

Biomimetic Synthesis of *N*-Nitroso-*N*-(1-methylacetyl)-3-methylbutylamine: An Unusual Carcinogenic Nitrosamine in Foods of Linxian, China

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The origin of NMAMBA [*N*-nitroso-*N*-(1-methylacetyl)-3-methylbutylamine], a carcinogen identified in moldy foods of Linxian, Henan, China, has been controversial. We find that it may arise by the interaction of isoamylamine, a decarboxylation product of leucine, and acetoin (3-hydroxy-2-butanone), a known constituent of corn. Oxidative nitrosation in dilute sulfuric acid leads directly from the amino alcohol to the nitrosamino ketone.

N-Nitroso-*N*-(1-methylacetyl)-3-methylbutylamine (1, NMAMBA) was first isolated from moldy corn cakes (Lu et al., 1979; Ji et al., 1985), which are an important part of the diet of the people of Linxian, Henan Province, China. This agricultural highland county in northwest Henan Province suffers from the highest recorded incidence of esophageal cancer in the world (150-300 cases/100 000 vs. a usual background incidence of 1.5-2/100 000; Yang, 1980). This cancer has been common in the area for at least 2000 years and is generally believed to be related to the diet as well as other underlying environmental factors.

The local food of the county has been shown to contain appreciably higher levels of volatile nitrosamines than food from other neighboring counties or food from villages in the Beijing area (Ji et al., 1985). The most common nitrosamines found in the food are *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA), but the presence of NMAMBA with its unusual structure was surprising, since no other nitrosamine with such a complex structure had previously been identified in environmental samples. A similar compound, *N*-nitroso-*N*-(1-methylacetyl)-2-methylpropylamine (2, NMAMPA), has recently been found in millet and wheat flour (Ji et al., 1986b).

It was proposed (Ji et al., 1986a; Ji et al., 1986b) that NMAMBA derives from isoamylamine, a decarboxylation product of leucine, an amino acid present in appreciable quantities in grains (Yang et al., 1956). The proposal suggested that the amine either underwent enzyme-mediated methylation, acetylation, and methylation or condensed with acetoin to produce the highly branched structure. Nitrosation from the high levels of nitrite in the water supply (Yang, 1980) could then lead to the nitrosamine.

During the course of several analytical studies of the Linxian cornmeal and steamed corn cakes, attempts have been made to isolate the amino ketone, *N*-(1-methylacetyl)-3-methylbutylamine, the presumed precursor of the nitrosamine (Ji et al., 1986b; Singer et al., 1986). It has never been found. We suggest now that the ketone is never present in the corn but that the amino alcohol, *N*-(1-methyl-2-hydroxypropyl)-3-methylbutylamine (3), is formed by direct reductive condensation of isoamylamine with acetoin and that this amino alcohol can be both ni-

trated and oxidized to the ketone by treatment with suitable nitrosating agents.

MATERIALS AND METHODS

Instrumentation. Infrared spectra were recorded on a Perkin-Elmer Model 297 infrared spectrometer. Mass spectra were recorded on a VG MS-7070 mass spectrometer in low-resolution mode in EI at 1K resolving power and in CI with ammonia gas also at 1K. For accurate mass measurements the resolving power was 3K. Melting points are uncorrected.

Chemicals. Isoamylamine was from Aldrich Chemical Co. Acetyl methyl carbinol (acetoin), as the dimer, was practical grade from Eastman. Other chemicals were reagent grade. All were used as received.

***N*-Nitroso-*N*-(1-methylacetyl)-3-methylbutylamine (1).** Acetoin (9.08 g, 0.1 mol) was dissolved in a mixture of ¹/₁₅ M phosphate buffer adjusted to pH 3.5 (25 mL) and water (25 mL). This solution was stirred at room temperature while a solution of isoamylamine (5.8 mL, 0.05 mol) in pH 3.5 phosphate buffer (25 mL) was added slowly. An oil formed rapidly. After the addition was complete, sodium cyanoborohydride (4.7 g, 0.075 mol) was added in one portion and the mixture was stirred for an additional 2 h at room temperature.

The reaction mixture was extracted with methylene chloride (3 × 25 mL) which was back-extracted with 2 N HCl (3 × 25 mL). The acid solution was basified with sodium carbonate and reextracted with methylene chloride (3 × 25 mL). This solution was dried (Na₂CO₃) and evaporated to a pale yellow oil: 3.3 g; IR (film) 3500 (br, OH), 3450 (NH), 1710 (ketone C=O).

(a) **Sodium Nitrite/Sulfuric Acid Procedure.** The crude amino alcohol 3 obtained as above (1.6 g, approximately 0.01 mol) was stirred at room temperature in 1 N sulfuric acid (72 mL). Sodium nitrite (6.9 g, 0.1 mol) was added in small portions, and the mixture was stirred for 2 h at room temperature. The mixture was extracted with methylene chloride (3 × 25 mL), which was washed with 5% sodium carbonate solution (2 × 15 mL), dried (Na₂CO₃), and evaporated to an orange oil. Thin-layer chromatography (TLC) on silica gel (1% acetone/methylene chloride) showed a prominent spot with an *R*_f equal to that of NMAMBA. The oil was chromatographed on a column of silica gel (25 g) with elution by methylene chloride. Aliquots (20 mL) were assayed by TLC and grouped accordingly.

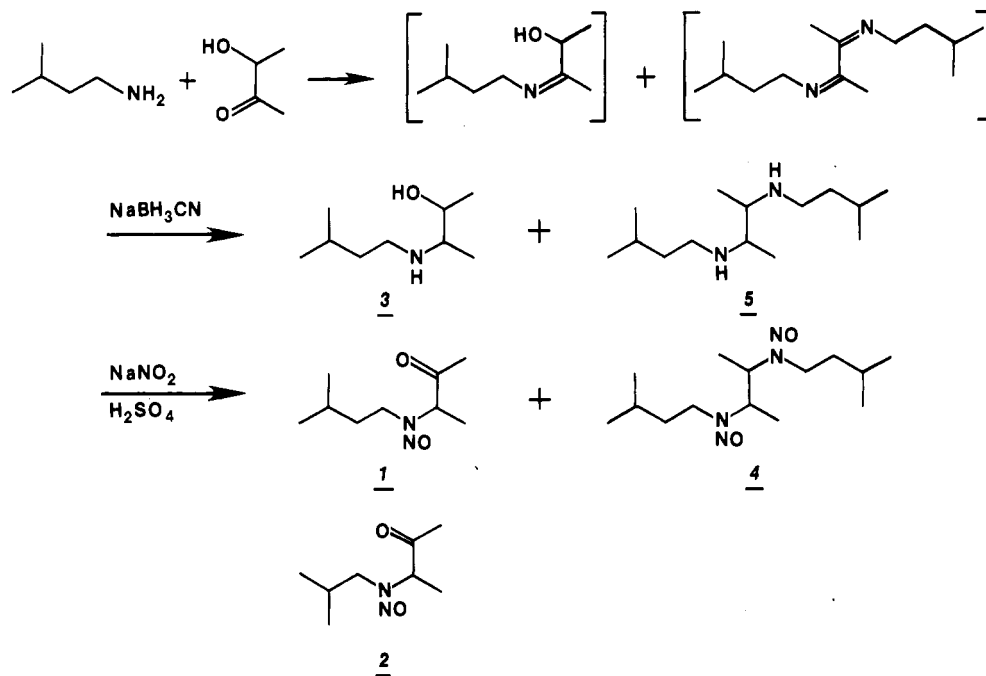
Two compounds eluted with incomplete resolution, but early fractions of the first component were pure as were late fractions of the second component. The first component (180 mg) was a low-melting solid (mp 87.5-89.5 °C) whose IR spectrum (KBr) showed no OH or carbonyl absorption. It appeared to be an aliphatic nitrosamine: MS

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Scheme I



(EI) m/z (rel intens) 286 (M^+ , 0%), 256 ($\text{M}^+ - \text{NO}$, 2.0), 226 ($\text{M}^+ - 2 \text{NO}$, 1.3), 171 (3.9), 143 (55.6), 114 (15.1), 112 (14.3), 98 (100), 71 (14.4), 57 (52.6), 56 (41), 55 (12.6), 45 (13.4), 44 (13.8), 43 (37.6); MS ($\text{NH}_3 \text{CI}$) m/e (rel intens) 304 ($\text{M}^+ + \text{NH}_4$, 24.3%), 258 ($\text{M}^+ + 2 \text{H}$, 19.9), 256 (16.3), 227 (7.7), 171 (19.3), 142 (13.7), 114 (100), 98 (10.5), 88 (18.2), 86 (16.4), 58 (14.6), 52 (54.5); ^{13}C NMR (CDCl_3) 62.38, 43.43, 35.10, 26.68, 22.23, 17.99 ppm. Anal. Calcd for $\text{C}_{14}\text{H}_{30}\text{N}_4\text{O}_2$: C, 58.71; H, 10.56; N, 19.56. Found: C, 58.85; H, 9.99; N, 19.54.

This component was identified as 2,3-bis[*N*-nitroso-*N*-(3-methylbutyl)amino]butane (4).

The second pure component (130 mg) was a yellow oil: IR (film) 1710 (ketone $\text{C}=\text{O}$), 1450 (NNO), no OH; MS, m/z (rel intens) 186 (M^+ , 0.3%), 156 ($\text{M}^+ - \text{NO}$, 2.6), 143 (30.3), 114 (26.8), 112 (11.0), 98 (100), 71 (12.6), 70 (10.6), 58 (16.1), 57 (66.3), 56 (48.2), 44 (26.5), 43 (64.4).

These spectra were identical with those of authentic NMAMBA (1).

(b) *Chromium Oxide/Sodium Nitrite Procedure*. The amino alcohol (2.35 g, 0.015 mol) was stirred in water (12 mL) at 0 °C while concentrated sulfuric acid (3 mL) was added. A solution of chromium(VI) oxide (3.25 g, 0.0325 mol) in water (8 mL) was added, and the reaction was allowed to warm to room temperature. After 5 h, chloroform (25 mL) and sodium nitrite (4.1 g, 0.059 mol) were added and the reaction was stirred overnight. The layers were separated, and the aqueous was extracted with chloroform (1 × 25 mL). The dark green combined chloroform solution was decolorized, dried (MgSO_4), and evaporated. The residual oil was filtered through a short silica gel column by elution with chloroform. The solvent was evaporated to leave a yellow oil (1.49 g) whose IR spectrum was identical with that of authentic NMAMBA (1).

RESULTS AND DISCUSSION

There has been considerable interest in the etiological factors responsible for the excessive incidence of esophageal cancer in the Linxian area of Henan Province in China. A number of related observations have strongly suggested that nitrosamines play a major role in the induction of these cancers. To that end, several studies have

examined the foods of Linxian, particularly in comparison with similar foods of areas without the high incidence of esophageal cancer. In addition to the nitrosamines that would be anticipated, NDMA and NDEA, nitrosamines with unusual structures, were isolated. NMAMBA (1) was originally found in the moldy steamed corn cakes that form a major part of the Linxian diet (Lu et al., 1979). More recently, NMAMBA has also been found in moldy millet and wheat flours along with a lower homologue NMAMPA (2) (Ji et al., 1986b). NMAMBA (1) has been shown to be carcinogenic to mice and rats (Li et al., 1980), and the homologous NMAMPA (2) may also be a carcinogen (Ji et al., 1986b).

The unusual structures of these nitrosamines have caused some consternation. Both contain branched alkyl chains and a ketone function, structural elements not previously observed in naturally occurring nitrosamines. It was proposed (Ji et al., 1986a; Ji et al., 1986b) that the alkyl portion could arise by decarboxylation of the amino acids leucine and valine, known to occur in appreciable quantities in grains (Yang et al., 1956). In fact, the corresponding primary amines, isoamylamine and isobutylamine, have been found in bread products (Golovnya, 1976). The hypothesis further suggested that the ketone portion could be added to the primary amine by a series of enzyme-mediated methylation, acetylation, and remethylation reactions, or by reductive condensation of the primary amine with acetoin. Since acetoin is a known constituent of corn (Boyko et al., 1977), this latter route seemed to be simpler and more probable.

Isoamylamine rapidly condenses with acetoin in aqueous acidic phosphate buffer to form an insoluble oil, presumably the Schiff base. In the presence of sodium cyanoborohydride, this product is quickly reduced to amino alcohol 3. A byproduct, 2,3-bis(isoamylamino)butane (5), is also formed in appreciable amounts (Scheme I). This product is reminiscent of the bis adducts formed as osazones from hydrazines and keto sugars. In fact, slowly adding a solution of the amine to a solution of excess (2-fold) acetoin did not completely suppress its formation.

Nitrosations are normally carried out in dilute hydrochloric acid [to take advantage of the catalytic activity of the halide ion (Ridd, 1961)] or in 30% acetic acid. The

acetic acid provides the ideal pH (3.5) for nitrosation and catalyzes the formation of N_2O_3 . It was felt that sulfuric acid, however, might provide a medium more conducive to both nitrosation and oxidation of the alcohol function. This proved to be the case. Nitrosation in sulfuric acid with excess nitrite (10-fold) proceeded smoothly to provide a mixture of the bis adduct 2,3-bis(*N*-nitroso-*N*-isoamylamino)butane (4) and NMAMBA (1). These products were separated on a silica gel column with some difficulty since even with 100% methylene chloride as eluent there was some overlap.

The NMAMBA was readily identified by comparison of IR and MS spectra with those of authentic material. The IR spectrum of the bis adduct contained no bands for OH, NH, or carbonyls. There was a band at 1450 cm^{-1} for the NNO function, and the spectrum suggested an alkyl nitrosamine. This was confirmed by the mass spectrum, both under EI and also under ammonia CI conditions. In EI, there was no M^+ but there was an $M^+ - 30$ for the loss of a NO and an $M^+ - 60$ for the loss of the second NO. The next major fragment was at m/e 143 for the symmetric cleavage into half of the molecule. The remaining fragmentations resemble those of NMAMBA. In the ammonia CI mass spectrum there was a prominent $M^+ + NH_4$.

The ^{13}C NMR spectrum shows only six lines, again indicating a simple symmetric structure. The line at 17.99 ppm is readily assigned to the two equivalent secondary methyl carbons. The intense line at 22.23 ppm represents the four isopropyl methyl carbons.

Oxidation of alcohols via nitrite esters under anhydrous conditions has previously been reported (Barton and Forbes, 1975). The formation of small amounts of nitrosamino ketones from amino alcohols under the usual aqueous nitrosation conditions has only occasionally been observed (J. E. Saavedra, unpublished observations).

It seems clear then that the original proposal of condensation of a primary amine with acetoin is indeed plausible and may very well be the route by which these unusual nitrosamines arise. It has been shown that the nitrosation step is not enzyme catalyzed (Ji et al., 1986a), but it is likely that the reductive condensation of the amine with the acetoin does require enzymic catalysis. This mode of formation should also account for the apparent absence of the amino ketone when precursors to nitrosamines are sought in the Linxian foods. It is never present except as

the nitrosamine. The amino alcohol is present but is probably too polar a species to be detected by the routine assays currently employed.

SAFETY

All nitrosamines should be presumed to be carcinogenic and should be handled with due care in well-ventilated fume hoods. Skin contact and inhalation should be avoided.

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Registry No. 1, 71016-15-4; 3, 105582-47-6; 4, 105582-45-4; 5, 105582-46-5; isoamylamine, 107-85-7; acetoin, 513-86-0.

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