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Automated synthesis, characterization, and structural analysis of oligonucleotide C-3′-radical precursors

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Abstract—C-3′-Acyl-3′-xylothymidine-containing oligonucleotides have been designed and synthesized for their use as radical precursors in the study of oxidative DNA damage initiated by a C-3′-radical. These oligomers were efficiently obtained using automated DNA synthesis techniques based on *H*-phosphonate chemistry. CD spectra and melting curves of the synthesized oligonucleotides were compared to those of their unmodified and xylomodified counterparts. The conformational analysis and hybridization studies indicate that the combination of the photolabile acyl group and the inversion of configuration at the sugar has no profound effect on the overall conformation of C-3′-acyl-2′-deoxy-3′-xylonucleotides as compared to their natural analogues. These systems should provide excellent tools for the elucidation of DNA damage processes.

1. Introduction

The damage suffered by DNA through oxidative assault contributes significantly to the development of disease and the manifestations of aging.[1–3] While lesions attributed to the direct modification of nucleobases have been widely accepted as primary players in nucleic acid damage processes, increasing evidence indicates the importance of deoxyribose oxidation in this mechanism.[4–6] To clearly elucidate the role that sugar derived oxidative damage plays in the etiology of disease, the nature and quantities of the various products that arise via oxidation of each site of the 2-deoxyribose ring of DNA must be thoroughly investigated. Site-selectively modified oligonucleotides (ODN) have proven their utility in the investigation of the fate of reactive intermediates in biological systems involving nucleic acids.[7] In the design of systems used to probe the effects of individual DNA damage events, modified nucleosides have been developed that participate in automated DNA synthesis, efficiently produce the reactive intermediate of interest, and have limited effects on the conformation and thermodynamic properties of the oligonucleotide under investigation. Modifications at the 2-deoxyribose moiety of nucleosides that efficiently initiate DNA damage through the generation of radicals include acyl groups[8,9] as well as aryl selenides.[10,11] These radical precursors participate in photochemical conversions to the radical of interest through exposure to ultraviolet (UV) light outside the range that usually damages DNA by other mechanisms.

This approach was applied to the design of a system utilized in the elucidation of the mechanisms involved in the damage of nucleic acids via the generation of a C-3′-thymidinyl radical.[12,13] The participation of this intermediate in DNA damage processes has been seldom indicated and its mechanism of degradation largely ignored. Due to the uniqueness of the fragments generated by the oxidation of DNA at each individual site of 2-deoxyribose, we believe that the infrequency of implication of the C-3′-radical in DNA damage may be due to a paucity of knowledge about the complete spectrum of fragments it creates. We have shown that (5-*O*-dimethoxytrityl-3-*C*-acetyl-2-deoxy-β-D-*threo*-pentofuranosyl)-thymidine (**1**) can be efficiently converted to its *H*-phosphonate monomer (Fig. 1) and manually incorporated into DNA strands using classical *H*-phosphonate chemistry.[12] The primary barriers to the synthesis of oligomers of this type are steric hindrance and the electronically negative impact created by inclusion of

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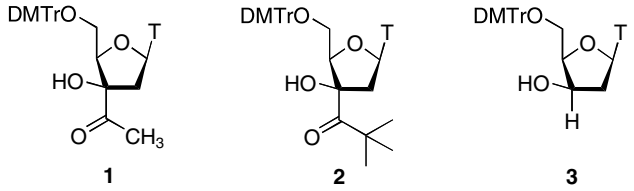


Figure 1. Nucleoside monomers.

an acyl group at the C-3′-position where coupling must occur to facilitate the growth of the strand. We now report the formation of DNA oligomers containing either **1** or (5-*O*-dimethoxytrityl-3-*C*-(2,2-dimethylpropanoyl)-2-deoxy-β-D-*threo*-pentofuranosyl)thymidine (**2**) using exclusively the *H*-phosphonate method of automated DNA synthesis. This method circumvents the problems associated with the inclusion of bulky phosphate-protecting groups such as those used in the phosphoramidite method[14] at the sterically encumbered 3′-site of the nucleoside monomer. Modified oligonucleotides obtained through these methods efficiently participate in photochemical generation of the C-3′-radical. To determine if the presence of the C-3′-acyl group as well as the inverted configuration at the site of modification has a profound effect on the conformation and hybridization properties of these sequences, circular dichroism (CD) and (ultraviolet–visible) UV–vis hybridization studies were performed. These studies are crucial for the validation of the use of these sequences as precursors for the radical of interest. To assess the contribution of configuration alone at the modified sugar on DNA synthesis and sequence conformation, oligonucleotides containing **3**[15] were also constructed using automated techniques and structural analyses were performed.

2. Results

2.1. Oligonucleotide synthesis and purification

Modified oligonucleotides were obtained using automated DNA synthesis on a 0.2 μmol scale following the *H*-phosphonate method. All modified nucleoside building blocks were prepared from thymidine primarily as described[13,16] and converted to their respective phosphonates using standard methods.[17] To test the efficiency of the automated synthetic protocol developed for the *H*-phosphonate method on our DNA synthesizer, several unmodified sequences were assembled. Coupling efficiencies for unmodified sequences were ⩾99% using the synthesis protocol described herein which employs 1.6 min coupling times and pivaloyl chloride as the activating agent (**ODN8**, Table 1). For the incorporation of **3** the same synthetic protocol was followed delivering coupling efficiencies of 94% at the site of incorporation and 92% for the overall sequence (**ODN6**, Table 1). These equal the coupling efficiencies observed for **ODN7**, which was obtained using our previously described manual coupling technique.

Due to the steric bulk and electronic influence of the acyl moieties at the position of chain elongation in building blocks **1** and **2**, several modifications were made to the protocol used for the construction of substrates containing these derivatives. First, in addition to the use of extended coupling times (5 vs 1.6 min) and higher monomer concentrations (100 mM as compared to 60–70 mM for standard monomers), a capping step was incorporated into the DNA synthesis protocol to limit the amount of truncated species. This method, based on the work by Andrus et al.,[18] utilizes standard *H*-phosphonate coupling chemistry with triethylammonium isopropyl phosphite to cap truncated sequences as their respective isopropylphosphonates. Employing this methodology, coupling efficiencies for the sequences were 50–72% (60–62% at the site of modification) for **1** and 68–73% (79–93% at the site of modification) in the case of **2**.

In standard automated DNA synthesis using *H*-phosphonate chemistry, a single oxidation is performed after assembly of the desired sequence.[17] When this approach was applied to the synthesis of oligonucleotides including **1** and **2**, the only products obtained were DNA oligomers absent of the acyl-modified monomer. Trityl values (not shown) obtained for this coupling step provided evidence that the modified thymidine was indeed coupled to the strand; however, its absence from the

Table 1. Oligonucleotide synthesis

	Sequence X = 1; Y = 2; Z = 3	CE (%) overall	CE (%) mod	MALDI-TOF MS $[M+H]^+$	
				Calcd	Found
ODN1	TCTATX*TTCACCTCCT	72	60	4776.19	4775.98
ODN2	CTACCTX*TCTATCTATCT	50	62	5393.59	5394.04
ODN3	CACTY*TACTTAT	73	93	3653.76	3653.9
ODN4	TCTATY*TTCACCTCCT	72	85	4818.27	4818.31
ODN5	CTACCTY*TCTATCTATCT	68	79	5435.68	5436.08
ODN6	TCTATZ*TTCACCTCCT	92	94	4734.15	4735.01
ODN7	CACTZ*TACTTAT	90	99[a]	3571.41	3571.49
ODN8	CACTTTACTTAT	99	99	3571.41	3571.49
ODN9[b]	TCTATTTTCACCTCCT	—	—	4734.15	—
ODN10[b]	CTACCTTTCTATCTATCT	—	—	5351.56	—

Detritylation with 3% trichloroacetic acid, 1.5 min; coupling with pivaloyl chloride (PvCl) as activator (100 mM × 240 μl). 1.6 min for *H*-phosphonates of unmodified nucleosides and **3** (60–70 mM), 5 min for **1** and **2** (100 mM); capping, triethylammonium isopropyl phosphite (44 mM), PvCl as activator 30 s; oxidation, 4% iodine in pyridine/H_2O/THF (1:1:8) and THF/H_2O/TEA (8:1:1) 30 min. X, acetyl; Y, pivaloyl; Z, xylo.

[a] Manual coupling.

[b] Purchased.

TT*ACTTAT
ODN11
TACTTAT
ODN12

Figure 2. Sequences for oxidation test. T* = **2**.

final sequence indicated its elimination during the removal of its 5′-dimethoxytrityl (DMTr)-protecting group using 3% trichloroacetic acid in dichloromethane. The inclusion of an oxidation step immediately following the coupling of **1** and **2** proved to prevent the loss of these modified nucleosides. In the synthesis of **ODN6** and **ODN7**, oxidation was smoothly performed after construction of the entire nucleotide sequence. It was anticipated that the *H*-phosphonate linkage at the modified nucleoside might possibly be shielded from oxidation due to its close proximity to the large dimethoxytrityl-protecting group on the upper face of the nucleoside. To ensure complete oxidation and retention of the radical precursor, several test sequences containing **2** were synthesized to determine the optimum conditions for this transformation. Sequence **ODN11** (Fig. 2) was synthesized using the above-described coupling conditions with oxidation times varied for each synthesis (15, 30, and 40 min.). As discussed earlier, it was assessed that the *H*-phosphonate linkage at the C-3′-modified thymidine is highly unstable to the acidic conditions used for detritylation. Based on this information, it was expected that incomplete oxidation would be quantitatively represented by the amount of sequence **ODN12** (TACTTAT) formed. The data below (Table 2) indicate 30 min to be the most favorable oxidation time with 15 min producing significantly more of the unmodified sequence as compared to the 30 and 40 min oxidations. The coupling efficiency of **2** in all three syntheses was identical (~85 ± 5%).

The purification of xylo-containing sequences was accomplished using oligonucleotide purification cartridges (OPC) and when necessary reversed-phase HPLC (RP-HPLC). In the case of C-3′-acyl-modified sequences, ion-exchange chromatography was required to separate small amounts of the sequences without the modification from full-length sequences.

2.2. Substituent effects on single-strand conformation

Preliminary investigations have shown that the C-3′-pivaloylthymidine delivers the radical of interest more efficiently and in shorter irradiation times than the previously utilized C-3′-acetyl derivative. This monomer is also a better participant in automated DNA synthesis delivering fewer oligonucleotide byproducts and higher coupling efficiencies. For these reasons, we have chosen oligonucleotides containing **2** as the optimum precursors for our oxidative damage studies. To determine if the conformation of the DNA is profoundly altered due to the presence of the inverted stereocenter and the large pivaloyl group at the modified nucleoside, single-stranded (ss) **ODN3-5** were probed by circular dichroism and compared to their unmodified counterparts (**ODN8-10**). Additionally, to determine if changes in the oligonucleotide conformation are due solely to inversion of stereochemistry at the 3′-carbon relative to natural DNA, **ODN6** and **7** were analyzed for comparison. As can be seen in Figure 3A the long wavelength CD bands of 12-mer oligonucleotides **ODN3**, **ODN7**, and **ODN8** are all very similar with their maxima centered around 280 nm and their minima at 250 nm. The intensities of the unmodified and the pivaloyl-substituted derivatives are almost identical. In the case of the 16-mer and 18-mer oligonucleotides, the maxima and minima are also the same. However, the intensities of the peaks are very different (Supplementary data). In the sequences shown, there appears to be no sequence-dependent change of conformation that can be detected by CD.

Table 2. Oxidation test

Oxidation test	Time (min)	Ratio **ODN12/ODN11**
I	15	0.17:1
II	30	0.08:1
III	40	0.09:1

Ratio **ODN12/ODN11** is representative of the relative amount of **ODN12** formed compared to **ODN11**. The amount of each oligonucleotide is determined from the peak areas divided by the calculated extinction coefficient of the corresponding oligonucleotides.

2.3. Substituent effects on duplex conformation and stability

The CD spectra of double-stranded (ds) **ODN3-5** (Fig. 4B) closely resemble those of normal B-form DNA with all having corresponding long-wavelength crossovers at ~260 nm. Differences in the peak intensities of the short wavelength CD bands can be seen in the case of **dsODN3** as compared to its unmodified and 3′-xylothymidine-containing counterparts (Fig. 4A). This conformational difference seems to however add to the stability of the sequence compared to the 3′-xylothymidine-containing oligomers as indicated by the melting temperatures in Table 3. The melting profile was obtained by plotting the increase in temperature against the α value that represents the relative fraction of the complex remaining in the initial (ds) state. T_m is determined as the temperature at which 50% of the DNA is in its duplex form. In the case of **dsODN7** and **dsODN6**, the presence of the 3′-xylothymidine destabilizes the duplex more than the sequences containing radical precursor **2** decreasing the melting temperature of these duplexes by 10.8 and 5.8 °C, respectively, relative to the unmodified derivatives.

3. Discussion

The automated synthesis of oligonucleotides requires efficient high-yielding reactions compatible to the intermediates formed during chain elongation. These steps include acid catalyzed detritylation of the nucleoside 5′-hydroxyl, coupling of the incoming monomer facilitated by some type of activating agent, followed by oxidation of the phosphonate linkages after each coupling step or after construction of the entire nucleotide sequence. Capping steps are usually included to limit the

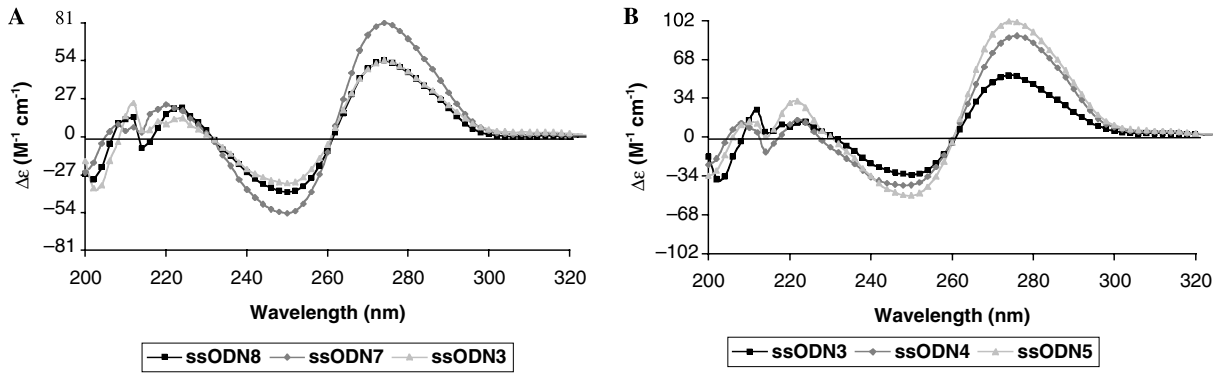


Figure 3. CDs of single-stranded oligonucleotides. The molar ellipticities are normalized. Each spectrum is an average of four scans. (A) Unmodified sequence **ODN8** (CACTTTACTTAT; closed squares) with overlays of the pivaloyl-substituted **ODN3** and 3′-xylothymidine-containing DNA **ODN7**. (B) CDs of all pivaloyl-modified oligomers (**ODN3–5**).

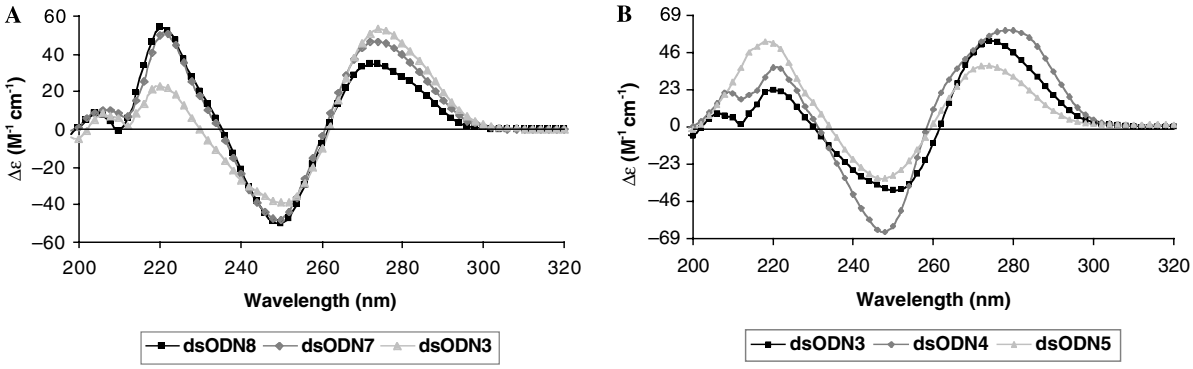


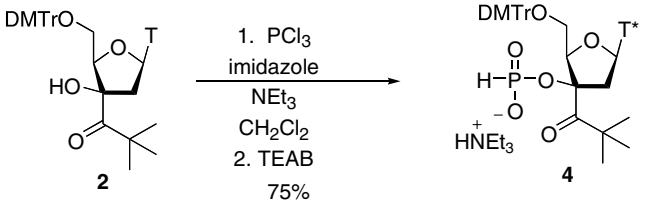
Figure 4. CDs of double-stranded oligonucleotides. The molar ellipticities are normalized. Each spectrum is an average of four scans. (A) Unmodified sequence **dsODN8** (CACTTTACTTAT; closed squares) with overlays of the pivaloyl-substituted **dsODN3** and 3′-xylothymidine containing DNA **dsODN7**. (B) CDs of all pivaloyl-modified oligomers (**dsODN3–5**).

Table 3. Melting temperatures of double-stranded oligonucleotides

	Sequence X = 1; Y = 2; Z = 3	T_m (°C)	ΔT_m (°C)
ODN8	CACTTTACTTAT	37.3	—
ODN7	CACTZ*TACTTAT	26.5	−10.8
ODN3	CACTY*TACTTAT	31.6	−5.7
ODN9	TCTATTTTCACCTCCT	51.1	—
ODN6	TCTATZ*TTCACCTCCT	45.3	−5.8
ODN4	TCTATY*TTCACCTCCT	48.8	−2.2
ODN10	CTACCTTTCTATCTATCT	51.6	—
ODN5	CTACCTY*TCTATCTATCT	50.3	−1.3

amount of truncated sequences in the final product mixture. Two of the primary obstacles in the development of the synthesis of modified oligonucleotides are the accessibility of monomeric building blocks and the coupling of the modification to the synthetic strand. The inclusion of a modification at the C-3′-position of a nucleoside requires the chemical manipulation of the 3′-hydroxyl of the monomer. Radical precursors belonging to this class of compounds with the unnatural xylo configuration are synthetically much more accessible than their ribo counterparts.[13] These substrates also participate more readily in the subsequent phosphorylation required for automated DNA synthesis. Additionally, C-3′-acyl-3′-xylothymidine *H*-phosphonates proved to be synthetically obtainable, whereas the more often used phosphoramidite derivatives were not. Several phosphoramidites have been synthesized, which are derived from C-3′-substituted nucleosides.[19–21] These derivatives contain alkyl groups that do not possesselectron-withdrawing character. This implies that the lack of reactivity of C-3′-pivaloylthymidine to 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite is likely both electronic and steric in nature. The higher reactivity of the phosphorus trichloride/imidazole/triethylamine reagent and smaller size of the phosphonate group facilitated the conversion of **2** to its *H*-phosphonate in a similar fashion to that of **1** (Scheme 1). The steric bulk introduced by the change to a *t*-butyl substituent did however necessitate the use of longer reaction times.

With an electron-withdrawing as well as bulky substituent at the position of chain elongation in monomer **4**, prolonged coupling times (5 min) were required for



Scheme 1. Synthesis of *H*-phosphonate **4**.

adequate reaction in oligonucleotide synthesis. Improvements were observed when the monomer concentration was increased from the standard 60–70 mM to 100 mM. With these improvements, coupling efficiencies as high as 62% were obtained for the *H*-phosphonate of **3** and 93% for **4**. The acid lability of the C-3′-acyl-3′-xylothymidine *H*-phosphonates, which necessitated the inclusion of an additional oxidation step in the automated protocol, is likely due to the proximity of the ketone moieties to the *H*-phosphonate linkage. When a hydrogen atom replaces the ketone, no hydrolysis of the newly incorporated monomer is observed. This substantiates the fact that the lability of the *H*-phosphonate linkage at **1** and **2** can be contributed to electronic effects associated with the presence of the keto group at the C-3′-position and not the conformation of the sugar. To accomplish a highly efficient synthesis, it was necessary to determine the optimum conditions for oxidation of the *H*-phosphonate linkage at the modified nucleoside. Through the oxidation test performed in the synthesis of **ODN11**, a shorter oxidation time of 15 min. was found to deliver twice as much unmodified sequence as longer oxidation times of 30 and 40 min. In the synthesis of unmodified oligonucleotides, oxidation times as short as 7 min have been reported with the same oxidizing reagent (http://www.thermohybaid.deknowledge/nucleicchem/index.html). With the high degree of steric crowding imposed upon the upper face of the nucleoside by the presence of the 5′-hydroxyl-protecting group and the newly formed *H*-phosphonate, it is not surprising that the oxidation at the modification requires longer reaction times. Purification of the desired sequences was greatly improved through the inclusion of a highly efficient capping step in the automated syntheses. This modification results in significantly lower levels of truncated species relative to the standard *H*-phosphonate method, which relies upon the activator, pivaloyl chloride, for capping. Through the use of ion-exchange chromatography the small amount of sequence, that is formed when coupling at the modification fails, was easily separated.

To validate the use of these precursors in the study of oxidative damage processes, it is significant to determine if the modifications placed in the oligonucleotide have a substantial effect on strand conformation. For this reason, we investigated the conformation of the modified oligonucleotides to determine if an inverted stereocenter at the modified nucleoside and/or the presence of the bulky radical precursor cause(s) conformational changes that may induce reactions specific to the precursor but not to the radical of interest. Comparison of the C-3′-acyloligonucleotides to substrates which possess only the inverted configuration at the site of modification provides valuable information as to the contribution of the acyl group to any differences seen in the substrates. Through NMR studies, Rosemeyer and Seela[15] showed that 3′,4′-*threo*-configured nucleosides display a significant amount (84%) of N-type sugar conformation making them 3′-endo, while the analogous erythro conformers exhibit S-type sugar puckering. NMR data and crystal structure analysis of C-3′-pivaloyl-3′-xylothymidine show that the ribose ring of the nucleoside is also 3′-endo but to a lesser extent than 3′-xylothymidine.[22] When the two are directly compared, the degree of N-type puckering in **2** is limited by the presence of the bulky group causing the sugar conformation to adapt less of a 3′-endo conformation than that of **3**. CD analysis of the 3′-pivaloylthymidine-containing oligomers shows that in single- and double-stranded DNA, small conformational differences can be observed when compared to the 3′-xylo and 3′-ribo containing nucleic acids. In the double-stranded systems, all exhibit maxima and minima at 275 and 250 nm, respectively, with crossovers at 260 nm. These are all features consistent with B-form DNA. CD measurements cannot however provide information as to the local conformational changes induced by the presence of the modified nucleotide. More telling is the information obtained from UV melting experiments from all modified systems and their unmodified counterparts (Fig. 5). Sequences containing **2** are stabilized by the presence of the pivaloyl moiety relative to those containing **3**. Melting temperatures for **dsODNs 6** and **7** are similar to those reported for a 14-mer sequence containing a single 3′-xylothymidine.[23] These data indicate that even in ds-DNA, the C-3′-pivaloyl-modified nucleoside does not distort the oligomer struc-

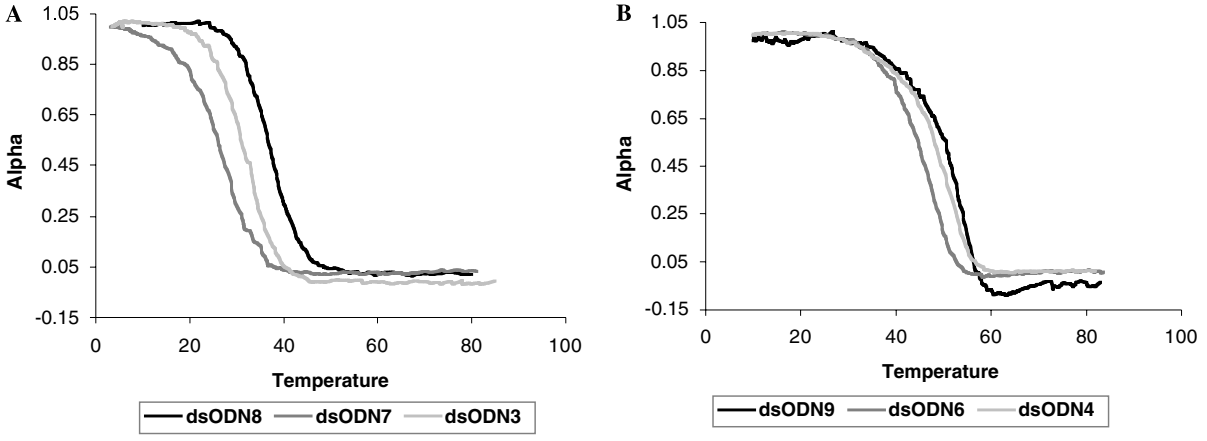


Figure 5. UV melting curves of all 12-mer sequences (A) and all 16-mer sequences (B). The α value, representing the relative fraction of the complex that remains in the initial state, was calculated at each temperature as follows: $\alpha(T) = [A_U(T) - A(T)]/[A_U(T) - A_L(T)]$, where $A_U(T)$ is the upper absorbance baseline, $A_L(T)$ the lower absorbance baseline, and $A(T)$ the absorbance as a function of the temperature T (in °C).

ture to the same extent as the 3′-xylothymidine or that the pivaloyl substituent compensates for any distortion created by the change in conformation. As the sequence becomes longer (**dsODN5**), the radical precursor has a negligible effect on duplex stability.

The ready availability and conformational similarities of these substrates make them ideal for the study of DNA damage processes relevant to biological systems. The structural similarities between these modified oligonucleotides and their natural counterparts, as demonstrated by CD and UV melting experiments, indicate that the modifications necessary for radical generation do not distort the duplex to any significant degree. Upon radical formation, this C-3′-acyl-modification will undergo photolytic bond scission and diffuse away from the oligomer, leaving behind the radical of interest. This process should not cause any major conformational changes within the duplex and will allow the radical to participate in chemical processes completely unrelated to the structure of the precursor. Preliminary studies indicate that oligomers containing **2** are efficient precursors for the radical of interest delivering fewer side products and requiring shorter irradiation times than precursor **1**. Based on these studies, we can anticipate that the use of these substrates in the study of oxidative damage processes, initiated by the C-3′-radical in both ss- and ds-DNA, will clearly represent the role of this reactive intermediate in such a biologically significant event.

4. Experimental

4.1. General

All glassware was oven-dried. Flash chromatography was performed using silica gel (Silica gel 60, 70–230 mesh, EM Science). TLC utilized silica gel plates (250 μm, F254, Whatman) with visualization using UV light and staining with anisaldehyde. All chemicals and reagents were of the highest purity available and used without further purification unless otherwise noted. Anhydrous reagents were obtained in sealed bottles. *H*-phosphonates derived from **1**[12] and **3**[15] were prepared as described. Tetrahydofuran was distilled from sodium/benzophenone and triethylamine from calcium hydride. Pivaloyl chloride was freshly distilled. N_2 was used as the inert atmosphere. ^1H, ^{13}C, and ^{31}P NMR spectra were obtained on a Bruker Avance 200 spectrometer. RP-HPLC and ion-exchange chromatography were performed on a Perkin Elmer LC-250 equipped with a 135 diode array detector. Mass spectral data for synthetic intermediates were obtained by fast atom bombardment (FAB, positive mode) on a Jeol HX-110 mass spectrometer (Michigan State University Mass Spectrometry Facility). Matrix-assisted laser desorption ionization time-of-flight spectra were obtained on a Bruker Daltonics Ultraflex spectrometer (Wayne State University Central Instrumentation Facility). CD spectra were recorded on a Jasco J-600 spectropolarimeter (Wayne State University Central Instrumentation Facility). Unmodified oligonucleotides were purchased from Integrated DNA Technologies, Coralville, IA or Synthegen, Houston, TX.

4.1.1. 1-[5-*O*-(4,4′-Dimethoxytrityl)-3-*C*-(2,2-dimethylpropanoyl)-3-*O*-(triethylammoniumphosphonyl)-2-deoxy-β-D-*threo*-pentofuranosyl]thymine (4). Imidazole (0.13 g, 1.90 mmol) was coevaporated twice with 10 ml each of dry acetonitrile and dissolved in anhydrous dichloromethane (7 ml). The solution was cooled to −10 °C and phosphorus trichloride (0.06 ml, 0.6 mmol) followed by freshly distilled triethylamine (0.30 ml, 2.16 mmol) in dichloromethane (0.30 ml) was added dropwise with vigorous stirring. The mixture was allowed to stir for 30 min at this temperature. The modified deoxyribonucleoside (**2**)[13] (0.11 g, 0.18 mmol) was coevaporated twice with anhydrous pyridine (25 ml) and dissolved in dichloromethane (4.5 ml). The solution was then added to the reaction mixture over 30 min at −10 °C. The mixture was slowly warmed to room temperature and stirred for 6 h. The mixture was then hydrolyzed with 1 M triethylammonium bicarbonate buffer TEAB (13 ml) and the organic layer was washed with an equal volume of 1 M TEAB. The organic layer was dried over magnesium sulfate and evaporated under reduced pressure. Flash chromatography (using a stepwise gradient of methanol (2–12%) in dichloromethane, containing 1% triethylamine) gave the *H*-phosphonate as a pale yellow foam in 75% yield.

^1H ($CDCl_3$, 200 MHz) 1.13 (t, 9H, [$(CH_3CH_2)_3$]), 1.22 (s, 9 H, [$(CH_3)_3CCO$], 1.74 (s, 3H, CH_3), 2.68–2.85 (m, 7H, [$(CH_3CH_2)_3NH+$] and H2′α), 3.14 (dd, 1H, H2′b, *J* H2′β–H2′α = 13.7 Hz, *J* H2′β–H2′α = 6.5 Hz), 3.44–3.60 (m, 2H, H5′), 3.78 (s, 6H, OCH_3), 4.38 (m, 1H, H4′), 5.23 (s, 0.5H, HPO_3^-), 6.06 (dd, 1H, H1′, *J* H1′–H2′β = 6.8 Hz), 6.81 (m, 4H, DMTr), 7.12–7.49 (m, 9H, DMTr), 7.92 (s, 1H, H6), 8.39 (s, 0.5H, HPO_3^-, *J* H–P = 631 Hz), 9.38 (s, 1H, NH).

^{13}C ($CDCl_3$, 200 MHz) 8.85 [$(CH_3CH_2)_3$], 12.50 [CH_3], 28.43 [$(CH_3)_3CCO$], 44.58 [C2′], 45.69 [$(CH_3CH_2)_3NH+$], 55.43 [CH_3O], 63.18 [C5′], 85.45 [C1′], 87.13 [C4′], 89.50 [C3′], 110.04 [C5], 113.37, 127.97, 128.06, 130.35, 136.12, 144.99, 158.72 [DMTr], 150.34 [C2], 163.99 [C4], 212.58 [$(CH_3)_3CCO$].

^{31}P ($CDCl_3$, 200 MHz) 1.01, J_{H-P} = 629 Hz (value relative to 0.05 M triphenylphosphine in $CDCl_3$).

MS *m/z* = 794.17 (M+H), 691.19(M−NH(C_2H_5)$_3$) HRMS: calcd. 794.9143, found 794.3793.

4.2. Oligonucleotide synthesis

Oligonucleotides containing monomers **1**–**3** were synthesized on an ABI Expedite 8909 DNA synthesizer at the 0.2 μmol scale using *H*-phosphonates with standard protecting groups (Glen Research, Sterling, VA). Deblocking was accomplished using 3% trichloroacetic acid in dichloromethane. Pivaloyl chloride was used as activator. The coupling time for unmodified nucleosides and **3** was 1.6 min. A capping step was employed based on the coupling of triethylammonium isopropylphosphite with pivaloyl chloride as activator. The incorporation of *H*-phosphonates **1** and **2** required coupling times of 5 min. Coupling yields for each monomer were deter-

mined by trityl assay. Unmodified sequences and those containing **3** were oxidized after complete construction of the desired sequence through simultaneous exposure of the oligomers to 4% I_2 in pyridine/H_2O/tetrahydrofuran THF (1:1:8) and THF/H_2O/triethylamine (8:1:1) for 30 min. C-3′-Acyl-modified oligonucleotides were oxidized twice under the same conditions, once immediately following the incorporation of **1** or **2** and then again after complete construction of the sequence.

4.3. Oligonucleotide deprotection, purification, and characterization

Oligomers were removed from the solid support and deprotected by exposure to 28–32% aqueous ammonia at 55 °C for 8 h. Purifications were performed using oligonucleotide purification cartridges (Poly-pak Cartridges, Glen Research or OPC Cartridges, Applied Biosystems, Foster, CA) followed by RP-HPLC (Merck RP-18 LiChrospher 124×5 mm column, Darmstadt, Germany) and/or ion-exchange chromatography (Dionex DNApac 100 analytical column, Sunnyvale, CA) and stored dry. Reversed-phase separations of trityl-protected oligonucleotides were performed using a linear gradient of 2–40% acetonitrile in 25 mM triethylammonium acetate (pH 7.0) over 30 min. Ion-exchange purifications of pivaloyl-modified oligomers were performed using linear gradients of Buffer A: 10% acetonitrile in 25 mM TEAA (pH 6.0) and Buffer B: Buffer A containing 1 M ammonium chloride. Acetyl-modified sequences were purified using Buffer A: 20% acetonitrile in 25 mM TEAB (pH 6.5) and Buffer B: 20% acetonitrile in 1 M TEAB (pH 7.8). Samples purified by ion-exchange chromatography were desalted using reverse-phase chromatography with a linear gradient of 5–60% acetonitrile in 25 mM triethylammonium acetate. Sequences were characterized using MALDI-TOF MS. Concentrations of oligonucleotide solutions were measured by UV absorption at 260 nm on a Varian Cary 50 Conc spectrometer, Palo Alto, CA. The C-3′-pivaloyl, -acetyl, and 3′-xylothymidines were assigned the molar absorptivity of thymidine.

4.4. Oxidation test

Modified nucleoside **2** was incorporated into DNA sequences at the penultimate position using the conditions described above for the synthesis of modified oligomers. The oxidation times employed directly after incorporation of **2** as well as post synthesis were 15, 30 or 40 min. The oligonucleotides were cleaved from the support using standard protocols and purified using OPCs as described above. The eluted oligomers were dried under vacuum and then dissolved in nanopure water. An aliquot (40 μl) of the solution was analyzed by ion-exchange chromatography (Dionex DNApac 100 analytical column) using a linear gradient from 8 to 45 % B over 30 min. The efficiency of oxidation was determined from the ratio of full-length oligonucleotide (retention time: 21.65 min) relative to the sequence lacking the modification (retention time: 18.85 min, Supplementary data).

4.5. Thermal denaturation studies

Thermal denaturation studies were performed on a Cary 50 Conc spectrometer, equipped with a Peltier temperature controller and a thermostated cuvette holder. To determine the melting temperature of each oligonucleotide, the oligomer was annealed with its complementary strand (1:1) in 10 mM potassium phosphate buffer, 140 mM NaCl, and 1 mM EDTA, pH 7.2 (2 μM each), by rapidly heating the solution to a temperature well above its melting temperature. The DNA was then slowly cooled to 10 °C and placed in the cuvette holder. The temperature was increased to 80 °C by increments of 1 °C min^{-1}. The absorbance at 260 nm was recorded every 0.5 to 1 min and the melting temperature (T_m) was determined. Data were truncated so that the upper and lower absorbance baselines reflecting the slopes in the transition region were determined. The α value, representing the relative fraction of the complex that remains in the initial state, was calculated at each temperature as follows:

$$\alpha(T) = [A_U(T) - A(T)]/[A_U(T) - A_L(T)],$$

where $A_U(T)$ is the upper absorbance baseline, and $A_L(T)$ the lower absorbance baseline, and $A(T)$ the absorbance as a function of the temperature T (in °C).

T_m is determined as the temperature at which $\alpha = 0.5$.

4.6. Circular dichroism measurement

Oligonucleotides (4 μM) were dissolved in 10 mM phosphate-buffered saline, 140 mM NaCl, and 1 mM EDTA (pH 7.2), and placed in a 1 cm quartz cuvette. Double-stranded experiments were performed by annealing the modified oligonucleotide with its complementary sequence as described above. The data were acquired at room temperature at wavelengths from 200 to 320 nm. Four readings per sample at 20 nm/min were recorded.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmc.2005.11.038.

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