Metabolism of 7-Fluoro-6-(3,4,5,6-tetrahydrophthalimido)-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (S-53482) in Rat. 1. Identification of a Sulfonic Acid Type Conjugate

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To examine the metabolic fate of 7-fluoro-6-(3,4,5,6-tetrahydrophthalimido)-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (S-53482), rats were given a single oral dose of [*phenyl*-^{14}C]-S-53482 at 1 (low) or 100 (high) mg/kg. The radiocarbon was almost completely eliminated within 7 days after administration in both groups. ^{14}C recoveries (expressed as percentages relative to the dosed ^{14}C) in feces and urine were 56−72 and 31−43%, respectively, for the low dose and 78−85 and 13−23%, respectively, for the high dose. S-53482 and seven metabolites were identified in urine and feces. Six of them were purified by several chromatographic techniques and identified by spectroanalyses (NMR and MS). Alcohol derivatives and an acetoanilide derivative were isolated from urine. Three sulfonic acid conjugates having a sulfonic acid group incorporated into the double bond of the 3,4,5,6-tetrahydrophthalimide moiety were isolated from feces. On the basis of the metabolites identified in this study, the metabolic pathways of S-53482 in rats are proposed.

Keywords: *Metabolism; herbicide; rat; identification; sulfonic acid conjugate; excretion; biotransformation*

INTRODUCTION

7-Fluoro-6-(3,4,5,6-tetrahydrophthalimido)-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (S-53482) is a new selective postemergence herbicide for control of annual broad-leaved weeds. In paticular, it offers excellent control of troublesome plants such as *Abutilon theophrasti*, *Amaranthus* spp., *Ambrosia artemisiifolia*, *Chenopodium album*, *Ipomoea* spp., *Sesbania exaltata*, and *Sida spinosa* (Yoshida et al., 1991). S-53482 provides excellent velvetleaf control at very low field rates of 0.75−1.5 oz of active ingredient (ai)/acre. This paper deals with (1) identification of the metabolites of S-53482 in rats and (2) absorption, excretion, and quantitative analysis of metabolites of S-53482 in rats after a single oral administration of low (1 mg/kg) or high (100 mg/kg) doses.

MATERIALS AND METHODS

Chemicals. S-53482 labeled uniformly in the phenyl ring ([*phenyl*-^{14}C]-S-53482, 5.46 GBq/mmol) was synthesized in our laboratory (Figure 1) and purified by preparative TLC with development in benzene/ethyl acetate, 4:1 (v/v), prior to use. The radiochemical purity of the labeled compound was confirmed to be >99% by TLC analysis. Unlabeled S-53482 (99.5% purity) and an unlabeled authentic standard, 6-amino-7-fluoro-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (APF), were also synthesized in our laboratory. Nuclear magnetic resonance (NMR) spectral data of S-53482 are summarized in Table 2. Mass (MS) spectral data were EI-MS (70 eV) *m*/*z* 354 (M^+). NMR data of APF are summarized in Table 3. MS spectral data were EI-MS (70 eV) *m*/*z* 220 (M^+).

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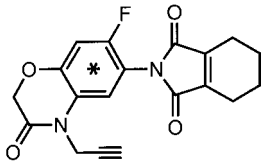

Figure 1. Chemical structure of [*phenyl*-^{14}C]-S-53482.

Table 1. TLC R_f Values of Authentic Standards and Identified Metabolites

		R_f value in solvent system[a]		
metabolite	compound	A	B	C
	S-53482	0.71	*b*	0.58
	APF	0.63	*b*	0.45
1	3-OH-SA	0.02	0.55	*c*
2	4-OH-SA	0.02	0.51	*c*
3	3-OH-A-SA	*c*	0.36	*c*
4	3-OH-S-53482	0.58	*b*	0.33
5	4-OH-S-53482	0.54	*b*	0.19
6	Ac-APFA	0.12	0.77	*c*

[a] Solvent systems: A, toluene/ethyl formate/formic acid = 5:7:1; B, ethyl acetate/acetone/water/acetic acid = 4:1:1:1; C, benzene/ethyl acetate = 1:1. [b] Solvent front. [c] Origin.

Chromatographic Procedures. Thin-layer chromatography (TLC) analyses were conducted essentially as described previously by Kaneko et al. (1988) and Saito et al. (1991). Precoated silica gel 60 F_{254} chromatoplates (Art. 5715, 20 × 20 cm, 0.25 mm thickness, and Art. 5744, 20 × 20 cm, 0.50 mm thickness, E. Merck, Darmstadt, Germany) and precoated silica gel RP-18F_{254}s chromatoplates (reversed phase, Art. 15389, 20 × 20 cm, 0.25 mm thickness, E. Merck) were used for analysis and isolation of metabolites. The following solvent systems were used: (A) toluene/ethyl formate/formic acid (5:7:1, v/v); (B) ethyl acetate/acetone/water/acetic acid (4:1:1:1, v/v); (C) benzene/ethyl acetate (1:1, v/v); (D) benzene/ethyl

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Table 2. ^{1}H and ^{13}C NMR Data for S-53482 and Metabolites 1−3

	S-53482		**1**		**2**		*3*	
	δ ($CDCl_3$)	*J* (Hz)	δ (CD_3OD)	*J* (Hz)	δ (CD_3OD)	*J* (Hz)	δ (CD_3OD)	*J* (Hz)
				^{1}H NMR Data				
proton no.								
2a	4.65 (2H,s)		4.75 (2H,s)		4.77 (2H,s)		4.05 (dd)	16.7, 3.3[a]
2b							3.81 (dd)	16.7, 3.3[a]
5	7.04 (d)	6.9[a]	7.31 (d)	6.6[a]	7.28 (d)	6.6[a]	7.33 (d)	7.9[a]
8	6.89 (d)	9.9[a]	7.04 (d)	9.9[a]	6.99 (d)	10.6[a]	6.98 (d)	10.6[a]
11a	4.67 (2H,d)	2.6	4.89 (dd)	17.8, 2.6	4.73 (2H,d)	2.6	4.80 (dd)	17.8, 2.6
11b			4.70 (dd)	17.8, 2.6			4.12 (dd)	17.8, 2.6
13	2.29 (t)	2.6	2.70 (t)	2.6	2.67 (t)	2.6	2.62 (t)	2.6
16	*b*		3.29 (d)	8.6	3.86 (m)		3.24 (d)	8.6
17a	2.44 (2H,m)		3.77 (br s)		2.12 (2H,m)		3.74 (brs)	
17b			*b*				*b*	
18a	1.83 (2H,m)		1.96−1.92 (m)		3.66 (brs)		1.98−1.92 (m)	
18b			1.50−1.34 (m)		*b*		1.49−1.32 (m)	
19a	1.83 (2H,m)		2.67 (m)		1.86 (m)		2.65 (m)	
19b			2.15 (m)		1.65 (m)		2.13 (m)	
20a	2.44 (2H,m)		1.96−1.92 (m)		2.57 (m)		1.98−1.92 (m)	
20b			1.50−1.34 (m)		2.29 (m)		1.49−1.32 (m)	
				^{13}C NMR Data				
carbon no.								
2	67.4		68.5		68.6		61.3	
3	162.9		165.0		165.0		173.9	
5	116.1 (d)	2.1[c]	117.4		118.0		123.7	
6	113.6 (d)	14.5[c]	115.4 (d)	14.5[c]	115.4 (d)	15.6[c]	112.6 (d)	13.8[c]
7	154.1 (d)	250.1[c]	155.5 (d)	249.1[c]	155.5 (d)	253.8[c]	160.0 (d)	242.2[c]
8	105.9 (d)	24.9[c]	106.3 (d)	23.8[c]	106.4 (d)	24.9[c]	105.3 (d)	22.8[c]
9	146.0 (d)	11.4[c]	148.2 (d)	11.3[c]	148.3 (d)	11.5[c]	157.5 (d)	10.4[c]
10	124.6 (d)	3.1[c]	126.1		126.2		132.3	
11	30.8		31.6		31.6		37.9	
12	76.7		78.0		78.1		78.9	
13	73.2		74.3		74.3		74.3	
15	169.0		174.8[d]		175.3		174.8[e]	
16	142.3		54.0		43.6		54.0	
17	21.2		72.1		32.8		72.3	
18	20.2		32.4		64.6		32.5	
19	20.2		25.6		29.7		25.5	
20	21.2		20.7		23.1		20.8	
21	142.3		70.4		72.7		70.5	
22	169.0		177.6[d]		175.3		177.7[e]	

[a] H−F coupling. [b] None. [c] C−F coupling. [d] Interchangeable. [e] Interchangeable.

acetate (1:2, v/v); (E) ethyl acetate/ethanol (6:1, v/v); and (F) acetonitrile/water (3:7, v/v, for reversed phase TLC).

Unlabeled standards on TLC plates were detected by viewing under UV light (254 nm). Radioactive spots were detected by placing X-ray films (SB-5, Kodak, Rochester, NY) on the plates for ∼1 week at 4 °C, followed by processing of the exposed films with a model M6B processor (Kodak).

High-performance liquid chromatography (HPLC) was carried out according to the methods reported previously (Tomigahara et al., 1994a) using the following systems: L-6200 HPLC Intelligent Pump (Hitachi Ltd., Japan), L-4000 UV detector (Hitachi Ltd.), and an LB 507A radioactivity monitor (Berthold, Germany). A YMC-Pack S-343-15 column (ODS, 20 mm i.d. × 250 mm, YMC Co., Ltd., Japan) and a YMC-Pack GI-340-15 (ODS, 20 mm i.d. × 50 mm, YMC Co., Ltd.) were used as the analytical column and the guard column, respectively.

Spectrometry. NMR spectra were obtained on a JEOL GSX-270 spectrometer (JEOL Ltd., 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; Aue et al., 1976) and C−H correlation spectroscopy (C−H COSY; Maudsley et al., 1977)] were obtained with the data processing program PLEXUS V1.6. Chemical shifts were determined in parts per million units relative to 0.00 in tetramethylsilane as an internal standard. Methanol-d_4 (99.5%, E. Merck), acetone-d_6 (99.8%, E. Merck), and chloroform-*d* (99.5%, E. Merck) were used as solvents for NMR. Direct electron impact (EI) and field desorption (FD) mass spectra were recorded on a Hitachi M-80B mass spectrometer (Hitachi Ltd.). 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. Secondary ion mass spectrometry (SI-MS) was performed with a Hitachi M-80B mass spectrometer. The accelerating voltage was 3.0 kV. Samples were introduced in a glycerol matrix. Thermospray mass spectrometry (TSP-MS) was performed with a Finnigan TSQ 700 mass spectrometer (Finnigan Mat Instrument Inc., San Jose, CA). The ion source and vaporizer temperatures were 250 and 80 °C, respectively. The electron multiplier was 1000 V. The discharge was set at 1000 V. Spectra were recorded in positive and negative ion modes.

Treatment of Animals. Charles River derived CD (Sprague−Dawley) male and female rats at the age of 6 weeks old were purchased from Charles River Japan Inc. (Japan) and maintained in an air-conditioned room at 22−24 °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., Japan) were provided ab libitum. Sixteen male rats were employed for the identification study. Totals of 10 males and 10 females were used for the ^{14}C excretion and ^{14}C tissue residue studies.

Identification Study. To collect sufficient amounts of unknown metabolites in urine and feces for spectroanalytical identification, a total of ∼9 g of [*phenyl*-^{14}C]-S-53482 was dissolved in corn oil and dosed orally to 16 rats for 5 consecutive days at ∼300 mg/kg/day. (This dose level and

Table 3. ^{1}H and ^{13}C NMR Data for Metabolites 4−6 and APFb

	4		5		6				APF	
	δ ($CDCl_3$)	J (Hz)	δ ($CDCl_3$)	J (Hz)	δ (CD_3OD)	J (Hz)	δ [$(CD_3)_2CO$]	J (Hz)	δ ($CDCl_3$)	J (Hz)
					^{1}H NMR Data					
proton no.										
2a	4.69 (2H,s)		4.68 (2H,s)		4.01 (d)	16.0	3.91 (d)	15.8	4.56	
2b					3.81 (d)	16.0	3.69 (d)	15.8		
5	7.05 (d)	6.8[a]	7.05 (d)	6.8[a]	7.70 (d)	7.9[a]	8.01 (d)	8.6[a]	6.65 (d)	7.9[a]
8	6.91 (d)	9.8[a]	6.90 (d)	9.8[a]	6.80 (d)	11.9[a]	6.84 (d)	11.9[a]	6.73 (d)	10.6[a]
11a	4.67 (2H,d)	2.4	4.67 (2H,d)	2.4	*b*		4.90 (dd)	17.2, 2.6	4.63 (2H,d)	2.6
11b					3.99 (d)	17.2	4.03 (dd)	17.2, 2.6		
13	2.30 (t)	2.4	2.30 (t)	2.4	2.61 (t)	2.4	2.67 (t)	2.6	2.28 (t)	2.6
17a	4.82 (br s)		2.05−1.93 (2H,m)		*c*		*c*		*c*	
17b	*c*									
18a	2.06−1.75 (2H,m)		4.37 (br s)		*c*		*c*		*c*	
18b			*c*							
19	2.57−2.37 (2H,m)		2.79−2.48 (2H,m)		*c*		*c*		*c*	
20	2.06−1.75 (2H,m)		2.79−2.48 (2H,m)		*c*		*c*		*c*	
CH_3CO^-	*c*		*c*		2.17 (3H,s)		2.12 (3H,s)		*c*	
NH_2^-	*c*		*c*		*c*		*c*		3.64 (2H,brs)	
					^{13}C NMR Data					
carbon no.										
2	67.5		67.5		61.4		60.9		67.9	
3	163.0		163.0		172.2		169.0		164.1	
5	116.1 (d)	2.1[d]	116.2		128.3 (d)	4.2[d]	126.2		103.8 (d)	4.1[d]
6	113.6 (d)	14.5[d]	113.5 (d)	14.5[d]	118.9 (d)	13.5[d]	119.8 (d)	13.5[d]	129.6 (d)	14.5[d]
7	154.0 (d)	251.2[d]	154.3 (d)	250.0[d]	157.0 (d)	249.2[d]	155.0 (d)	246.0[d]	147.6 (d)	237.8[d]
8	106.1 (d)	24.9[d]	106.1 (d)	24.9[d]	104.9 (d)	22.9[d]	104.7 (d)	22.9[d]	105.2 (d)	23.9[d]
9	146.3 (d)	11.4[d]	146.2		153.3 (d)	11.4[d]	151.8 (d)	10.4[d]	137.2 (d)	10.4[d]
10	124.7 (d)	3.1[d]	124.7 (d)	3.2[d]	122.9 (d)	3.1[d]	122.1		124.6 (d)	3.1[d]
11	31.0		31.0		37.9		37.5		31.0	
12	76.7		76.5		79.4		79.6		77.2	
13	73.4		73.4		73.8		73.8		72.6	
15	168.8[e]		168.7[g]		*c*		*c*		*c*	
16	144.5[f]		141.8[h]		*c*		*c*		*c*	
17	61.2		29.2		*c*		*c*		*c*	
18	30.2		65.0		*c*		*c*		*c*	
19	20.6		29.0		*c*		*c*		*c*	
20	18.3		17.4		*c*		*c*		*c*	
21	141.3[f]		139.7[h]		*c*		*c*		*c*	
22	168.4[e]		168.5[g]		*c*		*c*		*c*	
CH_3CO^-	*c*		*c*		173.8		172.9		*c*	
CH_3CO^-	*c*		*c*		23.0		23.6		*c*	

[a] H−F coupling. [b] Not observed. [c] None. [d] C−F coupling. [e] Interchangeable. [f] Interchangeable. [g] Interchangeable. [h] Interchangeable.

period were chosen because large amounts of metabolites were needed for purification and identification.) The specific activity of [^{14}C]-S-53482 was adjusted to 0.48 MBq/mmol by isotopic dilution with unlabeled S-53482. The animals were housed in Metabolica CO_2 cages (Sugiyamagen Iriki Co., Ltd., Japan) to allow separate collection of urine and feces.

^{14}C Excretion and ^{14}C Tissue Residue Studies. [*phenyl*-^{14}C]-S-53482 was dissolved in corn oil at 1 (low dose) or 100 (high dose) mg/5 mL and a single dose administered orally to five males and five females per dose at 5 mL/kg. These dose levels were chosen because S-53482 was found to be without obvious effects in rats at the dose of 1 mg/kg, whereas it produced slight suppression of body weight gain at the 100 mg/kg dose level in a toxicity study (unpublished observations). The dosed ^{14}C was adjusted to 9.25 MBq/kg in all cases. After administration of the labeled compounds, the animals were housed in Metabolica CO_2 cages. Urine and feces from each rat were collected 6 h (urine only) and 1, 2, 3, 5, and 7 days after administration. On the seventh day after dosing, rats were killed with collection of blood from the abdominal aorta under light anesthesia with diethyl ether and a total of 17−18 organs/tissues were removed from each animal.

Puification of Metabolites. A flow diagram for the procedures applied is given in Figure 2. All collected feces were mixed and homogenized with a 3-fold volume of acetonitrile using a Waring blender (Nihonseiki Co., Japan), and the homogenates were centrifuged at 3000 rpm (1500*g*) for 10 min using a Himac CR 20B3 Superspeed refrigerated centrifuge and an RR14A-159 rotor (Hitachi Ltd.). Residues were further extracted twice with acetonitrile. The fecal acetonitrile extracts were concentrated and partitioned three times between ethyl acetate and water. The aqueous solution was extracted three times with 1-butanol, and the resultant extract was subjected to silica gel (Kieselgel 60, 70−230 mesh, E. Merck) column chromatography using ethyl acetate (500 mL), acetone (500 mL), acetone/methanol [10:1 (500 mL) and 5:1 (500 mL), v/v], and methanol (500 mL) as the eluents (fractions A − E, respectively). As unknowns, metabolite **1** was detected in fraction B, **2** in fraction C, and **3** in fraction D. Fractions B−D were separately purified by preparative TLC (Art. 5744, solvent system B), and the portions containing **1**−**3** were purified further by preparative reversed phase TLC (Art. 15388, solvent system F). Each metabolite was then isolated by Sephadex LH-20 (Pharmacia, Japan) column chromatography using methanol as the eluent.

Collected urine samples were combined and then lyophilized using an FD-81 freeze-dryer (Tokyo Rikakikai Co., Ltd., Japan). After addition of water, the residue was chromatographed on Amberlite XAD-2 resin (Organo, Japan), washed with water, and eluted with methanol. The methanol eluates were evaporated and the residues partitioned three times between ethyl acetate and water. The ethyl acetate extract was concentrated and separated into two portions (containing **4** and **5**) by preparative TLC (Art. 5744, solvent system D). Metabolites **4** and **5** were purified further by HPLC after pretreatment with a SEP-PAK C18 cartridge (Waters, Milford, MA). The water extract from the partitioning was lyophilized, and the residue was absorbed on silica gel and chromatographed over 200 g of silica gel in a column. The column was then eluted with ethyl acetate (500 mL), ethyl acetate/acetone

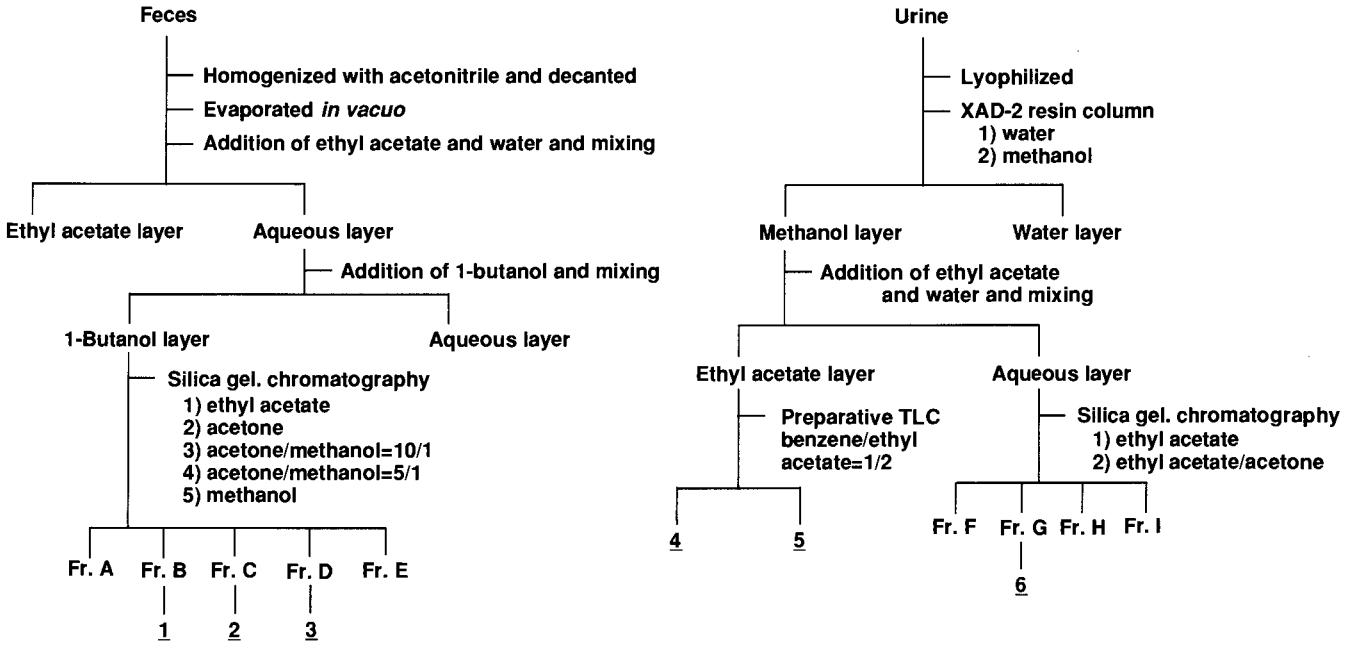

Figure 2. Flow diagram for steps in the purification of fecal and urinary metabolites.

[1:1, v/v (500 mL)], and methanol (300 mL) (fractions F−H, respectively). Fraction G was purified by preparative TLC (Art 5744, solvent system E), and the portion containing **6** was purified further with the HPLC system described above.

The isolated metabolites were identified by NMR and MS.

Radioanalysis. Radioanalysis was conducted as reported previously (Tomigahara et al., 1995) with radioactivity in organosoluble fractions, urine and silica gel being quantified by conventional liquid scintillation counting (LSC). Radioactivity of fecal homogenates, unextractable fecal residues, and tissues was quantified using the combustion method. These samples were combusted with a Tri-Carb 307 sample oxidizer (Packard, USA) with ^{14}C determined as $^{14}CO_2$ trapped in a scintillation solution of 8 mL of Oxisorb-CO_2 and 12 mL of Oxiprep-2 (New England Nuclear, Boston, MA) after being air-dried.

The urine was directly radioassayed by LSC. The 0−1, 1−2, and 2−3 day feces of rats were each extracted three times with 50−100 mL of acetonitrile and three times with 50−100 mL of water, and the extracts and air-dried unextractable residues were radioassayed. The other (the 3−5 and 5−7 day) feces were homogenized with water and the homogenates combusted for radioassay.

For the ^{14}C tissue residue determination, blood was divided into blood cells and plasma by centrifugation at 1000*g* for 10 min. Rat carcasses were minced with a meat chopper. One or two aliquots of each tissue sample were combusted for radioanalysis. The ^{14}C residue levels were expressed as microgram or nanogram equivalents of S-53482/g of wet tissue (ppm or ppb, respectively).

Radioactivity on TLC plates was measured by scraping the appropriate silica gel regions and counting in scintillation vials (scraping method).

Analysis of Metabolites in Excreta. The metabolites in 0−2 day urine and the 0−2 day fecal extracts were tentatively identified by TLC cochromatography (solvent systems A−C) with authentic standards and quantified by the TLC scraping method using solvent systems A and B.

RESULTS

Identification of Metabolites. The R_f values of the identified metabolites are summarized in Table 1. APF and the parent compound were identified by TLC cochromatography with authentic standards. The following six metabolites were purified and identified by spectroanalyses (NMR and MS). The NMR data are shown in Tables 2 and 3.

1. Metabolite **1** was isolated by reversed phase preparative TLC using solvent system F with a*n* R_f value of 0.50. Its H−H COSY and C−H COSY spectra are shown in Figure 3. Proton signals (in CD_3OD) at 7.31 ppm (1H, d, $J = 6.6$ Hz), 7.04 ppm (1H, d, $J = 9.9$ Hz), 4.75 ppm (2H, s), 4.89 ppm (1H, dd, $J = 17.8$, 2.6 Hz), 4.70 ppm (1H, dd, $J = 17.8$, 2.6 Hz), and 2.70 ppm (1H, t) indicated the presence of a benzoxazinone ring. Signals at 3.77 and 3.29 ppm in metabolite **1** were not detected in the ^1H NMR spectrum of the parent compound (Table 2). The findings from the H−H COSY and the C−H COSY spectra further suggested that the double bond of the 3,4,5,6-tetrahydrophthalimide moiety had a functional group, the signal at 3.29 ppm (d, $J =$ 8.6 Hz) could be ascribed to the angular proton (H-16), and one proton of the resultant 1,2-cyclohexanedicarboximide moiety was replaced with a functional group (Figure 3). This metabolite was very polar (high water solubility and low TLC R_f value in polar solvents), indicating the incorporated groups are polar groups. Negative ion mode TSP-MS and SI-MS showed a deprotonated molecular ion peak at *m/z* 451 $(M - H)^-$. The molecular weight of 452 was 98 mass units larger than that of the parent compound (354). These findings indicated that the replacement group was a hydroxy group (−OH−H: 16 mass units) and the group incorporated into the double bond of the 3,4,5,6-tetrahydrophthalimide moiety was a sulfonic acid group or $-OSO_2H$ (sulfurous ester) ($-SO_3H + H$ or $-OSO_2H + H$: 82 mass units). However, formation of the latter is generally unlikely because of its instability (Sato, 1960). Therefore, the incorporated group was concluded to be a sulfonic acid group. From interpretation of the H−H COSY and the C−H COSY, the position of the hydroxylation was assigned to the C-17 position (Figure 4). Consequently, **1** was identified as 7-fluoro-6-(3-hydroxy-1-sulfo-1,2-cyclohexanedicarboximido)-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (3-OH-SA). The MS data were as follows: TSP-MS (negative) *m/z* 451 $(M - H)^-$,

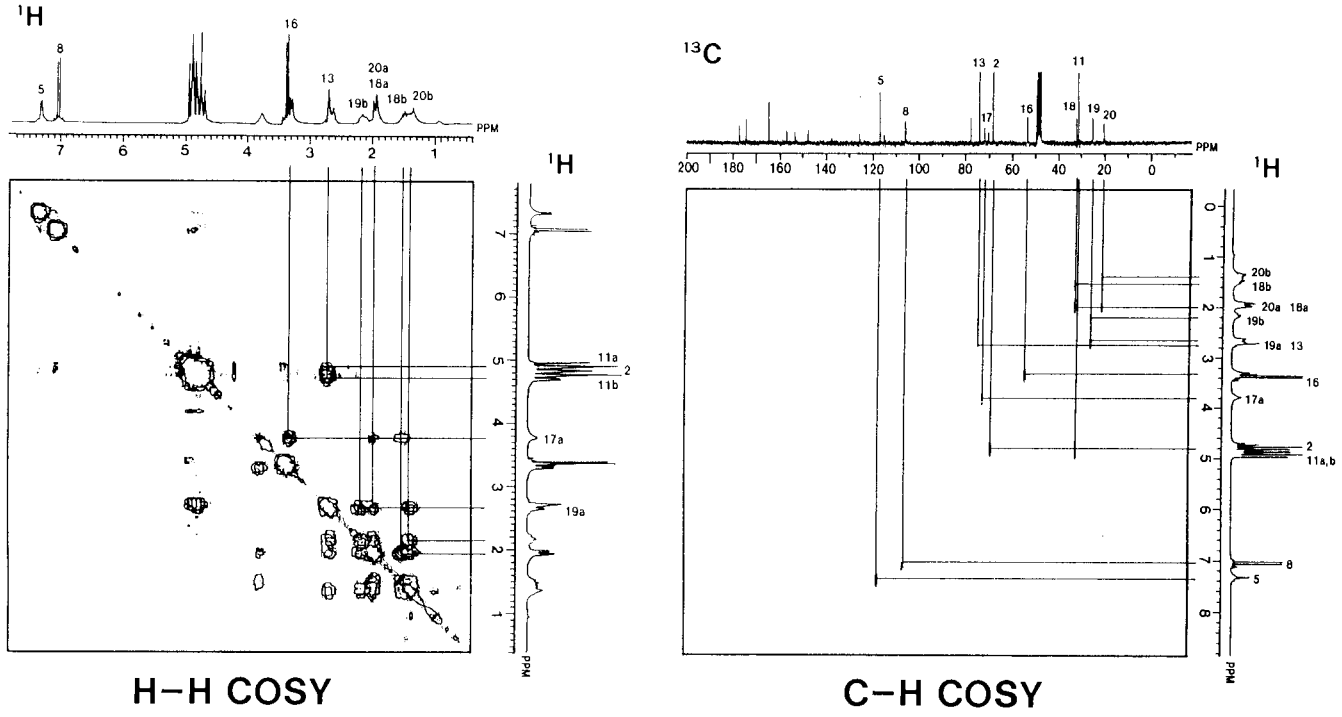

Figure 3. H−H COSY and C−H COSY spectra of metabolite **1**.

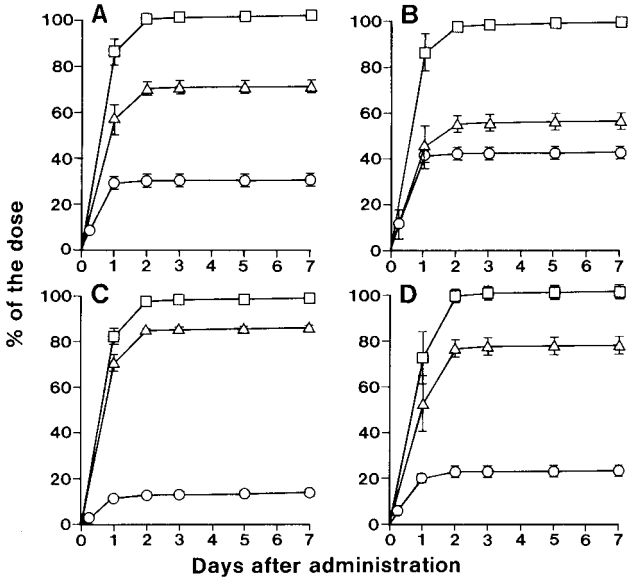

Figure 4. Cumulative ^{14}C excretion in feces and urine of rats after a single oral dose of [*phenyl*-^{14}C]-S-53482: (A) male, at a dose of 1 mg/kg; (B) female, at a dose of 1 mg/kg; (C) male, at a dose of 100 mg/kg; (D) female, at a dose of 100 mg/kg; (□) total; (△) feces; and (○) urine. Results are mean ± SD values ($n = 5$).

370 $(M - H - SO_3H)^-$, 352 $(M - H - SO_3H - H_2O)^-$; SI-MS (negative) *m/z* 451 $(M - H)^-$.

2. Metabolite **2** was isolated by reversed phase preparative TLC using solvent system F with an R_f value of 0.58. The ^1H NMR spectrum showed the presence of a benzoxazinone ring (Table 2). Proton signals of metabolite **2** were very close to those of metabolite **1**. However, the signal at 3.77 ppm in metabolite **1** was shifted to a higher magnetic field at 3.66 ppm in metabolite **2**. Negative mode SI-MS showed the same molecular ion peak at *m/z* 451 $(M - H)^-$ as for metabolite **1**. The incorporation group was considered to be a sulfonic acid moiety and the replacement group a hydroxyl group. From interpretation of the H−H COSY and the C−H COSY spectra, the hydroxyl group was assigned to the C-18 position. Therefore, metabolite **2** was identified as 7-fluoro-6-(4-hydroxy-1-sulfo-1,2-cyclohexanedicarboximido)-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (4-OH-SA). The MS data were as follows: SI-MS (negative) *m/z* 451 $(M - 1)^-$.

3. Metabolite **3** was isolated by reversed phase preparative TLC using solvent system F with an R_f value of 0.73. Proton signals of metabolite **3** were also very close to those of metabolite **1**. However, the signals at 4.75 ppm (H-2a,b), 4.89 ppm (H-11a), and 4.70 ppm (H-11b) in metabolite **1** were shifted to higher magnetic fields at 4.05 ppm (H-2a), 3.81 ppm (H-2b), 4.80 ppm (H-11a), and 4.12 ppm (H-11b), respectively, in metabolite **3** (Table 2). From the information above and the fact that this metabolite is more polar than metabolite **1**, it was considered that the amide linkage in the benzoxazinone ring of metabolite **3** was cleaved. The negative mode TSP-MS and SI-MS showed a deprotonated molecular ion peak at *m/z* 469 $(M - H)^-$. The molecular weight of 470 was 18 mass units larger than that of metabolite **1**. These findings indicated that a sulfonic group was incorporated (82 mass units), the replacement group was a hydroxyl group (16 mass units), and the amide linkage in the benzoxazinone ring was cleaved (18 mass units). From interpretation of the H−H COSY and C−H COSY spectra, the hydroxyl group was assigned to the C-17 position. Consequently, metabolite **3** was identified as 5-fluoro-2-(2-propynylamino)-4-(3-hydroxy-1-sulfo-1,2-cyclohexanedicarboximido)phenoxyacetic acid (3-OH-A-SA). The MS data were as follows: TSP-MS (negative) *m/z* 469 $(M - H)^-$, 388 $(M - H - SO_3H)^-$, 370 $(M - H - SO_3H - H_2O)^-$; SIMS (negative) *m/z* 469 $(M - 1)^-$.

4. Metabolite **4** was isolated by HPLC [flow rate = 3.5 mL/min; mobile phase = methanol/water/acetic acid (60:40:0.1, v/v), retention time = 42 min]. The ^1H NMR spectrum showed the presence of a benzoxazinone ring (Table 3) and seven protons of the tetrahydrophthalim-

ide moiety of the parent compounds with the remaining one shifted to a lower magnetic field, 4.82 ppm. These observations indicated that one proton of the tetrahydrophthalimide moiety was replaced with a functional group. From the molecular ion peak at *m/z* 370 (M^+) in the EI-MS and at *m/z* 371 $(M + 1)^+$ in the TSP-MS, the replacement was considered to be a hydroxyl group. From interpretation of the H−H COSY and the C−H COSY spectra, the position of the hydroxyl group was assigned to the C-17 position. Consequently, metabolite **4** was identified as 7-fluoro-6-(3-hydroxy-3,4,5,6-tetrahydrophthalimido)-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (3-OH-S-53482). The MS data were as follows: EI-MS (70 eV) *m/z* 370 (M^+); TSP-MS (positive) *m/z* 371 $(M + 1)^+$.

5. Metabolite **5** was isolated by HPLC (flow rate = 3.5 mL/min, mobile phase = methanol/water/acetic acid (60:40:0.1, v/v), retention time = 35 min]. The ^{1}H NMR spectrum showed the presence of a benzoxazinone ring (Table 3). The proton signals of metabolite **5** were very close to those of metabolite **4**. The difference in the ^{1}H NMR spectra between **4** and **5** was that the methine proton signal at 4.82 ppm in former was shifted to a higher magnetic field, 4.37 ppm. The EI-MS showed the same molecular ion peak at *m/z* 370 (M^+) as for metabolite **4**. These observations indicated the replacement to be to a hydroxyl group. From interpretation of the H−H COSY and the C−H COSY spectra, the position of the hydroxyl group was assigned to the C-18 position. Therefore, metabolite **5** was identified as 7-fluoro-6-(4-hydroxy-3,4,5,6-tetrahydrophthalimido)-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (4-OH-S-53482). The MS data were as follows: EI-MS (70 eV) *m/z* 370 (M^+); TSP-MS (positive) *m/z* 371 $(M + 1)^+$.

6. Metabolite **6** was isolated by HPLC [flow rate = 5.0 mL/min, mobile phase = methanol/water (50:50, v/v), retention time = 15 min]. In the ^{1}H NMR spectrum, proton signals for the tetrahydrophthalimide moiety were not detected, indicating that the imide linkage was cleaved. In addition, the chemical shifts of H-2 and H-11 protons were different from those of metabolites containing an intact benzoxazinone ring, and these signals were different from those chemical shifts of APF (Table 3). Therefore, it was considered that its amide linkage was cleaved. The proton signal at 2.12 ppm (3H) indicated the presence of an acetyl group. The molecular ion peak at *m/z* 280 (M^+) in the EI-MS and the FD-MS and interpretation of the H−H COSY and the C−H COSY spectra pointed to acetylation of the amino group of the aniline derivative. Consequently, metabolite **6** was identified as 4-(acetylamino)-5-fluoro-2-(2-propynylamino)phenoxyacetic acid (Ac-APFA). The MS data were as follows: EI-MS (70 eV) *m/z* 280 (M^+); FD-MS *m/z* 280 (M^+).

These metabolites were used in the subsequent study as authentic standards.

^{14}C Excretion. Data for ^{14}C excretion in feces and urine during the 7 days after a single oral administration of ^{14}C-S-53482 at low and high doses are shown in Figure 4.

The total ^{14}C recoveries (expressed as percentages relative to the dosed ^{14}C) within a 7 day period were 102.3% (feces, 71.5%; urine, 30.8%) in males and 99.2% (feces, 56.4%; urine, 42.8%) in females of the low-dose group and 98.2% (feces, 85.3%; urine, 13.0%) in males and 101.4% (feces, 78.1%; urine, 23.4%) in females of the high-dose group. The proportion of the urinary ^{14}C excretion in the high-dose group was significantly smaller than that in the low-dose group in both sexes. Urinary ^{14}C excretion in females of both dose groups was larger than that in males. Thus, for both dose groups, ^{14}C excretion was essentially almost complete after 7 days, although the sex- and dose-related differences were observed.

Table 4. ^{14}C Tissue Residues in Male and Female Rats on the Seventh Day after a Single Oral Administration of [*phenyl*-^{14}C]-S-53482 at 1 (Low Dose) or 100 (High Dose) mg/kg[a]

	μg or ng equivalents of S-53482/g of tissue			
	low dose (ppb)		high dose (ppm)	
tissue	male	female	male	female
adrenal	6 ± 0.9	6 ± 0.4	0.23 ± 0.078	0.21 ± 0.058
blood	22 ± 2.1	22 ± 3.5	1.68 ± 0.871	1.53 ± 0.187
blood cell	48 ± 5.3	49 ± 9.0	3.04 ± 1.406	2.82 ± 0.409
plasma	1 ± 0.2	1 ± 0.1	0.05 ± 0.026	0.04 ± 0.006
bone	1 ± 0.2	1 ± 0.1	0.04 ± 0.006	0.04 ± 0.006
bone marrow	3 ± 1.1	2 ± 0.3[b]	0.26 ± 0.171	0.19 ± 0.047
brain	4 ± 0.7	4 ± 0.6	0.07 ± 0.005	0.09 ± 0.016
carcass	6 ± 0.7	5 ± 0.8	0.27 ± 0.033	0.28 ± 0.086
fat	1 ± 0.2[c]	1 ± 0.9[c]	0.08 ± 0.011[d]	0.07 ± 0.014[b]
heart	14 ± 2.5	13 ± 2.9	0.24 ± 0.017	0.27 ± 0.034
kidney	16 ± 2.5	15 ± 2.5	0.40 ± 0.046	0.40 ± 0.035
liver	19 ± 2.5	12 ± 1.6	0.71 ± 0.138	0.52 ± 0.062
lung	9 ± 1.1	8 ± 1.0	0.27 ± 0.031	0.33 ± 0.052
muscle	5 ± 0.4	4 ± 0.8	0.12 ± 0.016	0.15 ± 0.027
pancreas	4 ± 0.3	3 ± 0.9	0.10 ± 0.004	0.17 ± 0.112
spleen	7 ± 0.6	6 ± 2.8	0.24 ± 0.030	0.28 ± 0.082
thyroid	10 ± 2.8[b]	11 ± 1.1[b]	0.33 ± 0.142[d]	0.81 ± 0.749[c]
testis	4 ± 0.3	−[e]	0.12 ± 0.016	−
ovary	−	2 ± 0.7	−	0.11 ± 0.020
uterus	−	1 ± 0.2	−	0.08 ± 0.015

[a] Data show mean ± SD values for five rats. However, data below the detection limit were in each case excluded from the calculations. [b] Values for four rats [c] Values for three rats. [d] Values for two rats. [e] −, not applicable.

^{14}C Tissue Residues. Data for ^{14}C tissue and blood residues on the seventh day after a single oral administration of ^{14}C-S-53482 are shown in Table 4. In both groups, blood cells showed the highest ^{14}C residue levels, which were 48−49 ppb in the low-dose and 2820−3040 ppb in the high-dose group. However, ^{14}C levels in the blood cells in the high-dose group did not increase in proportion to the dose. ^{14}C residue levels in liver were low, approximately one-third to one-fifth of those in the blood cells.

Amounts of Metabolites in Feces and Urine. S-53482 and 36 metabolites were detected and quantified by TLC. Table 5 show the amounts (percent of the dosed ^{14}C) of fecal and urinary metabolites.

Twenty-eight metabolites were detected in the fecal extract. Three fecal polar metabolites were identified as 3-OH-SA, 4-OH-SA, and 3-OH-A-SA (sulfonate metabolites) and five less polar metabolites as APF, 3-OH-S-53482, 4-OH-S-53482, Ac-APFA, and the parent compound. The amounts of the main fecal metabolites, 3-OH-SA, 4-OH-SA, and 3-OH-A-SA were 11.4−12.9, 3.9−4.9, and 1.4−2.8%, respectively, for the low-dose group and 5.7−6.0, 2.4, and 0.9−1.4%, respectively, for the high-dose group. The amounts of APF, 3-OH-S-53482, 4-OH-S-53482, and Ac-APFA were <1.5% in both groups.

Twenty-five metabolites were detected in urine. Major metabolites were APF, 3-OH-S-53482, 4-OH-S-53482, Ac-APFA, and 3-OH-SA, accounting for 0.9−5.4, 2.1−3.0, 1.1−2.1, 3.3−4.3, and 2.1−5.7%, respectively, in the

Table 5. Amounts of Metabolites in Urine and Feces within 2 Days after a Single Oral Administration of [*phenyl*-^{14}C]-S-53482 to Male and Female Rats at 1 (Low Dose) or 100 (High Dose) mg/kg[a]

	amount (% of dosed ^{14}C)			
	male		female	
metabolite	feces	urine	feces	urine
	Low Dose			
S-53482	0.2 ± 0.02	0.1 ± 0.05	0.2 ± 0.14	0.2 ± 0.04
APF	1.6 ± 0.26	0.9 ± 0.28	1.4 ± 0.30	5.4 ± 2.08
3-OH-S-53482	1.1 ± 0.36	2.1 ± 0.52	0.9 ± 0.28	3.0 ± 0.47
4-OH-S-53482	0.9 ± 0.10	1.1 ± 0.15	0.9 ± 0.18	2.1 ± 0.49
Ac-APFA	1.6 ± 0.22	4.3 ± 0.28	0.7 ± 0.12	3.3 ± 0.22
3-OH-SA	12.3 ± 1.49	2.1 ± 0.96	12.9 ± 0.99	5.7 ± 1.64
4-OH-SA	4.1 ± 0.56	1.1 ± 0.23	4.9 ± 1.01	2.1 ± 0.46
3-OH-A-SA	3.0 ± 0.45	1.0 ± 0.13	1.4 ± 0.15	1.0 ± 0.17
others	36.7	17.6	29.1	19.5
unextractable	8.9 ± 1.83	−	2.8 ± 1.12	−
total	70.4 ± 2.92	30.3 ± 2.79	55.2 ± 3.71	42.3 ± 2.78
	High Dose			
S-53482	50.9 ± 2.57	0.1 ± 0.03	46.2 ± 5.58	0.3 ± 0.12
APF	0.9 ± 0.17	0.9 ± 0.40	0.7 ± 0.23	3.3 ± 1.37
3-OH-S-53482	0.8 ± 0.11	1.5 ± 0.44	0.6 ± 0.09	2.5 ± 0.47
4-OH-S-53482	0.8 ± 0.09	1.0 ± 0.14	1.0 ± 0.08	2.2 ± 0.80
Ac-APFA	0.9 ± 0.11	1.3 ± 0.20	0.5 ± 0.04	1.5 ± 0.14
3-OH-SA	5.7 ± 0.42	1.2 ± 0.16	6.0 ± 0.83	2.1 ± 0.45
4-OH-SA	2.4 ± 0.46	0.5 ± 0.03	2.4 ± 0.49	0.7 ± 0.07
3-OH-A-SA	1.4 ± 0.24	0.4 ± 0.06	0.9 ± 0.15	0.5 ± 0.14
others	17.0	5.9	14.9	9.8
unextractable	3.9 ± 0.39	−	3.6 ± 0.45	−
total	84.7 ± 0.83	12.8 ± 1.53	76.8 ± 3.82	22.9 ± 2.65

[a] Data show mean ± SD values for five rats.

low-dose group and 0.9−3.3, 1.5−2.5, 1.0−2.2, 1.3−1.5, and 1.2−2.1%, respectively, in the high-dose group. Other metabolites identified were 4-OH-SA and 3-OH-A-SA, and the amounts of their metabolites were <2.1% in both groups.

The parent compound predominated in both sexes receiving the high dose but not in the low-dose case.

DISCUSSION

In the present study, S-53482 and its metabolites were found to be rapidly and almost completely eliminated from the bodies of both sexes within 7 days, ^{14}C tissue residue levels 1 week post-treatment were generally very low.

On the basis of the metabolites identified in our experiments, the metabolic pathways shown in Figure 5 are proposed for S-53482 in rats. The major biotransformation reactions were concluded to be as follows: (1) hydroxylation of the cyclohexene ring of the 3,4,5,6-tetrahydrophthalimide moiety; (2) cleavage of the imide linkage; (3) cleavage of the amide linkage in the benzoxazinone ring; (4) acetylation of the amino group of the aniline derivative; and (5) incorporation of a sulfonic acid group into the C═C double bond of the 3,4,5,6-tetrahydrophthalimide moiety.

The main metabolites were found to be sulfonate derivatives in the feces and alcohol and acetoanilide derivatives, produced after cleavage of the imino bond, in the urine.

In a separate biliary excretion study, S-53482 was not detected in bile (Y. Tomigahara, 1992, unpublished observation). Therefore, the parent compound detected in the feces here can be considered to have been excreted without absorption into the body. This is likely from the higher proportion of intact S-53482 observed with the high dose, which was presumably beyond the capacity for absorption. Data for the extents of metabolic transformations are presented in Table 6. The fact that the high-dose group showed a lower extent of metabolism could be explained by a lower absorption rate of the test compound from the gastrointestinal tract.

Sulfonic derivatives were also found in metabolic studies of other compounds containing the same tetrahydrophthalimide moiety (Yoshino et al., 1993a,b; Tomigahara et al., 1994b,c; Matsunaga et al., 1996, 1997). Sulfonic acid conjugation makes chemicals more polar and therefore facilitates rapid excretion, like other

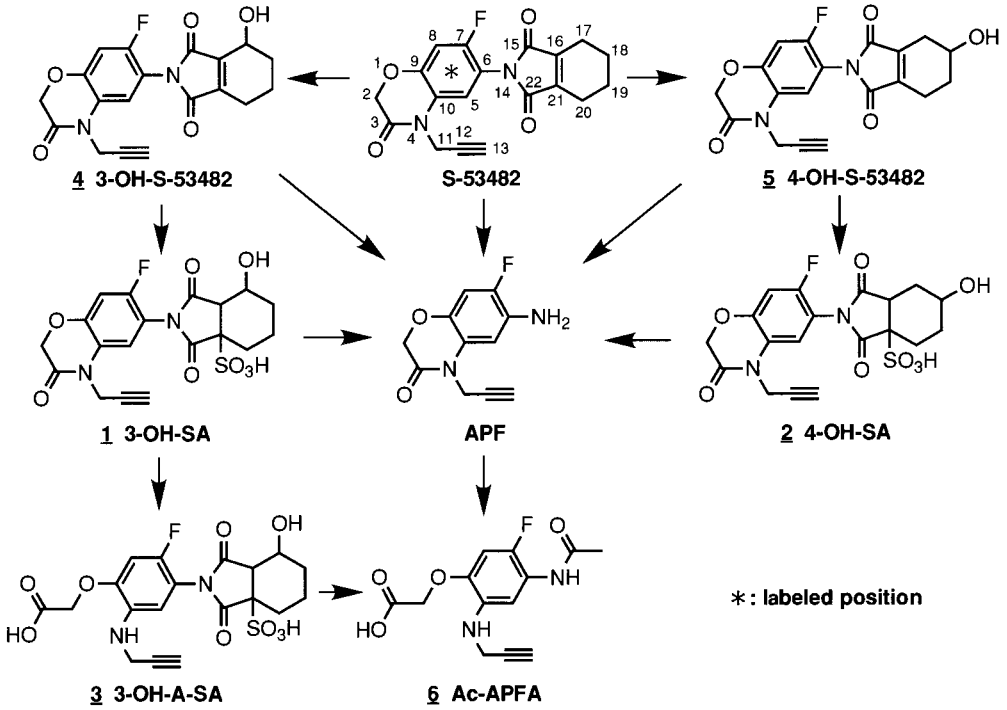

Figure 5. Proposed metabolic pathways for [*phenyl*-^{14}C]-S-53482 in the rat.

Table 6. Extent of Metabolic Transformations[a]

	% of dosed ^{14}C			
	low dose		high dose	
metabolic transformation	male	female	male	female
imide moiety cleavage	8.4	10.8	4.0	6.0
amide moiety cleavage	9.9	6.4	4.0	3.4
hydroxylation	28.8	34.9	15.7	18.9
acetylation	5.9	4.0	2.2	2.0
sulfonic acid conjugation	23.6	28.0	11.6	12.6

[a] Data were calculated from the values for identified metabolites in Table 5.

hydrophilic conjugation reactions. The mechanism of formation of sulfonic acid derivatives has been investigated using ^{35}S-labeled Na_2SO_4, cystein, and glutathione (Yoshino et al., 1993a). It was suggested that HSO_3^- is derived from SO_4^- (sodium or other cation salt) by probable microflora in a rat intestine and added nucleophilically to the C═C double bond nonenzymatically. Matsunaga et al. (1996) found that sulfurous acid nonenzymatically attacked the C═C double bond of the tetrahydrophthalimide moiety of some imide pesticides under alkaline conditions and supported the above proposed mechanism.

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