOF mass spectrometry. As indicated in Table 1, the observed molecular weights were consistent with the expected molecular weights for each cross-linked duplex.

Thermal Denaturation. The thermal stabilities of the cross-linked duplexes were examined by monitoring their UV absorbance at 260 nm as a function of temperature. The results are shown in Figure 4. In addition to studying the cross-linked duplexes, we also monitored absorbance vs temperature of d-C_4-$(A)_nC(T)_n$,which represent the component strands of the cross-linked duplexes, and non-cross-linked duplexes formed by d-$C_4(A)_nC(T)_n$/d-$C_4(A)_nG(T)_n$. The latter duplexes contain a C−G base pair in the position occupied by the C−C cross-link of the cross-linked duplexes.

Each of the d-$C_4(A)_nC(T)_n$ oligomers, where $n = 2$, 3, or 4, essentially showed a linear increase in A_{260} when heated over the temperature range 0−70 °C. This behavior is consistent with a lack of duplex formation. Duplex formation would require

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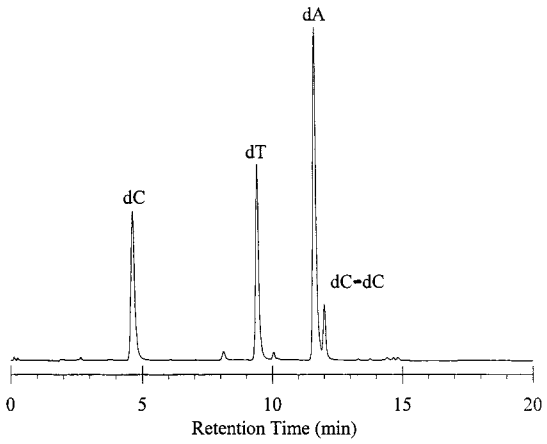

Figure 3. Reversed-phase HPLC analysis of duplex **1829** after digestion with snake venom phosphodiesterase and calf intestinal phosphatase. The HPLC conditions are given in the Experimental Section.

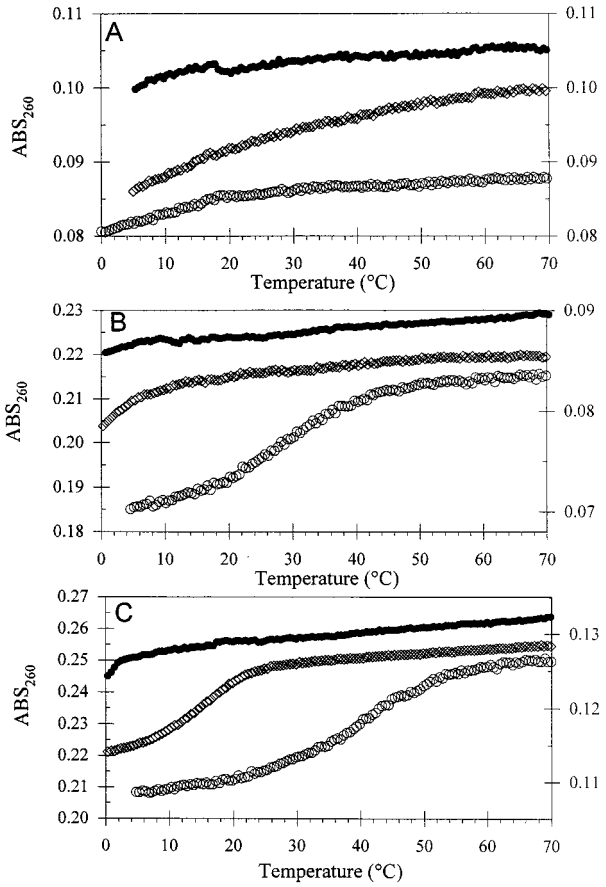

Figure 4. Thermal denaturation profiles of the cross-linked duplexes. (A) d-$C_4A_2CT_2$ (●); d-$C_4A_2CT_2$/d-$C_4A_2GT_2$ (□); and **1827** (○). (B) d-$C_4A_3CT_3$ (●); d-$C_4A_3CT_3$/d-$C_4A_3GT_3$ (□); and **1828** (○). (C) d-C_4A_4-CT_4 (●); d-$C_4A_4CT_4$/d-$C_4A_4GT_4$ (□); and **1829** (○). The experiments were carried out in buffer that contained 50 mM Tris, pH 7.2, 100 mM sodium chloride, and 0.5 mM ethylenediamine tetraacetate. The right axes show the A_{260} of the cross-linked duplexes, and the left axes show the A_{260} of the control oligomers.

the presence of an unstable C/C mismatch in the center of the duplex, and this most likely accounts for the failure of these oligomers to form duplexes under these conditions.

The cross-linked duplexes showed different behavior. Duplex **1827** displayed a nonlinear increase in absorbance over the temperature range 0−20 °C. This behavior suggests that minimal base-pairing interaction takes place in this short cross-linked duplex. In contrast, duplexes **1828** and **1829** each exhibited distinct sigmoidal transitions with inflection points at 30 and 39 °C, respectively. These transition curves are qualitatively similar to the melting curve observed for non-cross-linked duplex d-$C_4(A)_4C(T)_4$/d-$C_4(A)_4G(T)_4$, whose T_m is 16 °C. Thus, the shapes of the thermal transition curves of duplexes **1828** and **1829** suggest that the A/T base pairs of these cross-linked duplexes denature in a cooperative manner. Because the C−C cross-link is not hydrolyzed under these conditions, the strands of these cross-linked duplexes cannot completely separate as in the case of d-$C_4(A)_4C(T)_4$/d-$C_4(A)_4G(T)_4$. The presence of the cross-link increases the thermal stability of the adjacent A/T base pairs. Thus, the transition temperature of **1829** is 23 °C higher than the T_m of the non-cross-linked duplex d-$C_4(A)_4C(T)_4$/d-$C_4(A)_4G(T)_4$. Similar cooperative thermal denaturation and increases in stability have been observed in short DNA duplexes that contain 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen[34] or N2-G-mitomycin C−N2−G[19] interstrand cross-links.

Ligation Experiments. For repair studies and other biophysical characterization studies, it will be necessary to be able to ligate the cross-linked duplexes into longer duplex or plasmid DNA. The ability of the C−C cross-link duplexes to serve as substrates for DNA ligase was tested by following the ligation of 5′-^{32}P-labeled **1827**, **1828**, or **1829** to a 15-base pair acceptor duplex, **1831**, whose sequence is given in the Experimental Section. Because the cross-linked duplexes are symmetrical, successful ligation requires that each duplex undergo two individual ligation events. Thus both 5′-CCCC overhangs of the duplex are potential substrates for the ligation reaction.

The ligation reactions were carried out using a 4-fold molar excess of the acceptor duplex, which is a 2-fold ligation excess given that the cross-linked duplexes contain two ligation sites, under salt conditions similar to those used in the thermal denaturation studies. Each ligation reaction was incubated at 16 °C for 30 min prior to electrophoresis on a 20% denaturing polyacrylamide gel.

As shown in Figure 5, lane 2, a control duplex, **1830**, in which the C−C cross-link of **1829** has been replaced with a G/C base pair, is converted in the presence of T4 DNA ligase to two slower migrating species. These species presumably correspond to fully denatured 47-mer, the slowest moving band, and a hairpin, which can form because the sequence of the 47-mer is symmetrical. Under identical conditions, all three of the cross-linked duplexes are capable of serving as substrates for the ligation reaction, as shown by bands in lanes 4, 6, and 8. The mobilities of these ligated duplexes, whose sizes differ by two or four base pairs, are similar and, as expected, are less than the mobilities of the single-stranded ligation products of the non-cross-linked control duplex.

In separate experiments (data not shown), we found that a new product of intermediate gel mobility was formed when the ligation reactions were carried out using a 1:1 molar ratio of **1829** to acceptor duplex. This product was "chased" into a product of lower gel mobility when the ligation reaction was continued after addition of excess acceptor duplex. This behavior is consistent with first formation of half-ligated product consisting of one cross-link duplex and one acceptor duplex, followed by formation of the fully ligated product consisting of one cross-linked duplex and two acceptor duplexes. Thus, based on these titration experiments, the bands shown in lanes 4, 6, and 8 correspond to duplexes that contain one cross-linked duplex ligated to two acceptor duplexes.

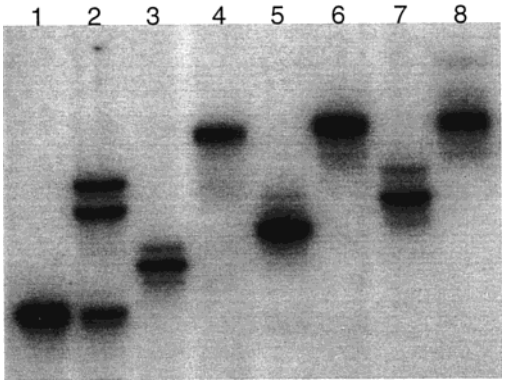

Figure 5. DNA ligation experiments. Control duplex **1830** (lanes 1 and 2) and cross-linked duplexes **1827** (lanes 3 and 4), **1828** (lanes 5 and 6), and **1829** (lanes 7 and 8) were each incubated with acceptor duplex **1831** in the absence (lanes 1, 3, 5, 7) or presence (lanes 2, 4, 6, 8) of T4 DNA ligase, and the reaction mixtures were subjected to electrophoresis on a 20% polyacrylamide gel run under denaturing conditions.

Each of the cross-linked duplexes was completely converted to its ligated product under the conditions of the reaction. This result suggests that the C−C cross-link does not interfere with the ligation reaction, even when the cross-link is located only two base pairs from the site of the ligation, as is the case with duplex **1827**. It is interesting to note that, although cross-linked duplex **1827** failed to exhibit any significant evidence of thermal denaturation, its ability to support ligation appears to be unhindered. While this behavior could be attributed to differences in buffer conditions between the two experiments, it seems more likely that T4 DNA ligase, an enzyme capable of catalyzing even blunt-ended ligations, stabilizes the interaction between this short duplex and the acceptor.

Conclusions

The results of our experiments show that the convertible nucleoside approach can be used in conjunction with orthogonal protecting groups to prepare interstrand cross-linked duplexes directly on a DNA synthesizer. Although duplexes having an ethyl cross-link between two opposing C residues were synthesized here, the method should be applicable to syntheses of other duplexes with opposed or staggered cross-links of varying chain length. Such duplexes, which could be incorporated enzymatically into longer pieces of DNA, should provide valuable substrates for a variety of biophysical and biochemical experiments.

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