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Chemical Modification of Deoxycytidine at Different Sites Yields Adducts of Different Stabilities: Characterization of N^3- and O^2-Deoxycytidine and N^3-Deoxyuridine Adducts of Butadiene Monoxide[1]

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Eight adducts were characterized from the reaction of deoxycytidine with the chemical carcinogen, butadiene monoxide (BM). They were identified as diastereomeric pairs of N^3-(2-hydroxy-3-buten-1-yl)deoxycytidine, N^3-(2-hydroxy-3-buten-1-yl)deoxyuridine, N^3-(1-hydroxy-3-buten-2-yl)deoxyuridine, and O^2-(2-hydroxy-3-buten-1-yl)deoxycytidine based on UV spectra, ^{1}H NMR, FAB/MS, and stability studies. The N^3-(2-hydroxy-3-buten-1-yl)deoxycytidine adducts were unstable at pH 7.4, 37°C, and deaminated to the corresponding N^3-deoxyuridine adducts with half-lives of 2.3 and 2.5 h. The N^3-(1-hydroxy-3-buten-2-yl)deoxycytidine diastereomers were not detected, apparently because of faster rates of deamination compared to theN^3-(2-hydroxy-3-buten-1-yl)deoxycytidine adducts. The corresponding four N^3-deoxyuridine adducts were stable for up to 168 h. The O^2-deoxycytidine adducts were unstable and decomposed with a half-life of 11 h. The N^3-(2-hydroxy-3-buten-1-yl)deoxycytidine adducts were initially the major adducts formed upon reaction of deoxycytidine with BM at 37°C in phosphate buffer (pH 7.4), but the corresponding N^3-deoxyuridine adducts showed a lag in formation due to the time needed for deamination. The N^3-(1-hydroxy-3-buten-2-yl)deoxyuridine and O^2-deoxycytidine adducts had linear formation rates, but were formed to a lesser extent. Heating the reaction mixture at 80°C for 1 h converted all N^3-deoxycytidine adducts to the stable N^3-deoxyuridine adducts. Incubation of deoxycytidine with an excess of BM at pH 7.4, 37°C, followed by the extraction and heating steps allowed calculation of the pseudo-first-order kinetic rate constants for the four uridine adducts. If the heating step was eliminated, then the pseudo-first-order kinetic rate constants could be calculated for the N^3-(2-hydroxy-3-buten-1-yl)deoxycytidine and O^2-(2-hydroxy-3-buten-1-yl)deoxycytidine adducts. The rate constants for N^3-(2-hydroxy-3-buten1-yl)deoxycytidine and the corresponding N^3-(2-hydroxy-3-buten-1-yl)deoxyuridine were five- to sixfold the rate constants for the N^3-(1-hydroxy-3-buten-2-yl)deoxyuridine and O^2-(2-hydroxy-3-buten-1-yl)deoxycytidine adducts. Thus, the results show that the reaction of deoxycytidine with BM yields adducts at different sites with different rates of formation and stabilities. Understanding the chemical interactions of deoxycytidine with BM and the stability of the various adducts may contribute to a better understanding of the molecular mechanisms of mutagenesis and carcinogenesis of BM and the development of useful biomarkers of exposure.

Key Words: **deoxycytidine adducts; deoxyuridine adducts; 1,3-butadiene; butadiene monoxide; DNA adducts; hydrolytic deamination; chemical carcinogenesis.**

Interactions of alkylating species with the purine and pyrimidine bases of DNA result in the production of a variety of adducts which are characteristic of the alkylating agent. These adducts have been impli-

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cated in the initiation of mutagenic and carcinogenic events (reviewed in 1, 2). While the majority of genotoxic agents react most readily with guanine residues, some extensively target one or more of the other bases (3–6).

BM,[3] a known mutagen (7) and carcinogen in mouse skin painting studies (8), has been shown to induce mutations at both GC and AT base pairs (7), suggesting that any of the four nucleoside bases of DNA may be involved in BM-induced mutation and carcinogenesis. We have previously identified eight adducts formed in the reaction of guanosine with BM (9). These adducts were characterized as diastereomeric N^1-(1-hydroxy-3-buten-2-yl)guanosine, N^2-(1-hydroxy-3-buten-2-yl)-guanosine, N^7-(1-hydroxy-3-buten-2-yl)guanosine, and N^7-(2-hydroxy-3-buten-1-yl)guanosine. While the N^1- and N^2-guanosine adducts were formed 10-fold less favorably than the N^7-guanosine adducts, they were stable for up to 1 week while the N^7 adducts had half-lives ranging from 48 to 96 h. Ten adducts formed in the reaction between adenosine with BM were also characterized (10). These adducts were characterized as diastereomeric pairs of N^1-(1-hydroxy-3-buten-2-yl)adenosine, N^1-(2-hydroxy-3-buten-1-yl)adenosine, N^6-(1-hydroxy-3-buten-2-yl)adenosine, N^6-(2-hydroxy-3-buten-1-yl)adenosine, and N^1-(1-hydroxy-3-buten-2-yl)inosine. The N^6-adenosine adducts were formed from the N^1-adenosine adducts through the Dimroth rearrangement. In a competing reaction, the N^1-(1-hydroxy-3-buten-2-yl)adenosine adducts also deaminated to the N^1-inosine diastereomers. The combined N^6-adenosine adducts were formed 3-fold more favorably than the N^1-inosine adducts, while the individual N^6-(2-hydroxy-3-buten-1-yl)adenosine diastereomers were formed only 2-fold less favorably than the individual N^7-guanosine adducts.

Cytosine contains multiple nucleophilic sites which may react with alkylating agents. In addition, cytosine is susceptible to deamination into uridine, a base not naturally found in DNA, which has base pairing properties different from cytosine. This deamination reaction appears to be enhanced by alkylation of deoxycytidine at the N^3 position by several aliphatic epoxides (11–15). Propylene oxide forms an N^3-deoxyuridine adduct (11) and propylene oxide-induced DNA adducts cause both transition and transversion mutations at cytosine residues when transfected into *Escherichia coli* on single-stranded M13 DNA (16). Ethylene oxide also forms an N^3-deoxyuridine adduct and, in a DNA template containing a single N^3-(2-hydroxyethyl)deoxyuridine, the adduct blocked DNA replication by the Klenow fragment of *E. coli* and bacteriophage T7 polymerase *in vitro* (17). In the absence of proofreading functions, the lesion could be bypassed and both A and T were incorporated opposite the adduct, resulting in GC → AT and GC → TA mutagenesis. Understanding the factors involved in these alkylation and deamination reactions is important for understanding of the DNA interactions of chemical carcinogens.

In this study we report the characterization of the adducts formed by the reaction between deoxycytidine and racemic BM. We find that alkylation of deoxycytidine at the N^3 position results in deamination of deoxycytidine to deoxyuridine. The pseudo-first-order kinetic rate constants of formation, stability, and effect of BM concentration on adduct formation were examined. Preliminary reports of these results have been presented (18).

MATERIALS AND METHODS

Materials. Racemic BM, deuterium oxide, D_6-Me_2SO, and trifluoroacetic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). 2′-Deoxycytidine and 2′-deoxyuridine were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade ACN was purchased from EM Science (Gibbstown, NJ). NMR supplies were obtained from Wilmad Glass Co. (Buena, NJ). All other chemicals were of the highest grade commercially available. *Caution: BM is a known mutagen and carcinogen in laboratory animals and should be handled using proper safety measures.*

Chemical synthesis of O^2- and N^3-deoxycytidine and N^3-deoxyuridine adducts of BM. Deoxycytidine (45 mg; 0.20 mmol) was dissolved in 3 ml of either H_2O (C-1, C-2, C-4, C-5, C-6, and C-7) or glacial acetic acid (C-3). All solutions were sonicated in a 4-ml vial with a screw-top Teflon-coated cap until dissolved. An excess of BM (0.16 ml; 2.0 mmol) was added and the reactions were heated at 50°C in a Dubnoff shaking water bath for 3 h (C-1, C-2, and C-3) or 24 h (C-4, C-5, C-6, and C-7). After removing reaction mixtures from the water bath, they were cooled to room temperature and extracted four times with 4 vol ethyl ether to remove unreacted BM. Reaction of deoxycytidine with BM in H_2O for 3 h produced two major peaks (C-1 and C-2), in addition to starting material, separated by HPLC. Extension of this reaction to 24 h resulted in four later-eluting peaks by HPLC separation (C-4, C-5, C-6, and C-7). Reaction of deoxycytidine with BM in glacial acetic acid for 3 h produced a major peak corresponding to C-3, as determined by UV spectra and retention time.

HPLC analysis and purification of deoxycytidine adducts of BM. HPLC separations and purification were performed as previously described for the guanosine and adenosine adducts of BM (9, 10). Briefly, analytical separations were performed with a 20-μl injection volume on a Beckman Ultrasphere 5 μm ODS reverse-phase analytical column (250 × 4.6 mm i.d.) using a gradient controlled HPLC system equipped with a diode array detector and UV detection at 260 and 280 nm. HPLC analysis involved use of a linear gradient program starting at 8 min from 10 to 100% pump B over 5 min [pump A 1% ACN (pH 2.5); pump B 10% ACN (pH 2.5)], at a flow rate of 1 ml/min. Semipreparative HPLC was performed using an isocratic solvent system pumping 75% B using a 100-μl injection volume on a Beckman Ultrasphere 5-μm ODS reverse-phase semipreparative column (250 × 10 mm i.d.) and a flow rate of 3 ml/min. Separation of synthetic mixtures revealed a total of seven adducts from the

[3] Abbreviations used: BM, butadiene monoxide; ACN, acetonitrile; FAB/MS, fast atom bombardment–mass spectrometry; C-1 and C-2, diastereomeric N^3-(2-hydroxy-3-buten-1-yl)deoxycytidine; C-3, diastereomeric O^2-(2-hydroxy-3-buten-1-yl)deoxycytidine; C-4 and C-5, diastereomeric N^3-(2-hydroxy-3-buten-1-yl)deoxyuridine; C-6 and C-7, diastereomeric N^3-(1-hydroxy-3-buten-2-yl)deoxyuridine.

reactions of deoxycytidine with BM whose retention times on a typical analytical chromatogram were 18.5, 19.3, 21.1, 23.2, 23.5, 24.2, and 25.0 min. These peaks were named C-1, C-2, C-3, C-4, C-5, C-6, and C-7 in order of their retention times, respectively. All adducts were fractionated two to four times by semipreparative HPLC until they were isomerically pure by analytical HPLC. The purified adducts were then used to obtain standard curves (r were greater than 0.99). The limits of detection using the analytical HPLC method were 0.5 μg/ml for N^3-deoxycytidine and N^3-deoxyuridine adducts and 1 μg/ml for O^2-deoxycytidine adducts.

Identification of BM–deoxycytidine adducts. UV spectra at pH 2.5 were performed using a Beckman diode array detector. Positive ion FAB/MS were performed using a Kratos MS-50 ultrahigh-resolution mass spectrometer as described previously (10). Spectra were matrix subtracted if the matrix was interfering. Proton NMR spectra were obtained for each adduct in D_2O, and in $[D_6]Me_2SO$ for confirmation of the structure of selected adducts. Homonuclear decoupling experiments were carried out on several samples to confirm proton assignments. Chemical shifts are reported in ppm with the H_2O peak as an internal standard. Circular dichroism spectra were recorded at 25°C on an Aviv circular dichroism spectrometer, Model 62A DS, using a 1-cm pathlength and an adduct concentration of 3×10^{-5} M.

Deoxyuridine (10 mM) was reacted with BM (750 mM) in phosphate buffer (pH 7.4) at 37°C for 4 h in order to compare UV spectra and retention times of the products to products identified as BM–deoxyuridine adducts from the reaction of deoxycytidine with BM.

To characterize the stability of the adducts, mixtures of all seven adducts (prepared by lyophilization of reaction mixtures) were dissolved in 2 ml of 0.1 N HCl, 0.1 N NaOH, or water and stabilities were monitored at 24°C by HPLC analysis. Aliquots were withdrawn at intervals, pH was adjusted to neutrality with 1 N HCl or 1 N NaOH, and they were immediately analyzed by HPLC for the disappearance of the parent adduct peaks.

Adduct stability at 37°C in phosphate buffer (pH 7.4) was examined with purified adduct (0.3 mM). Samples were withdrawn at timed intervals and analyzed by HPLC for disappearance of the parent adduct peak.

The reaction of BM with deoxycytidine under physiologic conditions. Adduct formation under *in vitro* physiologic conditions (37°C, pH 7.4) was investigated. Deoxycytidine (3.1 or 9.6 mM) and BM (750 mM) were reacted in 100 mM phosphate buffer (pH 7.4) containing 100 mM potassium chloride in a shaking water bath at 37°C for 4 h to calculate a pseudo-first-order rate constant. Aliquots were withdrawn at intervals, extracted with ethyl ether to remove unreacted BM, and either incubated at 80°C for 1 h to convert the unstable N^3-deoxycytidine adducts to more stable N^3-deoxyuridine adducts (C-4 and C-5) or not incubated (C-1, C-2, C-3, C-6, and C-7). In the case where the unstable adducts were analyzed, the samples were stored at 0°C until immediately before analysis by HPLC. The storage at 0°C was found to prevent the decomposition and deamination of the unstable adducts. Analysis for adduct formation was performed by HPLC as described above.

To investigate the effect of BM concentration on adduct formation, deoxycytidine (10 mM) and BM (10, 25, 50, and 500 mM) were reacted in phosphate buffer (pH 7.4) at 37°C. Reactions were stopped at 1 h by extraction with ethyl ether and incubated at 80°C for 60 min to allow conversion of unstable N^3-deoxycytidine adducts to stable N^3-deoxyuridine adducts. The samples were analyzed for the formation of the deoxyuridine adducts by HPLC.

RESULTS AND DISCUSSION

Identification of BM–deoxycytidine and BM–deoxyuridine adducts. Reaction of deoxycytidine with BM under *in vitro* physiologic conditions for 4 h resulted in the formation of seven product peaks as detected by HPLC. These products were designated C-1, C-2, C-3, C-4, C-5, C-6, and C-7 in order of their retention times (Fig. 1). All seven adducts were characterized by comparisons of the retention times and UV spectra to adducts prepared under synthetic conditions, which were also characterized by analysis of their ^{1}H NMR and FAB/MS spectra. Protons were assigned to NMR signals by comparing chemical shifts, multiplicities, integration ratios, and the results of homonuclear decoupling experiments with the published spectra of the characterized BM–guanosine and BM–adenosine adducts (9, 10). Spectra of cytidine (19), 2′-deoxycytidine, and 3-buten-1,2-diol were also used as references (data not shown). Products C-1 and C-2, C-4 and C-5, and C-6 and C-7 have nearly identical ^{1}H NMR spectra, respectively, suggesting they are diastereomeric pairs. Spectra representing each regioisomer are shown in Fig. 2 and chemical shifts and J values for all adducts are given in Table I.

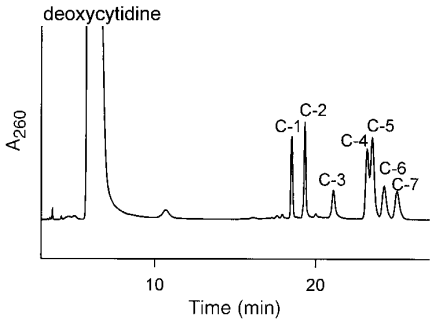

FIG. 1. HPLC chromatogram of the products formed by the reaction of racemic BM with deoxycytidine. Peaks C-1 and C-2 are diastereomeric pairs of N^3-(2-hydroxy-3-buten-1-yl)deoxycytidine, C-3 is a diastereomeric pair of O^2-(2-hydroxy-3-buten-1-yl)deoxycytidine, C-4 and C-5 are a diastereomeric pair of N^3-(2-hydroxy-3-buten-1-yl)-deoxyuridine, and C-6 and C-7 are diastereomers of N^3-(1-hydroxy-3-buten-2-yl)deoxyuridine. The peak eluting at 10.7 min was also present in the deoxycytidine control.

C-4 and C-5 were determined to be diastereomers of N^3-(2-hydroxy-3-buten-1-yl)deoxyuridine based on the following data. With a maxima of 263 nm (Fig. 3A), the UV spectra of C-4 and C-5 were consistent with the spectral shape and maxima of other N^3-substituted uridine adducts (20). NMR spectra of C-5 performed in $[D_6]Me_2SO$ showed loss of the N^4 proton between 6 and 7 ppm (data not shown) as previously reported for other N^3-substituted deoxycytidines (21). The BM moiety was determined to be attached to the N^3 of deoxyuridine on the terminal carbon based on the shift of H^7 (Figs. 2 and 4). H^7 was shifted farther upfield in C-4 and C-5 (4.46 and 4.45 ppm) than in their regioisomers C-6 and C-7 (5.55 and 5.55 ppm), suggesting it is adjacent to the hydroxyl group rather than the more deshielding N^3 of deoxyuridine. We have seen similar deshielding effects of nitrogen atoms versus hydroxyl moieties in

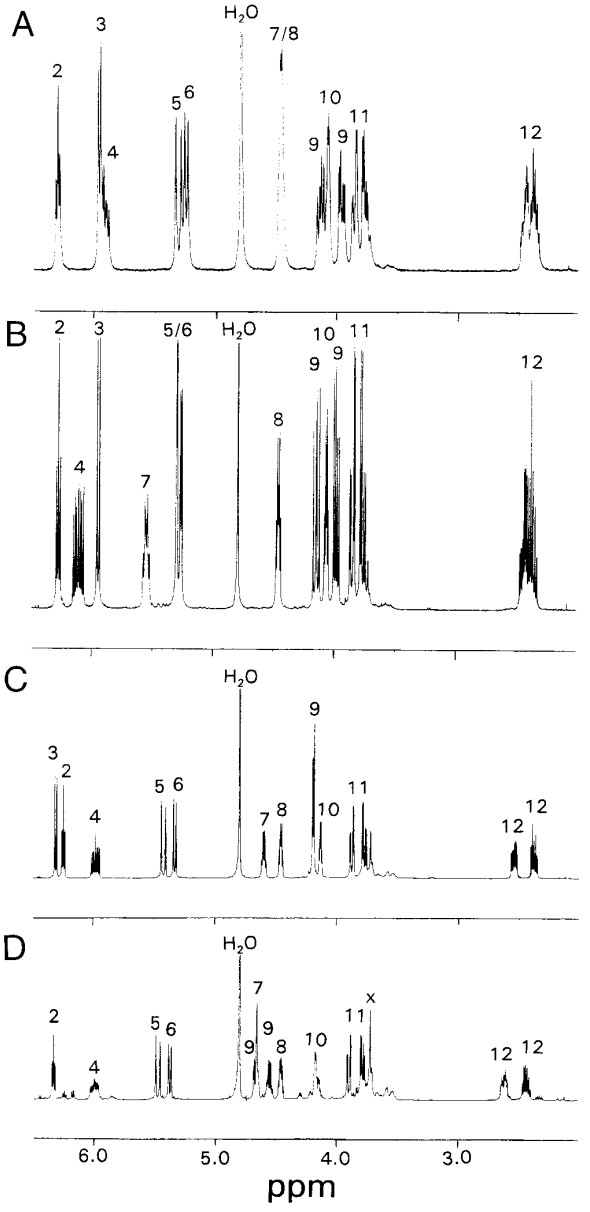

FIG. 2. ^{1}H NMR for (A) N^3-(2-hydroxy-3-buten-1-yl)deoxyuridine, C-5, (B) N^3-(1-hydroxy-3-buten-2-yl)deoxyuridine, C-7, (C) N^3-(2-hydroxy-3-buten-1-yl)deoxycytidine, C-1, and (D) O^2-(2-hydroxy-3-buten-2-yl)deoxycytidine, C-3. Peak numbers correspond to assignments in Fig. 4.

the adducts we have previously characterized with guanosine and adenosine (9, 10). Small differences in the shifts of $H^{9'}$s when attached to the hydroxyl group or the nitrogen are also consistent with what we have observed with the guanosine and adenosine adducts, and with what others have observed with styrene oxide adducts (22). Specific assignment of protons from the NMR spectra in D_2O was confirmed by homonuclear decoupling experiments. Saturation of H^1 affected the

TABLE I

500 MHz ^{1}H NMR Data for the Deoxycytidine and Deoxyuridine Adducts of BM in D_2O (J Values Reported in Hz, Chemical Shifts Reported in ppm)

	$H^{1\,a}$	H^2	H^3	H^4	H^5	H^6	H^7	H^8	H^9	H^{10}	H^{11}	H^{12}
C-1	8.12	6.25	6.31	5.98	5.42	5.33	4.60	4.46	4.19	4.13	3.87/3.77[c]	2.55/2.38[c]
	J = 7.5	J = 6.5, 6.5	J = 8.0	J = 17.0, 10.5, 6.5	J = 17.0	J = 10.5	J = 6.5, 6.0	J = 6.0, 4.5	J = NA[b]	J = 4.5, 4.0	J = 12.5, 3.25/5.0[d]	J = 14.0, 6.5, 4.75/6.5[d]
C-2	8.12	6.25	6.31	5.98	5.42	5.33	4.60	4.46	4.19	4.13	3.88/3.78[c]	2.55/2.38[c]
	J = 8.0	J = 6.5, 6.5	J = 8.0	J = 17.0, 10.5, 6.5	J = 17.0	J = 10.5	J = 6.25, 6.0	J = 6.5, 4.5	J = NA	J = 4.0, 4.0	J = 12.5, 3.25/5.5[d]	J = 14.0, 6.25, 4.5/6.0[d]
C-3	8.26	6.33	6.54	5.99	5.47	5.37	4.66	4.46	4.66/4.55[c]	4.17	3.89/3.78[c]	2.62/2.43[d]
	J = 7.5	J = 6.5, 6.0	J = 8.0	J = NA	J = 17.5	J = 10.5	J = NA	J = 6.5, 4.5	J = NA	J = 4.0, NA	J = 12.75, 3.5/5.0[d]	J = 13.5, 6.5/NA,[d] 6.5/NA[d]
C-4	7.86	6.30	5.96	5.92	5.31	5.25	4.46	4.46	4.14/3.95[c]	4.07	3.85/3.77[c]	2.46/2.37[c]
	J = 8.0	J = 6.8, 6.4	J = 8.0	J = 17.2, 10.8, 6.4	J = 17.2	J = 10.4	J = NA	J = NA	J = 13.6, 8.0/5.2[d]	J = 5.2, 3.6	J = 12.4, 3.6/5.2[d]	J = 14.2, 6.4/6.8,[c] 4.4/6.8[d]
C-5	7.86	6.30	5.96	5.92	5.31	5.25	4.45	4.45	4.14/3.96[c]	4.08	3.86/3.77[c]	2.45/2.37[c]
	J = 8.0	J = 6.8, 6.4	J = 7.6	J = 17.2, 10.4, 6.4	J = 17.2	J = 10.4	J = NA	J = NA	J = 13.1, 8.0/5.8[d]	J = 3.6, NA	J = 12.1, 3.2/5.4[d]	J = 13.6, NA/6.8,[d] NA/6.8[d]
C-6	7.86	6.28	5.95	6.11	5.28	5.29	5.55	4.46	4.15/3.98[c]	4.07	3.85/3.77[c]	2.44/2.36[c]
	J = 8.0	J = 6.8, 6.4	J = 8.0	J = 16.4, 10.8, 6.1	J = 16.4	J = 12.4	J = 7.6, NA	J = 6.4, 4.4	J = 11.2, 8.8/5.6[d]	J = 4.8, 3.8	J = 12.4, 3.6/5.2[d]	J = 14.2, 6.4/6.8,[d] 4.4/6.8[d]
C-7	7.85	6.28	5.95	6.11	5.28	5.29	5.55	4.46	4.15/3.98[c]	4.07	3.85/3.77[c]	2.44/2.37[c]
	J = 8.0	J = 6.8, 6.4	J = 8.0	J = 17.6, 10.4, 6.0	J = 18.0	J = 11.2	J = 8.4, NA	J = 6.4, 4.2	J = 11.5, 8.8/5.8[d]	J = 4.8, 4.0	J = 12.4, 3.6/5.2[d]	J = 14.0, 6.4/7.0,[d] 4.4/6.8[d]

[a] Proton numbering refers to assignments given in Fig. 4. Multiplicities can be found on the spectra, Fig. 2.
[b] NA = J values not available from spectra.
[c] Two chemical shifts are given corresponding to the two protons of the methylene group.
[d] Two J values are given corresponding to the two protons of the methylene group.

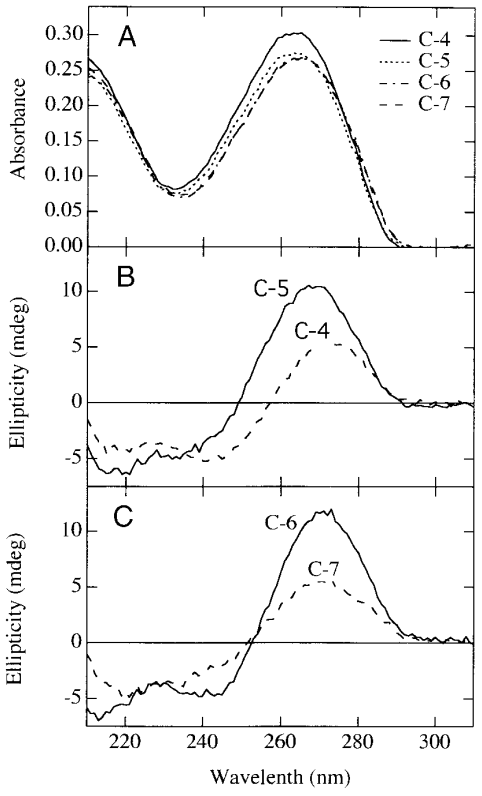

FIG. 3. UV spectra of the purified BM–deoxyuridine adducts have a UV maxima at 263 nm (A). CD spectra of the purified diastereomers of N^3-(2-hydroxy-3-buten-1-yl)deoxyuridine (C-4 and C-5, B) and N^3-(1-hydroxy-3-buten-2-yl)deoxyuridine (C-6 and C-7, C). Ellipticities were reported as ellipticity divided by absorbance at 263 nm, making them independent of concentration.

signal of H^3, while saturation of H^7 and H^8 affected H^4, H^9, H^{10}, and H^{12}.

The FAB/MS fragmentation patterns of C-4 and C-5 were very similar and are consistent with the proposed structure (Fig. 4). The expected molecular ion of *m/z* 299 (M + 1) was present in both spectra (Fig. 5A), as was the *m/z* 183 fragment which represents the typical nucleoside fragmentation to bH_2, loss of the sugar peak. Also present were the fragments *m/z* 165, corresponding to loss of H_2O from bH_2, and the sugar peak, *m/z* 117.

C-6 and C-7 were determined to be diastereomers of N^3-(1-hydroxy-3-buten-2-yl)deoxyuridine based on the following data. With a maxima of 263 nm (Fig. 3A), the UV spectra of C-6 and C-7 are consistent with the spectral shape and maxima of the C-4 and C-5 regioisomers, and other N^3-substituted uridine adducts (20). NMR of both diastereoisomers suggested that the BM moiety is attached to the N^3 of deoxyuridine at the carbon adjacent to the double bond based on the shift of H^7. As stated above, H^7 was shifted farther downfield in C-6 and C-7 (5.55 and 5.55 ppm) than in the regioisomers C-4 and C-5 (4.46 and 4.45 ppm), suggesting that it is adjacent to the deshielding N^3 of deoxyuridine. Specific assignment of protons from the NMR spectra was confirmed by homonuclear decoupling experiments. Saturation of the signal of H^7 affected the signals assigned to H^4 and H^9, saturation of H^{10} affected H^8 and H^{11}, and saturation of H^{12} affected H^2 and H^8.

The FAB/MS fragmentation patterns of C-6 and C-7 (Fig. 5B) were again very similar and are consistent with the proposed structure (Fig. 4). The expected molecular ion of *m/z* 299 (M + 1) was present in both spectra, as was, again, the 183 fragment representing the loss of the sugar. In addition, there was a small fragment at *m/z* 165, and the sugar peak at *m/z* 117. Loss of the entire BM side chain from bH_2 with H transfer back to the ring gave *m/z* 113.

The CD spectra of the N^3-deoxyuridine adducts (Figs. 3B and 3C) exhibit similar overall shapes, with the diastereomeric pairs demonstrating significant differences in magnitude and shape at certain wavelengths. These results suggest that the CD spectra of the adducts were dominated by the uridine ring chromophore rather than on the BM moiety containing the chiral center of interest. However, these differences are consistent with diastereomeric separation.

Further evidence for the characterization of C-4, C-5, C-6, and C-7 as deoxyuridine adducts was obtained when four similar peaks with identical retention times and UV spectra were obtained (data not shown) from the reaction of BM with deoxyuridine at 37°C in buffer (pH 7.4).

C-1 and C-2 were determined to be diastereomers of N^3-(2-hydroxy-3-buten-1-yl)deoxycytidine based on the following results. With a maxima of 282 nm, the UV spectra of C-1 and C-2 are consistent with the spectral shape and maxima of other N^3-substituted cytidine adducts (20), and distinct from the N^3-deoxyuridine adducts described above. When incubated at room temperature or higher, purified adducts C-1 and C-2 were unstable and converted to peaks with retention times and UV spectra consistent with peaks C-4 and C-5, respectively. This suggested C-1 and C-2 are N^3-deoxycytidine adducts that undergo hydrolytic deamination to N^3-deoxyuridine adducts. Previous studies have also found that N^3-deoxycytidine adducts readily deaminate to N^3-deoxyuridine adducts (11–15). NMR spectra performed in D_2O suggested that the BM moiety is attached to the N^3 of deoxycytidine at the terminal carbon based upon the shift of H^7 (Fig. 2, Table I), and this assignment was confirmed by the deamination to C-4 and C-5, the N^3-deoxyuridine adducts attached at the terminal carbon. The shift of H^7 was similar in C-1, C-2, C-4, and C-5 (4.60, 4.60, 4.46, and 4.45 ppm, respectively), while H^7 was shifted farther upfield in C-6 and C-7 (5.55 and 5.55 ppm, respectively). This suggested that in C-1 and C-2, H^7 is also adjacent to

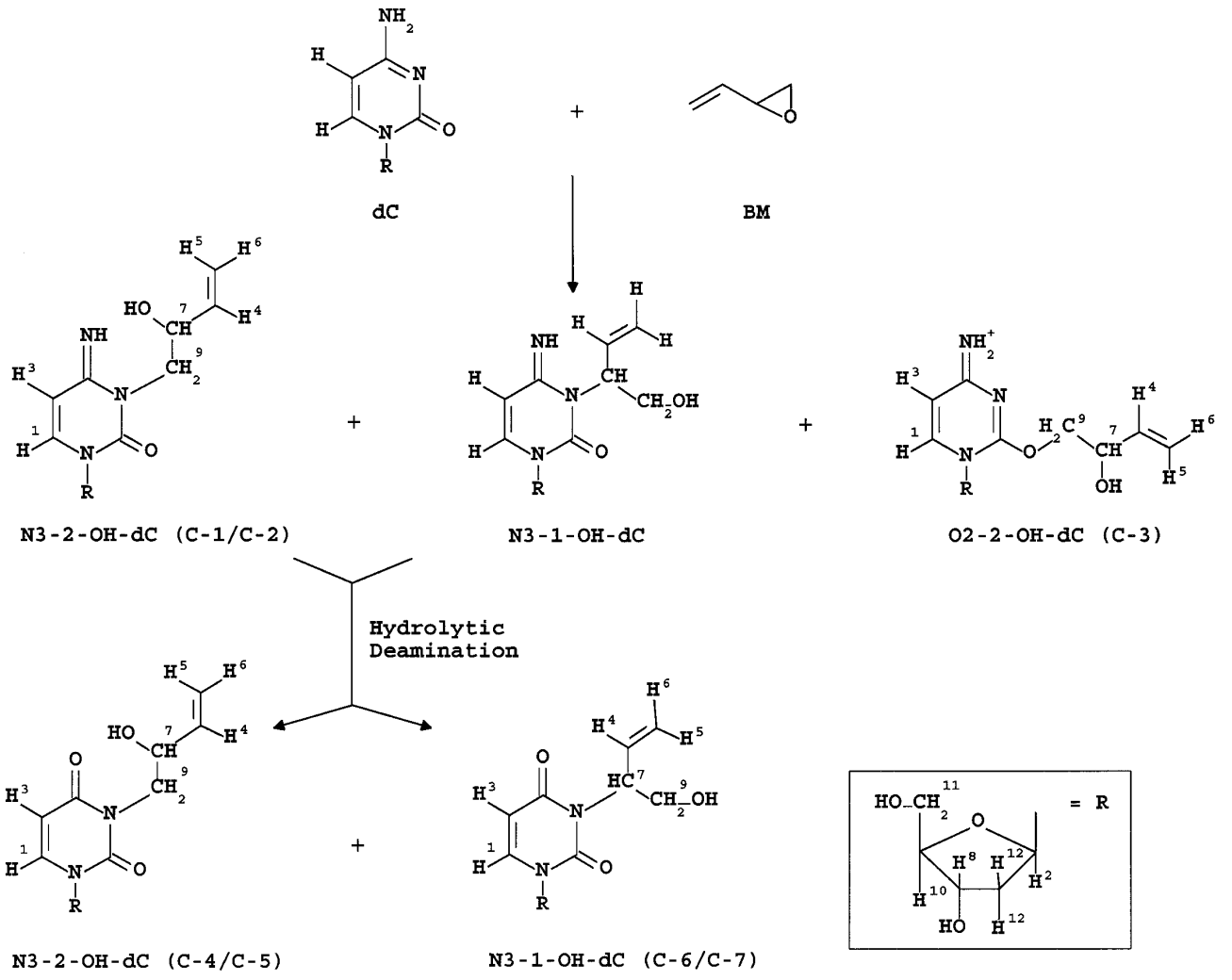

FIG. 4. BM reacts with deoxycytidine to form one O^2-deoxycytidine regioisomer and two N^3-deoxycytidine regioisomers which both undergo hydrolytic deamination to form the corresponding N^3-deoxyuridine regioisomers. Proton numbering is based on the chemical shifts of the first ^{1}H NMR spectrum solved (C-7).

the hydroxyl group rather than the more deshielding N^3 of deoxycytidine. Specific assignment of protons from the NMR spectra in D_2O was confirmed by homonuclear decoupling experiments. Saturation of the H^9 signal affected the signal for H^7; saturation of H^4 affected H^5, H^6, and H^7; saturation of H^7 affected H^4 and H^9; saturation of H^8 affected H^{10} and H^{12}; and saturation of H^2 affected H^{12}.

The FAB/MS fragmentation patterns of C-1 and C-2 were similar and are consistent with the proposed structure (Fig. 4). The expected molecular ion of *m/z* 298 (M + 1) was present in both spectra (Fig. 5C) and is suggestive of a deoxycytidine adduct compared to the molecular ion of *m/z* 299 for the deoxyuridine adducts, since the exocyclic NH had been converted to an oxygen. The *m/z* 182 fragment represents the typical nucleoside fragmentation to bH_2, loss of the sugar peak. Loss of the BM moiety from bH_2 is observed at *m/z* 112, while loss of H_2O from bH_2 is *m/z* 164.

C-3 was determined to be an unseparable pair of diastereomers of O^2-(2-hydroxy-3-buten-1-yl)deoxycytidine. Several attempts to separate these distereomers were unsuccessful. With a maxima of 235 and 266 nm, the UV spectra of C-3 was consistent with the unique spectral shape and maxima of other O^2-substituted cytidine adducts (20). ^{1}H NMR of C-3 performed in both D_2O (Fig. 2D) and $[D_6]Me_2SO$ (not shown) suggested the presence of two diastereomers by the increased splitting patterns of several signals (H^4, H^8, H^{10}, and H^{12}). NMR of C-3 performed in $[D_6]Me_2SO$ showed the presence of two singlets at 8.96 and 8.93 ppm which integrated as two protons, suggesting the diprotonation of the N^4 of deoxycytidine. The chemical shift of these N^4 protons is shifted significantly downfield of the re-

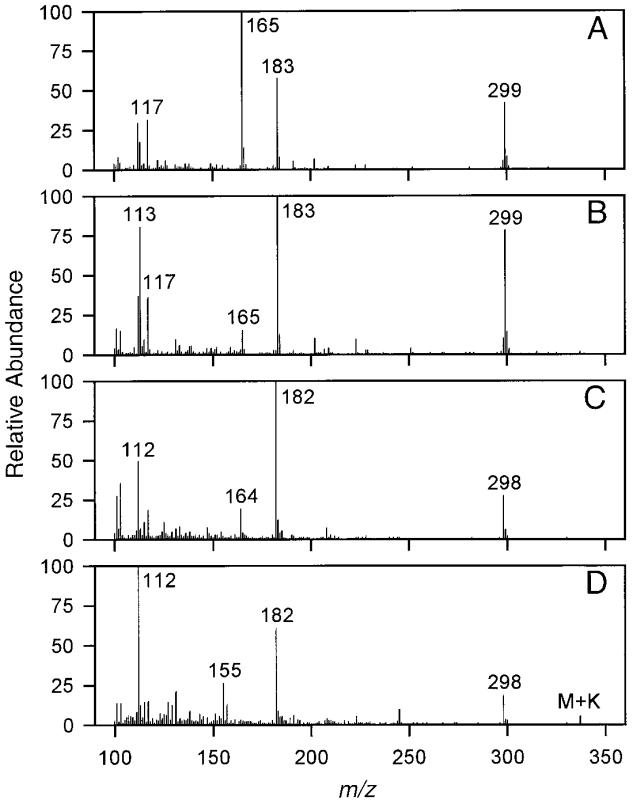

FIG. 5. FAB/MS of (A) N^3-(2-hydroxy-3-buten-1-yl)deoxyuridine, C-4, (B) N^3-(1-hydroxy-3-buten-2-yl)deoxyuridine, C-6, (C) N^3-(2-hydroxy-3-buten-1-yl)deoxycytidine, C-1, (D) O^2-(2-hydroxy-3-buten-1-yl)deoxycytidine, C-3.

ported values of other O^2-substituted cytidines having only a single N^4 proton (5–7.5 ppm, 21) and may be due to the positive charge on the nitrogen. Characterization of the N^3-deoxycytidine adduct of ethylene oxide also reported the diprotonation of the N^4 position with chemical shifts of 9.35 and 10.10 ppm (14). C-3 was determined to be an O^2-deoxycytidine adduct, as opposed to an O^2- or O^4-deoxyuridine adduct based on the presence of these two exocyclic N^4 protons. The BM moiety was determined to be attached to the O^2 of deoxycytidine on the terminal carbon based on the shift of H^7. H^7 was shifted to 4.66 ppm which is similar to the shifts of H^7 in C-1, C-2, C-4, and C-5, (4.60, 4.60, 4.46, and 4.45 ppm) which are all attached at the terminal carbon of BM. Attachment at the allylic carbon of BM would be expected to produce a much less stable product prone to decomposition. Again, specific assignment of protons from the NMR in D_2O was confirmed by homonuclear decoupling experiments. Saturation of H^8 affected the signal of H^{10} and H^{12}, saturation of H^7 affected H^4, and saturation of H^4 affected H^5, H^6, H^7, and H^9.

The FAB/MS fragmentation pattern of C-3 was consistent with the proposed structure (Fig. 4). The expected molecular ion of *m/z* 298 (M + 1) was present in the spectra (Fig. 5D), as was the *m/z* 182 fragment representing the bH_2 fragment also seen in C-1 and C-2, representing the loss of the sugar from *m/z* 298.

Stability of purified BM–deoxycytidine and BM–deoxyuridine adducts. O^2- and N^3-alkylated deoxycytidine can be further characterized by the lower stability of O^2-alkylated deoxycytidine under acid and alkali conditions (23, 24). Incubation of all adducts in 0.1 N HCl, 0.1 N NaOH, or H_2O confirmed the assignments made above. In 0.1 N HCl, C-1, C-2, C-4, C-5, C-6, and C-7 were stable for up to 96 h, while the peak C-3, corresponding to the O^2-alkyldeoxycytidine, had decreased by 40% in 96 h at 24°C. In 0.1 N NaOH, all adducts were unstable; however, peak C-3 was completely absent by 1 h at 24°C, while C-4, C-5, C-6, and C-7 had half-lives of approximately 8.8 h (C-4 and C-5) and 6.3 h (C-6 and C-7), and C-1 and C-2 were the most stable adducts in 0.1 N NaOH with half-lives of approximately 24 h. In H_2O, the N^3-alkylated deoxyuridine adducts were completely stable, while C-1, C-2, and C-3 had half-lives of 29, 24, and 34 h, respectively.

Incubation of purified regioisomeric N^3-deoxyuridine adducts (C-4, C-5, C-6, and C-7) at 37°C in phosphate buffer (pH 7.4) demonstrated that they were completely stable for 7 days (Fig. 6). The N^3-deoxycytidine adducts of BM are unstable under physiologic

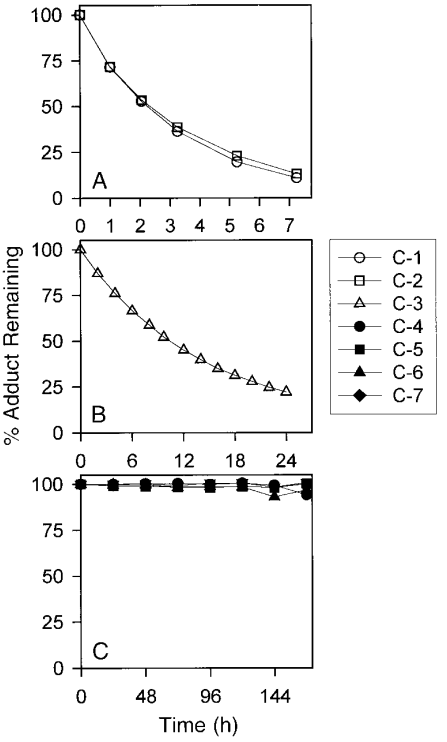

FIG. 6. Stability of purified BM–deoxycytidine adducts in phosphate buffer (pH 7.4) at 37°C.

conditions and readily convert to the N^3-deoxyuridine adducts. Incubation of the purified N^3-(2-hydroxy-3-buten-1-yl)deoxycytidine adducts (C-1 and C-2) resulted in conversion to the N^3-(2-hydroxy-3-buten-1-yl)deoxyuridine adducts (C-4 and C-5, respectively) with half-lives of 2.3 and 2.5 h, respectively (Fig. 6). The N^3-(1-hydroxy-3-buten-2-yl)deoxycytidine diastereomers were not detected, while the corresponding deoxyuridine adducts (C-6 and C-7) were, suggesting that the rate of deamination of this regioisomer was too rapid to detect. Thus the rate of hydrolytic deamination of these regioisomeric deoxycytidine adducts to deoxyuridine adducts occurs at different rates, possibly due to the involvement of the hydroxyl group of the alkylating agent in the deamination reaction as proposed by Solomon *et al.* (11). We see a more rapid deamination event in the regioisomer with the hydroxyl group attached at the terminal carbon (C-6 and C-7). Similarly, in characterizing the reaction between adenosine and BM, we found that only the N^1-adenosine adduct regioisomer with the hydroxyl group attached to the terminal carbon underwent deamination to the N^1-inosine adduct; however, both the N^1-α- and the N^1-β-adenosine adducts of styrene oxide deaminate with the primary hydroxyl-containing α-adduct deaminating at a 35-fold greater rate (22).

The N^3-deoxyuridine adducts could be important mutagenic precursors because uridine is not a natural component of DNA and base-pairs differently than cytidine. BM-induced GC → AT transition mutations have been observed (8). The purified O^2-deoxycytidine diastereomers (C-3) are also unstable at 37°C (pH 7.4) and dealkylate with a half-life of 11 h, which may be long enough to also be potentially mutagenic.

Effect of BM concentration on the formation of adducts. A roughly linear formation of adducts was found when deoxycytidine (10 mM) was reacted with varying amounts of BM (10, 25, 50, and 500 mM) for 1 h in phosphate buffer (pH 7.4) at 37°C. When the 80°C conversion step was included, the major N^3-deoxyuridine regioisomer adducts (C-4 and C-5) were detected at all concentrations (Fig. 7). The other regioisomer (C-6 and C-7) was detected only at 50 and 500 mM BM. When no conversion step was included, the N^3-deoxycytidine adducts (C-1 and C-2) were detected all the way down to 5 mM BM, the O^2-deoxycytidine adducts were detected at 2.5-fold and higher concentrations of BM, and N^3-deoxyuridine adducts were detected at 5-fold and higher BM concentrations (data not shown). At concentrations lower than those stated, adducts were undetectable by this HPLC method because the amounts of adducts were below the limits of detection. The ratios of adducts formed remained consistent over the range of BM concentrations tested, with the N^3-(2-hydroxy-3-buten-1-yl)deoxyuridine regioisomer formed 3.5-fold more favorably than the N^3-(1-hydroxy-3-buten-2-yl)deoxyuridine isomer. These results suggest that there is no threshold BM concentration for the formation of adducts with deoxycytidine near the range tested, and all of the adducts may be formed at lower BM concentrations even though they would be below the limits of detection of our assay.

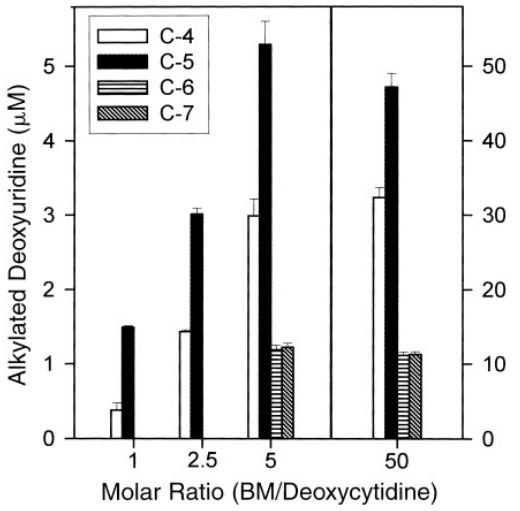

FIG. 7. Effect of BM concentration on the formation of the adducts formed when BM and deoxycytidine (10 mM) are reacted for 1 h at 37°C in phosphate buffer (pH 7.4). All N^3-deoxycytidine adducts were converted to N^3-deoxyuridine adducts following an 80°C conversion step prior to analysis. Values represent means ± SD of the results obtained from three experiments.

Reaction of deoxycytidine (3.1 or 9.6 mM) with an unlimiting BM concentration (750 mM) under physiologic conditions (pH 7.4, 37°C) allowed calculation of pseudo-first-order rate constants. C-3, C-6, and C-7 had linear rates of formation throughout the 4-h study, while C-1 and C-2, the N^3-deoxycytidine diastereomers, were initially linear, but plateaued at later time points as they deaminated to C-4 and C-5 (Figs. 8A and 8B). Meanwhile, C-4 and C-5 demonstrated an initial lag in formation which is probably due to the time needed for deamination. C-6 and C-7 did not show similar lags in formation possibly because the deamination event to convert the N^3-deoxycytidine adducts to N^3-deoxyuridine adducts is much faster and thus the precursor N^3-deoxycytidine adducts are never observed. In this case, C-1, C-2, C-3, C-6, and C-7 demonstrated linear formation from 0 to 120 min (0 to 60 min for C-1 and C-2). Evidence that the reaction was occurring under pseudo-first-order conditions was demonstrated by a similar rate constant calculation at both concentrations of deoxycytidine, thus pseudo-first-order rate constants were calculated for the formation of each of these adducts. These rate constants were 6.0×10^{-3} h^{-1} (C-1), 6.8×10^{-3} h^{-1} (C-2), 1.4×10^{-3} h^{-1} (C-3), 9.4×10^{-4}

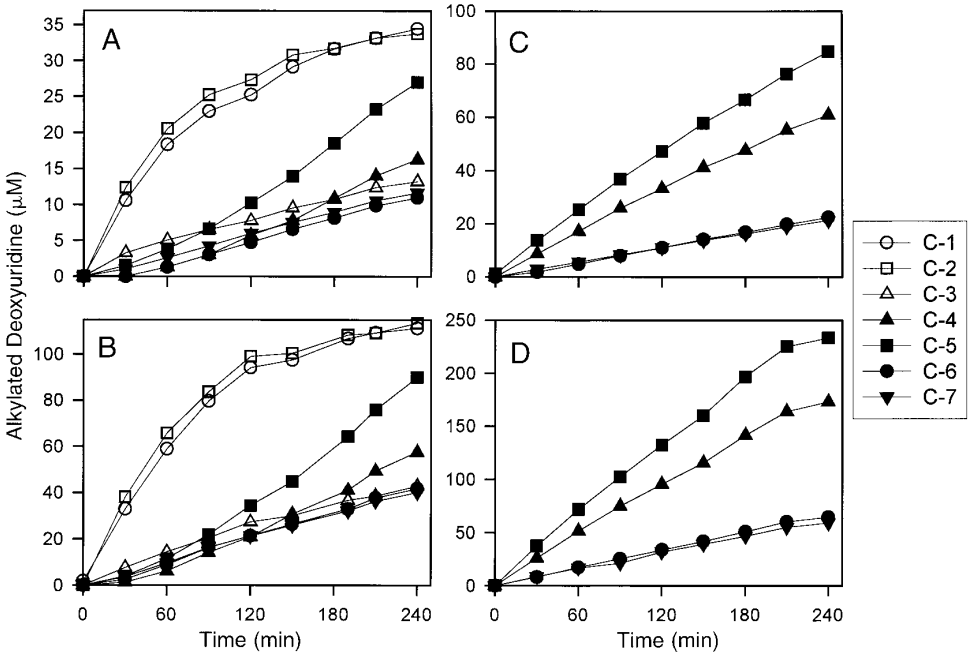

FIG. 8. Formation of the products of the reaction of deoxycytidine with BM at 37°C in phosphate buffer (pH 7.4) either before (A and B) or after (C and D) an 80°C heat-treatment step. To guarantee pseudo-first-order kinetic conditions, the BM concentration (750 mM) was much higher than the deoxycytidine concentrations (A, C: 3.1 mM; B, D: 9.6 mM).

h^{-1} (C-6), and 1.0×10^{-3} h^{-1} (C-7). Because C-1 and C-2 are the immediate precursors of C-4 and C-5, addition of a 80°C conversion step, which results in the conversion of all N^3-deoxycytidine precursors to N^3-deoxyuridine adducts, results in a linear rate of formation for C-4 and C-5 (Figs. 8C and 8D) and should give rates of formation similar to the initial rates of C-1 and C-2 formation. These rate constants were 5.2×10^{-3} h^{-1} (C-4) and 7.2×10^{-3} h^{-1} (C-5). Rate constants for C-6 and C-7 with the conversion step were similar to those calculated without the conversion step. Comparison of the rate constants for the reactions of BM with guanosine (9), adenosine (10), and deoxycytidine shows that, under *in vitro* physiologic conditions, the major adducts formed in the reaction with deoxycytidine (C-1, and C-2, and thus C-4 and C-5) have rate constants similar to the major N^6-adenosine regioisomer. These rate constants are approximately twofold those of the minor N^6-adenosine regioisomer, as well as the N^1-inosine and the N^1 and N^2 adducts of guanosine. The rate constants for the O^2-deoxycytidine adduct and the minor N^3-deoxyuridine regioisomer were the lowest measured this far. The N^7-guanosine adducts are the major adducts formed *in vitro.*

SUMMARY

Previous studies with other reactive epoxides have resulted in formation of deoxycytidine adducts. In a reaction between deoxycytidine and styrene oxide, Savela *et al.* (25) describe the primary adducts formed as N^3- and O^2-deoxycytidine adducts, but did not describe any deamination to deoxyuridine adducts. They reported the reaction with deoxyguanosine produced the greatest yield of adducts, while the deoxycytidine reaction produced a slightly lower yield of adducts but was significantly above that for deoxyadenosine and thymidine. The reaction of 2-cyanoethylene oxide with deoxynucleosides also resulted in a similar reactivity of the nucleosides (12). Deamination reactions similar to the one described here have previously been observed in the reaction between several two- and three-carbon epoxides and deoxycytidine. These reactions usually form a single N^3-deoxyuridine adduct (11–13, 15). Reaction with ethylene oxide resulted in the isolation of the unstable N^3-(2-hydroxyethyl)deoxycytidine adduct as well as the N^3-deoxyuridine adduct (14). However, no O^2-deoxycytidine adducts were characterized in those studies. Solomon *et al.* reported a 10-h half-life for the deamination reaction of the N^3-(2-hydroxyethyl)deoxycytidine adduct, which is considerably longer than the unmeasurable or 2.3- to 2.5-h half-lives for the regioisomeric N^3-deoxycytidine adducts reported here. Thus, different deoxycytidine adducts deaminate to deoxyuridine adducts at different rates. In this regard, we believe our study is the first to characterize regioisomeric adducts of the epoxide ring opening and observe a difference in the rate of deamination between the two.

In conclusion, we have characterized the four diastereomeric products formed in the reaction between deoxycytidine and the reactive epoxide, BM, under *in vitro* physiologic conditions as N^3-(2-hydroxy-3-buten-1-yl)-deoxycytidine, O^2-(2-hydroxy-3-buten-1-yl)deoxycytidine, N^3-(2-hydroxy-3-buten-1-yl)deoxyuridine, and N^3-(1-hydroxy-3-buten-2-yl)deoxyuridine. The N^3- and O^2-deoxycytidine adducts are the initial products formed. The N^3-deoxycytidine regioisomers deaminate to N^3-deoxyuridine adducts at different rates, but the regioisomeric deoxyuridine adducts are then extremely stable. The O^2-deoxycytidine adducts may also be stable enough to be of mutagenic significance. Characterization of these adducts and their physicochemical properties may contribute to a better understanding of the molecular mechanisms of mutagenesis and carcinogenesis. In addition, the stability and abundance of the deoxyuridine adducts may be useful in the development of biomarkers of exposure to BM and related compounds.

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