Metabolism of Pentyl 2-Chloro-4-fluoro-5-(3,4,5,6-tetrahydrophthalimido)phenoxyacetate (Flumiclorac Pentyl, S-23031) in Rats. 1. Identification of Metabolites in Feces and Urine of Rats

Haruyuki Matsunaga,* Naohiko Isobe, Hideo Kaneko, Iwao Nakatsuka, and Hirohiko Yamada

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

Male rats were orally given pentyl 2-chloro-4-fluoro-5-(3,4,5,6-tetrahydrophthalimido)phenoxyacetate (flumiclorac pentyl, S-23031) labeled with ^{14}C at 250 (mg/kg)/day for 6 consecutive days, and several 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, MS, and/or IR), and sulfonic acid conjugates were found to be predominant. These conjugates were easily synthesized by mixing conjugate precursors with sulfurous acid.

Keywords: *Flumiclorac pentyl; metabolism; rat; identification; and sulfonic acid conjugate*

INTRODUCTION

Pentyl 2-chloro-4-fluoro-5-(3,4,5,6-tetrahydrophthalimido)phenoxyacetate (flumiclorac pentyl, S-23031, Resource) is a new postemergence herbicide for broadleaved weed control in soybeans which is particularly effective against velvet leaf, *Abutilon theophrasti* (Kamoshita *et al*., 1992).

The metabolic fate of S-23031 in rats was investigated along with toxicological studies for safety evaluation. The present report deals with identification of fecal and urinary metabolites of S-23031 in rats. Absorption, distribution, and excretion of ^{14}C, and quantification of fecal and urinary metabolites are to be described elsewhere.

MATERIALS AND METHODS

Spectroscopy. NMR spectra were obtained on a JNM-GSX 270 spectrometer (JEOL, Tokyo, Japan) operating at 270 MHz for ^1H and 67.5 MHz for ^{13}C. Two dimensional (2D) spectra from $^1H-^1H$ chemical shift correlation spectroscopy ($^1H-^1H$ COSY), $^1H-^1H$ double quantum filtered COSY ($^1H-^1H$ DQF-COSY; Piantini *et al*., 1982), $^1H-^1H$ DQF phase-sensitive COSY (Marion and Wüthrich, 1983; Rance *et al*., 1983), and $^{13}C-^1H$ COSY were obtained with the data process program PLEXUS V1.6 (JEOL). Chemical shifts were generated in parts per million units relative to tetramethylsilane in methanol-d_4 (CD_3OD) and dimethylformamide-d_7 (DMF-d_7), or 2,2,3,3-tetradeutero-3-(trimethylsilyl)propionic acid sodium salt in deuterium oxide (D_2O) which gave 0 ppm for ^1H NMR. Dioxane in D_2O gave 67.3 ppm, and the methyl signal of *tert*-butyl alcohol gave 30.7 ppm for ^{13}C NMR.

SI-MS was performed with an Hitachi M-80B mass spectrometer at an accelerating voltage of 3.0 kV. Samples were introduced in a glycerol matrix, and spectra were recorded in a positive or a negative ion mode. A high-resolution SI-MS spectrum was obtained in a positive ion mode with similar conditions as described above. FAB MS was performed with a JEOL AX-505 mass spectrometer. The analytical conditions employed were essentially the same as those mentioned above.

IR spectra were recorded on a Fourier transfer infrared spectrophotometer FTIR-4100 (Shimadzu, Kyoto, Japan) in KBr pellets.

Chemicals. S-23031 labeled uniformly in the phenyl group, [^{14}C]S-23031, with a specific activity of 7.22 GBq/mmol (195 mCi/mmol) was synthesized in this laboratory (Figure 2).

Unlabeled S-23031 (purity > 99%) was prepared in Sumitomo Chemical Co., Ltd. (Osaka, Japan).

2-Chloro-4-fluoro-5-(3,4,5,6-tetrahydrophthalimido)phenoxyacetic acid (IMCA) was synthesized by Sumica Chemical Analysis Service (Osaka, Japan) and used as a standard for comparative spectroanalysis. NMR and MS data are as follows. ^1H NMR (270 MHz, $(CD_3)_2CO$): δ 1.81 (4H, m), 2.38 (4H, m), 4.84 (2H, s), 7.14 (1H, d), 7.49 (1H, d). EI-MS: m/z 353(M^+), 308.

Animal Treatment. Sprague-Dawley male and female rats were purchased at 6 weeks of age from Charles River Japan Inc. and acclimatized for 1 week before use. They were allowed free access to the pelleted diet (CRF-1, Oriental Yeast Inc., Tokyo, Japan) and water *ad libitum*.

To collect sufficient amounts of urinary and fecal metabolites for spectroanalytical identification, a total of approximately 5 g [^{14}C]S-23031 was suspended in corn oil (ca. 50 mg/mL) and administered to 14 male rats for 6 consecutive days at 250 (mg/kg)/day. The specific activity of [^{14}C]S-23031 was adjusted to 0.31 MBq/mmol by isotopical dilution with unlabeled S-23031. The treated rats were placed in Metabolica-CO_2 cages (Sugiyamagen Iriki, Co., Ltd., Tokyo, Japan) during the 6 days of treatment to allow the separate collection of urine and feces.

Sample Processing. All collected feces were combined and homogenized in a 3-fold volume of methanol−water (9:1 (v/v)) using a Waring blender (Nihonseiki Co., Tokyo, Japan). The homogenate was centrifuged at 3000 rpm (ca. 1500*g*) for 10 min, followed by decanting to obtain the supernatant. Residues were further extracted twice with methanol−water. The combined methanol−water extract was concentrated and subjected to sequential chromatographic purification.

Collected urine was lyophilized, and the residue was extracted with methanol. The methanol extract was concentrated and purified by HPLC after pretreatment with a SEP-PAK C18 cartridge (Millipore Co., Milford, MA).

Chromatography. Thin layer chromatography (TLC) was performed using precoated silica gel 60 F_{254} chromatoplates (20 × 20 cm, 0.25 mm layer thickness, E. Merck) with solvent I (6:1:1 1-butanol/water/acetic acid) (Kaneko *et al*., 1988).

Open column chromatography was performed with silica gel 60 (70−230 mesh, E. Merck), Amberlite XAD-2 resin (Organo, Tokyo, Japan), and Sephadex LH-20 (Pharmacia).

HPLC was carried out with a system consisting of a 638-50 pump (Hitachi, Ltd., Tokyo, Japan) fitted with a YMC-Pack S-343-15 column (ODS, 20 mm i.d. × 250 mm; YMC Co., Ltd., Kyoto, Japan) and YMC-Pack GI-340-15 (ODS, 20 mm i.d. × 50 mm; YMC) as a guard column, a 635M LC UV detector

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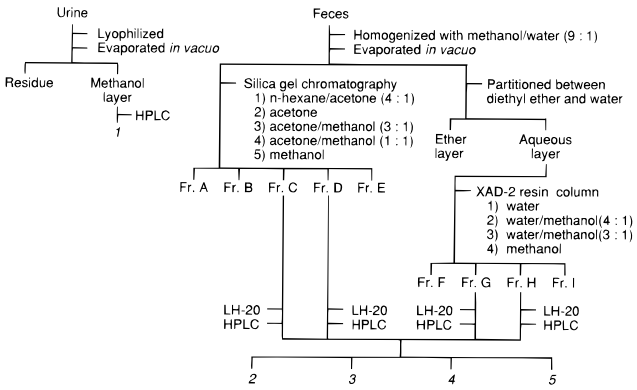

Figure 1. Flow diagram of purification procedures for fecal and urinary metabolites.

(Hitachi), and a LB 503 radioactivity monitor (Berthold, Germany). The conditions used are as follows: flow rate = 4 mL/min, mobile phase A (40:60:1 methanol/water/acetic acid) for the urinary metabolite; flow rate = 5 mL/min; mobile phases B (2:3 methanol/water) and C (3:97 methanol/water) for the fecal metabolites.

Formation of Sulfonic Acid Derivatives. The following is typical of the procedures employed. To a 50 mL round-bottom flask equipped with a magnetic stirrer were added 0.1−0.5 mmol of the imide derivative, 2−4 mL of methanol, 1.5−2 mL of triethylamine (large excess), and 0.1−0.2 mL (1.3−2.5 mmol) of sulfurous acid. The mixture was stirred overnight or for 15 min at room temperature. Solvents were removed with an N_2 gas stream. The residue was redissolved in methanol and subjected to preparative TLC developed in solvent II (4:1:1:1 ethyl acetate/acetone/water/acetic acid). Each silica gel region corresponding to an anticipated product was scraped off, and the product was eluted with methanol.

RESULTS

Isolation of Metabolites. The flow diagram for the purification of fecal and urinary metabolites is shown in Figure 1.

The fecal methanol−water extract was concentrated *in vacuo* by evaporation and divided into two portions. One was subjected to silica gel column chromatography using the following eluents: 1:1 *n*-hexane/acetone, 100% acetone, 3:1 acetone/methanol, 1:1 acetone/methanol, and 100% methanol (corresponding to fractions A−E, respectively). The other was partitioned three times between diethyl ether and water. After concentration, the aqueous phase was subjected to a column of Amberlite XAD-2 resin, which was washed with water, and the metabolites were eluted with 1:4 or 1:3 methanol/water and 100% methanol (corresponding to fractions G−I, respectively). Throughout these chromatographic fractionations, metabolites were tracked by silica gel TLC developed in solvent I. The target metabolites were detected in fractions C, D, G, and H. Each fraction was separately concentrated and purified by Sephadex LH-20 column chromatography eluted with 100% methanol as the eluent. The portions containing metabolites which gave R_f values of about 0.2−0.3 on TLC with solvent I were further purified by HPLC, and four metabolites (metabolites 2−5) were isolated.

One urinary metabolite (metabolite 1) was isolated by HPLC after prepurification.

Identification of Metabolites. The chemical structure of each purified metabolite from excreta was identified by spectroanalyses (NMR, MS, and/or IR). Chemical structures of the parent compound and the identified metabolites are shown in Figure 2.

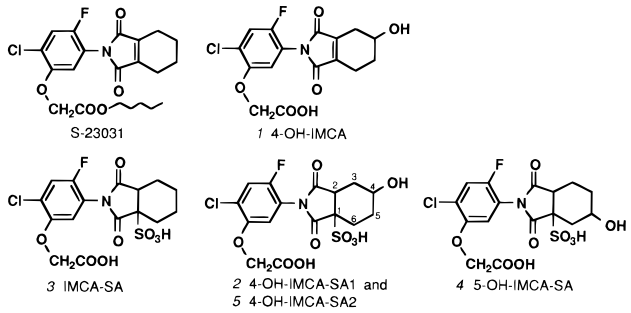

Figure 2. Chemical structures of S-23031 and identified metabolites.

*Metabolite **1**.* Metabolite **1** was isolated by HPLC with mobile phase A at the retention time of 34 min.

Proton NMR signals at 7.01 and 7.41 ppm indicated the presence of a phenyl moiety. A singlet at 4.72 ppm (two protons) showed $-OCH_2COO-$. There was no signal indicating a pentyl group. Signals at 1.9−4.2 ppm demonstrated seven protons. These regions were further analyzed by $^{13}C-^1H$ COSY and $^1H-^1H$ DQF-COSY. The $^{13}C-^1H$ COSY spectrum suggested the presence of the following carbon units: $3 \times >CH_2$ and $>CHO-$. These units were connected by analyzing the $^1H-^1H$ DQF-COSY spectrum. The positive ion mode SI-MS showed a protonated molecular ion at m/z 370 $(M + H)^+$. The molecular weight of 369 was 16 mass units larger than that of IMCA (MW = 353), indicating that one of the hydrocarbons might have been hydroxylated. On the basis of these findings, metabolite **1** was concluded to have a hydroxyl group at the 4-position of a 3,4,5,6-tetrahydrophthalimide (THP) moiety and was identified as 2-chloro-4-fluoro-5-[4-hydroxy-(3,4,5,6-tetrahydro)phthalimido]phenoxyacetic acid (4-OH-IMCA). ^1H NMR (CD_3OD): δ 1.92 (2H, m), 2.37 (1H, m), 2.49 (1H, m), 2.54 (1H, m), 2.68 (1H, m), 4.18 (1H, quint, J = 4.9 Hz), 4.72 (2H, s), 7.01 (1H, d, J = 6.3 Hz), 7.41 (1H, d, J = 9.3 Hz); ^{13}C NMR (CD_3OD): δ 18.4, 29.6, 29.8, 65.6, 67.7, 116.2, 119.0 (d, J = 25.0 Hz), 119.7 (d, J = 15.2Hz), 125.0 (d, J = 9.3 Hz), 141.3, 143.0, 151.7 (d, J = 7.3 Hz), 153.6 (d, J = 237.2 Hz), 169.7, 169,9, 171.9. SI-MS (positive ion): m/z 370 $(M + H)^+$.

*Metabolite **2**.* Metabolite **2** was isolated by HPLC with mobile phase C at the retention time of 40 min. Proton signals at 6.95 and 7.55 ppm indicated the presence of a phenyl moiety. A singlet at 4.55 ppm (two protons) showed $-OCH_2COO-$. There was no signal indicating a pentyl group. Signals at 1.8−4.0 ppm demonstrated eight protons, and these regions were further characterized by 2D NMR as described above. The $^{13}C-^1H$ COSY spectrum and the partial expansion spectrum of $^1H-^1H$ DQF-COSY are presented in Figure 3. The $^{13}C-^1H$ COSY spectrum suggested the presence of the following carbon units: $3 \times >CH_2$, $>CH-$, and $>CHOH$. The presence of a hydroxy group was confirmed by acetylation: a portion of metabolite **2** was acetylated with acetic anhydride in pyridine and the signal ascribed to $>CHO-$ shifted to about 0.9 ppm in the lower magnetic field. Five carbon units listed above were connected by analyzing the $^1H-^1H$ DQF-COSY spectrum, and the resultant linkage indicated that these carbon units comprized the THP moiety and the position of the hydroxyl group was assigned to C-4 (4-position). The findings from $^{13}C-^1H$ COSY and $^1H-^1H$ DQF-COSY spectra further suggested that the $>C{=}C<$ in the THP moiety had an addition of HX (X: a functional group identified below), and the signal at 3.71 ppm could be ascribed to the angular proton. This metabolite was

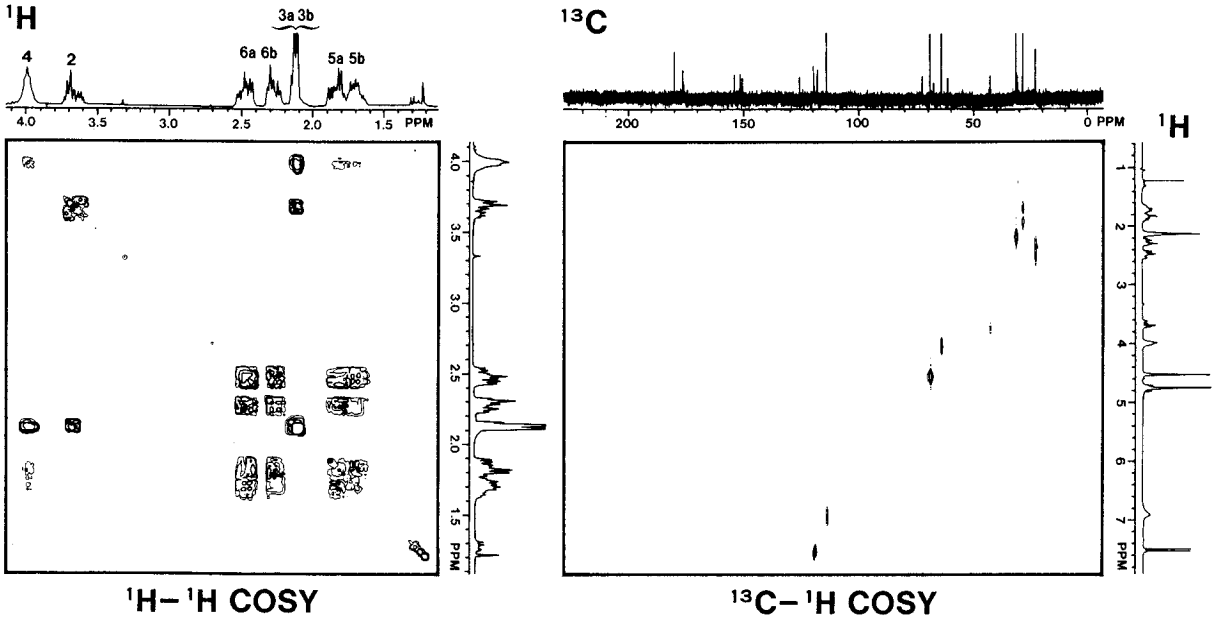

Figure 3. $^1H-^{13}C$ COSY spectrum and partial $^1H-^1H$ NMR spectrum (enlargement) of metabolite **2**.

very polar (high water solubility and low TLC R_f value with polar solvents), indicating the incorporated group X to be a polar group. The negative ion mode SI-MS showed a deprotonated molecular ion peak at m/z 450 $(M - H)^-$. The molecular weight (MW) of 451 was 82 mass units larger than that of metabolite **1** (4-OH-IMCA) (MW = 369). These findings indicated that a sulfonic acid group was incorporated into the >C=C< double bond in the THP moiety. The IR spectrum showed a strong peak at 1200 cm^{-1} which could be ascribed to S=O (stretching) of $-SO_3H$, supporting the presence of a sulfonic acid group. Consequently, metabolite **2** was identified as 2-chloro-4-fluoro-5-(4-hydroxy-1-sulfo-1,2-cyclohexenedicarboximido)phenoxyacetic acid (4-OH-IMCA-SA1). ^1H NMR (D_2O): δ 1.76 (1H, m), 1.86 (1H, m), 2.16 (2H, m), 2.30 (1H, dt, J = 4.9, 14.7 Hz), 2.51 (1H, ddd, J = 4.9, 10.3 Hz), 3.71 (1H, t, J = 6.8), 4.02 (1H, m), 4.55 (2H, s), 6.95 (1H, brs), 7.55 (1H, d, J = 9.3 Hz). ^{13}C NMR (D_2O): δ 23.0, 28.5, 31.5, 43.0, 64.3, 67.5, 69.1, 114.2, 118.0 (d, J = 15.7 Hz), 119.7 (d, J = 23.5 Hz), 125.8 (d, J = 9.8 Hz), 151.5, 152.2 (d, J = 248.5 Hz), 175.8, 176.4, 180.0. SI-MS (negative ion): m/z 450 $(M - H)^-$, 472 $(M + Na - 2H)^-$, 488 $(M + K - 2H)^-$. IR (KBr): 1200, 1046 cm^{-1}.

*Metabolite **3***. Metabolite **3** was isolated by HPLC with mobile phase B at the retention time of 17 min. The proton signals in a magnetic field higher than 4.6 ppm were very similar to those of metabolite **2**, except that there was no signal at about 4 ppm corresponding to the >C*H*OH (Figure 4). Signals at 1.4–3.5 ppm demonstrated nine protons, suggesting that the >C=C< in the 3,4,5,6-tetrahydrophthalimide (THP) moiety might have an added HX. The findings from $^{13}C-^1H$ COSY and $^1H-^1H$ DQF-COSY (phase sensitive) suggested the following carbon units: 4 × $>CH_2$ and one >CH–, connected so that the resultant linkage comprises the THP moiety. The negative ion SI-MS showed a deprotonated molecular ion peak at m/z 434 $(M - H)^-$, 16 mass units (oxygen) smaller than the corresponding peak of metabolite **2**. From the IR spectrum, absorption at 1202 cm^{-1} supported the presence of a sulfonic acid group. These findings indicated sulfonic acid group incorporation into the >C=C< double bond in the THP moiety. Thus, metabolite **3** was identified as 2-chloro-4-fluoro-5-(1-sulfo-1,2-cyclohexanedicarboximido)phenoxyacetic acid (IMCA-SA). ^1H NMR (D_2O): δ 1.38 (1H, m), 1.55 (1H, m), 1.66 (1H, m), 1.79 (1H, m), 1.92 (1H, m), 2.18 (1H, ddd, J = 4.8, 11.9, 14.1 Hz), 2.29 (1H, m), 2.50 (1H, ddd, J = 4.8, 4.8, 14.1 Hz), 3.53 (1H, t, J = ca. 8 Hz), 4.60 (2H, s), 6.98 (1H, brs), 7.60 (1H, d, J = 9.4 Hz). ^{13}C NMR (D_2O): δ 19.5, 19.6, 24.5, 24.7, 43.7, 67.8, 68.1, 113.1, 117.1 (d, J = 14.7 Hz), 118.6 (d, J = 25.4 Hz), 124.7 (d, J = 9.8 Hz), 150.4, 151.2 (d, J = 247.5 Hz), 174.9, 175.3, 179.8. SI-MS (negative ion): m/z 434 $(M - H)^-$, 456 $(M + Na - 2H)^-$, 472 $(M + K - 2H)^-$. IR (KBr): 1202, 1067 cm^{-1}.

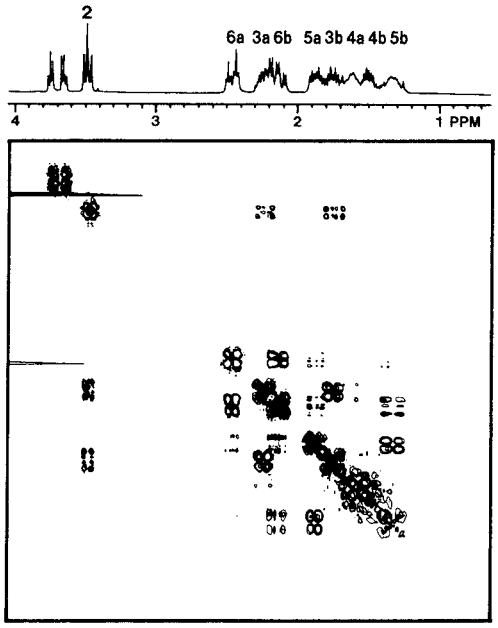

Figure 4. Partial $^1H-^1H$ COSY spectrum (enlargement) of metabolite **3**.

*Metabolite **4***. Metabolite **4** was isolated by HPLC with mobile phase C at the retention time of 21 min. The ^1H NMR spectrum of metabolite 4 in D_2O did not show well-separated signals. However, each signal could be adequately resolved in DMF-d_6 at 70 °C. A ^{13}C NMR spectrum was not feasible due to the paucity of this metabolite. The negative ion mode SI-MS showed a deprotonated molecular ion at m/z 450 (M –

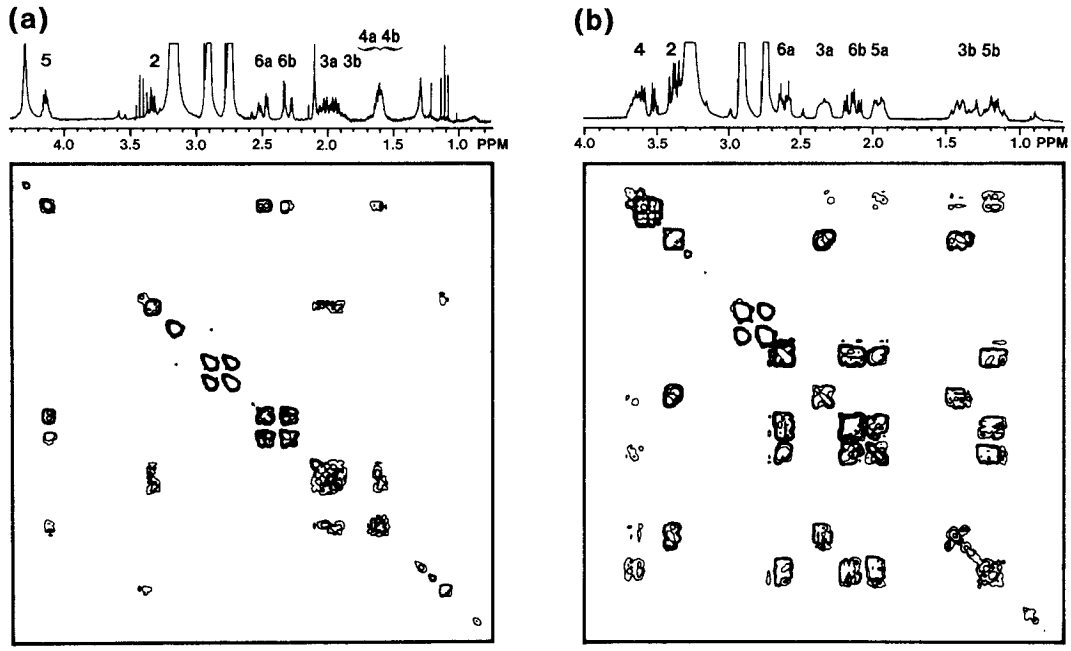

Figure 5. Partial $^1H-^1H$ DQF COSY spectra (enlargement) of metabolites **4** (a) and **5** (b).

$H)^-$, the same as for metabolite **2**, indicating that metabolite **4** was probably an isomer of metabolite **2**. From comparison of the pattern of ^1H NMR spectra of metabolite **2** and the $^1H-^1H$ DQF-COSY spectrum of metabolite **4** (*left* of Figure 5), hydroxylation at the 5-position of the THP moiety was concluded. Consequently, metabolite **4** was identified as 2-chloro-4-fluoro-5-(5-hydroxy-1-sulfo-1,2-cyclohexanedicarboximido)phenoxyacetic acid (5-OH-IMCA-SA). ^1H NMR (DMF-d_7, at 70 °C): δ 1.6 (2H, m), 1.9 (1H, m), 2.0 (1H, m), 2.3 (1H, dd), 2.5 (1H, dd), 3.3 (1H, dd), 4.1 (1H, m), 4.3 (2H, s), 7.0 (1H, d), 7.4 (1H, d). SI-MS (negative): m/z 450 $(M - H)^-$, 472 $(M + Na - 2H)^-$, 488 $(M + K - 2H)^-$. Metabolite **4** is considered to be a regioisomer of metabolite **2**.

Metabolite 5. Metabolite **5** was isolated by HPLC with mobile phase C at the retention time of 19 min. The ^1H NMR spectrum of metabolite **5** in D_2O, like that of metabolite **4**, did not show well-separated signals. However, each signal could be adequately resolved in DMF-d_6 at 60 °C. The ^{13}C NMR spectrum signals could not be assigned due to the paucity of this metabolite. The negative ion mode SI-MS showed a deprotonated molecular ion at m/z 450 $(M - H)^-$, the same as for metabolites **2** and **4**, indicating that metabolite **5** might be a further isomer. From interpretation of the $^1H-^1H$ DQF-COSY spectrum (*right* of Figure 5), metabolite **5** was concluded to have a hydroxyl group at the 4-position of the THP moiety and was identified as 2-chloro-4-fluoro-5-(4-hydroxy-1-sulfo-1,2-cyclohexanedicarboximido)phenoxyacetic acid (4-OH-IMCA-SA2). ^1H NMR (DMF-d_7, at 60 °C): δ 1.2 (1H, m), 1.4 (1H, m), 2.0 (1H, m), 2.1 (1H, m), 2.3 (1H, m), 2.6 (1H, m), 3.4 (1H, dd), 3.6 (1H, m), 4.4 (2H, brs), 7.0 (1H, d), 7.4 (1H, d). SI-MS (negative): m/z 450 $(M - H)^-$, 472 $(M + Na - 2H)^-$, 488 $(M + K - 2H)^-$. Metabolites **2** and **5** had the same planar structure, being stereoisomers. However, discrimnation between the two was possible by HPLC or by NMR spectra.

Formation of Sulfonic Acid Derivatives. Two imide herbicides, *N*-[4-chloro-2-fluoro-5-[(1-methyl-2-propynyl)oxy]phenyl]-3,4,5,6-tetrahydrophthalimide (S-23121, Yoshino *et al.*, 1994) and S-23031, and one insecticide, *trans*-tetramethrin (Tomigahara *et al.*, 1994) were mixed with sulfurous acid, resulting in the formation of 23121-sulfonic acid (SA), IMCA-SA, and *t*-tetramethrin-SA with 15−40% yields. MS and NMR spectral data are as follows.

23121-SA. ^1H NMR: δ 1.58 (1H, m), 1.7−2.1 (3H, d, J = 6.4 Hz), 2.16 (1H, m), 2.48 (2H, m), 2.75 (1H, ddd, J = 3.9, 4.4, 14.2 Hz), 3.32 (1H, d, J = 2.0 Hz), 3.77 (1H, t, J = 7.3 Hz), 5.37 (1H, q, J = 6.4 Hz), 7.55 (1H, d, J = 6.4 Hz), 7.84 (1H, d, J = 9.3 Hz). ^{13}C NMR: δ 22.5, 22.6, 23.9, 27.3, 27.9, 46.7, 70.0, 71.1, 79.1, 120.1, 120.3, 121.4 (d, J = 23.8 Hz), 129.2 (d, J = 10.4 Hz), 152.0, 154.9 (d, J = 249.1 Hz). FABMS (negative ion): m/z 428 $(M - H)^-$. High-resolution SI-MS (positive ion): m/z 430.0522 $(M + H)^+$ (Calcd for $C_{18}H_{18}FNClO_6S$: 430.0526).

IMCA-SA. The same as those of the fecal metabolite described above.

t-tetramethrin-SA: ^1H NMR: δ 1.14 (3H, s), 1.1−1.3 (2H, m), 1.4−1.6 (5H, m), 1.73 (3H, s), 1.83 (1H, m), 2.0−2.2 (3H, m), 2.34 (1H, m), 3.33 (1H, dd, J = 7.3, 7.3 Hz), 5.03 (1H, brd, J = 7.8 Hz), 5.57 (2H, m). SI-MS (negative ion): m/z 412 $(M - H)^-$.

DISCUSSION

Sulfonic acid derivatives were also found in metabolic studies of S-23121 (Yoshino *et al.*, 1994) and tetramethrin (Tomigahara *et al.*, 1994) which share the same THP moiety. The mechanism of incorporation of the sulfonic acid group was investigated previously by Yoshino *et al.* using ^{35}S-labeled Na_2SO_4, cysteine, and glutathione. It was suggested that HSO_3^- is derived from SO_4^{2-} (sodium or other cation salt) by the microflora in the intestine and added to the C=C double bond of the THP moiety. Our finding that sulfurous acid nonenzymatically attacks the C=C bond of the THP moiety of some imide pesticides under alkaline conditions supports the above proposed mechanism. HSO_3^- might react nucleophilically with THP in a Michael-like addition (Figure 6). Metabolic studies *in vitro* using gastrointestinal tract preparations should provide a clarification of this question.

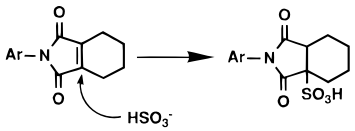

Figure 6. Site of addition of HSO_3^- to the THP moiety.

Although metabolites **2** and **5** showed the same planar structure, they could be separated by HPLC and showed different NMR spectra. Neither the relative nor the absolute stereochemical feature of these isomers has been determined as yet, but it is likely that the difference between them is due to variation in the configuration of the hydroxyl group at the 4-position in the THP moiety. When the hydroxymethine proton signal of metabolite **2** was compared with that of metabolite **5**, the half-band width of metabolite **2** was found to be smaller than that of metabolite **5**, indicating that the hydroxymethine proton of metabolite **5** has a large coupling constant (probably $J \geq 9$ Hz). The ^{1}H signal decoupling experiment supported this conclusion (data not shown). Therefore, the hydroxymethine proton of metabolite **5** is likely to be in the *axial* position, while that of metabolite **2** is in the *equatorial* position.

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