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REDUCTION OF METHYL 5-NITRO-2-FUROATE BY XANTHINE OXIDASE: AN EVIDENCE FOR HYDROXYLAMINOFURAN FORMATION

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After methyl 5-nitro-2-furoate was incubated with milk xanthine oxidase, two reduction products were isolated from the incubation mixture. Metabolite 1 was positive to a pentacyanoammineferroate reagent and data obtained from mass spectrometry of its acetylated derivative indicated that the metabolite was the hydroxylaminofuran derivative (methyl 5-hydroxylamino-2-furoate). Metabolite 2 was identified as the aminofuran derivative (methyl 5-amino-2-furoate) by comparison with a synthetic sample.

KEYWORDS —— nitrofuran derivative; methyl 5-nitro-2-furoate; *in vitro* metabolism; milk xanthine oxidase-hypoxanthine system; reduction products; methyl 5-hydroxylamino-2-furoate; methyl 5-amino-2-furoate

Reduction of the nitro group of nitrofuran derivatives seems to be biologically important, because it has been regarded as activation and detoxication steps. End products may be inactive, whereas unstable intermediates in the process may be reactive. It is known that nitrofuran derivatives are reduced enzymatically into nitrofuran anion-free radicals,[1-6] aminofuran derivatives,[7-10,16] open-chain cyano derivatives,[9-15] pyrrole derivatives[9,14] or dimer-type derivatives.[16] Hydroxylaminofuran derivatives seem to be possible candidates of reactive nitrofuran metabolites. However, isolation and identification of such metabolites have not been reported yet. In the present study, the enzymatic reduction of methyl 5-nitro-2-furoate was investigated in a xanthine oxidase system. We provide the first evidence of formation of a hydroxylaminofuran derivative.

Metabolite 1 revealed the UV absorption maximum at 303 nm in ethanol (the UV absorption maximum of the parent nitro compound: 295 nm in ethanol). The metabolite gave blue-violet color on a thin-layer plate when sprayed with 1% sodium pentacyanoammineferroate, which is a color reagent for the detection of nitroso or hydroxylamino compounds.[17] Prolonged incubation of the metabolite with xanthine oxidase led to its further reduction and/or its degradation. These facts suggest that the metabolite is an intermediate, probably methyl 5-nitroso or 5-hydroxylamino-2-furoate, produced during enzymatic reduction of the nitrofuran derivative.

The metabolite was labile, but could be stabilized by acetylation with acetic anhydride. As shown in Fig. 1, the acetylated metabolite gave a molecular

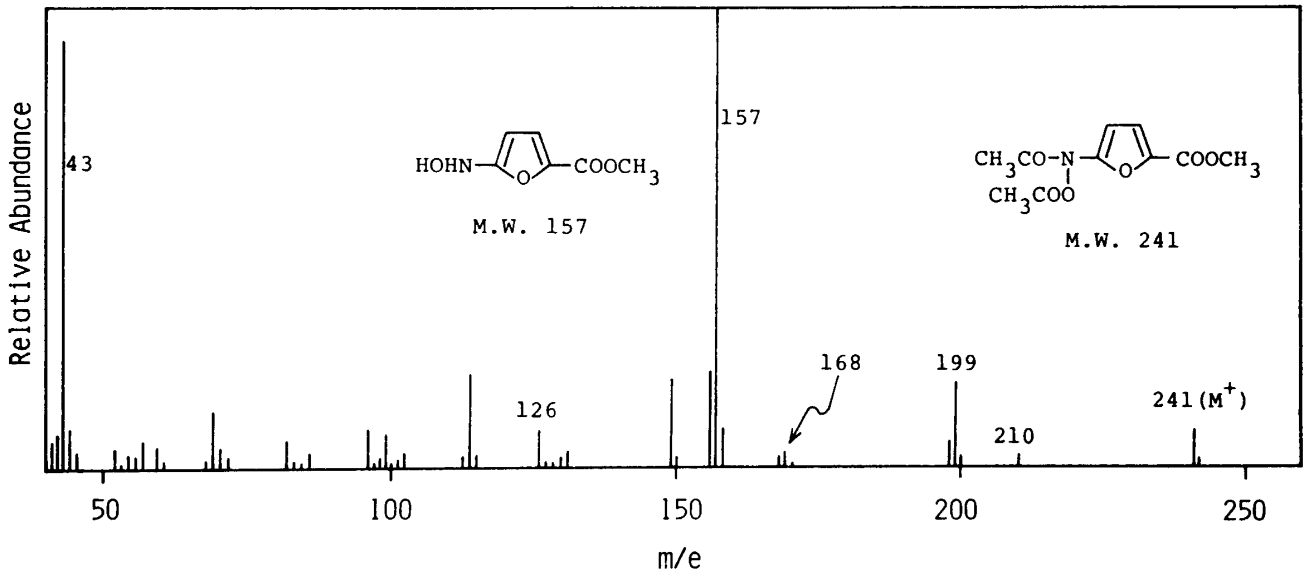

Fig. 1. Mass Spectrum of the Diacetyl Derivative of Methyl 5-Hydroxylamino-2-furoate (metabolite 1)

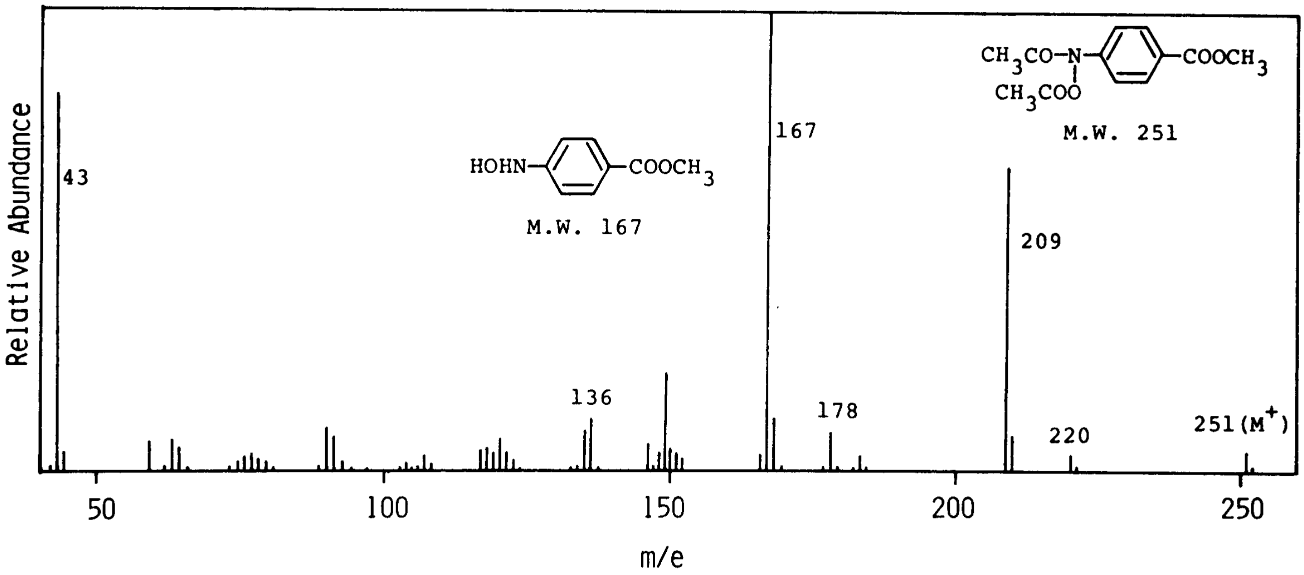

Fig. 2. Mass Spectrum of the Diacetyl Derivative of Methyl p-Hydroxylaminobenzoate

ion at m/e 241 and probably is the diacetyl derivative of methyl 5-hydroxylamino-2-furoate. The fragment ion at m/e 199 and the base peak at m/e 157 may represent the consecutive loss of acetyl groups from the molecular ion to form the monoacetyl derivative and then the hydroxylamino derivative. The fragment ions at m/e 210, 168 and

126 most likely result from the loss of methoxy group from methoxycarbonyl groups of the diacetyl, the monoacetyl and the hydroxylamino derivatives, respectively. The fragment ion at m/e 43 may be attributed to acetyl groups from these acetyl derivatives.

For confirmation of these assignments, synthetic methyl p-hydroxylaminobenzoate was acetylated under the same conditions as described in metabolite 1 and then subjected to mass spectrometry. As shown in Fig. 2, the molecular ion of the acetylated derivative was observed at m/e 251, indicating that it is the diacetyl derivative of the hydroxylamino compound, like metabolite 1 described above. The base peak at m/e 167 may be due to the loss of two acetyl groups from the molecular ion to form the hydroxylamino derivative. The fragment ion at m/e 209 may be attributable to the loss of an acetyl group from the molecular ion to form the monoacetyl derivative. Furthermore, fragment ions at m/e 220, 178 and 136 may be due to the loss of methoxy groups from methoxycarbonyl groups of the diacetyl, the monoacetyl and the hydroxylamino derivatives, respectively. These mass spectrometries indicate that metabolite 1 is a new-type nitrofuran metabolite, methyl 5-hydroxylamino-2-furoate.

An attempt was made to chemically reduce methyl 5-nitro-2-furoate as follows: The nitrofuran derivative in methanol was hydrogenated in the presence of 5% palladium on charcoal at room temperature. The crude reduction product was purified by silica gel (Kiesel gel 60, Merck) column chromatography and by recrystallization from ethanol. By this procedure, methyl 5-amino-2-furoate, but not methyl 5-hydroxylamino-2-furoate, was obtained as pale yellow plates, mp 135-136°C. Anal. Calcd for $C_6H_7NO_3$: C,51.07; H,5.00; N,9.93. Found: C,50.98; H,5.03; N,9.77. The UV spectrum in ethanol showed an absorption maximum at 316 nm. The mass spectrum gave a molecular ion at m/e 141. Comparison of the UV spectrum and the thin-layer chromatographic behavior of metabolite 2 with those of the synthetic aminofuran derivative showed that this metabolite is methyl 5-amino-2-furoate.

Thus, the present study demonstrated formation of the hydroxylaminofuran as well as the aminofuran derivative from methyl 5-nitro-2-furoate. As described previously,[18)] this nitrofuran derivative exhibited very weak mutagenicity against Salmonella typhimurium TA100, as compared with other nitrofuran derivatives such as 3-(5-nitro-2-furyl)-2-(2-furyl)acrylamide (AF-2) and 5-nitro-2-furanacrylamide. Further studies are in progress in our laboratories on the formation of 5-hydroxylaminofuran derivatives from other strongly mutagenic nitrofuran derivatives.

EXPERIMENTAL

Methyl 5-nitro-2-furoate[18)] and methyl p-hydroxylaminobenzoate[19)] were synthesized as described previously. The incubation mixture consisted of methyl 5-nitro-2-furoate (150 μmol), hypoxanthine (300 μmol) and milk xanthine oxidase (10 units) in a final volume of 120 ml of 33 mM Na·K-phosphate buffer (pH 7.4) containing acetone at a concentration of 0.5%. The incubation was carried out at 37°C for 30 min under an atmosphere of nitrogen in the dark. After incubation, the mixture was extracted twice with an equal volume of ethyl acetate. The combined ethyl acetate extract was dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo. The residue was subjected to thin-layer chromatography (TLC) (silica gel plates coated with Wako gel B-5FM, Wako Pure Chemical Industries,Ltd) using $CHCl_3$-MeOH (9:1) and spots were visualized under UV light. Two main spots due to metabolite 1 (Rf 0.40) and metabolite 2 (Rf 0.54) were scraped and extracted with acetone.

A small amount of metabolite 1 or methyl p-hydroxylaminobenzoate in 1 ml of ethyl acetate was reacted with 0.5 ml of acetic anhydride for 1 hr at room temperature. The mixture was diluted with 5 ml of water, neutralized with $NaHCO_3$ and then extracted twice with 10 ml of ethyl acetate. The crude product obtained from the ethyl acetate extracts was purified by TLC using $CHCl_3$-MeOH (9:1) for metabolite 1 (Rf 0.52) or benzene-acetone (9:1) for methyl p-hydroxylaminobenzoate (Rf 0.31).

Mass spectra were recorded with a Japan Electron Optics (JEOL) JMS-01SG mass spectrometer. UV spectra were taken with a Hitachi 340 spectrophotometer.

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