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

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Species differences in the metabolism of acetylenic compounds commonly used in the formulation of pharmaceuticals and pesticides have not been investigated. To better understand the in vivo reactivity of this bond, the metabolism of propargyl alcohol (PA), 2-propyn-1-ol, was examined in rats and mice. An earlier study (Banijamali, A. R.; Xu, Y.; Strunk, R. J.; Gay, M. H.; Ellis, M. C.; Putterman, G. J. *J. Agric. Food Chem.* **1999**, *47*, 1717−1729) in rats revealed that PA undergoes extensive metabolism primarily via glutathione conjugation. The current research describes the metabolism of PA in CD-1 mice and compares results for the mice to those obtained for rats. [1,2,3-^{13}C;2,3-^{14}C]PA was administered orally to the mice. Approximately 60% of the dose was excreted in urine by 96 h. Metabolites were identified, directly, in whole urine by 1- and 2-D ^{13}C NMR and HPLC/MS and by comparison with the available reference compounds. The proposed metabolic pathway involves glucuronide conjugation of PA to form 2-propyn-1-ol−glucuronide as well as oxidation of PA to the proposed intermediate 2-propynal. The aldehyde undergoes conjugation with glutathione followed by further metabolism to yield as final products 3,3-bis[(2-acetylamino-2-carboxyethyl)thio]-1-propanol, 3-[(2-acetylamino-2-carboxyethyl)thio]-3-[(2-amino-2-carboxyethyl)-thio]-1-propanol, 3,3-bis[(2-amino-2-carboxyethyl)thio]-1-propanol, 3-[(2-amino-2-carboxyethyl)thio]-2-propenoic acid, and 3-[(2-formylamino-2-carboxyethyl)thio]-2-propenoic acid. A small portion of 2-propynal is also oxidized to result in the excretion of 2-propynoic acid. On the basis of urinary metabolite data, qualitative and quantitative differences are noted between rats and mice in the formation of the glucuronide conjugate of PA and in the formation of 2-propynoic acid and metabolites derived from glutathione. These metabolites represent further variation on glutathione metabolism following its addition to the carbon−carbon triple bond compared to those described for the rat.

Keywords: *2-Propyn-1-ol mouse metabolism; glutathione conjugates; glucuronide conjugate*

INTRODUCTION

Species differences in the metabolism of acetylenic compounds, commonly used in the formulation of pharmaceuticals and pesticides, have not been elucidated. To understand the in vivo reactivity of this bond, we examined the metabolism of propargyl alcohol (PA), an acetylenic primary alcohol, which is used as a reactive and versatile intermediate in the synthesis of agricultural chemicals and pharmaceuticals. Our recent publication (Banijamali et al., 1999) described the elimination pattern and the biotransformation pathway of PA in male Sprague−Dawley rats using ^{13}C-PA and ^{13}C NMR spectroscopy to characterize metabolites, directly, in urine. The metabolites were then isolated, purified, and identified by mass spectrometry and NMR spectroscopy. It was found that in rats PA was metabolized via a primary pathway involving its oxidation to 2-propynoic acid and glutathione conjugation involving Michael addition at the terminal acetylenic carbon.

The objective of this study was to extend our knowledge of PA metabolism by determining the elimination pattern and biotransformation pathway of PA in male CD-1 mice using ^{13}C-PA and^{13}C NMR and mass spectrometry to identify metabolites directly in urine without the isolation of individual metabolites and to compare results for the two rodent species. The same dose level and oral gavage route of administration was selected for mice as was used for rats. Oral exposure was selected because ingestion is the most probable accidental route for human exposure, and gavage is a commonly used route of administration for metabolism studies.

MATERIALS AND METHODS

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 ppm (J = 70 and 14 Hz) and 77 ppm (J = 169 and 14 Hz) and a quartet at 85 ppm (J = 168 and 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 the dose solution) and was prepared by acid hydrolysis of an ester provided by Chemsyn Science Laboratory (Lenexa, KS). The radiolabeled material was mixed with ^{13}C-PA to give a specific activity of 8100 dpm/μg (2.04×10^{-1} mCi/mmol) and dissolved in deionized water to a concentration of 8 mg/mL.

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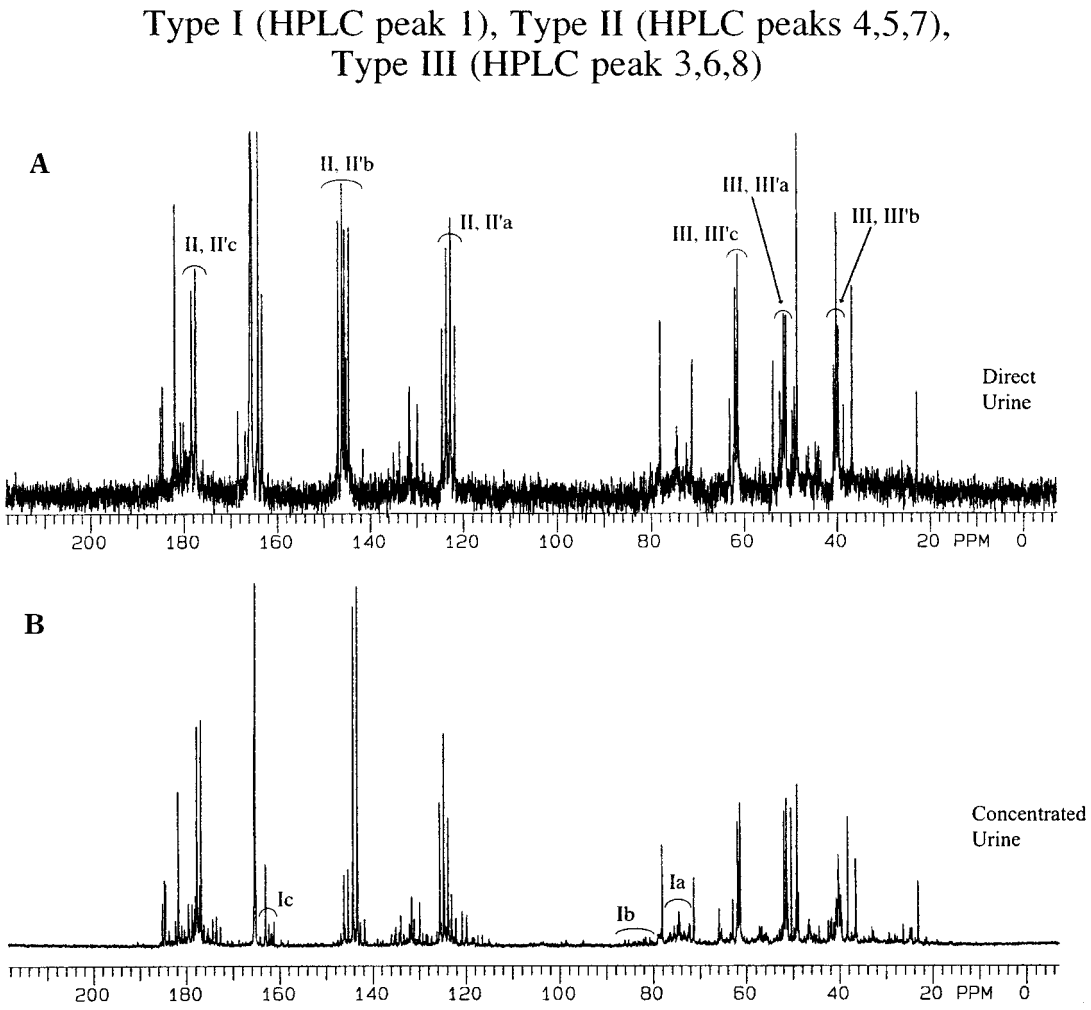


Figure 1. ^{1}H-decoupled ^{13}C NMR spectrum of direct urine (A) and concentrated urine (B) collected for 24 h after administration of 40 mg/kg [1,2,3-^{13}C]PA. Signals for metabolites are labeled according to metabolite number (see Table 1) and the letter of the carbon derived from PA (a, b, or c for the 3-, 2-, or 1-carbon, respectively).

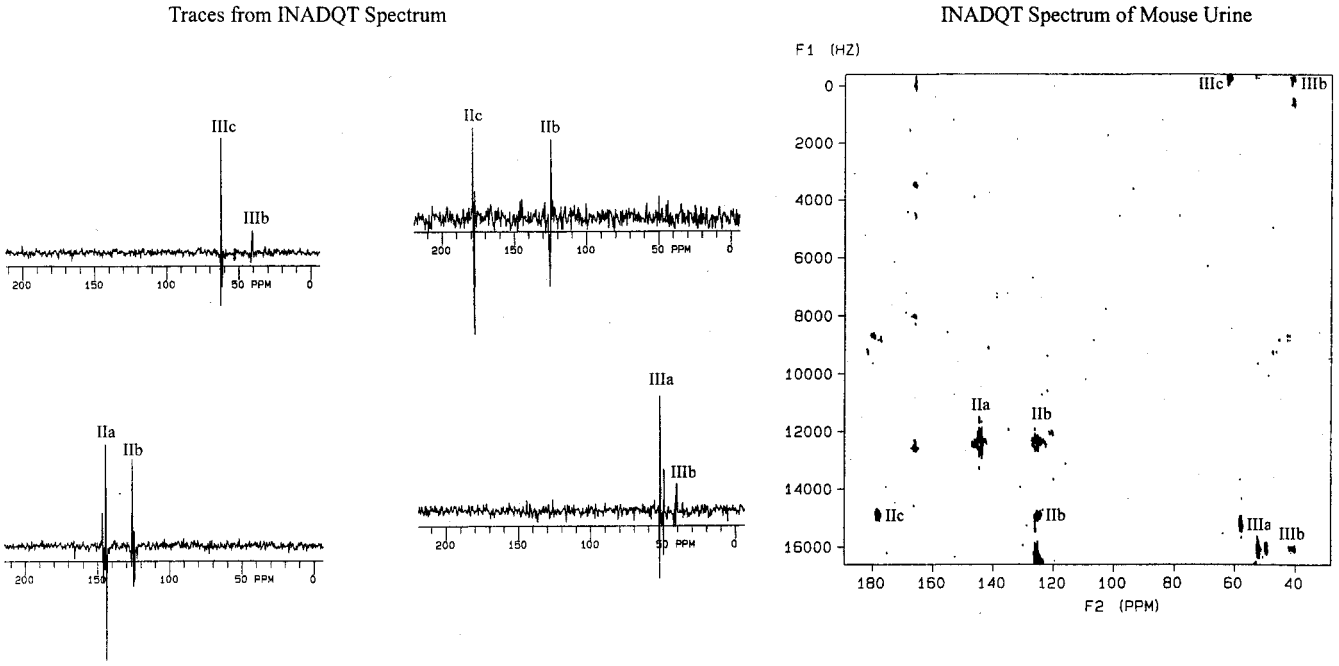


Figure 2. Carbon−carbon connectivities were established via INADEQUATE spectroscopy. The contours and traces are labeled with the Roman numeral representing the metabolite type and a letter designating the carbon derived from ^{13}C-PA (refer to Table 1).

Table 1. Chemical Shifts, Carbon−Carbon Coupling Constants, and Multiplicities for Metabolites of [1,2,3-^{13}C]Propargyl Alcohol in Mouse Urine

metabolite type[a]		chemical shift[b] (ppm)	carbon coupling (Hz)	carbon−carbon multiplicity	HPLC peak (Figure 3)	ACD/Labs[c]	ref compd[d] (ppm)
type I					1		
	Ia	73.32	164, 18	d, d		79	74.0
	Ib	82.40[e]	164, 100	d, d		74	78.6
	Ic	162.45[e]	100, 18	d, d		166	157
type II					4, 5, 7		
	IIa	145.04	71	d		148	149.8 *E*
	II′a	146.39	71	d			152.7 *Z*
	II″a	145.56	71	d			
	IIb	122.64	70, 71	d, d		113	118.1 *E*
	II′b	123.59	70, 71	d, d			117.2 *Z*
	II″b	122.84	67, 67	d, d			
	IIc	177.95	67	d		165	171.5 *E*
	II′c	177.78	67	d			172.7 *Z*
type III					3, 6, 8		
	IIIa	51.22	35	d		49	51.2
	III′a	51.13	35	d			
	IIIb	40.19	35, 35	d, d		41	40.2
	III′b	40.17[e]	37, 37	d, d			
	IIIc	61.76	37	d		59	61.5
	III′c	61.66	37	d			
type IV					2		
	IVa	49.38	36	d		54	
	IVb	nd[f]				83	
	IVc	nd				74	

[a] Type I, peak 1, HC≡CCOOH; type II, peaks 4, 5, 7, RS−CH═CHCOOH; type III, peaks 3, 6, 8, $(RS)_2$−$CHCH_2CH_2OH$ (R derived from GSH conjugation); type IV, peak 2, HC≡$CHCH_2$-*O*-glucuronide. [b] Center of multiplet pattern. Urea referenced to 165.5 ppm. The sample was prepared by adding 100 μL of D_2O to 400 μL of urine. The carbon atom is assigned a Roman numeral corresponding to the metabolite type and a letter, which designates its derivation from PA (a, b, or c corresponding to the 3-, 2-, or 1-carbon of PA, respectively). [c] Values of shift were obtained from ACD/Labs software. [d] Reference compounds were prepared in D_2O. [e] III′b is resolved from IIIb in spectra of concentrated urine. Small differences in shift are apparent for the center of multiplet patterns in concentrated urine as compared with direct urine and are attributed to differences in the matrix (i.e., pH). [f] Signals could not be definitively attributed to multiplet patterns from a PA-derived glucuronide conjugate directly in urine or in concentrated urine samples. However, signals with low intensity are present in the 70−85 ppm region of the spectrum that may arise from such a conjugate.

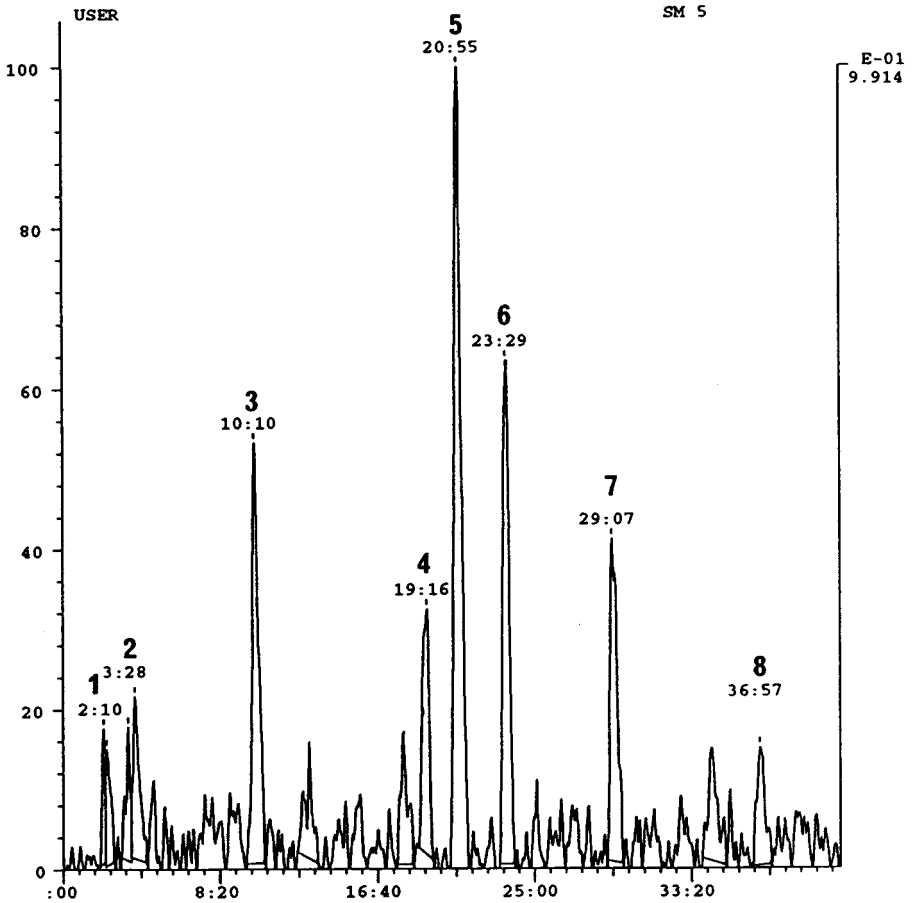


Figure 3. HPLC radiochromatogram of 24 h urine obtained from mice treated with PA (40 mg/kg) separated on a Hypercarb column utilizing a gradient of 50 mM formic acid and methanol. Retention time and peak number are shown. The approximate relative abundances are as follows: peak 1, 2%; peak 2, 6%; peak 3, 15%; peak 4, 10%; peak 5, 31%; peak 6, 17%; peak 7, 13%; peak 8, 6%.

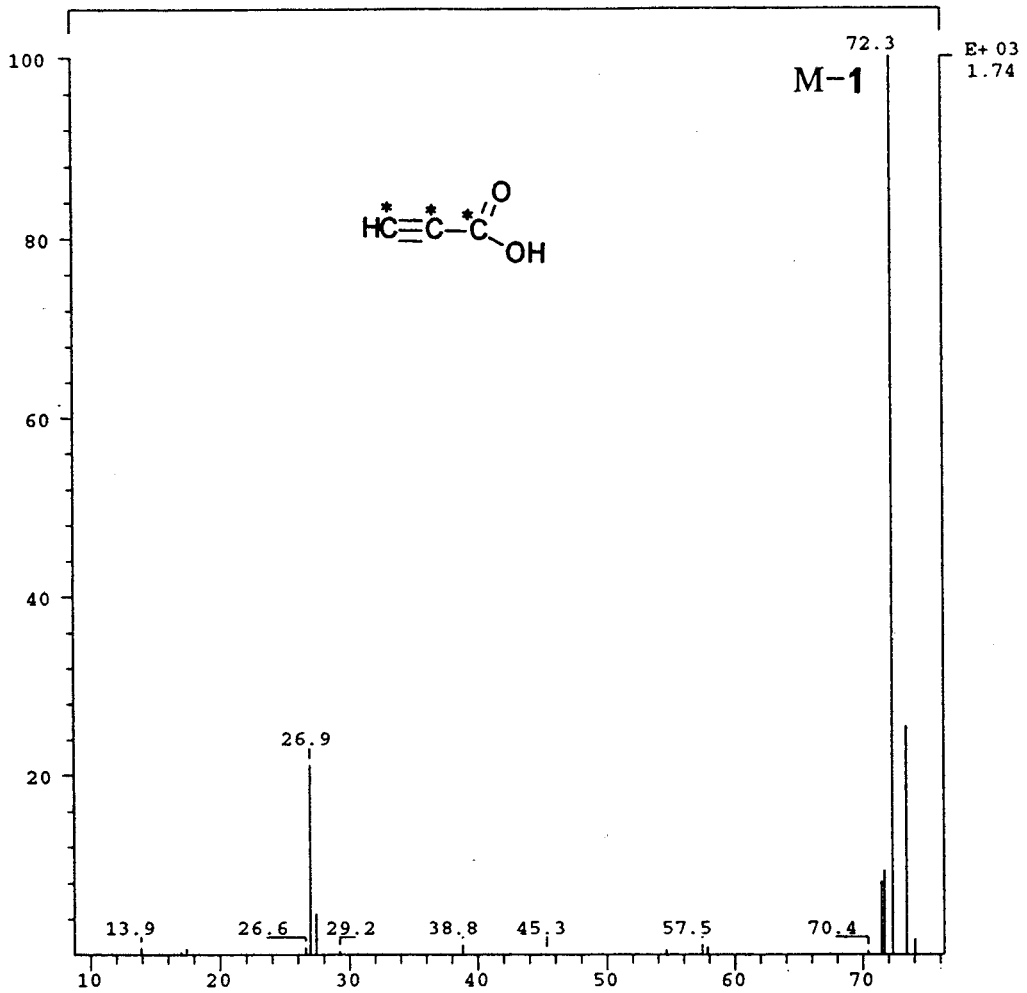


Figure 4. ESI LC/MS/MS of peak 1 (Figure 3) in mouse urine collected 24 h after administration of ^{13}C-PA, identified as 2-propynoic acid.

Animals: Treatment and Sample Collection. Male CD-1 mice (25), weighing ~23 g and ~9 weeks old, were purchased from Charles River Laboratories, Raleigh, NC. The mice were quarantined for at least 7 days and acclimated to metabolism cages for 7 days. Rodent Lab Chow 5002 (PMI Feeds, St. Louis, MO) and tap water were supplied ad libitum. Animals were housed in groups in plastic metabolism cages (Nalgene, Rochester, NY), which allowed separation and collection of urine and feces. During acclimation six or seven mice were housed in each cage, and following treatment, six mice were placed in each cage. Vials for urine and feces were supported in insulated containers of dry ice to freeze the excreta immediately upon collection. The mice were observed at least daily and twice within 5 h following treatment for signs of toxicity. Food and water consumption and urine and feces production were monitored daily for 4 to 6 days before treatment and until termination.

A mixture of [1,2,3-^{13}C]PA (95%) and [2,3-^{14}C]PA (5%) was administered by gavage to 24 mice at a dose of 40 mg/kg (5 mL/kg). Animals were returned to the metabolism cages, and urine and feces were collected at 24, 48, 72, and 96 h following treatment, at which time the mice were sacrificed.

Analytical Methods. *(1) Liquid Scintillation Counting (LSC).* Levels of radioactivity in urine were measured by LSC, as described previously (Banijamali et al., 1999).

(2) Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectra were acquired with a 5-mm dual proton−multinuclear probe on a Varian (Palo Alto, CA) XL-300 or VXR-300 spectrometer. 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 at 3 and 5 s, and a 45° (or 60°) pulse width. Spectra for urine samples were acquired with ~10000 transients and were referenced to urea at 165.5 ppm. Chemical shifts are expressed in parts per million (ppm).

Incredible natural abundance double-quantum transfer (INADEQUATE) spectra (Bax et al., 1981) were acquired on a sample of concentrated urine using the INADQT program from the Varian pulse sequence library. A relaxation delay time of 5 s and a τ value corresponding to a coupling constant of 70 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 32 complex points in t_1. Connectivities were established by examination of countour plots and traces (as shown in Figure 2).

Values of shift for carbons of potential metabolites were calculated using incremental substituent effects for alkynes, alkenes, and alkanes (Pretsch et al., 1989; Wehrli and Wirthlin, 1976; Breitmaier and Volter, 1987) and using the ^{13}C NMR database from Advanced Chemistry Development, Inc. (Toronto, Canada).

(3) 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, as described previously (Banijamali et al., 1999).

Sample Preparation. The frozen urine, collected 0−24 h after dose administration, was thawed at room temperature and centrifuged at 10000*g* for 10 min. Samples were prepared for NMR studies by adding ~100 μL of D_2O to 400 μL of the centrifuged urine. Samples of reference compounds were

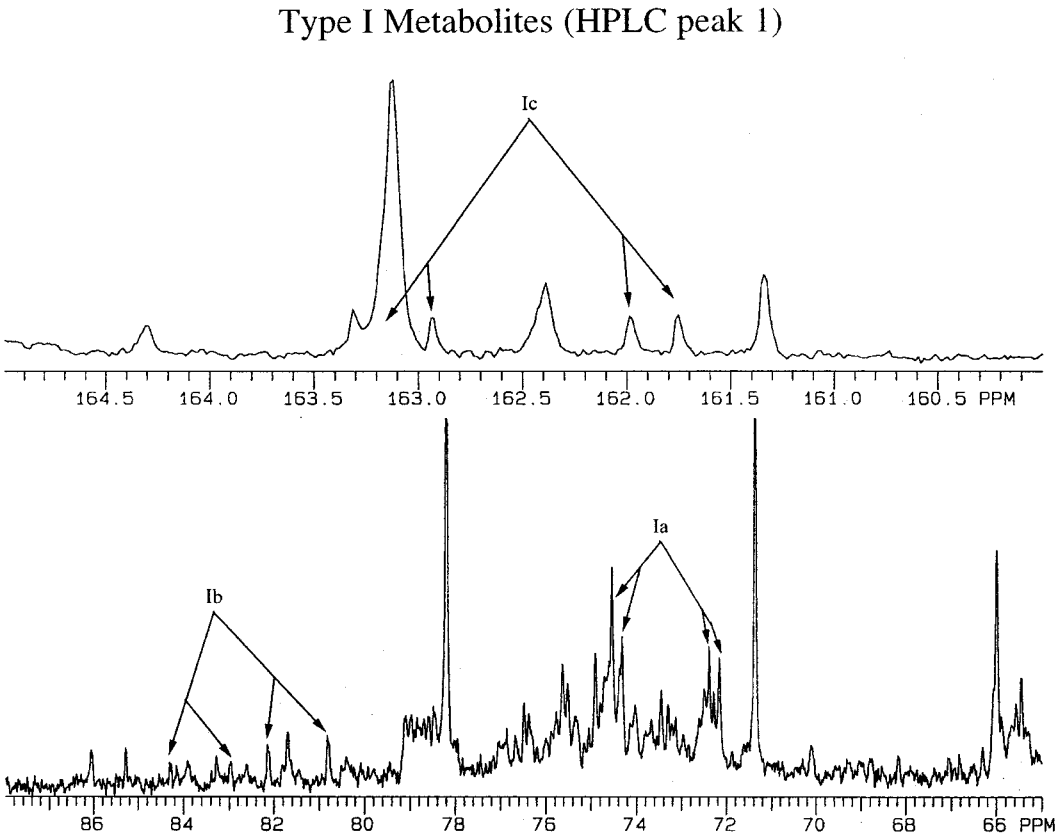


Figure 5. Expanded regions of the ^{13}C NMR spectrum of concentrated mouse urine. Signals labeled for the type I metabolite (HPLC peak 1) are at the same chemical shift positions as those previously detected in spectra obtained for rat urine (higher intensity) and also correspond to shifts for an authentic standard.

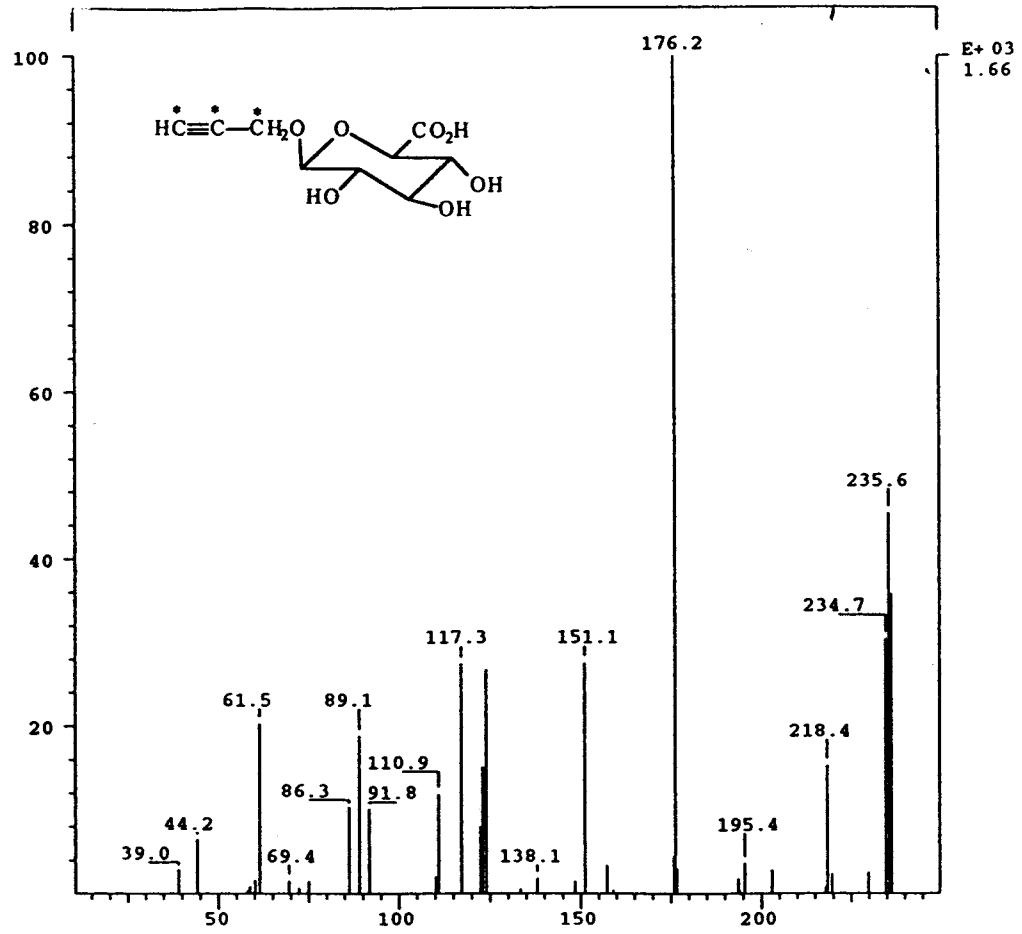


Figure 6. ESI LC/MS/MS of peak 2 (Figure 3) in mouse urine identified as propargyl alcohol−glucuronide.

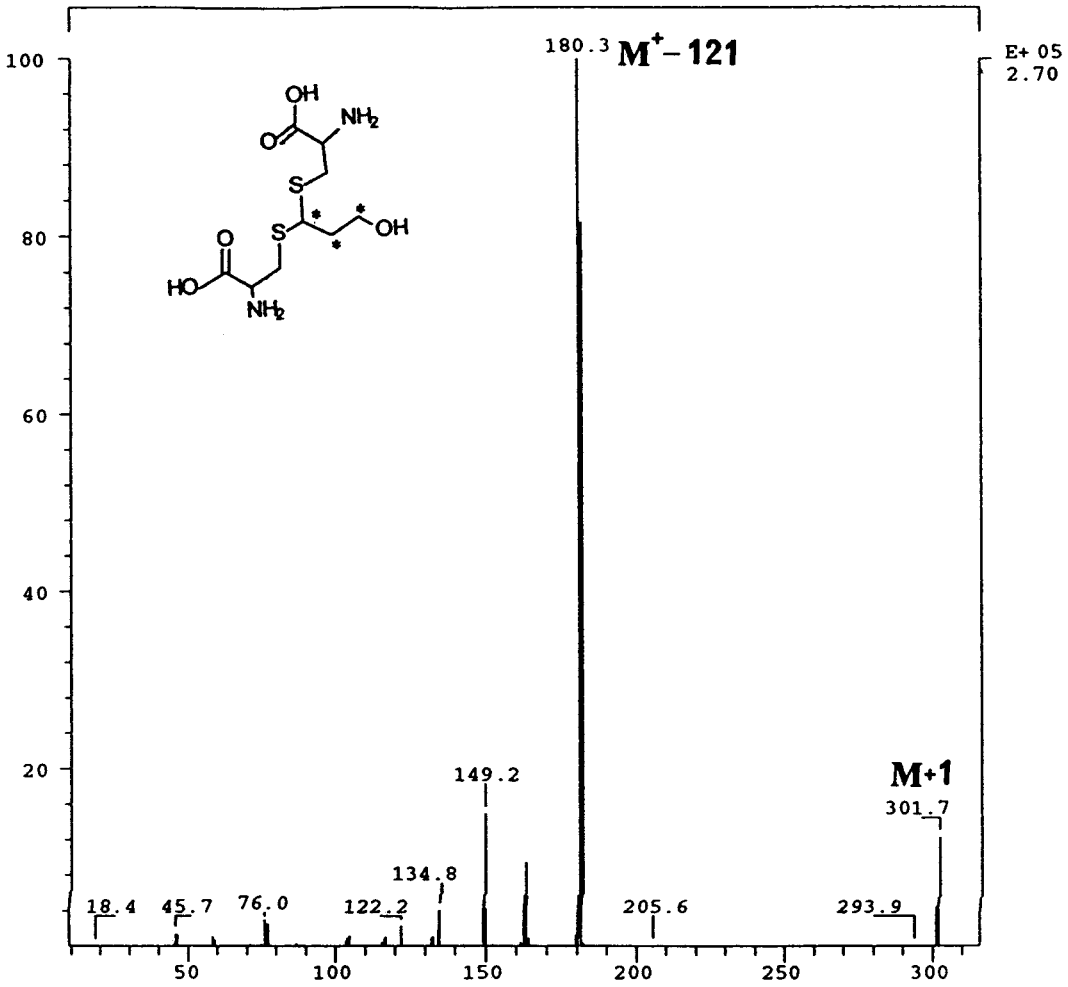


Figure 7. ESI LC/MS/MS of peak 3 (Figure 3) in mouse urine identified as 3,3-bis[(2-amino-2-carboxyethyl)thio]-1-propanol.

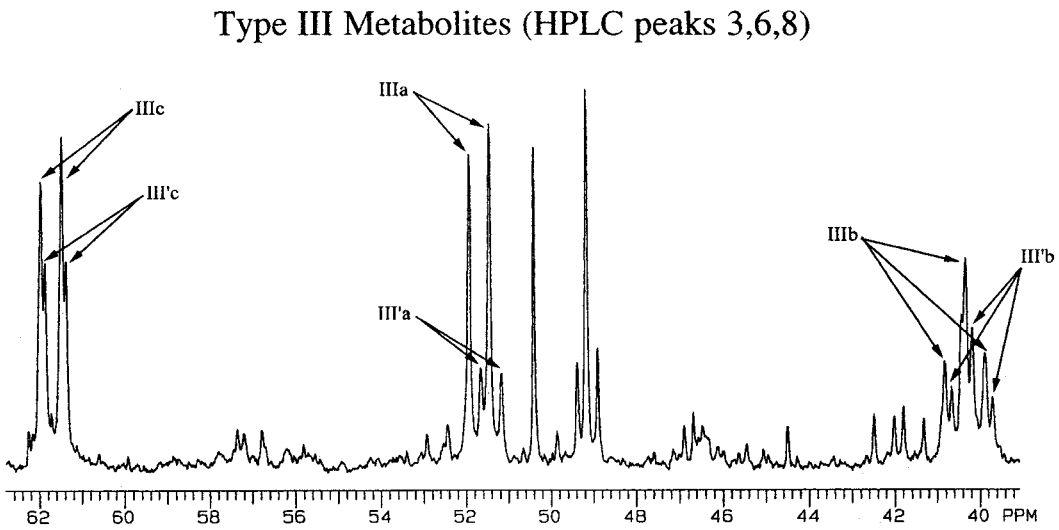
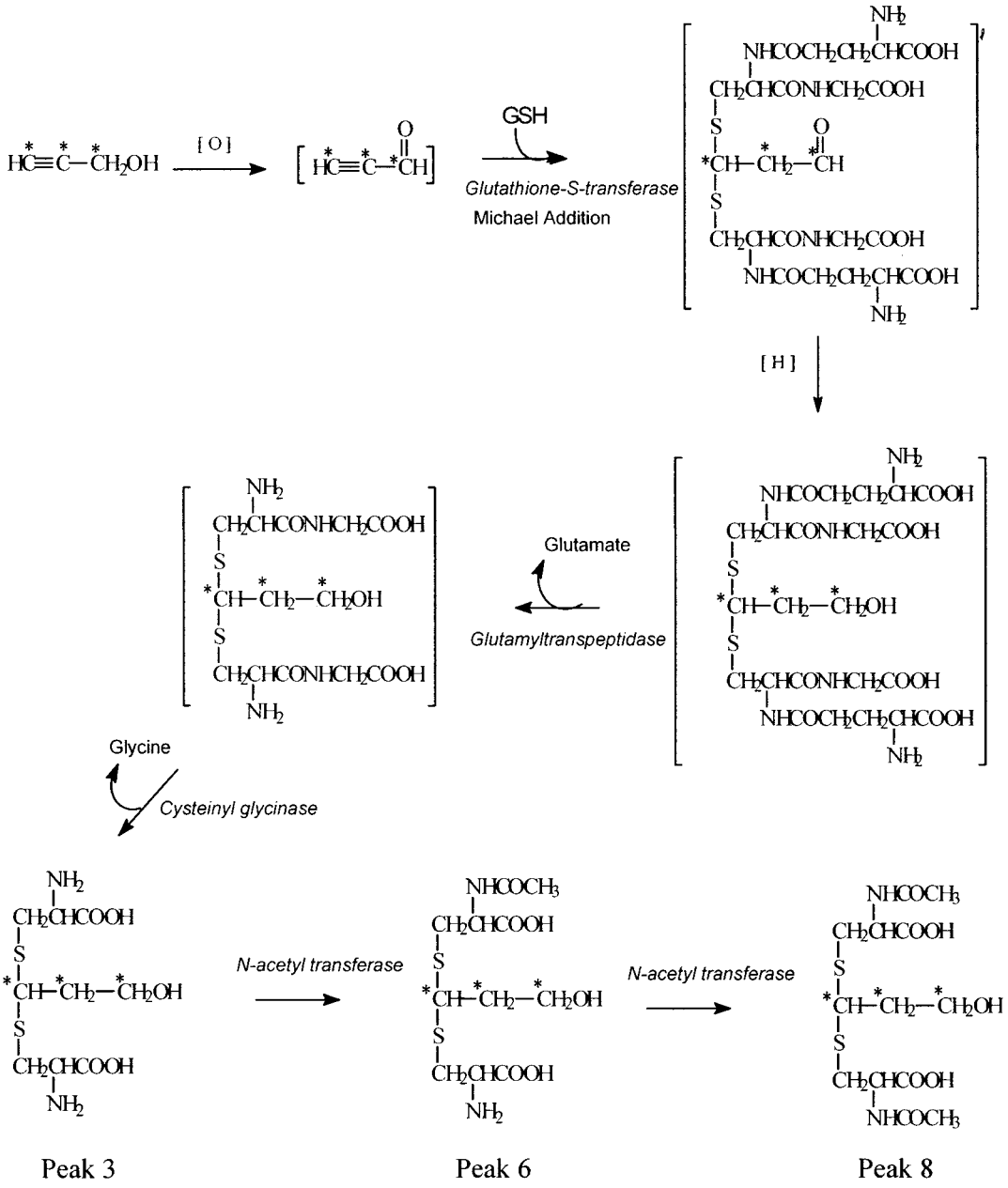


Figure 8. Expanded region of ^{1}H-decoupled ^{13}C NMR spectrum of mouse urine collected 24 h after administration of ^{13}C-PA, indicating the presence of type III metabolites (HPLC peaks 3, 6, and 8): 3,3-bis[(2-amino-2-carboxyethyl)thio]-1-propanol, 3-[(2-acetylamino-2-carboxyethyl)thio]-3-[(2-amino-2-carboxyethyl)thio]-1-propanol, and 3,3-bis[(2-acetylamino-2-carboxyethyl)thio]-1-propanol.

prepared by dissolving appropriate quantities in 500 μL of D_2O or $CDCl_3$. A concentrated sample of urine was prepared for NMR studies by the addition of 3× volume of methanol and vigorous swirling on a vortex. The precipitate was removed via centrifugation at 12000*g* for 15 min. The supernatant was decanted and evaporated to dryness under nitrogen, and the residue was reconstituted in 500 μL of D_2O. Centrifuged samples of mouse urine were used, directly, for mass spectral identification of metabolites.

Synthesis and Characterization of Reference Compounds. Several reference compounds were synthesized, and their structures were fully characterized by ^{1}H and ^{13}C NMR and mass spectrometry. The detailed procedure for their synthesis along with the MS and NMR data was presented in

Scheme 1. Proposed Metabolic Pathway for Formation of Peaks 3, 6, and 8[a]



[a] An asterisk denotes the position of ^{13}C label; structures in brackets are hypothetical intermediates.

a previous publication (Banijamali et al., 1999). Synthesis of (*E,Z*)-3-[(2-amino-2-carboxyethyl)thio]-2-propenoic acid had not been described previously.

Synthesis of (*E,Z*)-3-[(2-Amino-2-carboxyethyl)thio]-2-propenoic Acid. To a stirred solution of 4.1 g (33.0 mmol) of L-cysteine (Aldrich, Milwaukee, WI) and 2.0 g (20 mmol) of potassium hydrogencarbonate in 30 mL of water was added at 20 °C 2.0 mL (33 mmol) of propiolic acid (Aldrich) over 10 min. After stirring at ambient temperature overnight, the reaction mixture was cooled in an ice bath and acidified to pH 3. The product was filtered to give, on drying under vacuum, 2.1 g of a white solid: mp 178 °C (dec); ^{1}H NMR (D_2O) δ 3.30−3.55 (2H, 6d, SCH_AH_B), 4.04 (1H, m, NCH), 5.97 (0.2 H, d, J = 15 Hz, =CHCO), 6.00 (0.8H, d, J = 10 Hz, =CHCO), 7.33 (0.8H, d, J = 10 Hz, CHS=), 7.75 (0.2H, d, J = 15 Hz, SCH=) (the product is a 20:80 mixture of *E*/*Z* isomers); ^{13}C NMR (D_2O) δ 35.24, 38.64 ($\underline{C}H_2S$), 56.07, 56.90 ($\underline{C}HN$), 117.2, 118.1 (=$\underline{C}$HCO), 149.8, 152.7 (S$\underline{C}$H=), 171.5, 172.7, 174.5 ($\underline{C}$=O); LC-MS (ESI+Q1MS) 192 (M + 1), 175, 132 (100), 103, 86, 70.

RESULTS AND DISCUSSION

[^{13}C,^{14}C]Propargyl alcohol was administered to CD-1 mice by oral gavage. Food intake and fecal output did not change significantly following treatment. Water consumption was variable during acclimation and increased slightly following treatment. Urine production increased markedly within 24 h following treatment.

In contrast to rats that showed CNS depression characterized by ataxia and increased startle sensitivity following treatment with PA (Banijamali et al., unpublished observations), mice were nearly unaffected. Although mice showed only a slight decrease in startle response for 24 h, rats displayed piloerection, erythemia, and exophthalmos, in addition to CNS depression, for >48 h following treatment (Banijamali et al., unpublished observations).

Urine and feces were collected at 24, 48, 72, and 96 h following administration of PA. The highest concentra-

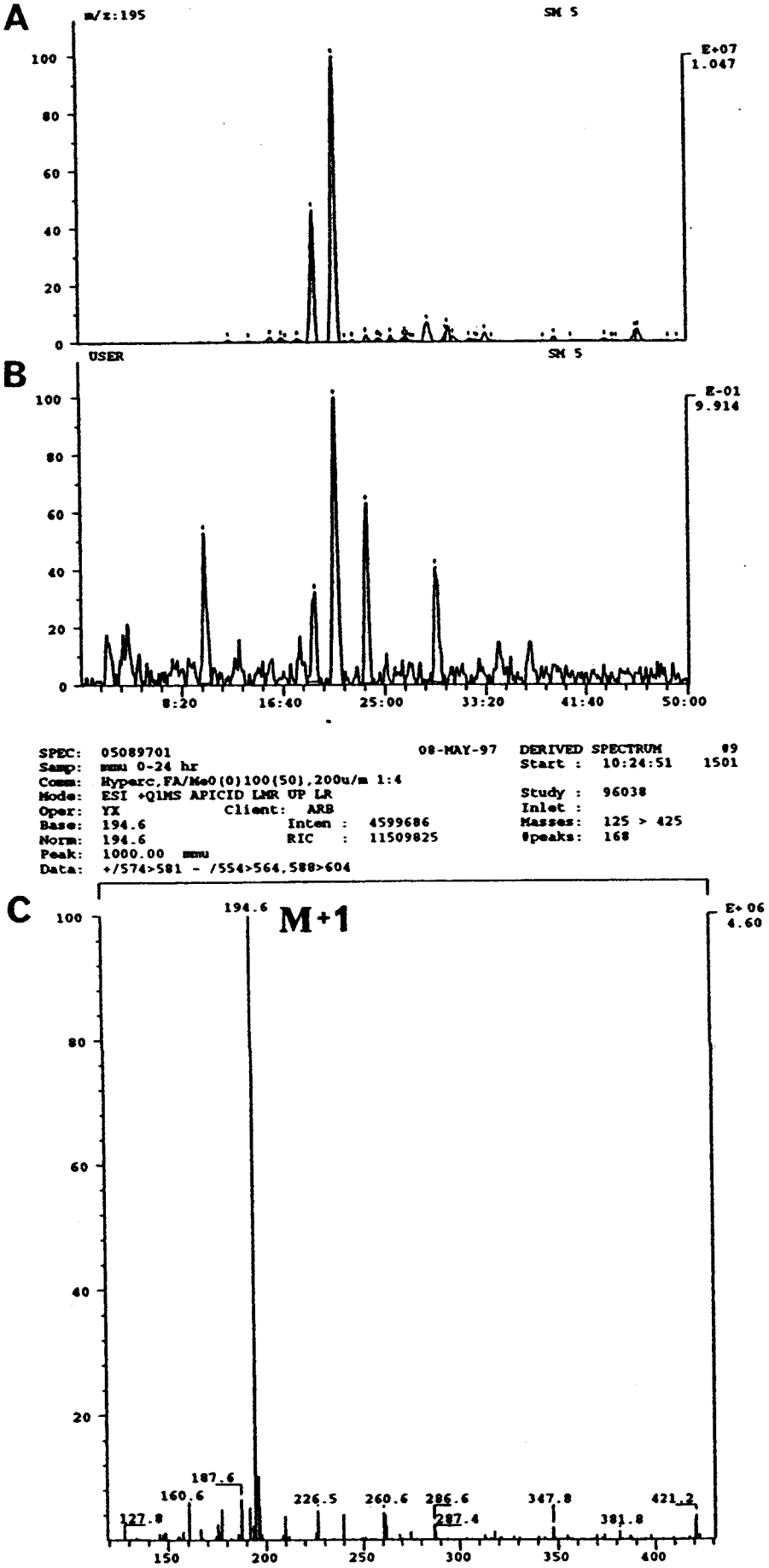


Figure 9. Positive ion ESI LC/MS of peaks 4 and 5 (Figure 3) in mouse urine: (A) selected ion chromatogram at *m*/*z* 195; (B) RAM trace; (C) full-scan Q1 MS.

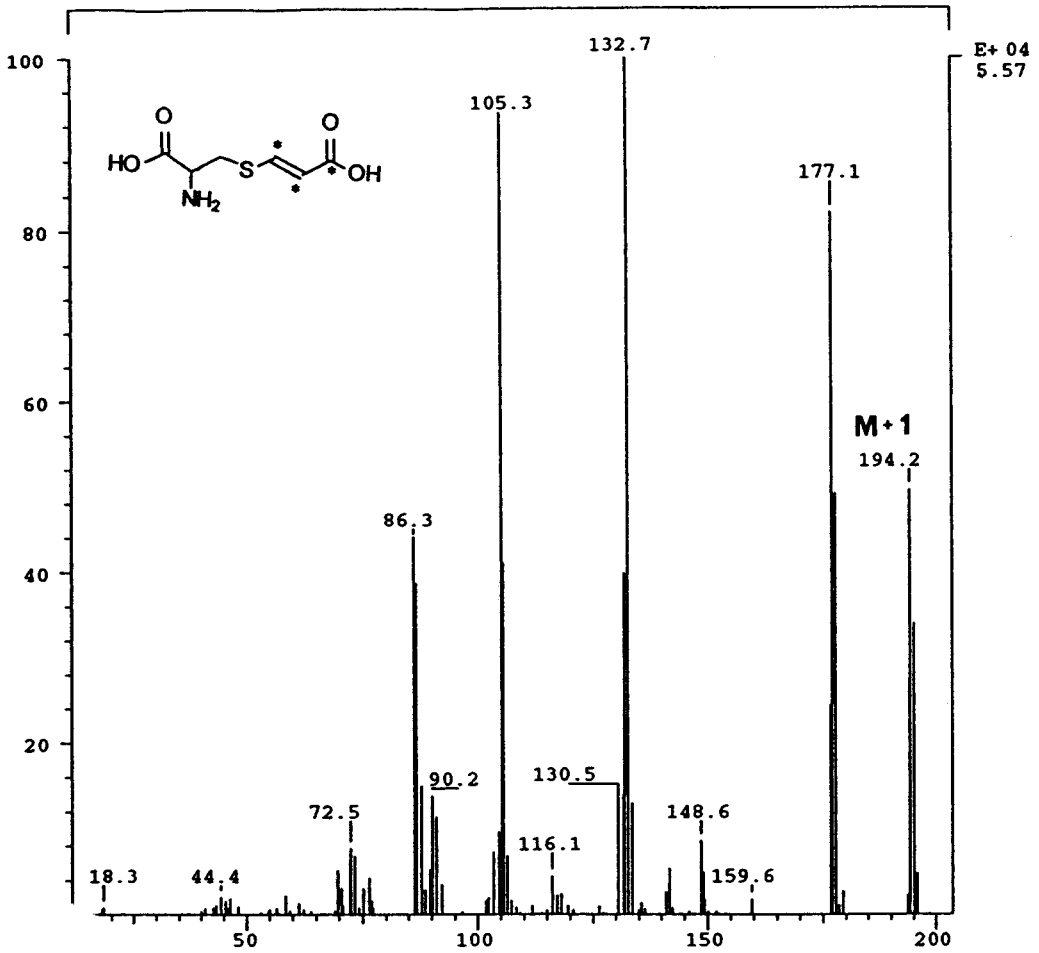


Figure 10. ESI LC/MS/MS of peaks 4 and 5 (Figure 3) in mouse urine identified as (*E*+*Z*)-[(2-acetylamino-2-carboxyethyl)thio]-2-propenoic acid.

tion of ^{14}C-PA equivalents (337 μg/g) was observed in the urine collected within 24 h of PA administration. About 60% of the administered radioactivity had been excreted in the urine by 96 h. Levels of radioactivity were much lower (2.65 μg/g) 4 days after administration of PA.

Identification of Metabolites. The identification of PA urinary metabolites was achieved by ^{13}C NMR (directly in urine) and by HPLC and mass spectrometry. Carbon-13 NMR was used to detect and identify partial structures of metabolites derived from ^{13}C-labeled portions of PA metabolites in the urine collected from CD-1 mice that received an oral dose of [1,2,3-^{13}C]PA. This approach avoids selective isolation of metabolites and enables determination of the presence of volatile and chemically labile metabolites that may occur on sample preparation by conventional techniques, through comparison of the NMR spectrum of urine before and after its concentration. A concentrated sample of urine was used for NMR studies. The proton-decoupled ^{13}C NMR spectra of urine and concentrated urine shown in Figure 1, panels A and B, respectively, were qualitatively similar. Qualitative differences between spectra were related to a better signal-to-noise ratio for the concentrated urine (Figure 1B). Some quantitative differences in the proportion of metabolites are indicated by differences in relative signal intensity between the two spectra.

The proton-decoupled ^{13}C NMR spectrum of control mouse urine (not shown) showed an intense singlet at 165.5 ppm that is assigned to urea; the less intense signals were consistent with sugars, hippurate, citrate, and creatine (Nicholson et al., 1987). The ^{13}C NMR spectrum of urine from mice administered ^{13}C-PA (Figure 1) contains signals from endogenous compounds and signals that appear as multiplet patterns not present in the spectrum 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 that appear as singlets because of the low incidence of adjacent ^{13}C nuclei. Major metabolites of PA give rise to intense signals, whereas many metabolites that are formed in lower concentration give rise to less intense signals. On the basis of chemical shift position and analysis by 2-D NMR (Figure 2), the metabolites were divided into four types (types I−IV). The type I metabolite is consistent with shifts for propynoic acid. The shifts for type II metabolites are consistent with substituted propenoic acid such as RS−CH=CHCO$_2$H. The shifts for type III metabolites are consistent with substituted propyl alcohols such as (RS)-2-CHCH$_2$CH$_2$-OH. The shifts for the type IV metabolite are consistent with a direct conjugation to the hydroxyl group of PA.

The above information was used together with MS data to determine the specific structure for each metabolite separated on HPLC. The chemical shifts (recorded for the center of the multiplet patterns) for signals derived from the labeled carbons of PA and the carbon−carbon coupling constants are listed in Table 1. The number and letters in Table 1 assigned to a metabolite designate the HPLC peak number (see

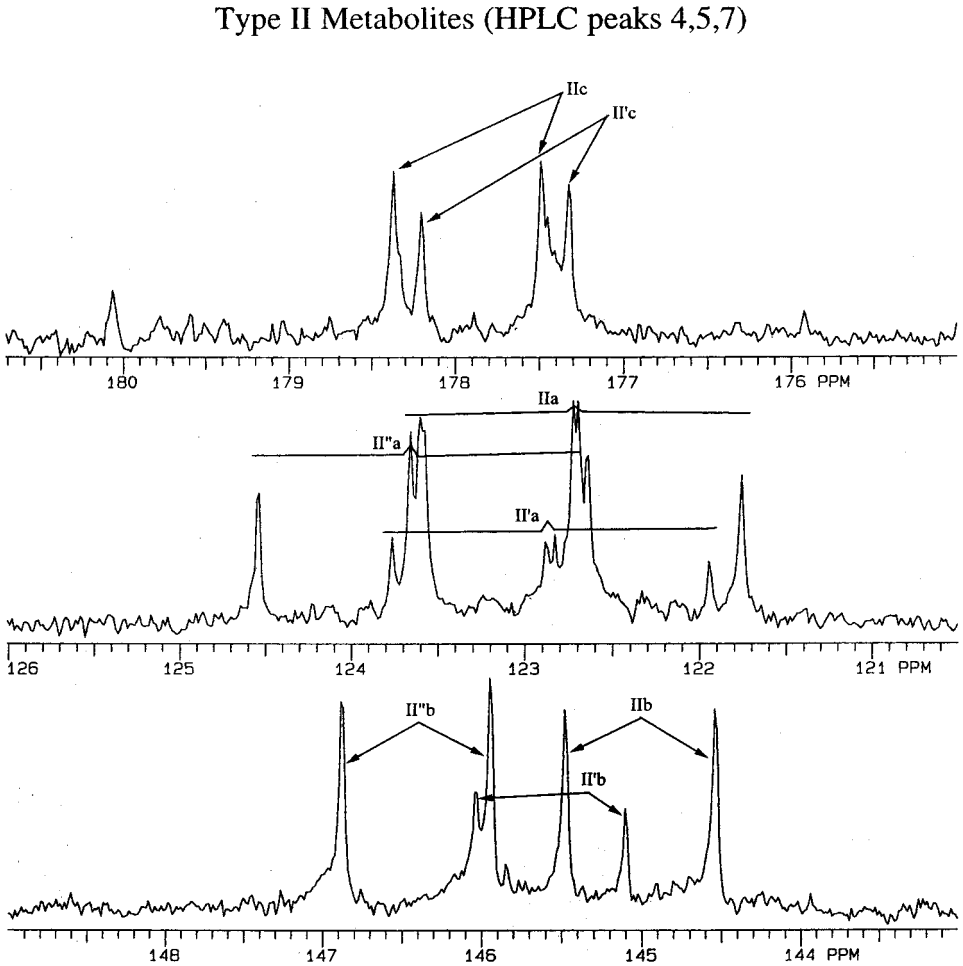


Figure 11. Expanded regions of the ^{13}C NMR spectrum of mouse urine showing resolved signals for at least two type II metabolites (HPLC peaks 4, 5, and 7).

below) and the carbon atoms derived from PA (a, b, or c for the 3-, 2-, or 1-carbon of PA), respectively.

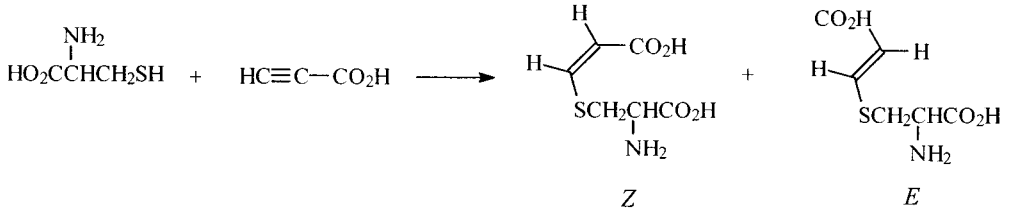
The NMR data are obtained for the portion of each metabolite derived from the ^{13}C-labeled carbons of PA; the remainder of each structure was assigned by comparing the experimental shifts with calculated values for a possible metabolite and/or with shifts obtained for reference compounds. Although this method does not enable definitive identification of the unlabeled portion of each metabolite, it facilitated development of appropriate techniques for mass spectral identifications.

The definitive identification of each metabolite was then made using HPLC/MS and HPLC/MS/MS. Mass spectral identification of metabolites was made by a multistep approach as described earlier. 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. The HPLC radiochromatogram of 24 h urine obtained from mice treated with PA separated on a Hypercarb column is shown in Figure 3. Retention time, peak number, and approximate relative abundances are also shown in Figure 3. The NMR type for each peak number is indicated in Table 1.

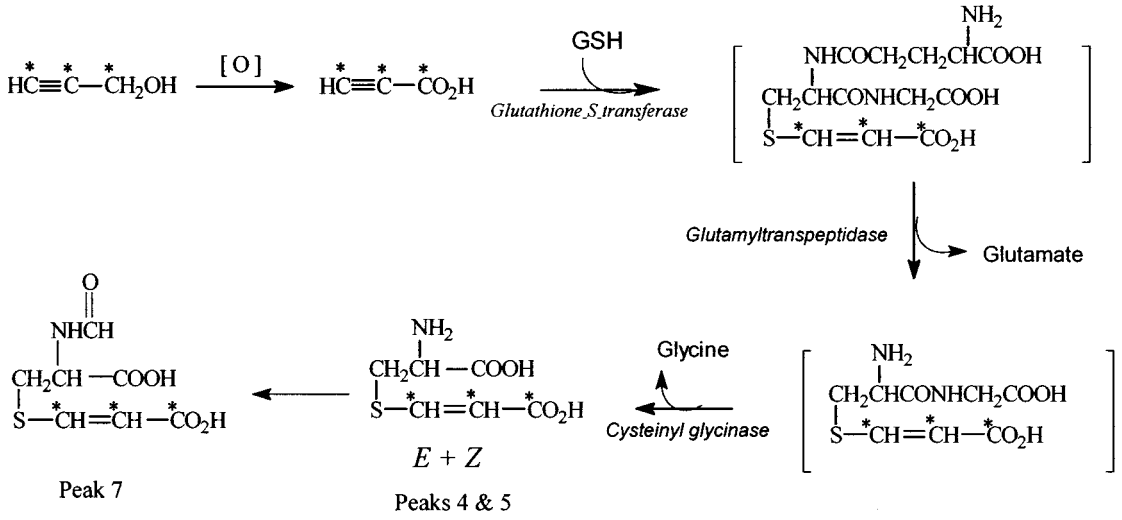
HPLC Peak 1 (Type I Metabolite). The negative ion ESI LC/MS of mouse urine showed a match of the retention time between RAM trace (peak 1) and the $[M - H]^-$ peak at *m/z* 72, indicating a molecular weight of 73 for peak 1. The ESI LC/MS/MS spectrum (Figure 4) of this ion showed only a prominent fragment ion at *m/z* 27, resulting from the neutral loss of carbon dioxide (*m/z* 45, $^{13}CO_2$) from the M − H peak, indicating the identity of HPLC peak 1 as 2-propynoic acid, an oxidation product of PA. Due to the low concentration [~2% total radioactive residue (TRR)] of this metabolite in the mouse urine, as opposed to ~27% TRR in the rat urine, the ^{13}C NMR signals for this metabolite were of low intensity, and all signals for 2-propynoic acid were detectable only in concentrated mouse urine. Two sets of signals, corresponding to *sp* hybridized carbons of 2-propynoic acid, were centered near 73 ppm (d,d 164, 18 Hz) and 82 ppm (d,d 164, 100 Hz), and a carbonyl carbon was located at ~163 ppm (d,d 100, 18) (Figure 5).

HPLC Peak 2 (Type IV Metabolite). The positive ion ESI LC/MS of mouse urine showed a match of the retention time between the RAM trace (peak 2) and the $[M + H]^+$ peak at *m/z* 236, indicating a metabolite with a molecular weight of 235. The daughter ion spectrum of the $[M + H]^+$ peak (Figure 6) showed only a prominent fragment ion at *m/z* 176, consistent with the molecular weight of glucuronic acid, resulting from the neutral loss of ^{13}C-PA (*m/z* 59, $H^{13}C{\equiv}{}^{13}C^{13}CH_2OH$) from the M + H peak. NMR spectra were examined to determine the presence of peaks that would arise from the proposed glucuronide conjugate. A doublet with low signal intensity was present at 49 ppm that could be

Scheme 2. Synthesis of Reference Compounds (*E*+*Z*)-3-[(2-Amino-2-carboxyethyl)thio]-2-propenoic Acid



Scheme 3. Proposed Metabolic Pathway for Formation of Peaks 4, 5, and 7[a]



[a] An asterisk denotes the position of ^{13}C label; structures in brackets are hypothetical intermediates.

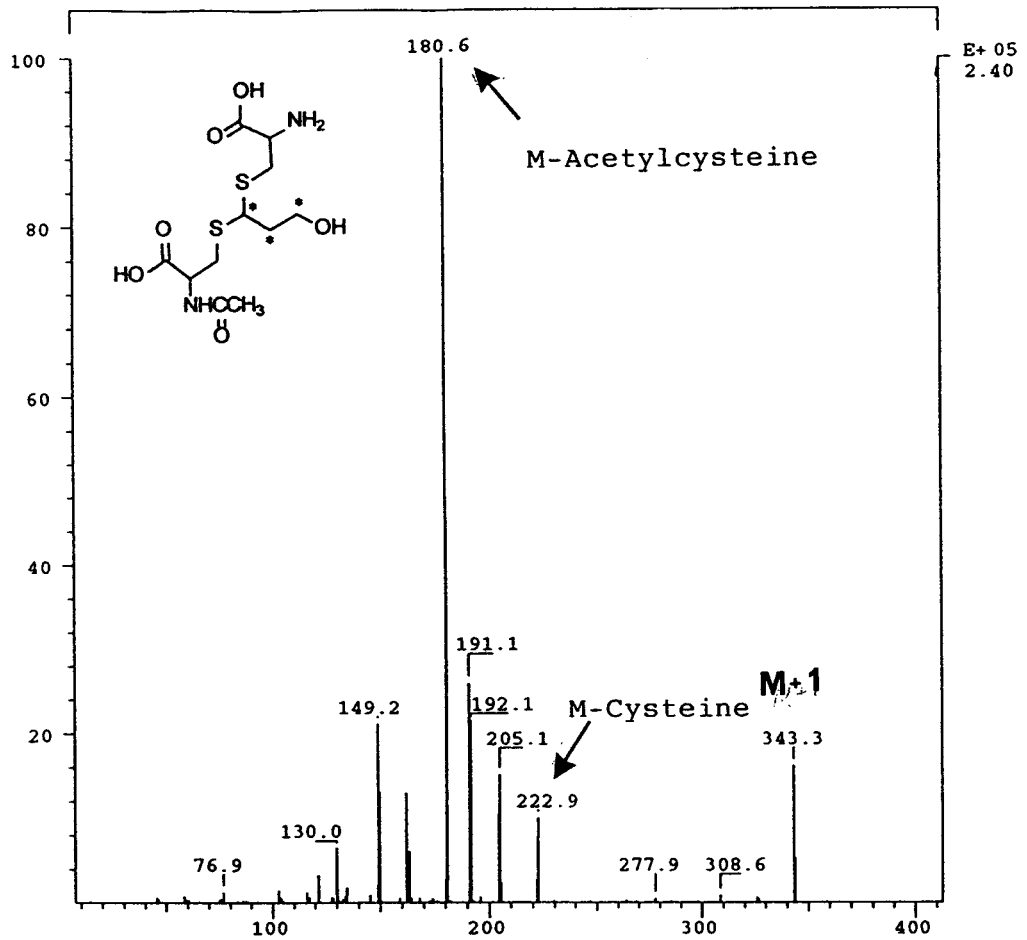


Figure 12. ESI LC/MS/MS of peak 6 (Figure 3) in mouse urine identified as 3-[(2-acetylamino-2-carboxyethyl)thio]-3-[(2-amino-2-carboxyethyl)thio]-1-propanol.

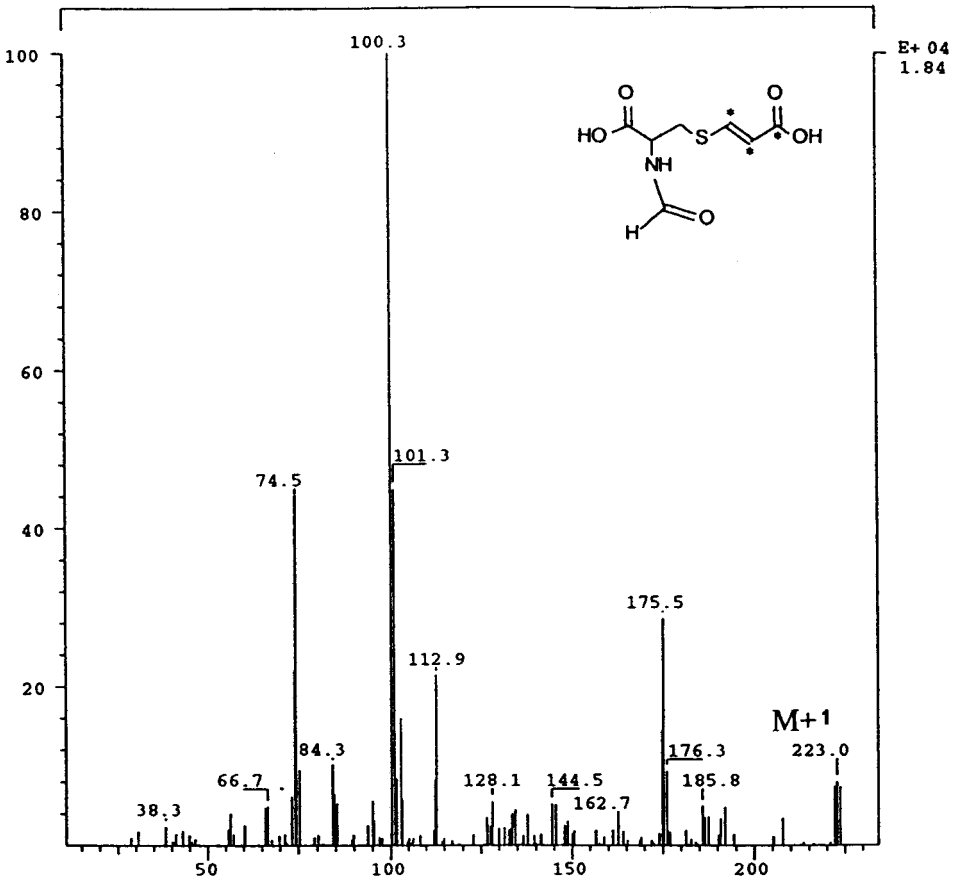


Figure 13. ESI LC/MS/MS of peak 7 (Figure 3) in mouse urine identified as 3-[2-(formylamino-2-carboxyethyl)thio]-2-propenoic acid.

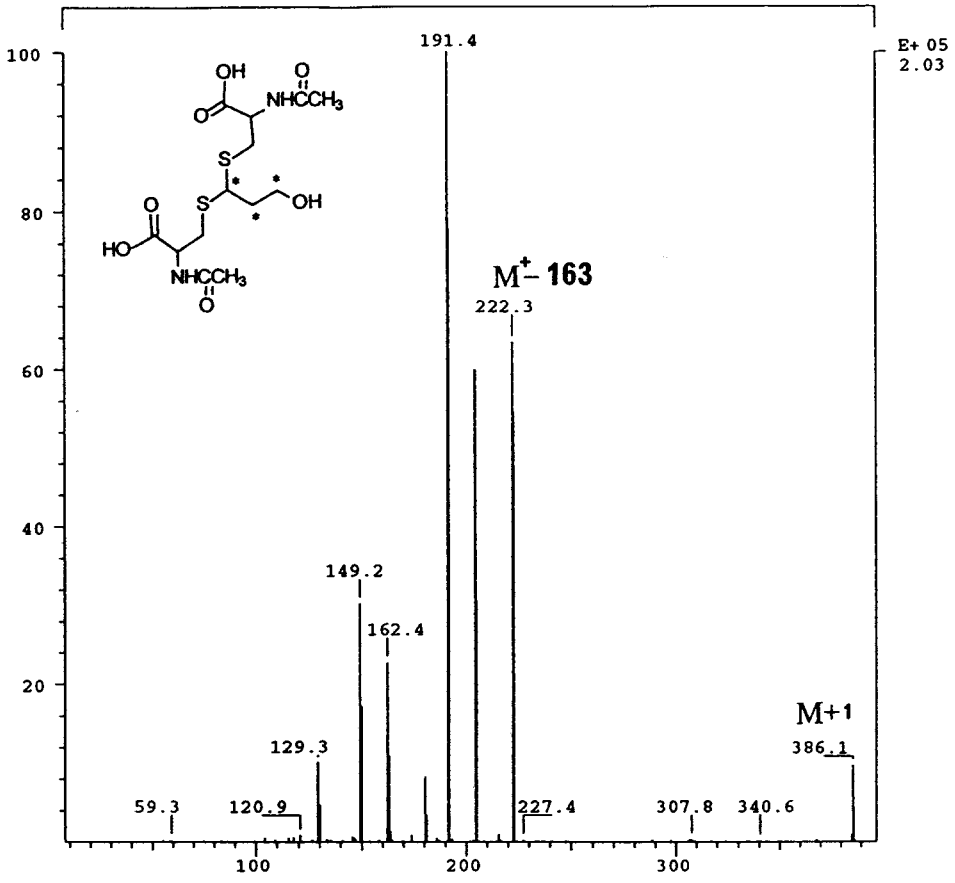


Figure 14. ESI LC/MS/MS of peak 8 (Figure 3) in mouse urine identified as 3,3-bis[(2-acetylamino-2-carboxyethyl)thio]-1-propanol.

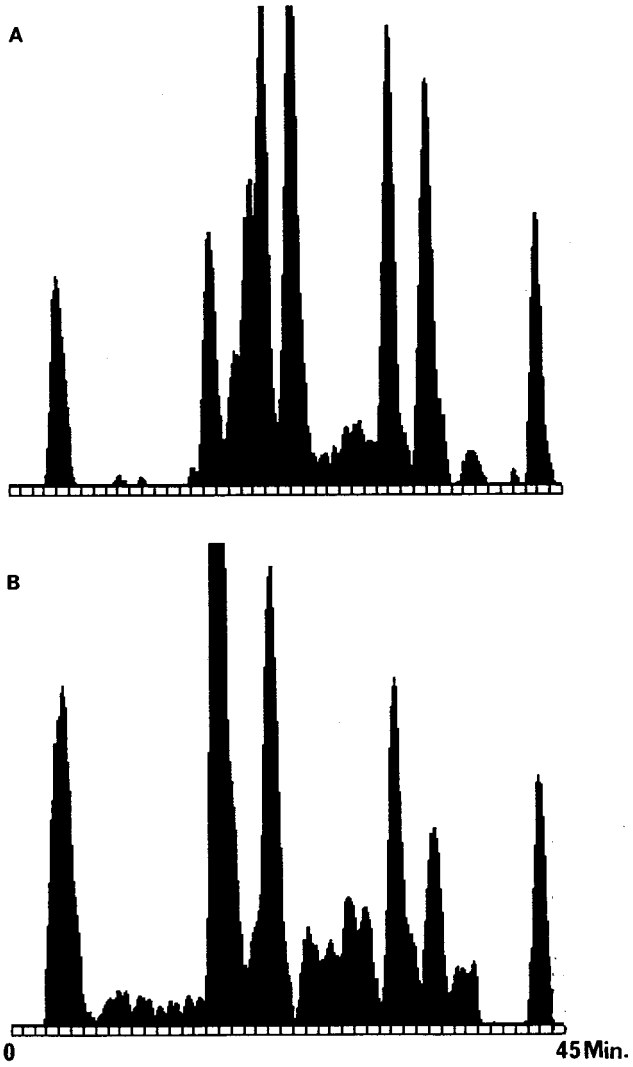


Figure 15. HPLC radiochromatograms of mouse (A) and rat (B) urine obtained on a SAX column.

attributed to the CH_2 carbon. Signals could not be definitely assigned to the C≡C carbons. However, signals with low intensity are present in the 70−85 ppm region of the spectrum that may arise from the carbons in such a conjugate. Thus, peak 2 was identified as propargyl alcohol−glucuronide. This metabolite, which was absent in rat urine, accounted for ∼6% TRR in 0−24 h mouse urine.

HPLC Peak 3 (Type III Metabolite). The positive ion ESI LC/MS of mouse urine showed a match of the retention time between the RAM trace (peak 3) and the $[M + H]^+$ peak at *m/z* 302, indicating a metabolite with a molecular weight of 301. The daughter spectrum of the $[M + H]^+$ peak (Figure 7) showed characteristic ion peaks at *m/z* 181 due to the loss of cysteine (*m/z* 121) and at *m/z* 149 resulting from the subsequent neutral loss of the $^{13}CH_2OH$ (*m/z* 32) portion of the PA moiety. The position of cysteine in the conjugate was determined from ^{13}C NMR as described below.

The expanded region of the carbon-13 NMR spectrum of urine (Figure 8) displayed shifts centered at 40 ppm (dd; J = 35 and 35 Hz), 51 ppm (d, J = 35 Hz), and 62 ppm (d, J = 37 Hz). At least two structurally similar metabolites (type III) that had chemical shift values for the 1-, 2-, and 3-carbons derived from ^{13}C-PA were resolved. The chemical shifts and coupling constant (J) of 35−37 Hz are indicative of sp^3 hybridized carbons. The doublet of doublets at 40 ppm (overlapping of the middle peak results in a triplet) was assigned to the center carbon of the PA-derived metabolite and arises from coupling to two adjacent ^{13}C atoms. The doublets at 51 and 62 ppm indicated carbons attached to only one other ^{13}C. Carbon−carbon connectivities were established via INADEQUATE spectroscopy between signals at 40 and 62 ppm and between signals at 51, 40, and 62 ppm (Figure 2). The chemical shift at 62 ppm is consistent with an attachment of a methylene carbon atom to a hydroxyl group. Thus, the structure $X-^{13}CH^{13}CH_2{}^{13}CH_2-Y$ was indicated. To fit the experimental values of 51, 40, and 62 ppm and assuming that functional group Y is OH, 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 X group must be RS, resulting in a structure of $(RS)_2-{}^{13}CH^{13}CH_2{}^{13}CH_2OH$. The chemical shifts for the synthetic reference compound, 3,3-bis[(2(acetylamino)-2-carboxyethyl)thio]-1-propanol (Banijamali et al., 1999), and calculated value (ACD/Labs) are consistent with the experimental shift values for this metabolite. As indicated above, R was determined by mass spectrometry to be a cysteinyl moiety resulting in the identification of this metabolite as 3,3-bis[(2-(amino)-2-carboxyethyl)-thio]-1-propanol, formed by the addition of 2 mol of glutathione (GSH) to 2-propynal, presumably catalyzed by glutathione *S*-transferase, in a reaction analogous to a Michael addition (March, 1985). Subsequent reduction of the aldehyde followed by losses of glutamate, catalyzed by γ-glutamyltranspeptidase, and glycine, catalyzed by cysteinyl glycinase (Hillenweck et al., 1997), produced the dicysteinyl conjugate as shown in Scheme 1. Peak 3 accounted for ∼15% TRR in 0−24 h urine.

HPLC Peaks 4 and 5 (Type II Metabolites). The positive ion ESI LC/MS of mouse urine showed an exact match of retention times between the RAM traces (peaks 4 and 5) and the $[M + H]^+$ at *m/z* 195 (Figure 9), confirming identical molecular weights of 194 for peaks 4 and 5. With both metabolites, the daughter spectrum of *m/z* 195 (Figure 10) showed characteristic ion peaks at *m/z* 149 due to the neutral loss of $H^{13}CO_2H$ (*m/z* 46) and at *m/z* 105 characteristic of an $S-^{13}CH=^{13}CH^{13}CO_2H$ moiety, indicating that the two metabolites are cysteine-conjugated isomers. The position of the cysteinyl moiety in the conjugate was determined from ^{13}C NMR as described below.

The expanded region of the ^{13}C NMR spectrum of mouse urine displayed several doublet of doublets (J_{cc} = 70 Hz) located at ∼123 ppm. Several doublets (J_{cc} = 70 Hz) were located near 145−146 ppm and near 178 ppm. At least three structurally similar type II metabolites were resolved (Figure 11, labeled as type II metabolites). The signals positioned at ∼123 and ∼146 ppm indicated a $H^{13}C=^{13}CH$ structure. The signal at ∼123 ppm shows connectivity to ∼178 ppm, indicating an $X-^{13}CH=^{13}CH^{13}CO_2H$ structure. Incremental shift values for substitution of functional groups (Pretsch et al., 1989) on alkenes revealed that the X group must be RS, resulting in a structure of $RS-^{13}CH=^{13}CH^{13}CO_2H$.

Two sets of reference compounds were prepared consistent with the proposed structure: one contained *N*-acetylcysteinyl for the R group (Banijamali et al.,

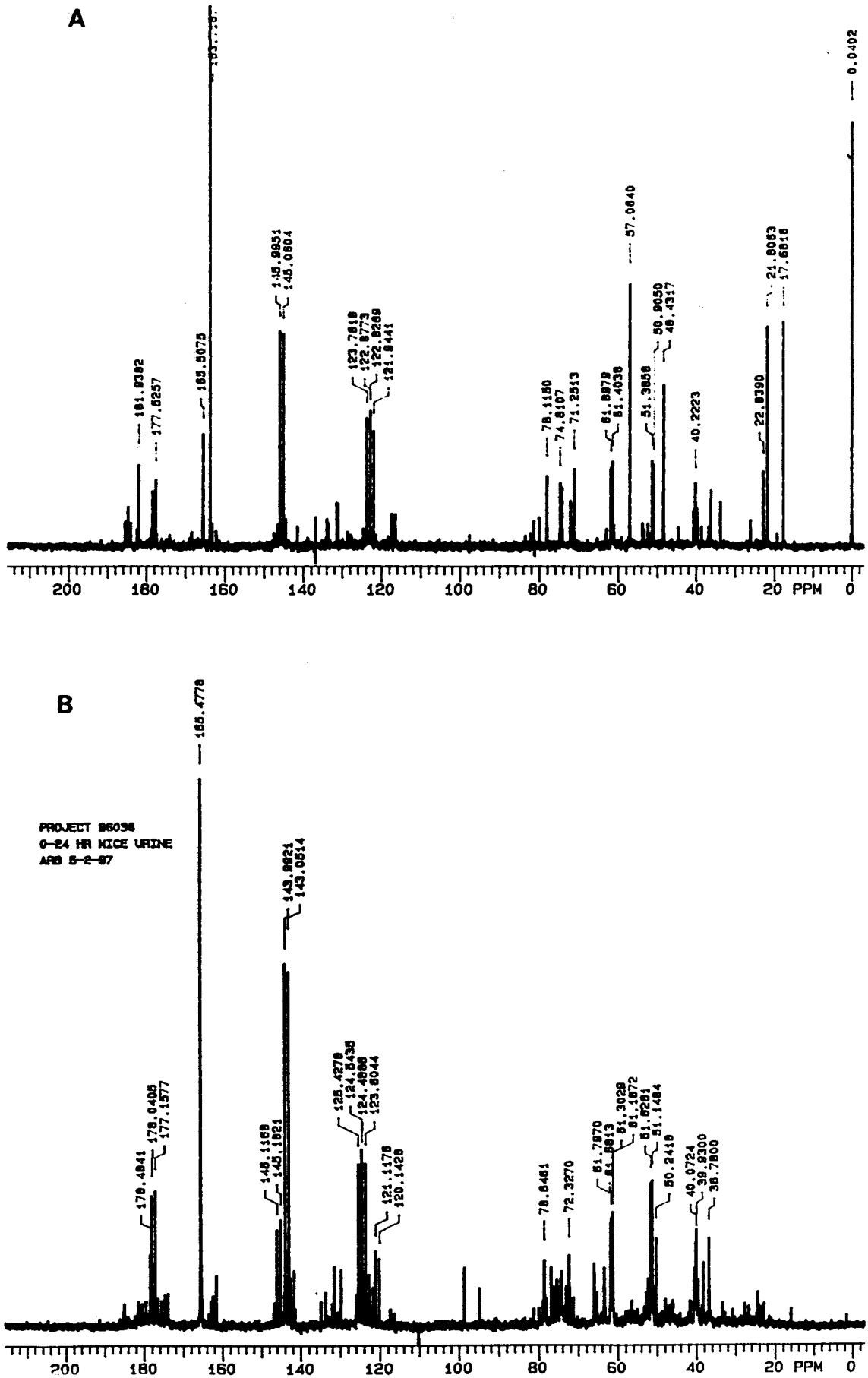


Figure 16. ^{1}H-decoupled ^{13}C NMR spectra of urine, collected for 24 h after administration of 40 mg/kg [1,2,3-^{13}C]PA from rats (A) and mice (B).

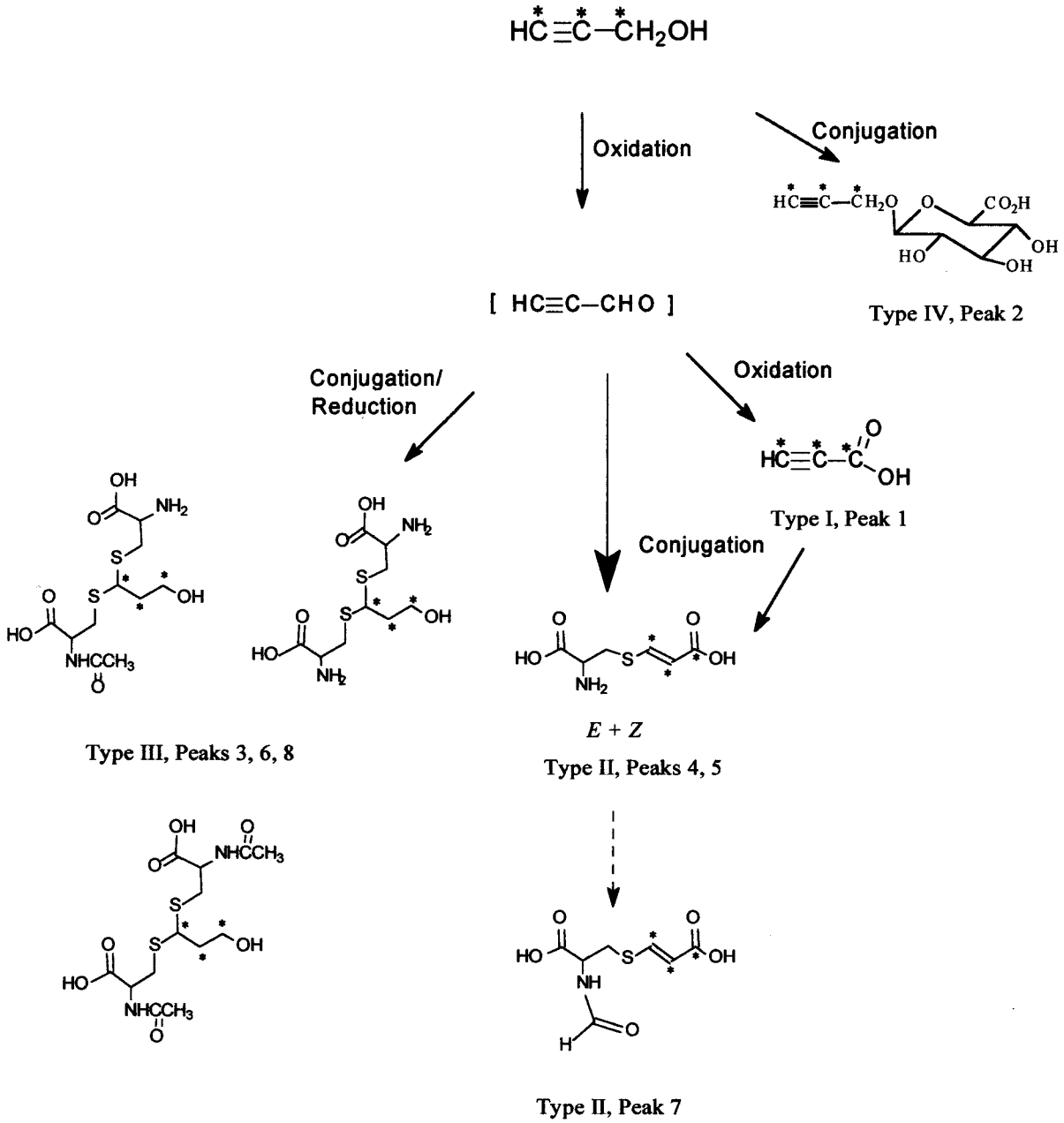


Figure 17. Pathway for the metabolism of PA in mice. An asterisk denotes the position of the ^{13}C label. The structure in brackets is the proposed intermediate. Peak numbers correspond to metabolites separated by HPLC.

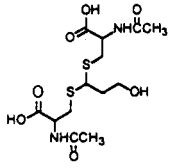
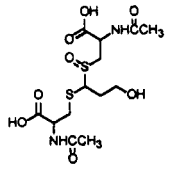
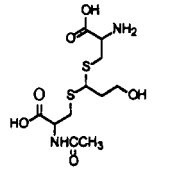
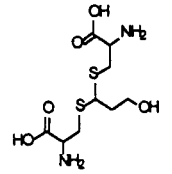
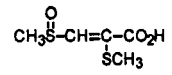
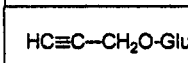
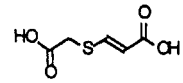
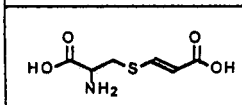
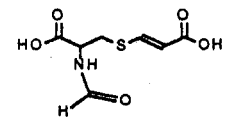
1999) and the other contained cysteinyl (Scheme 2). The chemical shifts for the synthetic standards, (*E*+*Z*)-3-[(2-amino-2-carboxyethyl)thio]-2-propenoic acid, and calculated values (ACD/Labs) are consistent with the experimental shift values for this metabolite.

Thus, peaks 4 and 5 were identified as (*E*+*Z*)-3-[(2-amino-2-carboxyethyl)thio]-2-propenoic acid, formed as shown in Scheme 3. Peaks 4 and 5 together accounted for ~41% TRR in 0−24 h urine.

HPLC Peak 6 (Type III Metabolite). The positive ion ESI LC/MS of mouse urine showed a match of retention time between the RAM trace (peak 6) and the $[M + H]^+$ ion at *m/z* 344, indicating a metabolite with a molecular weight of 343, 42 mass units higher than for peak 3 (Figure 7). The daughter ion spectrum of *m/z* 344 showed prominent fragment ions at *m/z* 223 and 181 resulting from the loss of cysteine (*m/z* 121) and acetylcysteine (*m/z* 163) from the molecular ion peak (Figure 12). The ^{13}C NMR chemical shifts of this metabolite were consistent with type III mtabolites. At least two type III metabolites were resolved in the ^{13}C NMR spectrum (Figure 8). Thus, peak 6 was identified as 3-[[2-(acetylamino)-2-carboxyethyl]thio]-3-[(2-amino-2-carboxyethyl)thio]1-propanol, formed from peak 3 (see Scheme 1) and accounted for ~17% TRR in 0−24 h urine.

HPLC Peak 7 (Type II Metabolite). The positive ion ESI LC/MS of this peak showed an $[M + H]^+$ ion at *m/z* 223, giving a molecular weight of 222, 28 mass units higher than for peaks 4 and 5 (Figure 9). The daughter ion spectrum of the parent peak with *m/z* 223 (Figure 13) showed characteristic ion peaks at 176 due to the neutral loss of $H^{13}CO_2H$ (47 *m/z*) and at 75 *m/z*, attributed to $H^{13}C{=}{}^{13}CH^{13}CO_2H$. The base peak in the spectrum at *m/z* 100 resulted from the loss of *m/z* 123 from the molecular ion peak and was assigned to the $[N{\equiv}CCH_2{-}S{-}H^{13}C{=}{}^{13}CH]^+$ moiety. The expanded region of the ^{13}C NMR spectrum of mouse urine showed

Table 2. Structure, Chemical Name, and Percent Total Radioactive Residue Attributed to Each Metabolite in Rat and Mouse Urine

STRUCTURE	CHEMICAL NAME	% TRR	
		Rat	Mouse
	3,3-bis[(2-(acetylamino)-2-carboxyethyl)thio]-1-propanol	20	6
	3-[[2-(acetylamino)-2-carboxyethyl]sulfinyl]-3-[[2-(acetylamino)-2-carboxyethyl]-thio]1-propanol	15	0
	3-[[2-(acetylamino)-2-carboxyethyl]thio]-3-[[2-(amino)-2-carboxyethyl]thio]1-propanol	8	17
	3,3-bis[(2-(amino)-2-carboxyethyl)thio]-1-propanol	0	15
	2-(methylsulfinyl)-3-(methylthio)-2-propenoic acid	7	0
	Propargyl alcohol-glucuronide	0	6
	3-(carboxymethylthio)-2-propenoic acid	20	0
	(E+ Z)- 3-[(2-amino-2-carboxyethyl)thio]-2-propenoic acid	0	41
	3-[(2-formylamino-2-carboxyethy)thio]-2-propenoic acid	0	13

multiplets adjacent to the signals attributed to peaks 4 and 5 (Figure 11). Small changes in the chemical shifts of carbons derived from the ^{13}C portion of PA compared with peaks 4 and 5 may be attributed to attachment of formylcysteinyl versus cysteinyl conjugation. The derivation of formylated cysteine is unknown. It is possible that it is a further metabolic product of the glutathione conjugate ($GSH-CH=CHCO_2H$) after degradation of

GSH to GLU−CYS or formed by formylation of the amino group of the cysteinyl moiety of peaks 4 and 5 (Scheme 3). Thus, on the basis of NMR and mass spectrometry, this metabolite was identified as 3-[(2-formylamino-2-carboxyethyl)thio]-2-propenoic acid This peak accounted for ~13% TRR in mouse urine.

HPLC Peak 8 (Type III Metabolite). 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 daughter spectrum of the peak with *m/z* 386 (Figure 14) showed characteristic ion peaks at *m/z* 223 due to the loss of acetylcysteinyl (*m/z* 163) and at *m/z* 191 resulting from the subsequent neutral loss of the $^{13}CH_2OH$ (*m/z* 32) portion of the PA moiety. The positions of the acetylcysteinyl moieties in the conjugate was determined from ^{13}C NMR, as described for peak 3, and by comparison with a synthetic reference compound (Banijamali at al., 1999). This metabolite, identified as 3,3-bis[(2-(acetylamino)-2-carboxyethyl)thio]-1-propanol, was formed from peak 6 (Scheme 1) and accounted for ~6% TRR in 0−24 h urine.

Other Metabolites. In addition to the assigned metabolites, multiplet signals with low intensity are present in NMR spectra of urine or concentrated urine that indicated the formation of additional metabolites. These signals may be attributed to the cysteinyl derivative, *N*-acetylcysteinyl derivative, *cis/trans* isomers, and/or diastereomers. A doublet near 120 ppm may suggest $H_2C{=}C(SR){-}COOH$-type metabolites (i.e., addition to the center carbon of the PA). These additional peaks constitute a small percentage of urinary metabolites derived from PA, as judged by their relative intensities.

Comparison of Radiochromatograms and NMR Spectra for Mouse and Rat Urine. The HPLC radiochromatograms of mouse and rat urine collected 24 h following administration of PA [using a strong anion-exchange (SAX) column] are shown in Figure 15. Retention times and percent relative abundance of each peak are shown in the chromatograms. The radiochromatograms were similar and suggested the same types of metabolites for the two species.

The ^{13}C NMR spectra of rat and mouse urine (Figure 16) have signals consistent with type I, type II, and type III metabolites. However, differences in the exact shift positions indicate differences in the specific identity for metabolites in the type II and type III families. Different relative intensities for signals in mouse urine, as compared with rat urine, suggest a quantitative species effect on metabolism.

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 by characterization of metabolites in mouse urine. Metabolites were identified, directly, in the whole urine by ^{13}C NMR and mass spectrometry and in some cases by comparison of the spectral data with those of synthesized reference compounds.

Two pathways of metabolism are proposed for PA in the mouse that include direct glucuronide conjugation of PA and oxidation of PA to the hypothetical intermediate 2-propynal (Figure 17). The aldehyde undergoes conjugation with glutathione and reduction to yield the type III metabolites: 3,3-bis[(2-acetylamino-2-carboxyethyl)thio]-1-propanol, 3-[[2-(acetylamino-2-carboxyethyl)thio]-3-[(2-amino-2-carboxyethyl)thio]-1-propanol, 3,3-bis[(2-amino-2-carboxyethyl)thio]-1-propanol. A small portion of the aldehyde is subsequently oxidized to result in the excretion of 2-propynoic acid. Type II metabolites (*E*+*Z*)-3-[(2-amino-2-carboxyethyl)thio]-2-propenoic acid and 3-[(2-formylamino-2-carboxyethyl)-thio]-2-propenoic acid are also excreted in the urine and could be formed either after direct GSH conjugation with 2-propynoic acid or after glutathione conjugation with aldehyde followed by oxidation to carboxylic acids. The mechanism appears to involve Michael addition at the terminal carbon.

Quantitative comparison of metabolites in rats and mice are shown in Table 2. Rats (43%) and mice (38%) excrete similar percentages of the dose (40 mg/kg) via GSH diconjugation/reduction of the reactive aldehyde intermediate (type III metabolites). The percentage of type I metabolite, 2-propynoic acid, was substantially higher in rat (27%) than in mouse (2%), indicating a higher capacity for oxidation of the PA aldehyde to propynoic acid in rats than in mice, whereas mice have a higher capacity for GSH monoconjugation/oxidation of the PA aldehyde (type II metabolites: 54% mice; 27% rats). However, it is also possible that the type II metabolites are formed following oxidation of the aldehyde to 2-propynoic acid followed by conjugation. This being the case, the flux through oxidation followed by GSH conjugation is similar between rats (54%) and mice (54%). A higher percentage of type II metabolites and a lower percentage of 2-propynoic acid for the mice, as compared with rats, may result from mice having a lower K_m and/or higher V_{max} for GSH conjugation with 2-propynoic acid. Another possibility is that the lower hepatic concentration of glutathione *S*-transferases in rats than in mice (Mannervik and Jemth, 1999) results in less conjugation with GSH and more aldehyde in rats being available to be oxidized to 2-propynoic acid.

A major difference between the metabolism of PA by rats and mice is the disposition of the cysteinyl conjugate of propynoic acid. In rats the amino group appears to undergo oxidative deamination or transamination to form the thiopyruvate intermediate, which is oxidized to thioacetate (20% of urinary total radioactive residue). In mice the cysteinyl conjugate is either unchanged (41%) or N-formylated (13%). The formation of a glucuronide conjugate of the parent compound was evident only in urine from mice (6%).

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