RELATIONSHIP BETWEEN CHEMICAL FUNCTIONAL GROUPS ON *Eucalyptus* SECONDARY METABOLITES AND THEIR EFFECTIVENESS AS MARSUPIAL ANTIPEEDEANTS

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Abstract—*Eucalyptus* displays strong intraspecific variation in resistance to browsing by marsupial folivores that can be attributed to variation in the concentration and type of diformylphloroglucinol compounds (DFPCs) in the foliage. In this study, we ask which functional groups of diformylphloroglucinol compounds determine their effectiveness in deterring feeding. We used a simple and highly deterrent compound, jensenone, as a model DFPC and compared its activity to structural variants that differ in the types of functional groups on the phloroglucinol molecule. Torquatone, a naturally occurring compound in the steam volatile fraction of *Eucalyptus torquata* foliage, has neither the aldehyde nor phenol groups that are believed to contribute to the antifeedant actions of jensenone. From the naturally occurring compounds we have synthesized two intermediates, a capped phenol/free aldehyde compound (acetyl-jensenone) and a free phenol/no aldehyde compound (demethyl-torquatone). Addition of jensenone and acetyl-jensenone to diets of common ringtail possums (*Pseudocheirus peregrinus*) substantially reduced their food intakes. Torquatone showed less activity, and there was little reduction in food intake when demethyl-torquatone was added to the diet. We conclude that at least the aldehyde groups attached to the aromatic nucleus are important in determining whether these compounds deter feeding by common ringtail possums, whereas the phenol groups may play only a minor role.

Key Words—*Eucalyptus*, *Pseudocheirus peregrinus*, jensenone, torquatone, plant secondary metabolite, feeding deterrent.

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INTRODUCTION

There have been many studies of the role of plant secondary metabolites (PSMs) in deterring feeding by mammalian herbivores. However, most have not quantified specific compounds but have used broad-scale assays of groups of compounds that share a similar functional group (such as phenolics (Oates et al., 1980; Cork, 1992; Kool, 1992; Hodar and Palo, 1997)) and/or react in a particular way with other dietary constituents (e.g., proteins and tannins (Provenza et al., 1990; Hume and Esson, 1993; McArthur and Sanson, 1993; Dearing, 1997)). Use of these measures has rarely resulted in clear-cut patterns because of differences in the activity of individual compounds that arise directly as a consequence of the particular molecular structure (Zucker, 1983; Waterman and Kool, 1994; Ayres et al., 1997). Increased understanding of the importance of PSMs in plant–herbivore interactions requires that we both identify and quantify, individually, the most active compounds.

Recent work on interactions between Eucalyptus and its marsupial folivores has shown that a newly discovered group of PSMs, the diformylphloroglucinol compounds (DFPCs), plays an important role in deterring feeding by marsupials (Lawler et al., 1998a,b; 1999a,b; Pass et al., 1998). Where our methods have progressed far enough to quantify individual compounds precisely [currently jensenone (Figure 1a) and sideroxylonals, dimers of jensenone (Figure 1e)], we have been able to show that in Eucalyptus species in which these are the predominant DFPCs they explain the majority of variation in feeding by marsupial folivores among individual trees (Lawler et al., 1999a; Lawler, unpublished data). However, in species where we cannot quantify all individual components, estimates of total DFPCs do not correlate nearly so well with feeding (Lawler et al., 1998a), suggesting that there is significant variation between different DFPCs in their deterrent activities.

A range of compounds based on acylated phloroglucinol with an isoprene or terpene side chain has been isolated from Eucalyptus foliage (Ghisalberti, 1996). All of the compounds thus far shown to be active against marsupial feeding have been DFPCs, characterized by two formyl (aldehyde —CHO) groups attached to the phloroglucinol molecule, but differing in the identity of and nature of bonding to the isoprene/terpene side chain (Figure 1a, e, f, g). Similarities in the levels of activity of macropcarpal G (Figure 1g), sideroxylonals, and jensenone (Lawler et al., 1998a,b, 1999a) and the much lower activity of torquatone (Figure 1b) have led us to suspect that it is the presence of aldehyde and/or phenol groups that determines and increases the deterrent activity of the DFPCs. The lower activity of Euglobals (Pass et al., 1998) that have an ether linkage to the terpene side chain (Figure 1f), relative to macropcarpals that have free hydroxyls in both positions ortho to the terpene, also may indicate that the nature of the linkage between the terpene side chain and the phloroglucinol molecule and its effects on H-bonding are important.
Fig. 1. Structures of compounds used in this study and related compounds known to act as antifeedants against folivorous marsupials.

In this study, we compared the effectiveness of four structurally similar and simple acylphloroglucinol derivatives in deterring feeding by common ringtail possums (*Pseudocheirus peregrinus*). Two of the compounds occur naturally in *Eucalyptus* foliage (jensenone and torquatone) (Figure 1a and b), whereas the
other two have been synthesized from these starting materials (Figure 1c and d). In these syntheses, we have removed or added functional groups with the intention of creating a series of compounds having the same basic structure and having both aldehyde and phenol groups (Figure 1a), neither aldehyde nor phenol groups (Figure 1b), phenol groups only (Figure 1d, demethyl-torquatone), and aldehyde groups only (Figure 1c, acetyl-jensenone). Note that the structure provided for acetyl-jensenone shows the \((\text{HO})_2\text{HC}\) group where the aldehyde group is expected. This is a \textit{gem}-diol, a group that is characterized by the ease with which it undergoes acidic cleavage (such as in an acid stomach) to revert to the aldehyde.

We conclude that the specific molecular structure is an important determinant of deterrent activity and that certain combinations of functional groups (e.g., aldehydes attached to aromatics) are fundamental to activity, but we do not yet have sufficient information on the metabolism of these compounds to identify the underlying mechanism that causes them to be such effective antifeedants.

**METHODS AND MATERIALS**

\textit{Animals.} This research was approved by the Animal Experimentation Ethics Committee of the Australian National University and conforms to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Twelve common ringtail possums (\textit{Pseudocheirus peregrinus}) were collected from \textit{Eucalyptus} woodlands and kept individually in metabolism cages as described by Lawler et al. (1998a) and maintained on a basal diet made fresh each day. The diet consisted of (% wet matter) 55.5% grated apple, 28.3% banana pulp, 4.7% lucerne hay ground to pass a 2-mm sieve, 5.5% ground rice hulls, 4.7% ground Weetbix (a wheat-based breakfast cereal), and 1.6% acid casein. All animals maintained body mass on this basal diet.

The effectiveness of each compound as a feeding deterrent was tested by offering a diet treated with each of the compounds separately in varying concentrations in a no-choice protocol (see below for experimental design). Treated food was offered at 18:00 hr and removed at 06:00 hr the following morning, when all animals were given the untreated basal diet. On alternating nights, the basal diet alone was offered to prevent carryover effects between experimental nights.

\textit{Analysis.} The four compounds tested were jensenone, torquatone, acetyl-jensenone, and demethyl-torquatone (Figure 1a–d). These represent a series of compounds with the same basic structure, but they vary in having phenol and aldehyde groups (jensenone), phenol (demethyl-torquatone), or aldehyde (acetyl-jensenone) groups, or neither phenol nor aldehyde groups (torquatone).

Jensenone and torquatone occur naturally in the foliage of \textit{Eucalyptus}
jensenii and E. torquata, respectively (Bowyer and Jefferies, 1959; Boland et al., 1992), and they were extracted directly from leaves, while the two other compounds were each synthesized from one of the naturally occurring compounds.

**Jensenone.** Eucalyptus jensenii foliage, air-dried and ground to pass a 2-mm screen, was extracted in 20% acetone–light petroleum in a Soxhlet apparatus. The extracts were concentrated and combined with 1 liter of diethyl ether and washed three times with 0.3 M NaOH. These washes were acidified with hydrochloric acid (10 M), and the precipitate was washed with ethanol and recrystallized from acetone to give spectroscopically pure jensenone as a colorless solid (approximately 1.0%) (see Lawler et al., 1998b for details).

**Torquatone.** Torquatone was extracted from fresh E. torquata foliage by steam distillation with cohabation (Foley et al., 1987). The resulting oil was loaded onto silica and eluted with 10% ether–light petroleum. The pure torquatone-bearing fractions were identified by thin layer chromatography (TLC) and combined. The solvent was then removed in vacuo to yield torquatone as a low-melting-temperature solid. Spectroscopic data were as for the published method (Ghisalberti et al., 1995).

**Acetyl-jensenone.** To a stirred solution of jensenone (20.0 g, 0.075 mol) and pyridine (22.5 ml) in dichloromethane (dry, 200 ml) was added acetic anhydride (57 ml, 7 eq). The reaction was monitored by thin-layer chromatography and, when all the jensenone was consumed, 200 ml water were added. The water layer was removed and the organic layer washed with water (2 × 200 ml), sodium bicarbonate solution (2%, 2 × 200 ml), water (2 × 200 ml), hydrochloric acid (1.0 M, 2 × 150 ml) and water (2 × 200 ml). The organic layer was dried (sodium sulfate), the solvent removed, and the residue further dried under high vacuum to give jensenone triacetate (21.26 g, 66%) as a pale oily solid. Spectroscopic data closely corresponded with published data (Boland et al., 1992).

**Demethyl-torquatone.** A solution of torquatone (38.20 g, 0.136 mol) was stirred in concentrated sulfuric acid (120 ml) for 5 hr, after which TLC (10% methanol–dichloromethane) indicated that the reaction had gone to completion. The reaction mixture was poured onto water–ice (200 g), and the resultant slurry stirred in an ice bath. The mixture was adjusted to pH 12 by direct addition of KOH pellets (caution!), allowed to warm to room temperature, and washed with dichloromethane (3 × 300 ml). The aqueous layer was adjusted to pH 4 by the careful addition of sulfuric acid (50%, ca. 160 ml) and extracted with dichloromethane (3 × 400 ml), dried (magnesium sulfate), and the solvent removed in vacuo to yield demethyl-torquatone as a reddish oil (32.06 g, 99%). $^1$H NMR (CDCl$_3$, $\delta$ 0.98 [6H, d, $-\text{C(O)CH}_2\text{CHCH}_3$], 2.07 [2H, d, $-\text{C(O)CH}_2\text{CH}_2\text{CH}_3$], 2.17 [1H, m, $-\text{C(O)CH}_2\text{CH}_2\text{CH}_3$], 2.23 (3H, s, Ar–CH$_3$), 2.25 [3H, s, ArCH$_3$]; MS (ES-MS) $m/z$ 239 (M+H$^+$) and 261 (M+Na$^+$_).
was dose-dependent, five concentrations of each plus a solvent-treated control for each compound were offered to the 12 animals over eight nights in a balanced alpha-crossover design, giving four replicates for each treatment (John and Williams, 1995). All compounds were tested together to prevent confounding of time with treatments. The extra controls were used to statistically balance the design. Parameters of the model, with compound and concentration as fixed effects, were estimated by Restricted Maximum Likelihood (REML) theory (Cunningham, personal communication). Data were tested first for linearity across concentrations within each compound and, when found not to depart significantly from linearity ($\chi^2 = 15.3, df = 12, P = 0.225$), the slopes of the dose-dependent linear relationships were compared.

Concentrations of each compound offered were chosen primarily on the basis of preliminary data for deterrence of each of the naturally occurring compounds, as there was insufficient material of each of the synthesized compounds available for substantial preliminary testing. Jensenone is highly deterrent to feeding (Lawler et al., 1998b, 1999b) and so was offered at low concentrations, whereas torquatone is substantially less deterrent (see below, Lawler unpublished) and was added at higher concentrations. The concentrations of both these compounds in the artificial diet were similar to those found in Eucalyptus foliage. We assumed that the alteration in numbers of functional groups on the benzene ring would alter the activity of the synthesized compounds and thus varied the concentrations of the two synthesized compounds relative to their parent compounds. We expected that acetyl-jensenone would be less active than jensenone, and we used slightly higher concentrations than those for jensenone. We expected that the phenol groups on demethyl-torquatone would increase its activity relative to torquatone, and we used slightly lower concentrations. Concentrations of each compound are summarized in Table 1.

RESULTS AND DISCUSSION

Clearly, the particular chemical structure of a compound is important in determining its effectiveness as a deterrent to feeding by common ringtail possums. There were distinct differences in the activity of each of the four compounds (Figure 2, $\chi^2 = 32.97, df = 3, P < 0.001$). Jensenone was the most effective feeding deterrent, while acetyl-jensenone also was highly deterrent. Torquatone showed substantially less activity, and demethyl-torquatone the least. The compounds tested here differ only in the nature of the functional groups attached to the benzene ring, yet there is an order of magnitude difference in the amount of compound required to produce the same reduction in food intake.

Given such variation in activity among these structurally similar compounds, it is apparent that conventional "total" phenolic assays are not appropr-
### Table 1. Concentrations of Each Compound Used in No-Choice Bioassay Experiment with Captive Ringtail Possums

<table>
<thead>
<tr>
<th></th>
<th>Jensenone</th>
<th>Acetyl-jensenone</th>
<th>Demethyl-torquatone</th>
<th>Torquatone</th>
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<tbody>
<tr>
<td>Concentration</td>
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<td>Concentration</td>
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<td>% wet matter</td>
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<td>μmol/g dry</td>
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<tr>
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<tr>
<td>266</td>
<td>7.52 0.06</td>
<td>428 13.63 0.18</td>
<td>238 70.03 0.50</td>
<td>280 119.04 1.00</td>
</tr>
<tr>
<td>15.04</td>
<td>0.12</td>
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</table>
Fig. 2. Dose-dependent relationships between food intakes by common ringtail possums and the molar concentrations of potential antifeedant compounds. Solid squares are jensenone, open circles are acetyl-jensenone, solid circles are torquatone, open squares are demethyl-torquatone, and solid triangles are controls.

ate to assess the resistance of foliage samples to marsupial folivory. Such assay techniques (e.g., those based on Folin reagents) indicate only the presence of the particular functional group assayed and give no information on other functional groups found on the molecule. There is a wide variety of different phenolic compounds found in the foliage of eucalypts (Hillis, 1966). If differences as subtle as those among the compounds used in the experiments reported here can cause such powerful differences in activity, then assays that cannot discriminate between a tannin and a simple phenolic clearly do not have the resolution to explain variation in marsupial feeding among trees. For example, both jensenone and demethyl-torquatone contain the same number of phenolic groups per mole and, thus, would give the same response in a “total” phenolics assay, yet the difference in feeding activity between them is great. It should also be noted that jensenone and other DFPCs active against marsupial folivores are not extracted in conventional phenolic assays (Lawler et al., 1998a).

A range of similar compounds (including jensenone) has been investigated for other biological activity. Several macrocarpals exhibit antibacterial activity (Murata et al., 1990; Yamakoshi et al., 1992; Osawa et al., 1996), and siderox-
ylonal A inhibits attachment of mussel larvae (Singh et al., 1996). Grandinol, a simple acylphloroglucinol derivative (similar to jensene but lacking one of the formyl groups) extracted initially from *E. grandis*, is a potent germination inhibitor, while similar compounds showed no such effect (Bolte et al., 1984). The euglobals (Figure 1f) have been investigated as potential antitumor agents, with some variation between euglobals of different structure. Detailed structure/activity studies have been carried out with a wide range of variations on the basic acylphloroglucinol structure (including DFPcs) to determine their importance in germination inhibition (Bolte et al., 1985) and inhibition of Epstein-Barr virus activation (as a de facto measure of antitumor activity) (Takasaki et al., 1990). These studies have been more comprehensive (i.e., covered a wider range of structural variation) than has been possible here because of the lower quantities of compound required for the bioassays. In both cases, they found substantial variation in activity associated with subtle changes in structure and were able to identify with reasonable precision the requirements for activity. Interestingly, the structural models for both germination and Epstein-Barr virus inhibition have similar requirements for the acylphloroglucinol structure with a formyl and a ketone group, which may apply also to deterrence of marsupial feeding. However, again, there are subtle differences in the requirements for activity. Jensenone was an effective Epstein-Barr virus inhibitor (Takasaki et al., 1990), but was less effective in inhibiting germination (Bolte et al., 1985; Boland et al., 1992).

A free phenolic group ortho to the isoprene unit was identified as important for the germination-inhibiting capacity of grandinol. That it may also be important in deterring feeding by marsupial folivores is suggested by the relative inactivity of torquatone. The reduced activity of euglobals, relative to macrocarpals (Pass et al., 1998), may indicate the need for free phenolic groups in both positions ortho to the isoprene/terpene group. Euglobals have an ether linkage at that position masking one of the phenolic groups, while this is lacking in the macrocarpals. Grandinol has not been tested against marsupials, but the groups of compounds shown to be active against marsupial feeding (macrocarpals, sideroxylonal, jensene) all share this feature. Further support for this contention is provided by the close correspondence in the molar thresholds shown by common ringtail possums of jensene and sideroxylonal. The molar threshold for jensene in this experiment is similar to the molar threshold for sideroxylonal of Lawler et al. (1999a), although it should be noted that the threshold for jensene might vary significantly between populations (Lawler et al., 1998b). Sideroxylonals are dimers of jensene, and one might, therefore, expect the molar threshold for sideroxylonal to be half that of jensene (while the threshold in milligrams would be similar). However, only one jensene monomer has all three phenolic groups free, while the other has one bound in an ether linkage (similar to that found in euglobals), which may eliminate the deterrent effect of that part of the molecule.
The results obtained in this study were somewhat counter to those expected. As the synthesized compounds were intended to be intermediate in structure between the two naturally occurring compounds, intermediate activities were expected. This was the case with acetyl-jensenone, although both aldehyde and phenol groups were capped. However, as we noted, the gem-diol groups revert easily to aldehyde groups in acidic conditions. We suspected also that the high activity shown by this compound was due to its also losing the acetyl groups and reverting to the parent compound in the acid stomach conditions of common ringtail possums. Support for this hypothesis is given by the observation that this occurred while attempting to purify acetyl-jensenone after the acetylation reaction by column chromatography. Significant proportions of the resulting product were shown by TLC and NMR to be jensenone, while no jensenone was evident in the reaction end-product before chromatography (Eschler, unpublished). This result is most likely due to the acidic nature of the silica gel used in the column. However, only small amounts of jensenone were produced over several days when acetyl-jensenone was stirred in 0.1 M HCl (Eschler, unpublished). Our original intention was to produce a more appropriate intermediate by methylating jensenone to produce a compound with the aldehydes present and phenols absent, but our attempts to do so in sufficient quantity failed.

The lower activity of demethyl-torquatone relative to its parent compound may be a function of its increased polarity. It contains two of the features of the active compounds (phenol groups, ketone) and, therefore, may be expected to show greater activity than torquatone. However, the presence of the phenol groups also makes the demethyl-torquatone more polar and, thus, may reduce the likelihood of its being absorbed across lipid membranes. If the action of jensenone (and other DFPCs) is at the surface of the gut wall, then lipophilicity may be irrelevant, as may be indicated by the similar deterreny exhibited by jensenone, sideroxylonal, and macrocarpal G, which vary in polarity. There are currently few data on the fate of any DFPC after ingestion. We know that jensenone causes the release of serotonin, possibly from the gut wall, but cannot say whether this is a result of cell damage or stimulation of specific receptors (Lawler et al., 1998b). However, when jensenone is incubated in an isolated section of guinea pig gut, it disappears rapidly from the lumen, and debris accumulates, indicating cell damage. No jensenone or apparent metabolites can subsequently be found either in the lumen, the gut tissue, or the surrounding fluid. It appears that jensenone binds to proteins, but it is not yet known if this is specific to certain protein types, such as receptors in the gut wall (S. McLean and S. Brandon, personal communication). Thus, it is not possible at this stage to say whether absorption across the gut wall is necessary for these compounds to exert their effects.

Another feature of the DFPCs that may be important in determining the antiherbivore activity, and thus interpreting ecological importance, may be the
identity of the terpene moiety. In the only relevant study to date, Takasaki et al. (1990) compared the antitumor activity of a range of euglobals that differ only in the terpenoid part of the molecule. While there was some variation among compounds, all were active, and they concluded that the terpene part of the molecule was less important than the features of the acylphloroglucinol component described above. We suspect this also to be the case when considering deterrence of marsupial feeding, in light of the similarities in activity between Jensenone, sideroxyonal, and macrocarpals. However, to date we have not been able to compare directly, for example, two macrocarpals with different terpene moieties, and we believe this is necessary before a firm conclusion is reached.

In conclusion, this structure--activity study has provided information that is useful in an ecological sense, illustrating the importance of identifying the specific molecular structures governing the plant--herbivore interaction. However, the quantities of each compound required for bioassays with whole animals, especially vertebrates, severely limit the number of compounds that can be synthesized in appropriate amounts, and this is perhaps reflected by the paucity of such studies in other plant--mammalian herbivore systems. In the only other study that we know of testing structural variants of a PSM against an herbivore that encounters it in the wild, only three compounds were tested (Clausen et al., 1986). In that study phenol groups were identified as being important for activity, but another cooccurring PSM with a phenol functionality was inactive. No attempt was made to understand the importance of other parts of the structure or the physiological effects of the compounds. Clearly, for plant--vertebrate herbivore systems, these limitations will, in most cases, prevent testing an extensive range of variations of the appropriate structure. This approach is unlikely, therefore, to lead to a complete understanding of the structural requirements. Detailed pharmacological studies of the metabolism of those compounds and the means by which animals can metabolize and tolerate or detoxify them may be a more profitable approach.

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