Structural Characterization of [^{14}C]Propargite Metabolites in Goat Urine by High-Resolution FT-NMR and Mass Spectrometry

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Urinary metabolites of [*phenyl*-U-^{14}C]propargite, 2-[4-(1,1-dimethylethyl)phenoxy]cyclohexyl-2-propynyl sulfite, a nonsystemic acaricide, in a lactating goat were identified. The goat received three consecutive daily doses equivalent to 340 ppm of propargite in the daily diet. The animal was sacrificed 8 h after the last dosing. A major route of elimination was found to be via the urine. Five major metabolites were isolated and characterized by FT-NMR and mass spectrometry. These compounds resulted from the hydrolysis of propynyl sulfite and subsequent hydroxylatioin of the *tert*-butyl portion of the molecule. Additional metabolites were formed by further oxidation or sulfation of the *tert*-butyl portion of the molecule and oxidation of the cyclohexyl moiety. The latter reaction yielded different stereoisomers with identical HPLC retention times.

INTRODUCTION

Propargite, 2-[4-(1,1-dimethylethyl)phenoxy]cyclohexyl-2-propynyl sulfite, is a nonsystemic acaricide with predominantly contact action and long residual activity developed by the Uniroyal Chemical Co. It is registered under the trade names Omite and Comite. Propargite is utilized to control many species of phytophagous mites (particularly motile stages) on a variety of crops such as vines, fruit trees (including citrus), hops, nuts, vegetables, ornamentals, cotton, figs, maize, cucumbers, groundnuts, and sorghum. The metabolic fate of [*phenyl*-^{14}C]propargite in laboratory rats after both a single dose and a subchronic oral dose was determined, and an extensive metabolism followed by a rapid elimination of the administered dose was observed (Banijamali and Tortora, 1988). To learn more about the comparative metabolism of this novel compound, [^{14}C]propargite was dosed orally to a lactating goat as described below and urine, feces, and milk were collected during the treatment period. Eight hours post last dose, the animal was sacrificed and tissue samples were collected. Approximately 16.5% of the administered dose was excreted in the urine and about 14% in the feces. Less than 1% and 0.1% of the administered dose were detected in the tissues and milk, respectively. About 67% of the administered dose is presumed to have remained in the gastrointestinal tract of the animal. This paper describes the isolation and identification of urinary metabolites of propargite.

EXPERIMENTAL PROCEDURES

Test Material. Propargite uniformly labeled with ^{14}C in the phenyl ring and having a specific activity of 30 mCi/mmol and a radiochemical purity of 98% was provided by Chemsyn Science Laboratories, Lenexa, KS. The radiolabeled material was diluted with cold propargite to a suitable specific activity (1 mCi/mmol) prior to dosing.

Animal Treatment and Sample Collection. To one female Alpine dairy goat (36 kg) were administered three consecutive daily doses of [^{14}C]propargite via a balling gun after each morning milking. Each dose contained approximately 22 mg of labeled and 652 mg of nonradioactive propargite. The dosed animal was maintained in a metabolism stall that allowed for the separate collection of urine and feces. Water and feed were provided ad libitum.

Urine was collected daily during the treatment period and the animal was sacrificed approximtely 8 h post last dose.

Analytical Methods. High-pressure liquid chromatography (HPLC) was performed with Waters instruments 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 a Berthold Model LB 506B radioactivity detector to monitor the HPLC column effluent. The analytical HPLC column was a C_8 IB-SIL, 5-μm, 150 × 4.6 mm (Phenomenex). The semipreparative HPLC column was a C_{18} Spherex, 5 μm, 250 × 10 mm (Phenomenex). Sample was applied by means of a Rheodyne Model 7125 injector equipped with a 200-μL injection loop. Data acquisition of the RAM and UV outputs was done by using Nelson Model 2600 software revision 5.1 (PE Nelson). The radioactivity on silica gel plates was visualized by using AMBIS Radioanalytic Imaging System 1125, with AST 286 computer and color probe software (V.1.74).

Radiocarbon was quantified in 10 mL of Scinti Verse II (Fisher) by dissolving an aliquot of the sample and counting in a Beckman Model LS 7500 liquid scintillation counter (LSC).

Nuclear magnetic resonance (NMR) spectra were obtained on a Varian XL-300 spectrometer. Metabolite samples (0.2–0.5 mg) dissolved in deuterium oxide (0.2 mL) were placed in 5-mm NMR tubes equipped with matched susceptibility plugs (Varian, part no. 00-969055-00) that allowed significant reduction of solvent volume (Varian, 1987). The residual H_2O protic solvent absorbances were used as internal references. Chemical shifts (δ) are expressed in parts per million.

Both electron ionization (EI) and chemical ionization (CI) mass spectra (MS) were obtained on a Finnigan 4500 GC/MS with a direct exposure probe (DEP) for sample introduction. The metabolites were placed as 1 μg/μL acetonitrile solutions on the probe. The solvent was evaporated before the sample was introduced into the MS. The spectrometer was set to scan up to 500 Da at a rate of 0.45 scans/s. All chemical ionizations were performed with ammonia as the reagent gas. Fast atom bombardment mass spectrometry (FABMS) analyses were performed with a Kratos MS890 double-focusing mass spectrometer using xenon gas for positive ion FABMS. The xenon gun voltage was set at 8 kV and the ion current at approximately 2 μA. Low-resolution FAB mass spectra were obtained at 1:1000 resolution. High-resolution FAB peak matching analysis was obtained at 1:9000 resolution. Calibration of low-resolution mass spectrometry data was accomplished by using cesium iodide positive ion clusters. Calibration of high-resolution peak matching was accomplished by using glycerol matrix ions and cesium iodide cluster ions as references. Tandem MS analyses for structural elucidation of selected parent ions were performed on the Kratos MS890 hybrid mass spectrometer in the EBQQ configuration. The collisionally induced dissociation of sample ions was promoted through the use of argon collision gas and a potential of

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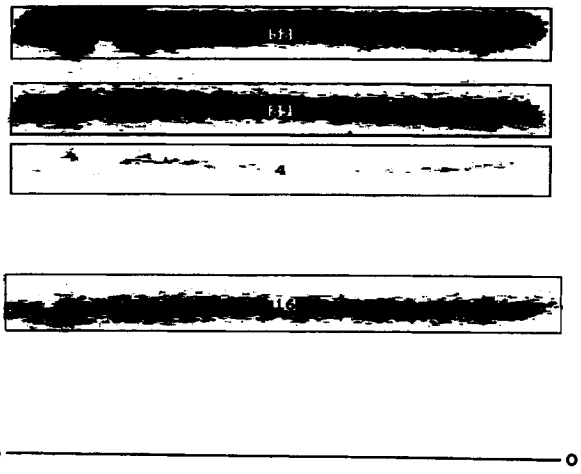


Figure 1. Radioautogram of goat urine metabolites separated on a silica gel TLC plate. Numbers in the bands indicate relative percentages of radioactivity as determined by the AMBIS system. The origin is marked by the letter O. The R_f values are 0.78 (band 1), 0.64 (band 2), 0.54 (band 3), and 0.29 (band 4).

20 eV. Samples of metabolites were dissolved in 1:2 methanol–glycerol for all FABMS analyses.

Extraction, Separation, and Purification of Goat Urinary Metabolites. LSC indicated that 16.5% of the applied dose was excreted in urine. Quantitative separation and purification of the urinary metabolites were achieved through a series of steps as follows.

1. Amberlite XAD-4. The preliminary extraction/cleanup of urinary metabolites consisted of adsorption onto activated Amberlite XAD-4 resin. The resin was activated by extensive washing with acetone, methanol, and water prior to use. The pooled urine sample was diluted with water (1:1 ratio), mixed with (approximately 2 mL/g) resin, and stirred for about 8–10 h. After filtration, the resin was washed with water and dried at room temperature. Samples were eluted by washing with acetonitrile, methanol, and acetone.

2. TLC. Concentrated organic extracts from the resin (approximately 6 million dpm in 3 mL of methanol) were applied to each preparative silica gel plate (Whatman PLK5F, 20 × 20 cm, 1000 μm) and developed in methanol–chloroform (30:70 by volume). Four distinct zones of radioactivity were distinguished by radioautography as shown in Figure 1. The plates were sprayed with water, and the radioactive bands were carefully scraped and extracted by stirring with acetonitrile. Extracts were filtered and concentrated, and the radioactivity was quantitated by LSC.

3. HPLC. Quantitative separation and purification of urinary metabolites were achieved by applying concentrated aqueous solutions of each of the TLC separated bands to a semipreparative HPLC column. Samples were chromatographed with a gradient system of ammonium formate (0.05 M, pH 6.5) and acetonitrile. The gradient was from 0 to 30% acetonitrile over 30 min (using curve profile 5) followed by a steep rise to 100% acetonitrile in 15 min (using curve profile 6) at a flow rate of 1.5 mL/min. The UV monitor was set at 238 nm, while the radioactivity monitor was set to 3K full scale. All runs were at ambient temperature. The individual metabolite peaks were collected, concentrated, and rechromatographed by HPLC (using water as solvent A) to obtain samples of high purity for spectral studies.

Methanolysis. To metabolite II (about 5 μg) in a test tube was added anhydrous methanolic HCl (1 M, 0.5 mL) prepared as described (Tang and Crone, 1989). The test tube was capped and incubated at 60 °C for 5 min. The solvent was evaporated under nitrogen and the residue dissolved in water for HPLC analysis. The radiochromatogram clearly indicated the quantitative disappearance of the sulfate conjugate (metabolite II) and formation of the hydrolysis product, 1-[4-(1,1-dimethyl-2-hydroxyethyl)phenoxy]-2,*x*-cyclohexanediol.

RESULTS AND DISCUSSION

Distribution of Radioactivity in Urine. The HPLC radiochromatogram of a urine sample before extraction indicated the presence of six radioactive peaks. The retention time and percent abundance for each peak are shown in Figure 2. With the HPLC conditions used, the metabolites were eluted in the order of decreasing polarity (i.e., the most polar metabolite eluted first). The absence of [14C]propargite in the urine indicated extensive propargite metabolism to more polar and more readily excretable components. (Propargite elutes with a retention time of about 40 min in this HPLC system.)

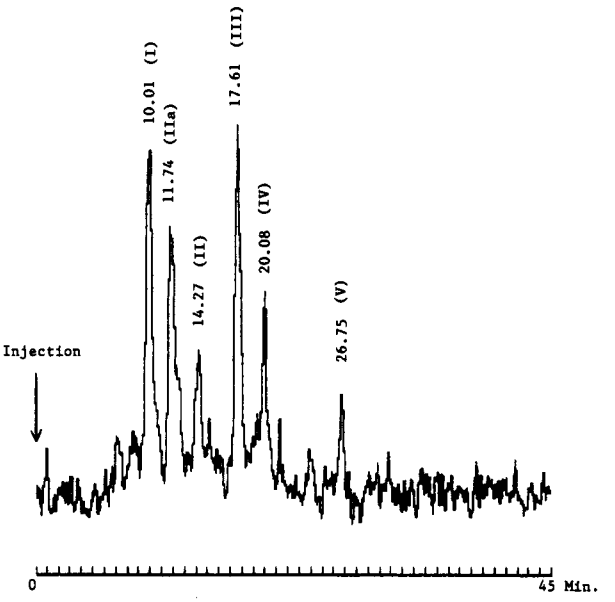


Figure 2. HPLC radiochromatogram of propargite metabolites in goat urine. Metabolite numbers and retention times are shown. The relative abundances in percent as determined by the PE Nelson system are I, 28%; IIa, 23%; II, 7%; III, 23%; IV, 12%; V, 6.5%.

Isolation and Purification of Urinary Metabolites. Over 95% of the radioactivity in the urine was adsorbed onto Amberlite XAD-4 resin and subsequently eluted from the resin by washing with acetonitrile, methanol, and acetone. These concentrated extracts were then subjected to preparative TLC. Areas of the preparative TLC plates corresponding to the radioactive bands were removed, and radioactive materials were eluted from the silica gel with acetonitrile. HPLC radiochromatograms of the bands separated by TLC demonstrated the effectiveness of the prepurification procedure. HPLC revealed the presence of two radioactive peaks in band 1 (tr = 17.61 and 20.08 min), one radioactive peak in band 2 (tr = 10.01 min), one radioactive peak of relatively low abundance in band 3 (tr = 26.75 min), and one radioactive peak in band 4 (tr = 14.27 min). Comparison of the relative abundance of radioactive peaks on HPLC with their corresponding radioactive bands on TLC suggested the conversion of unstable metabolite IIa to either metabolite II or metabolite IV upon TLC purification.

The products obtained from the initial preparative TLC separation required additional cleanup and further chromatographic separation, to remove unlabeled contaminants, prior to spectral analyses. Subjecting each band to a second preparative TLC and subsequent purification on HPLC gave samples of metabolites in high purity suitable for spectral analyses. For metabolites I–IV, between 0.5 and 1 mg of each was obtained. Metabolite V (tr = 26.75 min), found in TLC band 3 (Figure 1), was the least abundant in goat urine (about 100 μg).

Characterization of the Urinary Metabolites. The identification of propargite urinary metabolites was achieved by high-resolution NMR and mass spectrometry.

NMR. The metabolites gave preliminary ^{1}H NMR spectra similar to that of propargite glycol ether, a

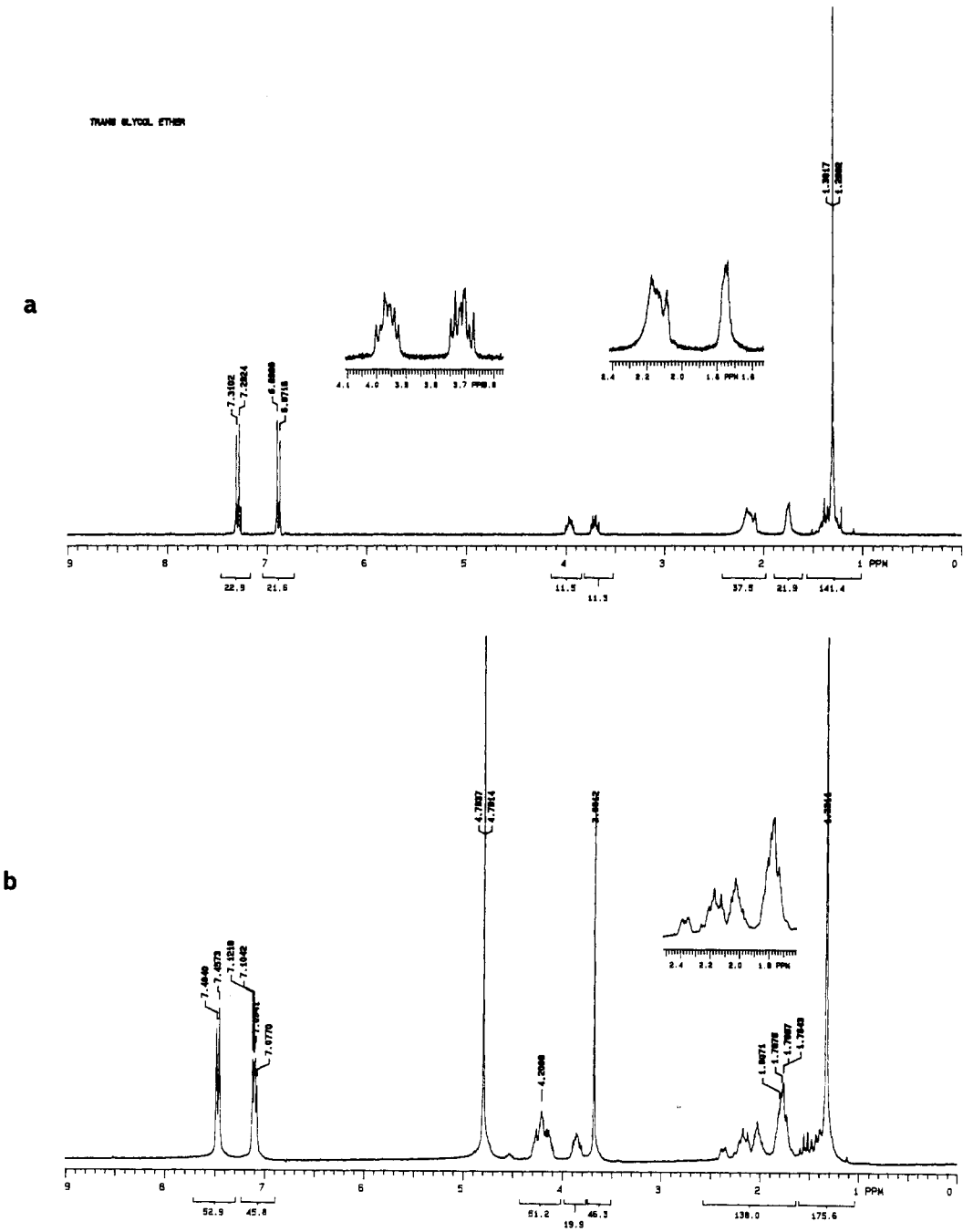


Figure 3. ^{1}H NMR (300 MHz) spectra of *trans*-propargite glycol ether (a) and metabolite IV (b).

breakdown product of propargite formed by hydrolysis of its propynyl sulfite chain (Figure 3). In general, integration of four protons in the aromatic region of these metabolites eliminated the possibility of aromatic substitution (e.g., phenol formation). The aromatic protons appeared as two doublets centered at δ 7.1 and 7.5, a characteristic of paradisubstitution. Hydroxylation of the cyclohexyl ring was supported by changes in both integration and chemical shifts of protons and carbons in the environment where hydroxylation had occurred. Hydroxylation of the cyclohexyl ring can produce different stereoisomers because the hydroxyl groups can assume either axial or equatorial conformations. Since HPLC retention times would remain unchanged, mixtures could be found in each peak. The complexity and multiplicity of the NMR signals corresponding to cyclohexyl protons and carbons suggest the production of isomeric mixtures that, in turn, prevent the determination of the position of the hydroxyl group and its stereochemistry on the ring. Therefore, the NMR spectra presented for metabolites hydroxylated on the cyclohexyl ring (metabolites I, II, and IV) represent more than one isomer. The peak for the *tert*-butyl group at δ 1.2, which appears as a broad singlet and integrates for nine protons in the ^{1}H NMR of propargite glycol ether, integrated for only six protons with the metabolites. Thus, with each metabolite, one of the *tert*-butyl methyl groups had been metabolized to another structure.

Mass Spectrometry. Mass spectrometry provided molecular weights and some structural information on urinary metabolites. All of the metabolites, with the exception of metabolite II, produced molecular ions under electron impact (EI) conditions and ammonium adduct ions with chemical ionization (CI). Metabolite II did not provide solid probe EI or CI ion current due to its low vapor pressure. Fast atom bombardment (FAB) analyses of metabolite II produced ion species corresponding to hydrogen, sodium, and glycerol adducts. These results are described in detail below.

Since NMR spectra indicated that for all the metabolites one of the *tert*-butyl methyl groups had been modified,

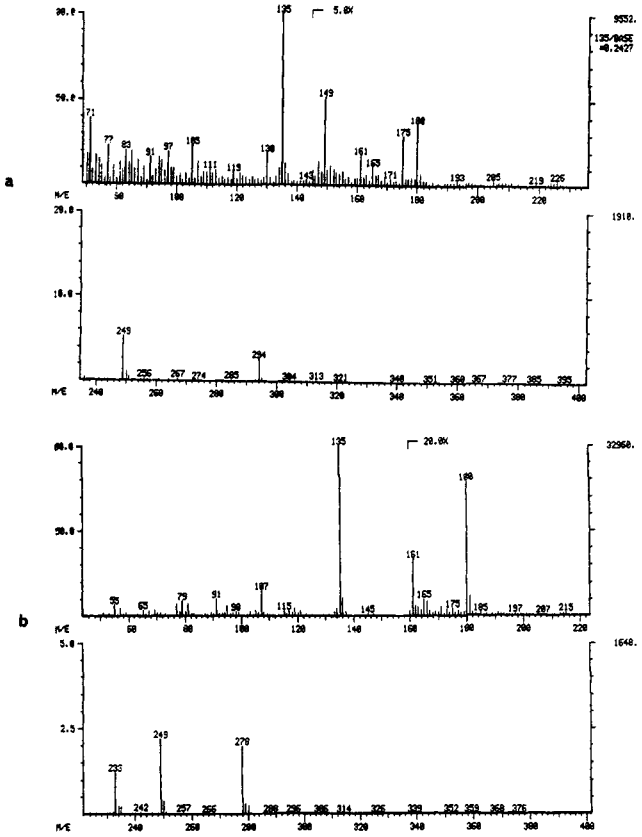
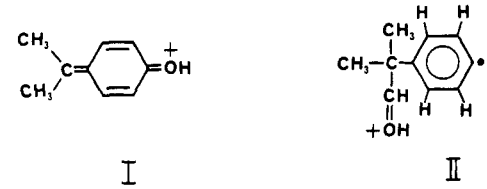


Figure 4. EI mass spectra of metabolite I (a) and metabolite III (b) obtained by direct-insertion probe.

the four metabolites analyzed by EI could be classified into two groups: two metabolites as well as the hydrolysis product of metabolite II had a hydroxyl group (alcohols) and two had a carboxyl group. The molecular weights of the alcohols differed by 16 mass units (264 and 280 m/z). They gave ions corresponding to M, M – H_2O, M – CH_2-OH and at 148 m/z (see below). The molecular weights of the acids also differed by 16 mass units (278 and 294 m/z). The two acids produced characteristic ion species resulting from the loss of COOH and at 135 m/z as described below. None of the CI mass spectra contained MH^+ adduct ions due to the high proton affinity (PA) of ammonia compared to the alcohols and acids (Harrison, 1983). All of the CI spectra contained M + NH_4 adduct ions. These studies established the molecular weights of metabolites I–V as 294, 382, 278, 280, and 264 m/z, respectively.

Identification of Metabolites. *Metabolites I and III.* The EIMS of metabolites I and III shown in Figure 4 displayed the highest molecular ion peaks at 294 and 278 m/z, respectively, as indicated by the following evidence. A significant peak at 45 m/z lower than the molecular ion peak, characteristic of loss of carboxylic acid, was evident at 249 m/z and at 233 m/z for metabolites I and III, respectively. The base peak at 135 m/z in both spectra was attributed to the loss of both carboxylic acid and cyclohexyl groups and subsequent formation of the isopropenylphenol cation (Figure 5, structure I). This ion is known to be the base peak in the EI spectrum of *p-tert*-butylphenol (Heller and Milne, 1978). In addition, the positive ion CIMS of these metabolites with an ammonium adduct ion peak for metabolite I at 312 m/z and at 296 m/z for metabolite III (spectra are not shown) provided further evidence for the molecular weight assignments of these metabolites.

The most characteristic feature in ^1H NMR spectra of these metabolites was found to be integration of six H's



I II

Figure 5. Structures of the base peaks in EIMS of metabolites.

at the *tert*-butyl group region of the spectra. Furthermore, the methylene group singlet peak at δ 3.85 in metabolite IV (Figure 3) and at δ 4.1 in metabolite II (Figure 6) was absent in ^1H NMR spectra of metabolites I and III. In addition, the ^{13}C NMR spectra of these metabolites as shown in Figure 7 for metabolite I gave a low-resonance signal at 190 ppm characteristic of a carbonyl type of carbon. Thus, these metabolites were identified as 1-[4-(2,*x*-dihydroxycyclohexoxy)phenyl]-2,2-dimethylacetic acid (metabolite I) and 1-[4-(2-hydroxycyclohexoxy)phenyl]-2,2-dimethylacetic acid (metabolite III).

Metabolites IV and V. The EIMS shown in Figure 8 displayed the highest molecular ion peak at 280 m/z for metabolite IV and at 264 m/z for metabolite V as indicated by the following evidence. In both spectra, a significant peak at 31 m/z lower than the molecular ion peak was evident. This was attributed to the loss of CH_2OH from the parent compound and subsequent formation of the stable tertiary carbonium ion peak at 249 m/z and at 233 m/z for metabolites IV and V, respectively. The base peak in the spectra of these metabolites occurred at 148 m/z. A precursor ion tandem mass spectrometry (parent MS/MS) experiment performed on metabolite IV confirmed the origin of the 148 m/z ion as the 262 m/z ion which in turn resulted from the neutral loss of water from the molecular ion. Since primary and secondary alcohols are capable of losing water, this loss could occur at either the *tert*-butyl or the cyclohexyl group. Both metabolites showed the loss of two water molecules: 244 m/z for metabolite IV and 288 m/z for metabolite V. The low abundance of the M – 2(H_2O) ion in the spectrum of metabolite V as compared to the same loss in metabolite IV would indicate an unfavorability of the loss of water from the *tert*-butyl group. It is therefore proposed that the neutral loss of water forming the 262 m/z ion in metabolite IV is from the cyclohexane ring and that the 148 daughter ions, from the 262 m/z ion, contain the hydroxy moiety on the *tert*-butyl group. The proposed structure of this fragment is hydroxylated *tert*-butylbenzene as a distonic radical cation (Figure 5, structure II). Further support for the molecular weight determinations of these metabolites was obtained from their CIMS studies (spectra not shown), which revealed ion species for metabolites IV and V at 298 and 282 m/z corresponding to ammonium adduct formation (M + NH_4) and at 280 and 264 m/z resulting from M + NH_4 – H_2O, respectively.

The ^1H NMR spectrum of metabolite IV (Figure 3) revealed the presence of a characteristic singlet resonance at δ 3.85 integrating for two protons. This peak arises as a result of aliphatic hydroxylation of a *tert*-butyl methyl group. Further hydroxylation on the cyclohexyl ring was evident from the changes in both integration and the chemical shift of that moiety. On the basis of this information, metabolite IV was identified as 1-[4-(1,1-dimethyl-2-hydroxyethyl)phenoxy]-2,*x*-cyclohexanediol.

Due to the relatively low abundance of metabolite I in the goat urine, NMR experiments could not be performed. However, by use of the MS information stated above, this metabolite is proposed to be 1-[4-(1,1-dimethyl-2-hydroxyethyl)phenoxy]-2-cyclohexanol.

Metabolite II. Low-resolution FABMS of metabolite

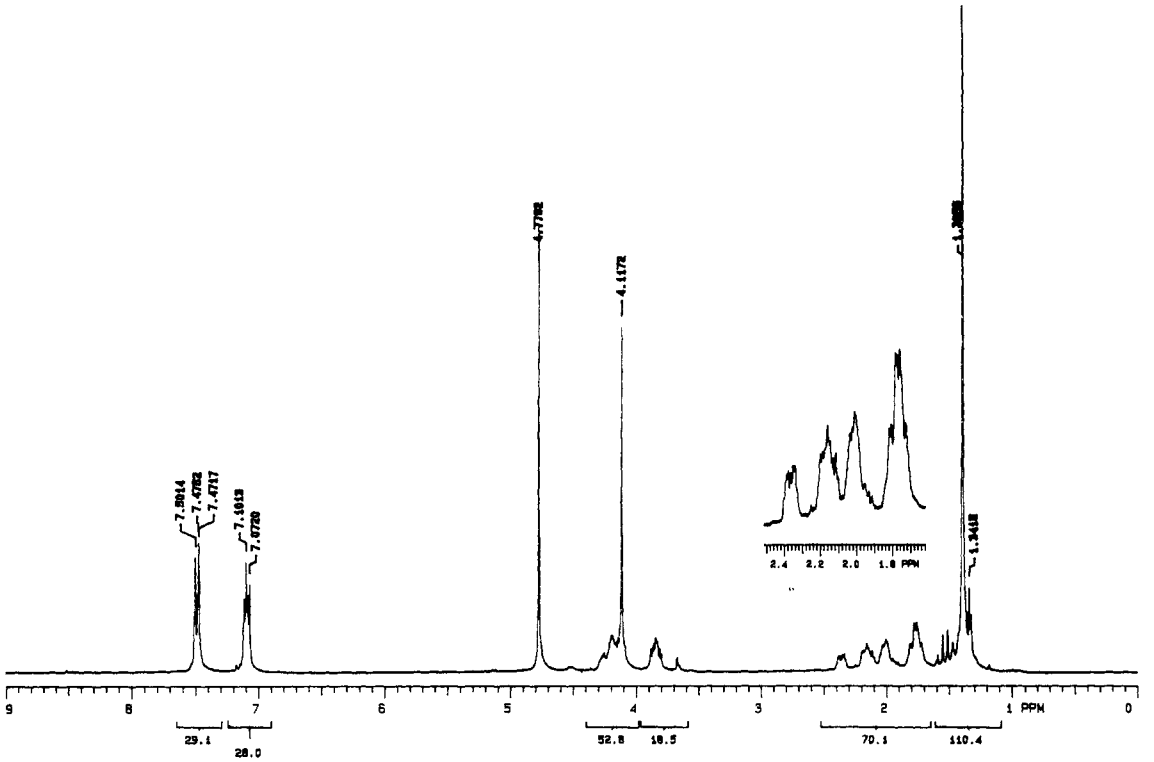


Figure 6. ^1H NMR (300 MHz) spectrum of metabolite II.

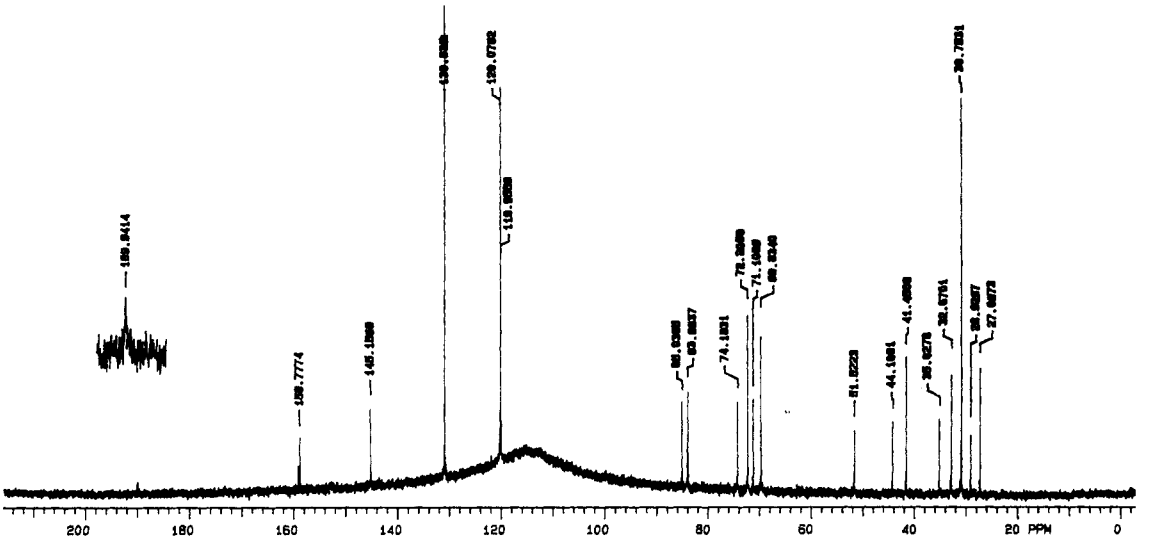


Figure 7. ^{13}C NMR (300 MHz) spectrum of metabolite I.

II (Figure 9) displayed a prominent ion at 383 m/z. Sodium, potassium, and glycerol adducts were observed at 405 m/z, 421 m/z, and 475 m/z, respectively, indicating the molecular weight of metabolite II is 382. Significant glycerol FABMS matrix ions were observed at 185 m/z (protonated glycerol dimer), 207 m/z (glycerol dimer–sodium adduct), and 223 m/z (glycerol dimer–potassium adduct). The ether cleavage is observed at the 251 m/z ion as is the [M – SO_4Na] ion at 263 m/z and the adduct ion at 497 m/z assigned to M + glycerol + sodium, which was formed in the FABMS source. The proposed MH^+ ion at 383 m/z (Figure 9) was accurately mass measured by peak matching at a resolution of 9000. The theoretical molecular weight for $C_{16}H_{23}O_7SNa$ (protonated) is 383.1138 m/z. The mass of protonated metabolite II was found to be 383.1125, which represents an error of 3 ppm. In addition, the hydrolysis product of metabolite II was subjected to EI and ammonia CI solid probe analysis. The predicted mass of the hydrolysis product, 280 m/z, was obtained. In addition, peaks indicative of a characteristic loss of methanol and water from the hydrolysis product were also observed.

The ^1H NMR spectrum of metabolite II is shown in Figure 6. Similar to metabolite IV, integration of the cyclohexyl ring protons clearly revealed a hydroxylation at this group. The presence of a relatively downfield singlet at δ 4.1 integrating for two protons, the most distinct feature of this metabolite, was indicative of attachment of the *tert*-butyl methyl group to a stronger electron withdrawing group than that of metabolites IV and V. This was found to be an acid sulfate sodium salt. The corresponding methylene peak after hydrolysis of sulfate was shifted about δ 0.25 upfield (3.85 ppm) to give a spectrum identical with that of metabolite IV. Thus, metabolite II was identified as 1-[4-(2,*x*-dihydroxycyclohexoxy)phenyl]-2,2-dimethyl sodium sulfate.

CONCLUSION

When propargite is given to a goat, it is degraded through a primary metabolic pathway involving hydrolysis of the

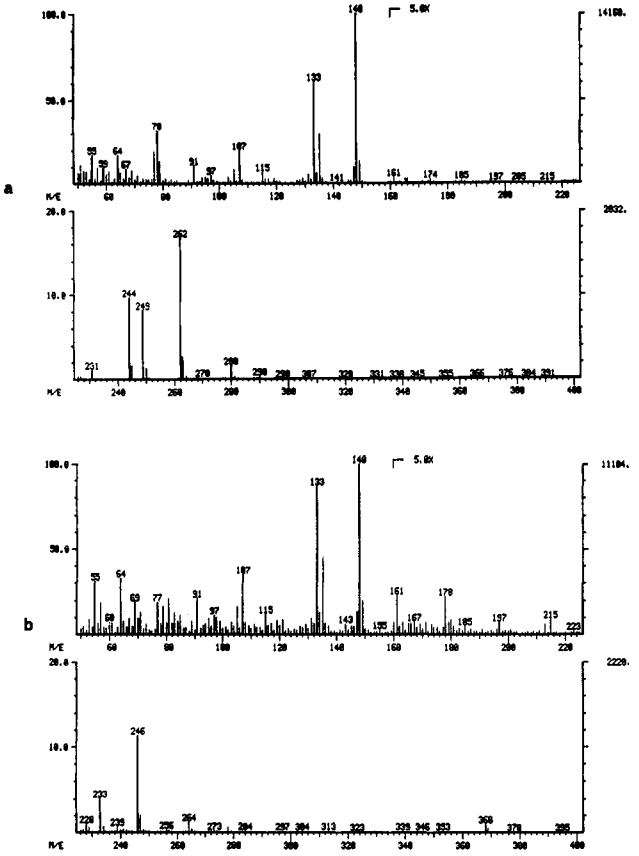


Figure 8. EI mass spectra of metabolite IV (a) and metabolite V (b) by direct-insertion probe.

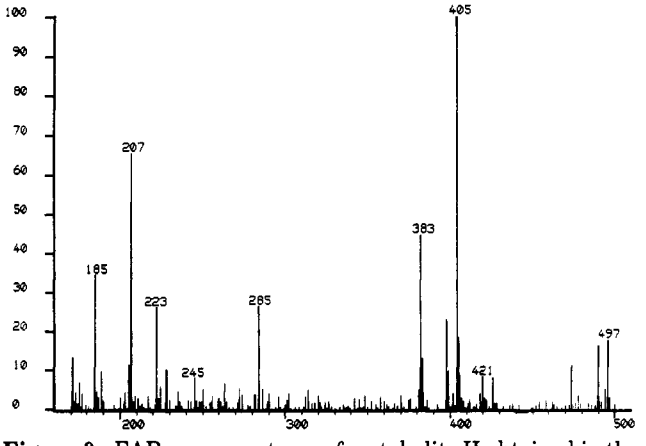


Figure 9. FAB mass spectrum of metabolite II obtained in the presence of glycerol.

propynyl sulfite side chain followed by aliphatic hydroxylation of the *tert*-butyl methyl group to form metabolite V. Subsequent hydroxylation at the cyclohexyl moiety of metabolite V yields metabolite IV as a mixture of axial/equatorial hydroxy isomers with identical HPLC retention times. This metabolite in turn undergoes sulfate conjugation to produce metabolite II, whose presence was verified by NMR, MS, and hydrolysis to form metabolite IV. Metabolite V can also undergo further oxidation of the hydroxylated *tert*-butyl group to produce the corresponding acid metabolite III. The most polar propargite urinary metabolite, metabolite I, was formed either by a second hydroxylation of metabolite III on the cyclohexyl ring or by further oxidation of metabolite IV at the hydroxylated *tert*-butyl group. The proposed pathway for the metabolism of propargite in the goat is shown in Figure 10.

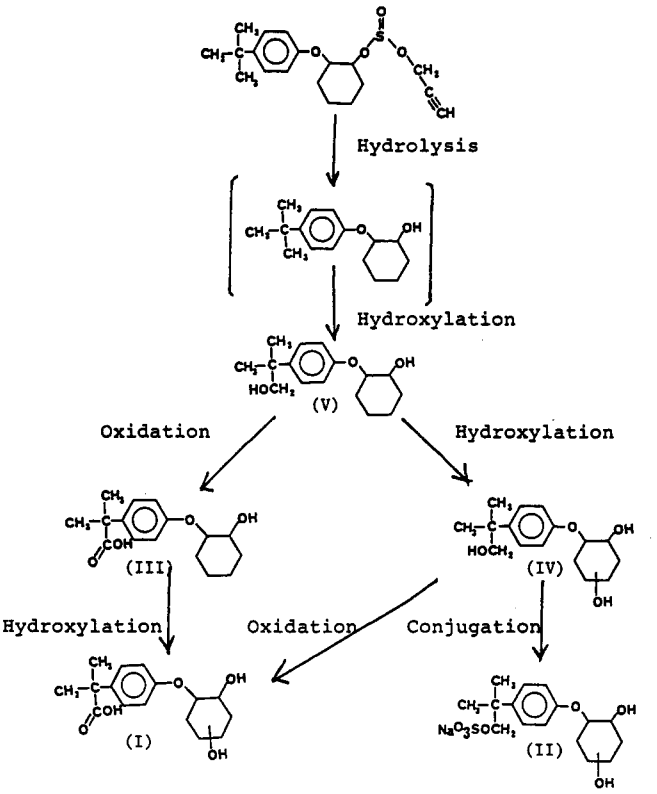


Figure 10. Proposed pathway for metabolism of propargite in goat.

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