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## Formation of 1,4-dioxo-2-butene-derived adducts of 2'-deoxyadenosine and 2'-deoxycytosine in oxidized DNA

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### Abstract

Oxidation of deoxyribose in DNA produces a variety of electrophilic residues that are capable of reacting with nucleobases to form adducts such as M<sub>1</sub>dG, the pyrimidopurinone adduct of dG. We now report that deoxyribose oxidation in DNA leads to the formation of oxadiazabicyclo(3.3.0)octamine adducts of dC and dA. We previously demonstrated that these adducts arise in reactions of nucleosides and DNA with *trans*-1,4-dioxo-2-butene, the β-elimination product of the 2-phosphoryl-1,4-dioxobutane residue arising from 5'-oxidation of deoxyribose in DNA and with *cis*-1,4-dioxo-2-butene, a metabolite of furan. Treatment of DNA with enediyne antibiotics capable of oxidizing the 5'-position of deoxyribose (calicheamicin and neocarzinostatin) led to a concentration-dependent formation of oxadiazabicyclo(3.3.0)octamine adducts of dC and dA, while the antibiotic bleomycin, which is capable of performing only 4-oxidation of deoxyribose, did not give rise to the adducts. The non-specific DNA oxidant, γ-radiation, also produced the adducts that represented ~0.1% of the 2-phosphoryl-1,4-dioxobutane residues formed during the irradiation. These results suggest that the oxadiazabicyclo(3.3.0)octamine adducts of dC and dA could represent endogenous DNA lesions arising from oxidative stresses that also give rise to other DNA adducts.

### INTRODUCTION

The base and deoxyribose moieties of DNA are subject to attack by reactive radical species from a variety of sources, including reactive nitrogen and oxygen species, xenobiotics and ionizing radiation. Relative to direct damage to DNA bases, such as the formation of 8-oxo-dG, the genetic toxicology of oxidation of deoxyribose in DNA has only recently become the focus of significant study. Deoxyribose oxidation results in the formation of oxidized abasic sites, strand breaks terminated with a variety of sugar residues, and freely diffusible degradation products. Many of these products are electrophilic and capable of reacting with local nucleophiles to form both DNA and protein adducts. Examples include the formation of DNA-protein cross-links when repair proteins interact with the deoxyribonolactone abasic site arising from 1'-oxidation of deoxyribose in DNA (1,2), the formation of M<sub>1</sub>dG, the pyrimidopurinone

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<sup>1</sup> Abbreviations Bp, base pair; dA, 2'-deoxyadenosine; dC, 2'-deoxycytidine; dG, 2'-deoxyguanosine; nt, 2'-deoxynucleotides; LC/MS, liquid chromatography/mass spectrometry.

adduct of dG, in reactions with base propenals arising from 4'-oxidation of deoxyribose (3–5), and the formation of glyoxal adducts of dG from the 3'-phosphoglycolaldehyde residue that arises from 3'-oxidation of deoxyribose (6). We now extend these observations to include DNA adducts derived from electrophilic products of 5'-oxidation of deoxyribose in DNA (7).

As shown in Scheme 1, the chemistry of 5'-oxidation of deoxyribose in DNA by a variety of DNA-cleaving agents, including enediynes (8) and manganese porphyrins (9), partitions to form strand breaks containing 3'-phosphate and 5'-nucleoside-5'-aldehyde residues, or a 3'-formylphosphate species accompanied by a four-carbon fragment that we and others have identified as a 5'-(2-phosphoryl-1,4-dioxobutane) residue (7,10). We recently showed that this dialdehydic residue undergoes  $\beta$ -elimination to form *trans*-1,4-dioxo-2-butene (7), a highly reactive  $\alpha,\beta$ -unsaturated dicarbonyl species. Further, the *cis*-isomer of 1,4-dioxo-2-butene is a metabolite of furan (11), an industrial solvent and component of food, smog and cigarette smoke (12,13) that has been shown to be carcinogenic in rodents (12,13). Interestingly, analogous species were identified in the  $\gamma$ -radiolysis of thymidine by Dizdaroglu *et al.* (14). We have demonstrated that both of these electrophilic isomers react with nucleophiles in DNA *in vitro* to form novel, stable oxadiazabicyclo(3.3.0)octamine adducts of dC, dG and dA under biologically relevant conditions (15–17) (Scheme 2). The dC and dA adducts were also detected in DNA from bacteria exposed to mutagenic concentrations of *cis*-1,4-dioxo-2-butene, indicating that these adducts are likely to be mutagenic (18,19).

Using a recently developed LC/MS method (18), we have now observed and quantified the formation of the 1,4-dioxo-2-butene-derived oxadiazabicyclo(3.3.0)octamine adducts of dC and dA (Scheme 2) in DNA oxidized by agents known to produce 5'-oxidation of deoxyribose in DNA. These results broaden the repertoire of DNA-derived electrophiles that react with DNA to form adducts and further complicate the spectrum of potentially endogenous DNA lesions that contribute to the mutagenic burden of human cells.

## EXPERIMENTAL PROCEDURES

### Materials

All chemicals and reagents were of highest purity available and were used without further purification unless noted otherwise. Calf thymus DNA was purchased from Sigma Chemical Company (St. Louis, MO) and dialyzed four times against potassium phosphate buffer (50 mM, pH 7.4, Chelex-treated and autoclaved; ref. 20) at 4 °C. Bleomycin was purchased from BioWorld (Dublin, OH). Calicheamicin was obtained from Wyeth Research (Cambridge, MA) and neocarzinostatin was obtained from Kayaku Co., Ltd. (Tokyo, Japan; no longer available from Kayaku, but now available from Sigma Chemical Co., St. Louis, MO). Acid phosphatase, deoxyribonuclease II, and phosphodiesterase II were purchased from MP Biomedical (Irvine, CA). The isotopically labeled standards for the *O*-benzyloxime derivatives of the dA and dC adducts, [<sup>15</sup>N<sub>3</sub>,<sup>13</sup>C<sub>4</sub>]-11-(acetaldehyde-*O*-benzyloxime)-1,*N*<sup>6</sup>-etheno-2'-deoxyadenosine and [<sup>15</sup>N<sub>3</sub>,<sup>13</sup>C<sub>4</sub>]-7-hydroxy-8-(acetaldehyde-*O*-benzyloxime)-3,*N*<sup>4</sup>-ethano-2'-deoxycytidine, respectively, were synthesized as previously described (18).

### Treatment of DNA with oxidants

Solutions of calf thymus DNA (0.5 ml, 300  $\mu$ g) in Chelex-treated potassium phosphate buffer (50 mM, pH 7.4) were aliquoted into silylated glass vials and treated with oxidants under the following conditions. For  $\gamma$ -radiation, solutions of calf thymus DNA were irradiated in a <sup>60</sup>Co source for various times (0–256 min) to produce doses up to 300 Gy. For calicheamicin and neocarzinostatin, aliquots (10  $\mu$ L) of methanolic stock solutions of the drugs were added to calf thymus DNA solutions containing 5 mM glutathione (drug activator) and the reaction allowed to proceed at ambient temperature for 1 h in the dark. For bleomycin, aliquots of a

stock of bleomycin freshly mixed with 0.9 molar equivalents of  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$  were added to the calf thymus DNA solutions and the reactions allowed to proceed at ambient temperature for 1 h, followed by Sephadex G25 chromatography to remove the Fe-bleomycin species. In all cases, DNA was precipitated with ethanol (0.3 M sodium acetate, pH 7), resuspended in water and frozen at  $-80^\circ\text{C}$ .

### Quantification of the oxadiazabicyclo(3.3.0)octamine adducts of dA and dC

These adducts were quantified using a recently developed LC-MS method (18) in which DNA (0.5 mg) was reacted with *O*-benzylhydroxylamine (2.9  $\mu\text{mol}$ ) in potassium phosphate buffer (44 mM, pH 8) in a total volume of 572  $\mu\text{L}$  at  $37^\circ\text{C}$  for 18 h. DNA was recovered by precipitation with ethanol and washed twice with 70% ethanol. Enzymatic digestion of DNA (0.5 mg/mL) was performed in ammonium acetate buffer (83 mM, pH 5) containing 174 mM magnesium sulfate, 392 mM ammonium chloride, 0.26 mg/mL deoxyribonuclease II, and 0.15 U/mL phosphodiesterase II in a total volume of 1 mL. After 20 min at  $37^\circ\text{C}$ , wheat germ acid phosphatase type I (0.55  $\mu\text{g}$ ) and the internal standards (150 fmol each) were added to each sample and they were incubated for an additional 2 h. The samples were then filtered through an acrodisc LC 13 mm syringe filter with a 0.45  $\mu\text{m}$  PVDF membrane (PALL, East Hills, NY). A portion (50  $\mu\text{L}$ ) of this solution was reserved for dG analysis. The DNA adducts were isolated by solid phase extraction (Strata-X 30 mg cartridges, Phenomenex, Torrance, CA) with sequential water, 20% methanol, and 100% methanol washes (1 mL each). The methanol wash, which contained the adducts, was concentrated to approximately 30  $\mu\text{L}$  under reduced pressure for analysis by capillary LC-MS (18). Capillary LC-MS analyses were performed with an Agilent capillary LC system linked to a ThermoFinnigan Quantum triple quadrupole mass spectrometer with an electrospray ionization source in positive mode as previously described (18).

The amount of adduct in each sample was determined from the product of the area ratio (unlabeled/labeled) and the amount of internal standard added, as described in detail elsewhere (18). The concentration of DNA in each sample was determined by measuring the concentration of dG in each DNA hydrolysate by HPLC with UV detection (21). Adduct levels were then normalized to the total amount of unmodified dG present in the sample.

## RESULTS

To explore the formation of oxadiazabicyclo(3.3.0)octamine adducts of dA and dC in DNA following 5'-oxidation of deoxyribose in DNA, levels of these adducts were measured in DNA that had been treated with several agents known to oxidize deoxyribose in DNA with different defined chemistries. For example, the diradical forms of calicheamicin and neocarzinostatin cause oxidation of the 4'- and 5'-positions of deoxyribose in double-stranded DNA (8,22,23), while bleomycin causes only 4'-oxidation (8).  $\gamma$ -Radiation is known to oxidize all positions in deoxyribose (24), but the spectrum of products differs from the enediynes and bleomycin (3, 8,25).

Adduct levels were determined by LC-MS/MS using the method recently established in our laboratory (18). The treated DNA was reacted with *O*-benzylhydroxylamine, which forms a stable oxime with the aldehydic functionality of the adducts (Scheme 2). Under the conditions of the derivatization reaction, the dA adducts undergo dehydration to form a substituted etheno adduct and are detected as 11-(acetaldehyde-*O*-benzyloxime)-1, $N^6$ -etheno-2'-deoxyadenosine.(Scheme 2). The dC adduct does not undergo the final dehydration (18). Enzyme hydrolysates of these samples were then analyzed by capillary electrospray tandem mass spectrometry (LC-ESI/MS/MS) with selected reaction monitoring for the *O*-benzyloxime derivatives of the adducts. Under the conditions of the derivatization and enzymatic hydrolysis,

we only detect the modified dA and dC adducts. The comparable dG adducts are unstable to the derivatization and hydrolysis conditions (18).

Treatment of DNA with neocarzinostatin and calicheamicin led to concentration dependent increases in the 1,4-dioxo-2-butene-derived adducts of both dA and dC in the DNA (Figure 1A and B). Only minor amounts of these adducts were detected in DNA treated with bleomycin, a compound that does not oxidize the 5'-position of the sugar (Figure 1C). Relative to the enediynes,  $\gamma$ -radiation produced relatively modest amounts of the oxadiazabicyclo(3.3.0)octamine adducts of dC (Figure 1D). In all cases, the levels of the dC adducts were substantially higher than the levels of the dA adduct, a phenomenon similar to that observed in DNA treated with *cis*-1,4-dioxo-2-butene (18). In addition, the dA adducts may be underestimated if there was not complete conversion of the initial oxime derivative of the oxadiazabicyclo(3.3.0)octamine adduct of dA to the substituted etheno derivative of this adduct (18) (Scheme 2).

## DISCUSSION

As we have demonstrated in previous studies, oxidation of deoxyribose in DNA leads to the formation of a variety of reactive electrophiles capable of forming DNA adducts (3–6,15–17). The results of the present studies extend this phenomenon to include adducts arising from products of 5'-oxidation of deoxyribose in DNA. We demonstrated previously that both *cis*- and *trans*-1,4-dioxo-2-butene, the latter a  $\beta$ -elimination product of the 2-phosphoryl-1,4-dioxobutane product of 5'-oxidation of deoxyribose in DNA (Scheme 1) (7), react with dG, dA and dC to form oxadiazabicyclo(3.3.0)octamine adducts (Scheme 2) (15–17). The present results are consistent with facile formation of these adducts in DNA exposed to a variety of oxidants capable of performing 5'-hydrogen atom abstraction in DNA.

Our conclusion that the formation of the oxadiazabicyclo(3.3.0)octamine adducts of dA and dC is the result of 5'-oxidation of deoxyribose is supported by the negative results obtained with bleomycin. This DNA-cleaving antibiotic specifically oxidizes the 4'-position of deoxyribose in DNA (8,26) and thus does not give rise to the 2-phosphoryl-1,4-dioxobutane product of 5'-oxidation. The minor amount of adducts observed with bleomycin may have resulted from DNA oxidation by  $\text{Fe}^{+2}$  released from or not chelated by bleomycin. Indeed, the enediynes calicheamicin and neocarzinostatin both cause 5'-oxidation of deoxyribose (7,8) and both produce the 2-phosphoryl-1,4-dioxobutane precursor of *trans*-1,4-dioxo-2-butene, so it is not surprising that these agents caused the formation of the oxadiazabicyclo(3.3.0)octamine adducts of dA and dC (Figure 1A and B). The levels of these adducts are likely to be reduced by the presence of glutathione in the reaction mixtures, since glutathione is an effective trap for 1,4-dioxo-2-butene (27,28).

The efficiency of formation of the adducts of dA and dC can be estimated from previous studies of the efficiency of formation of the 2-phosphoryl-1,4-dioxobutane residues of 5'-oxidation of deoxyribose. Previous studies revealed that the 2-phosphoryl-1,4-dioxobutane formed to the extent of 6 lesions per  $10^6$  nt per Gy of  $\gamma$ -radiation (7). Considering that we observed dC and dA adduct formation in  $\gamma$ -irradiated DNA to the extent of  $\sim 8$ – $20$  per  $10^9$  nt per Gy (Figure 1D), we estimate that about one in every  $\sim 1000$  2-phosphoryl-1,4-dioxobutane residues formed in the purified DNA leads to 1,4-dioxo-2-butene-derived DNA adducts. This value of 0.1% does not take into account any dG adducts that are likely to form, but we do not expect a dramatically higher yield of these adducts that those of dC (18). The mechanistic basis for this level of conversion of the 2-phosphoryl-1,4-dioxobutane residues to DNA adducts is unclear, but there are several possibilities. For example, sequence context could affect the local nucleobase and sugar residue orientations for the formation of these adducts. It is also possible that there are competing reactions of the 2-phosphoryl-1,4-dioxobutane residue or the eliminated *trans*-1,4-dioxo-2-butene, such reactions with glutathione or simply diffusion out of the helix. Whether

this level of conversion of the 2-phosphoryl-1,4-dioxobutane residue to DNA adducts occurs in cells and tissues remains to be established.

The relatively high proportion of C adducts compared to A adducts produced by all of the oxidants used in the present studies (Figure 1) is similar to results obtained in DNA treated with *cis*-1,4-dioxo-2-butene (18). However, the level of C adducts obtained with neocarzinostatin is 2- to 4-fold higher than that observed with either calicheamicin or  $\gamma$ -radiation (Figure 1A, D). One possible explanation for the differences between the oxidants is sequence selectivity of the deoxyribose oxidation. Neocarzinostatin produces 5'-oxidation at the T of ACT and GCT sequences of double-stranded DNA lesions (8), unlike calicheamicin and  $\gamma$ -radiation for which the site of deoxyribose oxidation is relatively sequence non-specific (23,29). Thus, it is possible that formation of either the 2-phosphoryl-1,4-dioxobutane or *trans*-1,4-dioxo-2-butene residues adjacent to C leads to higher levels of the oxadiazabicyclo(3.3.0)octamine adduct of C with neocarzinostatin compared to the other agents. This is similar to the higher levels of M<sub>1</sub>dG formed at the G of GC and GT sequence motifs in DNA following 4'-oxidation of the C and T by bleomycin (3–5).

In conclusion, we have demonstrated formation of the oxadiazabicyclo(3.3.0)octamine adducts of dA and dC in DNA oxidized by a variety of agents. The results suggest that these lesions could also represent endogenous DNA lesions arising from oxidative stresses that also give rise to other DNA adducts. Since they can potentially cause DNA-DNA and DNA-protein cross-links (17) and are likely to miscode (18), they may contribute to the overall toxicological effects of oxidative stress.

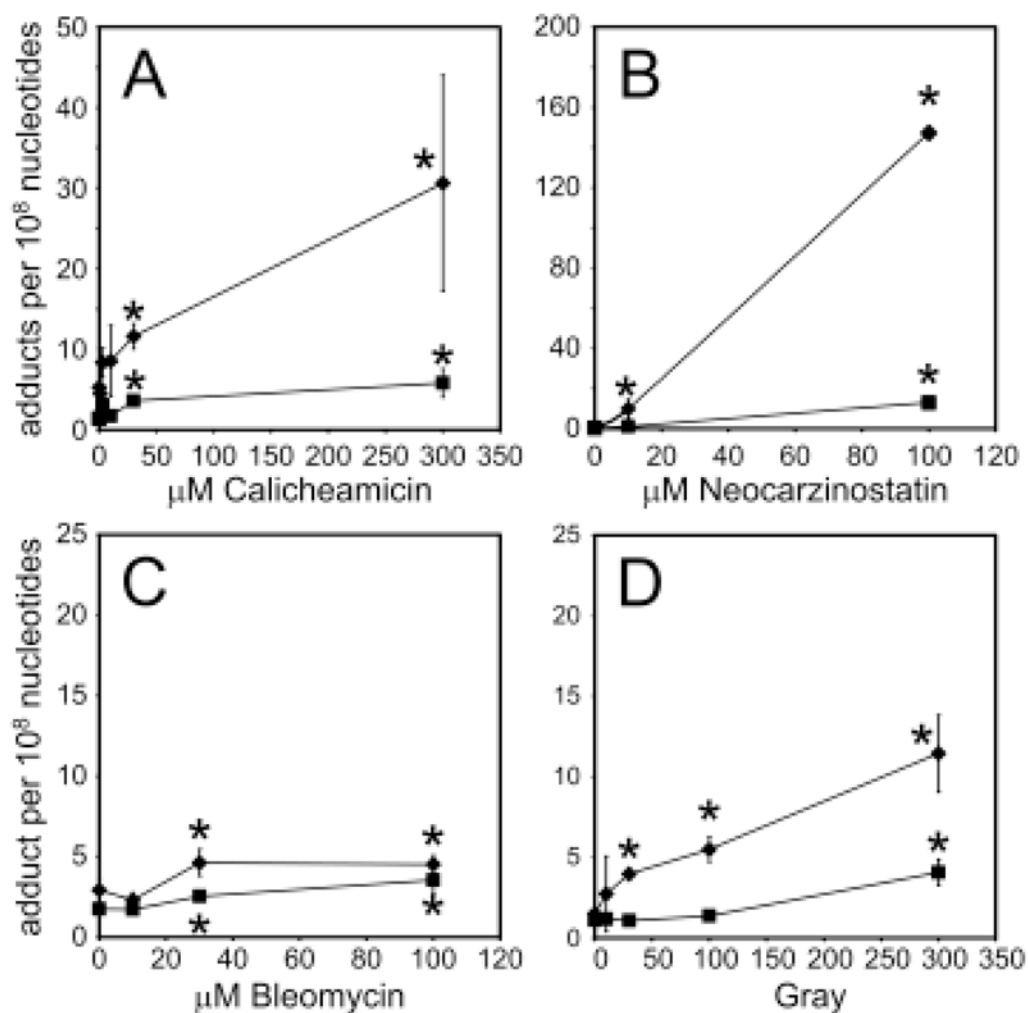
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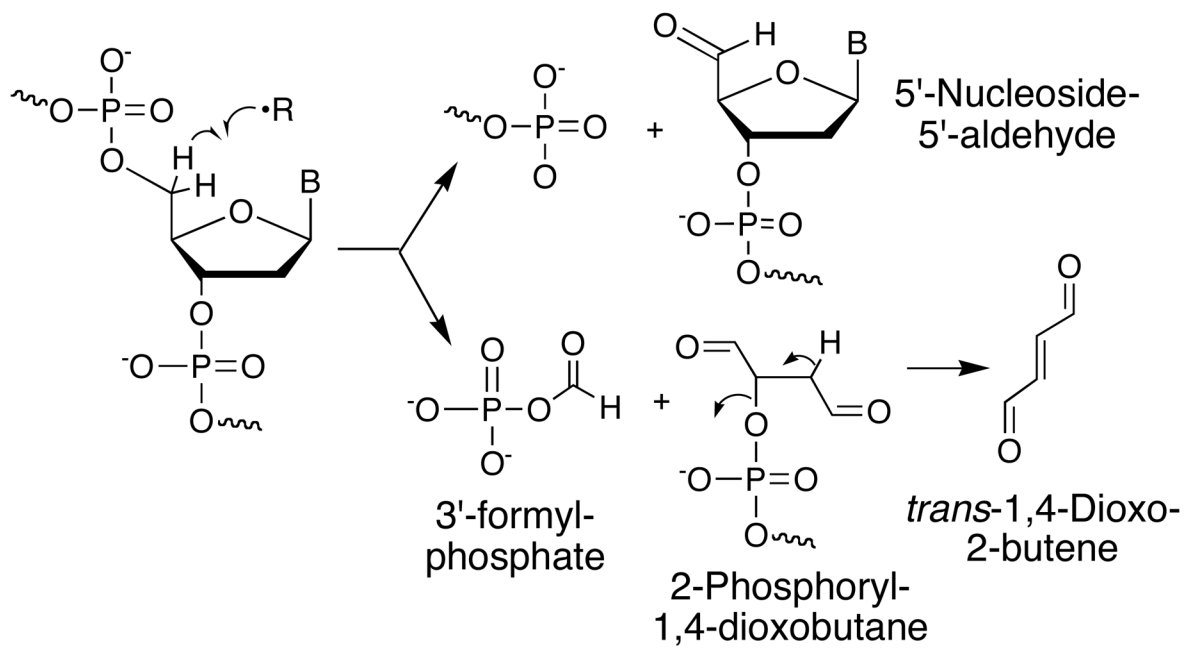
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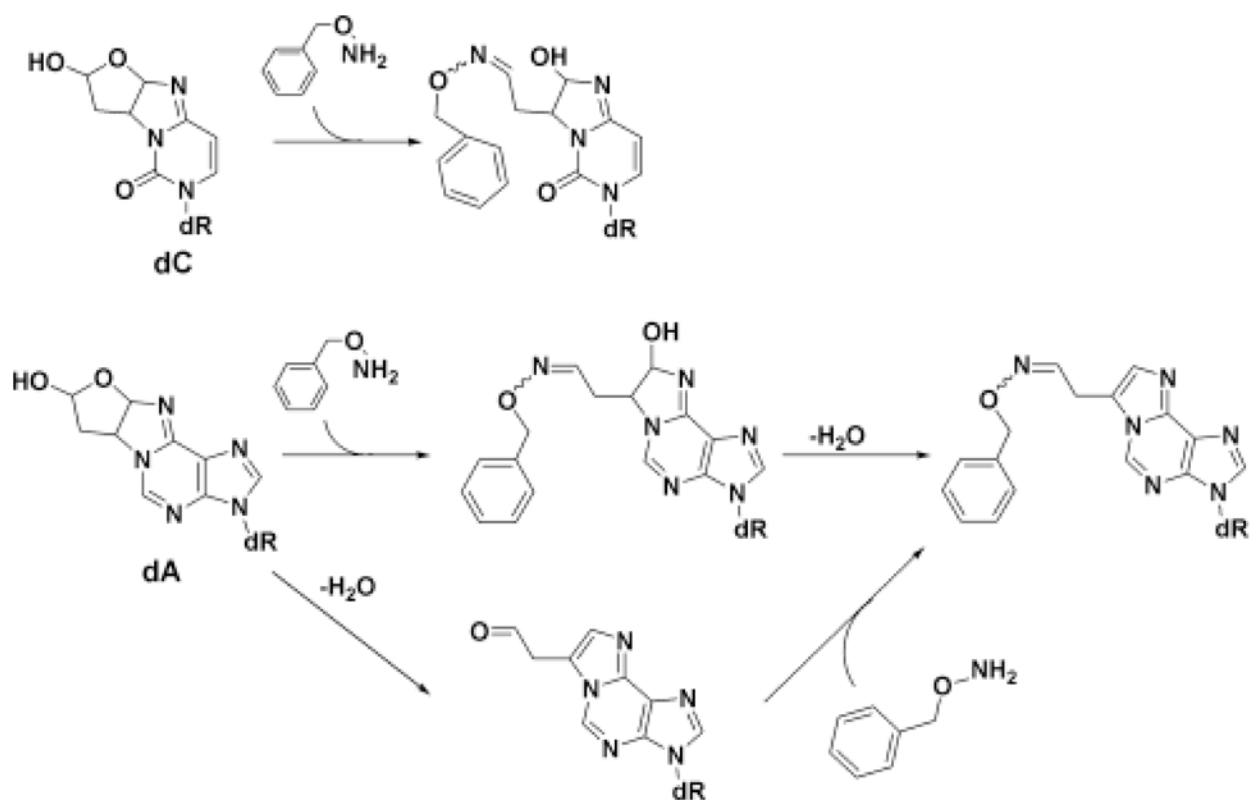
**Figure 1.** Levels of oxadiazabicyclo(3.3.0)octamine adducts of dA (square) and dC (diamond) in DNA treated with (A) calicheamicin, (B) neocarzinostatin, (C) bleomycin or (D)  $\gamma$ -radiation. Each data point represents mean  $\pm$  S.D. for three independent experiments, except for the 100  $\mu$ M neocarzinostatin data point that represents an average of two samples. \*Statistically significant difference from control levels ( $p < 0.05$ ; Student's t-test).



**Scheme 1.**

Generation of *trans*-1,4-dioxo-2-butene from 5'-oxidation of deoxyribose in DNA.





**Scheme 2.**

1,4-Dioxo-2-butene-derived adducts of dC and dA, and the formation of the benzylhydroxylamine derivatives.