Metabolism of 7-Fluoro-6-(3,4,5,6-tetrahydrophthalimido)-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (S-53482, Flumioxazin) in the Rat: II. Identification of Reduced Metabolites

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On single oral administration of ^{14}C-S-53482 [7-fluoro-6-(3,4,5,6-tetrahydrophthalimido)-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one, Flumioxazin] labeled at the 1- and 2-positions of tetrahydrophthaloyl group to rats at 1 (low dose) or 100 (high dose) mg/kg, the radiocarbon was almost completely eliminated within 7 days after administration in both groups with generally very low residual ^{14}C tissue levels. The predominant excretion route was via the feces. The major fecal and urinary metabolites involved reduction or sulfonic acid addition reactions at the 1,2-double bond of the 3,4,5,6-tetrahydrophthalimide moiety and hydroxylation of the cyclohexene or cyclohexane ring. One urinary and four fecal metabolites were identified using chromatographic techniques and spectroanalyses (NMR and MS). Three of five identified metabolites were unique forms, reduced at the 1,2-double bond of the 3,4,5,6-tetrahydrophthalimide moiety. On the basis of the metabolites identified in this study, the metabolic pathways of S-53482 in rats are proposed. To specify tissues forming reduced metabolites, an in vitro study was conducted. Reduction was found to take place in red blood cells.

Keywords: *Metabolism; flumioxazin; identification; reduction; double bond; reduced metabolite; blood cell; biotransformation*

INTRODUCTION

7-Fluoro-6-(3,4,5,6-tetrahydrophthalimido)-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (S-53482, Flumioxazin) is a new selective post-emergence herbicide for the control of annual broad-leaved weeds. In particular, 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). Major crops of usage of S-53482 are soybeans. In an earlier report (Tomigahara et al., 1999), we documented findings for metabolism of S-53482 in rats using ^{14}C-preparations labeled in the phenyl ring. However, extensive identification of metabolites has not been previously performed. Therefore, the present study was conducted with the objectives of (1) identifying urinary and fecal metabolites derived from the 3,4,5,6-tetrahydrophthalimide moiety and (2) comparing metabolic fates for radioactive label in the tetrahydrophthalimide moiety and the phenyl ring. This report deals with reduced metabolites of S-53482 in rats.

MATERIALS AND METHODS

Chemicals. S-53482 labeled at the 1- and 2-positions of the tetrahydrophthaloyl group ([*THP*-^{14}C]-S-53482, 3.93 GBq/mmol) was synthesized in our laboratory (Figure 5). The labeled preparation was purified by preparative TLC development in benzene/ethyl acetate 4/1 (v/v) prior to use. The radiochemical purity was more than 99% as confirmed by TLC analysis. Unlabeled S-53482 (99.6% purity) was also synthesized in our laboratory. 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), 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), 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), 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), and 5-fluoro-2-(2-propynylamino)-4-(3-hydroxy-1-sulfo-1,2-cyclohexanedicarboximido)phenoxyacetic acid (3-OH-A-SA) were purified from feces and urine of rats administered S-53482 labeled with ^{14}C in the phenyl ring ([*phenyl*-^{14}C]S-53482), identified in our previous study (Tomigahara et al., 1999) and used as authentic standards for the present investigation. The authentic standards were purified by preparative TLC prior to use, and their radiochemical purities were more than 98% as confirmed by TLC analysis.

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) 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 acetate (1/2, v/v) ×2, and (E) benzene saturated with formic acid/ethyl acetate/diethyl ether (5/2/1, 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 using SB-5 films (Kodak, Rochester, NY) or imaging plates (Fuji Photo Film Co. Ltd., Japan). The SB-5 films were placed in contact with TLC plates for about 1 week at 4 °C and then developed with a Model M6B processor (Kodak). The imaging plates were exposed to the TLC plates for 1 h or 1 day at room temperature and then processed 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 (Tomi-

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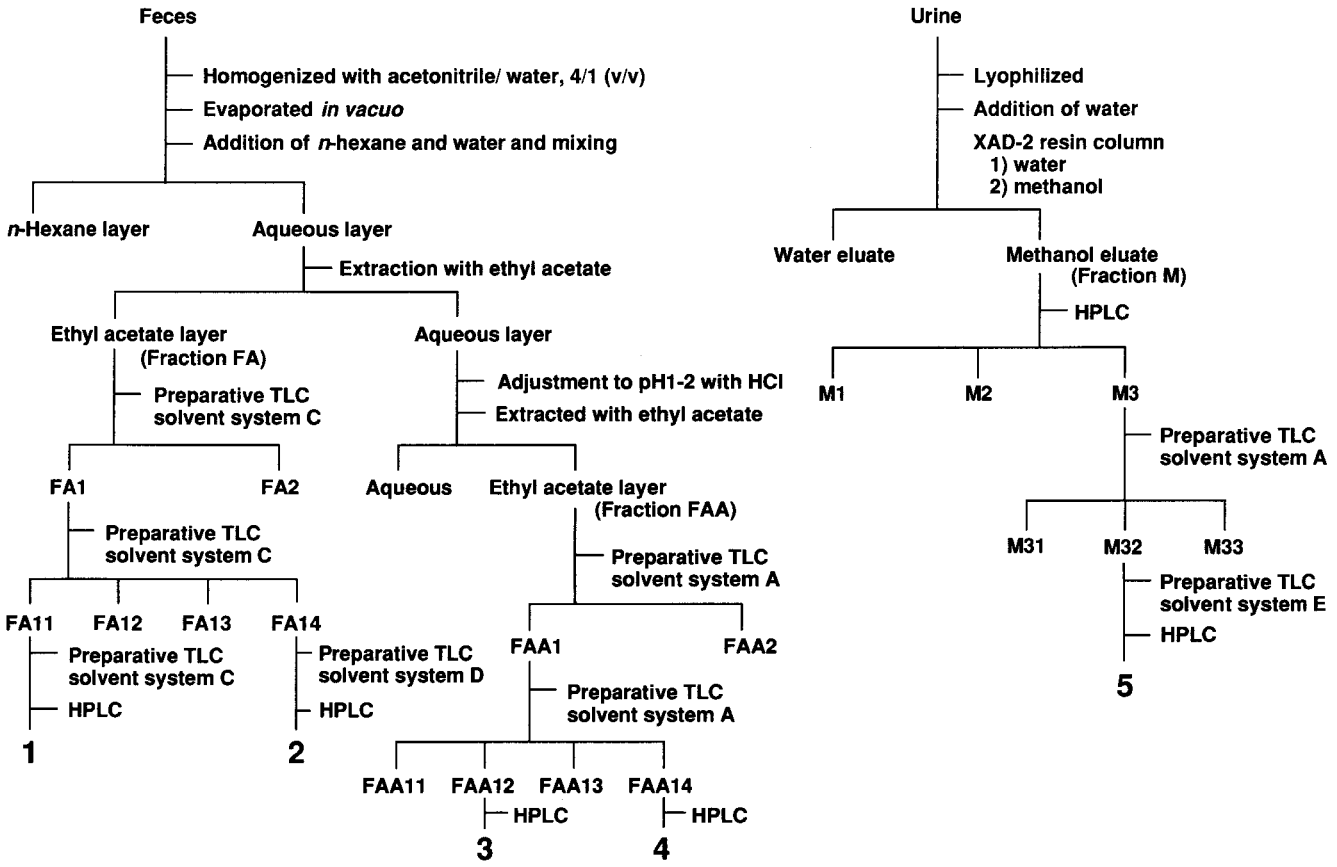

Figure 1. Flow diagram for steps in the purification of fecal and urinary metabolites.

gahara 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 id × 250 mm, YMC Co., Ltd., Japan) and a YMC-Pack GI-340-15 (ODS, 20 mm id × 50 mm, YMC Co., Ltd., Japan) were used as the analytical column and the guard column, respectively.

Spectrometry. Nuclear magnetic resonance (NMR) spectra were obtained on a JEOL GSX-270 spectrometer (JEOL Ltd., Japan) operating at 270 MHz for ^{1}H and 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), C−H Correlation Spectroscopy (C−H COSY, Maudsley et al., 1977), and Heteronuclear Multiple Quantum Coherence Spectroscopy (HMQC, Mueller, 1979)] were obtained with the data process program PLEXUS V1.6 (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), benzene-d_6 (99.8%, E. Merck), and chloroform-*d* (99.5%, E. Merck) were used as solvents for NMR. Secondary ion mass spectrometry (SI-MS) was performed with a Hitachi M-80B mass spectrometer (Hitachi Ltd.). The accelerating voltage was 3.0 kV. Samples were introduced in a glycerol matrix. Spectra were recorded in positive or negative ion mode.

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. Ten 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. Tissues were removed from four males for the in vitro study.

Identification Study. To collect sufficient amounts of unknown metabolites in urine and feces for spectroanalytical identification, we dissolved a total of about 1.5 g of [*THP*-^{14}C]S-53482 in corn oil and dosed it orally to 10 male Sprague−Dawley rats at the age of 7 weeks old for 7 consecutive days at 100 mg/kg/day. The specific activity of [*THP*-^{14}C]S-53482 was adjusted to 13.1 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. [*THP*-^{14}C]S-53482 was dissolved in corn oil at 1 (low dose) or 100 (high dose) mg/5 mL and administered orally to five males and five females per dose at 5 mL/kg. 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 (Sugiyamagen Iriki Co., Ltd.) to allow collection of expired air, urine, and feces separately. Expired air was passed through a 10% NaOH solution, and the included radioactive carbon dioxide was trapped for 2 days. On the seventh day after dosing, rats were killed by collection of blood from the abdominal aorta under light anesthesia with diethyl ether and a total of 23−24 organs/tissues were removed from each animal.

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

All collected feces were mixed and homogenized with a 3-fold volume of a mixture of acetonitrile/water, 8/2 (v/v) 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 a RR14A-159 Rotor (Hitachi Ltd.). Residues were further extracted twice with acetonitrile/water, 8/2 (v/v) in the same manner. At the time, the qualitative analysis was conducted and it was confirmed that the kinds of metabolites were the same as the metabolites obtained from the single dose studies (low dose and high dose). The fecal acetonitrile extracts were concentrated and partitioned three times between *n*-hexane and water to remove the parent compound and fecal materials. In addition, the aqueous solution was extracted three times with ethyl acetate (fraction FA) and extracted three times with

Table 1. Conditions Used for Final Isolation (Purification) of Metabolites[a]

metabolite	solvent (methanol/water/formic acid) (v/v)	flow rate (mL/min)	retention time (min)
1	68/32/0	5.0	26
2	55/45/0	5.0	16
3	45/55/1	5.0	29
4	45/55/1	5.0	27
5	55/45/1	5.0	24

[a] Column size: 20 mm id × 250 mm (ODS).

ethyl acetate (fraction FAA) after adjustment to pH 1−2 by addition of 1 N HCl aqueous solution. Fraction FA was separated into two portions by preparative TLC using a solvent system C (fractions FA1 and FA2). Fraction FA1 was then separated further into four portions by the same method (fractions FA11−FA14). As unknowns, metabolite **1** was detected in fraction FA11 and **2** in fraction FA14. Metabolite **1** was purified by preparative TLC using solvent system C and then further by HPLC after pretreatment with a SEP-PAK C18 cartridge (Waters, Milford, MA). Metabolite **2** was purified by preparative TLC using solvent system D and purified further by HPLC. Fraction FAA was separated into two portions by preparative TLC using solvent system A (fractions FAA1 and FAA2). Fraction FAA1 was then separated further into four portions by the same method (fractions FAA11-FAA14). Fractions FAA12 and FAA14 were subsequently purified by HPLC to give metabolites **3** and **4**, respectively.

Collected urine samples were combined and then lyophilized using a FD-81 freeze-dryer (Tokyo Rikakikai Co., Ltd., Japan). After addition of water (200 mL), the residue was chromatographed on Amberlite XAD-2 resin (Organo, Japan), washed with water (1000 mL) and eluted with methanol (1000 mL). At the time, the qualitative analysis was conducted and it was confirmed that kinds of metabolites were the same as the metabolites obtained from the single dose studies. The methanol eluates were evaporated, and the residue (fraction M) was separated into three portions (fractions M1 (10−17 min), M2 (17−23 min), and M3 (23−30 min)) by HPLC. The gradient conditions used were the following: start with 30% methanol/70% water, hold for 5 min, a linear gradient to 90% methanol/10% water from 5 to 15 min, to 100% methanol from 15 to 25 min, and then hold at 100% methanol for 25 to 30 min. The flow rate was 5 mL/min. Fraction M3 was separated into three portions by preparative TLC using solvent system A (fractions M31−M33), and fraction M32 was then purified using solvent system E and finally by HPLC to give metabolite **5**.

The conditions used for final isolation (purification) of metabolites are summarized in Table 1. Purified 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 directly by conventional liquid scintillation counting (LSC). The radioactivity of fecal homogenates and unextractable fecal residues was quantified using the combustion method. For this purpose, the 0−1 and 1−2 day feces of rats were extracted three times on each collection day with 50−100 mL of acetonitrile/water, 8/2 (v/v), and the extracts and residues were radioassayed. The other feces were homogenized with water and the homogenates combusted for radioassay.

^{14}C-Amounts in 10% NaOH solutions of collected expired CO_2 were measured as follows: the total amount of 10% NaOH solution was measured and two aliquots (1 mL) were radioassayed by LSC using HIONIC−FLUOR (Packard, Meriden, CT) as a scintillator.

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 (ca. 200 mg) was combusted for radioanalysis. Tissue samples were analyzed individually. The ^{14}C-residue levels were expressed as microgram or nanogram equivalents of S-53482/g wet tissue (ppm or ppb, respectively).

Table 2. TLC R_f Values of Authentic Standards and Identified Metabolites

metabolite	compound	R_f value[a] solvent system A	solvent system B	solvent system C
	S-53482	0.71	*b*	0.76
	3-OH-S-53482	0.52	*b*	0.35
	4-OH-S-53482	0.47	*b*	0.20
	3-OH-SA	0.03	0.43	*c*
	4-OH-SA	0.02	0.37	*c*
	3-OH-A-SA	*c*	0.24	*c*
1	SAT-482	0.62	0.99	0.53
2	4-OH-SAT-482	0.34	0.96	0.11
3	THPA	0.40	0.70	0.01
4	1-OH-HPA	0.30	0.58	*c*
5	3-OH-SAT-482	0.43	0.99	0.20

[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.

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 co-chromatography (solvent systems A−C) with authentic standards and quantified by the TLC scraping method using solvent systems A and B.

***In Vitro* Metabolism Study To Specify Tissues Forming Reduced Metabolites.** Blood, cells and plasma, liver, and kidneys were isolated to investigate which tissues formed reduced metabolites. Samples were prepared as reported previously (Tomigahara et al., 1997).

Blood was obtained from four male rats by decapitation with heparin as an anticoagulant, and separated into blood cell and plasma by centrifugation with a HimacR CR5DL centrifuge and a RS3S rotor at 3000 rpm (1500*g*) for 10 min (Hitachi Ltd., Japan). Each was then diluted with 50 mM phosphate buffer to give a final concentration of 50% (w/v).

The liver and kidneys of each animal were removed after decapitation. Four rat livers or kidneys were combined and homogenized in a Teflon-glass Potter-Elvehjem homogenizer with 50 mM phosphate buffer at a final concentration of 20% (w/v).

To specify tissues forming reduced metabolites, the following in vitro study was conducted: aliquots of about 5 μmol of [*THP*-^{14}C]S-53482 in 10 μL of acetone and/or NADPH (3 mg) were applied to 1 mL samples of 20% tissue homogenate or 50% blood cell or plasma preparations followed by incubation for 2 h at 37 °C. The reaction mixture without the 20% tissue homogenates or 50% blood cell or plasma preparations was added to 1 mL aliquots of 50 mM phosphate buffer and incubated as a control. The reactions were terminated by adding 2 mL of ice-cold ethanol. Denatured proteins were removed by centrifugation at 1500*g* for 10 min. Aliquots of the supernatant fraction were subjected to TLC using solvent systems A and C to allow qualitative analysis.

RESULTS

Identification of Metabolites. The following 5 metabolites were purified and identified by spectroanalyses (NMR and MS). The R_f values and chemical structures of the identified metabolites are summarized in Table 2 and Figure 5, respectively. The NMR data of the identified metabolites are shown in Tables 3 and 4.

Metabolite 1. Proton signals at 7.01 ppm (1H, d, J = 6.6 Hz), 6.91 ppm (1H, d, J = 9.2 Hz), 4.68 ppm (2H, s), 4.66 ppm (2H, d, J = 2.6 Hz), and 2.30 ppm (1H, t, J = 2.6 Hz) indicated the presence of a benzoxazinone ring. The ^1H NMR spectrum exhibited the presence of eight protons (1.94 ppm; 4H, m, and 1.51 ppm; 4H, m)

Table 3. ^{1}H and ^{13}C NMR Data for S-53482 and Metabolites 1, 3, and 4

^{1}H NMR Data

	S-53482		**1**		**3**		**4**	
proton no.	δ ($CDCl_3$)	J (Hz)	δ ($CDCl_3$)	J (Hz)	δ (CD_3OD)	J (Hz)	δ (CD_3OD)	J (Hz)
2	4.65(2H,s)		4.68(2H,s)		*a*		*a*	
5	7.04(d)	6.9[b]	7.01(d)	6.6[b]	*a*		*a*	
8	6.89(d)	9.9[b]	6.91(d)	9.2[b]	*a*		*a*	
11	4.67(2H,d)	2.6	4.66(2H,d)	2.6	*a*		*a*	
13	2.29(t)	2.6	2.30(t)	2.6	*a*		*a*	
16	*a*		3.10(m)		*a*		2.92(dd)	9.9, 4.5
17a	2.44(2H,m)		1.94(2H,m)		2.37(2H,m)		2.02(brd)	14.3
17b							1.93−1.30(m)	
18	1.83(2H,m)		1.51(2H,m)		1.72(2H,m)		1.93−1.30(2H,m)	
19	1.83(2H,m)		1.51(2H,m)		1.72(2H,m)		1.93−1.30(2H,m)	
20	2.44(2H,m)		1.94(2H,m)		2.37(2H,m)		1.93−1.30(2H,m)	
21	*a*		3.10(m)		*a*		*a*	

^{13}C NMR Data

	S-53482		**1**		**3**		**4**	
carbon no.	δ ($CDCl_3$)	J (Hz)	δ ($CDCl_3$)	J (Hz)	δ (CD_3OD)	J (Hz)	δ (CD_3OD)	J (Hz)
2	67.4		67.5		*c*		*c*	
3	162.9		163.0		*c*		*c*	
5	116.1(d)	2.1[d]	115.6(d)	2.1[d]	*c*		*c*	
6	113.6(d)	14.5[d]	114.1(d)	14.5[d]	*c*		*c*	
7	154.1(d)	250.1[d]	153.9(d)	256.5[d]	*c*		*c*	
8	105.9(d)	24.9[d]	106.1(d)	25.0[d]	*c*		*c*	
9	146.0(d)	11.4[d]	146.5(d)	11.5[d]	*c*		*c*	
10	124.6(d)	3.1[d]	124.8		*c*		*c*	
11	30.8		30.8		*c*		*c*	
12	76.7		78.0		*c*		*c*	
13	73.2		73.4		*c*		*c*	
15	169.0		178.0		171.6		177.7[e]	
16	142.3		40.4		136.6		62.3	
17	21.2		24.0		27.3		26.1	
18	20.2		21.8		22.4		18.3	
19	20.2		21.8		22.4		25.6	
20	21.2		24.0		27.3		36.5	
21	142.3		40.4		136.6		73.5	
22	169.0		178.0		171.6		183.2[e]	

[a] No proton. [b] H−F coupling. [c] No carbon. [d] C−F coupling. [e] Interchangeable.

of the tetrahydrophthaloyl moiety. The signal at 3.10 ppm (2H, H-16, and H-21) in metabolite **1** was not detected in the ^{1}H NMR spectrum of the parent compound. In the ^{13}C NMR spectrum, two carbon signals (C-16 and C-21) at 142.3 ppm of the parent compound are shifted to 40.4 ppm. The observations indicated that the double bond of the 3,4,5,6-tetrahydrophthalimide moiety was saturated. Positive or negative ion mode SI-MS gave a protonated or a deprotonated molecular ion peak at m/z 357 $(M + H)^+$ or 355 $(M - H)^-$. The molecular weight of 356 was 2 mass units larger than that of the parent compound (354). Consequently, **1** was identified as 6-(1,2-cyclohexanedicarboximido)-7-fluoro-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (SAT-482).

Metabolite 2. H−H COSY and HMQC spectra for metabolite **2** are shown in Figure 2. The ^{1}H NMR spectrum showed a presence of the benzoxazinone ring. The signal at 3.13 ppm (2H, H16, and H21) was the same as that of metabolite **1** (3.10 ppm) in the ^{1}H NMR spectrum, and in the ^{13}C NMR spectrum, signals at 39.2 ppm (C-16) and 38.5 ppm (C-21) were close to those of metabolite **1** (both, 40.4 ppm), again indicating that the double bond of the 3,4,5,6-tetrahydrophthalimido moiety was saturated. The proton signal at 4.05 ppm was not detected in the ^{1}H NMR spectrum of the parent compound or the metabolite **1**. This indicated that one proton of the resultant 1,2-cyclohexanedicarboximido moiety was replaced with a functional group. From the protonated molecular ion peak at m/z 373 $(M + H)^+$ in the positive ion mode SI-MS and the deprotonated molecular ion peak at m/z 371 $(M - H)^-$ in the negative ion mode SI-MS, the replacement group was considered to be a hydroxyl group. (The molecular weight of 372 was 16 mass units larger than that of metabolite **1** (356).) From the interpretation of the H−H COSY and the HMQC spectra, the position of the hydroxyl group was assigned to the C-18 position (Figure 2). Consequently, the metabolite **2** was identified as 7-fluoro-6-(4-hydroxy-1,2-cyclohexanedicarboximido)-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (4-OH-SAT-482).

Metabolite 3. The ^{1}H NMR spectrum showed the absence of a benzoxazinone ring group, while exhibiting the presence of eight protons (2.37 ppm; 4H, m, and 1.72 ppm; 4H, m). The ^{13}C NMR spectrum indicated the presence of eight carbons. Negative ion mode SI-MS showed a deprotonated molecular ion peak at m/z 169 $(M - H)^-$. The signals of NMR and MS spectra were the same as those of 3,4,5,6-tetrahydrophthalic acid (THPA) purified from the feces of rats administered *trans*-[alcohol-^{14}C]tetramethrin in a previous study (Tomigahara et al., 1994a). Consequently, **3** was identified to be THPA.

Metabolite 4. In the ^{1}H NMR spectrum, the proton signals of a benzoxazinone ring group were not detected and no UV absorption (230−320 nm) was observed. The ^{1}H NMR spectrum exhibited nine protons. The signal at 2.92 ppm (1H) indicated that a functional group was incorporated into the double bond of the 3,4,5,6-tetrahydrophthalimide moiety so that it was saturated. The ^{13}C NMR spectrum exhibited eight carbons. Negative ion

Table 4. ^{1}H and ^{13}C NMR Data for Metabolites 2 and 5

^{1}H NMR Data

	2				5	
proton no.	δ ($CDCl_3$)	J (Hz)	δ (C_6D_6)	J (Hz)	δ (C_6D_6)	J (Hz)
2	4.68(2H,s)		3.88(2H,s)		3.88(2H,s)	
5	7.03(d)	6.6[a]	7.10(d)	6.6[a]	7.04(d)	7.3[a]
8	6.90(d)	9.2[a]	6.55(d)	8.6[a]	6.54(d)	9.9[a]
11	4.66(2H,d)	2.6	4.10(2H,d)	3.3	4.09(2H,d)	3.3
13	2.29(t)	2.6	1.78(t)	2.6	1.76(t)	2.6
16	3.13(m)		2.28(m)		2.41(m)	
17a	2.38−1.20(2H,m)		2.30−1.00(2H,m)		3.76(brs)	
17b					*b*	
18a	4.05(brs)		3.29(brs)		2.10−0.90(2H,m)	
18b	*b*		*b*			
19	2.38−1.20(2H,m)		2.30−1.00(2H,m)		2.10−0.90(2H,m)	
20	2.38−1.20(2H,m)		2.30−1.00(2H,m)		2.10−0.90(2H,m)	
21	3.13(m)		2.28(m)		2.41(m)	

^{13}C NMR Data

	2		5	
carbon no.	δ ($CDCl_3$)	J (Hz)	δ (C_6D_6)	J (Hz)
2	67.5		67.6	
3	162.9		162.6	
5	115.5		115.9	
6	112.6		112.0	
7	153.8(d)	256.5[c]	156.0(d)	250.1[c]
8	106.4(d)	24.9[c]	106.5(d)	24.9[c]
9	147.2(d)	11.5[c]	147.2(d)	11.5[c]
10	124.7		124.7	
11	31.0		31.6	
12	77.9		77.9	
13	73.4		73.6	
15	177.5		177.2	
16	39.2		40.6	
17	31.0		69.5	
18	65.4		31.0	
19	29.7		19.8	
20	31.6		22.9	
21	38.5		49.4	
22	177.5		177.2	

[a] H−F coupling. [b] No proton. [c] C−F coupling.

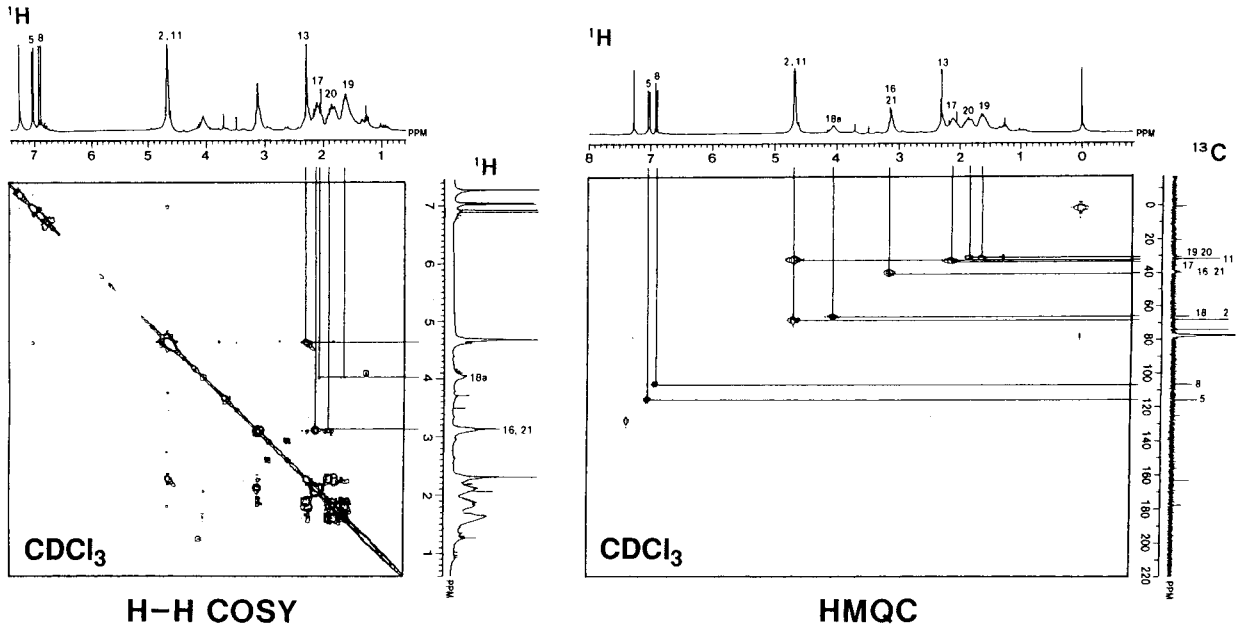

Figure 2. H−H COSY and HMQC spectra of metabolite 2.

mode SI-MS showed a deprotonated molecular ion peak at m/z 187 ($M - H)^-$. These findings indicate the functional group incorporated into the double bond to be a hydroxyl group. (The molecular weight of 188 was 18 mass units (−OH + H) larger than that of metabolite **3** (170).) The signals of NMR and MS spectra were the same as those of 1-hydroxy-1,2-cyclohexanedicarboxylic acid (1-OH-HPA) purified from the feces of rats adminis-

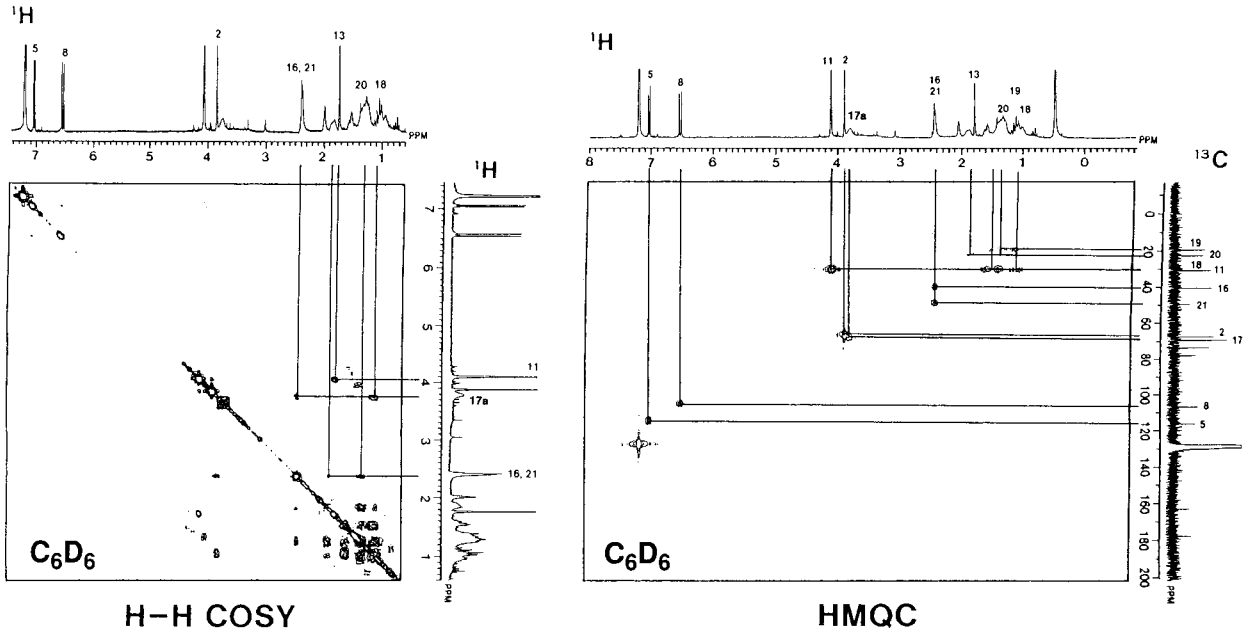

Figure 3. H−H COSY and HMQC spectra of metabolite **5**.

tered *trans*-[alcohol-^{14}C]tetramethrin in the previous study (Tomigahara et al., 1994a). Consequently, **4** was identified to be 1-OH-HPA.

Metabolite 5. H−H COSY and HMQC spectra for metabolite **5** are shown in Figure 3. The ^{1}H NMR spectrum showed the presence of a benzoxazinone ring. The proton signals of metabolite **5** were very close to those of metabolite **2** in C_6D_6. The observation indicated that the double bond of the 3,4,5,6-tetrahydrophthalimido moiety was saturated and one proton of the resultant 1,2-cyclohexanedicarboximido moiety was replaced with a functional group. From the protonated molecular ion peak at *m*/*z* 373 $(M + H)^+$ in the positive ion mode SI-MS and the deprotonated molecular ion peak at *m*/*z* 371 $(M - H)^-$ in the negative ion mode SI-MS, the replacement group was considered to be a hydroxyl group. (The molecular weight of 372 was the same as that of metabolite **2**.) In the ^{13}C NMR spectrum, the C-17 signal at 31.0 ppm in metabolite **2** was shifted to a lower magnetic field, 69.5 ppm, in metabolite **5** and the C-18 signal at 65.4 ppm in metabolite **2** was shifted to 31.0 ppm in metabolite **5**. From that mentioned above and the interpretation of the H−H COSY and the HMQC spectrum, the hydroxyl group was assigned to the C-17 position. Consequently, metabolite **5** was identified to be 7-fluoro-6-(3-hydroxy-1,2-cyclohexanedicarboximido)-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (3-OH-SAT-482).

^{14}C-Excretion. Data for ^{14}C-excretion in feces and urine during the 7 days after a single oral administration of [*THP*-^{14}C]S-53482 at low and high doses are shown in Table 5. For both dose groups, ^{14}C-excretion was essentially almost complete after 7 days. These data are very similar to those obtained in a [*phenyl*-^{14}C]S-53482 study (Tomigahara et al., 1999). No ^{14}C was found in expired air at any time point.

^{14}C-Tissue Residues. Data for ^{14}C-tissue and blood residues on the seventh day after a single oral administration of [*THP*-^{14}C]S-53482 are shown in Table 6. In both groups, blood cells showed the highest ^{14}C-residue levels, but direct proportionability with the dose was not observed. ^{14}C-Residue levels in kidney, heart, and liver were low, approximately one-third to one-fifth of those in the blood cells. In the other tissues values were lower than 11 ppb in the low dose group and 0.29 ppm in the high dose group. These data were very similar to those obtained in the [*phenyl*-^{14}C]S-53482 study.

Amounts of Metabolites in Feces and Urine. S-53482 and 29 metabolites were detected and quantified in urine and feces. The results are summarized in Table 7 (% of the dosed ^{14}C).

When the 0−2 day pooled fecal extracts after administration of [*THP*-^{14}C]S-53482 were subjected to TLC analyses using solvent systems A and B, at least 11 and 17 metabolites were detected, respectively. Polar metabolites retained at the origin on TLC using solvent system A were well separated using solvent system. Five fecal polar metabolites were identified as THPA, 1-OH-HPA, 3-OH-SA, 4-OH-SA, and 3-OH-A-SA (-SA; sulfonate metabolites), and six less polar metabolites as SAT-482, 3-OH-S-53482, 4-OH-S-53482, 3-OH-SAT-482, 4-OH-SAT-482 (-SAT-; reduced metabolites), and the parent compound. The amounts of the main fecal metabolites, 3-OH-SA and 4-OH-SA, were 11.4% and 5.2%, respectively, in males and 11.5% and 5.4% in females for the low dose group, and 3.8% and 1.6% in males and 3.9% and 1.5% in females for the high dose group. The amounts of the SAT-482, 3-OH-S-53482, 4-OH-S-53482, 3-OH-SAT-482, 4-OH-SAT-482, THPA, 1-OH-HPA, and 3-OH-A-SA were less than 2.5% in both groups. The amounts of the parent compound were 1.4−2.2% in the low dose group and 62.7−65.9% in the high dose group. A tendency for metabolism was also observed in the [*phenyl*-^{14}C]S-53482 study.

When the 0−2 day pooled urine was subjected to TLC using solvent systems A and B, at least 11 and 16 metabolites were detected, respectively. Major metabolites were 3-OH-S-53482, 4-OH-S-53482, and 3-OH-SA, accounting for 4.1%, 4.2%, and 4.8%, respectively, in males and 3.9%, 4.6%, and 6.1% in females for the low dose group, and 2.1%, 1.6%, and 1.9% in males and 2.0%, 2.3%, and 2.3% in females for the high dose group. Other metabolites identified were 3-OH-SAT-482, THPA, 1-OH-HPA, 4-OH-SA, and 3-OH-A-SA, their amounts being less than 2.4% in both groups. The amounts of

Table 5. Cumulative ^{14}C-Excretion into Feces and Urine within 7 Days after Single Oral Administration of [*THP*-^{14}C]S-53482 to Rats at 1 (Low Dose) or 100 (High Dose) mg/kg[a]

	% of dosed ^{14}C (time after administration)					
	0−6 hr	0−1 day	0−2 day	0−3 day	0−5 day	0−7 day
			Low Dose			
male						
feces	*b*	47.2 ± 12.33	64.3 ± 2.47	65.3 ± 2.11	65.7 ± 2.09	65.8 ± 2.09
urine	16.2 ± 2.98	29.0 ± 1.83	30.0 ± 1.89	30.2 ± 1.91	30.6 ± 1.92	30.7 ± 1.92
e.air[c]	*b*	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
total	16.2 ± 2.98	76.3 ± 11.51	94.3 ± 0.62	95.5 ± 0.53	96.2 ± 0.54	96.5 ± 0.53
female						
feces	*b*	36.1 ± 13.94	57.4 ± 5.05	59.1 ± 3.81	59.5 ± 3.77	59.6 ± 3.76
urine	19.9 ± 4.80	34.5 ± 3.12	35.8 ± 3.56	36.1 ± 3.58	36.6 ± 3.58	36.8 ± 3.58
e.air[c]	*b*	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
total	19.9 ± 4.80	70.6 ± 11.51	93.2 ± 1.58	95.2 ± 0.33	96.1 ± 0.30	96.4 ± 0.28
			High Dose			
male						
feces	*b*	72.8 ± 10.33	87.1 ± 4.08	87.3 ± 4.04	87.5 ± 4.01	87.5 ± 4.00
urine	3.4 ± 0.84	10.8 ± 0.96	11.6 ± 1.29	11.7 ± 1.32	11.8 ± 1.34	11.8 ± 1.34
e.air[c]	*b*	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
total	3.4 ± 0.84	83.6 ± 9.66	98.7 ± 3.30	99.0 ± 3.29	99.3 ± 3.29	99.4 ± 3.29
female						
feces	*b*	56.5 ± 13.22	82.6 ± 2.08	83.3 ± 1.80	83.4 ± 1.79	83.4 ± 1.79
urine	4.5 ± 1.06	12.5 ± 1.17	13.7 ± 1.42	13.8 ± 1.42	14.0 ± 1.42	14.1 ± 1.41
e.air[c]	*b*	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
total	4.5 ± 1.06	69.0 ± 13.50	96.2 ± 1.83	97.1 ± 1.61	97.3 ± 1.60	97.5 ± 1.60

[a] Data show mean ±S. D. values for five rats. [b] Not analyzed. [c] Expired air was collected until 2 days after administration.

Table 6. ^{14}C-Tissue Residues in Male and Female Rats on the Seventh Day after Single Oral Administration of [*THP*-^{14}C]S-53482 at 1 (Low Dose) or 100 (High Dose) mg/kg[a]

	μg or ng S-53482/g of tissue			
	low (ppb)		high (ppm)	
tissue	male	female	male	female
adrenal	7 ± 0.9	8 ± 2.6	0.20 ± 0.095	0.13 ± 0.017
blood	25 ± 1.6	24 ± 6.1	1.03 ± 0.116	1.14 ± 0.177
blood cell	46 ± 3.1	47 ± 4.5	2.18 ± 0.264	2.27 ± 0.251
bone	2 ± 0.2	2 ± 0.2	0.04 ± 0.010	0.05 ± 0.015
bone marrow	4 ± 0.6	4 ± 0.9	0.12 ± 0.021	0.15 ± 0.022
brain	4 ± 0.5	4 ± 0.3	0.06 ± 0.021	0.05 ± 0.007
carcass	7 ± 0.6	6 ± 0.1	0.24 ± 0.057	0.21 ± 0.024
eye	2 ± 0.3	2 ± 0.5	0.07 ± 0.020	<0.06[b]
fat	1 ± 0.2	1 ± 0.2	<0.07[b]	<0.08[b]
heart	15 ± 1.5	14 ± 1.0	0.23 ± 0.024	0.23 ± 0.021
kidney	16 ± 1.8	19 ± 1.3	0.39 ± 0.053	0.46 ± 0.086
liver	15 ± 2.4	14 ± 0.5	0.32 ± 0.028	0.30 ± 0.041
lung	10 ± 0.8	10 ± 0.8	0.25 ± 0.024	0.29 ± 0.061
muscle	6 ± 0.6	6 ± 0.8	0.14 ± 0.022	0.14 ± 0.018
ovary	*c*	4 ± 0.6	*c*	0.12 ± 0.021
pancreas	4 ± 0.5	4 ± 0.2	0.11 ± 0.021	0.11 ± 0.018
pituitary	7 ± 1.2	<8[b]	<0.98[b]	<0.79[b]
plasma	5 ± 0.7	8 ± 1.8	0.24 ± 0.054	0.31 ± 0.073
spleen	8 ± 0.7	8 ± 0.7	0.20 ± 0.044	0.21 ± 0.028
sub. gland[d]	5 ± 0.6	6 ± 0.6	0.11 ± 0.015	0.12 ± 0.019
testis	5 ± 0.5	*c*	0.12 ± 0.011	*c*
thymus	1 ± 0.1	1 ± 0.2	0.04 ± 0.005	0.05 ± 0.011
thyroid	11 ± 1.9	10 ± 1.5	<0.33[b]	<0.45[b]
uterus	*c*	4 ± 0.8	*c*	0.13 ± 0.048

[a] Figures represent mean ±SD values of five rats. [b] Below the detection limit. [c] Not analyzed. [d] Sub.gland: Submaxillary gland.

Table 7. Amounts of Metabolites in Urine and Feces within 2 Days after Single Oral Administration of [*THP*-^{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	2.2 ± 0.53	0.6 ± 0.08	1.4 ± 0.96	0.5 ± 0.24
SAT-482	0.6 ± 0.11	*c*	0.6 ± 0.11	*c*
3-OH-S-53482	2.1 ± 0.34	4.1 ± 0.40	1.4 ± 0.34	3.9 ± 0.86
4-OH-S-53482	1.5 ± 0.26	4.2 ± 0.31	1.5 ± 0.39	4.6 ± 1.12
3-OH-SAT-482	1.0 ± 0.12	1.2 ± 0.11	0.8 ± 0.09	1.2 ± 0.35
4-OH-SAT-482	2.3 ± 0.16	*c*	2.0 ± 0.47	*c*
THPA	0.3 ± 0.08	0.6 ± 0.16	0.2 ± 0.03	1.1 ± 0.17
1-OH-HPA	0.5 ± 0.14	0.8 ± 0.14	0.4 ± 0.08	1.0 ± 0.17
3-OH-SA	11.4 ± 1.31	4.8 ± 0.86	11.5 ± 1.54	6.1 ± 1.56
4-OH-SA	5.2 ± 0.45	2.2 ± 0.34	5.4 ± 0.43	2.4 ± 0.54
3-OH-A-SA	2.5 ± 0.44	0.7 ± 0.10	1.5 ± 0.32	1.0 ± 0.17
others[b]	29.0	10.8	25.9	14.0
unextractable	5.7 ± 1.42	*d*	4.8 ± 0.93	*d*
total	64.3 ± 2.47	30.0 ± 1.89	57.4 ± 5.05	35.8 ± 3.56
		High Dose		
S-53482	65.9 ± 4.13	0.2 ± 0.04	62.7 ± 4.11	0.3 ± 0.08
SAT-482	0.2 ± 0.03	*c*	0.3 ± 0.08	*c*
3-OH-S-53482	0.4 ± 0.05	2.1 ± 0.37	0.4 ± 0.05	2.0 ± 0.16
4-OH-S-53482	0.5 ± 0.11	1.6 ± 0.21	0.4 ± 0.05	2.3 ± 0.38
3-OH-SAT-482	0.3 ± 0.05	0.4 ± 0.05	0.3 ± 0.04	0.5 ± 0.14
4-OH-SAT-482	0.5 ± 0.12	*c*	0.5 ± 0.07	*c*
THPA	0.1 ± 0.01	0.3 ± 0.05	0.1 ± 0.02	0.4 ± 0.02
1-OH-HPA	0.2 ± 0.01	0.5 ± 0.11	0.2 ± 0.03	0.6 ± 0.09
3-OH-SA	3.8 ± 0.64	1.9 ± 0.35	3.9 ± 0.69	2.3 ± 0.44
4-OH-SA	1.6 ± 0.15	0.7 ± 0.14	1.5 ± 0.28	0.9 ± 0.17
3-OH-A-SA	0.7 ± 0.11	0.1 ± 0.03	0.5 ± 0.14	0.2 ± 0.03
others[b]	9.3	3.8	8.1	4.2
unextractable	3.6 ± 0.15	*d*	3.7 ± 0.82	*d*
total	87.1 ± 4.08	11.6 ± 1.29	82.6 ± 2.08	13.7 ± 1.42

[a] Data show mean ±SD values for five rats. [b] The data show the sum of the amounts of unidentified metabolites. [c] Not detected. [d] Not applicable.

the parent compound were 0.5−0.6% in the low dose group and 0.2−0.3% in the high dose group.

***In Vitro* Metabolism Study To Specify Tissues Forming Reduced Metabolites.** A representative TLC autoradiogram developed in solvent system C is shown in Figure 4. The reduced metabolite, SAT-482, of [*THP*-^{14}C]S-53482 is only present in the supernatant of the blood cell sample (irrespective of the presence or absence of NADPH as a cofactor). The other tissues and plasma had no reduction activity. In the liver and kidney samples containing NADPH, hydroxylated forms (3-OH-S-53482 and 4-OH-S-53482) were detected as the major metabolites. The majority of origin regions for TLC solvent system C for all samples with the exception of rat feces was considered to be THPA and a half amide

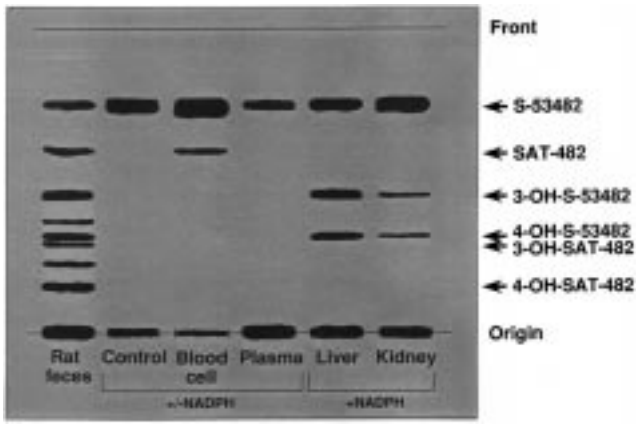

Figure 4. TLC autoradiogram of supernatants of blood cell, plasma, and other tissue samples treated with [*THP*-^{14}C]S-53482 in *in vitro* study. Solvent system C (benzene/ethyl acetate = 1/1 (v/v)) was used.

derivative of S-53482 (*N*-[7-fluoro-3-oxo-4-(2-propynyl)-2*H*-1,4-benzoxazin-6-yl]-3,4,5,6-tetrahydrophthalic acid, degradative compound) by results of qualitative TLC analysis using solvent system A.

DISCUSSION

In the present study, S-53482 was rapidly and almost completely eliminated from the bodies of both sexes within 7 days with ^{14}C-tissue residue levels one week post-treatment being generally very low. Even in blood cells, which showed the highest ^{14}C-value, the total residues amounted to no more than 0.2% of the given dose for both groups.

On the basis of the metabolites described previously and the present findings, the metabolic pathways shown in Figure 5 are proposed for S-53482 in rats. The major biotransformation reactions of S-53482 in rats 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) reduction at the 1,2-double bond of the 3,4,5,6-tetrahydrophthalimide moiety, (5) incorporation of a sulfonic acid group into the C=C double bond of the 3,4,5,6-tetrahydrophthalimide moiety, and (6) acetylation of the amino group of the aniline derivative. The main metabolites were sulfonate derivatives in feces and urine, and alcohol derivatives in urine. Small amounts of reduced metabolites, SAT-482, 3-OH-SAT-482, and 4-OH-SAT-482, were found in urine and feces.

The parent compound detected in feces was considered to be excreted without absorption into the body as reported previously (Tomigahara et al., 1999). Thus, the high dose level presumably exceeded the capacity for absorption. Similar findings were also obtained in the [*phenyl*-^{14}C]S-53482 study. The ^{14}C-excretion profile including the rate and the proportions in the feces and urine were similar to those in our [*phenyl*-^{14}C]S-53482 study. In both cases, ^{14}C-tissue and blood levels were generally very low. However, ^{14}C-residues in plasma were slightly higher in the [*THP*-^{14}C]S-53482 dosed groups than with [*phenyl*-^{14}C]S-53482. The same metabolites retaining the imide linkage were found in both studies in similar amounts. The extents of metabolic transformations are presented in Table 8. The high dose group showed lower extents of metabolism, in agreement with incomplete absorption rate of the test com-

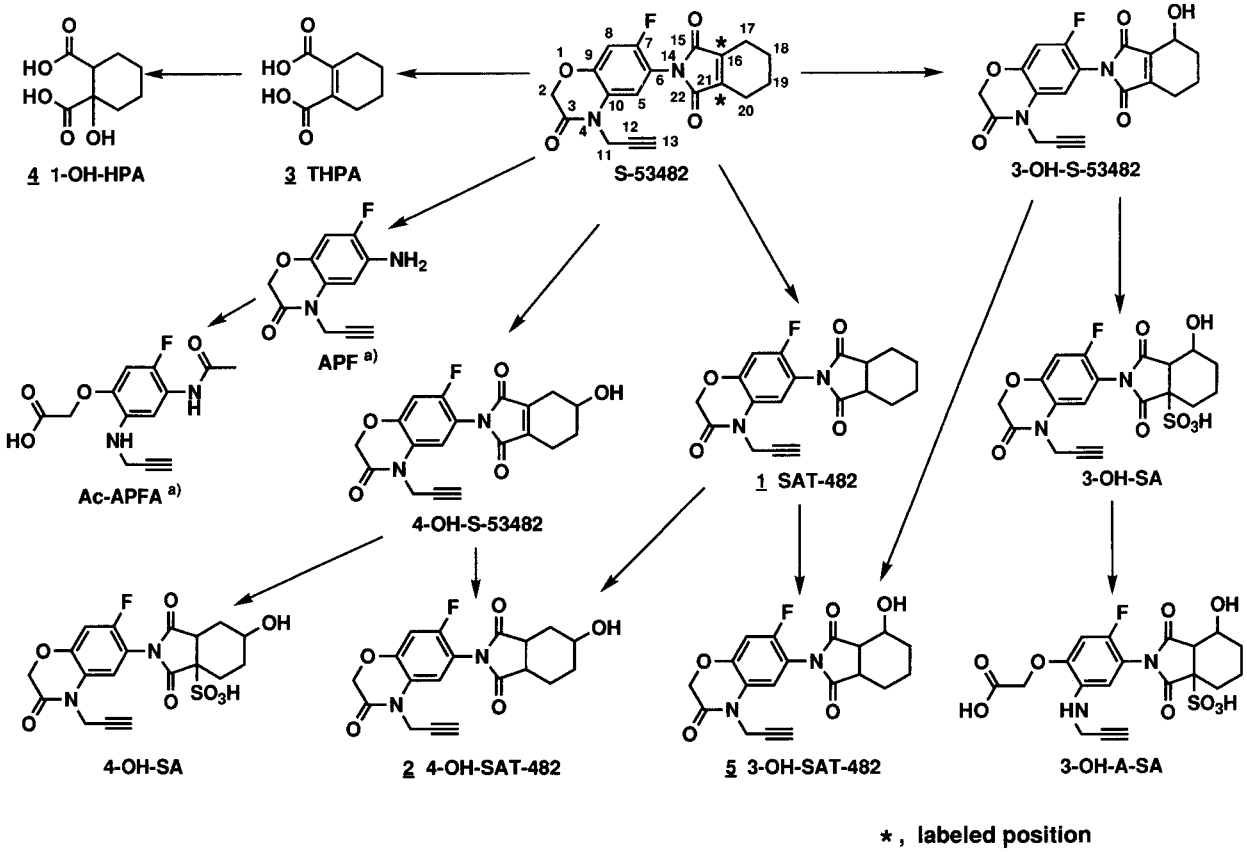

Figure 5. Proposed metabolic pathways for S-53482 in the rat. (a) Data were taken from the [*phenyl*-^{14}C]S-53482 study (Tomigahara et al., 1999).

Table 8. Extent of Each Metabolic Transformation[a]

	% of dosed ^{14}C			
	low		high	
metabolic transformation	male	female	male	female
non (parent)	2.8	1.9	66.1	63.0
imide moiety cleavage	2.2	2.7	1.1	1.3
amide moiety cleavage	3.2	2.4	0.8	0.7
hydroxylation	44.5	44.6	15.4	16.4
reduction	6.5	5.9	2.2	2.3
sulfonic acid conjugation	26.7	27.8	8.8	9.2
acetylation[b]	5.9	4.0	2.2	2.0

[a] Data were calculated from the values for identified metabolites in Table 7. [b] Data were taken from the [*phenyl*-^{14}C]S-53482 study (Tomigahara et al., 1999).

pound from the gastrointestinal tract. Main metabolic transformations were found to be hydroxylation and sulfonic acid conjugation. From the results of the present in vitro study, we can speculate that the hydroxylation reaction was cytochrome P-450s-dependent. As for the sulfonic acid conjugation, a few examples have been reported recently (Matsunaga et al., 1996 and 1997; Yoshino et al., 1993a,b; Tomigahara et al., 1994a−c, and 1998). Metabolites of the 3,4,5,6-tetrahydrophthalimide moiety (1-OH-HPA and THPA) detected here were also a feature of tetramethrin metabolism (Tomigahara et al., 1994a). In our previous report (Tomigahara et al., 1997), we argued that 1-OH-HPA may be formed by direct addition of H_2O to the double bond of THPA (hydration) in the intestinal tract (probable gut flora) or the liver (fumarase). As for reduced metabolites of S-53482, they have been newly established in the present study. Reduction reactions of 1,2-double bonds in mammalian species are rather rare, but a few examples have been reported (Fraser et al., 1967; Peters and Fraser, 1972; Tomigahara et al., 1994a). Fraser et al. (1967) and Peters and Fraser (1972) described an enzyme (α,*β*-unsaturated ketone reductase) catalyzing the reduction of α,*β*-unsaturated ketones by NADPH, which was found mainly in the human liver and dog erythrocytes but with greater activity in rat liver than in rat erythrocytes. However, in our previous study (Tomigahara et al., 1997), we concluded that reduction of the 1,2-double bond of tetramethrin having a 3,4,5,6-tetrahydrophthalimide moiety was due to another, totally different enzyme, existing mainly in rat blood cells. The reduction in this study might have had the same basis, in line with our earlier report (1997), because it was limited to the blood cell case and S-53482 has partially the same structure (3,4,5,6-tetrahydrophthalimide moiety) as tetramethrin.

Though neither relative nor absolute configuration of any chiral metabolites has been determined as yet, the relative configuration of reduced metabolites, SAT-482, 3-OH-SAT-482, and 4-OH-SAT-482, was estimated by half-bandwidth ($W_{1/2}$) of the H-16, H-21, H-17a, and H-18a signals. In the ^1H NMR spectrum of SAT-482, the H-16 and H-21 signals were observed as a multiplet at 3.10 ppm ($W_{1/2}$=10 Hz). These half-bandwidths indicate that one is in the *equatorial* and another is in the *axial* position but not both are in the *axial* position. Therefore, the relative configuration of the H-21 and H-16 was considered to be cis. As for other metabolites, 3-OH-SAT-482 and 4-OH-SAT-482, the relative configuration of the H-21 and H-16 also seemed to be cis (Table 9). Therefore, we speculated that the configuration of the additional protons of the reduced metabolites (SAT-482, 3-OH-SAT-482, and 4-OH-SAT-482) was in the *cis*-form (addition of protons from the same direction as the 1,2-double bond of the 3,4,5,6-tetrahydrophthalimide moiety) in line with earlier findings (Tomigahara et al., 1996 and 1997). The H-17a of 3-OH-SAT-482 and the H-18a of 4-OH-SAT-482 were considered to be both *axial* positions because of the large half-bandwidth.

For 3-OH-S-53482, 3-OH-SA, 3-OH-A-SA, 4-OH-S-53482, and 4-OH-SA, which are metabolites purified previously, the hydroxymethine proton (H-17a or H-18a) is likely to be in the *axial* position, in a similar way (by half-bandwidth).

The relative configuration of the H-16 and the hydroxy group (position C-21) of 1-OH-HPA was trans because its spectral data corresponded to those of an authentic standard for which stereo structure was determined (data not shown).

We also estimated that the configuration of the additional proton and the sulfonic acid group of sulfonic acid type conjugates was in the *cis*-form, because they were obtained easily by an organic reaction of the 1,2-double bond of the 3,4,5,6-tetrahydrophthalimide moiety (fused 5,6-bicyclic system) and sulfurous acid (Matsu-

Table 9. Estimated Positions of H-16, H-21, H-17A, and H-18A of Chiral Metabolites of S-53482 in Rats

	H-16		H-21		H-17a		H-18a	
metabolite	*J* or $W_{1/2}$ (Hz)	position	*J* or $W_{1/2}$ (Hz)	position	*J* or $W_{1/2}$ (Hz)	position	*J* or $W_{1/2}$ (Hz)	position
SAT-482	m, $W_{1/2}$ = 10	axial or equatorial	m, $W_{1/2}$ = 10	equatorial or axial	na[a]		na	
3-OH-SAT-482	m, $W_{1/2}$ = 10	axial or equatorial	m, $W_{1/2}$ = 10	equatorial or axial	brs, $W_{1/2}$ = 20	axial	na	
4-OH-SAT-482	m, $W_{1/2}$ = 10	axial or equatorial	m, $W_{1/2}$ = 10	equatorial or axial	na		brs, $W_{1/2}$ = 20	axial
1-OH-HPA	dd, *J* = 9.9, 4.5	axial	*b*		na		na	
3-OH-S-53482	*b*		*b*		brs, $W_{1/2}$ = 12	axial	na	
4-OH-S-53482	*b*		*b*		na		brs, $W_{1/2}$ = 15	axial
3-OH-SA	d, *J* = 8.6	axial	*b*		brs, $W_{1/2}$ = 20	axial	na	
3-OH-A-SA	d, *J* = 8.6	axial	*b*		brs, $W_{1/2}$ = 20	axial	na	
4-OH-SA	m, $W_{1/2}$ = 12	axial	*b*		na		brs, $W_{1/2}$ = 20	axial

[a] Not applicable. [b] None.

naga et al., 1996), and speculated that the sulfonic acid group and the additional proton are in the *equatorial* and *axial* positions, respectively, due to the fact that its large functional group tends to assume the *equatorial* position and from the fact that coupling constants between the H-16 and H-17a of 3-OH-SA and 3-OH-A-SA are ~9 Hz (Table 9).

ACKNOWLEDGMENT

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LITERATURE CITED

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