Metabolism of Furametpyr. 1. Identification of Metabolites and in Vitro Biotransformation in Rats and Humans

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Urinary and fecal metabolites in male rats treated with a ^{14}C-labeled fungicide, furametpyr [*N*-(1,3-dihydro-1,1,3-trimethylisobenzofuran-4-yl)-5-chloro-1,3-dimethylpyrazole-4-carboxamide, Limber], were purified by a combination of chromatographic techniques, and chemical structures of 14 metabolites were identified by spectroanalyses (NMR and MS). The major biotransformation reactions of furametpyr in rats were found to be (1) *N*-demethylation, (2) oxidation of the methyl group at C3 of the pyrazole ring, (3) oxidation of the methyl group at C1 of the 1,3-dihydroisobenzofuran ring, (4) hydroxylation at C3 of the 1,3-dihydroisobenzofuran ring, and (5) hydroxylation at C7 of the 1,3-dihydroisobenzofuran ring. In vitro metabolism by recombinant human cytochrome P450 revealed that a major biotransformation in humans is *N*-demethylation, catalyzed by CYP1A1, 1A2, 2C19, and 3A4.

Keywords: *Metabolism; furametpyr; identification; rats; human; cytochrome P450; biotransformation*

INTRODUCTION

Furametpyr [*N*-(1,3-dihydro-1,1,3-trimethylisobenzofuran-4-yl)-5-chloro-1,3-dimethylpyrazole-4-carboxamide, Limber] is a fungicide used to control rice sheath blight (Mori et al., 1997; Oguri, 1997). Metabolism of furametpyr has been investigated in conjunction with toxicological studies for safety evaluation. The present paper deals with the identification of metabolites of furametpyr in rats and humans. ^{14}C excretion and tissue concentrations in rats after oral administration of furametpyr are described in the following paper (Nagahori et al., 2000).

MATERIALS AND METHODS

Chemicals. [*phenyl*-^{14}C]-Labeled furametpyr (specific activity = 2.01 GBq/mmol) and unlabeled furametpyr (purity = 99.8%) were synthesized in our laboratory. The labeled compound was purified by preparative thin-layer chromatography (TLC) developed by chloroform/methanol, 10:1 (v/v), prior to use.

Chromatographic Procedures. TLC analyses were conducted essentially as described previously (Nagahori et al., 1997). Precoated silica gel 60 F_{254} chromatoplates (Art. 5715, 20 × 20 cm, 0.25 mm layer thickness, Merck, Darmstadt, Germany) were used for analyses and isolation of metabolites with the following solvent systems: (A) ethyl acetate/formic acid/water (35:2:2); (B) ethyl acetate/ethanol/water (10:2:1); and (C) toluene/ethyl formate (5:9, developed twice). The radioactive spots on TLC plates were detected by placing X-ray films (SB-5, Kodak, Rochester, NY) on plates at 4 °C, followed by processing of the exposed films with a model M6B processor (Kodak), or by placing imaging plates (Fuji film, Tokyo, Japan) on plates, followed by analyzing the imaging plates with a BAS-2000 (Fuji film). High-performance liquid chromatography (HPLC) was carried out with a system consisting of an L-6200 HPLC intelligent pump (Hitachi, Tokyo, Japan), an L-4200 UV−vis detector (Hitachi), and an LB 507A radioactivity monitor (Berthold, Calmbacher, Germany) fitted with a YMC-Pack column (ODS, 20 mm i.d. × 250 mm, YMC Co., Kyoto, Japan) (column A), a Cosmosil packed column 5C18 (4.6 mm i.d. × 250 mm, Nacalai Tesque, Kyoto, Japan) (column B), or a Zorbax SIL (4.6 mm × 250 mm, P.N.) (column C).

Spectrometry. Nuclear magnetic resonance (NMR) spectra were obtained on a JEOL GSX-270 spectrometer (JEOL, Tokyo, Japan) operating at 270 MHz for ^{1}H and at 67.5 MHz for ^{13}C at room temperature. Spectra were processed with the program PLEXUS V1.6 (JEOL). Methanol-d_4 (99.5%, Merck) was used as a solvent. Direct electron impact (EI) and field desorption (FD) mass spectra in the positive ionization mode were recorded on a Hitachi M-80B mass spectrometer (Hitachi). The ionizing energy and accelerating voltage for EI mass spectrometry (EI-MS) were 70 eV and 3.0 kV, respectively. The accelerating voltage for FD mass spectrometry (FD-MS) was 3.0 kV.

Treatment of Animals. Male Crj:CD(SD) rats at the age of 6 weeks old were purchased from Charles River Japan Inc. (Kanagawa, Japan) and maintained in an air-conditioned room at 21−25 °C with an alternating 12-h light and 12-h dark cycle for 1 week before use. To collect sufficient amounts of unknown metabolites in urine and feces for spectroanalytical identification, unlabeled furametpyr was dissolved in corn oil at 60 mg/mL and dosed orally to 10 rats for 4 consecutive days at ∼5 mL/kg/day (300 mg/kg/day) and then [*phenyl*-^{14}C]furametpyr adjusted to 0.1 MBq/mmol with unlabeled furametpyr was orally dosed at 300 mg/kg on the fifth day. A total of ∼3.4 g of furametpyr was dosed. The animals were housed in Metabolica CO_2 cages (Sugiyamagen Iriki Co., Tokyo, Japan) to allow the separate collection of urine and feces.

Purification of Metabolites. A flow diagram of the purification procedures is given in Figure 1. Urine was lyophilized, mixed with 500 mL of methanol, shaken, and centrifuged at 1000*g* for 10 min. The supernatant was collected by decantation. The residual fraction was washed twice with methanol. The supernatants were combined for concentration in vacuo and applied to an Amberlite XAD-2 resin column (10

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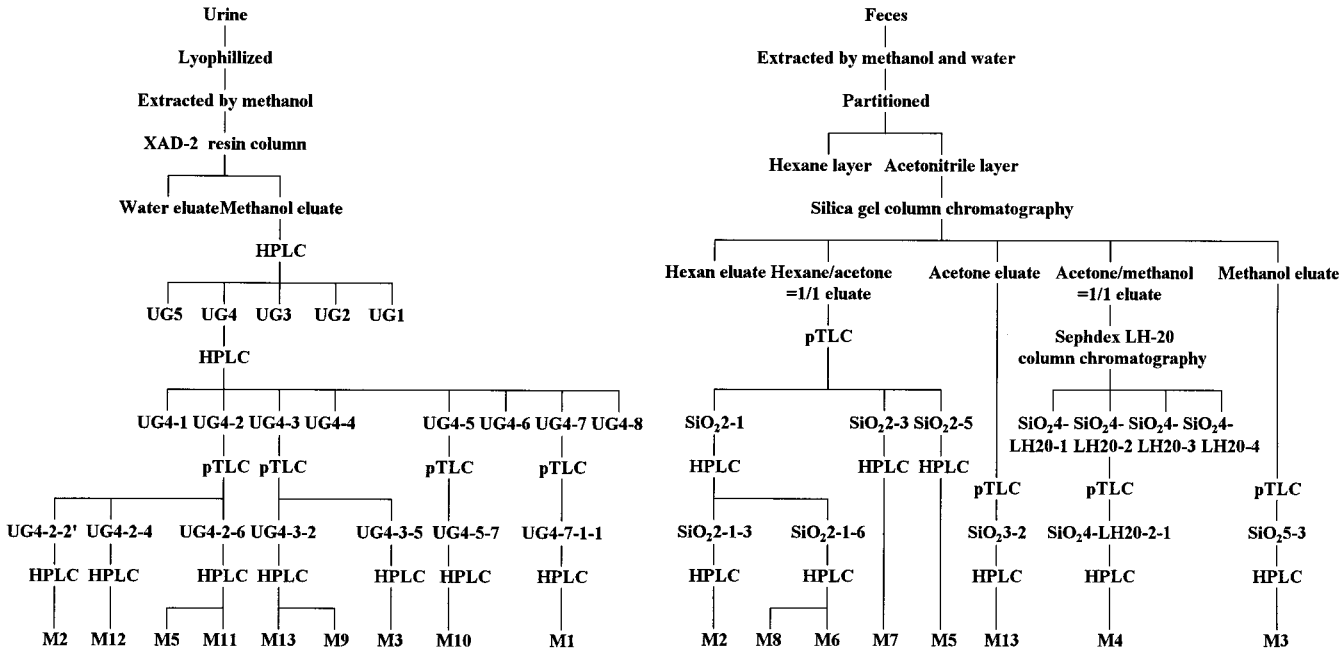


Figure 1. Flow diagram of purification procedures for urinary and fecal metabolites.

Table 1. HPLC Conditions Used for Isolation of Furametpyr Metabolites

metabolite	solvent (water/methanol) (v/v)	flow rate (mL/min)	column[b]	retention time (min)
M1	60:40	1.0	B	22
M2	70:30	1.0	B	50
	40:60	5.0	A	39
M3	55:45	1.0	B	41
	50:50[a]	1.0	C	16
M4	55:45	5.0	A	13
M5	40:60	5.0	A	59
	60:40	1.0	B	11
M6	55:45	1.0	B	18
M7	45:55	5.0	A	50
M8	35:65	5.0	A	65
M9	50:50[a]	1.0	C	19
M10	60:40	1.0	B	38−52
M11	60:40	1.0	B	15
M12	60:40	1.0	B	10
M13	45:55	5.0	A	59
	50:50[a]	1.0	C	12

[a] Hexane/ethyl acetate (v/v). [b] Column: (A) YMC-Pack column (ODS, 20 mm i.d. × 250 mm); (B) Cosmosil packed column 5C18 (4.6 mm i.d. × 250 mm); (C) Zorbax SIL (4.6 mm × 250 mm).

cm i.d. × 20 cm, Organo, Tokyo, Japan). The column was washed with water (1000 mL) and eluted with methanol (1000 mL). The methanol eluates were concentrated in vacuo and applied to HPLC or preparative TLC. Finally, nine metabolites (M1, M2, M3, M5, M9, M10, M11, M12, and M13) were purified by HPLC under the conditions described in Table 1.

All collected feces were mixed and homogenized with a 3-fold volume of methanol using an Excel Auto homogenizer (Nihonseiki Co., Tokyo, Japan), and the homogenates were centrifuged at 1000*g* for 10 min. The supernatants were obtained by decantation. Residues were further washed twice with methanol and once with water. The methanol and water extracts of feces were combined, concentrated in vacuo, and partitioned three times between *n*-hexane and acetonitrile. The acetonitrile layer was concentrated in vacuo and subjected to silica gel column chromatography (20 mm i.d. × 200 mm, Kieselgel 60, 70−230 mesh, Merck) using *n*-hexane (500 mL), *n*-hexane/acetone (1:1, v/v, 500 mL), acetone (500 mL), acetone/methanol (1:1, v/v, 500 mL), and methanol (500 mL) as solvents in sequence, and the eluates were designated $SiO_2$1 to $SiO_2$5, respectively. The fraction $SiO_2$4 was concentrated in vacuo and separated further by column chromatography using a Sephadex LH-20 (20 mm i.d. × 200 mm, Pharmacia, Uppsala, Sweden) into $SiO_2$4-LH20-1−4. Each fraction was further purified by HPLC or preparative TLC, and eight metabolites (M2, M3, M4, M5, M6, M7, M8, and M13) were isolated. The HPLC conditions used for the isolation of furametpyr metabolites are summarized in Table 1.

In Vitro Metabolism. Microsomes containing human CYP 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, or 3A4 expressed from cDNA in yeast were obtained from Sumika Chemical Analysis Service (Osaka, Japan). Fifty picomoles of each P450 was incubated at 37 °C in a final volume of 0.5 mL of 0.1 mM KH_2PO_4, pH 7.4, containing 1.5 μmol of NADPH and 1−80 nmol of furametpyr. Preincubations were performed for 5 min without the addition of furametpyr. Incubations were initiated by the addition of 5 μL of furametpyr solution in ethanol and terminated after 5−15 min by the addition of 5 mL of ethanol. Metabolites were extracted twice with ethanol, and portions of extracts were applied to TLC with solvent system A, followed by analysis of plates with a BAS-2000.

RESULTS

Identification of Metabolites. The R_f values for furametpyr and 14 identified metabolites are summarized in Table 2. The metabolites were purified from urine and feces as described under Materials and Methods. M2, M3, M3′, M5, and M13 were purified from both urine and feces, M1 and M9−M12 only from urine, and M4 and M6−M8 only from feces. Their chemical structures were identified by spectroanalyses (NMR and MS). Chemical structures and spectroanalysis data are shown in Table 3.

M1. M1 was suggested to have lost the *N*-methyl group of furametpyr. No signal for the three protons of the *N*-methyl group at the C1 of the pyrazole ring was observed, whereas furametpyr showed a singlet signal at 3.35 ppm on ^{1}H NMR. Similarly, the carbon signal of the *N*-methyl group at 36.5 ppm in the ^{13}C NMR spectrum disappeared with this metabolite. A molecular ion peak at *m*/*z* 319 [M^+] was observed by EI-MS. On the basis of its spectrum, M1 was identified as *N*-(1,3-dihydro-1,1,3-trimethylisobenzofuran-4-yl)-5-chloro-3-methylpyrazole-4-carboxamide (DM-Fur).

Table 2. TLC R_f Values for Furametpyr and Its Metabolites

	metabolite	R_f values with solvent systems[a] A	B	C
	furametpyr	0.79	−[b]	0.58
M1	DM-Fur	0.87	−	0.55
M2	3-CH_2OH-DM-Fur-OH	0.66	0.83	0.16
M3	Fur-COOH	0.53	0.23	0.01
M3′	Fur-COOH lactone	0.66	0.79	0.44
M4	DM-Fur-COOH	0.68	0.41	0.01
M5	3-CH_2OH-DM-Fur-CH_2OH	0.46	0.69	0.05
M6	DM-Fur-HK	0.79	0.88	0.62
M7	DM-Fur-CH_2OH	0.54	0.73	0.12
M8	DM-Fur-HK-OH	0.75	0.87	0.19
M9	Fur-OH	0.64	0.78	0.32
M10	DM-Fur-HK-CH_2OH	0.65	0.79	0.15
M11	3-CH_2OH-DM-Fur-COOH lactone	0.71	0.83	0.24
M12	DM-Fur-CH_2OH-OH	0.57	0.76	0.08
M13	DM-Fur-OH	0.70	0.72	0.27

[a] Solvent systems: (A) ethyl acetate/formic acid/water = 35:2:2 (v/v/v); (B) ethyl acetate/ethanol/water = 10:2:1 (v/v/v); (C) toluene/ethyl formate = 5:9 (v/v), developed twice. [b] −, not analyzed.

M2. M2 had no *N*-methyl group on the basis of NMR analysis. Furthermore, the ^{1}H and ^{13}C NMR spectra showed that the methyl group at C3 of the pyrazole ring was oxidized to a hydroxymethyl group. This proton signal of a hydroxymethyl group was observed at 4.85 ppm on ^{1}H NMR and at 58.9 ppm on ^{13}C NMR, and signals for the methyl group at C3 of the pyrazole ring of furametpyr (2.42 ppm on ^{1}H NMR and 13.6 ppm on ^{13}C NMR) disappeared. C−H coupling of two protons of the hydroxymethyl group with the carbon atom at the C3 of the pyrazole ring was confirmed by CH-COSY (data not shown). In addition, this metabolite was suggested to have a hydroxyl group at C7 of the isobenzofuran ring. Two doublet signals were observed in the benzene region of the ^{1}H NMR spectrum (6.74 and 7.19 ppm). The observed chemical shifts of carbons at C5, C6, and C7 of the isobenzofuran ring (127.2, 116.0, and 151.5 ppm) in the ^{13}C NMR spectrum were similar to values calculated (126.5, 117.2, and 146.5 ppm) by substituent chemical shift method (Stothers, 1972), if the hydroxyl group was not at the C5 or C6 but at the C7 of the isobenzofuran ring. A molecular ion peak was observed at *m*/*z* 351 [M^+] by FD-MS. On the basis of these results, M2 was identified as *N*-(1,3-dihydro-7-hydroxy-1,1,3-trimethylisobenzofuran-4-yl)-5-chloro-3-hydroxymethylpyrazole-4-carboxamide (3-CH_2OH-DM-Fur-OH).

M3. The ^{1}H and ^{13}C NMR spectra of M3 showed that one of the two methyl groups at C1 of the isobenzofuran ring was oxidized to carboxylic acid. The signals of the methyl group at C1 of the isobenzofuran ring of M3 were observed at 1.79 ppm (3H, singlet) in the ^{1}H NMR spectrum and at 26.3 ppm in the ^{13}C NMR spectrum. The signals of one methyl group of furametpyr disappeared. The FD-MS spectrum showed a molecular ion peak at *m*/*z* 363 [M^+]. On the basis of these data, the structure of M3 was identified as 4-(5-chloro-1,3-dimethylpyrazole-4-carbonylamino)-1,3-dihydro-1,3-dimethylisobenzofuran-1-carboxylic acid (Fur-COOH).

M3 was suggested to be converted to a lactone form (M3′) through the artificial mechanism proposed in Figure 2 and showed two bands on TLC analysis. The IR spectrum of M3′ showed a characteristic absorption band at 1735 cm^{-1} implying the presence of a carbonyl group of the lactone. Typical characteristic absorption bands (νOH) at 3000−2500 cm^{-1} attributable to a carboxyl group were not detected. Fur-COOH (M3) and 3-CH_2OH-DM-Fur-COOH (M11) were suggested to be converted to lactone forms, whereas DM-Fur-COOH (M4) was not, because the R_f values of M4 were similar to those of M3 and not to those of M3′ and M11.

M4. M4 was demonstrated to be an *N*-demethylated 1-carboxy derivative. The FD-MS spectrum showed a molecular ion peak at 349 [M^+]. On the basis of these results, the metabolite was identified as 4-(5-chloro-3-methylpyrazole-4-carbonylamino)-1,3-dihydro-1,3-dimethylisobezofuran-1-carboxylic acid (DM-Fur-COOH). It was not considered to be a lactone as described above. A lactone form of M4 was not detected, but we cannot clarify the reason.

M5. M5 was demonstrated to be an *N*-demethylated 3-hydroxymethyl derivative. Additionally, one of the two methyl groups at C1 of isobenzofuran ring had undergone oxidation to a hydroxymethyl group, because the signals for methyl and hydroxymethyl groups were observed at 1.54 ppm (3H), 3.61 ppm (1H, doublet, J = 11.2 Hz), and 3.67 ppm (1H, doublet, J = 11.2 Hz), respectively, on ^{1}H NMR and at 69.8 ppm on ^{13}C NMR, and these signals were suggested to be coupled with each other by CH-COSY (data not shown). Methylene protons in the hydroxymethyl group at C1 of the isobenzofuran ring showed geminal coupling. This is in line with the fact that chemical shifts of methylene protons near chiral atoms are different (Oki, 1985), because the carbon at C1 of the isobenzofuran ring of these metabolites is a chiral center. FD-MS and EI-MS spectra showed molecular ion peaks at 352 [$M + H]^+$ and 351 [M^+], respectively. On the basis of these data, the structure of M5 was identified as *N*-(1,3-dihydro-1-hydroxymethyl-1,3-dimethy1isobenzofuran-4-y1)-5-chloro-3-hydroxymethylpyrazo1e-4-carboxamide (3-CH_2OH-DM-Fur-CH_2OH).

M6. M6 was demonstrated to be an *N*-demethylated derivative. Additionally, C3 of the isobenzofuran ring was hydroxylated. A proton signal for the C3 methylene of the isobenzofuran ring disappeared on ^{1}H NMR, and a carbon signal shifted to a lower magnetic field on ^{13}C NMR (106.6 ppm). Furthermore, the protons of the methyl group at C3 of the isobenzofuran ring of M6 showed a singlet signal at 1.56 ppm without coupling with the C3 methylene proton of the isobenzofuran ring. The EI-MS spectrum showed a peak at 317 [$M - 18]^+$. On the basis of these results, M6 was identified as *N*-(1,3-dihydro-3-hydroxy-1,1,3-trimethylisobenzofuran-4-y1)-5-chloro-3-methyl-pyrazo1e-4-carboxamide (DM-Fur-HK).

M7−M13. These metabolites were identified by NMR and MS analyses, and their chemical structures and spectroanalysis data are shown in Table 3.

In Vitro metabolism. In vitro metabolism studies of furametpyr by human recombinant P450 isoforms indicated that furametpyr was mainly metabolized to DM-Fur (M1) by *N*-demethylation. CYP1A1, 1A2, 2C19, and 3A4 metabolized furametpyr efficiently to DM-Fur. All other P450s (CYP2A6, -2B6, -2C8, -2C9, -2C18, -2D6, and -2E1) failed to catalyze the reaction. Further studies on the kinetics of furametpyr metabolism by four P450 isoforms (CYP1A1, -1A2, -2C19, and -3A4) indicated that CYP1A1 and CYP2C19 possess higher affinity for furametpyr than CYP1A2 or CYP3A4, the highest clearance being obtained with CYP2C19 (Table 4).

Table 3. Chemical Structures and Spectroanalysis Data for Furametpyr and Isolated Metabolites[a]

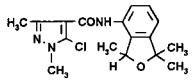
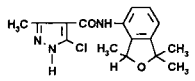
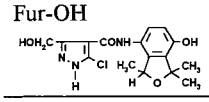
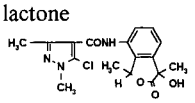
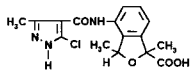
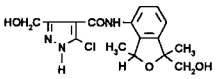
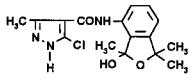
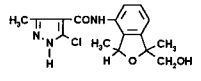
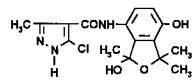
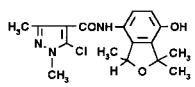
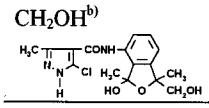
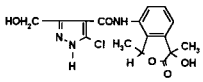
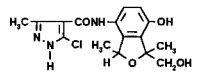
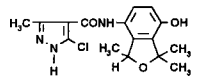
Compound		NMR (ppm)	MS (m/z)
Furametpyr 	^{1}H NMR (CD_3OD)	1.44 (3H, d, J = 6.5Hz), 1.51 (3H, s), 1.59 (3H, s), 2.42 (3H, s), 3.35 (3H, s), 5.48 (1H, q, J = 6.5Hz), 7.13 (1H, d, J = 7.0Hz), 7.26 (1H, d, J = 7.8Hz), 7.37 (1H, dd, J = 7.0, 7.8Hz)	EI-MS 333 (M^+)
	^{13}C NMR (CD_3OD)	13.6, 21.5, 29.4, 30.5, 36.5, 79.0, 86.1, 113.9, 119.6, 124.7, 129.1, 129.8, 132.3, 137.7, 149.7, 150.4, 162.9	
M1: DM-Fur 	^{1}H NMR (CD_3OD)	1.46 (3H, d, J = 6.8Hz), 1.50 (3H, s), 1.59 (3H, s), 2.52 (3H, s), 5.48 (1H, q, J = 6.8Hz), 7.12 (1H, d, J = 7.3Hz), 7.32 (1H, d, J = 7.8Hz), 7.37 (1H, dd, J = 7.3, 7.8Hz)	EI-MS 319 (M^+)
	^{13}C NMR (CD_3OD)	11.3, 21.6, 29.4, 30.5, 79.0, 86.1, 112.4, 119.6, 124.7, 129.8, 132.3, 137.5, 146.2, 149.7, 163.0	
M2: 3-CH_2OH-DM-Fur-OH 	^{1}H NMR (CD_3OD)	1.45 (3H, d, J = 6.5Hz), 1.57 (3H, s), 1.67 (3H, s), 4.85 (2H, s), 5.43 (1H, q, J = 6.5Hz), 6.74 (1H, d, J = 8.6Hz), 7.19 (1H, d, J = 8.6Hz)	FD-MS 351 (M^+)
	^{13}C NMR (CD_3OD)	22.2, 27.4, 29.0, 58.9, 79.2, 87.0, 111.3, 116.0, 123.4, 127.2, 133.6, 140.1, 150.0, 151.5, 163.0	
M3[1]: Fur-COOH lactone 	^{1}H NMR (CD_3OD)	1.46 (3H, d, J = 5.9Hz), 1.79 (3H, s), 2.43 (3H, s), 3.87 (3H, s), 5.63 (1H, q, J = 5.9Hz), 7.32 (3H, m)	FD-MS 363 (M^+)
	^{13}C NMR (CD_3OD)	13.6, 20.7, 26.3, 36.5, 81.4, 87.9, 113.7, 121.1, 125.8, 129.3, 130.0, 138.5, 144.0, 150.4, 162.9, 175.2	
M4: DM-Fur-COOH 	^{1}H NMR (CD_3OD)	1.53 (3H, d, J = 6.3Hz), 1.74 (3H, s), 2.53 (3H, s), 5.63 (1H, q, J = 6.3Hz), 7.42 (3H, m)	FD-MS 349 (M^+)
	^{13}C NMR (CD_3OD)	11.3, 20.9, 26.7, 81.1, 88.6, 112.4, 121.4, 125.6, 129.8, 132.2, 138.1, 144.4, 146.3, 162.9, 177.2	
M5: 3-CH_2OH-DM-Fur-CH_2OH[b] 	^{1}H NMR (CD_3OD)	1.47 (3H, d, J = 6.2Hz), 1.54 (3H, s), 2.53 (3H, s), 3.61 (1H, d, J = 11.2Hz), 3.67(1H, d, J = 11.2Hz), 4.86 (2H, s), 5.54 (1H, q, J = 6.2Hz), 7.13 (1H, d, J = 7.6Hz), 7.38 (1H, dd, J =7.6, 8.0Hz), 7.52 (1H, d, J =8.0Hz)	FD-MS 352 $(M+H)^+$ 351(M^+)
	^{13}C NMR (CD_3OD)	22.0, 56.8, 69.8, 79.8, 88.9, 120.2, 124.7, 129.6, 132.3, 138.3, 146.2, 162.5	
M6: DM-Fur-HK 	^{1}H NMR (CD_3OD)	1.56 (3H, s), 1.61 (3H, s), 1.75 (3H, s), 2.61 (3H, s), 7.08 (1H, d, J = 7.6Hz), 7.47 (1H, dd, J = 7.6, 8.1Hz), 8.20 (1H, d, J = 8.1 Hz)	EI-MS 317 $(M-18)^+$
	^{13}C NMR (CD_3OD)	12.2, 27.5, 28.9, 29.7, 85.7, 106.6, 117.9, 121.8, 131.8, 133.9, 149.2, 149.9, 162.2	
M7: DM-Fur-CH_2OH[b] 	^{1}H NMR (CD_3OD)	1.47 (3H, d, J = 6.5Hz), 1.55 (3H, s), 2.52 (3H, s), 3.62 (1H, d, J = 11.2Hz), 3.68 (1H, d, J = 11.2Hz), 5.53 (1H, q, J = 6.5Hz), 7.16 (1H, d, J = 8.6Hz), 7.38 (2H, m)	FD-MS 336 $(M+H)^+$
	^{13}C NMR (CD_3OD)	11.3, 21.8, 25.2, 69.7, 79.9, 88.7, 112.4, 120.5, 125.1, 129.6, 132.3, 138.9, 146.3, 163.0	
M8:DM-Fur-HK-OH 	^{1}H NMR (CD_3OD)	1.60 (3H, s), 1.67 (3H, s), 1.73 (3H, s), 2.59 (3H, s), 6.80 (1H, d, J = 8.6Hz), 7.73 (1H, d, J = 8.6Hz)	FD-MS 333 $(M-18)^+$
	^{13}C NMR (CD_3OD)	12.1, 27.7, 28.4, 28.5, 85.8, 106.7, 111.2, 116.8, 125.4, 135.5, 138.2, 147.6, 150.4, 162.3	
M9: Fur-OH 	^{1}H NMR (CD_3OD)	1.44 (3H, d, J = 6.1Hz), 1.57 (3H, s), 1.67 (3H, s), 2.41 (3H, s), 3.87 (3H, s), 5.43 (1H, q, J = 6.1Hz), 6.74 (1H, d, J = 8.2Hz), 7.06 (1H, d, J = 8.2Hz)	EI-MS 349 (M^+)
	^{13}C NMR (CD_3OD)	13.6, 22.0, 27.3, 29.0, 36.4, 79.3, 86.8, 111.2, 116.0, 123.4, 127.5, 133.7, 150.2, 151.7	
M10: DM-Fur-HK-CH_2OH[b] 	^{1}H NMR (CD_3OD)	1.52 (3H, s), 1.81 (3H, s), 2.61 (3H, s), 3.69 (2H, m), 7.10 (1H, d, J = 7.6Hz), 7.45 (1H, t, J = 7.6, 8.1Hz), 8.08 (1H, d, J = 8.1Hz)	FD-MS 351 (M^+)
	^{13}C NMR (CD_3OD)	12.2, 23.8, 69.6, 88.3, 118.9, 123.2, 131.1, 133.9, 146.0	

Table 3. (Continued)

Compound		NMR (ppm)	MS (m/z)
M11: 3-CH_2OH-DM-Fur-COOH lactone 	^1H NMR (CD_3OD)	1.48 (3H, d, J = 6.2Hz), 1.80 (3H, s), 4.86 (2H, s), 5.65 (1H, q, J = 6.2Hz), 7.28 (1H, d, J = 7.4Hz), 7.41 (1H, dd, J = 7.4, 7.8Hz), 7.49 (1H, d, J = 7.8Hz)	EI-MS 365 (M^+)
	^{13}C NMR (CD_3OD)	21.0, 26.4, 56.7, 81.2, 88.1, 114.0, 118.1, 120.8, 125.6, 130.0, 132.5, 137.8, 143.8, 149.2, 162.4, 175.3	
M12: DM-Fur-CH_2OH-OH[b] 	^1H NMR (CD_3OD)	1.47 (3H, d, J = 6.3Hz), 1.61 (3H, s), 2.51 (3H, s), 3.73 (1H, d, J = 10.9Hz), 3.73 (1H, d, J = 10.9Hz), 5.48 (1H, q, J = 6.3Hz), 6.76 (1H, d, J = 8.5Hz), 7.15 (1H, d, J = 8.5Hz)	EI-MS 351 (M^+)
	^{13}C NMR (CD_3OD)	11.3, 22.4, 24.0, 68.6, 80.4, 89.5, 112.4, 116.2, 123.5, 128.1, 130.5, 141.9, 146.0, 152.0, 163.4	
M13: DM-Fur-OH 	^1H NMR (CD_3OD)	1.46 (3H, d, J = 6.8Hz), 1.57 (3H, s), 1.67 (3H, s), 2.51 (3H, s), 5.43 (1H, q, J = 6.8Hz), 6.74 (1H, d, J = 8.4Hz), 7.10 (1H, d, J = 8.4Hz)	EI-MS 335 (M^+)
	^{13}C NMR (CD_3OD)	11.3, 22.1, 27.4, 29.0, 79.3, 86.9, 112.4, 116.0, 123.4, 127.6, 133.7, 140.6, 151.7, 163.3	

[a] M3 data were not obtained because M3 was converted to M3′ with the artificial mechanism proposed in Figure 2. [b] The two protons of the hydroxymethyl group show geminal coupling with each other, so they are shown as two different signals at ~3.6−3.7 ppm on ^1H NMR.

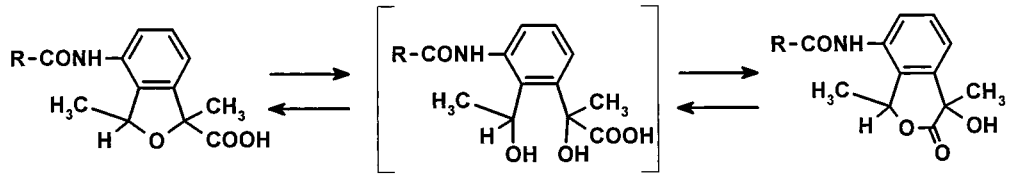


Figure 2. Proposed conversion reaction between furancarboxylic acid and hydroxyl lactone.

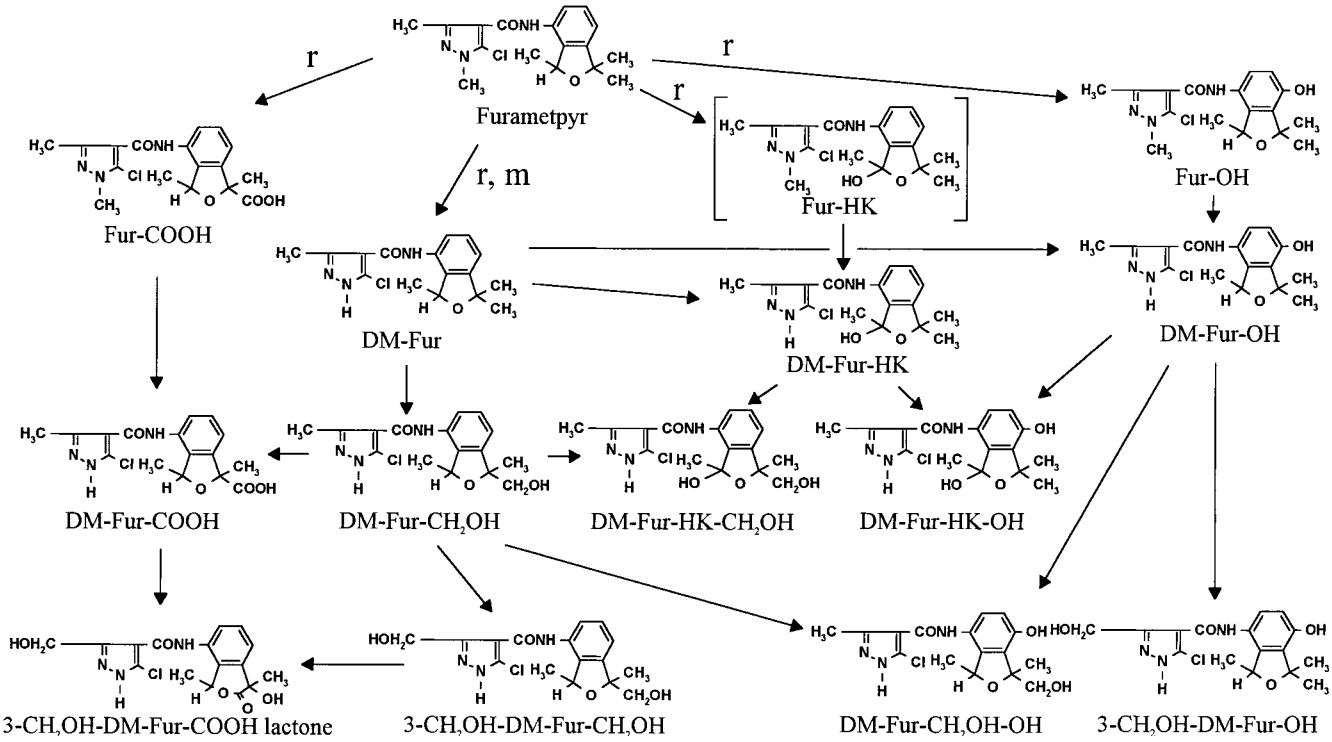


Figure 3. Proposed metabolic pathways of furametpyr in rats (r) and man (m).

DISCUSSION

In the current study, furametpyr is revealed to be extensively metabolized to >14 metabolites in rats. The major biotransformation reactions in rats were concluded to be as follows: (1) *N*-demethylation; (2) oxidation of the methyl group at C3 of the pyrazole ring; (3) oxidation of the methyl group at C1 of the 1,3-dihydroisobenzofuran ring; (4) hydroxylation at C3 of the 1,3-dihydroisobenzofuran ring; and (5) hydroxylation at C7 of the 1,3-dihydroisobenzofuran ring. Proposed metabolic pathways of furametpyr are shown in Figure 3.

Table 4. Michaelis−Menten Kinetic Parameters for *N*-Demethylation of Furametpyr to DM-Fur Obtained by Using Microsomes from Yeast Expressing Human Cytochrome P450s

isoform	K_m (μM)	V_{max} (pmol/pmol of P450/min)	CL_{int} (mL/min/pmol of P450)
1A1	16	3	0.17
1A2	53	12	0.22
2C19	19	16	0.85
3A4	37	2	0.05

Site-specific oxidation of methyl groups was revealed by the present study. Two methyl groups at C3 of the pyrazole ring and a methyl group at C1 of the 1,3-dihydroisobenzofuran ring were found to be oxidized to hydroxymethyl, but the methyl group at the C3 of the 1,3-dihydroisobenzofuran ring was not oxidized. This may be because of steric hindrance.

Furametpyr is a racemic substance at C3 of the isobenzofuran ring. When two methyl groups at C1 of the isobenzofuran ring of furametpyr are oxidized to hydroxymethyl or carboxylic acid, two diastereomers are formed. M5, M7, M10, and M12 are 1-hydroxymethyl derivatives, and M3, M4, and M11 are 1-carboxy derivatives, but it remains unclear whether these metabolites are *R*- or *S*-forms.

The main metabolic reaction of furametpyr was concluded to be *N*-demethylation. Most furametpyr is considered to undergo *N*-demethylation to DM-Fur and formaldehyde. In general, *N*-demethylation proceeds by three steps: (1) hydroxylation of the methyl group; (2) decomposition of unstable intermediate metabolite; and (3) formation of the first or second amine and the aldehyde (Karki and Dinnocenzo, 1995). The first stage (step 1) is reported to be catalyzed by microsomal cytochrome P-450-dependent mixed-function oxygenase.

P450-dependent *N*-demethylation is reported in some compounds, such as diazepam metabolized by CYP2C19 and CYP3A4 (Ono et al., 1996; Jung et al., 1997), caffeine by CYP1A2 (Miners et al., 1996), *S*-mephenytoin by CYP2B6 (Heyn et al., 1996), and nitrosodimethylamine by CYP2E1 (Yamazaki et al., 1992). In the present study, it was revealed that the major biotransformation of furametpyr in an in vitro human system is *N*-demethylation catalyzed by CYP1A1, -1A2, -2C19, and -3A4. Furametpyr is thus a novel compound that is extensively metabolized through *N*-demethylation by CYP1A1.

In conclusion, furametpyr primarily undergoes *N*-demethylation to form many kinds of metabolites (~40 in total). In vitro metabolism of furametpyr by human recombinant P450s showed that CYP1A1, -1A2, -2C19, and -3A4 contributed to the major biotransformation by *N*-demethylation. Findings for amounts of metabolites in urine and feces of rats administered furametpyr are published in the following paper (Nagahori et al., 2000).

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