Journal of Chemical Ecology, Vol. 26, No. 5, 2000

FATE OF THE DIETARY TERPENE, *p*-CYMENE, IN THE MALE KOALA

REBECCA BOYLE,[1,*] STUART McLEAN,[1] WILLIAM J. FOLEY,[2] BEN D. MOORE,[2] NOEL W. DAVIES,[3] and SUE BRANDON[1]

[1]*Tasmanian School of Pharmacy, University of Tasmania*
GPO Box 252-26, Hobart 7001, Tasmania, Australia

[2]*Division of Botany and Zoology*
Australian National University
Canberra 0200, ACT, Australia

[3]*Central Science Laboratory, University of Tasmania*
GPO Box 252-74, Hobart 7001, Tasmania, Australia

(Received June 28, 1999; accepted December 27, 1999)

Abstract—The fate of the dietary terpene, *p*-cymene, is reported in a specialist *Eucalyptus* leaf folivore, the koala (*Phascolarctos cinereus*). Six male koalas were given two single oral doses of *p*-cymene (0.37 mmol/kg and 1.49 mmol/kg). Urine and feces were collected for two 24-hr periods after each dose and analyzed for metabolites by extraction, gas chromatography, and mass spectrometry. A total of six metabolites were detected in the urine. A novel, extensively oxidized metabolite, 4-(1,2-dihydroxy-1-methylethyl)-benzoic acid, was identified and its structure elucidated by high resolution and chemical ionization mass spectrometry. Minor amounts of two glycine conjugated metabolites were also detected. Five metabolites were present in sufficient amounts to quantify, using isolated urinary metabolites as reference standards. The mean fractional recovery of administered *p*-cymene was 0.77 ± 0.09 and 0.84 ± 0.12 for the low and high dose, respectively. The major metabolite excreted was the novel carboxy diol compound. No fecal metabolites were found. Thus, the koala employs a strategy in the metabolism of *p*-cymene that promotes the production of extensively oxidized metabolites that consequently have increased polarity and enhanced renal excretion. This strategy is compared with that employed by other *Eucalyptus* leaf folivores.

Key Words—*Phascolarctos cinereus* (Goldfuss), koala, *p*-cymene, metabolism, detoxification, *Eucalyptus*, terpene.

*To whom correspondence should be addressed.

0098-0331/00/0500-1095$18.00/0

INTRODUCTION

A major issue in understanding the interactions between mammals and their food plants has been the importance of the detoxification of ingested plant secondary metabolites (PSMs). Freeland and Janzen (1974) argued 25 years ago that limitations and interspecific differences in enzyme detoxification would be important determinants of feeding niches of mammals. This idea has never been challenged, yet few data have been accumulated in the interim to support it. Nonetheless, recent syntheses of mammal–plant interactions assume that detoxification processes and capacities are crucial to our understanding of current ecological patterns.

Specialization on a single plant type is the exception rather than the rule among mammalian herbivores (Freeland and Janzen, 1974; Freeland, 1991). These authors argued that the array of PSMs occurring in plants usually necessitates the sampling and browsing behavior of herbivores to allow ingestion of limited amounts of many PSMs from a variety of plants. This effectively spreads the toxin load across the range of detoxification pathways available. The advantage of this stratagem is avoidance of saturation of enzymatic detoxification pathways.

The koala, however, feeds exclusively on leaves from relatively few species of *Eucalyptus*. Although there are quantitative and qualitative differences in PSM profiles, all eucalypts contain similar types of terpenes (Southwell, 1973; Eberhard et al., 1975; Morrow and Fox, 1980; Lawler et al., 1998). These are potentially toxic nonnutrients and typically require metabolism to enable their excretion from the body. Therefore, marsupials like the koala that feed exclusively on *Eucalyptus* leaves have overcome significant metabolic challenges by this specific group of PSMs.

We were interested in understanding the metabolic strategies employed in detoxifying monoterpenes that are encountered in *Eucalyptus* leaves. By studying the fate of the commonly occurring monoterpene, *p*-cymene, we can begin to understand the detoxification processes.

The metabolic fate of a number of monoterpenes has been studied in folivorous marsupials, and a large number of oxidized metabolites has been reported. Southwell (1975) reported novel α- and β-pinene lactone metabolites in the koala and also the metabolic fate of α- and β-pinene, *p*-cymene, and 1,8-cineole in brushtail possums (Southwell et al., 1980). Southwell et al. (1980) suggested greater oxidase activity in koalas, compared to brushtail possums, based on the metabolic products of α- and β-pinene. A detailed study of urinary metabolic products of 1,8-cineole has also been reported for brushtail possums (Carman and Klika, 1992; Bull et al., 1993; Carman et al., 1994; Carman and Rayner, 1994; Carman and Garner, 1996). The metabolic fate of several dietary terpenes has also been described for the ringtail possum (McLean et al., 1993).

Recently our group reported on a detailed comparative study examining the metabolic fate of *p*-cymene in two other marsupial folivores that specialize on *Eucalyptus* leaves, the greater glider (*Petauroides volans*) and the ringtail possum (*Pseudocheirus peregrinus*), as well as in a generalist herbivore, the brushtail possum (*Trichosurus vulpecula*) (Boyle et al., 1999). A species-specific pattern of urinary metabolite excretion was found, reflecting the natural occurrence of terpenes in the diet. The specialists each excreted three extensively oxidized metabolites, while subsequent conjugation with glucuronic acid was unimportant in the excretion of these metabolites. In contrast, the generalist brushtail excreted eight metabolites, of which the less oxidized metabolites were more abundant. Glucuronidation played an important role in the elimination of the less oxidized metabolites in the brushtail possum.

Both oxidation and conjugation increase the polarity of metabolites, thereby enhancing their renal elimination. Therefore, the greater the degree of oxidation, the greater the rate of elimination.

We hypothesized that the oxidative metabolic pathways of *Eucalyptus* specialists have evolved to become highly efficient at producing extensively oxidized metabolites that can be readily excreted without needing conjugation. On the other hand, the brushtail possum appeared to require less efficient oxidative pathways, as it excreted a greater proportion of partially oxidized metabolites. The less oxidized metabolites are metabolic precursors of the more extensively oxidized compounds. The brushtail then uses the glucuronidation pathway to enhance the renal excretion of these partially oxidized metabolites.

The present study extends our previous work on the comparative metabolism of *p*-cymene. The aim was to determine whether the koala conforms to the pattern of metabolism hypothesized for eucalypt specialists. It also completes a direct comparison of the metabolic fate of a single PSM, *p*-cymene, across the complete spectrum of marsupial eucalypt leaf-eaters in Australia.

METHODS AND MATERIALS

Materials. All reagents used were obtained from commercial suppliers and were of analytical grade. *p*-Cymene, cumic acid, and glycine were purchased from Aldrich Chemical Company, Inc (Castle Hill, NSW, Australia).

Animals. Six male koalas [weight 8.8 ± 1.0 kg (mean ± SD)] were captured on French Island, Victoria, Australia. They were housed at the Phillip Island Nature Park, Victoria, and released back to their point of capture at the end of procedures. Approval for this study was obtained from the appropriate institutional ethics committees. Koalas were held in purpose-built koala enclosures (6.5 × 3.5 m) during their captivity and placed into metabolism cages (1.3 × 0.6 × 0.9 m) within their enclosures for dosing experiments.

Koalas were fed *E. cephalocarpa* during the experiment, as its leaf was found to be low in *p*-cymene. Fresh leaf was supplied daily and the intake measured. Water was supplied ad libitum.

Dosing and Urine Collection. Koalas were given two single oral doses of *p*-cymene: 0.37 mmol/kg (50 mg/kg) and 1.49 mmol/kg (200 mg/kg). This was the same dosage regimen administered to animals in our earlier work and was chosen to allow a direct dose comparison between species.

p-Cymene was combined in a paste of Portagen powder (an infant milk formula; Mead Johnson & Company, Evansville, Indiana) and water, and the koalas licked the dose from a syringe. *p*-Cymene concentration in the paste was 15 mg/g for the lower dose and 45 mg/g for the higher dose.

Urine and feces were collected for 24 hr prior to and 24 hr and 48 hr after the administration of *p*-cymene. Urine was collected into containers positioned in Dry Ice and frozen immediately. Urine volume and pH were measured. The floor of each metabolism cage was washed with distilled water to remove residual urine, and the washings were combined with the day's urine. Fecal pellets were collected daily from the wire mesh floor of the metabolism cages and weighed before freezing.

Leaf Analysis. A leaf diet containing no, or minimal *p*-cymene and preferably few other terpenes was required for feeding koalas during experiments. Leaf samples of *E. cephalocarpa*, *E. globulus*, and *E. ovata* were analyzed for their terpene profiles to identify the most appropriate diet. To extract terpenes, a weighed amount of leaf (approximately 5 g) was placed into a 50-ml plastic centrifuge tube with 45 ml of ethanol (99%) and allowed to stand for 48 hr at room temperature (Ammon et al., 1985).

Gas chromatography–mass spectrometry (GC-MS) was used to analyze the leaf extracts with a Hewlett-Packard 5890 GC coupled to a HP 5970B MSD, using HP 59970A Chemstation software (Hewlett-Packard Australia Ltd., Melbourne). Chromatography was carried out on an HP-1 capillary column (25 m $\times$ 0.32 mm ID, coated with 0.52 μm cross-linked methyl silicone). GC-MS operating conditions were splitless injector 250°C, He carrier gas (15 psi), oven temperature 60–190°C at 5°C/min, then 190–290°C at 30°C/min, and held for 5 min, detector 290°C.

GC-MS was also used to estimate the *p*-cymene content of the chosen *E. cephalocarpa* leaf diet. A sample of leaf fed to each koala was collected daily and frozen immediately. Dry weight was determined on samples of fresh leaf by drying at 70°C to a constant weight. Very low levels of *p*-cymene were detected by selected ion monitoring (SIM) of the ethanol extract by GC-MS. Because the levels of *p*-cymene in leaf samples were so low, they were quantified as the relative proportion of 1,8-cineole (i.e., *p*-cymene was 0.3% of the 1,8-cineole concentration). The abundances of the diagnostic ions for both *p*-cymene (m/z 119) and 1,8-cineole (m/z 154) were standardized as a fraction of their respective

total ion current (TIC), and the appropriate factors (2.8 and 35.7, respectively) were applied to the diagnostic ion counts. A calibration curve for 1,8-cineole was prepared with α-pinene as the internal standard.

Urine Analyses. Urine samples were analyzed for both free and total metabolites, and the levels of each major metabolite were quantified. The method used is described in detail by Boyle et al. (1999). Gas chromatography resulted in the *p*-cymene-related peaks being overwhelmed by peaks derived from leaf terpene metabolites (mainly 1,8-cineole metabolites). It was, therefore, necessary to extract mass chromatograms of diagnostic ions to quantify metabolites (Table 1). Metabolites were identified by their GC retention times and mass spectra. Mass spectral data for all metabolites, except Cy 13, have been previously published (Southwell et al., 1980; Ishida et al., 1981, 1989; Walde et al., 1983).

Metabolites Cy 5 and 13 chromatographed poorly as their methyl ester derivatives and required further derivatization of the alcohol groups to their trimethylsilyl (TMS) derivatives by using *N*,*O*-bis(trimethyl) trifluoracetamide [BSTFA; Alltech Associates (Australia) Pty Ltd, Baulkham Hills, NSW]. Therefore, all samples were analyzed as the methyl esters only and the methyl esters plus TMS derivatives.

Isolated metabolites Cy 2, 5, and 6 (Figure 1) were available from previous work (Boyle et al., 1999). Metabolite Cy 13 was quantified by using the calibration curve prepared for Cy 5, since their respective structures differed only in a hydroxyl group. Cy 10 was synthesized from cumic acid. The acid chloride of cumic acid was prepared and then reacted with glycine [method based on Vogel (1959)]. GC-MS confirmed the structure of the product to be Cy 10.

Calibration curves were linear over the required concentration ranges for each metabolite.

Identification of Novel Metabolite (Cy 13). GC-MS monitoring of an ion at m/z 179 (which was diagnostic for the methyl ester of Cy 2) disclosed a previously unknown, major *p*-cymene metabolite at a significantly longer retention time than Cy 2. After additional derivatization (with BSTFA) to improve its chromatography, a tentative structure was elucidated by interpretation of the mass spectrum from first principles. Electron (EI) and chemical (CI) ionization and high-resolution mass spectrometry (HR-MS) provided information on the structure of the metabolite. EI mass spectra were obtained from the GC-MS instrument described above. CI and HR-MS spectra were obtained from a Kratos Concept ISQ spectrometer with an accelerating voltage of 5.3 kV, a scan rate of 0.9 sec/scan, m/z range of 100–550, and resolution of 1000 (3000 for accurate mass determinations). Perfluorokerosene (PFK) was used as an internal calibrant for accurate mass measurements, and ammonia was used as the chemical ionization reagent gas.

Urinary Glucuronic Acid Analysis. The total urinary glucuronic acid was measured for each of the six koalas before and after the high dose of *p*-cymene.

TABLE 1. *p*-CYMENE METABOLITES EXCRETED BY KOALA

Metabolite	Chemical name	Derivative[a]	R_t GC-MS (min)	Quantified (Y/N)	Diagnostic m/z	Purity (%)[b]
Cy 2	2-*p*-carboxyphenylpropan-2-ol	M	12.85	Y	179	81.2
Cy 5	2-*p*-carboxyphenylpropan-1-ol	M + TMS	15.14	Y	163	91.8
Cy 6	2-*p*-carboxyphenylpropionic acid	M	14.27	Y	163	95.3
Cy 10	*p*-isopropylbenzoylglycine (cuminuric acid)	M	17.86	Y	147	98.8
Cy 11	*p*-isopropenylbenzoylglycine	M	18.01	N		
Cy 13	4-(1,2-dihydroxy-1-methylethyl)-benzoic acid	M + TMS	16.39	Y	179	

[a] Methyl ester (M), trimethylsilyl ether (TMS).
[b] Purity of standards used for metabolite quantitation were estimated by GC analysis. Cy 13 was quantified by using the Cy 5 calibration curve.

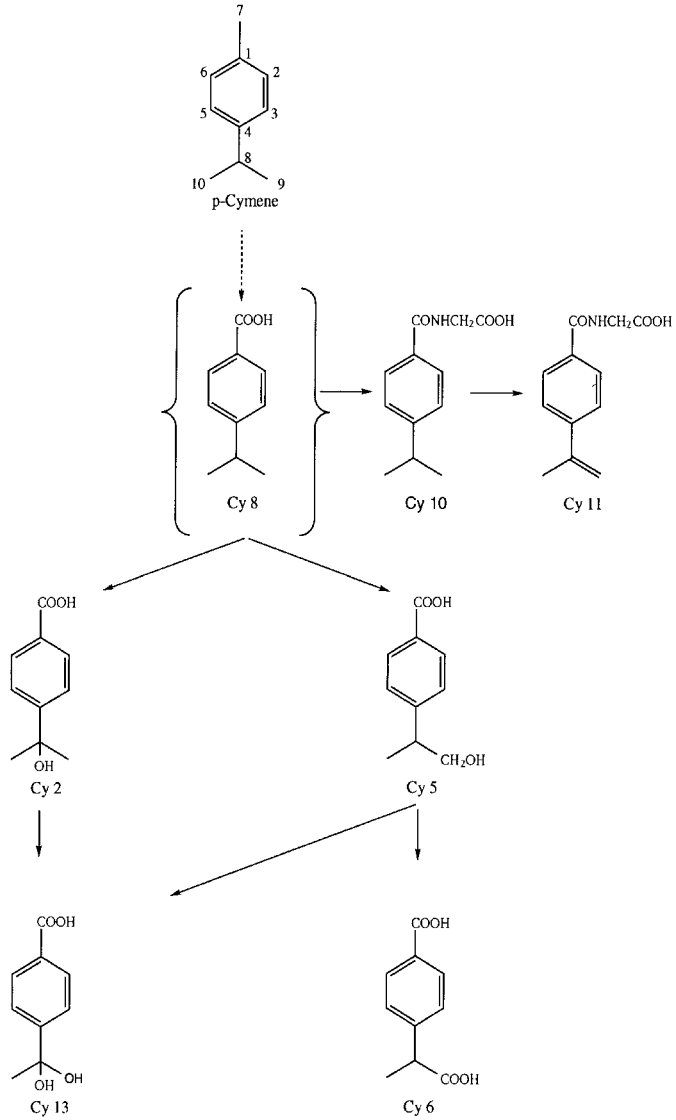


FIG. 1. Chemical structures of *p*-cymene metabolites and probable metabolic pathway in the male kaola. Metabolites are shown in their underivatized forms. Dashed arrow indicates where an intermediate metabolite has not been detected. Cy 8 was not found in the koala, but may be a common precursor to all metabolites.

The method used was based on that described by Blumenkrantz and Asboe-Hansen (1973). A 200-fold dilution with distilled water was suitable for most urine samples; however, some required further dilution.

Fecal Analysis. One fecal sample before and two after dosing of two koalas were analyzed individually. The fecal pellets were ground in a mortar and pestle and weighed. A slurry was made from 5 g of each fecal sample and 20 ml of distilled water. A weighed amount (approximately 5 g) of each sample was placed into two large centrifuge tubes. The first sample was hydrolyzed to measure total metabolites by adding 5 ml of 1.1 M (pH 5.2) acetate buffer and 250 μl extract of *Helix pomatia* (β-glucuronidase/arylsulfatase; Boehringer Mannheim, Germany), and incubated overnight in a waterbath at 37°C. The second sample was analyzed for free metabolites only. After overnight incubation, both samples were treated in the same manner. The pH was adjusted to 1 with 5 M HCl, then the mixture was extracted sequentially with three washes of ethyl acetate and three washes of dichloromethane–*n*-propanol (80 : 20). The two solvent extractions were treated separately. Each extract was evaporated to dryness and redissolved in 2 ml of ethyl acetate or 2 ml of methanol for the ethyl acetate and dichloromethane–*n*-propanol extract, respectively. A sample of each of the final extracts was methylated with diazomethane and analyzed by GC-MS under conditions described for urine analysis.

RESULTS

Qualitative Results. Six metabolites were identified in the urine of all koalas. The major metabolite was a novel compound and assigned the name Cy 13 (see Figure 1 and Table 1 for chemical structures and names). Cy 13 accounts for about half (Table 2) of all metabolites excreted. It is an extensively oxidized compound, acquiring a total of four oxygen atoms, and has undergone oxidation at three different sites.

The remaining five metabolites (Figure 1) excreted by the koala have been identified in other animal species and reported in the literature (Ishida et al., 1981, 1989; Matsumoto et al., 1992; Southwell et al., 1980; Walde et al., 1983; Boyle et al., 1999).

Mass Spectral Determination of Cy 13. Chromatography with SIM monitoring of the ion at m/z 179 revealed two *p*-cymene metabolite peaks, resulting from a β-cleavage on the isopropyl group. The first peak was Cy 2; the later eluting compound, Cy 13, had not previously been reported. The EI-GC-MS of the methyl ester of Cy 13 revealed a fragmentation pattern similar to Cy 2. Two possible structures were initially considered for Cy 13, both differing from Cy 2 by having undergone oxygenation on a third site at C-9. The first possibility was a dihydroxy acid, Cy 13, (Figure 1), and the second, a hydroxy diacid. CI-

TABLE 2. URINARY METABOLITES OF *p*-CYMENE IN KOALA[a]

		Total urinary metabolites excreted in 48 hr [% (mean ± SD)]			
		0.37 mmol/kg[b]		1.49 mmol/kg[b]	
Metabolite	O_n	Free	Total	Free	Total
Cy 2	3	9.1 ± 4.7	9.1 ± 3.7	8.0 ± 1.6	7.3 ± 1.8
Cy 5	3	11.3 ± 5.1	7.2 ± 3.3	7.7 ± 3.8	8.1 ± 4.5
Cy 6	4	24.6 ± 3.2	26.0 ± 7.0	24.2 ± 4.1	20.7 ± 3.0
Cy 10	2 + gly	6.8 ± 4.1	8.0 ± 3.8	7.5 ± 4.0	7.3 ± 3.6
Cy 11	2 + gly	tr	tr	tr	tr
Cy 13	4	48.1 ± 7.4	49.7 ± 7.6	52.6 ± 1.0	56.3 ± 5.2

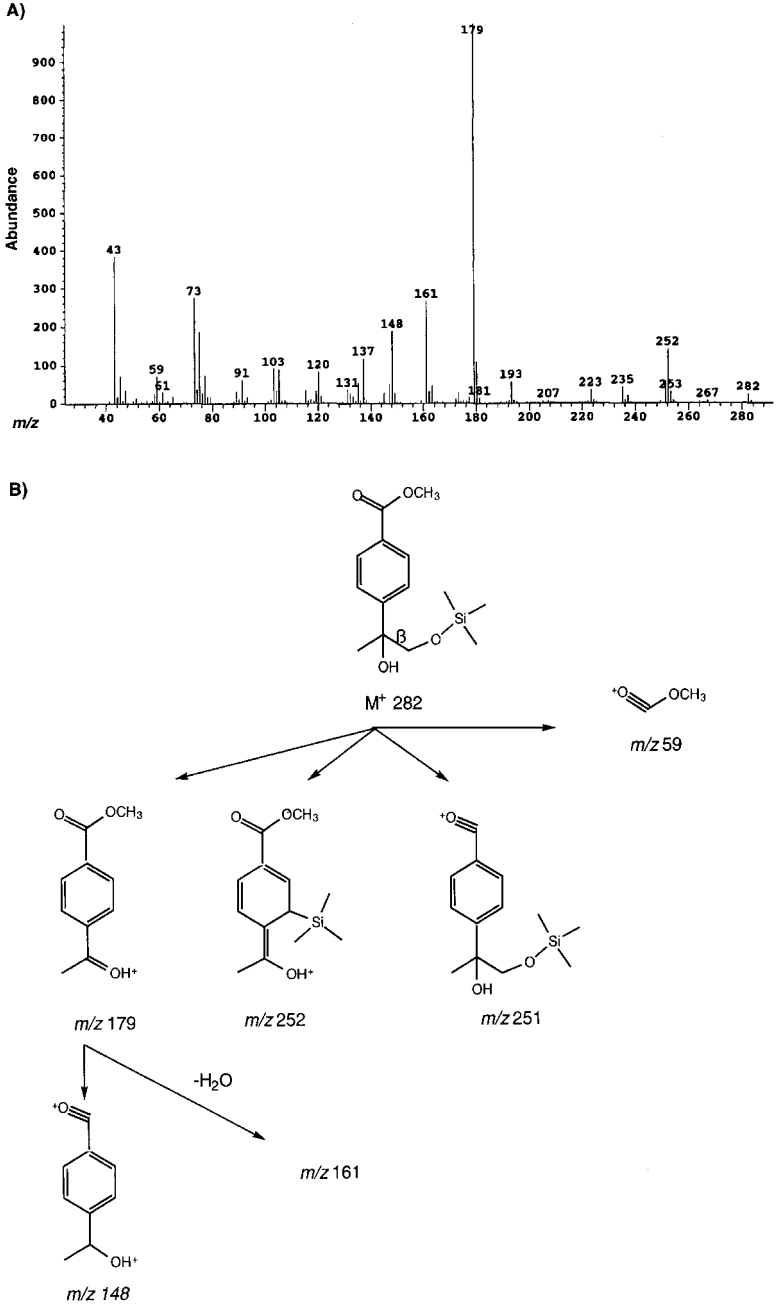
[a] O_n = number of oxygen atoms introduced by oxidation of p-cymene; gly = glycine conjugated metabolite; tr = trace (detectable, but not quantified). Student's *t* test comparing free and total metabolite recovery within dose and between doses. $P > 0.05$ in every case.
[b] *p*-Cymene dose. $N = 6$.

GC-MS indicated a molecular weight of 210 from the intense ion at m/z 228 ($M+NH_4^+$), and EI-GC-MS showed a trace of the M^+ ion at m/z 210.

Cy 13 chromatographed poorly after methylation alone, and the peak shape readily deteriorated. Chromatography improved dramatically with further derivatization with a TMS group, suggesting an additional hydroxy grouping. The C-8 hydroxy group of Cy 13 (and Cy 2) does not accept the TMS group due to steric hindrance.

The EI mass spectrum of Cy 13 (methyl ester and TMS derivative) was m/z (%): 282 [282.1229, $C_{14}H_{22}SiO_4$ calculated mass 282.1287, M^+(tr)], 252 [252.1173, $C_{13}H_{20}SiO_3$ calculated mass 252.1182, M^+-30 (18)], 251 [M-OCH_3, (9)], 179 [179.0717, $C_{10}H_{11}SiO_3$ calculated mass 179.0708, M^+-103 (100)], 161 (24), 148 (16), 137 (9), 120 (8), 105 (8), 103 (9), 91 (5), 77 (8), 75 (23), 73 (36), 59 (7), and 43 (41) (Figure 2). The elimination of CH_2O (M^+-30) from the molecular ion requires intramolecular rearrangement involving the TMS group. The rearrangement is illustrated in Figure 2 (inset). The methyl ester of an analogous compound, α-{[(trimethylsilyl)oxy]methyl} benzeneacetic acid, undergoes the same rearrangement, thus providing a model (NIST 1998).

The metabolite Cy 5 also has a primary alcohol at the C9 position. It would, therefore, be expected that the molecular ion of its TMS derivative would undergo similar rearrangement and fragmentation as Cy 13 (methyl ester and TMS derivative) and α-{[(trimethylsilyl)oxy]methyl} benzene acetic acid (methyl ester). Again, the mass spectral data confirmed this pattern, although no molecular ion at 266 was detected for Cy 5. The EI mass spectrum of Cy 5 (methyl ester and TMS derivative) was m/z (%): 251 (26), 237 (12), 236


A)
Abundance
m/z
179
43
73
161
148
252
59
91
103
120
137
131
193
181
207
223
235
253
267
282
B)
OCH3
Si
β
OH
M+ 282
+O
OCH3
m/z 59
OH+
m/z 179
m/z 252
m/z 251
-H2O
m/z 161
m/z 148

[236.1257, $C_{10}H_{11}SiO_3$ calculated mass 236.1233, M-CH_2O (61)], 235 (10), 132 (46), 104 (14), 103 (41), 91 (10), 77 (14), 75 (22), 74 (11), 73 (100), 59 (12), and 45 (14).

The combined mass spectral data provide strong evidence for the structure of Cy 13 being 4-(1,2-dihydroxy-1-methylethyl)benzoic acid.

Quantitative Results. Metabolites Cy 2, 5, 6, 10, and 13 were all present in amounts sufficient to quantify. Cy 11 was detected as a trace metabolite and not quantified. Table 1 reports the chemical names of metabolites, plus the derivatization required for chromatography and the purity of standards isolated from urine used for quantitation of metabolites. The ions used to identify and quantify metabolites by GC-MS are also reported in Table 1.

The fractions of the administered dose of 0.37 mmol/kg *p*-cymene recovered in 48 hr as free and total were, respectively, 0.82 ± 0.08 (mean ± SD) and 0.77 ± 0.09 ($N = 5$).

The fractions recovered for the dose of 1.49 mmol/kg were 0.77 ± 0.14 and 0.84 ± 0.12 for free and total metabolites, respectively ($N = 6$).

Individual metabolites are reported as the percent of total metabolites excreted (Table 2). Except for koala 1, which produced no urine for 24 hr after each dose, most of the metabolites found were excreted in the first 24 hr (median 77% for both doses; range: 0–97% for the lower dose, 0–100% for the higher dose). Results from the two 24-hr urine collections after dosing were combined for the total metabolite excretion.

The second most abundant metabolite, the dicarboxylic acid, Cy 6, also acquired four oxygen atoms. Together the 4-oxygen metabolites accounted for about 75% of the recovered metabolites. A further 7% of the recovered dose was excreted as the glycine conjugate, Cy 10. Cumic acid is likely to be the precursor of Cy 10, and it loses a hydroxyl group during glycine conjugation. A small amount of a second glycine conjugate, Cy 11, was also found.

There was no significant difference (Student's paired *t* test) between the recovery of free and total metabolites within each dose or between the two doses (Table 2). This suggests no hydrolyzable conjugation of *p*-cymene metabolites with either glucuronic acid or sulfate.

Leaf Analysis. The *p*-cymene content of *E. cephalocarpa* represented a daily background intake of about 10 mg/day (0.075 mmoles/day). The effect of this background intake was considered to be negligible.

FIG. 2. (Opposite) (A) Mass spectrum of Cy 13 (methyl ester and TMS derivative). (B) Proposed fragmentation pattern of Cy 13 (methyl ester and TMS derivative) as depicted in the mass spectrum (A). The rearrangement of silicon onto the aromatic ring results in an ion at m/z at M^+-30.

TABLE 3. URINARY GLUCURONIC ACID (GA)[a]

	Urinary glucuronic acid (mmol/kg)					
	0.37 mmol/kg[b]			1.49 mmol/kg[b]		
Koala	Day 0[c]	Day 1	Day 2	Day 0[c]	Day 1	Day 2
1	2.39	0[d]	1.820	1.30	0[d]	1.65
2	0.97	0.899	0.397	0.55	0.64	0.65
3	0.69	0.489	0.774	0.72	0.53	0.49
4	0[d]	1.449	1.378	1.48	1.04	0.96
5	0.70	3.405	1.435	1.13	0.39	2.49
6	0.69	1.358	1.211	1.28	0.92	0.65
Mean	1.09	1.52	1.17	1.08	0.70	1.15
SD	0.74	1.12	0.51	0.36	0.27	0.78
P value[e]		0.33	0.89		0.07	0.82

[a] 1.0 mmol GA = 194 mg.
[b] *p*-Cymene dose.
[c] Day 0 is the predose control.
[d] No urine voided.
[e] Student's *t* test comparing post *p*-cymene dose GA with control urine GA.

Glucuronic Acid. Table 3 reports the levels of glucuronic acid measured in the koala. Although the koalas excreted variable quantities of glucuronic acid daily, there was no statistically significant increase in glucuronic acid excretion after *p*-cymene was administered.

Fecal Analysis. GC-MS analysis of fecal extracts revealed no unchanged *p*-cymene, oxidized metabolites, or their conjugates.

DISCUSSION

The results of this study demonstrate that the male koala employs a metabolic strategy in the detoxification of *p*-cymene that promotes production of extensively oxidized metabolites that consequently have increased polarity and enhanced renal excretion. The koala excreted six metabolites of *p*-cymene, all of which were either extensively oxidized at multiple sites (Cy 13, 6, 5, and 2) or oxidized to a precursor carboxylic acid (cumic acid) and then conjugated with the amino acid, glycine (Cy 10 and 11). No significant conjugation of metabolites with glucuronic acid was detected. Urinary glucuronic acid excretion (Table 3) also indicates a lack of any statistically significant increase after the *p*-cymene doses, although the large SD indicates that these determinations would not be sensitive enough to detect *p*-cymene conjugation. Both observations combined provide strong evidence that glucuronidation is not important in the elimination of *p*-cymene in male koalas.

The absence of glucuronidation of *p*-cymene metabolites is noteworthy, considering the large amount of glucuronic acid found in the urine. The koalas excreted 1.7 ± 1.3 g (mean ± SD) of glucuronic acid daily (Table 3), suggesting that glucuronidation probably has an important role in the elimination of some PSM component of *E. cephalocarpa*.

Examining the chemical structures of metabolites provides insight into the enzymatic pathways involved in detoxification (Figure 1). All six metabolites were oxidized to a carboxylic acid at the C-7 position. It would, therefore seem that this is the most reactive site for oxidation. The C-7 oxidized metabolites, Cy 8, Cy 2, Cy 5, and Cy 6, have been reported as metabolites of cuminaldehyde (Ishida et al., 1989). About 7% was excreted as the glycine conjugate of cumic acid (4-isopropyl benzoic acid). Although unconjugated cumic acid was not excreted in the koala, it was a significant urinary metabolite in the rat, brushtail possum, and rabbit, and underwent partial conjugation with glucuronic acid in these species (Ishida et al., 1981, 1989; Walde et al., 1983; Boyle et al., 1999).

The remainder of the metabolites had also undergone oxidation at one or more alternative sites. Simple hydroxylation at the C-8 and C-9 positions produced Cy 2 and 5, respectively. The alcohol at the C-9 position of Cy 5 was further oxidized to a carboxylic acid, resulting in the dicarboxylic acid metabolite, Cy 6. Cy 6 has been reported as a *p*-cymene metabolite in other animal species and accounted for about 25% of the recovered dose in the koala. Cy 13 was oxidized at three different sites, forming alcohols at both the C-8 and C-9 positions. Together Cy 6 and Cy 13 accounted for the majority of the metabolites recovered.

Cy 13 was found, despite its poor chromatography, because of its abundance in koala urine. By using the double derivatization method developed here, urine samples from the other animal species used in our previous study (Boyle et al., 1999) were reanalyzed. Cy 13 was detected in all species (rat, brushtail possum, ringtail possum, and greater glider), but in minor amounts.

Ishida et al. (1982) tentatively assigned the Cy 13 structure to a metabolite of perilla aldehyde, based on EI mass spectral data alone. However, their EI data do not support the structure now assigned to Cy 13, which is based on considerably more extensive MS data and the fragmentation behavior of related model compounds. It is therefore proposed that this is a novel metabolite of *p*-cymene.

Little is known about the enzymes involved in the oxidation of terpenes in koalas. Cytochrome P-450 enzymes are located on the endoplasmic reticulum and catalyze aliphatic oxidations and aromatic hydroxylations of endogenous and xenobiotic compounds, producing more polar and, therefore, more readily excreted metabolites (Alvares and Pratt, 1990). Stupans et al. (1999) reported that the total CYP content of koala liver was similar to that found in the rat. Test substrates indicated that the koala possesses hepatic CYPs that correspond

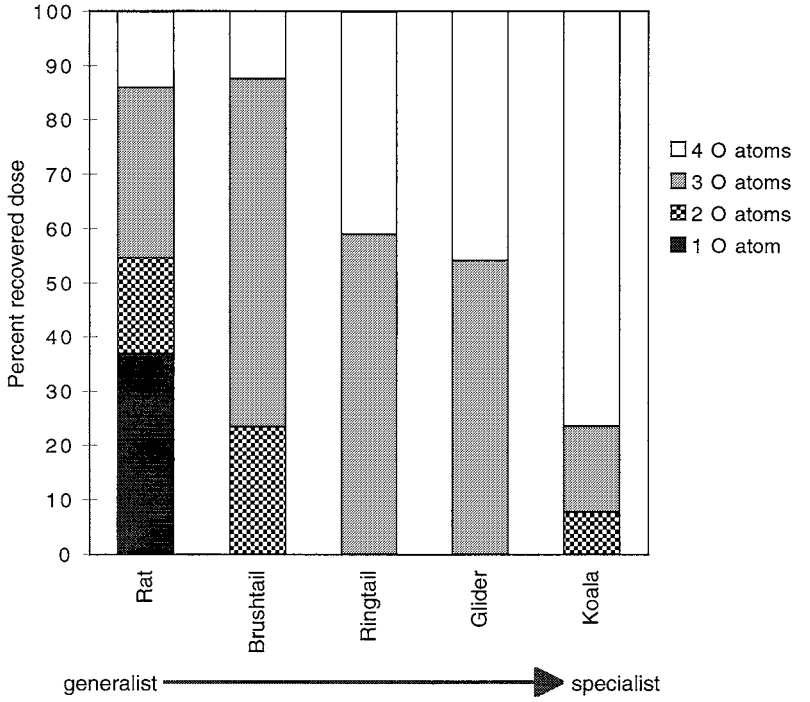


FIG. 3. Comparative oxidation of *p*-cymene in generalist and specialist eucalypt folivores and the eutherian rat. Metabolites are categorized by number of oxygen atoms acquired. Where two doses were administered, data were averaged (Boyle et al., 1999). Note that the 2-oxygen metabolites in the koala were conjugated with glycine, possibly preventing further oxidation.

to several CYP families (CYP 2A, 2B, 2C, 2E, and 3A) from rat livers. However, without data to relate rat and koala CYPs, it cannot be said which CYPs are responsible for *p*-cymene oxidation.

Combining the results from this study with our earlier work (Boyle et al., 1999), valuable insight is provided into the comparative detoxification mechanisms employed by all marsupials that consume leaves of eucalypts, from the generalist browser (the brushtail possum) through the specialists (the ringtail possum and greater glider) and to the most specialized (the koala). The rat was also included for comparison with a naive generalist eutherian. Figure 3 shows the difference in overall *p*-cymene excretion patterns among these species. The total free plus glucuronidated metabolites have been grouped according to the number of oxygen atoms acquired during oxidation (prior to any subsequent conjugation). Thus, CY 10 was scored as having received two oxygens (as cumic acid). It

should be noted that in the koala, glycine conjugation of cumic acid may prevent its further oxidation. Without this conjugation, there would probably have been no 2-oxygen metabolites of *p*-cymene (since no free cumic acid was excreted). Progression from generalist to specialist shows a distinct increase in the proportion of more extensively oxidized metabolites. Given that monoterpenes such as *p*-cymene are always present in the eucalypt leaf diet of these folivores, this difference suggests an adaptation of detoxification mechanisms to diet.

There was also an important difference in conjugation between the specialist and generalist feeders. Glucuronic acid is excreted in the urine conjugated to xenobiotic or endogenous compounds to increase polarity and, therefore, enhance renal excretion. There was no evidence of glucuronidation of *p*-cymene metabolites in any of the specialists, yet significant amounts of metabolites were glucuronidated in the brushtail possum and rat (Boyle et al., 1999). Evidently in the specialists, the preferred method of elimination of *p*-cymene is extensive oxidation rather than glucuronidation.

It now seems clear that many simple monoterpenes require no conjugation to be eliminated by the koala, ringtail possum, or greater glider. Conservation of the glucuronidation pathway in the excretion of simple monoterpenes would preserve the available glucuronidating capacity to assist in the elimination of other PSMs. The glucuronic acid excreted by the koala suggests that glucuronidation is important in the elimination of some PSMs.

The glycine-conjugated metabolites Cy 10 and 11 accounted for about 7% of the recovered metabolites in the koala, but were not detected at all in the ringtail or glider. Yet, the glycine conjugation pathway was active in all species, as hippuric acid, the glycine conjugate of benzoic acid, was excreted in each case.

Detoxification mechanisms may be considered in the following way. For each species, the ultimate aim of detoxification is to produce the most readily cleared metabolites within the available metabolic budget. After initial oxidation, each species balances further oxidation with conjugation to produce a metabolic excretion pattern, optimizing metabolic costs for the diet selection. It seems the brushtail can afford to conjugate less oxidized metabolites with glucuronic acid rather than opting for further oxidation.

We can only postulate on the reasons why specialists use a different strategy in the elimination of *p*-cymene metabolites compared to the generalist. Since renal excretion is enhanced by increased polarity, it is reasonable to assume that the greater the degree of oxidation, the greater the rate of elimination of the metabolite. Increased polarity, whether achieved by extensive oxidation or conjugation, presumably results in an overall enhanced capacity to excrete metabolites. It would, therefore, seem that the different strategies employed by specialist's compared to generalists must be based on some other factor, such as a balance of dietary energetics or enzyme availability.

If oxidation proceeds via a series of single-step reactions, then depending on the location of reactive sites on the *p*-cymene molecule, a large number of precursor metabolites are possible. As the efficiency of the oxidative enzymes increases, fewer of the precursor metabolites would be expected. The rat and brushtail possum excreted as many as nine different metabolites, whereas the ringtail and glider excreted only three metabolites, with a trace of a fourth in the ringtail. The koala excreted six, but two were as glycine conjugates, which may have prevented further oxidation to one of the other four metabolites.

Wild animals are difficult to work with and must be handled and housed carefully to minimize stress. Administering an oral dose to any animal may be stressful, particularly with standard procedures such as gavaging. The method devised for dosing koalas in this experiment appeared to cause little stress in the animals. The koalas participated in the dosing procedure, voluntarily licking the dose from a syringe. The accuracy and reproducibility of the dose received were acceptable, with only an occasional messy licker, after which the koala would lick the paste containing the dose from its fur.

Single bolus doses allow preliminary quantitative studies of the fate of a compound in a species. However, it is not normal feeding behavior, particularly for herbivores, and in this case does not allow direct comparison to *p*-cymene intake in wild koalas feeding from leaf. Presumably, wild koalas tolerate much larger daily intakes while browsing on eucalypt species with high oil yield. Nonetheless, this study has provided the essential tools necessary for us to examine constraints on feeding and detoxification in mammals.

Acknowledgments—We would like to thank Margaret Healy, Miranda Ebbers, and Kathrine Handasyde for their advice and assistance with koala care and handling. We would also like to thank the Phillip Island Nature Park for allowing us to use their koala holding facilities and eucalypt plantation.

REFERENCES

AMMON, D. G., BARTON, A. F., CLARKE, D. A., and TJANDRA, J. 1985. Rapid and accurate determination of terpenes in the leaves of *Eucalyptus* species. *Analyst* 110:921–924.

ALVARES, A. P., and PRATT, W. B. 1990. Pathways of drug metabolism, pp. 365–422, *in* W. B. Pratt and P. Taylor (eds.). Principles of Drug Action—The Basic of Pharmacology. Churchill Livingston, New York.

BLUMENKRANTZ, N., and ASBOE-HANSEN, G. 1973. New method for quantitative determination of uronic acids. *Anal. Biochem.* 54:484–489.

BOYLE, R., MCLEAN, S., FOLEY, W. J., and DAVIES, N. W. 1999. Comparative metabolism of the dietary terpene, *p*-cymene, in generalist and specialist folivorous marsupials. *J. Chem. Ecol.* 26:2109–2126.

BULL, S. D., CARMAN, R. M., CARRICK, F. N., and KLIKA, K. D. 1993. 7-Hydroxy-1,8-cineole and 7-cineolic acid. Two new possum urinary metabolites. *Aust. J. Chem.* 46:441–447.

CARMAN, R. M., and GARNER, A. C. 1996. 7,9-Dihydroxy-1,8-cineole and 2α,7-dihydroxy-1,8-cineole: two new possum urinary metabolites. *Aust. J. Chem.* 49:741–749.

CARMAN, R. M., and KLIKA, K. D. 1992. Partially racemic compounds as brushtail possum urinary metabolites. *Aust. J. Chem.* 45:651–657.

CARMAN, R. M., and RAYNER, A. C. 1994. 2-α,4-Dihydroxy-1,8-cineole—a new possum urinary metabolites. *Aust. J. Chem.* 47:2087–2097.

CARMAN, R. M., GARNER, A. C., and KLIKA, K. D. 1994. 2,9-Dihydroxy-1,8-cineole and 2,10-dihydroxy-1,8-cineole—two new possum urinary metabolite. *Aust. J. Chem.* 47:1509–1521.

EBERHARD, I. H., MCNAMARA, J., PEARSE, R. J., and SOUTHWELL, I. A. 1975. Ingestion and excretion of *Eucalyptus punctata* D.C. and its essential oils by the koala. *Phascolarctos cinereus* (Goldfuss). *Aust. J. Zool.* 23:169–179.

FREELAND, W. J. 1991. Plant secondary metabolites: biochemical coevolution with herbivores, pp. 61–81, *in* R. T. Palo and C. T. Robbins (eds.). Plant Defenses Against Mammalian Herbivores. CRC Press, Boca Raton, Florida.

FREELAND, W. J., and JANZEN, D. H. 1974. Strategies in herbivory by mammals: The role of plant secondary compounds. *Am. Nat.* 108:268–289.

ISHIDA, T., ASAKAWA, Y., TAKEMOTO, T., and ARATANI, T. 1981. Terpenoid biotransformation in mammals iii: Biotransformation of α-pinene, β-pinene, pinane, 3-carene, carane, myrcene, and *p*-cymene in rabbits. *J. Pharm. Sci.* 70:406–415.

ISHIDA, T., ASAKAWA, Y., and TAKEMOTO, T. 1982. Metabolism of myrtenal, perilla aldehyde and dehydroabietic acid in rabbits. *Hiroshima Kogyo Daigaku Kenkyu Kiyo* 16:79–91.

ISHIDA, T., TOYOTA, M., and ASAKAWA, Y. 1989. Terpenoid biotransformation in mammals. V. Metabolism of (+)-citronellal, (±)-7-hydroxycitronellal, citral, (−)-perillaldehyde, (−)-myrtenal, cuminaldehyde, thujone, and (±)-carvone in rabbits. *Xenobiotica* 19:843–855.

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

MATSUMOTO, T., ISHIDA, T., YOSHIDA, T., TERAO, H., TAKEDA, Y., and ASAKAWA, Y. 1992. The enantioselective metabolism of *p*-cymene in rabbits. *Chem. Pharm. Bull.* 40:1721–1726.

MCLEAN, S., FOLEY, W. J., DAVIES, N. W., BRANDON, S., DUO, L., and BLACKMAN, A. J. 1993. Metabolic fate of dietary terpenes from *Eucalyptus radiata* in common ringtail possums (*Pseudocheirus peregrinus*). *J. Chem. Ecol.* 19:1625–1643.

MORROW, P. A., and FOX, L. R. 1980. Effects of variation in *Eucalyptus* essential oil yield on insect growth and grazing damage. *Oecologia* 45:209–219.

NIST. 1998. NIST Mass Spectral Library. US Department of Commerce, Gaithersburg, Maryland.

SOUTHWELL, I. A. 1973. Variation in the leaf oil of *Eucalyptus punctata. Phytochemistry* 12:1341–1343.

SOUTHWELL, I. A. 1975. Essential oil metabolism in the koala (iii) novel urinary monoterpene lactones. *Tetrahedron* 24:1885–1888.

SOUTHWELL, I. A., FLYNN, T. M., and DEGABRIELE, R. 1980. Metabolism of α- and β-pinene, *p*-cymene and 1,8-cineole in the brushtail possum, *Trichosurus vulpecula. Xenobiotica* 10:17–23.

STUPANS, I., KONG, S., KIRLICH, A., MURRAY, M., BAILEY, E. L., JONES, B. R., and MCKINNON, R. A. 1999. Hepatic microsomal enzyme activity in the koala and tammar wallaby: high 17β-hydroxysteroid oxidoreductase activity in koala liver microsomes. *Comp. Biochem. Physiol. Part C* 123(1):67–73.

VOGEL, A. I. 1959. A Textbook of Practical Organic Chemistry Including Qualitative Organic Analysis, 3rd ed. Longmans, Green, London.

WALDE, A., VE, B., and SCHELINE, R. R. 1983. *p*-Cymene metabolism in rats and guinea pigs. *Xenobiotica* 13:503–512.