Articles

A Diazonium Ion Cascade from the Nitrosation of Tolazoline, An Imidazoline-Containing Drug

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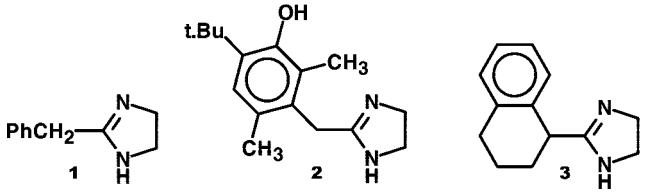
Tolazoline (1-benzylimidazoline), a representative imidazoline-containing drug, reacts readily with nitrite in acetic acid to produce a complex product mixture. Fourteen compounds have been identified as products of this transformation when an 8-fold excess of HNO_2 is used. The products, which include *N*-nitrosoamides, esters, alcohols, and phenylacetic acid, are rationalized as arising from a cascade of reactive diazonium ions. *N*-Nitrosotolazoline can be isolated from the nitrosation reaction in good yield when the mixture is extracted with CH_2Cl_2 as the transformation progresses. It nitrosates much more rapidly (50×) than tolazoline to give, among other products, the oxime [1-(*N*-nitroso-2-imidazolinyl)benzylidene]hydroxylamine, which can also be produced in good yield from the reaction of tolazoline with isopropyl nitrite. At low substrate and nitrite concentrations, the main reaction products are *N*-nitrosotolazoline, its decomposition product *N*-2-hydroxyethylphenylacetamide, the above-mentioned oxime, phenyl acetic acid, and 2-hydroxyethyl phenylacetate. The tolazoline nitrosation rate in three buffer systems has been determined at pH 3.4 and 37 °C ($k_{obs} = 6.25 \times 10^{-5}$ s^{-1} in 0.5 M acetate buffer with a $10 * [NO_2^-] = 250$ mM). Because *N*-nitrosotolazoline exhibits the chemical properties of a direct-acting mutagen and carcinogen, we have used the rate data to estimate its level of formation at nitrite concentrations <3 mM. Cursory examination of the nitrosation chemistry of oxymetazoline, a related drug, is primarily focused at its electron-rich aromatic ring.

Introduction

Here, we report the nitrosation chemistry of tolazoline **1**, a common drug used both in prescription form and as the active constituent in several over-the-counter medications (*1, 2*). Tolazoline contains a 2-substituted imidazoline moiety that is found in numerous drugs and drug candidates (*3*). Several imidazoline receptor proteins have been identified and cloned (*3*). Tolazoline and several structurally similar drugs, including oxymetazoline **2** and tetrahydrozoline **3**, are also ligands for several adrenergic receptors, which gives rise to their extensive use as decongestants and vasodilators (*1*). The structures and primary uses of a set of representative imidazoline-based drugs are given in Table 1.



Our inquiry into the nitrosation chemistry of tolazoline from a toxicological perspective has been motivated by several facts and lines of thought. All humans excrete *N*-nitrosoproline and several other nitrosamino acid derivatives (*4–7*). A significant percentage of these highly polar, easily excreted, noncarcinogenic nitrosamines arise from endogenous nitrosation processes (*4–7*). Acid-catalyzed nitrosation occurs in the stomach (*7–10*). Endogenous nitrosation also occurs at sites of inflammation through the production of NO and subsequent oxidative chemistry that produces nitrosating agents, as well as through some nitrate-reducing bacterial processes (*11–16*). Many of the nitrosamines produced from endogenous nitrosation are not detected because of P450-mediated metabolism, which converts them into progenitors of biomolecular-damaging electrophiles, particularly highly reactive diazonium ions (*13, 17–19*). Tolazoline and the imidazoline-containing drugs in question are secondary amidines and possess a free N−H that should be susceptible to nitrosation. We have shown that the nitrosation of tertiary amidines results in the facile production of diazonium ions (*20–22*) but were surprised to find that little is known about either secondary amidine nitrosation or the properties of the *N*-nitrosoamidines expected of them. From a chemical perspective, *N*-nitrosoamidines belong to a family of reactive *N*-nitroso compounds where the N−N═O group is bound to a carbonyl or C═NR group. These compounds, direct-acting mutagens and carcinogens, include *N*-nitrosoamides, *N*-nitrosourethanes, and *N*-nitrosoureas, of which the chemotherapeutic agents bischloroethynitrosourea (BCNU)[1] and lomustine (CCNU, chloroethylcyclohexylnitrosourea) are exemplary. Thus, it seems likely that the endogenous nitrosation of tolazoline and like drugs could result in reactive DNA-damaging electrophiles, which may have adverse toxicological consequences if the dosing is relatively high and the drugs are taken chronically.

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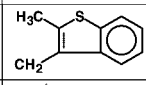
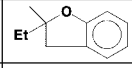
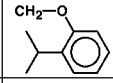
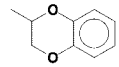
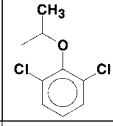
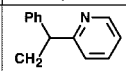
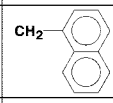
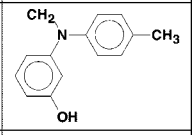
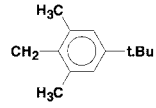
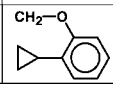
Table 1. Representative Imidazoline Drugs[a]

Drug/Compound Name	Action/Use	Structure (R)
Antazoline	Ophthalmic & antihistamine	$CH_2N(Ph)CH_2Ph$
Mitizoline	alpha 2-adrenoceptor antagonist/promoter of insulin secretion	
Cibenzoline	antiarrhythmic	
Efaraoxan	alpha 2-adrenoceptor antagonist/promoter of insulin secretion	
Fenoxazoline	Sympathomimetic	
Idazoxan	alpha 2-adrenoceptor antagonist/promoter of insulin secretion	
Lofexidine	Antihypertensive	
Midaglizole		
Naphazoline	Nasal decongestant	
Phentolamine	Antihypertensive	
Xylometazoline	Nasal Decongestant/adrenergic	
Cirazoline	alpha 1-adrenergic receptor agonist	

[a] All of these compounds can be found in the USAN formulary, but all may not be approved for human use.

We show here that tolazoline nitrosates readily under several different conditions. In acidic media, *N*-nitrosotolazoline is detected only under conditions where low NO_2^- concentrations are used because *N*-nitrosotolazoline nitrosates much more rapidly than tolazoline. This chemistry results in the formation of a variety of products that are indicative of the production of a number of reactive electrophiles.

Experimental Procedures

Caution: *Most nitrosamines are potent carcinogens. Considerable care should be taken in their use so as to avert exposure to humans and to avoid environmental contamination. We routinely performed all operations with these substances, except for dilute solutions thereof, in well-ventilated fume hoods. We rinsed all nitrosamine-contaminated glassware with a solution of concentrated HBr in glacial acetic acid, which is effective in cleaving the NO group from the amine nitrogen atom. The action of this agent in aprotic solutions is also effective in nitrosamine destruction. Aqueous solutions were treated with either Ni(R) or Al in concentrated sodium hydroxide. This process may produce hydrazines.*

General. Melting points were determined on a Thomas-Hoover capillary tube apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra of solutions ($CDCl_3$ unless otherwise stated) were recorded on Bruker ARX 250, DRX 300, or DRX 500 spectrometers. HPLC was performed with Waters system equipped with model 510 solvent delivery pumps, Waters model 490 programmable multiwavelength detector, Waters model 712 WISP autosampler, FC 203 Gilson fraction collector, and Millennium control software (Version 2.15). Two types of Agilent HPLC columns were used, a 4.6 mm × 25 cm Zorbax SB-C8 analytical column and a 10 mm × 25 cm Zorbax SB-C8 semipreparative column. Gas chromatography was performed on a HP 5890 gas chromatography fitted with a 30 m × 0.25 mm Alltech DB-5 column. GC/MS analysis was performed on an Agilent 6890 series GC system with Agilent 5973 network mass selective detector. Thin-layer chromatography was performed on EM Science aluminum plates precoated with 0.20 mm silica gel. Merck Kieselgel 230–400 mesh silica gel was used for flash column chromatography. UV–visible spectra were obtained on a Hewlett-Packard 8453 UV–visible spectrophotometer. LC/MS analysis was performed on a Finnigan triple-stage quadrupole TSQ 7000 mass spectrometer equipped with the following components: mobile phase pumps (TSP 4000, Quaternary pump), autosampler (SpectraSYSTEM AS 3000), UV detector (TSP UV 6000LP), and MS detector (Finnigan TSQ MS). The LC/MS data were acquired and processed with Xcalibur software. High-resolution mass spectrometry was provided by the Washington University Mass Spectrometry Resource, an NIH Research Resource (Grant P41RR00954). Combustion analyses were obtained from Dessert Analytics (Tucson, AZ). Solvents were dried and distilled by common procedures. All other chemicals were reagent grade and were used as purchased without further purification. HPLC solvents were degassed and filtered prior to use.

Nitrosation of Tolazoline and Isolation of Nitrosation Products. Aqueous sodium nitrite (1.4 mL, 7 M) was added in one portion to a solution of 250 mg of tolazoline hydrochloride (1.27 mmol) in 2 mL of glacial acetic acid. The reaction proceeded for 1 h at room temperature and then was quenched by adding saturated Na_2CO_3 solution. The reaction mixture was extracted with EtOAc (10 mL × 3). The extracts were combined, dried with anhydrous Na_2SO_4, concentrated, and separated by flash chromatography on silica gel. Some fractions from flash chromatography were purified by semipreparative HPLC equipped with a fraction collector.

***N*-Nitroso-*N*-(2-hydroxyethyl)phenylacetamide (10).** *N*-(2-Hydroxyethyl)phenylacetamide **13** (*23*), (1.79 g, 0.01 mol) was nitrosated at 0 °C according to the method of White (*24*) in a mixture of acetic acid (10 mL) and acetic anhydride (50 mL). Sodium nitrite (15 g, 0.22 mol) was added over a period of 5 h. Cold water (50 mL) was then added to quench the reaction. The reaction mixture was extracted with methylene chloride (50 mL × 3), and the combined organic layers were washed with 5% aqueous Na_2CO_3 and water. After the organic layer was dried over anhydrous Na_2SO_4 for 2 h, the solvent was evaporated, and the crude product was purified by flash chromatography with hexane and ethyl acetate as eluent solvents (1:1, $R_f = 0.5$). The pure product was obtained as pale yellow oil (1.37 g, 66% yield) but was too unstable for the determination of elemental composition. ^{1}H NMR: δ 7.31 (m, 5 H), 4.50 (s, 2 H), 3.97 (t, $J = 5.61$ Hz, 2 H), 3.56 (t, $J = 5.61$ Hz,

[1] Abbreviations: BCNU, bis-chloroethylnitrosourea; CCNU, chloroethylcyclohexylnitrosourea (lomustine); NAP, World Heath Organization (WHO)−International Agency for Research in Cancer (IARC) amine nitrosation protocol; BHT, 2,6-di-*t*.butyl-4-methylphenol; FDA, U.S. Food and Drug Administration; NTol, *N*-nitrosotolazoline.

2 H), 2.03 (b, 1 H). ^{13}C NMR: δ 175.8, 133.0, 129.5, 128.7, 127.4, 59.3, 41.3, 40.9. IR (neat): 3399, 2919 (b), 1724 (s), 1496, 1368, 1024 cm^{-1}.

***N*-(2-Acetoxyethyl)phenylacetamide (12).** *N*-(2-Acetoxyethyl)phenylacetamide **12** was prepared from **13** by acetylation with acetic anhydride followed by aqueous quenching, which gave a crude solid. Recrystallization from ethanol gave **12** as white solid (92% yield); mp 68–69 °C. ^{1}H NMR: δ 7.32 (m, 5 H), 5.89 (b, 1 H), 4.09 (t, J = 5.5 Hz, 2 H), 3.56 (s, 2 H), 3.49 (t, J = 5.5 Hz, 2 H,), 2.03 (s, 3 H). ^{13}C NMR: δ 171.1, 170.8, 134.7, 129.3, 128.8, 127.2, 62.8, 43.6, 38.6, 20.6. EIMS: 65, 88, 91, 92, 118, 130, 161, 179, 221. Anal calcd for $C_{12}H_{15}NO_3$: C, 65.14; H, 6.83; N, 6.33. Found: C, 65.05; H, 6.84; N, 6.25.

***N*-Nitroso-*N*-(2-acetoxyethyl)phenylacetamide (9).** The nitrosamide **9** was prepared from **12** in a method similar to the preparation of **10** (82% yield). The compound was too unstable for the determination of elemental composition. ^{1}H NMR: δ 7.31 (m, 5 H), 4.48 (s, 2 H), 4.03 (m, 4 H), 1.89 (s, 3 H). ^{13}C NMR: δ 174.9, 170.5, 133.1, 129.5, 128.7, 127.4, 59.9, 41.3, 37.3, 20.40. IR (neat): 3449 (w), 2912, 1736 (s), 1469, 1453, 1364, 1221 (s), 1012 cm^{-1}.

***N*-Nitroso-*N*-(2-chloroethyl)phenylacetamide (11).** The title nitrosamide **11** was prepared from *N*-(2-chloroethyl)phenylacetamide (*23*) by a procedure similar to that used to prepare **10**. The pure product was obtained as pale yellow oil after chromatography in a yield of 76%. ^{1}H NMR: δ 7.31 (m, 5 H), 4.49 (s, 2 H), 4.09 (t, J = 6.6 Hz, 2 H), 3.41 (t, J = 6.6 Hz, 2 H). ^{13}C NMR: δ 174.7, 129.5, 128.7, 127.5, 41.3, 39.3, 38.6. IR (neat): 2919 (s), 1736 (s), 1507 (s), 1453, 1372, 1260, 1078, 1028, 718 cm^{-1}.

***N*-Nitroso-2-imidazolinyl Phenyl Ketoxime (17) (Nitrosation of Tolazoline with Isopropyl Nitrite).** A round-bottom flask was charged with tolazoline free base (3.2 g, 20 mmol). Isopropyl nitrite (17.8 g, 200 mmol) was added at room temperature. The mixture was stirred at room temperature for 16 h. The excess isopropyl nitrite and 2-propanol were removed using a rotary evaporator. The pure product **17** (3.18 g, 73% yield) was obtained by recrystallization in a mixture of acetone and acetonitrile; mp 93 – 95 °C. ^{1}H NMR (acetone-d_6): δ 11.39 (s, 1 H), 7.76 (m, 2 H), 7.42 (m, 3 H), 4.29 (t, J = 7.2 Hz, 2 H), 3.97 (t, J = 7.2 Hz, 2 H). ^{13}C NMR (acetone-d_6): δ 152.2, 147.8, 132.1, 130.5, 129.3, 126.7, 55.2, 43.8. MS (EI): 77, 103, 116, 129, 144, 159, 172, 188, 189, 218. IR (KBr): 2839 (b), 1662 (s), 1458 (s), 1204 (s), 1009 cm^{-1}. HRMS (EI, protonated molecular ion followed by loss of NO): *m*/*z* calcd for $C_{10}H_{11}N_3O$, 189.0902; found, 189.0892. Anal. calcd for $C_{10}H_{10}N_4O_2$: C, 55.04; H, 4.62; N, 25.68. Found: C, 54.98; H, 4.60; N, 25.36.

***N*-Nitroso-2-imidazolinyl Phenyl Ketone *O*-Phenylacetyloxime (20).** The title compound was prepared from **17** by acylating it with phenylacetyl chloride. The product **20** is a yellow solid (yield, 93%); mp 73–74 °C. ^{1}H NMR: δ 7.79 (m, 2 H), 7.45 (m, 3 H), 7.27(m 3 H), 7.15(m, 2 H), 4.03 (t, J = 8.2 Hz, 2 H), 3.76 (s, 2 H), 3.60 (t, J = 8.2 Hz, 2 H). ^{13}C NMR: δ 166.9, 154.2,151.3, 132.7, 132.2, 129.6, 129.2, 128.9, 128.5, 127.6, 127.1, 54.6, 43.1, 40.1. HRMS (EI, protonated molecular ion followed by loss of NO): *m*/*z* calcd for $C_{18}H_{17}N_3O_2$, 307.1320; found, 307.1316. IR (neat): 1785 (s), 1657,1604, 1450 (s), 1215 (b) cm^{-1}. Anal. calcd for $C_{18}H_{16}N_4O_3$: C, 64.28; H, 4.79; N, 16.66. Found: C, 64.21; H, 4.84; N, 16.35.

***Z*- and *E*-2-Imidazolinyl Phenyl Ketoximes: The Denitrosation of 17.** The *Z* isomer of the title compound **18** was prepared by the dropwise addition of 0.4 mL of hydrogen bromide (30 wt % in acetic acid) at room temperature to a solution of **17** (0.218 g, 1 mmol) in 2 mL. The oxime (hydrobromide salt) precipitated. Recrystallization from ethanol gave 0.179 g of **18** (95% yield) as a white solid; mp 230 –233 °C. ^{1}H NMR (DMSO-d_6): δ 12.92 (s, 1 H), 10.66 (s, 2 H), 7.54 (m, 5 H), 4.05 (s, 4 H). ^{13}C NMR (DMSO-d_6): δ 160.4, 142.3, 130.4, 129.1, 125.6, 44.8. The *E* isomer **19** of the title compound was prepared by denitrosation of **17** with aqueous hydrochloride acid. After 0.218 g of **17** (1 mmol) was stirred in 2 M HCl for 12 h, the residue was evaporated to dryness and purified by flash chromatography with CH_2Cl_2 and MeOH as eluent solvent (9:1, v/v). Pure **19** (hydrochloride) was obtained as a sticky oil with a yield of 82%. ^{1}H NMR (DMSO-d_6): δ 13.44 (s, 1 H), 10.05 (s, 2 H), 7.49 (m, 5 H), 3.90 (s, 4 H). ^{13}C NMR (DMSO-d_6): δ 162.3, 143.1, 130.6, 129.3, 128.8, 127.5, 44.7.

2-Methyl-4-phenyl-5-oxazolone-3-oxide (29). According to the procedure reported by Seebach (*25*), **29**, as a white solid, was prepared from acetaldehyde and phenylglyoxylic acid oxime **30** in a yield of 83%; mp 72 −73 °C. ^{1}H NMR (in $CDCl_3$): δ 8.63 (m, 2 H), 7.50 (m, 3 H), 5.96 (q, J = 6.0 Hz, 1 H), 1.87 (d, J = 6.0 Hz, 3 H). ^{13}C NMR (in DMSO-d_6): δ 164.1. 131.2, 128.6, 126.3, 125.2, 124.7, 94.5, 18.0. IR (KBr): 3409 (b), 1789 (s), 1576 (s), 1490, 1375, 1248, 1104, 786 cm^{-1}. Anal. calcd for $C_{10}H_9NO_3$: C, 62.82; H, 4.74; N, 7.33. Found: C, 62.74; H, 4.78; N, 7.37.

***N*-Nitrosotolazoline (36).** Tolazoline hydrochloride (1.96 g, 10 mmol) was dissolved in 20 mL of aqueous buffer (NaH_2PO_4, 0.5 M, pH 4). To this solution, $NaNO_2$ (3.45 g, 50 mmol) in 20 mL of water was added slowly over a 2 h period while constantly stirring at room temperature. Methylene chloride (2 mL) was added to the above reaction mixture every 5 min and then removed with the replenishment of fresh solvent. Concentrated hydrochloric acid was added every few minutes to keep the pH approximately 4. The combined CH_2Cl_2 extracts were washed with $NaHCO_3$-saturated solution and NaCl-saturated solution and dried over Na_2SO_4. The solvent was then evaporated, and the residue was chromatographed on silica gel flash column with hexane and EtOAc as eluent solvents (1:2, v/v, R_f = 0.6). Pure **36** was obtained as pale yellow oil (1.5 g, 79% yield). ^{1}H NMR: δ 7.35 (m, 5 H), 4.26 (s, 2 H), 3.95 (t, J = 6.5 Hz, 2 H), 3.72 (t, J = 6.5 Hz, 2 H). ^{13}C NMR: δ 158.9, 133.9, 129.3, 128.6, 127.2, 52.8, 43.6, 34.2. IR (neat): 3031, 2877, 1684 (s), 1389 (s), 1277, 1200, 1024, 722 (s) cm^{-1}. HRMS (EI): *m*/*z* calcd for $C_{10}H_{11}N_3O$, 189.0902; found, 189.0898.

HPLC Quantification of UV-Absorbing Nitrosation Products. A nitrosation mixture obtained as described above was injected to the HPLC after dilution with CH_3CN/H_2O. Two wavelengths, 220 and 254 nm, were set for the UV detection of the eluting substances. A gradient program with two solvents CH_3CN (A) and NaH_2PO_4 buffer (0.025 M, pH 4.0) (B) was utilized for the mobile phase. HPLC program (min, eluent) at 1 mL/min: 0–40, 10−90% A; 40–45, 90% A.

Quantification of Ethylene Glycol and Its Derivatives in the Nitrosation Reaction Mixture. The ethylene glycol in the reaction mixture was determined by a quantitative ^{13}C NMR spectral method. Generally, 400 μL of the nitrosation mixture was mixed with 100 μL of CD_3CN (for magnetic field locking signal) and 50 μL of 0.6 M THF aqueous solution as an internal standard. The ^{13}C spectrum was collected for 30 min. The concentration was calculated based on the methylene group peak heights of ethylene glycol and THF (δ 62.73 and 67.51, respectively). Ethylene glycol monoacetate and ethylene glycol

diacetate were quantified by GC/MS using calibration curves based on peak areas from external standards (m/z = 74 for monoacetate and 116 for diacetate).

Quantification of Aldehyde Products in the Reaction Mixture. The aldehydes from the nitrosation mixture were derivatized as follows: A 500 μL aliquot was taken from the nitrosation mixture and mixed with 500 μL of 2,4-dinitrophenylhydrazine (2 mM in 18% phosphoric acid). After the sample was shaken for 5 min, the sample was diluted with CH_3CN to 100 mL and aliquots were then injected onto the HPLC. Hydrazone standards were prepared by refluxing the respective aldehydes (1 mmol) with 2,4-dinitrophenylhydrazine (1 mmol) in ethanol, containing 10% phosphoric acid. The precipitated hydrazones were filtered, washed, dried, and dissolved in acetonitrile. Six hydrazone derivatives from formaldehyde, acetaldehyde, glyoxal, and glycol aldehyde were prepared as authentic standards. A gradient program with two solvents CH_3CN (A) and H_2O (B) was utilized for the mobile phase. HPLC program (min, eluent) at 1 mL/min: 0–34, 10−40% A; 34–50, 40−80% A; 50–65, 80% A.

Nitrosation of Tolazoline with Nitric Oxide (NO) in O_2 Atmosphere. A round-bottom flask, sealed with a septum, was evacuated and flushed with NO three times and then filled with NO at a pressure of 1 atm at 25 °C (8.6 mL, 0.35 mmol). To this flask, oxygen (one-fourth volume of nitric oxide) and 7.0 mL of tolazoline solution (25 mM in aqueous phosphate buffer, pH 7.2) were added through the septum via a syringe. The reaction mixture was stirred at 37 °C for 1 h and then injected directly to HPLC for identification and quantification of the nitrosation products. *N*-Nitrosotolazoline (0.13%) was the only observed product under these conditions.

Tolazoline Nitrosation Kinetics. To a solution of tolazoline (10 mL, 25 mM) in acetate buffer (0.5 M, pH 3.4), a 7 M $NaNO_2$ solution (35.7 uL, 10 equiv) was added in one portion. The reaction mixture was sealed and stirred at 37 °C. An aliquot of 0.2 mL of solution was taken out via a syringe at different time intervals and diluted to 5.0 mL with acetonitrile for HPLC analysis. HPLC program (min, eluent) at 1 mL/min: 0–40, 10−90% A; 40–45, 90% A. Quantitation utilized a standard curve derived from known concentrations of *N*-nitrosotolazoline.

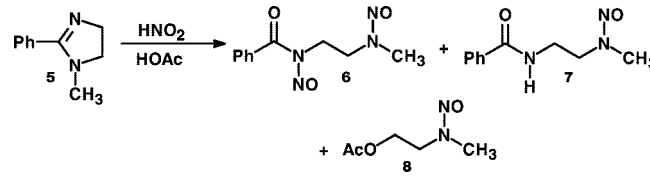
2,6-Dimethyl-3-hydroxy-4-t-butylphenylacetonitrile **53** was prepared by a literature method (*26*). ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.97 (s, 1H), 4.75 (s, 1H), 3.60 (s, 2H), 2.30 (s, 3H), 2.25 (s, 3H), 1.38(s, 9H). ^{13}C NMR (250 MHz, $CDCl_3$): δ 151.08, 135.97, 127.64, 126.52, 125.62, 122.82, 117.41, 34.24, 29.66, 19.92, 18.12, 12.25.

Nitrosation of Tolazoline in the Presence of 2,6-Dimethyl-3-hydroxy-4-*t*-butylphenylacetonitrile. In separate experiments, 0.5, 1, 2, or 4 equiv of aqueous sodium nitrite (4 M) was added to a 1:1 solution (0.6 M) of tolazoline hydrochloride and 2,6-dimethyl-3-hydroxy-4-*t*-butylphenylacetonitrile in glacial acetic acid. After a reaction time of 1 h at 25 °C, 50 μL aliquots were diluted to 1 mL with CH_3CN:H_2O (1:1) and injected onto a Zorbax ODS analytical HPLC column (4.6 mm × 25 cm) to separate and quantify tolazoline and the nitrile. Mobile phases: CH_3CN and NaH_2PO_4 buffer, linearly increasing CH_3CN 7−30% in 8 min, then from 72 to 75% from 9 to 14 min at a flow rate of 1 mL/min).

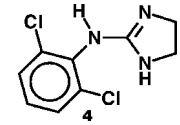
Results and Discussion

At the outset, we wish to emphasize that the chemistry described here in a generalized context does not necessarily apply to compounds that have a nitrogen atom as a substituent at the 2-position of the imidazoline ring. These substances, of

Scheme 1



which the antihypertensive drug clonidine **4** is an example, are guanidine derivatives and are expected to have a different chemistry toward nitrosating agents.



Clonidine

To our knowledge, there is no literature on the nitrosation chemistry of *N*-1 unsubstituted imidazolines. Iley et al. have prepared four 2-aryl-*N*-nitrosoimidiazolines by amide nitrosation chemistry methods (N_2O_4) and examined the kinetics of their denitrosation and hydrolysis in moderately strong acid (*27*). They were unable to prepare the *N*-nitrosoimidazolines by nitrous acid nitrosation. We have reported on the nitrosation chemistry of a tertiary imidazoline, 1-methyl-2-phenylimidazoline **5** (*22*). The transformation gives the products shown in Scheme 1. The nitrosamine **8** is a decomposition product of **6**, and **7** results from the denitrosation of **6**.

Tolazoline Nitrosation; Excess NO_2^-. In our previous research on amidine nitrosation, we found that a relatively large excess of nitrous acid was required to generate significant yields (*20, 21*). As a result, we initiated this nitrosation under "forcing conditions" where an 8-fold excess of NO_2^- was employed. We will first describe the characterization of the products formed from the reaction under these conditions and then describe the results from nitrosation reactions, which are closer to what may be encountered in vivo. The nitrosation of tolazoline was allowed to go for 1 h at 25 °C. A sample of the reaction mixture was taken for HPLC and gave the chromatogram shown in Figure 1. After basification, the mixture was extracted with EtOAc. The resulting mixture was subjected to both silica gel column and semiprep HPLC to isolate the products. This involved several batches and some variation in procedures to identify the peaks labeled A−K. The first peak in the chromatogram is due to NO_2^-.

Identification of Peaks F, I, and K. Flash column chromatography permitted the isolation of peaks F, I, and K as pure

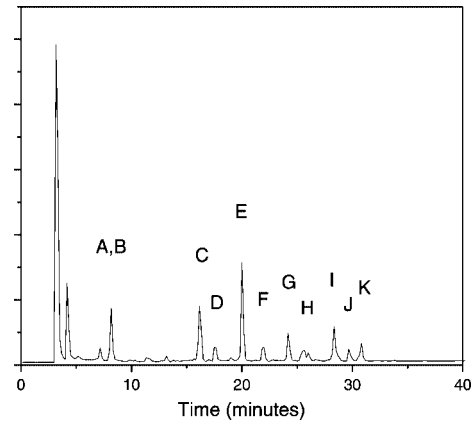
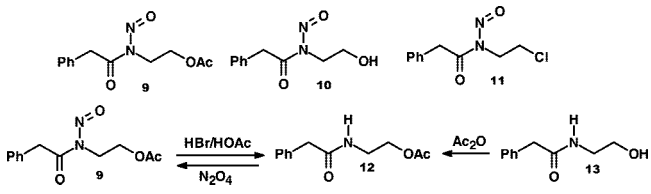


Figure 1. HPLC (reversed phase) of the nitrosation reaction mixture produced from the addition of an 8-fold excess of aqueous, concentrated $NaNO_2$ to a solution of tolazoline hydrochloride in glacial acetic acid at 25 °C after a reaction time of 1 h.

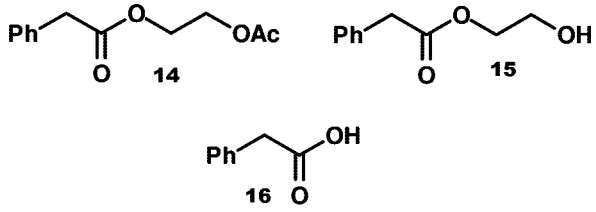
Scheme 2



compounds. Each of these substances gave a positive Griess test (*28*), indicating the presence of an *N*-nitroso group, and each also exhibited intense UV absorption at 240–260 nm, a characteristic of *N*-nitroso compounds. Peak I was assigned structure **9**, on the basis of its spectral data. This assignment was confirmed by unambiguous synthesis.

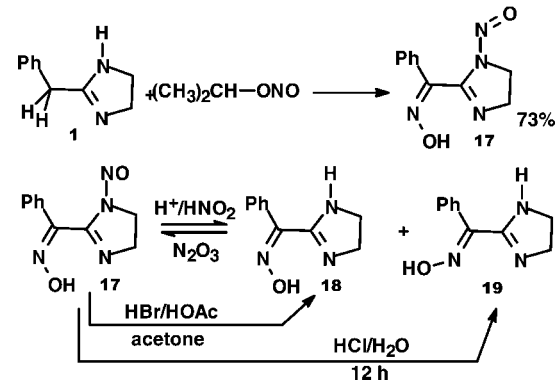
The ^{1}H NMR spectrum of **9** clearly indicates the existence of a monosubstituted benzene ring (C_6H_5), an isolated methylene group (CH_2) at δ 4.49 (singlet), two adjacent methylene groups ($-CH_2CH_2-$), which are coupled with each other, and a methyl group (CH_3-) at δ 2.06. The ^{13}C spectrum shows two C═O groups at δ 171.1 and 170.8. While nitrosamines often exhibit sets of peaks due to the presence of *Z/E* isomers due to restricted rotation around the N−NO bond, this is not true for *N*-nitrosoamides. No such isomers were observed for **9**. This suggests that the *N*-nitroso group is in the form of nitrosoamide. To further confirm the structural assignment of **9**, it was denitrosated by treating with HBr/HOAc as shown in Scheme 2. The mass spectrum and NMR of the denitrosated product were the same as the unambiguously synthesized *N*-2-acetoxyethylphenylacetamide **12**. The amide **12** was converted into **9** using White's procedure (Scheme 2) (*24*). Using similar procedures, we identified peak F as the alcohol **10** and peak K as the corresponding chloride **11**. The *N*-nitrosoamides **10** and **11** were synthesized from their known amides.

Peaks H, D, and C. The identification of the *N*-nitrosoamides **9** and **10** suggested that products of their decomposition may also be present. From White's extensive research, we know that *N*-nitrosoamides decompose by effective intramolecular migration of the *N*-acyl substituent to the *N*-nitroso oxygen to yield an unstable diazotate (*29*). In additon to diazonium-derived products, an acid and an ester are common products of *O*-acyldiazotate decomposition. We synthesized the esters **14** and **15** and showed that they had the same HPLC retention volumes as peaks H and D, respectively. These two substances were each isolated from the ethyl acetate extract of the reaction mixture by flash chromatography and shown to be identical with the respective synthesized compounds. Acidification of the basicified aqueous layer from the original ethyl acetate extraction of the reaction mixture led to a mixture from which phenylacetic acid **16** (peak C) was extracted with ethyl acetate and subsequently crystallized from ethanol. It was identical to the authentic compound and obtained in 30% yield.



Peak E. The substance giving rise to this peak was also obtained by flash SiO_2 chromatography. The compound, $C_{10}H_{10}N_4O_2$, gave a positive Griess test and showed strong UV absorption in the region 240–260 nm. Its ^{1}H NMR spectrum gave evidence of a monosubstituted benzene ring, did not reveal a benzylic CH_2 group, but did show two coupled CH_2, which suggested a conserved imidazoline ring, and exhibited an

Scheme 3



exchangeable proton at δ 11.39. Neither its ^{13}C NMR spectrum nor its IR spectrum showed a C═O, but the ^{13}C spectrum did show quaternary carbons at δ 152.2 and 147.8. On this basis, the compound was tentatively assigned the structure of the oxime **17** (Scheme 3), which was subsequently confirmed by X-ray crystallography (Figure 2). It has been produced by both

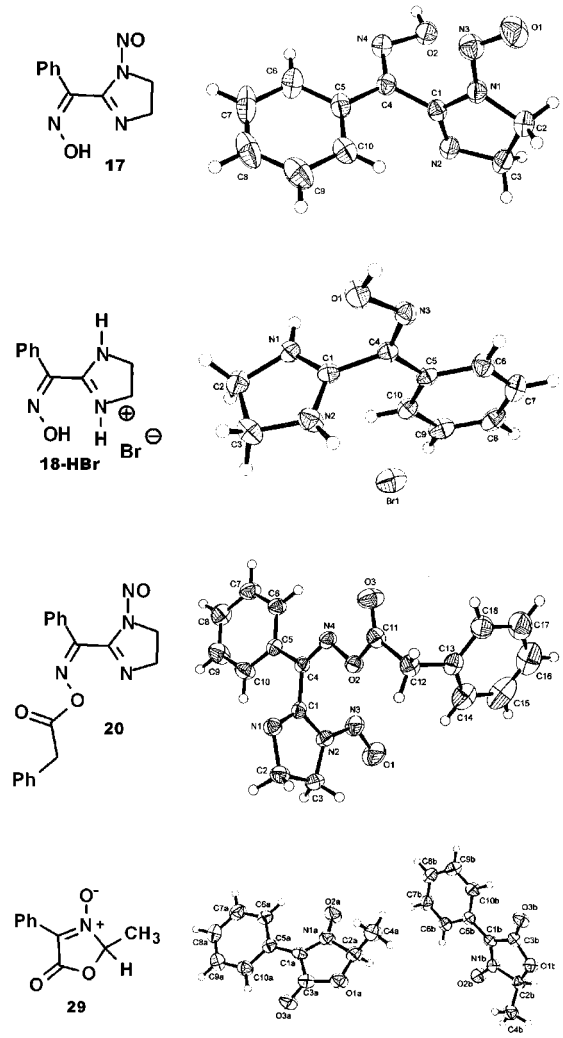


Figure 2. Ortep drawings produced from X-ray crystallographic structure determinations of compounds isolated from the reaction of tolazoline with excess HNO_2 in HOAc. The separate enantiomers of **29** are shown as they appear in the crystal of the racemic mixture.

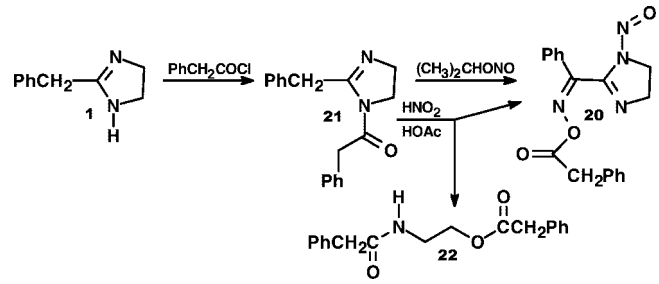
N- and C-nitrosation of tolazoline. Even in solution, **17** exists as only one of four possible *Z*/*E* isomers. The reaction of tolazoline with neat isopropyl nitrite gave **17** in 73% yield as the only significant product after crystallization. This permitted further experimentation.

Peaks A and B. Because the N atoms contained within either of the C═N groups of **17** could possibly be nitrosated further (*20–22, 30*), we reacted it with nitrous acid in acetic acid. HPLC examination of the reaction mixture showed two peaks, which had the same retention volumes as peaks A (32%) and B (68%) of the original tolazoline-derived reaction mixture. Neither compound gave a positive Griess test on TLC. Their NMR spectra, obtained following chromatographic isolation, were similar and showed that the two CH_2 groups of the imidazoline ring were equivalent. These data suggested that these compounds were isomeric denitrosation products of **17**. This hypothesis was proven to be correct by the following experiments. The nitrosation of either substance produced **17**. Two more preparative scale denitrosation experiments were performed. Reaction of **17** with HBr/HOAc in acetone led to the precipitation of **18** as a hydrobromide salt (Scheme 3). Conversion of this to free base and HPLC showed this substance to be peak A. X-ray crystallography revealed that this was the *Z* isomer as shown in Figure 2. On the other hand, stirring of **17** with 2 M HCl for 12 h followed by chromatographic isolation gave the hydrochloride **19**, the free base of which exhibited the HPLC characteristics of peak B. We reasonably presume that this is the *E* isomer of the oxime, but we do not have a crystal structure to prove it. Its ^1H and ^{13}C NMR spectra are quite similar to **18**.

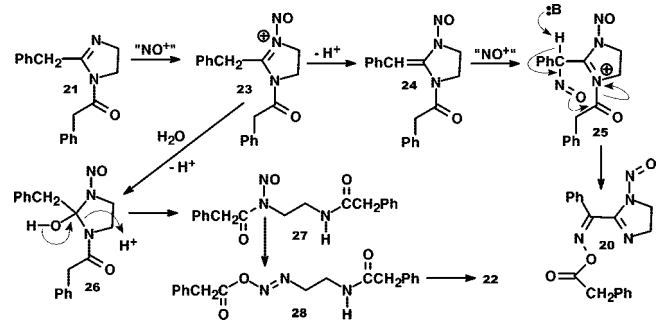
The denitrosation of *N*-nitrosoamides and isoectronically related compounds, in contrast to nitrosamines, under the acidic nitrosating conditions employed in the reaction is not uncommon. The adjacent carbon double bond to an electronegative atom (N or O) withdraws electrons from the *N*-nitroso-substituted N atom, making nucleophilic attack at the nitroso N and subsequent denitrosation more facile. The fact that the two denitrosation methods (Scheme 3) give different oxime isomers is understandable to a point. The configuration of the oxime in **17** is *Z*. HBr-catalyzed denitrosation in acetic acid/acetone is fast and involves NO protonation followed by nucleophilic attack of Br^- at N. These conditions do not lead to equilibration of the stereochemistry of the oxime functional group. On the other hand, the other two conditions for denitrosation, HNO_2/HOAc and aqueous HCl, permit not only protonation of the oxime N but reversible nucleophilic attack (likely H_2O) at its attached carbon, a process that will permit rotation around the C−N bond, resulting in a change in oxime stereochemistry.

Peak J. Chromatographic isolation of the material giving rise to peak J resulted in a solid that could be recrystallized from ethanol. This substance had the formula $C_{18}H_{16}N_4O_3$. It gave a positive Griess test, indicating an NNO group, and its NMR spectra revealed the presence of two benzene rings, one of which appeared to be associated with a phenylacetyl group. A set of adjacent coupled CH_2 groups were present. Ultimately, the structure of the material giving rise to peak J was established as **20** by X-ray crystallography (Figure 2). However, several experiments conducted in the course of establishing the structure of **20** are of interest. The fact that phenylacetic acid is a reaction product suggested the possible intermediacy of a reactive phenylacetylating agent. Reaction of tolazoline with phenylacetyl chloride gave the *N*-acylimidazoline **21** as expected. Its nitrosation with isopropyl nitrite gave **20**. Nitrosation of **21** with HNO_2/HOAc gave **20** and the open chain amide ester **22** (see

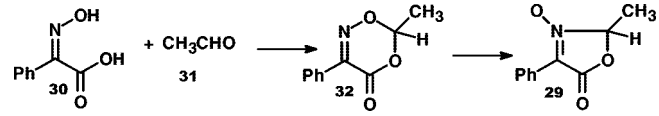
Scheme 4



Scheme 5



Scheme 6



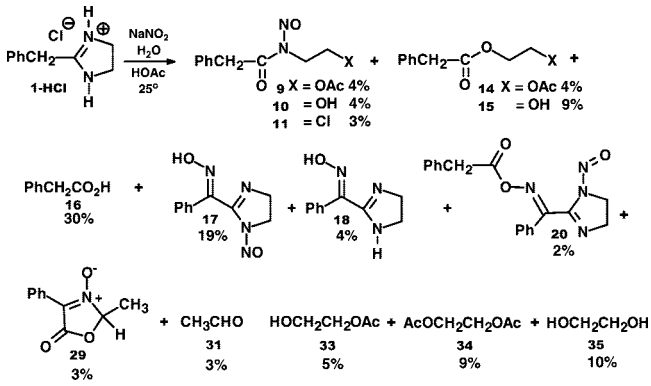
Scheme 4). Obviously, in these experiments, the *N*-bound phenylacetyl group migrated from N to what was a C-nitroso O under the conditions of nitrosation.

The mechanistic rationale for the acyl migration and the competitive chemistry are shown in Scheme 5. Regardless of the nitrosating agent, **23** is formed. In the case of isopropyl nitrite, no water is present and C-deprotonation occurs with facility because of the cogeneration of the basic isopropoxide. C-Nitrosation of the resulting alkene **24** produces **25**. Acyl migration of **25** occurs with C-deprotonation to give **20**. In the case of aqueous acidic nitrosation, the mechanism varies somewhat, and there is also competition for the intermediate **23**. Nucleophilic attack of water at C-2 of the imidazoline ring gives **26** after deprotonation. This undergoes ring opening to the *N*-nitrosoamide **27**. Rearrangement produces the unstable diazoacetate **28**, which extrudes N_2 to produce **22**. We will see below that similar processes are in action when tolazoline is nitrosated. The amide ester **22** is not present in the nitrosation mixture produced from tolazoline. This suggests that **21** is not an intermediate in tolazoline nitrosation (see below). We propose that **20** forms from the acylation of **17** as depicted in Scheme 11 below.

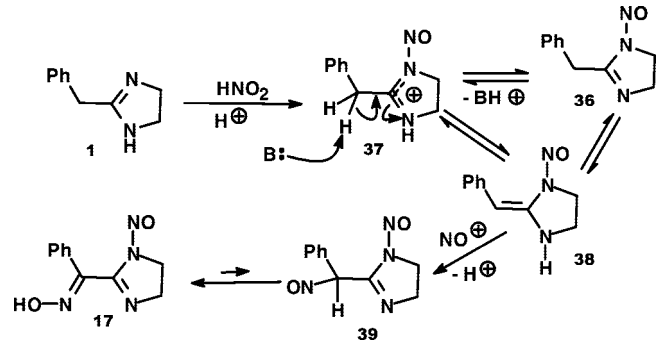
Peak G. The substance producing this peak was also isolated by chromatography and subsequently crystallized. Because its ^1H NMR spectrum, which showed only a monosubstituted benzene ring and a CH_3CH group with the CH at δ 5.69, was puzzling, the compound was subjected to X-ray crystallographic analysis, which revealed it to be 2-methyl-4-phenyl-1,3-oxazol-3-ine-3-oxide, **29** (Figure 2). We synthesized this compound by a method envisioned to give rise to it in the reaction mixture as shown in Scheme 6. The rearrangement that converts **32** to **29** has been well-established (*31*).

Isolation of Non-UV-Adsorbing Compounds. As is evident from both mechanistic considerations related to the decomposi-

Scheme 7



Scheme 8



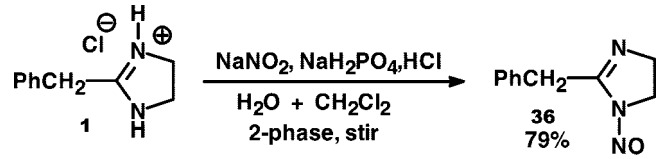
tion pathways of the *N*-nitrosoamides (see Schemes 10 and 11 below) and the structure of **29**, acetaldehyde **31** is produced in the reaction mixture. This was confirmed by treating the aqueous nitrosation mixture with 2,4-dinitrophenylhydrazine and the isolation of the corresponding hydrazone of acetaldehyde. We also used this method to quantify the amount of acetaldehyde in the mixture (3%). GC/MS analysis of the ethyl acetate extract of the tolazoline nitrosation mixture in combination with standards also showed the presence of the mono- (**33**) and diacetate (**34**) of ethylene glycol. The presence of ethylene glycol **35** itself was suspected but could not be proven with the GC/MS method being employed. Its presence and quantitation were proven by using ^{13}C NMR of an aliquot of aqueous reaction mixture and THF as a standard (see the Experimental Procedures for details).

Product Quantitation. With the identity of the reaction products in hand, we were able to quantify their amounts in the reaction mixture using standards. In the case of the UV-absorbing materials, HPLC was used. As stated, acetaldehyde was determined as its 2,4-dinitrophenylhydrazone. The acetate esters of ethylene glycol were assayed by GC/MS, and ethylene glycol was determined as noted above. The results are given in Scheme 7. Although there is an obvious overlap in the accounting, the material balance for the tolazoline-derived methylene groups is 84% and that of the phenyl groups is 87%. We had anticipated the isolation of *N*-nitrosotolazoline **36** from the nitrosation mixture but did not find it. Instead, we isolated compounds that are likely derived from it as we explain further below. The large number of products and their relatively low yields indicate the production of a number of reactive intermediates. The compounds formed in the largest amount are phenylacetic acid and the oximinonitrosamine **17**. This product is clearly produced from both the N- and the C-nitrosation of tolazoline (Scheme 5).

Preparation of *N*-Nitrosotolazoline. We were able to prepare *N*-nitrosotolazoline **36** by extracting it from the nitrosation mixture with CH_2Cl_2 as it formed. A solution of tolazoline hydrochloride in sodium phosphate buffer (pH 4) was rapidly stirred with CH_2Cl_2 (two phase). A concentrated solution of $NaNO_2$ was added along with additional HCl to maintain the pH. Separation of the phases permitted isolation of *N*-nitrosotolazoline in 79% yield.

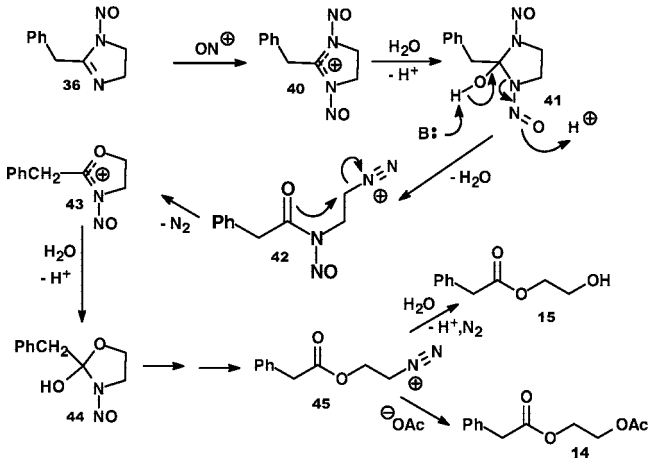
Properties of *N*-Nitrosotolazoline. The nitrosation of *N*-nitrosotolazoline under the same conditions used for the nitrosation of tolazoline gave the same reaction mixture as judged by comparison of their respective HPLC chromatograms. We discuss the properties of *N*-nitrosotolazoline in detail in an accompanying paper (*32*) but mention several pertinent items here. We considered that some of the products observed from the nitrosation of tolazoline might arise from the acid-catalyzed decomposition of *N*-nitrosotolazoline, but a major product of the latter transformation is the amide **13** (Scheme 2), which is not found in the nitrosation reaction mixture. This observation and the rationale presented below strongly suggest that most of the products observed in the tolazoline nitrosation mixture arise from the further nitrosation of *N*-nitrosotolazoline.

Mechanistic Rationale. The initial nitrosation process of tolazoline can be envisioned to proceed as illustrated in Scheme 8. The nitrosammonium ion **37** can lose a proton either from nitrogen to give *N*-nitrosotolazoline **36** or from the benzylic carbon to give **38**. The formation of **38** can also be perceived as a tautomerization of **36**. C-Nitrosation of **38** leads to **39** and thence to **17** by means of the well-known C-nitroso-oxime tautomerization. As discussed above, we did not observe any products that pointed to the further nitrosation of **17**, although it does undergo reversible *N*-denitrosation to **18** and **19** (Scheme 3).

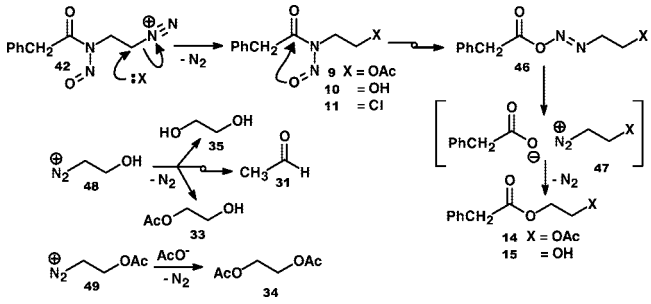


The nitrosation of basic nitrogen compounds is inversely related to their basicity because *N*-protonation robs them of their nucleophilic character toward nitrosating agents, which we write in our schemes as NO^+ for the sake of simplicity. (In our reactions, it is likely that N_2O_3 is the principal NO^+ donor.) The nitroso group is a very electron-withdrawing substituent, so *N*-nitrosotolazoline is certainly much less basic than tolazoline. Its effective nitrosation rate is correspondingly faster because a much greater fraction of the free base is present. We have shown that the C=N of amidines is very susceptible to nitrosation (*20–22*). Nitrosation of the imino nitrogen of *N*-nitrosotolazoline likely occurs very rapidly to generate **40** (Scheme 9). Reaction of **40** with H_2O at C-2 of the imidazoline ring then sets off a cascade of reactions that generate both stable products and more reactive intermediates. The intermediate **41** is essentially a tetrahedral intermediate such as are involved in carbonyl group reactions (*20*). Scission of either C−N bond leads to the diazonium ion **42**, which can be captured either by intramolecular nucleophilic attack to give **43** or by intermolecular attack involving either AcO^-, H_2O, or Cl^- to give the *N*-nitrosoamides **9**, **10**, or **11**, respectively (Scheme 10). The formation of **43** leads to another reactive diazonium ion **45** through **44** as shown. Decomposition of **45** (Scheme 9) by nucleophilic attack will give **14** and **15**, but this may not be the main pathway to these products. As shown in Scheme 10, the *N*-nitrosoamides **9** and **10**, produced from intermolecular nucleophilic attack on the diazonium ion **42**, can rearrange to the *O*-phenacyl diazotate **46**, which yields the ion pair **47**.

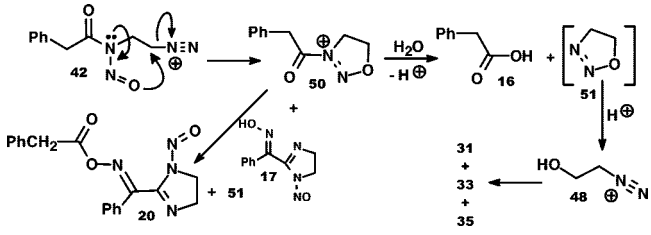
Scheme 9



Scheme 10



Scheme 11



Collapse of this species with loss of N_2 gives **14** and **15**. However, the ion pair can also separate to give phenylacetic acid **16** and the reactive diazonium ions **48** and **49**. The properties of **48**, the hydroxyethyl diazonium ion, are well-known. Rearrangement by hydride migration as N_2 leaves to give acetaldehyde **31**. Nucleophilic displacement of N_2 produces **35** and **33** (Scheme 10). Similarly, the formation of the diacetate **34** arises from **49**. It is difficult to conceive how this diester could arise from another path, and its presence leads credence to the interpretation.

Several of these products and intermediates can arise by another pathway as is shown in Scheme 11. In analogy with the carbonyl-induced intramolecular displacement of N_2 from the diazonium ion **42** to convert it to **43** (Scheme 9), the nitroso O of **42** can participate in a similar transformation in the conversion of **42** to **50**, a potent acylating agent, as shown in Scheme 11. Reaction of **50** with H_2O gives phenylacetic acid and 1,2,3-oxadiazoline **51**. This molecule has been the subject of several computational investigations and is unstable (*33, 34*). It also has been suggested as an intermediate in the decomposition of 3-alkyl-1,2,3-oxadiazolinium ions (*34, 35*) and clinically employed chloroethylnitrosoureas (*36, 37*). The transformation of **51** to **48** is predicted and reasonable. Fragmentation of **51** to diazomethane and formaldehyde has also been predicted. We did detect a trace of methyl phenylacetate, which can be explained by the reaction of diazomethane with phenyl acetic acid. The formation of the *O*-phenacyloxime **20** by the proposed reaction of **17** and **50** provides the best evidence for this pathway.

Table 2. Products from the Nitrosation of Tolazoline under NAP Conditions

conditions		product[a]				
pH	time (h)	**13**	**16**	**15**	**17**	**36**
3	1	0.55	0.05	0.14	0.05	0.03
3	4	1.08	0.09	0.30	0.02	0.01
3.5	1	0.52	0.04	0.12	0.06	0.02
3.5	4	1.19	0.09	0.28	0.04	0.02
4	1	0.47	0.04	0.10	0.05	0.03
4	4	0.96	0.06	0.19	0.04	0.02

[a] Percent yields.

Nitrosation with NO + O_2. Nitrosation at sites of inflammation through N_2O_3 production from NO could be important because the work of the MIT group has shown that intrinsic rate nitrosation rate constants for morpholine under modeled NO generation conditions are essentially the same as those found from aqueous acidic nitrosation (*38*). Obviously, adjustments must be made for the pH and the pK_a of the substrate regardless of the nitrosation environment. We made a cursory investigation of the reaction of tolazoline with NO + O_2. A 0.18 mM solution of tolazoline in pH 7.2 phosphate buffer at 37 °C was reacted with a mixture of 0.35 mmol of NO into which 0.09 mmol of O_2 had been introduced into the headspace and the mixture stirred for 1 h. A 0.13% yield of *N*-nitrosotolazoline, the only product observed by HPLC, resulted. It should be noted that under these conditions, there is significant conversion of the N_2O_3 formed to NO_2^- because of the higher pH. Regardless, the demonstration of the ready production of *N*-nitrosotolazoline is significant. With higher concentrations of NO + O_2 in organic solvents, a more complex array of products, which we have chosen to not discuss here, emerge. These show that both nitrosation at the imidazoline N and nitration at both the benzylic and the aromatic carbons of tolazoline occur. An investigation of the reaction of imidazolines with NO, which was directed at the preparation of diazeniumdiolates, has been reported since the completion of our research (*39*).

Nitrosation under Limiting Conditions. We recognize, of course, that the chemistry described above is far from representative of what may happen in vivo because of the large excess of nitrite employed and the relatively high substrate concentration. To better assess the toxicologically relevant nitrosation chemistry that could occur, we have also examined the nitrosation of tolazoline under more limiting conditions. In preliminary experiments, we found that the nitrosation of 120 mM tolazoline in buffer at pH 3 with 8 equiv of NO_2^- for 1 h gave *N*-nitrosotolazoline **36** in 8.7%, the ester **15** in 2.2%, and the oxime **17** in 1.4%. A 20 mM solution of tolazoline reacted with 1 equiv of NO_2^- under similar conditions to give *N*-nitrosotolazoline (0.02%).

Some years ago, a WHO advisory committee recommended that nitrogen-containing drugs be evaluated for nitrosatability under a set of specific conditions now referred to as the NAP [World Heath Organization (WHO)−International Agency for Research in Cancer (IARC) amine nitrosation protocol] test (*40, 41*). A 40 mM solution of the drug in buffered acetic acid at 37 °C is nitrosated with 4 equiv of NO_2^- for 1 and 4 h. The results of the application of this test to tolazoline are given in Table 2. Under these conditions, the acid-catalyzed decomposition product **13** of *N*-nitrosotolazoline **36** is formed. Products **15**–**17** all result from the further nitrosation of *N*-nitrosotola-

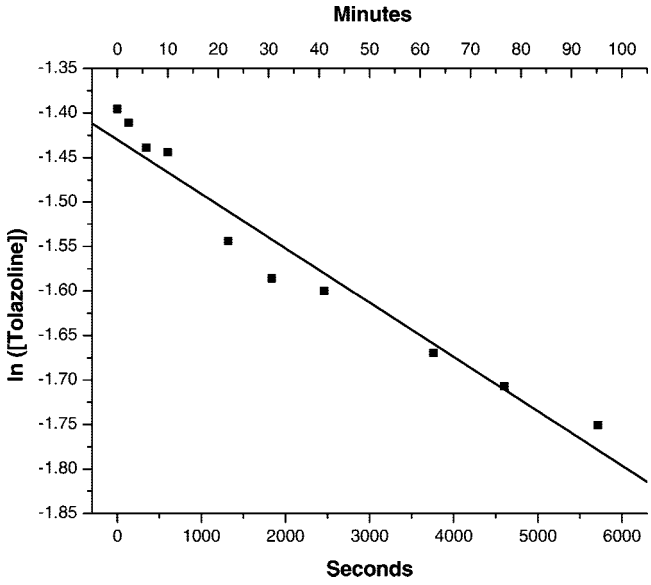


Figure 3. Kinetics plot of ln ($[Tol]_T$) vs time for the nitrosation of 25 mM tolazoline with 10 × $[NO_2^-]$ at 37 °C in 0.5 M acetic acid buffer at pH 3.4. The rate constant ($k_{obs} = 6.3 \pm 0.5\ 10^{-5}\ s^{-1}$) was obtained by linear regression (line, $r^2 = 0.95$).

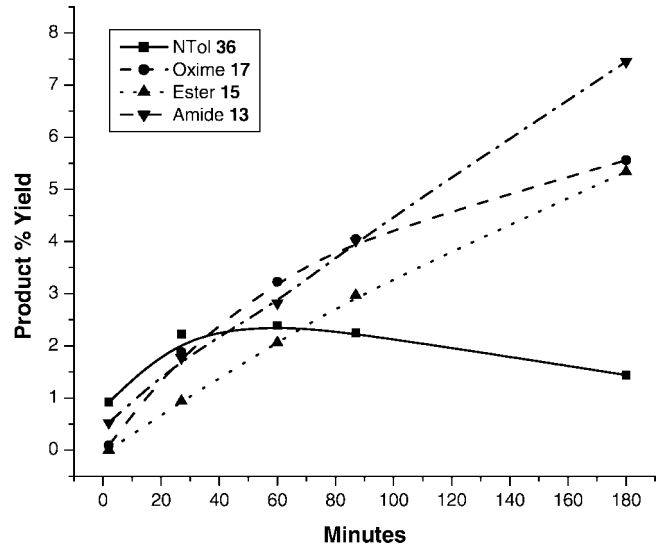


Figure 4. Product yields arising from the nitrosation (250 mM NO_2^-) of tolazoline (25 mM) in 0.3 M HOAc buffer at 37 °C.

zoline, showing that this chemistry and the reactive intermediates involved are significant. Except for **17** and **36**, yields are increased by longer reaction times, although we have not established error limits. Because its yield decreases with longer reaction times, the oxime **17** must be undergoing further transformations. The formation of **29** is the only indicator of such chemistry that we have discovered so far, but we did not detect the formation of **29** under these more limiting conditions.

Kinetics of Tolazoline Nitrosation. The kinetics of tolazoline nitrosation were examined in acetic acid buffer solutions of various strengths. Reactions in glacial acetic acid were too rapid to provide meaningful data. Because of the relatively high $[NO_2^-]$ (10 × [tolazoline]) used to ensure pseudofirst-order kinetics, we chose to work at a 0.5 M buffer concentration with relatively low substrate concentrations (25 mM tolazoline) at pH 3.4 where reasonable data could be obtained. Tolazoline concentrations were determined using internal standards at two different HPLC detector wavelengths to compensate for a peak overlap problem. The reactions in acetic acid, formic acid, and chloroacetic acid buffers were followed by HPLC determination of tolazoline concentration at 37 °C. Rate constants were obtained by linear regression of ln ($[Tol]_T$) vs time, where $[Tol]_T$ is the total analyzable tolazoline concentration, protonated and free base, at any time. A typical plot is shown in Figure 3. The rate constants (k_{obs}, $10^{-5}\ s^{-1}$) in the three buffer systems are as follows: CH_3COOH, 6.3 ± 0.5; HCOOH, 6.9 ± 0.5; and $ClCH_2COOH$, 6.9 ± 0.3. The determinations in acetic acid were the most error prone for unknown reasons. Our data do not shown any significant effect of buffer, although the rate constant obtained in acetic acid buffer is slightly less than those determined in the buffers of the more acidic acids.

$$k = \frac{k_{obs}(1 + [H^+]/K_a)(1 + K_1/[H^+])^2}{K_2[NO_2^-]_0^2} \qquad (1)$$

Under the conditions of our kinetic study, the active nitrosating agent can reasonably be assumed to be N_2O_3. This results in an effective rate dependence on $[NO_2^-]^2$, which we did not test. It follows then that the intrinsic pH-independent rate constant, k, corrected for both the $N_2O_3-NO_2^-$ equilibria and the amount of tolazoline free base determined by its pK_a (10.3), is given by eq 1 as determined at pH 3.4, where K_1 is the K_a for HNO_2, and $K_2 = [N_2O_3]/[HNO_2]^2$. The value of k so determined for the reaction in $ClCH_2COOH$ is $1.14 \times 10^7\ s^{-1}\ M^{-1}$. This value is near, but slightly less (<10×) than, those determined for other secondary amine nitrosations, a process that has been determined to be close to diffusion limited (*42, 43*). Observed nitrosation rates depend upon both the basicity of the substrate and the concentration of the nitrosating agent. The intrinsic rate constant k permits the estimation of nitrosation rates for other imidazoline-based drugs through the application of eq 1.

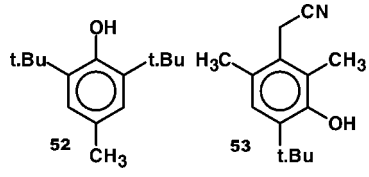
We also determined the nature of the products and their yields as a function of time for the initial portion of the nitrosation reaction in buffer with low substrate concentration but, again, at high $[NO_2^-]$, as in our kinetic studies. The data are shown in Figure 4. Under these conditions, the major products are *N*-nitrosotolazoline **36**, the secondary nitrosation products **15** and **17**, and the amide **13**, which we believe to come from the acid-catalyzed decomposition of **36** but could also arise from the denitrosation of the *N*-nitrosoamide **10**. The concentration of *N*-nitrosotolazoline **36** goes through a maximum, which is in agreement with our mechanistic rationalization of product origins. Both the ester **15** and the oxime **17** are produced from the further nitrosation of *N*-nitrosotolazoline. The fact that these secondary nitrosation products appear rapidly in the mixture is significant. The ester **15** arises from the decomposition of a highly reactive diazonium ion (Schemes 9 and 10). Thus, potent DNA and protein-alkylating agents are produced immediately from the nitrosation of tolazoline. We discuss the properties, particularly the acid-catalyzed decomposition, of *N*-nitrosotolazoline in an accompanying paper (*32*). However, we note here that the amide **13** also arises from a diazonium ion produced from the hydrolytic decomposition of *N*-nitrosotolazoline. Phenylacetic acid **16** is also a product of further nitrosation of **36**, but we did not quantitate it in this particular study.

We attempted to determine the nitrosation kinetics for *N*-nitrosotolazoline, but the reaction is extremely rapid. As a result, we can only provide estimates under these conditions: 25 mM *N*-nitrosotolazoline; 0.5 M buffered HOAc at pH 3.4; 37 °C, variable $[NO_2^-]$ as noted. When a 10× excess of NO_2^- was employed, 95% of the *N*-nitrosotolazoline was consumed in 8 min. When a single equivalent of NO_2^- was employed,

79% of the *N*-nitrosotolazoline reacted in 8 min. The estimated $t_{1/2}$ for the latter process is 3.5 min, while the $t_{1/2}$ for the nitrosation of tolazoline with a 10× excess of NO_2^- is 184 min. Thus, even ignoring the huge disparity in nitrite concentration, we see that the nitrosation of *N*-nitrosotolazoline is approximately 50 times faster than that of tolazoline by this comparison. It is evident why we were unable to isolate *N*-nitrosotolazoline from the nitrosation of tolazoline when a 8× excess of NO_2^- was employed. The much greater nitrosation rate of *N*-nitrosotolazoline likely arises from the fact that it is a much weaker base than tolazoline because of the electron-withdrawing capacity of the *N*-nitroso group. As a result, the fraction of *N*-nitrosotolazoline present as the nitrosatable free base in the acidic media is much greater than it is for tolazoline.

Oxymetazoline Nitrosation. We have made a cursory investigation of the nitrosation chemistry of another imidazoline-based drug, oxymetazoline **2**. This substance is an active constituent of many over-the-counter nasal decongestant spray products sold in the United States. Its nitrosation chemistry provides an interesting contrast to that of tolazoline. Several experiments showed that oxymetazoline disappears rapidly from nitrous acid solutions. The application of a variety of analytical techniques indicated that this compound was principally reacting through its electron-rich aromatic ring rather than at the imidazoline ring nitrogens. Attempts to separate the reaction mixture by HPLC or gain useful information from GC/MS were not fruitful. Numerous compounds were formed. NMR experiments showed that most of the products were arising from reactions with the aromatic ring. Not only were numerous peaks seen in the aromatic region of the ^{13}C spectra of the product mixtures, but a number of new methyl peaks were observed.

The aromatic ring of oxymetazoline, 3-hydroxy-2,6-dimethyl-4-*t*.butylphenyl, is structurally similar to that found in common phenolic antioxidants such as BHT, 2,6-di-*t*.butyl-4-methylphenol **52**. Antioxidants are known to rapidly reduce nitrosating agents to NO with the concomitant formation of phenolic radical cations and radicals and produce aromatic ring nitrosation and nitration products, among others (*44, 45*). Numerous products arise from the radicals. This NO-producing redox chemistry is expected to be much more rapid than *N*-nitrosation in the imidazoline ring, in part because of extensive ring *N*-protonation in acid. To prove this point, we compared the nitrosation of 1:1 mixtures of 3-hydroxy-2,6-dimethyl-4-*t*.butylphenylacetonitrile **53**, a synthetic progenitor of oxymetazoline, and tolazoline by assaying for the amount of each compound remaining after 1 h using 0.5–4 equiv of NO_2^-. The results shown in Figure 5 demonstrate that **53** is much more reactive toward HNO_2 than is tolazoline. These experiments and the intractable nitrosation mixtures produced from oxymetazoline nitrosation strongly suggest that its electron-rich aromatic ring determines the direction of the nitrosation chemistry for this substrate.



Toxicological Perspective. Here, we have shown that tolazoline **1**, a prototypical imidazoline-containing drug, undergoes nitrosation readily. Although there is very little published information on nitrosation chemistry of cyclic amidines such as imidazolines, the ready nitrosation of tolazoline is not unexpected because most basic secondary nitrogen compounds undergo facile nitrosation. However, the most common of these substrates are secondary amines and produce chemically stable nitrosamines, which require metabolic activation to produce their carcinogenic and adverse toxicological activities. *N*-Nitrosotolazoline **36** is a chemically reactive substance. Here, we have shown that it undergoes rapid secondary nitrosation. It competes effectively with tolazoline for available nitrosating equivalents. C-Nitrosation of *N*-nitrosotolazoline gives the isolable α-oximino-*N*-nitrosoimidazoline **17** (Scheme 8). *N*-Nitrosation of *N*-nitrosotolazoline produces a cascade of reactive electrophiles as shown in Schemes 9 and 10. The ester **14** and alcohol **15** are exemplary end products of the torrent of reactive diazonium ions and related electrophiles produced from the *N*-nitrosation of *N*-nitrosotolazoline. As shown in Table 2 and Figure 4, both **17** and **15** are significant nitrosation products even under the limiting NAP test conditions. In separate papers (*32, 46*), we present and discuss the facile acid-catalyzed decomposition of *N*-nitrosotolazoline to reactive electrophiles under physiological conditions, and the DNA−base-binding properties of electrophiles so derived. Although we have not probed its chemistry extensively, **17** is also expected to be the progenitor of electrophiles because it is an *N*-nitrosoimidazoline. With the exception of compounds like oxymetazoline, all secondary imidazolines are expected to possess nitrosation chemistry similar to tolazoline. All of the *N*-nitrosoimidazolines derived from such processes are expected to readily produce electrophiles under physiological conditions without further bioactivation. On the basis of voluminous literature precedent arising from *N*-nitroso compounds and other carcinogens and mutagens, *N*-nitrosoimidazolines are expected to be direct-acting mutagens and carcinogens. Thus, the nitrosation chemistry of imidazoline-based drugs should be seriously considered.

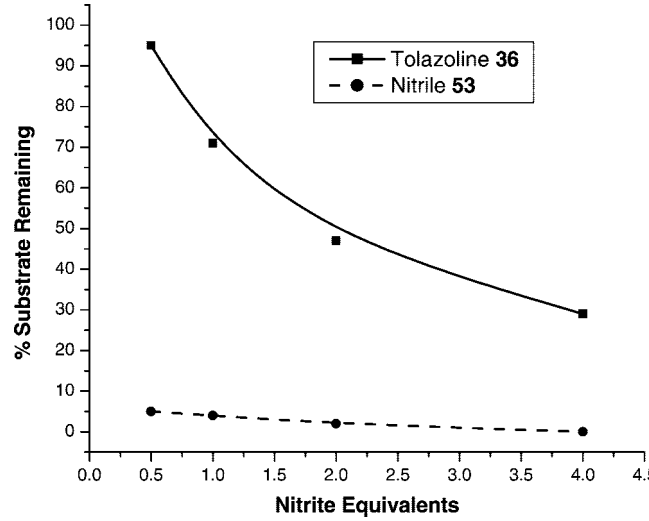


Figure 5. Mixtures (1:1) of tolazoline (600 mM) and **53** were nitrosated at 25 °C in HOAc for 1 h using variable equivalents of NO_2^-. The quantity of each substrate remaining in these competition experiments was determined by HPLC. The much greater reactivity of **53** toward HNO_2 is evident.

From the viewpoint of adverse toxicology, we are less concerned with the product contamination by *N*-nitrosoimidazolines such as *N*-nitrosotolazoline. Although this kind of nitrosation chemistry is a very serious and common problem that arises during the preparation, formulation, and packaging of nitrosatable drugs, excellent methods exist for the detection and elimination of these *N*-nitroso compounds (*47*). Here, we are more interested in the potential endogenous nitrosation of

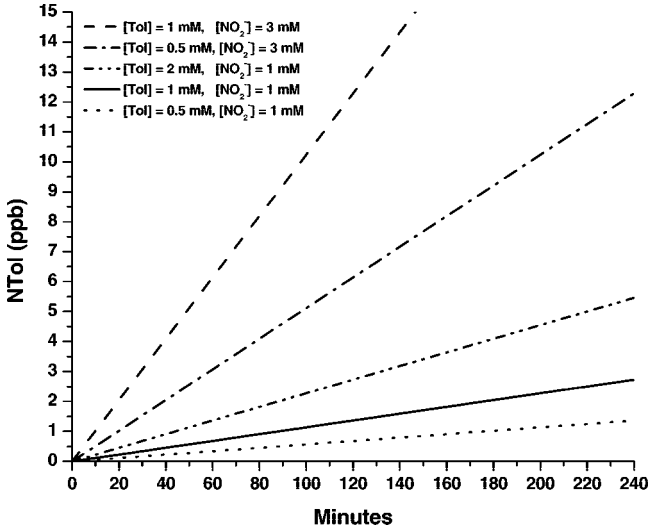


Figure 6. Estimates of *N*-nitrosotolazoline (NTol) formation (ppb) as a function of reaction time at various initial reactant concentrations are presented (pH 3.4, $T = 37$ °C).

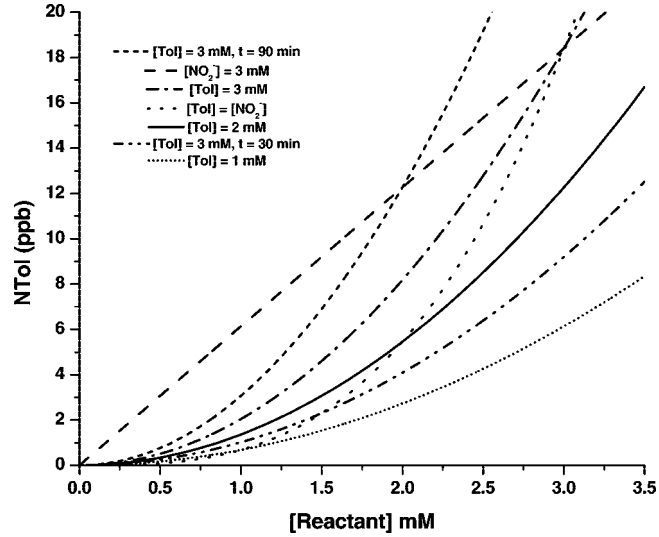


Figure 7. Estimates of NTol yields (ppb) at 1 h, unless noted in the legend, as a function of initial reactant concentration are shown (pH 3.4, $T = 37$ °C). The reactant concentration not given in the graph legend is the *X*-axis variable.

imidazoline-containing drugs, of which tolazoline is a model. Endogenous acid-mediated gastric nitrosation has been well-documented and extensively modeled (*5, 11, 12, 16, 41, 48–51*). The NAP test can be used to compare the nitrosatability of various substrates but utilizes greater concentrations of nitrite than those now accepted to represent the human physiologic range (*41*).

The main nitrosation agent under endogenous conditions, both gastric and those derived from profuse NO production in the presence of O_2, is N_2O_3 (*38*). In acid, such as exists in the stomach, the generation of N_2O_3 depends on $[NO_2^-]^2$. Thus, gastric nitrosation rates depend significantly upon the concentration of nitrite. While nitrite is a common ingredient of some foods, particularly cured meats and similar "salt-preserved" meats and fish, a major source of gastric nitrite is nitrate (*52–56*), which is reduced in human saliva by bacteria to nitrite. This process has been carefully documented. Basal fasting gastric nitrate and nitrite concentrations are about 1.2 and 0.2 mM, respectively (*41, 55*). These levels can rise rapidly and significantly following the ingestion of nitrate-rich vegetables; for example, a pulse dose of 400 mg of NO_3^- in vegetable juice results in a gastric NO_2^- concentration of 2.2 mM within 1–2 h, and levels remain significantly elevated for up to 6 h or longer (*41, 55*). Although the nitrate concentration of potable water in the United States is regulated and is commonly significantly below 10 ppm (0.2 mM), it is often much higher in some European countries and elsewhere because of intensive agriculture and more permissive regulation. Gastric nitrite concentrations also vary with stomach pH (increase with pH), diet, disease state, and other factors (*41*). A range of basal concentrations from 0 to 1.6 mM have been measured in one study (*57*). As a conservative refinement of the NAP test, Walters utilized $[NO_2^-]$ values of 25 μM as a typical gastric concentration in the fasting human stomach but acknowledges that much higher concentrations have been measured (*58*).

We have used our kinetic data to provide reasonable estimates of *N*-nitrosotolazoline concentrations in ppb under likely human drug and nitrite concentrations. Our estimates are presented in graphical form in Figures 6 and 7. For the sake of comparison, the U.S. FDA (U.S. Food and Drug Administration) action level for dimethylnitrosamine in malted beverages is 5 ppb. Molar mass adjustments for *N*-nitrosotolazoline yield an equivalence of 9–10 ppb. Initial rate plots for the formation of *N*-nitrosotolazoline are given in Figure 6 for an exemplary range of nitrite and tolazoline initial concentrations. Although significant gastric nitrite concentrations may be maintained for more than 4 h, the drug's reaction time is probably less than an hour in most cases because of absorption and emptying. The estimates depicted in Figure 6 show that significant concentrations of *N*-nitrosotolazoline are only achieved when $[NO_2^-] = 3$ mM. Similarly, Figure 7 shows how *N*-nitrosotolazoline yields at 1 h (except for two cases) depend on initial reactant concentrations. One hour yields greater than 5 ppb are achieved only when $[NO_2^-] > 1$ mM.

As discussed above, we determined the tolazoline nitrosation rates under pseudofirst-order conditions. The instability of *N*-nitrosotolazoline under the nitrosation conditions requires that the reaction be followed by determining the tolazoline concentration change, and this limitation requires that we use reactant concentrations greater than those likely to occur under endogenous conditions. Therefore, we calculated our estimates in the following way. In accordance with literature precedent (*42, 43*) and our observations, the nitrosation reaction was reasonably assumed to involve the reaction of the unprotonated substrate and N_2O_3. The rate constant expression k_o (1.00×10^{-3}), derived from our data (pH 3.4, 0.5 M acetate buffer, 37 °C), is given in eq 2, where k is the intrinsic rate constant as determined above. The integral form of the differential equation is given by eq 3 where [NTol] is the concentration of *N*-nitrosotolazoline. This equation cannot be solved explicitly for [NTol], but values of [NTol] can be obtained through the application of numerical methods using the Newton–Raphson algorithm and a simple computer program. We used this method to validate our approach by generating data, which could be compared with our experimental data. Explicit determination of [NTol] when the initial substrate concentrations are equal is possible using eq 4. However, for the concentrations and reaction times being considered in our estimates, it is possible to calculate [NTol] from the simple initial rate relationship given in eq 5. Comparisons with the more rigorous solutions showed that errors did not exceed 1%.

$$k_o = \frac{kK_2}{(1 + [H^+]/K_a)(1 + K_1/[H^+])^2} \quad (2)$$

$$k_o t = \frac{1}{([Tol]_0 - [NO_2^-]_0)^2} \times \mathrm{Ln}\left\{\frac{[Tol]_0([NO_2^-]_0 - [NTol])}{[NO_2^-]_0([Tol]_0 - [NTol])}\right\} + \frac{[NTol]}{[NO_2^-]_0([Tol]_0 - [NO_2^-]_0)([NO_2^-]_0 - [NTol])} \quad (3)$$

$$2k_o t = \frac{1}{([Tol]_0 - [NTol])^2} - \frac{1}{[Tol]_0^2} \quad (4)$$

$$[NTol] = [Tol]_0[NO_2^-]_0^2 k_o t \quad (5)$$

Summary and Conclusions

The nitrosation of tolazoline generates a complex array of products, most of which are derived from reactive diazonium ions produced from the further nitrosation of *N*-nitrosotolazoline. While *N*-nitrosotolazoline can be isolated through the application of special procedures, it nitrosates more rapidly than tolazoline because of its lower basicity. This secondary nitrosation occurs even at low substrate concentrations. Our succeeding papers (*32, 46*) show that *N*-nitrosotolazoline possesses the chemical properties typical of a direct-acting carcinogen. Nitrosation rates are comparable to what have been observed for various secondary amines. With the exception of compounds having an electron-rich aromatic ring substituent, other imidazoline-based drugs are expected to exhibit nitrosation chemistry similar to tolazoline. The nitrosation of oxymetazoline, which contains such an aromatic substituent, appears to result in transformations in the aromatic ring rather than exclusive reaction of the imidazoline moiety. Our estimates of endogenous nitrosation rates suggest that problematic levels of *N*-nitrosotolazoline or a related *N*-nitrosoimidazoline could arise when gastric nitrite levels, produced from the dietary consumption of either nitrate or nitrite, exceed 1 mM. These estimates are obviously dependent on drug dose and pH. We have also demonstrated that *N*-nitrosotolazoline forms from the reaction of tolazoline with a mixture of NO and O_2 at neutral pH. This observation is relevant to situations such as those that involve chronic inflammation where N_2O_3 is formed from these substances. From the perspective of endogenous nitrosation, occasional use of imidazoline-based drugs is not likely to be problematic, but their possible use for chronic disorders such as hypertension should be subject to careful risk−benefit analysis.

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Supporting Information Available: Selected ^1H and ^{13}C NMR spectra, enlarged Ortep drawings of X-ray determined structures, crystallographic data for these structures, and CIF files. This material is available free of charge via the Internet at http://pubs.acs.org.

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