Identification of Metabolites of [1,2,3-^{13}C]Propargyl Alcohol in Rat Urine by ^{13}C NMR and Mass Spectrometry

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Little is known about the metabolism of acetylenic (C≡C) compounds commonly used in the formulation of pesticides. To better understand the in vivo reactivity of this bond, we examined the metabolism of propargyl alcohol (PA), 2-propyn-1-ol, used extensively in the chemical industry. [1,2,3-^{13}C, 2,3-^{14}C]PA was administered orally to male Sprague−Dawley rats. Approximately 56% of the dose was excreted in urine by 96 h. Major metabolites were characterized, directly, in the whole urine by one- and two-dimensional ^{13}C NMR. To determine the complete structures of metabolites of PA, rat urine was also subjected to TLC followed by purification of separated TLC bands on HPLC. The purified metabolites were identified by ^{13}C NMR and mass spectrometry and by comparison with available synthetic standards. The proposed metabolic pathway involves oxidation of propargyl alcohol to 2-propynoic acid and further detoxification via glutathione conjugation to yield as final products: 3,3-bis[(2-(acetylamino)-2-carboxyethyl)thio]-1-propanol, 3-(carboxymethylthio)-2-propenoic acid, 3-(methylsulfinyl)-2-(methylthio)-2-propenoic acid, 3-[[2-(acetylamino)-2-carboxyethyl]thio]-3-[(2-amino-2-carboxyethyl)thio]-1-propanol and 3-[[2-(acetylamino)-2-carboxyethyl]sulfinyl]-3-[2-(acetylamino)-2-carboxyethyl]thio]-1-propanol. These unique metabolites have not been reported previously and represent the first example of multiple glutathione additions to the carbon−carbon triple bond.

Keywords: *Propargyl alcohol metabolism; metabolism of carbon−carbon triple bond; dicysteinyl conjugates; 2-propyn-1-ol*

INTRODUCTION

Limited literature exists regarding the metabolism of acetylenic (C≡C) compounds. To better understand the in vivo reactivity of this site, we examined the metabolism of propargyl alcohol (PA), an acetylenic primary alcohol, which is used as a reactive and versatile intermediate to synthesize agricultural chemicals, pharmaceuticals, and other chemical products.

The metabolism of PA and other acetylenic compounds has not been investigated extensively. Although a number of commercial pyrethroid insecticides containing a carbon−carbon triple bond (Matsuo, 1998) have been synthesized, the only literature cited has been a report by Tomigahara et al. (1994) who presented evidence for the addition of one molecule of acetylcysteine to the internal carbon of the triple bond of the pyrethroid insecticide, Etoc. However, attempts at preparing the metabolite by chemical synthesis resulted in addition to the external carbon of the triple bond.

PA is toxic in rats (LD_{50} = 70 mg/kg body weight), and it is an irritant to the skin, eyes, and respiratory tract. The mechanisms by which PA exerts its adverse effects have not been elucidated. These effects may be mediated by PA, itself, or by its metabolites.

The objectives of this study were to identify the metabolites of PA in rat urine and to determine the biotransformation pathway of PA administered to the rats by oral gavage.

Because metabolites of PA might be volatile and/or have a low molecular weight and be highly polar, giving them physical and chemical properties very similar to those of many natural products, a direct method (not requiring metabolite isolation) of metabolite characterization in the whole urine using one- and two-dimensional ^{13}C NMR spectroscopy was desired. Because ^{13}C is present at low natural abundance (1.1%), its sensitivity for NMR detection is low; therefore, the administration of ^{13}C-enriched propargyl alcohol was used to enhance the sensitivity of metabolite detection in rat urine enabling characterization of polar metabolites directly in urine without requiring the isolation and purification of individual metabolites. This approach was used by Fennell et al. (1991) and Sumner et al. (1992a,b) to characterize metabolites excreted by laboratory animals administered ^{13}C-labeled acrylonitrile, acrylamide, and methoxyethanol, respectively.

To determine the complete structures of metabolites of PA, rat urine was also subjected to thin-layer chromatography (TLC) followed by purification of the separated TLC bands on HPLC. Repeating these steps resulted in the isolation and purification of metabolites

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which were identified subsequently by mass spectrometry and NMR spectroscopy.

EXPERIMENTAL PROCEDURES

Test Material. Carbon-13-enriched (99%) propargyl alcohol ([1,2,3-^{13}C]PA) with a chemical purity of 98% was obtained from Cambridge Isotope Laboratories, Andover, MA, and gave a ^{1}H-decoupled ^{13}C NMR spectrum in D_2O consisting of two doublet of doublets at 52.1 ppm (J = 70, 14 Hz) and 77 ppm (J = 169, 14 Hz) and a quartet at 85 ppm (J = 168, 69 Hz). Radioactive propargyl alcohol ([2,3-^{14}C]PA) having a specific activity of 26.8 mCi/mmol and a radiochemical purity of 98% was used as a tracer (<5% in dose solution) and was obtained by acid hydrolysis of an ester provided by Chemsyn Science Laboratories, Lenexa, KS. To prepare the dose solution, the radiolabeled material was diluted with ^{13}C-PA to give a suitable specific activity (1.13×10^{-2} mCi/mmol) and dissolved in deionized water to a concentration of 21 mg/mL.

Animals: Treatment and Sample Collection. Male Sprague−Dawley rats (CD/BR), approximately 225 g and 7 weeks old, were purchased from Charles River Laboratories, Kingston, NY. Rats were quarantined for at least 7 days and acclimated to metabolism cages for a minimum of 3 days. Rodent Lab Chow and tap water were supplied ad libitum. Eight animals were housed in Nalgene metabolism cages which allowed separation and collection of urine and feces. Collection vials for urine and feces were supported in insulated containers of dry ice to freeze samples immediately. Food and water consumption and urine and feces production were monitored daily from 1 to 3 days before treatment until termination.

Labeled PA (21 mg/mL) was administered by gavage at a dose of 40 mg/kg (1.90 mL/kg). The specific activity was 5022 dpm/μg. Animals were returned to the metabolism cages, and urine was collected at 24, 48, 72, and 96 h following treatment at which time the rats were terminated.

Analytical Methods. 1. Combustion/LSC. Levels of radioactivity in urine were measured by direct liquid scintillation counting (LSC). Sample aliquots were mixed with Ultima Gold liquid scintillation cocktail and analyzed in a Wallac Model 1409 LSC (Gaithersburg, MD).

2. Thin-Layer Chromatography (TLC). Urine was lyophilized and the resulting solid dissolved in methanol (MeOH). The resulting solution (approximately 5 million dpm in 1 mL of methanol) was applied to each preparative silica gel plate (Whatman PLK5F, 20 × 20 cm, 1000 μm) and developed in a solvent system consisting *n*-propanol (*n*-PrOH):water:acetic acid (AcOH):triethylamine (TEA) (50:7.5:2.5:1 by volume). The plates were air-dried, and the radioactivity on the plates was visualized by means of an AMBIS Radioanalytic Imaging System 1125. The radioactive bands on the plates were marked with pencil, the plates were sprayed with acetonitrile (ACN), and the radioactive bands were scraped off and extracted individually by stirring with ACN and/or MeOH. Extracts were filtered and concentrated for subsequent purification/analysis.

3. High-Pressure Liquid Chromatography (HPLC). High-pressure liquid chromatography was performed with a Waters system (Milford, MA), composed of two model 510 pumps coupled to a model 680 system controller, and a Waters Lambda-Max model 481 UV spectrophotometer that was followed by an EG & G Berthold model LB 507B radioactivity monitor (RAM) (Gaithersburg, MD). The analytical HPLC column was PRP-X100, 7 μm, 250 × 4.6 mm (Hamilton); Brownlee guard column cartridges (C-8 and C-18) were supplied by Applied Biosystems (San Jose, CA). Samples were applied by means of a Rheodyne (Cotati, CA) model 7125 injector equipped with 500 μL injection loop. Data from the RAM and UV outputs were acquired using Nelson model 2600 software, revision 5.1 (PE Nelson, Cuppertino, CA), and recorded by BBC model SE 120 dual-channel, strip-chart recorders.

Quantitative separation and purification of metabolites were achieved by applying concentrated aqueous solutions of bands isolated from TLC plates to an anion-exchange HPLC column (PRP-X100, Hamilton) and using water as solvent A and 25% trifluoroacetic acid in ACN as solvent B. A gradient profile consisted of 100% solvent A for 5 min followed by a rise to 9% solvent B over 30 min which was then followed by a steep rise to 100% solvent B over 10 min. A flow of 1 mL/min was maintained throughout the run.

Individual peaks from the anion-exchange HPLC column were collected, concentrated, and rechromatographed to obtain samples of high purity for spectral studies. All runs were at ambient temperature with the UV monitor set at 254 nm and the radioactivity monitor set to 5K full scale.

4. Nuclear Magnetic Resonance (NMR) Spectroscopy. Nuclear magnetic resonance spectra were acquired with a 5 mm dual proton-multinuclear probe on Varian XL-300 or VXR-300 spectrometers. Carbon-13 NMR spectra were acquired in the double-precision mode with an acquisition time of 0.9 s, 30K data points, a relaxation delay ranging between 3 and 5 s, and a 45° (or 60°) pulse width. Spectra for urine samples were acquired with approximately 10 000 transients and were referenced to urea at 165 ppm. To obtain NMR spectra of purified metabolites, 5 mm NMR tubes equipped with matched susceptibility plugs (Varian, part no. 00-969055-00) that allowed significant reduction of solvent volume (Varian, 1987) were used. Samples were scanned for 12−48 h. Chemical shifts are expressed in parts per million (ppm).

Incredible natural abundance double quantum transfer (INADEQUATE) spectra (Bax, 1981) were acquired on the sample of concentrated urine using the INADQT program from the Varian pulse sequence library. Relaxation delays ranging between 5 and 10 s and F values corresponding to coupling constants of 37, 70, and 165 Hz were used to acquire data over the entire spectral window. Broad band decoupling was employed throughout the pulse sequence, and spectra were acquired in the phase-sensitive mode with 1024 complex points in t_2 and 16−32 complex points in t_1.

Two-dimensional heteronuclear *J*-resolved (Muller, 1975) spectra were acquired using the HET2DJ program from the Varian pulse sequence library with gated decoupling applied to the proton spin system. NMR spectra were obtained with 2048 points in t_2 and 64 points in t_1.

Values of shift for carbons of feasible metabolites were calculated using incremental substituent effects for alkynes, alkenes, and alkanes (Pretsch, 1989; Wehrli, 1976; Breitmaier, 1987), using the ^{13}C NMR database from Advanced Chemistry Development, Inc. (ACD) (Toronto, Canada), and by comparison with the available synthetic standards.

5. High-Performance Liquid Chromatography/Mass Spectrometry (HPLC/MS and HPLC/MS/MS). Mass spectral identification was performed on a Finnigan TSQ 7000 triple quadrupole mass spectrometer equipped with an electrospray ionization source. A Hewlett-Packard 1090 liquid chromatograph was used for separation and introduction of samples into the mass spectrometer. Effluent from the HPLC was split (4:1 for a 2.0 mm i.d. column) to a RAM and the mass spectrometer such that the time at which radioactive metabolites were introduced into the mass spectrometer could be determined accurately.

Mass spectral identification of unknown metabolites was made by a multistep approach. First, isolated metabolites were chromatographed on a Hypercarb HPLC column, 5 μm, 15 cm × 2.0 mm i.d., and the radioactive components in the effluents were detected by the RAM while full scan mass spectra were obtained in the first quadrupole (LC/MS). Some pure standards were introduced into the mass spectrometer (without an HPLC column) for flow injection analysis (FIA). The mobile phase for positive electrospray ionization (ESI) was MeOH and 50 mM formic acid; for the negative ESI mode the mobile phase was MeOH and 50 mM ammonium acetate. From the full scan spectra, the $[M + H]^+$ or $[M - H]^-$ ion of a radioactive metabolite was determined. In a second experiment, these ions were selected in the first quadrupole (MS-1) and fragments were produced by collision-induced dissociation (CID) at a collision energy of −20 to −35 eV; the full scan mass spectra

acquired in the third quadrupole (MS-3) yielded daughter ions from the dissociation of only the selected precursor ions (MS/CID/MS).

Spectra are presented as derived spectra and indicate the mathematical manipulation of the ions acquired from the chromatographic peak minus background ions as shown in the equation:

$$\text{derived spectrum} = \sum(\text{ions during LC peak}) - \sum[(\text{ions before peak}) + (\text{ions after peak})]$$

Extracted ion chromatograms indicate the ion intensity of a single mass-to-charge ratio (m/z) as a function of time.

Sample Preparation. The frozen urine samples were thawed at room temperature and centrifuged at 10000*g* for 10 min. Samples were prepared for NMR studies by adding about 100 μL of D_2O to 400 μL of the centrifuged urine. A concentrated sample of urine was prepared for two-dimensional NMR studies by adding an equivalent volume of MeOH and vigorously swirling on a vortex mixer. The resulting precipitate was removed via centrifugation at 12000g for 15 min. The supernatant was decanted and evaporated to dryness under nitrogen, and 500 μL of D_2O was added followed by centrifugation.

Synthesis of Metabolite Standards. Synthesis of (*E,Z*)-3-{[2-(Acetylamino)-2-carboxyethyl]thio}-2-propenoic Acid. To a stirred solution of 5.0 g (30.6 mmol) of *N*-acetyl-L-cysteine in 25 mL of pyridine, previously dried over molecular sieve, was added at 22 °C 2.5 g (35.6 mmol) of 2-propynoic acid. A water bath was put in place to moderate the exothermic reaction, and the reaction mixture was stirred at ambient temperature for 3 h. Concentration of the reaction mixture on a rotary evaporator gave a dark resinous material which was dissolved in 400 mL of water. Decolorization of the dark solution with activated carbon and concentration of the pale yellow solution to dryness, by rotary evaporation, afforded 6.55 g of a golden foam. Thin-layer chromatography (see above) yielded an intense spot, visible by UV at R_f 0.51. ^{1}H NMR (D_2O) revealed that the product was an 80/20 mixture of *E*/*Z* isomers which were isolated by preparative reverse-phase HPLC with a 47 × 300 mm cartridge containing Bondapak C_{18}, 15−20 μm particle size, 0.1% trifluoroacetic acid in water−ACN gradient.

(*E*)-3-{[2-(Acetylamino)-2-carboxyethyl]thio}-2-propenoic Acid: ^{1}H NMR (D_2O) δ 2.01 (3H, s, CH_3), 3.26 (1H, dd, J_{AX} = 8.0 Hz, J_{AB} = 14.3 Hz, H_A), 3.45 (1H, dd, J_{BX} = 4.5 Hz, J_{AB} = 14.3 Hz, H_B), 4.71(1H, m, H_X), 5.89 (1H, d, J = 15.4 Hz, =C*H*C(O)), 7.76 (1H, d, J = 15.4 Hz, =C*H*S); ^{13}C NMR (D_2O) δ 24.3 (*C*H_3), 35.8 (*C*H_2), 54.8 (*C*H), 116.6 (=*C*HCO), 151.6 (=*C*HS), 171.4, 175.8, 176.9 (*C*=O); LC/MS (ESI + Q1MS) 234 (M + 1), 216 (M + 1 − H_2O), 192, 174 (100), 132, 103, 88, 86, 84.

(*Z*)-3-{[2-(Acetylamino)-2-carboxyethyl]thio}-2-propenoic Acid: ^{1}H NMR (D_2O) δ 2.01 (3H, s, CH_3), 3.20 (1H, dd, J_{AX} = 8.0 Hz, J_{AB} = 14.5 Hz, H_A), 3.37 (1H, dd, J_{BX} = 4.6 Hz, J_{AB} = 14.5 Hz, H_B), 4.67 (1H, m, H_X), 5.92 (1H, d, J = 10.0 Hz, =C*H*C(O)), 7.35 (1H, d, J = 10.0 Hz, =C*H*S); ^{13}C NMR (D_2O) δ 24.3 (*C*H_3), 39.3 (*C*H_2), 55.6 (*C*H), 115.8 (=*C*HCO), 154.5 (=*C*HS), 172.5, 175.8 176.9, (*C*=O); LC/MS (ESI + Q1MS) 234 (M + 1), 216 (M + 1 − H_2O), 192, 174 (100), 132, 103, 88, 86, 84.

Preparation of (*E,Z*)-3-(Carboxymethylthio)-2-propenoic Acid. To a stirred solution of 6.5 g (68.5 mmol) of mercaptoacetic acid and 4.5 g (45.2 mmol) of potassium hydrogencarbonate in 30 mL of water, was added at 20 °C 5.0 g (68.5 mmol) of 2-propynoic acid over 5 min. After being stirred at ambient temperature for 21 h, the reaction mixture was neutralized with 3.8 mL (45 mmol) of concentrated hydrochloric acid and taken to dryness on a rotary evaporator. Analysis of the crude product by reverse-phase HPLC using a 0.1% TFA in H_2O showed 40% *Z* and 60% *E* isomers of the title compound. *E* and *Z* isomers were isolated from the product by preparative reverse-phase HPLC using a 47 × 300 mm cartridge containing Bondapak C_{18}, 15−20 μm particle size. The isomers were separated into two fractions using a reverse-phase HPLC and a gradient of 0.1% trifluoroacetic acid in water (A) and ACN (B) (100% A, 5 min; 97% A, 3% B, 20 min; 94% A, 6% B, 25 min, 25% A, 75% B, 5 min). The HPLC analysis of the two fractions showed one to be 100% *E* and other a mixture of 80.5% *Z* and 19.5% *E*.

(*E*)-3-(Carboxymethylthio)-2-propenoic Acid: ^{1}H NMR (D_2O) δ 3.83 (2H, s, SCH_2), 5.86 (1H, d, J = 15 Hz, =CHCO), 7.83 (1H, d, J = 15 Hz, SCH=); ^{13}C NMR (D_2O) δ 29.50 (*S*CH_2), 109.3 (=*C*HCO), 143.1 (S*C*H=), 164.1, 168.3 (*C*=O); LC/MS (ESI + Q1MS): 163 (M + 1), 145(M + 1 − H_2O), 117, 103 (100).

(*Z*)-3-(Carboxymethylthio)-2-propenoic Acid: ^{1}H NMR (D_2O) δ 3.71 (2H, s, SCH_2), 6.01 (1H, d, J = 10 Hz, =CHCO), 7.42 (1H, d, J = 10 Hz, SCH=); ^{13}C NMR (D_2O) δ 31.08 (*S*CH_2), 107.6 (=*C*HCO), 145.3 (S*C*H=) 163.9,167.9 (*C*=O); LC/MS (ESI + Q1MS): 163 (M + 1), 145(M + 1 − H_2O), 117, 103 (100).

Preparation of 3,3-Bis[(2-(acetylamino)-2-(methoxycarbonyl)ethyl)thio]-1-propyl Acetate. Utilizing a recently reported method (Komatsu et al., 1995) for the preparation of dithioacetals, 2.52 g (20.0 mmol) of freshly distilled 3-acetoxypropanal (White et al., 1962) and 7.1 g (40.0 mmol) of *N*-acetyl-L-cysteine methyl ester (Jones et al., 1966) were sequentially added to a solution of 0.64 g (2.0 mmol) of bismuth(III) chloride in 20 mL of ACN with stirring at 22 °C. ^{1}H NMR analysis of the reaction mixture after 5 days showed the absence of 3-acetoxypropanal. After evaporation of solvent, the residue was dissolved in ethyl acetate, washed with 5% aqueous sodium bicarbonate and water, and dried ($MgSO_4$). Evaporation of solvent gave 4.3 g of a pale yellow viscous oil which was shown by TLC to be a mixture. Purification of the product by preparative HPLC utilizing a mobile phase of 1% methanol in ethyl acetate gave 1.5 g of the title compound as a viscous oil: ^{1}H NMR ($CDCl_3$) δ 2.04, 2.05, 2.06 (8H, 2s + m, 2CH_3,CH_2), 2.92−3.22 (4H, m, CH_2S), 3.76, 3.77 (6H, 2s, CH_3O), 3.93 (1H, t, SCHS), 4.19 (2H, t, CH_2O), 4.83 (2H, m, CHN), 6.62 (2H, br, NH); ^{13}C NMR ($CDCl_3$) δ 20.92 (*C*H_3CO_2), 22.90, 22.96, 23.04 (*C*H_3CON), 32.37, 32.67 (*C*H_2S), 35.10 (C*C*H_2C), 49.04 (S*C*HS), 51.66, 51.74, 51.95, 52.65, 52.74, 52.78 (*C*HN + *C*H_3O), 61.43 (*C*H_2O), 170.1, 170.9, 171.0, 171.1, 171.2 (*C*=O); LC/MS (ESI + Q1MS) 453 (M + 1), 276 (M + 1 − 177), 216 (100).

Preparation of 3,3-Bis[(2-(acetylamino)-2-carboxyethyl)thio]-1-propanol. To a stirred solution of 1.40 g (3.09 mmol) of 3,3-bis[(2-(acetylamino)-2-(methoxycarbonyl)ethyl)-thio]-1-propyl acetate in 10 mL of MeOH was added a solution of 0.46 g (11.5 mmol) of sodium hydroxide in 30 mL of water. After 4 h at 22° C, the reaction mixture was acidified with 2 N HCl to a pH of 3. Methanol was removed by rotary evaporation, and the aqueous solution was then lyophilized to remove water. The crude product (1.6 g) was stirred in 30 mL of ethanol for 3 h and filtered to remove sodium chloride. Rotary evaporation of the filtrate gave 1.0 g of a white foam. Purification of the product using reverse-phase flash chromatography (O'Neil, 1991) (34 g of C_{18} bonded silica, eluted with water) afforded 0.12 g of the title compound as a highly viscous oil: ^{1}H NMR (D_2O) δ 2.02 (2H, m, CH_2), 2.05 (6H, s, CH_3), 3.00 (2H, dd, J_{AX} = 8.0 Hz, J_{AB} = 13.7 Hz, SCH_A), 3.20 (2H, dd, J_{BX} = 4.5 Hz, SCH_B), 3.75 (2H, t, CH_2O), 4.11 (1H, t, SCHS), 4.55 (2H, m, NCH_X); ^{13}C NMR (D_2O) δ 24.48 (*C*H_3), 34.18, 34.23 (*C*H_2S), 40.21 (C*C*H_2C), 51.23 (S*C*HS), 55.62, 55.90 (N*C*H), 61.48 (*C*H_2O), 176.6, 176.7, 177.3 (*C*=O); LC/MS (ESI + Q1MS): 383 (M + 1), 220 (M + 1 − 163), 202, 190(100).

RESULTS AND DISCUSSION

^{13}C,^{14}C-Propargyl alcohol was administered to rats by oral gavage, and urine and feces were collected at 24 h intervals for 96 h. About 56% of the administered radioactivity was found in the urine after 96 h. The highest concentration of PA residues (272 ppm) was measured in the urine collected within 24 h of PA administration. Residue concentrations in urine were much lower (3 ppm) 4 days after administration of PA.

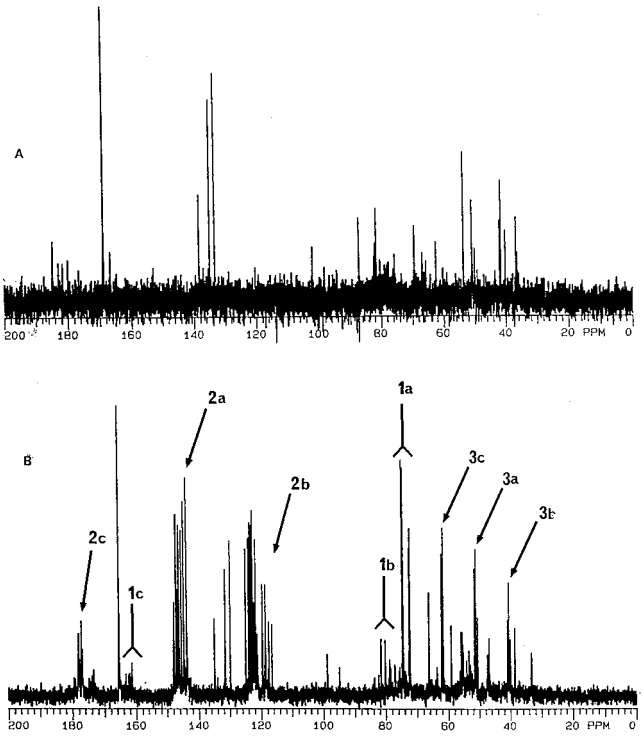


Figure 1. ^{13}C NMR spectrum of control urine (A) and urine collected for 24 h after administration of 40 mg/kg [1,2,3-^{13}C]-propargyl alcohol (B) indicating three major components, designated metabolites 1−3. The carbon atom is assigned a number corresponding to its metabolite number and a letter which designates its derivation from PA (a, b, or c corresponding to the 3-, 2-, and 1-carbon atoms, respectively, of propargyl alcohol).

Characterization of Metabolites Based on NMR of Urine. One- and two-dimensional NMR experiments were used to characterize the carbon−carbon correlations and proton multiplicity for the ^{13}C-labeled carbons in each metabolite of PA. While this approach does not provide definitive identification of the unlabeled portion of each metabolite, it delineates important structural aspects of each metabolite, which in turn enables the development of the appropriate chromatographic techniques for isolation and purification of the individual metabolites. In addition, this approach avoids the possibility of selective isolation and loss of volatile and chemically labile metabolites that may occur during sample preparation by conventional techniques. (After metabolites were characterized with such an approach, definitive identifications were made using other analytical methods as described in the section entitled "Identification of Urinary Metabolites".)

A concentrated sample of urine was used for 2D NMR studies. The one-dimensional ^{13}C NMR spectra obtained for the urine and concentrated urine were qualitatively similar. Differences between the spectra are due to a better signal-to-noise ratio for concentrated urine. Characterization of metabolites is based on NMR data obtained for the 0−24 h concentrated urine.

The proton-decoupled ^{13}C NMR spectra of control rat urine and of urine collected 24 h following administration of ^{13}C-PA are shown in panels A and B of Figure 1, respectively. The spectrum of control urine (Figure 1A) shows an intense singlet at 165 ppm that is assigned to urea; the less intense signals are consistent with sugars, hippurate, citrate, and creatine (Nicholson et al., 1987). The ^{13}C NMR spectrum of urine from rats

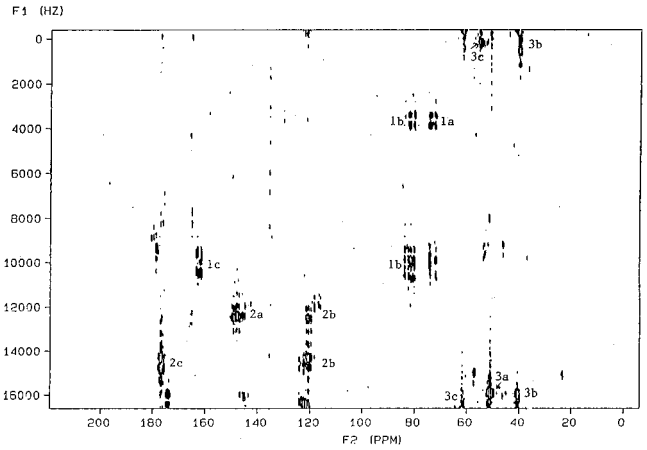


Figure 2. INADEQUATE spectrum of rat urine collected for 24 h following administration of [1,2,3-^{13}C]-propargyl alcohol. The spectrum was plotted to show carbon−carbon connectivities for metabolites. Correlation of each signal for each metabolite is determined by tracing the connectivities for signals at different chemical shifts (along the *x*-axis) which have the same double quantum frequency along the *y*-axis.

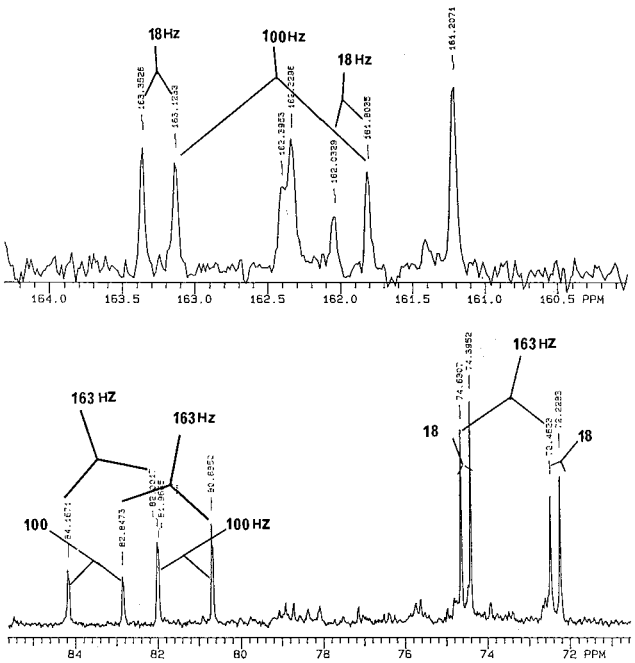


Figure 3. Partial ^{1}H-decoupled ^{13}C NMR spectrum of urine collected 24 h after administration of ^{13}C-PA, indicating the presence of 2-propynoic acid in the urine.

administered ^{13}C-PA (Figure 1B) contains signals from endogenous compounds and also contains signals that appear as multiplet patterns and are not present in spectra of control urine. These patterns arose from coupling between the labeled carbons of each metabolite. The carbons of compounds in control urine (1.1% natural abundance of ^{13}C) give rise to signals which appear as singlets because of low incidence of adjacent ^{13}C nuclei. Three major metabolites of PA give rise to the intense signals (labeled 1, 2, and 3) in Figure 1B, while many metabolites that are formed in lower concentrations give rise to the less intense signals.

Incredible natural abundance double quantum transfer (INADEQUATE) spectroscopy was used to correlate carbon signals in the complex urinary mixture, allowing for the determination of connectivity for carbon signals that arise from each metabolite of PA. The number of

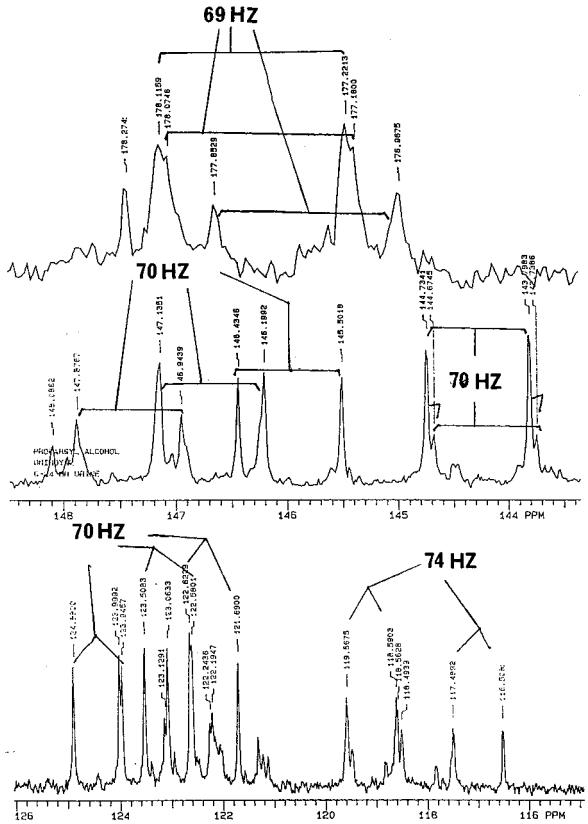
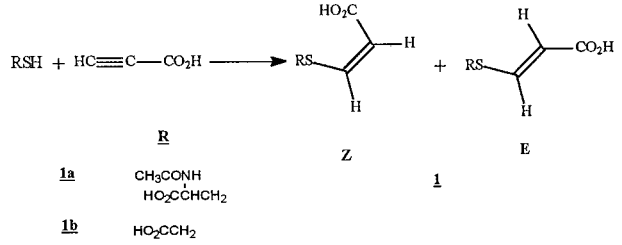


Figure 4. Partial ^{1}H-decoupled ^{13}C NMR spectrum of urine collected 24 h after administration of ^{13}C-PA, indicating the presence of 3-(carboxymethylthio)-2-propenoic acid in the urine.

Scheme 1. Synthesis of Metabolite Standards 3-(Carboxymethylthio)-2-propenoic Acid and 3-{[2-(Acetylamino)-2-carboxyethyl]thio}-2-propenoic Acid



hydrogens attached to each labeled carbon was determined by heteronuclear *J*-resolved (HET2DJ) spectroscopy. The number of hydrogens attached to a carbon is equal to the number of contours at the carbon's chemical shift position minus 1. The rationale behind the assignment of structure of each metabolite is presented below. The number and letters assigned to a metabolite designate its number and the letter of the carbon derived from PA (a, b, or c for the 3, 2, 1 carbons of PA, respectively).

Metabolite 1. The INADEQUATE spectrum of a concentrated sample of rat urine (Figure 2) has carbon peaks aligned along the same double quantum frequency (*y*-axis) between 73 ppm (1a) and 82 ppm (1b) and between 82 ppm (1b) and 163 ppm (1c). These connectivities are also indicated in the 1D spectrum (Figures 1B and 3) where two doublets with long-range splitting are present at 73 ppm (J = 163, 18 Hz) and 163 ppm (J = 100, 18 Hz), and a doublet of doublets is present at 82 ppm (J = 163, 100 Hz). The signals centered at 73 and 82 ppm are consistent with the

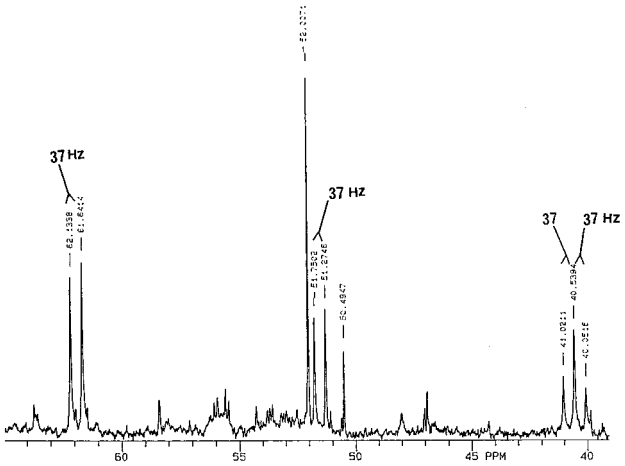


Figure 5. Partial ^{1}H-decoupled ^{13}C NMR spectrum of urine collected 24 h after administration of ^{13}C-PA, indicating the presence of 3,3-bis[(2-acetylamino)-2-carboxyethyl)thio]-1-propanol in the urine.

chemical shifts of the acetylenic carbons, C≡C, and the signal centered at 163 ppm is consistent with that of a carbonyl carbon. Moreover, the signal at 82 ppm shows connectivity to 163 ppm, indicating an attachment to a carboxyl group resulting in a HC≡C−CO_2H type structure, which could be formed readily with oxidation of propargyl alcohol. The chemical shifts for the standard 2-propynoic acid and theoretical values are consistent with the experimental shift values for this metabolite.

Metabolite 2. For metabolite 2, the connectivities are established between the peaks near 120 ppm (dd; J = 70, 70 Hz) and peaks near 145 ppm (d, J = 70 Hz) and 176 ppm (d, J = 70 Hz) (Figure 2). These connectivities are also indicated in the 1D spectrum (Figures 1B and 4) where doublets with coupling constants of 70 Hz are present at about 120−125, 145−150, and 175−180 ppm. These data reveal the structure of the metabolite as follows: the signals positioned at about 120 and 145 ppm indicate a HC=CH structure which was supported further by HET2DJ (spectrum is not shown) which showed two contours for each of the intense signals between 120 and 125 ppm and between 145 and 150 ppm. The signal at 120 ppm shows connectivity to 176 ppm, indicating an X−CH=CH−CO_2H structure for the ^{13}C-labeled position of the PA metabolite. Using incremental shift values for substitution of functional groups on alkenes, the X group is RS (the only feasible substituent for a metabolite in urine which agrees with the experimental chemical shift values of 145 and 120 ppm) resulting in a structure of RSCH=CHCO_2H. This type of structure may result from conjugation of glutathione with 2-propynoic acid followed by conversion to metabolites that can be excreted, such as cysteine or *N*-acetylcysteine derivatives. Two sets of standards were prepared consistent with the proposed structure: one contained *N*-acetylcysteine for the R group (Scheme 1; 1a) and the other contained thioacetic acid (Scheme 1; 1b). The chemical shifts for the synthetic standard, (*Z*)-3-(carboxymethylthio)-2-propenoic acid, and calculated values are consistent with the experimental shift values for this metabolite.

Metabolite 3. Connectivities for metabolite 3, determined by the INADEQUATE spectrum (Figure 2), are present between 40 (dd; J = 37, 36 Hz), 51 (d, J = 36 Hz), and 62 ppm (d, J = 37 Hz). These connectivities are also indicated in the 1D spectrum (Figures 1B and

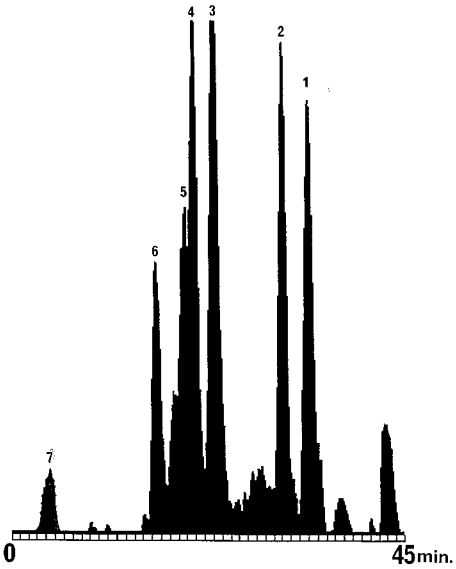
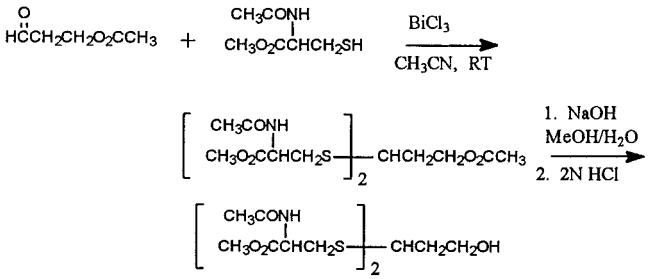


Figure 6. HPLC radiochromatogram of ^{14}C components in rat urine. Peak numbers with their approximate relative abundances, as determined by PE Nelson system, are as follows: peak 1, 20%; peak 2, 20%; peak 3, 15%; peak 4, 8%; peak 5, 27%; peak 6, 7%; peak 7, 1%.

Scheme 2. Synthesis of 3,3-Bis[(2-(acetylamino)-2-carboxyethyl)thio]-1-propanol



5) where doublets with coupling constant (J) of ~37 Hz are present at 40 (doublet of doublet, overlapping of the middle peak shows it as a triplet), 51 (doublet), and 62 ppm (doublet). The coupling constant of ~37 Hz between carbons is consistent with sp^3 hybridized carbons, and the chemical shift at 62 ppm is consistent with an attachment of a carbon to a hydroxyl group. Together, these data indicate an $XCH_2CH_2CH_2Y$ structure for the portion of the metabolite derived from PA. To fit the experimental values of 51.2, 40.2, and 61.5 ppm, and assuming the functional group Y is OH, the functional group X on a propane carbon must have α and β effects of 40 and 0, respectively. Using incremental shift values for substitution of functional groups on alkanes, the group X can be determined. An RS group is the only feasible substituent which satisfies these conditions, resulting in a structure of $(RS)_2CHCH_2CH_2OH$. The chemical shifts for the synthetic standard, 3,3-bis[(2-(acetylamino)-2-carboxyethyl)thio]-1-propanol, prepared in two steps (Scheme 2), and calculated values are consistent with the experimental shift values for this metabolite, as shown above.

Isolation and Purification of Urinary Metabolites. Since the NMR data described above are obtained only for the portion of each metabolite derived from the ^{13}C-labeled carbons of PA, the remainder of each structure was assigned tentatively by comparing the experimental shifts with calculated values of shifts for a possible metabolite and/or with shifts obtained for

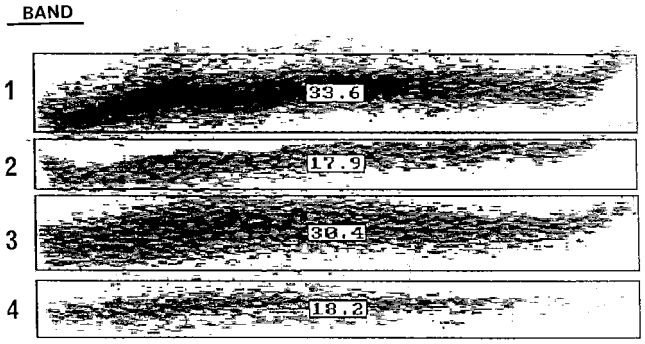


Figure 7. Autoradiogram of rat urinary metabolites separated on a normal-phase preparative TLC plate (PLK5F). Numbers in the bands indicate relative percentages of radioactivity as determined by the AMBIS system. The R_f values are as follows: 0.62 (band 1); 0.47 (band 2); 0.36 (band 3); and 0.21 (band 4).

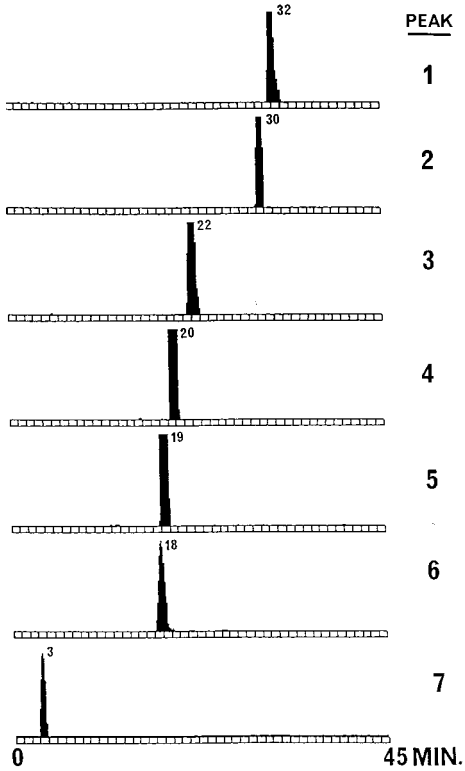


Figure 8. HPLC radiochromatograms of the metabolites of propargyl alcohol isolated from rat urine. Numbers on the peaks indicate the retention times on the anion-exchange column.

synthetic standards. The definitive identification of each isolated metabolite was then made by mass spectrometry and ^{13}C NMR spectroscopy as described below.

The HPLC radiochromatogram of urinary metabolites separated by an anion-exchange column showed several radioactive peaks. The peak number and percent abundance for each peak are given in Figure 6.

Metabolites were isolated and purified by lyophilizing the urine and subjecting the residue to preparative TLC. The radioactive bands (Figure 7) were subsequently analyzed by HPLC which showed two radioactive peaks in band 1 (tr = 30 and 19 min), two radioactive peaks in band 2 (tr = 30 and 18 min), three radioactive peaks in band 3 (tr = 32, 22, and 20 min), and two radioactive peaks in band 4 (tr = 22 and 3 min). Subjecting each band to a second preparative TLC and subsequent purification on an anion-exchange HPLC column gave

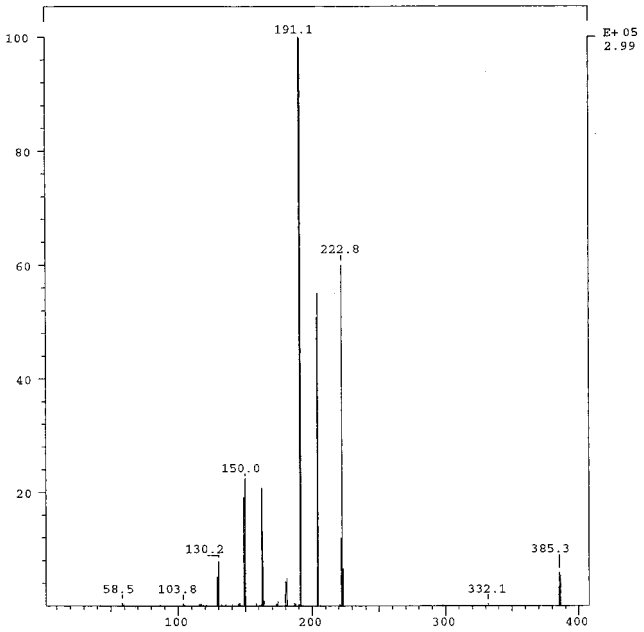


Figure 9. ESI LC/MS/MS of purified peak 1 (Figure 8) from rat urine.

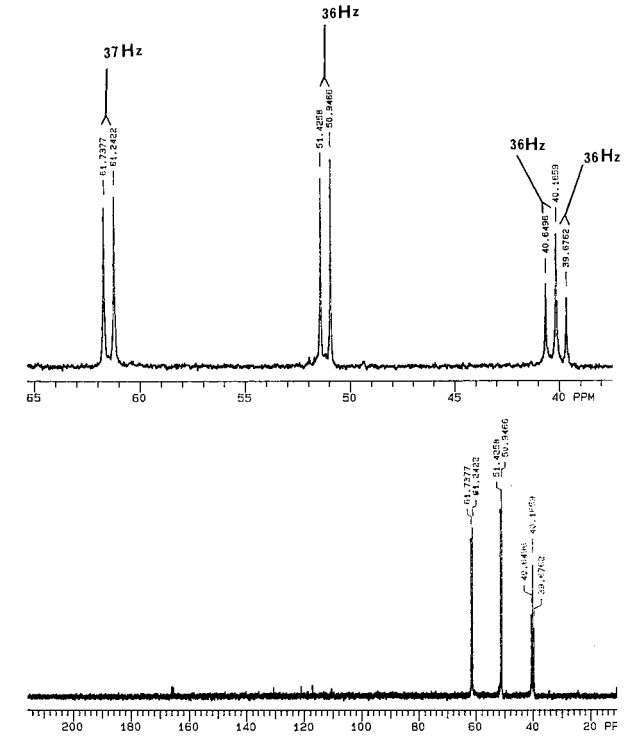


Figure 10. ^{1}H-Decoupled ^{13}C NMR spectrum of purified peak 1 (Figure 8) from rat urine identified as 3,3-bis[(2-acetylamino)-2-carboxyethyl)thio]-1-propanol.

isolated metabolites in high purity suitable for spectral analyses. The HPLC radiochromatograms of the PA urinary metabolites after final purification are shown in Figure 8.

Identification of Urinary Metabolites. The identification of PA urinary metabolites was achieved by ^{13}C NMR and mass spectrometry. To ensure unequivocal identification of molecular ion adducts ($[M + H]^+$) in the full scan Q1 mass spectrum, two prerequisites were established: (a) an exact match of retention time for the RAM peak and the molecular ion adduct peak; (b) the molecular ion adduct is the base peak in the Q1 mass spectrum.

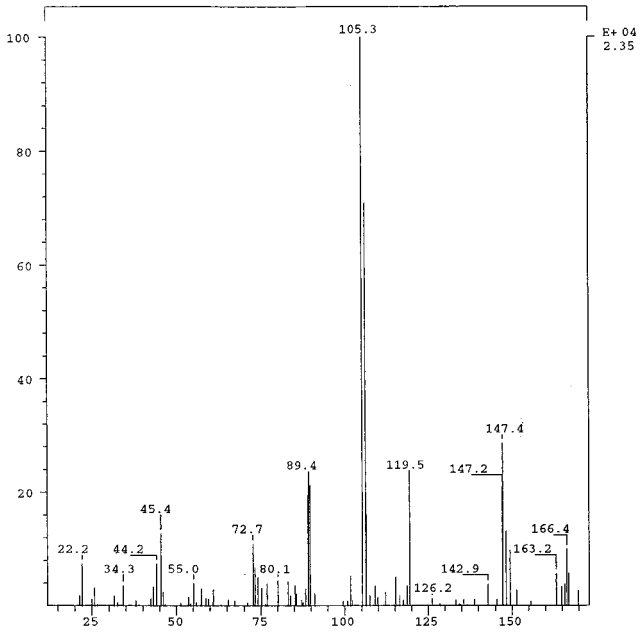
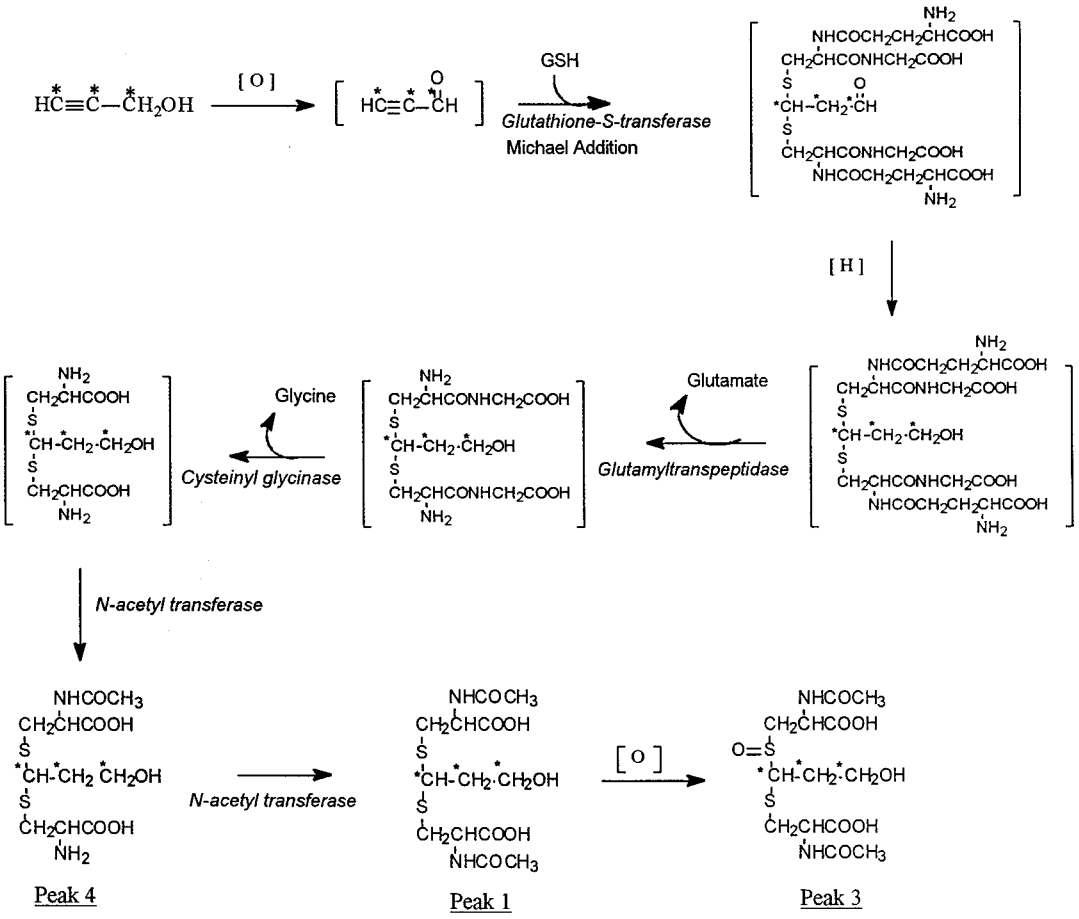


Figure 11. ESI LC/MS/MS of purified peak 2 (Figure 8) from rat urine.

Peak 1 eluted with an HPLC retention time of about 32 min (Figure 8) and accounted for about 20% of total radioactive residue (TRR) in 0−24 h urine (Figure 6). The positive ion ESI LC/MS of this peak showed an $[M + H]^+$ ion for the parent compound at *m/z* 386, suggesting a molecular weight of 385. The ESI LC/MS/MS spectrum of that ion (Figure 9) showed a prominent fragment ion at *m/z* 223, suggesting loss of acetylcysteine (*m/z* 163) from $[M + H]^+$. Further loss of water and $^{13}CH_2O$ (31 *m/z*) results in fragments at *m/z* 205 and 192, respectively.

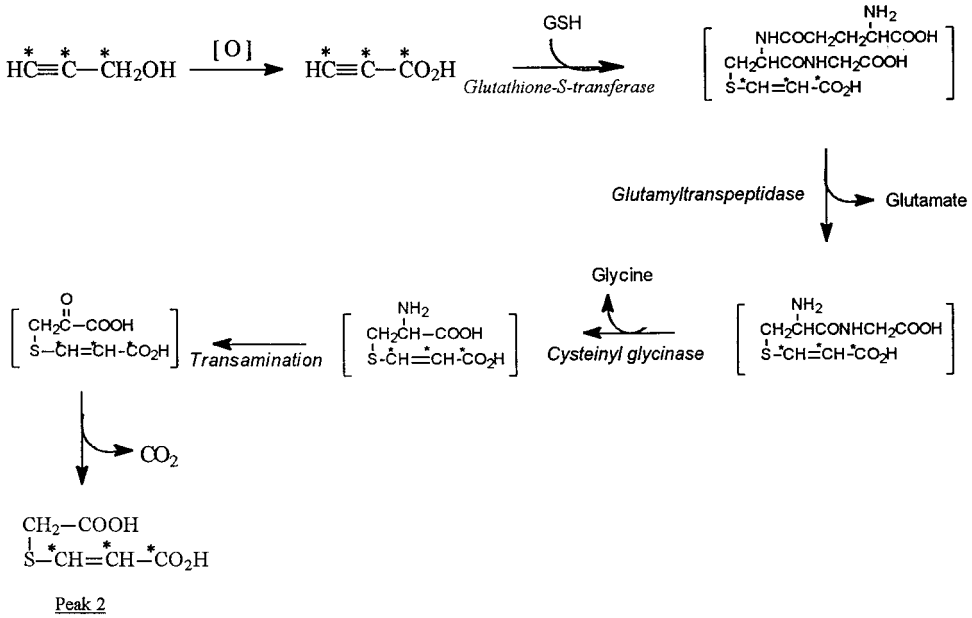
The ^{13}C NMR spectrum of peak 1 (Figure 10) displayed three sets of signals centered at 40.5 (dd, $J = 36$ Hz), 51.5 (d, $J = 36$ Hz), and 61.5 ppm (d, $J = 37$ Hz), similar to those shown for metabolite 3 identified directly in the urine (see Figure 5), consistent with the chemical shift of aliphatic carbons. Furthermore, the coupling constant of 36−37 Hz between these splits suggested an sp^3 hybridization for all three carbons of the metabolite derived from the ^{13}C-labeled portion of PA. The doublet of doublet was assigned to the middle carbon by virtue of its coupling to two sp^3 hybridized carbons resulting in four lines with the coupling constant (*J*) value of 37 Hz between the splits (overlapping of the middle peaks shows it as a triplet). The doublet at 51.5 ppm with the coupling constant of 36 Hz was attributed to the carbon bearing the conjugate moieties, two acetylcysteinyl units. (Signals from the carbon atoms of the *N*-acetylcysteine moiety, which are present at natural abundance, were not seen above the background signal.) This assignment agrees with the experimental shift values of 51.5 ppm and the mass spectral results. The doublet at 61.5 ppm ($J = 37$ Hz) was assigned to the carbon carrying the hydroxyl group. The chemical shift assignment, multiplicity pattern, and coupling constants are the same as those observed in urine, directly, for this metabolite and are in agreement with that of the synthetic standard, 3,3-bis[(2-(acetylamino)-2-carboxyethyl)thio]-1-propanol. Therefore, peak 1 was identified unequivocally as 3,3-bis[(2-(acetyl-

Scheme 3. Proposed Pathway for the Formation of Metabolites Eluted as Peaks 1, 3, and 4 (Figure 8)[a]



[a] The asterisk (*) denotes the position of the ^{13}C label. Structures in the brackets are the hypothetical intermediates.

Scheme 4. Proposed Pathway for the Formation of the Metabolite Eluted as Peak 2 (Figure 8)[a]



[a] The asterisk (*) denotes the position of the ^{13}C label. Structures in the brackets are the hypothetical intermediates.

amino)-2-carboxyethyl)thio]-1-propanol, resulting from addition of 2 mol of glutathione (GSH) to 2-propynal, catalyzed by *glutathione-S-transferase*, in a reaction analogous to a Michael addition (March, 1985). Subsequent reduction of the aldehyde to the corresponding alcohol followed by losses of glutamate, catalyzed by *γ-glutamyltranspeptidase*, and glycine, catalyzed by *cysteinyl glycinase* (Hillenweck et al., 1997), produced the dicysteinyl conjugate which in turn was acetylated via *N*-acetyl transferase to yield the diacetylcysteinyl metabolite as shown in Scheme 3.

Peak 2 eluted with an HPLC retention time of about 30 min (Figure 8) and accounted for about 20% of TRR in 0−24 h urine (Figure 6). On the basis of the ^{13}C NMR

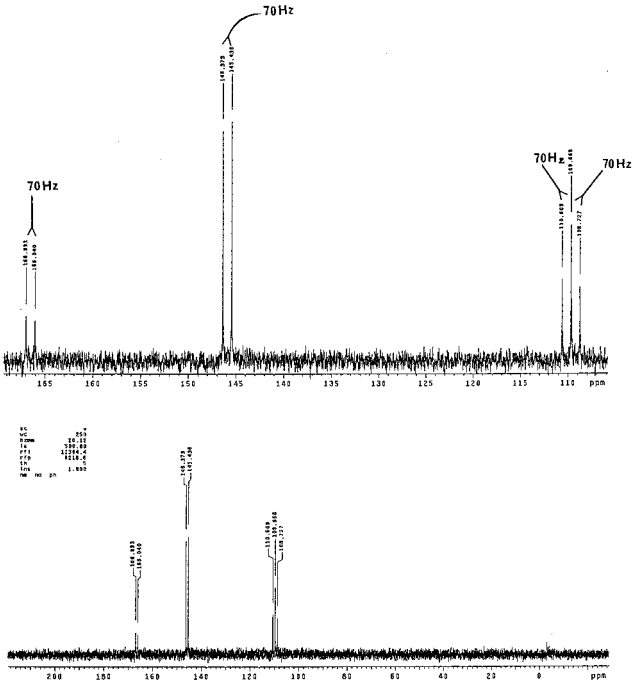


Figure 12. ^{1}H-Decoupled ^{13}C NMR spectrum of purified peak 2 (Figure 8) from rat urine identified as 3-(carboxymethylthio)-2-propenoic acid.

data obtained on whole urine and comparison of the chemical shifts with two sets of standards, this peak was characterized as RS−CH=CH−COOH. The positive ion ESI LC/MS of this peak showed an $[M + H]^+$ ion for the parent compound at *m/z* 166 and a sodium adduct [M + Na] ion at *m/z* 188, confirming the molecular weight as 165. The ESI LC/MS/MS spectrum of the parent peak with *m/z* 166 (Figure 11) showed characteristic ion peaks at *m/z* 148 due to the loss of water (*m/z* 18) and at *m/z* 120 resulting from the neutral loss of a carboxyl group ($^{13}CO_2H$, *m/z* 46). The base peak in the spectrum appeared at *m/z* 106 and was formed by a neutral loss of acetic acid (*m/z* 60) from the non-^{13}C-labeled portion of the metabolite to form $(S-{}^{13}CH={}^{13}CH-{}^{13}COOH)^+$.

The carbon-13 NMR spectrum of peak 2 (Figure 12) displayed shifts centered at 110 (d,d, $J = 70$ Hz), 146 (d, $J = 70$ Hz), and 167 ppm (d, $J = 70$ Hz) indicating the presence of two methine carbons and one ^{13}C-carboxyl carbon, consistent with metabolite 2 characterized directly in the whole urine. The spectrum is identical to the spectrum of the synthetic standard 3-(carboxymethylthio)-2-propenoic acid. Thus, peak 2 is 3-(carboxymethylthio)-2-propenoic acid, resulting from oxidation of PA to 2-propynoic acid followed by conjugation with GSH, catalyzed by *glutathione-S-transferase*. Sequential losses of glutamate, catalyzed by *γ-glutamyltranspeptidase*, and glycine, catalyzed by *cysteinyl glycinase*, results in the formation of the cysteinyl conjugate which undergoes oxidative deamination or transamination to form the corresponding thiopyruvate; subsequent loss of carbon dioxide yields the thioacetic acid metabolite, as shown in Scheme 4.

Peak 3 eluted with an HPLC retention time of 22 min (Figure 8) and accounted for about 15% of TRR in 0−24 h urine (Figure 6). The positive ion ESI LC/MS of this peak (Figure 13) showed an $[M + H]^+$ ion for the parent compound at *m/z* 402, suggesting a molecular weight of 401. The ESI LC/MS/MS spectrum of that ion (Figure

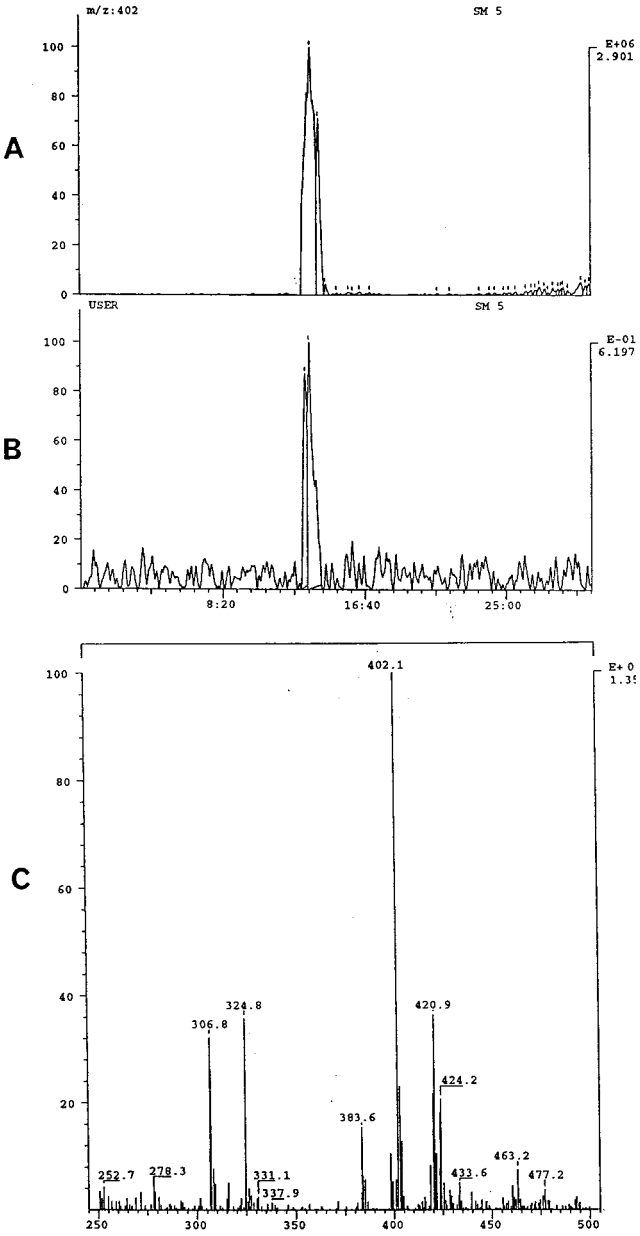


Figure 13. ESI LC/MS of purified peak 3 (Figure 8) from rat urine: (A) selected ion chromatogram at *m/z* 402, (B) RAM trace, and (C) full scan Q1 MS.

14) showed a loss of 179 mass units, attributed to acetylcysteinesulfoxide, to give a fragment of *m/z* 223; further loss of water and $^{13}CH_2O$ (31 *m/z*) gave fragments at *m/z* 205 and 192, respectively, similar to fragments observed for peak 1 (Figure 9).

The ^{13}C NMR spectrum of peak 3 (Figure 15) displayed signals centered at 32 (multiplets, $J = 37$ Hz), 60.5 (3 sets of doublets, $J = 37$ Hz), and 64.5 ppm (multiplets, $J = 37$ Hz). For this metabolite, the multiplet centered at about 32 ppm was assigned to the middle^{13}C-enriched carbon (carbon 2, $^{13}CH_2$) by virtue of its coupling to two sp^3 hybridized carbons, resulting in lines with the coupling constant (*J*) value of 37 Hz between the splits. The multiplet centered at 64.5 ppm with the coupling constant of 37 Hz was attributed to the carbon attached to the conjugates (i.e., $R_2{}^{13}CH$). Additional splits (multiplets) from ^{13}C-enriched nuclei in the spectrum of peak 3 suggest the presence of diastereoisomers. That its molecular weight was 16 mass units higher than that of peak 1 was attributed

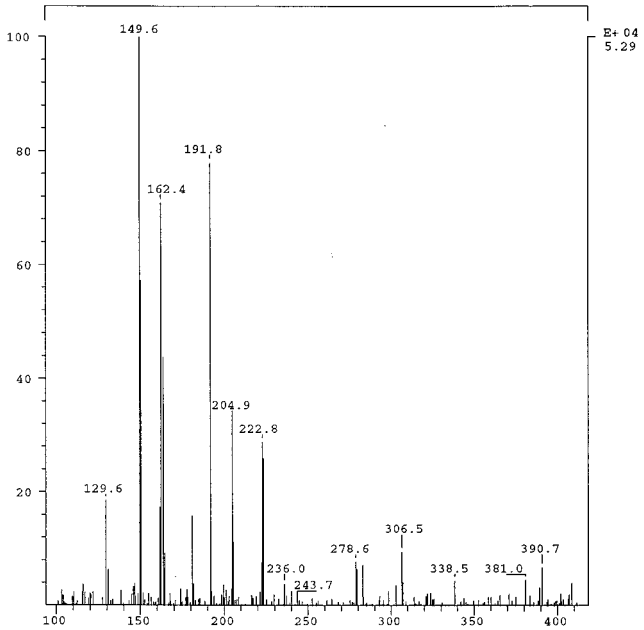


Figure 14. ESI LC/MS/MS of purified peak 3 (Figure 8) from rat urine.

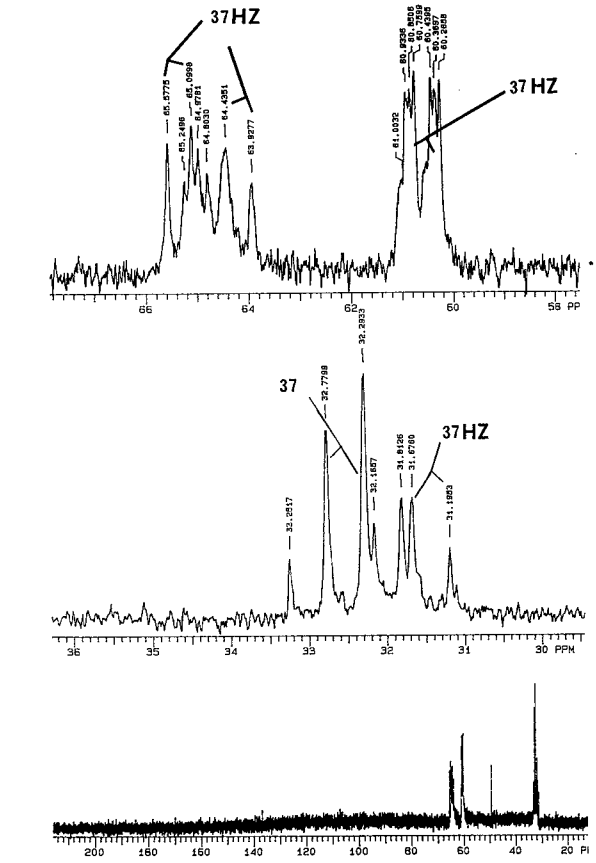


Figure 15. ^{1}H-Decoupled ^{13}C NMR spectrum of purified peak 3 (Figure 8) from rat urine identified as 3-[[2-(acetylamino)-2-carboxyethyl]sulfinyl]-3-[[2-(acetylamino)-2-carboxyethyl]-thio]-1-propanol.

to oxidation of a sulfur atom to form a sulfoxide, resulting in a chiral carbon and diastereomeric metabolites with the same HPLC retention time and very close chemical shifts in its ^{13}C NMR spectrum. In addition, oxidation of sulfur gave a spectrum with a downfield chemical shift of the adjacent carbon to about 15 ppm compared to the spectrum for peak 1. The doublet at

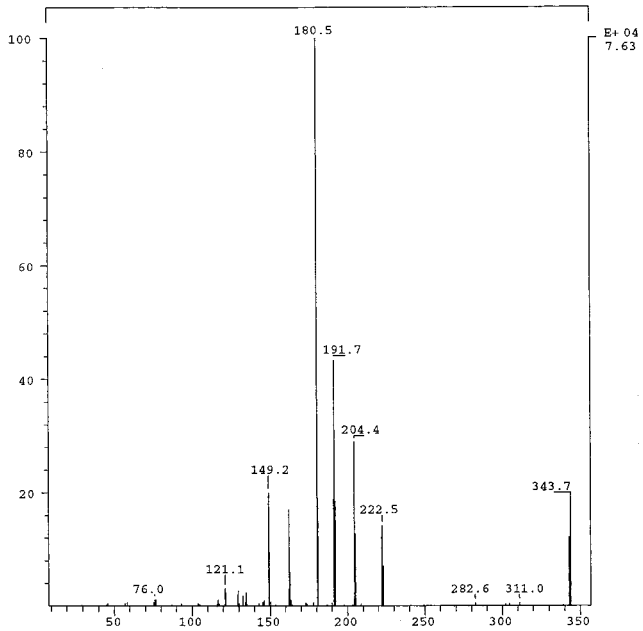


Figure 16. ESI LC/MS/MS of purified peak 4 (Figure 8) from rat urine identified as 3-[[2-(acetylamino)-2-carboxyethyl]thio]-3-[(2-amino-2-carboxyethyl)thio]-1-propanol.

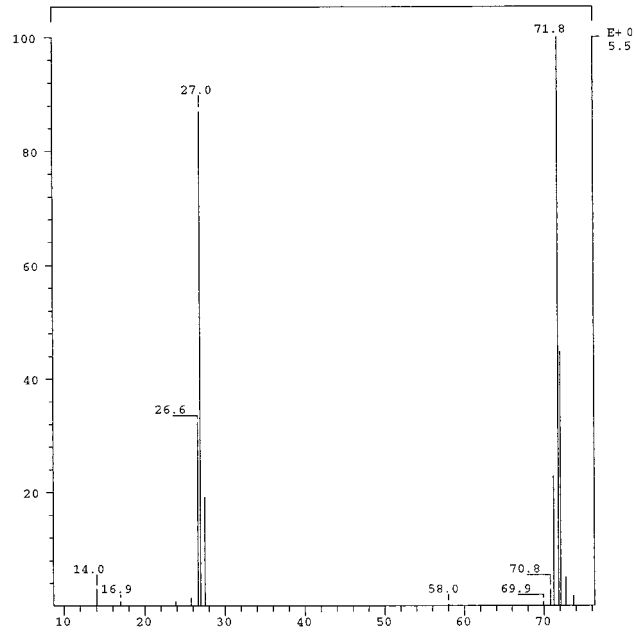
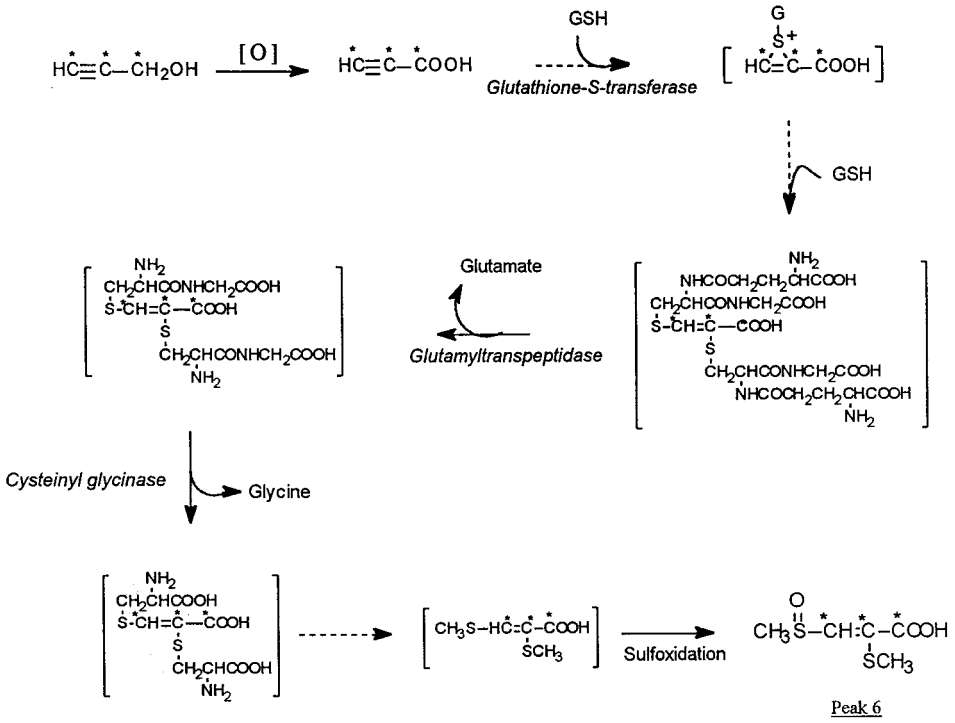


Figure 17. ESI LC/MS/MS of purified peak 5 (Figure 8) from rat urine.

60.5 ppm (J = 37 Hz) was assigned to the carbon bearing the hydroxyl group. Thus, peak 3 was identified unequivocally as 3-[[2-(acetylamino)-2-carboxyethyl]sulfinyl]-3-[[2-(acetylamino)-2-carboxyethyl]thio]-1-propanol, formed by sulfoxidation of peak 1 (Scheme 3).

Peak 4 eluted with an HPLC retention time of 20 min (Figure 8) and accounted for about 8% of TRR in 0−24 h urine (Figure 6). The positive ion ESI LC/MS of this peak showed an $[M + H]^+$ ion at m/z 344 giving a molecular weight of 343, 42 mass units lower than peak 1 (Figure 9). The ESI LC/MS/MS spectrum (Figure 16) showed prominent fragment ions at m/z 223 and at m/z 181 resulting from the loss of cysteine (m/z 121) and acetylcysteine (m/z 163) from the molecular ion peak. Thus, peak 4 was identified as 3-[[2-(acetylamino)-2-

Scheme 5. Proposed Pathway for the Formation of the Metabolite Eluted as Peak 6 (Figure 8)[a]



[a] The asterisk (*) denotes the position of the ^{13}C label. Structures in the brackets are the hypothetical intermediates.

carboxyethyl]thio]-3-[(2-amino-2-carboxyethyl)thio]1-propanol, formed in a similar manner as described for peak 1 (see Scheme 3).

Peak 5 eluted with an HPLC retention time of 19 min (Figure 8) and is the most abundant metabolite accounting for about 27% of TRR in 0−24 h urine (Figure 6). The negative ion ESI LC/MS of peak 5 showed an exact match of retention time between the RAM trace and $[M - H]^-$ peak at *m/z* 72 which gave a molecular weight of 73. The ESI LC/MS/MS spectrum (Figure 17) of this metabolite showed only a prominent fragment ion at *m/z* 27, resulting from the neutral loss of carbon dioxide (*m/z* 45, $^{13}CO_2$) from the M − H peak.

The ^{13}C NMR spectrum of peak 5 (Figure 18) displayed signals consisting of two doublets with long-range splitting centered at 73 (163, 18 Hz) and 163 ppm (100, 18 Hz) and a doublet of doublets at 82 ppm (163, 100 Hz). The signals centered at 73 and 82 ppm are consistent with the chemical shifts of the acetylenic carbons, C≡C, and the signal centered at 163 ppm is consistent with that of a carbonyl carbon. Furthermore, these signals are identical in chemical shifts, multiplicity, and coupling constants to those described for metabolite 1, directly identified in urine, and with that of the standard, 2-propynoic acid. Thus, on the basis of NMR and mass spectral data, peak 5 was identified as 2-propynoic acid, an oxidation product of propargyl alcohol.

Peak 6 eluted with an HPLC retention time of 18 min (Figure 8) and accounted for about 7% of TRR in 0−24 h urine (Figure 6). The positive ion ESI LC/MS of this peak showed an exact match of retention time between the RAM trace and the molecular ion adduct peak, $[M + H]^+$, at *m/z* 184 and showed that the $[M + H]^+$ adduct is the base peak in the full scan Q1 mass spectrum, indicating a molecular weight of 183. The ESI LC/MS/MS spectrum of that ion (Figure 19) showed prominent

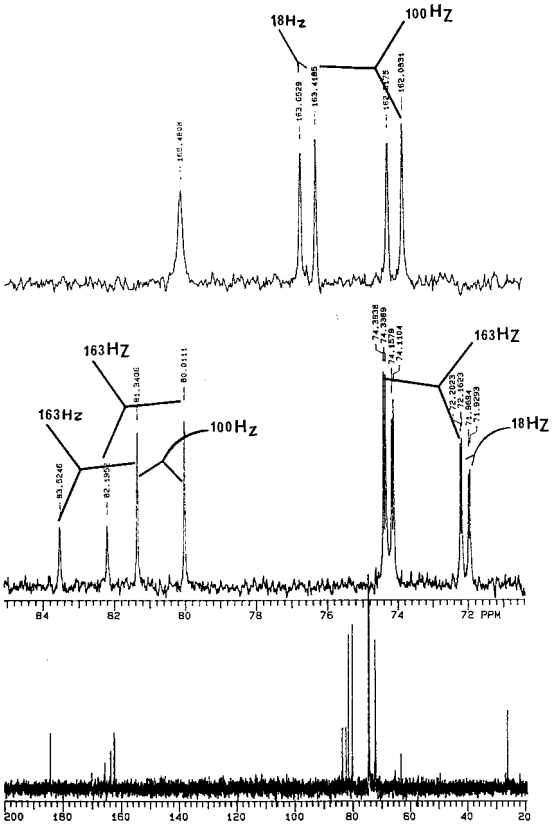
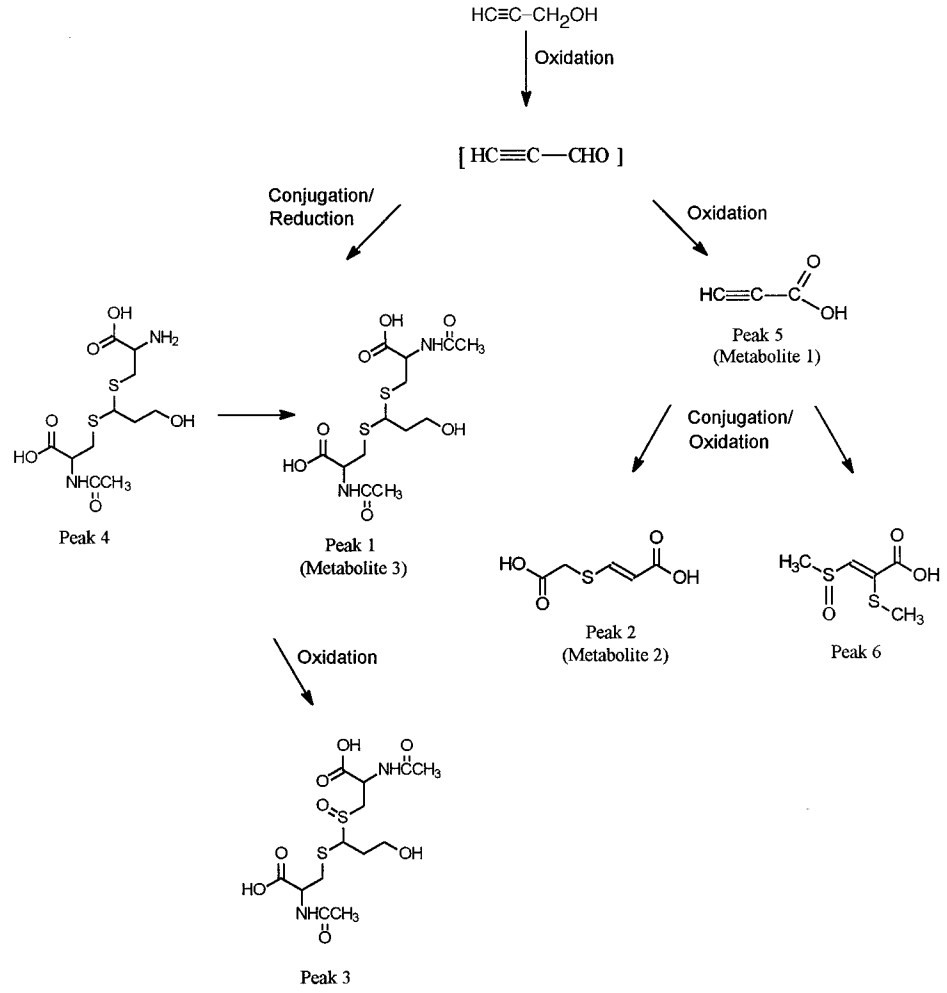


Figure 18. Expanded region of ^1H-decoupled ^{13}C NMR spectrum of purified peak 5 (Figure 8) from rat urine identified as 2-propynoic acid.

fragment ion peaks at *m/z* 166 due to the loss of water (*m/z* 18) and at *m/z* 138 resulting from the neutral loss of methyl sulfinyl (S═CH_2, *m/z* 46). The base peak in the spectrum appeared at *m/z* 122 and was attributed to the neutral loss of methyl sulfoxide (O═S═CH_2, *m/z*

Scheme 6. Pathway for the Metabolism of Propargyl Alcohol in Rats[a]



[a] Metabolite numbers indicate major metabolites characterized in situ (see Figure 1). Peak numbers indicate metabolites purified by HPLC (see Figure 8).

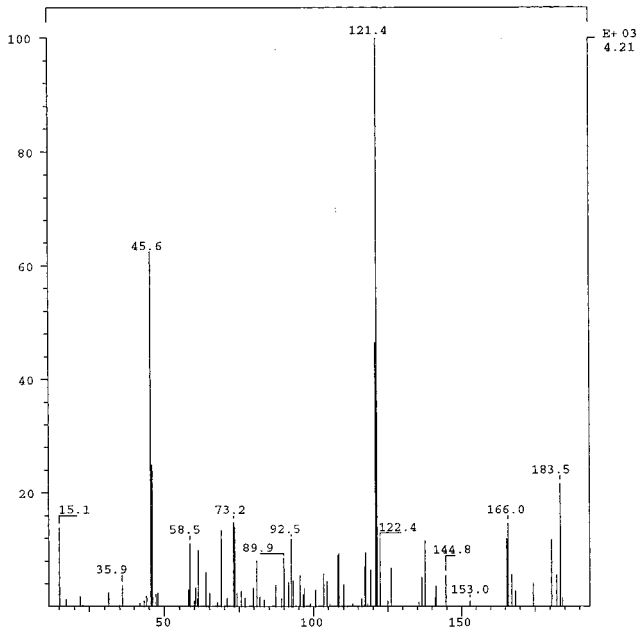


Figure 19. ESI LC/MS/MS of purified peak 6 (Figure 8) from rat urine.

62). Other characteristic fragment ion peaks were observed at *m/z* 74 assigned to $(H^{13}C{=}^{13}C{-}^{13}COOH)^+$ and at *m/z* 46 assigned to $^{13}COOH$.

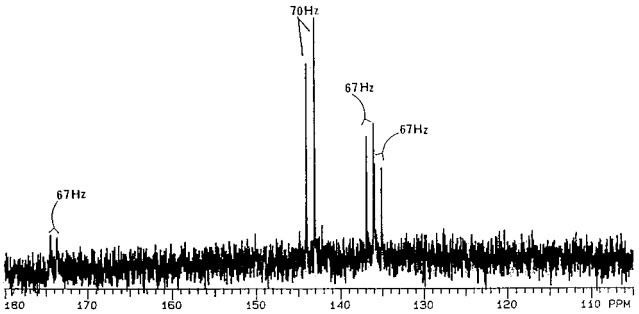


Figure 20. Expanded region of ^1H-decoupled ^{13}C NMR spectrum of purified peak 6 (Figure 8) from rat urine identified as 3-(methylsulfinyl)-2-(methylthio)-2-propenoic acid.

The carbon-13 NMR spectrum of peak 6 (Figure 20) displayed shifts centered at 136 (d,d, $J = 67$ Hz), 143.5 (d, $J = 70$ Hz), and 174 ppm (d, $J = 67$ Hz) indicating sp^2 hybridized carbons. The spectrum resembles the spectrum of peak 2 (Figure 12) which showed two methine carbons (signals centered at 110 and 146 ppm) and a carbonyl carbon (signal at 167 ppm) as evidenced by their chemical shifts. The only difference between the spectra was that the chemical shifts of the C_2 carbon for peak 6 (doublet of doublets was assigned to the middle ^{13}C portion of the metabolite derived from propargyl alcohol) appeared at about 136 ppm (i.e., shifted by about 26 ppm further downfield compared to peak 2). This downfield shift indicated an attachment

to an electron-withdrawing substituent, suggesting an RXCH=C(XR)−COOH type of structure for the portion of peak 6 derived from ^{13}C-PA. Using incremental shift values for substitution of functional groups on alkenes, the X group was determined to be RS for the middle carbon and RS=O for attachment to the terminal carbon. These were the only feasible substituents which agreed with the experimental shift values and resulted in an ROSCH=CSR−COOH type structure. Thus, peak 6 was identified as 3-(methylsulfinyl)-2-(methylthio)-2-propenoic acid, formed by oxidation of PA to 2-propynoic acid which then undergoes conjugation with GSH, catalyzed by *glutathione-S-transferase*; subsequent losses of glutamate, catalyzed by *γ-glutamyltranspeptidase*, and glycine, catalyzed by *cysteinyl glycinase*, results in the formation of the dicysteinyl conjugate which is degraded and oxidized further to form the 3-(methylsulfinyl)-2-(methylthio)-2-propenoic acid metabolite, as shown in Scheme 5.

Peak 7 eluted with an HPLC retention time of 3 min (Figure 8) and accounted for about 1% of TRR in 0−24 h urine (Figure 6). It was identified by GC/MSD as unchanged propargyl alcohol (spectrum is not shown).

CONCLUSION

The^{13}C-labeling/NMR tracer technique using [1,2,3-^{13}C]-PA provided a very convenient method to investigate the metabolism of PA in rat urine. Major metabolites were characterized, directly, in the whole urine by one- and two-dimensional ^{13}C NMR. To facilitate isolation and purification of metabolites, urine was lyophilized and extracted with methanol. The extract was subjected to TLC, and the zones of radioactivity were collected and purified further on HPLC. Identifications were confirmed on the individually purified metabolites by ^{13}C NMR and mass spectrometry and in some cases by comparison of the spectral data with those of chemically synthesized compounds. The bands in the^{13} C NMR spectrum of isolated metabolites were identical to those assigned to a metabolite in the whole urine, indicating that the metabolites had been isolated intact.

The proposed metabolic pathway involves oxidation of propargyl alcohol to 2-propynoic acid and glutathione conjugation to yield as final products: 3,3-bis[(2-(acetylamino)-2-carboxyethyl)thio]-1-propanol, 3-(carboxymethylthio)-2-propenoic acid, 3-(methylsulfinyl)-2-(methylthio)-2-propenoic acid, 3-[[2-(acetylamino)-2-carboxyethyl]thio]-3-[(2-amino-2-carboxyethyl)thio]-1-propanol, and 3-[[2-(acetylamino)-2-carboxyethyl]sulfinyl]-3-[[2-(acetylamino)-2-carboxyethyl]thio]-1-propanol. The mechanism appears to involve Michael addition at the terminal carbon. These unique metabolites have not been reported previously and represent the first example of multiple glutathione additions to the carbon−carbon triple bond. The proposed pathway for the metabolism of propargyl alcohol by rats is shown in Scheme 6.

LITERATURE CITED

Bax, A.; Freeman, R.; Frenkiel, T. A. An NMR technique for tracing out the carbon skeleton of organic molecule. *J. Am. Chem. Soc.* **1981**, *103*, 2102−2104.

Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy: high resolution methods and applications in organic chemistry and biochemistry*; VCH: New York, 1987.

Fennell, T. R.; Kedderis, G. L.; Sumner, S. J. Urinary metabolites of [1,2,3-^{13}C]Acrylonitrile in rats and mice detected by ^{13}C NMR spectroscopy. *Chem. Res. Toxicol.* **1991**, *4*, 678−687.

Hillenweck, A.; et al. Chlorothalonil Biotransformation by Gastrointestinal Microflora: *In Vitro* Comparative Approach in Rat, Dog, and Human. *Pestic. Biochem. Physiol.* **1997**, *58*, 34−48.

Jones, J. B.; Wigfield, D. C. Steroids and Steroidases. III Dicyclohexylcarbodiimide-Sulfoxide Oxidations of Alcohols and Thiols. *Can. J. Chem.* **1966**, *44*, 2517−2523.

Komatsu, N.; Uda, M.; Suzuki, H. Bismuth(III) Halides and Sulfate as Highly Efficient Catalyst for the Sulfenylation of Carbonyl and Related Compounds. *Synth. Lett.* **1995**, 984−986.

March, J. *Advanced Organic Chemistry*, 3rd ed.; John Wiley & Sons: New York, 1985.

Matsuo, R. Synthetic Pyrethroids containing a C−C Triple Bond. *Pestic. Sci.* **1998**, *52*, 21−28.

Muller, L.; Kumar, A.; Ernest, R. R. Two-dimensional carbon-13 NMR spectroscopy. *J. Chem. Phys.* **1975**, *63*, 5490−5491.

Nicholson, J. K.; Wilson, I. H. High-Resolution NMR Spectroscopy of biological samples as an aid to drug development. *Prog. Drug Res.* **1987**, *31*, 427−479.

O'Neil, I. A. Reverse Phase Flash Chromatography: A Convenient Method for the Large Scale Separation of Polar Compounds. *Synth. Lett.* **1991**, 661−662.

Pretsch, E.; Clerc, T.; Seibl, J.; Simon, W. *Tables of Spectral Data for Structure Determination of Organic Compounds*; Fresenius, W., et al., Ed.; Springer-Verlag: New York, 1989.

Sumner, S. J.; MacNeela, J.; Fennell, T. R. Characterization and Quantitation of Urinary Metabolites of [1,2,3-^{13}C]-Acrylamide in Rats and Mice using ^{13}C NMR Spectroscopy. *Chem. Res. Toxicol.* **1992a**, *5*, 81−89.

Sumner, S. J.; Stedman, D. B.; Clarke, D. O.; Welsch, F.; Fennell, T. R. Characterization of urinary metabolites from [1,2-methoxy-^{13}C]-Methoxyethanol in mice using ^{13}C NMR spectroscopy. *Chem. Res. Toxicol.* **1992b**, *5*, 553−560.

Tomigahara, Y.; Shiba, K.; Isobe, N.; Kaneko, H.; Nakatsuka, I.; Yamada, H. Identification of two new types of S-linked conjugates of Etoc in rat. *Xenobiotica* **1994**, *24*, 839−852.

Varian Publication 87-146635-00 Rev. A1087, Matched Susceptibility Plugs, Sept. 29, 1987.

Wehrli, F. W.; Wirthlin, T. *Interpretation of carbon-13 NMR spectra*; Heyden & Son: New York, 1976.

White, E. R. *Acrolein*; Smith, C. W., Ed.; John Wiley & Sons: New York, 1962; p 139.

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