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An Oxidized Abasic Lesion as an Intramolecular Source of DNA Adducts

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Abstract

5'-(2-Phosphoryl-1,4-dioxobutane) (DOB) is a lesion produced in DNA via a variety of damaging agents. The DOB lesion spontaneously generates *cis*- and *trans*-but-2-en-1,4-dial (**1**) via β -elimination. *Cis*- and *trans*-but-2-en-1,4-dial forms exocyclic adducts with nucleosides. We used chemically synthesized DNA containing tritiated DOB incorporated at defined sites to examine the reactivity of *cis*- and *trans*-but-2-en-1,4-dial. Although the local DNA sequence does not appear to influence the distribution of nucleoside adducts, we find that DOB generates relatively high yields of *cis*- and *trans*-but-2-en-1,4-dial nucleoside adducts that likely are promutagenic.

The DNA nucleobases readily react with electrophiles and the products of such reactions can be detrimental or useful. Reactions with electrophiles are exploited for analyzing nucleic acid structure, such as the selective methylation of purines by dimethyl sulfate.^[1] Other alkylating agents, such as chlor-ambucil and mitomycin, which form cross-links, are useful as therapeutic agents.^[2,3] A variety of bis-electrophiles form cyclic adducts with adenine, cytosine and guanine.^[4] Some of these damaging agents such as 4-hydroxy-non-2-en-1-al, a product of lipid peroxidation, are produced endogenously.^[5] Exogenous agents such as acrolein and other α,β -unsaturated aldehydes exhibit similar reactivity. Several of these cyclic adducts have been shown to be promutagenic, indicating a possible role in carcinogenesis for the agents that produce them.^[6] We wish to report how the generation of a bis-electrophile, *trans*-but-2-en-1,4-dial (**1**) from a DNA lesion affects its ability to form exo-cyclic adducts.

Cis-But-2-en-1,4-dial (*cis*-**1**) is a metabolite of the carcinogen furan that forms exocyclic adducts with 2'-deoxyadenosine (dA), 2'-deoxycytidine (dC), and 2'-deoxyguanosine (dG) (**2–4**, Scheme 1).^[7–10] The free nucleosides react in the order dC $\gg$ dA > dG.^[7] The *trans* isomer (*trans*-**1**), which also forms adducts with nucleotides in DNA, is released from 5'-(2-phosphoryl-1,4-dioxobutane) (DOB), an oxidized abasic lesion resulting from C5'-hydrogen-atom abstraction.^[8,11] The DOB lesion is produced by several antitumour agents and by γ radiation.^[11–14] This lesion is a potent irreversible inhibitor of DNA polymerase β ,

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NMR spectra of the mixture of ³H-**8** and ³H-**9**, ³H-**7**, ³H-**5** and a sample HPLC trace of **11a** after digestion and spiking with independently prepared **2–4** are available from the Journal's website.

an enzyme that is a primary component of base excision repair that is overexpressed in many cancers.^[15–19] Hence, DOB formation may provide a chemical basis for the potent cytotoxicity of the antitumor agents that produce it.

The half-life of DOB in DNA is as short as ~11 h at 37°C and pH 7.2 (10 mM phosphate).^[20] Reversible interstrand cross-linking with dA competes with elimination in duplex DNA, but the biochemical effects of cross-linking are unknown.^[21] Although *cis*- and *trans*-**1** form exocyclic adducts with the three nucleosides containing exocyclic amines, the corresponding adduct with dG was unstable to the analytical conditions.^[8,22] Consequently, only dA and dC adducts of *trans*-but-2-en-1,4-dial (*trans*-**1**) were detected in oxidatively damaged DNA.^[8] We took advantage of our ability to chemically synthesize oligonucleotides containing DOB from a stable precursor (**6**, Scheme 2) to enhance detection of but-2-en-1,4-dial (**1**) adducts of dA, dC, and dG (**2–4**) by using tritium labeling.^[20]

Results and Discussion

Using DOB as a precursor to *cis/trans*-but-2-en-1,4-dial (**1**) is a more efficient source of the corresponding nucleoside adducts than furan because the DNA lesion is essentially an intramolecular source of the bis-electrophile.^[23] Hence, a greater fraction of **1** reacts with DNA rather than other cellular nucleophiles (e.g. proteins, thiols). We rationalized that synthesizing a variety of ternary complexes containing DOB tritiated in a non-exchangeable position would be useful for probing this possibility by enabling us to examine the distribution of nucleoside adducts as a function of nucleotide sequence proximal to the lesion. In addition, using tritiated DOB as a source of *cis/trans*-but-2-en-1,4-dial (**1**) obviates the need for derivatizing the nucleoside adducts before HPLC analysis. We anticipated that this would facilitate detection of the dG adducts of **1**, as well as those of dA and dC.

Synthesis of Oligonucleotides Containing Tritiated DOB

The oligonucleotides were prepared as previously described using the tritiated phosphoramidite (³H-**5**).^[20] Phosphoramidite ³H-**5** was synthesized from **7**, which was an intermediate in the synthesis of the unlabeled phosphoramidite (Scheme 3).^[20] Tritium was introduced by reducing the aldehyde with NaB³H₄. This reaction yielded an inseparable 1:1 mixture of the desired product (³H-**8**) and that in which the silyl group migrated to the primary alcohol (³H-**9**) in 95% yield. Oxidation of the mixture of ³H-**8** and ³H-**9** with Dess–Martin periodinane yielded the desired aldehyde (³H-**7**). The tritiated aldehyde was then carried on to ³H-**5** (350 Bq nmol^{–1}) in the manner described by Kodama.^[20]

Oligonucleotides (**10a–d**, Fig. 1, 350 Bq nmol^{–1}) containing the DOB precursor (³H-**6**) were synthesized on solid-phase support using standard synthesis cycles, with two exceptions. The coupling time for ³H-**5** was extended to 5 min and the detritylation procedure (3% trichloroacetic acid) was not carried out after this phosphoramidite was coupled at the 5'-terminus of the oligonucleotide. The oligonucleotides were deprotected using concentrated aqueous ammonia (55°C) and purified by denaturing polyacrylamide gel electrophoresis (PAGE). The DOB lesion was revealed after hybridizing with the respective

oligonucleotides via photolysis at 350 nm (1 h). The corresponding ternary complexes (**11a–d**, Figs 1–3) were used immediately in incubation experiments.

Oligonucleotide Elimination Conditions

DOB undergoes β -elimination to form 2-butene-1,4-dial with a half-life on the order of 11 h under physiologically relevant conditions.^[20] This rate of formation of *cis/trans*-but-2-en-1,4-dial (**1**) is too slow for detecting the nucleoside adducts. The nucleoside adducts themselves are chemically unstable, indicating that incubating the ternary complexes for several half-lives of the elimination reaction would be counterproductive.^[7,24] However, incubating for a shorter time would yield insufficient quantities of adducts for quantification. Consequently, *cis/trans*-but-2-en-1,4-dial (**1**) elimination from 3'-³²P-**12** was quantified over 2 h between pH 7.2 and 10 at 37°C by denaturing PAGE (Fig. 3). The highest pH in this range was chosen to carry out subsequent reactions with the tritiated ternary DOB complexes, because it yielded the most rapid elimination and adducts **2–4** were stable under these conditions (data not shown).

Cis/trans-But-2-en-1,4-dial Adduct Formation

Adduct formation was analyzed in four ternary complexes containing ³H-DOB (**12**, Fig. 4). The sequence in the vicinity of DOB was varied to probe for intramolecular effects on reactivity. In two of the ternary complexes, DOB was surrounded by dG and dC. The other two complexes were rich in dA and dT in the vicinity of the lesion. After incubating for 2 h as described above, the DNA was precipitated and digested to nucleosides. The samples were spiked with diastereomeric mixtures of unlabeled nucleoside adducts (**2–4**) that were independently synthesized as previously described.^[7,24] Each nucleoside's adducts were separated into two peaks by reverse-phase HPLC. For quantification purposes, these peaks were collected separately and the amount of tritium determined by liquid scintillation counting. The tritium levels were corrected for background by collecting equal volumes of eluent immediately before and after the corresponding HPLC peaks and subtracting the average amounts of radiation in these from the radiation in the peaks containing the nucleoside adducts. The radioactivity in the fractions containing adducts were usually two to threefold higher than the background fractions.

The total adduct yields in the various ternary complexes ranged between 4.6 and 7.5% in the various ternary complexes (Table 1). Peterson and coworkers detected ~0.1% of dC (**2**) and dA (**3**) adducts arising from the oxidation of calf thymus DNA (~10 nM), which was oxidized by the antitumour antibiotics calicheamicin and esperamicin (100 μ M) or γ radiation.^[8] In their study, the yields were determined using a previously determined amount of DOB in DNA exposed to the same reaction conditions. They were unable to detect the dG adduct owing to its instability to the derivatization and analysis conditions.^[22] It is important to consider why the adduct yields observed in our experiment are as much as 75-fold higher. One difference in our experiments is that we incubated the DOB-containing DNA before adduct analysis (2 h, 37°C, pH 10) in order to induce elimination of **1** and enhance adduct formation. Peterson, Dedon and coworkers immediately subjected their damaged DNA to the derivatization conditions. Although their derivatization was carried out for 18 h at 37°C in pH 8.0 buffer, highly nucleophilic *O*-benzylhydroxylamine (~5 mM),

which would be expected to trap any **1** that is free in solution and trap any DOB that had not yet undergone elimination, was present. Hence, the disparity in adduct yields between the two experiments could be due to our incubating DNA containing DOB, which produces greater quantities of *cis/trans*-but-2-en-1,4-dial (**1**), before enzyme digestion of the DNA (Fig. 5).

The amounts of individual *cis/trans*-but-2-en-1,4-dial nucleoside adducts (**2–4**) also did not vary in the manner predicted by reactions of the monomeric nucleosides.^[7] The 2'-deoxycytidine adduct (**2**) did not dominate the product distribution. The relative amounts were more consistent with those reported when DNA was treated with various oxidants.^[8] When *cis/trans*-but-2-en-1,4-dial (**1**) was released from DOB within ternary complexes in which the surrounding nucleotides were rich in dC (**11a,b**, Fig. 2), adduct **2** was the major product but significant amounts of dA (**3**) and dG (**4**) adducts were also detected. Furthermore, when the sequence in the vicinity of DOB was rich in dA, we did not observe consistently high yields of the corresponding dA adducts (**3**). Overall, we conclude that the DNA sequence in the vicinity of DOB has little if any effect on the distribution of *cis/trans*-but-2-en-1,4-dial adducts.

However, the exocyclic adduct formation arising from DOB is clearly more efficient than when the adducts were generated in bacteria from exogenous but-2-en-1,4-dial.^[22] In the experiments described above, the concentration of DOB-containing DNA (4 μ M) represents the maximum possible concentration of but-2-en-1,4-dial. In contrast, but-2-en-1,4-dial was present at 1 mM in experiments in bacteria.^[22] The greater efficiency for formation of **2–4** in our experiments is attributed in part to the lack of competition for but-2-en-1,4-dial (**1**) with cellular nucleophiles that are present in the bacterial cells. We postulate formation of **1** from DOB within DNA provides higher yields of the corresponding nucleoside adducts owing to effective higher concentration of the electrophile and the absence of other cellular nucleophiles. The relatively hydrophobic organic molecule may diffuse along the major groove, minor groove, or both before ultimately being released into solution. However, the rate constant for reaction of *cis/trans*-but-2-en-1,4-dial is too small to compete with diffusion, which is why we do not observe a strong dependence in adduct distribution as a function of the DNA sequence surrounding DOB.

Conclusions

The DOB lesion is a more efficient source of *cis/trans*-but-2-en-1,4-dial nucleoside adducts (**2–4**) than the carcinogen furan. This observation is similar to that reported by Dedon et al., who found that base propenals produced in DNA were a more significant source of 2'-deoxyguanosine-malondialdehyde adducts than lipid peroxidation was.^[25] Whether this remains true in cells depends in part on the lifetime of the DOB lesion in this environment. Efficient DOB repair will reduce its ability to produce *cis/trans*-but-2-en-1,4-dial (**1**), as well as its possible reaction with the nucleophilic lysine residues within the histone proteins that make up nucleosomes.^[26,27]

Experimental

Materials and General Methods

Oligonucleotides were prepared on an Applied Biosystems Inc. 394 DNA synthesizer. Commercially available DNA synthesis reagents were obtained from Glen Research Inc. Snake venom phosphodiesterases were obtained from USB and Antarctic phosphatase was obtained from New England Biolabs. NaB^3H_4 (8.2 Ci mmol^{-1} , 100 mCi) was obtained from Perkin-Elmer. HPLC was carried out using a Waters system equipped with 515 pumps, and a 2487 multi-wavelength detector controlled by *Empower* software. Liquid scintillation counting was carried out using a Beckman LS6500 counter. Oligonucleotide photolyses were carried out in a Rayonet photoreactor equipped with 16 lamps with maximum emission at 350 nm.

Phosphoramidite ^3H -5

The tritium-labelled phosphoramidite of the DOB precursor ^3H -5 was synthesized from compound ^3H -7.^[20] The ^1H NMR (CDCl_3) and ^{31}P NMR (CDCl_3) spectra (OK) of ^3H -5 match previously reported data.^[20]

Preparation of ^3H -7

MeOH was refluxed with NaBH_4 while gently bubbled through with argon for 24 h at 30°C . Sodium was then added and the solution was refluxed for another 24 h in order to more completely dry the solvent. MeOH was distilled and stored over 4-Å molecular sieves. Compound **7**^[20] (200 mg, 0.645 mmol) in distilled MeOH (2 mL) was added to NaB^3H_4 (8.2 Ci mmol^{-1} , 100 mCi). NaBH_4 (30 mg, 0.8 mmol) was added after stirring the reaction at 25°C for 1 h. The resulting mixture was stirred at the same temperature for an additional 0.5 h. The reaction mixture was diluted with diethyl ether and washed with saturated NH_4Cl , followed by brine. The organic phase was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was purified by column chromatography (EtOAc /hexanes, 1:4) to give **8** and **9** (192 mg, 0.623 mmol, 95% total) as a 1:1 mixture. δ_{H} (CDCl_3) 0.08–0.15 (s, 6H), 0.91–0.92 (s, 12H), 1.78–2.05 and 2.47 (m, 4H), 2.12 (m, 1H), 2.86 (m, 4H), 3.46 (m, 1H), 3.63 (m, 1H), 3.98 and 4.31 (m, 1H), 4.07 (m, 1H).^[20]

A mixture of **8** and **9** (192 mg, 0.623 mmol), Dess–Martin periodinane (396 mg, 0.94 mmol) and anhydrous K_2CO_3 (138 mg, 1 mmol) in CH_2Cl_2 (6 mL) was stirred at 25°C for 30 min. A mixture of saturated $\text{Na}_2\text{S}_2\text{O}_3$ and saturated NaHCO_3 (1:4) was added to the reaction mixture and stirred vigorously for 5 min. The organic phase was washed with saturated NaHCO_3 , followed by brine, and dried over Na_2SO_4 . The solvent was removed under reduced pressure and the residue was purified by column chromatography (EtOAc /hexanes, 1:6) to give ^3H -7 (91.8 mg, 48%, 554 Bq nmol^{-1}). δ_{H} (CDCl_3) 0.11 (s, 6H), 0.95 (s, 12H), 1.97–2.08 (m, 2H), 2.21 (m, 2H), 2.77–2.80 (m, 6H), 4.08 (m, 1H), 4.26 (m, 1H), 9.68 (s, 1H).^[20]

Preparation of 1,4-Butenedial Adducts 2–4^[7,24]

The 1,4-butenedial adducts were prepared by incubating dC, dA or dG (5 mM) with 1,4-butenedial (25 mM) in phosphate buffer (50 mM, pH 7.4) at 37°C overnight. The adducts

were separated and collected from reverse-phase HPLC (Delta-Pak C₁₈ 150- μ m column, 300 $\times$ 7.8 mm, Waters) (method: the reverse-phase column was eluted with a linear gradient from 100% solvent A (H₂O) to 75% A and 25% B (acetonitrile) over 30 min with a flow rate of 2 mL min⁻¹). The adducts were lyophilized to dryness, resuspended in H₂O, and stored at -80°C. The concentration of each adduct was determined by measuring the absorbance at 260 nm and using the extinction coefficient of its native nucleoside precursor, dC, dA or dG. **2** *m/z* (electrospray ionization (ESI)-MS) [M⁺] for C₁₃H₁₇N₃O₆: calc. 311.29. Found 311.82. **3**: *m/z* (ESI-MS) [M⁺] for C₁₄H₁₇N₅O₅: calc. 335.12. Found 335.78. **4**: *m/z* (ESI-M) [M⁺] for C₁₄H₁₇N₅O₆: calc. 351.12. Found 389.87 [M⁺ + Mg].

Effect of pH on DOB Elimination

The ternary complex 3'-³²P-**12** (1 μ M) in phosphate-buffered saline (PBS, 100 mM, 100 mM NaCl, pH 7.2, 8.0, 9.0 or 10.0) was incubated at 37°C for 2 h. Aliquots (5 μ L) were removed and quenched with NaBH₄ (2 μ L, 500 mM) at 25°C for 1 h. The samples were mixed with formamide loading buffer (15 μ L, 90%, 10 mM EDTA). Aliquots (5 μ L) of the mixture were loaded onto a 20% denaturing polyacrylamide gel and the product was analyzed using a phosphorimager.

Detection of 1,4-Butenedial Adducts in Ternary Complexes

The ternary complex **11a-d** (10 μ M, 2 nmol) in PBS buffer (200 μ L, 10 mM, 100 mM NaCl, pH 7.2) was incubated at 37°C overnight. Alternatively, the ternary complex **11a-d** (10 μ M, 2 nmol) in PBS buffer (200 μ L, 100 mM, 100 mM NaCl, pH 10.0) was incubated at 37°C for 2 h. DNA was then precipitated from NaOAc (0.3 M, pH 5.6) and EtOH. The sample was resuspended in Antarctic phosphatase buffer (200 μ L, 50 mM bis-Tris/propane/HCl, 1 mM MgCl₂, 0.1 mM ZnCl₂, pH 6.0) and incubated with 10 units of nuclease P1, 0.28 units of snake venom phosphodiesterase, and 50 units of Antarctic phosphatase at 37°C for 2 h. The solution was filtered through YM-10 Microcon filters (Millipore) and washed with water (100 μ L). The filtrate was mixed with **2** (100 nM), **3** (20 nM), and **4** (80 nM). Each adduct was separated and collected from reverse-phase HPLC (Microsorb-MV C₁₈ 5- μ m column, 250 $\times$ 4.6 mm). The same volume of background fractions before and after the adduct peak was also collected. The HPLC method started at 100% solvent A (10 mM NH₄OAc, pH 6.0) maintained for 5 min with a flow rate of 1 mL min⁻¹. Then the method changed to a rapid linear gradient from 100% A to 98% A and 2% B (MeCN) over 1 min, followed by a linear gradient starting with 98% solvent A, to 80% A over 60 min. The sample was mixed with liquid scintillation cocktail (4 mL) and equilibrated for 3 h. The radioactivity of each sample was counted for 2 min using the liquid scintillation counter.

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5'-d(6NC GTA ATG CAG TCT)

10a N = G

10b N = C

10c N = A

10d N = T

Fig. 1.
Structures **10a–d**.

5'-d(TAA TGG CTA ACG CN₄₄N₄₅ **DOB** N₄₇ C GTA ATG CAG TCT)
 3'-d(ATT ACC GAT TGC GN₁₇N₁₆ N₁₅ N₁₄ G CAT TAC GTC AGA)

Substrate   N₄₄ N₄₅ **DOB** N₄₇
                   N₁₇ N₁₆   N₁₅ N₁₄

11a	A G DOB G
	T C C C
11b	A C DOB C
	T G G G
11c	A A DOB A
	T T T T
11d	T T DOB T
	A A A A

Fig. 2.
 Structures **11a-d**.

Fig. 3.

DOB ($3'$ - ^{32}P -**12**) elimination as a function of pH in phosphate buffered saline (100 mM, 100 mM NaCl). Each sample was treated with NaBH_4 to stabilize the remaining DOB before gel electrophoresis analysis. The control (Ctl) was reduced before incubation.

Fig. 4.
Structure **12**.

Fig. 5.
Yield of adducts **2–4** following incubation of ternary complexes (**11a–d**) containing DOB (5'-(2-phosphoryl-1,4-dioxobutane)).

Scheme 1.

Scheme 2.

Scheme 3.

(a) NaB^3H_4 , MeOH then NaBH_4 , MeOH; (b) Dess–Martin periodinane.

Table 1

Total adduct yield as a function of DNA sequence surrounding DOB (5'-(2-phosphoryl)-1,4-dioxobutane))

5'-d(TAA TGG CTA ACG CN ₄₄ N ₄₅ DOB N ₄₇ C GTA ATG CAG TCT) 3'-d(ATT ACC GAT TGC GN ₁₇ N ₁₆ N ₁₅ N ₁₄ G CAT TAC GTC AGA)									
Substrate	N ₄₄ N ₁₇	N ₄₅ N ₁₆	DOB N ₁₅	N ₄₇ N ₁₄	Total adduct yield [%]				
11a	A T	G C	DOB C	G C	7.5				
11b	A T	C G	DOB G	C G	6.0				
11c	A T	A T	DOB T	A T	5.2				
11d	T A	T A	DOB A	T A	4.6				