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(+)-δ-CADINENE IS A PRODUCT OF SESQUITERPENE CYCLASE ACTIVITY IN COTTON

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Key Word Index—*Gossypium hirsutum*; Malvaceae; upland cotton; biosynthesis; sesquiterpenes; phytoalexins; (+)-δ-cadinene; (*E*, *E*)-farnesyl pyrophosphate; 2,7-dihydroxycadalene; lacinilene C; desoxyhemigossypol; bacterial blight of cotton.

Abstract—Glandless cotton cotyledons stimulated to produce sesquiterpenoid phytoalexins by inoculation with *Xanthomonas campestris* pv. *malvacearum*, or by injection of oligogalacturonide elicitors, generated a hydrocarbon that was absent in mock-inoculated or non-inoculated cotyledons. Enzyme preparations from the same cotton cotyledons catalysed cell-free reactions which converted (*E*, *E*)-[1-^{3}H]farnesyl pyrophosphate into a predominant tritium-labelled hydrocarbon product. Large-scale cell-free reactions catalysed by enzyme preparations from cotton cotyledons previously inoculated with *Xanthomonas campestris* pv. *malvacearum* converted nonradioactive (*E*, *E*)-farnesyl pyrophosphate into the hydrocarbon product, which was identified as (+)-δ-cadinene by chiral GC-mass spectrometry. *In planta* incorporation of tritium into the sesquiterpenoid phytoalexins 2,7-dihydroxycadalene, lacinilene C, lacinilene C7-methyl ether and structurally related terpenoids occurred following injection of [^{3}H] (+)-δ-cadinene into previously inoculated glandless cotton cotyledons. The accumulation of (+)-δ-cadinene in bacteria-inoculated or elicitor-treated cotton cotyledons and the results of the incorporation experiment suggest that (+)-δ-cadinene is an early enzymatic intermediate in the biosynthesis of the sesquiterpenoid phytoalexins by upland cotton.

INTRODUCTION

2,7-Dihydroxycadalene (DHC) (**1**), 2-hydroxy-7-methoxycadalene (HMC) (**2**), lacinilene C (LC) (**3**) and lacinilene C7-methyl ether (LCME) (**4**) are sesquiterpenoid compounds produced in leaves and cotyledons of upland cotton (*Gossypium hirsutum* L.) during the hypersensitive response to incompatible races of the bacterial pathogen *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye (*Xcm*) and to pathovars of *X. campestris* that are not pathogenic to cotton [1, 2]. The hypersensitive response enables resistant lines of upland cotton to withstand infection with *Xcm*, the causal agent of bacterial blight of cotton, with little tissue damage. Compounds **1**, **3** and **4** exhibit antibacterial activity in aqueous solution and hence are phytoalexins [1, 2]. The terpenoid origin of DHC and the folding pattern of its farnesyl (or equivalent) precursor have been established [3].

In previous work [4], we showed that [1-^{3}H]farnesyl pyrophosphate should be an appropriate substrate for cell-free assay of cyclase activity involved in the biosynthesis of DHC. To avoid the complex mixtures of terpenes found in glanded cotton [5–8], we identified a glandless cotton line (WbM*gl*) whose healthy cotyledons lack the constitutive terpene production of glanded cotton, but still accumulate **1** after inoculation with a strain of *Xcm* to which this cotton line is resistant. We have now detected in cell-free extracts from *Xcm*-inoculated WbM*gl* cotyledons a cyclase activity which appears to catalyse the first reaction in biosynthesis of sesquiterpenoid phytoalexins from (*E*, *E*)-farnesyl pyrophosphate (FPP) (**5**). The tritiated product generated from (*E*, *E*)-[1-^{3}H]farnesyl pyrophosphate (**6**) was infiltrated into *Xcm*-inoculated WbM*gl* cotyledons, resulting in the labelling of **1**, **3** and **4** and structurally related terpenoids *in planta*. The identification of the product of the cotton cyclase activity as (+)-δ-cadinene (**7**) is now reported.

RESULTS AND DISCUSSION

Identification of an infection-induced hydrocarbon from cotyledons

Hexane extracts of non-inoculated and mock-inoculated glandless cotton (WbM*gl*) cotyledons eluted from silica gel with hexane revealed a virtual absence of sesquiterpene hydrocarbons upon analysis by gas chromatography–electron impact mass spectrometry (GC–EIMS). In contrast, GC–EIMS analysis of similar

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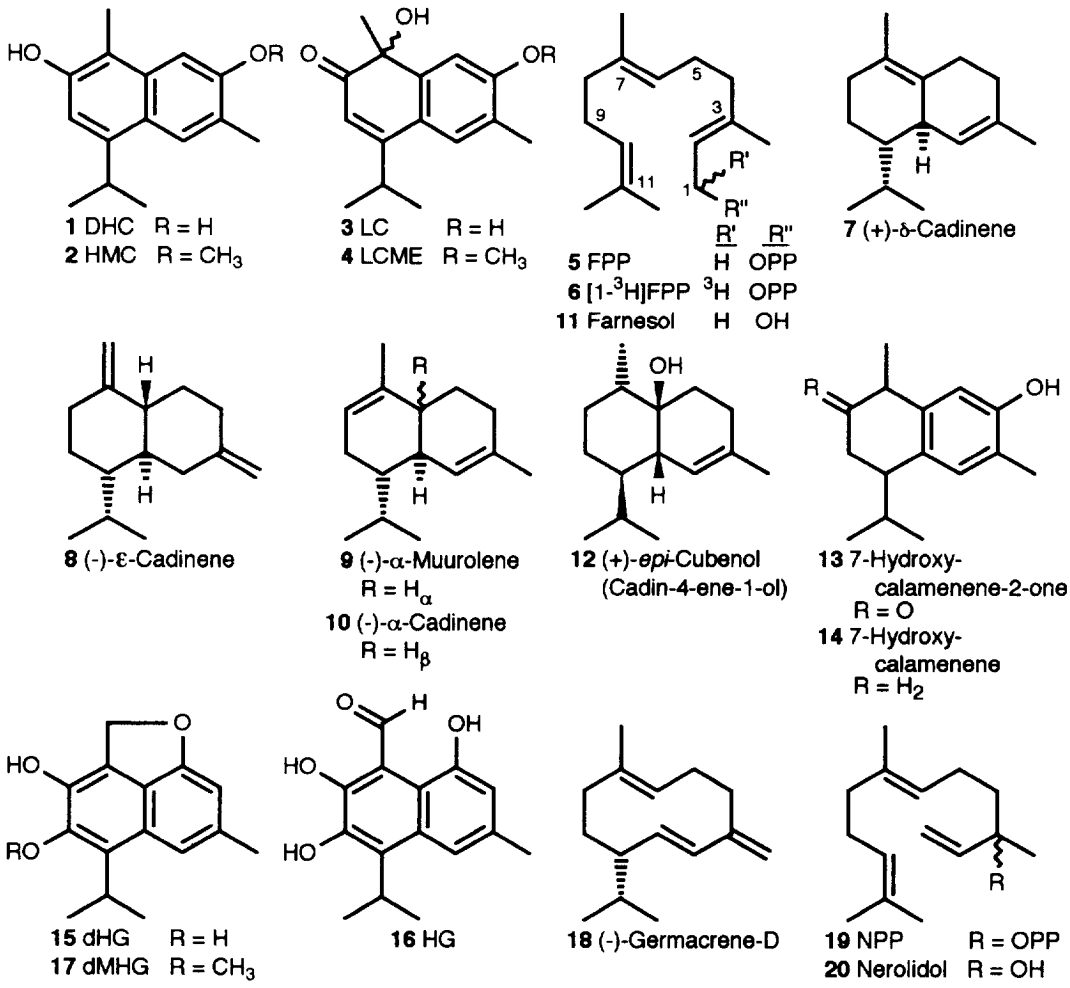

hexane extracts of *Xcm*-inoculated WbM*gl* cotyledons revealed the presence of three infection-induced compounds in the extracts, each compound exhibiting a mass spectrum characteristic of a sesquiterpene hydrocarbon. The major volatile sesquiterpene was δ-cadinene (**7**), while ε-cadinene (**8**) and α-muurolene (**9**) were tentatively present in smaller amounts. [The identification of **8** and **9** remains tentative because known standards for comparison were not available. The structures of **8** and **9** are shown arbitrarily as the (−)-enantiomers because chiral analysis was not performed on these compounds.]

To obtain more of the major sesquiterpene for further characterization, 1300 g of *Xcm*-inoculated WbM*gl* cotyledons were extracted, yielding *ca* 500 μg of a compound exhibiting chromatographic characteristics of a hydrocarbon: weak retention by silica gel and strong retention by octadecylsilane. The mass spectrum of the compound exhibited an *m/z* 204 $[M]^+$ and most closely matched the reference mass spectrum of δ-cadinene from literature sources [9, 10]. However, the mass spectrum was not diagnostic for δ-cadinene, because a number of sesquiterpene hydrocarbons of M_r 204 (e.g. β-cadinene [9]) exhibit very similar mass spectra. The ^{1}H NMR spectrum of the compound was consistent with the incomplete ^{1}H NMR spectral data for δ-cadinene [11–13].

To assure identification of the major infection-induced volatile sesquiterpene hydrocarbon by comparison to a known standard, authentic δ-cadinene (22 mg) was isolated from cade oil (from *Juniperus oxycedrus*), analysed and compared to published descriptions of the following characteristics: EIMS [9, 10], ^{1}H NMR [11–13], ^{13}C NMR [14, 15], IR [13, 16], CD [17] and Kovats' indices (R_I) from retention on gas chromatographic phases [18–20]. The UV spectrum (200–400 nm) of the purified compound exhibited only a shallow curve without maxima, consistent with the isolated double bonds of δ-cadinene. The cade oil component was further characterized by 1D and 2D NMR spectroscopic methods permitting unambiguous assignment of all ^{1}H and ^{13}C signals (Davis, G. D. *et al.*, unpublished results). The rigorously characterized δ-cadinene from cade oil co-chromatographed with the infection-induced hydrocarbon compound isolated from *Xcm*-inoculated cotton in four GC phases of varying polarities. The gas chromatographic analysis yielded Kovats' index values (R_I) [21] that corresponded well with literature values

Table 1. Comparison of Reference Kovats' Index [21] values for δ-cadinene (7) with experimentally determined values from co-injection of the induced hydrocarbon component from *Xcm*-inoculated cotton cotyledons and δ-cadinene from cade oil onto gas chromatographic phases of varying polarity

Compound(s)	100% Methyl (SE-30) Ref. [18]	Exp.	5% Phenyl–95% methyl (SE-54)* Ref. [19]	Exp.†	50% Phenyl–50% methyl Ref. [20]	Exp.	Polyethylene glycol (Carbowax 20M) Ref. [18]	Exp.
Co-injected cotton hydrocarbon and δ-Cadinene (7) from cade oil‡	1513	1517	1538	1565	1655	1658	1756	1755
β-Caryophyllene	1415	1415	1418	1440	1521	1521	1595	1591
α-Humulene	1448	1448	1456	1473	No lit. value		1667	1665

[Marker compounds (β-caryophyllene and α-humulene) included to assure confidence in calculated values.]

*Experimental (Exp.) values determined with a 120 m 5% phenyl–95% methyl (SE-54) column.

†Experimental values matched reference (Ref.) values when know sesquiterpenes (β-caryophyllene and α-humulene) were used to construct a Kovats' Indices standard curve [21] which could produce a self-consistent Kovats' index value [13, 22] for δ-cadinene.

‡In all GC phases the co-injected mixture of *ca* equal amounts produced a single, symmetrical peak.

for δ-cadinene (Table 1) when employing co-injected β-caryophyllene and α-humulene to generate self-consistent Kovats' indices [13, 22]. The co-chromatography studies and similarities of the ^{1}H NMR spectrum and the mass spectral data between the cotton infection-induced hydrocarbon from *Xcm*-inoculated glandless cotton and the δ-cadinene from cade oil demonstrated that δ-cadinene was accumulated during the course of the hypersensitive response of cotton to the pathogen *Xcm* to a level of *ca* 300 ng g^{-1} of inoculated cotyledons [by gas chromatography–flame ionization detection (GC–FID)]. In contrast, δ-cadinene was not detected in mock-inoculated and noninoculated cotton cotyledons, even though as little as 5 ng δ-cadinene g^{-1} of cotton cotyledon tissues (< 2% of *Xcm*-inoculated cotyledon tissue content) could be detected by GC–FID analysis.

Identification of the hydrocarbon product of cell-free reactions employing radioactive farnesyl pyrophosphate (**6**) *or non-radioactive farnesyl pyrophosphate* (**5**)

Enzyme preparations made from the *Xcm*-inoculated WbM*gl* cotton cotyledons which accumulated δ-cadinene catalysed conversion of **6** in cell-free reactions to a predominant radioactive product displaying the same chromatographic characteristics as the infection-induced hydrocarbon: weak retention by silica, strong retention by octadecylsilane. The radioactive product from this cell-free reaction and the non-radioactive δ-cadinene isolated from the *Xcm*-inoculated cotton cotyledons co-chromatographed in both analytical-scale sub-ambient temperature normal phase [23] and ambient temperature tandem column [24] reversed phase HPLC systems. Additionally, the radioactive product co-chromatographed with the δ-cadinene from cade oil in both analytical-scale HPLC systems. These findings support the identification of the radioactive product generated by the cell-free reaction as [^{3}H]δ-cadinene. The enzyme preparations exhibited specific activity in the range of 0.3–3 μkat kg protein^{-1} (i.e. 1–10 nmol δ-cadinene hr^{-1} mg^{-1} protein).

Further support for identification of the cell-free reaction product as tritium-labelled δ-cadinene was given by results from cell-free reactions utilizing **5** as substrate. The cell-free reactions were conducted with three controls [25] which consisted of GC–EIMS analyses of: (1) a hexane extract of a non-incubated aliquot of the enzyme preparation, (2) a hexane extract of the enzyme preparation incubated without substrate and (3) a boiled enzyme preparation incubated with **5**. The GC–EIMS analyses of the three controls demonstrated that the enzyme preparation (1) did not contain any endogenous δ-cadinene (XAD-4 resin [26] had removed endogenous non-polar terpenoids from the enzyme preparation), (2) did not generate δ-cadinene when incubated without **5** and (3) did not convert **5** to δ-cadinene after heat denaturation, respectively. It can be reasonably concluded from the first two controls that the δ-cadinene isolated from the cell-free reactions does not arise from endogenous δ-cadinene or endogenous substrate present in the enzyme preparation. The third control implies that the conversion of **5** to δ-cadinene requires enzyme activity. Thus, the controls assured that the measured biosynthesis was derived only from enzymatic action on the added substrate, **5**.

After incubation with **5**, the enzyme preparation was extracted to yield a sample for GC–EIMS analysis which revealed that **5** had been converted into a substance which matched the GC–EIMS characteristics of the δ-cadinene from cade oil. Co-injection of *ca* equal amounts of the enzymatic product and the δ-cadinene from cade oil resulted in a single homogeneous peak exhibiting a mass spectrum that matched that of δ-cadinene. Additionally, the GC–EIMS analysis revealed the presence of

minor amounts of a component tentatively identified as α-cadinene (**10**) [shown arbitrarily as the (−)-enantiomer] and farnesol (**11**) which presumably was generated by the action of phosphohydrolases [26] on the added substrate, **5**. The evidence supports the identification of the product of the cell-free conversion of **6** as tritium-labelled δ-cadinene and the identification of the hydrocarbon accumulated in the *Xcm*-inoculated glandless cotton cotyledons and the product of the cell-free conversion of **5** as δ-cadinene. The chiral GC–EIMS analysis described below confirmed this identification of the product of the cell-free conversion of **5**, as well as showing further that it is the specific enantiomer (+)-δ-cadinene (**7**).

Identification of the hydrocarbon product from elicitor-treated cotyledons

The results presented to this point support the hypothesis that δ-cadinene is biosynthesized during the hypersensitive response of cotton cotyledons to a bacterial pathogen. It has been reported [27] that several species of the bacterial genus *Streptomyces* produce *epi*-cubenol (cadin-4-ene-1-ol) (**12**), a sesquiterpene structurally similar to δ-cadinene. Although some lines of glanded cotton constitutively biosynthesize δ-cadinene and structurally related compounds [5–8], the report [27] heightened concern that *Xcm* could possibly be the organism that biosynthesized the δ-cadinene accumulated in inoculated glandless cotton cotyledons. To test this possibility, we performed an experiment to determine if the enzymes of the cotton cotyledons could generate δ-cadinene in the absence of the bacterial enzymes of *Xcm*. The treatment we chose was injection of a filter-sterilized mixture of oligogalacturonides obtained by hydrolysis of the plant cell wall component polygalacturonic acid that contains no bacteria or bacterial products, yet elicits phytoalexin accumulation in cotton cotyledons [28].

GC–FID and GC–EIMS analyses of hexane extracts of elicitor-treated WbM*gl* cotyledons showed that δ-cadinene was indeed present in the cotyledons at *ca* 60 ng g^{-1} of elicitor-treated cotyledons (*ca* 20% of the level in *Xcm*-inoculated cotyledons). There was no detectable accumulation of δ-cadinene in non-inoculated and mock-inoculated WbM*gl* control cotyledons. Additionally, GC–FID analysis showed that δ-cadinene does not accumulate in *Xcm* cells (nor in its culture medium) prior to inoculation. The elicitor-treated cotyledons were used as a source of enzyme preparations for cell-free reactions utilizing **6** as substrate. The predominant radioactive product isolated from these cell-free reactions chromatographed in both analytical-scale normal phase and reversed phase HPLC systems in a manner identical to that of the product of cell-free reactions catalysed by enzyme preparation made from *Xcm*-inoculated cotyledons. The enzyme preparations exhibited specific activity of *ca* 1 μkat kg^{-1} protein (i.e. *ca* 3 nmol δ-cadinene hr^{-1} mg^{-1} protein). The results of these experiments do not eliminate the possibility that *Xcm* may participate in the biosynthesis of δ-cadinene in the cotyledons. However, the constitutive production of δ-cadinene in glanded cotton [5, 8] and the evidence presented here demonstrate that cotton cotyledons are capable of accomplishing the biosynthesis of δ-cadinene in the absence of *Xcm* cells or compounds produced by *Xcm* cells.

Identification and enantiomeric assignment of the product of cyclase activity by chiral GC–EIMS analysis

Further proof of the chemical identity and enantiomeric assignment of the product (δ-cadinene) of cyclase activity was obtained for a number of reasons. δ-Cadinene has been isolated, in general, as the (+)-isomer from vascular plants [17] while the (−)-isomer has been found in liverworts [17], insects, marine sources and other plants [29]. Furthermore, an optically inactive (i.e. 'nearly racemic' [30]) sample of δ-cadinene named 'dysoxylenene' was isolated from the essential oil of the wood of *Dysoxylon frazeranum* Benth. ('rosewood'). It has been shown [2] that the cotton lines Im 216 and OK 1.2 accumulate predominantly, though not exclusively, the (+)-enantiomers of **3** and **4**, whereas the WbM(0.0) line accumulates predominantly the (−)-enantiomers. Thus, an investigation into the chirality of the δ-cadinene from cotton was necessary to determine if WbM*gl* cotton cotyledons generate (+)-δ-cadinene, (−)-δ-cadinene, or a mixture of the two enantiomers.

The identification and enantiomeric assignment of a natural product (or enzyme product) such as δ-cadinene can present practical challenges. Chemical identification can be accomplished by the time-proven method of dilution of a radioactive enzyme product with a non-radioactive standard, followed by recrystallization of the mixture (or a derivative) to constant specific activity and determination of melting point of the crystal. This method can be extended to include chiral assignment by use of a chiral non-radioactive standard. A good example of this combined chemical identification and chiral assignment has been employed for analysis of the enantiomeric products of α-pinene cyclases [31]. Alternatively, a radioactive enzyme product can be co-chromatographed with a non-radioactive chiral standard to constant specific activity under conditions capable of resolving enantiomeric standards. Recrystallization or co-chromatography requires modest amounts of enantiomeric standards, often obtained by chemical synthesis or isolation from phytochemical sources (e.g., essential oils). Chemical synthesis of a standard may be expensive in terms of time and consumption of reagents and often results in a low yield of a racemic product, as for the published synthesis of (±)-δ-cadinene [32]. Even if a relatively simple route to an enantiomer is found {e.g. acid-catalysed isomerization of (−)-α-cubebene or (−)-α-copaene to (+)-δ-cadinene [33]}, often purification from a product mixture is required.

Alternate sources of natural products may be prohibitively expensive or unavailable, as is the case for the essential oil source of (+)-δ-cadinene, cade oil [17] (the product of the destructive distillation of *Juniperus oxycedrus*). The recent imposition of governmental regula-

tions effectively prevents harvest of *Juniperus oxycedrus*; distillers have turned to *Juniperus phoenicea* as a substitute source material, thus generating a 'cade oil' rich in α-cedrene, but much poorer in δ-cadinene than traditional cade oil [34]. Other essential oil sources can be excessively expensive (e.g. ylang-ylang oil for δ-cadinene), especially if the desired natural product is contained in low abundance.

While co-chromatography of a radioactive enzyme product with non-radioactive standard may be performed with small amounts of standard (compared to recrystallization), there are potential problems. A major problem is the possible inability to resolve structurally similar products, even when multiple chromatographic systems are employed. Additionally, isotopic fractionation [35, 36] may occur, which could lead to errors in identification such as false negatives (i.e. radioactive enzyme product separates from non-radioactive analogue) or false positives (i.e. radioactive enzyme product co-chromatographs with non-radioactive, non-equivalent standard).

Chemical identification and chiral assignment by GC–EIMS can be a pragmatic solution to many of these practical problems. Aside from the instrumental requirement of a GC–MS unit, chiral analysis by GC–EIMS requires only small amounts (*ca* 1 mg or less) of enantiomeric standards and of the natural product to be characterized. Another advantage of this method is that the unknown compound and standard need not be of very high chemical purity, since the chromatography separates components and provides mass spectral identification of injected compounds. Derivatization of the standards and the enzyme product (or natural product) can generate a mixture of compounds which can yield a constellation of data, including the number of derivatives formed, retention indices, and mass spectral data. Thus, a combination of different types of data may be used to increase confidence in the chiral GC–EIMS analysis of the enzyme product or natural compound. The increasing number of chiral stationary phases now commercially available for capillary GC columns ensures that virtually any pair of enantiomers can be resolved. A good example of this resolving power is found in the chiral GC resolution of the enantiomers of δ-cadinene, a diolefin which has a paucity of the functional groups often required for successful resolution in other modes of chiral chromatography.

To obtain sufficient sample of the cyclase product, **5** was converted by cell-free reaction. To assist the determination of the enantiomeric constitution of the product of cyclase activity, samples of (+)-δ-cadinene, (−)-δ-cadinene and a 70:30 mixture of (+)-δ-cadinene and (−)-δ-cadinene were generated for use as standards. The δ-cadinene from cade oil was not epoxidized (because additional cade oil from *J. oxycedrus* was unavailable), but was useful because it has been characterized as (+)-δ-cadinene [17]. Chiral GC–FID analyses revealed that the (+)-δ-cadinene from cade oil co-chromatographed with the (+)-δ-cadinene standard [generated from (−)-α-cubebene] and with the product of cyclase activity. Additionally, the (+)-δ-cadinene standard, the δ-cadinene from cade oil, and the product of cyclase activity each resolved from the (−)-δ-cadinene standard during chiral GC–FID analyses.

The non-radioactive cyclization product and the three chiral standard preparations were each epoxidized [30, 37, 38] with monoperphthalic acid [39]. For chiral GC–EIMS, each δ-cadinene sample was mixed with the derived monoepoxide and diepoxide products, as well as the *n*-paraffins, pentadecane and nonadecane, which served as Kovats' index retention markers.

Chiral GC–EIMS analysed of the δ-cadinene standards and the derived epoxides revealed that each enantiomer of δ-cadinene was converted into two diastereomeric monoepoxide derivatives (designated as monoepoxide 1 and monoepoxide 2) and three diastereomeric diepoxide derivatives (designated as diepoxide 1, diepoxide 2 and diepoxide 3). Previous descriptions of the products recovered from the epoxidation of δ-cadinene vary from an epoxydiol, a monoepoxide and a single, crystalline diepoxide [30], to an epoxydiol and a single, crystalline diepoxide [37], to two diastereomeric monoepoxides [38]. Our detection by GC–EIMS of a larger number of epoxide derivatives is similar to the recent reports of multiple epoxidation products of α-humulene [40, 41], and for α-muurolene, γ-muurolene and γ-cadinene [42] in contrast to earlier reports [30, 43] in which fewer epoxide derivatives were generated from sesquiterpene hydrocarbons containing multiple double bonds. Presumably the other epoxidation products were in the supernatants from recrystallization of some of the epoxides [40, 41], or were converted to a predominant epoxide product(s) by adjustment of reaction conditions, or were only detectable by more modern methods such as GC–EIMS.

Gas chromatograms of the δ-cadinene enantiomers and the epoxidation derivatives demonstrated that (−)-δ-cadinene eluted before (+)-δ-cadinene; likewise, each epoxide derived from (−)-δ-cadinene eluted before the corresponding epoxide generated from (+)-δ-cadinene. The sample composed of a 70:30 mixture of (+)- and (−)-δ-cadinene enantiomers and derived epoxidation products revealed excellent resolution of the enantiomers of δ-cadinene, monoepoxide 1, diepoxide 1 and diepoxide 3, and partial resolution of the enantiomers of monoepoxide 2 and diepoxide 2. The calculated Kovats' indices for the two δ-cadinene enantiomers [generated from the 70:30 mixture of (+)-α-copaene and (−)-α-copaene] and the epoxide derivatives are listed in Table 2. The variation in the chiral separation of the enantiomers of each enantiomeric pair is not suprising since the resolution of the enantiomers of structurally related compounds by chiral gas chromatography often varies widely [44, 45]. The mass spectra of each enantiomeric pair of compounds were identical. The epoxides derived from the δ-cadinene enantiomers exhibited fragmentation patterns generally expected for cadinene compounds [46, 47] and for epoxide derivatives [47, 48].

The product of cyclase activity and its five epoxide derivatives co-chromatographed with the (+)-δ-

Table 2. Kovats' indices (R_I) of δ-cadinene enantiomers and enantiomers of derived monoepoxides and diepoxides separated on a 30 m × 0.25 mm i.d. fused silica capillary column coated with permethylated β-cyclodextrin

Compound	Source	Calculated R_I
(−)-δ-Cadinene	70:30 [(−):(+)]-α-Copaene	1572
(+)-δ-Cadinene	70:30 [(−):(+)]-α-Copaene	1590
Monoepoxide 1	(−)-δ-Cadinene	1676
Monoepoxide 1	(+)-δ-Cadinene	1689
Monoepoxide 2	(−)-δ-Cadinene	1741
Monoepoxide 2	(+)-δ-Cadinene	1742
Diepoxide 1	(−)-δ-Cadinene	1797
Diepoxide 1	(+)-δ-Cadinene	1804
Diepoxide 2	(−)-δ-Cadinene	1815
Diepoxide 2	(+)-δ-Cadinene	1817
Diepoxide 3	(−)-δ-Cadinene	1826
Diepoxide 3	(+)-δ-Cadinene	1836

cadinene standard [generated from (−)-α-cubebene] and its derivatives and resolved from the (−)-δ-cadinene standard [generated from (+)-α-copaene] and its derivatives. The mass spectra obtained from the cyclized product and from the (+)-δ-cadinene and (−)-δ-cadinene standards were identical to each other and matched literature mass spectral data for δ-cadinene [9, 10]. The mass spectrum for each of the five epoxide derivatives generated from the cyclized product was identical to those of the corresponding epoxide derivatives generated from the (+)-δ-cadinene and (−)-δ-cadinene standards [e.g. the mass spectrum of monoepoxide 1 generated from the cyclized product was identical to the mass spectra of monoepoxide 1 generated from the (+)-δ-cadinene standard and of monoepoxide 1 from the (−)-δ-cadinene standard].

Chiral GC–EIMS has allowed resolution and identification of the enantiomers of δ-cadinene and of the five epoxide derivatives from each enantiomer. The chiral GC–EIMS analyses have revealed that the partially purified cyclase activity converted **5** into one predominant product, (+)-δ-cadinene. Only one other unidentified compound (possibly a contaminant) was recovered [at *ca* 2% of the amount of (+)-δ-cadinene]. Although multiple product formation by a cyclase is possible [49], the tentatively-identified compounds **8**–**10** were not detected during the chiral GC–EIMS analyses of the extracts of this cell-free reaction catalysed by semi-purified cyclase.

The combined evidence of co-chromatography and of identical mass spectra from multiple derivatives has confirmed the identification of the product of cyclase activity as (+)-δ-cadinene. The use of chiral capillary GC–EIMS has allowed chromatographic separation and chiral assignment of the cyclized product and the constellation of epoxide derivatives, employing small amounts of cyclization product, standard compounds, and chemical waste and without radiochemicals.

Incorporation of tritium from δ-cadinene into cotton phytoalexins

To further explore the role of **7** in the biosynthesis of cotton sesquiterpenoids, an *in planta* incorporation experiment was performed. Sixty hours after inoculation with *Xcm*, cyclase activity and the accumulation of sesquiterpenoid phytoalexins (**1**, **3** and **4**) in glandless cotton cotyledons were strongly induced (time-course of cyclase activity, Tsuji, personal communication; time course of sesquiterpenoid accumulation, Davis, G.D., unpublished results). At that time, tritium-labelled **7** (in aqueous soln of 1.0% Tween 80 [50]) was injected into the cotyledons. After eight hr, the cotyledons were harvested, quick-frozen with liquid N_2, and extracted by aqueous methanolic and hexane solutions. The methanolic and hexane extracts were mixed and chromatographed by reversed phase HPLC. Figure 1 displays the portion of the chromatogram showing elution of labelled compounds of interest. Although preparation of the HPLC sample in the starting solvent (MeCN–H_2O–H_3PO_4, 40:60:0.1) may have provided preferential dissolution of more polar compounds relative to non-polar compounds, residual non-polar [^{3}H]δ-cadinene (i.e. not converted to labelled products) was distinctly present in the full-length version of the chromatogram shown in Fig. 1 (eluting at *ca* 126 min). Thus, a representative array of labelled compounds of polar to non-polar character was recovered from the [^{3}H]δ-cadinene-injected cotton cotyledons for detection during a single chromatographic separation.

Of the 19 μCi of precursor (tritium-labelled **7**) injected into the *Xcm*-inoculated cotyledons, *ca* 1.0% of the tritium was recovered in the labelled compounds shown in Fig. 1. Non-radioactive analogues of the labelled compounds were isolated by a parallel extraction and chromatography of *Xcm*-inoculated glandless cotton cotyledons (which had not been injected with tritium-labelled **7**). The radioactive compounds and the non-

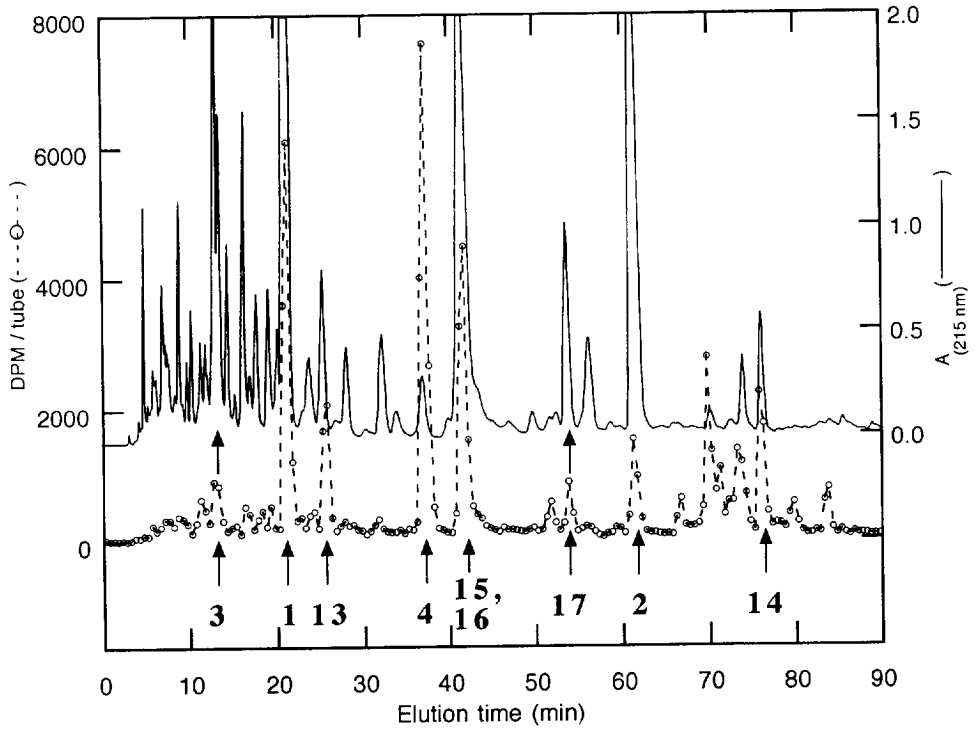

Fig. 1. Reversed phase HPLC of labelled compounds recovered from glandless cotton cotyledons injected with [^{3}H]δ-cadinene and harvested at 68 hr post *Xcm*-inoculation. (Total time of full chromatogram was 150 min.)

radioactive analogues were identified by similarly of the UV and mass spectral data and elution characteristics of the isolated non-radioactive analogues to literature reference (see Experimental). The elution order of **13** and **14** in relation to **1–4** and to **15–17** by reversed phase chromatography was reasonable. Although **13** and **14** were not further characterized as *cis*- or *trans*-isomers or as (+)- or (−)-enantiomers in this study, the *cis*- and *trans*-isomer of **13** and the *trans*-isomer of **14** have been isolated from *Xcm*-inoculated glanded (OK 1.2) cotton [51]. The UV and mass spectral analyses revealed that the sample fractions eluting at *ca* 42 min in Fig. 1 were composed of unresolved mixtures of greater amounts of **15** and lesser amounts of **16**. The relative levels of labelling of **15** and **16** could not be determined because the compounds were unresolved; however it is possible that labelled **16** may have been generated from **15** by enzymatic conversion *in planta* or by the previously documented non-enzymatic conversion of **15** to **16** [52]. Subsequent chromatography of extracts from *Xcm*-inoculated glandless (WbM*gl*) cotton cotyledons based on the method of Lee *et al.* [53] has permitted partial resolution of **15** and **16** (Abraham and Essenberg, unpublished results). Although possible precursors (**15** and **16**) of gossypol were present, neither labelled nor unlabelled gossypol was identified during chromatography of the incorporation experiment products. (Commercial gossypol from cotton eluted at *ca* 90 min in this HPLC system.)

Thus, the results presented in Fig. 1 revealed that tritium had been incorporated into the antibacterial sesquiterpenoid phytoalexins **1**, **3** and **4**, and the structurally related compounds **2**, **13**, **14**, **17** and the mixture of **15** and **16**. The presence of desoxyhemigossypol (dHG) (**15**) is of particular interest, since dHG functions as a phytoalexin in *Verticillium dahliae* resistance exhibited in infected xylem vessels of glanded cotton [54], but has not until now been reported as part of the foliar response to *Xcm*.

Because δ-cadinene is the most commonly documented sesquiterpene hydrocarbon produced in healthy glanded cotton cotyledons that has the cadinane carbon skeleton [5, 8], it is a plausible precursor to the cadinane-type phytoalexins **1**, **3** and **4** which are prominent in the hypersensitive response of *Xcm*-inoculated cotton plants. The accumulation of δ-cadinene in *Xcm*-inoculated (and elicitor-treated) glandless cotton cotyledons and generation of **7** in cell-free reactions are consistent with such a role. Furthermore, there is a temporal coincidence of the accumulation of (+)-δ-cadinene with the accumulation of the compounds **1–4** and **13–17** (Davis, G. D. *et al.*, data not shown). While irrefutable proof that a compound is a true biological intermediate is difficult to obtain [55], the *in planta* incorporation of tritium from [^{3}H]δ-cadinene into these structurally related compounds supports our hypothesis that δ-cadinene is a precursor to the numerous cadinene-based compounds found in cotton.

Mechanisms proposed for cyclization of farnesyl pyrophosphate to δ-cadinene

Although it is geometrically possible for (*E*, *E*)-FPP (**5**) to cyclize to a 10-membered ring, its 2,3-*trans* double bond would prevent formation of the C-1 to C-6 bond. This problem and two types of solution to it were described by Arigoni [56]. One possible solution involves cyclization of **5** to form a 10-membered ring, followed by deprotonation to yield an intermediate, germacrene-D, which can undergo a conformational change to cisoid configuration at carbons 2 and 3 (i.e. the carbons numbered as 2 and 3 in **5**) and, upon reprotonation, cyclize to δ-cadinene. Germacrene-D and δ-cadinene often occur together in plants [29] and nonenzymic, acid-catalysed stereospecific isomerization of (−)-germacrene-D (**18**) to (+)-δ-cadinene (**7**) in high yield has in fact been observed [57]. However, there is no direct evidence that germacrene-D is an intermediate in biosynthesis of δ-cadinene. We detected no accumulation of free germacrene-D in cell-free reactions which generated **7**, nor was it detected by GC–FID in extracts of inoculated cotton cotyledons. If it is an intermediate, it does not accumulate above *ca* 5 ng g^{-1} cotyledon tissue.

The second possible solution to the geometric problem posed by the 2,3-*trans* double bond involves initial isomerization of **5** to nerolidyl pyrophosphate (NPP) (**19**), following by rotation of the 2,3 bond to bring C-1 within bonding distance of C-6 [56]. [Compounds **19** and **20** (nerolidol) are shown as *trans*- isomers.] NPP could ionize to a pyrophosphate–germacrene cation pair, and progress through a series of cationic intermediates [58] involving a 1,3-hydride shift from C-1 to C-11 and the second cyclization, ending with deprotonation to δ-cadinene. There is strong evidence for the intermediacy of NPP in the enzymatic cyclization of **5** to trichodiene [59] and for a corresponding 10-carbon tertiary allylic intermediate in monoterpene cyclizations [60]. Therefore, biochemical precedents make NPP a more likely intermediate than germacrene-D. No nerolidol (**20**) was detected in the cell-free reactions utilizing **5**, while farnesol was found, presumably generated from FPP by the phosphohydrolases which are common in crude enzyme preparations [26]. However, the absence of nerolidol is not surprising, since Cane *et al.* were unable to detect any release of NPP from trichodiene synthase [59]. The tritium transfer from C-1 to C-11 that we demonstrated during biosynthesis of **1** and **2** [4] is predicted by both mechanisms.

A reasonable hypothesis based on our experimental evidence is that δ-cadinene is an early intermediate involved in the biosynthesis of the sesquiterpenoid phytoalexins of cotton. However, the isolation and correct identification of sesquiterpenes, and terpenes in general, is often complicated by the tendency of these compounds to undergo isomerization [23] or alteration during distillation [58] or gas chromatography with certain stationary phases [24]. While we have attempted to avoid these problems by using gentle methods of isolation and multiple forms of analysis, it should be noted that δ-cadinene has been proposed to be a thermodynamically stable product derived from mixtures of muurolenes and other cadinenes during distillation of plant materials [61]. However, simple batchwise purification of the crude enzyme preparation yielded a fraction which cyclized FPP to a single compound, (+)-δ-cadinene. Purification of the cyclase will be communicated in a future publication.

EXPERIMENTAL

Radiochemicals, chemicals and seed. (*E*, *E*)-[1-^{3}H]FPP (**6**) (sp. act. 32.14 mCi $mmol^{-1}$) was provided as a gift by Joseph Chappell, University of Kentucky. Compound **5** was synthesized [62]. The cotton line designated as 'Westburn M *glandless*' (WbM*gl*), a bacterial blight-resistant line lacking lysigenous glands was developed by crossing 'Westburn M' (WbM), a bacterial blight-resistant cultivar developed and released by the Oklahoma Agricultural Experiment Station [L. M. Verhalen personal communication] with the glandless line '21D111-112', which is homozygous in each of the recessive alleles gl_2 and gl_3 [63]. The initial cross was followed by four backcrosses to WbM with selection for the glandless phenotype in the first segregating generation after each cross and backcross (Greenhagen and Verhalen, unpublished work). The elicitor was prepared from polygalacturonic acid by autoclaving as previously described [64].

Acid-catalysed isomerizations [33] converted commercial (−)-α-cubebene, (+)-α-copaene and commercial '(−)-α-copaene' [actually a 70:30 mixt. of (−)-α-copaene and (+)-α-copaene] to product mixts containing (+)-δ-cadinene, (−)-δ-cadinene and a 70:30 mixt. of (+)-δ-cadinene and (−)-δ-cadinene, respectively. The δ-cadinene standards were isolated by routine normal phase and reversed phase HPLC. The (+)-α-copaene [65] was isolated from the essential oil of the root of angelica (*Angelica archangelica*) by distillation, CC with $AgNO_3$–silica (1:5) eluted with hexane, and semi-prep. reversed phase HPLC employing octadecylsilane eluted with MeCN–H_2O (3:1).

To assure synthesis of the proper stereoisomeric δ-cadinenes, values of $[\alpha]_D$ of the source materials were obtained for comparison to cited values: (−)-α-cubebene {$[\alpha]_D^{25}$ − 21.3° (in substance) found; $[\alpha]_D^{30}$ − 20.0° ($CHCl_3$; *c* 0.874) lit. [66]}, (+)-α-copaene {$[\alpha]_D^{25}$ + 6.2° (hexane; *c* 50) found; $[\alpha]_D^{23}$ + 6.4° ($CHCl_3$; *c* 1.2) lit. [65]}, and the 70:30 mixt. (−)-α-copaene and (+)-α-copaene {$[\alpha]_D^{25}$ − 0.7° (in substance) found; $[\alpha]_D^{20}$ − 0.7° (in substance) [determined by commercial supplier]}. [The commercial source term 'in substance' denotes a neat (undiluted) sample.] An infrared spectrum obtained for each of the three samples matched literature examples [16, 65, 66]. For the (+)-α-copaene, a 1D ^{1}H NMR spectrum was obtained that agreed with lit. description [65, 67]. Additionally, the derived δ-cadinene enantiomer samples were analysed by gas chromatography employing a capillary column capable of resolving the δ-cadinene enantiomers (see below). The 1D ^{1}H spectrum and the ^{13}C NMR spectrum of each isolated δ-cadinene enantiomer matched those of (+)-δ-cadinene isolated from the cade oil.

Cautionary notes. Analysts performing extractions similar to those described here may simplify GC elution patterns by use of CH_2Cl_2 as a polar solvent, thus avoiding the introduction into extracts of 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene, BHT) commonly added by many commercial vendors to Et_2O as a preservative. Exclusion of BHT is beneficial because it displays retention on the 100% methyl (SE-30) and 5% phenyl–95% methyl (SE-54) [19] gas chromatographic phases similar to that of many sesquiterpene hydrocarbons.

The concn of dilute solns (e.g. of δ-cadinene or other compounds or of extracts of cotton cotyledons) was performed at the lowest practical temp (*ca* 0° for hexane solns) in order to avoid possible losses of low M_r compounds by volatilization.

Workers wishing to use cade oil as a source of δ-cadinene should note the current practice [34] of substitution of *Juniperus phoenicea* for *Juniperus oxycedrus*, the traditional botanical source materials for cade oil [17]. The essential oil (cade oil) generated from *J. phoenicea* contains elevated amounts of α-cedrene and diminished amounts of δ-cadinene in comparison to the cade oil generated from *J. oxycedrus*.

Isolation and characterization of δ-*cadinene from* Xcm-*inoculated glandless cotton cotyledons.* Entire cotyledons of 11- or 12-day-old WbM*gl* seedlings were infiltrated with a suspension of *ca* 5×10^6 colony-forming units ml^{-1} of *Xanthomonas campestris* pv. *malvacearum* strain 3631 as previously described [68]. At 42 hr post-inoculation, the cotyledons were harvested, quick-frozen in liquid N_2 and stored at −70°. Thirteen hundred g fr. wt of inoculated cotyledons were processed by repetition of the following procedure to recover hexane-extractable compounds: 100 g of cotyledons were homogenized with 300 ml of chilled (4°) HPLC-grade Et_2O–hexane (5:95) in a glass blender body used with a spark-proof blender (30 sec on, 30 sec off; ×4). The combined supernatants were concd by rotary evapn (0° bath). The extract was dried with Na_2SO_4 to improve chromatography on silica gel.

The conc. hexane extract was passed through coarse silica gel (70–230 mesh). HPLC-grade hexane eluted fats, waxes and sesquiterpenes, while more polar compounds, including pigments, were retained on the silica. Frs containing substances with GC retention times characteristic of sesquiterpene hydrocarbons were combined and concd. Fats and waxes ppd by overnight storage of the conc. at −20° were removed by centrifugation. The extract was further concd to minimal vol., then suspended in MeCN–H_2O (35:65), and loaded onto a crude column constructed of 4 octadecylsilane cartridges attached in tandem (total wt of packing 4 g). Flow-through was discarded. Retained polar compounds were eluted with MeCN–H_2O (35:65) and discarded. Hydrocarbons were eluted with HPLC-grade MeCN which had been used to rinse the sample container to enhance recovery. After addition of water, the hydrocarbon eluate was back-extracted into hexane, concd by rotary evapn and diluted with hexane. The extract was subjected to sub-ambient temp. HPLC [23] using 4 3.9 mm i.d. × 300 mm silica columns (10 μm particle diameter) attached in tandem and submerged in an acetone bath maintained at −30°. Flow rate during elution with 100% HPLC-grade hexane was 1 ml min^{-1} or less due to elevated back-pressure (> 4000 psi) encountered at low column temp. Detection employed for HPLC sepns was UV absorption at 215 nm.

Frs containing the hydrocarbon were identified by GC–FID and combined, concd by rotary evapn and diluted in MeCN–H_2O (85:15). The sample was chromatographed at 1.0 ml min^{-1} in MeCN–H_2O (85:15), at ambient temp. (*ca* 23–26°) through a tandem arrangement [24] of 2 octadecylsilane columns (4.0 mm i.d. × 150 mm and 4.0 mm i.d. × 250 mm; both columns contained 5 μm media; back-pressure *ca* 1800 psi). Frs containing the desired hydrocarbon were combined; water was added, and the hydrocarbon was back-extracted into hexane. Following concn by argon gas stream at 0°, the hydrocarbon soln was stored at −70° under argon. The final yield of hydrocarbon was low (*ca* 500 μg), but was sufficient for GC–EIMS, 400 MHz ^{1}H NMR, GC–FID and HPLC co-chromatography with δ-cadinene from cade oil.

Isolation of δ-*cadinene* (**7**) *from cade oil.* Cade oil (50 g) was diluted in 200 ml hexane. Black polar material was partially removed by twice extracting the hexane soln with 500 ml H_2O. Silica (28–200 mesh) was added to decolorize the hexane soln to a light tan colour. The cade oil extract was purified by the protocol used for the extracts of cotton cotyledons except for the addition of a semi-prep. HPLC sepn [10 mm i.d. × 250 mm column, 5 μm octadecylsilane media; back-pressure *ca* 2300 psi at 2.5 ml min^{-1} when eluting with MeCN–H_2O (85:15)] prior to analytical-scale HPLC purification. δ-Cadinene (22 mg) was obtained (purity by GC–FID > 96%). The δ-cadinene was stable for 2–3 months at −20° in $CDCl_3$ under argon gas blanket, displaying no perceptible change in the ^{1}H NMR spectrum.

Enzyme prepns. Xcm-inoculated cotton cotyledons were homogenized in 50 mM HEPES (pH 7.2), 5 mM dithiothreitol, 10 mM sodium metabisulphite, 30 mM $MgCl_2$ and 10% glycerol (5 ml buffer g^{-1} cotyledon tissue) plus 0.3 g of insoluble polyvinylpyrrolidone and 0.3 g of XAD-4 resin g^{-1} of cotyledon tissue. The cotyledons were homogenized with a motorized homogenate a full speed (30 sec on, 30 sec off; ×4) at 4°. The homogenate was strained through 6 layers of cheesecloth, and the filtrate was centrifuged for 20 min at 27 000 *g* and 4°. The protein content of the supernatant was determined by the method of Bradford [69] with BSA as standard.

Cell-free reactions catalysing conversion of **6**. Cell-free reaction buffer was 50 mM HEPES (pH 7.2), 5 mM dithiothreitol, 30 mM $MgCl_2$ and 10% glycerol. An aliquot of the enzyme prepn (27 000 *g* supernatant) containing 10–60 μg of protein was diluted into this buffer to a total vol. of *ca* 490 μl in an 8-ml screw cap tube and pre-incubated at 30° for 5 min. (*E*, *E*)-[1-^{3}H]FPP (**6**) (*ca* 0.3 μCi in 10 μl) was added to give a final concn of

ca 20 μM FPP and a final vol. of 500 μl. The capped tube was then incubated at 30° for 30 min (conditions were determined to give linear kinetics), and the mixt. was extracted twice with 1.5 ml of hexane. The hexane extracts were pooled and mixed with 200 mg silica gel. After the silica gel settled, 1 ml of the hexane extract was added to 5 ml of scintillation fluid. Radioactivity of the sample was then determined by liquid scintillation counting. One ml of the hexane extract was concd by argon gas at 0° for identification of radioactive product(s) by analytical-scale normal phase or reversed phase HPLC. Virtually all of the radioactivity attributable to unconverted **6**, farnesol generated by cotton phosphohydrolases [26] or non-enzymatic hydrolysis, and other polar compounds was either retained by the aq. phase during extraction or by the 200 mg of silica gel subsequently added to the hexane extract.

Cell-free reactions catalysing conversion of **5**. These reactions were performed in the same manner as the cell-free reactions catalysing the conversion of **6**, except for an increased amount of cotyledons, use of (*E*, *E*)-FPP (**5**) as substrate and addition of the controls [25] described in Results and Discussion. *Xcm*-inoculated WbM*gl* cotyledons were homogenized; by use of reduced amounts of homogenization buffer, extracts containing 30 mg of protein in 24 ml of 27 000 *g* supernatant were obtained. The supernatant was divided into two 12-ml portions and pre-incubated at 30° for 5 min in screw-cap tubes. One-half ml of a soln of (*E*, *E*)-FPP (**5**) (1 mg ml^{-1} in cell-free reaction buffer) was added to each of the tubes. The capped tubes were incubated at 30° for 1.5 hr. The sample was then extracted in a manner similar to that of the cell-free reaction which catalysed conversion of **6**, but larger amounts of hexane were used to enhance recovery of non-radioactive cyclized product; thus, the reaction mixt. was extracted (×4) in a separatory funnel with 30 ml of hexane. The hexane extract was then concd by rotary evapn (0° bath). Silica gel was not employed so that intermediate polarity compounds (e.g. farnesol) would be recovered for GC analyses. The conc. extract was analysed by GC–FID and by GC–EIMS (70 eV) to obtain a mass spectrum for comparison to δ-cadinene ref. spectra from EPA/NIH [9] and another lit. source [10].

HPLC co-chromatography of radioactive product of cell-free reaction with cotton cotyledon hydrocarbon or with δ-cadinene from cade oil. The hexane extract of the cell-free reaction which utilized **6** was mixed with purified sesquiterpene hydrocarbon isolated from *Xcm*-inoculated glandless cotton cotyledons; the mixt. was then co-chromatographed by analytical-scale normal phase and reversed phase HPLC. The cell-free reaction product generated from **6** which was mixed with δ-cadinene from cade oil was co-chromatographed by analytical-scale normal phase and reversed phase HPLC. Elution in both HPLC systems was monitored by UV absorption at 215 nm and scintillation counting of sample frs. The void volume (V_0) of the analytical-scale normal phase column system was found to be *ca* 13.1 ml by injection of pentane [70]. Variability in capacity factors (k') observed in this subambient temp. normal phase HPLC system was probably due to fluctuations in the temp. (*ca* −30°) of the acetone bath used to cool the HPLC columns; capacity factors (k') are influenced by variations of cryogenic conditions in this type of HPLC system [23]. Typical capacity factors (k') determined from co-injections of commercial α-pinene, δ-cadinene (from cade oil or cotton), and commercial β-caryophyllene were 0.15, 0.64, and 0.71, respectively. The void volume (V_0) of the analytical-scale reversed phase column system was found to be 2.4 ml by injection of 1×10^{-5} M $NaNO_3$ [71]. The capacity factors (k') determined from co-injections of α-pinene, α-humulene and β-caryophyllene from commercial sources with (+)-δ-cadinene (from cade oil or cotton) were 4.2, 7.1, 8.3 and 9.9, respectively.

GC co-chromatography of δ-cadinene from cade oil with cotton cotyledon hydrocarbon or with the product of the cell-free conversion of **5** Co-chromatography of δ-cadinene from cade oil with the infection-induced hydrocarbon from *Xcm*-inoculated cotton cotyledons was conducted on 4 different gas chromatographic phases in WCOT capillary columns [0.25 mm i.d. × 30 m 100% methyl (SE-30); 0.25 mm i.d. × 60 m 5% phenyl–95% methyl (SE-54) × 2 (2 of the 60 m columns were joined by glass union to make a 120 m column); 0.25 mm i.d. × 30 m 50% phenyl–50% methyl; and 0.25 mm i.d. × 30 m polyethylene glycol (Carbowax 20M equivalent)]. The FID signal was plotted and integrated. Detector temp. was 300°; on-column injection of 0.2–1.0 μl was made at injector temp. of 55° and oven temp. of 85°; carrier gas was He, and linear flow rate was 28 cm sec^{-1} (equivalent to 1.0 ml min^{-1}). A typical oven gradient was 2 min hold at 85° followed by a gradient of 85° to 210° at 1° min^{-1}. Experimental Kovats' index values were obtained by use of a linear regression line calcd from retention times of co-injected *n*-paraffin hydrocarbon standards [21]. β-Caryophyllene and α-humulene were co-injected with samples as retention marker compounds on each column and as standards in place of *n*-paraffins to construct the Kovats' indices plot with self-consistent Kovats' indices [13, 22] for the 5% phenyl–95% methyl (SE-54) column.

Tentative identification of low abundance compounds in crude hexane extracts of Xcm*-inoculated glandless cotton cotyledons and of the soln from the cell-free reaction catalysing conversion of* **5**. GC–EIMS analysis of the crude hexane extract of *Xcm*-inoculated glandless cotton cotyledons was conducted on two different gas chromatographic phases in WCOT capillary columns [0.25 mm i.d. × 30 m 5% phenyl–95% methyl (SE-54) and 0.25 mm i.d. × 30 m polyethylene glycol (Carbowax 20M equivalent)]. On-column injections of 0.5–1.5 μl were made at an ambient temp. of *ca* 25° and oven temp. of 85°. Carrier gas was He, and linear flow rate was 28 cm sec^{-1} (equivalent to 1.0 ml min^{-1}). The oven gradient used for both columns was 2 min hold at 85° followed by a gradient of 85° to 220° at 2° min^{-1}. GC–EIMS analysis (70 eV) of low abundance compounds was performed with a VG TS-250 system. The tentative identification of ε-cadinene was based upon a close match with the published Kovats' index value for ε-cadinene chromatographed on a Car-

bowax 20M GC column [18]; the experimental mass spectrum was similar, but not identical, to the published mass spectrum of racemic ε-cadinene [72]:

tentatively-identified ε-cadinene: GC–EIMS 70 eV, *m/z* (rel. int.): 204[M]$^+$ (61), 189 [M − Me]$^+$ (31), 176 [M − C_2H_4]$^+$ (8), 161 [M − *iso*-Pr]$^+$ (100), 147 (52), 133 (66), 119 (55), 105 (67), 93 (71), 91 (73), 81 (53), 79 (61), 69 (61), lit.; ε-cadinene: GC–EIMS 70 eV, *m/z* (rel. int.): 204 [M]$^+$ (57), 189 [M − Me]$^+$ (10), 176 [M − C_2H_4] (61), 161 [M − *iso*-Pr]$^+$ (100), 133 [176 − *iso*-Pr] (79), 119 (27), 105 (26), 93 (44), 91 (49), 81 (54) [72].

The tentative identification of α-muurolene was based upon a close match with the Kovats' index value calcd from the published retention time for chromatography of α-muurolene on an SE-54 GC column [19] and ref. mass spectral data for α-muurolene from lit. [10, 19]. GC–FID and GC–EIMS (VG TS-250 system; 70 eV) analyses of the extract from the cell-free reaction which catalysed the conversion of **5** were conducted with a 0.25 mm i.d. × 30 m 50% phenyl–50% methyl WCOT capillary GC column. On-column injection of 0.5–1.5 μl was made at ambient temp. of *ca* 55° (GC–FID) or *ca* 25° (GC–EIMS); carrier gas was He, and linear flow rate was 28 cm sec^{-1} (equivalent to 1.0 ml min^{-1}). The oven gradient was 2 min hold at 85° then a gradient of 85°–170° at 3° min^{-1}, followed by a second gradient from 170° to 270° at 10° min^{-1}. The identification of α-cadinene was based upon a published mas spectrum [19]. Although no published retention value for α-cadinene was found for the intermediate polarity GC phase we employed [(50%-phenyl)-methylpolysiloxane], the tentatively identified α-cadinene eluted after δ-cadinene; similar elution order was seen for chromatography on polar Carbowax 20M GC columns [73].

Identification of sesquiterpene hydrocarbon from elicitor-infiltrated cotton; lack of hydrocarbon in mock-inoculated and noninoculated control cotyledons. The elicitor prepn was the hydrolysate of polygalacturonic acid prepd by the method of Robertsen [64]. The elicitor soln was filter-sterilized by 0.22 μm filter prior to use. WbM*gl* cotyledons were infiltrated with elicitor (5 mg ml^{-1}) in inoculation medium which was sterile, satd $CaCO_3$ soln. At the same time, separate WbM*gl* cotyledons were mock-inoculated by infiltration with the sterile, satd $CaCO_3$ soln or inoculated with an *Xcm* suspension (*ca* 5 × 10^6 colony-forming units ml^{-1}) in the same soln. The *Xcm*-, elicitor- and $CaCO_3$-infiltrated cotyledons and noninoculated cotyledons were cultivated in the same growth chamber and harvested at 42 hr post-infiltration. The cotyledons were immediately frozen in liquid N_2 and stored at −70° until analysis. Each type of cotyledon (500 mg) was separately extracted by hand homogenizer (Duall) with 10 ml, then 5 ml of Et_2O–hexane (5:95). Each soln was centrifuged, and the supernatant was concd to small vol. (*ca* 0.5 ml) by evapn (0° bath). Each sample was chromatographed on a small homemade silica gel column (70–230 mesh silica gel in a pasteur pipette with glass wool plug) with hexane until the first coloured (yellow) pigment started to elute from the column; most of the plant pigments were retained on the silica gel. Any yellow pigment in the eluted hexane was removed by addition of silica gel. The decolorized hexane solutions were concd to *ca* 2 ml by rotary evapn (0° bath) and then to dryness with Ar gas stream with sample container at 0°. Samples were suspended in hexane (10–20 μl) and on-column injections of 0.2–1.0 μl at an injector temp. of 55° and oven temp. of 85° were made for GC–FID analysis on the 0.25 mm i.d. × 30 m polyethylene glycol column (Carbowax 20M equivalent). Detector temp. was 300°. Carrier gas was He, and linear flow rate was 28 cm sec^{-1} (equivalent to 1.0 ml min^{-1}). The oven gradient used was 2 min hold at 85° followed by a gradient of 85–220° at 2° min^{-1}. Identity of any possible δ-cadinene peak was confirmed by co-chromatography of co-injected δ-cadinene from cade oil and GC–EIMS identification of the sesquiterpene hydrocarbon using the 0.25 mm i.d. × 30 m polyethylene glycol column (Carbowax 20M equivalent) with a VG TS-250 mass spectrometer.

Cell-free reaction catalysed by enzyme prepn made from elicitor-infiltrated cotton cotyledons. A separate aliquot of the cotyledons used for the preceding GC–EIMS identification of δ-cadinene in elicitor-infiltrated cotyledons was utilized for this expt. Cotyledon tissue (2 g) from each treatment were homogenized as described for the enzyme prepns above, and cell-free reactions using (*E*, *E*)-[1-^{3}H]FPP (**6**) as substrate were also prepd, incubated and extracted as described above for *Xcm*-inoculated cotyledons. One-sixth of the extract from each cell-free reaction (0.5 ml) was added to 5 ml of scintillation fluid; then radioactivity was determined by liquid scintillation counting. The remaining extract (*ca* 2.5 ml) was concd to near dryness by Ar gas at 0°. The extracts were each suspended in hexane containing α-pinene, β-caryophyllene and the δ-cadinene from cade oil as time retention markers detected by UV absorption at 215 nm during fractionation by the sub ambient temp. analytical-scale normal phase HPLC system. A 0.5 ml aliquots of each fr. was added to 5 ml of scintillation fluid, radioactive frs were detected by liquid scintillation counting. A second series of homogenizations, cell-free reactions, and hexane extracts were made, but the dried extracts were resuspended in MeCN–H_2O (85:15), containing α-pinene, α-humulene, β-caryophyllene, and δ-cadinene from cade oil as time retention markers detected by UV absorption at 215 nm during fractionation by the analytical-scale reversed phase HPLC system at room temp. An aliquot of 0.5 ml from each fr. was added to 5 ml of liquid scintillation fluid, then the radioactivity was determined by liquid scintillation counting.

GC-FID analysis of Xanthomonas campestris *pv.* malvacearum *bacteria and nutrient broth. Xanthomonas campestris* pv. *malvacearum* strain 3631 was cultured in nutrient broth (120 ml) to a concn of *ca* 5 × 10^6 colony-forming units ml^{-1}. The bacteria and nutrient broth were sepd by centrifugation (27 000 *g* for 20 min). The sepd bacteria and broth were each extracted with CH_2Cl_2–hexane (5:95). Each extract was concd by rotary evapn (0° bath), chromatographed on 70–230 mesh silica gel with hexane to remove polar components and

the hexane eluant was then concd by rotary evapn (0° bath) and Ar at 0° to *ca* 10–20 μl. On-column injection of 0.5 μl was made at injector temp. of 55° oven temp. of 85° of each extract allowed for GC–FID analysis employing 0.25 mm i.d. × 30 m 5% phenyl–95% methyl (SE-54) or 0.25 mm i.d. × 30 m polyethylene glycol (Carbowax 20M equivalent) WCOT capillary GC columns. The FID signal was plotted and integrated. The detector temp. was 300°; carrier gas was He, and linear flow rate was 28 cm sec^{-1} (equivalent to 1.0 ml min^{-1}). The oven gradient was 2 min hold at 85° followed by a gradient of 85–220° at 2° min^{-1}.

Determination of chirality of δ-cadinene from various sources. Samples of the non-radioactive cyclized product and the standards of (+)-δ-cadinene, (−)-δ-cadinene and the 70:30 mixt. of (+)-δ-cadinene with (−)-δ-cadinene were each epoxidized [30, 37, 38] with excess monoperphthalic acid [39] dissolved in Et_2O (3 × stoichiometric amount for both double bonds of δ-cadinene). The predominant epoxidation products at 4° after 2 hr or after 24 hr were monoepoxides or diepoxides, respectively. For chiral analysis, the injected samples (in CH_2Cl_2) contained the δ-cadinene enantiomer(s), the derived epoxidation products, and C-15 (pentadecane) and C-19 (nonadecane) *n*-paraffins (retention markers) in amounts that placed *ca* 20–50 ng of each component on-column. GC–EIMS analyses were performed at 70 eV, with sepns made by a 0.25 mm i.d. × 30 m WCOT capillary gas chromatographic column with a stationary phase of permethylated β-cyclodextrin. Injections of 1.0 to 2.0 μl with a 1:2 to 1:4 split were made at an injector temp. of 220° and oven temp. of 85°; carrier gas was He, and linear flow rate was 17 cm sec^{-1} (equivalent to 0.6 ml min^{-1}). Temp. gradient was initial hold at 85° for 1 min, then 5 min gradient at 10° min^{-1}, then a gradient of 1° min^{-1}. The chromatographic elution patterns and mass spectra of the four different samples were compared. The Kovats' indices [21] of the eluted compounds were calcd from the chromatogram produced from the sample generated by epoxidation of the 70:30 mixt. of (+)- and (−)-δ-cadinene; thus Kovats' indices for both enantiomers of δ-cadinene and the derived epoxidation products could be compared from one chromatographic analysis. The standard curve used to calculate R_I was generated by plotting the log of the corrected retention time for the co-injected pentadecane (C-15) and nonadecane (C-19) *n*-paraffin standards vs the arbitrary R_I values of 1500 and 1900, respectively. The calcd R_I for each compound is listed in Table 2. The mass spectra for both enantiomers of each enantiomeric pair were identical. The mass spectrum from each of the enantiomers of δ-cadinene was compared to reported mass spectra for δ-cadinene [9, 10]. The mass spectral patterns of the monoepoxides and diepoxides generated from δ-cadinene samples were compared with previously described patterns for cadinene compounds [46, 47] and epoxide compounds [47, 48]. The mass spectral data for the two monoepoxide derivatives and the three diepoxide derivatives from (+)-δ-cadinene [generated from (−)-α-cubebene] are listed below.

δ-Cadinene monoepoxide 1. GC–EIMS 70 eV, *m/z* (rel. int.): 220 $[M]^+$ (21), 205 $[M - Me]^+$ (8), 177 $[M - iso\text{-}Pr]^+$ (100), 159 $[M - iso\text{-}Pr - H_2O]^+$ (33), 149 (15), 135 [M–Me–CH_2 = $CHCHMe_2$, RDA]$^+$ (38), 119 (45), 110 (39), 91 (43), 43 (39).

δ-Cadinene monoepoxide 2. GC–EIMS 70 eV, *m/z* (rel. int.): 220 $[M]^+$ (23), 205 $[M - Me]^+$ (5), 177 $[M - iso\text{-}Pr]^+$ (100), 159 $[M - iso\text{-}Pr - H_2O]^+$ (31), 149 (15), 135 [M–Me–CH_2 = $CHCHMe_2$, RDA]$^+$ (42), 119 (52), 110 (42), 91 (45), 43 (42).

δ-Cadinene diepoxide 1. GC–EIMS 70 eV, *m/z* (rel. int.): 236 $[M]^+$ (0.4), 218 $[M - H_2O]^+$ (1), 208 (4), 193 $[M - iso\text{-}Pr]^+$ (6), 175 $[M - iso\text{-}Pr - H_2O]^+$ (23), 133 [M–Me–H_2O–CH_2 = $CHCHMe_2$, RDA]$^+$ (19), 110 (100), 109 (72), 107 (22), 95 (44) 93 (32), 43 (76).

δ-Cadinene diepoxide 2. GC–EIMS 70 eV, *m/z* (rel. int.): 236 $[M]^+$ (0.4), 208 (47), 193 $[M - iso\text{-}Pr]^+$ (20), 175 $[M - iso\text{-}Pr - H_2O]^+$ (14), 133 [M–Me–H_2O–CH_2 = $CHCHMe_2$, RDA]$^+$ (23), 110 (15), 109 (32), 107 (54), 95 (35) 93 (57), 43 (100).

δ-Cadinene diepoxide 3. GC–EIMS 70 eV, *m/z* (rel. int.): 236 $[M]^+$ (0.8), 208 (72), 193 $[M - iso\text{-}Pr]^+$ (30), 175 $[M - iso\text{-}Pr - H_2O]^+$ (11), 133 [M–Me–H_2O–CH_2 = $CHCHMe_2$, RDA]$^+$ (19), 110 (24), 109 (41), 107 (20), 95 (40) 93 (29), 43 (100).

Further GC–FID analysis of the δ-cadinene from cade oil and the δ-cadinene enantiomers [the cyclized product, (+)-δ-cadinene generated from (−)-α-cubebene, and (−)-δ-cadinene generated from (+)-α-copaene] was performed by injections onto the same capillary column. The FID signal was plotted and integrated. Detector temp. was 300°; on-column injection of 0.5–1.5 μl was made at injector temp. of 50° and oven temp. of 50°; carrier gas was He, and linear flow rate was 28 cm sec^{-1} (equivalent to 1.0 ml min^{-1}). The temp. gradient employed was: initial hold at 50° for 1 min, then 5 min gradient at 10° min^{-1}, then a gradient of 1° min^{-1}.

Incorporation of tritium label from δ-cadinene into cotton metabolites. Glandless cotton cotyledons (WbM*gl*) were inoculated with a suspension of *Xcm* (*ca* 5 × 10^6 cfu ml^{-1} in satd $CaCO_3$ soln). To obtain enzyme activity for conversion of **6** to tritium-labelled δ-cadinene, an enzyme prepn was made from 100 g of *Xcm*-inoculated glandless cotton cotyledons as described above for '*Enzyme preparations*', except for the deletion of sodium metabisulphite and $MgCl_2$ from the buffer soln. DEAE-modified cellulose media (50 g) was washed and equilibrated in 50 mM HEPES (pH 7.2), 5 mM dithiothreitol and 10% glycerol (HEPES buffer). The crude enzyme prepn was slurried with the DEAE-modified cellulose and allowed to sit for 30 min. The supernatant was discarded. Cyclase activity was eluted from the DEAE-modified cellulose by mixing with HEPES buffer containing 250 mM KCl (100 ml). After allowing the DEAE-modified cellulose to settle, the supernatant was mixed with 25 g of hydroxyapatite [the hydroxyapatite was previously washed with 100 mM potassium phosphate (pH 7.0), 5 mM dithiothreitol and 10% glycerol, then washed and equilibrated with a buffer of 5 mM potassium phosphate (pH 7.0), 5 mM dithiothreitol and 10% glycerol]. After the hydroxyapatite settled, the

supernatant was discarded. Cyclase activity was eluted with 50 ml 200 mM potassium phosphate (pH 7.0), 5 mM dithiothreitol and 10% glycerol. After 1 hr, the hydroxyapatite had settled and the supernatant containing the cyclase activity was decanted. The elution of cyclase activity from the hydroxyapatite was repeated with a second 50 ml batch of buffer. The 2 aliquots of eluting buffer were pooled to yield a soln of *ca* 85 ml. After the soln was made 30 mM in $MgCl_2$ and equilibrated for 10 min in a 30° water bath, (*E*, *E*)-[1-^{3}H]FPP (**6**) (50 μCi; sp. act. 26.9 Ci mmol^{-1}) was added. The container was stoppered and shaken occasionally. At 90 min, the cell-free reaction was quenched by addition of and extraction with hexane (4 × 50 ml). The hexane extract was gently concd by rotary evapn at 0°, exposed to silica gel to remove any polar contaminants or unconverted **6** and concd to minimal volume by rotary evapn, followed by Ar gas stream at 0°. The compound was dissolved in 200 μl of MeCN–H_2O (85:15) and chromatographed at 1.0 ml min^{-1} in MeCN–H_2O (85:15), at ambient temp. (*ca* 23–26°) on an octadecylsilane column (4.0 mm i.d. × 150 mm, 5 μm media; back-pressure *ca* 800 psi). Addition of water to desired frs (identified by liquid scintillation counting) and back extraction and concn allowed isolation of the tritium-labelled δ-cadinene. The tritium-labelled δ-cadinene (19 μCi) was resuspended in aq. soln containing 1% Tween 80 detergent (10 ml) to assist resuspension [50] of the labelled δ-cadinene. At 60 hr after injection of a suspension of *Xcm* (*ca* 5 × 10^6 colony-forming units ml^{-1} satd $CaCO_3$) into 9 cotyledons, the tritium-labelled δ-cadinene soln was injected. After 8 hr, the cotyledons (*ca* 5 g fr. wt) were harvested and immediately quick-frozen with liquid N_2 and stored at −70° until extracted. Two cotyledons were extracted with aq. MeOH soln as previously described [74]; the extracted cotyledons and solvents used to obtain the aq. MeOH extract were also extracted (2 × 20 ml) with hexane and concd by rotary evapn. The aq. MeOH extract back-extracted into $CHCl_3$ [74] was concd to minimal vol. with Ar gas stream at 0°. The hexane extract was added, and the combined extracts were concd to near dryness with Ar gas stream at 0°.

Resuspension of the combined extract with 200 μl of a soln of MeCN–H_2O–H_3PO_4 (40:60:0.1) yielded the sample for HPLC analysis. The sample was chromatographed by HPLC with a tandem arrangement of a 4.0 mm i.d. × 150 mm and 4.6 mm i.d. × 300 mm HPLC octadecylsilane columns, eluting at a flow rate of 1.0 ml min^{-1} with a starting eluent of MeCN–H_2O–H_3PO_4 (40:60:0.1; 5 μm media; back pressure of *ca* 1800 psi) isocratically for 30 min, followed by a linear gradient to MeCN–H_3PO_4 (100:0.1) at 150 min post-injection. The eluting compounds were collected in 0.5 ml frs; aliquots were analysed for radioactivity by liquid scintillation counting and monitored by UV absorption at 215 nm. Similar aliquots of analogous non-radioactive compounds were obtained by similar treatment of *Xcm*-inoculated glandless (WbM*gl*) cotton cotyledons (which were not injected with tritium-labelled δ-cadinene). The experimental mass spectra of the isolated non-radioactive compounds were compared with mass spectral data in the lit. for **1** [1, 75], **2** [75], **3** [1, 76], **4** [76], **13** [51] and **14** [51, 77], **15** [52, 78], **16** [79] and **17** [52, 78]. Similar comparisons were made for the UV spectral data of the isolated non-radioactive compounds to **1** [1], **2** [75], **3** [1, 76], **4** [76], **13** [51], **14** [51, 77], **15** [52, 78], **16** [79, 80] and **17** [52, 78]. The order of elution in the reversed phase chromatography was compared with that previously described for reversed phase or normal phase chromatography of **1**–**4** [1, 75, 76, 81] and **15**–**17** [52, 53, 80].

Other analytical methods employed to identify δ-cadinene from cade oil. CD was performed at ambient temp. (23–26°); 16 scans accumulated on a 1 × 10^{-5} M soln of δ-cadinene from cade oil in pentane were processed by personal computer. FT–IR analysis was performed at room temp. by accumulation of 192 scans on a sample of 50–100 μg of δ-cadinene from cade oil spread on a compressed zinc sulphide plate. UV absorption was recorded in hexane.

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