Detection of DNA Adducts Derived from the Reactive Metabolite of Furan, *cis*-2-Butene-1,4-dial

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Furan is a toxic and carcinogenic compound used in industry and commonly found in the environment. The mechanism of furan's carcinogenesis is not well-understood and may involve both genotoxic and nongenotoxic pathways. Furan undergoes oxidation by cytochrome P450 to *cis*-2-butene-1,4-dial, which is thought to mediate furan's toxic effects. Consistently, *cis*-2-butene-1,4-dial readily reacts with glutathione, amino acids, and nucleosides. To determine the importance of DNA alkylation in furan-induced carcinogenesis, we developed an assay for the detection of *cis*-2-butene-1,4-dial-derived DNA adducts. DNA samples were treated with *O*-benzyl-hydroxylamine, which reacts with the aldehyde functionality of the DNA adducts. Enzyme hydrolysates of these samples were then analyzed by capillary electrospray tandem mass spectrometry with selected reaction monitoring. The dCyd and dAdo adducts were detected in digests of DNA treated with nanomolar concentrations of *cis*-2-butene-1,4-dial. In addition, these adducts were present in DNA isolated from Ames assay strain TA104 treated with mutagenic concentrations of *cis*-2-butene-1,4-dial. These data support the hypothesis that *cis*-butene-1,4-dial is a genotoxic metabolite of furan. This method will allow us to explore the role of these adducts in furan-induced carcinogenesis.

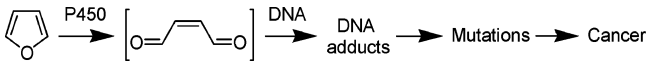
Introduction

Furan is an important environmental compound found in food, cigarette smoke, and air pollution (*1*, *2*). Furan is used extensively in industry, both as a solvent and as an intermediate in the synthesis of several important compounds (*3*). The National Toxicology Program reported extensive liver and kidney damage and an increased rate of hepatocellular carcinomas in rats and mice treated with furan (*3*). On the basis of these observations, they concluded that furan is "reasonably anticipated to be a human carcinogen" (*4*).

The toxicological activity of furan requires P450-mediated activation (Scheme 1) (*5*−*7*). Furan's toxicity in hepatocytes is inhibited by the P450 inhibitor, imidazole, and induced by acetone pretreatment (*7*). Consistent with the involvement of a reactive metabolite in the toxicity, the levels of in vitro microsomal protein binding of furan were enhanced in microsomes from rats pretreated with P450 inducers (*6*). The postulated reactive intermediate is the α,*β*-unsaturated dialdehyde, *cis*-2-butene-1,4-dial, which has been detected in microsomal incubations of furan (*8*−*10*) and reacts with amino acids and glutathione (*9*, *10*).

The underlying mechanism of furan-induced carcinogenesis is not well-understood. Several studies have suggested a potential genotoxic mechanism. *cis*-2-Butene-1,4-dial is mutagenic in the Ames assay (*11*). Furan was also clastogenic in Chinese hamster ovary cells (*12*), and *cis*-2-butene-1,4-dial has been shown to induce DNA single-strand breaks and cross-links in these cells (*13*). Tumors from mice treated with furan have different *ras* mutations than spontaneous tumors in untreated animals (*14*, *15*). However, other studies have suggested that furan is not genotoxic and that another mechanism, such as toxicity and compensatory cell proliferation, is responsible for furan-induced carcinogenesis. Furan did not induce reversions in *Salmonella typhimurium* TA100, TA1535, TA1537, and TA98 strains in the presence or absence of an S9 activating system (*16*). There was also no initiation of DNA repair in the livers of rats and mice treated with furan (*17*). However, furan induces a significant increase in cell proliferation in both rats and mice (*17*, *18*).

Scheme 1. Potential Genotoxic Mechanism of Furan-Induced Carcinogenesis



The lack of consistency in the mechanistic data for furan-induced carcinogenesis indicates the need for further research. Cellular reaction products such as DNA adducts can provide insights into the mechanisms of carcinogenesis and toxicity. Previous research has shown that the microsomal metabolite of furan, *cis*-2-butene-1,4-dial, reacts with dCyd to form an oxadiazabicyclooctaimine adduct (**1**, Scheme 2) (*19*) and with dAdo and dGuo to form initial ethano adducts (**2** and **3**, respectively) (*20*) that rearrange to form etheno-acetaldehyde adducts (**4** and **5**, respectively) (*21*). The current study describes the development of a sensitive liquid chromatography electrospray tandem mass spectrometry (LC/ESI-MS/MS) assay for the detection of *cis*-2-butene-1,4-dial-derived adducts in DNA

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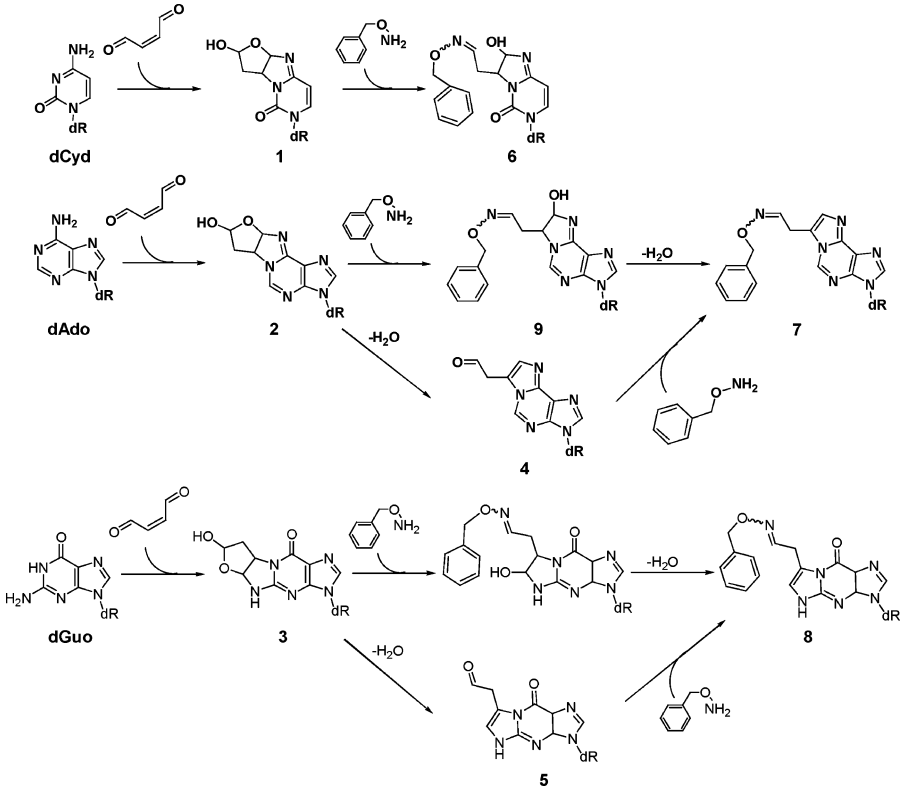
Scheme 2. Formation of Nucleoside Adducts by *cis*-2-Butene-1,4-dial and Derivatization with *O*-Benzylhydroxylamine



and its application to study their formation in DNA from *cis*-2-butene-1,4-dial-treated bacteria.

Experimental Procedures

Caution: *cis-2-Butene-1,4-dial is toxic and mutagenic and should be handled with proper safety equipment and precautions*.

Materials. dCyd, dAdo, dGuo, and *O*-benzylhydroxylamine were purchased from Sigma (St. Louis, MO). Acid phosphatase, DNase II, phosphodiesterase II, RNase A and proteinase K were purchased from MP Biomedical (Irvine, CA). $^{13}C_4$-Furan was synthesized using a published method (*22*). *cis*-2-Butene-1,4-dial and $^{13}C_4$-*cis*-2-butene-1,4-dial were prepared by oxidizing either unlabeled or $^{13}C_4$-furan with dimethyldioxirane (*23*). Solution concentrations were determined as previously reported (*11*). $^{15}N_3$-dCyd,$^{15}N_3$-dAdo, and $^{15}N_3$-dGuo were synthesized as previously described (*24*−*26*).

Instrumental Analysis. HPLC analyses were performed with a Shimadzu HPLC system linked to a dual channel Shimadzu UV detector, employing one of two methods. For method 1, a Prodigy C18 column (5 μm, 150 mm × 4.6 mm; Phenomenex, Torrence, CA) was eluted with a 40 min linear gradient from 0 to 80% acetonitrile in water. Method 2 employed a 25 min linear gradient from 0 to 25% acetonitrile in water. ^{1}H NMR analyses were performed with a Varian VI-500 MHz NMR and are reported in ppm relative to an external standard.

Characterization of the *O*-Benzyloxime Derivatives of the Nucleoside Adducts. *cis*-2-Butene-1,4-dial (0.06 mmol) was reacted with dCyd, dAdo, or dGuo (0.09 mmol) in 50 mM sodium phosphate, pH 7, for 24 (dCyd and dAdo) or 6 h (dGuo) at 37 °C (total volume, 5 mL). Then, *O*-benzylhydroxylamine (∼0.3 mmol) in 100 mM sodium phosphate, pH 7 (1 mL), was added and the solutions were incubated at 37 °C for 1 (dCyd and dAdo) or 24 h (dGuo). Acetone (100 μL) was added to remove excess *O*-benzylhydroxylamine. The resulting derivatized ethano-dCyd (**6**), etheno-dAdo (**7**), and etheno-dGuo (**8**) adducts were then collected from HPLC (method 1) and concentrated under reduced pressure. The samples were desalted via solid phase extraction with Strata-X 6 mL/500 mg cartridges (Phenomenex). After the sample was loaded onto the cartridge (4 mL), the cartridge was washed once with 17.5% MeOH in H_2O (6 mL) and twice with 100% MeOH (6 mL). The first methanol fraction was then concentrated to dryness under reduced pressure.

Efforts to weigh the resulting products were unsuccessful due to their hydroscopic nature. Instead, quantitative ^{1}H NMR analysis was performed on aliquots of the purified adducts (∼1 mg), using 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard (7.57 mM for **6**, 0.473 mM for **7**, or 1.20 mM for **8**) in D_2O (600 μL). The concentrations of the adduct solutions were determined by integrating ^{1}H signals of the adducts relative to those of DSS. This analysis indicated that the dAdo and dCyd adducts were >99% pure and the dGuo adducts were >95% pure. The resulting solutions were then diluted 100-fold in 50 mM potassium phosphate, pH 7, and the UV absorbance at 281, 279, or 284 nm was measured for **6**, **7**, and **8**, respectively, against a blank containing the appropriate concentration of DSS in D_2O. The extinction coefficient for each adduct was determined with Beer's law using the UV absorbance and the experimentally determined concentration.

7-Hydroxy-8-(acetaldehyde-O-benzyloxime)-3,N^4-ethano-2′-deoxycytidine (6). ^{1}H NMR (D_2O, ppm) major isomer 1: δ 7.48 (d, J = 8.3 Hz, 1H, H-6), 7.39−7.15 (m, 5H, C_6H_5), 6.74 (m, 1H, H-10), 6.03−5.96 (m, 1H, H-1′), 5.85 (d, J = 8.0 Hz, 1H, H-5), 5.22 (m, 1H, H-7), 4.93−4.81 (m, 2H, CH_2), 4.25−4.19 (m, 2H, H3′, H-8), 3.85−3.80 (m, 1H, H-4′), 3.62−3.54 (m, 2H, H-5′), 2.99−2.50 (m, 2H, H-9), 2.15−1.88 (m, 2H, H-2′). Major isomer 2: δ 7.42 (m, 1H, H-6), 7.41 (m, 1H, H-10), 7.39−7.15 (m, 5H, C_6H_5), 6.03−5.96 (m, 1H, H-1′), 5.75 (dd, J = 8.8, 4.0 Hz, 1H, H-5), 5.17 (m, 1H, H-7), 4.93−4.81 (m, 2H, CH_2), 4.25−4.19 (m, 2H, H-3′, H-8), 3.85−3.80 (m, 1H, H-4′), 3.62−3.54 (m, 2H, H-5′), 2.99−2.50 (m, 2H, H-9), 2.15−1.88 (m, 2H, H-2′). MS *m*/*z* (relative intensity): 417 (M + 1, 100). MS/MS *m*/*z* (relative intensity): 301 (M + 1 − 2′-deoxyribose, 100). Extinction coefficient (281 nm) = 8.6×10^3 M^{-1} cm^{-1}.

11-(Acetaldehyde-O-benzyloxime)-1,N^6-etheno-2′-deoxyadenosine (7). ^{1}H NMR (D_2O, ppm) major isomer (trans): δ 8.49 (s, 1H, H-2), 8.33 (s, 1H, H-8), 7.60 (t, J = 4.6 Hz, 1H, H-13), 7.26 (s, 1H, H-10), 7.06−6.81 (m, 5H, C_6H_5), 6.38 (dd, J = 6.8 Hz, 1H, H-1′), (H-3′ and oxime CH_2 obscured by water), 4.07 (m, 1H, H-4′), 3.83 (d, J = 4.6 Hz, 2H, H-12), 3.74−3.65 (m, 2H, H-5′),

2.79 (m, 1H, H-2′), 2.51 (m, 1H, H-2′). Minor isomer (cis): δ 8.65 (s, 1H, H-2), 8.30 (s, 1H, H-8), 7.60 (t, J = 4.6 Hz, 1H, H-13), 7.26 (s, 1H, H-10), 7.06−6.81 (m, 5H, C_6H_5), 6.45 (dd, J = 6.8 Hz, 1H, H-1′), (3′ and oxime CH_2 obscured by water), 4.07 (m, 1H, H-4′), 3.89 (d, J = 4.6 Hz, 2H, H-12), 3.74−3.65 (m, 2H, H-5′), 2.79 (m, 1H, H-2′), 2.51 (m, 1H, H-2′). MS *m/z* (relative intensity): 423 (M + 1, 100). MS/MS *m/z* (relative intensity): 307 (M + 1 − 2′-deoxyribose, 100). Extinction coefficient (279 nm) = 5.9×10^3 M^{-1} cm^{-1}.

11-(Acetaldehyde-O-benzyloxime)-1,N^2-etheno-2′-deoxyguanosine (8). ^{1}H NMR (D_2O, ppm): δ 7.92 (s, 1H, H-8), 7.59 (t, J = 4.2 Hz, 1H, H-13), 6.94−6.76 (m, 5H, C_6H_5), 6.82 (s, 1H, H-10), 6.25 (dd, J = 7.1 Hz, 1H, H-1′), (3′ and oxime CH_2 obscured by water), 4.02 (m, 1H, H−C4′), 3.78 (d, J = 3.9 Hz, 2H, C12−H_2), 3.66−3.62 (m, 2H, H-5′), 2.76 (m, 1H, H-2′), 2.42 (m, 1H, H-2′). MS *m/z* (relative intensity): 439 (M + 1, 100). MS/MS *m/z* (relative intensity): 323 (M + 1 − 2′-deoxyribose, 100). Extinction coefficient (284 nm) = 1.57×10^4 M^{-1} cm^{-1}.

Synthesis of Internal Standards. $^{13}C_4$-*cis*-2-Butene-1,4-dial (2 mM) was reacted with 1 mM $^{15}N_3$-dCyd, $^{15}N_3$-dAdo, or $^{15}N_3$-dGuo in 10 mM sodium phosphate for 24, 10, or 6 h, respectively, at 37 °C (total volume, 1 mL). The primary products were isolated by HPLC (method 2) and concentrated to 750 μL under reduced pressure. *O*-Benzylhydroxylamine (40 mM) in 100 mM sodium phosphate, pH 7 (250 μL), was added, and the solution was incubated for 1 ($^{15}N_3$-dCyd) or 24 h ($^{15}N_3$-dAdo and $^{15}N_3$-dGuo) at 37 °C. Acetone (50 μL) was then added, and the resulting peaks were isolated by HPLC (method 1) and concentrated under reduced pressure. The concentrations of the solutions were determined by UV analysis. Their structures were confirmed by MS analysis.

$^{15}N_3$,$^{13}C_4$-**6** MS *m/z* (relative intensity): 424 (M + 1, 100). MS/MS *m/z* (relative intensity): 308 (M + 1 − 2′-deoxyribose, 100). $^{15}N_3$,$^{13}C_4$-**7** MS *m/z* (relative intensity): 430 (M + 1, 100). MS/MS *m/z* (relative intensity): 314 (M + 1 − 2′-deoxyribose, 100). $^{15}N_3$,$^{13}C_4$-**8** MS *m/z* (relative intensity): 446 (M + 1, 100). MS/MS *m/z* (relative intensity): 330 (M + 1 − 2′-deoxyribose, 100).

Preparation of *cis*-2-Butene-1,4-dial-Treated Calf Thymus DNA. DNA (0.3 mg) and *cis*-2-butene-1,4-dial (0, 0.08, 0.4, 2, 10, or 50 μM) were incubated in 50 mM potassium phosphate, pH 7, at 37 °C (total volume, 500 μL). After 6 h, DNA was precipitated with ethanol, washed with 70% ethanol to remove excess *cis*-2-butene-1,4-dial, and dried under N_2. The samples were then analyzed for DNA adduct formation using the method described below.

Treatment of *S. typhimurium* TA104 Bacteria with *cis*-2-Butene-1,4-dial. Cultures of *S. typhimurium* TA104 were grown overnight in oxoid #2 nutrient broth, and the concentration was checked by its optical density at 650 nm (1.7 o.d.). For each sample, 100 mL of the culture was centrifuged at 4000*g* for 10 min. The resultant pellet was suspended in 200 mM sodium phosphate buffer, pH 7.4, containing 0, 1.42, 2.14, or 2.86 mM *cis*-2-butene-1,4-dial (total volume, 7 mL). After 30 min at 30 °C, the bacteria were then pelleted at 4000*g* for 10 min. They were washed with 7 mL of sodium phosphate buffer to remove any remaining *cis*-2-butene-1,4-dial and collected by centrifugation (4000*g* for 10 min). DNA was isolated with a modification of the method of Wang et al. (*27*). The bacterial pellet was suspended in a lysis buffer containing 1% w/v SDS, 5 mM EDTA, and 10 mM sodium phosphate, pH 7.5 (total volume, 8 mL). RNase A was added to a final concentration of 0.2 mg/mL, and the solution was incubated for 10 min at 37 °C. Then, proteinase K (1 mg/mL) was added and the solution was incubated for 1 h at 37 °C. DNA was precipitated with sodium iodide and 2-propanol. It was redissolved in 50 mM potassium phosphate, pH 7 (5 mL), and extracted with an equal volume of 24:1 chloroform/isoamyl alcohol. DNA was precipitated with ethanol, washed with 70% ethanol, dried under N_2, and analyzed for *cis*-2-butene-1,4-dial-derived DNA adducts with the protocol described below.

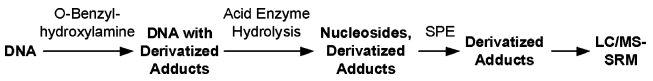
Assay for *cis*-2-Butene-1,4-dial-Derived DNA Adducts. DNA (300 μg) was dissolved in 50 mM potassium phosphate, pH 8, and 2.88 μmol of *O*-benzylhydroxylamine was added (total volume, 572 μL). Reactions were performed in triplicate. Following 18 h of incubation at 37 °C, the samples were precipitated with ethanol at ambient temperature and washed twice with 70% ethanol. This DNA was then subjected to enzymatic hydrolysis, employing the method reported by Dennehy and Loeppky (*28*, *29*). Digestions were performed with 0.3 mg/mL DNA in 83 mM ammonium acetate, pH 5, containing 8.3 mM magnesium sulfate, 20 mM ammonium chloride, 660 U/mL deoxyribonuclease II, and 0.49 U/mL phosphodiesterase II. After a 20 min incubation at 37 °C, wheat germ acid phosphatase type I (1.8 U/mL) and the internal standards (90 fmol of $^{13}C_4$,$^{15}N_3$-**6**, 60 fmol of $^{13}C_4$,$^{15}N_3$-**7**, and ∼90 fmol of $^{13}C_4$,$^{15}N_3$-**8**) were added to each sample. These solutions were then incubated for an additional 24 h. Then, each sample was filtered through a nylon membrane syringe filter (0.45 μm pore, 0.4 mm diameter, Millex-HN; Millipore Corporation, Billerica, MA). A portion was retained for dGuo analysis (50 μL). The DNA adducts were isolated by solid phase extraction (Strata-X 30 mg cartridges, Phenomenex) with sequential water, 20% methanol, and 100% methanol washes (1 mL each). The methanol wash, which contained the adducts, was concentrated to approximately 25 μL under reduced pressure for analysis by capillary LC-MS.

Capillary LC-MS analyses were performed with an Agilent capillary LC system linked to a ThermoFinnigan Quantum triple quadrapole mass spectrometer with an electrospray ionization source in the positive mode. The capillary temperature was set to 265 °C, the spray voltage was set to 4 kV, and the sheath gas pressure was set to 35. Direct injection of standards was accomplished with the standards dissolved in 50% acetonitrile in 10 mM ammonium acetate injected onto the source by a syringe pump with a flow rate of 15 μL/min. Standards were then analyzed in full scan mode followed by MS/MS analysis of the predominant ions with the collision energy set to 15 V. LC-MS/MS selected reaction monitoring (SRM) was performed with a scan time of 0.2 s and an isolation width of 0.2 *m/z* under one of two conditions: The fragmentations resulting from loss of the 2′-deoxyribose [417→301 (**6**), 424→308 ($^{15}N_3$,$^{13}C_4$-**6**), 423→307 (**7**), 430→314 ($^{15}N_3$,$^{13}C_4$-**7**), 439→323 (**8**), and 446→330 ($^{15}N_3$,$^{13}C_4$-**8**)] were monitored with the collision energy set at 15 V, or the fragmentations resulting from the sequential loss of 2′-deoxyribose, water, and the *O*-benzyloxyl group from the dCyd adducts [417→176 (**6**) and 424→183 ($^{15}N_3$,$^{13}C_4$-**6**)] and the sequential loss of 2′-deoxyribose followed by formaldehyde-*O*-benzyl-oxime from the dAdo adducts [423→173 (**7**) and 430→180 ($^{15}N_3$,$^{13}C_4$-**7**)] were monitored with the collision energy set at 25 V. Liquid chromatography was performed with a ZORBAX SB-C18 capillary column (5 μm, 150 mm × 0.5 mm; Agilent Technologies, Palo Alto, CA) eluted at 15 μL/min with a gradient of 20−50% acetonitrile in 10 mM ammonium acetate over 10 min followed by an 80% acetonitrile wash for 5 min.

Quantification with the $^{15}N_3$,$^{13}C_4$-**6** and $^{15}N_3$,$^{13}C_4$-**7** internal standards was validated by preparing solutions of a single concentration of the $^{15}N_3$,$^{13}C_4$-labeled standard (1.5 fmol/μL for **7** and 2.25 fmol/μL for **6**) with varying concentrations of unlabeled standards (0.03, 0.15, 0.75, 3.75, and 18.75 fmol/μL for each). These solutions were then analyzed by capillary LC-MS with selective reaction monitoring as described above. The relationship between the predicted and the observed ratios of labeled to unlabeled compound was plotted and fit to a linear regression. Because of the presence of a coeluting peak with the same mass as $^{13}C_4$,$^{15}N_3$-**6** in the bacterial DNA hydrolysates, the adducts were quantified with the collision energy set to 25 V (417→176 for **6** and 423→173 for **7**).

The concentration of DNA in each sample was determined by measuring the concentration of dGuo and dAdo in each DNA hydrolysate. This was achieved through HPLC with UV detection by comparison of the dGuo or dAdo peak areas to standard curves (*30*). Adduct levels were then normalized to the total number of DNA bases or unmodified parent nucleoside present in the sample. We assumed that the quantity of dCyd equaled that of dGuo.

Scheme 3. Overall Strategy for the Detection of *cis*-2-Butene-1,4-dial-Derived DNA Adducts



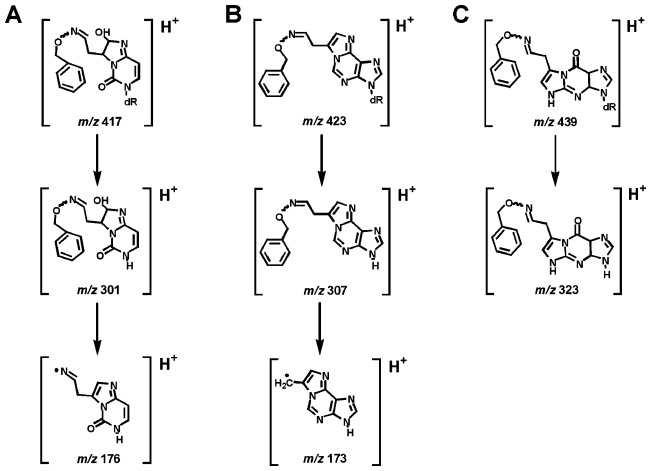
Results

Development of LC/ESI-MS/MS Method for the Detection of *cis*-2-Butene-1,4-dial-Derived DNA Adducts. To detect *cis*-2-butene-1,4-dial-derived adducts in DNA, we proposed to develop an LC/ESI-MS/MS assay similar to those reported for other carcinogen−nucleoside adducts (*31*). These methods achieve sensitive detection of modified nucleosides by monitoring the characteristic loss of the sugar, $[M + H - 116]^+$. Initial attempts to directly detect *cis*-2-butene-1,4-dial-derived DNA adduct formation via this approach met with little success due to weak ionization of the adducts (data not shown). We suspected that the poor sensitivity resulted from suppression of their ionization by coeluting unmodified nucleosides as well as likely gas phase reactions involving the aldehydic functionalities of the molecules (*32*). To improve the separation of *cis*-2-butene-1,4-dial-derived adducts from the unmodified nucleosides as well as prevent side reactions, we elected to derivatize the aldehyde groups with *O*-benzylhydroxylamine. A similar method, utilizing the hydroxylamine aldehyde reactive probe *N*′-aminoxymethylcarbonylhydrazino D-biotin, has been reported for the sensitive detection of the major malondialdehyde-derived deoxyguanosine adduct (*33*). Our previous observation that methoxyamine reacted readily with the secondary dGuo adducts **5** suggested that all of the *cis*-2-butene-1,4-dial-derived adducts will react with substituted hydroxylamines to form *O*-alkyloxime derivatives (*21*). These products lack the reactivity of the parent compound. Therefore, we explored the use of *O*-benzylhydroxylamine in the derivatization reactions described below. This particular reagent was chosen for its nonpolar nature, which will allow for greater separation of the adducts from unmodified nucleosides. The overall plan for the detection of *cis*-2-butene-1,4-dial-derived DNA damage is outlined in Scheme 3.

As predicted, *O*-benzylhydroxylamine reacted with each of the *cis*-2-butene-1,4-dial−nucleoside adducts. Derivatized nucleoside adducts (**6**−**8**) were isolated by HPLC from reaction mixtures containing the primary *cis*-2-butene-1,4-dial−nucleoside adducts (**1**−**3**) and *O*-benzylhydroxylamine (Scheme 2). Their identities as the *O*-benzyloxime derivatives of the primary dCyd adducts (**6**) and the secondary dAdo and dGuo adducts (**7** and **8**) were confirmed through MS and ^{1}H NMR analysis. MS analysis of each of the purified compounds indicated the parent ion corresponding to the expected M + 1 for each compound (*m*/*z* 417 for **6**, *m*/*z* 423 for **7**, and *m*/*z* 439 for **8**). ^{1}H NMR analyses of **6** and **7** indicated the replacement of the aldehyde functionality of the primary dCyd adducts (**1**) and secondary dAdo adduct (**4**) with an *O*-benzyloxime group in either a cis or a trans conformation. The ^{1}H NMR of the derivatized dGuo adduct (**8**) was similar to the previously described ^{1}H NMR of the *O*-methyloxime derivative of the secondary dGuo adduct (**5**) with the signals corresponding to the benzyl group replacing those of the CH_3 group (*21*).

The mass spectra of **6**−**8** all displayed a major fragment ion resulting from the loss of the 2′-deoxyribose group (**6**, 417→301; **7**, 423→307; and **8**, 439→323) (Scheme 4). When higher collision energies were used (25 V), the mass spectra of **6** and **7** also displayed further fragmentation of the adduct resulting from the additional loss of water and the benzyloxyl group from **6** (417→176) and loss of formaldehyde *O*-benzyl oxime from

Scheme 4. Fragmentation Patterns Observed by Capillary LC/MS-SRM for the Derivatized *cis*-2-Butene-1,4-dial Adducts of (A) dCyd, (B) dAdo, and (C) dGuo



7 (423→173) (Scheme 4). Both the low energy and the high energy transitions were utilized for the quantification and identification of these adducts in DNA exposed to *cis*-2-butene-1,4-dial.

Quantification of the adducts by LC/ESI-MS/MS required isotopically labeled standards. Initial studies using standards, which were only labeled at the nucleoside, indicated that there was a small amount (≤0.1%) of transfer of unlabeled 2-butene-1,4-dial from the labeled standard to unlabeled nucleosides present in the hydrolysates. This resulted in the artificial formation of unlabeled adducts that complicated the analysis, particularly when higher amounts of standards were employed. To avoid this problem, we prepared dually labeled standards by reacting $^{13}C_4$-*cis*-2-butene-1,4-dial with $^{15}N_3$-dCyd, $^{15}N_3$-dAdo, or $^{15}N_3$-dGuo. The resulting *cis*-2-butene-1,4-dial-derived nucleoside adducts were purified and subsequently reacted with *O*-benzylhydroxylamine to achieve $^{15}N_3$,$^{13}C_4$-**6**, $^{15}N_3$,$^{13}C_4$-**7**, and $^{15}N_3$,$^{13}C_4$-**8**. The mass spectra of these compounds were shifted by 7 amu from that observed for the unlabeled standards. Any transfer of 2-butene-1,4-dial from the standards to unlabeled nucleosides resulted in the formation of adducts with a 4 amu mass shift, which would not be detected during the analysis.

Standard curves were generated for the derivatized dCyd (**6**) and dAdo (**7**) adducts with known ratios of the unlabeled to $^{13}C_4$,$^{15}N_3$-labeled standards. A plot of the ratio of labeled to unlabeled adducts observed with capillary LC-MS to the expected ratio was linear across the observed range of ratios [**6** (417→301): $y = 0.9725x - 0.1406$, $R^2 = 0.9968$; **6** (417→176): $y = 1.0255x - 0.1661$, $R^2 = 0.9964$; **7** (423→307): $y = 1.0108x - 0.0059$, $R^2 = 1.000$; and **7** (423→173): $y = 0.9601x + 0.0264$, $R^2 = 0.9999$]. The limit of detection for both **6** and **7** was 0.24 fmol. Standard curves developed with the unlabeled and $^{13}C_4$,$^{15}N_3$-labeled dGuo adducts (**8**) varied considerably between preparations, particularly when the standards had been stored at −20 °C for greater than a month.

Determination of Adduct Levels in *cis*-2-Butene-1,4-dial-Treated Calf Thymus DNA. To test our detection method, calf thymus DNA was treated with increasing concentrations of *cis*-2-butene-1,4-dial for 6 h. We then tried two different protocols for the derivatization reaction. One involved initial enzymatic hydrolysis of the DNA followed by derivatization with *O*-benzylhydroxylamine. The second method reversed these two steps. The first approach was unsuccessful since the excess *O*-benzylhydroxylamine interfered with the subsequent mass

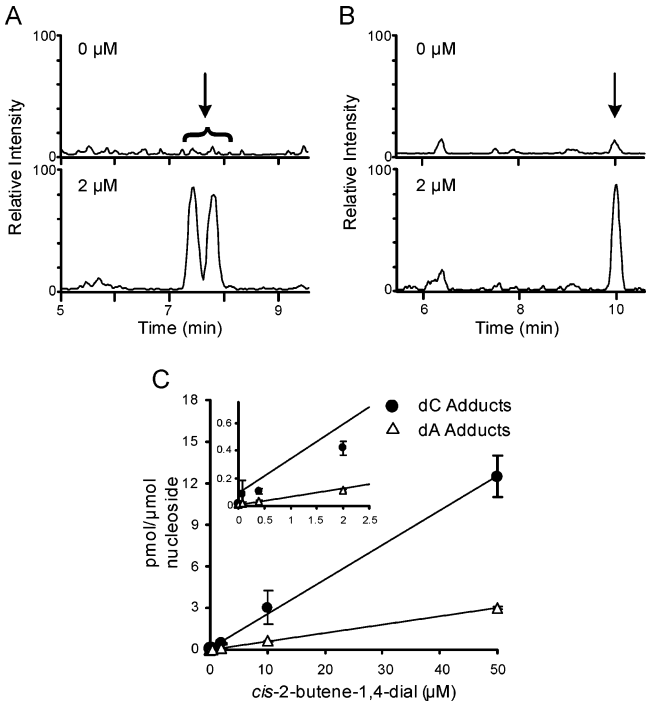


Figure 1. Formation of DNA adducts in calf thymus DNA treated with *cis*-2-butene-1,4-dial. (A) Representative capillary LC-MS traces obtained when monitoring the *m*/*z* 417→301 low energy transition for the derivatized *cis*-2-butene-1,4-dial−dCyd adducts in enzyme hydrolysates of 0 or 2 μM *cis*-2-butene-1,4-dial-treated calf thymus DNA. (B) Representative capillary LC-MS traces obtained when monitoring the *m*/*z* 423→307 low energy transition for the derivatized *cis*-2-butene-1,4-dial−dAdo adducts in enzyme hydrolysates of 0 or 2 μM *cis*-2-butene-1,4-dial-treated calf thymus DNA. (C) Graph demonstrating the linear relationship between the concentration of *cis*-2-butene-1,4-dial and the levels of the derivatized dCyd and dAdo adducts formed.

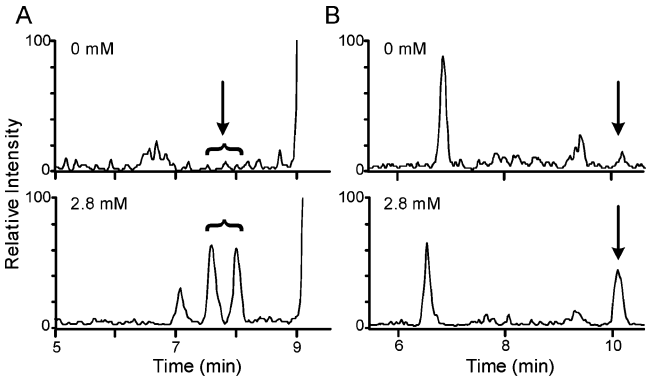


Figure 2. Detection of DNA adducts in DNA isolated from *S. typhimurium* TA104 bacteria treated with 0 or 2.8 mM *cis*-2-butene-1,4-dial for 30 min. (A) Representative capillary LC-MS traces obtained when monitoring the *m*/*z* 417→176 high energy transition of the derivatized *cis*-2-butene-1,4-dial−dCyd adducts (**6**). (B) Representative capillary LC-MS traces obtained when monitoring the *m*/*z* 423→173 high energy transition of the derivatized *cis*-2-butene-1,4-dial−dAdo adducts (**7**).

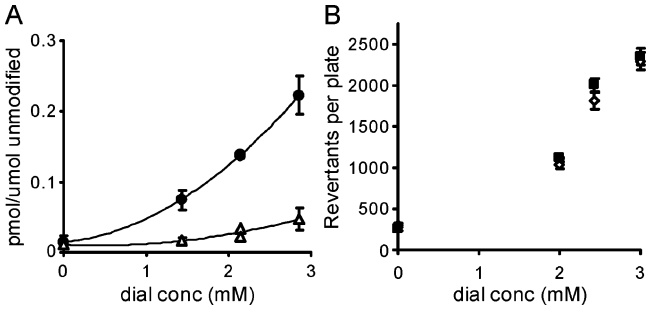


Figure 3. Formation of DNA adducts in *S. typhimurium* TA104 bacteria treated with *cis*-2-butene-1,4-dial. (A) Relationship between the concentration of *cis*-2-butene-1,4-dial and the levels of the derivatized dCyd (●) and dAdo (△) adducts formed in DNA isolated from treated TA104 bacteria ($n = 3$, mean ± SD, except 2.1, where $n = 2$). (B) Relationship between the concentration of *cis*-2-butene-1,4-dial and the Ames assay reversion rate (modified from ref *11*). The data points represent two different experiments (mean ± SD, $n = 4$).

spectral analysis, and efforts to remove this reagent failed. When the derivatization step was performed first, we were able to remove any unreacted *O*-benzylhydroxylamine by precipitation of the DNA with ethanol prior to the enzymatic hydrolysis step. Experiments were performed by varying the length of time for both the derivatization and the enzyme digestion reactions in order to optimize the detection of the three adducts. Levels of adducts **6** and **7** were maximized when the reaction with *O*-benzylhydroxylamine was conducted for 18 h and the enzymatic digestion proceeded for 24 h (data not shown). Under these conditions, adduct **8** was lost. This adduct was detected when the derivatization was performed for 1 h with a 4 h enzyme digestion period. However, the levels of this adduct varied substantially. Because the chemistry of the dGuo adducts prevented reliable analysis of this adduct, we elected to focus on the detection of **6** and **7**.

There was a linear relationship between the formation of the adducts and the concentration of *cis*-2-butene-1,4-dial (Figure 1). The identity of these adducts was confirmed by monitoring both transitions for each adduct (data not shown). The limits of detection of this assay were less than 0.02 pmol/μmol dCyd (<5 adduct per 10^9 total bases) for **6** and 0.01 pmol/μmol dAdo for **7** (<2.5 adducts per 10^9 total bases). There was a baseline level of these adducts in control DNA (≤0.01 pmol/μmol unmodified nucleoside), which might be the result of endogenous generation of these adducts.

Determination of Adduct Levels in DNA from Bacteria Treated with *cis*-2-Butene-1,4-dial. We predicted that *cis*-2-butene-1,4-dial would form adducts in DNA from *S. typhimurium* TA104 bacteria treated with *cis*-2-butene-1,4-dial at the concentrations and conditions where mutagenicity was observed (*11*). To test this hypothesis, adduct levels were determined in DNA isolated from *S. typhimurium* TA104 bacteria treated with increasing concentrations of *cis*-2-butene-1,4-dial. There was a dose-dependent increase in the levels of both **6** and **7** in DNA isolated from TA104 bacteria treated with *cis*-2-butene-1,4-dial (Figure 2), which roughly corresponded to the previously published mutagenicity results (Figure 3) (*11*). In untreated bacteria, a baseline level of the dAdo adduct **7** (~0.01 pmol/μmol dAdo) was detected, but the dCyd adduct **6** was below the limits of detection (<0.01 pmol/μmol dCyd).

Discussion

We have developed a sensitive assay for the detection of DNA adducts derived from *cis*-2-butene-1,4-dial, a reactive metabolite of the environmental carcinogen furan. As predicted based on its reactivity with nucleosides, *cis*-2-butene-1,4-dial reacts with DNA in vitro to form adducts with dCyd, dAdo, and dGuo in DNA. In addition, the dCyd and dAdo adducts were detected in DNA isolated from *S. typhimurium* TA104 bacteria treated with mutagenic concentrations of *cis*-2-butene-1,4-dial.

cis-2-Butene-1,4-dial readily reacts with calf thymus DNA, leading to the formation of DNA adducts that can be sensitively detected through derivatization with *O*-benzylhydroxylamine and subsequent analysis by mass spectrometry. This assay works

well for the analysis of the dCyd and secondary dAdo adducts but not for the detection of adducts formed with dGuo. Our inability to detect the dGuo adduct with this assay is likely the result of its reactive nature. The primary dGuo adducts have the shortest half-lives of the initial *cis*-2-butene-1,4-dial-derived adducts, rapidly dehydrating to form the secondary adduct **9** (*20*, *21*). The chemical characterization of **5** was complicated by its reactivity (*21*); this adduct appeared to form reversible cross-links when concentrated. If **5** exists as a cross-link in DNA, the aldehyde will be resistant to derivatization. The detection of the dGuo adducts is further complicated by the observation that the oxime derivatives of **3** and **5** are still somewhat unstable and subject to further reactions. We are currently investigating strategies to stabilize this adduct.

Although derivatization with *O*-benzylhydroxylamine provides a useful tool for the sensitive detection of DNA adducts formed by *cis*-2-butene-1,4-dial, there are several limitations to this assay. This assay is at best semiquantitative since we do not know the yield of the derivatization reaction within DNA. Also, it is likely that the levels of overall dAdo adducts are underestimated because this assay only detects the secondary form of the dAdo adduct (**7**). Under the conditions used in this study, we were unable to detect derivatized primary adduct, **9** (Scheme 2). This adduct can either undergo dealkylation to yield unmodified dAdo or dehydration to produce **7** (*20*). The relative contribution of these two pathways to the overall fate of **9** in DNA is not known.

Despite these limitations, this remains a useful assay for the detection of *cis*-2-butene-1,4-dial-derived DNA adducts. Derivatization with *O*-benzylhydroxylamine removes the reactive aldehyde from the adducts, preventing reactions between the adducts and other nucleophiles that would otherwise limit their detection. In addition, the presence of the benzyl group allows for easy separation of the derivatized adducts from the unmodified nucleosides through solid phase extraction, removing another obstacle to the detection of the adducts. Derivatization of the adducts also improves their ionization, enhancing the sensitivity of this assay. Swenberg and co-workers recently reported a similar assay for the dGuo adduct of malondialdehyde, pyrimido[1,2-*a*]purin-10(3*H*). Derivatization of this adduct with a hydroxylamine linked to biotin greatly increased its detection by MS (*33*).

The observation of DNA adducts in *cis*-2-butene-1,4-dial-treated bacteria supports the hypothesis that furan is a potentially genotoxic carcinogen (*11*). The reaction of *cis*-2-butene-1,4-dial with DNA was less efficient in bacteria than with calf thymus DNA in vitro. While differences in reaction conditions (time and temperature) could partially explain this discrepancy, there are many potential competing cellular nucleophiles in the bacterial cell, substantially reducing the amount of externally added *cis*-2-butene-1,4-dial that reaches DNA.

The correlation between the formation of *cis*-2-butene-1,4-dial-derived DNA adducts and the mutagenicity in the Ames assay suggests that the formation of these adducts is responsible for the mutagenicity of *cis*-2-butene-1,4-dial. Substitutions at both TA and GC base pairs can lead to phenotype reversion in TA104 (*34*, *35*), so any of the described DNA adducts induced by *cis*-2-butene-1,4-dial could be responsible for its mutagenesis. Future studies will examine the spectrum of mutations induced by *cis*-2-butene-1,4-dial and the contribution of the various DNA adducts to its mutagenic activity.

The development of an assay for the detection of *cis*-2-butene-1,4-dial-derived DNA adducts will provide an important tool for the analysis of DNA adduct formation as a result of furan exposure. Their detection in DNA from bacteria treated with *cis*-2-butene-1,4-dial validates the utility of this derivatization strategy for the analysis of *cis*-2-butene-1,4-dial-derived DNA damage.

The presence of low levels of **6** and **7** in untreated DNA suggests that these adducts could form through an endogenous pathway. Dedon and co-workers have proposed that *cis*- or *trans*-2-butene-1,4-dial formation could result from radical attack to deoxyribose in DNA (*36*). Therefore, radical reactions with DNA may be responsible for the background levels of these adducts.

In conclusion, the reactive metabolite of furan, *cis*-2-butene-1,4-dial, reacts with DNA to modify dAdo, dCyd, and dGuo. These adducts were observed in DNA isolated from bacteria treated with mutagenic concentrations of *cis*-2-butene-1,4-dial. These results demonstrate that this reactive metabolite is a genotoxic compound and may be involved in the carcinogenic mechanism of furan.

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Supporting Information Available: ^{1}H NMR spectra of **6**−**8** with DSS in D_2O. This material is available free of charge via the Internet at http://pubs.acs.org.

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