Nitrosation by Nitro–Nitroso Derivatives of Olefins: A Potential Mechanism for *N*-Nitrosamine Formation in Fried Bacon

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The mechanism of nitrosation of morpholine (MOR) by 2,3-dimethyl-2-nitro-3-nitrosobutane (DNNB) was studied as a model for N-nitrosation of amines by nitro–nitroso compounds. DNNB was mixed with excess MOR in hexane and heated in sealed ampules at temperatures of 148, 169, and 180 °C. The reaction produced 2 mol of *N*-nitrosomorpholine (NMOR)/mol of DNNB consumed, leaving 1 mol of 2,3-dimethylbutene (DMB) as a byproduct. Linear pseudo-first-order kinetic plots demonstrated that the reaction rate had a first-order dependence in DNNB concentration and a zero-order dependence in MOR concentration. This suggests a mechanism where DNNB thermally dissociates to N_2O_3 and DMB in a rate-limiting step, followed by rapid nitrosation of MOR. When MOR was nitrosated with the products of N_2O_3 addition to *cis*-3-heptene, the only 3-heptene found in the reaction mixture was the trans isomer. This and the fact that *p*-methoxyaniline inhibited the formation of NMOR suggest a free-radical mechanism. With these results, we present a hypothesis on the mechanism of nitrosamine formation during the frying of nitrite-cured meats such as bacon.

The formation of carcinogenic *N*-nitrosamines in nitrite-cured meats such as bacon has been of considerable research interest (Hotchkiss, 1987). Much of the work has focused on the source of amine precursors such as proline and thiazolidinecarboxylic acid. These amines are indirect precursors to *N*-nitrosopyrrolidine (NPYR) and *N*-nitrosothiazolidine (NTHZ), respectively. The mechanism by which proline reacts to form NPYR has been investigated in detail (Lee et al., 1983) as has the formation of NTHZ (Pensabene and Fiddler, 1985; Sen et al., 1985).

We have investigated the agent responsible for nitrosating the amine precursor when bacon is fried (Hotchkiss et al., 1985). This work demonstrated that the nitrosating agent could not be extracted or purged from the raw or fried-out fat, eliminating oxides of nitrogen (NO_x) and nitrite as the nitrosating agent. We suggested that the NO_x, which was formed from the added nitrite, reacted with unsaturated lipids during the curing process to form unknown compounds. These compounds could decompose during frying to form nitrosating agents. This hypothesis was consistent with the data showing that raw bacon does not contain appreciable amounts of *N*-nitrosamine and that the formation of *N*-nitrosamines is a thermally induced process.

Goutefongea et al. (1977) first suggested that nitrite could react with lipids during curing to form compounds that might be nitrosating agents. Later Walters et al. (1979) showed that NO_x derivatives of cyclohexene would nitrosate morpholine. We demonstrated that dinitrogen trioxide (N_2O_3) facilely reacted with methyloleate to form several addition products, some of which were capable of N-nitrosation under conditions similar to frying bacon (Ross et al., 1987). The nitro nitroso (pseudo-nitrosite) derivative demonstrated the greatest capacity for N-nitrosation. Mirvish and Sams (1984) observed that nitrogen dioxide (NO_2) also reacted with unsaturated fatty acids to form uncharacterized nitrosating agents.

The goal of the present work was to study the chemistry of N-nitrosation by nitro–nitroso compounds in detail in order to determine whether it is consistent with what is known about the chemistry of N-nitrosation in fried bacon. We chose to investigate the nitrosation of morpholine (MOR) by 2,3-dimethyl-2-nitro-3-nitrosobutane (DNNB). The latter compound lacks α-protons and cannot rearrange to the oxime. Addition of NO_x to olefins has been reported in the literature (Schechter, 1964; Park and Williams, 1972), but the products have not been characterized in detail nor has their nitrosation chemistry been investigated. It is possible that a similar chemistry is responsible for the formation of *N*-nitrosamines when bacon is fried.

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METHODS

Safety. *N*-Nitrosamines are potent animal carcinogens and must be handled with appropriate safety precautions.

Reagents. Dichloromethane (DCM), hexane, and water were glass-distilled and analyzed for positive peaks. *N*-Nitrosopyrrolidine (NPYR), *N*-nitrosodimethylamine (NDMA), *N*-nitrosomorpholine (NMOR), and morpholine (MOR) were purchased from Sigma Chemical Co. (St. Louis, MO); and 2,3-dimethyl-2-butene, *cis*-3-heptene, *trans*-3-heptene, and *p*-methoxyaniline were purchased from Aldrich Chemical Co. (Milwaukee, WI). 2,3-Dimethyl-2-nitro-3-nitrosobutane (DNNB) was synthesized by the procedure of Ross et al. (1987). After recrystallization from ether and sublimation, the product was characterized by IR (Perkin-Elmer Model 281), GC–MS (Hewlett-Packard Model 5995C), NMR (Varian Model XL-400), and elemental analysis.

Analyses. Volatile *N*-nitrosamines were determined by gas chromatography–thermal energy analyzer (GC–TEA). Chromatographic conditions were as follows: Model 5890A GC (Hewlett-Packard, Avondale, PA), equipped with a 10 ft × 2 mm (i.d.) glass column packed with 10% Carbowax 20M on 80–100 Chromosorb WHP; He flow rate, 25 mL/min; injector temperature, 185 °C; column temperature, 165 °C; Model 543 TEA (Thermedics, Woburn, MA); interface temperature, 200 °C; pyrolyzer temperature, 550 °C; trap temperature, −160 °C; analyzer pressure, 1.9 Torr.

N-Nitrosamines in fried-out pork fat and condensed frying vapors were determined by the method of Hotchkiss et al. (1985).

2,3-Dimethyl-2-butene was quantified by GC–TEA as above, except that the TEA was used as an olefin chemiluminescence detector by lowering the pyrolyzer to 200 °C and removing the cold trap. The GC was equipped with a 6 ft × 0.125 in. (o.d.) ss column packed with 3% OV-17 on 80–100 Chromosorb WHP (He flow rate, 10 mL/min; injector temperature, 175 °C; column temperature, 30 °C). The linearity of the response was tested over 2 orders of magnitude. The limit of detection (3:1 = S:N) was 2 ng. Hexanes gave no response.

cis- and *trans*-3-heptene were separated on a Model 5880A GC equipped with a 25 m × 0.32 mm (i.d.) HP-5 capillary column and FID (Hewlett-Packard) (He linear

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velocity, 22 cm/s; split ratio, 40:1; injector temperature, 100 °C; column temperature, 27 °C; detector temperature, 200 °C).

Electron impact mass spectra (70 eV) of DNNB were obtained with the following GC/MS conditions: 30 m × 0.32 mm Heliflex Bonded FSOT Superox capillary column (Alltech, Waukegan, IL); He flow rate, 1 mL/min (125 °C); split ratio, 50:1; injector temperature, 125 °C; column temperature, 90 °C (1 min) and then 5 °C/min to 125 °C (7 min); transfer line temperature, 150 °C; ion source temperature, 200 °C; mass analyzer temperature, 200 °C.

N-Nitrosation of Morpholine. DNNB or the products of N_2O_3 and *cis*-3-heptene were reacted with morpholine in 1 mL of hexane in sealed glass ampules in a heating block. The reaction was stopped by cooling in liquid N_2 and analyzed for NMOR, DNNB, and dimethylbutene or *cis*- and *trans*-3-heptene. Pseudo-first-order kinetics were determined by monitoring the formation of NMOR until completion ($5t_{1/2}$) with [MOR]/[DNNB] ≥ 20. Rate constants were determined by plotting ln ([NMOR]$_f$ − [NMOR]$_t$) versus time (min). The E_a was estimated by plotting ln k versus $1/T$ (K) for several temperatures. The [MOR] was increased to determine the order of the reaction in MOR. Unheated reagent mixtures did not result in the formation of a significant amount of NMOR during analysis.

RESULTS AND DISCUSSION

Characterization of DNNB. Elemental analysis of the pale blue solid isolated from the reaction of N_2O_3 and dimethylbutene gave the following. Anal. Calcd for DNNB; C, 45.0; N, 17.5; H, 7.50. Found: C, 45.1; N, 18.5; H, 7.57. This analysis was supported by a 2:1 molar TEA response ratio for DNNB to NMOR when the TEA was operated at a pyrolyzer temperature of 950 °C (nitro mode).

Electron impact GC–MS gave a base peak of m/z 57 with the next 10 largest peaks at m/z (relative abundance) 41 (99), 69 (93), 39 (58), 55 (47), 30 (43), 100 (39), 84 (31), 56 (19), 85 (17), and 46 (8). The highest significant mass was m/z 101 (3). When DNNB enriched 25% with [^{15}N]nitrogen was analyzed, the m/z 30 and 46 peaks decreased by 25% and the m/z 31 and 47 peaks increased. This confirmed the presence of the nitro group and suggested a nitroso group because the m/z 30 ion was larger than would be expected for compounds containing only nitro groups (Silverstein et al., 1981). When DNNB was analyzed by chemical ionization MS, using isobutane as the reagent gas, the base peak was m/z 130. This corresponds to the loss of HNO from the (M + 1) peak of DNNB.

IR analyses ($CHCl_3$) gave absorbances at 1535, 1459, 1392, 1377, 1342, and 850 cm^{-1}, which are in the ranges expected of aliphatic nitro and/or *N*-nitroso compounds (Lambert et al., 1976). Notably absent was an absorbance in the range of 1680–1650 cm^{-1}, which would be expected if the compound contained an aliphatic nitrite instead of a nitro group (Lambert et al., 1976). The pseudo-nitrosite structure, instead of the nitrosite (nitrite–nitroso), agrees with the structure assigned to a similar compound by Pfab (1977). The X-ray crystalline structure of the dimeric nitro–nitroso adduct of cyclohexene has been reported (Chiu et al., 1985).

NMR analyses ($CDCl_3$) suggested a structure that exhibits C_{2v} symmetry. ^{1}H NMR gave a singlet at δ 1.72 ($CHCl_3$, δ 7.24) that demonstrated the equivalence of the two sets of methyl protons. ^{13}C NMR furnished two singlet resonances, δ 23.0 and 91.4 ($CDCl_3$, δ 77.0) corresponding to the methyl and tertiary carbons, respectively. DNNB

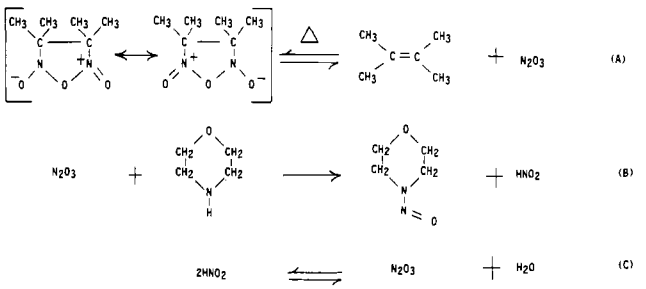

Figure 1. Proposed resonance structure for 2,3-dimethyl-2-nitro-3-nitrosobutane (DNNB) and proposed mechanism for the nitrosation of morpholine (MOR) by DNNB.

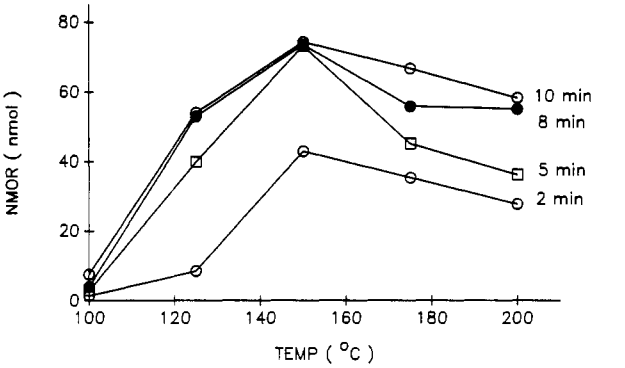

Figure 2. Formation of NMOR in hexane from 0.14 mM DNNB and 2.6 mM MOR for times of 2–10 min and temperatures of 75–200 °C.

synthesized with 25% enrichment in [^{15}N]nitrogen gave a single resonance in the ^{15}N NMR, which remained a singlet at temperatures as low as −80 °C.

The NMR data indicated that the electronic environment around each nitrogen atom in DNNB is equivalent. This could not be explained by a structure that is drawn with a separate nitroso and nitro group across the two carbons that made up the double bond before the addition of N_2O_3. Rather, a resonance structure (Figure 1) showing a partial sharing of one of the oxygen atoms on the nitro group by the nitroso group better represents a symmetrical structure and would explain the NMR spectra.

N-Nitrosation by DNNB. The amount of NMOR formed from 0.14 mM DNNB and 2.6 mM MOR increased with temperatures above 100 °C up to 200 °C when heated for 2–10 min (Figure 2). At 75 °C, no detectable NMOR was formed. When 1.6 μmol of DNNB was added to 50 g of fresh pork fat and fried, NDMA and NPYR were formed in amounts (steam + fat + solid) similar to those found when cured bacon is fried. The amount of volatile *N*-nitrosamine formed during frying increased with temperature as it does when bacon is fried (Figure 3). These data suggest that the thermally induced nitrosation by DNNB is similar to that observed when bacon is fried.

Pfab (1977) reported that thermolysis of 2-methyl-1-nitro-2-nitrosopropane yielded the olefin 2-methyl-1-nitropropene. We, therefore, investigated the formation of olefins as products of the nitrosation of MOR by DNNB. Using the TEA as an olefin detector (pyrolyzer temperature of 200 °C and no liquid N_2 trap), we found that 2,3-dimethyl-2-butene, but not the nitro olefin, was a product of the nitrosation of MOR by DNNB. This was supported by material balance experiments in which the moles of NMOR formed were approximately twice the moles of dimethylbutene formed (Figure 4).

These results indicated the following stoichiometry:

DNNB + 2MOR → 2NMOR + dimethylbutene

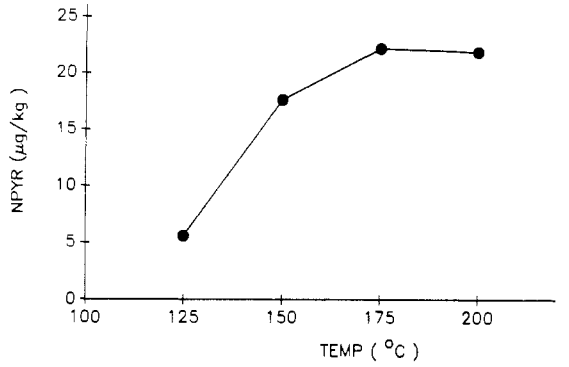

Figure 3. Formation of NPYR when raw ground pork fat that had 1.6 μmol of DNNB added per 50 g of fat was fried for 10–20 min at different temperatures.

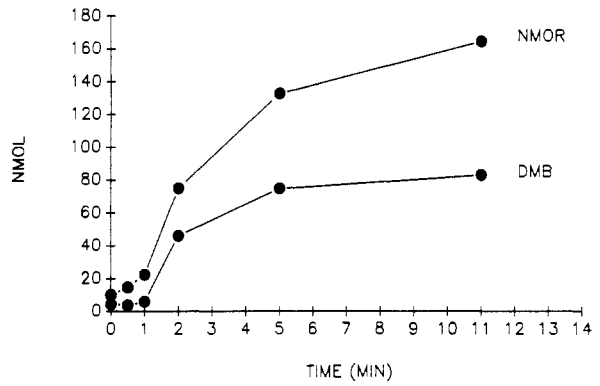

Figure 4. Concurrent formation of NMOR and dimethylbutene from 1.27 mM DNNB and 73.4 mM MOR when heated at 175 °C.

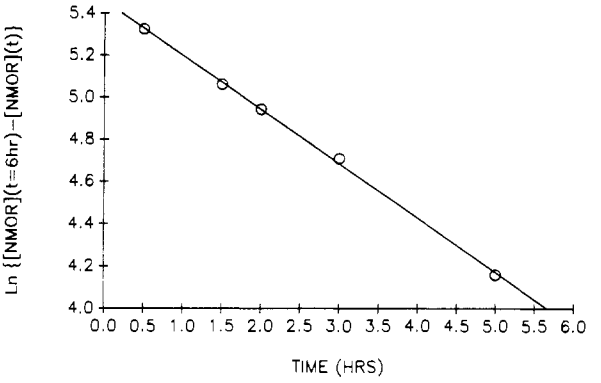

Figure 5. Pseudo-first-order plot for the formation of NMOR from 5.2 mM MOR and 0.038 mM DNNB at 169 °C.

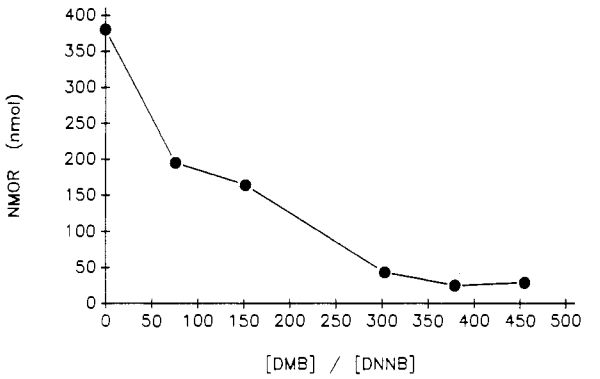

Figure 6. Inhibition of NMOR formation by 0–642 mM dimethylbutene when 1.41 mM DNNB and 4.2 mM MOR were heated at 170 °C for 5 min.

In order to determine whether the reaction proceeded by a direct transfer of the nitrosyl group to MOR (transnitrosation) or by the release of NO_x followed by nitrosation, we conducted a series of kinetic studies. A linear pseudo-first-order (excess MOR) kinetic plot showed the reaction to be first order in DNNB (Figure 5). Increasing the concentration of MOR in these experiments did not increase the pseudo-first-order rate constant, which indicated a zero-order dependence on MOR concentration. A reaction mechanism in which there was a direct transfer of a nitrosyl radical (transnitrosation) would be first order in MOR concentration. This suggested that the rate-determining step is the thermally induced dissociation of DNNB into dimethylbutene and N_2O_3, followed by rapid nitrosation of MOR. We propose a mechanism (Figure 1) in which DNNB thermally dissociates into dimethylbutene and N_2O_3 in a rate-limiting step (Figure 1A). In the presence of secondary amines, the N_2O_3 rapidly reacts to form *N*-nitrosamine and nitrous acid by the well-known nitrosation mechanism (Figure 1B). Two molecules of nitrous acid can combine in a rapid equilibrium to form another N_2O_3, which can further nitrosate available amine (Figure 1C). In addition to explaining the observed kinetic data, this mechanism also explains the empirical stoichiometry observed in our reactions.

Estimation of the energy of activation (E_a) for nitrosation of MOR by DNNB at temperatures 148, 169, and 180 °C gave a value of 32 kcal/mol. However, at 148 °C the reaction was less than 35% complete at 12 h. Data above 190 °C were not used because NMOR decomposition complicated the kinetic determinations. At 169 °C, which corresponds to the temperature at which bacon is fried, the first-order rate constant was 5.0×10^{-3} min^{-1} (Figure 5). We estimate raw bacon which contained 47 μmol of amine/kg (Lakritz et al., 1976) and fried at 169 °C would form *N*-nitrosamine at approximately 0.43 μmol/kg per min, assuming that 10% of the 120 μg/kg of sodium nitrite permitted in bacon reacted with unsaturated lipids to form pseudo-nitrosite (Cassens et al., 1979). This amount of *N*-nitrosamine is approximately 4 times the total (steam + fat + solid) *N*-nitrosamine typically found after frying (Hotchkiss and Vecchio, 1985), indicating sufficient pseudo-nitrosite may be formed from current nitrite levels to account for the *N*-nitrosamine content of fried bacon. We have previously shown that the amine is the limiting reagent in *N*-nitrosamine formation in fried bacon (Hotchkiss et al., 1985).

The addition of increasing amounts of dimethylbutene at the beginning of the reaction inhibited the amount of NMOR formed when the ampules were heated for 5 min at 170 °C (Figure 6). A similar inhibition of NMOR formation was observed when dimethylbutene was added to ampules that were heated for increasing times at the same temperature (data not shown). These experiments, along with the zero-order dependence on MOR concentration, further support a mechanism in which the rate-limiting step is the thermal dissociation of DNNB into N_2O_3 and olefin and in which dimethylbutene competes with MOR for nitrosating agent.

This mechanism raises the question of retention or inversion of the double-bond configuration when the olefin is regenerated from the nitro–nitroso adduct. When *cis*-3-heptene was reacted with N_2O_3 and the products used to nitrosate MOR, only *trans*-3-heptene was found in the reaction mixture, as would be expected for the mechanism in Figure 1. This also suggests that cis fatty acids in foods that are treated with nitrite could be converted to the trans isomer after cooking. The occurrence of trans fatty acids in the diet has been of concern (Life Sciences Research

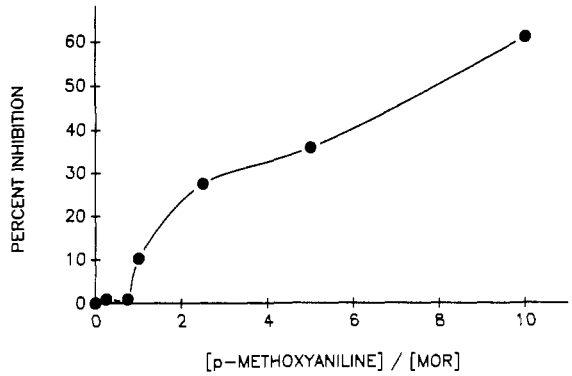

Figure 7. Inhibition of NMOR formation by 0–48.1 mM *p*-methoxyaniline from 4.8 mM MOR and 1.28 mM DNNB at 175 °C for 15 min.

Office, 1985). The use of nitrite in curing meats may represent a previously unknown source of trans fatty acids.

N-Nitrosamine formation during bacon frying can be inhibited by *p*-alkoxyanilines, which compete for the nitrosyl radical (Bharucha et al., 1986). These authors showed that *p*-methoxyaniline gave a 93% reduction in the formation of NPYR in fried bacon. When we added *p*-methoxyaniline to the DNNB–MOR reaction mixture, we also demonstrated inhibition of NMOR formation. Inhibition increased at ratios of methoxyaniline to MOR greater than 1 while ratios of less than 1 gave less that 2% inhibition (Figure 7). This indicated that methoxyaniline was not inhibiting the rate-limiting dissociation of DNNB but was merely competing with MOR for the N_2O_3.

CONCLUSIONS

An explanation of *N*-nitrosamine formation when bacon is fried must rationalize the following observations:

1. Uncooked bacon does not contain sufficient *N*-nitrosoproline or volatile *N*-nitrosamines to account for the *N*-nitrosamines found after frying, and longer frying times at higher temperatures result in more *N*-nitrosamine formation. Therefore, *N*-nitrosamine formation is a thermally induced process that does not occur until cooking temperatures are reached (Bharucha et al., 1979).
2. *N*-Nitrosamine formation occurs primarily in the lipid phase of bacon and can be effectively inhibited by lipophilic free-radical scavengers. This implies a free-radical mechanism that may not be a direct transfer of a nitroso group between the nitrosating agent and the amine (Bharucha et al., 1980).
3. The amount of *N*-nitrosamine formed does not correlate with residual nitrite levels but does increase with increasing amounts of added nitrite (Sen et al., 1974). Also, the free proline content and the amount of *N*-nitrosamine formed in any given sample increases with storage time while the amount of residual nitrite decreases (Pensabene et al., 1980). This suggests that the amine is limiting and that nitrite is converted to a nitrosating agent during curing and storage.
4. Raw and fried-out fat contain a nonvolatile, nonextractable, lipid–nitrite product that is capable of forming large amounts of *N*-nitrosamine when heated with excess amine (Hotchkiss et al., 1985).

We believe that our data are consistent with these observations and present the best available hypothesis on the mechanism of *N*-nitrosamine formation during the frying of cured meats. Taken as a whole, our data suggest that nitrite forms N_2O_3 when added to meats by a well-known equilibrium (Challis and Kyrtopoulos, 1978). The N_2O_3 reacts with the unsaturated groups in the fats to form the nitro–nitroso adduct. This adduct decomposes during frying to release oxides of nitrogen (e.g., NO, NO_2, N_2O_3), which nitrosate available amines. Our findings do not prove that this mechanism is responsible for *N*-nitrosamine formation in cured meats, however. We are in the process of determining whether the nitro–nitroso adduct of unsaturated fatty acids is formed in meats during curing and to what extent it might be responsible for *N*-nitrosamine formation.

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Registry No. MOR, 110-91-8; NMOR, 59-89-2; DNNB, 97029-24-8.

LITERATURE CITED

Bharucha, K. R.; Cross, C. K.; Rubin, L. J. *J. Agric. Food Chem.* **1979**, *27*, 63.
Bharucha, K. R.; Cross, C. K.; Rubin, L. J. *J. Agric. Food Chem.* **1980**, *28*, 1274.
Bharucha, K. R.; Cross, C. K.; Rubin, L. J. *J. Agric. Food Chem.* **1986**, *34*, 814.
Cassens, R. G.; Greaser, M. L.; Ito, T.; Lee, M. *Food Technol.* **1979**, *33*(7), 46.
Challis, B. C.; Kyrtopoulos, S. A. *J. Chem. Soc., Perkin Trans. 2* **1978**, 1296.
Chiu, K. W.; Savage, P. D.; Wilkinson, G.; Williams, D. J. *Polyhedron* **1985**, *4*, 1941.
Goutefongea, R.; Cassens, R. G.; Woolford, G. *J. Food Sci.* **1977**, *42*, 1637.
Hotchkiss, J. H. In *Advances in Food Research*; Chichester, C. O., Mrak, E. M., Schweigert, B. S., Eds.; Academic: New York, 1987; Vol. 31.
Hotchkiss, J. H.; Vecchio, A. J. *Food Technol. (Chicago)* **1985**, *39*(1), 67.
Hotchkiss, J. H.; Vecchio, A. J.; Ross, H. D. *J. Agric. Food Chem.* **1985**, *33*, 8.
Lakritz, L.; Spinelli, A. M.; Wasserman, A. E. *J. Food Sci.* **1976**, *41*, 879.
Lambert, J. B.; Shurvell, H. F.; Verbit, L.; Cooks, R. G. *Organic Structural Analysis*; Macmillan: New York, 1976.
Lee, M.-L.; Gray, J. I.; Pearson, A. M.; Kakuda, Y. *J. Food Sci.* **1983**, *48*, 820.
Life Sciences Research Office *Health Aspects of Dietary Trans Fatty Acids*; Federation of American Societies for Experimental Biology: Bethesda, MD, 1985.
Mirvish, S. S.; Sams, S. S. *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer*; O'Neill, I. K., Von Borstel, R. C., Miller, C. T., Long, J., Bartsch, H., Eds.; International Agency for Research on Cancer: Lyon, 1984; p 283.
Park, J. R.; Williams, D. L. H. *J. Chem. Soc., Perkin Trans. 2* **1972**.
Pensabene, J. W.; Fiddler, W. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 1077.
Pensabene, J. W.; Fiddler, W.; Miller, A. J.; Phillips, J. G. *J. Agric. Food Chem.* **1980**, *28*, 966.
Pfab, J. *J. Chem. Soc., Chem. Commun.* **1977**, 766.
Ross, H. D.; Henion, J.; Babish, J. G.; Hotchkiss, J. H. *Food Chem.* **1987**, *23*, 207.
Sen, N. P.; Iyengar, J. R.; Donaldson, B. A.; Panalaks, T. *J. Agric. Food Chem.* **1974**, *22*, 540.
Sen, N. P.; Seaman, S. W.; Baddoo, P. A. *Food Technol. (Chicago)* **1985**, *39*(1), 84.
Shechter, H. *Rec. Chem. Prog.* **1964**, *25*, 54.
Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. *Spectrometric Identification of Organic Compounds*, 4th ed.; Wiley: New York, 1981.
Walters, C. L.; Hart, R. J.; Perse, S. *Z. Lebensm. Unters. Forsch.* **1979**, *168*, 177.

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