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Residue Potential of Norsesquiterpene Glycosides in Tissues of Cattle Fed Austral Bracken (*Pteridium esculentum*)

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ABSTRACT: Austral bracken, *Pteridium esculentum*, occurs widely in Australian grazing lands and contains both the known carcinogen ptaquiloside and its hydroxy analogue, ptesculentoside, with untested carcinogenic potential. Calves were fed a diet containing 19% *P. esculentum* that delivered 1.8 mg of ptaquiloside and 4.0 mg of ptesculentoside per kilogram of body weight (bw) per day to explore the carcass residue potential of these compounds. Concentrations of ptaquiloside and ptesculentoside in the liver, kidney, skeletal muscle, heart, and blood of these calves were determined as their respective elimination products, pterosin B and pterosin G, by HPLC-UV analysis. Plasma concentrations of up to 0.97 μg/mL ptaquiloside and 1.30 μg/mL ptesculentoside were found, but were shown to deplete to <10% of these values within 24 h of bracken consumption. Both glycosides were also detected in all tissues assayed, with ptesculentoside appearing to be more residual than ptaquiloside. Up to 0.42 and 0.32 μg/g ptesculentoside was present in skeletal muscle and liver, respectively, 15 days after bracken consumption ended. This detection of residual glycosides in tissues of cattle feeding on Austral bracken raises health concerns for consumers and warrants further investigation.

KEYWORDS: ptaquiloside, ptesculentoside, bracken, *Pteridium esculentum*, cattle, carcase residues

INTRODUCTION

Bracken (genus *Pteridium*) is a ubiquitous fern occurring in forests and pastures across the globe. Bracken ferns have been linked with significant health concerns for both grazing domestic animals and human populations due to the presence of the norsesquiterpene glycoside ptaquiloside, **1a**, in this genus.[1−3] Ptaquiloside has long been suspected of causing human neoplasia, either from direct consumption of bracken or from indirect environmental exposure through inhalation of spores, leaching of ptaquiloside into groundwater, and ptaquiloside residues in milk from dairy cattle grazing bracken-infested pastures.[2] Ptaquiloside residues have also been detected in the meat of cattle consuming bracken as a high proportion of their diet,[3] and the potential human exposure to such ptaquiloside residues is significant as ptaquiloside has been reported to withstand cooking conditions.[4]

In recent studies we have determined that Austral bracken (*Pteridium esculentum*) contains both ptaquiloside,**1a**, and the related hydroxyptaquiloside ptesculentoside,**1b**, at concentrations up to 5.4 and 10.6 mg/g DW, respectively, along with lesser amounts of caudatoside, **1c** (Figure 1).[5,6] Ptesculentoside, **1b**, has a chemical reactivity similar to that of ptaquiloside, **1a**, and presumably has similar biological activity.[6] Consequently, the possibility of ptesculentoside, **1b**, residues entering the human food chain is of similar concern.

We report here an investigation of the potential formation and persistence of residues of both ptaquiloside, **1a**, and ptesculentoside, **1b**, in tissues of calves fed a diet containing *P. esculentum* of measured ptaquiloside and ptesculentoside content. Ptaquiloside, **1a**, and ptesculentoside, **1b**, residues in liver, kidney, muscle, heart, and blood of these calves were determined as their respective elimination products pterosin B, **2a**, and pterosin G, **2b**, by adaptation of a previously described HPLC-UV plant analysis method[5] to the analysis of animal tissues.

MATERIALS AND METHODS

Plant Material. Young unexpanded *P. esculentum* fronds ("crosiers") (Queensland Herbarium voucher AQ610813) were collected from Bribie Island, Queensland in October−November 2005, chopped into small pieces, and kept frozen at −20 °C until required.

Calf Feeding Trial. Thawed bracken was incorporated into a base diet of chaffed Rhodes grass (*Chloris gayana*) and lucerne (*Medicago sativa*) with a small quantity of molasses (approximately 50 mL) included to improve palatability. This diet was fed daily to four calves, approximately 7 months of age and 110 kg initial body weight (bw), and delivered 6 g bracken/kg bw/day (wet weight), corresponding to approximately 19% of the diet for either 18 days (two calves) or 24 days (two calves). Bracken was introduced incrementally over the initial 4 days (days 1−4, 25, 50, 75, and 100% of the daily dose, respectively). This feeding trial was conducted taking full account of the welfare of the calves under DPI&F Animal Ethics Committee Approval SA 2005/11/74. The health of the animals was monitored daily throughout the trial, with respiration and heart rate and rectal temperature recorded daily until day 24.

Jugular blood samples (3 × 10 mL) were obtained every 1−3 days until day 22 and then immediately before euthanasia. Blood for hematology was collected into EDTA anticoagulant; that for clinical biochemistry testing and toxin assay was collected into lithium heparin

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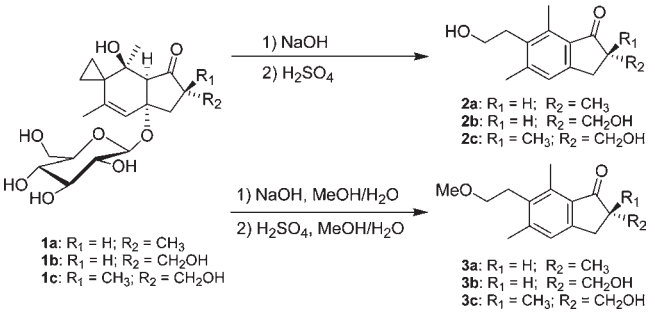

Figure 1. Conversion of ptaquiloside, **1a**, ptesculentoside, **1b** and caudatoside, **1c** to pterosin B, **2a**, pterosin G, **2b**, and pterosin A, **2c**, and related methoxypterosins **3a**, **3b**, and **3c**.

and centrifuged at 3200*g*, and separated plasma was stored at −40 °C before toxin assay. Hematological values measured by use of an automated hematology analyzer (ABC Vet Analyzer) were hemoglobin concentration, packed cell volume, erythrocyte, platelet, total and differential leucocyte counts, and erythrocyte indices; chemical concentrations and enzyme activities measured in plasma using an Olympus AU400 were aspartate aminotransferase, creatine kinase, glutamate dehydrogenase, γ-glutamyl transferase, total protein, albumin, globulin, calcium, magnesium, bilirubin (total and conjugated), urea, and creatinine. Skeletal muscle biopsies were taken from the rump muscles of all calves on days 6, 8, 10, and 12 (alternating between left and right rump muscles) of the feeding trial. After local analgesia was established using injected lignocaine, a scalpel was used to make a stab incision through the skin and subcutaneous tissue in the rump, and 0.2−0.4 g of muscle was removed with a scalpel and forceps. Muscle samples were blotted dry and immediately frozen (liquid nitrogen) and stored at −40 °C before assay. Incisions were closed with a single nylon suture, the wound was sprayed with an antiseptic tinctur, and animals were given a single intramuscular injection of a long-acting antibiotic.

Two calves (D1 and D2) were selected for a depletion study after termination of bracken feeding at day 18. After a further 14 days on the base diet without bracken, they were killed on the morning of day 33 of the trial by stunning with a captive bolt pistol and exsanguination and immediately necropsied. The remaining calves (F1 and F2) continued to be fed the bracken until day 24, and they were killed as above on the morning of day 25. At necropsy of all calves, samples of liver, kidney, heart, and skeletal muscle were collected and frozen immediately (liquid nitrogen) and stored at −40 °C before assay. Tissue samples were also collected at necropsy into 10% buffered neutral formalin for histopathology from sternal bone marrow, liver, kidney, spleen, and heart from all calves and from gall bladder, lung, adrenal gland, and alimentary tract from calves D1 and D2.

Tissue Extraction with Pterosin Conversion. Aqueous suspensions from tissue (5 g) homogenized (Polytron) in water (15 mL) or plasma (2 mL) in water (15 mL) were treated in an identical manner. The aqueous mixture was treated with 1 M NaOH (1 mL) and incubated at 40 °C for 1 h. The cooled solution was treated with 5 N H_2SO_4 (1 mL) and mixed, and then MeOH (20 mL) was added. The shaken suspension was kept for 10 min and then centrifuged at 5000*g* for 20 min. The decanted supernatant was extracted with CH_2Cl_2 (3 × 20 mL) and evaporated to dryness under vacuum at 30 °C. The residue was taken up in CH_3CN (0.5 mL) and filtered (0.22 μm PVDF) for HPLC-UV analysis.

Tissue Extraction without Pterosin Conversion. Tissue samples were treated as above, but omitting the NaOH−H_2SO_4 treatment.

Tissue Extraction with Methoxypterosin Conversion. Skeletal muscle (5 g) was homogenized (Polytron) in water (25 mL) and mixed with methanol (75 mL). The suspension was treated with 1 M NaOH and incubated at 40 °C for 1 h. The cooled solution was treated with 5 N H_2SO_4 in 50% methanol/water (2 mL), mixed, and centrifuged at 5000*g* for 20 min. The decanted supernatant was extracted with CH_2Cl_2 (3 × 40 mL) and evaporated to dryness under vacuum at 30 °C. The residue was taken up in CH_3CN (0.5 mL) and filtered (0.22 μm PVDF) for HPLC-UV analysis. An aqueous *P. esculentum* extract from the same batch of plants fed to the calves and containing ptaquiloside, **1a**, ptesculentoside, **1b**, and caudatoside, **1c**, was similarly treated to provide a solution of methoxypterosins **3a**, **3b**, and **3c** for comparison.

Table 1. Mean Recoveries from Blank Bovine Plasma and Muscle Spiked with Various Concentrations of Glycosides 1a and 1b (μg/mL Plasma and μg/g Skeletal Muscle, *n* = 3)

	ptaquiloside		ptesculentoside	
tissue	spike **1a**	recovery **1a** % (SD)	spike **1b**	recovery **1b** % (SD)
bovine plasma	1.1	65 (6)	1.4	73 (7)
	5.5	86 (2)	6.9	77 (2)
	34.3	92 (0)	43.1	77 (1)
bovine muscle	1.1	68 (3)	1.3	81 (3)
	5.5	86 (2)	6.6	74 (1)
	11.0	87 (2)	13.3	72 (2)
	32.9	88 (1)	39.8	72 (1)

HPLC-UV Analysis. HPLC separations were performed using a 250 × 4.6 mm i.d. Luna, 5 μm, C18 column (Phenomenex, Sydney, Australia) at 35 °C with a flow rate of 1 mL/min and an injection volume of 10 μL. The mobile phase was a mixture of (A) 40:60 CH_3CN/water (v/v) and (B) CH_3CN with a gradient as follows: 0−9 min, held at 100% A; 9−11 min, 100% A−100% B; 11−16 min, held at 100% B; 16−16.5 min, 100% B−100% A; 16.5−25 min, held at 100% A. Detection of individual components was by PDA at 220 nm for the pterosin conversion products pterosin G, **2b** (retention time 3.7 min), and pterosin B, **2a** (11.1 min). The minor component pterosin A, **2c**, eluted at 4.8 min but could not be quantitated in tissue extracts due to an interfering peak in these extracts. Methoxypterosin, **3b**, eluted at 9.1 min and **3a** and **3c** both eluted after 12 min during solvent ramp phase (when elution was performed isocratically with 100% solvent A, **3c** eluted at 14.2 min and **3a** at 19.2 min).

Reference Standards and Quantitation. Ptaquiloside and pterosin B were provided by Lars Rasmussen (Denmark). Standard solutions for HPLC analysis were prepared by conversion of a 3 μg/mL solution of ptaquiloside, **1a**, to pterosin, **2a**, as described.[5] The concentration was calculated on the basis of 100% conversion of ptaquiloside, **1a**, to pterosin B, **2a**, and expressed as ptaquiloside equivalents.

Quantitation of ptaquiloside, **1a**, in sample extracts was by direct comparison of pterosin B product UV absorbance with this standard. Quantitation of ptesculentoside, **1b** in sample extracts was performed by comparison of measured UV absorbance of pterosin G, **2b**, with ptaquiloside-derived pterosin B standard and allowing for relative absorbance factor of 0.73 (in glycoside equivalents) for this pterosin with respect to pterosin B, **2a**.[5]

Recoveries from Bovine Tissue Spiked with Glycosides 1a and 1b. An aqueous extract of dried and milled *P. esculentum* (Queensland Herbarium voucher AQ840152, collected at Casino, New South Wales) was filtered and passed through polyamide 6 as previously described,[5] and the ptaquiloside, **1a**, and ptesculentoside, **1b**, content of the cleaned extract was determined by pterosin conversion. Bovine plasma and skeletal muscle were spiked with aliquots of this cleaned extract, and the homogenized tissue was extracted and analyzed

Table 2. Molecular Ions and Selected MS/MS Fragment Ions of Pterosins and Methoxypterosins

compound	$[M + H]^+$	major fragment ions (relative abundance, %)
2a	219	219 (36), 201 (100), 186 (22), 183 (23), 173 (41), 168 (31), 145 (45)
2b	235	235 (3), 217 (91), 199 (21), 189 (37), 171 (81), 156 (100), 143 (29)
2c	249	249 (2), 231 (17), 203 (100), 185 (45), 170 (21), 157 (35)
3a	233	233 (6), 201 (100), 183 (32), 173 (18), 145 (39)
3b	249	249 (3), 231 (94), 203 (41), 199 (87), 171 (95), 156 (100)
3c	263	263 (15), 245 (26), 217 (77), 203 (31), 185 (100), 157 (53)

by HPLC-UV as described above, with three replicates at each concentration and mean recoveries calculated (Table 1).

LC-MS/MS Equipment and Conditions. Samples were analyzed using a Waters 2777C Sample Manager, and the liquid chromatographic separations were carried out on a Waters 1525 μ Binary HPLC pump system. LC separations were performed using a 250 × 4.6 mm i.d. Luna, 5 μm, C18 column (Phenomenex) at 35 °C with a flow rate of 1 mL/min. The mobile phase was a mixture of (A) 40% CH_3CN in water (v/v) and (B) CH_3CN with a gradient as follows: 0−12 min, held at 100% A; 12−12.01 min, 100% A−100% B; 12.01−20 min, held at 100% B; 20−20.01 min, 100% B−100% A; 20−25 min, held at 100% A.

MS/MS detection was made using a Quattro Premier Micromass triple-quadrupole mass spectrometer, equipped with an electrospray ionization (ESI) source used in positive mode. The capillary voltage was 3.0 kV; the desolvation and cone gases of nitrogen flow were set at 595 and 47 L/h, respectively. The desolvation and source temperatures was set at 350 and 150 °C, respectively. Argon was used as collision gas for MS/MS with a flow rate of 0.3 mL/min, and the collision energy was set at 20 eV and the cone voltage at 30 V for pterosins **2a** and **3a** and at 35 V for pterosins **2b**, **2c**, **3b**, and **3c**. Prominent MS/MS transitions were recorded for pterosins **2a** (218.9 → 201.1), **2b** (235.1 → 155.9), **2c** (249.1 → 203.1), **3a** (233.1 → 201.1), **3b** (249.1 → 231.2), and **3c** (263.1 → 185.2), with further MS data shown in Table 2.

RESULTS AND DISCUSSION

Norsesquiterpene Glycoside Residue Analysis in Bovine Tissues. We recently described a method to analyze ptaquiloside, **1a**, ptesculentoside, **1b**, and caudatoside, **1c**, in bracken ferns by HPLC-UV,[5] as their respective elimination products pterosin B, **2a**, pterosin G, **2b**, and pterosin A, **2c** (Figure 1). In the present study we have adapted this method to the analysis of the major glycosides **1a** and **1b** in tissues from calves consuming *P. esculentum* by aqueous extraction followed by NaOH−H_2SO_4 conversion to pterosins **2a** and **2b**. The identity of pterosin products was confirmed by LC-MS/MS. The minor glycoside caudatoside, **1c**, could not be quantitated by HPLC-UV in tissue extracts as pterosin A, **2c**, coeluted with an interfering peak in these extracts (Figure 2).

To verify the efficiency and reproducibility of this method we have spiked blank tissues with known quantities of ptaquiloside, **1a**, and ptesculentoside, **1b**, from a *P. esculentum* extract (AQ840152) with a measured glycoside concentration.[5] Bovine

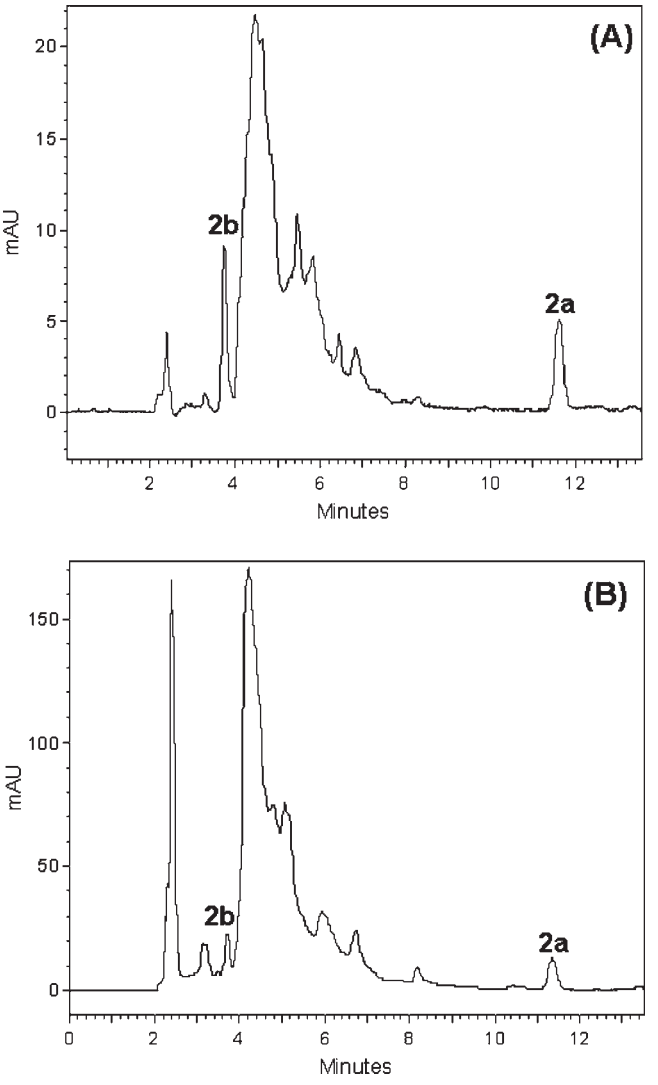

Figure 2. HPLC-UV chromatograms of pterosin converted extracts of (A) plasma and (B) kidney from fully fed calf F1.

plasma was spiked with glycosides **1a** and **1b** at three concentrations (three replicates at each concentration) with average recoveries of 81 and 76%, respectively (Table 1). Bovine muscle tissue was spiked with **1a** and **1b** at four concentrations (three replicates at each concentration) with average recoveries of 82 and 75%, respectively (Table 1).

The detection limits (LOD, S/N = 3) for ptaquiloside, **1a**, by this HPLC-UV method were 0.04 μg/mL in plasma and 0.02 μg/g in tissue samples, with limits of quantitation (LOQ, S/N = 7) of 0.10 μg/mL in plasma and 0.04 μg/g in tissue. For ptesculentoside, **1b**, the LODs were 0.03 μg/mL in plasma and 0.013 μg/g in tissue and the LOQs were 0.07 μg/mL in plasma and 0.03 μg/g in tissue.

Feeding Experiment. *P. esculentum* (Austral bracken), sourced from Bribie Island, contained 2.45 mg of ptaquiloside, **1a**, 5.50 mg of ptesculentoside, **1b**, and 1.33 mg of caudatoside, **1c**, per gram plant dry weight by HPLC-UV analysis.[5] Fresh plant material contained 12.1% dry matter and hence was calculated to contain 0.30 mg of ptaquiloside, **1a**, 0.66 mg of ptesculentoside, **1b**, and 0.16 mg of caudatoside, **1c**, per gram plant wet weight.

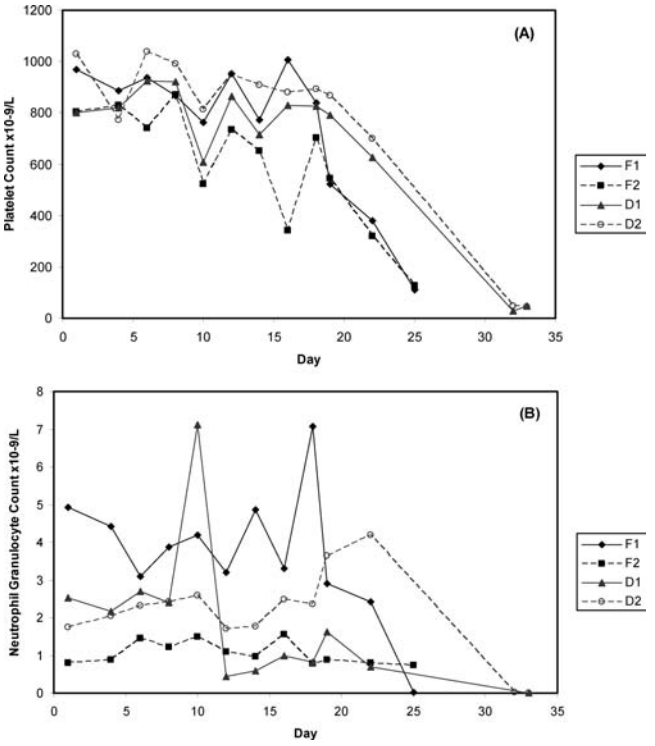

Figure 3. Platelet counts (A) and neutrophil granulocyte counts (B) measured in jugular blood during bracken feeding of calves: fully fed calves (F1 and F2) and "depletion" calves (D1 and D2).

This bracken was fed to calves daily for either 18 days (calves D1 and D2) or 24 days (calves F1 and F2), at a rate to supply 1.8 mg of ptaquiloside, **1a**, 4.0 mg of ptesculentoside, **1b**, and 1.0 mg of caudatoside, **1c**, per kilogram bw/day. This dose was chosen with the aim of avoiding poisoning of the calves and was based on previous experimental evidence.[7] Two calves showed some feed refusal (rejecting up to 20% of the feed offered) after 10 days, and these calves (calves D1 and D2) were selected for a depletion study following termination of bracken feeding at day 18 and thereafter were fed on a normal bracken-free diet for a further 15 days before euthanasia. Calves F1 and F2 were euthanized at day 25 while on the bracken diet. Plasma and skeletal muscle obtained by biopsy during feeding, as well as a range of tissues taken at necropsy, were assayed for ptaquiloside, **1a**, and ptesculentoside, **1b** (as corresponding pterosins **2a** and **2b**) by HPLC-UV (Figure 2).

Animal Health and Pathology. All calves gained weight while being fed the bracken and appeared healthy, but evidence of poisoning did emerge. Rectal temperatures, heart and respiratory rates, and all clinical biochemistry plasma values remained within normal reference ranges throughout. The fully fed calves F1 and F2 had a marked reduction in circulating platelet numbers in the days immediately before euthanasia, and calf F1 had a similar parallel decline in circulating neutrophil granulocyte numbers (Figure 3). All other hematological values were within normal reference ranges. No lesion was found in either calf F1 or F2 at necropsy. In histological sections from both calves, the sternal bone marrow appeared hypoplastic with very few myelocytes (granulocyte precursor cells) and a few scattered megakaryocytes (platelet precursor cells) present. No lesion was seen in histological sections of liver, kidney, spleen, or heart from either calf. These findings were consistent with known early effects of bovine poisoning by norsesquiterpene glycosides.[3]

Table 3. Concentrations of Ptaquiloside, 1a and Ptesculentoside, 1b in Plasma of Calves F1 and F2 over a 24 h Period before (t = 0) and after Feeding of Bracken Diet on Day 20

	calf F1		calf F2	
time (h)	**1a** (μg/mL)	**1b** (μg/mL)	**1a** (μg/mL)	**1b** (μg/mL)
0	<LOD	0.07	<LOD	0.12
3	0.97	1.30	0.67	1.07
8	0.29	1.07	0.21	1.10
24	<LOD	0.08	<LOD	0.11

In the period after bracken feeding ended, depletion calves D1 and D2 developed mild persistent diarrhea and on day 32 became depressed and anorexic, and fresh unclotted blood was seen in the feces. At this point these calves were immediately humanely killed to prevent serious adverse and unintended impacts on their welfare. Necropsy revealed lesions of similar type and severity in both calves D1 and D2. Numerous scattered small hemorrhages were seen in the abdominal and intercostal muscles, in the omentum and the mesentery, and beneath the external surfaces of the esophagus, the rumen, and the intestines. There were a few small ulcers in the mucosa of the abomasum and intestines with hemorrhage from these into the lumen of the alimentary canal. Some of the intestinal ulcers had penetrated to the peritoneum, where a small amount of fibrinous exudate adhered to the serosal surfaces and the adjacent omentum. These hemorrhagic and inflammatory lesions were also seen in histological sections from these tissues. Additionally, sections of sternal bone marrow had a histological appearance similar to those from calves F1 and F2. In intestinal sections, necrotic material in the mouths of intestinal ulcers was invaded by bacteria, which had penetrated into the submucosa. Adjacent submucosal blood vessels were thrombosed. In some cases, the mucosal ulceration was accompanied by edema and inflammation of the full thickness of the wall and a fibrinous peritonitis of the adjacent serosa. Neutrophil granulocytes appeared to be absent from the inflammatory response to this tissue damage despite bacteria having invaded the tissues. Small, clinically insignificant numbers of the common coccidial parasite *Eimeria* sp. were seen in the intestinal mucosa. Blood vessels in the livers were congested. No histological lesions were seen in the other tissues examined. The activity of coccidia in the intestinal lining cells may have initiated much of the ulceration seen by rupturing individual lining cells. Then the lack of effective neutrophil granulocyte defense against subsequent bacterial invasion of affected tissues allowed the damage to extend further.

The unintended onset of bleeding and bacterial infection consistent with the consequences of bone marrow damage expected from bovine poisoning by norsesquiterpene glycosides[3] in calves D1 and D2 was unfortunate. However, it does establish that our residue results reflect the upper limit of what could be expected in seemingly healthy cattle that had been eating sublethal amounts of bracken.

Feeding Trial Bovine Tissue Analysis. During the feeding trial glycosides were measured in plasma at variable levels from below LOD to 0.97 μg/mL for **1a** and from below LOD to 1.30 μg/mL for **1b** (results not shown), with the variation seemingly dependent on time elapsed after bracken consumption. Studies of plasma glycoside concentrations in calves F1 and F2 for which

Table 4. Concentrations of Ptaquiloside, 1a, and Ptesculentoside, 1b, in Tissues at Necropsy of "Depletion" Calves D1 and D2 and of Calves F1 and F2 Necropsied While Still Consuming Bracken (μg/mL Plasma and μg/g Other Tissues)

	calf D1		calf D2		calf F1		calf F2	
tissue	1a	1b	1a	1b	1a	1b	1a	1b
plasma	<LOD	<LOD	<LOD	<LOD	0.27	obs[a]	0.42	0.49
muscle[b]	<LOD	0.42	trace[c]	0.32	0.07	1.29	0.12	1.21
heart	<LOD	trace	<LOD	trace	0.17	0.88	0.15	0.89
liver	<LOD	0.25	trace	0.32	0.55	1.59	0.75	1.59
kidney	trace	trace	trace	trace	0.94	0.85	1.16	1.36

[a] Obscured, not able to be determined due to coeluting peak. [b] Skeletal muscle. [c] Between LOD and LOQ.

sequential samples were taken over a 24 h period (Table 3) demonstrated initially increased concentrations of **1a** and **1b** to a maximum approximately 4 h after bracken consumption followed by a marked decline to essentially prefeeding ($t = 0$) levels within 24 h. Glycosides **1a** and **1b** were not detected in plasma of "depletion"' calves D1 and D2 after 2 days of bracken-free diet.

Glycosides **1a** and **1b** were not detectable in the small muscle biopsy samples obtained during the feeding trial (<0.4 g). Ptaquiloside, **1a**, was detected in the larger tissue samples obtained at necropsy with up to 0.75 and 1.16 μg/g in liver and kidney and smaller amounts (<0.2 μg/g) in skeletal and cardiac muscles of calves F1 and F2 (Table 4). Interestingly the amount of ptesculentoside, **1b**, in tissues, particularly muscle and liver (up to 1.21 and 1.59 μg/g, respectively), was markedly higher than that of ptaquiloside, **1a**, and persisted in "depletion" calves D1 and D2 after bracken was withdrawn from the diet (Table 4). Muscle and liver from calves D1 and D2 contained 0.3−0.4 μg/g at slaughter after 15 days on a normal diet.

Confirmation of the Origin of Pterosins in Tissue Extracts. The identification of the elimination products pterosin B, **2a**, and pterosin G, **2b**, in tissue extracts was further confirmed by LC-MS/MS. LC-MS/MS analysis of pterosin products of a *P. esculentum* extract was performed on the same HPLC column as HPLC-UV analysis[5] and provided MS/MS spectra for pterosins **2a**−**2c**. MS/MS fragmentation of the protonated molecular ion of pterosin B, **2a** ($[M + H]^+$ 219), produces the dominant fragment ion m/z 201 as previously reported,[8] with other major fragment ions as shown in Table 2. Pterosin G, **2b** ($[M + H]^+$ 235), undergoes similar fragmentation with a number of ions 16 Da higher than those seen in pterosin B, and similarly pterosin A (**2c**) ($[M + H]^+$ 249) provides several fragment ions 14 Da higher than pterosin G (Table 2).

SRM transitions based on dominant fragment ions for **2a** (218.9 → 201.1), **2b** (235.1 → 155.9), and **2c** (249.1 → 203.1) together with confirmatory transitions were employed to detect these pterosins in tissue extracts at low levels and confirmed the presence of all three pterosins in base−acid-treated feeding trial tissue extracts, particularly kidney and liver. Tissue extraction without NaOH−H_2SO_4 conversion followed by HPLC-UV and LC-MS/MS analysis confirmed that negligible native pterosins existed in the tissues, implying that the glycosides **1a**, **1b**, and **1c** (rather than the pterosins) were present in the samples.

Attempts to detect unconverted ptaquiloside, **1a**, and ptesculentoside, **1b**, at microgram per gram concentrations in tissue were not successful by LC-MS/MS, as detection limits for these compounds are considerably higher than for the pterosins. As we were unable to directly confirm the presence of glycosides **1a**, **1b**, and **1c** in tissue samples, there was some uncertainty regarding the origins of the detected pterosin products, namely whether they are derived from residual glycosides **1a**, **1b**, and **1c** or from some bound pterosin product and, for example, released from DNA-−pterosin adducts by our conversion method. To confirm that pterosins are derived from glycosides **1a**, **1b**, and **1c**, we performed the glycoside/pterosin conversion in the presence of methanol to produce methoxypterosins **3a**, **3b**, and **3c** (Figure 1). The formation of methoxypterosin **3a** from ptaquiloside, **1a**, has been previously documented with methanol/water (2:1) treatment of a *Pteridium aquilinum* extract, forming **3a** as the predominant product (**3a**/**2a**, 5:1 by GC).[9] Similar treatment of a *P. esculentum* (AQ610813) extract provided a solution containing authentic **3a**, **3b**, and **3c** for comparison, with LC-MS/MS data consistent with these structures (Table 2). Such methoxy derivatives are formed by nucelophilic ring-opening of an intermediate dienone[9] and would not arise from the cleavage of tissue pterosin adducts in the presence of methanol. Our LC-MS/MS analysis of similarly methanol-treated tissues demonstrated the presence of methoxypterosin derivatives **3a**, **3b**, and **3c**, which is confirmation of the presence of the corresponding glycosides in these tissues.

Residue Risk in Grazing Animals. The presence of residues of ptaquiloside, **1a**, and ptesculentoside, **1b**, in animal tissues is of interest in the context of the health of human consumers. Our results indicate that calves eating *P. esculentum* have plasma concentrations of both compounds (up to 0.97 μg/mL **1a** and 1.30 μg/mL **1b** in this study), but that these deplete within 24 h of bracken consumption, with <10% of peak values remaining. Glycosides **1a** and **1b** were also detected in a range of tissue samples, with ptesculentoside, **1b**, appearing more residual with levels up to 0.42 and 0.32 μg/g present in muscle and liver 15 days after cessation of bracken consumption. Ptesculentoside has chemical reactivity similar to that of the known carcinogen ptaquiloside and potentially similar biological activity,[6] but this activity has not yet been investigated. The potential for ptesculentoside to form persistent residues in beef warrants further investigation in light of the potential toxicity and carcinogenicity of this compound.

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REFERENCES

(1) Vetter, J. A biological hazard of our age: bracken fern [*Pteridium aquilinum* (L.) Kuhn] — a review. *Acta Vet. Hung.* **2009**, *57*, 183–196.

(2) Yamada, K.; Ojika, M.; Kigoshi, H. Ptaquiloside, the major toxin of bracken, and related terpene glycosides: chemistry, biology and ecology. *Nat. Prod. Rep* **2007**, *24*, 798–813.

(3) Smith, B. L. The toxicity of bracken fern (genus *Pteridium*) to animals and its relevance to man. In *Handbook of Plant and Fungal Toxicants*; D'Mello, F., Ed.; CRC Press: New York, 1997; pp 63−76.

(4) Smith, B. L.; Seawright, A. A. Bracken fern (*Pteridium* spp.) carcinogenicity and human health — a brief review. *Nat. Toxins* **1995**, *3*, 1–5.

(5) Fletcher, M. T.; Brock, I. J.; Reichmann, K. G.; McKenzie, R. A.; Blaney, B. J. Norsesquiterpene glycosides in bracken ferns (*Pteridium esculentum* and *Pteridium aquilinum* subsp. *wightianum*) from eastern Australia: reassessed poisoning risk to animals. *J. Agric. Food Chem.* **2011**, *59*, 5133–5138.

(6) Fletcher, M. T.; Hayes, P. Y.; Somerville, M. J.; De Voss, J. J. Ptesculentoside, a novel norsesquiterpene glucoside from the Australian bracken fern *Pteridium esculentum*. *Tetrahedron Lett.* **2010**, *51*, 1997–1999.

(7) Alonso-Amelot, M. E.; Castillo, U.; Smith, B. L.; Lauren, D. R. Excretion, through milk, of ptaquiloside in bracken-fed cows. A quantitative assessment. *Lait* **1998**, *78*, 413–423.

(8) Jensen, P. H.; Jacobsen, O. S.; Hansen, H. C. B.; Juhler, R. K. Quantification of ptaquiloside and pterosin B in soil and groundwater using liquid chromatography−tandem mass spectrometry (LC-MS/MS). *J. Agric. Food Chem.* **2008**, *56*, 9848–9854.

(9) Bonadies, F.; Borzacchiello, G.; Dezzi, S.; Nicoletti, R.; Roperto, S. Mass spectrometric analysis of ptaquiloside, the toxic sesquiterpene from bracken fern. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 825–828.