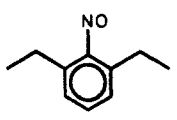
Properties and Decomposition of 2,6-Diethylnitrosobenzene

Stephen J. Wratten,* Hideji Fujiwara, and R. Thomas Solsten

2,6-Diethylnitrosobenzene (1), proposed as a toxic metabolite of 2,6-diethylaniline, was shown to decompose rapidly in solution or during GC analysis. This unexpected instability combined with the formation of an equilibrating mixture of monomeric and dimeric forms in solution resulted in very complex analytical data that appear to have been misinterpreted in the previous metabolic studies. A group of related compounds were synthesized and used to identify the thermal decomposition products of 1 based on their spectral and chromatographic properties. The major compounds identified are *N*-(2,6-diethylphenyl)hydroxylamine and 3-methyl-7-ethylanthranil, whose structures were confirmed by synthesis, and an unusually substituted azobenzene derivative.

Metabolic transformations of anilines and their acylated derivatives have been a subject of continuing interest to our group because of the important herbicidal properties of several 2-chloroacetanilides. A recent publication (Kimmel et al., 1986) described the identification of 2,6-diethylnitrosobenzene (1) as a rat metabolite of alachlor



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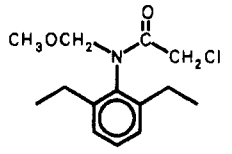

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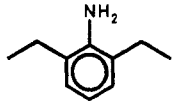

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(2) and reported than 1 was a mutagen in the Ames salmonella assay. Although nitrosobenzene and its derivatives have sometimes been detected as metabolites of anilines (Venulet and VanEtten, 1970), it was surprising to learn that 1 had been identified as arising from incubation of 2,6-diethylaniline (3) with rat liver preparations, since we had just completed a thorough study of the metabolism of ^{14}C-labeled diethylaniline by rat liver enzymes, under a variety of conditions, and had not detected 1 as a metabolite (Feng and Wratten, 1987). In order to clarify the basis for this discrepancy, an authentic sample of 1 was prepared as described (Kimmel et al., 1986) to facilitate attempts to detect it in the metabolic mixtures.

During these experiments, it was discovered that 1 is remarkably unstable, which may have led to a misinterpretation of the analytical and biological data by the previous workers, and upon decomposition it produced some unexpected products that are chemically intriguing. This paper reports the results of further studies that have defined the properties of 1 and its chemical fate when it was allowed to decompose at room temperature.

EXPERIMENTAL SECTION

Instrumentation. High-performance liquid chromatography (HPLC) was performed with a system assembled from components manufactured by Waters Associates (two Model 6000A pumps, a Model 720 controller, and a Model 440 UV detector operated at 254 nm), equipped with a Waters μ-Bondapak C_{18} column (3.9 × 300 mm), using a linear gradient (15 min) of 25–75% acetonitrile in 0.5% ammonium acetate buffer (pH 3.25) at 1 mL/min. Gas chromatography (GC) was conducted on a Varian Model 3700 chromatograph with a flame ionization detector using a 50% (phenylmethyl)silicone column (10 m × 0.53 mm) manufactured by Hewlett-Packard, programmed 80–250 °C at 10°/min. NMR spectra were recorded on a Varian XL-300 spectrometer in $CDCl_3$, with ^1H chemical shifts (δ) reported relative to $CHCl_3$ at δ 7.26 unless otherwise stated. Mass spectrometry (MS) was performed on a Finnigan MAT 4535 spectrometer equipped with an INCOS data system and a Finnigan gas chromatograph or a VG ZAB-HF spectrometer with a VG 11/250 data system and a Hewlett-Pckard Model 5890 gas chromatograph. HPLC/MS was conducted by interfacing an identical HPLC system as described above to the Finnigan MAT 4535 using a Vestec thermospray source operated with a vapor temperature of 325 °C and a vaporizer temperature of 110–120 °C in the filament-ionization mode; the chromatographic conditions were identical with those above except the buffer was replaced with water. Low-resolution MS analyses were conducted on the Finnigan MAT 4535 by direct probe or by GC using a cross-linked methylsilicone column (10 m × 0.32 mm) from Hewlett-Packard (isothermal 50 °C for 1 min, 20°/min to 130 °C, 10°/min to 250 °C; injector 150–250 °C) in the chemical ionization (CIMS, isobutane) or in the electron impact (EIMS) modes. High-resolution mass spectrometry was performed in the EIMS mode on the VG ZAB-HF either by peak matching or in the scanning mode during GC elution using a DB-5 column (25 m × 0.32 mm) from J & W Scientific (isothermal 100 °C for 1 min, 10°/min to 250 °C; injector 200–250 °C). Infrared spectra (IR) were recorded on an IBM Model 85 FTIR spectrometer by diffuse reflectance on a KCl matrix. UV spectra were recorded on a Beckman DU-7 spectrophotometer.

Chemicals. 3, 5, 7, and *m*-chloroperoxybenzoic acid (MCPBA) were purchased from Aldrich Chemical Co., and the other solvents and reagents were purchased from Fisher Scientific. These materials were all used without further purification.

2,6-Diethylnitrosobenzene (1). 2,6-Diethylaniline (3; 100 mg, 0.67 mmol) was dissolved in CH_2Cl_2 (3 mL) and the resultant mixture was stirred at −5 °C during the addition of MCPBA (288 mg of 82% purity, 1.36 mmol) in CH_2Cl_2 (3 mL) over 2 min. After being stirred for 5 min, the reaction mixture was evaporated, and the product was suspended in 10% diethyl ether in petroleum ether and added to a Florisil (15 g) column (1.5 × 20 cm) in the same solvent. 1 (73 mg, 0.45 mmol, 67% of theoretical) was eluted in the fourth 25-mL fraction as 50-mL portions of 10% and 20% diethyl ether in petroleum ether were used to develop the column. Alternatively, the crude reaction

Metabolism Section, Monsanto Agricultural Company, St. Louis, Missouri 63198.

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mixture in CH_2Cl_2 could be washed with 0.5 N NaOH (2 × 5 mL), dried (Na_2SO_4), and evaporated to yield a white solid, which was recrystallized from petroleum ether to yield white plates: mp 101–104 °C dec; ^1H NMR (as a 0.006 M solution) 7.49 (t, 0.5 H, monomer), 7.43 (t, 0.5 H, dimer), 7.27 (d, 1 H, dimer), 7.24 (d, 1 H, monomer), 2.97 (q, 2 H, monomer), 2.77 (dq, 2 H, dimer), 1.32 (t, 3 H, dimer), 1.22 (t, 3 H, monomer); HPLC/MS (peak at 19.3 min) m/z 164 (MH^+); EIMS, m/z (relative intensity) 163.0998 (27, $M^{\cdot+}$) $C_{10}H_{13}NO$ requires 163.0997, 148 (100), 144 (21), 134 (14), 130 (19), 120 (47), 105 (82), 91 (74), 79 (58), 77 (59); IR 2970, 1450, 1060, 780, 740 cm^{-1}.

2,6-Dimethylnitrosobenzene (6) was prepared by oxidation of 2,6-dimethylaniline (**5**) with MCPBA in the same way as above. **6** was obtained as crystals from ethanol: mp 131–133 °C (lit. mp 133.5–134 °C) (Azoulay et al., 1981); ^1H NMR (dimer) 7.29 (t, 1 H), 7.19 (d, 2 H), 2.47 (s, 6 H), (monomer) 7.31 (t, 1 H), 7.19 (d, 2 H), 2.65 (s, 6 H); EIMS, m/z (relative intensity) 135 (50, $M^{\cdot+}$), 105 (75), 79 (100), 77 (95).

2-Ethyl-6-methylnitrosobenzene (8). **8** was prepared by oxidation of 2-ethyl-6-methylaniline (**7**) with MCPBA as above. Crystals (mp 97–99 °C) of **8** were obtained from ethanol: ^1H NMR 7.1–7.5 (m, 3 H), 3.30 (q, 0.6 H, monomer), 2.78 (m, 1.4 H, dimer), 2.48 (s, 2.1 H, dimer), 2.38 (s, 0.9 H, monomer), 1.32 (t, 3 H); EIMS, m/z (relative intensity) 149.0833 (18, $M^{\cdot+}$) $C_9H_{11}NO$ requires 149.0841, 134 (73), 91 (100), 77 (75).

4-Amino-3,5-diethylphenol (9) and 3,5-diethyl-4-iminobenzoquinone (10) were prepared as described by Feng and Wratten (1987).

2,6-Diethylnitrobenzene (11). **3** (250 mg, 1.68 mmol) was dissolved in CH_2Cl_2 (30 mL) and the resultant mixture stirred at −5 °C. MCPBA (1131 mg of 82% purity, 5.38 mmol) in CH_2Cl_2 was added, and the mixture was warmed to 4 °C for 64 h. Additional MCPBA (350 mg, 1.68 mmol) was added, and stirring was continued for 7 h at room temperature. Dimethyl sulfoxide (0.5 mL) was added, and the mixture was extracted with 1 N NaOH (2 × 25 mL), 1 N HCl (50 mL), and water (2 × 25 mL). The organic layer was dried (Na_2SO_4) and evaporated, and the resulting crude product was added to a Florisil (10 g) column (1 × 23 cm) in 1% acetone in petroleum ether. The column was developed with the same solvent while 5–10-mL fractions were collected. Compound **11** (130 mg, 0.72 mmol, 43% of theoretical) was obtained as a yellow oil from fractions 4 and 5: ^1H NMR 7.35 (t, 1 H), 7.17 (d, 2 H), 2.60 (q, 4 H), 1.24 (t, 6 H); EIMS, m/z (relative intensity) 179.0960 (8, $M^{\cdot+}$), $C_{10}H_{13}NO_2$ requires 179.0946, 163 (30), 162 (36), 147 (67), 134 (22), 120 (69).

2-Amino-3-ethylacetophenone (12). 2-Ethylaniline (220 g, 1.82 mol) was mixed with concentrated HCl (78.2 mL, 0.91 mol) followed by 3-hydroxy-2-butanone (94.1 g, 0.91 mol) as an 85% solution in water. The reaction mixture was heated with a Dean–Stark trap to remove the water followed by refluxing 2 h. The resulting mixture was partitioned between 2 N HCl (1 L) and diethyl ether (1.5 L), and the product in the organic layer was distilled [bp 120 °C (1.5 torr)] to yield 2,3-dimethyl-7-ethylindole as a pink oil that slowly crystallized to a low-melting solid (130.6 g, 0.75 mol, 82% of theoretical): EIMS, m/z (relative intensity) 173.1224 (80, $M^{\cdot+}$), $C_{12}H_{15}N$ requires 173.1204, 158 (100).

2,3-Dimethyl-7-ethylindole (52.0 g, 0.3 mol) in methanol (1.9 L) was treated with sodium *m*-periodate (128.5 g, 0.6 mol) in water (1.2 L) at 0–7 °C, and the mixture was allowed to warm to room temperature and partitioned between water and CH_2Cl_2. The organic layer was dried ($MgSO_4$) and evaporated to yield a crude solid that was triturated with cold diethyl ether to obtain *N*-(2-acetyl-6-ethylphenyl)acetamide (47.8 g, 0.23 mol, 78% of theoretical) as a white solid, mp 122–123 °C (ethanol). Anal. Calcd for $C_{12}H_{15}NO_2$: C, 70.22; H, 7.37; N, 6.82. Found: C, 69.93; H, 7.50; N, 6.87.

N-(2-Acetyl-6-ethylphenyl)acetamide (10.0 g, 48.7 mmol) was refluxed in 4 N HCl (250 mL) for 2.5 h, cooled, diluted with water (500 mL), and basified with 50% NaOH. 2-Amino-3-ethylacetophenone (**12**; 8.0 g, 48.7 mmol, 100% of theoretical) was recovered by extraction with CH_2Cl_2 as a brown liquid and used without purification, although chromatography on silica gel using 12% diethyl ether in hexane could be used to obtain white crystals: mp 40–41 °C; ^1H NMR 7.64 (d, 1 H), 7.23 (d, 1 H), 6.42 (t, 1 H), 2.60 (s, 3 H), 2.51 (q, 2 H), 1.27 (t, 3 H); EIMS, m/z (relative intensity) 163.0989 (54, $M^{\cdot+}$), $C_{10}H_{13}NO$ requires 163.0997, 148 (100), 120 (7). Anal. Calcd for $C_{10}H_{13}NO$: C, 73.59; H, 8.03; N, 8.58. Found: C, 73.33; H, 7.97; N, 8.60.

2-Ethyl-6-(1-hydroxyethyl)aniline (13). Ketone **12** (12.0 g, 73.5 mmol) in tetrahydrofuran (THF, 25 mL) was added dropwise (15 min) to lithium aluminum hydride (1.40 g, 36.8 mmol) in THF (60 mL) at room temperature. The reaction was stirred an additional 5 min and partitioned between water and CH_2Cl_2. The organic layers were dried ($MgSO_4$), concentrated, and recrystallized from diethyl ether in hexane to yield **13**: 9.30 g, 56.4 mmol, 77% of theoretical; mp 60–61 °C; ^1H NMR 7.02 (m, 2 H), 6.71 (t, 1 H), 4.95 (br q, 1 H), 2.53 (q, 2 H), 1.61 (d, 3 H), 1.26 (t, 3 H); EIMS, m/z (relative intensity) 165.1123 (19, $M^{\cdot+}$), $C_{10}H_{15}NO$ requires 165.1154, 147 (72), 132 (100), 117 (35).

3-Methyl-7-ethylanthranil (14). Ketone **12** (100 mg, 0.61 mmol) was stirred in chloroform (10 mL) at room temperature during the addition (5 min) of MCPBA (250 mg of 82% purity, 1.2 mmol) and for 4 h afterward. The reaction mixture was washed with 0.1 N $NaHCO_3$ (2 × 10 mL), dried, and evaporated to yield a crude product (110 mg) that was purified on a Florisil (10 g) column (1 × 24 cm) using 3% acetone in petroleum ether as an eluant. **14** (55 mg, 0.34 mmol, 56% of theoretical) was obtained as a clear oil: ^1H NMR 7.26 (d, 1 H), 7.01 (d, 1 H), 6.86 (dd, 1 H), 2.94 (q, 2 H), 2.77 (s, 3 H), 1.36 (t, 3 H); CIMS, m/z (relative intensity) 162 (100, MH^+); EIMS, m/z (relative intensity) 161.0866 (23, $M^{\cdot+}$), $C_{10}H_{11}NO$ requires 161.0841, 146 (37), 118 (35), 91 (20), 43 (100).

***N*-(2,6-Diethylphenyl)hydroxylamine (15).** Freshly prepared **1** (274 mg, 1.68 mmol) was dissolved in ethanol (25 mL) and the resultant mixture was diluted with 0.5 N NH_4Cl (15 mL). This cloudy mixture was stirred vigorously with zinc dust (110 mg, 1.7 mmol) at room temperature. Two additional portions (18 mL) of 0.5 N NH_4Cl were added 2 min apart as the mixture became clear. After 15 min, the mixture was diluted with water (50 mL), and a fluffy precipitate later shown to be **1** was removed. The filtrate was extracted with CH_2Cl_2 (3 × 50 mL) to yield after drying (Na_2SO_4) and evaporation a crude product mixture (270 mg), which was dissolved in petroleum ether (3 mL) at −20 °C. The resulting crystals were recrystallized from petroleum ether to yield **15** (25 mg, 0.16 mmol, 9% of theoretical): mp 106–108 °C; IR 3320, 3200, 2965, 2873, 1492, 1450, 1411 cm^{-1}; ^1H NMR 7.10 (m, 3 H), 2.80 (q, 4 H), 1.28 (t, 6 H); EIMS, m/z (relative intensity) 165.1154 (54, $M^{\cdot+}$), $C_{10}H_{15}NO$ requires 165.1154, 148 (100), 132 (14), 120 (33). **15** was also present in samples prepared by reduction of **11** with 2 equiv of zinc in methanol–0.5 N NH_4Cl (1:2).

Azobenzene Derivative 16. A 1% solution of **1** in acetonitrile was allowed to decompose at room temperature

for 72 h, and repetitive HPLC injections were made under the above conditions while the peak at 15.6 min was collected. Larger amounts of **16** were later obtained by column chromatography of the decomposition mixture on Florisil using a gradient of acetone in petroleum ether: UV, λ_{max} (methanol) 464 nm, 323. IR 3440, 2930, 1695, 1460, 1350, 1270, 1110 cm^{-1}; ^1H NMR (CD_3CN) 7.65 (br d, 1 H), 7.55 (m, 2 H), 7.42 (t, 1 H), 7.30 (br d, 1 H), 7.25 (dd, 1 H), 5.05 (q, 1 H), 3.10 (q, 2 H), 2.72 (dq, 2 H), 2.15 (s, 3 H), 1.32 (d, 3 H), 1.21 (t, 3 H), 1.12 (t, 3 H); ^{13}C NMR ($CDCl_3$) δ 202.8, 148.5, 148.1, 144.2, 140.2, 138.4, 133.9, 131.2, 130.7, 130.5, 129.1, 124.9, 124.1, 65.0, 30.5, 25.6, 25.5, 23.7, 16.5, 16.4; CIMS, m/z (relative intensity) 325 (100, MH^+); EIMS, m/z (relative intensity) 324.1837 (<1%, $M^{\bullet+}$), $C_{20}H_{24}N_2O_2$ requires 324.1838, 307 (19), 306 (8), 291 (87), 148 (14), 43 (100).

Azobenzene Derivative 18. Analysis of **16** using GC/MS under the above conditions produced a single peak (elution at 15.4 min relative to Table I), tentatively assigned as **18**: CIMS, m/z (relative intensity) 307 (100, MH^+); EIMS, m/z (relative intensity) 306 (19, $M^{\bullet+}$), 291 (100).

Dihydroanthranil 19. Analysis of **1** using GC/MS under the above conditions produced a peak of variable intensity as a shoulder following the peak due to **14** tentatively assigned as **19**: CIMS, m/z (relative intensity) 164 (100, MH^+); EIMS, m/z (relative intensity) 163 (92, $M^{\bullet+}$), 148 (100), 120 (62), 103 (25).

3,7-Dimethylanthranil (20). A 1% solution of **8** in acetonitrile was allowed to decompose at room temperature for 6 days. Analysis of the resulting product mixture indicated that it contained a single major product tentatively assigned as **20**: ^1H NMR 7.27 (br d, 1 H), 6.99 (br d, 1 H), 6.85 (dd, 1 H), 2.78 (s, 3 H), 2.58 (s, 3 H); EIMS, m/z (relative intensity) 147.0687 (68, $M^{\bullet+}$), C_9H_9NO requires 147.0684, 132 (12), 118 (26), 104 (30), 43 (100).

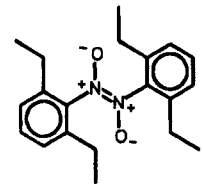
7-Ethylanthranil (21). During the GC/MS analyses of **8** and its degradation products, a peak due to a second very minor isomer was observed and tentatively assigned as **21**: CIMS, m/z (relative intensity) 148 (100, MH^+); EIMS, m/z (relative intensity) 147.0693 (39, $M^{\bullet+}$), C_9H_9NO requires 147.0684, 118 (100).

2-Amino-3-methylacetophenone (22). During the GC/MS analysis of the decomposed mixture derived from **8**, a peak that eluted later than **7**, **20**, and **21** was detected and tentatively assigned as **22**: CIMS, m/z (relative intensity) 150 (100, MH^+); EIMS, m/z (relative intensity) 149 (71, $M^{\bullet+}$), 134 (100), 106 (47).

RESULTS AND DISCUSSION

Properties of 2,6-Diethylnitrosobenzene (1). **1** was prepared by the oxidation of 2,6-diethylaniline (**3**) with 2 equiv of *m*-chloroperoxybenzoic acid (MCPBA) in methylene chloride or acetonitrile. The reaction was essentially instantaneous, and no intermediates were detected when the reaction was conducted in CD_3CN and monitored by ^1H NMR during stepwise addition of the MCPBA. After removal of the chlorobenzoic acid byproduct by chromatography on Florisil, the freshly prepared white solid was analyzed by mass spectroscopy (MS) in both the high-resolution electron impact mode (HRMS) using the direct probe and by combination with reversed-phase high-performance liquid chromatography (HPLC/MS). The results of these experiments confirmed that the reaction had produced a single product whose molecular formula and protonated molecular ions were those expected for **1**. In contrast, analysis of the fresh sample of **1** by gas chromatography (GC) or GC/MS produced a cluster of peaks with at least three major components, none of which could be clearly assigned as **1**, which indicated that **1** is too thermally unstable to allow reliable analysis by these techniques. **1** could be recrystallized from petroleum ether, but extensive handling or heating during attempts at crystallization sometimes produced less pure samples of **1** than were available from the Florisil chromatography.

^1H NMR analysis of a freshly prepared sample of **1** in $CDCl_3$ revealed the presence of two sets of nonequivalent ethyl signals [δ 2.97 (q) and 1.22 (t), coupled; δ 2.77 (dq) and 1.32 (t), coupled], in a ratio of about 1:1, accompanied by several aromatic multiplets. This unexpected result was clarified by reference to published reports that nitroso compounds, particularly aromatic derivatives with ortho substituents (Holmes et al., 1965; Dieterich et al., 1974) undergo a well-studied reversible dimerization. In solution, an equilibrium exists between a monomeric form such as **1** and a dimeric form, usually written as **4**, which is presumably in the trans configuration (Azoulay et al., 1981).



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^1H NMR analysis of concentrated solutions of these substances often contain signals for both forms, which reequilibrate upon dilution, in favor of larger amounts of the monomer (Sundberg, 1967). Indeed, when samples of **1** were analyzed by ^1H NMR, diluted, and analyzed again, the signals at δ 2.97 and 1.22 increased substantially and could therefore be assigned to the monomeric form. The chemical shifts of the ^1H NMR signals assigned by Kimmel et al. (1986) to **1** in CD_3CN correspond to those of the dimer **4**; the smaller signals due to the monomeric form apparently were incorrectly attributed to impurities at other oxidation states such as hydroxylamine or nitro derivatives by those authors. Kimmel et al. reported two overlapping quartets in their ^1H NMR data, which were also observed in our spectra as noted above at δ 2.77; this signal can now be assigned to the methylene protons of **4** and the additional multiplicity apparently arises because of hindered rotation about the C–N bond.

The position of the equilibrium was solvent dependent to some extent. Solutions of 2,6-diethylnitrosobenzene (0.006 M) in $CDCl_3$ were found to contain approximately equal concentrations of the monomeric and dimeric forms. ^1H NMR analysis of CD_3CN or CD_3OD solutions of **1** at this concentration contained somewhat larger amounts of the dimer than were present in $CDCl_3$. The presence of these two equilibrating forms also appeared to contribute to a broadening of the chromatographic peak produced by **1** during HPLC analysis and greatly complicated interpretation of the infrared and ultraviolet spectra as described for other nitroso aromatics (Rao and Bhaskar, 1969). Throughout the following discussion, the structural formula **1** will be used to refer to 2,6-diethylnitrosobenzene even though an equilibrium mixture of **1** and **4** exists in solution.

In contrast to the data produced by analysis of freshly prepared **1**, solutions that had been allowed to incubate at room temperature for even 2 h produced noticeably different results. After 24 h, ^1H NMR analysis revealed than almost none of the original compound remained; the half-life of **1** under these conditions was estimated to be 8–10 h. In contrast to its facile decomposition in solution,

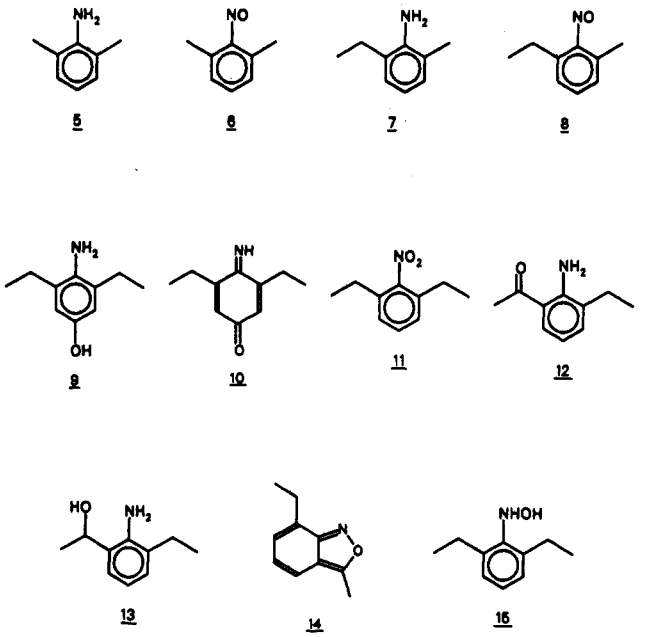


Figure 1. Structures of the synthetic compounds prepared for comparison with the decomposition products.

solid samples of 1 were stable at room temperature, suggesting that decomposition may be initiated by the monomeric free nitroso group, since crystalline samples of nitrosoaromatics are known to exist totally as the dimer (Azoulay et al., 1981).

Several brief experiments were conducted to determine which physical factors might affect the decomposition of 1 in solution. The results suggested that neither the absence of fluorescent room light nor varying the concentration of 1 altered the process significantly. The solvent used and the presence (5%) of either triethylamine or trifluoroacetic acid appeared to effect the distribution of products somewhat but did not appreciably change the rate of decomposition. The most important parameter investigated was temperature, since acetonitrile solutions of 1 showed very little change after 3 weeks at −20 °C, decomposed slowly at 4 °C, and lasted less than 1 h at temperatures of 40–50 °C. This thermal instability further substantiates the conclusion that GC is an inappropriate method for analysis of 1 in metabolic samples. HPLC/MS was not plagued by any detectable decomposition of 1 during many analyses, even though the Vestec thermospray interface was heated above 200 °C; apparently the temperature environment experienced by the analyte molecules during solvent vaporization was much below that of the instrument or occurs for too short of a time to elicit significant decomposition.

Preparation and Characterization of Reference Standards. On the basis of these initial experiments, the identities of the major products produced from 2,6-diethylnitrosobenzene during thermal decomposition at room temperature and during GC analysis were of interest. To facilitate these structural studies, the group of compounds shown in Figure 1 was obtained from commercial sources or prepared as described below and in the Experimental Section. The chromatographic and spectral properties of these compounds are summarized in Table I.

2,6-Dimethylnitrosobenzene (6) was prepared by oxidation of 2,6-dimethylaniline (5) with 2 equiv of MCPBA and purified by chromatography on Florisil and recrystallization from ethanol. Comparison of its melting point with that of Azoulay et al. (1981) confirmed the identity of 6. Interestingly, solutions of 6 did not show any significant decomposition after storage at room temperature for 7 days and eluted as a single peak that produced the proper molecular ions during GC/MS analysis. As expected, 6 also undergoes a reversible dimerization in solution, and formation of the dimer of 6 appears to be somewhat more favored than that of 1. The ^{1}H NMR chemical shift reported by Kimmel et al. (1986) for the aromatic methyl signal of 6 corresponded to that of the dimer; the signal they assigned as that of the corresponding nitro derivative agrees with that of monomeric 6.

Table I. Summary of the Chromatographic and Spectral Properties of the Synthetic Standards

structure	HPLC ret time,[a] min	GC ret time,[a] min	$M^{\cdot+}$,[b] amu	^{1}H NMR posn of benzylic protons[c]
3	13.4	4.5	149	2.54 (g)
5	9.4	2.7	121	2.21 (s)
6	14.2	2.0	135	2.47 (s)[d]
				2.65 (s)[e]
7	11.2	3.6	135	2.55 (q),
				2.21 (s)
8	16.0	2.5	149	2.78 (m),
				2.48 (s)[d]
				3.30 (q),
				2.38 (s)[e]
9	4.9	8.6	165	2.51 (q)
10	9.2	6.4	163	2.59 (q)
11	15.9	4.6	179	2.60 (q)
12	12.0	6.8	163	2.51 (q)
13	8.5	6.8	165	2.53 (q),
				4.95 (br q)
14	12.4	5.6	161	2.96 (q),
				2.78 (s)
15	10.4	unstable	165	2.80 (q)

[a] Conditions without MS as in the Experimental Section. [b] GC/MS in EI mode. [c] Position (δ) in $CDCl_3$. [d] Dimer. [e] Monomer.

The same reaction was used to convert 2-ethyl-6-methylaniline (7) into 2-ethyl-6-methylnitrosobenzene (8), which has not been reported in the literature other than by Kimmel et al. (1986) in which the reported ^{1}H NMR signals again corresponded to those of the dimer of 8. The stability of 8 was intermediate between that of 1 and 6. Decomposition of 8 at room temperature was slower than with 1, and the half-life was estimated to be about 36 h on the basis of the ^{1}H NMR data. GC analysis produced some decomposition although a significant amount of 8 eluted from the column unchanged.

4-Amino-3,5-diethylphenol (9) and its oxidation product 10 were prepared according to the reaction sequence described in the accompanying paper (Feng and Wratten, 1987). The iminoquinone 10 was sufficiently stable to be chromatographed by either GC or HPLC with only minor decomposition to the corresponding dioxygenated benzoquinone.

2,6-Diethylnitrobenzene (11) was prepared by further oxidation of 1 using MCPBA, initially at 0 °C with slow warming to room temperature. Since this oxidation was much slower than that which produced 1 from 3, the decomposition of 1 was competitive with its oxidation, and it was necessary to chromatographically remove the degradation products of 1 from the crude sample of 11.

Since ^{1}H NMR analyses had revealed the presence of products in the crude decomposition mixtures produced from 1 which appeared to have undergone benzylic oxidation of the ethyl groups, compounds 12–14 were also prepared. Oxidative cleavage of the heterocyclic ring of 2,3-dimethyl-7-ethylindole according to Roth and Lepke (1972) followed by acid-catalyzed hydrolysis of the resulting acetanilide produced 2-amino-3-ethylacetophenone (12). Reduction of 12 with lithium aluminum hydride

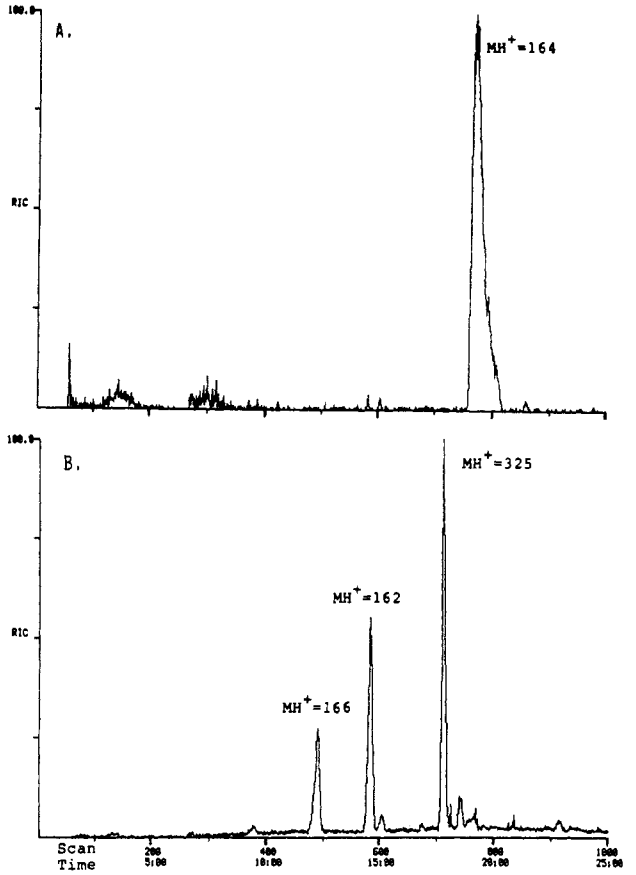


Figure 2. Total ion current chromatograms produced by HPLC/MS analyses of 1 before (A) and after (B) decomposition in acetonitrile solution.

afforded the corresponding alcohol **13**, while further oxidation with MCPBA produced 3-methyl-7-ethylanthranil (**14**) (2,1-benzisoxazole; Smalley, 1981).

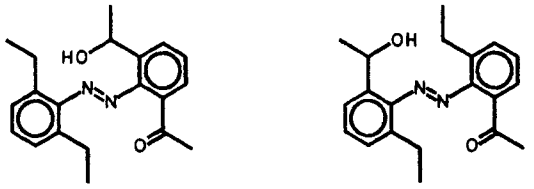
N-(2,6-Diethylphenyl)hydroxylamine (**15**) was prepared as a mixture with **3** by reduction of either **1** or **11** using zinc under neutral conditions. Pure **15** was obtained by crystallization from the crude product mixture using petroleum ether.

Products of Decomposition of 1 in Solution. The mixture resulting from decomposition of diethylnitrosobenzene **1** in acetonitrile solution was chosen for further study. HPLC/MS analysis of this mixture in the positive-ion mode revealed the presence of three components, all of which were more polar than **1** (Figure 2). The protonated molecular ions of the three components were observed at m/z 166, 162, and 325 in order of elution. Each of these components was isolated by preparative HPLC, and additional spectral data were recorded to establish their structures.

The ^{1}H NMR spectrum of the first-eluting compound contained three sets of signals at δ 1.28 (t, 6 H), 2.80 (q, 4 H), and 7.10 (m, 3 H), which were assigned to the diethylaromatic ring, unchanged from that of **1**. These ^{1}H NMR data and the HPLC retention time of this product were identical to those of **15** (Table I), allowing its structural assignment as *N*-(2,6-diethylphenyl)hydroxylamine. Analysis of the purified **15** by GC/MS in the EI mode revealed a cluster of peaks, corresponding to **3**, **9**, **14**, and a fourth peak that produced ions at m/z 165 ($M^{\cdot+}$) and 148 ($M^{\cdot+}$ - OH), appropriate for **15** (Coutts and Mukherjee, 1970). This result indicated that **15**, like **1**, undergoes substantial degradation during GC analysis. Attempts to purify additional samples of **15** by Florisil chromatography of the degradation mixture were unsuccessful and generated a number of secondary degradation products. The formation of hydroxylamine derivatives by thermal disproportionation of nitrosoaromatics has been observed previously (Boyer, 1969).

The second-eluting degradation product of **1**, which had produced protonated molecular ions at m/z 162 during HPLC/MS analysis, had chromatographic and spectral properties identical with those of an authentic sample of **14**. This product is apparently the thermal degradation product shown in the chromatograms of Kimmel et al. (1986), with a molecular weight 2 amu lower than that of **1**, for which a structure had not been assigned. This unexpected formation of a substituted anthranil directly from a nitrosobenzene derivative without prior benzylic substitution is potentially useful, since traditional syntheses have usually required a benzaldehyde or acetophenone derivative possessing appropriate nitrogen substituents (Smalley, 1981).

The third degradation product was isolated as an orange oil with a visible absorption maximum at 464 nm in methanol. HRMS established its molecular formula as $C_{20}H_{24}N_2O_2$, based on the molecular ions at m/z 324.1837 ($C_{20}H_{24}N_2O_2$ requires 324.1838), which corresponded to a dimerization of two molecules of **1** accompanied by a loss of two hydrogens. The infrared spectrum contained signals for carbonyl and hydroxyl groups at 1695 and 3440 cm^{-1}, respectively, which accounted for both of the oxygen atoms. The ^{1}H NMR spectrum in CD_3CN contained resonances assigned to two intact ethyl groups [δ 1.12 (t, 3 H), 1.21 (t, 3 H), 2.72 (dq, 2 H, J = 2, 7 Hz), 3.09 (q, 2 H)], an acetyl methyl singlet at 2.15 ppm, a fourth two-carbon fragment in which a heteroatom had been introduced at the benzylic position [δ 1.32 (d, 3 H), 5.05 (q, 1 H)], and six aromatic protons. Consideration of these data suggested two possible structures, **16** and **17**. Further comparison of the ^{1}H NMR data with those of the synthetic samples of **12** and **13** allowed assignment of the structure of the degradation product as **16**, primarily because of the marked nonequivalence of the two unoxidized ethyl groups, which was not consistent with structure **17**. Further evidence for the structure of **16** was provided by the ^{13}C NMR spectrum, which contained appropriate distinct signals for each of the 20 carbon atoms. GC/MS analysis of **16** in the electron ionization mode produced a single chromatographic peak whose mass spectrum contained molecular ions at m/z 306, which indicated that **16** had undergone dehydration during the analysis to produce a product that was probably **18**. The formation of an azobenzene product from the decomposition of a nitrosobenzene derivative was not unexpected (Boyer, 1969), but



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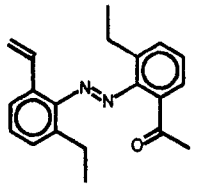
16



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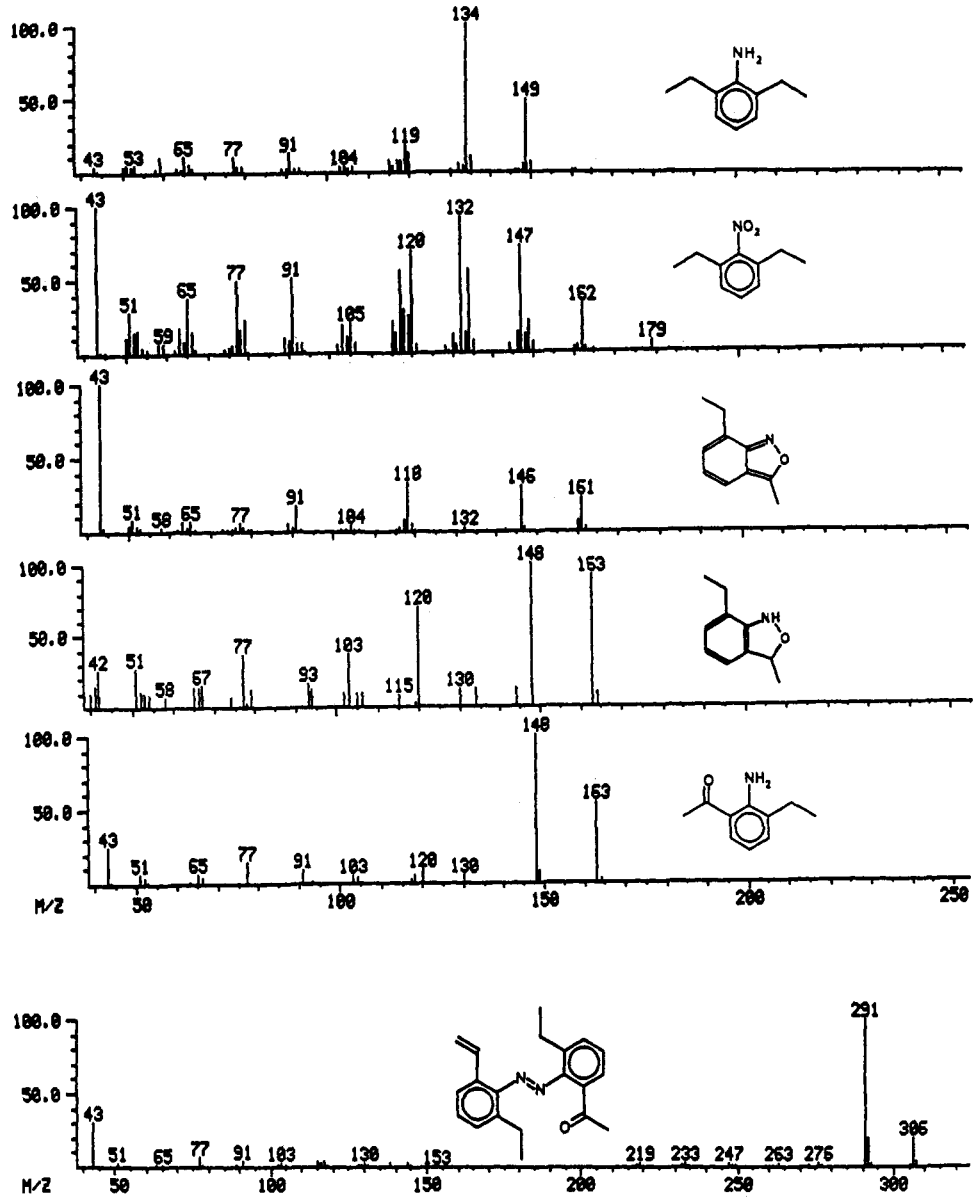


Figure 3. EI mass spectra of the six decomposition products produced from 1 during GC/MS analysis.

conversion only to 16, which possessed several different types of functionalities with each in a single specific oxidation state, is noteworthy.

The ^{1}H NMR spectrum of the decomposition mixture produced from 1 contained the appropriate signals for 14–16 as the only major components in approximately a 1:2:1 ratio based on integration. Since the formation of 14 and 16 from three molecules of 1 requires two formal oxidation steps, two additional molecules of 1 must apparently have been concomitantly reduced to produce 15, which is consistent with the stoichiometry observed in the resulting mixture.

A search of the chemical literature for reports of the instability of other 2,6-dialkylnitrosobenzenes was conducted. With one exception, no other references to compounds with 2,6-dialkyl substituents ranging in size from C_1 to C_4 other than methyl or *tert*-butyl were found; in one instance, the preparation of 2,6-diisopropylnitrosobenzene as a solid with melting point 98.5–100 °C was described with no mention of instability in solution (Hirota and Itano, 1978). 2,4,6-Tri-*tert*-butylnitrosobenzene, 2,3,5,6-tetramethylnitrosobenzene, and pentamethylnitrosobenzene are widely used as spin-trapping reagents without notable problems due to instability. On the basis of this evidence and the stability of 6, it appears that neither *tert*-butyl substituents, which of course lack benzylic protons, nor methyl substituents adjacent to the nitroso group are subject to decomposition to the extent observed for ethyl groups. Additional studies will be required to determine which other alkyl groups participate in this process.

Decomposition of 1 and 8 during GC Analysis. When solutions of freshly prepared 1 were analyzed by GC or GC/MS using standard splitless capillary conditions, six decomposition products were observed. The relative amounts of these products were highly variable when repetitive analyses were made on different instruments or under different chromatographic conditions. Their mass spectra were recorded in the EI (Figure 3) and chemical ionization (CI) modes, including scanning HRMS, and these data were compared with those of the reference standards 3 and 5–14 (Table I). This process allowed identification of 3, 11, 12, and 14 as products of the thermal degradation of 1 during GC analysis. A much later eluting peak was also detected that produced the mass spectra assigned earlier to 18. The sixth component, which appeared as a later eluting shoulder on the peak assigned as 14, appeared to be a dihydro derivative of 14 tentatively

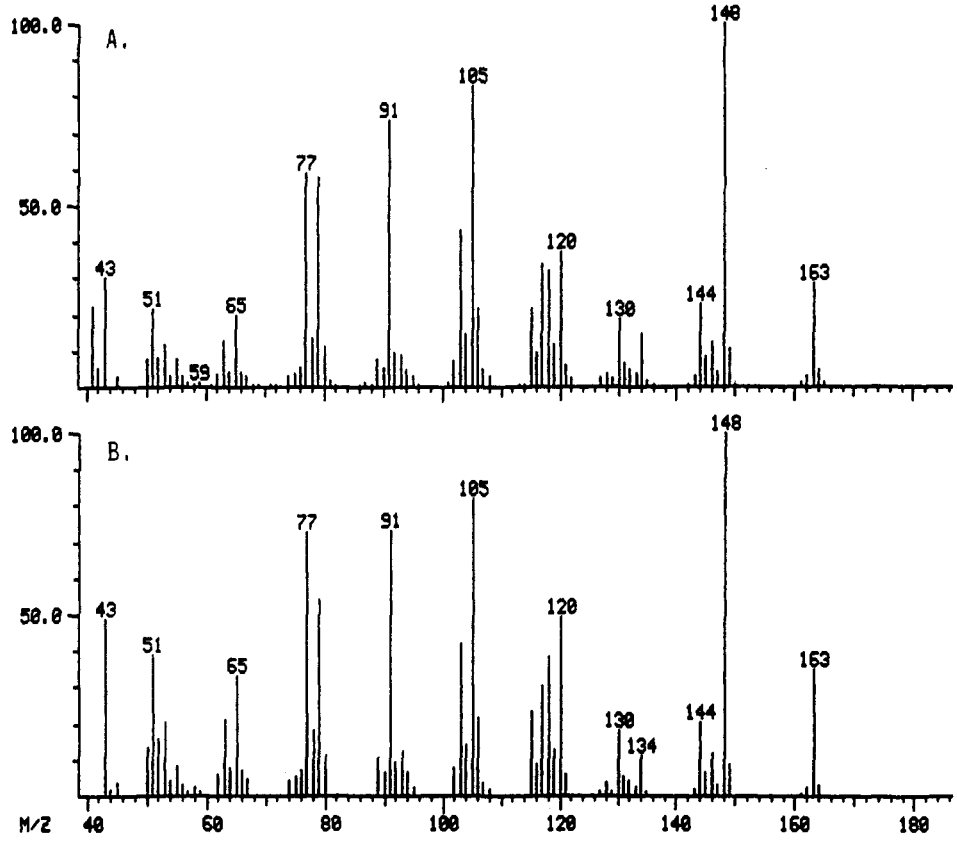
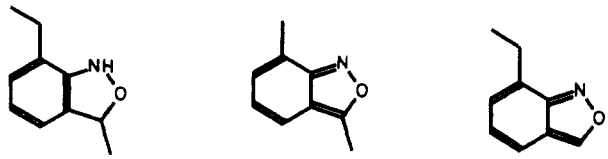


Figure 4. EI mass spectra of 2,6-diethylnitrosobenzene (1) as a solid (A) and during GC analysis (B).

formulated as **19**. This last component is isomeric with 1 and may represent the first step in the degradation process. In addition to these six decomposition products, during some analyses, peaks that produced mass spectra appropriate for 9 and 15 were also observed.



19 20 21

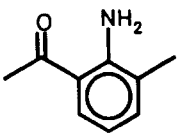
Similarly, GC/MS analysis of freshly prepared solutions of 8 indicated the formation of an analogous series of degradation products in lesser amounts, although the authentic reference compounds were not prepared for structural confirmation. In this case, the two isomeric anthranil derivatives **20** and **21** were both logical products for consideration, and peaks appropriate for both of these substances were observed. The predominant isomer was assigned as **20** on the basis of HRMS data that showed that a major fragment at m/z 104 resulted from loss of CH_3CO from the molecular ions at m/z 147 as had been observed for **14**. The mass spectrum of the minor isomer **21** contained ions at m/z 118 as the base peak, resulting from an analogous loss of CHO. This assignment was consistent with the ^1H NMR spectrum of the mixture produced by decomposition of 8 for 6 days at room temperature in acetonitrile in which the major decomposition product was tentatively assigned as **20** on the basis of the presence of two prominent methyl singlets at δ 2.53 and 2.78. As in the diethyl series, an anthranil, **20**, appears to be the thermal degradation product detected by Kimmel et al. (1986), with a molecular weight 2 amu lower than 8.

As stated earlier, during GC or GC/MS analysis, substantial amounts of 8 passed through the column unchanged in addition to the degradation products discussed above. 8 was detected as a peak that eluted earlier than 2-ethyl-6-methylaniline (**7**), **20**, or **21** and produced EI, CI, and high-resolution mass spectral data consistent with its structural assignment as 8. To further confirm this peak assignment, a solution of 8 was allowed to decompose at room temperature prior to GC/MS analysis, which then showed that the peak assigned as 8 was no longer present. These results are in contrast to the peak assignments made by Kimmel et al. (1986) using very similar chromatographic conditions, in which the peak assigned as 8 eluted later than either **7** or **20**. Further inspection of the chromatograms presented by those investigators also revealed that, in the case of diethylnitrosobenzene **1**, the same elution sequence was proposed, in which **1** eluted later than aniline **3** or the peak now assigned as anthranil **14**. Both of these elution patterns for **1** and **8** are the reverse of the sequence observed with the other nitrosobenzene derivatives that they studied and with our own data for **5** and **6** presented in Table I. This evidence suggested that perhaps Kimmel et al. (1986) had misassigned the peaks attributed to the two unstable nitrosobenzene derivatives **1** and **8**.

To further establish the elution sequence of nitrosobenzene derivatives as compared to the corresponding anilines, the GC/MS analysis of freshly prepared **1** was repeated several times with different concentrations, different injection port temperatures, and different instruments. In several instances, particularly at low concentrations and low injection port temperatures, a small peak (retention time 3.2 min relative to those in Table I) was detected that produced an EI mass spectrum identical with that obtained with solid **1** (Figure 4). This peak has therefore been assigned as intact **1**. During this portion of the study, the elution sequence of **1**, **8**, and their degradation products was examined with three different stationary phases [cross-linked methylsilicone, 5% (phenylmethyl)silicone, 50% (phenylmethyl)silicone], and the

elution sequence was that presented above and in Table I in all cases. This evidence confirms that, in all of the examples reported by Kimmel et al. (1986) and in the current study, the GC peak that should be assigned as the nitroso compound elutes earlier than the corresponding aniline. This elution sequence is also consistent with the relative position of 2,6-diethylnitrobenzene (11) (Table I), since 11 is expected to behave similarly to undecomposed 1 on the GC but elute somewhat later due to its greater molecular weight.

On the basis of the relative peak positions reported by Kimmel et al. (1986), it appears that they may have misassigned the peak caused by 12, which has been positively identified in the present study by comparison with an authentic sample as described above, as the nitroso derivative 1. Possibly the analogous compound 22 may have also been misassigned as 8. Such an error would be easy to make without a thorough study of the decomposition process since these pairs of compounds are isomers with very similar mass spectra except for the fragments resulting from loss of CH_3CO from the molecular ions.



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CONCLUSIONS

In the present study, 2,6-diethylnitrosobenzene (1) has been shown to have physical properties and an unexpected instability in solution, which complicate its analysis. Methods that rely on GC separations are inappropriate, especially because the extensive decomposition of 1 during the analysis produces substances that are isomeric with 1, resulting in possible misinterpretations even when GC/MS is used. In contrast, HPLC/MS analysis of freshly prepared solutions of 1 produce excellent results and can be accomplished with only minor peak broadening due to the equilibrium between the monomeric and dimeric forms 1 and 4. The application of this methodology to metabolic samples that may contain 1 is discussed in the accompanying paper by Feng and Wratten (1987). The instability of 1 in solution is also likely to complicate the interpretation of results from biological tests requiring days to complete, such as bacterial mutagenicity testing. Positive results from such testing may arise from the decomposition products 14–16 rather than from 1 itself. Similarly, studies with 2-ethyl-6-methylnitrosobenzene (8) are also subject to uncertainties due to decomposition, which are less severe than for 1, while 2,6-dimethylnitrosobenzene (6) is almost entirely exempt from these phenomena. The extent to which studies of other alkylated nitrosobenzene derivatives of potential toxicological or metabolic interest are plagued by such concerns remains unknown.

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In Vitro Oxidation of 2,6-Diethylaniline by Rat Liver Microsomal Enzymes

Paul C. C. Feng* and Stephen J. Wratten

Incubation of 2,6-diethylaniline (DEA) with NADPH-fortified rat liver microsomal enzymes produced 4-amino-3,5-diethylphenol (ADEP) as the major product of oxidation. ADEP was shown to undergo further oxidation to 3,5-diethylbenzoquinone 4-imine (DEBQI), which was isolated as a minor metabolite during DEA oxidation. Metabolites resulting from N-oxidation or alkyl hydroxylations were not observed.

N-Oxidation of arylamines is generally believed to result in the formation of reactive metabolic intermediates that are responsible for some of their toxic effects (Weisburger and Weisburger, 1973). Kimmel et al. (1986) recently reported the in vitro and in vivo metabolic conversion of several 2,6-dialkylchloroacetanilide herbicides to the corresponding 2,6-dialkylanilines by rats. The anilines were in turn reported to be converted to the corresponding 2,6-dialkylnitrosobenzenes, which were shown to be direct-acting mutagens by the Ames assay. These studies

Metabolism Section, Monsanto Agricultural Company, Chesterfield, Missouri 63198.

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