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FOLIAGE SESQUITERPENES OF *DACRYDIUM CUPRESSINUM*: IDENTIFICATION, VARIATION AND BIOSYNTHESIS*

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Key Word Index—*Dacrydium cupressinum*; Podocarpaceae; infraspecific variation; sesquiterpenes; biosynthesis; 9βH-caryophyllene.

Abstract—The major sesquiterpenes in the foliage of *Dacrydium cupressinum* are α-longipinene, longifolene, longibornyl acetate, caryophyllene, caryophyllene oxide, humulene, α- and β-selinene, β- and δ-elemene, aromadendrene and the rare 9βH-caryophyllene. Sesquiterpene levels vary greatly from tree to tree. As this variation is largely independent of environmental factors, genetic control is proposed. Longifolene and α-longipinene levels are closely correlated, as are those of caryophyllene and humulene. The biosynthetic implications of these correlations are discussed.

INTRODUCTION

Although the diterpenes of the New Zealand rimu tree, *Dacrydium cupressinum* Sol. *ex* Lamb. (Podocarpaceae), have attracted considerable attention [1], little interest has been shown in the sesquiterpenes. Early reports note the presence of an unidentified sesquiterpene in the leaf oil [2] and, subsequently, longiborneol (**1**) was reported as a constituent of the sawdust [3]. When we began to study diterpene levels in *D. cupressinum* foliage extracts by gas chromatography (GC), it was immediately obvious that not only were several sesquiterpenes present, but also, their levels varied as dramatically as did the diterpene levels. This led to a study of the causes of sesquiterpene variation parellel to that already reported for the diterpenes [1].

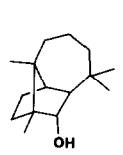
Another motive for examining the sesquiterpene components was the close structural similarity between the diterpene, lauren-1-ene (**2**) [4, 5], which is peculiar to *D. cupressinum*, and the 'triquinane' sesquiterpenes such as silphinene (**3**) [6]. The discovery of any such sesquiterpenes in rimu foliage could point to some overlap of sesqui- and diterpene biosynthetic routes.

RESULTS

Identification

Comparisons of GC retention index data with published values were very useful in the preliminary identification of several of the sesquiterpenes in rimu foliage. The values which were found in this work, and those reported previously [7–9], are compared in Table 1. Although in many cases GC retention data seemed to provide good evidence for the presence of a particular sesquiterpene, wherever possible the compound was isolated and its absolute configuration determined by measuring its specific rotation. With the aid of various spectral techniques (for details see Experimental) (+)-α-longipinene (**4**), (+)-longifolene (**5**), (−)-caryophyllene (**6**), (−)-caryophyllene oxide (**7**), humulene (**8**), (−)-α-selinene (**9**), (+)-β-selinene (**10**), δ-elemene (**11**), (+)-longibornyl acetate (**12**) and (−)-alloaromadendrene (**13**) were readily identified.

One of the sesquiterpene hydrocarbons which was isolated from rimu foliage had a rather confused 90 MHz ^{1}H NMR spectrum. Integration indicated that there were seven olefinic protons, but the signals of six of these overlapped. Addition of silver trifluoroacetate and a ytterbium shift reagent [10, 11] simplified the spectrum and allowed the identification of (−)-β-elemene (**14**).



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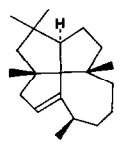

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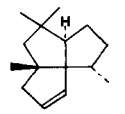

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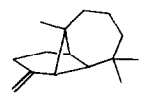
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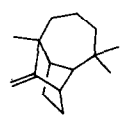
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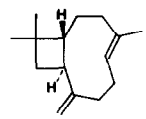

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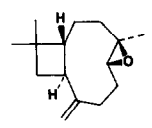
*Part 2 in the series "Foliage Components of New Zealand Gymnosperms". For Part 1 see ref. [1].

Table 1. Sesquiterpenes from rimu

Peak No.	Identity	Retention indices*			Levels†	
		A	B	C	Median	Range
1	δ-elemene (**11**)	1341	1506	1515	1	0–9
2	α-longipinene (**4**)	1358 (1359.5)	1527 (1524.5)	1541	2	0–4
3	β-elemene (**14**)	1390	1621	1631	7	0–45
4	longifolene (**5**)	1408 (1404)	1627 (1620)	1647 (1643)	22	0–45
5	caryophyllene (**6**)	1419 (1417.5)	1642 (1638)	1658 (1655.5)	20	2–56
6	?	1426	—	—	0	0–3
7	?	1436	—	—	1	0–4
8	humulene (**8**)	1449 (1446.8)	1706	1722 (1719)	5	0–16
9	alloaromadendrene (**13**)	1455	1686	1702	8	0–18
10	9βH-caryophyllene (**15**)	1461	1712	1730	8	0–31
11	?	1467	—	—	2	0–4
12	β-selinene (**10**)	1477	1751 (1749)	1768 (1766.5)	1	0–9
13	α-selinene (**9**)	1488	1753 (1750.5)	1770	6	0–14
14	?	1495	—	—	1	0–9
15	caryophyllene oxide (**7**)	1556	—	—	20	0–26
16	?	—	—	—	2	0–8
17	longibornyl acetate (**12**)	1647	—	—	2	0–8

*Literature values are in parentheses; A = SE-30, 130°; B = CW-20 M, 150°; C = CW-20 M, 165°.
†Expressed as % of total sesquiterpenes. Figures are from 47 adult rimu.



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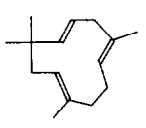

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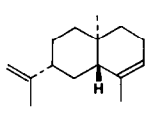

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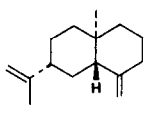

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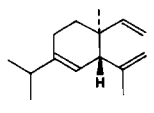

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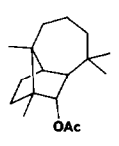

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One other sesquiterpene was isolated whose identification presented some problems. Only two of the known sesquiterpene skeleta, caryophyllane and himachalane, could accommodate all the features found by ^{1}H and ^{13}C NMR spectroscopy [12]. The ^{13}C NMR spectrum of the unknown closely resembled that of caryophyllene (**6**) [13, 14]. (+)-9βH-Caryophyllene (**15**), with a *cis* ring junction, is the only variant on the caryophyllane skeleton that could be simply derived from either (*E*,*E*)- or (*Z*,*E*)-farnesyl pyrophosphate and this was proposed as the structure of the unknown. This compound has only been reported once before, from the roots of an angiosperm *Euryops breviopapposus* M. D. Hend. (Compositae) [15]. Our ^{1}H NMR data matched the published values, but there were discrepancies in the positions of the ^{13}C NMR resonances for C-10 and C-11. However, figures reported in this paper for caryophyllene have also been disputed by other workers [13, 14]. 9βH-Caryophyllene is probably also present in the oleoresin of *Abies magnifica* A. Murr. (Pinaceae) as a compound from this source had similar GC retention, ^{1}H NMR and IR spectra to those found in this work [16]. However, none of this material was available for comparison. The absolute configuration of 9βH-caryophyllene is presently under investigation.

Variation

The variation of sesquiterpene levels in the foliage of 47 rimu trees is summarized in Table 1 and illustrated for longifolene (**5**) in Fig. 1. The same factors were examined as for variation of the diterpene levels and non-parametric statistics were once again used [1].

The sesquiterpene levels did not vary greatly (95% confidence limits ±1%) at different levels on each of three different adult rimu. Two of these were growing with their trunks almost fused together, but differed completely in the levels of most of their sesquiterpene components. The ranges of levels found in trees from the Otago Coast Forest were as great as the ranges in the combined samples of these trees with others from Westland and from the central North Island.

The levels of β-elemene (**14**), longifolene (**5**), caryophyllene (**6**), alloaromadendrene (**13**) and 9βH-caryophyllene (**15**) were compared at four sampling times for nine different trees. The Friedman two-way analysis of variance did not show any significant differences, and the Wilcoxon signed-rank test showed only two [17, 18]. The levels of β-elemene were lower in February, 1982 than in November, 1981 ($p = 0.01$), and the levels of alloaroma-

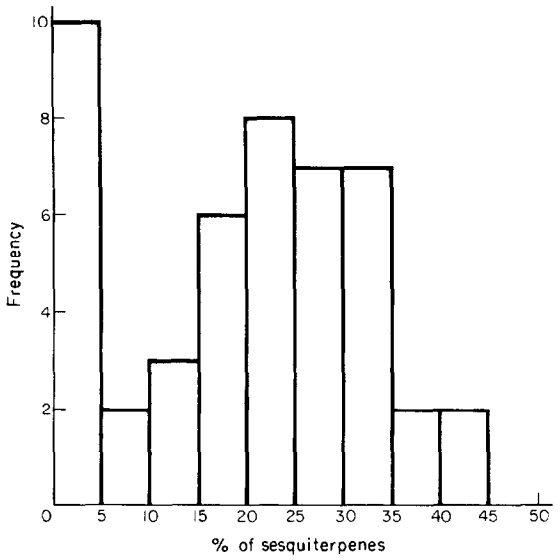
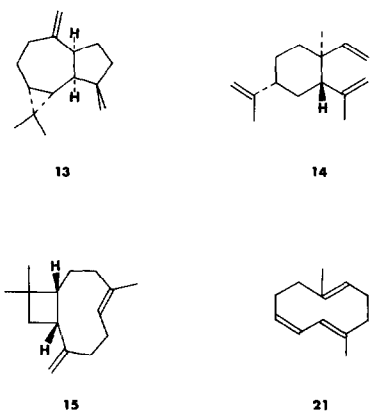


Fig. 1. Longifolene levels in 47 rimu.



dendrene were lower in November, 1981 than in the same month in 1982 ($p = 0.04$).

No significant differences in terpene levels were found between ten male and eight female rimu from the Otago Coast Forest. Nor were there any significant differences in levels of the major sesquiterpenes between adult and juvenile foliage samples from the same area. Longifolene (5), α-selinene (9) and caryophyllene oxide (7) showed significant regional variations as is shown for longifolene in Table 2.

Combination of the data for all the samples studied, revealed groups of sesquiterpenes whose levels were closely inter-related. To test the statistical significance of these associations, which were not normally distributed (see Fig. 1), Kendall rank correlation coefficients [17] were calculated. To display these results in a comprehensible form, these correlation coefficients were treated by a single linkage cluster analysis [18, 19] (Fig. 2). All the correlations linking the clusters were positive and statistically significant ($p < 0.05$). The biosynthetic significance of these findings is discussed below. Lauren-1-ene levels were more closely correlated with the other diterpenes than with any of the sesquiterpenes.

DISCUSSION

Twelve sesquiterpenes have been identified in foliage extracts of *D. cupressinum* but none shows any structural

Table 2. Longifolene levels in 47 rimu

	% of sesquiterpenes		
Trees	Median	Range	Rank sum
18 Otago Coast	15	0–47	250.0
10 Pureora	29	1–38	222.0
9 Hokitika	9	3–26	231.0

Kruskal–Wallis Statistic = 8.30217; $p = 0.0157$.

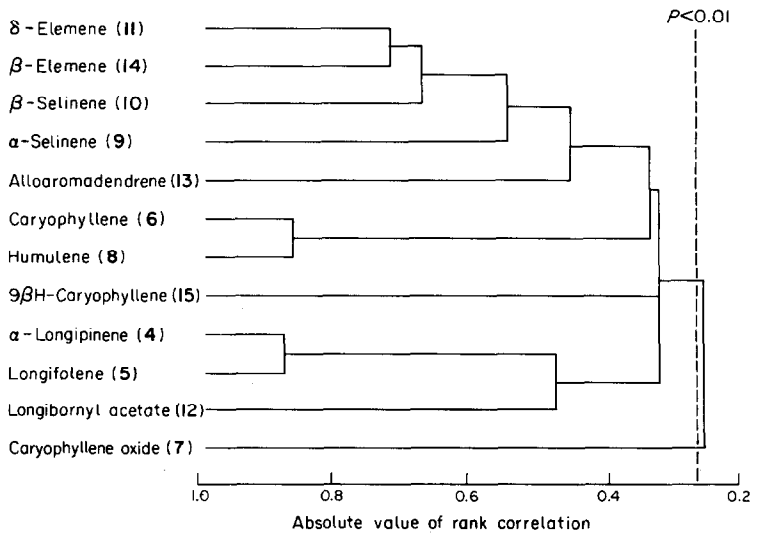


Fig. 2. Cluster analysis of sesquiterpene correlations. Data from 47 rimu.

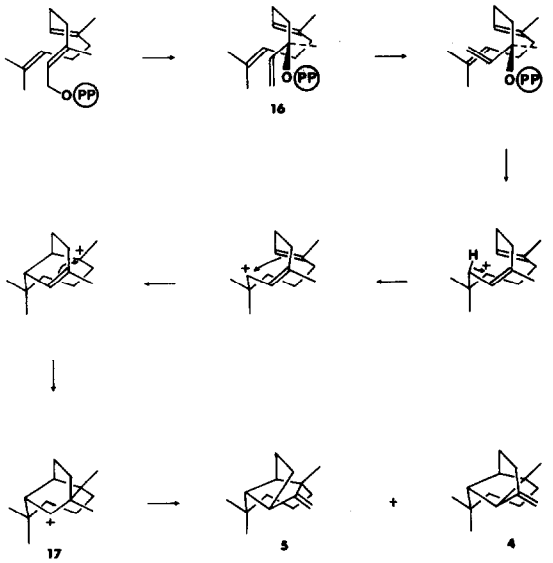
similarity to lauren-1-ene (**2**). All of these compounds varied greatly in level from tree to tree (Table 1) but levels were quite uniform in a given tree over a period of eighteen months. The only exceptions to this were β-elemene (**14**) and alloaromadendrene (**13**), whose levels each differed significantly between two sampling dates. Von Rudloff found that sesquiterpene levels varied greatly in the current year's leaves of *Picea glauca* Voss. (Pinaceae) but that levels in older leaves were constant [20]. No effort was made while sampling rimu foliage to distinguish old and new growth, and the samples taken in the Spring of 1981 may well have included some new growth.

Since sesquiterpene levels are largely unaffected by environmental factors, we conclude that the sesquiterpene levels in rimu foliage, like diterpene levels [1], are genetically controlled. No direct evidence is available from controlled breeding of *D. cupressinum*, but genetic control of sesquiterpene levels has been shown, by Marpeau *et al.*, in such a study on maritime pine, *Pinus pinaster* Ait. (Pinaceae) [21]. They found a clearly bimodal distribution of longifolene (**5**) levels, similar to that which we report here for rimu foliage (Fig. 1). In their case, the longifolene levels were found to be controlled by a single gene which also controls the biosynthesis of α-longipinene (**4**) [21]. Zavarin has suggested that biogenetic linkage of terpenes should be reflected by quantitative correlations [22], and the fact that the levels of longifolene and α-longipinene are closely correlated in *D. cupressinum* foliage (Fig. 2) further supports the close biosynthetic relationship between these two sesquiterpenes. The same phenomenon has been noted in the foliage of *Pseudotsuga* (Pinaceae) species [23]. How this linkage could arise is shown in Scheme 1, which is based on Arigoni's proposed biosynthesis of longifolene, a scheme supported by detailed isotopic labelling studies [24, 25]. An alternative alkyl shift would lead to α-longipinene as shown. This process has been reversed in the laboratory; acid treatment of α-longipinene gave longifolene and longiborneol (**1**) [26]. Scheme 1 retains the conformational and stereochemical implications of Arigoni's original, but the initial cyclization step is shown as the *anti* allylic displacement of pyrophosphate from *R*-nerolidyl pyrophosphate (*R*-NPP, **16**). This is a known biosynthetic reaction [27] and eliminates the second *syn* allylic rearrangement previously proposed [24]. The beauty of this mechanism is that once the appropriate *R*-NPP conformation has been achieved on the enzyme surface, cyclization to longifolene and α-longipinene can occur with minimal conformational change. This approach was also applied to the other sesquiterpenes found in *D. cupressinum* foliage.

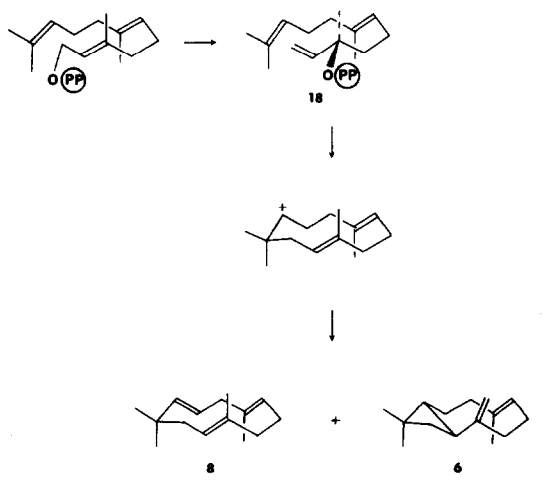
Longibornyl acetate (**12**) has a rank correlation of 0.47 with longifolene. This compound could be derived from carbocation **17** by acetate attack, or from longifolene, as has been achieved in the laboratory [26].

In the study of the genetic control of sesquiterpene levels in the needles of *P. pinaster* cited above, a single gene was found to control caryophyllene (**6**) levels [21]. This gene was independent of that which controlled longifolene levels, so an independent enzyme system must be responsible for caryophyllene biosynthesis. In rimu foliage, caryophyllene levels were only slightly correlated with the levels of longifolene and α-longipinene (Fig. 2), in accord with this result.

The levels of humulene (**8**) and caryophyllene (**6**) in rimu foliage are closely correlated (Fig. 2). Similar correlations have been noted in other species [28, 29]. Despite caryophyllene's status as one of the most common sesquiterpene hydrocarbons, its biosynthesis has not been studied in detail. The formation, and further cyclization, of humulene in one of its two most stable conformations has been invoked to explain the biosynthesis of several fungal and bacterial metabolites [25]. In the biogenesis of caryophyllene and humulene which is suggested in Scheme 2, the enzyme system catalyses the same reactions as in Scheme 1 (*syn* allylic rearrangement and *anti* allylic displacement), but uses *S*-NPP (**18**) as the cyclization substrate. The conformation of *S*-NPP shown would lead directly to caryophyllene and humulene in their most stable conformations [14, 30]. Nucleophilic attack of C-2 on C-10, as suggested in Scheme 2, has been achieved in the three step *in vitro* conversion of humulene to caryophyllene [31]. Furthermore, X-ray analysis of the



Scheme 1. Synthesis of longifolene and α-longipinene.



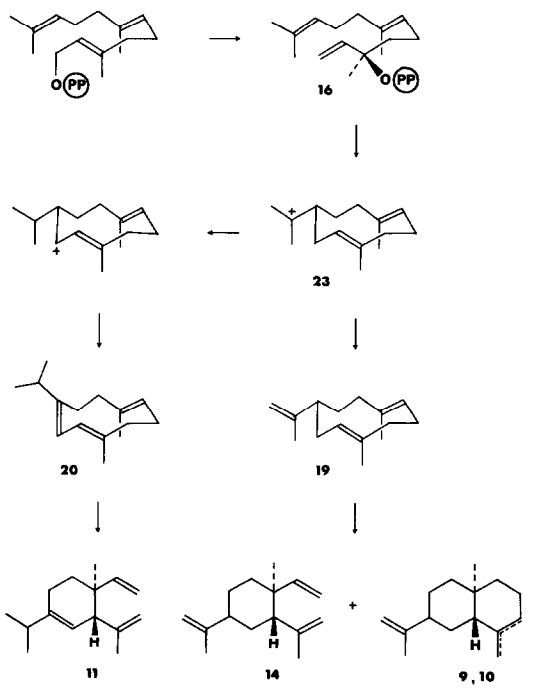
Scheme 2. Synthesis of caryophyllene and humulene.

humulene–silver nitrate complex found a C-2–C-10 distance of only 2.89 [32]. Caryophyllene oxide (**7**) was only slightly correlated with the other sesquiterpenes (Fig. 2), and was probably produced by non-enzymatic oxidation.

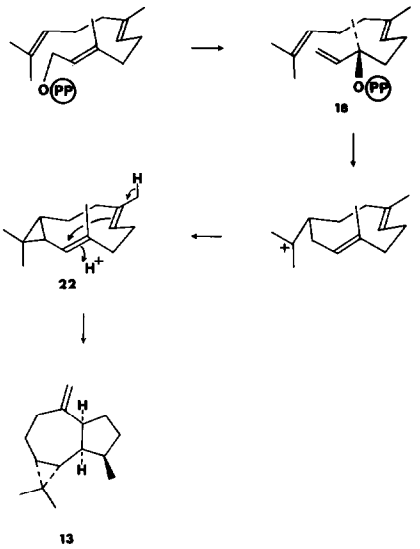
The remaining clusterings in Fig. 2 are less clear-cut than those which are discussed above, but a similar approach to the analysis of the biosynthetic interrelationships may be applied [33]. The basis of these arguments is as follows:

1. The general precursor of the sesquiterpenes, (*E,E*)-farnesyl pyrophosphate, is isomerized to either *R*-NPP (**16**) or *S*-NPP (**18**) by a *syn* allylic rearrangement.
2. Nerolidyl pyrophosphate undergoes cyclization by attack of either the 6,7- or the 10,11-double bond on C-1 in an *anti* allylic displacement of pyrophosphate.
3. The conformation of the enantiomer of nerolidyl pyrophosphate that undergoes this cyclization determines the stereochemistry of the product(s). In other words, the various carbocation-like reactions proceed without major conformational changes.

Andersen *et al.* suggested that NPP is the immediate substrate for enzymatic cyclization [34] and pointed out that this eliminates the mechanistic distinction between derivatives of (*E,E*)- and (*Z,E*)-farnesyl pyrophosphate [25, 35]. The concept that the conformation of the cyclization substrate determines the product stereochemistry is due to Arigoni [24]. The biogenetic hypothesis presented here can accomodate all the current evidence on sesquiterpene biosynthesis while invoking a minimum amount of enzymic control. In this work, twelve sesquiterpenes, with nine different carbon skeletons, were identified from the foliage of *D. cupressinum*. Five independent enzyme systems are proposed as responsible for their biosynthesis [33]. The central postulate of this hypothesis, the role of nerolidyl pyrophosphate as the general cyclization substrate, requires proof by detailed mechanistic studies on isolated enzymes.



Scheme 3. Synthesis of elemenes and selinenes.



Scheme 4. Synthesis of alloaromadendrene.

EXPERIMENTAL

General. Sampling, extraction, GC analytical and data handling methods are described in reference [1]. Unless otherwise stated, specific rotations were recorded as $CHCl_3$ solutions. ^{13}C NMR spectra were recorded as $CDCl_3$ solutions.

Bulk extractions. Individual sesquiterpenes were isolated by bulk extraction of foliage which had been shown to have a high level of the desired component. The following description of the isolation of 9βH-caryophyllene (**15**) serves to illustrate the methods used. Rimu foliage (260 g) was ground in a Wiley mill and slurried with *ca* 500 ml of water containing a small amount of silicone anti-foam agent. Steam distillation/extraction into 25 ml of *n*-hexane for 1 day gave, after removing the solvent, 1.9 g of a blue-green oil. Most of the sesquiterpenes distilled between 110° and 180° at 20 mmHg. Extractions of two further lots of foliage gave a total of 2.3 g of a green oil containing > 90% sesquiterpenes. Column chromatography on 100 g of $AgNO_3$–silica gel (*n*-hexane–EtOAc), monitored by GC, followed by further purification by $AgNO_3$–silica gel prep. TLC (Et_2O eluent) gave 9βH-caryophyllene (140 mg) as a waxy solid. IR and ^1H NMR spectra were as described in ref. [15]; ^{13}C NMR (22.5 MHz): δ18.7 (*q*, C-15), 25.2 (*t*, C-2), 25.4 (*q*, C-13), 27.3 (*t*, C-6), 29.9 (*q*, C-12), 32.9 (*s*, C-11), 38.2 (*t*, C-10), 39.1 (*t*, C-3), 41.6 (*t*, C-7), 43.0 (*d*, C-9), 53.8 (*d*, C-1), 112.7 (*t*, C-14), 121.8 (*d*, C-5), 136.5 (*s*, C-4), 149.7 (*s*, C-8); EIMS (probe) 70 eV, *m/z* (rel. int.): 204 $[M]^+$ (20), 189 (15), 161 (15), 133 (35), 93 (60), 79 (45), 69 (80), 55 (35), 41 (100); $[\alpha]_D$ +196°; mp < 35°.

The other known sesquiterpenes were isolated in a similar fashion. α-Longipinene (**4**), longifolene (**5**), caryophyllene (**6**), humulene (**8**), α-selinene (**9**) and β-selinene (**10**) were identified by their GC retention indices (Table 1). Further spectral confirmation was as follows.

α-Longipinene (**4**) was not isolated free from longifolene, but was further identified by ^1H NMR [36].

Longifolene (**5**) was identified by IR [37], ^1H NMR [38] and ^{13}C NMR [38] spectra; $[\alpha]_D$ +46° (lit. +42° [12]).

Caryophyllene (**6**) *and humulene* (**8**) were identified by com-

parison (IR and ^{1}H NMR) with authentic samples. Caryophyllene had $[\alpha]_D$ −12° (lit. −14° [12]).

Caryophyllene oxide (7) was identified by IR [39] and ^{1}H NMR [40] spectra; ^{13}C NMR (15.04 MHz): δ17.0 (*q*), 21.6 (*q*), 27.2 (*t*), 29.8 (*q* + *t*), 30.2 (*t*), 34.0 (*s*), 39.2 (*t*), 39.7 (*t*), 48.7 (*d*), 50.7 (*d*), 59.6 (*s*), 63.6 (*d*), 112.7 (*t*), 151.7 (*s*); mp (uncorr.) 62° (lit. 62–63° [41]).

α-Selinene (9) was identified by IR [42] and ^{1}H NMR [40] spectra; $[\alpha]_D$ −7° (lit. −14.5° [40]).

β-Selinene (10) was identified by IR [42] and ^{1}H NMR [43] spectra; $[\alpha]_D$ +19° (lit. +38° [12]).

δ-Elemene (11) *and β-elemene* (14) were identified by IR [42]; ^{1}H NMR [44] (The spectrum of β-elemene was made more amenable to analysis by the addition of tris[6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedionato)ytterbium]. β-elemene had $[\alpha]_D$ −25° (lit. −17° [12]).

Longibornyl acetate (12) was identified by IR and ^{1}H NMR spectra [45]; ^{13}C NMR (15.04 MHz): δ13.3 (*q*), 21.4 (*q*), 22.4 (*q* + *t*), 27.5 (*t*), 28.7 (*q*), 28.9 (*q*), 30.2 (*t*), 33.4 (*s*), 35.2 (*t*), 40.7 (*t*), 44.1 (*d*), 50.5 (*s*), 51.5 (*s*), 60.7 (*d*), 80.6 (*d*), 170.6 (*s*). Reduction with $LiAlH_4$ gave longiborneol (1) which was identical (IR, ^{1}H NMR) with an authentic sample; ^{13}C NMR: δ13.0 (*q*), 22.7 (*q* + *t*), 26.5 (*t*), 29.0 (*q*), 29.3 (*q*), 30.4 (*t*), 33.4 (*s*), 35.2 (*t*), 41.2 (*t*), 44.0 (*d*), 50.2 (*s*), 51.4 (*s*), 64.5 (*d*), 79.6 (*d*); $[\alpha]_D$ +23° (EtOH) (lit. +25° [12]); mp (uncorr.) 106–108° (lit. 105–107° [46]).

Alloaromadendrene (13) was identified by IR [37], ^{1}H NMR [47] and ^{13}C NMR [48] spectra; $[\alpha]_D$ −17° (lit. −22° [12]).

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