Glutathione and N-Acetylcysteine Inactivations of Mutagenic 2(5H)-Furanones from the Chlorination of Humics in Water

Robert T. LaLonde* and Shan Xie

Department of Chemistry, State University of New York, College of Environmental Science & Forestry, Syracuse, New York 13210-2786

Received December 28, 1992

The mutagenic 2(5H)-furanones resulting from the chlorination of lignohumic substances in water disinfection and paper pulp bleaching are known to be inactivated by thiols. The objectives of the present study were to characterize the kinetics of an inactivating reaction, isolate and characterize products, and determine their mutagenicity in relation to the starting, mutagenic 2(5H)-furanones. The *Salmonella typhimurium* (TA100) mutagenicity of mucochloric acid (MCA) had a mean value of 2800 revertants/μmol from four assays and was twice as potent as the C-5 isopropyl ether of MCA (MCA-IPE), whose mutagenicity was determined in the same four assays. A second-order reaction of MCA with GSH at pH 7 was observed. The major product, making up 70% of the total product mixture, was identified as a 1.5:1 mixture of two diastereomers formed by sulfur displacement of the C-4 Cl atom from MCA. The major diastereomer was isolated from the 1.5:1 mixture. Connectivity of GSH to the MCA moiety in the product was established by 2D long-range coupling NMR and fully coupled ^{13}C NMR. On the basis of circular dichroism, the major diastereomer had the *S* configuration at the hydroxyl-bearing, C-5 ring carbon. MCA-IPE reacted with GSH and *N*-acetylcysteine (NAC), giving 1:1 mixtures of two diastereomers, again by displacement of the C-4 Cl atom from MCA. A single diastereomer was isolated from the 1:1 MCA-IPE plus NAC reaction. Its structure, determined by X-ray crystallography, had the 5*R*,8*R* configuration and was in agreement with the gross structure deduced from the NMR analysis. Products from MCA and MCA-IPE reacting with GSH and NAC were observed to be nonmutagenic. Therefore, the inactivation of mutagens MCA and MCA-IPE by the displacement of their C-4 chlorine atoms by sulfur was concluded. In contrast to the reactions of MCA, the action of GSH and NAC on the potent bacterial mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) and MX-IPE produced intractable mixtures having the odor of reduced sulfur.

Introduction

The action of chlorine on the phenolic substances in humic acids and lignin, a process occurring in water disinfection and paper pulp prebleaching, results in the formation of a number of genotoxic, chlorine-substituted 2(5H)-furanones (*1–10*). One of these is 3,4-dichloro-5-hydroxy-2(5H)-furanone, or mucochloric acid (MCA)[1] (compound 1, Figure 1). Its mutagenicity (M_m) has been reported to range from 1000 to 10 000 rev/μmol (*9, 11–13*) in the standard plate incorporation assay of Maron and Ames (*14*) using *Salmonella typhimurium* (TA100). Another member of this class of genotoxins is 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX, **2**). It is roughly 1000 times more potent, having a mutagenicity reported from 1000 to 10 000 rev/nmol in the TA100 assay (*2, 8, 11, 15*).

We reported recently (*13*) that MCA was inactivated by *N*-acetylcysteine (NAC) and earlier (*11*) that MX was inactivated by a similar thiol, GSH, an important molecule which in vivo is often involved in the detoxication of xenobiotics. In the recent study of MCA, the major (**3**) and one of the more abundant minor products (**4**) were isolated as their methylated derivatives, characterized,

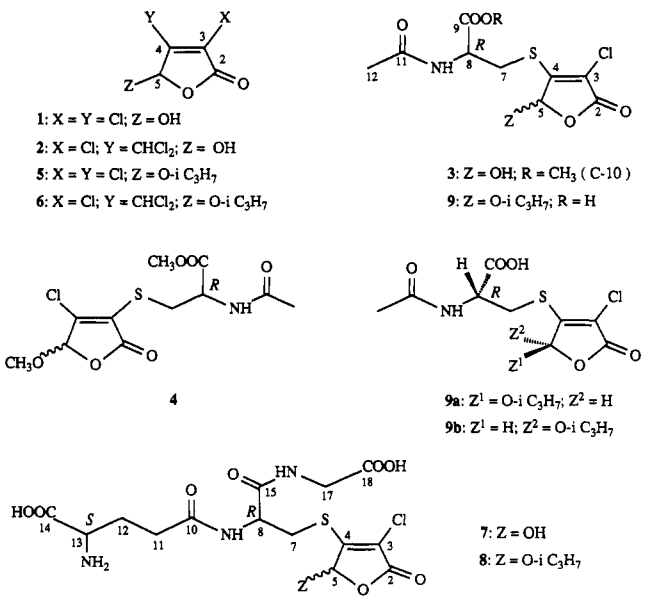

Figure 1. Structures of chlorine-substituted 2(5H)-furanones and their reaction products from GSH and NAC.

[1] Abbreviations: CIMS, chemical ionization mass spectrometry; COLOC, correlation spectroscopy via long-range couplings; COSY, correlated spectroscopy; 2D, two dimensional; DEPT, distortionless enhancement by polarization transfer; HETCOR, heteronuclear shift correlation; IPE, an isopropyl ether derivative; MCA, mucochloric acid; MCA-IPE, mucochloric acid isopropyl ether; M_m, molar mutagenicity; $\bar{M}_m$, mean molar mutagenicity; MX, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone; MX-IPE, 3-chloro-4-(dichloromethyl)-5-isopropoxy-2(5H)-furanone; NAC, *N*-acetylcysteine; ODS, octadecylsilane; ORTEP, Oak Ridge thermal ellipsoid program; rev, revertants; TA100, *Salmonella typhimurium* (TA100); TOCSY, total correlated spectroscopy.

0893-228x/93/2706-0445$04.00/0

and assayed against TA100. The major product, resulting from displacement of the C-4 Cl by the sulfur of NAC, was nonmutagenic, whereas the minor product, resulting from the displacement of the C-3 Cl, was still mutagenic, though significantly less so than MCA. This work demonstrated that both nonmutagenic and weakly mutagenic NAC conjugates of MCA could be formed.

To gain a more complete evaluation of the thiol inactivation chemistry of halogen-substituted 2(5*H*)-furanones, we have extended our studies to include the action of (1) GSH on MCA and the C-5 isopropyl ether of MCA (MCA-IPE, **5**); (2) NAC on MCA-IPE; and (3) NAC and GSH on MX and MX-IPE (**6**). Inclusion of the C-5 IPE derivatives limited the reaction species to only the closed-ring form of the 2(5*H*)-furanone by preventing the ring–chain tautomerism that can occur when a hydroxyl group is located at C-5.

The particulars of this paper deal with the kinetics of the reaction of MCA with GSH, the characterization of the isolable major conjugates resulting from MCA and MCA-IPE, and the comparative TA100 mutagenicities of reactants and conjugates. Our attempts to obtain isolable products from MX and MX-IPE were frustrated. Nevertheless, we describe briefly our procedures and observations.

Experimental Procedures

Chemicals. (*R*)-(+)-*N*-Acetylcysteine (NAC) and 2,3-dichloro-5-hydroxy-2(5*H*)-furanone (mucochloric acid, MCA) were purchased from Aldrich Chemical Co. (Milwaukee, WI), and GSH was obtained from Sigma Chemical Co. (St. Louis, MO). 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) was prepared by the method of Padmapriya *et al.* (*16*). MCA and MX were converted respectively to 2,3-dichloro-5-isopropoxy-2(5*H*)-furanone (MCA-IPE) and 3-chloro-4-(dichloromethyl)-5-isopropoxy-2(5*H*)-furanone (MX-IPE) by the method of Fishbein and Moore (*17*). Thus, in the preparation of previously unknown MX-IPE, a solution of 40 mg of MX (0.18 mmol) and 60 μL of 2-propanol (0.78 mmol) in 7.5 mL of benzene containing 1 drop of concentrated H_2SO_4 was heated to reflux for 24 h with separation of water as it was formed. The reaction solution was cooled and washed with saturated, aqueous $NaHCO_3$ solution, and then brine. Evaporation of the solvent left 40 mg of liquid residue, which was chromatographed from a column of silica gel eluted with ether–hexanes (1:1) to obtain 20 mg of liquid MX-IPE (**6**) (42%): ^{1}H NMR ($CDCl_3$): δ 1.35 (d, *J* 6.20, C-8 H_3), 1.36 (d, *J* 6.18, C-9 H_3), 4.20 (m, C-7 H), 6.17 (s, C-5 H), 6.60 (s, C-6 H); ^{13}C NMR: δ 21.87 (q, C-8), 22.86 (q, C-9), 60.71 (d, C-6), 75.58 (d, C-7), 100.25 (d, C-5), 125.14 (s, C-3), 149.01 (s, C-4), 163.86 (s, C-2); GC EIMS: *m/z* 243 (Cl_3, M-CH_3), 199 base peak (Cl_3, M – $OCH(CH_3)_2$), 165 (Cl_2, M – [$OCH(CH_3)_2$ + Cl]). Anal. Calcd for $C_8H_9Cl_3O_3$: C, 37.21; H, 3.50; Cl, 40.98. Found: C, 37.29; H, 3.70; Cl, 41.07. MCA, MCA-IPE, MX, MX-IPE, and products **7** and **9** were at least 99% pure as demonstrated by ^{1}H NMR.

Caution: *Compounds* **1**, **2**, *and* **5** (*Figure 1*) *have tested positive in the Salmonella mutagenicity assay with (TA100) without metabolic activation (see Mutagenesis Assay). Compound* **6** *is untested, but because of its structural similarly to* **2** *is a suspected direct-acting mutagen. Although none of these compounds are as yet known carcinogens, caution should be exercised in their handling and disposal.*

Chromatography. TLC was performed on Merck silica gel 60F-254 (No. 5554) sheets in the solvent systems indicated in the following subsections. Flash chromatography used Merck Kieselgel 60 (230–400 mesh). HPLC was carried out on a Shimadzu LC-6A at ambient temperature using a 150 × 4.6 mm Shimadzu octadecylsilane (ODS) column unless indicated otherwise. The detector wavelength was set at 254 nm, and the particular column was eluted isocratically. Retention times (t_R) are given in minutes.

Spectra, Elemental, and X-ray Analyses. ^{1}H (300 MHz) and ^{13}C NMR (75.45 MHz) and 2D NMR were determined in the solvents indicated with a Bruker AMX 300 spectrometer. Chemical shift values (δ) are relative to TMS (δ = 0.00 ppm). Coupling constants, *J*, are given in Hz. The determination of CH_3, CH_2, CH, or quaternary carbons was achieved by ^{13}C off-resonance or fully coupled spectra or distortionless enhancement by polarization transfer (DEPT) experiments and are indicated as q, t, d, and s, respectively, in reported NMR spectral properties. In reporting ^{13}C NMR data, only multiplicities from one-bond carbon–hydrogen couplings are indicated in the Experimental Procedures section. The descriptions of two-dimensional (2D) NMR experiments represented by abbreviations can be found in recent texts dealing with the subject, as, for example, Martin and Zektzer (*18*). UV spectra of compounds **7**–**9** and the rate study reaction mixtures of MCA and GSH were recorded on a Kontron UVIKON 860 spectrophotometer in the same solvents indicated earlier (*13*). Optical rotations were taken on a Perkin-Elmer 141 polarimeter. Elemental analyses were performed by Desert Analytics (Tucson, AZ). The X-ray structure of the MCA-IPE plus NAC single diastereomeric adduct (**9a**) was determined by Mr. P. J. Carroll, Chemistry Department, University of Pennsylvania, Philadelphia, PA. CD spectra were determined on a Jasco Model ORD/UV5 spectropolarimeter modified for CD by Sproul Scientific with part no. SS-107.

Kinetics. The procedures for preparing the reagent solutions, obtaining kinetic data, and analyzing these data were the same as previously reported (*13*) except that GSH replaced NAC.

Reaction of MCA (1) with GSH. MCA (40 mg, 0.24 mmol) and GSH (74 mg, 0.24 mmol) were dissolved in 8 mL of 0.1 M aqueous phosphate buffer (K_2HPO_4/KH_2PO_4 in a ratio adjusted to pH 7), and the resulting solution was kept under N_2 at 37 °C overnight. Thereafter, the solution was acidified with 10% aqueous HCl. The aqueous phase was freeze-dried. That the residue was a mixture of the two diastereomers of **7** was indicated by the ^{1}H NMR (D_2O): δ 1.85 (q, *J* 7.14, C-12 H_2), 2.22 (dt, *J* 3.20, 6.92, C-11 H_2), 3.01 (dd, *J* 8.51, 14.01, C-7 H_a), 3.17 (dd, *J* 8.39, 14.09, C-7 H_a), 3.30 (dd, *J* 4.96, 14.24, C-7 H_b), 3.46 (dd, *J* 5.50, 14.31, C-7 H_b), 3.52 (t, *J* 6.80, C-13 H), 3.53 (t, *J* 6.80, C-13 H), 3.64 (s, C-17 H_2), 3.66 (s, C-17 H_2), 4.32 (dd, *J* 5.25, 8.41, C-8 H), 4.47 (dd, *J* 5.40, 8.40, C-8 H), 6.07 (s, C-5 H), 6.10 (s, C-5 H); ^{13}C NMR (D_2O): δ 26.68 (t, C-12), 31.88 (t, C-7), 31.98 and 32.89 (t, C-11), 42.40 (t, C-17), 53.87 and 54.02 (d, C-8), 54.39 (d, C-13), 97.98 (d, C-5), 117.81 (s, C-3), 158.19 (s, C-4), 167.84 (s, C-2), 172.17 and 172.43 (s, C-15), 174.06 (s, C-14 and C-18), 175.42 and 175.49 (s, C-10); HPLC: t_R 6.20 and 6.71 min [major (70% of the total components)] (solvent: $CH_3CN/THF/H_2O$, 18:2:80, at 0.3 mL/min). Addition of the pure major diastereomer (see below) to the mixture of diastereomers resulted in the enhancement of the peak with the greater t_R value (6.71).

Recrystallization from methanol–water of the freeze-dry residue gave 20 mg of a solid, single diastereomer of **7** (19%): mp 181–183 °C dec; $[\alpha]_D$ –6.50° (*c* = 0.0037, CH_3OH); UV λ_{max} (H_2O): 285 nm (ε = 9.99 × 10^3); ^{1}H NMR (D_2O): δ 1.84 (q, *J* 7.18, C-12 H_2), 2.22 (dt, *J* 2.74, 7.61, C-11 H_2), 3.16 (dd, *J* 8.40, 14.11, C-7 H_a), 3.46 (dd, *J* 5.38, 14.07, C-7 H_b), 3.49 (t, *J* 6.48, C-13 H), 3.65 (s, C-17 H_2), 4.46 (dd, *J* 5.46, 8.42, C-8 H), 6.09 (s, 6.48, C-13 H), 3.65 (s, C-17 H_2), 4.46 (dd, *J* 5.46, 8.42, C-8 H), 6.09 (s, C-5 H); ^{13}C NMR (D_2O): δ 26.29 (t, C-12), 31.86 (t, C-7), 31.96 (t, C-11), 42.31 (t, C-17), 53.85 (d, C-8), 54.48 (d, C-13), 97.76 (d, C-5), 117.78 (s, C-3), 158.18 (s, C-4), 167.82 (s, C-2), 172.14 (s, C-15), 174.19 (coincidental singlets, C-14 and C-18), 175.50 (s, C-10). In addition to the NMR spectral characteristics reported above, DEPT, H–H correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), C–H heteronuclear chemical shift correlation (HETCOR), correlated spectroscopy via long-range couplings (COLOC), and fully coupled ^{13}C NMR determinations were made. HPLC: t_R 6.71 min (>99.1% of the total components) (solvent: $CH_3CN/THF/H_2O$, 18:2:80, at 0.3 mL/min). Anal. Calcd for $C_{14}H_{18}ClN_3O_9S$: C, 38.27; H, 4.10; N, 9.57. Found: C, 38.63; H, 3.98; N, 9.30. Details of the CD are given

in connection with the discussion of structure in the Results and Discussion section.

Reaction of MCA-IPE (5) with GSH. MCA-IPE (105 mg, 0.5 mmol) was dissolved in 2 drops of acetonitrile. To the resulting solution was added 154 mg (0.5 mmol) of GSH in 10 mL of 0.1 M aqueous phosphate buffer (pH 7.0) solution. The resulting mixture was allowed to stand under N_2 at 37 °C for 10 h. The 60 mg of resulting solid, a 1:1 mixture of two diastereomers (8), was collected by filtration: mp 175–177 °C; $[\alpha]_D$ −12.54° (c = 0.0015, CH_3OH); UV λ_{max} (CH_3OH): 279.3 nm ($\epsilon = 2.01 \times 10^4$); ^1H NMR (CD_3OD): δ 1.30 (dd, J 6.14, CH_3 of i-C_3H_7), 1.31 (dd, J 6.00, CH_3 of i-C_3H_7, H_3), 2.09 (m, C-12 H_2), 2.14 (m, C-12 H_2), 2.53 (t, J 6.61, C-11 H_2), 2.54 (t, J 6.92, C-11 H_2), 3.34 (dd, J 9.25, 13.67, C-7 H_a), 3.40 (dd, J 8.32, 13.56, C-7 H_a), 3.64 (t, J 6.16, C-13 H), 3.64 (t, J 6.18, C-13 H), 3.71 (dd, J 5.47, 13.59 C-7 H_b), 3.76 (dd, J 5.29, 13.59, C-7 H_b), 3.89 (s, C-17 H_2), 3.90 (d, J 2.37, C-17 H_2), 4.19 (m, CH of i-C_3H_7, H), 4.21 (m, CH of i-C_3H_7), 4.68 (dd, J 5.19, 9.13, C-8 H), 4.74 (dd, J 5.50, 8.35, C-8 H), 6.32 (s, C-5 H), 6.32 (s, C-5 H); ^{13}C NMR (CD_3OD): δ 22.44 and 22.53 (q, CH_3 of i-C_3H_7), 23.56 and 23.58 (q, CH_3 of i-C_3H_7), 27.61 (t, C-12), 32.51 (t, C-7), 32.75 (t, C-11), 42.16 (t, C-17), 54.17 and 54.45 (d, C-8), 55.21 (d, C-13), 75.51 and 75.68 (d, CH_3 of i-C_3H_7), 101.65 and 101.76 (d, C-5), 118.28 (s, C-3), 157.38 (s, C-4), 166.18 (s, C-2), 171.75 and 171.86 (s, C-15), 172.99 (s, C-14 or C-18), 173.59 (s, C-18 or C-14), 175.18 (s, C-10). ^{13}C NMR CH_3, CH_2, and CH resonances were distinguished by DEPT. HPLC: t_R 5.12 and 5.93 min (solvent: $CH_3CN/THF/H_2O$, 38:2:60, at 0.3 mL/min). Anal. Calcd for $C_{17}H_{24}ClN_3O_9S$: C, 42.41; H, 4.99; N, 8.73. Found: C, 42.46; H, 4.84; N, 8.45.

Reaction of MCA-IPE (5) with NAC. A solution of MCA-IPE (210 mg, 1.0 mmol), NAC (164 mg, 1.0 mmol), and $KHCO_3$ (200 mg, 2 mmol) in 40 mL of dry acetone was kept under N_2 and at 37 °C overnight. The solvent was evaporated on the rotary evaporator, and the residue was dissolved in 3 mL of 0.25 N aqueous HCl. The solution was extracted with ethyl acetate, and the extract was washed with brine and dried over anhydrous Na_2SO_4. Evaporation of the solvent on the rotary evaporator gave 320 mg of **9** (95%), a 1:1 mixture of two diastereomers: mp 155–157 °C; $[\alpha]_D$ +1.81° (c = 0.0048, CH_3OH); UV λ_{max} (CH_3OH): 280.7 nm ($\epsilon = 1.33 \times 10^4$); IR (KBr): 3300, 2960, 1760, 1594, 1572, 1533, 1370, 1310, 1226, 1204, 1012, 874 cm^{-1}; ^1H NMR (CD_3OD): δ 1.30 (dd, J 5.99, 6.00, CH_3 of i-C_3H_7, H_6), 1.31 (t, J 6.30, CH_3 of i-C_3H_7, H_6), 2.00 (s, C-12 H_3), 2.01 (s, C-12 H_3), 3.42 (dd, J 8.29, 13.58, C-7 H_a), 3.42 (dd, J 8.79, 13.62, C-7 H_a), 3.78 (dd, J 5.05, 13.57, C-7 H_b), 3.81 (dd, J 4.68, 13.63, C-7 H_b), 4.18 (m, CH of i-C_3H_7), 4.19 (m, CH of i-C_3H_7, H), 4.70 (dd, J 5.48, 7.77, C-8 H), 4.71 (dd, J 4.66, 8.68, C-8 H), 6.28 (s, C-5 H), 6.27 (s, C-5 H); HPLC: t_R 22.62 min (solvent: $CH_3CN/THF/H_2O$, 18:2:80, at 1 mL/min). Anal. Calcd for $C_{12}H_{16}ClNO_6S$: C, 42.73; H, 4.75; N, 4.15. Found: C, 42.84; H, 4.87; N, 4.00.

A 220-mg sample of **9** was dissolved in MeOH (2 mL), and the resulting solution was treated with a solution of diazomethane in ether. Evaporation of the solvent gave 230 mg of methyl ester. HPLC: t_R 23.92 and 24.85 min [solvent: $CH_3CN/THF/H_2O$ (pH 3.25), 24:1:75, at 1 mL/min].

Repeated recrystallization from methanol–water of a 140-mg portion of an initially 1:1 mixture of diastereomers gave a 20-mg sample of diastereomer **9a**: mp 180–181 °C; $[\alpha]_D$ −359.7° (c = 0.0050, CH_3OH); ^1H NMR (CD_3OD): δ 1.31 (dd, J 6.27, CH_3 of i-C_3H_7, H_6), 2.01 (s, C-12 H_3), 3.42 (dd, J 8.79, 13.63, C-7 H_a), 3.81 (dd, J 4.69, 13.62, C-7 H_b), 4.20 (m, CH of i-C_3H_7, H), 4.71 (dd, J 4.68, 8.78, C-8 H), 6.27 (s, C-5 H); ^{13}C NMR (CD_3OD): δ 24.75 (q, C-14), 25.97 (q, C-12), 35.03 (t, C-7), 55.85 (d, C-8), 77.99 (d, C-13), 103.97 (d, C-5), 121.02 (s, C-3), 159.72 (s, C-4), 168.45 (s, C-2), 174.70 (s, C-11), 175.87 (s, C-9). ^{13}C NMR CH_3, CH_2, and CH resonances were determined by DEPT. Details of the CD are given in connection with the discussion of structure in the Results and Discussion section.

A 10-mg sample of **9a** was treated with CH_2N_2 in the same manner as **9**. The resulting methyl ester showed a single peak on HPLC, t_R 25.03 min (same column, solvent, and rate as used for **9**). Addition of the methyl ester of **9a** to the mixture of diastereomeric methyl esters **9** enhanced the peak with the greater t_R value (25.02 min).

Action of NAC on MX (2). Conditions previously employed (*13*) for the reaction of NAC with MCA were applied to the action of NAC on MX. Thus, MX (30 mg, 0.14 mmol) was dissolved in several drops of acetonitrile, and the solution was made up to 6 mL with 0.1 M phosphate buffer (50 mL of 0.1 M K_2HPO_4 and 31 mL of 0.1 M KH_2PO_4, pH 7.0) containing 45 mg of NAC (0.28 mmol). The resulting solution was kept overnight at 37 °C under nitrogen. Thereafter, the solution was acidified with 10% aqueous HCl to pH 2 and extracted with ethyl acetate. The extract was washed with brine, dried over Na_2SO_4, and reduced to dryness at reduced pressure. The residue was dissolved in methanol and treated with diazomethane in the usual manner (*13*) to obtain 30 mg of a complex mixture that had the strong odor of reduced sulfur and could not be rectified by chromatographic methods. Similar results were obtained when MX was treated with NAC in acetone or methanol at 37 °C in the presence of $KHCO_3$ for periods of 6–24 h. MX (120 mg) was recovered unchanged, as determined by HPLC, t_R 8.25 min (column: 250 × 10 mm Spherisorb ODS; solvent: $CH_3CN/THF/H_2O$, 58:40:2, at 1.5 mL/min), and TLC, from a solution of NAC in acetonitrile–aqueous phosphate buffer (pH 7.0) after standing at 4 °C for 4 days in the dark.

Action of NAC on MX-IPE (6). A suspension of MX-IPE (20 mg, 0.08 mmol), NAC (16 mg, 0.08 mmol), and $KHCO_3$ (16 mg, 0.16 mmol) in 10 mL of acetone was stirred for 24 h at 37 °C under nitrogen. The contents of the reaction flask were evaporated to dryness under vacuum, and the resulting residue was dissolved in methanol, acidified with 10% aqueous HCl to pH 2, and processed further as described in the section immediately preceding. The resulting material (15 mg) was a complex mixture having the strong odor of reduced sulfur, and rectification of it by TLC or HPLC failed.

Mutagenesis Assay. The histidine-requiring (his$^-$) *S. typhimurium* tester strain TA100 supplied by Dr. Bruce Ames (University of California, Berkeley) was used in the standard plate incorporation mutagenesis assay performed according to Maron and Ames (*14*) for 72-h periods. The mutagenic activities of the four compounds (**1**, **5**, **7**, **9**), added in freshly prepared Me_2SO solutions to the top agar, were determined without activation by rat liver homogenate fraction S9. Compounds **7** and **9** were assayed as the diastereomeric mixtures. Three plates per dose level were prepared, except for the zero dose level, for which five plates were prepared. Mutagenic responses were determined simultaneously for three or four compounds in a single assay. In addition to the zero dose (the positive Me_2SO control), each assay included quintuplicate negative Me_2SO control, crystal violet, ampicillin, and sodium azide controls. Effects of bacterial toxicity on mutagenic response were minimized by imposing the statistical treatment of Bernstein *et al.* (*19*). Values for mutagenicity as rev/μg were obtained as the positive linear regression slopes from the linear portion of the dose/response plots. Multiplication of the slopes by the molecular weights in units of μg/μmol gave the molar mutagenicities (M_m) in units of rev/μmol.

Results and Discussion

The Mutagenicity of MCA and MCA-IPE and Their Thiol Reaction Products. The *S. typhimurium* (TA100) mutagenicities of the chloro-2(5*H*)-furanones and their compounds formed with GSH and NAC are summarized in Table I. Relevant statistical indicators from the linear regression treatment of the dose/response data and the mean molar mutagenicities ($\bar{M}_m$) are included. $\bar{M}_m$ for MCA (**1**) was statistically significantly smaller than were the $\bar{M}_m$ values of 6800 (SD = 216) and 8180 (SD = 268) reported (*12*, *13*) for this compound in two previous studies. However, the $\bar{M}_m$ value reported for MCA in Table I is well within a power of 10 of all the $\bar{M}_m$ values obtained in

Table I. Summary of Results and Comparison of the *S. typhimurium* (TA100) Mutagenicities of MCA,[a] MCA-IPE, and Products of These Two Compounds with NAC and GSH

MCA, MCA-IPE, or adduct	assay	spontaneous rev[b]	slope[c]	intercept[d]	n[e]	r^2[f]	M_m	
MCA (1)	1	153	12.6	182	23	0.808	2130	
	2	153	16.0	169	20	0.919	2710	
	3	144	13.9	141	20	0.902	2310	
	4	158	23.8	147	17	0.895	4030	
$\bar{M}_m$[g]							2800	(SD = 858, n = 4)
MCA-IPE (5)	1	153	6.03	167	17	0.947	1120	
	2	153	5.28	169	20	0.964	1160	
	3	144	6.17	153	17	0.920	1490	
	4	158	6.23	163	17	0.682	1320	
$\bar{M}_m$[g]							1270	(SD = 169, n = 4)
MCA-IPE + GSH (7)	3	144	*h*		20			
	4	158	*h*		20			
MCA-IPE + NAC (9)	1	144	*h*		20			
	2	158	*h*		20			

[a] MCA = mucochloric acid. [b] rev = revertants. [c] In units of rev/μg. [d] In units of rev/plate. [e] The number of cases. [f] The correlation coefficient squared. [g] Mean molar mutagenicities for n cases in units of rev/μmol. [h] The compound is nonmutagenic since it failed the criteria for mutagenicity of M. J. Prival and V. C. Dunkel (Environ. Mol. Mutagen. **1989**, *13*, 1–24).

Table II. Summary of the Initial Concentration Ratios, Least Squares Slopes, and Rate Constants for the Reaction: MCA[a] + GSH[b] → Products (P), Carried Out at 25 °C and pH 7

	slope × 10^3 absorbance (A) at		rate constants × 10^3			
[MCA]/[GSH]	261 nm	310 nm	k_2[c]	$\bar{k}_2$[d]	SD[e]	k_1[f]
1	18.6[g]		18.6			20.8[h]
2	11.2[i]		18.5[j]			10.7[h]
0.5	25.3[k]		21.8[l]			48.2[h]
				19.6	1.9	
1		21.5[m]	21.5			
2		26.6[n]	20.9[o]			
0.5		51.2[p]	21.1[q]	21.2	0.3	

[a] MCA = mucochloric acid = 2,3-dichloro-5-hydroxy-2(5*H*)-furanone. [b] GSH = glutathone. [c] The second-order rate constant in units of L mol^{-1} min^{-1}. [d] The mean second-order rate constant. [e] The standard deviation of the mean second-order rate constant. [f] The first-order rate constant, in units of min^{-1}, for the disappearance of MCA. [g] Obtained by plotting the time in min (t) against $1/A$, where A is the absorbance value at 261 nm at t. [h] Obtained by plotting t against ln A. [i] Obtained by plotting t against $\ln[A/(2A - A_0)]$, where A_0 is the initial absorbance at 261 nm. [j] Calculated by multiplying the corresponding tabulated slope value by $2/A_0$. [k] Obtained by plotting t against $-\ln[2A/(A_0 - A)]$. [l] Calculated by multiplying the corresponding tabulated slope by $1/A_0$. [m] Obtained by plotting t against $1/(2.3A_0 - P_t)$, where P_t is the absorbance at 310 nm at t and the factor 2.3 is the ratio of extinction coefficients ($\epsilon_P/\epsilon_{MCA}$). [n] Obtained by plotting t against $\ln[(2.3A_0 - P_t/(2.3A_0 - 2P_t)]$. [o] Calculated by multiplying the corresponding tabulated slope by $2/2.3A_0$. [p] Obtained by plotting t against $\ln[(4.6A_0 - P_t)/(4.6A_0 - P_t)]$. [q] Calculated by multiplying the corresponding tabulted slope by $1/2.3A_0$.

our repeated assays of MCA conducted over an extended period.

Replacement of the hydroxyl group of MCA (1) by the isopropoxy group in MCA-IPE (6) lowered mutagenicity by 2-fold. Displacement of the C-4 chlorine atom by the sulfur of GSH or NAC, a structural result evident from results to follow, produced sulfur-containing compounds that were judged nonmutagenic on the basis of the lack of a positive response of revertants to increasing dose of adduct. Thus, compound 7 was assayed in five doses in each of two assays: from 20 to 100 μg/plate in assay 1 and from 50 to 250 μg/plate in assay 2. In both assays the lowest dose resulted in an increase of 30–40 revertants/plate relative to spontaneous revertants, but all larger doses resulted in no more, and sometimes slightly fewer revertants, than the lowest dose. A similar result was obtained for 9 when assayed in the same manner. Compounds 7 and 9 were assayed as the mixtures of their two diastereomers.

Thus, it can be concluded that the displacement of the C-4 chlorine by the sulfur of GSH and NAC resulted in the loss of TA100 mutagenicity for both MCA and MCA-IPE. This result is in agreement with our earlier observation that the major product of MCA and NAC, isolated and assayed as its C-9 methoxy derivative (3), was nonmutagenic (*13*).

Kinetics. The set of time-dependent UV scans given in Figure 2 were obtained from three different solutions containing MCA reacting with GSH. These plots demonstrate the decreasing absorption at 261 nm occurring simultaneously with increasing product absorption at 311 nm. Absorbance values taken at 2-min intervals from similar scans allowed the second-order rate constants given in Table II to be calculated from 261-nm band disappearance and 311-nm band appearance. The three solutions differed in the initial [MCA]/[GSH] values from 1 to 2 to 0.5. Second-order kinetics were observed for each of the three concentration ratios. Differences in the mean second-order constants obtained by following absorbance at 261 and 311 nm were not statistically significant. First-order constants for the rate of disappearance of the 261-nm band were calculated for each initial concentration ratio and are included in Table II to demonstrate the dependence of the rate of the 261-nm band disappearance on GSH concentration. Compared to its previously studied reaction with NAC (*13*), MCA is significantly more reactive with GSH, by 5–6 times.

Characterization of Reaction Products. The reaction of MCA and MCA-IPE with GSH and the reaction of MCA-IPE with NAC resulted in the formation of a mixture of two diastereomers in each case. In the case of the MCA + GSH product, the two diastereomers, 7, occurred in a ratio of 1.5:1 as determined by HPLC. Together, these two diastereomers accounted for 70% of the product as determined by HPLC. In the cases of the two product mixtures resulting from MCA-IPE reacting with GSH and NAC, 1:1 mixtures of diastereomers (8 and 9, respectively) were obtained as determined also by HPLC.

Recrystallization of the MCA + GSH mixture of diastereomers from methanol–water gave a single pure diastereomer of 7. The latter was identified as the major

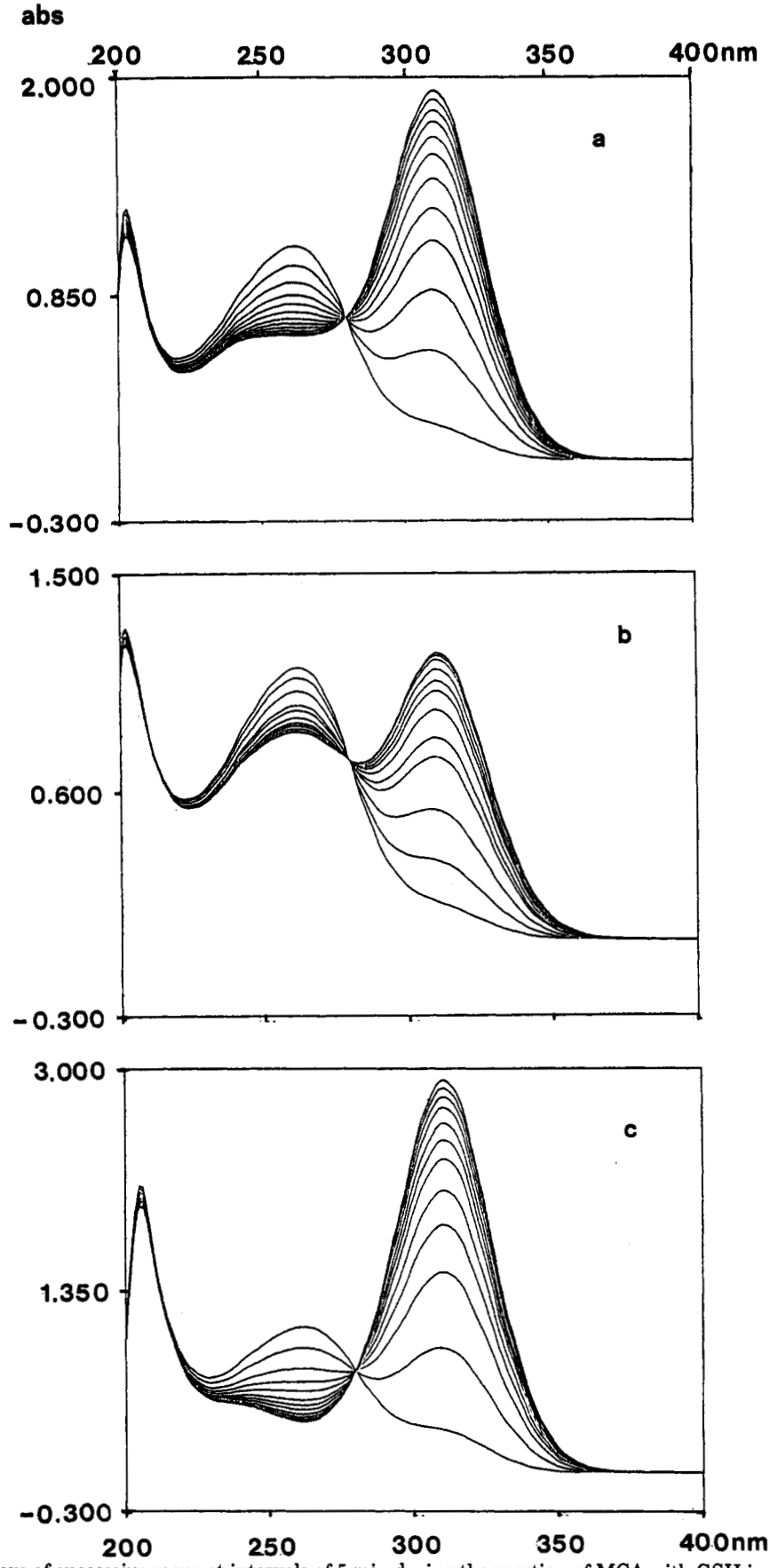

Figure 2. Curve overlays of successive scans at intervals of 5 min during the reaction of MCA with GSH in 0.1 M phosphate buffer solution (pH 7) at 25 °C. The initial [MCA] = 1.93×10^{-4} mM, and [MCA]/[GSH] = 1, 2, and 0.5 for plots a, b, and c, respectively.

diastereomer of the 1.5:1 mixture through HPLC by spiking the mixture with the single pure diastereomer. The ^1H NMR of the major diastereomer in D_2O solution showed no change in 3 days at 25 °C. Recrystallization of the 1:1 diastereomeric mixture resulting from the action of NAC on MCA-IPE also yielded a single diastereomer, **9a**.

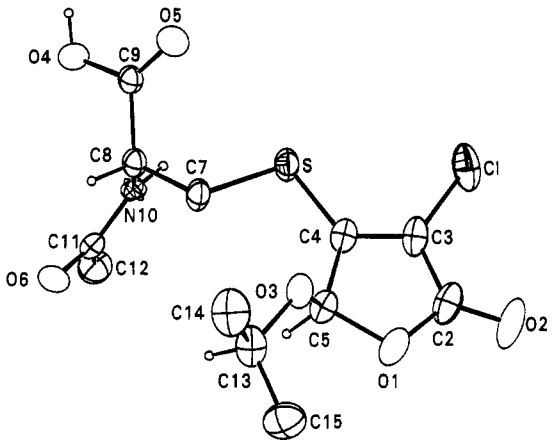

Figure 3. ORTEP representation of the X-ray model of the 5*R*,8*R* diastereomer obtained from the reaction of MCA-IPE with NAC.

The reactions of MX and MX-IPE with GSH and NAC were attempted at 37 °C and under the same conditions as employed for the MCA and MCA-IPE reactions. Resulting were faintly opaque, yellow mixtures having a strong odor of reduced sulfur. The complex nature of these mixtures was indicated by TLC and HPLC analyses, and their development in time could be followed by repetitive UV scans which showed none of the regular features such as shown in Figure 2 for the reaction of MCA with GSH. In contrast, at 4 °C no change in MX by NAC was observed in 4 days in the dark.

Our earlier NMR spectral identification of the product from MCA and NAC (*13*) served as a guide for characterizing the major diastereomer isolated from the reaction of MCA with GSH. Thus, all the CH_3, CH_2, CH, and quaternary carbon provided by MCA and GSH could be observed in the DEPT of **7**. The H–H COSY and the ^1H–^1H splitting patterns indicated that the branched chain of C, N, and S atoms of GSH had remained intact. C-5 H in the 2(5*H*)-furanone ring and C-17 H_a and H_b in the GSH moiety were not coupled to other protons. C-8 H was identified as the lowest field proton in the three-proton spin system consisting of C-7 H_a and H_b and C-8 H. C-13 H was part of the five-proton spin system consisting of C-11, C-12, and C-13 hydrogens that was evident in the TOCSY. The inverse HETCOR associated carbon with the assigned hydrogens directly attached to these carbon atoms. Quartenary carbons were assigned with the aid of the inverse detected heteronuclear spectrum, optimized for long-range couplings to hydrogen. Most importantly, this spectrum showed the coupling of C-7 H through sulfur to C-4 (δ 158.18). Also observed were the couplings C-5 H/C-2 (δ 167.82) and C-5 H/C-3 (δ 117.92), which, together with the C-7 H/C-4 coupling, accounted for all the carbons of the 2(5*H*)-furanone and the point of GSH attachment to this ring. C-4 appeared as a triplet in the fully coupled ^{13}C NMR spectrum as a result of the coupling of this carbon to C-7 H_a and H_b. The assigned δ values for the quaternary ring carbons were consistent with the well-established observation for 2(5*H*)-furanones: C-2 is located at the lowest field, C-3 at the highest, and C-4 in between.

For the products MCA-IPE + GSH (**8**) and MCA-IPE + NAC (**9**), the structures were assigned on the basis of the ^1H and ^{13}C NMR δ value concordance with δ values for MCA + GSH, just discussed. Also, the δ values for **3**, the major product from the reaction of MCA + NAC, studied earlier (*13*), were used for comparison. As expected, the exceptions to this δ value concordance were the δ values for C-5 and C-5 H, which were shifted downfield in the spectra of the IPE derivatives by 4 and 0.2 ppm, respectively. The X-ray crystallographic structure analysis of **9a** produced the Oak Ridge thermal ellipsoid program (ORTEP) view given in Figure 3. This structure determination established the 5*R* configuration of this product and confirmed that C-4 was the point at which the NAC moiety had become attached to MCA-IPE.

Because the configuration of both chiral centers of **9a** was now known, **9a**'s CD spectrum could be helpful in deducing the C-5 configuration of other 5-alkoxy- or stable 5-hydroxy-2(5*H*)-furanones possessing the same chromophore as **9a**. Establishing the C-5 configuration in thiol conjugates of MCA and its derivatives was conducted in anticipation of future studies with glutathione transferases, whose mediation of inactivation through conjugate formation may result in stereoselectivity dependent on the configuration of C-5 in the 2(5*H*)-furanone. As a consequence, one diasteriomeric conjugate may be formed from

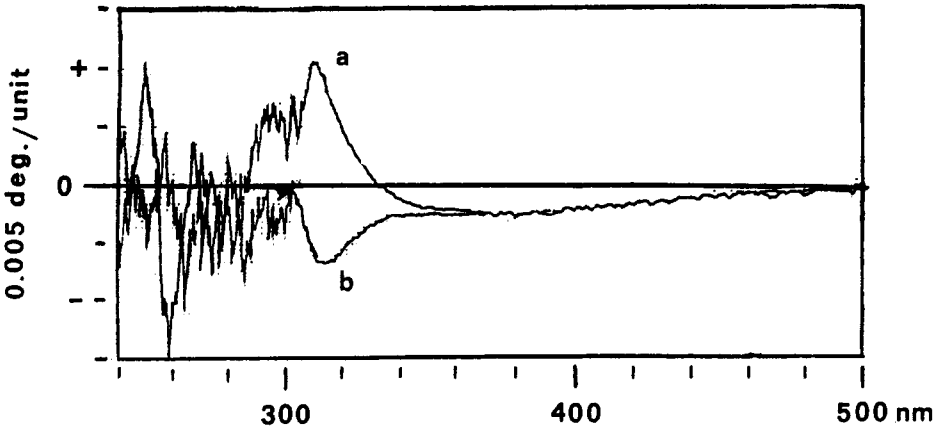

Figure 4. CD spectrum taken in the region 500–240 nm for (a) the solid major diastereomer from the 1.5:1 diastereomeric mixture (**7**) obtained from the MCA plus GSH reaction (curve a); (b) the 5*R*,8*R* diastereomer (**9a**) from the 1:1 diastereomeric mixture obtained from the MCA-IPE plus NAC reaction (curve b). The concentration of the major diastereomer of **7** was 2.05 mg/2.0 mg/mL of water while that of **9a** was 4.74 mg/2.0 mL of methanol. As the wavelength is shortened from 500 to 360 nm, curve a becomes gradually negative. Below 360 nm, the curve becomes positive, reaching a positive maximum at 380 nm, and then descends to a positive minimum at ~300 nm. Curve b also becomes negative in the region 500–360 nm, but then descends more abruptly, reaching a negative maximum at 312 nm before ascending to a negative minimum of close to 0° at ~300 nm.

a racemic 2(5*H*)-furanone, leaving one mostly unconverted, mutagenic enantiomer. Therefore, the CD spectrum of **9a** was determined and is shown in Figure 4 (curve b). The negative maximum at 312 nm for **9a** corresponds to the positive maximum at 308 nm (curve a) for the major diastereomer isolated from the 1.5:1 mixture of diastereomers represented by structure **7**. This observation indicates that the major diastereomer of **7** has the 5*S* configuration. At shorter wavelengths, both curves lack the clear definition of the curves at higher wavelengths. Nevertheless, additional opposing maxima can be made out.

In summary, the changes in MCA and MCA-IPE structure correlating with inactivation can be stated simply as follows. The major products from both GSH and NAC reactions resulted from the sulfur displacement of the C-4 Cl. A minor product resulting from the displacement of the C-3 Cl had been identified earlier (*13*) in the mixture from the MCA + NAC reaction, but the corresponding compounds could not be found in the product mixtures resulting from any of the reactions reported here. Products resulting from the displacement of C-3 and C-4 Cl of MCA by NAC have been attributed to the reactions of this 5-hydroxy-2(5*H*)-furanone in its open- and closed-ring forms, respectively (*13*). Thus, we conclude that MCA reacts with GSH in the same mode as MCA-IPE, the MCA model incapable of undergoing ring–chain tautomerism. Compared to NAC, GSH is the more effective inactivating agent of MCA because GSH is not only more reactive, but also more specific for reacting with the closed-ring form. Consequently, the more mutagen-enhancing chlorine atom is displaced.

Acknowledgment. Research supported by the U.S. Geological Survey, Department of the Interior, under USGS Award 14-08-0001-G1912. The views and conclusions contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies, either expressed or implied, of the U.S. Government. Support of the National Science Foundation (8821239) and the Research Foundation of the State University of New York for instrumentation used in this work is gratefully acknowledged. We also thank David J. Keimle for determining the NMR spectra and Patrick J. Carroll, X-Ray Facility, Chemistry Department, University of Pennsylvania, for structure determination.

References

(1) Holmbom, B. R., Voss, R. H., Mortimer, R. D., and Wong, A. (1981) Isolation and identification of an Ames-mutagenic compound present in kraft chlorination effluents. *Tappi* **64**, 172–174.

(2) Holmbom, B. R., Voss, R. H., Mortimer, R. D., and Wong, A. (1984) Fractionation, isolation and chlorination of Ames mutagenic compounds in kraft chlorination effluents. *Environ. Sci. Technol.* 18, 333–337.

(3) Kronberg, L., Holmbom, B., and Tikkanen, L. (1985) Fractionation of mutagenic compounds found during the chlorination of humic water. *Sci. Total Environ.* **48**, 343–347.

(4) Meier, J. R., Ringhand, H. P., Coleman, W. E., Munch, J. W., Streich, R. P., Kaylor, W. H., and Schenck, K. M. (1985) Identification of mutagenic compounds formed during chlorination of humic acid. *Mutat. Res.* **157**, 111–122.

(5) Kronberg, L., Holmbom, B., and Tikkanen, L. (1985) Mutagenic activity in drinking water and humic water after chlorine treatment. *Vatten* **41**, 106–109.

(6) Hemming, J., Holmbom, B., Reunanen, M., and Kronberg, L. (1986) Determination of the strong mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone in chlorinated drinking and humic waters. *Chemosphere* **5**, 549–556.

(7) Kronberg, L., Holmbom, B., Reunanen, M., and Tikkanen, L. (1988) Identification and quantification of the Ames mutagenic compound 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone and its geometric isomer (E)-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid in chlorine-treated humic water and drinking water extracts. *Environ. Sci. Technol.* **22**, 1097–1103.

(8) Meier, J. R., Knohl, R. B., Coleman, W. E., Ringhand, H. P., Munch, J. W., Kaylor, W. H., Streicher, R. P., and Kopfler, F. C. (1987) Studies on the potent mutagen, 3-chloro-4-(dichloromethyl)-5-hydroxyl-2(5H)-furanone; aqueous stability, XAD recovery and analytical determination in drinking water and in chlorinated humic acid solutions. *Mutat. Res.* **189**, 363–373.

(9) Meier, J. R., Ringhand, H. P., Coleman, W. E., Schenk, K. M., Munch, J. W., Streicher, R. P., Kaylor, W. H., and Kopfler, F. C. (1986) Mutagenic by-products from chlorination of humic acid. *Environ. Health Perspect.* **69**, 101–107.

(10) Kronberg, L., Christman, R. F., Singh, R., and Ball, L. M. (1991) Identification of oxidized and reduced forms of the strong bacterial mutagen (Z)-2-chloro-3-dichloromethyl)-4-oxobutenoic acid (MX) in extracts of chlorine-treated water. *Environ. Sci. Technol.* **25**, 99–104.

(11) Ishiguro, Y., LaLonde, R. T., Dence, C. W., and Santodonato, J. F. (1987) Mutagenicity of chlorine-substituted furanones and their inactivation by reaction with nucleophiles. *Environ. Toxicol. Chem.* **6**, 935–946.

(12) LaLonde, R. T., Perakyla, H., Cook, G. P., and Dence, C. W. (1990) The contribution of the 5-hydroxyl group to the mutagenicity of mucochloric acid. *Environ. Toxicol. Chem.* **9**, 687–691.

(13) LaLonde, R. T., and Xie, S. (1992) A Study of inactivation reactions of *N*-acetylcysteine with mucochloric acid, a mutagenic product of the chlorination of humic substances in water. *Chem. Res. Toxicol.* **5**, 618–624.

(14) Maron, D. M., and Ames, B. N. (1983) Revised methods for the Salmonella mutagenicity test. *Mutat. Res.* **113**, 173–215.

(15) LaLonde, R. T., Cook, G. P., Perakyla, H., Dence, C. W., and Babish, J. C. (1991) *Salmonella typhimurium* (TA100) mutagenicity of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone and its open- and closed-ring analogs. *Environ. Mol. Mutagen.* **17**, 40–48.

(16) Padmapriya, A. A., Just, G., and Lewis, N. G. (1985) Synthesis of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone: A potent mutagen. *Can. J. Chem.* **68**, 828–832.

(17) Fishbein, P. L., and Moore, H. W. (1990) 7-Chloro-7-cyanobicyclo-[4.2.0]octan-8-one. In *Organic Synthesis* (Paquette, L. A., Ed.) Vol. 69, pp 205–211, Organic Synthesis, Inc., Division of Organic Chemistry, American Chemical Society, Indiana University, Bloomington.

(18) Martin, G. E., and Zektzer, A. S. (1988) *Two-Dimensional NMR Methods for Establishing Molecular Connectivity*, VCH Publishers, New York.

(19) Bernstein, L., Kaldor, J., McCann, J., and Pike, M. C. (1982) An empirical approach to the statistical analysis of mutagenesis data from the Salmonella test. *Mutat. Res.* **97**, 267–281.