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A Lactone Metabolite Common to the Carcinogens Dioxane, Diethylene Glycol and N-Nitrosomorpholine: Aqueous Chemistry and Failure to Mediate Liver Carcinogenesis in the F344 Rat

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Abstract

1,4-Dioxan-2-one, **1**, was synthesized and the equilibrium constant between it and the hydrolysis product 2-(2-hydroxyethoxy) acetic acid, **2**, was determined as $K_O = 70 \pm 3.5$ in acidic aqueous media, 25 °C, ionic strength 1M, (KCl), 5% by volume acetonitrile. The carboxylic acid dissociation constant of **2** was determined under the same conditions to be $pK_a = 3.31 \pm 0.02$. On the basis of these two determinations, the equilibrium constant between **1** and carboxylic acid anion, **3**, and the proton was calculated to be $K_{OA} = 0.034 \pm 0.002$ M. The stability of **1** was determined in the range of pH between 1 and 8.5 in buffered aqueous solutions under the conditions above by UV spectrophotometric methods and exhibited simple first order kinetics of decay. On the basis of buffer dilution plots, the values of k_o, the rate constant for solvent mediated decomposition, were determined. The plot of log k_o against pH is consistent with a three term rate law for solvolysis with a hydrogen ion catalyzed rate constant $k_{H+} = 1.1\ (\pm 0.1)\ M^{-1}\ min^{-1}$, a water catalyzed rate constant, $k_w = 9.9\ (\pm 0.5) \times 10^{-4}\ min^{-1}$, and a hydroxide ion catalyzed rate constant, $k_{OH} = 4.1\ (\pm 0.3) \times 10^4\ M^{-1}\ min^{-1}$. The $t_{1/2}$ for decay at pH = 7.0, at 25 °C, is ~2 h. Treatment of F344 rats with aflatoxin B_1 (AFB_1) (positive control) elicited the expected preneoplastic foci in the livers of each rat tested, while subsequent administration of **1** (a total of 1.32 g over 12 weeks) failed to statistically increase focal number or focal volume percent. In 8 rats administered **1** (1.32 g, 12 weeks) alone, no increase above background foci was detected. This study does not support compound **1** as a common carcinogen.

Introduction

The powerful rodent liver carcinogen N-nitrosomorpholine,[1–3] the much weaker rodent liver carcinogen dioxane[4–6] and the weak rodent bladder/mammary carcinogen diethyleneglycol[7, 8] are all to a significant degree metabolized in vivo to a common set of compounds, Fig. 1, at the carboxylic acid level of oxidation.[9–11] Studies with these carcinogens did not identify all of the metabolites depicted in Fig. 1, but their existence and distribution is dictated by the thermodynamic cycle depicted within Fig. 1. The lactone **1**,

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1,4-dioxan-2-one, Fig. 1, was purported to be the major metabolite from dioxane in rats.[11] The chemistry and biological activity of **1** is also of interest as monomeric **1** is used in the synthesis of polymers employed as resorbable sutures.[12]

The role of lactone **1** in N-nitrosomorpholine carcinogenesis or its promotion has not been considered. DNA damage from a major metabolite of N-nitrosomopholine, 3-hydoxy-N-nitrosomorpholine, has been recently demonstrated via the formation of covalent adducts with DNA derived of diazonium ions intermediates.[13]

Lactone **1** has been speculated to be a genotoxic agent in dioxane and dietheylene glycol carcinogenesis, on the basis of structural analogy with other lactones that manifest DNA damage,[11] but evidence of genotoxicity by either agent or their metabolites is weak. In 2007, the U.S. Agency for Toxic Substances and Disease Registry released the "Draft Toxicological Report for 1,4-Dioxane"[14] that summarizes that only 2 of 17 in vitro studies, most employing xenobiotic metabolizing fractions, elicited positive indications of genotoxicity in the form of mutation, DNA damage, chromosomal aberrations or sister chromatid exchange (SCE). One of these two studies was considered weakly positive for SCE. A study not included in the Draft reiterates a lack of mutagenesis in the Ames assay with or without S9 fraction.[15] As summarized,[14] in vivo studies, involving doses with rats at, or above, the gram/kilogram body weight range, are somewhat more positive with respect to genotoxicity. Five of 15 such studies document mainly clastogenic activity.

The present report in part summarizes work carried out to determine the stability of lactone **1** preparatory to its *in vivo* testing, additionally reported here, regarding its ability to induce or promote liver carcinogenesis in the rat.

In the F344 rat, hepatic cancer and the process of cancer formation (i.e., liver carcinogenesis) can be initiated by a number of chemicals. The liver carcinogen aflatoxin B1 (AFB_1) is a secondary metabolic product of the mold *Aspergillus flavus* and AFB_1 is a potent hepatotoxin and carcinogen in the F344 rat and many other experimental animals, livestock and humans.[16–19] The F344 rat is highly sensitive to AFB_1 and the cancer process (*i.e.,* liver carcinogenesis) has been extensively studied. The early putative preneoplastic foci that are induced by AFB_1 can readily be quantified[20, 21] and both foci size and focal number in the liver can be measured. Experiments with the F344 rat provide examples of the growth of AFB_1-induced foci being enhanced[22] or suppressed[20, 21, 23–26] by feeding chemicals subsequent to damaging DNA with AFB_1. There is additional evidence that these agents ultimately modify (increase or suppress) the occurrence of actual liver cancer and not just the precursor foci.[27, 28] Lactone **1** was evaluated both for its ability to induce early putative preneoplastic lesions (henceforth termed foci) that ultimately give rise to frank hepatocellular cancers as well as the ability of lactone **1** to enhance or promote the growth of foci that have been induced by AFB_1. In neither case did **1** exhibit measurable activity.

Experimental

Chemicals

Except as noted, chemicals, solvents and isotopically enriched reagents employed in synthetic and analytical procedures were obtained from readily available commercial sources. Deionized water was obtained in house and filtered for HPLC. Acetonitrile was dried and distilled from calcium hydride. Tetrahydrofuran was dried and distilled from sodium. HPLC solvents were purchased as 'HPLC grade" and were not further purified.

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Analytical determinations

Measurements of pH were performed on an Orion pH meter, model SA720, with a combination electrode. Two point calibrations were done before pH values were recorded. Calibrations were performed using commercially available standards or 0.10 M HCl, pH = 1.10, as appropriate. Elemental analyses were performed by Atlantic Microlabs, Inc., Norcross, GA.

The equilibrium constant between **1** and its open-chain acid form was determined by measuring, by means of HPLC, the amount of **1**, calculated by interpolation from a standard curve, that remained after equilibration of an acidic aqueous solution of **1**. The initial concentration was gravimetrically determined. The equilibrium constant was determined as a function of ionic strength, by addition of KCl and hydrogen ion concentration in aqueous solutions that were 5% by volume acetonitrile and initially contained 0.05 M lactone.

The pK_a of (2-hydroxyethoxy)acetic acid was determined by measuring the pH of solutions of the carboxylic acid anion (Na^+ salt) that were partially neutralized by HCl. The pK_a measurement was made at various ionic strengths between 0.05 and 1M with a concentrated KCl stock. Final solutions contained 5% by volume acetonitrile. The resulting buffers varied in concentration between 0.07 and 0.08 M. Values of pH were recorded at two temperatures, the meter standardized at pH = 7.00 and 1.10 at the appropriate temperatures. Average values of pK_a were calculated from the Henderson-Hasselbalch equation. No correction was made for the existence of the lactone, which would have necessitated changes that were within the standard deviation of the initial determinations.

Kinetics

The decay of **1** was initially monitored in D_2O by observing, by ^{1}H-NMR, the loss of intensity of the overlapping resonances (4.78 ppm) for the hydrogens attached to C-6 at 25 °C in 0.005 M DCl, 0.005 M **1** and 0.005 M acetonitrile as an internal standard. The kinetics of decay, under identical conditions, of absorbance at 225nm was monitored, for comparison, by UV spectrometry. The good agreement, see Results, of these two measurements validated an extended kinetic analysis by UV spectrometry monitoring absorbance at 220, 225, 230, or 235 nm. The reaction conditions for the extended study were 5% by volume acetonitrile in water (H_2O), ionic strength 1 M (KCl), 25 °C and **1** at an initial concentration of 0.0013 M. Solutions were buffered with formic, acetic, cacodylic and ethylphosphonic acid buffers. Buffer dilution plots of k_{obsd} against buffer concentration, containing at least 4 buffer concentrations, were constructed from first order decay curves monitored for at least 3 $t_{1/2}$ of reaction. Such plots were linear and, from extrapolation to zero buffer concentration, the buffer independent rate constant, k_o, was derived. The associated buffer pH was taken as the average pH value of the measured values, at reaction endpoint, of all the buffer concentrations in a given experiment. Occasional checks of the pH, at t ~ 0, indicated no significant change in pH in the course of the reaction.

Synthesis. 1,4-dioxane-2-one (1)

A modification of an earlier preparation was employed.[12] A 2 L three-neck flask chilled in an ice bath and containing 134 g of 2-(2-methoxyethoxy)acetic acid (2 mol) was fitted with a reflux condenser and a stopper. To the remaining neck of the flask was fitted a chilled (0 °C) addition funnel containing 113 mL (3 mol eq) of chilled (0 °C) 62 % aqueous hydrobromic acid solution, which was immediately added dropwise to the stirred round bottom flask. The reaction mixture was maintained on ice for another 30 min. The reaction mixture was then stirred at room temperature for 1 hr, and subsequently heated with the temperature gradually rising to reach 150 °C over 2 hr. The temperature was then increased to 180 °C and the solution was refluxed overnight. The reaction was subsequently cooled to

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room temperature, and the pH was adjusted to 3 with $NaHCO_3$. The lactone was purified by subsequent vacuum distillation at bp 70–75 °C at 0.8 mm Hg to yield a product of 90–95 % purity based on NMR. Further recrystallization from tetrahyrofuran yielded 60 % (61 g) of colorless needles (T_m = 26.7 °C). ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ = 4.49 (*t*, 2H), 4.37 (s, 2H), 3.87 (*t*, 2H). ^{13}C NMR: 166.62, 68.63, 66.42, 62.70. *Anal.* Calcd for $C_4H_6O_3$: C, 47.06; H, 5.92; Found: C, 46.90; H, 5.94.

Sodium 2-hydroxyethoxy acetate (3-Na$^+$)

The procedure for synthesis of **1**, above, was initiated. Subsequent to the overnight reflux, the solution was cooled to room temperature and neutralized with 50 % aqueous NaOH. The mixture was diluted with 150 mL absolute ethanol and heated at 80 °C for 15 min. The solution was allowed to cool to room temperature with stirring. Stirring was continued until a white precipitate formed. Crystallization was allowed to proceed at 4 °C. The white needles were collected and rinsed with cold absolute ethanol to yield the desired product in 66% final yield. Mp = 206.5 °C. ^{1}H NMR (400 MHz, D_2O, ppm): δ = 3.83 (s, 2H), 3.60 (*t*, 2H), 3.50 (*t*, 2H). ^{13}C NMR: 178.19, 71.80, 60.50. *Anal.* Calcd for $C_4H_7O_4Na$: C, 33.81; H, 4.97; Found: C, 33.84; H, 5.00.

Animal Studies

Male F344 rats (90–100g) were purchased from Charles River Laboratories (Wilmington, MA) and allowed 2 weeks to acclimatize to the animal facility prior to treatment. Rats were fed the highly purified diet AIN-76A (American Institute of Nutrition) without the added antioxidant ethoxyquin (Harlan, Madison, WI). Throughout the experiments, both food and water were available *ad libitum*. The experimental protocol is schematically illustrated in Figure 2. At 5 weeks of age (about 125g body weight), all rats were gavaged with either AFB_1 (25 μg/rat) or vehicle (tricaprylin) 5 days per week for 2 successive weeks for a cumulative dose of 250 μg AFB_1 per rat. Commencing the week immediately following AFB_1 treatment, rats were gavaged with lactone **1**, dissolved in water immediately prior to use, three days (MWF) per week for 12 weeks. In the first week, the doses of dioxane were each 0.02g/rat for each of the three days. This dose was equal to or less than 15% of the reported LD_{50} dose.[29] In the subsequent 11 weeks, all doses were 0.04g/rat and they were equal to or less than 26% of the reported LD_{50} dose.

At 12 hours prior to autopsy, food was withdrawn to reduce the glycogen accumulation in the livers. From the left lateral hepatic lobe, multiple 2 mm thick sections were cut by hand, fixed in acetone at 4°C, embedded in paraffin, and processed by routine histological methods. Hepatic sections (5 μm thick) were stained by standard immunohistochemical methods for expression of glutathione S-transferase-placental isoform P (GST-P) foci and the foci were identified and analyzed by light microscopy. As with previous analyses[21, 24, 26], the observed focal data of number of foci per unit area of tissue examined and their individual focal transactional areas were subjected to morphometric transformation resulting in foci per cm^3 liver tissue, mean focal diameter and the volume percent of liver occupied by GST-P positive foci, a parameter analogous to tumor burden. Extensive details of these procedures have been published previously.[20, 26, 30]

Statistical analyses

Focal data (foci per cubic cm, mean diameter, and volume %) were statistically analyzed using a t-test (Stata Corp., College Station, TX).

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Results

Equilibria

Values for the equilibrium constant K_O, Fig. 1, at 25 °C under a variety of ionic strength and acid conditions, are listed in Table 1. The data were obtained by allowing **1** and **2** to equilibrate, the initial concentration of **1** having been gravimetrically determined, and measuring the amount of **1** remaining by reverse phase HPLC on the basis of interpolation from a standard curve for **1**.

Values for the pK_a of **2** under a variety of conditions of ionic strength and temperature are listed in Table 2. The values of pK_a were determined by measuring values of pH of partially neutralized solutions of the sodium salt of **3** the concentration of which was gravimetrically determined. On the basis of the observed pH, the ratio HA/A- was adjusted for the fractional ionization and the pK_a was calculated therefrom by use of the Henderson-Hasselbalch equation. No correction was made for the presence of **1** (see Discussion).

Kinetics

The kinetics of decay of **1** were initially determined by ^{1}H-NMR by monitoring the disappearance of hydrogen resonances at position 6 of the lactone in D_2O solutions containing 0.005 M DCl at 25 °C. In addition, decay of a shoulder of absorbance between 220 and 240 nm was monitored by UV spectrophotometry under identical conditions. Both processes exhibited good first order decay for 2.5 $t_{1/2}$ of decay, data not shown, and rate constants that were the same within experimental error: 0.017 ± 0.002 min^{-1} (NMR) and 0.016 ± 0.001 min^{-1} (UV). The good agreement validated a more extensive study using the more facile UV method with reactions in H_2O solutions containing 5% by volume acetonitrile.

Figure 3A depicts the decrease in the UV absorbance spectrum as a function of time in a cacodylic acid buffer, 90% acid form, spanning more than three $t_{1/2}$ of decay. The change in absorbance at 225 nm against time for this experiment is indicated in Fig. 3B and includes an overlaid fit to a simple firstorder decay process. Plots of k_{obsd} as function cacodylic acid concentration are plotted in Fig. 3C. Generally these plots contained a minimum of four points at buffer concentrations of buffer of less than 0.2 M total buffer concentration. Such plots were typically linear and permitted assignment, by extrapolation of a value of k_o, the buffer independent rate constant of hydrolysis. For any given plot, the largest value of k_{obsd} generally did not exceed the value of k_o by more than a factor of 7.

Carcinogenesis

The results of the animal studies are shown in Table 3. Large numbers of GST-P positive foci were observed in all rats treated with AFB_1 (groups 1 (AFB_1) and 2 (AFB_1 + **1**)) and the average size of these observed foci (focal transactional areas) was similar for the two groups treated with AFB_1 (>200μm - data not shown). Only one of 8 rats in Group 3 (**1** only) had a single focus and that individual focus was small (65 μm diameter) compared to the average foci induced by AFB_1 (>200μm). Rats treated with vehicle only, Group 4, had no foci. Observed focal data are inherently biased as large foci are over represented; thus, these data were subjected to morphological transformation. For number of foci per volume of liver or the mean focal diameter of the foci, there was no statistically significant difference between groups 1 and 2. Livers of rats not receiving AFB_1 (groups 3 and 4) had few if any foci. The volume % of liver occupied by GST-P positive foci (i.e., focal volume %) is the most robust measure and is analogous to tumor burden. There was no statistically significant difference in foci number, size or volume % between groups 1 and 2 and groups 3 and 4.

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Discussion

Equilibria

The equilibrium constant K_o, Table 1, is comparatively large and in favor of the open chain carboxylic acid, relative to the lactone. A lower limit value of $K_O > 50$, not inconsistent with the values in Table 1, was obtained by monitoring the disappearance of the lactone multiplets in D_2O, 1 M $DClO_4$, to equilibrium. At endpoint the lactone multiplets were barely visible above background preventing the determination of an actual value. The values determined under the varying conditions establish that there is a negligible effect of varying ionic strength on the equilibrium constant K_O, as was to have been expected given the absence of charged species on either side. The values of K_O, Table 1, are slightly to significantly larger than those reported previously for a set of permethylated hexose- δ-lactones[31] and glucono- δ -lactone[32] which range from 54 to 1.0 in aqueous media. In contrast, smaller ring lactones can be more thermodynamically favorable than their open chain forms.[33, 34] The large value of K_O means that even in the most favorable, acidic, environment, were the acid and lactone to reach equilibrium, the concentration of the lactone would be just 1.4% of the sum of both forms.

The value of the apparent pK_a for ionization of the open chain acid was determined under a differing conditions of ionic strength and/or temperature on the basis of the measured pH of partially neutralized solutions of acid; and, inspection of the values in Table 2 suggests expected values and small changes in pK_a with conditions. In calculating the value, inclusion of the lactone concentration was neglected because the small concentration value affected the absolute value of pK_a by less than the experimental error in the determinations. By comparison, the value of the apparent pK_a of methoxyacetic acid has been reported as 3.31 at 25 °C, ionic strength 1M (KCl). Further, the apparent pK_a values at 25 °C at ionic strength 1 M ($NaClO_4$ or NaCl) for the permethylated hexose-δ-lactones and glucono-δ-lactone range from 3.31 to 4.20.[31, 32, 35] The effect of changes in ionic strength and temperature are small, consistent with expectations on the basis of what is known for the effects upon acetic acid ionization.[36, 37]

The determination of K_O and the pK_a permits the calculation of K_{OA}, Fig.1, as $K_{OA} = K_a \times K_O = 0.034 \pm 0.002$ M, in which the uncertainty is calculated as the error propagated in the multiplication. Thus, at equilibrium, at pH = 7, the lactone represents $\sim 3 \times 10^{-4}$ percent of the total species represented in Fig 1.

Kinetics

The kinetics of decay of lactone **1** were monitored in parallel by UV spectrophotometry and ^{1}HNMR in solutions of D_2O and the values of k_{obsd} were the same within the error of the determinations. This validated a more extended investigation in buffered H_2O solutions using the more facile UV method.

Generally the value of k_{obsd} varied as a function of buffer concentration according to equation 1, in which k_{buf} is the second order rate constant for

$$k_{obsd} = k_o + k_{buf}[\text{buffer}]_{total} \quad (1)$$

acceleration of the decay by total buffer species, while k_o is the buffer-independent rate constant, obtained by linear extrapolation of plots such as in Fig. 3c. While stimulation by the oxygen acid buffers studied was significant, relative to the buffer independent rate constants, in the range of buffer concentrations studied, a more detailed study and analysis of these constants was not attempted.

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The variation of the value of log k_o as a function of solution pH is presented in Fig. 4 in which the solid circles represent the actual values of k_o determined in this study. The data in Fig. 4 are best described by a rate law for the variation of log k_o with pH that contains three kinetic terms, as indicated in equation 2, one involving the hydrogen ion, k_{H+}, a pH-independent term, k_w,

$$k_o = k_{H+}[H^+] + k_w + k_{OH}[OH^-] \quad (2)$$

and one involving hydroxide ion, k_{OH}. The best fit to the data of the rate law of equation 2 is indicated by the solid line in Fig. 4 with rate constant of k_{H+} = 1.1 (± 0.1) M^{-1} min^{-1}, k_w = 9.9 (± 0.5) × 10^{-4} min^{-1} and k_{OH} = 4.1 (± 0.3) × 10^4 M^{-1} min^{-1}. The best fit to an alternative two-term rate law containing only terms for the hydrogen and hydroxide ions is indicated by the dashed line in Fig. 4 and underestimates the rate constants observed in pH range from 3.5 – 6 by rate factors as large ~4, well outside the experimental error of the determinations.

The reactivity of lactone **1** with solvent species is comparable with what has been observed for other lactones.[31, 32] D-Glucono-δ-lactone is more reactive than **1** by less than a factor of 3 in the hydrogen ion, hydroxide ion and water-catalyzed hydrolyses. Similarly, in all but one case, the rate constants for the hydrolysis reactions of permethylated glycono-δ-lactones are within a factor of five of those determined here for **1**. In the case of tetra-O-methylmannono-δ-lactone, the rate constant for hydrogen ion catalyzed hydrolysis of 1 is faster by a factor of 12, though a reason for this is not clear. Surprisingly, **1** is ~1500 more reactive toward hydroxide ion than valerolactone,[38] perhaps reflecting greater electrophilicity and better leaving group ability in the former, and possibly a difference in rate limiting step on the reaction coordinate.

Though the equilibria determined above substantially disfavor the lactone **1**, the kinetics indicate a chemical stability that is sufficient to transit the extremes of the digestive tract. The $t_{1/2}$ for decay of **1** at pH 2 and 7 is 57 min and 127 min, respectively. The $t_{1/2}$ at pH = 7 is within a factor of three of those for decay of N-nitroso-N-methylurea[39] and N-methyl-N'-nitro-N-nitrosoguanidine[40] while the $t_{1/2}$ at pH = 2 is within a factor of 3 of trimethyl-N-nitrosourea.[39] Each of these N-nitroso compounds is a documented rodent carcinogen when orally administered.[1, 41–43] These solvolysis studies further established the feasibility for designing protocols for the animal studies described below.

Carcinogenesis in the rat

As expected with this rat model of liver carcinogenesis, the small total dose and short dosing schedule of AFB_1 (see Fig 2) resulted in large numbers of hepatic foci (Table 3, group1); however, subsequent exposure to lactone **1** did not modify the number of foci (group 1 vs group 2) that developed nor were their average focal size altered. Additionally, exposure to only lactone **1** resulted in no significant initiation of hepatic foci. One very small focus was encountered in the tissue sections of 1 of 8 rats receiving the cumulative total dose of 1.32 g of lactone **1**. Small spontaneous foci are occasionally observed in control rat livers.[30] For example, in a group of control rats exposed to no known carcinogen, but similar in age and sex to rats of this study, one spontaneous focus was observed and it was 2.5 times as large as the one observed in this study.

It is certainly possible that larger doses of lactone **1** treated over a more lengthy study period with examination of rats living to much older ages might have provided evidence of hepatic carcinogenesis, but this study does not presage such an outcome.

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Alternative genotoxins by which diethylene glycol, dioxane and N-nitrosomorpholine might manifest their carcinogenicity are the aldehyde preceding formation of **1**, aldehydes from the further metabolism of **1–3** and, in the case of N-nitrosomorpholine, the diazonium ion that has been proven to alkylate DNA and other aldehydes derived from diazonium ion fragmentation.[44]

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Non-standard Abbreviations

AFB_1	aflatoxin B1
HPLC	high-performance liquid chromatography
MWF	Monday-Wednesday-Friday
GST-P	glutathione S-transferase-placental isoform P

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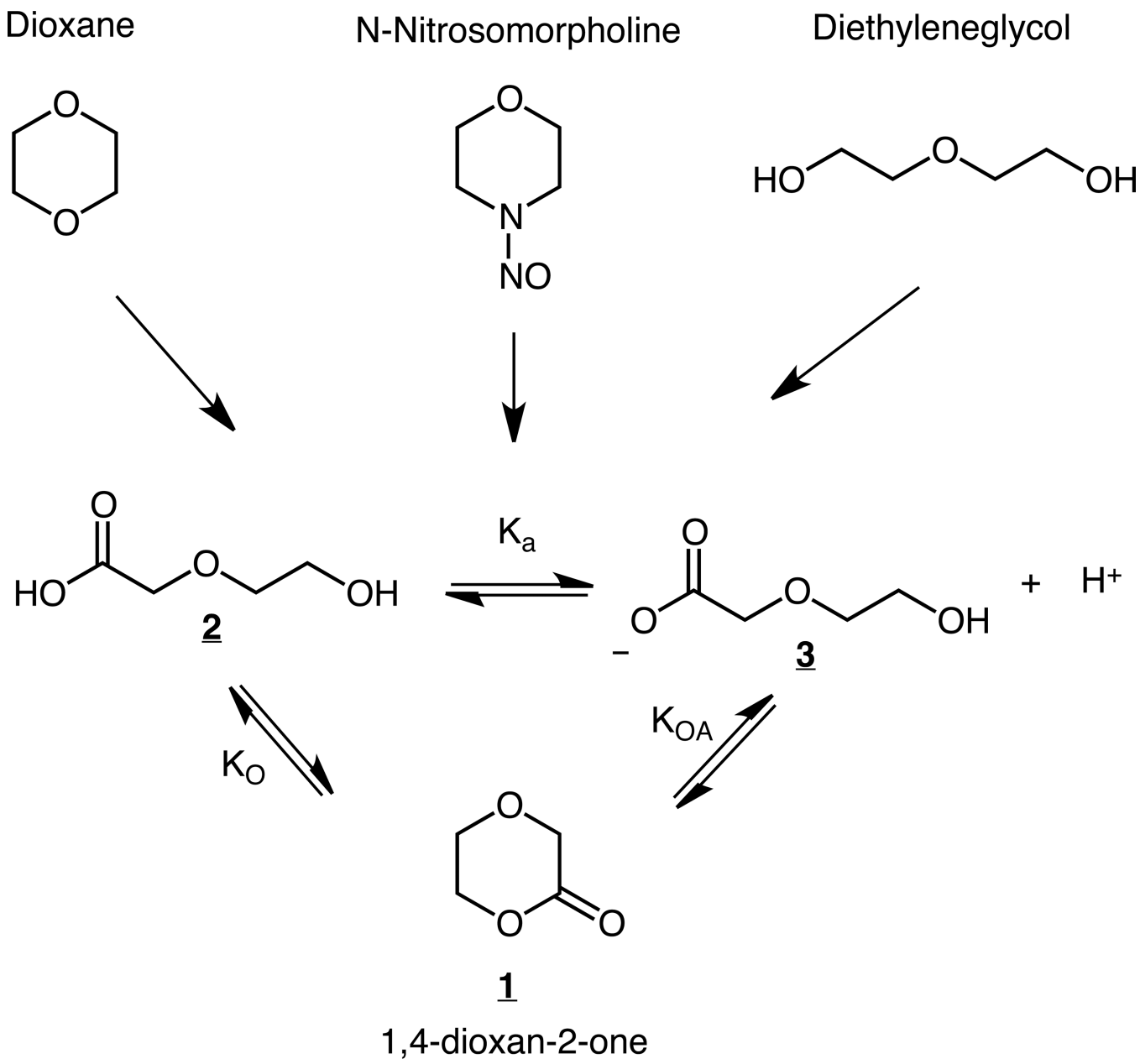

Figure 1.
Three carcinogens share a common set of metabolites.

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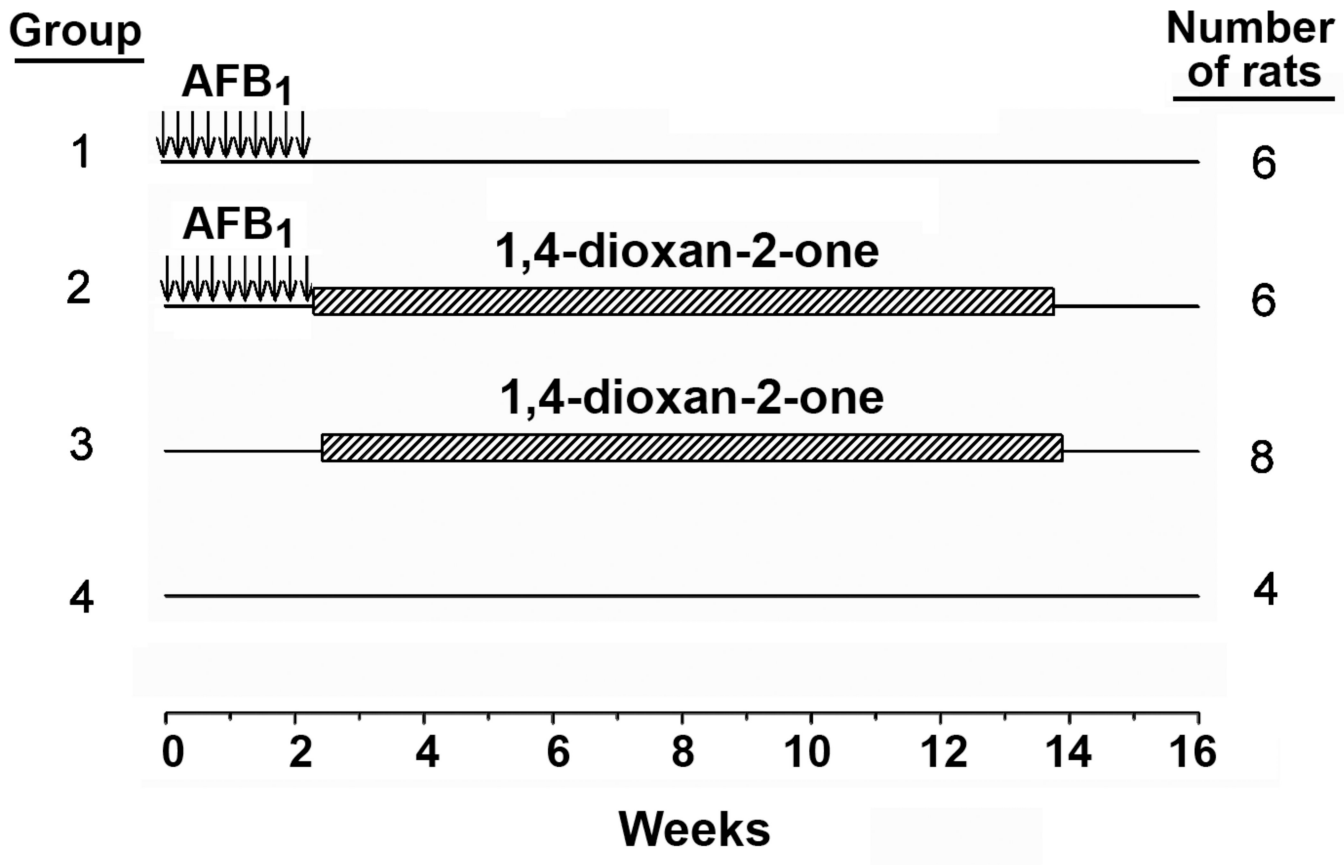

Figure 2.
Carcinogenesis treatment protocol. Aflatoxin B_1 (AFB_1) treatment is indicated by the arrows (groups 1 and 2) and 1,4-dioxane-2-one treatment is indicated by the hatched bars (groups 2 and 3). Group 4 is a vehicle treatment group.

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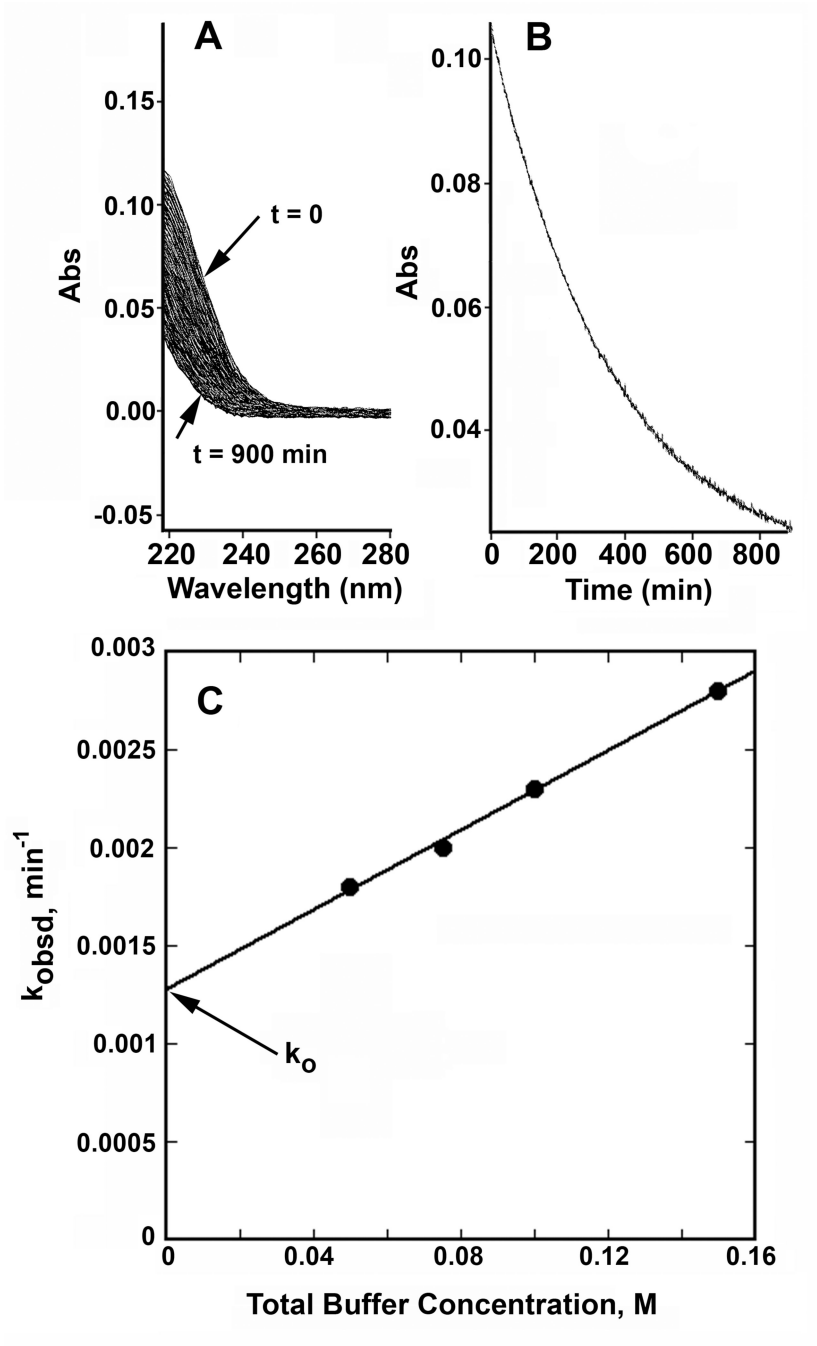

Figure 3.

Kinetics of decay of 1,4-dioxan-2-one in aqueous solutions, 5% by volume acetonitrile, ionic strength 1 M, KCl, cacodylic acid buffer, 90% acid form. A. Change in UV spectrum as a function of time, total buffer concentration = 0.08 M. B. Semi-logarithmic plot of absorbance change in A as a function of time, fit to a first order process is overlaid on the data. C. Change in k_{obsd} as a function buffer concentration extrapolated to the intercept to derive the value of k_o, the buffer independent rate constant.

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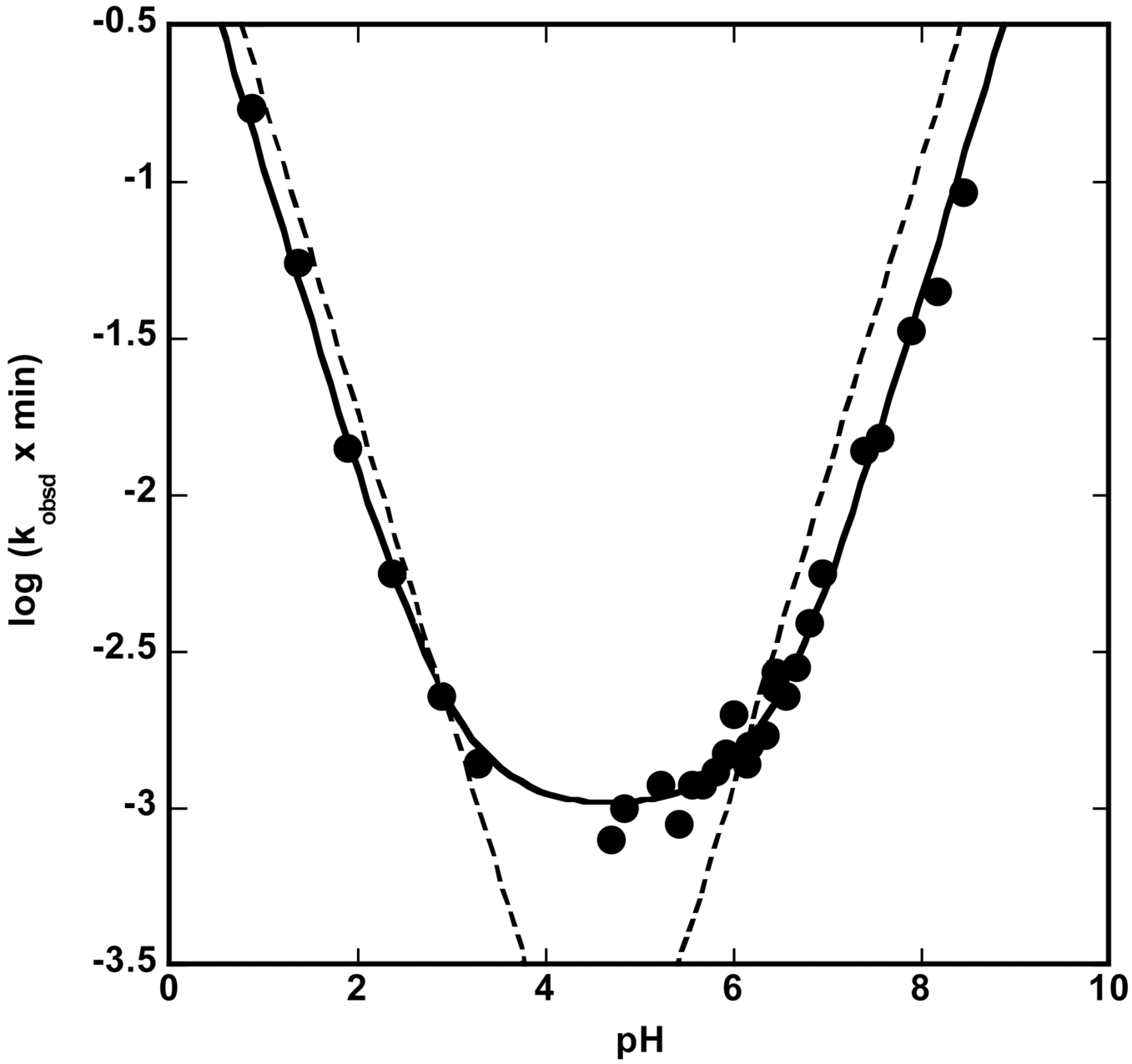

Figure 4.

Plot of the log of k_o as function of pH. The solid line is a best fit to a three-term rate law, eq 2, for the solvent mediated hydrolysis. The dashed line is the best fit to a two-term rate law for solvent mediated hydrolysis.

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Table 1

Values of K_o, the equilibrium constant for lactone **1** and its open-chain acid form, determined at 25 °C.

[HCl], M	Total Ionic Strength[a], M	K_o[b]
0.05	0.05	71
0.05	0.15	72
0.05	0.25	71
0.05	0.45	71
0.05	0.55	70
0.10	1.0	70
0.20	1.0	70

[a] Beyond [HCl], the balance of the ionic strength was maintained with KCl.

[b] The standard deviation of each determination is <5% on the basis of triplicate injections.

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Table 2

Determination of pK_a values for **2** under varying conditions of ionic strength and temperature.

Ionic Strength, M [a]	Observed pH [b]	Calculated pK_a [c]
0.05	3.44 ± 0.01	3.43
0.25	3.37 ± 0.01	3.36
0.35	3.37 ± 0.01	3.36
0.45	3.37 ± 0.01	3.36
0.55	3.39 ± 0.01	3.38
1.0 [d]	3.31, 3.58, 3.81 [e]	3.31 ± 0.02 [f]
1.0 [d]	3.27, 3.54, 3.76 [g]	3.27 ± 0.01 [h]

[a] Unless otherwise stated, the total buffer concentration was 0.05 M, at a prepared buffer ratio of AH/A- = 1.0. The balance of the ionic strength was maintained with KCl.

[b] Unless otherwise stated the value is the mean and standard deviation of duplicate determinations at 25 °C.

[c] Unless otherwise stated, the value is the pK_a calculated, for T= 25 °C, from the observed pH, the buffer ratio, corrected for the fractional dissociation of the acid form, and the Henderson-Hasselbalch equation.

[d] For buffer concentrations between 0.070 and 0.080 M. The balance of ionic strength was maintained with KCl.

[e] The pH values refer to measured values, at 25 °C, for buffers prepared at a buffer ratio of HA/A- = 1.0, 35/65 and 1/3, respectively.

[f] The value of pK_a, at 25 °C, is the mean and standard deviation of the pK_a values calculated from the observed pH values at the three buffer ratios, corrected for the fractional dissociation of the acid form, using the Henderson-Hasselbalch equation.

[g] The pH values refer to measured values, at 37 °C, for buffers prepared at a buffer ratio of HA/A- = 1.0, 35/65 and 1/3, respectively.

[h] The value of pK_a, at 37 °C, is the mean and standard deviation of the pK_a values calculated from the observed pH values at the three buffer ratios, corrected for the fractional dissociation of the acid form, using the Henderson-Hasselbalch equation.

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Table 3

Observed and morphometric data for F344 rats dosed with AFB_1 (group 1), AFB_1 followed by **1** (group 2), **1** only (group 3) and vehicle (group 4).

		Observed		Morphometric Data	
Group	Number of Rats	Mean Foci Counted[a]	Foci per cubic cm[a]	Mean Diameter[a] (microns)	Volume %[a]
1	6	44 ± 8	1230 ± 410	227 ± 35	3.1 ± 1.0
2	6	40 ± 3	1190 ± 230	210 ± 32	2.1 ± 0.7
3	8	0.1 ± 0.1	6.1 ± 6.1	65	0.001
4	4	0	0	---	0

[a]Values reported are mean ± standard error of the mean.