JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Metabolism of *N*-[(*R*)-1-(2,4-Dichlorophenyl)ethyl]-2-cyano-3,3-dimethylbutanamide (Delaus, S-2900) and Its Isomer, *N*-[(*S*)-1-(2,4-Dichlorophenyl)ethyl]-2-cyano-3,3-dimethylbutanamide (S-2900S), in Rats. 1. Identification of Metabolites in Feces and Urine

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Rats were orally dosed with a 1:1 diastereomixture of *N*-[(*R*)-1-(2,4-dichlorophenyl)ethyl]-2-cyano-3,3-dimethylbutanamide (Delaus, S-2900) and *N*-[(*S*)-1-(2,4-dichlorophenyl)ethyl]-2-cyano-3,3-dimethylbutanamide (S-2900S), both labeled with ^{14}C, at 200 mg/kg/day for 5 consecutive days, and 16 metabolites in urine and feces were purified by a combination of several chromatographic techniques. The chemical structures of all isolated metabolites were identified by spectroanalyses (NMR and MS). Several of them were unique decyanated and/or cyclic compounds (lactone, imide, cyclic amide, cyclic imino ether forms). Major biotransformation reactions of the mixture of S-2900 and S-2900S in rats are proposed on the basis of the metabolites identified in this study.

KEYWORDS: Metabolism; rat; fungicide; identification; biotransformation; decyanation; ring formation

INTRODUCTION

N-[(*R*)-1-(2,4-Dichlorophenyl)ethyl]-2-cyano-3,3-dimethylbutanamide (S-2900, Delaus) is a new fungicide for rice *Pyricularia griseaf* sp. *oryzae*. Its isomer, *N*-[(*S*)-1-(2,4-dichlorophenyl)-ethyl]-2-cyano-3,3-dimethylbutanamide (S-2900S), is also present at <5% in commercial grade preparations. The fungicidal activity of S-2900 is ∼4 times greater than that of S-2900S.

The metabolic fates of S-2900 and S-2900S in rats have been investigated along with toxicological studies conducted for safety evaluation. This publication describes the identification of fecal and urinary metabolites of a mixture of S-2900 and S-2900S from rats. Absorption, distribution, and excretion of ^{14}C and quantification of fecal and urinary metabolites are to be described elsewhere.

MATERIALS AND METHODS

Chemicals. The diastereomixture of S-2900 and S-2900S (1:1) (designated S-2558 hereafter) labeled uniformly in the phenyl group, [^{14}C]-S-2558, with a specific activity of 2.42 GBq/mmol, was synthesized in our laboratory (**Figure 2**). The labeled preparation was purified by preparative TLC development in benzene/ethyl acetate, 3:2 (v/v), prior to use. The radiochemical purity was >99% as confirmed by TLC analysis. Unlabeled S-2558 (99.9% purity) was synthesized by Sumitomo Chemical Co., Ltd. (Osaka, Japan). Unlabeled reference standard, *N*-[1-(2,4-dichlorophenyl)ethyl]-2-cyano-3-hydroxymethyl-3-methylbutanamide (tBuOH-S-2900, **Figure 2**), was also synthesized by Sumitomo Chemical Co., Ltd.

Animal Treatment. Charles River derived-CD (Sprague−Dawley) male and female rats at the age of 6 weeks old were purchased from Charles River Japan Inc. (Yokohama, 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. Water and pelleted diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) were provided ad libitum.

To collect sufficient amounts of urinary and fecal metabolites for spectroanalytical identification, a total of ∼2 g of [^{14}C]-S-2558 was dissolved in corn oil (50 mL) and dosed orally to eight male and four female rats for 5 consecutive days at 200 mg/kg/day. The specific activity of [^{14}C]-S-2558 was adjusted to 3.34 MBq/mmol by isotopic dilution with unlabeled S-2558.

For enzyme hydrolysis experiments, [^{14}C]-S-2558 was administered orally to one male and one female rat at 2 mg/kg. The specific activity of [^{14}C]-S-2558 was adjusted to 1.45 GBq/mmol.

The animals were housed in Metabolica CO_2 cages (Sugiyamagen Iriki Co., Ltd., Tokyo, Japan) to allow separate collection of urine and feces for 3 days after the last dosing.

Sample Processing. For metabolite purification experiments, all collected feces were mixed and homogenized with a 3-fold volume of a mixture of acetonitrile/water, 4:1 (v/v), using a Waring blender (Nihonseiki Co., Tokyo, Japan). The homogenates were centrifuged at 3000 rpm (∼1500*g*) for 10 min, followed by decanting to obtain the supernatant. Residues were further extracted twice with acetonitrile/water, 4:1 (v/v), in the same manner. The combined acetonitrile/water

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10.1021/jf0600509 CCC: $33.50
Published on Web 05/23/2006

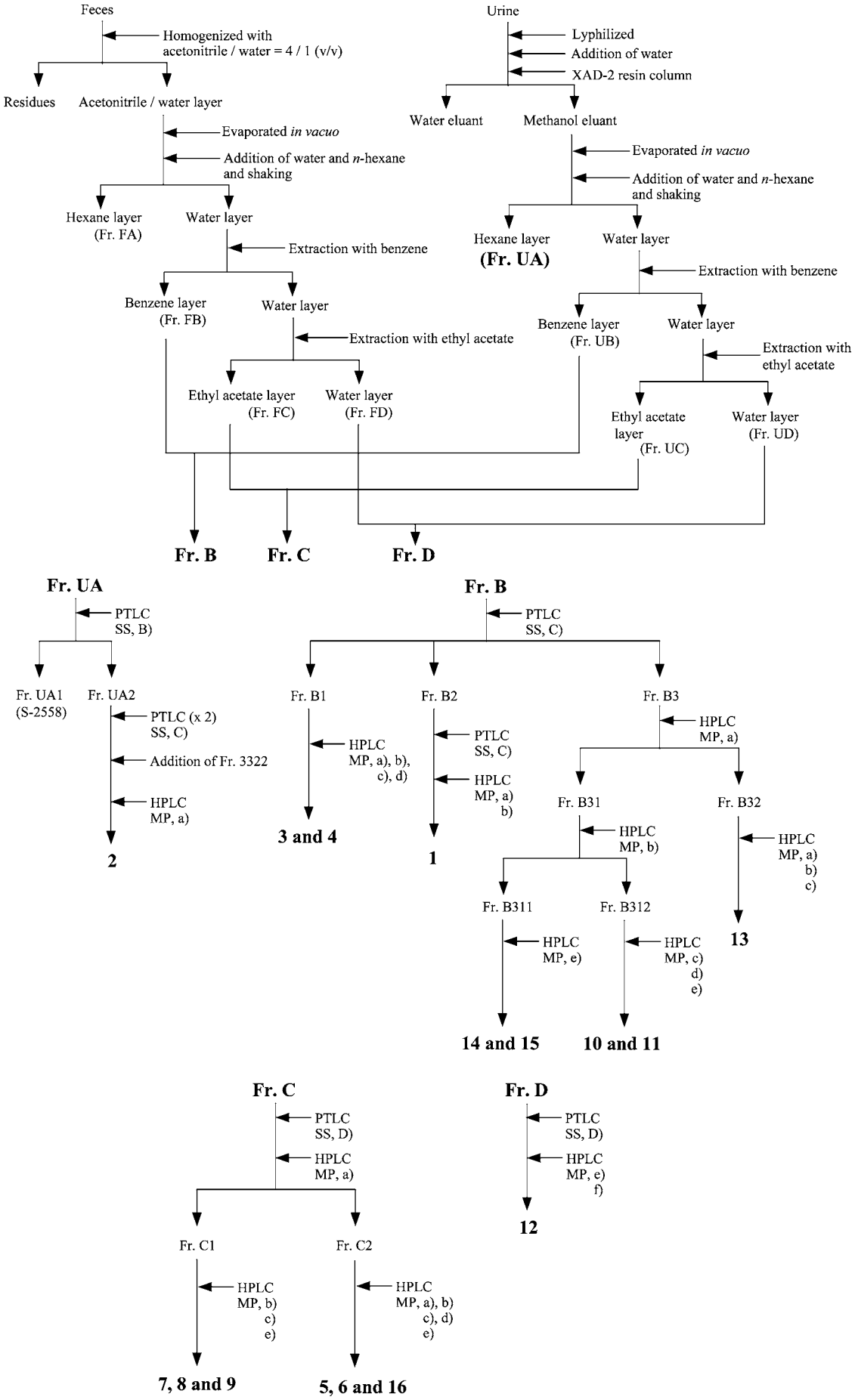

Figure 1. Flow diagram for steps in the purification of fecal and urinary metabolites. Abbreviations: SS, solvent system; MP, mobile phase.

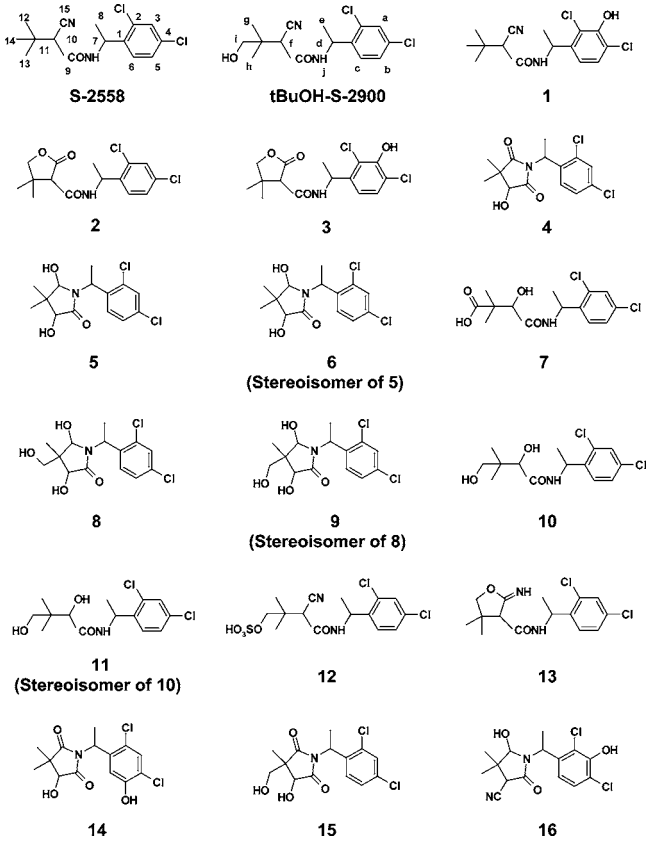

Figure 2. Chemical structures of S-2558, tBuOH-S-2900, and identified metabolites.

extract was concentrated and subjected to sequential chromatographic purification. Collected urine samples were combined and then lyophilized. After the addition of water (400 mL), the solution was subjected to chromatography.

Chromatography. Thin-layer chromatography (TLC) was conducted using precoated silica gel 60 F254 chromatoplates (article 5715, 20 × 20 cm, 0.25 mm thickness, and article 5744, 20 × 20 cm, 0.50 mm thickness, E. Merck, Darmstadt, Germany). The following solvent systems were used: (A) benzene/ethyl acetate (3:2, v/v); (B) benzene/ethyl acetate (2:3, v/v); (C) chloroform/methanol (9:1, v/v); and (D) ethyl acetate/acetone/water/acetic acid (10:1:1:1, v/v). Unlabeled standards on TLC plates were assessed by viewing under UV light (254 nm). The radioactive spots on TLC plates were detected by autoradiography with imaging plates (Fuji Photo Film Co. Ltd., Tokyo, Japan), which were then used to expose TLC plates for 1 day at room temperature, followed by processing with a BAS2000 Bio-image Analyzer (Fuji Photo Film Co. Ltd.).

High-performance liquid chromatography (HPLC) was carried out according to the methods reported previously (*1*) using the following systems: L-6200 HPLC Intelligent Pump (Hitachi Ltd., Tokyo, Japan), L-4000 UV detector (Hitachi Ltd.), and LB 507A radioactivity monitor (Berthold, Bad Wildbad, Germany). A YMC-Pack ODS-AM column (ODS, 10 mm i.d. × 300 mm, 5 μm, YMC Co., Ltd., Yawata, Japan) and a Guard-Pack Holder and Insert (Nova-Pack C18, Millipore Co., Tokyo, Japan) were employed as the analytical column and the guard column, respectively. The flow rate was 2 mL/min. The isocratic solvent systems used were as follows: (a) acetonitrile/water = 55:45; (b) acetonitrile/water = 50:50; (c) acetonitrile/water = 45:55; (d) acetonitrile/water = 40:60; (e) acetonitrile/water = 35:65; and (f) acetonitrile/water = 30:70.

Spectrometry. Nuclear magnetic resonance (NMR) spectra were obtained on a JEOL GSX-270 spectrometer (JEOL Ltd., Tokyo, Japan) operating at 270 MHz for ^{1}H and at 67.5 MHz for ^{13}C. The measurements were carried out at room temperature. Two-dimensional spectra [H−H correlation spectroscopy (H−H COSY) (*2*), C−H correlation spectroscopy (C−H COSY) (*3*), heteronuclear multiple quantum coherence spectroscopy (HMQC) (*4*), and ^{1}H detected heteronuclear multiple bond connectivity (HMBC) (*5*, *6*)] were obtained with the data process program PLEXUS V 1.6 (JEOL) on a JEOL GSX-270 spectrometer. Chemical shifts are given in parts per million units relative to 0.00 in tetramethylsilane as an internal standard. Methanol-d_4 (99.5%, E. Merck) and chloroform-*d* (99.5%, E. Merck) were used as solvents for NMR.

Fast atom bombardment mass spectrometry (FAB-MS) (*7*, *8*) was performed with a JEOL AX505 mass spectrometer (JEOL Ltd.). Samples were introduced in a glycerol or a thioglycerol matrix. Electrospray ionization mass spectrometry (ESI-MS) (*9*) was performed with a Finnigan TSQ700 mass spectrometer (Finnigan Mat Instrument Inc., San Jose, CA) fitted with a Hitachi L-6200 HPLC Intelligent Pump. Samples were introduced by the HPLC pump (mobile phase, methanol/water = 1/1 (v/v); flow rate = 0.6 mL/min). Atmosphere chemical ionization mass spectrometry (APCI-MS) (*10*) was performed with a Hitachi M1000 LC API mass spectrometer (Hitachi Ltd.) fitted with an HPLC pump (mobile phase, methanol or acetonitrile/0.1% formic acid = 75:25 (v/v); flow rate = 1 mL/min.). Spectra were recorded in positive or negative ion mode.

Purification of Metabolites. A flow diagram for the procedures applied is given in **Figure 1**.

The fecal acetonitrile/water extracts were concentrated and partitioned three times between *n*-hexane and water to remove the parent compound and nonpolar fecal natural products (*n*-hexane extracts, fraction FA). In addition, the aqueous solution was further separated into three portions by solvent extraction using benzene and ethyl acetate (benzene extract, fraction FB; ethyl acetate extract, fraction FC; and residual aqueous layer, fraction FD).

The urine sample was chromatographed on Amberlite XAD-2 resin (Organo, Tokyo, Japan), washed with water (2000 mL), and eluted with methanol (2000 mL). The methanol eluates were concentrated, and the residue was separated into four portions by solvent extraction (*n*-hexane extract, fraction UA; benzene extract, fraction UB; ethyl acetate extract, fraction UC; and residual aqueous layer, fraction UD).

Because the metabolites in urine were similar to those in feces, fractions FB and UB (designated fraction B), fractions FC and UC (fraction C), and fractions FD and UD (fraction D), respectively, were combined.

Further purification procedures were not conducted for fraction FA because it contained mainly parent compounds.

Fraction UA was separated into two portions by preparative TLC using solvent system B (fractions UA1 and UA2). Metabolite **2** was detected in fraction UA2 and purified further by preparative TLC using solvent system C and then further by HPLC (mobile phase a) after pretreatment with a Sep-Pak C18 cartridge (Waters, Milford, MA).

Fraction B was separated into three portions (fractions B1−B3). Metabolites **3** and **4** were purified from fraction B1 by several HPLC procedures (using mobile phases a−d). Metabolite **1** was purified from fraction B2 by HPLC procedures (using mobiles phases a and b). Fraction B3 was separated into two portions by HPLC (mobile phase a, fractions B31 and B32). Metabolite **13** was purified from fraction B32 by HPLC using mobile phases a−c. Metabolites **10**, **11**, **14**, and **15** were purified from fraction B31 according to the same method (using mobile phases b−e).

Fraction C was separated into two portions by preparative TLC (solvent system D) and HPLC (mobile phase a) (fractions C1 and C2). Metabolites **7**−**9** were purified from fraction C1 by using several HPLC procedures (mobile phases b, c, and e). Metabolites **5**, **6**, and **16** were purified from fraction C2 according to the same method (mobile phases a−e).

Fraction D was purified by preparative TLC (solvent system D) and further by HPLC (mobile phases e and f) to obtain metabolite **12**.

Chemical Reactions. *Acetylation of the Purified Metabolite 5.* An acetate derivative of metabolite **5** (**5Ac**) was synthesized in a manner similar to that reported by Shriner et al. (*11*) (**Figure 6A**). Metabolite **5** (5 mg, 0.016 mmol) was dissolved in 0.5 mL of acetic anhydride (Kanto Chemical Co., Inc., Tokyo, Japan) at 0 °C, and then 0.5 mL of pyridine (Kanto Chemical Co., Inc.) was added to the reaction mixture followed by stirring for 16 h at room temperature. After the addition of ice water (10 g), the reaction mixture was extracted three times with

Table 1. TLC R_f Values for S-2558, tBuOH-S-2900, and Identified Metabolites

metabolite	compound	R_f in solvent system[a] A	B
	S-2558	0.78	*b*
	tBuOH-S-2900	0.63	0.94
1	PhOH-S-2900	0.61	*b*
2	S-2900-lactone	0.84	*b*
3	PhOH-S-2900-lactone	0.63	0.96
4	αOH-S-2900-imide	0.70	*b*
5	αOH-S-2900-amido-alc.A	0.34	0.84
6	αOH-S-2900-amido-alc.B	0.50	0.9
		0.44	
7	tBuCOOH-αOH-S-2900	0.08	0.87
8	tBuOH-αOH-S-2900-amido-alc.A	0.24	0.70
		0.20	
9	tBuOH-αOH-S-2900-amido-alc.B	0.21	0.62
10	tBuOH-αOH-S-2900A	0.40	0.89
11	tBuOH-αOH-S-2900B	0.45	0.86
12	tBuOH-S-2900-sulfate	0.02	0.33
			0.30
13	S-2900-imino-ether	0.43	0.94
14	5-PhOH-αOH-S-2900-imide	0.38	0.82
15	tBuOH-αOH-S-2900-imide	0.33	0.77
16	PhOH-S-2900-amido-alc	0.39	0.84

[a] Solvent systems: A, chloroform/methanol, 9:1 (v/v); B, ethyl acetate/acetone/water/acetic acid, 10:1:1:1, (v/v). [b] Solvent front.

Table 2. HPLC t_R Values for S-2558 and Purified Metabolites

metabolite	t_R (min) in mobile phase[a] a	b	c	d	e	f
S-2558	32.8	–	–	–	–	–
	33.7	–	–	–	–	–
1	15.9	17.9	–	–	–	–
	16.8	19.1	–	–	–	–
2	24.0	–	–	–	–	–
3	13.2	14.8	18.7	25.8	–	–
	13.9	15.8	–	–	–	–
4	20.2	–	–	–	–	–
	20.9	–	–	–	–	–
5	11.9	14.4	16.1	21.1	27.0	–
6	11.9	15.1	17.1	22.8	–	–
7	11.0	12.0	–	–	–	
8	7.9	8.6	9.6	–	14.0	–
	8.4	9.5	10.8	–	17.8	–
9	8.1	8.9	–	–	16.3	–
10	15.1	18.4	22.8	31.0	54.0	–
11	15.1	18.6	23.0	31.6	54.8	–
12	–	–	–	–	–	14.8
13	18.0	23.9	31.0	–	–	–
	–	–	31.8	–	–	–
14	–	17.1	20.4	27.0	45.0	–
15	–	17.1	21.4	28.1	48.2	–
16	10.6	–	–	19.7	24.2	–
	11.4	–	–	–	–	–

[a] Mobile phases: see Materials and Methods. –, not analyzed.

ethyl acetate (20 mL). The extracts were concentrated in vacuo and subjected to preparative TLC in chloroform/methanol = 15:1 (v/v) to obtain the compound, **5Ac**. **5Ac** was further purified by HPLC [yield = 4 mg (0.010 mmol); column, see Materials and Methods; mobile phase, acetonitrile/water = 77:23(v/v); flow rate = 2 mL/min; retention time = 13.2 min].

Sulfation of the Reference Standard, tBuOH-S-2900. The sulfate conjugate of tBuOH-S-2900 was synthesized as reported by Matsuda et al. (*12*) and Chelebicki et al. (*13*) (**Figure 6B**). The reference standard, tBuOH-S-2900 (15 mg, 0.046 mmol), was dissolved in a mixture of ethyl acetate (0.5 mL) and CCl_4 (10 mL). Chlorosulfuric acid ($ClSO_3H$, 0.5 g, 4.3 mmol; Kanto Chemical Co., Inc.) was added slowly to the solution at 0 °C followed by stirring for 16 h at 0 °C. The reaction mixture was neutralized with 0.5 N NaOH and the solvent evaporated in vacuo. The residue was dissolved in water and purified by preparative TLC (solvent system D) and HPLC [column, see Materials and Methods; mobile phase, acetonitrile/water = 30:70 (v/v); flow rate = 2 mL/min; retention time = 14.5 min] to obtain a sulfate conjugate (10 mg, 0.025 mmol).

Enzyme Hydrolysis. For enzyme hydrolysis, urine and feces from each rat were collected for 3 days, and feces were extracted three times by acetonitrile/water, 4:1 (v/v). The extracts and the urine samples of one male and one female were subjected to preparative TLC using solvent system D. The polar portions of each sample (R_f < 0.5) were obtained by preparative TLC and then extracted from the silica gel with methanol. The methanol extracts were concentrated in vacuo, and the residues were treated with β-glucuronidase (bovine liver, type H-1, Sigma, St. Louis, MO) or aryl sulfatase (limpets, type VII, Sigma) in 0.1 M acetate buffer (pH 5.0) or 0.1 M phosphate buffer (pH 7.4) at 37 °C overnight. The released aglycones were analyzed by TLC. Metabolite **12** was also subjected to enzyme hydrolysis using sulfatase in a similar manner.

RESULTS

Identification of Metabolites. The following 16 metabolites were purified and identified by spectroanalyses (NMR and MS). The R_f values, the HPLC t_R values, and chemical structures of the parent compound, the reference standard, and the identified metabolites are summarized in **Tables 1** and **2** and **Figure 2**, respectively. The NMR and MS data of the parent compound, the reference standard, and the identified metabolites are summarized in **Tables 3**−**5**. For the parent compound (S-2558) and some of its metabolites, two chemical shifts are listed for some protons, because the parent compound and some metabolites were obtained as diastereomixtures.

*Metabolite **1**.* The ^1H NMR spectrum of **1** was similar to that of the parent compound, except that one of the three aromatic proton signals at 7.51−7.35 ppm in the parent compound was not detected in **1**. This indicated replacement of one proton of the 2,4-dichlorophenyl group in the parent compound with a functional group. Positive (pos) or negative (neg) ion mode FAB-MS and APCI-MS gave protonated and deprotonated molecular ion peaks at *m*/*z* 329 $(M + H)^+$ and 327 $(M - H)^-$. The molecular weight (328) was 16 mass units larger than that of the parent compound (312), indicating a hydroxyl group substitution. From interpretation of the H−H COSY, HMQC, and HMBC spectra, the hydroxyl group was assigned to the C3 position. Consequently, **1** was identified as *N*-[1-(2,4-dichloro-3-hydroxyphenyl)ethyl]-2-cyano-3,3-dimethylbutanamide (PhOH-S-2900).

*Metabolite **2**.* The HMBC spectrum for **2** is shown in **Figure 3**. In the ^1H NMR spectrum in CD_3OD, the signals for one methyl group of the *tert*-butyl group and the cyanomethyne proton signal were absent, and new AB quartet signals of a methylene (4.04 and 3.05 ppm, each 1H, d, J = 8.6 Hz) were observed. However, in $CDCl_3$, the cyanomethyne signal was observed. In the ^{13}C NMR spectrum, the signal of the cyano group (C15) in the parent compound was absent in **2**, and one additional carbonyl signal was observed instead. Therefore, it was concluded that one methyl group of the *tert*-butyl group was hydroxylated and the cyano group was oxidized to the carboxylic acid. However, positive or negative ion mode FAB-MS and APCI-MS gave protonated and deprotonated molecular ion peaks at *m*/*z* 330 $(M + H)^+$ and 328 $(M - H)^-$, respectively, which are 18 mass units smaller than those of the postulated compound. In the HMBC spectrum, formation of the lactone ring in this metabolite was confirmed by finding of a cross-peak (the long-range coupling) between the methylene protons (Hi) and the carbonyl carbon (C15) (**Figure 3**). Consequently,

Table 3. ^{1}H and ^{13}C NMR and MS Data for S-2558, tBuOH-S-2900, and Metabolites **1**–**4**

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

	S-2558				tBuOH-S-2900		**1**		**2**				**3**		**4**	
proton	δ (CD_3OD)	J (Hz)	δ ($CDCl_3$)	J (Hz)	δ ($CDCl_3$)	J (Hz)	δ (CD_3OD)	J (Hz)	δ (CD_3OD)	J (Hz)	δ ($CDCl_3$)	J (Hz)	δ ($CDCl_3$)	J (Hz)	δ (CD_3OD)	J (Hz)
Ha	7.51–7.35 (m)		7.40–7.25 (m)		7.42–7.20 (m)		*b*		7.49–7.35 (m)		7.37–7.20 (m)		*b*		7.48 (s)	
Hb	7.51–7.35 (m)		7.40–7.25 (m)		7.42–7.20 (m)		7.32 or 7.30 (d)	8.6	7.49–7.35 (m)		7.37–7.20 (m)		7.23 (d)	8.6	7.40 (d)	9.3
Hc	7.51–7.35 (m)		7.40–7.25 (m)		7.42–7.20 (m)		6.94 or 6.92 (d)	8.6	7.49–7.35 (m)		7.37–7.20 (m)		6.87 or 6.84 (d)	8.6	7.51 (d)	9.3
Hd	5.40 or 5.32 (q)	7.0	5.33 (m)		5.38 (m)		5.39 or 5.32 (q)	6.6	5.35 (q)	6.6	5.37 (q)	6.6	5.36 (q)	6.6	5.58 (q)	6.6
He	1.51 or 1.49 (3H, d)	7.0	1.53 (3H, m)		1.53 (3H, m)		1.49 or 1.47 (3H, d)	6.6	1.50 (3H, d)	6.6	1.49 or 1.47 (3H, d)	6.6	1.49 or 1.47 (3H, d)	6.6	1.73 (3H, d)	6.6
Hf	3.44 or 3.42 (s)		3.17 or 3.14 (s)		3.55 (s)		3.45 or 3.43 (s)		*c*		3.09 or 3.05 (s)		3.10 or 3.06 (s)		4.29 or 4.24 (s)	
Hg	1.20 or 1.12 (3H, s)		1.18 or 1.14 (3H, s)		1.08 (3H, s)		1.20 or 1.12 (3H, s)		1.18 or 1.05 (3H, s)		1.16 or 1.03 (3H, s)		1.16 or 1.04 (3H, s)		1.14 (3H, s)	
Hh	1.20 or 1.12 (3H, s)		1.18 or 1.14 (3H, s)		1.22 (3H, s)		1.20 or 1.12 (3H, s)		1.20 or 1.12 (3H, s)		1.42 (3H, s)		1.43 (3H, s)		1.29 (3H, s)	
Hi	1.20 or 1.12 (3H, s)		1.18 or 1.14 (3H, s)		3.30–3.60 (2H, m)		1.20 or 1.12 (3H, s)		4.22 or 4.17 (1H, d)	8.6	4.04 (1H, d)	8.6	4.04 (1H, d)	8.6	*b*	
									4.05 or 4.02 (1H, d)	8.6	3.95 (1H, d)	8.6	3.95 (1H, d)	8.6		
Hj	*c*		6.40 (br s)		6.71 (br s)		*c*		*c*		8.07 (br s)		8.06 (br s)		*b*	

(B) ^{13}C NMR Data

	S-2558		tBuOH-S-2900	**1**	**2**		**3**	**4**
carbon	δ (CD_3OD)	δ ($CDCl_3$)	δ ($CDCl_3$)	δ (CD_3OD)	δ (CD_3OD)	δ ($CDCl_3$)	δ ($CDCl_3$)	δ (CD_3OD)
15	118.1	118.1	118.0	118.1	176.5	176.9	175.9	*b*
14	28.0	28.0	69.6	28.0	80.1	78.6	78.6	181.9
13	28.0	28.0	23.0	28.0	27.0	24.7	24.7	22.3
12	28.0	28.0	22.3	28.0	21.1	21.1	21.2	20.3
11	35.7	35.7	40.4	35.6	41.6	41.0	41.0	46.3
10	49.8	49.8	45.2	50.1	57.0	54.1	54.1	76.3
9	166.0	166.0	165.9	165.9	168.0	163.8	163.9	177.9
8	20.7	20.7	20.7	20.7	20.9	20.7	20.7	16.8
7	47.8	47.8	49.3	48.1	47.6	46.4	46.6	49.2
6	128.6	128.6	130.4	118.9	130.4	129.8	118.2	132.0
5	129.0	129.0	128.9	129.0	129.0	127.7	127.9	128.0
4	129.6	129.6	128.7	150.8	129.5	127.5	148.0	135.3
3	130.4	130.4	129.5	118.2	128.6	127.4	117.9	130.1
2	134.7	134.7	134.5	122.3	134.5	133.4	119.8	137.0
1	140.7	140.7	141.1	141.8	141.0	139.2	140.8	136.2

(C) MS Data

	m/z					
mode	S-2558	tBuOH-S-2900	**1**	**2**	**3**	**4**
FAB(pos)	313 $(M+H)^+$	329 $(M+H)^+$	329 $(M+H)^+$, 351 $(M+Na)^+$	330 $(M+H)^+$	346 $(M+H)^+$, 368 $(M+Na)^+$	316 $(M+H)^+$
FAB(neg)	311 $(M-H)^-$	327 $(M-H)^-$	327 $(M-H)^-$	328 $(M-H)^-$	*d*	*d*
ESI(pos)	313 $(M+H)^+$	*d*	*d*	*d*	*d*	316 $(M+H)^+$
APCI(pos)	*d*	*d*	329 $(M+H)^+$	330 $(M+H)^+$	*d*	316 $(M+H)^+$
APCI(neg)	*d*	327 $(M-H)^-$	327 $(M-H)^-$	329 $(M-H)^-$	*d*	*d*

[a] s, singlet; br s, broad singlet; br d, broad doublet; d, doublet; dd, double-doublet. [b] None. [c] Not observed. [d] Not analyzed.

Table 4. ^{1}H and ^{13}C NMR and MS Data for Metabolites **5**, **5Ac**, and **6**–**9**

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

	5		**5Ac**		**6**		**7**		**8** (isomer I)		**8** (isomer II)		**9**	
proton	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)
Ha	7.56 (s)		7.44 (s)		7.53 or 7.46 (s)		7.45 (s)		7.47 (s)		7.52 (s)		7.57 (s)	
Hb	7.46 (d)	8.6	7.35–7.28 (m)		7.44 or 7.34 (d)	8.6	7.35 (d)	8.6	7.35 (d)	8.6	7.42 (d)	8.6	7.46 (d)	8.6
Hc	7.68 (d)	8.6	7.53 (d)		7.78 or 7.69 (d)	8.6	7.47 (d)	8.6	7.79 (d)	8.6	7.68 (d)	8.6	7.68 (d)	8.6
Hd	5.61 (q)	6.6	5.57 (q)		5.37 or 5.32 (q)	6.6	5.35 (q)	6.6	5.30 (q)	6.6	5.38 (q)	6.6	5.61 (q)	6.6
He	1.67 (3H, d)	6.6	1.66 (3H, d)		1.72 or 1.67 (3H, d)	6.6	1.46 (3H, d)	6.6	1.68 (3H, d)	6.6	1.72 (3H, d)	6.6	1.67 (3H, d)	6.6
Hf	4.22 (s)		5.51 (s)		4.21 or 3.78 (s)		4.22 (s)		4.36 (s)		4.19 (s)		4.36 (s)	
Hg	0.79 (3H, s)		0.87 (3H, s)		0.93 (3H, s)		1.15 (3H, s)		0.98 (3H, s)		0.88 (3H, s)		0.84 (s)	
Hh	1.11 (3H, s)		1.10 (3H, s)		1.19 or 1.01 (3H, s)		1.20 (3H, s)		3.85 (1H, d)	11.1	3.39 (2H, s)		3.78 (1H, d)	11.3
									3.66 (1H, d)	11.1			3.58 (1H, d)	11.3
Hi	4.04 (1H, s)		5.46 or 4.10 (1H, s)		4.76 or 4.19 (1H, s)		*b*		4.95 (1H, s)		4.56 (1H, s)		4.22 (1H, s)	
Hj	*b*		*b*		*b*		*c*		*b*		*b*		*b*	
CH_3CO^-	*b*		2.20 (3H, s)		*b*		*b*		*b*		*b*		*b*	
CH_3CO^-	*b*		2.09 (3H, s)		*b*		*b*		*b*		*b*		*b*	

(B) ^{13}C NMR Data

	5	**5Ac**	**6**	**7**	**8** (isomer I)	**8** (isomer II)	**9**
carbon	δ (CD_3OD)	δ (CD_3OD)	δ (CD_3OD)	δ (CD_3OD)	δ (CD_3OD)	δ (CD_3OD)	δ (CD_3OD)
15	*b*	*b*	*b*	*b*	*b*	*b*	*b*
14	87.9	87.3 or 86.6	88.5	184.8	87.7	83.4	86.9
13	20.7	20.9	24.1 or 20.4	22.0	65.2	64.4	65.1
12	20.4	20.7	20.7 or 15.6	22.0	15.4	11.2	15.2
11	43.7	42.1	44.2	49.6	48.7	48.7	48.7
10	76.9	75.9 or 75.5	78.1 or 76.7	78.4	72.8	72.3	73.1
9	175.8	170.4	176.7 or 175.3	174.6	176.5	175.3	175.6
8	18.5	18.3 or 17.6	17.7	21.8	17.7	19.3	18.5
7	48.4	48.1	50.7	47.1	50.9	49.6	49.5
6	131.5	129.9	131.0	129.3	131.9	131.1	131.5
5	128.2	127.3	128.3	128.7	128.3	128.3	128.2
4	130.6	134.5	135.3	129.5	135.2	134.7	129.9
3	130.6	129.6	130.1	130.1	130.3	129.9	130.6
2	135.7	135.0	134.7	134.2	136.2	136.2	135.7
1	136.4	135.2	139.5 or 136.6	141.7	139.5	136.6	136.4
CH_3CO^-	*b*	170.3	*b*	*b*	*b*	*b*	*b*
CH_3CO^-	*b*	170.0	*b*	*b*	*b*	*b*	*b*
CH_3CO^-	*b*	20.0	*b*	*b*	*b*	*b*	*b*
CH_3CO^-	*b*	20.0	*b*	*b*	*b*	*b*	*b*

(C) MS Data

	m/z						
mode	**5**	**5Ac**	**6**	**7**	**8** (isomer I)	**8** (isomer II)	**9**
FAB(pos)	318 $(M+H)^+$	402 $(M+H)^+$	318 $(M+H)^+$	334 $(M+H)^+$, 356 $(M+Na)^+$	334 $(M+H)^+$	334 $(M+H)^+$	334 $(M+H)^+$, 356 $(M+Na)^+$
FAB(neg)	316 $(M-H)$	418 $(M-H+H_2O)$	*d*	332 $(M-H)$	*d*	*d*	332 $(M-H)$, 370 $(M-2H+K)$
ESI(pos)	318 $(M+H)^+$, 340 $(M+Na)^+$	*d*	*d*	*d*	*d*	*d*	*d*
APCI(pos)	318 $(M+H)^+$	*d*	318 $(M+H)^+$	334 $(M+H)^+$	*d*	*d*	*d*
APCI(neg)	316 $(M-H)^-$	*d*	316 $(M-H)^-$	332 $(M-H)^-$	332 $(M-H)^-$	332 $(M-H)^-$	332 $(M-H)^-$

[a] s, singlet; br s, broad singlet; br d, broad doublet; d, doublet; dd, double-doublet. [b] None. [c] Not observed. [d] Not analyzed.

2 was identified as *N*-[1-(2,4-dichlorophenyl)ethyl]-(4,4-dimethyl-2-oxo-tetrahydrofuran-3-yl)carboxamide (S-2900-lactone).

Metabolite 3. The ^{1}H NMR spectrum of **3** was similar to that of **2** except for the absence of one aromatic proton in **2**. FAB-MS(pos) showed a protonated molecular ion peak at *m/z* 346 $(M + H)^+$, which is 16 mass units larger than that of **2**. These findings indicated that one proton of the 2,4-dichlorophenyl group for **2** was substituted by a hydroxyl group. From interpretation of the H−H COSY, HMQC, and HMBC spectra, the compound has a lactone ring, and the hydroxyl group was assigned to the C3 position. Therefore, **3** was identified as *N*-[1-(2,4-dichloro-3-hydroxyphenyl)ethyl]-(4,4-dimethyl-2-oxo-tetrahydrofuran-3-yl)carboxamide (PhOH-S-2900-lactone).

Metabolite 4. The HMBC spectrum for **4** is shown in **Figure 4**. In the ^{1}H NMR spectrum, the signal for one methyl group of the *tert*-butyl group disappeared and the signal of the cyanomethyne proton (Hf) in the parent compound (3.44 or 3.42 ppm) was shifted to the lower magnetic field resonating at 4.29 or 4.24 ppm. The ^{13}C NMR spectrum exhibited 14 carbons, 1 carbon fewer than the parent compound. In the ^{13}C NMR spectrum of **4**, the signals of the cyano group (C15) and one methyl group (C14) of the *tert*-butyl group in the parent compound disappeared and one additional carbonyl signal (181.9 ppm, C14) was observed. Furthermore, the cyanomethyne signal (49.8 ppm, C10) in the parent compound was shifted to the lower magnetic field resonating at 76.3 ppm in **4**. Therefore, it was postulated that one methyl group of the *tert*-butyl group was oxidized to acarboxylic acid and the cyano group was replaced with a functional group. In the HMBC spectrum, cross-peaks between the methyne proton (Hd) and two carbonyl carbons (C9 and C14) were observed (**Figure 4**). Thus, it was evident that this metabolite had an imide ring. The protonated molecular ion peak at *m/z* 316 $(M + H)^+$ observed in the positive ion mode MS supported the chemical structure of **4** shown in **Figure 4**. Consequently, **4** was concluded to be *N*-[1-

Table 5. ^{1}H and ^{13}C NMR and MS Data for Metabolites **10**–**16**

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

	10		11		12		13		14		15		16			
proton	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 (CDCl3)	J (Hz)	δ (CD_3OD)	J (Hz)	δ (CD_3OD)	J (Hz)
Ha	7.47 (s)		7.47 (s)		7.58–7.37 (m)		7.50–7.35 (m)		7.34 (s)		7.47 (s)		*b*		*b*	
Hb	7.35 (d)	8.6	7.35 (d)	8.6	7.58–7.37 (m)		7.50–7.35 (m)		*b*		7.37 (d)	8.6	7.36 (d)	7.8	7.36 (d)	7.8
Hc	7.50 (d)	8.6	7.49 (d)	8.6	7.58–7.37 (m)		7.50–7.35 (m)		7.25 (s)		7.70 (d)	8.6	7.06 (d)	7.8	7.06 (d)	7.8
Hd	5.40 (q)	6.6	5.41 (q)	6.6	5.33 (q)	6.7	5.39 or 5.32 (q)	6.5	5.52 (q)	6.6	5.58 (q)	7.3	5.58 (q)		5.54 (q)	6.5
He	1.49 (3H, d)	6.6	1.49 (3H, d)	6.6	1.50 or 1.48 (3H, d)	6.7	1.49 (3H,d)	6.5	1.71 (3H, d)	6.6	1.74 (3H,d)	7.3	1.65 (3H,d)	7.0	1.66 (3H,d)	6.5
Hf	3.97 (s)		3.94 (s)		*c*		*c*		4.30 (s)		4.67 (s)		*c*	7.0	3.58 (s)	
Hg	0.95 (3H, s)		0.98 (3H, s)		1.20 or 1.05 (3H, s)		1.13 or 1.02 (3H, s)		1.15 (3H, s)		1.06 (3H, s)		1.03 (3H, s)		1.08 (3H, s)	
Hh	0.95 (3H, s)		0.98 (3H, s)		1.26 or 1.23 (3H, s)		1.17 or 1.13 (3H, s)		1.30 (3H, s)		3.86 (1H, d)	11.1	1.15 (3H, s)		1.18 (3H, s)	
											3.48 (1H, d)	11.1				
Hi	3.50 (1H, d)	10.8	3.52 (1H, d)	11.0	3.86 (1H, d)	10.5	3.55–3.28 (2H, m)		*b*		*b*		4.14 (1H, s)		4.20 (1H, s)	
	3.38 (1H, d)	10.8	3.43 (1H, d)	11.0	3.73 (1H, d)	10.5										
Hj	*c*		*c*		*c*		*c*		*b*		*b*		*b*		*b*	

(B) ^{13}C NMR Data

	10	11	12	13	14	15	16	
carbon	δ (CD_3OD)	δ (CD_3OD)	δ (CD_3OD)	δ (CD_3OD)	δ (CD_3OD)	δ (CD_3OD)	δ (CD_3OD)	δ ($CDCl_3$)
15	*b*	*b*	117.7	*c*	*b*	*b*	116.2	114.4
14	70.3	70.2	74.0	69.6	181.4	180.5	88.7	87.7
13	21.4	21.5	23.6	22.7	22.2	64.6	21.2	21.0
12	21.4	21.4	21.9	22.3	20.4	16.9	23.8	23.4
11	40.6	40.5	39.0	40.4	46.4	52.2	42.6	41.6
10	77.4	77.3	45.5	45.2	76.4	70.7	46.3	44.8
9	175.1	175.1	165.4	166.9	178.2	178.7	167.8	165.4
8	21.0	20.8	20.7	20.7	16.8	14.9	18.5	18.2
7	47.0	46.9	49.3	49.3	49.4	49.7	49.7	48.8
6	129.6	129.4	130.3	130.4	118.2	131.8	119.1	120.0
5	128.5	128.6	128.7	128.9	153.8	128.1	128.7	128.1
4	129.6	129.4	129.1	128.7	121.9	135.2	152.2	148.5
3	130.2	130.3	129.6	129.5	130.9	130.1	119.6	120.2
2	134.4	132.5	134.5	134.6	123.8	137.0	124.3	121.4
1	141.6	141.2	141.1	140.9	137.9	135.2	136.6	135.6

(C) MS Data

mode	10	11	12	13	14	15	16
FAB(pos)	320 $(M+H)^+$, 342 $(M+Na)^+$	320 $(M+H)^+$, 342 $(M+Na)^+$	407 $(M-H)^-$	329 $(M+H)^+$	332 $(M+H)^+$	332 $(M+H)^+$	343 $(M+H)^+$, 365 $(M+Na)^+$
FAB(neg)	*d*	*d*	*d*	327 $(M-H)^-$	330 (M − H)	330 (M − H)	341 $(M-H)^-$
ESI(pos)	*d*	*d*	409 $(M+H)^+$, 431 $(M+Na)^+$	329 $(M+H)^+$, 351 $(M+Na)^+$	*d*	*d*	343 $(M+H)^+$, 365 $(M+Na)^+$
ESI(neg)	*d*	*d*	407 $(M-H)^-$	*d*	*d*	*d*	*d*
APCI(neg)	318 $(M-H)^-$	318 $(M-H)^-$	*d*	327 $(M-H)^-$	*d*	*d*	*d*

[a] s, singlet; br s, broad singlet; br d, broad doublet; d, doublet; dd, double-doublet. [b] None. [c] Not observed. [d] Not analyzed.

(2,4-dichlorophenyl)ethyl]-2-hydroxy-3,3-dimethylsuccinimide (αOH-S-2900-imide).

Metabolite 5. The HMBC spectrum for **5** is shown in **Figure 5**. The ^{1}H NMR spectrum of **5** was similar to that of **4** with the exception of the observation of one additional methyne proton (4.04 ppm, Hi). The ^{13}C NMR spectrum exhibited 14 carbons, indicating the disappearance of the cyano group. In the ^{13}C NMR spectrum, one carbonyl signal (C14) in **4** disappeared in **5** and one additional methyne signal (87.9 ppm, C14) was observed instead. Therefore, it was considered that one methyl group of the *tert*-butyl group was oxidized to an alcohol or an aldehyde and the cyano group was replaced with a functional group. From interpretation of the H−H COSY, HMQC, and HMBC spectra and FAB-MS, ESI-MS, and APCI-MS, the chemical structure in **Figure 5** was concluded. Consequently, **5** was identified as 1-[1-(2,4-dichlorophenyl)ethyl]-3,5-dihydroxy-4,4-dimethyl-2-pyrrolidone (αOH-S-2900-amido-alc.A). Formation of the cyclic amide ring was confirmed by the amide carbonyl carbon (C9) coupling to two methyne protons (Hf and Hi) in the HMBC spectrum. To substantiate further this structure, the acetate derivative (**5Ac**) of **5** was obtained by acetylation (**Figure 6A**). In the ^{1}H NMR and ^{13}C NMR spectra of **5Ac**, two acetyl methyl and two acetyl carbonyl signals were observed. Therefore, it was confirmed that **5** has two hydroxyl groups and that the proposed structure was correct.

Metabolite 6. The ^{1}H NMR spectrum of **6** was similar to that of **5**, the differences being that two methyne signals at 4.22 and 4.04 ppm in **5** were observed of 4.21 ppm (0.7H) or 3.78 ppm (0.3H) and 4.76 ppm (0.7H) or 4.19 ppm (0.3H) in **6**. The ^{13}C NMR spectrum of **6** was almost the same as that of **5**. Furthermore, the molecular weights were the same. Therefore, **6** was the 7:3 mixture of stereoisomers of **5** and identified as 1-[1-(2,4-dichlorophenyl)ethyl]-3,5-dihydroxy-4,4-dimethyl-2-pyrrolidone (αOH-S-2900-amido-alc.B).

Metabolite 7. The ^{1}H NMR and the ^{13}C NMR spectra of **7** were similar to those of **4**. Positive and negative ion mode FAB-MS and APCI-MS gave protonated and deprotonated molecular ion peaks at *m/z* 334 $(M+H)^+$ and 332 $(M-H)^-$, which were 18 mass units larger than those of **4**. In the HMBC spectrum, the methyne signal (Hd) showed coupling to only one carbon (C9) in **7**, although the Hd showed coupling to two carbonyl carbons (C9 and C14) in **4**. Therefore, **7** was considered to be the imide linkage cleaved metabolite of **4**. Consequently, **7** was identified as *N*-[1-(2,4-dichlorophenyl)-

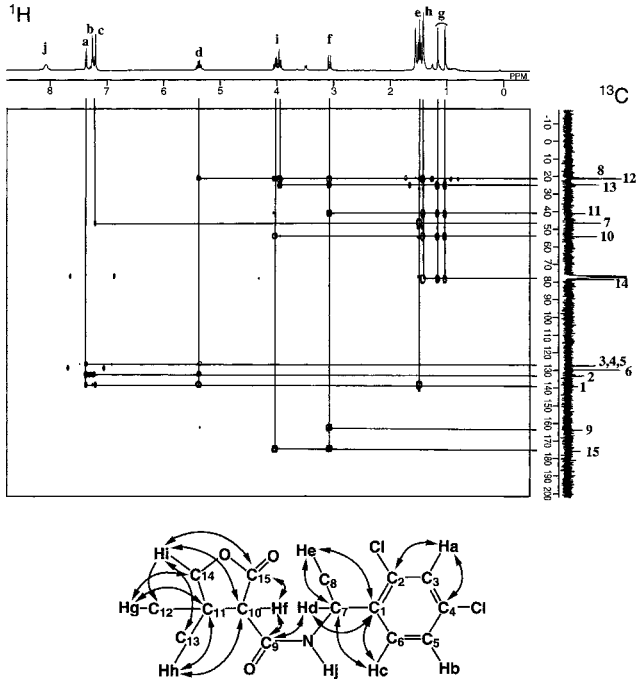

Figure 3. HMBC spectrum and chemical structure of metabolite **2**.

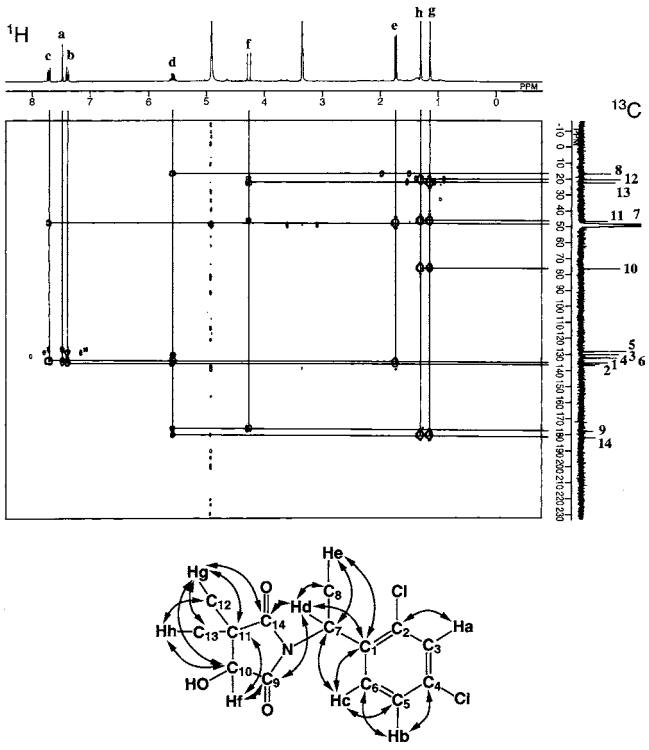

Figure 4. HMBC spectrum and chemical structure of metabolite **4**.

ethyl]-3-hydroxy-2,2-dimethylsuccinamic acid (tBuCOOH-αOH-S-2900).

*Metabolite **8***. The ^{1}H NMR and ^{13}C NMR spectra showed the existence of two stereoisomers. From the results of HMQC and HMBC measurement, it was confirmed that this metabolite was the 2:3 mixture of stereoisomers (designated isomers I and II). As for isomer I, its ^{1}H NMR spectrum was very close to that of **5** with the exception of the disappearance of one methyl group and the observation of one additional AB quartet methylene proton instead (3.85 and 3.66 ppm, each 1H, d, J = 11.1 Hz). The ^{13}C NMR spectrum was also the same as that of **5**, except that the magnetic field of C13 carbon for **8** (65.2 ppm)

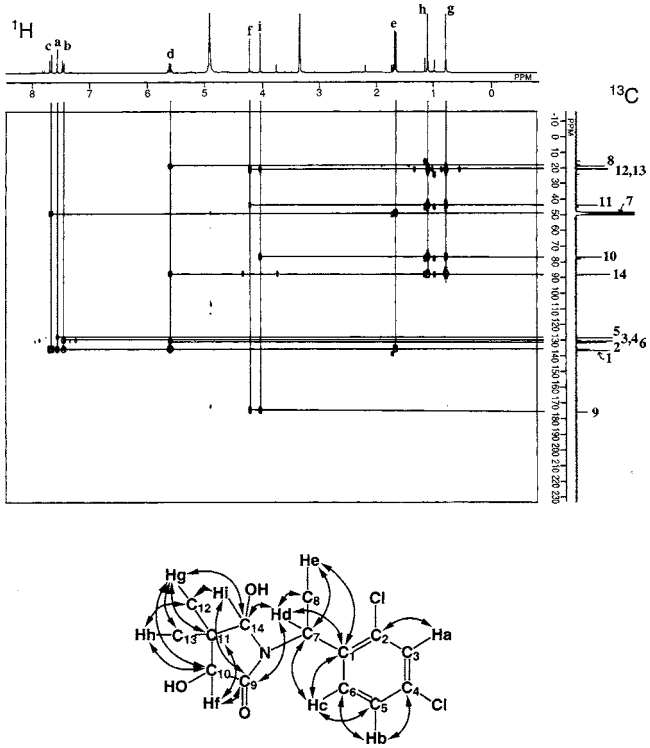

Figure 5. HMBC spectrum and chemical structure of metabolite **5**.

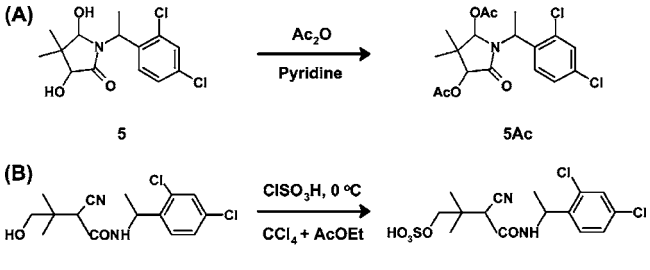

Figure 6. Chemical reactions: (**A**) acetylation of the purified metabolite **5**; (**B**) sulfation of the reference standard, tBuOH-S-2900.

was lower than that for **5** (20.7 ppm). Therefore, it was considered that one methyl group of the *geminal*-dimethyl group for **5** was oxidized to an alcohol. Amide carbonyl carbon (C9) coupling to two methyne protons (Hf and Hi) in the HMBC spectrum demonstrated this metabolite to have a cyclic amide ring. These findings were also observed for isomer II. From the above-mentioned results and the molecular weight of 333, which was 16 mass units larger than that of **5**, **8** was consequently identified as 1-[1-(2,4-dichlorophenyl)ethyl]-3,5-dihydroxy-4-hydroxymethyl-4-methyl-2-pyrrolidone (tBuOH-αOH-S-2900-amido-alc.A).

*Metabolite **9***. The ^{1}H NMR spectrum of **9** was similar to that of isomer I of **8**, the difference being that one methyne signal at 4.95 ppm (Hi) in **8** (isomer I) was observed at 4.22 ppm in **9**. The ^{13}C NMR spectrum of **9** was almost the same as that of isomer I. Furthermore, the molecular weights were the same as that of **8**. Therefore, **9** is a stereoisomer of **8**, identified as 1-[1-(2,4-dichlorophenyl)ethyl]-3,5-dihydroxy-4-hydroxymethyl-4-methyl-2-pyrrolidone (tBuOH-αOH-S-2900-amido-alc.B).

*Metabolite **10***. The ^{1}H NMR spectrum showed AB quartet methylene signals (3.50 and 3.38 ppm, each 1H, d, J = 10.8 Hz) in addition to the signals of **7**. From a comparison of the ^{13}C NMR spectrum of **7** with that of **10**, the C14 carbon in **7** was in a lower magnetic field at 184.8 ppm than that of **10**

(70.3 ppm), indicating the C14 of **10** to be a secondary carbon. Because the carbonyl carbon (C9) did not show coupling to the methylene protons (Hi) in the HMBC spectrum, this metabolite does not form the cyclic amide ring. FAB-MS(pos) gave a protonated molecular ion peak at *m*/*z* 320 $(M + H)^+$, and APCI-MS(neg) gave a deprotonated molecular ion peak at *m*/*z* 318 $(M - H)^-$, 14 mass units smaller than those of **7**. These show that one methyl group of the *tert*-butyl group in the parent compound is oxidized to an alcohol and the cyano group is replaced to a hydroxyl group in this metabolite. Consequently, **10** was identified as *N*-[1-(2,4-dichlorophenyl)ethyl]-2,4-dihydroxy-3,3-dimethylbutanamide (tBuOH-αOH-S-2900A).

*Metabolite **11***. The ^{1}H NMR and ^{13}C NMR spectra of **11** were similar to those of **10**. Furthermore, the molecular weights of **10** and **11** were the same. Therefore, **11** is a stereoisomer of **10**, identified as *N*-[1-(2,4-dichlorophenyl)ethyl]-2,4-dihydroxy-3,3-dimethylbutanamide (tBuOH-αOH-S-2900B).

*Metabolite **12***. The ^{1}H NMR and ^{13}C NMR spectra of **12** were similar to those of the reference standard, tBuOH-S-2900, suggesting one methyl group of the *tert*-butyl group was oxidized to an alcohol in **12**, as in tBuOH-S-2900. However, because this metabolite was very polar (low TLC R_f value in polar solvent), it was suspected that it was a conjugate. With FAB-MS(pos) and ESI-MS(neg), protonated and deprotonated molecular ion peaks were found at *m*/*z* 409 $(M + H)^+$ and *m*/*z* 407 $(M - H)^-$, respectively, 80 mass units larger than those of tBuOH-S-2900. This difference corresponds to one sulfur and three oxygen atoms. Therefore, **12** is the sulfate of tBuOH-S-2900, identified as (3-{*N*-[1-(2,4-dichlorophenyl)ethyl]carbamoyl}-3-cyano-2,2-dimethylpropyl hydrogen sulfate (tBuOH-S-2900-sulfate). Because enzyme hydrolysis using sulfatase does not occur with this metabolite, to further confirm the proposed structure is correct, the sulfate of tBuOH-S-2900 was obtained by chemical reaction (**Figure 6B**). The ^{1}H NMR spectrum of the synthetic compound corresponded to that of **12**; therefore, sufficient evidence of the correction of the proposed strcuture was obtained. Because this metabolite is not the aryl sulfate, enzyme hydrolysis by aryl sulfatase did not occur.

*Metabolite **13***. The ^{1}H NMR and ^{13}C NMR spectra of **13** were also similar to those of the reference standard, tBuOH-S-2900, except that the cyano carbon (C15) was not observed. Therefore, it was considered that one methyl group of the *tert*-butyl group was oxidized to an alcohol and the cyano group was changed in **13**. Positive and negative ion mode MS gave a protonated molecular ion peak at *m*/*z* 329 $(M + H)^+$ and a deprotonated molecular ion peak at 327 $(M - H)^-$, the same as those of tBuOH-S-2900. From the above and interpretation of the H−H COSY and HMQC spectra, **13** had a cyclic imino ether and was identified as *N*-[1-(2,4-dichlorophenyl)ethyl]-(2-imino-4,4-dimethyltetrahydrofuran-3-yl)carboxamide (S-2900-imino-ether).

*Metabolite **14***. The ^{1}H NMR spectrum of **14** was similar to that of **4** except for the absence of one aromatic proton. The aromatic protons each showed singlet signal, indicating para position relative to another and the existence of a functional group at C5. Positive and negative ion mode FAB-MS revealed protonated and deprotonated molecular ion peaks at *m*/*z* 332 $(M + H)^+$ and *m*/*z* 330 $(M - H)^-$, respectively, 16 mass units larger than those of **4**. Therefore, the functional group was concluded to be a hydroxyl group, and **14** was identified as *N*-[1-(2,4-dichloro-5-hydroxyphenyl)ethyl]-2-hydroxy-3,3-dimethylsuccinimide (5-PhOH-αOH-S-2900-imide). Formation of the imide ring was confirmed by the observation of long-range coupling between the methyne proton (Hd) and two carbonyl carbons (C9 and C14).

*Metabolite **15***. The ^{1}H NMR spectrum of **15** was similar to that of **9** but lacked one methyne proton signal (Hi for **9**). In the ^{13}C NMR, C14 in **9** (86.9 ppm) was shifted to a lower magnetic field resonating at 180.5 ppm, indicating a carbonyl carbon. From the above, the results of FAB-MS, and interpretation of the H−H COSY, HMQC, and HMBC spectra, **15** was identified as *N*-[1-(2,4-dichlorophenyl)ethyl]-2-hydroxy-3-hydroxymethyl-3-methylsuccinimide (tBuOH-αOH-S-2900-imide).

*Metabolite **16***. The ^{1}H NMR spectrum (CD_3OD) of **16** was similar to that of **5** but lacked the cyanomethyne proton (Hf) and one aromatic proton. However, the ^{1}H NMR spectrum in $CDCl_3$ showed the methyne proton. In the ^{13}C NMR, although C15 was lacking in **5**, it was observed at 116.2 ppm (in CD_3OD) or 114.4 ppm (in $CDCl_3$), meaning **16** has the cyano group. From this, interpretation of the H−H COSY, HMQC, and HMBC spectra, and the FAB-MS and ESI-MS spectra, **16** has a hydroxyl group at the C3 position and a cyclic amide ring. Consequently, **16** was identified as 1-[1-(2,4-dichloro-3-hydroxyphenyl)ethyl]-3-cyano-5-hydroxy-4,4-dimethyl-2-pyrrolidone (PhOH-S-2900-amido-alc). Formation of the cyclic amide ring was confirmed by cross-peaks between the two methyne protons (Hf and Hi) and the carbonyl carbon (C9).

Enzyme Hydrolysis. With the fecal extracts, enzyme hydrolysis did not occur. However, the main metabolite was considered to be metabolite **12**, which was not hydrolyzed by sulfatase as stated above. In the urine samples, polar metabolites were considered to be glucuronides and sulfates of several identified metabolites (**1**, **4**, **5**, etc.) and tBuOH-S-2900 in both sexes. The main metabolite in males was the glucuronide of metabolite **1**. In females, the main metabolites were the glucuronide and the sulfate of metabolite **1**. Representative TLC autoradiograms of urinary and fecal metabolites are shown in **Figure 7**.

DISCUSSION

In the present study, S-2558 was widely metabolized in the rat. Several kinds of ring formation (lactone, imide, cyclic amide, cyclic imino ether) were observed. For the generation of an imide ring, it was considered that the carbon (C14) of the carboxylic acid combined with the nitrogen of the amide group. Similarly, the C14 of the aldehyde combined with the nitrogen of the amide group for the formation of the cyclic amide, the oxygen of the alcohol (C14) attacked the carbon (C15) of the cyano group for the cyclic imino ether formation, and as for the lactone ring formation, the C15 of the carboxylic acid reacted with the oxygen of the alcohol (C14) or an imino group (−C═NH) of the cyclic imino ether was oxidized to the carbonyl group (−C═O). Most of the identified metabolites were decyanated compounds. Decyanation reactions are rather rare, but in the metabolism of pyrethroid insecticides having a cyano group, such as fenvalerate (*14*), cyphenothrin (*15*), and cypermethrin (*16*), decyanated metabolites have been detected. With such decyanation, cyanide ions could be released (nonenzymatically) during ester bond cleavage (by the action of a carboxylesterase). In the present study, the mechanisms of decyanation were not clarified. However, an intermediate having a structure like a cyanohydrine moiety is likely to exist, because the cyano groups of all decyanated metabolites were replaced with hydroxyl groups.

On the basis of the present findings, the major biotransformation reactions of S-2558 in rats were (1) oxidation of the

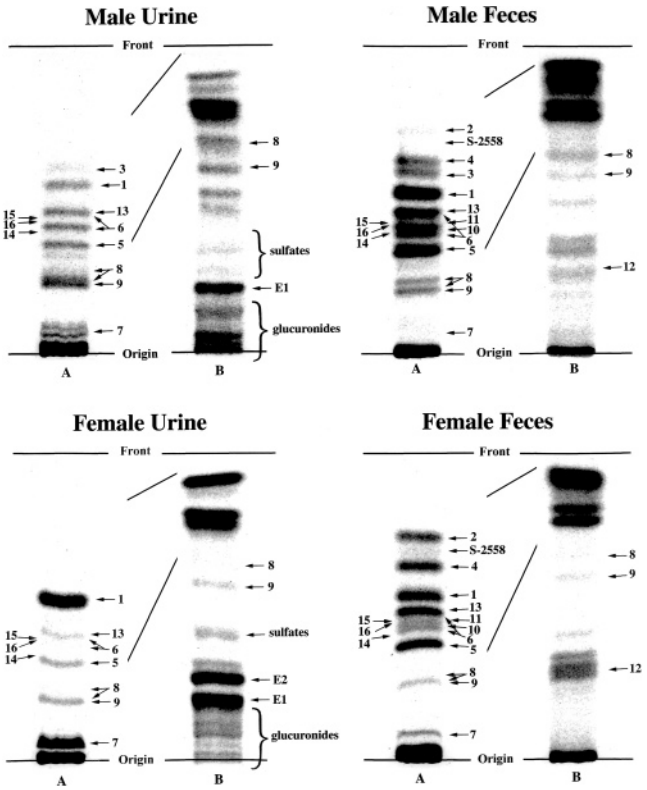

Figure 7. TLC autoradiograms of fecal and urinary metabolites. Solvent systems: A, chloroform/methanol = 9:1 (v/v); B, ethyl acetate/acetone/water/acetic acid = 10:1:1:1 (v/v) Abbreviations: E1, PhOH-S-2900-glucuronide; E2, PhOH-S-2900-sulfate.

methyl group of the *tert*-butyl group to an alcohol; (2) oxidation of the methyl group of the *tert*-butyl group to an aldehyde; (3) oxidation of the methyl group of the *tert*-butyl group to a carboxylic acid; (4) hydrolysis of the cyano group to a carboxylic acid; (5) replacement of the cyano group with a hydroxyl group; (6) hydroxylation at the C3 position of the aromatic ring; (7) hydroxylation at the C5 position of the aromatic ring; (8) formation of the lactone; (9) formation of the amide alcohol ring; (10) formation of the imide ring; (11) formation of the imino ether ring; and (12) glucuronidation and sulfation of the hydroxyl group. Metabolic pathways of S-2900 and S-2900S in rats will be described elsewhere.

ACKNOWLEDGMENT

We thank Kaeko Tanaka and Sayaka Yamada for expert technical assistance.

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Received for review January 6, 2006. Revised manuscript received March 20, 2006. Accepted March 23, 2006.

JF0600509