Synthesis of Oligonucleotides

Synthesis and Characterization of Oligonucleotides Containing the C4′-Oxidized Abasic Site Produced by Bleomycin and Other DNA Damaging Agents**

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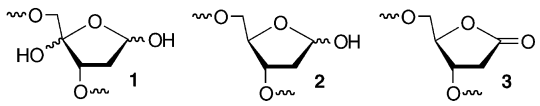
Exposure of DNA to oxidative stress produces strand scission and a variety of damaged nucleotides, which can be mutagenic and/or cytotoxic. The determination of the effects of specific lesions on nucleic acid structure, stability, and their interaction with polymerase and repair enzymes provide a chemical basis for the biological effects of DNA damage.[1,2] The execution of such investigations is greatly facilitated by the preparation of oligonucleotides containing lesions at defined sites.[3] Oxidized abasic site **1** results from formal abstraction of the C4′-hydrogen atom of a nucleotide in DNA. This alkali-labile lesion accounts for ≈40% of the DNA damage induced by the antitumor antibiotic bleomycin.[4] The C4′-oxidized abasic lesion is also produced following exposure of DNA to the neocarzinostatin chromophore, the enediynes, γ-radiolysis, and a variety of other damaging agents.[5] This oxidized abasic lesion is believed to be mutagenic and cytotoxic, and gives rise to deletions and substitutions in bacteria and mammalian cells.[6] Investigations of **1** have relied upon its sequence nonspecific and nonexclusive generation by the oxidizing agents mentioned above. In contrast, studies concerning related abasic sites (**2**, **3**) are facilitated by the accessibility of oligonucleotides containing them at defined sites.[7–9] Herein we report the first method for chemically synthesizing oligonucleotides that contain **1** at a defined site, and preliminary investigation of the lesion's effects on DNA.



The mechanism by which **1** is produced during DNA oxidation by bleomycin was investigated, and information regarding the source of oxygen at the C4 position was obtained.[4d] To our knowledge there are no reports that describe the ultimate stereochemistry of **1** in DNA, let alone

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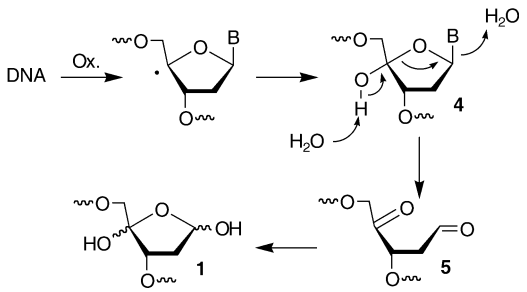
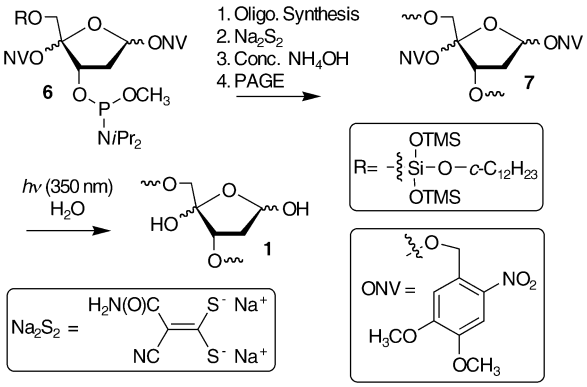
the stepwise mechanism by which it is formed from **4**. A plausible mechanism that was put forth suggests that initial oxidation of the C4′ position activates hydrolysis of the glycosidic bond (Scheme 1).[4c,d] The formation of **1** from the



Scheme 1. Proposed mechanism for the activation of the glycosidic bond.

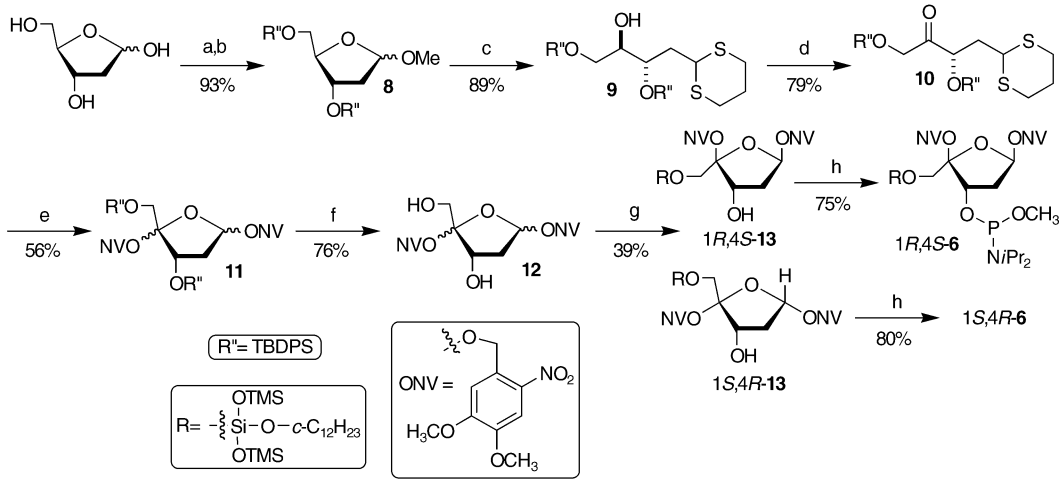
acyclic precursor **5** should result in a mixture of diastereomers.[4c,d] This expectation is consistent with NMR characterization of **2** in DNA, which also leads us to believe that the stereoisomers of **1** should exist in equilibrium with each other and small amounts of **5**.[10] As there was no reason for us to expect that **1** will exist in DNA as a single isomer, we saw no reason to attempt to generate it from individual stereoisomers of a stable precursor. While devising our approach, we also took into account that chemical syntheses of biopolymers that contain **2** and **3** are able to utilize the alkaline deprotection conditions typically employed during oligonucleotide synthesis by unmasking the alkali-labile lesions in a final photochemical step.[7,8] These considerations led us to design **6** as the phosphoramidite used during the automated synthesis to ultimately synthesize **1** in DNA (Scheme 2). Notable features of **6** include the *o*-nitroveratryl moiety (ONV), which serves as the alkali-resistant phototrigger, and the silyloxy protecting group for the primary alcohol. The latter allows us to synthesize protected oligonucleotides



Scheme 2. Synthesis of **1** in DNA.

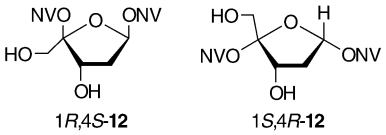
without exposing the bisacetal (**7**) to acid.[11] The *o*-nitroveratryl group is a member of the class of *o*-nitrobenzyl photolabile protecting groups that are of general use in synthesis, and oligonucleotide synthesis in particular.[12] It was anticipated that oligonucleotides containing **7** would be purified, stored, and used to generate **1** as needed.

Phosphoramidite **6** was prepared from the 3′,5′-*O*-silyloxy deoxyribose acetal **8** (Scheme 3). Cleavage of the methyl acetal in the presence of 1,3-propane dithiol provided the dithiane **9** with a C4-hydroxy group, which was then oxidized.[13] Subsequent oxidative cleavage of the dithiane ketone, **10**, in the presence of 3,4-dimethoxy-6-nitrobenzyl alcohol provided **11** as a mixture of four stereoisomers.[14] Although separation of the isomers was not practical at this step, the corresponding ^{1}H NMR spectrum indicated that two were formed in significantly greater amounts. Analytical samples of the individual diastereomers were obtained upon desilylation (**12**). ^{1}H NMR spectroscopy was used to determine that 1*R*,4*S*-**12** and 1*S*,4*R*-**12** accounted for $>80\,\%$ of the mixture of cyclized compounds. In practice, the major diastereomers were separated upon preparation of the cyclododecyloxy bis-trimethylsilyloxy silyl ethers (1*R*,4*S*- 1*S*,4*R*-



Scheme 3. a) Me_2SiCl_2, MeOH; b) *tert*-butyldiphenylsilylchloride (TBDPSCl), imadazole; c) propane dithiol, $BF_3{\cdot}Et_2O$, CH_2Cl_2, −10 °C; d) oxalyl chloride, dimethyl sulfoxide (DMSO), Et_3N, −78 °C; e) *N*-bromosuccinimide, 3,4-dimethoxy-6-nitrobenzyl alcohol (NVOH), CH_3CN:CH_2Cl_2 (2:1), −10 °C; f) tetrabutylammonium fluoride (TBAF), THF; g)cyclododecyloxy-bis-trimethylsilyloxysilyl chloride (RCl), diisopropylamine, CH_2Cl_2 0–25 °C; h) methyl *N*,*N*-diisopropylchlorophosphoramidite, diisopropylethylamine, CH_2Cl_2, 0–25 °C.

13), which were carried on to their respective phosphoramidites (1*R*,4*S*- 1*S*,4*R*-**6**).



1*R*,4*S*-**12** 1*S*,4*R*-**12**

The use of fluoride ions to unmask the primary hydroxy group during synthesis required that oligonucleotides be prepared on polystyrene solid-phase supports by using *O*-methyl phosphoramidites.[11] Solid-phase oligonucleotide synthesis was carried out in the 3′- to 5′-direction on 1 μmol scale by using modified versions of previously reported cycles.[11] With the exception of **6**, all phosphoramidites are commercially available, and were used to prepare oligonucleotides containing as many as 30 nucleotides (**14**–**16**). 5′-*O*-Dimethoxytrityl phosphoramidites containing "fast deprotecting" exocyclic amine protecting groups were used prior to incorporating **6**. This enabled us to monitor the progress of the synthesis by measuring the amount of the dimethoxytrityl cation released. Because of an absence of a similar UV-absorbing chromophore, it was not possible to directly measure the coupling of **6** or subsequent silylated phosphoramidites of the native nucleotides. Consequently, the coupling efficiency of 1*R*,4*S*-**6** was determined indirectly by comparing the isolated yield of **14a** to an otherwise identical oligonucleotide prepared entirely with 5′-*O*-dimethoxytrityl phosphoramidites, which contained thymidine in place of **7**. After the resin loading had been taken into account, the yield of **14a** was 89%, which was as much as the control oligonucleotide, and this yield can be used to estimate the lower limit for the coupling yield of **6**. No differences in coupling yields were observed when a mixture of 1*R*,4*S*- and 1*S*,4*R*-**6** was used, and for simplicity oligonucleotide synthesis with this mixture is currently the method of choice. All oligonucleotides were deprotected in two steps (Scheme 2). Phosphate triester demethylation by using Na_2S_2 preceded concentrated aqueous NH_3 deprotection/cleavage. After deprotection, photolabile oligonucleotides, **14**–**16**, were isolated by denaturing polyacrylamide gel electrophoresis, and characterized by ESIMS.[15]

5′-d(CGC ATG XGT AGT)
14a, b
5′-d(GAA GAC CCX GGC GCC)
15a, b
5′-d(GTC ACG TGC TGC AXA CGA CGT GCT GAG CCT)
16a, b
a: X = **7**; **b**: X = **1**

Photolytic liberation of **1** proved to be extremely efficient. HPLC analysis of photolyzed **14** showed 100% conversion in five minutes of irradiation to a single faster eluting product that did not absorb at 360 nm (Figure 1). In all cases 100% conversion of **7** was achieved during 20 min photolyses. Mass spectral analysis of the photolysates of **14**–**16** verified

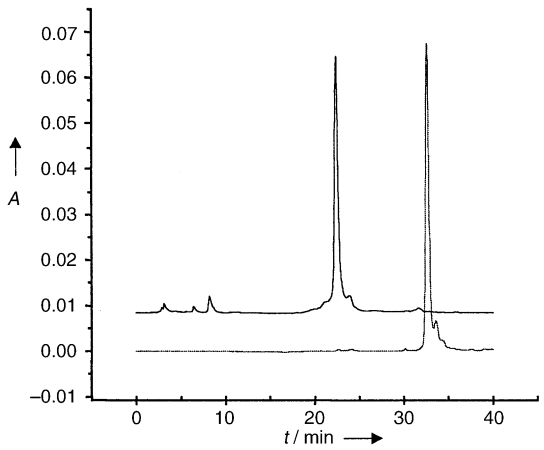


Figure 1. HPLC analysis of (bottom) **14a** (prior to photolysis) and (top) **14b** (after 20 min photolysis at $\lambda_{max} = 350$ nm; intensity: 4.2 mW cm^{-2} @ 360 nm). UV detection at 260 nm.

formation of the desired products, which were observed as a mixture of the cyclic and acyclic (-H_2O) forms.[15] The relative amounts of the cyclic and acyclic forms varied from sample to sample.

The stability of **1** in an oligonucleotide (5′-^{32}P-**15b**) towards cleavage (phosphate buffer (10 mM), pH 7.5; NaCl (100 mM)) was measured as a function of temperature by using denaturing gel electrophoresis (Figure 2). The C4′-

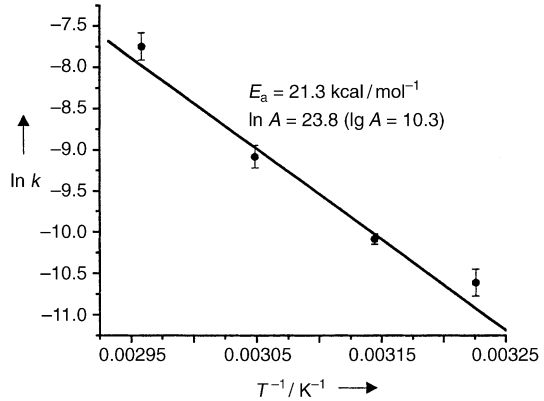
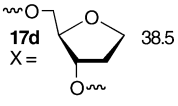


Figure 2. Arrhenius plot describing the cleavage of **1** in **15b**.

oxidized abasic site ($k = 2.46 \times 10^{-5}$ s^{-1}, $t_{1/2} = 7.8$ h) was > 10 times as susceptible to cleavage than **3** under comparable conditions (37 °C, pH 7.5), which was found to be 20 times more reactive than a non-oxidized abasic site (**2**).[16] Despite the relative lability of **1**, these studies clearly indicate that **1**

5′-d(CGC ATG XGT AGT)
3′-d(GCG TAC ACA TCA)
17a-d

	X	T_m (°C)
17a	T	54.3
17b	**1**	37.1
17c	**7**	46.6



17d 38.5

will be sufficiently long-lived to warrant attention by DNA repair enzymes.

A preliminary characterization of the effect of **1** on duplex thermal stability was carried out with the dodecamer **14b**. The lability of **1** at 55 °C led us to investigate the hybridization of **14b** to a complement containing A opposite the lesion at 37 °C to avoid cleaving **1** during hybridization. The suitability of the hybridization conditions was established by using the tetrahydrofuran abasic site analogue, **17d**. Identical T_m's were obtained for **17d** whether the duplex was hybridized at 37 °C (30 min, followed by 2 h at 25 °C) or at 95 °C (5 min, followed by slow cooling to 25 °C). Consequently, the mild conditions (37 °C) were used for the hybridization of **14b**, and the temperature was not allowed to go above 55 °C during the melting process. The T_m for **17b** (37.1 °C, 2.2 μM) did not change over three annealing/melting cycles.[15] Furthermore, no decomposition of **14b** subjected to the identical heating conditions side-by-side with **17b** was detected by HPLC.[15] The T_m for the resulting duplex containing **1** (**17b**) was comparable to that for **17d**, as might be expected for the structurally similar abasic sites. The higher T_m measured for **17c** may reflect the stabilization of the duplex due to π-stacking of the *o*-nitroveratryl groups.

In conclusion, we have developed an efficient method for synthesizing oligonucleotides that contain the C4′-oxidized abasic site, **1**, a common alkali-labile lesion. Despite the lability of **1**, the studies described above clearly indicate that the lesion is amenable to routine handling necessary for carrying out biochemical and biophysical characterization. Specifically, the C4′-oxidized abasic site **1** is sufficiently stable to enable examination of its interactions in a variety of environments such as those that include polymerases and DNA repair enzymes. The ability to store oligonucleotides that contain a stable, convenient photochemical precursor for the C4′-oxidized abasic site **6** in oligonucleotides will facilitate such studies on the lesion.

Experimental procedures for the synthesis of **6**, a description of oligonucleotide synthesis cycles, deprotection, photolysis, melting experiments, and HPLC analysis conditions are available in the Supporting Information. A table of the UV-melting data and sample melt for **17b**, HPLC traces of **14b** before and after 3 annealing/melting cycles, mass spectra of **14**–**16** are also available.

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