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FOLIAGE DITERPENES OF *DACRYDIUM INTERMEDIUM*: IDENTIFICATION, VARIATION AND BIOSYNTHESIS*

NIGEL B. PERRY and REX T. WEAVERS

Department of Chemistry, University of Otago, Box 56, Dunedin, New Zealand

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Key Word Index—*Dacrydium intermedium*; *D. fonkii*; Podocarpaceae; infraspecific variation; biosynthesis; diterpenes; *ent*-rosadiene; *ent*-sclarene; co-occurrence of enantiomers; biochemical systematics; *Lepidothamnus*.

Abstract—The major diterpenes in the foliage of *Dacrydium intermedium* have been identified as rimuene, *ent*-rosadiene, *ent*-beyerene, phyllocladene, *ent*-kaurene, sclarene and *ent*-sclarene. *ent*-Rosadiene and *ent*-sclarene have not been reported previously from natural sources. Considerable tree-to-tree variations are encountered and genetic control is proposed. Biosynthetic mechanisms are put forward to explain the presence of diterpenes of both enantiomeric series. A lack of mono- and sesquiterpenes in both *D. intermedium* and *D. fonkii*, which ties in with Quinn's proposed revision of the *Dacrydium* genus, is also noted.

INTRODUCTION

Dacrydium intermedium T. Kirk (Podocarpaceae) is a small tree endemic to New Zealand (*intermedium* refers to the long-persisting juvenile and intermediate forms [2]). It occurs in lowland, montane and subalpine forests and is sometimes dominant in bog forests in the west of the South Island. Quinn suggested that it should be regarded as a member of the genus *Lepidothamnus*, i.e. *Lepidothamnus intermedius* (T. Kirk) C. J. Quinn, together with one other New Zealand native and a species native to South America [3].

The diterpene hydrocarbons in a foliage extract have been examined once by GC [4]. Kaurene, rimuene and 'cupressene' were reported as major components, with a trace of isokaurene. These compounds were not isolated and the identifications were based on GC retentions on one stationary phase at one temperature. The identity of 'cupressene' is not clear since it has been identified as either beyerene (also known as hibaene and *ent*-stachene) or 13α-beyerene (isohibaene) [5, 6].

We have now examined this species as part of our examination of the foliage of New Zealand gymnosperms [1, 7].

RESULTS AND DISCUSSION

Small scale extractions

Mature foliage samples were taken from ten specimens of *D. intermedium* and these were initially examined by small-scale extraction and GC analysis, as for *D. cupressinum* samples [7]. No sesquiterpenes were detected in any of these extracts and monoterpenes were only present at low levels (< 1%).

Kaurene, rimuene, isokaurene, sclarene, isophyllocladene and phyllocladene were identified by their retention indices [8]. The levels of each diterpene component varied greatly from tree to tree (Table 1). For example, rimuene ranged between 1 and 60% of the total diterpenes. The compounds, with the exception of isophyllocladene and isokaurene, were isolated to confirm their identities and to determine specific rotations and hence absolute configurations. Furthermore, later GC runs performed to measure retention indices showed the presence of components which were not resolved under the conditions employed initially.

Bulk extractions

A bulk extraction was carried out on foliage of a tree shown by GC to contain mainly rimuene (**1**), sclarene (**2**) and phyllocladene (**3**). These compounds were separated by preparative TLC on silver nitrate impregnated silica gel. Their identities and normal absolute configurations were confirmed from their IR and ^{1}H NMR spectra, and their specific rotations.

A bulk extraction of the foliage of another tree led to the isolation of *ent*-kaurene (**4**). Although GC analysis had indicated that sclarene (**2**) was the only other major diterpene in this extract, ^{1}H NMR spectroscopy clearly showed the presence of a third major component which was not easily separated from sclarene on silver nitrate impregnated silica. GC analyses using a different stationary phase showed that this unknown component was present in several other *D. intermedium* foliage samples.

ent-*Rosadiene*

Treatment of the mixture of the unknown and sclarene (**2**) with tetracyanoethylene rapidly gave the Diels–Alder adduct of sclarene (**5**) [9]. The unknown was unaffected. The polar adduct (**5**) could be removed by filtering through silica.

^{1}H NMR and IR spectroscopy showed the presence of a

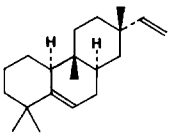
*Part 3 in the series "Foliage Components of New Zealand Gymnosperms". For Part 2 see ref. [1].

Table 1. Diterpene levels* in *D. intermedium*

	Tree†									
	1	2	3	4	5	6	7	8	9	10
Rimuene	(+)59	53	19	1	48	21	(+)25	19	42	24
Rosadiene	1	1	9	(−)18	1	11	(−)5	0	4	0
Beyerene	1	0	1	0	0	0	(+)16	0	2	0
Sclarene	(+)20	9	23	(−)56	11	7	7	15	10	(+)16
Isophyllocladene	2	2	2	0	1	1	1	0	1	2
Isokaurene	0	0	1	1	0	2	0	0	0	0
Phyllocladene	(+)17	24	21	0	26	29	22	44	18	46
Kaurene	0	10	23	(−)24	12	28	24	23	23	13

*Expressed as % of total diterpenes. Figures were deduced from GC analyses on SE-30 and CW 20 M capillary columns [8].

†Figures in parentheses indicate sign of optical rotation of isolated sample.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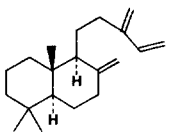
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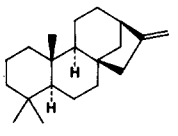
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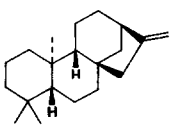
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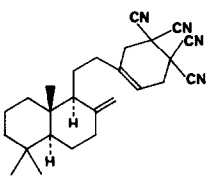
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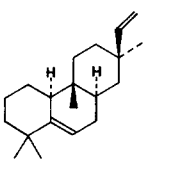
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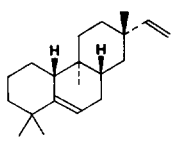
vinyl group and a trisubstituted double bond in the unknown. This indicated a tricyclic diterpene but the spectral data did not fit any such compounds reported as occurring naturally. The ^{1}H NMR spectrum contained an unusually high-field methyl resonance (δ0.68) similar to that in rimuene (**1**) (δ0.62), which has been attributed to the C-9 methyl group which is shielded by the endocyclic double bond [10]. These observations suggested that the unknown could be the C-13 epimer of rimuene, i.e. rosadiene (**6**) or its enantiomer (**7**).

Ireland and Mander synthesized (±)-rosadiene in parallel with their total synthesis of rimuene, but they only described the IR spectrum [11]. A more recent synthesis of *ent*-rosadiene (**7**), from an oxygenated natural product, has also been reported [12]. The specific rotation, IR spectrum and ^{1}H NMR spectrum of the material from *D. intermedium* were identical with the published data. The identity of the unknown was further confirmed by its acid-catalysed conversion to *ent*-rosa-5(10),15-diene (**8**) with the same specific rotation, GC retention index, and IR and ^{1}H NMR spectra as previously reported [13, 14]. This is the first report of the natural occurrence of *ent*-rosadiene (**7**).

ent-*Beyerene*

The GC conditions used to separate sclarene (**2**) and *ent*-rosadiene (**7**) revealed the presence of another diterpene hydrocarbon as a major component (15%) in one of the *D. intermedium* foliage extracts. This compound's retention behaviour suggested that it was beyerene (**9**), 13α-beyerene (**10**), or an enantiomer of either [8]. These compounds can be distinguished on the basis of their retention indices but not in the presence of co-eluting diterpenes as found in this extract. Comparison of a sample of this diterpene, isolated from a foliage extract with an authentic sample (IR and ^{1}H NMR) and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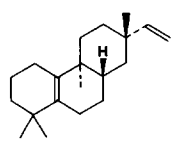
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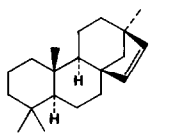
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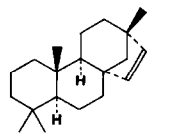
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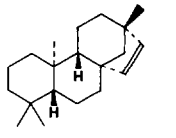
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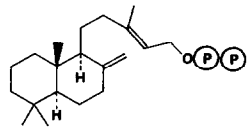
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measurement of its specific rotation, showed that it was *ent*-beyerene (**11**) [15].

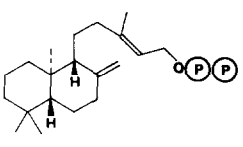
The danger of relying on GC retentions (especially at just one temperature on one stationary phase) are illustrated by the similar retentions of beyerene, rosadiene and sclarene on SE-30 at one temperature [8]. The peak identified as 'cupressene' by Aplin *et al.* in their *D. intermedium* extract [4], could have been any combination of these three compounds. Furthermore absolute configurations cannot be assumed as beyerene (**9**), enantiomeric to the material isolated here, has been found in the foliage of another gymnosperm [15]. An extreme instance of this problem is given below.

ent-*Sclarene*

The diterpenes thus far identified in *D. intermedium* foliage extracts fall into two biosynthetic classes:

1. Sclarene (**2**), rimuene (**1**) and phyllocladene (**3**) are in the 'normal' series derived from labda-8(17),13-dien-15-yl pyrophosphate (**12**) [16].
2. *ent*-Rosadiene (**7**), *ent*-beyerene (**11**) and *ent*-kaurene (**4**) are in the 'enantio' series derived from *ent*-labda-8(17),13-dien-15-yl pyrophosphate (**13**) [16].

Rimuene (**1**) and *ent*-rosadiene (**7**) have the same configuration at C-13, but opposite configurations at C-8, C-9 and C-10 whilst phyllocladene (**3**) and *ent*-kaurene (**4**) have the same configurations at C-8 and C-13 but opposite configurations at C-5, C-9 and C-10. It seemed possible that the corresponding configurational isomers of *ent*-beyerene (**9**) and sclarene (**2**) could be present in some specimens of *D. intermedium*. The corresponding compound to *ent*-beyerene in the 'normal' series is 13α-beyerene (**10**), but none of the ten trees sampled produced an appropriate GC peak at a practicable level for isolation. The corresponding compound to sclarene (**2**) in the 'enantio' series is simply *ent*-sclarene (**14**). Instances of the co-occurence of enantiomers in a single plant species are fairly common for the monoterpenes [17], but only one previous diterpene example could be found. Again a labdane derivative was involved: labda-8(17),13-dien-15-oic acid (**15**) and its enantiomer were both present in an extract of the wood of a West African angiosperm [18].



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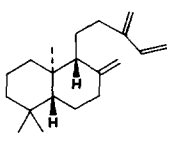

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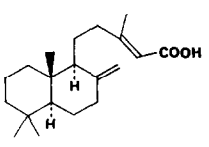

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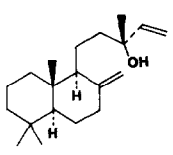

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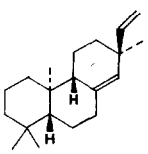

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One of the foliage samples contained only *ent*-rosadiene (**7**), *ent*-kaurene (**4**) and a peak identified by GC as sclarene, so this seemed the most likely source of *ent*-sclarene (**14**). Repeated preparative TLC on silver nitrate imp gel gave a sample of sclarene with no *ent*-rosadiene (**7**) detectable by ^{1}H NMR. This material was identical by ^{1}H NMR and IR spectroscopy to sclarene prepared by dehydration of manool (**16**) [19], but had a specific rotation of $-22°$ rather than $+30°$. The identification of *ent*-sclarene (**14**) was confirmed by the preparation of its tetracyanoethylene Diels–Alder adduct [9]. This had the same ^{1}H NMR, IR and mass spectra and the same melting point as the Diels–Alder adduct of sclarene (**5**), but a rotation of $-39°$ rather than $+40°$. This is the first report of the isolation of *ent*-sclarene (**14**).

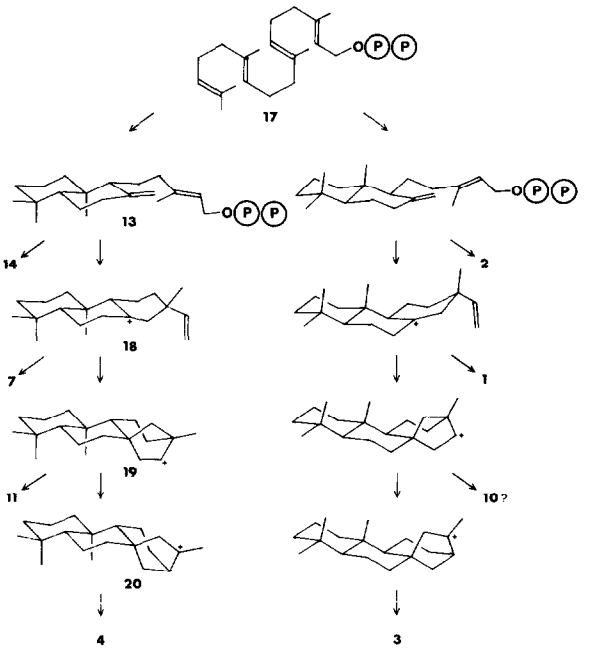
The specimen of *D. intermedium* from which sclarene (**2**) was isolated contained only 'normal' diterpenes. It seemed likely that foliage containing diterpenes from both biogenetic series would contain a mixture of sclarene enantiomers. However, the specific rotation of the tetracyanoethylene adduct of sclarene from such a tree indicated the presence of (+)-sclarene (**2**) (+35°).

Diterpene biosynthesis

Diterpene levels vary greatly from tree to tree in the foliage of *D. intermedium* (Table 1); however, no significant correlations were found between the levels of the major diterpenes in the ten trees analysed. Although the factors affecting this variation were not studied, it is unlikely that environmental factors are important, as the ten samples collected were from a single location. The diterpene levels would thus seem to be under genetic control, as we concluded for *D. cupressinum* [7].

The variations reported here for *D. intermedium* have a close parallel in the Japanese gymnosperm, sugi, *Cryptomeria japonica* D. Don (Taxodiaceae), where varying levels of rimuene, sclarene, phyllocladene and kaurene have been found [20]. The inheritance of the main diterpene components, kaurene, phyllocladene and sclarene was investigated by controlled breeding studies, and two genes, each with two alleles, were found to control the biosynthesis of these compounds [20, 21].

The polycyclic diterpene whose biosynthesis has been studied in most detail in *ent*-kaurene (**4**) because of this compound's importance as a precursor of the gibberellins. The current biosynthetic theory, recently reviewed by West [16] is presented in Scheme 1. The first step is the proton initiated cyclization of a suitable conformation of geranylgeranyl pyrophosphate (GGPP) (**17**) to *ent*-labda-8(17),13-dien-15-yl pyrophosphate (**13**). Ring C is formed by *anti* allylic displacement of pyrophosphate to give the carbocationic intermediate (**18**). Nucleophilic attack of the vinyl group on C-8 then gives **19**, which undergoes a 1,2-alkyl shift and deprotonation to give *ent*-kaurene (**4**). Reactions modelling steps **17** to **13**, **13** to **18** and **19** to **20** have been achieved *in vitro* [22]. One group has even managed to produce tricyclic diterpenes directly from an acyclic precursor [23]. However, non-enzymatic conversions of the type **18** to **19** have not been detected despite numerous attempts [22, 24] and the mechanism of this step is in doubt [24].



Scheme 1. Biosynthesis of *D. intermedium* diterpenes.

Enzyme preparations capable of synthesizing *ent*-kaurene (**4**) have been isolated from a few different organisms and their properties have been studied. Two enzymic activities have been distinguished [16]: '*a* activity' which involves the proton initiated cyclization of GGPP to the bicyclic intermediate **12**; and '*b* activity' which includes all the subsequent steps to *ent*-kaurene. In one case, two different *b* enzymes have been resolved. One synthesized *ent*-kaurene (**4**), while the other produced *ent*-sandaracopimaradiene (**21**) [25]. It has been proposed that these different *b* enzymes can operate in conjunction with a common *a* enzyme [16].

The *ent*-diterpenes found in *D. intermedium* foliage represent all the intermediates postulated in the biogenesis of *ent*-kaurene (**1**), as shown in Scheme 1. Furthermore, three of the corresponding intermediates in the 'normal' series are also represented. The following biosynthetic mechanisms in *D. intermedium* are consistent with these findings:

1. Two different *a* enzymes, which produce the enantiomeric bicyclic pyrophosphates (**12** and **13**) can be present. These can eliminate pyrophosphoric acid to give sclarene (**2**) and *ent*-sclarene (**14**) respectively, or undergo further cyclization.
2. Three different types of *b* activity can occur. These give rise to the following pairs of compounds by acting on either of the two enantiomers of the bicyclic precursors (**12** and **13**): (i) *ent*-rosadiene (**7**) or rimuene (**1**); (ii) *ent*-beyerene (**11**) or 13α-beyerene (**10**); (iii) *ent*-kaurene (**4**) or phyllocladene (**3**).

It is not clear whether the *b* enzymes have low substrate specificities or whether there are pairs of stereospecific enzymes. This could be tested with the kaurene synthetase preparations which are now available [16].

Biochemical systematics

No sesquiterpenes were detected in any of the extracts of *D. intermedium* foliage and monoterpenes were only present at low levels. All but one other member of the Podocarpaceae have been reported to have mono- and sesquiterpenes at comparable levels to the diterpenes in their foliage. The foliage oils of all the New Zealand *Podocarpus* and *Phyllocladus* species which have been examined in detail [26], and those of *D. cupressinum* [1, 7], *D. colensoi* [27], *D. biforme* [28], *D. kirkii* [29] and *D. franklinii* [30] were all found to contain more than 10% of monoterpenes and detectable amounts of sesquiterpenes. By contrast foliage from a specimen of *D. laxifolium* Hook., the pigmy pine, gave a solid steam-distillate which was largely phyllocladene (**3**). Monoterpenes made up less than 5% of the distillate and no sesquiterpenes were reported [31].

In his revision of the genus *Dacrydium* Quinn suggested that *D. intermedium* and *D. laxifolium* should be grouped with *D. fonkii* (Phil.) Benth. in the revived genus *Lepidothamnus* Phil. [3]. "... the species are united by their distinctive cone morphology with its erect ovule, the absence of resin ducts in the leaves which occur universally elsewhere in the family ... and a large number of cupressoid cross-fields not found elsewhere in the family. Chemically, these species are also unique in the family, having cupressoflavone as their major biflavonoid constituent ..."

The foliage of *D. fonkii*, a shrub found in southern Chile, has not previously been analysed for terpene components [32]. Mature foliage from a single specimen was subjected to small scale steam-distillation/solvent extraction as before. The major component was rimuene (**1**) from its GC retention indices, ^{1}H NMR and IR spectra, and specific rotation. No mono- or sesquiterpene components were detected (< 1%). These results further support Quinn's conclusions on grouping *D. intermedium*, *D. laxifolium* and *D. fonkii* in *Lepidothamnus*.

The lack of resin ducts in the foliage of these species is interesting since monoterpene biosynthesis has been associated with specialized cells surrounding the resin canals in the foliage of another gymnosperm, *Pinus pinaster* Ait. (Pinaceae) [33, 34]. Sesquiterpene biosynthesis was found to be a distinct process involving the endoplasmic reticulum and occurring throughout the leaves [33, 35].

EXPERIMENTAL

General. Small scale extractions and GC analyses were performed as detailed in ref. [7]. Additional quantitative data were obtained from isothermal GC analyses on a CW 20 M capillary column. GC retention indices were measured as in ref. [8]. Specific rotations (Table 2) and NMR spectra were obtained as $CHCl_3$ and $CDCl_3$ solutions respectively.

Sample collection. D. intermedium adult foliage samples were collected by B. J. Gilbertson, New Zealand Forest Service, from Kaniere State Forest, grid ref. 638472 (S58), altitude 100 m. Two collections were made, one in June 1982 and the other a month later. A sample of adult *D. fonkii* foliage was collected by Dr. Rodolfo Gajardo, Universidad de Chile, Santiago, in December 1982 from the Cordillera de los Pavillos, 41° 03′, S, 73° 50′ W, altitude 750 m. Samples have been lodged with the University of Otago Herbarium but reference numbers are not yet available.

Bulk extractions. D. intermedium foliage (85 g) was Soxhlet

Table 2. Specific rotations of diterpenes involved in this study

Compound	Tree	$[\alpha]_D$,°	lit.
Rimuene (1)	1	+51	(+56 [10])
	7	+42	
ent-Rosadiene (7)	4	−40	(−42 [12])
	7	−39	
ent-Beyerene (11)	7	+31	(+33 [36])
Sclarene (2)	1	+30	(+28 [9])
ent-Sclarene (14)	4	−22	
Phyllocladene (3)	1	+18	(+16 [36])
ent-Kaurene (4)	4	−70	(−80 [36])
ent-Rosa-5(10),15-diene (8)	—	+123	(+117 [13])

extracted into Me_2CO (750 ml) for 6 hr. This extract was concd to 250 ml, diluted with H_2O (250 ml) and extracted with Et_2O (2 × 250 ml). The combined extracts were washed with H_2O (250 ml), dried over $MgSO_4$ and the solvent removed to leave a dark green paste (6 g). This was preadsorbed on to alumina (20 g) from $CHCl_3$. The adsorbed material was placed on top of a dry column of alumina (80 g) and eluted with *n*-hexane (150 ml) to give a mixture (280 mg) of diterpene hydrocarbons (the carotenoids and chlorophylls were retained). $AgNO_3$–silica gel prep. TLC (C_6H_6–*n*-hexane, 1:4) separated rimuene (1, 62 mg) R_f 0.6, phyllocladene (3) contaminated with rimuene (53 mg) R_f 0.5, and sclarene (2, 36 mg) R_f 0.2. Phyllocladene was purified by recrystallization from Et_2O.

Similar methods were used to isolate the other diterpenes, but steam distillation, with co-extraction of the steam volatiles into *n*-hexane, was quicker as the chlorophylls and carotenoids were not extracted. Specific rotations for the diterpenes isolated are given in Table 2.

ent-*Rosadiene* (7). A mixture of *ent*-rosadiene and sclarene (68 mg) obtained from *D. intermedium* foliage was dissolved in deuterated THF (0.5 ml) and tetracyanoethylene (20 mg) added. ^{1}H NMR showed that the sclarene had all reacted within 10 min [δ6.35 (*q*) had disappeared]. Filtration through alumina and elution with hexane gave *ent*-rosadiene (30 mg): IR [12]; ^{1}H NMR [12].

ent-*Rosa*-5(10),15-*diene* (8). Dry hydrogen chloride was bubbled through a soln of *ent*-rosadiene (10, 30 mg) in $CHCl_3$ (3 ml) for 10 min and stirring was continued for 2 hr. Removal of the solvent then gave a yellow oil (29 mg) which was purified by $AgNO_3$–silica gel prep. TLC (C_6H_6–*n*-hexane, 1:4) to give, at medium R_f, *ent*-rosa-5(10),15-diene (9 mg): IR [13]; ^{1}H NMR [13].

ent-*Sclarene* (14). *D. intermedium* foliage (400 g) was extracted to give a yellow oil (1.2 g). This was filtered through alumina and 0.36 g of the *n*-hexane eluates (0.98 g total) were subjected to repeated $AgNO_3$–silica gel prep. TLC (*n*-heptane–MeCOEt, 95:5) to give *ent*-sclarene (18 mg): IR ν_{max} cm^{-1}: 3100, 1650, 1605, 1000, 900; ^{1}H NMR (90 MHz): δ0.74 (3H, *s*), 0.85 (3H, *s*), 0.93 (3H, *s*), 4.54 (1H, *s*, H-17), 4.82 (1H, *s*, H-17), 4.96 (2H, *s*, H-16), 4.8–6.6 (3H, ABX pattern, H-14, H-15); $[\alpha]_D$ −22°. The IR and ^{1}H NMR spectra were superimposable on those of authentic sclarene (2) prepared from manool (16) as follows: A soln of $POCl_3$ (7 ml) in pyridine (10 ml) was added to a stirred soln of manool (16, 1.0 g, $[\alpha]_D$ +32°) in pyridine (20 ml) at −10°. After stirring overnight this mixture was poured cautiously into satd $NaHCO_3$. Subsequent hexane extraction and filtration through alumina gave a clear oil (87 mg). Prep. $AgNO_3$–silica gel TLC (*n*-heptane–MeCOEt, 95:5) gave sclarene (2, 21 mg), $[\alpha]_D$ +28° (lit. +28° [9]).

TCNE adducts. The tetracyanoethylene (TCNE) adduct of *ent*-sclarene (14) was prepared by adding a soln of TCNE in THF to a mixture of diterpenes extracted from foliage. After 5 min, removal of solvent gave a brown oil which was filtered through alumina. The hexane eluates gave diterpene hydrocarbons and the Et_2O eluates gave a white solid which was recrystallized from Et_2O–MeOH to give the pure TCNE-adduct of *ent*-sclarene [9], mp 117° (Found: C, 77.9; H, 8.5; N, 13.6. Calc. for $C_{26}H_{32}N_4$: C, 78.0; H, 8.1; N, 14.0%): IR ν_{max} cm^{-1}: 3080, 2260 (weak), 1640, 1435, 890, 870; ^{1}H NMR (90 MHz): δ0.73 (3H, *s*), 0.84 (3H, *s*), 0.91 (3H), *s*), 3.00 (2H, *s*), 4.41 (1H, *s*, H-17), 4.84 (1H, *s*, H-17), 5.52 (1H, *s*, $W_{1/2}$ = 9 Hz): EIMS (probe) 70 eV, *m/z* (rel. int.): 400 $[M]^+$ (100), 385 (70), 257 (30), 205 (80), 149 (30), 137 (100), 123 (100), 109 (60), 95 (75), 81 (75), 69 (75). The TCNE adduct of sclarene (2) from the dehydration of manool was prepared similarly, mp 115°.

Extraction of D. fonkii *foliage.* Extraction of ground foliage (5 g) by steam distillation with concurrent extraction into hexane for 2 hr gave an extract (9.6 mg) that was largely rimuene, $[\alpha]_D$ +41°.

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