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Preparation of *N*3-thymidine–butylene–*N*3-thymidine interstrand cross-linked DNA via an orthogonal deprotection strategy

Gang Sun, Anne Noronha, Christopher Wilds *

Department of Chemistry and Biochemistry, Concordia University, 7141 Sherbrooke St. West, Montréal, Québec, Canada

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ABSTRACT

A DNA duplex containing an *N*3-thymidine–butylene–*N*3-thymidine interstrand cross-link (ICL) was prepared using an on-column orthogonal deprotection strategy to permit different nucleotide sequence composition around the cross-linked site. The conditions used to remove 5′-*O*-allyloxycarbonyl and 3′-*O*-*tert*-butyldimethylsilyl protective groups for various on-column oligonucleotide intermediates did not affect the cross-linked lesion. Efficient removal of these groups enabled successful coupling of 2′-deoxyphosphoramidites to produce the desired duplex with a 31% yield after deprotection and purification.

1. Introduction

Cellular DNA, on exposure to various environmental and chemotherapeutic agents, can undergo damage with numerous modifications identified including interstrand cross-links (ICLs). ICLs threaten genomic and cellular integrity because they present formidable blocks to essential metabolic processes that require obligate strand separation (replication and transcription). Even though cells must efficiently detect and remove damage for survival, ICL-inducing agents continue to be employed as chemotherapeutic treatments for many cancers.[1,2] However, resistance to ICL-inducing agents due in part to efficient DNA repair, can result in recurrent malignancies, which are unresponsive to treatment,[3,4] in addition to possibly increasing the risk of secondary cancers likely due to their mutagenic processing in normal cells.[5] Despite the substantial progress in the fields of ICL repair in bacteria and yeast, which occur primarily via a combination of processes including nucleotide excision repair (NER), homologous recombination (HR), and translesion synthesis (TLS)[6–9] a complete understanding of such repair in mammalian systems is still unclear.[10–17] In mammalian cells, it is thought that ICLs are repaired by the coordination of proteins from several pathways, including NER,[18,19] base excision repair (BER),[20] mismatch repair (MMR),[21,22] HR,[23,24] TLS,[25] and proteins involved in Fanconi anemia (FA).[26] Therefore, efforts to better understand the cellular responses to and repair of ICL-inducing lesions in mammalian cells offer the potential to improve the efficacy of these drugs in cancer therapy.

One continuing approach to investigate the role that repair pathways play in removing ICL is to use chemically synthesized oligonucleotides, which contain adducts that represent the lesions introduced by ICL-inducing agents. Differing strategies have been developed to obtain ICL-containing DNA. One avenue to ICL synthesis involves the incorporation of stable ICL forming precursors into single stranded DNA using standard DNA synthesis after which hybridization triggers ICL formation.[27,28] We and others have established an approach (mono and bidirectional) to synthesize cross-linked nucleosides to directly introduce the ICL, which enables exact placement of the desired ICL, using a combination of solution and solid-phase synthesis.[29–37] However, several of these model ICL systems to date have contained symmetrical nucleotide sequence composition (either complete or partial) around the cross-linked site (Fig. 1).

We have previously reported the synthesis of N3-thymidine–butylene–N3-thymidine (N3T–butylene–N3T) ICL duplexes with either partial or complete symmetry around the site of the cross-link using nucleoside dimers containing DMT and TBS protecting groups on the 5′- and 3′-*O* functionalities using mono- and bis-phosphoramidite approaches.[33,34] In order to accomplish the synthesis of asymmetric sequences it is imperative to design the dimer phosphoramidite to contain three different protective groups around the 5′- and 3′-*O* functionalities that are compatible with each other for selective removal without compromising the ICL lesion to introduce the cross-link into the desired ICL duplexes.[38]

* Corresponding author. Tel.: +1 514 848 2424x5798; fax: +1 514 848 2868; e-mail address: cwilds@alcor.concordia.ca (C. Wilds).

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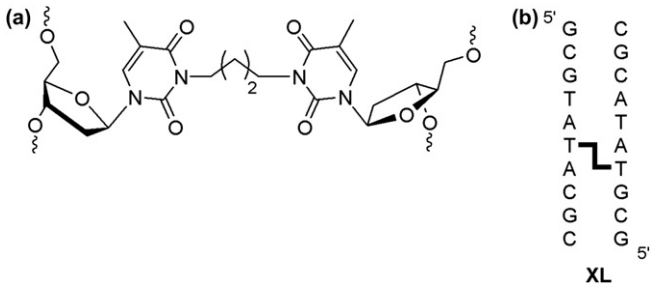


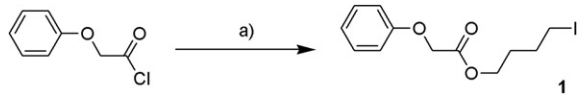
Fig. 1. (a) Chemical structure of the N3T–butylene–N3T ICL and (b) sequence of ICL oligonucleotide prepared (**XL**).

In this report, we describe the synthesis of 1-{*N*3-[5′-*O*-(dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-thymidylyl]}-4-{*N*3-[5′-*O*-(allyloxycarbonyl)-thymidylyl-3′-*O*-(β-cyanoethyl *N*,*N*′-diisopropyl)phosphoramidite]}butane (**6**), an asymmetric phosphoramidite that enables the synthesis of the *N*3T–butylene–*N*3T ICL and detailed procedures of the orthogonal deprotection strategy that allows for the selective nucleotide sequence assembly around the cross-linked site.

2. Results and discussion

2.1. *N*3T–butylene–*N*3T cross-linked phosphoramidite synthesis

The cross-linked dimer phosphoramidite (**6**) was synthesized in solution as illustrated in Schemes 1 and 2. The linker, 4-iodobutyl 2-phenoxyacetate (**1**), was prepared in a single step in quantitative yields by reacting 2-phenoxyacetyl chloride and potassium iodide in tetrahydrofuran (THF). The protected alkyl linker was coupled to 5′-*O*-dimethoxytrityl-3′-*O*-(*tert*-butyldimethylsilyl)-thymidine to produce *N*3-(4-iodobutyl)-5′-*O*-dimethoxytrityl-3′-*O*-(*tert*-butyldimethylsilyl)-thymidine in 85% yield.[33]

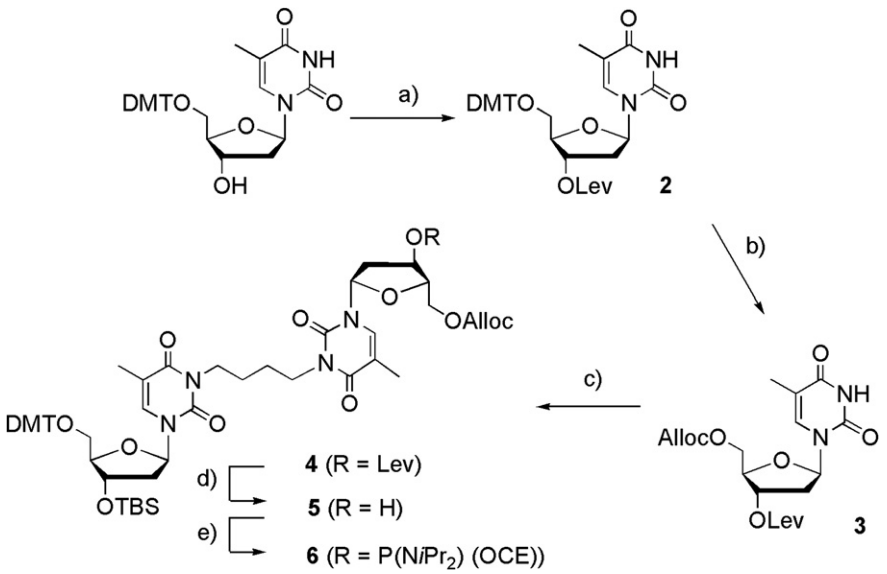


Scheme 1. Reagents and conditions: (a) KI, 2.5 equiv, THF, overnight, rt (95%).

Introduction of the levulinoyl (Lev) group at the 3′-OH of 5′-*O*-dimethoxytrityl-thymidine with levulinic acid, in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 4-dimethylaminopyridine (DMAP) in dioxane at rt, resulted in compound (**2**) in quantitative yields. The 5′-*O*-dimethoxytrityl group was removed by *p*-toluene sulfonic acid (*p*-TsOH) to release a free 5′-OH followed by its conversion to the allyloxycarbonyl (Alloc) group by reacting it with Alloc–OBt and DMAP in pyridine at rt overnight to produce compound (**3**) (85%).[39] Dimerization was accomplished by coupling a slight excess of compound (**3**) with *N*3-(4-iodobutyl)-5′-*O*-dimethoxytrityl-3′-*O*-(*tert*-butyldimethylsilyl)-thymidine in acetonitrile (MeCN) in the presence of 1,8-diazabicyclo-(5.4.0)-undec-7-ene (DBU) for 2 days at rt, which yielded the desired compound (**4**) in 55% yield. The 3′-*O*-Lev protective group was removed by treatment with 0.5 M hydrazine in a pyridine/acetic acid buffer (v/v 9:1) for 10 min at rt to yield preamidite (**5**) (96%). This was then converted to the cross-linked phosphoramidite (**6**) using a slight excess of *N*,*N*-diisopropylamino cyanoethyl phosphonamidic chloride in the presence of Hünigs base. The phosphoramidite was isolated by hexane precipitation in 71% yield and further analyzed by mass spectrometry, which gave the expected molecular mass (1261.5638). ^{31}P NMR analysis of the phosphoramidite revealed the presence of two signals for (**6**) (146.71 and 146.97 ppm) in the region diagnostic for a phosphoramidite.

2.2. Solid-phase synthesis of the *N*3T–butylene–*N*3T ICL

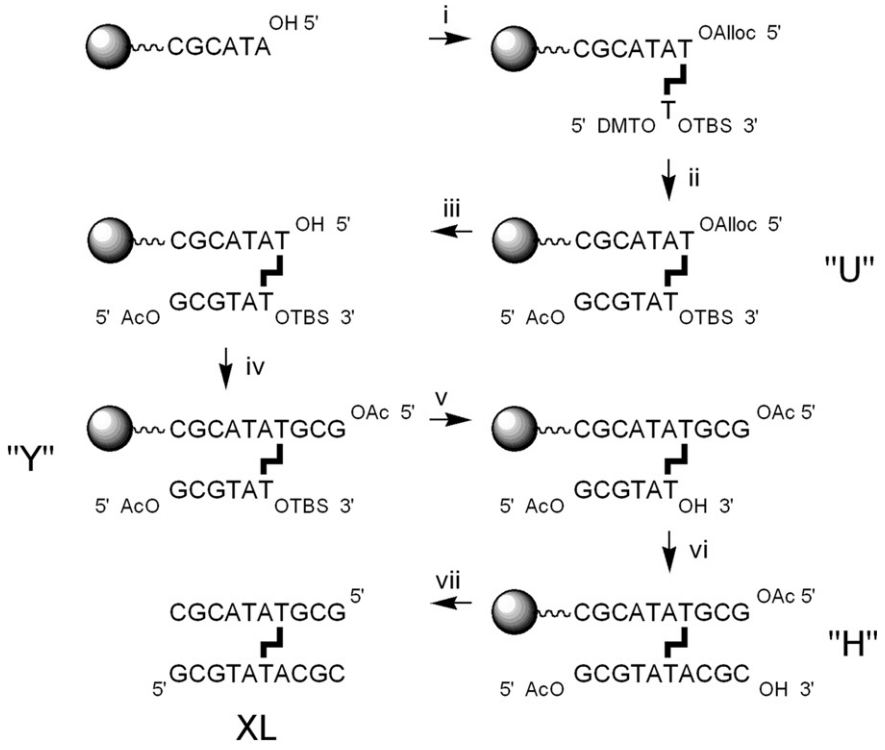
The ICL duplex containing the dimer was synthesized on 1 μmol scale employing an ABI 3400 solid-phase DNA synthesizer using polystyrene (PS) rather than controlled-pore glass (CPG) solid-support due to the incompatibility of triethylamine·trihydrofluoride (TEA·3HF) with the latter. As an added precaution, the cyanoethyl protecting groups were removed using triethylamine (TEA) as it has been observed that prolonged fluoride treatment using tetrabutylammonium fluoride (TBAF) could lead to chain cleavage.[34] The assembly of the ICL (Fig. 1) utilizing



Scheme 2. Reagents and conditions: (a) 2 equiv levulinic acid, 2 equiv EDC, 0.01 equiv DMAP, in 1,4-dioxane at rt for 16 h (quantitative yield); (b) 2.2 equiv *p*-TsOH, in CH_2Cl_2/CH_3OH (4:1) at rt for 1 h followed by 1.33 equiv Alloc–OBt, 0.2 equiv DMAP, in THF/pyridine (9:1) at rt for 24 h (85%); (c) 1.0 equiv *N*3-(4-iodobutyl)-5′-*O*-dimethoxytrityl-3′-*O*-(*tert*-butyldimethylsilyl)-thymidine, 2.0 equiv DBU, in CH_3CN at rt for 48 h (55%); (d) 0.5 M hydrazine hydrate, in pyridine/acetic acid (1:1) at rt for 10 min (96%); (e) 1.2 equiv ClP(OCE)(N*i*-Pr_2), 1.5 equiv DIPEA, in THF at rt for 1 h (71%).

phosphoramidite (**6**) is illustrated in Scheme 3. The position of the cross-link introduces asymmetry into the duplex (i.e., the nucleotide composition of the oligonucleotide strands around the cross-linked site is different). Intermediate U was assembled continuously on solid support by coupling 0.15 M of (**6**) directly to a short linear segment of DNA for 10 min, followed by removal of the DMT on the ICL dimer, around which the second nucleotide chain was grown. Chain assembly in the 3′ to 5′ direction proceeded smoothly using 3′-*O*-2′-deoxyphosphoramidites (dissolved to a concentration of 0.1 M in MeCN). A higher concentration and extended coupling time for (**6**) were necessary to ensure an optimal coupling efficiency due to its larger size compared to the standard 3′-*O*-2′-deoxyphosphoramidites. This step was followed by capping on the synthesizer to prevent undesired chain growth in subsequent steps. scavenger step results in oligonucleotide degradation as illustrated by IEX HPLC analysis of an U intermediate prepared for another ICL duplex (see Fig. 2b without scavenger-washing). The 3′-*O*-TBS group was then removed from the Y intermediate by treating the support with anhydrous TEA overnight followed by TEA·3HF twice for 30 min at rt. Reversed-phase HPLC analysis of the deprotected intermediate revealed complete removal of the silyl group with the shift of the major peak from 13.0 to 9.5 min in the case of the cross-link. Continued synthesis with repetitive coupling of 5′-*O*-2′-deoxyphosphoramidites at the 3′-end of intermediate Y gave the full-length ICL duplex.

The full-length cross-linked H-duplex was cleaved from the solid support and deprotected by treating the support with a mixture of ethanolic ammonia (1:3 v/v, 0.5 mL) at 55 °C, using standard



Scheme 3. Solid-phase synthesis procedure of oligonucleotide **XL** involving *N*3T—butylene—*N*3T phosphoramidite (**6**). (i) Coupling of phosphoramidite (**6**); (ii) removal of 5′-*O*-DMT on the machine, extension with 3′-*O*-2′-deoxyphosphoramidites and capping (to afford 'U'); (iii) removal of 5′-*O*-Alloc protective group; (iv) extension with 3′-*O*-2′-deoxyphosphoramidites and capping (to afford Y); (v) removal of the 3′-*O*-TBS protective group; (vi) extension with 5′-*O*-2′-deoxyphosphoramidites (to form H); (vii) Cleavage from the solid support and deprotection with NH_4OH/C_2H_5OH to give crude **XL**.

At each stage of the ICL duplex assembly, 1—2 mg of solid support was deprotected with ethanolic ammonia (1:3 v/v, 0.5 mL) at 55 °C for 4 h and the crude analyzed using Ion Exchange (IEX) HPLC. This was done to monitor amidite coupling and removal of a specific protecting group at each stage of the solid-phase synthesis. The HPLC profiles (shown in Fig. 2) illustrate the successful coupling of dimer phosphoramidite (**6**) with a major species at ca. 8 min corresponding to the desired U intermediate (Fig. 2a). After subjecting the oligomer-bound support to TEA treatment as described above, the Alloc protective group was removed with tetrakis(triphenylphosphine)palladium(0) [$Pd(PPh_3)_4$] in a butylamine—formic acid buffer solution for 3 h at 35 °C to free the 3′-OH group (Scheme 3). The successful removal of the Alloc group was determined indirectly through subsequent oligonucleotide assembly to produce intermediate oligomer Y from intermediate U (see Scheme 3). It is essential that all traces of remnant palladium (Pd) be removed using a Pd scavenger, namely sodium *N*,*N*-diethyldithiocarbamate to enable the successful synthesis of the Y intermediate.[40–42] The effect of avoiding this Pd deprotection conditions.[33] The crude oligomer was purified by IEX HPLC, using a gradient buffer of 20—55% B in 30 min, followed by desalting to afford the pure cross-linked duplex in 31% yield. This ICL duplex was digested to the constituent nucleosides with a combination of snake venom phosphodiesterase and calf intestinal phosphatase in a buffer containing 10 mM Tris (pH 8.1) and 2 mM magnesium chloride at 37 °C for 16 h and analyzed by C-18 reversed-phase HPLC (see Supplementary data for the HPLC trace). In addition to the four standard 2′-deoxynucleosides, one additional peak was observed with a retention time of 14 min identical to the completely deprotected dimer (**6**). The ratios of the component 2′-deoxynucleosides and cross-linked nucleosides were consistent with the theoretical composition of ICL duplex (see Supplementary data). The molecular weight of the cross-link duplex as determined by ESI-TOF mass spectrometry was in close agreement with the expected theoretical value (6110.0 Da).

The overall yield of the cross-linked oligonucleotide was 0.31 μmol (31% from a 1 μmol scale synthesis) after purification and

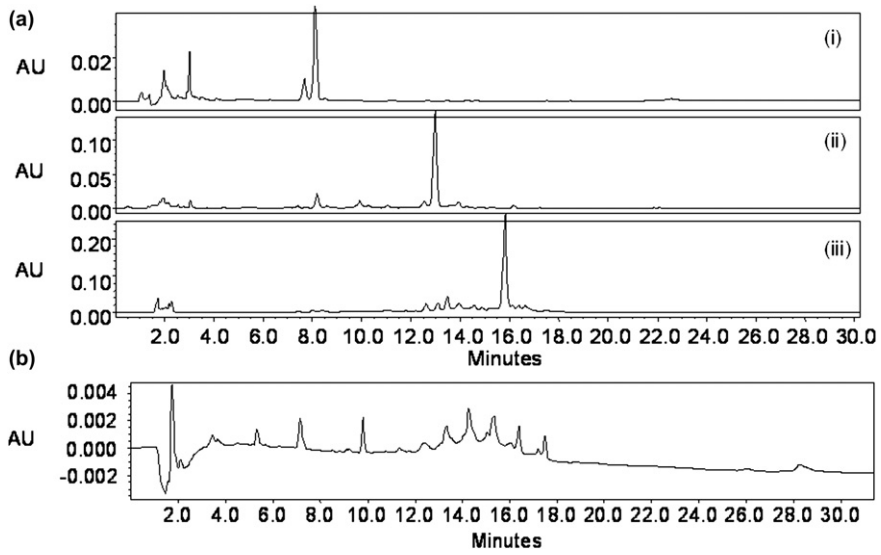


Fig. 2. (a) IEX HPLC chromatographs for intermediates in the synthesis of **XL** prepared with phosphoramidite (**6**). (i) 'U' intermediate; (ii) 'Y' intermediate; (iii) Crude **XL**; (b): IEX HPLC chromatograph of an 'U' intermediate without residual Pd scavenging treatment. The column, which was monitored at 260 nm, was eluted at rt with a gradient of 20–55% buffer B in 30 min. Buffer A: 10% acetonitrile, 100 mM Tris–HCl (pH 7.8); buffer B: 10% acetonitrile, 100 mM Tris–HCl (pH 7.8), 1 M NaCl.

desalting. Cross-linked duplexes prepared by methods described by Kishi and co-workers were reported to afford 0.15 μmol of material based on a 1 μmol after HPLC purification.[43] Among the dimers they investigated, one contained a 5′-*O*-*tert*-butyldiphenylsilyl (TBDPS) group, reported to couple with similar efficiency to other phosphoramidites studied. One explanation for the higher yield of final product we observed may be attributed the use of the 3′-*O*-*tert*-butyldimethylsilyl (TBS) protective group, which is easier to remove, enabling more efficient extension during phosphoramidite coupling to produce the desired ICL.

Preliminary attempts to prepare the ICL with an orthogonal deprotection strategy involving the levulinoyl (Lev) and phenoxyacetyl (Pac) groups present in 3′-*O*-phenoxyacetyl-5′-*O*-levulinoyl-thymidine, were unsuccessful (see Supplementary data for the synthesis and characterization of these compounds).[33,44–47] We believe the 3′-*O*-Pac group was partially removed from the dimer under conditions to remove the *tert*-butyldimethylsilyl (TBS) group during one of the assembly steps in duplex construction. The Pac group has been shown to be extremely labile under mild alkaline conditions (0.001 mol/L Na_2CO_3 in CH_3OH/CH_2Cl_2).[44,48,49] In our hands, attempts to remove the 5′-*O*-Lev group on the oligomer-bound solid support with hydrazine resulted in several undesired products on sequential extension, which could be due to the removal of benzoyl protected cytidine residues as reported previously.[50]

The stability of the **XL** duplex was assessed by ultraviolet thermal denaturation experiments (see Supplementary data). The profiles for the ICL and control duplex were sigmoidal indicating normal cooperativity and they displayed thermal melts of about 66 and 25 °C, respectively.

The CD spectra of the **XL** duplex as well as the non-cross-linked control were recorded at 10 °C. The CD spectra of the duplex exhibited signatures characteristic of B-form DNA with a positive maximum peak centered around 280 nm, a negative peak at approximately 250 nm, and a cross over around 270 nm.[51,52] In general the CD spectra were red shifted relative to the non-cross-linked controls (see Supplementary data). Previously, Webba da Silva and co-workers[53] have shown that duplexes containing the *N*3T–butylene–*N*3T ICL display dramatic widening of the major groove of the B-DNA stem without disruption of Watson–Crick base pairing due to the dominant contribution of cooperative and cumulative base stacking to the stability of the ApT compared to the TpT step suggesting that the latter is more deformable within a DNA stem. The constraints of the butyl tether perturb the structure very slightly, resulting in accommodation in a region between major and minor grooves that is in a staggered fashion, which deviates from being perpendicular to the stem axis. However, the canonical B-DNA local structure throughout the stem is observed.

3. Conclusion

An ICL duplex containing an *N*3T–butylene–*N*3T ICL has been successfully synthesized using a novel phosphoramidite (**6**) and solid-phase synthesis to afford the duplex in a 31% yield from a 1 μmol scale synthesis. The orthogonal deprotection strategy may be valuable for the synthesis of other ICL duplexes to produce substrates for various DNA repair studies.

4. Experimental section

4.1. General

5′-*O*-Dimethoxytrityl-thymidine, *N*,*N*-diisopropylamino cyanoethyl phosphonamidic chloride, and 3′-*O*-dimethoxytrityl-2′-deoxyribonucleoside-5′-*O*-(β-cyanoethyl-*N*,*N*′-diisopropyl)phosphoramidites were purchased from ChemGenes Inc. (Wilmington, MA). 5′-*O*-Dimethoxytrityl-2′-deoxyribonucleoside-3′-*O*-(β-cyanoethyl-*N*,*N*′-diisopropyl)phosphoramidites, protected 2′-deoxyribonucleoside–polystyrene supports, and ancillary reagents for solid-phase synthesis were purchased from Glen Research (Sterling, Virginia). Allyl chloroformate, *n*-butylamine, 1,8-diazabicyclo [5.4.0]undec-7-ene (DBU), formic acid, 1-hydroxybenzotriazole hydrate, hydrazine hydrate, levulinic acid, phenoxyacetyl chloride, *tert*-butyldimethylsilyl chloride (TBS–Cl), *p*-toluene sulfonic acid (*p*-TsOH), diisopropylethylamine (DIPEA), 4-dimethylaminopyridine (DMAP), triphenylphosphine (PPh_3), sodium *N*,*N*-diethyldithiocarbamate, tetrakis(triphenyl phosphine)palladium(0), and all other chemicals and solvents for synthesis were purchased from the Aldrich Chemical Company (Milwaukee, WI).

5′-*O*-Dimethoxytrityl-3′-*O*-(*tert*-butyldimethylsilyl)-thymidine, *N*3-[4-(phenoxyacetyl)butyl]-5′-*O*-dimethoxytrityl-3′-*O*-(*tert*-butyldimethylsilyl)-thymidine, *N*3-(4-iodobutyl)-5′-*O*-dimethoxytrityl-

3′-*O*-(*tert*-butyldimethylsilyl)-thymidine, 5′-*O*-dimethoxytrityl-3′-*O*-phenoxyacetyl-thymidine were all prepared with minor modifications to published procedures.[33]

Flash column chromatography (FCC) was performed using silica gel 60 (230–400 mesh) obtained from Silicycle (Quebec City, QC). Thin layer chromatography (TLC) was performed using precoated TLC plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) purchased from EMD Chemicals Inc. (Gibbstown, NJ). All solvents for column chromatography were obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). ^{1}H and ^{13}C NMR spectra were recorded on a Varian 500 MHz NMR spectrometer at rt at frequencies of 500 and 125.7 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts were reported in parts per million downfield from tetramethylsilane. ^{31}P NMR spectra (^{1}H decoupled) were recorded at a frequency of 202.4 MHz with H_3PO_4 used as an external standard.

4.2. Synthesis

*4.2.1. 4-Iodobutyl-1-phenoxyacetate (**1**).* Phenoxyacetyl chloride (3.412 g, 20.00 mmol) and potassium iodide (8.300 g, 50.00 mmol) were dissolved in THF (50 mL) at room temperature (rt). After 24 h, $NaHCO_3$ (1.681 g, 20.00 mmol) was added slowly to the solution. This was filtered and the solvent was removed in vacuo. The crude product was taken up in CH_2Cl_2 (100 mL) and the reaction quenched with aqueous $NaHCO_3$ (3%, 100 mL). The organic layer was washed with distilled water (100 mL×2), dried over sodium sulfate, and concentrated to give a viscous liquid. The crude product was purified by flash column chromatography (FCC) using a hexane/ethyl acetate (1:1) mixture to afford compound (**1**) as a yellowish oil (4.26 g, 95%). R_f (SiO_2 TLC): 0.76 in hexane/ethyl acetate (1:1). ^{1}H NMR ($CDCl_3$, ppm): δ 1.63–1.71 (tt, J=8.0, 6.5 Hz, 2H, CH_2), 1.72–1.79 (tt, J=8.0, 6.5 Hz, 2H, CH_2), 3.08–3.10 (t, J=6.5 Hz, 2H, CH_2), 4.15–4.17 (t, J=6.5 Hz, 2H, CH_2), 4.56 (s, 2H, OCH_2 of Pac), 6.83–6.85 (dd, J=8.5, 0.5 Hz, 2H, Ph of Pac), 6.93–6.94 (dd, J=7.0, 0.5 Hz, 1H, Ph of Pac), 7.19–7.23 (dd, J=8.5, 7.0 Hz, 2H, Ph of Pac). ^{13}C NMR ($CDCl_3$, ppm): 5.9, 29.4, 29.8, 64.0, 65.3, 114.6, 114.6, 121.8, 129.6, 129.6, 157.8, 169.0. ESI-MS (M+K^+): 372.9655 (calcd 372.9703).

*4.2.2. 5′-O-Dimethoxytrityl-3′-O-levulinoyl-thymidine (**2**).* To a solution of 5′-*O*-dimethoxytrityl-thymidine (5.446 g, 10.00 mmol) in 1,4-dioxane (60 mL) at rt were added EDC (3.820 g, 20.00 mmol), DMAP (0.012 g, 0.10 mmol), and levulinic acid (2.322 g, 20.00 mmol). After 12 h the solvent was removed in vacuo, the residue was taken up in CH_2Cl_2 (100 mL) and the solution washed with aqueous $NaHCO_3$ (3%, 100 mL). The organic layer was dried over sodium sulfate, purified by FCC to yield compound (**2**) as a colorless foam (6.42 g, 99%). R_f (SiO_2 TLC): 0.40 in hexane/ethyl acetate (1:9). ^{1}H NMR ($CDCl_3$, ppm): δ 1.29 (s, 3H, C5–CH_3), 2.12 (s, 3H, CH_3 of Lev), 2.32–2.38 (ddd, J=13.5, 7.0, 6.0 Hz, 1H, H2′), 2.58–2.62 (ddd, J=13.5, 5.5, 4.5 Hz, 1H, H2″), 2.63–2.66 (t, J=6.5 Hz, 2H, CH_2 of Lev), 2.67–2.70 (J=6.5 Hz, 2H, CH_2 of Lev), 3.35–3.38 (dd, J=13.0, 2.5 Hz, 1H, H5′), 3.39–3.42 (dd, J=13.0, 2.5 Hz, 1H, H5″), 3.72 (s, 6H, OCH_3 of DMT), 4.06 (ddd, J=5.0, 2.5, 2.5 Hz, 1H, H4′), 5.38–5.40 (ddd, J=6.0, 5.0, 4.5 Hz, 1H, H3′), 6.36–6.38 (dd, J=7.0, 5.5 Hz, 1H, H1′), 6.75–6.77 (m, 4H, Ph of DMT), 7.17–7.31 (m, 9H, Ph of DMT), 7.53 (s, 1H, H6). ^{13}C NMR ($CDCl_3$, ppm): 11.6, 28.0, 29.8, 37.8, 37.9, 55.3, 63.7, 75.7, 84.0, 84.3, 87.2, 111.6, 113.3, 127.2, 128.0, 128.1, 130.1, 130.1, 135.1, 135.2, 135.5, 144.2, 150.4, 158.8, 158.8, 163.6, 172.2, 206.3. ESI-MS (M+Na^+): 665.2483 (calcd 665.2475).

*4.2.3. 5′-O-Allyloxycarbonyl-3′-O-levulinoyl-thymidine (**3**).* To compound (**2**) (2.310 g, 3.594 mmol), dissolved in a mixture of CH_2Cl_2/CH_3OH (4:1, 75 mL), was added *p*-TsOH (1.505 g, 7.910 mmol) at rt. After 30 min, the solution was diluted with CH_2Cl_2 (300 mL) and then washed with aqueous $NaHCO_3$ (3%, 150 mL). The organic layer was washed with distilled water (150 mL), dried over sodium sulfate, and concentrated to give a colorless gum. The residue was purified by FCC using CH_3OH/CH_2Cl_2 (1:9) to afford the intermediate. This intermediate (0.620 g, 1.822 mmol) was then dissolved in a mixture of pyridine/THF (9:1, 70 ml) to which were added allyl 1-hydroxybenzotriazole carbonate (0.531 g, 2.423 mmol) and DMAP (0.045 g, 0.364 mmol) at rt. After 24 h, the solvent was removed in vacuo, the residue was taken up in CH_2Cl_2 (100 mL), and the solution was washed with aqueous $NaHCO_3$ (3%, 100 mL). The organic layer then was dried over sodium sulfate and purified by FCC using hexane/ethyl acetate (7:3) as eluent to afford compound (**3**) as a colorless foam (1.285 g, 85%). R_f (SiO_2 TLC): 0.72 in hexane/ethyl acetate (7:3). ^{1}H NMR ($CDCl_3$, ppm): δ 1.93 (s, 3H, CH_3–C5), 2.21 (s, 3H, CH_3 of Lev), 2.22–2.27 (ddd, J=14.0, 8.5, 6.5 Hz, 1H, H2′), 2.41–2.45 (ddd, J=14.0, 5.5, 1.5 Hz, 1H, H2″), 2.58–2.61 (t, J=6.5 Hz, 2H, CH_2 of Lev), 2.77–2.80 (t, J=6.5 Hz, 2H, CH_2 of Lev), 3.76–3.81 (dd, J=11.5, 2.5 Hz, 1H, H5′), 4.24–4.26 (ddd, J=5.0, 2.5, 2.5 Hz, 1H, H4′), 4.41–4.44 (dd, J=11.5, 2.5 Hz, 1H, H5″), 4.66–4.68 (d, J=6.0 Hz, 2H, CH_2 of Alloc), 5.26–5.30 (ddd, J=6.5, 5.0, 1.5 Hz, 1H, H3′), 5.30–5.33 (dd, J=10.5, 1.0 Hz, 1H, CH of Alloc), 5.37–5.40 (dd, J=17.0, 1.0 Hz, 1H, CH of Alloc), 5.90–5.96 (ddt, J=17.0, 10.5, 6.0 Hz, 1H, CH of Alloc), 6.40–6.43 (dd, J=8.5, 5.5 Hz, 1H, H1′), 7.42 (s, 1H, H6). ^{13}C NMR ($CDCl_3$, ppm): 12.6, 27.9, 29.7, 37.2, 37.8, 67.3, 69.0, 74.7, 82.1, 84.4, 111.7, 119.7, 131.0, 135.0, 150.6, 154.4, 163.8, 172.4, 206.4. ESI-MS (M+Na^+): 447.1375 (calcd 447.1380).

*4.2.4. 1-{N3-[5′-O-(Dimethoxytrityl)-3′-O-(tert-butyldimethylsilyl)-thymidylyl]}-4-{N3-[5′-O-(allyloxycarbonyl)-3′-O-(levulinoyl)-thymidylyl]}butane (**4**).* *N*3-(4-Iodobutyl)-5′-*O*-dimethoxytrityl-3′-*O*-(*tert*-butyldimethylsilyl)-thymidine (0.452 g, 0.537 mmol) and a slight excess of compound (**3**) (0.251 g, 0.592 mmol) were dissolved in MeCN (20 mL) to which was added DBU (0.164 g, 1.074 mmol) at rt. After 48 h, the solvent was removed in vacuo, the residue was taken up with CH_2Cl_2 (100 mL), and the solution was washed with aqueous $NaHCO_3$ (3%, 100 mL). The organic layer was dried over sodium sulfate and purified by FCC with a gradient of hexane/ethyl acetate (5:5 to 1:9) as eluent, to produce compound (**4**) as a colorless foam (0.336 g, 55 %). R_f (SiO_2 TLC): 0.76 in hexane/ethyl acetate (1:9). ^{1}H NMR ($CDCl_3$, ppm): δ 0.00 (s, 3H, $SiCH_3$), 0.06 (s, 3H, $SiCH_3$), 0.86 (s, 9H, $SiC(CH_3)_3$), 1.53 (s, 3H, C5–CH_3), 1.71–1.74 (m, 4H, CH_2 of butylene), 1.95 (s, 3H, C5–CH_3), 2.24 (s, 3H, CH_3 of Lev), 2.23–2.25 (m, 1H, H2′), 2.23–2.27 (m, 1H, H2″), 2.36–2.39 (m, 1H, H2′), 2.46–2.50 (m, 1H, H2″), 2.62–2.64 (t, J=6 Hz, 2H, CH_2 of Lev), 2.80–2.83 (t, J=6 Hz, 2H, CH_2 of Lev), 3.28–3.30 (dd, J=10.5, 1.5 Hz, 1H, H5′), 3.50–3.52 (dd, J=10.5, 1.5 Hz, 1H, H5″), 3.83 (s, 6H, OCH_3 of DMT), 3.99–4.02 (m, 1H, H4′), 3.99–4.02 (t, J=5.5 Hz, 4H, CH_2 of butylene), 4.21–4.24 (m, 1H, H4′), 4.43–4.48 (m, 2H, H5′ and H5″), 4.54–4.57 (m, 1H, H3′), 4.70–4.72 (d, J=6.0 Hz, 2H, OCH_2 of Alloc), 5.31–5.34 (m, 1H, H3′), 5.33–5.36 (dd, J=10.5, 1.0 Hz, 1H, CH of Alloc), 5.40–5.44 (dd, J=17.0, 1.0 Hz, 1H, CH of Alloc), 5.95–6.02 (ddt, J=17.0, 10.5, 6.0 Hz, 1H, CH of Alloc), 6.38–6.42 (m, 2H, 2×H1′), 6.87–6.89 (m, 4H, Ph of DMT), 7.28–7.44 (m, 9H, Ph of DMT), 7.46 (s, 1H, H6), 7.67 (s, 1H, H6). ^{13}C NMR ($CDCl_3$, ppm): −4.9, −4.7, 12.7. 13.3, 17.9, 25.3, 25.7, 27.9, 29.7, 37.2, 37.8, 41.0, 41.2, 41.6, 55.3, 62.9, 67.3, 69.0, 72.0, 74.8, 82.0, 85.2, 85.5, 86.6, 86.8, 110.2, 110.8, 113.3, 113.3, 119.6, 127.1, 128.0, 128.2, 130.1, 130.1, 131.1, 132.9, 133.5, 135.5, 135.6, 144.4, 150.9, 151.0, 154.5, 158.7, 163.2, 163.5, 172.3, 206.1. ESI-MS (M+Na^+): 1159.4929 (calcd 1159.4923).

*4.2.5. 1-{N3-[5′-O-(Dimethoxytrityl)-3′-O-(tert-butyldimethylsilyl)-thymidylyl]}-4-{N3-[5′-O-(allyloxycarbonyl)-thymidylyl]}butane (**5**).* Compound (**4**) (0.441 g, 0.388 mmol) was treated with 10 mL of HPAA (0.5 M hydrazine in a buffer composed of 9 mL pyridine and 1 mL glacial acetic acid) at rt. After 10 min, the solution was diluted with CH_2Cl_2 (100 mL) and washed with aqueous $NaHCO_3$

(3%, 100 mL). The water phase was extracted twice with CH_2Cl_2 (100 mL). The combined organic layer was dried over sodium sulfate and purified by FCC using a solvent system of hexane/ethyl acetate (1:9) to afford compound (**5**) as a colorless foam (0.387 g, 96%). R_f (SiO_2 TLC): 0.56 in hexane/ethyl acetate (1:9). ^{1}H NMR ($CDCl_3$, ppm): δ 0.00 (s, 3H, $SiCH_3$), 0.06 (s, 3H, $SiCH_3$), 0.86 (s, 9H, $SiC(CH_3)_3$), 1.53 (s, 3H, C5–CH_3), 1.71–1.73 (m, 4H, CH_2 of butylene), 1.95 (s, 3H, C5–CH_3), 2.23–2.29 (m, 2H, H2′ and H2″), 2.35–2.38 (m, 1H, H2′), 2.41–2.45 (m, 1H, H2″), 3.27–3.31 (dd, J=14.0, 3.0 Hz, 1H, H5′), 3.50–3.54 (dd, J=14.0, 3.5 Hz, 1H, H5″), 3.83 (s, 6H, OCH_3 of DMT), 3.97–4.00 (m, 1H, H4′), 3.98–4.00 (t, J=6.0 Hz, 4H, CH_2 of butylene), 4.15–4.18 (m, 1H, H4′), 4.45–4.48 (m, 2H, H5′ and H5″), 4.50–4.53 (m, 2H, 2×H3′), 4.69–4.71 (d, J=6.0 Hz, 2H, OCH_2 of Alloc), 5.52–5.55 (dd, J=10.5, 1.0 Hz, 1H, CH of Alloc), 5.58–5.62 (dd, J=17.0, 1.0 Hz, 1H, CH of Alloc), 5.94–6.01 (ddt, J=17.0, 10.5, 6.0 Hz, 1H, CH of Alloc), 6.39–6.42 (m, 2H, 2×H1′), 6.87–6.89 (m, 4H, Ph of DMT), 7.29–7.44 (m, 9H, Ph of DMT), 7.46 (s, 1H, H6), 7.68 (s, 1H, H6). ^{13}C NMR ($CDCl_3$, ppm): −4.9, −4.7, 12.7, 13.3, 15.3, 18.0, 25.3, 25.7, 40.6, 41.0, 41.1, 41.7, 55.3, 62.8, 66.7, 69.0, 71.2, 83.8, 85.3, 85.4, 86.6, 86.8, 110.2, 110.4, 113.2, 113.3, 119.7, 127.1, 128.0, 128.2, 130.0, 130.1, 131.1, 133.2, 133.6, 135.5, 135.5, 144.3, 150.8, 150.9, 154.8, 158.7, 163.4, 163.5. ESI-MS ($M+Na^+$): 1061.4567 (calcd 1061.4555).

*4.2.6. 1-{N3-[5′-O-(Dimethoxytrityl)-3′-O-(tert-butyldimethylsilyl)-thymidylyl]}-4-{N3-[5′-O-(allyloxycarbonyl)-thymidylyl-3′-O-(β-cyanoethyl N,N′-diisopropyl)phosphoramidite]}butane (**6**).* To compound (**5**) (0.317 g, 0.305 mmol) in THF (1.25 mL) was added DIPEA (0.059 g, 0.458 mmol) followed by *N*,*N*-diisopropylamino cyanoethyl phosphonamidic chloride (0.087 g, 0.366 mmol). After 30 min, the reaction mixture was diluted with ethyl acetate (50 mL). The organic layer was washed with aqueous $NaHCO_3$ (3%, 100 mL×2) followed by saturated NaCl (100 mL), then dried over sodium sulfate. After concentration in vacuo, the gum was precipitated from hexane to yield compound **6** as a colorless foam (0.269 g 71%). R_f (SiO_2 TLC): 0.81 in hexane/ethyl acetate (2:8). ^{31}P NMR (acetone-d_6, ppm): 146.71, 146.97. ESI-MS ($M+Na^+$): 1261.5638 (calcd 1261.5634).

4.3. Assembly of cross-linked DNA duplexes, purification, and characterization

4.3.1. Assembly of cross-linked DNA duplexes. The cross-linked duplex (shown in Fig. 1) was assembled using an Applied Biosystems Model 3400 synthesizer on a 1 μmol scale employing standard β-cyanoethyl phosphoramidite cycles supplied by the manufacturer with slight modifications to coupling times described below. Solutions of the nucleoside phosphoramidites containing standard protecting groups were prepared in anhydrous MeCN at a concentration of 0.1 M for the 3′-*O*-2′-deoxyphosphoramidites, 0.15 M for the cross-linked phosphoramidite (**6**), and 0.2 M for the 5′-*O*-2′-deoxyphosphoramidites. Assembly of sequences first involved detritylation (3% trichloroacetic acid [TCA] in CH_2Cl_2), followed by nucleoside phosphoramidite coupling with commercial 3′-*O*-2′-deoxyphosphoramidites (2 min), 5′-*O*-2′-deoxyphosphoramidites (3 min) or cross-linked phosphoramidite (**6**) (10 min); Subsequent capping with acetic anhydride/pyridine/tetrahydrofuran (1:1:8, v/v/v) and *N*-methyl-imidazole/tetrahydrofuran (16:84 w/v) and oxidation (0.02 M iodine in tetrahydrofuran/water/pyridine 2.5:2:1) followed every coupling. To form the Y intermediate, the cyanoethyl groups were removed from the polystyrene-linked oligomers by treating the support with 1 mL of anhydrous triethylamine (TEA) for at least 12 h. The support was then washed with 30 mL of anhydrous MeCN followed by anhydrous THF. The 5′-*O*-alloc group was removed from the partial duplex by treating the support with $Pd(PPh_3)_4/PPh_3$ (10:20 equiv to the support bound oligonucleotide) in a buffer solution of butylamine/formic acid (1:1, 100 equiv to the support bound oligonucleotide) for 3 h at 35 °C. The support was then washed with 30 mL each of anhydrous THF and MeCN followed by drying via high vacuum (30 min). The support bound oligomers was then treated with the Pd scavenger *N*,*N*-diethyldithiocarbamate as a saturated solution in MeCN (3 mL×2) for a total of 1 h at rt. This was followed by washing with 30 mL each of CH_2Cl_2 and MeCN followed by drying via high vacuum (30 min). Chain assembly was continued using 3′-*O*-2′-deoxyphosphoramidites followed by detritylation and an additional capping step for the 5′-OH group. The polystyrene-linked oligomers were treated with 1 mL of anhydrous TEA for 12 h. Then, the support was washed with 30 mL of anhydrous MeCN followed by anhydrous THF. Then, the TBS group was removed from the partial duplex by treating the support with 2×1 mL TEA·3HF for a total of 1 h. The support was then washed with 30 mL each of anhydrous THF and MeCN followed by drying via high vacuum (20 min). The final extension of the cross-linked duplex was then achieved using 5′-*O*-2′-deoxyphosphoramidites with a total detritylation time of 130 s with removal of the 3′-terminal trityl group by the synthesizer to yield duplexes on the solid support.

The oligomer-derivatized polystyrene was transferred from the reaction column to screw cap microfuge tubes fitted with Teflon lined caps and the oligomer released from the support and protecting groups removed by treatment with a mixture of concentrated ammonium hydroxide/ethanol (0.3 mL:0.1 mL) for 4 h at 55 °C. The cross-linked final product was separated from preterminated products by IEX HPLC using a Dionex DNAPAC PA-100 column (0.4 cm×25 cm) purchased from Dionex Corp, (Sunnyvale, CA) with a linear gradient of 20–55% buffer B over 30 min (buffer A: 100 mM Tris–HCl, pH 7.5, 10% MeCN and buffer B: 100 mM Tris–HCl, pH 7.5, 10% MeCN, 1 M NaCl) at 40 °C. The columns were monitored at 260 nm for analytical runs or 280 nm for preparative runs. The purified oligomer was desalted using C-18 SEP PAK cartridges (Waters Inc.) as previously described.[34]

4.3.2. Enzymatic digests. The cross-linked oligomer (0.1 A_{260} units) was characterized by enzymatic digestion (0.28 units of snake venom phosphodiesterase and 5 units of calf intestinal phosphatase in a buffer containing 2 mM magnesium chloride and 10 mM Tris at pH 8.1) for a minimum of 36 h at 37 °C.[34] The resulting mixture of nucleosides was analyzed by reversed-phase HPLC carried out using a Symmetry® C-18 5 μm column (0.46×15 cm) purchased from Waters Inc., Milford, MA. The C-18 column was eluted with a linear gradient of 0–60% buffer B over 30 min (buffer A, 50 mM sodium phosphate, pH 5.8, 2% MeCN and buffer B, 50 mM sodium phosphate, pH 5.8, 50% MeCN). The resulting peaks were identified by co-injection with the corresponding standards and eluted at the following times: dC (4.6 min), dG (6.8 min), dT (7.4 min), dA (7.9 min), and cross-linked dimer (14.2 min) and the ratio of nucleosides was determined. The molar extinction coefficient of *N*3-thymidine–butylene–*N*3-thymidine was determined previously.[33]

4.3.3. Mass spectrometry. ESI mass spectra for small molecules were recorded at the McGill University Department of Chemistry Mass Spectrometry Facility with a Finnigan LCQ DUO mass spectrometer in methanol or acetone. ESI mass spectra for oligonucleotides were obtained at the Concordia University Centre for Biological Applications of Mass Spectrometry (CBAMS) using a Micromass Qtof2 mass spectrometer (Waters) equipped with a nanospray ion source. The mass spectrometer was operated in full scan, negative ion detection mode.

4.3.4. UV thermal denaturation studies. Molar extinction coefficients for the oligonucleotides were calculated from those of the mononucleotides and dinucleotides according to nearest neighbor

approximations.[54,55] Non-cross-linked duplexes were prepared by mixing equimolar amounts of the interacting strands and lyophilizing the mixture to dryness. The resulting pellets (both controls and cross-linked duplex) were then re-dissolved in 90 mM sodium chloride, 10 mM sodium phosphate, 1 mM EDTA buffer (pH 7.0) to give a final concentration of 2.8 μM for control and cross-linked duplex. The solutions were then heated to 90 °C for 10 min, cooled slowly to rt, and stored at 4 °C overnight before measurements. Prior to the thermal run, samples were degassed by placing them in a speed-vac concentrator for 2 min. Denaturation curves were acquired at 260 nm at a rate of heating of 0.5 °C/min, using a Varian CARY Model 3E spectrophotometer fitted with a 6-sample thermostated cell block and a temperature controller. The data were analyzed in accordance with the convention of Puglisi and Tinoco[54] and transferred to Microsoft Excel™.

4.3.5. Circular dichroism (CD) spectroscopy. Circular dichroism spectra were acquired on a Jasco J-815 spectropolarimeter equipped with a Julaba F25 circulating bath. Samples were allowed to equilibrate for 5–10 min at 10 °C in 90 mM sodium chloride, 10 mM sodium phosphate, 1 mM EDTA (pH 7.0), at a final concentration of 2.8 μM for the cross-linked duplex and ca. 2.8 μM for the control duplexes. Each spectrum was an average of five scans. Spectra were collected at a rate of 100 nm/min, with a bandwidth of 1 nm and sampling wavelength of 0.2 nm using fused quartz cells (Starna 29-Q-10). The CD spectra were recorded from 350 to 200 nm at 10 °C. The molar ellipticity was calculated from the equation $[\theta]=\varepsilon/Cl$, where ε is the relative ellipticity (mdeg), C is the molar concentration of oligonucleotides (mol/L), and l is the path length of the cell (cm). The data were processed on a PC computer using Windows™ based software supplied by the manufacturer (JASCO, Inc.) and transferred into Microsoft Excel™ for presentation.

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Supplementary data

These data include ^{1}H, ^{13}C, and COSY NMR spectra, HPLC traces, mass spectra, UV thermal denaturation profiles, CD spectra, and additional synthetic procedures. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2012.07.043.

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