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JENSENONE: BIOLOGICAL REACTIVITY OF A MARSUPIAL ANTIFEEDANT FROM *Eucalyptus*

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Abstract—The resistance of *Eucalyptus* to browsing mammals has been related to the level and type of formylated phloroglucinol compounds (FPCs) present in the leaf. The antifeedant activity of FPCs appears to depend on their aldehyde groups, but little else is known of their mode of action. We have sought to elucidate this further by examining the biological reactivity and disposition of jensenone, a model FPC. Neither jensenone nor any metabolites were detected in urine or feces of marsupial brushtail or ringtail possums that had ingested up to 725 $mg \cdot kg^{-0.75}$. When jensenone was incubated in rat gastrointestinal segments *in vitro*, it rapidly disappeared. Jensenone also reacted rapidly with glutathione, cysteine, glycine, ethanolamine, and trypsin, and more slowly with acetylcysteine and albumin. Sideroxylonal, a more complex FPC, exhibited the same reactivity. Torquatone, a related compound that lacks both aldehyde groups and antifeedant activity, was unreactive. Mass spectroscopic analysis indicated that the adducts were Schiff bases formed between the aldehyde groups of FPCs and amine groups of the conjugating molecules. Successive adducts were formed with the two aldehyde groups of jensenone, and the four groups of sideroxylonal. The jensenone bis-glutathione adduct appeared to cyclize to the disulfide form. These findings suggest that the antifeedant effects of FPCs are due to their facile binding to amine groups on critical molecules in the gastrointestinal tract, leading to a loss of metabolic function. The consequent toxic reaction, probably involving chemical mediators such as 5-hydroxytryptamine (5HT), may cause colic, nausea, and a general malaise, resulting in anorexia.

Key Words—Antifeedant, plant secondary metabolite, *Eucalyptus*, jensenone, sideroxylonal, formyl phloroglucinols, glutathione conjugate, aldehyde.

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INTRODUCTION

Secondary compounds are ubiquitous in the diets of browsing mammals and there is much evidence that they can restrict the types and amount of plants eaten (Foley et al., 1999). For most ecologically important plant secondary metabolites (PSMs), we have little appreciation of the tissue targets that are affected by the PSM or of the pathways of detoxification and elimination of these compounds in consumers (Foley et al., 1999; McLean et al., 2001). If we are able to understand how PSMs are metabolized, we will better appreciate how variability in both plant and animal responses allow for the evolution of complex interactions between plants and herbivores. This is our objective in studies of *Eucalyptus* and its vertebrate herbivores in Australia.

Recent studies (Lawler et al., 1998a; Stapley et al., 2000) have demonstrated that the major factor that limits the amount of *Eucalyptus* leaves eaten by folivorous marsupials (e.g., koala, common ringtail, and common brushtail possum) is the concentration of formylated phloroglucinol compounds (FPCs) e.g., jensenone, sideroxylonal A (Figure 1). FPCs are highly diverse, but characterized by at least

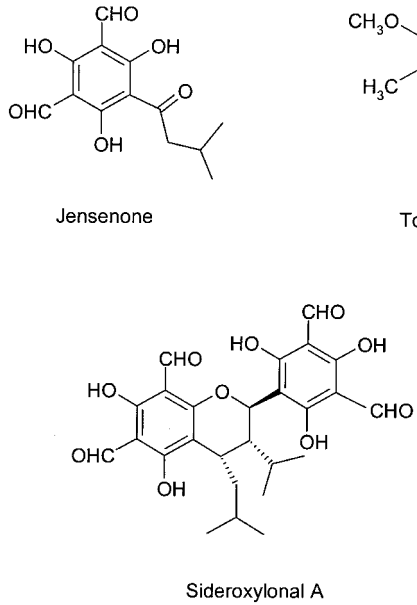

FIG. 1. Structures of phloroglucinol derivatives. Jensenone and sideroxylonal are formyl phloroglucinols (FPCs).

one fully substituted phenolic ring with one or two aldehyde groups, which are hydrogen bonded to the phenol group (Boland et al., 1992). Limited structure–function studies (Lawler et al., 1999a) have shown that the antifeedant effects of FPCs depend on the presence of the aldehyde group, but other correlational studies suggest other structural features are also important (B. D. Moore unpublished data).

Previous studies have shown that marsupials closely regulate their consumption of FPCs around a threshold, but that there is significant variation between species and among individual animals (Lawler et al., 1998a, 2000; Stapley et al., 2000). However, administration of the selective $5HT_3$-receptor antagonist, ondansetron (a powerful antiemetic), leads to significantly higher intakes of jensenone (Lawler et al., 1998b). This suggests that animals detect nauseous sensations from the ingestion of jensenone and so are able to titrate their intake in response to this. However, exactly how this occurs remains unknown.

In an attempt to understand why FPCs are such effective antifeedants and why some species and individual animals are more sensitive to their effects than others, we studied the biological disposition of jensenone in two species of marsupials, the common ringtail possum (*Pseudocheirus peregrinus*) and the common brushtail possum (*Trichosurus vulpecula*), by using a combination of *in vivo* and *in vitro* techniques. The findings led to an investigation of the biological reactivity of jensenone and its effects on the gastrointestinal tract.

METHODS AND MATERIALS

Animals. Details of the capture and care of brushtail (*Trichosurus vulpecula*) and ringtail possums (*Pseudocheirus peregrinus*) have been reported previously (Lawler et al., 1998a; Stapley et al., 2000). Rats (Hooded-Wistar) and guinea pigs were supplied by the Central Animal House, University of Tasmania. All animal procedures were approved by the Animal Experimentation Ethics Committee of the institution where each experiment was conducted.

Materials. Jensenone and torquatone were extracted and purified from *Eucalyptus jensenii* and *E. torquata* foliage, respectively (Lawler et al., 1999a). Sideroxylonal was extracted from *E. melliodora* foliage (Eschler and Foley, 1999). Extract of *Helix pomatia* (a mixture of β-glucuronidase 141,000 units/ml and aryl sulfatase 3950 units/ml) was obtained from Boehringer Mannheim (Germany) and *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) from Alltech Associates (Australia). Other chemicals and solvents were of analytical reagent grade. Trypsin and glutathione were from Boehringer Mannheim (Germany) and acetylcysteine, albumin and glycine from Sigma (Australia).

Jensenone Solutions. A standard solution of jensenone (50 mg in 2 ml 2% sodium carbonate) was prepared and diluted to give other concentrations. It had a

pH of 7–8 and was stable for at least 1 week by gas chromatographic (GC) analysis. Jensenone was readily extracted into ethyl acetate from aqueous solutions after acidification with 5 M HCl.

GC–FID. Extracts in ethyl acetate were dried under nitrogen and derivatized with BSTFA (20 μl, heated at 70°C for 5 min). GC analyses were conducted on a Varian 3300 instrument fitted with a 30 m Econocap capillary column (0.25 μm SE-54, 0.32 mm i.d.; Alltech Associates) and flame ionization detector (FID). GC conditions were: injection volume 1 μl, split ratio 1:20, carrier He at 9 psi, injector 250°C, detector 300°C, oven 150–290°C 10°C/min, held at 290°C for 9 min. Retention times of jensenone TMS derivatives were: major peak 12.2 min, minor peaks at 9.0, 9.4, and 13.4 min.

GC–MS. Details of the instrument and operating conditions for combined gas chromatography–mass spectrometry (GC–MS) have been reported previously (Boyle et al., 2000a). Jensenone gave a GC peak with a MW of 238 (rather than 266), corresponding to a degraded form.

LC–UV. Samples were either extracted into ethyl acetate, or simply filtered (0.45 μ) before analysis by liquid chromatography with UV detection (LC–UV). A Varian 9010 solvent delivery system with Rheodyne injector model 7161 was connected to a Varian 9050 UV–Vis absorbance detector and Star workstation (Varian Instruments, Melbourne). The column was a reverse-phase C18 Nova-Pak (3.9 $\times$ 150 mm; Waters Associates, Sydney), and the injection volume was 10 μl. The absorption maxima were: jensenone 268 nm; jensenone adducts 332 nm; sideroxylonal 280 nm; and torquatone 210 nm.

Several different mobile phase systems were required because of the variety of experimental procedures. System I was acetonitrile–1% acetic acid (85:15), 0.7 ml/min (jensenone eluted in 4.8 min). System II was methanol–1% acetic acid (55:45), 0.8 ml/min, programmed to 100% methanol at 15 min (jensenone eluted in 14.5 min). System III, used for sideroxylonal, was 2% acetic acid in methanol–2% acetic acid in water (96:4), 1 ml/min. Sideroxylonal exists in three isomeric forms (Eschler and Foley, 1999). It eluted as two peaks, at 2.29 min (sideroxylonal A/C) and 3.42 min (sideroxylonal B), and was quantitated by the sum of the two peaks.

LC–MS. Chromatography conditions for the analysis of jensenone reactions by liquid chromatography–mass spectrometry (LC–MS) were the same as LC–UV System II. Negative ion electrospray ionization (ESI) was used, and the instrument and operating conditions were as previously described (Boyle et al., 2001). MS/MS analyses were used to confirm the origin of product ions. Sideroxylonal reactions were analyzed by System IV: 2% acetic acid in methanol–2% acetic acid in water (80:20), programmed to 2% acetic acid in methanol in 10 min, 0.8 ml/min. Sideroxylonal peaks eluted at 7.58 min (A/C) and 10.02 min (B). MS of each peak produced ions at m/z 499 ($[M-H]^-$) and a fragment at m/z 249 (effectively half the molecule) (Neve et al., 1999).

Oral Dosing. Jensenone was incorporated into the feed of six brushtail and six ringtail possums, at concentrations of 0.0025–0.04% dry matter (Lawler et al., 1999b; Stapley et al., 2000). Intakes ranged from 428 to 725 $mg \cdot kg^{-0.75}$ (brushtails) and 8–48 $mg \cdot kg^{-0.75}$ (ringtails). In addition, two ringtail possums were gavaged with jensenone (dissolved in 2% sodium carbonate) at doses of 50 and 100 $mg \cdot kg^{-1}$, and two brushtails at a dose of 50 $mg \cdot kg^{-1}$. Urine and feces were collected for 24 hr and analyzed by LC and GC using methods similar to those used previously to characterize terpene metabolites (Boyle et al., 1999, 2000a,b, 2001). Jensenone was readily extracted from aqueous systems, although calibration curves were not prepared from excreta.

Disappearance of Jensenone from Rat Gastrointestinal Tissue. The rat gut tissue used was either the stomach or a segment (7–8 cm) of ileum, taken from a freshly-killed rat and washed and kept in Tyrode's solution (37°C, bubbled with air). One end of the tissue was ligated and the other end tied to a glass tube, from which the tissue was suspended in an organ bath. Jensenone (15 mg in 1 ml 1% sodium carbonate, pH 9) was placed in the tissue and samples of the contents for analysis were withdrawn through the glass tube at different times from 1 min onwards; the outer bath was also sampled. The gastrointestinal samples were centrifuged and filtered before analysis by LC–UV, or extracted into ethyl acetate and analyzed by GC–FID.

Attempts were made to recover jensenone from the gut tissue. After incubation with jensenone, segments of ileum were frozen in liquid nitrogen and ground with a mortar and pestle. Aliquots (100 mg) of the ground tissue and samples (50 μl) of the luminal contents were placed in a glass vial, 200 μl 5 M NaOH added, and the vial sealed and flushed with nitrogen. The contents were heated (60°C, 10 min), acidified, and extracted with ethyl acetate and analyzed for jensenone.

Reactivity of Jensenone. Jensenone (7.5 mg in 0.5 ml 2% sodium carbonate) was incubated at 37°C with 25 mg trypsin, albumin, glutathione, or acetylcysteine. Aliquots (10 or 100 μl) were taken, diluted, filtered, and analyzed by LC–UV. Experiments were conducted in duplicate and half-life calculated from the slope of the disappearance curve.

Formation of Adducts. Glutathione (25 mg) and jensenone (7.5 mg) were each dissolved in 0.5 ml 1% sodium carbonate and the solutions mixed to start the reaction. Aliquots (100 μl) were taken at different times, acidified with 2 drops 5 M HCl forming a precipitate that was removed by centrifugation and dissolved in methanol (2 ml). The methanol was filtered and analyzed by LC–UV and LC–MS. The temperature was lowered to 20°C in order to slow the reaction and follow product formation.

Glycine, L-cysteine, acetylcysteine, and ethanolamine were also tested for their ability to form adducts with jensenone. Jensenone (5 mg in 0.25 ml 1.0% sodium carbonate) was added to the test substance (15 mg in 0.25 ml 1.0% sodium carbonate) and incubated at 37°C for 60 min. Aliquots (10 μl) were removed

at different times, diluted with 0.1 ml distilled water and 0.9 ml methanol, and analyzed by LC–UV and LC–MS. Similarly, adduct formation was investigated by incubating glycine with sideroxylonal, torquatone or benzaldehyde.

Isolation of Adducts. Larger quantities of jensenone were reacted with glutathione, ethanolamine, or acetylcysteine, and the products acidified and extracted into ethyl acetate. Purification was by thin layer chromatography (silica gel) and crystallization (from ethyl acetate or methanol), as appropriate.

Rates of Reaction of Jensenone and Sideroxylonal with Glycine: Effect of pH. Because sideroxylonal is less soluble than jensenone in aqueous systems, lower concentrations and nonaqueous solvents were used to compare the reactivities of jensenone and sideroxylonal with glycine at different pH values. Two buffers were used: 0.067 M phosphate buffer for pH 7 and 8, and 0.1 M carbonate buffer for pH 9, 10, and 11. Jensenone (0.3 mg in 1.2 ml buffer) was added to 1.8 ml buffer containing 0.9 mg glycine and incubated at 25°C. Because of its insolubility, sideroxylonal was dissolved in ethyl acetate (1 mg/ml). Reactions were started by mixing 0. 3 ml (0.3 mg) of this solution with 0. 9 ml methanol, and adding it to 1.8 ml buffer containing 0.9 mg glycine and incubating as above. Methanol was omitted from the jensenone reaction because it caused LC peak broadening. Aliquots (100 μl) of each reaction mixture were removed at different times, mixed with methanol (200 μl), and analyzed by LC–UV. Sufficient 0.5 M HCl was included in the 200 μl methanol to neutralize the reaction mixture. As a check on stability, controls were run with glycine omitted.

Response of the Guinea Pig Ileum to Jensenone. A segment of ileum was taken from a guinea pig which had just been euthanized with pentobarbitone, and suspended in a 10 ml organ bath in Tyrode's solution at 37°C and gassed with air. Sublimed jensenone was dissolved in 0.5% sodium carbonate (10 mg $\cdot$ ml^{-1}) and diluted with buffer (pH 7). Histamine solutions were prepared in Tyrode's solution.

Effects of Jensenone on Histology of Rat Tissues. Hooded Wistar rats (male, 200–250 g) were starved overnight and gavaged the next morning with jensenone (10 mg in 1 ml 0.5% sodium carbonate, pH adjusted to 7–8) or vehicle (controls). The animals were euthanized (pentobarbitone) after 2, 6, and 24 hr, and tissues (stomach, duodenum, ileum, liver, kidney) taken for examination and placed in buffered formalin. Tissues were stained with haematoxylin and eosin before examination by light microscopy.

RESULTS

Metabolic Fate of Jensenone In Vivo. In several experiments, no trace of jensenone or related products was detected in the urine or feces of possums that had ingested jensenone. Excreta were extracted (before and after incubation with β-glucuronidase/arylsulfatase) and analyzed by GC–FID, GC–MS, and LC–UV.

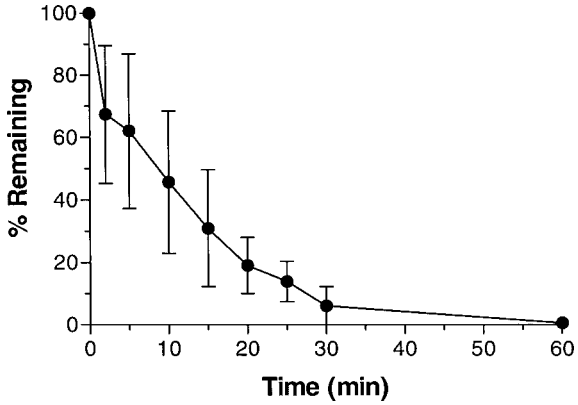

FIG. 2. Disappearance of jensenone from rat ileum (mean ± SE, $N = 3$).

Comparison with excreta from undosed animals showed that there were no new peaks in chromatograms from animals that had ingested jensenone.

Disappearance of Jensenone from Rat Gastrointestinal Tissue In Vitro. Having failed to find evidence of the excretion of jensenone or its metabolites, the fate of jensenone was investigated in isolated segments of rat ileum. The gut responded with vigorous activity and the contents became thick with mucus. Jensenone disappeared rapidly from the lumen (Figure 2), although none was detected in the outer bath. In similar experiments, jensenone also rapidly disappeared from the isolated rat stomach. After the disappearance of jensenone, variable amounts (8–33%) could be recovered after heating samples of tissue with sodium hydroxide.

Reactivity of Jensenone. Jensenone was stable in 2% sodium carbonate solution, but disappeared slowly when stored in methanol. In order to explore the possible causes of the disappearance of jensenone from gastrointestinal segments, it was incubated with various endogenous molecules: a gastrointestinal enzyme (trypsin), a protein (albumin), and two nucleophilic thiols (glutathione and acetylcysteine) known to protect cells against reactive electrophiles (De Vries and De Flora, 1993; Dickinson and Forman, 2002). Figure 3 shows that jensenone disappeared rapidly when incubated with trypsin (half life, $t_{1/2} = 5.4$ min) or glutathione ($t_{1/2} = 2.6$ min), and more slowly with albumin ($t_{1/2} = 91$ min) or acetylcysteine ($t_{1/2} = 69$ min).

Formation of Adducts. The reaction of glutathione and jensenone was analyzed after 60 min by LC–MS (Figure 4A). The monoadduct of glutathione and jensenone eluted at 6.9 min. It was characterized by the following ions: m/z 554 ($[M-H]^-$), 510 ($[M-H-CO_2]^-$), 476 ($[M-H-CO_2-H_2S]^-$), and sodium adducts m/z 576 and 598 (Figure 4B). The loss of H_2S indicated that the thiol

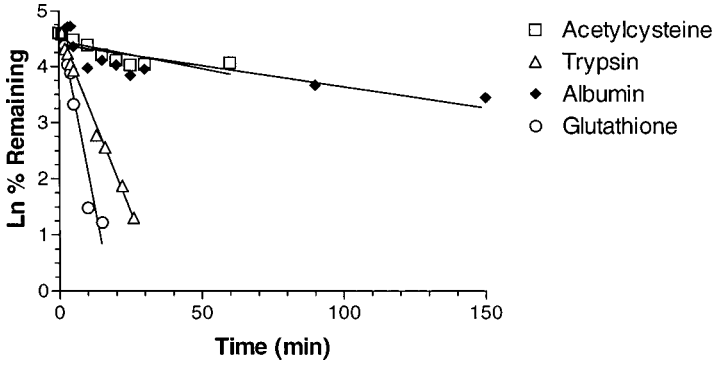

FIG. 3. Disappearance of jensenone when incubated with various compounds.

group was not involved in the bond between jensenone and glutathione. There was also a bis-adduct (jensenone and two glutathiones), which eluted at 3.01 min and showed characteristic ions in the same pattern. These were: m/z 843 ($[M-H]^-$), 799 ($[M-H-CO_2]^-$), 755 ($[M-H-2CO_2]^-$), and 721 ($[M-H-2CO_2-H_2S]^-$). However, the major LC peak (eluting at 2.68 min) had a molecular weight two daltons less than this and was interpreted as the bis-adduct with an internal disulfide bond, formed by oxidative linkage of the two glutathione sulphur atoms (Figure 5). It gave ions analogous to the bis-adduct following the loss of two protons: m/z 841 ($[M-H]^-$) and sequential losses of CO_2 to give ions at m/z 797 and 753, followed by loss of H_2S to give an ion at m/z 719 (Figure 4C). Figure 6 shows the sequential formation of mono- and bis-adducts of glutathione and jensenone. The presence of methanol resulted in the formation of methyl esters of the glutathione carboxylate groups, which were identified by MS.

Jensenone readily reacted with L-cysteine, glycine, and ethanolamine, in each case giving one major product by LC–MS, with the general formula: (jensenone + 2 amine $-2H_2O$). With acetylcysteine, jensenone produced one major product with an elution time of 2.56 min (LC System I). The negative ion electrospray mass spectrum gave an $[M-H]^-$ion at m/z 237 corresponding to a molecule consistent with (jensenone − CO).

Glycine readily reacted with sideroxylonal. The sideroxylonal–monoglycine adduct (MW 557) produced an ion at m/z 556 ($[M-H]^-$) and fragments at m/z 249 (without glycine) and 305 (i.e., m/z 249 with glycine). Sideroxylonal has four aldehyde groups, and there was clear evidence of sideroxylonal linking to two and three glycines, producing $[M-H]^-$ ions at m/z 613 and 670, respectively.

There was no reaction when benzaldehyde was incubated with glycine at 37°C (pH 7 or 8) for 24 hr. There was also no reaction of glycine with torquatone, a phloroglucinol derivative that lacks aldehyde groups (Figure 1).

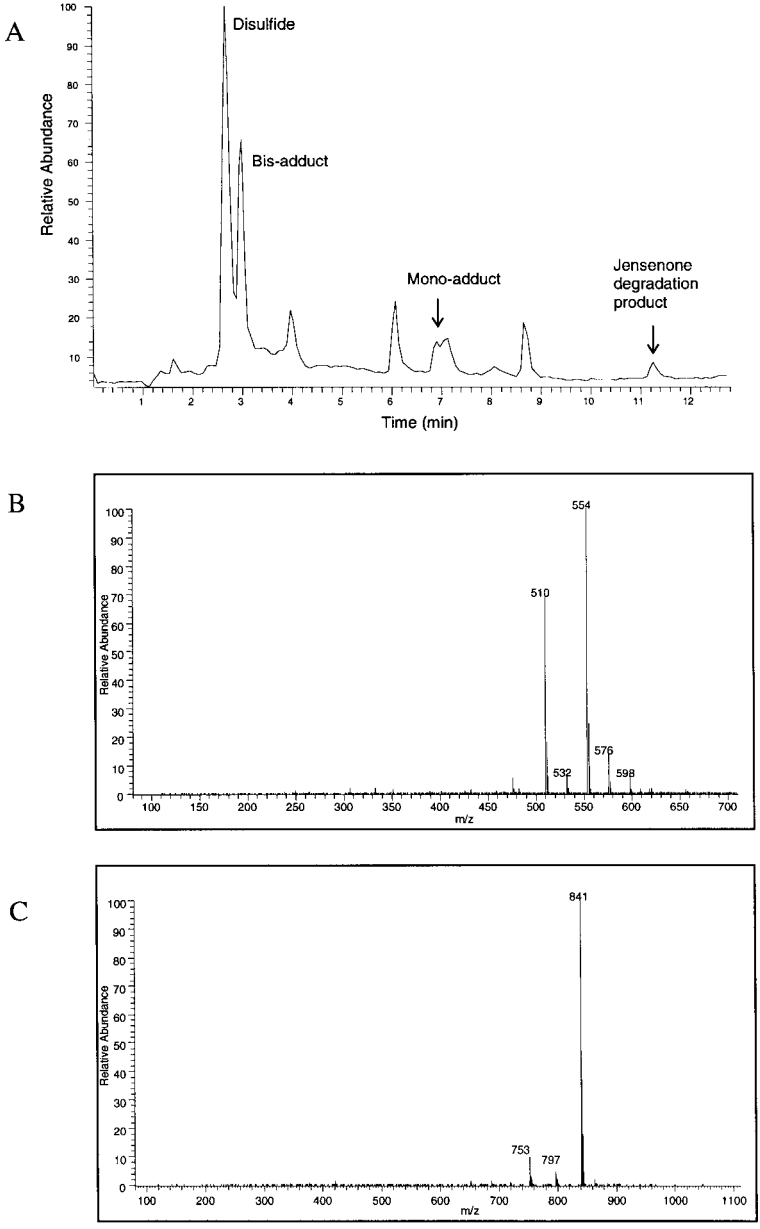

FIG. 4. (A) Negative ion ESI LC-MS chromatogram of glutathione adducts of jensenone. (B) Mass spectrum of mono-adduct (6.9 min). (C) Mass spectrum of bis-glutathione adduct with disulfide bridge (2.7 min).

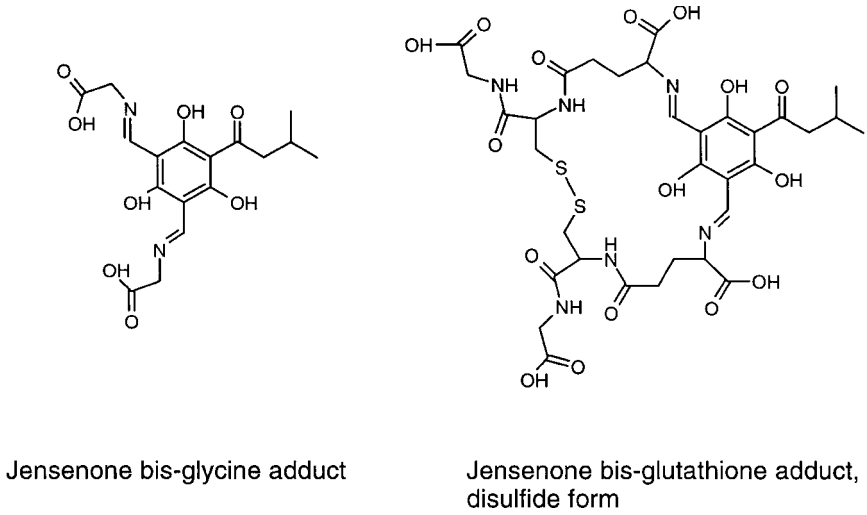

FIG. 5. Proposed structures of jensenone adducts with glycine and glutathione.

Isolation of Adducts. Attempts were made to prepare sufficient quantities of the adducts to enable NMR analysis. This was attempted with the adducts formed with glutathione and ethanolamine. However, these jensenone derivatives proved to be unstable during workup and storage. Also, the NMR data obtained indicated that the derivatives existed in two interchangable forms, resulting in a lack of a clear NMR spectrum. As this interchangability probably involves the phenolic protons that can hydrogen-bond with the carbonyl oxygens in two forms (as in jensenone

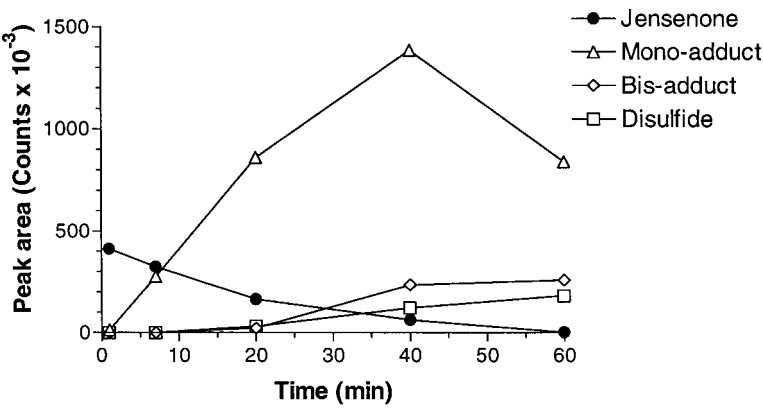

FIG. 6. Rates of formation of adducts of jensenone and glutathione.

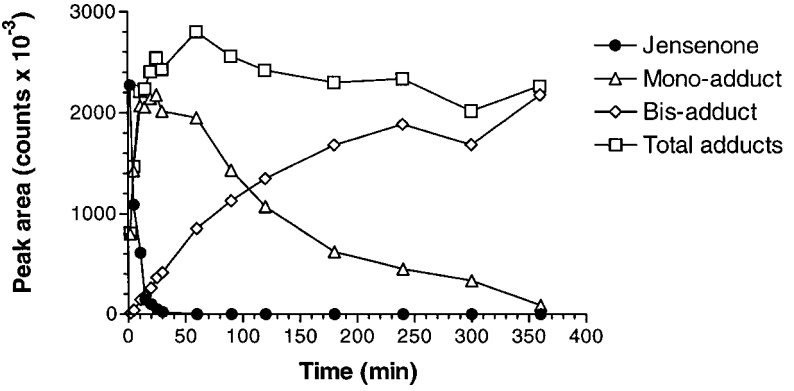

FIG. 7. Reaction of jensenone with glycine at pH 8.

itself; Boland et al., 1992; Ghisalberti, 1996), attempts were made to remove them by methylation, but this resulted in the methyl esters of the glutathione portion. Acetylation of the phenolic groups was also attempted, but this gave rise to multiple products.

Attempts were also made to prepare and characterize the product of acetylcysteine and jensenone. However, it was an orange oil that did not give a clear NMR signal.

Rates of Reaction of Jensenone and Sideroxylonal with Glycine: Effect of pH. The reactivity of jensenone and sideroxylonal were compared at different pH values, using glycine as a simple biological nucleophile. As an example, the reaction of jensenone and glycine at pH 8 is shown in Figure 7. The sequential formation of mono- and bis-adducts followed the disappearance of jensenone. The glycine adducts of sideroxylonal did not resolve by LC so the reaction was followed as the disappearance of sideroxylonal. Control incubations in buffer without glycine showed that there was no significant loss of jensenone or sideroxylonal during the incubation period.

Jensenone and sideroxylonal reacted with glycine over the pH range 7–11, and reacted faster at higher pH values. The half-life of jensenone fell from 234 min at pH 7 to 33 min at pH 11, while the corresponding half-lives for sideroxylonal were 140 min and 41 min, respectively. In each case, a plot of ln (half-life) versus pH gave a straight line: jensenone slope -0.521 ± 0.056 (mean $\pm$ SD, $N = 5$), $r^2 = 0.967$ and sideroxylonal slope -0.291 ± 0.026, $r^2 = 0.967$. These slopes were significantly different ($P < 0.01$, ANCOVA).

Response of the Guinea Pig Ileum to Jensenone. Because of the increased motor activity observed in the rat gut segments containing jensenone, the contractile response to jensenone was studied by using the standard guinea pig ileum preparation.

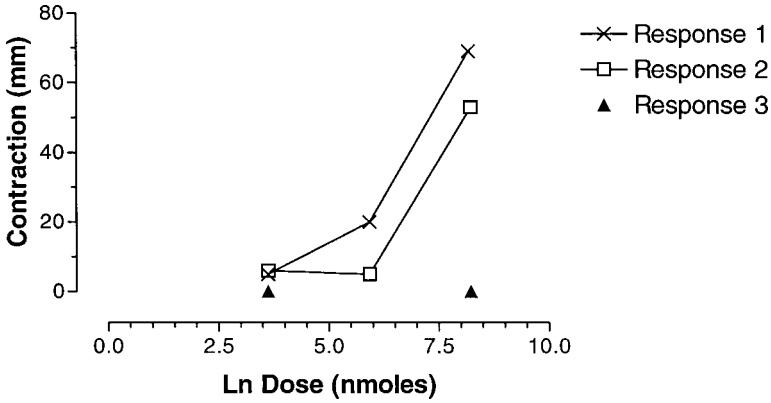

FIG. 8. Response of the guinea pig ileum to three doses of jensenone. The low dose (38 nmoles) was given three times, the middle dose (376 nmoles) was given twice, then the high dose (3760 nmoles) was given three times.

The ileum contacted when jensenone was injected into the bath water, but high concentrations were required (3.8 μmoles in the 10 ml organ bath). The same maximal contraction was produced by only 1.0 nmole histamine. The response to a dose of jensenone was not sustained (i.e., the contraction diminished with time) whereas the histamine response stayed constant until the tissue was washed. Repeated dosing with jensenone resulted in a diminishing response (Figure 8). In another segment of ileum, repeated administration of the high dose (1 mg, 3760 nmoles) produced a response that fell progressively from a maximum to zero in four repeats. The response to histamine was also decreased by jensenone treatment (data not shown).

The rapidly-decreasing response to jensenone made it difficult to assess the effect of possible antagonists. However, there was no evidence that a large dose of atropine (100 nmoles; sufficient to abolish the maximum response elicited by histamine, 1.0 nmoles) decreased the response to jensenone.

Effects of Jensenone on Histology of Rat Tissues. In comparison with controls, the jensenone-dosed rats lay quietly, flat on the stomach, and tended to have a wet muzzle, evidently due to salivation. These effects developed soon after the treatment, but the animals had recovered normal behavior by 6 hr. Upon opening the abdomen after euthanasia at 2 and 6 hr, the stomach and small intestine appeared distended by the jensenone dose. Tissue slides indicated some vacuolation at the tip of the villi, possible loss of villi and sloughing of cells. However, these findings were equivocal, and all other tissues appeared normal.

DISCUSSION

The initial objective of this study was to describe the metabolic fate of jensenone, a plant secondary metabolite (PSM) with a powerful antifeedant effect. Jensenone was considered to be a model for other antifeedant FPCs such as sideroxylonal. We have previously used the same general analytical methodology to describe the metabolites of terpene and phenolic PSMs in marsupial folivores (Boyle et al., 1999, 2000a,b, 2001; McLean et al., 2001, 2003). However, despite a thorough investigation, no evidence of jensenone or related compounds was found in the urine or feces of brushtail or ringtail possums that had ingested large amounts of jensenone. Either the jensenone was so chemically changed that it was unrecognizable, or it was sequestered at tissue sites. Incubation of jensenone in isolated segments of rat gastrointestinal tissue showed that it rapidly disappeared, indicating that there was a chemical reaction between jensenone and tissue sites.

Jensenone possesses two aldehyde and three phenolic groups. Aldehydes are reactive electrophilic molecules that undergo nucleophilic addition reactions (March, 1992) and are known to form covalent adducts with biological molecules. For example, the toxicity of ethanol is in part mediated by its metabolite, acetaldehyde, which forms adducts with proteins, leading to tissue injury (Lieber, 1991). Several potential target molecules were examined for their reaction with jensenone. The pancreatic enzyme, trypsin, is a protein that ingested jensenone would encounter in the small intestine. Albumin was assessed as another protein and one that jensenone would come into contact with if it were absorbed into the bloodstream. Both these proteins reacted with jensenone, albeit slowly in the case of albumin. Reaction with these proteins cannot explain the rapid disappearance of jensenone from the *in vitro* gut system, where they are absent, although other proteins could be involved. Smaller biological nucleophiles were then examined to elucidate the chemistry of jensenone reactivity.

Glutathione is a tripeptide that reacts with an enormous array of electrophilic xenobiotics to protect cellular nucleophiles (proteins and nucleic acids) from attack by toxic molecules (Parkinson, 2001). Electrophiles generally bind to the thiol group of reduced glutathione giving rise to a thio-adduct that can be subsequently converted into a mercapturic acid or other thioether metabolites (Parkinson, 2001). Although the initial reaction is catalyzed by a family of glutathione-*S*-transferases that are present at high levels, in the gut, liver, and other tissues, the electrophilic substrates typically also react nonenzymatically with glutathione (Ketterer, 1982, 1986). Acetylcysteine is another thiol nucleophile that undergoes similar reactions to glutathione and is used therapeutically to protect against electrophilic toxicity, most notably in the case of paracetamol overdose (Forrest et al., 1982; De Vries and De Flora, 1993). Jensenone reacted very rapidly with glutathione, and this was

initially interpreted as thioadduct formation. Jensenone reacted readily with the thio-amino acid cysteine that like glutathione, has both a thiol and amino group free. However, jensenone reacted much more slowly with acetylcysteine. The product of this reaction, equivalent to (jensenone − CO), was not analogous to the adducts formed with other nucleophiles. Acetylcysteine has a free thiol group, but the amine is blocked by acetylation. This was the first indication that the reaction with jensenone may involve amines rather than thiols, and was confirmed by the observation that jensenone reacted rapidly with the thiol-free amines, glycine, and ethanolamine.

MS analyses were consistent with the sequential formation of Schiff base adducts on each of the aldehyde groups of jensenone and sideroxylonal. There was MS evidence that the bis-glutathione–jensenone adduct cyclized to form the intramolecular disulfide, which also indicated that the glutathione thiols remained free after adduct formation.

Although aldehydes can react with thiols to form thiohemiacetals, they are also able to undergo addition reactions with amines to form a Schiff base (March, 1992). Schiff bases are most stable when conjugated with an aromatic group, but they also occur between nonaromatic biological molecules, for example acetaldehyde and lysine (Braun et al., 1995). On the other hand, glutathione reportedly forms a hemithioacetal with acetaldehyde (Ketterer, 1982), dichloroacetaldehyde (Guengerich and Liebler, 1985), and formaldehyde (Mason et al., 1986; Naylor et al., 1988). More often, though, the carbonyl group activates an adjacent double bond leading to a Michael addition reaction and thioether formation (Parkinson, 2001). This can occur at an aromatic ring, as in the reactive metabolite of paracetamol, *N*-acetylbenzoquinoneimine. We found no nonenzymatic reaction between benzaldehyde and glycine, although there is evidence that rat liver preparations can catalyze the formation of the *S*-benzyl glutathione conjugate from benzaldehyde (Mutlib et al., 2002).

The two aldehyde groups of ortho-phthalaldehyde react under alkaline conditions with both the amine and thiol groups of glutathione to form a cyclic derivative, which can be used to quantitate glutathione (Neuschwander-Tetri and Roll, 1989). An extensive search of the literature did not reveal any other reports of Schiff base formation with glutathione.

The sesquiterpene 1,4-dialdehyde polygodial reacts rapidly with cysteine, and its antifeedant properties, and hot taste, have been attributed to interactions between its aldehyde groups and tissue thiols (Kubo and Ganjian, 1981). However, analysis of the product of this reaction indicated that it first formed a Schiff base between an aldehyde group and the cysteine amine, followed by addition of the cysteine thiol group to the double bond, giving a thiazolidine ring (D'Ischia et al., 1982). This is a known reaction of aldehydes with cysteine (Jocelyn, 1972).

The question of why jensenone reacts preferentially with the amine rather than the thiol group of glutathione may be explained by the hard–soft theory

of reactivity (Guengerich and Liebler, 1985; Ketterer, 1986). Hard electrophiles (or nucleophiles) have a dense localization of charge, whereas the soft forms have a low charge density that is readily polarizable. Thiols are soft nucleophiles because of the large atomic volume of sulphur, whereas amines are relatively hard nucleophiles. Carbonyls are hard electrophiles, which react more readily with hard nucleophiles, while soft nucleophiles react with soft electrophiles, in both cases because the energy of activation is lower (Ketterer, 1986).

The adduct formation was faster at higher pHs for jensenone and sideroxylonal, supporting a base-catalyzed mechanism, with the amine in the nonprotonated form (March, 1992). The reactions proceeded rapidly over the pH range tested (pH 7–11), indicating that adduct formation will occur at all pHs likely to be encountered in the mammalian intestine.

The chemical (and biological) activity of aldehydes varies greatly, even within a structurally-similar group such as the unsaturated 1,4-dialdehydes (Jonassohn and Sterner, 1997). In these experiments, we found that benzaldehyde did not react with glycine under the conditions where jensenone and sideroxylonal reacted rapidly and completely. On the other hand, the sesquiterpene 1,4-dialdehyde polygodial reacts readily with primary amines to form stable pyrrole derivatives (Brooks et al., 1989; Jonassohn and Sterner, 1997). Gossypol, an insect antifeedant compound found in cotton plants, is a sesquiterpene dimer with two aldehyde groups that readily form imines with the amino groups of proteins (Gershenzon and Croteau, 1991). It is proposed that gossypol acts by binding dietary protein (making it less digestible) and/or inhibiting digestive enzymes.

The relatively high dose of jensenone required for its biological effects suggests a nonspecific mechanism, involving reaction with amino groups on various biological molecules causing enzyme inhibition and toxicity. Inflammatory mediators such as 5HT have been implicated in the antifeedant effects of jensenone (Lawler et al., 1998b). However, some sesquiterpene unsaturated dialdehydes appear to activate sensory neurons by stimulation of vanilloid receptors (VR) (Szallasi and Blumberg, 1999). VR respond to painful chemical stimuli, such as capsaicin, leading to pain and inflammation. It is possible that at least part of the effects of jensenone are mediated via VR.

The contractile response of the guinea pig ileum did not appear to be mediated through acetylcholine or histamine receptors, since it was not sustained nor was it blocked by a large dose of atropine. In fact, the low potency of jensenone suggests a nonspecific mechanism, and tachyphylaxis suggests rapid depletion of component(s) necessary for the response. Since the histamine response was also diminished, this points to a general mechanism, possibly involving the depletion of enzymes or energy required for contraction.

Although rats appeared sick after an oral dose of jensenone, they had regained normal behavior by 6 hr, and there was no clear histological evidence of damage to the upper gastrointestinal tract. Therefore, although jensenone (and presumably

related FPCs) can covalently bind to tissue amines, its effects were not irreversible, at least at the dose assessed.

Reactive electrophiles can form adducts with proteins via either of the two major nucleophilic groups, the cysteinyl thiol and lysinyl ε-amino group (Guengerich and Liebler, 1985). Glutathione possesses both these nucleophilic groups, and so may be able to react with both hard and soft electrophiles. Glutathione is present at high concentrations in cells (e.g., about 10 mM in the liver) and its capacity to form amine-adducts may indicate a hitherto unrecognized capability to inactivate hard electrophiles such as certain aldehydes like the FPCs. This appears to be the first report of glutathione forming an adduct with an electrophilic xenobiotic via its amine group rather than the thiol.

The findings of this study indicate that the cellular target of antifeedant FPCs are amino groups on critical molecules, such as lysinyl ε-amino groups on enzymes. The facile binding of FPCs, and absence of their metabolites in excreta, suggest that the antifeedant effects occur in the gastrointestinal tract itself. Loss of enzyme function may lead to altered cellular metabolism and the release of chemical mediators such as 5HT, resulting in contraction of gastrointestinal smooth muscle and stimulation of sensory neurons. The animal may experience colic, nausea, and a general malaise, all contributing to the antifeedant effect of FPCs.

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REFERENCES

BOLAND, D. J., BROPHY, J. J., and FOOKES, C. J. R. 1992. Jensenone, a ketone from *Eucalyptus jensenii*. *Phytochemistry* 31:2178–2179.

BOYLE, R., MCLEAN, S., and DAVIES, N. W. 2000a. Biotransformation of 1,8-cineole in the brushtail possum (*Trichosurus vulpecula*). *Xenobiotica* 30:915–932.

BOYLE, R., MCLEAN, S., FOLEY, W. J., and DAVIES, N. W. 1999. Comparative metabolism of dietary terpene, *p*-cymene, in generalist and specialist folivorous marsupials. *J. Chem. Ecol.* 25:2109–2126.

BOYLE, R., MCLEAN, S., FOLEY, W., DAVIES, N. W., PEACOCK, E. J., and MOORE, B. 2001. Metabolites of dietary 1,8-cineole in the male koala (*Phascolarctos cinereus*). *Comp. Biochem. Physiol. C-Toxicol. Pharmacol.* 129:385–395.

BOYLE, R., MCLEAN, S., FOLEY, W. J., MOORE, B. D., DAVIES, N. W., and BRANDON, S. 2000b. Fate of the dietary terpene, p-cymene, in the male koala. *J. Chem. Ecol.* 25:1095–1111.

BRAUN, K. P., CODY, R. B., JONES, D. R., and PETERSON, C. M. 1995. A structural assignment for a stable acetaldehyde-lysine adduct. *J. Biol. Chem.* 270:11263–11266.

BROOKS, C. J. W., COLE, W. J., and BRINDLE, P. A. 1989. Characterization of products formed by condensations of primary amines with polygodial, and their scope for enantiomer analyses. *J. Chromatogr.* 468:201–213.

DE VRIES, N. and DE FLORA, S. 1993. N-Acetyl-l-cysteine. *J. Cell. Biochem.* 17:270–277.

DICKINSON, D. A. and FORMAN, H. J. 2002. Cellular glutathione and thiols metabolism. *Biochem. Pharmacol.* 64:1019–1026.

D'ISCHIA, M., PROTA, G., and SODANO, G. 1982. Reaction of polygodial with primary amines: An alternative explanation to the antifeedant activity. *Tetrahedron Lett.* 23:3295–3298.

ESCHLER, B. M. and FOLEY, W. J. 1999. A new sideroxylonal from *Eucalyptus melliodora*. *Aust. J. Chem.* 52:157–158.

FOLEY, W. J., IASON, G. R., and MCARTHUR, C. (1999). Role of plant secondary metabolites in the nutritional ecology of mammalian herbivores: How far have we come in 25 years? pp. 130–209, *in* H.-J. G. Jung and G. C. Fahey (eds.). Nutritional Ecology of Herbivores. Proceedings of the vth International Symposium on the Nutrition of Herbivores, San Antonio, TX. American Society of Animal Science, Savoy, IIL.

FORREST, J. A. H., CLEMENTS, J. A., and PRESCOTT, L. F. 1982. Clinical pharmacokinetics of paracetamol. *Clin. Pharmacokinet.* 7:93–107.

GERSHENZON, J. and CROTEAU, R. 1991. Terpenoids, pp. 165–219, *in* G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores: Their Interactions with Secondary Plant Metabolites. Academic Press, San Diego, CA.

GHISALBERTI, E. L. 1996. Bioactive acylphloroglucinol derivatives from *Eucalyptus* species. *Phytochemistry* 41:7–22.

GUENGERICH, F. P. and LIEBLER, D. C. 1985. Enzymic activation of chemicals to toxic metabolites. *Crit. Rev. Toxicol.* 14:259–307.

JOCELYN, P. C. 1972. Chemical reactions of thiols, pp. 63–93, *in* Biochemistry of the SH Group. Academic Press, London.

JONASSOHN, M. and STERNER, O. 1997. Terpenoid unsaturated 1,4-dialdehydes, occurrence and biological activities. *Trends Org. Chem.* 6:23–43.

KETTERER, B. 1982. The role of nonenzymatic reactions of glutathione in xenobiotic metabolism. *Drug Metab. Rev.* 13:161–187.

KETTERER, B. 1986. Detoxication reactions of glutathione and glutathione transferases. *Xenobiotica* 16:957–973.

KUBO, I. and GANJIAN, I. 1981. Insect antifeedant terpenes, hot-tasting to humans. *Experientia* 37:1063–1064.

LAWLER, I. R., ESCHLER, B. M., SCHLIEBS, D. M., and FOLEY, W. J. 1999a. Relationship between chemical functional groups on *Eucalyptus* secondary metabolites and their effectiveness as marsupial antifeedants. *J. Chem. Ecol.* 25:2561–2573.

LAWLER, I. R., FOLEY, W. J., and ESCHLER, B. M. 2000. Foliar concentration of a single toxin creates habitat patchiness for a marsupial folivore. *Ecology* 81:1327–1338.

LAWLER, I. R., FOLEY, W. J., ESCHLER, B. M., PASS, D. M., and HANDASYDE, K. 1998a. Intraspecific variation in *Eucalyptus* secondary metabolites determines food intake by folivorous marsupials. *Oecologia* 116:160–169.

LAWLER, I. R., FOLEY, W. J., PASS, G. J., and ESCHLER, B. M. 1998b. Administration of a 5HT(3) receptor antagonist increases the intake of diets containing *Eucalyptus* secondary metabolites by marsupials. *J. Comp. Physiol. B-Biochem. Systemic Environ. Physiol.* 168:611–618.

LAWLER, I. R., STAPLEY, J., FOLEY, W. J., and ESCHLER, B. M. 1999b. Ecological example of conditioned flavor aversion in plant–herbivore interactions: Effect of terpenes of *Eucalyptus* leaves on feeding by common ringtail and brushtail possums. *J. Chem. Ecol.* 25:401–415.

LIEBER, C. S. 1991. Hepatic, metabolic and toxic effects of ethanol—1991 update. *Alcohol. Clin. Exp. Res.* 15:573–592.

MARCH, J. 1992. Advanced Organic Chemistry: Reactions, Mechanisms and Structure. Wiley, New York.

MASON, R. P., SANDERS, J. K. M., CRAWFORD, A., and HUNTER, B. K. 1986. Formaldehyde metabolism by *Escherichia coli*. Detection by *in vivo* carbon-13 NMR spectroscopy of S-(hydroxymethyl)glutathione as a transient intracellular intermediate. *Biochemistry* 25:4504–4507.

MCLEAN, S., BRANDON, S., DAVIES, N. W., BOYLE, R., FOLEY, W., MOORE, B., and PASS, G. J. 2003. Glucuronuria in the koala. *J. Chem. Ecol.* 29:1465–1477.

MCLEAN, S., PASS, G. J., FOLEY, W. J., BRANDON, S., and DAVIES, N. W. 2001. Does excretion of secondary metabolites always involve a measurable metabolic cost? Fate of plant antifeedant salicin in common brushtail possum, *Trichosurus vulpecula*. *J. Chem. Ecol.* 27:1077–1089.

MUTLIB, A. E., DICKENSON, P., CHEN, S. Y., ESPINA, R. J., DANIELS, J. S., and GAN, L. S. 2002. Bioactivation of benzylamine to reactive intermediates in rodents: Formation of glutathione, glutamate, and peptide conjugates. *Chem. Res. Toxicol.* 15:1190–1207.

NAYLOR, S., MASON, R. P., SANDERS, J. K. M., WILLIAMS, D. H., and MONETI, G. 1988. Formaldehyde adducts of glutathione. Structure elucidation by two-dimensional NMR spectroscopy and fast-atom-bombardment tandem mass spectrometry. *Biochem. J.* 249:573–579.

NEUSCHWANDER-TETRI, B. A. and ROLL, F. J. 1989. Glutathione measurement by high-performance liquid chromatography separation and fluorometric detection of the glutathione-orthophthalaldehyde adduct. *Anal. Biochem.* 179:236–241.

NEVE, J., LEONE, P. D., CARROLL, A. R., MONI, R. W., PACZKOWSKI, N. J., PIERENS, G., BJORQUIST, P., DEINUM, J., EHNEBOM, J., INGHARDT, T., GUYMER, G., GRIMSHAW, P., and QUINN, R. J. 1999. Sideroxylonal C, a new inhibitor of human plasminogen activator inhibitor type-1, from the flowers of *Eucalyptus albens*. *J. Nat. Prod.* 62:324–326.

PARKINSON, A. 2001. Biotransformation of xenobiotics, pp. 133–224, *in* C. D. Klaasen (ed.). Casarett and Doull's Toxicology: The Basic Science of Poisons. McGraw-Hill, New York.

STAPLEY, J., FOLEY, W. J., CUNNINGHAM, R., and ESCHLER, B. 2000. How well can common brushtail possums regulate their intake of *Eucalyptus* toxins? *J. Comp. Physiol. B-Biochem. Systemic Environ. Physiol.* 170:211–218.

SZALLASI, A. and BLUMBERG, P. M. 1999. Vanilloid (capsaicin) receptors and mechanisms. *Pharmacol. Rev.* 51:159–211.