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Metabolites of dietary 1,8-cineole in the male koala (*Phascolarctos cinereus*)

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Abstract

The in vivo metabolic fate of 1,8-cineole was investigated in six male koalas. Koalas were fed ad lib a diet of *Eucalyptus cephalocarpa* leaf with a 1,8-cineole concentration of $2.53 \pm 0.70\%$ dry mass of leaf, corresponding to a 1,8-cineole intake of 2.4 ± 1.1 mmol/kg (3.1 ± 1.3 g). Urine and faeces were collected for 24 h and metabolites identified by GC-MS and LC-MS. Metabolites were quantified before and after hydrolysis with β-glucuronidase to give free and total levels, respectively. Fractional recovery of ingested 1,8-cineole was 1.3 ± 0.4 and 1.4 ± 0.4 (mean ± S.D.) for free and total measurements, respectively. Seven metabolites were identified and quantified: 9- and 7-hydroxy-cineole, 9- and 7-cineolic acid, 7-hydroxy-9-cineolic acid, 9-hydroxy-7-cineolic acid and 7,9-dicineolic acid. The hydroxy-cineolic acids dominated the metabolite profile (85%). 7,9-Dicineolic acid, a novel metabolite of 1,8-cineole, accounted for almost 10% of the recovered dose making it the second most abundant metabolite after 7-hydroxy-9-cineolic acid (77%). Together, the less oxidised metabolites, the hydroxycineoles and cineolic acid, accounted for only 5% of the cineole consumed. Significant conjugation only occurred with four minor, less oxidised, alcohol and carboxylic acid metabolites. We have shown that the koala detoxifies and eliminates 1,8-cineole primarily by extensive oxidation without utilising conjugation pathways.

Keywords: 1,8-Cineole; Detoxification; *Eucalyptus*; Koala; Metabolism; *Phascolarctos cinereus*; Terpene

1. Introduction

All herbivores experience dietary challenges from a plethora of physical and chemical defences, evolved by plants for the purpose of deterring herbivores. The chemical challenge to many herbivores, and particularly folivores, is such that many ingest quantities of plant toxins that would be toxic to unadapted or naive species of mammals, including man. Their ability to subsist on such diets has historically been ascribed to an enhanced capability for detoxification (Freeland and Winter, 1975). However, until recently there has been little physiological evidence to support such claims. We report here the metabolic fate of 1,8-cineole, a common eucalypt monoterpene, in the koala (*Phascolarctos cinereus*) as part of a series of studies aimed at understanding detoxifi-

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cation strategies employed by eucalypt folivores and herbivores in general. These studies have been an important first step in understanding the detoxification processes involved with specific dietary toxins as well as demonstrating inter-species adaptations that reflect the occurrence of dietary toxins in the respective diets of the species studied.

Monoterpenes were chosen as the model for plant toxins as they are a relatively simple class of compound which are ingested by many herbivores in large quantities with a potentially major impact. For example, the rate of food intake may be influenced as extensive biotransformation is required before the terpenes are excreted from the body. Those Australian marsupials that feed on *Eucalyptus* foliage are classified as either specialist feeders [greater glider (*Petauroides volans*) and koala (*Phascolarctos cinereus*)] or generalist browsers [e.g. common brushtail possum (*Trichosurus vulpecula*)] that combine eucalypts with other plant species. Comparison of these species gives a useful system for understanding the adaptation of biotransformation mechanisms in response to dietary exposure to plant toxins.

We have reported on the fate of another terpene found commonly in *Eucalyptus* leaves, *p*-cymene, in a comparative study of four marsupial folivores (Boyle et al., 1999, 2000b). These previous studies showed an adaptive preference by specialist feeders (i.e. the koala and greater glider) to excrete only a few monoterpene metabolites, of which all had been oxidised at two or more different sites (i.e. hydroxy carboxylic acids and dicarboxylic acid). On the other hand, the generalist brushtail possum excreted a greater number of metabolites. The majority of the metabolites excreted had undergone oxidation at only one or two sites (to alcohols, carboxylic acids, hydroxy carboxylic acids and a dicarboxylic acid).

Furthermore, we found that the specialists did not utilise conjugation pathways extensively in the detoxification of monoterpenes, despite the excretion of large quantities of glucuronic acid in the urine (Boyle et al., 2000b). The generalist, however, did utilise conjugation pathways to enhance the excretion of less oxidised metabolites.

In the past the metabolic fate of terpenes such as 1,8-cineole have been reported in a number of animals, the majority being standard laboratory species such as rats, rabbits and guinea pigs (Ishida et al., 1979, 1981, 1982, 1989; Walde et al., 1983; Asakawa et al., 1988; Miyazawa et al., 1989) as well as in the brushtail possum (Southwell et al., 1980; Carman and Klika, 1991, 1992; Bull et al., 1993; McLean et al., 1993; Carman and Rayner, 1994; Carman et al., 1994; Carman and Garner, 1996). Our group has reported a comprehensive investigation of the detoxification products of 1,8-cineole in brushtail possums (Boyle et al., 2000a). The studies reported by our group have found that standard laboratory species have a relatively simple pattern of excretion compared to marsupial folivores.

Once the pattern of plant toxin metabolite excretion is known then subsequent pharmacokinetic studies can be performed. Such studies have the potential to contribute greatly to the assessment of detoxification capabilities and foraging behaviour of herbivores.

2. Methods and materials

2.1. Animals

The metabolic products of 1,8-cineole were investigated in urine from six male koalas (weight 8.79 ± 1.01 kg) being maintained on a diet of *Eucalyptus cephalocarpa* leaf, in which 1,8-cineole is the major terpene. The metabolic fate of *p*-cymene was investigated concurrently and details of animal husbandry, including ethical approval, housing, care and experimentation procedures have been reported previously (Boyle et al., 2000b). For the present study 1,8-cineole metabolite analyses were made on urine and faecal samples collected 24–48 h after a single oral dose of *p*-cymene (1.49 mmol/kg). There was minimal interference from *p*-cymene metabolites in the samples as the majority of the *p*-cymene dose had been excreted in the previous 24 h. Samples were stored at -18°C until analysed.

Koalas had been feeding on *E. cephalocarpa* for at least 3 days before samples were collected. Leaf was picked on a weekly basis from a selection of 12 trees in a purpose grown plantation. Animals were offered fresh leaf each day. The amount of 1,8-cineole ingested was assumed to be proportional to the amount of leaf consumed. Daily leaf intakes were measured and the amount of 1,8-cineole ingested estimated from the measured leaf concentration.

2.2. Leaf analysis

Leaf samples from each tree were collected, stored frozen at −18°C and analysed individually. Dry mass (DM) was determined on samples of fresh leaf by drying at 70°C to a constant weight.

The ethanol extractable component of *E. cephalocarpa* leaves was analysed to determine the terpene profile plus the 1,8-cineole content as previously described (Boyle et al., 2000b). Verification of terpene content by hydrodistillation of the volatile component of leaf samples from two trees confirmed effectiveness of the ethanol extraction method (Boland et al., 1991).

2.3. Urine analysis

Urine samples were analysed for both free (unconjugated) and total (unconjugated and conjugated) 1,8-cineole metabolite levels using the method developed for the brushtail possum (Boyle et al., 2000a). Briefly, 100 μl of urine was diluted to 500 μl with distilled water and internal standard (0.5 mg 2,5-dimethylbenzoic acid in 50 μl methanol) added. The sample was acidified to pH 1 with 20 μl of 5 M HCl and then extracted with three sequential volumes of ethyl acetate (1×1 ml and 2×0.5 ml). After vortexing for 30 s and subsequent centrifugation (2000 rev./min for 5 min) to ensure thorough mixing and separation of urine and ethyl acetate, the ethyl acetate fractions were combined. Each sample was then doubly derivatised: firstly with diazomethane to methylate carboxylic acid groups and secondly with *N*,*O*-bis(trimethyl) trifluoroacetamide (BSTFA; Alltech Associates Inc., Deerfield, IL, USA) to form trimethylsilyl (TMS) derivatives of hydroxyl groups.

Total metabolites were measured after an initial hydrolysis procedure. Diluted urine samples were mixed with 100 μl of 1.1 M acetate buffer and 25-μl extract of *Helix pomatia* (β-glucuronidase 141 000 units/ml plus aryl sulphatase 3950 units/ml; Boehringer Mannheim, Germany) and heated at 37.5°C overnight. After incubation, samples were treated as above except that 40 μl of 5 M HCl was required to acidify the acetate buffer. The formation of emulsions in the ethyl acetate phase after vortexing required physical disruption by stirring with a glass pipette and re-centrifuging to dissipate the emulsion.

Details of the gas chromatography-mass spectrometry (GC-MS) conditions used for identification and quantitation of 1,8-cineole metabolites in urine extracts have been reported previously (Boyle et al., 2000a). Glucuronic acid in urine samples was measured by colourimetry (Blumenkrantz and Asboe-Hansen, 1973; Boyle et al., 2000b).

Liquid chromatography-mass spectrometry (LC-MS) with mass spectrometry–mass spectrometry (MS/MS) capability was used to provide further mass spectral data on 1,8-cineole metabolites. Urine samples were filtered (0.45 μm) after dilution (1:10 in water).

The LC-MS used was a Waters Alliance 2690 HPLC coupled to a Finnigan LCQ using positive ion atmospheric pressure chemical ionisation (APCI) and both positive ion and negative ion electrospray ionisation. The chromatography was carried out on a Waters NovaPak 150×3.9 mm C18 analytical column. Mobile phase was 10% methanol/90% water (with 2% acetic acid) and graded to methanol 100% at 40 min, flow rate of 0.8 ml/min and injection volume 15 μl.

APCI MS conditions were as follows: vaporiser, 400°C; heated capillary, 170°C; capillary voltage, 24 V; current, 4 μA; sheath gas, 50 psi; and auxillary gas, 15 psi. Alternate data-dependent MS/MS scans from the most intensive ion were acquired using 23% of the maximum collision energy. Positive electrospray ionisation conditions were as follows: needle, 4.5 kV; heated capilliary, 270°C; sheath gas, 90 psi; auxillary gas, 50 psi; capillary voltage, 40 V; and data-dependent scans as for APCI. Negative ESI conditions were the same as for positive ESI except for polarity reversal on various focusing lenses.

2.4. Faecal analysis

Faecal samples were also analysed for both free and total 1,8-cineole metabolites using the method described previously (Boyle et al., 1999). Faecal pellets were ground with a mortar and pestle and 1 g mixed with 10 ml of water to form a slurry. A 500-mg aliquot of slurry was acidified to pH 1 with three drops of 5 N HCl and extracted into three washes (1×2 and 2×1 ml) of dichloromethane/*n*-propanol (80:20). A sample of the dichloromethane/*n*-propanol extract was evaporated to dryness and redissolved in 500 μl of ethyl acetate. Extracts were then derivatised in the same manner as for urine samples. Total

metabolite analyses were performed on faecal slurries which had first been incubated at pH 5.3 (200 μl of 1.1 M acetate buffer) with 50 μl of extract of *Helix pomatia* overnight at 37.5°C.

2.5. Identification of 1,8-cineole metabolites

Most metabolites were already known from the study of 1,8-cineole metabolism in brushtail possums (Boyle et al., 2000a). The structure of 7,9-dicarboxylic acid (Ci 22) was assigned from interpretation of the mass spectral data.

7-Hydroxy-9-cineolic acid (Ci 13) was isolated from koala urine to allow nuclear magnetic resonance (NMR) analysis. Both ^{1}H-NMR and ^{13}C-NMR were performed. They were recorded on a Varian 400-MHz Widebore Inova system on a 4-nucleus 5-mm solution probe and *z*-axis inverse-pulsed field gradient probe. Tetramethylsilane was used as an internal standard. The isomeric structure and relative stereochemistry were determined on the basis of short and long range gradient-COSY, gradient-HMQC/HMBC, DEPT and gradient-NOESY experiments.

2.6. Isolation of 7-hydroxy-9-cineolic acid (Ci 13)

Urine collected from the koala was fractionated into strong and weak acids and neutral compounds (Boyle et al., 2000a). The concentrated strong acid fraction was methylated with excess diazomethane and applied to 75-cm-wide preparative thin layer chromatography (pTLC) plates and developed in 100% ethyl acetate. As discussed by Boyle et al. (2000a) it was not possible to locate 1,8-cineole and its metabolites directly on TLC and so we relied on GC analysis of sequential zones (1–10) visualised by UV quenching or vanillin reaction. Each zone was dissolved in sequential methanol washes (2×15 ml and 1×10 ml) and the extract evaporated to dryness and the residue redissolved in ethyl acetate before GC analysis.

Final purification was by preparative HPLC (pHPLC). The instrument used was a Waters M-45 Solvent Delivery System coupled to a Waters Series 440 Absorbance Detector (214 nm) (Waters Associates, Inc, Milford, MA, USA) and LDC/Milton Roy CI-10B Integrator. Chromatography was carried out on a Waters PrepPak Cartridge 25×100 mm, prep NovaPak HR C18 6-μm 60-Å using a GuardPak Cartridge as a guard column. The column was radially compressed in a Waters PrepLC 25-mm Module at < 1500 psi. The HPLC pump was operated at a flow rate of 9 ml/min with water/methanol (55:45) as the mobile phase.

The Ci 13 residue from pTLC was dissolved in 5 ml of methanol, diluted with 50% mobile phase (water/methanol, 55:45), filtered (0.45-μm nylon filter; Aventec MFS, CA, USA) and 200 μl injected onto the column. Four fractions were collected accounting for all HPLC peaks. A sample (2-ml) of each fraction was acidified to pH 1 with 5 M HCl and extracted with 1 ml of ethyl acetate and the content analysed by GC-MS.

The same fractions from a further two pHPLC runs were combined in a large flask and the volume reduced to approximately 300 ml on a rotary evaporator. The remaining aqueous solution was acidified with 5 M HCl and extracted with three washes of ethyl acetate. The ethyl acetate extract was then evaporated to dryness.

3. Results

3.1. Terpene profile in E. cephalocarpa

Analysis of the ethanol extract of *E. cephalocarpa* showed that the major extractable component was 1,8-cineole (Fig. 1). Detectable amounts of limonene, *p*-cymene and α- and β-pinene were also present. This profile is similar to that reported by others for *E. cephalocarpa* (85.6% of its essential oil has been reported to be 1,8-cineole; Boland et al., 1991).

3.2. 1,8-Cineole leaf concentrations

The dry mass (DM) of leaves from the 12 trees fed to koalas was $42.0 \pm 2.1\%$. The dry leaf 1,8-cineole concentration was $2.53 \pm 0.70\%$ (wet leaf concentration of $1.07 \pm 0.32\%$).

3.3. 1,8-Cineole ingestion

The average daily intake of leaf (DM) by koalas during the sample period was 131 ± 45 g ($n = 6$). This equates to an intake of 1,8-cineole equivalent to 2.4 ± 1.1 mmol/kg (3.1 ± 1.3 g).

3.4. Isolation of 7-hydroxy-9-cineolic acid (Ci13)

Ci 13 was present in two zones after pTLC of

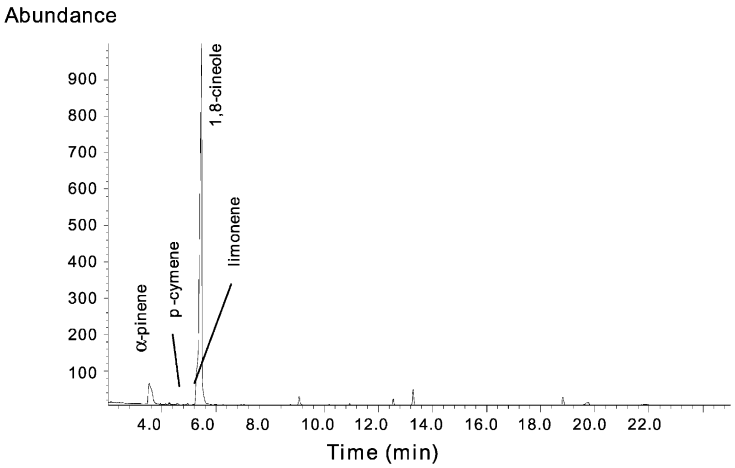

Fig. 1. TIC chromatogram from GC-MS analysis of ethanolic extract of *E. cephalocarpa* leaf.

the strong acid fraction ($R_f = 0.46–0.56$). The Ci 13 yield at this stage was approximately 400 mg and purity, measured by GC-MS, was 85%.

Further purification by pHPLC resulted in Ci 13 eluting in the final fraction collected, with no corresponding major peak. The final yield of Ci 13 was 105 mg (purity, by GC-MS, was 96%).

3.5. Identification and quantification of urinary 1,8-cineole metabolites

Urine output was 101 ± 41 ml (mean $\pm$ S.D.) and pH was 5.4 ± 0.2 (mean $\pm$ S.D.) for the urine samples analysed ($n = 6$).

Seven metabolites of 1,8-cineole were identified and quantified in the koala (Fig. 2). Fig. 3 shows the total ion chromatogram (TIC) from GC-MS analysis of hydrolysed urine extract. Note that the abbreviations used for metabolites reflect the order in which they elute from the GC and are consistent with the abbreviations applied to metabolites found in the brushtail possum (Boyle et al., 2000a). The 9- and 7-hydroxycineole (Ci 3 and 5), 9- and 7-cineolic acid (Ci 2 and 4), 7-hydroxy-9-cineolic acid and 9-hydroxy-7-cineolic acid (Ci 13 and 17) were common to both the koala and brushtail possum. Identification of Ci 2, 3, 4, 5 and 17 metabolite structures was described in a previous publication (Boyle et al., 2000a). The 7,9-dicineolic acid (Ci 22) was a new metabolite found only in the koala. The structure of Ci 22 was based on interpretation of the mass spectral data. The electron ionisation (EI) mass spectrum for methylated Ci 22 was as follows: *m/z* 183 (100), 151 (59), 123 (28), 81 (17), 79 (18), 77 (13), 45 (53) and 43 (41). The chemical ionisation (CI) mass spectrum gave an $[M + H]^+$ ion at *m/z* 243 consistent with methylated dicarboxylic acid. As only the methyl groups at positions 7, 9 and 10 in 1,8-cineole are capable of oxidation through to a carboxylic acid, and the intense ion at *m/z* 183 was characteristic of 7-carboxylation (the MS of methylated Ci 22 was very similar to that of methylated 7-cineolic acid), Ci 22 was tentatively assigned as 7,9-dicineolic acid.

The EI mass spectrum for the methylated and TMS derivative of Ci 13 was as follows: *m/z* 271 (11), 227 (23), 211 (7), 137 (68), 119 (12), 109 (11), 94 (10), 93 (100), 91 (15), 79 (13), 75 (44), 73 (49), 59 (20), 45 (33) and 43 (54). CI MS found the protonated molecular weight to be 287. NMR results were as follows: ^{1}H-NMR, 399.714 MHz, $CDCl_3$ δ (ppm): 3.66 (*s* 3H, $-OCHc_3$); 3.33 (pseudoquartet[AB] 2H, H_a, $J = 12$ Hz); 3.05 (broad *s* 1H, −OH); 1.93 (*m* 1H, H_g); 1.87 (*m* 1H, H_m, $J = 3$ Hz); 1.83 (*m* 1H, H_f, $J = 3$ Hz); 1.66 (*m* 1H, H_e); 1.52 (*m* 1H, H_j); 1.49 (*m* 2H, H_k H_l); 1.31 (*s* 3H, CH_{b3}); 1.29 (*m* 1H, H_h); 1.28 (*m* 1H, H_j) and for ^{13}C-NMR, 100.575 MHz, $CDCl_3$ δ (ppm): 177.23 (C=O); 78.88 (C_α); 73.64 (C_β); 67.80 (CH_{a2}); 51.98 ($-OCH_{c3}$); 30.95 (CH_m); 26.34 (H_eCH_h); 25.22 (H_fCH_i); 24.28 (CH_{b3}); 22.62 (H_kCH_l); 19.95 (H_gCH_j). See Fig. 4a for positions of labelled protons.

From NMR results the structure of Ci 13 was determined to be 7-hydroxy-9-cineolic acid (Fig. 4). An NOE interaction between the methoxy methyls and H_m prove this isomeric structure.

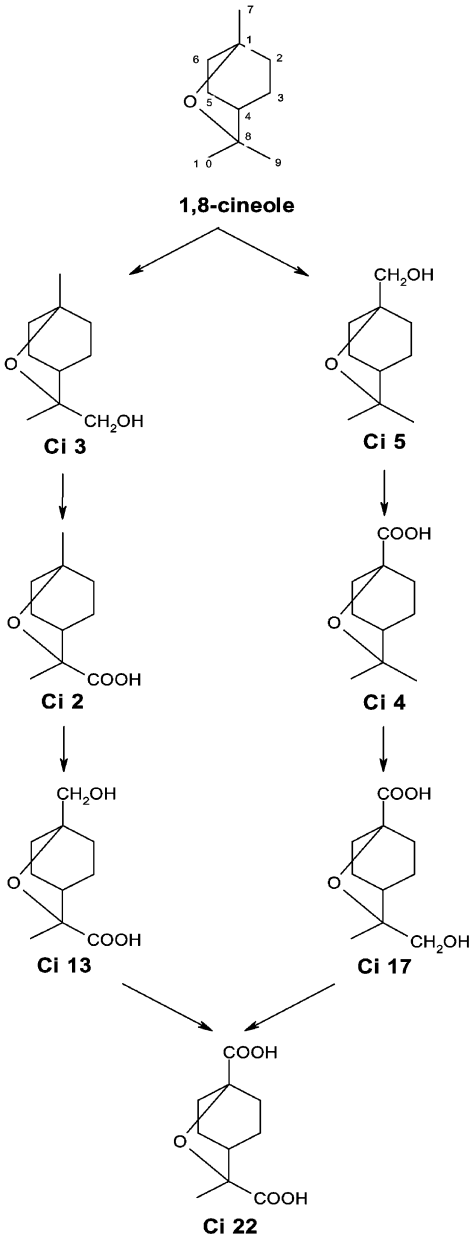

Fig. 2. Chemical structures of 1,8-cineole metabolites and probable metabolic pathways in the koala. Metabolite names were as follows: Ci 3, 9-hydroxy cineole; Ci 5, 7-hydroxy-cineole; Ci 2, 9-cineolic acid; Ci 4, 7-cineolic acid; Ci 13, 7-hydroxy-9-cineolic acid; Ci 17, 9-hydroxy-7-cineolic acid; Ci 22, 7,9-dicineolic acid.

From the chemical equivalence of H_k and H_l it could be suggested that the molecule is in a conformation with these ring protons bent down and away from the deshielding influence of the ether moiety. Energy calculations were performed which showed this to be the energetically preferred stereoisomer.

Fig. 5 shows the APCI LC-MS of a diluted koala urine sample. The hydroxyacids Ci 13 and Ci 17 were readily identified as in the brushtail possum (Boyle et al., 2000a). Although the dicarboxylic acid Ci 22 was not seen in positive ion APCI, analysis by negative ion electrospray LC-MS showed a peak consistent with the molecular ion at m/z 213 $[M-H]^-$ and an MS/MS daughter ion at m/z 169, due to loss of CO_2 from a carboxy group. Glucuronide conjugates were also found in the positive ion APCI analysis, as detailed previously for the brushtail possum (Boyle et al., 2000a) although the amounts were less in the koala. The hydroxycineole glucuronides were readily seen by monitoring the ion m/z 347 $[M+H]^+$ (Fig. 5). The conjugated acid metabolites were not as clear, but showed the water adduct ion at m/z 378 (Boyle et al., 2000a).

The abundance of each metabolite is shown in Fig. 6 for both free and total measurements. Overall, hydrolysis did not significantly increase the total recovery. However, the recovery of the minor, less oxidised, metabolites Ci 2, 3, 4 and 5 increased significantly between the two measurements [ANOVA (two-factor with replication) d.f. = 1, $F = 9.01$, $P = 0.005$].

The dose of 1,8-cineole was calculated from the estimated 24-h leaf consumption. The total urinary metabolites were used to calculate the fraction of the estimated dose recovered. The estimated fractional recovery was 1.3 ± 0.4 and 1.4 ± 0.4 (mean $\pm$ S.D.) for free and total measurements, respectively, and although > 1.0 was consistent between the two measurements.

As with previous studies where the pattern of metabolite excretion is complex, metabolites were grouped according to the degree of oxidation undergone (Boyle et al., 1999, 2000a,b). The groups of 1,8-cineole metabolites common to the brushtail possum and koala are the hydroxycineoles, cineolic acids and the hydroxycineolic acids. Ci 22 required a new grouping: dicineolic acid. Table 1 reports the percentages of total recovered metabolites for each group. Approximately 94% of total recovered metabolites had acquired three or four oxygens during oxidation, resulting in highly polar metabolites. As expected from the molar recoveries of metabolites (Fig. 6), the hydroxycineolic acids dominated the metabolite pro-

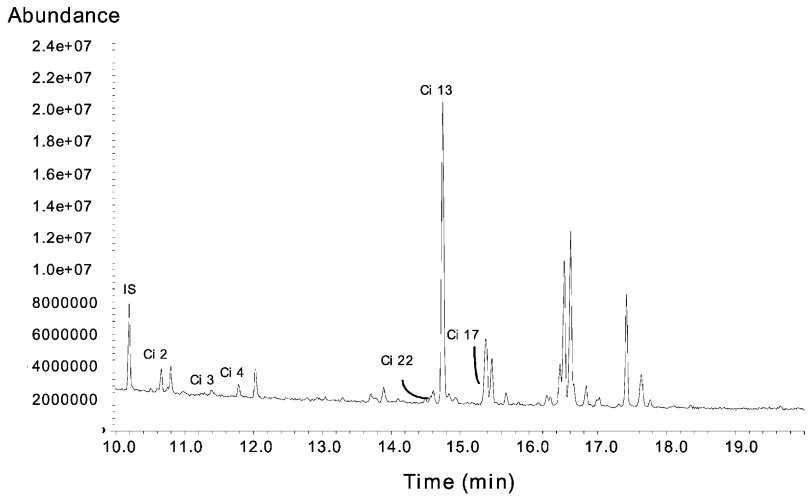

Fig. 3. TIC chromatogram from GC-MS of hydrolysed koala urine extract (methylated and TMS derivative). See Fig. 2 for metabolite structures.

file. The dicineolic acid metabolite Ci 22, the most oxidised of metabolites, accounted for almost 10% of the recovered dose and was the second most abundant metabolite after Ci 13 (77% dose).

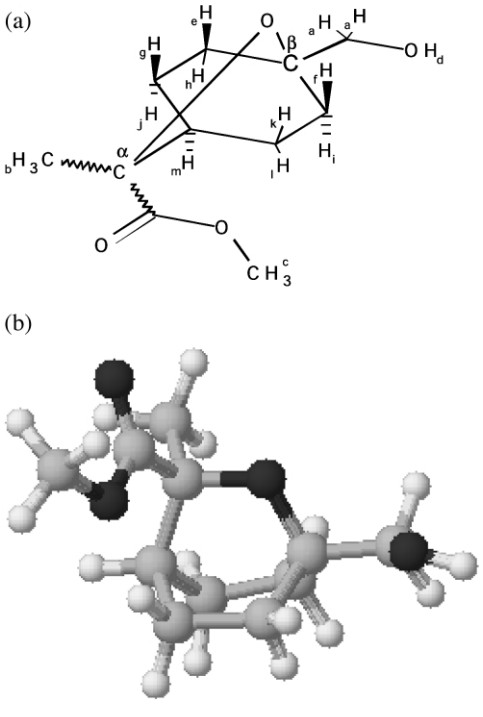

Fig. 4. (a) Simplified structure of Ci 13, 7-hydroxy-9-cineolic acid, showing labelling of protons and carbons used for NMR interpretation; and (b) optimal three-dimensional structural conformation of Ci 13 interpreted from ^{1}H- and ^{13}C-NMR. Dark shaded atoms are oxygen, lightly shaded atoms are carbon and white atoms are hydrogen.

3.6. Glucuronic acid

The amount of glucuronic acid measured in urine samples was 1.15 ± 0.78 mmol/kg (223 ± 151 mg/kg; $n = 6$). 1,8-Cineole conjugated metabolites accounted for only approximately 10% of this value (sum of conjugated metabolites was 125 ± 91 μmol/kg).

3.7. Faecal metabolites

No terpenes or terpene metabolites were detected in the faecal extracts analysed.

4. Discussion

By studying the metabolic pathways of 1,8-cineole in the koala we have demonstrated that this species employs a metabolic strategy which is highly specific and promotes rapid production and elimination of extensively oxidised metabolites. This strategy is similar to that employed for the detoxification of *p*-cymene (Boyle et al., 2000b).

The koala excreted seven metabolites of 1,8-cineole and although all seven were quantified, two (7-hydroxycineole and 7-cineolic acid) were present in only trace amounts. Furthermore, 9-hydroxycineole and 9-cineolic acid each accounted for only 2–3% of the recovered dose. One

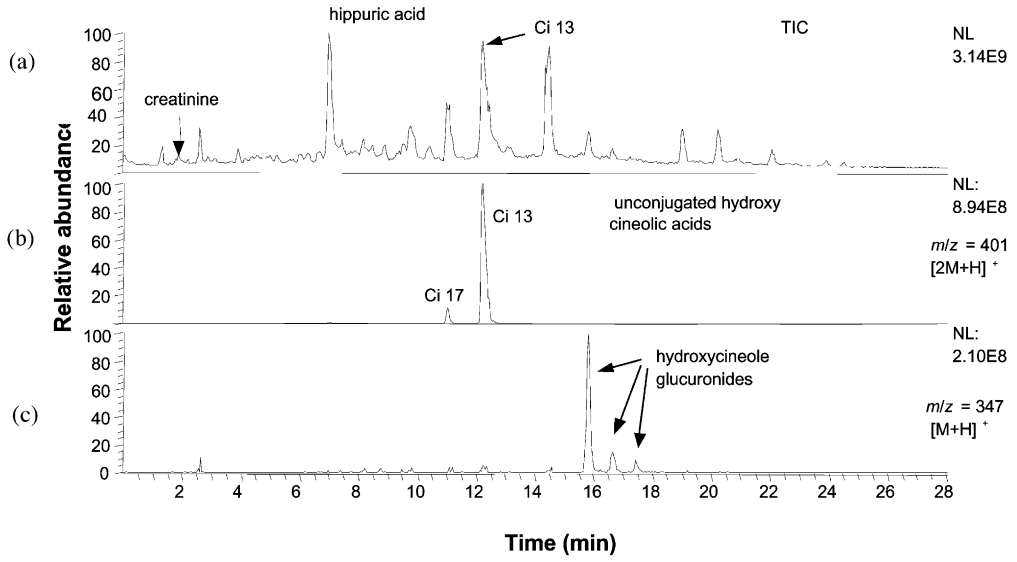

Fig. 5. APCI LC-MS of diluted koala urine sample. The traces are of the TIC (a) and selected ion chromatograms showing hydroxycineolic acids (the major metabolites, b) and hydroxycineole glucuronides (c). NL represents the normalisation levels for each trace. Two other common urinary compounds, creatinine and hippuric acid, are also marked on the TIC. Note that in this method of analysis peak areas do not indicate the relative quantities of metabolites.

metabolite, Ci 13, dominated the metabolite profile. When combined with the other hydroxy cineolic acid metabolite, Ci 17, they accounted for 85% of the recovered dose. The most extensively oxidised metabolite was the dicarboxylic acid metabolite, Ci 22, which accounted for a further 10% of the recovered dose.

The minor, less oxidised, metabolites underwent extensive conjugation with glucuronic acid. However, excretion of conjugated 1,8-cineole metabolites accounted for only a small percent (~5%) of the total recovered dose.

The specificity of the enzymatic pathways employed to detoxify 1,8-cineole is evident from the metabolite structures (Fig. 2). The koala oxidises 1,8-cineole only on the C7 and C9 positions. This is in contrast to the brushtail possum which oxi-

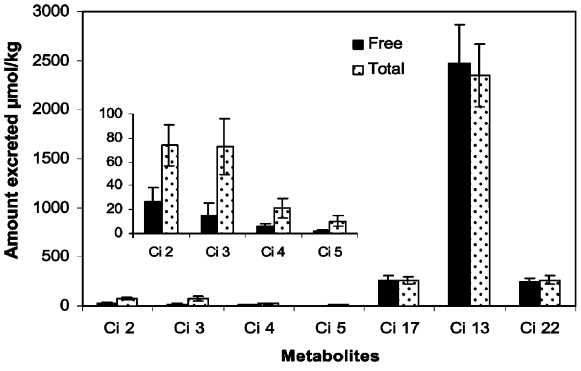

Fig. 6. Amounts (μmol/kg) of free and total levels of 1,8-cineole metabolites in koala urine (mean ± S.E., $n = 6$). Overall, hydrolysis did not increase the total recovery [ANOVA (two factor with replication) d.f. = 1, $F = 0.004$, $P = 0.95$]. Inset: minor metabolite recoveries scaled up to show that hydrolysis did increase the recovery of Ci 2, 3, 4 and 5 [ANOVA (two factor with replication) d.f. = 1, $F = 9.01$ $P = 0.005$].

Table 1
Excretion of free, conjugated and total 1,8-cineole metabolites expressed as the percentage of total recovered metabolites in koalas eating *E. cephalocarpa* leaves

Metabolite group	Percent (mean ± S.D.) of total urinary metabolites ($n = 6$)		
	Free	Conjugated	Total
Hydroxycineoles	0.4 ± 0.6[a]	2.1 ± 1.3	2.5 ± 1.5[a]
Cineolic acids	0.9 ± 0.7[a]	2.3 ± 1.6	3.3 ± 1.5[a]
Hydroxy cineolic acids	88.2 ± 14.1	3.3 ± 6.3	85.4 ± 2.1
Dicineolic acid	7.9 ± 2.6	0.9 ± 0.8	8.8 ± 2.6
Sum	97.4 ± 14.6	6.7 ± 10.2	100

Metabolites were grouped according to extent of oxidation. Free and total metabolites were determined, respectively, before and after hydrolysis, the difference was considered to be due to conjugated metabolites. In cases where metabolite free levels were slightly greater than total, the value of the difference was considered to be '0'. Fractional recovery of 1,8-cineole was 1.4 ± 0.4 (mean ± S.D.).

[a] Comparison of free and total metabolite recovery, $P < 0.05$ (Student's paired *t*-test).

dised many of the available carbons, including those on the ring structure (Carman and Klika, 1992; Bull et al., 1993; Carman and Rayner, 1994; Carman et al., 1994; Carman and Garner, 1996). For each metabolite group, the dominant metabolite had undergone the most extensive oxidation at the C 9 position (i.e. Ci 2, 3 and 13), indicating regioselective preference of the oxidative enzymes for this position in the koala.

Further oxidation of cytochrome P450 enzyme derived metabolites (e.g. hydroxyl metabolites (Pass et al., 1999)) to their corresponding carboxylic acid metabolites could possibly be catalysed by cytosolic alcohol and aldehyde dehydrogenase enzymes (Alvares and Pratt, 1990). That the koala excreted greater than 95% of 1,8-cineole as carboxylic acid metabolites suggests that if this enzyme pathway is in fact responsible for further oxidations then it is very efficient and has a high capacity. However, investigation into these enzymes in marsupials is required to confirm its involvement in the detoxification of terpenes.

The absence of significant quantities of precursor metabolites in the urine (i.e. the hydroxycineoles and dihydroxycineoles) implies that the additional oxidative reactions occur rapidly. For example, 7,9-dihydroxycineole, the immediate precursor of the major metabolite, was not detected at all. Rapid production of extensively oxidised metabolites would allow little opportunity or necessity for subsequent conjugation and excretion of the less oxidised, precursor metabolites.

In striking contrast to the koala and brushtail possum, micro-organisms appear to metabolise 1,8-cineole by oxidation of ring carbons (mainly C2 and C3) with great regio- and stereospecificity (Carman et al., 1986; Liu and Rosazza, 1990; Southwell et al., 1995). For example, *Pseudomonas flava* attacked 1,8-cineole predominantly at C2 to form 2-*endo*-hydroxy-1,8-cineole (Carman et al., 1986) while *Bacillus cereus* formed optically pure 2-*exo*-hydroxy-1,8-cineole (Liu and Rosazza, 1990). The Pyrgo beetle (*Paropsisterna tigrina*) formed the 2-*endo*-hydroxy metabolite with a trace of the 2-*exo* isomer (Southwell et al., 1995).

Recovery of 1,8-cineole exceeded the estimated amount of 1,8-cineole ingested by koalas. The fractions of the dose recovered for two koalas (numbered 2 and 5) were 1.95 and 1.67, respectively, and accounted for the majority of the overall excess recovery. If absorption, metabolism and excretion of ingested 1,8-cineole occurred over a longer period than the 24-h feeding and urine collection, then carryover from the previous day's intake may affect recovery. The amount of leaf ingested by koala 2 was half of that ingested on the previous day. Therefore carryover metabolites from the previous day's intake could contribute to the increased recovery. Similarly, koala 5 excreted minimal urine (26 ml compared to the mean of 101 ml) during the previous day's collection. Therefore, metabolites from the previous day's ingestion may have accumulated and been included in the current day's sample. Furthermore, the estimation of 1,8-cineole leaf concentrations and subsequently the ingested dose of 1,8-cineole, is subject to unpredictable natural variation. Terpene profile and content can be variable at many scales, including leaves within the same tree (Southwell, 1973; Boland et al., 1991), so extrapolating leaf concentrations from one leaf sample from a tree to all leaf collected from that tree will only provide an estimate. Finally, 1,8-cineole concentration was analysed on unselected samples of leaves whereas animals may be more selective in their leaf browsing. Thus the amount of 1,8-cineole ingested is nominal and therefore variable recovery is to be expected.

We are now able to make an interspecies comparison of the metabolic fate of 1,8-cineole between a specialist eucalypt folivore, the koala (the present study), and the generalist brushtail possum (Boyle et al., 2000b). The daily intake of 1,8-cineole differed between these animals in these two studies, with koalas consuming 2.4 ± 1.1 mmol/kg from a leaf diet compared to the possum intake of 4.5 ± 1.0 mmol/kg from a spiked artificial diet (Boyle et al., 2000b). However, in both cases intakes were within the range expected from a natural foraging diet (Cork, 1984; Foley et al., 1987; Hume and Esson, 1993). Furthermore, the pattern of metabolism of 1,8-cineole was unchanged across a large dietary concentration range in the brushtail possum (Boyle, 2000). This suggests that detoxification pathways are therefore unlikely to be overloaded at these intakes and the comparison of metabolite excretion patterns between species is valid.

The strategy employed by the koala to detoxify 1,8-cineole contrasts with that utilised by the brushtail possum. Both species excreted the majority of the recovered dose as hydroxy cineolic acids. However, the brushtail possum excreted 11 different metabolites in varying proportions com-

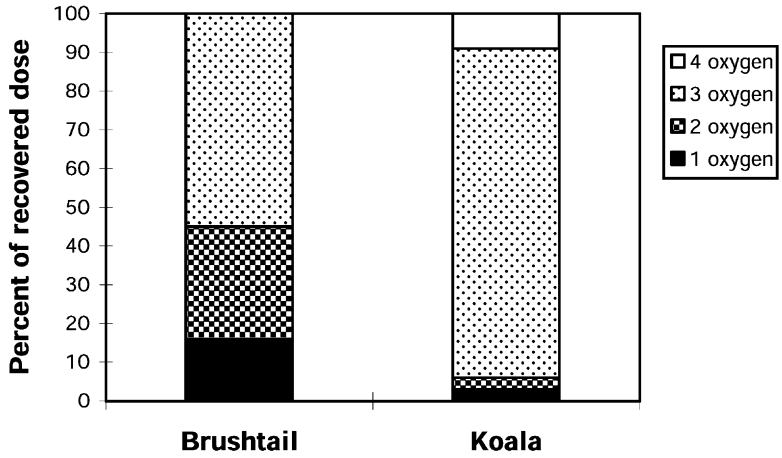

Fig. 7. Comparative oxidation of 1,8-cineole in the brushtail possum and the koala. Metabolites are categorised by the number of oxygen atoms acquired before conjugation. The values for the brushtail possum were reported in a separate study (Boyle et al., 2000a)

pared to the koala's two, of which Ci 13 alone accounted for the majority of the recovered dose. The differences in the pattern of oxidation between the two species can be summarised by grouping metabolites according to the number of oxygen atoms acquired (Fig. 7). The koala excreted the majority of the recovered 1,8-cineole dose ($>90\%$) as extensively oxidised metabolites (i.e. three or more oxygen atoms) compared to 60% in the possum. In both species, conjugation of the less oxidised metabolites was significant, but a much smaller percentage of the total metabolites was excreted in this form by the koala. In both species of marsupial, it appeared that the less oxidised metabolites were not readily excreted without further alteration, by either glucuronidation or further oxidation. The brushtail possum utilises the conjugation pathway more than does the koala, which prefers the production of extensively oxidised metabolites. These patterns of excretion are similar to those found in the metabolism of *p*-cymene (Boyle et al., 1999, 2000b).

The minor role of glucuronidation of both *p*-cymene and 1,8-cineole metabolites in the koala was surprising considering the large amount of glucuronic acid found in their urine. The koala excreted approximately 2 g of glucuronic acid daily (Boyle et al., 2000b) and this value is comparable to those reported by other studies (Hinks and Bolliger, 1956, 1957; Southwell, 1975). Since glucuronidation was not significantly involved in the elimination of terpenes, it must have an important role in the elimination of other components of their diet. This is the subject of a further investigation.

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