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Identification of Fonofos Metabolites in *Latuca sativa*, *Beta vulgaris*, and *Triticum aestivum* by Packed Capillary Flow Fast Atom Bombardment Tandem Mass Spectrometry

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The metabolism of fonofos, a thiophosphonate insecticide, was investigated in mature lettuce (*Latuca sativa*), beet (*Beta vulgaris*), and wheat (*Triticum aestivum*). Six new metabolites were identified by LC-MS and LC-MS-MS analysis using fast atom bombardment (FAB) and packed capillary LC columns with application of the on-column focusing technique. These methods provided the sensitivity required to identify unknown metabolites that were present in the mature plants at only 20−230 ppb. Structural elucidation was facilitated by use of fonofos labeled with both carbon-14 and carbon-13 in the phenyl ring. In all three plants fonofos was converted to a glucose conjugate of thiophenoxylactic acid. Oxidation of the glucose conjugate gave isomeric sulfoxides in all species examined. Thiophenoxylactic acid was found esterified to malonic acid in lettuce. In beets, *S*-phenylcysteine was found as its malonic acid amide. A second metabolite unique to beets was *N*-(malonyl)-[2[(ethoxyethylphosphinothionyl)oxy]phenyl]cysteine. This novel structure was confirmed by synthesis.

KEYWORDS: Fonofos; metabolism; lettuce; wheat; beet; mass spectrometry; structure elucidation; stable isotopes; synthesis; cysteine conjugate β-lyase

INTRODUCTION

Fonofos (*O*-ethyl *S*-phenyl ethylphosphonodithioate) is a dithiophosphonate insecticide used to control lepidopterous insects in corn, potatoes, and peanuts. Considerable work has been done on the metabolism of fonofos in animals. Rat liver microsomes convert fonofos to fonofos oxon, which then yields *O*-ethyl ethylphosphonate (EOP) by hydrolysis (*1*). Rats treated with (*ethyl*-1-^{14}C)fonofos excreted 54.5 and 32.7% of the dose in urine as *O*-ethyl ethylphosphonothioate (ETP) and EOP, respectively (*2*). Rats metabolized (*phenyl*-U-^{14}C)fonofos to methylphenyl sulfone (9.9% of dose in urine) and to water-soluble conjugates, which upon acid hydrolysis gave 3-(hydroxyphenyl) methyl sulfone (24.9% of dose) and 4-(hydroxyphenyl) methyl sulfone (23.4% of dose). The same metabolites were found when thiophenol was administered to rats (*3*). Thiophenol is thus an intermediate in the metabolism of fonofos that is formed by hydrolysis of either fonofos or its oxon.

Plant metabolism of fonofos has been studied only in *Solanum tuberosum* (*4*). In mature tubers grown in soil treated with (*ethyl*-1-^{14}C)fonofos, 32% of the total residue was extracted as EOP. An additional 7% of EOP resulted from treatment of bound residues with 1 N HCl. No ETP was detected. Tubers grown with soil containing (*phenyl*-U-^{14}C)fonofos had only 3.8% of the total residue present as methylphenyl sulfone. The majority of the residue (86.8%) was present as water-soluble unknowns of which 57% could be cleaved by β-glucosidase to an unknown aglycon that could be methylated with diazomethane but that did not cochromatograph with any of the three isomeric hydroxyphenyl methyl sulfone derivatives.

The use of mass spectrometry in the analysis of conjugates and other metabolites of xenobiotics has been recently reviewed (*5*). The development of continuous-flow FAB was a major advance in the identification of polar and thermally labile metabolites by LC-MS (*6*). The solvent flow requirements of this interface matched those of packed capillary HPLC columns with internal diameters of 0.1−0.3 mm. An injection technique called on-column focusing has been developed that allows use of large (10−100 μL) injection volumes with packed capillary HPLC columns without significant deterioration of chromatographic resolution (*7*, *8*). The application of this injection technique to the identification of metabolites of xenobiotics by packed capillary LC-MS has been described in detail (*9*, *10*).

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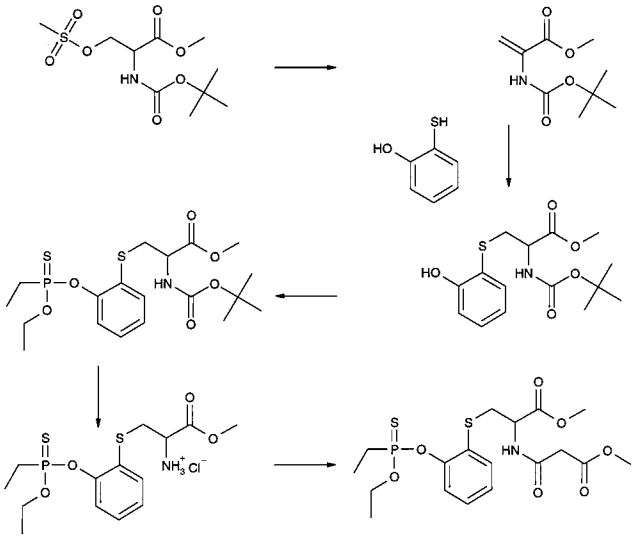
Scheme 1. Synthesis of DL-Cysteine, *N*-(Methoxycarbonylacetyl)-[2-[(ethoxyethylphosphinothionyl)oxy]phenyl]-, Methyl Ester



It is the purpose of this study to use LC-MS and LC-MS-MS with packed capillary HPLC and continuous-flow FAB to identify the water-soluble metabolites of fonofos found in mature plants. These experiments were done as part of a larger crop rotation study done for reregistration of fonofos with the EPA.

MATERIALS AND METHODS

Chemicals. *Fonofos*. *O*-Ethyl *S*-phenyl-U-^{14}C-$^{13}C_6$-ethylphosphonodithioate was synthesized by Zeneca Ag Products (Richmond, CA). The material was prepared with a $^{12}C/^{13}C$ ratio of about 2:1 and a specific activity of 28 Ci/mol. The synthesis has previously been described (*11*). Structural confirmation was done by H NMR and EI-GC-MS. The material was found to have a purity of 99% by GC-MS.

DL-Cysteine, N-(Ethoxycarbonylacetyl)-[2[(ethoxyethylphosphinothionyl)oxy]phenyl]-, Methyl Ester. See **Scheme 1**.)

N-tert-Butoxycarbonylserine (20.5 g, 0.10 mol), Cs_2CO_3 (16.5 g, 50.6 mmol), and CH_3I (12 mL, 27.3 g, 0.192 mol, 1.9 equiv) were stirred in DMF (250 mL) overnight. The mixture was then diluted with water, and the product was extracted with EtOAc (three times). The combined extracts were washed with brine (saturated), dried ($MgSO_4$), filtered, and concentrated to give 17.05 g (78% isolated) of *N-tert*-butoxycarbonylserine methyl ester as an oil: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.45 (s, 9H), 2.79 (br t, 1H), 3.78 (s, 3H), 3.91 (m, 2H), 4.4 (br d, 1H), 5.75 (br d, 1H).

Methane sulfonyl chloride (6.0 mL, 77.5 mmol) in CH_2Cl_2 (25 mL) was added dropwise to an ice-cold solution of *N-tert*-butoxycarbonylserine methyl ester (15.3 g, 69.8 mmol) and Et_3N (10.7 mL, 7.70 g, 77.0 mmol) in CH_2Cl_2 (150 mL). The ice bath was removed, and the solution was stirred for 2.5 h, then partitioned with brine (saturated) and extracted with CH_2Cl_2 (three times), dried (MgSO4), filtered, and concentrated to give 19.56 g (94% isolated) of the mesylate of *N-tert*-butoxycarbonylserine methyl ester: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.46 (s, 9H), 3.03 (s, 3H), 3.81 (s, 3H), 4.48 (m, 1H), 4.59 (m, 2H), 5.47 (br d, 1H).

Et_3N (8.0 mL, 5.80 g, 57.5 mmol) in THF (25 mL) was added dropwise to a solution of the mesylate (15.23 g, 51.27 mmol) in THF (250 mL) and allowed to stir for 6 h, diluted with brine (saturated), and extracted with CH_2Cl_2 (two times). The combined extracts were dried ($MgSO_4$), filtered, and concentrated to give 11.4 g of crude *N-tert*-butoxycarbonyldehydroalanine methyl ester. This crude ester was combined with 2-hydroxythiophenol (6.53 g, 51.8 mmol), and Et_3N (1.40 mL, 1.01 g, 10.0 mmol) and stirred in degassed CH_3OH (200 mL), under N_2, for 3 days. The solution was diluted with brine (saturated) and extracted with EtOAc (three times). The combined extracts were dried ($MgSO_4$), filtered, and concentrated. Column chromatography (SiO_2: hexane/EtOAc, gradient elution) gave 8.56 g of DL-cysteine, *N*-(*tert*-butoxycarbonyl)-2-hydroxyphenyl-, methyl ester as an oil: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.42 (s, 9H), 3.10 (dd, 1H), 3.22 (dd, 1H) or 3.16 (d of AB quartet, 2H), 3.62 (s, 3H), 4.53 (br, 1H), 5.40 (br, 1H), 6.85 (t, 1H), 6.98 (d, 1H), 7.16 (t, 1H), 7.27 (d, 1H).

The phenol (8.56 g, 26.2 mmol), *O*-ethyl-ethylphosphonochloridothioate (9.93 g, 57.5 mmol), Et_3N (10 mL, 7.26 g, 71.9 mmol), and (dimethylamino)pyridine (catalyst) were heated to reflux in THF (250 mL) for 4 h. The solution was cooled, diluted with water, and extracted with EtOAc (three times). The combined extracts were washed with brine (saturated), dried ($MgSO_4$), filtered, and concentrated to give 14 g of an oil. Column chromatography (SiO_2: hexane/EtOAc, gradient elution) afforded 9.15 g (75%) of DL-cysteine, *N*-(*tert*-butoxycarbonly)-[2-[(ethoxyethylphosphinothionyl)oxy]phenyl]-, methyl ester as an oil: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.31 (m, 6H), 1.41(s, 9H), 2.27 (br sextet, 2H), 3.36 (br d, 2H), 3.55 (s, 1.5H), 3.57 (s, 1.5H), 4.18 (m, 1H), 4.38 (m, 1H), 4.57 (broad, 1H), 5.46 (br m, 1H), 7.14 (t, 1H), 7.23 (m, 2H), 7.44 (d, 1H).

HCl gas was passed through a solution of the Boc ester (5.70 g, 12.3 mmol) in ether (200 mL). The solid was collected, washed with ether, and dried to give 2.02 g (45%) of DL-cysteine, [2-[(ethoxyethylphosphinothionyl)oxy]phenyl]-, methyl ester amine hydrochloride: ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.18 (t, 1.5H), 1.26 (m, 4.5H), 2.29 (sextet, 2H), 3.53 (s, 3H), 3.56 (m, 1H), 3.42 (m, 1H), 4.21 (m, 3H), 7.26 (m, 3H), 7.38 (d, 1H), 8.99 (br s, 3H).

Methyl malonyl chloride (0.75 g, 5.5 mmol) in CH_2Cl_2 (5 mL) was added dropwise to a ice bath cooled solution of the amine salt (1.00 g, 2.73 mmol) and Et_3N (1.5 mL, 1.09 g, 10.7 mmol) in CH_2Cl_2 (25 mL). The solution was allowed to warm to room temperature and stirred overnight. The mixture was diluted with $NaHCO_3$ (saturated) and extracted with CH_2Cl_2 (three times). The combined extracts were washed with brine (saturated), dried ($MgSO_4$), filtered, and concentrated. Column chromatography (SiO_2: hexane/EtOAc gradient elution) afforded 0.82 g (65%) of DL-cysteine, *N*-(methoxycarbonylacetyl)-[2-[(ethoxyethylphosphinothionyl)oxy]phenyl]-, methyl ester as an oil: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.29 (m, 6H), 2.25 (sextet, 2H), 3.16 (d, 1H), 3.20 (d, 1H), 3.44 (m, 2H), 3.61 (s, 3H), 3.48 (s, 3H), 4.23 (m, 2H), 4.83 (m, 1H), 7.17 (m, 1H), 7.22 (m, 2H), 7.46 (br d, 1H), 7.78 (br t, 1H).

Soil Treatment and Planting. A sandy loam soil obtained from Visalia, CA (57% sand, 34% silt, 9% clay, 0.8% organic matter, pH 7.4, cation exchange capacity 7.6 mequiv/100 g), was put in containers and treated with radiolabeled fonofos dissolved in 50:50 acetone/water at an application rate of 49 μg/cm^2 of soil surface (4.7 lb/acre). The soil was maintained at ambient temperature for 32 days in a greenhouse in Richmond, CA, before the crops were planted. Crops were harvested at maturity (3 months for *Latuca sativa*, 3.5 months for *Triticum aestivum*, and 4 months for *Beta vulgaris*).

Extraction. The mature lettuce leaves, beet leaves, beet roots, and wheat straw were pulverized with dry ice and then extracted with 50:50 (v/v) acetonitrile/water. The acetonitrile was removed by rotary evaporation, and the residual aqueous solution was extracted with ethyl acetate to remove nonpolar neutrals. The aqueous extracts were analyzed by HPLC-RAM and were the source of all of the metabolites identified in this study.

HPLC-RAM Analysis. HPLC-RAM analysis was done on a Hewlett-Packard model 1090 HPLC with a Raytest Ramona model 5-LS radioactivity monitor equipped with a solid scintillation cell. A PhaseSep Spherisorb S5 ODS2 column (4.6 mm i.d., 250 mm length) was used at 1 mL/min with the following gradient: 5−25% A in 60 min; 25−55% A in 20 min; 55% A for 10 min; 55−100% A in 10 min with A = acetonitrile containing 0.1% trifluoroacetic acid and B = 0.1% aqueous trifluoroacetic acid.

Metabolite Isolation. Metabolite A15 was isolated from beet leaf. The aqueous extract was acidified with 1 M HCl and extracted with ethyl acetate. The ethyl acetate extract was chromatographed on preparative HPLC. A PhaseSep Spherisorb S5 ODS2 column (4.6 mm i.d., 250 mm length) was used at 1 mL/min with the following gradient: 15−20% A in 15 min; 20−25% A in 10 min; 25−30% A in 5 min; 30−100% A in 20 min with A = acetonitrile containing 0.1% trifluoroacetic acid and B = 0.1% aqueous trifluoroacetic acid. The

fractions containing metabolite A15 were methylated with excess ethereal diazomethane. The methylated sample was further purified before LC-MS analysis by HPLC using 45:55 (v/v) acetonitrile/0.1% aqueous trifluoroacetic acid.

Metabolites A4, A5, and A14 were isolated from lettuce leaf. A Phenomenex Spherisorb 5 ODS2 column (10 mm i.d., 250 mm length) was used at 3 mL/min to purify the aqueous plant extract using the following gradient: 0−50% A in 25 min; 50% A for 10 min; 50−100% A in 5 min with A = acetonitrile containing 0.1% trifluoroacetic acid and B = 0.1% aqueous trifluoroacetic acid. This gave an A4/A5 fraction and an A14 fraction. The A4/A5 fraction was purified by HPLC using 7.5:92.5 (v/v) acetonitrile/water to give fractions A4 and A5. Metabolite A4 was methylated with excess ethereal diazomethane and analyzed by LC-MS. Metabolite A5 was methylated with excess ethereal diazomethane and was further purified before LC-MS analysis by HPLC using 12:88 (v/v) acetonitrile/0.1% aqueous trifluoroacetic acid. The A14 fraction from the preparative chromatography was further purified by HPLC (20:70 acetonitrile/water) and then methylated with excess ethereal diazomethane. The methylated material was further purified before LC-MS analysis by HPLC using 40:60 (v/v) acetonitrile/water.

Metabolite A12 was isolated from beet root. The aqueous extract was acidified with 1 M HCl and extracted with ethyl acetate. The ethyl acetate extract was chromatographed on a PhaseSep Spherisorb S5 ODS2 column (4.6 mm i.d., 250 mm length) at 1 mL/min with the following gradient: 0% A for 5 min; 0−50% A in 20 min; 50% A for 5 min; 50−100% A in 5 min with A = acetonitrile containing 0.1% trifluoroacetic acid and B = 0.1% aqueous trifluoroacetic acid. The fractions containing metabolite A12 were methylated with excess ethereal diazomethane before LC-MS analysis.

Metabolite A10 was isolated from wheat straw. The aqueous extract was chromatographed on preparative HPLC using the same column and method used to chromatograph the lettuce extract. The A10 fraction was further purified by HPLC using 25:75 (v/v) acetonitrile/0.1% aqueous trifluoroacetic acid and then again using 15:85 (v/v) acetonitrile/0.1% aqueous trifluoroacetic acid. Metabolite A10 was methylated with excess ethereal diazomethane and was further purified before LC-MS analysis by HPLC using 20:80 (v/v) acetonitrile/water.

NMR and Mass Spectrometry. NMR spectra were obtained on a General Electric model QE-300 Fourier transform spectrometer.

Packed capillary LC flow FAB was done on a Finnigan-MAT model TSQ 700 triple-quadrupole mass spectrometer (San Jose, CA) equipped with a flow FAB source. Mobile phases were prepared that contained acetonitrile, aqueous 0.1% trifluoroacetic acid, and 5% glycerol. The mobile phase was pumped with a Perkin-Elmer model 250 HPLC (Norwalk, CT) to a splitter connected to (1) an HPLC column (1 mm diameter by 200 mm long ABI Spheri-5 RP-18, 5 μm) that flowed to waste and (2) a Rheodyne model 8125 injector (Cotati, CA) with a 5.0 μL sample loop. Prior to injection, purified methylated metabolite samples (10−200 ng/5 μL injection) were concentrated to dryness and then redissolved in LC-MS mobile phase that had been diluted 2-fold with water to achieve solvent focusing. A 0.322 mm i.d. × 150 mm capillary HPLC column packed with Spherisorb ODS-2 (3 μm) from LC Packings (San Francisco, CA) was connected to the outlet of the Rheodyne injector. A flow rate of 8−10 μL/min through the packed capillary HPLC column was achieved by adjusting the total flow from the Perkin-Elmer pump from 0.15 to 0.2 mL/min. The packed capillary HPLC column effluent flowed through an ABI model 785A UV detector (Foster City, CA) that was operated at 210 nm and was equipped with a 60 nL flow cell, model UZ-View, from LC Packings. The flow from the UV detector was connected to the flow FAB source of the TSQ 700. The source was operated at 20−30 °C and bombarded with 6−7 kV xenon atoms. Source stability was achieved with the use of a Bausch and Lomb model Stereo 4 binocular microscope. MS-MS experiments were done with 0.5 mTorr of argon as collision gas. Ions were accelerated prior to collision-induced dissociation (CID) by applying a 20 V potential difference between the first quadrupole and the collision cell. Scans of 1 s duration were acquired in all cases.

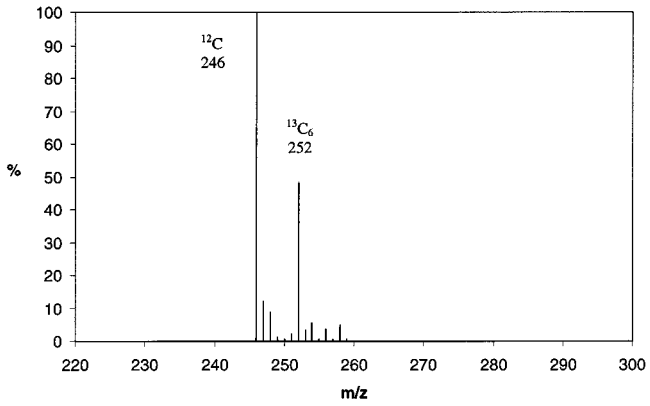


Figure 1. Molecular ion region from the electron impact mass spectrum of [^{14}C-UL-*phenyl*]-[^{13}C$_6$-*phenyl*]-*O*-ethyl-*S*-phenylethyl-phosphonodithioate.

RESULTS AND DISCUSSION

To identify metabolites of fonofos in mature plants, U-^{14}C-^{13}C$_6$-labeled fonofos (*O*-ethyl *S*-phenyl-U-^{14}C-^{13}C$_6$-ethylphosphonodithioate) was synthesized. The label incorporated ^{14}C to allow quantitation and to monitor purification of metabolites. The purpose of the ^{13}C$_6$ -label was (1) to provide a recognizable isotope pattern to readily distinguish fonofos metabolites from unlabeled natural products and (2) to allow tandem mass spectrometric experiments of both labeled and unlabeled precursor ions to aid in structure elucidation. **Figure 1** shows the molecular ion region of the mass spectrum obtained by electron impact GC-MS of the U-^{14}C-^{13}C$_6$-labeled fonofos used in the metabolism studies. The most abundant peak was observed at *m*/*z* 246 corresponding to fonofos, where all carbons are carbon-12 ($^{12}C_{10}H_{15}POS_2$). The next most abundant peak was observed at *m*/*z* 252 ($^{13}C_6{}^{12}C_4H_{15}POS_2$). This peak is from the ^{13}C$_6$-phenyl-labeled material. Despite the high specific activity of this material (28 Ci/mol), ions corresponding to molecules of fonofos containing four, five, and six atoms of carbon-14 (at *m*/*z* 254, 256, and 258) are of low abundance (4−8% of the intensity of the ion of *m*/*z* 246) and were not useful for the recognition of fonofos-related ions of unknown metabolites.

Soil treated with [U-^{14}C-^{13}C$_6$]fonofos was kept at ambient temperature for 32 days before seeds of lettuce (*Latuca sativa*), beet (*Beta vulgaris*), or wheat (*Triticum aestivum*) were planted. The plants were grown to maturity and the lettuce leaves, beet leaves, beet roots, and wheat straw were extracted with acetonitrile/water. HPLC-RAM analysis of the various extracts gave the radiochromatograms shown in **Figure 2**. Six major and a variety of minor radioactive peaks (named A1−A15) were found. All of the metabolites were more polar than fonofos, which was not detected in mature plant tissue.

Metabolite A8 (see **Figure 2**) cochromatographed with methylphenyl sulfone. The other labeled metabolites were identified by LC-MS. Extensive purification was required before LC-MS analysis due to the low concentration of metabolites in plants grown in soil containing biologically relevant amounts of fonofos. For example, the maximum application rate used in agriculture (4 lb/acre) corresponds to a concentration of fonofos in the top 7 cm of soil of ~5 ppm. At maturity the plants contained only 0.3−1.7% of the applied radioactivity. Concentrations in the plant tissues used to isolate metabolites A4, A15, A5, A14, and A12 were only 20, 40, 60, 70, and 230 ppb, respectively.

FAB gave significantly larger MH^+ intensities from the methylated samples than for the free acids. Consequently, all of the metabolites were analyzed as the free acid and as their

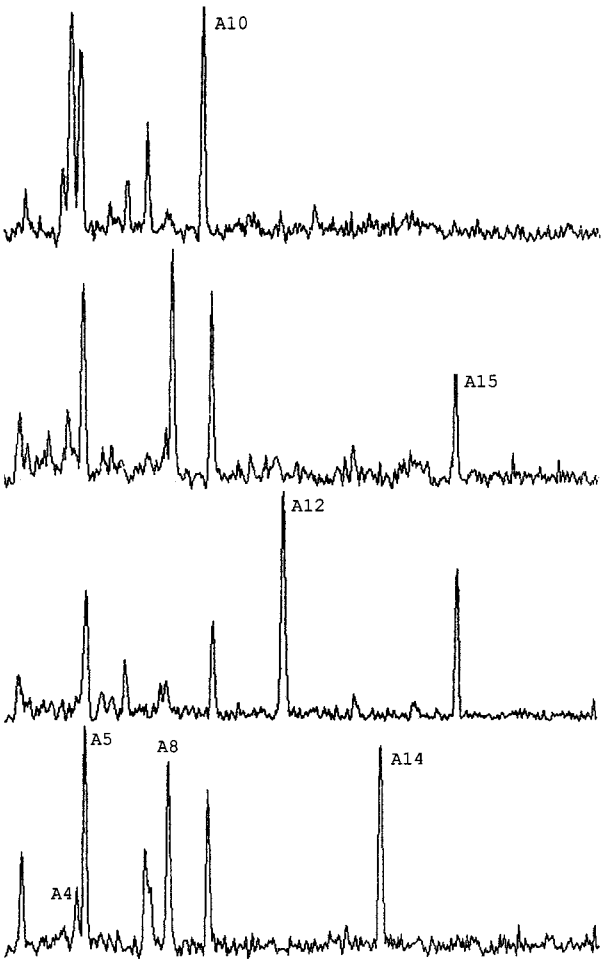


Figure 2. Radiochromatogram (^{14}C, *y*-axis) of lettuce leaf (bottom), beet root, beet leaf, and wheat straw (top) extracts by gradient HPLC. The *x*-axis is time after injection from 0 to 100 min.

methyl esters. LC-MS analysis of native metabolite A15 gave an MH^+ ion of *m*/*z* 436. Metabolite A15 was then methylated with diazomethane and examined by packed capillary LC-MS. The corresponding mass spectrum showed an MH^+ ion of *m*/*z* 464, which indicates that the peak is a dimethyl ester of the native metabolite. The MH^+ ion of the methylated metabolite showed the same isotopic pattern as for the starting fonofos, demonstrating the presence of the intact phenyl group in the unknown metabolite. Furthermore, the intensity of the $MH^+ + 2$ ion relative to MH^+ (11.8 ± 0.6% for the mean ± SD of three independent observations) suggests the presence of two sulfur atoms in metabolite A15.

Subsequent analysis of methylated metabolite A15 by LC-MS-MS observing product ions of *m*/*z* 464 resulted in the CID spectrum shown in **Figure 3** (bottom). The fragment ion of *m*/*z* 109 was first attributed to C_6H_5S; however, no reasonable structure for A15 could be assembled that contained this substructure. Next the methylated metabolite was analyzed by LC-MS-MS observing product ions of the $^{13}C_6$ isotope peak at *m*/*z* 470. This gave the CID spectrum shown in the top of **Figure 3**. By comparing the two CID spectra in **Figure 3** it is possible to determine which fragment ions contain the phenyl ring. For example, the CID fragments at *m*/*z* 125, 211, 233, 261, 263, 364, and 432 contain all six carbons of the phenyl group because these ions are observed at *m*/*z* values 6 amu higher in the analogous CID spectrum of the $^{13}C_6$-labeled MH^+ ion. The ions of *m*/*z* 109, 137, 170, and 202 were found at the same *m*/*z* value

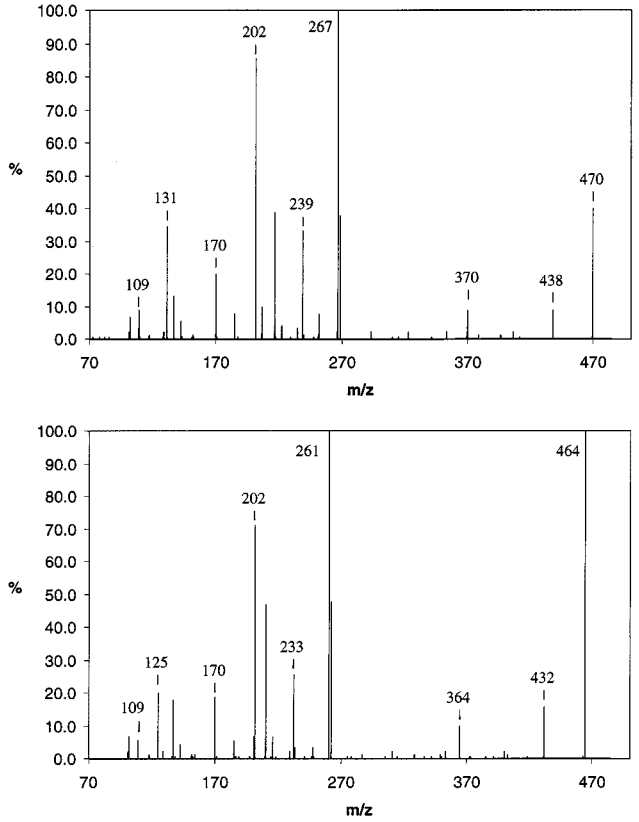


Figure 3. CID spectra of *m*/*z* 470 (top) and 464 (bottom) from the LC-MS-MS analysis of methylated metabolite A15.

in both CID spectra and therefore contain none of the phenyl carbons.

To determine the number of sulfur atoms in the CID fragment ions, methylated metabolite A15 was analyzed by LC-MS-MS observing product ions of the ^{34}S isotope peak at *m*/*z* 466. Fragment ions containing no sulfurs should appear at the same *m*/*z* value in CID spectra of either *m*/*z* 464 or 466. Fragments containing one sulfur will produce two equal intensity peaks separated by 2 amu in the CID spectrum of *m*/*z* 466, because the precursor ions contain the ^{34}S atom in either one of two possible positions. Fragment ions that contain both sulfurs will produce a single peak in the CID spectrum of *m*/*z* 466, but because the fragment contains the ^{34}S atom of the precursor, it will have an *m*/*z* ratio 2 amu higher than the analogous ion in the CID spectrum of the *m*/*z* 464 ion. The presence of ^{18}O and $^{13}C_2$ complicates the analysis somewhat. In the CID spectrum of *m*/*z* 466, the observed ratio of intensities of *m*/*z* $x + 2$ to *m*/*z* *x* is shown in **Figure 4** for nine fragment ions (x = 109, 125, 137, 170, 202, 211, 233, 261, and 364). Also shown in **Figure 4** are the theoretical intensity ratios for the nine ions of interest assuming zero, one, or two sulfurs per ion. By comparing the observed-to-predicted ratios it is clear that the fragment ions of *m*/*z* 170 and 202 contain no sulfur atoms, the fragment ions of *m*/*z* 109, 125, 137, and 211 contain one sulfur atom, and the fragment ions of *m*/*z* 233, 261, and 364 contain two sulfur atoms.

The CID fragment ion from A15 of *m*/*z* 125 contained one sulfur and all six phenyl carbons, suggesting the empirical formula of C_6H_5OS and that metabolite A15 contained hydroxythiophenol as a substructure. The fragment ions of *m*/*z* 109 and 137 contained one sulfur atom and no phenyl carbons. Furthermore, ions of *m*/*z* 109 and 137 are found as fragment ions of fonofos (electron impact data, not shown), which suggests the substructures C_2H_5PSOH and $C_2H_5PSOC_2H_5$ for the ions of *m*/*z* 109 and 137. Assembling these substructures provided the

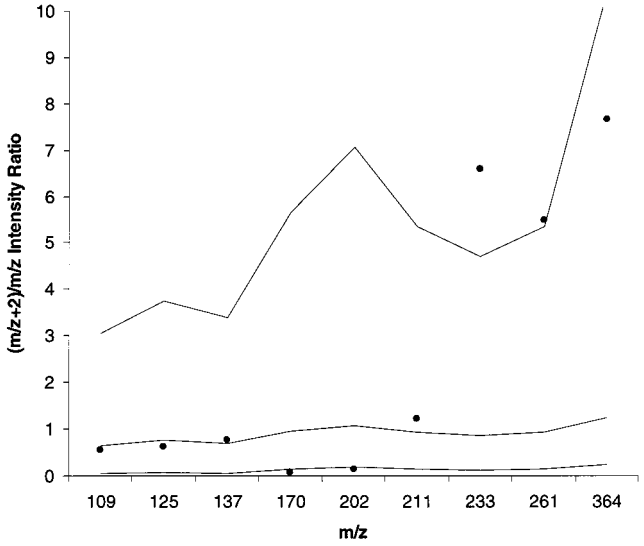


Figure 4. Predicted (solid lines) vs observed (●) ion intensity ratios for ions of *m*/*z* 109 ($C_2H_6OPS_x$), 125 ($C_6H_5OS_x$), 137 ($C_4H_{10}OPS_x$), 170 ($C_7H_8NO_4S_x$), 202 ($C_8H_{12}NO_5S_x$), 211 ($C_{10}H_{13}NO_2S_x$), 233 ($C_8H_{10}O_2PS_x$), 261 ($C_{10}H_{14}O_2PS_x$), and 364 ($C_{14}H_{23}NO_4PS_x$) from the LC-MS-MS product ion analysis of *m*/*z* 466 of methylated metabolite A15 where $x = 0$ (bottom), 1 (middle), or 2 (top). Predicted ion intensity ratio $= [(a + b + c)/((a(\text{no. of C in ion}/18) + b(\text{no. of O in ion}/7) + c(x/2))] - 1$, where $a = 0.005(1.08 \times 18)^2$, $b = 0.02(7)$, and $c = 4.21(2)$.

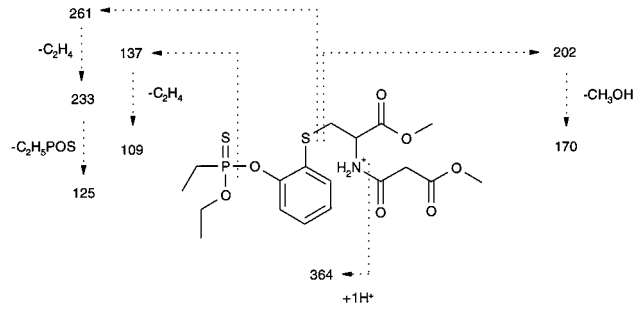


Figure 5. CID fragmentation scheme for methylated metabolite A15.

structure proposed for the dimethyl ester of metabolite A15. **Figure 5** shows this structure as well as the origin of the major CID fragmentation ions. Further work was needed to determine the hydroxythiophenol substitution pattern in metabolite A15. Due to biosynthetic mechanistic reasons the 1,2-substituted isomer was considered to be most likely. A proposed pathway is shown in **Scheme 2**. Metabolite A15 could arise via glutathione addition to a benzene epoxide, rearrangement, and elimination of H_2S to give the glutathione conjugate of the fonofos oxon isomer. Subsequent hydrolysis to lose glycine and glutamate followed by malonylation would give metabolite A15. The proposed rearrangement occurs via a five-member transition state and results in biosynthesis of a 1,2-substituted hydroxythiophenol. Consequently, the 1,2-substituted isomer of A15 was synthesized (see **Scheme 1**). The synthetic material cochromatographed with the methylated metabolite on HPLC. Furthermore, the CID spectrum obtained by LC-MS-MS analysis of synthetic A15M was nearly indistinguishable from the analogous CID spectra of the metabolite, demonstrating that the proposed structure is correct.

Packed capillary LC-MS analysis of methylated metabolite A14 showed an MH^+ ion of *m*/*z* 313. Analysis of methylated peak A14 by LC-MS-MS was done with observation of product ions of *m*/*z* 313. The CID spectrum (see **Figure 6**) obtained

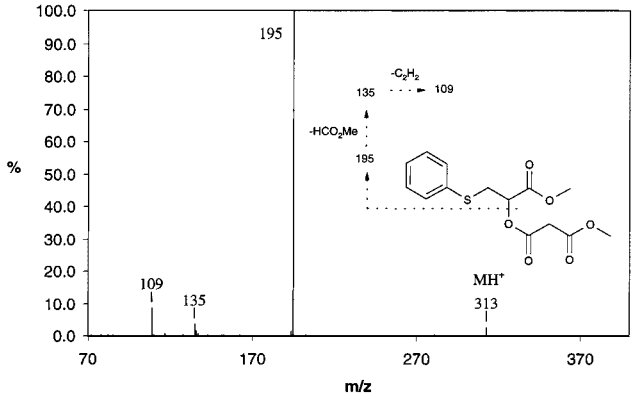
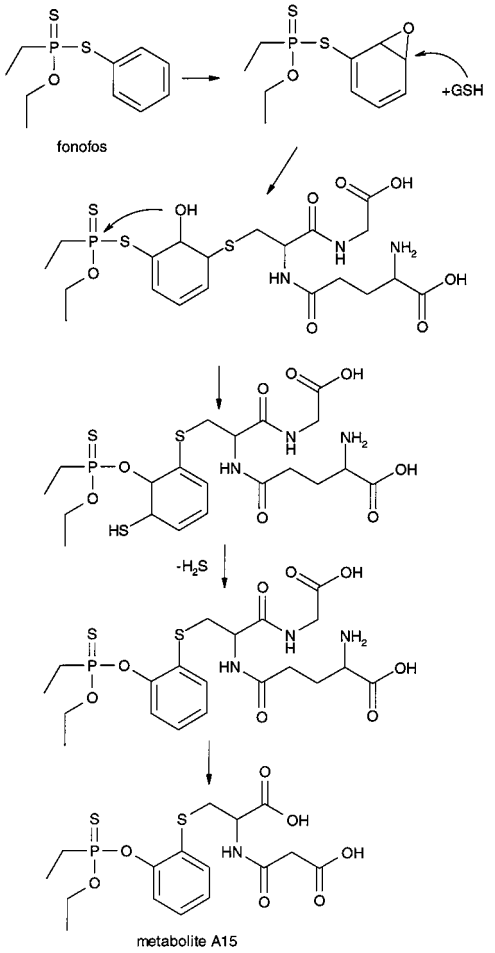


Figure 6. CID spectrum of *m*/*z* 313 from the LC-MS-MS analysis of methylated metabolite A14.

Scheme 2. Proposed Mechanism for Biosynthesis of Metabolite A15



contained fragment ions of *m*/*z* 109, 135, and 195. This suggested the structure of a malonate ester of thiophenoxylactic acid.

Packed capillary LC-MS analysis of methylated metabolite A12 showed an MH^+ ion of *m*/*z* 312. Analysis of methylated peak A12 by LC-MS-MS was done with observation of product ions of *m*/*z* 312. The CID spectrum obtained (see **Figure 7**) contained fragment ions of *m*/*z* 101, 118, 135, 195, 202 (as in A15), 252, and 280, suggesting the malonic acid amide of *S*-phenylcysteine. This structure and the origin of the major CID fragment ions are also shown in **Figure 7**.

Packed capillary LC-MS analysis of methylated metabolite A10 showed an MH^+ ion of *m*/*z* 375. Analysis of methylated

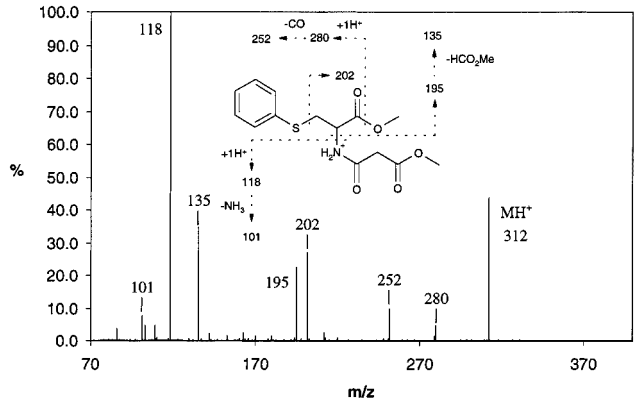


Figure 7. CID spectrum of *m*/*z* 312 from the LC-MS-MS analysis of methylated metabolite A12.

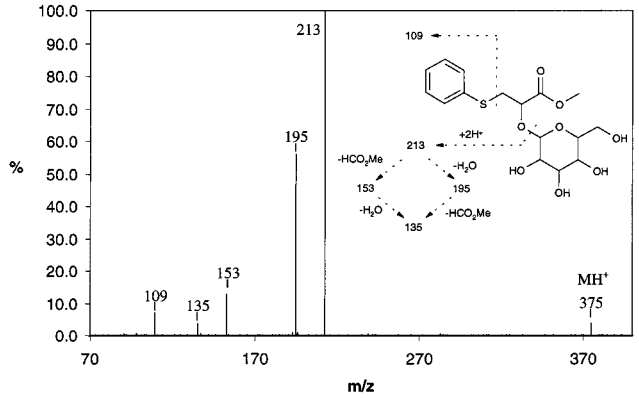


Figure 8. CID spectrum of *m*/*z* 375 from the LC-MS-MS analysis of methylated metabolite A10.

peak A10 by LC-MS-MS was done with observation of product ions of *m*/*z* 375. The CID spectrum obtained contained fragment ions of *m*/*z* 109, 135, 153, 195, and 213, suggesting the glucoside of thiophenoxylactic acid. This structure and the origin of the major CID fragment ions are shown in **Figure 8**.

Packed capillary LC-MS analysis of methylated metabolites A4 and A5 showed both to have an MH^+ ion of *m*/*z* 391. Analysis of methylated peak A4 and methylated peak A5 by LC-MS-MS was done with observation of product ions of *m*/*z* 391. The CID spectra obtained were nearly indistinguishable and contained fragment ions of *m*/*z* 103, 127, and 229, suggesting isomers of molecular weight 16 amu higher than that of methylated metabolite A10. Oxidation of metabolite A10 with hypochlorite gave material that comigrated on HPLC with metabolites A4 and A5. This suggested that metabolites A4 and A5 are sulfoxides of metabolite A10. These structures and the origin of the major CID fragment ions are shown in **Figure 9**. Furthermore, methylation gave only monomethyl derivatives for metabolites A4 and A5. (The alternative isomers with the phenyl ring oxidized to a phenol should have methylated to give dimethyl derivatives of molecular weight 404.) Finally, the analysis of methylated peak A4 and methylated peak A5 by LC-MS-MS was done with observation of product ions of *m*/*z* 229, which corresponds to the protonated aglycon. The CID spectrum obtained contained fragment ions of *m*/*z* 71, 103, 109, 125, 127, 169, and 197. The fragment of *m*/*z* 109 (C_6H_5S) is much more likely to arise from the proposed sulfoxides than from the alternative phenolic isomers.

Prior to this study, the metabolism of fonofos in plants has been studied only in *Solanum tuberosum* (*4*). Fonofos is reported to be metabolized to thiophenol, which is converted to methyl

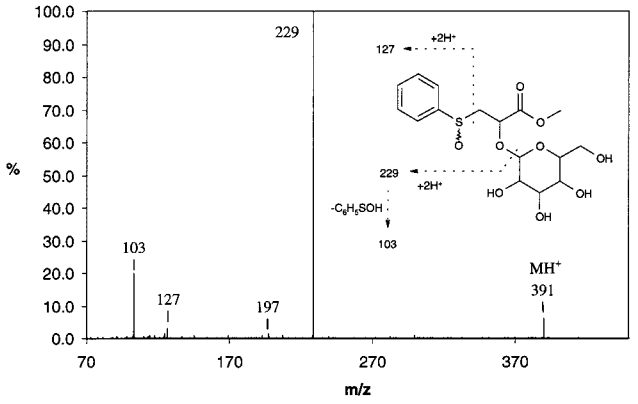
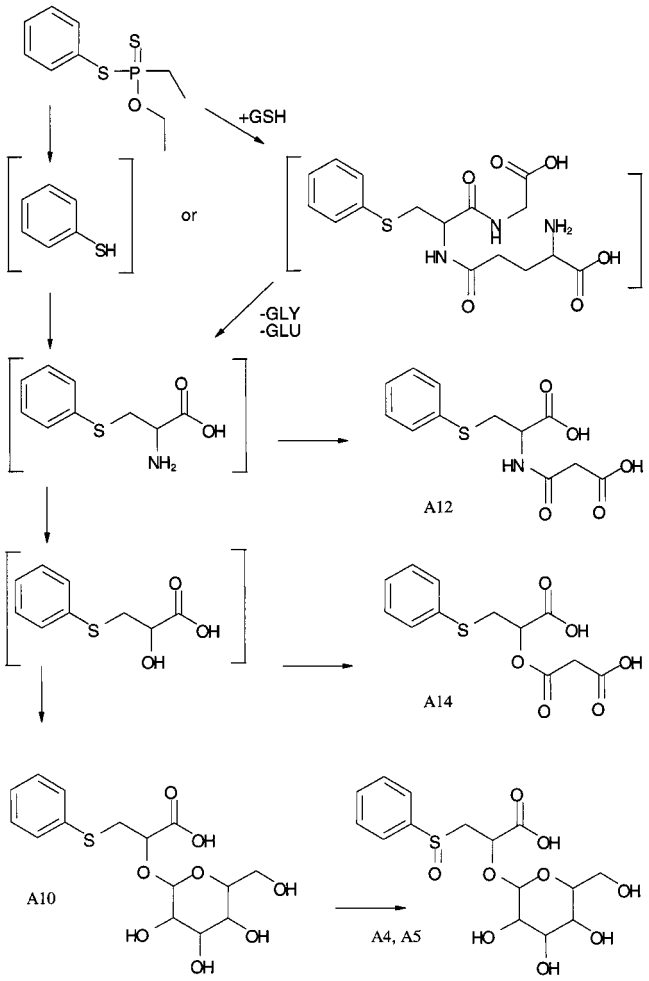


Figure 9. CID spectrum of *m*/*z* 391 from the LC-MS-MS analysis of methylated metabolite A4.

Scheme 3. Proposed Pathway for Metabolism of Fonofos



phenyl sulfide, methyl phenyl sulfoxide, and ultimately methylphenyl sulfone.

The cysteine and lactic acid derivatives found in this study (A4, A5, A10, A12, and A14) appear to be typical plant metabolites of a glutathione conjugate (*12*). The metabolic pathway proposed for formation of the fonofos metabolites identified in this paper is shown in **Scheme 3** and shows how these metabolites could be formed from a glutathione conjugate of thiophenol. **Scheme 3** also shows an alternative route to these metabolites via hydrolysis of fonofos to thiophenol and then synthesis of the cysteine conjugate of thiophenol. The intermediacy of thiophenol is supported by the identification of

methylphenyl sulfone, formed most likely by methylation and oxidation of thiophenol. Synthesis of the cysteine conjugate of thiophenol could be accomplished by addition of pyruvate and NH_3 to thiophenol by a cysteine conjugate β-lyase (EC 4.4.1.13) operating in reverse (C−S synthesis, not C−S cleavage). Cysteine conjugate β-lyases have been well documented in the rat (*13*) and in plants (*14*). To our knowledge, this enzyme has never been shown to act in the biosynthesis of cysteine conjugates. Study of the metabolism of [$^{34}S_2$]fonofos could prove whether the glutathione- or the thiophenol-related pathway proposed in **Scheme 3** is operative in plants and provide evidence for a C−S lyase acting in the biosynthesis of cysteine conjugates.

ABBREVIATIONS USED

LC-MS, liquid chromatography−mass spectrometry; GC-MS, gas chromatography−mass spectrometry; FAB, fast atom bombardment; EOP, *O*-ethyl ethylphosphonate; ETP, *O*-ethyl ethylphosphonothioate; HPLC, high-pressure liquid chromatography; DMF, dimethylformamide; NMR, nuclear magnetic resonance; THF, tetrahydrofuran; RAM, radioactivity monitor; UV, ultraviolet; TIC, total ion current; CID, collision-induced dissociation; GSH, glutathione.

SAFETY

Diazomethane is an explosive (use safety shield), insidious poison (a well-ventilated hood is absolutely necessary) and a strong irritant, which does not cause discernible reaction at the time of contact but later, even in minute amounts, produces an inflammatory response.

N-Methyl-*N*′-nitro-*N*-nitrosoguanidine is the starting material for the preparation of diazomethane. It is a highly flammable, toxic mutagen that may cause cancer, may cause heritable genetic damage, is a possible teratogen, is toxic if swallowed, is harmful by inhalation, and is irritating to the eyes, respiratory system, and skin.

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