Identification of a Novel Animal Metabolite of Methomyl Insecticide†

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In studies conducted to determine the fate of methomyl in goat, chicken, and monkey, an unknown highly polar, acidic metabolite was found in the urine/excreta of all three animals. The metabolite was purified by HPLC and analyzed by microcolumn LC/MS with electrospray ionization and by NMR. A Hypercarb porous graphitic carbon HPLC column was used for purification and LC/MS, since the unknown was unretained on conventional reversed phase HPLC columns. [^{14}C]Methomyl was used as radiotracer, and [^{13}C]methomyl was added at the 50% level to facilitate metabolite identification. The molecular weight of the metabolite was determined by LC/MS-electrospray, using positive and negative ionization, and structurally useful fragment ions were obtained in both modes by in-source collision-induced dissociation (CID). High-resolution accurate mass measurements of the MH^+ doublet allowed assignment of the elemental composition. NMR data showed the presence of methyl, methylene, and methine protons with coupling observed between ^{13}C and the methyl and methine protons. From these data, the structure was determined to be an unusual double conjugate of acetonitrile (a degradate of methomyl), involving conjugation with both cysteine and sulfate.

Keywords: *Methomyl; animal metabolism; identification; double conjugate*

INTRODUCTION

Methomyl [*S*-methyl-*N*-[(methylcarbamoyl)oxy]thioacetimidate] is the active ingredient in DuPont's Lannate insecticide. Methomyl is one of the leading insecticides in the world, in terms of both amount used and acreage. It has been registered since 1967. It is registered for use on over 100 crops in 70 countries. It is highly effective for control of over 100 insects as an ovicide, larvicide, and adult insecticide. Methomyl is a polar, water soluble compound, and it is rapidly and extensively metabolized in animals (Harvey et al., 1973) and plants (Harvey and Reiser, 1973). Isolation and purification of the highly polar metabolites is very difficult, because their physical and chemical properties are very similar to those of many natural products. In earlier studies on the metabolism of methomyl in the rat (Harvey et al., 1973), it was found that essentially all of the radioactivity was eliminated in 24 h in the ratio of 1 part of carbon dioxide, 2 parts of acetonitrile, and 1 part of urinary metabolites. The urinary metabolites were not identified. More recently, the metabolic fate of methomyl has been investigated in the monkey, goat, and hen to support the U.S. EPA reregistration of this insecticide. The major metabolites in these studies were also acetonitrile and carbon dioxide, accounting for 5−34% and 19−34% of the administered dose, respectively. This paper discusses the technology used in the identification of a novel unknown highly polar metabolite excreted by all three animals. This metabolite represents 1−4% of the initial dose of parent to the animals. It is one of numerous excreted metabolites, and information on the identification of other metabolites and the absorption, distribution, and excretion of [^{14}C]methomyl and the quantitation of metabolites in the various animals will be described elsewhere.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]- and [1-^{13}C]Methomyl, *S*-methyl *N*-[(methylcarbamoyl)oxy]thioacetimidate, were obtained from DuPont NEN Research Products, Boston, MA. Chemical and radiochemical purities were >98%. [1-^{13}C]Methomyl was incorporated into the radiolabeled test substance at approximately 40−50 atom %.

Animal Treatment. Five leghorn hens (wt ∼1.5 kg each) and a lactating goat (Toggenburg/Alpine cross), wt ∼50 kg) were dosed with 5 and 160 mg of [1-^{14}C/^{13}C]methomyl, respectively, by capsule for 3 days at a specific activity of 23.3 and 11.0 μCi/mg, respectively. Four male cynomolgus monkeys (wt ∼2.2 kg) were dosed once with [1-^{14}C/^{13}C]methomyl at approximately 5 mg/kg (15.1 μCi/mg) using sodium acetate buffer (0.1 M, pH 5) as the dosing vehicle. Animals were placed in metabolism chambers and excreta collected daily and stored frozen until analysis.

Metabolite Isolation. The metabolite was isolated and purified from various excreta matrices using similar HPLC methodology after the initial extraction, concentration, and/or cleanup steps. Chicken excretus was combined and homogenized with 2-fold methanol/water (1:1 v/v) using a Tekmar Tissumizer. The homogenate was centrifuged, and the supernatants were decanted and saved. The pellet was further extracted twice using the same procedures. The methanol/water extracts were combined and concentrated by rotary evaporation to a water only containing fraction. Initial cleanup was achieved by passing the concentrated extract through a 10-g C_{18} Mega Bond Elut solid phase extraction cartridge. The metabolite of interest was not retained but separated from other components retained on the cartridge. The partially purified metabolite was next injected onto a Hamilton PRP-1 column (7.0 mm × 305 mm) and eluted with water containing 0.1% trifluoroacetic acid at a flow rate 2.0 mL/min. While the metabolite eluted in the void volume using the acidic mobile phase, it was again separated from other acidic more nonpolar components using this column. The

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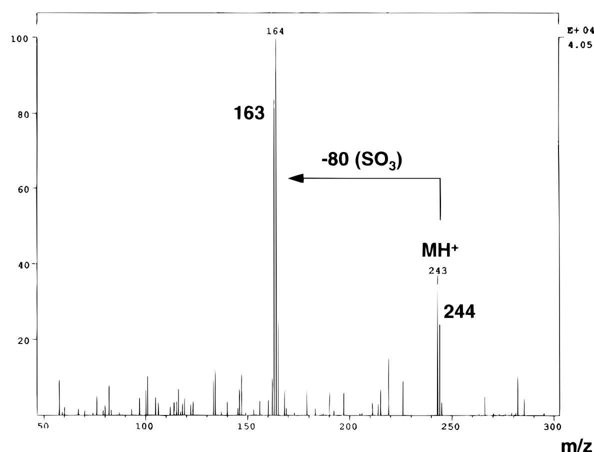

Figure 1. Electrospray ionization mass spectrum of metabolite, positive ion mode with in-source CID.

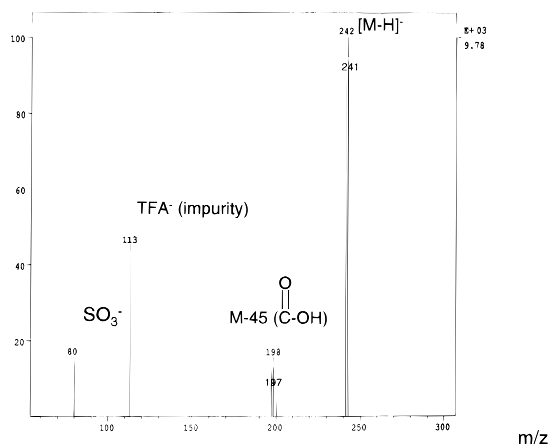

Figure 2. Electrospray ionization mass spectrum, negative ion mode with in-source CID.

unretained fraction was further purified using a Shandon Hypercarb porous graphitic carbon HPLC column (10 mm × 250 mm). HPLC gradient conditions varied slightly depending on the matrix, but, in general, initial mobile phase conditions were 0.1% trifluoroacetic acid in water for 9−10 min, followed by a linear gradient to 100% methanol over 25−30 min, at a flow rate of 4.5 mL/min. The metabolite of interest was retained and eluted with approximately 40−50% methanol. Initial cleanup of goat urine involved using the C_{18} solid phase extraction cartridge cleanup and PRP-1 HPLC fractionation, followed by further purification on Zorbax Rx C_{18} HPLC and the graphitic carbon HPLC columns using gradients of acidified water (trifluoroacetic or formic acid) and methanol or acetonitrile. Monkey urine was lyophilized, resuspended in a small amount of water, applied directly to PRP-1 for initial fractionation, and further purified as described above.

LC/MS. The isolated metabolite samples, typically 1−2 μg, were dissolved in 20−40 μL of water containing 0.1% formic acid, and 1 μL (∼50 ng of metabolite) was injected with a Valco C14W injection valve into a 15 cm × 0.32 mm i.d. Hypercarb S (7 μm particles) column (LC Packings, Inc.). The initial mobile phase was water + 0.1% formic acid for 3 min and then an acetonitrile gradient to 90% acetonitrile at 40 min using a Micro-Tech Scientific gradient pump. Flow rate was 10 μL/min. A UV detector (Linear UVIS 200), on-line between the column and MS, was set at 210 nm. The transfer line was fused silica tubing, 50 μm i.d. × 200 μm o.d. A Finnigan MAT 900 magnetic sector mass spectrometer (E-B geometry) was used with a magnetic scan from *m/z* 45 to 1000 at 3 s/decade. Electrospray ionization (Finnigan ESI-2, heated metal capillary) was used, with 8- kV spray voltage. Sheath gas (nitrogen at 4 bar) and sheath liquid (acetonitrile at 10 μL/min) were used.

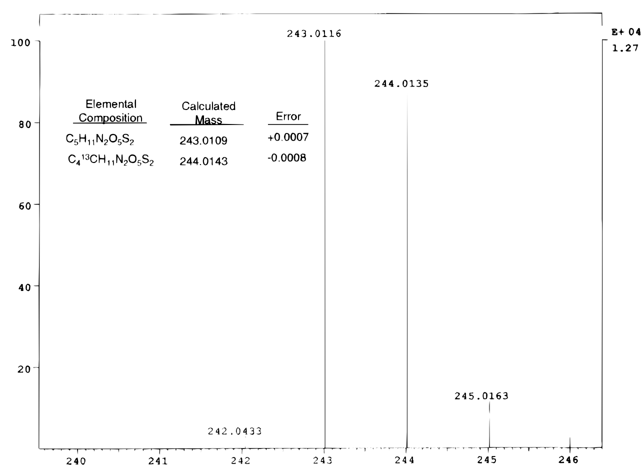

Figure 3. High-resolution accurate mass results obtained on MH^+ doublet.

High-resolution accurate mass measurements were made at 6000 resolution using an electric scan from *m/z* 235 to 255 with a 2-s cycle time. Linuron (MW 248) was used as a reference standard, and a 0.05 mg/mL solution of linuron in acetonitrile was added through the sheath liquid tube at 10 μL/min.

NMR. All NMR data were acquired using a Bruker AMX-360 spectrometer equipped with an inverse detect probe. About 5−10 μg of the sample was dissolved in 200 μL of D_2O. The temperature was 17 °C, which helped minimize interference from the water resonance by moving it to a higher field than is typical at room temperature.

Proton 1D data were acquired using a standard one-pulse experiment with continuous wave (CW) presaturation of the water resonance.

Proton−proton correlated (correlated spectroscopy, COSY) data were acquired using a standard Bruker pulse sequence for double quantum filtering (DQF) with time proportional phase incrementation. The sequence was modified in-house to include CW presaturation of the solvent resonance.

Proton−carbon correlated data were also acquired using a standard Bruker pulse sequence for proton detection of heteronuclear interaction (heteronuclear multiple bond correlation, HMBC). Globally optimized alternating-phase rectangular pulses (GARP) decoupling of the carbon frequency was used during the acquisition time period to eliminate proton−carbon splitting of the observed signals. A delay of 100 ms was used for evolution of the heteronuclear interaction. This corresponds to a coupling constant of 5 Hz.

RESULTS AND DISCUSSION

The key to isolation of this highly polar metabolite was the use of a Hypercarb HPLC column. In initial work using C_{18} and PRP-1 columns for isolation, the unknown metabolite eluted in the void volume and isolated samples did not give useful MS or NMR data. PRP-1 columns often retain polar metabolites, but not in this case. A Hypercarb column retained the metabolite and separated it from many polar natural products. This column gives enhanced retention of highly polar compounds and is a particularly good adsorbent for compounds with many functional groups (Shandon, 1994). It uses standard RPLC mobile phases and separates analytes in their ionized forms. Even Hypercarb column-purified samples did not give electrospray mass spectra by LC/MS with a C_{18} column, apparently due to coelution of other components in the solvent front that suppressed ionization of the metabolite. Use of a Hypercarb column for LC/MS resulted in successful analyses.

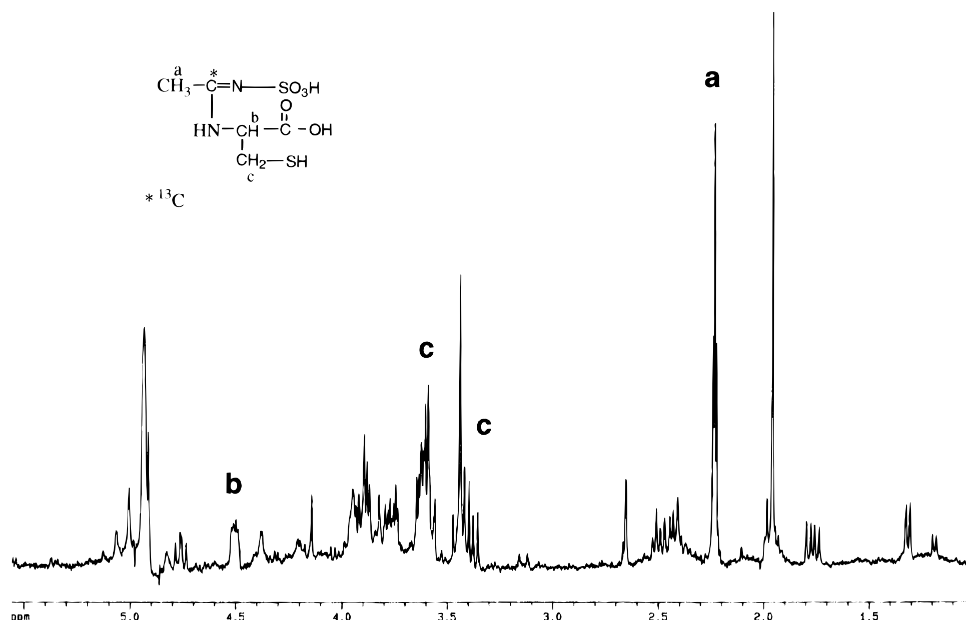

Figure 4. Proton NMR spectrum.

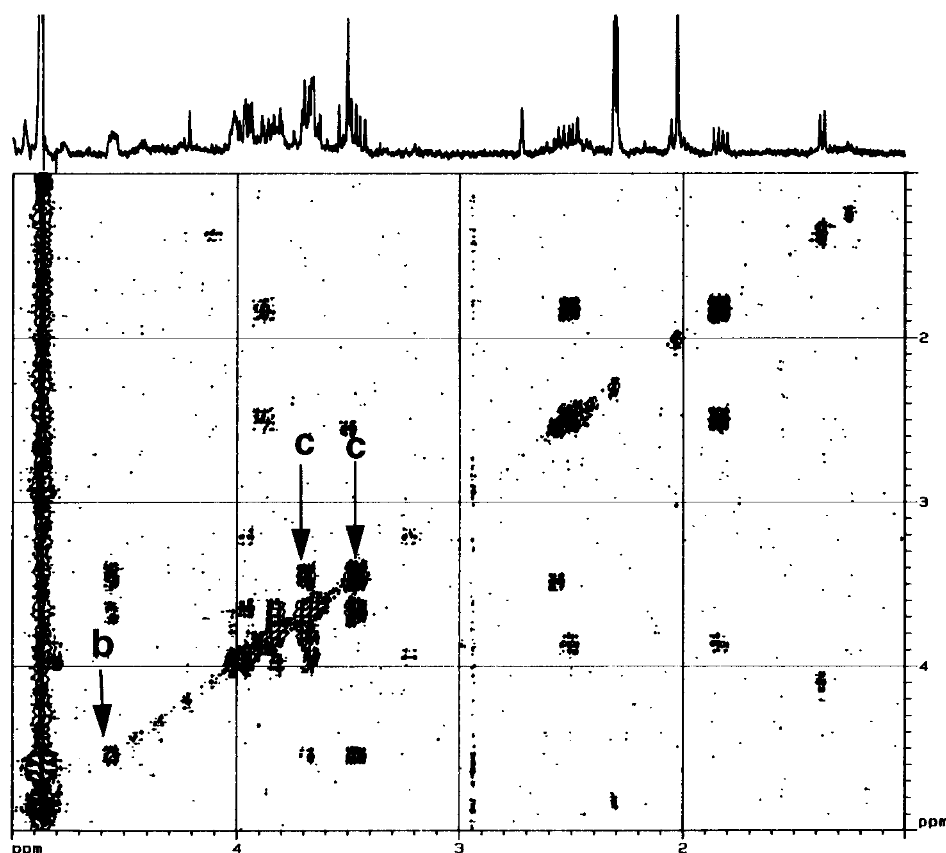

Figure 5. NMR: proton/proton correlated, DQF-COSY.

Incorporation of ^{13}C was very helpful for interpretation of LC/MS and NMR data. Characteristic 1:1 doublets, 1 amu apart, were obtained in the mass spectra, and long-range coupling of certain protons with ^{13}C was observed in the NMR spectra.

Analysis of the unknown metabolite by LC/MS with electrospray ionization (ESI) in the positive ion mode shows an MH^+ doublet at *m/z* 243/244, indicating a molecular weight of 242. The CID mass spectrum (Figure 1) shows a prominent fragment ion doublet at *m/z* 163/164, suggesting loss of SO_3 from the MH^+. No other fragment ions were observed. The ESI negative ion CID mass spectrum (Figure 2) gives an $[M - H]^-$ doublet at *m/z* 241/242, which confirms the molecular weight as 242, and fragments at *m/z* 197/198, indicating loss of a carboxyl group, and *m/z* 80, indicating an SO_3^- suggesting a sulfate conjugate. The absence of a fragment ion at *m/z* 97 due to HSO_4^- is evidence the unknown is not a sulfate ester, as these typically give both *m/z* 97 and 80 by negative ion electrospray (Metzger et al., 1995). High-resolution accurate mass data (Figure 3) were obtained by LC/MS (50-ng injection) in the positive ion mode on the MH^+ doublet. Excellent agreement with the calculated mass for the elemental

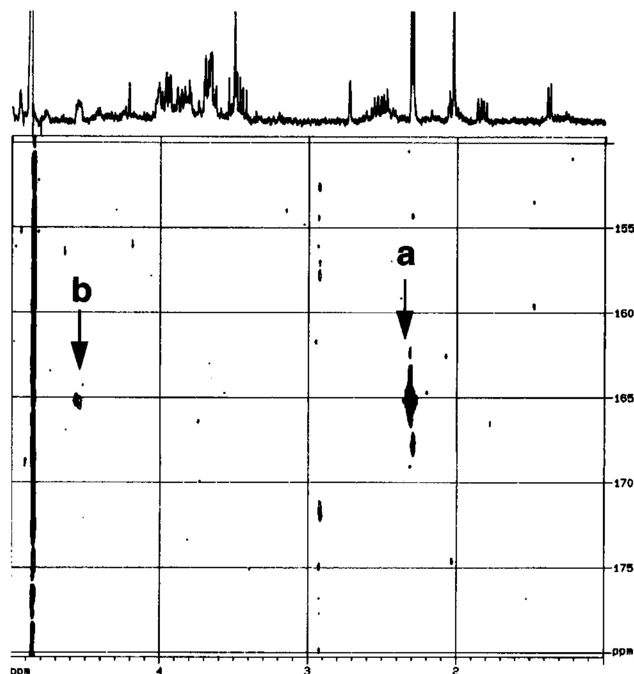

Figure 6. NMR: proton/carbon correlated, HMBC.

composition of both ions was obtained. No other logical elemental compositions were found by computer search within a ±0.002 mass unit tolerance, and the following element limits: C and H, 0−100; N, 0−4; O, 0−6; S, 0−3. These results indicate the elemental composition of the metabolite is $C_5H_{10}N_2O_5S_2$, after a hydrogen is subtracted from the protonated molecule that was mass measured.

The 1D proton NMR spectrum (Figure 4) is complicated due to impurities in the sample, but a methyl group derived from the parent is discernible by chemical shift (2.23 ppm) as is the characteristic pseudo-triplet, which is due to the superposition of the resonances from the ^{13}C-coupled protons, and the protons associated with the ^{12}C species. In addition, the loss of the methyl carbamate moiety from the parent is clear on the basis of the absence of the methyl singlet at 2.85 ppm. Other assignments based on the 1D spectrum were not made.

The combination of DFQ-COSY (Figure 5) and HMBC (Figure 6) allowed the assignment of the cysteinyl protons, as well as the mode of conjugation. From the HMBC, the α-methyl group (a) is shown to couple to the amidine carbon at a ^{13}C chemical shift of 165 ppm. Also evident is the coupling from the proton (b) at 4.5 ppm to the same ^{13}C chemical shift. This same proton is coupled to two others (c) in the COSY spectrum at 3.45 and 3.38 ppm, and these two are coupled to each other. These data are consistent with an N-linked cysteine conjugate of acetonitrile and are in agreement with the mass spectral data. While other correlations may be noted in the COSY spectrum, the 1D intensities do not allow that they are part of this system.

A proposed metabolic pathway for formation of this unusual metabolite is given in Figure 7. The proposed pathway through acetonitrile is based on finding acetonitrile as a major metabolite in rats, monkeys, goats, and hens and the logical chemistry of the proposed conjugation and hydrolysis reactions. Other pathways are possible, such as direct conjugation of methomyl with glutathione by displacement of the S−CH_3 by *S*-glutathione as the initial step, but this would require a reduction of the thiohydroxamate N−O bond in a

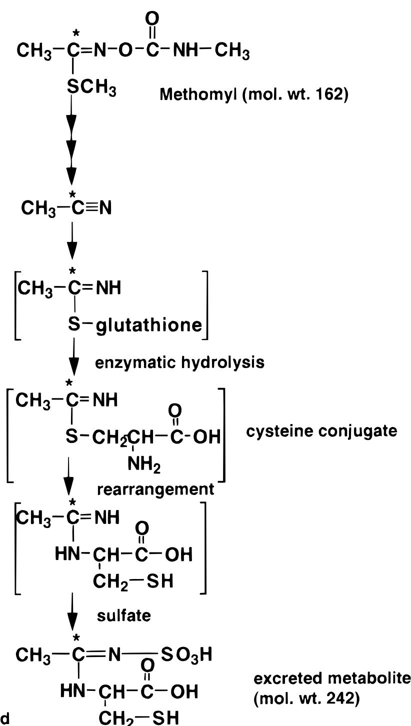

Figure 7. Proposed metabolic pathway.

subsequent step that is chemically unlikely. Rearrangement of the S-linked cysteine conjugate to the N-linked is unusual, but has been observed (Kondo et al., 1981; Lamoureaux et al., 1973). In vitro studies with atrazine have shown this to be a fast, nonenzymatic rearrangement (Lamoureaux et al., 1973). This metabolic sequence is in competition with mercapturic acid formation, which would require acetylation of the cysteine amino group, and is more commonly found in mammals. Only a few double conjugates in animals have been reported in the literature (Quistad et al., 1986). Sulfate−glutathione conjugates of 1-bromopentane (Grasse and James, 1972) and 4-(dimethylamino)-azobenzene (Coles et al., 1983) have been reported. We are not aware of any *N*-sulfate−cysteine conjugates previously published.

In conclusion, elucidation of this unusual structure required a collaborative effort and the use of new technology, such as porous graphitic carbon HPLC, incorporation of ^{13}C, LC/MS with electrospray ionization, high-resolution accurate mass measurement in the LC/MS mode, and two-dimensional NMR techniques.

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