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ONTOGENETICAL CHANGES IN MONOTERPENOIDS OF *MELALEUCA ALTERNIFOLIA* LEAF

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Key Word Index—*Melaleuca alternifolia*; Myrtaceae, tea tree, essential oil, steam distillation; solvent extraction; biogenesis, *cis*-sabinene hydrate, terpinen-4-ol, artifact formation

Abstract—Individual leaves on *Melaleuca alternifolia* branches were examined for variation in volatile oil composition. Gas chromatography of solvent extracts revealed flush growth rich in sabinene, *trans*-sabinene hydrate and *cis*-sabinene hydrate. Sequential examination of apical to basal single leaf extracts showed a dramatic decrease in thujane precursors and concomitant increase in the concentration of *p*-menthanes γ-terpinene, terpinen-4-ol and α-terpineol. Steam distillation of flush growth gave an oil rich only in the *p*-menthanes typical commercial oil of *Melaleuca*. The significance of these *in vivo* and *in vitro* transformations is discussed.

INTRODUCTION

Melaleuca alternifolia Cheel (Tea tree) (family Myrtaceae) is a paperbark tree which follows water courses and flourishes in swampy conditions in the north coast region of New South Wales, Australia [1]. The leaves of one chemical variety give an essential oil (1–3%) with bactericidal and fungicidal properties which have secured it a place in the commercial oil market for more than 50 years [2] The biological activity is assumed to be related to (+)-terpinen-4-ol (**1**) [2, 3], the major component of the steam-distilled oil. In a recent review, both Lamparsky [4] and Koedam [5, 6] imply that terpinen-4-ol may arise either as an artifact in essential oil production or as a genuine biogenetic product. In both cases, the likely precursors were identified as sabinene (**2**) or monoterpene alcohols (**3** and **4**) Thus a terpinen-4-ol rich species such as *M. alternifolia* provides an excellent species for the assessment of these implications.

A close chemical relationship between the thujanes and *p*-menthanes has been understood ever since Wallach reported the acid catalysed conversion of sabinene (**2**) to terpinen-4-ol (**1**) in 1906 [7–9]. Subsequently, other products including sabinene hydrates (**3** and **4**) were also detected in the reaction mixture [10–14]. Acid solvolysis of alcohols (**3** and **4**) gave identical products in similar ratios to sabinene [15, 16]

In contrast with these conversions, the reverse chemical transformations from the *p*-menthanes to the thujanes, have not been recorded Reactions proceeding through intermediate ion **5** yield *p*-menthane rather than thujane derivatives [17]. Biogenetic theory, however, beginning with Ruzicka's early proposals, has suggested the formation of thujanes from *p*-menthane cation **5** rather than the reverse [17–20] Recent *in vivo* experimental evidence indicates that these transformations are reversible, at least in some plant systems [17].

In the absence of isotopic labelling, some information on monoterpene biosynthesis can be gained by studying ontogenetical changes in leaf oil composition [19, 21]. In this way Loomis [19], Maarse [22], Clark and Menary [23, 24] and Attaway [25] were able to outline probable biogenetic pathways for origanum, peppermint, and tangerine monoterpenoids.

This communication reports the variation in terpene composition of ethanolic extracts from the developing leaves of *Melaleuca alternifolia*, contrasts extract composition with steam distilled oil composition and discusses implications for sabinene–sabinene hydrate–terpinene–terpinen-4-ol biogenetic transformations.

RESULTS AND DISCUSSION

A recently developed method for the examination of the volatile constituents of *Eucalyptus* leaf [26] was used to follow the development of the oil in successive *Melaleuca alternifolia* leaves from the apex to the base of the branch. A single mature leaf, weighing as little as one milligram, when immersed in ethanol, gave a solution shown by GC to be almost identical to a solution of the steamed distilled oil. In contrast, however, flush growth (leaves close to the apex which were lighter green in colour than the mature leaf) gave a GC trace indicating the presence of different major components. When subject to LC, PLC, GC and GC–MS analysis, this flush growth consisted predominantly of monoterpenoid constituents (Table 1) with *ca* 15% sesquiterpenoids and higher boiling components. The major component (39%) of the extract was *cis*-sabinene hydrate (**4**) with *trans*-sabinene hydrate (**3**) and sabinene (**2**) occurring as significant minor constituents Thujanes (**2**–**4**) contributed 52 2% to the extract but only 0 5% to a typical commercial steam distilled oil (Table 1) Terpinenes and terpineols in contrast contributed 16.2% to the extract and 74 2% to the oil Both skeletal types then accounted for 68.4% of the extract and 74 7% of the oil.

That the thujane skeleton is indeed a precursor of the terpinenes and terpineols in *M. alternifolia* was established by steam distilling a sample of flush tip extract and

Table 1 A comparison between the monoterpenoid constituents of (a) the diethyl ether extract of flush growth and (b) the steam distilled oil of a commercial cut from *M alternifolia*

Constituent	R_I*	(a) %	Extract ident	(b) %	Oil ident
1 α-Thujene	920	1 9	a,	1 6	a
2 α-Pinene	923	1 5	a, b,	3 6	a, b
3 Sabinene (**2**)	960	5 6	a, b, c	0 5	a, b
4 β-Pinene	960	0 6	a,	0 9	a b
5 Myrcene	984	0 7	a,	1 2	a b
6 α-Phellandrene	992	0 3	a, b	0 7	a, b
7 α-Terpinene	1003	0 3	a, b	12 4	a, b
8 *p*-Cymene	1007	0 3	a, b	2 6	a, b
9 Limonene[f]	1013	0 5	a, b	0 9	a, b
10 1,8-Cineole[f]	1013	4 3	a, b	7 2	a, b
11 β-Phellandrene[f]	1013	0 5	a,	0 9	a, b
12 γ-Terpinene	1044	6 3	a, b	24 4	a, b
13 *trans*-Sabinene hydrate (**3**)	1050	7 6	a, b, c, d		
14 Terpinolene	1073	1 4	a, b	4 1	a, b
15 *cis*-Sabinene hydrate (**4**)	1076	39 0	a, b, c, d, e		
16 *trans*-Menth-2-en-1-ol	1104	0 3	a, c	tr	a,
17 *cis*-Menth-2-en-1-ol	1121	0 3	a, c	tr	a
18 Terpinen-4-ol (**1**)	1154	7 6	a, b	31 2	a, b
19 α-Terpineol	1167	0 6	a, b	2 1	a, b
20 *trans*-Piperitol	1175	tr	a, c		
21 *cis*-Piperitol	1186	1 0	a, c		
22 Unknown	1203	1 4	- -		
Total		82 0			94 3

*Calculated from FSOT BPI (10 m × 0 2 mm) column

a = R_I, b = GC/MS, c = CO–GC, d = ^{1}H NMR, e = IR, f = separated on FSOT BP 21 (50 m × 0 25 mm) column, tr = trace

obtaining an oil almost identical to the steam distilled oil The thujanes sabinene (**2**), *trans*-sabinene hydrate (**3**) and *cis*-sabinene hydrate (**4**) were now almost absent (0 1, 0 9 and 2 3%, respectively) Thus the terpinenes and terpineols of *M alternifolia* are artifacts of the steam distillation when flush growth is processed. The formation of terpinen-4-ol (**1**) from sabinene hydrate has been suggested by Taskinen from observations with steam distilled sweet marjoram oil [27] and Koedam with Leyland cypress oil [6]

The varying levels of thujane and terpinene derivatives were seen as individual leaves along a branch were examined The most significant variations reflected a decrease in *cis*-sabinene hydrate (**4**) concentration and concomitant increases in γ-terpinene and terpinen-4-ol concentrations as the branch was analysed from apex to base (Fig 1a) The concentrations of *trans*-sabinene hydrate (**3**) and sabinene (**2**) also fell as the concentration of α-terpineol increased (Fig 1b) Some of the minor constituents *trans*- and *cis*-menth-2-en-1-ol and *trans*- and *cis*-piperitol increased concentration initially, peaked to coincide with the inflection point of other constituents and then decreased in concentration (Fig 1c) The decrease in thujane concentration, the corresponding increase in terpinene derivatives and the combined levels are shown in Fig 1d Analysis of six branches at various stages of development in different seasons gave similar plots with the point of inflection moving on the *x* axis in relation to the point of cessation of flush growth Analysis of stem adjacent to each leaf node revealed extracts identical to the leaf extracts

Although the concentrations of α-terpinene and terpinolene fluctuated, maximum levels for the extract were considerably lower than for the steam distilled oil These may then arise as artifacts of the steam distillation process Terpenes β-ocimene [28], pregeijerene [29], germacrene C [30], hedycaryol [31, 32], chrysanthenone [33] and sabinene hydrate [15] have all been similarly implicated in artifact formation by steam distillation In aqueous medium most plant material reaches a pH value between 4 and 7, [5, 6], so artifact formation is to be expected when even mildly acid labile components are steam distilled. Determination of pH values for *M alternifolia* still pot liquours and condensation waters showed mild acidity with values of 4 6 and 4 4, respectively Steam distillation in pH 7 0 buffer gave higher proportions (12 3%) of *cis*-sabinene hydrate indicating that terpinen-4-ol formation was at least partially pH dependent

The concentrations of α-thujene, α-pinene, β-pinene, myrcene, α-phellandrene, limonene 1, 8-cineole and β-phellandrene, did not vary significantly during the course of development of the leaves These components with the exception of myrcene, are stereospecific at C-4 and generally possess greater stability Although α-thujene, for example, reacts through the same intermediate ion (**6**) as sabinene (**2**) [34], the latter reacts considerably faster *in vitro* [11, 12] and *in vivo* is much more efficient at transferring tracer than α-thujene [35]

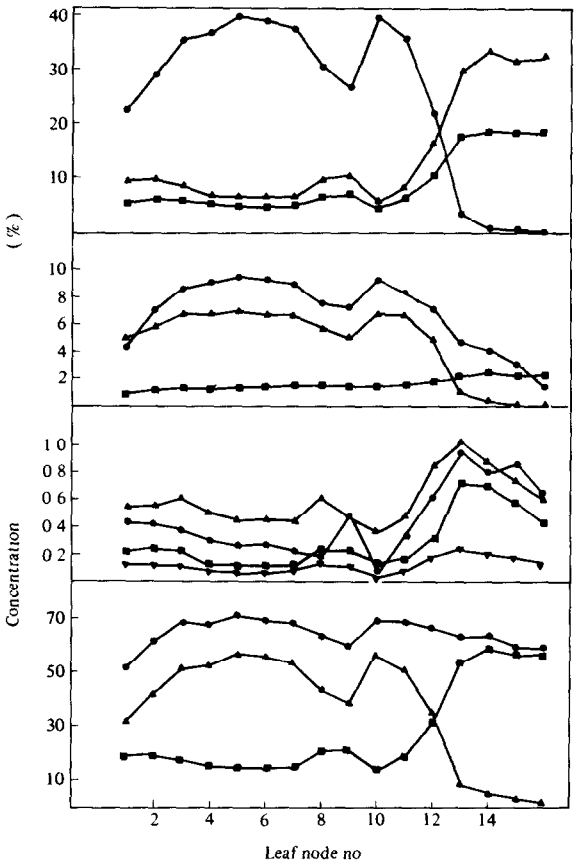
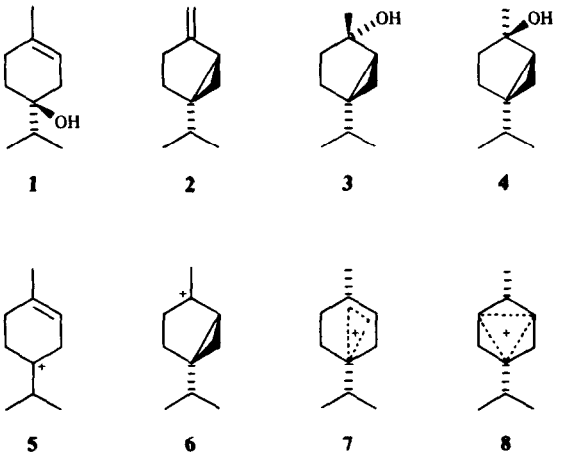


Fig 1 Ontogenetical variation in the percentage contribution to *M alternifolia* leaf extracts by (a) *cis*-sabinene hydrate (4) (●), terpinen-4-ol (1) (▲), γ-terpinene (■), (b) sabinene (2) (●), *trans*-sabinene hydrate (3) (▲), α-terpineol (■), (c) *trans*-(▲) and *cis*—(●) menth-2-en-1-ol, *trans*-(▼) and *cis*-(■) piperitol, (d) total thujanes (▲), total terpinenes (■) and total thujanes + terpinenes (●)

Similar ontogenetical changes have been observed with peppermint (*Mentha piperita*), origanum (*Origanum vulgare*) and tangerine (*Citrus reticulata*) The change observed in *Melaleuca alternifolia* in this investigation, however, represents, not a simple reduction, as in peppermint [19, 23, 24, 36, 37] but a 1,3-diaxial hydroxyl shift as *cis*-sabinene hydrate (**4**) converts to terpinen-4-ol (**1**) The changes noted in origanum [22], where groups of three leaf pairs were analysed, were not as significant as those in either peppermint or *M. alternifolia* With tangerine (*Citrus reticulata*) peel oil, the seasonal increase in (+)-limonene was matched inversely by a decrease in linalool concentration indicating that the latter was possibly a biogenetic precursor of the former [25]. The plot of this relationship is almost superimpossible upon that for the *cis*-sabinene hydrate—terpinen-4-ol relationship in *M. alternifolia* (Fig. 1a).

The ratio of terpinene derivatives in the oil of *M. alternifolia* [38] was seen to be remarkably similar to the steam distilled oils of majoram [27, 39] and Leyland cypress [6] and also the acid catalysed products of sabinene and the sabinene hydrates [15, 39]. This is in contrast to the acid catalysed equilibrium of *p*-menthadienes which forms predominantly α-terpinene [14]



Hence, the proposal of a cyclopropylcarbinyl ion intermediate such as **7** in preference to trishomocyclopropenyl cation **8** or classical relative **5** accounts for the preferred ratios of terpinenes and the high degree (80%) of stereospecificity associated with formation of terpinen-4-ol (**1**) [11, 12] in this and other species Comparisons of the optical rotations of the volatile oils obtained from *M alternifolia* flush tips (+7 6°) and mature leaf (+8 5°) indicated the stereospecificity of this transformation.

A comparison of *M alternifolia* oil and extract with those of sweet marjoram (*Majorana hortensis*) showed many similarities including the predominance of *cis*-sabinene hydrate in the extract and terpinen-4-ol in the steam distilled oil [27] Our tea tree results when compared with the marjoram literature results prompted us to propose that the terpinene derivatives from the latter were in fact artifacts from the steam distillation of thujane precursors such as sabinene (**2**) and the sabinene hydrates (**3**) and (**4**). Subsequently Fischer *et al* [39] published a comparison of marjoram extracts and oils and concluded similarly that the biosynthetic capacity of the marjoram plant with regard to monoterpenoids seems to be confined to the synthesis of the thujane skeleton. They also isolated the unstable *cis*-sabinene hydrate acetate which in an even less stable precursor form (presumably a glycoside) was proposed as the source of marjoram constituents

Melaleuca alternifolia, (family Myrtaceae) is similar to marjoram *Majorana hortensis* (family Laminaceae) only at the flush growth stage. The *Melaleuca* has the capacity to biogenetically convert the sabinene precursors to the terpinene final products as the leaf matures. A similar leaf node development investigation of marjoram showed that this conversion does not occur biogenetically in the marjoram plant as the leaf matures. The prominent *cis*-sabinene hydrate acetate peak present in the gas chromatogram of marjoram was absent in the tea tree extract chromatograms even when flush growth apical leaves were examined using the Fischer procedure [39].

The occurrence of *trans* and *cis*-*p*-menth-2-en-1-ol and *trans* and *cis*-piperitol observed in *M. alternifolia* was significant Although it is tempting to propose such structures as biogenetic intermediates, it is also likely that their presence relates to artifact formation [40].

The formation of terpinene derivatives from the bicyclic thujane skeleton has significant biogenetic implica-

tions Theoretical considerations, beginning with Ruzickas's early proposals [17–20] have indicated that thujanes are formed from *p*-menthane cation **5** rather than the reverse as is observed here with *M alternifolia* In marjoram the sabinene hydrate precursors have been traced back to the sabinene hydrate acetate and even back further to an unstable hydrophilic precursor [39] In *M alternifolia* the absence of the acetate indicates a different pathway, the details of which await further investigation On the other hand the thujane to terpinene conversion lends support to recent experimental evidence indicating that these transformations are reversible at least in some plant systems [17] For example, tracer experiments with *Tanacetum vulgare* have shown that [^{3}H]-geranyl pyrophosphate is incorporated into thujanes and *p*-menthanes [35] Also, label in α-terpineol and terpinen-4-ol is readily transferred to thujanes [35, 41] Our studies with *M. alternifolia*, however, suggest that at least γ-terpinene and terpinen-4-ol are formed subsequent to the thujane skeleton

From a quantitative and stereochemical viewpoint, *cis*-sabinene hydrate (**4**) appears to be the logical biogenetic precursor of some but not all of both terpinen-4-ol (**1**) and γ-terpinene in *M. alternifolia* As the 1,3-migration of the equatorial hydroxyl of the C-4 epimer (**3**) is less likely, *trans*-sabinene hydrate may contribute via the classical intermediate ion **5** the racemic contribution to the terpinen-4-ol and the remainder of the other terpinenes. Confirmation of these biogenetic speculations awaits radiolabelling investigations

EXPERIMENTAL

Optical rotations were measured on the oils as neat liquids, IR spectra were recorded as liquid films and ^{1}H NMR spectra in $CDCl_3$ GC was performed on a using H_2 as carrier gas with either open tubular (FSOT) BP1 (10 m) or BP21 (50 m) 0 22 mm i d columns Individual runs were temp programmed from 40° (1 min) to 250° at 10° per min for BP1 and from 50° (1 min) to 180° at 10 per min for BP 21 Percentage compositions (Standard error of mean <0 08) were computed with a HP 7673 A controller and HP 3393 A integrator Retention indices were calculated with respect to straight chain hydrocarbon standards GC–MS was conducted through an all glass straight split interface at 70 eV ionising voltage and 8000 V accelerating voltage with ion source at 180° The chromatographic separation was performed using a 70 m × 0 5 mm FFAP SCOT column with He as carrier gas and programming at 3° per min from 65° to 180° Spectra were acquired every 6 sec and processed by a VG Digispec Display data system

Plant material Melaleuca alternifolia leaf was collected from young trees (*ca* 12 months) during periods of abundant flush growth (summer–autumn) in a plantation situation at the North Coast Agricultural Institute, Wollongbar, Australia Trees were propagated from seed collected in the Richmond River region of northern N S W Voucher specimens (383092-94) are lodged with Dr B Barlow (Division of Plant Industries Herbarium, CSIRO, Canberra)

Leaf analysis An individual leaf or leaf node cluster was added to a preweighted glass GC vial with insert (cap 0 05 ml), weighed and submerged in EtOH containing tridecane (0 1 mg/ml) Extraction for 3–4 days showed that 30 hr was the minimum extraction time required for complete extraction of volatiles Trituration extraction with liquid N_2–pentane [39] gave identical results For the isolation and identification of precursors flush tips (48 5 g) were dried (48 hr) and the dried leaf (13 6 g) extracted with EtOH (200 ml) Extraction with Et_2O (to avoid the formation of ethyl ether artifacts as occurs with marjoram [27] and juniper [42] ethanolic extracts) gave a soln with a near identical GC trace Cold vacuum concn gave a green wax which decomposed on both silica and alumina flash columns Effective separation was achieved on a PLC plate by elution with a petrol–MeOH–EtOAc (8 1 1) to give a band containing 87% sabinene hydrates (*trans*-**3** *cis*-**4** 1 4) IR ν_{max}^{film} cm^{-1} 3340, 1130, 1050, 990, 950, 930 (lit [43]), ^{1}H NMR ($CDCl_3$) *cis* δ 0 32 (1H, *dd* J_{trans} = 5 Hz J_{cis} = 8 Hz, collapsed with irrad. at 1 06, (5-H), 0 64 (1H, *dd*, J_{trans} = 5 Hz, J_{gem} = 4 Hz, collapsed with irrad at 0 32, 6-H_{endo}), 0 88 (3H, *d*, *J* = 7 Hz, 8-H_3), 0 92 (3-H, *d*, *J* = 7 Hz, 9-H_3), 1 06 (1-H, *dd*, J_{cis} = 8 Hz, J_{gem} = 4 Hz, collapsed with irrad at 0 32, 6-H_{exo}), 1 33 (3-H, *s*, 10-H_3), 1 63 (*m*, modified with irrad at 0 92 7-H) (lit [44]), *trans* 0 23 (1H, *dd*, J_{trans} = 5 Hz, J_{gem} = 4 Hz, 6-H_{endo}) 0 39 (1H, *ddd*, J_{cis} = 8 Hz, J_{trans} = 5 Hz, $J_{1,7}$ 1 Hz, 5-H), 0 91 (3H, *d*, *J* = 7 Hz, 8-H_3), 0 97 (3H, *d*, *J* = 7 Hz, 9-H_3) (lit [44]), EIMS (GC) 70 eV, *m/z* rel int) *cis* 154 (2), 139 (3), 137 (3), 136 (26), 121 (30), 93 (100), 91 (42), 79 (28), 77 (34), 43 (20), 41 (25), 39 (15) (lit [52]), *trans* 154 (4), 139 (5), 137 (4), 136 (20), 121 (30), 93 (100), 91 (42), 79 (25), 77 (38), 71 (27), 43 (30), 41 (28), 39 (18) (lit [45])

Isolation of steam volatile oils M alternifolia flush growth (178 g) was hydrodistilled in an all glass apparatus with cohobation for 2 hr to yield 2 5 ml (1 4%) of volatile oil ($[\alpha]_D^{25}$ +7 6°) Buffered distillation was carried out at pH 7 0 in 0 1 M KH_2PO_4 (100 ml)–0 1 M NaOH (58 2 ml) buffer soln

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