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Formation of Fused-Ring 2'-Deoxycytidine Adducts from 1-Chloro-3-buten-2-one, an in Vitro 1,3-Butadiene Metabolite, under in Vitro Physiological Conditions

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Abstract

1-Chloro-3-buten-2-one (CBO) is a potential metabolite of 1,3-butadiene (BD), a carcinogenic air pollutant. CBO is a bifunctional alkylating agent that readily reacts with glutathione (GSH) to form mono-GSH and di-GSH adducts. Recently, CBO and its precursor 1-chloro-2-hydroxy-3-butene (CHB) were found to be cytotoxic and genotoxic in human liver cells in culture with CBO being approximately 100-fold more potent than CHB. In the present study, CBO was shown to react readily with 2'-deoxycytidine (dC) under in vitro physiological conditions (pH 7.4, 37 °C) to form four dC adducts with the CBO moieties forming fused rings with the N3 and N4 atoms of dC. The four products were structurally characterized as 2-hydroxy-2-hydroxymethyl-7-(2-deoxy-β-D-erythro-pentofuranosyl)-1,2,3,4-tetrahydro-6-oxo-6H,7H-pyrimido[1,6-a]pyrimidin-5-ium (**dC-1** and **dC-2**, a pair of diastereomers), 4-chloromethyl-4-hydroxy-7-(2-deoxy-β-D-erythro-pentofuranosyl)-1,2,3,4-tetrahydro-6-oxo-6H,7H-pyrimido[1,6-a]pyrimidin-5-ium (**dC-3**), and 2-chloromethyl-2-hydroxy-7-(2-deoxy-β-D-erythro-pentofuranosyl)-1,2,3,4-tetrahydro-6-oxo-6H,7H-pyrimido[1,6-a]pyrimidin-5-ium (**dC-4**). Interestingly, **dC-1** and **dC-2** were stable under our experimental conditions (pH 7.4, 37 °C, 6 h) and existed in equilibrium as indicated by HPLC analysis, whereas **dC-3** and **dC-4** were labile with the half-lives being 3.0 ± 0.36 and 1.7 ± 0.06 h, respectively. Decomposition of **dC-4** produced both **dC-1** and **dC-2**, whereas acid hydrolysis of **dC-1/dC-2** and **dC-4** in 1 M HCl at 100 °C for 30 min yielded the deribosylated adducts **dC-1H/dC-2H** and **dC-4H**, respectively. Because fused-ring dC adducts of other chemicals are mutagenic, the characterized CBO-dC adducts could be mutagenic and play a role in the cytotoxicity and genotoxicity of CBO and its precursors, CHB and BD. The CBO-dC adducts may

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Supporting Information Available

The UV absorption, NMR, and high-resolution ESI-mass spectra of **dC-1/dC-2**, **dC-3**, and **dC-4**, the UV absorption spectra of **dC** and **dC-1H/dC-2H**, and the high-resolution ESI-mass spectrum of **dC-4H**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

also be used as standards to characterize CBO-DNA adducts and to develop potential biomarkers for CBO formation in vivo.

INTRODUCTION

1-Chloro-3-buten-2-one (CBO), an α,β -unsaturated ketone, is the product of 1-chloro-2-hydroxy-3-butene (CHB) bioactivation by alcohol dehydrogenase or rat liver cytosol.¹ CHB, a chlorohydrin, can be formed from 1,3-butadiene (BD) through a myeloperoxidase (MPO)-mediated oxidation in the presence of hydrogen peroxide and high concentrations of chloride anion (Scheme 1).² Thus, CBO is a potential metabolite of BD. BD itself is a petrochemical primarily used to manufacture synthetic rubber and plastics. However, BD is also an air pollutant; it is included in the "Air Toxics" list by the U.S. Environmental Protection Agency (EPA),³ with automobile exhaust and cigarette smoke as major environmental sources.⁴ Importantly, BD is classified as a human carcinogen by both the EPA and the International Agency for Research on Cancer.^{4,5} Human exposure has been associated with lymphatic and hematopoietic cancers in industrial workers occupationally exposed to BD.⁴ Long-term BD exposure also causes cancers in multiple organs in mice and rats.^{4,6}

Since BD is a carcinogen and is ubiquitous in the environment, several studies have been conducted to investigate the mechanisms of its carcinogenicity.⁷ BD itself is not a direct-acting carcinogen; rather, its mutagenicity and carcinogenicity are caused by the metabolites. It is well established that BD can undergo cytochrome P450-mediated bioactivation to form 3,4-epoxy-1-butene (EB).⁸ Further metabolism of EB leads to the formation of other reactive metabolites, including 1,2,3,4-diepoxybutane (DEB), 3,4-epoxy-1,2-butanediol (EBD), and hydroxymethyl vinyl ketone (HMK).⁸ These metabolites are reactive toward DNA, leading to formation of distinct adducts at different purine and pyrimidine sites.⁷

Whereas the above-described bioactivation of BD is presumably active in tissues such as liver, lung, and kidney, because these tissues contain cytochrome P450s, an alternative BD bioactivation pathway may be active in the immune and bone marrow cells, in particular, neutrophil granulocytes. These cells contain abundant MPO, whose major function is to produce hypochlorous acid (HOCl) from hydrogen peroxide and chloride anion when the cells are stimulated by invading microorganisms or upon oxidative burst as part of the inflammatory response.^{9,10} If BD is present under these conditions, an addition reaction between BD and HOCl can occur, leading to the formation of a different metabolite, that is, CHB (Scheme 1).² CHB, in turn, can then be biotransformed to CBO.¹

CBO is a bifunctional alkylating agent that has high reactivity toward intracellular nucleophiles. CHB and CBO were both cytotoxic and genotoxic in human liver cells in culture with CBO being approximately 100-fold more potent than CHB.¹¹ CHB was also found to be mutagenic by the Ames test, however, the high toxicity of CBO prevented a reliable assessment of CBO mutagenicity.¹¹ Collectively, the results suggested that CHB and CBO could play a role in the mutagenicity/carcinogenicity of BD, in particular, because human exposure to BD has been linked to an increased incidence of lymphatic and hematopoietic cancers.⁴ Currently, the molecular basis of CHB and CBO genotoxicity is unclear.

Genotoxicity of CBO indicates that it causes DNA damage. CBO is expected to form DNA adducts by reacting with DNA bases, which could be part of the underlying reasons of CBO genotoxicity. The formation of DNA adducts is central to initiating the mutagenic process for DNA-reactive chemicals. It is thus important to identify DNA adducts of CBO to better

understand the molecular mechanisms of its genotoxicity. In addition, DNA adducts of CBO have potential to be used as biomarkers of CBO formation after BD exposure. Therefore, as the first-step toward characterizing nucleoside and DNA adducts of CBO, we investigated the reaction of CBO with 2'-deoxycytidine (dC) under in vitro physiological conditions (pH 7.4, 37 °C) and structurally characterized the formed adducts. In addition, the stabilities of these adducts under in vitro physiological conditions were examined, and their decomposition products at pH 7.4 and 37 °C were characterized. Furthermore, the acid hydrolysis products of these adducts in 1 M HCl at 100 °C were also identified.

EXPERIMENTAL PROCEDURES

Caution

CHB and CBO are cytotoxic and genotoxic and must be handled using proper safety measures.

Materials

BD (99%) was obtained from Dalian Da-Te Gas Ltd. (Dalian, China). Calcium hypochlorite was obtained from Alfa Aesar (Ward Hill, MA, U.S.). Chromium oxide was obtained from Adamas Reagent Company (Shanghai, China). dC was purchased from Sigma-Aldrich (St. Louis, MO, U.S.). Other reagents were purchased from Sinopharm Chemical Reagent Company (Shanghai, China) and were of analytical reagent grade. CHB was synthesized through bubbling a mixture of BD and carbon dioxide into a calcium hypochlorite suspension in an ice-water bath as reported previously.¹² CBO was prepared by mild oxidation of CHB using Jones' reagent.¹ The crude CBO product had a good purity (~90%). Pure CBO (> 98%) was obtained through purification of the crude CBO on a silica gel column with dichloromethane as the eluent. The concentrations of all phosphate buffers were 100 mM and the buffers additionally contained 100 mM KCl to mimic the KCl concentrations in blood or plasma.

Instruments and Methods

Mass spectra were recorded on a Bruker microTOF II mass spectrometer with electrospray ionization (ESI). NMR spectra were collected on a Bruker Instruments Avance AV500 spectrometer (500 MHz). HPLC separation was performed on a Waters Alliance 2695 HPLC system (Milford, MA, U.S.). The detector was a Waters 2996 diode array detector with the wavelength range from 210 to 400 nm. Analytical separation was carried out on an Agilent ZORBAX 80Å StableBond SB-C18 analytical column (4.6 × 250 mm, 5 μm). A linear gradient program was used, starting at 1 min from 0% pump B to 12% pump B over 11 min [pump A, 1% (v/v) methanol with the pH being adjusted to 2.5 with trifluoroacetic acid (TFA); pump B, 10% (v/v) methanol at pH 2.5] at a flow rate of 1 mL/min and then at 12 min from 12% to 100% pump B over 2 min, at 35 min from 100% to 0% pump B over 5 min, and stopped at 40 min. Preparative separation was performed on a Waters SunFire C18 preparative column (10 × 150 mm, 5 μm). The mobile phases were the same as those used for analytical separation. The above gradient program was also used for preparative separation of **dC-1**, **dC-2**, and the acid hydrolysis products (**dC-1H/dC-2H** and **dC-4H**), except the flow rate was changed to 3 mL/min. For isolation of **dC-3** and **dC-4**, a 10-min isocratic program with 100% pump B at a flow rate of 3 mL/min was used. Fractions were collected manually and were lyophilized on a CHRIST ALPHA 1-4 LD-2 freeze dryer (Osterode am Harz, Germany).

Reactions of CBO with dC at Different pH Values and Different CBO:dC Molar Ratios. Time-Course Experiments

dC (10 mg, 0.044 mmol) was dissolved in 2 mL of phosphate buffer with the specified pH value (4.0, 7.4, and 10.0) and pure CBO (20 μ L, 0.22 mmol, CBO:dC molar ratio of 5:1) was added. The mixture was incubated at 37 °C. Aliquots (100 μ L) were withdrawn every hour and were subjected to HPLC analyses. For the reactions at pH 7.4 and 10.0, the pH values of the aliquots needed to be adjusted to ~ pH 5 using HCl prior to HPLC analyses. For the reaction with the CBO:dC molar ratio of 2:1, dC (5 mg, 0.022 mmol) was dissolved in 2 mL of pH 7.4 phosphate buffer and pure CBO (3.6 μ L, 0.039 mmol) was added. The mixture was then incubated at 37 °C and subjected to HPLC analyses as described above.

Isolation of the Reaction Products for Structural Characterization

For isolation of the products, the reactions were performed at larger scales. To prepare **dC-1** and **dC-2**, dC (53 mg, 0.23 mmol) was dissolved in 3 mL of pH 7.4 phosphate buffer and pure CBO (100 μ L, 1.1 mmol) was added. The solution was incubated at 37 °C for 3 h. After extraction by ethyl ether, **dC-1** and **dC-2** were isolated using preparative HPLC. To isolate **dC-3** and **dC-4**, dC (5.3 mg, 0.023 mmol) was dissolved in 2 mL of pH 4 phosphate buffer and pure CBO (10 μ L, 0.11 mmol) was added. After incubation at 37 °C for 3 h and extraction by ethyl ether, **dC-3** and **dC-4** were purified using preparative HPLC.

Stabilities of the Reaction Products and Determination of Their Half-Lives. General Procedures

A small quantity of purified reaction products (1-2 mg) was dissolved in pH 7.4 phosphate buffer and incubated at 37 °C. Aliquots were withdrawn every hour and were subjected to HPLC analyses. The peak areas of the compounds to be examined were determined and were regressed against time using exponential decay ($y = a \times e^{-bx}$). The half-lives were calculated using the data obtained after regression. The experiments were carried out in triplicate and the reported values were the means and standard deviations of three independent experiments.

Decomposition of Products at in Vitro Physiological Conditions. General Procedures

A small quantity of purified products (1-2 mg) was dissolved in 1 mL of pH 7.4 phosphate buffer and incubated at 37 °C for time specified. The solution was then analyzed on HPLC.

Acid Hydrolysis of the Reaction Products

A small quantity of purified products (1-2 mg) was dissolved in 1 mL of 1 M HCl and was then heated in a boiling water bath for 30 min. After cooling down to ambient temperature and the pH being adjusted to ~6, the solution was analyzed on HPLC. As needed, the acid hydrolysis products were isolated using preparative HPLC.

RESULTS

Reaction of CBO with dC

CBO readily reacted with dC under in vitro physiological conditions (pH 7.4, 37 °C). This was evident by dC exhibiting rapid consumption that correlated well with the rapid appearance of reaction products (see below). The chromatogram of the reaction mixture showed four major product peaks with the retention times at 14.4, 17.2, 24.3, and 26.4 min, respectively (Figure 1). The compounds that the four peaks represent were designated **dC-1**, **dC-2**, **dC-3**, and **dC-4** in the order of the retention times. **dC-1**, **dC-2**, and **dC-3** appeared as sharp peaks, however, **dC-4** was observed as a broad peak (Figure 1). These products exhibited UV absorption spectra similar to that of dC with the exception of **dC-3**, which

exhibited a different spectrum but had the absorption maxima almost identical to those of the other three products (Figure S1). The absorption maxima were 217 and 284 nm for **dC-1**, 216 and 284 nm for **dC-2**, 216 and 285 nm for **dC-3**, and 217 and 285 nm for **dC-4** (the dC absorption maxima were 212 and 280 nm at the same conditions). Thus, the four compounds were expected to be the reaction products of CBO and dC.

Dependence of the Formation of the Reaction Products on the CBO:dC Molar Ratio and the Incubation pH. Time-Course Experiments

To determine the optimal conditions to prepare the reaction products, a series of time-course experiments with different CBO:dC molar ratios and at different incubation pH was performed.

The reaction was first carried out with the CBO:dC molar ratio of 2:1 at pH 7.4 and 37 °C. Under these conditions, the dC peak area exhibited a rapid decrease at the early stage of the reaction (within 2 h) whereas little change in the dC peak area was observed from 3 to 8 h (Figure 2). The results suggested that CBO has been completely consumed by both the reaction with dC and degradation reactions within the first two hours of the incubation. The observed four products exhibited distinct rates of formation and stabilities under the incubation conditions. The amounts of **dC-1** and **dC-2** increased over time during the entire reaction, whereas that of **dC-4** increased steeply within 1 h, reached the maximum at 2 h, and then decreased over time (Figure 3A). On the other hand, the amount of **dC-3** showed a moderate increase within 2 h and then little change over time (Figure 3A). That is to say, **dC-1**, **dC-2**, and **dC-3** were formed with no significant decomposition under the reaction conditions, whereas **dC-4** underwent formation at the early stage of the reaction and then exhibited decomposition. The peak area of dC did not change significantly after 2 h (Figure 2), whereas that of **dC-2** exhibited a steeper increase only after 3 h (Figure 3A), suggesting that **dC-2** was not a direct reaction product of CBO and dC. On the other hand, the peak area of **dC-3** did not increase after 2 h, suggesting that **dC-3** was a direct reaction product of CBO and dC.

When the incubation pH was kept unchanged but the CBO:dC molar ratio was increased to 5:1, the profiles of the products were similar with the yields of all products increasing approximately by two folds (Figure 3B). At pH 10.0 and the CBO:dC molar ratio of 5:1, the pattern of the formation and decomposition of the products was qualitatively similar to that observed at pH 7.4 and the same CBO:dC molar ratio, although the yields of **dC-3** and **dC-4** were approximately halves of those at pH 7.4 (Figure 3C). However, at pH 4.0, the profiles of the products changed radically; **dC-4** became the predominant product (Figure 3D) [it should be noted that under these conditions **dC-4** appeared as a large broad peak, and **dC-3** became a small unresolved shoulder peak of **dC-4** (Figure 4), therefore the peak areas of **dC-3** and **dC-4** were combined as shown in Figure 3D]. Decomposition of **dC-4** at pH 4.0 was very slight, making the reaction of CBO with dC at pH 4.0 almost exclusively yield **dC-4**. Among the three pH values, the yield of **dC-4** was the highest at pH 4.0; the peak area of **dC-4** at 4.5 h was ~3 fold as great as that of **dC-4** at pH 7.4 and 1.5 h (Figure 3B and 3D). Meanwhile, the yields of **dC-1** and **dC-2** were the lowest at pH 4.0; their peak areas at 6 h were only 10% and 4% of those at pH 7.4 and the same CBO:dC molar ratio (Figure 3B and 3D), respectively.

Characterization of dC-4

The high-resolution ESI-mass spectrum of **dC-4** showed a peak at m/z 332.1001 and its isotope peak at m/z 334.0979 with ~1/3 the intensity (Figure S2A), which was consistent with an expected molecular formula $C_{13}H_{19}N_3O_5Cl$ (the calculated formula weight was 332.1013). The peak was very weak with the intensity being only 2.6% of the base peak.

The base peak, which also contained a chlorine atom, appeared at m/z 216.0528 (the calculated value for the formula $C_8H_{11}N_3O_2Cl$ was 216.0540) and was apparently the daughter ion of the ion at m/z 332.1001 after loss of a deoxyribose moiety. Therefore, the mass spectral data indicated that **dC-4** was a reaction product of CBO and dC after addition of one molecule of CBO to dC.

To determine the chemical structure of **dC-4**, its ^1H NMR, heteronuclear multiple quantum coherence (HMQC), and heteronuclear shift correlations via multiple bond connectivities (HMBC) spectra in dimethyl sulfoxide ($DMSO-d_6$) and the ^1H NMR spectrum in D_2O were recorded. The ^1H NMR spectrum in $DMSO-d_6$ (Figure S3) showed the presence of 19 protons, indicating that the ion at m/z 332.1001 observed in the ESI-mass spectrum of **dC-4** was actually its molecular ion rather than the protonated molecular ion. That is to say, **dC-4** was a quaternary ammonium salt. Four singlet peaks at 10.97, 7.30, 5.36, and 5.16 ppm were not observed when the spectrum was obtained in D_2O (data not shown), indicating that these peaks were the signals of protons on amino and hydroxyl groups. The signal at 10.97 ppm could be assigned only to the proton on the amino group. The observation that the peak contained only one proton, together with the remarkable feature that it appeared at such an unusually low field, indicated that both $N3$ and N^4 positions were alkylated. Alkylation at the $N3$ position produces a positive charge on the pyrimidine ring, which can move to the N^4 position via tautomerization. This tautomerization results in the formation of an imine structure with the N^4 atom carrying the positive charge, which explained why the N^4 proton appeared at such an unusually low field. That is to say, the CBO moiety formed a fused ring with the pyrimidine ring.

Based on the reactivity of CBO, it was expected that the vinyl end of CBO first reacted with one of the nitrogen atoms of dC, and then the other nitrogen reacted with the carbonyl group to form a six-membered ring. Depending on whether the $N3$ and N^4 atoms of dC reacted with the vinyl end of CBO, two chemical structures could be formed (Scheme 2) and needed to be distinguished using the obtained NMR spectra.

On the basis of the ^{13}C chemical shift information obtained from the HMQC spectrum (Figure S4) and the $^1H/^{13}C$ data reported in the literature, the signals of the two protons at the pyrimidine ring were identified.¹³ Similarly, the signals of the protons in the deoxyribose moiety, which exhibited only small shifts from the data of the corresponding protons in DEB-2'-deoxyguanosine adducts, were assigned by comparison with the data published previously.¹⁴ The remaining six protons were hydrogen atoms of three CH_2 in the CBO moiety, which appeared at 4.20 and 3.74 (2H), 3.88 (2H), and 2.14 ppm (2H) based on the HMQC spectrum. The corresponding ^{13}C chemical shifts were 37.6, 48.2, and 26.8 ppm, respectively. In the CBO moiety, apparently $C3''$ (the numbering of carbon atoms is shown in the structure of **dC-4** in Scheme 3) should appear at the highest field as it was connected to two other carbon atoms, thus the lowest chemical shift (26.8 ppm) was assigned to $C3''$. The two $H1''$ were expected to appear as either a singlet peak or two doublet peaks. Although it could not be determined if the signal at 3.88 ppm was a singlet peak due to overlapping with the signal of $H4'$, the possibility that the signals at 4.20 and 3.74 ppm were those of $H1''$ could be excluded because these signals showed complex multiplicity. Consequently, the signal at 3.88 ppm was assigned to $H1''$, and those at 4.20 and 3.74 ppm to $H4''$ (Table 1). In addition, the assignment was consistent with the coupling information in the HMBC spectrum (see below).

The HMBC spectrum of **dC-4** (Figure S5) indicated that the proton at 8.29 ppm ($H6$) coupled to four carbon atoms at 86.8, 93.4, 146.5, and 155.0 ppm, and the proton at 6.26 ppm ($H5$) coupled to three carbon atoms at 86.8, 142.1, and 155.0 ppm. Thus, the carbon at 146.5 ppm was assigned to $C2$, and that at 155.0 ppm to $C4$. Further support for these

assignments was provided by the coupling information of H1', which coupled to the carbon atoms at 142.1 and 146.5 ppm, but not the carbon atom at 155.0 ppm. The signal at 4.20 ppm (one of the two H4'' atoms) was observed to couple to both carbon atoms at 146.5 and 155.0 ppm (i.e., C2 and C4) besides coupling to two other carbon atoms at 26.8 and 78.3 ppm. The other H4'' atom, which appeared at 3.74 ppm, did not show coupling to C2, but did show a weak coupling to C4. No other protons exhibited coupling with C2 and C4. Therefore, the coupling information in the HMBC spectrum indicated that C4'' was connected to N3. Namely, **dC-4** was the product with the vinyl end of CBO reacting with N3. Therefore, the structure of **dC-4** was determined as 2-chloromethyl-2-hydroxy-7-(2-deoxy- β -D-*erythro*-pentofuranosyl)-1,2,3,4-tetrahydr o-6-oxo-6*H*,7*H*-pyrimido[1,6-*a*]pyrimidin-5-ium (Scheme 3).

Characterization of dC-3

Like **dC-4**, in the high-resolution ESI-mass spectrum of **dC-3** (Figure S2B), the molecular ion was observed at m/z 332.0990 with the isotope peak at m/z 334.0974 with $\sim 1/3$ the intensity. Thus, **dC-3** had identical molecular formula to **dC-4**. Very similarly to **dC-4**, the molecular ion peak was very weak (only 3.9% of the base peak). The base peak, which appeared at m/z 216.0513 and contained a chlorine atom, was the daughter ion of the molecular ion after loss of the deoxyribose moiety. Therefore, **dC-3** was an isomer of **dC-4**.

Since **dC-3** appeared as a relatively minor product in comparison with **dC-4** with the yield being approximately 10% of **dC-4** at most (estimated during preparative separation by the peak areas), the quantity of **dC-3** isolated was very small. As a result, its HMBC spectrum was not recorded. Instead, its ^1H NMR spectrum, together with the correlation spectroscopy (COSY) and HMQC spectra in DMSO- d_6 , were obtained. The ^1H NMR and COSY spectra were of good quality, although a very strong water peak at 3.36 ppm in the ^1H NMR spectrum was observed due to the very low concentration of **dC-3** in DMSO- d_6 (Figure S6). The HMQC spectrum exhibited poor signal-to-noise ratios (Figure S7).

The ^1H NMR spectrum of **dC-3** showed signals of 17 protons with two protons missing (Figure S6). Just as **dC-4**, the signal of the proton at the exocyclic amino group, which appeared at 10.75 ppm, indicated that the CBO moiety formed a fused ring with N3 and N⁴. The COSY spectrum (Figure S8) showed that the peak at 10.75 ppm coupled to a signal at 3.89 ppm, which, in turn, coupled to a peak at 2.97 ppm. Obviously, the coupling information indicated that the vinyl end of CBO reacted with the exocyclic amino group and the signals at 3.89 and 2.97 ppm were those of H4'' and H3'', respectively (Table 1, Scheme 3). Furthermore, based on the coupling information obtained from the COSY spectrum, it was possible to identify the signals of H1' (6.05 ppm), H2' (2.25 and 2.11 ppm), H3' (4.22 ppm), H4' (3.87 ppm), and H5' (3.60 ppm). The two protons on the pyrimidine ring appeared at 8.13 (H6) and 6.24 ppm (H5), and the COSY spectrum confirmed that they coupled each other. The signal of the two H1'' atoms was missing; it was very likely that the signal was masked by the very strong water peak at 3.36 ppm. Because H1'' did not couple to other protons, the COSY spectrum was not helpful to their identification. Moreover, the signal of H1'' in the HMQC spectrum (Figure S7) was also not observed probably due to the poor signal-to-noise ratios of the spectrum. Nonetheless, the missing signal did not affect structural characterization of **dC-3**. Based on the information from the ESI-MS and NMR data, clearly the structure of **dC-3** was established as 4-chloromethyl-4-hydroxy-7-(2-deoxy- β -D-*erythro*-pentofuranosyl)-1,2,3,4-tetrahydr o-6-oxo-6*H*,7*H*-pyrimido[1,6-*a*]pyrimidin-5-ium (Scheme 3).

Characterization of dC-1 and dC-2

dC-1 and **dC-2** appeared as two separate peaks in the chromatogram of the reaction mixture (Figure 1). However, we failed to isolate pure **dC-1** or **dC-2**. The two compounds were fraction-collected separately on preparative HPLC several times, but every time each compound obtained after lyophilization was still a mixture of **dC-1** and **dC-2**, and their ratios [$\sim 24:76$ (**dC-1:dC-2**) by peak areas] virtually kept unchanged. Therefore, it appeared that there existed a rapid equilibrium between **dC-1** and **dC-2**. As a result, the two compounds were co-collected and the mixture was subjected to MS and NMR analyses.

The high-resolution ESI-mass spectrum of the **dC-1/dC-2** mixture exhibited a weak peak at m/z 314.1310 (Figure S2C), whose intensity was only 6.7% of the base peak at m/z 198.0856. The data were consistent with a molecular formula $C_{13}H_{20}N_3O_6$ (the calculated formula weight is 314.1352) and the base peak was the daughter ion of the molecular ion after loss of the deoxyribose moiety. Thus, **dC-1** and **dC-2** were expected to be products with CBO being added to dC and then the chlorine atom being replaced by a hydroxyl group.

The ^1H NMR, HMQC, and HMBC spectra of the **dC-1/dC-2** mixture in $DMSO-d_6$ were recorded. The ^1H NMR spectrum showed the presence of 20 protons (Figure S9). Just as **dC-4**, this number was the same as that in the molecular formula obtained from the ESI-MS data, indicating that **dC-1** and **dC-2** were quaternary ammonium salts. The chemical shifts of the protons in the pyrimidine ring and deoxyribose moiety were similar to those in **dC-4** (Table 1), and thus were easily identified. To identify the signals of the protons of the three CH_2 in the CBO moiety, their ^{13}C chemical shifts were determined by the HMQC spectrum (Figure S10). $C3''$ and $C4''$ were assigned because their chemical shifts changed little in comparison with the corresponding values in **dC-4** (Table 1). The remaining signals (^{13}C , 65.5 ppm; ^1H , 3.49 and 3.61 ppm, see Table 1) were thus those of $1''-CH_2$. The chemical shift of $C1''$ was significantly higher than that in **dC-4** (48.2 ppm), consistent with the chlorine atom at position $1''$ being replaced by a hydroxyl group.

The HMBC spectrum of **dC-1/dC-2** mixture (Figure S11) resembled that of **dC-4**. The chemical shifts of $C2$ and $C4$ were similarly determined as 146.6 and 155.0 ppm on the basis of the coupling information of $H5$ and $H6$. As **dC-4**, the signal at 4.25 ppm (one of the two $H4''$ atoms) coupled to the carbon atoms at 146.6 and 155.0 ppm, and that at 3.69 ppm (the other $H4''$ atom) exhibited a very weak coupling with the carbon atom at 146.6 ppm. The coupling information established the connection of $C4''$ with $N3$. Therefore, **dC-1** and **dC-2** had the same ring structure as **dC-4**. In fact, the only difference between **dC-1/dC-2** and **dC-4** was that the chlorine atom at position $1''$ being replaced by a hydroxyl group. Therefore, **dC-1** and **dC-2** were a pair of diastereomers that rapidly converted to each other. Their structures were determined as 2-hydroxy-2-hydroxymethyl-7-(2-deoxy- β -D-erythro-pentofuranosyl)-1,2,3,4-tetrahydro-6-oxo-6*H*,7*H*-pyrimido[1,6-*a*]pyrimidin-5-ium (Scheme 3).

Stabilities and Decomposition of the Isolated Reaction Products

At pH 7.4 and 37 °C, **dC-1** and **dC-2** were stable with no decomposition being observed within 6 h. However, **dC-3** and **dC-4** were found to be labile and undergo decomposition. Their half-lives were 3.0 ± 0.36 and 1.7 ± 0.06 h, respectively.

Incubation of **dC-3** at pH 7.4 and 37 °C led to the formation of a product eluted at 12.9 min with UV absorption at 214 and 280 nm. This product was not characterized further since **dC-3** itself was a relatively minor product in comparison with **dC-4**. On the other hand, decomposition of **dC-4** yielded two products, which were identified as **dC-1** and **dC-2** by

co-elution experiments. Thus, the decomposition of dC-4 occurred primarily by the replacement of the chlorine atom by a hydroxyl group (Scheme 3).

Acid Hydrolysis Products of dC-1, dC-2, and dC-4

To prepare standards that can be used as potential biomarkers of exposure to CBO (or its precursors CHB and BD), the acid hydrolysis products of the dC-CBO adducts were characterized.

Acid hydrolysis of the **dC-1/dC-2** mixture in 1 M HCl at 100 °C for 30 min gave several products with one product being predominant (data not shown). The predominant product, which should be the mixture of a pair of enantiomers and was designated as **dC-1H/dC-2H**, was eluted at 5.1 min and exhibited the UV maximal absorption at 214 and 280 nm (Figure S1F). **dC-1H/dC-2H** was expected to be the product of **dC-1/dC-2** after losing the deoxyribose moiety. The structures of **dC-1H/dC-2H** were confirmed through incubation of the deribosylated acid hydrolysis product of **dC-4** (**dC-4H**) at pH 7.4 and 37 °C (see below).

Acid hydrolysis of **dC-4** in 1 M HCl at 100 °C for 30 min yielded a major product eluted at 11.8 min with the UV absorption maxima at 214 and 281 nm, which was designated **dC-4H**. The high resolution ESI-mass spectrum of **dC-4H** exhibited a peak at m/z 216.0511 (which was the base peak, see Figure S2D). The isotope peak appeared at m/z 218.0480 with its intensity being approximately one third of the base peak, indicating that the product contained a chlorine atom. The result was consistent with the expectation that **dC-4H** was the product of **dC-4** after losing the deoxyribose moiety. After incubation at pH 7.4 and 37 °C for 24 h, **dC-4H** was completely converted to a product with the retention time at 5.1 min. This converted product was confirmed to be the same compound as **dC-1H/dC-2H** through the co-elution experiment. Therefore, acid hydrolysis of **dC-1**, **dC-2**, and **dC-4** occurred simply through cleavage of the glycosidic bonds and the loss of deoxyribose moieties to form the corresponding deribosylated adducts.

DISCUSSION

CBO, a known metabolite of CHB and potential metabolite of BD, is a bifunctional alkylating agent that exhibited strong cytotoxicity and genotoxicity in human liver cell culture. CBO produced alkali-labile sites on DNA and was capable of directly generating single-strand breaks on DNA as examined by the comet assay.¹¹ As the first-step toward characterizing the DNA adducts of CBO and their potential roles in CHB mutagenicity and genotoxicity, the present study focused on investigating the reactivity of CBO toward dC at different incubation conditions, characterizing the adducts produced at physiological conditions, and the stabilities, decomposition, and acid hydrolysis products of the adducts. Similar studies with other nucleosides and DNA are presently ongoing in our laboratories.

The results obtained from the time course (Figure 3) and stability studies provided evidence that **dC-3** and **dC-4** are direct products of the reaction of CBO and dC, whereas **dC-1** and **dC-2** are decomposition diastereomeric products of **dC-4**, resulting from a hydrolysis reaction to replace the chlorine atom by a hydroxyl group. This hydrolysis reaction was enhanced as the incubation pH was increased from pH 4.0 to pH 10.0. Because of the rapid formation of **dC-3** and **dC-4**, and their rapid decomposition resulting in the formation of **dC-1** and **dC-2** from **dC-4**, all of these adducts could be formed in vivo upon exposure to CBO, or its precursors CHB and BD.

For CBO, the initial reaction exclusively occurred at the vinyl end likely because of its higher reactivity in comparison with the chloromethyl or carbonyl moieties of CBO. For dC, the reaction predominantly occurred at N3, which was consistent with the reactivity of dC in

the reaction of dC with EB or acrolein.^{15,16} The CBO-dC initial products should have structures with the CBO moieties being open-chained (Scheme 2). However, such open-chained products were not observed, indicating that the ring-closing reactions to form **dC-3** and **dC-4** (Scheme 2) occurred very fast. Evidence for the formation of optical isomers in the ring-closing reactions was provided by the characterization of **dC-1/dC-2** mixture, i.e., when the carbonyl group in the CBO moiety was attacked by a nitrogen atom to form a fused ring, a chiral carbon atom was created. Two configurations of this chiral carbon atom (R and S) were expected to be formed and the obtained products should be a pair of diastereomers. Although **dC-1** and **dC-2** were the only pair of diastereomers identified, other identified products (**dC-3** and **dC-4**) were likely to be mixtures of diastereomers unresolved on HPLC. Evidence for this hypothesis is provided by the finding that **dC-4** decomposed to **dC-1** and **dC-2** at physiological conditions, suggesting that the isolated **dC-4** product was a pair of diastereomers unresolved on HPLC. The rapid equilibrium existing between **dC-1** and **dC-2** can be explained by an intermediate with an open-chained CBO moiety (Scheme 4). Similar equilibria between the ring-opened and ring-closed structures were reported for the acrolein-deoxyguanosine adducts.^{17,18}

dC has been reported to form adducts carrying *N*3,*N*4-fused rings with acrolein,¹⁶ vinyl chloride,^{19,20} and lipid peroxidation products, such as 4-oxo-2-nonenal.^{21,22} Similarly, dC could be a major target of DNA modification for lipid peroxidation products, such as 4-oxo-2-nonenal, which preferentially reacts with dC residues in double-stranded DNA.^{23,24} Furthermore, the fused-ring dC adducts were already detected in vivo, including in human saliva,²⁵ in the C57BL/6JAPC(min) mouse model of colorectal cancer,²⁴ in rats exposed to vinyl chloride,²⁰ and even in unexposed mice and rats.²⁶ These adducts in unexposed animals were probably produced from endogenously formed lipid peroxidation products. Thus, the fused-ring dC adducts can be used as specific biomarkers of endogenous lipid hydroperoxide-mediated DNA damage,²⁷ or biomarkers for oxidative DNA damage.²⁸ The levels of the fused-ring dC adducts in human urine had potential to be used as cancer risk biomarkers for thalassemic diseases.²⁹

Besides their use as biomarkers of toxicant exposure and disease state, the fused-ring dC adducts may be persistent and cause mutations. These adducts were highly persistent in rats exposed to vinyl chloride up to 14 days post-exposure.^{20,30} In another study, Regulus et al. found that the repair kinetic of these dC adducts in a human lymphocyte cell line was rather slow, with a half-life of 10 h.³¹ The fused-ring dC adducts were found to be potentially miscoding lesions,³² causing CG → AT transversions and CG → TA transitions.³³ They were mutagenic in bacteria and mammalian cells,^{34,35} and were genotoxic in *E. Coli* and human cell lines.²⁴ Additionally, the fused-ring dC adducts might be involved in the clastogenicity of tris(2,3-dibromopropyl)phosphate.³⁶ Like acrolein and 4-oxo-2-nonenal, CBO is also an α,β -unsaturated carbonyl compound and can form fused-ring dC adducts and thus might cause mutations in exposed animals and humans. Therefore, further studies are warranted to characterize the in vivo formation, persistence, and mutagenicity of the CBO-dC adducts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ADH	alcohol dehydrogenase
BD	1,3-butadiene
CBO	1-chloro-3-buten-2-one
CHB	1-chloro-2-hydroxy-3-butene
COSY	correlation spectroscopy
dC	2'-deoxycytidine
DEB	1,2,3,4-diepoxybutane
DMSO	dimethyl sulfoxide
dR	2-deoxyribose
EB	3,4-epoxy-1-butene
EBD	3,4-epoxy-1,2-butanediol
ESI	electrospray ionization
GSH	glutathione
HMBC	heteronuclear shift correlations via multiple bond connectivities
HMQC	heteronuclear multiple quantum coherence
HMVK	hydroxymethylvinyl ketone
MPO	myeloperoxidase
TFA	trifluoroacetic acid

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Figure 1.

A typical chromatogram of the reaction mixture of CBO and dC under in vitro physiological conditions (the molar ratio of CBO:dC was 5:1, the incubation time was 3 h, and the monitoring wavelength was 260 nm)

Figure 2.
Consumption of dC over time with the CBO:dC molar ratio of 2:1 at pH 7.4 and 37 °C
(monitoring wavelength: 260 nm)

Figure 3.
Dependence of the formation of the reaction products **dC-1**, **dC-2**, **dC-3**, and **dC-4** on the CBO:dC molar ratio and the incubation pH

Figure 4.

The chromatogram of the reaction mixture of CBO and dC at pH 4.0 and 37 °C (the molar ratio of CBO:dC was 5:1, the incubation time was 3 h, and the monitoring wavelength was 260 nm)

Scheme 1.

The Pathway of BD Metabolism via Myeloperoxidase (MPO) and the Formation of CHB and CBO (ADH, alcohol dehydrogenase)

Scheme 2.

The Speculated Mechanisms for the Formation of **dC-3** and **dC-4** (dR = 2'-deoxyribosyl)

Scheme 3.

The Structures of the CBO-dC Nucleoside Adducts Formed under in Vitro Physiological Conditions and the Decomposition and Acid Hydrolysis Products of the Major Reaction Product **dC-4**

Scheme 4.

The Equilibrium between **dC-1** and **dC-2** via an Open-Ring Carbonyl-Containing Intermediate

Table 1

Assignment of ^1H and ^{13}C NMR signals of **dC-1/dC-2**, **dC-3**, and **dC-4**^a **dC-1/dC-2** **dC-3** **dC-4**

	dC-1/dC-2		dC-3		dC-4	
	^1H	$^{13}\text{C}^b$	^1H	$^{13}\text{C}^b$	^1H	$^{13}\text{C}^b$
H2		146.6				146.5
H4		155.0				155.0
H5	6.25 (d, 1H, $J = 8$ Hz)	93.6	6.24 (d, 1H, $J = 8$ Hz)	95.9	6.26 (d, 1H, $J = 8$ Hz)	93.4
H6	8.23 (d, 1H, $J = 8$ Hz)	141.3	8.13 (d, 1H, $J = 8$ Hz)	139.9	8.29 (d, 1H, $J = 8$ Hz)	142.1
H1'	6.08 (t, 1H, $J = 6$ Hz)	86.6	6.05 (t, 1H, $J = 6$ Hz)	86.9	6.08 (t, 1H, $J = 6$ Hz)	86.8
H2'	2.26 (m, 1H)	40.1	2.25 (m, 1H)	39.6	2.28 (m, 1H)	40.1
	2.16 (m, 1H)		2.11 (m, 1H)		2.17 (m, 1H)	
H3'	4.22 (m, 1H)	69.2	4.22 (m, 1H)	69.4	4.22 (m, 1H)	69.4
H4'	3.87 (m, 1H)	87.9	3.87 (m, 1H)	87.9	3.88 (1H) ^f	88.1
H5'	3.60 (m, 2H)	60.4	3.60 (m, 2H)	60.5	3.61 (m, 2H)	60.3
H1''	3.61 (m, 1H) 3.49 (m, 1H)	65.5	--- ^c	--- ^d	3.88 (2H) ^f	48.2
H2''		79.1				78.3
H3''	2.03 (m, 1H) 1.95 (m, 1H)	26.4	2.97 (m, 2H)	41.7	2.14 (m, 2H)	26.8
H4''	4.25 (m, 1H) 3.69 (m, 1H)	37.5	3.89 (m, 2H)	39.4	4.20 (m, 1H) 3.74 (m, 1H)	37.6
NH	10.78 (br s, 1H)		10.75 (s, 1H)		10.97 (s, 1H)	
2''-OH	6.66 (br s, 1H)		7.22 (s, 1H) ^e		7.30 (s, 1H)	
OH	5.34 (br s, 3H)		5.14 (br s, 1H) 5.35 (br s, 1H)		5.16 (br s, 1H) 5.36 (br s, 1H)	

^a See Scheme 3 for the numbering of the atoms.

^b The data of the chemical shifts of carbons except C2, C4, and C2'' were obtained from the HMQC spectra. The values of C2, C4, and C2'' were obtained from the HMBC spectra. The chemical shifts of C2, C4, and C2'' in **dC-3** were not available because the HMBC spectrum of **dC-3** was not recorded.

^c The signal was probably masked by the strong water peak and thus was unable to be identified.

^d The cross-peak was not observed in the HMQC spectrum due to poor signal-to-noise ratio of the spectrum. As a result, the ^{13}C chemical shift of the atom was not given.

^eThe assignment was tentative because two similar peaks, which might come from impurities, appeared at 7.02 and 7.12 ppm.

^fThe multiplicities of the signals of H4' and H1'' were unclear due to overlapping of their signals.