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# Quantitative co-occurrence of sesquiterpenes; a tool for elucidating their biosynthesis in Indian sandalwood, *Santalum album*

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**Abstract**

A chemotaxonomic approach was used to investigate biosynthetic relationships between heartwood sesquiterpenes in Indian sandalwood, *Santalum album* L. Strong, linear relationships exist between four structural classes of sesquiterpenes; α- and β-santalenes and bergamotene; γ- and β-curcumene; β-bisabolene and α-bisabolol and four unidentified sesquiterpenes. All samples within the heartwood yielded the same co-occurrence patterns, however wood from young trees tended to be more variable. It is proposed that the biosynthesis of each structural class of sesquiterpene in sandalwood oil is linked through common carbocation intermediates. Lack of co-occurrence between each structural class suggests that four separate cyclase enzymes may be operative. The biosynthesis of sandalwood oil sesquiterpenes is discussed with respect to these co-occurrence patterns. Extractable oil yield was correlated to heartwood content of each wood core and the oil composition did not vary significantly throughout the tree.


*Keywords:* Essential oil; *Santalum album*; α-Santalene; β-Santalene; α-*trans*-Bergamotene; *epi*-β-Santalene; Santalols; Heartwood

## 1. Introduction

Indian sandalwood, *Santalum album* L., is a hemi-parasitic tree native to India and Indonesia that is highly valued for its fragrant heartwood. The heartwood of mature trees (>10 years old) contains essential oils, chiefly the sesquiterpene alcohols *cis*-α-santalol (**1**), *cis*-β-santalol (**2**) (Verghese et al., 1990), α-*trans*-bergamotol (**3**), *epi-cis*-β-santalol (**4**) (Fig. 1) along with small quantities of *trans*-β-santalol and *cis*-lanceol (**5**) (Howes et al., 2004). The parent hydrocarbons, α-santalene (**6**), β-santalene (**7**), α-bergamotene (**8**) and *epi*-β-santalene (**9**) are also present in the oil, as well as α-curcumene (**10**), γ-curcumene (**11**), β-curcumene (**12**), β-bisabolene (**13**) and α-bisabolol (**14**) (Figs. 2 and 3) (Adams et al., 1975; Braun et al., 2003; Howes et al., 2004). Compositional differences have been noted throughout the roots and stem of *Santalum spicatum* (Piggot et al., 1997) and *S. album* (Shankaranarayana et al., 1998). Although a route to **1** and **2** from farnesyl diphosphate was proposed almost 40 years ago (Parker et al., 1967), it has not been studied thoroughly due to slow onset of oil formation and the location of key enzymes within woody tissue. The former renders *in vivo* isotopic feeding experiments impractical, while the latter makes extraction of functional enzyme systems difficult due to the low number of living cells in wood.

Terpenoid variation can be used to explore hypothetical biogenic pathways. A quantitative, mathematical relationship following the general linear form $y = ab + C$ between individual compounds in an oil profile would suggest that

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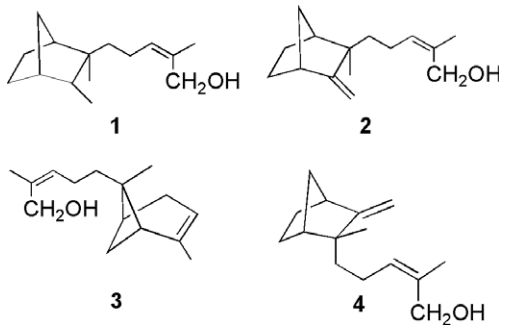


Fig. 1. Main sesquiterpene alcohols of *Santalum album*.

the biosynthesis of such chemicals is not only closely related, but may involve the same enzyme system or at least a common intermediate (Zavarin, 1970). The resulting proportionality of these compounds would be determined by flexibility at the active site of an enzyme (allowing more than one isomer to be produced), and the mechanistic probability of hydride shifts or Wagner–Meerwein type rearrangement of the intermediate. Multiple product formation has been noted in various sesquiterpene synthases extracted from plants (Colby et al., 1998; Munck and Croteau, 1990), and by site-directed mutation of active site residues in functional enzymes (Hyatt and Croteau, 2005; Little and Croteau, 2002). *In vivo* essential oil profiling, with the objective of identifying co-occurrences in these species, has not been widely performed on these species.

Alternatively, the co-occurrence of structurally similar compounds may be due to several enzymes working in unison. In this instance, the correlation would most likely be weaker than the common intermediate hypothesis, as several complicating factors would be involved. Quantitative co-occurrence would best explain structural isomers that share a common intermediate. Correlation between product and functionalised precursor may be apparent, however the strength of correlation is likely to be considerably lower than between structural isomers. Such relationships may yield negative linear slopes, where substitution of a precursor compound results in an increase in the proportion of product (Zavarin, 1970; Zavarin et al., 1970).

In this study, plantation grown *S. album* of various ages was analysed for heartwood oil content and composition. Variation in composition was considered both within individual trees, age groups and amongst the entire population. The biosynthesis of sandalwood sesquiterpenes is described with respect to these co-occurrences.

## 2. Results and discussion

Both qualitative and quantitative co-occurrence patterns were discovered in the essential oil of *S. album* heartwood. Percent composition data was the most informative for investigating quantitative co-occurrence patterns, as peak area data revealed only qualitative relationships. Product-precursor relationships were apparent, but correlation was weak. Samples in which the concentration of oil was very low (particularly 100 cm cores from young trees) gave more variable co-occurrence ratios. When a single age group of trees were considered, slight differences could be seen in the essential oil composition between younger and older wood. A higher proportion of sesquiterpene alcohols was observed for 30 cm cores, while 100 cm cores had a slightly lower proportion. The amount of heartwood in each core sample was correlated to the overall oil yield. The total oil content of central and transition zone heartwood was fairly consistent at 3.1% to 3.6% dry wt.

Zavarin (1970) noted that both quantitative and qualitative co-occurrence patterns may exist in the essential oil composition of conifers and many other plants. Here, an example of qualitative co-occurrence in sandalwood oil was α-santalol (**1**) and β-santalol (**2**). Both shared a strong, positive linear correlation ($r = 0.99$) when peak area data was used. However, when percent composition data was used (Fig. 5), a cluster of points arises around 53% and 23% for α-santalol and β-santalol, respectively, with no significant correlation. This highlights the importance of using percent composition data when examining co-occurrences. Percent composition data is essentially adjusted by the presence of everything else in the oil, whereas peak area data is not. For this reason, only co-occurrences which arose from percent composition data were investigated.

Striking co-occurrence patterns were present amongst the olefinic sesquiterpenes (**6**–**9**) (Fig. 6A). Generally, older wood cores tended to follow the trendline very closely, but cores form younger trees were more variable. An example of this variation is illustrated in Fig. 6B. Oil deposition most probably initiates in the heartwood-sapwood transition zone and after a certain concentration of oil is reached, biosynthesis ceases. The oxidation of sesquiterpenes to sesquiterpenols must also occur in this period and would affect the overall composition of the oil for this sample. Younger heartwood, or wood which has only recently been synthesised may contain lower amounts of alcohols **1**–**4**.

A number of mono- and sesquiterpene synthases are remarkable in the fact that they can transform a single substrate into multiple products. Examples of synthases that can utilise both enantiomers of an acyclic terpene 3-diphosphate *R*-**15** and *S*-**15** (Fig. 2) have also been described (Colby et al., 1998; Munck and Croteau, 1990; Wise and Croteau, 1999). Moreover, some cyclases can generate enantiomers from the corresponding l-diphosphate (**16**, **17**) (Fig. 2) due to the inherent chirality arising from the folding of an acyclic substrate into a right- and left-handed helical conformation (Colby et al., 1998; Munck and Croteau, 1990).

The proposed biosynthetic pathway to the santalenes and α-bergamotene (Fig. 1) has been adapted from the model pathways proposed for mono- (Wise and Croteau, 1999) and sesquiterpene examples (Kollner et al., 2004) with the added constraint imposed by the known absolute configuration of these sesquiterpenes (Krotz and Helmchen, 1994; Chapuis et al., 1998). Interestingly, *epi*-β-santa-

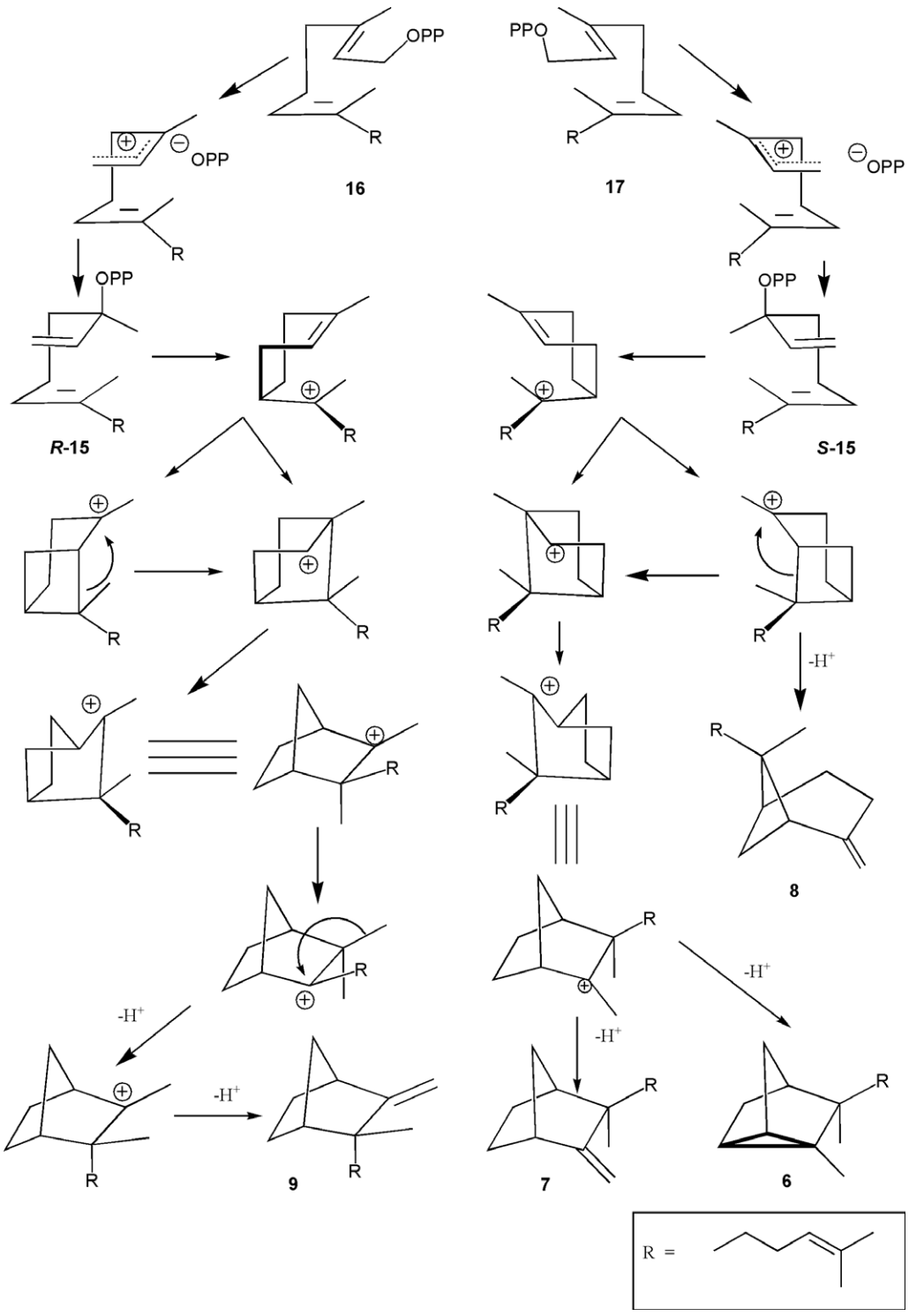


Fig. 2. Proposed biosynthesis of the santalenes and bergamotene (**6**–**9**).

lene (**9**) appears to be generated from a sequence that leads to products antipodal to those that include **6**, **7** and **8**. Another possibility is that some amount of 3*S*-nerolidyl diphosphate (*S*-**15**) may cyclise in the *anti-exo* conformation as observed for the monoterpene analogues (Fig. 4) (Wise and Croteau, 1999). In either case, one would expect to see *epi*-α-santalene and *epi*-α-bergamotene in the oil profile, unless these isomers were not resolved under the chosen chromatographic conditions.

Correlation was also found between γ-curcumene (**10**) and β-curcumene (**11**) ($y = 0.588x + 0.28$, $r = 0.88$). It is presumed that these two monocyclic sesquiterpenes are synthesised from the bisabolyl cation (**18**) via a 1,2-hydride shift followed by loss of a proton (Fig. 3). As neither of

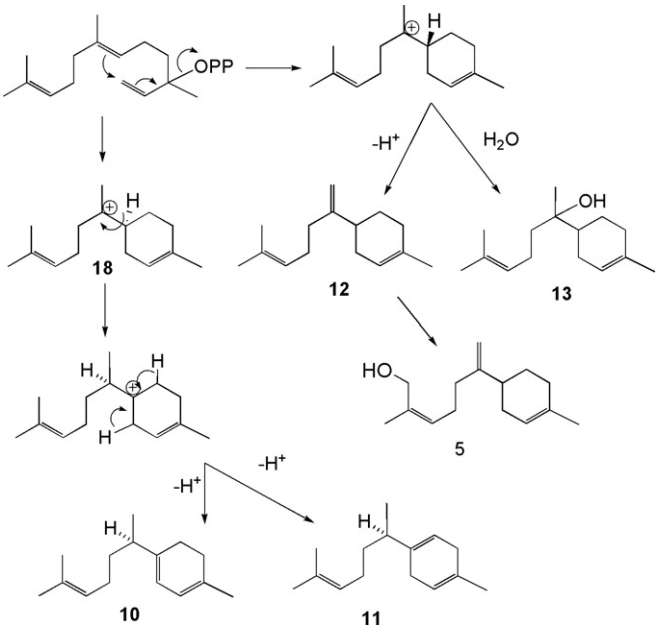


Fig. 3. Proposed biosynthetic pathway of farnesyl diphosphate to the curcumenes (**10** and **11**), bisabolene and bisabolol (**12** and **13**) and *cis*-lanceol (**5**).

these compounds shared a linear relationship with either the santalene series or β-bisabolene, the biosynthesis of the curcumenes may be a separate process unrelated to the other sesquiterpenes which utilises a different cyclase.

Although only minor components, the percent composition of β-bisabolene (**12**) was correlated ($r = 0.76$) to that of α-bisabolol (**13**). The synthesis of **12** probably occurs via the initial cyclisation product from farnesyl or nerolidyl diphosphate, followed by loss of a proton from C-14 (Fig. 3). It would appear that the formation of curcumenes **11** and **12** could be closely related to that of **13** and **14**, but no correlation between these compounds was observed. It is possible that different cyclases are involved and generate the enantiomeric bisabolyl intermediate, whereby the 6*R*-compound leads to **12** and **13** and the 6*S*- to **10** and **11** following a 6, 7-hydride shift.

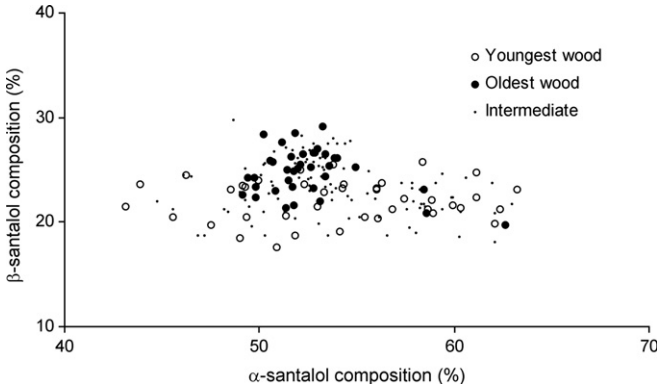


Fig. 5. Co-occurrence of α- and β-santalol (**1** and **2**, respectively) in *S. album* oil.

The fourth series of sesquiterpenols in *S. album* heartwood eluted between α-curcumene and α-bisabolol and had a molecular ion of 220 $m/z$ (data not shown). Strong linear correlations were present between these three compounds, while having no correlation with the santalenes, curcumenes or bisabolene. The full identity of these compounds is not known, but based on their correlations being independent from the other sesquiterpenes it seems probable that a separate cyclase/synthase is responsible for their synthesis.

Cores extracted from wood deemed younger (5–8 year-old trees at 100 cm above ground) tended to be further from the main trendlines than those derived from mature wood (14–17 year-old trees at 30 cm above ground). Sapwood contained almost no extractable oil with only the main component of sandalwood oil, α-santalol, occasionally present in very small concentrations. Transition zone wood contained an average of $36 \pm 6.0$ mg g$^{-1}$ dry wt. of extractable oil, while the central heartwood yielded $30 \pm 5.1$ mg g$^{-1}$ dry wt. of oil. The maximum oil yield for both transition zone and heartwood was 90.6 mg g$^{-1}$ dry wt. and 60.5 mg g$^{-1}$ dry wt., respectively. No differences were observed in either the yield or composition of the extracted oil from both zones. The current under-

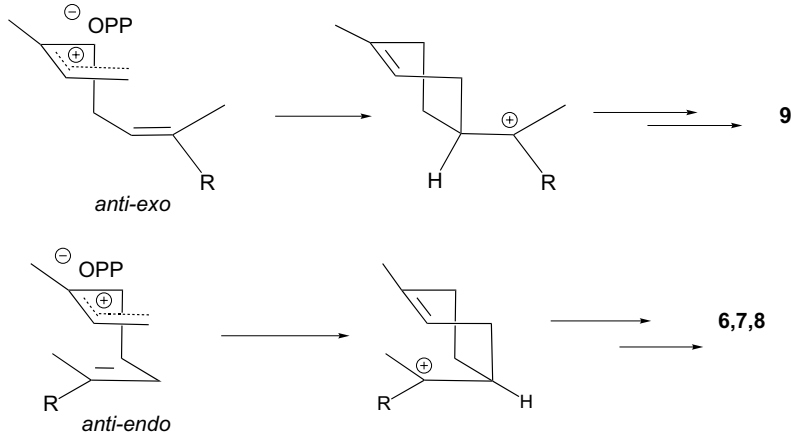


Fig. 4. Alternative folding of acyclic sesquiterpene precursor farnesyl diphosphate in *Santalum album*.

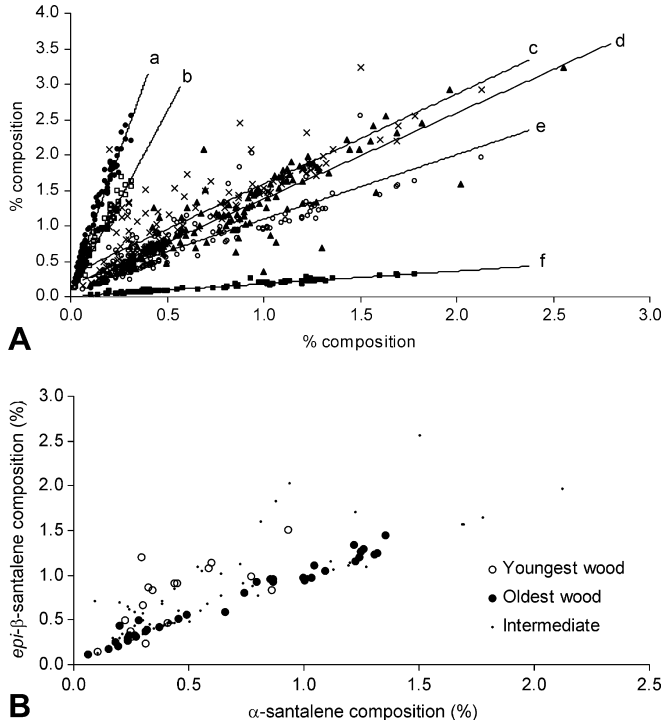


Fig. 6. A: Co-occurrence of compounds **6**–**9** in *S. album* oil: (a) **7** vs. **8** ($y = 7.54x + 0.13$, $r = 0.98$); (b) compounds **8** vs. **9** ($y = 5.08x + 0.74$, $r = 0.98$); (c) compounds **6** vs. **8** ($y = 1.28x + 0.31$, $r = 0.88$); (d) compounds **9** vs. **7** ($y = 1.21x + 0.16$, $r = 0.90$); (e) compounds **6** vs. **9** ($y = 0.92x + 0.18$, $r = 0.85$); (f) compounds **6** vs. **8** ($y = 0.18x + 0.002$, $r = 0.98$). B: Co-occurrence of compounds **6** and **9** with the age of wood cores highlighted.

standing of heartwood formation suggests that as the secondary xylem ages, water conducting tissues stop functioning or become blocked (Zimmermann, 1983). This occurs as the tree grows, presumably in seasonal bursts. Seasonal growth was difficult to identify in wood discs as these trees had been flood irrigated regularly throughout the dry season. Due to the slow-growing habit of sandalwood, seasonal changes in composition or yield may not have been obvious when using a 9 mm wide sample of transition wood.

Total essential oil yield from *S. album* trees varied considerably amongst the sampled population. Presence or absence of essential oil depended mostly on tree age, and generally trees under 8 years yielded little fragrant heartwood. Extractable oil yield was compared to the proportion of heartwood in cores from both heights, resulting in the equation: oil yield $= 42.803 \times$ proportion of heartwood $- 0.4501$, $r = 0.87$, $P < 0.01$. Despite this general trend, many samples from 15 year old trees yielded very little fragrant heartwood. Several factors involving the structure of storage tissues and the location of essential oils within the heartwood undoubtedly contribute to the variation seen in this relationship, and these in turn may be related to growing conditions, host tree species and genotype. Increased rates of sesquiterpene biosynthesis, either genetically or environmentally induced, may also contribute to this disparity. Further work is required in order to fully understand the process of heartwood formation and secondary metabolite biosynthesis in wood.

## 3. Experimental

### 3.1. Site description

*S. album* trees were growing at the Frank Wise Agricultural Research Station, Kununurra, Western Australia. Soil was heavy clay, very plastic when wet and very hard when dry. The Northcote key classifies the soil type as Ug 5.29, coloured dark brown (2.5 Y 4/2) with neutral to slightly alkaline pH (Radomiljac, 1998). Kununurra has a tropical climate with a very distinct monsoon wet season from December through to April, and an average annual rainfall of 790 mm. Mean temperature ranges from 15 to 30 °C in winter and 25 to 38 °C in summer (Australian Bureau of Meteorology, Commonwealth Government Perth). The plot was within the Ord River Irrigation Area where flood irrigation was carried out monthly during dry seasons. Sandalwood trees were growing alongside various hosts, including *Acacia trachycarpa*, *Azaderachta indica*, *Dalbergia* sp. *Khaya* sp. *Melaleuca* sp. *Pongamia* sp. *Sesbania formosa*, and *S. grandifolia*. Both host and sandalwood trees were form pruned.

### 3.2. Within-tree essential oil variation (destructive sampling)

Discs (5 cm thick) from ground level were cut from four 10 year-old sandalwood trees in May 2003. These were dried and stored. The upper surface of each disc was sanded using a belt sander so that central heartwood, sapwood and the transitional zone between these two zones could be easily distinguished. Air-dried wood samples were taken from these zones by drilling parallel to the grain of the wood and collecting the shavings. Three replicates of shavings were taken from the sapwood, transition zone (to the inside of the transition line) and central heartwood using a drill equipped with a 9 mm drill bit. This yielded enough wood shavings for subsequent analyses. These were stored at room temperature in plastic bags until analysis.

### 3.3. Core sampling of plantation trees (non-destructive sampling)

Core sampling was performed in spring 2004. Cores (diameter 12 mm) were extracted from the diameter of 98 trees of ages ranging from 5 years to 17 years. Cores from two heights, 30 and 100 cm above ground level were taken using a petrol-powered drill with a specialised coring bit. Trees were re-sealed with caulking polymer to prevent infection. In total, 196 cores were extracted at two heights (30 and 100 cm) from 98 trees, ranging in age from 9 to 18 years. Heartwood from trees aged 5–8 years, and at 100 cm above ground was deemed 'younger'. Heartwood from

trees aged 14–17 years and 30 cm above ground was deemed 'older'. Once removed these were placed into paper bags and transported to the laboratory where they were air-dried and stored at ambient temperature (20 ± 5 °C). Oven drying of all wood samples was not performed as this risked volatilisation of the heartwood oils.

Core length and heartwood proportion was measured. In some instances the heartwood was difficult to distinguish from sapwood. Wood cores were ground in a Rhetz–Mhule grinder to 500 μm and stored in re-sealable plastic bags until analysis. Heartwood was not separated from sapwood prior to grinding.

### 3.4. Solvent extraction of wood material

Sandalwood shavings (4–5 g) were placed into 50 ml volumetric flasks and a 1 ml aliquot of internal standard, (±)-camphor (4.00 g $l^{-1}$) was added. The flasks were then filled just short of the mark with spectroscopic grade hexane. Volumetric flasks were stored at 21 °C for at least 2 days and periodically shaken before being topped up to the mark and analysed by GC-FID.

### 3.5. Gas chromatographic analysis of sandalwood extracts

GC analysis was performed using a Shimadzu GC-17 A instrument equipped with an AT-WAX column (Alltech, 30 m, 0.25 mm inside diameter, 0.25 μm film thickness) and flame ionisation detection. Injection volume was 1.5 μl, the injection port temperature was 200 °C and detector 250 °C. Helium (2.4 ml $min^{-1}$) was the carrier gas an and a split ratio of 20:1 was used. Oven temperature was ramped from 80°C at 4 °C $min^{-1}$ to 180 °C and held for 20 min (total run time 45 min). Peak identification was facilitated by application of GC–MS using a Shimadzu QP2010 instrument under identical column conditions. Integration was performed using Shimadzu GC-Solutions software. Areas were recorded for all detectable peaks, and percent composition was calculated by taking area of peak divided by total chromatogram area × 100. Samples which contained small amounts of total oil tended to overestimate the proportion of the major components (**1**–**4**).

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