Production of 3-(1,2-Epoxypropyl)-5,6-dihydro-5-hydroxy-6-methylpyran-2-one by *Aspergillus ochraceus* Wilhelm

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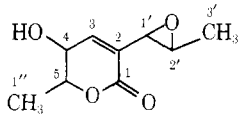
The metabolite 3-(1,2-epoxypropyl)-5,6-dihydro-5-hydroxy-6-methylpyran-2-one has been isolated from the culture medium of an ochratoxin-producing isolate of *Aspergillus ochraceus*. This metabolite was also produced in trace amounts on corn and rye.

Aspergillus ochraceus has been isolated from a variety of sources, including agricultural products (Raper and Fennell, 1965). Several isolates of this fungus produce the mycotoxin ochratoxin A (Hesseltine *et al.*, 1972). Other known metabolites of the isolate, *A. ochraceus* Wilhelm, include isocoumarins (Cole *et al.*, 1971; Yamazaki *et al.*, 1972b), and pyrazines (Kodaira, 1961; Yamazaki *et al.*, 1972a).

The lactone 3-(1,2-epoxypropyl)-5,6-dihydro-5-hydroxy-6-methylpyran-2-one (I) has been isolated from fungi of



I

the *A. ochraceus* group (Mills and Turner, 1967; Rosenbrook and Carney, 1970; Yamamoto *et al.*, 1970). We report here the production and isolation of this lactone from the culture medium of the isolate *A. ochraceus* Wilh., and the production of trace amounts on corn and rye under laboratory conditions.

MATERIALS AND METHODS

A. ochraceus Wilh., NRRL3174, was maintained at 25° on Czapek's agar slants with 20% sucrose and 0.7% Difco yeast extract added (Davis *et al.*, 1969). The culture medium (Moore *et al.*, 1972) was modified to the following composition on a gram per liter basis: sucrose, 40.0 g; L-glutamic acid, 6.0 g; KH_2PO_4, 1.0 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $ZnSO_4 \cdot 7H_2O$, 1.0 mg; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2.5 mg. Erlenmeyer flasks (1000 ml) containing 450 ml of medium (pH 5.2) or 250-ml flasks containing 50 g of corn or rye plus 25 ml of water were stoppered with cotton plugs and autoclaved at 121° for 15 min. Flasks were inoculated with conidia from 14- to 21-day-old cultures of *A. ochraceus*. Liquid cultures were incubated for 14 days and solid substrates were incubated for 28 days as stationary cultures at 25°.

Isolation. The mycelium from 18 l. of the liquid medium was removed by filtration and the filtrate extracted three times with equal volumes of chloroform. The mycelium was extracted separately by stirring briskly with chloroform and decanting. The combined extracts were dried (Na_2SO_4) and the excess solvent removed under reduced pressure to yield 12 g of crude extract. The extract was chromatographed over 150 g of Grace desiccant grade silica gel (60–200 mesh) using a 3 × 125 cm column. Elution was started with hexane, successive hexane–chloroform mixtures, chloroform, and finally successive mixtures of chloroform–methanol. Fractions were monitored using thin-layer chromatography (tlc) on glass plates coated with Merck silica gel G and eluted with chloroform–acetone (93:7, v/v). The metabolite was detected as a dark spot under ultraviolet light and also with I_2 vapor. Metabolite I came off the column in fractions eluted with chloroform–hexane (80:20, v/v) through fractions eluted with chloroform–methanol (95:5, v/v). Fractions containing I were combined to afford 1.81 g of reasonably pure material and purified to a constant mp (109–110°) by recrystallization from acetone–hexane mixtures.

Spectral Analysis. The infrared (ir), ultraviolet (uv), and 100-MHz proton magnetic resonance (pmr) spectra in $CDCl_3$ of I were identical in all respects with previously published spectral data for 3-(1,2-epoxypropyl)-5,6-dihydro-5-hydroxy-6-methylpyran-2-one (Mills and Turner, 1967; Rosenbrook and Carney, 1970). We also report the ^{13}C nuclear magnetic resonance (cmr) spectrum for this compound which affords additional evidence for the previously reported structure. The cmr spectrum of I was obtained using a Varian XL-100 spectrometer equipped with a pulsed Fourier transform accessory.

Spectra were taken with 250 mg of sample in 4 ml of acetone-d_6 using 12-mm sample tubes. Proton decoupled spectra required approximately 12,000 scans. Chemical shifts are assigned on the basis of selected model compounds (Johnson and Jankowski, 1972; Levy and Nelson, 1972) as well as splitting patterns and hydrogen–carbon coupling constants derived from gated decoupling experiments. These values are reported in parts per million from TMS: 17.17 (C-1″ or C-3′); 17.65 (C-1″ or C-3′); 54.56 (C-2′); 58.10 (C-1′); 67.35 (C-5); 79.22 (C-4); 128.53 (C-2); 142.39 (C-3); 162.64 (C-1).

Isolation from Solid Substrates. Each corn and rye sample was homogenized for 2 min in a Waring Blendor and extracted twice with chloroform. The chloroform was evaporated to a small volume. Subsequent tlc analyses of the crude extracts plus a sample of authentic I revealed a trace of the metabolite I in each substrate.

RESULTS AND DISCUSSION

Based upon previously reported physical constants and spectral data, this new metabolite of *A. ochraceus* has been identified as 3-(1,2-epoxypropyl)-5,6-dihydro-5-hydroxy-6-methylpyran-2-one (I).

This metabolite was tested against several species of bacteria and showed weak, broad-spectrum, antibiotic properties as reported previously (Rosenbrook and Carney, 1970). Since trace amounts of this lactone were produced on corn and rye under arbitrarily selected laboratory conditions, it is possible that significant quantities might be produced in naturally infested feed grains. Additional studies are planned to investigate its biological activity in animals because moldy grains such as these might become incorporated into animal feeds.

It has been reported (Turner, 1971) that incorporation of labeled acetate into I gives an anomolous distribution of radioactivity at the junction of C-2 and -1′. Such a labeling pattern is not in agreement with normal polyketide biosynthesis (Richards and Hendrickson, 1964). Our cur-

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rent chemical studies are, therefore, directed to employment of ^{13}C-enriched growth medium for *A. ochraceus* Wilh. in order to study further the biosynthesis of I and related compounds using cmr.

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LITERATURE CITED

Cole, R. J., Moore, J. H., Davis, N. D., Kirksey, J. W., Diener, U. L., *J. Agr. Food Chem.* **19,** 909 (1971).
Davis, N. D., Searcy, J. W., Diener, U. L., *Appl. Microbiol.* **17,** 742 (1969).
Hesseltine, C. W., Vandergraft, E. E., Fennell, D. I., Smith, M. L., Shotwell, O. L., *Mycologia* 64, 539 (1972).
Johnson, L. F., Jankowski, W. C., "Carbon-13 NMR Spectra," Wiley, New York, N. Y., 1972.
Kodaira, Y., *Agr. Biol. Chem.* **25,** 261 (1961).
Levy, G. C., Nelson, G. L., "Carbon-13 Nuclear Magnetic Resonance for Organic Chemists," Wiley, New York, N. Y., 1972.
Mills, S. D., Turner, W. B., *J. Chem. Soc. C,* 2242 (1967).
Moore, J. H., Davis, N. D., Diener, U. L., *Appl. Microbiol.* **23,** 1067 (1972).
Raper, K. B., Fennell, D. I., "The Genus Aspergillus," Williams and Wilkins, Baltimore, Md., 1965.
Richards, J. H., Hendrickson, J. B., "Biosynthesis of Steroids, Terpenes, and Acetogins," W. A. Benjamin, New York, N. Y., 1964.
Rosenbrook, W., Jr., Carney, R. E., *Tetrahedron Lett.,* 1867 (1970).
Turner, W. B., "Fungal Metabolites," Academic Press, New York, N. Y., 1971.
Yamamoto, I., Suide, H., Henmi, T., Yamano, T., *Takeda Kenkyusho Ho* **29,** 1 (1970); *Chem. Abstr.* **73,** 52347V (1971).
Yamazaki, M., Maebayashi, Y., Miyaki, K., *Chem. Pharm. Bull.* **20,** 2274 (1972a).
Yamazaki, M., Maebayashi, Y., Miyaki, K., *Chem. Pharm. Bull.* **20,** 2276 (1972b).

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Formation of *N*-Nitrosopyrrolidine from Pyrrolidine Ring Containing Compounds at Elevated Temperatures

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The formation of *N*-nitrosopyrrolidine from various pyrrolidine ring containing compounds and sodium nitrite at elevated temperatures was investigated. *N*-Nitrosopyrrolidine was formed when dry samples of L-proline, glycyl-L-proline, L-prolylglycine, and pyrrolidine were heated with nitrite at 170° for 2 hr and when proline was heated for 2 hr with sodium nitrite at 170° in a pH 6.2 buffer solution. Buffered collagen samples produced *N*-nitrosopyrrolidine at temperatures of 120° and above. L-Hydroxyproline did not produce *N*-nitrosopyrrolidine. The identity of *N*-nitrosopyrrolidine was confirmed in the heated samples by gas–liquid chromatography coupled with mass spectrometry.

N-Nitrosopyrrolidine (NPY), which has been shown to be carcinogenic in animal feeding trials (Druckrey *et al.,* 1967), has been identified in cooked but not in raw bacon. Fazio *et al.* (1973) reported levels of NPY from eight commercial brands of fried bacon ranged from 10 to 108 ppb. Crosby *et al.* (1972) and Sen *et al.* (1973) detected NPY in 13 of 24 samples and in 7 of 8 samples of fried commercial bacon, respectively.

Lijinsky and Epstein (1970) suggested cooking proteinaceous food might cause pyrolysis of protein and produce amino acids and the nitrosatable secondary amine pyrrolidine (PY). The greatest source of pyrrolidine ring compounds in foods would be the two α-imino acids proline (PR) and hydroxyproline (HP). Proline and HP are abundant amino acid residues in collagen which is the most abundant protein in mammalian muscle tissue (Price and Schweigert, 1971). In a recent report, Bills *et al.* (1973) identified *N*-nitrosoproline, pyrrolidine, spermidine, proline, and putrescine as potential precursors for the formation of NPY.

The purpose of this study was to investigate the heat-induced formation of NPY by reacting sodium nitrite with various pyrrolidine ring containing compounds. Included in this study were PR, HP collagen, and two proline containing dipeptides.

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EXPERIMENTAL PROCEDURES

Chemicals. L-Proline, HP, *N*-nitrosodipropylamine (NDPA), and NPY were purchased from the Eastman Kodak Co., Rochester, N. Y. Glycyl-L-proline (GP), L-prolylglycine (PG), and collagen from bovine Achilles tendon were purchased from the Sigma Chemical Co., St. Louis, Mo. Pyrrolidine was obtained from the Aldrich Chemical Co., Milwaukee, Wis. The GP, PG, and collagen were shown by thin-layer chromatography (Brenner and Niederwieser, 1960) to contain no free proline. Control samples of GP, PG, and collagen spiked with 1% free proline were easily detected by the thin-layer procedure. Since NDPA and NPY are potent carcinogens, caution was taken in handling nitrosamine solutions, spiked samples, heated vials, and concentrates, and work was done in efficient fume hoods whenever possible.

Preparation of Samples. Weighed amounts of compounds under investigation and powdered sodium nitrite were added to 35-ml test tubes. PR and HP samples were heated in the dry state and also in pH 6.2 buffer. For the collagen samples which were heated in the dry state, nitrite was added in solution and the collagen–nitrite mixture was subsequently freeze-dried. For the samples which were heated in solution, 1 ml of buffer was added to the test tube prior to sealing. The test tubes were shaken to completely mix the components, sealed in an oxygen–methane flame, and immediately placed in the oven. After heating, the samples were removed from the oven and allowed to cool. Samples not analyzed immediately were stored at −15° until extraction.