Ovine Urinary Metabolites of Hymenoxon, a Toxic Sesquiterpene Lactone Isolated from *Hymenoxys odorata* DC

Martin K. Terry, Howard G. Williams, Hyeong L. Kim,* Lynn O. Post, and E. Murl Bailey, Jr.

A urinary metabolite of hymenoxon was isolated from both hymenoxon- and bitterweed-treated sheep. Three groups of sheep were administered by stomach tube an aqueous slurry of finely ground hymenoxon, a toxic sesquiterpene lactone isolated from bitterweed (*Hymenoxys odorata* DC.), at dosages of 50, 75, and 100 mg/kg. In addition, three groups of sheep were given equivalent dosages of hymenoxon by administering ground bitterweed (2.37% hymenoxon content) through rumen fistulas. Both feeding regimens gave an epimeric mixture of 4-desoxy-11,13-dihydrohymenoxon (3a and 3b) as major metabolites, which were excreted as their glucuronides (2a and 2b). The purification of 3a and 3b by preparative gas chromatography resulted in the isolation of both of their dehydration products, 4a and 4b. The structures of the metabolites 3a and 3b were deduced from the proton NMR spectrum of 4a.

Hymenoxon (1, $C_{15}H_{22}O_5$) is a toxic sesquiterpene lactone (Kim et al., 1975; Pettersen and Kim, 1976) isolated from *Hymenoxys odorata* DC. (bitterweed), which grows in the semiarid region of Texas. Bitterweed toxicity in sheep and goats has been known for a long time (Clawson, 1931), and poisoned sheep and goats are usually found from late December through early May during which time bitterweed is the only green plant available, especially in drought years. The toxicity of hymenoxon is essentially identical with that of bitterweed in sheep (Terry et al., 1981), and the toxicity of bitterweed depends on its hymenoxon content. The intraperitoneal and oral LD_{50}'s of hymenoxon in sheep are about 7 and 75 mg/kg, respectively (Kim et al., 1975; Terry et al., 1981). Reported herein is the isolation of two epimers of 2,3-dehydro-4-desoxy-11,13-dihydrohymenoxon, 4a and 4b, from the urine of sheep that were fed both hymenoxon and bitterweed as described by Terry et al. (1981).

EXPERIMENTAL SECTION

Instrumentation. Gas chromatography–mass spectrometry (GC–MS) was performed by a Hewlett-Packard Model 5992 instrument with data system using a 180 cm × 2 mm glass column packed with 3% OV-01 on Chromosorb W-HP (100–200 mesh) (column A). The injector temperature was 250 °C; the column temperature was initially maintained at 235 °C for 1 min and then was increased at 5 °C/min to 270 °C. The flow rate of the carrier gas, helium, was 30 mL/min. The ion monitored was at m/e 121.

Proton NMR spectra were recorded on a Varian XL-200 FT instrument, and high-resolution mass spectra were recorded on a Du Pont 21-110C double-focusing instrument.

Preparative GC separations were carried out on a Tracor Model 550 instrument using a 180 cm × 4 mm i.d. glass column packed with 5% OV-101 on Chromosorb G-HP (80–100 mesh) (column B). Injector and detector temperatures were 260 °C and the column temperature was kept at 190 °C. The flow rate of the nitrogen, carrier gas, was 60 mL/min. The eluates were collected in glass capillary tubes, 30 cm × 1.3 mm o.d., by using a Brownlee-Silverstein thermal gradient collector (Brownlee and Silverstein, 1968).

Treatment of Animals. Seventeen of twenty-eight mixed-breed wether lambs (Terry et al., 1981) weighing

Department of Veterinary Physiology and Pharmacology (M.K.T., H.L.K., L.O.P., and E.M.B.) and Department of Entomology (H.G.W.), Texas A&M University, College Station, Texas 77843.

23–34 kg were randomly divided into six groups. Three groups (three, three, and two sheep) were given finely ground hymenoxon as an aqueous slurry by stomach tube at dosages of 50, 75, and 100 mg/kg, respectively. The remaining three groups of three sheep each were given ground bitterweed (2.37% hymenoxon) through rumen fistulas at dosages of 1.90, 2.85, and 3.80 g/kg, respectively. Hymenoxon (mp 135.5–136.5 °C) was isolated from bitterweed (Kim et al., 1975) that was collected in June 1980 near San Angelo, TX. Urine samples were collected from each sheep at ambient temperature (20–30 °C) for 48 h following dosing with hymenoxon or bitterweed. The upper part of a screw-capped 3.8 L size plastic container such as a bleach bottle was cut and taped to each sheep and the collected urine was drained several times a day into individual collection bottles. The total volume of urine collected from each sheep was not determined accurately; however, it varied considerably (10 mL/2.0 L) probably due to the spillage and/or other reasons. Each urine sample was filtered through filter paper (Whatman No. 1) to remove the contaminated solid debris and kept frozen until the isolation of metabolites. Urine and bile samples were also collected from the bladder and gall bladder of the sheep that died (five hymenoxon fed and two bitterweed fed) at the time of necropsy, and they were stored in a freezer. No preservative has been added to urine or bile during collection or storage. Feces were collected from the treated sheep and dried at room temperature in a laboratory fume hood.

Isolation of Hymenoxon Metabolites. A urine sample collected at ambient temperature was extracted 3 times with volumes of ethyl acetate equal to 20% of the volume of the urine. The pooled ethyl acetate extract was dried in vacuo, leaving a dark red to brown oil, which was redissolved in a small volume of acetone.

The initial separation of urinary metabolites was accomplished by thick-layer (2.0-mm) silica gel plates, which were developed once in ethyl acetate. By GC–MS analysis of zonal scrapings, compounds of interest were located in a band, R_f 0.26–0.47. The scrapings of this band were eluted with acetone, which was concentrated to a small volume. This concentrated eluate was applied to thin-layer (0.25-mm) plates, which were developed twice in ethyl acetate. From these plates successive 1.0 cm wide bands were scraped, eluted with acetone, and analyzed by GC–MS to locate the sesquiterpene lactones or related compounds.

Four large GC peaks with same base peak (m/e 121) in their respective mass spectra were designated A (3a), B (3b), C, and D and their GC retention times on column A were 4.3, 4.6, 5.6, and 6.1 min, respectively. Further

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purification of the two predominant metabolites, A (**3a**) and B (**3b**), obtained from a band (R_f 0.64–0.72) in the thin-layer plates was accomplished by preparative GC preceded by a treatment with excess diazomethane–ether until the evolution of N_2 ceased. Preparative GC separation resulted in dehydration of **3a** and **3b**, yield **4a** and **4b** collected at 7.5–9.3 min and 9.3–10.0 min, respectively. **3a**: MS (70 eV) m/e 268.1 (M^+). **3b**: MS (70 eV) m/e 268.1 (M^+). **4a**: ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.20 (1 H, dd, J = 2.7, 6.2 Hz, H-3), 4.59 (1 H, dd, J = 1.7, 6.2 Hz, H-2), 1.98 (1 H, t, J = 2.2 Hz, H-1), 4.87 (1 H, m, H-8), 3.68 (1 H, d, J = 10.4 Hz, H-4a or H-4b), 3.43 (1 H, d, J = 10.4 Hz, H-4a or H-4b), 1.01 (3 H, s, C-5 methyl), 1.11 (3 H, d, J = 2.0 Hz, C-10 methyl), 1.27 (3 H, d, J = 7.6 Hz, C-11 methyl), 1.32 (1 H, d, J = 3.5 Hz, H-6a or H-6b), 1.39 (1 H, d, J = 3.5 Hz, H-6a or H-6b), 2.41 (1 H, m, H-7), 1.74 (1 H, m, H-9a or H-9b), 1.81 (1 H, m, H-9a or H-9b), 1.56 (1 H, m, H-10), 2.19 (1 H, m, H-11); MS (70 eV) m/e 250 (M^+: found, 250.1566; calcd, 250.1596 as $C_{15}H_{22}O_3$).

Effect of β-Glucuronidase on Urinary Metabolites. Prior to extraction, a 20-mL sample of urine from a bitterweed-treated sheep was diluted with 2 volumes of phosphate buffer (0.1 M, pH 6.8). Half of the buffered urine was treated with β-glucuronidase (Type IX from *Escherichia coli*, 2000 units/mL) for 24 h at 37 °C, while the other half was maintained at 0 °C without the enzyme during the 24-h incubation interval. Other urine samples were treated in the same manner. The effect of β-glucuronidase treatment on the number of sesquiterpene lactones and their relative abundances was determined by GC–MS after extraction with ethyl acetate.

**Analysis of Bile and Feces for Metabolites of Hymenoxon.** Bile (18 mL) was collected from the gallbladder of a sheep killed by bitterweed. This sample was first extracted with ethyl acetate, and then (after the evaporation of the residual ethyl acetate from the aqueous phase in vacuo) the aqueous portion was divided into two 9-mL aliquots. One of these aliquots was buffered with 2 volumes of phosphate buffer (0.1 M, pH 6.8) and incubated with β-glucuronidase (Type IX, 2000 units/mL) for 24 h at 37 °C. The other aliquot was likewise diluted with phthalate buffer (0.1 M, pH 5.0) and incubated with arylsulfatase (Type IV from limpets, 50 units/mL) for 24 h at 37 °C. Other bile samples were also treated in the same manner. The two enzyme-treated aliquots were then extracted with ethyl acetate, and the extracts were concentrated and analyzed for hymenoxon or hymenoxon metabolites by GC–MS.

Feces from a bitterweed-treated and a hymenoxon-fed sheep were air-dried and extracted with acetone or ethyl acetate in a Waring blender. The extracts were filtered, concentrated, and analyzed by GC–MS.

RESULTS AND DISCUSSION

Mortality occurred from 15 to 45 h after treatment among mid- and high-dose groups; two of three and two of two sheep died with hymenoxon doses of 75 and 100 mg/kg, respectively, and two of three sheep died with 3.80 g/kg ground bitterweed, as reported (Terry et al., 1981).

Four metabolites of hymenoxon were detected by GC–MS in the ethyl acetate extracts of urine samples collected at ambient temperature from hymenoxon- and bitterweed-treated sheep. A typical gas chromatogram is shown in Figure 1. Two pairs of GC peaks were present with the retention times on column A of 4.3–4.6 min (metabolites A and B) and 5.6–6.1 min (metabolites C and D). Hymenoxon showed a retention time of 5.1 min under the same GC conditions. All four metabolites and hymenoxon had common fragment ions in their respective mass
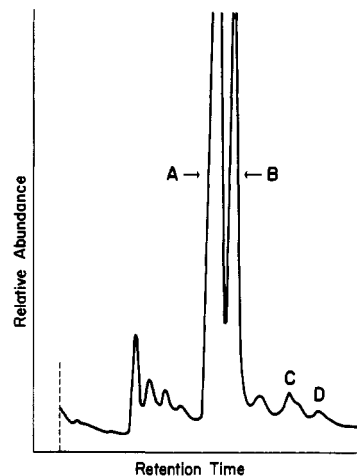
Figure 1. A typical chromatogram of the urinary extracts. Chromatographic conditions are described under Experimental Section, and the retention time of each metabolite is listed in Table I.

spectra. No hymenoxon was found in the extracts of urine, bile, and feces of hymenoxon- or bitterweed-treated sheep.

Two predominant metabolites, A (**3a**) and B (**3b**), were present (usually in a ratio of 2:1 to 3:1 on GC) in all the urine samples collected from both hymenoxon- and bitterweed-treated sheep following β-glucuronidase treatment, suggesting that they are excreted as glucuronides (Figure 1 and Table I). In urine samples collected from the bladder of sheep that died of bitterweed, or hymenoxon intoxication, neither metabolite A (**3a**) nor metabolite B (**3b**) was detected until the sample was treated with β-glucuronidase; such treatment increased the yield of metabolites from urine samples collected at ambient temperature. None of the above-mentioned urinary metabolites were detected in the extracts of bile or feces, before or after treatment with arylsulfatase or β-glucuronidase. The mass spectra of A (**3a**) and B (**3b**) were essentially identical (m/e 268, M^+). The separation of GC peaks of A (**3a**) and B (**3b**) by column B was achieved only after the ethyl acetate extract of urine was treated with excess diazomethane, which did not alter either the GC retention times or mass spectra on GC–MS (column A) but did sharpen trailing impurity peaks.

The separation of metabolites A (**3a**) and B (**3b**) by preparative GC, however, caused the dehydration of both A (**3a**) and B (**3b**). The molecular ion of the dehydrated A (**4a**) or B (**4b**) was found at m/e 250 and the GC retention time was 2.7 and 2.9 min, respectively. The dehydrated B (**4b**) sample collected by preparative GC was slightly contaminated with dehydrated A (**4a**) and its mass fragmentation pattern was essentially identical with that of dehydrated A (**4a**). The high-resolution MS of the dehydrated A (**4a**) gave the elemental composition of $C_{15}H_{22}O_3$. Metabolite A (**3a**) lost 1 mol of water under the preparative GC conditions employed. The presence of this dehydrated A (**4a**) has not been detected in the extracts of urine, bile, or feces before or after the treatment with β-glucuronidase.

The structure of the dehydration product of metabolite A shown in formula **4a** is based on the analysis of its proton NMR spectrum, which revealed the presence of an –O–CH=CH–CH– system. A low-field one-proton signal (H_3) at 6.20 (dd, J = 2.7, 6.2 Hz) is coupled to another one proton signal (H_2) at 4.59 (dd, J = 1.7, 6.2 Hz), and further coupled to an allylic proton (H_1) at 1.98 (t, J = 2.2 Hz), which appeared as a triplet due to the overlapping of two doublets. Decoupling experiments confirmed the inter-

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Table I. Retention Time, Relative Abundance, and Base Peak of Urinary Metabolites Separated by GC–MS (See Figure 1)

spectrum	retention time, min	relative abundance, %	base peak
A (3a)	4.32	100	120.95
B (3b)	3.56	55.8	121.05
C	5.62	2.6	120.95
D	6.12	1.0	120.95

actions between these three protons.

A low-field multiplet (1 H) at δ 4.87 was assigned to be H-8 on the basis of chemical shift and decoupling experiments. On irradiation of H-8, a multiplet (1 H, H-7) at δ 2.41 and two multiplets (1 H each, H-9a and H-9b) at δ 1.81 and 1.74 simplified. The low-field region, in addition to H-2, H-3, and H-8 signals, contained two doublets of a typical AB system at δ 3.68 and 3.43 (J = 10.4 Hz), which could be ascribed to two protons of –0–CH₂–C in the six-membered ring.

The three-proton singlet at δ 1.01 was assigned to the C-5 methyl group and the three-proton doublet at δ 1.11 (J = 7 Hz) assigned to the C-10 methyl group as previously reported in the ¹H NMR spectrum of hymenoxon. The other three-proton doublet, at δ 1.27 (J = 7.6 Hz), is the C-11 α-methyl signal compared with the ¹H NMR spectrum of hymenolane (Petterson and Kim, 1976) whose C-11 β-methyl signal appeared at δ 1.05 (J = 7 Hz) as a doublet (Herz et al., 1980). The low-field region of the ¹H NMR spectrum of dehydrated B (4b) is essentially identical with that of dehydrated A (4a), indicating these two are epimers at C-11. The C-5 and C-10 methyl signals of dehydrated B (4b) appeared at δ 1.05 and 1.11 as a singlet and a doublet (J = 6.8 Hz), respectively, and the third three-proton signal due to C-11 methyl appeared at δ 1.04 as a doublet (J = 7.3 Hz). If it is assumed that the relative stereochemistry of the C-3 hydroxyl has not been modified in the metabolism of hymenoxon, the structure of metabolite A is consistent with the structure 3a.

Very little is known regarding the metabolism of sesquiterpene lactones. On the basis of the in vitro observations of the facile reactivity of the exocyclic methylene groups of five- or six-membered lactones with nucleophiles such as thiols, the toxicity of hymenoxon is assumed to be the result of indiscriminate binding of hymenoxon to critical cellular thiols. The indirect evidence supporting this hypothesis includes the following: (1) Cysteine protects dogs and sheep from hymenoxon toxicity (Kim et al., 1975; Rowe et al., 1980). (2) Pretreatment with hepatic thiol inducers, e.g., butylated hydroxyanisole (BHA) or ethoxyquin (EQ), reduces the toxicity of hymenoxon or bitterweed in mice (Kim et al., 1981) and in sheep (Kim et al., 1982). (3) Sesquiterpene lactones with no exocyclic methylene group are far less toxic than hymenoxon (Kim, 1980). However, the assumed in vivo reaction products of hymenoxon and thiols such as 5 or its subsequent metabolites have not been isolated. Furthermore, the role of the bis(hemiacetal) moiety in the toxicity of hymenoxon has not been elucidated.

It appears that hymenoxon (1) is metabolized to epimers of 4-desoxy-11,13-dihydrohymenoxon (3a and 3b), which are excreted as glucuronides (2a and 2b). These glucuronides are partially hydrolyzed to 3a and 3b by bacterial glucuronidase when the urine samples are collected at ambient temperatures and contaminated with microorganisms. The metabolites 3a and 3b are not detected in the urine samples taken directly from the bladder or a hymenoxon- or bitterweed-intoxicated sheep but can be
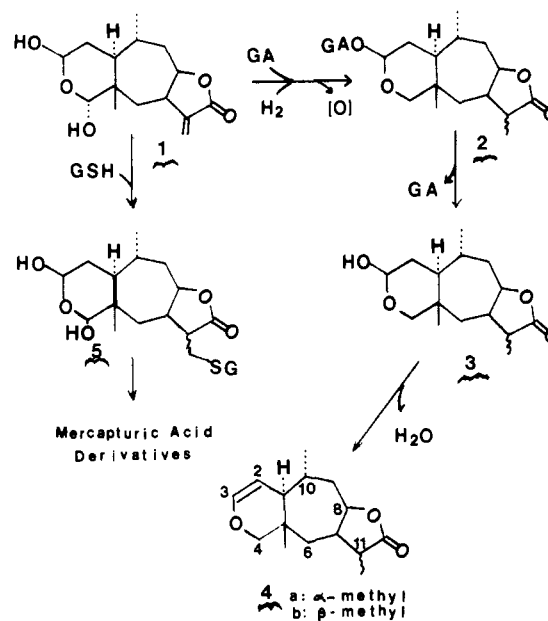

Figure 2. A proposed metabolic pathway of hymenoxon. Compounds 4a and 4b are artifacts formed during the preparative GC separation. GSH = glutathione; GA = glucuronic acid.

detected when the same urine samples are treated with β-glucuronidase. The metabolites 3a and 3b are dehydrated to 2,3-dehydro-4-desoxy-11,13-dihydrohymenoxon (4a and 4b) during the preparative gas chromatographic separation. A proposed metabolic pathway of hymenoxon is illustrated in Figure 2.

It is of interest that the hydrogenation of the exocyclic methylene moiety in vivo is not stereoselective; the dehydrated metabolites A (4a) and B (4b) are epimers. The mechanisms of hydrogenation and deoxygenation of hymenoxon have not been elucidated but they would appear to constitute a minor pathway, in view of the very low yields of these metabolites and the probable reaction with thiols in vivo as indicated above. Further research on the metabolism and mechanism of action of hymenoxon and related compounds is in progress.

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