

ISOLATION AND IDENTIFICATION OF URINARY METABOLITES OF AF-2
(3-(5-NITRO-2-FURYL)-2-(2-FURYL)ACRYLAMIDE) IN RABBITS

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SUMMARY. After oral administration of AF-2 (3-(5-nitro-2-furyl)-2-(2-furyl)acrylamide) to rabbits, the two unique metabolites, M-I and M-II, were isolated from the urine. M-I, yellow needles of mp 117^o, was identified as a new type metabolite of nitrofurantoin derivative, 2-(β -carboxypropionyl)-3-(5-methylthio-2-furyl)acrylamide by its mass, ir and nmr spectrometries. M-II, yellow solid, appears to be cis-trans isomer of M-I considering from its uv and mass spectral data, and the behavior on tlc.

AF-2, which had been used as a food preservative in Japan, is known to have carcinogenic activity in mice (1,2) and rats (2), and mutagenic activity in a variety of organisms (3). Previously, we reported the metabolic fate of nitrofurantoin derivatives including AF-2 in rats (4-7). More recently, the organic solvent-extractable reduction products of AF-2 and nitrofurazone by milk xanthine oxidase, rat liver xanthine oxidase and rat liver microsomes were identified as 2-(2-furyl)-3-(5-oxo-2-pyrrolidin-2-yl)acrylamide and 5-amino-2-furaldehyde semicarbazone, respectively (8). However, any metabolite of AF-2 has not been isolated yet in vivo. This paper describes the isolation and identification of unique metabolites of AF-2 in the urine of rabbits for the first time.

MATERIALS AND METHODS

Chemicals. AF-2 (mp 151 - 152^o) and its [¹⁴C] labelled compound (acrylamide-3-¹⁴C) which was radiochemically pure were kindly donated by Ueno Pharmaceutical Co., Ltd.

Synthesis of α -chloro-5-methylthio-2-furylacrylamide. This compound was prepared by heating a mixture of potassium monochloroacetate (2 g) and 5-methylthio-2-furaldehyde (2 g) which was synthesized by a procedure of Carro et al. (9), in Ac₂O (9 g) at 130^o

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The abbreviation used is: AF-2, 3-(5-nitro-2-furyl)-2-(2-furyl)-acrylamide.

for 4 h, and then converted to its amide by successive treatment with thionylchloride and NH_3 in benzene. The crude compound was recrystallized from aqueous EtOH to form yellow needles, mp 96° . The uv spectrum: λ_{max} EtOH 336 nm. High resolution mass spectral analysis: Calcd. for $\text{C}_8\text{H}_8\text{O}_2\text{NSCl}$ (M^+ , m/e): 216.9964. Found: 216.9960.

Thin-layer chromatography. Tlc was conducted on silica gel plates (Wako gel B-5 FM, 0.25 mm thick) developing in (A) CHCl_3 -acetone-MeOH-AcOH (5:3:1:1, v/v), (B) benzene- CHCl_3 -AcOH (2:1:1, v/v) and (C) benzene-acetone (7:3, v/v). The chromatograms were visualized under uv light (3650 Å).

Analytical procedures. Ir spectra were obtained using KBr pellets with a Japan Spectroscopic DS-701 G ir spectrophotometer. Nmr spectra were determined with a Japan Electron Optics (JEOL) JNM PS-100 spectrometer using tetramethylsilane as an internal standard. Mass spectra were recorded with a Japan Electron Optics (JEOL) JMS-01SG mass spectrometer. Uv spectra were obtained with a Hitachi 124 spectrophotometer.

Micro tests for sulfur and nitrogen atoms in the metabolite molecule. This was carried out according to Feigl (10).

Administration of drug. Male albino rabbits weighing about 3.5 kg were given [^{14}C] AF-2 (0.27 $\mu\text{Ci}/\text{mg}$) at a single oral dose of 100 mg/kg as suspension and 24-h urine was collected. In other experiments, non-labelled AF-2 was given orally at a dose of 100 mg/kg once a week for four weeks and each 24-h urine was collected.

Isolation of metabolites. Prior to isolation of AF-2 metabolites, preliminary experiment was carried out with radioactive 24-h urine following administration of [^{14}C] AF-2. The urine was extracted three times with ethyl acetate at pH 2. After removal of solvent, the extract was placed on silica gel column (Merck Kieselgel 60) and eluted stepwise with CHCl_3 , CHCl_3 -acetone (1:1, v/v) and MeOH. Each fraction was subjected to radioactivity measurement. High radioactivity was detected in CHCl_3 -acetone (1:1, v/v) eluate, which was rechromatographed similarly as above. This crude metabolites fraction showed a radioactive, yellow spot (dark yellow under uv light) at R_f 0.6 on tlc with the solvent system A, but was separated into two radioactive, yellow spots with R_f values of 0.28 (M-I) and 0.35 (M-II) in the solvent system B. M-II revealed lower radioactivity than M-I.

Next, the 24-h urine, which was obtained from rabbits given nonlabelled AF-2, was treated similarly as above. The crude metabolites obtained by repeated silica gel column chromatography were successively purified by preparative tlc with the solvent systems A and B. M-I which was eluted from the band of R_f 0.28 with acetone was recrystallized from CHCl_3 to form yellow needles, mp 117° . The thin-layer chromatogram of this crystals showed one yellow spot at R_f 0.28 in the solvent system B. No attempt to crystallize the M-II was made because its small quantity.

RESULTS AND DISCUSSION

The metabolite I of AF-2 (M-I) was shown to possess both sulfur and nitrogen atoms in its molecule by the micro tests. The uv spectrum in EtOH had the absorption maximum at 347 nm.

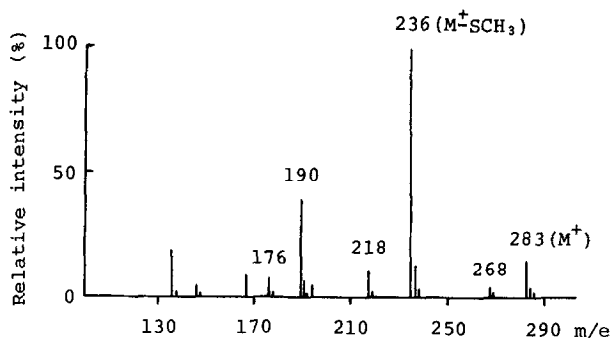


Fig.1. Mass spectrum of AF-2 metabolite (M-I).

No bathochromic shift was observed in alkaline EtOH differing from AF-2 and other nitrofuran derivatives. The mass spectrum (Fig.1) gave a molecular ion at m/e 283, accompanying fragment ions at m/e 236 ($M^+ - 47$). The high resolution mass spectrum showed the following:

	m/e Found	Calcd. for
Molecular ion	283.0506	$C_{12}H_{13}O_5NS$, 283.0514
$M^+ - 47(SCH_3)$	236.0545	$C_{11}H_{10}O_5N$, 236.0558

The ir spectrum (KBr) had bands at 3300 (NH), 1710 (COOH), 1660 (CH=CH-CO), 1650 (amide I) and 1620 (CH=CH) cm^{-1} .

The nmr spectrum is shown in Fig.2. The doublets at δ 6.60 and 7.02 ppm are attributed to the furan ring protons and the singlet at δ 7.23 ppm must result from vinyl proton. These assignments were strongly supported by the spectrum of synthetic methylthiofuran derivative, α -chloro-5-methylthio-2-furylacrylamide (furan ring protons, δ 6.68 (d, $J=3.5$ Hz) and 7.21 (d, $J=3.5$ Hz) ppm; vinyl proton, δ 7.63 (s) ppm). Furthermore, the protons of methylthio group of both M-I and the synthetic methylthiofuran derivative were observable at δ 2.55 ppm overlapping on multiplets attributed to the solvent, DMSO- d_6 . These evidences indicate that M-I of AF-2 is 2-(β -carboxypropionyl)-3-(5-methylthio-2-furyl)acrylamide.

The presence of carboxyl group in its molecule, which was evident from the ir spectrum, was further confirmed by the facts that this metabolite remained at origin on tlc with a neutral solvent system C, while after methylation with diazomethane it moved up to R_f 0.20 in the same solvent system. The compound

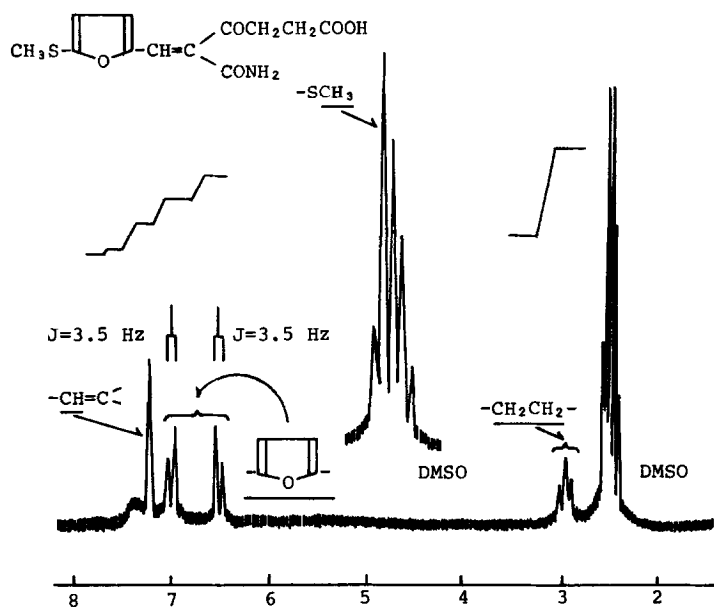


Fig.2. Nuclear magnetic resonance spectrum of AF-2 metabolite (M-II).

with Rf value of 0.20 gave a molecular ion at m/e 297 corresponding to the methyl ester of M-I. So far, the two different mechanisms are proposed for the enzymatic formation of the methylthio metabolites; one is direct introduction of methylthio group from methionine (e.g. in the metabolism of 2-acetylaminofluorene (11)), and the other is two steps introduction, in which SH group is firstly introduced from cysteine and then S-methylated with S-adenosylmethionine (e.g. in the metabolism of bromazepam (12)). However, the formation mechanism of 5-methylthiofuran derivatives from AF-2 has not been elucidated yet.

Although the minor metabolite of AF-2 (M-II) was not isolated as crystals, it was assumed that M-II might be cis-trans isomer of M-I, because M-I and M-II had the identical uv and mass spectra, showing close Rf values on tlc, and in addition M-II was always converted in part to M-I on repeating preparative tlc. The occurrence of such steric isomers is reasonable considering from our previous finding (13) of enzymatic cis-trans isomerization of AF-2.

The preliminary mutation test showed that methylthiofuran derivatives such as M-I and α -chloro-5-methylthio-2-furylacrylamide are inactive against *Salmonella typhimurium* TA 100.

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