JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Metabolism of Diethofencarb (Isopropyl 3,4-Diethoxyphenylcarbamate) in Rats: Identification of Metabolites in Urine

HIROKAZU TARUI,* YOSHITAKA TOMIGAHARA, MASAYOSHI MATSUI, NAOHIKO ISOBE, HIDEO KANEKO, AND NOBUYOSHI MIKAMI

Environmental Health Science Laboratory, Sumitomo Chemical Company, Ltd., 1-98 3-Chome, Kasugade-Naka, Konohana-Ku, Osaka 554-8558, Japan

Rats were orally given a diethofencarb (isopropyl 3,4-diethoxyphenylcarbamate) labeled with ^{14}C, at 300 mg/kg/day, for 4 consecutive days, and 11 metabolites in urine were purified by a combination of several chromatographic techniques. The chemical structures of all isolated metabolites were identified by spectroanalyses (NMR and MS). Ten of them were newly identified forms. Five of them were *S*-conjugates: three mercapturic acid conjugates, one *S*-methyl conjugate, and one *SO*-methyl conjugate. The others were two phenoxyacetic acids, hydroxyacetanilide, hydroxyisopropyl carbamate, and oxazolinone derivatives. From the results, the existence of the following reactions in rats can be concluded: (1) deethylation of the 4-ethoxy group; (2) conjugation of phenols with glutathione, γ-glutamyltranspeptidation and depeptidation of the glutathione to form cysteine conjugates, and *N*-acetylation of the cysteine; (3) cleavage of the *C*−*S* linkage of cysteine conjugates followed by methylation; (4) oxidation of the *S*-methyl group; (5) cleavage of the carbamate linkage; (6) acetylation of the resultant amino group; (7) oxidation of the acetyl group; (8) oxidation of the isopropyl group; (9) cyclization of the oxidized isopropyl carbamate group; and (10) oxidation of the 4-ethoxy group.

KEYWORDS: Metabolism; rat; fungicide; identification; biotransformation; conjugation; mercapturic acid

INTRODUCTION

Diethofencarb (POWMYL, isopropyl 3,4-diethoxyphenylcarbamate, DFC), a fungicide, is very effective for the control of various fungal species, especially *Botrytis* spp., *Cercospora* spp., and *Venturia* spp., that are resistant to benzimidazole fungicides (*1*).

Investigations of the metabolic fate of diethofencarb in rats have been conducted along with toxicological studies conducted for safety evaluation (*2*). The present paper deals with the identification of urinary metabolites of diethofencarb in rats. Absorption, distribution, and excretion of ^{14}C and quantification of fecal and urinary metabolites are described elsewhere.

MATERIALS AND METHODS

Chemicals. Diethofencarb labeled uniformly in the phenyl group with a specific activity of 8.88 MBq/mg, [*phenyl*-^{14}C]diethofencarb (radiochemical purity = 98.7% and chemical purity = 98.8%) was synthesized in our laboratory (**Figure 1**) and stored in a refrigerator (<10 °C). The labeled preparation was purified by preparative TLC development in benzene/ethyl acetate, 3:2 (v/v), prior to use, and the radiochemical purity was cofirmed to be >99%.

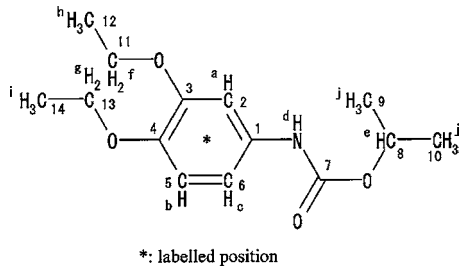

Figure 1. Chemical structure of diethofencarb.

Unlabeled diethofencarb (chemical purity = 98.2%) and the authentic standard, 4-OH-DFC (chemical purity = 99.5%) were prepared in our laboratory.

Other chemicals were of reagent grade unless otherwise noted in the text.

Animal Treatment. Charles River derived-CD (Sprague−Dawley) male rats at the age of 10 weeks were housed in polypropylene cages (290 × 340 × 170 mm, Clea Japan Inc., Tokyo, Japan) on sawdust bedding (White Flake, Charles River Japan Inc.) at the density of five or fewer animals/cage during a quarantine and acclimation period (7 days). The animal cages were exchanged for clean ones three times a week. The in-life portion of the study was conducted under the following environmental conditions: room temperature, 21−25 °C; relative humidity, 40−70%; ventilation, 10 air exchanges per hour; and artificial lighting from 8:00 a.m. to 8:00 p.m. Animals had free access to the diet (CRF-1, Oriental Yeast, Tokyo, Japan) and water throughout

* Author to whom correspondence should be addressed. Telephone: 81-6-6466-5321. Fax: 81-6-6466-5442. E-mail: taruih@sc.sumitomo-chem.co.jp.

10.1021/jf034779i CCC: $27.50
Published on Web 01/09/2004

Table 1. R_f Values

compound	solvent system[a] A	B	C	D	E	F
diethofencarb	0.47	0.38	0.77	1.00	0.62	–[b]
4-OH-DFC	–	–	0.65	0.98	0.59	–
1	–	–	0.08	0.89	–	–
2	–	–	0.07	0.81	–	–
3	–	–	0.80	1.00	0.66	–
4	–	–	0.38	1.00	0.33	–
5	–	–	0.01	0.53	0.10	–
6	–	–	0.16	0.85	–	–
7	–	–	0.30	1.00	0.32	–
8	–	–	0.36	0.95	0.39	0.90
9	–	–	0.21	0.87	–	–
10	–	–	0.04	0.68	0.17	–
11	–	–	0.16	0.92	–	–

[a] A, toluene/ethyl acetate = 5:2 (v/v); B, hexane/diethyl ether = 1:1 (v/v); C, chloroform/ethanol/acetic acid = 18:1:1 (v/v/v); D, ethyl acetate/acetone/water/acetic acid = 4:1:1:1 (v/v/v/v); E, toluene/ethyl formate/formic acid = 5:7:1 (v/v/v); F, ethyl acetate/acetone/water/acetic acid = 9:1:1:1 (v/v/v/v). [b] –, not analyzed.

the study. The water had been passed through a water filtration device (type: PTS-3, 5D-C-1, AMF, Tokyo, Japan). Rats dosed with the ^{14}C-labeled compound were housed individually in glass metabolism cages (Metabolica CO_2, Sugiyamagen Iriki, Tokyo, Japan) designed for the separation and collection of urine and feces.

Administration. Corn oil was used as a vehicle, as in a previous study (*1*). To collect sufficient amounts of urinary metabolites for spectroanalytical identification, (~2 g of [*phenyl*-^{14}C]diethofencarb), four male rats at the age of 11 weeks were given repeated oral doses of the ^{14}C-labeled compound at 300 mg/5 mL of corn oil/kg using a glass syringe (2 mL volume, Sougo Rikagaku Glass, Kyoto, Japan) equipped with a stainless steel gastric probe (Natsume Product, Tokyo, Japan) daily for 4 consecutive days. The specific activity of the [*phenyl*-^{14}C]diethofencarb was adjusted to 4.4×10^5 dpm/mg by isotopic dilution with unlabeled diethofencarb.

Thin-Layer Chromatography (TLC). Precoated silica gel 60 F_{254} TLC plates (20 × 20 cm, 0.25 or 0.5 mm thickness, Merck, Darmstadt, Germany) were used for the separation and identification of urinary metabolites. The 0.5 mm thickness plates were used only for separation. Plates were developed using the following solvent systems: (A) toluene/ethyl acetate = 5:2 (v/v); (B) hexane/diethyl ether = 1:1 (v/v); (C) chloroform/ethanol/acetic acid = 18:1:1 (v/v/v); (D) ethyl acetate/acetone/water/acetic acid = 4:1:1:1(v/v/v/v); (E) toluene/ethyl formate/formic acid = 5:7:1(v/v/v); (F) ethyl acetate/acetone/water/acetic acid = 9:1:1:1 (v/v/v/v); (G) hexane/diethyl ether = 1:2 (v/v).

Un-radiolabeled test compounds and authentic standards on TLC plates were detected by viewing under UV light at 254 nm. The radioactive metabolites on TLC plates were detected by autoradiography using imaging plates (Fuji Photo Film, Tokyo, Japan), placed in contact with TLC plates for 1 h to 4 days at room temperature and then processed with a BAS2000 Bio-image Analyzer (Fuji Photo Film). R_f values for un-radiolabeled test compounds and authentic standards are shown in **Table 1**.

High-Performance Liquid Chromatography (HPLC). Instruments used in this study were as followss: pump, Hitachi L-6200 type intelligent pump (Hitachi, Tokyo, Japan); UV detector, Hitachi L-4000; RI detector, LB 507A HPLC radioactivity monitor (Perkin-Elmer Life Science); recorder, Millennium^{32} (Waters, Milford, MA).

An ODS column (YMC packed column, ODS-AM, 10 mm i.d. × 250 mm, YMC, Kyoto, Japan) was used for preparative isolation with a Guard-Pack holder and insert (Nova-Pack C18, Millipore Co.) as a guard.

Acetonitrile (0.2% formic acid) (MP 1), methanol (0.2% formic acid) (MP 2), and water (0.2% formic acid) (MP 3) were used for the mobile phase with the flow rate set as 1.5 mL/min for all solvent systems. Analysis systems [elution system and ratio (v/v) of mobile phase] were as follows: (a) isocratic elution, MP 1/MP 3 = 50:50; (b) linear gradient elution, MP 1/MP 3 = 20:80 (at 0 min), 100:0 (at 40 min); (c) isocratic elution, MP 2/MP 3 = 35:65; (d) isocratic elution, MP 1/MP 3 = 20:80; (e) linear gradient elution, MP 1/MP 3 = 60:40 (at 0 min), 85:15 (at 25 min), 100:0 (at 25.1−35 min); (f) linear gradient elution, MP 1/MP 3 = 20:80 (at 0 min), 60:40 (at 20 min), 100:0 (at 20.1−35 min); (g) isocratic elution, MP 2/MP 3 = 30:70; (h) isocratic elution, MP 1/MP 3 = 30:70; (i) linear gradient elution, MP 1/MP 3 = 20:80 (at 0 min), 60:40 (at 30 min), 100:0 (at 30.1−35 min); (j) isocratic elution, MP 2/MP 3 = 50:50; (k) linear gradient elution, MP 1/MP 3 = 20:80 (at 0 min), 60:40 (at 20 min), 100:0 (at 20.1−25 min); (l) isocratic elution, MP 1/MP 3 = 25:75; (m) isocratic elution, MP 2/MP 3 = 40:60; (n) isocratic elution, MP 2/MP 3 = 60:40.

Spectrometry. ^{1}H NMR, H−H COSY, and HMBC were determined with a Varian UNITY plus 400 NMR spectrometer (Varian Inc., Harbor City, CA) with CD_3OD and $CDCl_3$ as solvents. Chemical shifts are given in parts per million units relative to 0.00 in tetramethylsilane as an internal standard.

ESI-MS spectra were obtained with an LCQ DECA XP MASS spectrometer (Thermo Finnigan Corp., San Jose, CA).

Purification of Metabolites. Urine collected within 1 day after the final administration of ^{14}C-labeled dosing solution from each rat was subjected to the following treatment. All urine (~300 mL) was combined, lyophilized, and extracted three times with 200 mL of methanol. Each extract was combined and concentrated to dryness under a gentle stream of nitrogen gas. The residue was dissolved with 30 mL of water and extracted three times with the same volume of hexane using a 100 mL separation funnel. Each extract was combined and concentrated to dryness under a gentle stream of nitrogen gas (this fraction was designated fraction a). The water layer was then extracted three times with the same volume of ethyl acetate. Each extract was combined and concentrated to dryness under a gentle stream of nitrogen gas (this fraction was designated fraction b). The water layer was subjected to enzyme hydrolysis with 30 g of 0.2 M acetate buffer (pH 5.0) and 0.5 g of sulfatase [type H-1 (containing a β-glucuronidase activity), Sigma, St. Louis, MO]. The mixture was incubated for 19 h in a shaker at 37 °C and then extracted four times with 60 mL of ethyl acetate. Each extract was combined and concentrated to dryness under a gentle stream of nitrogen gas (this fraction was designated fraction c). The remaining water layer was adjusted to pH 2 with 36% HCl and extracted four times with 60 mL of ethyl acetate. Each extract was combined and concentrated to dryness under a gentle stream of nitrogen gas (this fraction was designated fraction d).

Preparative TLC. Fractions a, b, c, and d were applied to preparative TLC with solvent systems G, C, C, and D, respectively, and separated into three, four, four, and five fractions, respectively (designated a-1 to a-3, b-1 to b-4, c-1 to c-4, and d-1 to d-5). Each fraction was scraped from the TLC and eluted with methanol. Fractions a-2, a-3, b-1, b-3, b-4, c-1, d-1, d-4, and d-5 were not used for the following isolation.

Preparative HPLC. Fractions a-1, b-2, c-2, c-3, c-4, d-2, and d-3 were subjected to preparative HPLC to isolate metabolites.

a-1 was applied to HPLC using analysis system a. The main peak at t_R = 41−43 min (designated metabolite **3**) was collected and applied to spectroanalyses.

b-2 was applied to HPLC using analysis system b. The peak at t_R = 20−22 min (b-2-1) was collected and further applied to HPLC using analysis system c. The main peaks at t_R = 19−22 min (designated metabolite **8**) and 39−43 min (b-2-1-2) were collected, and the latter was further applied to HPLC using analysis system d. The main peak at t_R = 36 min (designated metabolite **7**) was collected. Metabolites **7** and **8** were applied to spectroanalyses.

c-2 was applied to HPLC using analysis system e. The peak at t_R = 15−16 min (4-OH-DFC) was collected, applied to spectroanalyses, and used for chemical reactions.

c-3 was applied to HPLC using analysis system f. The peak at t_R = 18−19 min (designated metabolite **4**) was collected and applied to spectroanalyses.

c-4 was applied to HPLC using analysis system b. The peaks at t_R = 16−17 min (c-4-1), 17−18 min (c-4-2), and 29−31 min (c-4-3) were collected. c-4-1 and c-4-2 were applied to HPLC using analysis system g, and the main peaks of each fraction at t_R = 20−22 min (designated metabolite **11**) and 36−37 min (designated metabolite **6**) were collected

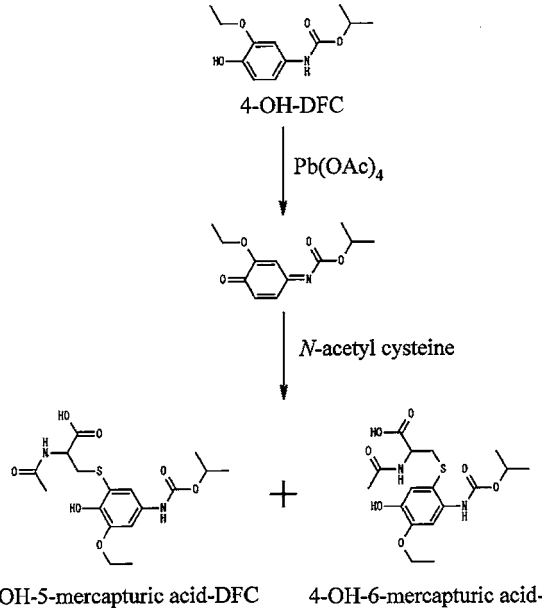

Figure 2. Chemical reactions; synthetic routes for metabolites **1** and **2**.

and applied to spectroanalyses. c-4-3 was further applied to HPLC using analysis system h. The main peak of the fraction at t_R = 78−80 min (designated metabolite **9**) was collected and applied to spectroanalyses.

d-2 was applied to HPLC using analysis system i. The peak at t_R = 29−31 min (d-2-1) was collected. d-2-1 was further applied to HPLC using analysis system j. The main peaks at t_R = 44−47 min (designated metabolite **1**) and 65−68 min (designated metabolite **2**) were collected and applied to spectroanalyses.

d-3 was applied to HPLC using analysis system k, and the peak at t_R = 16−20 min (d-3-1) was collected and further applied to HPLC using analysis system l. The main peaks at t_R = 15−17 min (d-3-1-1) and 17−18 min (d-3-1-2) were collected. d-3-1-1 was applied to HPLC using analysis system m, and the main peak at t_R = 19−22 min was collected (designated metabolite **5**). d-3-1-2 was applied to HPLC using analysis system m. The main peak at t_R = 20−23 min (d-3-1-2-1) was collected and further applied to HPLC using analysis system d. The main peak at t_R = 24−25 min (designated metabolite **10**) was collected. Metabolites **5** and **10** were applied to spectroanalyses.

Chemical Reactions: [*phenyl*-^{14}C]-4-OH-5-(*N*-acetylcysteine)-DFC and [*phenyl*-^{14}C]4-OH-6-(*N*-acetylcysteine)-DFC (*3*−*7*). Synthetic routes for these two compounds are shown in **Figure 2**. [*phenyl*-^{14}C]-4-OH-DFC (25 μmol) purified from urine (fraction c-2) was dissolved in 100 μL of dichloromethane (Kanto Chemical Co., Inc.) at room temperature, and then lead tetraacetate (27.5 μmol) in 100 μL of dichloromethane was added to the reaction mixture followed by stirring for 0.5 h. After the addition of *N*-acetylcysteine (50 μmol) in 100 μL of water, the reaction mixture was vigorously stirred for 3 min. The reaction mixture was concentrated under a gentle stream of nitrogen gas and subjected to preparative TLC with solvent system D. The resultant crude sample was purified by HPLC using analysis system n (retention times = 31 and 32 min) to obtain the compounds [*phenyl*-^{14}C]-4-OH-5-(*N*-acetylcysteine)-DFC (yield = 0.50 μmol) and [*phenyl*-^{14}C]-4-OH-6-(*N*-acetylcysteine)-DFC (yield = 0.25 μmol), respectively.

RESULTS

Identification of Metabolites. The following 11 metabolites were purified and identified by spectroanalyses (NMR and MS). The R_f values and chemical structures of the parent compound, the reference standard, and the identified metabolites are summarized in **Table 1** and **Figure 4**, respectively. The NMR and MS data of metabolites are given in **Tables 2** and **3**, along with those for the authentic standards, DFC and 4-OH-DFC, as basic data for identification of metabolites.

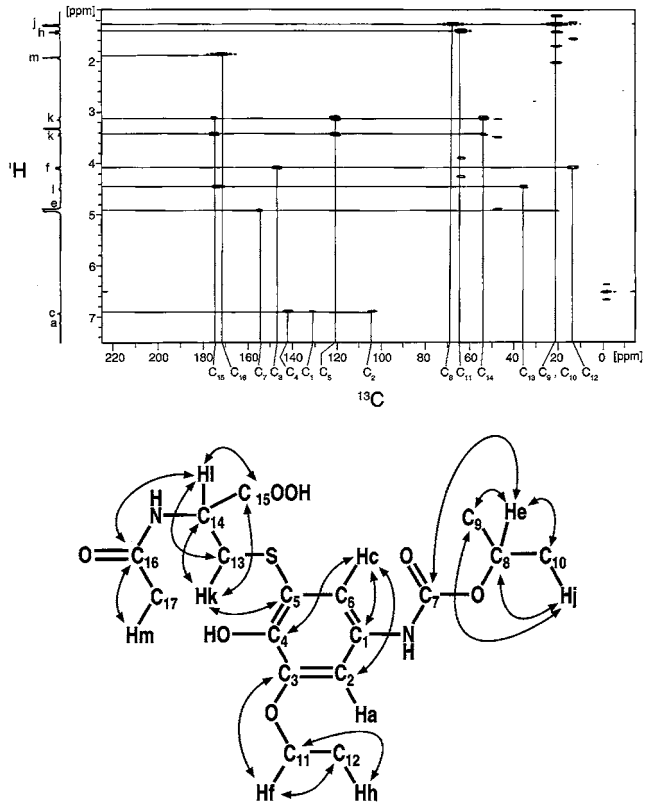

Figure 3. HMBC spectrum and chemical structure of metabolite **1**.

Metabolite 1. This metabolite was identified by TLC cochromatography with a ^{14}C-labeled authentic standard, [*phenyl*-^{14}C]-4-OH-5-(*N*-acetylcysteine)-DFC. Structural identification was made using the following criteria.

The presence of a mercapturic acid moiety was shown by the detection of characteristic aliphatic protons (H_k, 2H; H_l, 1H; H_m, 3H) in the ^{1}H NMR. By comparison with the spectrum of the authentic standard, 4-OH-DFC, it was shown that the two aromatic protons (H_a, 1H; H_c, 1H) were at the 2- and 6-positions. Therefore, it was clear that the phenyl proton of 4-OH-DFC at the 5-position (H_b, 1H) was replaced by the sulfur atom of mercaputuric acid. H−H COSY, HSQC, and HMBC (**Figure 3**) spectra supported this conclusion. The spectra showed the existence of an isopropyl group and the disappearance of the ethyl moiety of the 4-ethoxy group. The ESI-MS spectrum showed a $[M - H]^-$ ion peak at *m*/*z* 399, which implied the sum of the molecular weights of 4-OH-DFC (M239) and mercapturic acid (M163). On the basis of these results, **1** was considered to be a mercapturic acid conjugate of 4-OH-DFC and identified as 2-acetamino-3-{3-ethoxy-2-hydroxy-5-[(isopropoxycarbonyl)amino]phenylthio}propionic acid.

Metabolite 2. As with **1**, this metabolite was also identified by TLC cochromatography with the ^{14}C-labeled authentic standard, [*phenyl*-^{14}C]4-OH-6-(*N*-acetylcysteine)-DFC.

The ^{1}H NMR spectrum showed the addition of a mercapturic acid group (H_k, 2H; H_l, 1H; H_m, 3H) to the phenyl ring. All spectra were similar to those of **1** except for the phenyl proton signals, showing a difference in the position replaced by mercapturic acid. The ESI-MS spectrum showed a $[M - H]^-$ ion peak at *m*/*z* 399. On the basis of these results, **2** was considered to be a mercapturic acid conjugate of 4-OH-DFC and identified as 2-acetamino-3-{4-ethoxy-3-hydroxy-2-[(isopropoxycarbonyl)amino]phenylthio}propionic acid.

Metabolite 3. The ^{1}H NMR spectrum showed the existence of an isopropyl group and the disappearance of the ethyl moiety

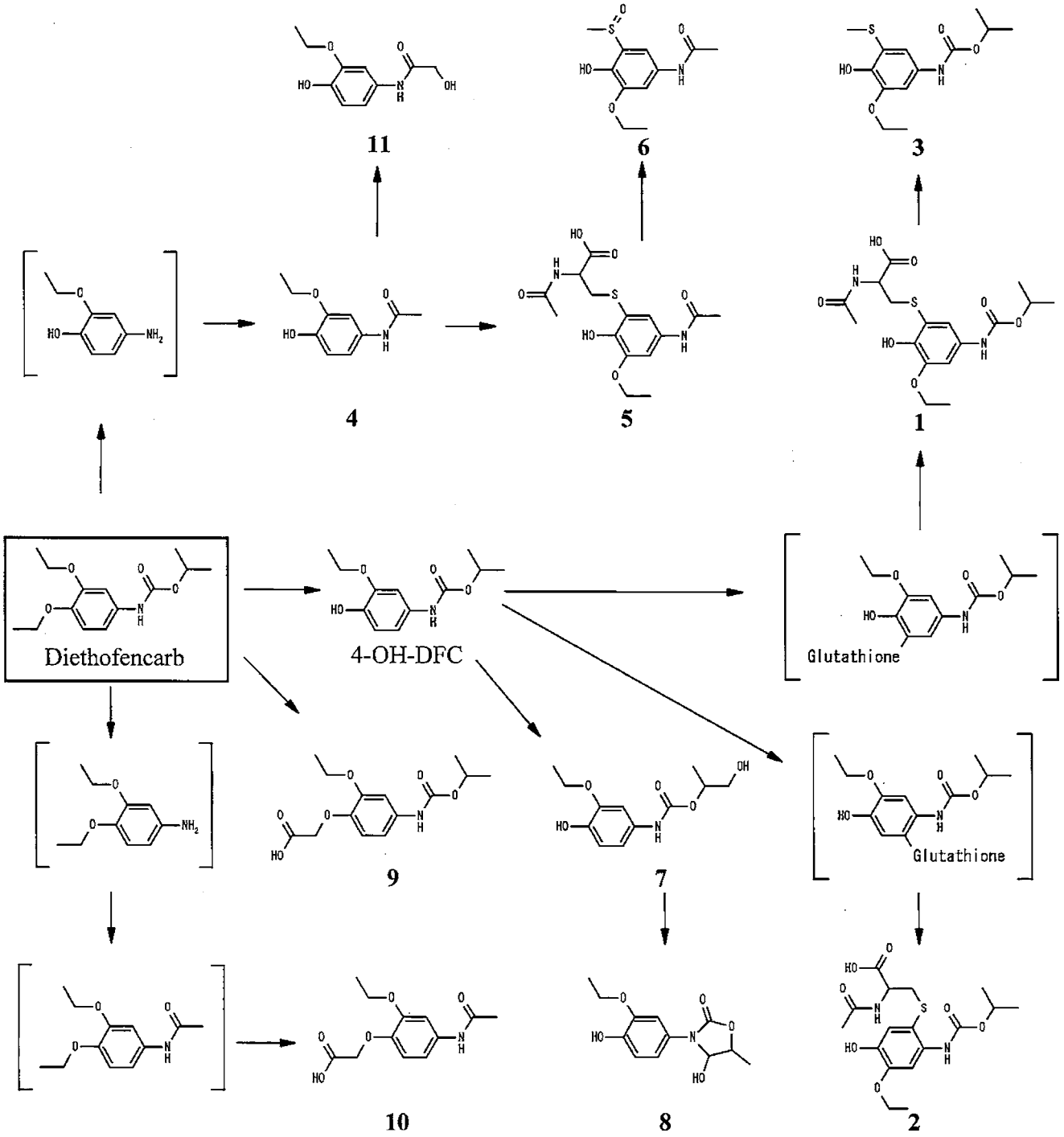

Figure 4. Proposed metabolic pathways for the identified metabolites of diethofencarb in rats.

of the 4-ethoxy group. Furthermore, one additional methyl proton signal (H_k, 3H) was observed, and one phenyl proton signal had disappeared. Judging from the chemical shift of H_k and the correlation between H_k and the phenyl carbon in the HMBC spectrum, it was considered that the additional methyl group was bonded with the sulfur atom and this *S*-methyl moiety was present in the phenyl ring. Two aromatic protons (H_a, 1H; H_c, 1H) showed replacement of the phenyl proton at the 5-position, compared with the coupling pattern and chemical shift of **1**. The ESI-MS spectrum showed a $[M - H]^-$ ion at *m*/*z* 284, which implied the sum of the molecular weights of 4-OH-DFC (M239) and the *S*-methyl moiety (M47). Therefore, **3** was identified as isopropyl 3-ethoxy-4- hydroxy-5-(methylthio)carbanilate.

Metabolite 4. This metabolite had already previously been identified in rat urine (*1*). The ^1H NMR spectrum showed the disappearance of the ethyl moiety of the 4-ethoxy group and the transformation by cleavage of the carbamate linkage followed by acetylation of the resultant amino group. The ESI-MS spectrum showed a $[M - H]^-$ ion peak at *m*/*z* 194. On the basis of these results, **4** was identified as *N*-(3-ethoxy-4-hydroxyphenyl)acetamide.

Metabolite 5. The ^1H NMR spectrum was similar to that of **1** except for signals for isopropyl protons, showing conjugation with mercapturic acid at the 5-position in the phenyl ring and transformation of the (isopropoxycarbonyl)amino group to an acetamino group, as with **4**. The ESI-MS spectrum showed a $[M - H]^-$ ion peak at *m*/*z* 355, which implied the sum of the

Table 2. Spectral Data of Diethofencarb, 4-OH-DFC, and Metabolites **1**–**6**

(A) ^{1}H NMR Data[a]

	diethofencarb		4-OH-DFC		**1**		**2**		3		**4**		**5**		**6**	
proton	δ ($CDCl_3$)	*J* (Hz)	δ (CD_3OD)	*J* (Hz)	δ (CD_3OD)	*J* (Hz)	δ (CD_3OD)	*J* (Hz)	δ (CD_3OD)	*J* (Hz)	δ (CD_3OD)	*J* (Hz)	δ (CD_3OD)	*J* (Hz	δ (CD_3OD)	*J* (Hz)
H_a	7.2 (br s)		7.1 (d)	2.4	7.1 (br s)		7.4 (br s)		7.3 (br s)		7.2 (d)	2.4	7.3 (d)	2.4	7.5 (d)	2.4
H_b	6.8 (d)	8.4	6.7 (d)	8.6	- - - - -		7.0 (s)		- - - - -		6.7 (d)	8.6	- - - - -		- - - - -	
H_c	6.7 (dd)	8.4, 2.4	6.8 (dd)	8.6, 2.4	6.9 (s)		- - - - -		6.9 (s)		6.8 (dd)	8.6, 2.4	7.0 (d)	2.4	7.2 (d)	2.4
H_d	6.5 (br s)		*****		*****		*****		*****		*****		*****		*****	
H_e	5.0 (m)		4.9 (m)		4.9 (m)		4.9 (m)		4.9 (m)		- - - - -		- - - - -		- - - - -	
H_f	4.1 (2H, q)	7.1	4.1 (2H, q)l	7.1	4.1 (2H, q)	7.1	4.1 (2H, q)	7.1	4.1 (2H, q)	7.1	4.1 (2H, q)	7.1	4.1 (2H, q)	7.1	4.1 (2H, q)	7.1
H_g	4.1 (2H, q)	7.1	- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -	
H_h	1.4 (3H, t)	7.1	1.4 (3H, t)	7.1	1.4 (3H, t)	7.1	1.4 (3H, t)	7.1	1.4 (3H, t)	7.1	1.4 (3H, t)	7.1	1.4 (3H, t)	7.1	1.4 (3H, t)	7.1
H_i	1.4 (3H, t)	7.1	- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -	
H_j	1.3 (6H, d)	6.4	1.3 (6H, d)	6.2	1.3 (6H, d)	6.2	1.3 (6H, d)	6.2	1.3 (6H, d)	6.4	- - - - -		- - - - -		- - - - -	
	- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -	
	- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -	
-*S*-MA																
H_j	- - - - -		- - - - -		4.5 (m)		4.4 (m)		- - - - -		- - - - -		4.5 (m)		- - - - -	
H_k	- - - - -		- - - - -		3.4 (dd)	13.9, 4.4	3.4 (m)		- - - - -		- - - - -		3.4 (dd)	13.8, 4.4	- - - - -	
H_k	- - - - -		- - - - -		3.1 (dd)	13.9, 8.4	3.0 (m)		- - - - -		- - - - -		3.1 (dd)	13.8, 8.4	- - - - -	
H_m	- - - - -		- - - - -		1.9 (3H, s)		1.9 (3H, s)		- - - - -		- - - - -		1.9 (3H, s)		- - - - -	
SCH_3	- - - - -		- - - - -		- - - - -		- - - - -		2.3 (3H, s)		- - - - -		- - - - -		- - - - -	
$SOCH_3$	- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		2.8 (3H, s)	
$NCOCH_3$	- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		2.1 (3H, s)		2.1 (3H, s)		2.1 (3H, s)	
phenyl-OH	- - - - -		*****		*****		8.4 (br s)		8.5 (s)		*****		*****		*****	
isopropyl-OH	- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -	
	- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -		- - - - -	

(B) MS Data

mode	diethofencarb	4-OH-DFC	**1**	**2**	**3**	**4**	**5**	**6**
ESI (positive)	*m/z* 268 ([M + H])	*m/z* 240 ([M + H])	- - - - -[b]	- - - - -	*m/z* 286 ($[M + H]^+$)	- - - - -	- - - - -	- - - - -
ESI (negative)	- - - - -	- - - - -	*m/z* 399 ([M − H])	*m/z* 399 ([M − H])	*m/z* 284 ([M − H])	*m/z* 194 ([M − H])	*m/z* 355 ([M − H])	*m/z* 256 ([M − H])

[a] s, singlet; br s, broad singlet; br d, broad doublet; d, doublet; dd, double-doublet; t, triplet; q, quartet; m, multiplet; *****, not observed; - - - - -, none; -*S*-MA, -*S*-mercapturic acid. [b] - - - - -, not analyzed.

Table 3. Spectral Data of Metabolites **7**–**11**

(A) ^{1}H NMR Data[a]

	7		**8** (*cis*)		**8** (*trans*)		**9**		**10**		**11**	
proton	δ ($CDCl_3$)	*J* (Hz)	δ (CD_3OD)	*J* (Hz)	δ (CD_3OD)	*J* (Hz)	δ (CD_3lOD)	*J* (Hz)	δ (CD_3OD)	*J* (Hz)	δ (CD_3OD)	*J* (Hz)
H_a	7.3 (br s)		7.1 (d)	2.4	7.1 (d)	2.3	7.2 (d)	2.4	7.3 (d)	2.4	7.3 (d)	2.4
H_b	6.8 (d)	8.4	6.8 (d)	8.6	6.8 (d)	8.6	6.8 (d)	8.6	6.9 (d)	8.6	6.7 (d)	8.6
H_c	6.6 (dd)	8.4, 2.4	6.9 (dd)	8.6, 2.4	6.9 (dd)	8.6, 2.3	6.8 (dd)	8.6, 2.4	7.0 (dd)	8.6, 2.4	6.9 (dd)	8.6, 2.4
H_d	6.5 (br s)		-----		-----		*****		*****		*****	
H_e	5.0 (m)		4.4 (dq)	6.6, 2.3	4.7 (dq)	6.6, 5.4	4.9 (m)		-----		-----	
H_f	4.1 (2H, q)	7.1	4.1 (2H, q)	7.1	4.1 (2H, q)	7.1	4.1 (2H, q)	7.1	4.1 (2H, q)	7.1	4.1 (2H, q)	7.1
H_g	-----		-----		-----		4.5 (2H, s)		4.6 (2H, s)		-----	
H_h	1.4 (3H, t)	7.1	1.4 (3H, t)	7.1	1.4 (3H, t)	7.1	1.4 (3H, t)	7.1	1.4 (3H, t)	7.1	1.4 (3H, t)	7.1
H_i	-----		-----		-----		-----		-----		-----	
H_j	3.8 (dd)	12.1, 3.2	5.2 (d)	2.3	5.5 (d)	2.3	1.3 (6H, d)	6.6	-----		-----	
	3.6 (dd)	12.1, 6l6	1.4 (3H, d)	6.6	1.4 (3H, d)	6.6						
	1.3 (3H, d)	6.4	-----		-----		-----		-----		-----	
-*S*-MA	-----		-----		-----		-----		-----		-----	
H_j	-----		-----		-----		-----		-----		-----	
H_k	-----		-----		-----		-----		-----		-----	
H_k	-----		-----		-----		-----		-----		-----	
H_m	-----		-----		-----		-----		-----		-----	
SCH_3	-----		-----		-----		-----		-----		-----	
$SOCH_3$	-----		-----		-----		-----		2.1 (3H, s)		4.1 (2H, s)	
$NCOCH_3$	5.5 (1H, br s)		8.5 (s)		8.5 (s)		-----		-----		*****	
phenyl-OH	2.9 (0.5H, dd)	7.6, 6.5	*****		*****		-----		-----		*****	
isopropyl-OH	2.7 (0.5H, dd)	8.2, 6.6	*****		*****		-----		-----		-----	

(B) MS Data

mode	**7**	**8** (cis)	**8** (trans)	**9**	**10**	**11**
ESI (positive)	-----[b]	-----	-----	-----	-----	-----
ESI (negative)	*m*/*z* 254 ([M – H])	*m*/*z* 252 ([M – H])	*m*/*z* 252 ([M – H])	*m*/*z* 296 ([M – H])	*m*/*z* 252 ([M – H])	*m*/*z* 210 ([M – H])

[a] s, singlet; br s, broad singlet; br d, broad doublet; d, doublet; dd, double-doublet; t, triplet; q, quartet; m, multiplet; *****, not observed; -----, none; -*S*-MA, -*S*-mercapturic acid. [b] -----, not analyzed.

molecular weights of **4** (M195) and mercapturic acid (M163). On the basis of these results, **5** was considered to be a mercapturic acid conjugate of **4** and identified as 2-acetamino-3-[5-acetamino-3-ethoxy-2-hydroxyphenylthio]propionic acid.

*Metabolite **6***. The ^{1}H NMR spectrum showed the disappearance of the ethyl moiety of the 4-ethoxy group and the transformation by cleavage of the carbamate linkage followed by acetylation of the resultant amino group as with **4**. Furthermore, in the ^{1}H NMR spectrum, one additional methyl proton signal at 2.8 ppm ($SOCH_3$) was observed and one phenyl proton signal (H_b, 1H) had disappeared. Two aromatic protons (H_a, 1H; H_c, 1H) showed replacement of the phenyl proton at the 5-position, compared with the coupling pattern and chemical shift of **5**. The ESI-MS spectrum showed a $[M - H]^-$ ion peak at *m*/*z* 256, which implied the sum of the molecular weightsof **4** (M195) and the $SOCH_3$ moiety (M73). On the basis of these results, it was considered that the additional methyl group was an incorporated sulfine group and the resultant methylsulfinyl group was incorporated at the 5-position of the phenyl ring. Therefore, **6** was identified as *N*-(3-ethoxy-4-hydroxy-5-methylsulfinylphenyl)acetamide.

*Metabolite **7***. The ^{1}H NMR spectrum showed the disappearance of the ethyl moiety of the 4-ethoxy group. Change of the signal for the methyl proton (H_j, 3H) to a low-field methylene proton indicated hydroxylation of one methyl group in the isopropyl moiety, and the chemical shift difference of hydroxymethylene protons was caused by an effect of the derived chiral carbon and amide system. The ESI-MS spectrum showed a $[M - H]^-$ ion peak at *m*/*z* 254. On the basis of these results, **7** was identified as 1-hydroxypropan-2-yl 3-ethoxy-4-hydroxycarbanilate.

*Metabolite **8***. The HPLC analysis and ^{1}H NMR spectrum showed this metabolite to be a mixture of two isomers in equilibrium (approximately 7:3 = *cis*-:*trans*-isomer). The ^{1}H NMR spectrum showed the disappearance of the ethyl moiety of the 4-ethoxy group and one methyl group in the isopropyl moiety and the existence of one methine proton (H_j, 1H). In the HMBC spectrum, a methyl proton (H_j, 3H) correlated with both methine carbons (*cis*, δ 88/C_9 and δ 78/C_8; and *trans*, δ 84/C_9 and δ 75/C_8). The ESI-MS spectrum showed a $[M - H]^-$ ion peak at *m*/*z* 252. On the basis of these results, it was considered that an (isopropoxycarbonyl)amino group had been transformed to the oxazolidine ring. Therefore, **8** was identified as 3-(3-ethoxy-4-hydroxyphenyl)-4-hydroxy-5-methyloxazolidin-2-one.

*Metabolite **9***. The ^{1}H NMR spectrum showed the disappearance of one methyl proton (H_h, 3H) at the 4-ethoxy group, indicating oxidation. Other signals were the same as for 4-OH-DFC. The ESI-MS spectrum showed a $[M - H]^-$ ion at *m*/*z* 296, and the ESI-MS/MS showed a fragment ion at *m*/*z* 238 attributed to $[M - CH_3COOH]^-$. On the basis of these results, **9** was identified as 2-{2-ethoxy-4-[(isopropoxycarbonyl)amino]-phenoxy}acetic acid.

*Metabolite **10***. The ^{1}H NMR spectrum showed the disappearance of a methyl proton, as with **9**, and transformation of an (isopropoxycarbonyl)amino group to an acetamino group, the same as **4**. The ESI-MS spectrum showed a $[M - H]^-$ ion at *m*/*z* 252. On the basis of these results, **10** was identified as (4-acetamino-2-ethoxyphenoxy) acetic acid.

*Metabolite **11***. The ^{1}H NMR spectrum showed the disappearance of the ethyl moiety of the 4-ethoxy group and transformation by cleavage of the carbamate linkage followed by acetylation of the resultant amino group as with **4**, and the methyl group at the acetyl moiety was oxidized to a hydroxyacetyl group. The ESI-MS spectrum showed a $[M - H]^-$ ion

at *m*/*z* 210. On the basis of these results, **11** was identified as *N*-(3-ethoxy-4-hydroxyphenyl)-2-hydroxyacetamide.

DISCUSSION

In the present study, diethofencarb was found to be widely metabolized in the rat body and 10 metabolites were identified in urine. Sulfate and/or glucuronide conjugates of 4-OH-DFC and sulfate and/or glucuronide conjugates of **4** were the major forms (*1*). S-Containing metabolites such as **1**−**3**, **5**, and **6** were newly identified.

In general, glutathione and cysteine conjugates of xenobiotics are not excreted in the urine and feces in mammals and are subjected to further metabolism prior to excretion. Cysteine conjugate is one of the metabolites of glutathione conjugate. Four metabolic pathways of cysteine conjugates have been reported: (1) *N*-acetylation reaction to give mercapturic acid conjugates (*8*−*10*); (2) cleavage reaction of the *C*−*S* bond to form thiol-containing metabolites, which are further converted to *S*-methyl conjugates, *S*-glucuronides, and sulfenic acids (*11*−*15*); (3) *S*-oxidation reactions to give the cysteine conjugate *S*-oxide (*16*); and (4) transamination reactions to yield 3-mercaptopyruvic acid conjugates (*17*, *18*), which are further converted to 3-mercaptolactic acid conjugates (*7*, *19*−*23*). On the basis of the present results, metabolic pathways of DFC in rats can be proposed (**Figure 4**).

In this study, five S-containing metabolites were detected; mercapturic acid conjugates exemplified by **1**, **2**, and **5** and *S*-methyl conjugates such as **3** and subsequent metabolites of **3** such as **6**. The existence of these S-containing metabolites demonstrated that diethofencarb is conjugated with glutathione and further metabolized to S-containing metabolites via cysteine conjugates.

ACKNOWLEDGMENT

We thank Kaeko Tanaka, Rie Omori, and Tomoko Tsuda for expert technical assistance.

LITERATURE CITED

(1) Shiba, K., et al. Metabolism of diethofencarb in rats. *J. Pestic. Sci.* **1990**, *15*, 395−403.

(2) Baker, F. C.; Kimmel, E. C.; Arndt, T. A *Metabolism Study with [phenyl-^{14}C]-Diethfencarb and [isopropyl-^{14}C]-Diethofencarb on Grapes*; Technical Report (PTRL Report 996W); PTRL West, Inc.: 2002.

(3) Potter, D. W.; Hinson, J. A. Mechanisms of Acetaminophen Oxidation to *N*-Acetyl-*P*-benzoquinone Imine by Horseradish Peroxidase and Cytochrome P-450. *J. Biol. Chem.* **1987**, *262*, 966−973.

(4) Potter, D. W.; Miller, D. W.; Hinson, J. A. Horseradish Peroxidase-Catalyzed Oxidation of Acetaminophen to Intermediates that Form Polymers or Conjugate with Glutathione. *Mol. Pharmacol.* **1985**, *29*, 155−162.

(5) Potter, D. W.; Hinson, J. A. The 1- and 2-Electron Oxidation of Acetaminophen Catalyzed by Prostaglandin *H* Synthase. *J. Biol. Chem.* **1987**, *262*, 974−980.

(6) Lau, S. S.; Hill, B. A.; Highet, R. J.; Monks, T. J. Sequential Oxidation and Glutathione Addition to 1,4-Benzoquinone: Correlation of Toxicity with Increased Glutathione Substitution. *Mol. Pharmacol.* **1988**, *34*, 829−836.

(7) Eckert, K.-G.; Eyer, P.; Sonnenbichler, J.; Zetl, I. Activation and detoxication of aminophenols. III. Synthesis and structural elucidation of various glutathione addtion products to 1,4-benzoquinone. *Xenobiotica* **1990**, *20*, 351−361.

(8) Bernauer, U.; Birner, G.; Dekant, W.; Henschler, D. Biotransformation of trichloroethene: dose-dependent excretion of 2,2,2-trichloro-metabolites and mercapturic acids in rats and humans after inhalation. *Arch. Toxicol.* **1996**, *70*, 338−346.

(9) Birner, G.; Vamvakas, S.; Dekant, W.; Henschler, D. Nephrotoxic and Genotoxic *N*-Acetyl-*S*-dichlorovinyl-L-cysteine Is a Urinary Metabolite after Occupational 1,1,2-Trichloroethene Exposure in Humans: Implications for the Risk of Trichloroethene Exposure. *Environ. Health Perspect.* **1993**, *99*, 281−284.

(10) James, S. P.; Needham, D. Some Metabolites of *S*-Pentyl-L-cysteine in the Rabbit and Other Species. *Xenobiotica* **1973**, *3*, 207−218.

(11) Commandeur, J. N. M., et al. Enzymes and transport systems involved in the formation and disposition of glutathione S-conjugates. *Pharmacol. Rev.* **1995**, *47*, 271−330.

(12) Tomisawa, H.; Ichihara, S.; Fukazawa, H.; Ichimoto, N.; Tateishi, M.; Yamamoto, I. Purification and characterization of human hepatic cysteine-conjugate β-lyase. *Biochem. J.* **1986**, *235*, 569−575.

(13) Tomisawa, H.; Fukazawa, H.; Ichihara, S.; Tateishi, M. A novel pathway for formation of thiol-containing metabolites from cysteine conjugates. *Biochem. Pharmacol.* **1986**, *35*, 2270−2272.

(14) Tomisawa, H.; Ichimoto, N.; Ichihara, S.; Fukazawa, H.; Tateishi, M. Involvement of alkane-thiols from corresponding cysteine conjugates. *Xenobiotica* **1988**, *18*, 1029−1037.

(15) Lappin, G. J.; Pritchard, D.; Moore, R. B.; Laird, W. J. D. Metabolism of 2,3,5,6-tetrachloronitrobenzene (tecnazene) in rat. *Xenobiotica* **1996**, *26*, 65−77.

(16) Tomisawa, H.; Hayashi, M.; Fukushima, M.; Iida, S.; Uda, F.; Hattori, K.; Tateishi, M. A novel pathway for formation of thiol metabolites and cysteine conjugate sulphoxides. *Biochem. Pharmacol.* **1993**, *46*, 1113−1117.

(17) Stevens, J. L.; Hatzinger, P. B.; Hayden, P. J. Quantitation of multiple pathways for the metabolism of nephrotoxic cysteine conjugates using selective inhibitors of L-α-hydroxy acid oxidase (L-amino acid oxidase) and cysteine conjugate β-lyase. *Drug Metab. Dispos.* **1989**, *17*, 297−303.

(18) Stevens, J. L.; Robbins, J. D.; Byrd, R. A. A Purified Cysteine Conjugate β-Lyase from Rat Kidney Cytosol. *J. Biol. Chem.* **1986**, *261*, 15529−15537.

(19) Kinuta, M.; Ubuka, T.; Yao, K.; Yamada, S.; Yukihiro, K.; Tomozawa, M. Preparation and characterization of *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]glutathione and its derivatives as proposed precursors of *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-cysteine, a compound found in human urine. *Biochim. Biophys. Acta* **1993**, *1157*, 192−198.

(20) Tomisawa, H.; Okamoto, A.; Hattori, K.; Ozawa, N.; Uda, F.; Tateishi, M. Purification and Characterization of 3-Mercaptopyruvic Acid S-Conjugate Reductases. *Biochem. Pharmacol.* **1990**, *40*, 2047−2057.

(21) Bloemen, L. J.; Monster, A. C.; Kezic, S.; Commandeur, J. N. M.; Veulemans, H.; Vermeulen, N. P. E.; Wilmer, J. W. Study on the cytochrome P-450- and glutathione-dependent biotransformation of trichloroethylene in humans. *Int. Arch. Occup. Environ. Health* **2001**, *74*, 102−108.

(22) Kinuta, M.; Ubuka, T.; Yao, W.-B.; Zhao, Y.-Q.; Shimizu, H. Isolation of *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-thiolactic acid, a new metabolite of histidine, from normal human urine and its formation from *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-cysteine. *Biochem. J.* **1994**, *297*, 475−478.

(23) Tomisawa, H.; Hayashi, M.; Uda, F.; Okamoto, A.; Hattori, K.; Ozawa, N.; Tateishi, M. Purification and characterization of 3-mercaptolactic acid *S*-conjugate oxidases. *Biochem. Pharmacol.* **1992**, *44*, 703−714.

Received for review July 15, 2003. Revised manuscript received November 18, 2003. Accepted December 4, 2003.

JF034779I