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Synthesis and Aqueous Chemistry of α-Acetoxy-*N*-nitrosomorpholine: Reactive Intermediates and Products

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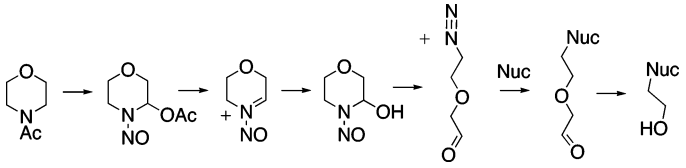

α-Acetoxy-*N*-nitrosomorpholine (**7**) has been synthesized starting by the anodic oxidation of *N*-acetylmorpholine in methanol. The 55% yield of *N*-nitrosomorpholinic acid, after cyanide-for-methoxy group exchange and hydrolysis, is an improvement of ∼10-fold over our original 10-step method, and this is readily converted to **7**. A study of the kinetics of decomposition of **7** in aqueous media at 25 °C and 1 M ionic strength was conducted over the pH range from 1 to 12. The reaction exhibited good first-order kinetics at all values of pH, and a plot of the log of k_0, the buffer-independent rate constant for decomposition, against pH indicated that a pH-independent reaction dominates in the neutral pH region whereas acid- and base-catalyzed reactions dominate in the low and high pH regions, respectively. Reaction at neutral pH in the presence of increasing concentrations of acetate ion results in a decrease in the value of k_{obsd}, to an apparent limiting value consistent with a common-ion inhibition by the capture, and competing base-catalyzed hydration of, an *N*-nitrosiminium ion intermediate. The 100-fold smaller reactivity of **7** at neutral pH compared with its carbon analogue, α-acetoxy-*N*-nitrosopiperidine, is also consistent with the electronic effects expected for such a reaction. The dinitrophenylhydrazones derived from pH-independent and acid-catalyzed reactions are identical in kind and quantity, within experimental error, to those observed in the decay of α-hydroxy-*N*-nitrosomorpholine. Decay of **7** in the presence of benzimidazole buffer results in the formation of 2-(2-(1*H*-benzo[*d*]imidazol-1-yl)ethoxy)acetaldehyde (**12**) and 2-(1*H*-benzo[*d*]imidazol-1-yl)ethanol (**13**). Independent synthesis and study of **12** indicates that it is stable at 80 °C in 0.1 M DCl, but it slowly decomposes to **13** in neutral and basic media in a reaction that is stimulated by primary and secondary amines, but not by tertiary amines and carbonate buffer. The benzimidazole trapping studies and those of the stability of **12** indicate the possibility that metabolic activation of *N*-nitrosomorpholine by hydroxylation α to the nitroso nitrogen can result in the deposition of a metastable ethoxyacetaldehyde adduct on the heteroatoms of DNA.

Introduction

Nitrosomorpholine (NMOR, 1, Scheme 1) is a potent liver and esophageal carcinogen in experimental animals[1,2] to which there is human exposure through foods,[3] groundwater,[4] personal care products,[5] and endogenous formation,[6,7] as well as in certain workplace environs such as the rubber industry.[8−10] Epidemiological evidence indicates that exposure to nitrosamines in rubber manufacture correlates with incidence of a variety of cancers.[11]

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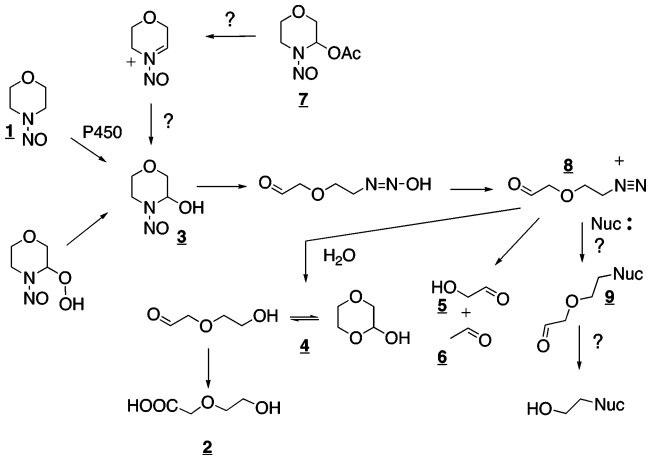
SCHEME 1



There is evidence, in the isolation of **2** from rats dosed with NMOR, that NMOR is metabolized in part through the classical p450 hydroxylation reaction, α to the nitroso group, as indicated in Scheme 1 (compound **3**).[12] There is also evidence that **1** undergoes hydroxylation adjacent the oxygen, the consequences of which have been a focus of the Loeppky group.[13−20] It was recently demonstrated, in contrast to what had earlier been reported,[21] that the unstable **3**, generated from the hydroperoxide (Scheme 1), forms the signature fragment **4** as the major product of decomposition in aqueous media, as well as lesser amounts of the products of diazonium ion rearrangement and fragmentation, **5** and **6**.[22]

To further study the chemistry of the metabolite **3**, a stable source is of interest, and the α-acetoxynitrosamine **7** is an obvious candidate. α-Acetoxynitrosamines are widely used as presumed equivalents to the α-hydroxynitrosamines.[1,2,23] Unadorned cyclic and acyclic α-acetoxynitrosamines generally decompose by S_N1 solvolysis via short-lived *N*-nitrosiminium ion intermediates that, upon hydration with attack on carbon, give rise to the α-hydroxy compounds, as hypothetically indicated for **7** in Scheme 1.[24−31] Attack on the cation at the nitroso nitrogen can be competitive with attack at carbon, and consequent denitrosation instead yields the imine with no diazonium ion-generating potential. Attack on the nitrogen atom of the nitrosiminium ion is generally a minor reaction, the extent varying, but not predictably, with structure. In some cases, attack at nitrogen becomes a substantial pathway, so that the α-acetoxy compound is not a true "equivalent" to the α-hydroxynitrosamine.[32] As we have a need to study the reaction products of the decomposition of the unstable **3** in the presence of biomolecules, use of **7** as a precursor requires validation that **7** indeed decomposes via **3**, in good yields.

Potential nucleoside and protein adducts of the diazonium ion **8**, derived from the decomposition of **3**, are of interest, as they might be of biological significance and could be important as biomarkers of nitrosomorpholine exposure, but their existence is uncertain. The related diazonium ions derived from *N*-nitrosopyrrolidine (NPYR) and *N*-nitrosopiperidine (NPIP) exhibit divergent behaviors in this regard, according to the work of Hecht and colleagues.[33−36] The diazonium ion from α-hydroxylation of NPYR reacts directly with a number of purine heteroatoms. To date, however, that derived from the α-hydroxylation of NPIP has not been shown to undergo direct substitution by nucleosides, possibly because of the rate at which it undergoes intramolecular cyclization by carbonyl (or hydrate) attack to give the cyclic form analogous to **4**.

Little is known of the existence and source of DNA adducts from NMOR. It has been demonstrated that N7-(hydroxyethyl)-Gua forms in rats dosed with NMOR.[37] This lesion could be derived from alternative modes of metabolic activation of NMOR, according to the extensive work of the Loeppky group,[16−18,38] but some reflection suggests the possibility that such an adduct might derive after deposition of the ethoxyacetaldehyde adduct, **9**, derived from the diazonium ion **8** (Scheme 1).

In the present report, the following three facts relevant to the question marks in Scheme 1 are established: (a) α-Acetoxy-*N*-nitrosomorpholine generates **3** in essentially quantitative yield

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TABLE 1. Pseudo-First-Order Rate Constants for the Solvolysis of α-Acetoxy-*N*-nitrosomorpholine at Varying Concentrations of Cacodylic Acid (pH = 5.16), 2-(Cyclohexylamino)ehane-2-sulfonic Acid CHES (pH = 8.51), and Bicarbonate (pH = 9.78) Buffers at 25 °C, 1 M Ionic Strength (KCl)

	$10^5 \times k_{obsd}$ (s^{-1})		
buffer concentration (M)	cacodylate	CHES	bicarbonate
0.05	6.1	6.3	18.4
0.1	5.9	6.3	18
0.15	6	6.2	17.6
0.2	5.9	6.2	17.1

via S_N1 solvolysis. (b) The diazonium ion **8** is trapped by the model nucleophile benzimidazole, presaging the formation of analogous adducts derived from nucleosides and proteins. (c) The resulting ethoxyacetaldehyde **9** indeed converts to the hydroxyethyl adduct (Scheme 1) at a low rate in aqueous solution at physiological pH in a reaction that is stimulated by primary and secondary amines. Last, a number of literature procedures have been refined and combined to permit an order-of-magnitude improvement in the efficiency of the synthesis of the α-acetoxy-*N*-nitrosomorpholine **7**.

Results

Synthesis. Anodic oxidation of *N*-acetylmorpholine, as reported earlier,[39] proved to be a simple and efficient method for functionalizing the morpholine ring to the 3-methoxy-substituted derivative (**10**). The subsequent BF_3-catalyzed nitrile substitution also proceeded in good crude yield as indicated by ^1H and ^{13}C NMR analysis. Though previously reported to occur under acidic conditions, the nitrile hydrolysis proved futile in our hands under the given conditions.[39] We therefore reverted to a basic hydrolysis, which rapidly generated the desired carboxylate, and the amide was subsequently hydrolyzed with acid. The overall yield, from acetyl morpholine to the *N*-nitrosomorpholine carboxylic acid (**11**), was 55%.

Kinetics. α-Acetoxy-*N*-nitrosomorpholine (7). Over the pH range 1−12, solvolysis of **7**, monitored at 325 nm, 25 °C, 1 M ionic strength (KCl), exhibited good first-order kinetics of decay for 3−5 half-lives. The values of k_{obsd} varied only slightly with buffer concentration at constant buffer ratio, with the exception of acetate buffer (addressed below), typically changing by less than 10% over a concentration range of 0.05−0.2 M. Typical effects of changes in buffer concentration are indicated by the data summarized in Table 1. The values of k_0, the apparent first-order rate constant for buffer-independent decay, were obtained as the *y* intercepts of plots of k_{obsd} vs buffer concentration. The values of k_0 varied with pH and are reported in Table 2.

The effect of acetate ion concentration on the solvolysis of α-acetoxy-*N*-nitrosomorpholine was studied at 25 °C, 4 M constant ionic strength (KCl), over the range of acetate ion concentration of 0−3 M, pH 6.53. Acetate ion inhibits the solvolysis, as indicated by the solid circles in Figure 1, apparently to a limiting degree indicated by the leveling of the effect at high acetate ion concentration. Data previously published for α-acetoxy-*N*-nitrosopyrrolidine (squares) and α-acetoxy-*N*-nitrosopiperidine (triangles) are included in Figure 1 for comparison.[30]

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TABLE 2. Values of k_0, the Buffer-Independent First-Order Rate Constant for the Solvolysis of α-Acetoxy-*N*-nitrosomorpholine as a Function of pH at 25 °C, 1 M Ionic Strength (KCl)

pH	$10^5 \times k_0$ (s^{-1})	pH	$10^5 \times k_0$ (s^{-1})
0.99	47	6.31	5.8
1.1	35	8.16	6.1
1.24	27	8.49	7
1.61	19.5	8.73	6.7
2.11	10.1	9.02	7.5
2.56	6.8	9.37	9.4
3.49	6.1	9.78	19.3
3.71	6.8	10.3	33
4.51	5.7	12.1	1520
5.25	5.8		

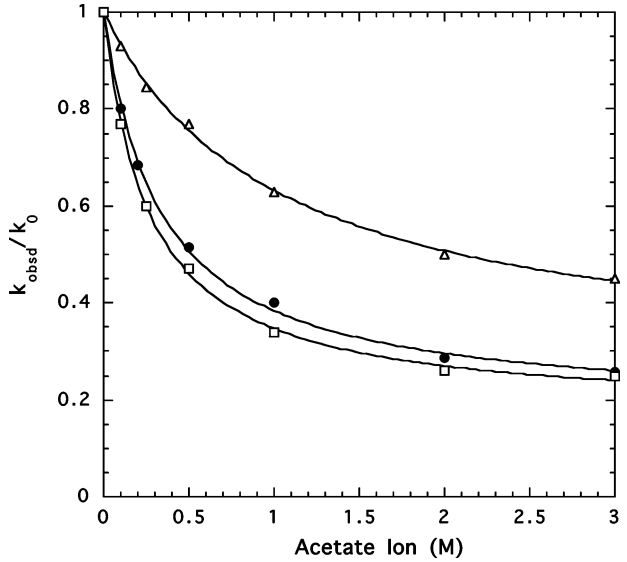


FIGURE 1. Plot of k_{obsd} against acetate ion concentration for the decay of α-acetoxy-*N*-nitrosomorpholine (circles) in aqueous media, pH = 6.21, 1% acetonitrile by volume at 25 °C, 4 M ionic strength (KCl). Data previously published[30] for α-acetoxy-*N*-nitrosopiperidine and α-acetoxy-*N*-nitrosopyrrolidine are included as triangles and squares, respectively.

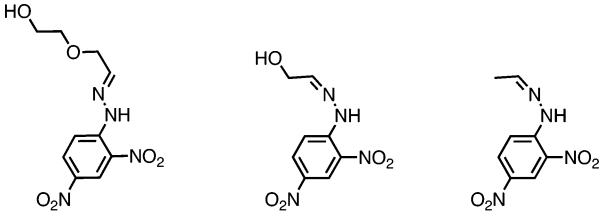
2-(2-(1H-Benzo[*d*]imidazol-1-yl)ethoxy)acetaldehyde (12). The stability of this product of benzimidazole capture of the diazonium ion **8** (Scheme 1) was first examined by NMR spectroscopy in D_2O solutions. In D_2O, ^1H NMR analysis indicates that **12** exists to the extent of >95% in the hydrate form. The results indicate that **12** is stable in acidic media (0.1 M DCl) at 80 °C for 24 h, but decays over the course of 1 h in basic media (0.1 M NaOD) at room temperature. The decay of **12** was then studied by initial-rate kinetic experiments, at 25 °C, in aqueous media employing HPLC by monitoring the decay of the UV/vis signal of **12** against a stable internal standard, 5-benzimidazole carboxylic acid. Experiments with ethanolamine, glycine, and morpholine buffers indicated that a change in buffer concentration from 0 to 0.3 M increased the value of k_{obsd} by factors typically not less than 10. Experiments with 1,4-diazabicyclo[2.2.2]octane (DABCO) and carbonate buffers exhibited a change in the values of k_{obsd} by less than 10% when the buffer concentration was varied from 0.05 to 0.3 M. For the primary and secondary amines studied, plots of k_{obsd} against buffer concentration were linear with slopes, k_2, of the observed second-order rate constant. These values varied with pH and are summarized in Table 3.

TABLE 3. Second-Order Rate Constants, k_2, for 1-(2-Ethoxyacetaldehyde)benzimidazole in Aqueous Buffered Solutions at 25 °C, 1 M Ionic Strength (KCl)

buffer	pH	$10^6 \times k_2$ (M^{-1} s^{-1})
ethanolamine	5.98	16.5
	6.97	41.2
	7.74	121
	8.05	135.9
	8.64	95.8
morpholine	8.6	69.2
	7.75	73.5
glycine	7.95	52.8
	8.9	165.4
	9.82	126.1
	10.79	107.7
DABCO	6.99	<0.005
	8.99	<0.17
	10.49	<1.3
carbonate	8.79	<0.14
	9.69	<0.11
	10.29	<2.5

2-(1H-Benzo[*d*]imidazol-1-yl)ethanol (13) Formation. The initial-rate kinetics of the formation of **13** from the decay of **12** were monitored by HPLC and, velocities of formation were determined using standard curves generated from the authentic standard. For experiments in 0.1 M glycine (pH = 8.90 and 10.0, μ = 1 M KCl), the rate constants determined under intial-rate conditions at 39 °C were just under a factor of 10 less that the rate constants of the decay of **12** that were determined at 25 °C, assuming a theoretical yield of **13** of 100%.

Products. α-Acetoxy-*N*-nitrosomorpholine: Solvolysis. The yields for the reaction in 0.1 M cacodylic acid buffer (pH 6.54) of the dinitrophenylhydrazones **14**−**16** were 61 ± 2%, 34 ± 6%, and 33 ± 4%, respectively. The yields for the reaction in 0.01 M $HClO_4$ (pH 1.93) of the dinitrophenylhydrazones **14**−**16** were 60 ± 3%, 34 ± 4%, and 37 ± 3%, respectively.



α-Acetoxy-*N*-nitrosomorpholine: Benzimidazole Trapping. Decomposition of α-acetoxy-*N*-nitrosomorpholine (25mM) in the presence of 0.10 M benzimidazole buffer, pH 6.20, for 48 h at 37 °C gave a 0.099% yield of **12** and a 0.062% yield of **13**, which were identified by LC/MS and co-chromatography with authentic standards. Reactions at longer times indicated a relatively smaller proportion of **12** and a larger proportion of **13**. This suggested that the latter might be derived from the former and prompted the independent study of the chemistry of **13**.

Product of Decay of 12. The decay of **12** at 25 °C was followed, on a longer time scale (above), by the formation of **13**. This product **13** was identified by MS analysis and coelution with the authentic standard. The decay at this temperature was followed to 35% completion, and at this point, the yield of **13** was 15% of the original amount of **12**, indicating a 43% (= 100 × 15/35) yield. Reaction at 100 °C, 0.2 M glycine buffer, pH 8.88, led to complete decay of **12**, as indicated in Figure 2 in which chromatogram 2a indicates $t = 0$, and chromatograms 2b and 2c were recorded at an intermediate point and the end point, respectively, indicating the formation of **13** (Figure 2b). The corresponding MS data of **12** and **13** are presented in Figure 2d and e, respectively. After 48 h, with no further measurable increase in the amount of **13**, the yield of this product was determined to be 72%. This product exhibited less than 5% decomposition under these conditions with an additional 24 h of incubation.

Discussion

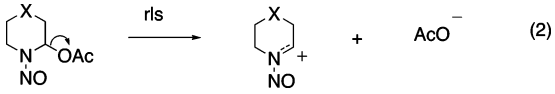
A. Synthesis. The present preparation of α-acetoxy-*N*-nitrosomorpholine is a substantial improvement over our initial method. The prior 10-step synthesis of nitrosomorpholinic acid, penultimate precursor to α-acetoxy-*N*-nitrosomorpholine, began from a serine derivative and proceeded with an overall yield of 6%.[22] The present procedure is relatively facile. After anodic oxidation of the α position of *N*-acetylmorpholine, the three-step formation of the *N*-nitrosomorpholine carboxylic acid is a one-pot procedure. Final $Pb(OAc)_4$-mediated oxidative decarboxylation[40] occurs in exceptionally good yield, 84%, to give the desired α-acetoxy-*N*-nitrosomorpholine.

B. α-Acetoxy-*N*-nitrosomorpholine. 1. Kinetics of Decay. The values of log k_0, the first-order rate constants for the buffer-independent decay of α-acetoxy-*N*-nitrosomorpholine, are plotted against pH as the solid circles in Figure 3. Also included in the plot are previously published data[30] for the solvolysis of α-acetoxy-*N*-nitrosopiperidine and the analogous pyrrolidine derivative in triangles and squares, respectively. As can be seen, the decay of α-acetoxy-*N*-nitrosmorpholine obeys the same three-term rate law that characterizes the decay of the other cyclic compounds, and acyclic compounds as well, as is summarized in eq 1.

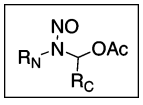
$$k_0 = k_1 + k_{H^+}[H^+] + k_{OH}[OH^-] \quad (1)$$

Values for the rate constants were obtained from a nonlinear least-squares fit of the data and are presented in Table 4, along with values for the other cyclic compounds that have been studied.

The smaller magnitude of the dominant rate constant for decay in the physiological pH range, k_1, for α-acetoxy-*N*-nitrosomorpholine compared to that for α-acetoxy-*N*-nitrosopiperidine is consistent with what is expected for a mechanism involving rate-limiting formation of an *N*-nitrosiminium ion. The morpholine derivative solvolyzes slightly more than 100-fold more slowly than the piperidine derivative. This is expected for the reaction of eq 2.



In the case of acyclic α-acetoxynitrosamines, it has been demonstrated that the transition state for reaction analogous to eq 2 engenders positive charge buildup on both the α carbon and the nitroso nitrogen atoms.[28] For several different families of acyclic compounds, differing at R_C (structure below), the values of ρ^*, for varying R_N in each family, range from −1.6 to −2.88.



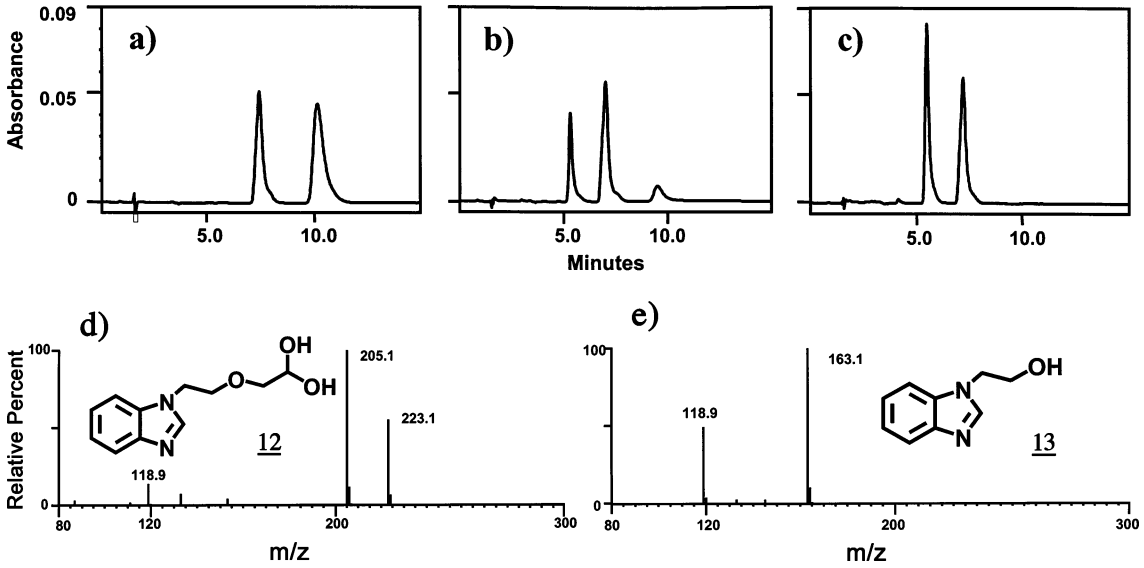


FIGURE 2. HPLC analysis of 1-(2-ethoxyacetaldehyde)benzimidazole reaction in 0.2 M glycine buffer, pH 8.875, at 100 °C and 1 M ionic strength (KCl) (a) at the initial time point, (b) after 2 h, and (c) after 21 h. LC/MS analysis of peaks with elution times of (d) 10.0 and (e) 5.5 min. The peak at 7.5 min is the internal standard, benzimidazole-5-carboxylic acid.

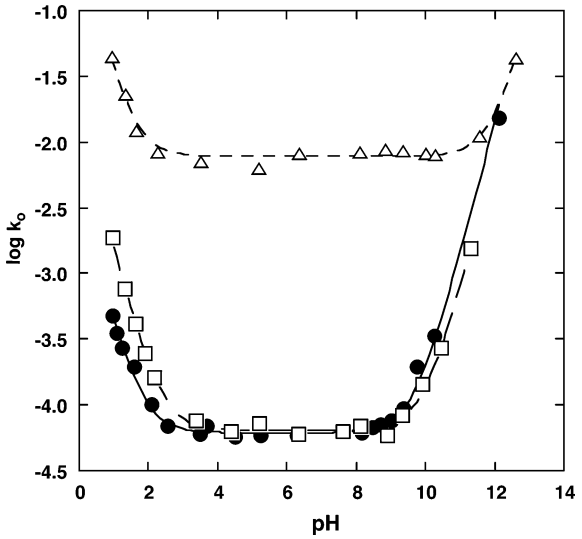
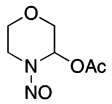
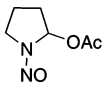
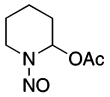


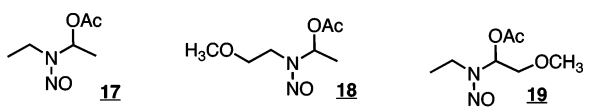
FIGURE 3. Plots of log k_0, the buffer-independent rate constant for decay of cyclic α-acetoxynitrosamines as a function of pH for reaction at 25 °C, 1 M ionic strength (KCl), 1% MeCN by volume. Data for α-acetoxy-*N*-nitrosomorpholine, α-acetoxy-*N*-nitrosopiperidine, and α-acetoxy-*N*-nitrosopyrrolidine are given by circles, triangles, and squares, respectively.

Similarly for $R_N = -CH_3$ and $-CH_2CH_3$, $\rho^* = -2.82$ and -2.60, respectively. A more specific comparison is enlightening. Structures and rate constants for the pH-independent solvolysis of analogous acyclic compounds are indicated below. It can be seen by comparison of compounds **17** and **18** that oxygen substitution for the carbon two carbons removed from the nitrogen slows the reaction by a factor of 3, whereas by comparison of **19** with **17**, substitution of oxygen for the hydrogen one carbon removed from the α-substituted carbon slows the solvolysis by a factor of ~140. The decrease by a factor of 126 in changing from the piperidine to the morpholine derivative is thus reasonable; a precise concordance of the substituent effects in the cyclic and acyclic systems is not expected. Finally, the similar magnitudes of substituent effects suggest that there is little in the way of anchimeric assistance to solvolysis by the endocyclic oxygen.

TABLE 4. Rate Constants[a] for the Decay of Cyclic α-Acetoxynitrosamines in Aqueous Media, 1 Vol % Acetonitrile, at 25 °C, 1 M Ionic Strength (KCl)[a]

Compound	k_{H+} (M^{-1} s^{-1})	$10^2 \times k_I$ (s^{-1})	k_{OH} ($M^{-1}s^{-1}$)
	0.004[b]	0.0062[b]	1.39[b]
	0.0153	0.0064	0.74
	0.29	0.78	0.87

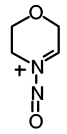
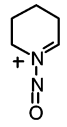
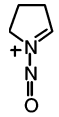
[a] Values calculated as previously reported.[30] Standard error <5%. [b] Determined in this work.



k_1 0.001 s^{-1} 0.00037 s^{-1} 0.0000072 s^{-1}

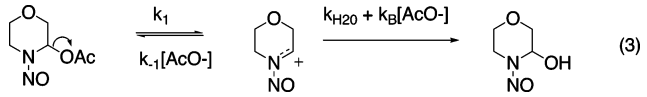
(40) Saavedra, J. E. *J. Org. Chem.* **1979**, *44*, 4511−4517.

TABLE 5. Rate Constant Ratios, Eq 3, for Reactions of Cyclic *N*-Nitrosiminium Ions with Acetate Ion in Aqueous Solution at 25 °C, pH 6.53, 4 M Ionic Strength (KCl)

	Acetate Ion Reactions[a]	
Cation	k_{-1}/k_{H2O} (M^{-1})	k_b/k_{H2O} (M^{-1})
	2.47±0.09	0.54±0.06
	3.12±0.06	0.61±0.03
	0.79±0.05	0.31±0.05

[a] Rate constant ratios based on fits using the equations previously reported[30] for eq 3.

Further evidence consistent with an S_N1 mechanism of solvolysis is the observation (Figure 1) of the "common-ion rate depression" that occurs with increasing concentration of acetate ion at constant ionic strength. This was observed previously with both the piperidino and pyrrolidino analogues, and the leveling effect of concentration was ascribed to a competing general base-catalyzed hydration reaction, as indicated in the mechanism of eq 3.[30]

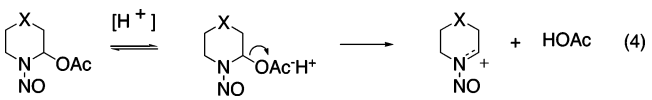


(3)

An alternative possibility that has not been specifically ruled out here is that acetate acts at the nitroso nitrogen, but this is typically a minor pathway in related systems.[24,25,28−30,41] Fitting of the data to the appropriate equation[30] results in the fits indicated by the lines in Figure 1 using values of the ratios of the rate constants reported in Table 5. These values are of reasonable magnitude compared to those of the other two systems studied.

On the basis of the above analysis then, α-acetoxy-*N*-nitrosomorpholine would appear to react, at physiological pH, via the "normal" mechanism involving rate-limiting formation of an *N*-nitrosiminium ion.

The acid- and base-catalyzed reactions of α-acetoxynitrosamines have been less well studied, but here too, the morpholine derivative does not seem unusual. The observation that acid catalyzes group exchange α to the ring nitrogen suggests leaving-group protonation as in the mechanism of eq 4.[42−46]



(4)

The morpholine derivative is more sluggish than the piperidine derivative, consistent with the diminution of equilibrium constant for protonation and the increase in the barrier for formation of the *N*-nitrosiminium ion, because of the electronegative oxygen atom in the morpholine ring. There is more evidence that the mechanism of the k_{OH} reaction entails carbonyl group attack by hydroxide ion.[26] Nucleophilic attack and subsequent decay of the tetrahedral intermediate should both be slightly accelerated by electron withdrawal by the morpholino oxygen, consistent with the slightly greater value of k_{OH} for α-acetoxy-*N*-nitrosomorpholine.

A final point respecting reactivity of the ester compared to that of the α-hydroxynitrosomorpholine formed upon decomposition of the morpholine derivative is warranted: the latter is orders of magnitude more reactive in all regions of pH, in contrast to the analogous pair of piperidino derivatives. In the case of α-acetoxy-*N*-nitrosopiperidine, it is nearly equally reactive as α-hydroxy-*N*-nitrosopiperidine in the acidic to near-neutral range of pH, whereas the latter has a substantially larger rate constant for base-catalyzed decay.[30] The substituent effects on k_{H^+} and k_1 for the esters are such that α-hydroxynitrosomorpholine is $>10^2$ more unstable than α-acetoxy-*N*-nitrosomorpholine across the entire range of pH studied.

2. Products. Solvolysis. The nearly quantitative yield of the elements of the carbon skeleton derived from **7** is consistent with the mechanism outlined in Scheme 1. The fact that there is good agreement between the yields reported here and those derived directly from the α -hydroxynitrosamine,[22] namely, 62%, 26%, and 26% for **14**, **15**, and **16**, respectively, is consistent with the intermediacy of the latter in the decay of the ester and the absence of significant denitrosation of the nitrosiminium ion, as might have been encountered.

Benzimidazole Capture of the Diazonium Ion, 8. The detection of the hydrate of **12** in reactions in which **7** is decomposed in aqueous solutions containing benzimidazole is consistent with nucleophilic trapping of the diazonium ion intermediate (Scheme 1). It indicates that the diazonium ion is not completely scavenged by intramolecular nucleophilic attack by the carbonyl group (or hydrate), a possibility to which we alluded earlier (Introduction). The low yield of **12** is not unexpected because of the weak nucleophilic selectivity of primary diazonium ions. Reaction with solvent water, rearrangement, and presumably intramolecular attack compete quite favorably with benzimidazole attack on **8**.[47] The observation of the product **12** indicates that the "signature" fragment of α-hydroxynitrosomorpholine, the ethoxyacetaldehyde moiety, is likely deposited at the heteroatoms of DNA and proteins.

C. Decomposition Chemistry of 12. The observation that **13** forms in increasing amounts, at the expense of **12**, with increasing incubation time of aqueous solutions of **7** that was previously decomposed in the presence of benzimidazole suggested the unstable nature of the ethoxyacetaldehyde functionality. Stable in 0.1 M HCl at 80 °C for 24 h, it readily decomposes in basic media, and its more sluggish decay in more

(41) Chahoua, L.; Mesic', M.; Revis, C.; Fishbein, J. C. *J. Org. Chem.* **1997**, *62*, 2500−2504.

(42) Mochizuki, M.; Anjo, T.; Takeda, K.; Suzuki, E.; Sekiguchi, N.; Huang, G. F.; Okada, M. *IARC Sci. Publ.* **1980**, *41*, 553−559.

(43) Mochizuki, M.; Anjo, T.; Sekiguchi, N.; Ikarashi, A.; Suzuki, A.; Wakabayashi, Y.; Okada, M. *Chem. Pharm. Bull.* **1986**, *34*, 4.

(44) Mochizuki, M.; Anjo, T.; Wakabayashi, Y.; Sone, T.; Okada, M. *Tetrahedron Lett.* **1980**, *21*, 1761−1764.

(45) Mochizuki, M.; Anjo, T.; Okada, M. *Chem. Pharm. Bull.* **1978**, *26*, 6.

(46) Okada, M.; Mochizuki, M.; Anjo, T.; Sone, T.; Wakabayashi, Y.; Suzuki, E. *IARC Sci. Publ.* **1980**, *31*, 71−82.

(47) Lu, X.; Heilman, J. M.; Blans, P.; Fishbein, J. C. *Chem. Res. Toxicol.* **2005**, 1462−1470.

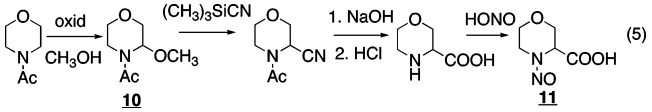
neutral media is markedly stimulated by primary and secondary amines, although not by the tertiary amine DABCO or by carbonate buffers. The lack of concordance between the rate constants of decay of the ethoxyacetaldehyde adduct and those derived for formation of the hydroxyethyl adduct (Results) require the formation of a non-steady-state intermediate, possibly an imine or enamine.[48] Although some effort was expended to detect such species, this was unsuccessful. Regardless, the present results clearly indicate the potential, under physiological conditions, for an ethoxyacetaldyde adduct, initially deposited from nitrosomorpholine that was metabolically activated by hydroxylation α to the nitroso nitrogen, to decompose to a chemically more stable hydroxyethyl adduct. Whether the ethoxyacetaldehyde adduct deposited in DNA is chemically stabilized by potential cross-link formation with exocyclic amino groups of the bases, or destabilized as a result of the intermediacy of such structures in the formation of the hydroxyethyl adducts, is not presently clear.

Experimental Section

Warning! Many nitrosamines are powerful carcinogens. Precautions taken in handling include use of frequently changed double pairs of disposable gloves and a well-ventilated hood. Contaminated, and potentially contaminated, materials were treated with 50% aqueous sulfuric acid containing a commercially available oxidant.

Materials. Typically, organic solvents were dried and purified by distillation with CaH_2 before use. The chemicals for synthesis and kinetics were ACS grade or better. Organic chemicals were typically purified prior to use in synthesis or kinetic experiments. Deionized water was used in all analytical procedures.

Synthesis. α-Acetoxy-N-nitrosomorpholine was prepared as previously reported by $Pb(AcO)_4$-catalyzed oxidative decarboxylation of N-nitrosomorpholine carboxylic acid.[22,40] Aspects of the synthesis of morpholine carboxylic acid, beginning with anodic oxidation in methanol of acetyl morpholine, as in eq 1, have also been previously published,[39] but the additions and alterations described below were essential for success in our hands.



3-Methoxy-*N*-acetylmorpholine (10). To a solution of tetraethylammonium perchlorate (510 mg, 2.2 mmol) in 100 mL of methanol, contained in a water-jacketed titration vessel the inner wall of which was lined with a reticulated carbon anode, was added 5 g of N-acetylmorpholine (38 mmol). The temperature was maintained at 14 °C by means of a refrigerated recirculating bath, and electrolysis was carried out by passing a constant current of 370 mA through the solution, the cathode being suspended in the center of the vessel. After 7 h, electrolysis was stopped. The solvent was stripped in vacuo, and to the residue was added 100 mL of ethyl acetate. The solution was washed with 15 mL of water and dried over Na_2SO_4. The product was purified by distillation, bp 97 °C at 0.25 mmHg. NMR ($CDCl_3$): 2.10 and 2.15 (2s, 3H), 3.27 and 3.30 (2s, 3H), 3.45−4.2 (m, 6H), 4.74 and 5.53 (2s, 1H).

***N*-Nitrosomorpholine-3-carboxylic Acid (11).** To a solution of **10** (2.02 g, 12.8 mmol) in 50 mL of CH_2Cl_2 were added 3.4 mL of trimethylsilyl cyanide and a few drops of borontrifluoride etherate. The reaction mixture was stirred for 15 h at room temperature under Ar. Further purification was not effected prior to the next two hydrolysis reactions.

The crude 220 mg of N-acetyl-3-cyanomorpholine was dissolved in 0.1 M NaOH and stirred for 3 h at room temperature in order to effect nitrile hydrolysis. The base was subsequently neutralized with HCl, and additional HCl was added to a final concentration of ∼3.5 M. Amide hydrolysis was effected over 16 h at 100 °C, after which the product was purified by recrystallization in H_2O, yielding 171 mg of crude morpholine carboxylic acid. This was then nitrosated[22] without prior purification to give, after purification by silica gel column using hexane/ethyl acetate (6/4), 181 mg of spectroscopically pure material. E form[49] (56.8%). ^{1}H NMR ($CDCl_3$): 5.47 (1H, d, J = 4 Hz), H5 4.65 (1H, m), 4.42 (1H, d, J = 12.35 Hz), 4.10 (1H, m), 4.11 (1H, m), 3.63 (1H, td), 3.50 (1H, dd, J = 12.35, 4.1 Hz), 11.59 (1H, s). ^{13}C NMR ($CDCl_3$) δ: 171.6, 67.3, 67.0, 51.1, 47.9. Z form (43.2%). ^{1}H NMR ($CDCl_3$): 5.41 (1H, d J = 3.68 Hz), 4.65 (1H, m), 4.51 (1H, d, J = 11.91 Hz), 3.98 (1H, dd, J = 11.91, 4.6 Hz), 3.81 (1H, dd, J = 11.91, 3.68 Hz), 3.32 (1H, td), 3.02 (1H, td), 11.59 (1H, s). ^{13}C NMR ($CDCl_3$): δ 173.6, 68.3, 65.8, 60.7, 39.0.

Methanesulfonic Acid 2-Allyloxyethyl Ester (20).[50] 2-Allyloxyethanol (50.0 g, 0.49 mol) was dissolved in pyridine (80 mL). Methane sulfonyl chloride (84.12 g, 0.73 mol) was slowly added to the stirring solution at 0 °C. The mixture was allowed to stir at 0 °C for 1 h and was then stirred at room temperature for 2 h. The reaction mixture was diluted with deionized water (100 mL) and 1 N HCl (300 mL). The mixture was extracted with CH_2Cl_2 (3 × 100 mL) and washed with water (100 mL), 10% $NaHCO_3$ (100 mL), and brine (100 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo (71% yield). ^{1}H NMR ($CDCl_3$): 3.01 (3H, s), 3.66 (2H, t, J = 4.6 Hz), 3.99 (2H, d, J = 5.9 Hz), 4.33 (2H, t, J = 5.3 Hz), 5.22 (2H, dd, J = 1.4, 10.5 Hz), 5.83 (1H, m). ^{13}C NMR ($CDCl_3$): δ 134.1, 117.8, 72.3, 69.4, 67.8, 37.8. Anal. Calcd for $C_{12}H_{14}N_2O\cdot0.2H_2O$: C, 70.01; H, 7.05; N, 13.61. Found: C, 70.29; H, 7.14; N, 13.64.

1-(2-(Allyloxy)ethyl)-1H-benzo[*d*]imidazole (21). Sodium hydride (4.4 g, 0.11 mol, 60 wt %) was added in portions to a cold (0 °C) solution of benzimidazole (13.1 g, 0.11 mol) in DMF (50 mL). After the mixture had been stirred at room temperature for 1 h, **20** (5.0 g, 0.028 mol) was added. The mixture was heated at 65 °C for 2 h. After being allowed to cool, the mixture was concentrated in vacuo. The crude mixture was purified by silica gel column chromatography ($CHCl_3/CH_3CN$, 7/3) followed by distillation, bp 162 °C at 1.5 mmHg (89% yield). ^{1}H NMR (CD_2Cl_2): 3.76 (2H, t, J = 5.3 Hz), 3.93 (2H, d, J = 5.3 Hz), 4.33 (2H, t, J = 5.3 Hz), 5.14 (2H, ddd, J = 1.4, 10.6 Hz), 5.81 (1H, m), 7.27 (2H, m), 7.44 (1H, d, J = 7.3 Hz), 7.75 (1H, d, J = 7.3 Hz), 7.95 (1H, s). ^{13}C NMR (CD_2Cl_2): δ 144.36, 144.25, 134.9, 134.6, 123.0, 122.2, 120.5, 117.2, 110.2, 72.5, 68.8, 45.5.

2-(2-(1H-Benzo[*d*]imidazol-1-yl)ethoxy)acetaldehyde (12). ***21*** (520 mg, 2.6 mmol) was dissolved in CH_2Cl_2 (50 mL) and cooled to −78 °C. Ozone was bubbled through the solution until a blue color appeared. The solution was purged with oxygen, and $(Ph)_3P$ (674 mg, 2.6 mmol) was added. The solution was allowed to warm to room temperature and stirred for 24 h. The cloudy solution was diluted with water (50 mL). The aqueous layer was collected, washed with ether (2 × 100 mL), and concentrated in vacuo. The resulting red oil (550 mg) was dissolved in 1.0 M TFA (2.5 mL). The product, in the hydrate form, was purified as the TFA salt by HPLC (25% yield). ^{1}H NMR (D_2O): 3.45 (2H, d, J = 5.1 Hz), 4.03 (2H, t, J = 4.6 Hz), 4.70 (2H, t, J = 4.6 Hz), 5.03 (1H, t, J = 5.1 Hz), 7.64 (2H, m), 7.84 (2H, m), 9.23 (1H, s). ^{13}C NMR (D_2O): δ 140.4, 130.9, 130.4, 126.9, 126.5, 114.5, 112.8, 88.0, 73.3, 68.1, 46.6. Anal. Calcd for $C_{13}H_{13}F_3N_2O_4$: C, 49.06; H, 4.12; N, 8.8. Found: C, 49.25; H, 4.22; N, 8.57.

***tert*-Butyl-(2-chloroethoxy)dimethylsilane (22).[51]** Imidazole (5.07 g, 75 mmol) was added to a cold (0 °C) solution of

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(49) Karabatsos, G. J.; Taller, R. A. *J. Org. Chem.* **1964**, *29*, 4373.

(50) Barton, J. E.; Clinch, K.; O'Hanlon, P.; Ormrod, J.; Rice, M.; Turnbull, M. International Patent Application WO 9319599, 1993.

(51) Hill, S. T.; Mokotoff, M. *J. Org. Chem.* **1984**, *49*, 1441−1442.

2-chloroethanol (6.0 g, 75 mmol) in CH_2Cl_2 (30 mL). The solution was stirred for 10 min, and then tert-butyldimethylsilyl chloride (TBDMSCl) (11.22 g, 75 mmol) was added. The solution was stirred at 0 °C for 1 h and then at room temperature for 24 h. The solution was diluted with CH_2Cl_2 (30 mL) and washed with water (3 × 50 mL) and brine (1 × 25 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo. The product was purified by distillation, bp 66 °C at 9 mmHg (84% yield). ^{1}H NMR ($CDCl_3$): 0.06 (6H, s), 0.88 (9H, s), 3.51 (2H, t, J = 6.4 Hz), 3.81 (2H, t, J = 6.4 Hz). ^{13}C NMR ($CDCl_3$): δ 63.9, 45.3, 26.0, 18.5, −5.1.

1-[2-*tert*-Butyldimethylsilanyloxy)ethyl]benzimidazole (23).[52] Sodium hydride (0.82 g, 20.5 mmol, 60 wt %) was added to a solution of benzimidazole (2.43 g, 20.5 mmol) in DMF (30 mL). After the solution had been stirred at room temperature for 0.5 h, **22** (1.0 g, 5.1 mmol) and KI (0.85 g, 5.1 mmol) were added. The mixture was heated at 80 °C for 2 h. After being allowed to cool, the mixture was concentrated in vacuo. The crude mixture was purified by silica gel column chromatography (CH_2Cl_2/CH_3CN, 8/2) (79% yield). ^{1}H NMR (CD_2Cl_2): 0.0 (6H, s), 0.80 (9H, s), 3.93 (2H, t), 4.27 (2H, t), 7.27 (2H, m), 7.42 (1H, dd), 7.73 (1H, dd), 7.92 (1H, s). ^{13}C NMR (CD_2Cl_2): δ 144.5, 144.4, 134.6, 123.0, 122.2, 120.5, 110.3, 62.2, 47.9, 26.0, 18.5, −5.5.

2-(1H-Benzo[*d*]imidazol-1-yl)ethanol (13).[44] Tetrabutylammonium fluoride (10.6 mL, 10.6 mmol, 1 M in THF) was added to a cold (0 °C) solution of **23** (0.98 g, 3.5 mmol) in THF (10 mL) under Ar and stirred for 1.5 h. The solution was diluted with ethyl acetate (25 mL) and washed with water (2 × 25 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo. The product was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 9.5/0.5) (42% yield). ^{1}H NMR (CD_2Cl_2): 2.65 (1H, s), 3.98 (2H, t, J = 5.1 Hz), 4.25 (2H, t, J = 5.1 Hz), 7.08 (1H, t, J = 7.3 Hz), 7.20 (1H, t, J = 7.4 Hz), 7.40 (2H, dd, J = 8.3 Hz), 7.73 (1H, s). ^{13}C NMR (CD_2Cl_2): δ144.0, 143.5, 133.9, 123.2, 122.5, 119.8, 110.2, 60.8, 48.5. Anal. Calcd for $C_9H_{10}N_2O$: C, 66.65; H, 6.21; N, 17.27. Found: C, 66.65; H, 6.26; N, 17.30.

Kinetic Measurements. α-Acetoxy-*N*-nitrosomorpholine (7). Temperature was maintained at 25 °C by means of an attached recirculating bath. The buffer solution in each reaction cell was made up by diluting a concentrated stock buffer solution. Reaction solutions were maintained at ionic strength μ = 1.0 M, unless otherwise noted, using KCl. Values of pH were obtained using a pH meter equipped with a combination electrode. Two-point calibrations were done before pH values were recorded. Calibrations were carried out using commercially available standards or those prescribed by the Merck Index.[53] Kinetic runs were initiated when solutions containing substrate dissolved in acetonitrile were injected into the cuvettes or injected mechanically into the observation cell of a stopped-flow instrument, to give a final substrate concentration of (1−2) × 10^{-4} M. The final acetonitrile concentration was 4 vol %.

2-(2-(1H-Benzo[*d*]imidazol-1-yl)ethoxy)acetaldehyde (12). These kinetic runs were carried out using an HPLC instrument with an autosampler, a temperature-controlled carousel, and a photodiode array detector monitoring at 250 nm. Separations were performed on a reverse-phase C18 5 μ 250 × 4.6 mm column with 100% isocratic 20 mM acetic acid pH 3.2 as the eluant. The temperature was maintained at 25 °C unless otherwise noted. The buffer solution in each experiment was made by diluting a concentrated stock buffer solution. The final buffer concentration ranged from 0.05 to 0.3 M. Reaction solutions were maintained at ionic strength μ = 1.0 M, using KCl. Values of pH were obtained as described above. Kinetic experiments were initiated when a solution containing the substrate freshly dissolved in water was injected into the reaction vial and then mechanically injected onto the HPLC. The final substrate concentration was (1−2) × 10^{-4} M. 5-Benzimidazole carboxylic acid was used as an internal standard. The experiments were deemed complete when less than 10% of the substrate had decayed.

Product Analyses. Products from the decay of **7** were quantitated as 2,4-dinitrophenylhydrazone (DNPH) derivatives after separation by HPLC.[22] The products of decomposition of α-acetoxy-N-nitrosomorpholine at pH 1.93 and 6.54 were quantitated after 10 half-lives of reaction. The reactant concentrations were typically in the range of 10^{-4}−10^{-5} M. After the reaction, the pH was adjusted to ∼2 with HCl, and a volume of DNPH stock was added. The derivatized products were separated and quantified by HPLC using an acetonitrile/0.01 M acetic acid eluent over a 4.6 mm × 25 cm C18 column. Quantitation of the products was accomplished by interpolation from standard curves containing at least two points.

Product analysis in the case of the decay of **12** was carried out by means of LC/MS and/or UV/vis/LC methods, and product identification was confirmed by MS and coelution with authentic standards under two different conditions of chromatography.

Acknowledgment. This work was supported by Grant RO1 CA52881 from the National Cancer Institute, National Institutes of Health.

JO051936Z

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(53) Stecher, P. G., Ed. *The Merck Index*, 8th ed.; Merck & Co.: Rahway, NJ, 1968.