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VERTEBRATE HERBIVORY ON *Eucalyptus*—IDENTIFICATION OF SPECIFIC FEEDING DETERRENTS FOR COMMON RINGTAIL POSSUMS (*Pseudocheirus peregrinus*) BY BIOASSAY-GUIDED FRACTIONATION OF *Eucalyptus ovata* FOLIAGE

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Abstract—Factors determining the acceptance of *Eucalyptus ovata* foliage by common ringtail possums (*Pseudocheirus peregrinus*) were studied. Bioassay-guided fractionation was used with foliage from both browser-susceptible and browser-resistant trees to identify the chemical components underlying the resistance. In foliage from browser-resistant trees, the deterrent principles were contained in the base-soluble fraction of the chloroform extract. Further fractionation of this material yielded polar and nonpolar fractions that contained acylphloroglucinol derivatives, and from the polar fraction we isolated macrocarpal G. Addition of this compound to an artificial diet at a concentration of 2.1% of dry matter resulted in a 90% reduction of voluntary food intake compared with solvent-treated controls. This is the first time that a specific compound in *Eucalyptus* has been shown to inhibit feeding of any marsupial folivore.

Key Words—Plant secondary metabolites, terpenes, feeding, folivory, bioassay, marsupial, *Eucalyptus ovata*, *Pseudocheirus peregrinus*.

INTRODUCTION

Herbivory in *Eucalyptus* forests and woodlands varies widely (Landsberg and Cork, 1997). *Eucalyptus* dominates more than 90% of Australian forests, and

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studies have shown that some of the 600 species suffer little damage (e.g., Pahl, 1987). Even more striking are variations within species, with some trees being killed while an adjacent tree is apparently untouched (Edwards et al., 1993; Pahl, 1987). An inability to identify the factors controlling herbivory in *Eucalyptus* forests impedes our ability to manage these environments or to contribute to testing many recent theories about herbivory. Consequently, there have been many attempts to identify the chemical basis for this difference in palatability. A number of studies have sought correlations between food choice and primary constituents such as leaf nitrogen, leaf sugars, and fibre (Degabriele, 1981; Cork and Pahl, 1984; Geritz, 1987; Kavanagh and Lambert, 1990; Osawa, 1992; Hume and Esson, 1993). Few trends have emerged other than a weak influence of water and total nitrogen on feeding by koalas, but even so, this result explains little of the between-tree variation.

Other studies have attempted to relate the concentration of plant secondary metabolites (PSMs) in the leaves to the level of herbivory. *Eucalyptus* trees appear to be well defended plants because terpenoids, tannins, and associated phenolics can comprise up to 40% of the leaf dry matter (Fox and Macauley, 1977; Morrow and Fox, 1980; Foley, 1992), but again no consistent patterns of food choice or feeding behavior in relation to PSMs have been found. For example, in koalas (*Phascolarctos cinereus*), Southwell (1978) found no association between browsing and cineole concentrations in foliage, Betts (1978) suggested that the ratio of cineole to sesquiterpenes best explained the use of *E. globulus* foliage by koalas, and Hume and Esson (1993) concluded that koalas use foliar terpenoids as a positive feeding cue, preferring those with a relatively high concentration of monoterpenes. Taken together, these studies suggest either that an important component has been omitted from the analyses or that we have little understanding of what actually constitutes high nutritional quality in *Eucalyptus*.

The difficulty of this approach, which correlates food choice or feeding pressure with the chemical composition of the foliage, is that the analyses are not necessarily those that are relevant to the animal. Bryant et al. (1983) and Reichardt et al. (1984, 1990) showed the value of bioassay-guided fractionation in their studies of food choice of snowshoe hares in boreal ecosystems. This approach allowed specific compounds to be identified and tested for their repellent properties. Recognizing that correlative approaches had been exhausted, we adopted a bioassay-guided fractionation protocol to identify the specific constituents of *Eucalyptus ovata* foliage that determine its acceptability for a small marsupial browser, the common ringtail possum (*Pseudocheirus peregrinus*).

METHODS AND MATERIALS

Animals and Basal Diet. This research was approved by the Animal Experimentation Ethics Committee of James Cook University and conforms with the

Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Common ringtail possums (*Pseudocheirus peregrinus*) were used in all bioassays. They were caught by hand in *Eucalyptus* woodland near Townsville and Melbourne and maintained individually in large outdoor cages (preliminary experiments) or in metabolism cages as described by Foley (1992). A period of at least four weeks was allowed for acclimation to captivity, during which time the animals were fed mixed *Eucalyptus* foliage, fruit, and bread. In preliminary experiments (described below) they were offered only *Eucalyptus* foliage, but in all bioassay experiments, they were maintained on a palatable artificial diet made from cereals and fruit (Foley, 1992). The diet was made consistently to be 32% dry matter, and all animals maintained mass when offered this diet. Drinking water was supplied ad libitum.

Preliminary Identification of Resistant Trees and Susceptible Trees. Foliage was collected from 24 individual *E. ovata* trees and offered to captive ringtail possums. Initially, branches from six individual trees were weighed and placed in buckets of water in the cages at 18:00 hr. The weight of control branches did not change by more than 0.2%, and thus no correction for water uptake or loss was necessary. All branches were reweighed at 08:00 hr the next day, and any plants that were uneaten were noted. This procedure was repeated until we had preliminary identifications of four resistant and four susceptible trees. We then provided each foliage individually to ringtail possums and measured wet matter intake (Foley, 1992). Resistant trees were taken to be those of which animals ate less than 15 $g/kg^{0.75}$/day, whereas susceptible trees were defined as those of which animals ate at least 90 $g/kg^{0.75}$/day.

Extraction and Purification of Plant Secondary Metabolites. Foliage was collected from four resistant trees, mixed thoroughly, immediately frozen in liquid nitrogen, and stored at −20°C. A similar collection was made from four susceptible trees. The dry matter content of this foliage was 32–41% dry matter. Volatile constituents were extracted by steam distillation in an all-glass apparatus for 12 hr. The product of this distillation was extracted with diethyl ether and the organic layer dried over Na_2SO_4 and evaporated in vacuo to yield the steam-distillate fraction.

Crude methanol extracts of both resistant and susceptible foliages were prepared, and these extracts were partitioned until specific feeding deterrents were identified. *E. ovata* foliage was extracted in a Soxhlet apparatus with methanol for 24 hr. The methanol was filtered and reduced under vacuum to near dryness. This was designated as the methanol extract. A methanol extract was then partitioned between water (water extract) and chloroform. The organic layers were combined and evaporated to yield the chloroform extract. Extraction of the chloroform-soluble fraction with base (1.0 M NaOH, 3 × 200 ml) followed by reacidification and back-extraction of the caustic layer [5.0 M to pH 2.0, 3 × 200 ml dichloromethane (DCM)] afforded a mixture of low-molecular-weight (< 1000 daltons) phenolic compounds and fatty acids. This was designated the crude phenolic extract.

Further fractionation of the crude phenolic extract by repeated vacuum-assisted silica gel chromatography (90:10 DCM–methanol) (Coll and Bowden, 1986) resulted in a polar and a nonpolar fraction. Examination of these fractions by ^{1}H NMR suggested that the nonpolar fraction comprised compounds similar to the euglobals isolated from *E. globulus* by Kozuka et al. (1982a,b) and some fatty acids, whereas the polar fraction contained compounds similar to the macrocarpals isolated by Nishizawa et al. (1992) together with fatty acid.

Individual compounds were purified from these fractions by a combination of column chromatography on silica and Sephadex LH-20 and reversed phase HPLC. Structures of the known compounds reported here were confirmed by comparison of ^{1}H, ^{13}C, and high-resolution mass spectra with published data.

Bioassay-Guided Fractionation. Two types of bioassay were used to identify which fractions acted as antifeedants. The first was a two-choice protocol used by past workers (e.g., Bryant et al., 1983; Reichardt et al., 1984). Each bioassay used an extract from an original 600-g wet mass of foliage and was applied to 600 g wet mass of the basal diet described above (see bioassay conditions below). Extracts were dissolved in a minimum volume of hot methanol, then thoroughly mixed with the dry components of the basal diet. The solvent was removed under vacuum and the diet then made up as normal to produce the test food. Controls were prepared in exactly the same manner using methanol alone, and the position of the control and treated food in the cage was randomized each day. Each experiment was performed over four days with six common ringtail possums. We followed Sinclair et al. (1988) and calculated the proportion of the total intake resulting from the test and control diet each day and tested whether this was significantly different using a Wilcoxon matched pairs, signed rank test. When there was a statistically significant difference between treated food and controls in this protocol, we conducted a no-choice experiment, in which the animals were offered treated food alone. Comparisons were then made between the amount of food eaten when animals had no choice and the amount of food consumed on the previous day when control diets alone were offered.

Analytical. ^{1}H NMR spectra were recorded at 300.133 MHz and ^{13}C NMR at 75.47 MHz using a Bruker AM-300 spectrometer. Chemical shifts are quoted as parts per million relative to $CHCl_3$ set to δ7.26 (^{1}H NMR) and 77.0 (^{13}C NMR), unless otherwise stated. Capillary gas–liquid chromatography (GLC) was performed on a Hewlett-Packard 5890 gas chromatograph with flame ionization detection and on bonded-phase silica columns (BP 1.0.25). Waters Radial Pac 25×10 C18 reversed-phase cartridges were used for all high-performance liquid chromatography (HPLC).

Cyanogenic glycosides were isolated and characterized as described by Brimer and Dalgaard (1984) and Cardona et al. (1992) and quantified using the procedure of Lambert et al. (1975). Feigl-Anger papers were used as a quali-

tative test for cyanogenic glycosides in both whole foliage and foliar extracts prior to purifications of the cyanogenic principle.

RESULTS

Summary figures showing the path taken during the bioassay procedures and the intake of each fraction by common ringtail possums are shown in Figures 1 and 2.

Intake of Foliage from Resistant and Susceptible Trees. When provided a choice between foliage cut from resistant or susceptible trees, common ringtails strongly preferred that from the susceptible trees ($P < 0.001$). Intake of foliage from susceptible trees was 105 ± 7 g wet matter (32.1 ± 2.3 g dry matter), whereas only 5 ± 4 g (wet mass) of foliage from the resistant trees was consumed. In a no-choice test with resistant foliage, animals consumed 10 ± 6 g wet matter.

Methanol Extracts from Resistant and Susceptible Foliage. In a two-choice test there was no significant difference between controls and food treated with a methanol extract of susceptible foliage ($P = 0.14$). In contrast, controls were preferred over food treated with a methanol extract from resistant foliage ($P = 0.04$).

Steam Distillate from Resistant and Susceptible Foliage. The yield of steam distillate from susceptible foliage was 0.1% wet matter and that from resistant foliage was 1.0%. Gas–liquid chromatography showed that 1,8-cineole was the dominant component of the oil from mature foliage from resistant trees, comprising about 80% of the total extract. Although cineole was also the dominant fraction of the mature foliage from the extract of the susceptible foliage, linalool was the principal component in young leaf from the same trees again comprising about 80% of the extract. No young leaves were produced on the resistant trees during the study for comparison.

Animals preferred ($P = 0.05$) controls over the food treated with the steam distillate of resistant trees applied to the basal diet at a concentration of 1.0% (wet mass). Animals showed no preference for controls over food treated either with the steam-distillate fraction of the susceptible foliage or with a 0.1% concentration of steam distillate from resistant foliage ($P = 0.29$).

Pure cineole (>99% by GLC) added to the basal diet at a concentration of 0.8% was a strong deterrent ($P = 0.01$) to feeding in a two-choice test. However, when the same concentration was offered in a no-choice protocol, there was no significant difference ($P = 0.16$) between food intake during the test days and in the days both before and after the test when only control food was offered. Pure linalool at a concentration of 0.1% and 0.8% (equal to the range of cineole concentrations tested) did not have a significant effect on food intake in a two-choice test ($P = 0.40$ and $P = 0.15$, respectively).

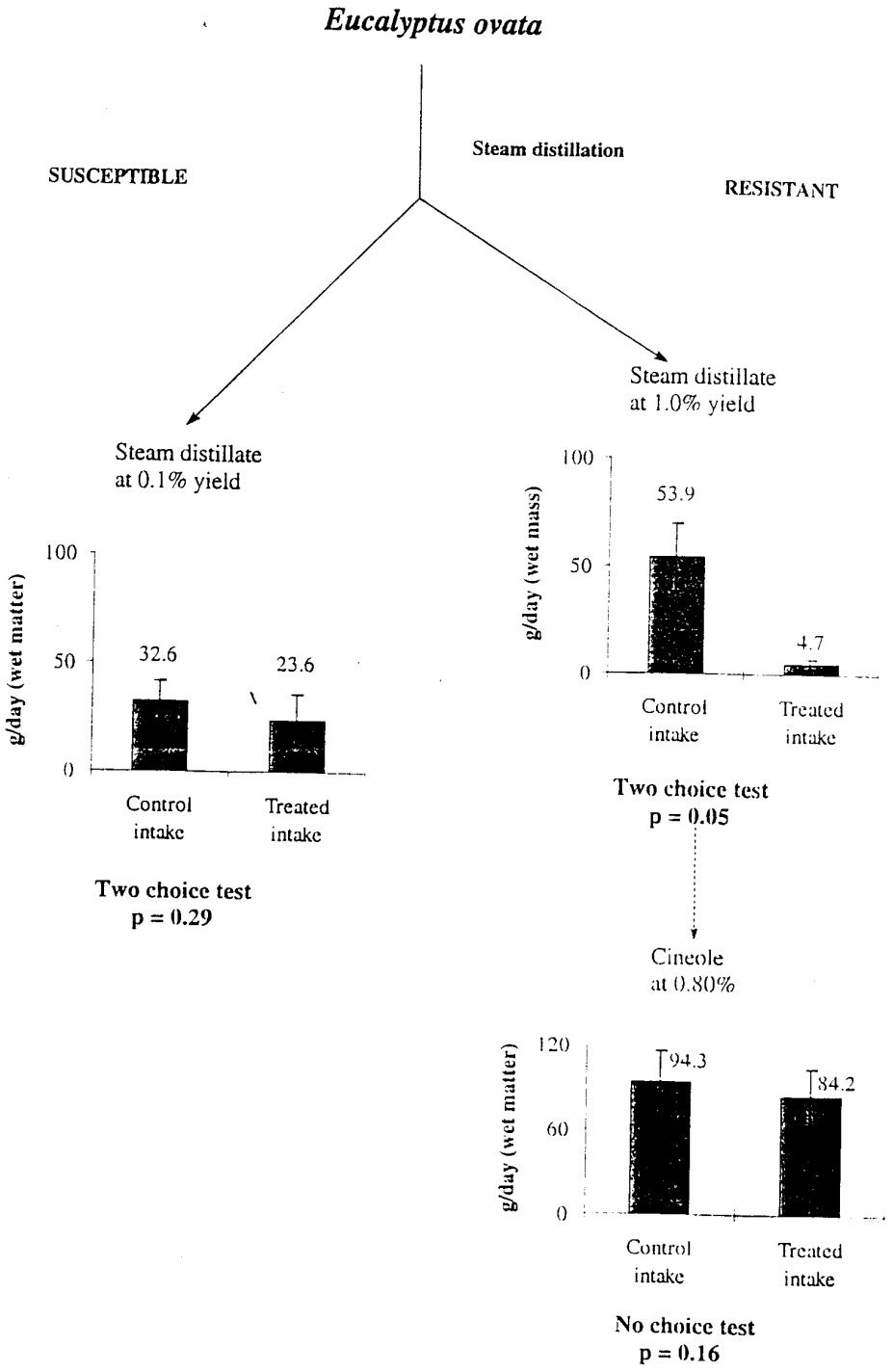


FIG. 1. Bioassay-guided fractionation of volatile constituents of browsing-resistant and susceptible foliage of *Eucalyptus ovata*. Each graph shows the mean ± SE of wet matter intake for six common ringtails. All results are from two choice tests except where indicated. *P* values are the significance of the difference in intake between control and treated food.

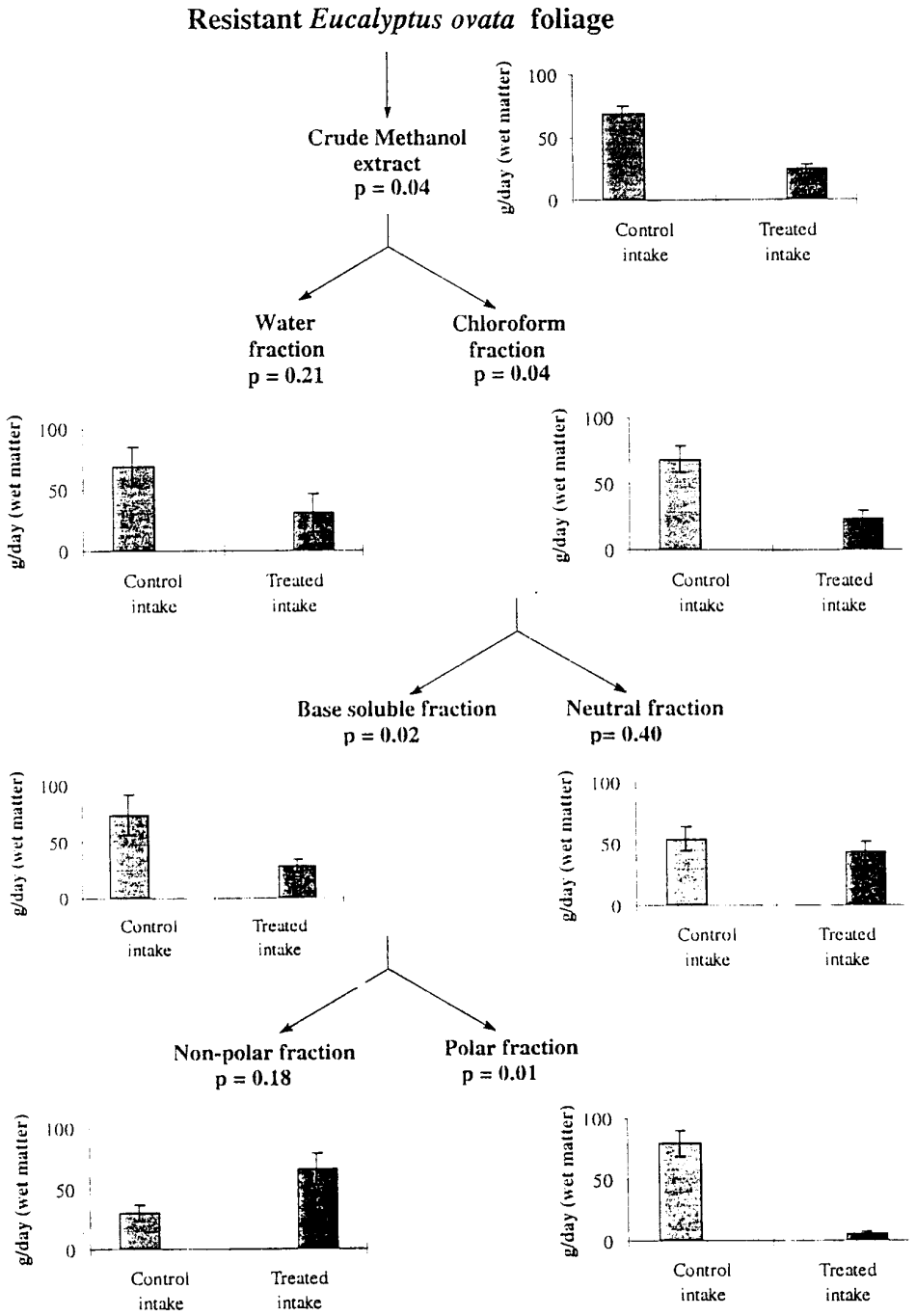


FIG. 2. Bioassay-guided fractionation of nonvolatile constituents of browsing-resistant foliage of *Eucalyptus ovata*. Each graph shows the mean ± SE of wet matter intake for six common ringtails and results from two choice tests. *P* values are the significance of the difference in intake between control and treated food.

Water-Soluble Fraction of Methanol Extract of Resistant Foliage. The presence of a cyanogenic compound was detected by the use of Feigl-Anger papers in the water-soluble fraction of resistant foliage. This cyanogenic activity was isolated and shown by ^{1}H NMR to be prunasin by comparison with literature values (Cardona et al., 1992). Prunasin occurred at a concentration 44 $\pm$ 5 μg cyanide/g fresh leaf in resistant foliage but no cyanide was detected in the foliage from the susceptible trees. Despite this, the water-soluble extract containing these glycosides was not significantly deterrent to the animals, and there was no significant difference between the intake of food treated with the water-soluble extract or controls ($P = 0.21$) in a two-choice test.

Chloroform-Soluble Fraction of Methanol Extract of Resistant Foliage. Controls were preferred over the chloroform-soluble material from the methanol extract ($P = 0.04$) in a two choice test.

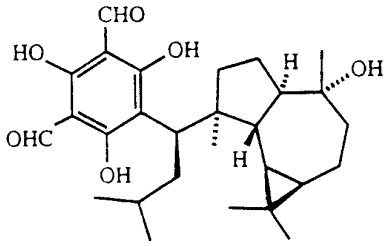
Base-Soluble and Neutral Extracts of Chloroform Solubles from Resistant Foliage. Animals strongly preferred controls ($P = 0.02$) over food treated with the base soluble extract of chloroform solubles from resistant foliage in a two-choice test. In contrast, there was no significant difference in food intake between controls and food treated with the acid-soluble fraction. This confirmed that the deterrent fractions were likely to be phenolic in nature.

Polar and Nonpolar Fractions of Base-Soluble Extract. The base-soluble extract was chromatographed (see conditions above) to yield two fractions of differing polarity. Animals showed no significant preference ($P = 0.18$) between controls and food treated with the nonpolar fraction in two-choice tests. In contrast, controls were strongly preferred ($P = 0.01$) over food treated with the polar fraction of the base-soluble extract of resistant foliage.

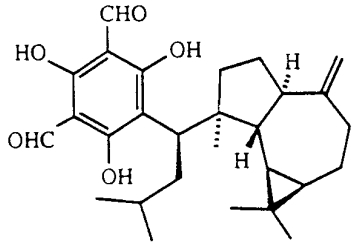
Identification of PSMs. Examination of both the polar and nonpolar fractions of the base solubles by ^{1}H NMR suggested the presence of hydrogen-bonded hydroxyls on a fully substituted aromatic chromophore attached to a hydrocarbon/terpene moiety. These features closely matched the structures of a group of acylphloroglucinol–terpene adducts previously identified in *Eucalyptus*, namely the euglobals (Kozuka et al., 1982a,b) and the macrocarpals (Nishizawa et al., 1992; Yamakoshi et al., 1992).

Chromatography on Sephadex LH-20 and reverse-phase HPLC (95:4.9:0.1 acetonitrile–water–acetic acid) of the nonpolar fraction afforded a number of euglobals (euglobal Ib, Ic, IIa, BI_1, III, IVb, and V) and another simpler phloroglucinol derivative [compound 1 of Qi and Snyder (1991)]. Euglobal III (Figure 3) was the dominant component of this fraction.

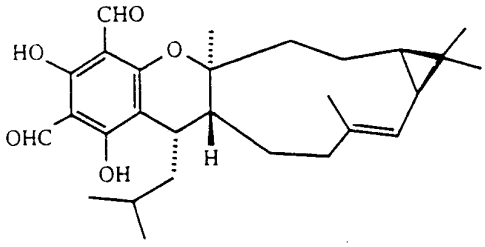
The dominant component of the polar mixture was isolated by chromatography on Sephadex LH-20 and reverse-phase HPLC (97:2.5:0.5 acetonitrile–dichloromethane–acetic acid). Comparison of the ^{13}C NMR data (in d_4-methanol) with published values (Yamakoshi et al., 1992) allowed this compound to be assigned as macrocarpal G (Figure 3).



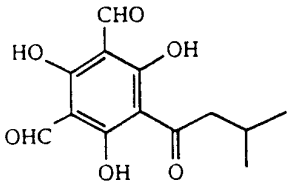
Macrocarpal A



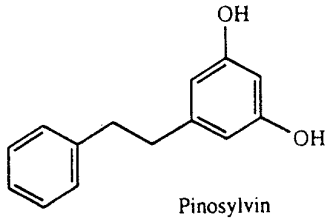
Macrocarpal G



Euglobal III



Jensenone



Pinosylvin

FIG. 3. Planar structures of acylphloroglucinol-terpene adducts of *Eucalyptus* foliage and of pinosylvin.

Macrocarpal G. The macrocarpal fraction was refined by repeated chromatography over silica (0.5:99.5 acetic acid–light petroleum) to yield macrocarpal G (Figure 3) in sufficient purity (>95% by HPLC) for bioassay. The impurities were long-chain fatty acids that proved very difficult to separate. We added this material to the diet at a concentration of 0.7% of wet matter (2.1% dry matter) and used a no-choice protocol. Animals ate too little of the treated food to maintain themselves, and the intake was significantly different from controls ($P = 0.03$), demonstrating that macrocarpal G is at least partially responsible for the selective feeding behavior of common ringtail possums on *E. ovata* foliage.

DISCUSSION

The striking differences in food intake of different *E. ovata* trees quantifies the field observations of many biologists (e.g., Hindell and Lee, 1987; Pahl, 1987; Geritz, 1987) that *Eucalyptus* displays interspecific variation in its susceptibility to vertebrate herbivores, but this study is the first to explain how this is mediated. Although the trees that we subsequently examined in detail were collected 20 km apart, resistant and susceptible *E. ovata* trees can be found side by side (Geritz, 1987; W. Foley, personal observation).

Bioassay-guided fractionation provided clear evidence that common ringtail possums fed little on resistant *E. ovata* foliage because of the presence of the phloroglucinol-terpene adduct known as macrocarpal G. This is the first time that a specific molecule in *Eucalyptus* has been identified as a feeding deterrent for either insects or mammals. Macrocarpal G was first isolated in 1992 (Yamakoshi et al., 1992) and is one of several related bioactive phloroglucinol derivatives with widespread biological activity (Ghisalberti, 1996).

There remains some confusion in published work regarding the name macrocarpal G since the same planar structure has been assigned to both macrocarpal G (Yamakoshi et al., 1992) and macrocarpal C (Nishizawa et al., 1992). Since the NMR experiments described in these two reports were carried out in different solvents, direct comparisons are not possible. Our NMR spectra were obtained in methanol as were those of Yamakoshi et al. (1992), and so we have followed those workers and used the name macrocarpal G.

The structure of both euglobals and macrocarpals suggests that they are derived from two separate biosynthetic pathways. Both contain a terpene moiety coupled to a fully substituted aromatic ring. How these pieces are coupled has been the subject of some speculation. Kozuka et al. (1982a,b) argued for a Diels-Alder-based mechanism for the biosynthesis of the euglobals, whereas the addition of the appropriate benzylic cation to a sesquiterpene has been proposed to generate macrocarpals. Bicyclogermacrene is the terpene involved in the biosynthesis of macrocarpals A and G. Macrocarpal G is presumed to be the

dehydrated product of macrocarpal A or one of its isomers. Both euglobal III and macrocarpal G can, however, be envisaged as being derived from addition of bicyclogermacrene to the same benzylic carbocation. Euglobal IVa and IVb could also arise by similar condensations (see Figure 4).

We presume that the terpene moiety confers a high degree of lipid solubility on the macrocarpals and so serves to carry the reactive aldehyde and phenol groups across membranes. If this is true, then we would expect that the level of deterrence of these compounds is a function of both the number of reactive groups on the molecule and the degree of lipid solubility conferred by the terpene moiety. Evidence for this suggestion comes from the relative antifeedant activity of the polar (principally macrocarpal G) and nonpolar fractions (principally euglobal III) in our experiments. Both compounds have the same acylphloroglucinol skeleton but the macrocarpals have a free hydroxyl where the euglobals have an ether linkage connecting the aromatic portion with the terpene. It is possible that the free hydroxyl of the macrocarpal species contributes significantly to the antifeedant activity. Preliminary studies (D. M. Pass and W. Foley, unpublished) using jensenone (Boland et al., 1992) (Figure 3), a related acylphloroglucinol compound from *Eucalyptus jensenii* support this hypothesis, although a directed structure–activity study is in progress.

Lipophilic phenols have been implicated as feeding deterrents for other mammalian browsers (e.g., snowshoe hares, *Lepus americanus*). For example, Clausen et al. (1986) showed that foliar pinosylvin was a significant factor in the choice of snowshoe hares for green alder, and the relative activities observed between pinosylvin and its mono- and dimethylated derivatives parallel the differences in the antifeedant activities demonstrated here for euglobal III and macrocarpal G. Interestingly, there are structural and biosynthetic similarities between compounds such as pinosylvin and dihydrochalcone [which is also deterrent to snowshoe hares; (Clausen et al., 1986)] and macrocarpal G. This raises the possibility that aspects of the chemical structures of these compounds provides some deterrent properties against a wide range of mammalian browsers.

We do not know at present what effects the macrocarpals have on common ringtail possums and thus why they are so strongly avoided. Preliminary evidence (Lawler, Foley and G. J. Pass unpublished data) is that acylphloroglucinols cause nausea in marsupials and so lead to the development of a conditioned food aversion but whether this occurs as a result of the native compound or its metabolites is not yet known.

Previous correlative studies of diet choice in both vertebrate and insect browsers of *Eucalyptus* have implicated the terpenoid 1,8-cineole as the deterrent molecule (Betts, 1978, Edwards et al., 1993) but our study is the first to use bioassays to test that hypothesis. It is therefore significant that we found that cineole was not deterrent when the animals were given no choice at a concentration of cineole similar to that in resistant foliage.

High concentrations of cineole were measured in the unpalatable *E. ovata*

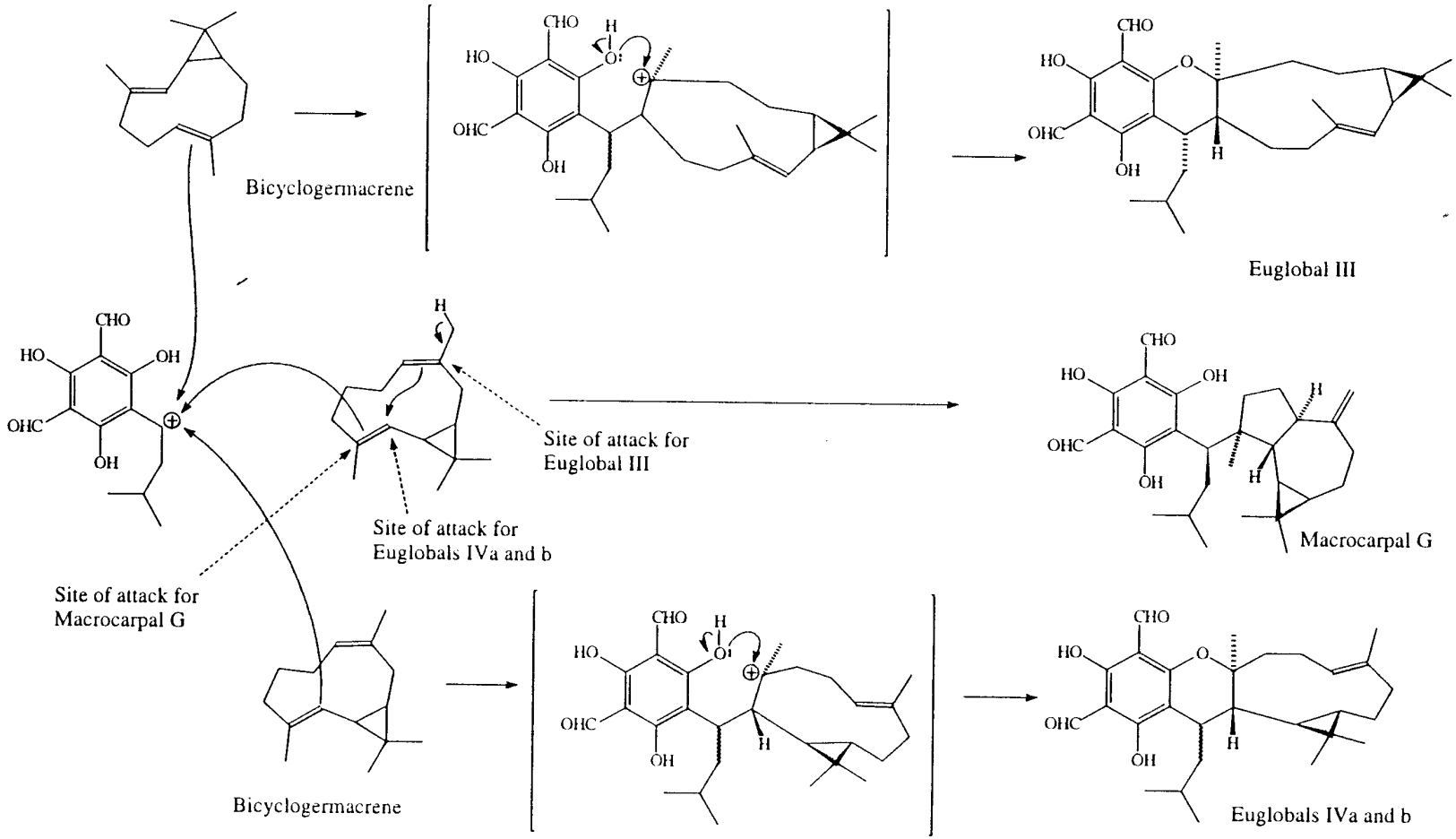


FIG. 4. Proposed addition of bicyclogermacrene to the appropriate carbocation for generation of euglobals III, IVa, IVb, and macrocarpal G.

as well as in a number of other unpalatable trees within other species of *Eucalyptus* [e.g., in *E. polyanthemos* (D. M. Pass unpublished data) and in various other *Eucalyptus* species (Edwards et al., 1993)]. This suggests the possibility that cineole may be used as a cue by some animals to the presence of high levels of macrocarpal G and related compounds. Both the euglobals and the macrocarpals are composed of a (poly)acylated phloroglucinol moiety with either a monoterpene or sesquiterpene attached through a C-5 unit. In most cases the monoterpene functionality appears to derive from β-pinene (or a related monoterpene), whereas sesquiterpene functionalities are derived from bicyclogermacrene (see Figure 4). Cineole has never been recorded as part of the acylphloroglucinol–terpene adduct, and we suggests that any involvement of cineole as an adjunct to deterrent macrocarpals must be only indirect. It is possible that high levels of cineole signal to animals that macrocarpals are present, but this idea requires testing.

In these studies it was found that animals chose to eat little cineole when offered a choice between food containing cineole and food that did not. However, when no choice was offered, animals consumed food with 0.8% (wet mass) cineole readily. This implies that ingestion of cineole involves a cost for the animals but one that they are willing to pay when no other food is available. However, the nature of this cost is unclear. For example, Krockenberger (1988) found no difference in the digestibility or metabolizability of food when common ringtails were fed cineole for a prolonged period of time at a concentration of 1.4% dry matter. Lawler (unpublished) found that at concentrations of 12% dry matter common ringtails could maintain body mass on high intakes of artificial diets. Costs may be related to general disturbances to acid–base metabolism as described by Foley (1992) and Foley et al. (1995), in which biotransformation of ingested PSMs leads to production of a proton that must in turn be excreted. However, Bull et al. (1993) have shown that urinary metabolites of cineole may act as pheromones in common brushtail possums, allowing discrimination between the scent marks of female and male possums, and so in this instance the animal is deriving some value from the PSM.

The acylphloroglucinol backbone of the macrocarpals is present in a variety of related compounds found within different species of *Eucalyptus* (Ghisalberti, 1996; D. M. Pass, unpublished). Given the demonstration of antifeedant activities and other wide-ranging biological actions [e.g., tumor suppressant, and antibacterial and antifouling activities (Kozuka et al., 1982a,b; Nishizawa et al., 1992; Yamakoshi et al., 1992)], we postulate that this group of compounds are significant herbivore defenses within the whole genus. Given the dominance of *Eucalyptus* in Australian forests, these compounds are likely to significantly modulate other plant–animal interactions within the continent.

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